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The Effects of Dehydrozingerone on Growth, Biofilm Formation, and Ergosterol Biosynthesis of *Candida albicans*

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ABSTRACT

Introduction: *Candida albicans* can cause various diseases, which might lead to various cases of life-threatening diseases. Biofilm is a specific feature of *C. albicans* formed on mucosal surfaces and medical devices. Moreover, biofilm protects *Candida* cells from antifungals and makes the treatment challenging. Here, we studied the effects of dehydrozingerone on *C. albicans* growth, ergosterol biosynthesis, biofilm formation, and the expression of an essential gene involved in yeast-hypha transition. **Methods:** *C. albicans* cells were treated with serial two-fold concentrations of dehydrozingerone (0.125-2 mg/ml) for 48 h at 35 °C. The weights of the fungal cells were estimated as a sign of fungal growth. Biofilm formation was evaluated by a tetrazolium salt (XTT) reduction assay. The expression of the *HWP1* gene was assayed by real-time PCR. **Results:** Dehydrozingerone inhibited *C. albicans* growth in the range of 3.57% to 84.28%, dose-dependently. The ergosterol content of yeast cells was reduced by 50% in the highest concentration. The biofilm formation was also inhibited by more than 50% at the highest concentration. The expression of the *HWP1* gene was suppressed by dehydrozingerone at different concentrations. **Conclusion:** Our results indicate that dehydrozingerone displayed effective activity against growth, biofilm formation, and ergosterol biosynthesis in *C. albicans in vitro*.

INTRODUCTION

Candida albicans is an opportunistic fungus that causes infection in immunodeficient individuals. Various essential factors contribute to *C. albicans* pathogenesis, including biofilm formation. Biofilm is composed of various microorganisms that are fastened to the medical devices or mucosal surface, possibly leading to superficial and systemic infections. *C. albicans*, one of the members of the genus *Candida*, is commonly related to biofilm development on medical devices [1, 2]. Compared to the planktonic cells, the *Candida* biofilm structures are more resistant to antifungal agents [3, 4].

Several adhesion families play an important role in adhesion and biofilm formation. Hyphal wall protein (Hwp), one of the essential family of the adhesions, includes several members, *Hwp1*, *Hwp2*, *Rbt1*, *Eap1*, and *Ywp1*. The *Hwp1*, a mannoprotein in the cell wall of hyphal cells and germ tubes, plays a crucial role in biofilm formation [5, 6].

Ergosterol is an essential component of membrane lipids and controls fungi membrane permeability, fluidity, and integrity [7]. Current available antifungal agents for treating candida infections can be grouped into several chemical curriculums with various cellular targets. Enzymes of the ergosterol biosynthetic pathway are essential targets for numerous antifungals used in candidiasis, with the polyenes and the azoles having the central position among them [8].

Several studies attempted to find efficient strategies for controlling fungi growth and biofilm formation [6]; moreover, various attempts were made to find compounds or products with a natural base with antifungal and anti-biofilm activity. For example, Wong *et al.* (2014) recognized functional agents with inhibitory activity on growth and biofilm formation of *C. albicans* [9]. Phenazines produced by *P. aeruginosa* modulate *C. albicans* metabolism, hyphal transition, and biofilm formation [10].

Dehydrozingerone (DHZ) is a phenolic compound isolated from ginger rhizomes. This compound is the structural half analog of curcumin (essential compound of turmeric rhizomes [11] with antioxidant, antimutagen, anti-inflammatory, antimalarial, antifungal/antifeedant, and antiplatelet activity as well as properties against Alzheimer's disease [12]. This study surveyed the effects of dehydrozingerone on growth, biofilm formation, and ergosterol biosynthesis in *C. albicans*. We also

investigated one of the critical genes, *HWP1*, involved in the biofilm formation of *C. albicans*.

MATERIAL AND METHODS

Chemicals. RPMI 1640 was obtained from Biosera (France). 3-bis (2-methoxy-4-nitro- 5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and dehydrozingerone (CAS. 1080-12-2) were purchased from Sigma (St. Louis, MO, USA). Sabouraud dextrose agar (SDA) was obtained from Merk (E. Merck, Germany). Sabouraud dextrose broth (SDB) was purchased from Scharlau Chemie (S. A., Barcelona, Spain). GITC (Guanidium isothiocyanate) reagent, RNase-free DNase, random hexamer primers, Revert Aid M-MuLV reverse transcriptase, and SYBR Green master mix were obtained from Thermo Fisher Scientific (USA). *n*-Hexan (CAS. 110-54-3) was purchased from Sigma (St. Louis, MO, USA). The stock solution of dehydrozingerone was organized in dimethyl sulfoxide, purchased from Sigma-Aldrich.

Strain and culture condition. *C. albicans* ATCC10231 from pathogenic fungi culture collection (PFCC) of the Pasteur Institute of Iran was cultured on Sabouraud dextrose agar and incubated at 28 ℃ for 48 h. The fungal suspensions were prepared in final inoculum sizes of 10^5cells/ml for the growth inhibition analyses and 0.5-2.5×10^3 cells/ml for the biofilm formation.

Determination of the minimum inhibitory concentrations. The antifungal activity of dehydrozingerone (DHZ) was done by the broth microdilution method according to CLSI standard M27-A3 [13]. Different concentrations of DHZ (0.125 to 2 mg/ml) were arranged, and RPMI 1640 with MOPS (0.165 M, pH 7.0) was used to establish the test. Antifungal activity assay was done with final inoculum concentrations of 0.5-2.5×10^3 cells/ml in a 96-well U-bottomed microtitration plate followed by incubation at 37°C for 48 h. The optical density was measured at 600 nm to analyze minimum inhibitory concentrations (MICs) with a plate reader (BioTek, USA). The inhibitory concentration of 50% (MIC_{50}) was determined by comparing the growth of *C. albicans* in treated samples alongside the controls [14]. The experiments were repeated in triplicate.

Biofilm formation. *C. albicans* 10231 was cultured on Sabouraud dextrose agar plates for 24 h at 37°C. The fungal suspension at 10^6cells/ml concentrations in RPMI-1640 was added into flat-bottom 96-well microtiter plates, treated with different concentrations of DHZ (0.125 to 2mg/ml) to a final volume of 200 µl per well. The biofilm formation was determined after 24 h at 37°C using 2,3-bis (2-methoxy-4-nitro- 5-sulphophenyl)-2H-tetrazolium-5-carboxanilide. The metabolic activity was measured spectrophotometrically at 490 nm using a microplate reader [15].

Ergosterol extraction and quantitation. Fungal sterols were extracted according to Breivik and Owades (1957) with slight modifications [16]. Briefly, spore suspension was cultured in the presence of different concentrations of DHZ. The culture was incubated 72 h at 35 °C at 180 rpm on a shaking incubator; after incubation time, the fungal mass was harvested by centrifugation (10000 rpm for 5 min) and washed three times by distilled water. The weight of the yeasts was measured, and all samples were equiponderated (samples had the same weight) and transferred to the test tube. Saponification was performed by adding 3 mL of 25% alcoholic KOH solution (25 g of KOH and 35 mL of sterile distilled water, brought to 100 mL with 100% ethanol) to each tube and mixing for 1 min by vortexing followed by incubating in an 85 °C water bath for 1 h. After incubation, the tubes were allowed to cold at room temperature. Nonsaponifiable sterols were extracted by adding a mixture of 1 ml distilled water and 3 mL *n*-hexane and shaken for 3 min. The hexane layer was collected and transferred to another clean tube and stored at −20 °C for 18–24 h. After incubation, the sterol extracts were diluted fivefold in 100% ethanol. For analysis, the ergosterol content was scanned by spectrophotometry between 200 and 300 nm with a Perkin–Elmer EZ 301 spectrophotometer. The amount of ergosterol and the late sterol intermediate, 24 (28)-dehydroergosterol (DHE), were estimated in a percentage of the fungal weight and quantified by the following formula:

$$\%\text{ergosterol}+\%24\ (28)\ \text{DHE} = [(A_{281.5}/290) \times F] / \text{pellet weight},$$

$$\%24\ (28)\ \text{DHE} = [(A_{230}/518) \times F] / \text{pellet weight},$$

$$\text{and } \%\text{ergosterol} = [\%\ \text{ergosterol}+\%24(28)\text{DHE}] - \%24\ (28)\ \text{DHE}$$

F is the factor for dilution in ethanol, and 290 and 518 are the *E* values (in percentages per centimeter) for crystalline ergosterol and 24 (28) - DHE.

Gene expression by real-time PCR. Fungal cells were treated with different concentrations (0.125 to 2 mg/ml) of DHZ in RPMI 1640 and incubated at 37°C for 24 h. The yeast cells were homogenized by glass beads, and the total RNA was extracted using GITC reagent and treated with RNase-free DNase [17]. The cDNA was prepared using1000 ng RNA with random hexamer primers and Revert Aid M-MuLV reverse transcriptase. The expression of the *HWP1*gene was determined by Real-Time quantitative RT-PCR. The amplification was performed using the SYBR Green master mix in 25µl reactions containing 20 ng cDNA and 0.2μ M of each primer in a Rotor gene 6000 (Corbett) sequence detection system. The specific primer sets for real-time PCR were:

HWP1…F5'- CTCCAGCCACTGAAACACCA-3', R5'- GGTGGAATGGAAGCTTCTGGA-3', and

ACT1…F5'-CGTTGTTCCAATTTACGCTGGT-3', R5'-TGTTCGAAATCCAAAGCAACG-3' [18].

Real-time RT-PCR program included an initial incubation at 95°C for 10 min, followed by 35 cycles of 95 °C for 60 sec, 60 °C for 60 sec and 72 °C for 45 sec. The reactions were repeated in triplicate. The *β-actin* gene was used as a reference, and the folding changes were determined using the relative threshold method ($2^{-\Delta\Delta CT}$) [19].

Statistical analysis. The biofilm formation and gene expression data were analyzed by one-way analysis of variance (ANOVA) using GraphPad PRISM 6 (GraphPad Prism Software Inc, San Diego, CA, USA). The differences with $P<0.05$ were considered significant.

RESULTS

Effects of dehydrozingerone on the fungal growth. The results revealed that DHZ inhibited the fungal growth dose-dependently with increasing concentrations. DHZ was a growth inhibitor with an MIC_{50} of approximately >1mg/ml concentration at 48 h (Table 1).

Table 1. Inhibitory effect of dehydrozingerone on *C. albicans* growth

dehydrozingerone (mg/ml)	Fungal mass (mg)	Fungal growth (%)	Growth inhibition (%)
0	0.175 ± 0.02	100	0.00
0.125	0.168 ± 0.04	96.42	3.57
0.25	0.153 ± 0.06	87.85	12.14
0.5	0.123 ± 0.02	70.71	29.28
1	0.100 ± 0.07	57.15	42.85
2	0.027 ± 0.003	15.71	84.28

Effects of dehydrozingerone on *Candida* biofilm formation. Yeast cells were treated with various concentrations of DHZ, and biofilm formation of *C. albicans* ATCC10231 was defined after 24 h. Biofilm formation suppression was observed dose-dependently at all concentrations of DHZ, with~50% inhibition at a concentration of 1mg/ ml (Fig. 1).

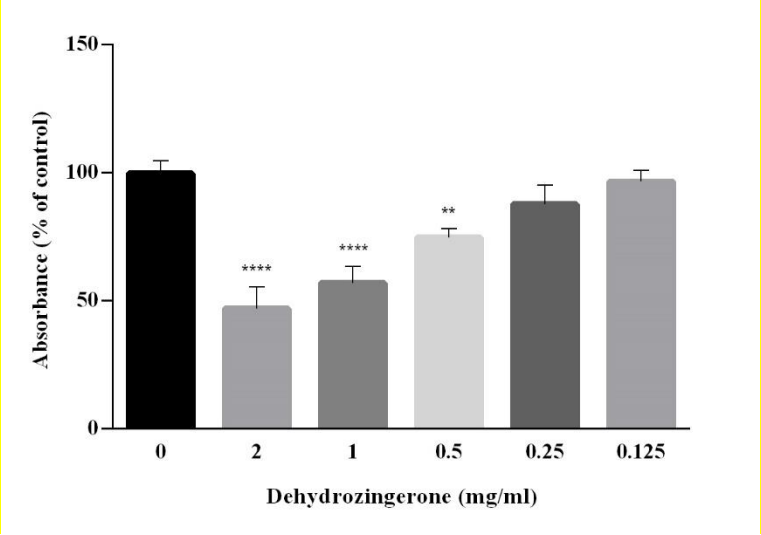

Fig. 1. The effect of dehydrozingerone on *C. albicans* biofilm formation in various concentrations. Biofilm viability was estimated using the XTT assay. The mean of XTT values of the growth control was set to 100%. The results are presented as the means ± standard deviations of three independent experiments. (* *) $P<0.01$ and (* * * *) $P<0.001$ compared to the control (0 mg/ml).

Effects of dehydrozingerone on ergosterol synthesis. Table 2 shows the effect of DHZ on ergosterol production in the plasma membrane of *C. albicans*. The ergosterol amount was inhibited dose-dependently by DHZ. The inhibition percentage of ergosterol was estimated at 31.34% to 1.91% in the highest and lowest concentration, respectively, after treatment with different concentrations of DHZ.

Effect of dehydrozingerone on the expression of *HWP1* gene. Total RNA was extracted from *C. albicans* after treatment with different concentrations of DHZ. The expression of the *HWP1* gene, related to biofilm formation, was evaluated by a real-time PCR.

Confirmation of the result was done by displaying a single peak in the melt curves and the correct size of the amplicons, verified by agarose gel electrophoresis. The results indicated suppression of the gene expression in *C. albicans* (P<0.0001) after treatment with DHZ and reductions at the transcriptional level of the gene in a dose-dependent manner. The gene expression was suppressed by ~45% at the highest DHZ concentration (Fig.2).

Table 2. Effect of dehydrozingerone on *C. albicans* ergosterol synthesis

Dehydrozingerone (mg/ml)	Ergosterol content per fungal mass (µg/mg)	Ergosterol Inhibition (%)
0	0.105 ± 0.01	0.00
0.125	0.103 ± 0.02	1.91
0.25	0.0986 ± 0.03	6.09
0.5	0.0845 ± 0.001	19.52
1	0.0765 ± 0.05	27.14
2	0. 0721 ± 0.002	31.34

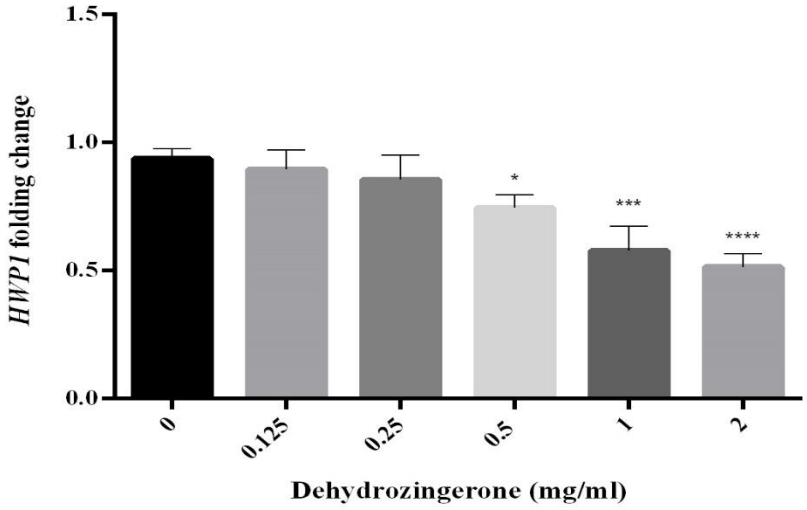

Fig. 2. The level of *HWP1* mRNA expressions in treated (0.125 to 2 mg/ml) and non-treated *C. albicans* cells. Each sample was normalized for the amount of template to the levels of *b-actin.* Asterisks show significant differences in mRNA levels between the treated and the control (0 mg/ml) fungal cells. The bars represent the mean ± standard deviation of three independent experiments, (P<0.0001)..

DISCUSSION

Since ancient times natural products have been used for the treatment of various diseases. Among them, plants, a unique source of numerous traditional medicines worldwide for millennia, have now been indicated as templates for improving novel drugs [12].

Studies demonstrated that biofilm formation is the most crucial step to trigger an infection in humans [20]. Therefore, many attempts were made to find molecules of natural origin with antifungal and antibiofilm properties [21].

In this study, we investigated the antifungal and antibiofilm activity of DHZ on *C. albicans*; moreover, we evaluated the effect of this compound on ergosterol synthesis. Microbiological activities of DHZ toward some strains of bacteria and fungi have been evaluated; for example, Kubra *et al*. (2012) studied the antifungal activity of DHZ on various filamentous fungi such as *Aspergillus*, *Fusarium*, and *Penicillium* spp, with MIC and MFC, ranging from 755 to 911 μM and 880 to 1041 μM, respectively, they showed that the mycelia and spore germination were significantly inhibited [11]. Burmudzija *et al*. (2017) reported that some derivatives of DHZ represented substantial microbiological activities against some strains of bacteria and fungi. Moreover, these compounds demonstrated *in vitro* cytotoxic activity against some cancer cell lines (HeLa, LS174, and A549) [22]. Svetaz *et al*. (2014) have studied biotransformation and antifungal activity of DHZ and two of its derivatives on some filamentous fungi, including *Aspergillus, Fusarium, Geoterichum, Cunninghamella, Mucor, Penicillium, Rhizopus,* and *Trichosporum* [23]. In the present study, we showed that the growth and biofilm

formation of *C. albicans* was inhibited approximately 50% at about 1 mg/ml concentrations of DHZ (Table 1, Fig.1); moreover, ergosterol synthesis was reduced by about 31% at the highest concentration (Table 2).

Filamentation is a crucial ability and one of the critical virulence factors of *Candida* spp. The *HWP1*, encoding hyphal wall protein (HWP) and cell wall glycoproteins, has a significant role in filamentation and biofilm formation [24]. Therefore, any suppression or elimination of *HWP1* leads to significant defects in the biofilm formation process [25].

Many studies on the effect of different compounds on biofilm formation involved genes. Purpurin strongly inhibited biofilm formation in *C. albicans* and suppressed the critical genes, *ECE1*, *ALS3*, *HYR1*, *HWP1*, and *RAS1*, responsible for filamentation of this pathogenic yeast [26]. Abd El-Baky *et al*. (2016) studied the effect of anti-inflammatory drugs and dexamethasone on biofilm formation and expression of some adhesion-related genes of *C. albicans* and *Staphylococcus aureus*. They reported that the expression of the critical genes, *HWP1* and *ALS1*, in biofilm formation were affected by these agents [27]. It has been demonstrated that allicin represented a substantial effect on the expression of the *HWP1* gene in *C. albicans* [28].

Here we showed that DHZ suppresses the expression of the *HWP1* gene under the *in vitro* test conditions. Furthermore, our results demonstrated that the suppression in the *HWP1* gene expression was in a dose-dependently manner. In the previous study, we showed that ellagic acid suppressed *HWP1* gene expression by up to 80% [29], but here, the expression of the *HWP1* gene did not reach 50% of the highest concentration of DHZ in comparison with control (Fig. 2). Meanwhile, we showed that DHZ, in *C. albicans*, had inhibitory activity on ergosterol biosynthesis, the most important mechanism of antifungal effect on the fungal cell.

Since DHZ exhibited an inhibitory effect on fungal growth, ergosterol synthesis, and biofilm formation by suppressing a gene responsible for biofilm formation, it can be a valuable natural-based substance in pharmaceutical use to prevent biofilm formation and treatment *Candida* infection.

CONFLICT OF INTEREST

The authors have not declared any conflict of interest.

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