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EXPERIMENTAL STUDY

Preparation and characterization of ZnO nanoparticles coated by chitosan-linoleic acid; fungal growth and biofilm assay

Barad S[1], Roudbary M[2], Nasrollahi Omran A[1], Porgham Daryasari M[3]

Department of Medical Mycology and Parasitology, School of Medicine, Iran University of Medical Science, Tehran, Iran. **roudbari.mr@iums.ac.ir**

ABSTRACT

INTRODUCTION: This study has been carried out on reviewing the use of new synthetic component of zinc oxide nanoparticles (ZnO NPs) coated by Chitosan-linoleic acid (CS-LA) and to assess Minimum inhibitory concentration (MIC) of nanoparticles on clinical samples and biofilm formation in vitro.

MATERIALS AND METHODS: At first the synthesized ZnO NPs coated by CS-LA were identified with X-ray powder diffraction (XRD), Scanning electron microscope (SEM), Transmission electron microscope (TEM) and Fourier Transform Infrared Spectroscopy analysis (FTIR).Through in vitro tests, the value of MIC and Minimum fungicide concentration (MFC) of nanoparticles and standard and clinical strains of *candida* were evaluated in comparison with fluconazole as the control group using the CLSI-M27 method. Finally, biofilm formation was studied using MTT assay.

RESULT: The results showed that MIC50 of fluconazole and nanoparticle in clinical strains was 64 µg/ml and 128 µg/ml, respectively. The MIC of fluconazole and nanoparticle in *C. albicans* (ATCC10231) was 8 µg/ml and 32 µg/ml respectively. The MFC of nanoparticle and fluconazole for clinical samples was recorded at similar level (128 µg/ml). MTT results indicated that the capacity of inhibition of biofilm formation was 43.07 % and 36.68 % by ZnO NPs and fluconazole, respectively.

CONCLUSION: It is concluded that the new synthesized nanoparticle has appropriate efficacy compared with fluconazole in inhibitory activity on *C. albicans* growth and biofilm formation. As a result, ZnO NPs can be introduced as an effective agent for diminishing adhesion capacity of *C. albicans (Tab. 1, Fig. 4, Ref. 26)*. Text in PDF *www.elis.sk.*

KEY WORDS: zinc oxide, chitosan, linoleic acid, *Candida* biofilm, antifungal.

Introduction

Candida species is a potential pathogen due to various unique characteristics such as the ability to change morphology i.e. changing from yeast to hyphal, hydrolytic enzymes and forming biofilm which may caused many opportunistic fungal disease. Different factors including immunological deficiency, use of extended spectrum of drugs and corticosteroids may intensify the disease. On the other hand, regular administration of antifungal drugs e.g. fluconazole causes pharmaceutical resistance due to creating genetic mutant strains and biofilm resistant structures (1).

Biomedicine is one of the areas that experienced a drastic advance through the use of nano scale material. Among these, Drug delivery nanosystems have been the center of attention during the past few years. The objective behind developing these systems which mostly include drugs, carrier, targeting ligands and surface modifications is drug controlled delivery, maintaining drug concentrationin therapeutic range within a proper period of time and drug specific transfer to the targeted tissue. Liposomes, micelles, nanoparticles, antibody conjugates and polymer conjugates, are samples of nano medicine systems (2).

Nowadays, various nanoparticles are produced with antifungal properties although several studies have proven the antifungal property of ZnO nanoparticles (3); ZnO, one of the most common ones, is of physical and chemical property with high energy bond (60 MV). These nanoparticles are produced with different structures by different methods as hydrothermal dissolution which at suitable size can inhibit fungal growth (4–6).

Chitosan (CS) is of a broad application in nano medicine and pharmacology due to non-toxicity and incomparable physiochemical and biological characteristics. It has been shown that Chitosan positive charges react with cellular DNA of some bacteria (6) and have antimicrobial activity. Additionally, Linoleic acid (LA) with Trans-11 and Cis-9 arrangement is one of the effective acids on fungal growth (7).

Researchers showed the property and resistance of nano particles increase when they bind with these fatty acids (8). It was found in some studies that these fatty acids are effective on pathogenic fungi in plants and human. However, some materials like

[1]Department of Medical Mycology, Faculty of Medicine, Tonekabone Branch, Islamic Azad University, Tonekabon, Iran, [2]Department of Medical Mycology and Parasitology, School of Medicine, Iran University of Medical Science, Tehran, Iran, and [3]Department of Chemistry, Kerman Branch, Islamic Azad University, Kerman, Iran

Address for correspondence: M. Roudbary, Department of Medical Mycology and Parasitology, School of Medicine, Iran University of Medical Science, Post Code: 14496-14530, Tehran, Iran.

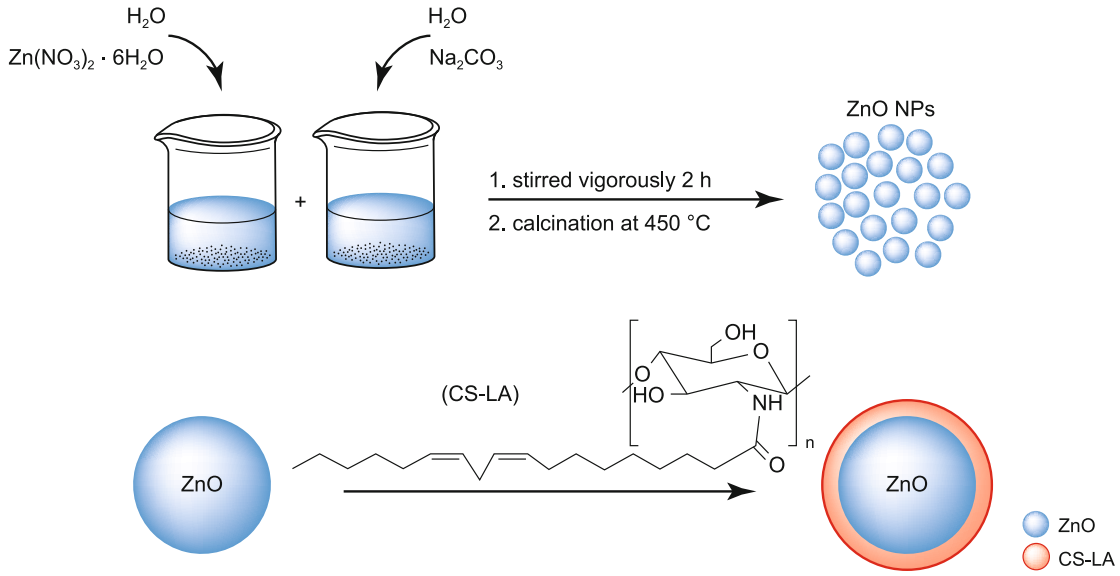

Fig. 1. Preparation and schematic overview of ZnO NPs coating procedure.

Timol, Linanol and Cymene with antifungal and antibacterial effects have been discovered previously (9).

Collectively, although several data established the antifungal property of linoleic acid, chitosan and ZnO nanoparticle distinctly, but the evidence demonstrating these materials as unique Nano compounds is not understood completely, on the other hand developing the novel antifungal agents is an urgent demand. For this, ZnO NPs were synthesized and coated by Linoleic acid conjugated with Chitosan to enhance their antifungal properties. Finally, its capacity was evaluated through inhibiting *candida* growth and biofilm formation in vitro conditions.

Material and methods

Culture of fungal samples

In this study, *C. albicans* standard strain (ATCC10231) and the fluconazole resistant clinical strains of *C. albicans* from vulvovaginal Candidiasis were used. Isolates were cultured on Sabouraud Dextrose Agar (SDA, Sigma) then incubated at 37 °C for 48 hours for appropriate growth of *Candida*. After this time and appearance of the colony they were used to prepare a suspension.

Preparation of Candida yeast cell suspension

Some *Candida* yeast cells were collected by sterile loop from surface of SDA medium and diluted with 1 ml of sterile Phosphate buffer saline (PBS). The 0.5 MC Farland was prepared according to references (10). The yeast concentration in this stiffness is about $1x10^6$–$5x10^6$ CFU/mL (11).

Preparation of fluconazole dilution

10 ml of Dimethyl solphoxide (DMSO) was added to 0.0128 mg of fluconazole (sigma) to prepare a solution of 1280 µg/ml concentration. The solution was then sterilized with filtration. Finally a serial concentration of 128–0.25 µg/ml was prepared.

Preparation of ZnO nanoparticles

ZnO nanoparticles were prepared according to the following method (6, 12): Concisely, 12.71 g Na_2CO_3 was dissolved in 240 mL deionized water. Consequently, 29.75 g Zn $(NO_3)_2.6H_2O$ was dissolved in 200 ml deionized water and added to the Na_2CO_3 solution drop wisely and the mixture were stirred vigorously for 2 h. Finally, the white precipitates were washed with distilled water and ethanol for several times and dried 6 h under vacuum at 100 °C lastly, white precipitates were calcinated at 450 °C for 3 h to obtain ZnO nanoparticles.

Conjugation of LA to CS

In order to conjugate LA to CS the following method was used (13). Briefly, a solution of 200 mg CS in 20 ml deionized water was prepared. Consequently, LA was dissolved in 10 ml of ethanol and added to CS solution. The mixture was stirred at 80 °C. The EDC solution in deionized water was added into the mixture (EDC and LA molar ratio was 100:1, respectively) and stirred for 12 h at 80 °C. Finally, the mixture was dialyzed first against 50 % ethanol solution for 24 h and then against deionized water for 48 h. Finally, the LA conjugated CS was lyophilized and kept for follow up study.

Preparation of ZnO nanoparticles coated by CS-LA

A solution of 25 mg CS-LA in 5 mL 3 % acetic acid was prepared and the suspension was stirred for 24 h. Consequently, a solution of 10 mg of ZnO nano particles in 5 ml ethanol (96 %) was ultra-sonicated for 10 min and the solution pH adjusted to 3.5–4.5 by acetic acid. Subsequently, 5 ml of CS-LA solution added into the ZnO nano particles solution and mixture was stirred at room temperature for 24 h. Finally, the CS-LA coated ZnO nano par-

Tab. 1. The value of MIC and MFC of ZnO NPs and fluconazole against *C. albicans.*

Substance	MTT mean of OD	Percent inhibition of biofilm	MIC50*		MIC range*	MFC	
			ATCC	Clinical isolates		ATCC	Clinical isolates
Fluconazole	3.15	36.68 %	8	64	0.25_128	16	128
ZnO NPs	2.48	43.07 %	32	128	0.25_128	64	128

*µg/mL

ticles were collected by centrifugation at 10,000 rpm and washed with excessive distilled water and ethanol before freeze-drying 1(4). Figure 1 shows the sequence of steps for the preparation of CS-LA coated ZnO NPs.

Determination of MIC of ZnO NPs and fluconazole

Micro dilution broth method was used to determine MIC. For this, 96 sterile flat-bottom-well microplates (Nunc) were used with 100 µl of RPMI1640 medium (gibco) supplemented with (3-Nmorpholino propane sulfonic acid) MOPS buffer. Then, serial dilutions (0.25–128) µg/ml were prepared out of ZnO NP sand poured in 96-flat-well microplates, with 100 µl in each well. Next 100 µl of standardized suspension was added to each well to reach the final volume to 300 µl. The lowest concentration of nanoparticles that inhibited the *Candida* growth is considered as its MIC (10). Using the same procedure and similar dilution, fluconazole MIC was determined according to CLSI M27–M3 (15). To compare the effect of Nanoparticle with Fluconazole two wells were used in microplate as positive and negative controls. There was 100 µl of media plus 100 µl of *Candi*da suspension as positive control and 100 µl of media plus 100 µl of ZnO NPs as negative control.

To determine the MFC, 50 µl from each clear well with no fungus growth in micro plates was subcultured on SDA plates. All plates were incubated at 37 °C for 48 h. The least concentration showing no visible colony on sub culturing was taken as MFC (10).

Biofilm formation and MTT Assay

The biofilm formation ability of *Candida* species was assessed by MTT (5 mg/ml in PBS, Sigma) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test. For this, clinical isolates of *C. albicans* treated MIC concentration of nanoparticles and then, $1x10^6$ yeast cells were inoculated into 96 sterile flat-bottom-well microplates. Then it was incubated at 37 °C for 48 h. Free-living cells formed on the plate surface were washed and eliminated with sterile buffer (PBS PH: 7.4,). After this time, Biofilm formation was evaluated by adding YNB (Yeast Nitrogen Base) medium with 50 mM glucose. The plates were incubated at 37 °C for 48 h, and the biofilm was made by *candida*. Afterwards, the YNB medium was detached and washed with 4 ml of PBS.

Then under sterile conditions, MTT test with Tetrazolium salt was carried out on cells bound to the bottom of the Microplate. After adding salt and a 4-hour incubation, DMSO was added and optical density (OD) was determined by Elisa reader (Memmert, Germany) at 540 nanometer. The same procedure was conducted for isolates which were treated by fluconazole. There is a direct correlation between amount of OD and living adherence cells (16).

MIC, MFC and MTT tests were performed in triplicate with similar effects for the nanoparticle.

Statistical analysis

Statistical analysis of data was conducted by IBM SPSS Statistics version 23. To this end, statistical test of Wilcoxon Signed Ranks test was used. In all statistical tests significant level was considered $p < 0.05$.

Results

Zinc oxide nanoparticles were prepared by precipitation method. Here we synthesized a new zinc oxide nanoparticles targeted for antifungal activity against *C. albicans* using microdilution test and was characterized by FT-IR, SEM,TEM and XRD.

MIC50 of ZnO NPs was 32 µg/ml and 128 µg/ml for standard and clinical isolates of *Candida*. The MIC of fluconazole was 8

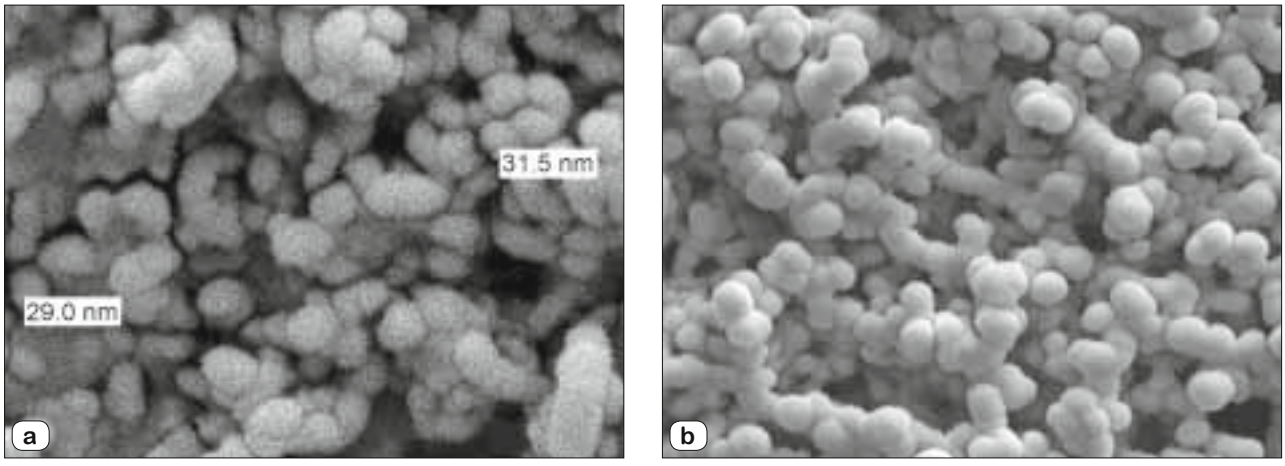

Fig. 2. FE-SEM images of ZnO nanoprticles (a) and (b).

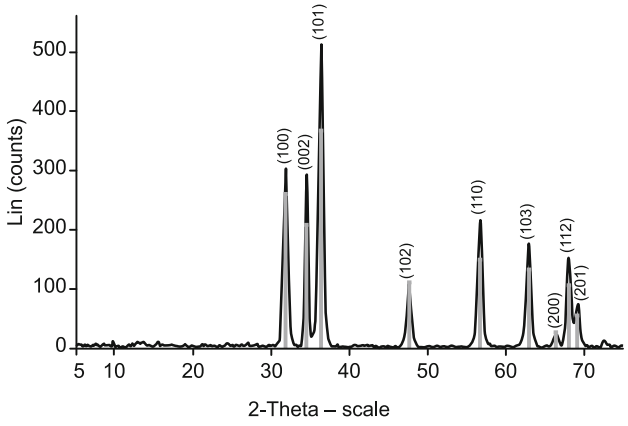

Fig. 3. XRD pattern of the ZnO nanoparticles.

μg/ml and 64 μg/ml for standard and clinical isolates of *Candida,* respectively.

MFC of ZnO NPs and fluconazole was similar for clinical samples (128 μg/ml). MFC of nanoparticle and fluconazole was 64 and 16 μg/ml for standard and clinical isolates.

The results of MIC and MFC for the ZnO NPs and fluconazole are shown in Table 1.

Wilcoxon test showed that regarding the amount of MTT and MFC, there is a significant difference between fluconazole and ZnO NPs ($p < 0.05$). With regard to averages it can be stated that nanoparticles have caused a higher decrease of *Candida* adherence and viability of *Candida* and MFC than fluconazole. Regarding the value of MIC, there was no significant difference between fluconazole and ZnO NPs ($p > 0.05$). The result are shown in Table 1.

Characterization of synthesized Nanoparticles (NPs) are elucidated as follows:

1. *Morphological Analysis of NPs*
   Morphological characterization of ZnO nanoparticles was performed by FE-SEM and TEM analysis. As shown in Figure 2, uniform particle size distribution and spherical shape of ZnO nanoparticles could be seen perceptibly. The result showed size of Mono dispersed ZnO nanoparticles with a diameter about 30 nm.
2. *XRD analysis of NPs*
   In order to confirm the formation of ZnO nanoparticles structure, XRD patterns were obtained. As shown in Figure 3, the result showed characteristic peaks of ZnO crystalline structure observed in 100, 002, 101, 102, 110 and 103 (2θ) region. The results are in agreement with published reports and confirmed the high purity of ZnO nanoparticles obtained (17).
3. *FT-IR analysis*
   FT-IR spectroscopy was employed to confirm the successful preparation of ZnO nanoparticles. As shown in Figure 4, the broad band absorption peak at around 3100–3400 $cm^{-1}$was assigned to hydroxyl groups (ν_{O-H}) and absorption peaks at around 500 cm^{-1} can be assigned to Zn-O stretching vibrations (ν_{Zn-O}). Successful conjugation of Linoleic acid (LA) to chitosan (CS) could be also confirmed by FT-IR spectra. As shown in (Fig. 4),

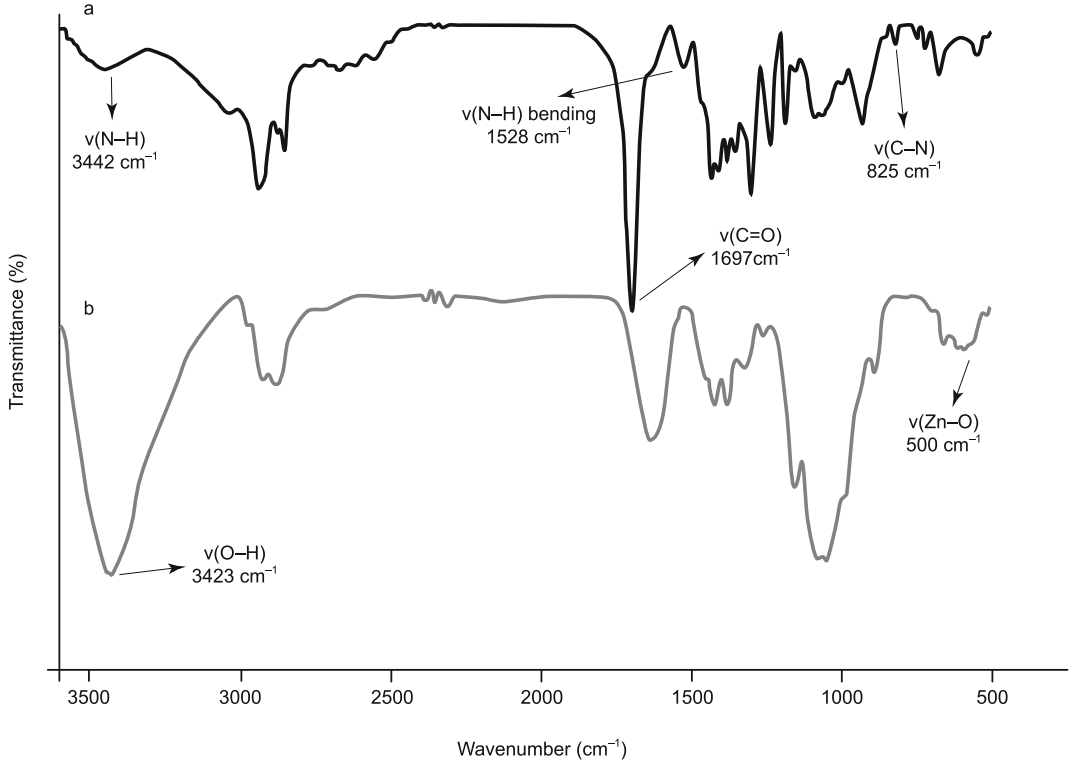

Fig. 4. FT-IR spectra of conjugated linoleic acid (LA) to chitosan (CS) (a), ZnO nanoparticles (b).

a strong absorption peak in 1650–1690 cm^{-1} can be assigned to C=O stretching ($\nu_{C=O}$) and absorption peak at around 3100–3500 cm^{-1} can be attributed to the stretching vibration of N-H bonds (ν_{N-H}) and N-H bending vibration also can be observed at around 1550–1640 cm^{-1} in amid groups. Moreover, the peak appeared at around 830 cm^{-1} and was related to the stretching vibrations of C-N bonds (ν_{C-N}). Therefore the obtained result confirmed successful preparation of ZnO NPs and conjugation of Linoleic acid (LA) to chitosan (CS) (18, 19).

Discussion

Nowadays systemic infections caused by pathogenic yeasts are on the rise due to immunosuppressive diseases like AIDS (Acquired immunodeficiency diseases), hematological malignancies as well as mortality causes, in particular, for hospitalized patients (20).

As a result, designing the new drug delivery system via nanotechnology which is able to inhibit biofilm formation through controlling expression of effective genes in pathogenesis, can be one of the drug delivery system objectives to improve treatment with minimum side effects and optimized quality.

In the present research, for the first time, antifungal effect of new synthesized nanoparticles was investigated. Moreover, efficacy in comparison with fluconazole was studied.

Our findings indicated that nanoparticles can considerably inhibit the growth of fluconazole-resistant clinical strains at concentrations of 128 µg/ml, and completely stop their growth at this concentration that is surprising as compared to that of fluconazole (MIC; 64 µg/ml).

Interestingly, the inhibition percent of biofilm formation, for nanoparticles was greater than that of fluconazole (43.07 % vs 36.68 %).

On the other hand, first type Hydroxyl and Amine groups located on Chitosan structure give it permission for chemical modification to control the physical properties. Chemical bonding of drug to Chitosan through functional groups can produce beneficial products. Fatty acids are also of antifungal properties that normally can penetrate fungal lipid membrane to disrupt structure and fluidity of membrane which are preferred in the nanoparticle (21).

Since fluconazole-resistant clinical strains were used in this study, our findings showed the synthesized nanoparticles can have desirable effects on these resistant samples with regard to MIC, MTT results compared to fluconazole.

Also significant differences between fluconazole and nanoparticles ($p < 0.05$) were found in MFC and MTT assays.

Regarding the obtained results, synthesized nanoparticle has anti-fungal effect and can inhibit the biofilm construction and act as a proper agent in medical instruments.

In addition, other effective properties of this nanoparticle in *candida* growth inhibition can be referred to Chitosan antifungal property and Linoleic acid that was supported by several experimental studies . Although different studies are existing regarding the effect of nanoparticles on *candida* species and bacteria, the information about antifungal effects of the conjugated nanoparticles is limited.

Maribel Plascecio et al (22), showed Chitosan has been referred to as an appropriate antimicrobial substance as well as a substance with changing susceptibility to nanofiber. Haghighi et al (23) studied the role of Titanium oxide nanoparticles to control *Candida* biofilm formation on Catheter surfaces. The results showed this nanoparticle is of antifungal property and can inhibit biofilm formation on medical instruments.

Hassan Nageh et al (24) searched on assessment of antibacterial and antifungal property of Chitosan nanofiber in 2014 and used Ciprofloxacin and drug delivery was studied on inhibition growth of C. *albicans, Klebsiella* and *E. coli*.

Fernandez et al (25) studied the nanofibers antifungal property made of Chitosan in comparison with Chitosan in 2014. Finally, through producing Alfa-chitin nanofibers, it was proved that Chitosan nanofibers had more powerful inhibitory effect against *Aspergillus niger* fungal strain than Chitosan itself.

In another study it was shown that the ZnO and CuO nanoparticles have anti *C. albicans* properties and MIC and MFC of ZnO NPs was 200 µg/ml and 400 µg/ml (4), respectively, supporting our finding , however the low MIC level of the nanoparticle in our study may be related to

The result of research performed by Singh showed that zinc oxide nanoparticles do have strong antibacterial and antifungal activity against selected strains of bacteria and fungi in comparison with conventional zinc oxide particles (26).

It would be beneficial for future studies to investigate the safety, and detailed mechanisms of this nanoparticles should be further studied in vitro and in vivo. In summary, it is suggested this nanoparticles can be combined with fluconazole for more effective properties.

Conclusion

Taken together, more interest is focused on using drugs sources with less side effects and in some cases better efficacy. This study explains that inhibitory activity of nanoparticles has a compatible antifungal effect with fluconazole. Monitoring the side effects of these nanoparticles on animal models and showing how safe they are, they can enable their wide use.

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