Original Article

Teasaponin suppresses *Candida albicans* filamentation by reducing the level of intracellular cAMP

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Background: Candidiasis has long been a threat to human health, but cytotoxicity and resistance always block the usefulness of antifungal agents. The ability to switch between yeast and hypha is one of the most discussed virulence trait attributes of the human pathogenic fungus *Candida albicans*. The morphological transition provides a novel target for developing antifungal drugs. The aim of the present study was to explore the activity and mechanism of teasaponin (TS), a generally regarded as safe natural product, in inhibiting filamentation of *C. albicans*, hoping to provide an experimental basis for its clinical application.

Methods: The effect of TS on filamentation and biofilm formation of *C. albicans* was evaluated by XTT reduction assay and microscopy. The level of intracellular cAMP was measured to further explore the underlying mechanism. In addition, cytotoxicity of TS was evaluated by using MTT assay *in vitro* and *Caenorhabditis elegans* model *in vivo*. The potential of TS-resistance induction was tested by a serial passage experiment.

Results: TS displayed a moderate antifungal activity against the wild type, efflux pump mutant and multi-resistance *C. albicans* strains, and could effectively retard filamentation and biofilm formation with a low MIC value. Further mechanism investigation revealed that the reduced cAMP level inhibited filamentation and biofilm formation. In addition, TS showed no significant cytotoxicity *in vitro* or *in vivo*, and had little potential to develop resistance during long-time induction.

Conclusions: Our work evaluated the antifungal activity of TS against filamentation and biofilms formation of *C. albicans* and disclosed the underlying mechanism, which might provide useful clues for the potential clinical application of TS in fighting clinical fungal infections by targeting the virulence factors.

Keywords: Teasaponin; *Candida albicans*; filamentation; cAMP

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Introduction

Some risk factors, including the use of immunosuppressive, cytotoxic drugs, powerful antibiotics and various implanted devices, make opportunistic fungal pathogens liable to attack immunocompromised or some other debilitated hosts (1). *Candida* species are the fourth most common pathogens causing hospital-acquired systemic infections in the United States, with crude mortality rates as high as 50% (2). Meanwhile, it is estimated that up to 75% of women experienced at least one episode of vulvovaginal candidiasis (VVC) in their lifetime (3), of whom, 5–8% experienced

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Table 1 The MIC of TS and FLC against different genotypes of *C. albicans in vitro*

Drugs and strains[α]	MICs of drugs (μg/mL)[β]	
	TS	FLC
ATCC10231	64	1
SC5314	64	2
YEM30	64	2
DSY448	64	0.5
DSY1050	64	0.5
CA10	64	>128
CA148	64	>128
CCF3	64	1
HLY3426	64	1

[α], ATCC10231, SC5314 and YEM30 are wild-type strains. The genotype of DSY448 is Δ*cdr1*::*hisG-URA3-hisG*/Δ*cdr1*::*hisG*; The genotype of DSY1050 is Δ*cdr1*::*hisG*/Δ*cdr1*::*hisG* Δ*cdr2*::*hisG*/Δ*cdr2*::*hisG* Δ*mdr1*::*hisG-URA3-hisG*/Δ*mdr1*::*hisG* (14). CA10 and CA148 are clinical isolated azoles multi-resistance *C. albicans* isolates (15). The genotype of CCF3 is *ura3::1 imm434/ura3::1 imm434 flo8::hisG/flo8::hisG-URA3-hisG*; The genotype of HLY3426 is *ura3::1 imm434/ura3::1 imm434 flo8::hisG/flo8::hisG RP10::ACT1p-FLO8–13MYCFLAG-URA3* (16). [β], TS, teasaponin; FLC, fluconazole.

at least four episodes of chronic recurrences per year (4). *Candida albicans* is the most common human fungal pathogen, causing both superficial and invasive candidiasis (5). Usually, *C. albicans* is a benign member of the human gastrointestinal and genital tracts. However, if the host immune function is impaired or the environmental niche is available, *C. albicans* can cause debilitating mucosal or fatal systemic infections via exerting its own virulence factors (6).

The dimorphic phenotype, ranging from budding yeast to hyphae, is a striking feature of *C. albicans*. Yeast cells, as a disseminating form, play a critical role in the early stage of the infection process for adhesion on the tissue surface. Hyphal cells, as an important pathogenic form, have the advantage of escaping from the host immune system and invading the tissue in the pathogenesis (7). This morphological plasticity is a crucial perpetrator of candidiasis. In addition, structured biofilm consists of yeast, hyphae and self-produced extracellular matrix with high drug-resistance up to 1,000 folds (8). *C. albicans* is equipped with a variety of virulence traits and escape strategies, helping them colonize within the commensal microbiota and invade the host tissue during infection. The expression of adhesins and invasins, the plasticity of morphology, the formation of biofilm, contact sensing, phenotypic switching and the secretion of hydrolytic enzymes are some important virulence properties (9). Thus, antivirulence, especially the formation of hyphae and biofilm, is a powerful therapeutic strategy for the management of *C. albicans* infection (10).

Like antibiotics, antifungal drugs also face the problem of drug resistance and toxicity that block the treatment of fungal infection. Natural products have long been recognized as an excellent source of new drugs. Statistically, from 1981 to 2014, nearly 60% of small-molecule drugs approved by the U.S. Food and Drug Administration (FDA) were either natural products or based thereon (11). Teasaponin (TS), generally regarded as a safe compound, is a tea seed-derived natural surfactant with various bioactivities. Previous studies (12,13) have demonstrated that TS possesses many pharmacological functions and fine foaming and emulsifying activity for pesticides. In this study, we found that TS could effectively inhibit the formation of hyphae and biofilm of *C. albicans*. Further mechanism research showed that the reduction of cyclic adenosine monophosphate (cAMP) level might contribute to the inhibitory effect of TS.

Methods

Strains, culture and chemicals

C. albicans strains used in this study are shown in *Table 1* (14-16). *C. albicans* strain ATCC10231 was obtained from the ATCC Essentials of Life Science Research, USA. Wild type *Caenorhabditis elegans* strain N2 was obtained from the Caenorhabditis Genetics Center, USA. Clinical derived *C. albicans* azole-resistant isolates CA10 and CA148 were donated by Shandong Provincial Qianfoshan Hospital (15). All strains were stocked and prepared as our lab previously described (17). Fluconazole (FLC) and dibutyryl-cAMP (db-cAMP) were purchased from Sigma (St Louis, USA), fetal bovine serum (FBS) was purchased from Gibco (USA), and TS was purchased from Aladdin (Shanghai, China). They were dissolved in dimethyl sulfoxide (DMSO) (Sigma, St Louis, USA) at 10 or 50 mg/mL. In each assay, the content of DMSO was below 0.5%.

Minimal inhibitory concentration (MIC) test

The MIC value of TS against different genotypes of *C. albicans* was determined by the broth microdilution method

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according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (M27-A3) (18). The minimal concentration of zero visible growth was considered as the endpoint value.

Inhibitory curve of proliferation

Wild type strain YEM30 was diluted in synthetic medium, plus dextrose (SD medium) with an initial inoculum of 1×10^5 cells/mL. TS was added to the final concentrations of 0, 16, 32, and 64 μg/mL, and 2 μg/mL FLC served as positive control group. Cells were cultured at 30 ℃ with shaking at 200 rpm for 48 hours. Every 2 hours, 100-μL aliquots of samples were transferred to a 96-well flat-bottom plate and detected for absorbance at 600 nm with a Bio-Rad microplate reader (Bio-Rad Laboratories, Richmond, CA).

Inhibition of filamentation

The effect of TS on the filamentation of wild type strain YEM30 in different culture media was detected using a modified broth microdilution method as previously described (19). Cells containing various doses of TS (0, 16, 32 and 64 μg/mL) were diluted in RPMI1640 medium (pH7.0, buffered with MOPS), RPMI1640 plus 5% FBS medium, spider medium (1% nutrient broth, 0.5% mannitol, 0.1% K_2HPO_4, pH 7.2) and spider plus 5% FBS medium, respectively, at 1×10^5 cells/mL and incubated at 37 ℃ without shaking. Hyphal development was monitored by a microscope (Olympus IX71, Olympus, Tokyo, Japan) in the bright field mode at specific times (3, 6, 9 and 12 h).

Inhibition of biofilm formation

The effect of TS against biofilm formation was measured using an XTT reduction assay and microscopic observation as previously described (20). 100 μL YEM30 (wild type strain), CCF3 (*flo8/flo8* mutant) and HLY3426 (*flo8/flo8* + *FLO8* mutant) (16) cell suspensions (1×10^6 cells/mL in RPMI1640 medium or RPMI1640 plus 5% FBS medium) containing various doses of TS (0, 32, 64 and 128 μg/mL) were seeded into a 96-well flat-bottom plate. The plate was incubated at 37 ℃ for 24 h in static culture. After three washes with sterile PBS (0.01 M, pH 7.2), biofilm formation was measured with an XTT Cell Proliferation Assay Kit (BestBio, Shanghai, China) according to the provided protocol and observed under an Olympus microscope in the bright field mode.

Determination of intracellular cAMP content

The effect of TS on the intracellular cAMP concentrations was measured as previously described with some modifications (21). YEM30 cells (1×10^6 cells/mL) with various doses of TS were cultured in RPMI1640 medium at 37 ℃ for 12 h, and 2 μg/mL FLC served as positive control group. Cells were harvested by centrifugation, washed 3 times with deionized water, weighed the dry weight, frozen in liquid nitrogen and thawed at room temperature repeatedly, and finally suspended in deionized water with 5% trichloroacetic acid. Following breaking by ultrasonication, the supernatant was neutralized with water-saturated ether five times and then subjected to freeze-drying. The content of intracellular cAMP was measured using a cAMP ELISA Kit (Elabscience Biotechnology Co., Ltd, Guangdong, China) according to the provided protocol. The concentrations of cAMP were modified by their cell weights.

cAMP rescue test

To test the effect of cAMP on hyphal formation after TS treatment, YEM30 cells were prepared as the above filamentation experiment. db-cAMP was added into 32 μg/mL TS and drug-free treatment group at a final concentration of 1 mM. The db-cAMP free group served as control. After 6-h incubation at 37 ℃, the morphology of formed hyphae was observed under an Olympus microscope.

The effect of cAMP on biofilm formation in the presence of TS was tested as previously described (20) with some modifications. YEM30 cells were prepared as the above biofilm formation experiment. db-cAMP was added to 64 μg/mL and drug-free treatment groups at a final concentration of 1 mM. The db-cAMP free group served as control. After 24-h incubation at 37 ℃, the formed biofilm was detected by XTT reduction assay and microscopic observation.

Transcriptional levels of hypha-specific genes

Quantitative real-time RT-PCR was used to investigate the transcriptional levels of hypha-specific genes under TS treatment. As previously described (20), YEM30

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Table 2 Gene-specific primers used for real-time RT-PCR

Primers	Sequence
RAS1-F	GGCCATGAGAGAACAATATA
RAS1-R	GTCTTTCCATTTCTAAATCAC
ALS3-F	CTAATGCTGCTACGTATAATT
ALS3-R	CCTGAAATTGACATGTAGCA
HWP1-F	TGGTGCTATTACTATTCCGG
HWP1-R	CAATAATAGCAGCACCGAAG
CDC35-F	TTCATCAGGGGTTATTTCAC
CDC35-R	CTCTATCAACCCGCCATTTC
PDE2-F	ACCACCACCACTACTACTAC
PDE2-R	AAAATGAGTTGTTCCTGTCC
EFG1-F	TATGCCCCAGCAAACAACTG
EFG1-R	TTGTTGTCCTGCTGTCTGTC
ECE1-F	GCTGGTATCATTGCTGATAT
ECE1-R	TTCGATGGATTGTTGAACAC
18S rRNA-F	AATTACCCAATCCCGACAC
18S rRNA-R	TGCAACAACTTTAATATACGC

cells were diluted in RPMI1640 medium with or without 32 μg/mL of TS. After an incubation of 6-h at 37 ℃, cells were harvested and washed with sterile water. The hot phenol method was used to extract the total RNAs, and the cDNA was synthesized by a cDNA synthesis kit (TaKaRa Biotechnology, Dalian, China). PCR reactions were performed using a SYBR Green master mix (TaKaRa Biotechnology, Dalian, China) in a Roche Real-Time PCR System. Primer sequences are listed in *Table 2*. The *18S rRNA* served as the internal control gene.

Induction of TS resistance

The capacity of TS resistance induction was tested as previously described with some modifications (19). Briefly, YEM30 cells were inoculated in YPD broth and cultured at 30 ℃, 200 rpm with 32 μg/mL TS, a concentration half of MIC inhibiting hyphal formation. Every day, 100 μL of each culture was serially transferred into 10 mL YPD broth (yeast extract 1%, peptone 2% and dextrose 2%) containing 32 μg/mL TS, and the everyday transfer was performed for 40 days. Each day, cultures were withdrawn to test their MICs.

Toxicity assay

Two normal cell lines and wild type *C. elegans* were used to evaluate the cytotoxicity of TS.

The effect of TS against the proliferation of cell line HT22 (mouse hippocampal cell line) and HEK 293T (human embryonic kidney cell line) was detected by MTT assay as previously described (22). After 24-h incubation, the treated cells were incubated with MTT (0.5 mg/mL) for additional 4 h in the dark. The spectrophotometric absorbance of each well was measured at 570 nm using a Bio-Rad plate reader. The IC_{50} value was calculated based on the percentage of viable cells.

The effect of TS on the survival of wild type *C. elegans* N2 was also tested as previously described (20). Briefly, different concentrations of TS were added to the prepared nematodes in a 96-well plate. After 2-day incubation at 25 ℃, the treated worms were monitored by microscopic observation, and the number of living and dead worms were recorded to calculate the survival rate.

Statistical analysis

All experiments were assayed in triplicate on a given day and repeated on additional two days. All results are represented as the mean values with the corresponding standard deviations (SD) of triplicate measurements from three independent experiments. Statistical significance was analyzed by Student's *t*-test (two-tailed, unequal variance). $P<0.05$ was considered as statistically significant.

Results

TS inhibits the proliferation of C. albicans

To evaluate the effect of TS against the proliferation of *C. albicans*, the MIC value of TS against different genotypes of *C. albicans* was assayed *in vitro*. As shown in *Table 1*, TS displayed moderate antifungal activity against *C. albicans*, and the MIC values of all tested strains was 64 μg/mL, whether it be wild type strain (ATCC10231, SC5314 and YEM30) or efflux pump mutant strain (DSY448 and DSY1050) (14) or multi-resistance strain (CA10 and CA148) (15) or avirulent strain (CCF3, *flo8/flo8* mutant) and its complementation strain (HLY3426, *flo8/flo8* + *FLO8* mutant) (16). The MIC of FLC (positive control) was 0.5–>128 μg/mL for different strains.

In addition, the proliferation inhibitory curve of TS was

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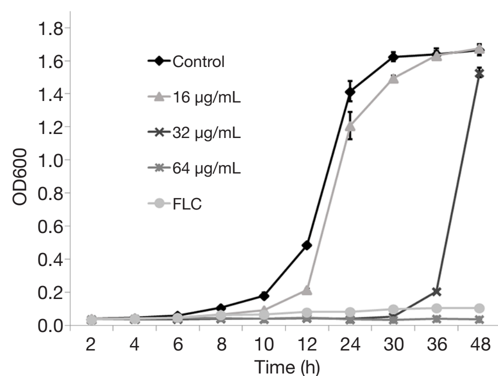

Figure 1 The growth curve of wild type *C. albicans* strain YEM30 under TS treatment. YEM30 cells were diluted in the SD medium and incubated with different doses of TS at 30 ℃ with shaking. OD600 of each group was detected at a 2-h interval using a Bio-Rad microplate reader. The results are shown as means ± standard deviation (SD).

also plotted over 48 h. The results showed that 16 μg/mL TS (1/4 MIC) could retard the growth of *C. albicans* as compared with drug-free control group over 24 h. The growth curve of 32 and 64 μg/mL was similar to that of 2 μg/mL of FLC (positive control), almost completely suppressing the proliferation in 24 h, and during the treatment with 32 μg/mL TS, *C. albicans* resumed growth after 36 h (*Figure 1*). We collected the treated cells with 32 or 64 μg/mL TS for 48 h and measured their MIC values of TS. Meanwhile, the collected cells were stained by propidium iodide (PI) to determine viable or dead cells. Results showed that the MIC values of TS had no change after TS treatment for 48 h, implying no resistance formation. PI could stain most cells of 64 μg/mL TS treated cells in red fluorescence, while few cells treated by 32 μg/mL TS could be penetrated by PI (data not shown). These results implied that TS could inhibit the proliferation of *C. albicans*, the resumed growth of 32 μg/mL TS group might due to the degradation of TS reducing its antifungal activity, and high concentration of TS exerted fungicidal activity against *C. albicans*.

TS retards the hyphal growth of C. albicans

Knowing that hyphal growth is the most recognized virulence factor during the *C. albicans* infection process, we examined the effect of TS on the hyphal growth *in vitro* by microphotography. As illustrated in *Figure 2A*, hyphal initiation and elongation were observed in control and 16 μg/mL groups in RPMI 1640 medium within 9 h, and extensive biofilm formation were observed at 12 h. In contrast, cells in 32 μg/mL group grew as a yeast form within 9 h, and the length of formed hyphae at 12 h was much shorter than that in control and 16 μg/mL groups. The yeast form remained unchanged within 12 h in the 64 μg/mL and FLC treatment (positive control) groups.

We also examined the hyphal growth in other hypha-induced cultures, RPMI 1640 plus 5% FBS medium, spider medium and spider plus 5% FBS medium. After 9-h culture at 37 ℃, hyphal growth in the spider medium was similar as that in RPMI1640 medium. Meanwhile, most cells of 64 μg/mL TS group in plus FBS medium still retained yeast form just like those in the positive control FLC group. In addition, the number and length of hyphae in 32 and 64 μg/mL groups were much smaller and shorter than those in control group (*Figure 2B*). This phenomena implied that TS had the capacity to inhibit hyphal formation of *C. albicans*.

TS inhibits the biofilm formation of C. albicans

Knowing that hyphal growth plays a crucial role in biofilm formation, and high drug resistance and host immune escape are key virulence factors contributing to biofilm formation (23), we examined whether TS had an impact on the biofilm formation of wild type and avirulent strains in different medium using microphotography and XTT reduction assay. Microscopic observation revealed that the formed biofilm in wild type strain YEM30 were composed of a mixture of hyphae and yeast cells in non-treated and 32 μg/mL groups. In contrast, only clusters of yeast or short hyphae were observed on the bottom of the well without biofilm structures in 64 and 128 μg/mL groups (*Figure 3A*). The XTT assay result showed that the inhibitory effect was dose-dependent, as reflected by a decrease in metabolic activity. Specifically, 64 μg/mL TS could significantly prevent about 80% biofilm formation (*Figure 3B*, $P<0.01$). Under the filamentous-induced condition (RPMI 1640 plus FBS medium), although there were more formed biofilm or cells in 64 or 128 μg/mL groups as compared with the control group under the filamentous-induced condition (plus FBS) (*Figure 3A*), 64 and 128 μg/mL TS could significantly inhibit biofilm formation (*Figure 3B*, $P<0.01$). Meanwhile, the inhibitory tendency of non-filamentous strain CCF3 and its complementation strain HLY3426 was similar to the wild type strain. These data implied that TS had a negative effect on biofilm formation of *C. albicans*.

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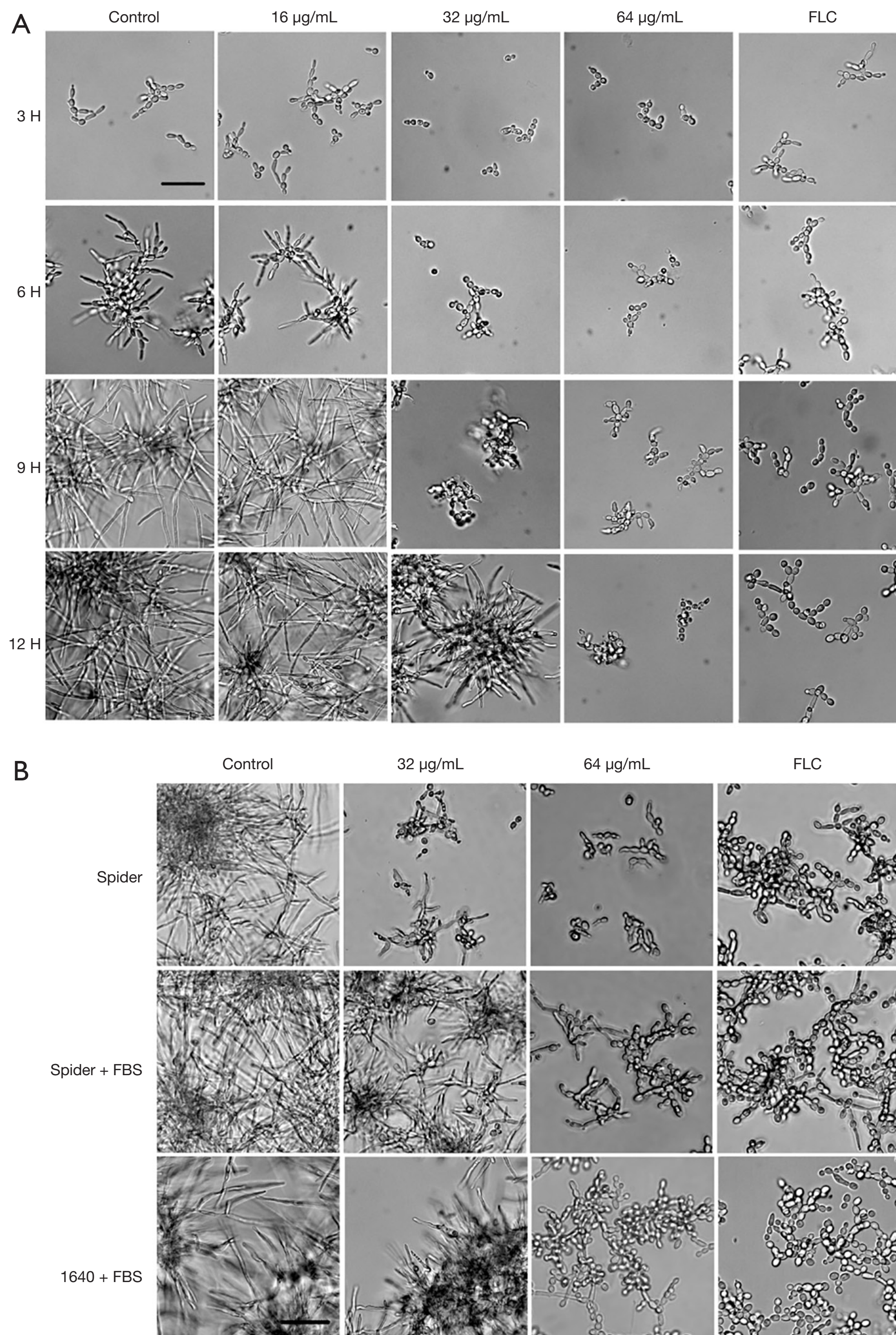

Figure 2 The effect of TS on the filamentation of *C. albicans*. (A) YEM30 cells were diluted in the RPMI1640 medium and incubated with different doses of TS at 37 ℃ without shaking. Every three hours, cells were imaged under a microscope in the bright field. 2 μg/mL FLC served as positive control. (B) YEM30 cells were diluted in spider medium, spider plus 5% FBS medium and RPMI1640 plus 5% FBS medium, respectively, with 32 and 64 μg/mL of TS and incubated at 37 ℃ without shaking for 9 h. Hyphal growth was observed under a microscope. 2 μg/mL FLC served as positive control. The bar indicates 20 μm.

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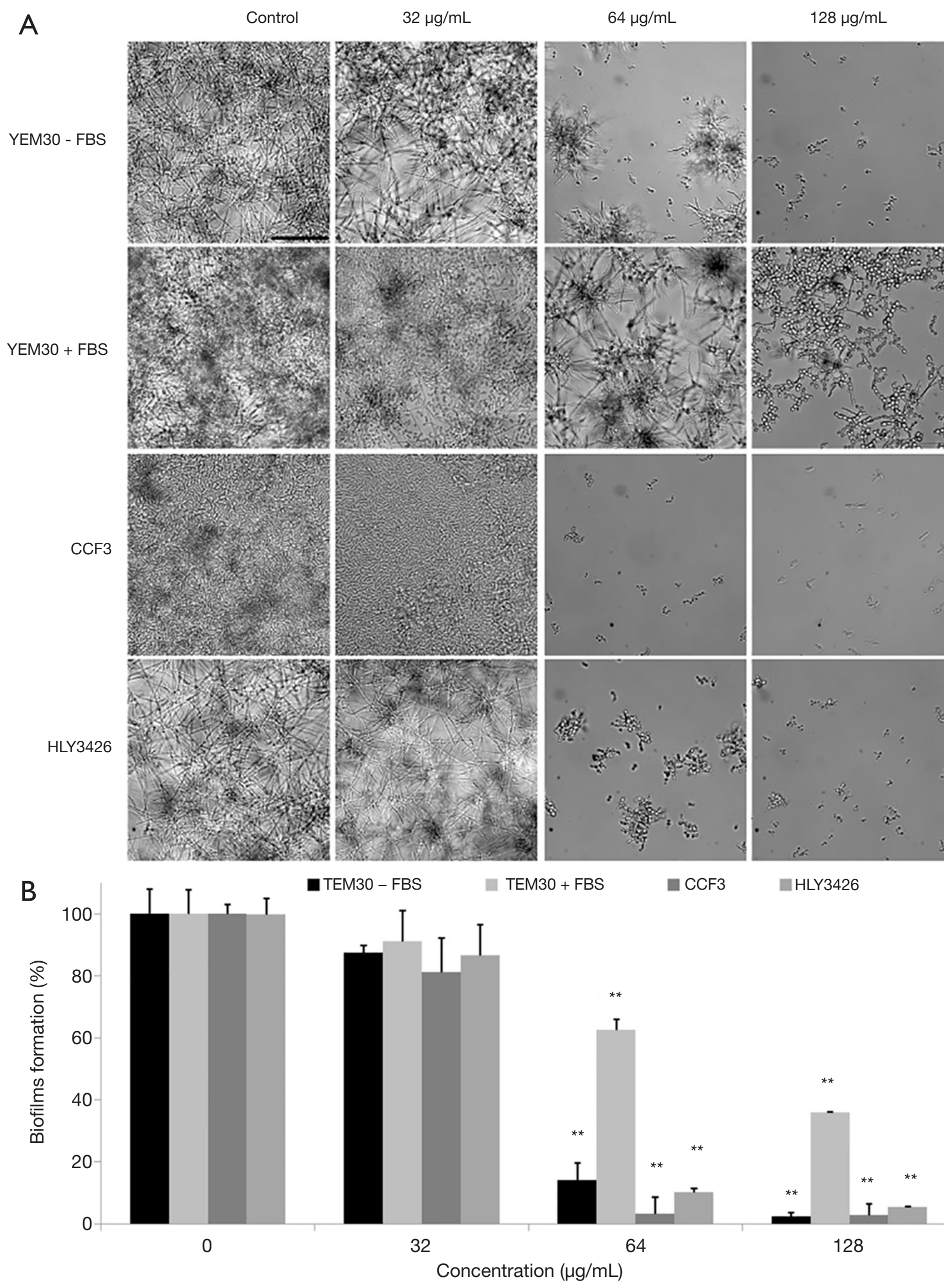

Figure 3 The inhibitory effect of TS against biofilm formation of *C. albicans*. YEM30, CCF3 and HLY3426 cells were diluted in the RPMI1640 medium or RPMI1640 plus 5% FBS medium and incubated with different doses of TS at 37 ℃ without shaking for 24 h. Microscopic observation (A) and XTT reduction assay (B) were used to detect the formed biofilm in different treatment groups. The bar in (A) indicates 50 μm. The results in (B) are shown as means ± standard deviation (SD). Asterisks (**) represent significance with P<0.01.

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TS inhibits the hyphal and biofilm formation via reducing the level of cAMP content

Knowing that cAMP occupies the central position in the regulatory network of hyphal development. The decrease of the intracellular cAMP level can weaken the yeast-to-hyphal morphology switch (8), we examined the intracellular cAMP level of *C. albicans* in the presence of TS. As shown in *Figure 4A*, TS treatment reduced the cAMP level in a dose-dependent manner, and positive control FLC had no significantly effect on the cAMP content. Subsequently, we conducted a cAMP rescue experiment to confirm whether cAMP was involved in TS-induced inhibition. The result showed that 1 mM of exogenous db-cAMP restored the hyphal growth of *C. albicans* in the present of 32 µg/mL TS treatment (*Figure 4B*). In addition, exogenous db-cAMP significantly recovered the growth of TS-treated biofilm from 24% to 48% (*Figure 4C*, $P<0.01$) and rebuilt the hyphal and biofilm formation as shown by microscopic observation (*Figuire 4D*). These results implied that the inhibitory effect of TS against flamentation and biofilm formation was attributed to the decrease of intracellular cAMP content.

TS decreases the transcriptional levels of hypha-specific genes

Since TS could inhibit the hyphal formation via reducing the cAMP content, the underlying molecular mechanisms were investigated by assessing the transcriptional levels of hypha-specific genes involved in Ras-cAMP-PKA signaling pathway (20). Results showed that TS could significantly downregulate all tested genes (*RAS1*, *ALS3*, *HWP1*, *CDC35*, *EFG1*, *ECE1*) except *PDE2*, encoding Pde2 protein to hydrolyze intracellular cAMP (*Figuire 4E*). These results implied that TS could inhibit flamentation via suppressing the expression of hypha-specific genes.

Repeated exposure does not result in TS-resistance

Knowing that resistance always complicates the management of fungal infection and limits the use of antifungal drugs, we evaluated the development of TS resistance upon 40-day repeated transfers and exposures to 32 µg/mL (half of MIC) of TS. The results showed that the MIC of TS remained at the level of 64 µg/mL during the 40-day experiment (*Figure 5*), implying that TS had little potential to cause *C. albicans* to develop resistance after long-time and repeated exposures.

TS shows low cytotoxicity

Tow normal cell lines and *C. elegans* were utilized to evaluate the toxicity of TS. The results showed that the IC_{50} of TS against HT22 (mouse hippocampal cell line) and HEK 293T (human embryonic kidney cell line) was 91.17 and 80.69 µg/mL, respectively, both of which were lower than the MICs of *C. albicans* (*Table 3*). In addition, neither 32 nor 64 µg/mL TS displayed a significantly effect on the survival of worms after 2-day incubation (*Figure 6*). These results implied that TS had low cytotoxicity *in vitro* and *in vivo*.

Discussion

A long-term therapy is necessary for most fungal infections due to their relapse and obstinacy. However, the adverse effects and drug-resistance usually hinder the management of infection and the usefulness of antifungal agents (24). Meanwhile, they also restrict the clinical application of many antifungal leading compounds. In this study, although TS showed moderate antifungal activity against *C. albicans*, it is worth noting that TS could exert its antifungal action independent of the ATP-binding cassette (ABC) transporters Cdr1 and Cdr2, and the major facilitator Mdr1 as well. Overexpression of efflux pump gene is a vital reason for drug resistance (25), implying that TS should have little chance to form resistance. The following subsequent serial passage experiment showed no significant change of MIC value during the 40-day induction, thus confirming the low resistant potential of TS. What's more, TS, known as a safe compound, indeed displayed low cytotoxicity against normal cell lines and *C. elegans*. Both low resistance and low toxicity of TS encouraged us to do in-depth research on its further clinical application.

C. albicans is known as the most common opportunistic pathogenic fungus which possesses a number of virulence factors facilitating colonization and infection of the host. The yeast-to-hyphal morphological transition (dimorphism) is considered to represent the most important virulence factor of *C. albicans* (26). Although both the budding yeast and filamentous hyphal form seem to be important for the pathogenicity of *C. albicans*, hyphal growth is a more critical driver of pathogenesis in that it is more invasive and contributes to host tissue damage (27). In symptomatic mucosal infections, the infected tissues often contain invading hyphae; likewise, hyphal defective mutants of *C. albicans* were found to have low virulence (28). Given the

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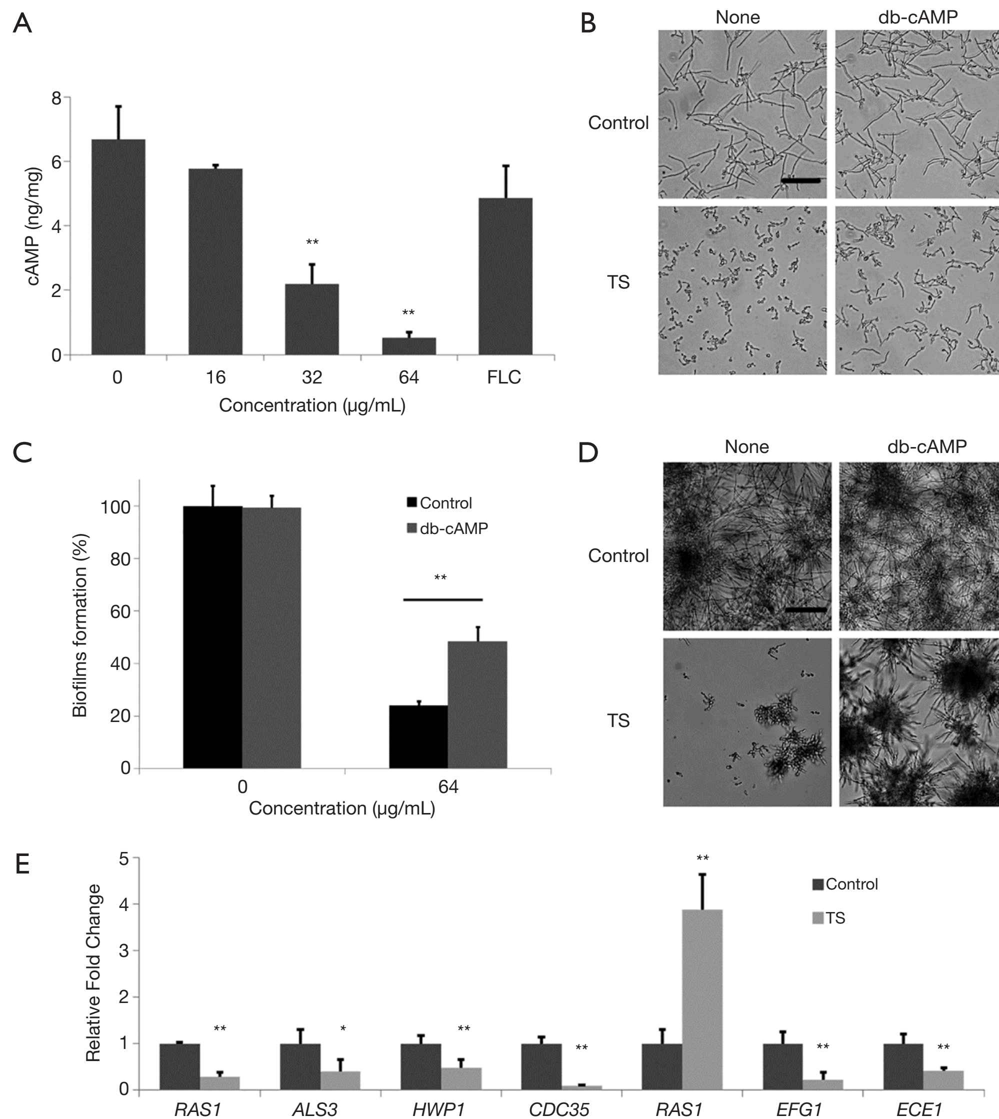

Figure 4 The effect of cAMP content on hyphal and biofilm formation in the presence of TS treatment. (A) TS reduced the intracellular cAMP level. Cells were incubated in RPMI1640 medium with different concentrations of TS or FLC for 12 h at 37 ℃. A cAMP Elisa Kit was used to measure the content of intracellular cAMP. (B) Exogenous cAMP restored TS-inhibited hyphal formation. YEM30 was diluted in RPMI1640 medium with or without 1 mM of db-cAMP and 32 μg/mL of TS. After 6-h incubation at 37 ℃, cells were imaged under a microscope in the bright field. (C) and (D) exogenous cAMP partly restored TS-inhibited biofilm formation. The XTT reduction assay (C) and microscopic observation (D) were used to detect the formed biofilm under different treatments. (E) TS suppressed the expression of hypha-specific genes involved in Ras-cAMP-PKA signaling pathway. The total RNAs of TS treated cells were extracted by the hot phenol method, and the transcriptional levels of hypha-specific genes was measured by quantitative real-time RT-PCR. The bars in (B) and (D) indicate 50 μm. Results in (C) are shown as means ± standard deviation (SD). Asterisks (*) represent significance with $P<0.05$. Asterisks (**) represent significance with $P<0.01$.

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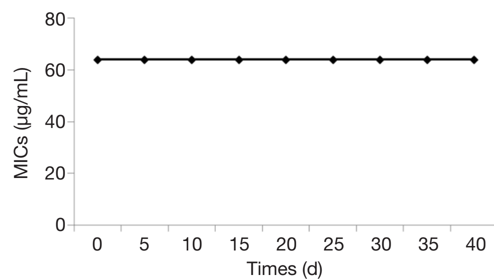

Figure 5 The capacity of TS-resistance formation. Prepared YEM30 cells were diluted in YPD broth and incubated with 32 μg/mL of TS at 30 ℃, 200 rpm. Every day, cells were transferred and measured by serial passage for 40 days. The results are shown as means ± standard deviation (SD).

Table 3 The IC_{50} value of TS against different cell lines

Cell lines	IC_{50} (μg/mL)
HT22 (mouse hippocampal cell line)	91.17
HEK 293T (human embryonic kidney cell line)	80.69

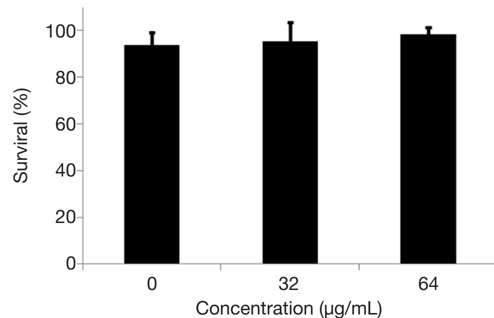

Figure 6 The toxicity of TS against *C. elegans*. Wild type *C. elegans* N2 was incubated with different doses of TS in the BHI medium at 25 ℃. After 2-day culture, the worms were observed using a microscope to calculate the survival rates. Results were shown as means ± standard deviation (SD).

importance of hyphae in virulence, morphological transition of *C. albicans* has been regarded as an important therapeutic target (27).

In the present study, we found that TS could inhibit the yeast-to-hyphal transition *in vitro*. Since TS treated cells displayed a growth reduction, we used FLC, which is a fungistatic drug affecting growth, also has an effect on hyphae growth because of the growth arrest during the germ tube formation, as a positive control to explored the inhibition of hyphal formation due to the growth reduction or not. Results showed that TS had a similar effect as FLC on the TS treated filamentation, and TS could effectively inhibit the hyphal formation in the hypha-induced serum medium as well. We detected the effect of TS and FLC on the cellular level of cAMP. Results showed that FLC had no significantly effect on the cAMP content, on the contrary, TS could significantly decrease the intracellular cAMP level, which implied that TS acted different modes from FLC to inhibit filamentation and the inhibitory effect was at least not all due to the suppression of proliferation. In addition, in the presence of serum, a strong promoter of filamentation, TS-treated cells could proliferate and remain in the yeast form or develop short hyphae at a low dose, and TS could significantly inhibit biofilm formation at the same concentrations of serum-free medium. These phenomena confirm that this inhibitory effect of TS on filamentation is at least not all due to the suppression of proliferation.

Hyphal growth is triggered by multiple environmental cues and mediated by a series of signal transduction pathways (8). cAMP, known as a second messenger, is crucial for the Ras1-cAMP-Efg1 signaling pathway, which regulates the yeast-to-hyphal transition via controlling the synthesis of cAMP (29). A decrease in intracellular cAMP level could block *C. albicans* in the yeast form irrespective of whether it is under the hypha-induced condition or not (30), implying that a certain level of cAMP is required for filamentation. It was found in our study that TS could decrease the intracellular cAMP level, and exogenous cAMP could rescue the inhibition of hyphae. In addition, the decreased transcriptional levels of hypha-specific genes involved in Ras1-cAMP-Efg1 pathway induced by TS was also obtained. Especially, the downregulation of *CDC35* expression, the cAMP synthetic gene, and the upregulation of *PDE2*, the cAMP hydrolase gene give a molecular reason for the cAMP content reduction. These results suggest that TS could decrease the content of cAMP by affecting the expression of hypha-specific genes to inhibit the yeast-to-hyphal transition of *C. albicans*.

Biofilm is structured microbial communities attaching to the surface of biomaterial or non-biomaterial, which consist of mixture of yeast, hyphae and matrix. Hyphal formation often occurs in *Candida* infections on implanted devices such as indwelling catheters or prosthetic heart valves (31). Crucially, they are seriously resistant to antimicrobial agents due to their limited penetration and high antimicrobial tolerance. Biofilm formation enhances the difficulty of treatment and represents one of the most

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well-known virulence factors. Our work showed that TS could significantly retard biofilm formation. To detect the inhibitory effect via inhibition of filamentation or proliferation, the non-filamentous and its complementation strains were used to test the effect of TS on their biofilm formation. As TS has a similar inhibitory effect on the two strains, the suppression of biofilm formation induced by TS is partial to the suppression of proliferation. Moreover, exogenous cAMP could rescue the inhibition of biofilm formation induced by TS as well, implying that both the inhibition of proliferation and filamentation result in the suppression of biofilm formation.

In summary, we evaluated the cytotoxicity and antivirulence activity of TS against *C. albicans*, and preliminarily explored its action mode of hyphal inhibition. Our work may provide a friendly antifungal natural product to expand the current antifungal agent arsenal via blocking the virulence factors of *C. albicans*.

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Footnote

Conflicts of Interest: GB serves as an unpaid managing editor of Annals of Translational Medicine. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the ethics committee at Affiliated Hospital of Xuzhou Medical University, the number of the approval was XXYFY2019-KL141-01.

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