

## EVALUATION OF ANTICANDIDAL EFFECTS OF ESSENTIAL OILS OF COMMERCIAL LAVENDER (*Lavandula angustifolia* Miller) IN COMBINATION WITH KETOCONAZOLE AGAINST SOME *Candida* Berkhout STRAINS

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**Abstract:** Application of combination studies with essential oils and standard antifungal drugs may reduce adverse effects of synthetic drugs and serve as alternative approach against various pathologies including candidiasis. The aim of the present study was to determine the interaction of two commercial lavender (*Lavandula angustifolia* Miller) essential oils in combination with ketoconazole against clinical and standard strains of the human pathogens *Candida albicans* ATCC 10231, *C. krusei* NRRL Y-7179 and *C. glabrata* ATCC 66032. The chemical compositions of the investigated essential oils were confirmed both by gas chromatography/flame ionization detector (GC/FID) and gas chromatography/mass spectrometry (GC/MS) methods. Anticandidal activities of the essential oils were screened *in vitro* by the microdilution method. The resulting interaction of combining lavender essential oils and ketoconazole was tested using the checkerboard method. The results showed that the interaction between essential oils and ketoconazole revealed additive and indifferent effects against the tested strains. In conclusion, the effects observed by the combination of lavender essential oils and ketoconazole may be encouraging to be used against pathogenic *Candida* strains.

**Özet:** Uçucu yağların standart antifungal maddeler ile kombinasyonları, sentetik ilaçların olumsuz etkilerini azaltabilir ve kandidiyaz dahil olmak üzere çeşitli patolojilere karşı alternatif bir yaklaşım olarak kullanılabilir. Bu çalışmanın amacı, Farmakope kalitesindeki Lavanta (*Lavandula angustifolia* Miller) uçucu yağlarının ketokonazol ile kombinasyonlarının patojen *Candida albicans* ATCC 10231, *C. krusei* NRRL Y-7179 ve *C. glabrata* ATCC 66032 suşlarına karşı antikandidal etkinliğini belirlemektir. İncelenen uçucu yağların kimyasal içerikleri hem gaz kromatografisi/alev iyonlaşma dedektörü (GK / AİD) hem de gaz kromatografisi/kütle spektrometresi (GK/KS) yöntemleriyle belirlenmiştir. Esansiyel yağların antikandidal aktiviteleri, mikrodilüsyon metodu ile *in vitro* olarak incelenmiştir. Lavanta uçucu yağları ve ketokonazol kombinasyon çalışmaları dama tahtası yöntemi kullanılarak test edilmiştir. Lavanta uçucu yağları ile ketokonazol kombinasyon çalışması sonuçları, test edilen tüm *Candida* suşlarına karşı “aditif” ve “indiferent” etkili olduğu görülmüştür. Sonuç olarak, lavanta uçucu yağlarının ketokonazol kombinasyonu ile gözlemlenen etkiler, patojenik *Candida* suşlarına karşı kullanımda etkili olabilir.

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### Introduction

Infectious diseases have recently increased the prolonged use of various antifungal drugs, contributing to the development of drug resistance against some species of the fungal genus *Candida* Berkhout, such as the azole-resistant *Candida albicans* (Robin) Berkhout strains along with *C. krusei*. In addition, many cases have been attributed to *Candida* infections particularly in persons with hematologic malignancies and transplant recipients patients (Pfaller *et al.* 2008).

The antifungal effect of azoles is due to their actions in inhibition of ergosterol biosynthesis on fungal cell membranes (Casalnuovo *et al.* 2004, Sardi *et al.* 2013). Currently used antifungal drugs may have the risk of toxicity, may result drug–drug interactions and lack of fungicidal efficacy. Furthermore, resistance to these drugs often develops rapidly (Metzger & Hofmann, 1997, Pinto *et al.* 2009).



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Plants are known to produce a great variety of compounds. Particularly, aromatic plants have been used in folk medicine as antimicrobial agents since ancient times. (Hosseinzadeh *et al.* 2015). Essential oils (EOs) of many plants are known to possess a large number of pharmacologically active compounds and have antimicrobial activity. EOs show a wide-spectrum antimicrobial activity, including disruption of cell-membrane structures, leading to an alteration in membrane permeability and a consequent leakage of cell contents (Cox *et al.* 2000, Chen *et al.* 2013). For instance, recent studies on antifungal activities of some EOs have shown disruption of the fungal membrane (Pinto *et al.* 2006, Khan *et al.* 2010, Samber *et al.* 2015).

With this in mind, combination of antifungal therapies with natural sources may be achieved to overcome the resistance against *Candida* species. Specifically, EOs are known to be used in therapeutic preparations for centuries (Hosseinzadeh *et al.* 2015). The EOs of various *Lavandula* species from Lamiaceae are well known to have antimicrobial activities along with other effects they possess (Cavanagh & Wilkinson 2002, Fismer & Pilkington 2012). Thus, using the combinations of EOs with standard antifungal drugs employ as an alternative approach against various pathologies including candidiasis and may reduce adverse side effects, lower the dose and decrease toxicity (Cottarel & Wierzbowski 2007). EOs of various plants have been previously investigated for their antifungal efficacy in combination with some antifungal drugs and proved to be successful (Shin & Kang 2003, Shin 2003, Amber *et al.* 2010, Göger *et al.* 2018). Shin & Kang (2003) reported that EO fraction of *Agastache rugosa* combined with ketoconazole was found to be synergistic against the opportunistic fungus *Blastoschizomyces capitatus*. *Pelargonium graveolens* EO fractions were demonstrated to have additive effects with amphotericin B and ketoconazole against *Aspergillus* species (Shin 2003). In more recent studies, *Ocimum sanctum* EO and its combination with two azoles, fluconazole and ketoconazole, were studied against resistant isolates (Amber *et al.* 2010). *Matricaria recutita* EO was combined with fluconazole against *C. albicans* (Göger *et al.* 2018). It thus appears that, although proved to have antimicrobial activities when tested alone, studies on combination of lavender EOs with antifungal drug are rather limited. In one recent study, nystatin was tested in combination with *Lavandula angustifolia* EO against *C. albicans* ATCC 10231 and an indifference effect was observed for the combination (De Rapper *et al.* 2016)

This study was performed with the aim of identification of chemical compositions of two EOs of lavender (*L. angustifolia* Miller) by GC/FID and GC/MS analytical methods and evaluate their anticandidal activities with ketoconazole by microdilution and checkerboard methods against *C. albicans* ATCC 10231, *C. krusei* NRRL Y-7179 and *C. glabrata* ATCC 66032 strains.

## Materials and Methods

### Materials

Two commercial Lavender EOs were purchased from Sevil (Turkey) and Caesar & Lorethz GmbH (Hilden, Germany) companies and were labeled as L1 and L2, respectively. The standard powders of ketoconazole and resazurin were obtained from Sigma-Aldrich.

Anticandidal activities of the EOs of L1 and L2 were tested against standard (*C. albicans* ATCC 10231, *C. krusei* NRRL Y-7179 and *C. glabrata* ATCC 66032) and clinical (*C. albicans*, *C. krusei* and *C. glabrata*) isolates provided from Akdeniz University, Faculty of Medicine, Antalya.

### GC/FID and GC/MS Analyses

#### *GC/FID analysis*

The EOs were analyzed by capillary GC using the Shimadzu GC 2010 system. The column and analysis conditions used for GC/MS were also applied for GC. FID detector temperature was 250°C. In order to get the same elution order with GC/MS, injections were done by using the same column and operational parameters.

#### *GC/MS analysis*

The GC/MS analysis was realized with the Shimadzu GC 2010 system. CP-Sil 5 CB column (25 m x 0.25 mm Ø, 0.25 µm film thickness) was used with helium as the carrier gas (1 mL/min). GC oven temperature was kept at 60°C for 10 min and programmed to 260°C at a rate of 4°C/min, and kept constant at 260°C for 5 min. Split ratio was adjusted to 50:1. The injector temperature was at 250°C. MS were recorded at 70 eV, where the mass range was from *m/z* 35 to 450.

#### *Identification of the volatile components*

Mass spectrometry (MS) was used to characterize the volatiles by comparing their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes. Computer matching against commercial (Wiley GC/MS Library, MassFinder 3 Library) (McLafferty & Stauffer 1989, Koenig *et al.* 2004) and *in-house* “Başer Library of Essential Oil Constituents” built up by genuine compounds and components of known oils, as well as MS literature data (Joulain & Koenig 1998, ESO (1998)) were also used for the identification.

#### *Determination of minimum inhibitory concentration (MIC)*

Anticandidal activities were performed according to the adapted guidelines of Clinical and Laboratory Standards Institute (CLSI) M27-A2 standard method. Minimum inhibitory concentrations (MIC) of EOs of L1 and L2 and ketoconazole were determined using the standard and clinically isolated *Candida* isolates (see above).

Stock solutions of EOs and ketoconazole were dissolved in DMSO. EOs were diluted two-fold initially, with a final concentration range 2560 - 5 µg/mL, whereas the ketoconazole was diluted from 64 to 0.125 µg/mL.

*Candida* cultures with a final inoculum size of  $1 \times 10^3$  colonies forming units (CFU/mL) were prepared. A 10  $\mu$ L of the suspensions at  $10^3$  CFU/mL were inoculated into a two-fold diluted solutions of the tested EOs and ketoconazole in 96 well microplates. The microplates were incubated at 35°C for 48 h. Microbial growth was visualized by adding 20  $\mu$ L resazurin (0.01%). MIC was defined as the lowest concentration, which did not result in any visible growth compared with the control plates. Microplates free of antifungal standards were used as the growth controls. Negative controls were used to confirm sterility and the effects of the solvents. Assays were undertaken in triplicate.

#### Combination of Lavender EOs and ketoconazole by the checkerboard method

The antifungal interaction between ketoconazole and EOs of L1 and L2 were studied by the checkerboard method using a 96-well plate using an 8-by-8 well configuration (Van Vuuren *et al.* 2009). EOs of L1 and L2 and ketoconazole were prepared as serial dilutions using the same solvents (medium) in the MIC test.

50  $\mu$ L aliquots of each EOs were added to the wells of a 96-well plate in a vertical orientation and 50  $\mu$ L aliquots of each ketoconazole dilution were added in a horizontal orientation so that the plate contained various concentration combinations of the EOs and ketoconazole. Growth controls were applied in wells free of antifungal standards. Negative control was also applied to confirm sterility. Each well was inoculated with 100  $\mu$ L ( $5 \times 10^3$  CFU per well) of one of the six *Candida* suspensions and incubated at 35°C for 48 hours. Following the incubation, 20  $\mu$ L resazurin was added to the wells and the wells were left at 35°C for 2 h. Growth in wells was indicated by the color change from blue to pink.

*In vitro* interaction between Lavender EOs and ketoconazole were determined by using the fractional inhibitory concentrations (FIC). The FIC is expressed as the interaction of two compounds, where the concentration of each test compound in combination is expressed as a fraction of the concentration that would produce the same effect when used singularly (Vuuren *et al.* 2007, Stanojevic *et al.* 2010) and may be classified as follows:

$$\text{FIC of EO} = \frac{\text{MIC of EO in combination with antifungal drug}}{\text{MIC of EO alone}}$$

$$\text{FIC of ketoconazole} = \frac{\text{MIC of antifungal drug in combination with EO}}{\text{MIC of ketoconazole}}$$

The FIC index (FICI) was calculated by adding both FICs:

FICI = FIC of essential oil + FIC of ketoconazole and the FICI was interpreted as representing as;

FICI  $\leq$  0.5 = synergism effect

FICI  $0.5 \leq$  1 = additive effect

FICI 1 to 4 = indifferent effect

FICI  $\geq$  4 = antagonism effect

## Results

In our present study, lavender EOs were analyzed by GC/FID and GC/MS methods and confirmed at the quality of Pharmacopoeia grade. The EOs are characterized by high amounts of linalyl acetate (30.5-42%) and linalool (35.9- 46%) (Figures 1 and 2). The major constituents of EOs and their relative percentages are listed in Table 1. The chemical composition of lavender EOs have previously been revealed by different studies with which the EOs were reported to be generally characterized by high levels of linalool, linalyl acetate, lavandulyl acetate, terpinen-4-ol and lavandulol (Cassella *et al.* 2002, Cavanagh & Wilkinson 2002, Shellie *et al.* 2002, Dauria *et al.* 2005, Lodhia *et al.* 2009, Sokovic *et al.* 2010, Danh *et al.* 2013, Jianu *et al.* 2013, De Rapper *et al.* 2013, Yap *et al.* 2014, De Rapper *et al.* 2016, Kırmıer *et al.* 2017). The amount of 1,8-cineole and camphor, on the other hand, often varies between very low to moderate (Cavanagh & Wilkinson 2002, Shellie *et al.* 2002).

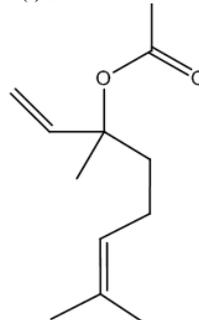
**Table 1.** Major constituents of lavender EOs.

*KI	Compounds	L1 (%)	L2 (%)
0939	$\alpha$ -Pinene	-	3.2
1029	Limonene	-	trace
1031	1,8-cineole	4.8	0.7
1097	<b>Linalool</b>	<b>44.0</b>	<b>35.8</b>
1146	Camphor	4.8	0.5
1169	Borneol	1.6	2.9
1177	Terpinen-4-ol	-	1.7
1189	$\alpha$ -Terpineol	1.4	1.4
1257	<b>Linalyl acetate</b>	<b>30.5</b>	<b>36.0</b>
1381	Lavandulyl acetate	1.04	1.0
<b>TOTAL</b>	<b>%</b>	<b>88.14</b>	<b>83.2</b>

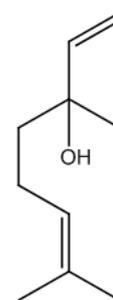
\*KI: Kovats indices calculated according Adams (2001)

L1 and L2 correspond to two purchased lavender Eos

(-) Not detected



**Fig. 1.** Linalyl acetate



**Fig. 2.** Linalool

Resistance of yeast isolates to antifungal drugs, such as fluconazole and ketoconazole were reported to increase in serious fungal infections making patient management a complicate case (Sanglard & Odds 2002, Jamil *et al.* 2017). *Candida species* are considered as one of the most important causes of human infections. They may become pathogenic and cause candidiasis under some conditions, such as prolonged antifungal therapy and reduced immunity. Candidiasis range from mild infection such as

onychomycosis or perish to potentially fatal systemic candidiasis (Akortha *et al.* 2009).

Azoles are by far the most commonly used antifungals in clinical treatment with lower cytotoxicity and perfect efficacies. However, their prolonged use has led to the development of drug resistance in *Candida* species (Hasanbeigi *et al.* 2005, Magill *et al.* 2006, Zomorodian *et al.* 2011).

Many essential oils have been proven to possess antifungal properties and thus they can be potentially used as antifungal agents. As proven *in vitro*, EOs show effective antifungal activities even against resistant clinical isolates of *Candida* species. The use of combinations of EOs and their isolated components are thus new approaches to increase their efficacy in fungal treatment, for taking advantage of their synergistic and additive effects (Cleff *et al.* 2010, Pozzatti *et al.* 2010, Stringaro *et al.* 2014).

From this point of view, EOs of lavender tested for their anticandidal activities against *Candida* strains (Table 2) were found to have a MIC range of 160-640 µg/mL compared to ketoconazole MIC range of 1-4 µg/mL. In particular, the inhibitory effect of L2 EO represented with a MIC value of 160 µg/mL was more pronounced than that of L1 EO when tested with standard and clinical *C. krusei* strains (Table2).

**Table 2.** MIC values (µg/mL) of EOs and ketoconazole against *Candida* isolates.

Tested <i>Candida</i> isolates	MIC values of L1 EOs	MIC values of L2 EOs	MIC values of Ketoconazole
<i>C. albicans</i> ATCC 10231	320	320	2.0
<i>C. krusei</i> NRRLY-7179	320	160	2.0
<i>C. glabrata</i> ATCC 66032	320	640	2.0
<i>C. albicans</i> clinical isolate	320	320	2.0
<i>C. krusei</i> clinical isolate	320	160	2.0
<i>C. glabrata</i> clinical isolate	320	320	2.0

The checkerboard results of EOs and ketoconazole combinations were listed in Tables 3 and 4. The L1 + ketoconazole combination against both clinical and standard *C. albicans* ATCC 10231, *C. krusei* NRRL Y-7179 and *C. glabrata* ATCC 66032 showed additive effects (FICI= 0.53 to 0.56) (Table 3). The L2 + ketoconazole combination against *C. albicans* (FICI= 1.0) and *C. glabrata* (FICI= 0.56) clinical isolates also resulted in an additive activity pattern (Table 4).

In combination therapy, interactions between EOs and antifungals can display four possible types of effects as synergistic, additive, indifferent and antagonistic. Synergism is observed when the effect of the combined substances is greater than the sum of their individual

effects. An additive effect is observed when the combined effect is equal to the sum of the individual effects. Indifference is observed when there is no interaction. Antagonism is observed when the effect of one or both compounds is less when they are applied together than when applied individually (Bassole *et al.* 2012, Yap *et al.* 2014).

The different interactions observed in antifungal activity between EOs of L1 and L2 can be explained by the chemical compositions EOS and the ratio of their components. Since EOs contain many chemical mixtures, differences can be observed in their antimicrobial effects and the type of effect mechanisms. Most of the antimicrobial activity effect for EOs was attributed to their chemical compositions derived from hydrocarbons, oxygenated terpenoids, particularly phenolic terpenes, phenylpropanoids and alcohols (Burt, 2004).

Lavender EOs have been previously demonstrated to be active against a broad spectrum ranging from Gram positive and Gram negative bacteria to *Candida* strains (Dauria *et al.* 2005, Lodhia *et al.* 2009, Sokovic *et al.* 2010, Danh *et al.* 2013, Jianu *et al.* 2013), and thus they were suggested to be useful in treating bacterial and fungal infections including those related with drug resistant strains. Along with their antimicrobial activity, lavender EOs were used in the treatment and relieve of symptoms of skin conditions such as psoriasis, dermatitis and eczema. It has also been suggested that topical application of lavender EOs may inhibit parts of the allergic pathway (Cavanagh & Wilkinson 2002).

Synergistic antifungal activity of combination of lavender EOs and tea tree oil (*Melaleuca alternifolia*) against dermatophyte infections were reported (Cassella *et al.* 2002). De Rapper *et al.* (2013) investigated the combination of *L. angustifolia* EO with forty five different aroma therapeutic essential oils against *C. albicans* ATCC 10231, *P. aeruginosa* ATCC 27858 and *S. aureus* ATCC 6538. The most favourable interactions were displayed with *L. angustifolia* and *Cinnamomum zeylanicum* or with *Citrus sinensis* EOs against *C. albicans* and *S. aureus*.

*L. angustifolia* EO was also combined with the commercial antimicrobial drugs nystatin, chloramphenicol, ciprofloxacin and fusidic acid against *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 27858 and *C. albicans* ATCC 10231. The best interaction was shown between *L. angustifolia* EO with chloramphenicol and tested against the pathogen *P. aeruginosa* (FICI= 0.29).

The mode of action of lavender EO on antimicrobial activity against multi-drug resistant *Escherichia coli* J53 R1 when used singly and in combination with piperacillin was demonstrated (Yap *et al.* 2014). The time-kill analysis showed synergistic interaction between lavender EO and piperacillin at 4 h. The results of this study indicate that lavender EO is able to disrupt membrane structures of *E. coli* and has the potential to inhibit bacterial quorum sensing.

In conclusion, we reported here for the first time the anticandidal effects of *L. angustifolia* EOs with ketoconazole combination against the standard and clinical strains of the human pathogens *C. albicans*, *C. glabrata* and *C. krusei* in a comparative manner. As revealed by the results, the absence of antagonism may be promising for combination of lavender EOs and ketoconazole in anticandidal use. The effective dose of

ketoconazole use in combination may be reduced, rapid drug resistance may be prevented or combination use may provide a solution to drug-drug resistance problems and can be considered a promising alternative pharmaceutical agent for topical application in surface infections. Therefore, lavender EOs should be combined with different antibiotic/antifungal drugs and tested against different pathogens.

**Table 3.** Fractional inhibitory concentration index (FICI) for L1 EO.

<i>Candida</i> isolates	L1 MIC (alone) (µg/ml)	Ketoconazole MIC (alone) (µg/ml)	L1 MIC (combination) (µg/ml)	Ketoconazole MIC (combination) (µg/ml)	FICI	The resulting interaction type
<i>C. albicans</i> ATCC 0231	320	2.0	160	0.06	0.53	Additive
<i>C. krusei</i> NRRL Y-7179	320	2.0	10	1.0	0.53	Additive
<i>C. glabrata</i> ATCC 6032	320	2.0	20	1.0	0.56	Additive
<i>C. albicans</i> clinical isolate	320	2.0	160	0.06	0.53	Additive
<i>C. krusei</i> clinical isolate	320	2.0	160	0.06	0.53	Additive
<i>C. glabrata</i> clinical isolate	320	2.0	10	1.0	0.53	Additive

**Table 4.** Fractional inhibitory concentration index (FICI) for L2 EO.

<i>Candida</i> isolates	L2 MIC (alone) (µg/mL)	Ketoconazole MIC (alone) (µg/mL)	L2 MIC (combination) (µg/mL)	Ketoconazole MIC (combination) (µg/mL)	FICI	The resulting interaction type
<i>C. albicans</i> ATCC 10231	320	4.0	320	2.0	1.5	Indifferent
<i>C. krusei</i> NRRL Y-7179	160	1.0	10	2.0	2.06	Indifferent
<i>C. glabrata</i> ATCC 66032	640	4.0	640	4.0	2.0	Indifferent
<i>C. albicans</i> clinical isolate	320	4.0	160	2.0	1.0	Additive
<i>C. krusei</i> clinical isolate	160	1.0	80	2.0	2.05	Indifferent
<i>C. glabrata</i> clinical isolate	320	4.0	20	2.0	0.56	Additive

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