

RESEARCH PAPER

Voriconazole-Loaded Nanoliposomes against *Cryptococcus neoformans*: Antifungal Activity, Biofilm Biomass Reduction, and Expression of *CXT1* and *LAC1*

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ABSTRACT

Objective(s): *Cryptococcus neoformans* is an opportunistic fungal pathogen whose virulence is associated with capsule formation, melanin production, and biofilm development. This pilot study evaluated the biological activity of voriconazole-loaded nanoliposomes (VCZ-NLs), with emphasis on biofilm biomass reduction and potential modulation of the virulence-associated genes *CXT1* and *LAC1*.

Materials and Methods: A reference strain of *C. neoformans* was used in this pilot study. VCZ-NLs were prepared and characterized using dynamic light scattering, zeta potential analysis, and transmission electron microscopy. Antifungal susceptibility of yeast-phase cells was determined by minimum inhibitory concentration (MIC) testing. Biofilm biomass was quantified using the crystal violet microtiter assay. Urease activity was qualitatively assessed using Christensen's urea agar. Relative expression of *CXT1* and *LAC1* was evaluated by quantitative real-time PCR using the $2^{-\Delta\Delta Ct}$ method, with untreated cells as the calibrator. Experiments were performed in technical triplicate as part of a pilot design.

Results: *C. neoformans* showed a VCZ MIC of 4 µg/mL, and liposomal encapsulation did not alter the observed yeast-phase MIC. Urease activity showed no apparent change, while *CXT1* expression showed a modest downward trend and *LAC1* expression remained largely unchanged under the tested conditions. VCZ-NLs produced a greater reduction in crystal violet-measured biofilm biomass than free VCZ under the conditions tested.

Conclusion: VCZ-NLs preserved planktonic antifungal activity and produced a greater reduction in crystal violet-measured biofilm biomass under the tested conditions. Because crystal violet measures biomass rather than viability, these findings do not establish a biofilm-specific or enhanced antifungal effect. Further studies with biological replication and viability-based assays are warranted.

Keywords: *Cryptococcus neoformans*; Voriconazole; Biofilms; Urease; Nanoliposomes

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INTRODUCTION

Cryptococcus neoformans is a predominant opportunistic fungal pathogen responsible for life-threatening infections, particularly among immunocompromised individuals (1). Cryptococcal meningoencephalitis remains a serious clinical concern worldwide, with substantial morbidity and mortality despite antifungal therapy (2, 3). The pathogenicity of *C. neoformans* is driven by a complex array of virulence determinants, including the polysaccharide capsule, melanin production, and biofilm formation (4). The cryptococcal capsule, primarily composed of glucuronoxylomannan and galactoxylomannan, plays a critical role in immune

evasion and fungal survival (5). Capsule biosynthesis depends on several glycosyltransferases, including a β -1, 2-xylosyltransferase encoded by the cryptococcal xylosyltransferase (*CXT1*) gene (6, 7). Another important virulence factor is melanin, synthesized via the laccase enzyme encoded by *LAC1*, which protects fungal cells against oxidative stress and enhances intracellular survival (8). In addition to these classical determinants, biofilm development has emerged as an important contributor to persistence and reduced susceptibility to antifungal treatment in *C. neoformans* (9, 10). Current treatment of cryptococcosis relies mainly on azoles

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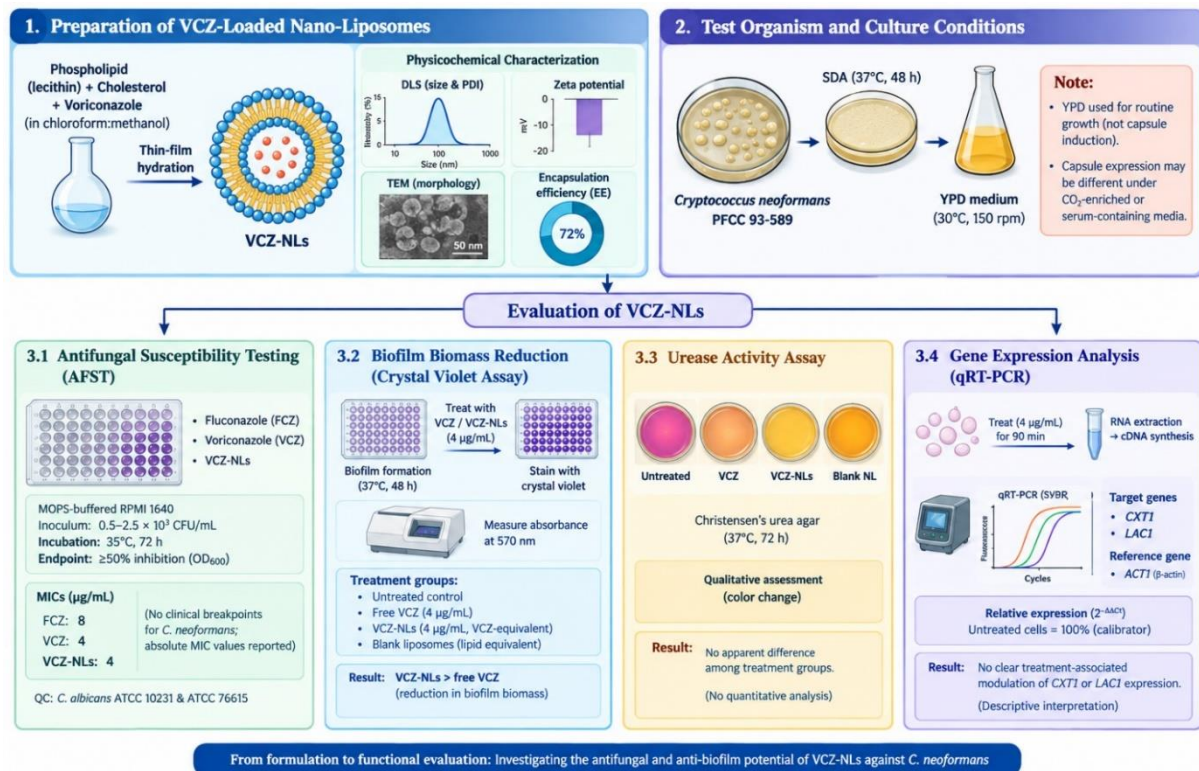


Fig. 1. Schematic overview of VCZ-loaded nanoliposomes (VCZ-NLs) and their evaluation against *Cryptococcus neoformans*. VCZ-NLs preserved planktonic susceptibility and produced a greater reduction in crystal-violet-measured biofilm biomass than free VCZ, with no apparent changes in urease activity or *CXT1/LAC1* expression.

and polyenes; however, toxicity, pharmacokinetic limitations, and emerging resistance may limit therapeutic effectiveness. Voriconazole (VCZ), a second-generation triazole, exhibits broad antifungal activity but still faces challenges in drug delivery and therapeutic performance (11). Nano-based delivery systems, particularly nanoliposomes (NLs), have attracted attention as versatile platforms for antimicrobial drug delivery because they can facilitate controlled drug release, allow physicochemical and surface modification, and potentially improve drug delivery to biofilm-associated infections (12, 13). Lipid-based nanocarriers have also been investigated as potential approaches for improving antimicrobial drug delivery, including delivery to biofilm-associated targets (14, 15). Building upon our previous findings, which demonstrated the efficacy of VCZ-NLs against *Candida albicans* and *Aspergillus flavus* (16, 17), this study aims to expand this framework to *C. neoformans*. Here, we evaluated the antifungal activity of VCZ-NLs against yeast-phase *C. neoformans* and their effect on crystal violet-measured biofilm biomass, urease activity, and the relative expression of the virulence-associated genes *CXT1* and *LAC1*. The overall experimental workflow and rationale of the study

are illustrated in Figure 1, highlighting the development of VCZ-NLs and their evaluation across planktonic susceptibility, biofilm biomass reduction, urease activity, and gene expression profiling.

MATERIALS AND METHODS

Study and experimental design

All experiments were performed using a well-characterized reference strain of *C. neoformans*. The experimental workflow comprised four principal assays: (i) antifungal susceptibility testing (AFST) against fluconazole (FCZ), free VCZ, and VCZ-NLs; (ii) biofilm biomass quantification using the crystal violet microtiter plate assay; (iii) qualitative assessment of urease activity using Christensen's urea agar; and (iv) quantitative analysis of the relative expression of the virulence-associated genes *CXT1* and *LAC1* by quantitative real-time PCR (qRT-PCR). Each experimental condition was assessed in technical triplicate. This pilot proof-of-concept study included no independent biological replicates. Thus, the three measurements obtained for each condition represent technical replicates of the same experimental preparation rather than independent biological experiments. Data were therefore interpreted descriptively, and no

inferential statistical testing was used to support definitive biological conclusions. The findings should consequently be considered preliminary and require validation using independent biological replicates and multiple clinical isolates in future studies.

Organism, media, and growth conditions

For this study, *C. neoformans* strain PFCC 93-589 was obtained from the Pathogenic Fungi Culture Collection at the Pasteur Institute of Iran (<http://fa.pasteur.ac.ir/VisitDetails.aspx?Id=1311>). The strain was initially cultivated on Sabouraud dextrose agar (SDA) plates and incubated at 37°C for 48 hours. Fungal cells were subsequently cultured in yeast extract-peptone-dextrose (YPD) medium, composed of 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) dextrose, and incubated at 30°C under moderate agitation (150 rpm)(18). It should be noted that YPD medium was used for routine growth conditions rather than optimal capsule induction.

Antifungal Susceptibility Testing (AFST)

The antifungal susceptibility of *C. neoformans* PFCC 93-589 was determined by the broth microdilution method according to the CLSI M27 reference method (19). Briefly, standardized yeast suspensions were prepared in MOPS-buffered RPMI 1640 medium, and the final inoculum was adjusted to $0.5\text{--}2.5 \times 10^3$ CFU/mL. Two-fold serial dilutions of fluconazole (FCZ; 0.125–64 µg/mL), voriconazole (VCZ; 0.031–16 µg/mL), and voriconazole-loaded nanoliposomes (VCZ-NLs; 0.031–16 µg/mL, expressed as VCZ-equivalent concentrations) were prepared. After inoculation, the microdilution plates were incubated at 35°C for 72 h. MICs were determined based on both visual assessment of fungal growth and spectrophotometric measurements at 600 nm, with the MIC endpoint defined as $\geq 50\%$ inhibition of growth compared with the drug-free growth control. Appropriate growth and sterility controls were included, and each condition was tested in technical triplicate. Quality-control assays were performed using *Candida albicans* ATCC 10231 and ATCC 76615. The current CLSI M27M44S reference was followed for the MIC interpretive framework and quality-control assessment. Since clinical MIC breakpoints for *C. neoformans* are not established for fluconazole or voriconazole, the results were reported as absolute MIC values rather than categorized as susceptible, intermediate/SDD, or resistant. Where applicable, epidemiological cutoff values (ECVs) were considered only as population-

based reference values and were not interpreted as clinical susceptibility or resistance categories.

Preparation of voriconazole-loaded nanoliposomes and physicochemical characterization

VCZ-NLs were prepared using a modified thin-film hydration method based on previously reported protocols (16, 17). Briefly, lecithin and cholesterol, at a weight ratio of 45:5 (w/w), together with VCZ, were dissolved in chloroform: methanol (2:1, v/v). The organic solvent was evaporated under reduced pressure to form a thin lipid film on the inner wall of a round-bottom flask. The dried film was hydrated with distilled water to generate multilamellar vesicles, followed by probe sonication in an ice bath to obtain nanosized liposomes. Particle size, polydispersity index (PDI), and zeta potential were determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments, UK). Morphological evaluation was performed by transmission electron microscopy (TEM) (LEO 906; Zeiss, Germany) operating at 120 kV. Blank liposomes were prepared using the same procedure without VCZ. The VCZ-to-total-lipid ratio used in the formulation was 0.25 (w/w).

Encapsulation efficiency

Encapsulation efficiency (EE) was determined according to previously described methods (16, 17). The nanoliposomal suspension was centrifuged to separate unencapsulated VCZ from the liposomal fraction. Subsequently, VCZ was extracted by adding chloroform (500 µL chloroform to 1 mL liposomal suspension), followed by vortexing and phase separation. The absorbance of the organic phase was measured at 256 nm using a UV-Vis spectrophotometer. Encapsulation efficiency was calculated using the standard formula described previously(20): $EE (\%) = (\text{amount of encapsulated VCZ} / \text{total amount of VCZ added}) \times 100$.

Biofilm formation assay

Biofilm formation was quantified using the crystal violet microtiter plate assay (21). Standardized fungal suspensions (1×10^6 CFU/mL prepared in RPMI 1640 medium) were inoculated into flat-bottom 96-well polystyrene plates and incubated at 37 °C for 48 h to allow biofilm development. Following biofilm establishment, wells were treated with free VCZ or VCZ-NLs at 4 µg/mL, with the VCZ-NL concentration expressed as the VCZ-equivalent concentration. In addition, blank liposomes (Blank NL), prepared identically to VCZ-NLs but without drug, were included as a vehicle control at a lipid concentration equivalent to that used in the VCZ-NL formulation. After treatment, non-adherent cells were removed

Table 1. Sequences of primers.

Primers Name	Sequence (5'-3')	PCR Product Size (bp)	Reference
Cxt1 -F Cxt1 -R	CGGAATGGTATGCCTATGTC TCTCTTCTCCAGGTTGCTC	163	(18)
LAC1 -F LAC1 -R	AGAAGGGAAGGAAGGTGA TATACCTCACAACGCCAA	85	(8)
ACT1-F ACT1-R	TGC CTCTGGTCGTACCACTG GCGAAACCTTCGTAGATGGG	85	(8)

by gentle washing with sterile phosphate-buffered saline (PBS). Biofilms were fixed with methanol and stained with 0.1% crystal violet. After staining, excess and unbound crystal violet was removed by gently washing the wells with sterile distilled water. The bound dye was then solubilized using 95% ethanol. Absorbance was measured at 570 nm using a microplate reader. All experiments were performed in technical triplicate.

Urease activity assay

Urease activity was evaluated using Christensen's urea agar as previously described (22). Freshly cultured *C. neoformans* colonies were inoculated onto the medium and exposed to free VCZ or VCZ-NLs at a VCZ-equivalent concentration of 4 µg/mL. Untreated cells and the blank nanoliposome formulation were included as controls. The inoculated media were incubated at 37 °C for 72 h. Urease activity was assessed qualitatively based on the development of the characteristic color change in the medium. No growth-zone diameter measurements or quantitative statistical analyses were performed.

RNA extraction, cDNA synthesis, and quantitative RT-PCR

C. neoformans cells were exposed to 4 µg/mL voriconazole (VCZ) or VCZ-NLs, expressed as the VCZ-equivalent concentration, for 90 min before RNA extraction. Total RNA was extracted from treated and untreated *C. neoformans* cells using established protocols (18). RNA was reverse-transcribed into cDNA using the Sensiscript Reverse Transcription Kit (QIAGEN, Germany). Primer sequences are listed in Table 1. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Master Mix (AMPLIQON, Denmark) in 10 µL reactions containing 0.5 µL cDNA, 0.25 µL of each primer, 5 µL master mix, and nuclease-free water. Thermal cycling consisted of 95 °C for 5 min followed by 45 cycles of 95 °C for 10 s, 58 °C for 35 s, and 70 °C for 20 s. Relative expression of *CXT1* and *LAC1* was calculated using the $2^{-\Delta\Delta Ct}$ method with *ACT1* (β -actin) as the reference gene. All reactions were performed in technical triplicate (23, 24). Untreated cells were

used as the calibrator. As this was a preliminary pilot study, amplification efficiency, melt-curve analysis, and independent biological replication were not evaluated.

Statistical analysis

All experimental conditions were assessed in technical triplicate. Data are presented as mean $\pm$ standard deviation (SD) of the technical replicates. Because no independent biological replicates were included, the findings were interpreted descriptively, and exploratory statistical inference was not used to support definitive biological conclusions.

RESULTS

Quantitative measurements are presented as mean $\pm$ SD of three technical replicates per condition. Qualitative findings are described descriptively. As this study was a preliminary pilot investigation using a single reference strain, biological replicates were not included, and the findings should be interpreted with caution.

Physicochemical characterization of VCZ-loaded nano-liposomes

The physicochemical properties of the prepared VCZ-NLs were evaluated by dynamic light scattering (DLS) and zeta potential analysis. The mean particle size was 110.0 ± 3.08 nm, with a polydispersity index (PDI) of 0.359 ± 0.11 . The particle-size distribution was centered approximately around 100 nm, with a minor fraction of larger particles/aggregates (Figure 2). The measured zeta potential was -9.30 ± 0.48 mV, indicating a mildly negative surface charge. These findings were consistent with the formation of nanoscale liposomal vesicles with a relatively narrow particle-size distribution under the tested conditions.

Transmission electron microscopy

Transmission electron microscopy (TEM) analysis showed predominantly spherical, bilayered vesicles consistent with the nanoliposomal structure of VCZ-NLs (Figure 3). The observed vesicular morphology was consistent with the nanoscale dimensions determined by DLS.

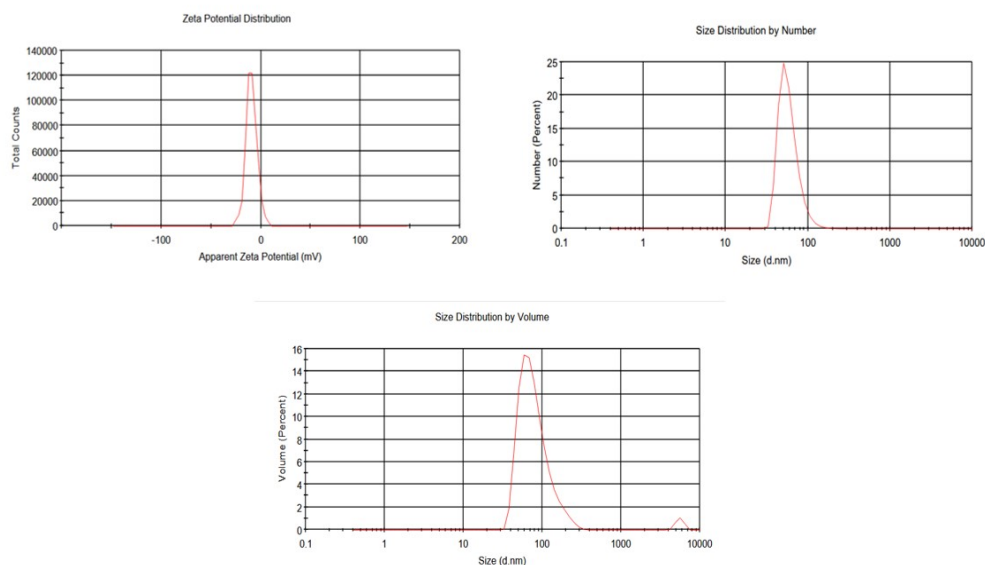


Fig. 2. Zeta potential and particle size distribution of voriconazole-loaded nanoliposomes. The VCZ-NLs showed a mildly negative surface charge, with a mean zeta potential of -9.30 ± 0.48 mV, and a particle-size distribution centered at approximately 100 nm. The mean particle size was 110.0 ± 3.08 nm, with a PDI of 0.359 ± 0.11 . A minor population of larger particles/aggregates was also observed.

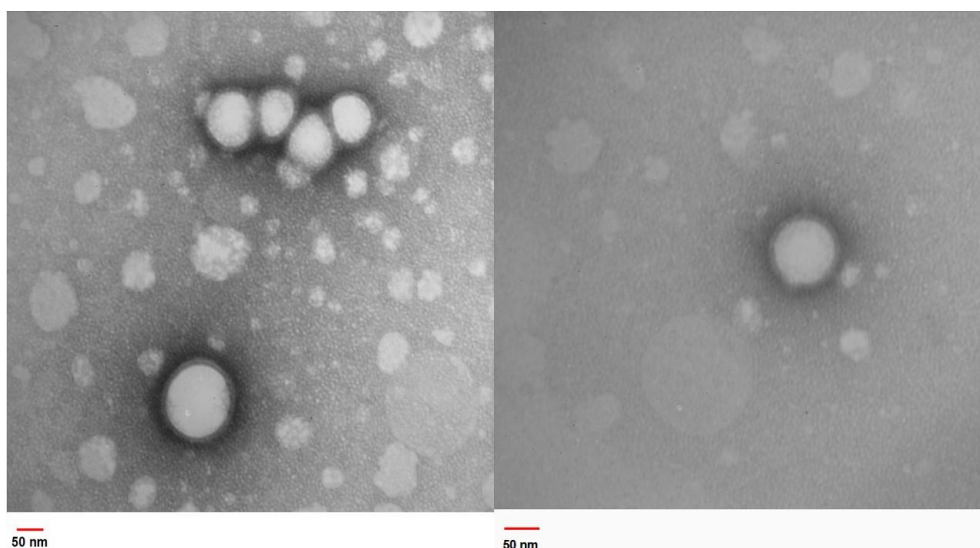


Fig. 3. Transmission electron microscopy (TEM) image of voriconazole-loaded nanoliposomes (VCZ-NLs). The VCZ-NLs appeared predominantly spherical and showed a discernible bilayer membrane structure. Scale bar: 50 nm.

Drug encapsulation efficiency

The EE analysis indicated that approximately $72 \pm 3\%$ of the NL vesicles successfully entrapped VCZ, confirming efficient drug loading within the lipid bilayer. This level of encapsulation is consistent with previously reported liposomal antifungal formulations.

Antifungal Susceptibility Testing

The MICs of FCZ, VCZ, and VCZ-NLs against *C. neoformans* PFCC 93-589 were 8, 4, and 4 $\mu\text{g/mL}$,

respectively (Table 2). The MIC of VCZ-NLs was expressed as the voriconazole-equivalent concentration. Because CLSI M27M44S, 4th edition (2026), does not provide clinical MIC breakpoints for *C. neoformans* for fluconazole or voriconazole, the observed MIC values were reported without assignment to susceptible or resistant categories. Under the conditions tested, nanoencapsulation therefore did not alter the observed planktonic VCZ MIC.

Table 2. Minimum inhibitory concentrations of fluconazole, voriconazole, and voriconazole-loaded nanoliposomes against *Cryptococcus neoformans* PFCC 93-589.

Antifungal treatment	MIC ($\mu\text{g/mL}$)	Clinical interpretation M27M44S-Ed4
FCZ	8	Not categorized
VCZ	4	Not categorized
VCZ-NLs	4	Not categorized

* MIC is expressed as the voriconazole-equivalent concentration. **Abbreviation:** MIC, minimum inhibitory concentration; FCZ, fluconazole; VCZ, voriconazole; VCZ-NLs, voriconazole-loaded nanoliposomes. **Note:** CLSI M27M44S, 4th edition (2026), does not provide clinical MIC breakpoints for *Cryptococcus neoformans* for fluconazole or voriconazole. Therefore, the MIC values are reported descriptively and are not assigned to susceptible, intermediate/SDD, or resistant categories. Epidemiological cutoff values, when available, should not be interpreted as clinical susceptibility or resistance breakpoints.

Effect of VCZ and VCZ-NLs on biofilm biomass

The effects of free VCZ and VCZ-NLs on preformed *C. neoformans* biofilms were evaluated using the crystal violet assay, which provides an estimate of total biofilm biomass rather than cell viability. Treatments were applied at 4 $\mu\text{g/mL}$, corresponding to the planktonic VCZ MIC, with the VCZ-NL concentration expressed as the VCZ-equivalent concentration. Free VCZ produced a reduction in crystal-violet-measured biofilm biomass compared with the untreated control, whereas the blank nanoliposome formulation showed no apparent reduction under the tested conditions. VCZ-NLs produced a greater reduction in crystal-violet-measured biofilm biomass than free VCZ under the conditions tested (Figure 4). Because the experiments included technical triplicates but no independent biological replicates, this comparison was interpreted descriptively and should be considered preliminary rather than definitive evidence of a biofilm-specific or enhanced antifungal effect. Moreover, because

treatment was performed at the planktonic VCZ MIC, a contribution of effects on fungal growth or survival to the observed reduction in measured biomass cannot be excluded.

Urease activity

Qualitative evaluation of urease activity showed no apparent difference in the characteristic color change of the urea medium among cells treated with free VCZ, VCZ-NLs, the blank nanoliposome formulation, and the untreated control under the conditions tested (Figure 5). The treatments were applied at 4 $\mu\text{g/mL}$, corresponding to the planktonic VCZ MIC, with the VCZ-NL concentration expressed as the VCZ-equivalent concentration. Thus, VCZ-NLs did not show an apparent effect on urease activity compared with free VCZ in this qualitative assay. Because the assay was qualitative, no quantitative comparison or statistical analysis of urease activity was performed.

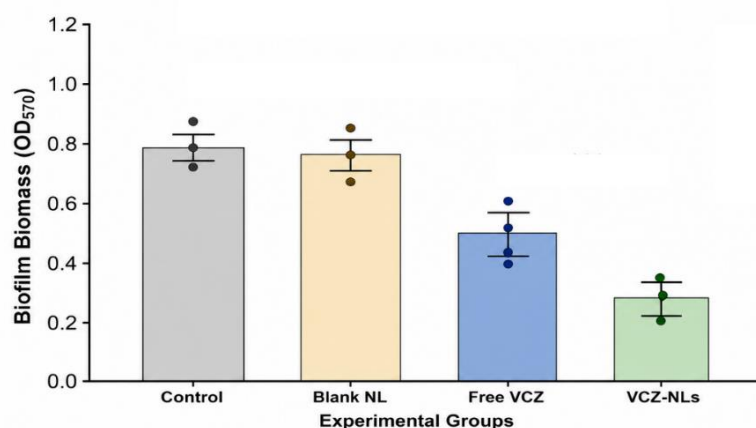


Fig. 4. Effect of free VCZ and VCZ-NLs on preformed *C. neoformans* biofilm biomass. Biofilm biomass was quantified using the crystal violet microtiter assay following treatment with free VCZ or VCZ-NLs at 4 $\mu\text{g/mL}$ VCZ-equivalent concentration. Blank nanoliposomes (Blank NL) were included as a vehicle control. Crystal violet measurements represent total biofilm biomass rather than cell viability. Data are presented as mean $\pm$ SD of technical triplicates. VCZ-NLs produced a greater reduction in crystal violet-measured biofilm biomass than free VCZ under the tested conditions. Because no independent biological replicates were included, the comparison is presented as a descriptive, exploratory observation.

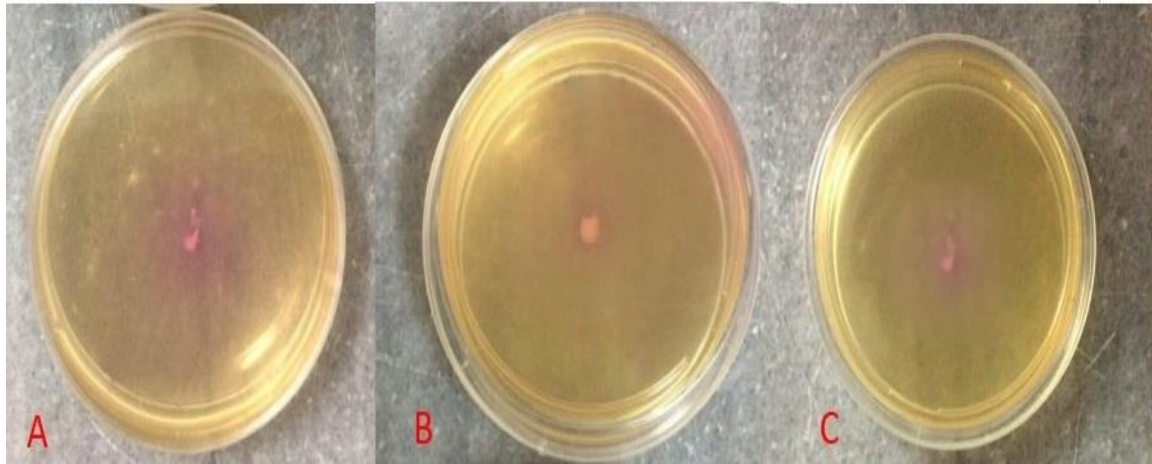


Fig. 5. Urease activity of *C. neoformans* cultured on Christensen's urea agar following exposure to antifungal treatments. (A) Untreated control; (B) free VCZ; (C) VCZ-NLs. Treatments were applied at a VCZ-equivalent concentration of 4 $\mu\text{g/mL}$, corresponding to the planktonic VCZ MIC. Urease activity was qualitatively assessed based on the characteristic color change of the medium. No apparent difference in urease activity was observed among the tested groups.

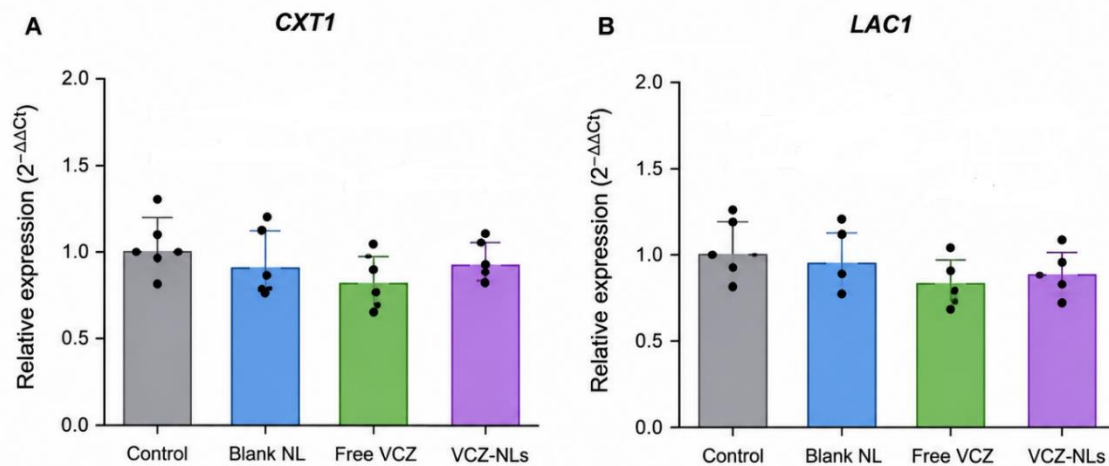


Fig. 6. Relative expression of *CXT1* and *LAC1* following treatment with free VCZ and VCZ-NLs. Gene expression was determined using the $2^{-\Delta\Delta C_t}$ method with *ACT1* as the reference gene and untreated cells as the calibrator. Data are presented as mean $\pm$ SD of technical triplicates. The observed differences are presented descriptively because no independent biological replicates were included. No definitive biological inference was made from the technical replicate data.

Gene expression analysis

Total RNA was successfully extracted from *C. neoformans* cells treated with free VCZ, VCZ-NLs, and untreated controls. RNA integrity and purity were confirmed by electrophoresis and spectrophotometric analysis before cDNA synthesis. Relative expression of *CXT1* and *LAC1* was quantified by qRT-PCR following normalization to *ACT1*, with untreated cells set as the calibrator (100%). Compared with untreated cells, *CXT1* expression showed a modest downward trend following treatment, with relative expression levels of approximately 85% and 84% in the VCZ and VCZ-NL groups, respectively. In contrast, *LAC1* expression remained largely unchanged, with only minor fluctuations of less than 10% among the

tested conditions (Figure 6). Given the absence of independent biological replicates, these differences were interpreted descriptively and should not be considered evidence of statistically supported changes in gene expression. Overall, the pilot data did not indicate a clear treatment-associated modulation of *CXT1* or *LAC1* expression under the conditions tested.

DISCUSSION

The present pilot study evaluated the physicochemical properties and biological activity of VCZ in free and NL forms against *C. neoformans*. Overall, the NL formulation demonstrated appropriate nanoscale characteristics and a measurable encapsulation efficiency, supporting its suitability as a lipid-based delivery system (25, 26).

The VCZ-NLs showed a mean particle size of approximately 110 nm with a moderate PDI, indicating acceptable particle-size distribution following drug incorporation. Comparable nanoliposomal formulations reported in the Nanomedicine Journal have also demonstrated nanoscale particle sizes, measurable encapsulation efficiency, negative zeta potential, and predominantly spherical bilayer morphology, supporting the use of these physicochemical parameters for characterization of lipid-based nanocarriers (27). The PDI = 0.359 indicates a moderate size distribution of the NL formulation. While values below 0.3 are generally considered indicative of a narrow distribution, slightly higher PDI values have been reported for lipid-based nanocarriers prepared by conventional methods. This level of polydispersity may reflect heterogeneity in vesicle size; however, the present study did not directly evaluate whether this characteristic affected biological activity. As reported in comparable liposomal systems (16, 17, 25), drug loading typically increases vesicle diameter relative to blank liposomes, which was also observed here. The zeta potential of VCZ-NLs was -9.30 ± 0.48 mV, indicating a mildly negative surface charge and relatively limited electrostatic repulsion between the nanoliposomes. Such surface-charge values have been reported for neutral or slightly anionic liposomal antifungal systems. However, zeta potential alone should not be interpreted as evidence of physical or long-term formulation stability, particularly because stability over time was not systematically evaluated in the present study. Dispersion stability in liposomal systems may also be influenced by non-electrostatic mechanisms, including steric effects associated with the lipid components. Thus, the observed zeta potential provides information about the surface characteristics of VCZ-NLs, whereas dedicated stability studies assessing changes in particle size, PDI, zeta potential, and drug retention over defined storage periods would be required to establish the stability profile of the formulation. Previous reports indicate that incorporation of hydrophobic drugs such as VCZ can partially alter surface charge through lipid head-group reorganization, which may influence nanoparticle cell interactions (16, 17). Despite the generally favorable nanoscale size for drug delivery, the relatively large polysaccharide capsule of *C. neoformans* may still limit nanoparticle access to the cell surface (28). Capsule thickness can reach several micrometers and is known to restrict the penetration of macromolecules, which may partly explain why liposomal encapsulation did not

enhance yeast-phase VCZ susceptibility under the tested conditions. This finding suggests that further investigation of VCZ-NLs in cryptococcal biofilm models may be warranted, particularly using viability-based assays and sub-MIC exposures. However, the present data do not establish a biofilm-specific effect of the nanoliposomal formulation. Future optimization may therefore benefit from further size reduction or surface modification strategies to improve capsule penetration.

AFST demonstrated identical MIC values for free and VCZ-NLs against the tested *C. neoformans* strain, indicating that liposomal encapsulation preserved the intrinsic antifungal activity of the drug under yeast-phase cell conditions (29). This observation is consistent with prior studies showing that lipid carriers often maintain baseline azole potency while primarily improving pharmacokinetics, tissue distribution, or toxicity profiles rather than MIC values (17). Although enhanced activity was not observed in this strain, previous work, including our earlier studies, has shown improved efficacy of liposomal VCZ in certain *Candida* and *Aspergillus* isolates, suggesting that the benefit of nano-delivery may be species- or permeability-dependent and potentially more evident *in vivo* (16, 17).

In contrast to the yeast-phase cell results, the biofilm assay revealed a greater reduction in crystal violet-measured biofilm biomass following treatment with VCZ-NLs compared with free VCZ. Because the crystal violet assay primarily reflects biofilm biomass rather than cell viability, this finding should be interpreted as a greater reduction in biofilm biomass rather than direct evidence of enhanced fungal killing or reduced cell viability. The greater reduction in biofilm biomass observed with VCZ-NLs may be related to properties of liposomal drug delivery systems that have been proposed to facilitate antimicrobial delivery in biofilm-associated infections, including controlled drug release and opportunities for physicochemical or surface modification (13). However, penetration into the biofilm matrix and local drug retention were not directly assessed in the present study and should therefore be regarded as possible explanations rather than demonstrated mechanisms. Cryptococcal biofilms are recognized to exhibit increased tolerance to antifungal agents (9, 10), highlighting the importance of evaluating biofilm-associated responses separately from planktonic susceptibility. Importantly, the inclusion of a blank liposome control enabled differentiation between the effect of the active drug and that of the carrier system. The absence of an apparent

reduction in the blank liposome group suggests that the observed reduction in biofilm biomass was not visibly attributable to the lipid vehicle alone under the tested conditions. The dissociation between unchanged MIC values and reduced biofilm biomass highlights the importance of evaluating biofilm-specific responses when assessing nano-formulated antifungals (14, 15). Notably, this finding was not accompanied by apparent changes in the virulence markers evaluated in this study, as no clear treatment-associated changes were observed in *CXT1*, *LAC1*, or urease activity under the tested conditions. However, the observed reduction in crystal-violet-measured biomass should not be interpreted as definitive evidence of enhanced antifungal or biofilm-specific activity. From a clinical perspective, formulations that reduce biofilm biomass may have potential therapeutic relevance even when conventional planktonic susceptibility metrics remain unchanged, but this possibility requires further validation.

Nevertheless, the interpretation of these findings should be approached with caution. The study was conducted using a single reference strain and without independent biological replication, limiting assessment of biological variability and the generalizability of the findings. In addition, because the biofilm treatment was performed at 4 µg/mL, corresponding to the planktonic VCZ MIC, a contribution of the antifungal effect on fungal growth or survival to the observed reduction in crystal violet-measured biofilm biomass cannot be excluded. Thus, the present findings should be considered preliminary and hypothesis-generating rather than definitive evidence of enhanced efficacy. Future studies incorporating viability-based assays, including metabolic or CFU-based approaches, and direct assessment of nanoliposome penetration and drug retention within the biofilm matrix, together with independent biological replication and sub-MIC exposures, are warranted to confirm and mechanistically characterize the observed effect.

Assessment of urease activity showed no apparent modulation following exposure to either free VCZ or VCZ-NLs. Qualitative assessment based on the characteristic color change of Christensen's urea agar indicated no apparent difference in urease activity across the tested groups. Because urease contributes to cryptococcal virulence and central nervous system invasion, the absence of an apparent change in the qualitative assay provides no indication of altered urease-associated activity under the present experimental conditions. Reports directly examining urease modulation by

nano-formulated azoles are limited, and thus, our findings contribute preliminary data to this underexplored area. Future studies using quantitative urease assays, prolonged exposure times, or sub-MIC conditions may better clarify whether nanocarriers can influence urease-associated virulence mechanisms.

Gene expression analysis of the virulence-associated genes *CXT1* and *LAC1* showed no clear treatment-associated modulation under the tested conditions. Although *CXT1* showed a modest downward trend, *LAC1* expression remained largely unchanged. Because the analysis was based on technical replicates without independent biological replication, these observations should be interpreted descriptively rather than as evidence of statistically supported changes. This pattern contrasts with earlier reports in *C. albicans*, where VCZ-NLs significantly modulated resistance-related genes (17). The difference may reflect species-specific regulatory responses, differences in target genes, or characteristics of the tested *C. neoformans* strain. However, the present pilot data do not establish whether the observed biofilm biomass reduction is related to transcriptional modulation of *CXT1* or *LAC1*. Expanded gene-expression analyses using independent biological replicates will be required to investigate this possibility.

Several limitations should be acknowledged. This work was designed as a proof-of-concept pilot study using a single reference strain and technical replicates, which limits assessment of biological variability and generalizability. In addition, only a single concentration and exposure condition were evaluated for the biofilm and gene-expression experiments. Furthermore, the gene expression analysis was limited to two virulence-associated markers (*CXT1* and *LAC1*), which may not fully capture the complexity of regulatory pathways involved in biofilm formation and pathogenicity; additional genes such as *CAP* family members, *URE1*, and *BZP4* were not assessed. Moreover, biofilm assessment was based solely on crystal violet staining, which quantifies total biomass but does not distinguish between viable and non-viable cells. Complementary approaches such as *XTT* metabolic assays, *CFU* enumeration, or *LIVE/DEAD* staining were not performed and would be required to confirm the impact of VCZ-NLs on biofilm viability. In addition, physicochemical characterization of the nanoliposomal formulation, including drug release kinetics and stability over time, was not evaluated, limiting mechanistic interpretation of the observed biofilm biomass reduction. Therefore,

the greater reduction in biofilm biomass observed with VCZ-NLs cannot be definitively attributed to controlled release, improved stability, enhanced penetration, or increased local drug retention. Future investigations incorporating biological replicates, multiple clinical isolates, time-kill analyses, in vivo models, and comprehensive formulation characterization will be essential to fully define the clinical and therapeutic potential of VCZ-NLs against cryptococcosis.

CONCLUSION

The present findings demonstrate that liposomal encapsulation of VCZ preserved the observed antifungal activity against *C. neoformans* at the yeast-phase level, with no clear treatment-associated modulation of the examined virulence genes (*CXT1* and *LAC1*) under the tested conditions. Notably, the NL formulation produced a greater reduction in crystal violet-measured biofilm biomass despite an unchanged planktonic MIC. However, this finding should not be interpreted as direct evidence of reduced cell viability or a biofilm-specific effect, particularly because biofilm treatment was performed at a concentration corresponding to the planktonic VCZ MIC. Improved nanoliposome penetration or local drug retention may contribute to the greater reduction in biofilm biomass observed with VCZ-NLs, but these mechanisms were not directly examined in the present study. Although this pilot study was limited to technical triplicates and a single reference strain, the findings provide preliminary observations supporting further investigation of VCZ-NLs as a potential approach for targeting cryptococcal biofilm biomass. Further studies incorporating biological replicates, multiple clinical isolates, viability-based biofilm assays, direct assessment of nanoliposome penetration and drug retention, and in vivo models are warranted.

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AUTHORS' CONTRIBUTIONS

S.N. and P.H. conceived and designed the study, contributed to data collection, and drafted the manuscript. Z.R., B.B., and H.H. performed the experiments, conducted the statistical analyses, and critically revised the manuscript for important intellectual content. All authors read and approved the final version of the manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests or personal relationships that could have influenced the work reported in this paper.

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ETHICAL CONSIDERATIONS

The study was approved by the ethics committee of Tabriz University of Medical Science (Grant no. IR.TBZMED.VCR.REC.1398.441).

AI STATEMENT

We acknowledge the use of ChatGPT (OpenAI) to improve the linguistic quality, grammar, and readability of the manuscript. Additionally, AI-assisted tools were utilized in the design and generation of the Graphical Abstract (Figure 1). The authors have thoroughly reviewed, verified, and edited all AI-generated text and imagery, and they assume full responsibility for the final content and scientific integrity of this manuscript.

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