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ORIGINAL PAPER

# Study on the antifungal activity and mechanism of tea saponin from *Camellia oleifera* cake

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## Abstract

The purpose of this study was to isolate tea saponin from defatted *C. oleifera* cake and explore its potential antifungal activity and mechanism. UHPLC–MS/MS identified the compounds, and the antibacterial activity of tea saponin was determined by the inhibition zone method and double dilution method. In addition, the influence of tea saponin on the cell membrane, hyphae, and biofilm was studied to explore the antifungal mechanism of tea saponin. The results showed that the purity of tea saponin was 90.61%, and the main components of *C. oleifera* saponins were oleiferasaponin $D_3$. Tea saponin has an apparent inhibitory effect on fungus. The minimum inhibitory concentrations (MIC) of the tea saponin against *C. albicans*, *S. cerevisiae*, and *Penicillium* were 0.078, 0.156, and 0.156 mg/mL, while the minimum fungicidal concentrations (MFC) were 0.312, 0.625, and 0.625 mg/mL, respectively. Tea saponin could destroy the cell membrane structure, which led to the leakage of cell contents and inhibited the growth of mycelium, reduced cell adhesion and aggregation, and effectively inhibited the formation of biofilm of *C. albicans*. Transcriptomic analyses indicated that tea saponin could down-regulate the expression of several hyphae- and biofilm-related genes (ALS3, ECE1, HWP1, EFG1, and UME6). This study confirmed that tea saponin from *C. oleifera* cake can be used as an effective source of natural antifungal agents and provide guidance on their utilization in the field of food safety.

**Keywords** Tea saponin · *Camellia oleifera* cake · Antifungal activity · Antifungal mechanism

## Abbreviations

| | |
|---|---|
| *C. oleifera* | *Camellia oleifera* Abel |
| *C. albicans* | *Candida albicans* |
| *S. cerevisiae* | *Saccharomyces cerevisiae* |
| AO/EB | Acridine orange/ethidium bromide |
| SEM | Scanning electron microscope |
| MIC | Minimum inhibitory concentrations |
| MFC | Minimum fungicidal concentrations |
| UHPLC–MS/MS | Ultrahigh pressure liquid chromatography–high-resolution mass spectrometer |

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## Introduction

Microorganisms breed easily in food during processing and storage, resulting in food contamination, deterioration, and even harm to human health. Diseases caused by foodborne pathogenic microorganisms have seriously endangered public safety [1]. According to the World Health Organization (WHO) statistics, 600 million people (almost 1 in 10 people in the world) fall ill after eating contaminated food, and 420 000 die every year. Fungi, one of the main microorganisms that cause food spoilage, can grow in almost all kinds of food. The toxins produced by fungus and the growth of the pathogenic fungus will damage human health [2]. According to the reports, there are more than 5 million species of fungi in the world, and about 300 species can cause human diseases that result in a large number of morbidity and mortality [3]. *Penicillium*, *Saccharomyces cerevisiae*, and *Candida albicans* were considered common species of spoilage microorganisms. *Penicillium* spp. were considered as great producers of mycotoxins and other secondary metabolites, which can be toxic and harmful to humans [4]. Moreover, yeast is known to play an important

role in food fermentation, but they may also be one of the main causes of food spoilage. A rising number of yeast species, such as *S. cerevisiae* that are found in food, are being considered as potential pathogens [2]. *Candida albicans*, a pathogenic fungus widely found in nature, can cause invasive infections and harm to people's lives [5], especially for people with low immunity (such as AIDS patients, cancer patients) such that the fatality rate is as high as 40% [6]. It is generally believed that the pathogenicity of the *C. albicans* is closely related to hyphal and biofilm formation [7, 8]. *Candida albicans* can adhere to the surface of cells or materials to proliferate and form hyphae, followed by combining with extracellular polymers to form a biofilm, increasing the cells' resistance to antifungal drugs and reducing the damage of the host defense [9]. For reducing the harm of foodborne pathogenic microorganisms, the food industry uses a variety of synthetic antimicrobial agents (orbates, nitrates, benzoates, etc.) to inhibit the growth of microorganisms. Nevertheless, artificial antimicrobial agents may have adverse effects on humans and the environment [10]. Natural antimicrobial agents are bioactive substances derived from plants, animals and beneficial microorganisms (essential oils and extract derived from plants, enzymes obtained from animal sources, bacteriocins from microbial sources, organic acids, etc.). Compared to synthetic antimicrobial agents, natural antimicrobial agents are relatively safer and have become more popular [11].

*Camellia oleifera Abel* (*C. oleifera*) is one of the four largest woody oil crops in the world [12]. *Camellia oleifera* cake, a by-product, is produced after oil extraction from *C. oleifera* seeds and contains abundant active substances such as tea saponin [13], polysaccharide [14], protein [15], and polyphenol [16]. However, only a small amount of *C. oleifera* cakes are used to clean fish ponds or low value-added fertilizers, while most of them are discarded, resulting in a waste of resources [17]. Tea saponin is a mixture of oleanane-type pentacyclic triterpenoid saponins with similar structures and a kind of natural surfactant with excellent performance. Tea saponin also has better biological activities, such as antioxidative, antiinflammatory [18], anticancer [19], antibacterial [20], and antitumor [2] acions and can be widely used in food and medicine. As a triterpene saponin, tea saponin has been proved to have an apparent inhibitory effect on the growth of some fungi (*Fusarium*, *Penicillium*, yeast) [21, 22]. However, the current research of antimicrobial activity on tea saponin is mainly limited to its antimicrobial effect, and relatively little is known about its antifungal mechanisms.

In this study, tea saponin was isolated from defatted *C. oleifera* cake and identified by UHPLC–MS/MS. Then, the antifungal activity of the tea saponin and the antifungal mechanism were studied through the influence of the tea saponin on the cell membrane, mycelial growth, and biofilm of *C. albicans*, and the expression of hypha- and biofilm-related genes was investigated by qRT-PCR, providing theoretical guidance for the development of tea saponin as a natural antifungal agent and improving the utilization value of camellia cake and tea saponin.

## Materials and methods

### Preparation of *Camellia oleifera* cake

Fresh *Camellia oleifera* fruits were collected from Jieyang City, Guangdong Province, China, on November 17, 2020. The *C. oleifera* seeds were dried in a heat pump at 60 °C and pressed in an oil press. *Candida oleifera* cake was collected after camellia seed oil was removed.

### Fungal strains

Fungal strains include *S. cerevisiae* (ATCC9763), *Penicillium*, and *C. albicans* (CMCC98001 and ATCC10231), among which *S. cerevisiae* and *C. albicans* were from Shanghai Preservation and Biotechnology Center, China. *Penicillium* was isolated from raisins in the laboratory.

### Extraction and isolation of tea saponin

*Camellia oleifera* cake was crushed in a pulverizer and passed through a 60-mesh screen. The powders were cryopreserved at − 20 °C. *Camellia oleifera* cake powders were extracted with 60% ethanol solution at the ratio of 1:20.5, the extraction temperature was 80 °C, and the extraction time was 3.2 h. Then, the supernatant was collected after centrifugation as a crude extract. The activated AB-8 macroporous resin was filled into the chromatographic column with a volume of 200 mL. The crude extract of tea saponin was passed through a chromatography column under the following conditions: the concentration of tea saponin was 50 mg/mL, the pH was 6, and the flow rate was 2 mL/min. Then, the impurities in turn were removed with distilled water and 30% ethanol solution successively, and, finally, tea saponin was eluted with 80% ethanol solution at a flow rate of 2 mL/min. The purified tea saponin solution removed the ethanol by rotary evaporator and then dried in an oven at 60 °C to get tea saponin powder. A proper amount of tea saponin powder was weighed and prepared into 1 mg/mL solution by 80% ethanol. The content of tea saponin was determined by the concentrated sulfuric acid–vanillin method [23]. According to Formula (1), the purity of tea saponin was calculated:

$$\text{The purity of tea saponin } (\%) = \frac{A + 0.0113}{0.9061C}. \quad (1)$$

## Ultrahigh pressure liquid chromatography–high resolution mass spectrometry (UHPLC–MS/MS)

The purified tea saponin was identified and analyzed by UHPLC–MS/MS with ACQUITYUPLCBEHC18 column (1.7 μm). The mobile phase contains 0.1% formic acid solution (A) and acetonitrile (B). The gradient elution program was as follows: 2% B (0–5 min); 98% B (5–20 min); 2% B (20–25 min); 2% B (25–30 min). The flow rate was 0.4 mL/min and the injection volume was 2 μL. MS analysis was performed with an electrospray ionization (ESI) source in the negative ion mode to analyze the purified tea saponin. The mass scan range was 100–1400 m/z, sheath gas velocity 45 arb, auxiliary gas velocity 15 arb, the capillary temperature 320° C, spray voltage 3.2 kV, auxiliary gas heater temperature 300° C, and atomization pressure 30 Psi.

## The antifungal activity of tea saponin

### The inhibition zone method

Tea saponin was dissolved in sterile water to prepare solutions at 0.625, 1.25, 2.5, 5, and 10 mg/mL. Medium (20 mL) and 100 μL cell suspension ($10^7$ CFU/mL) were added to the Petri dish (*S. cerevisiae* and *Penicillium*-added PDA medium, *C. albicans*-added SDA medium). The Oxford cups were placed on the medium, 100 μL of tea saponin solution was added into the Oxford cup, and sterile water was used as a control group. *Candida albicans* and *S. cerevisiae* were cultivated at 28 °C for 2 days, and *Penicillium* was cultivated at 28 °C for 5 days. After incubation, the inhibitory zone diameter was measured.

### Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

MIC and MFC experiments were performed using the double dilution method [24]. Tea saponin was dissolved in SDB medium to prepare solutions at 0, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5, and 10 mg/mL. 5 mL tea saponin solution and 50 μL cell suspension ($10^7$ CFU/mL) were added to the different test tubes, respectively, and culture at 28 °C in a shaker (*C. albicans* cultured for 1 day, *S. cerevisiae* and *Penicillium* for 2 days). The lowest concentration that was not visibly turbid was the minimum inhibitory concentration (MIC). After determination of the MIC, 50 μL fungus suspension from all test tubes with no growth was added to the solid medium and culturing continued for 48 h. The lowest concentration without fungi growth was the minimum fungicidal concentration (MFC).

### Time-inhibition curve of *C. albicans*

Tea saponin was dissolved in an SDB medium to obtain different concentrations of tea saponin solution (0, 0.5 MIC, 1 MIC, and 1 MFC). The *C. albicans* suspension ($10^7$ CFU/mL) was added to the medium which contains different concentrations of tea saponin and the inoculation amount was 1% of the medium. The OD600 values were measured after being cultured for a while.

## Antifungal mechanism

### Assay of relative electrical conductivity and nucleic acid leakage

The relative electric conductivity was determined by referring to the method of Cai slightly modified [25]. The *C. albicans* suspension ($10^7$ CFU/mL) was centrifuged and washed with sterile water until the conductivity of the suspensions was close to that of sterile water, then resuspended and mixed with an equal volume of tea saponin solution (0 MIC, 2 MIC, and 2 MFC). After being cultured at 28 °C for a while and centrifuged, the electric conductivity of the supernatant was determined.

The effect of tea saponin on nucleic acid release from *C. albicans* was determined by referring to the method of Dong, slightly modified [1]. The *C. albicans* suspension ($10^7$ CFU/mL) was centrifuged and washed with PBS buffer three times, then resuspended and mixed with an equal volume of tea saponin solution (0 MIC, 2 MIC, and 2 MFC). After being cultured at 28 °C for a while and centrifuged, the OD260 value of the supernatant was determined.

### The observation of the hyphae and cells' surface morphology

Mycelium growth was observed by an optical microscope. Circular glass slides were put into a 12-well culture plate. 1 mL *C. albicans* suspension ($10^6$ CFU/mL) which was resuspended by RPMI-1640 medium and 1 mL tea saponin solution (0 MIC, 2 MIC, and 2 MFC) were added to the 12-hole culture and cultured at 28 °C for 8 h. The growth of mycelium was observed under a microscope.

The surface morphology of *C. albicans* was observed by scanning electron microscope (SEM). The activated cell solution ($10^7$ CFU/mL) was resuspended by PBS buffer. Then, an equal volume of tea saponin solution (0 MIC, 2 MIC, and 2 MFC) was added and cultured for 24 h. After centrifugation, the cells were washed three times with PBS buffer and then fixed with 2.5% glutaraldehyde solution overnight at 4 °C. After centrifugation, the cells were washed three times with PBS buffer, followed by dehydration using a series of ethanol solutions in eight steps of 15 min each:

30% ethanol, 50% ethanol, 70% ethanol, 80% ethanol, 90% ethanol, 100% ethanol, 100% ethanol, and 100% ethanol. The dehydrated and dried cells were gold coated with the help of a vacuum coating instrument and finally the surface morphology of the *C. albicans* was observed by using SEM.

#### Test for inhibition and eradication of biofilm

Biofilms of *C. albicans* were produced on a 96-well polystyrene plate. The *C. albicans* suspension ($10^7$ CFU/mL) was resuspended with RPMI-1640 medium, and tea saponin was dissolved in RPMI-1640 medium. 100 µL *C. albicans* suspension and 100 µL tea saponin solution (0–2.5 mg/mL) were added to each well, and the plate was incubated at 28 °C for 24 h to evaluate the inhibitory effect of tea saponin on the biofilm. In the new 96-well polystyrene plate, 200 µL *C. albicans* suspension was added to each well, and the plate was cultured at 28 °C for 24 h. Then it was sucked out of the medium, 200 µL tea saponin solution (0–2.5 mg/mL) was added and continued to culture for 24 h to evaluate the eradication effect of tea saponin on mature biofilm.

According to the description of Liang, slightly modified, the amount of biofilm was determined by crystal violet staining [26]. After cultivation, the medium was sucked out, and the cells were washed with PBS buffer, dried in a ventilated place and stained with 0.4% crystal violet for 60 min. After repeatedly washing with PBS buffer and air drying, 30% glacial acetic acid (150 µL) was added to each well and stood at room temperature for 90 min. The OD595 value was measured by an enzyme standard instrument.

#### Evaluation of cell adhesion and aggregation

The adhesion of *C. albicans* was determined by referring to the description of Yang, slightly modified [27]. 100 µL *C. albicans* suspension ($10^6$ CFU/mL) and 100 µL tea saponin solution (0, 1 MIC, 2 MIC, 1 MFC, and 2 MFC) were added to each well of a 96-well polystyrene plate and incubated at 28 °C for 90 min. The amount of cells was determined by crystal violet staining.

The aggregation of *C. albicans* was observed with a microscope. The method was the same as above after *C. albicans* was treated with tea saponin solution (0 MIC, 0.5 MIC, 1 MIC, and 1 MFC), and the medium was sucked out. The cells were washed with PBS buffer, dried in a ventilated place and stained with 0.4% crystal violet for 60 min. After washing with PBS buffer and air dried, the aggregation of *C. albicans* was observed by microscope.

#### Fluorescence microscope assay

1 mL of *C. albicans* suspension (10^7 CFU/mL) and 1 mL of tea saponin solution (0 MIC, 2 MIC, and 2 MFC) were added to the laser confocal Petri dish and incubated at 28 °C for 24 h. After cultivation, the medium was sucked out and the cells washed with PBS buffer. The biofilm cells were dealt with AO/EB dye for 20 min in a dark environment and washed with PBS buffer after sucking out the dye and observed under an inverted fluorescence microscope.

#### Quantitative real-time PCR analysis

Quantitative real-time PCR was used to explore the expression of hypha- and biofilm-related genes (ECE1, EFG1, ALS3, HWP1, and UME6) of *C. albicans* under treatment with tea saponin. *Candida albicans* ($10^7$ CFU/mL) was resuspended in RPMI-1640 medium and mixed with the same volume of tea saponin solution (2 MFC) to culture at 28 °C and 220 r/min for 6 h. After cultivation, the cells were washed three times with PBS buffer and then centrifuged to collect the cells. The total RNA was isolated by the CTBA method [28], and cDNA was synthesized using a cDNA synthesis kit (iScript™ gDNA Clear cDNA Synthesis Kit). PCR was conducted with the SYBR Premix EX Taq in an Applied Biosystems QuantStudio 6 Flex system. The primer sequences used for gene amplification are listed in Table 1. 18SrRNA, a housekeeping gene, was used as an internal control.

### Statistical analysis

The data were analyzed by SPSS Statistics 26.0 software, and the results were expressed as mean ± standard deviation (SD). Single-factor variance and Duncan test were used to determine whether there was a significant difference in the group.

**Table 1** Primers used in qRT-PCR analysis

| Gene | Primer design |
|---|---|
| ALS3 | F-CAACTTGGGTTATTGAAACAAAAACA |
| | R-AGAAACAGAAACCCAAGAACAACCT |
| HWP1 | F-GCTCAACTTATTGCTATCGCTTATTACA |
| | R-GACCGTCTACCTGTGGGACAGT |
| ECE1 | F-GCTGGTATCATTGCTGATAT |
| | R-TTCGATGGATTGTTGAACAC |
| EFG1 | F-CATCACAACCAGGTTCTACAACCAAT |
| | R-CTACTATTAGCAGCACCACCC |
| UME6 | F-GACTAAGATGGCTGGGTCTCA |
| | R-TGGAGTCGGAATTACCAGTGT |
| 18S rRNA | F-AATTACCCAATCCCGACAC |
| | R-TGCAACAACTTTAATATACGC |

## Results and discussion

### Antifungal activity of tea saponin

#### Qualitative analysis of tea saponin from *C. oleifera* cake

The chemical constituents of saponins isolated from *C. oleifera* cake were identified by UHPLC–MS/MS in negative ion model and the chromatogram as shown in Fig. 1. Ten compounds were initially detected and the results are shown in Table 2. The main component of *C. oleifera* saponins was compound 8, and its mass spectrum is shown in Fig. 2a. Compound 8 (tR 15.33 min) contained the parent ion at m/z 1201.56494 and characteristic fragment ions at m/z 1021.50812 ([M-H-C6H11O6]-). A prominent peak at m/z 665.39453 corresponded with the triterpenoid moiety fragmented from the parent molecule [17]. Therefore, its molecular formula was postulated as C58H89O26. Compound 8 had been named oleiferasaponin $D_3$ [29] whose structure was determined to be 3-*O*-*β*-D-xylopyranosyl-(1 → 2)-*β*-D-galactopyranosyl-(1 → 3)-[*β*-D-galactopyranosyl-(1 → 2)]-*β*-D-glucuronopyranosyl-22α-tigloyloxyolean-12-ene-23-al-16α,28-diol (Fig. 2b).

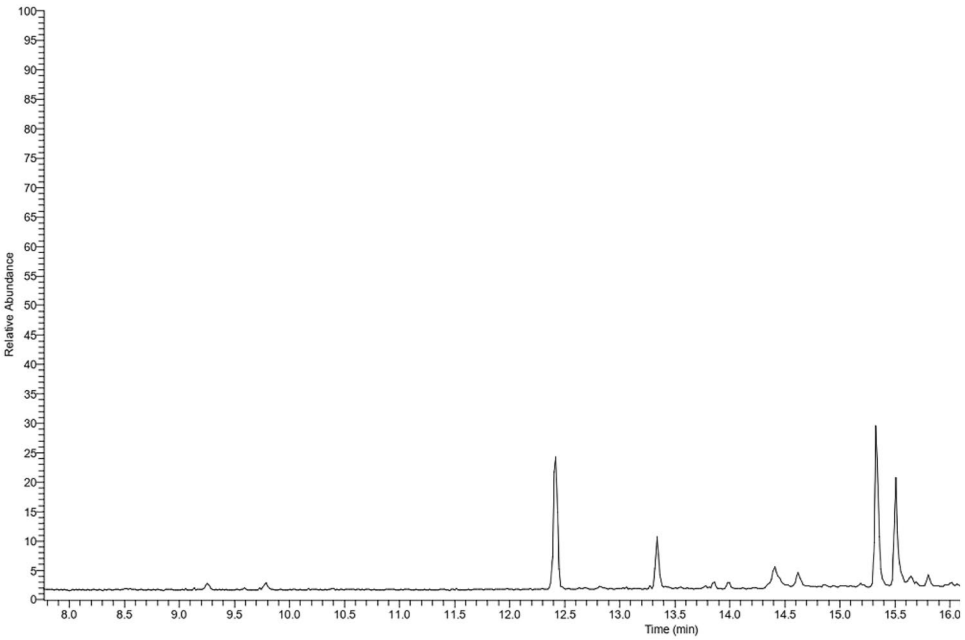


**Fig. 1** Base peak chromatogram of tea saponins from *C. oleifera* cake

**Table 2** Characterization of chemical compounds in saponins isolated from *C. oleifera* cake by UHPLC–MS/MS in negative mode

| NO | tR(min) | Molecular formula | Measured mass (m/z) | Proposed compound |
|---|---|---|---|---|
| 1 | 8.52 | C15 H10 O6 | 287.05487 | Kaempferol(isomers) |
| 2 | 8.52 | C33 H40 O20 | 757.21844 | 3-{[4,5-dihydroxy-6-(hydroxymethyl)-3-{[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxy}oxan-2-yl]oxy}-5-hydroxy-2-(4-hydroxyphenyl)-7-{[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}-4H-chromen-4-one |
| 3 | 9.26 | C21 H20 O11 | 449.10803 | Trifolin |
| 4 | 9.26 | C27 H30 O15 | 595.16571 | 2-(3,4-Dihydroxyphenyl)-5-hydroxy-4-oxo-4H-chromen-7-yl 6-*O*-(6-deoxy-alpha-L-mannopyranosyl)-beta-D-glucopyranoside |
| 5 | 9.26 | C15 H10 O6 | 287.05493 | Kaempferol(isomers) |
| 6 | 13.98 | C15 H10 O6 | 287.05490 | Kaempferol(isomers) |
| 7 | 14.62 | C22 H26 O6 | 387.18015 | Bis(methylbenzylidene)sorbitol |
| 8 | 15.33 | Unkown | 1201.56494 | |
| 9 | 15.51 | C24 H30 O6 | 415.21149 | Bis(4-ethylbenzylidene)sorbitol |
| 10 | 15.51 | C30 H46 O4 | 471.34711 | 18-*β*-Glycyrrhetinic acid |

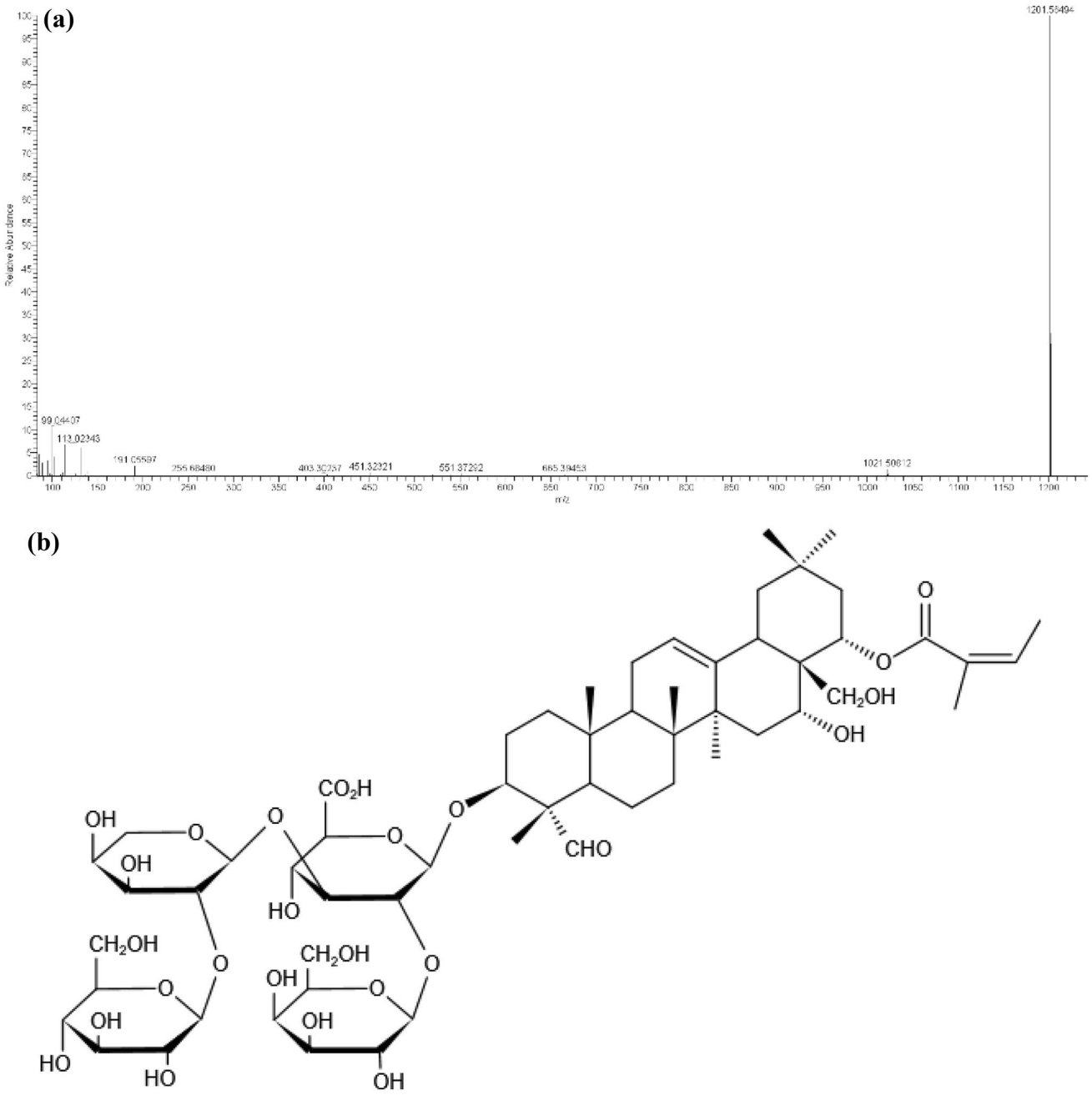


**Fig. 2** Mass fragment of oleiferasaponin $D_3$

### Determination of antifungal activity

The antifungal effect of tea saponin was investigated by measuring the inhibition zone diameter using the Oxford cup method. The antifungal results of tea saponins are shown in Table 3. In the range of 0–10 mg/mL concentration, tea saponin had an apparent inhibitory effect on *S. cerevisiae*, *Penicillium*, and *C. albicans*, and the size of the inhibition zone increased with increasing concentration. The MIC values of tea saponins against *C. albicans*, *S. cerevisiae*, and *Penicillium* were 0.078, 0.156, and 0.156 mg/mL, respectively, and the MFC values were 0.312, 0.312, and 0.625 mg/mL, respectively. These results showed that tea saponin had a pronounced antifungal effect against *C. albicans*, *S. cerevisiae*, and *Penicillium*, which was similar to that of previous studies [21, 30]. The time–inhibition curve of tea saponin against *C. albicans* is shown in Fig. 3. *Candida albicans* began to proliferate after culturing for 4 h and tended to grow slowly after 24 h without tea saponin treatment. With the increase in tea saponin concentration, the growth of *C.*

**Table 3** Antifungal activity results of tea saponin (determination of inhibition zone size, MIC and MFC)

| Species | Inhibition zone diameter (mm) mean ± SD | | | | | MIC | MFC |
|---|---|---|---|---|---|---|---|
| | 10 mg/mL | 5 mg/mL | 2.5 mg/mL | 1.25 mg/mL | 0.625 mg/mL | | |
| *Penicillium* | 21.57 ± 0.07a | 18.26 ± 0.07c | 15.77 ± 0.37ef | 13.78 ± 0.24 h | - | 0.156 | 0.625 |
| *S. cerevisiae* | 18.57 ± 0.32c | 15.58 ± 0.11efg | 13.67 ± 0.42 h | 12.06 ± 0.38i | 10.82 ± 0.22j | 0.156 | 0.312 |
| *C. albicans* (CMCC98001) | 19.41 ± 0.29b | 18.15 ± 0.24c | 16.24 ± 0.16de | 14.77 ± 0.24 g | 10.63 ± 0.75j | 0.078 | 0.312 |
| *C. albican s*(ATCC10231) | 18.35 ± 0.13c | 16.90 ± 0.50d | 15.19 ± 0.67 fg | 13.20 ± 0.81 h | 11.35 ± 0.58ij | 0.078 | 0.312 |

The values of different letters (mean ± standard deviation) are significantly different ($P < 0.05$)

-, no inhibition zone

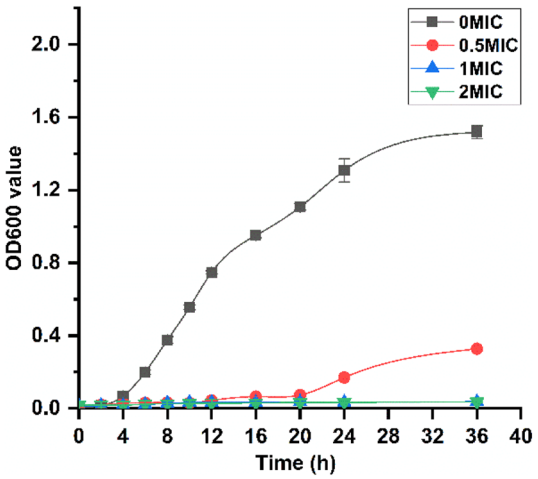


**Fig. 3** Effect of tea saponin on the growth of *C. albicans*

*albicans* was inhibited. When the concentration of tea saponin reached 0.5 MIC, *C. albicans* began to grow slowly after 20 h. When the concentration of tea saponin reached 1 MIC and 2 MIC, *C. albicans* almost did not grow within 0–36 h. The results further confirmed the inhibitory effects of tea saponin on the proliferation of *C. albicans*.

### Effect of tea saponin on mycelial growth of *C. albicans*

The transformation of *C. albicans* from yeast to mycelial type is one of the main virulence factors, and hyphae play a vital role in the formation of biofilm and the pathogenesis of fungal infection [31]. We had carried out a study about the effect of tea saponin on the mycelial growth of *C. albicans*. The result is shown in Fig. 4, and cells were gathered and formed hyphae in the blank group. At the same time, most of the cells treated with tea saponin were scattered, and it was observed that the growth of hyphae was inhibited. When the tea saponin concentration was 0.5 MIC, the aggregation of the cell was decreased and formed a small number of hyphae. When the tea saponin concentration reached 1 MIC or 1 MFC, there was nearly no hyphae formation. The above results showed that tea saponin could also inhibit the transformation of *C. albicans* from yeast cells to hyphal cells. It was widely accepted that the prerequisite for biofilm formation was the transformation of *C. albicans* from yeast cells to hyphal cells [31]. Therefore, tea saponin might also inhibit the biofilm formation. It had been shown that saponins could damage the cell wall and cell membrane of *C. albicans* and induce cell structural changes, which led to cell lysis and death. At the same time, saponins could also inhibit mycelium growth and biofilm formation, leading to reduction in the pathogenicity of *C. albicans* [32, 33]. Therefore, we hypothesized that tea saponin inhibited *C. albicans* proliferation might by damaging the cell membrane, inducing apoptosis and inhibiting the mycelium growth and biofilm formation.

## The damage of tea saponin to cell membrane structure

Cell membrane played an essential role in controlling the ingress and egress of substances. The disruption of cell membrane would lead to the leakage of small molecules ($K^+$, $Na^+$), nucleic acid, and intracellular protein from the cell, and loss of function [34]. The change of the cell membrane permeability could be reflected by the relative electrical conductivity change. As shown in Fig. 5, relative conductivity in the blank group increased slowly and was 18.67% after culturing for 12 h, which might be caused by the normal apoptosis of some cells. In the tea saponin treatment groups, the relative conductivity increased significantly with the increase of time. After being treated with 1 MIC and 1 MFC for 12 h, the relative conductivity was 25.90% and 40.48%, respectively. Therefore, tea saponins could aggravate the leakage of cell contents, and the degree of leakage increased significantly with the increase of tea saponin concentration.

The result of nucleic acid leakage is shown in Fig. 6; compared with the blank group, the leaked content of nucleic acid increased significantly after tea saponin treatment, and the leaked degree of nucleic acid increased with the extension of time, which indicated that the cell membrane had

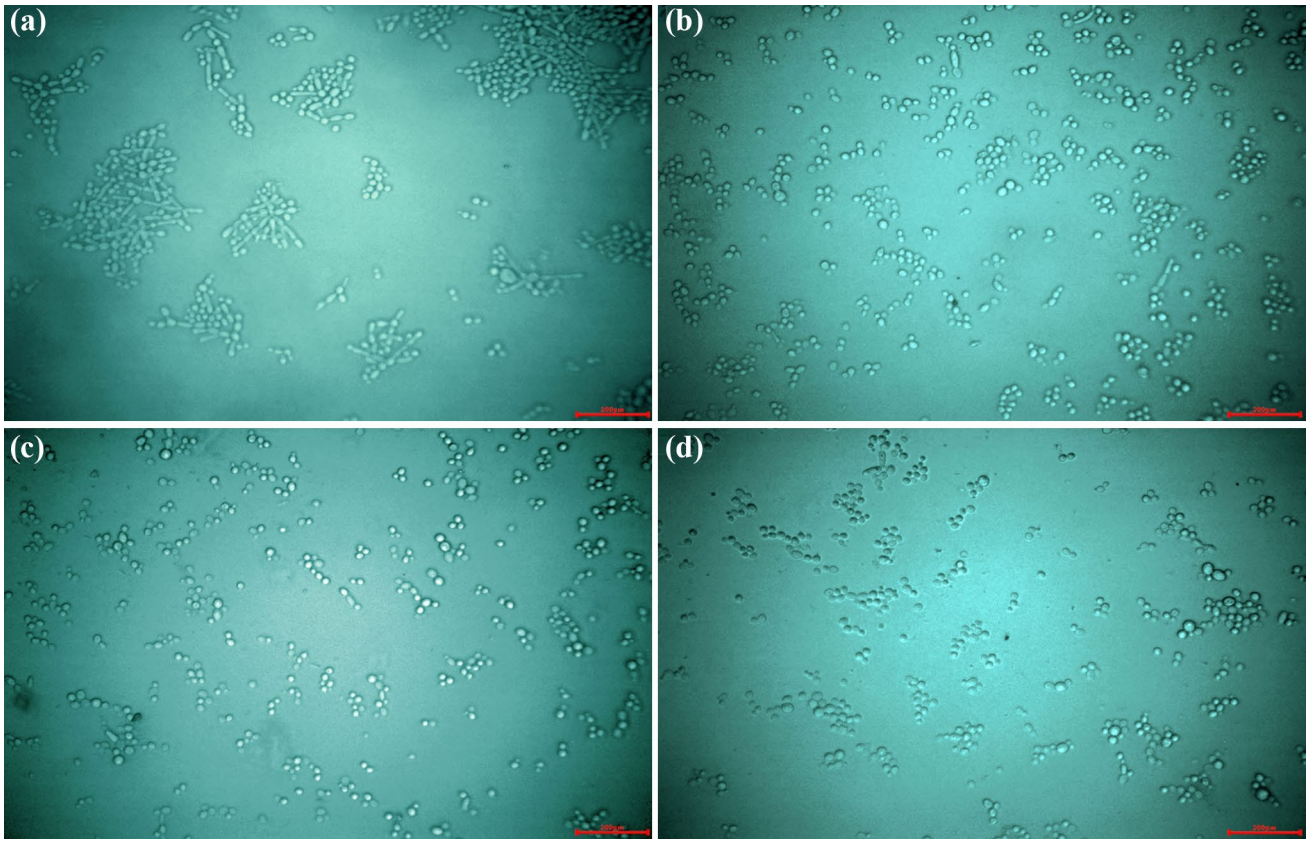


**Fig. 4** The inhibitory effect of tea saponin on the mycelial growth of *C. albicans* was observed under a microscope. **a** *C. albicans* treated with 0 MIC. **b** *C. albicans* treated with 0.5 MIC. **c** *C. albicans* treated with 1 MIC. **d** *C. albicans* treated with 1 MFC

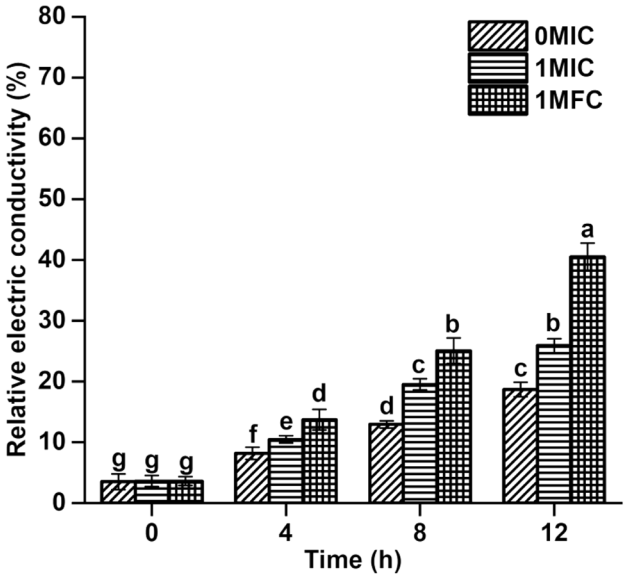


**Fig. 5** Relative conductivity of *C. albicans* after treatment with tea saponin (0 MIC, 1 MIC, 1 MFC) for different times

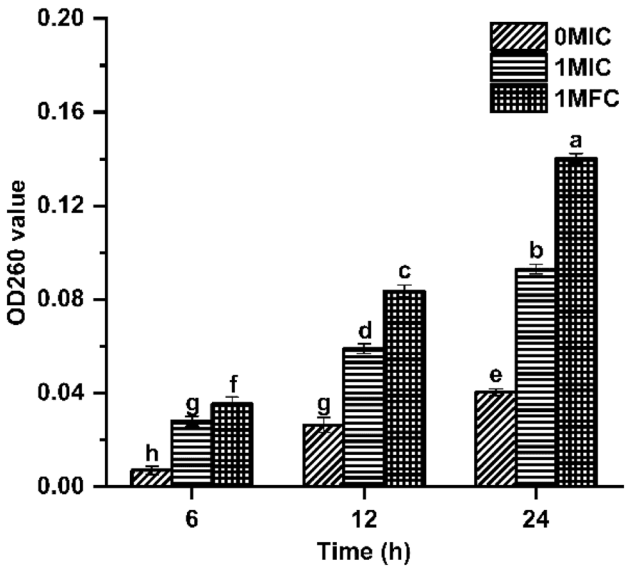


**Fig. 6** Nucleic acid release of *C. albicans* after treating with tea saponin (0 MIC, 1 MIC, 1 MFC) for different times

been destroyed, resulting in a large amount of nucleic acid leakage.

Scanning electron microscopy could intuitively reflect the morphological alterations of the cell surface. As shown in Fig. 7, *C. albicans* in the blank group showed ellipsoid or spherical shape, with no apparent wrinkles on the surface, and some cells had changed to the mycelial type. After treatment with tea saponin at 1 MIC, some cells had become wrinkled and deformed. At 1 MFC, the surface of most cells was deformed and wrinkled obviously, and some cells were ruptured. The above results indicated that tea saponin could damage the structure of the cell membrane, which led to small molecules ($K^+$, $Na^+$), nucleic acid and other macromolecular substance leakage, affecting the normal growth of cells and inhibiting the proliferation of *C. albicans*. As previous studies reported, saponin could destroy the structure of the cell membrane and lead to the leakage of substances in the cell [32, 35].

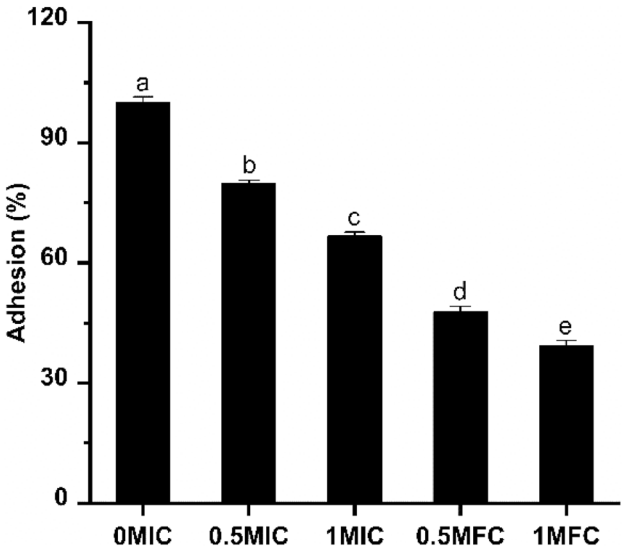


**Fig. 8** Effect of tea saponin on adhesion of *C. albicans*

## Effect of tea saponin on biofilm formation of *C. albicans*

### Effect of tea saponin on adhesion and aggregation of *C. albicans*

Biofilm, whose formation is a potential factor in developing drug resistance in fungi, is a complex of microorganisms and extracellular polymers (proteins, exopolysaccharides, DNA) [36]. One of the major virulence factors of *C. albicans* was the ability to form a biofilm, and adhesion was the first step for biofilm formation. We therefore tested the effect of tea saponin on adhesion, aggregation, and biofilm of *C. albicans*. As shown in Fig. 8, with the increase of the concentration of tea saponin, the adhesion of *C. albicans* decreased significantly. When the concentration of tea saponin was 0.5 MIC, the cell adhesion rate was 79.81%; at 1 MIC, the adhesion rate was 66.58%; and at 1 MFC, the adhesion rate was as low as 39.16%. As could be seen in Fig. 9, under the same visual field, many cells gathered together without tea saponin treatment. After being treated with tea saponin, the number of aggregated cells decreased. Especially when the concentration reached 1 MFC, the cells were significantly dispersed. Thus, tea saponin could reduce cell adhesion and prevent cell aggregation. Previous studies had found that the decrease of adhesion may be related to extracellular hydrolases such as phospholipase and protease. Substances with rich saponins could reduce the acid and alkaline phosphatase, phosphorolytic enzyme, and glucosaminidase of *C. albicans*. [37, 38]. Meanwhile, the result may also be related to the down-regulated expression of adhesion-related genes (HWP1, ALS3, ECE1) in *C. albicans*.

### Inhibition and eradication of tea saponin on *C. albicans* biofilm

The results of biofilm inhibition and eradication are shown in Fig. 10; the inhibitory effect of tea saponin on biofilm was pronounced. After treatment with tea saponin at 1.5

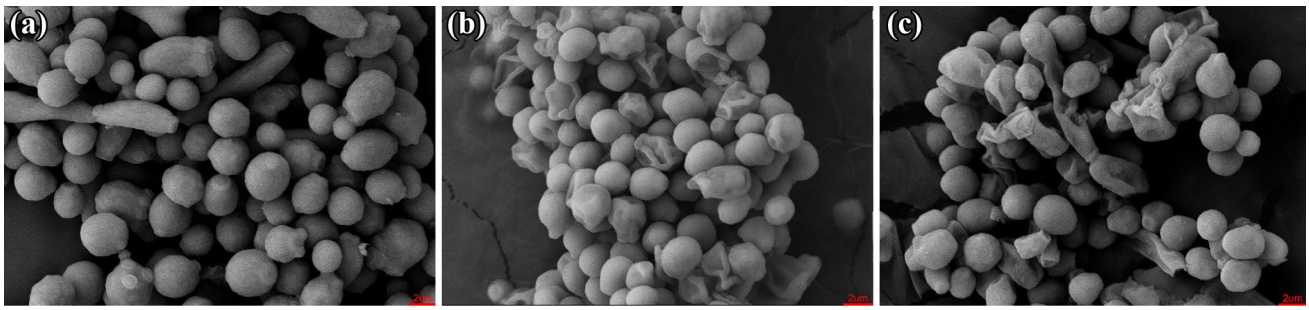


**Fig. 7** Scanning electron microscope was used to observe the surface morphology of *C. albicans*. **a** *C. albicans* treated with 0 MIC. **b** *C. albicans* treated with 1 MIC. **c** *C. albicans* treated with 1 MFC

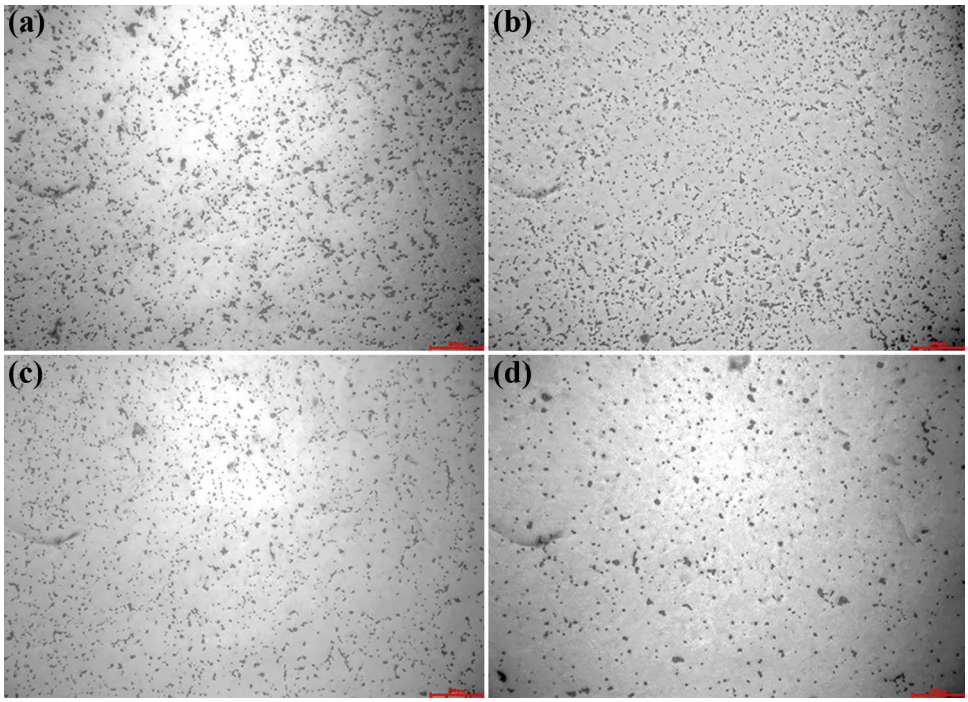


**Fig. 9** Effect of tea saponin on the aggregation of *C. albicans*. **a** *C. albicans* treated with 0 MIC.**b** *C. albicans* treated with 0.5 MIC. **c** *C. albicans* treated with 1 MIC. **d** *C. albicans* treated with 1 MFC

MIC, the inhibition rate reaches 89.82%, and when the concentration continues to increase, the inhibition rate does not increase much. At the same time, the eradication effect of tea saponin on biofilm was dose dependent, and the eradication rate of tea saponin on biofilm was much lower than the inhibition rate. The highest inhibition rate of tea saponin on biofilm was 92.65%, while the highest eradication rate of tea saponin on biofilm was 80.41%. When the concentration was 1.5 MIC, the inhibition rate was 89.82%, and the eradication rate was only 43.87%. Together, these results demonstrated that tea saponin had an excellent inhibitory effect on the biofilm formation of *C. albicans* at low concentration and a good eradication effect on the formed biofilm at high concentration. It echoed with the previous experimental results that hyphal formation and cell aggregation were prerequisites for biofilm formation. Tea saponin could inhibit the transformation of *C. albicans* from yeast cells to hyphal cells, reduce cell adhesion and prevent cell aggregation, and inhibit biofilm formation of *C. albicans*.

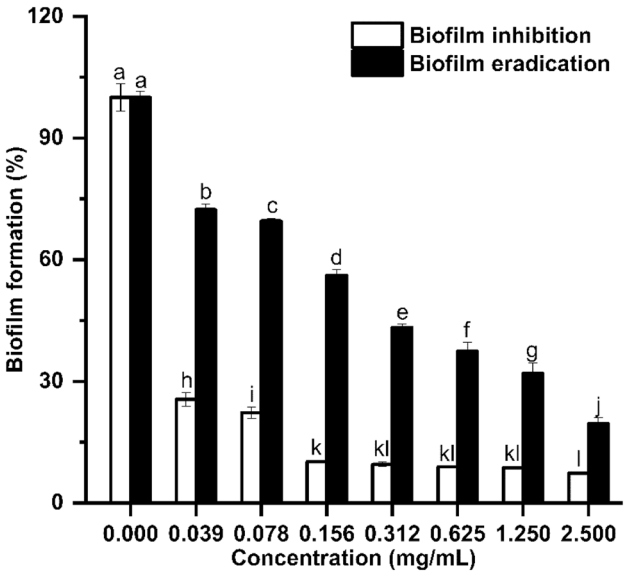


**Fig. 10** Effect of tea saponin on the inhibition and eradication of *C. albicans* biofilm

## Fluorescence microscope observation

Acridine orange can cross the cell membrane and stain nuclear DNA to make it emit bright green fluorescence. Ethidium bromide (EB) can only penetrate the damaged cell membrane and stain nucleus DNA, emitting orange-red fluorescence. Under a fluorescence microscope, living cells will show green fluorescence, while apoptotic cells show brighter orange or orange-green fluorescence [39]. As shown in Fig. 11, a large amount of densely green fluorescence was observed in the blank group, indicating that many cells had gathered and formed a dense biofilm. Compared with the blank group, when the concentration of tea saponin was 1 MIC, there was less and scattered green fluorescence, which indicated that the aggregation of cells decreased and formed sparser biofilm. At 1 MFC, some scattered orange fluorescence signals and few green fluorescence signals could be observed, indicating that many cells had apoptosis, and the

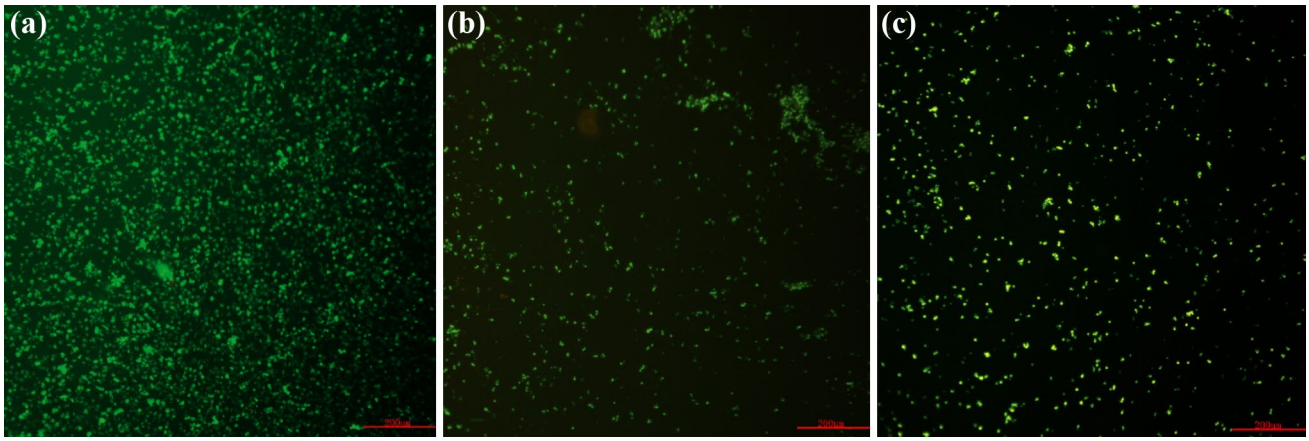


**Fig. 11** Fluorescence microscopic observation of *C. albicans* biofilm after treating with tea saponin. **a** *C. albicans* treated with 0 MIC. **b** *C. albicans* treated with 1 MIC. **c** *C. albicans* treated with 1 MFC

formation of biofilm was inhibited. The above experimental results indicated that tea saponin could destroy the cell membrane and reduce the aggregation of cells, which led to apoptosis and destroyed the biofilm.

### Effect of tea saponin on the expression of related genes in *C. albicans*

The virulence factors and pathogenicity of *C. albicans* are closely related to cell adhesion, hyphae and biofilm formation [7, 8]. In previous studies, tea saponin inhibited mycelium and biofilm formation of *C. albicans* and reduce cell adhesion. To further explain the possible mechanism that tea saponin inhibited *C. albicans* biofilms and filamentation, the expression of hypha-and biofilm-related genes was investigated by qRT-PCR. Compared with the control group, the related gene expression was down-regulated after treatment with tea saponin, in which ALS3, ECE1, and HWP1 were scarcely expressed, and EFG1 and UME6 were down-regulated by 1.28- and 1.39-fold, respectively (Fig. 12). ALS3, HWP1, and ECE1 were necessary for mycelial development [40], and their expressions were closely related to cell elongation, biofilm formation [41], and cell adhesion [42, 43]. UME6 was not only an important regulator of mycelial elongation and virulence of *C. albicans*, but also a key component of the downstream filamentous growth regulation pathways of Rfg1, Nrg1, and Tup1 [44]. EFG1, a central transcriptional regulator of morphogenesis and metabolism in *C. albicans*, was necessary to form a normal biofilm [45]. Alteration of these genes' expression affected adhesion, hyphal and biofilm formation. Therefore, the study showed that tea saponin could down-regulate the expression of these specific genes (ALS3, ECE1, HWP1, EFG1, and UME6) to prevent the transformation of *C. albicans* from yeast cells to hyphal cells, reduce cells adhesion and prevent cell aggregation, and inhibit biofilm formation of *C. albicans*.

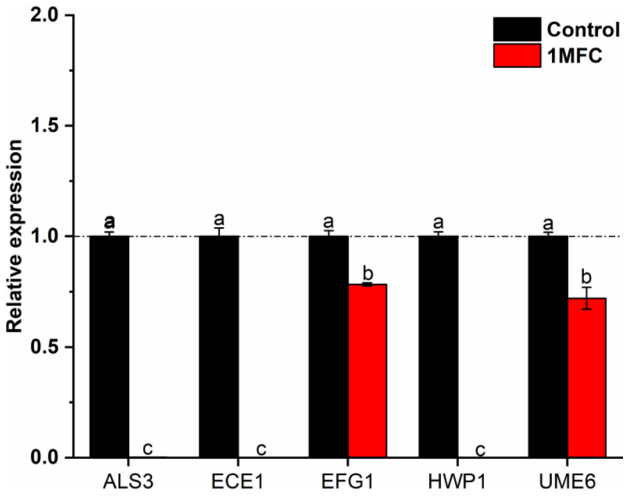


**Fig. 12** Differential expression of hypha- and biofilm-related genes of *C. albicans* treated with tea saponin (1 MFC)

## Conclusion

In this study, tea saponins were isolated from defatted *C. oleifera* cake, and UHPLC–MS/MS identified the main components of *C. oleifera* saponins were oleiferasaponin $D_3$. The results of the antifungal experiment showed that tea saponin had a good inhibitory effect on *C. albicans*, *S. cerevisiae*, and *Penicillium*. Among them, tea saponin had the most obvious inhibitory effect on *C. albicans*, and the MIC was 0.078 mg/mL. Tea saponin could destroy the structure of the cell membrane, lead to the leakage of the cell contents, and affect the normal growth of cells. Meanwhile, tea saponin

could inhibit the transformation of *C. albicans* from yeast cells to hyphal cells and reduce the extent of adhesion and aggregation of cells, thus inhibiting the formation of biofilm, and in turn inhibiting the *C. albicans* proliferation. In a word, tea saponin may become a natural antifungal agent and have a broad application prospect in the field of food safety.

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**Author contributions** ZY: writing—original draft, data curation, writing—review and editing, software. XW: conceptualization, formal analysis, methodology, resources, writing—review and editing, project administration, fund applicant. JH: investigation, instrumental analysis, data collection.

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**Availability of data and material** The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

**Code availability** Not applicable.

## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.

**Compliance with ethics requirements** This article does not contain any studies with human or animal subjects.

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