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EFFECTS OF ESSENTIAL OIL COMBINATIONS ON PATHOGENIC YEASTS AND MOULDS

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Essential oils (EOs) can be used as alternative or complementary antifungal agents against human pathogenic moulds and yeasts. To reduce the effective dose of antimicrobial agents, EOs are combined which can lead to synergistic or additive effect. In this study the anti-yeast and anti-mould activities of selected EOs were investigated, alone and in combinations, against clinical isolates of *Candida albicans*, *C. parapsilosis, Aspergillus fumigatus*, *A. terreus, Rhizopus microsporus*, *Fusarium solani* and *Lichtheimia corymbifera*. Minimum inhibitory concentrations (MICs) were determined for the EOs of cinnamon, citronella, clove, spearmint and thyme. To investigate the combination effect of the EOs, fractional inhibitory concentrations (FICs) were defined by the checkerboard method and the type of interaction was determined by the FIC index (FICI). FIC index below 0.5 was considered as synergism and between 0.5 and 1 as additive effect. Strongest antifungal activity was showed by thyme EO with MIC values below 1.0 mg/ml. Combination of EOs resulted in additive or indifferent effect, with occasional “borderline synergism”. The best combination was cinnamon with clove leading to additive effect in all cases.

Keywords: Essential oils – fungal pathogens – fractional inhibitory concentration – combined effect

INTRODUCTION

Fungal infections are severe diseases among immunocompromised and immunosuppressed patients, causing sometimes life-threatening conditions. Both yeasts and moulds are involved as causative agents and the symptoms range from topical to systemic. Among yeasts, *Candida* species were the most frequently isolated organisms. The main cause of the infection, called candidiasis, is *Candida albicans* but other *Candida* species are also involved [7]. Candidiasis can affect the mouth, the vagina, and rarely a serious invasive form can develop.

Inhalation of mould spores can lead to allergic symptoms but sometimes moulds can grow in the human body causing severe damage to the lung, eye and skin. Invasive aspergillosis is the most common filamentous fungal infection diagnosed in

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immunocompromised patients [29]. Species involved in this disease are *Aspergillus fumigatus*, *A. flavus*, *A. niger* and *A. terreus* [3, 20, 29]. Zygomycosis used to be very rare but has become an emerging infection because the growing numbers of risk factors like diabetes, neutropenia, bone marrow transplantation, long-term use of steroids, and chemotherapy in patients with haematological malignancy [2, 8, 31]. Species described as causative agents are *Rhizopus microsporus* and *Lichtheimia* (formerly *Absidia*) spp. The plant pathogen fungus *Fusarium solani* was reported causing mycotic keratitis in neutropenic patients [33]. Treatment of mycoses is not easy; it requires long time and agents may cause toxic side-effects on the human body. Resistance against antifungals is quite common among fungal pathogens; most *A. terreus* strains, e.g., are resistant to amphotericin B [3, 29], and the number of fluconazole-resistant *Candida* species is also increasing [1, 26]. The search for natural antifungal agents has gained interest recently because these could provide alternative solutions with less or no toxic side-effects and their combined use with standard therapeutic agents could enhance the effectiveness of treatment. Essential oils (EOs) are well-known antimicrobials and their use as preservatives, disinfectants or therapeutic agents has been under intensive research [6]. Combination of EOs could lead to synergism, decreasing the effective concentration. The aim of this study was to investigate the combined effect of selected EOs (cinnamon, citronella, clove, spearmint and thyme) on human pathogenic yeasts and moulds.

MATERIALS AND METHODS

Strains and culture conditions

The fungal strains used in this study were from the Szeged Microbiological Collection (SZMC, WDCM 987) and were isolated from human infections: *C. albicans* SZMC 1363 (vaginal mycosis); *C. parapsilosis* SZMC 1408 (CBS 604 – intestinal mycosis); *Aspergillus fumigatus* SZMC 2394 (keratomycosis); *A. terreus* SZMC 2394 (keratomycosis); *Fusarium solani* SZMC 11412 (keratomycosis), *Rhizopus microsporus* SZMC 13644 (CBS220.92 – human mycosis) and *Lichtheimia corymbifera* FSU 9682 (unknown origin). The yeasts and moulds were cultured on malt extract medium (0.4% malt extract, 1% glucose, 0.1% yeast extract) at 30 °C.

Essential oils

The EOs of cinnamon bark (*Cinnamomum zeylanicum* Presl.), clove (*Syzygium aromaticum* (L.) Merill et Perry), thyme (*Thymus vulgaris* L.), citronella (*Cymbopogon nardus* (L.) Rendle), and spearmint (*Mentha spicata* L.) were purchased from Aromax Zrt. (Budapest, Hungary). The quality of EOs used in this study met the standards described in the European Pharmacopoeia 4th edition. The EOs used in this study in

combination were chosen from 15 essential oils based on the results of previous studies [13].

Gas Chromatography

The percentage evaluation of the essential oil samples was made using a Fisons GC 8000 gas chromatograph (Carlo Erba, Milan, Italy), equipped with a flame ionization detector (FID). An Rt-β-DEXm (Restek) capillary column, 30 m × 0.25 mm i.d., 0.25 µm film thickness, was used. The carrier gas was nitrogen at 6.8 ml/min flow rate; 0.2 ml of a 0.1% solution was injected (1 ml essential oil in 1 ml chloroform). Splitless injection was carried out. The temperatures of the injector and detector were 210 °C and 240 °C, respectively. The oven temperature was increased at a rate of 8 °C/min from 60 °C to 230 °C, with a final isotherm at 230 °C for 5 min. Identification of peaks was made by retention time and standard addition; percentage evaluation was carried out by area normalization. We made three parallel measurements, RSD was below 4.5%.

GC-MS conditions

The essential oil compounds were identified with an Agilent 6890N/5973N GC-MSD (Santa Clara, CA, USA) system equipped with an Agilent HP-5MS capillary column (30 m × 250 µm × 0.25 µm). The GC oven temperature was programmed to increase from 60 °C (3 min isothermal) to 200 °C at 8 °C/min (2 min isothermal), from 200–230 °C at 10 °C/min (5 min isothermal) and finally from 230–250 °C at 10 °C/min (1 min isothermal). High purity helium was used as carrier gas at 1.0 ml/min (37 cm/s) in constant flow mode. The injections were made directly from the diluted essential oil, 1 ml (10 ml/ml essential oil in hexane) was injected at 0.7 mg/ml velocity, splitless type, with an Agilent 7683 autosampler. The injector temperature was 250 °C and the split ratio was 1:50. The mass selective detector was equipped with a quadrupole mass analyser and was operated in electron ionization mode at 70 eV in full scan mode (41–500 amu at 3.2 scan/s). The data were evaluated using MSD ChemStation D.02.00.275 software (Agilent). The identification of the compounds was carried out by comparing retention times and recorded spectra with the data of authentic standards, and the NIST 05 library was also consulted.

Determination of MIC (MFC) values

MIC values were determined by macrodilution for yeasts and by poisoned food method for moulds [24, 17]. Briefly: Into test tubes containing 1 ml malt extract medium 1 ml EO solutions were added in a concentration range of 0.4–50 mg/ml in twofold increments. EO solution were made from a stock solution (100 mg/ml in 50

v/v% ethanol) with malt extract medium containing 1% Tween 40 for the solubilisation of the EOs. Final concentration of EOs in the tubes ranged from 0.2 to 25 mg/ml. The tubes were inoculated with 0.1 ml 24 h old yeast suspension (10^5 cfu/ml). After incubation for 24 h growth was checked by the track dilution method [14]. 10 μl samples were dropped on plates containing malt extract medium and allowed to migrate through the plate. After drying the plates were incubated for 24 hours at 30 °C and number of colonies was evaluated. MIC was considered as the EO concentration where no colonies were detected. For moulds the essential oils were mixed in a concentration range of 0.2 to 25 mg/ml to 10 ml medium and poured into Petri dishes. After solidification of the medium spore suspension (10^5 cfu/ml) obtained from 72 h old mould cultures were spread on the plates and the plates were incubated for 1 week. MIC was considered as the lowest EO concentration where no growth was detected.

Experiments were repeated three times.

Combination of essential oils

Checkerboard method was used to investigate the effect of EO combinations. For yeasts test tubes were arranged in a 4×4 pattern and filled with 1 ml malt extract medium. 1 ml EO solutions were mixed to the medium in the following matter: the first member of an EO pair was added to the tubes in rows in 0; MIC/2; MIC and 2 MIC concentrations giving a final concentration of 0; MIC/4; MIC/2 and MIC. MIC for each EO was determined in the previous experiment. The other member of the EO pair was added to tubes in the columns also in 0; MIC/2; MIC and 2 MIC concentration. In this way all possible combinations can be checked. The first tube containing no EO ($0+0$ mg/ml for the EO pair) was considered as the positive control. The tubes were inoculated with 0.1 ml 24 h old yeast suspension (10^5 cfu/ml) then incubated for 24 h at 30 °C. After incubation 10 μl from each tube was used to check growth by the track dilution method. For moulds EOs were mixed to agar containing medium in the same way, poured into Petri dishes and inoculated with spore suspensions (10^5 cfu/ml). After incubation for 1 week growth was checked and the first combination where no growth occurred was used to determine the fractional inhibitory index (FICI). Fractional inhibitory concentration (FIC) was determined from MIC values determined for an EO alone or in combination: FIC = [MIC in combination]/[MIC alone]. $FICI = FIC_A + FIC_B$ where FIC_A and FIC_B are the fractional inhibitory concentrations for the two EOs of an EO pair. Results are interpreted as synergy ($FICI < 0.5$), addition ($0.5 \geq FICI \leq 1$), indifference ($1 > FICI \leq 4$) or antagonism ($FICI > 4$) [17].

Pairs of EOs were chosen based on their main components and low MIC values. EOs containing phenolic compounds (cinnamon, thyme and clove) were combined with each other or with citronella or spearmint containing mainly monoterpenes.

All experiments were repeated three times.

RESULTS

Composition of essential oils

The chemical composition of the EOs of cinnamon bark, clove, thyme, citronella and spearmint can be seen in Table 1. Cinnamaldehyde (74.0%) containing an aromatic ring was the main compound in cinnamon bark oil. Other important constituents identified in this EO were cinnamyl acetate (5.3%), linalool (3.8%) and eugenol (2.7%). In the clove oil eugenol (88.6%) was the main constituent, and the other relevant compound was identified as β-caryophyllene (8.6%). The phenolics thymol (46.3%) and p-cymene (22.1%) were the main compounds in thyme essential oil. Carvacrol was present in 3.2% in this oil. Citronella oil contained the oxygenated terpenes citronellal (36.2%), geraniol (25.3%), and citronellol (13.6%) and the non-oxygenated terpene limonene (3.5%) as the most relevant compounds. In the spearmint oil (–)-R-carvone (69.0%) another oxygenated terpene was the main compound, but limonene was also present in remarkable quantity (21.2%).

MIC/MFC values

MIC values for *Candida* species and moulds are presented in Table 2. The most sensitive strains seemed to be *C. albicans* and *C. parapsilosis* with MICs below 1.0 mg/ml for almost all EOs. In general, spearmint showed weak antifungal activity; in most cases MIC was not detected because in previous screening experiments with paper discs containing 100 mg/ml EO no inhibition zone was detected [13]. Best results were achieved with thyme EO showing MIC values below 1 mg/ml for all of the investigated fungi. In another study thyme EO also showed a broad fungitoxic spectrum on different aspergilli and *F. oxysporum* [17]. Cinnamon, citronella, and clove showed good antifungal activity against most of the investigated species. *Rhizopus* species are known to be insensitive to most of the common antifungals because of their numerous hydrolyzing enzymes can break down the active molecules [32]. In the study of Tzortzakis and Costas [32] lemongrass oil showed moderate inhibition on the colony growth of *R. stolonifer* but spore formation was totally inhibited at 500 ppm concentration. In our study, only the EOs of clove, spearmint and thyme showed inhibition activity against *R. microsporus*. The investigated aspergilli and *F. solani* were all sensitive to cinnamon, clove and thyme EOs and showed partial sensitivity to citronella. Pawar et al. [23] also found that cinnamon bark or leaf EO inhibited the growth of *A. niger*.

Combination of essential oils

Results of the combination of EOs are presented in Table 3. Most of the interactions were additive or indifferent. In the former case, FICI values were 0.5–0.75 and the

Table 1
The percentage composition of the investigated essential oils

t_R MS (min)	t_R FID (min)	Compound	Cinnamon	Citronella	Clove	Spearmint	Thyme
			%				
5.9	5.8	*α*-Pinene	0.5	–	–	0.5	0.9
6.3	6.2	Camphene	–	–	–	–	0.9
6.9	6.7	*β*-Pinene	–	–	–	0.6	1.4
7.7	7.2	*α*-Terpinene	–	–	–	–	2.2
8.0	7.4–7.5	Limonene	1.4	3.5	–	21.2	–
7.9	7.6	*p*-Cymene	1.2	–	–	–	22.1
8.0–8.1	7.9–8.0	1,8-Cineole	2.1	–	–	–	9.8
8.6	8.5	γ-Terpinene	–	–	–	–	0.3
9.5	10.1	Linalool	3.8	–	–	–	5.1
	10.2	Neral	–	1.0	–	–	–
10.5	11.0	Citronellal	–	36.2	–	–	–
10.7	11.2	Geranial	–	2.2	–	–	–
10.6	11.3	Menthone	–	–	–	1.1	–
11.5	11.7	Terpinen-4-ol	–	–	–	–	0.6
11.0	12.4	Borneol	–	–	–	–	1.0
11.5	12.5	Dihydro-carveol	–	–	–	1.3	–
11.9	12.9	Citronellol	–	13.6	–	–	–
12.3	13.4	(–)-R-Carvone	–	–	–	69.0	–
12.4	13.4	Geraniol	–	25.3	–	–	–
13.1	13.5	Anethol	2.3	–	–	–	–
13.7	13.9	Dihydro-carveyl acetate	–	–	–	0.9	–
14.1	14.1	Citronellyl acetate	–	2.3	–	–	–
14.5	14.2	Geranyl acetate	–	2.6	–	–	–
14.9	14.7	*β*-Elemene	–	2.9	–	–	–
12.9	14.9	Cinnamaldehyde	74.0	–	–	–	–
15.4	14.9	*β*-Caryophyllene	–	–	8.6	–	2.5
16.0	15.5	*α*-Humulene	–	–	2.2	–	–
13.2	15.6	Thymol	–	–	–	–	46.3
14.3	15.8	Eugenol	2.7	–	88.6	–	–
16.4	15.8	*β*-Cubebene	–	0.9	–	–	–
13.3	15.9	Carvacrol	–	–	–	–	3.2
17.0	16.0	*β*-Cadinene	–	1.0	–	–	–
17.4	16.4	Elemol	–	2.4	–	–	–
15.8	16.9	Cinnamyl acetate	5.3	–	–	–	–
19.1	18.3	Eudesmol	–	2.3	–	–	–
18.0	18.9	*β*-Caryophyllene oxyde	–	–	0.5	–	–
Total			93.3	93.9	99.9	94.6	96.3

Table 2
Minimal inhibitory concentrations (mg/ml) of the investigated essential oils

Essential oil	*Candida albicans*	*C. parapsilosis*	*Aspergillus fumigatus*	*Aspergillus terreus*	*Fusarium solani*	*Lichtheimia corymbifera*	*Rhisopus microsporus*
Cinnamon	0.19	0.78	0.19	0.19	0.19	3.13	>25
Citronella	0.39	0.78	0.78	–	1.56	>25	>25
Clove	0.78	0.39	1.56	3.13	3.13	3.13	6.25
Spearmint	>25	6.25	–	–	–	–	1.56
Thyme	0.78	0.19	0.39	0.78	0.78	0.19	0.19

– not detected, because of no inhibition zone was achieved during screening.

MIC values of the individual EOs were lower in combination than alone, while in case of indifference the EOs kept their MIC values also in combination. The type of interaction apparently depended not only on the type of EOs combined but also on the microbes against which they were used. Saprophytic moulds with a broad spectrum of degradation enzyme activity [32, 33] seemed to be less susceptible. Best results (additive effect in all cases) were achieved by the combination of cinnamon and clove, both having phenolic compounds as main components. The other EO combination (cinnamon, thyme) with phenolic components resulted in indifferent and additive effect based on the fungus investigated.

Table 3
Type of interaction between essential oil combinations basing on the evaluation of the fractional inhibitory concentration index (FICI)

Essential oil pairs	*Candida albicans*	*C. parapsilosis*	*Aspergillus fumigatus*	*Aspergillus terreus*	*Fusarium solani*	*Lichtheimia corymbifera*	*Rhisopus microsporus*
Cinnamon/ citronella	0.5 (A)	–	1.0 (I)	–	2.0 (I)	–	–
Cinnamon/ clove	–	–	–	0.75 (A)	–	0.75 (A)	–
Cinnamon/ thyme	–	–	2.0 (I)	–	–	0.5 (A)	–
Citronella/ clove	0.5 (A)	0.5 (A)	–	–	2.0 (I)	–	–
Citronella/ Thyme	0.5 (A)	0.5 (A)	–	–	2.0 (I)	–	–
Thyme/ Spearmint	–	–	–	–	–	–	0.5 (A)

A – Addition; I – indifference; – not detected.

DISCUSSION

EOs can have more than 50 active ingredients grouped into terpenes or aromatic compounds [4]. The antimicrobial activity of EOs is the result of several mechanisms. The main target of the EOs in pro- and eukaryotes is the cell membrane or the cell wall [4, 5, 9, 19]. The membrane loses its integrity while its permeability and fluidity are increased [11, 19]. In yeasts, the content of ergosterol, a major sterol in the membrane responsible for cell integrity, was reduced by the action thyme EO [25]. Thyme and thymol reduced the virulence of *Trichophyton* and *Aspergillus* spp. with 80% reduction of elastase activity in the investigated moulds [16]. EOs thus affect microbes in several different ways. It seems that the chemical type of EO ingredients plays a considerable role in the antimicrobial activity. Best results were achieved by EOs or EO compounds containing aromatic rings [10, 12, 15] and oxygenated terpenes [22]. Our MIC results also were lower for cinnamon, clove and thyme EOs containing aromatic compounds. Synergism has been reported for combinations of EOs or EO ingredients against *E. coli* [24, 27], *Staphylococcus aureus* [27], *Listeria monocytogenes*, *Bacillus cereus*, and *Yersinia enterocolitica* [10]. Fortification of cinnamon EO with cinnamaldehyde and thyme EO with thymol showed synergistic effect against *A. flavus*, and additive effect against *Candida albicans* and *Penicillium islandicum* [21]. In our experiments only "borderline synergism" (FICI values of 0.5 at the border of synergistic and additive effect) was found. Several samples are found in the literature for the synergistic activity of EOs and antifungal agents, also in case of resistant fungi [16, 28, 30]. We will also broaden our combination experiments to this field, because literature data and our results presented here both suggest that EOs in the right concentration can give an alternative or complementary therapy in the treatment of fungal infections.

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