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Paeonia lactiflora Inhibits Cell Wall Synthesis and Triggers Membrane Depolarization in *Candida albicans*

Heung-Shick Lee[1] and Younhee Kim[2*]

[1]*Department of Biotechnology and Bioinformatics, Korea University, Sejongsi 30019, Republic of Korea*
[2]*Department of Korean Medicine, Semyung University, Jecheon 27136, Republic of Korea*

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*Corresponding author
Phone: +82-43-649-1346;
Fax: +82-43-649-1341;
E-mail: ykim@semyung.ac.kr

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Fungal cell walls and cell membranes are the main targets of antifungals. In this study, we report on the antifungal activity of an ethanol extract from *Paeonia lactiflora* against *Candida albicans*, showing that the antifungal activity is associated with the synergistic actions of preventing cell wall synthesis, enabling membrane depolarization, and compromising permeability. First, it was shown that the ethanol extract from *P. lactiflora* was involved in damaging the integrity of cell walls in *C. albicans*. In isotonic media, cell bursts of *C. albicans* by the *P. lactiflora* ethanol extract could be restored, and the minimum inhibitory concentration (MIC) of the *P. lactiflora* ethanol extract against *C. albicans* cells increased 4-fold. In addition, synthesis of (1,3)-β-D-glucan polymer was inhibited by 87% and 83% following treatment of *C. albicans* microsomes with the *P. lactiflora* ethanol extract at their 1× MIC and 2× MIC, respectively. Second, the ethanol extract from *P. lactiflora* influenced the function of *C. albicans* cell membranes. *C. albicans* cells treated with the *P. lactiflora* ethanol extract formed red aggregates by staining with a membrane-impermeable dye, propidium iodide. Membrane depolarization manifested as increased fluorescence intensity by staining *P. lactiflora*-treated *C. albicans* cells with a membrane-potential marker, $DiBAC_4(3)$ ((*bis*-1,3-dibutylbarbituric acid) trimethine oxonol). Membrane permeability was assessed by crystal violet assay, and *C. albicans* cells treated with the *P. lactiflora* ethanol extract exhibited significant uptake of crystal violet in a concentration-dependent manner. The findings suggest that *P. lactiflora* ethanol extract is a viable and effective candidate for the development of new antifungal agents to treat *Candida*-associated diseases.

Keywords: Antifungal, *Candida albicans*, cell wall, membrane permeability, membrane potential, *Paeonia lactiflora*

Introduction

Candida albicans is a known leading fungal pathogen of fungal bloodstream infections, although a notable shift has been observed toward non-*albicans* species of *Candida*, including *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* [1, 2]. Frequently, *Candida* lives and persists as commensals when innate and acquired immune systems act together with the normal bacterial flora. However, *Candida* can be transformed into a pathogen by certain critical events, such as extensive antibacterial treatment or a dysfunction of the immune system, which may be life-threatening [3]. Recently, lethal systemic candidiasis has become more widespread because of the increasing population of seriously ill or immunocompromised patients, including HIV-infected patients, patients with cancer, transplant recipients, and patients undergoing more frequent use of invasive medical procedures [4].

At present, four molecular classes of antifungal drugs (polyenes, azoles, echinocandins, and nucleotide analogs) are available for targeting three metabolic pathways for the treatment of systemic fungal infections [5]. Polyenes, such as amphotericin B, target ergosterol, the analog of cholesterol found in fungal cell membranes, which is involved in

membrane integrity, membrane fluidity, and the function of many membrane-bound enzymes [6, 7]. Azoles target the ergosterol biosynthetic pathway by inhibiting lanosterol 14α-demethylase [8]. Echinocandins inhibit the biosynthesis of (1,3)-β-glucan, which is a major component of cell walls in fungi [9]. Nucleotide analogs, such as flucytosine, inhibit DNA or RNA synthesis [10].

In spite of extensive study exploring new antifungal agents, commonly used drugs for the treatment of candidiasis remain limited, largely because fungi have a eukaryotic nature and a close evolutionary relationship with their human hosts, along with the emergence of resistant yeasts [11, 12]. Therefore, approaches to treating *Candida* infections have become more challenging, and it is essential to explore new antifungals that possess both good therapeutic effects and a low toxicity. Traditionally, natural products were used in ethnomedicine owing to the presence of effective antifungal defense mechanisms in higher plants [13]. *Paeonia lactiflora* Pallas, white peony, belonging to the family Paeoniaceae, is a herbaceous perennial flowering plant, and indigenous to central and eastern Asia. In traditional Korean medicine, peeled 3- or 4-year-old *P. lactiflora* roots are parboiled in boiling water and dried under the sun for use owing to their pharmacological properties for the treatment of shigellosis and gynecological disorders [14]. *P. lactiflora* contains paeoniflorin, paeonel, paeonin, tannin, β-sitosterol, and triterpenoid, and it has been reported to be effective for the treatment of rheumatoid arthritis, systemic lupus erythematosus, hepatitis, and other inflammatory diseases [14, 15].

In the present study, we measured the MICs of an ethanol extract from *P. lactiflora* against medically important *Candida* species, including *C. albicans*, *C. glabrata*, *C. krusei*, and *C. tropicalis*, and evaluated the mode of antifungal action against *C. albicans*, which is a major fungal pathogen. The results provide a valuable understanding of the pharmacological utility of *P. lactiflora* ethanol extract as a potential antifungal agent for the treatment of *C. albicans* infections.

Materials and Methods

Candida Strains

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

Preparation of *P. lactiflora* Ethanol Extract

Plant materials used for the study included boiled and dried *P. lactiflora* root, with the bark removed, obtained from jchanbang.com, Korea. Thirty grams of dried *P. lactiflora* root was infused in 300 ml of 70% ethanol for 1 h, and then boiled for 2 h. After centrifugation of the suspension at 2,000 ×*g* for 20 min, the supernatant was concentrated using a vacuum evaporator and lyophilized to obtain the ethanol extract. The ethanol extract from *P. lactiflora* (2.6 g) was dissolved in dimethyl sulfoxide (DMSO) to 100 mg/ml, filter-sterilized, and kept at −20°C until used. Amphotericin B (Sigma, USA), the positive control, was dissolved in distilled water to 16 mg/ml, filter-sterilized, and further diluted in broth just before the assay.

Antifungal Susceptibility Test

The in vitro MICs of the ethanol extract from *P. lactiflora* were determined against the test strains via a modified CLSI M27-A3 protocol [16] of the colorimetric broth microdilution method in the presence of resazurin as a cell growth indicator [17]. Briefly, the wells holding the 2-fold serial dilutions of the *P. lactiflora* ethanol extract, or amphotericin B (100 μl), were prepared in round-bottom 96-well microplates with RPMI-1640 medium of L-glutamine without bicarbonate and buffered with MOPS (3-[N-morpholino]- propanesulfonic acid) buffer (pH 7.0). The inoculum suspension (100 μl) containing 0.1 mg/ml resazurin was added to give a final concentration of between 1×10^3 and 5×10^3 cells/ml for the assays. The plates were incubated at 35°C and the MICs were determined after 24 h. Colorimetric MIC end-points were considered as the lowest sample concentration that stayed blue, or the first sample that changed from blue to purple [17]. Solvent controls with DMSO used for the *P. lactiflora* extract preparation were prepared, and no inhibitory effects were detected in the presence of the solvent control at up to 1% (v/v). Assays were repeated three times, and the MIC was determined as the highest MIC of the three experiments.

Sorbitol Protection Assay

The sorbitol protection assay [18] was performed using a modified CLSI M27-A3 protocol containing resazurin, as was described above. In a 96-well plate, one row contained 2-fold dilutions of the *P. lactiflora* ethanol extract and another row contained the *P. lactiflora* ethanol extract and 0.8 M of sorbitol as an osmotic stabilizer. All the wells inoculated with *C. albicans* cell suspension were incubated at 35°C, and the MICs were determined at 24 and 72 h, respectively.

Cell Morphology Analysis

C. albicans ATCC 18804 cells (5×10^6 cells/ml), in the exponential phase, were incubated in either a YM medium (0.3% yeast extract, 0.3% malt extract, 1% peptone, 2% dextrose), or a YM medium containing 0.8 M of sorbitol with amphotericin B, or the *P. lactiflora* ethanol extract, at a final concentration equivalent to its 0 or 1× MIC at 35°C with shaking at 200 rpm for 4 h. The cells were stained with neutral red solution to a final concentration of 500 μg/ml for 5 min and were examined by phase-contrast microscopy.

Preparation of Microsomes and Activity Assay of (1,3)-β-D-Glucan Synthase

(1,3)-β-D-Glucan synthase activity was measured with microsome membranes and quantified using aniline blue assay. Microsome membranes were prepared from *C. albicans* ATCC 18804 cells in the exponential phase according to the method of Shedletzky *et al*. [19] with slight modification [20]. For the effect of *P. lactiflora* ethanol extract on (1,3)-β-glucan synthase activity, assays with aniline blue mix were conducted [18]. Fluorometric measurements were carried out in a black 96-well microplate using a spectrofluorometer (Bio-Tek) at 400 nm excitation and 460 nm emission wavelengths.

Calcofluor White and PI-Double Staining

C. albicans ATCC 18804 cells at the exponential phase (5×10^6 cells/ml) were incubated with the ethanol extract from *P. lactiflora* at a final concentration equivalent to its MIC with shaking at 200 rpm at 35°C for 4 h. The suspension was washed with phosphate-buffered saline (PBS, pH 7.4), and Calcofluor White (Sigma, USA) and PI were added to give final concentrations of 0.1 μg/ml and 10 μM, respectively. The cells were stained for 10 min in the dark at room temperature, and the effects of the ethanol extract from *P. lactiflora* on the cell walls and cytoplasmic membranes of *C. albicans* were evaluated by both fluorescence and bright-field microscopy.

Effect of the *P. lactiflora* Ethanol Extract on Membrane Potential

C. albicans ATCC 18804 cells in the exponential phase (5×10^6 cells/ml) were incubated at 35°C with shaking at 200 rpm for 3 h with the ethanol extract from *P. lactiflora* at concentrations corresponding to its 1× MIC for fluorescence microscopy, or its 1× MIC, 4× MIC, and 8× MIC for spectrofluorometry. Solvent (DMSO) controls were included for each treatment of the *P. lactiflora* ethanol extract. Harvested cells were mixed with an equal volume of $DiBAC_4(3)$ (20 μg/ml) and incubated for 30 min in the dark at room temperature. Stained *C. albicans* cells were examined under a fluorescence microscope, or fluorescence intensity was measured in a black 96-well microplate using a spectrofluorometer at 493 nm excitation and 516 nm emission wavelengths. The data represent the mean of the quintuplicate measurements from two independent assays.

Effect on Membrane Permeability

Alteration of membrane permeability was investigated by crystal violet assay with slight modification [21, 22]. *C. albicans* ATCC 18804 cells at the exponential phase were harvested by centrifugation at 4,500 ×*g* at 4°C for 5 min. The cells were washed twice and resuspended in 0.5% NaCl. The ethanol extract from *P. lactiflora*, corresponding to the concentrations of 0.5× MIC, 1× MIC, or 2× MIC, was added to the suspension and incubated at 37°C, 200 rpm for 8 h. Solvent (DMSO) controls were included for each treatment of the ethanol extract from *P. lactiflora*. Cells were harvested and washed in 0.5% NaCl, and then the cell density of each experimental group was adjusted to 1×10^8 cells/ml and resuspended in 0.5% NaCl solution containing 10 μg/ml crystal violet. The cell suspensions were incubated at 37°C, 200 rpm for 10 min. The cells were precipitated by centrifugation at 12,000 ×*g* at 4°C for 15 min, and the amount of crystal violet remaining in the supernatant was measured at 590 nm using a spectrophotometer. The OD values of the initial solution of crystal violet used in the assay were regarded as 100%. The percentage of crystal violet uptake of all the cells was calculated as follows: uptake of crystal violet (%) = (A_{590} of the sample)/(A_{590} of crystal violet solution) × 100.

Statistical Analysis

All experiments were carried out at least twice, in triplicates, or quadruplicates. For each outcome, the data were summarized as the mean ± standard error. The effect of *P. lactiflora* treatments, when compared with controls, was assessed using SigmaPlot 13.0 employing the Student-Newman-Keuls method for one-way analysis of variance. A p value of <0.05 was considered statistically significant.

Results

Antifungal Susceptibility Test

The antifungal activity of the ethanol extract from *P. lactiflora* was judged against the test strains *C. albicans* ATCC 18804, *C. krusei* ATCC 750, *C. glabrata* ATCC 2001, and *C. tropicalis* ATCC 32196 using the colorimetric broth microdilution assay (Table 1). The MICs of the ethanol extract from *P. lactiflora* were 196 μg/ml for *C. albicans* ATCC 18804, and 98 μg/ml for *C. krusei* ATCC 750 and *C. tropicalis* ATCC 32196. The MIC of *C. glabrata* ATCC 2001 was 25 μg/ml, revealing it to be relatively sensitive to the ethanol extract from *P. lactiflora* as compared with the other *Candida* strains.

Sorbitol Protection Assay

Damage to the physical integrity of fungal cell walls has

Table 1. Antifungal susceptibility test of the ethanol extract from *P. lactiflora* against *Candida* spp.

	MIC (μg/ml)	
	P. lactiflora ethanol extract	Amphotericin B
C. albicans ATCC 18804	196	0.25
C. krusei ATCC 750	98	0.25
C. glabrata ATCC 2001	25	0.13
C. tropicalis ATCC 32196	98	0.13

The in vitro MICs of the ethanol extract from *P. lactiflora* against *Candida* spp. were determined by the modified CLSI M27-A3 method containing resazurin. *C. albicans* ATCC 18804 cells were incubated with the ethanol extract from *P. lactiflora* in resazurin-containing RPMI-1640 medium (pH 7.0) at 35°C and the MICs were determined at 24 h.

Table 2. Sorbitol protection assay.

Incubation time (h)	MICs of the *P. lactiflora* ethanol extract (μg/ml)	
	No sorbitol	Sorbitol
24	196	196
72	196	784

The in vitro MICs of the ethanol extract from *P. lactiflora* against *C. albicans* ATCC 18804 cells were determined by the modified CLSI M27-A3 method containing resazurin. *C. albicans* ATCC 18804 cells were incubated with the ethanol extract from *P. lactiflora* without or with sorbitol (0.8 M) in resazurin-containing RPMI-1640 medium (pH 7.0) at 35°C and the MICs were determined at 24 and 72 h, respectively.

been associated with antifungals that led to fungal lysis. The harmful effects of an antimicrobial compound on cell walls can be reversed with an osmoprotectant, such as sorbitol [18]. To analyze whether the antifungal activity of the ethanol extract from *P. lactiflora* is related to the fungal cell wall, a colorimetric microdilution assay of CLSI M27-A3 was performed with the ethanol extract from *P. lactiflora* against *C. albicans* cells in the absence and presence of sorbitol. As shown in Table 2, the MIC values of the ethanol extract from *P. lactiflora* against *C. albicans* cells were the same, being 196 μg/ml at 24 h, irrespective of the presence of sorbitol. However, the MIC value of the ethanol extract from *P. lactiflora* against *C. albicans* cells increased to 784 μg/ml only in the same medium supplemented with sorbitol at 72 h. Therefore, the augmented MIC value in the sorbitol-protected assay is evidence that the ethanol extract from *P. lactiflora* is associated with cell wall integrity disruption in *C. albicans*.

Cell Morphology Analysis

C. albicans cells in the exponential phase were incubated with amphotericin B, or the ethanol extract from *P. lactiflora* at its MIC, in YM medium or sorbitol-containing YM medium, for 4 h with shaking, and the cells were observed after staining with vital dye neutral red under a phase-contrast microscope. The control *C. albicans* cells cultivated in YM, or sorbitol-containing YM medium, were stained with neutral red in their vacuoles, indicating that they were

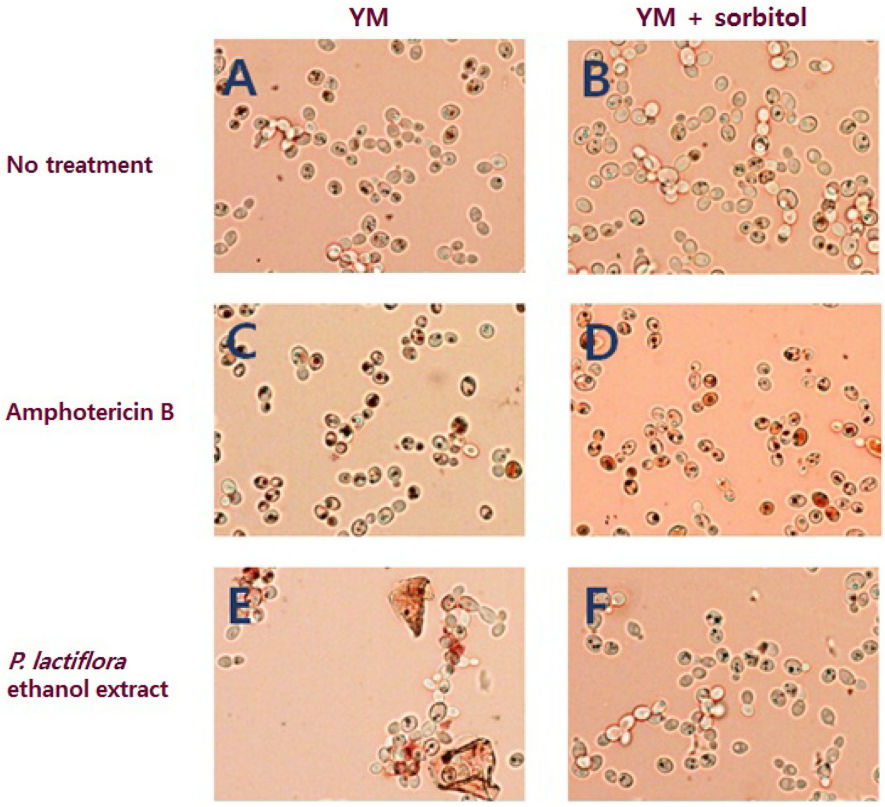

Fig. 1. Cell morphology analysis.

Exponential-phase *C. albicans* ATCC 18804 cells were treated without (**A** and **B**) or with amphotericin B (**C** and **D**) and the ethanol extract from *P. lactiflora* (**E** and **F**), respectively, at a final concentration equivalent to its MIC and incubated in YM medium (**A**, **C**, and **E**) or YM medium with 0.8 M sorbitol (**B**, **D**, and **F**) at 35°C with shaking for 4 h. The cells were stained with neutral red (500 μg/ml) and were examined by phase-contrast microscopy. Scale bars: 10 μm.

healthy in both media (Figs. 1A and 1B). Some dead *C. albicans* cells treated with amphotericin B were darkly stained with neutral red in their cytoplasm regardless of sorbitol (Figs. 1C and 1D), demonstrating that the target of amphotericin B is not related to the cell wall. However, the lysed cells often found in *P. lactiflora*-treated *C. albicans* cells grown in YM medium (Fig. 1E) were rarely observed in *P. lactiflora*-treated *C. albicans* cells grown in YM medium supplemented with sorbitol (Fig. 1F). In conclusion, *C. albicans* cells with defective cell walls caused by the *P. lactiflora* ethanol extract were protected from cell lysis in isotonic medium.

Effect of the *P. lactiflora* Ethanol Extract on (1,3)-β-D-Glucan Synthase

As judged from the results of the sorbitol protection assay (Table 2), and cell morphology analysis (Fig. 1), it is assumed that a plausible target of the ethanol extract from *P. lactiflora* on *C. albicans* is the cell wall assembly. Therefore, we tested whether the ethanol extract from *P. lactiflora* inhibits the (1,3)-β-D-glucan synthase of *C. albicans* cells. (1,3)-β-D-Glucan synthase activity was measured with microsome membranes prepared from *C. albicans* ATCC 18804 cells and quantified using aniline blue assay. Fluorescence, representing (1,3)-β-D-glucan synthase activity after treatment with the ethanol extract from *P. lactiflora*, was expressed as a percentage. Synthesis of (1,3)-β-glucan was inhibited by 87% and 83% following treatment of microsomes with the ethanol extract from *P. lactiflora* at their 1× MIC and 2× MIC, respectively, and the difference between the control cells and *P. lactiflora*-treated *C. albicans* cells at its 2× MIC was statistically significant ($p < 0.05$) (Fig. 2).

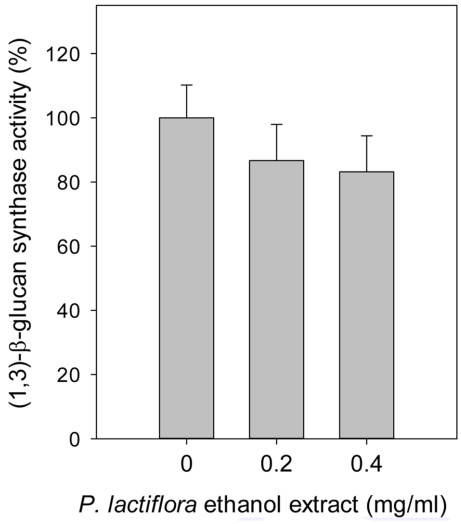

Fig. 2. Effect of the ethanol extract from *P. lactiflora* on (1,3)-β-D-glucan synthase activity.
Relative (1,3)-β-D-glucan synthase activity was expressed as a percentage to determine the changes in (1,3)-β-D-glucan synthase activity caused by each treatment. Each value represents the mean ± SE.

Calcofluor White and PI Double Staining

C. albicans ATCC 18804 cells at the exponential phase were incubated with the ethanol extract from *P. lactiflora* at a final concentration equivalent to its MIC for 4 h. The cells were double-stained with Calcofluor White and PI, and examined by both fluorescence microscopy (Figs. 3A and 3B) and bright-field microscopy (Fig. 3C). Calcofluor White is a blue fluorescent dye that stains fungal cell walls, including chitin and callose. PI is a cationic molecule, which binds to nucleic acids through a damaged cell membrane. Consequently, dead or dying cells, which have defective cell membranes, are characterized by a red fluorescence signal, whereas those with intact cell membranes are not stained with PI. As shown in Fig. 3A, the cell walls were stained blue with Calcofluor White, but nucleic acids were

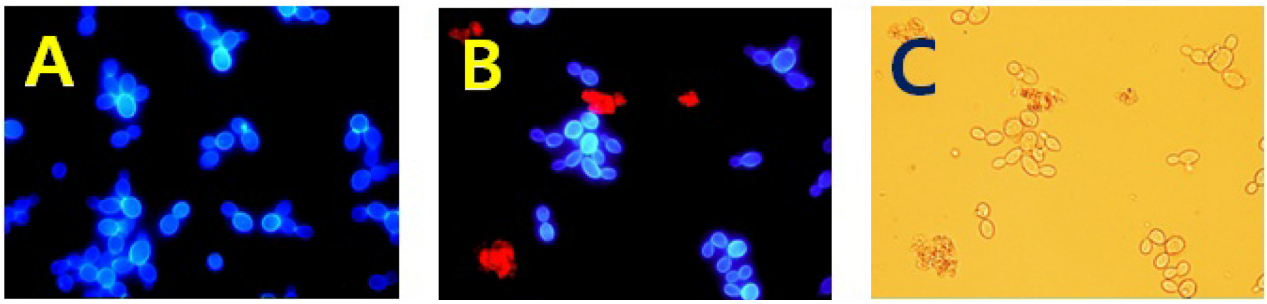

Fig. 3. Membrane-damaged *C. albicans* cells revealed by Calcofluor White and propidium iodide (PI) double staining.
C. albicans culture was incubated without (**A**) or with 196 μg/ml *P. lactiflora* ethanol extract (**B** and **C**) for 4 h, and then double-stained with Calcofluor White and PI. Fluorescent (**A** and **B**) and bright-field (**C**) images of the cells are shown. Blue fluorescence represents the cell wall stained by Calcofluor White, and red fluorescence represents PI-stained nucleic acids.

not stained with PI in control *C. albicans* cells. Thus, we can surmise that the control cells have intact cell membranes and cell walls. In contrast, some cells were stained red with PI in *P. lactiflora*-treated *C. albicans* cells at its MIC. Specifically, PI-stained cells formed aggregates with defective cell walls, so they were not stained with Calcofluor White (Fig. 3B). Additionally, the aggregates did not seem to have a cell boundary (Fig. 3C). Therefore, we can conclude that the integrity of *C. albicans* cell walls and cell membranes was compromised by the ethanol extract from *P. lactiflora*.

Effect of the *P. lactiflora* Ethanol Extract on Membrane Potential

In order to investigate whether the membrane damage in *C. albicans* cells by the ethanol extract from *P. lactiflora* is associated with the membrane potential, *C. albicans* cells were incubated with the ethanol extract from *P. lactiflora* for 3 h and stained with $DiBAC_4(3)$, which is a membrane-potential marker. Untreated *C. albicans* cells were rarely seen to be fluorescent green (Fig. 4A), but a high proportion of *C. albicans* cells treated with the ethanol extract from *P. lactiflora* at its MIC for 3 h were stained fluorescent green by $DiBAC_4(3)$ (Fig. 4B). The result indicates that the ethanol extract from *P. lactiflora* induced a change in the membrane potential of *C. albicans*. Therefore, the fluorescence of *C. albicans* cells was quantified, after treatment of *C. albicans* cells with variable concentrations of the ethanol extract from *P. lactiflora*, using a fluorescence filter at 493 nm excitation and 516 nm emission wavelengths (Fig. 4C). The fluorescence intensity of the cells treated with the ethanol extract from *P. lactiflora* at their 4× and 8× MIC increased 13% and 18%, respectively (Fig. 4C), and the difference between the DMSO control and each experimental group was statistically significant ($p < 0.01$).

Crystal Violet Assay

It has been suggested that an altered membrane potential is correlated with the enhancement of membrane permeability [23]. To evaluate if the ethanol extract from *P. lactiflora* also affects the membrane permeability of *C. albicans* cell membranes, a crystal violet assay was performed. As shown in Fig. 5, *C. albicans* cells treated with the ethanol extract from *P. lactiflora* at their 1× MIC, 2× MIC, and 4× MIC increased the uptake of crystal violet in a concentration-dependent manner, and the difference between the DMSO control and each experimental group was statistically significant ($p < 0.01$).

Discussion

Before advances in modern medicine and synthetic drugs, people used natural products to cure diseases and ailments. The merits of plant extracts used in traditional medicine are multifold, and there is often synergistic activity owing

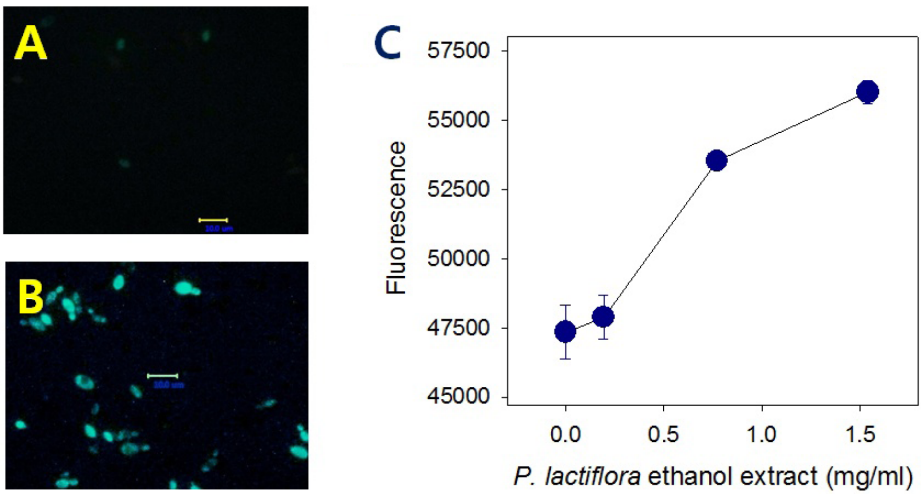

Fig. 4. Effect of the *P. lactiflora* ethanol extract on the membrane potential.
(**A** and **B**) Fluorescence microscopy: *C. albicans* culture was incubated without (**A**) or with 196 μg/ml *P. lactiflora* ethanol extract (**B**) for 3 h, and washed with PBS. $DiBAC_4(3)$ was added to the cells and fluorescent images of the cells are shown after using a green filter. Bars, 10 μm. (**C**) Spectrofluorometry: *C. albicans* cells (5×10^6 CFU/ml) were incubated with concentrations corresponding to 1× MIC, 4× MIC, or 8× MIC *P. lactiflora* ethanol extract for 3 h. A DMSO control was included for each treatment. Cells were stained with $DiBAC_4(3)$ for 30 min in the dark at room temperature, and fluorescence (arbitrary unit) was measured using a spectrofluorometer. The data represent the mean of the quintuplicate measurements from two independent assays. Each value represents the mean ± SE.

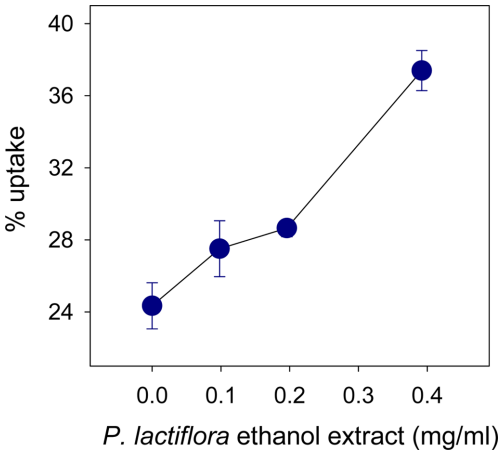

Fig. 5. Alteration of the membrane permeability, measured by crystal violet uptake (%).

C. albicans cells (1×10^8 CFU/ml) were incubated with 98, 196, or 392 μg/ml *P. lactiflora* ethanol extract at 37°C for 8 h and harvested. Solvent (DMSO) controls were included. At zero time, 0.5% NaCl containing 10 μg/ml crystal violet was added to the *C. albicans* cells and incubated at 37°C for 10 min, and the crystal violet uptake (%) was measured at 590 nm. Each value represents the mean ± SE.

to the presence of several active ingredients. The application of many of these natural remedies, used for centuries or even since ancient times, has been shown through the years to be safe, in addition to being effective. In a previous study of ours, the aqueous extract of *P. lactiflora* (Paeoniae Radix) exhibited antifungal activity against *C. albicans* with an MIC of 10 mg/ml [24]. In our present study, we have found the antifungal activity of the ethanol extract from *P. lactiflora* to be more effective (MIC of 0.196 mg/ml) than that of the aqueous extract from *P. lactiflora* against *C. albicans*. Herein, we also examined the mode of action of the ethanol extract from *P. lactiflora* against *C. albicans*.

The fungal cell wall surrounds the cell membrane, provides cells with rigidity and strength, and maintains osmotic support via the turgor pressure of the protoplast. The cell wall is composed of many macromolecules including β-glucans, chitin mannoproteins, and other proteins, and the enzymes that synthesize these constituents are potentially major targets for antifungals. Impairment of the essential cell wall components by antifungals causes cell lysis, but cells can survive with an appropriate osmotic stabilizer in the medium [20, 25]. The fact that the anticandidal effect of the ethanol extract from *P. lactiflora* was protected by isotonic media indicates that the *P. lactiflora* extract correlates with a disruption in the cell wall integrity of *C. albicans* (Fig. 1F and Table 2).

Although the sorbitol protection effect on *P. lactiflora*-treated *C. albicans* cells was apparently remarkable at 4 h of cultivation in YM medium in the cell morphology analysis (Fig. 1F), the effect was not noticeably observed at 24 h of cultivation in RPMI-1640 medium in the sorbitol protection assay (Table 2). The apparent discrepancy in the different responses of *P. lactiflora*-treated *C. albicans* cells to sorbitol in the two experiments could be explained as being the result of the different growth conditions of the *C. albicans* cells. *C. albicans* cells are routinely grown in YM medium with shaking for planktonic cells. YM medium is a selective medium, used mainly for the isolation of fungi and yeasts, because the growth of bacteria is prohibited by the acidic environment of the medium. By contrast, the sorbitol protection assay is based on the antifungal susceptibility test, according to CLSI M27-A3 guidelines (Table 2), and the assay employs RPMI-1640 medium buffered with MOPS (pH 7.0) in a static condition. RPMI-1640 medium is used for the research of *C. albicans* biofilm or adhered cells, and the medium mimics the composition of human fluids [26] containing limited nutritional sources with no protein, lipids, or growth factors. Owing to the optimal nutrient sources, aeration, and good physical conditions, exponential-phase *C. albicans* cells in YM medium for cell morphology analysis grow faster than lag-phase *C. albicans* cells in RPMI-1640 medium in the sorbitol protection assay. Hence, the newly formed *C. albicans* cells are prone to lysis by shaking, because they have defective cell walls attributable to the inhibitor of cell wall synthesis in the *P. lactiflora* ethanol extract. The acidic pH of the medium serves to enhance the lysis of *P. lactiflora*-treated *C. albicans* cells. By contrast, slow growers in RPMI-1640 medium somehow evade the burden of the *P. lactiflora* ethanol extract for 24 h of cultivation. It has been reported that penicillin, an inhibitor of bacterial cell wall synthesis, cannot kill nongrowing bacteria, but can rapidly kill the same bacteria by lysis during exponential growth [27]. Like penicillin, the *P. lactiflora* ethanol extract seems to be rather ineffective at inhibiting the cell wall synthesis of slow growers.

No apparent sorbitol effect on *P. lactiflora*-treated *C. albicans* cells was observed for MIC values at 24 h irrespective of the presence of sorbitol, but an effect was detected at 72 h of incubation in RPMI-1640 medium (Table 2). Even though the *P. lactiflora* ethanol extract appears to be slightly ineffective for slow growers, their cell walls are defective at 24 h of cultivation in RPMI-1640 medium, regardless of sorbitol. The antifungal susceptibility test employs colorimetric MIC endpoints, and the MIC was considered to be the lowest sample concentration that remained blue,

indicating no growth. Resazurin is an oxidation-reduction indicator, and the blue resazurin is reduced to bright pink resorufin by mitochondrial enzymes of viable cells [17]. The wells holding sorbitol-containing RPMI-1640 medium with 196 or 392 mg/ml *P. lactiflora* ethanol extract would contain just a few cells with defective cell walls, exhibiting low metabolic activity (blue) in sorbitol-containing RPMI-1640 medium at 24 h. Notwithstanding their environmental issues, they survive and continue to form new cells, or hyphae, with the help of sorbitol during the next 48 h (pink). The neutral pH and static culture conditions are helpful for the survivability of the *P. lactiflora* ethanol extract in RPMI-1640 medium. Inactivation or exhaustion of the active ingredients of *P. lactiflora* ethanol extract would support the formation of normal *C. albicans* cells as time passes.

Neutral red is a supravital dye that easily penetrates cell membranes and enters the lysosome to stain it red in viable cells [28]. However, neutral red leaks out into the cytoplasm, where it then stains the cytoplasm red if lysosomal membranes are damaged in dead cells [22, 29]. Yeast cells have numerous vacuoles that correspond functionally to mammalian lysosomes and plant vacuoles [30]. The control *C. albicans* cells survived since their vacuoles were stained with neutral red (Figs. 1A and 1B). Amphotericin B targets ergosterol, which is a component of the cell membranes of fungi. The cytoplasm of dead or dying *C. albicans* cells, after treatment with amphotericin B, were stained dark-red with neutral red with intact cell walls irrespective of sorbitol (Figs. 1C and 1D). The quantity of large membranous debris and darkly stained materials observed in *P. lactiflora*-treated *C. albicans* cells were fairly diminished in the *C. albicans* cells in YM medium supplemented with sorbitol (Fig. 1F).

The results from the sorbitol protection assay and cell morphology analysis suggest the possibility that *C. albicans* cells treated with the ethanol extract from *P. lactiflora* suffered an injury in their cell wall structure, with extensive changes in the osmotic pressure of *C. albicans* cell interior. Therefore, this conjecture was tested using aniline blue assay, and the data that (1,3)-β-D glucan synthase activity decreased in the presence of the ethanol extract from *P. lactiflora* demonstrate that the *P. lactiflora* ethanol extract inhibits the pathway of cell wall synthesis (Fig. 2).

Calcofluor White is a bluish fluorescent brightener and binds to β(1→3) and β(1→4) polysaccharides by ultraviolet radiation. PI is generally excluded from live cells, and binds DNA and RNA only through damaged cell membranes. As shown in Fig. 3B, PI-stained red cells were observed mainly in aggregates rather than isolated single red cells, and the cell walls were not stained blue with Calcofluor White in red aggregates. That is, the aggregates found in Figs. 3B and 3C are presumed to be the cells having damaged cell membranes, with no or defective cell walls. Cell aggregates were possibly formed by ionic interactions between protoplasts. The result of Calcofluor White-PI staining indicates that both cell walls and cell membranes are damaged by the ethanol extract from *P. lactiflora* in *C. albicans* cells.

In higher organisms, the inside of most cells maintains a negative charge relative to the outside of the cells, and the difference in charge is called the membrane potential. $DiBAC_4(3)$, which is a lipophilic anion, can go through eukaryotic cell membranes when their membrane potential has been compromised [31]. Therefore, depolarized cells display enhanced green fluorescence by binding of $DiBAC_4(3)$ to inner cell membranes or intracellular proteins. As shown in Fig. 4B, *P. lactiflora*-treated *C. albicans* cells were fluorescent green after $DiBAC_4(3)$ staining, and the fluorescence intensity increased significantly in a concentration-dependent manner (Fig. 4C). The membrane potential of fungal cells is formed and maintained by several types of transporters that move ions across the plasma membrane. The major protein in yeast cell membranes is the proton pump Pma1 (P-type H^+-ATPase), which pumps protons outside the cytoplasm and drives the membrane potential to more negative values [32]. In addition, many channels and transporters are found in yeast plasma membranes, such as the potassium-selective outward ion channel TOK1, non-selective cation channels, potassium transporters Trk1 and Trk2, sodium/potassium–proton antiporter Nha1, sodium phosphate symporter Pho89, and voltage-gated calcium channel Cch1 [32]. In many types of cells, the membrane potential powers the uptake of glucose and many other molecules against their concentration gradient by symporters and antiporters.

Alteration of the membrane potential is often associated with membrane permeability due to damage to membrane proteins in cell membranes. Changes in membrane permeability by the ethanol extract from *P. lactiflora* may be caused by the inactivation of one of the transporters of channel proteins. It has been reported that crystal violet, which is a lipophilic cation at a neutral pH, does not penetrate cells with intact cell membranes, but penetrates cells with impaired cell membranes. Therefore, the crystal violet assay is often used for the detection of membrane damage [33]. Thus, changes in membrane permeability were explored in *C. albicans* cells, after treatment with the ethanol extract from *P. lactiflora*, by crystal violet assay.

P. lactiflora-treated *C. albicans* cells exhibited an increased uptake of crystal violet in a concentration-dependent manner (Fig. 5). Uptake of monovalent cations by crystal violet indicates that there may be a movement of other monovalent cations into the cytoplasm: The accumulation of H^+ ions will induce the decreased pH in the cytoplasm and decreased membrane potential to lead to energy depletion. If not, uptake of Na^+ or K^+ will make the cells have a decreased membrane potential, or increased osmotic pressure, which will allow for more water to move into the cytoplasm and form strong turgor pressure. It is surmised that a swollen protoplast, due to increased osmotic pressure, would easily rupture, since *C. albicans* cells treated with the ethanol extract from *P. lactiflora* have defective cell walls. Moreover, it is thought that the protoplasts tend to form aggregates transiently by membrane-membrane fusion. In fact, moderately swollen or elongated cells and protoplasts escaping from the ruptured cell walls were found relatively easily in *C. albicans* cells treated with the ethanol extract from *P. lactiflora* (data not shown). Therefore, one can speculate that the ethanol extract from *P. lactiflora* induces substantial damage to the cell walls and cell membranes simultaneously. The multiple targets of the ethanol extract from *P. lactiflora* against *C. albicans* illustrate that it could be a valuable constituent in any potential future therapeutic drugs against *Candida*-associated infections.

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