



Research Article

Negative Regulators, *TUP1*, *NRG1*, and *TOR1*: Possible Molecular Targets of Antibiofilm Activity of Nano-Fe₃O₄ in *Candida albicans*

Mahbobeh Baghiat Esfahani¹, Alireza Khodavandi^{2*}, Fahimeh Alizadeh², Nima Bahador¹

¹Department of Microbiology, Shiraz Branch, Islamic Azad University, Shiraz, Iran.

²Department of Microbiology, Gachsaran Branch, Islamic Azad University, Gachsaran, Iran.

Article Info

Article History:

Received: 25 Feb 2024

Accepted: 17 Jul 2024

ePublished: 27 Jul 2024

Keywords:

- Biofilm
- Gene expression
- Magnetic iron oxide nanoparticles
- Regulators

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-Fe₃O₄) 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-Fe₃O₄.

Methods: This study examines the antifungal activity of nano-Fe₃O₄ 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-Fe₃O₄.

Results: Nano-Fe₃O₄ 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-Fe₃O₄ upregulates targets *TUP1*, *NRG1*, and *TOR1* in *C. albicans* pre-formed biofilms.

Conclusion: Our results suggest that nano-Fe₃O₄ 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-Fe₃O₄.

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-Fe₃O₄) 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-Fe₃O₄ when scrutinized for its antibiofilm activity, depicted robust potential against *C. albicans* biofilm formation.¹⁸⁻²¹ This study examined the antifungal activity of nano-Fe₃O₄ 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-

*Corresponding Author: Alireza Khodavandi, E-mail: alireza_khodavandi@yahoo.com

©2024 The Author(s). This is an open access article and applies the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>). Non-commercial uses of the work are permitted, provided the original work is properly cited.

Fe₃O₄. 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-Fe₃O₄.

Methods

Materials and microbial strains

The commercial nano-Fe₃O₄ (purity: 98+%, APS: 20 - 30 nm, SSA: 40-60 m²/g, color: dark brown, morphology: spherical, bulk density: 0.84 g/cm³ and true density: 4.8-5.1 g/cm³) 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-Fe₃O₄ 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-Fe₃O₄ 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 × 10³ 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-Fe₃O₄ 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-Fe₃O₄ based on MIC (2× MIC, 1× MIC, and ½× MIC). The microtiter plates contained approximately 1 × 10⁶ 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-Fe₃O₄

The effect of nano-Fe₃O₄ 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 × 10⁶ 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 Thermanox™ coverslips (SPL Life Sciences). The wells were then treated with 4 mL of nano-Fe₃O₄ 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-Fe₃O₄

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-Fe₃O₄ and fluconazole.^{25,30} Briefly, *C. albicans* pre-formed biofilms treated with different concentrations of nano-Fe₃O₄ 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 RNeasy 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 TGCTGCTCCTCCTTATCCCACATC	58 °C	176	This study
	R AGTTTGACCGTATTTTGGCATGA			
TOR1	F AGCTCTTCTGTGGCTTCTAGTGCAA	62 °C	158	This study
	R GTTCTCAAGGCCGCATCCCCTTT			
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 \pm 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-Fe₃O₄ on *C. albicans* and pre-formed biofilms

The MICs for the nano-Fe₃O₄ and fluconazole were 50 and 4 μ g/mL, respectively. Biofilm studies showed that nano-Fe₃O₄ 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-Fe₃O₄ dose-dependently inhibited *C. albicans* pre-formed biofilms by 37.87, 25.74, and 24.78% with 2 \times MIC, 1 \times MIC, and 1/2 \times MIC, respectively. The CV method also revealed that nano-Fe₃O₄ inhibited pre-formed biofilms by 26.22, 13.90, and 11.43% with 2 \times MIC, 1 \times MIC, and 1/2 \times MIC, respectively.

The microscopic architecture of *C. albicans* pre-formed biofilms revealed that nano-Fe₃O₄ reduced hyphae formation. Compared to the untreated control, which exhibited large hyphal cells, the nano-Fe₃O₄-treated biofilms contained more yeast cells and fewer hyphal elements. Additionally, the results of the filamentation index supported the antibiofilm activity of nano-Fe₃O₄ (Tukey's HSD; *p*<0.001 and *p*<0.0001). These findings suggest that nano-Fe₃O₄ inhibits yeast filamentation and biofilm maturation in *C. albicans* (Figure 2).

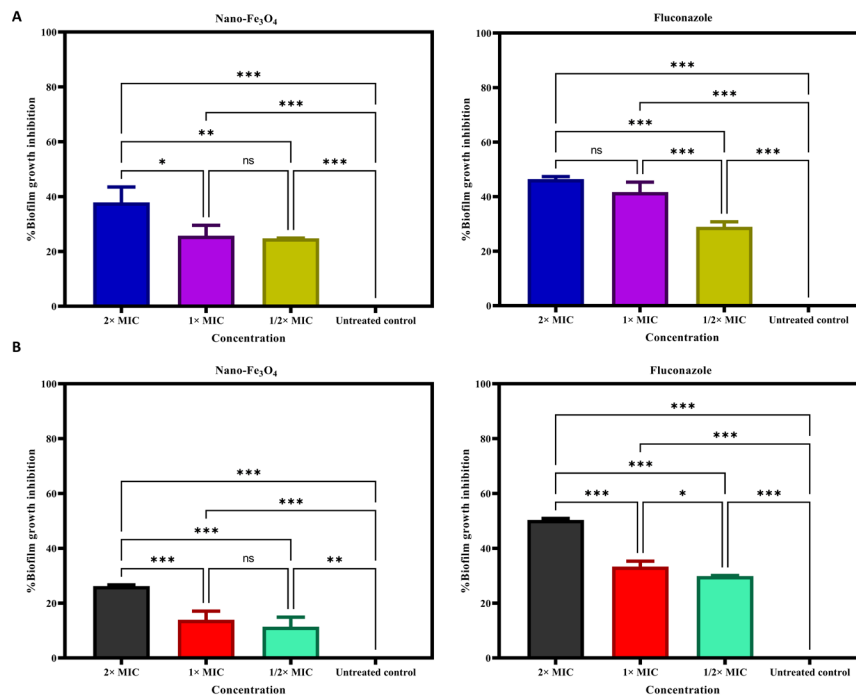


Figure 1. Effect of 2 \times MIC, 1 \times MIC, and 1/2 \times MIC concentrations of nano-Fe₃O₄ in *C. albicans* pre-formed biofilms using (A) XTT and (B) CV methods. The experiment was performed in three replicates (ns: not significant, **p*<0.05, ***p*<0.01, and ****p*<0.001), and graphs are plotted using mean \pm SD.

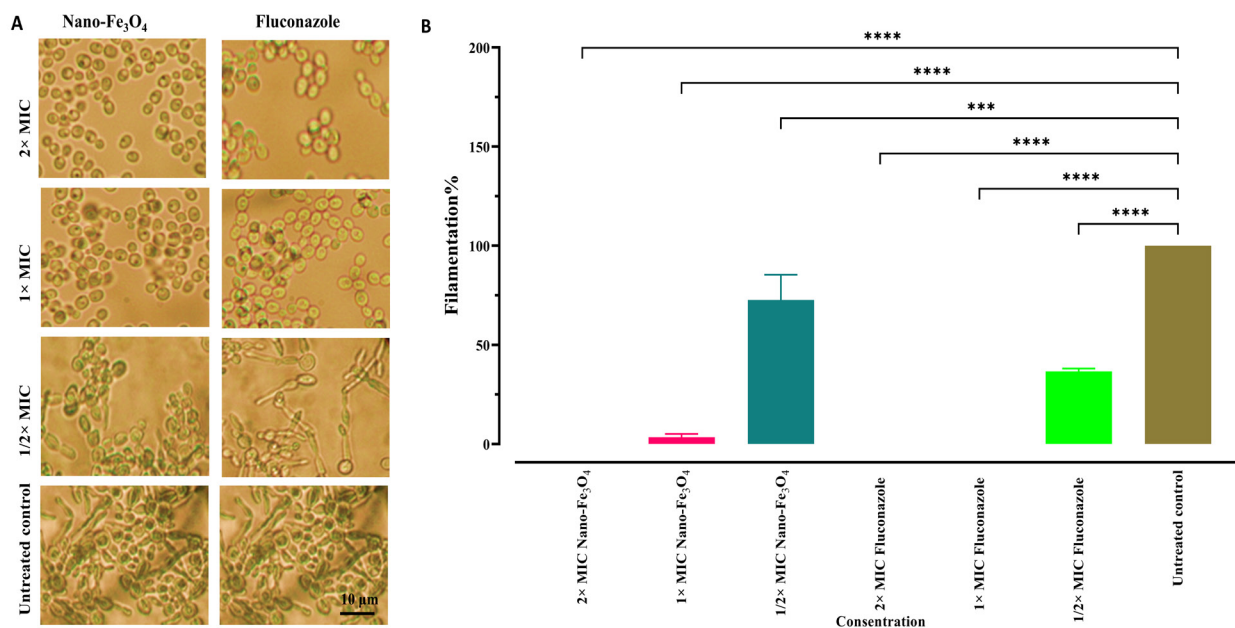


Figure 2. Effect of nano-Fe₃O₄ on the microscopic architecture of *C. albicans* pre-formed biofilms. (A) Illustrations and (B) filamentation index of *C. albicans* pre-formed biofilms after treatment with nano-Fe₃O₄ at different concentrations based on MIC (2× MIC, 1× MIC, and ½× MIC). Data are the mean 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-Fe₃O₄

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-Fe₃O₄ based on MIC (2× MIC, 1× MIC, and ½× MIC). Nano-Fe₃O₄ 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-Fe₃O₄, respectively (Figure 3). These results show that nano-Fe₃O₄ 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-Fe₃O₄ against pre-formed biofilms of *C. albicans*. Nano-Fe₃O₄ 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-Fe₃O₄ affected the expression levels of biofilm-associated genes in *C. albicans* pre-formed biofilms.

Nano-Fe₃O₄ has been reported to exhibit antifungal

activity against *C. albicans*.³¹⁻³⁴ However, the mechanisms responsible for the effect of nano-Fe₃O₄ on antimicrobial activity and its antibiofilm activity have been little studied. Previous reports have shown that the interaction of nano-Fe₃O₄ 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-Fe₃O₄ 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-Fe₃O₄ 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-Fe₃O₄ 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-Fe₃O₄ 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-Fe₃O₄. Various derivatives of nano-Fe₃O₄ have been shown to inhibit biofilm formation.^{20,35,36} Nano-Fe₃O₄ 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-Fe₃O₄ reduced the

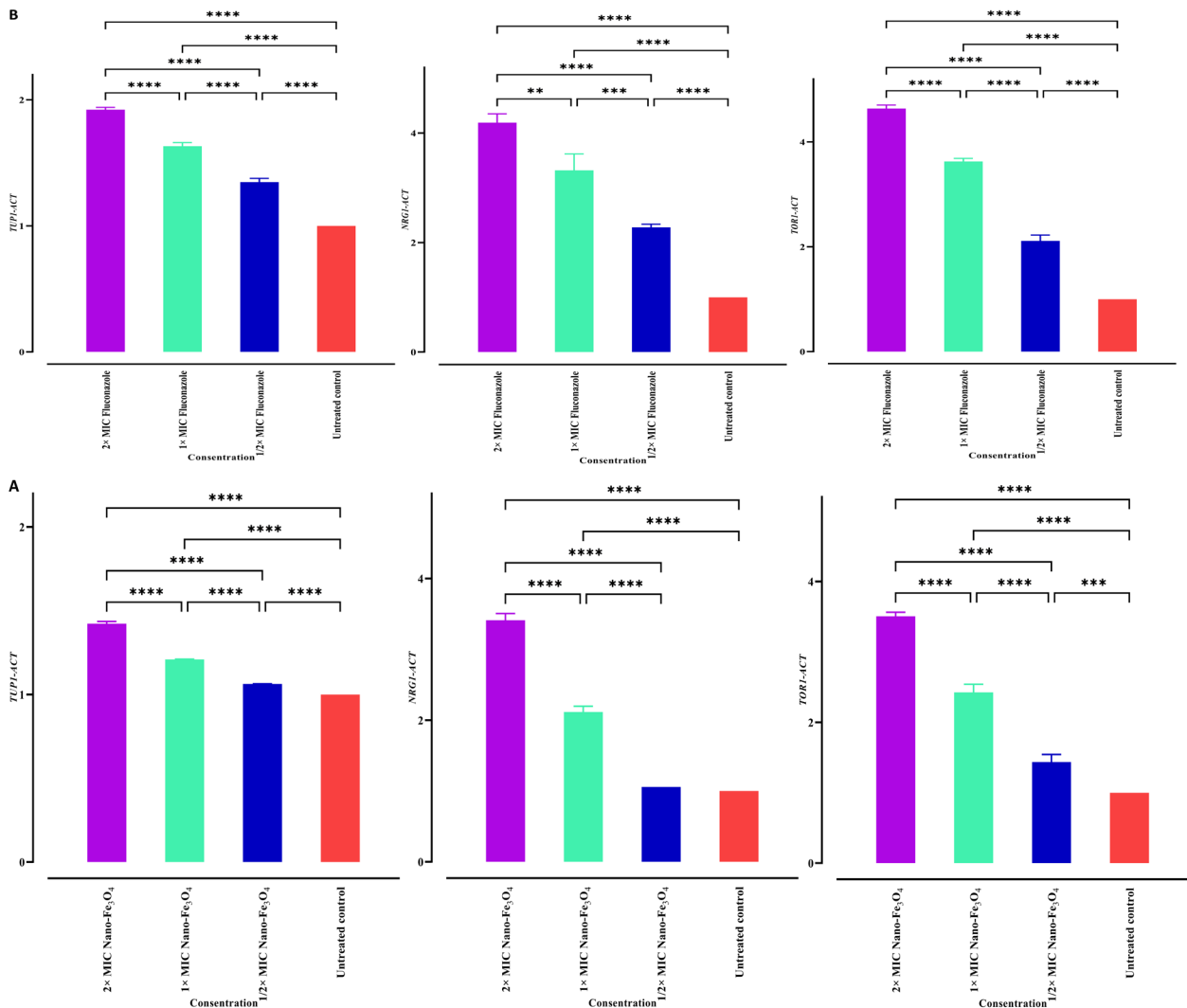


Figure 3. Relative expression levels of *TUP1*, *NRG1*, and *TOR1* in *C. albicans* pre-formed biofilms after treatment with (A) nano-Fe₃O₄ and (B) fluconazole at different concentrations based on MIC (2× MIC, 1× MIC, and 1/2× MIC). Data are the mean values ±SD of three replicates showing significant 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-Fe₃O₄ at 2× MIC, 1× MIC, and 1/2× 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-Fe₃O₄ at 2× MIC, 1× MIC, and 1/2× MIC significantly downregulated the expression of *BCR1*, *ALS1*, *ALS3*, *HWPI*, 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*, *HWPI*, *RAS1*, *ALS3* and *ALS4*.³⁷ Nano-Cu₂O 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 1/2× MIC, respectively. Nano-Ag at concentrations of 2× MIC, 1× MIC, and 1/2× MIC upregulated the expression of *TOR1* by 4.85-, 3.03-, and 1.87-fold, respectively. Taken together, our results show that nano-Fe₃O₄ inhibits biofilm formation by upregulating negative regulators. In this study, different concentrations of fluconazole (2× MIC, 1× MIC, and 1/2× 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-Fe₃O₄ 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-Fe₃O₄ 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.

Acknowledgements

The authors wish to thank the Islamic Azad University of Shiraz for infrastructure facilities. The results presented in this study are part of a PhD thesis (IR.IAU.SHIRAZ.REC.1401.034).

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>.

References

1. Atriwal T, Azeem K, Husain FM, Hussain A, Khan MN, Alajmi MF, Abid M. Mechanistic Understanding of *Candida albicans* biofilm formation and approaches for its inhibition. *Front Microbiol.* 2021;12:638609. doi:10.3389/fmicb.2021.638609
2. Lemberg C, Martinez de San Vicente K, Fróis-Martins R, Altmeier S, Tran VDT, Mertens S, et al. *Candida albicans* commensalism in the oral mucosa is favoured by limited virulence and metabolic adaptation. *PLoS Pathog.* 2022;18:e1010012. doi:10.1371/journal.ppat.1010012
3. Lachat J, Pascault A, Thibaut D, Le Borgne R, Verbavatz JM, Weiner A. Trans-cellular tunnels induced by the fungal pathogen *Candida albicans* facilitate invasion through successive epithelial cells without host damage. *Nat Commun.* 2022;13:3781. doi:10.1038/s41467-022-31237-z
4. Chow EWL, Pang LM, Wang Y. From jekyll to hyde: the yeast-hyphal transition of *Candida albicans*. *Pathogens.* 2021; 10:859. <https://doi.org/10.3390/pathogens10070859>
5. Su C, Lu Y, Liu H. Reduced TOR signaling sustains hyphal development in *Candida albicans* by lowering Hog1 basal activity. *Mol Biol Cell.* 2013;24:385-97. doi:10.1091/mbc.E12-06-0477
6. Sionov RV, Feldman M, Smoum R, Mechoulam R, Steinberg D. Anandamide prevents the adhesion of filamentous *Candida albicans* to cervical epithelial cells. *Sci Rep.* 2020;10:13728. doi:10.1038/s41598-020-70650-6
7. Das S, Goswami AM, Saha T. An insight into the role of protein kinases as virulent factors, regulating pathogenic attributes in *Candida albicans*. *Microb Pathog.* 2022;164:105418. doi:10.1016/j.micpath.2022.105418
8. Flanagan PR, Liu NN, Fitzpatrick DJ, Hokamp K, Köhler JR, Moran GP. The *Candida albicans* TOR-activating GTPases Gtr1 and Rhb1 coregulate starvation responses and biofilm formation. *MSphere.* 2017;2:e00477-17. doi:10.1128/mSphere.00477-17
9. Raut JS, Shinde RB, Chauhan NM, Karuppayil SM. Terpenoids of plant origin inhibit morphogenesis, adhesion, and biofilm formation by *Candida albicans*. *Biofouling.* 2013;29:87-96. doi:10.1080/08927014.2012.749398
10. Pistoia ES, Cosio T, Campione E, Pica F, Volpe A, Marino D, et al. All-trans retinoic acid effect on *Candida albicans* growth and biofilm formation. *J Fungi.* 2022;8:1049. doi:10.3390/jof8101049
11. Manoharan RK, Lee JH, Kim YG, Lee J. Alizarin and chrysazin inhibit biofilm and hyphal formation by *Candida albicans*. *Front Cell Infect Microbiol.* 2017;7:447. doi:10.3389/fcimb.2017.00447
12. Manoharan RK, Lee JH, Lee J. Efficacy of 7-benzyloxyindole and other halogenated indoles to inhibit *Candida albicans* biofilm and hyphal formation. *Microb Biotechnol.* 2018; 11:1060-9. doi:10.1111/1751-7915.13268
13. Lee JH, Kim YG, Choi P, Ham J, Park JG, Lee J. Antibiofilm and antivirulence activities of 6-gingerol and 6-shogaol against *Candida albicans* due to hyphal

- inhibition. *Front Cell Infect Microbiol.* 2018;8:299. doi:10.3389/fcimb.2018.00299
14. Lee JH, Kim YG, Khadke SK, Yamano A, Watanabe A, Lee J. Inhibition of biofilm formation by *Candida albicans* and polymicrobial microorganisms by nepodin via hyphal-growth suppression. *ACS Infect Dis.* 2019;5:1177-87. doi:10.1021/acscinfecdis.9b00033
 15. Khan F, Bamunuarachchi NI, Tabassum N, Jo DM, Khan MM, Kim YM. Suppression of hyphal formation and virulence of *Candida albicans* by natural and synthetic compounds. *Biofouling.* 2021;37:626-55. doi:10.1080/08927014.2021.1948538
 16. Kim YG, Lee JH, Park S, Khadke SK, Shim JJ, Lee J. Hydroquinones including tetrachlorohydroquinone inhibit candida albicans biofilm formation by repressing hyphae-related genes. *Microbiol Spectr.* 2022;10:e0253622. doi:10.1128/spectrum.02536-22
 17. Baghiat Esfahani M, Khodavandi A, Alizadeh F, Bahador N. Antibacterial and anti-biofilm activities of microbial synthesized silver and magnetic iron oxide nanoparticles against *Pseudomonas aeruginosa*. *IEEE Trans Nanobioscience.* 2023;22:956-66. doi:10.1109/TNB.2023.3268138
 18. Baghiat Esfahani M, Khodavandi A, Alizadeh F, Bahador N. Biofilm-associated genes as potential molecular targets of nano-Fe₃O₄ in *Candida albicans*. *Pharmacol Rep.* 2023;75:682-94. doi: 0.1007/s43440-023-00467-3
 19. Chifiriuc C, Grumezescu V, Grumezescu AM, Saviuc C, Lazăr V, Andronescu E. Hybrid magnetite nanoparticles/Rosmarinus officinalis essential oil nanobiosystem with antibiofilm activity. *Nanoscale Res Lett.* 2012;7:209. doi:10.1186/1556-276X-7-209
 20. Niemirowicz K, Durnaś B, Tokajuk G, Piktel E, Michalak G, Gu X, et al. Formulation and candidacidal activity of magnetic nanoparticles coated with cathelicidin LL-37 and ceragenin CSA-13. *Sci Rep.* 2017;7:4610. doi:10.1038/s41598-017-04653-1
 21. Vera-González N, Shukla A. Advances in biomaterials for the prevention and disruption of *Candida* biofilms. *Front Microbiol.* 2020;11:538602. doi:10.3389/fmicb.2020.538602
 22. Clinical and Laboratory Standards Institute: Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard. CLSI document M27-A3 and Supplement S. 2008.
 23. Clinical and Laboratory Standards Institute: Reference method for broth dilution antifungal susceptibility testing of yeasts; informational supplement. CLSI document M27-S4. 2012.
 24. Braga PC, Culici M, Alfieri M, Dal Sasso M. Thymol inhibits *Candida albicans* biofilm formation and mature biofilm. *Int J Antimicrob Agents.* 2008;31:472-7. doi:10.1016/j.ijantimicag.2007.12.013
 25. Khodavandi A, Harmal NS, Alizadeh F, Scully OJ, Sidik SM, Othman F, et al. Comparison between allicin and fluconazole in *Candida albicans* biofilm inhibition and in suppression of *HWPI* gene expression. *Phytomedicine.* 2011;19:56-63. doi:10.1016/j.phymed.2011.08.060
 26. Costa EM, Silva S, Tavaría FK, Pintado MM. Insights into chitosan antibiofilm activity against methicillin-resistant *Staphylococcus aureus*. *J Appl Microbiol.* 2017;122:1547-57. doi:10.1111/jam.13457
 27. Khodavandi A, Alizadeh F, Jafarzadeh M. Synergistic interaction of fluconazole/amphotericin B on inhibition of enzymes contributes to the pathogenesis of *Candida tropicalis*. *Pharm Sci.* 2018;24:280-90. doi:10.15171/PS.2018.41
 28. de Chaumont F, Dallongeville S, Chenouard N, Hervé N, Pop S, Provoost T, et al. Icy: an open bioimage informatics platform for extended reproducible research. *Nat Methods.* 2012;9:690-6. doi:10.1038/nmeth.2075
 29. Rocha FAC, Alves AMCV, Rocha MFG, Cordeiro RA, Brillhante RSN, Pinto ACMD, et al. Tumor necrosis factor prevents *Candida albicans* biofilm formation. *Sci Rep.* 2017;7:1206. doi:10.1038/s41598-017-01400-4
 30. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc.* 2008;3:1101-8. doi:10.1038/nprot.2008.73
 31. Seddighi NS, Salari S, Izadi AR. Evaluation of antifungal effect of iron-oxide nanoparticles against different *Candida* species. *IET Nanobiotechnol.* 2017;11:883-8. doi:10.1049/iet-nbt.2017.0025
 32. Zare-Khafri M, Alizadeh F, Nouripour-Sisakht S, Khodavandi A, Gerami M. Inhibitory effect of magnetic iron-oxide nanoparticles on the pattern of expression of lanosterol 14 α -demethylase (*ERG11*) in fluconazole-resistant colonising isolate of *Candida albicans*. *IET Nanobiotechnol.* 2020;14:375-81. doi:10.1049/iet-nbt.2019.0354
 33. Abbas HS, Ismaeil TAM. An analysis of iron oxide nanoclusters as antifungal agents and in silico model with lanosterol 14 α -demethylase in *Candida albicans*. *NanoWorld J.* 2022;8:36-41. doi:10.17756/nwj.2022-098
 34. Adhikari A, Chhetri K, Acharya D, Pant B, Adhikari A. Green synthesis of iron oxide nanoparticles using *Psidium guajava* L. leaves extract for degradation of organic dyes and anti-microbial applications. *Catalysts.* 2022;12:1188. doi:10.3390/catal12101188
 35. Thambirajoo M, Maarof M, Lokanathan Y, Katas H, Ghazalli NF, Tabata Y, et al. Potential of nanoparticles integrated with antibacterial properties in preventing biofilm and antibiotic resistance. *Antibiotics.* 2021;10:1338. doi:10.3390/antibiotics10111338
 36. Khalid HF, Tehseen B, Sarwar Y, Hussain SZ, Khan WS, Raza ZA, et al. Biosurfactant coated silver and iron oxide nanoparticles with enhanced anti-biofilm and anti-adhesive properties. *J Hazard Mater.* 2019;364:441-8. doi:10.1016/j.jhazmat.2018.10.049
 37. Joshi KM, Shelar A, Kasabe U, Nikam LK, Pawar

- RA, Sangshetti J, et al. Biofilm inhibition in *Candida albicans* with biogenic hierarchical zinc-oxide nanoparticles. *Biomater Adv.* 2022;134:112592. doi:10.1016/j.msec.2021.112592
38. Padmavathi AR, Das A, Priya A, Sushmitha TJ, Pandian SK, Toleti SR. Impediment to growth and yeast-to-hyphae transition in *Candida albicans* by copper oxide nanoparticles. *Biofouling.* 2020;36:56-72. doi:10.1080/08927014.2020.1715371
39. Baghiat Esfahani M, Khodavandi A, Alizadeh F, Bahador N. Possible molecular targeting of biofilm-associated genes by nano-Ag in *Candida albicans*. *Appl Biochem Biotechnol.* 2024;196:4205-33. doi:10.1007/s12010-023-04758-6
40. Halbandge SD, Jadhav AK, Jangid PM, Shelar AV, Patil RH, Karuppayil SM. Molecular targets of biofabricated silver nanoparticles in *Candida albicans*. *J Antibiot.* 2019;72:640-4. doi:10.1038/s41429-019-0185-9
41. Bojsen R, Regenber B, Gresham D, Folkesson A. A common mechanism involving the TORC1 pathway can lead to amphotericin B-persistence in biofilm and planktonic *Saccharomyces cerevisiae* populations. *Sci Rep.* 2016;6:21874. doi:10.1038/srep21874
42. Hamdy R, Soliman SSM, Alsaadi AI, Fayed B, Hamoda AM, Elseginy SA, et al. Design and synthesis of new drugs inhibitors of *Candida albicans* hyphae and biofilm formation by upregulating the expression of *TUP1* transcription repressor gene. *Eur J Pharm Sci.* 2020;148:105327. doi:10.1016/j.ejps.2020.105327
43. Patil SB, Sharma RK, Gavandi TC, Basrani ST, Chougule SA, Yankanchi SR, et al. Ethyl isothiocyanate as a novel antifungal agent against *Candida albicans*. *Curr Microbiol.* 2024;81:29. doi:10.1007/s00284-023-03542-3
44. Bastidas RJ, Heitman J, Cardenas ME. The protein kinase Tor1 regulates adhesin gene expression in *Candida albicans*. *PLoS Pathog.* 2009;5:e1000294. doi:10.1371/journal.ppat.1000294