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Antifungal Activity of *Salvia miltiorrhiza* Against *Candida albicans* Is Associated with the Alteration of Membrane Permeability and (1,3)-β-D-Glucan Synthase Activity

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Candidiasis has posed a serious health risk to immunocompromised patients owing to the increase in resistant yeasts, and *Candida albicans* is the prominent pathogen of fungal infections. Therefore, there is a critical need for the discovery and characterization of novel antifungals to treat infections caused by *C. albicans*. In the present study, we report on the antifungal activity of the ethanol extract from *Salvia miltiorrhiza* against *C. albicans* and the possible mode of action against *C. albicans*. The increase in the membrane permeability was evidenced by changes in diphenylhexatriene binding and release of both 260-nm-absorbing intracellular materials and protein. In addition, inhibition of cell wall synthesis was demonstrated by the enhanced minimal inhibitory concentration in the presence of sorbitol and reduced (1,3)-β-D-glucan synthase activity. The above evidence supports the notion that *S. miltiorrhiza* has antifungal activity against *C. albicans* by the synergistic activity of targeting the cell membrane and cell wall. These findings indicate that *S. miltiorrhiza* displays effective activity against *C. albicans* in vitro and merits further investigation to treat *C. albicans*-associated infections.

Keywords: Antifungal activity, *Candida albicans*, membrane permeability, (1,3)-β-D-glucan synthase, *Salvia miltiorrhiza*

Introduction

Candida species are mild commensals in humans, but opportunistic pathogens that can cause superficial and systemic infections in severely ill or immunocompromised patients [22]. Although more than 100 species of *Candida* have been described, most of all *Candida*-associated nosocomial bloodstream infections are associated with *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei* [22, 23], and *C. albicans* is known as a primary fungal pathogen [14].

Regardless of the extensive efforts to develop new antifungal agents, currently available drugs are very restricted for the treatment of *Candida* infections. There are four molecular groups targeting three different metabolic pathways that are available for the treatment of systemic fungal infections, including fluoropyrimidine analogs, polyenes, azoles, and echinocandins [29]. Polyene drugs such as amphotericin B target ergosterol. Ergosterol is the analog of cholesterol found in fungal cell membrane, which plays a role in membrane integrity, membrane fluidity, and the function of many membrane-bound enzymes [10, 32]. Azole drugs are involved in the ergosterol biosynthetic pathway by inhibiting lanosterol 14α-demethylase [3], and echinocandins target the biosynthesis of (1-3)-β-glucan, which is a major component of the cell wall in fungi [15]. Along with the emergence of resistant yeasts, approach to *Candida* infections is problematic due to the eukaryotic characteristics of fungi and the close evolutionary relationship between fungal cells and their human hosts, reducing the number of drug targets that can be applied to the pathogen selectively [26]. Thus, it is essential to develop new antifungal

agents with good therapeutic effect as well as low toxicity. Plant products have been used traditionally in ethnomedicine as effective antifungals, and are regarded as a part of the defense mechanism in higher plants [6]. *Salvia miltiorrhiza* Bunge, belonging to *Lamiaceae*, is a perennial plant that grows in China and East Asia. Roots of the plant have been used in ethnomedicine, and hold lipophilic tanshinone I, tanshinone IIA, cryptotanshinone, and dihydrotanshinone as well as the hydrophilic danshensu and salvianolic acid B as the active constituents [1, 16]. *S. miltiorrhiza* has been used in traditional medicines owing to its pharmacological properties, containing anti-hypertension, anti-platelet aggregation, anti-atherosclerosis, antitumor, anti-oxidative, antimicrobial, and antifungal activities [11, 12, 17, 28, 33].

This study was aimed to determine the minimal inhibitory concentration (MIC) of the *S. miltiorrhiza* ethanol extract against medically important *Candida* species, including *C. albicans*, *C. krusei*, *C. glabrata*, and *C. tropicalis*, and evaluate the possible mechanism of action against *C. albicans*, which is a major fungal pathogen, based on the results of propidium iodide (PI) uptake, release of A_{260}-absorbing materials and protein, changes in DPH binding, increased MIC in the presence of the osmoprotectant, and decreased (1,3)-β-D-glucan synthase activity. The results offer valuable insight into the pharmacological utility of *S. miltiorrhiza* as a promising antifungal agent against *C. albicans* infections.

Materials and Methods

Preparation of Ethanol Extract

Dried roots of *S. miltiorrhiza* were obtained from jchanbang.com, Korea. Dried *S. miltiorrhiza* roots (30 g) were infused in 300 ml of 70% ethanol for 1 h and boiled for 2 h. The supernatant obtained by centrifugation of the suspension at 2,000 ×*g* for 20 min was concentrated using a vacuum evaporator (Eyela, Japan) and lyophilized to yield an ethanol extract. The ethanol extract of *S. miltiorrhiza* (5.1 g) was dissolved in dimethyl sulfoxide (DMSO) to 100 mg/ml, and kept at −20°C until used (hereafter referred to as *S. miltiorrhiza*). Positive-control drug amphotericin B was dissolved in distilled water at the concentration of 16 mg/ml, filter-sterilized, and further diluted in broth just before the assay.

Candida Strains

C. albicans (ATCC 18804, KCCM 50235), *C. krusei* (ATCC 32196, KCCM 11426), *C. glabrata* (ATCC 2001, KCCM 50044), and *C. tropicalis* (ATCC 750, KCCM 50075) were purchased from the Korean Culture Center of Microorganisms (KCCM).

Antifungal Susceptibility Test

The in vitro minimum inhibitory concentrations of *S. miltiorrhiza* against *Candida* spp. were determined by the modified CLSI M27-A3 protocol of the colorimetric broth microdilution method in the presence of resazurin as a cell growth indicator, according to Liu *et al*. [18]. Growth and sterility controls in the presence of DMSO used in sample preparation were also included, and no inhibitory effects were observed in the presence of the solvent control at the highest concentration used (1% (v/v)). All assays were repeated at least three times.

Effect on Cytoplasmic Membrane

C. albicans ATCC 18804 cells at the exponential phase (1×10^6 cells/ml) were incubated with *S. miltiorrhiza*, at a final concentration equivalent to its MIC, at 35°C with shaking at 200 rpm for 3 h. The suspension was washed with phosphate-buffered saline (PBS, pH 7.4), and PI (10 μM) was added. After the incubation of the cell suspension for 30 min in the dark at room temperature, the effect of *S. miltiorrhiza* on the cytoplasmic membrane of *C. albicans* was evaluated using a confocal laser microscope (Olympus).

Loss of 260-nm-Absorbing Materials and Proteins

In order to investigate the antifungal effect of *S. miltiorrhiza* on the integrity of the *C. albicans* cell membrane, the release of 260-nm-absorbing materials and proteins was determined spectrophotometrically using the method of Kahn *et al*. [13] with slight modifications. Briefly, *C. albicans* cells (1×10^9 cells/ml) at the exponential phase were washed twice and dissolved in 0.85% NaCl. The suspension was treated with 4× MIC *S. miltiorrhiza* or the same volume of DMSO (control) as *S. miltiorrhiza*. At each time point (0, 30, 60, and 90 min), 0.5 ml of cell suspension was taken and filtered through a spin column containing a 0.22 μm filter (Costar, USA), by centrifugation at 12,000 ×*g* for 1 min. To measure the leakage of cellular materials that absorb at 260 nm, the absorbance of filtrate was read at 260 nm using a UV-microplate reader (Bio-Tek) and the absorbance of DMSO control was subtracted. To evaluate the protein leakage, filtrate was mixed with Bradford reagent according to the method provided by the manufacturer (Bio-Rad) and absorbance at 595 nm was read using a microplate reader. The data represent the absorbance at 595 nm of DMSO control subtracted from the absorbance at 595 nm of sample at each time point. The data are representative of three independent experiments, carried out in quadruplicates.

Effect of *S. miltiorrhiza* on DPH Binding to *C. albicans* Cells

C. albicans cells (5×10^7 cells/ml) were incubated with 2× MIC or 4× MIC *S. miltiorrhiza* at 35°C on a shaking incubator at 200 rpm for 120 and 150 min, respectively. The control cells were incubated with the same volume of DMSO as that of *S. miltiorrhiza*. The cells were separated from the growth medium and the plant extract by centrifugation at 4,000 ×*g* for 5 min, washed, and resuspended in PBS. Each suspension was adjusted so that the optical density at 595 nm would be 1×10^8 CFU/ml. To evaluate the effect of *S. miltiorrhiza* on 1,6-diphenyl-1,3,5-hexatriene (DPH) binding to the *C. albicans* membrane, *C. albicans* ATCC 18804 cells were incubated

with the fluorescence probe DPH at a final concentration of 2 μM at room temperature for 30 min in the dark. The samples were washed with PBS (pH 7.4), and fluorescence was measured in a black 96-well microplate using a spectrofluorometer (Bio-Tek) at 360 nm excitation and 460 nm emission wavelengths. The results represent the average of quadruplicate measurements from three independent assays.

Ergosterol Binding Assay

In order to assess whether *S. miltiorrhiza* binds ergosterol in *Candida* membranes, ergosterol binding assay was performed by the modified CLSI M27-A3 protocol [5]. Briefly, duplicate plates were prepared containing *S. miltiorrhiza* or amphotericin B (positive control). One plate included 2-fold dilutions of *S. miltiorrhiza* and the other plate included *S. miltiorrhiza* and 200 μg/ml ergosterol. Each well inoculated with 100 μl of cell suspension (1~5 × 10^3 cells/ml) was incubated at 35°C. MIC end-points were determined at 2 and 7 days.

Sorbitol Protection Assay

Sorbitol protection assay was carried out by the modified CLSI M27-A3 protocol as described above. Briefly, duplicate plates were prepared containing *S. miltiorrhiza.* One plate included 2-fold dilutions of *S. miltiorrhiza* and the other plate included *S. miltiorrhiza* and 0.8 M sorbitol as an osmotic protectant [8]. All the wells inoculated with cell suspension were incubated at 35°C.

Preparation and Quantification of (1,3)-β-D-Glucan Synthase

Preparation and analysis of (1,3)-β-glucan synthase from *C. albicans* cells were performed according to the methods of Shedletzky *et al.* [27] with little modifications. Briefly, *C. albicans* cells cultivated in 1 L of YM broth at 37°C for 16 h were harvested and washed with ice-cold 15 ml breakage buffer (50 mM Tris·Cl (pH 7.4), 1 mM EGTA, 20 μM phenylmethylsulfonyl fluoride, 4 mM dithiothreitol, and 5 μm leupeptin), and broken by 12 cycles of 1 min in a Bead Beater (BioSpec Products, USA) with 0.5 mm acid-washed glass beads. The homogenate was then centrifuged at 3,000 ×*g* for 10 min at 4°C, and the supernatant containing microsomes and membranes were gathered by centrifugation at 100,000 ×*g* for 1 h at 4°C. The pellet was resuspended in 15 ml of breakage buffer with 33% glycerol and stored at −27°C. Protein concentration was measured using the Bio-Rad protein assay kit. For (1,3)-β-glucan synthase assay [8], 100 μg of *C. albicans* membrane protein was mixed with 50 mM Tris-Cl (pH 7.4), 20 μM GTP, 4 mM EDTA, 0.5% Brij 35, 6.6% glycerol, and 2 mM UDP-glucose. All the reaction samples were incubated for 30 min at 25°C and stopped by adding 10 μl of 6 N NaOH. Glucans formed were solubilized by incubating the reaction samples at 80°C for 30 min followed by adding 210 μl of aniline blue mix (40: 21: 59 of 0.1% aniline blue, 1 N HCl, and 1 M glycine/NaOH (pH 9.5)). Reaction samples were further incubated at 50°C for 30 min and at room temperature for 30 min. Fluorescence was measured in a black 96-well microplate using a spectrofluorometer (Bio-Tek) at 400 nm excitation and 460 nm emission wavelengths. The data represent the mean of quadruplicate measurements from three independent assays.

Flow Cytometry Analysis on Fungal Cell Cycle

C. albicans ATCC 18804 cells (1 × 10^7cells/ml) at the exponential growth phase were incubated with 1× MIC *S. miltiorrhiza* at 35°C with shaking at 200 rpm for 3 h. The cells were collected and washed with PBS (pH 7.4) and then fixed with 70% cold ethanol overnight to permeabilize the cell membrane. The cells mixed with 200 μg/ml RNase A (Sigma, USA) was left to react for 1 h at 50°C. To stain DNA, PI in PBS was added to a final concentration of 10 μM, and the mixture was incubated for 30 min at room temperature in the dark. Cell cycle analysis was carried out utilizing the Muse Cell Analyzer from Millipore (Merck Millipore, USA) according to the manufacturer's instructions. Data obtained from one run out of two independent experiments, which were conducted in duplicate, are presented.

Statistical Analysis

The differences in relative fluorescence or (1,3)-β-D-glucan synthase activity (mean ± standard error) of each group treated with *S. miltiorrhiza* and control were calculated using Student's *t*-test, and were regarded as statistically significant when a *p*-value was less than 0.05. All experiments were performed at least three times in quadruplicates.

Results

Antifungal Susceptibility Assay

The antifungal activity of *S. miltiorrhiza* was judged against standard strains of *C. albicans* ATCC 18804, *C. krusei* ATCC 750, *C. glabrata* ATCC 2001 and *C. tropicalis* ATCC 32196 using a colorimetric broth microdilution assay (Table 1). The MICs of *S. miltiorrhiza* against the test strains were in close proximity to one another, ranging from 39 μg/ml for *C. albicans* ATCC 18804 and *C. krusei* ATCC 750 to 78 μg/ml for *C. glabrata* ATCC 2001 and *C. tropicalis* ATCC 32196. MICs of *S. miltiorrhiza* and amphotericin B against the tested strain of *C. albicans* ATCC 18804 were

Table 1. MIC values of *S. miltiorrhiza* against *Candida* spp.

	S. miltiorrhiza (μg/ml)	Amphotericin B (μg/ml)
C. albicans ATCC 18804	39	1
C. krusei ATCC 750	39	1
C. glabrata ATCC 2001	78	2
C. tropicalis ATCC 32196	78	1

The in vitro MICs of *S. miltiorrhiza* against *Candida* spp. were determined by the modified CLSI M27-A3 protocol of the colorimetric broth microdilution method containing resazurin. All assays were repeated three times.

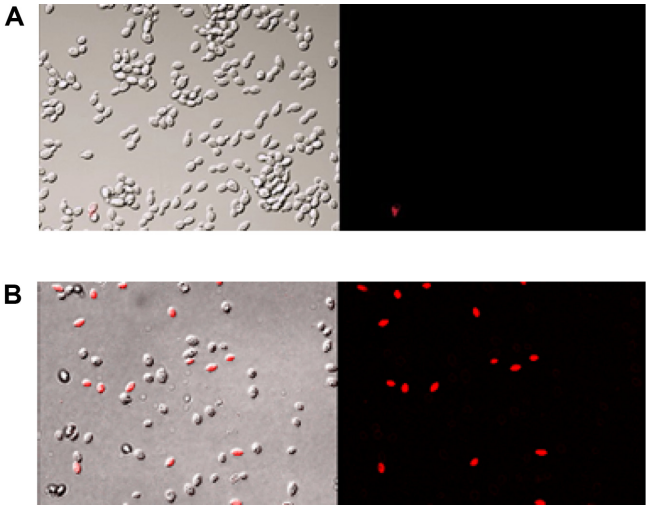

Fig. 1. Effect of *S. miltiorrhiza* on the *C. albicans* ATCC 18804 cell membrane.

(**A**) Untreated cells; (**B**) cells treated with 1× MIC *S. miltiorrhiza* for 3 h were stained with PI and fluorescent signals were examined using confocal laser microscopy. Superimposed fluorescent signals with Nomarski differential interference contrast images (left panes) and fluorescent images of PI (right panels) are shown .

39 μg/ml and 1 μg/ml, respectively.

Effect of *S. miltiorrhiza* on *C. albicans* Cell Membrane

Cells with severe membrane lesion will internalize PI. Once entering the cell, PI binds to nucleic acids. As a result, cells with damaged membranes present a red fluorescence signal, whereas those with intact membranes do not. Our data showed that 1× MIC *S. miltiorrhiza* was effective to damage the cell membrane of *C. albicans* ATCC 18804 cells with 3 h of incubation (Fig. 1).

Leakage of Cellular Materials Absorbing at 260 nm and Proteins

Disruption of the physical integrity of the fungal cell membrane has been associated with antifungal agents that exhibit rapid fungicidal activity. To assess if *S. miltiorrhiza* is involved in disrupting the integrity of *C. albicans* ATCC 18804 cell membranes, the leakage of intracellular contents was analyzed after treatment with *S. miltiorrhiza*. Suspensions of *C. albicans* ATCC 18804 cells in 0.85% NaCl were treated with 4× MIC *S. miltiorrhiza*, or DMSO as a control, at different time intervals. *S. miltiorrhiza* induced steady leakage of cellular materials in a time-dependent manner (Fig. 2), and treated cells showed significant release of intracellular nucleotide and proteins compared with DMSO-treated control ($p < 0.05$). This data are consistent with the loss of the plasma membrane integrity evidenced by PI uptake.

Measurement of DPH Binding to Cell Membrane

Binding of DPH and its derivatives into intramembranes is coupled with strong increment of their fluorescence [31]. Therefore, the influence of *S. miltiorrhiza* on the *C. albicans* plasma membrane was examined using fluorescence probe DPH (Table 2). After exposure of *C. albicans* ATCC 18804 cells to 2× MIC and 4× MIC *S. miltiorrhiza* for 120 or 150 min, respectively, the *C. albicans* cells were then incubated with

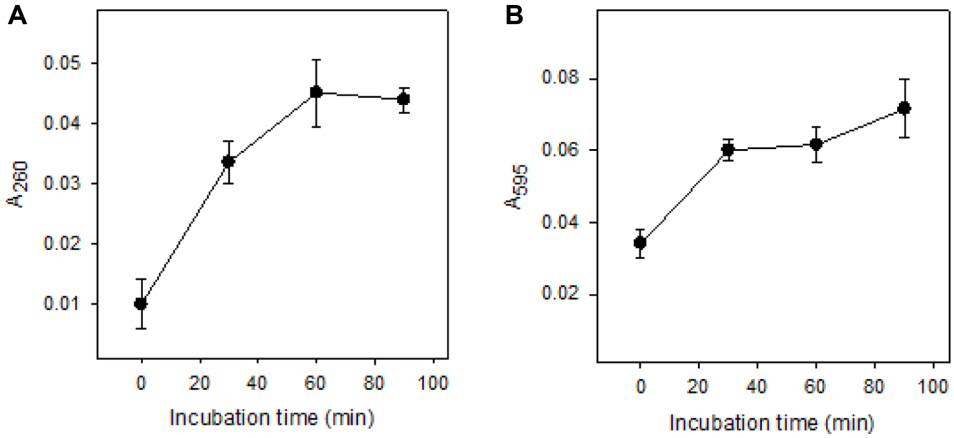

Fig. 2. Cell leakage analysis of *S. miltiorrhiza* against *C. albicans* ATCC 18804 cells.

S. miltiorrhiza (4× MIC) or DMSO (control) was added to *C. albicans* ATCC 18804 cells, and then samples taken at the indicated time points were filtered through a 0.22 μm filter. (**A**) Nucleotide leakage: absorbance at 260 nm of filtrate was subtracted from the background absorbance of control. (**B**) Protein leakage: absorbance at 595 nm of filtrate mixed with Bradford reagent was subtracted from the background absorbance of DMSO control. Each value represents the average ± SE of quadruplicates.

Table 2. Effect of *S. miltiorrhiza* on DPH binding to *C. albicans* ATCC 18804.

	S. miltiorrhiza					
	0		2× MIC		4× MIC	
Incubation time (min)	Fluorescence (AU)	Relative fluorescence (%)	Fluorescence (AU)	Relative fluorescence (%)	Fluorescence (AU)	Relative fluorescence (%)
120	21.30 ± 2.36	100	15.5 ± 1.29	72.8**	13.75 ± 0.50	64.7**
150	19.75 ± 0.50	100	8.5 ± 0.57	43.0**	6.75 ± 0.50	34.1**

Measurements were performed on whole cells of *C. albicans* ATCC 18804 with *S. miltiorrhiza*. Relative fluorescence was expressed as a percent to determine the change in DPH binding caused by each treatment. Each value represents the average ± SE of quadruplicates. AU: arbitrary unit. $^{**}p < 0.01$.

Table 3. Effect of exogenous ergosterol on the MIC of *S. miltiorrhiza* against *C. albicans* ATCC 18804.

Test agents	MIC (μg/ml)			
	2 days		7 days	
	-ergosterol	+ergosterol	-ergosterol	+ergosterol
S. miltiorrhiza	39	39	39	39
Amphotericin B	1	2	1	2

Table 4. Effect of sorbitol on the MIC of *S. miltiorrhiza* against *C. albicans* ATCC 18804.

Test agents	MIC (μg/ml)			
	2 days		7 days	
	-sorbitol	+sorbitol	-sorbitol	+sorbitol
S. miltiorrhiza	39	156	39	>1,248
Amphotericin B	1	1	1	1

DPH. As Table 2 presents, the fluorescence intensity of DPH exhibited a significant reduction in *S. miltiorrhiza*-treated *C. albicans* cells as compared with DMSO control ($p < 0.01$). In the presence of 2× MIC of *S. miltiorrhiza*, the fluorescence was reduced to 73% for 120 min and 43% for 150 min, respectively, in comparison with the DMSO control (100%). After 4× MIC *S. miltiorrhiza* treatment, the fluorescence intensity was decreased by about 65% and 34%, respectively. This result suggests that *S. miltiorrhiza* is involved in a manner that alters the membrane composition of *C. albicans* cells to lead to a change of membrane permeability and disruption of the physical integrity of the *C. albicans* cell membrane.

Ergosterol Binding Assay

To check whether *S. miltiorrhiza* binds membrane ergosterol, an essential lipid for many aspects of yeast cell physiology, in *C. albicans* cells, the MIC of *S. miltiorrhiza* against *C. albicans* ATCC 18804 was evaluated in the absence and presence of ergosterol in the broth. As shown in Table 3, the MIC of *S. miltiorrhiza* against *C. albicans* ATCC 18804 was not varied in the presence of ergosterol, but the MIC for the positive control, amphotericin B, increased 2-fold in the presence of ergosterol, revealing the binding of amphotericin B to the main sterol of yeast membranes. The data confirm that *S. miltiorrhiza* does not act in a manner that binds ergosterol directly in the *C. albicans* membrane to change membrane permeability.

Sorbitol Protection Assay

To analyze if *S. miltiorrhiza* affects the fungal cell wall, the MICs of *S. miltiorrhiza* and amphoterin B (negative control) were evaluated against *C. albicans* ATCC 18804 cells in the absence and presence of sorbitol. Damaging effects of an antimicrobial compound on cell walls can be recovered in the presence of osmoprotectants such as sorbitol. As shown in Table 4, the MIC of *S. miltiorrhiza* against *C. albicans* cells increased more than 30-fold at 7 days. In contrast, the MIC of the negative control, amphotericin B, was not changed.

Effect of *S. miltiorrhiza* on (1,3)-β-D-Glucan Synthase

The increased MIC values observed in the sorbitol protection assay (Table 4) implies that one of the possible

Table 5. Effect of S. *miltiorrhiza* on (1,3)-β-D-glucan synthase activity.

S. miltiorrhiza	Fluorescence (AU)	(1,3)-β-D-Glucan synthase activity (%)
0	42,851 ± 479	100
2× MIC	41,196 ± 1,375	96.1
4× MIC	37,688 ± 2,317	87.9
8× MIC	36,041 ± 1,434**	84.1

Relative (1,3)-β-D-glucan synthase activity was expressed as a percent to determine the changes in (1,3)-β-D-glucan synthase activity caused by each treatment. Each value represents the average ± SE of quadruplicates. AU: arbitrary unit. $^{**}p < 0.01$.

targets of *S. miltiorrhiza* is the cell wall in *C. albicans* cells. Therefore, it was examined if *S. miltiorrhiza* inhibits the (1,3)-β-glucan synthase in *C. albicans* ATCC 18804 cells. The quantity of (1,3)-β-glucan synthase activity after treatment with *S. miltiorrhiza* was expressed as a percentage of the DMSO control. The content of (1,3)-β-glucan in the cell wall decreased approximately 12% and 16% in 4× and 8× MIC *S. miltiorrhiza*-treated *C. albicans* cells, respectively, as compared with DMSO control cells (Table 5), and the changes by 8× MIC *S. miltiorrhiza* were statistically significant ($p < 0.01$).

Effect of *S. miltiorrhiza* on *C. albicans* Cell Cycle

Although *S. miltiorrhiza* seems to impair the function of both cell membrane and cell wall of *C. albicans* cells, the antifungal effect was not fungicidal in the preliminary test. Therefore, the effect of *S. miltiorrhiza* on the cell cycle progress of *C. albicans* was explored, as shown in Fig. 3. In our study, growth control showed 20% cells in the G_0/G_1 phase in 3 h, whereas *S. miltiorrhiza*-treated *C. albicans* cells showed a major peak (42% cells) in the G_0/G_1 phase, representing a 2.1-fold increase in 3 h. The data indicate that *S. miltiorrhiza* elicits prolonged G_0/G_1 arrest in *C. albicans* cells.

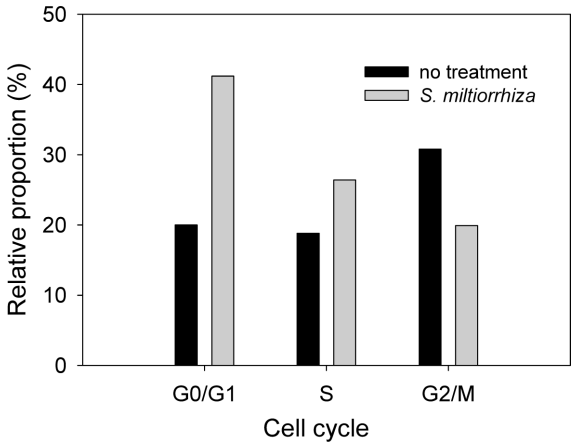

Fig. 3. Effect of *S. miltiorrhiza* on the cell cycle progress of *C. albicans* ATCC 18804.

Suspensions of *C. albicans* ATCC 18804 (1×10^8 cells/ml) were incubated without (black bars) or with 2× MIC *S. miltiorrhiza* (gray bars) for 3 h, and then washed with PBS. The cells were fixed in 70% ethanol overnight, and then stained with 10 μM PI. Cell cycle analysis was performed with the Muse Cell Analyzer.

Discussion

Plant extracts used in traditional medicine are thought to have the advantages of their several active components and their synergistic activity over conventional single component drugs: *S. miltiorrhiza* seems to contain a few active antifungal components against *C. albicans*, which affect the structure and function of both the cell membrane and cell wall in *C. albicans*.

Many cellular processes involved in growth and cell function are associated with changes in the membrane characteristics [2]. Although PI cannot penetrate intact cells since it carries two positive charges [25], it can go through the damaged cell membrane to bind nucleic acids. The results of treatment of *C. albicans* cells with *S. miltiorrhiza* in the presence of PI showed an enhancement of red fluorescence caused by membrane damage (Fig. 1). Leakage of cytoplasmic material is considered a serious destruction of the cytoplasmic membrane. The data that 260-nm-absorbing materials and protein of filtrates exposed to *S. miltiorrhiza* were significantly different from those of control confirm that *S. miltiorrhiza* acts in a manner that disrupts the physical integrity of the *C. albicans* cell membrane. Damage of the cell membrane could change the membrane permeability and disturb its ability of osmotic regulation or excluding toxic materials [4]. Fungal cell membrane is a typical lipid bilayer composed of 30–40% sterols, 10–20% sphingomyelin, low levels of glycosphingolipids, and some membrane proteins [20]. Ergosterol is a sterol in the fungal cell membrane, and changes in ergosterol synthesis affect membrane rigidity, fluidity, and permeability [21]. Amphotericin B, which targets ergosterol, is recognized to be involved in changes in membrane permeability of many organisms, bringing a leakage of cellular materials to cell lysis and death [7]. To know the reason why membrane permeability was changed, the ability of *S. miltiorrhiza* to bind membrane ergosterol was examined in *C. albicans* cells. The result of ergosterol binding assay suggests that *S. miltiorrhiza* does not bind to the ergosterol of fungal membranes. Alterations in lipid composition, such as the length, the degree of saturation, and branching of fatty acyl chains, lead to modification of the biophysical characteristics of the cell membrane [2]. Higher levels of polyunsaturated fatty acids were found in mycelial lipids than in *C. albicans* lipids [9]. Since DPH is soluble in the hydrocarbon tail region, and supposed to be oriented parallel to the axis of the lipid acyl chain, it is often used in measurements of fluorescence anisotropy and provides insight into membrane fluidity and lipid ordering [24]. To determine

whether *S. miltiorrhiza* influences the lipid composition of the *C. albicans* cell membrane, the ability of DPH binding to the *C. albicans* cell membrane was assessed. Both 2× and 4× MIC *S. miltiorrhiza* caused a significant reduction in the DPH fluorescence intensity as compared with control cells. Our data suggest that the lipid composition or fluid properties of the *C. albicans* membrane have been altered by *S. miltiorrhiza*. The fluidity of biomembranes is involved in a variety of functions of cells, such as growth of cell, membrane transport, signal transduction, and membrane-related enzymatic activities [19]. Therefore, alterations in membrane permeability in *S. miltiorrhiza*-treated *C. albicans* were possibly attributable to the changes in the composition of membrane lipid, such as fatty acids, but not ergosterol. The fungal cell wall surrounds the cell membrane and confers cell rigidity and strength, providing osmotic protection from protoplast turgor. The cell wall consists of many macromolecules such as β-glucans, chitin mannoproteins, and other proteins, and many of these macromolecules are essential to the fungi, and enzymes that synthesize these constituents could be important antifungal targets [30].

Damage to the essential cell wall components by antifungal agents will lyse cells in the absence of an osmoprotectant, but cells will continue to grow if a suitable stabilizer is present in the medium [8]. Increase of MIC in the presence of sorbitol demonstrates that *S. miltiorrhiza* is involved in cell wall synthesis. This conjecture was confirmed via aniline blue assay: the reduced activity of (1,3)-β-D glucan synthase proves that *S. miltiorrhiza* contains the component preventing the synthesis of cell wall.

Regardless of the synergistic activity of *S. miltiorrhiza* targeting the *C. albicans* cell wall and cell membrane, its antifungal effect on *C. albicans* cells seems to be modest, since the antifungal activity was significantly efficacious when *S. miltiorrhiza* was used at slightly high concentrations. Therefore, it was investigated whether *S. miltiorrhiza* has an influence on cell cycle progression in *C. albicans*. As shown in Fig. 3, *S. miltiorrhiza* arrested *C. albicans* cells at the G_0/G_1 phase, leading to growth inhibition.

In conclusion, it is hypothesized that the damage in cell membrane and cell wall, by modified cell membrane composition and obstruction of cell wall synthesis, in *S. miltiorrhiza*-treated *C. albicans* cells imposes severe stress on *C. albicans* cells, leading to the arrest of cell cycle progression by controlling signaling pathways and loss of health. Multiple targets of *S. miltiorrhiza* for the cell membrane and cell wall in *C. albicans* could be worthwhile to use as a potential drug for future therapeutic option against *Candida*-associated infections.

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