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Evaluating the Antifungal Activity of Nano-curcumin against *Candida* Species Isolated from Otomycosis Patients

Kashirifar Hossein[1], Kiakojouri Keyvan[2]✉, Mahdavi Omran Saeid[3], Hoseinnejad Akbar[4, 5], Jafarzadeh Jalal, Aminian Ahmad Reza[6], Jahangiri Mohammad[7], Bagheri Soghra[8], Taghizadeh Armaki Mojtaba[3]✉

1. Student Research Committee, Babol University of Medical Sciences, Babol, Iran
2. Department of Ear, Nose and Throat, School of Medicine, Roohani Hospital, Babol University of Medical Sciences, Babol, Iran
3. Infectious Diseases and Tropical Medicine Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran
4. Department of Medical Mycology, Faculty of Medicine, Jundishapur University of Medical Sciences, Ahvaz, Iran
5. Student Research Committee, Ahvaz Jundishapur University of Medical Science, Ahvaz, Iran
6. Department of Physiology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
7. Université de Lorraine, Inserm, Défaillance Cardiovasculaire Aigue et Chronique (DCAC), Université de Lorraine, Lorraine, France
8. Department of Medical Mycology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

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Abstract

Background & Objective: *Candida* species are one of the most common causes of otomycosis. Antifungal drugs commonly used in treatment often have a variety of side effects, including toxicity, cross-reactivity, and drug resistance. In recent years, along with the advances in nanotechnology and the motivation to find new antifungal drugs, there has been a growing interest in the use of nanoparticles such as nano-curcumin in the treatment of fungal infections. The aim of this study was to evaluate the antifungal sensitivity of nano-curcumin on *Candida* species isolated from otomycosis.

Materials & Methods: In this experimental study, 100 isolated *Candida* samples from patients with otomycosis were included. Synthesis of nano-curcumin using chitosan (CS) nanoparticles was performed by ionic gelation method. Antifungal susceptibility testing was performed using broth microdilution method according to CLSI-M27-S4 guidelines on all *Candida* isolates to miconazole, clotrimazole and nanocurcumin. Data were analyzed in SPSS 27 software with independent t-test and chi-squared tests.

Results: Curcumin nanoparticles showed antifungal activity against all *Candida* species. However, the mean MIC of miconazole and clotrimazole for different *Candida* species was significantly lower than curcumin nanoparticles, indicating the lower antifungal effect of nano-curcumin than these two antifungals. Also, the mean MIC of nano-curcumin was not significantly different among *Candida* species ($P < 0.05$), whereas it was significantly different for miconazole and clotrimazole ($P < 0.05$).

Conclusion: The results of the present study showed that nano-curcumin had a lower antifungal effect than miconazole and clotrimazole, but due to nano-curcumin's safety, it can be used as a potential antifungal drug for the treatment of otomycosis after further investigation.

Keywords: *Candida*, Otomycosis, Nanocurcumin, Miconazole, Clotrimazole

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Introduction

Today, fungal infections caused by opportunistic fungi are on the rise due to the overuse of antibiotics, antifungals, and corticosteroids in immunocompromised people (1, 2). One of these opportunistic fungal infections is otomycosis, an acute, subacute, or chronic infectious disease of the outer and middle ear that causes pain, inflammation, discharge, pus, itching, and deafness (3). Globally, the disease has spread and according to recent studies, its prevalence is increasing in countries near Iran such as Russia, Bahrain, Turkey, and Iraq (4-6).

✉**Corresponding Authors: 1. Taghizadeh Armaki Mojtaba,** Infectious Diseases and Tropical Medicine Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran
Email: mojtabataghizade@yahoo.com
✉**2. Kiakojouri Keyvan,** Department of Ear, Nose and Throat, School of Medicine, Roohani Hospital, Babol University of Medical Sciences, Babol, Iran
Email: kia_ko13358@yahoo.com

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Studies have shown that the causative agents of otomycosis are yeasts such as *Candida albicans* (*C. albicans*) and *non-albicans*, and various molds such as *Aspergillus niger*, *Aspergillus* flavus, *Aspergillus fumigatus*, and rarely *Penicillium*, *Mucor*, *Rhizopus*, *Scopulariopsis* and *dermatophytes* (7-9). Antifungals currently used to treat this disease include clotrimazole, tolnaftate, miconazole, econazole, and fluconazole, and in cases of failure to respond to local treatment or perforation of the tympanic membrane, voriconazole or systemic itraconazole can be used (10). Recent studies have shown that the use of these antifungals, especially the azoles, has many side effects, including liver problems, irregular heartbeat, cancer, and in some cases poisoning (11-13). Currently, resistance of fungal pathogens to these drugs is increasing, which will eventually lead to unsuccessful treatment and progression of the infection (14). Some natural compounds have a variety of anticancer, antimicrobial, antioxidant, and antifungal effects. Curcumin is a potent natural compound found in turmeric that has been reported to have many antifungal effects (15). As this compound has low solubility in water and biological fluids, many formulations have been developed to increase its solubility (16). One of the new solutions use in order to increase the solubility and improve the bioavailability of curcumin is the use of nanoparticles. Due to their special surface properties, these particles can have better therapeutic effects and fewer side effects compared to conventional drugs (17). A novel curcumin nanoparticle was prepared using chitosan polymer in the present study, and after characterization and particle stability studies, the antifungal activity of curcumin nanoparticles on different *Candida* species obtained from patients with otomycosis was evaluated in vitro.

Materials and methods

This experimental study included 100 *Candida* isolates (*C. albicans*, *Candida orthopsilosis* (*C. orthopsilosis*), *Candida* parapsilosis (*C. parapsilosis*), *Candida tropicalis* (*C. tropicalis*) *Candida glabrata* (*C. glabrata*) available in the Bank of Parasitology and Mycology Department of Babol University of Medical Sciences, Babol, Iran, which had already been identified microscopically, macroscopically, and molecularly.

Synthesis of nanocurcumin

In addition, curcumin was purchased from Sigma Aldrich Co. (USA). Nano-curcumin was synthesized based on Babaei et al's study at the Faculty of Health, Babol University of Medical Sciences, Babol, Iran (18).

Preparation of fungal suspension

Candida isolates were first cultured on Sabouraud Dextrose Agar (SDA). Next, a few (fresh) yeast colonies were removed and a yeast suspension was prepared using 5 mL of sterile saline and the concentration of yeast cells was read as 1-5 × 10^6 CFU/mL (transmission 75-77%) using a spectrophotometer with a wavelength of 530 nm. Finally, the stock suspension was diluted to 1:1000 with RPMI medium (Sigma Chemical Co.) to prepare the inoculation suspension.

Stock solution preparation of clotrimazole, miconazole and nanocurcumin

Based on the Clinical and Laboratory Standards Institute (CLSI) M27-S4 standard protocol for yeast fungi, 2.3 mg of pure powder of each drug (clotrimazole and miconazole Sigma-Aldrich, St. Louis, MO, USA) was dissolved separately in 1 mL of DMSO (dimethyl sulfoxide), and 5.52 mg of nano-curcumin powder was dissolved in 15 mL of water, and a drug stock with a concentration of 3200 µg/mL was prepared. After RPMI medium (containing glutamine and without bicarbonate, buffered with M-morpholinepropanesulfonic acid (MOPS) (Sigma) was prepared and its pH was adjusted to 7, the drug stock was diluted 1:100 with RPMI medium to a final dilution of 0.016 to 16 µg/mL for clotrimazole and miconazole, and 0.016 to 64 µg/mL for nanocurcumin.

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Antifungal susceptibility testing (AFST)

AFST was performed according to the CLSI-M27-S4 protocol using the broth microdilution method in flat-bottomed 96-well microplates (19). First, 100 µL of RPMI medium (Sigma Chemical Co.) was added to all wells of the 96-well microplate except the first column. 200 µL of stock solutions of nanocurcumin, clotrimazole and miconazole were added to the first column wells, then 100 µL was transferred from the first column wells to the second column and the serial dilution continued to the tenth column. Columns 11 and 12 were used as positive and negative controls for the assay. Then, 100 µL of yeast suspension with a transmission of 75-77 were added to all columns except the negative control. Microplates were incubated for 24 hours at 35-37˚C and visually observed. MIC values were reported as 50% growth inhibition compared to the positive control (20). Strains *C. parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258) were used as quality control measures.

Statistical analysis

Central indices (mean, median) and dispersion indices (range, standard deviation, interquartile range) of results were analyzed using SPSS version 27 software (IBM). The independent t-test was used for quantitative results and the chi-squared test for qualitative variables, with $P < 0.05$ being considered significant.

Results

Frequency and percentage of 100 *Candida* isolates based on descriptive statistics were 53 (53%) *C. albicans*, 19 (19%) *C. orthopsilosis*, 13 (13%) *C. parapsilosis*, 7 (7%) *C. tropicalis*, 8 (8%) *C. glabrata*. The mean MICs of the two antifungals, miconazole and clotrimazole, were 1.10 and 3.22 µg/mL respectively, which was significantly ($P<0.001$) lower than curcumin nanoparticles with a mean MIC of 4.92 µg/mL, showing that miconazole and clotrimazole have more antifungal activity than curcumin nanoparticles. However, in some species such as *C. parapsilosis* and *C. glabrata*, the mean MIC of curcumin nanoparticles was greater than or equal to the mean MIC of clotrimazole indicating that curcumin nanoparticles may be more effective than clotrimazole against the *C. parapsilosis* and *C. glabrata* species (Chart 1). Comparison of the MIC50 and MIC90 of two antifungal drugs, clotrimazole and miconazole, showed that miconazole had a better antifungal activity than clotrimazole against all *Candida* species tested ($P<0.001$). Notably, the MIC values of miconazole and clotrimazole were significantly different between various *Candida* species ($P < 0.05$), indicating that these two antifungals had the highest activity against *C. orthopsilosis* and *C. tropicalis* and the lowest activity against *C. glabrata*. Nanocurcumin also had the same activity against all *Candida* species and there was no statistically significant difference between its antifungal activity.

Table 1. Descriptive statistics of antifungals and nano-curcumin

Candida species	MIC50/MIC90/GM (µg/mL)	Nanocurcumin	Miconazole	Clotrimazole
C. albicans	**MIC50**	4	0.25	1
	MIC90	8	2	4
	GM	4.33	0.28	1.45
	Maximum	8	4	16
	Minimum	0.25	0.016	0.25

C. orthopsilosis	**Std. Deviation**	2.085374	1.115338	2.926345
	MIC50	4	0.25	1
	MIC90	8	0.5	16
	GM	4.46	0.32	1.54
	Maximum	8	8	16
	Minimum	2	0.125	0.25
	Std. Deviation	2.034785	1.770014	5.693852
C. parapsilosis	**MIC50**	4	0.5	1
	MIC90	8	4	16
	GM	4.69	0.68	2.47
	Maximum	8	8	16
	Minimum	2	0.125	1
	Std. Deviation	2.100061	2.361805	6.465887
C. tropicalis	**MIC50**	4	0.5	1
	MIC90	4	4	4
	GM	4.41	0.74	1.10
	Maximum	8	8	2
	Minimum	2	0.125	1
	Std. Deviation	1.799471	2.949601	0.377964

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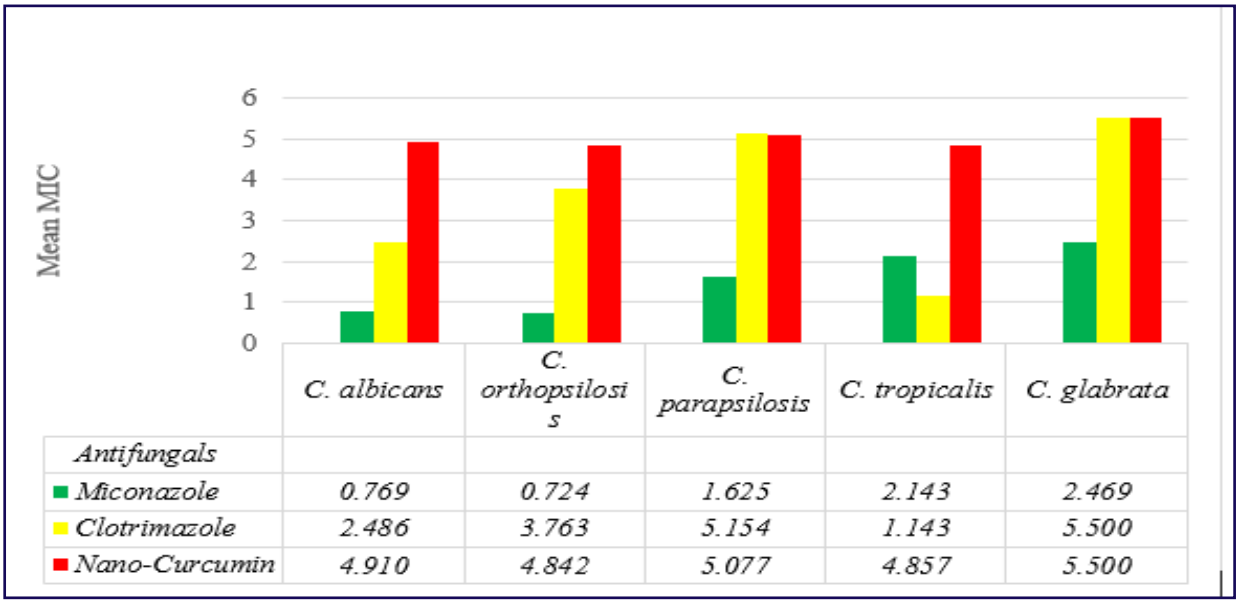
C. glabrata	**MIC50**	4	2	4
	MIC90	8	4	8
	GM	5.18	1.54	4.36
	Maximum	8	8	16
	Minimum	4	0.25	2
	Std. Deviation	2.070197	2.522886	4.629100
Total isolates	**MIC50**	4	0.25	1
	MIC90	8	4	8
	GM	4.46	0.411	1.72
	Maximum	8	8	16
	Minimum	0.25	0.032	0.25
	Std. Deviation	2.032468	1.796280	4.306428



Antifungals	*C. albicans*	*C. orthopsilosis*	*C. parapsilosis*	*C. tropicalis*	*C. glabrata*
Miconazole	0.769	0.724	1.625	2.143	2.469
Clotrimazole	2.486	3.763	5.154	1.143	5.500
Nano-Curcumin	4.910	4.842	5.077	4.857	5.500

Chart 1. MIC mean of nano-curcumin, miconazole and clotrimazole against different *Candida* species

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Discussion

In recent years, resistance of *Candida* species to common antifungal drugs has increased, ultimately leading to treatment failure and disease progression (21). The use of nano-origin materials, as well as non-toxic natural compounds with antifungal activity may lead to appropriate therapeutic solutions with a rational preventive/therapeutic approach to controlling *Candida* infections (22). Considering the biochemical properties of curcumin nanoparticles and their diverse biological activities, this study was conducted with the aim of investigating the antifungal effects of these nanoparticles against different *Candida* species isolated from otomycosis patients (23). Findings of the present study showed that miconazole and clotrimazole had lower MIC than nano-curcumin in all species. In other words, nano-curcumin had less antifungal effects than miconazole and clotrimazole. The MIC of nano-curcumin was the same between different *Candida* species and had no significant difference, which indicates the same antifungal effects of nano-curcumin on all *Candida* species. The findings of Nilufar et al. (2011) and Sadeghi et al. (2020), similar to our study, showed the appropriate antifungal activity of curcumin against *Candida* isolates, but unlike the present study, curcumin had better antifungal activity than the control drug fluconazole, which may be due to the type of antifungal drug used in the study (24, 25). Kumar et al. (2014) confirmed the antifungal effect of curcumin on *Candida* cells, stating that this compound can cause cell wall and membrane damage (26). Paul et al (2018) reported the MIC of silver curcumin nanoparticles for different *Candida* species, ranging from 31.2 for *C. glabrata* to 125 µg/mL for *C. parapsilosis* and*C. krusei*. The MIC values of nanoparticles were higher than those in the present study, and this difference in MIC could be due to the use of silver together with nano-curcumin (27). In the study by Hazzah et al. (2015), the MIC of curcumin nanoparticles was 0.187 and curcumin was 1.5 µg/mL against *C. albicans.* They stated that the incorporation of curcumin into lipid nanoparticles increased the stability and antimicrobial activity of the compounds. MICs obtained for nano curcumin were lower than the MICs of the present study and exhibited better antifungal properties. The reasons for this could be due to the difference in synthesis method, type, and size of nanoparticles (28). Babaei et al. (2016) showed that increasing the dose of curcumin improved its antifungal effect, but it was lower than the control drug nystatin, which is consistent with the results of the present study (29). In contrast to our study, Phuna et al. (2020) reported better antifungal effects of curcumin nanoemulsion in inhibiting the growth of *C. albicans*, *C. glabrata* and *C. krusei* compared to fluconazole (30). The reasons for the difference between the results of the above study and our study could be the different origins of isolation of *Candida* species from the infection, the type of control drug, and the intrinsic resistance of *C. glabrata* and *C. krusei* species to azoles. Most of the studies, as well as the present study, confirmed the efficacy of nano-curcumin on *Candida* species. However, there are differences in the activity of the curcumin nanoparticles used in the studies, which can also be related to the type of substances associated with the nanoparticles, method of synthesis, type of *Candida* species, source of infection, and the method used for antifungal susceptibility testing.

Conclusion

Results of the present study and previous studies regarding the antifungal effects of nano-curcumin showed that nano-curcumin can be

considered as a potential agent for the treatment of *Candida* infections. It is recommended that further studies be designed on the cytotoxic effects and combined effects of this nanoparticle on fungal pathogens (filamentous and yeast) together with antifungal drugs.

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Conflicts of interest

All authors declare having no conflicts of interest with regard to the publication of this paper.

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Data Availability

Data are available on request from the authors.

Ethical Considerations

This study was approved by the Research Ethics Committee of Babol University of Medical Sciences; Babol, Iran.

Code of Ethics

This article's code number is IR.MUBABOL.REC.1400.145.

Authors' Contributions

Conceived and designed the study: MTA, HK and KK. Performed the experiments: HK, JJ, AH and SB. Analyzed the data: AH. Contributed reagents/materials/analysis tools: AA, KK, JJ, MJ. Drafted and revised the manuscript: MTA, KK and AH.

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