Antifungal Activity of Carvacrol on Ergosterol Synthesis in Multidrug Resistant Candida albicans

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Original Article

Abstract

Introduction: Greatly increased use of antifungal therapies has resulted in the development of multidrug resistant. The phenolic compound carvacrol have been reported to have anti-Candida activity. This work is an attempt to examine effect of carvacrol on ergosterol synthesis against multidrug resistant Candida albicans.

Methods: This cross-sectional study has been conducted on 30 immune-compromised patients from vagina, mouth and skin surfaces during 2016-2017. Colonizing clinical isolates of C. albicans were identified and drug resistant isolates detected using WHONET software. The susceptibility tests for carvacrol were carried out in terms of disk diffusion, broth microdilution and time kill assays against multidrug resistant C. albicans. The ability to change from yeast to hyphal morphology exploited using a light microscopy. Ergosterol quantification has been investigated by spectrophotometric analysis. The expression profile of ERG11 gene was studied using quantitative real time RT-PCR to admit the possibility of further associated ergosterol pathway. Statistical analysis was performed with SPSS 21.0. Comparisons were performed using one-way ANOVA, with Tukey’s tests.

Results: Ten colonizing clinical isolates of C. albicans were identified. Multidrug resistant isolates of C. albicans were detected. Carvacrol was found to have MIC90 of 100-200 µg/ml for multidrug drug resistant isolates of C. albicans. The time kill curve results show that carvacrol could significantly inhibit the growth of C. albicans (P≤0.05). In multidrug resistant C. albicans treated with carvacrol there was a marked reduction of the transition of yeast cells to hyphal cells. Significant decrease of ergosterol content was noted in multidrug resistant C. albicans treated with carvacrol. Furthermore, significant down-regulation was observed on ERG11 gene in multidrug resistant C. albicans treated with carvacrol (P≤0.05).

Conclusion: Carvacrol show strong antifungal activity against multidrug resistant C. albicans. These results provide proof of concept for the implementation of carvacrol that may have potential applications in the treatment of drug resistance C. albicans infections.

Key words: Candida albicans, Ergosterol, Polymerase Chain Reaction

Introduction: Candida albicans is a part of the human normal microbiota, which becomes an opportunistic pathogen. Importantly, C. albicans is a potentially deadly human pathogen in immune-competent and immune-compromised hosts (1, 2). Antifungal drugs that are available for the treatment of Candida infections include the
The increased use of antifungal agents has led to multiple exposures of Candida species to these drugs with a resulting development of multidrug resistance. In addition, serious side effects include nephrotoxicity, visual disturbances, congestive heart failure caused by antifungal drugs. Moreover, some of these drugs are in limited clinical use due to high costs (2, 4-5).

Resistance isolates of C. albicans poses a serious threat to therapy and as a result has initiated a search for identifying new antifungal agents that could be used to treat the infections. In the recent years, antifungal compounds of natural origin, such as carvacrol, have received particular attention. They are a promising therapeutic strategy for fungal infections. Carvacrol \([\text{C}_8\text{H}_8\text{O}_3\text{H}[\text{C}_6\text{H}_3]]\), is a monoterpenoid phenol compound of the essential oils produced by of the Lamiaceae family. Results from in vitro and in vivo studies show that carvacrol possess antifungal activity against C. albicans (6-13).

Methods:

In this cross sectional study during a 6-month period in 2016-2017, various clinical samples such as vagina, mouth and skin surface swabs were collected from 30 immuno-compromised patients (diabetes, cancer and maintenance hemodialysis patients) who admitted in Shahid Beheshti hospital affiliated to Yasooj University of Medical Sciences and informed consent was obtained from patients. Clinical samples transported to the laboratory immediately. 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). Colonizing clinical isolates were routinely cultured in Sabouraud dextrose agar (SDA, Difco Laboratories, Detroit, Michigan). All the isolates were identified by conventional (including microscopic and macroscopic morphology, germ tube formation, carbohydrate assimilations, carbohydrate fermentation and urease test) and molecular method (using the universal fungal primers ITS1 and ITS4). Candida colonies were plated on CHROMagar™ Candida (CHROMagar, France) to check for purity and screening for green colonies (16). Reference strains of C. albicans ATCC 10231 and C. albicans CBS 5982 were employed. In addition, CLSI disk diffusion (M44-A2) and broth microdilution antifungal susceptibility tests (M27-A3 and M27-S4) were used to detect drug (amphotericin B, fluconazole, ketoconazole and miconazole) resistant isolates of C. albicans. The antimicrobial susceptibility test results were analyzed using WHONET software (17-19).

In vitro susceptibility to carvacrol (Sigma-Aldrich Co. St Louis, MO, USA) were determined using broth dilution antifungal susceptibility test in accordance with CLSI M27-A3. Inoculum suspensions were prepared in sterile 0.85% NaCl and standardized spectrophotometrically at 530 nm \((10^6 \text{ CFU/ml})\). This inoculum was diluted 1:100 in Roswell Park Memorial Institute medium 1640 (RPMI 1640) with 0.2% glucose.

Results from in vitro activity of carvacrol on 100 clinical isolates of C. albicans was evaluated MIC\(_{50}\) and MIC\(_{90}\) values of carvacrol were observed at 64 and 125 \(\mu\text{g/ml}\), respectively. Chami et al (13) demonstrated that the carvacrol and eugenol could be considered as strong antifungal agents in the treatment of experimental oral candidiasis induced by C. albicans in immune-suppressed rats.

Carvacrol exerts its effect on the cell membrane instability by targeting and binding ergosterol in C. albicans (6, 8, 14). The ergosterol and its biosynthetic pathway is essential for fungal growth. This pathway includes rate-limiting enzyme, lanosterol 14α-demethylase, which is essential in the ergosterol synthesis in fungi. Lanosterol 14-demethylase is a member of cytochrome P450 enzyme family encoded by the ERG11 gene. This enzyme is involved in the conversion of lanosterol to ergosterol, which catalyses the oxidative removal of the 14α-methyl group from lanosterol (15, 16).

In the present study, we attempted to evaluate the antifungal effect of carvacrol on ergosterol synthesis in multidrug resistant C. albicans. For this, we performed antifungal susceptibility, time kill assay, hypha formation, ergosterol content and gene expression profiling of multidrug resistant C. albicans.
water and 3 ml of n-heptane (Sigma-Aldrich) [buffered to pH 7.0 with 0.165 M hydroxide solution was added to harvested cells (Sigma-Aldrich) [buffered to pH 7.0 with 0.165 M hydroxide solution was added to harvested cells to achieve a final concentration of 0.5–5 × 10^3 CPU/ml, as verified by viable colony counts in SDA. The final concentration for carvacrol was 0.19–400 μg/ml. Test microtiter plates with 96 U wells (Moheb Qazvin, Iran) were incubated for 24 h at 35°C. The MIC endpoint for carvacrol was defined as the lowest concentration at which there was a 50 and 90% inhibition of growth as compared with the carvacrol-free growth control. Growth (carvacrol-free) and sterility (medium alone) control wells were established on each test. The time kill assay of carvacrol against C. albicans was determined at 0, 2, 4, 6, 8, 12, 24, and 48 h of incubation at 35°C by plating 10-fold dilutions on SDA. Carvacrol was used at concentrations ranging from 2×MIC to ¼×MIC (19).

The suspension of C. albicans cell with a density of 1×10^9 cells/ml was used to inoculate 6-well cell culture plates containing 100 μl carvacrol at concentrations ranging from 2×MIC to ¼×MIC. The cultures were incubated for 90 min at 35°C. Hyphal growth was induced by incubating cultures for 16 h at 35°C and 200 rpm. The hyphae were washed with PBS and visualized with a Nikon microscope, using a 40× objective (20).

The ergosterol quantification in the C. albicans cell membrane was performed by method as described by Santos et al. (21). Briefly, C. albicans colony (cell mass: 25 mg) from an overnight SDA plate culture was added to carvacrol at ranging from 2×MIC to ¼×MIC and incubated at 35°C for 24 h. Three ml of 25% alcoholic potassium hydroxide solution was added to harvested cells and incubated in a water bath at 85°C for 1 h. Thereafter the reaction was cooled to room temperature and a mixture of 1 ml of sterile water and 3 ml of n-heptane (Sigma-Aldrich) was added. The supematant was removed, and the reading was performed in spectrophotometer at 282 and 230 nm. A calibration curve with standard ergosterol was constructed and used to calculate the amount of ergosterol. The results were expressed as the percentage of ergosterol in comparison with the growth control.

Total RNA was extracted from C. albicans cells treated with carvacrol and fluconazole at concentrations of 2×MIC and 1×MIC using a RNeasy Mini Kit (Qiagen, Hilden, Germany) containing DNase I, as described by the manufacturer. RNA quality and quantity of different samples were estimated by spectrophotometric absorbance in a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). The integrity of total RNA was determined by electrophoresis on a formaldehyde-denaturing agarose gel. Total RNA was reverse transcribed into single-stranded cDNA using a M-MuLV reverse transcriptase and random hexamer oligonucleotides (Fermentas, USA), according to the manufacturer’s instructions. Real time PCR was performed using cDNA. Amplification, data acquisition and data analysis were performed on a Bio-Rad Mini Opticon™ system (USA) using™SYBR Green qPCR Master Mix (Fermentas, EU). The oligonucleotide primers for ERG11 (GenBank accession number X13296) and β actin were listed in Table 1. Relative quantitation of gene expression was obtained by the Pfaffl method (20, 22).

Data were analysed using the statistical software SPSS 21.0 for Windows (SPSS Inc, Chicago, IL, USA). Experiments were done in triplicate. Comparisons were performed using one-way ANOVA, with Tukey’s test. P≤0.05 was considered to be statistically significant.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERG11</td>
<td>Forward</td>
<td>5′ TGG AGA CGT GAT GCT G 3′</td>
<td>(22)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′ AGT ATG TGT GAC ACC CAT AA 3′</td>
<td></td>
</tr>
<tr>
<td>ACT</td>
<td>Forward</td>
<td>5′ GAG TGG CTC CAG AAG AAC TGC CAG 3′</td>
<td>(20)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′ TGA GTA ACA CGA TCA CCA GAA TCC 3′</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Oligonucleotide primers used for PCR

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Results:

Clinical isolates of *C. albicans* were phenotypically identified and their reliability were confirmed by DNA sequencing. The 10 isolates of *C. albicans* were obtained from 30 immuno-compromised patients. The antimicrobial susceptibility results for clinical isolates of *C. albicans* are presented in Table 2.

<table>
<thead>
<tr>
<th>Antibiotic Name</th>
<th>%R</th>
<th>%I</th>
<th>%S</th>
<th>%R 95% CI</th>
<th>MICs µg/ml</th>
<th>Geom. Mean</th>
<th>MIC range µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>90</td>
<td>0</td>
<td>10</td>
<td>541-99.5</td>
<td>4</td>
<td>283</td>
<td>1-4</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>66.5-100</td>
<td>64</td>
<td>27.86</td>
<td>16-64</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>66.5-100</td>
<td>8</td>
<td>746</td>
<td>4-8</td>
</tr>
<tr>
<td>Miconazole</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>66.5-100</td>
<td>8</td>
<td>8</td>
<td>8-8</td>
</tr>
</tbody>
</table>

Table 2. Antifungal susceptibility of clinical isolates of *C. albicans* from immuno-compromised patients

Table 3 summarizes the MIC (µg/ml) values of carvacrol against multidrug resistant isolates of *C. albicans*.

<table>
<thead>
<tr>
<th>Antifungal/Isolates</th>
<th>C. albicans ATCC 10231</th>
<th>C. albicans CBS 5982</th>
<th>Ca1</th>
<th>Ca2</th>
<th>Ca3</th>
<th>Ca4</th>
<th>Ca5</th>
<th>Ca6</th>
<th>Ca7</th>
<th>Ca8</th>
<th>Ca9</th>
<th>Ca10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carvacrol</td>
<td>MICo</td>
<td>50</td>
<td>25</td>
<td>200</td>
<td>100</td>
<td>100</td>
<td>200</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>MICo</td>
<td>25</td>
<td>12.5</td>
<td>100</td>
<td>50</td>
<td>100</td>
<td>50</td>
<td>100</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>MICo</td>
<td>16</td>
<td>0.125</td>
<td>64</td>
<td>16</td>
<td>32</td>
<td>32</td>
<td>64</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>MICo</td>
<td>1</td>
<td>0.0312</td>
<td>4</td>
<td>8</td>
<td>16</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>16</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Kappa coefficient showed that there was high degree of agreement between the results obtained by both disk diffusion and broth microdilution antifungal susceptibility tests. Multidrug resistance was observed in clinical isolates of *C. albicans* from immuno-compromised patients. The resistance rates of clinical isolates of *C. albicans* for amphotericin B, fluconazole, ketoconazole and miconazole were 90% (9.10), 100% (10.10), 100% (10.10), and 100% (10.10) respectively.

Table 3 summarizes the MICs of carvacrol compared with fluconazole against drug resistant isolates of *C. albicans*. Carvacrol was found to have MICo of 100-200 µg/ml for drug resistant isolates of *C. albicans*.

Figures 1A and B show the pattern of growth and kill by carvacrol and fluconazole of *C. albicans* ATCC 10231 and *C. albicans* CBS 5982, respectively, at concentrations ranging from 2×MIC to ¼×MIC. The results revealed that carvacrol reduced number of *C. albicans* cells after 2, 4, 6, 8, 12, 24 and 48 compared to untreated control (P≤0.05). In general, time kill curves for *C. albicans* ATCC 10231 showed decreases 3.73-, 2.99-, 2.47 and 2.23-fold in fungal population size (the log_{10} CFU) treated with 2×MIC, 1×MIC, 1/2×MIC and 1/4×MIC of carvacrol, respectively. *C. albicans* ATCC 10231 cells were not killed by fluconazole but instead continued to multiply, albeit at a lower rate than untreated control. At 48th, the log_{10} CFU value was reduced 4.67-, 3.69-, 3.19 and 3.14-fold in the *C. albicans* CBS 5982 treated with 2×MIC, 1×MIC, 1/2×MIC and 1/4×MIC of carvacrol, respectively. At concentrations ranging from 2×MIC to ¼×MIC, fluconazole reduced the counts by 7.82- to 4.42-fold log_{10} CFU/ml in *C. albicans* CBS 5982.

Figure 1. Time kill curves of carvacrol and fluconazole at concentrations ranging from 2×MIC to ¼×MIC against (A) *Candida albicans* ATCC 10231 and (B) *Candida albicans* CBS 5982. Data are means with standard error from three independent experiments in triplicate assays. C: Carvacrol, F: Fluconazole
To measure the changes in hypha formation we exploited a light microscopy to visualize yeast–hyphal transition indicated that the treatment of preformed hypha with carvacrol resulted in a subtle reduction in yeast-hyphal transition compared to untreated control. As shown in Figure 2, carvacrol decreased yeast-hyphal transition of C. albicans ATCC 10231.

Figure 2. Determination of hypha generation by Candida albicans ATCC 10231 treated with 2 × MIC, 1 × MIC, 1/2 × MIC and 1/4 × MIC of carvacrol after 16 h. (A) Untreated control, (B) 2 × MIC, (C) 1 × MIC, (D) 1/2 × MIC, (E) 1/4 × MIC. Magnification × 40, Bar = 50 µm

Our results indicate that the carvacrol exert their antifungal effect through inhibition of ergosterol biosynthesis in C. albicans ATCC 10231 and C. albicans CBS 5982. At 2 × MIC, 1 × MIC, 1/2 × MIC and 1/4 × MIC concentrations, carvacrol reduced 66.4%, 61%, 54.2% and 38.5%, respectively, of total ergosterol content in C. albicans ATCC 10231. No significant differences in mean ergosterol content were observed in C. albicans ATCC 10231 grown in the presence of fluconazole (P > 0.05). The decrease in ergosterol content of C. albicans CBS 5982 at 2 × MIC, 1 × MIC, 1/2 × MIC and 1/4 × MIC of carvacrol were 67%, 61.2%, 52.4% and 43%, respectively. Fluconazole was effective at concentrations ranging from 2 × MIC to 1/4 × MIC and caused 64 to 42% reduction in total ergosterol content in C. albicans CBS 5982 (Figure 3).

Figure 3. Percent ergosterol levels of (A) Candida albicans ATCC 10231 and (B) Candida albicans CBS 5982 after 24 h of treatment with different concentrations of antifungal agents based on MIC. Data are means with standard error from three independent experiments in triplicate assays. C: Carvacrol, F: Fluconazole

Figure 4. Relative quantitation of gene expression of ERG11 in Candida albicans ATCC 10231 and Candida albicans CBS 5982 treated with C: carvacrol and F: fluconazole at concentrations of 2 × MIC and 1 × MIC after 24 h. Data are means of fold changes with standard error from three independent experiments amplified in triplicates.

Conclusion:

Resistance to treatment with antifungal drugs has been extensive clinical problem. High
throughput screening has emerged as a tool for biological investigation that could be used to treat the infections (2, 5, 23). In this study, we show that carvacrol displays potential anti-Candida activity which has been earlier reported to active against C. albicans (6, 8). Our data supports influences of carvacrol in multidrug resistance clinical isolates of C. albicans. Several lines of evidence demonstrate that carvacrol was the most effective compound in killing drug resistant Candida species (24, 25).

The mechanisms of action of carvacrol was suggested to be the result of its inhibitory activity on membrane integrity of C. albicans and the disruption of ergosterol biosynthesis which may also prove lethal to the C. albicans. It was demonstrated that carvacrol exerts its antifungal effects by mechanisms resembling calcium stress and inhibition of the target of rapamycin signaling pathway (6, 9, 26). The time kill assays performed in this study provide information on the effect of carvacrol on drug resistance C. albicans. Our observation suggests that carvacrol, which not only active on drug resistance C. albicans, also influences growth and kill of Candida cells. The antifungal activity of carvacrol on the drug resistance C. albicans were able to reduced completely a significant number of yeast forms. In addition, this molecule inhibited the transition from yeast to hyphae cells and also ergosterol content that could decrease the ability of C. albicans cells to cause disease.

The major pathways that is essential for fungal growth include the ergosterol and its biosynthetic pathway. However, the ERG11 gene play key roles in ergosterol biosynthetic pathway (15, 16).

In this study, the relative gene expression results indicated the inhibitory effect of carvacrol on ERG11 gene that regulate ergosterol synthesis. Akins (26) indicated that the ergosterol biosynthesis genes ERG3 and ERG11 are responsible for antifungal resistance in C. albicans. Khodavandi et al. (19) were demonstrated that the fluconazole in combination with terbinafine significantly reduce the expression of ERG1, 3 and 11 in the cell membrane of C. albicans.

In conclusion, our study demonstrates that carvacrol exhibits potent antifungal activity against drug resistance C. albicans, reduce the number of yeast forms, inhibits hyphae formation, reduce ergosterol content and inhibitory effect of ERG11 gene expression. Because infections due to drug resistance C. albicans are an alarming health problem, these results suggest that carvacrol may have potential applications in the treatment of drug resistance C. albicans infections. Further investigation is needed to gauge the validity of the effect of carvacrol on other significant genes contributing to the cell membrane of C. albicans with different techniques such as next generation sequencing and microarray.

Acknowledgments:
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Antifungal Activity of Carvacrol

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چکیده

مقدمه: استفاده از داروهای ضد قارچی به میزان قابل توجهی افزایش یافته و منجر به ایجاد مقاومت دارویی چندگانه شده است. مطابق گزارش‌ها ترکیب فنلی کاروکرل دارای فعالیت ضد کاندیدیایی می‌باشد. در مطالعه حاضر، تلاش بر این است که تاثیر کاروکرول بر سنتز ارگوسترول کاندیدا آلبیکانس دارای مقاومت دارویی چندگانه قندگانه شده است.

روش کار: این مطالعه مقطعی طی سال‌های 2013-2015 بر روی 33 نمونه واژن، دهان و سطوح پوست بیماران مبتلا به ضعف سیستم ایمنی انجام شد. جدایه‌های بالینی کلونیزه کاندیدا آلبیکانس شناسایی شدند و جدایه‌های مقاوم به داروهای استخراج شده با استفاده از نرم‌افزار WHONET تشخیص داده شدند. تست حساسیت تاثیر کاروکرول علیه کاندیدا آلبیکانس دارای مقاومت WHONET استخراج شده از ترمیم‌های طبیعی با استفاده از روش نرم‌افزاری میکروکولونژی میکروبیولوژی، واحد یاسوج، دانشگاه آزاد اسلامی، یاسوج، ایران. نتایج

نتیجه‌گیری: نتایج حاصل از آزمون‌های تحلیل آماری با استفاده از نرم‌افزار SPSS 21.0 نشان داد که کاروکرول می‌تواند به طور معنی‌داری از رشد کاندیدا آلبیکانس دارای مقاومت، به دلیل کاهش تبدیل سلول‌های مخمری به ریسه و کاهش میزان ارگوسترول در نواحی ارگوسترول گسترش دهنده باعث کاهش سیستم ایمنی گردید.

کلماتهای کلیدی: کاندیدا آلبیکانس، ارگوسترول، واکنش زنجیره‌ای پلیمراز (PCR)