

## 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 MIC<sub>90</sub> 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

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### 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

polyenes, azoles, allylamines, echinocandins and miazines. Their target mechanisms are inhibiting or interfering with cell membrane or cell wall and inhibiting DNA synthesis (2-4).

The increased use of antifungal agents has led to multiple exposures of *Candida* species to these drugs with a resulting development of multidrug resistant. 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 [C<sub>6</sub>H<sub>3</sub>CH<sub>3</sub>(OH)(C<sub>3</sub>H<sub>7</sub>)], is a monoterpene 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). Vardar-Unlu *et al* (12) the *in vitro* activity of carvacrol on 100 clinical isolates of *C. albicans* was evaluated. MIC<sub>50</sub> and MIC<sub>90</sub> values of carvacrol were observed at 64 and 125 µg/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 14 $\alpha$ -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 14 $\alpha$ -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*.

#### 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, CSLI 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<sup>6</sup> CFU/ml). This inoculum was diluted 1:100 in Roswell Park Memorial Institute medium 1640 (RPMI 1640) with 0.2% glucose

(Sigma-Aldrich) [buffered to pH 7.0 with 0.165 M morpholinophosphonyl sulfate (MOPS)] to achieve a final concentration of  $0.5-5 \times 10^3$  CFU/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  $1 \times 10^6$  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 supernatant 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.  $P \leq 0.05$  was considered to be statistically significant.

Table 1. Oligonucleotide primers used for PCR

Primer	Orientation	Sequence	Reference
<i>ERG11</i>	Forward	5' TGG AGA CGT GAT GCT G 3'	(22)
	Reverse	5' AGT ATG TTG ACC ACC CAT AA3'	
<i>ACT</i>	Forward	5' GAG TTG CTC CAG AAG AAC ATC CAG 3'	(20)
	Reverse	5' TGA GTA ACA CCA TCA CCA GAA TCC 3'	

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

Antibiotic Name	%R	%I	%S	%R 95% CI	MIC <sub>90</sub> µg/ml	Geom. Mean	MIC rangeµg/ml
Amphotericin B	90	0	10	54.1-99.5	4	2.83	1-4
Fluconazole	100	0	0	66.5-100	64	27.86	16-64
Ketoconazole	100	0	0	66.5-100	8	7.46	4-8
Miconazole	100	0	0	66.5-100	8	8	8-8

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

Antifungal/Isolates	<i>C. albicans</i> ATCC 10231	<i>C. albicans</i> CBS 5982	Ca1	Ca2	Ca3	Ca4	Ca5	Ca6	Ca7	Ca8	Ca9	Ca10
Carvacrol	MIC <sub>90</sub>	50	25	200	100	200	100	200	200	100	100	100
	MIC <sub>50</sub>	25	12.5	100	50	100	50	100	100	50	50	50
Fluconazole	MIC <sub>90</sub>	16	0.125	64	16	32	32	32	64	16	32	16
	MIC <sub>50</sub>	1	0.0312	4	8	16	8	8	4	16	8	8

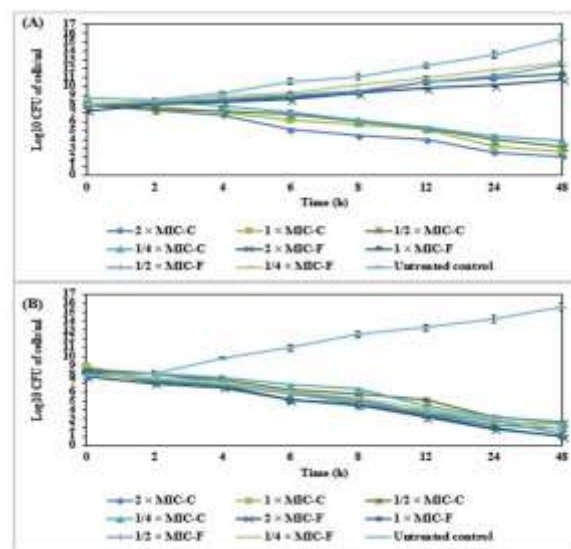
Ca: Multidrug resistant isolates of *C. albicans*.

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 immune-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 MIC<sub>90</sub>s of carvacrol compared with fluconazole against drug resistant isolates of *C. albicans*. Carvacrol was found to have MIC<sub>90</sub> 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<sub>10</sub> CFU) treated with 2×MIC, 1×MIC, 1/2×MIC and ¼×MIC of carvacrol, respectively. *C. albicans* ATCC 10231 cells were not killed by fluconazole but instead

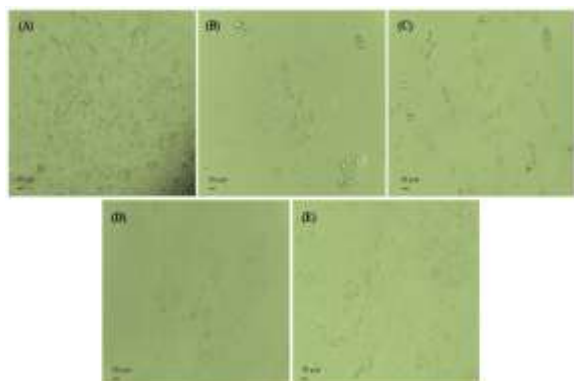
continued to multiply, albeit at a lower rate than untreated control. At 48<sup>th</sup>, the log<sub>10</sub>CFU value was reduced 4.67-, 3.69-, 3.19 and 3.14-fold in the *C. albicans* CBS 5982 treated with 2×MIC, 1×MIC, 1/2×MIC and ¼×MIC of carvacrol, respectively. At concentrations ranging from 2×MIC to ¼×MIC, fluconazole reduced the counts by 7.82- to 4.42-fold log<sub>10</sub> 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.

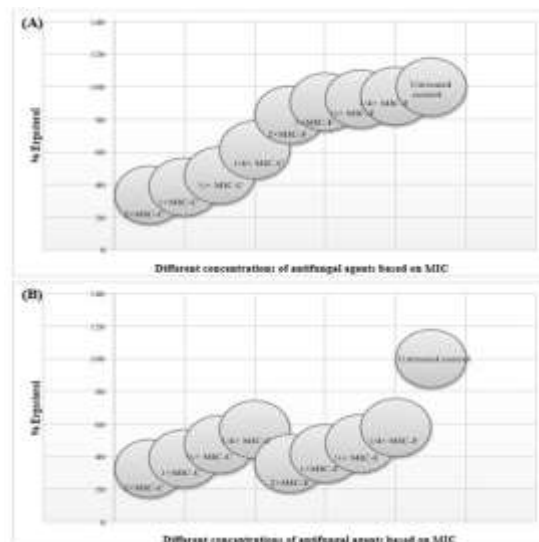


**Figure 2. Determination of hypha generation by *Candida albicans* ATCC 10231 treated with 2 × MIC, 1 × MIC, 1/2 × MIC and 1/4 × MIC of carvacrol after 16 h. (A) Untreated control, (B) 2 × MIC, (C) 1 × MIC, (D) 1/2 × MIC, (E) 1/4 × MIC. Magnification × 40, Bar = 50 μm**

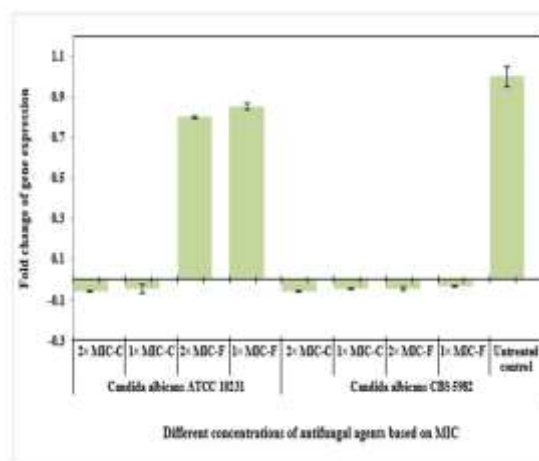
Our results indicate that the carvacrol exert their antifungal effect through inhibition of ergosterol biosynthesis in *C. albicans* ATCC 10231 and *C. albicans* CBS 5982. At 2×MIC, 1×MIC, 1/2×MIC and 1/4×MIC concentrations, carvacrol reduced 66.4%, 61%, 54.2% and 38.5%, respectively of total ergosterol 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 4 demonstrate the effect of carvacrol on expression levels of *ERG11* in *C. albicans* ATCC 10231 and *C. albicans* CBS 5982. The expression levels of *ERG11* were down regulated (P≤0.05) in *C. albicans* ATCC 10231 and *C. albicans* CBS 5982 cells treated with carvacrol at concentrations of 2×MIC and 1×MIC. The expression levels of

*ERG11* gene did not show any significant differences in *C. albicans* ATCC 10231 treated with fluconazole (P>0.05). While the expression levels of *ERG11* were down regulated (P≤0.05) in *C. albicans* CBS 5982.



**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

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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.

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## اثر ضد قارچی کارواکرول بر سنتز ارگوسترول کاندیدا آلیکانس دارای مقاومت دارویی چندگانه

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مجله پزشکی هرمزگان سال بیست و دوم شماره اول ۹۷ صفحات ۱۲۱-۱۱۳

### چکیده

**مقدمه:** استفاده از داروهای ضد قارچی به میزان قابل توجهی افزایش یافته و منجر به ایجاد مقاومت دارویی چندگانه شده است. مطابق گزارش ها ترکیب فنی کارواکرول دارای فعالیت ضد کاندیدیایی می باشد. در مطالعه حاضر، تلاش بر این است که تأثیر کارواکرول بر سنتز ارگوسترول کاندیدا آلیکانس دارای مقاومت دارویی چندگانه مورد بررسی قرار گیرد.

**روش کار:** این مطالعه مقطعی طی سال های ۲۰۱۶ تا ۲۰۱۷ بر روی ۳۰ نمونه واژن، دهان و سطوح پوست بیماران مبتلا به ضعف سیستم ایمنی انجام شد. جدایه های بالینی کلونیزه کاندیدا آلیکانس شناسایی شدند و جدایه های مقاوم به دارو با استفاده از نرم افزار WHONET شناسایی شدند. تست حساسیت سنجی تأثیر کارواکرول علیه کاندیدا آلیکانس دارای مقاومت دارویی چندگانه با استفاده از روش های انتشار دیسک، میکرودیالوژن برآش و سنجش زمان مرگ سلولی انجام شد. در کاندیدا آلیکانس دارای مقاومت دارویی چندگانه تیمار شده با کارواکرول توانایی تبدیل فرم مخمری به ریسبه با استفاده از میکروسکوپ نوری بررسی شد. با استفاده از روش اسپکتروفتومتری میزان ارگوسترول آنالیز شد. برای بررسی ارتباط بیشتر با مسیر سنتز ارگوسترول، با استفاده از *real time RT-PCR* کمی پروفیل بیان ژن *ERG11* مورد مطالعه قرار گرفت. تجزیه و تحلیل آماری با استفاده از نرم افزار SPSS 21.0 انجام شد. مقایسه داده ها با استفاده از آزمون های آنالیز واریانس یک طرفه و توکی انجام شد.

**نتایج:** ده جدایه بالینی کلونیزه کاندیدا آلیکانس شناسایی شد. جدایه های کاندیدا آلیکانس دارای مقاومت دارویی چندگانه شناسایی شد. کارواکرول دارای MIC<sub>90</sub> ۱۰۰-۲۰۰ میکروگرم/میلی لیتر برای جدایه های کاندیدا آلیکانس دارای مقاومت دارویی چندگانه بود. نتایج منحنی زمان مرگ نشان داد که کارواکرول می تواند به طور معنی دار از رشد کاندیدا آلیکانس جلوگیری نماید ( $P \leq 0.05$ ) کاهش معنی دار تبدیل سلول های مخمری به ریسبه در کاندیدا آلیکانس دارای مقاومت دارویی چندگانه تیمار شده با کارواکرول وجود داشت. علاوه بر این، کاهش میزان ارگوسترول کاندیدا آلیکانس دارای مقاومت دارویی چندگانه تیمار شده با کارواکرول به خوبی مشاهده شد. در نهایت، در کاندیدا آلیکانس دارای مقاومت دارویی چندگانه تیمار شده با کارواکرول بیان ژن *ERG11* کاهش پیدا کرد ( $P \leq 0.05$ )

**نتیجه گیری:** نتایج حاصل از آزمایش نشان داد که کارواکرول علیه کاندیدا آلیکانس دارای مقاومت دارویی چندگانه تأثیر داشت. نتایج حاصل اثباتی برای کارایی کارواکرول در درمان عفونت های ناشی از کاندیدا آلیکانس دارای مقاومت دارویی ارائه می دهد.

**کلیدواژه ها:** کاندیدا آلیکانس، ارگوسترول، واکنش زنجیره ای پلیمرز

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