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Development of *Candida albicans* Biofilms Is Diminished by *Paeonia lactiflora* via Obstruction of Cell Adhesion and Cell Lysis

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Candida albicans infections are often problematic to treat owing to antifungal resistance, as such infections are mostly associated with biofilms. The ability of *C. albicans* to switch from a budding yeast to filamentous hyphae and to adhere to host cells or various surfaces supports biofilm formation. Previously, the ethanol extract from *Paeonia lactiflora* was reported to inhibit cell wall synthesis and cause depolarization and permeabilization of the cell membrane in *C. albicans*. In this study, the *P. lactiflora* extract was found to significantly reduce the initial stage of *C. albicans* biofilms from 12 clinical isolates by 38.4%. Thus, to assess the action mechanism, the effect of the *P. lactiflora* extract on the adhesion of *C. albicans* cells to polystyrene and germ tube formation was investigated using a microscopic analysis. The density of the adherent cells was diminished following incubation with the *P. lactiflora* extract in an acidic medium. Additionally, the *P. lactiflora*-treated *C. albicans* cells were mostly composed of less virulent pseudohyphae, and ruptured debris was found in the serum-containing medium. A quantitative real-time PCR analysis indicated that *P. lactiflora* downregulated the expression of *C. albicans* hypha-specific genes: *ALS3* by 65% (p = 0.004), *ECE1* by 34.9% (p = 0.001), *HWP1* by 29.2% (p = 0.002), and *SAP1* by 37.5% (p = 0.001), matching the microscopic analysis of the *P. lactiflora* action on biofilm formation. Therefore, the current findings demonstrate that the *P. lactiflora* ethanol extract is effective in inhibiting *C. albicans* biofilms in vitro, suggesting its therapeutic potential for the treatment of biofilm-associated infections.

Keywords: Biofilm, *Candida albicans*, hypha-specific gene, pseudohypha, qPCR, *Paeonia lactiflora*

Introduction

Although *Candida* species are commensal in healthy humans, they become opportunistic pathogens that can cause superficial and systemic diseases in seriously ill or immunocompromised patients [1]. Biofilm formation by fungi plays a key role in pathogenesis [2], and most diseases caused by *Candida albicans* are associated with biofilm growth [3]. Fungal biofilms display a reduced susceptibility to available antifungal drugs, when compared with their planktonic counterparts [4]. This fungal biofilm resistance to antifungal drugs is derived from complex and multifactorial mechanisms, such as a reduced growth rate, enhanced cell density, diverse stress responses, presence of persister cells, secretion of an extracellular matrix, upregulation of the membrane transporter system and efflux pumps, and differential regulation of drug targets [4, 5]. In the case of *C. albicans*, the biofilm is initiated by the adhesion of blastospores to a solid surface or other cells, followed by a transition from yeast to hyphal forms. Biofilms advance through an increased cell density, the presence of multilayers of cells, and elongation of the filaments to form a mesh-like network consisting of yeast, hyphae, and pseudohyphae. Additionally, sessile cells accumulate an extracellular polymer matrix, resulting in mature biofilms [6].

Despite the ongoing development of new antifungal agents, most available antifungals are ineffective against *Candida* biofilms owing to the requirement of high

concentrations for activity [7] or they have significant side effects due to toxicity [8]. Moreover, the search for effective antifungals is hindered by the eukaryotic nature of fungal cells, since they evolve closely with their human hosts [9], limiting the number of drug targets that can be explored to selectively eradicate the pathogen. Thus, there is an urgent need to develop novel antifungals with low toxicity and high therapeutic activity. Plant products are generally used in traditional ethnomedicine, since they have effective antimicrobial and antifungal activities as a part of their defense mechanism [9]. Therefore, the development of phytochemicals against *Candida* biofilms could be an excellent strategy [10].

A previous study by the current authors demonstrated the antifungal activity of the ethanol extract from *Paeonia lactiflora* against *C. albicans*, which was associated with the synergistic actions of preventing synthesis of the cell wall (1,3)-β-D-glucan polymer and changing the membrane depolarization and permeability [11]. Therefore, this study investigated the ability of the *P. lactiflora* ethanol extract to reduce the development of *C. albicans* biofilms in vitro, and examined its mode of action against *C. albicans* biofilms using qRT-PCR analysis.

Materials and Methods

Fungal Strains and Growth Conditions

Candida albicans ATCC 18804 and *Candida albicans* SC5314 (ATCC MYA-2876) were procured from the Korean Culture Center of Microorganisms and American Type Culture Collection (USA), respectively. A total of 12 clinical *C. albicans* isolates, collected from candidiasis patients, were kindly provided by Prof. K.H. Lee [12]. The yeast strains were routinely cultivated in YM medium (yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, and dextrose 1%) at 37°C with agitation.

Plant Material and Extraction

The ethanol extract from *Paeonia lactiflora* was prepared by lyophilization of a 70% ethanol extract from dried roots of *P. lactiflora.* The *P. lactiflora* extract was then dissolved in dimethyl sulfoxide (DMSO) at 100 mg/ml, and kept at −20°C until used [11]. The amphotericin B was purchased from Sigma (USA). For all tests, DMSO was used in the controls (no treatment) at levels equivalent to those used in the antifungal agent treatment groups.

Antifungal Susceptibility Test

The minimum inhibitory concentration (MIC) of *P. lactiflora* against *C. albicans* SC5314 was determined according to the modified Clinical and Laboratory Standards Institute (CLSI) protocols M27-A3 for the colorimetric broth microdilution method using resazurin (Sigma, USA) as an indicator of cell viability [13]. The colorimetric MIC end points were taken as the lowest sample concentration that stayed blue or the first dilution that changed from blue to slightly purple. Amphotericin B was used as the reference standard for CLSI M27-A3. The inoculated plates were incubated at 35°C for 24 h. All the assays were repeated in triplicate.

Effect of *P. lactiflora* Ethanol Extract on *C. albicans* Viability

An overnight *C. albicans* ATCC 18804 culture was adjusted in the YM medium to a final concentration of 5×10^6 cells/ml and incubated in the absence or presence of 1× MIC (196 µg/ml) of the *P. lactiflora* ethanol extract [11] at 37°C for 3 h with agitation. The cells were then harvested and resuspended in 20 µl of YM. Next, the cell suspension (5 µl) was mixed with 2 µl of a 0.1% methylene blue solution (Sigma, USA) on a slide glass and covered with a cover slip. The cell viability and morphology were observed using an inverted microscope.

Effect of *P. lactiflora* Ethanol Extract on *C. albicans* Adhesion to Polystyrene Plates

An overnight *C. albicans* ATCC 18804 culture was adjusted in YNB medium with 50 mM glucose (YNB/glucose) to a final concentration of 5×10^6 cells/ml. *C. albicans* cell suspensions (0.2 ml) were added to a flat-bottom 96-well polystyrene plate and the suspensions were with or without the *P. lactiflora* extract at 1× MIC at 37°C. Three hours later, the liquid medium was gently aspirated and the wells were washed with phosphate-buffered saline (PBS) to remove any loosely adhered cells. Forty microliters of PBS was added to each well, and adherent cells were examined using an inverted microscope. To visualize the *C. albicans* cell walls, the *C. albicans* cells were stained with 5 µl of 0.05% Calcofluor-White (Sigma, USA) for 1 min and examined using an inverted epifluorescence microscope.

Effect of *P. lactiflora* Ethanol Extract on Germ Tube Formation

An overnight C. albicans ATCC 18804 culture was adjusted using RPMI 1640 medium (RPMI 1640 with L-glutamine and without sodium carbonate buffered with MOPS, pH 7) containing 10% fetal bovine serum (FBS) to a concentration of 5×10^6 cells/ml. *C. albicans* cell suspensions (0.2 ml) were added to a flat-bottom 96-well polystyrene plate with the *P. lactiflora* extract at 1× MIC. The plate was incubated at 37°C for 4 h and the effect of the *P. lactiflora* ethanol extract on germ tube formation was investigated using an inverted microscope.

Quantification of Antibiofilm Activity

The antibiofilm activity of *P. lactiflora* was measured using the biofilms from the 12 clinical *C. albicans* isolates based on an XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) reduction assay [14] with slight modifications. For each clinical *C. albicans* isolate, an overnight culture was adjusted using YNB/glucose to a concentration of 1×10^7 cells/ml. The *C. albicans* cells were allowed to form biofilms by aliquoting 0.2 ml of the

C. albicans cell suspensions into a flat-bottom 96-well polystyrene plate, which was then incubated at 37°C for 3 h. Thereafter, the liquid medium was discarded and the wells were gently washed twice with PBS to remove any loosely adhered cells. To check the effect of the *P. lactiflora* ethanol extract on the biofilms, fresh YNB/glucose medium (0.2 ml) without or with the *P. lactiflora* ethanol extract at 1× MIC was placed into each well holding the initial stage of the biofilms. The plate was then incubated for an additional 16 h at 37°C with moist air. Thereafter, the planktonic cells were discarded by gently aspirating and washing the plate twice with 0.2 ml of PBS. Finally, the biofilms were quantitated using a tetrazolium XTT reduction assay [15]. All the experiments were carried out in quadruplicate. The baseline values were normalized to 100 and the results are expressed in percentages of inhibition. The data from one of three independent experiments are presented.

RNA Purification and cDNA Synthesis

C. albicans SC5314 cells grown overnight in YM broth at 37°C with agitation were adjusted in RPMI 1640 medium to a concentration of 1 × 10^7 cells/ml. The *C. albicans* cells were allowed to form biofilms by aliquoting 2.5 ml of the *C. albicans* cell suspensions into a flat-bottom 6-well polystyrene plate, which was then incubated at 37°C for 4 h. Thereafter, the non-adhered cells including the liquid medium were gently aspirated and the wells were washed twice with PBS to remove any loosely adhered cells. To examine the changes in the biofilm-associated gene expression, 2 ml of fresh RPMI medium or RPMI medium containing 196 μg/ml of the *P. lactiflora* ethanol extract was added to each well holding the 4 h-aged biofilms, and the plate was incubated for an additional 90 min at 37°C with moist air. All the reactions were performed in duplicate. The biofilm cells were all collected using a sterile cell scraper. The total RNA was then extracted from the biofilms using the Trizol reagent (Invitrogen, USA) and an RNeasy Mini Kit (Qiagen, Germany). The harvested cells were resuspended in 0.8 ml of the Trizol reagent and transferred to a Lysing matrix C containing glass beads (MP Biochemicals, USA). The *C. albicans* SC5314 cells were then disrupted using a Minilys tissue homogenizer (Bertin, France) with 6 runs of 30 sec under strong agitation and cooling on ice for 1 min between each run. Cell lysis was checked by examining the extract using an inverted microscope. The lysate was mixed with 0.2 ml of chloroform/ml Trizol, incubated at room temperature for 5 min, and centrifuged at 4°C at 12,000 ×*g* for 15 min. The aqueous phase was then carefully removed and the RNA was purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The cDNA was synthesized using the ReverTra Ace qPCR RT master mix with a gDNA remover (Toyobo, Japan) according to the manufacturer's instructions.

Quantitative Real-Time PCR

Qiagen Rotogene Q was used as the gene analysis system. The amplification and detection of the biofilm genes were performed using a real-time PCR with the SYBR Green Realtime PCR master mix (Toyobo, Japan). The primers were designed from the *C. albicans* SC5314 genome sequence through CGD (Candida Genome Database) [16] using Primer3 software from NCBI Primer Blast [17], and synthesized by Macrogen (Korea). The primer sequences used for the gene expression analysis are shown in Table 1. All the reactions were carried out in triplicate. The final volume of each reaction was 20 μl, consisting of 10 μl of SYBR Green Master Mix, 1 μl of forward primer (0.5 μM), 1 μl of reverse primer (0.5 μM), 1 μl of cDNA, and 7 μl of RNase-free water. The PCR conditions comprised an activation step of 1 min at 95°C, followed by 40 cycles at 95°C for 15 sec, 60°C for 15 sec, and 72°C for 45 sec. Data were collected at the 72°C step of each cycle. The

Table 1. Primers used for qRT-PCR.

Gene	Primer	Sequence	Tm (°C)	Amplified length (bp)
ACT1	Forward	GACGCTCCAAGAGCTGTTTTC	59.8	108
	Reverse	GGATTGGGCTTCATCACCAAC	59.5	
ALS1	Forward	GCCACAACCACCACAGTTAC	59.3	136
	Reverse	AATGAGGACGGGAAAATGATGG	58.7	
ALS3	Forward	GCTGGTGGTTATTGGCAACG	60.1	142
	Reverse	ATGGTAAGGTGGTCACAGCG	60.0	
EAP1	Forward	CCAGCCCATCAGTTCCTACC	59.8	160
	Reverse	AGTGCAGAGCCAGATCCTTC	59.5	
ECE1	Forward	TGCCGTCGTCAGATTGCCAG	63.1	96
	Reverse	CCAACATCTGGAACGCCATC	59.3	
HWP1	Forward	CCGGAATCTAGTGCTGTCGTC	60.5	83
	Reverse	GCAGATGGTTGCATGAGTGG	59.6	
SAP1	Forward	AACCAATGAGGCTGCTGGTG	60.9	110
	Reverse	TGGCAGCATTGGGAGAGTTG	60.6	

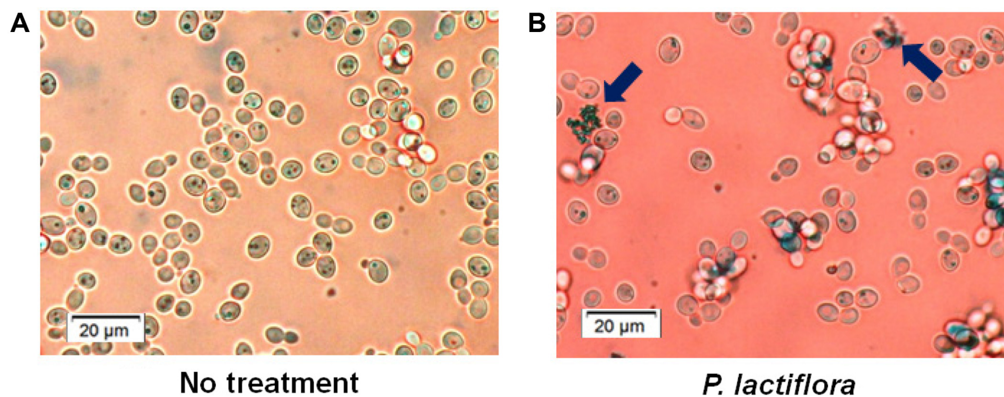

Fig. 1. Effect of *Paeonia lactiflora* ethanol extract on *Candida albicans* viability.

C. albicans ATCC 18804 cells were grown in a YM medium in the absence (**A**) or presence of 196 µg/ml *P. lactiflora* ethanol extract (**B**) at 37°C for 3 h with agitation. The harvested cells were stained with methylene blue and visualized under an inverted microscope at ×400 magnification. Arrows indicate cell debris. Bars, 20 µm.

fold regulation of each target gene was calculated using the comparative Ct method, using *ACT1* Ct to normalize the data, and the biofilm expression data were treated with a solvent control as the reference sample to determine the ΔΔCt values. The standard deviation (SD) of each gene expression value was calculated using Sigma plot 13.0. The results were normalized to *ACT1* RNA, and considered statistically significant at $p \leq 0.05$.

Statistical Analysis

The differences in the optical density (mean ± SD) of the individual biofilms incubated with the *P. lactiflora* ethanol extract and control were calculated using Student's *t*-test. The SD of each optical density was calculated using Sigma plot 13.0, and considered statistically significant when the *p*-value was less than 0.05.

Results

Antifungal Susceptibility Assay

C. albicans ATCC 18804 has been as the test organism for studying the antifungal activity of the test samples in this study, since it is a standard *C. albicans* strain used by many *Candida* researchers. In this study, the *P. lactiflora* ethanol extract was found to be effective for inhibiting *C. albicans* biofilm development. However, to clarify the action mechanism using a qPCR analysis is difficult as the genome of *C. albicans* ATCC 18804 has not yet been sequenced. Therefore, for the gene expression analysis, this study used *C. albicans* SC5314, as its whole genome, a 28 Mb diploid composed of eight sets of chromosome pairs, has already been sequenced and is available from the CGD [16]. The MIC of the ethanol extract from *P. lactiflora* was also determined for *C. albicans* SC5314, which was used for the qRT-PCR analysis, according to CLSI M27-A3. The MICs of the *P. lactiflora* ethanol extract and amphotericin B for *C. albicans* SC5314 were 49 and 0.25 µg/ml, respectively (Table 2). The MICs of the *P. lactiflora* extract against other *C. albicans* and non-*albicans* strains from our previous studies were also included for comparison [11]. The results indicated a 4-fold greater susceptibility to the *P. lactiflora* ethanol extract by *C. albicans* SC5314 compared with *C. albicans* ATCC 18804.

Effect of *P. lactiflora* Ethanol Extract on *C. albicans* Cell Viability

C. albicans ATCC 18804 cells were grown in YM in the presence of the *P. lactiflora* ethanol extract at 1× MIC at 37°C for 3 h. The harvested cells were then stained with methylene blue. In contrast to the healthy ovoid *C. albicans* cells, where the cytoplasm was not stained with methylene blue (Fig. 1A), dead cells with blue-stained cytoplasm and ruptured cell debris were detected (Fig. 1B). It is also noteworthy that the *P. lactiflora*-treated *C. albicans* cells tended to form aggregates (Fig. 1B).

Table 2. Minimum inhibitory concentrations (MICs) of Paeonia *lactiflora* ethanol extract against different *Candida* species and strains.

	MIC (µg/ml)		Reference
	P. lactiflora	Amphotericin B	
C. albicans SC5314	49	0.25	This study
C. albicans ATCC 18804	196	0.25	[11]
C. krusei ATCC 32196	98	0.25	[11]
C. glabrata ATCC 2001	25	0.13	[11]
C. tropicalis ATCC 750	98	0.13	[11]

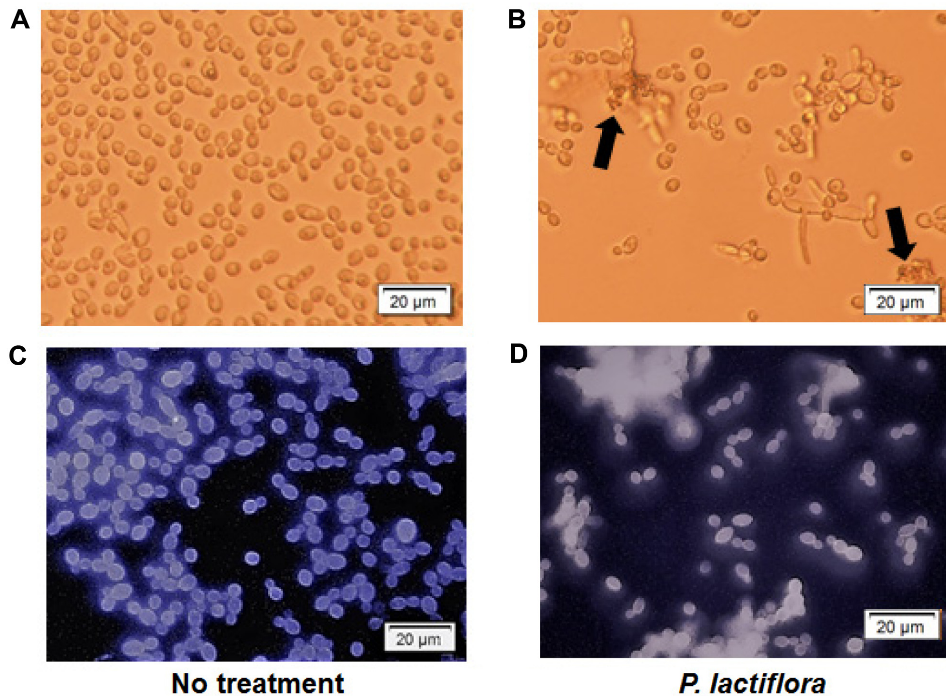

Fig. 2. Effect of *Paeonia lactiflora* ethanol extract on adhesion of *Candida albicans* cells to polystyrene.
After *C. albicans* ATCC 18804 cells were incubated in the absence (panels **A** and **C**) or presence of 196 µg/ml *P. lactiflora* ethanol extract (panels **B** and **D**) at 37°C for 3 h in a flat-bottomed polystyrene plate, the medium including nonadherent cells was removed and PBS added. The adherent cells were investigated under an inverted microscope (panels **A** and **B**), whereas the adherent cells stained with Calcofluor-White were observed under UV light using an inverted epifluorescence microscope at ×400 magnification (panels **C** and **D**). Cell debris is shown by arrows. Bars, 20 µm.

Effect of *P. lactiflora* on Adhesion of *C. albicans* Cells to Polystyrene Plates

The adhesion assay was carried out using a YNB/glucose medium according to an antibiofilm activity assay [14]. *C. albicans* ATCC 18804 cells were treated with the *P. lactiflora* ethanol extract at 1× MIC at 37°C for 3 h and the non-adhered cells were removed by washing with PBS. In contrast to the well-adhered and evenly distributed

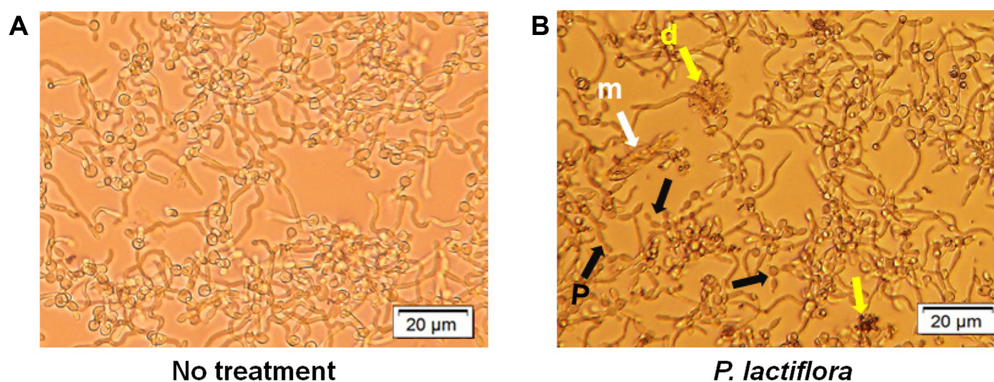

Fig. 3. Effect of *Paeonia lactiflora* ethanol extract on germ tube formation.
C. albicans ATCC 18804 cells were grown in RPMI 1640 medium containing 10% FBS in the absence (**A**) or presence of 196 µg/ml *P. lactiflora* ethanol extract (**B**) at 37°C for 4 h in a flat-bottomed 96-well polystyrene plate. The *C. albicans* germ tubes in the surrounding medium were visualized using an inverted microscope at ×400 magnification. In contrast to typical hyphal forms of *C. albicans* (panel **A**), the characteristic structures are indicated by arrows: p, pseudohyphae (black); d, ruptured debris (yellow); and m, membranous materials (white). Bars, 20 µm.

C. albicans cells in Figs. 2A and 2C, non-adhered cell debris and cell aggregates are clearly shown in Figs. 2B and 2D. When using Calcofluor-White, a fluorescent stain that binds to cellulose and chitin in fungal cell walls, non-adhered fluorescent cell aggregates were found (Fig. 2D), along with some pseudohyphae (Fig. 2B).

Effect of *P. lactiflora* Ethanol Extract on Germ Tube Formation

C. albicans cells are known to form thicker biofilms in RPMI 1640 medium than in YNB/glucose medium [18]. Therefore, germ tubes from *C. albicans* ATCC 18804 cells were induced in RPMI 1640 medium containing 10% FBS, and the effect of the *P. lactiflora* ethanol extract was investigated. Unconstricted filaments extending from unbudded *C. albicans* ATCC 18804 cells were formed (Fig. 3A). Hyphae are not constricted at the neck of the mother cell with parallel sides along their entire length [19]. As shown in Fig. 3B, the *P. lactiflora* extract did not seemingly inhibit germ tube formation, yet the lengths of the *P. lactiflora*-treated *C. albicans* hyphae were shorter than those of the control. Pseudohyphal cells are constricted at the neck between the mother cell and the bud, and are wider at the center than at the two ends, yet the width and length of a pseudohyphal cell can be extremely different [19, 20]. In this study, typical constricted pseudohyphal cells were predominantly found, as indicated by the arrows, along with cell debris or membranous materials (Fig. 3B). As previously reported, the *P. lactiflora* ethanol extract inhibits the synthesis of the *C. albicans* cell wall and damages the cell membrane function, resulting in cell swelling and subsequent bursting due to osmotic pressure [11].

In Vitro Antibiofilm Activity of *P. lactiflora* against Initial Stage of *C. albicans* Biofilms

Adherence is considered a critical stage in biofilm formation. The inhibitory effect of the *P. lactiflora* extract on *C. albicans* biofilms was studied at the early stage of *C. albicans* biofilm development. Three-hour-aged *C. albicans* biofilms were incubated without or with the *P. lactiflora* extract at 1× MIC for 16 h. Tetrazolium salt XTT was used to monitor the metabolic activity of the biofilms formed by the 12 clinical *C. albicans* isolates (Table 3). The experimental data showed that the *P. lactiflora* ethanol extract significantly inhibited *C. albicans* biofilm formation by 38.4% ($p < 0.01$), confirming its effective anti-biofilm activity against *C. albicans* biofilms.

Table 3. Inhibitory effect of the *P. lactiflora* ethanol extract on *C. albicans* biofilm development.

C. albicans strains	No treatment	*P. lactiflora*	Relative inhibition of biofilm formation (%)
	A_{492}		
1	0.444 ± 0.043	0.340 ± 0.101	23.4
2	0.269 ± 0.039	0.140 ± 0.017[a]	48.0
3	0.492 ± 0.024	0.326 ± 0.053[a]	37.7
4	0.329 ± 0.044	0.233 ± 0.019[a]	29.2
5	0.381 ± 0.047	0.317 ± 0.032	16.8
6	0.338 ± 0.043	0.216 ± 0.022[a]	36.1
7	0.453 ± 0.039	0.221 ± 0.046[a]	51.2
8	0.154 ± 0.019	0.086 ± 0.003[a]	44.2
9	0.477 ± 0.067	0.228 ± 0.019[a]	52.2
10	0.422 ± 0.071	0.209 ± 0.014[a]	50.6
11	0.368 ± 0.029	0.212 ± 0.022[a]	42.4
12	0.411 ± 0.037	0.291 ± 0.024[a]	29.2
Mean ± SD	0.378 ± 0.096	0.235 ± 0.075[a]	38.4 ± 11.7

Three hour-aged initial stage of *C. albicans* biofilms from 12 clinical isolates were incubated in the absence or presence of 196 μg/ml *P. lactiflora* ethanol extract for 16 h at 37°C. Metabolic activity was assessed using the XTT reduction assay measuring the absorbance at 492 nm. Values reported are the means of quadruplicate determinations ± standard deviations (SD). A p value of ≤0.01 indicates a significant difference between no and the *P. lactiflora* extract-treatment and is marked with a.

Biofilm-Dependent Expression of Adhesin Genes

Since the process of yeast cell adhesion to a surface is essential for biofilm development, this study analyzed the gene expression changes known to be involved in yeast adhesion and biofilm formation, such as *ALS1*, *ALS3*, *EAP1*, *ECE1*, *HWP1*, and *SAP1*, where the expression level of each gene was normalized with the housekeeping gene *ACT1* for both the *P. lactiflora*-treated and untreated biofilms.

The proteins Als1 (agglutinin-like sequence 1) and Als3 play a role in the surface attachment of a biofilm [21]. Eap1 (enhanced adherence to polystyrene protein 1) is a cell wall adhesin, which is a glycosylphosphatidylinositol-anchored glucan-linked protein, and it plays a role in cell adhesion to polystyrene and epithelial cells [22]. During hyphal formation, Ece1 (extent of cell elongation 1) is processed into a smaller peptide candidalysin, a cytolytic peptide toxin, to activate or damage epithelial cells as the hyphal factor [23]. Hwp1 (hyphal wall protein 1) is involved in host cell attachment, and is not expressed during the yeast phase, but is highly expressed on germ tubes and hyphal surfaces [24]. *SAP1* encodes the secreted aspartyl proteinase 1 protein, which is a member of the multigene *SAP* (secreted aspartic proteinase) family [25]. Sap activity is regarded as a virulence factor for *C. albicans*, and *SAP1* expression has been found during the initial invasion of the skin and

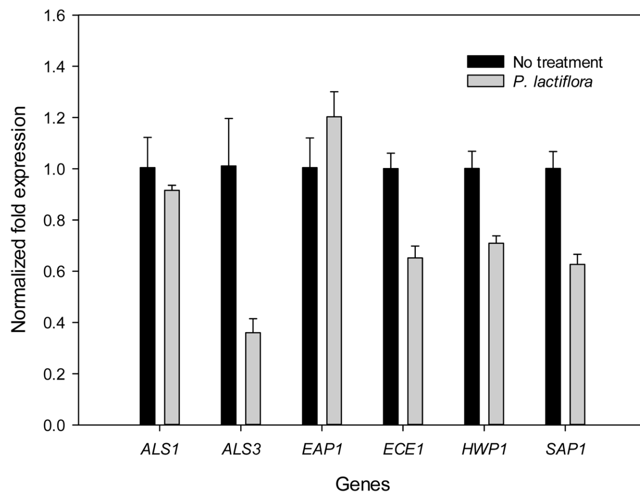

Fig. 4. Effect of *Paeonia lactiflora* ethanol extract on expression of *Candida albicans* hypha-specific genes.

C. albicans SC5314 biofilms formed in RPMI 1640 medium at 37°C for 4 h were treated without or with the *P. lactiflora* ethanol extract for 90 min at 37°C. The expression of the indicated genes was then analyzed by qRT-PCR. The expression level of each gene is shown after normalization with the housekeeping actin gene *ACT1*. The histogram shows the relative expression fold-changes of the genes following *P. lactiflora* treatment as compared with the control. Data are the mean of three independent experiments ± SD.

correlates with skin tissue damage [26]. As shown in Fig. 4, significant downregulation of *ALS3* by 65% (p = 0.004), *ECE1* by 34.9% (p = 0.001), *HWP1* by 29.2% (p = 0.002), and *SAP1* by 37.5% (p = 0.001) was observed after exposing the 4 h-aged *C. albicans* biofilms to 196 μg/ml *P. lactiflora* ethanol extract for 90 min. A reduced expression of *ALS1* was also found in the *P. lactiflora*-treated biofilms, but the difference was not significant (p = 0.269). Meanwhile, a higher expression of *EAP1* was found in the *P. lactiflora*-treated biofilms, but the difference was not significant (p = 0.086).

Discussion

Candida pathogenicity is determined by a variety of virulence factors, such as adherence to host surfaces, biofilm formation, and the secretion of hydrolytic enzymes, including proteases, phospholipases, and hemolysins [27]. When compared with conventional pharmaceutical drugs, plant extracts have synergistic advantages with multiple active components and fewer side effects, as proven by continued use in traditional medicine. The ethanol extract from *P. lactiflora* has already been shown to inhibit the synthesis of the *C. albicans* cell wall (1,3)-β-D-glucan polymer, leading to *C. albicans* cell lysis [11]. Moreover, the extract is also known to inhibit the function of the *C. albicans* cell membrane by depolarization and changing the permeability [11]. Therefore, in the current study, cell debris or membranous materials were clearly found among the *C. albicans* cells treated with the *P. lactiflora* ethanol extract (Figs. 1B, 2B, and 3B). It was also expected that the *P. lactiflora* extract would inhibit *C. albicans* biofilm formation, since it damages the cell wall integrity and hampers the functioning of the cell membrane. Thus, an XTT reduction assay demonstrated that the *P. lactiflora* extract at 1× MIC significantly reduced the average metabolic activity of the *C. albicans* cells within the biofilms by 38.4% (Table 3), suggesting that the extract has the potency to block biofilm development. However, *P. lactiflora* was not effective in reducing the germ tube formation of *C. albicans* (Fig. 2B). Rather, the *P. lactiflora*-treated cells were inclined to form pseudohyphae in the YNB/glucose medium (Fig. 2B), in which *Candida* cells normally grow as budding yeast forms due to the acidic pH of the medium. Therefore, the inhibitory effect of *P. lactiflora* on biofilm formation is clearly not due to the inhibition of germ tube formation. Notwithstanding, the density of adherent *C. albicans* cells in the *P. lactiflora*-treated group was lower than that of the control (Fig. 2B). One more notable phenomenon found in the *P. lactiflora*-treated *C. albicans* cells was that the *C. albicans* cells tended to adhere to each other to form cell aggregates or clumps without adhering to the plate (Figs. 1B, 2B, and 2D). Thus, to survive the harsh conditions resulting from a defective cell wall and damaged cell membrane due to the *P. lactiflora* extract, the *C. albicans* cells seemed to transit from a budding yeast to pseudohyphal forms, and adhered to each other to form cell aggregates.

C. albicans filaments are thought to be required for tissue invasion and yeast-form cells for dissemination in the host [28]. The stepwise evolution from yeast to pseudohyphae to hyphae is also believed to be related with increased virulence gene expression and the development of a variety of virulence properties [29]. The transition from yeast to filaments in *C. albicans* is regulated by multiple factors, such as the presence of serum, 37°C, pH, and depletion of nutrients [29].

The *ALS* gene family encoding cell-surface glycoproteins is known to be associated with adhesion to host surfaces and potentially other cellular processes [30]. In the *P. lactiflora*-treated biofilms, the expression of *ALS3* and *HWP1* was significantly reduced 0.650-fold and 0.292-fold, respectively, when compared with the control (Fig. 4). These data also agree well with the increased detachment of adherent cells

(Fig. 2B). Moreover, the predominance of pseudohyphal forms among the *C. albicans* cells treated with the *P. lactiflora* extract coincides with the reduced expression of *ALS3*, as it has been reported that *ALS3* is only transcribed in germ tubes and hyphae [31]. In contrast to the significantly reduced *ALS3* expression, the slightly reduced *ALS1* expression by 0.09-fold (p = 0.269) with the *P. lactiflora* treatment indicates that the importance of Als1 may not be greater than that of Als3 in biofilm development. Similarly, the previous report that *ALS1* expression depends on the growth conditions [30] suggests that the role of Als1 is not essential.

In the *P. lactiflora*-treated biofilms, the expression of *SAP1* was significantly decreased by 0.375-fold (p = 0.001), when compared with the control (Fig. 4). This also corresponds to the enhanced detachment of adherent cells (Figs. 2B and 2D). Moreover, the *P. lactiflora*-treated biofilms showed an upregulated expression of *EAP1* by 0.198-fold, when compared with the control (p = 0.086). Whereas the expression of *EAP1* has no apparent influence on the morphology of *C. albicans*, the N-terminal tandem repeat domain of Eap1 is known to support the pseudohyphal growth of *S. cerevisiae* [22]. Fungal glycoprotein adhesins aggregate into cell surface patches through amyloid-like interactions, and this adhesin clustering promotes cell-cell binding [32]. *C. albicans* Eap1 is a glycoprotein adhesin that forms insoluble amyloids, and amyloid formation is an important component of the cellular aggregation mediated by these proteins [33]. Thus, the enhanced expression of *EAP1* in the *P. lactiflora*-treated *C. albicans* biofilms may possibly have been associated with the increased formation of cell aggregates and pseudohyphal growth. The expression of *ECE1* was significantly inhibited by 0.349-fold (p = 0.001) in the *P. lactiflora*-treated *C. albicans* biofilms, suggesting that the virulence of *C. albicans* was also significantly weakened.

The switch from yeast to pseudohyphae to hyphae is regarded to be related to increased virulence gene expression and the development of different virulence properties [33]. Generally, pseudohyphae are ellipsoidal and are constricted at the septal junctions [19], whereas hyphae have parallel sides with a uniform width and possess true septa without constrictions [29]. Considering that the *P. lactiflora*-treated *C. albicans* cells often formed cell aggregates (Figs. 1B, 2B, and 2D) and transformed from budding yeast to pseudohyphae in an acidic medium (Fig. 2B), it would seem that the *P. lactiflora* treatment actually made the situation worse. Thus, although the *C. albicans* cells that encountered the *P. lactiflora* extract tried to switch their form from yeast to pseudohyphae to attach or invade surfaces or to form cell aggregates possibly to limit the surface area in the surrounding medium, the live ovoid or pseudohyphal cells became sick as their cell wall structure and membrane function were damaged as follows. First, the *C. albicans* cells in the cell aggregates were deemed dead, as some cells in the cell aggregates were stained blue with methylene blue (Fig. 1B). Second, the diameters of the hyphal tubes were narrower or the lengths of the hyphae were shorter than those of the control, meaning they were not vigorous (Fig. 3B). Finally, in a medium that induces germ tubes, the *P. lactiflora*-treated *C. albicans* cells did not transit from budding yeast forms to true hyphae, but rather to pseudohyphae, which are less virulent than hyphae (Fig. 3B). Therefore, it would seem that the *P. lactiflora* extract attenuated the virulence of *C. albicans* by obstructing the morphological change from a pseudohyphal to a hyphal filament.

In summary, the *P. lactiflora* ethanol extract showed a good inhibitory effect on biofilm formation by impeding cell adhesion to polystyrene via downregulation of the expression of Als3, Hwp1, Sap1, and Ece1, and obstructing the morphological transition from pseudohyphal to hyphal filaments. Furthermore, the extract hindered biofilm development by inhibiting cell wall synthesis and damaging the cell membrane function, which led to cell swelling and ultimately cell lysis due to osmotic pressure.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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