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# *In vitro* photodynamic inactivation effects of hypocrellin B on azole-sensitive and resistant *Candida albicans*

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ABSTRACT

*Background and aim:* The extensive use of antifungal drugs has led to resistance from *Candida albicans*. The search for alternative treatment against drug-resistant *C. albicans* is highly desirable. Antimicrobial photodynamic therapy (aPDT) is an emerging and promising approach for treating localized and superficial *C. albicans* infections. The aim of this study was to investigate the photodynamic inactivation (PDI) effects of hypocrellin B (HB) on azole-sensitive and resistant *C. albicans in vitro*.

*Methods:* The PDI efficacies of HB on standard *C. albicans* strain (ATCC 10231), azole-sensitive clinical isolate of *C. albicans*, and azole-resistant clinical isolate of *C. albicans* were assessed. The uptake of HB in *C. albicans* cells was investigated by confocal laser scanning microscopy (CLSM). The PDI effects on cellular structure and surface characteristics were investigated by transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

*Results:* HB exhibited no significant dark toxicity, but inactivated the azole-sensitive and resistant *C. albicans* in a light-dose and PS concentration-dependent manner. CLSM images indicated that PDI treated *C. albicans* cells showed stronger fluorescence compared to untreated cells. TEM images suggested that significant damage to the cell wall, membrane, and cytoplasm were induced by HB-mediated PDI. SEM analysis revealed that the surface of *C. albicans* cells became twisted and ruptured after PDI treatment.

*Conclusions:* Azole-sensitive and resistant *C. albicans* could be effectively inactivated by HB in the presence of light, and HB-mediated aPDT shows promise as an antifungal treatment for *C. albicans*.

## 1. Introduction

*Candida albicans* commonly lives in the human gastrointestinal tract, oral cavity, and vagina [1]. This species causes a broad range of diseases from superficial mucosal infections to life-threatening invasive candidiasis, particularly in devitalized patients undergoing treatment with chemotherapy and radiotherapy for cancer, or immune compromised patients with organ transplantation and acquired immune deficiency syndrome [2]. The prevalence of *C. albicans* is higher in diabetic, pregnant, and elderly individuals [3]. Antifungal agents such as azoles, polyenes, pyrimidine, and echinocandins are commonly used to treat systematic and invasive candidiasis, but they are toxic to the host and may interrupt cell functions [4,5]. More importantly, irregular and continuous administration of these antifungal drugs can result in resistance. Therefore, numerous studies have been focused on discovering alternatives to eradicate drug-resistant *C. albicans*, for which, the fungi will not be easily able to develop resistance.

Photodynamic therapy (PDT) is a light-based treatment used in dermatology, oncology, and ophthalmology, and has been developed as an antimicrobial therapy, named as antimicrobial photodynamic therapy (aPDT). This method utilizes light of an appropriate wavelength in combination with a photosensitizer (PS), and molecular oxygen to induce a phototoxic reaction. Under light illumination, the PS can be energized from the ground state to an excited state that can undergo molecular collisions with molecular oxygen present in and around targeted cells, resulting in the formation of superoxide anion ($O_2^{-\cdot}$), hydroxyl radicals ($OH^{\cdot}$), and hydrogen peroxide ($H_2O_2$) through a Type I reaction, or singlet oxygen ($^1O_2$) through a Type II reaction. These reactive oxygen species (ROS) can disrupt microbial structures and leading to microbe death [6]. Compared to conventional

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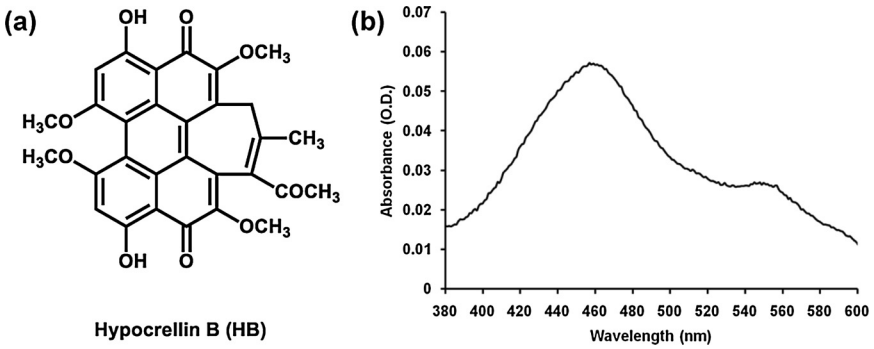


**Fig. 1.** Chemical structure and absorption spectrum of hypocrellin B (HB).

antifungal drugs, aPDT shows higher targeting through the accumulation of PS in fungi cells and site-specific light irradiation [7], fewer harmful chemicals effects to host cells [8], and lower potential for resistance because of the non-specific action of ROS [9].

Among the three essential elements in aPDT, PS plays an important role. Previous studies demonstrated that *C. albicans* can be effectively inactivated in the presence of light by a wide range of traditional PSs [10]. In addition to these chemically synthesized PSs, traditional Chinese herbs are a rich resource of antimicrobial drugs, and potential PSs. Hypocrellin (Fig. 1), a perylene quinone derivative isolated from natural fungus sacs of *Hypocrella bambusae* growing in the north-western region of Yunnan Province in China, consists hypocrellin A (HA) and hypocrellin B (HB), which are similar in structure, showing a difference of only one hydroxyl group [11]. Compared to first-generation PSs such as the hematoporphyrin derivative (HpD) and photofrin, hypocrellin has a distinct chemical structure, higher photodynamic efficiency, lower dark toxicity toward normal cells, and faster clearance rate from the tissues, and thus is promising as a PS [12].

In recent years, HB-mediated PDT has been evaluated as a potential clinical therapeutic method for treating several diseases. For instance, Jiang et al. demonstrated that the photodynamic action of HB significantly decreased the proliferation of ovarian cancer cells, and caused severe damage to their mitochondrial structure [13]. Hu et al. found that HB-mediated PDT induced significant keloid fibroblast apoptosis and decreased cell viability, suggesting that HB-PDT can be used to treat keloid [14]. Li et al. discovered that liposomal HB in the presence of light led to choroidal neovascularization occlusion in a rat model with minor damage to the collateral retina or retinal pigment epithelium [12]. Additionally, HB exhibited efficient photodynamic inactivation (PDI) in aPDT against pathogens. Jiang et al. found that HB-mediated aPDT inhibited the growth of *Escherichia coli* cells and remarkably damaged on their ultrastructure [15]. Hashimoto et al. indicated that HB combined with LED light effectively decreased antibiotic-resistant *Pseudomonas aeruginosa* in a burned mouse model [16]. However, the photodynamic effects of HB on fungi, particularly drug-resistant *C. albicans*, are unclear. Therefore, we assessed the potential of HB to mediate the PDI of drug-sensitive and resistant strains of *C. albicans* and evaluated the PDI effects on the cellular structure and surface characteristics.

## 2. Materials and methods

### 2.1. Fungi strains and culture conditions

A standard *C. albicans* strain (ATCC 10231), azole-sensitive clinical isolate of *C. albicans*, and azole-resistant clinical isolate of *C. albicans* were provided by the First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China. The minimum inhibitory concentration (MICs) values of the azole-resistant isolate for antifungal drugs are shown in Table 1, as determined by the broth microdilution reference method [17]. The three strains were cultured on Sabouraud dextrose agar (SDA, Solarbio, Beijing, China) at 37 °C for 36 h. Colonies were transferred into 15 mL Sabouraud dextrose broth (SDB, Solarbio, Beijing, China) and incubated at 37 °C for 12 h. Cell pellets were centrifuged at 4000 rpm for 15 min (Beckman, Brea, CA, USA), followed by two washes with sterile phosphate-buffered saline (PBS, pH 7.0). *C. albicans* were resuspended in PBS to a density of $1 \times 10^7$ colony forming units (CFU)/mL for subsequent experiments.

**Table 1**
Sensitivity of the azole-resistant isolate of Candida albicans to common antifungal drugs.

| Antifungal drugs | MIC (μg/mL) | Sensitivity |
|---|---|---|
| Fluconazole | 64 | Resistant |
| Voriconazole | 8 | Resistant |
| Itraconazole | 2 | Resistant |
| 5-fluorocytosine | ≤4 | Sensitive |
| Amphotericin B | 0.5 | Sensitive |

### 2.2. PS and light source

HB was prepared as described previously [18]. Briefly, natural fungus sacs of *Hypocrella bambusae* were placed in a sorbitic extractor and extracted with acetone. After removing the acetone, the obtained viscous solid was placed in a beaker containing petroleum ether, stirred while heating to boiling, cooled and suction-filtered. These steps were repeated several times until the viscous solid was a loose solid. Recrystallization from benzene and petroleum ether was used to produce HA. HA was dissolved in 1.5% potassium hydroxide aqueous (3.5 L) and stirred for 24 h without light. The solution was neutralized with a slight excess of dilute hydrochloric acid to produce a large amount of brownish red precipitate. The precipitate was dissolved in chloroform, washed eight times with 1% sodium bicarbonate aqueous solution and washed twice with water. The brownish red solid was dried and recrystallized from benzene and petroleum ether to afford HB.

Next, 1 mM HB stock solution was freshly prepared by completely dissolving HB in 50 μL dimethyl sulfoxide (DMSO), followed by dilution to 1 mL with sterilized PBS. The stock solution was filtered through a 0.22-μm filter disk and properly diluted with sterilized PBS before use. A 50 W xenon lamp (Ceaulight CEL-HXF300, Beijing, China) was employed for irradiation experiments. Light with wavelength of 400–780 nm was selected by an optical filter (Ceaulight CEL-UVIRCUT PD-145, Beijing, China). To avoid heating the samples, a 1-cm ice-cold water filter was placed between the samples and optical filter. The light fluence rate at the sample level was adjusted to 80 mW $cm^{-2}$ with a power meter (Ceaulight CEL-NP2000, Beijing, China).

### 2.3. PDI efficacy of HB on C. albicans

*C. albicans* suspension (2 mL, $1 \times 10^7$ CFU/mL) was collected by centrifugation (4000 rpm, 15 min) and resuspended in 2 mL PBS

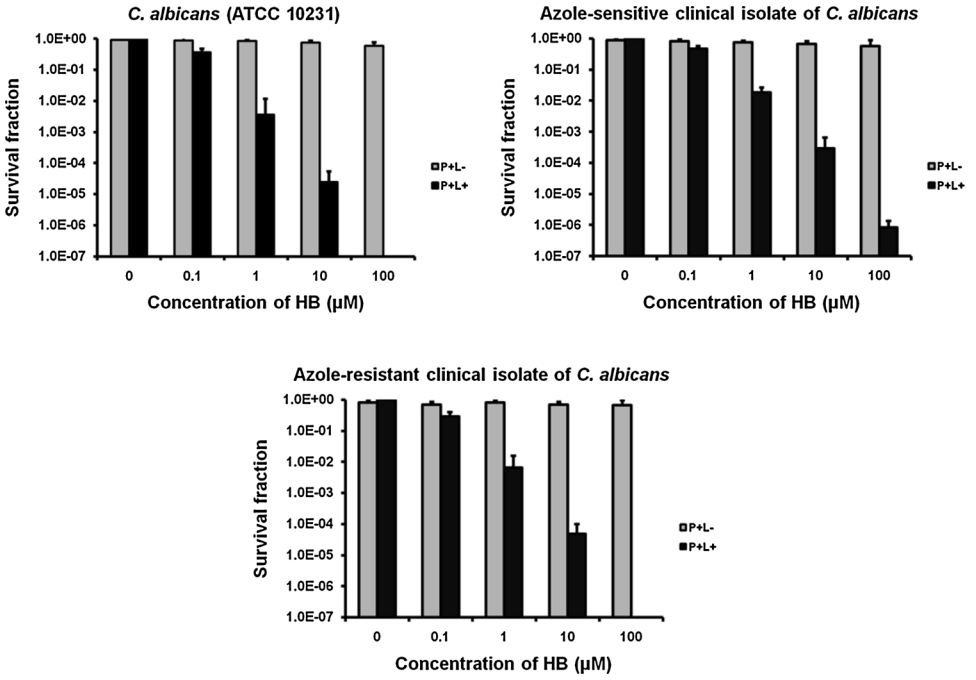


**Fig. 2.** Survival of *C. albicans* treated with different concentrations of hypocrellin B. (P + L-): represent the survival after incubation without irradiation (dark toxicity); (P + L+): represent the survival after 15 min irradiation (72 J $cm^{-2}$).

containing 0.1 (0.0005% DMSO, v/v), 1 (0.005% DMSO, v/v), 10 (0.05% DMSO, v/v), or 100 (0.5% DMSO, v/v) μM of HB. The suspension was incubated at 37 °C in the dark for 30 min in a shaking incubator (100 rpm), and then transferred into 35 mm polystyrene culture dishes. Yeast samples were irradiated with 400–780 nm light for 15 min, corresponding to an energy dose of 72 J $cm^{-2}$. Next, *C. albicans* cells were centrifuged, resuspended, and serially diluted by 10-fold with sterilized PBS. Thirty microliters of each dilution were spread in triplicate onto SDA, incubated at 37 °C for 48 h, and subjected to colony-counting. Survival was calculated as $N_{PDI}/N_0$, where $N_{PDI}$ was the number of CFU/mL after PDI, and $N_0$ was the number of CFU/mL without any treatment.

*C. albicans* cells were incubated with 100 μM of HB at 37 °C in the dark for 30 min and irradiated with 400–780 light for 5, 10, 15, and 20 min, corresponding to energy dose of 24, 48, 72, 96 J $cm^{-2}$, respectively. Additionally, *C. albicans* cells were incubated with 100 μM of HB at 37 °C in the dark for 10, 20, 30, and 40 min, and then irradiated with light for 15 min (72 J $cm^{-2}$). After illumination, the cells were centrifuged, serially diluted, and spread onto SDA. Colonies developed were counted, and survival was calculated as described above.

### 2.4. Confocal laser scanning microscopy (CLSM)

*C. albicans* was incubated with 10 μM of HB at 37 °C in the dark for 30 min, and then irradiated with 400–780 nm light for 15 min. After PDI treatment, the cells were washed three times with PBS and resuspended in 2 mL Hoechst 33342 (1 μg/mL, Aladdin, Shanghai, China) in PBS and incubated in the dark at room temperature for 10 min in a shaking incubator (100 rpm). Labeled cells were washed three times with PBS, spotted onto glass slides, and immobilized by the coverslips. Cell imaging was conducted on a CLSM (Leica SP8 STED, Wetzlar, Germany). CLSM images of HB and Hoechst 33342 fluorescence were collected using solid-state diode lasers, at excitation wavelengths of 458 and 351 nm, respectively, and with appropriate emission filters.

### 2.5. Transmission electron microscopy (TEM)

*C. albicans* was incubated with 100 μM of HB at 37 °C in the dark for 30 min, and then irradiated with 400–780 nm light for 15 min. The cells were collected by centrifugation, and fixed in 2.5% glutaraldehyde (Sinopharm Chemical Reagent Company, Beijing, China) at 4 °C for 3 h. The cell pellets were washed with PBS twice and incubated with 1% osmium tetroxide (Johnson Matthey, London, UK) at 4 °C for 3 h. The samples were dehydrated with 30% ethanol for 10 min, 50% ethanol for 10 min, 70% ethanol for 10 min, uranyl acetate in 70% ethanol for 2 h, 90% ethanol for 10 min, 100% ethanol for 10 min, and epoxypropane for 10 min, respectively, followed by embedding in Epon 812 epoxy resin (Structure Probe, Inc., West Chester, PA, USA) at 60 °C for 24 h. Next, 50–70 nm thin-sectioned samples were prepared using an LKB-V ultratome (LKB, Uppsala, Sweden). Uranyl acetate and lead citrate were used to stain the samples for 15 min. Finally, the samples were observed on a TEM (Hitachi H-7650, Tokyo, Japan).

### 2.6. Scanning electron microscopy (SEM)

*C. albicans* was incubated with 10 or 100 μM of HB and irradiated with light for 15 min as described above. After PDI treatment, suspensions were transferred into a 24-well polystyrene microplate (Corning, NY, USA) containing glass coverslips and incubated at 37 °C for 1 h. The coverslips were gently washed with PBS twice and fixed in 2.5% glutaraldehyde at 4 °C for 3 h. Next, the coverslips were gently washed with PBS three times and incubated with 1% osmium tetroxide at 4 °C for 3 h. After dehydration with 10–100% ethanol, the samples were freeze dried, sputter-coated with gold, and observed using a SEM (Hitachi TM-1000, Tokyo, Japan).

### 2.7. Statistics

SPSS 22.0 software (SPSS Inc, Chicago, IL, USA) was used to analyze the experimental data. The *t* test was conducted to validate the significance difference and *P* values of $< 0.01$ were considered as significantly different.

## 3. Results

### 3.1. PDI efficacy of HB on C. albicans

The three *C. albicans* strains were incubated with 0.1–100 μM of HB at 37 °C for 30 min in the dark and irradiated by 400–780 nm light for 15 min. As shown in Fig. 2, HB did not exhibit obvious dark toxicity against the three *C. albicans* strains at the concentrations tested, and light illumination alone had no cytotoxic effect. However, the irradiated groups showed a reduced number of viable yeast cells with increasing concentrations of HB. After irradiation with 72 J $cm^{-2}$ white light, 0.1 μM HB yielded 0.43, 0.33, and 0.54 $log_{10}$ reductions in the survival of *C. albicans* ATCC 10231, azole-sensitive clinical isolate of *C. albicans*, and azole-resistant clinical isolate of *C. albicans*, respectively. Additionally, 2.45, 1.73, 2.18 $log_{10}$ reductions in yeast survival were observed for the three *C. albicans* strains after treatment with 1 μM HB under the same light irradiation dose. A concentration of 10 μM HB caused 4.60, 3.54, and 4.32 $log_{10}$ reductions in these *C. albicans* strains. When the concentration of HB reached 100 μM, the azole-sensitive clinical isolate showed a 6.01 $log_{10}$ reduction, with no viable cells detected for *C. albicans* ATCC 10231 or azole-resistant clinical isolate of *C. albicans*, representing a 7 $log_{10}$ reduction.

To investigate the effects of light energy dose on the cell survival of *C. albicans*, fungi cells were incubated with 100 μM HB in the dark for 30 min and irradiated with 400–780 nm light for 5, 10, 15, and 20 min, corresponding to energy doses of 24, 48, 72, and 96 J $cm^{-2}$, respectively. As shown in Fig. 3, 100 μM HB with 24 J $cm^{-2}$ irradiation reduced the survival of *C. albicans* ATCC 10231 and azole-resistant *C. albicans* by over 2 $log_{10}$. Additionally, 100 μM HB with 48 J $cm^{-2}$ irradiation reduced the survival of these strains by 4 $log_{10}$. At a light energy dose of 72 J $cm^{-2}$, 100 μM HB killed all *C. albicans* ATCC 10231 and azole-resistant *C. albicans* cells. For the azole-sensitive clinical isolate of *C. albicans*, 100 μM HB with 24, 48, 72, and 96 J $cm^{-2}$ light irradiation caused 1.99, 3.66, 6.08, and 7.00 $log_{10}$ reductions in survival, respectively.

The effects of HB incubation time on the survival of *C. albicans* cells were also assessed. Cells were incubated with 100 μM HB in the dark for 0, 10, 20, 30, and 40 min, and illuminated with 72 J $cm^{-2}$ light. The results shown in Fig. 4 demonstrate that resuspending the cells in 100 μM HB and irradiation with 72 J $cm^{-2}$ light directly (0 min incubation) caused over 1 $log_{10}$ reductions in the survival of *C. albicans* ATCC 10231, azole-sensitive *C. albicans*, and azole-resistant *C. albicans*. As the incubation time increased, survival of the three strains decreased. When the incubation time reached 30 min, no viable cells were detected for *C. albicans* ATCC 10231 or azole-resistant *C. albicans*, and 6 $log_{10}$ reduction in the survival of azole-sensitive *C. albicans* was observed. Incubation with 100 μM HB for 40 min and irradiation with 72 J $cm^{-2}$ light caused 6.73 $log_{10}$ reductions in the survival of azole-sensitive *C. albicans*.

### 3.2. Fluorescence labeling

To investigate the uptake of HB by *C. albicans* cells, the three strains were incubated with 10 μM of HB at 37 °C in the dark for 30 min, and then stained with the DNA-specific fluorescent dye Hoechst 33342 (1 μg/mL) in the dark for 10 min. As shown in Fig. 5, spots of weak red fluorescence from HB were observed in the *C. albicans* cells (HB and Merge, P + L-). The nucleus was differentiated as punctate blue fluorescence (Hoechst 33,342 and Merge, P + L-). After irradiation with 72 J $cm^{-2}$ light, stronger red fluorescence from HB and blue fluorescence from Hoechst 33342 were observed in throughout the *C. albicans* cells (HB, Hoechst 33342, and Merge, P + L+).

### 3.3. Photodynamic effect on cellular structure

To examine whether the HB-mediated photodynamic effect caused any morphological changes in the cellular structure, the *C. albicans* cells were analyzed by TEM; representative results are shown in Fig. 6. The untreated cells of *C. albicans* ATCC 10231, azole-sensitive clinical isolate of *C. albicans*, and azole-resistant clinical isolate of *C. albicans* displayed a normal morphology with a characteristic thick cell wall, intact plasma membrane and irregularly shaped nucleus. The cell wall and plasma membrane were well-differentiated, and ribosomes were visible as dark particles dispersed in the cytoplasm (P-L-). After treatment with 100 μM HB and 72 J $cm^{-2}$ light irradiation, comparison with untreated cells revealed significant damage to the cell wall, membrane,

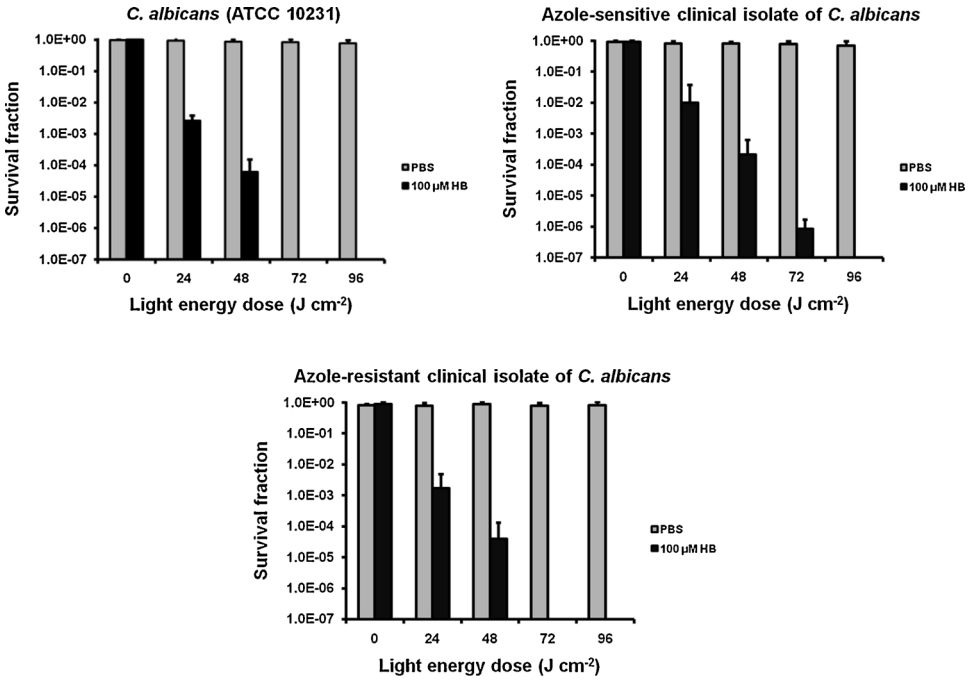


**Fig. 3.** Survival of *C. albicans* irradiated with different light energy doses. (PBS): represent the survival of *C. albicans* resuspended in PBS; (100 μM HB): represent the survival of *C. albicans* incubated with 100 μM HB for 30 min in the dark.

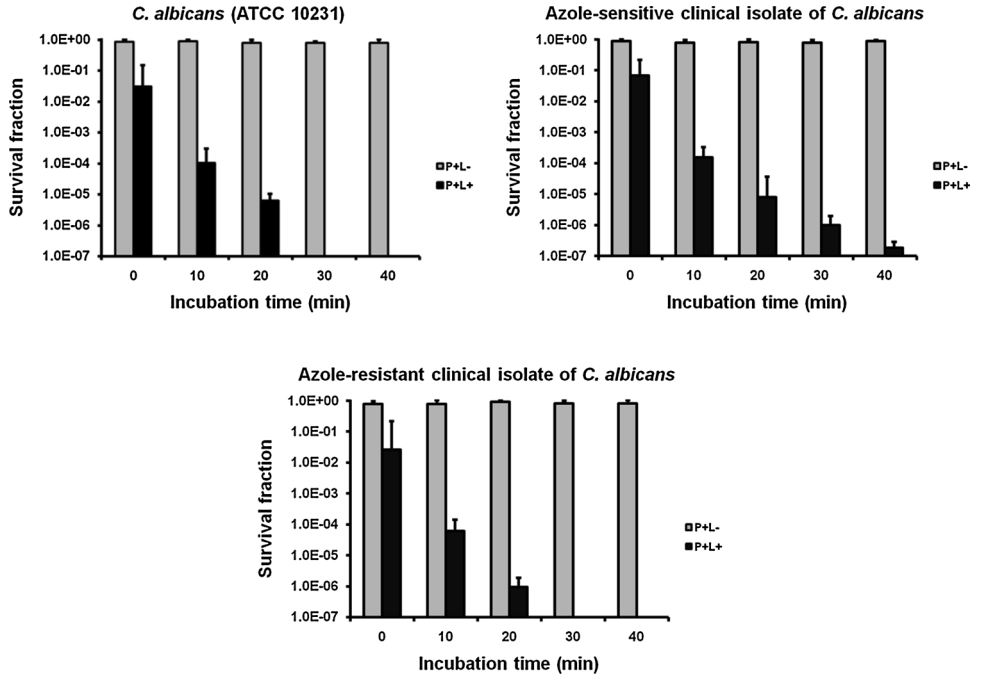


**Fig. 4.** Survival of *C. albicans* incubated with 100 μM HB for different period. (P + L-): represent the survival after incubation without irradiation (dark toxicity); (P + L+): represent the survival after 15 min irradiation (72 J $cm^{-2}$).

and cytoplasm. The cell envelopes showed shape changes characterized by cell wall swelling and membrane rupture. Obvious condensation of the cytoplasm was observed in *C. albicans* cells and the nuclei were not visible (P + L+).

### 3.4. Photodynamic effect on cell surface

Yeast cells were analyzed by SEM to determine whether the photodynamic effect mediated by HB altered the cell surface characteristics (Fig. 7). A normal round shape with a smooth surface was observed for untreated cells of *C. albicans* ATCC 10231, azole-sensitive clinical isolate of *C. albicans*, and azole-resistant clinical isolate of *C. albicans* (P-L-). However, 10 μM HB combined with 72 J $cm^{-2}$ light irradiation induced twisted surface on these *C. albicans* cells. After incubation with 100 μM HB and irradiated with 72 J $cm^{-2}$ white light, ruptured *C. albicans* cells were observed (P + L+).

## 4. Discussion

*C. albicans* is predominant on oral mucosal layers, in vaginal infections, and in invasive bloodstream infections [19]. It is not only the main etiological agent associated with oral candidiasis in humans but also the most common mycosis affecting patients with HIV, in whom oropharyngeal candidiasis is a major cause of morbidity. Overuse of antifungal drugs in these affected patients significantly increased their resistance, and approximately 81% of patients with HIV infection are estimated to be colonized with azole-resistant *C. albicans* strains [20]. aPDT is an emerging and promising approach for treating diseases caused by fungi, particularly in localized and superficial infections such as oral and vaginal candidiasis. aPDT is unlikely to induce resistance and can inhibit *C. albicans* virulence factors as well as reduce *in vivo* pathogenicity [21]. Previous studies demonstrated that *C. albicans* was effectively inactivated *in vitro* by a wide variety of traditional PSs such as 5-aminolaevulinic acid [22], methylene blue (MB) [23], toluidine blue O [24], rose bengal (RB) [25], porphyrin [26], and phthalocyanine [27]. Although these PSs were effective against *C. albicans*, indicating their potential for use in aPDT of localized and superficial infections caused by *C. albicans*, the PDI efficacy and merits of these PSs cannot be accurately compared because of differences in the PS structures and light sources used.

There is a long history of using traditional Chinese herbs as folk medicine for treating infectious diseases, and many active compounds isolated from traditional Chinese herbs have been shown to have anti-infection and anti-inflammation effects [28]. Hypocrellin, active pigment isolated from *H. bambuase*, has been used as a traditional Chinese medicine to treat rheumatoid arthritis, gastric diseases, and skin diseases related to fungal infections for several years [29]. Ma et al. evaluated the intrinsic antimicrobial activities of HB against *C. albicans* and found that its MIC value was 22.0 μg/mL [30]. Although HB showed weak antifungal activity for *C. albicans*, much more attention has been focused on its photodynamic applications. In this study, we demonstrated that HB combined with 400–780 nm white light was effective for photodynamic killing of *C. albicans*. In the presence of 72 J $cm^{-2}$ light, 10 and 100 μM HB yielded 3.54–4.60 and 6.01–7.00 $\log_{10}$ reductions in the survival of the three *C. albicans* strains. Compared to another second-generation anionic PS hematoporphyrin monoethyl ether developed in China, HB showed relatively lower PDI efficacy [31]. Cationic charges have been reported to enhance the binding affinity of PSs to bacteria and therefore increase PDI efficacy [32]. However, the opposite results might be obtained for *C. albicans*. Zhou et al. found that benzylidene cyclopentanone-based PS with an anionic group was more effective in PDI of *C. albicans* compared to the same PS with a cationic group [33].

Few studies have examined PDI efficacy towards drug-resistant *C. albicans*. Dovigo et al. found that azole-resistant *C. albicans* strains were more resistant to porphyrin-mediated aPDT compared to sensitive strains [34]. Opposite results were obtained by Mang et al., who demonstrated that fluconazole-resistant *C. albicans* were equally susceptible to Photofrin-mediated aPDT as compared to non-resistant strains [35]. Paz-Cristobal et al. demonstrated that the resistance mechanisms developed by *C. albicans* against azoles do not interfere with the mechanism of photodynamic cell death using hypericin. They suggested that the mechanisms of resistance to antifungal drugs affect the efficacy of PDI depending on the PS used [36]. The results of the present study showed that the susceptibility of *C. albicans* ATCC 10231 to HB was similar to that of the azole-resistant clinical isolate of *C. albicans*, which

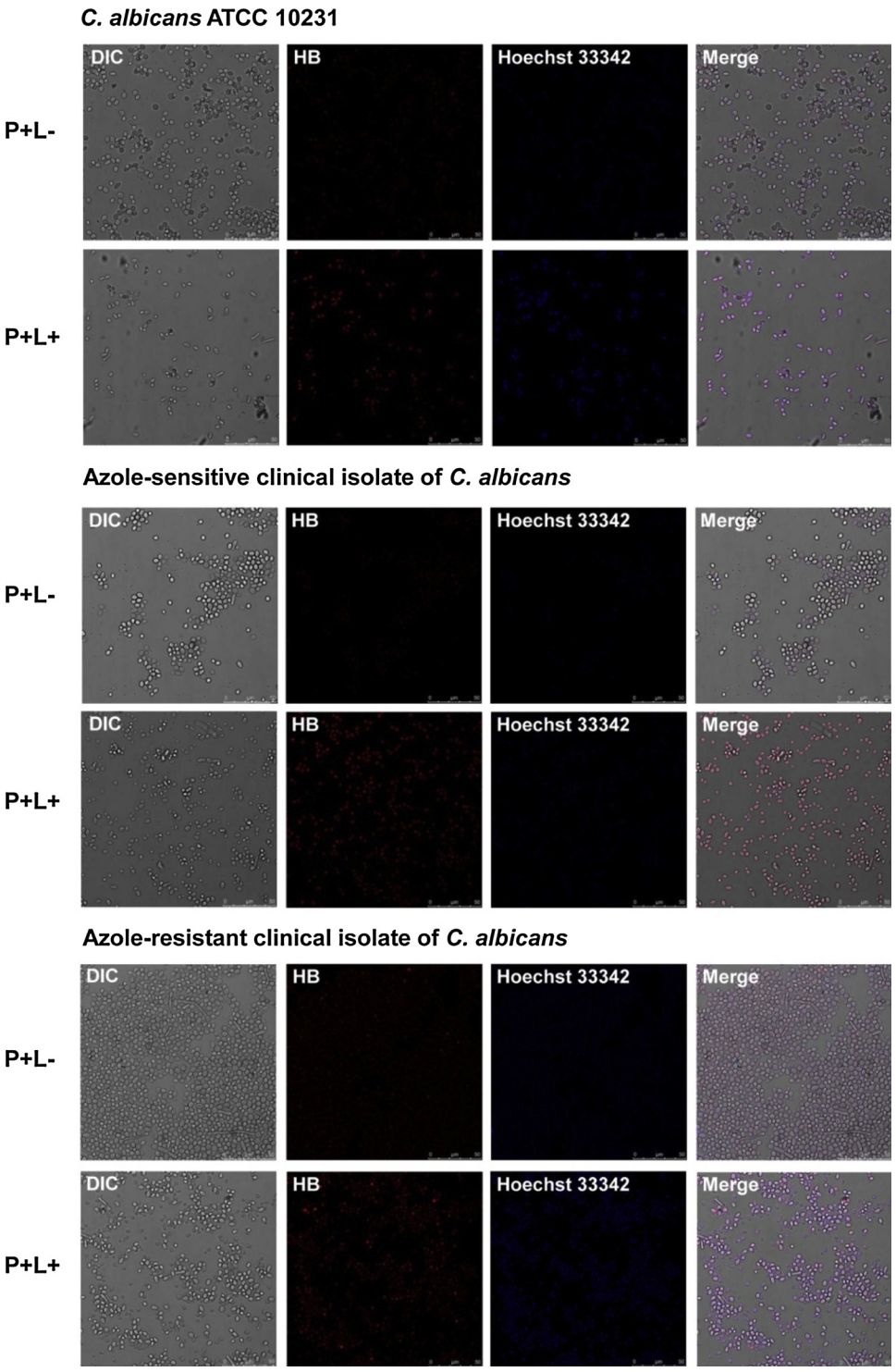


**Fig. 5.** Confocal laser scanning microscope (CLSM) images of *C. albicans* cells. (P + L-): *C. albicans* cells incubated with 10 μM HB in the dark for 30 min, and stained with Hoechst 33342 for 10 min in the dark. (P + L-): *C. albicans* cells incubated with 10 μM HB in the dark for 30 min, irradiated with light for 15 min (72 J $cm^{-2}$), and stained with Hoechst 33342 for 10 min in the dark.

agrees with previous studies [35,36]. However, the PDI efficacy of HB against the azole-sensitive clinical isolate of *C. albicans* was relatively lower than that of *C. albicans* ATCC 10231 and azole-resistant clinical isolate of *C. albicans*. Similar results were obtained in several previous studies [37–40]. Dovigo et al. evaluated aPDT mediated by curcumin against five clinical isolates of *C. albicans*, and found differences in the susceptibility of these isolates [38]. Oliveira-Silva et al. suggested that the different susceptibilities were related to the efflux systems of *C. albicans*, and isolates overexpressing ATP-binding cassette pumps tended to show decreased PS uptake and survive following PDI [39]. Rossoni et al. reported that biofilms of *C. albicans* serotypes A and B differed in their sensitivity to PDI, but the sensitivity of *C. albicans* to PDI in planktonic form was unclear [40]. We suggested that the different susceptibilities of various isolates resulted from several factors. To better understand this, further studies are needed to investigate the characteristics of these isolates in our subsequent research.

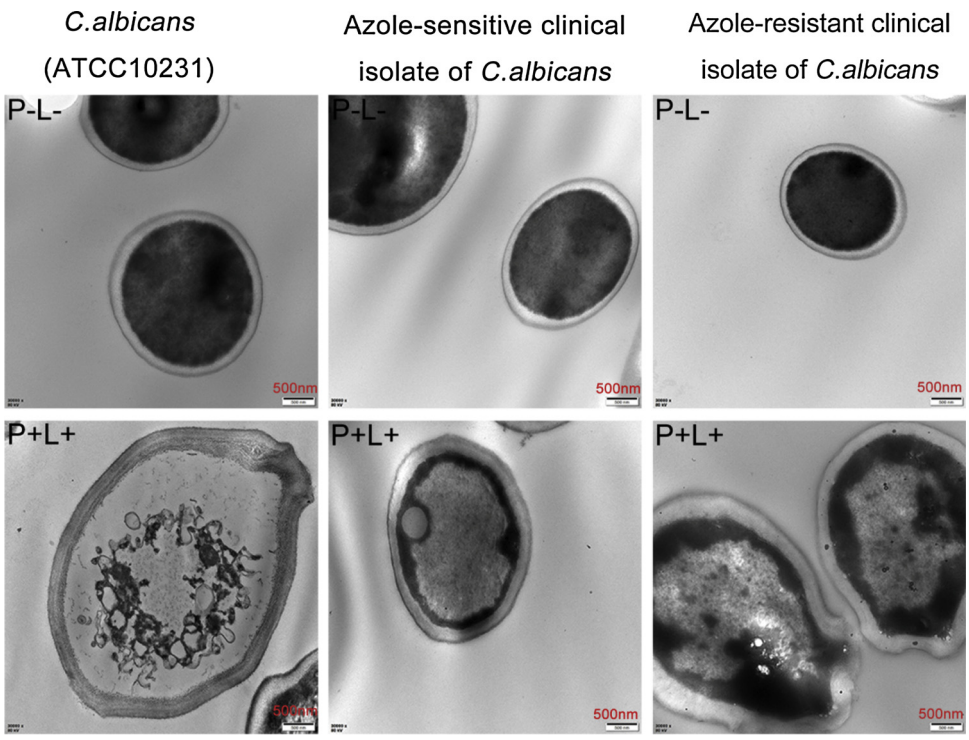


**Fig. 6.** Transmission electron microscopy images of *Candida albicans*. (P-L-): No treatment. (P + L+): Incubated with 100 μM hypocrellin B for 30 min and irradiated with 400–780 nm light for 15 min (72 J $cm^{-2}$).

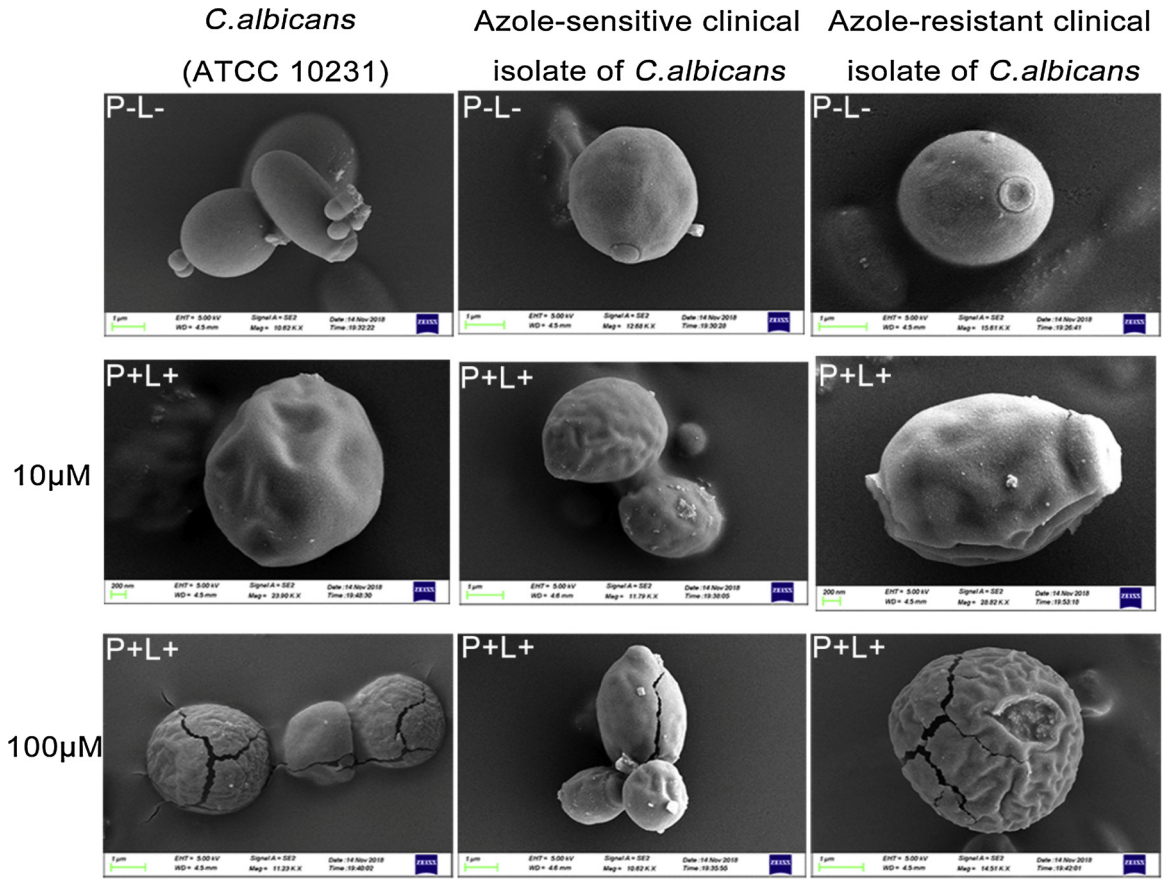


**Fig. 7.** Scanning electron microscopy images of *Candida albicans*. (P-L-): No treatment. (P + L+): Incubated with 10 or 100 μM hypocrellin B for 30 min and irradiated with 400–780 nm light for 15 min (72 J $cm^{-2}$).

The damage induced by HB-mediated PDI to the cell envelop and cytoplasmic organelles of *C. albicans* was clearly observed by TEM. HB-mediated PDI induced membrane ruptures and cell wall swelling of *C. albicans*, suggesting increased cell membrane permeability and impaired cell wall function. Similar results were also found in a previous study reported by Monfrecola et al., who employing 5-aminolaevulinic acid for PDI of *C. albicans* [22]. Zhou et al. found that benzylidene cyclopentanone-based PS with two anionic groups in the presence of light damaged the cell wall, preventing the cells from maintaining their normal size and shape. They predicted that PDI mediated by this PS induced cell wall and cell membrane damage which enhanced permeability, and then PS gradually penetrated the cells and caused further intracellular photodynamic damage. However, they observed no significant changes in cell morphology and size, and the cell wall thickness was normal compared to untreated cells after PDI mediated by benzylidene cyclopentanone-based PS with one anionic or cationic group, which could target and damage the mitochondria of *C. albicans* during the irradiation process [33]. Although the data reported by Zhou et al. suggested that the PDI mechanism towards *C. albicans* was dependent on the PS used, TEM images obtained in this study indicated that HB caused damage to the cell envelope which was in accordance with the results observed for benzylidene cyclopentanone-based PS with two anionic groups. Direct evidence provided by SEM images revealed the twisted and ruptured *C. albicans* cells after PDI treatment, suggesting that the cell envelope was severely damaged. Based on previously reported results and those obtained in the present study, ROS may be mainly responsible for the effects on the cell wall and cytoplasmatic components stimulating alterations in the cell envelope structure and accumulation of macromolecules. This process can prevent the *C. albicans* from multiplying and producing viable cells.

In conclusion, we investigated the PDI effects mediated by HB on azole-sensitive and azole-resistant *C. albicans in vitro*. HB showed no obvious dark toxicity, and effectively inactivated *C. albicans* cells in a light-dose and PS concentration-dependent manner. The susceptibility of *C. albicans* strains to HB-mediated PDI was not affected or impaired by the yeast being resistant to azole antifungal agents, suggesting that HB is a good PS for use in aPDT of drug-resistant *C. albicans* strains. TEM indicated that HB-mediated PDI induced significant damage to the cell wall, membrane, cytoplasm, and nuclei. SEM analysis revealed that the surface of *C. albicans* cells became twisted and ruptured after PDI treatment. These data suggest that ROS might be responsible for the damage to cytoplasmatic and cell wall components, and differs from the mechanism of antifungal drugs. The non-specific action of ROS produced by aPDT was unlikely to induce resistance of *C. albicans*. Based on these results, HB-mediated aPDT shows promise as an antifungal treatment.

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