Original Article

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Inhibitory Effect of Carvacrol on the Expression of Candida albicans Hyphae-Specific Gene *(HWP1)*

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ABSTRACT

BACKGROUND AND OBJECTIVE: *Candida albicans* infection is a problem of growing clinical importance, particularly in immunocompromised populations. This study aimed to investigate the inhibitory effect of carvacrol on the hyphae formation and expression of *C. albicans HWP1*.

METHODS: This cross-sectional study was done over a 6-month period in 2016-2017. Vaginal, mouth and skin surface swabs were obtained from immunocompromised patients. Colonizing clinical isolates of *C. albicans* were identified and drug susceptible isolates detected using WHONET software. The susceptibility test for carvacrol (range 6.25–300 µg/ml) were carried out with a broth microdilution according to the CLSI guidelines against drug susceptible *C. albicans*. The time kill assay of carvacrol (range 2 × MIC to ¼ × MIC) was determined. Hyphae inhibition was evaluated by light microscopy. We determined the expression levels of *HWP1* implicated in hyphae formation of *C. albicans* ATCC 14053 cells by quantitative RT-PCR.

FINDINGS: Ten colonizing clinical isolates of drug susceptible *C. albicans* were identified. Carvacrol was inhibited the growth of all drugs susceptible *isolates of C. albicans* (MIC range, 25-200 µg/ml). Time kill curve assay demonstrated that carvacrol could significantly inhibit the growth of *C. albicans* ($p \leq 0.05$). Carvacrol efficiently prevented hyphae formation in drug susceptible *C. albicans*. The expression levels of *HWP1* gene were down-regulated by 1.82- and 1.62-fold at concentrations of 2 × MIC and 1×MIC of carvacrol, respectively.

CONCLUSION: These results suggest that carvacrol could provide an improved and safe clinical approach in treating *Candida* infections by prevention of hyphae formation.

KEY WORDS: *Candida albicans, Gene Expression, Hyphae*.

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Introduction

Transition from yeast to hyphal morphology is not only potentiated by virulence factors such as the secretion of hydrolases but also necessary for dissemination of fungi to different host sites. *Candida albicans* is a polymorphic pathogen with ability to yeast–hyphal transition. The filamentous hyphal cells display increased resistance to host cell-mediated phagocytosis, enhanced adherence to host cell surfaces with the ability to invade epithelial cells. Adhesion leads to colonization, production of germ tube, elongation of filaments and involved in dissemination within tissues, resulting in tissue damage (1-4).

Host tissue invasion is mediated via surface-associated cell adhesion molecule expression. The specific and nonspecific interactions enable *C. albicans* attachment to a wide variety of tissues with a specific protein adhesins. The important adhesion molecules are agglutinin-like sequence (ALS) proteins and hyphal cell wall protein (Hwp1).

The N-terminus of Hwp1 is a substrate for mammalian transglutaminase enzymes and induces a bond between *C. albicans* hyphae and host cells. Although *Candida* species are commensal members of normal microbiota, transient and localised perturbations to the mucosal sites can contribute to *Candida* overgrowth infection (2,3,5). Evidence demonstrate that the expression pattern of *HWP1* gene in *C. albicans* pathogenesis is associated with a number of other putative virulence factors and variety of host risk factors (6,7). Polyenes, azoles, allylamines, echinocandins, 5-fluorocytosine and miazines are clinically important antifungal agents that inhibit or interfere with the cell membrane, cell wall, protein and DNA synthesis (8-11). High throughput screening has emerged as a powerful tool for the identification of antifungal mechanism of natural products that could be used to treat the infections.

During recent years, studies demonstrated that carvacrol can act as a membrane-disrupting agent by targeting and binding to fungal membrane ergosterol (10,11). Carvacrol exhibits antifungal activity against *C. albicans* (9,11-17). Dalleau et al., (18) showed the antifungal efficacy of carvacrol on *C. albicans* planktonic cells and biofilms.

C. albicans have emerged as major causes of human disease, which ranges from superficial to deep-seated invasive candidiasis, especially among the immunocompromised patients (19,20). The aim of this study is to investigate the antifungal activity of carvacrol on drug susceptible *C. albicans*. In particular, we investigated the inhibitory effect of carvacrol using time kill assay, hypha formation and gene expression profiling on the *C. albicans*.

Methods

Microorganisms, reagents and growth medium: This cross-sectional study conducted over a 6-month period in 2016-2017. Vaginal, mouth and skin surface swabs were collected from 60 immunocompromised patients (diabetes, cancer and maintenance hemodialysis patients). All immunocompromised patients admitted to the Shahid Beheshti hospital affiliated to Yasooj University of Medical Sciences, Iran, were eligible for the study. The exclusion criteria for patients was candidiasis infections.

These clinically derived samples were collected under consent processes and transported to microbiology laboratory, Islamic Azad University of Yasooj, Iran. This study was approved by Research Ethics Committee of our institute (Ethical code 1280679) (The study protocol conformed to the ethical guidelines of the 2008 Declaration of Helsinki). The clinical samples were plated out on Sabouraud dextrose agar (SDA, Difco Laboratories, Detroit, Michigan) and incubated at 35°C for 24 h.

The colonizing *C. albicans* isolates were correctly identified by microscopic and macroscopic morphology, germ tube formation, CHROMagar™ *Candida* (CHROMagar, France), carbohydrate assimilations, carbohydrate fermentation, urease test and PCR-based detection identification using the universal fungal primers (ITS1 and ITS4). *C. albicans* ATCC 14053 was purchased from the Iranian Research Organization for Science and Technology.

For susceptibility of *C. albicans* to amphotericin B, fluconazole, ketoconazole and miconazole, results obtained by Clinical and Laboratory Standards Institute (CLSI) disk diffusion (M44-A2) and broth microdilution antifungal susceptibility tests (M27-A3 and M27-S4) were analyzed using WHONET software (21,22). To prepare suspensions of *C. albicans*, all of

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the colonizing isolates were freshly subcultured onto SDA. Stock solutions of carvacrol, amphotericin B, fluconazole, ketoconazole and miconazole (Sigma-Aldrich, St Louis, MO, USA) were prepared in the solvent dimethyl sulfoxide (DMSO).

Susceptibility assays: One hundred µl of suspensions of C. albicans (1-5×106 CFU/ml) was poured on SDA. The antibiotic discs such as amphotericin B (10 µg), fluconazole (15 µg), ketoconazole (15 µg) and miconazole (10 µg) (Rosco, Denmark) were placed on the agar surface. The diameter of the inhibition zone observed around the discs was measured in millimetres after 24 h of incubation at 35°C.

Minimum inhibitory concentration (MIC) for *C. albicans* cells were determined using broth dilution antifungal susceptibility test in accordance with CLSI M27-A3 and M27-S4 guidelines. In brief, serial two-fold dilutions of each antifungal compound in RPMI-1640 with L-glutamine (Sigma-Aldrich) buffered to pH 7.0 with 0.165 M morpholinophosphonyl sulfate (MOPS) were prepared and added to 96 U wells microtiter plates (Moheb Qazvin, Iran). The test *C. albicans* isolates were suspended in RPMI 1640 medium at a final density of 0.5-5 × 10^3 CFU/ml and added to the test wells containing diluted test antifungal compounds.

Drug- and yeast-free controls were also prepared as growth and sterile controls, respectively. All the plates were incubated at 35°C for 24 h and the absorbance at 530 nm was measured using a Stat Fax 303 Reader (Awareness Technology, Inc., USA). The lowest concentration of the test antifungal compound which cause ≥50% or 90% reduction in the absorbance compared to that of control was considered the MIC(22).

Time kill assay: The time kill assays were determined as described previously (23). In brief, cell suspensions of 1 × 10^6 cells/ml in RPMI 1640 medium were prepared and were added to different concentrations of carvacrol (2 × MIC, 1 ×MIC, ½× MIC and ¼ × MIC). These tubes were incubated for 48 h at 35°C and colonies counted at 0, 2, 4, 6, 8, 12, 24 and 48 h after incubation by plating 10-fold dilutions on SDA.

Hypha formation and treatment: *C. albicans* hyphae were developed on the surface of plastic coverslips in 6-well cell culture plates as described previously (24). In brief, cell suspensions of 1-5 × 10^6 cells/ml in RPMI 1640 medium were prepared and 4 ml were added to select wells containing 4 ml of different concentrations of carvacrol (2 × MIC, MIC, ½× MIC and ¼ × MIC) and incubated at 35 °C for 90 min. Hypha formation was induced by incubating cultures for 16 h at 35 °C with shaking at 200 rpm. The coverslips were washed with PBS and viewed with a light field microscope (Nikon, Japan).

Relative quantitation of gene expression: Hyphae of *C. albicans* (ATCC 14053) were grown in the absence or presence of different concentrations of carvacrol (2 × MIC, and 1 ×MIC) in 6-well plates as described above. Hyphae cells were removed from the bottom of the plates and the total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. 0.5 µg of template was reverse-transcribed with M-MuLV reverse transcriptase (Fermentas, USA). Primers for expression of hyphae-specific gene (*HWP1)* were taken from the literature (Table 1).

PCR consisted of denaturation at 95°C for 4 min, followed by 26 cycles of amplification (94°C for 40 s, 56°C for 45 s, and 72°C for 45 s) and final extension at 72 °C for 10 min using a TPersonal thermocycler (Biometra- Germany). The expression of *β actin* was used for normalization and to analyse the relative changes in target gene expression. Gene expression is given in relative values from Quantity One 1-D Analysis software 4.6.5 (Bio-Rad), setting the fold change in target gene expression = target/reference ratio in experimental sample relative to target/reference ratio in untreated control sample. Gene expression with statistically significant variation (p≤0.05) and a fold change of ≥ 2- fold or ≤ 0.5 were classified as the significant up-regulated or down- regulated, respectively. The PCR products were purified using the QIAquick Gel Extraction kit (Qiagen, USA) and confirmed by DNA sequencing (First BASE Laboratories Sdn. Bhd., Malaysia) (22,24). Statistical analysis: Results are expressed as mean value±standard deviation of three replicates. Data were analysed by analysis of variance (ANOVA). The comparison two means was calculated using the Tukey's Post hoc test. P≤0.05 were considered significant. Statistical analysis was performed using the SPSS software (version 21; SPSS Inc., Chicago, IL).

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Table 1. Primers used in this study

Primer	Sequence	Reference
HWP1-F	F: 5´ GGTAGACGGTCAAGGTGAAACA 3´	(24)
HWP1-R	R: 5´ AGGTGGATTGTCGCAAGGTT 3´	
ACT -F	F: 5´ ACCGAAGCTCCAATGAATCCAAAATCC 3´	(24)
ACT -R	R: 5´ GTTTGGTCAATACCAGCAGCTTCCAAA 3´	

Results

Out of 60 clinical samples 20 samples yielded C. albicans growth. The reliability of clinical isolates of C. albicans were confirmed by phenotypically and DNA sequencing. Out of 20 C. albicans isolates, 10 isolates were identified as drug susceptible using WHONET software. Tables 2 and 3 summarizes the in vitro susceptibilities of the clinical isolates of C. albicans to antifungal drugs as measured by the CLSI reference disk diffusion and broth microdilution antifungal susceptibility tests. Comparison of the results of the disk diffusion and broth microdilution antifungal susceptibility tests by kappa score demonstrated high degree of agreement for amphotericin B, fluconazole, ketoconazole and miconazole.

Table 2. Antifungal susceptibility results against *clinical isolates of C. albicans*

Antibiotic Name	Code	Antibiotic class	%R	%I	%S
Amphotericin B	AMB	Antifungals	45	-	55
Fluconazole	FLU	Antifungals	50	-	50
Ketoconazole	KET	Antifungals	50	-	50
Miconazole	MIC	Antifungals	50	-	50

We tested antifungal activity of carvacrol against drug susceptible *C. albicans* using the MIC in broth microdilutions. Carvacrol showed remarkable antifungal activity against drug susceptible *C. albicans* and their MIC were compared with those of fluconazole ($p \leq 0.05$). The antifungal agent fluconazole was used as a positive control (Table 4). MIC of carvacrol ranged from 25-200 µg/ml; the growth of all drugs susceptible *isolates of C. albicans* was inhibited. The fungal population size (the $\log_{10}$ CFU) at different concentrations of carvacrol on *C. albicans* ATCC 14053 and drug susceptible isolate were investigated using the time kill assay. $\log_{10}$ CFU were reduced after 2, 4, 6, 8, 12, 24 and 48 h after incubation compared to untreated control ($p \leq 0.05$). The $\log_{10}$ CFU value was reduced 8.63-, 6.48-, 5.82 and 6.48-fold in the *C. albicans* ATCC 14053 treated with 2 × MIC, 1 × MIC, 1.2 × MIC and ¼ × MIC of carvacrol, respectively at 48 h after incubation. In addition, 7.13-, 6.08-, 4.92 and 4.54-fold of the fungal populations of drug susceptible isolate was killed by treating with 2 × MIC, 1 × MIC, 1/2 × MIC and ¼ × MIC of carvacrol, respectively (Fig. 1).

The effects of carvacrol on *C. albicans* hyphae were visually confirmed by light field microscope. For untreated control, *C. albicans* hyphae formed on the plastic coverslips consisted of filamentous cells and scattered colonies. The carvacrol-treated hyphae were reduced in number and density of filamentous cells and scattered colonies depending on concentration of carvacrol. Compared to the untreated control, no filamentous cells were observed in *C. albicans* culture treated with 2 × MIC of carvacrol and scattered colonies, mostly composed of yeast cells, were visualized by light field microscope (Fig. 2).

To elucidate the potential molecular mechanism behind the ability of carvacrol to prevent growth of *C. albicans* hyphae, we analysed the changes in the gene expression levels of *C. albicans* ATCC 14053 cells in hyphae exposed to carvacrol (Fig 3). The expression levels of hyphae-specific gene that encode products that function as surface adhesins, such as *HWP1* was suppressed by carvacrol treatment during the hyphae formation ($p \leq 0.001$). In *C. albicans* cells treated with carvacrol the expression levels of *HWP1* were down-regulated by 1.82- and 1.62-fold at concentrations of 2 × MIC and 1 × MIC, respectively.

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Table 3. Results of antifungal susceptibility against *clinical isolates of C. albicans by using the disk diffusion and broth microdilution methods*

Isolates/Antifungals	Amphotericin B		Fluconazole		Ketoconazole		Miconazole	
	Disk zone diameter (mm)	MIC_{90} (µg/ml)	Disk zone diameter (mm)	MIC_{90} (µg/ml)	Disk zone diameter (mm)	MIC_{90} (µg/ml)	Disk zone diameter (mm)	MIC_{90} (µg/ml)
C. albicans ATCC 14053	19.00±0.04	0.03	22.00±0.10	0.125	30.00±0.04	0.06	22.00±0.08	2
CI- 1*	9.50±0.20	1	12.00±0.10	16	19.50±0.20	1	8.00±0.10	8
CI- 2	14.70±0.06	0.5	18.70±0.10	4	24.00±0.10	0.06	21.70±0.06	2
CI- 3	16.00±0.10	0.5	21.00±0.01	4	25.70±0.06	0.06	20.00±0.10	2
CI- 4	12.00±0.10	0.06	18.00±0.03	2	25.70±0.10	0.03	18.00±0.10	2
CI- 5	17.00±0.10	0.5	15.70±0.02	4	24.00±0.10	0.125	20.30±0.06	1
CI- 6	15.10±0.01	1	11.00±0.03	16	18.50±0.04	1	10.00±0.08	8
CI- 7	15.70±0.06	1	16.00±0.10	4	28.00±0.10	0.06	20.00±0.10	2
CI- 8	14.30±0.06	1	18.00±0.02	4	31.00±0.10	0.06	20.00±0.10	1
CI- 9	8.10±0.01	4	11.00±0.05	32	17.50±0.20	0. 5	10.00±0.10	8
CI- 10	11.70±0.06	0.5	20.00±0.10	4	30.30±0.06	0.06	20.00±0.10	1
CI- 11	14.30±0.06	1	19.70±0.10	2	25.50±0.10	0.06	20.00±0.10	2
CI- 12	12.00±0.10	1	21.70±0.03	2	25.50±0.10	0.125	17.70±0.06	2
CI-13	16.00±0.10	1	16.80±0.04	1	26.60±0.10	0.03	19.70±0.06	2
CI- 14	8.60±0.01	4	9.00±0.03	16	18.50±0.04	1	9.00±0.08	8
CI- 15	9.50±0.05	4	10.00±0.07	16	19.50±0.20	1	11.00±0.10	8
CI- 16	8.50±0.01	4	10.00±0.10	16	18.50±0.04	1	10.00±0.08	8
CI- 17	8.00±0.01	8	11.00±0.05	32	17.50±0.20	0.5	10.00±0.10	8
CI- 18	8.50±0.01	8	9.00±0.04	16	19.50±0.04	1	9.00±0.08	8
CI- 19	8.50±0.01	4	10.00±0.03	16	17.50±0.20	1	10.00±0.10	8
CI- 20	9.00±0.02	4	9.00±0.10	64	18.50±0.04	1	10.00±0.08	8

*CI: *Clinical isolates of C. albicans*

Table 4. MIC (µg/ml) values of carvacrol against drug susceptible clinical isolates of *C. albicans*

Isolates / Antifungals	Carvacrol		Fluconazole	
	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}
C. albicans ATCC 14053	25	12.5	0.125	0.03
CI- 2*	100	50	4	2
CI- 3	25	12.5	4	2
CI- 4	200	100	2	1
CI- 5	100	50	4	2
CI- 7	100	50	4	2
CI- 8	100	50	4	2
CI- 10	50	25	4	2
CI- 11	100	50	2	1
CI- 12	100	50	2	1
CI- 13	50	25	1	0.5

*CI: Drug susceptible *C. albicans*

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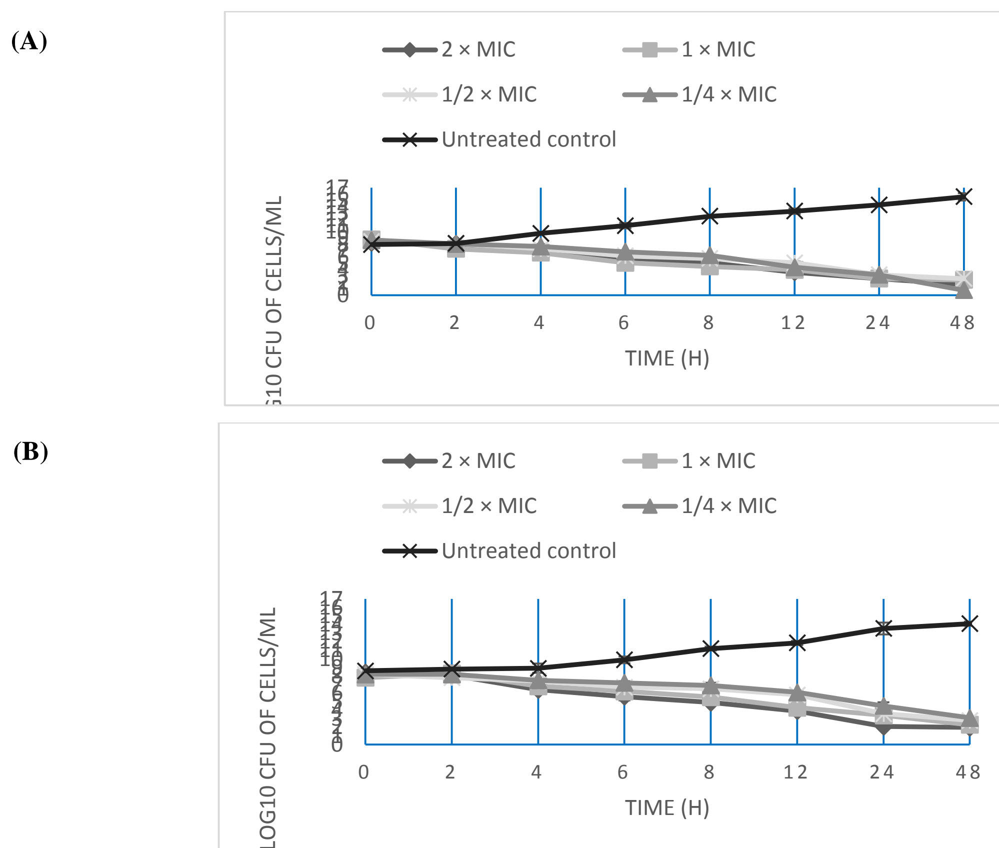

Figure 1. Effects of carvacrol at concentrations ranging from 2 × MIC to ¼ × MIC on the time kill curves. (A) *Candida albicans* ATCC 14053 and (B) *Candida albicans,* drug susceptible isolate. Data are the means ± SD of triplicate determinations

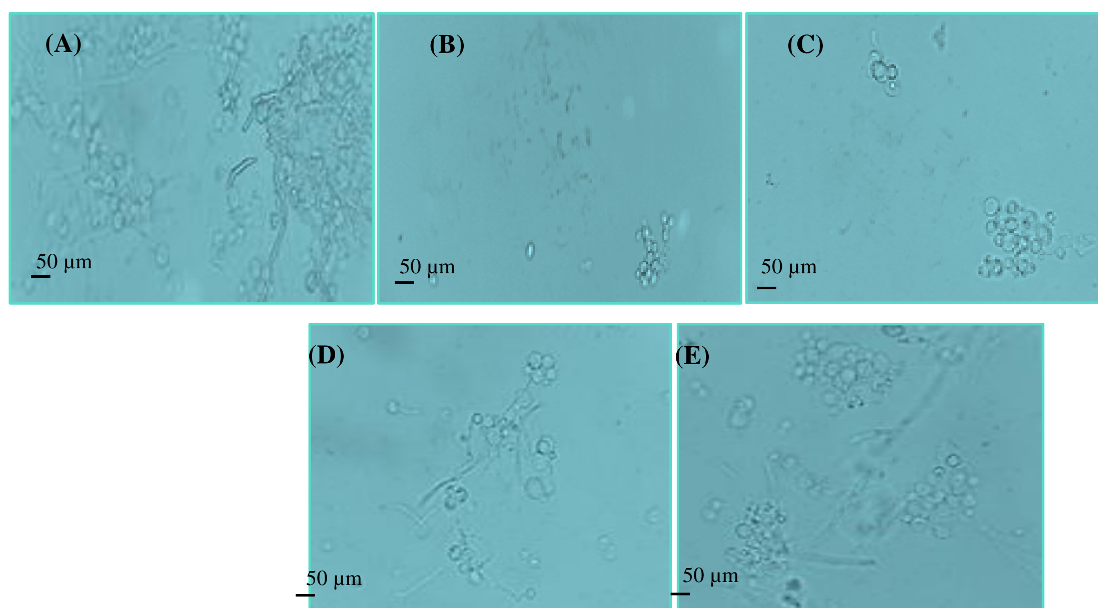

Figure 2. Representative light field microscope images showing the hyphae formation by *Candida albicans* ATCC 14053 treated with different concentrations of carvacrol after 16 h incubation. (A) Untreated control, the morphology was consisted of filamentous cells and yeast form (B) Treated with 2× MIC of carvacrol, complete destruction of filamentous cells (C) Treated with 1× MIC of carvacrol, major changes in hyphae formation, mostly yeast form observed (D) Treated with ½× MIC of carvacrol, reduction of filamentous and yeast cells observed (E) Treated with ¼× MIC of carvacrol, less filamentous cells were observed, with less number of yeast cells. Magnification × 40, Bar = 50 µm

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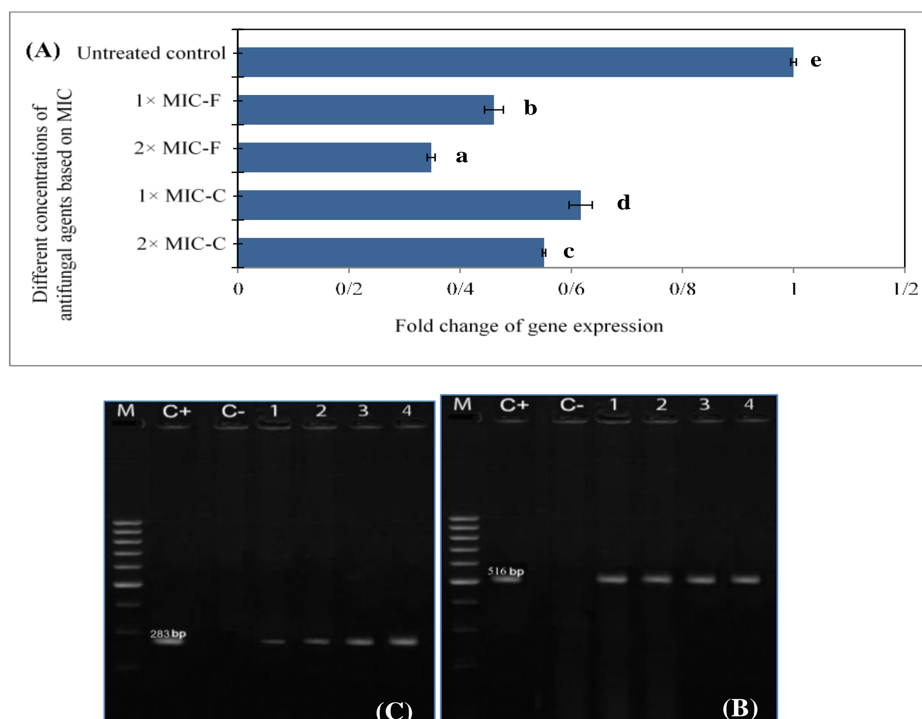

Figure 3. (A) Quantitative RT-PCR analysis of *Candida albicans* ATCC 14053 hyphae-specific gene (*HWP1* in the presence or absence of carvacrol (C) and fluconazole (F) at concentrations of 2 × MIC and 1 × MIC. Data are the means ± SD of triplicate determinations. Means with different letters indicate statistically significant differences at p≤0.05. (B) and (C) Expression of *HWP1* and *β actin* genes, respectively in *C. albicans* ATCC 14053 treated with different concentrations of carvacrol by quantitative RT-PCR. M: DNA Ladder, C+: *HWP1* or *β actin* without carvacrol (untreated control), C-: Control negative for PCR. 1: Treated with 2× MIC concentration of fluconazole, 2: Treated with 1× MIC concentration of fluconazole, 3: Treated with 2× MIC concentration of carvacrol, 4: Treated with 1× MIC concentration of carvacrol

Discussion

The present study evaluated the antifungal efficacy of carvacrol against *C. albicans* using the MIC in broth microdilutions, time kill assay, hypha formation and gene expression profiling. The carvacrol effectively inhibited the generation of *C. albicans* hyphae. Carvacrol have shown some effectiveness against planktonic cells and *C. albicans* biofilms (18,25). Such antifungal activity of carvacrol can be attributed to Ca^{2+} stress and inhibition of the TOR (Target of Rapamycin) pathway. The findings of the study were confirmed with time kill and hyphae formation of cells treated with carvacrol which showed a significant reduction in the number of cells. These reductions of filamentous cells and yeast form are more likely due to penetration of carvacrol into the cells resulting in the decrease in the number of cells with inhibitory effect of the yeast– hyphal transition. The activation of specific signaling pathways with carvacrol can disturb the integrity of the membrane and therefore blocking the ergosterol biosynthesis pathways leading to the result of damage to enzymatic cell systems, including those associated with energy production and synthesis of structural compounds (12,14,15, 26-28). Additionally, the above results were supported by the changes in the gene expression levels of *C. albicans* cells in hyphae exposed to carvacrol. The expression levels of specific gene including *HWP1* implicated in hyphae formation of *C. albicans* cells were determined by quantitative RT-PCR. These results corroborate to the literature research, where are cited the changes in the gene expression levels of *C. albicans HWP1* in hyphae exposed to antifungal agents (3,24,29,30). We demonstrate that carvacrol prevents hyphae formation against drug susceptible C. albicans. In addition, carvacrol showed reduction of filamentous cells and yeast form and inhibitory effect of HWP1 gene expression. These results suggest that carvacrol could provide an improved and safe clinical approach in treating *Candida* infections. Because infections due to drug resistance C. albicans are an alarming health problem. Hence future studies may investigate the efficacy of carvacrol against recalcitrant infections.

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