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In vitro inhibitory activities of magnolol against *Candida* spp.

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Abstract: *Candida* spp. cause various infections involving the skin, mucosa, deep tissues, and even life-threatening candidemia. They are regarded as an important pathogen of nosocomial bloodstream infection, with a high mortality rate. As a result of prolonged exposure to azoles, the therapeutic failure associated with azoles resistance has become a serious challenge in clinical situations. Therefore, novel, alternative antifungals are required urgently. In the present study, the CLSI M-27A broth microdilution method and the 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay were used to evaluate the antifungal effects of magnolol against various standard *Candida* strains in planktonic mode and biofilm formation, respectively. The antifungal activity of magnolol was demonstrated in planktonic *C. albicans* and non-albicans *Candida* species, especially fluconazole-resistant *Candida krusei*, with the minimum inhibitory concentrations ranging from 10 to 40 μg/mL. The $BMIC_{90}$ (minimum concentration with 90% *Candida* biofilm inhibited) values of magnolol ranged from 20 to 160 μg/mL, whereas the $BMIC_{90}$ values of fluconazole were more than 128 μg/mL. As an alternative and broad-spectrum antifungal agent, magnolol might be of benefit to the treatment of refractory *Candida* infection.

Keywords: magnolol, inhibition, *Candida* spp., biofilm

Introduction

The genus *Candida*, an opportunistic pathogen, is prone to attack immunocompromised hosts or those with debilities, causing the infection of the skin, mucosa, deep-tissues, or even the life-threatening candidemia.[1] With the use of potent antibiotics; immunosuppressive and cytotoxic agents; and implanted devices, as well as prolonged intensive care unit stays, the risk of *Candida*-associated nosocomial infections is increasing remarkably. According to a survey from the US National Nosocomial Infections Surveillance System, *Candida* species are the fourth most common cause of nosocomial bloodstream infection, with a mortality rate of 35%.[2]

Azoles, such as fluconazole and itraconazole, are the most frequently prescribed antifungals in candidiasis therapy, which destroy the cellular structures of fungi by inhibiting the biosynthesis of membranous ergosterol.[3] However, long-term or repeat exposure to azoles in refractory infection can induce the emergence of resistant strains.[4] Among *C. albicans* isolates from candidemia patients and human immunodeficiency virus (HIV)-positive patients with oropharyngeal candidiasis, 0%–4.3% and 9.5% were reported to be fluconazole resistant, respectively.[5–8] In recent years, the incidence of infections caused by non-albicans *Candida* species (NACS), including *C. glabrata*, *C. dubliniensis*, and *C. krusei*, increased.[9] Approximately 26% of *Candida* bloodstream infections investigated in the USA were attributed to *C. glabrata*,[10] and

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1.5%–32% of HIV-positive populations were infected with *C. dubliniensis*.[11,12] Azoles-induced *Candida* species screening is responsible for the increased infection by NACS. Under the stress of azoles, the species susceptible to azoles are inhibited, leaving the resistant species to grow richer.[13] *C. krusei* is intrinsically azoles-resistant, while the resistance of *C. glabrata* may be acquired. Their ability to take up exogenous sterols allows *C. glabrata* to grow in the presence of azoles.[14] Despite *C. dubliniensis* being mostly sensitive to azoles, it can develop azole resistance during antifungal treatment.[15] The incidence was reported to be 23% in HIV-positive individuals.[16] Therefore, a novel, alternative agent is needed against a broad range of fungi.

Magnolol, a lignin compound, was extracted initially in the 1930s from the dried bark of the stem, root, or branch of the traditional Chinese medicinal plant *Magnolia officinalis*. Previous studies demonstrated that magnolol could inhibit the growth of *Helicobacter pylori* remarkably,[17] as well as other pathogens localized in the oral cavity, including *Streptococcus mutans*,[18] *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Prevotella intermedia*.[19] The inhibitory activities of magnolol against *Cryptococcus neoformans*, *Aspergillus niger*, and *C. albicans* were also demonstrated.[18,20] Nevertheless, NACS-associated infections have increased notably, and the effect of magnolol on NACS, especially the resistant species, remains unclear. Therefore, in this study, the activities of magnolol against various *Candida* spp. were evaluated in planktonic mode and in biofilm formation.

Materials and methods

Organisms and culture condition

Five different standard strains of *C. albicans* (ATCC90028), *C. krusei* (ATCC6258), *C. dubliniensis* (MYA646), *C. glabrata* (ATCC90030), and *C. parapsilosis* (ATCC22019), obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA), were used in the study. *C. parapsilosis* (ATCC22019) was used as a quality control isolate.

All yeasts were cultured aerobically on Sabouraud dextrose agar (SDA) plates (BioMérieux Industry Co. Ltd., Shanghai, China) for 48 h at 37°C, and stored at 4°C ready for use.

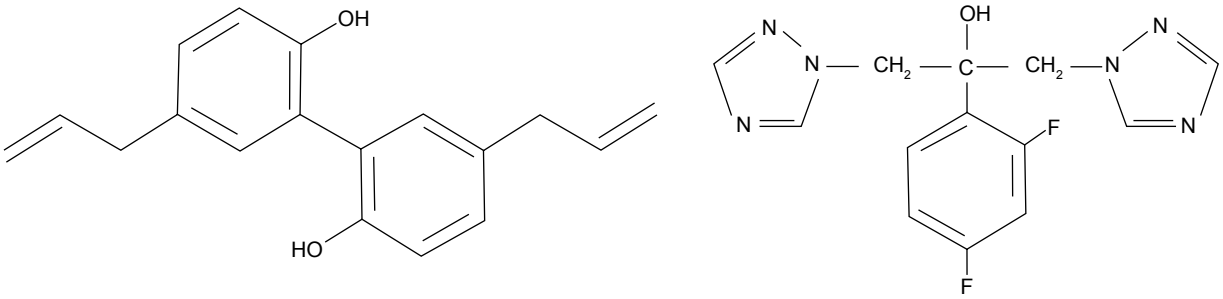
Drug preparation

Commercial powders of magnolol and fluconazole (Figure 1) were obtained from the National Institutes for Food and Drug Control (Beijing, China). The purity was measured by high-performance liquid chromatography and determined to be about 98.8% for magnolol, 99.8% for fluconazole. The drugs were dissolved in dimethyl sulfoxide (Sigma-Aldrich Co., St Louis, MI, USA), and stored at a concentration of 1.28×10^5 μg/mL for magnolol, and 1.28×10^4 μg/mL for fluconazole, at –80°C.

Antifungal activity of magnolol against planktonic *Candida* cells

Susceptibility testing of planktonic yeast cells to magnolol was performed following the CLSI M-27A broth microdilution method.[21] The frozen magnolol solution was thawed and diluted in Roswell Park Memorial Institute (RPMI) 1640 medium (containing L-glutamine) (Life Technologies Co., Madison, WI, USA), which was buffered to pH =7.0 using 0.165 M 3-morpholinopropane-1-sulfonic acid (Sigma-Aldrich Co.). The magnolol solution (100 μL of a 2-fold dilution) was pipetted into each well of a 96-well microtiter plate. The final concentration of magnolol ranged from 2.5 to 1,280 μg/mL.

Fresh yeast cells were harvested and washed twice with PBS (pH =7.2). Yeast suspensions at 1×10^4 cells/mL were prepared using RPMI 1640 medium. Aliquots of 100 μL of the yeast suspension was inoculated into each well containing the magnolol solution, and incubated for 48 h at 37°C. The



Magnolol **Fluconazole**

Figure 1 Structures of magnolol and fluconazole.

minimum inhibitory concentration (MIC) was determined on visual inspection. The MIC was defined as the lowest concentration, at which no yeast could be seen to grow. The experiment was performed in triplicate.

As a positive control, the MICs of fluconazole against the planktonic yeasts were determined in parallel, and the final concentration of fluconazole ranged from 0.25 to 128 μg/mL.

Preparation of standard yeast suspensions for biofilm studies

Yeast cells were grown on an SDA plate for 18 h at 37°C. A loopful of the yeast was then inoculated into yeast nitrogen base medium (YNB, Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) supplemented with 50 mM glucose in an orbital shaker at 80 rpm. After overnight incubation, the yeast cells were harvested. After washing twice in PBS, yeast suspensions at 1×10^7 cells/mL were prepared in YNB (pH =7.0) medium containing 100 mM glucose.

Biofilm formation

A previously described method was used for *Candida* biofilm formation.[22] Briefly, aliquots of 100 μL of the standard yeast suspensions were pipetted into each well of polystyrene microtiter plates and incubated for 90 min at 37°C in a shaker at 80 rpm, which allowed the yeast cells to attach to the well surface. Thereafter, the yeast suspensions were aspirated, and each well was washed gently with 100 μL of sterilized PBS. Following the pipetting of 200 μL of YNB medium supplemented with 100 mM glucose into each well, 4 μL of 2-fold dilutions of magnolol solutions were added to each well, the final concentrations of magnolol ranged from 5 to 2,560 μg/mL. The microtiter plates were subsequently incubated at 37°C in a shaker at 80 rpm. After 6, 12, 24, or 48 h of incubation, the yeast suspensions were aspirated. The 4 time-points were set up based on the developmental phases during the period of *Candida* biofilm formation. Each well was washed twice with sterilized PBS to remove unattached cells.

The influence of fluconazole on *Candida* biofilm production was also studied, and the final concentration of fluconazole ranged from 0.25 to 128 μg/mL.

XTT reduction assay

This assay was used to determine the biofilm activity by measuring the reduction of 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT).[23] XTT (Sigma-Aldrich Co.) was dissolved in PBS at 1 mg/mL. After sterilization through a 0.22-μm filter, the XTT solution was stored at −80°C until use. Menadione (0.4 mM; Sigma-Aldrich Co.) was prepared in acetone immediately before the assay. Before each assay, the thawed XTT solution was mixed with the menadione solution at a ratio of 5 to 1 by volume. Following the prewash, 200 μL of XTT-menadione-PBS reagent was added to each well containing adherent yeast cells, and incubated in the dark. Three hours later, 100 μL of the supernatant in each well was transferred to new wells. The color of the supernatants in each well was measured using a microplate reader (model: EL ×808) (BioTek Instruments, Inc., Waltham, MA, USA) at 490 nm. The absorbance value of each solution was read as the optical density (OD) value. The experiment was performed in triplicate and the average result was used. The yeast suspension without drug was regarded as the drug-free control.

The $BMIC_{90}$ was defined as the minimum concentration with 90% *Candida* biofilm inhibited, of which produced 90% reduction of OD value compared with the drug-free control.

Result

Antifungal activity of magnolol against planktonic *Candida* cells by broth microdilution

Magnolol demonstrated in vitro inhibitory activities against planktonic *C. albicans*, as well as non-albicans *Candida*, in terms of their MICs (Figure 2). Of the tested strains, *C. dubliniensis* was most susceptible to magnolol, with a MIC of 10 μg/mL, followed by *C. glabrata* with a MIC of 20 μg/mL, and *C. albicans* with a MIC of 40 μg/mL, which was equal to that of *C. krusei* (Table 1).

Corresponding to the standard recommended by National Committee for Clinical Laboratory Standards (NCCLS) [21], fluconazole showed antifungal activities against *C. albicans*, *C. dubliniensis*, and *C. glabrata* with MICs of 0.25, 0.5, and 2 μg/mL, respectively, while *C. krusei* was resistant to fluconazole, with a MIC of 32 μg/mL.

Inhibitory effects of magnolol on *Candida* biofilm formation by the XTT reduction assay

Magnolol was remarkably effective at inhibiting *Candida* spp. biofilm formation compared with fluconazole. At the mature-stage of biofilm (48 h), the $BMIC_{90}$ of magnolol against *C. albicans* was 160 μg/mL. *C. dubliniensis* and *C. glabrata* were more susceptible to magnolol, with the $BMIC_{90}$ values of 20 and 40 μg/mL, respectively. Even

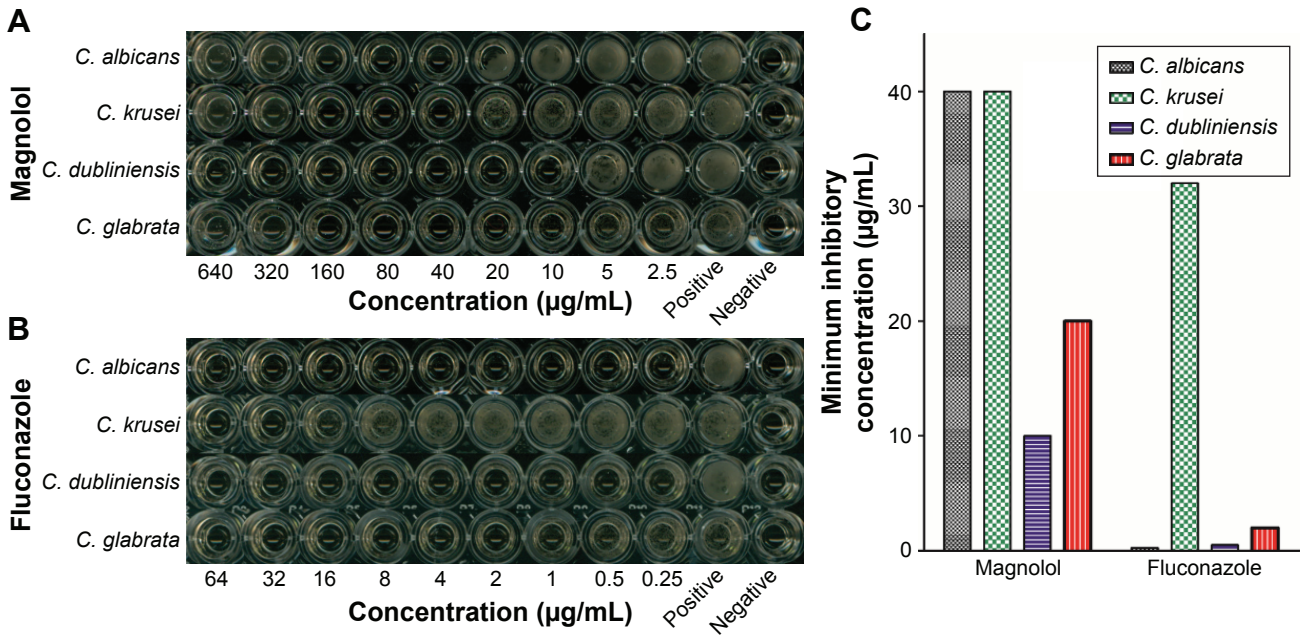


Figure 2 The inhibitory effects of magnolol on planktonic-mode *Candida* cells using the CLSI M-27A broth microdilution method.
Notes: The quantity of *Candida* cells was reduced as the drug concentrations of the wells increased. At the MICs or over, no yeast cells were observed to grow. (**A**) Results for magnolol; (**B**) results for fluconazole; (**C**) the MICs of magnolol and fluconazole for *C. albicans*, *C. krusei*, *C. dubliniensis*, and *C. glabrata*.
Abbreviation: MICs, minimum inhibitory concentrations.

C. krusei, the fluconazole-resistant strain, demonstrated sensitivity to magnolol, with a $BMIC_{90}$ of 80 μg/mL. In comparison, fluconazole showed higher $BMIC_{90}$ values for all strains, at over 128 μg/mL. No definitive values were identified because of the restricted concentrations of fluconazole used in the study (0.25–128 μg/mL) (Table 1).

Compared with the planktonic-mode, the $BMIC_{90}$ values of fluconazole increased by 4–500 times against 48 h yeast-biofilms, changing from 0.25 to 32 μg/mL to over 128 μg/mL. By contrast, the $BMIC_{90}$ values of magnolol ranged from 20 to 160 μg/mL for the 4 *Candida* spp., which were only 2–4 times higher than the MICs in planktonic form (ranging from 0.25 to 32 μg/mL; Table 1).

Based on the developmental phases during the period of *Candida* biofilm formation, 4 different time-points were chosen to evaluate the effect of magnolol on *Candida* biofilm formation. All yeast strains were most vulnerable to magnolol at the maturation age of 12 h. The $BMIC_{90}$ of *C. albicans* was 20 μg/mL, *C. krusei*, *C. dubliniensis* and *C. glabrata* were all 10 μg/mL. With the maturation of the biofilms, the $BMIC_{90}$ values showed a tendency to increase (Figure 3), changing from 10 to 20 μg/mL to 20–80 μg/mL (from 12 to 24 h). With the exception of *C. albicans*, the $BMIC_{90}$ values of the other strains reached a high level, which was maintained until 48 h. From 12 to 48 h of biofilm development, the $BMIC_{90}$ of *C. albicans* continued to increase. This result suggested that the inhibitory effect of magnolol on yeast biofilm production reached a peak at around 12 h, which coincided with the start of maturation, after which, the yeast biofilm became more tolerant to magnolol (Figure 3).

Using the XTT reduction assay, the biofilm metabolic activities of the *Candida* strains were identified by the microplate reader in terms of absorbance ($OD_{490\,nm}$) (Figure 4). At the 4 time-points observed, the decreases in absorbance correlated with the increasing magnolol concentrations. After 6 h of culture, the metabolic activities of the strains

Table 1 Activities of magnolol against planktonic-mode and 48-h biofilm production of various *Candida* spp.

	Planktonic-mode (MIC, μg/mL)				Biofilm ($BMIC_{90}$, μg/mL)			
	C. albicans	*C. krusei*	*C. dubliniensis*	*C. glabrata*	*C. albicans*	*C. krusei*	*C. dubliniensis*	*C. glabrata*
Magnolol	40	40	10	20	160	80	20	40
Fluconazole	0.25	32	0.5	2	>128	>128	>128	>128

Notes: MIC is the minimum concentration of the drugs that inhibited 100% of the yeast growth on visual inspection. $BMIC_{90}$ is the minimum concentration of the drugs that produced 90% reduction of optical density value compared with the drug-free control.
Abbreviations: MIC, minimum inhibitory concentration; $BMIC_{90}$, the minimum concentration with 90% *Candida* biofilm inhibited.

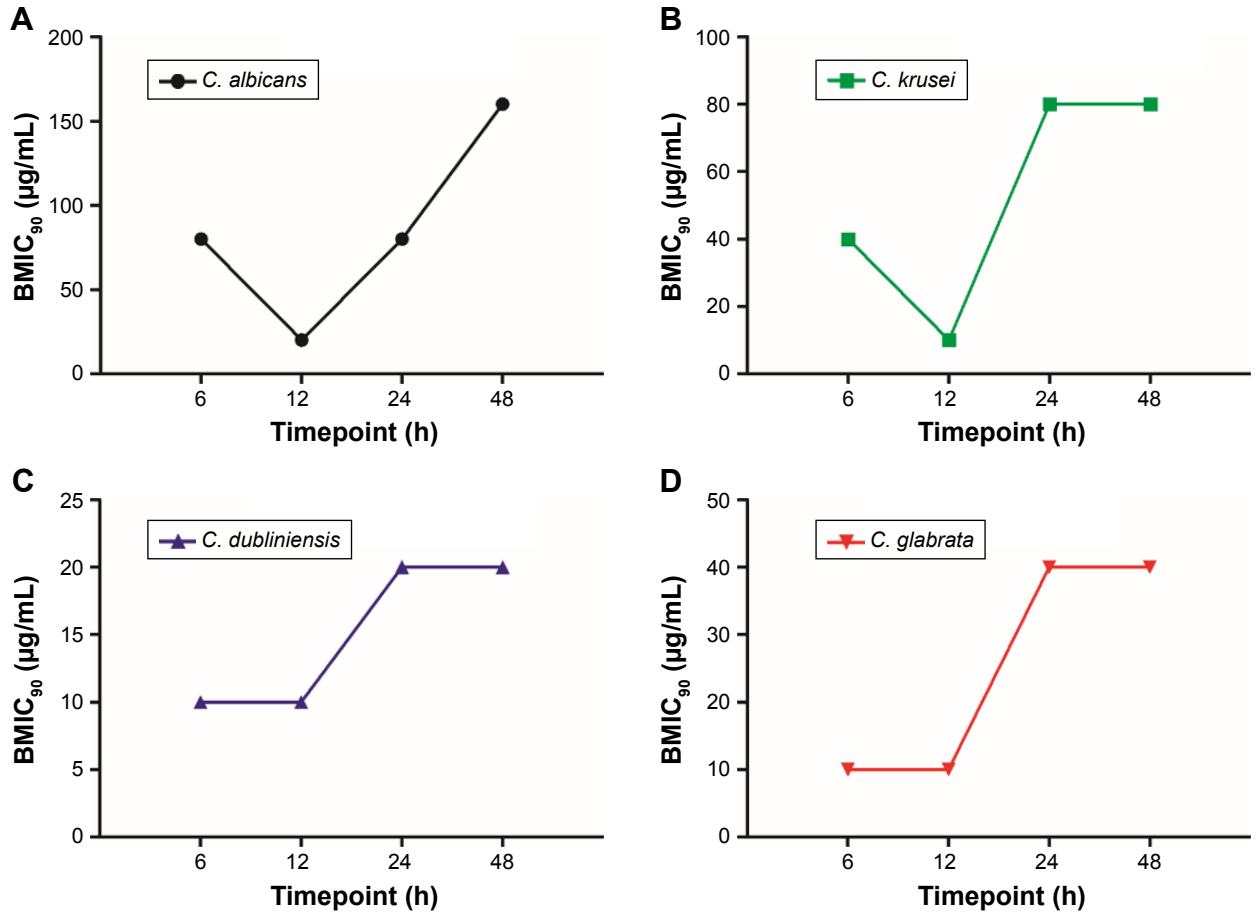


Figure 3 Inhibitory activities of magnolol against *Candida* biofilm formation at different time-points using the XTT reduction assay.
Notes: (**A**) *C. albicans*; (**B**) *C. krusei*; (**C**) *C. dubliniensis*; (**D**) *C. glabrata*.
Abbreviations: $BMIC_{90}$, the minimum concentration with 90% *Candida* biofilm inhibited; XTT, 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

except for *C. albicans*, decreased rapidly. At 12 h, greatly reduced metabolic activity was observed in all strains. Thereafter, the rate of decline slowed in all strains. This result implied that the fungicidal efficacy of magnolol was relatively high up to 12 h.

Discussion

Magnolol, the major chemical compound purified from the traditional Chinese medicinal plant *M. officinalis*, has been demonstrated to have various pharmacological functions in the treatment of illness, including antianxiety,[24] analgesic,[25] smooth muscle relaxing,[26] anti-tumorigenic,[27] and antimicrobial activities. Magnolol is active against Gram-positive and acid-fast bacteria,[17–19] *H. pylori*, and *Propionibacterium acne*.[17] By reducing the secretion of IL-8 and TNF-α induced by *P. acne*, magnolol may exhibit anti-inflammatory effects.[28] The inhibitory effects of magnolol against clinical isolates of *C. albicans* are remarkable, with the MIC values ranging from 16 to 32 μg/mL.[18,20] It was confirmed in the present study using the planktonic standard strain of *C. albicans* (ATCC90028), in which the MIC of magnolol was 40 μg/mL. Besides *C. albicans*, the potent antifungal effects of magnolol for NACS, including *C. krusei*, *C. dubliniensis*, and *C. glabrata* in the planktonic form, were determined with MICs ranging from 10 to 40 μg/mL. *C. krusei* is intrinsically fluconazole-resistant, and *C. dubliniensis*,[15] *C. glabrata*,[14] and *C. albicans*[29] may develop fluconazole-resistance in clinical antifungal therapy. Fluconazole targets mainly membranous ergosterol. Therefore, mutations of the ergosterol biosynthesis gene, *Erg11*, and the overexpression of drug efflux pumps Mdr1p and Cdr1p/Cdr2p, are responsible for fluconazole resistance in *C. albicans*.[30] In *C. glabrata*, the resistance to fluconazole is attributed to its ability to take up exogenous sterols instead of the altered cell membrane sterols. On the other hand, mutations in the *Pdr1* gene are also involved in fluconazole resistance of *C. glabrata*.[31] Although the precise mechanism is unclear, *C. krusei* is considered to resist fluconazole via the efflux pump activity,[32] as well

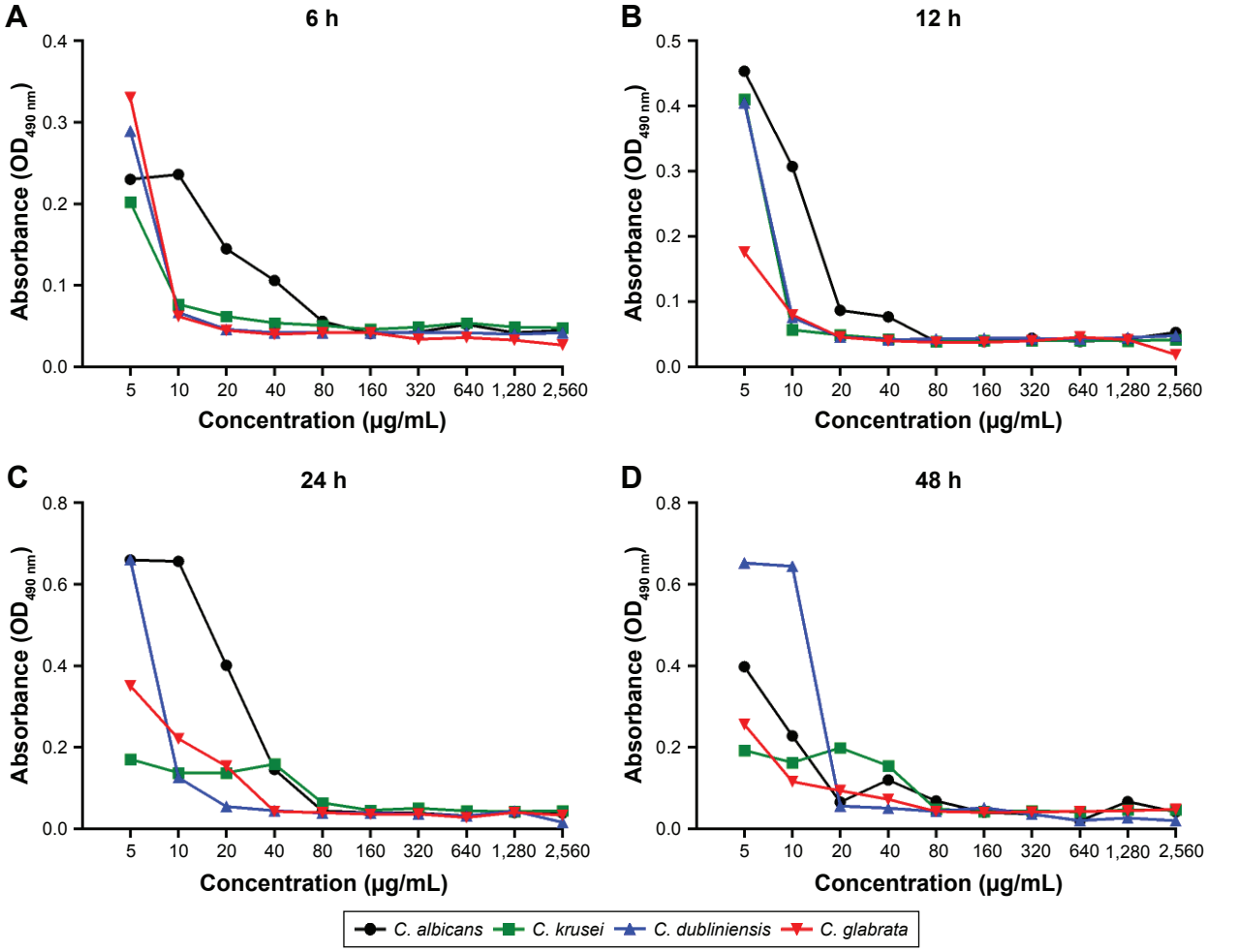


Figure 4 Influence of different concentrations of magnolol on *Candida* biofilm activity during biofilm maturation, as assessed by the XTT reduction assay.
Note: Assessed at 6 (**A**), 12 (**B**), 24 (**C**), and 48 (**D**) h.
Abbreviation: OD, optical density.

as the reduced azole affinity for Erg11p.[33] In this regard, our data suggested that magnolol had a broad antifungal spectrum mechanism that differs from that of fluconazole. Membranous ergosterol may be not the target of magnolol, or at least, not the only one.

In recent years, although *C. albicans* is still the predominant cause of candidiasis, NACS-associated infections have increased notably. *C. glabrata* is isolated frequently from patients with vulvovaginal or urinary candidiasis because of its affinity for epithelial cells of the vagina[33] and urethra.[34] Its rapid dissemination throughout the body contributes to *C. glabrata*'s increasing prevalence in candidemia cases.[35] In the USA, *C. krusei* was responsible for 2.7% of NACS-associated infections.[36] It is considered an important risk factor causing *Candida* infection among patients with hematological malignancies and bone marrow transplants.[37] *C. dubliniensis* was either the second or the third most commonly identified pathogenic fungi in patients with HIV/AIDS,[38,39] and was associated with 2%–7% of candidemia cases.[40,41] In oral infections, *C. glabrata* or *C. krusei* was responsible for 42% of Sjogren's syndrome cases combined with oral candidiasis.[42] For this reason, as an alternative antifungal agent, magnolol may potentially benefit the treatment of NACS-associated infections, particularly the infection caused by the azoles-resistant species.

In nature, most microorganisms prefer growth in the form of biofilm, in which one or more other species are embedded within an extracellular matrix (ECM), comprising a complex community.[43] Approximately 65% of all clinical infections are biofilm-associated.[44] A biofilm is significantly less susceptible to antimicrobial agents, being 10–1,000 times more resistant to antimicrobial agents than the planktonic form.[45] Notably, the concentrations of magnolol required in

the present study to reduce 90% of metabolic activity were just 2 to 4 times higher for biofilms than for planktonic cells, whereas the concentrations of fluconazole required increased by 4 to 500 times. Moreover, magnolol was more active than fluconazole in inhibiting biofilm formation of *Candida* spp. for the $BMIC_{90}$ values for fluconazole were all >128 μg/mL for *C. albicans*, *C. krusei*, *C. dubliniensis*, and *C. glabrata*, whereas the $BMIC_{90}$ values for magnolol ranged from 20 to 160 μg/mL for them. Despite the $BMIC_{90}$ of magnolol against *C. albicans* being 160 μg/mL, the other isolates were more susceptible, with $BMIC_{90}$ values in the range of 20–80 μg/mL, whereas the $BMIC_{90}$ of fluconazole against all strains were over 128 μg/mL.

Multiple mechanisms are responsible for biofilm-associated resistance, including drug efflux pumps, delayed penetration of the antimicrobial agent through the biofilm matrix, decrease of growth rate or cell metabolism.[46,47] Fluconazole-associated resistance attributes to the overexpression of drug efflux pumps genes *Mdr1* and *Cdr1/Cdr2*, as well as the alteration of ergosterol biosynthesis pathway.[48] However, a different mechanism of magnolol from fluconazole could be indicated because of a broad antifungal range of magnolol against *Candida* spp., including intrinsic fluconazole-resistant isolate. Few studies focus on the antifungal mechanisms of magnolol. In the report of Sun et al magnolol was considered to inhibit *C. albicans* biofilm formation through decreasing the yeasts' adhesive and morphological transitional abilities, and its fungicidal capabilities.[20] In addition, cell wall component β-1,3-glucan may also be a target of magnolol as echinocandin family antifungals.[49] Levels of β-1,3-glucan on *Candida* cell walls as well as in ECM were significantly elevated in biofilm-form than in planktonic-mode, which may benefit the biofilm fluconazole-resistance.[50] Moreover, since magnolol was demonstrated to take effect around the cellular logarithmic phase in the present study, the inhibitory effects of cell growth may also be a possible mechanism of magnolol against biofilm formation. However, further studies are needed.

Candida biofilm formation involves several specific stages: 1) The early phase (60–90 min). In this stage, round yeast cells adhere to the substrate; 2) The developmental phase: attached cells proliferate to form a basal layer, and biofilm formation begins; 3) The biofilm maturation stage: complex layers of polymorphic cells develop and become encased in an ECM (24 h); 4) The dispersal stage: some round yeast cells disperse from the biofilm to seed new sites.[51] In the different developmental stages, the biofilm has different biological behaviors. With the maturation of the biofilm, *Candida* spp. cells exhibited increased tolerance to magnolol. To obtain 90% biofilm reduction, increased amounts of magnolol were required. In the present study, the lowest dosage of magnolol required was at around 12 h of culture, after which the $BMIC_{90}$ values started to increase remarkably. It is reasonable to assume that magnolol attacks yeast cells in the logarithmic phase (16–18 h) when the cells are sensitive to environmental changes.[52] Using the XTT reduction assay, the biofilm metabolic activity of *Candida* spp. was assessed. Our data showed that with the increasing magnolol concentrations, the metabolic activities of the biofilms decreased. The reduction proceeded through the course of biofilm formation, which suggested that the effects of magnolol on *Candida* spp. biofilm formation were concentration-dependent.

In summary, in contrast to fluconazole, the antifungal spectrum of magnolol was broad. Various *Candida* spp., including *C. albicans*, *C. krusei*, *C. dubliniensis*, and *C. glabrata* were susceptible to magnolol, both in planktonic mode and biofilm form. Magnolol was more active than fluconazole at inhibiting biofilm formation of *Candida* spp. The effect was concentration-dependent, and might act during the logarithmic phase of yeast growth. As an alternative antifungal agent, magnolol might be beneficial to treat NACS-associated infections, particularly those caused by azoles-resistant species. Nevertheless, the safety and the antifungal effect in vivo require further evaluation.

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Disclosure

The authors report no conflict of interest in this work.

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