J HerbMed Pharmacol. 2016; 5(2): 78-84.

Journal of HerbMed Pharmacology

Journal homepage: http://www.herbmedpharmacol.com

Fungicidal effect of *Origanum vulgare* essential oil against *Candida glabrata* and its cytotoxicity against macrophages

Ghasem Vahedi[1], Ali Reza Khosravi[1*], Hojjatollah Shokri[2], Zahra Moosavi[3], Nowruz Delirezh[4], Aghil Sharifzadeh[1], Abbas Barin[5], Somayeh Shahrokh[1], Asad Balal[1]

[1]Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
[2]Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran
[3]Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
[4]Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
[5]Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

ARTICLE INFO

Article Type:
Original Article

Article History:
Received: 3 January 2016
Accepted: 16 March 2016

Keywords:
Origanum vulgare
Essential oil
Candida glabrata
Antifungal
Oropharyngeal candidiasis

ABSTRACT

Introduction: *Candida glabrata* is a yeast fungus regularly isolated from patients with impaired immunity who receive a routine antifungal therapy. Drug-resistant strains of *C. glabrata* have been emerged in recent years. The aim of this study was to examine the therapeutic efficacy *Origanum vulgare* essential oil (OVEO) against drug-resistant strains of *C. glabrata* and its cytotoxic effect on macrophages.

Methods: Specimens were collected from mucosal surfaces of the oral cavity of medically approved oropharyngeal candidiasis (OPC) in HIV-positive patients and volunteered healthy individuals using sterile swabs or mouthwashes. In vitro antifungal susceptibility testing was done using microdilution and disc diffusion methods. Chemical composition of OVEO was determined using gas chromatography mass spectrometry. The cytotoxic effect of essential oil on macrophages was examined using tetrazolium dye (MTT).

Results: Minimum inhibitory concentration (MIC) range of OVEO in healthy individuals and OPC patients was 150-200 and 150-250 µg/mL, respectively. OVEO efficiently inhibited growth of resistant isolates. In isolates obtained from HIV patients, both MIC50 and MIC90 of OVEO were 200 µg/mL while in healthy individuals were 150 and 200 µg/mL, respectively. Moreover, OVEO induced significant reduction in proliferation of murine RAW264.7 and peritoneal macrophages in concentrations higher than 100 and 300 µg/mL, respectively. Main constituents of OVEO were thymol (27.3%), γ-terpinene (20.7%) and carvacrol (16.1%).

Conclusion: OVEO could be used as a fungicidal agent against fungal infections caused by azole-resistant *C. glabrata*. A combination therapy along with standard antifungals is suggested to avoid its cytotoxic effects.

Implication for health policy/practice/research/medical education:
The results of this study can help to overcome increasing rate of resistance to current standard antifungal drugs.
Please cite this paper as: Vahedi G, Khosravi AR, Shokri H, Moosavi Z, Delirezh N, Sharifzadeh A, et al. Fungicidal effect of Origanum vulgare essential oil against Candida glabrata and its cytotoxicity against macrophages. J HerbMed Pharmacol. 2016;5(2):78-84.

Introduction

Candida spp. are normal yeast flora commensally present on the mucosal surfaces of about 60%-80% of healthy individuals (1). But in the case of impaired or suppressed immunity, Candida spp. could trigger the opportunistic fungal infections in spite of their nonpathogenic nature (2). Factors affecting immune system such as diabetes, long-term corticosteroid therapy, receiving solid-organ transplants, immunosuppressing diseases such as HIV and neutropenia might predispose the patients to fungal infections which are usually known as nosocomial fungal infections (3).

Intrinsic and acquired multi-drug antimicrobial resistance of fungi in immunocompromised patients has been frequently reported as an important medical issue in recent years (4). *Candida* (formerly *Torulopsis*) *glabrata* was recorded as the second leading cause of invasive candidiasis (IC) in immunosuppressed patients in the United States

***Corresponding author:** Ali Reza Khosravi; Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. Tell: +982161117099; Fax: +982166933222; Email: khosravi@ut.ac.ir

(5). Most of the *C. glabrata* infections occurred on mucosal surfaces (oral cavity, esophagus, vagina, or urinary system) or as a systemic blood stream infection (6). At least one episode of vulvovaginal candidiasis is observed in 75% of all women during lifetime, and 5%-10% of whom will acquire the disease again (7). Azole-resistance of *C. glabrata* and *Candida krusei* isolates in recurrent vulvovaginal candidiasis (RVVC) patients has been documented (8). The frequency of itraconazole-resistant *C. glabrata* isolates was reported 74.1% in RVVC patients (8). In recent years alternative nature-based therapies have been taken into consideration. Origanum species are one of the local medicinal plants growing wild in and endemic to Mediterranean countries. Most of the Origanum species grow in east Mediterranean regions. Unlike most Origanum species, *Origanum vulgare* is the only species widely spread in different regions and commonly occurs in the Irano-Turanian region (9). The blooming period of endemic *O. vulgare* species in Iran is between June and August. It grows in different geographical regions in Iran, mostly in west, northwest and north (10).

The main purpose of the present study is to examine potential antifungal attributes of essential oil (EO) of endemic Iranian *O. vulgare* and to determine its cytotoxic effect on a murine RAW264.7 macrophage cell line and murine peritoneal macrophages. We compared the EO fungicidal activity among *C. glabrata* isolates obtained from two groups of volunteers including healthy individuals and HIV-positive patients with oropharyngeal candidiasis (OPC) clinical manifestations.

Materials and methods

Plant materials and isolation of EO

Origanum vulgare Linn. ssp. vulgare in pre-flowering stage in June 2014 was collected from forests and highlands around Piranshahr, West Azarbaijan province, northwest Iran. The plant was authenticated by a herbal specialist. 250 g of ground aerial parts of plant was mixed in 1600 mL of distilled water and transferred into a Clevenger apparatus. The EO isolation was done by hydrodistillation for 3 hours according to instructions recommended by British pharmacopoeia. Water was removed from extracted EO by decantation and anhydrous sodium sulphate. Density of purified EO was obtained 0.92 g/mL by gravimetric method. Isolated purified EO was stored at 4°C in a dark container until use.

Characterization of EO chemical composition

Chemical composition of *O. vulgare* EO (OVEO) was determined by gas chromatography (GC)-flame ionization detector (FID) and GC-mass spectrometry (MS). A Thermoquest Finnigan (later Thermo Fisher Scientific) instrument equipped with a DB-5 capillary column (60 m × 0.25 m i.d., film thickness 0.25 µm) was used for GC-FID. Nitrogen was used as carrier gas at a fixed flowing rate of 1.1 mL/min. The ionization energy was 70 eV in a split mode (ratio 1:50). The oven temperature was gradually elevated from 60°C to 250°C at a rate of 5°C/min. The injector and detector (FID) temperature was set at 250°C and 280°C, respectively. GC-MC evaluation of OVEO was established using a Thermoquest Finnigan Trace GC-MS machine. The temperature program and capillary column used in this analysis were the same as GC-FID. Helium at a flowing rate of 1.1 mL/min was used for GC-MS in a split ratio of 1:50. The main components present in OVEO were characterized by measuring their retention indices resulted from programmed temperatures for n-alkanes (C6-C24) and capillary column. The components were detected by comparing their mass spectra to the internal reference mass spectra database in software provided alongside GC instrument. Quantification of identified components was obtained by calculating the area under the FID peaks.

Specimen collection from human participants

Before specimen collection, all participants received informed consent. All HIV positive patients included in this study had clinical manifestations of OPC and had received azole therapy. Also, healthy volunteers who had no history of receiving azole drugs were classified eligible to participate in the study. Samples were collected from oral mucosal surfaces using sterile swabs. Number, grouping and demographic characteristics of HIV-positive patients and healthy volunteers under this study can be found in our two previous studies (11,12). For some of the OPC patients, we used mouthwash (MW) method.

Yeasts growth conditions and identification

The swabs and MW samples were cultured on Sabouraud Dextrose Agar and incubated aerobically at 37°C for 72 hours. Yeast isolates were identified using morphologic, physiologic, biochemical and molecular methods.

In-vitro susceptibility testing – microdilution

Broth microdilution antifungal susceptibility testing (AST) for yeasts was done for yeasts based on National Committee for Clinical Laboratory Standards (NCCLS) (recently referred to as Clinical and Laboratory Standards Institute [CLSI]) M27-A2 protocol (13). OVEO along with two azole derivatives including fluconazole and itraconazole were incorporated in AST. OVEO dilutions were prepared in MOPS-buffered RPMI1640 medium containing 4% ethanol. Final concentrations of OVEO in yeast-inoculated wells were 10, 20, 50, 100, 150, 200, 250, 300, 400 and 500 µg/mL. Final concentration of ethanol was 2%. Concentration of OVEO solutions in ethanol was calculated based on density of OVEO which was 0.92 g/mL and density of ethanol which was 0.79-0.793 g/cm^3 (mean 0.7915 g/cm^3). Also, both fluconazole and itraconazole were dissolved in DMSO. The concentration range of two-fold solutions for fluconazole and itraconazole was 0.25-128 µg/mL and 0.0625-32 µg/mL, respectively. Final concentration of DMSO was 1% based on M27-A2 instructions (13). Inoculated microtiter plates were incubated at 35°C for 24 and 48 hours. MIC end points were read at 24 and 48 hours.

In vitro susceptibility testing-disc diffusion

Neo-Sensitabs™ fluconazole 25 µg tablets were used for antifungal disk diffusion susceptibility testing of yeasts based on CLSI M44-A protocol (14). Zone of inhibition was defined as the nearest whole millimeter at the point at which there was a prominent decrease in growth of yeast colonies around drug disc.

Macrophage cell culture conditions

Murine peritoneal RAW264.7 macrophage (RM) cell line was purchased from Human and Animal Cell Bank, Iranian Biological Resource Center. Cells were cultured using high glucose DMEM with 10% FBS and incubated in a humidified 5% CO2 incubator at 37°C. Eight-week-old female BALB/c mice were purchased from Razi Vaccine and Serum Research Institute, Tehran, Iran. Murine normal resident peritoneal macrophages (PMs) were isolated from peritoneal cavity of the mice and were cultured in DMEM supplemented with 10% FBS.

MTT cytotoxicity assay

RPMI 1640 medium without phenol red (RWP) was used for MTT assay. 10^4 cells in 100 µL of RWP medium were seeded into each well of 96-well microtiter plate. Final concentrations of OVEO in 10 wells of a row were 10, 100, 150, 200, 250, 300, 350, 400, 500, 1000 µg/mL. Triton-X100 was used as positive control. Last well was dispensed by only 100 µL of RWP medium (negative control). After 24-hour incubation the supernatant was removed and 100 µL of fresh RWP medium was replaced. 10 µL of 5 mg/mL MTT stock solution was added to each well. The plate was incubated at 37°C for 4 hours. After 4-hour incubation, the supernatant medium was gently aspirated out and 100 µL of DMSO was dispensed into each well and mixed thoroughly by pipetting. The plate was incubated at 37°C for 15 minutes. All wells were mixed thoroughly and the absorbance was read using an ELISA reader at 570 nm wavelength.

Statistical analysis

Mann-Whitney U test was used to investigate the differences in mean MIC of OVEO between two groups. Also we analyzed the differences between frequencies of resistant, susceptible and susceptible dose-dependent (SDD) isolates using the test of proportions. Inhibitory concentrations 50% (IC50) of OVEO were calculated using dose-response analysis and three-parametric nonlinear regression method. *P* values less than 0.05 were considered as significant.

Results

Monoterpenes and oxygenated monoterpenes were main components of OVEO

Main components of OVEO according to GC-FID and GC-MS analysis were thymol (27.3%), γ-terpinene (20.7%), carvacrol (16.1%), germacrene D-4-ol (9.5%), β-Pinene (6.4%), β-caryophyllene (3.2%) and p-cymene (3%). Oxygenated monoterpenes including cis-β-terpineol (1.3%), thymol (27.3%) and carvacrol (16.1%) were main group of components in OVEO. Two phenolic isomers thymol (27.3%) and carvacrol (16.1%) comprised 43.4% of OVEO as most abundant components followed by monoterpene hydrocarbons including α-thujene, α-pinene, sabinene, β-pinene, α-terpinene, γ-terpinene and p-cymene comprising 35.4% of OVEO and oxygenated sesquiterpenes including germacrene D-4-ol and caryophyllene oxide. Sesquiterpene hydrocarbons including β-caryophyllene, α-himachalene and α-candinene were other components of OVEO. Octen-3-ol (0.3%), an aromatic alcohol, was also detected in OVEO (Table 1).

Azole-resistant isolates of *C. glabrata* were more prevalent among patients with OPC

We could isolate six *C. glabrata* isolates from individuals people and 16 isolates from patients with OPC. All *C. glabrata* isolates obtained from healthy individuals were classified as susceptible based on fluconazole and itraconazole disc diffusion and microdilution test results (Table 2 and Figure 1). The results of fluconazole disc diffusion and fluconazole microdilution tests in isolates from healthy people were 100% identical (all isolates were classified susceptible by both methods). Also in the case of isolates from patients with OPC, only two differences were observed between the results of fluconazole disc diffusion and fluconazole microdilution. These two isolates were susceptible in fluconazole disc diffusion but were SDD in fluconazole microdilution. On the other hand, among isolates obtained from patients with OPC, 10 isolates in both fluconazole microdilution and disc diffusion

Table 1. Chemical composition of Origanum vulgare essential oil by GC-MS and GC-FID analysis

Components	RI	Area under peak (%)
α-Thujene	932	0.9
α-Pinene	943	1.3
Octen-3-ol	975	0.3
Sabinene	984	1.3
β-Pinene	988	6.4
α-Terpinene	1026	1.8
p-Cymene	1035	3
γ-Terpinene	1060	20.7
cis-β-Terpineol	1153	1.3
Thymol	1285	27.3
Carvacrol	1288	16.1
β-Caryophyllene	1380	3.2
α-Himachalene	1459	2.2
Germacrene D-4-ol	1493	9.5
α-Candinene	1531	1
Caryophyllene oxide	1582	2.8
Total		99.1
Monoterpene hydrocarbons		35.4
Oxygenated monoterpenes		44.7
Sesquiterpene hydrocarbons		6.4
Oxygenated sesquiterpenes		12.3
Others		0.3

Abbreviations: GC-MS, gas chromatography mass spectrometry; GC-FID, gas chromatography-flame ionization detector; RI, retention index.

Table 2. MIC ranges, MIC50 and MIC90 of azoles and *Origanum vulgare* essential oil and frequencies of azole-resistance in *Candida glabrata* isolates obtained from healthy individuals and OPC patients

Participants	Antifungals	MIC (µg/mL)			R	SDD	S
		50 (%)	90 (%)	Range	No. (%)	No. (%)	No. (%)
Healthy	Fluconazole	1	2	0.5-4	0 (0 %)	0 (0 %)	6 (100 %)
	Itraconazole	0.03125	0.0625	0.03125-0.0625	0 (0 %)	0 (0 %)	6 (100 %)
	OVEO	150	200	150-200	-	-	-
Patients with OPC	Fluconazole	64	64	4-64	10 (62.5 %)	3 (18.75 %)	3 (18.75 %)
	Itraconazole	0.125	1	0.0625-4	4 (25 %)	4 (25 %)	8 (50 %)
	OVEO	200	200	150-250	-	-	-

Abbreviations: MIC, minimum inhibitory concentration; OPC, oropharyngeal candidiasis; No, number; R, resistant; SDD, susceptible dose-dependent; S, susceptible.

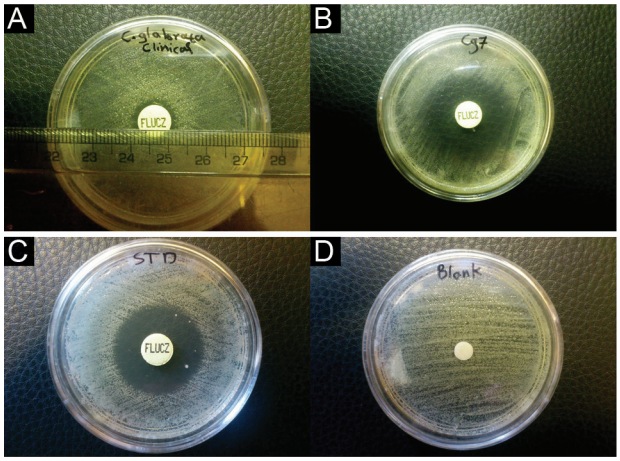

Figure 1. Antifungal susceptibility testing of *Candida glabrata* isolates by disc diffusion; fluconazole 25 µg tablets were used for disc diffusion assays; (A) disc diffusion testing of an azole-resistant isolate of a *C. glabrata* obtained from a patient with oropharyngeal candidiasis (OPC); (B) disc diffusion testing of a *C. glabrata* isolated from a healthy individual; (C) disc diffusion testing of a standard strain of *C. glabrata* (ATCC 90030); (D) disc diffusion testing of a blank disc on the standard strain of *C. glabrata* (ATCC 90030).

assays were classified as resistant in order to CLSI instructions (Table 2 and Figure 1). The frequencies of resistant isolates based on disc diffusion and micro dilution tests in patients with OPC were statistically higher than those in healthy individuals ($P=0.0322$, test of proportions). In addition, four isolates obtained from patients with OPC showed resistant properties in itraconazole microdilution AST. Four other isolates were SDD in itraconazole AST (Table 2). There was not any significant difference in itraconazole results between resistant isolates from healthy individuals and patients with OPC ($P=0.4633$, test of proportions). MIC50, MIC90 and MIC ranges of azole drugs in *C. glabrata* isolates obtained from patients with OPC were considerably higher than those obtained from healthy individuals (Table 2).

OVEO completely inhibits growth of both resistant and non-resistant isolates of *C. glabrata*

The MIC of OVEO for *C. glabrata* isolates obtained from healthy individuals ranged 150-200 µg/mL. Also the MIC range for isolates from OPC patients was 150-250 µg/mL (Table 2). No statistically significant difference was found between the mean MIC of isolates from healthy people and mean MIC of isolates from OPC cases ($P=0.0809$, Mann-Whitney U test). Also, the difference between the mean OVEO MIC of azole- resistant and non-resistant isolates obtained from OPC patients was not significant ($P=0.0873$, Mann-Whitney U test). However, MIC of OVEO in azole-resistant isolates of *C. glabrata* among OPC patients was slightly higher than those in azole- susceptible and -SDD isolates in both patients with OPC and healthy individuals.

OVEO exhibits its cytotoxic activity on RMs and PMs in concentrations higher than 100 and 300 µg/mL, respectively

Both macrophage cell types (cell line and normal cells) showed a descending pattern of proliferation rate in response to increasing dose of OVEO (Figure 2). Proliferation rate of murine RMs and PMs was significantly lower than negative control cells at concentrations 150 and 350 µg/mL of OVEO, respectively ($P=0.0215$ and $P=0.008$, respectively, Mann-Whitney U test). The significant reduction in proliferation of cells continued in all subsequent higher concentrations (Figure 2). IC50 of OVEO for RMs was 346.6 µg/mL while the IC50 of OVEO for PMs was calculated 630.4 µg/mL using a three-parametric nonlinear regression model. Moreover, the morphology of macrophages was affected by OVEO treatment. Macrophages treated with toxic doses of OVEO seemed to be detached from surface of the culture flask, floated and rounded (Figure 3).

Discussion

We have studied the chemical composition of OVEO using chemical analysis instruments. We found the following components in order of amount; thymol, γ-terpinene and carvacrol, as the most abundant in OVEO. Our findings are partially consistent with the only study of OVEO in the same geographical region (10).

Long-term azole administration for treatment of oral candidiasis in HIV patients could result in emergence of azole resistance. 81% of non-*C. albicans* isolates in azole-administered patients were fluconazole-resistant (15). These reports are in agreement with our results. We have found that 81.25% (13/16) of *C. glabrata* isolates obtained from patients with OPC were fluconazole- resistant or

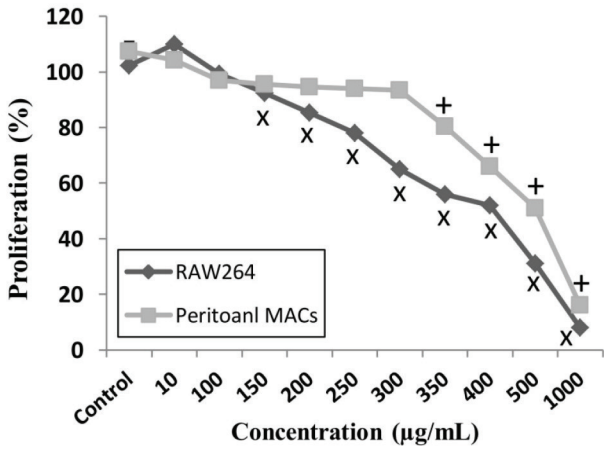

Figure 2. Antiproliferative effect of *Origanum vulgare* essential oil (OVED) on two types of murine macrophages including RAW264.7 and resident peritoneal macrophages. Cells were treated with different concentrations of *O. vulgare* essential oil for 24 hours then the percentage of cell growth inhibition was measured by MTT assay. + indicates significant difference in inhibition rate of cells proliferation between the cells treated with *O. vulgare* essential oil and negative control cells ($P < 0.05$, Mann-Whitney U test). X indicates significant difference in inhibition rate of cells proliferation between the cells treated with *O. vulgare* essential oil and negative control cells ($P < 0.05$, Mann-Whitney U test).

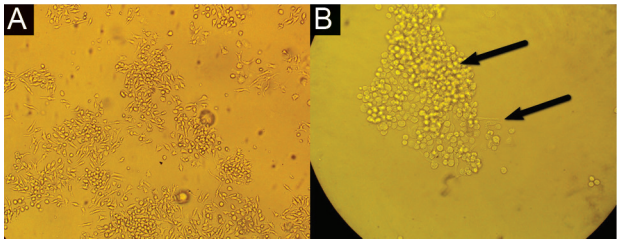

Figure 3. RAW264.7 macrophages (RMs) were imaged using an inverted microscope; (A) the negative control RMs after 24-hour incubation where most of the macrophages show normal morphology, are stretched and usually adhered; (B) RMs treated with 300 µg/mL *Origanum vulgare* essential oil (OVED) after 24-hour incubation; The upper arrow points to detached, floated, clumped and rounded RMs; lower arrow points to adhered and stretched RMs.

-SDD. Ten of 16 (62.5%) isolates were fluconazole-resistant and four (25%) were itraconazole-resistant. Moreover, the researchers observed that in none-immunosuppressed patients, only 1.2% and 0.9% of *C. albicans* isolates were fluconazole- and itraconazole- resistant, respectively. This means that approximately all *C. albicans* isolates obtained from immunocompetent/healthy individuals were azole-susceptible. But among *C. glabrata* isolates, 7% and 19.5% of isolates were resistant to fluconazole and itraconazole, respectively (16). This means that some of the *C. glabrata* isolates obtained from immunocompetent/healthy individuals were again azole-susceptible even if their hosts did not receive any azole-therapy regimen during lifetime. This finding could represent intrinsic-resistance to azoles in *C. glabrata* isolates obtained from healthy people. But, in our study, all isolates from healthy individuals were azole-susceptible. No resistance among the isolates from healthy individuals in the present study could be due to inclusion of a small population of healthy individuals in AST examinations.

The antimicrobial activity of EO constituents was ranked as follows: phenols > aldehydes > ketones > alcohols > esters > hydrocarbons (17). Thymol and carvacrol are phenolic monoterpenes. The mixture of thymol and carvacrol results in stronger antimicrobial activity (18). Also, γ-terpinene is a monoterpene hydrocarbon that has no considerable antibacterial or fungicidal activity (19). There has been only one study of antifungal efficacy of EO of an Iranian strain of *O. vulgare* against fluconazole-resistant and susceptible isolates of *C. glabrata* to date. But in contrast to our study the *C. glabrata* isolates in the referenced study were obtained from patients with vulvovaginal candidiasis and the mean MIC of OVEO on *C. glabrata* isolates was 340.2 µg/mL. The main components of *O. vulgare* strain in referenced study were linalool (42%), thymol (25.1%) and α-terpineol (10%) (20). A recent similar study has incorporated fluconazole-resistant and susceptible isolates of *C. glabrata* but *C. glabrata* isolates used in this study were not clinical and the fluconazole resistance was induced in vitro (21). Mean MIC and minimum fungicidal concentration for two fluconazole-susceptible and -resistant groups of *C. glabrata* isolates were significantly different (21). Our MIC results for fluconazole-resistant *C. glabrata* isolates were inconsistent with the referenced study. Also, in a similar study Candida yeast species were isolated either from OPC patients or immunocompromised patients with disseminated mycoses (22). The MIC range for both fluconazole-resistant and susceptible isolates of *C. albicans* was 200-800 µg/mL. The MIC90 for all fluconazole-resistant and susceptible subgroups of *C. albicans* and *Candida dubliniensis* isolates was 400 µg/mL. There was no difference in MIC ranges between resistant and susceptible isolates of Candida species (22). In our study, there was also no difference in OVEO MIC90 values between resistant and susceptible isolates but the MIC values of *C. glabrata* isolates were different from those in the referenced study. These variations might be explained by different EO chemical composition of Oregano strains used in these investigations. In the referenced study, carvacrol comprised 92.6% of OVEO (21,22). But in our study thymol and carvacrol together comprised 43.4% of OVEO with a thymol/carvacrol ratio of 1.7. The differences in MIC values between our study and other studies may relate to the reported evidences on the synergism between thymol and carvacrol. Combination of thymol and carvacrol has further antimicrobial activity (23). The best antimicrobial synergy was obtained in thymol and carvacrol ratio of 1:1 (23).

Thymol and carvacrol mode of action has been mostly related to their reaction with microbial cell membrane. They initiate modifying effects on the outer and inner membrane, interact with or misfold/unfold the outer membrane proteins, cause changes in cell membrane fluidity, increase the membrane permeability and leakage of

necessary ions and change the pH hemostasis (24). The mechanisms of their antifungal action have been related to disruption of the fungal cell wall integrity and weakening of the synthesis procedure of ergosterol (25). Mutations or alterations of enzymes have been involved ergosterol biosynthesis pathways, which are the targets of azole drugs, and fungal cell adaptation to azole drugs by regulating the efflux pumps has been known to be responsible for developing azole resistance in Candida spp. (26). The ergosterol-impairing effect of thymol and carvacrol indicates the potential synergistic activity of these natural antifungals with the standard ergosterol synthesis-targeting antifungal agents like azoles (27). In addition, the cell wall-disrupting action of thymol and carvacrol seems to possess a potential synergistic activity with well-known cell wall-disrupting activity of the polyenes including amphotericin B and nystatin (28).

To the best of our knowledge, only two studies have investigated the cytotoxic effect of OVEO, carvacrol or thymol on macrophages or peritoneal leukocytes (29,30). In one of these studies, human THP-1 macrophage cell line and human peripheral blood mononuclear cells (PBMCs) were treated with *Origanum syriacum* L. and *Thymus vulgaris* L. extracts. IC50 values of *O. syriacum* extract on THP-1 cells and PBMCs were 2126 and 425 µg/mL, respectively. The toxic effect of *O. syriacum* extract on normal PBMCs was significantly higher than that on THP-1 cell line (29). Our examinations of normal macrophages and cell line macrophages showed that the toxic effect of OVEO on cell line macrophages was about two times higher than that on normal macrophages. But we have used EO of a local strain of *O. vulgare* and the origin of both cell types were from mice. In a study, the toxic effect of *Thymus vulgaris* EO and its main components, thymol and carvacrol, was investigated on leukocytes isolated from peritoneal cavity of rats. *T. vulgaris* EO showed considerable reduction in cell viability at concentration ≥90 µg/mL. For carvacrol, the cell viability did not change at concentration as high as 90 µg/mL. In thymol MTT assay, no reduction in cell viability was observed at all concentrations, even 150 µg/mL (30). No reported toxicity of thymol and carvacrol mentioned in previous studies is consistent with our results. In our study, the main components of OVEO were phenolic monoterpenes including thymol and carvacrol and the significant reduction in viability of RMs and PMs was started at concentrations 150 and 350 µg/mL of OVEO, respectively.

Conclusion

In our experiments, OVEO showed fungicidal activity against both azole-resistant and non-azole-resistant strains of *C. glabrata* and also showed toxic effect against macrophages. Although its IC50 values (on both macrophage cell types) were higher than its fungicidal doses, but to gain a safe result we suggest a combination therapy of standard antifungal drugs such as Amphotericin B, azoles and nystatin whose mechanism(s) of action is (are) the same as and/or similar to those of OVEO and therefore prevent or ameliorate the cytotoxic and adverse effects of both standard drugs and any OVEO-based therapies.

Authors' contributions

All authors contributed to the conception of the study, confirmed the final version of the article and approved all aspects of the study.

Conflict of interests

The authors declare no competing interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission and redundancy) have been completely observed by the authors.

Funding/Support

This research was funded and supported by Research Council of University of Tehran. The results described and presented in this article were part of a PhD thesis project.

References

1. Cannon R, Chaffin W, Oral colonization by Candida albicans. Crit Rev Oral Biol Med. 1999;10:359-383.
2. Oksala E, Factors predisposing to oral yeast infections. Acta Odontologica. 1990; 48:71-74.
3. Wenzel RP. Nosocomial candidemia: risk factors and attributable mortality. Clin Infect Dis. 1995;20:1531-1534.
4. Farmakiotis D, Tarrand JJ. Kontoyiannis DP. Drug-resistant Candida glabrata infection in cancer patients. Emerg Infect Dis. 2014;20:1833.
5. Fidel PL, Vazquez JA. Sobel JD. Candida glabrata: review of epidemiology, pathogenesis, and clinical disease with comparison to C. albicans. Clin Microbiol Rev. 1999;12:80-96.
6. Li L, Redding S, Dongari-Bagtzoglou A. Candida glabrata, an emerging oral opportunistic pathogen. J Dent Res. 2007;86:204-215.
7. Mårdh PA, Rodrigues AG, Genç M, Novikova N, Martinez-de-Oliveira J, Guaschino S. Facts and myths on recurrent vulvovaginal candidosis—a review on epidemiology, clinical manifestations, diagnosis, pathogenesis and therapy. Int J STD AIDS. 2002;13:522-539.
8. Richter SS, Galask RP, Messer SA, Hollis RJ, Diekema DJ, Pfaller MA. Antifungal susceptibilities of Candida species causing vulvovaginitis and epidemiology of recurrent cases. J Clin Microbiol. 2005;43:2155-2162.
9. Letswaart JH, Letswaart J. A taxonomic revision of the genus Origanum (Labiatae). The Hague: Leiden University Press; 1980.
10. Pirigharnaei M, Zare S, Heidary R, Khara J, EmamaliSabzi R Kheiry F. The essential oils compositions of Iranian Oregano (Origanum vulgare L.) populations in field and provenance from Piranshahr district, West Azarbaijan province, Iran.

Avi J Phyt. 2011;1:106-114.
11. Vahedi G, Sharafi G, Vahedi A, Vahedi S, Mohajer T, Abbasi T. Role of denture-wearing on colonization and antifungal resistance of oral Candida albicans isolates in healthy people. J Mycol Res. 2014;1:43-53.
12. Sharifzadeh A, Khosravi A, Shokri H, Jamnani FA, Hajiabdolbaghi M, Tamami IA. Oral microflora and their relation to risk factors in HIV+ patients with oropharyngeal candidiasis. J Mycol Méd Mycol. 2013;23:105-112.
13. Pfaller MA, Standards NCFCL. Reference method for Broth dilution antifungal susceptibility testing of yeasts: Approved standard. National Committee for Clinical Laboratory Standards; 2002.
14. Sheehan DJ, Standards NCFCL: Method for antifungal disk diffusion susceptibility testing of yeasts: Approved guideline. NCCLS; 2004.
15. Johnson EM, Warnock DW, Luker J, Porter SR, Scully C. Emergence of azole drug resistance in Candida species from HIV-infected patients receivingprolonged fluconazole therapy for oral candidosis. J Antimicrob Chemother. 1995;35:103-114.
16. Hajjeh RA, Sofair AN, Harrison LH, et al. Incidence of bloodstream infections due to Candida species and in vitro susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. J Clin Microbiol. 2004;42:1519-1527.
17. Kalemba D, Kunicka A. Antibacterial and antifungal properties of essential oils. Curr Med Chem. 2003; 10:813-829.
18. Lambert R, Skandamis PN, Coote PJ, Nychas GJ. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J Appl Microbiol. 2001;91:453-462.
19. Rao A, Zhang Y, Muend S, Rao R. Mechanism of antifungal activity of terpenoid phenols resembles calcium stress and inhibition of the TOR pathway. Antimicrob Agents Chemother. 2010;54:5062-5069.
20. Khosravi A, Shokri H, Kermani S, Dakhili M, Madani M Parsa S. Antifungal properties of Artemisia sieberi and Origanum vulgare essential oils against Candida glabrata isolates obtained from patients with vulvovaginal candidiasis. Med Mycol. 2011;21:93-99.
21. Pozzatti P, Scheid LA, Spader TB, Atayde ML, Santurio JM, Alves SH. In vitro activity of essential oils extracted from condiments against fluconazole-resistant and -sensitive Candida glabrata. J Mycol Med. 2015;25:213-217.
22. Pozzatti P, Scheid LA, Spader TB, Atayde ML, Santurio JM Alves SH. In vitro activity of essential oils extracted from plants used as spices against fluconazole-resistant and fluconazole-susceptible Candida spp. Can J Microbiol. 2008;54:950-956.
23. Guarda A, Rubilar JF, Miltz J, Galotto MJ. The antimicrobial activity of microencapsulated thymol and carvacrol. Int J Food Microbiol. 2011;146:144-150.
24. Hyldgaard M, Mygind T, Meyer RL. Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. Front Microbiol. 2012;3:12.
25. Ahmad A, Khan A, Akhtar F, et al. Fungicidal activity of thymol and carvacrol by disrupting ergosterol biosynthesis and membrane integrity against Candida. Eur J Clin Microbiol Infect Dis. 2011;30:41-50.
26. Joseph-Horne T, Hollomon DW. Molecular mechanisms of azole resistance in fungi. FEMS Microbiol Lett. 1997;149:141-149.
27. Faria N, Kim JH, Gonçalves L, Martins ML, Chan K, Campbell B. Enhanced activity of antifungal drugs using natural phenolics against yeast strains of Candida and Cryptococcus. Lett Appl Microbiol. 2011;52:506-513.
28. de Castro RD, de Souza TM, Bezerra LM, Ferreira GL, de Brito Costa EM, Cavalcanti AL. Antifungal activity and mode of action of thymol and its synergism with nystatin against Candida species involved with infections in the oral cavity: an in vitro study. BMC Complement Altern Med. 2015;15:417.
29. Ayesh BM, Abed AA. In vitro inhibition of human leukemia THP-1 cells by Origanum syriacum L. and Thymus vulgaris L. extracts. BMC Res Notes. 2014; 7:612.
30. Fachini-Queiroz FC, Kummer R, Estevão-Silva CF, et al.: Effects of thymol and carvacrol, constituents of Thymus vulgaris L. essential oil, on the inflammatory response. Evid Based Complement Alternat Med. 2012;2012:657026.