In Vitro and *in Vivo* Antifungal Activities of the Eight Steroid Saponins from *Tribulus terrestris* L. with Potent Activity against Fluconazole-Resistant Fungal

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Antifungal activity of natural products is being studied widely. Saponins are known to be antifungal and antibacterial. We have isolated eight steroid saponins from *Tribulus terrestris* L., namely TTS-8, TTS-9, TTS-10, TTS-11, TTS-12, TTS-13, TTS-14 and TTS-15. TTS-12 and TTS-15 were identified as 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 and tigogenin-3-*O*-β-D-glucopyranosyl(1→2)-[β-D-xylopyranosyl(1→3)]-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside, respectively. The *in vitro* antifungal activities of the eight saponins against six fluconazole-resistant yeasts, *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei*, and *Cryptococcus neoformans* were studied using microbroth dilution assay. The results showed that TTS-12 and TTS-15 were very effective against several pathogenic candidal species and *C. neoformans in vitro*. It is noteworthy that TTS-12 and TTS-15 were very active against fluconazole-resistant *C. albicans* (MIC_{80}=4.4, 9.4 μg/ml), *C. neoformans* (MIC_{80}=10.7, 18.7 μg/ml) and inherently resistant *C. krusei* (MIC_{80}=8.8, 18.4 μg/ml). So *in vivo* activity of TTS-12 in a vaginal infection model with fluconazole-resistant *C. albicans* was studied in particular. Our studies revealed TTS-12 also showed *in vivo* activities against fluconazole-resistant yeasts. In conclusion, steroid saponins TTS-12 and TTS-15 from *Tribulus terrestris* L. have significant *in vitro* antifungal activity against fluconazole-resistant fungi, especially TTS-12 also showed *in vivo* activity against fluconazole-resistant *C. albicans*.

Key words *Tribulus terrestris* L.; fluconazole-resistant; saponins; *Candida albicans*

In recent twenty 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.[1—3] *Candida albicans* (*C. albicans*) is an organism that is most often associated with serious fungal infections, and can cause fungal diseases in immunocompromised patients, including cancer patients, organ transplant patients, and those with human immunodeficiency virus infections.[4] Candidal vaginitis is predominantly caused by strains of *C. albicans* (90%),[5—7] 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. Fluconazole was the mostly used azole drugs, and its use has resulted in clinically resistant strains of *Candida* spp.[8,9] Especially, 3.6—7.2% of vaginal isolates of *Candida* spp. from women with *Candida* vaginitis is resistant to fluconazole.[10] 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, the fruit of *T. terrestris* L. is used for treating cutaneous pruritus, edema, inflammation and tracheitis.[11] In our previous study, we isolated and identified eight steroid saponins from *T. terrestris*.[12] In the present report, eight steroid saponins were tested to investigate their properties against fluconazole-resistant yeasts often encountered clinically, especially *C. albicans*.

MATERIALS AND METHODS

Plant Materials *T. 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.

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, $CHCl_3$, and *n*-butanol. Our previous preliminary study on *C. 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 (10×50 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 **1** (TTS-8, 33 mg), **2** (TTS-9, 21 mg), **3** (TTS-10, 46 mg), **4** (TTS-11, 27 mg), **5** (TTS-12, 1 g), **6** (TTS-13, 72 mg), **7** (TTS-14, 43 mg), **8** (TTS-15, 52 mg).

Organisms Used A total of 62 clinical isolates of fluconazole-resistant *Candida* species and fluconazole-resistant

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Cryptococcus neoformans (*C. neoformans*) obtained from different hospitals in Shanghai, were tested (Table 1), and 80% minimal inhibitory concentrations (MIC_{80}) of all fluconazole-resistant isolates is greater than 64 μg/ml according to American National Committee for Clinical Laboratory Standards (NCCLS).[13] The collection included the following numbers of isolates: 47 clinical isolates of *C. albicans*, 2 isolates of *C. tropicalis*, 3 isolates of *C. glabrata*, 2 isolates of *C. parapsilosis*, 3 isolates of *C. neoformans*, and 5 isolates of *C. krusei*. 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.

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

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.[13] *C. krusei* (ATCC6258) and *C. parapsilosis* (ATCC22019) were quality controlled strains, and tested in each assay. Fluconazole (FLC) obtained from its manufacturers served as the positive control. The drug MIC_{80} 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, U.S.A.) with the final concentrations between 128.0 and 0.25 μg/ml (111.30—0.220 μmol/l), and the final concentrations of FLC was 64.0—0.125 μg/ml (209.15—0.410 μmol/l), depending on the MIC results from our preliminary study.

Laboratory Animals Forty female Sprague–Dawley (SD) rats weighing 100 to 120 g (Center of Experimental Animals, Second Military Medical University, Shanghai, China) were used for the study of vaginal infections with *C. albicans*. Everything was approved by an ethical committee.

Growth Curve Study The effect of TTS-12 and TTS-15 exposure in relation to time and concentration on clinical isolate Y0305433 of *C. albicans* was determined in YEPD liquid medium. The MIC_{80} value of clinical isolate Y0305433 of *C. albicans* was greater than 64 μg/ml. 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.

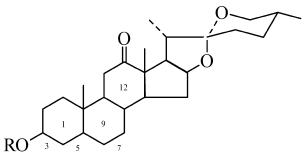
Vaginal Infection Model with *C. albicans* The vaginal infection animal model was established based on modified models previously described by Sobel *et al.*[14] to obtain a more chronic and homogeneous infection. Briefly, forty female animals were ovariectomized, and estrus was induced with subcutaneous administration of estradiol at a dose of 10 mg/kg 3 d before infection and maintained by subcutaneous estradiol at a dose of 4 mg/kg weekly throughout the experiment. Clinical isolate Y0305433 of *C. albicans* was inoculated intravaginally with 10^7 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 thirty-two infected animals were equally randomized into four groups: Group 1, control; Group 2, fluconazole (40 mg/kg), was served as the positive control; Group 3, TTS-12 (40 mg/kg); and Group 4, TTS-12 (80 mg/kg). The vaginal *C. albicans* load was evaluated at day 3 post-infection, and day 3, 7, 14 after initiation of drug administration. TTS-12 or FLC was administered to the infection animals for 14 consecutive days.

RESULTS

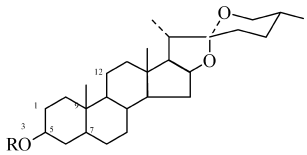
Identification of Ten Compounds Identification of the ten compounds showed that they were hecogenin-3-*O*-β-D-glucopyranosyl (1→4)-β-D-galactopyranoside (**1**, TTS-8), tigogenin-3-*O*-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside (**2**, TTS-9), hecogenin-3-*O*-β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside (**3**, TTS-10), hecogenin-3-*O*-β-D-xylopyranosyl(1→3)-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside (**4**, 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 (**5**, 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 (**6**, TTS-13), hecogenin-3-*O*-β-D-glucopyranosyl(1→2)-[β-D-xylopyranosyl(1→3)]-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside (**7**, TTS-14), tigogenin-3-*O*-β-D-glucopyranosyl(1→2)-[β-D-xylopyranosyl(1→3)]-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside (**8**, TTS-15). The chemical structures of the compounds **1**—**8** were shown in Fig. 1.

Antifungal Susceptibility Results Compounds **1**—**8** isolated from *Tribulus terrestris* L. were identified as steroid saponins, the *in vitro* activity of which were evaluated against six fluconazole-resistant yeasts (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. neoformans*, *C. krusei*) which are often encountered clinically. The results were showed in Table 1. Compounds **1**, **2**, **3**, **4** and **6** (TTS-8, TTS-9, TTS-10, TTS-11 and TTS-13) were inactive on yeasts tested, and compound **7** (TTS-14) had somewhat activities against *C. albicans*, *C. glabrata*, *C. neoformans* and *C. krusei*, with MIC_{80} values of 56.9, 74.7, 42.7 and 35.2 μg/ml respectively. Especially, compounds **5** and **8** (TTS-12 and TTS-15) had significant antifungal activities against six fluconazole-resistant yeasts tested, *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. neoforman*, *C. krusei*. Importantly, TTS-12 and TTS-15 clearly inhibited the growth of *C. albicans*, and the MIC_{80} value was determined to be 4.4 and 9.4 μg/ml respectively. They were also very effective against *C. neoforman* and *C. glabrata* at 10.7 and 18.7 μg/ml, especially showed activities against inherently fluconazole-resistant *C. krusei* (with the MIC values of 8.8 and 18.4 μg/ml) and *C. tropicalis* (with the MIC values of 32.0 and 64.0 μg/ml).

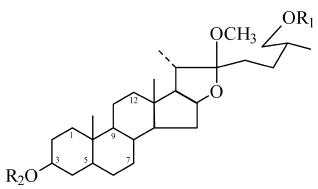
Growth Curve TTS-12 and TTS-15 activity showed dose and time dependency against the growth of fluconazole-resistant *C. albicans* (Fig. 2). By 8 h post-incubation significant inhibition was observed at concentrations as low as 8,



1. TTS-8: R=−¦Â−D−gal^{4}−¦Â−D−glu



2. TTS-9: R=−¦Â−D−gal^{4}−¦Â−D−glu



6. TTS-13: R_1=−¦Â−D−glu

R_2=−¦Â−D−gal^{4}(−¦Â−L−rha^{2})−¦Â−D−glu^{2}(−¦Â−D−xyl^{3})−¦Â−D−xyl

3. TTS-10: R=−¦Â−D−gal^{4}−¦Â−D−glu^{2}−¦Â−D−glu

4. TTS-11: R=−¦Â−D−gal^{4}−¦Â−D−glu^{3}−¦Â−D−xyl

5. TTS-12: R=−¦Â−D−gal^{4}(−¦Â−L−rha^{2})−¦Â−D−glu^{2}(−¦Â−D−xyl^{3})−¦Â−D−xyl

7. TTS-14: R=−¦Â−D−gal^{4}−¦Â−D−glu^{2}(−¦Â−D−xyl^{3})−¦Â−D−glu

8. TTS-15: R=−¦Â−D−gal^{4}−¦Â−D−glu^{2}(−¦Â−D−xyl^{3})−¦Â−D−glu

Fig. 1. The Chemical Structures of Steroid Saponins **1**—**8** from *Tribulus terrestris* L.

Table 1. *In Vitro* MIC_{80} Values of Eight Compounds from *Tribulus terrestris* L. against Six Fluconazole-Resistant Yeasts Tested

No.	Comp.	*C. albicans*	*C. glabrata*	*C. parapsilosis*	*C. tropicalis*	*C. neoformans*	*C. krusei*
1	TTS-8	>128.0	>128.0	>128.0	>128.0	>128.0	>128.0
2	TTS-9	>128.0	>128.0	>128.0	>128.0	>128.0	>128.0
3	TTS-10	≥128.0	>128.0	>128.0	>128.0	>128.0	>128.0
4	TTS-11	≥128.0	≥128.0	>128.0	>128.0	>128.0	>128.0
5	TTS-12	4.4±2.7	10.7±4.6	24.0	32.0	10.7±4.6	8.8±4.4
6	TTS-13	>128.0	>128.0	>128.0	>128.0	>128.0	>128.0
7	TTS-14	56.9±33.2	74.7±48.9	>128.0	>128.0	42.7±18.5	35.2±17.5
8	TTS-15	9.4±6.1	18.7±12.2	64.0	64.0	18.7±12.2	18.4±13.1
9	FLC	≥64.0	≥64.0	≥64.0	≥64.0	≥64.0	≥64.0

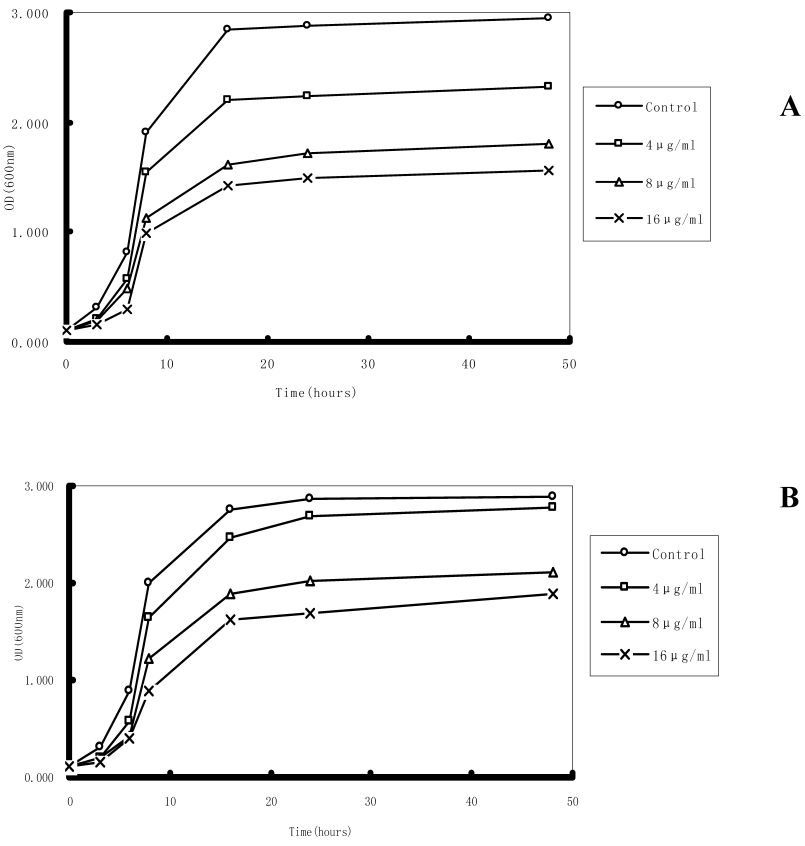


Fig. 2. The Effect of TTS-12 (A) or TTS-15 (B) from *Tribulus terrestris* L. on the Growth of Clinical Isolate Y0305433 of Fluconazole-Resistant *C. albicans*

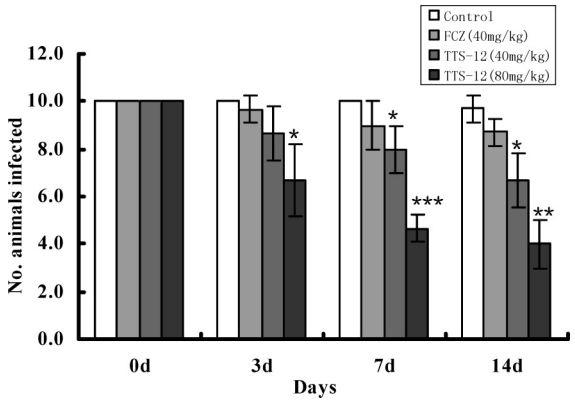


Fig. 3. The Effect of TTS-12 from *Tribulus terrestris* L. on the Number of Animals Infected Vaginally with Clinical Isolate Y0305433 of Fluconazole-Resistant *C. albicans*, along with Control and the Positive Control, FLC

Significance: $*p<0.05$, $**p<0.01$, $***p<0.001$ compared to the control.

16 μg/ml compared with the control.

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 rat vaginitis) was established, where the animals were challenged with fluconazole-resistant clinical isolate Y0305433 of *C. albicans*. All rats were infected with *C. albicans* and assessed mycologically 3 d after infection. Figure 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 *C. albicans*, the number of the animals infected significantly decreased after TTS-12 was administrated at the dose of 40 mg/kg and 80 mg/kg, and there was a statistically significant difference between the control and the TTS-12 treatment groups at all time-points.

DISCUSSION AND CONCLUSION

Candida species have emerged as clinically important pathogens associated with opportunistic infections, *C. albicans* of whom is an important opportunistic human pathogen causing common ailments such as thrush and vaginitis, as well as chronic conditions in immunocompromised patients.[6] So the azole drugs, especially fluconazole, are widely used to fight *C. albicans* infections. Not surprisingly, repeated fluconazole therapy for fungal infections in patients, such as vaginitis women with *C. albicans* infections has been associated with an increase in azole resistance, and the survey showed that 33.5% clinical *C. albicans* isolates from vaginitis women are resistant to fluconazole.[10] In addition, several other *Candida* species, such as *C. krusei* and *C. tropicalis* are inherently resistant to fluconazole.[15] So it is very important finding antifungal drugs with novel chemical structure.

In Traditional Chinese Medicine, the plant *T. 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.[11,16] Earlier studies showed that *T. terrestris* L. contained flavanoids, steroid saponins, alkaloids and polysaccharides.[17—22] In our previous studies, we isolated from *T. terrestris* L. eight steroid saponins, TTS-8, TTS-9, TTS-10, TTS-11, TTS-12, TTS-13, TTS-14 and TTS-15.[12] Many studies in the literature[23—26] reported that most steroid saponins have antifungal activities. In the present study, we therefore conducted a series of experiments to investigate activities of the eight steroid saponins we isolated from *T. terrestris* L. previously against fluconazole-resistant fungi, and did some pioneer work concerning the effects of steroid saponins against fluconazole-resistant fungi.

The results of our study showed that TTS-8, TTS-9, TTS-10, TTS-11 and TTS-13 were inactive, and TTS-14 had activities against *C. albicans*, *C. glabrata*, *C. neoformans* and *C. krusei*, and its MIC values was similar to fluconazole. However, TTS-12 and TTS-15 had significant antifungal activities against the six fluconazole-resistant yeasts tested, *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. neoforman* and *C. krusei*. Importantly, the present experiment showed that TTS-12 and TTS-15 had potent anti fluconazole-resistant *C. albicans* activity where MIC_{80} were 4.4 and 9.4 μg/ml respectively, by far lower than that of fluconazole and other steroid saponins reported. According to the literature MIC_{80} of most saponins against *C. albicans* is greater than >50 μg/ml and that of individual saponins is between 4 and 20.8 μg/ml.[23—27] The studies of Renault *et al.*[25] and De Lucca *et al.*[28] demonstrated that CAY-1, a steroid saponin, from the ground fruit of *Capsicum frutescens*, had antifungal activity on *C. albicans*, and the IC_{50} and IC_{90} were determined to be 3.8 μg/ml (3.1 μmol/l) and 7.7 μg/ml (6.2 μmol/l) resepctively. Extracts of *Eriocephalus africanus* L., *Felicia erigeroides* DC and *Helichrysum crispum* (L.) D. Don inhibited the growth of *C. albicans*.[29] Prosapogenin A of dioscin exhibited antifungal activity against human pathogenic yeasts *C. albicans* (MIC 20.8 μg/ml), *C. glabrata* (MIC 6.25 μg/ml) and *C. tropicalis* (MIC 25 μg/ml).[26] TTS-12 and TTS-15 were also very active on fluconazole-resistant *C. neoforman* and *C. glabrata* at 10.7 and 18.7 μg/ml, also by far lower than the antifungal activities of fluconazole, and similar to several other saponins reported with the MICs of 2—12.5 μg/ml.[23,24] Especially, TTS-12 and TTS-15 showed activities against inherently fluconazole-resistant *C. krusei* (with the MIC values of 8.8 and 18.4 μg/ml) and *C. tropicalis* (with the MIC values of 32.0 and 64.0 μg/ml). 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 *C. albicans* (90%),[5—7] and remains to be a common problem in immunocompetent or healthy women. So we also observed the *in vivo* activity of TTS-12 against fluconazole-resistant *C. albicans*. According to the literature, the candidal vaginitis rat model is a stable model that can be used for study of drugs on candidal vaginitis. Because the steroidal glycosides TTS-12 and TTS-15 are from the same chemical structures, and at the same time TTS-12 showed stronger antifungal activities, we studied the *in vivo* anti fluconazole-resistant *C. albicans* activities of TTS-12. 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 against fluconazole-resistant fungi.

The steroidal glycosides tested in the experiment are from the same chemical class, but only TTS-12 and TTS-15 sig-

nificantly exhibited anti fluconazole-resistant yeasts activity against *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. neoforman* and *C. krusei*. These results indicate that there are critical structural features that are responsible for the antifungal activity. While the furostanol-type steroidal glycosides, TTS-13 was inactive, confirming earlier observations.[30] 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 somewhat anti fluconazole-resistant yeasts activity. In addition, the chemical difference between TTS-12, TTS-14, TTS-15 and compounds TTS-8, TTS-9, TTS-10, TTS-11 was the number of connecting saccharides. So a carbonyl group at C-12 and the number of connecting saccharides are probably related to the anti fluconazole-resistant yeasts activity of compounds. So TTS-9, a saponin without a carbonyl base at C-12 did not also show antifungal activity.

Based on the results, 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. The relations of the number of oligosaccharide residue and the carbonyl base at C-12 and antifungal activities of compounds were not detailedly reported in the literature so far.

In conclusion, the results of the present study provide pharmacological reference for the traditional use of *T. terrestris* L. TTS-12 and TTS-15 isolated from *T. terrestris* L. are steroidal saponins with potent *in vitro* and *in vivo* properties against a number of fluconazole-resistant fungal pathogens, and identifying the mode of action and its *in vivo* toxicity warrants further study in the light of developing new antifungal drugs against human nosogenic fungi, especially against fluconazole-resistant fungi.

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