Anti-fungal Activity of Schinifoline Against *Candida albicans in Vitro*

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***Abstract*—Fungal infections are a major clinical cause of morbidity and mortality. *Candida albicans*, in particular, is an opportunistic pathogenic yeast that is commonly found in health people. Out of the misuse of broad-spectrum antibiotics and immunosuppressants, antifungal resistance is continually increasing and has become the biggest obstacle in clinical treatment of fungal infections. China is the biggest producer of *Zanthoxylum Bungeanum* and *Zanthoxylum schinifolium* that are widely used as traditional seasoning and traditional Chinese medicine. Schinifoline is the first 4-quinolinoid alkaloid found in Zanthoxylum. In this study, we purified schinifoline from *Zanthoxylum schinifolium* and tested its anti-fungal activity. *Z. schinifolium* was extracted for the total alkaloid, then separated and identificatied by ^{1}H and ^{13}CNMR spectra, EIMS and infrared spectra were determined. Column chromatography and thin layer chromatography were performed, 0.04g of schinifoline was identified. Our results showed that 100 and 200 mg L^{-1} of schinifoline could significantly inhibit the growth and biofilm formation of *C. albicans*. Moreover, we observed that at 100 and 200 mg L^{-1}, schinifoline effectively suppressed the transition of *C. albicans* from yeast to hyphae. The data were shown that schinifoline significantly inhibited the toxicity of *Candida albicans*. Our study uncovered valuable insights into the discovery of natural drugs that inhibit *Candida albicans in vitro*.**

Index Terms*—schinifoline, *Candida albicans*, *Zanthoxylum schinifolium*, fungistasis, *in vitro

I. Introduction

Candida albicans is a conditioned pathogenic fungus commonly found on the human oral, respiratory, gastrointestinal, vaginal, cutaneous and mucosal surfaces. It is one of the most common pathogens that are responsible for fungal infections [1]. In recent years, the infection rate of *C. albicans* has increased significantly with an increasing number of immunodeficiency patients [2], [3]. Furthermore, the widespread use of broad-spectrum antibiotics and immunosuppressive agents has contributed to the development of drug resistance in *C. albicans* and other species. Fluconazole (FLC), for example, has become the biggest obstacle to successful treatment of fungal infection [4]. Therefore, the need for high efficiency natural plant fungicides with little to no side effects is greater than ever before.

Zanthoxylum is a rutaceae shrub widely cultivated in many countries. Particularly, China has the largest area and the highest yield of *Zanthoxylum* cultivation, with *Zanthoxylum bungeanum* and *Zanthoxylum schinifolium* being the leading varieties according to early investigation [5]. The mature and dry pericarp of *Zanthoxylum* has been used as culinary condiments and in Chinese medicine for thousands of years [6]-[8]. The chemical constituents and bioactivities of *Zanthoxylum* have been extensively studied, including components such as flavonoids, terpenoids, volatiles, alkaloids, coumarins, amides, lignans and steroids [9]-[11]. The alkaloids found in *Zanthoxylum* have been isolated and identified, and the bioactive compounds, schinifoline and skimmianine, were effective against two stored-product insects, *T. castaneum* and *S. zeamais* [12]. However, other functions of schinifoline are rarely reported. In this study, we optimized the extraction process of schinifoline, and confirmed the anti-fungal ability of schinifoline against *Candida albicans in vitro*.

II. Materials and Methods

A. Materials

Figure 1. Fresh and dry epicarp of *Zanthoxylum schinifolium*.

Zanthoxylum schinifolium (dried epicarp, Fig. 1), was purchased from Chinese prickly ash base of Zhaotong City, Yunnan Province. PBS buffer solution, Yeast Extract Peptone Dextrose broth, RPMI-1640 medium, XTT-menadione solution, and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). *C. albicans* strains used in this study were the clinical isolates of SC5314, donated by the medical school of Southeast University. The strains were grown in liquid YPD broth [13].

B. Methods

1) Separation and identification of schinifoline

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Ethanol was used as a solvent to improve the yield of total alkaloids. Firstly, extraction based on single factors, such as ethanol concentration, pH, extraction temperature, and extracting time, was performed. Then, through the orthogonal experiment design method, total alkaloid in *Z. schinifolium* was investigated. Based on previous research, the extraction process of total alkaloid was optimized. *Z. schinifolium* (10 kg, dried epicarp) was extracted with 70% ethanol (3×60 L) in 60°C water bath for 3 h. The extracts were concentrated using a vacuum rotary evaporator, then pH was adjusted to 3 using HCl, let sit overnight, filtered, and removed from the precipitation. The aqueous layer was modified with $NH_3 \cdot H_2O$ ammonia to pH 9-10, then repartitioned with chloroform (3×5 L). Residue was formed after evaporation of chloroform, and total alkaloid was achieved after evaporation of the solvent. The total alkaloid was subjected to separation and identification at the test center of China Pharmaceutical University. Melting points, and ^{1}H and ^{13}CNMR spectra were recorded using alkaloid deuterium chloroform ($CDCl_3$) as solvent with tetramethylsilane (TMS) as the internal standard. EIMS were determined on a spectrometer. Infrared spectra were recorded in a spectrophotometer. Column chromatography was carried out with chloroform-methanol gradient, and then a thin layer chromatography was performed with GF254 chromatographic plates, and 0.04g of schinifoline was identifed. The ^{1}H and ^{13}CNMR result was in agreement with previous reports [12].

Schinifoline was dissolved in DMSO to prepare the stock solution, and then diluted into different working concentrations (50, 100 and 200 mg L^{-1}). The concentrations of schinifoline used for the experiments were based on previous experiments.

2) C. albicans growth curve assay

After activation, *C. albicans* was diluted into 1×10^6 cfu mL^{-1} in YPD broth, then treated with different concentrations of schinifoline (50, 100 and 200 mg L^{-1}). Samples were taken at 37°C at 0, 12, 24, 36, and 48 h. Yeast counts were determined using the platelet count method [13]. The growth of *C. albicans* treated with different concentrations of schinifoline was expressed as the curve. Experiments were performed in triplicate.

3) Biofilm formation - before the biofilm adhesion phase

Experiment was performed as previously described [13]. Schinifoline ranging from 50 to 200 mg L^{-1} was prepared in RPMI-1640 medium in 96 well plates. Wells without schinifoline served as negative control. Cell suspensions of 1×10^6 cfu mL^{-1} were prepared using RPMI-1640 medium. Then, 100 μL of solution was inoculated into each well of the 96-well plates. After incubation at 37℃ for 48 h, nonadherent cells were removed using PBS buffer, and biofilm growth was analyzed with XTT assay. The 96 wells plates were incubated at 35°C in the dark for 2 h. Absorbance of wells was measured with a BioTek Synergy 4 microplate reader at 490 nm. Experiments were performed in triplicate [13], [14].

4) Biofilm formation - after the biofilm adhesion phase

Cell suspensions of 1×10^6 cfu mL^{-1} were prepared using RPMI-1640 medium, and 100 μL of cell suspensions was inoculated into each well of the 96-well plates and incubated at 37℃ for 24 h. This incubation process allowed the cells to attach to the solid surface. After removing the nonadherent cells by washing with PBS buffer, 100 μL of schinifoline ranging from 50 to 200 mg L^{-1} in RPMI-1640 medium was added to each well. Plates were incubated at 37℃ for 24 to allow for biofilm formation. Biofilm growth was analyzed with XTT assay as described [14], [15]. The 96 wells plates were incubated at 35°C in the dark for 2 h. The absorbance of wells was measured with a BioTek Synergy 4 microplate reader at 490 nm. Experiments were performed in triplicate.

5) Filamentation assay

C. albicans was grown for 24 h in YPD medium, and washed with PBS buffer for three times. Then, 1×10^6 cfu mL^{-1} of *C. albicans* with or without schinifoline were incubated in RPMI-1640 medium at 37℃ for 12 h. Quantification of the inhibition of yeast to hyphal transition was performed by counting the number of individual budded cells versus the number of hyphae in the population. More than 100 cells were counted from each well [14], [15]. Experiments were performed in triplicate.

6) Statistical analysis

All data were presented as means ± standard error of the mean (S.E.M.). Graphs were generated using Microsoft Excel software (Microsoft Corp., Redmond, WA). Statistical analysis was performed using SPSS 12.0 software (SPSS Inc., Chicage, USA). Differences between groups were determined using analysis of variance (ANOVA). Probability levels of 0.05 and 0.01 were considered statistically significant.

III. Results and Discussion

A. Schinifoline Inhibits Growth of C. albicans

We investigated the effects of schinifoline on growth of *C. albicans*. The time-growth curve showed that, at 100 and 200 mg L^{-1}, schinifoline could significantly inhibit the growth of *C. albicans*, with 200 mg L^{-1} of schinifoline showing obviously superior effect compared to 100 mg L^{-1} of schinifoline. However, compared to the control group, 50 mg L^{-1} of schinifoline only showed moderate suppression of the growth of *C. albicans* (Fig. 2).

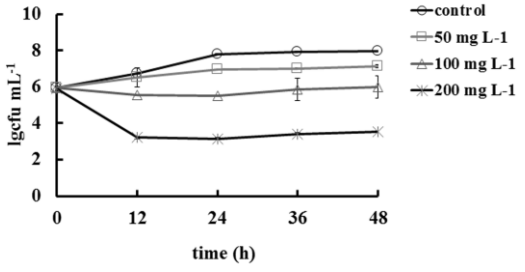


Figure 2. Time-growth curve of *C.albicans* treated with different concentrations of schinifoline. Bars represent means ± S.E.M.

B. Schinifoline Inhibits C. albicans Biofilm Formation at Different Developmental Stages

We investigated the effects of schinifoline on *C. albicans* biofilm formation and development. Schinifoline inhibited biofilm formation in a dose-dependent manner (Fig. 3 and Fig. 4). During the early phase of *C. albicans* biofilm formation immediately after adhesion, we examined the effects of schinifoline at concentrations of 50-200 mg L^{-1}. At 100 and 200 mg L^{-1}, schinifoline significantly inhibited biofilm formation (Fig. 3). Even during the biofilm maturation stage, schinifoline treatment at 100 and 200 mg L^{-1} significantly inhibited biofilm formation compared to negative control (Fig. 4).

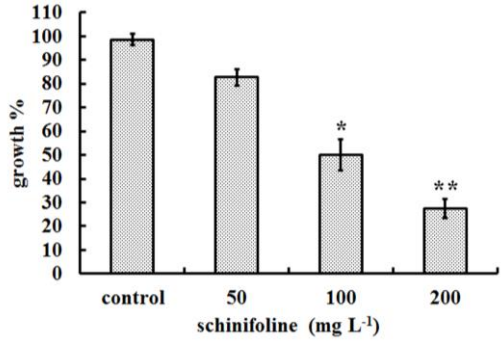


Figure 3. Inhibition of biofilm formation of *C. albicans* by schinifoline, *C. albicans* cells were incubated continuously in the presence of schinifoline at 37 ℃ for 48 h. Bars represent means ± S.E.M. *P < 0.05 vs control; **P < 0.01 vs control.

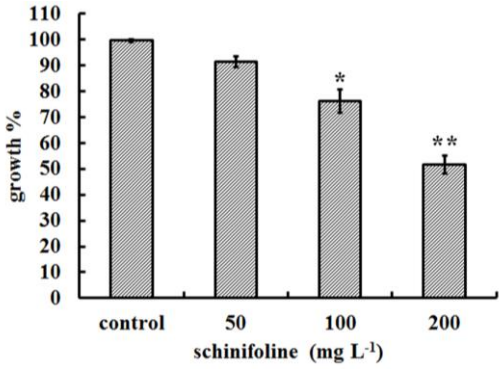
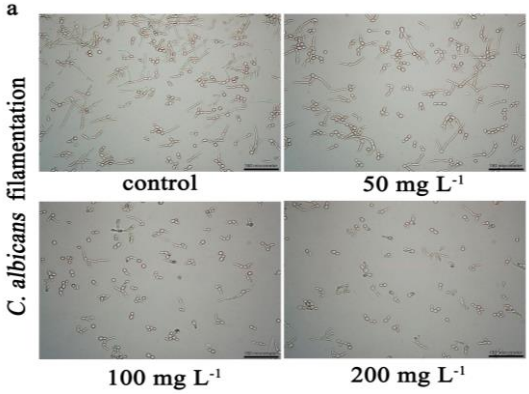


Figure 4. Inhibition of biofilm formation of *C. albicans* by schinifoline, *C. albicans* cells were allowed to adhere to plate for 24 h, and then schinifoline was added and incubated further for 24 h at 37 ℃. Bars represent means ± S.E.M. *P < 0.05 vs control; **P < 0.01 vs control.

C. Schinifoline Inhibits the Transition of C. albicans from Yeast to Hyphae



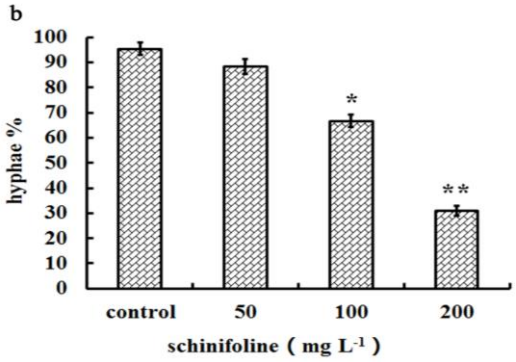


Figure 5. Schinifoline inhibits the transition of C. albicans from yeast to hyphae. Bars represent means ± S.E.M. *P < 0.05 *vs* control; **P < 0.01 *vs* control.

We found that at 100 and 200 mg L^{-1}, schinifoline was effective in inhibiting the transformation of *C. albicans* from yeast to the mycelium state (Fig. 5a). Moreover, we observed that at 100 and 200 mg L^{-1}, schinifoline effectively suppressed the transition of *C. albicans* from yeast to hyphae (Fig. 5b).

IV. Conclusion

In this study, the time-kill curve showed that at 50, 100 and 200 mg L^{-1}, schinifoline inhibited the growth of *C. albicans*. Additionally, our results demonstrated that 100 and 200 mg L^{-1} of schinifoline inhibited *C. albicans* biofilm formation at different developmental stages. In addition, 100 and 200 mg L^{-1} of schinifoline effectively inhibit the transition of *C. alibcans* form yeast to hyphae. Based on these findings, we selected 100 and 200 mg L^{-1} of schinifoline for inhibiting the growth of *C. albicans* in the *in vitro* experiment. In recent years, the population of immune deficiency has increased, and the wide use of broad-spectrum antibiotics, corticosteroids and immune inhibitors has resulted in a sharp rise in the incidence of fungal infection and mortality. Azole has exhibited good therapeutic effect on *Candida* infection due to its low toxicity, however, its extensive use has led to the increase in the lowest inhibitory concentration of azole and reports of clinical treatment failure [16]-[18]. Nowadays, drugs such as acanthocyte and doconazole are the main alternatives in cases of azole resistance, however, most patients cannot afford these drugs due to their high cost. Therefore, the emergence of drug-resistant fungi is a substantial challenge affecting clinical treatment. The use of a combination of drugs has become a hotspot in the research of antifungal drugs. Additionally, the search for new antifungal drugs with low-toxicity from natural plants is necessary to combat fungal infections [19], [20].

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