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Research Article

Synergistic Interaction of Fluconazole/Amphotericin B on Inhibition of Enzymes Contributes to the Pathogenesis of *Candida Tropicalis*

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ABSTRACT

Background: Candidiasis has gained much attention in recent decades due to its increasing prevalence in immunocompromised patients. Usually, antifungals such as fluconazole and amphotricin B are used for treatment of candidiasis, but one of the major clinical problems is the emergence of antifungal resistance. Combination antifungal therapy is one of the most commonly used methods to alleviate the problem of antifungal resistance.

Methods: The effect of fluconazole alone and in combination with amphotericin B on *C.* tropicalis isolates were performed using the Clinical and Laboratory Standards Institute (CLSI) reference method. Eventually hypha formation, time kill study, proteinase and phospholipase activity and expression of *PLB* and *SAP2* genes were carried out to investigate the enzymes inhibitory properties of antifungal tested against *C. tropicalis*.

Results: Results showed the significant synergic effect of fluconazole in combination with amphotericin B in inhibiting the growth of *C.* tropicalis isolates, with fractional inhibitory concentration indices ranging from 0.06 to 0.5. The combination of fluconazole with amphotericin B reduced the number of yeast form and inhibited the yeast to hyphae transition in *C. tropicalis*. The antifungals tested were able to show the effect of down regulating expression of the selected genes significantly in fluconazole/amphotericin B ranging from 1.42- to 2.27-fold.

Conclusion: Our results demonstrated that the synergistic interaction of fluconazole/amphotericin B would be worth exploring for the management of candidiasis. In addition, *PLB* and *SAP2* genes could be probable molecular targets in the synergistic interaction of fluconazole/amphotericin B in *C. tropicalis*.

Introduction

Candida tropicalis is one of the most widely encountered medical pathogen after *Candida albicans*. Over the past few decades, candidiasis due to *C. tropicalis* has been increased in frequency, particularly in people with lymphoma, leukemia and diabetes thus proclaiming this yeast to be emerging pathogen.[1-3] *C. tropicalis* has the highest similarity to *C. albicans* and shares several pathogenic features to the point where the two species have three specific key virulence factors such as proteinase, phospholipase and biofilm formation.[1,4-8]

There are significant expansions of virulence factors, such as adherence to host cells, the ability of this fungus to filamentation and penetrate into the cells, phenotypic switching, thigmotropism (contact sensing), the production of tissue-damaging hydrolytic enzymes (e.g. secreted aspartyl proteinases (SAPs), phospholipases and haemolysin), biofilm formation and a range of fitness attributes in pathogenic species.[1,4-14] Moreover, SAPs are now emerging as a prominent virulence factors in candidiasis that promotes host tissue damage. *C. tropicalis* possesses one subfamily of four genes encoding Saps (SAPT1-4), and phospholipases coding genes particularly *PLB*.[1,2,7,15,16]

Candidiasis is treated with several classes of antifungal agents, including azoles and polyenes. Azoles block the ergosterol biosynthesis pathway by inhibition of the enzyme lanosterol 14-α-demethylase, causing loss of fluidity and original function of the membrane and therefore halting the growth of fungi. While polyene antifungal agents such as amphoterecin B exhibit fungicidal effect by directly binding to ergosterol and disrupt the lipid composition of fungal membrane, forming membrane pores, which causing leakage of essential contents of the fungal cell. Although fluconazole and amphotericin B have been the standard antifungal agents for treatment of candidiasis, treatment failure with either agent has been reported. The innate and acquired antifungal resistance, the toxicity and the limited number of available agents, and the rise in infections incidence due to non-*albicans Candida* spp. support to develop more effective and less toxic therapeutic strategies to treat

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candidiasis.[17-18]

Recent advances in development of antifungal therapy include the use of antifungal agents in combination, for example, fluconazole/amphotericin B.[17] In the present study, *in vitro* antifungal effect of fluconazole alone and in combination with amphotericin B against *C. tropicalis* were examined. Subsequently, hypha formation, time kill study, proteinase and phospholipase activity and expression patterns of selected genes involved in the biosynthesis of the enzymes contributes to the pathogenesis of *C. tropicalis* such as *PLB* and *SAP2* were analyzed with fluconazole/amphotericin B combination treatments.

Materials and Methods

Candida tropicalis isolates and growth conditions

The present study was conducted with five clinical isolates of *C. tropicalis* and the reference strain ATCC 750. The five isolate of *C. tropicalis* were obtained from 185 immunocompromised patients that admitted in Shahid Beheshti hospital affiliated to Yasooj University of Medical Sciences. The informed consent was provided with patients for the use of their samples in study. All the isolates were identified by conventional[19] and molecular methods.[20] The reliability of *C. tropicalis* colonies were confirmed by CHROMagar *Candida* medium (CHROMagar Company, France) and DNA sequencing. All isolate of *C. tropicalis* were kept in sterile 20% (v/v) glycerol stocks at −80 °C. *C. tropicalis* plated on sabouraud dextrose agar (SDA, Difco Laboratories, Detroit, Michigan) containing 300 µg/mL of chloramphenicol at 35–37 °C for 24 h to ensure viability. This study was approved by Research Ethics Committee of our institute (no. 1205769). (The study protocol conformed to the ethical principles of the 2008 Declaration of Helsinki).

Antifungals

Fluconazole and amphotericin B were obtained from Sigma-Aldrich Chemicals Co. (St. Louis, MO).

Susceptibility testing of Candida tropicalis isolates

The minimal inhibitory concentrations (MICs) were determined with fluconazole (0.03125–64 mg/L) alone or in combination with amphotericin B (0.0313–16 mg/L), using broth microdilution antifungal susceptibility test.[21] *C. tropicalis* cell suspension (0.5 McFarland) was prepared by transferring five colonies of about 1mm size of a 24 h old culture in sterile 0.85% NaCl and diluted to achieve a final concentration of $5 \times 10^2 - 2.5 \times 10^3$ yeast cells/mL. According to CLSI document (CLSI M27-A3), 50 or 100 mL of the two-fold dilution of the fluconazole and amphotericin B alone or in combination, dissolved in a standard Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) with 0.2% glucose [buffered to pH 7.0 with 0.165 M morpholinophos- phonyl sulfate (MOPS)] using 96-well U-bottom microplates. Subsequently, 100 mL of a standardized cell suspension was added to the drug mixture. After incubation for 24 h at 35 °C the susceptibility endpoint was calculated as the lowest concentration of each drug (alone or in combination) that caused a 50 and 90% reduction of cell growth compared with that of untreated control. Drug interaction was regulated on the basis of the fractional inhibitory concentration (FIC) index using the results of MICs determined with the antifungal alone and in combination; the FIC was calculated for each antifungal combination according to the following formulas:

$$\left[\text{FIC index} = \text{FICx} + \text{FICy}\right]$$

$$\text{FIC index} = \frac{\text{MIC of drug x, in combination}}{\text{MIC of drug x, tested alone}} + \frac{\text{MIC of drug y, in combination}}{\text{MIC of drug y, tested alone}} \quad \text{Eq. (1)}$$

Two drugs are defined as having synergistic effect, if the FIC indexes are ≤ 0.5, while they act as partial synergy when the FIC > 0.5 but < 1.0, additive when FIC =1.0, indifferent when FIC > 1.0 but < 4.0, and antagonistic when FIC ≥ 4.0.[22,23]

In vitro time kill study

Foure mL of *C. tropicalis* cell suspension (1×10^6 cells/mL) was dissolved in RPMI 1640 and mixed with a concentration equal to the 1× MIC of fluconazole alone or in combination with amphotericin B. Time-kill samples were incubated at 35 °C. Hundred µL of each mixture was loaded after different time intervals, plated on SDA and incubated at 35 °C. The colony forming unit (CFU)/mL was calculated by observing and count colonies.[22]

Candida tropicalis hypha formation

C. tropicalis ATCC 750 was induced to form hypha formation according to the method by Khodavandi et al.[24] At first, 4 mL of a suspension of *C. tropicalis* cell with a density of 1×10^6 cells/mL was added to 4 µL of fluconazole and amphotericin B alone or in combination at different concentrations based on MIC (2× MIC, 1× MIC, ½× MIC and ¼× MIC) using 6-well cell culture plates and incubated at 35 °C for 90 min. After incubation for 16 h with gentle shaking at 35 °C, the hyphae were washed with PBS and viewed with a light microscope (Leica, DMRA II, Germany).

Candida tropicalis proteinase production assay

Proteinase activity of *C. tropicalis* treated with fluconazole and amphotericin B alone or in combination at different concentrations based on MIC (2× MIC and 1× MIC, ½× MIC and ¼× MIC) was performed by following method by Macdonald and Odds.[25] Briefly, *C. tropicalis* from RPMI 1640 cultures were grown in 5 mL YCB + BSA medium (11.7 g/L Yeast Carbon Base [Difco]; 10 g/L glucose; 5 g/L bovine serum albumin, fraction V [Sigma–Aldrich]) and placed into a shaking incubator at 30 °C of 200 rpm for 72 h. Proteolytic activity was also measured by the difference in trichloroacetic acid soluble products absorption at 280 nm in triplicate after 1 h

incubation of the culture supernatant with BSA substrate at 37 °C. The specific activity of proteinase was expressed as $OD_{280\ nm}/OD_{600\ nm}$ values of the culture. The OD readings equal to or less than 0.02 were considered negative.

Candida tropicalis phospholipase production assay

The method of Price et al.[26] was used; *C. tropicalis* cells treated with fluconazole and amphotericin B alone or in combination at different concentrations based on MIC (2× MIC, 1× MIC, ½× MIC and ¼× MIC) were grown overnight to stationary phase in RPMI 1640 at 37 °C. The cells were diluted and standardized to a concentration of 2×10^5 cells/mL. Triplicate of the suspension of cells was plated out on Phospholipase agar [10 g peptone, 40 g dextrose, 16 g agar, 80 mL Egg Yolk Emulsion (Fluka, Chemie AG, Buchs, Switzerland) was added to 1000 mL of distilled water] and incubated at 30 °C. After 72 h of incubation period, the diameters of the colonies and the formation of halo zones were measured. The phospholipase activity was calculated by dividing the colony diameter (mm) by the precipitation zone plus colony diameter. Four classes were described for phospholipase activity as follows, phospholipase zone (Pz) = 1 as negative phospholipase activity; $0.82 \leq Pz \leq 0.88$ as weak enzymatic activity; $0.75 \leq Pz \leq 0.81$ as moderate activity; $0.67 \leq Pz \leq 0.74$ meant strong phospholipase producers.

Expression analysis of Candida tropicalis PLB and SAP2 genes by reverse transcriptase (RT)-PCR

Expression of *C. tropicalis PLB* and *SAP2* genes were analyzed by RT-PCR. Total RNA was extracted from *C. tropicalis* cells treated with fluconazole and amphotericin B alone or in combination at different concentrations based on MIC (2× MIC, 1× MIC, ½× MIC and ¼× MIC) using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's manual with slight changes. Absorption ratio of A260/A280 and A260/A230 derived from spectrophotometric NanoDrop® ND-1000 (NanoDrop Technologies Inc., Wilmington, DE) analysis provided an estimate of quantity and purity of RNA. The integrity of total RNA was confirmed by formaldehyde-denaturing agarose gel electrophoresis. The RNA samples were treated with RNAse-free DNase I (Fermentas, USA) for removal of DNA contamination. Moloney-Murine Leukemia Virus (MMLV) reverse transcriptase and random hexamers (Fermentas) were used to convert 0.5 µg of total RNA into complementary DNA (cDNA). In each treatment, synthesized cDNA was amplified from the specific primers (Table 1). House-keeping gene (actin) was used to normalize the RT-PCR data. The PCR reaction was performed at 95 °C for 4 min, 26 cycles of 3-step cycling, denaturation at 94 °C for 40 s, annealing at 56 °C for 45 s, extension at 72 °C for 45 s and final extension at 72 °C for 10 min in a TPersonal thermocycler (Biometra- Germany).

The PCR products were separated on agarose gel electrophoresis and captured images by the AlphaImager HP system. The intensity of PCR products was quantitated by comparing to known DNA molecular weight marker (Fermentas, USA). The relative quantification of gene expression were determined as follows: fold change in target gene expression = target/reference ratio of experimental sample relative to target/reference ratio of untreated control sample. The expression of genes with statistical significance and a fold change of ≥2-fold or ≤0.5-fold were considered up-regulated or down-regulated, respectively. The excision PCR products of the agarose gel were purified using the QIAquick Gel Extraction Kit (Qiagen). The purified PCR products identity was confirmed by sequencing analysis (First BASE Laboratories Sdn. Bhd., Malaysia). The sequence similarity was queried by BLASTN in the GenBank database of NCBI.[27]

Statistical analysis

Raw data were subjected to statistical tests using the software SPSS 24.0 for windows (SPSS Inc. Chicago, IL, USA). Data were analyzed using analysis of variance (ANOVA) with Tukey's HSD test. Results are expressed as the average mean of the biological replicates ± standard deviations (S.D).

Results

C. tropicalis isolates were identified by morphological, biochemical and molecular methods. The reliability of *C. tropicalis* was confirmed by DNA sequencing. The nucleotide sequences were analyzed via the non-redundant nucleotide sequences in GenBank showed 100 % similarity with the respective gene sequences. The results of susceptibility testing of *C. tropicalis* isolates on fluconazole and amphotericin B revealed the inhibitory activity against *C. tropicalis*, except one isolate were resistant to amphotericin B. The breakpoint for amphotericin B was considered as ≥2.0 µg/mL.[28]

Table 1. Oligonucleotide primers used for PCR.

Primer	Orientation	Sequence	Length (bp)	Reference
PLB	Forward	5'CCCATACGATTTATGGAAT3'	501	[20]
	Reverse	5'CCATTGACACAAGCATTTAC3'		
ACT	Forward	5'TAGGTTTGGAAGCTGCTGGT3′	250	This study, GenBank: XM_002549283.1
	Reverse	5′GACAAGGAAGCCAAAATGGA3′		
SAP2	Forward	5'TAATGGTGCCGTTGCTGGTT3'	327	This study, GenBank: AF115320.1
	Reverse	5'ATTCTCAGCTTCAAGTGTTGTGT3'		
ACT	Forward	5'GGCTGGTAGAGACTTGACCG3'	502	This study, GenBank: XM_002549283.1
	Reverse	5'AGCCAAAATGGAACCACCGA3'		

Table 2. Relative MIC (µg/mL) and FIC values of fluconazole alone and in combination with amphotericin B against isolates of *Candida tropicalis.*

Isolates /Antifungals	Fluconazole		Amphotericin B		Fluconazole/Amphotericin B		
	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	FIC
C. tropicalis ATCC 750	4	0.5	0.5	0.031	1/0.125	1/0.25	0.5
CI-1	32	0.031	8	0.016	2/8	0.007/0.031	1.06
CI-2	1	0.062	0.5	0.007	0.125/0.05	0.007/0.031	0.23
CI-3	0.25	0.031	1	0.007	0.031/0.125	0.007/0.031	0.25
CI-4	1	0.031	4	0.016	0.031/0.125	0.016/0.0625	0.06
CI-5	16	0.062	0.5	0.007	0.125/0.05	0.007/0.031	0.11

CI: Clinical isolates of *C. tropicalis*
Data are means ± standard deviation of three independent experiments.

The MIC range of fluconazole and amphotericin B against *C. tropicalis* was subsequently 0.062–32 µg/mL and 0.007–8 µg/mL. From Table 2, it can be seen that all the isolates except one, the fluconazole in combination with amphotericin B had synergistic properties. Indifference was found for fluconazole in combination with amphotericin B in *C. tropicalis* amphotericin B-resistant isolate.

Figure 1 shows the potency of fluconazole alone and in combination with amphotericin B in decreasing the cell number of *C. tropicalis* ATCC 750 after 0, 2, 4, 6, 8, 10, 12, 24 and 48 h compared to untreated control. Fluconazole alone and in combination with amphotericin B showed a significant reduction in number of viable cells at different time intervals. Also the killing patterns of fluconazole alone and in combination with amphotericin B against *C. tropicalis* isolates significantly reduced the viable cell numbers at various time intervals (data not shown).

Findings from the hypha formation inhibitory properties of fluconazole alone and in combination with amphotericin B on growth of *C. tropicalis* ATCC 750 exhibited significant reduction in hypha formation compared to untreated control. Figure 2 shows *C. tropicalis* ATCC 750 hypha formation inhibitory properties of fluconazole/amphotericin B at different concentrations based on MIC (2× MIC, 1× MIC, ½× MIC and ¼× MIC) after 16 h.

The *C. tropicalis* isolates treated with fluconazole alone and in combination with amphotericin B showed decreased proteolytic activity compared to untreated control (Tukey's HSD, $P < 0.05$; Table 3). For *C. tropicalis* isolates treated with combination of fluconazole/amphotericin B, the mean value of OD_{280} nm/OD_{600} nm were 0.02 ± 0.001 which considered negative.

Findings from the phospholipase production-inhibitory properties *C. tropicalis* isolates treated with fluconazole alone and in combination with amphotericin B exhibited significant reduction in phospholipase activity compared to untreated control. As shown in Table 4, *C. tropicalis* isolates treated with combination of fluconazole/ amphotericin B could significantly (Tukey's HSD, $P < 0.05$) reduce phospholipase activity. Treatment of fluconazole alone and in combination with amphotericin B was found to be effective in reducing phospholipase activity of *C. tropicalis* isolates at all concentration.

Relative quantitative RT-PCR analysis of *PLB* and *SAP2* expression was conducted in *C. tropicalis* ATCC 750 treated with fluconazole alone and in combination with amphotericin B. *PLB* and *SAP2* gene expression profiles contained measurements of relative abundances of genes.

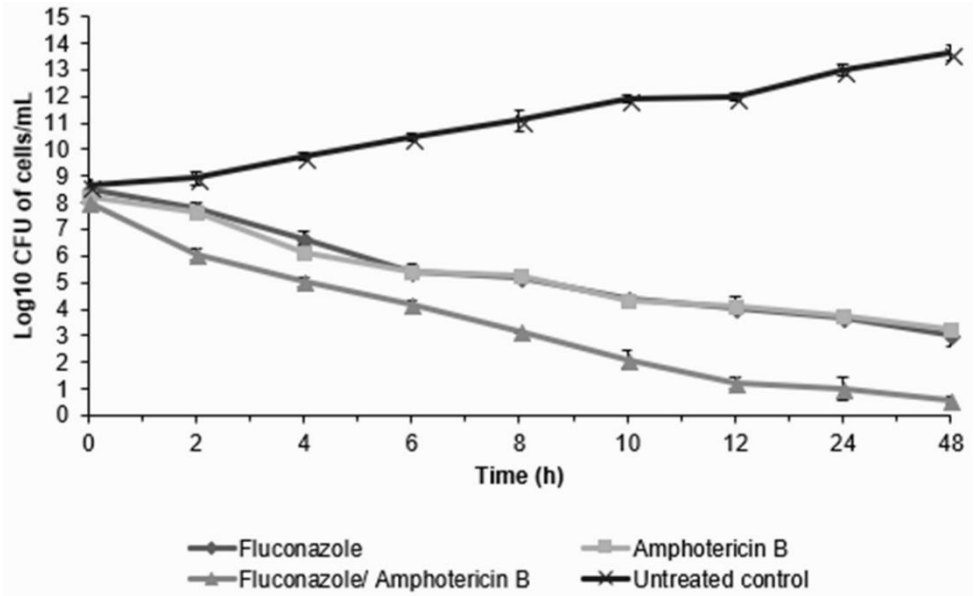

Figure 1. Time–kill curves of fluconazole alone and in combination with amphotericin B with a concentration equal to the 1× MIC against *Candida tropicalis* ATCC 750 at different time intervals.

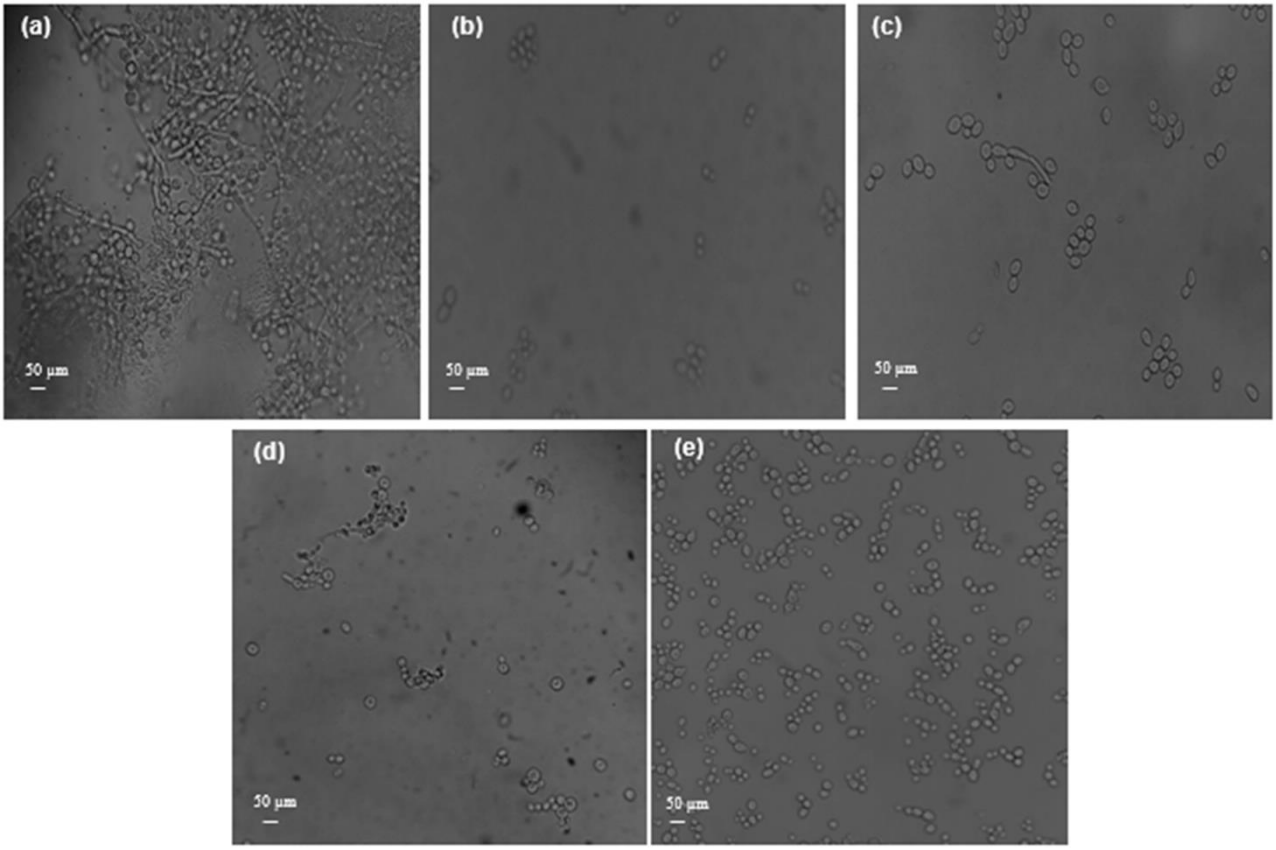

Figure 2. Light microscopic view of *Candida tropicalis* ATCC 750 treated with fluconazole/amphotericin B at different concentration based on MIC after 24 h. (a) Untreated control, (b) 2× MIC, (c) 1× MIC, (d) ½× MIC, (e) ¼× MIC. Magnification × 40, Bar = 50 µm.

Table 3. Results of proteinase activity assay (OD_{280}nm/OD_{600}nm) of *Candida tropicalis* isolates treated with fluconazole alone and in combination with amphotericin B in different concentration based on MIC.

Antifungals/ Isolates		*C. tropicalis* ATCC 750	CI-1	CI-2	CI-3	CI-4	CI-5
Fluconazole	Untreated control	0.06±0.001^{a}	0.06±0.00^{a}	0.06±0.02^{a}	0.04±0.02^{a}	0.06±0.001^{a}	0.06±0.005^{a}
	2× MIC	0.02±0.001^{bc}	0.02±0.001^{d}	0.02±0.41^{bc}	0.02±0.00^{c}	0.02±0.001^{bc}	0.02±0.004^{bc}
	1× MIC	0.03±0.007^{b}	0.03±0.007^{bc}	0.02±0.55^{bc}	0.03±0.01^{b}	0.03±0.002^{b}	0.03±0.003^{b}
	½× MIC	0.03±0.009^{b}	0.03±0.00^{bc}	0.03±0.05^{b}	0.03±0.10^{b}	0.03±0.002^{b}	0.03±0.002^{b}
	¼× MIC	0.03±0.002^{b}	0.04±0.007^{b}	0.03±0.005^{b}	0.03±0.01^{b}	0.03±0.002^{b}	0.03±0.002^{b}
Amphotericin B	Untreated control	0.06±0.001^{a}	0.06±0.00^{a}	0.06±0.002^{a}	0.04±0.02^{a}	0.06±0.001^{a}	0.06±0.005^{a}
	2× MIC	0.03±0.001^{b}	0.02±0.001^{bc}	0.02±0.00^{bc}	0.03±0.008^{b}	0.03±0.001^{b}	0.03±0.005^{b}
	1× MIC	0.03±0.001^{b}	0.03±0.002^{b}	0.03±0.00^{b}	0.03±0.00^{b}	0.03±0.02^{b}	0.03±0.007^{b}
	½× MIC	0.03±0.008^{b}	0.03±0.42^{b}	0.03±0.00^{b}	0.03±0.002^{b}	0.03±0.002^{b}	0.03±0.007^{b}
	¼× MIC	0.03±0.005^{b}	0.03±0.22^{b}	0.03±0.00^{b}	0.03±0.02^{b}	0.03±0.002^{b}	0.03±0.00^{b}
Fluconazole/ Amphotericin B	Untreated control	0.06±0.001^{a}	0.06±0.00^{a}	0.06±0.002^{a}	0.04±0.02^{a}	0.06±0.001^{a}	0.06±0.005^{a}
	2× MIC	0.02±0.001^{bc}	0.02±0.11^{bc}	0.02±0.00^{bc}	0.02±0.001^{b}	0.02±0.001^{bc}	0.02±0.00^{bc}
	1× MIC	0.02±0.001^{bc}	0.02±0.00^{bc}	0.02±0.003^{bc}	0.02±0.02^{b}	0.02±0.002^{bc}	0.03±0.007^{b}
	½× MIC	0.02±0.001^{bc}	0.02±0.10^{bc}	0.02±0.004^{bc}	0.02±0.03^{b}	0.02±0.001^{bc}	0.03±0.00^{b}
	¼× MIC	0.03±0.001^{b}	0.03±0.07^{b}	0.03±0.004^{b}	0.02±0.06^{b}	0.03±0.004^{b}	0.03±0.001^{b}

$^{a–d}$ 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.

The reliability of the PCR products was confirmed by DNA sequencing. The sequences displayed 100 % similarity with the respective gene when analyzed via the non-redundant nucleotide sequences in GenBank.

Relative expression levels of the *PLB* and *SAP2* genes were significantly different at all concentrations of tested antifungals based on MIC (Tukey's HSD, $P < 0.05$; Figures 3 and 4). The box plots allows comparison of *PLB/ACT* and *SAP2/ACT* ratio at different concentrations of fluconazole alone and in combination with amphotericin B based on MIC (Figure 5). The expression levels of the *PLB* and *SAP2* genes were significant ($P \leq 0.05$) down-regulated compared with untreated control.

Table 4. Results of phospholipase activity assay (colony diameter/ precipitation zone diameter) of *Candida tropicalis* isolates treated with fluconazole alone and in combination with amphotericin B in different concentration based on MIC.

Antifungals/ Isolates		*C. tropicalis* ATCC 750	CI-1	CI-2	CI-3	CI-4	CI-5
Fluconazole	Untreated control	0.57±0.05^{a}	0.58±0.15^{a}	0.60±0.00^{a}	0.58±0.64^{a}	0.57±0.04^{a}	0.60±0.00^{a}
	2× MIC	0.89±0.06^{d}	0.88±0.01^{d}	0.89±0.00^{d}	0.89±0.00^{d}	0.87±0.01^{d}	0.89±0.05^{c}
	1× MIC	0.85±0.00^{bc}	0.84±0.11^{b}	0.85±0.10^{bc}	0.83±0.60^{b}	0.85±0.01^{c}	0.85±0.03^{b}
	½× MIC	0.85±0.02^{bc}	0.85±0.00^{bc}	0.85±0.12^{bc}	0.84±0.00^{bc}	0.85±0.11^{c}	0.85±0.00^{b}
	¼× MIC	0.84±0.10^{b}	0.84±0.01^{b}	0.84±0.05^{b}	0.83±0.20^{b}	0.82±0.01^{b}	0.85±0.00^{b}
Amphotericin B	Untreated control	0.57±0.05^{a}	0.58±0.15^{a}	0.60±0.00^{a}	0.58±0.04^{a}	0.57±0.04^{a}	0.60±0.00^{a}
	2× MIC	0.86±0.06^{d}	0.87±0.02^{d}	0.88±0.06^{c}	0.88±0.02^{c}	0.88±0.01^{d}	0.88±0.05^{c}
	1× MIC	0.82±0.20^{b}	0.83±0.20^{bc}	0.85±0.00^{b}	0.82±0.00^{b}	0.84±0.11^{bc}	0.83±0.13^{b}
	½× MIC	0.82±0.00^{b}	0.83±0.03^{bc}	0.85±0.00^{b}	0.82±0.06^{b}	0.84±0.04^{bc}	0.83±0.10^{b}
	¼× MIC	0.83±0.10^{bc}	0.82±0.00^{b}	0.85±0.00^{b}	0.82±0.03^{b}	0.83±0.00^{b}	0.83±0.10^{b}
Fluconazole/ Amphotericin B	Untreated control	0.57±0.05^{a}	0.58±0.15^{a}	0.60±0.00^{a}	0.58±0.04^{a}	0.57±0.04^{a}	0.60±0.00^{a}
	2× MIC	0.99±0.04^{d}	0.97±0.01^{e}	0.96±0.00^{d}	0.95±0.00^{d}	0.95±0.05^{e}	0.95±0.09^{d}
	1× MIC	0.86±0.00^{bc}	0.86±0.00^{cd}	0.85±0.00^{bc}	0.85±0.10^{bc}	0.86±0.01^{cd}	0.87±0.02^{bc}
	½× MIC	0.84±0.00^{b}	0.84±0.00^{bc}	0.83±0.05^{b}	0.85±0.00^{bc}	0.85±0.11^{bc}	0.85±0.20^{b}
	¼× MIC	0.84±0.00^{b}	0.83±0.00^{b}	0.83±0.00^{b}	0.83±0.03^{b}	0.83±0.00^{b}	0.85±0.02^{b}

$^{a–e}$ 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.

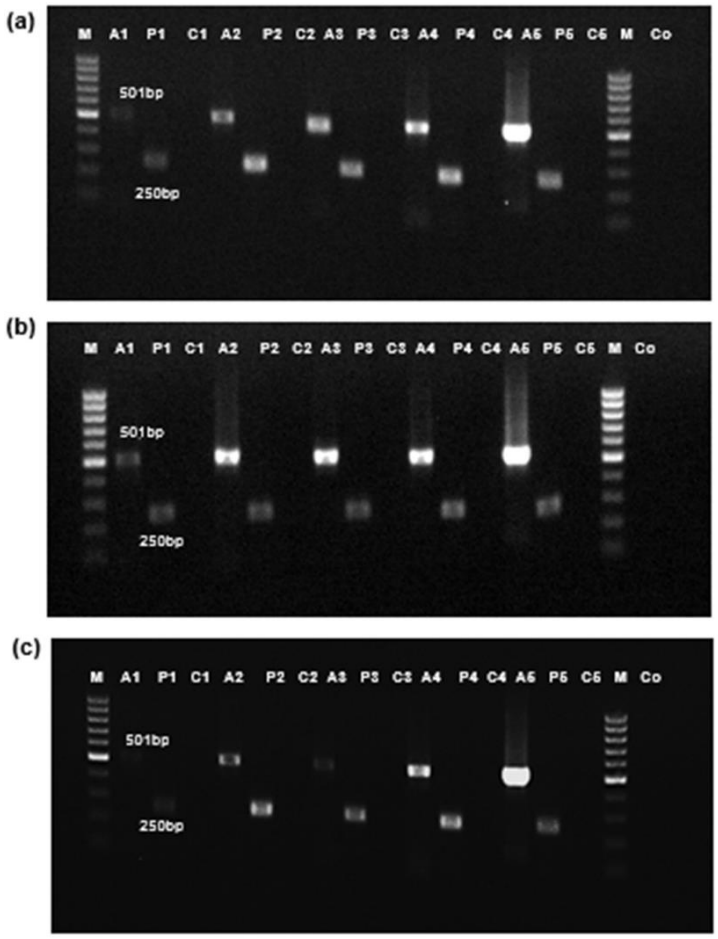

Figure 3. Gel electrophoresis of semi quantitative RT-PCR product of *PLB* gene from *C. tropicalis* ATCC 750 treated with fluconazole (A), amphotericin B (B) and fluconazole/amphotericin B (C). M: 100 bp DNA Ladder, A1: *Actin* with 2× MIC concentration of antifungals, P1: *PLB* with 2× MIC concentration of antifungals, C1: Internal control without M-MuLV reverse transcriptase, A2: *Actin* with 1× MIC concentration of antifungals, P2: *PLB* with 1× MIC concentration of antifungals, C2: Internal control without M-MuLV reverse transcriptase, A3: *Actin* with ½× MIC concentration of antifungals, P3: *PLB* with ½× MIC concentration of antifungals, C3: Internal control without M-MuLV reverse transcriptase, A4: *Actin* with ¼× MIC concentration of antifungals, P4: *PLB* with ¼× MIC concentration of antifungals, C4: Internal control without M-MuLV reverse transcriptase, A5: *Actin* without antifungals (untreated control), P5: *PLB* without antifungals (untreated control), C5: Internal control without M-MuLV reverse transcriptase, Co: Control negative for PCR.

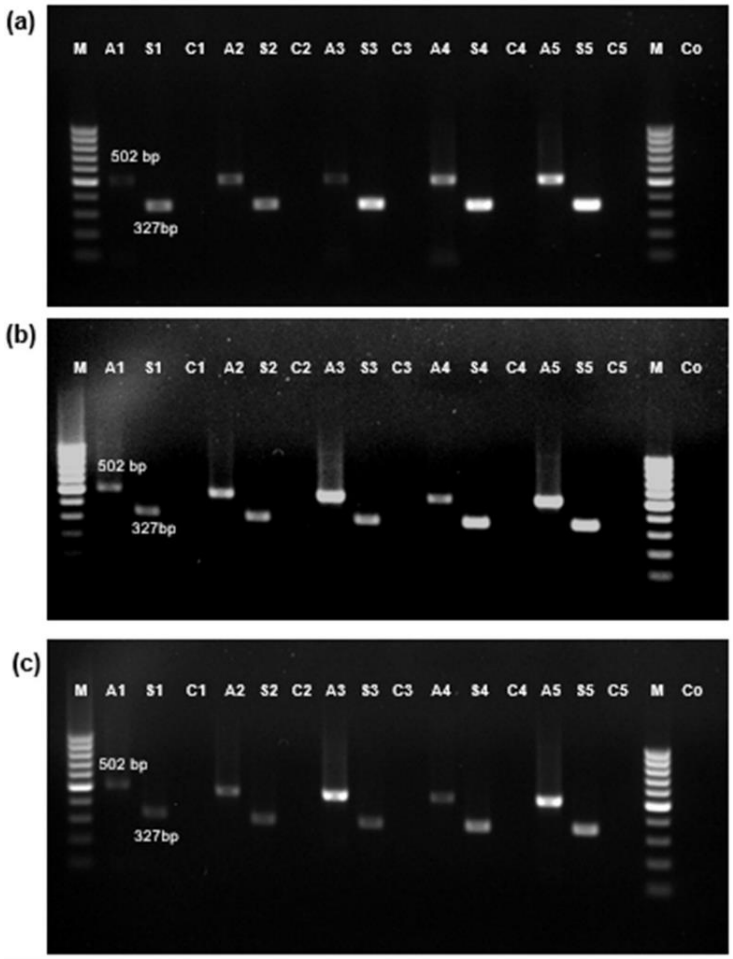

Figure 4. Gel electrophoresis of quantitative RT-PCR product of *SAP2* gene from *C. tropicalis* ATCC 750 treated with fluconazole (a), amphotericin B (b) and fluconazole/amphotericin B (c). M: 100 bp DNA Ladder, A1: *Actin* with 2× MIC concentration of antifungals, S1: *SAP2* with 2× MIC concentration of antifungals, C1: Internal control without M-MuLV reverse transcriptase, A2: *Actin* with 1× MIC concentration of antifungals, S2: *SAP2* with 1× MIC concentration of antifungals, C2: Internal control without M-MuLV reverse transcriptase, A3: *Actin* with ½× MIC concentration of antifungals, S3: *SAP2* with ½× MIC concentration of antifungals, C3: Internal control without M-MuLV reverse transcriptase, A4: *Actin* with ¼× MIC concentration of antifungals, S4: *SAP2* with ¼× MIC concentration of antifungals, C4: Internal control without M-MuLV reverse transcriptase, A5: *Actin* without antifungals (untreated control), P5: *SAP2* without antifungals (untreated control), C5: Internal control without M-MuLV reverse transcriptase, Co: Control negative for PCR.

The fold change values of *PLB* expression to untreated control for 2× MIC, 1× MIC, ½× MIC and ¼× MIC concentrations of fluconazole were 0.50 ± 0.007, 0.53 ± 0.002-, 0.56 ± 0.003- and 0.63 ± 0.004-fold, respectively. The fold change values of *PLB* expression for 2× MIC, 1× MIC, ½× MIC and ¼× MIC concentrations of amphotericin B were 0.52 ± 0.001-, 0.67 ± 0.006-, 0.69 ± 0.004- and 0.69 ± 0.007-fold, respectively. While, the *PLB* mRNA was down-regulated 0.44 ± 0.001-, 0.45 ± 0.002-, 0.46 ± 0.004- and 0.62 ± 0.006- fold at concentrations of 2× MIC, 1× MIC, ½× MIC and ¼× MIC of fluconazole/amphotericin B, respectively. Also, the fold change values regarding to *SAP2* expression for 2× MIC, 1× MIC, ½× MIC and ¼× MIC concentrations of fluconazole were 0.50 ± 0.01-, 0.53 ± 0.01-, 0.57 ± 0.01- and 0.62 ± 0.01-fold, respectively. The fold change values of *SAP2* expression for 2× MIC, 1× MIC, ½× MIC and ¼× MIC concentrations of amphotericin B were 0.52 ± 0.01-, 0.55 ± 0.01-, 0.61 ± 0.01- and 0.71 ± 0.01-fold, respectively. Moreover, the *SAP2* mRNA was down-regulated 0.48 ± 0.01-, 0.51 ± 0.01-, 0.57 ± 0.01- and 0.70 ± 0.01- fold at concentrations of 2× MIC, 1× MIC, ½× MIC and ¼× MIC of fluconazole/amphotericin B, respectively. Indeed the expression level of *PLB* and *SAP2* was down-regulated 1.45–2.27- and 1.41–2.08-fold, respectively, at different concentrations based on MIC of fluconazole alone and in combination with amphotericin B.

Discussion

C. tropicalis followed by *C. glabrata* is the most prevalent isolate from non-*albicans Candida* species and accounts for 4 to 25% of all cases of candidiasis. Although *C. tropicalis* are usually considered susceptible to fluconazole, but in the few years, increase in resistance to fluconazole has been observed. Inaddition, amphotericin B is relatively ineffective to the host environment, novel approaches to therapy are urgently needed.[29-30]

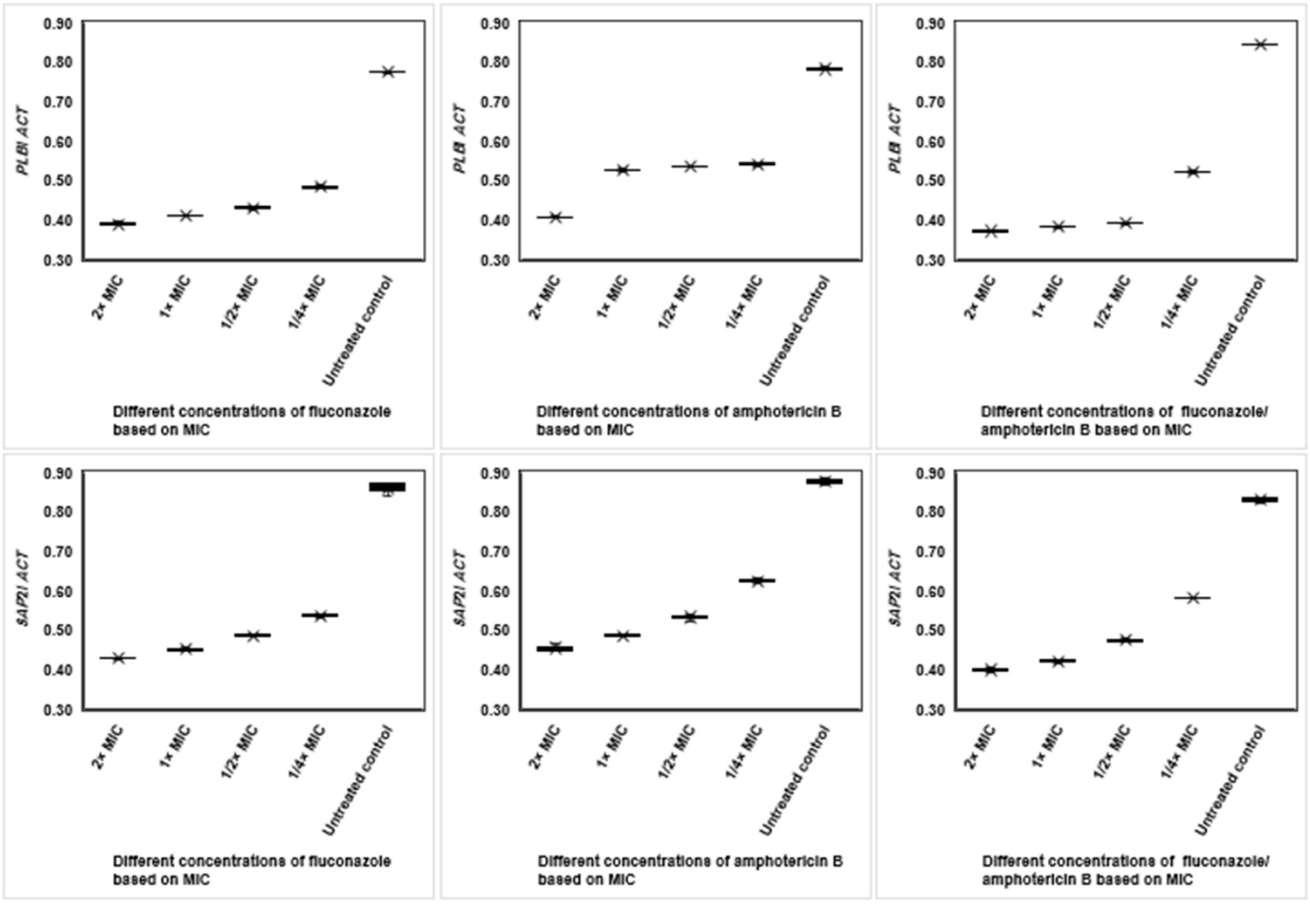

Figure 5. Box plots of *PLB/ ACT* and *SAP2/ ACT* ratio at different concentrations of fluconazole alone and in combination with amphotericin B based on MIC.

In the present work, we investigated the synergistic interaction of fluconazole/amphotericin B on inhibition of enzymes contributes to the pathogenesis of *C. tropicalis* obtained from immunocompromised patients in Yasooj, Iran. Most of all the isolates tested was synergistic interaction of fluconazole/amphotericin B. These results corroborate the most current literature research, where is cited the synergistic or indifferent interaction of fluconazole/amphotericin B in *Candida* spp.[31-33] Fluconazole inhibit the sterol biosynthetic pathways in fungi, while amphotericin B acts by binding to such sterols, creating pores in the fungal membrane. The mechanism proposed for potential synergism between fluconazole or amphotericin B is simultaneous inhibition of various components of fungal cell targets, especially cell membrane targets,[17,31,33] meaning that the potential for synergistic interaction of fluconazole/amphotericin B is possible. Indeed, the effects of fluconazole in combination with amphotericin B were shown to be significant in *Candida* by some reports.[34-35]

The primary factor in the *Candida* colonization is adherence to host cells and ability to filamentation and penetrate into the cells.[7,16] Concerning the production of virulence factors, we investigated the ability of *C. tropicalis* isolates treated with fluconazole alone and in combination with amphotericin B to produce hyphae cells. The tested antifungals on the *C. tropicalis* completely reduced the number of yeast form in time kill study. This work demonstrated the ability of fluconazole alone and in combination with amphotericin B to inhibit the yeast to hyphae transition represents the essential virulence factor of *Candida*, suggesting that tested antifungals could decrease the ability of *C. tropicalis* cells to cause disease. In addition, extracellular tissue-damaging hydrolytic enzymes appear to play a key role in the adherence of *Candida* to host surfaces, tissue penetration, invasion and possibly to destruction of host tissues. The two most significant hydrolytic enzymes produced by *C. tropicalis* are the SAPs and phospholipases.[4,6,16,36] The *C. tropicalis* isolates treated with fluconazole alone and in combination with amphotericin B decreased proteolytic and phospholipase activity in comparison with the untreated control. This finding may be due to the fact that tested antifungals could also reduce the ability of *C. tropicalis* cells to cause disease.

Down-regulated expression of *PLB* and *SAP2* genes was found in *C. tropicalis* ATCC 750 treated with fluconazole alone and in combination with amphotericin B. The down-regulated expression of *PLB* and *SAP2* genes in *C. tropicalis* ATCC 750 treated with fluconazole/ amphotericin B combination extends the findings of our initial study, where the expression of this gene was first reported. The down-regulated hydrolytic enzymes-

specific genes, consistent with the morphological yeast to hyphae switch which completely reduced the number of yeast form, inhibit the yeast to hyphae transition and reduced of proteolytic and phospholipase activity. Importantly, fluconazole and amphotericin B alone significantly reduced *C. tropicalis* pathogenic properties compared with untreated control and the fluconazole in combination with amphotericin B significantly reduced the *C. tropicalis* pathogenic properties compared with fluconazole and amphotericin B alone. Few studies have investigated the expression of *PLB* and *SAP2* genes in *C. tropicalis* treated with antifungals. This study, to the best of our knowledge, is the first one to be done on *PLB* and *SAP2* genes of *C. tropicalis* treated with antifungals. Our results are in partial agreement with Khodavandi et al.[23] revealed that fluconazole in combination with terbinafine significantly down-regulated the expression of *ERG*1, 3, and 11 genes in *C. albicans.* Ibrahim et al.[37] investigated the effect of high doses of fluconazole alone and in combination with voriconazole and amphotericin B on the expression levels of *CDR1*, *KRE1* and *SKN1* genes responsible for *Candida* biofilm resistance. Significant up-regulation of *SKN1* expression and to a lesser extent *KRE1* was observed in *Candida* biofilms treated with amphotericin B alone or in combination. Choi et al.[38] revealed up-regulation of *CDR1*, *MDR1*, and *ERG11* genes in fluconazole-nonsusceptible *C. tropicalis* isolates. Fernandes et al.[39] showed that *C. tropicalis* resistance to voriconazole is unable to control biofilms, and the up-regulation of *ERG* genes is likely to be probable molecular mechanism of *Candida* biofilm resistance. The azole resistant isolate of *C. tropicalis ERG11* was up-regulated, which found to be in agreement with the relatively larger amount of ergosterol in isolate. Moreover, up-regulation of *ERG11* associated with a missense mutation in this gene.[40] With regards the *SAP* genes, different expression profiles in *SAP* genes were obtained. Khodavandi et al.[24] revealed that the allicin had no significant effect on the expression levels of SAPs1-4 genes, whereas fluconazole was able to down-regulated the expression of *SAP4* gene.

Conclusion

The potential of synergistic interaction of fluconazole/amphotericin B on inhibition of enzymes contributes to the pathogenesis of *C. tropicalis.* In addition, *PLB* and *SAP2* genes could be probable molecular targets in combination of fluconazole with amphotericin B in *C. tropicalis*.

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Conflict of interests

The authors claim that there is no conflict of interest.

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