



Antifungal effects of zinc nanoparticles green synthesized by *Lavandula angustifolia* extract, alone and combined with nystatin against *Candida albicans*, a major cause of oral candidiasis

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ARTICLE INFO

Article Type:
Original Article

Article History:
Received: 4 May 2022
Accepted: 29 July 2022

Keywords:
Thrush
Candidiasis
Fungicidal effects
Cytotoxicity
Nanomaterial

ABSTRACT

Introduction: This work aimed to determine the antifungal effects of zinc nanoparticles (ZnNPs) green synthesized by *Lavandula angustifolia* extract, alone and along with nystatin against *Candida albicans*.

Methods: ZnNPs were green synthesized with *L. angustifolia* extract by microwaves method. Antifungal effects of ZnNPs were studied by measuring the minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) using the broth microdilution method based on the modified M27-A3 protocol on yeasts, recommended by the Clinical and Laboratory Standards Institute (CLSI). Effects of green synthesized ZnNPs against human normal fibroblast-like Gingiva (HGF1-PI1) and human epithelial-like oral cancer (KB) cell lines were studied by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method.

Results: The ZnNPs showed a spherical shape with some grains of different lengths. The best levels of MIC and MFC were connected to the combination of ZnNPs + nystatin with values of 0.204 and 0.250 µg/mL, respectively. The combination of ZnNPs with nystatin compared to the nystatin group had a significantly better antifungal effect on *C. albicans* ($P < 0.001$). The 50% cytotoxic concentrations of ZnNPs against normal (HGF1-PI1) and cancer (KB) cells were 172.3 and 83.2 µg/mL, respectively.

Conclusion: We found that ZnNPs plus nystatin had a potent antifungal effect against *C. albicans*. These findings indicated the cytotoxic effects of green synthesized ZnNPs on cancerous cells, whereas they were nontoxic for normal cells. Additional studies are necessary to explain the accurate mechanism and toxicity.

Implication for health policy/practice/research/medical education:

The results of the present in vitro study showed that green synthesized zinc nanoparticles (ZnNPs), especially with nystatin, had a potent antifungal effect against *Candida albicans* as the main cause of oral candidiasis. These findings indicate the cytotoxic effects of green synthesized ZnNPs on cancerous cells, whereas they were nontoxic for normal cells. Nevertheless, further investigations are required to elucidate the precise mechanism as well as systemic toxicity, especially in clinical settings.

Please cite this paper as: Nasiri S, Mahmoudvand H, Shakibaie M, Mousavi SAH, Sepahvand A. Antifungal effects of zinc nanoparticles green synthesized by *Lavandula angustifolia* extract, alone and combined with nystatin against *Candida albicans*, a major cause of oral candidiasis. J Herbmed Pharmacol. 2022;11(4):540-545. doi: 10.34172/jhp.2022.62.

Introduction

Candidiasis is an important and common fungal disease of the oral mucosa caused by different species of *Candida* (1). More than 300 different species of microorganisms,

including 20 species of *Candida*, have been recognized in the oral cavity that the presence of which can be very dangerous, causing widespread infections (2). *Candida* spp. are the measure of normal oral flora (25%-50%) of

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healthy individuals; this rate is even higher in certain conditions such as AIDS patients, the use of dentures, diabetic patients, chemotherapy, malignancies, and children (3).

Thrush is the most common form of candidiasis, which begins with increased growth and proliferation of different species of *Candida*, such as *C. albicans* in the mouth (4). The lesions of this fungus are in the form of prominent, soft, creamy, and cheese-shaped to yellowish plaques that easily peel off, leaving a reddish-brown background that leaves a sore and sensitive surface (5). A number of nonspecific mechanisms such as changes in the microbial flora of saliva following the use of antibiotics, smoking, poor oral hygiene, changes in the oral epithelium due to dry mouth, or inherited diseases are predisposing factors for the growth and reproduction of candidate species (6). Treatment of candidiasis is a complex issue due to the emergence of resistance to *Candida* strains and some adverse side effects (7,8). Therefore, it is necessary to find new antifungal drugs with high activities against *Candida* spp (9,10).

The application of nanotechnology is currently considered one of the remarkable advances in the field of dentistry (11-14). For nanotechnology, applications in the fields of medicine, environment, energy, chemistry, physics, etc., have been measured, which makes this technology an interdisciplinary and cross-sectoral field (15-18). Nanoparticles, especially metal nanoparticles with high ability to absorb and scatter light, high compatibility with living organisms and the ability to interact with biological molecules have many applications in life sciences, medicine, and dentistry. Extensive applications of metal nanoparticles have also led researchers to consider the use of simple, low-cost, and non-toxic methods for the synthesis of these materials (19,20).

The production of nanoparticles can be achieved through various physical and chemical methods, each with its own disadvantages (21). The use of plant extracts for the green synthesis of metal nanoparticles is superior to other biological processes and can be used on a large scale due to its economics and no need to maintain cell cultures (22,23). Nowadays, the microwave heating method has found increasing application in the synthesis of numerous nanomaterials due to some properties, uniform heat distribution, increased reaction speed, and reduced time and energy required (24). Due to the importance of the subject in this work, we studied the antifungal effect of nanoparticles with lavender (*Lavandula angustifolia*) synthesized by microwave method on *C. albicans*.

Materials and Methods

Preparation and characterization of Zn nanoparticles (ZnNPs)

After collecting the *L. angustifolia* (leaves) from the rural regions of Kerman, Iran, in October 2019 and identifying by a botanist, an herbarium sample was deposited at Razi

Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran (No. 1399.309). They were extracted by percolation method (methanol 8%) for 72 hours at the 21°C. Zn sulfate solution with a concentration of 1 mM was prepared with deionized water, and different amounts of the prepared plant extract were added to it separately. Then, the mixture was exposed to microwaves of different strengths and times in the microwave. Based on the color change of the mixture to dark gray, which is the color characteristic of ZnNPs production, and drawing the UV-Vis spectrum of the mixture using a spectrophotometer and observing the peak of ZnNPs at 266 nm, nanoparticle formation was investigated qualitatively (25,26). The physical and chemical properties of the synthesized nanoparticles were determined using various instrumental methods such as scanning electron microscope (SEM), energy dispersive x-ray analysis (EDX), X-ray diffraction (XRD), and Fourier-transform infrared spectroscopy (FTIR).

Determination of minimum inhibitory concentration (MIC)

A standard McFarland 0.5 suspension (1×10^8 CFU/mL) of *C. albicans* (ATCC5027) was obtained (27). The MIC of ZnNPs on standard strains of *C. albicans* was obtained by microbroth dilution method in 96 sterile plates according to the Clinical and Laboratory Standards Institute (CLSI) instructions (28). Briefly, various concentrations of ZnNPs were added to the semi-McFarland turbidity CFU/mL 1.5×10^8 and the plates were incubated for 24 hours at 37°C. Then, 2, 3, 5-tri-phenyltetrazolium chloride (visual indicator of fungal growth) was added to the plates. DMSO and nystatin were used as the negative and positive controls, respectively.

Determination of minimum fungicidal concentration (MFC)

To do this, a loop of the contents of the thin tubes was transferred to plates containing the medium. After 24-72 hours, the growth was observed and the medium containing the last concentration of ZnNPs with no colony growth was considered as the minimum concentration of MFC.

Cytotoxicity effects

Effect of green synthesized ZnNPs against human normal fibroblast-like Gingiva (HGF1-PII, Pasture Institute, Iran) and human epithelial-like oral cancer (KB, Pasture Institute Iran) cell lines were studied by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method (29,30). After culturing the cell lines in Dulbecco's modified eagle medium (Merck, Germany) enhanced with fetal bovine serum (15%, Merck, Germany), penicillin (100 IU/mL) and streptomycin (100 µg/mL), in microplates, were exposed with the ZnNPs (10-400 µg/mL) for 48 hours at 37°C with 5% CO₂. The

50% cytotoxic concentration (CC_{50}) value for each cell was separately calculated through the probit test in SPSS software (version 26.0).

Statistical analysis

SPSS software (version 26.0) was used to perform data analysis. $P < 0.05$ was considered a significant level.

Results

Characterization of ZnNPs

The ZnNPs synthesis was confirmed using UV-VIS spectral analysis, which shows the absorption peak in the range of 230-330 nm. Also, EDX analysis of ZnNPs showed that Zn adsorption peaks, including $ZnL\alpha_1$, $ZnK\alpha_1$, and $ZnK\beta_1$ were 1.01, 8.64, and 9.57 kg, respectively. The results of the XRD pattern indicated that 101, 201, 202, 203, and 300 were refractive peaks at 19.6, 36.9, 39, 43.9, and 54.8 degrees (Figure 1A). FTIR analysis revealed that ZnNPs had peaks at 1401, 1262, 1064, and 580 cm, which can be related to CC, CN, CN, and C- (F, Cl or Br). The highest adsorption at 1627 cm, 3418 cm, and 2923 cm can be caused by the polypeptide amide Bond-1, OH stretching (Figure 1B).

SEM analysis showed that ZnNPs had a spherical shape with some grains of different lengths (Figure 2A) with the size ranged from 30 to 80 nm, while most nanoparticles were between 50 and 60 nm (Figure 2B) of the phenol bond stretching of the carboxylic acid, carboxylic acid.

Determination of antifungal effects of nanoparticles by MIC and MFC

The values of MIC and MFC after 3 replications for ZnNPs as well as nystatin as a control drug are listed in Table 1. The lowest levels of MIC and MFC related to the combination of ZnNPs + nystatin were reported at 0.204 and 0.250 $\mu\text{g/mL}$, respectively. The results showed that the combination of ZnNPs with nystatin compared to the nystatin group as a control drug had a significantly better antifungal effect on *C. albicans* ($P < 0.001$).

Cytotoxicity effects of ZnNPs on normal and cancer cells

Figure 3 depicts the effects of the green synthesized ZnNPs on human normal (HGF1-PI1) and cancer (KB) cell lines after 48 hours incubation. The findings of MTT method indicated that the CC_{50} of ZnNPs against normal (HGF1-PI1) and cancer (KB) cells were 172.3 and 83.2 $\mu\text{g/mL}$, respectively.

Discussion

Candida albicans is the most common opportunistic pathogen of the oral cavity and part of the microflora of this cavity that its oral colonization occurs at birth or shortly thereafter; the number of carriers of *Candida* increases with age (3). The main site of colonization of *C. albicans* is in the posterior part of the dorsal surface of the tongue; this oral disease often occurs due to some local

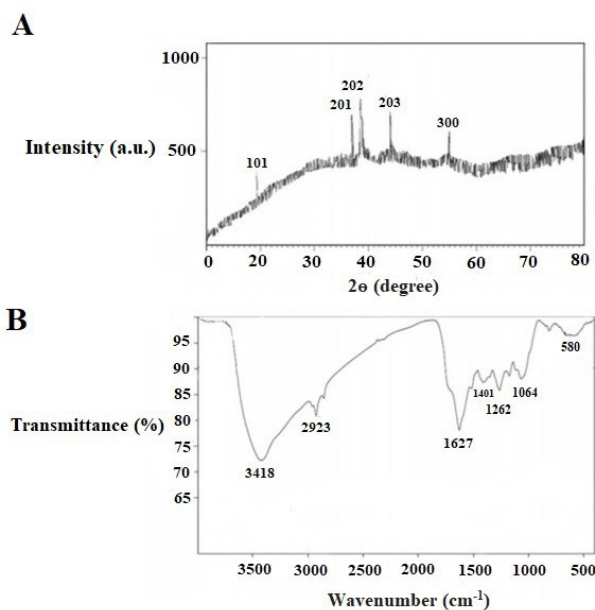


Figure 1. X-ray diffraction patterns (A) and Fourier-transform infrared spectroscopy (FTIR) (B) of zinc nanoparticles using extraction of lavender extract based on the microwave method.

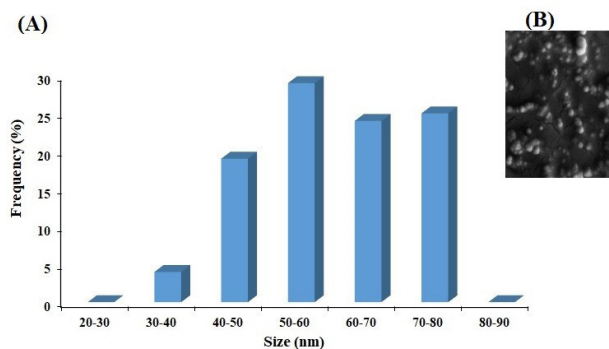


Figure 2. Particle size distribution (A) and scanning electron microscope (SEM) (B) of zinc nanoparticles using extraction of lavender extract based on microwave method.

or systemic factors. So, the elimination or reduction of candidates predisposing to *Candida* infection should be considered before initiating antifungal drug treatment (4).

Important antifungal drugs for the treatment of oral candidiasis are polyene and azoles that have topical and systemic forms. Topical medications have fewer side

Table 1. Antifungal effects of microwave-synthesized zinc nanoparticles (ZnNPs) in comparison with nystatin on *C. albicans* by determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) assay

Drug	MIC ($\mu\text{g/mL}$)	MFC ($\mu\text{g/mL}$)
ZnNPs	8.6 \pm 1.15	10.0 \pm 0.0
Nystatin	1.33 \pm 0.57	1.66 \pm 0.57
ZnNPs + nystatin	0.204 \pm 0.079*	0.250 \pm 0.0*

* $P < 0.001$ difference was statistically significant compared with nystatin group.

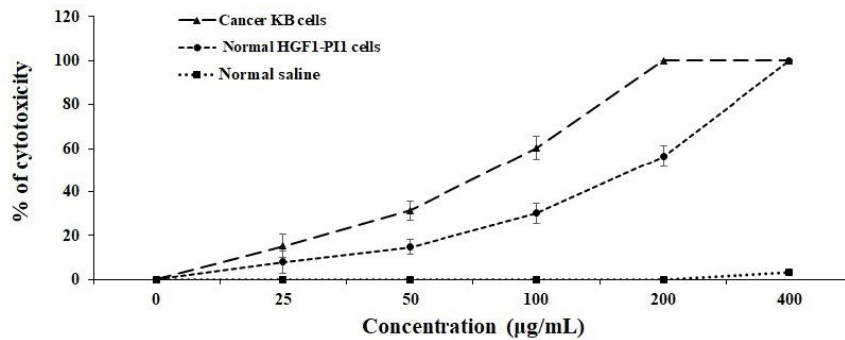


Figure 3. The cytotoxicity activity of zinc nanoparticles (ZnNPs) on human normal fibroblast-like Gingiva (HGF1-P11, Pasture Institute, Iran) and human epithelial-like oral cancer (KB) (Mean±SD; n=3).

effects and drug interactions than systemic forms. In addition, many topical medications are able to treat acute oral infections (7). When choosing a topical antifungal drug, factors such as taste, ease of use, sensitivity, or resistance to the drug and its price should be considered. Oral suspensions are also a better choice in patients with dry mouth. On the other hand, the existence of numerous mouthwashes due to less side effects and easiness of custom has caused many patients to be satisfied with them and has brought them more cooperation in this regard (7). The most famous polyene drug is nystatin, which is a dry powder and relatively insoluble and unstable in water; its suspension is obtained by dispersing the powder in hot water; in addition to being bitter in taste, it does not have much power to kill the infection and is not available for clinical use, because the patient must prepare it for use (31). Another important issue is that although antifungal antibiotics improve and eliminate infections but their long-term use causes the emergence of refractory fungi and the patient's weakness and disability (4).

Pharmaceutical nanotechnology is a global strategic approach that directly refers to the design and development of nanostructures with unique diagnostic and therapeutic properties (32). In addition, nano-knowledge-based drug delivery systems accelerate the motility of intracellular drugs, reduce the number of drug-resistant microorganisms, accelerate the possibility of aggregation of target organs through superficial functional changes, limitation of systemic adverse effects, and safety suppression (12).

In addition to the unique physical and chemical properties of nanomaterials, these materials act to detect sensitive and selective fungal markers and may have intrinsic antimicrobial properties (33). Today, nanoparticles, due to their various applications in various fields of medicine, industry, and health, are among the factors that are necessary for their antifungal properties to be further studied and researched because metal ions such as silver, copper, and zinc have long had significant functions in health and in the treatment of various diseases (34). On the other hand, the effect of ZnNPs has

been known from the past to the present, so the ability to deliver zinc through the structure of nanoparticles has dramatically increased its biological and antimicrobial value, because ZnNPs have a higher contact surface than zinc particles in the form of mass (35).

In order to obtain an effective substance in the treatment of oral candidiasis, the antifungal effect of ZnNPs synthesized with lavender on *C. albicans* was investigated. According to the findings, the lowest MIC and MFC belonged to ZnNPs + nystatin, and the highest amount belonged to ZnNPs. The combination of ZnNPs+ nystatin in comparison with nystatin alone showed a significant anti-*Candida* effect and caused growth inhibition and ultimately fungal mortality. Therefore, the combination of ZnNPs + nystatin, in comparison with ZnNPs and nystatin alone, exerts its inhibitory and lethal effect on *C. albicans* at lower concentrations. This means that ZnNPs have a synergistic effect on the antifungal properties of nystatin and combination therapy is a better option.

Similar studies have been performed to evaluate the anti-*Candida* effects of ZnNPs and other zinc nanoforms such as zinc nanoxide. In a study, the results of microdilution broth and agar well diffusion tests showed that chemically synthesized ZnONPs with MIC and MFC values of 64 and 128 µg/mL, respectively, had significant anti-candida effects against *C. albicans* yeast (36). In the study of Sharma et al, the diameters of *C. albicans* growth inhibition zones exposed to zinc oxide nanoparticles synthesized at concentrations of 5, 10, 15, and 20 mg/mL by disk diffusion method were 1.5, 5.3, 8.6, and 11.14 mm, respectively, which indicate the anti-candidal effects of these nanoparticles (37). In another study, MFC of ZnONPs was obtained at 0.1 mg/mL; this concentration inhibited the growth of *C. albicans* by more than 95%. ZnNPs also inhibited the growth of *C. albicans* when added in the logarithmic phase of growth. In addition, stimulation of zinc oxide by visible light increased yeast cell death (38). This study, consistent with the result of our study, emphasizes that ZnNPs and ZnONPs may provide a new family of fungicidal compounds.

In general, the results of previous studies have shown

that although zinc and zinc oxide inherently have positive effects on killing germs, but using them on a larger scale, such as micro and powder, will prevent them from working. A previous research has shown that the antibacterial activity of ZnO and its ability to induce reactive oxygen species (ROS) generation increase with decreasing particle size. Hence, it can be concluded that ZnNPs, compared to the mass form, have greater antifungal effects (39). Disruption of cell permeability, inhibition of cell growth, and induction of apoptosis as direct mechanisms and production of oxidative stress through the production and release of zinc ions in the environment that cause their penetration through the cell wall are suggested as possible mechanisms actions of this nanoparticle (40).

Concerning the cytotoxic activity of green synthesized ZnNPs by the MTT method, we found that the CC_{50} of ZnNPs against normal (HGF1-PI1) and cancer (KB) moth cells were 172.3 and 83.2 $\mu\text{g/mL}$, respectively. A previous study showed the cytotoxic effects of green synthesized ZnNPs on human lung (A549), breast (MCF7), colorectal (HT-29), and colon (Caco-2) cancer cell lines with CC_{50} values of 22.3, 86, 10.9, and 56.2 $\mu\text{g/mL}$ (41). These findings indicated the cytotoxic effects of green synthesized ZnNPs on cancerous cells, whereas they were nontoxic for normal cells.

Conclusion

ZnNPs, particularly with nystatin, displayed an antifungal effect against *C. albicans*. These findings indicated the cytotoxic effects of green synthesized ZnNPs on cancerous cells, whereas they were nontoxic for normal cells. However, more surveys are necessary to explain the exact mechanism and toxicity.

Authors' contributions

AS supervised the study, SN, SAHM, HM, and MS reviewed and contributed to data collection and preparation of the manuscript. The first draft was prepared by HM and AS. All authors read the final version and confirmed it for publication.

Conflict of interest

The authors declare no conflict of interest.

Ethical considerations

Ethical issues (including plagiarism, data fabrication, double publication etc.) have been completely observed by the authors. This project was approved by the ethics committee of Lorestan University of Medical Sciences with the ethics ID IR.LUMS.REC.1400.328.

Funding/Support

None.

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