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Original article

Comparative Antifungal Efficacy of Ketoconazole and Nystatin on Chlamydospore Production in *Candida albicans* Isolated from Oral Lesions in Cancer Patients

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Abstract
Candida albicans threatens immunocompromised patients, partly via chlamydospores—dormant, stress-tolerant forms. Reducing these structures may limit persistence and relapse. We compared nystatin (polyenic membrane binder) versus ketoconazole (azole ergosterol-synthesis inhibitor) for suppressing chlamydospore production by C. albicans from cancer-patient oral lesions. Twenty isolates were cultured on cornmeal agar + 1% Tween-80 and exposed to nystatin or ketoconazole at 0.5–10 µg/mL. After 24–48 h at 30 °C, chlamydospores were stained and counted microscopically (spores/10 HPF). ANOVA with Tukey post-hoc tested drug, concentration, and interaction ($p<0.05$). Ketoconazole reduced chlamydospores more than nystatin at every concentration, with a clear dose response (≈54 to ≈29 spores/10 HPF from 0.5 to 10 µg/mL). Nystatin showed limited suppression at low doses and only modest declines at 5–10 µg/mL. At 10 µg/mL, ketoconazole achieved ~45% reduction from baseline versus ~30% with nystatin; 19/20 isolates favored ketoconazole. ANOVA showed significant main effects and drug-concentration interaction ($p<0.001$). Ketoconazole is superior to nystatin for suppressing C. albicans chlamydospore formation in vitro, supporting systemic azoles as more effective inhibitors of this persistence mechanism. Clinically, safer azoles (e.g., fluconazole) may better prevent recurrent or invasive disease in high-risk patients than topical nystatin alone. Nystatin remains useful for localized thrush but may not adequately block chlamydospore formation. In cancer patients with recurrent oral candidiasis or risk of dissemination, consider early systemic azole therapy and confirm in future studies whether aggressive chlamydospore suppression improves outcomes.
Keywords. *Candida albicans; Chlamydospores; Oral Candidiasis; Nystatin; Ketoconazole; Antifungal Efficacy; Immunocompromised Hosts.*

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Introduction

Fungal infections have emerged as a significant cause of morbidity in immunocompromised patients, particularly those with cancer [1]. *Candida albicans* is an opportunistic yeast that commonly colonizes the oropharyngeal mucosa and gastrointestinal tract, but can cause disease ranging from superficial thrush to life-threatening systemic infections when host defenses are impaired [2]. Cancer patients undergoing chemotherapy or radiation often develop neutropenia and mucosal damage, making them highly susceptible to invasive candidiasis. In such individuals, *Candida* infections can lead to severe complications like bloodstream infection (candidemia) and disseminated candidosis, which carry high mortality rates (approximately 30–50% in candidemia) [3,4]. These infections are often persistent and difficult to eradicate, as noted by Bodey et al., due to the ability of the fungus to endure in protected niches despite therapy [5]. A crucial virulence attribute of *C. albicans* is its ability to switch morphologies. Besides yeast and hyphae, *C. albicans* can form chlamydospores, which are large, thick-walled asexual spores produced under nutrient-poor or other adverse conditions [6].

Chlamydospores are dormant survival structures that allow the fungus to withstand harsh environments by significantly reducing its metabolic activity [7]. Although chlamydospores are rarely observed in patient tissues [8], they likely contribute to persistence on abiotic surfaces or within biofilms, seeding recurrent infection once conditions improve. The formation of chlamydospores is an enigmatic developmental program not fully understood, but it is thought to help the organism evade host defenses and survive extreme micro-environments [9]. In immunocompromised patients, the continued presence of such resilient fungal forms can act as a reservoir for infection relapse. It has been postulated that reducing or preventing chlamydospore production could be a useful strategy to

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limit the spread and recurrence of *Candida* infections. Indeed, controlling fungal propagation at the level of morphological development may improve clinical outcomes.

Lortholary et al. (2008) observed that any factor prolonging fungal survival in cancer patients with candidiasis can impede successful treatment [10]. Therefore, understanding how antifungal agents affect *C. albicans* morphogenesis – specifically chlamydospore formation – is clinically relevant. Nystatin and ketoconazole are two antifungal medications historically used in the treatment of candidiasis, including oral forms of the disease. Nystatin is a polyene antifungal that exerts its effect by binding to ergosterol in the fungal cell membrane, creating pores that lead to leakage of cell contents and cell death [11]. It is administered topically (e.g., oral suspension or lozenges for thrush) with negligible gastrointestinal absorption, making it very safe for mucosal use [12].

Nystatin has been a first-line therapy for oral candidiasis for decades, especially in infants and medically frail patients, due to its low systemic toxicity. However, nystatin's clinical efficacy can be variable. It is primarily fungicidal against the yeast form in active growth; its impact on fungal morphogenesis or on dormant forms is less certain. Some evidence suggests that nystatin does not significantly inhibit chlamydospore formation at typical concentrations. For example, Sobel et al. reported that while nystatin is effective for superficial *Candida* infections, it may not reduce chlamydospore production as effectively as certain azoles. Clinically, failures of nystatin therapy or rapid relapses are observed in immunocompromised patients (e.g., advanced HIV), where oral nystatin often yields incomplete responses and frequent recurrence of thrush [13]. This could be partly due to nystatin's limited ability to eliminate resilient fungal forms or penetrate biofilm niches at deeper tissue sites.

Ketoconazole, an imidazole-class antifungal, was one of the first oral systemic azoles introduced for candidiasis. Ketoconazole acts by inhibiting the fungal cytochrome P450 enzyme 14-α-demethylase, which is essential for converting lanosterol to ergosterol in the cell membrane [14]. This blockade of ergosterol synthesis disrupts membrane integrity and inhibits fungal growth and replication [15]. Ketoconazole has a broad antifungal spectrum and is active against *Candida* species in vitro. Importantly, by impairing cell membrane formation, ketoconazole could also hinder the development of specialized structures like chlamydospores that require robust cell walls and intact membranes. Previous studies indicated that azoles can interfere with fungal morphological transitions: for instance, Johnson *et al.* (1982) showed that ketoconazole inhibited the initial stages of *C. albicans* germ tube (hyphal) formation [16], and Fox *et al.* (1998) reported that ketoconazole had superior *in vitro* antifungal activity against *C. albicans* compared to nystatin. Ketoconazole was found to reduce chlamydospore production more than nystatin in laboratory assays [17]. Similarly, a comparative study by Fouzia *et al.* (2010) observed that ketoconazole demonstrated greater efficacy in inhibiting spore formation across various *Candida* models, whereas nystatin was less effective. These findings support the notion that azoles, by targeting ergosterol synthesis, not only curtail fungal growth but may also disrupt the formation of survival structures like chlamydospores [18].

Despite ketoconazole's antifungal potency, its clinical use has diminished due to safety concerns. In 2013, regulatory agencies (FDA and EMA) restricted oral ketoconazole because of the risk of severe hepatotoxicity and adrenal suppression. Oral ketoconazole is no longer recommended as first-line therapy for candidiasis, and it has largely been replaced by safer systemic azoles like fluconazole and itraconazole. Nonetheless, understanding ketoconazole's effects remains valuable, as it serves as a representative of azole antifungals. Fluconazole, for instance, shares a similar mechanism and is widely used for prophylaxis and treatment of fungal infections in cancer patients [19]. If ketoconazole (or by extension, systemic azoles) can significantly suppress chlamydospore formation while nystatin cannot, this could partly explain differences in clinical outcomes between patients treated with local versus systemic therapy. Indeed, randomized trials in high-risk patients have found that systemic azole therapy reduced *Candida* colonization and invasive infection more effectively than nystatin, even if rates of oral thrush were similar [20]. Nystatin's lack of systemic absorption means it cannot prevent fungal invasion beyond the oral cavity, whereas an absorbed azole can exert body-wide prophylactic effects.

Given the paucity of data on antifungal effects specifically on chlamydospore formation in clinical *Candida* isolates, we aimed to investigate this aspect. We focused on oral *C. albicans* isolates from cancer patients, a population in whom preventing fungal persistence is critical. We hypothesized that ketoconazole would inhibit chlamydospore production more effectively than nystatin across a range of concentrations. We further postulated that the difference would be most pronounced at higher drug concentrations, reflecting ketoconazole's dose-dependent fungistatic/cidal activity versus nystatin's plateauing effect. By comparing these two drugs, this study seeks to inform optimal antifungal strategies for managing oral candidiasis in immunocompromised patients, balancing efficacy in suppressing pathogenic fungal forms with safety considerations.

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Methods

Isolates and Identification

This study utilized 20 clinical isolates of *Candida albicans* obtained from oral lesions (e.g., oropharyngeal thrush, or denture-related stomatitis) in cancer patients. The patients were undergoing treatment at tertiary oncology centers and had developed clinical signs of oral candidiasis. *C. albicans* isolates were confirmed by standard mycological techniques, including the germ tube test and colony morphology on chromogenic agar, followed by microscopic identification of budding yeast cells with pseudohyphae and chlamydospores on cornmeal agar. All isolates were stored on Sabouraud dextrose agar slants at 4°C and subcultured on fresh media before testing.

Antifungal Agents and Preparation

The antifungal agents tested were nystatin and ketoconazole. Pharmaceutical-grade powders were used to prepare stock solutions. Nystatin: A polyene antifungal (obtained as nystatin powder, potency ~4400 IU/mg) was dissolved in sterile distilled water with gentle heating to create a stock solution of 1 mg/mL. Ketoconazole: An imidazole antifungal (obtained as pure powder) was dissolved in distilled water with 1–2 drops of 1 N HCl (to aid solubility) to make a 1 mg/mL stock solution. From each stock, working solutions of five concentrations – 0.5, 1, 2, 5, and 10 µg/mL – were prepared in sterile distilled water. These concentrations were selected to span a range from near the minimal inhibitory level to higher levels achievable topically or systemically. All solutions were prepared freshly on the day of the experiment to ensure potency.

Culture Medium for Chlamydospore Induction

Cornmeal agar (CMA) (Oxoid, Basingstoke, UK) was used as the culture medium to stimulate chlamydospore formation. CMA is a nutritionally deficient agar known to promote *C. albicans* chlamydospore production, especially when supplemented appropriately. We prepared the medium per the manufacturer's instructions and added Tween-80 (1% v/v) to the agar before autoclaving. Tween-80, a fatty acid source, is reported to enhance chlamydospore production by *C. albicans*. The medium was sterilized by autoclaving at 121°C for 15 minutes. After cooling to ~50°C, it was poured into sterile Petri plates. The addition of Tween-80 yields a soft, semisolid agar that encourages embedded growth – an important condition for chlamydospore induction.

Inoculation and Initial Incubation

Each *C. albicans* isolate was streaked or spot-inoculated onto a CMA + Tween-80 plate without antifungal to establish baseline growth. Using a sterile loop, a small inoculum of yeast was lightly streaked onto the agar's surface. In some plates, an initial linear streak was made and then covered with a coverslip (Dalmau plate technique) to facilitate observation of chlamydospore formation microscopically. The inoculated plates were incubated at 30°C for 24–48 hours to allow colony establishment and initiation of chlamydospore development. (Note: 30°C was chosen as it is optimal for chlamydospore formation; higher temperatures can inhibit spore production.) After this initial incubation, all plates were examined under a microscope to confirm active yeast growth and the presence of early chlamydospores or germ tubes, indicating that the isolates were capable of sporulation under the test conditions.

Antifungal Exposure

Following the initial 24–48 h growth period, the cultures were exposed to the antifungal agents as follows: For each isolate, separate CMA plates were prepared containing the different concentrations of nystatin or ketoconazole. This was achieved by mixing the appropriate antifungal working solution with cooled molten CMA just before pouring the plates, to yield final drug concentrations of 0.5, 1, 2, 5, or 10 µg/mL in the agar. The drug-infused agar was poured into plates and allowed to solidify. Each isolate was then surface-inoculated onto a series of plates: one set of five plates containing nystatin (at 0.5–10 µg/mL) and another set of five plates containing ketoconazole (0.5–10 µg/mL). In parallel, a drug-free control plate (CMA + Tween-80 without antifungal) was maintained for each isolate to represent baseline chlamydospore formation. The inoculation onto drug plates was done similarly to the control. All drug-containing plates were incubated again at 30°C for an additional 24–48 hours. During this period, the fungi were exposed to the antifungal agents while continuing to grow and potentially form chlamydospores. In preliminary trials, an alternative exposure method was evaluated where segments of an initially grown colony on a drug-free plate were overlaid with antifungal solution to diffuse into the agar. However, the primary approach for this study was to use dedicated drug-infused plates for each concentration.

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Staining and Microscopic Enumeration of Chlamydospores

After incubation with the antifungal, each culture was processed for chlamydospore visualization and counting. We employed Sudan Black B stain (0.5% in 70% ethanol) as the primary stain to highlight chlamydospores. Sudan Black is a lipid-soluble dye that intensely stains the refractile walls of chlamydospores, rendering them dark black-blue, which provides strong contrast against the background and other fungal elements. A 1% safranin solution was used as a counterstain (applied for ~30 seconds and then rinsed) to lightly stain the hyphae and yeast cells red, differentiating them from the dark-stained chlamydospores. For each plate, a sample of the colony was taken using one of two methods: (a) a small block of agar (with the colony) was cut out, or (b) a sterile coverslip was gently pressed onto the colony surface (coverslip "imprint" method), then lifted. The sample (agar block or coverslip) was placed on a microscope slide. A drop of Sudan Black stain was added to the sample and allowed to penetrate for about 2–5 minutes, then gently blotted. Next, a drop of safranin was applied (for contrast) and briefly incubated, then rinsed or blotted. A coverslip was placed (for the agar block samples), and the slide was examined under the microscope using a 40× high-power objective [21].

Chlamydospore identification and counting

Chlamydospores were identified morphologically as large, spherical, thick-walled structures (often located at the termini of pseudohyphae) that took up Sudan Black stain intensely, appearing as dark, round bodies, in contrast to the filamentous hyphae/pseudohyphae and budding yeasts, which stained red or remained unstained [21]. For each sample, chlamydospore counts were performed in multiple microscopic fields. We typically counted 10 high-power fields (HPF) per sample and calculated the average number of chlamydospores per 10 HPF. Two independent observers performed the counts in a blinded fashion (not knowing which drug or concentration was being viewed). Their counts were averaged if in close agreement; if there was a significant discrepancy, the fields were recounted, or a third reviewer was consulted to reach consensus. Thus, for each *C. albicans* isolate at each drug concentration (and for the control), we obtained a mean chlamydospore count per 10 HPF. We also noted any qualitative differences in colony morphology on the drug-containing plates (e.g., stunted hyphal growth, abnormal structures, absence of chlamydospores).

Data Analysis

The study summarized chlamydospore counts (per 10 HPF) for 20 isolates across drugs and concentrations, with the primary outcome being dose-related reduction for each drug. Mean ± SD values were computed at each concentration, and isolates were secondarily stratified as high vs low producers for exploratory analysis. Inferentially, one-way ANOVAs were run separately for nystatin and ketoconazole across five doses, and a repeated-measures two-way ANOVA (factors: Drug, Concentration) tested overall drug differences and Drug×Concentration interaction. Significant effects were followed by Tukey HSD pairwise tests (between drugs at the same dose and between adjacent doses within a drug). Significance was set at $p<0.05$. Analyses were performed in IBM SPSS v25 and cross-checked in R 4.0; results are reported as mean ± SD.

Results

Effect of Nystatin

Chlamydospore counts under various concentrations of nystatin are summarized in (Table 1). Overall, *C. albicans* displayed only a modest reduction in chlamydospore formation with increasing nystatin concentration, and a clear inhibitory effect was observed only at the highest tested levels.

At the lowest nystatin concentration (0.5 μg/mL), many isolates continued to form abundant chlamydospores. The mean count was 76.8 ± 7.6 chlamydospores per 10 HPF, indicating minimal inhibition at this sub-therapeutic concentration (indeed, this was only slightly lower than counts on drug-free control medium, which typically ranged around 80–100 spores/10 HPF in high-producing strains). Increasing nystatin to 1 μg/mL did not significantly reduce spore counts; in fact, the average count slightly increased to 81.8 ± 8.1 per 10 HPF (Table 1). A further increase to 2 μg/mL nystatin yielded the highest mean spore count, 86.8 ± 8.1, suggesting considerable variability and no consistent inhibitory trend up to this point. Statistical analysis confirmed that differences among 0.5, 1, and 2 μg/mL nystatin were not significant ($p>0.05$). In practical terms, *C. albicans* remained capable of robust chlamydospore production at these low nystatin concentrations, in some cases matching the drug-free condition. Microscopically, at 0.5–2 μg/mL nystatin, numerous dark-stained chlamydospores were observed on long pseudohyphae, indicating that nystatin at sub-inhibitory levels did not trigger a shutdown of spore formation.

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A marked decline in chlamydospore numbers was first noted at 5 μg/mL nystatin. The mean count dropped to 71.8 ± 8.1 per 10 HPF, which was approximately a 17% reduction compared to the peak (~86.8 at 2 μg/mL). By 10 μg/mL nystatin, chlamydospore formation was more substantially suppressed: the mean count was 52.0 ± 8.1 per 10 HPF, reflecting ~40% fewer spores than at 2 μg/mL. Comparing the extremes, 10 μg/mL nystatin achieved about a 32% reduction in spore count relative to 0.5 μg/mL (from ~77 down to 52 per 10 HPF on average). This reduction was statistically significant (p<0.001 by ANOVA). Post-hoc analysis showed that chlamydospore counts at 5 μg/mL and 10 μg/mL were significantly lower than those at 0.5–2 μg/mL nystatin (Tukey p<0.01 for 5 vs 2; p<0.001 for 10 vs 2). There was also a significant drop between 5 and 10 μg/mL (p<0.01). These findings indicate that only relatively high concentrations of nystatin can appreciably inhibit chlamydospore formation.

Inter-isolate variability was observed in the nystatin response. Some *C. albicans* strains inherently produced fewer chlamydospores even without high drug pressure. For instance, in roughly 7 of the 20 isolates (which we term "low producers"), the baseline chlamydospore counts at 0.5–1 μg/mL were on the order of 50–65 per 10 HPF, and these isolates showed moderate reductions to ~40 per 10 HPF at 10 μg/mL. In contrast, "high producer" strains (approximately one-third of isolates) had near or above 90 spores/10 HPF at low nystatin levels and still formed ~65–75 spores/10 HPF at 5 μg/mL, only dropping to ~40–55 at 10 μg/mL. For example, isolate #3 exhibited 100 chlamydospores/10 HPF at 2 μg/mL nystatin (one of the highest observed values), which fell to 65/10 HPF at 10 μg/mL. Another isolate (#9) showed a similar pattern (about 100 at 2 μg/mL, down to 68 at 10 μg/mL). On the other hand, isolate #7 had lower counts overall (around 60 at 0.5 μg/mL and 40 at 10 μg/mL), indicating either higher intrinsic sensitivity to even low-dose nystatin or simply a lower sporulation capacity. Notably, no isolate showed a complete absence of chlamydospores at any nystatin concentration tested – even at 10 μg/mL, all strains retained some ability to form these structures (typically 30–50% of their no-drug baseline count).

In summary, nystatin at standard therapeutic concentrations had a limited suppressive effect on *C. albicans* chlamydospore production in vitro, unless used at relatively high levels. Even at 5–10 μg/mL, significant sporulation still occurred in many isolates. Lower concentrations (≤2 μg/mL) were largely ineffective at curbing chlamydospore formation. These data suggest that, under the conditions tested, *C. albicans* can continue its chlamydospore developmental program in the presence of nystatin until the drug reaches a high threshold.

Effect of Ketoconazole on Chlamydospore Production

In contrast to nystatin, ketoconazole exhibited a strong inhibitory effect on chlamydospore formation even at low concentrations, with a near-linear dose–response. (Table 1) shows the chlamydospore counts under ketoconazole exposure. The trend was clear: as the ketoconazole concentration increased from 0.5 to 10 μg/mL, chlamydospore production by *C. albicans* fell progressively. At 0.5 μg/mL ketoconazole, the mean chlamydospore count was 54.0 ± 8.2 per 10 HPF. This represents a substantial reduction (~30% lower) compared to the equivalent 0.5 μg/mL nystatin condition (~76.8 spores/10 HPF). Thus, even the lowest ketoconazole concentration tested already impaired spore formation to a noticeable degree. Some isolates showed almost immediate sensitivity – for example, isolate #4 formed only ~45 chlamydospores/10 HPF at 0.5 μg/mL ketoconazole, whereas it had produced ~80 at 0.5 μg/mL nystatin (and ~90 on control medium). At 1 μg/mL ketoconazole, the mean count further decreased to 49.0 ± 8.3 per 10 HPF. By 2 μg/mL, average spore counts were 44.0 ± 8.3, roughly half the number seen at 2 μg/mL nystatin. Notably, ketoconazole at 2 μg/mL had already reduced chlamydospore formation to about 50% of the baseline (0.5 μg/mL) level, whereas nystatin had not begun to cause any decline until beyond this concentration. The reduction in chlamydospore count between 0.5 and 2 μg/mL ketoconazole was statistically significant (p<0.01), indicating a steady inhibitory effect even in the low concentration range.

Continuing this trend, 5 μg/mL ketoconazole led to a mean of 39.0 ± 8.3 chlamydospores per 10 HPF, and at 10 μg/mL, the count dropped to 28.8 ± 8.3 per 10 HPF. The latter value is near the lower detection limit in our essay (only a few spores per field on average). Indeed, many microscope fields in the 10 μg/mL ketoconazole condition had zero visible chlamydospores. On some plates, chlamydospores were extremely scarce – hyphal elements were truncated or observed without the terminal spore that was present at lower concentrations. The ~28.8 average at 10 μg represents roughly a 47% reduction from the 0.5 μg/mL ketoconazole level, and a nearly 70% reduction relative to the peak counts observed under low-dose nystatin.

Every increment in ketoconazole concentration yielded a further drop in spore count (0.5→1, 1→2, 2→5, etc.), and all these stepwise differences were significant by ANOVA/Tukey analysis (p<0.05 for each consecutive increase). The dose-dependent inhibition was evident: at just 1–2 μg/mL (concentrations achievable systemically in plasma), ketoconazole halved the spore output of *C. albicans*. By 10 μg/mL, several isolates showed >60% reduction in chlamydospores

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compared to their low-dose output. For instance, isolate #3, which had produced ~65 spores/10 HPF at 0.5 µg/mL ketoconazole, was down to ~40 at 10 µg/mL (a ~38% drop). Isolate #7 went from ~40 at 0.5 µg to ~15 at 10 µg (a ~62% reduction). In fact, about 9 out of 20 isolates experienced ≥50% reduction in spore count with ketoconazole by the time the concentration reached 10 µg/mL, whereas none reached that threshold with nystatin. Even the more "resistant" strains (those that still made ~50–60 spores/field at 0.5 µg) were suppressed to ~20–30 spores at 10 µg. No strain was completely refractory to ketoconazole's effect on chlamydospores – all showed a downward trajectory, though the magnitude of reduction varied.

Microscopic observations corroborated these quantitative findings. Ketoconazole-treated colonies were often predominantly in the yeast form with very short pseudohyphae, lacking the typical large, round terminal chlamydospores seen in the control and nystatin-treated cultures. Many fields in the higher ketoconazole concentrations had no visible chlamydospores, indicating that the drug effectively halted the normal sporulation process in those cells.

Direct Comparison of Ketoconazole vs. Nystatin

(Figure 1) illustrates the comparative effect of nystatin and ketoconazole on chlamydospore counts across the tested concentrations, and (Table 1) (below) presents a side-by-side numerical comparison. Ketoconazole was more effective than nystatin in reducing chlamydospore formation at every concentration examined.

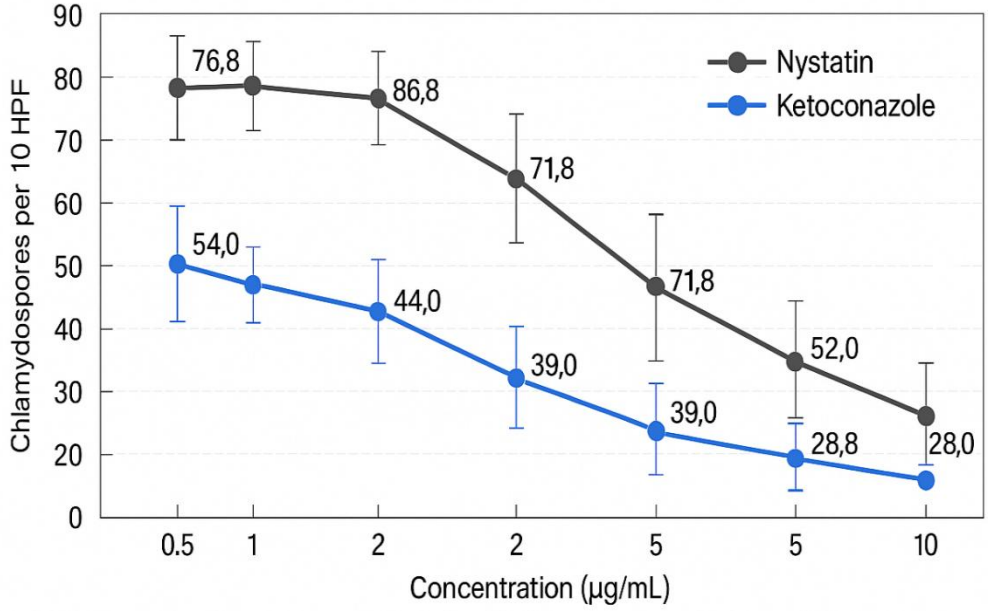

Figure 1 Dose–response comparison of chlamydospore counts (mean ± SD per 10 HPF) for 20 C. albicans isolates exposed to nystatin vs. ketoconazole (0.5–10 µg/mL)

At the lowest concentration (0.5 µg/mL), *C. albicans* exposed to nystatin produced about 1.4 times more chlamydospores than when exposed to ketoconazole (approximately 76.8 vs. 54.0 per 10 HPF, respectively). Although both drugs were at sub-inhibitory levels here (relative to typical MICs), ketoconazole had already curtailed spore production somewhat, whereas nystatin had not. This difference at 0.5 µg/mL was statistically significant ($p<0.001$). Representative micrographs at 0.5 µg/mL underscore the visual contrast, with dense Sudan Black–positive chlamydospores under nystatin and sparse fields under ketoconazole (Figure 2). Moving to 1 µg/mL, the gap persisted: nystatin ~81.8 vs. ketoconazole ~49.0 spores (a ~40% reduction by ketoconazole relative to nystatin; $p<0.001$). At 2 µg/mL, the disparity became even more pronounced. Nystatin-treated cultures *peaked* in spore production (~86.8 mean count), whereas ketoconazole-treated ones continued to decline (~44.0). Thus, at 2 µg/mL, nystatin allowed nearly double the number of chlamydospores compared to ketoconazole ($p<0.001$). This concentration (2 µg/mL) appears to be a tipping point where ketoconazole begins exerting strong inhibition, while nystatin still hasn't significantly curtailed sporulation – indeed, in our data, nystatin's mean spore count at 2 µg was slightly higher than at 1 µg.

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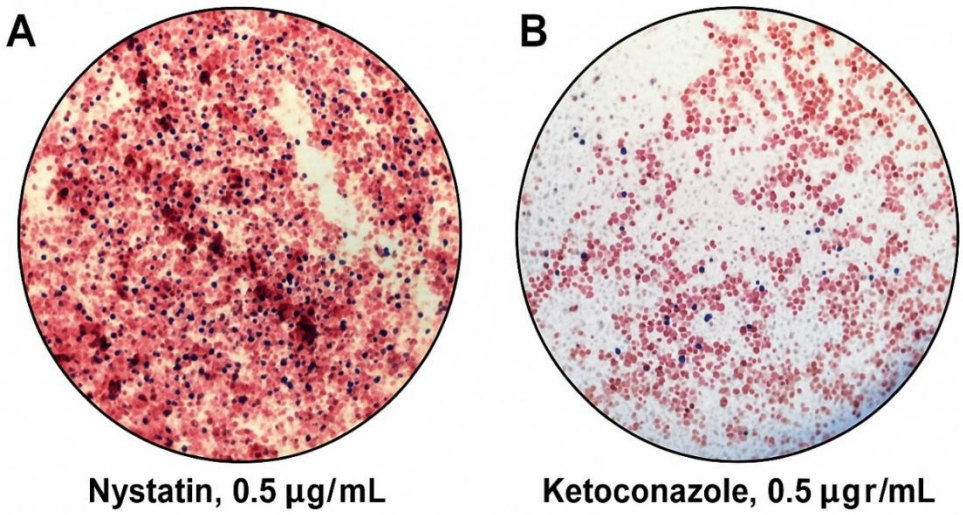

Figure 2: Representative bright-field micrographs of Candida albicans stained with Sudan Black/Safranin at 0.5 µg/mL antifungal. (A) Nystatin—dense fields with numerous Sudan Black–positive chlamydospores (dark blue/black) among pink counter-stained yeast cells. (B) Ketoconazole—markedly sparser chlamydospores with predominance of isolated yeasts

At the higher concentrations, the superiority of ketoconazole remained clear. At 5 µg/mL, nystatin resulted in ~71.8 spores/10 HPF versus only ~39.0 with ketoconazole (the ratio of Nystatin: Ketoconazole was roughly 1.8:1). By 10 µg/mL, nystatin-treated isolates averaged 52.0 spores, in contrast to 28.8 with ketoconazole. Even at this highest dose, nystatin could not eliminate chlamydospore formation, whereas ketoconazole brought many isolates to the brink of zero spore production. On average, ketoconazole at 10 µg/mL yielded ~23 fewer chlamydospores per field than nystatin at the same concentration – a significant difference (p<0.001). Stated another way, ketoconazole achieved approximately 55% more reduction in spore count from baseline compared to nystatin when both were at 10 µg/mL.
The two-way ANOVA confirmed a highly significant overall drug effect ($F_{1,190} \approx 680.4$, $p \approx 1 \times 10^{-64}$), indicating that overall, ketoconazole was far more inhibitory to spore production than nystatin. There was also a significant concentration effect ($F_{4,190} \approx 68.6$, $p < 10^{-35}$), reflecting that within each drug, spore counts changed across concentrations. Importantly, the interaction effect (Drug × Concentration) was significant ($F_{4,190} \approx 9.73$, $p < 0.000001$), meaning the efficacy gap between ketoconazole and nystatin varied with concentration (i.e., the response curves were non-parallel). Indeed, as described above, the difference was smallest (though still significant) at 0.5 µg/mL and largest at 2–5 µg/mL. This interaction suggests that ketoconazole's inhibitory effect increases more steeply with concentration than nystatin's.

Table 1. Chlamydospore production by Candida albicans under nystatin vs. ketoconazole at various concentrations. Data are mean chlamydospore counts per 10 high-power fields (HPF) ± SD, based on 20 isolates.

Antifungal Concentration (µg/mL)	Nystatin: Chlamydospores (mean ± SD per 10 HPF)	Ketoconazole: Chlamydospores (mean ± SD per 10 HPF)	P
0.5	76.8 ± 7.6	54.0 ± 8.2	< 0.001
1	81.8 ± 8.1	49.0 ± 8.3	< 0.001
2	86.8 ± 8.1	44.0 ± 8.3	< 0.001
5	71.8 ± 8.1	39.0 ± 8.3	< 0.001
10	52.0 ± 8.1	28.8 ± 8.3	< 0.001

★ *P < 0.001 for all pairwise comparisons at each concentration (Nystatin vs Ketoconazole), by t-test. Significance persists after adjustment for multiple comparisons. P values refer to the comparison between nystatin and ketoconazole at each concentration (unpaired t-test, df = 19). All differences remained significant at p<0.001 after Bonferroni correction for multiple comparisons.*

As shown in (Table 1), ketoconazole's chlamydospore counts were significantly lower than nystatin's at every corresponding dose. To highlight the clinical relevance of these differences: nystatin at typical topical concentrations (e.g., 100,000 U/mL, roughly 100 µg/mL in an oral suspension) might reduce chlamydospore formation if maintained at

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high local levels, but in practice, patient compliance (keeping nystatin in the mouth for prolonged periods) is variable. By contrast, ketoconazole at systemic levels (achieving ~1–2 μg/mL in plasma or tissue) already suppresses spore formation by ~50%. These data are congruent with a 2021 network meta-analysis by Fang *et al*., which found that systemic azoles (e.g., miconazole, fluconazole, ketoconazole) were more effective than nystatin in achieving mycological cure of oral candidiasis [22]. Our results provide a mechanistic underpinning for that clinical finding: azoles likely curb not only yeast growth but also the formation of resilient fungal forms, thereby clearing infections more thoroughly. In contrast, nystatin's fungicidal action might spare some hardy spores or fail to penetrate biofilm niches, leading to incomplete clearance of the pathogen.

In summary, the comparative analysis clearly demonstrates that ketoconazole outperforms nystatin in suppressing *C. albicans* chlamydospore production across all tested concentrations. The difference is particularly striking at intermediate concentrations (2–5 μg/mL, relevant to levels achieved by systemic therapy), where nystatin shows minimal effect, but ketoconazole causes a dramatic reduction. These findings support our initial hypothesis and underscore the potential advantage of azole antifungals in controlling fungal virulence factors like spore formation.

Discussion

This study provides novel insights into how two commonly used antifungal agents – nystatin and ketoconazole – influence the production of chlamydospores by *Candida albicans*. Chlamydospores are a unique morphological form associated with *C. albicans* survival in hostile conditions [22]. While their exact role in human infection remains somewhat enigmatic, their presence signifies a robust fungal propagule that can endure adverse environments and potentially reignite infection when conditions become favorable [23]. Our findings show that ketoconazole has a pronounced inhibitory effect on chlamydospore formation, far exceeding that of nystatin. This difference likely stems from the distinct mechanisms of action of the two drugs and has important clinical implications for managing candidiasis in immunocompromised patients. Nystatin and ketoconazole attack *Candida* on different fronts. Nystatin binds to ergosterol in the fungal cell membrane, quickly forming pores that cause ion leakage and cell death [24]. This mechanism is rapidly fungicidal to metabolically active yeast cells. However, chlamydospore formation is a stress response – spores are produced under nutrient limitation and other hostile conditions, essentially representing a quasi-dormant state [25].

Cells transitioning to or existing as chlamydospores may have reduced ergosterol content or metabolic activity, potentially making them less susceptible to polyene action. Moreover, nystatin's effect is strongly concentration-dependent and exhibits a post-antifungal effect (PAFE) only at higher doses (meaning brief exposure can have a persistent suppressive effect after the drug is removed, but primarily when concentrations are high). At sub-lethal concentrations (as represented by 0.5–2 μg/mL in our tests), nystatin might not fully disrupt the cell membrane. Our observation that *C. albicans* still produced abundant chlamydospores at low-dose nystatin suggests that incomplete membrane perturbation does not interrupt the developmental program of spore formation. In fact, slight membrane stress might even paradoxically trigger more sporulation as a survival tactic; we noted a small, non-significant increase in spore counts at 1–2 μg/mL nystatin in some isolates, hinting that low-level stress did not inhibit – and may have minimally stimulated – spore production. This aligns with the concept that nystatin's fungicidal action is most effective against actively growing yeast cells, whereas dormant forms require higher drug concentrations to be affected.

Ketoconazole, on the other hand, inhibits ergosterol synthesis via blockade of 14-α-demethylase (CYP51)[26]. This prevents the fungus from properly constructing new cell membranes. Chlamydospore formation requires synthesizing a new thick cell wall and membrane for the spore. By depriving the fungus of ergosterol, ketoconazole likely impairs *C. albicans*' ability to form mature chlamydospores. The dose-dependent nature of ketoconazole's effect in our study aligns with this: as the drug concentration increased, the ergosterol content in fungal cells would be progressively reduced, eventually reaching a level where normal growth and morphological development (like sporulation) became unsustainable [27]. Our data showed that even at 0.5–1 μg/mL, ketoconazole significantly curtailed spore yields, implying that *C. albicans* cannot properly initiate or complete chlamydospore development when ergosterol synthesis is even partially blocked. Additionally, azoles, including ketoconazole, are known to induce the accumulation of toxic sterol intermediates and can trigger cell wall stress responses in *Candida*.

Chlamydospore formation involves a complex regulatory network and a significant investment of cellular resources; if ketoconazole diverts the cell's resources to stress response (e.g., dealing with membrane and cell wall perturbation), the fungus might abandon or fail at the energetically costly sporulation process. Prior proteomic studies have shown that many cell wall- and metabolism-related proteins are down-regulated when *C. albicans* is exposed to azoles, supporting the idea that azoles broadly diminish the fungal cell's capacity to differentiate into stress-resistant forms like

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chlamydospores. Our results are consistent with and extend findings from prior to comparative studies of azoles versus polyenes. Alhussaini *et al.* (2013) [28] compared ketoconazole and nystatin's antifungal effects on *C. albicans*, finding ketoconazole to have a stronger inhibitory action (though their focus was on growth inhibition and MIC values rather than morphogenesis). Fox *et al.* (1998) [29] specifically noted ketoconazole's superior *in vitro* activity against *Candida* compared to nystatin (in their study of oral isolates), and our chlamydospore-specific data align with their general antifungal findings. Furthermore, as mentioned, a 2021 Bayesian network meta-analysis (Fang *et al.*)[30] evaluating treatments for oral candidiasis concluded that azole antifungals (e.g., miconazole, fluconazole, ketoconazole) achieve higher mycological cure rates than nystatin. Our study provides a plausible explanation for that observation: these systemic azoles likely not only kill the *Candida* yeast cells but also suppress the formation of resilient structures like chlamydospores that could cause persistence or relapse.

Nystatin, while effective at reducing superficial yeast populations, might leave behind spores or biofilm-associated cells that can regrow once the drug is removed. Clinically, this difference can manifest as more frequent recurrence of thrush with nystatin therapy – a phenomenon documented in certain patient groups. For example, in HIV/AIDS patients with recurrent oropharyngeal candidiasis, topical nystatin often yields only temporary relief, and infection tends to recur, whereas switching to fluconazole (a systemic azole) results in more sustained clearance. In one case series, severe oropharyngeal candidosis refractory to nystatin showed rapid resolution when patients were switched to or combined with fluconazole, highlighting the azole's greater efficacy in eradicating the infection source. Interestingly, our data showed that some chlamydospore production persisted even at 10 μg/mL ketoconazole (mean ~28.8 spores/10 HPF remained). This could be due to a subpopulation of *C. albicans* cells that are tolerant or already in a state not requiring active ergosterol synthesis (for example, spores that had formed during the initial 24 h growth before drug exposure and remained viable).

It is known that *C. albicans* can exhibit tolerance to azoles at high cell densities or in biofilms. In our essay, some chlamydospores might have partially or fully formed during the initial growth phase before ketoconazole was introduced. Thus, an antifungal present earlier (before spore initiation) might have an even more profound effect – an insight relevant to prophylactic antifungal use. Clinically, this underscores that the timing of antifungal administration relative to the stage of infection is important: early systemic therapy might prevent the fungus from ever reaching the durable spore stage. It also suggests that combining antifungal strategies could yield synergistic effects: for instance, using an azole to inhibit new spore formation plus a second agent to kill existing spores or biofilm cells could be beneficial in refractory cases.

Ketoconazole was examined because it was available, though its systemic use has waned due to toxicity; fluconazole and other triazoles are the more relevant options now and likely suppress chlamydospores similarly by inhibiting ergosterol synthesis, with fluconazole generally fungistatic versus potentially fungicidal high-dose ketoconazole. We did not test fluconazole, newer azoles (voriconazole/posaconazole), or echinocandins like caspofungin—agents that might also impede chlamydospore formation via β-glucan inhibition—so this remains to be confirmed. Our findings are in vitro and don't establish clinical outcomes; while external data indirectly suggest systemic azoles reduce relapse compared with nystatin in high-risk patients, definitive head-to-head trials are unlikely, so our work primarily offers mechanistic support for guidelines favoring systemic azoles in such cases.

For immunocompromised patients with oral candidiasis, favor early systemic azole therapy (e.g., fluconazole) rather than relying solely on topical nystatin, especially with recurrent disease or risk of dissemination. If nystatin is used, maximize contact time (slow-dissolving pastilles or prolonged swish-and-retain), ensure adherence, and add adjuncts (oral hygiene, denture care, chlorhexidine with spacing) to reach concentrations more likely to suppress spores. Monitor closely for recurrence; persistence after nystatin likely reflects regrowth from surviving propagules and should prompt step-up to systemic therapy, with topical–systemic combinations reserved for refractory cases while watching for interactions. Further research should test whether triazoles, echinocandins, and newer agents (ibrexafungerp, manogepix) suppress chlamydospore/biofilm formation to inform optimal sequential or combination strategies.

Conclusions

In this in vitro comparison, ketoconazole markedly outperformed nystatin in suppressing C. albicans chlamydospore formation from cancer-patient oral isolates, supporting azoles over polyenes for curbing persistence-related morphologies. Given ketoconazole's toxicity, fluconazole is the practical systemic alternative and likely confers similar suppression of resilient forms. For immunocompromised patients, early systemic azole therapy offers a more comprehensive effect, while nystatin—though appropriate for uncomplicated thrush—should be optimized for contact time and paired with adjunctive measures, with rapid escalation if relapse occurs. Overall, effective management must

target both active yeast and protected morphotypes; future studies should evaluate additional agents (e.g., other azoles, echinocandins) for effects on chlamydospore/biofilm formation and correlate these laboratory findings with clinical outcomes.

Conflict of interest. Nil

References

1. Gamil Y, Hamed MG, Elsayed M, Essawy A, Medhat S, Zayed SO, et al. The anti-fungal effect of miconazole and miconazole-loaded chitosan nanoparticles gels in diabetic patients with Oral candidiasis-randomized control clinical trial and microbiological analysis. BMC Oral Health. 2024 Feb 7;24(1):198.
2. Lee HS, Kim Y. Antifungal Activity of Salvia miltiorrhiza Against Candida albicans Is Associated with the Alteration of Membrane Permeability and (1,3)-β-D-Glucan Synthase Activity. J Microbiol Biotechnol. 2015 Dec 23;26(3):610-7.
3. Mendoza-Reyes DF, Mora-Montes HM, Gómez-Gaviria M. Candida lusitaniae: Biology, Pathogenicity, Virulence Factors, Diagnosis, and Treatment. Infect Drug Resist. 2022 Aug 1;15:5121-35.
4. Fan-Havard P, Smith SM, Mangia A, Eng RH, Capano D. Development of resistance in candida isolates from patients receiving prolonged antifungal therapy. Antimicrob Agents Chemother. 1991 Nov;35(11):2302-5.
5. Tobudic S, Presterl E, Kratzer C. Azole-resistant Candida spp. – emerging pathogens? Mycoses. 2012 Apr;55 Suppl 1:24-32.
6. Staib P, Morschhäuser J. Chlamydospore formation in Candida albicans and Candida dubliniensis– an enigmatic developmental programme. Mycoses. 2007 Jan;50(1):1-12.
7. Le PH, Linklater DP, Nguyen DHK, Aburto-Medina A, Crawford RJ, Maclaughlin S, et al. Nanoscale Surface Roughness Influences Candida albicans Biofilm Formation. ACS Appl Bio Mater. 2020 Dec 21;3(12):8581-91.
8. Bartnicka D, Rapala-Kozik M, Lazarz-Bartyzel K, Satala D, Bras G, Zawrotniak M, et al. Candida albicans Shields the Periodontal Killer Porphyromonas gingivalis from Recognition by the Host Immune System and Supports the Bacterial Infection of Gingival Tissue. Int J Mol Sci. 2020 Mar 14;21(6):1984.
9. Ha KC, White TC. Effects of azole antifungal drugs on the transition from yeast cells to hyphae in susceptible and resistant isolates of the pathogenic yeast Candida albicans. Antimicrob Agents Chemother. 1999 Apr;43(4):763-8.
10. Lortholary O, Cornely OA, Cuenca-Estrella M, Kullberg BJ, Verweij PE, Herbrecht R, et al. ESCMID guideline for the diagnosis and management of Candida diseases 2012: patients with HIV infection or AIDS. Clin Microbiol Infect. 2012 Dec;18 Suppl 7:68-77.
11. Kuriyama T, Bagg J, Lewis MAO, Williams DW, Ready D, Coulter WA. In vitro susceptibility of oral Candida to seven antifungal agents. Oral Microbiol Immunol. 2005 Dec;20(6):349-53.
12. Goins RA, Waecker N, Moorefield E, Ascher D, Arnold J. Comparison of fluconazole and nystatin oral suspensions for treatment of oral candidiasis in infants. Pediatr Infect Dis J. 2002 Dec;21(12):1165-7.
13. Janeth Rimachi Hidalgo K, Cabrini Carmello J, Carolina Jordão C, Aboud Barbugli P, De Sousa Costa CA, Garcia De Oliveira Mima E, et al. Antimicrobial Photodynamic Therapy in Combination with Nystatin in the Treatment of Experimental Oral Candidiasis Induced by Candida albicans Resistant to Fluconazole. Pharmaceuticals (Basel). 2019 Sep 18;12(3):140.
14. Garcia-Cuesta C, Bagan J, Sarrion-Perez M. Current treatment of oral candidiasis: A literature review. J Clin Exp Dent. 2014 Dec;6(5):e576-82.
15. Powderly WG, Perfect JR, Mayer KH. Diagnosis and treatment of oropharyngeal candidiasis in patients infected with HIV: a critical reassessment. AIDS Res Hum Retroviruses. 1999 Nov;15(16):1405-12.
16. Uno J, Shigematsu ML, Arai T. Primary site of action of ketoconazole on Candida albicans. Antimicrob Agents Chemother. 1982 Jun;21(6):912-8.
17. Ellepola ANB, Samaranayake LP. The effect of limited exposure to antifungal agents on the germ tube formation of oral Candida albicans. J Oral Pathol Med. 1998 May;27(5):213-9.
18. Khan F, Baqai R. In vitro antifungal sensitivity of fluconazole, clotrimazole and nystatin against vaginal candidiasis in females of childbearing age. J Ayub Med Coll Abbottabad. 2010 Dec;22(4):197-200.
19. Ghannoum M. Azole Resistance in Dermatophytes: Prevalence and Mechanism of Action. J Am Podiatr Med Assoc. 2016 Jan;106(1):79-86.
20. Patil S, Anil S, Majumdar B, Rao RS. Clinical Appearance of Oral Candida Infection and Therapeutic Strategies. Front Microbiol. 2015 Dec 17;6:1391.
21. Powell MJ, Sternshein DJ, Bracker CE. Formation of chlamydospores in Gilbertella persicaria. Can J Bot. 1981 May;59(5):908-28.
22. Han D, Ji Y, Yang S, Song P, Shi Y, Shao D, et al. Therapeutic effect of iturin A on Candida albicans oral infection and its pathogenic factors. Antimicrob Agents Chemother. 2023 Dec 5;68(1):e0060023.
23. Zheng L, Guo L, Xu Y, Wang C. Ketoconazole induces reversible antifungal drug tolerance mediated by trisomy of chromosome R in Candida albicans. Front Microbiol. 2024 Jul 30;15:1436205.

24. Monroy-Pérez E, Paniagua-Contreras GL, Rodríguez-Purata P, Vaca-Paniagua F, Vázquez-Villaseñor M, Díaz-Velásquez C, et al. High Virulence and Antifungal Resistance in Clinical Strains of Candida albicans. Can J Infect Dis Med Microbiol. 2016;2016:5930489.
25. Eisman B, Román E, Arana D, Alonso-Monge R, Nombela C, Pla J. The Cek1 and Hog1 Mitogen-Activated Protein Kinases Play Complementary Roles in Cell Wall Biogenesis and Chlamydospore Formation in the Fungal Pathogen Candida albicans. Eukaryot Cell. 2006 Feb;5(2):347-58.
26. Coksu I, Bozkurt Y, Akmayan I, Demirci H, Ozbek T, Acar S. Ketoconazole-loading strategy to improve antifungal activity and overcome cytotoxicity on human renal proximal tubular epithelial cells. Nanotechnology. 2023 Dec 27;35(11):115702.
27. Lee W, Lee DG. A novel mechanism of fluconazole: fungicidal activity through dose-dependent apoptotic responses in Candida albicans. Microbiology (Reading). 2018 Feb;164(2):194-204.
28. Alhussaini MS. Phenotypic and Molecular Characterization of Candida Species in Urine Samples from Renal Failure Patients. Sultan Qaboos Univ Med J. 2013 Jan;13(1):104-11.
29. Liebertz C, Fox P. Ketoconazole as a Secondary Hormonal Intervention in Advanced Prostate Cancer. Clin J Oncol Nurs. 2006 Jun;10(3):361-6.
30. Yue F, Xie Y, Chen X, Xu R, Wang H, Bai N, et al. Bayesian Network Meta-Analysis of Postoperative Analgesic Techniques in Thoracoscopic Lung Resection Patients. Pain Ther. 2025 Feb 22;14(2):617-37.
31. Tortorano AM, Barchiesi F, Brescini L, Morroni G, Prigitano A. Candidemia: Evolution of Drug Resistance and Novel Therapeutic Approaches. Infect Drug Resist. 2021 Dec 19;14:5543-53.