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In Vitro Antifungal Activity of a New Combination of Essential Oils Against Some Filamentous Fungi

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Abstract: The antifungal activities of essential oils and herbal extracts have been demonstrated against a range of filamentous fungi. In this study, *in vitro* antifungal activity of a combination of some essential oils (EO) extracted from the herbs (*Thymus vulgaris*, *Salvia officinalis*, *Eucalyptus globulus* and *Mentha piperita*) against some filamentous fungal strains (*Metrhizium sp.*, *Ophiostoma sp.*, *Trichoderma sp.* And *enicillium expansum*) was determined. The composition of oils was analyzed by gas chromatography (GC) and GC/mass spectrometry. The minimum inhibitory concentrations (MIC) and minimum fungicidal concentration (MFC) were determined by serial microdilution method. Based on the results, 1,8-cineol (21.37%), thymol (13.86%), camphor (7.92%), α -thujone (7.71%), menthon (6.8%) and menthol (6.2%) were the major constituents. The results indicated inhibitory effects of the combination of essential oils on the filamentous fungi tested. The MIC and MFC values were, respectively, 0.022 and 0.064 mg/ml for *Metrhizium* sp., 0.02 and 0.064 mg/ml for *Ophiostoma* sp., 0.018 and 0.048 mg/ml for *Trichoderma* sp. and 0.03 and 0.085 mg/ml for *Penicillium expansum*. *Penicillium expansum* showed the lowest inhibitory activity but was not significant ($p>0.05$). Also, *Trichoderma sp.* was the most sensitive species to this combination. According to this experiment, this combination was found to have a wide spectrum of activity against all filamentous fungi examined in this study and may be proposed for control of fungal diseases.

Key words: Antifungal Activity · Essential Oils · Filamentous Fungi

INTRODUCTION

Importance of fungal infections, the difficulties in their treatment and the increase in resistance to antifungal agents have increased research on therapeutic alternatives [1, 2, 3]. The essential oils (EO) and products of plant had a wide application in folk medicine, fragrance industries and food flavoring and preservation but only in recent years they have started to be recognized for their potential antimicrobial role [1, 4-6]. Although numerous studies have documented the EO antibacterial and anticandidal effect [7-12], there have been a few comprehensive *in vitro* studies of the effects exerted by EO on filamentous fungi, probably due to the difficulties encountered in standardized susceptibility methods for these mycetes [13-16]. EO antimicrobial activity *in vitro* could be tested using a micro dilution assay [1, 5, 11, 17].

The aim of this study was to determine *in vitro* antifungal activity of combination of four EOs (*Thymus vulgaris* (Thyme), *Salvia officinalis* (common sage), *Eucalyptus globulus* (blue gum eucalyptus) and *Mentha piperita* (peppermint)) against some filamentous fungi (*Metrhizium sp.*, *Ophiostoma sp.*, *Trichoderma sp.* and *Penicillium expansum*) by using modified serial microdilution assay.

MATERIALS AND METHODS

Combined Essential Oils: The combined essential oils (CEO) used in this study were extracted from the herbs; *Thymus vulgaris* (thyme), *Salvia officinalis* (common sage), *Eucalyptus globulus* (blue gum eucalyptus) and *Mentha piperita* (peppermint). The herbs were collected from an experimental field in the Zardband region located in the north eastern of Tehran, Iran.

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Air-dried leaves and stems of the herbs (90g from each herb) were subjected to hydro distillation for 4 hours using a Clevenger-type apparatus to produce essential oils according to the method recommended by the European Pharmacopoeia [17]. The final combination of essential oils was prepared using an emulsifier. It was composed of 30% *Salvia officinalis*, 30% *Thymus vulgaris*, 20% *Mentha piperita* and 20% *Eucalyptus globulus* extracts [5] The CEO was dried over anhydrous sodium sulfate and stored in a sealed vial at low temperature (5-10°C) before analysis.

Gas Chromatography and Mass Spectrometry (GC-MS) Analyses: The composition of the CEO was determined by gas chromatography (GC) coupled with mass spectrometry (MS). GC analysis was performed using a Shimadzu GC-9A gas chromatograph equipped with a DB-5 fused silica column (30m × 0.25 mm, film thickness 0.25 micron). Oven temperature was held at 40°C for 5 min and then increased to 250°C at a rate of 3°C/min. Injector and detector (FID) temperatures were 260°C. Helium was used as a carrier gas with a linear velocity of 32 cm/s. Percentages of different components were calculated by electronic integration of FID peak areas without the use of response factor correction. The different components within the CEO were identified by comparisons of their mass spectra with those of a database of known spectra [19] or with authenticated reference compounds [20]. Identities were confirmed by comparison of their retention indices either with those of authenticated compounds or with data published in the literature [19].

Fungal Strains and Culture: The test microorganisms included four isolates of the filamentous fungi (*Metrhizium sp.*, *Ophiostoma sp.*, *Trichoderma sp.* and *Penicillium expansum*). All of the fungal strains were derived from stock cultures held in the Molecular Biology Laboratory of the Biological Sciences Department, Macquarie University, NSW, Australia.

Fungi were identified according to macroscopic and microscopic morphological procedures and maintained on potato dextrose agar (PDA) slopes stored at room temperature. Before the experiments the fungi were transferred to fresh media and incubated for 7 days, in twice.

For preparation of non-germinated conidial suspensions, inocula were prepared by growing fungal strains on PDA (Merck, Darmstadt, Germany) slopes at room temperature for 7 days as described by the National Committee for Clinical Laboratory Standards (CLSI) [21]. Slopes were flooded with 0.85% saline and conidia gently probed. The resulting suspensions were removed and vortexed thoroughly. After the settling of the larger particles, suspensions were adjusted by nephelometry and diluted in saline to obtain inocula of 2×10^4 CFU/ml, as confirmed by colony counts in triplicate on SDA agar.

Broth Microdilution Method: Broth microdilution (BM) testing was based on National Committee for Clinical Laboratory Standards (CLSI) method [21], with some modifications. Minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) of the CEO were determined by the broth micro dilution assay using liquid cultures in 96-well microplates for measuring fungal growth. One hundred microliters of the CEO were added to the column 1 of each plate and then serially diluted as doubling dilutions up to the last well of first column (dilution factor 1:1). Each well of the first three rows of the plate was then inoculated with 100 microliters of inocula (2×10^4 cfu/ml). Three rows of each plate were inoculated just with conidial suspension without any CEO (positive control) and one row was incubated only with broth medium (negative control). The microplates were incubated for 7 days at 30°C. After incubation, 50 microliters (0.4 mg/ml in ethanol) of p-iodonitrotetrazolium was added to the wells and the absorbance was measured at 492 nm. Then, 100 microliters of each well was inoculated into the SDA plates and incubated for 3 days at 30°C. Data were calculated as the percentage of fungal growth relative to positive controls. Minimum inhibitory concentrations (MIC) were determined as the lowest concentrations that significantly inhibited target cell growth and minimum fungicidal concentrations (MFC) were calculated as the lowest concentrations that abrogated fungal growth. MIC and MFC were calculated in mg/ml by multiplying the results in 60% (percentage of herbal extract in CEO).

Statistical Analysis: MIC and MFC were compared between treatments using Student's T-test. P values lower than 0.05 ($P<0.05$) were considered to reflect significant differences between treatments.

RESULTS

The composition of the combined essential oils from each of the herbs used to formulate the CEO was determined by GC/MS analysis is shown in Table 1. Forty-six different components were detected. Based on the results, 1, 8-cineol (21.37%), thymol (13.86%), camphor (7.92%), a-thujone (7.71%), menthon (6.8%) and menthol (6.2%) were the major constituents.

Table 1: Composition of the combined essential oils extracted from *Salvia officinalis*, *Thymus vulgaris*, *Eucalyptus globulus* and *Mentha piperita*

	S. officinalis	*T. vulgaris*	*E. globulus*	*M. piperita*	CEO
Compound	%	%	%	%	%
Salvene	0.3	-	-	-	0.09
Tricyclene	0.2	1.2	-	-	0.42
α- pinene	3.7	-	2.18	0.4	1.626
Camphene	6.6	0.6	-	-	2.10
Verbenene	1.6	-	-	-	0.48
Sabinene	0.2	1.8	-	0.6	0.72
β-pinene	0.4	0.1	0.69	0.6	0.408
Myrcene	0.9	2.4	0.69	-	1.128
α- Terpinene	0.2	2.1	1.66	-	1.022
ñ-cymene	0.2	17.6	1.27	-	5.594
Limonen	2.4	1.2	1.45	4	2.17
1,8 –cineole	9.6	0.3	88	4	21.37
γ-Terpinolene	0.1	14.8	-	-	4.47
Terpinolene	0.2	-	0.25	-	0.11
β-Thujone	6.4	-	-	-	1.92
α-Thujone	24.7	1	-	-	7.71
Camphor	26.4	-	-	-	7.92
Isopulegol	0.2	-	-	-	0.06
Pinocamphone	1.1	-	-	-	0.33
Borneol	4.2	1.1	-	-	1.59
Terpinene-4-01	0.7	1.7	0.52	1	1.024
α-Terpineol	0.5	-	1.59	-	0.468
Myrtanol	1	-	-	-	0.03
Thymol	0.2	46	-	-	13.86
Bornyl acetate	1.3	-	-	-	0.39
Eugenol	0.1	-	-	-	0.03
α-Humulene	0.9	-	-	-	0.27
Caryophyllen oxide	0.2	-	-	-	0.06
Guaiol	3.3	-	-	-	0.99
α- Phellandrene	-	0.1	0.54	-	0.138
Trans sabinene	-	1	-	-	0.30
Linalool	-	2.3	0.16	-	0.722
Menthyl thymol	-	0.7	-	-	0.21
Menthyl carvacrol	-	0.4	-	-	0.12
Carvacrol	-	2.5	-	-	0.75
Cis β-ocimene	-	-	0.18	-	0.036
Trans pinocarveol	-	-	0.33	-	0.066
α -Terpinyl acetate	-	-	0.49	-	0.098
Menthone	-	-	-	34	6.80
Menthofuran	-	-	-	1.8	0.36
Isomenthone	-	-	-	6.15	1.23
Menthol	-	-	-	31	6.2
Carvone	-	-	-	0.4	0.08
Menthyl acetate	-	-	-	3	0.6
β -Cariyophyllene	-	-	-	2	0.4
Germacrene	-	-	-	1.3	0.26
Other minor components	-	-	-	-	3.27

The results shown in Fig. 1 compare the MICs and MFCs of the CEO in this experiment. The MIC and MFC values were, respectively, 0.022 and 0.064 mg/ml for *Metrhizium* sp., 0.02 and 0.064 mg/ml for *Ophiostoma* sp., 0.018 and 0.048 mg/ml for *Trichoderma* sp. and 0.03 and 0.085 mg/ml for *Penicillium expansum*. *Penicillium expansum* showed the lowest inhibitory activity but was not significant ($p>0.05$) and among the 4 fungal strains tested, the most susceptible was *Trichoderma* sp. but was not statistically significant ($p>0.05$).

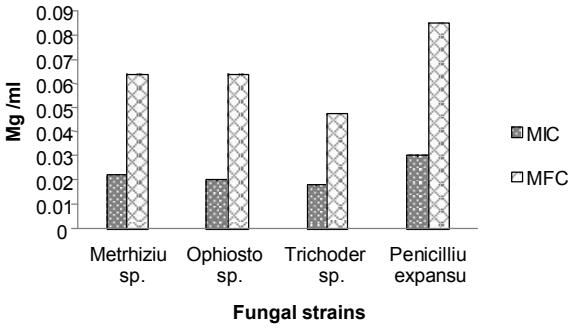

Fig. 1: Minimum inhibitory concentrations (MIC) (mg/ml) and minimum fungicidal concentrations (MFC) (mg/ml) of the combined essential oil (CEO) on fungal strains

DISCUSSION

Recently, the scientific interest into biological properties of essential oils and herbal extracts has been increasing. In the present study, the antifungal activity of a new combination of EOs was studied by microdilution assay against dermatophytes and other moulds.

The major components of the CEO used in the current study were 1,8-cineol, thymol, camphor, α-thujone, menthon and menthol. All of these compounds have been shown to have antimicrobial activities against common laboratory target strains in other studies [1, 4, 7, 20-23]. Their mechanisms of action have most often been attributed to the disturbance of microbial membranes, disrupting the proton motive force, electron flow, active transport and resulting in the coagulation of intracellular contents [7, 24]. However, more data will be necessary either to confirm *in vitro* efficacy or to explain the mechanisms of action of EO.

Our results demonstrated that CEO is very active on filamentous fungi. Similarly, the high sensibility of filamentous fungi was observed by other authors with other EOs [25, 28-32].

Some studies have concluded that combinations of essential oils have greater antimicrobial activity than their individual components [33, 34]. This fact led Mourey and Canillac [35] to suggest that the minor components in essential oils are also critical to antimicrobial activity providing synergistic or potentiating effects. The herbal extracts used in this research were found to have antimicrobial effects on bacterial and fungal strains [7, 22, 30, 36, 37]. Our findings showed that CEO was effective against filamentous fungi strains (*Metrhizium sp.*, *Ophiostoma sp.*, *Trichoderma sp.* and *Penicillium expansum*) which are often resistant to available antifungal agents. This is in agreement with findings of previous research on antimicrobial effects of combinations of essential oils on fungal species [1, 22, 25]. This result may open important perspectives in alternative antifungal therapies. Further studies are required to study the effect and toxicity of these compounds in experimental animals (*In vivo*) and to establish if they could be safely used as antifungal agents against these fungi. Moreover, this study could identify candidate CEO for developing alternative methods to control environmental undesirable filamentous fungi.

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