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Antibiofilm activity of selected plant essential oils from the *Lamiaceae* family against *Candida albicans* clinical isolates

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Abstract

Introduction. The virulence of *Candida albicans* is conditioned by several virulence factors, one of which is the formation of biofilm which reduces the sensitivity of the yeast to conventional antimycotics. This study determines the antifungal and antibiofilm activity of five essential oils (EOs) of the *Lamiaceae* family: *Salvia officinalis, Thymus vulgaris, Rosmarinus officinalis, Origanum vulgare,* and *Hyssopus officinalis.*

Materials and method. In the preliminary research, the antifungal effect of eachof the EOs was tested in the concentration range of 200–0.4 mg/mL on planktonic *Candida albicans* (*C. albicans*) cells. A total of 13 *C. albicans* clinical isolates and one reference strain were evaluated on biofilm formation.

Results. Nine isolates (69.2%) showed weak biofilm production and four strains (30.8%) were detected as moderate biofilm producers. The EOs of *Thymus vulgaris* and *Origanum vulgare* were seen as effective antifungal agents on planktonic cells with the MIC 0.4 mg/mL. The highest average MIC values were recorded in *Salvia officinalis* EO (24.0 and 14.8 mg/mL). All isolates were used to determine EOs efficacy on the inhibition of adherence phase and biofilm formation. The biofilm production of *C. albicans* after exposition by EOs was quantitatively examined by crystal violet dye.

Conclusions. The most effective for adherence phase and biofilm formation were EOs of *Origanum vulgare* (0.1 mg/mL and 0.3 mg/mL) and *Thymus vulgaris* (0.1 mg/mL and 0.4 mg/mL). The obtained results show that EOs of *Thymus vulgaris* and *Origanum vulgare* are potential agents for antifungal treatment or prophylaxis by reducing the resistance of pathogen.

Key words

biofilm, *Candida albicans*, antifungal effect, antibiofilm activity, *Lamiaceae* essential oils

INTRODUCTION

In recent decades, many cases of infections caused by opportunistic fungal pathogens have been reported in immunocompromised patients. A common pathogen initiating mycosis (usually candidiasis) is represented by *C. albicans* belonging to the kingdom *Fungi,* class: *Saccharomycetes* and family: *Saccharomycetaceae* [1, 2]. In addition, according to the WHO, 15% of hospitalized patients who in the last decade have suffered from nosocomial infections, the cause of infections were *C. albicans* and *Cryptococcus neoforman* [3].

This eukaryote organism is a common natural microbiota of gastrointestinal, urogenital and oral flora. However, when there is a microbial imbalance affecting the organism, *C. albicans* has a potential to proliferate and cause various infections, from affecting superficial mucous membrane (e.g., mucocutaneous candidiasis) to life-threatening systemic candidiasis [1, 4]. Candidaemia becomes a public health problem when *C. albicans* attacks the bloodstream and spreads to other organs. Invasive candidiasis is the most common infection in France (43%), and in 2010 reached mortality up to 40.6%. In 2010, invasive candidiasis was reported as the fourth most common cause of nosocomial infections in the United States [5].

A wide range of virulence factors, such as polymorphism, hydrolases secretion, metabolic adaptation, adhesins and invasins, or biofilm formation, play an important role in yeast pathogenicity. Most *Candida* infections are associated particularly with biofilm formation [6]. Biofilm consists of structured microbial communities bound to biotic or abiotic surfaces, surrounded by an extracellular matrix that they produce themselves. The most frequently colonized abiotic surfaces are catheters and dentures, and even the surface of artificial heart valves. Thus, the introduction of invasive treatment methods increases the risk of candidiasis. An example of a biotic surface is mucous membrane [7, 8]. Biofilm formation begins with adherence of *C. albicans* cells to a surface, followed by biofilm proliferation and maturation (Fig. 1). This phase is characterised by the transition between yeast cell and hyphal growth form. At the end of biofilm formation, non-adherent yeast cells are dispersed and colonise other surfaces. This is confirmed by the fact that hyphae are invasive and the yeast cells are responsible for their spreading [9, 10].

The increased incidence of mycotic infections is associated with the development of resistance to the antifungals used. In many cases, the antifungal resistance presents by over-expression of efflux pumps, built into the cell walls of pathogen which are able to expel the drug from the cell. The

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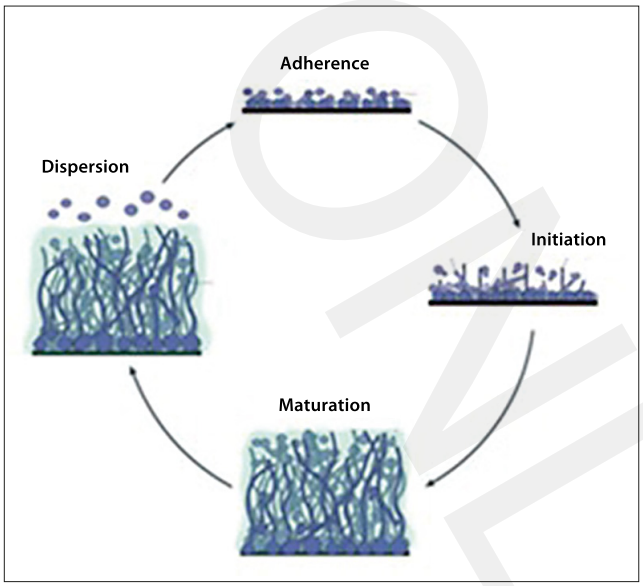

Figure 1. Process of biofilm forming: adherence, initiation, maturation, dispersion

main component of the cell plasma membranes is ergosterol. Mutations of the genes encoding for ergosterol biosynthesis (especially ERG11) lead to the drug resistance, especially to azoles. The high degree of resistance of the *C. albicans* to antifungals is also caused by biofilm production. Together with other limitations such as adverse side-effects, high costs and drug toxicity, they provide added challenges to the treatment [11, 12]. This is the reason for searching for alternative methods in the treatment of fungal infections. One possible solution is the use of plant products. EOs appear to be potential antifungal agents. EOs are mixtures of low molecular weight compounds extracted by steam distillation, hydrodistillation or solvent extraction of various plant parts (leaves, herb, flower). Terpenoids (monoterpenes, sesquiterpenes) and phenylpropanoids form the major constituents of EOs. Plant species of the *Lamiaceae* family are the most frequently studied due to their medicinal and pharmaceutical properties. The *Lamiaceae* family comprises 236 genera with about 7,000 botanical species. *Salvia* is the largest genus comprising 900 botanical species [13, 14].

Species belonging to *Lamiaceae* show variable biological activity, from antiviral, antimicrobial to anti-inflammatory and antifungal, as well as antioxidant and antitumoural or antiangiogenic potential [14, 15, 16]. Examples of the *Lamiaceae* family with medicinal importance are *Salvia officinalis* [17, 18], *Origanum vulgare* [15, 19], *Ocimum basilicum* [20], *Rosmarinus officinalis* [15, 21], *Melissa officinalis* [22, 23] and *Mentha piperita* [24, 25]. EOs produce a group of lipophilic substances, therefore the principle of its effect is based on membrane destabilization, and may even have an inhibitory effect on the cell cycle of *C. albicans* [26].

OBJECTIVE

The aim of this study was *in vitro* detection of an inhibitory effect of five EOs from the *Lamiaceae* family (*Origanum vulgare*, *Salvia officinalis*, *Rosmarinus officinalis*, *Hyssopus officinalis*, *Thymus vulgaris*) on *C. albicans* biofilm formation, as well as on its disintegration.

MATERIALS AND METHOD

Tested yeasts and growth conditions. A total of 13 clinical isolates were harvested from patients suspected of candidiasis – 8 isolates (61.5 %) from respiratory tract mucosa and 5 clinical isolates (38.5%) from sputum (Tab. 1). The tested clinical isolates of *C. albicans* were provided by the Department of Medical and Clinical Microbiology (Louis Pasteur University Hospital, Košice, Slovakia) and the testing verified using the reference strain *C. albicans* ATCC 10231 (Czech Collection of Microorganisms, Brno, Czech Republic). The isolates were cultivated onto Sabouraud dextrose agar-SDA (HiMedia, Laboratories Pvt., Ltd., Mumbai, India) and incubated for 24 hours at 37±1 °C.

Table 1. Clinical isolates *C. albicans*

	Men (Σ=8) n/%	Women (Σ=5) n/%	Total (n/%)
respiratory tract mucosa	7/53.8	1/7.7	8/61.5
sputum	1/7.7	4/30.8	5/38.5

Tested essential oils. Five certificated essential oils from the *Lamiaceae* family were procured (Calendula Co., Nová Ľubovňa, Slovakia) and analysed. EOs of *Origanum vulgare, Hyssopus officinalis, Thymus vulgaris*, *Salvia officinalis* and *Rosmarinus officinalis* were obtained by hydrodestillation from various parts of the plants, and their chemical composition identified by gas chromatography (Tab. 2).

The stock solutions of all the EOs tested at concentration 400 mg/mL were prepared as emulsions containing 30% gum Arabic from the EOs. Antibiofilm activity of EOs was detected in a different concentration range for each EO. This was depended on the MIC value reached in the testing on planktonic cells *C. albicans*.

Table 2. Examined essential oils

Essential oil	Plant Species	Origin	Content substances
Origani aetheroleum	*Origanum vulgare*	herb	carvacrol (85.0±3%)
Hyssopi aetheroleum	*Hyssopus officinalis*	herb	pinocamphone (50.0±2%) izopinocamphene (28.0±1%) α-pinene (11.0±1%)
Thymi aetheroleum	*Thymus vulgaris*	herb	*ρ*-cymene (40.0±3%) tymol (32.0±2%)
Salviae aetheroleum	*Salvia officinalis*	aerial parts	1,8-cineole (30.0±1%) thujone (3.0±0.2%) borneol (3.0±0.2%)
Rosmarini aetheroleum	*Rosmarinus officinalis*	leaves	1,8-cineole (25.0±1%) α-pinene (19.0±1%)

Susceptibility testing of *C. albicans* planktonic cells to EOs. The *in vitro* susceptibility testing of *C. albicans* to EOs was determined using broth microdilution method M27-A3 (CLSI – Clinical and Laboratory Standards Institute, 2008), with some modifications. The quality control of the test was verified by the sensitivity of the reference strain *Candida albicans* ATCC 10231 to fluconazole. The MIC of fluconazole was 4 µg/mL, which is in line with the criteria of the M27-A3 method (<8 µg/mL – sensitive, 16 – 32 µg/mL – dose-dependent, ≥ 64 µg/mL – resistant as emulsions containing 30% gum Arabic from the EOs).

The test was performed in 96-well microtiter plates, in duplicate. Briefly, stock solutions at concentration of 400 mg/mL of all the EOs tested were prepared. Each stock solution was diluted with Sabouraud-dextrose broth enriched with glucose (10 mM)_(SG) at concentrations of 200–0.4 mg/mL, by binary dilution. Afterwards, it was added to 100 µl inoculum of density 10^3 CFU/mL (McFarland 0.5), prepared from stock cultures (24 h., 37 °C) of 13 clinical isolates and one reference strain ATCC 10231 with SG.

The plates were incubated at 37 °C for 24 hours, and for better visualization 10 µL of 0.15% resazurin solution was added for 4 hours. The susceptibility of *Candida albicans* was characterized by MICs (minimum inhibitory concentrations) of EOs – the lowest concentration of agent required to delay growth of the pathogen. The resulting MIC value was determined as the average MIC of the EOs acting on clinical isolates.

Testing of antibiofilm activity. In the preliminary research, the biofilm formation of all 13 clinical isolates and the reference strain was determined according the method reported by Jin et al. (2003) [24]. Subsequently, the effect (on adherent cells – 0-h biofilm and on mature, 48-h biofilm) of antifungal EOs was observed on biofilm-forming isolates, also using the method described by Jin et al. (2003) [24] with some modifications. As the given clinical isolates obtained from the sputum and respiratory tract mucosa showed approximately the same values of MIC, they were evaluated together as one group. Finally, the reduction of the biofilm by the agents was evaluated and compared with the control.

Cells preparation for biofilm formation. The 24-hour-old cultures of 13 isolates and the reference strain incubated at 37 °C were used to prepare the inoculum by selecting approximately 10 colonies and inoculating 20 mL of SG. After 18 hours of incubation at 37 °C on an orbital shaker (80 rpm), the yeasts were harvested and washed with 5 mL of PBS (Phosphate-buffered saline, pH 7.2) in 2 centrifugation cycles for 10 min. at 1,000 rpm. Afterwards, the cellular density was adjusted to 10^7 cells/mL in sterile PBS for *C. albicans* isolates and the reference strain, corresponding to an optical density of 0.38 at 520 nm.

Detection of biofilm formation. Biofilm production was assessed in flat-bottom 96-well microtiter plates into which 100 µL of each cell isolate suspension was transferred in triplicate. Three wells of the microtiter plate were used as control, without *C. albicans* suspension. The adhesion phase lasted for 1.5 h at 37 °C, with continuous shaking (80 rpm), during which time the yeast cell adhered to the surface of the microtiter wells. The microtiter plate was then washed twice with 150 µL of PBS to remove non-adherent cells. Subsequently, 100 µL of SG was added to each well of the microtiter plate. For biofilm formation, the microtiter plate was incubated for 66 h at 37 °C, with shaking at 80 rpm. The spent medium was removed daily and fresh medium added.

Crystal violet staining. The crystal violet assay described by Dhale et al. (2014) [25] was used for quantification of biofilm formation. Following the 66 h incubation, the medium was aspirated, and the microtiter plate washed twice with 200 µL of PBS. After 45 min. of air drying, wells were stained with 110 µL of 0.15% crystal violet solution for 45 min. The plate was then washed 4 times with 350 µL of sterile distilled water and discoloured for 45 min. with 95% ethanol in the amount of 200 µL. 100 µL of destaining solution were transferred into the wells of a new microtiter plate, and absorbance was measured with a microtiter plate reader (Dynex Technologies, Inc., Virginia, USA) at 650 nm.

The intensity of biofilm formation was assessed on the basis of the optical density values (OD) of adherent yeast biofilm cells of all tested strains. The average OD of 3 measurements was determined separately for each strain. The cut-off value (ODc) for the microtiter plate test was calculated as 3 standard deviations above the mean OD of the negative control (without biofilm): average OD of the negative control using 3 measurements + (3x SD of negative control). Afterwards, OD value of the tested strain was formulated as the average OD value of the strain reduced by ODc value (OD= average OD of a strain – ODc). Based on the obtained results, the strains were divided into 4 groups (Tab. 3) [26].

Table 3. Interpretation of biofilm production. OD – optical density; ODc – cut-off value

Production of biofilm	Interpretation
Non-biofilm producer (0)	OD ≤ ODc
weak biofilm producer (+)	ODc < OD ≤ 2xODc
moderate biofilm producer (++)	2xODc < OD ≤ 4xODc
strong biofilm producer (+++)	4xODc < OD

Determination of EOs effect on biofilm production. All the studied isolates of *C. albicans* were used to determine the efficacy of selected EOs (Calendula, a.s., Nova Lubovňa, Slovak Republic) in inhibiting the adherent phase (0-h biofilm) and biofilm formation (48-h biofilm). This was performed at concentration range 25–0.05 mg/mL for *Origanum vulgare*, *Hyssopus officinalis*, and *Thymus vulgaris* EOs; 200 – 0.4 mg/mL for *Salvia officinalis* EO, and 100 – 0.2 mg/mL for *Rosmarinus officinalis* EO. The tested concentrations included MICx32, MICx16, MICx8, MICx4, MICx2, MIC, MIC:2, MIC:4, MIC:8, MIC:16, MIC:32, MIC:64.

EOs at the tested concentrations and 100 µL of SG were added to adherent cells (after 1.5 h at 37 °C, 80 rpm) in 96-well microtiter plates to test the inhibitory effect on the adhesion phase, prior to which all plates were washed twice with 150 µL PBS. Results were obtained after 48 h incubation, To test the effect of EOs on biofilm formation, 100 µL of each EO and 100 µL of SG were added to a 48 h biofilm (37 °C, 80 rpm) after washing the plates twice (150 µL PBS). *C. albicans* biofilm production after EOs exposure was quantified by crystal violet staining, and the percentage of biofilm reduction was determined by the following formula [27]:

$$biofilm\ reduction\ (\%) = \left[1 - \left(\frac{OD_{650}\ sample}{OD_{650}\ control}\right)\right] \times 100$$

Control – OD of sample without EO exposure, Sample – OD of sample after EO exposure.

Statistical analysis. The obtained results were evaluated by MS Excel statistical functions and the biofilm reduction assessed by statistical programme GraphPad Prism 5.0 (GraphPad Software Inc., San Diego (CA), USA), using a one-way ANOVA test, Dunnett's multiple comparison test ($p < 0.0001$).

RESULTS

Results of *C. albicans* planktonic cell susceptibility test are shown in Table 4 (isolates of respiratory tract musoca) and Table 5 (isolates of sputum). The sensitivity of clinical isolates from sputum and respiratory tract mucosa did not differ. The exception was *Salvia officinalis* EO, where clinical sputum isolates showed higher sensitivity (14.8±11.8) than mucosal isolates (24.0±18.6). Significant antifungal activity was found for *Origanum vulgare* and *Thymus vulgaris* EOs with MIC value 0.4 mg/mL. According to the average MIC value, *Salvia officinalis* EO appears to be the weakest antifungal EO.

Table 4. Antifungal effect of EOs (MIC; mg/mL) on planktonic cells of *C. albicans* clinical isolates of respiratory tract mucosa

MIC (mg/mL)	*Origanum vulgare*	*Rosmarinus officinalis*	*Salvia officinalis*	*Thymus vulgaris*	*Hyssopus officinalis*
min.-max.	0.4	1.6 – 3.13	1.6 – 50	0.4	0.8 – 1.6
x ± SD	0.4 ± 0	2.4 ± 0.8	24.0 ± 18.6	0.4 ± 0	0.9 ± 0.3
Mo	0.4	3.13	25	0.4	0.8
Me	0.4	2.4	25	0.4	0.8
MIC50	0.4	1.6	25	0.4	0.8
MIC90	0.4	3.13	50	0.4	0.8

Min.-max. – minimum and maximum MIC value (mg/mL); x – average (mg/mL); SD – standard deviation; Mo – modus; Me – median; MIC50/MIC90 – minimum inhibitory concentration inhibiting 50%, resp. 90% of the total of isolates (mg/mL).

Table 5. Antifungal effect of EOs (MIC; mg/mL) on planktonic cells of *C. albicans* clinical isolates of sputum

MIC (mg/mL)	*Origanum vulgare*	*Rosmarinus officinalis*	*Salvia officinalis*	*Thymus vulgaris*	*Hyssopus officinalis*
min.-max.	0.4	1.6 – 3.13	3.13 – 50	0.4	0.8
x± SD	0.4±0	2.4±0.9	14.8±11.8	0.4±0	0.8±0
Mo	0.4	1.6	25	0.4	0.8
Me	0.4	2.4	15.6	0.4	0.8
MIC50	0.4	1.6	6.3	0.4	0.8
MIC90	0.4	3.13	25	0.4	0.8

Min.-max. – minimum and maximum MIC value (mg/mL); x – average (mg/mL); SD – standard deviation; Mo – modus; Me – median; MIC50/MIC90 – minimum inhibitory concentration inhibiting 50%, resp. 90% of the total of isolates (mg/mL)

The intensity of biofilm production in 13 clinical isolates is evaluated in Table 6. Of the 13 isolates, 9 isolates (69.2%) showed weak biofilm production and 4 (30.8%) strains were detected as moderate biofilm producers.

Table 6. Results of biofilm producers

Production of biofilm	Mucosa of respiratory tract	Sputum	Total (n/%)
Non-biofilm producer (0)	0/0	0/0	0/0
weak biofilm producer (+)	5/38.5	4/30.8	9/69.2
moderate biofilm producer (++)	3/23.1	1/7	4/30.8
strong biofilm producer (+++)	0/0	0/0	0/0

Cut-off value (ODc) = 0.017

Table 7 shows the MICs of 5 EOs tested for inhibition in the adherence phase, and statistical analysis of EOs effect in the mature 48-h biofilm. Comparing the MIC values, all tested EOs were more effective (at lower concentration) on the adherence phase than on mature 48-h biofilm. The MICs of EOs affecting the yeasts biofilm formation (0-h biofilm) were in the concentration range 0.1–10.1 mg/mL, which is 1.5 – 3-fold less compared to the MIC range of tested EOs on 48- biofilm (Fig. 2). *Origanum vulgare* EO appears to be the most effective during the adherence phase with MIC 0.1 mg/mL, and for the already formed 48-h biofilm, although at a 3 times higher concentration (0.3 mg/mL).

Table 7. Statistical evaluation of EOs MIC (mg/mL) against 0-h, 48-h biofilm *C. albicans*

MIC (mg/mL)	*Origanum vulgare*		*Rosmarinus officinalis*		*Salvia officinalis*		*Thymus vulgaris*		*Hyssopus officinalis*	
	0 h	**48 h**	**0 h**	**48 h**	**0 h**	**48 h**	**0 h**	**48 h**	**0 h**	**48 h**
min.	0.1	0.2	3.1	6.3	3.1	6.3	0.1	0.2	1.6	3.1
max.	0.1	0.4	50	50	12.5	50	0.2	0.4	3.1	3.1
x	0.1	0.3	10.1	15.4	6.0	19.7	0.1	0.4	2.1	3.1
SD	0	0.1	13.3	11.3	3.2	11.4	0.1	0.1	0.7	0
Mo	0.1	0.4	6.25	12.5	6.25	12.5	0.1	0.4	1.6	3.13
Me	0.1	0.4	6.25	12.5	6.25	12.5	0.1	0.4	1.6	3.13
MIC50	0.1	0.4	6.25	12.5	6.25	12.5	0.1	0.4	1.6	3.13
MIC90	0.1	0.4	25	25	12.5	25	0.2	0.4	3.13	3.13

Min.–max. – minimum and maximum MIC value (mg/mL); x – average (mg/mL); SD – standard deviation; Mo – modus; Me – median; MIC50/MIC90 – minimum inhibitory concentration inhibiting 50% or 90% of the total of isolates (mg/mL).

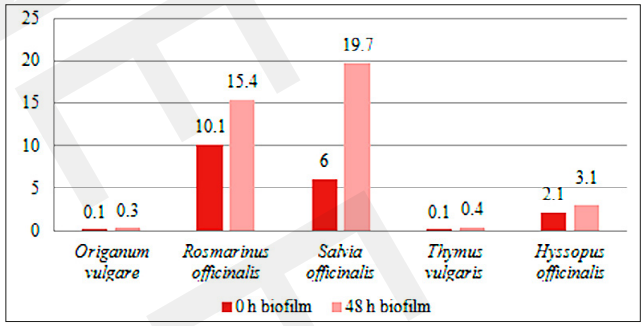

Figure 2. MICs (mg/mL) of tested EOs effective against biofilm-forming *C. albicans* strains

Origanum vulgare EO affected the adherence phase (0-h biofilm) at the same concentration 0.1 mg/mL of MIC50 and MIC90 value (Tab. 7). Inhibition of the formed 48-h biofilm was obtained at 0.4 mg/mL for both MIC50 and MIC90. *Thymus vulgaris* EO was effective at the same MIC50 and MIC90 value (0.4 mg/mL). The highest average MIC values were recorded in *Rosmarinus officinalis* EO for the biofilm-forming yeasts (10.1 mg/mL, and 48-h-old biofilm was obtained with MIC 15.4 mg/mL. This EO showed the least biofilm degradation (63.8%). Although *Salvia officinalis* EO was least effective for the 48-h biofilm (MIC 19.7 mg/mL), it obtained the highest reduction in biofilm formation (70.6%). All tested EOs differed significantly in reduction ($p < 0.0001$) of 48-h biofilm, compared to the control (without EO exposure) (Tab. 8).

Table 8. Reduction (%) of biofilm formation (48-h biofilm) after exposure to EOs

OD	Control	*Origanum vulgare*	*Rosmarinus officinalis*	*Salvia officinalis*	*Thymus vulgaris*	*Hyssopus officinalis*
x	0.0354	0.0108	0.0128	0.0104	0.0109	0.0112
Reduction (%)	-	69.49***	63.84***	70.62***	69.21***	68.36***

OD – optical density, *** $p < 0.0001$

DISCUSSION

Nowadays, the main problem is the increase in fungal infections, manifesting primarily in superficial lessions. However, *C. albicans* as a frequently isolated pathogen can attack the blood system or organs, sometimes with poor prognosis [31].

An important aspect in the development of *Candida albicans* infection is the yeast transformation from planktonic to biofilm-forming cells (sessile state). Biofilm production is a significant virulence factor of *C. albicans* yeast, which plays a key role in its resistance to conventional antimycotics [6, 7]. *Candida* biofilm consists 3 cell types: small oval yeast cells, long tubular hyphal cells, and oval pseudohyphal cells [32]. The biofilm is characterized by the production of an extracellular matrix, producing yeast protected against antimycotics [33].

Therefore, an important task arises – to find new, alternative methods for treating infection caused by *Candida* biofilm. The plant kingdom represents a potential for natural resources against various biological activities. In particular, the *Lamiaceae* family appearsto be a large source of EOs which are of interest to many researchers, and which are characterized by diverse bioactivity [15, 34].

The current study investigated the antibiofilm activity of 5 EOs against *C. albicans* produced by selected plant species from the *Lamiaceae* family: *Salvia officinalis*, *Thymus vulgaris*, *Rosmarinus officinalis*, *Origanum vulgare*, and *Hyssopus officinalis*. First, the effect of each EOs tested on *C. albicans* planktonic cells in the concentration range of 200 – 0.4 mg/mL was investigated. Significant antifungal activity of two EOs (*Thymus vulgaris* and *Origanum vulgare*) was found at the lowest tested concentration (MIC 0.4 mg/mL). These results are similar to those obtained in a recent study by Baj et al. [35], which reported the antifungal efficacy of *Thymus vulgaris* and *Origanum vulgare* EOs in the MIC range 0.5–0.25 mg/mL. In the presented study, *Salvia officinalis* EO, with the highest MIC value, was considered the agent with the weakest antifungal effect. Based on EO MIC values, concentration ranges were chose to study the inhibitory effect of biofilm formation.

All 13 *C. albicans* clinical isolates were able to form biofilm, up to 69.2% (9 isolates) showed weak biofilm production and 30.8% (4 strains) were detected as moderate biofilm producers. Weak biofilm formation was observed in *C. albicans*, which agrees with the study by Mohandas and Ballal [36]. The results obtained in the current study are in the contrast to the study by Udayalaxmi et al. [37], in which moderate to intense profound biofilm production in 55% of *C. albicans* isolates were found. In the same study, the biofilm production of non-albicans species was also investigated, and up to 63% of *C. tropicalis* isolates produced biofilm moderately to intensively. According to the study by Marak and Dhanashree [38], *C. parapsilosis* is an excellent biofilm producer with 100% biofilm production among non-albicans species. The intensity of the biofilm production depends on the localization and the type of colonized surface [38, 39].

Out of the 5 EOs tested, *Origanum vulgare,* showed the highest efficacy in inhibiting the adherence phase and biofilm formation of *C. albicans* with MIC 0.1 mg/mL and 0.3 mg/mL, respectively. Carvacrol and thymol, as the main components of *Origanum vulgare* EO, are probably responsible for the antibiofilm effect. This fact correlates with the study by Doke et el. [40] and the study of Vasconcelos et al. [41], both of which confirm the strong antibiofilm activity of monoterpenes, such as carvacrol, thymol and geraniol. In the presented study,a similar efficacy to *Origanum vulgare* EO was obtained with *Thymus vulgaris* EO, which was effective at MIC of 0.1 mg/mL (adherence phase) and 0.4 mg/mL (biofilm formation), respectively.

Among the tested EOs, *Rosmarinus officinalis* and *Salvia officinalis* were identified as the agents with the highest MICs to elicit the antibiofilm effect, similar to their effect on planktonic cells. The low efficacy of both *Rosmarinus officinalis* and *Salvia officinalis* EO could have been caused by the presence of the identical predominant component of the chemical spectrum, namely 1.8- cineole. *Rosmarinus officinalis* EO was observed as the least effective in the adherence phase, with an average MIC of 10.1 mg/mL. These results differ from the study by Cavalcanti et al. [42], in which the antiadherent activity of the *Rosmarinus officinalis* essential oil was evaluated. At the concentration of 2.25 mg/mL, a significant inhibition of adhesion and cell disruption were observed. The difference in the presented results may be due to the chemical composition. Limonene was not present in the chemical composition of EO, in comparison to the EO studied by Cavalcanti et al. [42]. The other main components (1.8-cineole, ρ-cymene, α-pinene, camphor) were identical.

The EO of *Salvia officinalis* was one of the EOs with high MIC values, which also determined its weak antibiofilm effect. In spite of the fact that *Salvia officinalis* EO showed antibiofilm effect (48-h biofilm) at high values of MIC (19.7 mg/mL), it was able to reduce biofilm formation the most intensively (70.62 %) among the EOs tested. In the study by Thaweboon and Thaweboon [43], up to 93.33% reduction was achieved at MIC 20 mg/mL. The result may have been affected by the predominant components of the essential oil, but the exact composition is not given in the study.

Knowledge of the toxicological profile of natural compounds is very important in drug research. The study by Potente et al. [44] describes the safety profile of selected EOs from the *Lamiaceae* family, as well as the toxicity of *Origanum vulgare* ssp. hirtum Link, at MIC 1080 μg/mL, corresponding to an IC_{50} 148.5 μg/mL. Nowadays, only a few studies, mention the carvacrol toxicity (major component of *Origanum vulgare* EO). Carvacrol (25 μM) in V79 Chinese hamster lung fibroblast cells did not cause any DNA damaging effects. In tests with human lymphocytes, plant compounds (thymol, carvacrol, and γ -terpinene) did not induce DNA strand breakage (50–100 μM). However, carvacrol as a flavouring ingredient is present in low concentrations in human food. It has been approved by the Federal Drug Administration (FDA) in the USA for use in food, and it is generally considered safe for human consumption [45].

CONCLUSION

In conclusion, *Lamiaceae* represents a family of high significance, with important medicinal properties, due to its richness in EOs. Although all the 5 EOs tested showed antifungal and antibiofilm activity, *Origanum vulgare* EO showed the best alternative for treatment or adjuvant therapy of mycoses. It was found, that it is not only the agent with the lowest MIC antifungal activity, but it also obtained low MIC values in the antibiofilm effect. On the other hand, *Salvia officinalis* EO, with the highest values of MICs, also obtained the highest biofilm reduction. *Thymus vulgaris* EO has been evaluated as another agent which appears to have potential in the prophylaxis or for the treatment of mycoses. Therapy is more often complicated due to resistance of *C. albicans,* the reason for which is the formation of biofilm. The use of EOs seems to be a future solution, but their impact on biofilm formation requires further investigation.

REFERENCES

1. Martins N, Ferreira IC, Barros L, et al. Candidiasis: predisposing factors, prevention, diagnosis and alternative treatment. Mycopathologia. 2014; 177(5–6): 223–240. https://doi.org/10.1007/s11046-014-9749-1
2. Moore D, Alexopoulos CJ, Ahmadjian V. Fungus, Encyclopædia Britannica, Encyclopædia Britannica, inc, (access 2020.04.05). https://www.britannica.com/science/fungus/Annotated-classification
3. Khan HA, Baig FK, Mehboob R. Nosocomial infections: Epidemiology, prevention, control and surveillance. Asian Pac J Trop Biomed. 2017; 7(5): 478–82. https://doi.org/10.1016/j.apjtb.2017.01.019
4. Gow NA, Yadav B. Microbe Profile: Candida albicans: a shape-changing, opportunistic pathogenic fungus of humans. Microbiology. 2017; 163(8): 1145–1147. https://doi.org/10.1099/mic.0.000499
5. D'agostino M, Tesse N, Frippiat JP, et al. Essential oils and their natural active compounds presenting antifungal properties. Molecules. 2019 Jan; 24(20): 3713. https://doi.org/10.3390/molecules24203713
6. Mayer FL, Wilson D, Hube B. Candida albicans pathogenicity mechanisms. Virulence. 2013; 4(2): 119–128. https://doi.org/10.4161/viru.22913
7. Fanning S, Mitchell AP. Fungal biofilms. PLoS Pathog. 2012; 8(4): e1002585. https://doi.org/10.1371/journal.ppat.1002585
8. Lohse MB, Gulati M, Johnson AD, et al. Development and regulation of single-and multi-species Candida albicans biofilms. Nat Rev Microbiol. 2018; 16(1): 19–31. https://doi.org/10.1038/nrmicro.2017.107
9. Höfs S, Mogavero S, Hube B. Interaction of Candida albicans with host cells: virulence factors, host defense, escape strategies, and the microbiota. J Microbiol. 2016; 54(3): 149–169. http://doi.org/10.1007/s12275-016-5514-0
10. Tsui C, Kong EF, Jabra-Rizk MA. Pathogenesis of Candida albicans biofilm. FEMS Pathog Dis. 2016; 74(4): ftw018. https://doi.org/10.1093/femspd/ftw018
11. Pristov KE, Ghannoum MA. Resistance of Candida to azoles and echinocandins worldwide. Clin Microbiol Infect. 2019; 25(7): 792–8. https://doi.org/10.1016/j.cmi.2019.03.028
12. Costa-de-Oliveira S, Rodrigues AG. Candida albicans Antifungal Resistance and Tolerance in Bloodstream Infections: The Triad Yeast-Host-Antifungal. Microorganisms. 2020; 8(2): 154. https://doi.org/10.3390/microorganisms8020154
13. Raja RR. Medicinally potential plants of Labiatae (Lamiaceae) family: an overview. Res J Med Plant. 2012; 6(3): 203–213. https://doi.org./10.3923/rjmp.2012.203.213
14. Carović-Stanko K, Petek M, Martina G, et al. Medicinal plants of the family Lamiaceaeas functional foods–a review. Czech J Food Sci. 2016; 34(5): 377. https://doi.org/10.17221/504/2015-CJFS
15. Nieto G. Biological activities of three essential oils of the Lamiaceae family. Medicines. 2017; 4(3): 63. https://doi.org/10.3390/medicines4030063
16. Nagy M, Mučaji P, Grančai D. Pharmacognosy. Biologically active plant metabolites and their sources. 2nd ed. Bratislava: HERBA, 2017. ISBN 978-80-89631-64-3.
17. Sookto T, Srithavaj T, Thaweboon S et al. In vitro effects of Salvia officinalis L. essential oil on Candida albicans. Asian Pac J Trop Biomed. 2013; 3(5): 376–380. https://doi.org/10.1016/S2221-1691(13)60080-5
18. El Euch SK, Hassine DB, Cazaux S, et al. Salvia officinalis essential oil: Chemical analysis and evaluation of anti-enzymatic and antioxidant bioactivities. S Afr J Bot. 2019; 120: 253–60. https://doi.org/10.1016/j.sajb.2018.07.010
19. Sakkas H, Papadopoulou C. Antimicrobial activity of basil, oregano, and thyme essential oils. J Microbiol Biotechnol. 2017; 27(3): 429–38. https://doi.org/10.4014/jmb.1608.08024
20. Gucwa K, Milewski S, Dymerski T, et al. Investigation of the antifungal activity and mode of action of Thymus vulgaris, Citrus limonum, Pelargonium graveolens, Cinnamomum cassia, Ocimum basilicum, and Eugenia caryophyllus essential oils. Molecules. 2018; 23(5): 1116. https://doi.org/10.3390/molecules23051116
21. Elansary HO, Abdelgaleil SA, Mahmoud EA, et al. Effective antioxidant, antimicrobial and anticancer activities of essential oils of horticultural aromatic crops in northern Egypt. BMC Complement Altern Med. 2018; 18(1): 214. https://doi.org/10.1186/s12906-018-2262-1
22. Alizadeh BB, Shahidi F. Melissa officinalis essential oil: Chemical compositions, antioxidant potential, total phenolic content and antimicrobial activity. Nutr Food Sci Res. 2019; 6(1): 17–25. https://doi.org/10.29252/nfsr.6.1.17
23. Serra E, Saubade F, Ligorio C, et al. Methylcellulose hydrogel with Melissa officinalis essential oil as a potential treatment for oral candidiasis. Microorganisms. 2020; 8(2): 215. https://doi.org/10.3390/microorganisms8020215
24. Desam NR, Al-Rajab AJ, Sharma M, et al. Chemical constituents, in vitro antibacterial and antifungal activity of Mentha× Piperita L. (peppermint) essential oils. J King Saud Univ Sci. 2019; 31(4): 528–33. https://doi.org/10.1016/j.jksus.2017.07.013
25. Modarresi M, Farahpour MR, Baradaran B. Topical application of Mentha piperita essential oil accelerates wound healing in infected mice model. Inflammopharmacol. 2019; 27(3): 531–7. https://doi.org/10.1007/s10787-018-0510-0
26. Raut JS, Karuppayil SM. A status review on the medicinal properties of essential oils. Ind Crops Prod. 2014; 62: 250–264. https://doi.org/10.1016/j.indcrop.2014.05.055
27. Jin YYHK, Yip HK, Samaranayake YH, et al. Biofilm-forming ability of Candida albicans is unlikely to contribute to high levels of oral yeast carriage in cases of human immunodeficiency virus infection. J Clin Microbiol. 2003; 41(7): 2961–2967. https://doi.org/10.1128/JCM.41.7.2961-2967.2003
28. Dhale RP, Ghorpade MV, Dharmadhikari CA. Comparison of various methods used to detect biofilm production of Candida species. J Clin Diagn Res: JCDR. 2014; 8(11): DC18-Dc20. http://doi.org/10.7860/JCDR/2014/10445.5147
29. Ruchi T, Sujata B, Anuradha D. Comparison of phenotypic methods for the detection of biofilm production in uro-pathogens in a tertiary care hospital in India. Int J Curr Microbiol App Sci. 2015; 4(9): 840–49.
30. Chemsa AE, Zellagui A, Öztürk EE, et al. Antibiofilm formation, antioxidant and anticholinesterase activities of essential oil and methanol extract of Marrubium deserti de Noé. J Mater Environ Sci. 2016; 7(3): 993–1000.
31. Invasive Candidiasis Statistics. Centers for Disease Control and Prevention. https://www.cdc.gov/fungal/diseases/candidiasis/invasive/statistics.html (access 07.08.2020)
32. Gulati M, Nobile CJ. Candida albicans biofilms: development, regulation, and molecular mechanisms. Microbes Infect. 2016; 18(5): 310–321. http://www.doi.org/10.1016/j.micinf.2016.01.002
33. Silva S, Rodrigues CF, Araújo D, et al. Candida species biofilms' antifungal resistance. J Fungi. 2017; 3(1): 8. https://doi.org/10.3390/jof3010008
34. Bekut M, Brkić S, Kladar N, et al. Potential of selected Lamiaceae plants in anti (retro) viral therapy. Pharmacol Res. 2018; 133: 301–314. https://doi.org/10.1016/j.phrs.2017.12.016
35. Baj T, Biernasiuk A, Wróbel R, et al. Chemical composition and in vitro activity of Origanum vulgare L., Satureja hortensis L., Thymus serpyllum L. and Thymus vulgaris L. essential oils towards oral isolates of Candida albicans and Candida glabrata. Open Chem. 2020; 18(1): 108–118. https://doi.org/10.1515/chem-2020-0011
36. Mohandas V, Ballal M. Distribution of Candida species in different clinical samples and their virulence: biofilm formation, proteinase and phospholipase production: a study on hospitalized patients in southern India. J Glob Infect Dis. 2011; 3(1): 4. https://doi.org/10.4103/0974-777X.77288
37. Udayalaxmi SJ, D'Souza D. Comparison between virulence factors of Candida albicans and non-albicans species of Candida isolated from genitourinary tract. J Clin Diagn Res. 2014; 8(11): DC15-DC17. http://doi.org/10.7860/JCDR/2014/10121.5137

38. Marak MB, Dhanashree B. Antifungal susceptibility and biofilm production of Candida spp. isolated from clinical samples. Int J Microbiol. 2018; 2018. https://doi.org/10.1155/2018/7495218
39. Sida H, Shah P, Pethani J, et al. Study of biofilm formation as a virulence marker in Candida species isolated from various clinical specimens. Int J Med Sci Public Health. 2016; 5(5): 842–846. https://doi.org/10.5455/ijmsph.2016.24082015139
40. Doke SK, Raut JS, Dhawale S, et al. Sensitization of Candida albicans biofilms to fluconazole by terpenoids of plant origin. J Gen Appl Microbiol. 2014; 60(5): 163–168. https://doi.org/10.2323/jgam.60.163
41. Vasconcelos LCD, Sampaio FC, Albuquerque ADJDR, et al. Cell viability of Candida albicans against the antifungal activity of thymol. Braz Dent J. 2014; 25(4): 277–281. https://doi.org/10.1590/0103-6440201300052
42. Cavalcanti Y, Almeida LDFDD, Padilha WWN. Anti-adherent activity of Rosmarinus officinalis essential oil on Candida albicans: an SEM analysis. Rev Odonto Ciênc. 2011; 26(2): 139–144. http://dx.doi.org/10.1590/S1980-65232011000200008
43. Thaweboon B, Thaweboon S. Inhibitory effect of Salvia officinalis L. oil on Candida biofilm. In Advanced Materials, Mechanical and Structural Engineering: Proceedings of the 2nd International Conference of Advanced Materials, Mechanical and Structural Engineering (AMMSE 2015), Je-ju Island, South Korea, September 18–20, 2015. 2016 Apr 14 (p. 1). CRC Press.
44. Potente G, Bonvicini F, Gentilomi GA, et al. Anti-Candida Activity of Essential Oils from Lamiaceae Plants from the Mediterranean Area and the Middle East. Antibiotics. 2020; 9(7): 395. https://doi.org/10.3390/antibiotics9070395
45. Suntres ZE, Coccimiglio J, Alipour M. The bioactivity and toxicological actions of carvacrol. Crit Rev Food Sci Nutr. 2015; 55(3): 304–18. http://dx.doi.org/10.1080/10408398.2011.653458