Article

Evaluation of The Antifungal Activity of *Mentha x piperita* (Lamiaceae) Of Pancalieri (Turin, Italy) Essential Oil and Its Synergistic Interaction with Azoles

Vivian Tullio [1]*, Janira Roana [1], Daniela Scalas, and Narcisa Mandras [1]

1 Dept. Public Health and Pediatrics, Microbiology Division, University of Turin, Turin (Italy); vivian.tullio@unito.it

2 Area Servizi Alla Ricerca Polo Agraria e Medicina Veterinaria, University of Turin, Turin (Italy)

* Correspondence: vivian.tullio@unito.it; Tel.: 0039 0116705637

Abstract: The promising antimicrobial activity of essential oils (EOs) led researchers to use them in combination with antimicrobial drugs in order to reduce drug toxicity, side effects, and resistance with single agents. In Pancalieri (Turin, Italy), there is a local production of *Mentha* x *piperita* worldwide known as "Mentha of Pancalieri". The EO from this *Mentha* is considered as one of the best peppermint EO in the world. In our research, we assessed the antifungal activity of "Mentha of Pancalieri" EO either alone or in combination with azole drugs (fluconazole, itraconazole, ketoconazole) against a wide panel of yeast and dermatophyte clinical isolates. The EO was analyzed by GC-MS and its antifungal properties were evaluated by MIC/MFC parameters, according to the CLSI guidelines, with some modifications. The interaction of peppermint EO with azoles was evaluated through the chequerboard and isobologram methods. Results suggest this EO exerts a fungicidal activity against yeasts, and a fungistatic activity against dermatophytes. Interaction studies with azoles indicate mainly synergistic profiles between itraconazole and peppermint EO vs. *Candida* spp., *Cryptococcus neoformans* and *Trichophyton mentagrophytes*. Peppermint of Pancalieri EO may act as a potential antifungal agent and may serve as a natural adjuvant for fungal infection treatment.

Keywords: essential oils; *Mentha x piperita*; "Mentha of Pancalieri"; azoles; antifungal activity; yeasts and dermatophytes; synergism

1. Introduction

With the wide use of synthetic and semi-synthetic antimicrobial drugs, advantages and disadvantages have been highlighted over the years, including the spread of drug-resistant pathogens, and have focused research on natural products as useful antimicrobial tools. Currently, there is evidence that EOs may exert remarkable biological activities against viruses, bacteria, fungi and parasites. Several EOs are generally recognized as safe, do not accumulate in the liver or kidneys, can stimulate the immune system, and cause no resistance, since microbes are unable to adapt to their heterogeneous structure. *Mentha x piperita* L. (peppermint) EO is one of the most widely produced and consumed EOs. Literature data have shown that peppermint EO and its main components (menthol and menthone) display antimicrobial effects, but their mechanism of action is still not elucidated [1-4]. Near Turin (Piedmont, Italy), in Pancalieri, there is a typical local production of *M.x piperita* (Huds) var. OFFICINALIS (Sole), form RUBESCENS (Camus) (Lamiaceae), worldwide known as "Mentha of Pancalieri" or "Mint Italo-Mitcham", of which it is used or the green plant that is dried for its conservation and used in herbal medicine, or the EO obtained from the steam current distillation of the whole plant green grass (not shredded) [5]. The cultivation and distillation processes of this plant boast a secular tradition. The EO from "Mentha of Pancalieri", thanks to its

high quality and its peculiarities, is actually considered by experts to be as one of the best peppermint EO in the world. Due to the continuous increase of fungal infections and development of azole-resistant fungal strains, in our research, we assessed the antifungal activity of "Mentha of Pancalieri" EO against a wide panel of yeast and dermatophyte clinical isolates. The EO possible synergistic interaction with azoles, drugs used in fungal infections treatment, was also evaluated.

2. Results

The phytochemical composition of "Mentha of Pancalieri" EO confirmed to be highly rich in oxygenated monoterpenes (menthol 41.7%; menthone 21.8%), in accord with data from the European Pharmacopoeia 8th Ed. (Table 1).

Table 1. Phytochemical composition of "Mentha of Pancalieri" essential oil in comparison with European Pharmacopeia indication for *Mentha x piperita* (Lamiaceae) essential oil.

Main components	Mentha of Pancalieri EO (area %)	Eur. Ph. 8th ed. (area %)
limonene	1.8	1-3.5
1,8-cineole	5.3	3.5-8
menthone	**21.8**[1]	**14-32**
isomenthone	1.5	1.5-10
menthil-acetate	4.8	2.8-10
isopulegole	0.16	max 0.2
menthol	**41.7**[1]	**30-55**

[1] Main components.

"Mentha of Pancalieri" EO was found to exert a good inhibitory activity against all tested fungal strains (Table 2), in comparison with azole drugs (Tables 3-5). Notably, this EO exerted the most remarkable antifungal activity against *Cryptococcus neoformans*, displaying the lowest Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) values (Table 2, range 0.06-0.125 %, v/v). A higher antimicrobial activity was also detected against other non-*Candida* yeasts, such as *Saccharomyces cerevisiae*, and *Pichia carsonii* (MIC range 0.125-0.5 and 0.125-0.25 %, v/v, respectively). Based on MFC results, "Mentha of Pancalieri" EO was found to display a fungicidal activity against yeast cells (MIC=MFC), and a fungistatic activity against dermatophytes. In fact, MFCs for dermatophytes were found to be one or two concentrations higher than MICs.

Table 2. MIC[1] and MFC[2] ranges of "Mentha of Pancalieri" essential oil towards yeasts and dermatophytes clinical strains.

Species	Isolates (n.)	MIC range (%,v/v)	MFC range (%,v/v)
Candida spp.			
Candida albicans	ATCC 90028	0.5	1
Candida albicans	6	0.5-1	0.5-1
Candida glabrata	ATCC 90030	0.5	0.5
Candida glabrata	2	0.5	0.5-1
Candida krusei	1	0.25	>1
Candida parapsilosis	1	0.5	0.5
Candida tropicalis	1	1	1
Candida valida	2	0.25-0.5	0.25-0.5
Candida lusitaniae	1	0.5	0.5
Candida norvegensis	2	0.25-0.5	0.25-0.5
non-*Candida spp.*			
Cryptococcus neoformans	7	0.06-0.125	0.06-0.125

Saccharomyces cerevisiae	4	0.125-0.5	0.25-1
Pichia carsonii	2	0.125-0.25	0.125-1
Sporobolomyces salmonicolor	1	0.5	1
Kloekera japonica	1	0.5	0.5
Dermatophytes			
Trichophyton mentagrophytes	2	0.5	>1
Microsporum canis	2	0.125	>1
Microsporum gypseum	1	0.125	>1

[1]MIC = Minimum inhibitory concentration; [2]MFC= Minimum fungicidal concentration

No significant differences in inhibitory and fungicidal concentrations were found between azole-susceptible (S) and azole-resistant (R) strains (Tables 3, 4).

By chequerboard testing, binary combinations of FLC with "Mentha of Pancalieri" EO were found to be additive (0.5<FICI≤1) against either azole-S *Candida albicans* or azole-S *C.glabrata* strains (Table 3). Notably, when synergistic or additive effects were not observed, no antagonism was reported, since binary mixtures of FLC/peppermint EO yielded indifferent effects on the azole-R *C.krusei* strain (FICI=2). Notably, the combinatorial effects between ITZ and "Mentha of Pancalieri" EO were found to be synergistic (FICI≤0.5) against all *Candida* spp. strains (Table 3).

Table 3. MIC[1] **and FICI**[2] **of "Mentha of Pancalieri" EO *plus* fluconazole or itraconazole against azole-S/R *Candida* spp. strains**

Antifungals		*C. albicans* azole-S strain[1]	*C. glabrata* azole-S*/R** strain[1]	*C. krusei* azole-R strain[1]
Mentha of Pancalieri EO	MIC (%, v/v) alone	0.5	0.5	0.25
Fluconazole	MIC (μg/ml) alone	0.12	4*	64
	FIC of EO	0.5	0.5	1
	FIC of FLC	0.12	0.12	1
	FICI	**0.62**	**0.62**	**2**
	Interpretation	**ADD[4]**	**ADD**	**IND[2]**
Mentha of Pancalieri EO	MIC (%, v/v) alone	0.5	0.5	0.25
Itraconazole	MIC (μg/ml) alone	0.5	2**	2
	FIC of EO	0.12	0.25	0.25
	FIC of ITZ	0.12	0.25	0.25
	FICI	**0.24**	**0.5**	**0.5**
	Interpretation	**SYN[4]**	**SYN**	**SYN**

[1]MIC = Minimum inhibitory concentration; [2]FICI= Fractional inhibitory concentration index

[3]Breakpoints determining susceptibility/resistance to fluconazole (FLC) and itraconazole (ITZ) according to the CLSI document: FLC S≤0.125 μg/ml and R≥4 μg/ml (*C.albicans*), FLC R≥64 μg/ml *C.glabrata)*; *C.krusei* is basically resistant to FLC*;* ITZ S≤0.125 μg/ml and R≥1 μg/ml (*C.krusei*, *C.glabrata*) [6,7]

[4]ADD: additive; IND: indifferent; SYN: synergy.

Moreover, the isobologram profiles confirmed a synergistic associations between ITZ and "Mentha of Pancalieri" EO (Figure 1 a,b,c).

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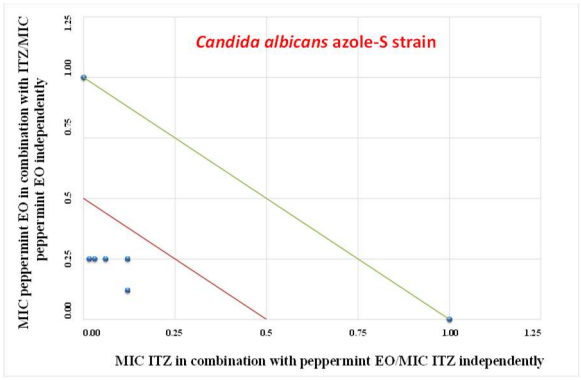
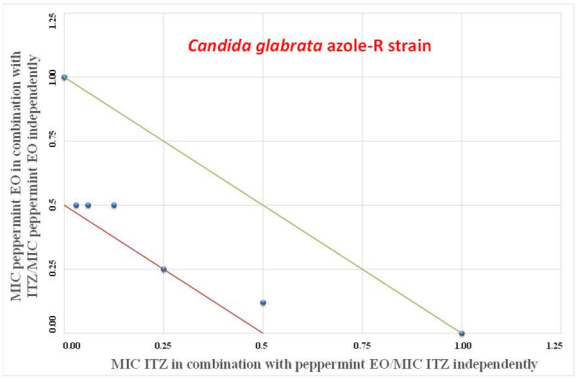


(a)



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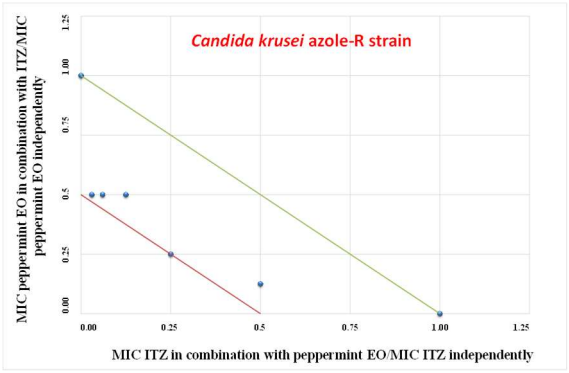

(c)

Figure 1. Isobolograms of synergistic interactions: plots of "Mentha of Pancalieri" EO and ITZ against *Candida* spp. (azole-susceptible/resistant strain). ITZ FIC data are drafted on x-axis, while EO FIC values are drafted on y-axis (**a**) Isobologram plot of EO and ITZ against *Candida albicans* azole-S strain; (**b**) Isobologram plot of EO and ITZ against *Candida glabrata* azole-R strain (**c**) Isobologram plot of EO and ITZ against *Candida krusei* azole-R strain.

In accord to the FIC indexes described for *C.neoformans* azole-S isolate, "Mentha of Pancalieri" EO was found to exert a synergistic profile (Table 4, FICI≤0.5), with a decrease of the MICs, as expressed by the isobologram in Figure 2. On the contrary, concerning *C.neoformans* azole-R strain, binary combination of ITZ/peppermint EO yielded additive effects (FICI=0.62).

Table 4. MIC[1] and FICI[2] of "Mentha of Pancalieri" EO *plus* itraconazole against azole-S/R *Cryptococcus neoformans.*

Antifungals		*C. neoformans* azole-S strain	*C. neoformans* azole-R strain
Mentha of Pancalieri EO	MIC (%, v/v) alone	0.06	0.06
Itraconazole	MIC (μg/ml) alone	0.5	2
	FIC of EO	0.12	0.5
	FIC of ITZ	0.25	0.12
	FICI	**0.37**	**0.62**
	Interpretation	**SYN[3]**	**ADD**

[1]MIC = Minimum inhibitory concentration; [2]FICI= Fractional inhibitory concentration index

[3]ADD: additive; SYN: synergy.

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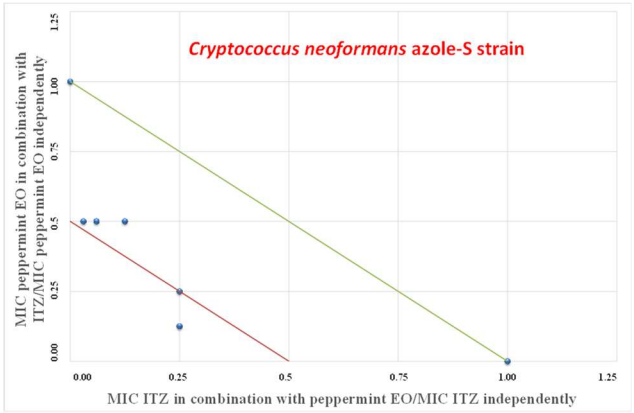

Figure 2. Isobologram of synergistic interactions: plot of "Mentha of Pancalieri" EO and ITZ against *Cryptococcus neoformans* azole-S strain. ITZ FIC data are drafted on x-axis, while EO FIC values are drafted on y-axis.

By chequerboard testing, binary combinations of ITZ/KTZ with "Mentha of Pancalieri" EO were found to be synergistic (Table 5, FICI≤0.5) against *Trichophyton mentagrophytes*, as represented by the corresponding isobologram in Figure 3 (a, b). Conversely, indifferent interactions of ITZ/KTZ and "Mentha of Pancalieri" EO binary mixtures were recorded against either *M.canis* or *M.gypseum* strains (Table 5, 1<FICI<4).

Table 5. MIC[1] and FICI[2] of "Mentha of Pancalieri" EO *plus* itraconazole or ketoconazole against dermatophytes

Antifungals		*Trichophyton mentagrophytes*	*Microsporum canis*	*Microsporum Gypseum*
Mentha of Pancalieri EO	MIC (%, v/v) alone	0.5	0.125	0.125
Itraconazole	MIC (µg/ml) alone	0.5	1	1
	FIC of EO	0.25	1	1
	FIC of ITZ	0.12	0.03	0.125
	FICI	**0.37**	**1.03**	**1.125**
	Interpretation	**SYN[3]**	**IND[3]**	**IND**
Mentha of Pancalieri EO	MIC (%, v/v) alone	0.5	0.125	0.125
Ketoconazole	MIC (µg/ml) alone	2	4	2
	FIC of EO	0.25	1	1
	FIC of KTZ	0.12	0.015	0.015
	FICI	**0.37**	**1.015**	**1.015**
	Interpretation	**SYN**	**IND**	**IND**

[1]MIC = Minimum inhibitory concentration; [2]FICI= Fractional inhibitory concentration index

[3]IND: indifferent; SYN: synergy.

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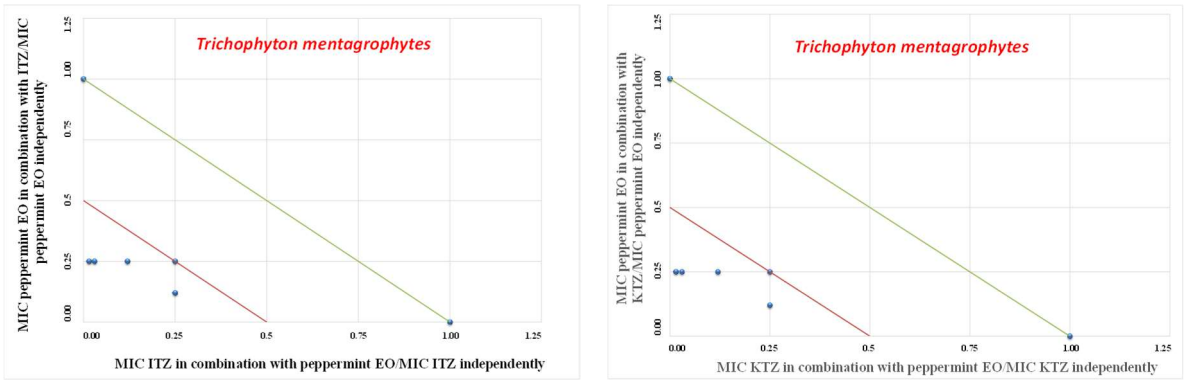

(a) (b)

Figure 3. Isobolograms of synergistic interactions: plots of "Mentha of Pancalieri" EO and ITZ or KTZ against *Trichophyton mentagrophytes*. ITZ/KTZ FIC data are drafted on x-axis, while EO FIC values are drafted on y-axis (**a**) Isobologram plot of EO and ITZ against *T.mentagrophytes*; (**b**) Isobologram plot of EO and KTZ against *T.mentagrophytes.*).

3. Discussion

Peppermint leaves (fresh and dried) and its EO are very well known herbal medicinal product, widely used since a long time, being a popular remedy inside and outside European countries, for its antispasmodic, choleretic and carminative properties [8,9]. Some studies demonstrated that *Mentha x piperita* EO possesses antimicrobial properties and antioxidant activity [9]. In addition, *Mentha x piperita* EO is capable to inhibit the production of *Candida* and other microbial biofilms [10-12], reducing at subMIC concentrations in a dose-dependent manner the amount of exoproteins associated to *Staphylococcus aureus* virulence [13]. In this study, "Mentha of Pancalieri" EO exhibited a higher antimicrobial activity towards *C. neoformans*, *S.cerevisiae*, and *P.carsonii*, and displayed a good antimicrobial activity towards *C. glabrata* and *C. krusei*, species often resistant to conventional drugs. *Mentha x piperita* L. EO is able to carry out anticandidal activity with consequent cell "death", by decreasing ergosterol amounts, producing intracellular acidification following PM-ATPase inhibition [4]. Recent studies have shown that *Mentha x piperita* L. EO inactivates *S. cerevisiae* that deteriorate fruit juices through the disorder of several physiological functions, i.e. enzymatic activity, efflux pump activity, early apoptosis [14]. "Mentha of Pancalieri" EO showed a higher activity against dermatophytes than azole drugs and displayed a fungistatic activity, in agreement with studies of Ibrahim *et al.* with *Mentha x piperita* L. EO [2].

The antimicrobial activity of "Mentha of Pancalieri" EO could be mainly related to menthol and/or menthone, the main compounds contained in the EO [4]. However, this activity may be due to a possible synergy between all the EO components [9, 10]. In fact, while our result about MICs for menthol (data not shown) indicated a better activity of this component against dermatophytes in comparison with EO *in toto*, MICs of menthone (data not shown) are twice higher those observed with the EO *in toto*.

The promising antimicrobial activity of EOs, has led researchers to use them in combination with available antimicrobial drugs, to assess their possible clinical use. Drug combinations represent a promising strategy for the development of new antifungal strategies, in order to overcome drug

resistance, limit the side effect and toxicity of drugs and increase therapeutic efficacy [10,15]. In this research, we assessed the combined effect of "Mentha of Pancalieri" EO and three antifungal azoles, such as FLC, ITZ, and KTZ. Our data showed a synergistic (against *Candida* spp., *C.neoformans,* and *Trichophyton mentagrophytes*) interaction between ITZ and "Mentha of Pancalieri" EO, with a noteworthy reduction in the concentration of the azoles at the MIC. However, our FICI result about menthol and menthone in combination with ITZ or KTZ (data not shown) indicated only an additive activity of these components against dermatophytes in comparison with EO *in toto*, suggesting once again that the activity of an EO is due to the phytocomplex that expresses its effectiveness.

4. Materials and Methods

4.1 *Essential oil*.

Commercial "Mentha of Pancalieri" EO, obtained from the fresh leaves of *M.x piperita* (Huds) var. OFFICINALIS (Sole), form RUBESCENS (Camus) (Lamiaceae) by steam distillation, was purchased from Erbe Aromatiche Essenzialmenta, Pancalieri (Turin, Italy). "Mentha of Pancalieri" EO composition was analyzed by GC-MS at Drug Science and Technology Department (University of Turin, Italy) with a Supelcowax capillary column (Supelco, Bellefonte, PA) as previously described [16]. For susceptibility testing, EO was dissolved in ethanol (1:2.5), and diluted (1:20) up to 2% (v/v) in RPMI-1640 medium with L-glutamine and without sodium bicarbonate (Sigma-Aldrich, Rome, Italy), as previously described [17]. Then, 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich) and 0.2% glucose were added to EO solution. Final pH was 7.0. The EO was protected from light and humidity and maintained at 4 °C just before use [17].

4.2 *Antifungal agents.*

Fluconazole (FLC; F8929), itraconazole (ITZ; I6657), and ketoconazole (KTZ; K1003) powders (≥98% purity by HPLC) were achieved from Sigma-Aldrich. FLC solutions were prepared in sterile distilled water, whereas ITZ and KTZ solutions were prepared in 100% DMSO (Sigma-Aldrich), and stored at -20°C just before use [15].

4.3 Fungal strains.

16 *Candida* spp., (*C.albicans, C.glabrata, C.krusei, C.parapsilosis, C.tropicalis, C.valida, C.lusitaniae, C.norvegensis*), 15 non-*Candida spp*. clinical strains (*Cryptococcus neoformans, Saccharomyces cerevisiae, Kloekera japonica, Pichia carsonii, Sporobolomyces salmonicolor*), and 5 dermatophyte clinical isolates (*Trichophyton mentagrophytes*, *Microsporum canis*, *M.gypseum*) were tested (Table 2). *C.albicans* ATCC 90028 and *C.glabrata* ATCC 90030 were also included.

Yeasts identification was determined by the API ID32C systems (BioMérieux, Rome, Italy). Then, strains were maintained at − 80 C in Microbanks™ (Pro-Lab Diagnostics, Neston, UK), and cultured twice on Sabouraud dextrose agar (SDA, Oxoid, Milan, Italy) at 35°C for 72h before the assays. Dermatophyte identification was carried out by macroscopic and microscopic observation of the colonies and reproductive structures after 15 days of incubation on Mycobiotic agar at 25°C (Merck, KGAA, Germany) [17,18]. Molds were stored in SDA at 4°C until use. For susceptibility testing non-germinated conidial suspensions were prepared as previously described [17].

4.4 *In vitro antifungal susceptibility assays*.

Fungal strains were assayed for susceptibility to "Mentha of Pancalieri" EO, and to azoles (FLC, ITZ, KTZ), by a broth microdilution method, in accordance to CLSI guidelines (CLSI M27-A3 and M27-S4) [19, 20] for yeasts, and CLSI M38-A2 [21] for dermatophytes, with some modifications for the EO [15,17]. Tween 80 (Sigma-Aldrich) (final concentration 0.001%, v/v) was employed to enhance EO solubility, without precluding fungal proliferation. Blastoconidia yeast suspensions and dermatophyte conidial suspensions were prepared to yield final inocula of ~$1.5x10^3$ CFU/ml, and ~$1.5x10^4$ CFU/ml, respectively [15, 17]. Microdilution plates were set up, as previously described

[15,17] and yeast strains were incubated at 35°C for 24h, whereas dermatophytes were incubated at 30°C for 7 days. MICs of FLC, ITZ, and KTZ were determined visually as the lowest concentration of drug that produced complete inhibition (dermatophytes) or a significant reduction (≥50% inhibition, yeasts) of growth, in comparison with control. MICs of EO were considered the lowest concentration with no visible growth. MFC was determined by inoculating 10µl from wells not turbid on SDA agar plates incubated for 3 (yeasts) and 4 (dermatophytes) days at 30°C or at 35°C (*Cryptococcus* sp). MFC was defined as the lowest concentration resulting in no growth on subculture from the MICs [15,17].

4.5 Checkerboard assays and evaluation of the fractional inhibitory concentration index (FICI).

A two-dimensional checkerboard with serial twofold dilutions below the MIC to 2xMIC of each compound was performed in a 96-well microtiter plate, as previously described [15, 22]. Microorganism suspensions were joined to each well containing mixtures of azole drug/EO. FICI values were calculated according to the following formula [22]: FICI = FICa+FICb = MICa in combination/MICa tested alone + MICb in combination/MICb tested alone; where MICa and MICb are the MICs of azole and of EO used alone. Synergy and antagonism were defined by FICI values of ≤0.5 and >4, respectively. A FICI value between 0.5 and 1.0 was interpreted as additive, whereas a value between 1.0 and 4.0 was interpreted as indifferent [22].

4.6. Isobolograms.

Data of the checkerboard assays were expressed graphically by isobolograms, as previously described [15,22]. FIC values of azole drug were reported on y-axis, whereas FIC values of peppermint EO on x-axis. The straight line that joins the intercept points is the line of additivity (FICI=1). Below this line we can find the additive area (0.5<FICI≤1) and synergistic (FICI≤0.5) effects, respectively. FICI values above of the straight line corresponded to indifferent (1<FICI<4) or antagonistic (FICI>4) interactions [22].

5. Conclusions

The EO of "Mentha of Pancalieri" contains mainly menthol and menthone, in according with other countries and European Pharmacopeia guidelines. Our data are difficult to compare with literature data because of different methodology used in experimental procedures and different *Mentha x piperita* L. EOs composition. For example, the EO acquired from the dried leaves of *Mentha x piperita* in Brasil contains 51% of linalool, as the main compound [23], probably due to different cultivation conditions. It is very important that mint cultivation and EO production be strictly controlled, because it is recognized that the chemical composition of mint EOs can be influenced by many factors, such as, humidity, nutrients, temperature, etc.,. From this point of view, "Mentha of Pancalieri" can guarantee controlled composition, and consequently high quality that make it an effective product for its exploitation.

"Mentha of Pancalieri" EO may act as a potential antifungal agent and may serve as a natural adjuvant for fungal infection treatment. Further researches are necessary to confirm these data.

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Sample Availability: Samples of the compounds Mentha of Pancalieri EO, fluconazole, itraconazole, ketoconazole, and fungal clinical strains (yeasts and dermatophytes) are available from the authors.