



# Fungicidal versus Fungistatic activity of five Iranian essences against fluconazole resistant *Candida* species

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## ABSTRACT

**Introduction:** Antifungal resistant is one of the causes of high mortality rates during invasive candidiasis. Since development of new antifungal agents is limited, researchers have focused on natural products including essential oils (EOs) with antifungal properties. In immunocompromised patients fungicidal activity is of benefit. This study was designed to evaluate chemical composition and fungicidal/fungistatic activities of five Iranian EOs and against fluconazole resistant *Candida* species.

**Methods:** To determine chemical composition of EOs gas chromatography-mass spectroscopy (GC/MS) was employed. Fluconazole resistant *Candida* species were chosen and minimum inhibitory concentration (MIC) values of studied EOs were determined by broth microdilution method. Minimum fungicidal concentration (MFC) was determined as the lowest concentration with no fungal growth on solid media. Fungicidal activity was calculated by MFC/MIC ratio.

**Results:** The results showed that *C. albicans* and *C. tropicalis* isolates were susceptible to itraconazole (ITC) and voriconazole (VRC) while one species of *C. glabrata* and *C. krusei* each was resistant to itraconazole; and itraconazole resistant *C. glabrata* isolate was resistance to voriconazole as well. Among tested EOs, the ones from *Cinnamomum cayennense*, *Origanum majorana* var. *majoranoides* and *Andropogon citratus* had the highest anti-*Candida* activity. *Artemisia aromatica* A. Nelson had the highest MIC value against *Candida* isolates. All EOs in this study had fungicidal activity.

**Conclusion:** In general, the tested natural compounds are suitable to be used as anti-*Candida*. However more studies are needed on each chemical compound to evaluate its antifungal activity alone or in combination with other agents.

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

The results of this study revealed that the tested plant essential oils might be used as alternatives to conventional antifungals for treatment of fungal infections.

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## Introduction

Incidence of invasive fungal infections (IFIs) has been increased in recent years (1). *Candida* species are the most frequent fungal pathogens isolated from IFIs. Although infections with *Candida albicans* have been the most prevalent, infections due to non-*albicans Candida* have been increased significantly in the last decades (2,3). The increase in prevalence of these species might be as a result of higher resistance to conventional antifungals (4).

Development of new antifungals is challenging; because there are only few targets for antifungal actions and toxicity level of many antifungals is relatively high

(5). In recent years, some researchers have focused on natural compounds including essential oils (EOs) and herbal extracts derived from medicinal plants and other biomaterials with antifungal activities (6). EOs are aromatic compounds derived from plant materials (7). EOs have shown to possess antimicrobial, insecticidal and antioxidant properties (8). It is believed that these properties are related to the functions of their components in plants (9). Iran is a country rich in medicinal plants and EOs from these plants could be potent antifungals (10). Conventional antifungals are divided into 2 groups of fungicidal and fungistatic compounds (11). Fungicidal

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activity of antifungals is a benefit in most IFIs, especially in early stages of the disease, including systemic candidiasis, cryptococcal meningitis in HIV patients and aspergillosis in immunocompromised individuals (12,13). This study was designed to evaluate the fungicidal and/or fungistatic activities of 5 Iranian EOs against fluconazole (FLC) resistant *Candida* species and to study the effect of EOs' chemical compositions on their antifungal properties.

## Materials and Methods

### Isolation of *Candida* species

*Candida* species were isolated from patients having candidiasis, referred to Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran, during 2016-2018. A total of 50 isolates from different organs were cultured on Sabouraud Dextrose agar (Merck, Germany) at 30°C for 7 days. *Candida* isolates were identified by germ tube test and RAPID yeast plus system (Remel Inc., Lenexa, KS, USA). Isolates were kept at 4°C before use (14).

### Preparation of essential oils

In the present study, leaves of *Artemisia aromatica* A. Nelson, *Andropogon citratus* and *Origanum majorana* var. *majoranoides*, bark of *Cinnamomum cayennense* and flower buds of *Syzygium aromaticum* were purchased from Pakan Bazr Company, Isfahan, Iran. The plants were authenticated taxonomically at Pharmacognosy Department, Faculty of Pharmacy, University of Tehran, Iran.

EOs were extracted by hydro-distillation of plant parts in a Clevenger-type apparatus at 100°C for 5 hours. The EOs were collected and dried over anhydrous sodium sulfate. The chemical composition of each EO was analyzed using a 6890 N Agilent Gas chromatograph coupled to a 5975 C Agilent Mass-selective detector (Agilent Technologies, Avondale, PA, USA) (15). EOs were stored in a dark glass bottle at 4°C until used.

### Preparation of antifungal agents

FLC, itraconazole (ITC) and voriconazole (VRC) standards were prepared from Sigma Company (Sigma, St. Louis, MO, USA). Antifungal stocks were prepared by dissolving 64 mg antifungal in 50 mL DMSO. Stocks were kept at -20°C before use.

### Determination of minimum inhibitory concentration

Broth microdilution method was used to study antifungal susceptibility of *Candida* isolates to mentioned antifungal agents and studied EOs according to Clinical and Laboratory Standards Institute (CLSI) M27-A3 document. In brief, 2 fold serial dilutions of EOs (15.6, 31.2, 62.5, 125, 175, 250, 350, 500, 700, 1000 and 2000 µg/mL) and 3 azole agents (0.031-64 µg/mL) were prepared in 96-well plates using RPMI-1640 media (Sigma, St. Louis, MO,

USA) buffered with MOPS (Sigma, St. Louis, MO, USA). *Candida* inoculums were adjusted to density of  $1 \times 10^3$ - $5 \times 10^3$  CFU/mL with the help of a hemocytometer and 0.1 mL of each inoculum was added to microplate wells which were incubated at 30°C for 24-48 hours. Non-inoculated medium (200 mL) was considered as negative control (blank), and the well with no EO/azole was spotted as positive control. The growth in each well was compared with the growth in the control well. Minimum inhibitory concentrations (MICs) were visually determined and defined as the lowest concentration of the EO/azole which inhibited growth in comparison with positive control. All tests were done in replicates of three (16).

### Determination of minimum fungicidal concentration

Wells with no visible growth after 48 hours of incubation were cultured on Sabouraud Dextrose Agar by surface plate count method. Plates were incubated at 30°C for 10 days. Minimum fungicidal concentration (MFC) was determined as the lowest concentration with no growth on solid media. All experiments were done with 3 replicates (17).

### MFC/MIC ratio

EOs were considered fungicidal if the ratio of MFC to MIC was less or equal to 4 and if the ratio was higher than four the EO was deemed fungistatic (18).

### Statistical analysis

Data were analyzed using SPSS version 21. Analysis of variance (ANOVA) and Dunnett post hoc test were done to compare different data. A *P* value of less than 0.05 was considered significant.

## Results

### Isolation of *Candida* species

Specimens were taken from upper respiratory tract, urine and blood. *Candida* isolates were initially tested for susceptibility to FLC by broth microdilution method. Isolates with MIC range of 64 or greater were considered resistant to FLC. A total of 12 *Candida* species that were resistant to FLC were selected for further studies. These isolates included *C. albicans* (6 isolates), *Candida glabrata* (3 isolates), *Candida krusei* (2 isolates) and *Candida tropicalis* (1 isolate).

### Analysis of essential oils by Gas chromatography/ Mass spectrometry

EOs were analyzed on a 6890 N Agilent Gas chromatograph coupled to a 5975 C Agilent mass-selective detector (Agilent Technologies, Avondale, PA, USA) with a 7683 Agilent auto sampler. In brief, 1 µL of each sample was injected in the split less mode at 250°C/into a 30 m × 0.25 mm × 0.5 µm DB-5 MS capillary column and operated by MSD Chemstation software (Agilent Technologies).

The temperature program used for the chromatographic

separation was 50°C/2 min; 25°C/1 min; 100°C/2 min; 5°C/1 min and 290°C/5 min. The carrier gas was helium (99.999%) with a constant flux of 1.0 mL/min. Mass spectrometry was operated in an electron impact ionization mode with the energy of 70 eV. Several unknown peaks were observed after injection. Peaks were identified using computer library. The main components of the EOs are summarized in Table 1.

Major compounds of *A. aromatica* A. Nelson were Anethole (22.02%), d-limonene (14.93%) and alpha pinene (16.44%). Isothymol (59.42%), thymol (15.23%) and trans-caryophyllene (10.18%) were the major constituents of *A. citratus*; while carvacrol (57.86%), thymol (13.54%) and trans-caryophyllene (11.52%) were the main compounds found in *O. majorana* var. *majoranoides*. *C. cayennense* was mainly composed of cinnamaldehyde (62.06%), cinnamaldehyde dimethyl acetate (8.14%) and eugenol (7.49%). We found eugenol (96.81%) as the main component of *S. aromaticum* followed by lower percentages of carvacrol (1.74%) and Trans-Caryophyllene (0.39%).

#### Determination of MIC and MFC

Antifungal susceptibility test of EOs in comparison with ITC and VRC against FLC resistant *Candida* species was done using broth micro dilution method (Table 2). All *C. albicans* and *C. tropicalis* isolates were susceptible to both ITC and VRC. Whereas, isolates of *C. krusei* and *C. glabrata* were resistant to ITC. ITC resistant *C. glabrata* was resistant to VRC as well. Among EOs, MICs of *A. aromatica* A. Nelson, *A. citratus*, *O. majorana* var. *majoranoides*, *C. cayennense* and *S. aromaticum* ranged between 1000 to 2000 µg/mL (mean value: 1454.5 ± 509.6 µg/mL), 125 to 175 µg/mL (mean value: 156.8 ± 24.6 µg/mL), 173 to 350 µg/mL (mean value: 208 ± 55.8 µg/mL), 125 to 175 µg/mL (mean value: 147.7 ± 25.5 µg/mL) and 700 to 1000 µg/mL (mean value: 740.9 ± 105.4 µg/mL), respectively. *O. majorana* var. *majorana* had significantly higher MICs for *C. glabrata* than other examined *Candida* species ( $P < 0.05$ ). The MIC ranges of other EOs against *Candida* species were not statistically significant ( $P > 0.05$ ). *C. cayennense* could significantly inhibit growth of *C. albicans* in lower concentrations than *O. majorana* var. *majoranoides*, *S. aromaticum* and *A. aromatica* A. Nelson ( $P < 0.05$ ). Whilst it was only more effective than *O. majorana* var. *majoranoides* against *C. glabrata* isolates ( $P < 0.05$ ). The most effective EOs on *C. krusei* isolates were *A. citratus* and *C. cayennense* ( $P < 0.05$ ); and *O. majorana* var. *majoranoides* was significantly more effective against *C. tropicalis* than *A. aromatica* A. Nelson and *C. cayennense* ( $P < 0.05$ ).

#### Determination of fungicidal activity

The ration of MIFC/MIC of studied EOs against *Candida* isolates is shown in Figure 1. Since the MIC/MFC ratios of all EOs in this study were less than 4, they could be

considered fungicidal. *C. cayennense* MIC/MFC ratio was similar for all *Candida* species. While *A. aromatica* A. Nelson had the nearest ratio against *C. krusei* and other essences had the lowest ratio for *C. tropicalis*.

#### Discussion

*Candida* species cause a variety of fungal infections from superficial candidiasis of oral and vaginal mucosa to disseminated and deep tissue infections. These infections are most often caused by *C. albicans* followed by *C. glabrata*, *C. parapsilosis*/*C. tropicalis* and *C. krusei* according to epidemiological studies in different geographical regions (19). FLC, an azole antifungal, is the most common antifungal used for treatment of candidiasis. It inhibits lanosterol 14 α demethylase enzyme encoded by *ERG11* gene. However, some non *albicans Candida* species have intrinsic resistant to azoles. In addition, *Candida* spp. are able to develop resistant to azoles (20,21). Azole resistance is seen especially in patients receiving azole prophylaxis (22).

In this study we examined the fungicidal activities of 5 Iranian EOs on FLC resistant to *Candida* species. *Candida* yeasts were isolated from candidiasis cases. Resistant to other conventional systemic azoles in Iran was investigated as well. In our study only one isolate of *C. glabrata* was resistant to VRC and one isolate of *C. glabrata* as well as *C. krusei* were dose dependently susceptible to ITC. According to recent studies, resistance to azole antifungals has increased in recent years. Widespread use of triazoles has resulted in emergence of resistant to non *albicans Candida* species including *C. glabrata* and *C. krusei* (23,24). These species have intrinsic resistant to FLC and it is said that this inherited resistance could be seen in VRC as well (23). In our study only one isolate of *C. glabrata* was resistant to VRC. Studies have shown that mutations in *ERG11* gene could induce azole resistance in *C. albicans* (24). Stephanie et al demonstrated that there is a combination effect between FLC and VRC susceptibility in *C. albicans* strains, while ITC susceptibility is not affected (24). We did not observe any correlation between FLC and VRC susceptibility among our *C. albicans* isolates. It might be due to different mutation regions in our isolates. Biological activity of EOs is highly related to their chemical compounds (25). In our study, *C. cayennense* was the most effective one against *Candida* isolates. GS/MS analysis revealed cinnamaldehyde, cinnamaldehyde dimethyl acetal and eugenol as its main ingredients. Other studies have demonstrated that the amount of cinnamaldehyde affect antifungal activity of *Cinnamomum* spp. The higher cinnamaldehyde content the higher antifungal activity would be (26). *O. majorana* var. *majoranoides* and *A. citratus* had the best anti *Candida* activity following *C. cayennense*. GC/MS analysis revealed that isothymol and thymol compromised approximately 50% of *A. citratus*. These compounds are known for

Table 1. Summary of the chemical composition (%) of essential oils evaluated by GC/MS

<i>Artemisia aromatica</i> A. Nelson			<i>Andropogon citratus</i>			<i>Origanum majorana</i> var. <i>majoranoides</i>			<i>Cinnamomum cayennense</i>			<i>Syzygium aromaticum</i>		
Component	Area (%)	RT (min)	Component	Area (%)	RT (min)	Component	Area (%)	RT (min)	Component	Area (%)	RT (min)	Component	Area (%)	RT (min)
Anethole	22.02	17.51	Isothymol	23.59	59.42	Carvacrol(or isothymol)	57.86	23.56	Cinnamaldehyde	62.06	22.20	Eugenol	96.81	25.98
Alpha-pinene	16.44	6.31	Thymol	22.54	15.23	Thymol	13.54	22.56	Cinnamaldehyde dimethyl acetal	8.14	26.79	Carvacrol	1.74	22.81
D-limonene	14.93	9.90	Trans-caryophyllene	27.38	10.18	Trans-caryophyllene	11.52	27.37	Eugenol	7.49	25.26	Trans-caryophyllene	0.39	27.31
Linalool L	9.47	13.07	Cymene	9.58	5.82	Cymene	6.78	9.58	Linalool L	4.84	13.02	Thymol	0.29	22.39
Allyl-6-methoxyphenol	7.14	25.05	Gamma-terpinen	10.99	2.70	Gama-terpinene	3.11	10.99	Trance-cinnamyl acetate	4.07	28.44	Chavicol	0.18	20.87
Trans-caryophyllene	4.34	27.25	Eugenol	25.12	1.79	3-Allyl-6-methoxyphenol	1.98	25.10	Alpha-terpineole	3.22	17.02	Benzene	0.09	9.50
Gamma-terpinene	3.55	11.02	Alpha-humulene	28.49	1.41	Alpha-humulene	1.57	28.48	Benzyl benzoate	2.23	38.08	Alpha-humulene	0.07	28.51
Beta-myrcene	2.47	8.24	Trans-Anethole	21.66	0.72	DL-Limonene	0.67	9.70	1,8-Cineole	1.10	9.75	DL-Limonene	0.04	9.66
Delta-3-Carene	2.35	8.95	2-Beta-pinene	7.62	0.53	2-Beta-pinene	0.61	7.62	Diepoxyp-menthane	0.88	22.87	Cis-jasmone	0.04	31.81
Limonene dioxide 4	2.00	22.32	Copaene	25.59	0.38	Copaene	0.43	25.58	(E)-Cinnamaldehyde	0.76	18.19	Camphor	0.03	14.64
Limonene dioxide 1	1.99	22.54	Caryophyllene oxide	32.76	0.25	Caryophyllene oxide	0.27	32.76	Isoterpinolene	0.48	17.25	Globulol	0.03	35.35

**Table 2.** Antifungal susceptibility test of selected essential oils in comparison with 3 conventional azole antifungals against *Candida* isolates

<i>Candida</i> isolates	Itraconazole		Voriconazole		<i>Artemisia aromatica</i> A. Nelson		<i>Andropogon citratus</i>		<i>Origanum majorana</i> var. <i>majoranoides</i>		<i>Cinnamomum cayennense</i>		<i>Syzygium aromaticum</i>	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
<i>C. albicans</i> 1	0.125	<0.5	0.125	<0.5	2000	2000	175	350	175	350	125	175	700	1000
<i>C. albicans</i> 2	0.062	<0.5	0.062	<0.5	1000	2000	175	350	175	350	125	175	700	1000
<i>C. albicans</i> 3	0.062	<0.5	0.062	<0.5	1000	2000	175	350	175	350	125	175	700	1000
<i>C. albicans</i> 4	0.062	<0.5	0.062	<0.5	2000	2000	175	350	250	350	125	175	700	1000
<i>C. albicans</i> 5	0.062	<0.5	0.062	<0.5	1000	2000	125	350	175	350	125	175	700	1000
<i>C. albicans</i> 6	0.062	<0.5	0.062	<0.5	1000	2000	125	175	175	350	125	175	700	1000
<i>C. glabrata</i> 1	4	<64	8	<64	1000	2000	175	250	175	250	175	250	700	1000
<i>C. glabrata</i> 2	0.125	<0.5	0.25	<1	1000	2000	175	250	175	250	175	250	700	700
<i>C. glabrata</i> 3	0.125	<0.5	0.125	<0.5	2000	2000	125	175	175	350	175	250	1000	1000
<i>C. krusei</i> 1	0.062	<0.5	0.062	<0.5	2000	2000	125	175	350	500	125	175	1000	1000
<i>C. krusei</i> 2	4	<64	0.5	<2	1000	2000	125	175	350	500	125	175	700	1000
<i>C. tropicalis</i>	0.5	<2	0.125	<0.5	1000	2000	175	175	175	175	175	250	700	700

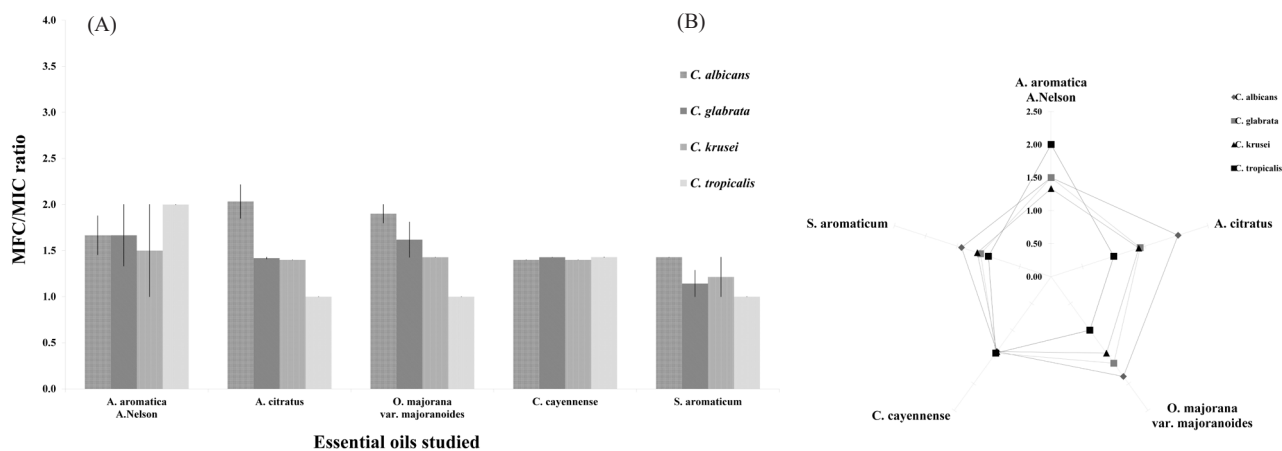
Data are shown as mean ( $\mu\text{g/mL}$ ).

their antifungal activities (27). Another compound with high value in *A. citratus* was Trans-Caryophyllene. It is sesquiterpene found in many EOs especially cloves (28). Trans-Caryophyllene is known for its anti-inflammatory and pain killer effects (29). *S. aromaticum* activity against FLC resistant *Candida* species was also suitable (MIC = 700  $\mu\text{g/mL}$ ). Only one isolate of *C. glabrata* and *C. krusei* had the MIC of 1000  $\mu\text{g/mL}$ . Other studies have demonstrated a suitable antifungal activity for eugenol *in vivo* and *in vitro* (30). *A. aromatica* A. Nelson was found to have the lowest antifungal activity against the studied *Candida* species in our study. Since the numbers of isolates were not similar in our study, we could not predict if the tested EOs act differently on various *Candida* species, although according to our results there was no difference between FLC resistant *Candida* species. All EOs in our study had fungicidal properties. It has

been documented that fungicidal activity of antifungals is related to more therapeutic success and less recurrent and persistent infection especially in invasive candidiasis and candidemia (12,13). It has been shown that fungicidal compounds inhibit morphogenic transformation of *Candida* spp while fungistatics do not affect morphogenesis (18).

## Conclusion

In conclusion, EOs in this study have potential antifungal effects against *Candida* species and there is no known resistant mechanism in *Candida* species against studied EOs. These EOs are probably suitable to be used as supplementary agents along with conventional antifungals. However more studies are needed to evaluate their safety on host cells and to examine the effects of their chemical compounds alone and/or in combination with



**Figure 1.** (A) Comparison the MIC/MFC ratio of different essential oils against *C. albicans*, *C. glabrata*, *C. krusei* and *C. tropicalis*. Data are shown as mean  $\pm$  SE. (B) A diagram on relative MIC/MFC ration of studied essential oils.

other agents.

### Authors' contributions

ARK contributed to study design and manuscript editing. DN and AS performed experiment and acquired data. DN prepared the manuscript draft. DN submitted the manuscript. All authors read and confirmed final version of the article for publication.

### Conflict of interests

Authors declare no conflict of interest.

### Ethical considerations

This study was approved by Deputy of Research and Technology, Faculty of Veterinary Medicine, University of Tehran (Ethical Code 30792/1/1). All ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission and redundancy) have been completely observed by the authors.

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