

Original Article

# Inhibition of *Candida albicans* yeast– hyphal transition by combination of fluconazole with amphotericin B

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## Abstract

**Introduction:** Candidiasis represents a major threat to the life and health in immune-compromised individuals. The number of antifungal drugs is limited for the treatment of candidiasis. Combination therapy is one of the most frequently used techniques to alleviate this problem.

**Methods:** Clinical isolates of *Candida albicans* were obtained from the immune-compromised patients. Antifungal susceptibilities to fluconazole and amphotericin B alone and in combination were performed by broth microdilution method. Eventually direct microscopic observation, time-kill kinetic assay, biomass and metabolic activity of the hypha, Sap enzyme activity and expression of *SAP3* gene were carried out in *C. albicans*.

**Results:** Combination of fluconazole with amphotericin B demonstrated synergistic and partial synergistic effects with fractional inhibitory concentration index ranged from 0.031 to 0.75. The data indicated that combination of fluconazole with amphotericin B exerted antifungal effects through reducing time-kill kinetic, yeast– hyphal transition, biomass and metabolic activity of the hypha and Sap enzyme activity in *C. albicans*. Additionally, the expression levels of the *SAP3* gene were significantly down regulated ( $P < 0.001$ ) in *C. albicans* treated with combination of fluconazole with amphotericin B.

**Conclusion:** Taken together, these events may confirm the potential uses of combination of fluconazole with amphotericin B against *C. albicans*. The results suggest that *SAP3* gene could be probable target of synergistic interaction of fluconazole and amphotericin B in *C. albicans*.

## Keywords:

Amphotericin B;  
*Candida albicans*;  
Fluconazole;  
*SAP3*

**Received:** 18 Jan 2018

**Accepted:** 3 Sep 2018

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## Introduction

*Candida albicans* is the most commonly recognized yeast pathogens that is responsible for opportunistic candidiasis in immune-compromised patients. *C. albicans*, selectively expresses several virulence factors that contribute to pathogenesis. Some virulence factors are of obvious importance, such as

recognition biomolecules, morphogenesis and biofilm formation, phenotypic switching, thigmotropism, metabolic adaptation and hydrolytic enzymes (Calderone and Fonzi, 2001; Haque et al., 2016; Mayer et al., 2013).

Following adhesion of the *C. albicans* to host cell surface and yeast– hyphal transition, *C. albicans* hyphae secreted of hydrolases are thought to enhance the efficiency of extracellular nutrient

acquisition. The secreted aspartyl proteinases (SAPs), degrades many human proteins on the lesion site, contributing to tissue penetration and tissue invasion in addition to evading immune responses. The proteolytic activity is attributed to a multigene SAP family of *C. albicans* with at least 10 members, SAP1– 10. *C. albicans* SAP1– 8 are secreted and released into the surrounding medium, whereas SAP9 and SAP10 remain bound to the cell surface (Chin et al., 2016; Dalle et al., 2010; Mayer et al., 2013). Research studies have demonstrated that there is differential expression pattern of the SAPs at various stages of the infection process. SAP1–3 appear to be essential for adherence of *C. albicans* to the host cell surface and tissue damage of localized infection, whereas SAP4–6 are involved in systemic disease. Little is known about SAP7 and SAP8 expression pattern in *C. albicans* pathogenesis. The SAP9 and SAP10 which are cell- surface associated proteases with function in cell surface integrity, cell separation and also indirectly contribute to adhesion (Borelli et al., 2007; Staniszewska et al., 2012).

The oldest class of antifungal drugs is the polyenes, of which amphotericin B is highly fungicidal and directly bind ergosterol and disrupt the fungal cell membrane (Gray et al., 2012). Fluconazole is the most widely-used yeast-active azole that inhibit ergosterol biosynthesis and, in general, are fungistatic (Lass-Flörl, 2011). Nowadays, increasing resistance of *C. albicans* to antifungal drugs is a major problem. There is important to find a powerful tool to treat infections with resistant *C. albicans*. Use of combination the antifungal drug is a cornerstone for treatment of resistant *Candida* infections (Pianalto and Alspaugh, 2016). Given the synergistic combination of fluconazole with amphotericin B against *C. albicans*, we hypothesized that it could augment the efficacy on inhibition of yeast– hyphal transition. To that end, we aimed to determine the effects of fluconazole in combination with amphotericin B, against *C. albicans* hyphae and conducted a series of follow-up studies to investigate on their antifungal activity.

## Materials and methods

### *Candida albicans* isolates

*C. albicans* ATCC 14053 was purchased from the Iranian Research Organization for Science and

Technology. Ten clinical isolates of *C. albicans* were identified using morphological, biochemical and molecular techniques from immune-compromised patients who admitted in Shahid Beheshti hospital affiliated to Yasooj University of Medical Sciences. Written informed consent was obtained from patients for the use of the samples in research. All isolates were kept at  $-80^{\circ}\text{C}$  in sterile 20% (v/v) glycerol stocks. This study was approved by Research Ethics Committee of our institute (Ethics No. 95-6). The study protocol conformed to the ethical guidelines of the 1995 Declaration of Helsinki. Clinical isolates of *C. albicans* were freshly subcultured onto Sabouraud dextrose agar (SDA, Difco Laboratories, Detroit, Michigan) supplemented with  $300\mu\text{g/ml}$  of chloramphenicol. *C. albicans* cells were passaged at least twice to ensure viability and purity. Subsequently, the turbidity equivalent to 0.5 McFarland standards prepared from a fungal suspension and quantified by spectrophotometer at 530nm wavelengths. The suspension was adjusted to a concentration of  $1-5\times 10^6$  yeast cells/ml and diluted the cells to a final concentration of  $0.5\times 10^3 - 5\times 10^3$  cells/ml. For each of the fungal isolates the viability of the yeast was  $>97\%$  by viable pour plate counting method (Alizadeh et al., 2017; Harmal et al., 2012; Wayne, 2008).

### Antifungal drugs

Fluconazole and amphotericin B powder were purchased from the Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). Fluconazole and amphotericin B were dissolved in dimethylsulfoxide (DMSO). The fluconazole and amphotericin B are mixed in 1:1 ratio (Khodavandi et al., 2010; Wayne, 2008).

### Antifungal drug assays

The concentration of fluconazole and amphotericin B were determined by broth microdilution antifungal susceptibility assay developed by the CLSI document (Wayne, 2008). The broth microdilution susceptibility test was performed using 96-well U-bottom tissue culture microplates containing  $100\mu\text{l/well}$  of RPMI-1640 with L-glutamine (Sigma-Aldrich). The 96-well U-bottom microplates were prepared with  $100\mu\text{l/well}$  of the twofold dilution of fluconazole ranged from 0.03 to  $64\mu\text{g/ml}$  and amphotericin B ranged from 0.03 to  $16\mu\text{g/ml}$ , alone or in combination in RPMI-1640 with L-glutamine buffered to pH 7.0 with 0.165M

morpholinophosphonyl sulfate. The microplates, including drugs and *C. albicans* cells, were kept at 4°C for 2h and incubated at 35°C. MICs were measured at 530nm using a Stat Fax 303 Reader (Awareness Technology, Inc., USA) after 24h (Mardani et al., 2018; Sharifynia et al., 2017; Shokohi et al., 2016; Wayne, 2008). Drug interaction was regulated as synergistic, additive, indifferent or antagonistic on the basis of the fractional inhibitory concentration (FIC) index using the results of MICs determined with the antifungal drugs (Khodavandi et al., 2010).

#### **Time-kill kinetic assay**

Yeast cells at a density of  $1 \times 10^6$  cells/ml were treated with the  $1 \times \text{MIC}$  of each antifungal drugs alone or in combination. Samples were incubated at 35°C. After different time intervals (0, 2, 4, 6, 8, 10, 12, 24 and 48h), the fungicidal activity of antifungal drugs or combination was measured by viable pour plate counting method. Fungistatic and fungicidal activity were considered to be achieved when the number of CFU/ml were  $<99.9\%$  and  $\geq 99.9\%$  compared with the starting inoculum size, respectively (Klepser et al., 1998).

#### **Effect of antifungal drugs on *Candida albicans* yeast– hyphal transition**

The yeast– hyphal transition was performed in 96-well U-bottom microplates. Briefly, the *C. albicans* ATCC 14053 cell suspension was provide in RPMI-1640 medium with a starting cell density of  $1 \times 10^6$  cells/ml and dispensed into the wells of a microplates at 100µl/well. Fluconazole and amphotericin B alone and in combination with the different MIC concentrations ( $2 \times \text{MIC}$ ,  $1 \times \text{MIC}$ ,  $\frac{1}{2} \times \text{MIC}$  and  $\frac{1}{4} \times \text{MIC}$ ) were added (100µl/well). Similarly, 100µl of RPMI-1640 medium containing 5% DMSO without antifungal drugs was added into the selected wells for control. Microplates were incubated at 35°C for 90min. Subsequently, the mixture was incubated at 35°C for 16h with gentle shaking. The metabolic activity of hypha was quantitatively measured by colorimetric XTT [2,3-bis (2- methoxy- 4- nitro- 5 sulfophenyl)- 5- [(phenylamino) carbonyl 2 – [H-tetrazolium hydroxide] reduction and also crystal violet assays (Peeters et al., 2008).

#### **Colorimetric XTT reduction assay**

Subsequent to the appropriate incubation of the microplates, medium was aspirated from the wells and unattached cells were removed by washing with sterile phosphate buffer saline (PBS). Colorimetric change in the XTT reduction assay of hypha was performed as previously reported (Peeters et al., 2008). Briefly, 100µl of the XTT-menadione (Sigma-Aldrich) solution was added into each well containing prewashed hypha and incubated in the dark at 37°C for 5h. Following incubation, colorimetric XTT reduction was measured in a microplate reader at 490nm.

#### **Crystal violet assay**

*C. albicans* yeast– hyphal transition was also measured by crystal violet assay. The 100µl of 99% methanol was added to the prewashed hypha. The wells were dried and stained with 100µl of crystal violet solution (Sigma-Aldrich) for 20min. Afterwards, each well was washed with tap water and immediately destained with 150µl of acetic acid 33% (Sigma-Aldrich). The absorbance values measured in a microplate reader at 590nm (Peeters et al., 2008).

#### **Effect of antifungal drugs on *Candida albicans* yeast– hyphal transition via light microscopy**

Effect of fluconazole and amphotericin B alone and in combination based on different concentrations of MIC ( $2 \times \text{MIC}$ ,  $1 \times \text{MIC}$ ,  $\frac{1}{2} \times \text{MIC}$  and  $\frac{1}{4} \times \text{MIC}$ ) on *C. albicans* ATCC 14053 yeast– hyphal transition was evaluated by growing the cells ( $1 \times 10^6$  cells/ml) in RPMI-1640 using cell culture plates and incubated at 35°C for 90min. The mixture was incubated at 35°C for 16h with gentle shaking and the hyphae were washed with PBS. Subsequently, aliquots of samples were viewed under light field microscope (Leica, DMRA II, Germany) and photographed. Samples without antifungal drugs were also examined under similar condition (Haque et al., 2016).

#### **Proteinase production assay**

Proteinase production assay was performed in 5ml YCB+BSA medium (11.7g/l Yeast Carbon Base [Difco]; 10g/l glucose; 5g/l bovine serum albumin, fraction V [Sigma-Aldrich]). *C. albicans* ATCC 14053 cell suspension ( $1 \times 10^6$  cells/ml) were grown in medium and incubated with different concentrations of fluconazole and amphotericin B alone and in combination ( $2 \times \text{MIC}$ ,  $1 \times \text{MIC}$ ,  $\frac{1}{2} \times \text{MIC}$  and  $\frac{1}{4} \times \text{MIC}$ ) at

**Table 1:** Oligonucleotide primers and their specifications used for PCR

Primer	Orientation	Sequence	Length (bp)	Reference
SAP3	Forward	5' TGCTACTCCAACAACCTTCA 3'	252	Khodavandi et al., 2011
	Reverse	5' GAATCAGGAACCCATAAATC 3'		
ACT	Forward	5'ACCGAAGCTCCAATGAATCCAAAAT 3'	516	Khodavandi et al., 2011
	Reverse	5'GTTTGGTCAATACCAGCAGCTTCCAA'		

30°C with agitation (200rpm) for 72h. Proteolytic activity was determined by measuring the increase in trichloroacetic acid soluble products absorbing at 280nm after incubation of the culture supernatant with BSA substrate for 1h at 37°C. The specific activity was expressed as OD<sub>280nm</sub>/OD<sub>600nm</sub> values of the culture. The OD readings equal to or less than 0.02 were considered negative (Macdonald and Odds, 1980).

#### Expression analysis of *SAP3* gene by reverse transcriptase-PCR

Effect of inhibitory concentration of fluconazole and amphotericin B alone and in combination on the expression of *SAP3* gene was evaluated by reverse transcriptase (RT)-PCR. Total RNA of the cells was extracted from fluconazole and amphotericin B alone and in combination treated (2×MIC, 1×MIC, ½×MIC and ¼×MIC) and untreated (0µg/ml) hyphal growth samples of RPMI-1640 medium using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Clearly, RNA integrity was assessed on formaldehyde-denaturing agarose gel. The quantity and quality of extracted RNA was measured using spectrophotometric NanoDrop® ND- 1000 (NanoDrop Technologies Inc., Wilmington, DE) analysis. Total RNA was treated with RNase- free DNase I (Fermentas, USA). cDNA was synthesized from DNase I treated total RNA (0.5µg) with Moloney- Murine Leukemia Virus reverse transcriptase and random hexamers (Fermentas). Primers for target (*SAP3*) and reference (*ACT*) genes were described in Table 1.

RT-PCR reaction was performed at 95°C for 4min, 26 cycles of -step cycling, denaturation at 94°C for 40s, annealing at 56°C for 45s, extension at 72°C for 45s and final extension at 72°C for 10min in a TPersonal thermocycler (Biometra- Germany). To check the DNA contamination in templates, DNase I treated

total RNA were included in each run. Sequencing analysis confirmed the identity of the purified product (First BASE Laboratories Sdn. Bhd., Malaysia). The sequences of predicted DNA products were queried in the NCBI by BLASTN similarity searching.

The amplification products were analyzed with the three volume-based analyses by Alphasampler HP imaging system. The abundance of transcripts was determined by volume-based analyses using the standard volumes and regression curve with logistic regression method. The intensity of amplification products was quantitated by comparing to DNA mass standard (Fermentas). The relative quantification of target transcripts was determined as follows: fold change in target gene expression= target/ reference ratio in experimental sample relative to target/ reference ratio in absolute control sample. Statistically significant of gene level variation and a fold change of ≥2- fold or ≤0.5 were classified as upregulated or down- regulated, respectively (Khodavandi et al., 2011).

#### Statistical analysis

One-way analysis of variance (ANOVA) was applied to test for the expression of *SAP3* gene in the *C. albicans* treated with fluconazole and amphotericin B alone and in combination. Tukey's HSD test was performed to compare treatment means where significant ( $P<0.05$ ) differences were found with the ANOVA. Analyses were done with SPSS 24.0 for windows statistical software (SPSS Inc., Chicago, IL, USA).

## Results

All the *C. albicans* isolates were produced a green color colony on CHROMagar *Candida*, and biochemical and molecular analyses confirmed to

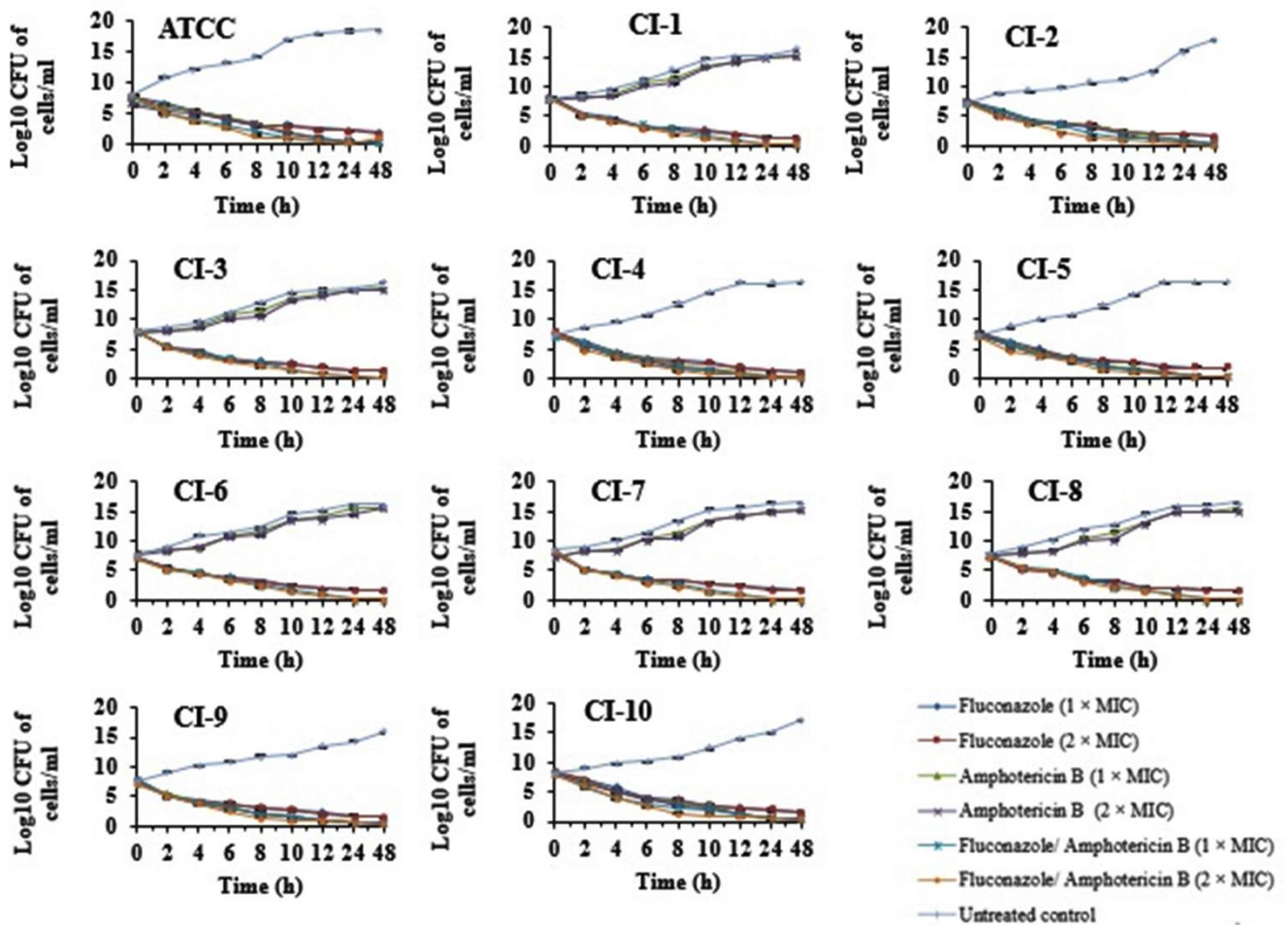


**Table 2:** MIC ( $\mu\text{g/ml}$ ) and FIC values of fluconazole and amphotericin B alone and in combination against clinical isolates of *C. albicans*

Isolates /Antifungal drugs	Fluconazole		Amphotericin B		Fluconazole/ Amphotericin B		
	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 14053	4	0.5	0.5	0.031	1/0.25	0.125/0.0313	0.75
CI- 1	32	0.031	8	0.016	2/4	0.007/0.031	0.56
CI- 2	8	0.031	2	0.016	0.5/0.125	0.007/0.031	0.125
CI- 3	32	0.125	8	0.031	1/0.25	0.016/0.0625	0.06
CI- 4	0.5	0.031	0.25	0.016	0.25/0.063	0.007/0.031	0.75
CI- 5	4	0.125	0.25	0.016	0.5/0.125	0.007/0.031	0.625
CI- 6	32	0.125	8	0.016	2/4	0.016/0.0625	0.56
CI- 7	32	0.031	8	0.016	2/4	0.007/0.031	0.56
CI- 8	32	0.031	8	0.016	2/4	0.007/0.031	0.56
CI- 9	16	0.125	0.25	0.007	0.5/0.125	0.016/0.0625	0.031
CI- 10	16	0.031	2	0.007	2/4	0.007/0.031	0.56

CI: Clinical isolates of *C. albicans*.

Data are means  $\pm$  standard deviation of three independent experiments.



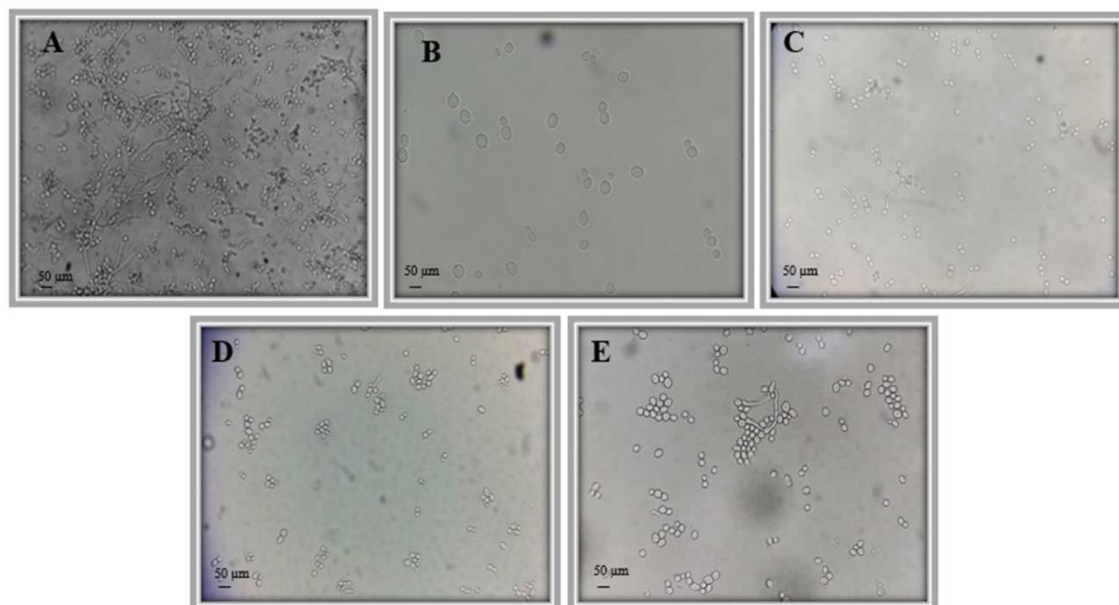
**Fig.1.** Time-kill curves of fluconazole and amphotericin B alone and in combination against *C. albicans* isolates at different time intervals. Antifungal drugs were tested at a concentration equal to 1x MIC and 2x MIC.

**Table 3:** Results of XTT and crystal violet assays for hypha-associated *C. albicans* ATCC 14053 treated with fluconazole and amphotericin B alone and in combination at different MIC concentrations

Concentration of antifungal drugs	Means absorbance at 490 nm $\pm$ SD using XTT assay			Means absorbance at 590 nm $\pm$ SD using CV assay		
	*Fluconazole	*Amphotericin B	*Fluconazole/Amphotericin B	*Fluconazole	*Amphotericin B	*Fluconazole/Amphotericin B
2 $\times$ MIC	0.15 $\pm$ 0.00 <sup>a</sup>	0.14 $\pm$ 0.01 <sup>a</sup>	0.09 $\pm$ 0.00 <sup>a</sup>	1.19 $\pm$ 0.10 <sup>a</sup>	1.18 $\pm$ 0.04 <sup>a</sup>	0.95 $\pm$ 0.00 <sup>a</sup>
1 $\times$ MIC	0.16 $\pm$ 0.00 <sup>b</sup>	0.16 $\pm$ 0.01 <sup>b</sup>	0.10 $\pm$ 0.00 <sup>b</sup>	1.21 $\pm$ 0.11 <sup>b</sup>	1.20 $\pm$ 0.01 <sup>b</sup>	0.97 $\pm$ 0.01 <sup>b</sup>
½ $\times$ MIC	0.18 $\pm$ 0.05 <sup>c</sup>	0.17 $\pm$ 0.01 <sup>c</sup>	0.11 $\pm$ 0.00 <sup>c</sup>	1.43 $\pm$ 0.08 <sup>c</sup>	1.41 $\pm$ 0.05 <sup>c</sup>	1.01 $\pm$ 0.04 <sup>c</sup>
¼ $\times$ MIC	0.21 $\pm$ 0.00 <sup>d</sup>	0.20 $\pm$ 0.01 <sup>d</sup>	0.13 $\pm$ 0.00 <sup>d</sup>	1.65 $\pm$ 0.09 <sup>d</sup>	1.63 $\pm$ 0.07 <sup>d</sup>	1.10 $\pm$ 0.04 <sup>d</sup>
Untreated control	0.52 $\pm$ 0.10 <sup>e</sup>	0.52 $\pm$ 0.10 <sup>e</sup>	0.52 $\pm$ 0.10 <sup>e</sup>	2.60 $\pm$ 0.04 <sup>e</sup>	2.60 $\pm$ 0.04 <sup>e</sup>	2.60 $\pm$ 0.04 <sup>e</sup>

\*  $\mu\text{g/ml}$ 

<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.

**Fig.2.** Effects of fluconazole and amphotericin B in combination against *C. albicans* ATCC 14053 hyphal formation after 16 h. (A) Untreated control, (B) 2 $\times$  MIC, (C) 1 $\times$  MIC, (D) ½ $\times$  MIC, (E) ¼ $\times$  MIC. Magnification  $\times$  40, Bar = 50  $\mu\text{m}$ .

be *C. albicans*. Antifungal evaluation of fluconazole and amphotericin B alone and in combination were carried out according to CLSI guidelines against yeasts. Fluconazole and amphotericin B in combination displayed synergistic and partial synergistic effects *in vitro* (FIC= 0.031-0.75). Clearly, fluconazole alone was shown to be active against all clinical isolates tested with MICs between 0.5-32 $\mu\text{g/ml}$ . Although amphotericin B alone revealed the inhibitory activity against 50% of *C. albicans* isolates it significantly ( $P < 0.001$ ) reduced the MICs of amphotericin B against the amphotericin B-resistant *C. albicans* isolates when used in combination (Table

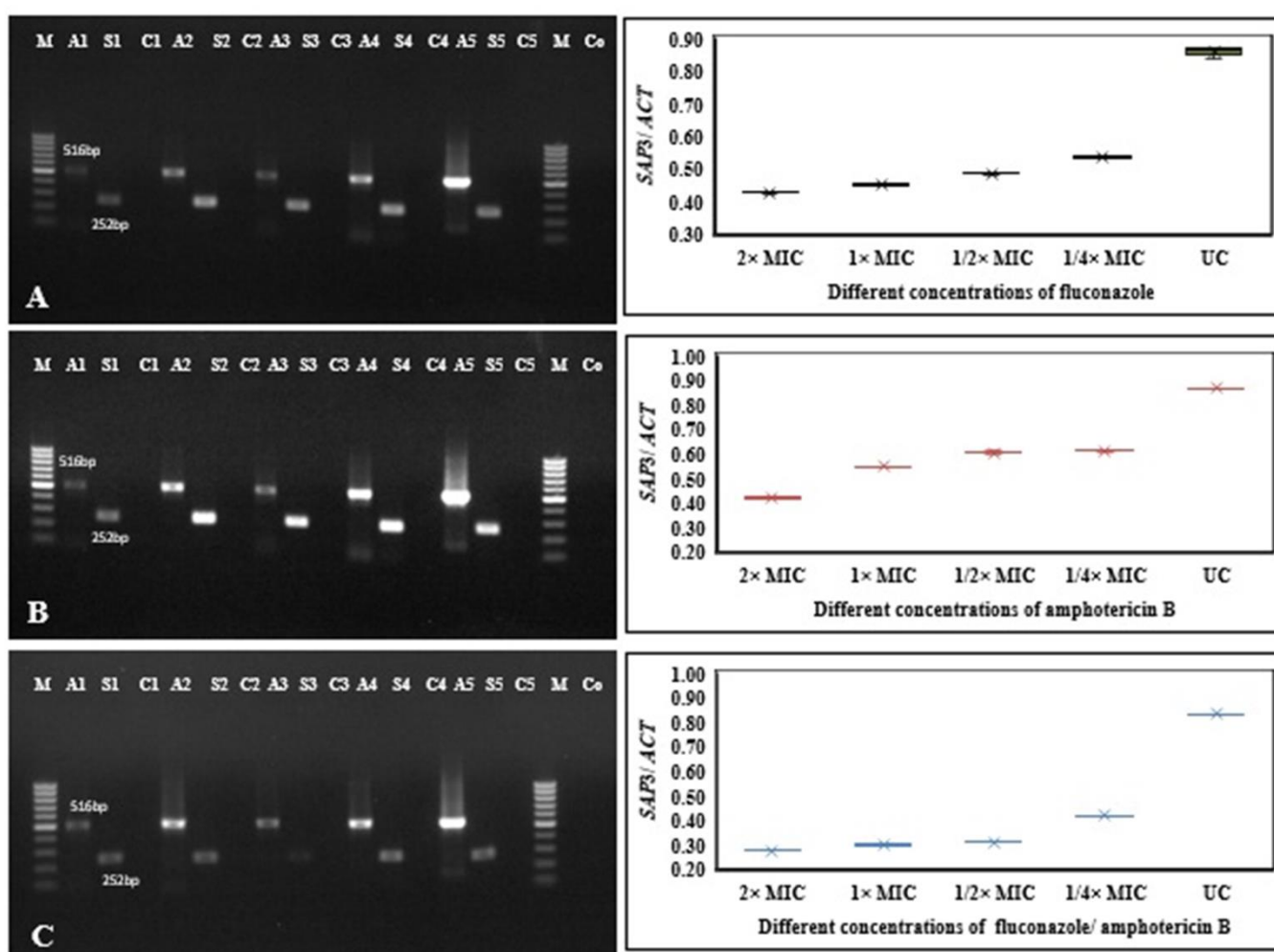
2).

The synergism of the fluconazole and amphotericin B against *C. albicans* isolates tested was confirmed by time-kill studies with numbers of CFU/ml as the  $y$  axis and time as the  $x$  axis (Fig. 1). At fluconazole concentrations equal to 2 $\times$ MIC and 1 $\times$ MIC fungistatic effect was observed against all *C. albicans* isolates. Our data showed at the concentration 2 $\times$ MIC, amphotericin B-sensitive *C. albicans* isolates demonstrated a cidal endpoint ( $\geq 99.9\%$  reduction in numbers of CFU/ml from the starting inoculum) activity of amphotericin B alone at 12h after inoculation, while the fluconazole and amphotericin B

**Table 4:** Results of proteinase production assay of *C. albicans* ATCC 14053 treated with fluconazole and amphotericin B alone and in combination at different concentration based on MIC

Concentration of antifungal drugs	Means OD <sub>280nm</sub> / OD <sub>600nm</sub> ± SD		
	Fluconazole	Amphotericin B	Fluconazole/ Amphotericin B
2 × MIC	0.02 ± 0.04 <sup>a</sup>	0.02 ± 0.05 <sup>a</sup>	0.01 ± 0.01 <sup>a</sup>
1 × MIC	0.03 ± 0.01 <sup>ab</sup>	0.03 ± 0.01 <sup>ab</sup>	0.02 ± 0.01 <sup>b</sup>
½ × MIC	0.03 ± 0.09 <sup>ab</sup>	0.04 ± 0.03 <sup>c</sup>	0.02 ± 0.00 <sup>b</sup>
¼ × MIC	0.04 ± 0.02 <sup>c</sup>	0.04 ± 0.04 <sup>c</sup>	0.02 ± 0.01 <sup>b</sup>
Untreated control	0.06 ± 0.05 <sup>d</sup>	0.06 ± 0.05 <sup>d</sup>	0.06 ± 0.05 <sup>c</sup>

<sup>a-d</sup> Means ± S.D in each treatment and column with different superscript differ significantly (Tukey's HSD,  $P < 0.05$ ). The results were performed in three independent experiments.



**Fig.3.** Expression analysis of *SAP3* gene in *C. albicans* ATCC 14053 treated with fluconazole (A), amphotericin B (B) and fluconazole and amphotericin B in combination (C) and box plots of *SAP3/ACT* ratio at different concentrations of fluconazole and amphotericin B alone and in combination based on MIC.

M: 100 bp DNA Ladder, A1: *Actin* with 2 × MIC concentration of antifungal drugs, S1: *SAP3* with 2 × MIC concentration of antifungal drugs, C1: Internal control without M-MuLV reverse transcriptase, A2: *Actin* with 1 × MIC concentration of antifungal drugs, S2: *SAP3* with 1 × MIC concentration of antifungal drugs, C2: Internal control without M-MuLV reverse transcriptase, A3: *Actin* with ½ × MIC concentration of antifungal drugs, S3: *SAP3* with ½ × MIC concentration of antifungal drugs, C3: Internal control without M-MuLV reverse transcriptase, A4: *Actin* with ¼ × MIC concentration of antifungal drugs, S4: *SAP3* with ¼ × MIC concentration of antifungal drugs, C4: Internal control without M-MuLV reverse transcriptase, A5: *Actin* without antifungal drugs (untreated control), S5: *SAP3* without antifungal drugs (untreated control), C5: Internal control without M-MuLV reverse transcriptase. UC: untreated control.

in combination exhibited fungicidal activity against *C. albicans* isolates at 8h after inoculation. The combination of fluconazole and amphotericin B showed the strongest synergism by time-kill kinetic assay. Amphotericin B-resistant *C. albicans* isolates were not killed by amphotericin B but instead continued to multiply, albeit at a lower rate and level of saturation than untreated control, while the fluconazole and amphotericin B in combination resulted fungicidal activity against *C. albicans* isolates resistance to amphotericin B at 12h after inoculation. Table 3 shows the significant results for reduction of both biomass and metabolic activity of the hypha, as determined by crystal violet and XTT assays respectively, in response to different concentrations of antifungal drugs based on MIC ( $P < 0.05$ ). The effect of combination has also significantly decreased yeast–hyphal transition in XTT and crystal violet assays ( $P < 0.05$ ).

Light microscopy used to visualize yeast–hyphal transition indicated that the treatment of preformed hypha with fluconazole and amphotericin B alone and in combination resulted in a subtle reduction in yeast–hyphal transition compared to untreated control. Figure 2 shows light microscopy pictures of growing *C. albicans* ATCC 14053 hypha treated with fluconazole and amphotericin B in combination with different concentrations of MIC after 16h. On the other hand, fluconazole and amphotericin B alone demonstrated activity against yeast–hyphal transition for *C. albicans* ATCC 14053 (data not shown).

Findings from the proteinase production assay of *C. albicans* ATCC 14053 treated with fluconazole and amphotericin B alone and in combination exhibited significant reduction ( $P < 0.05$ ) in proteolytic activity compared to untreated control (Table 4). Treatment with fluconazole and amphotericin B in combination was considered negative proteolytic activity (the mean value of  $OD_{280nm} / OD_{600nm}$  was  $0.018 \pm 0.02$ ).

The results of relative quantitative RT-PCR assays showed that fluconazole and amphotericin B alone and in combination at all concentrations based on MIC caused down-regulation in the expression levels of *SAP3* gene. The statistical analysis revealed significant difference in the expression levels of *SAP3* in three experimental groups. Moreover, the expression levels of *SAP3* were significant down-regulated compared to untreated control with the fluconazole challenge alone for 2xMIC, 1xMIC,

1/2xMIC and 1/4xMIC by 2.7- fold, 2.6- fold, 2.2- fold and 1.8- fold, respectively compared with those of the untreated control group (Tukey's HSD;  $P < 0.001$ ). Expression of *SAP3* was down-regulated on amphotericin B challenge for 2xMIC, 1xMIC, 1/2xMIC and 1/4xMIC compared with the untreated control group 2.04- fold, 1.6- fold, 1.4- fold and 1.4- fold, respectively ( $P < 0.001$ ).

The combination of fluconazole and amphotericin B significantly down-regulated the expression levels of *SAP3* compared with those for the fluconazole and amphotericin B challenge alone by 3.03- fold, 2.8- fold, 2.7- fold and 2- fold at concentrations of 2xMIC, 1xMIC, 1/2xMIC and 1/4xMIC, respectively ( $P < 0.001$ ; Fig. 3). The analysis of the nucleotide sequence of the predicted DNA products revealed a high similarity via nucleotide Blast in Gene Bank.

## Discussion

*C. albicans* is the most common cause of yeast infections, leading to a range of superficial mucocutaneous to life-threatening invasive infections, particularly in immune-compromised individuals (Dalle et al., 2010; Mayer et al., 2013). The current therapeutic options for *Candida* infections are extremely limited. Recent studies reported resistance to fluconazole and amphotericin B in yeast infections. Combination therapy is now considered to be the best option to minimize the risk of resistance and side effect of existing potent antifungal drugs to eradicate the candidiasis infection (Odds, 2003; Rex et al., 2003; Spampinato et al., 2013).

In the present study we showed the effects of fluconazole and amphotericin B alone and in combination on inhibition of *C. albicans* yeast–hyphal transition. *In vitro* studies have found conflicting results, with some groups reporting evidence of antagonism and some evidence of enhanced effects (Paterson et al., 2001). Sugar et al. (1995) using murine models have demonstrated an additive effect of giving amphotericin B with fluconazole. The synergistic interaction between fluconazole and amphotericin B has previously been shown with *C. albicans* cells in clinical experience (Odds, 2003; Paterson et al., 2001; Rex et al., 2003). Our findings, based on time-kill studies, consistently demonstrated fungicidal activity of amphotericin B against amphotericin B-sensitive *C. albicans* isolates.



Fungicidal activity of fluconazole and amphotericin B in combination was observed in clinical isolates of *C. albicans*. Similarly, Lewis et al. (1998), found that administration of fluconazole and amphotericin B in combination, fungicidal activity was similar those observed with amphotericin B alone.

The synergistic interaction between fluconazole and amphotericin B has previously been shown, but the concept of combination antifungal treatment can lead to important assumptions regarding the mechanisms of these interactions. Given that adherence is often the first stage of host colonization, we investigated the ability of *C. albicans* ATCC 14053 treated with fluconazole and amphotericin B alone and in combination to produce hyphae cells. Microscopic observation, biomass and metabolic activity of the hypha, Sap enzyme activity and expression of *SAP3* gene correlated well with the efficacies of the combination treatments. These results partly contradict those of Wu et al. (2000) who found a decrease in Sap activity in susceptible strains of *C. albicans* exposed to fluconazole.

Consistent with the observations in previous studies, *SAP3* gene could be probable molecular target in the synergistic interaction between fluconazole and amphotericin B against *C. albicans*. Our findings also showed that exposure of *C. albicans* to antifungal drugs effects on expression of *SAP3* secreted proteinase gene in a dose- dependent manner by down-regulating. Khodavandi et al. (2011) revealed that the allicin had no effect on *SAPs*1-4 expression, whereas fluconazole was able to suppress *SAP4* expression and an initial decrease in *SAP1* and *SAP3* expression. Gu et al. (2016) demonstrated that fluconazole plus fluoxetine caused down-regulation of *SAP1* to *SAP4* in resistant *C. albicans*. Hosseini et al. (2016) indicated that carvacrol is effective in reduction of *SAP1-3* expression in fluconazole - resistant and -susceptible isolates of *C. albicans*. Ripeau et al. (2002) showed that the, expression of *SAP1* to *SAP3*, *SAP7* to *SAP9*, *PLB1* and the *EFB1* was unaltered in *C. albicans* treated with caspofungin over a period of 7h.

## Conclusion

These findings may reflect the potential of combination of fluconazole and amphotericin B on inhibition of yeast– hyphal transition in *C. albicans*.

The probable mechanisms were related to down-regulation of *SAP3* gene with inhibition of biomass and metabolic activity of yeast– hyphal transition and Sap enzyme activity. The positive correlation existed between these factors in *C. albicans* treated with combination of fluconazole and amphotericin B, may be explained by a decrease in fungal virulence. However, greater knowledge of molecular mechanisms of antifungal drug effects could help in the development of new therapeutic strategies.

## Acknowledgments

The authors are thankful to the Islamic Azad University of Yasooj for financial support. Results of the current study are part of the MSc thesis.

## Conflict of interest

The authors claim that there is no conflict of interest.

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