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Anticandidal Activity and Anticandidal Mechanism of Essential Oil of *Cuminum cyminum* L. and *Myrtus communis* L. Mixture

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ABSTRACT

C. cyminum (cumin) and *M. communis* (myrtle) comprise many biologically active molecules. In this study, cumin seed and myrtle leaves were mixed then hydrodistilled and analyzed by GC-MS. The main components of the essential oil were cuminal (50.71%), 1,8 cineole (8.30%), O-cymene (7.88%), ß-pinene (7.62%), α-pinene (7.16%), γ-terpinene (6.09%) and α-terpinolene (2.19%). The antifungal activity of the essential oil against *C. albicans*, *C. parapsilosis* and *C. tropicalis* was investigated using spectrophotometric broth microdilution, colorimetric broth microdilution, and agar well diffusion tests. Antimicrobial mechanism of the EO was researched by TTC-dehydrogenase relative activity, protein and DNA leakage analysis. While the MIC values of the oil were 3.29 mg mL^{-1} for *C. albicans*, 3.57 mg mL^{-1} for *C. parapsilosis* and 3.65 mg mL^{-1} for *C. tropicalis*, the 50% cytotoxic concentration values were between 0.17 mg mL^{-1} and 2.61 mg mL^{-1} for *Candida* species. The inhibition values against yeasts in dark and light conditions were found as 42.1 mm on *C. parapsilosis* and 39 mm on *C. tropicalis* exposed dark and light, respectively. The inhibition zones of oil in water, glucose, glycerol and salt environment. As a result, the highest IZ was found as 26.6 mm on *C. albicans* and 26 mm on *C. tropicalis* in the presence of a 3% glycerol environment. The DNA and protein levels were increased when yeast was exposed to the EO. As a result, the oil of mixed *C. cyminum* and *M. communis* preserved its antimicrobial stability in different environmental conditions and should contribute to new antifungal research.

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Cuminum cyminum L. ve *Myrtus communis* L. Karışım Uçucu Yağının Antikandidal Aktivitesi ve Antikandidal Mekanizmasının Belirlenmesi

ÖZET

C. cyminum (kimyon) ve *M. communis* (murt) pek çok aktif bileşeni olan özellikle antimikrobiyal, antioksidan ve antidiyabetik özellikleriyle bilinmektedirler. Bu çalışmada, ilk kez olarak, kimyon tohumları ve murt bitkisinin yaprakları birlikte su distilasyonuna tab tutulmuş ve elde edilen uçucu yağın bileşenleri GC-MS cihazıyla belirlenmiştir. Uçucu yağın temel bileşenleri cuminal (%50.71), 1,8 sineol (%8.30), osimen (%7.88), ß-pinen (%7.62), α-pinen (%7.16), γ-terpinen (6.09%) ve α-terpinolen (%2.19) olarak belirlenmiştir. Antifungal aktiviteyi belirlemek amacıyla uçucu yağın *C. albicans*, *C. parapsilosis* and *C. tropicalis* mantarlarına karşı Spektrofotometrik Broth Mikrodilüsyon, Kolorimetrik Broth Mikrodilüsyon ve Agar Well Difğzyon metodlarıyla etkileri belirlenmiştir. Uçucu yağın animikrobiyal etkisini belirlemek amacıyla TTC-dehidrogenaz aktivite, protein kaçağı ve DNA aktiviteleri incelenmiştir. Yağlarin MIC değerleri *C. albicans* için 3.29 mg mL^{-1}, *C. parapsilosis* için 3.57 mg mL^{-1} ve *C. tropicalis* için 3.65 mg mL^{-1}, CC50 (%50 sitotoksisite konsantrasyonu) değerleri ise

Mikrobiyoloji

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çalışılan kandidalar için 0.17 mg mL^{-1} ve 2.61 mg mL^{-1} olarak belirlenmiştir. Yağların mayalara karşı karanlık ve ışık (standart LED ışık) koşullarında etkilerinin de belirlendiği çalışmada en yüksek inhibisyon 42.1 mm ile *C. parapsilosis* ve 39 mm ile *C. tropicalis*'de sırasıyla karanlık ve ışıkta bulunmuştur. Çalışmada ayrıca yağların su, glikoz, gliserol ve tuz koşullarında inhibisyon bölgeleri (IZ) 26.6 mm ile *C. albicans* ve 26 mm ile *C. tropicalis*'de %3 gliserol koşullarında bulunmuştur. Mayaların DNA ve protein seviyelerinde yağa maruz kalma durumlarına göre artış tespit edilmiştir. Sonuç olarak, kimyon ve murt birlikte distile uçucu yağının antimikrobiyal stabilite gösterdiği ileri çalışmalar yapılması gerektiği tespit edilmiştir.

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INTRODUCTION

Candidiasis is a common infection found in the gastrointestinal tract, oral cavity, and esophagus, skin, vagina, and vascular system. The most important species isolated is *Candida albicans* (Calderone et al., 2001). However, in the last two decades, infections caused by non-albicans *Candida* species have been observed to increase (Miguel et al., 2005). Candida species for instance *C. albicans*, *C. parapsilosis* and *C. tropicalis* could cause multiple nosocomial bloodstream infections with high morbidity and mortality rates (Wisplinghoff et al., 2004; Pappas et al., 2016). *C. parapsilosis* could cause invasive candida related disease (Trofa et al., 2008). The other emerging human pathogen is *C. tropicalis*, identified with more detailed screening methods and it causes serious infections with other Candida pathogens (Silva et al., 2012).

Many antifungal drugs are used in the treatment of candidal infections, and the side effects of these drugs occur especially in immunocompromised patients. In addition, candidal resistance emerges as another important problem even in healthy people (Whaley et al., 2017). For this reason, plants are being constantly being researched to discover new antifungal agents. Essential oils, (EOs), which have particularly strong effects against microbes, are extracted from a wide variety of plants and are widely used in antimicrobial research (Bakkali et al., 2008). Some of those are the essential oils of cumin, fennel, manuka (Elisa et al., 2019), thyme, pennyroyal, and lemon (Mahdavi et al., 2010), which have strong antifungal performance. Among medicinal plants, *C. cyminum* (cumin) and *M. communis* (myrtle) are known as two important aromatic plants with very high biological activity. *Myrtus communis* L., belongs to Myrtaceae family, is an evergreen aromatic tree in bush form, and widely grows throughout the Mediterranean region (Aleksic and Knezevic, 2014). Especially leaves of myrtle have been used in medicine for years as an antidiabetic, antiseptic, analgesic, emmenagogue, astringent, haemostatic, and cardiotonic agent, and in lithotripsy applications (Sumbul et al., 2011). Furthermore, myrtle leaves essential oil have been used perfume and cosmetic industry and, owing to high antimicrobial activity even in post-harvest applications (Zomorodian et al., 2013; Bahadirli et al., 2020). *M. communis* plants cultivated in the Mediterranean is for its fruits, but for leaves, demands consume mostly by collecting from nature. *C. cyminum* L., from the Apiaceae family, is an annual herbaceous plant, that has been used as a medicinal herb in Asia, Europe, and Africa (Johri, 2011; Al-Rubaye et al., 2017). Cumin is an important export crop of Turkey after poppy, oregano and bay laurel (Kırıcı et al., 2020). Cumin is cultivated in ca. 361.761 da area, especially in Central Anatolia (Kırıcı et al., 2020; Baydar, 2020). Dried seeds of *C. cyminum* are commonly used for culinary and medicinal purposes (VanderJagt et al., 2002). Many cumin types are used in traditional medicine and veterinary applications as antispasmodic, due to their appetizing, carminative, stimulant, and astringent effects and as a remedy against flatulence, diarrhea, and indigestion (Morton, 1976).

Aromatic plants are very active against microorganisms due to their bioactive molecules. Research conducted so far on the essential oil contents of these plants has made important contributions to microbiology. In recent years, studies have been investigating the antimicrobial effects of combinations of essential oils with different herbs and antibiotics to control pathogenic microorganisms and to reveal resistance mechanisms and multiple biochemical processes (Bassolé and Juliani, 2012; Bajpai and Dash, 2012). Also, essential oils used for pharmaceutical, food, or cosmetic purposes are generally not used alone. They are mostly found in

mixtures in the drugs or foods (Başer, 2009), and exposed to environmental factors such as pH, food additives (Gutierrez et al., 2008), and light (Erdoğan Eliuz, 2017). *C. cyminum* as a stimulant, carminative, therapeutic in respiratory diseases (Thippeswamy and Naidu, 2005) and *M. communis* as a reliever, therapeutic in lung diseases are indispensable (Asgarpanah and Ariamanesh, 2015) food and medicine sources used by the people.

This study, best of our knowledge is the first study to make essential oil extraction from two plants (*M. communis* and *C. cyminum*) mixed in equal proportions. The antifungal effect and antifungal mechanism of the essential oil obtained from the mixture was investigated against *C. albicans*, *C. parapsilosis*, and *C. tropicalis*. In addition, it was researched whether there was any change in the antifungal performance of this mixture oil in NaCl, glucose, glycerol, and LED light environments.

MATERIAL and METHOD

The Extraction Method and GC-MS Analysis

Cumin seeds were purchased from an herbalist in Hatay and myrtle leaves were collected from nature in Hatay/Turkey, 2020. Cumin seeds was grounded and myrtle leaf dried and cut into small pieces. The seeds of *C. cyminum* and *M. communis* leaves were mixed (1:1) and hydro-distilled with Clevenger-type apparatus for 4 hours. The mixture of essential oil was stored at 4 °C until the time of analysis. The essential oil composition was determined with GC-MS (Agilent 7890A-5975C) equipped with an Agilent 19091S-433 model column (30m X 250 µm film X 0.25 µm). Helium was a carrier gas. The detector transfer line temperature was 260 °C and the detector ionization temperature was 250 °C. The analysis program was as follows: starting with 60 °C increased to 150 °C with 5 °C min^{-1}, from 150 to 200 °C with 1.5°C min^{-1}, from 200 °C to 240°C with 4 °C min^{-1}. The essential oil components were defined using Wiley and NIST database. Retention indices were calculated against *n*-alkanes (C8–C40).

Antifungal Screening:

Antifungal activity of the essential oil of the plant mixture against *Candida albicans* (ATCC 90028), *C. tropicalis* (ATCC 750) and *C. parapsilosis* (ATCC 22019) were tested by Spectrophotometric Broth Microdilution and Agar Well Diffusion methods. Yeasts' inoculums were prepared in 4 mL of Sabouraud Dextrose Broth media and incubated at 35°C, overnight followed the directions of document M27-A of the NCCLS. After 24 hours the yeast cultures were adjusted to 0.5 McFarland Standard (NCCLS, 1997).

Spectrophotometric Broth Microdilution Method

This method was performed to determine MIC (Minimum Inhibitory Concentration) of *C. cyminum* and *M. communis* EO mixture on *C. albicans*, *C. tropicalis* and *C. parapsilosis* using a 96-well microplate. Firstly, a 50 µL portion of the Mueller Hinton Broth (MHB) was poured in all wells and then a 50 µL portion of the essential oil mixture (3.024 mg mL^{-1}) in %10 DMSO (Dimethyl sulfoxide) was poured into the first order wells. Columns 11 (media and yeast) was the negative control and 12 (fluconazole; FLC:128 µg mL^{-1}) was a positive control. Lastly, 5 µL cultures of yeast were inoculated on all the wells except negative control. Then, the two-fold serial dilution of the EO mixture was made and the final diluted was 0.05 mg mL^{-1}. The same procedure was applied for the antibiotic. The plates were incubated at 35°C for 24 hours and the optical density of growth was measured at 415 nm after incubation by spectrophotometer (Thermo Scientific, MULTISKAN GO). The MIC of the EO mixture, which results in 99.9% inhibition of growth, was calculated using the regression curve obtained from Eq. 1 and Eq. 2 (Erdoğan Eliuz, 2020).

$$\text{Growth (\%)} = \left(\frac{OD_{test}}{OD_{control}}\right) \times 100 \quad \text{Eq. 1}$$

$$\text{Inhibition (\%)} = \left[1 - \frac{OD_{test\ well}}{OD_{corresponding\ control\ well}}\right] \times 100 \quad \text{Eq. 2}$$

Cytotoxicity Assay for Candida Species

Colorimetric (MTT: 4,5 dimethyl-2-thiazole-2,5-diphenyl-2H-tetrazolium bromide) broth microdilution method was performed to determine the 50% cytotoxic concentration (CC50) of the oil based on the method previously applied for microorganisms (Wang et al., 2010; Cruz et al., 2018). Briefly, MTT (Sigma) solution was prepared to obtain the concentration of 5.0 g/L and stored -20 °C at until the experiment. MHB medium was loaded and then two-fold serial diluted of the essential oil was added to all wells. No essential oil was added to the negative control well alone. Then, 5 µl of the yeast prepared according to Mcfarland 0.5 was added to all wells. Subsequently, the plates were left to incubate for 24-48 hours and then 25 µl of MTT (1/10 diluted from stock solution) was added to all wells and the incubation was continued for 1 more hours at 37 °C. The metabolically active fungal cells disrupted the tetrazolium chain that gave MTT its yellow color and turned into purple-colored MTT-formazan crystals. By ensuring that these crystals were dissolved in 200 µl of DMSO added to each well, the resulting color density was measured in the spectrophotometer at 575 nm wavelength, and the % viability was determined by proportioning the optical densities of

the control and test wells. The CC50 reflects the concentration of the oil required to reduce the yeast cell viability by 50% and was calculated using the equation below (Eq 3 and Eq 4). All experiments were repeated three times in the study.

$$\text{Viability (\%)} = \left(\frac{OD_{test}}{OD_{control}}\right) \times 100 \quad \text{Eq. 3}$$

Cytotoxicity (%) = 100- Viability (%) Eq. 4

Agar Well Diffusion Method

The inhibition zones of the *C. cyminum* and *M. communis* mixture on *C. albicans*, *C. parapsilosis* and *C. tropicalis* were studied with AWD method, the analysis was replicated 3 times (Erdoğan Eliuz et al., 2017). The experiment was carried out under both light and dark incubation conditions. The 50 µL of the EO mixture add to the wells and incubated at 35°C for 24 hours (dark treatment). The same procedure was repeated for the light incubation experiments and a standard white LED source (470 Lumens / 40 watts) was used as the light (light treatment). After one day-incubation, caliper and sterile distilled water were measured as negative controls.

To determine the inhibition area of the EO against the yeast in different stress conditions, the test was performed in the presence of NaCl, Glucose, Glycerol. For this, 1 mL of 1%, 3% and 9% of NaCl, glucose, glycerol solutions and 100% distilled water were prepared in aseptic conditions. The pure essential oil (100 µL) of *C. cyminum* and *M. communis* mixture was added to each solution and mixed for 2 minutes at 100 rpm. The 50 µL of the samples were added to the wells in the MHA petri dishes which were prepared and where the *C. albicans*, *C. parapsilosis* and *C. tropicalis* were spread as in the first agar well diffusion experiment. All tests were performed three times and after 24-hours of incubation, the inhibition zones were measured. Solutions with no added oil were used as a negative control.

Antifungal Mechanism of the EO of *C. cyminum* and *M. communis* Mixture

The inhibition mechanism of the essential oil of the EO of *C. cyminum and M. communis* mixture on the yeast was also determined. The yeast exposed to the essential oils then TTC (Triphenyl tetrazolium chloride dye)-DRA (Dehydrogenase relative activity) of the yeasts were analyzed according to the Ding et al., (2016) and Ersoy et al., (2019) after some modifications. TTC-DR produces insoluble reddish colored TF (2,3,5-triphenyl formazan) which is reduced by the H^+ acceptor TTC dye in the living cells. Regarding this absorbance of the TF was evaluated at 485 nm to obtain dehydrogenase enzyme activity in the cells. A 0.2 mL of yeast (McFarland 0.5) was incubated with 0.2 mL of the mixture and glucose solution (0.1 mL) with TTC (0.1 mol/L glucose and 0.4% TTC solution) for 35°C during 2.5 hours. The mixture was centrifuged at 14,000×g for 3 min and the supernatant (50 µL) was transferred to the well (96-plate) and finally, 10 µL ethanol was added. The plates were kept for 5 min in a shaking incubator to develop red. The measurement of absorbance at 485 nm was taken every 10 minutes using the UV-Vis spectrophotometer. The experiments were repeated 3 times and DRA was formulated as follows:

$$\% = \left[\frac{ODx}{ODc}\right] * 100$$

where OD_x is the absorbance of treated and OD_c is the control samples.

Intracellular protein and DNA leakage in the yeast was studied according to Qi and Hung, (2019). The 0.2 mL of yeast culture (McFarland 0.5) was incubated with the 0.2 mL of the EO mixture for 37 ° C, for 2 hours. The sample was centrifuged at 14,000×g for 3 min and the supernatant (50 µL) was withdrawn, then stained with Bradford dye reagent (50 µL) for 15 minutes to determine leakage of protein. The absorbance of the culture was measured at 595 nm.

For DNA leakage, all procedures were the same as the protein leak test. No dye was used in the process and the absorbance was determined at 260 nm (Qi and Hung, 2019).

The increase of leakage of protein and DNA was formulated as follows:

$$\% = \left[\frac{ODx - ODc}{ODc}\right] * 100$$

where OD_x is the absorbance of treated and OD_c is the control samples

Statistical Analyses

The MICs, CC50, and IZ of the EO were measured by One-way ANOVA with posthoc Tukey HSD Test ($p<0.01$ and $p<0.05$). To compare IZs of dark treatment with light treatment was used T-Test for 2 Independent Means (<0.05).

RESULTS and DISCUSSION

The Components of the EO Mixed *C. cyminum* and *M. communis*

The compounds of the essential oil of mixed *C. cyminum* and *M. communis* were determined by GC-MS analysis and a total of 19 compounds were identified. The quality values of the compounds were higher than 83% (Table 1). The main compounds were found cuminal, which was determined at a peak ratio of 50.71% and with a peak quality of 98%. Cuminal was followed by 1,8 cineole (8.30%), O-cymene (7.88%), ß-pinene (7.62%), α-pinene (7.16%), γ-terpinene (6.09%) and α-terpinolene (2.19%). The

other compounds were camphene, 1-Terpinen-4-ol, nerol, ß-myrcene, p-cymen-7-ol, phellandral, α-thujene, isobutyl isobutyrate, terpinolene, α-terpinene, O-cymene, and 2,4-dimethyl-3-pentanone.

Table 1. Chemical composition of *C. cyminum* and *M. communis* mixture essential oil
Çizelge 1. C. cyminum ve *M. communis* karışımının uçucu yağ bileşenleri

No	[a]RT	[b]RRI	Compound	% [c]RA	MW	%Quality
1	3.133	616	2,4-Dimethyl-3-pentanone	0.03	$C7H_{14}O$	90
2	6.012	736	Isobutyl isobutyrate	0.12	$C_8H_{16}O_2$	90
3	6.469	937	α-Thujene	0.17	$C_{10}H_{16}$	87
4	6.754	943	α-Pinene	7.16	$C_{10}H_{16}$	96
5	8.469	979	ß-Pinene	7.62	$C_{10}H_{16}$	94
6	9.104	992	ß-Myrcene	0.28	$C_{10}H_{16}$	92
7	9.881	1008	P-Cymene	0.05	$C_{10}H_{16}$	93
8	10.226	1015	α-Terpinene	0.05	$C_{10}H_{16}$	97
9	10.736	1026	O-Cymene	7.88	$C_{10}H_{14}$	97
10	10.944	1030	1,8-Cineole	8.30	$C_{10}H_{18}O$	99
11	12.309	1059	γ-Terpinene	6.09	$C_{10}H_{16}$	97
12	13.680	1087	Terpinolene	0.08	$C_{10}H_{16}$	98
13	14.618	1107	α-terpinolene	2.19	$C_{10}H_{16}$	95
14	18.375	1185	1-Terpinen-4-ol	0.35	$C_{10}H_{18}O$	98
15	19.230	1202	Camphene	0.98	$C_{10}H_{16}$	91
16	22.007	1260	Cuminal	50.71	$C_{10}H_{12}O$	98
17	23.266	1286	Phellandral	0.21	$C_{10}H_{16}O$	91
18	27.486	1374	p-Cymen-7-ol	0.28	C10H14O	87
19	28.500	1395	Nerol	0.33	$C_{10}H_{18}O$	83
			Total-identified compounds	92.88		
			Non-identified compounds	7.12		

[a]Retention Time relative, [b]Calculated Kováts index, [c]Relative area (peak area relative to the total peak area).
MW: Molecular Weight (g mol^{-1})

In this study, cuminal was the most detected compound in the mixed oil, and it has been shown in previous studies that it is the basic component of cumin (Li and Jiang, 2004; Sahana et al., 2011; Chaudhary et al., 2014). The 1,8 cineole, which was detected at a high rate after cumin, is one of the most important components detected in both myrtus and cumin essential oil (Chalchat et al., 1998; Özek et al., 2000; Yadegarinia et al., 2006; Gachkar et al., 2007; Mahboubi and Bidgoli, 2010; Bahadirli et al., 2021). The other molecules such as O-cymene (Chalchat et al., 1998; Gachkar et al., 2007), ß-pinene (Li and Jiang, 2004; Chaudhary et al., 2014; Curini et al., 2003), α-pinene (Chalchat et al., 1998; Allaq et al., 2020; Yadegarinia et al., 2006), γ-terpinene (Li and Jiang, 2004; Gardeli et al., 2008; Sahana et al., 2011), and α-terpinolene (Chalchat et al., 1998; Curini et al., 2003, Li and Jiang, 2004) were previously detected by several studies in both *C. cyminum* and *M. communis* EO. In literature, the compound of 2,4-Dimethyl-3-pentanone was found only in *M. communis* (Mahboubi et al., 2010), but no record was found with *C. cyminum*.

The EOs of *C. cyminum* (Allaq et al., 2020) and *M. communis* (Aleksic et al., 2014; Cannas et al., 2013) have antifungal properties, about which a great amount of data has already been published. A broad-spectrum antifungal performance of the EO obtained from both plants is due to the high percentage of cumin molecule that cumin (Naeini et al., 2014; Petretto et al., 2018) contains and α-pinene and 1,8-cineole that myrtle has (Jamoussi et al., 2005; Bouzabata et al., 2013; Bahadirli et al., 2021). There is a great lack of literature on the combination of essential oils. This study is original in this respect. The only study, that combined cumin with another herb was Minooeianhaghighi's study. Minooeianhaghighi et al. (2017), found that *C. cyminum* with *Lavandula binaludensis* mixed oils were inhibited many *C. albicans* isolates than cumin alone. In that study, only essential oils were mixed. However, in this study, it was left to hydrodistillation with cumin and myrtle, together.

The evaluation of antifungal activity and cytotoxicity of the EO on Candida species

The mean MICs, CC50 and IZs were calculated and, the results showed that the essential oil of *C. cyminum* and *M. communis* mixture were effective against *C. albicans*, *C. parapsilosis* and *C. tropicalis* by spectrophotometric broth microdilution and agar well diffusion method (Table 2). The MICs of the EO against the yeasts were between 3.29 and 3.65 mg mL^{-1} and they were 3.29 for *C. albicans*, 3.57 mg mL^{-1} for *C. parapsilosis* and 3.65 mg mL^{-1} C. tropicalis. ($p<0.05$).

Table 2. MICs and IZs of the EO of *C. cyminum* and *M. communis* mixture against *C. albicans, C. parapsilosis* and *C. tropicalis.*

Çizelge 2. C. cyminum ve M. communis karşımının uçucu yağının C. albicans, C. parapsilosis ve C. tropicalis'e karşı MIC ve IZ değerleri

Species	EO-MIC (mg mL^{-1})	FLC-MIC (µg mL^{-1})	EO-CC$_{50}$ (mg mL^{-1})	IZ (mm) in dark environment	EO-IZ (mm) in LED environment
C. albicans	3.29^{a} ±2.07	17.9 ^{a} ± 1.8	2.61^{a} ±1.4	19.3^{abcdT}±1.5	23^{efgT}±0.8
C. parapsilosis	3.57^{b} ±1.43	102.5^{ac} ±3.8	0.58^{b} ±0.5	42.1^{aeT}±0.8	33.01^{cT} ±6.6
C. tropicalis	3.65^{c} ±3.94	25.7^{bc} ± 2.8	0.17^{c} ±0.16	41.01^{bft} ±2.1	39^{dgt}±3.6

(ANOVA, p<0.05, Tukey HSD). Values on the same column with same superscript letters differ statistically (a,b,c,de,f,g). IZs in the same line (IZs in dark environment and IZs in LED environment) with "T" superscript letters: statistically significant, with "t" superscript letters: no statistically significant (T-Test for 2 Independent Means p<0.05). FLC: antibiotic

The cytotoxicity of different concentrations of the essential oils (0.38-3 mg mL^{1}) was studied on *C. albicans*, *C parapsilosis* and *C. tropicalis* (Table 2). The EO significantly decreased the viability of the yeasts and IC50 of the essential oil was 2.61 mg mL^{-1} for *C. albicans*, 0.58 mg mL^{-1} for *C. parapsilosis* and 0.17 mg mL^{-1} for *C. tropicalis* (p<0.05). When the MTT dye was reduced by the viable yeast cells, the yeast cells and was in the form of clusters of beads-like cells and completely covered with dye (Figure 1).

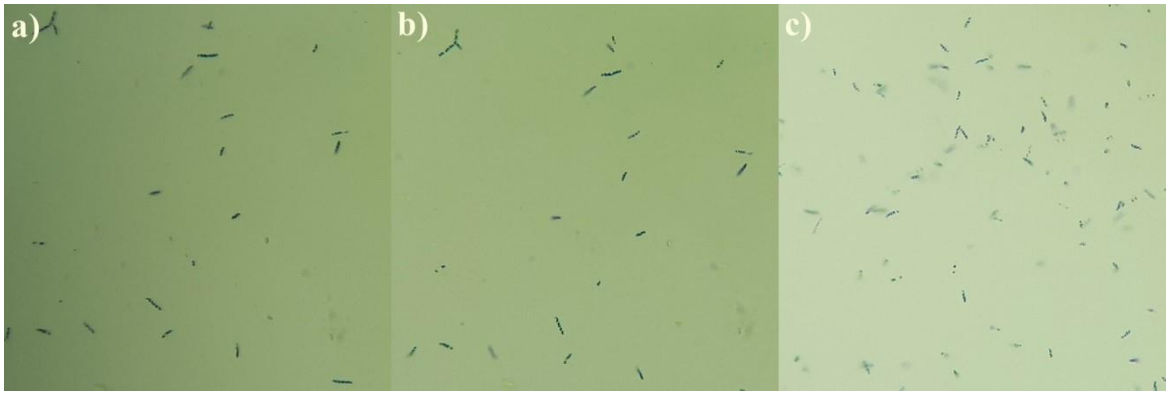

Figure 1. The micrograph in the light microscope (40X) of yeast cell-formazan crystal complexes formed in the MTT reduction solution

Şekil 1. MTT indirgeme solüsyonunda oluşan maya hücresi-formazan kristal komplekslerinin ışık mikroskobundaki (40X) mikrografı

In the present study, strong antifungal activity of the EO of cumin and myrtle part mixture was determined in standard condition (in dark incubation) against *C. albicans*, *C. parapsilosis*, and *C. tropicalis* (Figure 2). The essential oil exhibited different levels of antifungal activities depending on the dark and light treatments applied. The inhibition zones of the EO were 19.3 mm against *C. albicans*, 42.1 mm against *C. parapsilosis* and 41.01 mm against *C. tropicalis* in dark treatment, while IZs were 23 mm on *C. albicans*, 33.01 mm on *C. parapsilosis* and 39 mm on *C. tropicalis* in light treatment (p<0.05).

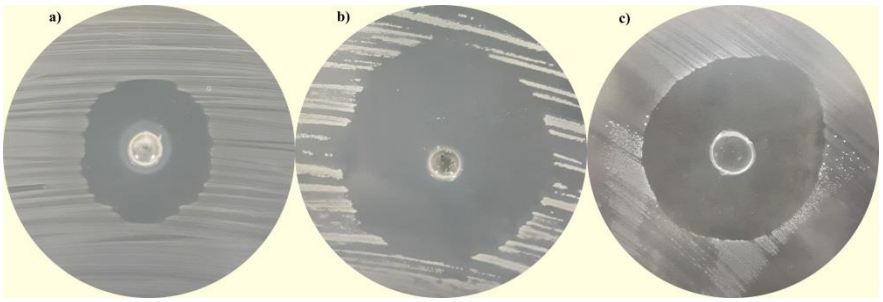

Figure 2. The images of inhibition zone of the EO of the plant mixture on C. albicans (a), C. parapsilosis (b) and C. tropicalis (c)

Şekil 2. Uçucu yağ karışımının *C. albicans* (a), *C. parapsilosis* (b) ve *C. tropicalis*'e karşı inhibisyon bölgeleri

There were any MTT study found on the effects of cumin and myrtle against Candida species, however, the CC50 values of the oil obtained from both plants can be expressed in the following order: *C. tropicalis*

(0.17 mg mL^{-1}) < *C. parapsilosis* (0.58 mg mL^{-1}) < *C. albicans* (2.61 mg mL^{-1}). When compared with the data in the literature, it is stated that cumin and myrtle alone were active against Candida-type fungi. The MICs of cumin alone were 4.375 mg mL^{-1} for many *C. albicans* strains and 2.188 mg mL^{-1} for *C. parapsilosis* because of cuminaldehyde, γ-terpinene, and ß-pinene components of cumin (Vieira et al., 2019). Considering the MIC value (3.29 mg mL^{-1}) of the mixed plants against *C. albicans* in this study, it can be said that the MIC in *C. albicans* decreased due to a synergistic effect between plants. For *Candida parapsillosis*, (3.57 mg mL^{-1}), an antagonistic effect can be mentioned because the MIC value increased with mixing the cumin and myrtle. In another article, the MICs of *M. communis* EO, the main components of which were 1,8-cineole, a-pinene, and limonene, were 2.5 mg mL^{-1} for Candida albicans ATCC 10231, *C. krusei*, C. tropicalis ATCC 13803, and *C. parapsilosis* ATCC 90018 (Bouzabata et al., 2015). In this study, the MICs of the mixture were between 3.65 and 3.29 mg mL^{-1} for Candida species. In this case, it was determined that when the cumin was mixed into the myrtle plant, the MICs increased. Similarly, the MICs of *C. cyminum* oil was 289 mg L-1 against *C. albicans* and *C. dubliniensis* (Naeini et al., 2014). Compared to Naeini's study, the enrichment of cumin with myrtle in this study may have enhanced its antimicrobial effect against *C. albicans*. In this case, an antagonistic effect can be mentioned among the oils.

The compounds of essential oil are highly affected by environmental alterations. In this study, changes in IZs were controlled by adding the EO into different solutions. As a result, it was observed that the effects of the essential oil on yeast continued in different concentrations of the solutions, but differences occurred between inhibition zones. This may be due to the interactions of the essential oil with glycerol, glucose, and NaCl. or, IZs for each fungal strain may have changed depending on their resistance status.

Antifungal activity in the stress conditions

The inhibitory effect of the EO of *C. cyminum* and *M. communis* mixture, at different NaCl, glucose, glycerol concentrations and distilled water added the EO on the yeasts is shown in Table 3. No inhibition was observed against *C. albicans*, *C. parapsilosis* and *C. tropicalis* in the presence of pure water, 1%, 3%, 9% NaCl, glucose, glycerol without EO as negative control. The maximal IZs of the EO tested in different stress conditions can be presented as: 3% glycerol (26.6 mm)> 3% glucose (24 mm)> 3% NaCl (20 mm)> Water (19 mm) for *C. albicans*; 9% glucose (20 mm)> 3% NaCl (19.6 mm)> 9% NaCl (18 mm)> 9% glycerol (17.6 mm)> water (14.3 mm) for *C. parapsilosis*; 3% glycerol (26 mm)> 1% NaCl (21.3 mm)> water (21.1 mm)> 1% glucose (17 mm) for *C. tropicalis*. The lowest IZs were 13.3 mm and 10 mm for *C. albicans* and *C. parapsilosis*, respectively, at 1% NaCl concentration, while the lowest IZ was 7.6 mm at 9% glucose for *C. tropicalis*.

Table 3. Anticandidal effect of the essential oil (10%) in the presence of NaCl, glucose, glycerol and water environments

Çizelge 3. Uçucu yağların (%10) NaCl, glikoz, gliserol ve su ortamlarında antikandidal aktiviteleri

		C. albicans			*C. parapsilosis*			*C. tropicalis*		
	C %	With EO		NG	With EO		NG	With EO		NG
NaCl	1	13.3^{abc}	±1.07	*	10^{ab}	±0.9	*	21.3^{a}	±0.08	*
	3	20^{f}	±2.0	*	19.6^{a}	±1.07	*	19.3^{b}	±0.09	*
	9	16^{d}	±2.8	*	18^{c}	±2.3	*	11.6^{e}	±1.09	*
Glucose	1	22.6^{a}	±3.0	*	16^{d}	±2.2	*	17^{f}	±1.27	*
	3	24^{b}	±3.0	*	16^{e}	±2.1	*	16.6^{g}	±2.31	*
	9	20^{g}	±1.08	*	20^{f}	±0.09	*	7.6^{abc}	±0.07	*
Glycerol	1	19.6^{h}	±1.08	*	14^{g}	±2.02	*	17.6^{h}	±1.3	*
	3	26.6^{cde}	±1.9	*	13.3^{h}	±1.11	*	26^{cd}	±1.07	*
	9	16^{e}	±1.011	*	17.6^{i}	±1.08	*	12.3^{d}	±2.04	*
Water	100	19^{i}	±2.13	*	14.3^{b}	±2.03	*	21.1^{i}	±2.07	*

C: The concentration of NaCl, Glucose, Glycerol, and water. NG: negative control, *: no inhibition zone. The mean IZs were expressed with the standard deviation (±) and significance level (ANOVA, $p<0.05$, Tukey HSD). Values on the same column with same superscript letters differ statistically at the level of 0.05.

Antifungal mechanism of the EO of *C. cyminum* and *M. communis* mixture

When a microorganism is exposed to an antimicrobial agent, the cell structure damaged and therefore many intracelluler macromolecules are left out. In this study, it was investigated the effect of the EO of *C. cyminum* and *M. communis* mixture on yeast cells (Table 4). All the treatments showed the DRA dropped from 100% to near 75% within 15 min. In yeast treated with the EO, DRA dropped to 75.01%, 81.9%, 80.1% in supernatant of *C. albicans*, *C. parapsilosis* and *C. tropicalis*, respectively, within 20 min.

Table 4. The dehydrogenase relative activity (DRA%) and the percentage increase (%) of intracellular DNA and protein leakage of *C. albicans*, *C. parapsilosis* and *C. tropicalis* after the EO of *C. cyminum and M. communis* mixture treatment

Çizelge 4. *C. cyminum* ve *M. communis* karışım uçucu yağına maruz bırakılan *C. albicans*, *C. parapsilosis* ve *C. tropicalis*'in dehidrogenaz bağıl aktivite (DRA%) ve Hücre içi DNA ve protein kaçışının ortalama artış (%) değerleri

	Time	DRA (%)	Protein leakage (%)	DNA leakage (%)
C. albicans	5. min.	90.18	6.76	1.006
	10. min	80.12	7.78	2.03
	15.min.	75.01	8.01	3.01
C. parapsilosis	5. min.	93.7	0.08	3.78
	10. min.	82.6	1.25	3.04
	15.min.	81.9	5.25	3.04
C. tropicalis	5. min.	90.1	2.46	3.83
	10. min.	80.2	3.25	4.1
	15.min.	80.1	4.01	5.2

All experiments were done in triplicate ($P \leq 0.05$). For EO activation, Standard deviation of all 3 replicate experiments was between 0.1 and 0.001. Min: minute.

After treatment by the EO at MIC, the fastest increase in protein and DNA leakage in supernatant of yeast was found in the first 20 minutes compared to the control. Percent increase in the water-soluble proteins of yeast exposed to the EO was 6.76%-8.01% for *C. albicans*, 0.08%-5.25% for *C. parapsilosis*, and 2.46%-4.01% for *C. tropicalis* in 15 min. Percent increase in DNA leakage of the yeast exposed to the EO was 1.006%-3.01% for *C. albicans*, 3.78%-3.04% for *C. parapsilosis*, and 3.83%-5.2% for *C. tropicalis* in 15 min. These results implied the cell structures of the yeast were destroyed after treatment by the EO, causing protein and DNA leakage.

The mechanism of action of most antibiotics used recently destroys the microorganism by targeting specific regions such as the cell wall of microorganisms, nucleic acids, and proteins. However, after a while, yeast gain resistance against these new antibiotics by changing the structures targeted by the antibiotic (Yao and Moellering, 2011). Therefore, it is necessary to understand the antimicrobial mechanism correctly and to reveal the effects of new antimicrobial compounds. In this study, when the yeasts were exposed to EO, there was cell destruction due to the increase in protein and DNA in the environment.

CONCLUSION

Essential oils are widely used in various fields such as the cosmetics, pharmaceutical, and food industries, and antimicrobial activity of them are their antimicrobial effect is the most powerful biological activity known. The stability of these oils alone or in mixtures is a powerful alternative in the fight against resistant strains. Therefore, we have to develop various methods against antibiotic resistance, which will be the most important problem of the future. Antimicrobial agent combinations are one of these methods. Essential oils are often not found alone in foods and medicines. With this study, it was shown that there may be changes in the antimicrobial activity of the oil, depending on the amount of salt, water, and light. When aromatic oils are hydro distilled alone or with another plant, it has been shown that there may be changes in their active ingredients. This situation can be turned into an advantage for biological activity studies. Cumin and myrtle are among the most significant herbs of traditional medicine and are the two recommended plants especially for diabetes. In diabetic studies, new drug formations can be created using these plants. These two herbs can be used in skin surface infections. Finally, environmental factors such as heat, light, and water should be included and investigated in more detail in future studies.

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Author's Contributions

The contribution of the authors is equal.

Statement of Conflict of Interest

The authors have declared no conflict of interest.

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