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Research Article

Negative Regulators, *TUP1*, *NRG1*, and *TOR1*: Possible Molecular Targets of Antibiofilm Activity of Nano-Fe_3O_4 in *Candida albicans*

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Abstract

Background: *Candida albicans* is an opportunistic pathobiont that manifests as candidiasis. Drug-resistant biofilms hinder current treatment of candidiasis. The morphological control of filamentous growth and biofilm formation is vital for the pathogenicity of *C. albicans*. This study aimed to investigate the antifungal activity of magnetic iron oxide nanoparticles (nano-Fe_3O_4) against *C. albicans*, their ability to inhibit pre-formed biofilms, and to examine the expression levels of negative regulators, transcriptional repressors thymidine uptake 1 (*TUP1*), the negative transcriptional regulator of glucose repressed genes (*NRG1*), and target of rapamycin (*TOR1*) in *C. albicans* pre-formed biofilms after treatment with nano-Fe_3O_4.

Methods: This study examines the antifungal activity of nano-Fe_3O_4 against *C. albicans*, its ability to inhibit pre-formed biofilms, and investigates the expression levels of negative regulators, *TUP1*, *NRG1*, and *TOR1* in *C. albicans* pre-formed biofilms after treatment with nano-Fe_3O_4.

Results: Nano-Fe_3O_4 at concentrations of 2× minimum inhibitory concentration (MIC), 1× MIC, and ½× MIC showed significant inhibitory effects on *C. albicans* pre-formed biofilm formation by 2,3-bis (2-methoxy-4-nitro-5 sulfophenyl)-5-[(phenylamino) carbonyl])-2H-tetrazolium hydroxide (XTT), crystal violet staining and light field microscopy with a MIC of 50 µg/mL. Gene expression profiling showed that nano-Fe_3O_4 upregulates targets *TUP1*, *NRG1*, and *TOR1* in *C. albicans* pre-formed biofilms.

Conclusion: Our results suggest that nano-Fe_3O_4 diminishes pre-formed biofilms and may subsequently reduce the pathogenicity of *C. albicans*, which can be responsible for biofilm-associated infections. *TUP1*, *NRG1*, and *TOR1* may be possible molecular targets in *C. albicans* pre-formed biofilms after treatment with nano-Fe_3O_4.

Introduction

Candida albicans is an important member of the human mycobiota, but can become a pathobiont in various anatomical sites and cause candidiasis.[1,2] *C. albicans* invades susceptible host tissues leading to tissue damage.[3] It possesses various virulence characteristics, especially a morphological switch between round yeast cells and a hyphal form, which contributes to invasion and destruction of host tissues.[3,4] Hyphal morphogenesis plays a crucial role in *C. albicans* biofilm formation, and is tightly regulated by many molecules, with kinases being the most important.[4-7] *C. albicans* morphogenesis is negatively regulated by transcriptional repressors thymidine uptake 1 (Tup1), the negative transcriptional regulator of glucose repressed genes (Nrg1), and repressor of filamentous growth (Rfg1),[4] while the target of rapamycin (Tor1) is a negative regulator of filamentous growth.[8]

Biofilm formation involves the attachment and encasement of microbial communities with extracellular polymeric substances, preventing efficient penetration of antifungal agents into cells. *C. albicans* cells embedded in biofilms are less sensitive or insensitive to antimicrobial agents and the host immune response.[1,9,10] Therefore, eliminating biofilm formation is considered an effective alternative treatment strategy to target the virulence characteristics of *Candida*. This study focuses on the antibiofilm activity of magnetic iron oxide nanoparticles (nano-Fe_3O_4) against *C. albicans* and aims to identify possible molecular targets of biofilm-associated genes.

The inhibition of *C. albicans* biofilm formation by up- or down-regulating of several biofilm-related genes has been reported.[11-17] However, nano-Fe_3O_4 when scrutinized for its antibiofilm activity, depicted robust potential against *C. albicans* biofilm formation.[18-21] This study examined the antifungal activity of nano-Fe_3O_4 on planktonic growth and biofilm formation in *C. albicans*. Light field microscopy was used to observe the microscopic architecture of *C. albicans* pre-formed biofilm after treatment with nano-

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Fe_3O_4. Quantitative real-time reverse transcription PCR (qRT-PCR) was used to study the expression levels of the negative regulators, *TUP1*, *NRG1*, and *TOR1* in *C. albicans* pre-formed biofilm after treatment with nano-Fe_3O_4.

Methods

Materials and microbial stains

The commercial nano-Fe_3O_4 (purity: 98+%, APS: 20 - 30 nm, SSA: 40-60 m^2/g, color: dark brown, morphology: spherical, bulk density: 0.84 g/cm^3 and true density: 4.8-5.1 g/cm^3) was procured from Nanosany Corporation (#NS3220, Mashhad, Razavi Khorasan, Iran), and fluconazole (#F8929) was purchased from Sigma Aldrich (St. Louis, MO, USA). *C. albicans* PTCC 5027 was obtained from the Iranian Biological Resource Center (Tehran, Iran).

Antifungal activity of nano-Fe_3O_4 against C. albicans

The minimum inhibitory concentration (MIC) was established based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI, M27-A3; M27-S4) broth dilution method,[22,23] using the round-bottomed 96-well microtiter plates (SPL Life Sciences Co., Ltd., Pocheon-si, Korea). Nano-Fe_3O_4 was serially diluted in dimethyl sulfoxide, and concentrations of 0.0312 to 200 µg/mL were added to the wells of a 96-well microtiter plate. Each well contained 0.5-5 × 10^3 colony forming units (CFU)/mL of *C. albicans* suspension in Roswell Park Memorial Institute (RPMI) 1640 medium L-glutamine, 2% glucose, and 0.165 M morpholinepropanesulfonic acid buffer without sodium bicarbonate (Sigma-Aldrich). The plates were then incubated at 35 ˚C for 24 and 48 hours. The MIC was defined as the lowest concentration that completely inhibited fungal growth, as determined by a microplate reader (DA3200, DANA Co., Iran) at a wavelength of 530 nm. Positive (no antifungal agent) and negative (complete medium alone) growth controls were included. Fluconazole was employed as a reference standard.

Antibiofilm potency of nano-Fe_3O_4 against C. albicans

The pre-formed biofilm assays were conducted using the 2,3-bis (2-methoxy-4-nitro-5 sulfophenyl)-5-[(phenylamino) carbonyl])-2H-tetrazolium hydroxide (XTT) and crystal violet (CV) staining methods in 96-well microtiter plates (SPL Life Sciences Co., Ltd., Pocheon-si, Korea), and cultured *C. albicans* PTCC 5027 with different concentrations of nano-Fe_3O_4 based on MIC (2× MIC, 1× MIC, and ½× MIC). The microtiter plates contained approximately 1 × 10^6 CFU/mL of fungal suspension in RPMI 1640 medium with L-glutamine, 2% glucose, and 0.165 M morpholinepropanesulfonic acid buffer without sodium bicarbonate (Sigma-Aldrich). Antifungal agents were added and incubated at 35 ˚C for 90 min. Biofilm formation continued with further incubation at 35 ˚C for 24 h at 150 rpm. Colorimetric changes in the absorbance of XTT and CV were recorded at wavelengths of 490 nm and 590 nm, respectively.[24,25] Fluconazole was used as a reference standard. The percentage inhibition was evaluated using the formula previously described by Costa *et al.*[26]

The microscopic architecture of C. albicans pre-formed biofilms after treatment with nano-Fe_3O_4

The effect of nano-Fe_3O_4 and fluconazole on pre-formed biofilm architecture was examined on *C. albicans* PTCC 5027 using a light field microscope (Nikon Corporation. Tokyo, Japan) as previously described.[27] Briefly, 1 × 10^6 CFU/mL of *C. albicans* suspension was added to the wells of a 6-well cell culture plate containing RPMI 1640 medium with L-glutamine, 2% glucose, and 0.165 M morpholinepropanesulfonic acid buffer without sodium bicarbonate (Sigma-Aldrich) and ThermanoxTM coverslips (SPL Life Sciences). The wells were then treated with 4 mL of nano-Fe_3O_4 at different concentrations based on the MIC (2× MIC, 1× MIC, and ½× MIC) and incubated at 35 ˚C for 90 min, followed by further incubation at 35 ˚C for 24 h at 150 rpm. The pre-formed biofilms were observed under a light field microscope and analyzed using Icy Bioimage Analysis software. The results were expressed as the number of hyphae/total cell counts (hyphae and planktonic cells) ×100.[28,29]

Gene expression profiles in C. albicans pre-formed biofilms after treatment with nano-Fe_3O_4

Real-time quantitative reverse transcription PCR (qRT-PCR) was used to analyze gene expression profiles of negative regulators, *TUP1*, *NRG1*, and *TOR1* in *C. albicans* pre-formed biofilms after treatment with nano-Fe_3O_4 and fluconazole.[25,30] Briefly, *C. albicans* pre-formed biofilms treated with different concentrations of nano-Fe_3O_4 based on MIC (2× MIC, 1× MIC, and ½× MIC) were centrifuged at 3000 rpm for 10 min. Total RNA was then isolated and purified using the Iraizol RNA kit (RNA Biotechnology Company, Isfahan, Iran), and genomic DNA was removed using the enzyme DNase. Results were verified using an Implen Nanophotometer NP 80 (Munich, Germany) and agarose gel electrophoresis. The primers (shown in Table 1) were created using the OLIGO 7 Primer Analysis Software (DBA Oligo, Inc., USA) and synthesized by Macrogen, Inc. (Seoul, South Korea). The *β-actin* gene was used as a housekeeping gene and internal control to normalize the varying concentrations of RNA during RNA isolation. Two internal-technical and negative controls (without M-MuLV reverse transcriptase and without template) were included in each sample to ensure that the PCR products originated from cDNA, and not genomic DNA. The qRT-PCR was evaluated with SYBR Green I dye (Biofact Co., South Korea) using an Applied Biosystems StepOne Real-Time PCR System (Darmstadt, Germany). The cycling conditions were as follows: 95 ˚C for 5 min; then 40 cycles of 95 ˚C for 15 s, 58 to 62 ˚C for 20 s and 72 ˚C for 15 s, followed by a melting curve analysis running from 65 to 95 ˚C, 0.5 ˚C per 5 s increments. The expression levels of each target gene were calculated using the double-delta CT

Table 1. Primer sequences used in this study.

Target gene		Sequence primer (5'-3')	Annealing temperature	PCR product length (bp)	Reference
TUP1	F	AGACGGCAAGTTCATCGCCACC	62 ˚C	197	This study
	R	CTTCTGCACCTGTCGCCAAGAGT			
NRG1	F	TGCTGCTCCTCCTTATCCCCATC	58 ˚C	176	This study
	R	AGTTTGACCGTATTTTGGCATGA			
TOR1	F	AGCTCTTCTGTGGCTTCTAGTGCAA	62 ˚C	158	This study
	R	GTTCTCAAGGCCGCATCCCGTTT			
ACT	F	ATGTTCCAGCTTTCTACGTTTCCA	58 ˚C	175	[18]
	R	GTCAAGTCTCTACCAGCCAAATCA			

method. Figures S1-S4 show the melting curve of RT-PCR and the amplification plot of *TUP1*, *NRG1*, and *TOR1*.

Statistical analysis

Normality was judged by the Shapiro-Wilk test. One-way analysis of variance followed by Tukey's multiple comparisons test was used to analyze the data. GraphPad Prism (Version 9) was used to analyze the data. Mean values ± SD are presented as results. p values of less than 0.05 are considered statistically significant. All experiments were performed in at least triplicates.

Results

Effects of nano-Fe_3O_4 on C. albicans and pre-formed biofilms

The MICs for the nano-Fe_3O_4 and fluconazole were 50 and 4 µg/mL, respectively. Biofilm studies showed that nano-Fe_3O_4 significantly inhibited *C. albicans* biofilm formation (Tukey's HSD; $p<0.05$, $p<0.01$, and $p<0.001$; Figure 1). The XTT method showed that nano-Fe_3O_4 dose-dependently inhibited *C. albicans* pre-formed biofilms by 37.87, 25.74, and 24.78% with 2× MIC, 1× MIC, and ½× MIC, respectively. The CV method also revealed that nano-Fe_3O_4 inhibited pre-formed biofilms by 26.22, 13.90, and 11.43% with 2× MIC, 1× MIC, and ½× MIC, respectively.

The microscopic architecture of *C. albicans* pre-formed biofilms revealed that nano-Fe_3O_4 reduced hyphae formation. Compared to the untreated control, which exhibited large hyphal cells, the nano-Fe_3O_4-treated biofilms contained more yeast cells and fewer hyphal elements. Additionally, the results of the filamentation index supported the antibiofilm activity of nano-Fe_3O_4 (Tukey's HSD; $p<0.001$ and $p<0.0001$). These findings suggest that nano-Fe_3O_4 inhibits yeast filamentation and biofilm maturation in *C. albicans* (Figure 2).

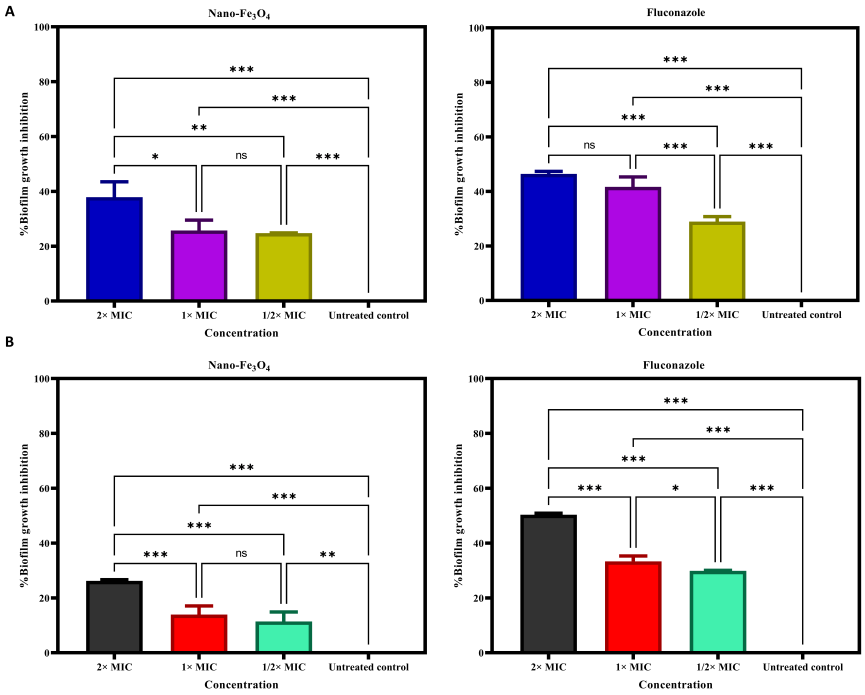

Figure 1. Effect of 2× MIC, 1× MIC, and ½× MIC concentrations of nano-Fe_3O_4 in *C. albicans* pre-formed biofilms using (A) XTT and (B) CV methods. The experiment was performed in three replicates (ns: not significant, *p < 0.05, **p < 0.01, and ***p < 0.001), and graphs are plotted using mean ± SD.

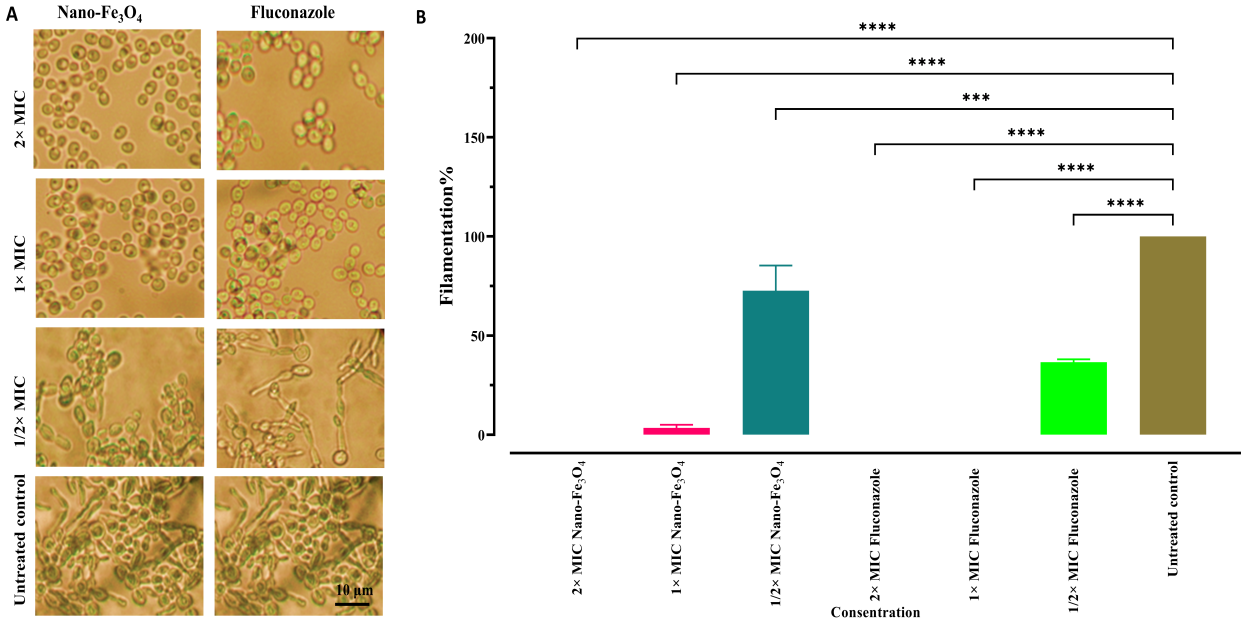

Figure 2. Effect of nano-Fe_3O_4 on the microscopic architecture of *C. albicans* pre-formed biofilms. (A) Illustrations and (B) filamentation index of *C. albicans* pre-formed biofilms after treatment with nano-Fe_3O_4 at different concentrations based on MIC (2× MIC, 1× MIC, and ½× MIC). Data are the mean values ± SD of three replicates (***$p < 0.001$ and ****$p < 0.0001$).

Gene expression profiles in C. albicans pre-formed biofilms after treatment with nano-Fe_3O_4

In this study, we used a qRT-PCR assay to investigate the expression of negative regulators *TUP1*, *NRG1*, and *TOR1* in *C. albicans* after treatment with different concentrations of nano-Fe_3O_4 based on MIC (2× MIC, 1× MIC, and ½× MIC). Nano-Fe_3O_4 significantly upregulated the expression of negative regulator genes: *TUP1* (1.42-, 1.21-, and 1.06-fold), *NRG1* (3.41-, 2.11-, 1.06-fold), and *TOR1* (3.51-, 2.43-, and 1.44-fold) compared to the untreated control in pre-formed biofilms treated with 2× MIC, 1× MIC, and ½× MIC of nano-Fe_3O_4, respectively (Figure 3). These results show that nano-Fe_3O_4 has a significant impact on the expression levels of key negative regulator genes, supporting the inhibition of *C. albicans* biofilm formation (Tukey's HSD; $p<0.001$ and $p<0.0001$). Furthermore, the expression levels of *TUP1*, *NRG1*, and *TOR1* in *C. albicans* pre-formed biofilms treated with fluconazole at concentrations of 2× MIC, 1× MIC, and ½× MIC were also significantly upregulated compared to the untreated control (Tukey's HSD; $p<0.01$, $p<0.001$, and $p<0.0001$).

Discussion

Here we report the inhibitory activity of nano-Fe_3O_4 against pre-formed biofilms of *C. albicans*. Nano-Fe_3O_4 at different concentrations based on the MIC (2× MIC, 1× MIC, and ½× MIC) significantly inhibited hyphal development and biofilm formation. Also notably consistent with its biofilm inhibitory activity, nano-Fe_3O_4 affected the expression levels of biofilm-associated genes in *C. albicans* pre-formed biofilms.

Nano-Fe_3O_4 has been reported to exhibit antifungal activity against *C. albicans*.[31-34] However, the mechanisms responsible for the effect of nano-Fe_3O_4 on antimicrobial activity and its antibiofilm activity have been little studied. Previous reports have shown that the interaction of nano-Fe_3O_4 with microorganisms resulted in a significant increase in the production of hydroxyl radicals, singlet oxygen, hydrogen peroxide, superoxide anion, and generation of electrons and holes. The molecules produced during the interaction between nano-Fe_3O_4 and microorganisms can degrade cell wall polysaccharides, initiate cellular lipid peroxidation, inactivate enzymes, and damage DNA, leading to the death of microbial cells. The interaction of nano-Fe_3O_4 with microorganisms can also affect the functions of proteins in the cell membrane via electrostatic interactions. There are multiple mechanisms that can inhibit *C. albicans* biofilm formation, including accumulation of nanoparticles in the cytoplasm or periplasm.[34] Furthermore, nano-Fe_3O_4 exhibits antibiofilm activity due to direct contact with microbial cells and the formation of reactive oxygen species, which limits surface attachment, damaging specific components of cells and destruction of biofilm architecture.[20,35]

The antibiofilm activity of nano-Fe_3O_4 was confirmed through microscopic analysis of *C. albicans* pre-formed biofilms. These results were consistent with XTT and CV analysis of pre-formed biofilms, confirming the effectiveness of nano-Fe_3O_4. Various derivatives of nano-Fe_3O_4 have been shown to inhibit biofilm formation.[20,35,36] Nano-Fe_3O_4 coated with cathelicidin LL-37 and ceragenin CSA-13 effectively inhibited *C. albicans* biofilm formation by disrupting the cell wall, leading to loss of biofilm structure.[20] Rhamnolipid coated nano-Fe_3O_4 reduced the

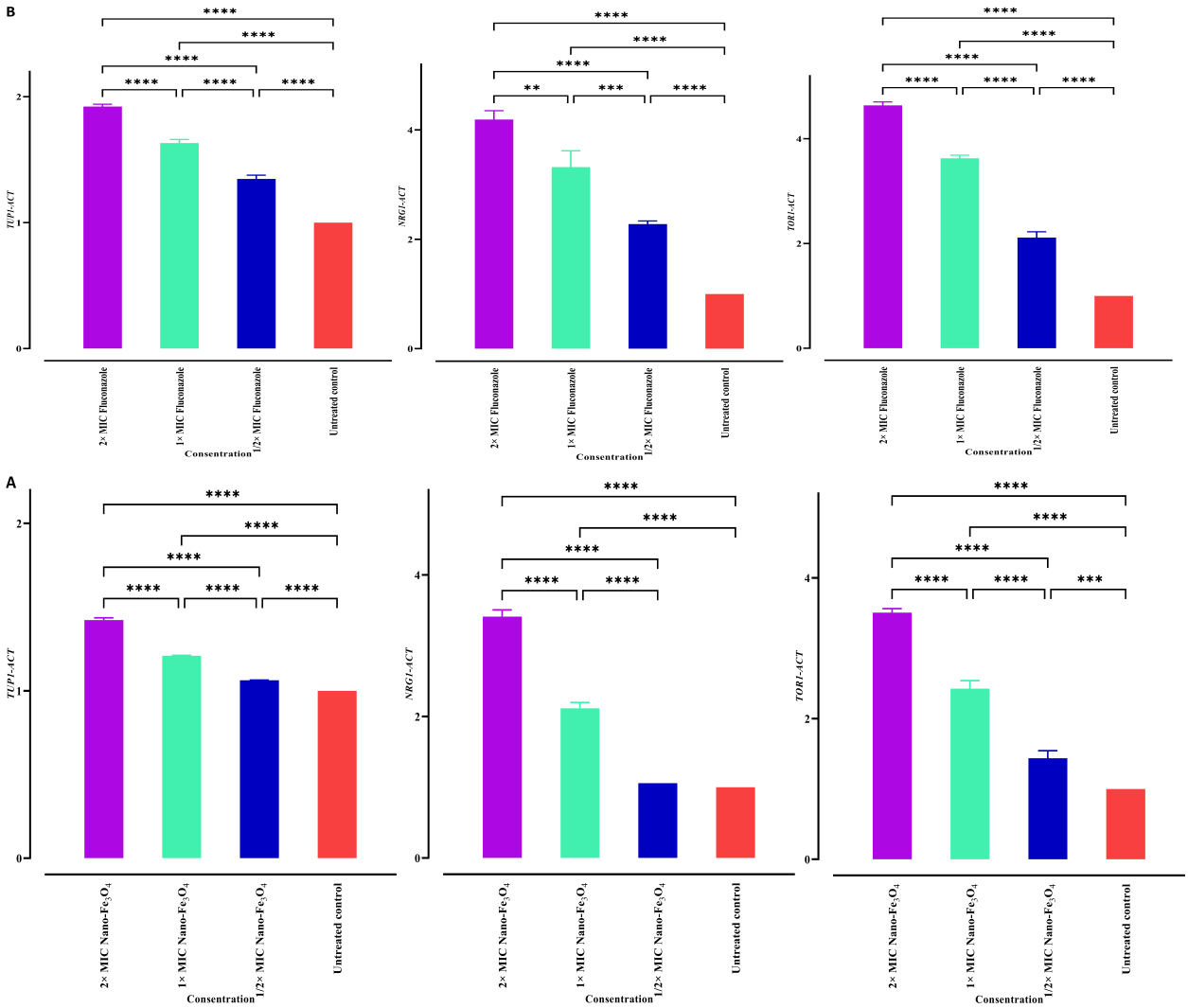

Figure 3. Relative expression levels of *TUP1*, *NRG1*, and *TOR1* in *C. albicans* pre-formed biofilms after treatment with (A) nano-Fe_3O_4 and (B) fluconazole at different concentrations based on MIC (2× MIC, 1× MIC, and ½× MIC). Data are the mean values ± SD of three replicates showing significant differences (**p < 0.01, ***p < 0.001, and ****p < 0.0001) as compared using one-way analysis of variance and Tukey's HSD tests.

cell adhesion by altering surface hydrophobicity, which contributes to the antibiofilm activity.[36]

Our qRT-PCR results were in line with our antibiofilm outcomes. Nano-Fe_3O_4 at 2× MIC, 1× MIC, and ½× MIC significantly upregulated the gene expression of negative regulators, *TUP1*, *NRG1*, and *TOR1* in *C. albicans*, according to qRT-PCR results. We previously reported that nano-Fe_3O_4 at 2× MIC, 1× MIC, and ½× MIC significantly downregulated the expression of *BCR1*, *ALS1*, *ALS3*, *HWP1*, and *ECE1* genes in *C. albicans* biofilm formation.[18] Our results also confirm the findings of previous studies examining the inhibitory effects of nanoparticles on *C. albicans*. For example, nano-ZnO inhibits biofilm formation by upregulating the gene expressions of *BCY1*, *NRG1*, and *TUP1* and downregulating *PHR1*, *PHR2*, *EFG1*, *HWP1*, *RAS1*, *ALS3* and *ALS4*.[37] Nano-Cu_2O and nano-CuO upregulated the gene expressions of *NRG1* and *TUP1* and downregulated *RAS1*, *CPH1* and *HST7*.[38] Nano-Ag upregulated the expression of *TUP1*, *NRG1*, and *TOR1*,[39] but biofabricated nano-Ag did not upregulate the expression of the *NRG1* and *TUP1* genes.[40] Bojsen *et al.*[41] investigated whether growth arrest by chemical inhibition of TORC1 significantly increased the proportion of amphotericin tolerant persister cells in *C. albicans* and *Candida glabrata* in both planktonic and biofilm growth modes, and our qRT-PCR results may be consistent with these results. Baghiat Esfahani *et al.*[39] showed that the nano-Ag could cause an upregulation of *NRG1* and *TUP1* genes by 3.86-, 2.21-, 1.17-, 1.53-, 1.27-, and 1.06-fold at concentrations of 2× MIC, 1× MIC, and ½× MIC, respectively. Nano-Ag at concentrations of 2× MIC, 1× MIC, and ½× MIC upregulated the expression of *TOR1* by 4.85-, 3.03-, and 1.87-fold, respectively. Taken together, our results show that nano-Fe_3O_4 inhibits biofilm formation by upregulating negative regulators. In this study, different concentrations of fluconazole (2× MIC, 1× MIC, and ½× MIC) displayed upregulation of *TUP1*, *NRG1*, and *TOR1* in *C. albicans*. Several studies have investigated the use of drug

inhibitors for *C. albicans* biofilm formation by upregulating the expression of *TUP1*, *NRG1*, and *TOR1* transcription repressor genes. Hamdy *et al.*[42] described the activation of *TUP1* in *C. albicans* treated with 5-[3-substitued-4-(4-substituedbenzyloxy)-benzylidene]-2-thioxo-thiazolidin-4-one derivatives. Additionally, Patil *et al.*[43] showed that ethyl isothiocyanate inhibited *C. albicans* morphogenesis by upregulating *TUP1, MIG1,* and *NRG1* by 3.10-, 5.84-, and 2.64-fold, respectively. Bastidas *et al.*[44] demonstrated that rapamycin did not inhibit filamentation in the *TOR1-1*/*TOR1* rapamycin-resistant strain.

Conclusion

The emergence and spread of multidrug-resistant *C. albicans* has promoted the development and progression of antibiofilm agents. The present study shows that nano-Fe_3O_4 inhibits *C. albicans* biofilm formation by upregulating negative regulators. *TUP1*, *NRG1*, and *TOR1* may be potential molecular targets in *C. albicans*. Further research is necessary to assess the therapeutic potential of nano-Fe_3O_4 in treating biofilm-associated infections.

Ethical Issues

This study was approved by the Ethics Committee of Islamic Azad University of Shiraz, Shiraz, Iran (code: IR.IAU.SHIRAZ.REC.1401.034) in accordance with the Helsinki Declaration (2008) and the ethical standards of the National Research Committee.

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Authors Contribution

Mahbobeh Baghiat Esfahani: Methodology, Software, Formal analysis, Data curation, Resources, Visualization. Alireza Khodavandi, Fahimeh Alizadeh, and Nima Bahador: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data Curation, Project Administration, Visualization, Supervision, Writing - Original Draft, Writing - Review & Editing.

Conflict of Interest

All of the authors declare that there are no conflicts of interest in this manuscript.

Supplementary Data

Supplementary data (Figures S1-S4) are available at https://doi.org/10.34172/PS.2024.22.

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