Quercetin Assists Fluconazole to Inhibit Biofilm Formations of Fluconazole-Resistant Candida Albicans in In Vitro and In Vivo Antifungal Managements of Vulvovaginal Candidiasis

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Key Words
Quercetin • Fluconazole • Candida albicans • Vulvovaginal candidiasis • Biofilm

Abstract

Background: Vulvovaginal candidiasis (VVC) is a common gynecological disease. Candida albicans is believed to be mainly implicated in VVC occurrence, the biofilm of which is one of the virulence factors responsible for resistance to traditional antifungal agents especially to fluconazole (FCZ). Quercetin (QCT) is a dietary flavonoid and has been demonstrated to be antifungal against C. albicans biofilm. Methods: 17 C. albicans isolates including 15 clinical ones isolated from VVC patients were employed to investigate the effects of QCT and/or FCZ on the inhibition of C. albicans biofilm. Results: We observed that 64 µg/mL QCT and/or 128 µg/mL FCZ could (i) be synergistic against 10 FCZ-resistant planktonic and 17 biofilm cells of C. albicans, (ii) inhibit fungal adherence, cell surface hydrophobicity (CSH), flocculation, yeast-to-hypha transition, metabolism, thickness and dispersion of biofilms; (iii) down-regulate the expressions of ALS1, ALS3, HWP1, SUN41, UME6 and ECE1 and up-regulate the expressions of PDE2, NRG1 and HSP90, and we also found that (iv) the fungal burden was reduced in vaginal mucosa and the symptoms were alleviated in a murine VVC model after the treatments of 5 mg/kg QCT and/or 20 mg/kg FCZ. Conclusion: Together with these results, it could be demonstrated that QCT could be a favorable antifungal agent and a promising synergist with FCZ in the clinical management of VVC caused by C. albicans biofilm.

Introduction

Vulvovaginal candidiasis (VVC), firstly termed "acute Candida vaginitis", is a common gynecological disease with a morbidity in 70-75% women at least once during their lifetime [1]. The clinical symptoms of VVC include vulva erythema, edema, excoration, and...
fissure formation as well as introital and vaginal erythema [2]. Several lines of evidence indicated that the aetiology of VVC might not be attributed to an opportunistic infection or an immunodeficiency, but might be associated with a hypersensitivity response to a genetically defined commensal organism [3-5]. Although VVC is usually not a fatal factor for woman life, the (re)occurrence of VVC challenges the life quality of the infected women [6]. Multiple factors including long-term overuse of over-the-counter antifungal drugs, one-dose managements, low exposure to azoles together with the growing immunocompromised patients (such as diabetics and HIV women) contribute to the ever-rising incidence of VVC [7, 8].

*Candida albicans*, a dimorphic opportunistic fungal pathogen commonly colonized on human mucosal membrane and skins, was believed to be mainly responsible for the occurrence of VVC in more than 85% of cases [9]. *C. albicans* is able to adhere to biotic (e.g. epithelial or endothelial cells) and abiotic (e.g. central venous and types of catheters) surfaces to form biofilm, a complex three-dimensional structure of fungal cell agglomerates. A biofilm is a special phenotype of *C. albicans* composed of extracellular matrix, the chemical constitute of which consists of proteins, polysaccharides as well as DNA [10], and encased fungal cells which are supposed to have altered susceptibility to antifungal agents and metabolism compared with its planktonic counterparts [11]. A biofilm phenotype provides a potent shield of fungal cells from the attacks of host immune system and extraneous antifungal chemicals. The biofilm formation of *C. albicans* is artificially divided into at least three stages: adherence stage (0-11 h) in which the fungal pathogens adhere reversibly (0-4 h) and irreversibly (5-11 h) to an abiotic/biotic surface, development stage (12-30 h) in which the extracellular polymath substances (EPS) are secreted and the yeast-to-hypha transition is under way, and maturation stage (31-72 h) in which the yeast and hyphal forms of fungal pathogens were enclosed by excretive matrixes [11]. During the three stages, the fungal cells are continuously dispersing from the settlement to the environment for searching new colonization [12]. *C. albicans* biofilms have been considered to be one of the critical factors accounting for fungal resistance to traditional antimycotic drugs, including azoles, polyenes, echinocandins, etc. [13].

Fluconazole (FCZ) is the most commonly used first-line drug of theazole family in clinical prophylaxis and treatment of mucosal and invasive *Candida* infections including VVC. The target of FCZ is believed to be the lanosterol 14-α demethylase which is an essential enzyme encoded by *ERG11* gene in the ergosterol biosynthesis process of *C. albican* [14]. Although FCZ was effective in 71% of the patients with VVC and the success cure rate could reach 90.6% in FCZ-sensitive *C. albican* isolates, it was reported that the failure rate could be up to 100% in FCZ-resistant *C. albican* isolates [15]. Of note, the resistance rate to *C. albicans* was between 10-20% for FCZ in patients with VVC [16, 17]. Therefore, it is necessary to seek novel medicines to use alone or in combination with FCZ in the treatment of FCZ-resistant *C. albicans* isolated from VVC.

Quercetin (QCT), a dietary flavonoid, has been demonstrated to possess the antifungal function to manage clinical *C. albicans* biofilms and sensitize the susceptibility of FCZ-resistant *C. albicans* isolates to FCZ [18, 19]. However, there were no *in vitro* and/or *in vivo* reports of QCT alone/or in combination with FCZ on inhibiting biofilm formation of FCZ-resistant *C. albicans* isolated from VVC patients. In this work, we employed microdilution method, checkerboard assay, time-kill test, quantitative reverse transcription polymerase chain reaction (qRT-PCR) as well as animal test to investigate the antibiofilm activities of QCT and/or FCZ in VVC-originated FCZ-resistant *C. albicans* isolates.

**Materials and Methods**

**Strains and culture conditions**

*C. albicans* reference strains SC5314 and ATCC10231 as well as fifteen clinical isolates from VVC patients were obtained from the Department of Gynecology, the First People's Hospital of Shangqiu and...
stored in yeast extract-peptone-dextrose (YPD, Oxoid Ltd., Cambridge, UK) medium with 10% (v/v) of glycerol at -80°C. These isolates were confirmed by API®32C biochemical testing panel (bioMérieux UK Ltd., Basingstoke, UK). Then the isolates were propagated in YPD medium for 24 h at 37°C, washed by phosphate-buffered saline (PBS) (Sigma-Aldrich, Shanghai, China), collected by centrifugation and then resuspended in RPMI-1640 medium (Sigma-Aldrich, Shanghai, China) for use.

Susceptibility tests

The antifungal susceptibilities of QCT and FCZ (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) were evaluated by broth microdilution method in terms of Clinical and Laboratory Standards Institute (CLSI) guidelines M27-A3 [20] with a final inoculum of $2 \times 10^3$ CFU/mL in RPMI-1640 medium. The stock solutions of QCT and FCZ were prepared in dimethyl sulphoxide (DMSO) with the final concentration < 5% (v/v). The antifungal activities of QCT and FCZ against planktonic cells were represented by minimum inhibitory concentration (MIC), which was defined as the lowest drug concentration inhibiting visible growth. The combined antifungal activity of QCT and FCZ were performed by the checkerboard assay and represented by the fractional inhibitory concentration index (FICI), which was equal to $(\text{MIC}_\text{QCT} \text{ in combination}/\text{MIC}_\text{QCT} \text{ alone}) + (\text{MIC}_\text{FCZ} \text{ in combination}/\text{MIC}_\text{FCZ} \text{ alone})$ with interpretation of synergism as FICI ≤ 0.5, indifference as 0.5 < FICI ≤ 4.0, and antagonism as FICI > 4.0 [21]. The antibiofilm activities of QCT and FCZ were represented by sessile MIC$_{50}$ (SMIC$_{50}$), which was determined as a ≥ 50% reduction of optical density (OD) at the wavelength of 492 nm in metabolic activity by 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT, Amherst, MA, USA) assay compared with the control, with a final inoculum of $1 \times 10^6$ CFU/mL in RPMI-1640 medium. The combination of QCT and FCZ against biofilm cells were also performed by the checkerboard assay and represented by FICI as described above [22].

XTT assay

The metabolic activity was performed as described before with a few modifications [23]. The XTT power (= 50 mg) was firstly dissolved in a Riger’s solution (= 100 mL), blended with freshly menadione solution to the final concentration of 1.72 mg/mL, and filtered by 0.22 μm millipore filters (Millipore, Billerica, MA, USA) before use. After incubations with antifungal drug, the supernatant was pipetted out and each well was washed by pH 7.2 PBS. Subsequently, the newly-prepared XTT solution was pipetted into the pre-washed wells. After 2 h of incubation at 37°C, a certain volume of colored solution (= 100 μL) was transferred to a new 96-well plate and the OD value was measured at a wavelength of 492 nm by a 318-microplate reader (Sanco Instruments, Shanghai, China).

Time-kill (T-K) test

The T-K test were performed according to a previous procedure with a small modifications [24]. Briefly, a final inoculum of $1 \times 10^6$ CFU/mL in RPMI-1640 medium was used for the ten FCZ-resistant C. albicans isolates in a 96-well bottom-flat polystyrene microtiter plate. The corresponding MICs in combination were used in the ten isolates for 48 h of incubation at 37°C. At 8, 16, 24, 32, 40, and 48 h, the strain broth was pipetted out and spread on sabouraud dextrose agar (SDA, Gibco, Invitrogen, Carlsbad, CA, USA) to evaluate colony forming unit (CFU) for another 48 h of incubation at 37°C. The control contained fungal cells and broth medium with no drug. The interpretations were based on the 48-h of results as follows: the synergism was defined as an increase of no less than 100-fold in killing for the combined agents compared with the most active agent used alone, antagonism was defined as a decrease of no less than 100-fold in killing for the combined agents compared with the most active agent alone, and the indifference was defined as a less than 100-fold in killing for the combined agents compared with any agents used [25].

Cell adhesion assay

A quantity of drug-containing broth (= 100 μL, 64 μg/mL QCT and/or 128 μg/mL FCZ) was co-incubated with C. albicans 04 solution (= 100 μL, $1 \times 10^6$ CFU/mL) in a 96-well bottom-flat polystyrene microtiter plate for 2 and 4 h at 37°C. The control included no drug. After incubations, the supernatant was discarded and the wells were washed three times by sterile PBS. The adhered cells were detected by XTT assay as described above and observed by SEM as described below.
Scanning electron microscope

After the 4 h of treatments of 64 µg/mL QCT and/or 128 µg/mL FCZ, the isolate samples were fixed by 2.5% glutaraldehyde overnight, and post-fixed with 0.1% osmium tetroxide for 1 h. The samples were then dehydrated by 30, 50, 70, 90 and 100% ethanol for 10 min each. After critical point drying, the samples were sputter coated with gold in a vacuum evaporator, and the morphologies of C. albicans 04 were observed by a scanning electron microscope (SEM, JSM-6700F, Japan).

Cell surface hydrophobicity (CSH)

The CSH of C. albicans 04 was surveyed by the water-hydrocarbon twophase assay followed by a previous report [26]. Briefly, the drug-dissolving solution (= 2 mL, 64 µg/mL QCT and/or 128 µg/mL FCZ) was co-incubated with the same volume of strain solution (= 1 × 10^6 CFU/mL) in a glass tube for 24 h at 37°C. After 10 min 3000 g of centrifugation, the fungal cells were re-suspended with sterile PBS and adjusted OD_{600} to 1. An aliquot of strain solution (= 1.2 mL) was transferred into another clean glass tube and overlaid with 0.3 ml of octane (Sinopharm, Shanghai, China). After the mixed solution was separated into two definite phases by 3 min vortex, the OD_{600} of the aqueous phase was detected. The control was set as the OD_{600} of the aqueous phase without the octane overlay. The relative hydrophobicity was calculated as follows: [(OD_{600} of the control - OD_{600} after octane overlay)/OD_{600} of the control] × 100.

Flocculation

The flocculation procedures were followed by a previous description with minor modifications [27]. Briefly, the adjusted C. albicans 04 broth (OD_{600} = 0.5) was co-cultured with 64 µg/mL QCT and/or 128 µg/mL FCZ in newly prepared RPMI-1640 for 4 h of incubation at 37°C with gentle shaking. Then the suspension was vortexed for several seconds and photographed immediately. The sediments of cells were observed by inverted microscope (OLYMPUS, Tokyo, Japan) after 2 min.

Yeast-to-hypha transition on solid and semisolid plates

As for the test on solid plate, the strain broth of C. albicans 04 (= 1 × 10^6 CFU/mL) was inoculated on a YPD agar plate which was freshly prepared supplemented with 64 µg/mL QCT and/or 128 µg/mL FCZ for 4 h of incubation at 37°C. The colony shape on solid plate was photographed by a digital camera (Olympus, Tokyo, Japan). Before the test on semisolid plate, GM-BCP medium (10 g peptone, 5 g glucose, 0.012 g bromcresol purple, pH 8.0, Huankai, Guangzhou, Guangdong, China) was immediately prepared and adjusted to pH 8.0 by 1 M NaOH. Then the GM-BCP semisolid plate was mixed with QCT and/or FCZ and inoculated by the strain broth of C. albicans 04 (= 1 × 10^6 CFU/mL) for 5 h of incubation at 37°C. Finally, the plate was dissected and the transection of colony was photographed laterally.

Fluorescent microscope

The 1 cm^2 slides were firstly incubated in 75% (v/v) ethanol for 24 h and washed several time by sterile PBS. Then the slides were placed in wells of a 6-well plate containing both the strain broth of C. albicans 04 (= 1 × 10^6 CFU/mL) and antifungal agent solutions (64 µg/mL QCT and/or 128 µg/mL FCZ). The stock solution of fluorescein diacetate (FDA, Sigma, Shanghai, China) was prepared by dissolving acetone to a concentration of 10 mg/mL and stored at -20 °C before use. The working solution of FDA (1:50, 100 μL) freshly diluted in sterile PBS was added into each pre-rinsed well of 96-well microtiter plate with 100 μL fungal solution. The plate was incubated in the dark at 37 °C for 30 min on a rocking table, and observed by a fluorescent microscope (Olympus IX81, Tokyo, Japan) at the emission wavelength of 460 nm.

Biofilm biomass

The biofilm biomass was measured by crystal violet staining. Briefly, the C. albicans 04 broth (= 100 μL, 1 × 10^6 CFU/mL) was co-incubated with RPMI-1640 medium containing 64 µg/mL QCT and/or 128 µg/mL FCZ for 24 h of incubation at 37°C in a 96-well bottom-flat polystyrene microtiter plate. The supernatant was discarded, and freshly-prepared crystal violet solution (= 100 μL, 0.5%, m/v) was added into each well. After 10 min of dicing, the staining solution was pipetted out gently and the loose cells were removed by sterile PBS. Finally, the OD value was recorded at the wavelength of 560 nm by a 318-microplate reader.
Confocal scanning laser microscope (CLSM)
The CLSM procedure were undertaken as described before with a few modifications [28]. Briefly, the C. albicans 04 (1 × 10⁶ CFU/mL) was incubated with/without 64 µg/mL QCT and/or 128 µg/mL FCZ in RPMI-1640 medium at 37°C for 48 h. Then the supernatant was aspirated and the wells were rinsed by sterile PBS for several times. Before staining, the FUN1 stock solution (= 4 µL, Invitrogen, Shanghai, China) was diluted in 10 mL GH solution which consisted of 23.8% (m/v) HEPES (Sigma-Aldrich, Shanghai, China) and 2% D-glucose (Sigma-Aldrich, Shanghai, China) with pH 6.8-8.2 adjusted by 1 M NaOH. Subsequently, the freshly-prepared FUN1 solution was added into each well for 30 min of incubation at darkness. The images were taken by confocal laser-scanning microscope (Olympus Fluoview FV1000), and a detailed three-dimensional image of biofilm was performed using Z-stacks (depending on the height of the biofilm).

Biofilm dispersion
PVC catheter pieces were firstly cut into pieces (1 cm × 1 cm) and sterilized by soaking in 75% (v/v) ethanol for 2 h. After washing by PBS, the pieces were pre-incubated with fetal calf serum (FCS) at 37°C for 4 h, and then transferred to a clean 24-well microtiter plate, co-incubated with C. albicans 04 broth (= 1 × 10⁶ CFU/mL) at 37°C for 24 h. The biofilm-coated pieces were washed with sterile PBS three times and treated by 64 µg/mL QCT and/or 128 µg/mL FCZ in a bottom-clean 24-well polystyrene microtiter plate at 37°C for another 24 h of incubation. After incubation, the next process were divided into two steps: on one hand, the supernatant (100 µL) was aspirated into a new 96-well microtiter plate, and the OD value was detected at the wavelength of 600 nm; on the other hand, the pieces were fetched out by forceps, rinsed by PBS, put into glass tubes with 1 mL PBS, and vortexed rigorously to detach the biofilm completely. The solutions containing detached biofilm cells were serially 10 × diluted and spread on SDA plate for CFU counting.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis
The qRT-PCR were performed as previously described with several modifications [29]. Briefly, the C. albicans 04 broths (=1 × 10⁶ CFU/mL) were treated by 64 µg/mL QCT and/or 128 µg/mL FCZ at 37°C for 24 h. The total RNA was extracted from C. albicans 04 using MagExtractor-RNA kit (ToyoBo, Tokyo, Japan). The absorption was measured at 260 and 280 nm to confirm the purity of RNA and to calculate the amount of RNA. Approximately 1 µg of the extracted total RNA was used to synthesize cDNA by ReverTra Ace qPCR RT Master Mix with gDNA Remover kit (ToyoBo, Tokyo, Japan). The primers of ALS1, ALS3, HWPI, SUN41, UME6, ECE1, PDE2, NRG1, HSP90 and ACT1 were listed in Table 1. The procedures of qRT-PCR was performed on ABI7000 fluorescent quantitative PCR system (Applied Biosystem) including 95°C for 5 min, 40 cycles of 95°C for 15 s, 48°C for 15 s, 72°C for 45 s. All data were normalized to housekeeping gene ACT1 (reference gene). The relative target-gene expression was calculated as a fold change of 2⁻ΔΔCt value as previously described [30].

Murine model
The murine VVC model was established according to the previous procedures described in [31]. The animal care and use committee of the First People’s Hospital of Shangqiu approved the designs and procedures of the experiment. Sixty KM clean mice (= 15-20 g) were purchased from the animal center of Shanghai Biological Science Institution (Shanghai, China) and divided into five groups with 12 per each group. All mice were housed in the animal center, given standard food and water provided ad libitum at room temperature for at least a week prior to next experiments. Three days prior to inoculation, mice were injected with 100 µl of sesame oil containing 0.1 mg of β-estradiol (Sigma-Aldrich, Shanghai, China) subcutaneously in the lower abdomen. Estrogen injections were administered once every other day for three times in a week. A loopful of C. albicans

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<th>Table 1. Primers for PCR</th>
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04 blastoconidia from Sabouraud-dextrose agar was added into 10 ml of YPD broth supplemented with 0.1% glucose for 18 h of incubation to stationary phase at 30°C with gentle shaking. After centrifugation at 1000 g for 5 min and three-time washing by sterile PBS, the stationary-phase cultures containing viable blastoconidia were resuspended in sterile PBS and adjusted to the concentration of $1 \times 10^8$ CFU/mL counted by a hemocytometer. An aliquot of 20 μl of the stationary-phase suspension, generating an inoculum size of $2 \times 10^6$ blastoconidia, was injected into the vaginal lumen of the estrogen-treated mice. The normal controls were inoculated with 20 μl of sterile PBS. The infected mice underwent vaginal lavage immediately with 0.1 ml of sterile PBS containing protease inhibitors at day 1 postinoculation to confirm the establishment of the murine VVC model. After the establishment of murine model, the mice were treated with 5 mg/kg QCT and/or 20 mg/kg FCZ by gavage once a day for consecutive 7 days. The normal control and model control were treated with PBS. At day 1 postinoculation, the vaginal lumens of mice were lavaged by PBS with repeated aspiration and agitation with a pipette tip. The lavage fluids were combined on ice during processing, were removed to perform fungal colony counting, morphology and HE staining.

Statistical analysis

All experiments were performed triplicate in three different occasions. The data were presented as means ± standard deviation and calculated by SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Differences between groups were determined using analysis of variance (ANOVA). A $p$-value < 0.05 was defined as statistically significant.

Results

Anti-planktonic and anti-biofilm activities of QCT and/or FCZ

The susceptibilities of 17 $C. albicans$ isolates including two standard (SC5314 and ATCC10231), 10 FCZ-resistant (MIC ≥ 64 μg/mL) and five FCZ-susceptible (MIC < 64 μg/mL) isolates from VVC patients to QCT and FCZ were assessed under planktonic (represented by MIC) and biofilm (represented by SMIC$_{50}$) states. The MICs of QCT ranged from 128 to 512 μg/mL with FICI ranging 0.188-1, while the SMIC$_{50}$ of QCT were ≥ 1024 μg/mL accompanying with FICI between 0.188 - 0.5 (Table 2). The susceptibility test showed synergism in FCZ-resistant isolates under planktonic state, and both FCZ-sensitive and –resistant isolates under biofilm states when QCT and FCZ were combined. Due to their susceptibilities to the

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combination of QCT and FCZ under both planktonic and biofilm states, the 10 FCZ-resistant isolates were used in T-K test. It could be observed that the antifungal activities of QCT and FCZ alone were weaker than that of their combination (Fig. 1). The following experiments were performed in *C. albicans* 04 isolate due to its strong susceptibility to the combination of QCT and FCZ.

**Combination of QCT and FCZ inhibits the adherence, CSH and flocculation of *C. albicans* 04**

The adherence is the first step of fungal biofilm formation and the initial reversible adherence is especially important for subsequent biofilm development. We found that QCT
(= 64 µg/mL) in combination with FCZ (= 128 µg/mL) could significantly inhibit the initial adherence in *C. albicans* 04 (*p* < 0.05), while the effects of QCT and FCZ alone on the initial adherence were negligible (*p* > 0.05, Fig. 2A). The similar conclusions could be made from the SEM results (Fig. 2B). The Candida CSH and flocculation were also of essential indicators of fungal adherence. Compared with the control, the combination of QCT (= 64 µg/mL) and FCZ (= 128 µg/mL) could significantly inhibit CSH (Fig. 3A) and flocculation (Fig. 4A) in *C. albicans* 04 isolate, as the results evidently displayed that the organic phase was nearly transparent (Fig. 3B) and almost no agglomerates could be seen (Fig. 4B).

**Combined use of QCT and FCZ inhibits the yeast-to-hypha transition of *C. albicans* 04**

Yeast-to-hypha transition is a critical process of fungal biofilm development and an important virulence factor. In the drug-free control, the colony of *C. albicans* 04 isolate was

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**Fig. 2.** Effects of 64 µg/mL QCT and/or 128 µg/mL FCZ on adherence in *C. albicans* 04 by (A) XTT assay after 2 h and 4 h incubations and (B) SEM after 4 h incubation. *, *p* < 0.05, compared with the control.

**Fig. 3.** Histograms (OD value at 600 nm) and images of CSH when 64 µg/mL QCT and/or 128 µg/mL FCZ were used in combination in *C. albicans* 04. *, *p* < 0.05, compared with the control.
wrinkled (Fig. 5A) with abundant lathy fila extending out the fungal cell (Fig. 5B), most of which were filamentous (Fig. 5C). After the treatment of QCT (= 64 µg/mL) combined with FCZ (= 128 µg/mL), the colony became smooth (Fig. 5A) with no fila (Fig. 5B) and the fluorescein-dyed fungal cells appeared almost to be yeast (Fig. 5C). The antifungal activity of QCT seemed to be stronger than that of FCZ as the former could be more efficient to inhibit yeast-to-hypha transition in *C. albicans* 04 isolate (Fig. 5).

Metabolism and thickness of biofilm as well as the dispersion of pre-formed biofilm were affected by QCT and/or FCZ in *C. albicans* 04 isolate

Compared with the control, QCT (= 64 µg/mL) alone could cause significant decrease of metabolism (*p* < 0.05), and the combination of QCT (= 64 µg/mL) and FCZ (= 128 µg/mL) nearly decreased 2/3 metabolic activity in *C. albicans* 04 isolate (*p* < 0.05, Fig. 6A). In CLSM results, the fluorescent intensity (FI) was between 140-160 and the FIs were even more than 180 in several biofilm areas in the control. After management of QCT (= 64 µg/mL) or FCZ (= 128 µg/mL), the FIs dropped to 60-80 and 100-120 respectively. The FI further declined to less than 20 once QCT (= 64 µg/mL) in combination with FCZ (= 128 µg/mL) was used (Fig. 6B). The dispersion was monitored in 24-h pre-formed biofilm in *C. albicans* 04 isolate after drug treatments. Compared with the control, the biofilm cells were stimulated to disperse significantly (*p* < 0.05, Fig. 7A) and the biofilm cells attached on the surface were dramatically reduced (*p* < 0.05, Fig. 7B) when QCT (= 64 µg/mL) and FCZ (= 128 µg/mL)
were used concomitantly. However, QCT (= 64 µg/mL) or FCZ (= 128 µg/mL) alone seemed to be ineffective to induce biofilm dispersion and detachment (p > 0.05, Fig. 7A and 7B).

**Effects of QCT and/or FCZ on the expressions of biofilm-related genes**

To further investigate the effects of QCT and/or FCZ on *Candida* biofilm development, the expressions of 9 biofilm-related genes were analyzed by qRT-PCR. It was observed that when QCT (= 64 µg/mL) or FCZ (= 128 µg/mL) was used alone, the expressions of *ALS1*, *ALS3*, *HWPI*, *SUN41*, *UME6* and *ECE1* were down-regulated and those of *PDE2*, *NRG1* and *HSP90* were up-regulated. However, the differences were not significant compared with the control (p > 0.05, Fig. 8). Of note, the alterations of the 9 gene expressions had significant differences (p < 0.05, Fig. 8) when QCT (= 64 µg/mL) and FCZ (= 128 µg/mL) was used in combination. It could be observed that the down-regulations of *ALS1*, *ALS3*, *HWPI*, *SUN41*, *UME6* and *ECE1* were of 2.27-, 3.13-, 4.17-, 3.70-, 2.78- and 3.03-fold respectively, and the up-regulations of *PDE2*, *NRG1* and *HSP90* were of 2.45-, 2.32- and 2.35-fold respectively (Fig. 8).
Therapeutic treatment of QCT and/or FCZ in a murine VVC model

The anti-VVC potential of QCT and/or FCZ were surveyed in a murine model infected with *C. albicans* 04 isolate. As shown, the fungal burden were largely relieved when QCT (= 5 mg/kg) was used alone compared with the control (*p* < 0.05, Fig. 9A). Impressively, the fungal cells seemed to be wiped out as the fungal quantity decreased to approximately 17 CFU/mL when QCT (= 5 mg/kg) and FCZ (= 20 mg/kg) were administered together (*p* < 0.05, Fig. 9A). From the images of lavage, we observed abundantly criss-cross filamentous cells in the control (Fig. 9B). After HE staining, it could be observed that the vaginal mucosa was nearly destroyed, internal hyperaemia and adema were widespread, and the infiltration of inflammatory cells were notable in/beneath vaginal mucosa in the control (Fig. 9B). By the management of QCT (= 5 mg/kg) or FCZ (= 20 mg/kg), the cellular symptoms were relieved to different degrees (Fig. 9B). Noticeably, when exposed to QCT (= 5 mg/kg) and FCZ (= 20 mg/kg), the hyphal cells disappeared and only a few of yeast-form cells were visible. More importantly, the integrity of vaginal mucosa was largely recovered, internal hyperaemia and adema were alleviated greatly, and there were no evident visions of the infiltration of inflammatory cells in the case of combined use of QCT (= 5 mg/kg) and FCZ (= 20 mg/kg, Fig. 9B).
Discussion

QCT has been demonstrated to possess a series of functions as well as antifungal potentials [19, 32-34]. In this study, the MIC\textsubscript{90} of QCT were generally > 200 µg/mL consistent with a previous report [19], and the antibiofilm effect of QCT alone was poor as the SMIC\textsubscript{50} were ≥ 1024 µg/mL (Table 2). However, QCT appeared to have strong synergism with FCZ in FCZ-resistant *C. albicans* isolates (Table 2 and Fig. 1). Intriguingly, the synergism of QCT and FCZ was independent of strain phenotype of resistance/susceptibility. As a matter of fact, most of the flavonoids were good at inhibiting the growth of FCZ-susceptible free-living cells and bad at dealing with FCZ-resistant clinical isolates and biofilms in *C. albicans*, but they usually performed satisfactorily in combination with FCZ in biofilm removal [18, 28, 35, 36].

Combinatorial therapy has become a universal approach in the prevention and management of *C. albicans* biofilms [37]. *C. albicans* biofilms represent a new type of mode of fungal survival and confer to fungal pathogen greater resistance that was even up to hundreds of folds greater than their planktonic counterparts, resulting in single use of currently available antifungal agents futile. Both SMIC\textsubscript{50} and SMIC\textsubscript{90} of FCZ are usually above 1024 µg/mL against *C. albicans* biofilms under static state demonstrated in a previous and our studies (Table 2), and can be even higher under flow condition [38, 39]. Owing to sluggish exploration of novel antifungal chemicals, the "old" drugs (such as flavonoids) with antifungal potentials become an alternative choice to sensitize the antifungal activity of FCZ when they are used in combination with FCZ.

Fungal adherence was one of the most important determinants of pathogenesis. Concerning VVC infection, the clinical *C. albicans* isolates from VVC patients have been shown in vitro the ability to adhere to the surfaces of both microtiter plates and intrauterine contraceptive devices [40, 41]. Herein, the combination of QCT and FCZ could significantly inhibit the adherence of VVC isolate (Fig. 2) and the expressions of adherence-related genes (i.e. *ALS1, ALS3* and *HWP1*, Fig. 8). *ALS1* and *ALS3* were members of the Agglutinin-Like Sequence (ALS) gene family and had great impacts on biofilm formation in the early stage (i.e. adherence stage) [42-44]. *HWP1* was a well-characterized genes encoding an important cell surface protein in *C. albicans* and mediating mainly tight binding to mucosal cells (such as oral/vaginal epithelial cells) [45, 46]. The cell surface hydrophobicity (CSH) of *C. albicans* was reported to be implicated in the adhesion, biofilm formation and FCZ resistance, as greater CSH mostly resulted in higher ability of adherence to surface, biofilm formation, resistance to FCZ [47, 48]. Apart from cell-surface adherence, the adhesins (e.g. Als1, Als3 and Hwp1) also conferred a property of cell-cell adhesion in *C. albicans*, i.e. flocculation [49]. It could be observed that combined use of QCT and FCZ reduced the flocculation and CSH in VVC-originated *C. albicans* isolate (Fig. 3 and 4).

The yeast-to-hypha transition was a well-known virulence factor to cause tissue damage and a mark of biofilm development in *C. albicans* [50]. In this experiment, concomitant used of QCT and FCZ evidently inhibited the yeast-to-hypha transition (Fig. 5), the biofilm metabolism (Fig. 6A) and the thickness of biofilm matrix (Fig. 6B) in a VVC isolate consistent with the previous results [18]. Notably, it was reported that *ALS1* and *HWP1* were also hypha-specific genes as the expression of former mRNA was correlated well with hyphal growth and the protein product encoded by the latter was only expressed on hyphae [51-53]. Sun41, a putative cell wall glycosidase, is required in biofilm formation, cell wall integrity, and virulence [54], while UME6-mediated regulation of hyphal growth and biofilm development of *C. albicans* was dependent on the expression of *SUN41* [55]. The expressions of *ECE1* and *PDE2* were closely related with hyphal growth of *C. albicans* dependent on cAMP-PKA signaling pathway [56]. Our results indicated that QCT in combination with FCZ could dramatically influence the expressions of hypha-, biofilm-related genes (i.e. *ALS1, HWP1, SUN41, UME6, PBS2* and *PDE2*, Fig. 8).

Dispersion was also believed to be an important stage in *C. albicans* biofilm development, and the dispersion process occurred throughout the whole biofilm development. Fungal dispersion resulted in an initiation of fungal recolonization and a new round of *Candida* biofilm
formation accompanying with disseminated candidiasis [12]. Although the dispersed fungal cells had different susceptibility and metabolic pattern, a hypothesis was accepted that the cells free from the protection of biofilm matrix would be more easily to be eradicated by host immune system and antifungal agents. We found that QCT could assist FCZ to facilitate the dispersion of biofilm cells (Fig. 7A) and to decrease fungal cells on microtiter surface (Fig. 7B). It has been reported that NRG1 and HSP90 were negatively in charges of fungal biofilm dispersion [57, 58]. From our results, the expressions of both genes were upregulated by more than two folds after exposure to QCT and FCZ (Fig. 8).

To our knowledge, this was the first report of in vivo effect of QCT and/or FCZ on VVC. A previous study demonstrated that C. albicans could adhere the vaginal mucosa and form a complex three-dimensional architecture of a typical biofilm with abundant extracellular matrix after 24-48 h of infection [59], and several key transcriptional regulators (e.g. NRG1 and UME6) were implicated in the immunopathogenesis of Candida vaginitis via the yeast-to-hypha switch and the associated morphogenetic response [60]. After the treatments of QCT and FCZ, the fungal loading decreased (Fig. 9A), the hypha-form cells disappeared and the inflammation of mucosal epithelial cell were greatly alleviated (Fig. 9B).

Together with these results, it could be demonstrated that QCT could be a favorable antifungal agent and a promising synergist with FCZ in the clinical management of VVC caused by C. albicans biofilms.

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Disclosure Statement

None.

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