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ANTIMICROBIAL AND ANTIOXIDANT PROPERTIES OF THYME (*Thymus vulgaris* L.), ROSEMARY (*Rosmarinus officinalis* L.) AND LAUREL (*Lauris nobilis* L.) ESSENTIAL OILS AND THEIR MIXTURES

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Abstract

In this study, the antimicrobial and antioxidant properties of thyme (TEO), rosemary (REO) and laurel essential oils (LEO) and their mixtures (TEO/REO, TEO/LEO, REO/LEO, 1/1, v/v and TEO/REO/LEO, v/v/v, 1/1/1) were investigated. The antimicrobial activity was measured by the agar well diffusion method, while antioxidant capacity was measured using the FRAP and DPPH scavenging activity methods. All essential oils and their mixtures showed antimicrobial activity and antioxidant capacity. The highest antimicrobial activity against *S. aureus*, *E. coli* O157:H7 and *L. monocytogenes* was determined in TEO with zone diameters of 39.33, 28.00 and 30.67 mm, respectively. In general, essential oil mixtures negatively affected the antimicrobial activity compared to essential oils alone, and *E. coli* O157:H7 was less sensitive to the inhibitory activity of essential oils and their mixtures than *S. aureus* and *L. monocytogenes*. The FRAP values of all essential oils and mixtures ranged from 3.67 (REO) to 40.30 mg/mL (LEO), while the DPPH scavenging activity values ranged from 21.31 (REO) to 89.48% (TEO/LEO). These results suggest that essential oils obtained from thyme, rosemary, laurel and their mixtures have potential to be used as natural antimicrobial and antioxidant agents in the food industry.

Keywords: Antimicrobial activity, antioxidant capacity, thyme essential oil, rosemary essential oil, laurel essential oil

KEKİK (*Thymus vulgaris* L.), BİBERİYE (*Rosmarinus officinalis* L.) VE DEFNE (*Lauris nobilis* L.) UÇUCU YAĞLARININ VE KARIŞIMLARININ ANTİMİKROBİYAL VE ANTİOKSİDAN ÖZELLİKLERİ

Öz

Bu çalışmada, kekik (KUY), biberiye (BUY) ve defne uçucu yağlarının (DUY) ve karışımlarının (KUY/BUY, KUY/DUY, BUY/DUY, 1/1, v/v ve KUY/BUY/DUY, v/v/v, 1/1/1) antimikrobiyal ve antioksidan özellikleri araştırılmıştır. Uçucu yağların antimikrobiyal aktiviteleri agar kuyu difüzyon metodu ile belirlenirken, antioksidan kapasiteleri, FRAP ve DPPH radikal söndürücü kapasite yöntemleri ile belirlenmiştir. İncelenen tüm uçucu yağlar ve karışımları antimikrobiyal aktivite ve antioksidan kapasite göstermiştir. *S. aureus, E. coli* O157: H7 ve *L. monocytogenes*'e karşı en yüksek antimikrobiyal aktivite, sırasıyla 39.33, 28.00 ve 30.67 mm zon çapı ile KUY'de belirlenmiştir. Genel olarak, uçucu yağ karışımları, tek başına uçucu yağlara oranla antimikrobiyal aktiviteyi negatif etkilemiş ve *E. coli* O157: H7, uçucu yağ ve karışımlarının inhibitör etkisine *S. aureus* ve *L. monocytogenes*'den daha az duyarlı olmuştur. Uçucu yağ ve karışımlarının FRAP değerleri 3.67 (BUY) ile 40.30 mg/mL (DUY), DPPH radikal söndürücü kapasite değerleri ise %21.31 (BUY) ile 89.48 (KUY/DUY) arasında belirlenmiştir. Bu sonuçlar kekik, biberiye ve defneden elde edilen uçucu yağların ve karışımlarının gıda endüstrisinde doğal antimikrobiyal ve antioksidan ajan olarak kullanım potansiyeline sahip olduğunu ortaya koymuştur.

Anahtar kelimeler: Antimikrobiyal aktivite, antioksidan kapasite, kekik uçucu yağı, biberiye uçucu yağı, defne uçucu yağı

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INTRODUCTION

In recent years there has been increasing concern over the safety of synthetic food additives including the possible toxicity of those synthetic chemicals that are used as antimicrobials and antioxidants. Therefore, interest has been focused on the use of natural antimicrobial and antioxidants compounds to prolong the shelf life of food products in the food processing sector (Burt, 2004; Sacchetti et al., 2005; Santoyo et al., 2006). When compared to synthetic food additives, natural additives are readily acceptable by consumers. They are considered to be safe, no safety tests are required by legislation, they are identical to the food that people have eaten for hundreds of years or have been mixing with food, and they not only prolong the shelf life of foods but also add to the nutraceutical value of the foods (Pokorny, 1991).

Essential oils are plant secondary metabolites (Zaouali et al., 2010) and are extracted from different parts of plants such as leaves, barks, flowers, seeds, buds, twigs and fruits. They can be obtained from plant material by extraction, expression, fermentation or enfleurage but distillation is the most common method for the production of essential oil (Burt, 2004). It is reported that many different essential oils are important sources of natural antioxidants and antimicrobials (Sacchetti et al., 2005; Lin et al., 2009) and they have long been used in pharmacology, medicine, food and the cosmetic industry (Zaouali et al., 2010; Bayaz, 2014). The efficiency of essential oils depends on the nature of active ingredients (Toroğlu and Çenet, 2006). The composition and amount of essential oil vary depending on the harvesting season (Burt, 2004), the type and part of plant, the geographical structure and climate of the region where it is cultivated (Bayaz, 2014) and the extraction method (Mith et al., 2014).

Thyme (*Thymus vulgaris* L.), rosemary (*Rosmarinus officinalis* L.) and laurel (*Lauris nobilis* L.) grow naturally in Turkey (Dadalioğlu and Akdemir Evrendilek, 2004; Önenç and Açıkgöz, 2005). Among these plants, thyme, a plant of the Labiatea family (Önenç and Açıkgöz, 2005), possesses many beneficial effects such as carminative, antiseptic, antioxidant and antimicrobial properties (Lee et al., 2005; Bozin et al., 2006). Thyme essential oil (TEO) contains various terpenoids such as α-pinene, myrcene, p-cymene, γ-terpinene, linalool, thymol and carvacrol (Youdim and Deans, 2000). Quantitatively, thymol and carvacrol are major components of thyme extract (Lee et al., 2005). Thymol and other phenolic components inhibit microorganisms causing an increase in the permeability of the cell membrane and reduction of vital intracellular substances or disruption bacterial enzyme systems (Ouattara et al., 2001). The antioxidant capacity of TEO comes from its active compounds such as thymol and carvacrol (Ruberto and Baratta, 2000).

Rosemary, a member of the Lamiaceae family (Perez et al., 2007), grows naturally on dry rocky slopes and hillsides or in pine forests and is used fresh, dried or as an essential oil (Özcan and Chalchat, 2008). It is well known that rosemary essential oil (REO) has antimicrobial and antioxidant properties (Sacchetti et al., 2005; Gachkar et al., 2007; Genena et al., 2008; Zaouali et al., 2010; Ojeda-Sana et al., 2013). Rosemary contains flavones, steroids, diterpenes and triterpenes. Carnosol and carnosic acid are primarily responsible for its antioxidant capacity, while α-pinene, bornyl acetate, camphor and 1,8-cineole are related to antimicrobial activity (Genena et al., 2008).

Laurel, a plant of the Lauraceae family (Erkmen and Özcan, 2008), is an evergreen bush native to the Mediterranean region and its essential oil is used as a flavoring additive in the culinary and food industry (Santoyo et al., 2006). It is reported that the laurel plant has antimicrobial and antioxidant effects (Ramos et al., 2012; El et al., 2014) and the predominant components of its essential oil are 1,8-cineole, α-terpinene and sabinene (Dadalioğlu and Akdemir Evrendilek, 2004). The antioxidant capacity of laurel essential oil (LEO) comes from its eugenol and methyl eugenol contents (El et al., 2014).

There are many studies on the antimicrobial and antioxidant properties of thyme, rosemary and laurel plants and their essential oils. However, there is no study on the antimicrobial activity and antioxidant capacity of mixtures of TEO, REO and LEO in the literature. Hence, this study aimed to determine antimicrobial and antioxidant properties of TEO, REO and LEO and their mixtures. The determinations included antimicrobial activity against *S. aureus, E coli* O157:H7 and *L. monocytogenes* and antioxidant capacity tests such as FRAP and DPPH scavenging activity.

MATERIALS AND METHODS

Plant materials

Dried thyme (*Thymus vulgaris* L.), rosemary (*Rosmarinus officinalis* L.) and laurel (*Lauris nobilis* L.) plants were purchased from a local market in Samsun, Turkey in June 2015, powdered using a household coffee grinder (Sinbo, 2909 model, Istanbul, Turkey) and kept in bottles under cool conditions until use.

Bacterial strains

Staphylococcus aureus (ATCC 25923), *Escherichia coli* O157:H7 (NCTC 12900) and *Listeria monocytogenes* (ATCC 19111) were used as test organisms. These microorganisms were provided by The Food Control Laboratory Directorate, Samsun, Turkey.

Essential oil extraction

The essential oils used in this study were extracted by hydro-distillation using a Clevenger apparatus (Sesim Kimya Laboratuvar, Ankara, Turkey). For this purpose, a 50 g sample of each plant powder was mixed with 500 mL distilled water and placed in the apparatus for 3 h of distillation. The extracted essential oils were dehydrated using anhydrous sodium sulphate and then stored in dark glass bottles at +4 °C until use. The essential oil yields (v/w) of dried thyme, rosemary and laurel plants were 1.35, 0.98 and 1.04%, respectively.

Determination of antimicrobial activity

The antimicrobial activity of essential oils alone and their mixtures (TEO/REO, TEO/LEO, REO/LEO, 1/1, v/v and TEO/REO/LEO, v/v/v, 1/1/1) were determined by the agar well diffusion method according to Rather et al. (2012). Firstly, *S. aureus, E coli* O157:H7 and *L. monocytogenes* were grown in Brain Heart Infusion (BHI) broth at 37 °C for 24 h. After incubation, each of the bacterial suspensions was adjusted to a turbidity of 0.5 Mc Farland units in BHI. 0.1 mL from the bacterial suspension was spread on the surface of Mueller Hinton Agar (MHA, Oxoid) and the plates were allowed to dry. Then, 5 mm diameter wells were punched into the agar plate surfaces under aseptic conditions and 50 µL of each essential oil and essential oil mixture were placed in the well on the inoculated plates. These plates were incubated at 37 °C for 24 h. After incubation, total diameters of inhibition zones (bacterial growth free diameter, mm) were measured in mm, including diameter of the well (5 mm). All tests were performed in triplicate and results are expressed as average values of zone diameter.

Determination of antioxidant capacity

The antioxidant capacity of essential oils and their mixtures was determined using two different methods: the ferric reducing antioxidant power (FRAP) method according to Gao et al. (2000) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity method according to Nakajima et al. (2004) with some modifications.

For the FRAP determination, the essential oils and their mixtures were diluted with methanol for a suitable concentration and then 50 µL of diluted samples were mixed with 0.95 mL of ferric-2,4,6-tripyridyl-s-triazine (TPTZ) reagent (prepared by mixing 300 mM acetate buffer, pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$ in the ratio 10/1/1). The absorbance was measured with a UV-VIS spectrophotometer (Helios Gamma, Thermo Spectronic, USA) at 593 nm. Trolox standard curves (12.5-125 ppm, R^2=0.999) were used to calculate FRAP values and antioxidant capacity of the samples defined as mg Trolox/mL.

For the DPPH scavenging activity determination, essential oils and their mixtures were dissolved in methanol and then diluted samples were added to 1 mL of DPPH methanol solution (6 x 10^{-5} M). After vigorous shaking, the mixture was left to stand for 30 min at room temperature and the absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Helios Gamma, Thermo Spectronic, USA). DPPH scavenging activity was calculated by:

$$\text{DPPH scavenging activity (\%)} \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100 \quad (1)$$

where A_{blank} is the absorbance of the control, and A_{sample} is the absorbance of the sample.

Statistical analysis

Values were expressed as mean ± standard deviation. Data were subjected to analysis of variance (ANOVA) and significant differences of the mean values were compared using the Duncan's multiple range test. Analysis was performed using the SPSS statistical package program (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL, USA). A significance level of 0.05 was chosen.

RESULTS AND DISCUSSION

Antimicrobial activity

The antimicrobial activity results of the essential oils and their mixtures against the test microorganisms are shown in Fig. 1. As can be seen, all of the essential oils and their mixtures showed an inhibitory effect against all the test microorganisms and the inhibition zones ranged from 9.67 to 39.33 mm. Among the essential oils studied, the highest antimicrobial activity against *S. aureus* was determined in TEO with a zone diameter of 39.33 mm, followed by REO with a zone diameter of 20.00 mm, and the lowest activity was determined in LEO with a zone diameter of 17.67 mm (P <0.05). Among the essential oil mixtures, the highest antimicrobial activity was determined in the mixture of TEO/LEO, while the lowest activity was determined in the mixture of REO/LEO (P <0.05). Similar to *S. aureus*, the highest antimicrobial activity against *E. coli* O157:H7 and *L. monocytogenes* was determined in TEO with inhibition zone diameters of 28.00 and 30.67 mm, respectively (P <0.05). In general, essential oil mixtures negatively affected the antimicrobial activity compared to essential oils alone.

The antimicrobial properties of plant essential oils are due to the phenolic compounds present in their composition. In general, essential oils containing phenolic compounds such as carvacrol, eugenol and thymol at high levels show a strong antimicrobial effect against pathogenic microorganisms (Cosentino et al., 1999; Lambert et al., 2001). These compounds may inactivate the essential enzymes, react with the cell membrane activity, or disturb the genetic material functionally and disturb energy production and structural component synthesis (Celikel and Kavas, 2008). Diameters of the inhibition zones of essential oils

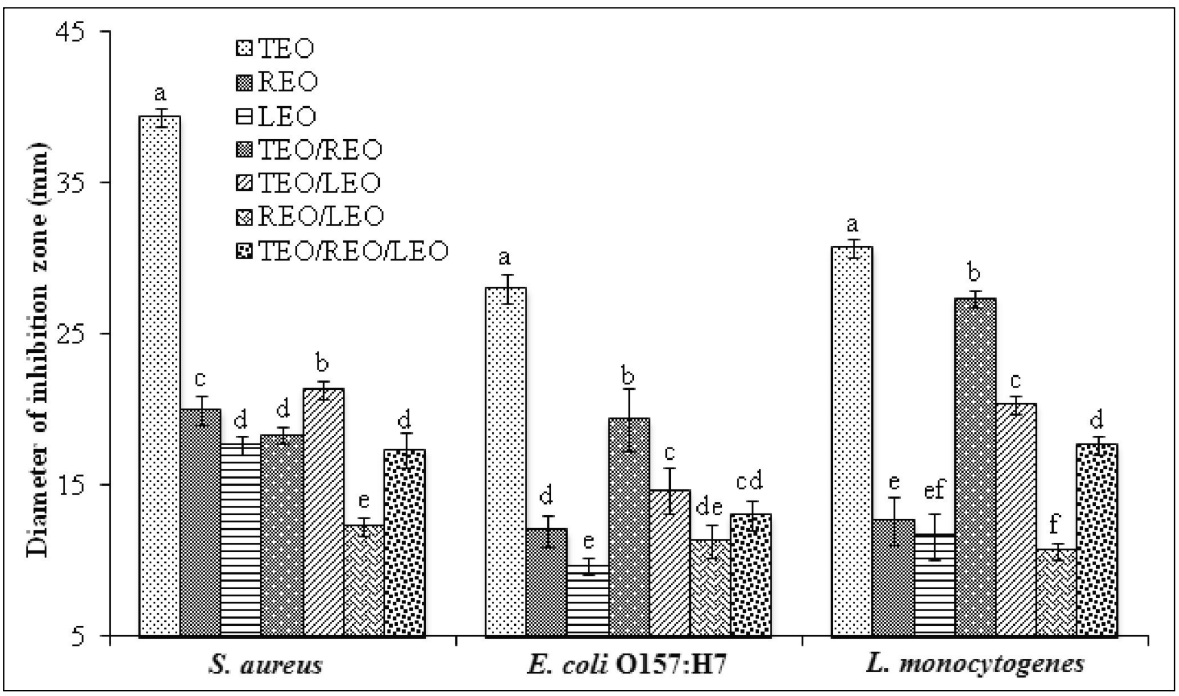

Fig. 1. Antimicrobial activity of thyme (TEO), rosemary (REO) and laurel essential oils (LEO) and their mixtures against *S. aureus*, *E coli* O157:H7 and *L. monocytogenes*. Bars represent means ± SD of three replicates. Different letters (a-f) on the bars in each group indicate significantly differences (P <0.05).

and their mixtures vary according to their components (Abdullah et al., 2015) and bacteria strains (Lambert et al., 2001). The higher antimicrobial activity of TEO could be attributed to its thymol content, a monoterpene with a phenolic ring (Miladi et al., 2013). However, the main component of REO and LEO is 1,8-cineole belonging to the ethers group (Celikel and Kavas, 2008; Miladi et al., 2013). Similar to our findings, Abdollahzadeh et al. (2014) showed that TEO oil had a higher antimicrobial activity than REO against *L. monocytogenes* (PTCC 1163).

Antimicrobial activity of TEO, REO and LEO has also been reported by various researchers. The inhibitory effect of two thyme (*Thymus vulgaris* L. and *Thymus serpyllum* L.) hydrosols, widely used in food products against pathogenic bacteria, was tested by Sağdıç (2003) who found that hydrosols had inhibitory effects against *E. coli* (ATCC 25922), *E. coli* O157: H7 (ATCC 33150) and *S. aureus* (ATCC 2392) with inhibition zone diameters of 14-14, 12-12 and 17-18 mm, respectively. Gachkar et al. (2007) examined the chemical and biological characteristics of REO and reported that it showed 16.67, 8.33 and 16 mm inhibition zones against *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923) and *L. monocytogenes* (PTCC 1298). Also, Fu et al (2007) reported that REO showed inhibitory effect against S. aureus (ATCC 6538) and *E. coli* (ATCC 8739) and the diameters of the inhibition zone were measured as 18.5 and 100 mm respectively.

Dadalioğlu and Akdemir Evrendilek (2004) reported that the inhibitory effect of LEO on the pathogens was in the following order: *E. coli* O157:H7 > *S. aureus* > *L. monocytogenes*. The inhibitory effect of LEO against various microorganisms using the agar well diffusion method was investigated by Yilmaz et al. (2013) who reported that LEO showed antibacterial activity against *E. coli* O157:H7 (33 mm), *S. aureus* (ATCC 25923) (10 mm) and *L. monocytogenes* (ATCC 7644) (22 mm). Antimicrobial activity of essential oil obtained from laurel against *L. monocytogenes* (ATCC 7644), *E. coli* O157:H7 (ATCC 8739) and *S. aureus* (ATCC 25923) was also determined by Ekren et al (2013). Kon and Rai (2012) researched the antibacterial activity of TEO alone and in combination with other essential oils. The results showed that REO produced inhibition zones of 7.0 mm (*S. aureus*) and 7.4 mm (*E. coli*) while LEO produced inhibition zones of 10.9 mm (*S. aureus*) and 6.9 mm (*E. coli*). However, TEO alone had 22.7 and 22.5 mm inhibition zones against S. aureus (ATCC 25923) and *E. coli* (ATCC 25922), in combination with REO had 14.6 and 15.7 mm inhibition zones, and in combination with LEO had 17.6 and 16.2 mm inhibition zones, respectively. LEO or REO were demonstrated higher antimicrobial activity when used in combination with TEO. The results of the present study are similar to the above results with slight differences. The differences could be attributed to varied environmental and ecological characteristics of the plants, extraction method of the essential oils, method of analysis and bacterial strains.

As seen in Fig. 1, in general *E. coli* O157:H7 was less sensitive to the inhibitory activity of TEO, REO and LEO and their mixtures than *S. aureus* and *L. monocytogenes*. Generally, Gram-positive bacteria are known to be more susceptible to the essential oils or antibacterial compounds than Gram-negative bacteria, which are in a good agreement with previous findings (Miladi et al., 2013; Mith et al., 2014). This resistance could be attributed to the structure of cellular walls of Gram-negative bacteria, mainly with regard to the presence of lipoproteins and lipopolysaccharides that form a barrier to restrict entry of hydrophobic compounds (Mith et al., 2014).

Antioxidant capacity

In general, the single method is not recommended for the determination of the antioxidant activities of plant extracts because of their complicated composition (Bozin et al., 2006). Therefore, two different assays were applied to determine the antioxidant activities of essential oils: FRAP and DPPH scavenging activity. These assays have different mechanisms. The DPPH method is based on the ability of antioxidants to act as radical scavengers while the FRAP method measures the ability of antioxidants to perform as reducing agents (Prusinowska and Smigielski, 2015). The antioxidant capacity results of the essential oils and their mixtures are shown in Fig. 2.

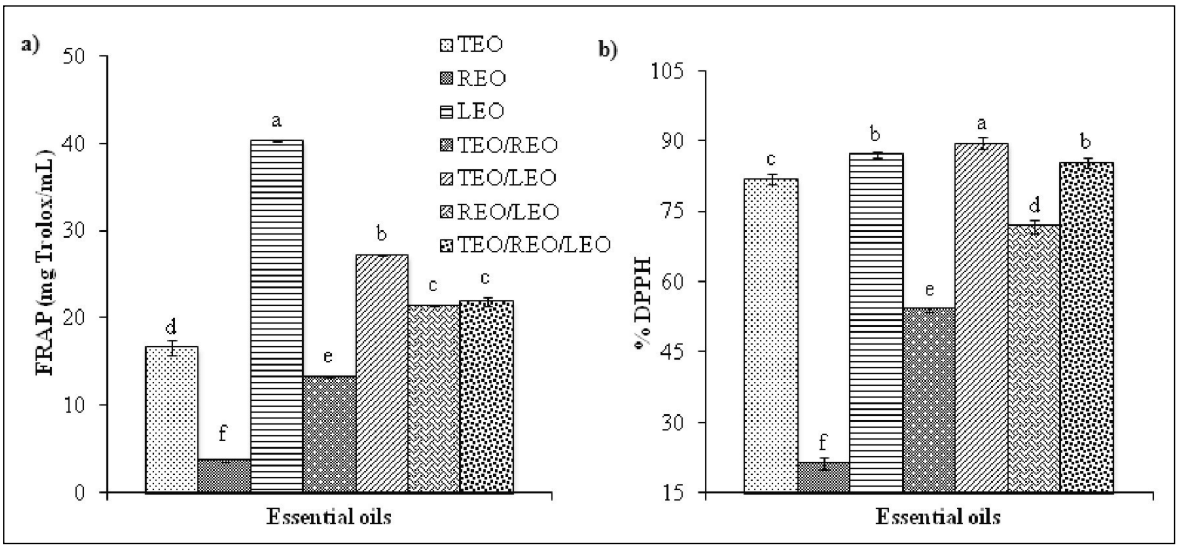

Fig. 2. Antioxidant capacity [a) FRAP and b) DPPH scavenging activity] of thyme (TEO), rosemary (REO) and laurel essential oils (LEO) and their mixtures. Bars represent means ± SD of three replicates. Different letters (a-f) on the bars indicate significantly differences (P <0.05).

As can be seen, all the essential oils and their mixtures exhibited antioxidant capacity while the highest antioxidant capacity among the essential oils alone was determined in LEO in both methods, followed by TEO and the lowest antioxidant capacity was observed in REO (P <0.05). However, the highest antioxidant capacity among the essential oil mixtures was obtained in the mixture of TEO/LEO. When the essential oils and their mixtures were evaluated together, the highest antioxidant capacity was determined in LEO based on the FRAP assay (Fig. 2a), while the highest value was determined in the mixture of TEO/LEO based on the DPPH test (Fig. 2b) (P <0.05). Despite this difference, the antioxidant capacity values determined by both methods were generally parallel to each other.

It is considered that the antioxidant capacity of LEO is related to its eugenol and methyl eugenol content (El et al., 2014), while the antioxidant capacity of TEO may be explained by its thymol and carvacrol content, two phenolic compounds with known antioxidant capacity (Ruberto and Baratta, 2000). However, the antioxidant capacity of REO comes from its epirosmanol, carnosol, rosmanol, carnosic acid, rosmaridiphenol, rosmadial, rosmarinic acid, isorosmanol and rosmariquinone content (Yanishlieva-Maslarova and Heinonen, 2001). These compounds delay oxidation by inhibiting the formation of free fatty acid radicals at the beginning with giving hydrogen from phenolic hydroxyl groups (Üstün and Turhan, 1999). Several researchers have also reported that essential oils and extracts obtained from laurel, thyme and rosemary are effective antioxidants (Bozin et al., 2006; Santoyo et al., 2006; Gachkar et al., 2007; Lie et al., 2009; Basmacıoğlu Malayoğlu et al., 2011; Yilmaz et al., 2013; Fadda et al., 2014).

In an earlier study, REO showed about 23% DPPH inhibition while DPPH inhibition of TEO was determined as 69.3% (Gachkar et al., 2007). Basmacıoğlu Malayoğlu et al. (2014) reported that DPPH inhibition for LEO and REO was 39.70 and 32.00%, respectively. El et al. (2014) studied the antioxidant capacity of essential oils extracted from laurel leaves using solvent-free microwave and hydrodistillation and reported that DPPH inhibition of LEO obtained by hydrodistillation was 83.3%. Similar to our results, Sachetti et al. (2005) reported that the DPPH scavenging activity of TEO was higher than REO. In another study, FRAP values of the essential oils obtained from six different rosemary varieties were found to be between 16.53 and 21.77 mmol/L (Zaouali et al., 2010). The results of the present study are similar to those in the above literature with some differences. These differences may be due to the variety and harvesting time of plants, environmental and regional conditions, amount of active substance, extraction method and solvent type.

CONCLUSION

In recent years, studies have focused on natural additives such as essential oils because of the toxicity and carcinogenicity of synthetic additives. The results of this study showed that all the essential oils studied and their mixtures exhibited both antimicrobial activity and antioxidant capacity. Among the essential oils, the highest antimicrobial activity was determined in TEO while the highest antioxidant capacity was determined in LEO. According to these results, TEO, REO and LEO alone and their mixtures can be used as natural antimicrobials and antioxidants in food processing.

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