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Copper Nanoparticles Reduce Expression of Key Virulence Genes in Vaginal *Candida albicans* Infection: Implications for Novel Antifungal Therapies

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ABSTRACT

Introduction: Vulvovaginal candidiasis (VVC) is a common and often chronic condition affecting approximately 75% of women worldwide. *Candida albicans* is a primary fungal pathogen responsible for a significant proportion of VVC cases. This cross-sectional study investigated the expression levels of two critical virulence genes, *ALS1* and *HWP1*, in *C. albicans* isolates from women diagnosed with VVC. Moreover, we examined the effect of copper nanoparticles on the expression of these genes, exploring their potential as a novel antifungal therapy for VVC treatment. **Methods**: This study recruited 30 patients diagnosed with VVC from Razi Hospital, Iran. We employed polymerase chain reaction (PCR) to confirm the presence of the *ALS1* and *HWP1* genes in *C. albicans* isolates. Subsequently, we extracted RNA from the isolates and assessed the effect of copper nanoparticles on the expression of *ALS1* and *HWP1* genes using quantitative real-time PCR (qRT-PCR). **Results**: Of the 30 *C. albicans* clinical isolates analyzed, 17 (56.7%) harbored both *HWP1* and *ALS1* virulence genes. Copper nanoparticles significantly downregulated the expression of these genes. Notably, treatment with 8.8 µg/mL copper nanoparticles resulted in a significant reduction of *HWP1* gene expression, while 3.23 µg/mL copper nanoparticles led to a significant decrease in *ALS1* gene expression. **Conclusion**: This study identified the presence of *ALS1* and *HWP1* virulence genes in *C. albicans* isolates from women with VVC and demonstrated the potential of copper nanoparticles to downregulate their expression. These findings offer promising insights into the development of novel antifungal therapies for VVC treatment. However, further investigations with larger, more diverse cohorts and comprehensive analyses are necessary to fully understand the effects of copper nanoparticles on *C. albicans* gene expression and their potential clinical applications for VVC management.

INTRODUCTION

C. albicans, a ubiquitous commensal fungus that colonizes the gastrointestinal tract, oral mucosa, and vagina, can undergo a transition to a pathogenic state, leading to mucosal infections. In immunocompetent individuals, the immune system, particularly neutrophils, effectively counteracts *C. albicans* infections. However, under specific conditions, candidiasis can shift from commensalism to pathogenesis, resulting in the colonization of internal organs and systemic dissemination, which can lead to potentially life-threatening complications. VVC, primarily caused by *Candida* species, is a prevalent fungal infection affecting a significant proportion of women, particularly during pregnancy. Epidemiological studies have reported that approximately 75% of pregnant women experience at least one episode of VVC, with 40-50% developing recurrent infections. Moreover, 25-40% may harbor asymptomatic VVC, detectable only through positive fungal cultures. Notably, *Candida* species, with *C. albicans* being the most frequent causative agent, are responsible for the majority of vaginal infections in Europe, as documented by several studies [1-3].

Biofilms are complex, structured communities of microorganisms that adhere to surfaces and each other, exhibiting distinct properties compared to their planktonic counterparts. *C. albicans* can form multicellular biofilms composed of yeast cells, hyphae (including true and pseudohyphae), and an extracellular matrix. The agglutinin-like sequence (*ALS*) gene family and the hyphal wall protein 1 (*HWP1*) gene are key contributors to *C. albicans* biofilm formation. While the wall protein 1 (*WPI*) gene may also play a role in *C. albicans*

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pathogenicity, further investigation is needed to determine its specific contribution. The *ALS* family exhibits the strongest binding affinity to host cells and proteins, with *ALS1* expression increasing during biofilm growth, suggesting a critical role in this process. The *ALS* gene family encodes fungal cell wall glycoproteins, facilitating adhesion and initial stages of fungal development. Notably, biofilms are associated with reduced susceptibility to antifungal drugs, including fluconazole, and the *ALS* gene family is believed to play a role in this resistance mechanism. Similarly, the HWP1 protein, a mannoprotein-binding glycosylphosphatidylinositol (GPI)-anchored cell wall protein, facilitates covalent binding to host cells [4-5].

Copper nanoparticles possess antimicrobial and antibacterial properties due to their high surface-to-volume ratio, making them a promising agent against vaginitis. Upon contact with water or biological fluids, copper nanoparticles release copper ions, which contribute to their antimicrobial effects. Building on the success of copper nanoparticles against various microorganisms, researchers are exploring their potential as a novel treatment for vaginitis. Metal nanoparticles, encompassing silver, gold, palladium, and copper, have demonstrated a range of properties, including anti-cancer, antibacterial, antifungal, and catalytic effects [6-11].

This study explores the potential of copper nanoparticles as a novel therapeutic strategy for vaginal candidiasis, with a focus on their impact on the expression of the *ALS1* and *HWP1* genes in patient samples. Through a comprehensive research approach, we investigate the antimicrobial properties of copper nanoparticles against *C. albicans* and their potential to influence the expression of *ALS1* and *HWP1* genes, which are crucial for biofilm formation and potentially contribute to drug resistance. Building upon previous research on nanoparticle applications in medicine, this study addresses previously identified limitations and focuses on specific research questions and well-defined hypotheses related to the use of copper nanoparticles for vaginal candidiasis treatment. Our findings have the potential to contribute to the development of more effective treatments for vaginal candidiasis and potentially other fungal infections, addressing a significant unmet clinical need.

MATERIAL AND METHODS

Patient recruitment and questionnaire. This study enrolled 30 participants diagnosed with vaginal candidiasis at Razi Hospital and the Mycology Department, Pasteur Institute of Tehran, based on pre-defined eligibility criteria. The A standardized questionnaire was used to collect information on demographics (age, race, place of birth, education level, occupation), clinical symptoms (itching, discharge, odor), medical history (diabetes, hormone therapy, corticosteroid therapy, contraceptive use, antifungal medication use, dietary supplement use, underlying diseases, pregnancy), lifestyle habits (history of drug, tobacco, and alcohol use, duration of substance use), and family history (cancer or specific diseases).

Diagnostic and laboratory procedures. The diagnosis of *C. albicans* was confirmed using standard microbiological techniques. Vaginal swab samples were cultured on Sabouraud dextrose agar (SDA) medium, and subsequent isolates were identified through microscopic examination, as described in established protocols [12-15].

Antifungal susceptibility testing. The susceptibility of *C. albicans* isolates to fluconazole was determined using the disk diffusion method on SDA medium, as described previously [16-20]. Briefly, *C. albicans* isolates were cultured in Brain Heart Infusion Broth (BHIB) medium (BioMaxima, Poland) for 24 h to obtain a standardized inoculum.

Preparation and application of copper nanoparticles. A suspension of pure copper nanoparticles (US Research Nanomaterials Inc, Houston, USA) was prepared at a concentration of 0.01 g/mL in sterile physiological saline. The suspension was mixed for 5 min using a CORTEX mixer to ensure uniform distribution of the nanoparticles.

Cultures of *C. albicans* isolates in Brain Heart Infusion Broth (BHIB) medium were standardized to an optical density (OD) of 0.5 at 600 nm using a spectrophotometer. Aliquots of 10 µL of each standardized culture were transferred to four separate fresh BHIB media. Then, copper nanoparticle suspensions (320 µL, 640 µL, and 1280 µL) were added to separate culture tubes containing standardized *C. albicans* cultures. The tubes were incubated at 37°C for 24 h alongside negative control tubes containing only fresh BHIB medium.

The selection of nanoparticle concentrations was informed by previous research demonstrating the antifungal efficacy of copper nanoparticles against *Candida* species. Fungal growth inhibition was employed as the primary metric to assess the efficacy of copper nanoparticles, following established protocols [21-22].

RNA extraction and PCR. An aliquot of the culture was transferred to a microtube containing 200 µL of sterile saline solution and incubated at 100°C for 10 min for subsequent RNA extraction. After centrifugation at 9,910 × g, the supernatant was collected, and the pellet was discarded. The supernatant was then stored at -70 °C until further analysis [21].

PCR was performed to detect the presence or absence of the HWP1 and ALS1 genes in each *C. albicans* isolate. RNA quality and quantity were evaluated using a NanoDrop spectrophotometer and agarose gel electrophoresis. Primers for PCR amplification were selected based on established specificity and sensitivity for these genes. Quantitative real-time PCR (qPCR) was employed to measure RNA concentration and purity. The

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RNA concentration was measured using a real-time PCR instrument, and the sample was then reverse transcribed into cDNA for subsequent analysis.

Real-time PCR reaction mixtures contained 10 µL of RealQ Plus 2X Master Mix (Green), 2 µL of cDNA, 1 µL of each forward and reverse primer for the target genes, and 6 µL of distilled water, prepared in a final volume of 20 µL [19-23].

Statistical analysis. Statistical analysis was performed using IBM SPSS Statistics version 21 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) was employed to evaluate significant differences between groups, followed by the least significant difference (LSD) post hoc test to identify specific group differences. Statistical significance was defined as $P < 0.05$. Prior to analysis, all assumptions of the statistical tests were verified, including normality and homoscedasticity of the data.

Ethical considerations. The study was approved by the relevant institutional review board (IRB code: IR.IAU.CTB.REC.1401.017) and informed consent was obtained from all participants prior to their enrollment in the study. Participants' privacy and confidentiality were ensured throughout the study, and their personal information was anonymized and de-identified.

RESULTS

Fluconazole disk test. The results of the fluconazole susceptibility testing are presented in Table 1 and Figure 1. Table 1 displays the sample numbers and corresponding fluconazole zone diameters in millimeters, indicating the degree of susceptibility to fluconazole. Figure 1 provides a visual representation of the fluconazole zone diameters, with the yellow highlighted areas illustrating the size of each sample's zone of inhibition.

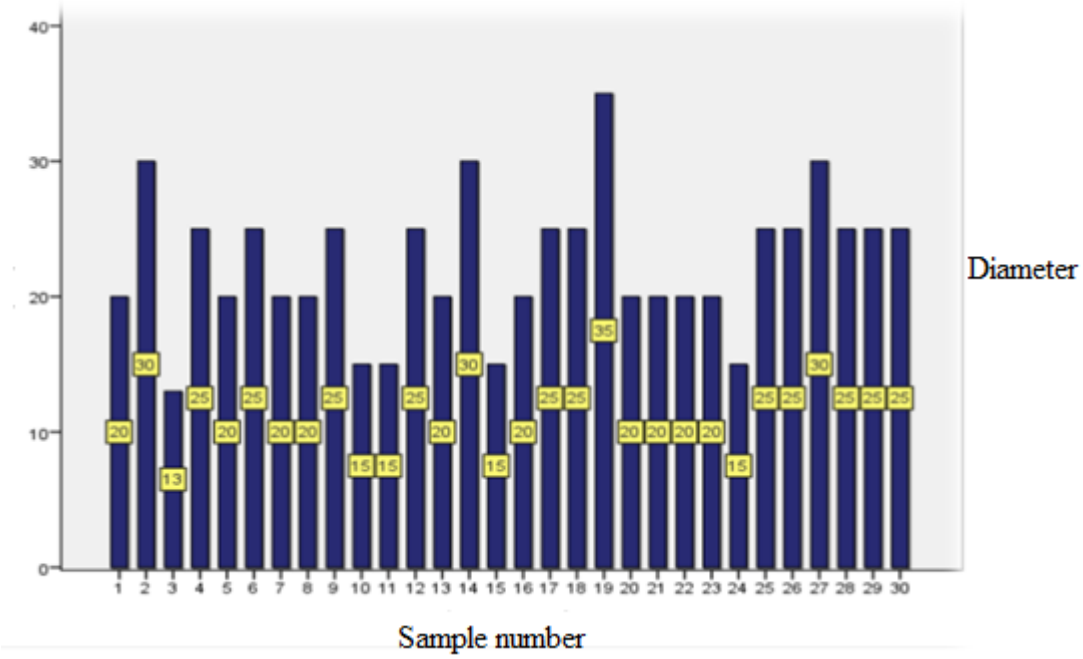

Fig. 1. Fluconazole susceptibility testing results, displaying the diameter (in millimeters) of inhibition zones for each sample. The figure uses a color-coded system to indicate the presence or absence of key virulence genes: blue bars represent samples positive for the *HWP1* gene, green bars represent samples positive for the *ALS1* gene, beige represents samples lacking both genes, and purple represents samples positive for both genes.

PCR results. The presence or absence of specific genes in each sample is presented in Figure 2 and Table 1. The results show that only two samples (16 and 18) tested positive for the *HWP1* gene, while six samples (4, 6, 11, 12, 15, and 28) tested positive for the *ALS1* gene. Fifteen samples (2, 5, 7, 8, 9, 10, 13, 17, 19, 20, 21, 22, 23, 25, and 26) tested positive for both the *ALS1* and *HWP1* genes. Five samples (1, 3, 14, 24, and 27) did not express either gene.

Effects of copper nanoparticles on gene expression. As shown in Table 1 and Figure 3, treatment with copper nanoparticles significantly reduced the expression of the ALS1 and HWP1 genes in vaginal *C. albicans* isolates compared to the control group ($P < 0.01$). The results demonstrate a statistically significant downregulation of these key virulence genes. Furthermore, Figure 4 illustrates the altered expression levels of the ALS1 and HWP1 genes in response to copper nanoparticle treatment, highlighting the potential of this novel antifungal therapy to modulate gene expression and attenuate virulence.

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Table 1. Relative expression levels of *ALS1* and *HWP1* genes in vaginal *C. albicans* isolates, analyzed using IBM SPSS Statistics version 21

Gene	Type	Reaction efficiency	Expression	Standard error	95% C.I.	P(H1)	Result
ACT1	REF	0.98	1.000	-	-	-	-
ALS1	TRG	0.99	0.309	0.077 - 1.255	0.027 - 5.668	0.021	DOWN
HWP1	TRG	0.99	0.113	0.022 - 0.666	0.005 - 2.544	0.003	DOWN

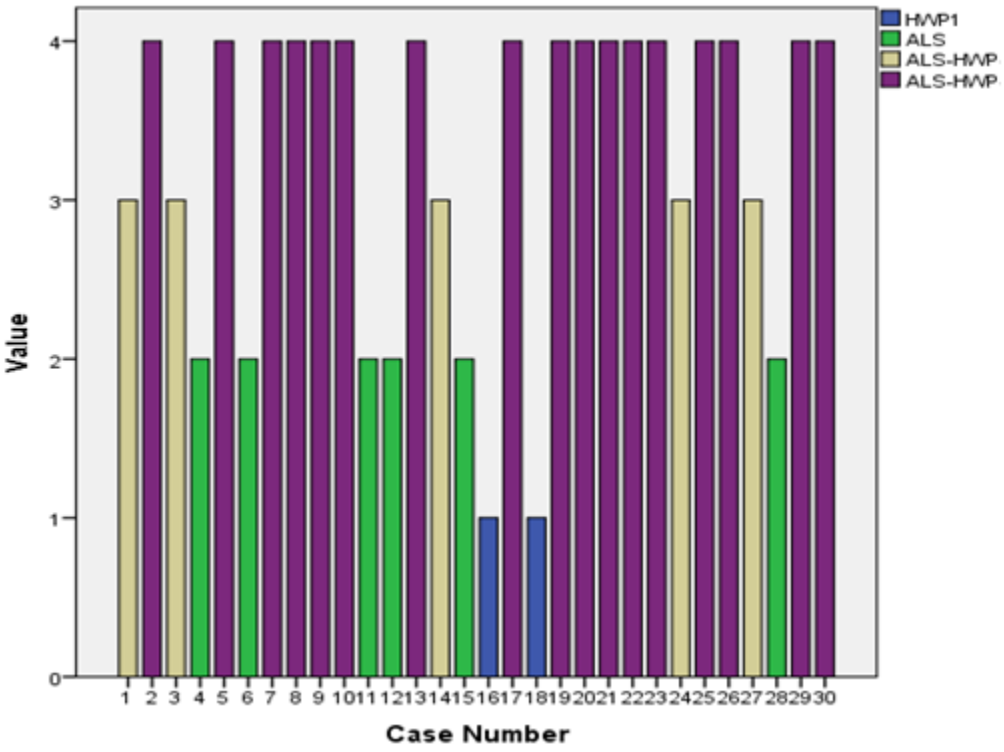

Fig. 2. PCR amplification of ALS1 and HWP1 genes in vaginal *C. albicans* isolates

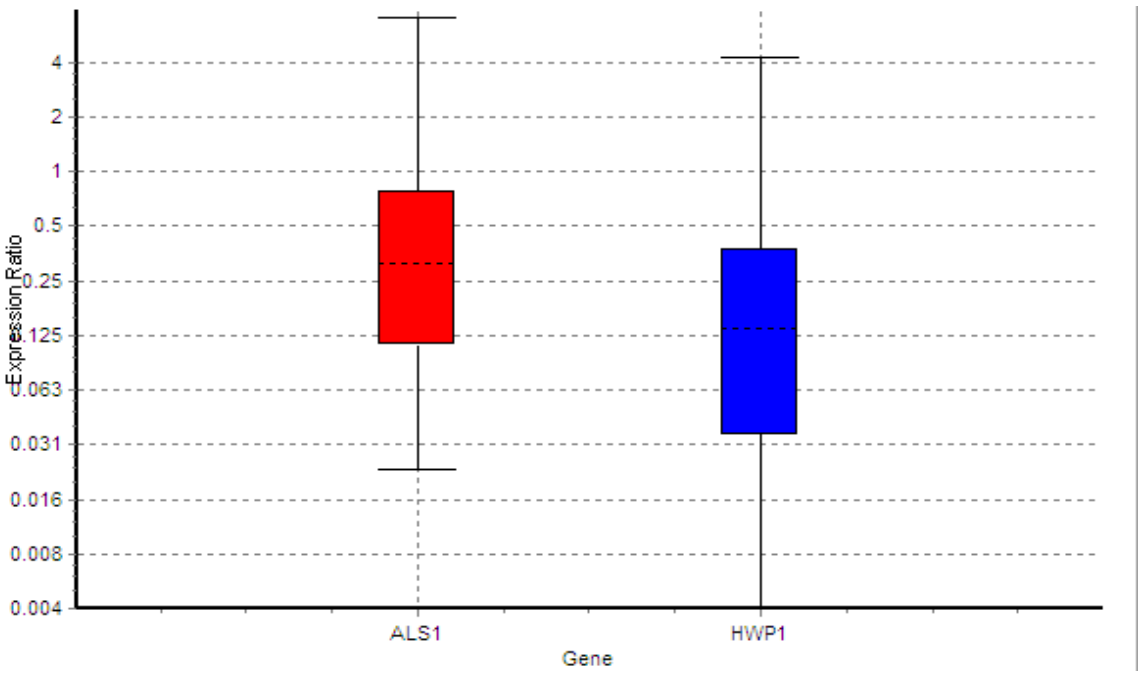

Fig. 3. The gene expression of *ALS1* and *HWP1*

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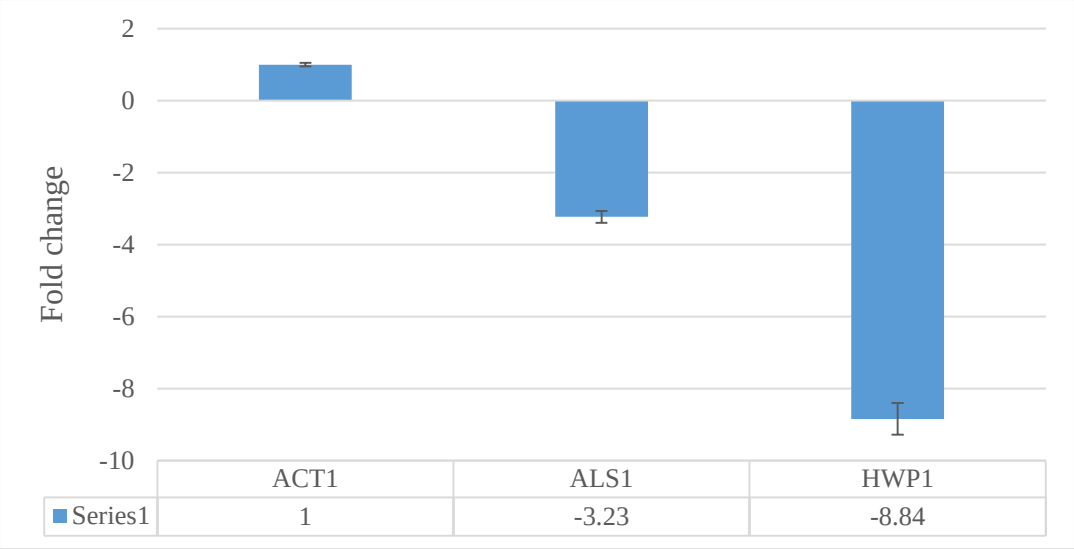

Fig. 4. Changes in the expression levels of the *ALS1* and *HWP1* genes in response to treatment with copper nanoparticles

Our results demonstrate that treatment with copper nanoparticles significantly downregulates the expression of key virulence genes in vaginal *C. albicans* isolates, suggesting a potential therapeutic strategy for the treatment of *Candida* vaginitis. These findings are consistent with previous studies that have reported the antifungal properties of copper nanoparticles, further supporting their potential as a novel antifungal therapy.

DISCUSSION

Candidiasis vaginitis is a common gynecological disorder affecting an estimated 75% of women at some point in their lifetime, with *C. albicans* accounting for approximately 80% of these infections. As a significant public health concern, candidiasis vaginitis is compounded by the growing threat of antifungal resistance among *Candida* species [2]. The management of candidiasis has become increasingly complex due to the emergence of antifungal-resistant *Candida* strains, which poses a significant clinical challenge. The current antifungal armamentarium is limited by toxicity, high costs, and restricted efficacy, underscoring the urgent need for innovative therapeutic approaches. The rising incidence of *Candida* infections, particularly those caused by opportunistic and antifungal-resistant strains, places a substantial burden on healthcare systems, highlighting the need for novel therapeutic strategies with enhanced antifungal activity and broader coverage [23, 24].

An international cross-sectional survey of 6,010 women aged 16 years and above, conducted across six countries, found that a substantial proportion (ranging from 29% to 49%) reported having experienced at least one instance of vaginal candidiasis (yeast infection) during their lifetime. More than 9% of participants reported recurrent vulvovaginal candidiasis (RVVC), highlighting the significant burden of this condition. The survey revealed a striking age-related difference in the probability of primary vaginal yeast infection, with women aged 50 being twice as likely (25%) to experience it compared to those aged 25 (10%) [16].

Our study identified the presence of both *ALS1* and *HWP1* genes in patient samples, implicating their role in vaginal *C. albicans* infections. Notably, treatment with copper nanoparticles significantly downregulated the expression of *ALS1* and *HWP1* genes ($P < 0.01$), suggesting a key mechanism underlying their antifungal activity. This downregulation of virulence genes by copper nanoparticles reveals a novel antifungal mechanism, which may offer advantages over existing agents by targeting critical virulence pathways and potentially reducing the risk of resistance. Furthermore, this approach may help combat the growing threat of antifungal resistance by exploiting a new target for therapeutic intervention.

The downregulation of *ALS1* and *HWP1* gene expression observed in this study is particularly significant, as these genes play a crucial role in the pathogenesis of *C. albicans*. Specifically, they mediate the adhesion of the fungal pathogen to host cells and facilitate biofilm formation, two critical steps in the establishment and progression of vaginal candidiasis. The downregulation of these genes by copper nanoparticles suggests a novel antifungal mechanism, potentially leading to improved clinical outcomes for individuals suffering from *Candida* vaginitis. By targeting and downregulating key virulence factors, copper nanoparticles may offer a promising therapeutic approach for the treatment of vaginal candidiasis, warranting further investigation.

Previous studies have investigated the antifungal potential of various nanoparticles, with copper nanoparticles showing significant promise in inhibiting *C.*

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albicans. Research by Soltani *et al*. (2017) assessed the antifungal properties of silver and copper nanoparticles, as well as their combination with amphotericin B, for developing a treatment against *C. albicans*. The study found that silver and copper nanoparticles had minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) of 31 and 62.50 ppm, respectively, while their combination exhibited concentrations of 15.50 and 31 ppm, respectively. Notably, silver and copper nanoparticles demonstrated a greater impact than amphotericin B, which showed the least effect. These findings suggest that silver and copper nanoparticles, used alone or in combination, may be more effective against *C. albicans* than amphotericin B [25-27].

This study's findings on the downregulation of virulence genes by copper nanoparticles align with recent research exploring the immunomodulatory effects of nanoparticles on the host-pathogen interaction. For instance, a study by Mohammadi et al. (2022) demonstrated that silver nanoparticles modulated the immune response to C. albicans infection, enhancing the production of pro-inflammatory cytokines and reducing fungal burden [9].

Copper nanoparticles have been found to reduce expression of key virulence genes in vaginal *C. albicans infections*, which has implications for novel antifungal therapies [28].This study aligns with other research on the immunomodulatory effects of nanoparticles on the host-pathogen interaction [29]. For instance, silver nanoparticles have been shown to modulate the immune response to *C. albicans* infection, enhancing the production of pro-inflammatory cytokines and reducing fungal burden [30]. Similarly, copper oxide nanoparticles have been found to induce the expression of antimicrobial peptides in human keratinocytes, leading to enhanced antifungal activity against *C. albicans* [30]. These studies highlight the potential of nanoparticles to not only directly target fungal pathogens but also modulate the host immune response, offering a multifaceted approach to combat *Candida* infections.

This study has limitations, including a small sample size and no placebo control group, and its findings require cautious interpretation. While the comprehensive patient questionnaire is a strength, further investigation is needed to address limitations and validate findings. The observed gene expression reduction warrants further exploration in more complex in vivo models and clinical trials to assess its true clinical significance. To advance these findings towards clinical application, future studies with larger, more diverse cohorts and robust placebo controls are crucial, and should explore optimal nanoparticle formulation, delivery methods, and efficacy in well-designed clinical trials for *Candida vaginitis*.

This study identifies the presence of *ALS1* and *HWP1* genes, crucial virulence factors in *C. albicans*, in patient samples. Notably, copper nanoparticle treatment significantly downregulated the expression of these genes, suggesting a potential novel mechanism for antifungal therapy. While these initial findings are promising, further research with larger, more diverse patient populations is essential to confirm the observed downregulation of virulence genes by copper nanoparticles and translate these findings into clinically meaningful outcomes. Additionally, exploring potential synergistic effects through combination therapies with established antifungal agents could further enhance the therapeutic potential of copper nanoparticles, offering a promising avenue for combating *Candida* infections.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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