

Original Article

Ginseng stem-leave saponins (GSLs) could enhance the activity of fluconazole (FLC) against genital candidiasis

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Received January 21, 2017; Accepted February 16, 2017; Epub April 15, 2017; Published April 30, 2017

Abstract: Background: Limitations of antifungal agents used in the treatment of genital candidiasis, as the emergence of resistant strains, more efforts are needed to find new antifungal agents. Methods: we performed in vitro drug susceptibility testing for GSLs against 31 *Candida* spp. clinical isolates following Clinical and Laboratory Standard Institute (CLSI) M27-A3 guidelines. Flow cytometer (FCM) and transmission electron microscopy (TEM) were used to assess the alterations induced by GSLs in *C. parapsilosis* cells. Experiments in mice were performed to determine the antifungal activity of GSLs against genital candidiasis. Results: The 24 h of minimum inhibitory concentration (MIC₅₀) of *C. albicans*, *C. tropicalis*, *C. krusei*, *C. glabrata* were < 0.49 µg/ml, 0.98 µg/ml, < 0.49 µg/ml, 1.95 µg/ml, respectively. A series of events related to alterations were observed in GSLs-treated *C. parapsilosis*, including the proportion of G₀/G₁ phase cells increased, the proportion of S-G₂-M phase cells decreased, proliferation index (PI) decreased, extensive chromatin condensation, nuclear fragmentation, and abundant cell sap released. Additionally, at day 3 post-medication, the clone forming unit (CFU) counts of group C (treated with FLC) and D (treated with FLC + GSLs) were 0.16 ± 0.24, 0.06 ± 0.13, respectively. At day 7 post-medication, the CFU counts of group C and D were both 0.00 ± 0.00. Conclusions: GSLs possesses activity against genital *Candida* spp. clinical isolates in vitro and in vivo. GSLs could be a potential candidate to fight against genital candidiasis by inhibiting the synthesis of DNA, cellular division and the proliferation activity of *Candida*.

Keywords: Ginseng stem-leave saponins, fluconazole, genital candidiasis, mechanism

Introduction

Genital candidiasis mainly includes the female vulva and vaginal *Candida*, *Candida* balanitis, urethritis in male. Vulvovaginal candidiasis (VVC) is a common opportunistic fungal infection in women at reproductive age, which caused by *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis* etc, accounting for 95% in genital candidiasis, the main pathogen was *C. albicans*, about 70%~90% [1-3]. According to reports [4, 5] in the world about 70%~75% of all women of reproductive age had at least one case of VVC during their lifetime, 40%~50% of the patients had recurrent experience, while 5%~8% women developed recurrent Vulvovaginal Candidiasis (RVVC). Sexual transmission was considered the main reason for the frequent recurrence of genital candidiasis. *C. albicans* and non-*albicans* species tend-

ed to exhibit decreased susceptibility to azoles and even resistance was another difficulty for the treatment of VVC [2, 6]. It has the characteristics of obvious symptoms, sexual partners with the same patient, easy to relapse etc, which have exerted a serious influence on the physical and mental health and quality of life of patients and their families [7].

Recently, the non-*albicans* species morbidity has increased noticeably especially in post-menopausal women [8]. It has been reported that VVC induced by *C. albicans* were prone to drug resistance due to overusing and misusing of antifungal agents, and non-*albicans* species such as *C. krusei*, were resistant to FLC [2, 9]. Unsatisfied results are often achieved when using conventional treatment for VVC. Therefore, more anti-genital candidiasis agents with low toxicity and high efficiency are urgently

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needed. Traditional Chinese Medicines (TCM) have a long history in the treatment of fungal infections, and have accumulated rich experience. So the selected drugs with anti-*Candida* activity from TCM, and combined with western medicines, which not only increase therapeutic efficacy, but also reduce toxicity, side effects and prevent the emergence of drug resistance [10, 11].

In this study, we demonstrated that GSLs had the activity against genital *Candida* spp. clinical isolates in vitro susceptibility testing. GSLs could induce the alteration of the cell cycle of *C. parapsilosis* and change the ultrastructural of *C. parapsilosis* by FCM assay and TEM. Animal experiments further identified that GSLs could enhance the activity of FLC against genital candidiasis.

Materials and methods

Reagents

FLC powder was provided by Shanghai three dimensional Pharmaceutical Co., Ltd (purity 99.9%, Shanghai, China). GSLs was purchased from National Institute for the Control of Pharmaceutical and Biological Products. MOPS was purchased from Sigma company. DMSO was obtained from American Amersco Corporation.

Animals

ICR strain of female mice (8-9 weeks) were obtained from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. All procedures performed on mice were approved by the Animal Care and Use Committee of Shanghai Tongji Hospital, China.

Fungi and identification of fungal species

The 31 strains were isolated from patients with genital *Candida* infection in the Department of Dermatology of Tongji Hospital Affiliated to Tongji University. Isolates were obtained from the male and female external genital organs and other parts of the female vagina. *C. krusei* (ATCC6258) and *C. parapsilosis* (ATCC22019) were selected as quality control strains, which were obtained from Centre of fungal preservation of the Changzheng Hospital (Second Military Medical University, Shanghai). The clinical isolates were identified with CHROMagar and API-20CAUX.

In vitro susceptibility testing

GSLs susceptibility testing was performed following Clinical and Laboratory Standard Institute (CLSI) M27-A3 guidelines [12]. GSLs was diluted twofold so as to make a final concentration ranging from 0.49~250 µg/ml. Prepared the inoculum by using sterile 0.85% saline. Adjusted the cell density to a 0.5 McFarland turbidity standard with spectrophotometric methods. Using filtered Rosewell Park Memorial Institute (RPMI) 1640 broth to diluted suspensions 1000-fold to a concentration of $1\sim5 \times 10^6$ CFU/ml. Plated 100 µl of the suspensions into each well containing 100 µl of GSLs. Incubated trays at 35°C for 24 h and 48 h. MIC values were determined as the GSLs concentration at which 50% fungal growth was inhibited (MIC_{50}) [13].

Determination of the cell cycle and PI of *C. parapsilosis* by FCM

C. parapsilosis (ATCC22019) suspensions were treated with different doses of GSLs and were washed 3 times. Mixed the precipitates and re-suspended them with PBS. After 3 times freeze-thaw, washed the suspensions twice with PBS and adjusted to a concentration of $1\sim5 \times 10^6$ CFU/ml. Subsequently, added 1 ml propidium iodide, held the suspensions at 4°C for 12 h and 24 h, then washed away propidium iodide, filtered the samples with 300-well nylon net [14, 15]. Finally, the alteration of cell cycle and PI of *C. parapsilosis* were detected by flow cytometry (BD, FACS) [16, 17]. PI formula is as follows:

$$PI = (S + G_2/M)/(G_0/G_1 + S + G_2/M) \times 100\%.$$

The ultrastructure of *C. parapsilosis* under TEM

C. parapsilosis (ATCC22019) were incubated with GSLs or normal saline (NS) for 12 h and 24 h at 35°C. Collected the samples in due time and centrifugated at 3000 rpm for 10 min. Discarded the supernatant and washed the precipitate 3 times with PBS. Then samples were pre-fixed with 2% glutaraldehyde, fixed with 1% osmium tetroxide and underwent a gradient of dehydration with ethanol and acetone. Subsequently, embedded samples with resin and double-stained. Ultimately, observed ultra-thin sections under TEM (Hitachi H-7000, Japan) [15].

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Table 1. Effects of GSLS against genital *Candida* spp. clinical isolates ($\mu\text{g/ml}$)

Species (Number)	MIC ₅₀	
	24 h ^a	48 h
<i>C. albicans</i> (16)	< 0.49	0.98
<i>C. tropicalis</i> (4)	0.98	31.25
<i>C. krusei</i> (5)	< 0.49	1.95
<i>C. glabrata</i> (6)	1.95	15.63

^ah, MIC₅₀ s of GSLS against genital *Candida* spp. clinical isolates were determined at 24 and 48 h of incubation.

In vivo animal study

Murine vulvovaginal candidiasis model: ICR strain of female mice (8-9 weeks) were injected subcutaneously with estradiol benzoate (0.05 mg/day) for 6 days before inoculation with *C. albicans* standard strain (ATCC64548) until the end of the experiment. Mice were inoculated in the vaginas with 20 μl of a suspension containing indicated live *C. albicans* ($5 \times 10^4/\text{CFU}$), then inverted mice for 1~2 min. After 3 days and 7 days of infection, 40 μl sterile PBS was injected into the vaginas for 4~5 times, respectively. Lavage fluid was dissolved in 1 ml sterile PBS, 200 μl suspension was inoculated in the Sabouraud culture dish containing Gentamicin at 35°C for 48 h. Then CFU count was performed. When $\text{CFU} \geq 10 \times 10^3 \text{ CFU/ml}$, indicating that the model building was successful. Degree of edema and characters of secretions of the vulva in model mice were observed. Taking the vaginal lavage fluid 20 μl , 1000 rpm centrifugal for 15 s, used for sediment image microscopy. Mice were monitored daily and were killed after 3 days or 7 days of infection, The vaginal tissues were removed, and then homogenized in 0.5 ml PBS for fungal burdens measurement or fixed in 10% neutral formalin for Hematoxylin and eosin (H&E) and Periodic acid-Schiff (PAS) staining [18, 19].

Treatment of mouse model with Candida vaginitis

Successfully induced mice with vulvovaginal candidiasis, female ICR mice were randomly allocated to four different groups. Group A, oral NS 0.2 ml/day, vaginal lavage with NS 10 $\mu\text{l}/\text{day}$; group B, oral NS 0.2 ml/day containing FLC 7 mg/kg, vaginal lavage with NS 10 $\mu\text{l}/\text{day}$; group C, oral NS 0.2 ml/day containing FLC 7 mg/kg, vaginal lavage with NS 10 $\mu\text{l}/\text{day}$ con-

taining FLC 13 mg/kg; group D, oral NS 0.2 ml/day containing FLC 7 mg/kg, vaginal lavage with NS 10 $\mu\text{l}/\text{day}$ containing GSLS 50 mg/kg. After treatment for 3 days and 7 days, CFU count, H&E and PAS staining were performed.

Statistics analysis

Data were expressed as Mean \pm standard deviation (SD). All statistical analyses were performed using GraphPad Prism V6.02 (GraphPad Software, Inc) and IBM SPSS Statistics Version21 (IBM Corp., USA). One-way ANOVA with Dunnett test was employed to analyze the differences between sets of data. Statistical significance was set at a *p*-value in the figures as: *, *P* < 0.05; **, *P* < 0.01.

Results

In vitro susceptibility testing

The results of GSLS *in vitro* susceptibility testing for 24 h and 48 h showed that GSLS has activity against genital *Candida* spp. clinical isolates (**Table 1**).

Influence of GSLS on the cell cycle and PI of C. parapsilosis

The result of this study indicated that the GSLS group compared with the negative control, generally the proportion of G₀/G₁ phase increased, the proportion of *C. parapsilosis* S-G₂-M phase decreased, PI decreased (**Figure 1**). The treatment of 125 $\mu\text{g/ml}$ and 250 $\mu\text{g/ml}$ GSLS had significant inhibitory effect on *C. parapsilosis* for 12 h, S phase was markedly inhibited compared with the negative control (*P* < 0.01 and *P* < 0.01, **Figure 1A, 1C**). After 24 h, the inhibitory effect of GSLS at 62.5 $\mu\text{g/ml}$ on S phase of *C. parapsilosis* was more obvious (*P* < 0.01, **Figure 1B, 1D**) and the inhibitory effect of GSLS at 125 $\mu\text{g/ml}$ and 250 $\mu\text{g/ml}$ on S phase of *C. parapsilosis* were also significant (*P* < 0.01 and *P* < 0.05, respectively; **Figure 1B, 1D**). The proportion of S phase in GSLS groups were significantly lower than that of negative control for 12 h and 24 h (**Figure 1A-D**). Additionally, PI of *C. parapsilosis* decreased with the increase of GSLS concentration (**Figure 1E**). The treatment of 125 $\mu\text{g/ml}$ and 250 $\mu\text{g/ml}$ GSLS for 12 h and 24 h, PI values were significantly lower compared to that of the negative control, respec-

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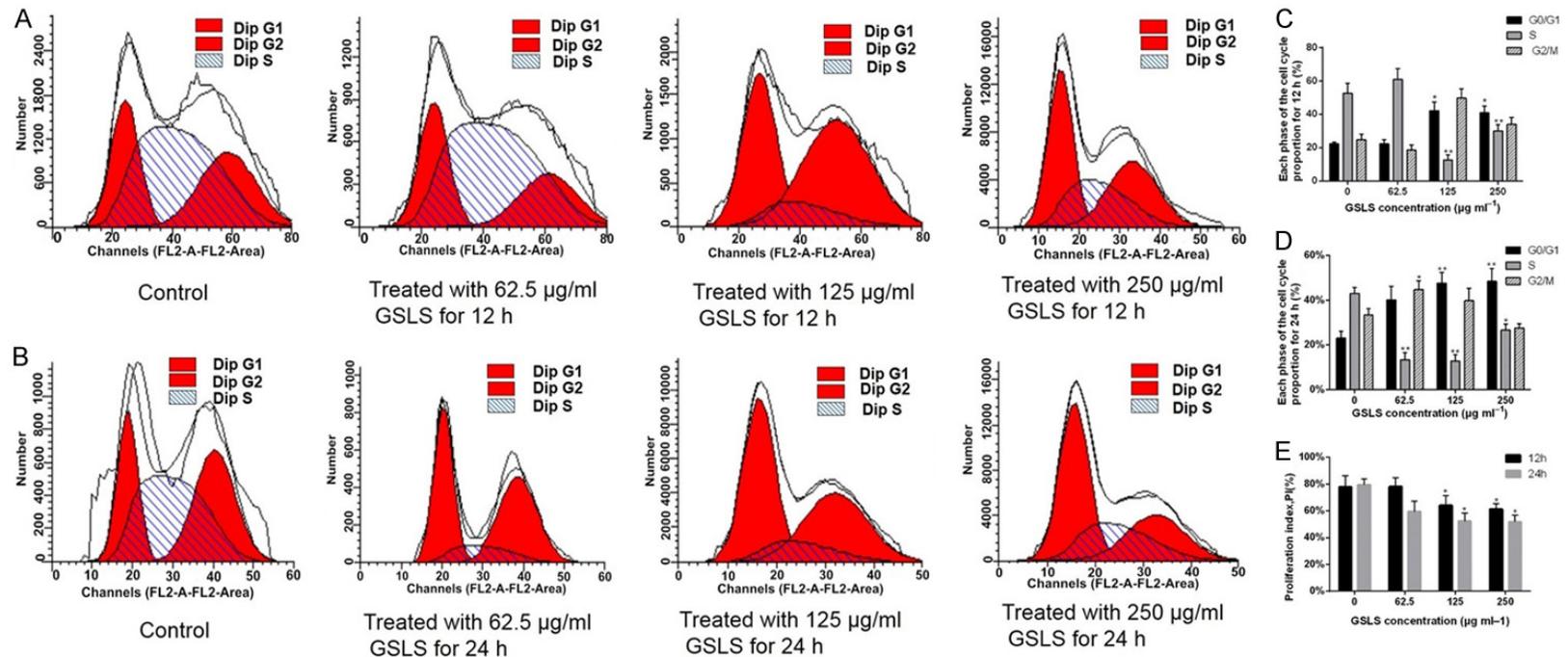


Figure 1. Influences of GSLS on the cell cycle of *C. parapsilosis*. A. Effect of different GSLS concentrations on the cell cycle of *C. parapsilosis* for 12 h; B. Effect of different GSLS concentrations on the cell cycle of *C. parapsilosis* for 24 h; C. Quantitative analysis of the influence of GSLS on the cell cycle of *C. parapsilosis* for 12 h ($\bar{x} \pm s$); D. Quantitative analysis of the influence of GSLS on the cell cycle of *C. parapsilosis* for 24 h ($\bar{x} \pm s$); *C. parapsilosis* fungi suspensions were treated with different doses of GSLS and incubated at 35 °C for the corresponding period. The changes of cell cycle of *C. parapsilosis* were detected by FCM. E. PI value of *C. parapsilosis* after exposure to 62.5, 125 and 250 µg/ml GSLS for 12 h and 24 h, respectively, the group with no treatment of agent was set as control. Compared to the control, *, $P < 0.05$; **, $P < 0.01$.

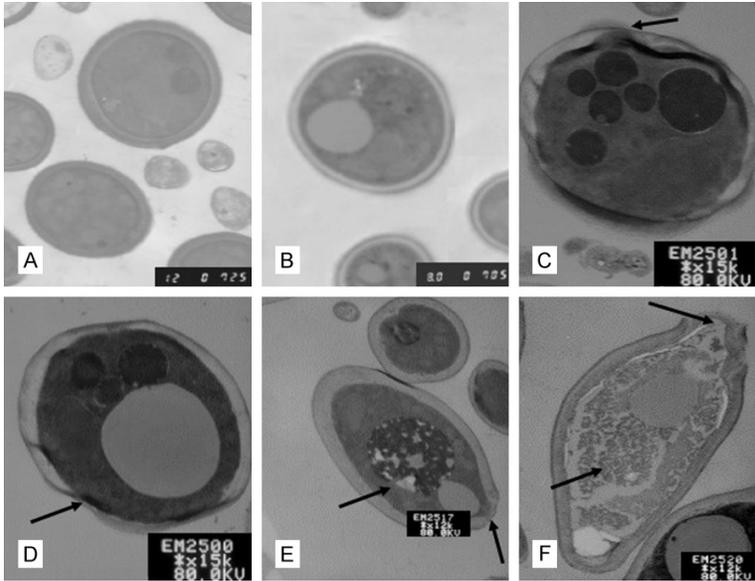


Figure 2. Influences of GSLs on the ultrastructure of *C. parapsilosis* were observed by TEM. (A) Treated with NS ($\times 20000$, 12 h); (B) Treated with NS ($\times 20,000$, 24 h); (C) Treated with 62.5 $\mu\text{g}/\text{ml}$ GSLs ($\times 20000$, 12 h); (D) Treated with 125 $\mu\text{g}/\text{ml}$ GSLs ($\times 20000$, 12 h); Arrows indicate a slight shrinkage in the wall and membrane of *C. parapsilosis* in (C and D); (E) Treated with 62.5 $\mu\text{g}/\text{ml}$ GSLs ($\times 20,000$, 24 h). Arrows indicate the damaged wall, non-homogenous cytoplasm, bubble formation and patchy areas with low electron density; (F) Treated with 125 $\mu\text{g}/\text{ml}$ GSLs ($\times 20,000$, 24 h). Arrows indicate extensive chromatin condensation, nuclear fragments, dissolved organelles and broken cytoplasm.

tively ($P < 0.05$, $P < 0.05$, $P < 0.05$, $P < 0.05$, respectively; **Figure 1E**). These suggested GSLs could inhibit the synthesis of *C. parapsilosis* DNA, suppress cellular division, regulate cell cycle and inhibit the proliferation activity of *C. parapsilosis*.

Effects of GSLs on the ultrastructure of *C. parapsilosis*

In control group, incubation with NS for 12 h, *C. parapsilosis* was round or oval with intact wall and membrane, an intact nuclear membrane, nucleus and nucleoli could be clearly observed, (**Figure 2A**). After 24 h incubation, no obvious changes were found in the ultrastructure of *C. parapsilosis* (**Figure 2B**). Treated with 62.5 $\mu\text{g}/\text{ml}$ and 125 $\mu\text{g}/\text{ml}$ GSLs for 12 h (**Figure 2C, 2D**), a slight shrinkage in the wall and membrane of *C. parapsilosis* was observed, intracellular lipid droplets increased significantly, vacuoles increased, and its nucleus was intact, clear but irregular. Up to 24 h incubation with GSLs at 62.5 $\mu\text{g}/\text{ml}$, the wall was damaged, the cytoplasm was non-homogenous, bubble was formation and patchy areas with low elec-

tron density were exhibited in *C. parapsilosis* (**Figure 2E**). The cells exposed to 125 $\mu\text{g}/\text{ml}$ GSLs for 24 h showed the wall border of *C. parapsilosis* was blurred and dissolved, extensive chromatin condensation, nuclear fragments, dissolved organelles and broken cytoplasm (**Figure 2F**).

Murine vulvovaginal candidiasis model

After inoculation of *C. albicans* in mice for 3 days, some hyphae and spores could be seen by vaginal lavage fluid smear fungal microscopic examination (**Figure 3A**). At day 7 post-infection, massive hyphae and spores were discovered (**Figure 3B**). Obvious swelling of the vulva, milk white paste secretions increased significantly. Moreover, with prolonged infections, HE staining revealed that inflammatory influx of vaginal tissues were aggravated in mice (**Figure 4A**).

PAS staining also identified a large number of hyphae and spores (**Figure 4C**).

Effects of GSLs combined with FLC in the treatment of *Candida vaginitis* in mice

After treatment for 3 days, vaginal lavage fluid smear microscopic examination in group A is basically the same as before administration; group B, C (**Figure 3C**) and D showed a small amount of fungal pseudohyphae, blastospores and a large number of vaginal epithelial cells. At day 7 post-medication, more pseudohyphae and blastospores, a large number of vaginal epithelial cells and milk white paste secretions can be observed in group A; group B, C and D indicated that a small number of vaginal epithelial cells, no fungal pseudohyphae and blastospores were found. HE staining suggested a small number of inflammation cells infiltration in the dermis of vaginas. No inflammatory influx of the superficial layer of the vaginal wall was observed (**Figure 4B**). PAS staining showed no hyphae and spores (**Figure 4D**). In addition, the results of vaginal CFU count in mice confirmed the effect of GSLs combined with FLC could

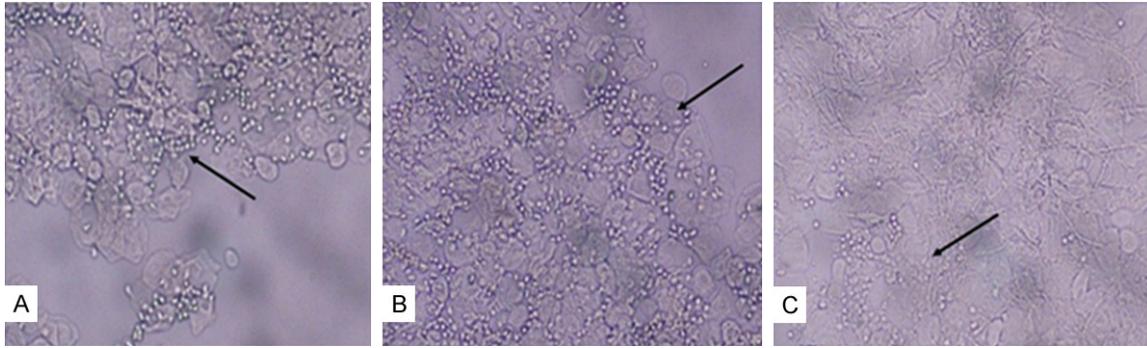


Figure 3. Mice vaginal lavage smear in group C (A. Mice was inoculated with *C. albicans* for 3 days; B. Mice was inoculated with *C. albicans* for 7 days; C. Mice medication for 3 days). Arrows indicate hyphae and spores.

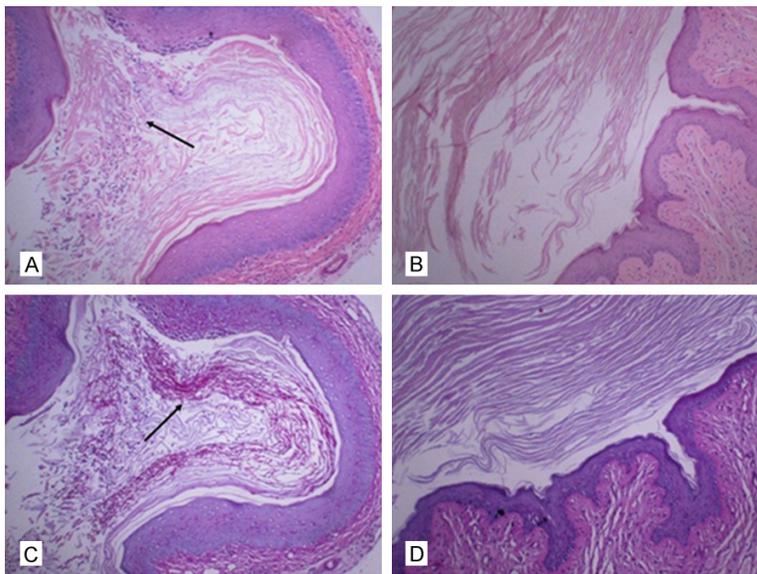


Figure 4. The representative pathological changes of vaginal tissue section of group C. (A) Group C was inoculated with *C. albicans* for 7 days. Arrow indicate inflammatory cells influx; (B) Group C received medication for 7 days. (HE staining in A and B, Magnification = 100 ×); (C) Group C was inoculated with *C. albicans* for 7 days. Arrow indicate *C. albicans* filaments in the tissues; (D) Group C received medication for 7 days. (PAS staining in C and D, Magnification = 100 ×).

against *Candida* vaginitis. No significant difference in the levels of fungal burden of vaginas was observed between group C (FLC + FLC) and group D (FLC + GSLS) ($P > 0.05$, **Figure 5**). Fungal burdens in the vaginas of group D significantly lower as compared to group A (NS + NS) and B (FLC + NS) ($P < 0.01$ and $P < 0.05$, respectively; **Figure 5**).

Discussion

Genital candidiasis is one of the most common diseases of reproductive tract infection [20].

Recently, researches have indicated that the proportion of *C. glabrata* increased continuously in VVC [21, 22]. Richer *et al.* [23] studies also found that the infection rate of non-*albicans* species in the culture of vaginal secretion of RVVC patients was as high as 42%.

In vitro susceptibility test demonstrated that GSLS has antifungal activity against genital *Candida* including *C. albicans* and non-*albicans Candida* species. It has been reported that *C. krusei* isolates were resistant to FLC and the resistant rate was 100% [2]. However, the 24- and 48-hours MIC₅₀ of GSLS against *C. krusei* were $< 0.49 \mu\text{g/ml}$ and $1.95 \mu\text{g/ml}$, respectively in our study. It means that GSLS could be a potential candidate to fight

against *C. krusei*-related infections. Subsequently, we found that GSLS could inhibit the synthesis of *Candida* DNA, suppress cellular division, regulate cell cycle and inhibit the proliferation activity of *Candida* by FCM. Observed from TEM, GSLS could destroy the ultrastructural of *Candida*. We speculated that the antifungal mechanism of GSLS was similar to that of most antifungal drugs, which acted on the cell membrane or cell wall of fungi. Destroyed selective permeability of cell membrane, decreased intracellular osmotic pressure, resulting in the cell content leakage.

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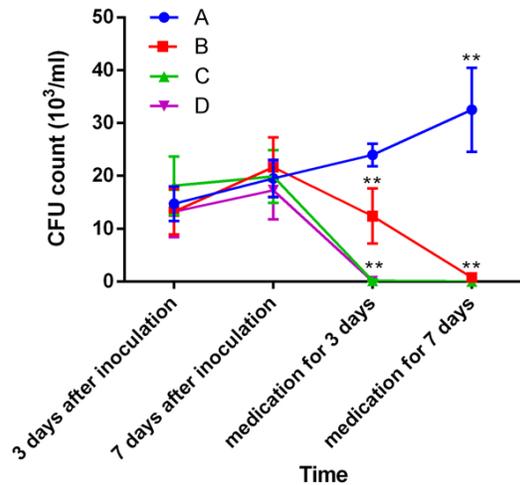


Figure 5. CFU count of mice vaginal lavage fluid at different times. A. Oral NS 0.2 ml/day + vaginal lavage with NS 10 μ l/day (n = 8 per group), compared with group D, **, $P < 0.01$; B. Oral NS 0.2 ml/day containing FLC 7 mg/kg + vaginal lavage with NS 10 μ l/day (n = 8 per group), compared with group D, *, $P < 0.05$; C. Oral NS 0.2 ml/day containing FLC 7 mg/kg + vaginal lavage with NS 10 μ l/day containing FLC 13 mg/kg (n = 8 per group); D. Oral NS 0.2 ml/day containing FLC 7 mg/kg + vaginal lavage with NS 10 μ l/day containing GSLS 50 mg/kg (n = 8 per group). CFU count were detected on the vaginal smear at the 3rd day and the 7th day after inoculation with *C. albicans* and after treatment for 3 days and 7 days, respectively.

Disturbed their normal metabolism. The cell death was promoted, and the purpose of inhibiting fungi was achieved. However, we found out that GSLS had the function of inhibiting DNA synthesis and cell division by FCM. Therefore, it can not be ruled out GSLS can directly destroy the nuclear DNA, and then affect the structure and function of the cell organelles, disrupting its normal metabolism, resulting in cell death. So, it is necessary to further study the effect of GSLS on the target site of *Candida*.

The morphological and physiological changes were remarkable after GSLS treatment in the present study, including the alteration of the cell cycle of *C. parapsilosis*, the proportion of G_0/G_1 phase cells increased, the proportion of S- G_2 -M phase cells decreased, PI decreased, extensive chromatin condensation, nuclear fragmentation, and abundant cell sap released. These changes suggested that GSLS could inhibit the synthesis of DNA of *C. parapsilosis*. Recently, Yang [15] *et al.* studies exhibited that baicalin could inhibit the activities of Ca^{2+} - Mg^{2+}

ATPase and SDH, increase cytosolic Ca^{2+} content, and hasten apoptosis rate. Moreover, study performed by Wang [24] *et al.* indicated that baicalin may induce cell death via apoptosis to inhibit the development of *C. albicans* bio-films. Compared with these studies, our work mainly focused on the alteration of the cell cycle of *C. parapsilosis*, and used varied experimental methods and parameters to survey antifungal activity of GSLS. In particular, we carried out animal experiment of GSLS combined with FLC in the treatment of *C. albicans* vaginitis in mice. From the results of these works, it was evident to observe that GSLS could enhance the activity of FLC against genital candidiasis in mice.

Actually, previous studies have reported that some TCM do not have strong antifungal activities (e.g. baicalein [25], gallic acid [26], matrine [27]), but usually owned favourable in vivo antifungal functions (e.g. sodium houttuynonate [28], Panax Notoginseng Saponins and GSLS [11]). Our findings are consistent with previous studies performed by Keyal Uma [11] *et al.* that FLC used in combination with GSLS is effective against resistant strains of *C. albicans* in vitro. The antifungal activity presented here was relatively strong. Although the combination therapy has become a common clinical practice, as it could significantly improve the antifungal efficacy of each drug compared to each drug used alone and reduce adverse side effects, before widely used in the clinic, we still need for further screening of such combinations [11, 29].

In conclusion, this study demonstrated the activity of GSLS combination with FLC in vivo against genital *Candida*, and the inhibition synthesis of DNA of *Candida*. Therefore, GSLS might be a drug of choice in the treatment of genital Candidiasis infections.

Acknowledgements

This work was supported by the Natural Science Foundation of Science and Technology Commission of Shanghai Municipality (No. 17ZR-1426300); the Natural Science Foundation of Science and Technology Commission of Shanghai Municipality (No. 13ZR1437900); the National Natural Science Foundation of China (No. 81671989); the National Natural Science Foundation of China (No. 81601745).

Disclosure of conflict of interest

None.

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