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Determination of Antimicrobial Activity of Different Essential Oils Obtained from Plants on Staphylococcus aureus Strains Isolated from Foods

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ARTICLE INFO	ABSTRACT
Research Article	Essential oils (EOs) are known for its antimicrobial activity against several pathogenic bacteria. The present work evaluated the antimicrobial activity of 15 different EOs on survival of different strains of different <i>Staphyloccocus aureus</i> strains isolated from traditional cheeses by disc diffusion method.
Received : 08/02/2020 Accepted : 23/03/2020	The most antimicrobial activity on the strains was found as oil thyme oil (mean zone diameter 23.203 mm). Clove oil and black seed oil had the highest antimicrobial activity after thyme oil with average zone diameters of 13.698 mm and 11.267 mm respectively. <i>Hypericum perforatum</i> L. oil (mean zone 6.209 mm), ginger oil (mean zone 6.250 mm) and garlic oil (mean zone 6.267 mm) were the lowest antimicrobial activity. New studies about antimicrobial effect of EOs in vivo conditions are
Keywords: Staphyloccocus aureus, Antimicrobial activity Essential oils Disc Diffusion Method Food	recommended.

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Bitkilerden Elde Edilen Çeşitli Uçucu Yağların Gıda Kaynaklı Staphyloccus aureus Suşları Üzerindeki Antimikrobiyel Etkinliğinin Belirlenmesi

MAKALE BİLGİSİ	ÖZ
Araştırma Makalesi	Uçucu yağlar (EO), çeşitli patojenik bakterilere karşı antimikrobiyel aktivitesi ile bilinir. B çalışmada, 15 farklı EO'nun geleneksel peynirlerden izole edilmiş olan farklı <i>Staphyloccocus aureu</i> suşları üzerine göstermiş olduğu antimikrobiyel aktivite disk difüzyon yöntemi ile belirlenmiştir. E
Geliş : 08/02/2020 Kabul : 23/03/2020	yüksek antimikrobiyel aktivite gösteren EÖ, kekik yağı (ortalama zon çapı 23,203 mm) olara bulunmuştur. Karanfil yağı ve çörekotu yağının ise sırasıyla 13,698 mm ve 11,267 mm'lik ortalam zon çapları ile kekik yağından sonra en yüksek antimikrobiyel aktiviteyi gösterdiği saptanmıştır. San kantaron yağı (ortalama zon 6,209 mm), zencefil yağı (ortalama zon 6,250 mm) ve sarımsak yağ
Anahtar Kelimeler: Staphylococcus aureus Antimikrobiyel aktivite Uçucu yağ Disk difüzyon yöntemi Gıda	(ortalama zon 6,267 mm) ise çalışmadaki en düşük antimikrobiyel aktivite gösteren yağla EO'ların <i>in vivo</i> koşullarda antimikrobiyel etkisi hakkında yeni çalışmalar önerilmektedir.
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Introduction

Essential oils (EOs) are volatile and fragrant compounds produced by plants with oily consistency (Bassole et al., 2012). EOs are mixtures of hydrocarbons and oxygenated hydrocarbons originating from the isoprenoid pathways, mainly composed of monoterpenes and sesquiterpenes (Sharifi-Rad et al., 2017). They are synthesized in plant organs such as flower, stem, fruit, seed and secreted by secretion cells, ducts, and glands (Cicioğlu-Arıdoğan et al., 2002; Bassole et al., 2012; Sharifi-Rad et al., 2017; de Aguiar et al., 2018).

Although the EOs obtained from different plants are in the chemical composition and the proportions of the substances in the composition obtained by different methods of extraction, their basic components belong to the same chemical classes as mono and sesquiterpene, aldehyde, ketone, ether and ester, alcohol and hydrocarbons (Sharifi-Rad et al., 2017). For example, thyme and carvarol are the predominant constituents in thyme oil. The dominant active ingredient of clove essential oil is eugenol; most active substances found in black seed oil are thymokinone and p-sinene (Bulca et al., 2014; Han et al., 2017; Omonjio et al., 2018). Eucalyptus oil, laurel oil and sage oil is the dominant active ingredient 1.8 cineol (dos Santos et al., 2007; Barbosa et al., 2016; Gölükçü et al., 2017). Similarly, rosemary essential oil contains 1.8 cineol and camphor; α pinene and limonene in cumin oil, α felladren and limonene in dill oil; α pinene and α felladren in ginger oil and limonene and α pinene in pine oil are predominantly active substances (Hussain et al., 2010; Radulescu et al., 2010; Sultan et al., 2005; Demirci et al., 2015).

Although their practical use dates back to hundreds of years, it has only recently been scientifically approved. EOs are often used in cosmetics, medicine and food industry. However, EOs are known to have antimicrobial, antifungal and insecticidal activities (Cicioğlu-Arıdoğan et al., 2002). Antimicrobial activity of EOs is depended on the source of plant, concentration of the target microorganism, the composition of the substrate, the production and storage conditions of the food (Faydaoğlu et al., 2013). The mechanism of action of EOs is realized by the ability of phenolic monoterpenes to alter microbial cell permeability, cytoplasmic damage, interfere with cellular energy system and disrupt proton motility. Cytoplasmic membrane damage leads to cell death. (Burt et al., 2004; Li et al., 2011; Calo et al., 2014). Some EOs have been reported to inhibit the growth of microorganisms in food sources and prolong the shelf life of processed foods (Cosentino et al., 1999; Barbosa et al., 2016; Mohammadpour et al., 2012). In recent years, EOs have attracted scientific interest because of their potential as a source of biologically active compounds with antimicrobial and antiviral activities that can replace traditional protectives, antibiotics and pesticides (Cicioğlu-Arıdoğan et al., 2002; Herman et al., 2015; de Aguiar et al., 2018).

Salmonella enterica, Staphylococcus aureus, Bacillus cereus, Clostridium perfringens, C. botulinum, Campylobacter jejuni, Escherichia coli O157: H7 and Listeria monocytogenes may cause foodborne outbreaks (Seow et al., 2013; de Aguiar et al., 2018). Although antibiotic use is required for the treatment of these bacteria, the development of antibiotic resistance is the most important factor in treatment failure (Seow et al., 2013; Sevindik et al., 2017). Concerns about consumers' negative perceptions of chemical preservatives have encouraged the food industry to look for new and reliable alternatives, and natural preservatives have begun to be developed (Güner et al., 2012; Seow et al., 2013; Mohammed et al., 2018; Pehlivan and Sevindik, 2018).

In this way, the objectives of this study were to evaluate the *in vitro* activity of 15 different EOs, by disc diffusion methods, against field isolates of *S. aureus* strains isolated from traditional cheeses in Ankara.

Materials and Methods

Essential Oils

In this study, 15 different commercial pure oils (purity \geq 98%) (BalenTM, Ankara) were purchased from herbalists was tested. The purity and composition of which was determined by the manufacturer. These EOs were: *Hypericum perforatum* L, Ginger, Garlic, Laurel, Juniper, Cumin, Pine, Eucalyptus, Dill, Rosemary, Mint, Sage, Black seed, Clove and Thyme. All the oils were stored at room temperature in the dark prior to testing.

Bacterial Isolates

In this study, 85 different *S. aureus* strains isolated from traditional cheeses and the reference strain used in this study (*S. aureus* ATCC6538) were obtained from the culture collection of Food Microbiology Laboratory, Department of Food Engineering, Ankara University, Ankara, Turkey. These strains inoculated on Tryptic Soy Broth (TSB) (SigmaTM, Germany) and Brain Heart Infusion (BHI) broth (MerckTM, Germany) and incubated at 35°C for 24 h. All of the strains used in this study were stored at -20°C with 30% (v/v) glycerol (MerckTM, Germany).

Preparation of Essential Oils for Testing

A sterile 6 mm diameter white disk (Oxoid Ltd, ES) impregnated with 15 μ L of pure EO was prepared. The discs were soaked for a night.

Disc Diffusion Method

As a preliminary step, the antibacterial activity of the EOs was determined by the disk diffusion method. Briefly, steril 90 ml TSA medium was prepared. Meanwhile, from an overnight culture, a suspension of 1×10^8 CFU/mL was prepared in sterile saline solution. Ten mL bacterial suspensions were transfered onto TSA medium, mixed with shaker and poured into sterile petri dishes. After ensuring that the media solidified, the discs impregnated with different oils numbered 1 to 15 were placed in the petri dishes and allowed to incubate at 37 °C for 24 hours. Furthermore, the effect of the volatile fraction of every EO was studied with the inverted Petri dish method (de Aguiar et al. 2017). The antibacterial activity was evaluated by measuring in millimeters the diameter of the inhibitory zone. All experiments were conducted in duplicate.

Statistical Analysis

The results were evaluated by repeated measures ANOVA. The obtained measurements were tested by twoway ANOVA analysis. Post-Hoc (Tukey-HSD) tests were performed for factors that were found to be significant as a result of variance analysis.

Results and Discussion

In our study, the antimicrobial activity of commercially available pure EOs obtained from 15 different plants on *S. aureus* strains was investigated. Zone measurements were made by taking into account the completely transparent diameter around the disk. Zone formation around the disc is shown in Figure 1. The values comparing the mean measurements of the zone diameters for different types of EOs are shown in Table 1 and the graph of the same results is shown in Figure 2. EOs susceptibility of *S. aureus* strains is also showed in Table 2. Within the scope of the study, the diameters of 15 different EOs were investigated by means of a ruler and averages of parallels were obtained for 86 strains of *S. aureus*.

As a result of the study, significant differences were found in the 95% confidence interval between the activities of EOs. According to the results, the most effective antimicrobial EO was determined as thyme oil (average zone 23.203 mm). The highest antimicrobial activity after thyme oil was found as clove (mean zone 13.698 mm) and black seed oil (mean zone 11.267 mm) respectively. *H. perforatum* oil (mean zone 6.209 mm), ginger oil (mean zone 6.250 mm) and garlic oil (mean zone 6.267 mm) were the lowest antimicrobial activity in the study.

The highest antimicrobial activity was found in tyme oil with a zone diameter of 23.203 mm. According to our results, all strains tested were sensitive to thyme oil. The antimicrobial activity of thyme oil was measured as zone diameter above 20 mm in 47 (54.7%) of 86 S. aureus strains and these strains were found to be very sensitive to thyme oil. 35 (40.7%) of the strains were moderately sensitive and zone ranges were 15 mm-20 mm. 4 (4.7%) strains were found to be less sensitive to this oil (9 mm-14 mm zone diameter). Thyme oil is an oil with high antimicrobial activity due to its predominant components, thymol and carvocrole (Bayaz et al., 2014). Carvacrol and thymol breakdown the bacterial cell membrane and causes the cell material to leave the cell (Chaieb et al., 2017). In our studies, the antimicrobial activity of thyme oil was similar to different studies (Con et al., 1998; Ertürk et al., 2010; Radünz et al., 2019). We found that antimicrobial activity on thyme against S. aureus was determined as a zone diameter of 29 mm. In previously both studies (Con et al., 1998; Ertürk et al., 2010), thyme oil showed activity above 20 mm zone diameter. In this context, the results of this study were consistent with them. Lambert et al. (2001) studied the antimicrobial activity of thymol and carvocrol on S. aureus and P. aureginosa, and found that the combined use of thymol and carvocrol showed higher antimicrobial activity than when used alone. These results suggest that thyme oil may be the cause of the higher inhibitory effect than a volatile oil containing only one of thymol or carvocrole.

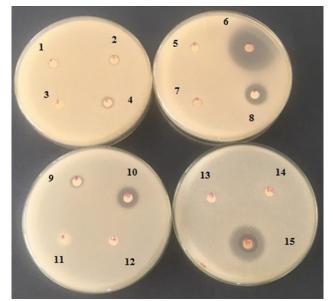


Figure 1. Inhibition zones formed by volatile oils around the disc (*Staphylococcus aureus* ATCC6538) (Disc 1: eucalyptus oil, disc 2: dill oil, disc 3: juniper oil, disc 4: rosemary oil, disc 5: ginger oil, disc 6: thyme oil,disc 7: *Hypericum perforatum* oil, disc 8: mint oil, disc 9: sage oil, disc 10: clove oil, disc 11: garlic oil, disc 12: pine oil, disc 13: Laurel oil, disc 14: cumin oil, disc 15: black seed oil).

Table 1. Average zone di	ameters	and standard deviation
values of Eos*		
Essential oils	Mean	Standard Deviation

Essential oils	Mean	Standard Deviation				
Hypericum perforatum	6.21ª	0.65				
Ginger	6.25 ^a	0.78				
Garlic	6.27 ^a	0.72				
Laurel	6.33 ^{a,b}	0.95				
Juniper	6.49 ^a , ^b	1.31				
Cumin	6.51 ^b , ^c	1.35				
Pine	6.85°, ^d	1.72				
Eucalyptus	7.08 ^d	1.41				
Dill	7.52 ^e	2.12				
Rosemary	8.47 ^f	2.15				
Mint	8.63 ^f	3.23				
Sage	9.44 ^g	1.61				
Black seed	11.27 ^h	5.57				
Clove	13.7 ⁱ	6.82				
Thyme	23.2 ^j	8.67				
F: 164,709; P: <0.001						

*The difference between the mean values of oil types was statistically significant (F=164,709; P<0.001). The smallest significant difference test (LSD: Least Significant Difference) was performed for multiple comparisons to determine the source of the differences. In case of no significant difference between the groups, the mean values were indexed with the same letter.

In our study, clove oil was found to be the most effective oil after thyme oil. The average zone diameter of clove oil was 13.698 mm. While a total of 85 strains were sensitive to clove oil, only one strain had no antimicrobial activity. In this study, 8 (9.3%) strains were very sensitive to clove oil and 20 (23.3%) strains were found to be moderately sensitive 57 (63.3%) strains.

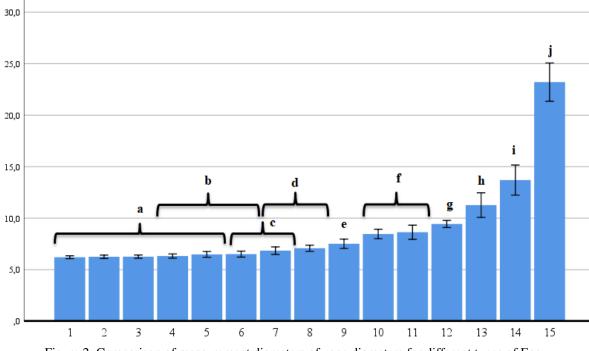


Figure 2. Comparison of measurement diameters of zone diameters for different types of Eos

Essential Oils	_ ^a		$+^{b}$		++ ^c		+++ ^d	
Essential Olis	n	%	n	%	n	%	n	%
Hypericum perforatum	82	95.3	4	4.7	0	0.0	0	0.0
Ginger	81	94.2	5	5.8	0	0.0	0	0.0
Garlic	84	97.7	2	2.3	0	0.0	0	0.0
Laurel	80	93.0	6	7.0	0	0.0	0	0.0
Juniper	78	90.7	7	8.1	1	1.2	0	0.0
Cumin	77	89.5	9	10.5	0	0.0	0	0.0
Pine	72	83.7	14	16.3	0	0.0	0	0.0
Eucalyptus	66	76.7	20	23.3	0	0.0	0	0.0
Dill	58	67.4	28	32.6	0	0.0	0	0.0
Rosemary	39	45.3	46	53.5	1	1.2	0	0.0
Mint	46	53.5	33	38.4	7	8.1	0	0.0
Sage	12	14.0	73	84.9	1	1.2	0	0.0
Black seed	37	43.0	22	25.6	19	22.1	8	9.3
Clove	1	1.2	57	66.3	20	23.3	8	9.3
Thyme	0	0.0	4	4.7	35	40.7	47	54.7

Table 2. EOs susceptibility of Staphylococcus aureus strains

a: ≤8 mm; b: between 8 mm and 14 mm; c: between 14 mm and 20 mm; d: >20 mm

Moreira et al. (2005) in their study of eucalyptus, tea tree, rosemary, mint, rose, clove, lemon, thyme, pine and basil plants obtained from the essential oils of different E.coli O157: H17 strains tested on the antimicrobial activity. They found that clove oil was the highest activity oil. Metin et al. (2016) investigated the antimicrobial activity of 100% pure commercial oils of clove, mint and lavender plants against some bacterial fish pathogens. As a result of their study, it was found that clove oil was the highest antimicrobial activity and certain concentrations of the other two oils showed inhibition properties. Antimicrobial activity in cloves has been reported to be due to eugenol in oil structure (Chaieb et al., 2017; Yücel-Şengün et al., 2018). Quattara et al. (1997) found that the most effective essential oils belong to cloves, cinnamon, allspice and rosemary cinnamon, cloves, allspice, rosemary, black pepper, marjoram, garlic and cumin essential oils of different dilutions were identified against 2 Gram negative and 4 Gram positive bacteria. They stated that the common feature of these oils was eugenol and cinnamaldehyde. Radünz et al. (2019) investigated the antimicrobial efficacy of clove oil on S. aureus, E. coli, L. monocytogenes and S. typimurium and determined the antimicrobial activity as 28.3 mm, 28.1 mm, 24.7 mm and 22.2 mm, respectively. It was thought that the zone value found for S.aureus is considerably higher than the average zone value found in our study. Around the disc, two different zones can be formed: a fully transparent zone and a fuzzy transparent zone. In the evaluation section, only the evaluation of the transparent zone and the evaluation of the zone formation cannot make the total results mathematically different (Radünz et al., 2019).

In our study, the highest activity after black seed oil, oregano and clove oil was determined as the third oil. The average zone value of black seed oil was found to be 11.267 mm. A total of 49 strains were found to be sensitive to black seed oil. In 8 (9.3%) of these strains, values above 20 mm zone diameter were determined and these strains were determined as very sensitive. Nineteen (22.1%) of them were found to be moderately sensitive to black seed oil and 22 (25.6%) were less sensetive. The antimicrobial activity of black seed oil is supported by different studies. Nair et al. (2005), the seed oil of black seed plant on L. monocytogenes investigated the antimicrobial activity. In this study, a disc containing impregnated black seed oil and sunflower oil and a disc containing gentamicin were used. They found that the highest antimicrobial activity was shown by black seed oil with an average zone value of 31.5 mm (Nair et al., 2005). It is thought that the difference between the zone value in the study of Nair et al. (2005) and the zone value in our study stems from the difference in microorganism tested. Antimicrobial efficacy of black seed oil has been reported in different studies similar to our study. Thymokinone, the dominant substance found in black seed oil, has been shown to exhibit antimicrobial activity against bacterial strains such as E. coli, S. aureus, P. aureginosa and B. subtilis and especially bactericidal activity against gram positive cocci (Khan et al., 2003; Chaieb et al., 2011; Güzelsoy et al., 2018).

Antimicrobial activity of sage, mint and rosemary oils on S. aureus strains was also determined in our study. The mean antimicrobial activity of sage oil on S. aureus was 9.44 mm, while the mean peppermint oil was 8.63 mm and the mean antimicrobial activity of rosemary oil was also 8.47 mm. While 12 (14%) strains, 46 (53.5%) strains and 39 (45.3%) strains were insensitive to these fats, 73 (84.9%) strains, 33 (38.4%) strains and 46 (53.5%) strains were less sensitive, respectively. Among them, 1 (1.2%) strains, 7 (8.1%) strains and 1 (1.2%) strains were identified as medium susceptible. None of the strains were found in the very sensitive category for all three fats. The results obtained from the study were consistent with the results of different researchers. Moreira et al. (2005), Metin et al. (2016) and Quattara et al. (1997)'s results were in parallel with our results.

In our study, mean zone values of dill, pine, cumin and ginger oils were determined as 7.52 mm, 6.85 mm, 6.51 mm and 6.25 mm respectively. While 58 (67.4%) of the strains were found insensitive to dill oil, 28 (32.6%) were found to be less sensitive. While pine oil was not effective in 72 (83.7%) strains, 9mm-14mm zone diameter was determined on 14 (16.3%) strains. Similarly, while 77 (89.5%) strains were insensitive to cumin oil, the remaining 9 (10.5%) strains were found to be less sensitive (9 mm-14 mm zone diameter). While 81 strains (94.2%) were insensitive to ginger oil, only 5 strains (5.8%) were less sensitive (9 mm-14 mm). According to our results, ginger oil was determined as the lowest antimicrobial activity oil after H. perforatum oil. Cumin, dill, ginger and pine oils contain at least one common substance. α - pinene and limonene in cumin oil, α -felladren and limonene in dill oil; α -pinene and α -felladren in ginger oil and limonene and α - pinene in pine oil are the predominant active ingredients (Sultan et al., 2005; Hussain et al., 2010; Radulescu et al., 2010; Mohammadpour et al., 2012; 1016

Demirci et al., 2015). Low levels of antimicrobial activity suggest that α -pinene and limonene and felladren alone or in combination have no effect on *S. aureus* strains.

In our study, the lowest antimicrobial activity oils were H.perforatum (average zone diameter: 6.21 mm), ginger (average zone diameter: 6.25 mm), garlic (average zone diameter: 6.27 mm), laurel (average zone diameter 6.33 mm), juniper (average zone diameter 6.49 mm) and cumin (6.51 mm). On a total of 86 S. aureus strains, 82 (95.3%), 81 (94.2%), 84 (97.7%), 80 (93.0%), 78 (90.7%) and 77 (89.5%) strains were found insensitive to these five fats. None of the strains had a zone diameter greater than 20 mm. Except for juniper oil (1 strain), no strain was found to be moderately sensitive to these oils. 4 strains (4.7%) were used for *H. perforatum* oil, 5 strains (5.8%) for ginger oil, 2 strains (2.3%) for garlic oil, 6 strains (7.0%) for laurel oil, 7 strains (8.1%) for juniper oil and 9 strains (10.5%) was found to be less sensitive to cumin oil. Our study results differ from other studies. In our study, antimicrobial activity of garlic and cumin oil on S. aureus was determined. Quattara et al. (1997) found that antimicrobial activity of garlic and cumin oil. The difference between the two studies is thought to be due to the difference in the bacterial strain tested. Biondi et al. (1993) on the other hand, the dilution of 1/2 of the laurel oil in 1/2, 1/5 and 1/10dilutions of laurel oil has an antimicrobial effect on S. aureus and they do not observe any efficacy in other dilutions. In our study, pure laurel oil had very limited antimicrobial activity on S.aureus. In this context, between our results and Biondi et al. (1993)'s result were not compatible. It is thought that this difference may be caused by different varieties and virulence characteristics of the strains even if they are the same species.

Conclusion

Aromatic plants and the essential oils obtained from these plants have been used for various purposes such as the treatment of diseases among the people and preservatives and flavors in the food for centuries. The use of synthetic additives and antimicrobials that have increased with the advancing technology have had many side effects on health and the need to investigate new sources such as the resistance of microorganisms to synthetic antimicrobials. As a result, the use of medicinal and aromatic plants and the essential oils obtained from them has come to the fore again and efforts to improve the use of these products in medicine, cosmetics, agriculture and food have accelerated. The results of the studies on the antibacterial, antifungal, antiviral, insecticide and food protective effects of the essential oils obtained from plants are generally positive. Therefore, an alternative way of using herbal essential oils is thought.

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