

## Antifungal activities and action mechanisms of compounds from *Tribulus terrestris* L.

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### Abstract

Antifungal activity of natural products is being studied widely. Saponins are known to be antifungal and antibacterial. We used bioassay-guided fractionation to have isolated eight steroid saponins from *Tribulus terrestris* L., which were identified as hecogenin-3-*O*- $\beta$ -D-glucopyranosyl (1  $\rightarrow$  4)- $\beta$ -D-galactopyranoside (TTS-8), tigogenin-3-*O*- $\beta$ -D-glucopyranosyl (1  $\rightarrow$  4)- $\beta$ -D-galactopyranoside (TTS-9), hecogenin-3-*O*- $\beta$ -D-glucopyranosyl (1  $\rightarrow$  2)- $\beta$ -D-glucopyranosyl (1  $\rightarrow$  4)- $\beta$ -D-galactopyranoside (TTS-10), hecogenin-3-*O*- $\beta$ -D-xylopyranosyl (1  $\rightarrow$  3)- $\beta$ -D-glucopyranosyl (1  $\rightarrow$  4)- $\beta$ -D-galactopyranoside (TTS-11), tigogenin-3-*O*- $\beta$ -D-xylopyranosyl (1  $\rightarrow$  2)- $\beta$ -D-xylopyranosyl (1  $\rightarrow$  3)- $\beta$ -D-glucopyranosyl (1  $\rightarrow$  4)-[ $\alpha$ -L-rhamnopyranosyl (1  $\rightarrow$  2)]- $\beta$ -D-galactopyranoside (TTS-12), 3-*O*-{ $\beta$ -D-xylopyranosyl (1  $\rightarrow$  2)-[ $\beta$ -D-xylopyranosyl (1  $\rightarrow$  3)]- $\beta$ -D-glucopyranosyl (1  $\rightarrow$  4)-[ $\alpha$ -L-rhamnopyranosyl (1  $\rightarrow$  2)]- $\beta$ -D-galactopyranosyl}-26-*O*- $\beta$ -D-glucopyranosyl-22-methoxy-(3 $\beta$ ,5 $\alpha$ ,25R)-furostan-3,26-diol (TTS-13), hecogenin-3-*O*- $\beta$ -D-glucopyranosyl (1  $\rightarrow$  2)-[ $\beta$ -D-xylopyranosyl (1  $\rightarrow$  3)]- $\beta$ -D-glucopyranosyl (1  $\rightarrow$  4)- $\beta$ -D-galactopyranoside (TTS-14), tigogenin-3-*O*- $\beta$ -D-glucopyranosyl (1  $\rightarrow$  2)-[ $\beta$ -D-xylopyranosyl (1  $\rightarrow$  3)]- $\beta$ -D-glucopyranosyl (1  $\rightarrow$  4)- $\beta$ -D-galactopyranoside (TTS-15). The in vitro antifungal activities of the eight saponins against five yeasts, *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Cryptococcus neoformans* were studied using microbroth dilution assay. In vivo activity of TTS-12 in a *Candida albicans* vaginal infection model was studied in particular. The results showed that TTS-12 and TTS-15 were very effective against several pathogenic candidal species and *Cryptococcus neoformans* in vitro. It is noteworthy that TTS-12 and TTS-15 were very active against *Candida albicans* (MIC<sub>80</sub> = 10 and 2.3  $\mu$ g/mL) and *Cryptococcus neoformans* (MIC<sub>80</sub> = 1.7 and 6.7  $\mu$ g/mL). Phase contrast microscopy showed that TTS-12 inhibited hyphal formation, an important virulence factor of *Candida albicans*, and transmission electron microscopy showed that TTS-12 destroyed the cell membrane of *Candida albicans*. In conclusion, TTS-12 has significant in vitro and in vivo antifungal activity, weakening the virulence of *Candida albicans* and killing fungi through destroying the cell membrane.

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**Keywords:** *Tribulus terrestris* L.; Saponins; Antifungal; *Candida albicans*

### 1. Introduction

In recent 20 years, the risk of opportunistic fungal infections has greatly increased in patients who are severely immunocompromised due to cancer chemotherapy, organ or bone marrow transplantation and human immunodeficiency virus infection (Wingard et al., 1979, 1991, 1993). *Candida albicans* is an organism that is most often associated with serious fungal infections, and can cause fungal diseases in immunocompromised patients, including cancer patients,

**Abbreviations:** MICs, minimal inhibitory concentrations; TEM, transmission electronic microscope

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organ transplant patients, and those with human immunodeficiency virus infections (Fridkin and Jarvis, 1996). Candidal vaginitis is predominantly caused by strains of *Candida albicans* (90%) (Sobel et al., 1995, 1998a,b, 2001), and remains to be a common problem in immunocompetent or healthy women.

Despite advances in antifungal therapies, many problems remain to be solved for most antifungal drugs available. For example, the use of amphotericin B, known as the “gold standard”, is limited because of its infusion-related reactions and nephrotoxicity (Grasela et al., 1990; Fanos and Cataldi, 2000). The use of azoles, such as fluconazole, ketoconazole and miconazole, has resulted in clinically resistant strains of *Candida* spp. (Lyman and Walsh, 1992; Sojakova et al., 2004). A 3.6–7.2% of vaginal isolates of *Candida albicans* from women with candidal vaginitis is resistant to fluconazole (Sobel et al., 2003). This situation highlights the need for advent of safe, novel and effective antifungal compounds.

Plants provide abundant resources of antimicrobial compounds and have been used for centuries to inhibit microbial growth. *Tribulus terrestris* L. (Zygophyllaceae) is an annual creeping herb widely growing in China. It is also distributed in Japan, Korea, western Asia, southern Europe and Africa. In traditional Chinese pharmaceuticals, *Tribulus terrestris* L. is used for treating cutaneous pruritus, edema, inflammation and tracheitis (Jiangsu New Medical College, 1977). In our previous study, we isolated and identified 10 compounds from *Tribulus terrestris* L. (Xu et al., 2000). In the present report, eight of the 10 saponins were tested to investigate their antifungal properties, especially against *Candida albicans*.

## 2. Materials and methods

### 2.1. Plant materials

*Tribulus terrestris* L. was collected from Henan Province, China. It was identified by Prof. H.C. Zheng of the Department of Pharmacognosy, College of Pharmacy of the Second Military Medical University, Shanghai. A voucher specimen (950812) is available in the herbarium of this Department.

### 2.2. Extraction and purification

The air-dried and powdered plant (10.7 kg) was extracted three times with an excess of 80% EtOH at room temperature. After removal of the solvent by evaporation, the residue was extracted with petrol,  $\text{CHCl}_3$  and *n*-butanol. Our previous preliminary study on *Candida albicans* showed that the *n*-butanol extract using the macrobroth dilution method had antifungal activity. The *n*-butanol layer was chromatographed over a macroporous resin column (10 cm × 50 cm, 2 kg), and first eluted successively with water and then with 50%, 70% and 90% EtOH. The four fractions were separated by a combination of chromatography over silica gel, reversed phase RP-18 chromatography, sephadex G-25 and HPLC to yield pure compounds TTS-8 (33 mg), TTS-9 (21 mg), TTS-10 (46 mg), TTS-11 (27 mg), TTS-12 (1 g), TTS-13 (72 mg), TTS-14 (43 mg), TTS-15 (52 mg), TTS-16 (56 mg) and TTS-18 (42 mg).

### 2.3. Organisms used

A total of 69 American Type Culture Collection (ATCC) and clinical isolates of *Candida* species and *Cryptococcus neoformans* obtained from different hospitals in China, or commercially, or kindly donated were tested (Table 1). The collection included the following numbers of isolates: 51 isolates of *Candida albicans* (ATCC76625, ATCC64550 isolates and 49 clinical isolates), 4 isolates of *Candida tropicalis* (4 clinical isolates), 5 isolates of *Candida glabrata* (ATCC11006 isolate and 4 clinical isolates), 5 isolates of *Candida parapsilosis* (ATCC18062 isolates and 4 clinical isolates) and 3 isolates of *Cryptococcus neoformans* (ATCC32609 isolate and 2 clinical isolates). All isolates were identified by Shanghai Changhai Hospital. The isolates were stored as water suspensions until use. Prior to test, each isolate was passaged on potato dextrose agar (Sangon, Shanghai, China) to ensure purity and viability.

*Candida albicans* SC5314, a strain most often used in the study of virulence and genetics of *Candida albicans*, was kindly donated by White TC from the University of Washington, and Spencer Redding from the University of Texas Health Science Center at San Antonio.

Table 1  
In vitro MIC<sub>80</sub> values of eight compounds from *Tribulus terrestris* L. against different yeasts

No.	Compound	<i>Candida albicans</i>	<i>Candida glabrata</i>	<i>Candida parapsilosis</i>	<i>Candida tropicalis</i>	<i>Cryptococcus neoformans</i>
1	TTS-8	>128.0	>128.0	>128.0	>128.0	>128.0
2	TTS-9	>128.0	>128.0	>128.0	>128.0	>128.0
3	TTS-10	>128.0	>128.0	>128.0	>128.0	>128.0
4	TTS-11	>128.0	>128.0	>128.0	>128.0	>128.0
5	TTS-12	1.0 ± 0.6	8.8 ± 4.4	45.3 ± 21.3	21.3 ± 8.3	1.7 ± 0.6
6	TTS-13	>128.0	>128.0	>128.0	>128.0	>128.0
7	TTS-14	41.7 ± 20.5	57.6 ± 41.7	>128.0	>128.0	48.0 ± 27.7
8	TTS-15	2.3 ± 1.0	19.2 ± 12.1	74.7 ± 26.1	106.7 ± 33.0	6.7 ± 2.3
9	FLC	1.3 ± 0.7	1.4 ± 0.5	5.2 ± 3.3	1.5 ± 0.6	1.33 ± 0.58
10	AMB	0.29 ± 0.15	0.28 ± 0.21	0.75 ± 0.27	0.63 ± 0.25	0.21 ± 0.07
11	ICZ	0.12 ± 0.06	0.35 ± 0.14	0.38 ± 0.14	0.38 ± 0.14	0.17 ± 0.07

#### 2.4. Media

All strains used in this study were grown in two complete media consisting of a YEPD liquid medium (1% Bacto Peptone [Difco, USA], 0.5% yeast extract [Difco], 2% glucose [Sangon]), and a solid medium prepared by adding 2% agar (Sangon).

#### 2.5. Laboratory animals

Forty female Sprague–Dawley (SD) rats weighing 100–120 g (Center of Experimental Animals, Second Military Medical University, Shanghai, China) were used for the study of vaginal infections with *Candida albicans*. This experiment was approved by the Bioethic Committee of the Second Military Medical University, and the procedures of the experiment were strictly according to generally accepted international rules and regulations.

#### 2.6. Antifungal susceptibility test

The in vitro minimal inhibitory concentrations (MICs) of the compounds were determined by the micro-broth dilution method according to the methods defined by the National Committee for Clinical Laboratory Standards (NCCLS, 2002). *Candida krusei* (ATCC6258) and *Candida parapsilosis* (ATCC22019) were quality controlled strains, and tested in each assay. Fluconazole (FLC), itraconazole (ICZ) and amphotericin B (AMB) obtained from their respective manufacturers served as the positive control. The drug MIC<sub>80</sub> was defined as the first well with an approximate 80% reduction in growth compared to the growth of the drug-free well.

The eight compounds to be tested were dissolved in dimethyl sulfoxide (DMSO), and the stock solutions of the serial two-fold dilutions were prepared in RPMI 1640 medium (Gibco, USA) with the final concentrations between 128.0 and 0.25 µg/mL (111.30–0.220 µmol/L), and the final concentrations of FLC, ICZ and AMB were 64.0–0.125 µg/mL (209.15–0.410 µmol/L), 2.0–0.004 µg/mL (2.83–0.006 µmol/L) and 2.0–0.004 µg/mL (2.16–0.004 µmol/L), respectively, depending on the MIC results from our preliminary study.

#### 2.7. Growth curve study

The effect of TTS-12 and TTS-15 exposure in relation to time and concentration on *Candida albicans* SC5314 was determined in YEPD liquid medium. TTS-12 and TTS-15 solutions (in DMSO) were added to the cultures to form an optical density of 0.1 (measured at a wavelength of 600 nm), the final concentrations of which were 0, 2, 4, 8 or 16 µg/mL (0, 1.74, 3.48, 6.96, 13.91 µmol/L). The growth was monitored by measuring the optical density (600 nm) of the cultures during the subsequent 48 h.

#### 2.8. Vaginal infection model with *Candida albicans*

The vaginal infection animal model was established based on modified models previously described by Sobel et al. (1998) to obtain a more chronic and homogeneous infection. Briefly, 40 female animals were ovariectomized, and estrus was induced with subcutaneous administration of estradiol at a dose of 10 mg/kg 3 days before infection and maintained by subcutaneous estradiol at a dose of 4 mg/kg weekly throughout the experiment. *Candida albicans* was inoculated intravaginally with 10<sup>7</sup> yeast cells per 0.1 mL of sterile saline and 0.1 mL per rat. Inoculation was performed using a micropipette with disposable tips. The 32 infected animals were equally randomized into four groups: Group 1, control; Group 2, miconazole (MCZ, 30 mg/kg); Group 3, TTS-12 (30 mg/kg); Group 4, TTS-12 (60 mg/kg). MCZ was served as the positive control. The vaginal *Candida albicans* load was evaluated at day 3 post-infection, and day 3, 7, 14 after initiation of drug administration. TTS-12 or MCZ was administered to the infection animals for 14 consecutive days.

#### 2.9. Hyphal induction

*Candida albicans* SC5314 cells were induced to form hyphae in medium 199 (10 × M199, Gibco). The medium was pre-warmed to 37 °C. The cells from a 48 h stationary-phase culture were transferred to 5 mL of 1 × M199 to a final concentration of 3 × 10<sup>6</sup> cells/mL, and TTS-12 solution was added to the growth medium to final concentrations of 0, 4 and 16 µg/mL (0, 3.48 and 13.91 µmol/L), and the cultures were incubated for 6 h at 37 °C, 5% CO<sub>2</sub>. The hyphal formation of *Candida albicans* SC5314 was seen with an inverted phase contrast microscope with the magnification of 400. FLC was used as a positive control with the final concentration of 4 µg/mL.

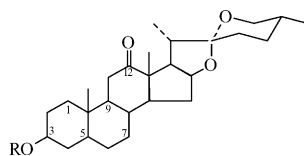
#### 2.10. Ultrastructure analysis by transmission electron microscopy

Transmission electronic microscopy was performed to observe the effect of TTS-12 on cell ultrastructure. *Candida albicans* SC5314 cells (1 × 10<sup>8</sup> cells/mL) were collected after being treated with TTS-12 at 8 µg/mL for 16 h, washed twice with PBS solution, centrifuged for 10 min at 3000 rpm, fixed in 2% glutaraldehyde at 4 °C for 72 h, and then placed in 1% phosphotungstic acid. The cells were desiccated with gradients, and embedded with EPON-812. Ultrathin sections were prepared and observed after double staining with uranium and plumbum under a transmission electron microscope (HITACHI H-800, Japan) with 2 × 10<sup>4</sup> magnification. At the same time, the untreated cells were used as control, and FLC (8 µg/mL) was served as the positive control.

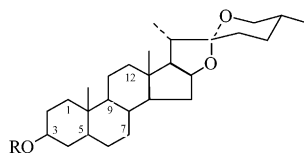
### 3. Results

#### 3.1. Identification of 10 compounds

Identification of the 10 compounds showed that they were hecogenin-3-*O*-β-D-glucopyranosyl (1 → 4)-β-D-galactopyranoside (TTS-8), tigogenin-3-*O*-β-D-glucopyranosyl (1 → 4)-β-D-galactopyranoside (TTS-9), hecogenin-3-*O*-β-D-glucopyranosyl (1 → 2)-β-D-glucopyranosyl (1 → 4)-β-D-galactopyranoside (TTS-10), hecogenin-3-*O*-β-D-xylopyranosyl (1 → 3)-β-D-glucopyranosyl (1 → 4)-β-D-galactopyranoside (TTS-11), tigogenin-3-*O*-β-D-xylopyranosyl (1 → 2)-[β-D-xylopyranosyl (1 → 3)]-β-D-glucopyranosyl (1 → 4)-[α-L-rhamnopyranosyl (1 → 2)]-β-D-galactopyranoside (TTS-12), 3-*O*-[β-D-xylopyranosyl (1 → 2)-[β-D-xylopyranosyl (1 → 3)]-β-D-glucopyranosyl (1 → 4)-[α-L-rhamnopyranosyl (1 → 2)]-β-D-galactopyranosyl]-26-*O*-β-D-glucopyranosyl-22-methoxy-(3β,5α,25*R*)-furostan-3,26-diol (TTS-13), hecogenin-3-*O*-β-D-glucopyranosyl (1 → 2)-[β-D-xylopyranosyl (1 → 3)]-β-D-glucopyranosyl (1 → 4)-β-D-galactopyranoside (TTS-14), tigogenin-3-*O*-β-D-glucopyranosyl (1 → 2)-[β-D-xylopyranosyl (1 → 3)]-β-D-glucopyranosyl (1 → 4)-β-D-galactopyranoside (TTS-15), 2*S*,3*S*,4*S*,5*S*-hexitol (TTS-16), inorganic salt (a mixture of NaNO<sub>3</sub>, KNO<sub>3</sub> and K<sub>2</sub>NO<sub>2</sub>F·HF in a ratio of 46.1:36.4:17.4) (TTS-18). The chemical structures of the eight compounds were shown in Fig. 1.



1. TTS-8: R=-| $\hat{A}$ -D-gal<sup>4</sup>-| $\hat{A}$ -D-glu
3. TTS-10: R=-| $\hat{A}$ -D-gal<sup>4</sup>-| $\hat{A}$ -D-glu<sup>2</sup>-| $\hat{A}$ -D-glu
4. TTS-11: R=-| $\hat{A}$ -D-gal<sup>4</sup>-| $\hat{A}$ -D-glu<sup>3</sup>-| $\hat{A}$ -D-xy
7. TTS-14: R=-| $\hat{A}$ -D-gal<sup>4</sup>-| $\hat{A}$ -D-glu<sup>2</sup>-| $\hat{A}$ -D-glu<sup>3</sup>-| $\hat{A}$ -D-xy



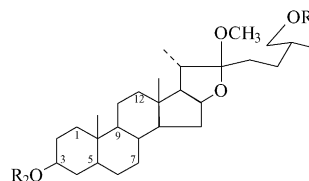
2. TTS-9: R=-| $\hat{A}$ -D-gal<sup>4</sup>-| $\hat{A}$ -D-glu
5. TTS-12: R=-| $\hat{A}$ -D-gal<sup>4</sup>-| $\hat{A}$ -D-glu<sup>2</sup>-| $\hat{A}$ -D-xy  
| $\hat{A}$ -L-rha | $\hat{A}$ -D-xy
8. TTS-15: R=-| $\hat{A}$ -D-gal<sup>4</sup>-| $\hat{A}$ -D-glu<sup>2</sup>-| $\hat{A}$ -D-glu<sup>3</sup>-| $\hat{A}$ -D-xy

#### 3.2. Antifungal susceptibility results

Of the 10 compounds isolated from *Tribulus terrestris* L., 8 compounds were identified as steroid saponins, the in vitro activity of which were evaluated against five human pathogenic yeasts (*Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata*, *Cryptococcus neoformans*) which are often encountered clinically. The results were showed in Table 1. TTS-8, TTS-9, TTS-10, TTS-11 and TTS-13 were inactive against fungi tested, and compound TTS-14 had somewhat activities against *Candida albicans*, *Candida glabrata*, *Cryptococcus neoformans*, with MIC<sub>80</sub> values of 41.7, 57.6, 48.0 μg/mL, respectively. Especially, TTS-12 and TTS-15 had significant antifungal activities against the five yeasts tested, *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Cryptococcus neoformans*. Importantly, TTS-12 and TTS-15 clearly inhibited the growth of *Candida albicans*, and the MIC<sub>80</sub> value was determined to be 1.0 and 2.3 μg/mL, respectively. They were also very effective against *Cryptococcus neoformans* at 1.7 and 6.7 μg/mL.

#### 3.3. Growth curve

TTS-12 and TTS-15 activity showed dose and time dependency against the growth of *Candida albicans* SC5314 (Fig. 2). By 8 h post-incubation significant inhibition was



6. TTS-13: R<sub>1</sub>=-| $\hat{A}$ -D-glu  
R<sub>2</sub>=-| $\hat{A}$ -D-gal<sup>4</sup>-| $\hat{A}$ -D-glu<sup>2</sup>-| $\hat{A}$ -D-xy  
| $\hat{A}$ -L-rha | $\hat{A}$ -D-xy

Fig. 1. Chemical structures of compounds 1–8.

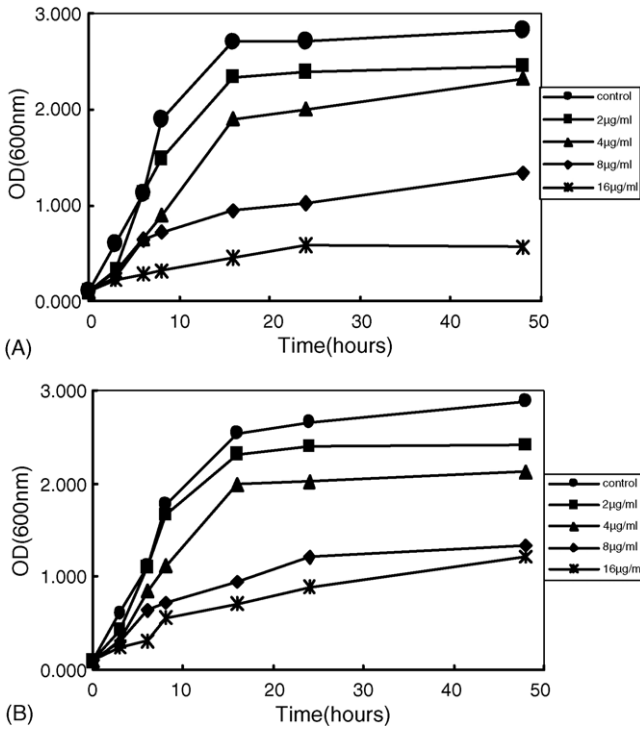


Fig. 2. The effect of TTS-12 or TTS-15 on the growth of *Candida albicans*. Yeast cells were treated with TTS-12 (0, 2, 4, 8 and 16 µg/mL) (A) or TTS-15 (0, 2, 4, 8 and 16 µg/mL) (B) in the subsequent for 48 h.

observed at concentrations as low as 8.16 µg/mL compared with the control.

3.4. The vaginal infection model

After completing the experiment with in vitro activity, we examined the activity of TTS-12 in vivo. For this purpose, an experimental vaginal infection model (oestrogen-dependent

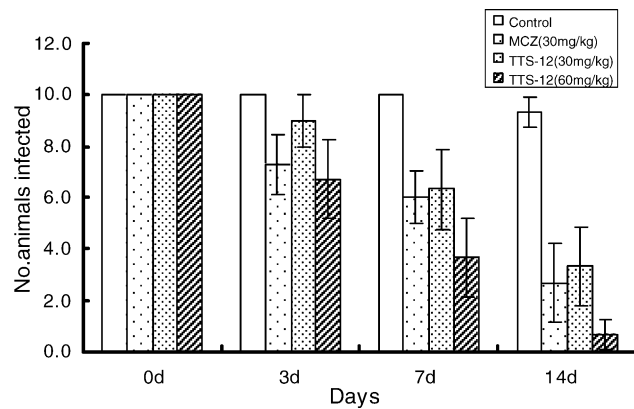


Fig. 3. The number of vaginal infection animals. The rats were infected vaginally with *Candida albicans*, and then treated with MCZ (30 mg/kg), TTS-12 (30 mg/kg) and TTS-12 (60 mg/kg) in various treatment days (0 days, 3 days, 7 days and 14 days). Significance: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to the control.

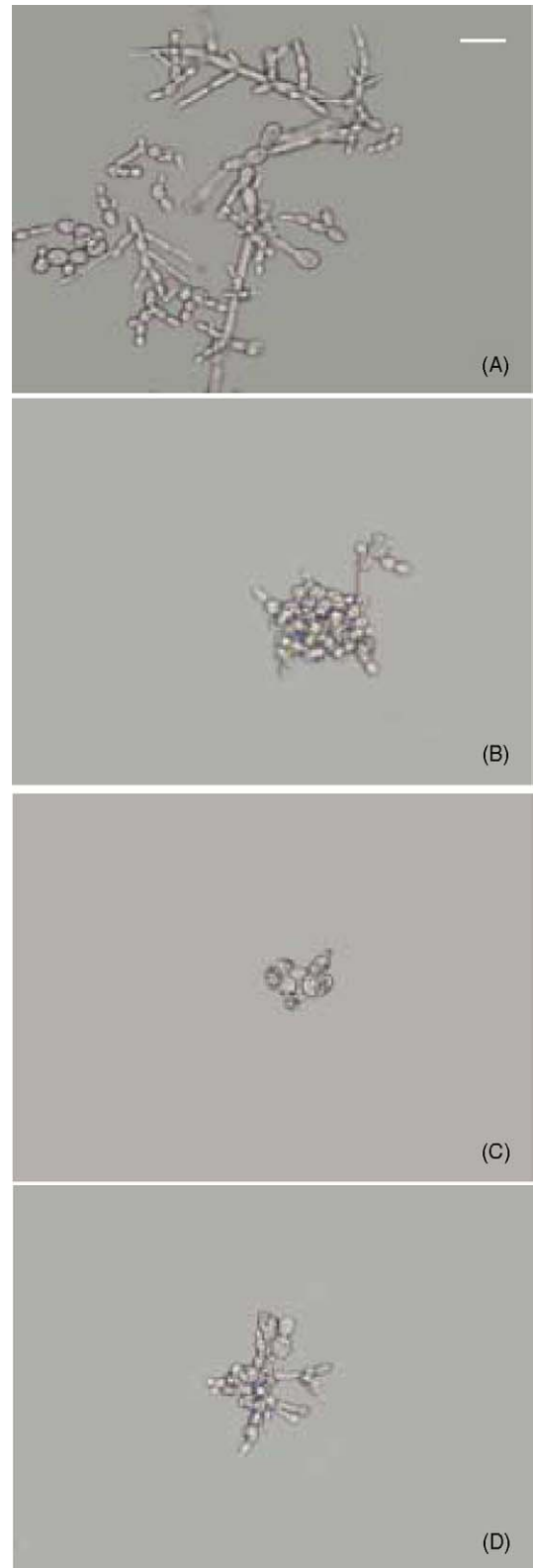


Fig. 4. Hyphalformation of *Candida albicans* cells. *Candida albicans* SC5314 cells were induced to form hyphae in medium 199, and then were treated with TTS-12 (A, control; B, 4 µg/mL; C, 16 µg/mL), and FLC (D, 4 µg/mL) as the positive control. Hyphal formation of *Candida albicans* cells was obviously inhibited by TTS-12. The white bar represents a length of 20 µm (A).

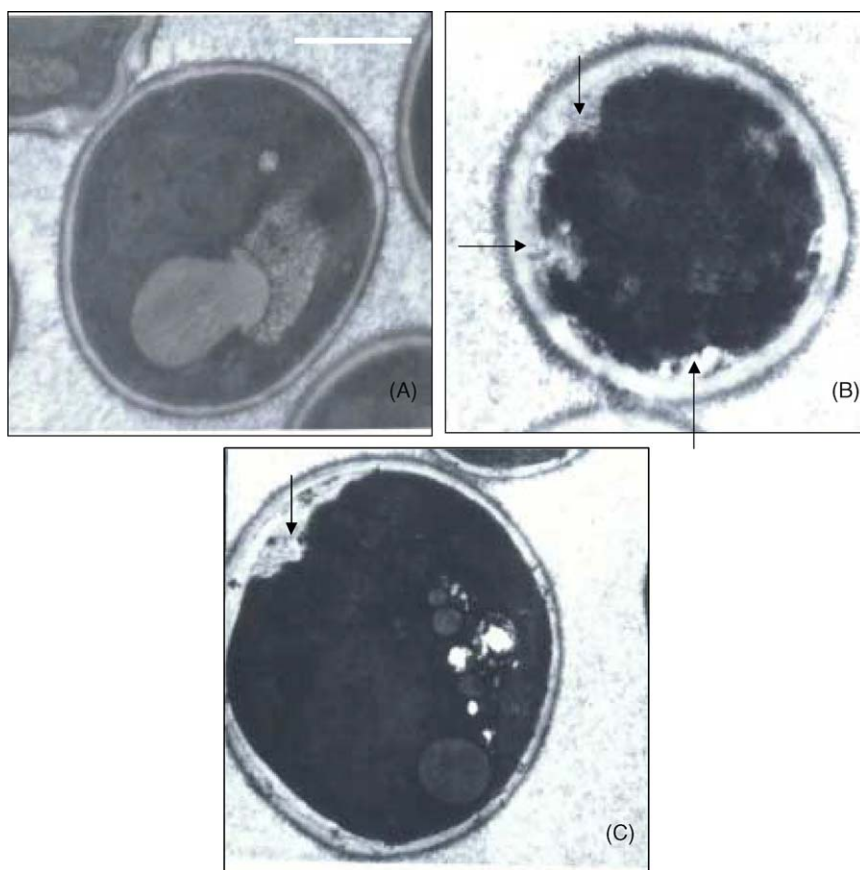


Fig. 5. Ultrastructure of *Candida albicans* cell. *Candida albicans* cells were treated with TTS-12, and were observed by transmission electron microscopy. (A) Normal ultrastructure of *Candida albicans* cell; (B) ultrastructure of *Candida albicans* treated with TTS-12 ( $2 \mu\text{g/mL}$ ); (C) ultrastructure of *Candida albicans* treated with FLC ( $8 \mu\text{g/mL}$ ) as the positive control. The cell membrane of *Candida albicans* was seriously destroyed by TTS-12 or FLC (arrows indicate destroyed cell membrane). The white bar represents a length of  $2 \mu\text{m}$  (A).

rat vaginitis) was established, where the animals were challenged with *Candida albicans* strain. All rats were infected with *Candida albicans* and assessed mycologically 3 days after infection. Fig. 3 shows that TTS-12 caused a rapid clearance of the strain from the vagina of the experimentally infected rats. In the vaginal infection model with *Candida albicans*, the number of the animals infected significantly decreased after TTS-12 was administrated at the dose of 30 and 60 mg/kg, and there was a statistically significant difference between the control and the TTS-12 treatment groups at all time-points.

### 3.5. Hyphal induction

*Candida albicans* SC5314 cells were incubated for 6 h in the presence of either DMSO (control), or 4 and  $16 \mu\text{g/mL}$  TTS-12, or  $4 \mu\text{g/mL}$  FLC, and then observed by phase contrast microscopy. In the absence of the drug, hyphal formation was observed in the isolate SC5314, while in the 4 and  $16 \mu\text{g/mL}$  TTS-12 or FLC groups, hyphal formation of *Candida albicans* was inhibited markedly (Fig. 4).

### 3.6. Ultrastructure analysis

In TEM photographs, the cell membrane and cell wall of *Candida albicans* SC5314 were clearly seen in normal *Candida albicans* (Fig. 5A). TTS-12 and FLC strongly destroyed the cell membrane of *Candida albicans* SC5314 (Fig. 5B and C).

## 4. Discussion and conclusion

In traditional Chinese Medicine, the plant *Tribulus terrestris* L. has long been used for the treatment of cutaneous pruritus, edema and inflammation, but no detailed studies concerning the related active components have been reported (Jiangsu New Medical College, 1977; Chu et al., 2003). Earlier studies showed that *Tribulus terrestris* L. contained flavanoids, steroid saponins, alkaloids and polysaccharides (Bourke et al., 1992; Wu et al., 1996; Yan et al., 1996; Li et al., 1998; Liu et al., 2003; Conrad et al., 2004). In our previous studies, we isolated from *Tribulus terrestris* L. eight steroid saponins, TTS-8, TTS-9, TTS-10, TTS-11, TTS-12, TTS-13, TTS-14 and TTS-15, but did not study their biological activ-

ities in detail (Xu et al., 2000). Many studies in the literature (Ekabo et al., 1996; Mshvildadze et al., 2000; Renault et al., 2003; Sautour et al., 2004) reported that most steroid saponins have antifungal activities. In the present study, we therefore conducted a series of experiments to investigate antifungal activities of the eight steroid saponins we had isolated previously, and did some pioneer work concerning the effects of steroid saponins on the ultrastructure and the hypha, an important factor of fungal virulence.

The results of our study showed that TTS-8, TTS-9, TTS-10, TTS-11 and TTS-13 were inactive, and TTS-14 had insignificant activities against *Candida albicans*, *Candida glabrata*, *Cryptococcus neoformans*. It is noteworthy that TTS-12 and TTS-15 had significant antifungal activities against the five yeasts tested: *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Cryptococcus neoformans*. Among these fungi, *Candida albicans* is the most common infection-causing fungus; about 45% of clinical fungal infections were caused by *Candida albicans* (Gupta et al., 2004). The present experiment showed that TTS-12 and TTS-15 had potent anti-*Candida albicans* activity, where MIC<sub>80</sub> was 1.0 and 2.3 µg/mL, respectively, by far lower than that of the saponins previously reported. According to the literature, MIC of most saponins against *Candida albicans* is greater than >50 µg/mL and that of individual saponins is between 4 and 20.8 µg/mL (Ekabo et al., 1996; Mshvildadze et al., 2000; Renault et al., 2003; Sautour et al., 2004). The studies of Renault et al. (2003) and De Lucca et al. (2002) demonstrated that CAY-1, a steroid saponin, from the ground fruit of *Capsicum frutescens*, had antifungal activity on *Candida albicans*, and the IC<sub>50</sub> and IC<sub>90</sub> were determined to be 3.8 µg/mL (3.1 µmol/L) and 7.7 µg/mL (6.2 µmol/L), respectively. Extracts of *Eriocephalus africanus* L., *Felticia erigeroides* DC and *Helichrysum crispum* (L.) D. Don inhibited the growth of *Candida albicans* (Salie et al., 1996). Prosapogenin A of dioscin exhibited antifungal activity against human pathogenic yeasts *Candida albicans* (MIC 20.8 µg/mL), *Candida glabrata* (MIC 6.25 µg/mL) and *Candida tropicalis* (MIC 25 µg/mL) (Sautour et al., 2004). TTS-12 and TTS-15 were also very active on *Cryptococcus neoformans* (MIC<sub>80</sub> = 1.7 and 6.7 µg/mL, respectively), similar to the antifungal activities of several other saponins reported with the MICs of 2–12.5 µg/mL (Ekabo et al., 1996; Mshvildadze et al., 2000). The time course study indicated that TTS-12 and TTS-15 reduced the fungal viability rapidly at a dose dependent rate (Fig. 2). Candidal vaginitis is predominantly caused by strains of *Candida albicans* (90%) (Sobel et al., 1995, 1998a,b, 2001), and remains to be a common problem in immunocompetent or healthy women. So we also observed the in vivo antifungal activity of TTS-12. According to the literature, the candidal vaginitis rat model is a stable model that can be used for study of drugs on candidal vaginitis. Our result showed that vaginal administration of TTS-12 had a marked therapeutic effect on candidal vaginitis. Above all, steroid saponin TTS-12 has marked in vitro and in vivo antifungal activities.

The steroidal glycosides tested in the experiment are from the same chemical class, but only TTS-12 and TTS-15 exhibited significant antifungal activity against *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis* and *Cryptococcus neoformans*. These results indicate that there are critical structural features that are responsible for the antifungal activity. The chemical difference between the aglycons of TTS-12, TTS-15 and compounds TTS-8, TTS-10, TTS-11, TTS-14 was the presence of a carbonyl group at C-12 for TTS-8, TTS-10, TTS-11 and TTS-14. Only TTS-14 showed some antifungal activity. Another chemical difference between TTS-12, TTS-14, TTS-15 (which connect more than four oligosaccharides) and TTS-8, TTS-9, TTS-10, TTS-11 (which connect three or fewer oligosaccharides) is the number of connecting saccharides. So the absence of a carbonyl group at C-12 and the number of connecting saccharides are probably related to the antifungal activity of compounds. That is probably the reason why TTS-9, a saponin without a carbonyl base at C-12, did not show antifungal activity. Our result also showed that TTS-13 was inactive against fungi, which confirmed the earlier observations that furostanol-type steroidal glycosides were fungally inactive (Hufford et al., 1988).

Bedir et al. (2002) also studied the antifungal activities of seven steroid saponins from *Tribulus terrestris* L., and the chemical structure of spirostanol saponin 2 (Compound 2) in their study was identical with that of TTS-14. Their results showed that four furostanol-type steroidal glycosides 4–7 (Compound 4–7) were inactive against fungi, which was coincident with our results. Without a carbonyl base at C-12, spirostanol saponin 1 (Compound 1) in their study, which connects three oligosaccharides, failed to show antifungal activity. This finding is similar to the antifungal activity of TTS-9, which connects two oligosaccharides. In addition, spirostanol saponins 2 and 3 of their study with carbonyl base at C-12 had strong antifungal activities, their MIC values against *Candida albicans* were all 6.25 µg/mL, and MIC values against *Cryptococcus neoformans* were 2.00 and 3.12 µg/mL, respectively. However, our result failed to show that TTS-14 had strong activity against *Candida albicans* and *Cryptococcus neoformans*, with MIC being 41.7 and 48.0 µg/mL, respectively. We do not know the reason causing the difference, which needs further experiments.

Based on our results and the literature, spirostanol framework and the number of oligosaccharide residue attached at C-3 of aglycon seem closely related to antifungal effects of steroid saponins, but further studies are required to confirm the relation of carbonyl base at C-12 and strong antifungal activity.

Hyphae are an important factor of fungal virulence. It is through hyphae that *Candida albicans* invades human tissues. There have been few studies reporting the effect of saponins on hyphae. The observations of phase contrast microscopy showed that TTS-12 clearly inhibited hyphal formation during the hyphal induction of *Candida albicans* (Fig. 4). *Candida albicans* is a dimorphic yeast. Its ability to switch from

yeast cells to hyphae is considered to be important for the interactions of *Candida albicans* with its host (Cutler, 1991). Hyphae are long, slender, continuous tubules with septae that separate each of the nuclei without distinct indentation at the septae. Both yeast cells and hyphae are present in the host during commensal growth and during infection. Hyphae are thought to be an important virulence factor that promotes invasion of cells into the mucosa, allowing candidal cells to resist macrophage and neutrophil engulfment (Yang, 2003).

The action mechanisms of saponins may lie in damage to the membrane and leakage of cellular materials, ultimately leading to cell death (Mshvildadze et al., 2000). This activity has been documented in a number of saponins, and the damaging effects have been shown against a variety of fungi, including *Candida albicans*, *Saccharomyces cerevisiae*, *Trichodemta viride*, *Acremonium* spp. and *Cryptococcus neoformans* (Lalitha and Venkataraman, 1991; Polacheck et al., 1991). For example, medicagenic acid 3-*O*-beta-D-glucopyranoside, an antimycotic saponin from alfalfa root, formed stable complexes with ergosterol, causing lethal leakage of ions out of yeast cells (Polacheck et al., 1991). The present study clearly revealed the antifungal activity of the steroidal saponins. TTS-12 destroyed the yeast cell membrane through TEM (Fig. 5), so that the cytoplasm components leaked out of the cells and the yeast cells were killed by TTS-12.

The results of the present study provide pharmacological reference for the traditional use of *Tribulus terrestris*, documenting that saponins exert antifungal activity by inhibiting fungal hyphae and destroying the ultra structure of fungi in particular.

In conclusion, TTS-12 and TTS-15 are steroidal saponins with potent properties against a number of fungal pathogens, and identifying the mode of action and its in vivo toxicity warrants further study in the light of developing new antifungal drugs. Further studies are also required to confirm the structure-activity relation of steroid saponins we propose in the paper.

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