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Research Article

Changes in Composition, Antibacterial and Antifungal Activities of *Mentha piperita* Essential Oil After Storage

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Abstract

In this study, the effect of storage on the antibacterial and antifungal activities of *Mentha piperita* L. essential oil was investigated. Essential oils of oven-dried plant samples were obtained by hydro-distillation, analyzed by gas chromatography/mass spectrometry and stored in a refrigerator at 4 ⁰ C and in a freezer at -20 ⁰ C for one, three and six months. Antibacterial and antifungal activity of the essential oils against some gram-positive (*Bacillus cereus*, *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*), gram-negative (*Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Yersinia enterocolitica*) bacterial strains and fungi (*Candida albicans*, *Aspergillus niger*) were evaluated by agar well diffusion method. The antibacterial and antifungal effects of essential oils increased during storage. In general, essential oils stored at -20 ⁰ C produced more antimicrobial activity than those stored at +4 ⁰ C. The most affected microorganisms by essential oils were *Staphylococcus aureus*, *Proteus vulgaris* and *Aspergillus niger*. The results of the present study revealed that antibacterial and antifungal properties of *Mentha piperita* L. essential oil could be preserved and even improved when stored in proper temperature and period.

Keywords: Agar diffusion assay, peppermint, essential oil

Depolama Sonucunda *Mentha piperita* Uçucu Yağ Kompozisyonunun, Antibakteriyel ve Antifungal Aktivitesinin Değişimi

Öz

Bu çalışmada, *Mentha piperita* L. uçucu yağının antibakteriyel ve antifungal aktiviteleri üzerine depolamanın etkisi araştırılmıştır. Kurutma fırınında kurutulmuş bitki örneklerinin uçucu yağları, hidrodistilasyon yöntemiyle elde edilmiş, gaz kromatografisi/kütle spektrometrisi ile bileşen analizi yapılmış ve buzdolabında 4 ⁰ C'de, derin dondurucuda ise -20 ^{0}C'de bir, üç ve altı ay süreyle depolanmıştır. Uçucu yağlar bazı gram pozitif (*Bacillus cereus*, *Staphylococcus aureus*, *Bacillus subtilis* ve *Micrococcus luteus*), gram negatif (*Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumonia*, *Yersinia enterocolitica*) bakteri suşları ve mantarlar (*Candida albicans*, *Aspergillus niger*) üzerinde agar kuyusu difüzyon yöntemi ile denenmiştir. Depolama süresi ile uçucu yağların antibakteriyel ve antifungal aktiviteleri artmış, genel olarak -20 ⁰ C'de depolanan uçucu yağlar, +4 ⁰ C'de depolananlardan daha fazla antimikrobiyal aktivite ortaya çıkartmıştır. Uçucu yağlardan en çok etkilenen mikroorganizmalar *Staphylococcus aureus*, *Proteus vulgaris* ve *Aspergillus niger* olmuştur. Bu çalışmanın sonuçları, *Mentha piperita* L. uçucu yağının antibakteriyel ve antifungal özelliklerinin uygun sıcaklık ve sürede saklandığında korunabileceğini ve hatta geliştirilebileceğini ortaya koymuştur.

Anahtar Kelimeler: Agar difüzyon yöntemi, mentol nanesi, uçucu yağ

1. Introduction

Medicinal and aromatic plants have been used in traditional folk medicine for hundreds of years for their broad-range biological potentials due to naturally occurring bioactive compounds they possess (Rao et al., 2004; Miguel, 2010). Among these chemicals synthesized in medicinal plants, essential oils with high therapeutic properties are of considerable importance (Calo et al., 2015). Essential oils play a critical role in plant defense system as they have a variety of well-known strong antimicrobial and antioxidant behaviors (Hyldgaard et al., 2012; Seow et al., 2014). During the long time span since their first discovery, it has proven that essential oils and their constituents show antibacterial (Dorman and Deans, 2000; Nazzaro et al., 2013), antiparasitic (Anthony et al., 2005), antifungal (Cavanagh, 2007), antiviral (Astani et al., 2010), and antioxidant (Ozkan et al., 2007) activities.

Mentha piperita, a member of the family Lamiaceae and known as medicinal peppermint, is one of the oldest known multipurpose plant species used since the ancient times (McKay and Blumberg, 2006; Herro and Jacob, 2010; Dhifi et al., 2013). Peppermint provides one of the most popular and widely used essential oils, mostly due to its major compounds menthol and menthone, which are commonly used in oral caring products, medicines, cosmetics, and food industry (Scavroni et al., 2005; Tejesh et al., 2007; Kızıl et al., 2010; Kamatou et al., 2013). Several studies are available in the literature indicating carminative, diuretic, stimulatory, anesthetic, inflammatory, antifungal, and antibacterial properties of peppermint essential oil (Ribeiro-Santos et al., 2017; Singh and Pandey, 2018).

The content and composition of essential oils are affected by several factors such as climate and soil conditions, production practices, plant growth stage at harvest and postharvest conditions (Arabhosseini et al., 2007; Figueiredo et al., 2008; Barra, 2009). The composition of essential oils, on the other hand, might change due to the storage conditions of the isolated oil (Rosado et al., 2013; Mehdizadeh et al., 2017). The constituents of essential oils are generally heat-sensitive and the degree of alterations is often a function of duration and temperature of the storage (Choi and Sawamura, 2002; Rosado et al., 2013). Furthermore, changes may also be occurred even in less stable constituents due to chemical interactions with other constituents. Therefore, changes in the chemical composition of essential oil during storage reduce its shelf-life and customer satisfaction (Misharina et al., 2003; Fennell et al., 2004; Rowshan et al., 2013).

In previous studies, the effect of different storage conditions on the chemical composition of essential oils of *Citrus tamurana* (Choi and Sawamura, 2002), *Majorana hortensis* (Misharina et al., 2003), *Rosa damascena* (Mohamadi et al., 2011), *Rosmarinus officinalis* (Turek and Stintzing, 2012), *Ocimum basilicum* (Rosado et al., 2013), *Melissa officinalis* (Najafian, 2014), *Lavandula officinalis* (Najafian, 2016) and *Cuminum cyminum* (Mehdizadeh et al., 2017) were studied. However, the scientific literature is lack of information about the compositional changes of *Mentha piperita* essential oil during storage. Furthermore, to the best of our knowledge, there is no study regarding the effect of storage conditions on antimicrobial activity of the essential oils. Therefore, the present study was aimed to

determine the effect of storage temperature and duration on the composition and antibacterial and antifungal activities of *Mentha piperita* essential oil.

2. Material and Method

2.1. Plant material

Mentha piperita plants used in this study were gathered from the collection plots of medicinal and aromatic plants at Field Crops Department, Faculty of Agriculture of Ordu University, Ordu, Turkey. The plants were harvested at the beginning of flowering and dried in a drying oven at 35 ℃.

2.2. Extraction of essential oils

The dried leaf sample (100 grams) was taken from the Clevenger type distillation device and boiled at 100 degrees for 3 hours. The hydro-distilled essential oils were dried over anhydrous sodium sulfate and put in tightly closed dark vials for further analysis.

2.3. Essential oils storage conditions

The oil samples were subjected to different storage treatments to determine the effect of storage conditions on the constituents and antimicrobial activities of peppermint essential oil. The oil samples were stored at 4 ºC and -20 ºC temperatures for one, three and six successive months in a refrigerator until analysis. An oil sample which was analyzed immediately after the extraction was used as the control (unstored).

2.4. Gas chromatography and mass spectrophotometry analysis

Essential oil components were determined with the aid of GC-MS (GC/MS-QP2010 Shimadzu brand) device. TRB-5MS column with thickness of 0.25 m, length of 30.0 m and diameter of 0.25 µm was used for separation of target compounds. The column flow rate using pure helium (99.999%) was set at 1 mL/min, the temperature at injection port is 250 °C; split injection was adopted. The ion source temperature was fixed as 200°C. The oven temperature was programmed as follows: initial temperature was 40 °C at which point it was held for 2 min before finally being increased by 4 °C/min to 240°C (and again held for 3 min). Total run time was 55 min. Simultaneously; the full scan mode was obtained over a mass range from m/z 40 to 400 to confirm the identification of the analytes

2.5. Essential oil constituents

In *Mentha piperita* essential oil, components such as α-pinene, β-pinene, 1,8 cineole, menthone, menthol, pulegone, piperitone, caryophyllene are the main components. In the study, the proportional change that occurs as a result of storage in these main components is taken as a basis.

2.6. Determination of antimicrobial activity

The antimicrobial activities of essential oils were studied using eight bacteria (four gram-negative; *Escherichia coli* ATCC® 25922, *Klebsiella pneumoniae* ATCC® 13883, and *Yersinia enterocolitica* ATCC® 27729 and four gram-positive *Bacillus cereus* ATTC ®11778, *Staphylococcus aureus* ATCC® 6538, *Bacillus subtilis* ATCC® 6051, and *Micrococcus luteus* B1018) and two fungi (*Candida albicans* ATCC® 10231 and *Aspergillus niger* ATCC® 9642).

Mueller Hinton Agar (MHA, Merck), Mueller Hinton Broth (MHB, Merck) and Sabouraud Dextrose Broth (SDB, Difco), Sabouraud Dextrose Agar (SDA, Oxoid) were used for growing bacterial and fungal cells, respectively. The test microorganisms were obtained from culture repository of Biology Department culture collection at Ordu University, Faculty of Arts and Sciences, Ordu, Turkey.

For bacteria, MHA medium (Merck, 40 ml) and for fungi, SDA medium (Oxoid, 40 ml) were poured into each petri dish. All bacterial strains were grown in MHB (Merck) for 24 h at 37 ºC and the yeast and fungal strains were grown in SDB (Difco) at 27 ºC for 48 h. Overnight cultures were diluted with broth and final bacterial and yeast/fungal cell concentrations were adjusted to 10^8 and 10^7 cells/mL by measuring spectrophotometrically at 600 nm, respectively. 100 µL of each diluted suspension was placed over agar in petri dishes and dispersed. Then, sterile paper dishes (6 mm diameter) were placed on agar to load 25 µL of each essential oil sample (110g/100ml). For the fungi Nystatin and for the bacteria Ampicillin and Cephazolin were used as a positive control. Alcohol and acetone were also used as negative control. Inhibition zones which formed on the medium were measured in millimeter after incubation for antibacterial 24 h at 37 ºC and 48 h at 28 ºC for antifungal activities, respectively. All tests were made in triplicate (Taş et al., 2015).

3. Result and Discussion

3.1. Essential oil constituents

In the present study, the constituents of peppermint essential oil samples were identified in response to different storage periods and temperatures. The top eight essential oil constituents determined by GC-MS analysis were given in Table 1, but some compounds with trace amounts were not reported here. The composition of stored peppermint essential oil displayed some changes during storage which was comparable to that of the control (unstored). As shown here, menthone (33.12%), menthol (18.01%) and 1,8-cineole (11.36%) were the three main components of unstored peppermint essential oil.

Table 1 indicated that the storage of essential oils generally resulted in a decrease in the concentrations of α-pinene, β- pinene, 1,8-cineole, and piperitone as compared to the control (unstored), whereas the content of pulegone increased in all storage conditions. The contents of menthone and menthol increased with all storage periods and temperatures except for the storage at -20 ºC for 6 months and +4 ºC for 3 months, respectively. Irregular changes were

detected in the content of caryophyllene depending on the temperature and duration of storage.

Table 1. Major constituents (%) detected in *Mentha piperita* essential oils after 1, 3, and 6 months of storage at 4 and -20 ^{0}C, along with the control.

Storage	Essential oil constituents (%)							
	α-pinene	β- pinene	1,8 cineole	Menthone	Menthol	Pulegone	Piperitone	Caryophyllene
Control	**1.60**	**3.00**	**11.36**	**33.12**	**18.01**	**3.34**	**1.25**	**3.71**
1 m +4 ^{0}C	1.38	2.49	10.39	33.75	23.18	4.71	1.04	3.69
3 m +4 ^{0}C	1.54	2.68	11.20	30.46	11.94	4.90	1.11	4.15
6 m +4 ^{0}C	1.12	1.44	10.28	33.86	20.08	4.88	1.05	3.69
1 m -20 ^{0}C	1.22	2.34	10.31	35.51	23.78	4.31	1.04	2.93
3 m -20 ^{0}C	1.65	2.93	11.27	35.39	14.80	4.64	1.18	3.97
6 m -20 ^{0}C	1.20	1.42	10.55	29.20	27.10	4.30	0.95	3.81

3.2. Antibacterial and antifungal activity of the essential oils

Antimicrobial activities of the essential oils were qualitatively assessed by comparing the inhibition zone diameters obtained through disc diffusion assay; the larger the zone diameters, the stronger the antimicrobial activity is. The values of the inhibition zone diameters (mm) were given in Table 2 as the mean ± SD of triplicate experiments. The stored and unstored *Mentha piperita* essential oils presented strong antibacterial and antifungal activities against all tested microorganisms noted by high inhibition zone diameters. Zone diameters of the stored essential oils oscillated between 15.3±0.03 (for Bacillus cereus) to 46.6±0.01 (for *Aspergillus niger*). On the other hand, the unstored essential oil samples (the control) mostly exhibited less inhibition with lower zone diameters. All assayed bacteria and fungi were sensitive to the tested essential oils. In comparison to the commercial antibiotic nystatin, the antifungal activities of the stored essential oils against *Candida albicans* and *Aspergillus niger* were much higher. On the other hand, stored essential oils particularly at -20 ºC presented high antibacterial activities against both gram-negative and gram-positive bacteria compared to the comparator antibiotics ampicillin and cephazolin.

These results indicated that storage at both 4 ºC and -20 ºC for one, three and six months substantially increased antimicrobial activities of the studied essential oils. There were noticeable differences in antimicrobial activities of the essential oils stored at 4 ºC and -20 ºC temperatures. Essential oils that stored at -20 ºC showed much higher antimicrobial activities showing higher inhibition zone diameters, except for *Bacillus cereus*, compared with those of the essential oils stored at 4 ºC. In general, the differences among the duration of one, three and six months were greater at -20 ºC storage than those at 4 ºC.

All storage temperatures and periods increased inhibition zone diameters of the stored essential oils against nearly all tested microorganisms as compared to the unstored control, indicating high potential of antimicrobial activity. However, the inhibition zone diameter in *Bacillus cereus* reduced with the storage at -20 ºC for 1, 3, and 6 months. Regarding the gram-positive and gram-negative bacteria studied, *Mentha piperita* essential oils stored for different durations and temperatures displayed relatively same inhibitory activities. The essential oils evaluated in this study were less active on *Escherichia coli* and *Bacillus cereus*, with lower diameters of growth inhibition zones. On the other hand, the most affected microorganisms by essential oils were *Staphylococcus aureus*, *Proteus vulgaris* and *Aspergillus niger.*

Table 2. Diameters of inhibition zones (mm), including disc diameter of 6 mm, of *Mentha piperita* essential oils after 1, 3, and 6 months of storage at 4 and -20 ° C, along with the control

	Bacteria & Fungus				
Storage	*E. coli*	*P. vulgaris*	*K. pneumoniae*	*Y.enterocoliti*	*B. cereus*
Control	**15.3±0.05**	**21.8±0.09**	**21.4±0.03**	**25.5±0.01**	**21.0±0.08**
1 m +4 ° C	17.2±0.02	24.5±0.05	23.3±0.03	27.7±0.01	22.6±0.01
3 m +4 ° C	15.9±0.02	26.1±0.03	24.0±0.03	28.3±0.03	23.4±0.02
6 m +4 ° C	18.2±0.03	27.3±0.02	25.2±0.02	28.5±0.03	23.6±0.04
Mean +4 ° C	10.10	25.97	24.17	28.20	23.17
1 m -20 ° C	24.4±0.01	40.5±0.05	39.5±0.01	38.5±0.03	15.3±0.03
3 m -20 ° C	25.3±0.02	42.3±0.03	39.0±0.03	39.2±0.03	16.7±0.05
6 m -20 ° C	29.7±0.04	46.5±0.12	44.9±0.07	44.2±0.09	16.1±0.06
Mean -20 ° C	26.47	43.10	41.13	40.63	16.03
Ampicillin	19.0±0.00	29.0±0.00	15.2±0.01	26.6±0.57	26.0±0.97
Cephazolin	19.0±0.00	6.0±0.00	17.2±0.01	34.3±0.57	28.7±0.34
Nystatin	NT	NT	NT	NT	NT
Solvents	-	-	-	-	-

Table 2 (continue). Diameters of inhibition zones (mm), including disc diameter of 6 mm, of *Mentha piperita* essential oils after 1, 3, and 6 months of storage at 4 and -20 ° C, along with the control

	Bacteria & Fungus				
Storage	*S. aureus*	*B. subtilis*	*M. luteus*	*C. albicans*	*A. niger*
Control	**22.4±0.04**	**18.6±0.03**	**22.6±0.00**	**22.5±0.07**	**32.5±0.05**
1 m +4 ° C	25.6±0.02	21.6±0.03	26.9±0.00	25.5±0.03	35.5±0.03
3 m +4 ° C	32.5±0.03	26.4±0.02	24.2±0.05	27.1±0.03	39.1±0.03
6 m +4 ° C	33.4±0.03	27.4±0.02	25.3±0.03	26.2±0.03	44.2±0.03
Mean +4 ° C	30.50	25.13	25.43	26.27	39.60
1 m -20 ° C	41.5±0.03	36.5±0.03	39.4±0.01	32.5±0.02	36.1±0.04
3 m -20 ° C	43.3±0.03	38.2±0.00	39.1±0.03	33.2±0.03	39.6±0.10
6 m -20 ° C	45.5±0.03	42.9±0.06	43.6±0.05	37.6±0.01	46.6±0.01
Mean -20 ° C	43.43	39.17	40.70	34.43	40.73
Ampicillin	10.0±0.00	35.6±0.00	6.0±0.00	NT	NT
Cephazolin	6.0±0.00	38.6±0.11	35.7±0.02	NT	NT
Nystatin	NT	NT	NT	17.0±0.00	17.0±0.00
Solvents	-	-	-	-	-

-: no inhibition, NT: Not tested, *Escherichia coli* ATCC® 25922 gram (-), *Proteus vulgaris* ATCC® 7829 gram (-), *Klebsiella pneumoniae* ATCC® 13883 gram (-), *Yersinia enterocolitica* ATCC® 27729 gram (-), *Bacillus cereus* ATTC ®11778 gram (+), *Staphylococcus aureus* ATCC® 6538 gram (+), *Bacillus subtilis* ATCC® 6051 gram (+), *Micrococcus luteus* B1018 gram (+), *Candida albicans* ATCC® 10231, *Aspergillus niger* ATCC® 9642

To our knowledge, this study is the first attempt regarding storage-induced changes in the composition and antimicrobial activity of stored *Mentha piperita* essential oils. The major compounds identified were menthol and menthone in both unstored and stored oil samples which is in accordance with the previous findings (Iscan et al., 2002; Kızıl et al., 2010; Derwich et al., 2010; Ahmad et al., 2014; Pľuchtová et al., 2018). Menthol and menthone were the most sensitive compounds for the storage and the total contents of menthol and menthone were higher in all storage treatments except for the one at +4 ºC for 3 months than those of the control. α-pinene, 1,8-cineole and pulegone producing less changes during storage were found to be the most stable compounds. It was demonstrated that the biosynthesis of menthone and menthol started with geranyl diphosphate (the precursor of all monoterpenes) and proceeded via limonene and pulegone as intermediate constituents, and

finally menthone was reduced to menthol (Croteau et al., 2005; Davis et al., 2005). Our results were in agreement with this findings showing that the content of menthol reached its highest value (27.10%) with the storage at -20 ºC for six months, but the content of its precursor menthone (29.20%) showed the lowest record among all the treatments.

A number of studies have recently indicated that the constituents of essential oils are subject to significant changes during storage with temperature, storage period and light are the main factors (Choi and Sawamura, 2002; Sawamura et al., 2004; Mockutë et al., 2005; Najafian 2014; Mehdizadeh et al., 2017). Rosado et al., (2013), on the other hand, detected no differences in the quantities of the major components of leaf essential oil of *Ocimum basilicum* L. that were stored for one year at -20°, 4° or 25 °C, while the concentrations of the minor constituents changed significantly. The authors also stated that storage of the oil in the dark did not significantly affect the content of the oil constituents. Misharina (2001) in coriander and Misharina et al. (2003) in marjoram reported similar results indicating that storage in the dark had no significant alterations in composition, but storage in the light resulted in substantial changes. There have been no data indicating the effect of storage conditions on the composition of *Mentha piperita* essential oil, with which to compare the results of this study.

In the present study, the antimicrobial activity of *Mentha piperita* essential oil against Gram (+) and Gram (-) bacterial and fungal strains were evaluated by measuring the diameter of growth inhibition zones using disc diffusion assay. The results of the present study revealed that *Mentha piperita* essential oils had significant activity against all the bacteria and fungi tested. Although there are several papers reporting the antimicrobial activity of *Mentha piperita* essential oil, there is no study dealing with the effect of storage on antimicrobial activity (Ahmad et al., 2014; Vergis et al., 2015; Sing and Pandey, 2018). A number of studies have confirmed that the antimicrobial activity of *Mentha piperita* essential oil is mostly a function of menthol and menthone which are often the most predominant constituents of peppermint essential oil in accordance with the findings of this study (Iscan et al., 2002; Mimica-Dukic et al., 2003; Hussain et al., 2010; Kızıl et al., 2010; Kamatou et al., 2013).
It is noteworthy to mention that the antimicrobial activity of the stored essential oils underwent remarkable changes during storage. The storage at both at 4 ºC and -20 ºC for different periods considerably increased antibacterial and antifungal activities of the tested essential oils as compared to the unstored control. The storage at -20 ºC, however, was more effective than the storage at 4 ºC, which was proven with higher growth inhibition zone diameters. The biggest (231%, for *B. subtilis*) and the smallest (-23%, for *B. cereus*) changes in the antimicrobial activity noted by larger growth inhibition zone diameters were found for the essential oils stored at -20 º C for 6 months.

Interestingly, *Mentha piperita* essential oils stored for different durations and temperatures showed rather similar activities against gram-positive and gram-negative bacteria. Generally, it was stated that gram-negative bacteria are generally less susceptible than gram-positive bacteria as their outer membrane provided a barrier toward hydrophobic antimicrobial

compounds like those found in essential oils (Iscan et al, 2002; Trombetta et al., 2005; Nazzaro et al., 2013). Pľuchtová et al. (2018), however, reported Slovak peppermint and Italian spearmint essential oils showed relatively the same antibacterial activity against the selected gram-negative bacteria, but there was a variation against gram-positive bacteria.

4. Conclusion

In the present study, the changes in constituents and antimicrobial activity of *Mentha piperita* essential oils stored for 1, 3 and six months at 4 and -20 ºC were studied. The results indicated that the contents of menthol and menthone as the major components of peppermint essential oil were affected by the temperature and duration of storage. Furthermore, antibacterial and antifungal activities of peppermint essential oil were substantially increased with the storage. In conclusion, the results of the present study revealed that antimicrobial and antifungal properties of *Mentha piperita* essential oil could be preserved and even improved when stored in proper temperature and period. To the best of our knowledge, this is the first report regarding storage-induced changes in the composition and antimicrobial activity of *Mentha piperita* essential oil. Therefore, further studies are needed to determine the effect of storage on the compositional changes and antimicrobial activities of *Mentha piperita* essential oil in detail.

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