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Effect of loureirin A against *Candida albicans* biofilms

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[ABSTRACT] Loureirin A is a major active component of *Draconis sanguis*, a traditional Chinese medicine. This work aimed to investigate the activity of loureirin A against *Candida albicans* biofilms. 2, 3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay and scanning electron microscopy were used to investigate the anti-biofilm effect. Minimal inhibitory concentration testing and time-kill curve assay were used to evaluate fungicidal activity. Cell surface hydrophobicity (CSH) assay and hyphal formation experiment were respectively carried out to investigate adhesion and morphological transition, two virulence traits of *C. albicans*. Real-time RT-PCR was used to investigate gene expression. *Galleria mellonella*–*C. albicans* and *Caenorhabditis elegans*–*C. albicans* infection models were used to evaluate the *in-vivo* antifungal effect. Human umbilical vein endothelial cells and *C. elegans* nematodes were used to evaluate the toxicity of loureirin A. Our data indicated that loureirin A had a significant effect on inhibiting *C. albicans* biofilms, decreasing CSH, and suppressing hyphal formation. Consistently, loureirin A down-regulated the expression of some adhesion-related genes and hypha/biofilm-related genes. Moreover, loureirin A prolonged the survival of *Galleria mellonella* and *Caenorhabditis elegans in C. albicans* infection models and exhibited low toxicity. Collectively, loureirin A inhibits fungal biofilms, and this effect may be associated with the suppression of pathogenic traits, adhesion and hyphal formation.

[KEY WORDS] *Candida albicans*; Loureirin A; Anti-biofilm; Morphological transition

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Introduction

Draconis sanguis (*Xue Jie* in Chinese) is a deep red resin obtained from stems of *Dracaena cochinchinensis* and other plant sources [1]. It is a rare and precious traditional medicine used by China and some other countries since ancient times [2]. There are many kinds of preparations containing *Draconis sanguis*, and they are clinically used for the treatment of blood stasis syndrome, trauma, gynecopathy, and allergic dermatitis [3-4]. Modern pharmacological studies have found that *Draconis sanguis* has anti-microbial activities [1]. According to the records in "Great Dictionary of Chinese Medicine" and "Chinese materia medica", water extracts of *Draconis sanguis* exhibited inhibitory effect against *Trichophyton violaceum*, *Trichophyton gypseum*, and *Trichophyton schoenleinii*. Moreover, CAI *et al*. reported the antimicrobial effect of Guangxi *Draconis sanguis* [5]. At a concentration of 0.25 mg·mL^{-1}, *Draconis sanguis* could inhibit the growth of *Staphylococcus aureus, Diphtheria bacilli* and *Bacillus anthracis*. While at 50 mg·mL^{-1}, *Draconis sanguis* could inhibit the growth of *Candida albicans* [5].

Candida albicans is a major opportunistic fungal pathogen causing superficial to life-threatening infections, especially in immunocompromised patients [6]. More specifically, *C. albicans* is the fourth leading cause of vascular catheter-related infections and disseminated bloodstream infections accounts for a mortality rate of above 40% [7-10]. Moreover, *C. albicans* is the third leading cause of urinary catheter-related infections [7, 11-13]. It has a high propensity to develop biofilms on the surfaces of medical devices and various types of catheters [14]. *C. albicans* biofilms exhibits complex three-

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dimensional structure, and its formation attributes to the adhesion to biomaterial surfaces, growth to form an anchoring layer, and yeast-to-hypha morphological transition of *C. albicans* [15-16]. *C. albicans* biofilms contribute to virulence [17] through the invasion of tissues, harboring persister cells, and the barring of antibiotics/antifungals through physical exclusion and efflux pumps. Unlike planktonic cells, *C. albicans* biofilms display resistance to a wide variety of clinical antifungal agents, including most frequently used amphotericin B and fluconazole [18-19]. Of note, in a report by Vila and colleagues, at a concentration as high as 16 × MIC, fluconazole could not inhibit the development of biofilms [20]. Therefore, there is an urgent need to identify or develop new agents that effectively inhibit or prevent *C. albicans* biofilms.

Although *Draconis sanguis* has been reported to have anti-*C. albicans* activity at high concentrations, its anti-biofilm activity has not yet been evaluated. In this study, we evaluated the activity of loureirin A (Lou A), a main active component of *Draconis sanguis* [1] (Fig. 1A) against *C. albicans* biofilms, and investigated the underlying mechanism.

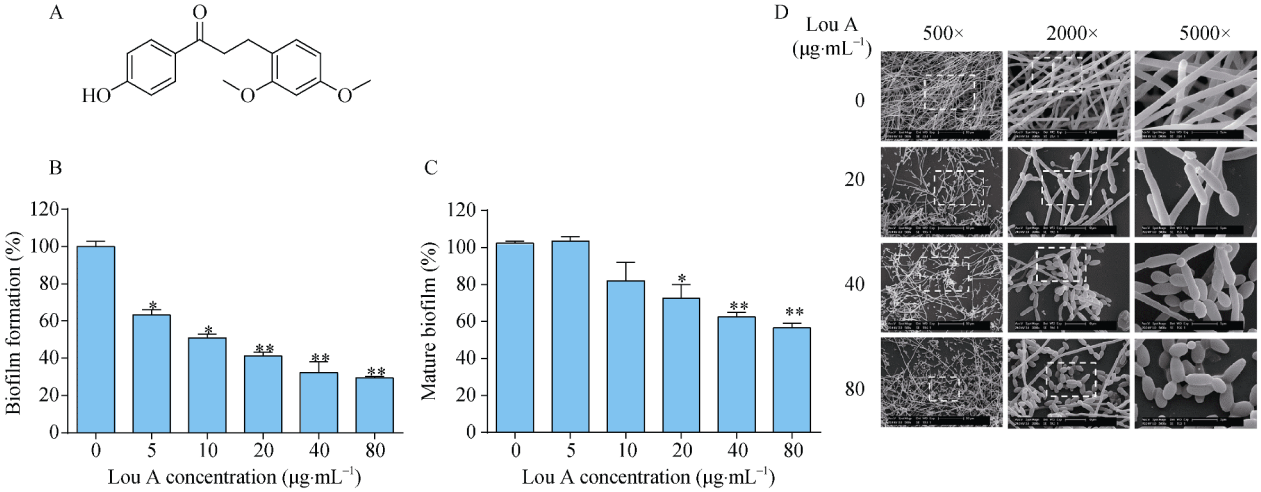

Fig. 1 (A) Chemical structure of Lou A. (B) Effects of increasing concentrations of Lou A on biofilm formation. (C) Effects of different concentrations of Lou A on mature biofilms. Biofilm formation was evaluated by XTT reduction assay, and the results were presented as the percentage compared to the control biofilms formed without Lou A treatment. Biofilm formation results represent the mean ± SD for five independent experiments. *$P < 0.05$, **$P < 0.01$ *vs* the control biofilms. (D) Effects of different concentrations of Lou A on biofilm formation imaged with SEM. Images in the dashed boxes are enlarged and the enlarged images are shown to the right

Materials and methods

Strains, culture and agents

C. albicans strains, including SC5314, Y0109, 13, 19, 21, 100, 103, 805, 0710922 were routinely grown in Yeast extract Peptone Dextrose (YPD) (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Lou A was purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China (Shanghai, China), and the level of purity was ≥ 98%. Other media used included YPD + 10% (*V/V*) Fetal Calf Serum (FCS, Gibco, Bethesda, MD, USA) [21], Spider medium [21], Lee medium (pH = 6.8) [22], and RPMI 1640 medium (Gibco, Bethesda, MD, USA) [21]. *Caenorhabditis elegans glp-4; sek-1* strain was propagated on nematode growth medium on lawns of *Escherichia coli* OP50 by using standard methods [23].

Biofilm formation assay

The assay was carried out as described previously [21, 24]. Briefly, *C. albicans* SC5314 was used to form biofilms. Serial concentrations of Lou A were added to fresh RPMI 1640 after *C. albicans* adhesion to the surface of 96-well tissue culture plates. The plate was further incubated at 37 °C for 24 h and the formation of biofilm was measured with a 2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay [25]. To detect the effect of Lou A on mature biofilms, *C. albicans* biofilms were formed without drug treatment and Lou A was then added and treated for 24 h.

Scanning electron microscopy (SEM) assay

The assay was carried out as described previously [21, 24]. Briefly, *C. albicans* biofilms were developed on sterile glass disks coated with poly-L-lysine hydrobromide (Sigma, Cat. No. P6282). The biofilm samples were observed through a Philips XL-30 SEM (Philips, The Netherlands) in high vacuum mode [21].

Antifungal susceptibility testing

Antifungal susceptibility test was carried out in 96-well microtiter plates (Greiner, Germany) as described previously [21, 24], using the broth microdilution protocol of the Clinical and Laboratory Standards Institute methods (M27-A3) [21, 24] to determine minimal inhibitory concentration (MIC) of Lou A.

Time-kill curve assay

Time-kill curve assay was carried out as described previously [21]. Three independent experiments were carried out.

Cellular surface hydrophobicity (CSH) assay

C. albicans CSH was measured by water-hydrocarbon two-phase assay as described previously [21, 25].

Hyphal formation assay in liquid and solid media

1×10^6 CFU·mL^{-1} *C. albicans* SC5314 cells were incubated in liquid hypha-inducing medium RPMI 1640, Spider or Lee at 37 °C for 3 h to grow hyphae. The hyphae were photographed using inverted phase contrast microscope (Amersham Pharmacia, German). As for filamentation test in solid media, about 10 *C. albicans* cells were plated on agars containing Spider, YPD + FCS, or Lee's media. Cells in Spider were incubated at 37 °C for 5 d, YPD + FCS at 37 °C for 3 d, and Lee's at 25 °C for 14 d.

Real-time RT-PCR assay

C. albicans SC5314 cells were incubated in cell culture dishes for 90 min to adhere, the medium was removed and replaced with medium containing 40 μg·mL^{-1} Lou A or DMSO as the control. The cultures were then incubated statically at 37 °C for an additional 1 h. Then the *C. albicans* cells were collected immediately after the incubation. RNA extraction and real-time PCRs were performed as described previously [23]. RNA was extracted using a fungal RNAout kit (TIANZ, China) according to the manufacturer's instructions. A reverse transcription reaction was performed to transform 500 ng RNA into cDNA for each group with a reverse transcription kit (TaKaRa, Japan). Real-time PCR was conducted using a 7500 real-time PCR system (Applied biosystems, USA) with SYBR green I (TaKaRa) as the monitor. A total volume of 20 μL reaction system was prepared in accordance with the manufacture's instructions. The PCR program consisted of initial denaturation stage (95 °C, 30 sec), 40 cycles of PCR stage (95 °C, 5 sec; 60 °C, 34 sec), and melt curve stage (60−95 °C). The threshold cycle (C_T) above background for each reaction was calculated The C_T value of each gene was normalized to that of 18S rRNA in its group and marked as ΔC_T. Then $\Delta\Delta C_T$ was calculated by subtracting ΔC_T of corresponding gene in control group from ΔC_T value in Lou A treated group. Gene expression data in the results was indicated as $2^{-\Delta\Delta CT}$, and the gene whose result < 0.5 was considered to be down-regulated compared with the control group. Triplicate independent experiments were conducted.

Galleria mellonella survival assay

G. mellonella−*C. albicans* infection model was used to evaluate the antifungal effect of Lou A, and survival assay was performed as described previously [26-27]. Briefly, *C. albicans* SC5314 inoculum was injected at the last left pro-leg of *G. mellonella* larvae using a Hamilton syringe. 2 mg·kg^{-1} Lou A was injected at the last right pro-leg. A control group received sterile water. The number of dead larvae was scored daily.

Caenorhabditis elegans survival assay

C. elegans was infected by *C. albicans* SC5314 as described previously [28]. For Lou A treatment groups, 20 μg·mL^{-1} Lou A was added, and DMSO solvent group was included as a control. Worms were scored daily and dead worms were removed from the assay.

Toxicity evaluation using human umbilical vein endothelial cells (HUVECs) and C. elegans nematodes

HUVEC Cells were treated with various concentrations of Lou A for 24 h. Then the medium was removed and replaced with medium containing 500 μg·mL^{-1} XTT and 3.8 μg·mL^{-1} phenazine methosulfate. After treatment at 37 °C for additional 4 h, absorbance at 450 nm was measured with the Multilabel Counter (Thermo Fisher Scientific, Massachusetts, USA).

C. elegans nematodes were also used to evaluate the toxicity of Lou A as described previously [29]. Briefly, the nematodes were transferred to liquid medium containing 160 μg·mL^{-1} Lou A or the solvent DMSO at the same volume. The worms were incubated at 25 °C for 6 d and observed daily.

Statistical analysis

Survival was examined by using the Kaplan-Meier method and differences were determined by using the log-rank test (STATA 6; STATA, College Station, TX). A P value of < 0.05 was considered statistically significant [26-27].

Results

Lou A inhibits the formation of C. albicans biofilms

In this study, we evaluated the effect of Lou A on *C. albicans* biofilms. Lou A inhibited biofilm formation in a dose dependent manner (Fig. 1B). Biofilm formation was inhibited by 37% ($P < 0.05$) in the presence of 5 μg·mL^{-1} of Lou A, with extended inhibition with increasing concentrations of the compound. In the presence of 80 μg·mL^{-1} Lou A, the biofilm formation was 30% of structure formed in the negative control group. Notably, Lou A also inhibited mature biofilms dose dependently (Fig. 1C). At 20 μg·mL^{-1}, Lou A inhibited mature biofilms by 26% ($P < 0.05$). The effect was more pronounced with increasing concentrations of Lou A. When 80 μg·mL^{-1} Lou A was added to the fungal biofilm, the measured material was 44% ($P < 0.01$) compared to the negative control. Collectively, Lou A exhibited a remarkable anti-biofilm effect, both preventing formation and inhibiting mature structures.

Consistent results were obtained in SEM assay where increasing concentrations of the compound were observed to inhibit the collection or formation of filaments. Provision of 20 μg·mL^{-1} of Lou A caused defect in filamentation and destroyed the 3D structure of the *C. albicans* biofilms (Fig. 1D). These alterations were more pronounced with increasing concentrations of Lou A. Provision of 80 μg·mL^{-1} of Lou A inhibited biofilm formation and most *C. albicans* cells were in yeast form.

Anti-biofilm effect of Lou A is not attributed to its antifungal activity

We evaluated the antifungal capacity of Lou A on *C. albicans* by evaluating the minimal inhibitory concentration (MIC) of the compound using a collection of both drug sus-

ceptible and drug resistant strains of *C. albicans*. Our panel included 5 fluconazole-susceptible strains and 4 fluconazole-resistant strains. The MIC_{50} and MIC_{80} of Lou A on all the 9 strains tested were no less than 80 μg·mL^{-1} (Table 1), which was much higher than the anti-biofilm concentrations of Lou A. These results indicated that the anti-biofilm effect of Lou A was not attributed to its antifungal activity.

The results of a time-kill curve assay confirmed the MIC findings, showing that ≤ 80 μg·mL^{-1} Lou A had no significant inhibitory effect on *C. albicans* (Fig. 2). At a concentration as high as 80 μg·mL^{-1} of Lou A, there was a modest antifungal activity, but no significant difference compared with the control group (P = 0.3723). Thus, our results suggest that Lou A affects an aspect of biofilm formation without adversely affecting the planktonic cells at concentrations less than 80 μg·mL^{-1}.

Table 1 MICs of Lou A against fluconazole-susceptible and fluconazole-resistant *C. albicans*

C. albicans strains	MIC_{50} (μg·mL^{-1})		MIC_{80} (μg·mL^{-1})	
	FLC	Lou A	FLC	Lou A
Fluconazole-susceptible C. albicans				
SC5314	0.5	> 80	0.5	> 80
Y0109	0.125	80	0.125	> 80
13	0.5	> 80	0.5	> 80
19	0.5	> 80	0.5	> 80
21	0.5	> 80	0.5	> 80
Fluconazole-resistant C. albicans				
100	32	> 80	> 1024	> 80
103	32	> 80	> 1024	> 80
805	32	> 80	> 1024	> 80
0710922	32	> 80	> 1024	> 80

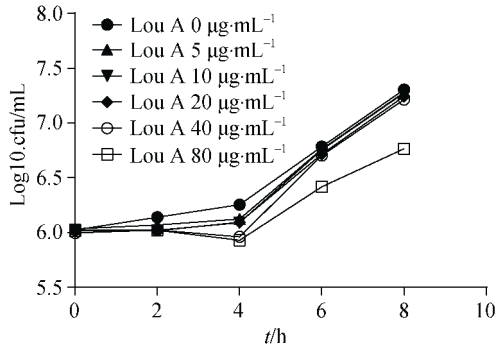

Fig. 2 Time-kill curves of various concentrations of Lou A on *C. albicans* strain SC5314. 10 mg/ml Lou A in DMSO was used as a stock and added to 1 × 10^6 cells·mL^{-1} *C. albicans* suspensions in RPMI 1640 medium to obtain the indicated Lou A concentrations. *C. albicans* suspension in RPMI 1640 medium with 0.8% DMSO was used as the control. The data are shown as mean ± SD from three independent experiments. Statistical significance among the groups was determined by the analyses of variance (ANOVA) and no significant difference was observed

Lou A decreases cell surface hydrophobicity (CSH) of C. albicans

Since cell adhesion and yeast-to-hypha morphological transition are two important aspects for *C. albicans* biofilm formation [15] and there is a positive correlation between CSH and cell adhesion of *C. albicans* [30-31], we examined the effect of Lou A on CSH. The normal CSH of *C. albicans* was found to be 0.93 (Fig. 3). 5 μg·mL^{-1} Lou A decreased CSH to 0.61 (P < 0.01; Fig. 4). We observed a dose-dependent reduction in CSH with increasing concentration of Lou A (Fig. 3). The CSH of *C. albicans* decreased to 0.01 in the presence of 80 μg·mL^{-1} Lou A (Fig. 3).

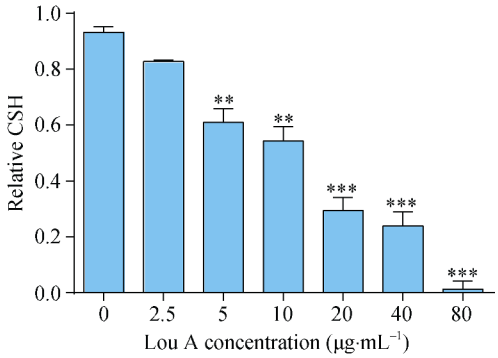

Fig. 3 Effects of various concentrations of Lou A on CSH of *C. albicans* SC5314. CSH was evaluated by using the water-hydrocarbon two-phase assay. The data are shown as mean ± SD from three independent experiments. **P < 0.01, ***P < 0.001 *vs* the control group without treatment

Lou A inhibits hyphal formation of C. albicans

We further studied the effect of Lou A on yeast-to-hypha morphological transition of *C. albicans*. For this assay, cells were grown in liquid hypha-inducing media, including RPMI 1640, Spider, or Lee. Without Lou A treatment, *C. albicans* cells formed true hyphae in all the media tested (Fig. 4A). In Spider medium, 20 μg·mL^{-1} Lou A could inhibit the yeast-to-hypha morphological transition. At 40 μg·mL^{-1}l, Lou A disrupted the hyphal formation totally (Fig. 4A). Consistently, Lou A inhibited *C. albicans* hyphal formation in RPMI 1640 and Lee media (Fig. 4A).

The inhibition effect of Lou A on *C. albicans* hyphal formation was further confirmed on solid hypha-inducing media, including Spider, YPD + FCS and Lee media. On Lou A free solid hypha-inducing media, *C. albicans* cells formed wrinkled colonies or radial hyphae on all the surfaces tested (Fig. 4B). Of note is that a previous study has revealed that wrinkled or radial colonies indicate pseudohyphal and mycelial cells inside the colonies while smooth colonies indicate budding yeast cells inside [32]. At 5 μg·mL^{-1}, Lou A inhibited the developing of radial colonies (on solid Spider or Lee media) or wrinkled colonies (on solid YPD + FCS medium) to some extent. The addition of Lou A inhibited the hyphal formation inside the colonies in a dose dependent manner, and only

smooth colonies were observed with 40 μg·mL^{-1} Lou A treatment.

Exposure to Lou A alters C. albicans gene expression

To explore the mechanism of biofilm disruption by Lou A, we investigated the expression changes of some known adhesion-related, hypha-related, and biofilm-related genes in response to Lou A treatment. The hypha-specific genes and biofilm-related genes such as *SAP4*, *SAP5*, *SAP6*, *ECE1*, *HWP1* and *PGA10*, were down-regulated after Lou A treatment. Adhesion-specific genes *IFF4* and *EAP1* and some regulation genes, including *RAS1*, *GPR1*, *CYR1*, *EFG1*, *CEK1*, *CPH1* and *UME6* were also down-regulated (Table 2). The results were consistent with the biofilm/filament defects induced by Lou A.

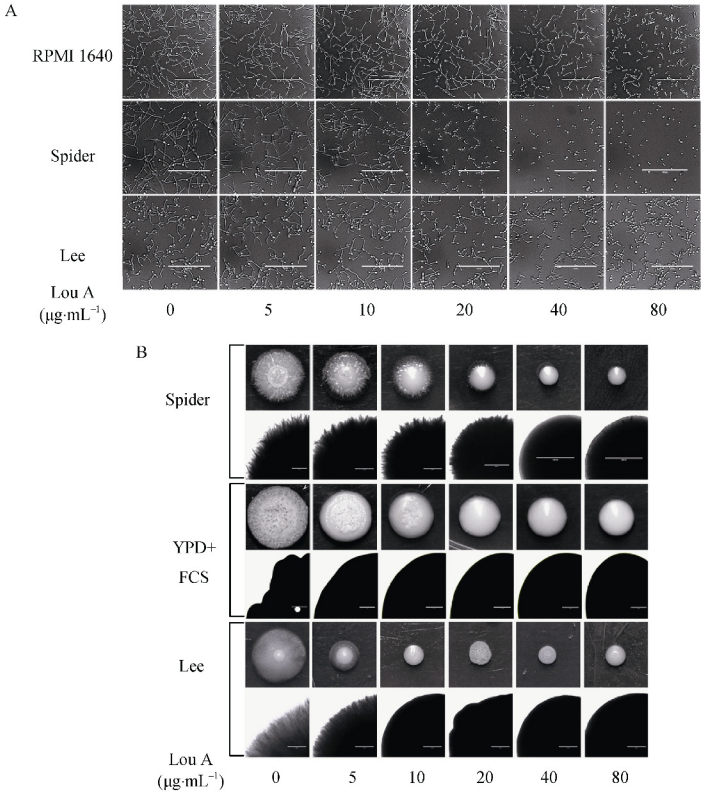

Fig. 4 Effects of Lou A on hyphal formation in hypha-inducing media. (A) Exponentially growing *C. albicans* SC5314 cells were transferred to hypha-inducing liquid media. The liquid media include RPMI 1640, Spider and Lee. The cellular morphology was photographed after incubation at 37 °C for 3 h. Scale bar = 100 μm. (B) Approximately 10 cells were plated on different solid media. YPD + FCS is a rich medium that induces true hyphae. Spider medium contains mannitol as the carbon source. Lee is a medium rich in amino acids that induces true hyphae. Temperature and time of incubation were as follows: Spider, 37 °C, 5 d; Lee, 25 °C, 14 d; YPD + FCS, 37 °C, 3 d

Table 2 Fold changes of some important genes related to biofilm formation in *C. albicans* after Lou A treatment

Gene	Fold change	Gene	Fold change
Hypha-related genes		Adhesion-related genes	
RAS1	0.07 ± 0.03****	*HWP1*	0.45 ± 0.04****
GPR1	0.17 ± 0.10****	*IFF4*	0.23 ± 0.01****
CYR1	0.19 ± 0.01****	*EAP1*	0.21 ± 0.01****
CEK1	0.23 ± 0.03****	Biofilm-related genes	
CPH1	0.11 ± 0.02****	*PGA10*	0.15 ± 0.03****
UME6	0.16 ± 0.03****	Transcription factors	
SAP4	0.18 ± 0.02****	*EFG1*	0.13 ± 0.07****
SAP5	0.19 ± 0.003****	*TEC1*	1.15 ± 0.10
SAP6	0.22 ± 0.06****	*BCR1*	1.14 ± 0.35
ECE1	0.26 ± 0.03****	*NDT80*	2.00 ± 0.28****
		ROB1	0.67 ± 0.13*
		BRG1	0.83 ± 0.20

$^{*}P < 0.05$; $^{****}P < 0.0001$ *vs* the control group without treatment (One-way analysis of variance followed by a *post hoc* Dunnett-*t* test)

Lou A exhibits antifungal effect in vivo in G. mellonella and C. elegans candidiasis models

Since Lou A inhibited yeast-to-hypha morphological transition, the most widely acknowledged pathogenic trait of *C. albicans*, we further investigated the *in vivo* antifungal activity of Lou A using a *G. mellonella–C. albicans* infection model and a *C. elegans–C. albicans* infection model. In *G. mellonella–C. albicans* infection model, 2 mg·kg^{-1} Lou A was administered. Lou A significantly protected *G. mellonella* from *C. albicans* infection ($P < 0.05$; Fig. 5A). Consistently, Lou A significantly protected *C. elegans* from *C. albicans* infection at the concentration of 40 μg·mL^{-1} ($P < 0.05$; Fig. 5B). Collectively, Lou A treatment could prolong the survival of *G. mellonella* and *C. elegans* with *C. albicans* infection, indicating the antifungal effect of Lou A *in vivo*.

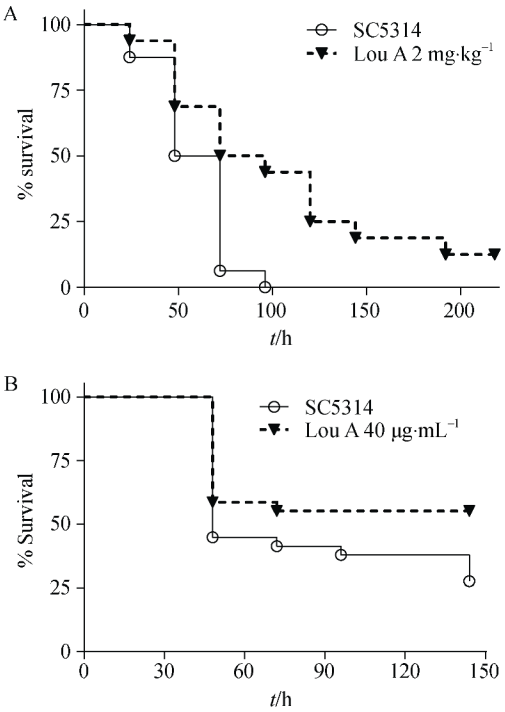

Fig. 5 Lou A prolongs the survival of *G. mellonella and C. elegans* infected by *C. albicans*. (A) *G. mellonella* larvae were infected with *C. albicans* SC5314 and then treated with 2 mg·kg^{-1} Lou A or drug free sterile water as the control. *G. mellonella* survival was observed over 216 hours (9 days) at 37 °C (n = 16 larvae per group). Dead larvae were counted and removed daily. $P < 0.01$ *vs* the control infection group without drug treatment. (B) Lou A prolongs the survival of *C. elegans glp-4; sek-1* nematodes infected by *C. albicans* SC5314. Nematodes were infected with *C. albicans* for 4 h and then transferred to pathogen-free liquid medium in the presence of 40 μg·mL^{-1} Lou A or DMSO. *C. elegans* survival was observed over 144 hours (6 days) at 25 °C (n = 60 worms per group). Dead worms were counted and removed daily. $P < 0.05$ *vs* the control infection group without drug treatment

Lou A exhibits low toxicity on HUVEC and C. elegans

We tested the toxicity of Lou A using HUVEC. Concentrations ranging from 0 to 160 μg·mL^{-1} were tested. No toxicity was observed of Lou A at concentrations below 80 μg·mL^{-1} (Fig. 6A). We further tested the toxicity of Lou A using *C. elegans* worms. At the concentration as high as 160 μg·mL^{-1}, no toxicity was observed of Lou A, and all the worms with Lou A treatment looked as healthy as those in the drug-free group (Fig. 6B).

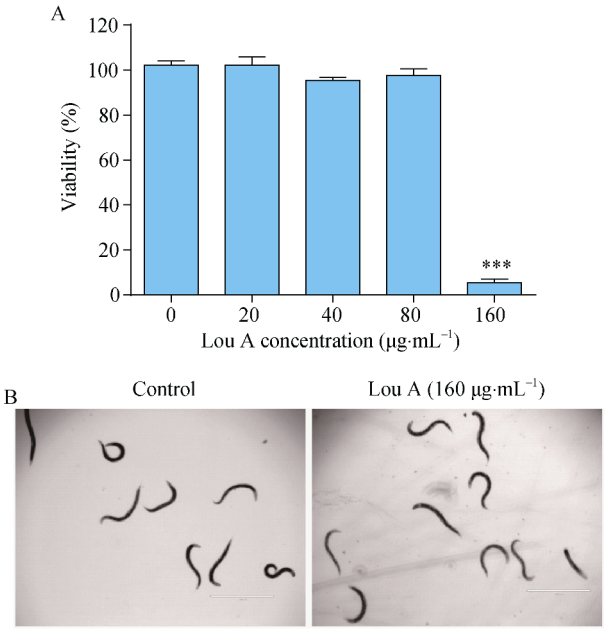

Fig. 6 Lou A exhibits low toxicity. (A) Lou A exhibited low toxicity on HUVEC. The data are shown as mean ± SD from three independent experiments. Statistical analyses were performed using ANOVA. $^{***}P < 0.001$ *vs* the control group without treatment. (B) Lou A exhibited low toxicity on *C. elegans* worms. 160 μg·mL^{-1} Lou A was used and solvent DMSO was used as the control. Scale bar = 1 mm

Discussion

Draconis sanguis has been widely used in different countries for ages. Its main chemical constituents are flavonoids. Lou A and loureirin B are two major active flavonoid compounds in *Draconis sanguis*, and both are used as markers to identify sources or control the quality of preparations containing *Draconis sanguis* [33]. Of note, Lou A has a better solubility in water, which presents advantages over loureirin B as an anti-biofilm agent. In this investigation, we evaluated the activity of Lou A against *C. albicans* biofilms.

Lou A presents a stronger anti-biofilm effect rather than the fungicidal effect. More specifically, it inhibits biofilm through suppressing virulence traits, adhesion and yeast-to-hypha morphological transition, rather than killing *C. albicans* cells. There are three known stages for biofilm formation: adhesion to biomaterial surfaces, growth to form an anchoring layer, and morphological transition to form a complex three-dimensional structure [15-16]. Our data indicate that

20 μg·mL^{-1} Lou A significantly inhibits biofilm formation, decreases CSH (the indicator of adhesion), and alters the yeast-to-hypha morphological transition, while concentrations as high as 80 μg·mL^{-1} Lou A could not affect the growth of *C. albicans*. Thus, the anti-biofilm formation effect of Lou A seems attributed to its anti-adhesion and anti-morphological-transition activities.

Our RT-PCR data also supports that Lou A inhibits biofilm through suppressing adhesion and yeast-to-hypha morphological transition. Some important adhesion/hypha-related genes, including *HWP1*, *IFF4*, *EAP1*, *ECE1*, *RAS1*, *GPR1*, *CYR1*, *EFG1*, *CEK1*, *CPH1*, *UME6*, and *PGA10* were down-regulated after Lou A treatment. *HWP1* [24], *IFF4* [34] and *EAP1* [35] are related to adhesion. *RAS1* [24], *GPR1* [36], *CYR1* [36], *EFG1* [37], *CEK1* [36] and *CPH1* [38] are involved in either cAMP pathway or MAPK pathway, two most important pathways regulating yeast-to-hypha transition. *UME6* [39], *ECE1* [37] and *HWP1* [37] are hypha-specific genes, and *PGA10* [40] is involved in biofilm formation. The down-regulation of these genes indicates that Lou A may inhibit biofilm through suppressing adhesion and hyphal formation.

The formation of biofilm is a virulence attribute to *C. albicans*. In particular, it contributes to the pathogenicity of two invertebrate infection models: *G. mellonella* and *C. elegans* [29, 41-42]. In this study, we use a *G. mellonella–C. albicans* infection model and a *C. elegans–C. albicans* infection model to evaluate the *in-vivo* antifungal activity of the precious Lou A. These models are reliable to evaluate both the efficacy and the toxicity of antifungal agents [26, 28]. Importantly, these models do not need as large amount of agents as the mammalian models require [26, 28] and thus make it possible for us to evaluate limited quantities of Lou A. Our data indicate that 2 mg·kg^{-1} Lou A could prolong the survival of *G. mellonella* with *C. albicans* infection. 40 μg·mL^{-1} Lou A significantly protected *C. elegans* from *C. albicans* infection, while the agent at this concentration could not inhibit the growth of *C. albicans*. The antifungal activity of Lou A *in vivo* may attribute to the inhibitory effect of Lou A on pathogenic traits of *C. albicans*. Adhesion on host cells is the first step for *C. albicans* to cause infection [43]. Yeast-to-hypha transition is the most widely acknowledged pathogenic trait of *C. albicans*, both in mammalian hosts and insect hosts [44-46]. Thus, our *in-vivo* data also suggest that Lou A can suppress pathogenic traits, adhesion and hyphal formation of *C. albicans*.

In conclusion, Lou A can inhibit *C. albicans* biofilms thus suppressing a pathogenic trait, and exhibits low toxicity. Further research work is needed to generate analogues with higher potency. Moreover, translational research is required to determine whether the effect of Lou A or analogues of the compound can be applicable in a clinical setting.

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