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ORIGINAL ARTICLE

Improved efficacy of eugenol and *trans*-anethole in combination with octenidine dihydrochloride against *Candida albicans* and *Candida parapsilosis*

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Abstract

Introduction and Objective. Candidiasis is a fungal infection caused by yeasts from the Ogenus Candida. Considering increasing antifungal resistance rates the activity was analyzed of natural compounds to eradicate *Candida* spp. The aim of the study was to check the antifungal activity of selected essential oil compounds (EOCs; thymol, menthol, eugenol [E], carvacrol, trans-anethole [TA]) alone, and in combination with octenidine dihydrochloride (OCT) against *C. albicans* and *C. parapsilosis* reference, and clinical strains.

Materials and method. Investigated clinical isolates were obtained from skin wounds of patients treated for superficial wounds candidiasis. The following parameters were studied: antifungal susceptibility testing using the VITEK system, antifungal activity of EOCs alone and in combination with OCT using microdilution and checkerboard assays, antifungal efficacy of selected chemicals using time-kill curve assay, and changes in cell permeability in the presence of selected chemicals using crystal violet assay.

Results. Clinical isolates of *C. albicans* and *C. parapsilosis* were resistant to fluconazole and voriconazole. The highest inhibition activity against Candida isolates was observed for E. The OCT – TA and OCT – E combinations showed synergistic and additive activities against all strains, respectively. These combinations also appeared to affect the rate of yeast cell killing and increasing the permeability of Candida cells.

Conclusions. The study indicates that E and TA potentially used in formulation with OCT might eradicate pathogenic yeasts; however, microbiological and clinical studies are still required.

Key words
Candida, eugenol, trans-anethole, octenidine dihydrochloride, synergistic activity

INTRODUCTION

Candidiasis, a fungal infection caused by yeasts from the genus *Candida*. *Candida albicans*, is the most common cause of fungal infections worldwide [1]. There are over 20 species of *Candida* spp. that can cause infection in human. Of this number, *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* are the five most common. Most infections are induced by yeast of the *Candida* genus which were previously present in the body (endogenous infections), and disturbance of the balance between yeasts and host contributes to the disease process [2].

The pathogenesis of candidiasis depends on virulence factors of the fungus which allow colonization and invasion of the tissues, as well as enabling avoidance of the host immune responses. *C. albicans* virulence factors include: complexity of cell wall structure, adhesion, pleomorphism, enzymatic activity, molecular mimicry, and phenotypic variation [3]. The ability of yeasts to form biofilms and secreted aspartyl proteinases make the treatment of patients with *Candida* spp. infections difficult. Biofilm formation increases both resistance to antifungal drugs and host immune response, while secreted aspartyl proteinases (SAP) – a degradative enzyme – enables and facilitates invasion [4]. Cutaneous

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candidiasis can occur in several clinical forms. *Candida* species are the secondary factors of dermatomycoses [5]. The most common form of yeast dermatitis is candidal intertrigo (candidosis in tertriginosa, intertrigo candidamycetica), which occurs in the skin folds, particularly in obese people, whereas candidal folliculitis (folliculitis candida – cetica) is more common in people with reduced immunity. Cutaneous candidiasis can be effectively treated with topical azoles and polyenes, including clotrimazole, miconazole and nystatin [6]. Local treatment of nail infections caused by *Candida* spp. is usually ineffective. In typical cases, oral terbinafine or itraconazole are used rather than oral griseofulvin as they appear to be more effective [7]. Terbinafine has a short and variable activity, which is confirmed by clinical trials. On the other hand, itraconazole seems to be effective, but treatment may take as long as 3 – 4 months [8]. *Candida* spp. sensitivity to antifungal drugs can be predicted on the basis of knowledge of the species; however, sensitivity of individual isolates may differ. Resistance to azoles and echinocandins is an important property.

Pfaller et al. [8] reported data for fluconazole and voriconazole susceptibility – 90.2% of the *Candida* spp. isolates were found to be susceptible to fluconazole; however, reduced fluconazole susceptibility (defined as <75.0% susceptible) was observed in 13 of 31 species. Resistance to fluconazole was shown to increase for *C. parapsilosis*, *C. guilliermondii*, *C. lusitaniae*, *C. sake* and *C. pelliculosa* throughout the whole period of time. Fluconazole resistance was found in isolates of *C. guilliermondii*, *C. inconspicua*, *C. rugosa* and *C. norvegensis*. Resistance to voriconazole was generally uncommon during the study. One-third of fluconazole-resistant isolates of *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. rugosa*, *C. lipolytica*, *C. pelliculosa*, *C. apicola*, *C. haemulonii*, *C. humicola*, *C. lambica* and *C. ciferrii* stayed susceptible to voriconazole.

Resistance to echinocandins is generally uncommon, with incidence rates ranging from 0 – 1.7%. In total, 38% of echinocandin-resistant *C. glabrata* isolates also appear to be resistant to fluconazole [9]. *C. albicans*, *C. tropicalis* and *C. parapsilosis* isolates are hardly ever resistant to amphotericin B, but *C. lusitaniae* strains often showed clinically significant resistance to this antifungal drug. Some data indicate that this drug should be applied at maximum doses in *C. glabrata*- and *C. krusei*-related infections [10]. However, with regards to other classes of antimicrobial drugs, there is growing awareness of increasing resistance to antifungal drugs. Administration of higher doses, prolonged therapy and a need for booster therapy generate further health problems for patients and, on the other hand, lead to increased resistance. For this reason, great attention is paid to healthy prebiotic food and a healthy lifestyle, with probiotics being recommended [11]. Due to recurrence of wounds and ulcers, which makes treatment difficult, researchers seek supporting treatment. Professional literature shows the benefits of probiotics, as well as essential oils or their compounds [12, 13]. Apart from possessing strong antimicrobial properties, probiotics are characterized by immunostimulatory, anti-inflammatory and analgesic properties, which are their greatest assets [14].

OBJECTIVE

The aim of the study was to check the antifungal activity of selected essential oil compounds (EOCs; thymol, menthol, eugenol [E], carvacrol, *trans*-anethole [TA]) alone, and in combination with octenidine dihydrochloride (OCT) against *C. albicans* and *C. parapsilosis* reference and clinical strains. Isolates were obtained from skin wounds of patients receiving treatment for superficial candidiasis.

MATERIALS AND METHOD

Characteristics of yeasts. Yeast strains investigated in this study were isolated from the skin wounds of patients treated for superficial candidiasis. Sabouraud Gentamicin Chloramphenicol 2 agar (SGC2, bioMérieux, Warsaw, Poland) was used for culturing yeast strains. The cultivation was carried out for 48 h at 37 °C under aerobic conditions. All chosen yeast isolates were identified with VITEK 2 YST ID Card (bioMérieux, Warsaw, Poland), which confirmed their affiliation with *C. albicans* and *C. parapsilosis* species. Susceptibility to recommended antimycotic agents (amphotericin B, capsofungin, fluconazole, flucytosine, micafungin, voriconazole), expressed by the value of the MIC of yeast strains, was investigated with AST-YS08 ID Card (bioMérieux, Warsaw, Poland), as recommended by the manufacturer. According to the references, the clinical *C. albicans* and *C. parapsilosis* isolates were resistant to fluconazole and voriconazole with MIC = 8 mg/L [15]. *C. albicans* ATCC 10231 and *C. parapsilosis* ATCC 22019 strains were used as the control. The strains were obtained from the collection of the Chair of Microbiology, Immunology and Laboratory Medicine at the Pomeranian Medical University in Szczecin, Poland. The study was approved by the Bioethics Committee of the Medical University in Łódź, Poland (Approval No. RNN/360/18/KE).

Chemicals. Concentrations of the following EOCs: thymol (≥98.5% purity, Sigma-Aldrich, Darmstadt, Germany), eugenol (E; >98 % purity, Ernesto Ventos S.A, Barcelona, Spain), carvacrol (98% purity, Sigma-Aldrich, Darmstadt, Germany), and *trans*-anethole (TA; 99% purity, Sigma-Aldrich, Darmstadt, Germany) from 500 – 0.12 µL/mL were dissolved in Tween 80 (Sigma-Aldrich, Darmstadt, Germany) (1%, *v/v*) and diluted with Sabouraud Dextrose Broth (SDB; Sigma-Aldrich, Darmstadt, Germany).

Concentrations of octenidine dihydrochloride (OCT; 98.0% purity, Schülke & Mayr GmbH, Norderstedt, Germany) from 500 – 0.12 µg/mL were prepared by dissolving the substance in DMSO (Loba Chemie, Mumbai, India) (2%, *v/v*) and diluting it using SDB.

Determination of minimum inhibitory concentration (MIC) of chemicals. Susceptibility testing for chemicals against *C. albicans* and *C. parapsilosis* strains expressed by MIC values was determined with the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI document M27-A3) [16]. The final concentrations of 1% (*v/v*) autoclaved Tween 80 (for EOCs) [17] and 2% (*v/v*) autoclaved DMSO (for OCT) were added into the SDB medium to enhance solubility. Then, two-fold dilutions (1,000 µg/mL – 2.44 µg/mL for OCT, and 500 – 0.02 µL/mL

for EOCs) were performed. In brief, each well contained 50 µL of OCT or EOCs and 50 µL of yeast suspension at the final concentration of 1×10^3 – 5×10^3 CFU/mL. All assays were performed in duplicate. The MIC values were assessed after 24 h of incubation at 37 °C. Control tests with SDB and SDB supplemented with Tween 80 (1%, *v/v*) and DMSO (2%, *v/v*) were performed. The final result of MIC was expressed in mg/mL based on the EOCs density.

Checkerboard method. The method of investigation has been described previously [18]. Briefly, two-fold dilutions (1,000 µg/mL – 2.44 µg/mL for OCT, and 500 – 0.02 µL/mL for EOCs) were performed. Finally, each well contained the following compounds: 25 µL of the appropriate concentration of EOCs, 25 µL of OCT concentration and 50 µL of yeast suspension with final concentration of 1×10^3 – 5×10^3 CFU/mL. The plates were incubated for 24 h at 37 °C. All assessments were performed in duplicate. MIC of both OCT and EOCs in combination was determined. The combined effects of chemicals were calculated and expressed in the fractional inhibitory concentration index (FICI), using the following formulas:

$$FIC = \frac{MIC\ of\ EOC\ and\ OCT\ in\ combination}{MIC\ of\ EOC\ or\ OCT\ alone}$$

$$FICI = FIC\ of\ EOC + FIC\ of\ OCT$$

Interpretation of results: synergy (FICI < 0.5), addition ($0.5 \le FICI \le 1.0$), indifference ($1.1 < FICI \le 4.0$) or antagonism ($FICI > 4.0$) [18].

Time-kill curve assay. Killing kinetics was assessed by a time-dependent killing assay according to Kang et al. [19] with a slight modification. Media (25 mL) were inoculated with C. albicans and C. parapsilosis to obtain bacterial cells concentrations of 0.5 on the McFarland scale, and incubated at 37 °C under shaking conditions (100 rpm). 100-µL samples were removed from the cultures at 0, 1, 2, 3, 4, 5, and 6 h, which were then subsequently diluted, spread on SGC2 plates and incubated for 24 h at 37 °C. The number of viable cells was determined by counting the formed colonies. Time-kill curves were constructed by plotting the mean colony counts (log10 CFU/mL) versus the time.

Changes in cell permeability – crystal violet assay. Alteration in cell permeability was detected using a crystal violet assay [20, 21]. OCT, E and TA were tested alone and in combination (OCT – E, OCT – TA). The following concentrations of chemicals were used: 0.1% (v/v); 0.5% (v/v); and 1.0% (v/v). The percentage of crystal violet uptake was calculated using the following formula:

$$Crystal violet uptake\ (\%) = \frac{(OD_{550}\ values of crystal\ violet - OD_{550}\ values of sample)}{OD_{550}\ values of crystal violet solution} \times 100$$

RESULTS

Antifungal effects of chemicals alone and in combination. It was shown that all strains were susceptible to tested EOCs and OCT. The highest and lowest inhibitory activity of EOCs against all strains was observed for carvacrol (Minimum inhibitory concentration (MIC) = 0.95 ± 0.00 mg/mL) and TA (MIC = 247.00 ± 0.00 mg/mL – 494.00 ± 0.00 mg/mL), respectively. The obtained MIC value for OCT against all strains was 3.91 ± 0.00 mg/mL.

It was noted that OCT – TA (FICI = 0.16 – 0.38) and OCT – E (FICI = 0.75 – 1.00) combinations showed synergistic and additive activities against all strains, respectively. Indifferent effect was found in OCT – thymol (FICI = 2.00 – 4.00), OCT – menthol (FICI = 3.00), and OCT – carvacrol (FICI = 3.00 – 4.00) combinations.

It was also observed that the addition of surfactants (Tween 80 and dimethyl sulfoxide [DMSO]) had no influence on the growth of any of the yeast strains. Results of MICs of EOCs and OCT alone and in combination against Candida spp. strains are summarized in Table 1.

Antifungal efficacy testing results. The time-kill kinetics profile for strains under OCT, E, TA, OCT – E, and OCT – TA influence is shown in Figure 1. Due to the indifference effect of OCT – thymol, OCT – menthol, and OCT – carvacrol combinations in the checkerboard assay against Candida strains, these EOCs were not considered in this assay.

It has been proved that the OCT – E combination showed the best antifungal efficacy against all tested strains; samples removed from culture at 0 h during incubation showed no growth. Furthermore, the OCT – TA combination also was effective for all yeast cell cultures. All strains cultured in medium supplemented with subinhibitory concentration (MIC_{50}) of OCT and MIC_{50} of TA showed a reduced number of viable cells within the first 1 h of incubation, compared to the medium supplemented with MIC_{50} of TA or MIC_{50} of OCT. Effects of DMSO and Tween 80 were also analyzed and no differences in antifungal efficacy results were found (data not shown).

Results of membrane permeability in Candida cells. Changes in the membrane permeability of Candida cells under OCT, E, TA, OCT – E, and OCT – TA influence are presented in Figure 2. Due to the indifference effect of the OCT – thymol, OCT – menthol, and OCT – carvacrol combination in the checkerboard assay against Candida strains, these EOCs were not considered in this test.

The uptake of crystal violet by C. albicans ATCC 10231 and C. parapsilosis ATCC 22019 and tested clinical isolates in the absence of OCT, E, and TA were used as the baseline reading. The percentage of crystal violet uptake reflected cell membrane permeability of tested strains.

In comparison to the untreated cells, it was found that different concentration of OCT, E, TA, and OCT – E, OCT – TA combinations increased crystal violet uptake by *C. albicans* and *C. parapsilosis* strains. Nevertheless, the OCT (1.0%, *v/v*) – E (1.0%, *v/v*) combination was the most effective. In this case, the increase of the crystal violet uptake ranged from 65.6 – 90.6% (mean = 78.1%) and from 30.9 – 37.1% (mean = 34.0%) for *C. albicans* and *C. parapsilosis* strains, respectively. Interestingly, despite the synergistic effect obtained between OCT and TA against all strains, crystal violet uptake results of the OCT (1.0%, *v/v*) – TA (1.0%, *v/v*) combination were lower than for the OCT (1.0%, *v/v*) – E (1.0%, *v/v*) combination. In this case, the increase of the crystal violet uptake ranged from 25.7 – 27.1% (mean = 26.4%) and from 24.2 – 28.3% (mean = 26.3%) for *C. albicans* and *C. parapsilosis* strains, respectively.

Table 1. Minimum inhibitory concentration (MIC) of essential oil compounds (EOCs) alone and in combination with octenidine dihydrochloride (OCT) against *Candida albicans* and *Candida parapsilosis* reference strains and clinical isolates obtained from skin wounds

Strains	OCT – EOCs	MIC_o	MIC_c	FIC	FICI	Type of interaction
C. albicans ATCC 10231	OCT – thymol					
	OCT (µg/mL)	3.91 ± 0.00	7.81 ± 0.00	2.0	4.0	I
	Thymol (mg/mL)	1.25 ± 0.00	2.50 ± 0.00	2.0		
	OCT – menthol					
	OCT (µg/mL)	3.91 ± 0.00	7.81 ± 0.00	2.0	3.0	I
	Menthol (mg/mL)	20.00 ± 0.00	20.00 ± 0.00	1.0		
	OCT – eugenol					
	OCT (µg/mL)	3.91 ± 0.00	1.95 ± 0.00	0.5	0.75	A
	Eugenol (mg/mL)	4.18 ± 0.00	1.04 ± 0.00	0.25		
	OCT – carvacrol					
	OCT (µg/mL)	3.91 ± 0.00	7.81 ± 0.00	2.0	4.0	I
	Carvacrol (mg/mL)	0.95 ± 0.00	1.91 ± 0.00	2.0		
	OCT – *trans*-anethole					
	OCT (µg/mL)	3.91 ± 0.00	0.98 ± 0.00	0.25	0.38	S
	trans-Anethole (mg/mL)	494.00 ± 0.00	61.75 ± 0.00	0.13		
C. albicans isolate	OCT – thymol					
	OCT (µg/mL)	3.91 ± 0.00	3.91 ± 0.00	1.0	3.0	I
	Thymol (mg/mL)	2.50 ± 0.00	5.00 ± 0.00	2.0		
	OCT – menthol					
	OCT (µg/mL)	3.91 ± 0.00	7.81 ± 0.00	2.0	3.0	I
	Menthol (mg/mL)	20.00 ± 0.00	20.00 ± 0.00	1.0		
	OCT – eugenol					
	OCT (µg/mL)	3.91 ± 0.00	1.95 ± 0.00	0.5	1.0	A
	Eugenol (mg/mL)	4.18 ± 0.00	2.09 ± 0.00	0.5		
	OCT – carvacrol					
	OCT (µg/mL)	3.91 ± 0.00	7.81 ± 0.00	2.00	4.0	I
	Carvacrol (mg/mL)	0.95 ± 0.00	1.91 ± 0.00	2.0		
	OCT – *trans*-anethole					
	OCT (µg/mL)	3.91 ± 0.00	0.98 ± 0.00	0.25	0.38	S
	trans-Anethole (mg/mL)	494.00 ± 0.00	61.75 ± 0.00	0.13		

Strains	OCT – EOCs	MIC_o	MIC_c	FIC	FICI	Type of interaction
C. parapsilosis ATCC 22019	OCT – thymol					
	OCT (µg/mL)	3.91 ± 0.00	3.91 ± 0.00	1.0	2.0	I
	Thymol (mg/mL)	1.25 ± 0.00	1.25 ± 0.00	1.0		
	OCT – menthol					
	OCT (µg/mL)	3.91 ± 0.00	7.81 ± 0.00	2.0	3.0	I
	Menthol (mg/mL)	20.00 ± 0.00	20.00 ± 0.00	1.0		
	OCT – eugenol					
	OCT (µg/mL)	3.91 ± 0.00	1.95 ± 0.00	0.5	1.0	A
	Eugenol (mg/mL)	4.18 ± 0.00	2.09 ± 0.00	0.5		
	OCT – carvacrol					
	OCT (µg/mL)	3.91 ± 0.00	7.81 ± 0.00	2.0	4.0	I
	Carvacrol (mg/mL)	0.95 ± 0.00	1.91 ± 0.00	2.0		
	OCT – *trans*-anethole					
	OCT (µg/mL)	3.91 ± 0.00	0.49 ± 0.00	0.13	0.19	S
	trans-Anethole (mg/mL)	247.00 ± 0.00	15.44 ± 0.00	0.06		
C. parapsilosis isolate	OCT – thymol					
	OCT (µg/mL)	3.91 ± 0.00	7.81 ± 0.00	2.0	3.0	I
	Thymol (mg/mL)	1.25 ± 0.00	1.25 ± 0.00	1.0		
	OCT – menthol					
	OCT (µg/mL)	3.91 ± 0.00	7.81 ± 0.00	2.0	3.0	I
	Menthol (mg/mL)	20.00 ± 0.00	20.00 ± 0.00	1.0		
	OCT – eugenol					
	OCT (µg/mL)	3.91 ± 0.00	1.95 ± 0.00	0.5	1.0	A
	Eugenol (mg/mL)	2.09 ± 0.00	1.04 ± 0.00	0.5		
	OCT – carvacrol					
	OCT (µg/mL)	3.91 ± 0.00	3.91 ± 0.00	1.0	3.0	I
	Carvacrol (mg/mL)	0.95 ± 0.00	1.91 ± 0.00	2.0		
	OCT – *trans*-anethole					
	OCT (µg/mL)	3.91 ± 0.00	0.49 ± 0.00	0.13	0.16	S
	trans-Anethole (mg/mL)	494.00 ± 0.00	15.44 ± 0.00	0.03		

I – indifference; A – additive; S – synergy

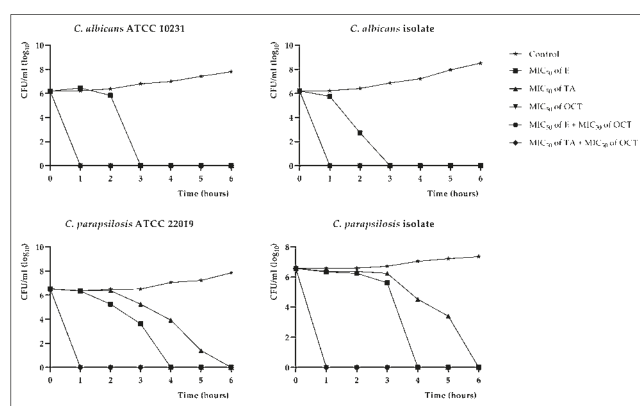

Figure 1. Antifungal efficacy of subinhibitory concentration (MIC50) of eugenol (E) and MIC50 of trans-anethole (TA) alone and in combination with MIC50 of octenidine dihydrochloride (OCT) against Candida strains

These observations indicate that both OCT – E and OCT – TA combinations had the ability to increase cell permeability of *C. albicans* and *C. parapsilosis* reference and clinical strains. The higher intake of the crystal violet dye indicated greater damage to the cells.

DISCUSSION

Among commensals of human skin, *Malassezia*, *Cryptococcus*, *Rhodotorula*, and *Candida* genera were identified. Although fungi are part of commensal skin microbiota, various species are also pathogenic, among which *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. orthopsilosis* are the most commonly known *Candida* species skin pathogens [5]. Fungal skin infections may affect up to about 25% of the population [22].

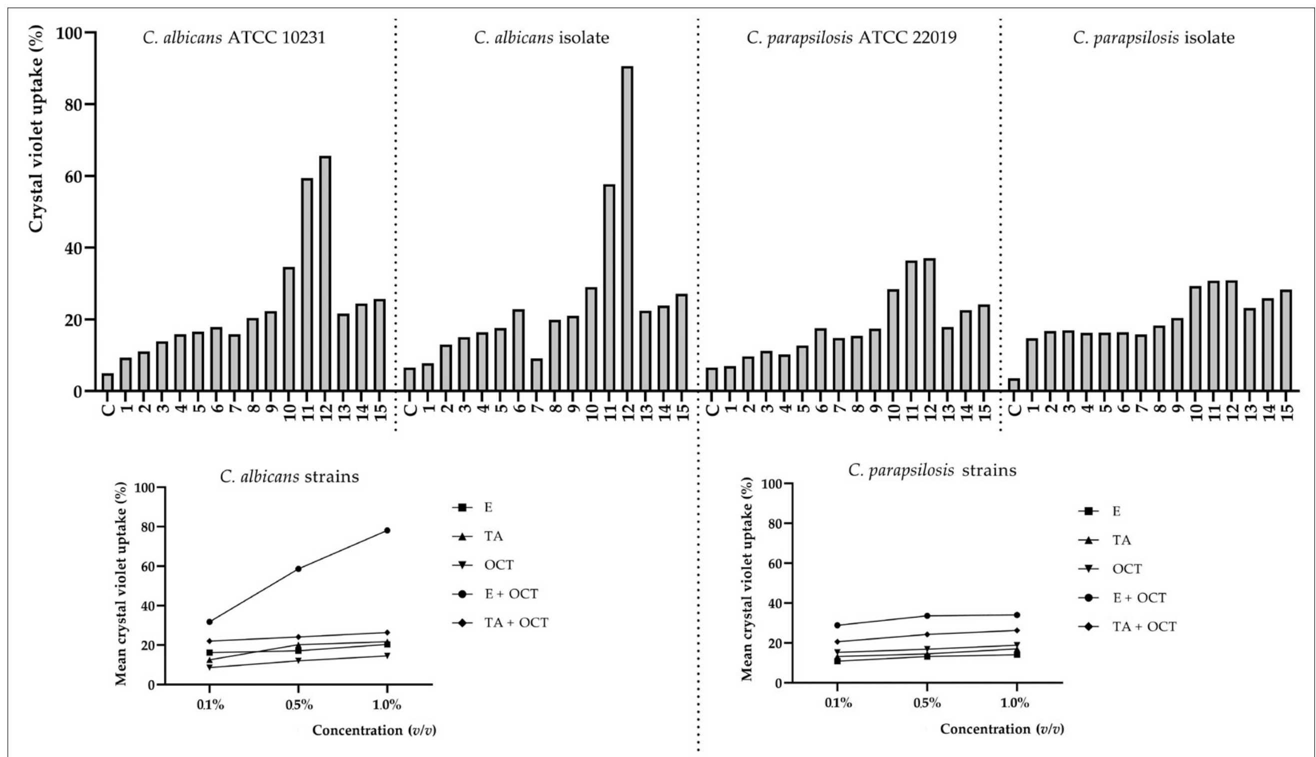

Figure 2. Changes in membrane permeability of Candida spp. cells untreated (C – control) and treated with: 1) octenidine dihydrochloride (OCT, 0.1%, v/v); 2) OCT (0.5%, v/v); 3) OCT (1.0%, v/v); 4) eugenol (E, 0.1%, v/v); 5) E (0.5%, v/v); 6) E (1.0%, v/v); 7) trans-anethole (TA, 0.1%, v/v); 8) TA (0.5%, v/v); 9) TA (1.0%, v/v); 10) OCT (0.1%, v/v) + E (0.1%, v/v); 11) OCT (0.5%, v/v) + E (0.5%, v/v); 12) OCT (1.0%, v/v) + E (1.0%, v/v); 13) OCT (0.1%, v/v) + TA (0.1%, v/v); 14) OCT (0.5%, v/v) + TA (0.5%, v/v); 15) OCT (1.0%, v/v) + TA (1.0%, v/v)

Candida infections are very often concomitant with common diseases, such as diabetes, immunodeficiency, cancer, or diseases of the digestive system associated with disorders of microbiome. Application of broad-spectrum antibiotics, performing blood transfusion, *Candida* spp. colonization, use of central venous catheter, and total parenteral nutrition are other risk factors [23]. Treatment of candidal infections becomes difficult due to the growing resistance of yeasts to drugs. A search for new, active compounds is also a challenge as these should effectively combat eukaryotic yeast cells and demonstrate hardly any toxicity to human body. *Candida* spp. can live in structure biofilm, which is associated with phenotypic expression of resistance genes, particularly those encoding efflux pumps, and presence of persister cells which play a big role. Point mutations in *ERG11* is one mechanism of resistance to fluconazole identified in *C. albicans*.. At least one mutation that resulted in amino acid substitutions was found within *ERG11* alleles., while the increased expression of *ERG11* is causes by mutations in the gene encoding the zinc-cluster transcriptional regulator Upc2p in *C. albicans* [24]. Another mechanism of fluconazole resistance in *C. albicans* is associated with over-expression of drug efflux pumps Mdr1p and Cdr1p/Cdr2p [25]. A relationship is also suspected between *CDR1* and *ERG11* and fluconazole resistance in *C. parapsilosis,* which is associated with the presence of fluconazole-resistant isolates in a single homozygous SNP (A395T) in *MMR1,* which corresponds to Y132F amino acid substitution. Moreover, this substitution can also be responsible for resistance to voriconazole of fluconazole-resistant strains [26]. Due to the spread of azole-resistant yeasts, especially in hospital settings, researchers are looking not only for effective antifungal drugs, but also compounds or formulations preventing the spread of resistant strains of yeasts, especially in medical care facilities.

Biocides such as benzalkonium chloride, chlorhexidine digluconate or sodium hypochlorite are antimicrobial chemicals applied to protect surfaces in hospitals conditions [27]. Effective application of disinfection is important in the prevention and control of hospital infections; however, prolonged use of disinfectants can also lead to resistance, with some microorganisms – including *Candida* spp., – showing biocide resistance [28]. Mechanisms of fungal resistance to antimicrobial chemicals are believed to be similar to those of fungi demonstrating against antifungal drugs. These mechanisms can be divided into acquired and intrinsic mechanisms [29]. Acquired resistance was found to be associated with the mutation or collection of genetic materials that allow cells to survive in harmful conditions. Intrinsic mechanisms include natural properties of a fungal cell, such as decreased permeability, stimulated activity of efflux pumps, repair mechanisms, phenotypic modulation, and inactivation of biocides [30]. Cuellar-Rufino et al. [31] reported that *C. albicans and C. glabrata* lacking catalase gene (*cta1*) and superoxide dismutase 1 and 2 double mutants (sod1Δ, sod2Δ) strains, exposed to 10 mM of lugol, showed a significant increase in antioxidant enzymes activity, similar to that induced with the presence of catalase and superoxide dismutase. Additionally, superoxide dismutase 1 and 2 double mutants (sod1Δ, sod2Δ) of *C. glabrata* were more susceptible to lugol treatment in relation to the parental strain. These results suggest that superoxide dismutase 1 and 2 activities can be involved in the resistance of *Candida* spp. to iodine.

The occurrence of both disinfectant and drug resistance is increasingly disturbing, rendering it highly important

to study the antifungal potential of new molecules. Many reports indicate that plant extracts could become a source of bioactive molecules. Some of them are essential oils, obtained from various parts of certain plants (leaves, flowers, seeds, bark, etc., which demonstrate numerous therapeutic properties, including antifungal activity [32]. According to Martinez et al. [33], oils, such as thyme, oregano, clove, nutmeg, cinnamon, mint, citronella, lavender and rosemary are among the essential oils which may be used in phytosanitary composition. Many of them are detailed in the European Pharmacopeia. Tomczykowa et al. [34] describe a novel gel formulation exhibiting antifungal action that contains essential oil from *Bidens tripartita* (BTEO) of the *Asteraceae* family. Hydrogel supplemented with 100 mg/g BTEO concentration demonstrated the highest activity against *C. tropicalis* and *C. krusei*, except for clinical strain of *C. parapsilosis*. The main EOCs, which have recently become very popular, are also worth mentioning. Patent US010813360B2 is a successful example of the search for effective phytosanitary compositions with fungicidal properties [33]. In described compositions, the effect of agents with known fungicidal properties is potentiated synergistically by carvacrol and thymol.

The current investigated selected EOCs: thymol, menthol, E, carvacrol and TA, which are the main ingredients of essential oils from *Thymus vulgaris* L., *Syzygium aromaticum* (L.) Merr. & Perry, *Origanum vulgare* L., *Foeniculum vulgare* Mill., respectively. These EOCs were for study due to numerous literature reports on their positive interaction with antifungal compounds [35, 36, 37]. In addition, the antifungal activity of OCT against *Candida* strains has been proved [38, 39]. The current study conducted with the use of the checkerboard method demonstrated the ability to enhance the antifungal activity of OCT by TA and E with synergistic additive effects against reference strains and clinical isolates, respectively. Efficacy results analyzed with the use of time-kill assay showed that the OCT – E and OCT – TA combinations were more effective in comparison to untreated cells. Moreover, OCT – E and OCT – TA combinations had the ability to increase cell permeability of *C. albicans* and *C. parapsilosis* strains. which is one of mechanisms of action that can lead to leakage of cellular components and loss of ions [40]. This is very important considering the fact that isolates with increased biocide MICs and MBCs, such as OCT or chlorhexidine, may commonly appear as a consequence of their introductioninto hospital environments, as occurs in cases of MRSA (Methicillin Staphylococcus aneus), a staph infection difficult to treat because of resistance to some antibiotics [41].

TA and E should be safe, e.g. as ingredients of *Candida* yeast eradication preparations. According to the literature, the lethal dose (LD_{50}) of TA and E in oral administration of laboratory animals is 2,090 – 3,050 mg/kg and 2,650 mg/kg, respectively [42, 43, 44]. Moreover, E is used for a long time within the oral mucosa in dentistry.

Although the search for new and effective means for the eradication of microorganisms, including yeasts of the *Candida* genus, is extremely important from the clinical aspect, there is also the need to prevent the spread of drug-resistant strains from long-treated infections in the environment.

CONCLUSIONS

Based on the obtained results, it can be concluded that E and TA in combination with OCT show a strong relationship against *C. albicans* and *C. parapsilosis* reference and clinical strains isolated from skin wounds of patients treated for superficial candidiasis. These combinations have also been shown to affect the rate of yeast cell killing, and increase the permeability of *Candida* cells. Studies indicate that E and TA in formulation with OCT can potentially eradicate pathogenic yeasts, but further microbiological and clinical studies are still required.

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