

Activity of Selected Essential Oils against *Candida* spp. strains. Evaluation of New Aspects of their Specific Pharmacological Properties, with Special Reference to Lemon Balm

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ABSTRACT

The aim was to investigate the antifungal effectiveness and some of pharmacological properties of essential oils (EOs), which had not yet been thoroughly studied in the planned scope. We first evaluated MIC/MFC of sixteen EOs against *C. albicans* ATCC 10231. Then, five most active EOs were tested, using 50 clinical *Candida* spp. strains and additional reference *C. albicans* ATCC 90028 strain. The time-kill curve, carryover, post-antifungal effects (PAFE), mutant prevention concentrations, the susceptibility of reference strains to the cell wall disrupting agents and tolerance to oxidative stress, were evaluated. For these detailed studies, we chose the following four essential oils. Clove oil, Geranium oil, Lemon balm and Citronella oil, with MICs of 0.097% (v/v), resulted concentration- and time-dependent killing and may be therapeutically safe, because they do not generate resistance. The best one was Lemon balm, which caused most extended PAFE, significantly reduced tolerance to oxidative stress and increased susceptibility to Calcofluor White, Congo Red and SDS. Phytochemical analysis of these four EOs has been performed and compared; looking for the reason that Lemon balm was the best.

Keywords: *Candida*; Cell Wall Sensitivity; Essential Oils PAFE; Mutant Generation; Oxidative Stress Tolerance; Time-Killing

1. Introduction

Numerous members of *Candida* spp. are commensal organisms colonizing several ecological niches of healthy individuals. On the other hand, they are also the most frequent pathogens in humans at risk. Depending on the immunological state of the host, candidiasis can develop as mild superficial infection or very dangerous, life threatening invasive one. Epidemiological data also show an increase in local fungal infections of chronic wounds (diabetic foot, burn, bed-sore, cancer ulceration), with the participation of *C. albicans* biofilm. Such infections, similarly to systemic ones, are very difficult to eradicate since *Candida* cells living as a biofilm community show extremely high resistance to most of the currently used

antifungal drugs [1,2]. Despite the discovery in the last decades of potent antimycotics, most types of systemic and local *Candida* infections still remain a serious medical problem. Thus, it is pivotal to discuss various strategies that may become therapeutic tools in future, in which the search for agents with novel mechanisms of action should be considered. The most intensively studied option is using plant products as factors modulating the resistance of microorganisms [3]. Indeed, many compounds of plant origin affect some structures and some of the mechanisms responsible for the resistance phenotype in bacteria and fungi. A very interesting trend of worldwide studies is the possible synergism that may occur between, for example, plant-derived agents and antibiotics or other chemotherapeutics. It can result in strong growth inhibition even of drug-resistant microbes (bacte-

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ria or fungi) and the ability to obtain a stronger post-antibiotic effect [4-9]. Generally, studies on the possible use of plant-derived compounds for combating human pathogens are being carried out worldwide and knowledge coming from *in vitro* studies in this field is already quite extensive. However, the modern approach to observations drawn from folk medicine is to clarify the mechanisms of their activity, forming the basis for safe use. The main objective of the present study was to investigate the possibility of using essential oils as potential medicinal substances active against *Candida* spp. For this purpose, the essential oils were chosen which had not yet been thoroughly studied in the planned scope. As target microorganisms we used *C. albicans* reference strains and a group of *C. albicans* and various non-*albicans* *Candida* clinical isolates. More detailed research, concerning time-kill curve, carryover and post-antifungal effects as well as the mutant prevention concentration of four selected the most active essential oils, was conducted. Also, the susceptibility of *C. albicans* to cell wall disrupting agents and yeast cell tolerance to oxidative stress induced by hydrogen peroxide, after treatment with essential oils were estimated. For these preparations the phytochemical analysis has also been performed.

2. Materials and Methods

2.1. *Candida* Strains and Culture Conditions

A total of 52 strains were tested for their susceptibility to essential oils. These comprised two reference *C. albicans* strains (ATCC 10231, ATCC 90028) and 50 clinical isolates: *C. albicans* (n = 20), *C. glabrata* (n = 13), *C. krusei* (n = 6), *C. parapsilosis* (n = 5), *C. tropicalis* (n = 6). The *Candida* isolates were obtained from cultures of blood, wounds swabs, stool and various mucosal tissue specimens of patients hospitalized at Dr Wł. Biegański Provincial Specialist Hospital in Lodz, Poland. Suspensions of the yeasts for each test were prepared from fresh (24 h-old) cultures grown at 35°C on Sabouraud Dextrose agar (SDA, Difco Laboratories, USA) or if necessary on YPG P-0035 medium (Yeast Extract Peptone Glucose, BTL, Poland).

2.2. Essential oils, MIC and MFC Determination

Essential oils (EOs) of plants listed in **Table 1**. were used, all purchased from Pollena Aroma, Poland. The initial screening of 16 EOs activity was performed by broth microdilution method, according to the guidelines of EUCAST with minor modifications [10,11], using *C. albicans* ATCC 10231 strain as a target organism. Then, the MIC/MFC (minimum inhibitory/fungicidal concentration) of 5 most active EOs against 50 clinical *Candida* spp. and additional reference (*C. albicans* ATCC 90028) strains was evaluated, using the same experimental pro-

Table 1. List of essentials oils tested at different stages of research.

Plant species	Essential Oil	1	2	3
<i>Lavandula angustifolia</i>	Lavender oil	•	•	
<i>Melaleuca alternifolia</i>	Tea tree oil	•		
<i>Citrus limon</i>	Lemon oil	•		
<i>Anthemis nobilis</i>	Anthemis oil	•		
<i>Melissa citrata indica</i>	Lemon balm	•	•	•
<i>Pinus sylvestris</i>	Scott pine	•		
<i>Ribes nigrum</i>	Black currant	•		
<i>Cymbopogon citratus</i>	Citronella oil	•	•	•
<i>Pimpinella anisum</i>	Oleum anisi	•		
<i>Pelargonium graveolens</i>	Geranium oil	•	•	•
<i>Eugenia caryophyllata</i>	Oleum Caryophylli (Clove oil)	•	•	•
<i>Hyssopus officinalis</i>	Hyssop oil	•		
<i>Mentha piperita</i>	Peppermint oil	•		
<i>Thymus vulgaris</i>	Thyme oil	•		
<i>Rosmarinus officinalis</i>	Rosemary oil	•		
<i>Abies sibirica</i>	Abies oil	•		

1—MIC/MFC evaluation; *C. albicans* ATCC 10231, 2—MIC/MFC evaluation; 50 clinical *Candida* spp. strains, *C. albicans* ATCC 90028, 3—tests evaluating selected pharmacological properties; *C. albicans* ATCC 10231, *C. albicans* ATCC 90028.

toocol. Briefly, EOs (concentrations at range 6.25% - 0.024%, v/v) were deposited (100 µL) in triplicate in the wells of flat-bottom polystyrene 96-well microplates (Nunc, Denmark). Then, 100 µL of yeast suspension (10^5 CFU/mL in RPMI-1640/0.5% Tween 20) was added. The positive control was a suspension of yeasts in the culture medium, and the negative control was the medium. After 48 h incubation at 35°C, the absorbance at A_{600} (multi-counter Victor 2, Wallac, Finland) was determined. The endpoint was defined as the lowest concentration of the compound resulting in total inhibition (MIC₁₀₀) of yeast growth, compared to the growth in the control wells. The lowest concentration of essential oils fungicidal to $\geq 99.9\%$ of the original inoculum (MFC) was determined parallelly, by subculturing 10 µL from the wells with suspected MIC, 2x, 4x MIC, on the SDA without any antimicrobial agents. No visible colony growth after subsequent 24 - 48 h incubation was accepted as MFC. All experiments were conducted in duplicate.

2.3. GC-FID-MS Analysis of Selected EOs

Essential oils were analyzed using Trace GC Ultra (Thermo Electron Corporation) equipment combined with

DSQ II mass spectrometer and with flame ionization detector (FID) throughout MS-FID Splitter. Analysis was provided using nonpolar chromatography column: Rtx-1 ms (Restek) 60 m length, inner diameter 0.25 mm, film thickness 0.25 μm . Temperature programme: 50°C (3 min), temperature rise 4°C/min; 310°C (10 min); injector temperature 280°C; detector temperature 310°C. Helium was used as a carrier gas which was pressurized to 300 kPa, ionization energy 70 eV, ion source temperature 200°C. Identification of components was based on the comparison of their MS spectra with those in a laboratory-made MS library, commercial libraries (NIST 98.1 and Mass Finder 4) along with the retention indices associated with a series of alkanes with linear interpolation (C8-C26). A quantitative analysis (expressed as percentages of each component) was carried out by peak area normalization measurements without correction factors.

2.4. EOs Killing Kinetics Assay

In time-kill curve studies, four most active essential oils were used against *C. albicans* ATCC 10231 and *C. albicans* ATCC 90028 strains. These are EOs obtained from the following plants: *Cymbopogon citratus* (Citronella oil), *Pelargonium graveolens* (Geranium oil), *Eugenia caryophyllata* (Clove oil), *Melissa citrata indica* (Lemon balm). The inoculum suspension (5×10^5 CFU/mL) was prepared in 10 mL of RPMI-1640 (Cytogen, Poland) with or without EOs in the concentrations range from 1/2 MIC to 2x MICs. At predetermined time points (0, 0.5, 1, 2, 4, 6, 8, 24 and 48 h) of incubation at 35°C, a 100 μL samples were serially diluted in sterile water. Then, each 100 μL aliquot was plated onto SDA plates for CFU counting, after 24 - 48 h incubation at 35°C. The results were reported as the mean percentage of survival \pm standard deviation of four replicates, conducted for each compound twice.

2.5. EOs Carryover Effect

Fungal suspensions (*C. albicans* ATCC 10231 and *C. albicans* ATCC 90028) of approximately 5×10^3 CFU/mL were prepared in RPMI-1640 medium, and 100 μL of each was added to 900 μL of sterile water without or with EO (at MIC to 4x MIC). Immediately after the addition of the fungal suspension to the agent solution, the test tubes were vortexed and 50 μL aliquots were plated on the SDA. Following 48 h of incubation at 35°C, the CFU of *Candida* was determined. The mean colony count at each multiple of the MIC tested was compared with the data for the control. A significant antifungal carryover was defined as $> 25\%$ reduction in CFU in comparison to the control level. The experiments were conducted in duplicate.

2.6. Post-Antifungal Effect (PAFE) of EOs

The PAFE of EOs against *C. albicans* ATCC 10231 and *C. albicans* ATCC 90028 strains was estimated. The inoculum suspensions (5×10^5 CFU/mL) were prepared in 10 mL of RPMI-1640 with or without EO (at MIC and 2x MIC). After 1 h exposure to the EOs, samples were diluted 1:100 in pre-warmed medium to effectively remove the EOs. The diluted cultures were then incubated with agitation (200 rpm) at 35°C. At the desired time points, 100 μL from each sample was serially diluted 10-fold in sterile water, and 100 μL was plated on SDA. Following incubation at 35°C for 48 h, the number of CFU was counted. The PAFE was calculated using the formula: $\text{PAFE} = T - C$, where T represents the time required for the CFU to increase 1 \log_{10} CFU/mL in the test culture above the CFU observed immediately after EO removal, and C represents the time required for the count of the untreated control tube to increase by 1 \log_{10} CFU/mL. The experiments were performed in duplicate.

2.7. Selection of Single-Step EOs-Resistant Mutants

In order to evaluate the frequency of spontaneous single-step mutations of *C. albicans* ATCC 10231 and *C. albicans* ATCC 90028 strains, a fungal suspension containing $\sim 10^{10}$ CFU was plated on SDA plates (24 cm in diameter) containing each EO at concentrations from 1 to 8x MIC. Mutation frequency was expressed as a number of resistant colonies per inoculum. It was calculated by counting the total number of colonies appearing after 7 days of incubation at 35°C on a plate containing EOs and by dividing this number by the total number of CFU plated. The experiment was repeated twice with three plates for each EO concentration.

2.8. *Candida* Susceptibility to Cell Wall Disrupting Agents and Oxidative Stress Tolerance under the Influence of Essential Oils

Fresh suspensions of *C. albicans* ATCC 10231 and *C. albicans* ATCC 90028 in RPMI-1640 medium, prepared from cultures on SDA, were exposed to EOs at MIC for 1 h, 35°C. Other set of suspensions were prepared after yeasts culture (24 h, 35°C) in the presence of 1/2 MIC oils (agar dilution). Control suspensions were left without EOs influence. Examination of *Candida* samples exposed to EOs at MIC, was preceded by washing three times in order to avoid carryover effect. The volume of 5 μL of ready-to-use suspensions with densities of 10^5 , 10^4 , 10^3 cells/mL were spotted on YPG plates containing one of the following cell surface disrupting agents: Calcofluor White (5 $\mu\text{g}/\text{mL}$, 10 $\mu\text{g}/\text{mL}$), sodium dodecyl sulfate (SDS) (0.02%, 0.03%, 0.05%) and Congo Red

(20 µg/mL, 50 µg/mL); all agents were purchased from Sigma, USA; the above concentrations were chosen after preliminary studies. The plates were incubated at 30°C and monitored for *Candida* growth during 3 days. Each of the experiments was performed in triplicate. To test the oxidative stress tolerance, the remainder of *Candida* cell suspensions (10^5), pre-exposed to essential oils or control suspensions, were transferred to Eppendorf tubes in a volume of 1 mL and treated with hydrogen peroxide (12.5, 25 or 50 mM) for 1 h at 35°C. Then, fungal suspensions were diluted (10^5 to 10^3 cells/mL) and spotted (5 µL) onto YPG plates. Their growth was monitored for 3 days and compared to the growth of control cultures not treated with EOs and untreated with hydrogen peroxide.

2.9. Statistical Analysis

If necessary, differences in parameters were tested for significance using “U” Mann-Whitney test and computer program Statistica 5.0.

3. Results

Screening of sixteen essential oils (EOs), listed in **Table 1**, against reference *C. albicans* ATCC 10231 strain has confirmed their known, usually high fungistatic/fungicidal activity. Of the preparations used, five EOs which were most active, and not so well-known when it comes to the scope of our research (Lavender oil, Lemon balm, Citronella oil, Geranium oil, Clove oil), were classified to the second step. In the further experiments, the clinical isolates representing *C. albicans* (n = 20), *C. glabrata* (n = 13), *C. krusei* (n = 6), *C. parapsilosis* (n = 5), *C. tropicalis* (n = 6) species and additional reference strain—*C. albicans* ATCC 90028, have been tested as targets. Similar ranges of MIC (0.024% - 0.39%, v/v) for *C. albicans*, and non-*albicans* clinical strains were noted. However, the fungicidal activity of tested EOs differed,

with respect to *C. albicans* and other species. The minimum fungicidal concentrations (MFCs) for *C. glabrata*, *C. krusei*, and *C. parapsilosis* individual strains varied but usually the concentration ranges were higher than that these active for *C. albicans* (**Table 2**). Then, the time kill curve, carryover, post-antifungal effect, mutant prevention concentrations, the susceptibility of *Candida* reference strains to the cell wall disrupting agents and yeast tolerance to oxidative stress, were evaluated. Clove oil, Geranium oil, Lemon balm and Citronella oil were used for these tests. The results of the experiments on killing kinetics of Lemon balm are shown in **Figure 1**. The fungicidal endpoint (99.9% CFU reduction) for *C. albicans* ATCC 90028 was achieved after 4 h at MIC and after 1 h at 2x MIC, while for *C. albicans* ATCC 10231, respectively, after 6 and 2 h. Similar kinetics of killing was showed by Citronella oil.

Clove oil and Geranium oil revealed a 99.9% fungicidal activity after a longer time (data not shown). Individual essential oils showed a concentration-dependent PAFE, which means inhibiting the re-multiplication of *Candida*. A 1-hour exposure to Lemon balm at MIC resulted in a long PAFE against both strains tested (3 h). This essential oil, when used at 2x MIC, caused PAFE which lasted 10 h against *C. albicans* ATCC 10231 and 5 h against *C. albicans* ATCC 90028. Citronella oil caused similarly strong PAFE, effectively inhibiting regrowth of *C. albicans* ATCC 10231 after 1 h at MIC and after 4 h at 2x MIC. The PAFE against *C. albicans* ATCC 90028 lasted even longer—5 h at MIC and 7 h at 2x MIC. The PAFE of Geranium oil and Clove oil, both used at MIC, was weaker (1 h and 3 h, against *C. albicans* ATCC 10231 and *C. albicans* ATCC 90028, respectively). These two EOs, when used at 2x MIC, caused PAFE which was comparable strong with that of the above described Lemon balm and Citronella oil.

Due to the specific physical and chemical properties of essential oils, such as low density and oiliness, investiga-

Table 2. Fungicidal activity (minimal fungicidal concentrations, MFCs—% v/v) of selected essentials oils: 1—Lavender oil, 2—Lemon balm, 3—Citronella oil, 4—Geranium oil, 5—Clove oil.

<i>Candida</i> strains	MFC range (% v/v)				
	1	2	3	4	5
<i>C. albicans</i> ATCC 10231	0.19	0.097	0.097	0.19	0.19
<i>C. albicans</i> ATCC 90028	0.19	0.097	0.097	0.19	0.19
<i>C. albicans</i> clin., n = 20	0.097 - 0.78	0.048 - 0.19	0.048 - 0.39	0.048 - 0.39	0.048 - 0.78
<i>C. glabrata</i> clin., n = 13	0.048 - 1.56	0.12 - 1.56	0.024 - 1.56	0.024 - 0.78	0.048 - 1.56
<i>C. krusei</i> clin., n = 6	0.39 - 1.56	0.78 - 1.56	0.048 - 1.56	0.019 - 0.78	0.39 - 1.56
<i>C. parapsilosis</i> clin., n = 5	0.048 - 1.56	0.048 - 0.78	0.048 - 1.56	0.097 - 1.56	0.097 - 1.56
<i>C. tropicalis</i> clin., n = 6	0.024 - 0.19	0.048 - 0.097	0.097 - 0.19	0.048 - 0.097	0.097 - 0.39

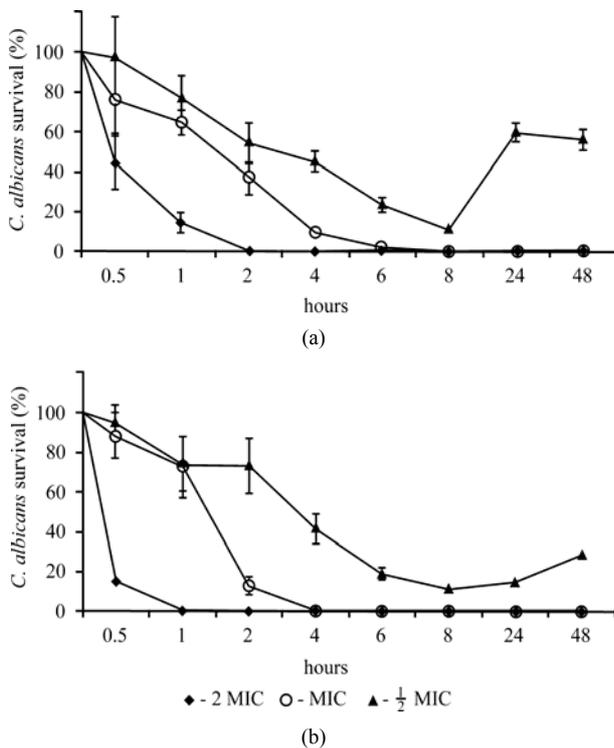


Figure 1. Time- and concentration-dependent effect of Lemon balm on *C. albicans* ATCC 10231. (a) or *C. albicans* ATCC 90028; (b) growth. The presented results are mean (% of yeast survival \pm S. D.) from two independent experiments performed in quadruplicate, according to protocol described in detail in the Material and Methods section.

tion of carryover effect was included in our study. It was evaluated over a range of essential oils concentrations from 1x to 8x MIC. A significant antifungal carryover defined as >25% reduction in CFU/ml compared to the control value was showed for all essential oils tested, however, with a different multiple of the MIC. For Citronella oil and Geranium oil this effect was visualized at a concentration of 4 - 8x MIC, depending on the strain of *Candida*. However, carryover effect of Clove oil and Lemon balm was found at a concentration of 2x MIC. To eliminate the carryover effect of EOs in “PAFE experiments” described above, intensive rinsing of fungal cells, after their short-term exposure to oils was performed. The selection of one-step resistant mutants of *C. albicans* ATCC 10231 and *C. albicans* ATCC 90028 was performed with increased concentrations of each essential oil. Using the inocula total of $\sim 10^{10}$ *Candida* CFU, none of the tested EOs was shown as a selector of mutants at 2x to 8x MIC concentration. The mutation frequency for fungal cultures grown with MIC of EOs was 6.7×10^{-9} only in the case of using Clove oil and Geranium oil against *C. albicans* ATCC 10231. However, these single colonies subcultured on media containing the same concentration of the essential oil did not grow back during

further 7 days of incubation.

C. albicans cells when exposed to essential oils exhibited lower oxidative stress tolerance after treatment with various doses of hydrogen peroxide (12.5, 25 and 50 mM) in comparison to the control cells. This effect was stronger in the case of *C. albicans* ATCC 90028 strain than ATCC 10231, regardless whether we used short-term exposure (1 h) to oils at MIC or long-term exposure (24 h) at half MIC. Among the studied oils the most significant increase in susceptibility to hydrogen peroxide was caused again by Lemon balm (Figure 2). Spot plating of yeasts (previously treated with essential oils) on media containing different concentrations of various cell wall damaging agents resulted in delays and growth reduction. Representative results documenting the increased sensitivity of *C. albicans* ATCC 10231 and ATCC 90028 strains to selected concentrations of Calcofluor White, Congo Red and SDS are shown in Figure 3. They concern the effect caused by the Lemon balm.

The phytochemical analysis of four EOs, tested in details as described above, has been performed by GC-mass spectrometry. A quantitative analysis has been carried out by peak area normalization measurements without correction factors and presented in Table 3, as percentages of each component. Lemon balm contained higher percentage of monoterpene hydrocarbons (10.9%) than Citronella oil (3.5%). The other two oils—Geranium oil and Clove oil do not contain such ingredients. On the other hand, only Clove oil contain phenylpropane fraction, which accounted for 86% of the total compounds. Quantitative analysis and comparison of the composition of the four EOs showed significant differences in the individual components which are the most well-known

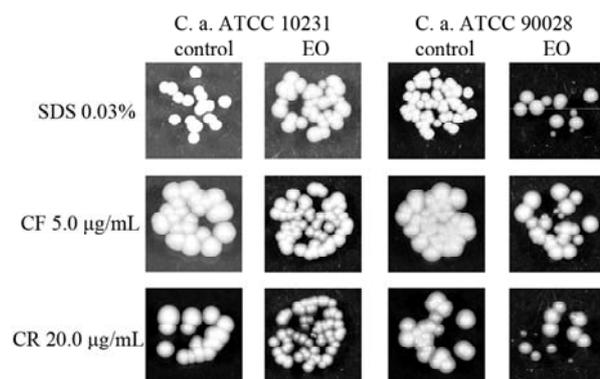


Figure 2. Susceptibility of *C. albicans* ATCC 10231 or *C. albicans* ATCC 90028 to cell wall disrupting agents. First panel-growth on 0.03% SDS containing medium; second panel-growth on Calcofluor White 5 µg/mL containing medium; third panel-growth on 20 µg/mL Congo Red containing medium. Strains were treated with Lemon balm (at MIC, 1 h, 35°C), then diluted (10^5 to 10^3 cells/mL), and spotted on YPG plates containing the cell surface disrupting agents.

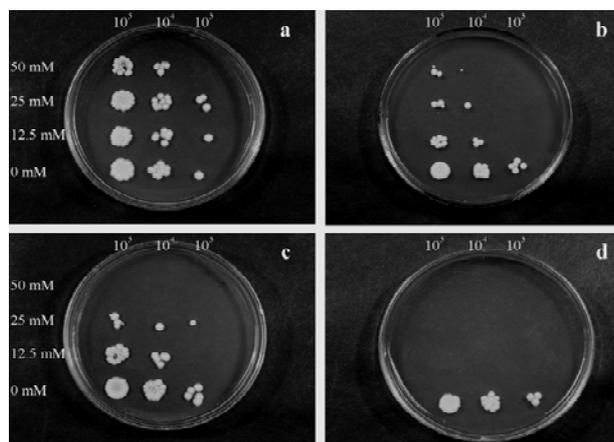


Figure 3. Oxidative stress assay. (a) *C. albicans* ATCC 10231, (c) *C. albicans* ATCC 90028) control plates (yeasts non-treated with Lemon balm); (b) *C. albicans* ATCC 10231, (d) *C. albicans* ATCC 90028 test plates (yeasts treated with MIC of EO, 1 h, 35°C). Then, strains were incubated with different doses of hydrogen peroxide (12.5 - 50 mM) for 1 h, diluted (10^5 to 10^3 cells/ml) and spotted on YPG plates (5 μ L). Yeast cultures, untreated with H_2O_2 , were spotted as controls.

for its antimicrobial activity. The highest concentration found in different oils was, as follows: eugenol (Clove oil, 86.2%), citronellol (Geranium oil, 44.0%); geraniol and citronellal (Citronella oil, 22.4% and 36.2%, respectively) and citral (neral + geranial) (Lemon balm, 8.6% + 10.1%).

4. Discussion

In our view, the most interesting field of world-wide study of plant-derived products is new applications of essential oils (EOs), such as alternative natural anti-fungal substances in some kinds of therapy, for example, topical administration. While the exact mechanism of most of them is still unclear, these herbal products have been widely explored in folk and traditional medicines and as an alternative to conventional chemotherapy. We consider the possibility of using them in the treatment of difficult to heal cancer ulceration, diabetic foot ulcers complicated by fungal, bacterial or mixed-etiology infections. Due to known toxic activity of EOs, their potential usage limited to the topical therapy is reasonable [2, 12-15]. Therefore, the main objective of the present study was firstly to investigate the antifungal effectiveness of EOs (listed in Table 1) and secondly, to establish some not described pharmacological properties, determining the impact of the most active among them, on the physiology of the yeasts. The obtained results indicate that essential oils chosen by us (Lavender oil, Clove oil, Geranium oil, Lemon balm and Citronella oil) showed a similar range of MIC but not MFC against *C. albicans* and non-*albicans* clinical strains, *i.e.* *C. krusei*, *C. gla-*

Table 3. Composition of selected essential oils: 1—Citronella oil, 2—Lemon balm, 3—Geranium oil, 4—Clove oil. RI—retention index, —not identified.

Compound	RI _{lit}	1 2 3 4			
		[%]			
α -Pinene	934	-	0.6	0.6	-
β -Pinene	974	-	3.2	-	-
Limonene	1025	3.5	6.6	-	-
Linalool	1087	0.7	-	5.5	-
Citronellal	1135	36.2	6.2	-	-
<i>iso</i> -Menthone	1146	-	-	7.5	-
Citronellol	1210	14.1	37.0	44.0	-
Neral	1218	0.2	8.6	-	-
Geraniol	1238	22.4	10.2	10.5	-
Geranial	1247	0.2	10.1	0.4	-
Citronellyl formate	1260	-	0.8	9.8	-
Geranyl formate	1284	-	-	2.2	-
Eugenol	1333	-	-	-	86.2
Citronellyl acetate	1335	3.2	t	0.1	-
Geranyl acetate	1363	2.8	0.1	t	-
β -Elemene	1390	2.7	0.2	-	-
(<i>E</i>)- β -Caryophyllene	1421	0.2	2.2	0.9	10.4
Elemol	1540	3.3	-	-	-
Caryophyllene oxide	1572	-	7.4	0.9	0.7
γ - <i>epi</i> -Eudesmol	1618	-	-	4.2	-
monoterpene hydrocarbons	3.5	10.9	-	-	-
oxygenated monoterpenes	81.0	74.2	86.3	-	-
sesquiterpene hydrocarbons	8.8	5.5	3.6	12.7	-
oxygenated sesquiterpenes	5.5	8.1	6.2	0.7	-
phenylpropanes	-	-	-	86.2	-

brata C. tropicalis and *C. parapsilosis* (Table 2).

Unfortunately, it coincides with the increasing resistance of these non-*albicans* species to antifungal drugs. The international surveillance of epidemiology and antifungal resistance of *Candida* spp. by SENTRY Antimicrobial Surveillance Program has found that the emergence of opportunistic pathogenic *Candida* spp. was continuously increasing in the order of *C. albicans* (48.7%) non-*albicans Candida*, *viz.*, *C. parapsilosis* (17.3%), *C. glabrata* (17.2%), *C. tropicalis* (10.9%), *C. krusei* (1.9%) and other *Candida* spp. (4.0%) [1].

Several of the 16 oils tested by us, which were further thoroughly examined (Clove oil, Geranium oil, Lemon balm and Citronella oil), showed strong effect on the cell wall and membrane of *C. albicans*. Furthermore, they caused long-lasting PAF effect and were shown as safe, because they did not generate resistance in one-step assay. Time-kill experiments revealed that all these EOs were fungicidal and acted quickly (starting from 0.5 h of co-incubation). The most effective was essential oil of *Melissa citrata indica* herb (Lemon balm), which caused the quickest decrease in cell density of *C. albicans* ATCC 90028 and less evident effect towards *C. albicans* ATCC 10231. What is more interestingly, a 1-hour exposure of *C. albicans* ATCC 90028 to Lemon balm resulted in the longest PAFE. To our knowledge, this report is the first one describing such effects of essential oils. Furthermore, MPC (mutant prevention concentration) of this oil, tested by single-step methodology was equal to or twice as high as their MICs, thus MSW (mutant selection window) was narrow [16-18].

One can ask a question what is so special in the physicochemical properties of *Melissa* essential oil that it exhibits such high activity against *Candida*. Until recently it was known that extracts and essential oils of *Melissa* sp. are used rather in traditional medicine to treat insomnia, anxiety, gastric conditions, psychiatric conditions, migraines, hypertension and bronchial conditions. Their antimicrobial/antiviral activity, although described, is definitely less known and no specific characterization has been provided, especially concerning anti-*Candida* activity. What was reported earlier the most important identified compounds of Lemon balm showing antimicrobial effects were geraniol, citral, citronellal, and trans-caryophyllene which are also present in the composition of other essential oils with proven antimicrobial activity [19-21]. Since different chemotypes of the same species may grow in the same place, both the composition and antimicrobial activity may differ, even in the case of commercial essential oils. Therefore, it is important that the composition of the essential oil tested has to be given each time [22]. The main components of *Melissa citrata indica* essential oils tested in this study were identified by GC-mass spectrometer analyses. The percentage compositions is listed in **Table 3**. Indeed, citral (neral and geranial) was the main component of Lemon balm and according to many authors these aldehydes show high antimicrobial activity [13,19,22-24]. It is possible, however, that these and other compounds quantity/quality composition is relevant to Lemon balm highest anti-*Candidal* activity. It is known that the efficacy of the whole herb extract/oil may lie on the low doses of the active constituents present in an herbal product altogether. Quantitative analysis and comparison of the composition of the four EOs tested in the present report showed sig-

nificant differences in the individual components which are the most well-known for its antimicrobial activity *i.e.* eugenol, citronellol, geraniol, citronellal, and citral (neral + geranial). Although oil of *Melissa* showed the most desirable properties, the other oils are also worthy of consideration as a potentially useful.

The results of our study concerning the action of Lemon balm and three other selected EOs clearly indicate, that the phenotypic features of *Candida* yeasts that decide about their success as invasive pathogens, were targets for their activity. The fungal cell wall is composed primarily of polysaccharides such as glucans, mannans, chitin and chitosan. It also contains proteins and lipids, which are often associated with these polysaccharides [25]. The knowledge of the participation of *C. albicans* products in the pathogenesis of infections has substantially expanded, but it is still incomplete in many aspects. Nevertheless, it is known that their multilayer, hard permeable cell wall is a strong barrier to the operation of antifungal drugs. Thus, it seems advisable to search for products which could weaken their structure. Essential oils, even if used at low concentrations have the desired characteristic, which has been proved in our study. It is generally assumed that the mechanisms by which the constituents of essential oils inhibit the growth of microorganisms may be partially dependent on their hydrophobicity. It enables them to embed in the cell wall, damage the lipid layer of the cell membrane and mitochondria, impair enzyme systems and exhibit side effects on various proteins [4,5,13,14]. Some of them inhibit microbial growth by causing also a global arrest in protein synthesis or inducing cytoplasm coagulation [13]. Our results have shown that essential oils cause changes in the composition of the cell wall of *Candida*, since its sensitivity to Calcofluor White, Congo Red, or SDS definitely have increased. It has been reported that Calcofluor binds to β -linked fibrillar polymers, interferes with chitin assembly resulting in growth rate reduction, and alters incorporation of mannoproteins into cell wall. Growth inhibition by detergent SDS was connected with an increase in wall porosity, solubilization of the plasma membrane, whereas Congo Red altered glucan synthesis and assembly [4,25,26]. However, by weakening the cell wall by essential oil action, effects of the above cell-wall disrupting agents increased, rendering the cell more susceptible to their lower concentrations, which we have shown in our report (**Figure 2**).

Another question which we asked was if an efficient oxidative stress response of *Candida* will be affected by essential oils action. The response may be of clinical interest, since it is important for *C. albicans* invasion and colonization of host tissues and survival within the host cells (phagocytes) in the course of an *in vivo* infection. *C. albicans* strains show *in vitro* a great natural resistance to

H₂O₂ (10 - 50 mM). It has been reported that various H₂O₂ treatments have distinct effects on antioxidant enzymes (catalase, superoxide dismutase, glutathione oxidase) [27-29]. Therefore, our observation that preincubation of *C. albicans* with essential oils (either short with MIC or longer with 1/2 MIC) decreased tolerance to oxidative stress induced by all concentrations of H₂O₂ used (12.5, 25.0, 50.0 mM) suggests, that the activity of various anti-oxidative enzymes could be decreased. It cannot be ruled out that the architecture of the cell wall proteome might be changed by possibly preventing correct positioning and anchoring of cell wall localized superoxide dismutase or other proteins that are directly or indirectly responsible for countering oxidative stress damage [14,20,27-29]. Our results indicate that some essential oils with antifungal activity, such as Lemon balm, can be considered in the future for more clinical evaluations and possible applications, other than the one currently used. For example, they could be introduced into modern palliative care in patients with cancer fungating wounds or diabetic foot [22,30-33].

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