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Original paper

Indifferent Effect in Combinations of Mentha piperita Essential Oil and Fluconazole against *C. albicans*

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Abstract In this study, it was evaluated the interaction of mint (*Mentha piperita* L.) essential oil (EO) and fluconazole (FLC) combinations using microdilution chequerboard technique. The mint oil were hydrodistilled and identified its chemical composition using GC-MS. The most representative compounds in mint oil were menthol (36.01%), menthone (14.8%), isomenthone (10.8%), neomenthol (8.7%), metilaseat (8.21%). The antifungal activity of *M. piperita* EO, alone or in combination with FLC determined by spectrophotometric chequerdoard microdilution assay. MIC of *M. piperita* EO and FLC against *C. albicans* were determined as 15.4 and 13.6 µg/mL, respecively. According to FICI (Fractional Inhibitory Concentration Index) interpretation models, indifferent effect were observed 100% for *C. albicans* in *M. piperita* EO and FLC combinations. When being exposed to *M. piperita* EO, the surface of *C. albicans* became disorganized to die eventually on SEM observation.

Keywords Indifferent effect, *Candida albicans*, *Mentha piperita* L., SEM.

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Introduction

Mentha piperita L., commonly named as mint or peppermint is a hybrid of *M. aquatica* L. and *M. spicata* L. species and grown as a culture plant. It is sharp-smelling, close-to-red body with 30-90 cm high, mostly hairless and dark green, full-leaf perennial herb. Secondary metabolites in *M. piperita* such as essential oils, carotenes, tocopherols polyphenols, flavonoids are used commercially cosmetic, food and pharmaceutical industries (K.V. PETER [1], R. SINGH & al [2]). Especially, the essential oil (EO) of *M. piperita* is commonly used in traditional medicine as antimicrobial (A. SARTORATTO & al [3]), antioxidant (D. YADEGARINIA & al [4]), cytotoxic and antiallergenic agent (D.L. MCKAY & al [5]). EO obtained from the leaves of the *M. piperita* is approximately 1-4% is menthol which economically valuable. It is an component of great importance used in flavor, toothpaste, oral spray, anemic balsam, cough, sugar and gum products (L. KARUZA & al [6], G. ISCAN & al [7], M.D. SOKOVIĆ & al [8]).

Candida albicans among many Candida species, is the lead actors of many diseases induced by fungal infection. They alone or in union with other species cause nosocomial bloodstream infections with high morbidity and mortality ratios. Although antibiotics such as fluconazole and amphotericin B are used for *C. albicans*, herby therapies are needed in the treatment of fungal diseases because of obstruction the spread of *Candida* types products (L. OSTROSKY-ZEICHNER & al [9], H. WISPLINGHOFF & al [10], D.J. DIEKEMA & al [11], B. OZCELIK & al [12]). Therefore, multi drug therapy may offer advantage in situations which natural antimicrobial agents such as essential oils were used in combinations with antibiotics against *C. albicans*.

Many studies have reported that various components of essential oils interact with antibiotics to either decrease or increase antimicrobial performance (P.J. DELAQUIS & al [13], A. MOUREY & al [14]). However, there are not enough studies with the interaction of *M. piperita* EO with antibiotics. Therefore, it was assessed anti-candidal properties of the mint EO against *C. albicans* by broth microdilution methods, and the synergistic effect (SynE), indifferent effect (IndE) or antagonistic effect (AntE) of FLC**Mp*EO combinations using checkerboard microdilution method. Additionally, it was observed some damaged parts in yeast cell surfaces observed by scanning electron microscopy (SEM) at the end of the 24-h yeast incubation with *M. piperita* EO.

Materials and Methods

1. Plant material

Aerial parts of *M. piperita* were collected in Mersin (Turkey), in May 2017 and the botanical identification of mint were done according to Davis's book (P.H. DAVIS & al [15]). Dried aerial parts of the plant (100 g) were ground in a waring blender and then subjected to hydrodistillation for 4 h according to the standard procedure defined in the European Pharmacopoeia. The essential oil of the plant was stored at 4°C in the dark until analyzed (E. ERDOGAN & al [16]). The chemical characterization of the oil was carried out in the Mersin University Advanced Technology Education Research and Application Center (MEU-MEITAM) by GC-MS analysis.

2. GC-MS analysis

The GC-MS analysis was done on an Agient 7890A; fitted with an apolar HP-5MS fused-silica capillary column (30 m × 0.25 mm i.d., 0.33 µm film thickness); Helium was used as carrier gas in the analyzes (at a flow rate of 1 mL/ min). Column temperature was performed according to 60°C, with 5 min initial hold, and then raised to 240°C at 15°C/min, and then to 250°C (20 min); injection mode Split 1/50. Analysis was coupled to an Agilent Mass Selective Detector MSD 5975; ionization energy voltage 70 eV. Mass spectra were scanned in the range 30-550 amu. Most components were identified by comparison of their Kovats retention indices (Ri) and NIST 02 and Wiley 275 libraries in mass spectra.

3. Antifungal screening (MIC, FIC and FIC index)

The combined antifungal activity of *M. piperita* EO (*Mp*EO) and antibiotic (FLC) were researched on *Candida albicans* using modified microdilution chequerdoard technique. Firstly, the inoculum of *C. albicans* was prepared in 4 mLSabouraud Dextrose Broth incubated at 37ºC, overnight. After 24 hours, the culture suspension were adjusted to 0.5 McFarland Standard Turbidity and stored at +4°C until use (J. MCFARLAND [17]). Mint EO were dissolved at 50 µL/mL(4.29 mg/mL) with dimethyl sulfoxide (10% DMSO). The experiment were performed on 96-well microtiter plates and firstly 50 µLof Mueller Hinton Broth (MHB) medium were added into all wells. Two-fold serial dilutions of 50 µLmint oil was made (A1-H1) on the y-axis along of chequerdoard plate. Two-fold serial dilutions of 128 µg/mLFLC was made x-axis along from 2nd to 10th column and mint oil (single concentration) was added to each row except A2-A10 row as follows Figure 1, in order to obtain the FIC final concentrations. Finally, 10 µl culture of microorganisms was inoculated on all wells except medium control wells.

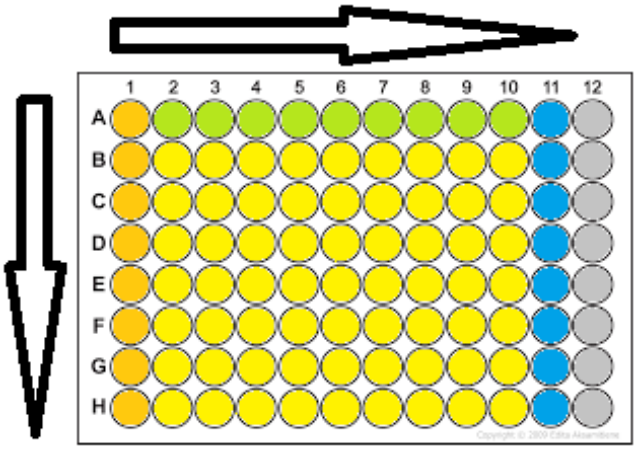

Figure 1. Design of the FICI experiment on a Eliza plate; orange wells: mint oil dilution and yeast, green wells: antibiotic dilution and yeast, yellow wells: antibiotic and mint oil combinations and yeast, blue wells: yeast growth control, gray wells: media growth control.

4. The calculation of MIC value

All plates were incubated at 37°C for 24 hours, the growth (turbidity) was measured at 415 nm. For MIC analysis, the optical density was read both before, T0 and after 24 hours-incubation, T24. For each plate, MIC were calculated using the following formula: The OD for each replicate at T0 was subtracted from the OD for each replicate at T24. The Percent growth = (ODtest/OD control)x100. Percent Inhibition = 100-(OD test well/OD of corresponding control well)x100 for each row of the 96-well plate. The dose–response curves obtained from plotting the linear of the concentration of the oils against the resulting percent inhibition of fungal growth were obtained with the regression analysis, giving an R^2 value. MIC (the lowest concentration of test material which results in 99.9% inhibition of growth) were calculated using the R^2 formula on inhibition curve (T. PATTON & al [18], Y. SICAK & al [19]).

5. The calculation of FICI value

For each plate, FIC and FIC index were calculated using the following formula: all of the wells of the microtitration plates that corresponded to an MIC, the sum of the FICs (ΣFIC) was calculated for each well with the equation; $\Sigma FIC = FIC_{A(Antibiotic)} + FIC_{M(Mint\ oil)} = (MIC_{(A+M)/A}) + (MIC_{(A+M)/M})$, where MIC_A and MIC_M are the MICs of antibiotic and mint oil alone, respectively, in all of the wells corresponding to an MIC (isoeffective combinations. The meaning of FICI; synergistic effect (SynE) when FICI value ≤ 0.5; an indifferent effect (IndE) when $0.5 < FICI \leq 4$ and an antagonistic effect (AntE) when FICI value>4 were described previously (T. STERGIOPOULOU & al [20], F.C. ODDS [21]).

6. Cell surface analysis using SEM on *C. albicans*

After 24 hours incubation of *Mp*EO with *C. albicans*, cultures in MIC wells were collected for scanning electron microscopy (SEM) examination. The yeast culture was centrifuged in a 1.5 mLeppendorf at 15000 rpm for 5 min and pellet was resuspended in 1.5 mL of 2.5% glutaraldehyde solution for 4 h. Then the sample were dehydrated by successive 10-min incubations in 35%, 50%, 70%, 95% and 100% ethanol. After the final incubation, the samples were allowed to dry on aluminum foil. Finally, before microscopic imaging, the speciments were dried at "critical point" (EMITECH K850) in liquid CO_2 under 60-mbar pressure. The samples were platin covered by sprying (Quorum 150R ES). The samples were examined with the SEM (ZEISS SUPRA 55) (S.C. CHAN & al [22], M.M. HARRIOTT & al [23]).

7. Statistical analysis

Statistical analyses and significance were measured by Student's *t*-test (for paired samples), or LSD test in one-way analysis of variance for MICs and three independent t-tests for SEM using SPSS 25. The experiment was repeated at least 3 times. Differences were considered significant at $p \leq 0.05$.

Results and Discussion

1. Chemical Composition

The components of the mint oil extracted mint leaves with its retention time (Rt) and quantity (%) were listed in Table 1. Analysis of the oil from mint were found that it contained major essential oils, menthol (36.01%), menthone (14.8%), isomenthone (10.8%), neomenthol (8.7 %), metilaseat (8.21%), limonene (3.25%), 1,8 cineole (2.98%), *α* pinene (1.68%), *β* pinene (0.69%), piperithone (0.63%), and trace amounts less than 0.3% with sabinene, *α* terpinene, *p*-cymene, *α* terpineol, *β* caryophyllene, germacrene D and thymol, which together comprised 93.55% of the essential oil in this report.

Similarly, Derwich and coworkers reported menthone, menthol, menthyl acetate as the dominant compound of *M. piperita* EO collected from Atlas median in the region of Meknes (Morocco) (E. DERWICH & al [24]) in agreement with other authors, such as Sun and coworkers (2014) identified menthol, menthone and menthy acetate occurring abundantly in mint collected in China (Z. SUN & al [25]). In another study, it was reported that *M. piperita* contained *α*-terpinene, isomenthone, *trans*-carveol, piperithone oxide, and *β*-caryophyllene as the main compounds (D. YADEGARINIA & al [4]). However, it was not detect carveol derivatives in *M. piperita* EO in the present study.

Table 1. Chemical composition of essential oil from *M. piperita*

RT (min)	Component	Quantity (%)	RT[a] (min)	Component	Quantity[b] (%)
8.09	*α*-pinene	1.68	15.81	menthone	14.8
9.65	sabinene	0.28	18.37	menthol	36.01
9.90	*β*-Pinene	0.69	19.33	methyl acetate	8.21
10.07	*α*-Terpinenee	0.21	21.82	*α*-terpineol	0.26
11.35	limonene	3.25	22.81	piperithone	0.63
11.68	1,8 cineole	2.98	23.15	*β*-caryophyllene	0.22
12.15	*p*-cymene	0.12	24.87	germacrene-D	0.10
13.14	neomenthol	8.7	25.22	thymol	0.11
14.64	isomenthone	10.8		**TOTAL**	**89.05%**

[a]RT: Retention Time
[b]Quantity (%): more than 0.1

2. Antifungal Activity and Evaluation of FICI value

According to the findings of this results, The MIC of *M. piperita* EO and FLC were 13.6 µg/mLand 15.4 µg/mLfor *C. albicans* (Table 2), respectively.

EO of *M. piperita*, whose major compound were menthol (53.28%), menthyl acetate (15.1%) and menthofuran (11.18%), were found to inhibit *C. albicans* at concentrations up to 2 µl/mL (M.J. SAHARKHIZ & al [26]). According to Iscan and coworker's study, (2002), *M. piperita*, whose dominant compounds were menthol and menthone, were found to inhibit to *C. albicans* at MIC of 0.625 mg/mL(G. ISCAN & al [7]). In contrast, In Sartoratto's work, MIC value from EO of *M. piperita* which comprise different dominant content such as linalool, carvone, and 3-octanol determined against *C. albicans* were 0.74 mg/mL(A. SARTORATTO & al [3]).

Essential oils as secondary metabolites extracted from plants were known affective many pathogen microorganisms for years. However, that natural antimicrobial agent were not known interactions with synthetic antimicrobials. For this, spectrophotometric checkerboard technique were used to detect interactions between oils and antibiotics (S. HEMAISWARYA & al [27], K.A. HAMMER & al [28], K.V. KON & al [29], F. LV & al [30]).

Table 2. MIC values of combinations of *M. piperita* and FLC alone and in combination and FICI values of the combination of them with *C. albicans* by 24 hours of incubation. The average MIC values were expressed with the standard deviation (±) and significance level (p<0.01). Statistically, (SPSS-ANOVA, LSD) there is no difference between the MIC groups (p<0.05). n = 3. FLCd + *Mp*EO$_{100}$: Antibiotic (FLC) dilution + 25 µLmint oil concentration (100 µg/mL). Interpretation of FICI values of FLC**Mp*EO combinations against *C. albicans* were found to be 100% Indifferent Effect (Ind).

	C. albicans		**Interpretation of FICI values of FLC*_Mp_EO**
	MIC (µg/mL)	**FICI**	
FLCd	$13.6_{\pm 2.1}$	-	
*Mp*EOd	$15.4_{\pm 7.4}$	-	
FLCd+ *Mp*EO $_{100}$	$16.2_{\pm 5.3}$	2.2	Ind
FLCd+ *Mp*EO $_{50}$	$16.1_{\pm 1.2}$	2.2	Ind
FLCd+ *Mp*EO $_{25}$	$18.3_{\pm 5.2}$	2.5	Ind
FLCd+ *Mp*EO $_{12.5}$	$19.6_{\pm 3.3}$	2.7	Ind
FLCd+ *Mp*EO $_{6.25}$	$20.1_{\pm 0.2}$	2.8	Ind
FLCd+ *Mp*EO $_{3.125}$	$18.6_{\pm 26.2}$	2.6	Ind
FLCd+ *Mp*EO $_{1.56}$	$18.4_{\pm 2.7}$	2.5	Ind

The interaction data in the form of the fractional inhibitory concentration indices (FICIs) were listed in Table 2. When the MIC values of FLC* *Mp*EO combinations were studied, the MICs of FLC* *Mp*EO combinations were found to be between 16.1 and 20.1 µg/mL(p<0.05). However, the values of the MIC in all FLC*MSO fractions were found is not statistically different from the MIC values determined from FLC and MSO alone against *C. albicans*. According to the current study, exposing *C. albicans* to FLC* *Mp*EO combinations during 24 h, only indifferent effect were occured as 100% for *C. albicans.* None syn. and ant. effects and remarkable indifferent action was observed against yeast according to FICI range of 2.2 to 2.8.

In literature, there were a few studies in which indifferent effect was detected on *C. albicans*, not related to essential oils. For instance, in a study about additive interaction of ginger starch on anticandidal activity of honey, 32% honey (v/v) with 4% starch and 36% honey (v/v) with 2% starch in media occured additive effect on *C. albicans* (A. MOUSSA & al [31]). *In vitro* interaction of terbinafine with fluconazole against 30 strains of *C. albicans*, indifference was observed in 60% of the terbinafine (antifungal agent)-fluconazole interactions *albicans* (F. BARCHIESI & al [32]). In another study, the combination of *Lasienthera africana* and *Heinsia crinata* (African medicinal plants) produced an additive effect (indifference) on *C. albicans* (I.E. ANDY & al [33]). In contrast, in studies where essential oils and antibiotic combinations are studied on *Candida anbicans*, the synergistic effect is mostly determined. Stringaro and coworkers noted that Fractional inhibitory concentrations indices (FICI=0.375) of antifungal drug fluconazole (FLC) combined with *Mentha suaveolens* EO against *C. albicans* were noted synergism (A. STRINGARO & al [34]).

The *M. piperita* EO showed synergistic activities with vancomycin, gentamycin, and amphotericin B on *C. albicans* with the FICI of 0.128 (M. MAHBOUBI & al [35]). *Thymus vulgaris* essential oil and amphotericin drug (antifungal agent) combinations were found synergistic

performance on *C. albicans* (B. SURESHA & al [36], R. GIORDANI & al [37]).

3. SEM analysis on *C. albicans*

Changes in *C. albicans* cell surface were observed in high magnification SEM images (Fig. 2). Mean size of *C.albicans* cells were calculated as 3353 $_{\pm 79.0}$ µm taken from non-treated and 4086 $_{\pm 58.5}$ µm incubated with mint oil. Although, the change in cell volume reported was not found to be statistically significant (p<0.05). It is rather clear that the yeast cells suffered from the mint oil. It was observed irregular spread patterns in treated *C. albicans* with *Mp*EO.

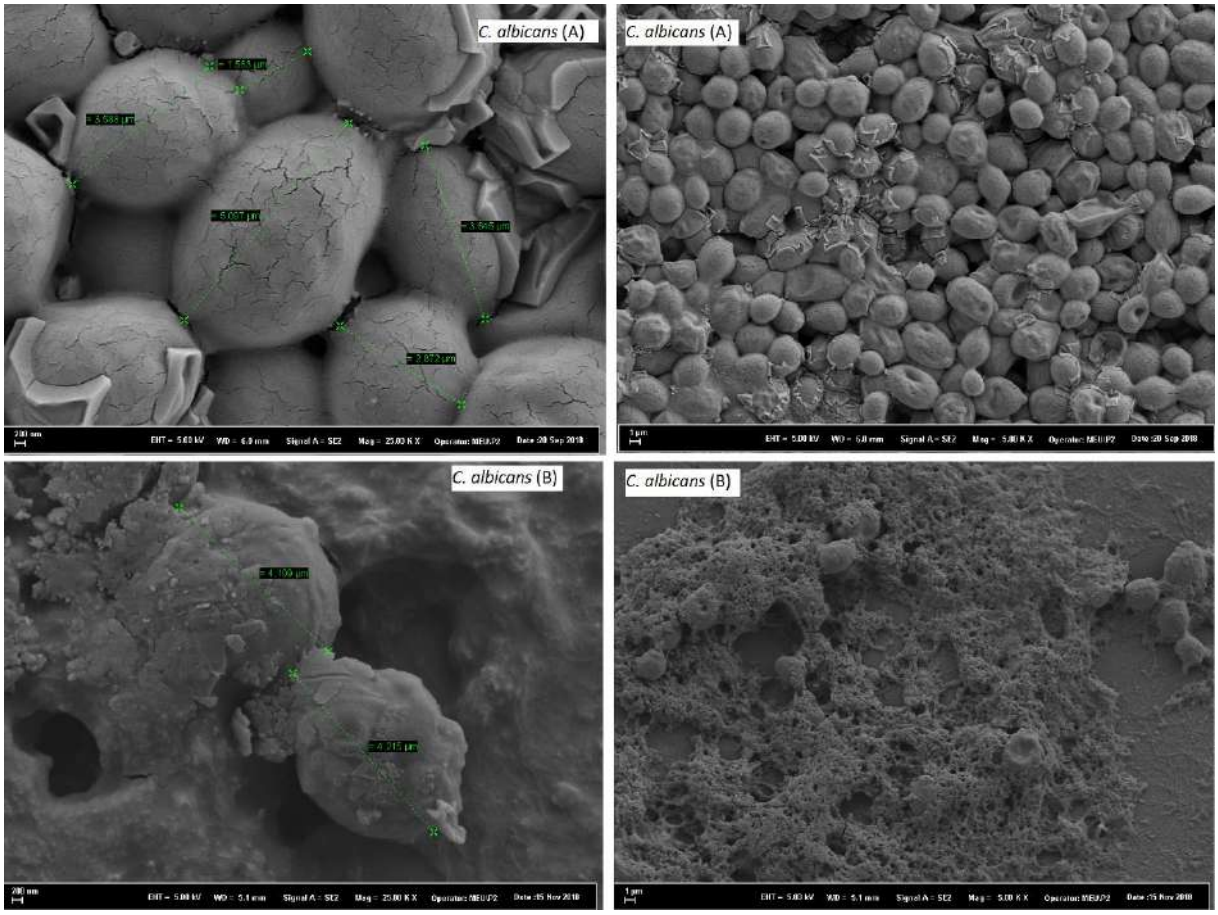

Figure 2. Representative SEM images of attached of *C. albicans*, (A) taken from non-treated (Mean cell size: 3353 $_{\pm 79.0}$ µm) and (B) incubated with *M. piperita* essential oil (Mean cell size: 4086 $_{\pm 58.5}$ µm) after 24-h incubation. B images show a general overview of the fungal attachment by deformation in cell surface. n=5, p<0.05.

Conclussion

In summary, this paper provided an example of indifferent interaction of FLC* *Mp*EO oil combinations against *C. albicans*. Peppermint oil with menthol and menthone as the major components exhibits high antifungal activity against *C. albicans*. It was clearly showed cell die due to damaged surface with images of SEM. The combination of peppermint oil with FLC should be used to treatment of antibiotic and herbal therapy at the real time. Thus, mint oil alone or in combination with other antifungal agents, may provide a promising new scheme in ethnobotanical antifungal treatments.

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