Enzymatic profile, adhesive and invasive properties of *Candida albicans* under the influence of selected plant essential oils*

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The influence of essential oils (EOs) used at sublethal level, on the presence and intensity of *Candida albicans* virulence factors was evaluated. Minimal inhibitory concentrations (MICs) of Lemon balm, Citronella, Geranium and Clove oils were established as 0.097% (v/v). Using the agar plates with substrates for proteases, phospholipases and hemolysins it was shown that *C. albicans* ATCC 10231 and *C. albicans* ATCC 90028 strains differed in the type and amount of enzymes produced. No significant difference in their total amount could be detected after pretreatment for 24 h with EOs at ½ MIC. However, the short-term (1 h) acting oils at MIC caused a statistically significant reduction in this activity. In the API ZYM test it was demonstrated that both strains exhibited activity of the same 9 out of 19 enzyme types and that EOs caused a significant decrease in the release of some of them. In the presence of subMIC of EOs, or when the fungus had previously been exposed to the MIC of oil, germ tubes formation was significantly and irreversibly reduced. Such *C. albicans* spotted on the Spider agar containing EOs at subMICs were unable to penetrate the agar. A significant decrease in the *C. albicans* adhesion to the fibroblast monolayer with respect to controls was also demonstrated when yeasts had been exposed to EOs at MIC (1 h) in liquid medium. Thus, it has been shown that tested oils, used even at subMIC, exhibit significant activity reducing the presence/quantity of important *C. albicans* virulence factors.

Key words: adhesion, *Candida albicans*, hydrolytic enzymes, germ tubes, invasion, mycelium

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INTRODUCTION

In recent years the prevalence of both local and systemic infections associated with *Candida* sp. and other fungi has increased as a result of the growing number of the immunocompromised patients population. Another reason is that despite the discovery of potent antimycotics, most of such infections remain a serious medical problem because of increasing fungal resistance (Messer *et al.*, 2006; Papon *et al.*, 2013; Tlamçani & Er-rami, 2013; Kabir & Ahmad, 2013). This highlights the need to develop new therapeutic strategies including, for example, a search for agents with novel mechanisms of action which could be used independently or in combination with conventional medicines. Various bioactive molecules obtained from diverse natural resources have been reported as potent antifungal compounds. Traditional medicines for the treatment of infectious diseases are in most cases based on products derived from plants, such as their secondary metabolites — alkaloids, tannins, flavanoids, peptides, glycoproteins, phenolic acids or multicomponent essential oils (Bakkali *et al.*, 2008; Reichling *et al.*, 2009; Alviano &Alviano, 2009; Rajeshkumar & Sundararaman, 2012). Despite the numerous studies showing effects of many of these products against *Candida*, none of them have gained acceptance as a drug. This is mainly because of insufficient data concerning their pharmacological properties, lack of components standardization and, among other things, vague knowledge of the possible side effects of their use. For example, various essential oils (EOs) proved to possess antimycotic properties, and might be potentially used as antifungal agents in medical practice, however, their usually low biocompatibility index is the main obstacle. Clinical studies with EOs are rare. These comprise at the moment only topical administration of products containing EOs in patients suffering from vaginal candidosis, tinea infections, onychomycosis, or in patients with cancer-related infected chronic ulceration (Martin & Ernst, 2004; Warnke *et al.*, 2006). There is some hope for the use of inhalation, but clinical evaluation for its efficacy and safety is needed. In the light of these doubts, essential oils still could be considered only in terms of their possible topical application by using them at the lower concentration than cytotoxic and fungicidal. Is this a reasonable approach? The answer could be yes, as it is evidenced by the fact that currently, one of the most exploited *in vitro* studies is the evaluation of the possibility of using essential oils and other plant-derived products, not directly as biocidal agents, but as products modulating the pathogen's resistance and/or influencing the expression of its virulence factors. *C. albicans* has a number of factors shaping its virulence, such as cell surface-associated adhesins, egzo-enzyme production, germ tube, mycelium and biofilm formation. Only recently the structures in *Candida* cells that enable adhesion to the surface of medical polymers or tissues, as well as factors responsible for endocytosis and for counteracting the oxidative stress generated by immunocompetent cells, have been identified (Hiller *et al.*, 2011; Naglik *et al.*, 2011; Wächtler *et al.*, 2012; Kabir & Ahmad, 2013). Although the knowledge concerning

the participation of particular products of *C. albicans* in the pathogenesis of infections has substantially expanded, it is still incomplete in many aspects and therefore needs to be supplemented. The main objective of the present study is the evaluation of the influence of selected essential oils used in low concentrations on *C. albicans* cells characteristics and the presence of their main virulence factors responsible for invasive properties.

MATERIALS AND METHODS

Essential oils (EOs). The EOs obtained from the following plants: *Melissa citrata indica* (Lemon balm), *Cymbopogon citratus* (Citronella oil), *Pelargonium graveolens* (Geranium oil), *Eugenia caryophyllata* (Clove oil), purchased from Pollena Aroma, Poland were tested. EOs were initially diluted in 96% ethanol (1:1, v/v) and later in RPMI-1640 medium (Cytogen, Poland) with 0.5% Tween 20 (Sigma, USA) as described (Budzyńska *et al.*, 2013). Essential oils were analyzed using Trace GC Ultra (Thermo Electron Corporation) equipment combined with DSQ II mass spectrometer and with flame ionization de- tec- tor (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 the peak area.

Organisms. Reference strains *Candida albicans* ATCC 10231 and *C. albicans* ATCC 90028 purchased from MicroBiologics (Mi, USA) were used. Strains were stored as stock cultures in Sabouraud Dextrose broth (SDB, Difco Laboratories, USA) with 15% glycerol as protective agent, at –75°C. Suspensions of the yeasts in RPMI-1640 medium (usually at a cell density of $1–2 \times 10^6$ cells /ml), used in each experimental procedures were freshly derived from the stock sample, prepared as the 24 h-old cultures, grown at 35°C on Sabouraud Dextrose agar (SDA, Difco Laboratories, USA).

Evaluation of minimal inhibitory concentration (MIC) of essential oils. The MICs of EOs against *C. albicans* ATCC 10231 and *C. albicans* ATCC 90028 strains were determined using the broth microdilution assay according to the guidelines of EUCAST, with minor modification, as described (Budzyńska *et al.*, 2013). The tested EOs concentration range was 0.024–6.25% (v/v). After 48 h incubation of the test system at 35°C, the endpoint was defined as the lowest concentration of each EO resulting in total inhibition (MIC_{95}) of yeast growth, compared to the untreated control, by measuring the absorbance at 600 nm (multi- counter Victor 2, Wallac, Finland). The experiments were conducted in duplicate.

Pre-treatment of *Candida* cells with essential oils. Fresh suspensions of both C. albicans strains were exposed to the action of EOs at MIC for 1 h, 35°C (in RPMI-1640 liquid medium) and then washed three times with PBS in order to avoid 'carryover' effect. Other set of suspensions to be tested were prepared from yeasts cultured for 24 h, 35°C h in the constant presence of ½ MIC of oils, incorporated into SDA plates ("agar dilution"). Control suspensions were left without EOs influence. *Candida* suspensions prepared in this way, if not indicated otherwise, were used in the assays described below conducted in duplicate on three separate occasions.

Overall enzymatic activity of *Candida* in the production of proteases, phospholipases and hemolysins. Effect of essential oils. Phospholipase activity (P_z) was examined using an egg yolk agar (EYA) assay. The test medium contained SDA with the addition of 1M NaCl, 1M $CaCl_2$ and 10% of egg yolk. Proteinase activity (Pr_z) was evaluated with the use of a bovine serum albumin (BSA) assay. The medium consisted of 0.2% BSA, 1.17% glucose, 0.01% yeast extract and 2% agar, which was adjusted to a pH of 5.0. Hemolysin activity (H_z) was assayed on SDA plates supplemented with 7% horse blood. An aliquot (5 μl) of the yeast suspension preincubated with EOs was inoculated onto the plates containing the above substrates and incubated at 37°C for 3 days (phospholipase, proteinase) or for 2 days (hemolysin). Prior to the measurement of proteinase activity, BSA plates were stained with amido black — 0.25% (w/v) in glacial acetic acid 49.75% (v/v), and immediately washed with distilled water. P_z, Pr_z and H_z were expressed as the proportion of *C. albicans* colony diameter compared to the diameter of the precipitation/hemolysis zone ±S.D. According to the applied contractual scale, P_z, Pr_z and H_z values = 1 meant negative reaction, while the values of 0.7–0.99 — represented weak activity; 0.5–0.69 — medium activity and the values of <0.5 — indicated strong secretory activity.

A detailed profile of the enzymes produced by *Candida*. Effect of essential oils. Activity of hydrolytic enzymes was measured by API ZYM (bioMerieux, France) strip tests containing substrates for the detection of 19 hydrolases. The suspensions of *C. albicans* ATCC 10231 and *C. albicans* ATCC 90028 (both pretreated with EOs) prepared in 0.85% saline were inoculated in the cupules of the API ZYM strips. Untreated *C. albicans* strains served as controls. The strips were incubated for 4 h at 37°C and the results were read according to the manufacturer's instructions. Enzymatic activity was determined in nanomoles of the hydrolysed substrate according to the intensity of the color reaction on the scale 1–5, i.e. 1–5 nanomoles, 2–10, 3–20, 4–30, and 5–40 and more nanomoles.

Germ tubes formation. To determine serum-induced filamentation in liquid media and mycelium-like growth of *C. albicans* ATCC 10231, blastoconidia were incubated in a RPMI-1640 medium containing 10% (v/v) of fetal calf serum (RPMI- FCS), without or with the addition of ½ or ¼ MIC of oils. After the following time points: 1, 2, 3, 4, 18 and 48 h, the proportion of germ tubes, hyphae or other forms of cell morphology was evaluated in each culture by a microscopic examination of an aliquot of a culture with a light microscope (ZEISS Primo Star, Germany). Cells were considered as germinated if they had a germ tube at least twice the length of the cell. In another experiment set *C. albicans* strains were exposed to the action of EOs at MIC for 1 h, 35°C (in RPMI-1640 liquid medium) and then washed three times with PBS in order to avoid 'carryover' effect or cultured for 24 h, 35°C h in the constant presence of ½ MIC of oils, incorporated into SDA plates ("agar dilution"). Afterwords, germ tube formation was evaluated as described above. The results of both experiments are expressed as GTF/100 cells (germ tube forming cells per 100 cells counted) ±S.D.

Spider agar-invasive hyphal growth. The test medium consisted of nutrient broth 1% (w/v), mannitol 1% (w/v), K_2HPO_4 0.2% (w/v) and agar 1.35% (w/v) with the addition of essential oils at ½ or ¼ MIC (agar dilution). Spider medium without essential oils served as control. Aliquots (2 µl) of *C. albicans* ATCC 10231 suspensions were spotted onto Spider agar plates and the micromorphology of growing colonies (mycelium formation) was monitored daily during 7 days of incubation at 25°C. The presence of hyphal growth at the colony edges was determined using a stereomicroscope (PZO, Poland) and photographed using a digital camera.

Adhesion of *Candida* to fibroblasts. The influence of essential oils. L929 mouse fibroblasts (ATCC cell line CCL 1, NCTC clone 929) were cultured and passaged in RPMI-1640 medium with L-glutamine and $NaHCO_3$ (Sigma, USA) supplemented with 10% heat inactivated FCS (Cytogen, Poland) and 1% penicillin/streptomycin (Polfa, Poland) at 37°C and 5% CO_2. Prior to the assay, *Candida* were exposed to the action of EOs at MIC for 1 h, 35°C or cultured for 24 h, 35°C h in the constant presence of ½ MIC of oils. For infection, the semi-confluent monolayers in 6-well plates were washed with PBS and 1.5×10^2 *C. albicans* ATCC 10231 cells were added to each well. After 45 min of incubation at 37°C and 5% CO_2, monolayers were washed once with PBS to remove unbound yeasts, and overlayered with melted SDA medium. After 24–48 h incubation at 37°C, *Candida* colonies were counted. The results are shown as a percentage of adherent yeasts (CFU, colony forming units), compared to the control ±S.D.

Statistical analysis. Most of the values are expressed as means ±S.D. When applicable, statistical differences were evaluated using STATISTICA 6.0, (USA). P<0.05 was considered significant.

RESULTS

Minimal inhibitory concentration (MIC) of essential oils: *Melissa citrata indica* (Lemon balm), *Cymbopogon citratus* (Citronella oil), *Pelargonium graveolens* (Geranium oil), *Eugenia caryophyllata* (Clove oil) tested in the broth microdilution assay was 0.097% (v/v). This concentration (MIC), half and one quarter MIC were further used in our experiments.

Enzymes/hemolysins production affected by essential oils

Using the agar plates containing substrates for particular enzymes, such as BSA, egg yolk or horse blood, it was shown that *C. albicans* reference strains tested in the present study differed in the type and amount of enzymes produced. Both *C. albicans* ATCC 10231 and *C. albicans* ATCC 90028 were found to be positive for the production of extracellular proteinases (respectively, $Pr_z = 0.34\pm0.01$; $Pr_z = 0.27\pm0.01$) and hemolysins, although the latter activity was assessed as medium ($H_z = 0.62\pm0.04$; $H_z = 0.57\pm0.03$). However, production of phospholipase occured only in *C. albicans* ATCC 90028 strain — $P_z = 0.44\pm0.02$. No significant difference in enzymatic and hemolytic activity could be detected between the control strains and those pretreated for 24 h with essential oils at ½ MIC, while the short-term (1 h) acting oils at MIC caused a statistically significant reduction in activity (Table 1). Therefore, we checked whether in this case the effect of lowering in cell viability by oils used at inhibitory concentrations occured. The assessment of the CFU number and the trypan blue exclusion assay showed that the viability of the test and control yeast suspension was at the same level (97–99%).

When a semi-quantitative micromethod API ZYM containing substrates for the detection of 19 hydrolases was used, it was demonstrated that both *C. albicans* reference strains exhibit the activity of the same 9 enzyme types, i.e. alcaline phosphatase, esterase (C4), esterase lipase (C8), leucine and valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, and N-acetyl-β-glucosaminidase. Analysis of the enzymatic activity of *Candida* treated with essential oils revealed a statistically significant decrease in the release of alkaline phosphatase, esterase, esterase lipase, leucine arylamidase and naphthyl-AS-BI-phosphohydrolase. In addition, the strongest release of acid phosphatase (40 nM of hydrolyzed substrate) in both control strains were significantly decreased or completely inhibited by EOs action. The activity of α-glucosidase and N-acetyl-β-glucosaminidase, recorded at the level of 20-30 nM in *C. albicans* ATCC 90028 was also significantly reduced. Table 2 presents the effect of Lemon balm used for the pretreatment of *Candida* at ½ MIC (24 h) or at MIC (1 h), on the enzymatic activity of *C. albicans* reference strains.

Table 1. Enzymatic activity of *C. albicans* reference strains (ATCC 10231 and ATCC 90028) pretreated for 1 h with essential oils at MIC.
Tests were performed using SDA plates supplemented with enzyme substrates, such as BSA (proteinases), egg yolk (phospholipases) and horse blood (hemolysins). Results are expressed as Pz, Prz and Hz indexes calculated as the proportion of *C. albicans* colony diameter compared to the diameter of the precipitation or hemolysis zone. According to the applied scale, values Pz, Prz and Hz = 1 means negative reaction, while values of 0.7–0.99 — weak activity; 0.5–0.69 — medium activity and value of <0.5 — means strong secretory activity.

Activity	Control	Lemon balm	Geranium oil	Citronella oil	Clove oil
C. albicans ATCC 10231					
Phospholipase (P_z)	Not active	Not active	Not active	Not active	Not active
Proteinase (Pr_z)	0.34±0.01	0.35±0.01	0.36±0.02	0.36±0.01	0.36±0.01*
Hemolysin (H_z)	0.62±0.04	0.65±0.03	0.66±0.03*	0.68±0.03*	0.63±0.04
C. albicans ATCC 90028					
Phospholipase (P_z)	0.44±0.02	0.65±0.04*	0.47±0.01*	0.53±0.04*	0.48±0.01*
Proteinase (Pr_z)	0.27±0.01	0.3*	0.29±0.01*	0.29*	0.28±0.01*
Hemolysin (H_z)	0.57±0.03	0.69±0.04*	0.64±0.04*	0.68±0.03*	0.67±0.08

*result statistically significant at the significance level p<0.05

Table 2. API ZYM test for *C. albicans* ATCC 10231 (A) and ATCC 90028 (B) enzymatic activity under the influence of essential oils.
Their mode of action towards these strains was as follows: MIC for 1 h [1] or ½ MIC for 24 h [2], details are described in Material and Methods.

A

C. albicans ATCC 10231	Hydrolytic enzymes activity [nmol]								
	E2	E3	E4	E6	E7	E11	E12	E16	E18
Control	10	30	40	30	10	40	30	10	10
Lemon balm [1]	0	5	5	5	0	20	10	0	0
Lemon balm [2]	0	5	5	10	0	0	10	0	0
Geranium oil [1]	0	10	10	10	0	20	10	0	0
Geranium oil [2]	5	5	5	20	0	0	10	0	0
Citronella oil [1]	10	5	10	10	10	20	10	0	0
Citronella oil [2]	0	5	5	30	0	0	20	0	0
Clove oil [1]	0	10	10	10	0	20	10	0	0
Clove oil [2]	0	20	5	10	0	0	0	0	0

B

C. albicans ATCC 90028	Hydrolytic enzymes activity [nmol]								
	E2	E3	E4	E6	E7	E11	E12	E16	E18
Control	20	30	10	30	10	40	20	20	30
Lemon balm [1]	5	5	5	5	0	20	5	0	0
Lemon balm [2]	5	5	5	30	0	0	10	0	0
Geranium oil [1]	5	5	5	5	0	20	5	0	0
Geranium oil [2]	5	10	5	30	0	0	10	0	5
Citronella oil [1]	5	5	5	5	0	20	10	0	0
Citronella oil [2]	5	5	5	40	0	0	5	0	0
Clove oil [1]	5	10	10	30	0	20	10	0	10
Clove oil [2]	5	10	5	30	0	0	5	5	0

E2 — alkaline phosphatase, E3 — esterase (C-4), E4 — esterase lipase (C-8), E6 — leucine arylamidase, E7 — valine arylamidase, E11 — acid phosphatase, E12 — naphthol-AS-BI-phosphohydrolase, E16 — α-glucosidase, E18 — N-acetyl-β-glucosaminidase

Effect of essential oils on the kinetic of the germ tubes formation and a mycelium-like growth

In the constant presence of subMIC (½ or ¼) of tested EOs, germ tubes formation (GTF) by *C. albicans* ATCC 10231 strain was significantly reduced (Fig. 1). The number of GT-positive cells per 100 cells counted after 4 h of co-incubation, dropped from 49.0±2.65 to 1–4, depending on the type of essential oil and its concentration (½ or ¼ MIC). Thus, in the presence of essential oils, the number of GTF cells was reduced by approximately 95–100%, when compared to the control. The most potent in this respect was Lemon balm. Interestingly, lowering the number of GT- positive cells was also observed in the case when the fungus had previously been exposed to the oil, washed out from it and induced to filamentation by being put in an optimal medium (RPMI-FCS) for 4 h. A similar prolonged effect was obtained for both, *C. albicans* cells preincubated for a short time (1 h) with oils at MIC, and yeasts grown prior to GT induction, in the presence of half the MIC for 24 hours (Fig. 2).

The observed reduction in *C. albicans* ability to form filaments by the action of essential oils was irreversible, as verified during further incubation of preincubated yeasts for a total of 18–48 hours. During this time, *C. albicans* control cells formed aggregates (microcolonies) in-terspersed with filaments and true hyphae (mycelium), while yeasts treated earlier with subinhibitory concentrations of oils took a form of short blastospore-budding chains (Fig. 3A, A₁). Also, the morphology of cells after different treatments, observed with a light microscope, indicated that *C. albicans* cells grown in the presence of
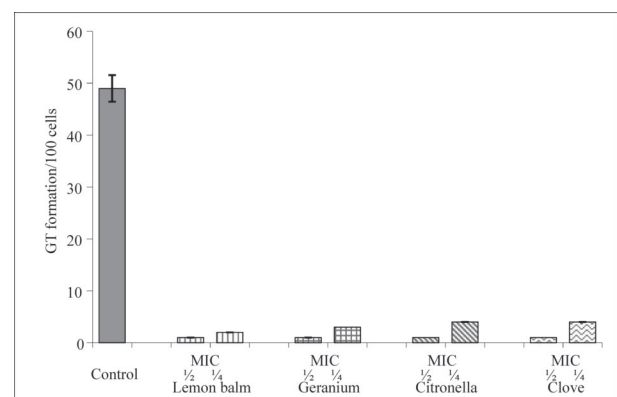

Figure 1. Germ tubes formation (GTF) by *C. albicans* induced by 10% of FCS in RPMI-1640 medium containing ½ or ¼ MIC of EOs, evaluated microscopically after 4 h of co-culture.
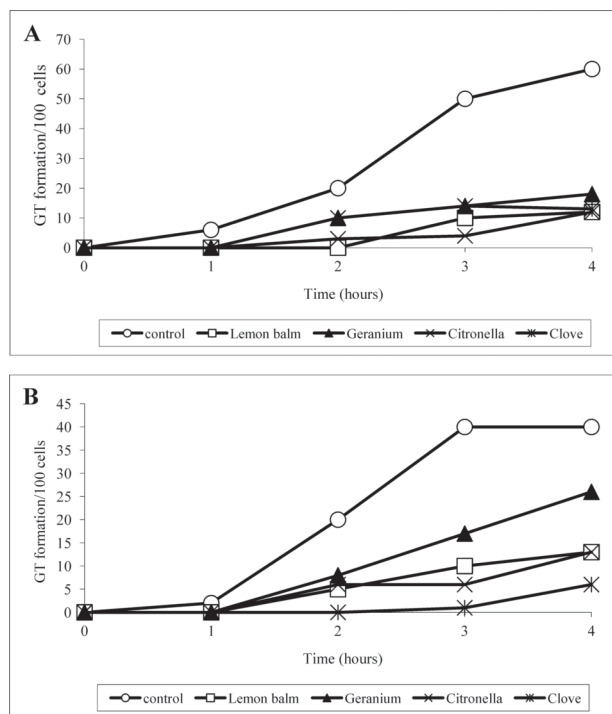The results are expressed as GTF/100 cells ±S.D.

Figure 2. Kinetics of germ tube formation (GTF) evaluated after 1, 2, 3, 4 h of incubation in RPMI-FCS.
The effect of EOs used for pretreatment of yeasts: at MIC for 1 h (**A**); at ½ MIC for 24 h (**B**). Details of experiment schedule is given in Materials and Methods. The results are expressed as GTF/100 cells ± S.D. in each time point.

essential oils had abnormal shapes and smaller sizes. It can be assumed that there was almost a total arrest of the morphological transformation ability, caused by the action of essential oil components.

This effect was correlated with the reduction in the invasive capacity of yeasts, as stated in the test simulating their penetration into tissues. It involved assessing the degree of hyphae invasion of *Candida* to a Spider agar medium evaluated after 7 days of incubation. Filaments
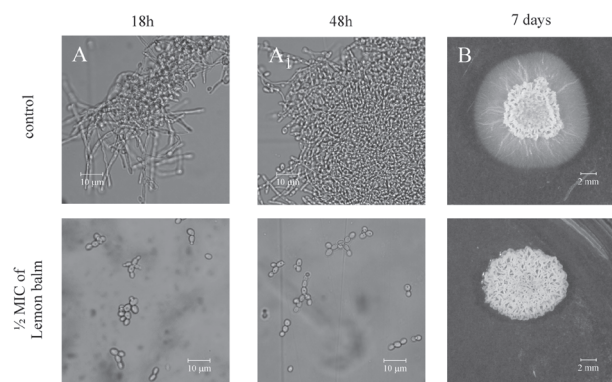

Figure 3. Mycelium formation by *C. albicans* ATCC 10231 in RPMI-10% FCS, in the absence or presence of Lemon balm at ½ MIC, for 18 (A) and 48 h (A₁).
At the indicated time points samples were withdrawn, evaluated microscopically (a light microscope) and photographed at 100× magnification; (**B**) *C. albicans* colonies grown for 7 days at 37°C on Spider medium containing Lemon balm at ½ MIC. The presence or lack of hyphal growth at the colony edges was determined using stereomicroscope and photographed using a digital camera at 12× magnification.
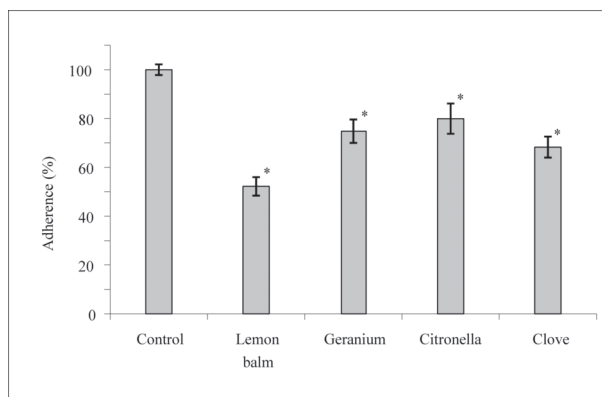

Figure 4. Adherence of *C. albicans* ATCC 10231 to L929 fibroblast monolayer under the influence of EOs (preincubation at MIC, 1 h).
The results are shown as percentage of adherent yeast cells (calculated on the basis of CFU number) ± S.D., comparing to the control which was considered as 100% of adherence, *result statistically significant at the significance level $p < 0.05$.

(hyphal growth), examined using a stereomicroscope, seen at the edges of *Candida* colonies grown without the influence of essential oils, were large and formed an extensive spider branched zone around a dense mass of mycelium, while *C. albicans* spotted on the Spider agar containing individual oils at subMIC were unable to penetrate the agar. Colonies were compact and branching filaments radiating the agar were absent. All oils were similarly effective in this test. An example result for the effect of Lemon balm is shown in Fig. 3B.

Adhesion of *C. albicans* to eukaryotic cells under the influence of essential oils

A statistically significant decrease in the *C. albicans* ATCC 10231 adhesion to the fibroblast monolayer with respect to controls was demonstrated in an experiment where yeasts had been exposed to EOs at MIC (1 h) in liquid medium (Fig. 4). Once again Lemon balm was the most potent (app. 50% inhibition of cell adhesion). When *C. albicans* cells, prior to the adherence assay, were grown in the presence of half the MIC for 24 hours, their adhesion ability was not significantly affected (data not shown).

DISSCUSSION

According to our knowledge, this complex study as a first one describes the pharmacological properties of selected essential oils (EOs) directed towards *Candida albicans*. Although the antifungal activity of several EOs and pure components has been earlier reported, the investigations focused mostly on the determination of their cidal activity. Relatively little research concerned the mechanisms of their action such as impact on the germ tubes/biofilm formation or morphology of a single cell (Hammer *et al.*, 2000; Agarwal *et al.*, 2008; Silva *et al.*, 2011; Carrasco *et al.*, 2012; Khan & Ahmad, 2012). This report includes the results proving the influence of Clove oil, Geranium oil, Lemon balm and Citronella oil on possible mechanisms reported to be relevant for *Candida* pathogenesis, namely germ tube and mycelium formation, adhesive and invasive properties and extracellular production of various enzymes. It has been shown that tested oils, used even at a sublethal concentration (½ MIC), exhibit significant biological activity reducing

the expression/quantity of these virulence factors. In our previous study it was shown that the same oils resulted in concentration- and time-dependent killing and also, that they did not generate resistance in a "one-step resistant mutant" assay. The most efficient one was Lemon balm, which caused the longest "PAFE" (post-antibiotic fungistatic effect), significantly reduced tolerance to oxidative stress induced by H_2O_2 and increased susceptibility to cell-wall damaging agents — Calcofluor White, Congo Red and SDS (Budzyńska et al., 2013). The current study is a continuation of this research. It is aimed at further explaining the mechanisms of essential oils action on Candida cells. Research onto new treatment options effective in combating bacterial or fungal infections, involves a search for substances with different types of activity. They can not only have direct antimicrobial activity, but also exhibit synergistic activity with classic pharmacological agents, restrict the expression of microbial virulence factors, prevent microbial adhesion and aggregation, or activate host immune defense mechanisms designed to combat infections. All essential oils used in our study fulfill the above expectations.

Why are essential oils so interesting? First of all, these volatile, chemically extremely complex products are characterized by a wide antimicrobial activity. Secondly, because of their mode of action affecting several targets at the same time, resistance or adaptation to EOs has been rarely observed. In order to combat infection their hydrophobic constituents contact directly the phospholipid bilayer of the microbial cell membrane, leading to an increase of ion permeability, leakage of vital intracellular constituents or impairment of the bacterial enzyme systems and their respiration, as well as inhibition of protein synthesis and assembly. Their antifungal properties are also related to the ability of the main constituents (terpenes) to pass through the fungal cell wall and settle between fatty acid chains of lipid bilayers, disrupting lipid packaging and altering the structure of the cell membrane (Edris, 2007; Bakkali et al., 2008; Reichling et al., 2009).

Very important and well known virulence factors secreted from the Candida cells are hydrolytic enzymes such as proteases, lipases and phospholipases. These enzymes play a role in nutrition, adhesion to host cells, and tissue destruction resulting in the spread of the pathogen. The most important are Saps (secreted aspartyl proteinases). Saps1-3 are secreted by blastospores, Saps 4–6 are released mainly by filamentous forms, and Sap 9 and 10 are strongly associated with the cell wall of both morphotypes. Protease activity is complemented by the action of phospholipases, which are enzymes responsible for the hydrolysis of one or more ester bonds in the cell membrane glycerophospholipids. Another important virulence factor of C. albicans is the efficient acquisition of iron provided by the action of a family of proteins with hemolytic activity — Rbt5 and mannan (mannoproteins), released from the cell wall (Naglik et al., 2004; Gácser et al., 2007; Meiller et al., 2009; Negri et al., 2010; Sorgo et al., 2013; Deorurkhar & Saini, 2013). In the present study it was demonstrated that enzymatic activity of Candida treated with essential oils was significantly decreased. It concerned both the general pool of enzymes detected by use of agar plates containing substrates such as BSA, egg yolk or horse blood, and the number of individual enzyme type evaluated using the API ZYM. The release of alkaline phosphatase, esterase, esterase lipase, leucine arylamidase and naphthyl-AS-BI-phosphohydrolase, acid phosphatase and N-acetyl-ß-glucosaminidase has been reduced. This observation is important in the light of the

results of other experiments on the formation of filaments. It is assumed that the most enzymatically active parts are apical tips of young hyphal cells which are best suited to adhere to and invade host tissues. Thus, the fact that essential oils almost completely and irreversibly inhibit the formation of germ tubes, followed by the creation of the mycelium, is a very interesting attribute of these products. Yeasts treated earlier with subMIC of the oils took at the tested end-points a form of short blastospore-budding chains. It is an important achievement since young buds might be more susceptible to essential oils and, in vivo to the activity of host immune effector mechanisms (Bakkali et al., 2008). Our observation does not fully corroborate the reports by Hammer et al. (2000), who used for the similar study the essential oil of Malaleuca alternifolia (TTO) and observed a reversible inhibition of the germ tube formation in C. albicans. Thus, our results are more spectacular, also because they correspond to the data obtained in previous studies (Budzyńska et al., 2013), which demonstrated that PAFE lasted up to 8–10 hours after exposure of yeasts to the essential oils.

Taken together, these results suggest that the morphological transformation of C. albicans cells under the influence of essential oils is completely blocked. At this stage of the study, however, we do not know what is the precise mechanism of their action. According to Biswas et al., (2007), Cleary & Saville, (2010) and Thomson et al., (2011) filamentation is controlled by a very complex network of regulatory pathways that converge onto specific transcriptional regulators. Anyway, the capacity of each compound to inhibit germ tube formation could be an important factor to assess its antifungal activity.

As was mentioned earlier, the filamentation process is accompanied by changes in the synthesis of enzymes and other important extracellular products as well as in the expression of adhesins. Indeed, the above observations on the inhibition of some C. albicans properties correspond with the results of other experiments on the influence of EOs on yeasts adhesion to epithelial cells. Strong adhesion of Candida, based primarily on specific interactions of surface receptors with ligands of the host cells, occurs already at the stage of tissue colonization. The most important group of proteins incudes Hwp1 and Eap1p adhesins belonging to Als family. The protein which plays a key role in multiple processes which are necessary for C. albicans to colonize a host is Als3. It is expressed only on pseudohyphae and hyphae but not on blastospores (Liu & Filler, 2011). Since the expression of adhesins is largely dependent on morphological forms of Candida, the obtained results suggest that the principal point of essential oils action occurs in the early stages of morphogenesis. It is evidenced by the fact that inhibition of germ tube formation correlates with decreased adhesion to epithelial cells (Fig. 4.). However, it must be noted that C. albicans, having numerous types of adhesins, has not completely lost the adhesion ability.

In our study, essential oils even if used at low concentrations, have been proved to possess desired antifungal characteristics. Our attempt to clarify some of the mechanisms of their action represents a modern approach to observations drawn from ethnomedicine. Essential oils from Melissa citrata indica (Lemon balm), Cymbopogon citratus (Citronella oil), Pelargonium graveolens (Geranium oil), Eugenia caryophyllata (Clove oil) tested in this study had a negative impact on the pathogenicity of C. albicans dependent upon the production of enzymes, decreased the expression of adhesins and blocked yeast-hyphae transition.

Conflict of Interest

The authors have declared that there is no conflict of interest.

Acknowledgements

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