

# Inhibitory Effect of Fluconazole Combined with Amphotericin B on Fluconazole-Resistant *Candida albicans* Biofilm Formation

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## Article Info

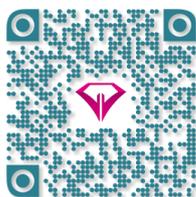
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## ABSTRACT

**Background & Objective:** The incidence of biofilm-related infections caused by *Candida albicans* has increased dramatically. *C. albicans* biofilm-related infections are more resistant to antifungal medications. This work was an attempt to examine inhibitory effects of fluconazole in combination with amphotericin B on fluconazole-resistant *C. albicans* biofilm.

**Materials & Methods:** Fluconazole-resistant *C. albicans* was identified among colonized clinical isolates of *C. albicans* obtained from the patients at Shahid Beheshti Hospital, Yasooj, Iran, between September 2016 and January 2017. The MICs of fluconazole alone and in combination with amphotericin B were determined on fluconazole-resistant *C. albicans* by broth microdilution method based on the CLSI document M27-A3 and the synergistic effects were interpreted by the FIC index. To address the inhibitory effects of drug combinations, we investigated the anti-biofilm activities of fluconazole alone and in combination with amphotericin B against fluconazole-resistant *C. albicans* using crystal violet staining, light field microscopy and expression analysis of *HWPI* using RT-PCR.

**Results:** Ten percent of the colonizing clinical isolates of *C. albicans* were found to be fluconazole-resistant. The fluconazole combined with amphotericin B exhibited synergistic and partial synergistic effects against fluconazole-resistant *C. albicans* isolates with FIC index ranging from 0.50 to 0.75. Our findings demonstrated that fluconazole combined with amphotericin B exerted a significant reduction and inhibition on biomass, biofilm formation and the expression levels of *HWPI* ( $P < 0.05$ ).

**Conclusion:** Fluconazole combined with amphotericin B showed potent inhibitory activity against fluconazole-resistant *C. albicans* biofilm formation. Other measures are important to determine the effectiveness of the drug combinations against *C. albicans* biofilm.

**Keywords:** Amphotericin B, Antifungal agents, Biofilm, *Candida albicans*, Fluconazole



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## Introduction

*Candida* biofilms have been associated with the severe resistance to antifungal agents compared to their planktonic cells (1,2). *Candida albicans* is the most common fungal pathogen associated with the biofilm-related infections. Biofilm formation of *Candida* has gained considerable attention due to its contributions to the unacceptably high rates of mortality in the immunocompromised hosts (3,4). The biofilms communities are organized, structured, coordinated and functional communities of cells, in which a matrix of exopolymeric substances is embedded (2,4,5).

A variety of transcriptional regulations determine the process of biofilm formation in *C. albicans*. Nine master regulatory genes; *BCR1*, *BRG1*, *EFG1*, *FLO8*, *GALA*, *NDT80*, *ROB1*, *RFX2* and *TEC1* are required to control both themselves and approximately 1000 target genes

involved in the biofilm formation in *C. albicans* such as hyphal morphogenesis (e.g., *HWPI*), extracellular matrix production (e.g., *GSCI* and *MNN1*), and drug resistance (e.g., *CDR1* and *MDR1*) (6-9). Hyphal Wall Protein 1 (encoded by *HWPI*) is a well-characterized *C. albicans* hypha-associated cell surface protein that mediates the adhesion to the epithelial cells, as well as biofilm formation (10,11).

A few classes of antifungal drugs such as polyenes, azoles, allylamines, echinocandins and miazines are in common use for the treatment of *Candida* species infections. Polyenes (e.g., amphotericin B) are fungicidal, intercalating into membranes containing ergosterol and form membrane pores that destroy the cell contents. The most widely used group is azoles (e.g., fluconazole) which possess fungistatic effect by inhibition of the lanosterol

14 $\alpha$ -demethylase which leads to an accumulation of the toxic sterol pathway intermediates (2,12). Due to the limited number of antifungal agents and increased resistance of biofilm cells, therapeutic options available in the treatment of *Candida* infections are exceedingly insufficient. The development of new class of antifungal agents is not easy; therefore, this problem might be alleviated using drug combinations. Moreover, reports showed that drug combination is highly effective for the eradication of *Candida* biofilm (13,14).

Given the synergistic efficacy of fluconazole along with amphotericin B against *C. albicans* (15), we hypothesized that fluconazole combined with amphotericin B could augment the efficacy on the biofilm formation inhibition on fluconazole-resistant *C. albicans*. In this study, we evaluated the *in vitro* efficacy of fluconazole alone and in combination with amphotericin B against fluconazole-resistant *C. albicans* and observed their antimicrobial effects on the biofilm formation. In addition, crystal violet (CV) staining, microscopic analysis of biofilm formation and the expression analysis of hypha specific gene (*HWPI*) were investigated on fluconazole-resistant *C. albicans*.

## Materials and Methods

### Organism

The reference strains of *C. albicans* ATCC 10231 strain 3147 [*C. albicans* Drug Resistance Panel (ATCC® MP-8™)] and *C. albicans* CBS 5982 were used in this study. Fluconazole susceptibility was studied on 100 colonized clinical isolates of *C. albicans* obtained from 100 pregnant women, diabetics, cancer and maintenance hemodialysis patients at Shahid Beheshti Hospital, Yasooj, Iran, between September 2016 and January 2017. Fluconazole susceptibility of clinical isolates of *C. albicans* was determined using CSLI M27-A3 document with slight modification (16).

### Combination Testing of Fluconazole with Amphotericin B on Fluconazole-Resistant *Candida albicans*

The effects of fluconazole alone and in combination with amphotericin B were tested against fluconazole-resistant *C. albicans* by broth microdilution method using CLSI document (M27-A3) with slight modification. Briefly, the inocula from 24 h *C. albicans* cultures on Sabouraud Dextrose Agar (SDA, Merck Research Laboratories, Germany) were prepared to a turbidity equivalent to a 0.5 McFarland standard with a spectrophotometer at 530 nm. The *C. albicans* suspensions were further diluted to yield an inoculum concentration of approximately  $5 \times 10^2 - 2.5 \times 10^3$  cells/mL in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich) with 0.2% glucose [buffered to pH 7.0 with 0.165 M morpholinophosphonyl sulfate (MOPS)]. The minimal inhibitory concentration (MIC) assay was performed with 96-well plates. *C. albicans* inoculum (100  $\mu$ L) was exposed to 100  $\mu$ L of the two-fold dilutions of the fluconazole (ranging 0.0313-64  $\mu$ g/mL) and amphotericin B (ranging 0.0313-16  $\mu$ g/mL) alone and in combination. The fluconazole and amphotericin B in combination were mixed in 1:1 ratio. The microplates were

kept at 4°C for 2 h and incubated at 35°C for 24 h to evaluate the MICs. The end points of  $\geq 50\%$  and  $\geq 90\%$  reduction in turbidity relative to the positive control were determined using a Stat Fax 303 Reader (Awareness Technology, Inc., USA) at 530 nm.

To evaluate the interaction between fluconazole and amphotericin B, the FIC index was calculated from the data obtained with relative MICs determination with the antifungal alone and in combination as described earlier (16,17).

### Combination Testing of Fluconazole with Amphotericin B on Fluconazole-Resistant *Candida albicans* Biofilm Formation

The biofilm formation assay was performed exactly as described previously (18-21). Combination testing of fluconazole with amphotericin B on fluconazole-resistant *C. albicans* biofilm formation was determined by crystal violet (cv) staining, microscopic analysis of biofilm formation and the expression analysis of *C. albicans* hypha specific gene.

The yeast cell suspension ( $1 \times 10^6$  cells/mL) was dispensed into the wells of 96-well plates (100  $\mu$ L/well). The wells were filled with 100  $\mu$ L of four different concentrations based on the MIC values (2 $\times$ MIC, 1 $\times$ MIC, 1/2 $\times$ MIC and 1/4 $\times$ MIC) of fluconazole alone or in combination with amphotericin B. After incubation at 35°C for 90 min, the plates were incubated again at 35°C for 24 h with gentle shaking. After fixation of biofilm with 99% methanol, they were stained by aqueous crystal violet for 20 min and de-stained using 33% acetic acid. The absorbance values were obtained at 590 nm using Stat Fax 303 Reader (18, 19).

The effect of fluconazole alone or in combination with amphotericin B on fluconazole-resistant *C. albicans* (ATCC 10231) biofilm formation was qualitatively analyzed by the light field microscope. The biofilms were formed on glass cover slips in 6-well cell culture plates by growing the cells ( $1 \times 10^6$  cells/mL) in RPMI-1640 treated with four different concentrations based on the MIC values (2 $\times$ MIC, 1 $\times$ MIC, 1/2 $\times$ MIC and 1/4 $\times$ MIC) of fluconazole alone or in combination with amphotericin B. The microplates were incubated at 35°C for 90 min, and then maintained for 24 h with gentle shaking at 35°C. Thereafter, the cover slips were washed with PBS and visualized with the Leica light field microscope using 40X objective lens (19,20).

Subsequently, the effect of fluconazole alone or in combination with amphotericin B was evaluated on the expression of hypha specific gene (*HWPI*) on fluconazole-resistant *C. albicans* (ATCC 10231) biofilm formation by two-step reverse transcription polymerase chain reaction (RT-PCR). The biofilms were formed on 96-well plates by growing the cells ( $1 \times 10^6$  cells/mL) in RPMI-1640 treated with four different concentrations based on the MIC values (2 $\times$ MIC, 1 $\times$ MIC, 1/2 $\times$ MIC and 1/4 $\times$ MIC) of fluconazole alone or in combination with amphotericin B and total RNA of the cells was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The cDNA was synthesized

from DNase I-treated total RNA using Moloney-Murine Leukemia Virus reverse transcriptase and random hexamers (Fermentas, USA). The RT-PCR reaction was conducted using the primers for the target *HWPI* and housekeeping internal control *Beta actin* genes (Table 1). The RT-PCR was performed in Personal thermocycler (Biometra- Germany) with an initial denaturation at 95°C (4 min), followed by 26 cycles of denaturation (94°C/40 sec), annealing (56°C/45 sec) and extension (72°C/45 sec), and final extension at 72°C for 10 min. The specificity of the primers was confirmed by the sequencing analysis (First BASE Laboratories Sdn. Bhd., Malaysia). The amplified products were analyzed by Quantity One 1-D Analysis (Bio-Rad, USA, version 4.6). The relative

quantification of the target transcripts were determined by the volume-based analysis comparing to DNA mass standard (Fermentas, USA) (19-21)

### Statistical Analysis

All the experiments were performed in triplicate. Data were analyzed using the statistical software SPSS 23.0 (SPSS Inc., Chicago, IL., USA). All data were expressed as mean values with the corresponding standard deviations (SD). The comparisons were performed using one-way ANOVA, with Tukey's HSD post hoc test. A P-value less than 0.05 was considered statistically significant.

**Table 1. Oligonucleotide primers used for RT-PCR**

Primer	Orientation	Sequence	Reference
<i>HWPI</i>	Forward	5' GGTAGACGGTCAAGGTGAAACA 3'	(21)
	Reverse	5' AGGTGGATTGTCGCAAGGTT 3'	
<i>Beta actin</i>	Forward	5' ACCGAAGCTCCAATGAATCCAAAATCC 3'	(20)
	Reverse	5' GTTTGGTCAATACCAGCAGCTTCCAAA 3'	

## Results

Hundred colonized *C. albicans* isolated from the clinical samples were identified and used for the fluconazole susceptibility testing. Ten fluconazole-resistant *C. albicans* were identified and used in the study. For fluconazole-resistant *C. albicans*, the relative MIC of fluconazole was  $\geq 16 \mu\text{g/mL}$  when used alone. The 90% of fluconazole-resistant *C. albicans* isolates were resistant to amphotericin B with MIC  $\geq 2.0 \mu\text{g/mL}$ . The susceptibility assay showed that the combination of fluconazole and amphotericin B was synergistic (20%), partial synergistic (20%), additive (30%) and indifferent (30%) as antifungal effects against fluconazole-resistant *C. albicans* (Table 2). Combination of fluconazole and amphotericin B exhibited synergistic and partial synergistic effects against fluconazole-resistant *C. albicans* isolates with FIC index ranged from 0.50 to 0.75

Table 3 shows the corresponding results for the reduction of biomass determined by the CV staining of the inhibitory effect of fluconazole combined with amphotericin B on fluconazole-resistant *C. albicans* biofilm at different concentrations based on the MIC values. There was no significant effect of fluconazole alone on the reduction of biomass on fluconazole-resistant *C. albicans* biofilm. Furthermore, no significant effect was observed of amphotericin B

alone on the reduction of biomass in amphotericin B-resistant isolates. The effect of fluconazole combined with amphotericin B significantly decreased the biomass on fluconazole-resistant *C. albicans* biofilm ( $P < 0.05$ ).

The combination testing of fluconazole with amphotericin B on fluconazole-resistant *C. albicans* (ATCC 10231) biofilm formation was determined by the microscopic analysis of biofilm. Figure 1 shows the corresponding results for the reduction of biofilm, which resulted in the reduction of biofilm formation, hyphae and planktonic cells on fluconazole-resistant *C. albicans* treated with fluconazole combined with amphotericin B. As shown in the Figure 1, the fluconazole alone could not decrease the biofilm formation, hyphae and planktonic cells of fluconazole-resistant *C. albicans* compared to the untreated control ( $P > 0.05$ ).

Our results indicated that the combination of fluconazole with amphotericin B on fluconazole-resistant *C. albicans* (ATCC 10231) biofilm formation exerted their antifungal effect through inhibition of the expression levels of *HWPI* ( $P \leq 0.05$ ). No significant difference was observed in the mean of expression levels of *HWPI* on fluconazole-resistant *C. albicans* grown in the presence of fluconazole and amphotericin B alone ( $P > 0.05$ ) (Figure 2 and Figure 3).

**Table 2. Relative MIC ( $\mu\text{g/mL}$ ) and FIC values of fluconazole alone and in combination with amphotericin B against fluconazole-resistant *C. albicans***

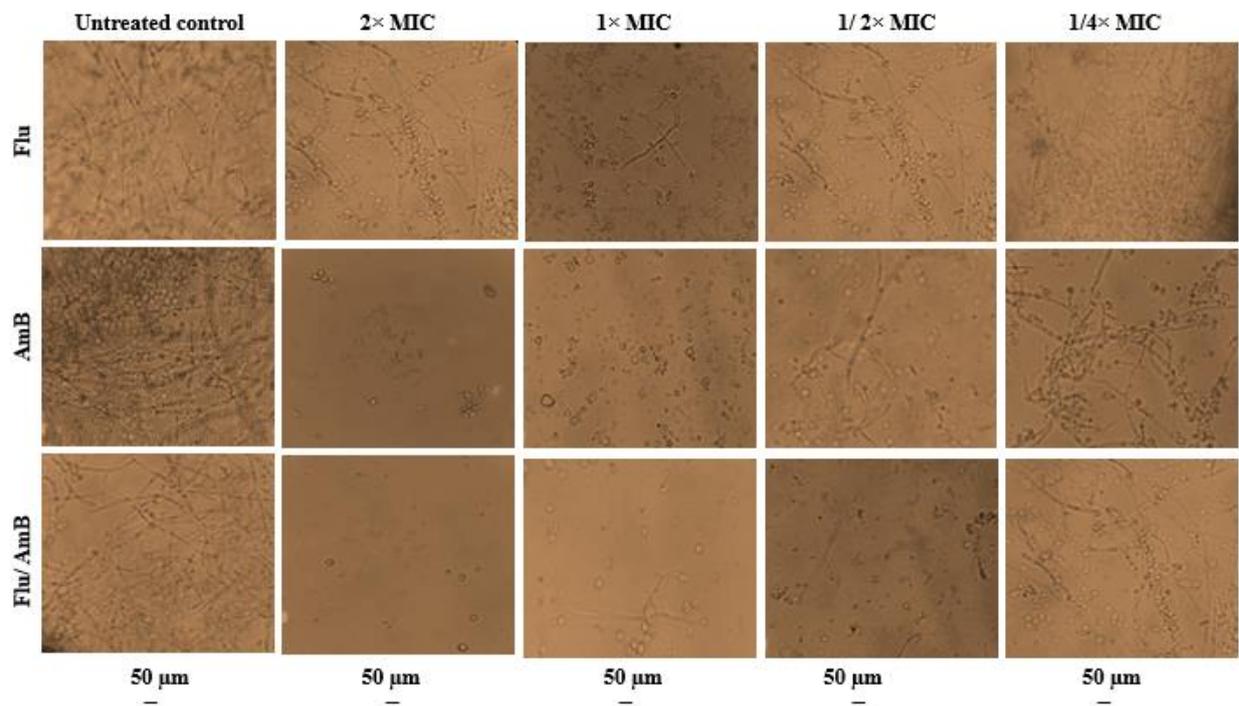
Isolates /Antifungals	Flu		AmB		Flu/ AmB			Interpretive
	MIC <sub>90</sub>	MIC <sub>50</sub>	MIC <sub>90</sub>	MIC <sub>50</sub>	MIC <sub>90</sub>	MIC <sub>50</sub>	FIC	
<i>C. albicans</i> ATCC 10231	16	1	1	0.0156	4/0.5	0.25/0.0313	0.75	PS
<i>C. albicans</i> CBS 5982	0.125	0.0321	0.0313	0.0078	0.0625/0.0078	0.0312/0.0039	0.74	PS
CI-1	64	4	4	1	16/2	2/0.5	0.75	PS
CI-2	16	8	4	0.5	16/2	4/0.5	1.5	I
CI-3	32	16	4	2	8/1	4/0.5	0.5	S
CI-4	32	8	2	0.5	8/1	2/0.25	0.75	PS
CI-5	32	8	4	0.5	16/2	0.25/0.0625	1	A
CI-6	64	4	1	0.5	16/2	0.5/0.25	2.25	I
CI-7	16	16	2	1	8/1	4/0.5	1	A
CI-8	32	8	4	2	8/1	4/0.5	0.5	S
CI-9	16	8	4	1	16/2	0.25/0.0313	1.5	I
CI-10	16	8	2	0.5	8/1	2/0.25	1	A

CI: Clinical isolates of *C. albicans*, Flu: fluconazole, AmB: amphotericin B PS: Partial synergy; S: Synergy; A: Additive; I: Indifference Data are means of three independent experiments

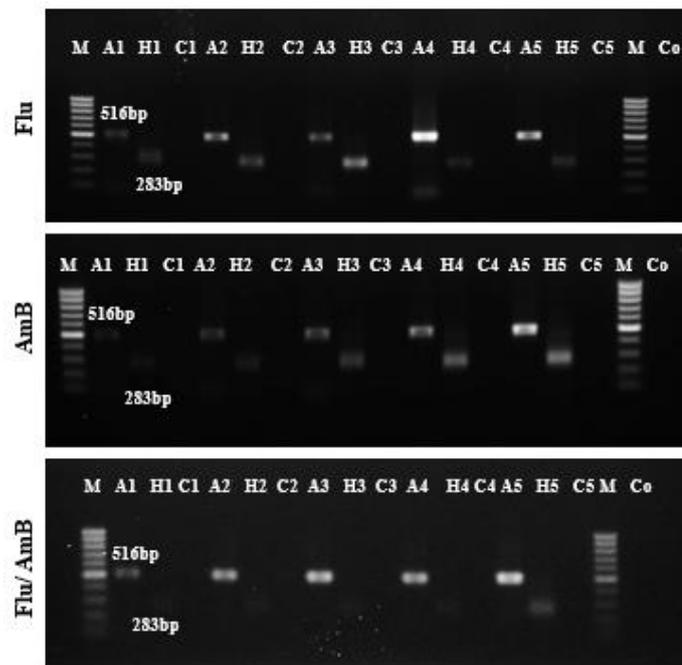
**Table 3. Biofilm formation assay using CV staining ( $\mu\text{g/mL}$ ) on fluconazole-resistant *C. albicans***

Concentration of antifungal agents/ Isolates	<i>C. albicans</i> ATCC 10231	<i>C. albicans</i> CBS 5982	CI-1	CI-2	CI-3	CI-4	CI-5	CI-6	CI-7	CI-8	CI-9	CI-10
Flu												
2 $\times$ MIC	0.32 $\pm$ 0.15 <sup>a</sup>	0.08 $\pm$ 0.15 <sup>a</sup>	0.37 $\pm$ 0.16 <sup>a</sup>	0.30 $\pm$ 0.15 <sup>a</sup>	0.35 $\pm$ 0.15 <sup>a</sup>	0.36 $\pm$ 0.18 <sup>a</sup>	0.33 $\pm$ 0.17 <sup>a</sup>	0.35 $\pm$ 0.15 <sup>a</sup>	0.38 $\pm$ 0.19 <sup>a</sup>	0.38 $\pm$ 0.18 <sup>a</sup>	0.34 $\pm$ 0.16 <sup>a</sup>	0.37 $\pm$ 0.19 <sup>a</sup>
1 $\times$ MIC	0.33 $\pm$ 0.17 <sup>b</sup>	0.14 $\pm$ 0.17 <sup>b</sup>	0.38 $\pm$ 0.17 <sup>b</sup>	0.32 $\pm$ 0.17 <sup>b</sup>	0.35 $\pm$ 0.15 <sup>a</sup>	0.34 $\pm$ 0.15 <sup>a</sup>	0.34 $\pm$ 0.17 <sup>b</sup>	0.36 $\pm$ 0.17 <sup>b</sup>	0.36 $\pm$ 0.17 <sup>b</sup>	0.33 $\pm$ 0.15 <sup>b</sup>	0.35 $\pm$ 0.15 <sup>b</sup>	0.37 $\pm$ 0.16 <sup>b</sup>
1/2 $\times$ MIC	0.38 $\pm$ 0.16 <sup>c</sup>	0.19 $\pm$ 0.16 <sup>c</sup>	0.38 $\pm$ 0.16 <sup>b</sup>	0.31 $\pm$ 0.17 <sup>b</sup>	0.36 $\pm$ 0.16 <sup>b</sup>	0.33 $\pm$ 0.17 <sup>c</sup>	0.34 $\pm$ 0.15 <sup>b</sup>	0.36 $\pm$ 0.19 <sup>b</sup>	0.35 $\pm$ 0.16 <sup>c</sup>	0.40 $\pm$ 0.18 <sup>c</sup>	0.35 $\pm$ 0.16 <sup>c</sup>	0.39 $\pm$ 0.16 <sup>c</sup>
1/4 $\times$ MIC	0.38 $\pm$ 0.16 <sup>c</sup>	0.20 $\pm$ 0.16 <sup>d</sup>	0.39 $\pm$ 0.16 <sup>c</sup>	0.32 $\pm$ 0.17 <sup>c</sup>	0.39 $\pm$ 0.16 <sup>c</sup>	0.37 $\pm$ 0.18 <sup>d</sup>	0.38 $\pm$ 0.16 <sup>c</sup>	0.37 $\pm$ 0.16 <sup>c</sup>	0.38 $\pm$ 0.17 <sup>d</sup>	0.41 $\pm$ 0.17 <sup>d</sup>	0.35 $\pm$ 0.16 <sup>d</sup>	0.41 $\pm$ 0.16 <sup>d</sup>
Untreated control	0.49 $\pm$ 0.16 <sup>d</sup>	0.49 $\pm$ 0.16 <sup>c</sup>	0.52 $\pm$ 0.19 <sup>d</sup>	0.48 $\pm$ 0.17 <sup>d</sup>	0.48 $\pm$ 0.15 <sup>d</sup>	0.52 $\pm$ 0.17 <sup>c</sup>	0.47 $\pm$ 0.19 <sup>d</sup>	0.49 $\pm$ 0.18 <sup>d</sup>	0.50 $\pm$ 0.17 <sup>c</sup>	0.52 $\pm$ 0.18 <sup>c</sup>	0.48 $\pm$ 0.15 <sup>c</sup>	0.51 $\pm$ 0.15 <sup>c</sup>
AmB												
2 $\times$ MIC	0.05 $\pm$ 0.16 <sup>a</sup>	0.06 $\pm$ 0.15 <sup>a</sup>	0.37 $\pm$ 0.15 <sup>a</sup>	0.36 $\pm$ 0.16 <sup>a</sup>	0.37 $\pm$ 0.17 <sup>a</sup>	0.37 $\pm$ 0.15 <sup>a</sup>	0.37 $\pm$ 0.18 <sup>a</sup>	0.37 $\pm$ 0.16 <sup>a</sup>	0.07 $\pm$ 0.17 <sup>a</sup>	0.36 $\pm$ 0.15 <sup>a</sup>	0.37 $\pm$ 0.15 <sup>a</sup>	0.36 $\pm$ 0.15 <sup>a</sup>
1 $\times$ MIC	0.14 $\pm$ 0.18 <sup>b</sup>	0.14 $\pm$ 0.18 <sup>b</sup>	0.35 $\pm$ 0.17 <sup>b</sup>	0.42 $\pm$ 0.17 <sup>b</sup>	0.38 $\pm$ 0.17 <sup>b</sup>	0.36 $\pm$ 0.17 <sup>b</sup>	0.35 $\pm$ 0.17 <sup>b</sup>	0.37 $\pm$ 0.16 <sup>a</sup>	0.12 $\pm$ 0.19 <sup>b</sup>	0.32 $\pm$ 0.15 <sup>b</sup>	0.36 $\pm$ 0.15 <sup>b</sup>	0.37 $\pm$ 0.15 <sup>b</sup>
1/2 $\times$ MIC	0.18 $\pm$ 0.16 <sup>c</sup>	0.19 $\pm$ 0.17 <sup>c</sup>	0.35 $\pm$ 0.16 <sup>b</sup>	0.42 $\pm$ 0.16 <sup>b</sup>	0.38 $\pm$ 0.17 <sup>b</sup>	0.36 $\pm$ 0.19 <sup>b</sup>	0.35 $\pm$ 0.14 <sup>b</sup>	0.37 $\pm$ 0.16 <sup>a</sup>	0.19 $\pm$ 0.15 <sup>c</sup>	0.33 $\pm$ 0.16 <sup>c</sup>	0.36 $\pm$ 0.16 <sup>b</sup>	0.37 $\pm$ 0.16 <sup>b</sup>
1/4 $\times$ MIC	0.20 $\pm$ 0.17 <sup>d</sup>	0.20 $\pm$ 0.18 <sup>d</sup>	0.41 $\pm$ 0.16 <sup>c</sup>	0.42 $\pm$ 0.17 <sup>b</sup>	0.39 $\pm$ 0.16 <sup>c</sup>	0.39 $\pm$ 0.18 <sup>c</sup>	0.40 $\pm$ 0.18 <sup>c</sup>	0.39 $\pm$ 0.16 <sup>b</sup>	0.20 $\pm$ 0.15 <sup>d</sup>	0.33 $\pm$ 0.17 <sup>c</sup>	0.36 $\pm$ 0.19 <sup>b</sup>	0.40 $\pm$ 0.17 <sup>c</sup>
Untreated control	0.48 $\pm$ 0.19 <sup>e</sup>	0.49 $\pm$ 0.18 <sup>c</sup>	0.48 $\pm$ 0.19 <sup>d</sup>	0.50 $\pm$ 0.18 <sup>c</sup>	0.49 $\pm$ 0.19 <sup>d</sup>	0.50 $\pm$ 0.23 <sup>d</sup>	0.50 $\pm$ 0.15 <sup>d</sup>	0.49 $\pm$ 0.16 <sup>c</sup>	0.49 $\pm$ 0.21 <sup>e</sup>	0.47 $\pm$ 0.19 <sup>d</sup>	0.49 $\pm$ 0.19 <sup>c</sup>	0.50 $\pm$ 0.19 <sup>d</sup>
Flu/ AmB												
2 $\times$ MIC	0.04 $\pm$ 0.18 <sup>a</sup>	0.04 $\pm$ 0.15 <sup>a</sup>	0.05 $\pm$ 0.18 <sup>a</sup>	0.04 $\pm$ 0.15 <sup>a</sup>	0.05 $\pm$ 0.15 <sup>a</sup>	0.04 $\pm$ 0.16 <sup>a</sup>	0.04 $\pm$ 0.18 <sup>a</sup>	0.03 $\pm$ 0.17 <sup>a</sup>	0.04 $\pm$ 0.16 <sup>a</sup>	0.04 $\pm$ 0.18 <sup>a</sup>	0.04 $\pm$ 0.15 <sup>a</sup>	0.05 $\pm$ 0.17 <sup>a</sup>
1 $\times$ MIC	0.11 $\pm$ 0.16 <sup>b</sup>	0.10 $\pm$ 0.16 <sup>b</sup>	0.11 $\pm$ 0.19 <sup>b</sup>	0.11 $\pm$ 0.15 <sup>b</sup>	0.10 $\pm$ 0.15 <sup>b</sup>	0.10 $\pm$ 0.17 <sup>b</sup>	0.10 $\pm$ 0.17 <sup>b</sup>	0.11 $\pm$ 0.17 <sup>b</sup>	0.11 $\pm$ 0.17 <sup>b</sup>	0.10 $\pm$ 0.18 <sup>b</sup>	0.12 $\pm$ 0.15 <sup>b</sup>	0.10 $\pm$ 0.17 <sup>b</sup>
1/2 $\times$ MIC	0.15 $\pm$ 0.16 <sup>c</sup>	0.13 $\pm$ 0.16 <sup>c</sup>	0.16 $\pm$ 0.16 <sup>c</sup>	0.18 $\pm$ 0.16 <sup>c</sup>	0.17 $\pm$ 0.15 <sup>c</sup>	0.17 $\pm$ 0.17 <sup>c</sup>	0.16 $\pm$ 0.15 <sup>c</sup>	0.18 $\pm$ 0.17 <sup>c</sup>	0.17 $\pm$ 0.17 <sup>c</sup>	0.19 $\pm$ 0.15 <sup>c</sup>	0.17 $\pm$ 0.15 <sup>c</sup>	0.17 $\pm$ 0.15 <sup>c</sup>
1/4 $\times$ MIC	0.16 $\pm$ 0.15 <sup>d</sup>	0.17 $\pm$ 0.17 <sup>d</sup>	0.18 $\pm$ 0.19 <sup>d</sup>	0.19 $\pm$ 0.15 <sup>d</sup>	0.19 $\pm$ 0.15 <sup>d</sup>	0.18 $\pm$ 0.15 <sup>d</sup>	0.18 $\pm$ 0.15 <sup>d</sup>	0.19 $\pm$ 0.16 <sup>d</sup>	0.18 $\pm$ 0.17 <sup>d</sup>	0.21 $\pm$ 0.19 <sup>d</sup>	0.20 $\pm$ 0.15 <sup>d</sup>	0.19 $\pm$ 0.15 <sup>d</sup>
Untreated control	0.48 $\pm$ 0.19 <sup>e</sup>	0.47 $\pm$ 0.15 <sup>c</sup>	0.48 $\pm$ 0.18 <sup>c</sup>	0.48 $\pm$ 0.15 <sup>c</sup>	0.46 $\pm$ 0.22 <sup>c</sup>	0.49 $\pm$ 0.15 <sup>c</sup>	0.49 $\pm$ 0.15 <sup>c</sup>	0.50 $\pm$ 0.17 <sup>c</sup>	0.50 $\pm$ 0.17 <sup>c</sup>	0.49 $\pm$ 0.15 <sup>c</sup>	0.48 $\pm$ 0.19 <sup>c</sup>	0.48 $\pm$ 0.15 <sup>c</sup>

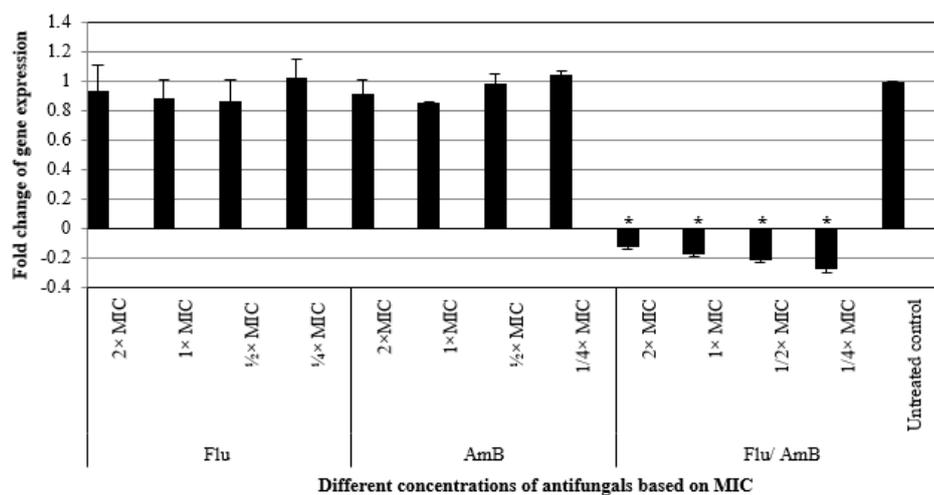
CI: Clinical isolates of *C. albicans*, Flu: fluconazole, AmB: amphotericin B <sup>a-e</sup> Means  $\pm$  SD in a column with different superscript differ significantly ( $P < 0.05$ ) using Tukey's HSD test. The results were performed in three independent experiments



**Figure 1.** Light field microscopy of the inhibitory effect of fluconazole combined with amphotericin B on fluconazole-resistant *C. albicans* (ATCC 10231) biofilm formation at different concentrations. Magnification  $\times 40$ , Bar = 50  $\mu\text{m}$ . Flu: fluconazole, AmB: amphotericin B.



**Figure 2.** *HWP1* expression in fluconazole-resistant *C. albicans* (ATCC 10231) biofilm treated with fluconazole combined with amphotericin B. M: 100 bp DNA Ladder, A1: *Beta actin* (2  $\times$  MIC), H1: *HWP1* (2  $\times$  MIC), C1: Internal control, A2: *Beta actin* (1  $\times$  MIC), H2: *HWP1* (1  $\times$  MIC), C2: Internal control, A3: *Beta actin* ( $\frac{1}{2}$   $\times$  MIC), H3: *HWP1* ( $\frac{1}{2}$   $\times$  MIC), C3: Internal control, A4: *Beta actin* ( $\frac{1}{4}$   $\times$  MIC), H4: *HWP1* ( $\frac{1}{4}$   $\times$  MIC), C4: Internal control, A5: *Beta actin* (untreated control), H5: *HWP1* (untreated control).



**Figure 3.** Relative gene expression levels of *HWP1* in combination of fluconazole with amphotericin B on fluconazole-resistant *C. albicans* (ATCC 10231) biofilm formation at different concentrations. Flu: fluconazole, AmB: amphotericin B.\* Significant downregulation. Data are means of fold changes  $\pm$  SD from three independent experiments amplified in triplicates.

## Discussion

In this study, we evaluated the inhibitory effect of fluconazole combined with amphotericin B against *C. albicans*. Our data supported the influences of fluconazole combined with amphotericin B against *C. albicans*. Although theoretically the use of fluconazole combined with amphotericin B may lead to the antagonism based on the specific modes of action of the azoles and polyenes, different reports have shown the inhibitory effect of fluconazole combined with amphotericin B against *C. albicans* (13,15,22,23).

The frequency of biofilm-related infections caused by *C. albicans* has been increased disproportionately in the last few decades and become incurable because of the increasing number of immunocompromised hosts (3,4). The current therapeutic options for the *Candida* infections are extremely limited as compared to those for the treatment of bacterial infections. The new formulations development for the antifungal drugs is not an easy task; only one new formulation of antifungal drugs has been created over the past 30 years. Several lines of evidence demonstrated that drug combinations are strategies to meet some of these challenges (13,24).

In the present study we investigated the inhibitory effect of fluconazole combined with amphotericin B on fluconazole-resistant *C. albicans* biofilm formation. Our findings, based on the biomass inhibitory effects, consistently demonstrated that fluconazole combined with amphotericin B exerted a significant reduction on fluconazole-resistant *C. albicans* biofilm formation. Moreover, the morphological alterations on fluconazole-resistant *C. albicans* biofilms correlated well with the inhibitory effect of the fluconazole combined with amphotericin B treatments. In addition, fluconazole

combined with amphotericin B indicated the inhibitory effect on *HWP1* gene that contributes to the biofilm formation. Since hyphal morphogenesis is a key virulence factor in *C. albicans* infection, the inhibition of *HWP1* could decrease the ability of *C. albicans* to cause the infection. *C. albicans* biofilms are intrinsically resistant to the various antifungal agents (2,25). Combination therapy is an option for the eradication of *Candida* biofilm (26). Haque *et al.* (13) showed that combination of antifungal agents was the most effective against *C. albicans* biofilm.

However, the results of the present study demonstrated that fluconazole alone was not effective against fluconazole-resistant *C. albicans* biofilms in biomass reduction, morphological changes and the gene expression alterations. Although fluconazole has been proved as highly effective agent against *Candida* biofilms (27), there was no inhibitory effect observed on the fluconazole-resistant *C. albicans* biofilms. Several possible mechanisms of resistance to fluconazole have been identified in *Candida* species including genes involved in the ergosterol biosynthesis pathway, drug transporters, ploidy changes and loss of heterozygosity (28). In addition, the cells in a biofilm structure display a reduced susceptibility to azoles that target ergosterol biosynthesis pathway via possibly causing the ineffectiveness blockage of the enzyme lanosterol 14 $\alpha$ -demethylase (26).

Our results demonstrated that the amphotericin B alone exhibited inhibitory effects against fluconazole-resistant and amphotericin B susceptible *C. albicans* (ATCC 10231) biofilm. The amphotericin B application alone resulted in the reduction in biomass of the biofilm and morphological alterations at different concentrations against fluconazole-

resistant *C. albicans* (ATCC 10231). Kuhn *et al.* (29) confirmed the resistance of mature *C. albicans* to the conventional antifungal agents. They demonstrated that lipid formulations of amphotericin B exhibited inhibitory effects against *C. albicans* biofilms. However, amphotericin B alone was not able to inhibit *HWP1* gene on fluconazole-resistant *C. albicans* biofilms. This result suggests that the amphotericin B alone may inhibit other genes involved in the biofilm development on fluconazole-resistant *C. albicans*.

## Conclusion

In conclusion, these findings demonstrated that fluconazole combined with amphotericin B exhibits potent inhibitory effects against fluconazole-resistant *C. albicans* biofilm formation. Evaluating the treatment efficacy of fluconazole combined with amphotericin B against fluconazole-resistant *C. albicans* biofilm with other measures is important in determining the antifungal effectiveness.

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## Conflict of Interest

Authors declared no conflict of interests.

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