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Original Article

Expression of Lanosterol 14-Demethylase (*ERG11*) Gene of Three-Drug Combinations in *Candida albicans*

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Abstract

Patients with impaired immunity are at particular risk of infections with *Candida albicans.* Antifungal drugs such as azoles commonly used for candidiasis treatment, but drug resistance is one of the most common problems for public health. The aim of this study was to evaluate the expression of lanosterol 14-demethylase (*ERG11*) gene for three-drug combinations in *C. albicans.* Disk diffusion and broth microdilution susceptibility tests were employed to evaluate the synergic effects of amphotericin B, fluconazole, voriconazole, ketoconazole and itraconazole. Quantification of *ERG11* gene expression was carried out in *C. albicans* treated with three-drug combinations of fluconazole/ ketoconazole/ voriconazole and fluconazole/ ketoconazole/ itraconazole. Three-drug combinations revealed synergistic and partial synergistic effect for all tested isolates (FIC index range of 0.27-0.77). The expression levels of *ERG11* were down-regulated by three-drug combination of fluconazole/ ketoconazole/ voriconazole treatment. Fluconazole synergizes with ketoconazole and voriconazole in three-drug combination against *C. albicans* by targeting of the *ERG11* gene.

Keywords: *Candida albicans,* Fluconazole, *ERG11,* Itraconazole, Ketoconazole, Synergic effect, Voriconazole.

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1. Introduction

Fungi are eukaryotes that their cell structure and cellular metabolism are similar to animal cells. Some species of the fungus can cause serious infections which are hard to both treat and diagnose. Candida albicans is a potentially deadly fungal pathogen, particularly in immune-compromised patients. The therapeutic options for fungal infections

are quite limited compared to bacterial infections [1, 2].

The antifungal drugs in use include polyenes, azoles, echinocandins, allylamines and miazines. Polyenes such as amphotericin B blocks the ergosterol biosynthetic pathway via selective disruption of fungal cell membranes by directly binding to ergosterol and forming membrane pores, causing leakage of essential contents of the cell. Azoles are the most widely used class of antifungal drugs, which inhibit the activity of the lanosterol 14α-demethylase (Erg11p) leads to block the ergosterol biosynthesis and accumulation of toxic sterol intermediates. The ultimate end result of effect of azole antifungals on fungi is lysis of the cell and death [3-7]. Various imidazole (diazole) antifungal drugs have been claimed to inhibit Candida growth. Moreover, triazoles represent wide spectra of antifungal activity against fungi. Although azole antifungals are members belonging to the same class of antifungal drugs, they have very different chemical properties that impact the pharmacokinetics and spectrum of activities. However, compared with imidazole antifungal drugs such as ketoconazole and miconazole, which characterized by five-membered rings with two nitrogen atoms, triazoles such as fluconazole, itraconazole and voriconazole are characterized by five-membered rings containing three nitrogen atoms. In addition, the extended hydro-phobic side chains of itraconazole and ketoconazole provide extra points of the pharmacokinetics and spectrum of activities [2, 8, 9].

The polyene and azole antifungal drugs interfere with ergosterol function. Ergosterol is the major product of sterol biosynthesis, which play an important role in structural and signaling functions [10-12]. In C. albicans, ergosterol is synthesized from lanosterol, albeit through various enzyme reactions and requires oxygen at multiple steps. The biosynthesis of ergosterol consists of 20 enzymes encoded by the specific genes. The lanosterol 14α-demethylase enzyme, encoded by ERG11, is suggested to be major rate-limiting enzyme in ergosterol biosynthetic pathway [7, 13].

The ever-increasing array of antifungal drug resistance of C. albicans continue to pose a serious life-threatening infection. Thus, it seems that there is important to find powerful methodical approaches that could be used to treat candida infections. The combination of two or more therapeutic antifungal drugs is a cornerstone for treatment of life-threatening infections. The use of combination strategy and the synergistic effect obtained by the combination of antifungal drugs has likely greatly improved efficacy of existing drugs such as azoles and reducing their harmful side effects on the host cell [9, 12, 14, 15]. In the current study, we have evaluated in vitro activity of amphotericin B, fluconazole, voriconazole, ketoconazole and itraconazole alone and in three-drug combinations against C. albicans. Moreover, the gene expression levels of ERG11 gene were assessed by RT-PCR in C. albicans treated with fluconazole, voriconazole, ketoconazole and itraconazole in three-drug combinations.

2. Materials and Methods

2.1. Materials and Organisms

All drugs were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). *Candida albicans* ATCC 14053 was obtained from the Iranian Research Organization for Science and Technology. Clinical isolates of *C. albicans* were isolated from immune-compromised patients (diabetes, cancer and maintenance hemodialysis patients) at the clinical center of Shahid Beheshti, Yasooj, Iran. Clinical isolates were routinely cultured on Sabouraud Dextrose Agar (SDA, Merck Research Laboratories, Darmstadt, Germany) supplemented with 300 µg/ml of chloramphenicol, and individual colonies were identified by microscopic and macroscopic morphology, germ tube formation and inoculated into freshly prepared CHROMagar *Candida* medium (CHROMagar Company, France). Once clinical isolates identified, culture was stored at − 80 °C in Sabouraud Dextrose Broth (SDB, Merck Research Laboratories) with chloramphenicol and sterile 20% (v/v) glycerol [16].

2.2. Antifungal Susceptibility Assays

One hundred µl of *C. albicans* cell suspensions (1×10^6 to 5×10^6 cells/ml) were poured into SDA plates. The prepared discs at concentrations of 5, 10 and 15 mg/ml of amphotericin B, fluconazole, voriconazole, ketoconazole and itraconazole placed on the agar surface. Moreover, the prepared discs at a final concentration of 15 mg/ml of amphotericin B, fluconazole, voriconazole, ketoconazole and itraconazole in three-drug combinations (fluconazole/ voriconazole/ amphotericin B, fluconazole/ ketoconazole/ amphotericin B, fluconazole/ itraconazole/ amphotericin B, fluconazole/ itraconazole/ voriconazole, fluconazole/ ketoconazole/ voriconazole, fluconazole/ ketoconazole/ itraconazole) placed on the agar surface and incubated at 35 °C for 24 h. The diameter of the inhibition zone observed around the discs was measured (Modified Clinical and Laboratory Standards Institute guidelines, CLSI -M44-A2) [17].

The minimum inhibitory concentrations (MICs) of antifungal drugs alone and in three-drug combinations were determined with standard broth microdilution assay according to the CLSI guidelines (M27-A3) [2, 18]. The 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)] was used to prepare *C. albicans* cell suspensions of final density $5 \times 10^2 – 2.5 \times 10^3$ cells/ml. One hundred µl of *C. albicans* cell suspensions were aliquoted into 96-well plates in the presence of 100 µl of the two-fold dilution of the fluconazole (ranging 0.03125–64 µg/ml), voriconazole, ketoconazole and itraconazole (ranging 0.0313–16 µg/ml) alone and in three-drug combinations. If, for example, *C. albicans* growth occurs between dilution of 2 µg/ml and 4 µg/ml for fluconazole, the MIC was determined with serial dilutions ranging of 2-4 µg/ml. The microplates, including antifungal drugs and *C. albicans* cell suspensions, were kept at 4 °C for 2 h and incubated at 35°C for 24 h. The

absorbance was measured at 530 nm using a Stat Fax 303 Reader (Awareness Technology, Inc., USA). The lowest concentration of the antifungal drugs which cause ≥50% or 90% reduction in absorbance compared to the positive control was considered the MIC. Eventually, the fractional inhibitory concentration (FIC) index was calculated as [(MIC of drug A in combination/MIC of drug A alone)]+[(MIC of drug B in combination/MIC of drug B alone)]+[(MIC of drug C in combination/MIC of drug C alone)]. Synergy was defined as FIC index ≤0.5; partial synergy when the FIC > 0.5 but < 1.0, additive as FIC = 1.0, indifferent when the FIC index > 1.0 but < 4.0, and antagonistic if the FIC ≥ 4.0 [19, 20].

2.3. RNA Extraction and RT-PCR

C. albicans (ATCC 14053) cells were exposed to three-drug combinations of fluconazole/ ketoconazole/ voriconazole and fluconazole/ itraconazole/ voriconazole at different concentrations based on MIC (2 × MIC, 1 ×MIC, 1/2 × MIC and 1/4 ×MIC). The mixture was centrifuged at 3000 rpm for 10 min. The pellet was washed three times with PBS. RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was reverse-transcribed with M-MuLV reverse transcriptase (Fermentas, USA). Reverse transcription was carried out in the presence of random hexamer oligonucleotides (Fermentas). PCR reactions were carried out using PCR Kit (Fermentas). For amplification of ERG11 transcripts, oligonucleotide primer was taken from the literature (Table 1). PCR products were resolved by QIAquick Gel Extraction kit (Qiagen) and sequence by First BASE Laboratories Sdn. Bhd., Malaysia [21].

2.4. Relative Quantitation of Gene Expression Assay

This assay was carried out as previously described [16]: the concentration of PCR products were compared to DNA mass standard (MassRuler Low Range DNA Ladder, Ready-to- Use, Fermentas)] according to manufacturer's operating instructions of Quantity One 1-D Analysis software 4.6.5 (Bio-Rad). The fold change in target gene expression was calculated as target/reference ratio of experimental sample relative to target/reference ratio of untreated control sample. Up-regulation was defined as statistically significant variation ($P \leq 0.05$) and a fold change of ≥ 2- fold and down-regulation if the fold change ≤ 0.5.

Table 1. Primers and their specifications used in this study.

Primer	Orientation	Sequence	Length (bp)	Reference
ERG11	Forward	5' TTGGTGGTGGTAGACATA 3'	163	[21]
	Reverse	5' TCTGCTGGTTCAGTAGGT 3'		
ACT	Forward	5' ACCGAAGCTCCAATGAATCCAAAATCC 3'	516	[21]
	Reverse	5' GTTTGGTCAATACCAGCAGCTTCCAAA 3'		

2.5. Ethical Issues

Research Ethics Committees of our institute approved the study (Ethics No. 9411). The study conformed to the ethical guidelines of the 2008 Declaration of Helsinki.

2.6. Statistical Analysis

Results are presented as the mean ± standard deviation of three independent experiments and statistical analysis was performed using analysis of variance (ANOVA) with Tukey's HSD test. Statistical significance was tested at the $P \leq 0.05$ levels. Statistical analyses were performed using SPSS 20.0 (SPSS Inc. Chicago, IL, USA).

3. Results and Discussion

The Antifungal Activity of Amphotericin B, Fluconazole, Voriconazole, Ketoconazole and Itraconazole Alone and in Three-Drug Combinations against C. albicans We employed 10 clinical isolates of C. albicans as well as a reference strain C. albicans ATCC 14053 to evaluate the antifungal effects of amphotericin B, fluconazole, voriconazole, ketoconazole and itraconazole alone and in three-drug combinations. Table 2 showed the inhibition zone of amphotericin B, fluconazole, voriconazole, ketoconazole and itraconazole alone against clinical isolates of C. albicans at 5, 10 and 15 mg/ml concentration. Itraconazole was the most active drug against all tested isolates following by voriconazole, ketoconazole and fluconazole. Drugs at 15 mg/ml concentration had the highest efficacy ($P \leq 0.05$) with inhibition zone range of 14–30 mm. Therefore, we chose 15 mg/ml concentration for further studies against *C. albicans*. The three-drug combination results are shown in Table 3. The inhibition zone of the amphotericin B, fluconazole, voriconazole, ketoconazole and itraconazole in three-drug combination ranged from 16–44 mm. Evaluation of the three-drug combination revealed a most active three-drug combination effect of fluconazole/ ketoconazole/ voriconazole and fluconazole/ ketoconazole/ itraconazole for all tested isolates, respectively. Our favorable findings were verified by broth microdilution assay. Table 4 summarizes the MIC values of the fluconazole, voriconazole, ketoconazole and itraconazole alone and in three-drug combinations against clinical isolates of *C. albicans.* The MIC range of fluconazole, voriconazole, ketoconazole and itraconazole when used alone against *C. albicans* were subsequently 2.30–3.60 µg/ml, 1.20–2.50 µg/ml, 1.10–2.50 µg/ml and 1.25–2.50 µg/ml. Results of the three-drug combinations of fluconazole/ itraconazole/ voriconazole, fluconazole/ ketoconazole/ voriconazole, fluconazole/ ketoconazole/ itraconazole revealed that 100% of the clinical isolates of *C. albicans* had synergistic (FIC= 0.27-0.54) or partial synergistic (FIC= 0.57-0.77) effects.

Table 2. Disk diffusion test results (mm) of amphotericin B (AMB), fluconazole (FLU), voriconazole (VORI), ketoconazole (KETO) and itraconazole (ITRA) at different concentrations (mg/ml) alone against clinical isolates of *C. albicans* after 24 h incubation at 35°C.

Antifungal agents/ Isolates		**Ca ATCC 14053**	**CI1**	**CI2**	**CI3**	**CI4**	**CI5**	**CI6**	**CI7**	**CI8**	**CI9**	**CI10**
AMB	5 mg/ml	8.00±0.01	9.00±0.02	8.00±0.01	7.00±0.00	8.00±0.01	9.00±0.00	9.00±0.01	10.00±0.01	7.00±0.00	7.00±0.01	9.00±0.02
	10 mg/ml	12.00±0.00	11.00±0.03	12.00±0.00	13.00±0.01	14.00±0.03	12.00±0.00	14.00±0.02	12.00±0.02	12.00±0.00	14.00±0.02	10.00±0.00
	15 mg/ml	16.00±0.01	15.00±0.03	16.00±0.01	17.00±0.03	14.00±0.02	16.00±0.01	18.00±0.00	15.00±0.01	17.00±0.02	16.00±0.00	14.00±0.01
FLU	5 mg/ml	10.00±0.00	10.00±0.01	12.00±0.02	11.00±0.01	10.00±0.01	11.00±0.02	12.00±0.01	12.00±0.02	10.00±0.00	9.00±0.00	11.00±0.01
	10 mg/ml	18.00±0.02	18.00±0.01	19.00±0.02	20.00±0.00	18.00±0.01	16.00±0.00	19.00±0.01	17.00±0.02	19.00±0.01	20.00±0.00	16.00±0.02
	15 mg/ml	24.00±0.01	22.00±0.01	23.00±0.03	24.00±0.02	22.00±0.01	21.00±0.00	23.00±0.01	24.00±0.02	23.00±0.00	24.00±0.01	22.00±0.00
VORI	5 mg/ml	12.00±0.00	12.00±0.00	11.00±0.01	10.00±0.01	10.00±0.02	10.00±0.01	13.00±0.01	12.00±0.00	12.00±0.01	10.00±0.01	14.00±0.02
	10 mg/ml	22.00±0.02	21.00±0.01	23.00±0.01	24.00±0.03	22.00±0.00	20.00±0.00	20.00±0.00	19.00±0.03	21.00±0.02	22.00±0.01	20.00±0.00
	15 mg/ml	30.00±0.01	27.00±0.02	29.00±0.03	30.00±0.00	26.00±0.01	25.00±0.00	28.00±0.01	27.00±0.01	29.00±0.03	28.00±0.02	24.00±0.00
KETO	5 mg/ml	12.00±0.00	11.00±0.01	13.00±0.02	12.00±0.00	12.00±0.01	12.00±0.00	10.00±0.01	11.00±0.02	11.00±0.00	11.00±0.01	12.00±0.00
	10 mg/ml	20.00±0.00	19.00±0.03	21.00±0.02	22.00±0.01	19.00±0.01	18.00±0.00	20.00±0.01	18.00±0.00	20.00±0.00	21.00±0.03	18.00±0.01
	15 mg/ml	28.00±0.00	26.00±0.03	28.00±0.00	27.00±0.01	26.00±0.00	24.00±0.02	27.00±0.01	26.00±0.00	26.00±0.02	27.00±0.01	23.00±0.01
ITRA	5 mg/ml	12.00±0.00	11.00±0.01	12.00±0.00	10.00±0.01	11.00±0.01	11.00±0.00	11.00±0.02	13.00±0.01	11.00±0.00	11.00±0.00	13.00±0.01
	10 mg/ml	22.00±0.02	20.00±0.00	22.00±0.00	23.00±0.01	21.00±0.01	19.00±0.03	21.00±0.01	19.00±0.02	20.00±0.00	21.00±0.03	19.00±0.03
	15 mg/ml	30.00±0.00	26.00±0.01	29.00±0.03	28.00±0.00	26.00±0.00	24.00±0.01	28.00±0.02	29.00±0.03	30.00±0.00	29.00±0.01	25.00±0.03

CI: Clinical isolates of *C. albicans*.

Data are means ± standard deviation of three independent experiments.

Table 3. Disk diffusion test results (mm) of amphotericin B (AMB), fluconazole (FLU), voriconazole (VORI), ketoconazole (KETO) and itraconazole (ITRA) in three-drug combinations against clinical isolates of *C. albicans* at 15 mg/ml concentration after 24 h incubation at 35°C.

Antifungal agents/ Isolates	**Ca ATCC 14053**	**CI1**	**CI2**	**CI3**	**CI4**	**CI5**	**CI6**	**CI7**	**CI8**	**CI9**	**CI10**
FLU/ VORI/ AMB	18.00±0.03	16.00±0.01	19.00±0.01	17.00±0.02	18.00±0.00	20.00±0.01	18.00±0.02	18.00±0.00	20.00±0.02	16.00±0.00	18.00±0.01
FLU/ KETO/ AMB	20.00±0.00	20.00±0.01	18.00±0.02	20.00±0.02	21.00±0.01	21.00±0.00	19.00±0.01	21.00±0.00	20.00±0.01	18.00±0.00	21.00±0.01
FLU/ ITRA/ AMB	18.00±0.00	18.00±0.00	20.00±0.01	18.00±0.00	17.00±0.01	20.00±0.02	21.00±0.00	18.00±0.00	17.00±0.02	21.00±0.00	17.00±0.00
FLU/ ITRA/ VORI	36.00±0.02	28.00±0.01	31.00±0.02	35.00±0.01	36.00±0.00	34.00±0.01	32.00±0.02	29.00±0.01	30.00±0.01	29.00±0.01	35.00±0.02
FLU/ KETO/ VORI	40.00±0.00	38.00±0.01	38.00±0.00	37.00±0.01	40.00±0.00	44.00±0.02	39.00±0.01	38.00±0.00	40.00±0.02	37.00±0.01	41.00±0.03
FLU/ KETO/ ITRA	38.00±0.01	37.00±0.02	39.00±0.01	36.00±0.00	36.00±0.01	35.00±0.00	38.00±0.01	37.00±0.01	39.00±0.00	38.00±0.02	34.00±0.03

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

Table 4. MIC (μg/ml) and FIC values of fluconazole (FLU), voriconazole (VORI), ketoconazole (KETO) and itraconazole (ITRA) alone and in three-drug combinations against clinical isolates of *C. albicans*.

Antifungal agents/ Isolates		**Ca ATCC 14053**	**CI1**	**CI2**	**CI3**	**CI4**	**CI5**	**CI6**	**CI7**	**CI8**	**CI9**	**CI10**
FLU	MIC_{90}	3.50±0.10	2.50±0.20	2.30±0.10	3.30±0.20	2.60±0.30	3.50±0.20	2.50±0.30	3.60±0.10	3.50±0.50	2.30±0.20	2.90±0.30
	MIC_{50}	0.50±0.08	0.80±0.09	0.60±0.02	0.60±0.01	0.50±0.00	0.70±0.08	0.50±0.02	0.60±0.01	0.50±0.02	0.70±0.01	0.80±0.07
VORI	MIC_{90}	1.20±0.09	2.00±0.02	2.50±0.20	2.00±0.09	1.80±0.40	2.50±0.10	2.00±0.01	2.50±0.10	1.90±0.02	2.20±0.08	2.50±0.10
	MIC_{50}	0.40±0.01	0.40±0.09	0.35±0.02	0.50±0.01	0.50±0.01	0.39±0.04	0.45±0.08	0.50±0.03	0.40±0.02	0.50±0.01	0.53±0.02
KETO	MIC_{90}	1.10±0.09	1.50±0.02	1.40±0.20	2.10±0.09	2.00±0.40	2.25±0.10	1.50±0.01	2.50±0.10	1.70±0.02	1.90±0.08	1.80±0.10
	MIC_{50}	0.35±0.05	0.40±0.02	0.39±0.00	0.50±0.09	0.32±0.01	0.38±0.08	0.47±0.02	0.50±0.01	0.40±0.02	0.35±0.09	0.43±0.01
ITRA	MIC_{90}	1.25±0.02	1.60±0.10	1.40±0.07	2.10±0.20	1.80±0.01	2.50±0.10	1.80±0.05	2.10±0.03	1.50±0.06	1.80±0.03	1.60±0.09
	MIC_{50}	0.35±0.01	0.36±0.02	0.35±0.01	0.42±0.04	0.38±0.09	0.40±0.03	0.32±0.05	0.39±0.01	0.36±0.09	0.39±0.02	0.38±0.06
FLU/ ITRA/ VORI	MIC_{90}	0.30±0.05	0.35±0.02	0.40±0.09	0.45±0.03	0.40±0.01	0.50±0.02	0.50±0.09	0.42±0.02	0.50±0.01	0.36±0.03	0.50±0.09
	MIC_{50}	0.075±0.09	0.09±0.01	0.08±0.02	0.083±0.09	0.07±0.01	0.08±0.09	0.095±0.01	0.088±0.05	0.096±0.09	0.095±0.01	0.085±0.06
	FIC	0.58	0.53	0.62	0.58	0.60	0.54	0.73	0.49	0.74	0.52	0.69
	Interpretation	PSYN	SYN	PSYN	PSYN	PSYN	SYN	PSYN	SYN	PSYN	SYN	PSYN
FLU/ KETO/ VORI	MIC_{90}	0.25±0.03	0.20±0.06	0.40±0.09	0.39±0.01	0.40±0.06	0.51±0.04	0.43±0.02	0.49±0.05	0.25±0.04	0.35±0.05	0.50±0.02
	MIC_{50}	0.062±0.09	0.063±0.01	0.091±0.02	0.085±0.09	0.064±0.01	0.08±0.09	0.081±0.01	0.063±0.05	0.09±0.09	0.085±0.01	0.075±0.06
	FIC	0.51	0.31	0.62	0.50	0.58	0.58	0.67	0.35	0.35	0.49	0.65
	Interpretation	SYN	SYN	PSYN	SYN	PSYN	PSYN	PSYN	SYN	SYN	SYN	PSYN
FLU/ KETO/ ITRA	MIC_{90}	0.20±0.09	0.30±0.05	0.20±0.03	0.40±0.02	0.20±0.04	0.50±0.05	0.25±0.01	0.40±0.02	0.50±0.02	0.18±0.05	0.30±0.01
	MIC_{50}	0.065±0.09	0.09±0.02	0.065±0.03	0.09±0.05	0.062±0.00	0.07±0.09	0.09±0.01	0.086±0.01	0.091±0.09	0.096±0.02	0.08±0.01
	FIC	0.40	0.51	0.37	0.50	0.29	0.57	0.41	0.46	0.77	0.27	0.46
	Interpretation	SYN	SYN	SYN	SYN	SYN	PSYN	SYN	SYN	PSYN	SYN	SYN

CI: Clinical isolates of *C. albicans*.
SYN: synergism, PSYN: partial synergism.

3.1. Relative Quantitation of Gene Expression Assay

RT- PCR was performed due to study the effect of three-drug combinations on a gene expression level of ergosterol biosynthesis pathway. The *C. albicans* ATCC 14053 cells were exposed to three-drug combinations of fluconazole/ ketoconazole/ voriconazole and fluconazole/ ketoconazole/ itraconazole (2× MIC, 1× MIC, ½× MIC and ¼× MIC values) for 24 h and their relative quantitation of gene expression was evaluated. The *ERG11* gene was found significantly down-regulated by 2.90-, 2.05-, 1.88- and 1.94-fold after 2× MIC, 1× MIC, ½× MIC and ¼× MIC fluconazole/ ketoconazole/ voriconazole treatment, respectively (Tukey's HSD, $P \leq 0.05$). No obvious change was found in the expression of *ERG11* in *C. albicans* cells treated with 2× MIC, 1× MIC, ½× MIC and ¼× MIC of fluconazole/ ketoconazole/ itraconazole by 1.05-, 0.85-, 0.93- and 1.10-fold, respectively (Figure 1). The box plots allow comparison of *ERG11/ACT* ratio at different concentrations of fluconazole/ ketoconazole/ voriconazole and fluconazole/ ketoconazole/ itraconazole in three-drug combinations based on MIC (Figure 2). The identity of the *ERG11* gene was confirmed by DNA sequencing and nucleotide BLAST software.

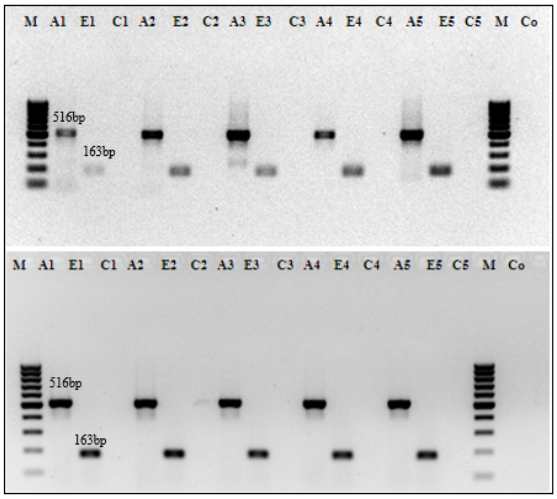

Figure 1. Expression pattern of *ERG11* gene in *C. albicans* ATCC 14053 treated with fluconazole (FLU), itraconazole (ITRA) and voriconazole (VORI) in three-drug combinations (A), fluconazole (FLU), ketoconazole (KETO) and voriconazole (VORI) in three-drug combinations (B). M: (100 bp) DNA mass standard marker, A1: *Actin* with 2× MIC concentration of three-drug combinations, E1: *ERG11* with 2× MIC concentration of three-drug combinations, C1: Internal control without M-MuLV reverse transcriptase, A2: *Actin* with 1× MIC concentration of three-drug combinations, E2: *ERG11* with 1× MIC concentration of three-drug combinations, C2: Internal control without M-MuLV reverse transcriptase, A3: *Actin* with ½× MIC concentration of three-drug combinations, E3: *ERG11* with ½× MIC concentration of three-drug combinations, C3: Internal control without M-MuLV reverse transcriptase, A4: *Actin* with ¼× MIC concentration of three-drug combinations, E4: *ERG11* with ¼× MIC concentration of three-drug combinations, C4: Internal control without M-MuLV reverse transcriptase, A5: *Actin* without antifungals (untreated control), E5: *ERG11* without antifungals, C5: Internal control without M-MuLV reverse transcriptase, Co: Control negative for PCR.

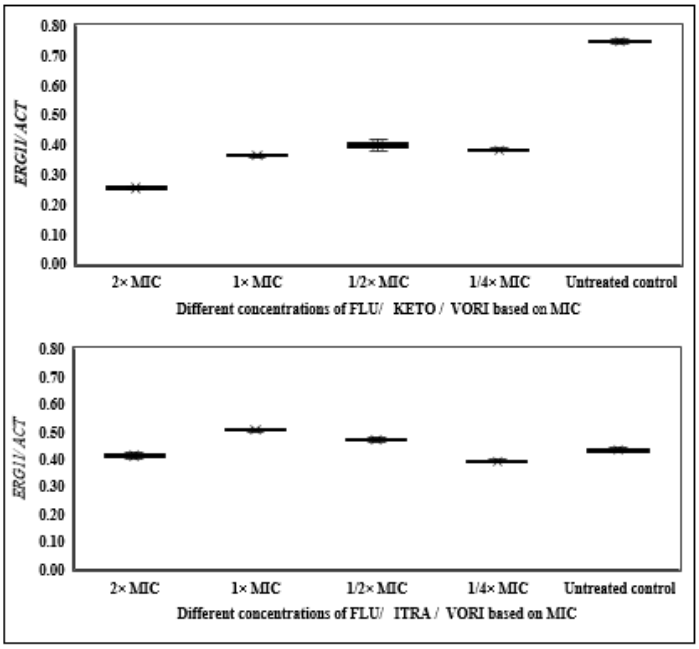

Figure 2. Box plots of *ERG11/ ACT* ratio at different concentrations of fluconazole (FLU), voriconazole (VORI), ketoconazole (KETO) and itraconazole (ITRA) in three-drug combinations based on MIC in *C. albicans* ATCC 14053.

3.2. Discussion

In the past few years, the antifungal armamentarium has significantly expanded; however, the substantial morbidity and mortality associated with invasive fungal infections remains unacceptably high in immune-compromised patients. These infections are often difficult to treat in high-risk patients, especially when antifungal resistance problem is one of the elements contributing to therapeutic failure. Recently, antifungal combinations are increasingly used in clinical practice as a promising strategy to improve outcomes for refractory some forms of invasive infections [14, 22-24]. Numerous studies indicated that the synergistic effect obtained by the antifungal compounds used in combination might improve the effectiveness of conventional antifungal drugs and reduce their side effects to host [9, 12, 14, 15, 24-26].

In the present work, we investigated the antifungal activity of amphotericin B, fluconazole, voriconazole, ketoconazole and itraconazole alone and in three-drug combinations on *C. albicans* obtained from immuno-compromised patients in Yasooj, Iran. Evaluation of the antifungal activity of amphotericin B, fluconazole, voriconazole, ketoconazole and itraconazole alone and in three-drug combinations displayed a most active three-drug combination effect of fluconazole/ ketoconazole/ voriconazole and fluconazole/ ketoconazole/ itraconazole for all tested isolates. All tested isolates revealed synergistic and partial synergistic effect in three-drug combinations. These results corroborate the most literature research, where

are cited the synergistic or indifferent interaction of antifungal drugs in *Candida* species [27-35]. Ghannoum et al [27] determine combinations of one-, two- and three-drug of amphotericin B, fluconazole and 5-fluorocytosine against *C. albicans*. The MICs of amphotericin B, fluconazole and 5-fluorocytosine were 0.23, 1.88 and 0.12 µg/ml, respectively against *C. albicans* ATCC 36082. Their results indicated that two-drug combinations (amphotericin B/ 5-fluorocytosine or fluconazole/ 5-fluorocytosine) had an indifferent effect and three-drug combinations (amphotericin B/ fluconazole/ 5-fluorocytosine) showed an additive effect on the growth of *C. albicans*. Paterson et al. [29] investigated the effect of the combination of amphotericin B with azoles (fluconazole, ketoconazole and itraconazole) against resistant *Candida* species in neutropenic patients. Results showed the percentage of patients colonized with *Candida* fell significantly due to a decrease in *Candida* species colonization. Characteristics identified for various azole antifungals revealed that they have very different physico-chemical properties that impact pharmacokinetics and spectrum of activities [2, 8, 9].

Between the three-drug combinations tested on a gene expression level of ergosterol biosynthesis (*ERG11*), fluconazole/ ketoconazole/ voriconazole combinations deserves more attention because this association has shown significant down-regulation at 2× MIC, 1× MIC, ½× MIC and ¼× MIC values. Several studies demonstrate down regulation of the *ERG11* gene in *C. albicans* treated with fluconazole [7, 16, 20, 36, 37]. This alteration could be due to fluconazole function in target gene coding major rate-limiting enzyme involved in the synthesis of ergosterol [38]. Alizadeh et al. [7] demonstrate the effect of fluconazole on down regulation of *ERG3* and *ERG11* genes. Also, Alizadeh et al. [16] revealed that expression pattern of *ERG11* gene was different in fluconazole-susceptible and -resistant *C. albicans*. Borecká-Melkusová et al [36] investigated the expression of the *ERG1*, *ERG3*, *ERG7*, *ERG9*, *ERG11* and *ERG25* genes in response to incubation with fluconazole in *C. albicans* and *Candida dubliniensis* clinical isolates. Low or moderate expression of the *ERG11* gene was observed and it was regulated by fluconazole. Khodavandi *et al* [20] demonstrated that, the fluconazole exhibited significant synergy with terbinafine against *C. albicans*. The expression levels of *ERG1, 3*, and *11* genes demonstrated significant reduction in comparison to fluconazole alone. The combination of *Thymus vulgaris* extracts with *Mentha spicata* revealed the decrease of *ERG11* in *C. albicans* [39].

4. Conclusion

In this study, we showed that fluconazole could be a candidate of synergist with voriconazole, ketoconazole and itraconazole in three-drug combinations against *C. albicans*. Whether these events reflect the potential of fluconazole/ ketoconazole/ voriconazole in three-drug combination for inhibition of *ERG11* gene in *C. albicans* which

differentially expresses specific gene requires further studies.

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