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Original Article

Interaction of *Candida albicans* with Fluconazole/ Clotrimazole: Effect on Hyphae Formation and Expression of Hyphal Wall Protein 1

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Background and Aims: *Candida albicans* (*C. albicans*) is the most common opportunistic human pathogen. Therapeutic options for *Candida* infections are limited to available antifungal drugs. The aim of this study was to investigate the effects of fluconazole/clotrimazole (FLU/CLT) on *C. albicans* hyphae formation.

Materials and Methods: We have established the effectiveness of the combination of FLU/CLT on *C. albicans* hyphae formation. Interaction of *C. albicans* with combination of FLU/CLT was performed using the CLSI guidelines and time-killing curves. We investigated the anti-hyphal activities of combination of FLU/CLT against *C. albicans* using XTT and crystal violet assays as well as scanning electron microscopy and expression of *HWP1* gene.

Results: The interaction of *C. albicans* with FLU/CLT resulted in synergistic, partial synergistic and indifferent effects. The interaction of FLU/CLT were confirmed by time-killing curves. FLU/CLT combined resulted in the reduction of metabolic activity and hyphae formation in *C. albicans.* Images taken by scanning electron microscopy indicated the effectiveness on hyphae disruption. According to relative real time polymerase chain reaction analysis, the mean Ct values revealed the significant decrease in expression level of the *HWP1* gene. A 2.86- and 2.33-fold decrease in *HWP1* gene expression was observed in combination of FLU/CLT treatment at 2× minimum inhibitory concentration and 1× minimum inhibitory concentration, respectively (p=0.002).

Conclusions: We confirmed that the hyphae is a target for the combination of FLU/CLT in *C. albicans*. *HWP1* gene is likely to be considered as a probable targets synergistic interaction of FLU/CLT against *C. albicans.*

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Introduction

Candida albicans (C. *albicans*) is a pleomorphic fungus that commonly exists as a normal resident microbiota of the human body. Nevertheless, this interaction between the host and microbe is not always benign, and as a pathogen, *C. albicans* is responsible for a wide range of infections from mucosal to invasive systemic candidiasis. However, pathogenesis of *C. albicans* is facilitated by environmental conditions [1-5]. Colonization of *C. albicans* occurs as a result of adherence to host epithelial cells [4, 6]. The adhesion of *C. albicans* to epithelial cells is a complex process and involve several different factors. Following adhesion of *C. albicans* to the cell surface, hyphae growing out of the yeast are thought to play a major role in the pathogenesis of the *C. albicans* and the infection progresses. There are a range of adhesins that bear the potential to interact with host epithelial cell receptors. One of the most studied adhesins to date is the hyphal wall protein 1 (HWP1). HWP1, which its N-terminal domain mimicing human epithelial cell transglutaminase substrates, is required for mucosal pathogenicity [2, 3, 7, 8]. *HWP1* is strongly induced during hyphae growing out of the yeast. The transcriptional activators and repressors coordinately regulate *HWP1* expression in *C. albicans* that control morphology [9, 10].

Much as considerable progress around antifungals has made adequate treatment of candidiasis, the available studies have demonstrated unacceptably high mortality associated with invasive systemic candidiasis [11-13]. For the treatment of candidiasis azole, antifungal agents are recommended. All of the azole antifungals inhibit the function of cytochrome P_{450} system to some degree of specificity [14]. The rapid emergence of azole drug-resistant of *C. albicans* has been reported [15, 16]. There are numerous evidences for effective combinations of antifungal agents [17, 18]. Mendling et al., revealed that combination of fluconazole (FLU) and clotrimazole (CLT) with the sequential dose of FLU is effective for the treatment of recurrent *Candida* vaginitis [19]. In the present study, we investigated the effect of combination of FLU/CLT on *C. albicans* hyphae formation. We have used gene expression profiling of hypha-specific gene (*HWP1)* to investigate the transcriptional responses of *C. albicans* hyphae exposed to combination of FLU/CLT at concentrations based on minimum inhibitory concentration (MIC).

Materials and Methods

C. albicans and chemicals

C. albicans ATCC 14053 reference strain was purchased from Iranian Research Organization for Science and Technology. Ten clinical isolates were obtained during recurrent vulvovaginal candidiasis infection (defined as 3 or more per year) in patients who had previously used FLU and CLT in the past 5 years. Frozen glycerol stock of the isolates were regularly revived on Sabouraud Dextrose Agar (SDA, Merck Research Laboratories, Darmstadt, Germany). Roswell Park Memorial Institute (RPMI)-1640 medium with L-

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glutamine without sodium bicarbonate (Sigma-Aldrich Co. St. Louis, MO, USA) was buffered with 0.165 M morpholine-propanesulfonic acid (MOPS, Sigma-Aldrich) to a pH of 7. Stock solutions of FLU (Sigma-Aldrich) and CLT (Sigma-Aldrich) were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich) according to recommendations provided by the Clinical and Laboratory Standards Institute (CLSI) M27-A3 guidelines and stored at -20˚C until use. The FLU and CLT were mixed in 1:1 ratio [20, 21].

Susceptibility testing

Antifungal activity of FLU and CLT alone and in combination against planktonic cells of clinical isolates of *C. albicans* was tested by broth microdilution method using CLSI M27-A3 guidelines. Serially double-diluted concentrations of FLU range 0.0312–64 µg/ml and CLT range 0.0313-16 µg/ml alone and in combination were prepared in RPMI-1640 medium. One hundred µl of each dilution was dispensed into the well of a presterilized, U-bottomed 96-well polystyrene microtiter plate in the presence of 100 µl of the *C. albicans* planktonic cell suspensions of final density 5×10^2- 2.5×10^3 cells/ml. RPMI-1640 medium containing 5% DMSO was included in control wells. Thereafter, microtiter plates were kept at 4˚ C for 2 h and incubated at 35˚ C for 24 h. After incubation, growth of cells was measured by Stat Fax 303 Reader (Awareness Technology, Inc., USA) at a wavelength of 530 nm. Totally, 90% and 50% inhibitory concentrations were taken as the relative MIC values [20, 21].

Interaction of fluconazole/clotrimazole

The interaction between FLU and CLT was determined on the basis of the fractional inhibitory concentration (FIC) index. The FIC index was defined as follows: [(MIC of drug A in combination/MIC of drug A alone)] + [(MIC of drug B in combination/MIC of drug B alone)]. The interaction was defined as synergistic if the FIC index was ≤0.5, partial synergy when the FIC> 0.5 but < 1.0, additive if the FIC = 1.0, indifferent when the FIC index > 1.0 but < 4.0, and antagonistic if the FIC ≥ 4.0 [22].

Time-killing test

In order to further confirm the effect of FLU and CLT alone and in combination, *C. albicans* ATCC 14053 and CI-5- isolate were used to determine the time-killing curves. Initial inoculum at a density of 1×10^6 cells/ml were treated with different concentrations (2×MIC and 1×MIC) of FLU and CLT alone and in combination. The number of viable cells was determined by colony counting at 0, 2, 4, 6, 8, 10, 12, 24, and 48 h after exposure to antifungal agents at 35˚C. The interaction between FLU and CLT tested by time-kill methods (at 24 to 48 h) were determined as follows: synergy, ≥2 $\log_{10}$ decrease in CFU/ml compared to the most active agent; additive effect <2 but >1 $\log_{10}$ decrease in CFU/ml compared to the most active agent; indifference, <2 but >1 $\log_{10}$ increase in CFU/ml compared to the least active agent; and antagonism, a ≥2 $\log_{10}$ increase in CFU/ml compare to the least active agent [23, 24].

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Combination of fluconazole/clotrimazole on *C. albicans* hyphae

The hyphae formation assay was performed in U-bottomed 96-well polystyrene microtiter plate as described previously [25]. Briefly, 100 µl of the *C. albicans* ATCC 14053 cell suspension (1×10^6 cells/ml) was dispensed into the wells of microtiter plates. Different concentrations (2×MIC, 1×MIC, ½×MIC and ¼×MIC) of FLU and CLT alone and in combination were added (100 µl per well) to each well and incubated at 35°C for 90 min without agitation. The microtiter plate was incubated at 35°C for 16 h with gentle shaking. The metabolic activity of the *C. albicans* hyphae was determined quantitatively using colorimetric XTT [2,3-bis (2-methoxy-4-nitro-5 sulfophenyl)-5-[(phenylamino) carbonyl 2-[H-tetrazolium hydroxide] reduction and crystal violet assays.

XTT reduction assay of *C. albicans* hyphae

At the end of appropriate incubation, the supernatant was aspirated from the wells and washed 3-times with sterile phosphate-buffered saline. The *C. albicans* cell viability was determined using colorimetric XTT reduction assay [25]. Sterilized XTT/menadione (Sigma-Aldrich) solution transferred to each well containing prewashed hypha and incubated in the dark at 37°C for 5 h. After the incubation, colorimetric change in the XTT reduction was measured in a microtiter microplate reader at 490 nm.

Crystal violet assay of *C. albicans* hyphae

The prewashed *C. albicans* hyphae was fixed with methanol and air dried. Then, *C. albicans* hyphae was stained with crystal violet solution (Sigma-Aldrich) for 20 min. Hundred µl of acetic acid 33% (Sigma-Aldrich) was distributed into the wells containing prewashed hyphae. The optical density was measured at 590 nm with Stat Fax 303 Reader (Awareness Technology) [25-27].

Scanning electron microscopy of *C. albicans* hyphae

Standard cell suspension of *C. albicans* was diluted (1×10^6 cells/ml) in RPMI-1640 medium supplemented with combination of FLU/CLT at MIC concentration on Thermanox™ plastic coverslips (Nunc, Denmark) in 6-well cell culture plates (Nunc). The cell culture plates were incubated at 35°C for 90 min (without agitation) and incubated again at 35° C for 16 h with gentle shaking. Thereafter, the prewashed hyphae was fixed in 2% (v/v) glutaraldehyde in phosphate-buffered saline (PH 7.2). Samples were washed with sodium cacodylate buffer and placed in 1% osmium tetroxide for 2 h at 4° C.

Samples were subsequently washed with sodium cacodylate buffer, dehydrated in a series of ascending ethanol solutions, put into critical point dryer and then stuck onto the stub. The specimens were coated with gold and viewed with a Philips XL30 (ESEM, UK) scanning electron microscope [25].

qRT-PCR analysis of *C. albicans* hypha-specific gene

C. albicans hyphae was performed in the presence of standard cell suspension of *C. albicans* and different concentrations

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(2×MIC and 1×MIC) of FLU and CLT alone and in combination in 6-well cell culture plates (Nunc).

Total RNA was extracted from *C. albicans* hyphae using RNeasy Mini Kit (Qiagen, Hilden, Germany) and 0.5 µg of total RNA was reverse transcribed with Moloney-Murine Leukemia Virus (MMLV) reverse transcriptase and random hexamers (Fermentas, USA). Resultant cDNAs were amplified by real time polymerase chain reaction (RT-PCR) using hypha specific and housekeeping internal control beta-actin gene primers (Table 1). Real time PCR reactions were run with ™SYBR Green qPCR Master Mix (Fermentas, USA) on Bio-Rad MiniOpticon™ system (USA). Relative expression was quantified by the Pfaffl method [23, 25].

The Research Ethics Committees of our institute (Ethical code 1213469) approved the study. The study protocol conformed to the ethical guidelines of the 2013 Declaration of Helsinki. Informed consent was obtained from patients.

Statistical analysis

Data were expressed as mean values of the biological replicates±standard deviations. One-way analysis of variance was applied to test the differences between treated and control groups. Tukey's HSD test was performed for a multiple comparison and statistical significance was tested at the $p<0.05$ levels. Statistical analyses were performed using the software SPSS 21.0 for windows (SPSS Inc. Chicago, IL, USA).

Results

Table 2 illustrates the susceptibility of clinical isolates of *C. albicans* and reference strains of *C. albicans* ATCC 14053 to FLU alone and in combination with CLT. The isolates were considered resistant at the MIC value ≥8.0 µg/ml for fluconazole, and ≥1.0 µg/ml for CLT as established by the CLSI M27-A3. As presented in table 2, all clinical isolates of *C. albicans* were resistant to FLU (MIC range 12.30-18.90 µg/ml), and CLT (MIC range 1.25-2.50 µg/ml).

The combined effects of the FLU and CLT against clinical isolates of *C. albicans* and reference strains of *C. albicans* ATCC 14053 are shown in table 3. A comparison between these values showed that the combination of FLU and CLT can be decreased from its MIC value of FLU and CLT by 6.79- to 61.50-fold and 1.04- to 6.25-fold, respectively. FLU showed synergistic (70%), partial synergistic (20%) and indifferent (10%) interaction with CLT against clinical isolates of *C. albicans*.The synergism of FLU/CLT against *C. albicans* ATCC 14053 and CI-5 isolate were confirmed by time-killing curves (Fig. 1). The combination of FLU/CLT at 2×MIC and 1×MIC concentrations against *C. albicans* ATCC 14053 revealed a 4.06, 4.10, 3.16 and 3.60 $\log_{10}$ CFU /ml decrease compared with FLU alone at 24 and 48 h, respectively. The results indicated that FLU and CLT action alone have little antifungal effect throughout 48 h in CI-5- isolate of *C. albicans*, but the combination yielded 2 × MIC and 1 × MIC a 6.03, 4.80, 7.02 and 5.95 $\log_{10}$ CFU/ml decrease compared with fluconazole alone at 24 and 48 h, respectively.

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Table 1. Primers for analysis of hyphae specific gene by RT-PCR

Primer	Orientation	Sequence	Reference
HWP1	Forward	5´ TCAGTTCCACTCATGCAACCA 3´	[28]
	Reverse	5´ AGCACCGAAAGTCAATCTCATGT 3´	
Beta-actin	Forward	5´ GAGTTGCTCCAGAAGAACATCCAG 3´	[29]
	Reverse	5´ TGAGTAACACCATCACCAGAATCC 3´	

Table 2. Susceptibility of clinical isolates of *C. albicans* in terms of MIC (µg/ml) to FLU and CLT

Isolates/ Antifungal agents	FLU		CLT	
	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}
***C. albicans* ATCC 14053 (Control)**	3.50±0.10	0.40±0.02	0.90±0.01	0.45±0.20
CI- 1	12.30±0.30	6.70±0.02	1.25±0.10	0.50±0.10
CI- 2	16.30±0.01	7.90±0.10	2.50±0.05	0.50±0.03
CI- 3	16.50±0.03	8.80±0.10	2.50±0.04	0.50±0.01
CI- 4	15.50±0.05	6.80±0.03	2.50±0.06	0.25±0.09
CI- 5	16.50±0.04	3.70±0.03	2.50±0.04	0.25±0.02
CI- 6	12.50±0.30	3.90±0.04	2.50±0.05	0.50±0.09
CI- 7	13.50±0.04	6.60±0.10	1.70±0.09	0.25±0.08
CI- 8	13.50±0.05	6.50±0.10	1.50±0.02	0.25±0.09
CI- 9	18.50±0.10	6.60±0.09	1.50±0.08	0.25±0.02
CI- 10	18.90±0.02	6.90±0.04	1.50±0.04	0.25±0.05

CI= Clinical isolates of *C. albicans*

Table 3. Interaction of FLU and CLT against clinical isolates of *C. albicans*

Isolates/ Antifungal agents	FLU/CLT				
	MIC_{90}	MIC_{50}	FIC_{90}	FIC_{50}	Outcome
***C. albicans* ATCC 14053 (Control)**	0.09±0.05	0.05±0.01	0.13	0.24	Synergy
CI- 1	0.20±0.01	0.05±0.04	0.18	0.11	Synergy
CI- 2	2.40±0.02	0.90±0.05	1.11	1.91	Indifferent
CI- 3	0.90±0.04	0.06±0.04	0.42	0.13	Synergy
CI- 4	0.90±0.05	0.07±0.09	0.42	0.29	Synergy
CI- 5	1.50±0.01	0.19±0.05	0.69	0.81	Partial Synergy
CI- 6	1.40±0.02	0.25±0.01	0.67	0.56	Partial Synergy
CI- 7	0.50±0.01	0.09±0.09	0.33	0.37	Synergy
CI- 8	0.50±0.05	0.09±0.02	0.37	0.37	Synergy
CI- 9	0.50±0.01	0.08±0.01	0.36	0.33	Synergy
CI- 10	0.50±0.09	0.07±0.00	0.36	0.29	Synergy

CI= Clinical isolates of *C. albicans*

Table 4 exhibits the significant results to reduce hyphae after treatment with antifungal agents using XTT and CV assays for FLU and CLT alone and in combination in all concentrations based on MIC (p=0.001). XTT and CV assays for hyphae quantification also indicated *C. albicans* ATCC 14053 hyphae treated with combination of FLU and CLT being more significant than FLU and CLT alone (p=0.001).

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Table 4. Results of XTT and CV assays against *C. albicans* ATCC 14053 hyphae treated with FLU and CLT alone and in combination in different concentrations based on MIC

Concentration of antifungal agents	Means absorbance at 490 nm±SD using XTT assay			Means absorbance at 590 nm±SD using CV assay		
	FLU	CLT	FLU/CLT	FLU	CLT	FLU/CLT
2 × MIC	0.13 ± 0.01	0.15 ± 0.06	0.10 ± 0.03	1.38 ± 0.01	1.44 ± 0.08	1.09 ± 0.03
1 × MIC	0.15 ± 0.01	0.18 ± 0.02	0.11 ± 0.02	1.44 ± 0.04	1.48 ± 0.09	1.11 ± 0.05
½ × MIC	0.16 ± 0.02	0.19 ± 0.04	0.12 ± 0.02	1.48 ± 0.02	1.49 ± 0.07	1.19 ± 0.06
¼ × MIC	0.18 ± 0.01	0.21 ± 0.01	0.13 ± 0.02	1.55 ± 0.04	1.61 ± 0.09	1.22 ± 0.05
Untreated control	0.43 ± 0.02	0.43 ± 0.03	0.43 ± 0.04	2.11 ± 0.01	2.11 ± 0.01	2.11 ± 0.01

The combination of FLU and CLT against *C. albicans* ATCC 14053 hyphae was visually verified by scanning electron microscopy (SEM) (Fig. 2). The growing hyphae was composed by yeast cells and hyphae form in untreated control group. The FLU and CLT combination-treated hyphae reduced in number and density of cells and also completely destroyed *C. albicans* hyphae.

In qRT-PCR, a variable Ct value of *HWP1* gene in *C. albicans* ATCC 14053 hyphae treated with FLU and CLT alone and in combination revealed their gene expression levels being affected by the treatments (Fig. 3). The mean Ct values revealed that the combination of FLU/CLT induces a significant decrease in expression level of the *HWP1* gene by 2.86- and 2.33-fold at 2× MIC and 1× MIC concentrations, respectively (p=0.002). Furthermore, the expression of *HWP1* gene in *C. albicans* ATCC 14053 hyphae treated with FLU and CLT FLU and CLT alone showed a variable level of expression compared to the untreated control.

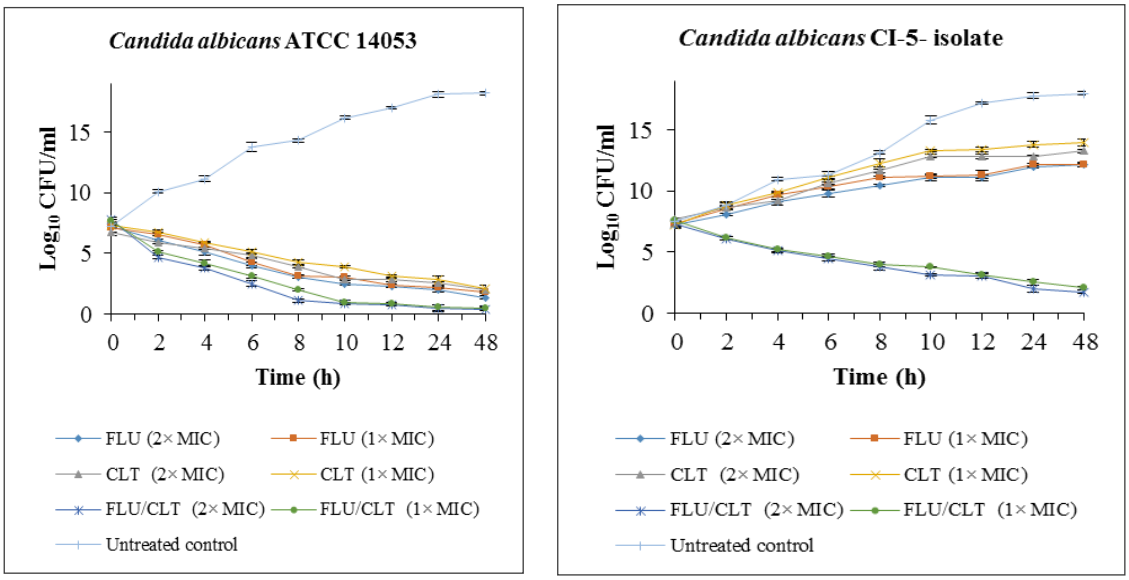


Fig. 1. Time-killing curves of FLU and CLT alone and in combination against *C. albicans* ATCC 14053 and CI-5 isolate at 0, 2, 4, 6, 8, 10, 12, 24, and 48 h. Data is represented as mean±SD.

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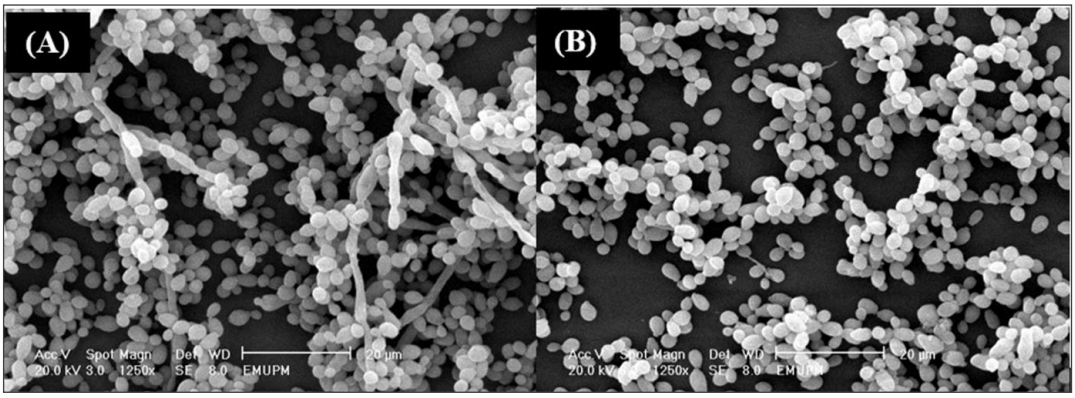


Fig. 2. Scanning electron microscopy of *C. albicans* ATCC 14053 hyphae. (A) Untreated control, the morphology is composed of yeast cells and hyphae form. (B) Treated with combination of FLU and CLT at MIC concentration, complete destruction of hyphae form and reduced yeast cells were observed. Magnification 1250×.

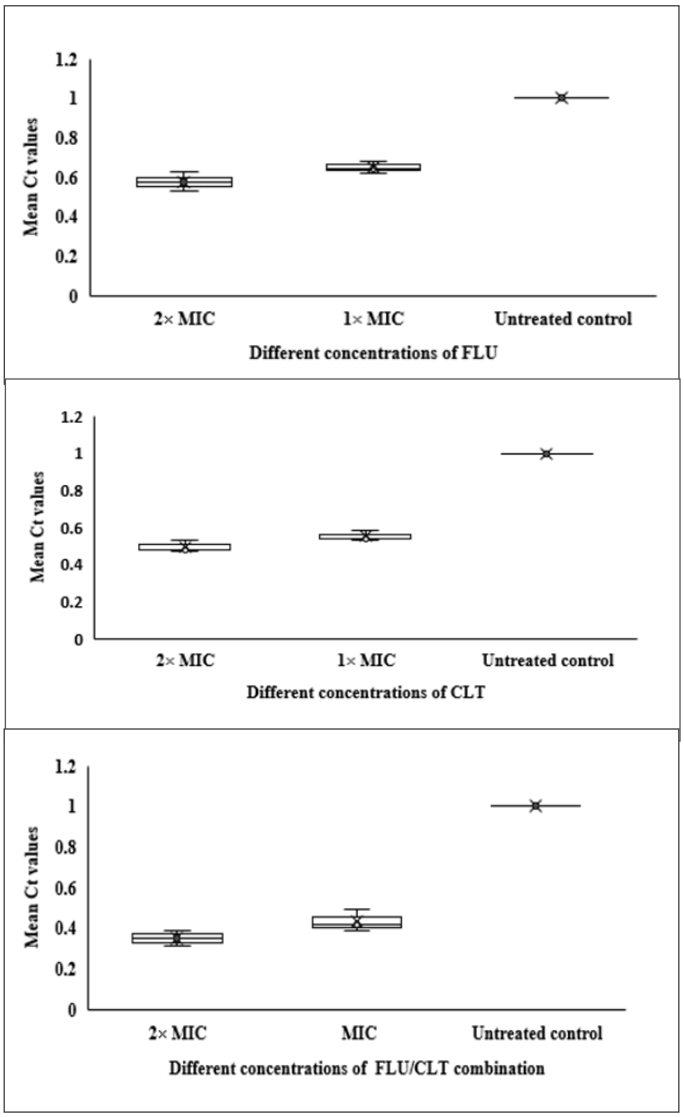


Fig 3. Whisker box plots of *HWP1* gene expression at different concentrations of FLU and CLT alone and in combination based on MIC in *C. albicans* ATCC 14053 hyphae. The boxes represent the mean Ct values and the line inside the box represents median values. The bars across the boxes show locations of the minimum and maximum Ct values. Whiskers in the plot represent 95% confidence intervals. Data represent mean values±SD.

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Discussion

Therapeutic strategies for fungal infections are limited by the small number of available antifungal drugs so that there is a need for efficient methods to identify novel drug targets and develop effective drug combinations [23, 30]. The switch between yeast and hyphae is a major role in the pathogenesis of *C. albicans* that could serve as target for treatment of infections. Several studies have proven useful in achieving that goal [23, 30-32]. In the current report, we demonstrate the combination of FLU/CLT on *C. albicans* and uncover their effective combinations on hyphae formation.

A synergistic effect was observed when FLU combined with CLT in clinical isolates proved resistant to FLU and CLT. FLU and CLT alone and in combination inhibited hyphal formation; they inhibited cell proliferation at higher concentrations. Combination of FLU/CLT is an extensively investigated drug combination that was shown to inhibit *Candida* cells [19, 33]. In an attempt to investigate the effectiveness combination of FLU/CLT on *C. albicans* hyphae, the growing hyphae was verified by XTT and CV assays and SEM. The combination of FLU and CLT destroyed hyphae and yeast form, suggesting their effective combinations on hyphae formation. We then analysed the expression level of hyphae specific gene in the *C. albicans* hyphae. Of three treatments, combination of FLU/CLT decreased and more variable expression of hyphae specific gene; in addition, FLU and CLT alone demonstrated a variable level of expression thus suggesting a change in the expression of *HWP1* gene involved in hyphae formation and pathogenesis. We confirmed that hyphae is a target of the combination of FLU/CLT by showing inhibition of cell proliferation and hyphae formation and molecular analysis of hyphae specific gene in *C. albicans*.

Microbial analysis has already indicated that azole antifungal drugs are effective on *C. albicans* hyphae formation [23, 31, 34, 35]. Likewise, a role for HWP1 in *C. albicans* hyphal growth had been demonstrated by adhesins to host epithelial cell receptors [2, 3, 7, 8]. Our study provides further support for this interaction by linking pharmacological inhibition of hyphae formation. The inhibition of hyphae formation was accompanied by a reduction in hyphae specific gene expression. Khodavandi et al. exhibited that combination of FLU/ amphotericin B reduced yeast-hyphal transition and biomass and metabolic activity of the hypha in *C. albicans* [23]. Wakabayashi et al. demonstrated that triazole antifungal agents combined with triazoles lactoferrin-related compounds inhibit the growth of hyphae, an important form of pathogenesis in azole-resistant *C. albicans* strains [30]. The synergistic antifungal effect of licofelone in combination with fluconazole against *C. albicans* strongly reduced hyphal formation. The expression of RAS/cAMP/PKA pathway related genes (*RAS1*, *CYR1*, *TPK2*), and biofilm formation related genes including biofilm and

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cell wall regulator 1 (*BCR1)*, *HWP1*, agglutinin-like sequence (*ALS*) 1 and *ALS3* genes were down-regulated with the presence of licofelone combined with fluconazole [31]. FLU in combination with CLT revealed significant synergistic effects against *C. tropicalis* with FIC indices ranging from 0.011 to 0.43. In addition, the combination of FLU with CLT could trigger a down-regulation of *C. tropicalis* agglutinin-like sequence (*ALS1* and *ALS3*), lipase (*LIP1* and *LIP4*), and secreted aspartyl protease (*SAP2* and *SAP4*) genes, respectively [36].

Conclusion

Our intention in assaying the effectiveness combination of FLU/CLT on *C. albicans* was to investigate the hyphae formation and transcriptional responses. Our results suggest that the *HWP1* might constitute useful targets for the combination of FLU and CLT. This raises the prospect of identifying molecular mechanisms of combination of FLU/CLT as potential inhibitors of *C. albicans* virulence. However, this study demonstrated that the antifungal effect of FLU in combination with CLT could be critical in developing therapeutic strategies against *C. albicans*.

Conflict of interest

The authors declared no conflict of interest.

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