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Original Article

Purpurin inhibits the adhesion of *Candida albicans* biofilms

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Abstract: Aim: Purpurin (1, 2, 4-trihydroxy-9, 10-anthraquinone) is one of the trailing herb extracts from madder root, one of the anthraquinone compounds, also known as hydroxyl madder pigment. Its role in combatting *C. albican*is not reported. As biofilm was the barrier for treatment, leading to resistance to antifungal drugs, purpurin could serve as a candidate modality for the dilemma. Methods: We here conducted a series of in vitro studies to evaluate the role of purpurin in suppressing *C. albican.* The inhibition on adhesion step for ATCC90028 was studied. The tetraolium (XTT) reduction assay and crystal violet assay as well as real time RT-PCR were used to detective the expression of genes ALS1, EFG1 and HWP1. Results: Our study demonstrated that purpurin could suppress *C. albicans* activity. Purpurin inhibited the adhesion process during the formation of biofilms in a dose-independent manner. The expressions of adhesion-related genes, namely ALS1, EFG1 and HWP1 were decreased compared with control. Conclusion: Purpurin could inhibit adhesion of *C. albicans* possibly via suppression of the expressions of ALS1, EFG1 and HWP1.

Keywords: Purpurin, adhesion, *Candida albicans*, biofilms

Introduction

Candida albicans is an opportunistic pathogen. It can form biofilm adhering to the cell or abiotic surface. Biofilm is highly complex and highly drug-resistant comprising of extracellular matrix, micro-colonies of yeast, hyphae and pseudohyphae. In comparison to the planktonic fungal, the biofilm is another presentation of *C. albicans* [1]. The formation of biofilm includes three stages. First, from 0 to 11 h, *C. albicans* adhere to the surface of the carrier, beginning to proliferate and agglomerate with each other. Then, starting from 12 to 30 h, the cells gather into groups and secrete extracellular matrix, forming hyphae meanwhile. At last, ending at 31 to 72 h, cells are at the maturation stage with longer hyphae and more extracellular matrix, forming the network structure of biofilm [2]. Biofilm formation is considered to be one of the most important pathogenic factors of *C. albicans* [3]. Amid all, the most critical step within biofilm formation is the adhesion stage, as it is the first stage of the biofilm formation and is thus considered to be an important factor for virulence [4, 5].

The adhesion of the biofilm is dependent on a variety of factors. The ALS1 gene encodes the glycoprotein molecules on the surface of cell wall of *C. albicans*, playing a pivotal role in the adhesion stage [6-10]. The HWP1 gene is also related to adhesion, it can mediate the formation of hyphae and promote *C. albicans* to adhere to the host cells [11, 12]. The EFG1 gene is also necessary for the formation of hyphae, as the strains deficient of EFG1 can not form hyphae, further affecting the adhesion of *C. albicans* to the host cells [13, 14].

To date, azoles remain the mainstay of antifungal treatments, and fluconazole is a classic and frontline antifungal drug. Nonetheless, compared to the planktonic presentation, the resistance of *C. albicans* in the form of biofilms to fluconazole is improved by 1000 times [15, 16]. Formation of biofilm is closely associated with treatment failure and novel modalities are at urgent need.

Purpurin (1, 2, 4-trihydroxy-9, 10-anthraquinone) is one of the trailing herbs extracted from madder root, also known as hydroxyl madder

Table 1. Primers used in real-time PCR to detect the expression of adhension-related genes

Gene	Primer sequence	PCR product size (bp)	Tm (°C)
5.8 s RNA	5-CGGATCTCTTGGTTCTCGC-3	135	58.04
	5-CAAACAGGCATGCCCTCC-3		58.71
ALS1	5-GCGGTTCTCATGAATCAGCATCC-3	120	62.14
	5-CAGGTGATGGAGCTTCTGTAGG-3		60.16
HWP1	5-CTCCAGCTGGTTCACAACCATC -3	163	61.45
	5-GGTTCAGTGGCAGGAGCTGATG-3		63.46
EFG1	5-GCTTCGGCTCCTCCACCTCCAC-3	146	67.07
	5-CCTGCACCAGAAGCACCAGACAC-3		65.85

pigment. It can combat the *C. albicans* in a dose-dependent manner [17]. However, the effect on fungal adhesion of purpurin has not been confirmed. In this study, the impact of purpurin on adhesion process of *C. albicans* in the biofilm formation has been studied, and the expression level of adhesion-related genes, namely ALS1, EFG1 and HWP1 was studied.

Material and methods

Yeast strains and growth conditions

The standard *C. albicans* strain ATCC90028 (Provided by fungal department of Huashan Hospital) was used. The strain was maintained on Sabouraud dextrose agar supplemented with Yeast Peptone and Dextrosemedium (YPD, 1% Yeast Extract, 2% Peptone, 2% Dextrose) for 24 h at 28°C in an incubator shaker (180 rpm). The yeast cells were harvested and washed three times with phosphate-buffered saline (PBS, pH 7.4).

Biofilm formation and quantification

A standard protocol reported by Ranage *et al* was followed with minor modifications [15]. Briefly 1.0×10^6 cells/mL resuspended in RPMI-1640 (Gibco, USA) were prepared. Then, 100 µL of suspension was added into 96-well plates (Corning Inc., NY) and were incubated at 37°C. The supernatant was aspirated and plates were washed with PBS using methanol. Staining was preformed using crystal violet at the dilution of 0.01%. The formation of biofilm was measured using 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay. The yeast was processed at 2 h, 4 h, 8 h, 12 h, 16 h, 20 h, 24 h, 36 h, 48 h, 60 h and 72 h to measure the kinetics of biofilm formation. The XTT (Sigma, USA) was prepared to a final concentration of 1 µM. A 100 µL aliquot of XTT/menadione was added to each pre-washed biofilm samples. The 96-well plates were incubated in dark for 2 h at 37°C and were subject to the plate reader for the measurement of absorbance of wavelength at 490 nm.

Antifungal effect on biofilm

Prior to the test, purpurin (Sigma, USA, with a purity of 90%) was dissolved in Dimethyl suffixed (DMSO). Yeast suspensions were adjusted to 1.0×10^6 cells/mL using RPMI-1640 medium. Gradient concentrations of purpurin (at final concentrations from 320 µg/mL to 0 µg/mL diluted by 2-fold each) were added into 96-well plates. All plates were incubated at 37°C for 48 h. The viability was measured by XTT reduction assay. The morphological structure of biofilm was observed via staining using crystal violet. Fluconazole was used as the control at final concentrations from 128 µg/mL to 0 µg/mL diluted by 2-fold.

Toxicity tests

The oral fibroblasts extracted from patients were cultured in F-12K medium with 10% FBS. Cells were trypsinized and cultured at the cell density of 5000 per well. Different concentrations of purpurin (at final concentrations from 320 µg/mL to 0 µg/mL diluted by 2-fold each) were added into the plates. The plates were incubated at 37°C for 48 h and the viability was measured by XTT reduction assay.

Adherence assays

Based on previous reported, 0 µg/mL, 10 µg/mL, 20 µg/mL and 40 µg/mL of purpurin were selected to treat the *C. albicans* for 2 h for adhesion assay. Also, fluconazole at 128 µg/mL was used as control. Cells suspended at 1.0×10^6 cells/mL in RPMI-1640 medium were treated with aforementioned concentrations of purpurin. Cells were then stained using crystal violet and quantified with methods abovementioned.

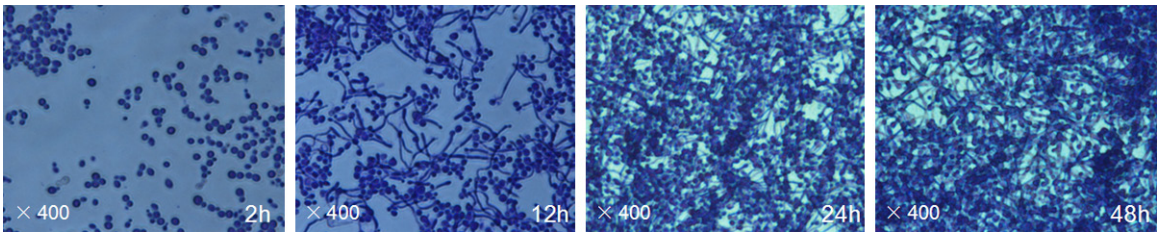

Figure 1. The biofilm morphology at 2 h, 12 h, 24 h and 48 h under an inverted phase contrast microscope with 0.01% crystal violet staining.

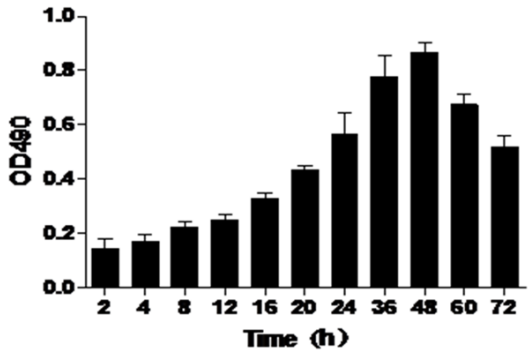

Figure 2. The growth curve of *C. albicans* biofilm from 2 h to 72 h. Data was measured with microplate reader with absorbance of wavelength at 490 nm.

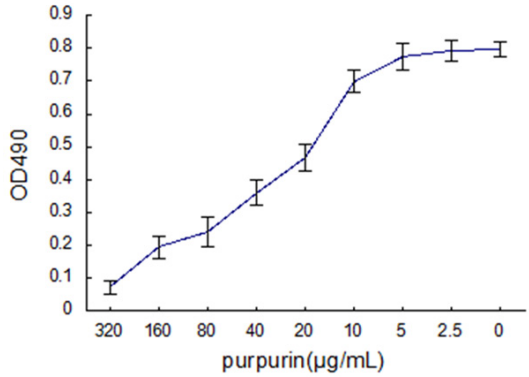

Figure 3. The effect of different concentrations of purpurin on biofilm at 48 h.

Expression of adhesion-related genes in biofilm

To investigate whether the expression of s adhesion-related genes in *C. albican* (ALS1, EFG1 and HWP1) were altered in response to20 μg/mL purpurin, the Real-time polymerase chain reaction (Real-time PCR) was used. Fuconazoleat 128 μg/mL were set as control. Total RNA was extracted from biofilms and was converted to cDNA. Primers were synthesized with sequences summarized in **Table 1** and the PCR reactions were run in triplicates.

Results

Different stages of biofilm were demonstrated in **Figure 1**, showing presentations at 2 h, 12 h, 24 h and 48 h. Single cells were predominantly seen at 2 h, with relatively few germ tube-like structure and no hyphae. After 12 h, the fungal cells began to congregate, and the hyphae began to appear. At 24 h, hyphae and extracellular matrix were predominantly observed. The interwoven reticular structure of hyphae and the typical biofilm structure were present at 48 h.

XTT reduction assays were used to measure the activity of *C. albicans* biofilm at different time points to minimize the deviations. The growth curve was measured (**Figure 2**). Along with increased time for incubation, the vitality of *C. albicans* showed a downward trend in general, despite a transient increase at 48 h.

The viability assay was used to determine whether purpurin induced cell death of *C. albicans*. As demonstrated in the XTT reduction assays, the growth of biofilm suppressed by purpurin in a dose-dependent manner, namely with the purpurin concentrations being reduced, the activity was increased (**Figure 3**). However, when the concentration of purpurin reached 5 μg/mL, the activity of *C. albicans* was almost unaffected as to normal levels (**Figures 4**, **5**). We also showed that purpurin inhibited adhesion process of the biofilm formation in a dose-independent manner (**Figure 6**).

We then studied expressions of adhesion-related genes (**Figure 7**). Expressions of ALS1, EFG1 and HWP1 were decreased compared with con-

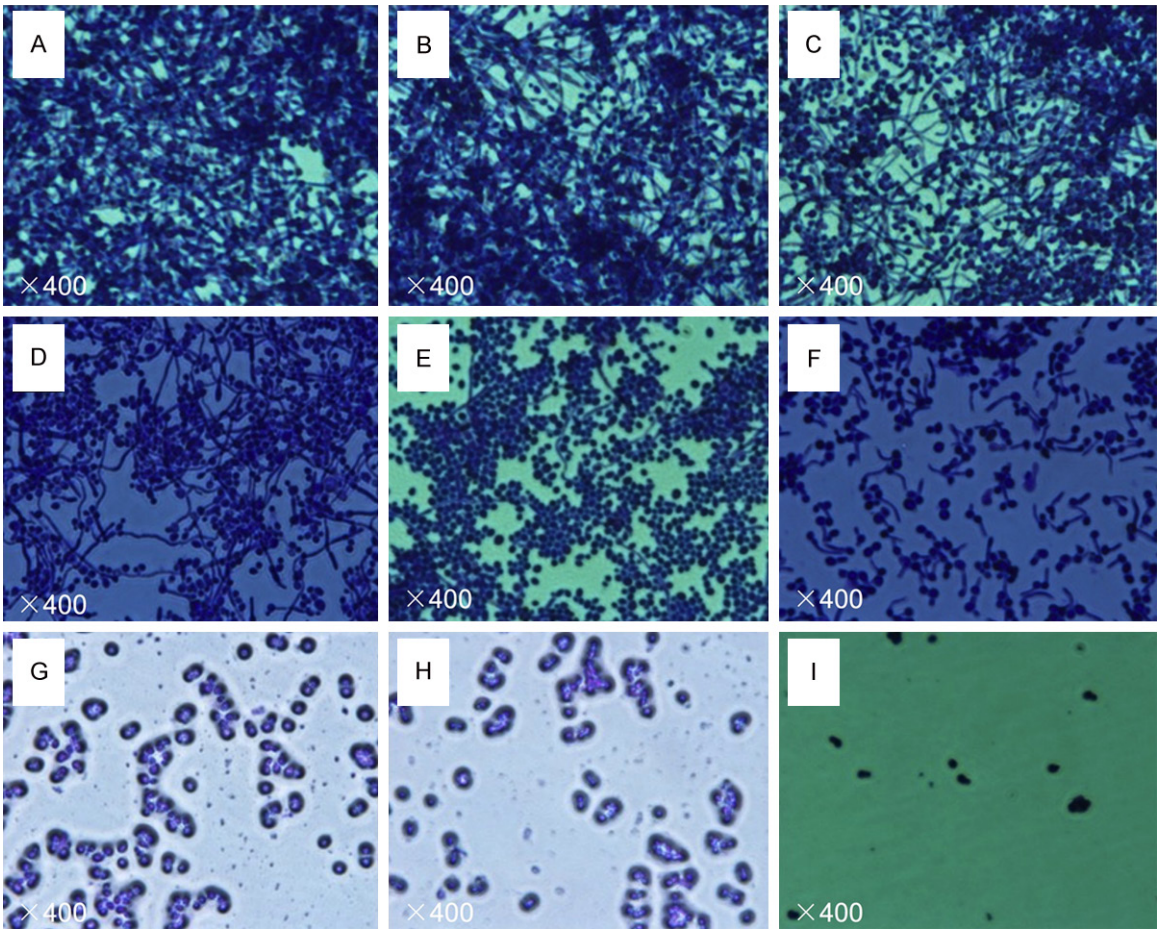

Figure 4. Light microscopy images of *C. albicans* ATCC90028 biofilms treated with different concentrations of purpurin captured at 48 h, with (A) Untreated control, the morphology was composed by compact multilayered structures including cells and hyphae. (B-I) Treated with different concentrations of purpurin (at final concentrations of from 0 μg/ml to 320 μg/ml diluted by 2-fold each), showing the higher concentration, the less and shorter the hyphae were, and the more scatter the biofilms.

trol, yet not as low as that treated with fluconazole (**Table 2**).

Discussion

The virulence of *C. albicans* includes hyphae production, adhesion, phenotypic switching and formation of some extracellular hydrolytic enzymes such as proteinase [18-20]. Colonization of *C. albicans* onto the surface of host cells is a primary step for infection, that is to say, the adhesion is the first and one of the most important stage for the biofilm formation. Inhibiting the adhesion process can prevent the fungus to combine with the host cells and interfere the biofilm formation.

Using the XTT reduction assays, we have shown that purpurin suppresses biofilm formation in a dose-dependent manner. Together with the microscopic morphologic changes of biofilms with different concentrations purpurin, we suggest that the optimal concentration is 20 μg/mL for purpurin. Therefore, 0 μg/mL, 10 μg/mL, 20 μg/mL and 40 μg/mL of purpurin were selected to explore the inhibition of adhesion step. **Figure 6** demonstrates that purpurin has an effect on adhesion in a dose-dependent manner. In order to confirm this conclusion, the gene expression analysis has been studied. **Figure 7** indicates that the expressions of ALS1, EFG1 and HWP1are lower in samples treated with 20 μg/mL purpurin compared to negative control ($P<0.05$). However, compared with fluconazole, the expression levels are not as much suppressed, indicating that there is more than one inhibitive mechanisms, where further studies are warranted.

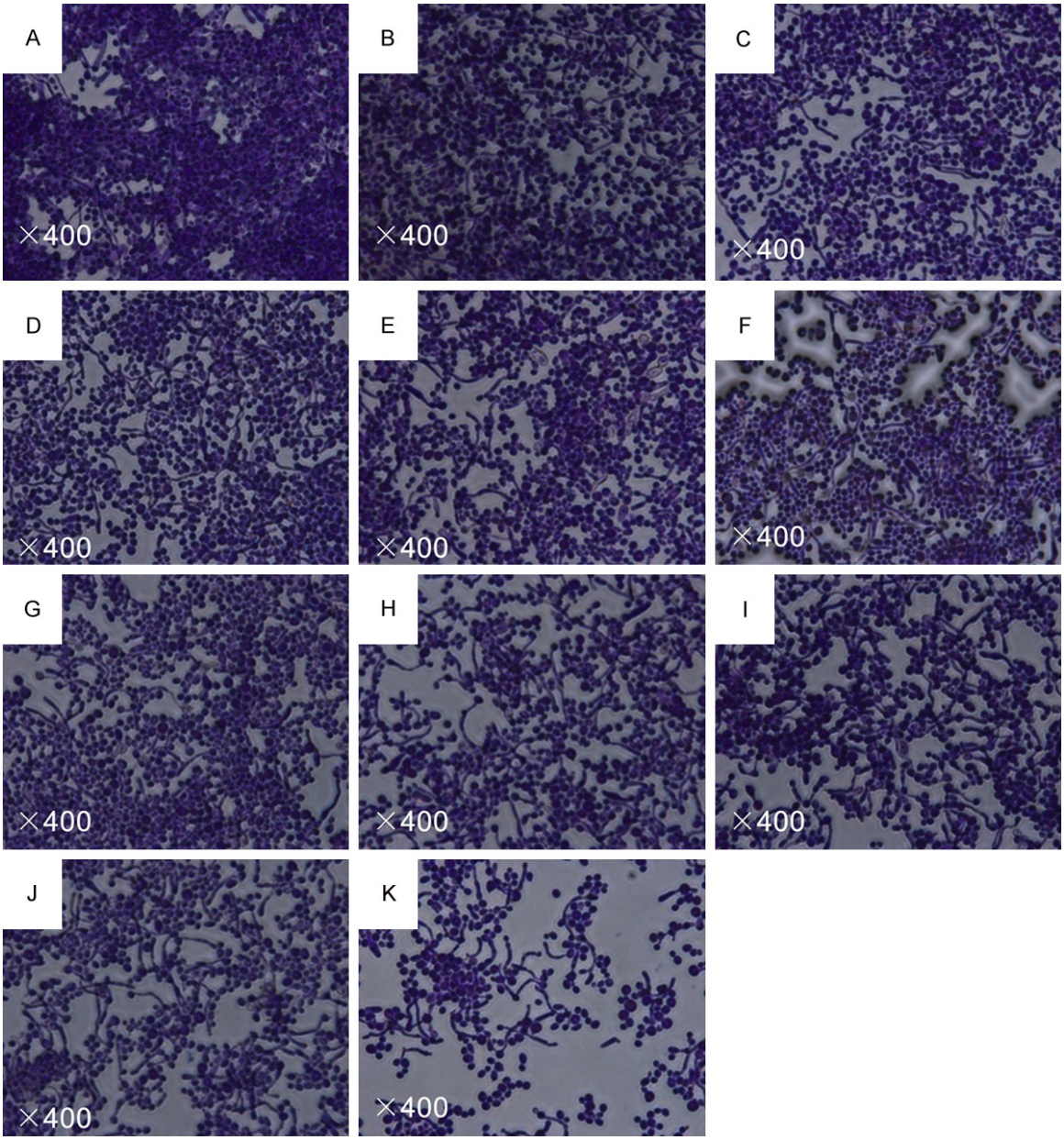

Figure 5. Light microscopic images of *C. albicans* ATCC90028 biofilms treated with different concentrations of fluconazole for 48 h. A. Untreated control, the morphology was composed by compact multilayered structures including cells and hyphae. B-K. Treated with different concentrations of fluconazole (at final concentrations of from 0 μg/ml to 128 μg/ml diluted by 2-foldeach), the higher concentration, the hyphae were less and shorter, and the biofilms were more scatter. Captured at 400×.

In this study, we have found that purpurin not only reduces the ability of strain ATCC90028 to form biofilm, but also reduces the ability to adhere to polystyrene plates in a dose-dependent manner. The inhibitory effect of purpurin on fungal adhesiveness suggests that it could be potent to suppress the pathogenicity of *C. albicans* by inhibiting the initial stages of biofilm formation. As preventing biofilm formation is a key for treating diseases caused by *C. albicans*, these findings should help to develop new antifungal treatment strategies.

In conclusion, our results have demonstrated that purpurin could have the potential of inhibiting the adhesion step of *C. albicans* and could suppress the expressions of ALS1, EFG1 and HWP1, which are potential target genes. We

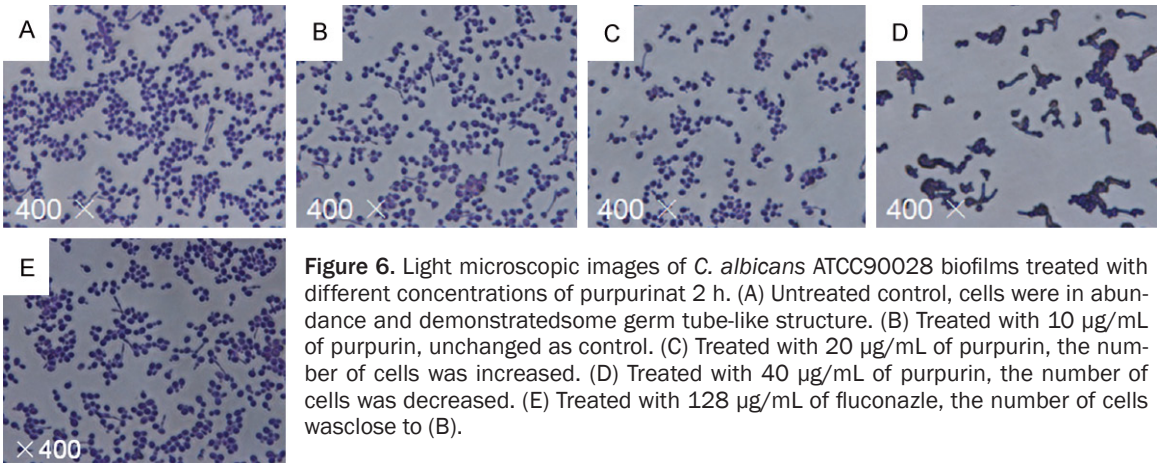

Figure 6. Light microscopic images of *C. albicans* ATCC90028 biofilms treated with different concentrations of purpurinat 2 h. (A) Untreated control, cells were in abundance and demonstratedsome germ tube-like structure. (B) Treated with 10 μg/mL of purpurin, unchanged as control. (C) Treated with 20 μg/mL of purpurin, the number of cells was increased. (D) Treated with 40 μg/mL of purpurin, the number of cells was decreased. (E) Treated with 128 μg/mL of fluconazle, the number of cells wasclose to (B).

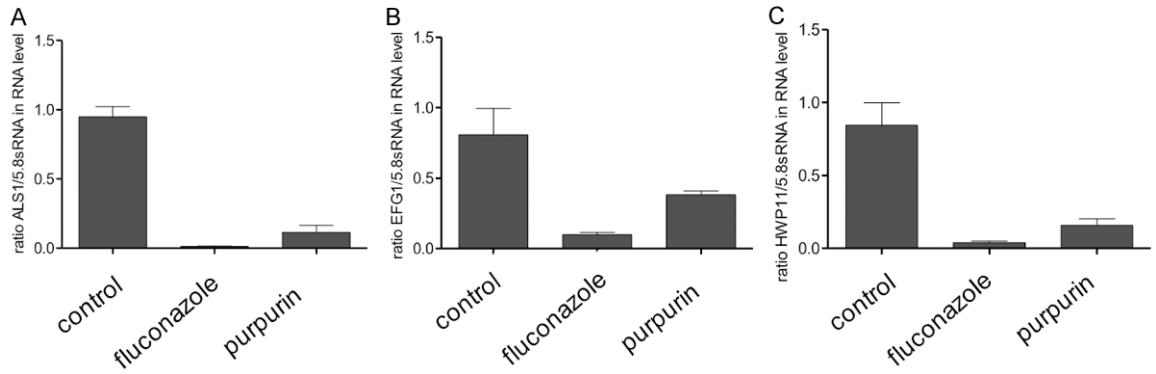

Figure 7. The analysis of adhesion-related genes by Real-time PCR. Expressions of (A) ALS1, (B) EFG1, and (C) HWP1 were downregulated compared with mock control ($P<0.05$ for all) but not as low as fluconazole ($P<0.05$ for all).

Table 2. The relative expressions of genes (Mean ± SD)

	ALS1	EFG1	HWP1
Control	0.9487897±0.0737194	0.8069176±0.1892303	0.8439643±0.1557576
Fluconazole	0.0135431±0.0014718	0.0979687±0.0166591	0.0403632±0.0098604
Purpurin	0.1147313±0.0494203	0.3813132±0.0273706	0.1577437±0.044496

thus speculate that purpurin can be developed into a supplementary therapeutic against biofilm formation of *C. albicans*.

Acknowledgements

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Disclosure of conflict of interest

None.

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