Infection and Drug Resistance

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ORIGINAL RESEARCH

Antifungal Activity of the Dichloromethane Extract of CaoHuangGuiXiang Formula Against *Candida auris* by in vitro and in vivo Evaluation

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Purpose: CaoHuangGuiXiang (CHGX) formula is a traditional Chinese medicine for the treatment of *Candida*-related infection. However, its antifungal mechanisms against the emerging fungal pathogen *Candida auris* remain unclear. This study aimed to evaluate the antifungal activity of the dichloromethane extract of CHGX (CHGX-DME) and clarified its antifungal mechanims against *C. auris*.

Methods: The major components of CHGX-DME were identified by ultra-performance liquid chromatography tandem mass spectrometry. Then, the minimal inhibitory concentration (MIC) assay and the time-kill kinetic assay were performed to investigate the in vitro antifungal activity of CHGX-DME against *C. auris*, including 8 isolates of 4 discrete clades and 2 special phenotypes (filamentous and aggregative). Furthermore, the effect of CHGX-DME on biofilm development was examined. In addition, the in vivo toxicity and efficacy of CHGX-DME were evaluated in a *Galleria mellonella* infection model.

Results: First, 20 major compounds in CHGX-DME were detected and characterized. The $MIC_{50\%}$ and $MIC_{90\%}$ of CHGX-DME against *C. auris* isolates ranged from 50–200 mg/L and 100–400 mg/L, respectively. At 400 mg/L, CHGX-DME was able to efficiently kill more than 70% and 90% of *C. auris* cells after 3 hours and 6 hours of treatment, respectively. This notable antifungal activity exhibited a dosage- and time-dependent manner. Moreover, CHGX-DME not only played a critical role in inhibiting the proliferation of filamentous and aggregative cells, but also showed restricting effect on biofilm development in *C. auris*. Importantly, it significantly improved the survival rate and reduced the fungal burden in *G. mellonella* infection models, suggesting a remarkable treatment effect against *C. auris* infection.

Conclusion: CHGX-DME exhibited potent antifungal activity against *C. auris* and significantly ameliorated this fungal infection in the *G. mellonella* model, confirming that it would be a promising antifungal drug for the troublesome and emerging fungal pathogen *C. auris*.

Keywords: *Candida auris*, multidrug resistance, CaoHuangGuiXiang formula, aggregative form, biofilm formation, antifungal efficiency

Introduction

Candida auris, an emerging multidrug resistant fungal pathogen first described in 2009 in Japan, has become a major public health threat due to its rapid and widespread prevalence over the past decade.[1] Until 2023, *C. auris* infection cases have been reported in more than 50 countries on 6 continents.[2,3] In China, a total of 312 cases of *C. auris* infection were detected in 18 hospitals in 10 provinces from 2016 to 2023.[4] *C. auris* is recognized as a life-threatening infection acquired in hospital and possesses a high transmission capacity between hospitalized individuals, posing great risks in nosocomial outbreaks.[5] A crude in-hospital mortality rate for *C. auris*-related candidemia ranges from 30% to 70%.[6–9] Given the high mortality and rapid prevalence, *C. auris* has not only been classified as an "urgent threat" to public health

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by the US Centers for Disease Control and Prevention (CDC), but ranked as a "critical priority" group of fungal pathogens list by the World Health Organization (WHO) in 2022.[3,4]

A prominent feature of *C. auris* is its intrinsic or acquired multidrug resistance, making it consider as a "superbug". Generally, most clinical isolates exhibit resistance to fluconazole and some are resistant to 2 or 3 major classes of antifungals, including azoles, echinocandins, and amphotericin B, leading the fungal management and treatment of *C. auris* infection extremely difficult.[10–12] Another unique property of *C. auris* is its persistent colonization tropism for human skin and abiotic surfaces, which facilitates its hospital transmission and nosocomial outbreaks in healthcare facilities. Similar to other *Candida* species, *C. auris* is capable of morphological transition, biofilm formation and developing unique aggregative form which are closely associated with its adaptation to environmental and host niches.[13–17] In particular, biofilm is a critical virulence factor for fungal pathogens due to its enhance colonization in host tissues and high resistance to antifungal drugs.[18] Although *C. auris* could develop weak biofilm compared to that formed by *C. albicans*, its biofilm still exhibited high levels of drug resistance compare with its planktonic cells.[13] Hence, enhanced efforts to elucidate its pathogenic mechanisms and exploit novel antifungal drugs are highly needed for the prevention and better outcomes of *C. auris* infection.

Traditional Chinese Medicines were regarded as a goldmine for exploring novel antifungal drugs due to its diversity, availability, and being less prone to drug resistance. With the anti-*Candida* effect on biofilm formation, morphological transition and host immune regulation, a variety of traditional herbal formulas and bioactive compounds have attracted widespread attention.[19–21] CHGX formula is an effective prescription used for the treatment of *Candida*-related infections in critically ill patients for at least two decades.[22] In a previous study, we validated that CHGX formula exhibited antifungal activity against *Candida* species and demonstrated that it could improve candidemia in the murine model. With further chemical extraction of CHGX formula, we found that the dichloromethane extract of CHGX formula (CHGX-DME) was the most effective active part against *Candida albicans*. However, its antifungal activity against multidrug resistance isolates of *C. auris* remains to be clarified.

In the current study, we first demonstrated that CHGX-DME exhibited a broad antifungal activity against *C. auris* isolates derived from 4 major phylogenetic clades. Further investigation indicated that CHGX-DME could inhibit the proliferation of specific cell types and improve the survival rates of *C. auris* infection in the *Galleria mellonella* model, suggesting that CHGX-DME would be a promising antifungal agent for *C. auris* infection and has great potential for novel antifungal development.

Materials and Methods

Strains and Cultivation

The fungal strains used in this study are listed in Table S1. The *C. auris* strains were generously provided by Prof. Guanghua Huang at Fudan University. Fungal cells were routinely cultured in a yeast extract peptone dextrose (YPD) medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose; and 20 g/L agar was added for solid medium). For the killing assay, a completely synthetic Lee's glucose medium was used. For the growth of filamentous cells, cells were plated onto each solid medium plates and incubated at 25 °C for 7 days.

Preparation of CHGX-DME

As showed in Figure 1, the CHGX formula (50 g) consists of the root and rhizomes of *Glycyrrhiza uralensis* Fisch. ex DC. [Fabaceae] (Gan Cao 15 g, Voucher number 211209002), the root and rhizomes of *Rheum palmatum* L. [Polygonaceae] (Da Huang 10 g, Voucher number 2103137), the bark of *Neolitsea ca ssia* (L). Kosterm. [Lauraceae] (Rou Gui 10 g, Voucher number 21081303), and the aerial part of *Pogostemon cablin* (Blanco) Benth (Guang Huoxiang 15 g, Voucher number 2108079). These herbs were provided by Beijing Hospital of Traditional Chinese Medicine (Beijing, China). The preparation of CHGX formula was performed as previously reported.[22] Briefly, the CHGX formula was soaked in deionized water for 30 minutes and then decocted for 30 minutes. The filtrate was collected, and the decoction was repeated once. The total filtrate was extracted twice with petroleum ether, dichloromethane, ethyl acetate, and N-butanol according to polarity gradients. The dichloromethane extract was evaporated by

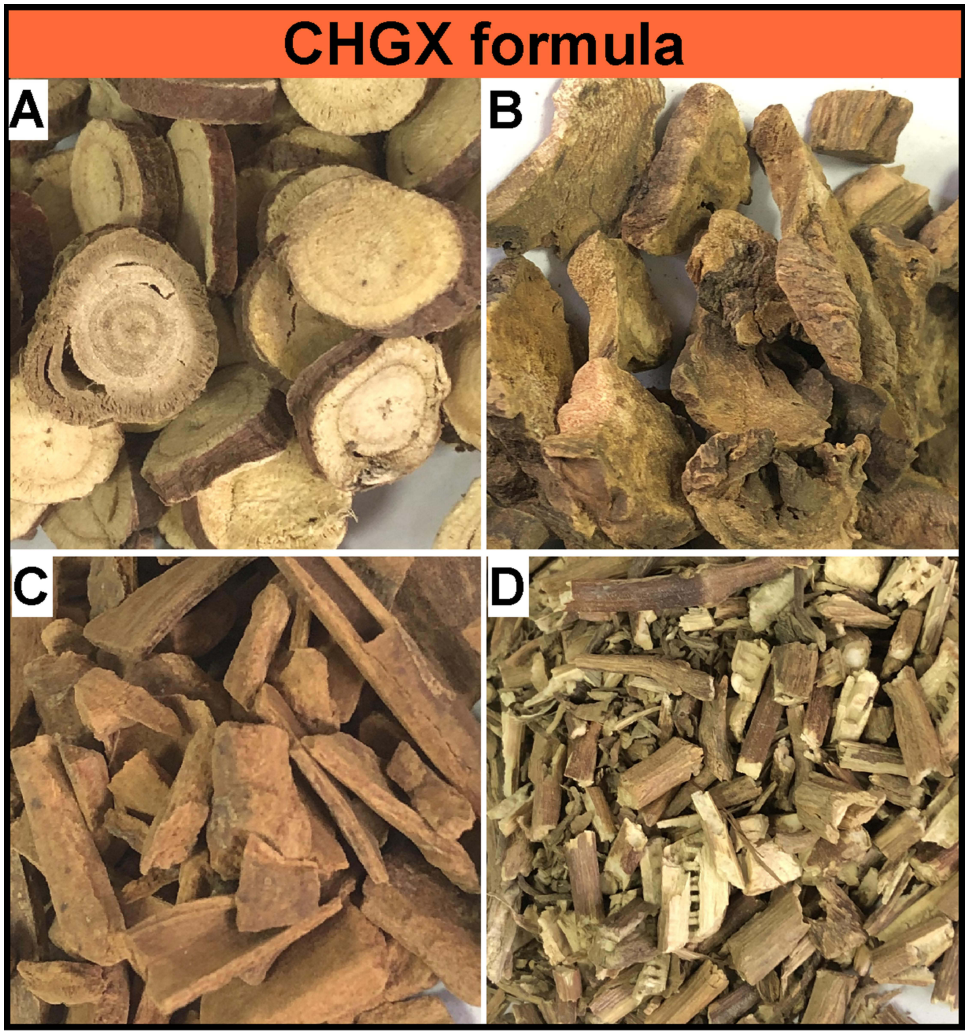

Figure 1 The images of CHGX formula. (**A**) *Glycyrrhiza uralensis* Fisch. ex DC. [Fabaceae] (Gan Cao); (**B**) *Rheum palmatum* L. [Polygonaceae] (Da Huang); (**C**) *Neolitsea cassia* (L.) Kosterm. [Lauraceae] (Rou Gui); (**D**) *Pogostemon cablin* (Blanco) Benth (Guang Huoxiang).

rotary evaporation at 60 °C, and then dried by nitrogen blowing concentrator into a dark brown paste with a yield of approximately 0.11%, known as CHGX-DME.

Ultra-Performance Liquid Chromatography Tandem Mass Spectrometry (UPLC-MS/MS)

An UPLC-MS/MS system equipped with Water ACQUITY UPLC and HESI quadrupole tandem linear ion trap mass spectrometry was employed to identify the main components of CHGX-DME according to previously described.[22,23]

The liquid chromatographic elution condition: A Water BEH Shield RP C18 1.7μm, 2.1 mm×100 mm column was used. The mobile phase was 0.1% (v/v) formic acid aqueous solution (A) and acetonitrile (B) with a flow rate of 0.3 mL/minute, and the injection volume was 2 μL. The samples and standards were eluted as follows: 0–1 minute: 95% A; 1–2.4 minutes: 95–90% A; 2.4–13.5 minutes: 90–68% A; 13.5–18.5 minutes: 68–10% A; 18.5–19 minutes: 10–95% A; and 19–21 minutes: 95% A.

The mass spectrometry condition: HESI-II in positive and negative ionization mode was used. The voltage of HESI+ and HESI- was 3.8 kV and 3.2 kV, and the ionization source temperature was 300 °C. Nitrogen was used in both the sheath gas and auxiliary gas. The collision gas with nitrogen was used at a pressure of 1.5 mTorr. Xcalibur 4.2 software (Waters Corp.) was used for data collection and processing.

Minimal Inhibitory Concentration (MIC) Assay

The MIC assays were performed according to the CCLS document M27-A3 and previous reports with slight adjustment.[22,24] Briefly, *C. auris* cells from each strain were initially patched on YPD plates at 37 °C for 24 hours. Single colonies were collected and washed twice with ddH_2O. Approximately 500 fungal cells of each strain were inoculated in 0.2 mL of RPMI1640 medium (w/v, 1.04% RPMI1640, 3.45% MOPs, pH adjustment to 7.0 with NaOH) with a serial two-fold concentration (from 12.5 to 800 mg/L) of CHGX-DME in a 96-well plate. Fungal cells were incubated at 37 °C for 24 hours for the MIC assay. For CHGX-DME, two reading breakpoints were defined for MIC determination: 50% ($MIC_{50\%}$) and 90% ($MIC_{90\%}$) inhibition of fungal growth compared to drug-free growth control. All tests were performed in duplicate. Fluconazole and amphotericin B were served as positive drug control. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control.

Killing Assay of Fungal Cells

The fungal cells from a single colony of each strain were initially inoculated in liquid YPD medium and cultured at 30 °C with shaking overnight. Cell cultures were collected and washed twice with ddH_2O. Then, the fungal cells were adjusted to 2×10^5 cells/mL in 3 mL of liquid Lee's glucose medium and treated with 100, 200, or 400 mg/L of CHGX-DME at 30 °C with shaking. The viable cells were determined using the colony-forming unit (CFU) plating assay at the indicated time points (0 hour, 3 hours, 4 hours, 6 hours or 24 hours). Strain RICU13 was used for assay of filamentous cells and strain SJ02 was used for assay of aggregative cells. Three independent repeats were performed for each test. The percentage of viable cells was calculated as follows: = (number of colonies of CHGX-DME treated sample / average number of colonies of the control group) × 100%.

Effects of CHGX-DME on Biofilm Formation

The microtiter plate biofilm assay was performed as previously described with slight modifications.[25,26] Two representative aggregative cells from the *C. auris* strains SJ02 and A103 were used. The aggregative cells of each strain were initially inoculated in liquid YPD medium at 30 °C with shaking. Overnight cultures were collected, washed and re-suspended into RPMI1640 medium. Cultures (OD_{600}=0.2) were added to a 96-well flat-bottom polystyrene plates (BD Falcon) and incubated at 37 °C for 90 minutes at 200 rpm for initial adhesion. The bottoms of the plates were gently washed with 1×PBS. Then fresh RPMI1640 medium supplemented with various concentrations (400, 200, 100, 50, 25 mg/L) of CHGX-DME was added into 96-well plates for an additional 48 hours of growth at 37 °C with shaking. The CHGX-DME untreated group was severed as control. The plates were imaged before and after being gently washed with 1×PBS. Biofilms were treated with 0.25% trypsin in 200 μL of 1×PBS at 37 °C for 1 hour, and then re-suspended and collected for the cell intensity (OD_{600}) assay by the Synergy 4 Gene 5 plate reader (Biotek, Potton, United Kingdom). Three independent biological replicates of each test were performed.

In vivo Toxicity Assay in the G. mellonella Model

The larvae of *G. mellonella* (0.3–0.4 g) were purchased from Tianjin Huiyu biological technology Co. LTD. (Tianjin, China). The larvae were randomly divided into 7 groups. The CHGX-DME were firstly dissolved in DMSO at a high concentration of 50000 μg/mL as the mother liquor. Then, the working concentrations of CHGX-DME were respectively diluted by 20 times (2500 μg/mL), 10 times (5000 μg/mL) and 5 times (10000 μg/mL) in 1×PBS buffer. Each larva was injected with CHGX-DME (25 μg/larva, 50 μg/larva and 100 μg/larva) or dimethyl sulfoxide (DMSO) (5%, 10% and 20%) in 10 μL of 1×PBS through the last two left-proleg using microliter syringes. 10 larvae were used in each group. After treatment, the larvae were placed in plastic culture dishes and incubated at 30 °C and 37 °C for 10 days. Death without movement after touching stimulus was monitored and recorded daily. The doses of CHGX-DME were calculated considering the MIC and the weight of the larva.

In vivo Antifungal Efficiency Assay

Evaluation of the antifungal efficiency of CHGX-DME in a *G. mellonella* infection model was performed as previously described with slight modifications.[27] For the survival curve assay, *C. auris* strains BJCA002 and RICU1 were used. The larva infected with approximately 1×10^7 fungal cells was placed at 30°C, while the larva infected with approximately 5×10^6 fungal cells was placed at 37°C. After 1 hour of infection, infected larvae were treated with 50 μg/larva of CHGX-DME in 10 μL of 1×PBS. 10% DMSO in 1×PBS were severed as untreated control. 10 larvae were used in each group and incubated for 10 days. Death without movement after touching the stimulus was monitored and recorded daily.

For the fungal burden assay, the strain BJCA002 was used. Approximately 2.5×10^6 cells were injected into each larva and incubated at 37°C, while approximately 2×10^6 cells were injected into each larva and incubated at 30 °C. 5 larvae were used in each group. After 1 hour of infection, infected larvae were treated with 50 μg/larva of CHGX-DME in 10 μL of 1×PBS. 10% DMSO in 1×PBS were severed as untreated control. The larvae were sacrificed after 1 days of infection at 37°C and 4 days of infection at 30°C. The internal body contents were squeezed, homogenized, diluted, and plated onto YPD medium at 30°C for the CFUs analysis.

Statistical Analysis

All statistical data were performed using the SigmaStat Package software (SPSS26.0 Inc). Values were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed to compare statistical differences with multiple groups and students *t*-test was performed between two groups. Survival curves were performed using log-rank analysis (Mantel-Cox). $*p < 0.05$ was considered statistically significant.

Results

Main Components of GHGX-DME

CHGX-DME was refined from the dichloromethane extract of CHGX water-decoction with a yield of approximately 0.11%. UPLC-MS/MS was employed for identifying the major ingredients of CHGX-DME. As illustrated in Figure 2, the composition of the ingredients in CHGX-DME was presented by total positive and negative ion chromatograms. In particular, 20 major compounds, including gallic acid, protocatechuic acid, ethyl-4-methoxycinnamate, isoliquiritin,

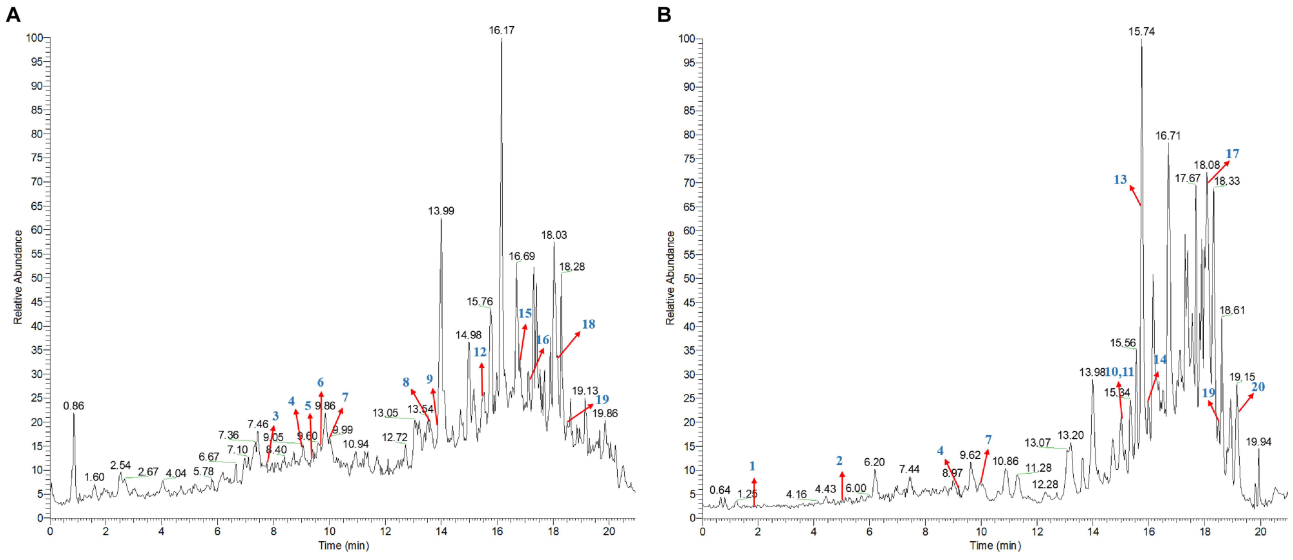

Figure 2 Total ion chromatogram of GHGX-DME by UPLC-MS/MS. The positive ion (**A**) and negative ion (**B**) chromatograms showed the major compounds in CHGX-DME: (1) Gallic acid; (2) Protocatechuic acid; (3) Ethyl-4-methoxycinnamate; (4) Isoliquiritin; (5) Coumarin; (6) Cinnamic acid; (7) Liquiritin; (8) Ononin; (9) Rhapontigenin; (10) Catechin; (11) Epicatechin; (12) Emodin; (13) Liquiritigenin; (14) Wogonin; (15) Physcion; (16) Retrochalcone; (17) Rheic acid; (18) Calycosin; (19) Pogostone; (20) Emodin.

coumarin, cinnamic acid, liquiritin, ononin, rhapontigenin, catechin, epicatechin, emodin, liquiritigenin, wogonin, physcion, retrochalcone, rheic acid, calycosin, pogostone, and emodin were detected and characterized in Table S2.

Antifungal Susceptibility of C. auris to CHGX-DME

C. auris was currently divided into 6 discrete clades according to its genetic lineages and geographical features.[2,28,29] *C. auris* isolates generally exhibit clade-specific properties related to its drug resistance and pathogenesis. In order to evaluate the antifungal susceptibility of different *C. auris* isolates to CHGX-DME, 8 strains derived from 4 major clades were selected for antifungal susceptibility assay. Except for BJCA001 (clade I) and CBS10913 (clade II) which were susceptible to fluconazole and amphotericin B, the other 6 strains were resistant to 1 or 2 antifungal drugs tested. As shown in Table 1, the $MIC_{50\%}$ of CHGX-DME against *C. auris* isolates ranged from 50 to 200 mg/L, and the $MIC_{90\%}$ of CHGX-DME varied from 100 to 400 mg/L. Remarkably, 2 strains of clade III, RICU1 (fluconazole resistant) and BJCA002 (fluconazole-amphotericin B resistant), had relatively lower $MIC_{50\%}$ (50 mg/L) and $MIC_{90\%}$ (200 mg/L), suggesting that CHGX-DME is a promising antifungal drug against multidrug resistant *C. auris*.

Antifungal Activity of CHGX-DME Against C. auris

To further verify the in vitro antifungal effect of CHGX-DME, we next performed the time-kill kinetics assay (Figure 3). At 400 mg/L, more than 90% of *C. albicans* cells were killed both after 3 hours and 6 hours of CHGX-DME treatment in Lee's glucose medium, which was in accordance with our previous report.[22] For *C. auris*, 2 isolates from clade III (BJCA002 and RICU1) were used for the killing assay. As expected, at 400 mg/L of CHGX-DME, the percentage of viable cells was less than 22% and 5% after 3 hours and 6 hours of treatment, respectively (Figure 3A). In addition, CHGX-DME exhibited notable anti-*C. auris* activity in a dosage- and time-dependent manner. As indicated in Figure 3B, the viability of *C. auris* was much higher at 200 mg/L of CHGX-DME than at 400 mg/L of CHGX-DME, meanwhile the viability was significantly lower at 6 hours than at 3 hours after treatment of CHGX-DME treatment. Consistently, CHXG-DME still exerted a potent antifungal effect after 24 hours of treatment, suggesting that it had an advantage on stability and high-efficiency of antifungal agents.

Effect of CHGX-DME on Filamentous and Aggregative Cells of C. auris

Morphological diversity and plasticity play critical roles in environmental adaptation, virulence, and drug resistance both in bacterial species and fungal species. Similar to *C. albicans, C. auris* is capable of switching between various phenotypic cells, such as yeast and filamentous growth, and non-aggregative (single-cell) and aggregative form.[14,26,30] Using a similar strategy, we attempted to investigate the antifungal activity of CHGX-DME against the four unique

Table 1 Antifungal Susceptibility of C. auris to CHGX-DME

Clades	Isolates	CHGX-DME		FLC (mg/L)	AMB (mg/L)
		$MIC_{50\%}$ (mg/L)	$MIC_{90\%}$ (mg/L)		
Clade I	BJCA001	200	400	2	0.25
	CBS12766	200	400	256	0.25
Clade II	CBS10913	100	100	2	0.25
	CBS12372	100	400	128	0.25
Clade III	BJCA002	50	200	64	4
	RICU1	50	200	256	0.5
Clade IV	AR bank #0386	200	400	128	0.25
	SZ-5	200	400	128	0.25

Abbreviations: MIC, minimal inhibitory concentration; FLC, fluconazole; AMB, amphotericin B.

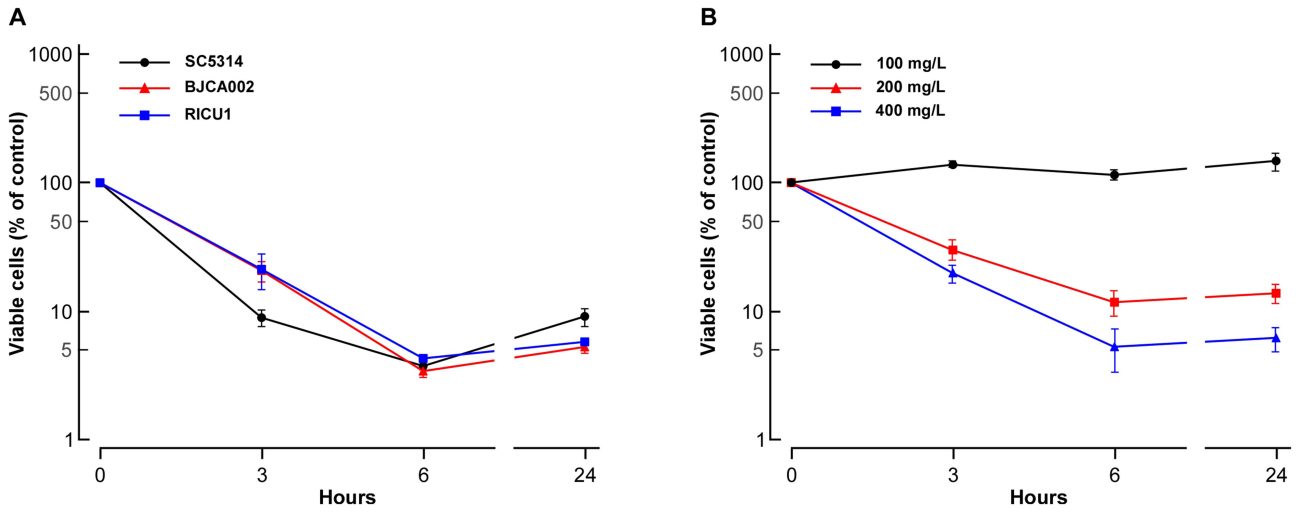

Figure 3 Killing effect of CHGX-DME against *C. albicans* and *C. auris*. (**A**) Fungal cells of *C. albicans* (SC5314) and *C. auris* (BJCA002 and RICU1) were incubated in Lee's glucose medium and treated with 400 mg/L of CHGX-DME at 30 °C. (**B**) Fungal cells of BJCA002 were incubated in Lee's glucose medium and treated with 100 mg/L, 200 mg/L and 400 mg/L of CHGX-DME at 30 °C. Cell viabilities were determined using the CFUs plating assay at the indicated time points. Three biological replicates were performed and the values were indicated as (mean ± SD) %.

phenotypes of *C. auris*. After 4 hours of treatment with 400 mg/L of CHGX-DME, the killing rate of yeast and filamentous cells of the representative strain RICU13 was 74% and 46%, respectively (Figure 4A). However, the killing effect of CHGX-DME on yeast cells was significantly higher than that on filamentous cells.

Thereafter, we examined the antifungal activity of CHGX-DME against the specific aggregative form of *C. auris*. The representative non-aggregative cells SJ01 and aggregative cells SJ02 were used. Interestingly, at 400 mg/L, CHGX-DME exhibited potent antifungal activity against aggregative cells of strain SJ02 after 4 hours of treatment, although the cell viabilities of the aggregative cells were evidently higher than that of the non-aggregative cells (Figure 4B). These results suggested that CHGX-DME exhibited unique advantages in the development of drug resistance as antifungal agents.

Inhibitory Role of CHGX-DME in Biofilm Formation of C. auris

Biofilm development is an important biological characteristic of fungal pathogens which closely associated to its antifungal resistance. Similar to *C. albicans, C. auris* is also able to develop biofilm.[16,17] However, the ability of biofilm

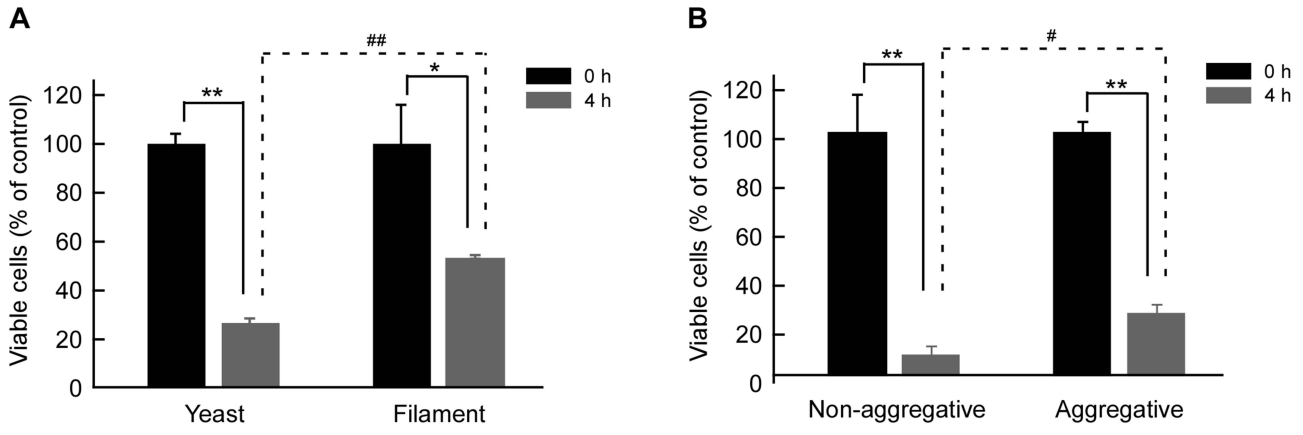

Figure 4 Antifungal activity of CHGX-DME on the filamentous and aggregative cells of *C. auris*. (**A**) The yeast and filamentous cells of strains RICU13. (**B**) Non-aggregative cells of strain SJ01 and aggregative cells of strain SJ02. Approximately 2×10^5 cells of each phenotype were incubated in Lee's glucose medium and treated with or without 400 mg/L of CHGX-DME at 30 °C. Cell viabilities were determined using the CUFS plating assays after 0 hour and 4 hours of the treatment. Three biological replicates were performed and the values were indicated as (mean ± SD) %.

Notes: $^{*}p<0.05$ and $^{**}p<0.01$ indicate significant differences of the cell viable rates between 0 hour and 4 hours; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ indicate significant differences of the cell viable rates between yeast and filamentous cells, or non-aggregative and aggregative cells.

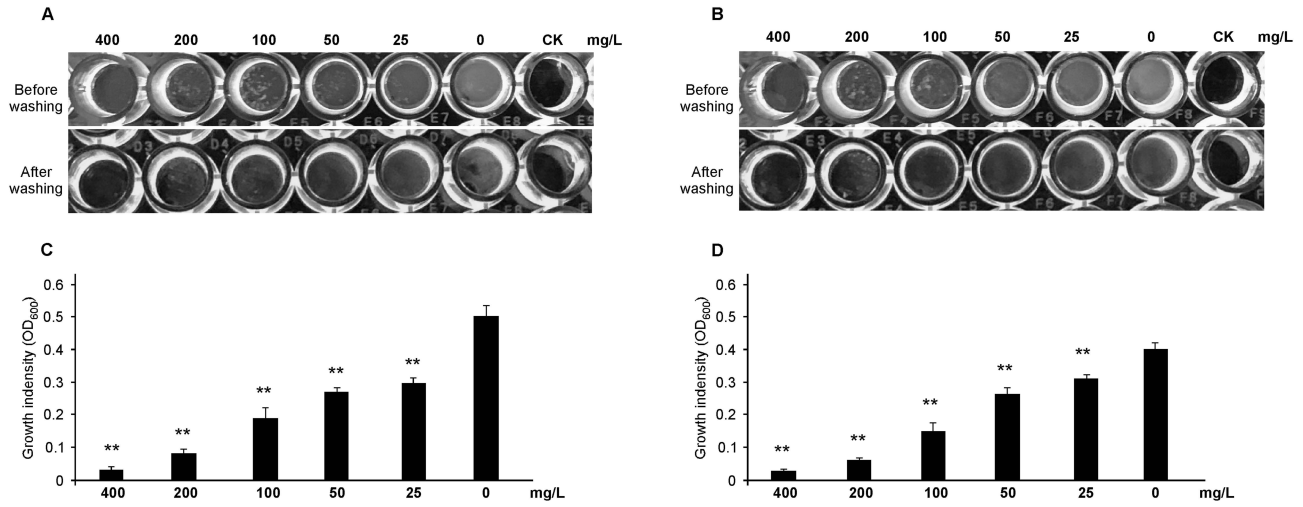

Figure 5 The inhibitory effect of CHGX-DME on biofilm formation by aggregative cells of *C. auris*. (**A**) Biofilm formation of strain SJ02. (**B**) Biofilm formation of strain A103. (**C** and **D**) The growth intensity of biofilm. The minimal inhibitory concentration of CHGX-DME on biofilm formation was determined in 96-well flat-bottom polystyrene plates by the micro-dilution method. The aggregative cells of each strain were initially incubated in RPMI1640 medium at 37 °C for 90 minutes. Then, cells were washed and treated with different concentration of CHGX-DME in RPMI1640 medium at 37 °C for 48 hours. The polystyrene plates were imaged before and after gently washing. The biofilms were treated with 0.25% trypsin for 1 hour, and then re-suspended and collected for growth intensity (OD_{600}) assay. Three biological replicates were performed and the values were indicated as (mean ± SD) %.

Notes: $^{**}p<0.01$, by one-way analysis of variance (ANOVA), indicates significant differences between CHGX-DME treated and untreated samples as indicated.

formation differs in various isolates and clades of *C. auris*.[16,17,26] Although both the non-aggregative and aggregative cells of C. auris are capable of biofilm formation, the latter was prone to develop more robust biofilm.[16,26] Next, we tested the anti-biofilm activity of CHGX-DME against 2 representative aggregative strains, SJ02 and A103. As shown in Figure 5A and B, at 400 mg/L, strains SJ02 and A103 failed to form mature biofilms with CHGX-DME treatment. Compared to the untreated groups, the aggregative cells developed much weaker biofilm under treatment with 200 mg/L and 100 mg/L of CHGX-DME. To further evaluate the anti-biofilm effect, we performed the biofilm growth intensity assay and verified the inhibitory role of CHGX-DME in the biofilm formation of *C. auris* (Figure 5C and D).

Evaluation of Toxicity and Antifungal Efficacy of CHGX-DME in G. mellonella Infection Models

In a previous study, we confirmed the safety and efficacy of CHGX water-decoction against *C. albicans* in a murine infection model.[22] Here, we attempted to evaluate the toxicity and antifungal potency of CHGX-DME against *C. auris* using a *G. mellonella* model. As described in Figure 6A, except for 1 larva death in the high concentration group (100 μg/larva) on the 5th day, no larva mortality was observed in both the middle (50 μg/larva) and low (25 μg/larva) concentration groups at 30 °C throughout 10 days of treatment. Similarly, there was no larva mortality in both the middle and low concentration groups at 37 °C (Figure 6B). Therefore, a safe dosage of CHGX-DME was chosen at 50 μg/larva for the following antifungal efficacy assay. In the BJCA002 infection model, there was no obvious improvement in the survival rate of larvae with CHGX-DME treatment both at 30 °C and 37 °C during 10 days of infection (Figure 7A and B). However, CHGX-DME could significantly improve the survival rate of larvae in the RICU1 infection model both at 30 °C and 37 °C (Figure 7C and D).

To further examine the antifungal efficacy of CHGX-DME, we next performed the fungal burden assay in the *G. mellonella* infection model (Figure 8). Surprisingly, with 50 μg/larva of CHGX-DME, the fungal burden of larvae infected with strain BJCA002 was significantly lower after both 1 days of infection at 37 °C and 4 days of infection at 30 °C in contrast to the untreated groups. These results confirmed that CHGX-DEM exhibited promising in vivo efficacy in the treatment of *C. auris* infections, despite its varied effect between different isolates of *C. auris*.

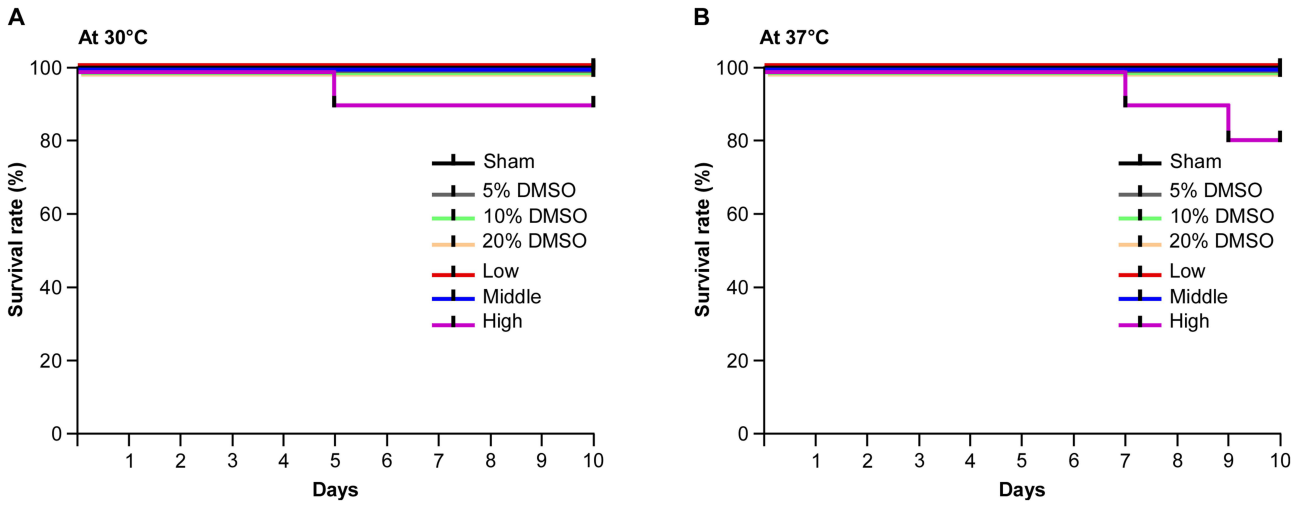

Figure 6 The toxicity assay of CHGX-DME in a *G. mellonella* model. Larvae were randomly divided into sham group, DMSO (5%, 10%, and 20%) groups, and CHGX-DME (low 25 μg/larva; middle 50 μg/larva, and high 100 μg/larva) groups. 10 larvae were used in each group and incubated at 30 °C (**A**) and 37 °C (**B**) for 10 days. Larvae death was monitored and recorded daily.

Discussion

The emerging fungal pathogen *C. auris* has increased global concern due to its widespread prevalence and high mortality. Although *C. albicans* still remains the predominant agent of in-hospital fungal infections, *C. auris* has surpassed all *Candida* species as the most intractable pathogen to treat as its pronounced clinical resistance to all available classes of antifungal agent, including azoles, echinocandins, and amphotericin B. Traditional Chinese Medicines, which contain plenty of medical plant-derived products, have become the mainstay in developing novel anti-infective drugs. CHGX is an empirical herbal prescription that has been used for decades in the treatment of *Candida*-related infections in clinical. In the present study, we reported that CHGX-DME not only showed potent and stable antifungal activity against *C. auris* isolates, but also exerted inhibitory roles in proliferation of specific cell forms and development of biofilm. Besides, we demonstrated its safety and efficacy in the *G. mellonella* infection model, providing an alternative and promising treatment for *C. auris* infections.

According to the geographical origin and genomic difference, *C. auris* has been divided into six major discrete clades, the South Asia Clade (I), the East Asia Clade (II), the South Africa Clade (III), the South America Clade (IV), the Middle East (Iran) Clade (V), and a possible sixth clade (VI) reported from Singapore.[8,28,29] At least 40% of *C. auris* isolates displayed resistance to two antifungal classes and approximately 4% were multidrug resistant to the three classes of drugs.[31,32] Particularly, Clade II isolates contains the highest percentage of drug-susceptible isolates; while a majority of Clade I isolates were drug resistant or multidrug resistant.[2,33,34] We found that CHGX-DEM exhibited potent and stable antifungal activity against four major clades of *C. auris* isolates at rationalized concentration (Table 1, Figure 3). Regarding the clade-specific drug-resistant property of *C. auris*, the broad spectrum of antifungal activity of CHGX-DME would have great advantages in prompt treatment and effective prevention of *C. auris* infection.

Currently, the reasons behind the emergence and prevalence of *C. auris* have not yet been elucidated. In addition to challenges in the identification and multidrug resistance, the evolution of virulence factors also leads *C. auris* extra problematic to handle. The formation of hyphae is an important feature of pathogenic *Candida* species for invasion of host tissue.[35] *C. auris* had long been considered incapable of filamentous growth. Recent studies showed that it was capable of filamentation under specific environmental conditions.[15,30] We reported that CHGX-DME also showed a noticeable fungicidal effect against filamentous cells (Figure 4A), suggesting that it played a certain inhibitory role in the pathogenesis of *C. auris*.

Unlike other pathogenic *Candida* species, *C. aruis* rarely colonizes the mucosal surfaces and the gastrointestinal tract as a commensal yeast. Actually, it tends to colonize human skin and abiotic surfaces for a prolonged period of time, promoting nosocomial transmission and outbreaks in hospital environments.[36,37] The peculiar adhesive capacity

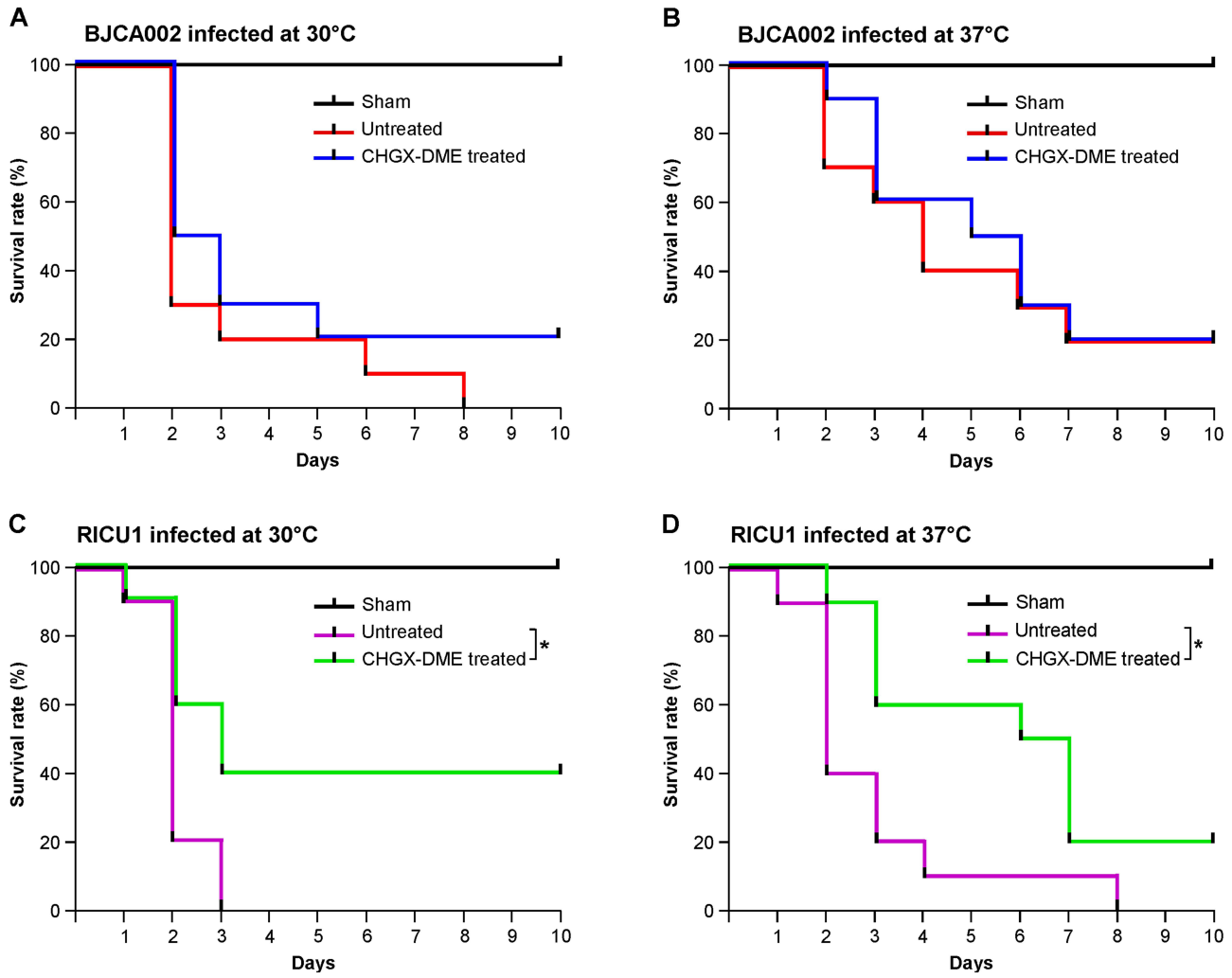

Figure 7 Antifungal efficiency of CHGX-DME in a *G. mellonella* infection model. (**A** and **B**) Survival curves of *G. mellonella* larvae infected with BJCA002. (**C** and **D**) Survival curves of *G. mellonella* larvae infected with RICU1. The larvae infected with 1×10^7 cells and 5×10^6 cells of *C. auris* were placed at 30 °C and 37 °C, respectively. Infected larvae were treated or untreated with 50 μg/larva of CHGX-DME. 10 larvae were used in each group and larvae death was monitored and recorded daily.
Notes: *$p<0.05$, by log rank test, indicates significant difference between untreated and CHGX-DME treated groups.

of *C. auris* is closely related to its special phenotypes, such as aggregative form and biofilm. In this study, we found that CHGX-DME could effectively inhibit the proliferation of aggregative form and biofilm development of *C. auris* (Figures 4B and 5), and indicated that the anti-adhesion and anti-biofilm activity of CHGX-DME would be less likely to develop drug resistance, contributing greatly to eradication and containment of *C. auris* infection with in clinical settings.

Mammalian models of fungal infection have long been considered as the gold standard for research on virulence, pathogenicity, and efficacy evaluation of antifungal drugs.[38] Recently, the larvae of the *G. mellonella* model exhibits vast potential as an optional invertebrate model of fungal infection, which possess advantages on similar cellular immune system with mammals, and easy maintenance for large-scale screening and replication.[39–41] Here, we employed the *G. mellonella* model to evaluate the toxicity of CHGX-DME and demonstrated that it exhibited a desirable in vivo efficacy against *C. auris* infection (Figures 6–8). These results not only underscored the promising candidate of CHGX-DME as an alternative antifungal agent for the emerging pathogen, but highlighted the utility of this *G. mellonella* model to investigate the efficacy of antifungal drugs.

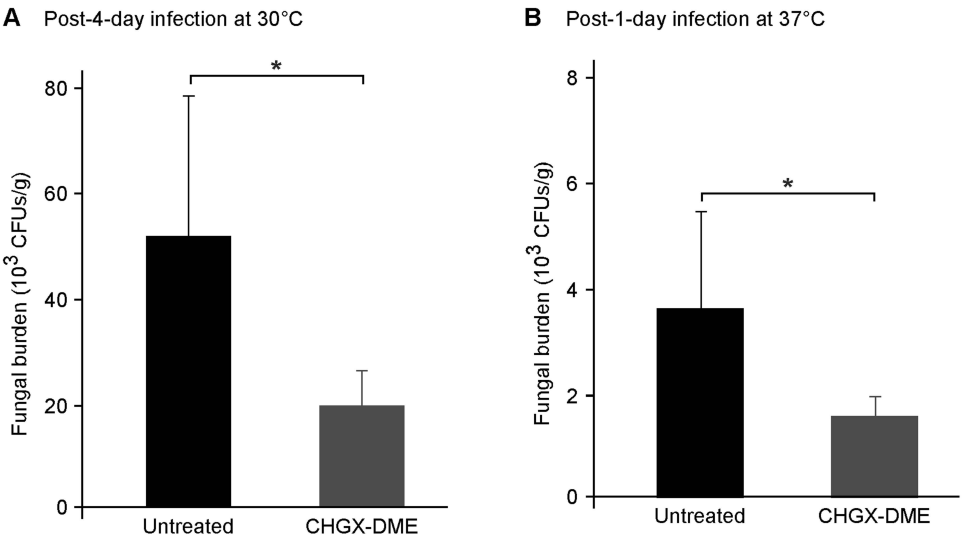

Figure 8 The fungal burden assay in a *G. mellonella* infection model. (**A**) The fungal burden of larvae post-4-day infection 30°C. Approximately 2×10^6 cells were injected into each larva. (**B**) The fungal burden of larvae post-1-day infection at 37°C. Approximately 2.5×10^6 cells were injected into each larva. The yeast cells of BJCA002 were used. After 1 hour of infection, larvae were treated with or without 50 μg/larva of CHGX-DME. The infected larvae were sacrificed after both 4 days of infection at 30 °C and 1 days of infection at 37 °C. The internal body contents were collected and homogenized for the fungal burden assay. The values were indicated as (mean ± SD) %.
Notes: Student's *t*-test was used to compare differences between untreated and CHGX-DME groups; *$p < 0.05$.

Conclusion

In conclusion, we demonstrated the in vitro and in vivo antifungal activity of CHGX-DME and provided new insights into developing novel antifungal drugs against *C. auris* as well as other important emerging fungal pathogens. However, many questions remain to be addressed. For example, what bioactive compounds of CHGX-DEM are responsible for antifungal activity? What are the drug targets of CHGX-DEM against *C. auris*? Is CHGX-DME involved in the regulation of host immune regulation, and did this contribute to its antifungal activity? Significant research efforts are needed to answer these questions. Moreover, large-scale clinical trials are necessary to further evaluate its clinical efficacy and pharmacological effect.

Ethics Statement

This study was approved and supervised by the Ethics Committee of Beijing Hospital of Traditional Chinese Medicine, Capital Medical University. Due to that this study was a retrospective study on *Candida* strains and did not contain any sensitive personal information, the need of informed consent was waived by the Ethics Committee of Beijing Hospital of Traditional Chinese Medicine, Capital Medical University. We confirmed that all methods were conducted in accordance with the declaration of Helsinki.

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Disclosure

The authors report no conflicts of interest in this work.

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