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Original article

Efficacy of *Lamiaceae* essential oils with selected azoles against *Candida albicans* clinical isolates

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Abstract

A current problem in candidiasis treatment is increasing resistance to azoles, which are often prescribed to patients. The study underlines the high resistance of yeasts to fluconazole, which achieved high MIC (minimal inhibitory concentration) values both alone and in combination with essential oils (EOs). Antifungal activity of *Hyssopus officinalis, Thymus vulgaris, Salvia officinalis* and *Rosmarinus officinalis* EOs was determined against 13 clinical isolates of *Candida albicans* and reference strain *Candida albicans* ATCC 10231. The synergistic effect was investigated for the combination of itraconazole and fluconazole with *Hyssopus officinalis* and *Thymus vulgaris* EOs. Based on the fractional inhibitory concentration index, the synergistic effect was achieved in all of the samples exposed to itraconazole with *Hyssopus officinalis* (FICI 0.3±0.06). On the other side, the additive effect was proven in use of itraconazole with *Thymus vulgaris* (FICI 0.75±0.35) and fluconazole with both EOs tested (FICI 0.81±0.19; 0.88±0.57) This study shows the importance of monitoring the synergistic effect of antifungals combined with EOs, because it is a possible solution for reducing the resistance and improving the disease prognosis.

Key words: *Candida albicans*, fluconazole, itraconazole, Lamiaceae essential oils, synergistic effect

Introduction

The yeast *Candida albicans* (*C. albicans*) is a part of human microbiome and its importance is related to protection against excessive multiplication of other pathogens. In terms of therapy, fungal infections can be divided into two groups. One of them is systemic mycosis, which contributes to mortality and morbidity in high-risk patients (Martins et al. 2014). The most common form of invasive candidiasis is candidaemia. In systemic candidiasis, infectious metastatic foci may be formed in various organs such as kidney, spleen and liver. Candidiasis subsequently causes the failure of organs, leading to mortality in approximately 50% of all cases, regardless of antifungal therapy. A serious problem is central nervous system infection, which

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is manifested as acute disseminated fungal encephalitis in infants and as chronic granulomatous meningoencephalitis in adults. Impairment of the cardiovascular system is also a serious problem. Mycotic endocarditis may occur after cardiac surgery and after catheterization (Dignani et al. 2009, CDC 2019).

On the other hand, there are local skin or mucosal infections, which, unlike systemic infections, do not endanger the patient's life. The classification of mucocutaneous candidiasis depends on the location of the outbreak and includes: genital, intrauterine, anal, nail and oral forms. *C. albicans* is the most common commensal in humans colonizing the mucous membranes of the urogenital tract, with vaginal mycoses being the most common infections. Oral candidiasis is also relatively common (Oksuz et al. 2007, Martins et al. 2014).

The problem of developing new antifungals is the little-known selective targets for action on *C. albicans* (Wall and Lopez-Ribot 2020). Based on the mechanism of action, antifungals are classified as ergosterol targeted (polyenes, azoles, allylamines, morfolines), β-1,3-D-glucan affecting (echinocandins) and intracellular (nucleoside analogues) compounds (Příborský 2018).

Azole antifungals, agents that inhibit C14-α-demethylase (the enzyme promoting the biosynthesis of fungal-specific membrane sterol – ergosterol), are the most frequently used for the treatment of candidiasis. Fluconazole and itraconazole are often used to treat mucosal mycoses as well as in cases requiring systemic treatment. The triazole compound, fluconazole is preferred due to its low toxicity, low cost and good availability in various dosage forms, but increasing resistance is a disadvantage of its use (Pfaller et al. 2010, Příborský 2018). Knowledge of the resistance of *C. albicans* to fluconazole is important to maintain its use in clinical practice. Resistance is based on several mechanisms, such as increased drug efflux, altered or increased drug target, or altered sterol biosynthesis. A benefit in this area is the study of natural resources, which are a potential alternative to conventional antifungals. The different mechanism of action, the low incidence of side effects and diverse activity predetermine the interest in investigating the benefits of essential oils in the treatment or prevention (Karpiński 2020). In addition, the effect of antifungals in combination with essential oils may contribute to the reduction of pathogen resistance to drugs thus making the treatment more effective (de Oliveira Santos et al. 2018). The researchers discovered an effect on *C. albicans* of several *Lamiaceae* essential oils namely *Salvia officinalis, Origanum vulgare, Hyssopus officinalis, Rosmarinus officinalis*, *Thymus vulgaris* (Raut and Karuppayil 2014, Elansary et al. 2018, Karpiński 2020). Cavalcanti et al. (2011) have shown that within the antifungal activity of *R. officinalis*, the oil has an antiadhesive effect on *C. albicans* and even affects its morphogenesis. Similarly, essential oil (EO) of *S. officinalis* inhibits the adhesion of yeast to the denture, thus preventing the development of candida stomatitis (Sookto et al. 2013). EO of *T. vulgaris* oil, which is effective against various oral pathogens, can also be used to prevent and treat oral infections caused by *C. albicans.* (Fani and Kohanteb 2017, Baj et al. 2020). Anticandidal agents also include EO of *H. officinalis* or *Ocimum basilicum* (Vlase et al. 2014).

The aim of this study was to investigate the antifungal effect of selected *Lamiaceae* EOs and subsequently to evaluate their synergistic effect with antifungal agents against *C. albicans in vitro*. The synergistic effect of the best antifungal EO in combination with an antifungal to which most strains of *C. albicans* were resistant, was evaluated.

Materials and Methods

Preparation of inoculum

The experiments were performed on thirteen clinical isolates of *C. albicans* (61.5% from males, 38.5% from females) from the respiratory tract mucosa of patients with confirmed candidiasis manifesting by typical symptoms of respiratory disease – fever, cough, shortness of breath, pain when breathing or coughing. Isolates were obtained from the Department of Medical and Clinical Microbiology, Louis Pasteur University Hospital, Košice. As the reference strain *C. albicans* ATCC 10231 (American Type Culture Collection) was used. Inoculum was prepared from 24 hour old cultures of *C. albicans* (37±1°C) grown on Sabouraud dextrose agar (HiMedia, Laboratories Pvt., Ltd., Mumbai, India). The stock suspension of 10^6 CFU/ml was prepared in PBS (phosphate-buffered saline) and diluted to 10^3 CFU/ml yeast inoculum in Sabouraud-dextrose broth (Hi Media Laboratories Pvt. Ltd., Mumbai, India) supplemented with glucose (10 mM).

Tested essential oils and antifungals

Four tested essential oils from the family *Lamiaceae* were procured by Calendula Company, Nová Ľubovňa, Slovak Republic with certificates of the quality. The following chemical composition was determined by the producer: *Hyssopi aetheroleum* (herb of *Hyssopus officinalis*, pinocamphone 50.0±2%; izopinocamphene 28.0±1%; α-pinene 11.0±1%), *Thymi aetheroleum* (herb of *Thymus vulgaris*, ρ-cymene 40.0±3%; tymol

32.0±2%), *Salviae aetheroleum* (aerial parts of *Salvia officinalis*, 1,8-cineole 30.0±1%; thujone 3.0±0.2%; borneol 3.0±0.2%) and *Rosmarini aetheroleum* (leaves of *Rosmarinus officinalis,* 1,8-cineole 25.0±1%; α-pinene 19.0±1%).

Among the azole antifungals used in clinical practice, three antifungals (Sigma Aldrich, Schnelldorf, Germany) were selected, namely itraconazole, clotrimazole and fluconazole.

Determination of minimal inhibitory concentration (MIC) of EOs and conventional antifungals

Antifungal activity of four EOs and three antifungals (ATFs) was investigated according to the standard broth microdilution method M27-A3 (CLSI 2008), CLSI with some modifications, in triplicate. The determination of MIC values of each agent against *C. albicans* clinical isolates was performed in 96-well microtiter plates with U-shaped bottom. Initially, tested concentration of EOs and antifungals were prepared directly in the microtiter plate by binary dilution. 100 µl of agents from the most concentrated well were transferred to the next well of the microtiter plate and then 100 µl of inoculum were added. In this way EO concentration from 2.10^5 µg/ml to 400 µg/ml and antifungal concentration from 16 to 0.0313 µg/ml were reached. Stock solutions of all tested EOs were prepared as solutions emulsified by gum arabic (30% of EO contain). Stock solutions of itraconazole and clotrimazole powders were prepared by dissolving in 2% DMSO (dimethyl sulfoxide, Sigma Aldrich, Schnelldorf, Germany). Distilled water was used to dilute fluconazole powder.

Column 11 served as a negative control (medium alone), and column 12 as the positive one (inoculum alone). The volatility of individual compounds was ensured by tight closing of the microtiter plates.

The susceptibility of yeasts to azoles was assessed according to the interpretation criteria of method M27-A3 (CLSI 2008) for fluconazole: susceptible (S) ≤ 8 µg/ml, susceptible dose dependent (S-DD) = 16 – 32 µg/ml, resistant (R) ≥ 64 µg/ml and itraconazole: S ≤ 0.125 µg/ml, S-DD = 0.25 – 0.5 µg/ml, R ≥ 1 µg/ml. Criteria by Nelson et al. (2013) (susceptible ≤ 0.5 µg/ml, resistant (R) > 0.5 µg/ml) were used to evaluate the clotrimazole efficacy. MICs were evaluated after 24-hour incubation (35°C).

For better visualization of the results, 0.15% solution of resazurin (15 µl) was added 4 hours before reading them subsequently. The violet colour indicated inhibition of yeast growth and the discoloration signalled yeast growth. To determine the synergistic effect, the most effective EO and antifungal to which *C. albicans* showed the highest resistance was selected. Testing was performed on resistant *C. albicans* strains.

Determination of the fractional inhibitory concentration (FIC) of EOs and conventional antifungals

A checkerboard method (Bolatchiev et al. 2020) was used to evaluate the effect of the combination of *Thymus vulgaris* (100 - 6250 µg/ml) or *Hyssopus officinalis* (200 - 6250 µg/ml) EOs with fluconazole (FLC) (0.25 - 64 µg/ml) and itraconazole (ITR) (0.125 - 64 µg/ml). Based on the MIC values from the previous testing, we determined the tested concentrations of substances in the ratios for EO: MICx8, MICx4, MICx2, MIC, MIC/2, MIC/4 and for antifungals: MICx8, MICx4, MICx2, MIC, MIC/2, MIC/4, MIC/8, MIC/16. Followed by 24 hour incubation of microtiter plates at 35±1°C, 0.15% resazurin solution (10 µl) was added for easier detection of the results, as in the previous testing. The effect of the combination was evaluated on the basis of the fractional inhibitory concentration index (FICI).

The FICI is defined by relation:

FICI = (MIC of EO in combination with ATF / MIC of EO alone) + (MIC of ATF in combination with EO / MIC of ATF alone)

FICI – fractional inhibitory concentration index;
EO – essential oil; ATF – antifungal;
FIC – minimal inhibitory concentration in combination;
MIC – minimal inhibitory concentration

FICI values indicated the following effects: synergistic (≤0.5), additive (0.5 – 1), indifferent (1 – 4) or antagonistic (>4).

Statistical analysis

The MIC values of EOs and conventional antifungals as well as the occurrence of the resistance were evaluated by the MS Excel statistical functions (average, SD, median, mode). The achieved FICI values were assessed by the statistical program GraphPad Prism 5.0 (GraphPad software Inc. CA, USA) by using a one-way ANOVA test, Tukey's Multiple Comparison Test with significance at a p-value of < 0.05.

Results

Based on MIC values (Table 1), the best antifungal effect was seen with *Thymus vulgaris* EO (400 µg/ml) followed by EOs of *Hyssopus officinalis* (800 µg/ml) and *Rosmarinus officinalis* EO (2400 µg/ml). EO of *Salvia officinalis* revealed the weakest anticandidal effect and the average MIC value was 6016 µg/ml.

Table 1. Statistical evaluation of minimal inhibitory concentrations (MICs) (µg/ml) of essential oils (EOs) and antifungals.

Tested agents	min. - max.	x	SD	Mo	Me
Hyssopus officinalis EO	800 - 1600	800	220	800	800
Rosmarinus officinalis EO	1600 - 3130	2400	790	3130	3130
Thymus vulgaris EO	400	400	0	400	400
Salvia officinalis EO	1600 - 12500	6016	4068	6250	6250
Clotrimazole	0.0313 - 1	1.6	4.34	1	0.25
Itraconazole	0.0625 - 16	4.4	5.84	16	2
Fluconazole	2 - 16	10.3	6.58	16	16

min.–max. – minimum and maximum of MIC value (µg/ml); x – average (µg/ml); SD – standard deviation; Mo – modus; Me – median

Table 2. Evaluation of *Candida albicans* resistance against to a specific azoles.

	Clotrimazole (∑=13) n/%	Itraconazole (∑=13) n/%	Fluconazole (∑=13) n/%
S	9/69.2	2/15.4	3/23.1
S-DD	0/0	4/30.8	2/15.4
R	4/30.8	7/53.8	8/61.5

S – susceptible strains, S-DD – susceptible dose dependent strains, R – resistant strains, n – number of strains

Table 3. Antifungal activity (µg/ml) of antifungals (ATFs), essential oils (EOs) and statistical analysis of index of the fractional inhibitory concentration (FICI).

ATF	EO	ATF (µg/ml)		EO (µg/ml)		FICI ± SD	Effect
		alone	combined	alone	combined		
itraconazole	*H. officinalis*	4.4	1.1	800	800	0.3^{a}±0.06	S
	T. vulgaris		2.1	400	228.6	$0.75^{a, b, c}$±0.35	A
fluconazole	*H. officinalis*	10.3	13.4	800	1682.5	$0.81^{b, d}$±0.19	A
	T. vulgaris		6.6	400	312.5	$0.88^{c, d}$±0.57	A

ATF – antifungal; EO – essential oil; FICI – index of the fractional inhibitory concentration; SD – standard deviation; S – synergistic effect; A – additive effect; $^{a, b, c, d}$ – values with different designations are statistically different from each other $p<0.05$

From tested azoles, up to eight strains of *C. albicans* were resistant to fluconazole and the lowest efficiency was underlined by the MIC value 10.3 µg/ml (Table 1 and 2).

The results in Table 3 confirm the more advantageous uses of the combination of both, *Hyssopus officinalis* and *Thymus vulgaris* EO with itraconazole, compared to the combination with fluconazole. The MIC of itraconazole was reduced in combination with *Hyssopus officinalis*, resulting in a strong synergistic effect and a significantly lowest FICI value 0.3±0.06 (Table 3). Itraconazole inhibited the yeast's growth at a concentration of 1.1 µg/ml and 2.1 µg/ml in combination with EO of *Hyssopus officinalis* (800 µg/ml) and EO of *Thymus vulgaris* (228.6 µg/ml), the MIC values for fluconazole and both EOs were higher.

For fluconazole, a predominantly additive effect (62.5%) was observed in combination with *Hyssopus officinalis* EO. The combination of fluconazole with *Thymus vulgaris* EO (Fig. 1) showed the synergistic effect in only 25%, the indifferent effect was manifested in 50% of isolates. The most effective appeared to be the combination of *Thymus vulgaris* EO with itraconazole, which achieved the additive effect in 57.1% of cases. The synergistic effect was recorded only in 14.3% of cases (Table 4).

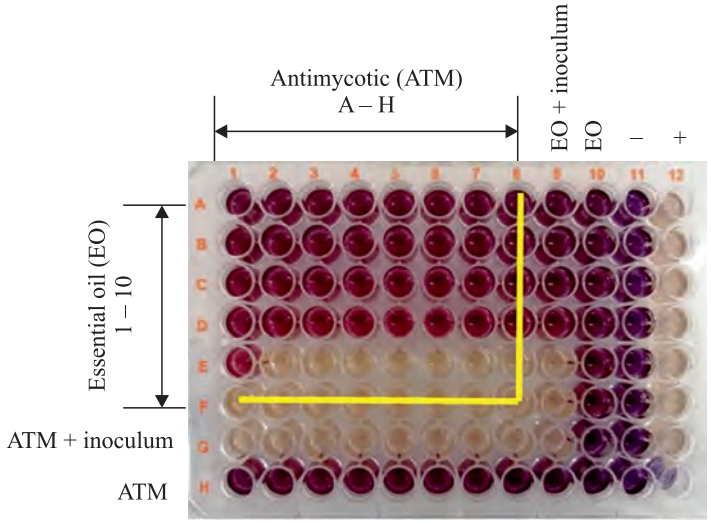

Fig. 1. Detection of synergistic effect of *T. vulgaris* EO with fluconazole against *C. albicans* reference strain by a checkerboard method after addition of 0.15 % resazurin.

Table 4. Evaluation of the efficacy (%) of *Hyssopus officinalis* EO and *Thymus vulgaris* EO in combination with azoles against *Candida albicans.*

Effect (%)	Fluconazole (Σ=8) n/%		Itraconazole (Σ=7) n/%	
	H. officinalis	*T. vulgaris*	*H. officinalis*	*T. vulgaris*
Synergistic	0/0	2/25	7/100	1/14.3
Additive	5/62.5	2/25	0/0	4/57.1
Indifferent	3/37.5	4/50	0/0	2/28.6
Antagonistic	0/0	0/0	0/0	0/0

n – number of the tested clinical isolates of *C. albicans*

Discussion

Due to the increase in yeast resistance, there is a need to search for new strategies in the treatment of candidiasis. One of the options is the use of essential oils, a complex mixtures of low molecular weight compounds, produced by plants of various genera belonging to 60 families (e.g., *Alliaceae, Apiaceae, Lamiaceae, Rutaceae, Myrtaceae, Asteraceae, Poaceae*). The cosmopolitan distribution is typical for the family *Lamiaceae* which includes plant species with a broad spectrum of bioactivity (Nuzhat and Vidyasagar 2014).

In this study, a potential pharmacological strategy in anti-candidal therapy was investigated, specifically the combination of conventional antifungals with some EOs of the *Lamiaceae* family. As we considered it to be a type of a pilot study, we used a relatively small number of samples. Accordingly, treatment with tested substances could not be applied, but *in vitro* conditions were proposed. Preliminary experiments confirmed the antifungal efficacy of all four tested EOs (*H. officinalis, R. officinalis, T. vulgaris, S. officinalis*). However, only the two most effective EOs were tested for the synergistic effect.

A quantitative method of susceptibility testing (MIC – the lowest concentration of an agent that will inhibit the visible growth of a microorganism after incubation) helps determine which substance is the most effective. The lowest MIC value (400 µg/ml) was found out in *Thymus vulgaris* EO, followed by *Hyssopus officinalis* EO with the MIC value 800 µg/ml. The study by Duarte et al. (2005) reports the antifungal activity of *Thymus vulgaris* EO at a concentration of 2000 µg/ml. However, our results are close to those obtained in the recent study revealing the antifungal effect of *Thymus vulgaris* EO in the concentration range of 500 – 250 µg/ml (Baj et al. 2020).

The pharmacological group of azoles is famous for its use for many years. In our study, among the three azoles (clotrimazole, fluconazole, itraconazole), two substances were used to test the synergistic effect – fluconazole and itraconazole. These were selected based on the highest incidence of resistant isolates tested. As results note, *C. albicans* showed the highest

resistance to fluconazole (eight isolates) and itraconazole (seven isolates).

Our results are comparable to those obtained by Bueno et al. (2010) although we have observed susceptibility of *C. albicans* over a wider range of antifungal concentration. Bueno et al. (2010) recorded susceptibility at concentrations of 0.031 - 0.5 µg/ml for itraconazole and at 0.125 - 8 µg/ml for fluconazole. In our study, clinical isolates of *C. albicans* were susceptible in the concentration range of 0.062 - 16 µg/ml for itraconazole and 2 - 16 µg/ml for fluconazole.

Three mechanisms of the resistance of yeasts are currently considered. Most important are multidrug pumps (efflux pumps) built into the cell wall of a pathogen which are able to expel the drug from the cell. The second mechanism is the alteration or up-regulation of ERG11 (the gene encoding the enzyme being targeted) which leads to the resistance. The last one are bypass pathways. They are the result of mutations in which the pathogen retains the functional membranes (Pfaller et al. 2010, Pristov and Ghannoum 2019). Among the mechanisms of the yeasts resistance, efflux pumps are responsible for the high degree of azole resistance. There are two types of active transporters involved in *C. albicans* resistance. The first type of transporters is encoded by drug resistance-CDR genes and multidrug resistance-MDR1 genes, whose overexpression is responsible especially for fluconazole resistance (de Oliveira Santos et al. 2018). Higher resistance of *C. albicans* to fluconazole in comparison to itraconazole was also confirmed in our study based on the number of resistant strains, MIC and FIC values.

The effect of itraconazole in combination with *Hyssopus officinalis* EO was surely synergistic (100 %), while the effect of fluconazole was additive (62.5 %) or indifferent (37.5 %). In combination with *Thymus vulgaris* EO, the additive effect of itraconazole (57.1%) prevailed over the effect of fluconazole, which was synergistic in 25% of isolates. However, in a study by Scalas et al. (2018), itraconazole combined with *T. vulgaris* EO achieved a synergistic effect against the yeast *Cryptococcus neoformans*.

The biological activity of EO is conditioned by the main component, but in some cases the total bioactivity is the result of the action of several components together (Raut and Karuppayil 2014). The antifungal effect of *Thymus vulgaris* EO is attributed to the main phenolic monoterpenes (carvacrol and thymol) (Bona et al. 2016, Alexa et al. 2018). The most effective EO found in our study was *Hyssopus officinalis* EO and its antifungal effect is due to cis-pinocampon and ß-pinene (Hristova et al. 2015).

Conclusion

This study provides one way to reduce resistance of *C. albicans* to antifungal compounds. It was concluded that the combination of itraconazole and *Hyssopus officinalis* clearly had a synergistic effect on yeast growth inhibition. Dose reduction of an antifungal is expected to help reduce its side effects and make the patient treatment more effective. On the basis of this *in vitro* pilot study, it can be concluded that some EOs could be anti-candidiasis medicines, but such assumption requires further research.

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