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Antifungal Activity of Schinifoline Against *Candida Albicans* in *Caenorhabditis Elegans*

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Abstract: *Zanthoxylum schinifolium* has been used as spices and traditional medicine in China for hundreds of years. A variety of active substances have been isolated from *Zanthoxylum schinifolium* using biological and chemical techniques. Among these substances, the effect of schinifoline has gradually attracted much attention. *Candida albicans* is one of the most common pathogens isolated from the gastrointestinal tract, vagina, and mouth in healthy individuals. In a healthy population, there are various mechanisms in host, such as the microbial flora, the epithelial barriers, and the innate immune system, that can control the presence of *Candida albicans*. However, when host immunity is compromised, an invasive fungal infection is more likely to occur. In this study, we explored the antifungal activity of schinifoline against *Candida albicans* in *Caenorhabditis elegans*. To determine the optimal concentration of schinifoline, we investigated the lifespan, defecation cycle and locomotion behavior of *Caenorhabditis elegans* after treatment with schinifoline. In addition, we examined colony formation in the intestine of *Caenorhabditis elegans* after *Candida albicans* infection. The results indicated that 100 and 200 mg/L of schinifoline could prolonged the lifespan, shorten the defecation cycle and increased the locomotion behavior of *Caenorhabditis elegans*, with 100 mg/L of schinifoline being the optimal concentration. Moreover, 100 mg/L of schinifoline increased the lifespan of *Caenorhabditis elegans* after infection and inhibited the colony formation of *Candida albicans* in *Caenorhabditis elegans* intestine. Therefore, we concluded that schinifoline exhibits anti-fungal effects and its potential use as natural drugs should be further explored in future studies.

Keywords: Schinifoline; fungistasis; anti-aging; *Candida albicans*; *Caenorhabditis elegans*

1 Introduction

China is the biggest producer of *Zanthoxylum schinifolium* in the world with the largest cultivated area. Sichuan pepper is the ripe and dry pericarp of *Zanthoxylum schinifolium*, which is a prickly tree or shrub of the rutaceae Figs. 1(A)-1(B). *Zanthoxylum* is widely used as food seasoning in the preparation of edible flavors such as papaya, mango and orange. In addition, *Zanthoxylum* also plays an important role in Traditional Chinese Medicine [1]. To date, studies found that the main chemical components of *Zanthoxylum* include alkaloids, amides, lignin, volatile oils, coumarin, flavonoids and fatty acids, all of which possess important effects on the cardiovascular system, digestive system and immune function, in addition to their anesthetic, bacteriostatic and anti-tumor pharmacological activities [2-4]. Among these components, schinifoline is a 4-quinolinone alkaloid that was first discovered in *Zanthoxylum* Fig. 1(C). Schinifoline has been demonstrated to possess optimal bacteriostatic activity and to selectively inhibit the activity of a gram-positive bacterium [5-6]. However, few studies have explored the effects of schinifoline *in vivo*.

Figure 1: The pericarp of *Zanthoxylum schinifolium* (A: fresh pericarp; B: dry pericarp) *and* structural formula of schinifoline

*Candida albican*s (*C. albicans*) is a dimorphic commensal yeast that commonly colonizes the human gastrointestinal tract, vagina, mouth. In recent years, *C. albicans* has become one of the most common and leading causative agents of fungal infection [7-8]. The pathogenicity of *C. albicans* is closely related to its morphological changes: *C. albicans* can switch between the budding yeast, pseudo hyphae and true hyphae formation according to environmental conditions. The formation of hyphae and biofilms, and the integrity of the cell wall also contribute to its virulence. Due to the widespread usage, including the cases of misuse, of broad-spectrum antibiotics and immunosuppressants, the increasing prevalence of antifungal resistance has become the biggest obstacle in clinical treatment of fungal infections. Since traditional Chinese medicine is known for its rich resources and low toxicity, there is a growing interest in the understanding potential mechanism and utility of Chinese herbs as new antifungal drugs with high efficiency and low toxicity [9-10].

Caenorhabditis elegans (*C. elegans*) is widely used in biological, medical and environmental studies due to its simple structure, convenient breeding, and large amounts of genetic homologies to higher animals [11]. *C. elegans* has been successfully used to identify and assess virulence factors of several human pathogens, e.g., *Salmonella enterica*, *Pseudomonas aeruginosa*, *Yersinia pseudotuberculosis*, and *Candida albicans*. In some studies, *C. elegans* was employed to identify the effectiveness of various chemicals or plant extracts on inhibiting *C. albicans* [12-14]. The purpose of this study is to assess the antimicrobial activity of schinifoline using *C. elegans* as a model organism. Furthermore, this study is designed to accelerate the efficient application of schinifoline against bacterial or fungal infections.

2 Materials and Methods

2.1 Drugs

Schinifoline is a natural 4-quinolinone alkaloid purchased from NanJing JianCheng Biological Co., Ltd. Schinifoline was extracted from the pericarp of *Zanthoxylum schinifolium*. The purity of schinifoline was 99%. Prior to the experiment, we conducted *in vitro* investigation, which showed that schinifoline could inhibit the activity of *Candida albicans*. The lowest bacteriostatic concentration was 50 mg/L, and the concentration associated with a significant difference was 200 mg/L. Therefore, we selected 50-200 mg/L as the antibacterial concentrations of schinifoline to be tested against *C. albicans in vivo* in this study. In brief, 2% DMSO was used to dissolve schinifoline into three working concentrations (50, 100 and 200 mg/L) [15-16]. All the other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd.

2.2 Strains and Maintenance

In this study, *C. elegans* strains used were wild-type N2 and the *C. albicans* strains studied were SC5314 and CaSA1 (ura3:: imm434/ura3:: imm434; CDR1-GFP-URA3) [17-18]. The CDR1 gene encodes a *C. albicans* ABC transporter that functions as an efflux pump to control the pathogenic adaptation system and the utilization of mammalian hormones and other environmental cues. The CaSA1

strain expresses a GFP gene under the control of CDR1 promoter [19]. All nematodes were maintained in nematode growth medium (NGM) plates with *Escherichia coli* OP50 at 20°C [17,20]. Gravid-stage nematodes were collected and then lysed in a bleach mixture (1 M NaOH, 10% HOCl). Age synchronous populations of L4 (larvae stage 4) were collected [20].

2.3 Examining the Effects of Schinifoline in Nematodes

In order to determine if treatment of schinifoline alone caused any deleterious or beneficial effects in *C. elegans*, different concentrations of schinifoline (50, 100, and 200 mg/L) were administrated to L4 nematodes for 24 h in the presence of food. After 24 h, nematodes were assessed for lifespan, defecation cycle and locomotion behavior. The methods used to examine the endpoints were performed as previously described. Defecation cycle was examined for a fixed number of cycles in *C. elegans*. A cycle period was defined as the interval between the initiations of two successive posterior body-wall muscle contraction steps. Locomotion behavior was evaluated by the number of head thrashes and body bends. Head thrash was defined as a change in the direction of bending at the mid body. Body bend was counted as a change in the direction of the part of the nematodes corresponding to the posterior bulb of the pharynx along the y axis, assuming that the nematode was traveling along the x axis. Thirty nematodes were examined per treatment and three replicates were performed for each concentration [21-23].

2.4 C. elegans Survival Assay

We chose 100 mg/L of schinifoline as the optimal concentration according to the assessment of schinifoline in nematodes. L4 nematodes were placed onto lawns seeded with *C. albicans* for 24 h to allow for infection. An inhibitory effect of schinifoline on *C. albicans* was observed after 48 h. Within 24 h, the inhibitory effect increased continuously and the best bacteriostatic effect was achieved in 24 h. No significant change in bacteriostatic effect was observed after this time point. Therefore, we chose 24 h as the duration of *C.albicans* infection. The worms were following treated with 100 mg/L of schinifoline for the next 24 h. Thirty nematodes were examined per treatment, and the lifespan of the nematodes was considered as endpoint [17,20].

2.5 Colony-forming Units Assay of C. albicans

L4 nematodes were respectively infected with *C. albicans* or CaSA1:: GFP labeled *C. albicans* for 24 h, and then treated with 100 mg/L of schinifoline for the following 24 h. Nematodes were then washed to remove all surface *C. albicans*. Fifty nematodes per condition that were infected with *C. albicans* were homogenized and plated on YPD agar. Plates were incubated at 37°C for 48 h. *C. albicans* colonies were counted to determine the CFU per worm. Meanwhile, the nematodes infected with CaSA1::GFP labeled *C. albicans* were photographed under a fluorescence microscopy to detect the distribution of *C. albicans* in nematode [13].

2.6 Statistical Analysis

All data in this article 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 18.0 software (SPSS Inc., Chicago, USA). Differences between groups were determined using analysis of variance (ANOVA). Probability levels of 0.05 and 0.01 were considered to be statistically significant.

3 Results

3.1 Effects of Schinifoline in C. elegans

L4 nematodes were treated with schinifoline for 24 h. Results showed that treatment with 50-200 mg/L of schinifoline significantly increased lifespan of nematodes compared with control, with 100 mg/L

of schinifoline showing the strongest effect compared to the other two concentrations. (Fig. 2). Treatment with 50-200 mg/L of schinifoline resulted in obvious alterations in the defecation cycle of nematodes compared with control (Fig. 3). As defecation cycle is related to the metabolic capacity of nematodes, our results indicated that schinifoline was effective in inhibiting the toxicity of *C. albicans* in nematodes. In addition, 100 and 200 mg/L schinifoline significantly improved the locomotion capacity of nematodes, which is related to nerve function, Therefore, schinifoline, at the examined concentrations, could play a positive role in nematodes by impacting endpoints such as lifespan, defecation cycle and locomotion behavior. Based on these findings, 100 mg/L of schinifoline was selected as the appropriate concentration for subsequent experiments.

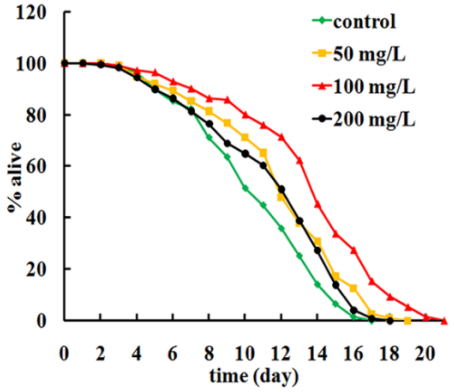

Figure 2: Effects of schinifoline treatment on lifespan of nematodes

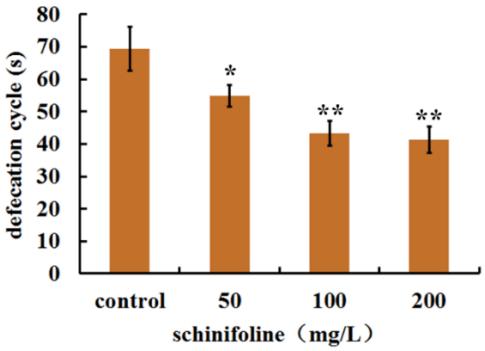

Figure 3: Effects of schinifoline treatment on defecation cycle of nematodes. **P* < 0.05, ***P* < 0.01

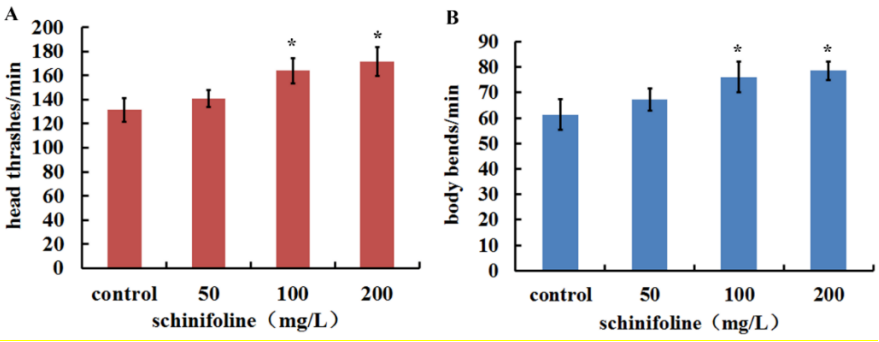

Figure 4: Effects of schinifoline treatment on locomotion behavior of nematodes (A: head thrash; B: body bend). **P* < 0.05

3.2 Schinifoline Inhibited C. albicans Virulence in Nematodes

The mean lifespan of *C. elegans* infected with *C. albicans* was shorten significantly Figs. 5(A)-5(B). After 24 h of 100 mg/L of schinifoline treatment, however, the mean lifespan of animals infected with *C. albicans* increased significantly from 7.0 ± 0.8 days to 12.3 ± 0.5 days. Therefore, schinifoline treatment could effectively reversed the adverse effects of *C. albicans* on nematodes.

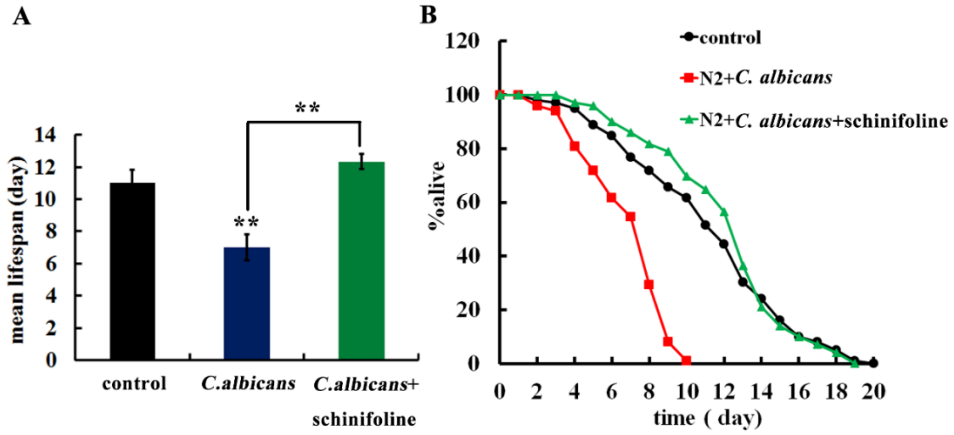

Figure 5: Schinifoline treatment extended the lifespan of nematodes infected with *C. albicans*. L4 nematodes were infected with C. *albicans* lawns for 24 h and then treated with 100 mg/L of schinifoline for 24 h. $^{**}P < 0.01$

3.3 Schinifoline Decreased C. albicans Colony Formation During C. albicans Infection in Nematodes intestine

We studied the effects of schinifoline treatment on *C. albicans* colony formation following *C. albicans* infection in nematodes. The data showed that treatment with 100 mg/L schinifoline after *C. albicans* infection significantly decreased the number of colony forming units (CFU) in nematodes intestine compared with control (Fig. 6). By using a CaSA1:: GFP labeled *C. albicans* strain, it was observed that 100 mg/L schinifoline treatment noticeably reduced the relative fluorescence intensity of CaSA1:: GFP in both the proximal and distal of nematode intestine (Fig. 7). Therefore, schinifoline treatment was demonstrated to significantly inhibit *C. albicans* colony formation in nematodes intestine after infection.

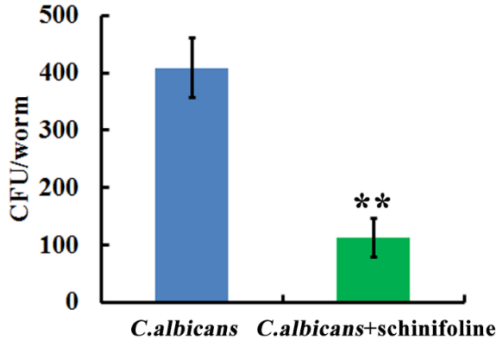

Figure 6: Effects of schinifoline treatment on colony-forming in nematodes infected with *C. albicans*. $^{**}P < 0.01$

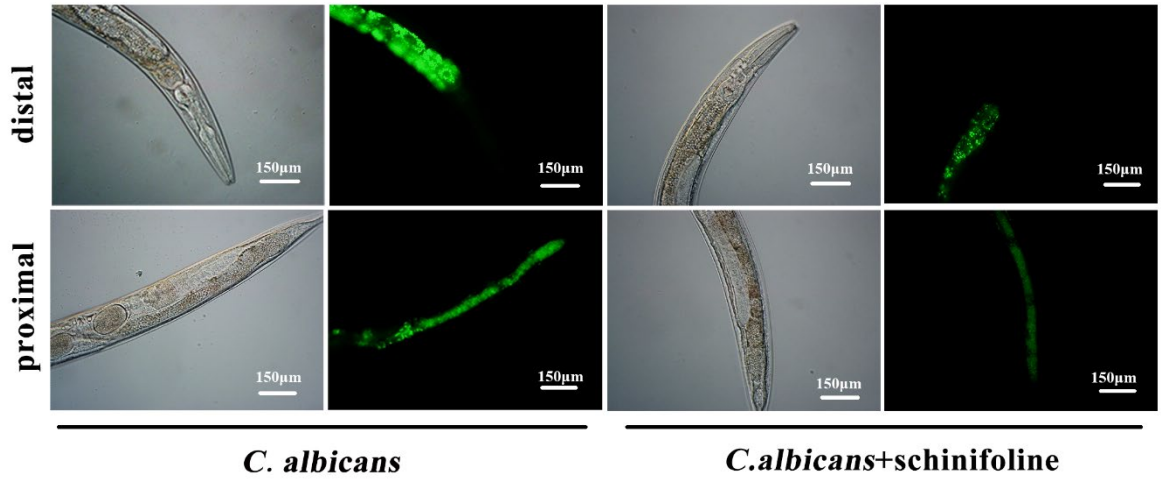

Figure 7: Effects of schinifoline treatment on the expression of CaSA1:: GFP in infected nematodes

4 Discussion

The use of traditional Chinese medicine as antifungal agents has attracted much attention due to its low toxicity, wide spectrum and long efficacy [24]. Since the chemical constituents and pharmacological effects of *Zanthoxylum* have been well studied, we asked if components in *Zanthoxylum* possess any antifungal effects on *C. albicans*. In the present study, we specifically studied the pharmacological effects of schinifoline at 50, 100 and 200 mg/L in *C. elegans*. We discovered that 100 and 200 mg/L schinifoline could increase the lifespan, shorten the defecation cycle and improved the locomotion behavior of *C. elegans*. However, no significant difference was found between the two concentrations. Therefore, we chose 100 mg/L of schinifoline as the optimal concentration for the subsequent experiments. L4 nematodes were treated with 100 mg/L of schinifoline for 24 h after *C. albicans* infection. The results showed that 100 mg/L of schinifoline increased the lifespan of *C. elegans* after infection and inhibited the colony formation of *C. albicans* in *C. elegans* intestine. Nevertheless, the anti-fungal mechanism of schinifoline against *C. albicans* in *C. elegans* remains unclear and require further investigations.

5 Conclusion

To date, more than 300 kinds of traditional Chinese herbs have been discovered to possess antifungal activities and some of them have been applied clinically with good outcomes. Due to the rapid development of traditional Chinese medicine extraction, separation and structure identification technology, increasing attention has been paid on researching the effective ingredients within traditional Chinese medicine to be used as antifungal drugs. Novel antifungal drugs with low-toxicity from natural plants would make a substantial difference in combating fungal infections. In this study, we demonstrated that schinifoline could be a promising anti-fungal therapy. As a country with a large planting area of *Zanthoxylum schinifolium*, China should look to improve the utilization of *Zanthoxylum schinifolium* to increase its values. The development and utilization of schinifoline would not only provide an effective alternative for the treatment of fungal diseases, but also promote the development of the characteristic agroforestry economy in China.

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