



## Analysis of ergosterol and gene expression profiles of sterol $\Delta^{5,6}$ -desaturase (*ERG3*) and lanosterol 14 $\alpha$ -demethylase (*ERG11*) in *Candida albicans* treated with carvacrol

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### ABSTRACT

**Introduction:** Usually, for treatment of fungal infections, antifungals such as azoles are used, but one of the biggest problems faced in clinical practice is the emergence of resistance for most of these drugs. Antifungal drugs derived from plants may alleviate this problem. The aims of this study were to analyse the ergosterol and gene expression profiles of *ERG* genes in *Candida albicans* treated with carvacrol.

**Methods:** We used carvacrol and conducted a series of follow-up studies to examine the inhibitors of *Candida* species isolated from immunocompromised patients. Antifungal susceptibility test, time-kill study, ergosterol binding assay and ergosterol content were investigated. Eventually, the expression of *ERG3* and *ERG11* genes was carried out to investigate the inhibitory properties of antifungal activity against *Candida albicans* using quantitative real time RT-PCR.

**Results:** Carvacrol was able to inhibit *Candida* species and reduce time-kill kinetic in *C. albicans*. This phytoconstituent acted by binding to ergosterol in the fungal membrane and caused a reduction of 52% of the ergosterol content compared to the untreated growth control. Finally, carvacrol displayed significant down-regulation of *ERG3* and *ERG11* genes in *C. albicans*.

**Conclusion:** These results provide proof of concept for the implementation of carvacrol inhibitors of *Candida* species. In addition, *ERG3* and *ERG11* genes could be probable target of carvacrol against *C. albicans*.

### Implication for health policy/practice/research/medical education:

The carvacrol showed promising anticandidal effects against *Candida* species, implying its potential in the therapy of *Candida* species infections. In addition, these results can provide insights into the mechanism of action of carvacrol against *C. albicans*.

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### Introduction

Outbreaks of fungal human disease are a serious and costly health problem in all immunocompromised patients (1,2). Five *Candida* species including *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* account for approx. 90% of identifiable invasive *Candida* infections. Clearly, it is demonstrated that *albicans* is the most important *Candida* species. On the other hand, several virulence factors are contributed to *Candida* pathogenicity (3-5).

Ergosterol is neutral lipids of the fungal cellular membranes that regulate fluidity, function and biogenesis

of membranes. Fungal ergosterol homeostasis is critical for many cellular processes including regulation of transcription control of ergosterol biosynthetic pathway genes and proteins involved in sterol processing and uptake. The ergosterol biosynthetic enzymes are essential for fungal growth and the primary target for several anticandidal agents. Antifungal agents in the azole class target the fungal cell membrane through inhibition of the enzyme 14 $\alpha$ -demethylase, product of the *CYP51/ERG11*, thus block the biosynthesis of ergosterol and lead to defects in cell membrane integrity and cellular integrity (6-8).

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About 20 genes involve in the biosynthesis of ergosterol in fungi and there are single-copy of the *C. albicans* *ERG* genes in this pathway. Sterol  $\Delta^{5,6}$ -desaturase (encoded by *ERG3*) is the second last step in the ergosterol biosynthetic pathway that adds a double bond at the C-5 carbon of the B-ring structure of episterol to produce ergosta-5,7,24(28)-trienol. The final step is the substrate of sterol  $\Delta^{22}$ -desaturase and sterol  $\Delta^{24}$ -reductase encoded by *ERG5* and *ERG4*, respectively (7,9,10). Lanosterol 14-demethylase (*ERG11*) gene is a member of cytochrome P450 enzyme family that is an essential enzyme for ergosterol synthesis. This enzyme converts lanosterol to ergosterol which catalyzes the oxidative removal of the 14 $\alpha$ -methyl group from lanosterol (11).

Plants have been a source of secondary metabolites. Carvacrol from the class of monoterpene phenols is a major component of essential oil extract from the *Lamiaceae* family with strong antimicrobial properties. It is demonstrated that carvacrol has been inhibitory activity against *C. albicans*. The antifungal mechanism action of carvacrol is cell membrane disruption by targeting and binding to membrane ergosterol (12-15).

During recent years, insight into the antifungal mechanism of natural products has markedly increased. In the current study, we conducted a series of follow-up studies to investigate the antifungal inhibitory effects of carvacrol against *Candida* species isolated from immunocompromised patients. In addition, ergosterol binding assay, ergosterol quantification and expression profiles of selected genes involved in the ergosterol biosynthetic pathway of *C. albicans* treated with carvacrol were analyzed.

## Materials and Methods

Clinical isolates of *C. albicans* (Ca2, Ca3, Ca5, Ca10), *C. krusei* (Ck1, Ck24, Ck25, Ck26) and *C. tropicalis* (Ct7, Ct9, Ct11, Ct17) were obtained from immunocompromised patients who admitted in Shahid Beheshti affiliated to Yasooj University of Medical Sciences. Written informed consent was obtained from patients for the use of the samples in research. Isolates were cultured in Sabouraud dextrose broth (SDB, Difco Laboratories, Detroit, Michigan). The identities of the clinical isolates of *Candida* were confirmed by phenotypic (16) and molecular techniques (17). Briefly, clinical isolates were plated on CHROMagar *Candida* medium (CHROMagar Company, France) to check for purity and the reliability of *Candida* species were confirmed by DNA sequencing. *C. albicans* ATCC 14053, *C. krusei* ATCC 6258 and *C. tropicalis* ATCC 750 were employed as the strain-controls.

### Phenotypic methods for clinical isolates identification

Phenotypic methods including microscopic and macroscopic morphology, germ tube formation, carbohydrate and nitrate assimilations, carbohydrate fermentation and urease test were carried out to identities

of the clinical isolates (16).

### DNA-sequencing techniques for clinical isolates identification

The universal fungal primers ITS1 (5'- TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-3') were used for DNA amplification (17). The amplified PCR products were confirmed by DNA sequencing. The sequence homology was detected using the nucleotide BLAST program.

### Antifungal agents and susceptibility testing

Carvacrol and fluconazole were obtained from Sigma Chemicals Co. (St. Louis, MO). Antifungal disk diffusion susceptibility test and broth microdilution antifungal susceptibility test were performed as described in the CLSI guideline (M44-A2 and M27-A3, method for antifungal disk diffusion susceptibility testing of yeasts and reference method for broth dilution susceptibility testing of yeasts, respectively) (18,19).

### Time-kill study

The time-kill study of carvacrol was performed with the methodology described by Khodavandi et al (20). The *C. albicans* ATCC 14053 was chosen from the results of susceptibility testing. To determine the kinetics of fungal death, 4 mL of  $1 \times 10^6$  *C. albicans* ATCC dissolved in RPMI 1640 and mixed with the different concentrations based on MIC (MIC and  $\frac{1}{2} \times$  MIC) of carvacrol. After 0, 2, 4, 6, 8, 10, 12, 24 and 48 hours incubation at 35°C, 100  $\mu$ L of each mixture was collected and 10-fold serial dilutions were made and plated on Sabouraud dextrose agar (SDA, Difco Laboratories). Eventually, colonies were counted after 24 hours incubation at 37°C and the CFU were calculated. Furthermore, fluconazole was used as a control drug.

### Ergosterol binding assay

Ergosterol binding properties of antifungal agents were performed using the following method by Escalante et al (21). The *C. albicans* ATCC 14053 was chosen from the results of susceptibility testing. The MIC of carvacrol against *C. albicans* ATCC was determined in accordance with the guidelines of CLSI M27-A3, at different concentrations (50-250  $\mu$ g/mL) of ergosterol (Sigma). Fluconazole was used as a control drug.

### Ergosterol quantification

The ergosterol quantification in the cell membrane of *C. albicans* was performed by method as described by Santos et al (22). At first, 25 mg of the *C. albicans* cell mass was added to carvacrol with different MIC concentrations (MIC and  $\frac{1}{2} \times$  MIC) in polypropylene tubes and incubated at 35°C for 24 hours. The *C. albicans* cells were harvested by centrifugation at 1643 g for 5 minutes at 4°C, and washed once in a sterile distilled water. The net wet weight

of the cell pellet was determined. Lipids were extracted with 3 mL of ethanolic solution of potassium hydroxide 25% in *C. albicans* cell mass and incubated in an 85°C water bath for 1 hours. After incubation, the tubes were incubated at room temperature. Finally, 1 mL of sterile water and 3 mL of *n*-heptane (Sigma) were added and vortexed for 3 minutes. The aqueous fraction was removed with a pipette, and the spectrophotometric measurements were performed at 282 and 230 nm. The calibration curve of standard ergosterol was constructed and used to determine the amount of ergosterol. The results of the quantitative determination of ergosterol were expressed as the percentage of ergosterol in treated cells compared to untreated control ones.

#### Quantitation of gene expression levels

Gene expression levels of *ERG3* (GenBank accession number AF069752) and *ERG11* (GenBank accession number X13296) were measured using quantitative real-time RT-PCR, as described previously (23,24) in *C. albicans* ATCC 14053 cells treated with carvacrol at different concentrations based on MIC (MIC and  $\frac{1}{2} \times$  MIC). Briefly, single-stranded cDNA was synthesized from 0.5  $\mu$ g extracted RNA using M-MuLV reverse transcriptase and random hexamer oligonucleotides (Fermentas, USA). Oligonucleotide primers were listed in Table 1. The primers for *ERG3*, *ERG11* and  $\beta$  *actin* were amplified by PCR using SYBR Green qPCR Master Mix (Fermentas, EU) in a Bio-Rad MiniOpticon™ system (USA), according to the manufacturer's protocol. Relative gene expression data were analyzed by the Pfaffl method. Fluconazole was also used as a control drug.

#### Statistical analysis

Data were analyzed using the statistical software SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA). Data were expressed as mean values  $\pm$  standard deviations (SD) or standard error (SE) from three independent replicate experiments. Results were statistically analyzed using one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test. *P* values of  $\leq 0.05$  were considered statistically significant.

## Results

#### Studies of phenotypic and genotypic characteristics

Clinical yeast isolates selected from SDA, were phenotypically identified to species level. Macroscopically,

colonies of *Candida* (on the surface of SDA) are cream to yellow in color. Phenotypic characteristics of three identified *Candida* species is shown in Table 2. The reliability of *C. albicans*, *C. krusei* and *C. tropicalis* was confirmed by DNA sequencing via the non-redundant nucleotide sequences from Gen Bank. The sequences displayed high homology and confirmed to have 100% identify with the respective gene sequence.

#### Antifungal susceptibility

The antimicrobial activity of carvacrol compared with standard drug (fluconazole), was assessed by the disk diffusion susceptibility test against clinical isolates of *Candida* species. The results of sensitivity to antifungal drugs (diameter of inhibition zone in mm) are summarized in Table 3. The results revealed that carvacrol could able to show antimicrobial properties against clinical isolates of *Candida* species compared to the standard antifungal agent (fluconazole) at 30  $\mu$ g/mL concentration. However, the antifungal activity of the carvacrol was found to be potent at 20  $\mu$ g/mL concentration for isolates of *C. albicans*.

Table 4 summarizes the MICs of the antifungal drugs tested against clinical isolates of *Candida* species. The MIC of the carvacrol was 25  $\mu$ g/mL for 100% of the clinical isolates of *C. albicans* evaluated. However, the MIC of the carvacrol for clinical isolates of *C. krusei* and *C. tropicalis* were ranged from 25-50  $\mu$ g/mL and 50-100  $\mu$ g/mL, respectively. From Table 4, it can be seen that 2 of 5 clinical isolates of *C. krusei* and 2 of 5 clinical isolates of *C. tropicalis* were resistant to fluconazole.

#### Time kill study

The dynamic antifungal effect of carvacrol against *Candida* species was confirmed by time kill studies. The antifungal effect of carvacrol was depicted by time kill curves with  $\log_{10}$  CFU of cells/mL as Y-axis and time as X-axis (Figure 1). The results show the potency of carvacrol in decreasing the cell number of *Candida* species after 0, 2, 4, 6, 8, 10, 12, 24 and 48 hours compared to untreated control ( $P \leq 0.05$ ). At 48<sup>th</sup>, the  $\log_{10}$  CFU value was reduced more than 25- and 20-fold in the *C. albicans* ATCC treated with MIC and  $\frac{1}{2} \times$  MIC of carvacrol, respectively. The  $\log_{10}$  CFU values were no significant difference among these three species.


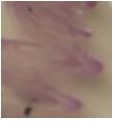

#### Effect of carvacrol on ergosterol binding properties

The results of the ergosterol binding assay showed

**Table 1.** Oligonucleotide primers used for PCR

Primer	Orientation	Sequence	Reference
<i>ERG3</i>	Forward	5' TCC AGT TGA TGG GTT CTT CC 3'	(24)
	Reverse	5' GGA CAG TGT GAC AAG CGG TA 3'	
<i>ERG11</i>	Forward	5' TGG AGA CGT GAT GCT G 3'	(23)
	Reverse	5' AGT ATG TTG ACC ACC CAT AA3'	
<i>ACT</i>	Forward	5' GAG TTG CTC CAG AAG AAC ATC CAG 3'	(24)
	Reverse	5' TGA GTA ACA CCA TCA CCA GAA TCC 3'	

**Table 2.** Phenotypic characteristics of *Candida albicans*, *Candida krusei* and *Candida tropicalis*

Isolates	Germ tube production	Carbohydrate and nitrate assimilations								Carbohydrate fermentation					Urease	CHROM-agar colony color
		GLU	MAL	SUC	LAC	TER	XYL	STA	KNO <sub>3</sub>	GLU	MAL	SUC	LAC	TER		
<i>C. albicans</i> ATCC	+	+	+	+	-	+	+	+	-	+	+	-	-	+	-	Blue-green
																
Ca2	+	+	+	+	-	+	+	+	-	+	+	-	-	+	-	Blue-green
Ca3	+	+	+	+	-	+	+	+	-	+	+	-	-	+	-	Blue-green
Ca5	+	+	+	+	-	+	+	+	-	+	+	-	-	+	-	Blue-green
Ca10	+	+	+	+	-	+	+	+	-	+	+	-	-	+	-	Blue-green
<i>C. krusei</i> ATCC	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	Pink-purple
																
Ck1	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	Pink-purple
Ck24	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	Pink-purple
Ck25	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	Pink-purple
Ck26	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	Pink-purple
<i>C. tropicalis</i> ATCC	-	+	+	+	-	+	+	+	-	+	+	+	-	+	-	Dark blue
																
Ct7	-	+	+	+	-	+	+	+	-	+	+	+	-	+	-	Dark blue
Ct9	-	+	+	+	-	+	+	+	-	+	+	+	-	+	-	Dark blue
Ct11	-	+	+	+	-	+	+	+	-	+	+	+	-	+	-	Dark blue
Ct17	-	+	+	+	-	+	+	+	-	+	+	+	-	+	-	Dark blue

GLU: Glucose; MAL: Maltose; SUC: Sucrose; LAC: Lactose; TRE: Trehalose; XYL: Xylose; STA: starch. Ca: Clinical isolates of *C. albicans*; Ck: Clinical isolates of *C. krusei*; Ct: Clinical isolates of *C. tropicalis*.

**Table 3.** Antimicrobial activities of carvacrol against isolates of *Candida albicans*, *Candida krusei* and *Candida tropicalis* at different concentration

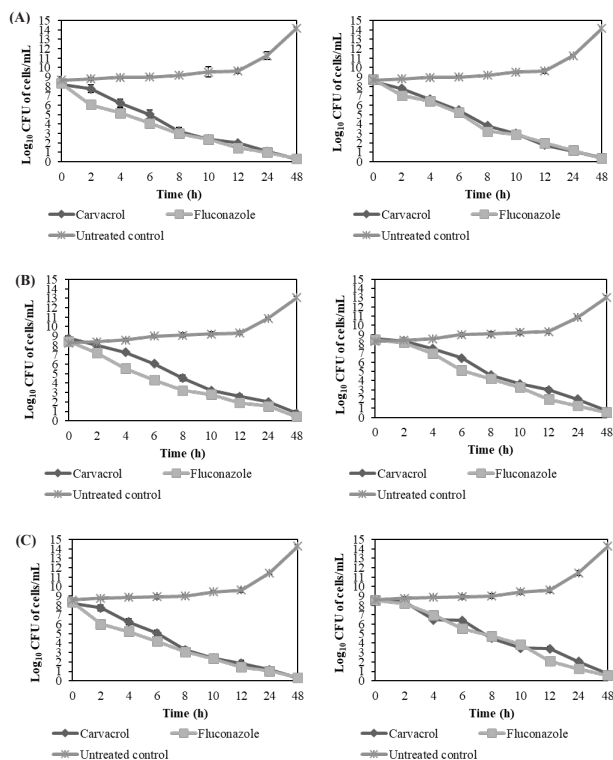
Isolates		<i>C. albicans</i> ATCC	Ca2	Ca3	Ca5	Ca10	<i>C. krusei</i> ATCC	Ck1	Ck24	Ck25	Ck26	<i>C. tropicalis</i> ATCC	Ct7	Ct9	Ct11	Ct17		
Inhibition zone (mm)	Carvacrol	10 µg/mL	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	
		20 µg/mL	11.00 ± 0.20	8.00 ± 0.20	8.00 ± 0.10	7.00 ± 0.20	10.00 ± 0.20	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
		30 µg/mL	16 ± 0.10	14.50 ± 0.11	14.00 ± 0.20	13.80 ± 0.22	15 ± 0.10	14.10 ± 0.11	10.10 ± 0.10	11.50 ± 0.24	13.40 ± 0.20	11.80 ± 0.10	14.70 ± 0.12	12.30 ± 0.21	13.50 ± 0.00	12.80 ± 0.11	11.50 ± 0.00	11.50 ± 0.00
	Fluconazole	25 µg/mL	15.70 ± 0.10	13.90 ± 0.20	14.70 ± 0.11	13.00 ± 0.20	13.00 ± 0.20	13.10 ± 0.23	9.00 ± 0.00	10.20 ± 0.10	12.50 ± 0.11	10.70 ± 0.00	14.70 ± 0.13	12.50 ± 0.10	13.70 ± 0.11	13.40 ± 0.00	12.00 ± 0.11	12.00 ± 0.11

Ca: Clinical isolates of *C. albicans*; Ck: Clinical isolates of *C. krusei*; Ct: Clinical isolates of *C. tropicalis*.  
Data are means ± standard deviation of three independent experiments.

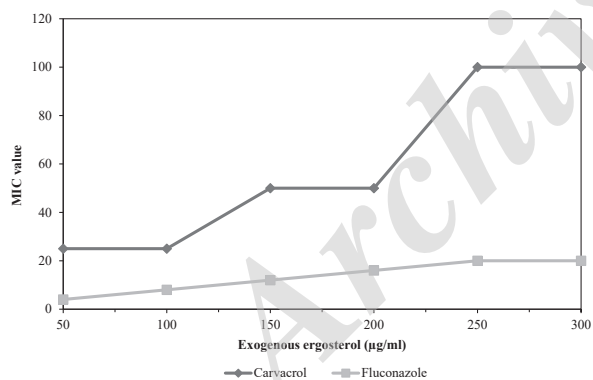
**Table 4.** Relative MIC (µg/mL) values of carvacrol against isolates of *Candida albicans*, *Candida krusei* and *Candida tropicalis*

Antifungals/ Isolates		<i>C. albicans</i> ATCC	Ca2	Ca3	Ca5	Ca10	<i>C. krusei</i> ATCC	Ck1	Ck24	Ck25	Ck26	<i>C. tropicalis</i> ATCC	Ct7	Ct9	Ct11	Ct17
Carvacrol	MIC <sub>50</sub>	6.25	12.5	6.25	12.5	6.25	12.5	12.5	12.5	12.5	12.5	25	25	25	25	50
	MIC <sub>90</sub>	25	25	25	25	25	25	50	50	25	25	50	50	50	50	100
Fluconazole	MIC <sub>50</sub>	0.125	0.125	0.25	0.25	0.25	0.25	2	1	0.25	0.25	0.5	1	2	1	2
	MIC <sub>90</sub>	4	8	8	16	8	16	64	64	32	32	8	16	64	16	64

Ca: Clinical isolates of *C. albicans*; Ck: Clinical isolates of *C. krusei*; Ct: Clinical isolates of *C. tropicalis*.  
Data are means ± standard deviation of three independent experiments.



**Figure 1.** Time kill curves of carvacrol and fluconazole in different concentration based on MIC (MIC and  $\frac{1}{2}\times$  MIC) against (A) *Candida albicans* ATCC 14053, (B) *Candida krusei* ATCC 6258 and (C) *Candida tropicalis* ATCC 750. Data are means with standard error from three independent experiments in triplicate assays. Statistically significant differences between the treatments and the control are indicated with an asterisk ( $P \leq 0.05$ ).

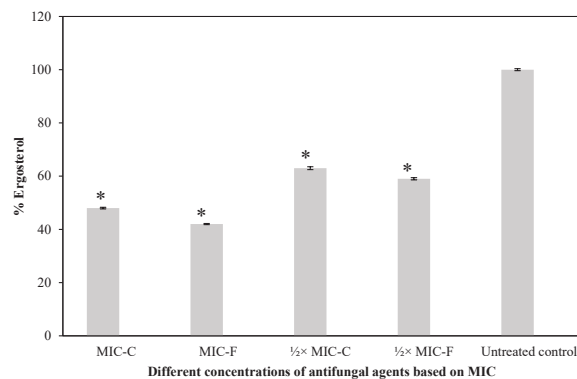


**Figure 2.** Effect of different concentrations of exogenous ergosterol (50-300  $\mu\text{g}/\text{mL}$ ) on the MIC of carvacrol and fluconazole against *Candida albicans* ATCC 14053.

(Figure 2) that the MIC values of carvacrol for *C. albicans* ATCC cells a 4-fold increase of MIC was observed in the presence of 250 and 300  $\mu\text{g}/\text{mL}$  of exogenous ergosterol. In addition, a 5-fold increase of MIC was observed for the control drug (fluconazole) in the presence of 250 and 300  $\mu\text{g}/\text{mL}$  of exogenous ergosterol.

#### Quantitation of *Candida albicans* ergosterol content

To further investigation of the action of the carvacrol on the *C. albicans* cell membrane, the ergosterol content



**Figure 3.** Percent ergosterol levels of *Candida albicans* ATCC 14053 after 24 hours of treatment with different concentrations of antifungal agents. Data are means with standard error from three independent experiments in triplicate assays. Statistically significant differences between the treatments and the control are indicated with an asterisk ( $P \leq 0.05$ ).

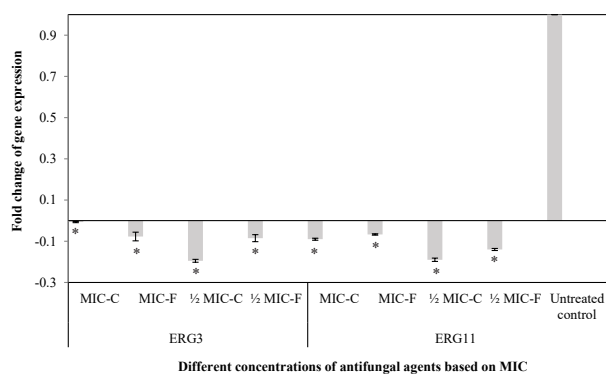
was evaluated in *C. albicans* ATCC 14053 cells treated with the different MIC-concentrations of carvacrol (MIC and  $\frac{1}{2}\times$  MIC). Figure 3 demonstrates treatment with carvacrol caused a reduction of 52% of the ergosterol content compared to the untreated growth control. The ergosterol content increased with the decrease of carvacrol concentration ( $P \leq 0.05$ ). The *C. albicans* cells treated with fluconazole reduced the ergosterol contents in 58%. When the fluconazole concentration equal to  $\frac{1}{2}$  MIC was used, leads to lower ergosterol levels.

#### Gene expression analysis

In the present study, *C. albicans* ATCC was treated with different concentrations of carvacrol and fluconazole (MIC and  $\frac{1}{2}\times$  MIC). We monitored the expression levels of *ERG3* in *C. albicans* cells by quantitative real-time RT-PCR. The expression levels of *ERG3*, a sterol  $\Delta^{5,6}$ -desaturase gene, down regulate ( $P \leq 0.05$ ) in the *C. albicans* cells treated with different concentrations of carvacrol and fluconazole (Figure 4). *C. albicans* gene expression patterns were similar in response to the antifungal activity of carvacrol and fluconazole. The expression levels of *ERG3* were demonstrated the lowest fold change expression in *C. albicans* treated with carvacrol compared to fluconazole at concentrations equal to MIC. While the fold changes in terms of *ERG3* expression to untreated control for  $\frac{1}{2}\times$  MIC of carvacrol was higher compared to fluconazole. The authenticity of the PCR products was verified by DNA sequencing. The sequences confirmed high homology to the related genes in Gene Bank.

#### Discussion

According to the literature previously demonstrated that carvacrol has been found to be active against *C. albicans* (12-15). Fluconazole was used as a positive control because it is one of the most commonly used antifungal agents for



**Figure 4.** Relative quantitation of *ERG3* and *ERG11* genes normalized to house-keeping gene, actin in *Candida albicans* ATCC 14053 after 24 h of treatment with different concentrations of antifungal agents by real time RT-PCR. Data are means of fold changes with standard error from three independent experiments amplified in triplicates. Statistically significant differences between the treatments and the control are indicated with an asterisk ( $P \leq 0.05$ ).

the treatment of *Candida* species infections (25). Our findings consistent with previous studies of antifungal activity of carvacrol could inhibit *Candida* species growth (12-15,26). The authors studied two clinical isolates of *C. krusei* and two clinical isolates of *C. tropicalis* which presented MIC= 64 µg/mL for fluconazole. However, the antimicrobial activity of the carvacrol was found to be potent against fluconazole-resistant *Candida* species. Our favorable results are, at least in partial, in agreement with Gallucci et al. ones (26), who demonstrated that carvacrol had the highest anti-*Candida* activity against fluconazole-resistant *Candida* species. These results parallel the work of Rao et al (27), showing that carvacrol was the most effective in killing *C. albicans*. In addition, the tested antifungals on the *C. krusei* and *C. tropicalis* completely reduced the number of yeast form in time kill study. In the literature, carvacrol has been found to be active against *C. krusei* and *C. tropicalis* (26).

The effect of ergosterol on carvacrol was investigated using the broth microdilution technique. Findings from the MIC values of carvacrol for *C. albicans* ATCC cells exhibited a 4-fold increase of MIC. This effect of ergosterol is in good agreement with the Lima et al (14) report.

The ergosterol quantification assay revealed that reduction of the ergosterol contents caused by carvacrol and fluconazole at different concentration of MIC. This effect helps explain the demonstrated concentration dependent on antifungals, since higher concentrations of carvacrol and fluconazole lead to lower ergosterol contents. The mechanism proposed for mode of actions of carvacrol and fluconazole are inhibition of fungal sterol biosynthesis. Considering ergosterol as an important sterol found in cell membranes of fungi, changes in its biosynthetic pathway may also prove lethal to the fungal cell (14,28,29).

Down-regulated expression of *ERG3* and *ERG11* genes were found in *C. albicans* ATCC treated with carvacrol

and fluconazole at different MIC concentrations. The down-regulated sterol  $\Delta^{5,6}$ -desaturase and lanosterol 14 $\alpha$ -demethylase genes were consistent with the ergosterol binding properties and ergosterol content. Importantly, carvacrol and fluconazole significantly reduced *C. albicans* ATCC ergosterol related properties compared with untreated control. Few studies have investigated the expression of *ERG3* and *ERG11* genes in *C. albicans* treated with antifungals. Our results are, in partial, in agreement with Khodavandi et al ones (20), who showed that fluconazole in combination with terbinafine significantly reduced the expression of *ERG1*, 3, and 11 in *C. albicans*. The effect of combination of tunicamycin and amphotericin B has significantly decreased the expression level of *MP65* and *ERG3* in *C. albicans* and *C. dubliniensis* (30). Yang et al (31) investigated the effect of citral on five ergosterol biosynthetic pathway genes (*ERG7*, *ERG11*, *ERG6*, *ERG3* and *ERG5*) in *Penicillium digitatum*. *P. digitatum* treated with citral greatly affected the expression levels of ergosterol biosynthetic pathway genes consistent with a decrease in ergosterol content and accumulation of massive lanosterol. Furthermore, *ERG3*, *ERG4* and *ERG5* were downregulated in *Saccharomyces cerevisiae* treated with fluconazole (32). A previous study showed that the five major genes: ABC transporter genes *CDR1* and *CDR2*, major facilitator efflux gene *MDR1* and ergosterol biosynthesis genes *ERG3* and *ERG11* are responsible for antifungal resistance in *C. albicans* (33).

This study supports the view that carvacrol exerts its antifungal activity on the cell membrane of *C. albicans* by inhibition of ergosterol biosynthesis. Whether these events reflect the potential of carvacrol for inhibition of ergosterol biosynthesis in *C. albicans* which differentially expresses a specific gene, requires further dissection. In addition, *ERG3* and *ERG11* genes could be probable molecular targets for carvacrol in *C. albicans*. Greater knowledge of molecular mechanisms of antifungal effects may serve as a guide for future in the development of new therapeutic strategies.

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#### Authors' contributions

All the authors contributed to design of the study, analyzed and interpreted the data. FA and AK managed the literature searches and produced the initial draft. All authors read and approved the final version of the manuscript for publication.

#### Conflict of interests

The authors declared no competing interests.

### Ethical considerations

Ethical issues in research have been completely observed by the authors (Ethical code 1180679). This study was approved by Research Ethics Committee of our institute (The study protocol conformed to the ethical guidelines of the 2008 Declaration of Helsinki).

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