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Design and Synthesis of Novel Thymoquinone-Zein Nanoparticles; Evaluation of the Inhibitory Effect on *Candida albicans* and Biofilm Formation *in Vitro*

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ABSTRACT

Backgrounds: *Candida albicans* (*C. albicans*) as a fungal pathogen is part of the normal flora of the human body, which could cause various infections in patients with defective immune systems. Nowadays, there is a need to design and synthesis new drug formulations to overcome drug resistance in this genus. Thymoquinone (TQ) is the main ingredient in *Nigella sativa*, which has considerable antifungal properties. The aim of this study was to investigate the inhibitory effects of thymoquinone-zein nanoparticles (TQ-ZNPs) on *C. albicans*.

Materials & Methods: In the current study, TQ was encapsulated in zein (as a biodegradable carrier) and polyethylene glycol (PEG). The antifungal activity of TQ-ZNPs against *C. albicans* (ATCC 10231; standard strain) and their inhibitory effects on biofilm formation were examined using standardized broth microdilution and MTT assays, respectively. The total oxidant status (TOS) of *C. albicans* was assessed using colorimetric method, and the toxic effect of nanoparticles on peripheral blood mononuclear cells (PBMCs) was evaluated by MTT assay.

Findings: The minimum inhibitory concentration (MIC) of TQ-ZNPs was significantly reduced compared to that of free TQ. MIC values of TQ-ZNPs and free TQ were determined to be 7.4 and 50 µg/mL, respectively. Biofilm formation was inhibited, and oxidant production by fungal cells was increased. The findings of this study showed that TQ-ZNPs had no toxic effect on PBMCs.

Conclusion: This study results revealed that the synthesized nanoparticles had a good antifungal activity without any toxicity. The results demonstrated the superior efficiency of TQ-ZNPs over free TQ. Hence, this structure could be used to load hydrophobic drugs. However, more studies are needed to evaluate the beneficial properties of TQ-ZNPs.

Keywords: *Candida albicans*, Antifungal agent, Nanoparticles, Thymoquinone, Zein.

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Introduction

In the last decade, *Candida* infections have considerably increased among high-risk patients. Excessive use of antifungal agents causes drug resistance in pathogenic species. *C. albicans* is the most prevalent fungal pathogen that causes numerous infections. This pathogen has a high adaptability which enables it to transit from commensal to pathogen due to many virulence factors, especially biofilm formation. Biofilms formed by *C. albicans* are intrinsically resistant to antifungal drugs and have little susceptibility to common treatments. This highlights the importance of conducting research to prevent and control the formation of these biofilm structures [1]. Researchers are currently trying to develop low-cost, less toxic, and highly effective antifungal agents. New antifungal drugs are produced at a much slower rate than antibacterial drugs. Nowadays, due to the rising number of hosts susceptible to fungal infections and the emergence of drug resistance, the tendency to use herbal medicines (with low side effects) has increased [2]. These herbs have been used for many years as alternative treatments for fungal infections. *Nigella sativa* is one of these herbs. Studies have shown that thymoquinone (TQ) as the main ingredient of *N. sativa* could be used to treat fibrosis [3], nervous [4] and cardiovascular diseases [5], liver and kidney disorders [6, 7], hyperlipidemia [8], and diabetes [9]. TQ reacts with the amino group of amino acids and is metabolized to semiquinone or thymohydro-quinone under a series of reduction-oxidation reactions. This leads to the production of reactive oxygen species (ROS) [10]. Increased ROS levels lead to cell death by affecting cytochrome c and activating caspases [11]. TQ is severely degraded upon exposure to light and is unstable in aquatic environments due to its hydrophobic groups. Hence, it is important to encapsulate it in a carrier. Zein is a biodegradable carrier, which is exclusively found in corn. Zein is a natural hydrophobic protein approved by the FDA to carry medications that require oral administration [12, 13].

Objectives: This study aimed to synthesize novel antifungal nanoparticles (i.e. TQ-ZNPs) pegylated with PEG (polyethylene glycol as a surfactant) to increase the stability of TQ. The antifungal activity of TQ-ZNPs against *C. albicans* (ATCC 10231) was assessed. In addition, the inhibitory activity of novel NPs against biofilm formation as well as TOS levels (i.e. ROS and nitric oxide (NO)) induced in *C. albicans* were evaluated.

Materials and Methods

Materials: Zein protein, thymoquinone, fluconazole powder, RPMI 1640, and DMEM were purchased from Sigma-Aldrich (USA). Polyethylene glycol (PEG: Mw400) and ethanol were obtained from Golriz Company (Iran). Standard strain of *C. albicans* (ATCC 10321) was purchased from Manassas (VA, USA). Sabouraud dextrose agar (SDA) was provided by Merck (Germany), and the kit for determining the total oxidant status was bought from Kiazist Company (Iran).

Synthesis of TQ-ZNPs: TQ encapsulation was performed based on previous studies with a minor modification [14]. For this purpose, 60 mg of zein was added to 5 mL of 70% ethanol. After stirring for 2 min at 150 rpm,4 mg of TQ powder was added to the above solution, and then 4 mL of PEG was added and stirred for 2 min. This solution was immediately added to sterilized distilled water (50°C). The pH of the distilled water was adjusted by 1 M potassium hydroxide and kept constant in the range of 9.5-10 during the synthesis process. After stirring for 3 hours, the solution was first centrifuged at slow speed (2500×g) for 5-10 min to remove large particles, and then the supernatant was centrifuged at high speed (12000×g) for 15-20 min. Subsequently,

the supernatant was washed with distilled water three times and eventually placed in a freezer (-20°C).

Antifungal susceptibility testing of TQ-ZNPs: Antifungal susceptibility testing was performed on TQ-ZNPs according to CLSI M27 A3/S4 protocol. For this purpose, 2.5×10^3 cells of the standard strain of *C. albicans* (ATCC 10231) were counted with a slide hemocytometer and prepared in RPMI 1640 culture medium. Then 100 µL of this suspension was seeded into a 96-well microplate [15]. Two-fold serial diluted concentrations of TQ-ZNPs (0.12–118 µg/mL), free TQ (0.78–400 µg/mL), and bare ZNPs (155.52–0.3037 mg/mL) were prepared in RPMI 1640. Then 100 µL of each of them was added to the wells. Fluconazole at different concentrations (0.5-128 µg/mL) was used as the standard drug. The microplate was incubated at 37°C for 24 hours, and the lowest concentration which inhibited the growth of fungal cells was defined as the MIC. For MFC determination, 10 µL of the contents of the wells that showed no growth after 48 hours were subcultured on SDA medium plates. Fungal cell suspension without treatment was used as a positive control, and the culture medium was used as a negative control.

Biofilm susceptibility assay: Biofilm formation was evaluated using MTT assay [16]. A suspension of 1×10^6 *C. albicans* cells was prepared. About 100 µL of this cell suspension was seeded into each well of a 96-well microplate, and then 100 µL of yeast nitrogen base (YNB) culture medium was added to the wells and incubated at 37°C for 2 hours. Different concentrations of TQ-ZNPs (0.12–118 µg/mL), free TQ (1.56–800 µg/mL), bare ZNPs (311.04–0.6075 mg/mL), and fluconazole (0.5–128 µg/mL) were prepared in YNB medium and poured into the wells. After 24-48 hours, 120 µL of each well was gently removed and washed via phosphate-buffered saline (PBS) to remove the free or planktonic cells from the wells. Afterwards 20 µL of tetrazolium salt was poured into the wells and incubated at 25°C for 4 hours in dark conditions. Then dimethyl sulfoxide (DMSO) was added to the wells and after 15-20 min, the wells were read by ELISA reader (STAT FAX 2100) at 570-600 nm. These experiments were repeated three times, and the absorption values were determined as the average of the values obtained for each sample. The controls included positive control (culture medium + fungal suspension), negative control (culture medium), and blank (tetrazolium salt + DMSO).

Determination of total oxidant status (TOS): Utilizing a TOS kit (Kiazist Company, Iran) and the colorimetric assay, the TOS levels were determined [17]. First, a cell suspension of *C. albicans* (5×10^4) was prepared. Then it was treated with 0.5×MIC, 1×MIC, and 2×MIC of TQ-ZNPs (3.7, 7.4, and 14.8 µg/mL), TQ (25, 50, and 100 µg/mL), and fluconazole (0.5, 1, and 2 µg/mL), respectively. Next, 100 µL of the treated cell suspensions were added to 200 µL of TOS reagent in a microplate. The microplate was incubated at 25°C for 10 min. Then the absorbance of the microplate was read at 560 nm. The reaction mechanism was based on the production of chromogens due to the oxidation of Fe^{+2} to Fe^{+3} via cellular oxidants. As a result, the higher the absorbance, the higher the production of oxidants. The test was calibrated with different concentrations of hydrogen peroxide.

Cytotoxicity assay on PBMCs: The mitochondrial function of PBMCs (peripheral blood mononuclear cells) was investigated using tetrazolium salt reduction using MTT method [18]. To prepare MTT dye, 50 mg of MTT powder was added to 10 mL of PBS, and the solution was sterilized using a 0.22 µm filter. At first, 1×10^6 cells were counted in the DMEM medium containing 10% FBS (fetal bovine serum) and 1% pen/strep. Then 100 µL of the suspension was seeded in a 96-well microplate and incubated at 37°C for 24 hours with 5% CO_2. Then by adding 100 µL of

fluconazole (2-64 µg/mL), TQ-ZNPs (2-10 µg/mL), bare-ZNP (4.86-311.04 mg/mL), and free TQ (2-10 µg/mL) to each well, the plate was incubated for 24 hours. Afterward 20 µL of MTT dye was poured into the wells, and the microplate was placed in dark conditions for 4 hours. Finally, DMSO was added to the wells and after 15 min, the absorbance was recorded via ELISA reader (STAT FAX 2100) at 590-600 nm. Cell suspension without exposure to the aforementioned agents was considered as a positive control, and culture medium was used as a negative control. The blank well contained tetrazolium salt and DMSO. All experiments were performed in triplicate. The results were obtained using the following equation:

Cell viability (%) = Absorbance of the test / Absorbance of the control × 100

Statistical analysis: All experiments were performed in triplicate. The results were obtained as the average of the measurements. Statistical analysis was done using GraphPad Prism software Version 9.0.1 (151) by employing one-way ANOVA, and a *p*-value of < .05 was assumed statistically significant.

Findings

Antifungal susceptibility testing: The MIC values of TQ-ZNPs, bare ZNPs, free TQ, and fluconazole were 7.40,9720 , 50, and 1 µg/mL, respectively. Moreover, their MFC values were 14.75, >77760, 150, and 2 µg/mL, respectively. Bare ZNPs showed no significant inhibitory effect on fungal cell growth. MIC and MFC values of TQ-ZNPs were significantly less than those of free TQ ($p< .05$) (Table 1).

Biofilm formation: Table 2 shows the susceptibility of the biofilms formed by *C. albicans* to different concentrations of TQ-ZNPs, bare ZNPs, free TQ, and fluconazole. The obtained values showed that TQ-ZNPs inhibited *Candida* biofilm formation more strongly than free TQ ($p< .05$). Biofilms formed by *C. albicans* showed susceptibility

Table 1) MIC (minimum inhibitory concentration) and MFC (minimum fungicidal concentration) values of fluconazole, free TQ, TQ-ZNPs, and bare-ZNPs against *Candida albicans* (ATCC 10231)

	MIC (µg/mL)	MFC (µg/mL)
FLZ	1	2
Free TQ	50	150
TQ-ZNPs	7.40	14.75
Bare-ZNPs	9720	>77760

Abbreviations: MIC: minimum inhibitory concentration, MFC: minimum fungicidal concentration, TQ: thymoquinone, ZNPs: zein nanoparticles, TQ-ZNPs: thymoquinone-zein nanoparticles, FLZ: fluconazole

Table 2) sMIC (sessile minimum inhibitory concentration) of fluconazole, free TQ, TQ-ZNPs, and bare-ZNPs against *Candida albicans* (ATCC 10231)

sMIC (Sessile Minimum Inhibitory Concentration)				
C. albicans	FlZ (µg/mL)	Free TQ (µg/mL)	TQ-ZNPs (µg/mL)	Bare-ZNPs (µg/mL)
	4	100	29.5	> 77760

Abbreviations: TQ: thymoquinone, ZNPs: zein nanoparticles, TQ-ZNPs: thymoquinone-zein nanoparticles, FLZ: fluconazole

to 4 and 29.5 µg/mL of fluconazole and TQ-ZNPs, respectively. Thus, there was no significant difference between fluconazole and TQ-ZNPs in inhibiting biofilm formation.

Total oxidant status (TOS): TOS levels induced in *C. albicans* upon exposure to fluconazole, free TQ, and TQ-ZNPs are presented in Figure 2. These levels were 2.35, 1.9, and 1.56 µM/mg in *C. albicans* treated with TQ-ZNPs, free TQ, and fluconazole, respectively. TQ-ZNPs induced more production of ROS and NO than fluconazole ($p< .05$).

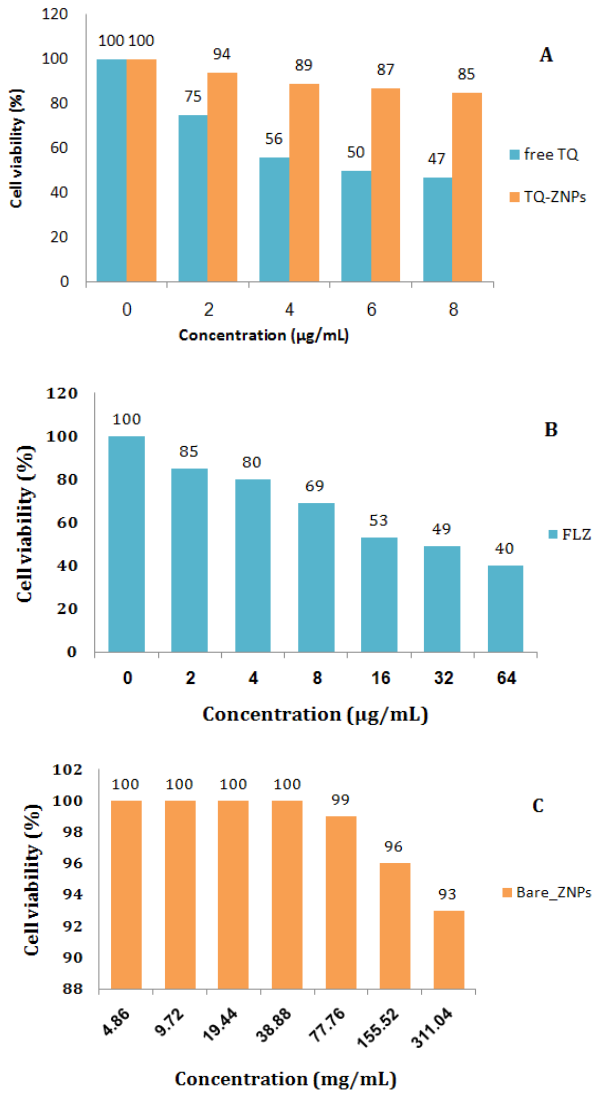

Figure 1) Assessment of the cytotoxic effects of A) TQ-ZNPs and free TQ, B) fluconazole, and C) bare ZNPs on PBMCs

Abbreviations: ZNPs: zein nanoparticles, TQ: thymoquinone, TQ-ZNPs: thymoquinone-zein nanoparticles, PBMCs: peripheral blood mononuclear cells

Cytotoxicity of TQ-ZNPs: The viability percentages of PBMCs exposed to TQ-ZNPs, bare ZNPs, free TQ, and fluconazole during 24 hours of incubation are presented in Figure 1. The viability percentages of cells treated with TQ-ZNPs (10 µg/mL), fluconazole (64 µg/mL), and free TQ (10 µg/mL) were 82, 40, and 41%, respectively. TQ-ZNPs showed no toxic effect. However, they had a dose-dependent effect on the viability of PBMCs

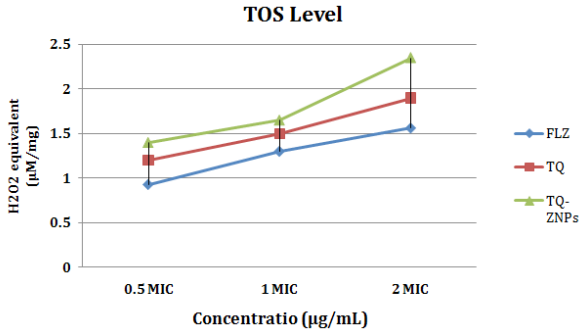

Figure 2) TOS levels in *C. albicans* treated with different concentrations of TQ-ZNPs, free TQ, and fluconazole (0.5 MIC, 1 MIC, and 2 MIC)

Abbreviations: TQ: thymoquinone, TQ-ZNPs: thymoquinone-zein nanoparticles, FLZ: fluconazole, TOS: total oxidative status

Discussion

Nowadays, the emergence of drug-resistant species has led to the development of new drugs to solve this problematic issue. The prevalence of opportunistic fungal infections, such as those caused by *Candida* species, is rising. Candidemia is considered as the most common form of invasive disease caused by *Candida* and as the fourth and sixth most common nosocomial infection in the United States and Europe, respectively [19, 20]. Furthermore, the mortality rate in patients with candidiasis is greatly increased due to the production of biofilm [21]. The biofilm formed by *C. albicans* is 4,000 times more resistant to fluconazole treatment than planktonic or free cells, which could lead to therapeutic failures when using common

antifungal drugs [16, 22, 23].

In recent years, alternative therapies such as herbal medicines have received more attention than ever before. The fungistatic activity of *N. sativa* against the *Candida* genus has been proved in some studies with MIC values of 10 [24] and 6.1-48.8 mg/mL [25]. However, previous studies have proven that the antifungal activity of TQ (the main component of *N. sativa*) is almost 1,000 times greater than that of *N. sativa*. In a study by Almshawit and Macreadie (2017), the MIC value of TQ against *C. albicans* was 50 µg/mL [26], which is compatible with the present study results. Nevertheless, in another study by Randhawa et al. (2015), the MIC value of nano-sized TQ was 160 µg/mL [27]. The major problem with using free TQ is that it is sensitive to light and insoluble in aquatic environments. Therefore, in the current research, a biodegradable protein (zein) was used to enhance the stability of free TQ and prevent its destruction. In addition, PEG was employed to increase its dispersion and solubility in aquatic media. To prove the compatibility of TQ-ZNPs with normal cells, their cytotoxicity was evaluated. TQ-ZNPs had significantly less toxic effects on PBMCs than free TQ. Bhattacharjee et al. (2020) reported that the viability (%) of PBMCs was 42% when the free TQ concentration was 9 µg/mL, which is compatible with this study results (41%) [28]. In another investigation, by adding PEG to TQ, cell viability increased to 80% [29]. In the present study, by combining zein, TQ, and PEG, the viability of cells increased to 89%. The cytotoxicity findings demonstrated that TQ-ZNPs were more biocompatible than free TQ, and that toxicity was greatly reduced when zein and PEG were used.

TQ as a fungicidal agent induces the production of oxidative stress which causes damage to biological molecules. Examination of the cell wall of *C. albicans* exposed to 30 µg/mL of TQ showed that the cytoplasmic membrane was irregular and detached from the cell wall due to the production of ROS and NO, finally leading to the disintegration of the cytoplasm and the nucleus [30]. Excessive production of oxidants leads to the death of fungal cells.

The more the biofilm produced by *Candida* genus, the more the resistance to common antifungal drugs such as amphotericin B and fluconazole. During biofilm formation, the number of drug efflux pumps increases, and the membrane sterol composition alters. These changes increase the resistance of biofilms. In a study, the effects of different concentrations of TQ on the biofilm formation of *C. glabrata* were investigated. The TQ concentration required to inhibit biofilm formation of *C. glabrata* was 50 times higher than that required for planktonic cells [26]. Free TQ may not be successful in inhibiting biofilm or treating *Candida* systemic infections because its activity is reduced by binding to serum proteins [31]. Therefore, encapsulation of TQ not only solves this problem but also improves the efficiency of TQ due to its gradual release from the zein structure. As a result, this structure could be used as a model for in vivo studies.

In this research, the cytotoxic effect of free TQ in TQ-ZNPs structure on PBMCs was greatly reduced due to the use of zein and PEG. TQ-ZNPs and free TQ had a fungicidal effect, and bare ZNPs had a weak fungistatic effect. Based on this study findings, the efficiency of TQ-ZNPs in inducing the production of oxygen and nitrogen mediators was higher than that of free TQ. It seems that proper interaction between PEG and *C. albicans* cell wall facilitates the entry of TQ into fungal cells and consequently induces the production of ROS and NO.

Conclusion

In conclusion, TQ-ZNPs exhibited favorable antifungal effects on *C. albicans* (standard

strain) in all assays performed and could be considered as a suitable candidate for developing new antifungal medications. This study results demonstrated that the cytotoxicity of free TQ was greatly reduced using zein and PEG. Since TQ-ZNPs were more biocompatible, safer, and more effective than free TQ, they could be used as an appropriate alternative to azole drugs which have many side effects. However, further in vitro and in vivo studies are needed to evaluate their efficacy.

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Ethical Permission: Since this research was conducted on microorganisms, there was no need for ethical approval.

Authors' Contributions: Conceptualization: SR, ShRM, HDH, MR, and ZMH; data curation and formal analysis: SR, ShRM, HDH, MR, and ZMH; investigation: SR and HDH; methodology and project administration: ShRM and HDH; supervision: Sh RM; validation: SR, ShRM, H DH, MR, and ZMH; writing the original draft: S R; writing, reviewing, and editing: SR, ShRM, HDH, MR, and ZMH.

Conflict of Interests: The authors declare no conflict of interest.

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