Analysis of ergosterol and gene expression profiles of sterol Δ^5,6-desaturase (ERG3) and lanosterol 14α-demethylase (ERG11) in Candida albicans treated with carvacrol

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A R T I C L E  I N F O
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A B S T R A C T
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 antifungal 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.


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 antifungal agents. Antifungal agents in the azole class target the fungal cell membrane through inhibition of the enzyme 14α-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 Δ^14-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)-tri-enol. The final step is the substrate of sterol Δ^12-desaturase and sterol Δ^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 14α-methyl group from lanosterol (11). Plants have been a source of secondary metabolites. Carvacrol from the class of monoterpen 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 Yasoju 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 × 10^6 C. albicans ATCC dissolved in RPMI 1640 and mixed with the different concentrations based on MIC (MIC and ½× MIC) of carvacrol. After 0, 2, 4, 6, 8, 10, 12, 24 and 48 hours incubation at 35°C, 100 µL 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 µg/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 ½× 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 μg 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 β 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 ± 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 ≤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% identity 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 antifungal properties against clinical isolates of Candida species compared to the standard antifungal agent (fluconazole) at 30 μg/mL concentration. However, the antifungal activity of the carvacrol was found to be potent at 20 μg/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 μg/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 μg/mL and 50-100 μg/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 log₁₀ 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 ≤ 0.05). At 48h, the log₁₀ 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 log₁₀ 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 2. Phenotypic characteristics of *Candida albicans*, *Candida krusei* and *Candida tropicalis*

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Germ tube production</th>
<th>Carbohydrate and nitrate assimilations</th>
<th>Carbohydrate fermentation</th>
<th>Urease</th>
<th>CHROM-agar colony color</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GLU MAL SUC LAC TER XYL STA KNO₃ GLU MAL SUC LAC TER</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em> ATCC</td>
<td>+</td>
<td>+ + + + - + + + + - + + - - + - + - -</td>
<td></td>
<td>+ + -</td>
<td>Blue-green</td>
</tr>
<tr>
<td>Ca2</td>
<td>+</td>
<td>+ + + + - + + + + - + + - - + - + - -</td>
<td></td>
<td>+ + -</td>
<td>Blue-green</td>
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<tr>
<td>Ca3</td>
<td>+</td>
<td>+ + + + - + + + + - + + - - + - + - -</td>
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<td>+ + -</td>
<td>Blue-green</td>
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<tr>
<td>Ca5</td>
<td>+</td>
<td>+ + + + - + + + + - + + - - + - + - -</td>
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<td>+ + -</td>
<td>Blue-green</td>
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<tr>
<td>Ca10</td>
<td>+</td>
<td>+ + + + - + + + + - + + - - + - + - -</td>
<td></td>
<td>+ + -</td>
<td>Blue-green</td>
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<tr>
<td><em>C. krusei</em> ATCC</td>
<td>-</td>
<td>+ - - - - - - - - - - - - - + - + - + + - - + - + - -</td>
<td></td>
<td>Pink-purple</td>
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<tr>
<td>Ck1</td>
<td>-</td>
<td>+ - - - - - - - - - - - - - + - + - + + - - + - + - -</td>
<td></td>
<td>Pink-purple</td>
<td></td>
</tr>
<tr>
<td>Ck24</td>
<td>-</td>
<td>+ - - - - - - - - - - - - - + - + - + + - - + - + - -</td>
<td></td>
<td>Pink-purple</td>
<td></td>
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<tr>
<td>Ck25</td>
<td>-</td>
<td>+ - - - - - - - - - - - - - + - + - + + - - + - + - -</td>
<td></td>
<td>Pink-purple</td>
<td></td>
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<tr>
<td>Ck26</td>
<td>-</td>
<td>+ - - - - - - - - - - - - - + - + - + + - - + - + - -</td>
<td></td>
<td>Pink-purple</td>
<td></td>
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<tr>
<td><em>C. tropicalis</em> ATCC</td>
<td>-</td>
<td>+ + + + - + + + + - + + - - + - + - -</td>
<td></td>
<td>Dark blue</td>
<td></td>
</tr>
<tr>
<td>Ct7</td>
<td>-</td>
<td>+ + + + - + + + + - + + - - + - + - -</td>
<td></td>
<td>Dark blue</td>
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<tr>
<td>Ct9</td>
<td>-</td>
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<tr>
<td>Ct11</td>
<td>-</td>
<td>+ + + + - + + + + - + + - - + - + - -</td>
<td></td>
<td>Dark blue</td>
<td></td>
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<tr>
<td>Ct17</td>
<td>-</td>
<td>+ + + + - + + + + - + + - - + - + - -</td>
<td></td>
<td>Dark blue</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Isolates</th>
<th>C. albicans ATCC</th>
<th>Ca2</th>
<th>Ca3</th>
<th>Ca5</th>
<th>Ca10</th>
<th>C. krusei ATCC</th>
<th>Ck1</th>
<th>Ck24</th>
<th>Ck25</th>
<th>Ck26</th>
<th>C. tropicalis ATCC</th>
<th>Ct7</th>
<th>Ct9</th>
<th>Ct11</th>
<th>Ct17</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 μg/mL</td>
<td></td>
<td></td>
<td></td>
<td>20 μg/mL</td>
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<td></td>
<td></td>
<td>30 μg/mL</td>
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<tr>
<td></td>
<td></td>
<td>Inhibition zone (mm)</td>
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<tr>
<td></td>
<td></td>
<td>6.00 ± 0.00</td>
<td>0.00</td>
<td>6.00 ±</td>
<td>0.00</td>
<td>6.00 ± 0.00</td>
<td>6.00 ±</td>
<td>0.00</td>
<td>6.00 ±</td>
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<td>6.00 ± 0.00</td>
<td>6.00 ±</td>
<td>0.00</td>
<td>6.00 ±</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16 ± 0.10</td>
<td>14.50 ±</td>
<td>0.11</td>
<td>14.00 ±</td>
<td>0.20</td>
<td>13.80 ±</td>
<td>0.10</td>
<td>10.0 ±</td>
<td>0.10</td>
<td>14.10 ± 0.11</td>
<td>12.50 ±</td>
<td>0.10</td>
<td>13.50 ±</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.90 ±</td>
<td>0.20</td>
<td>14.70 ±</td>
<td>0.11</td>
<td>13.00 ±</td>
<td>0.20</td>
<td>13.0 ±</td>
<td>0.23</td>
<td>9.00 ±</td>
<td>0.10</td>
<td>1250 ±</td>
<td>0.11</td>
<td>10.70 ±</td>
<td>0.00</td>
</tr>
</tbody>
</table>

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*

<table>
<thead>
<tr>
<th>Antifungals/ Isolates</th>
<th>C. albicans ATCC</th>
<th>Ca2</th>
<th>Ca3</th>
<th>Ca5</th>
<th>Ca10</th>
<th>C. krusei ATCC</th>
<th>Ck1</th>
<th>Ck24</th>
<th>Ck25</th>
<th>Ck26</th>
<th>C. tropicalis ATCC</th>
<th>Ct7</th>
<th>Ct9</th>
<th>Ct11</th>
<th>Ct17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carvacol</td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>6.25</td>
<td>12.5</td>
<td>6.25</td>
<td>12.5</td>
<td>6.25</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
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<td>6.25</td>
<td>12.5</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>25</td>
<td>25</td>
<td>25</td>
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<td>50</td>
<td>50</td>
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<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>0.125</td>
<td>0.125</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>2</td>
<td>1</td>
<td>0.25</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
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<tr>
<td></td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>64</td>
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<td>32</td>
<td>8</td>
<td>16</td>
<td>64</td>
<td>16</td>
<td>64</td>
</tr>
</tbody>
</table>

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.
Time kill curves of carvacrol and fluconazole in different concentration based on MIC (MIC and ½× 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 ≤ 0.05).

(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 µg/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 µg/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 was evaluated in C. albicans ATCC 14053 cells treated with different concentrations of carvacrol (MIC and ½× 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 ≤ 0.05). The C. albicans cells treated with fluconazole reduced the ergosterol contents in 58%. When the fluconazole concentration equal to ½ 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 ½× 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 Δ5,6-desaturase gene, down regulate (P ≤ 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 ½× 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
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 Δ^2,4-desaturase and lanosterol 14α-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 amphoteracin B has significantly decreased the expression level of MP65 and ERG3 in *C. albicans* and *C. dublinensis* (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 thoroughly 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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