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Determination of Antibacterial Activity of Essential Oils from Aromatic Plants Against Pathogenic and Antibiotic-Resistant Microorganisms in Animals and Animal Products

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Abstract: Dairy calf mastitis is a relatively common infectious condition that mostly results from *Staphylococcus aureus* infections. It causes significant economic losses for dairy farmers all over the world. Due to misuse of drugs, drug-resistant pathogens have started to emerge. Since these pathogens do not respond to commonly used treatments, the economic loss is increasing every year. In this study, the antimicrobial activity of three different plants (*Origanum onites, Teucrium polium*, and *Vitex agnus-castus*) collected from Aydın and its surroundings on six different antibiotic-resistant microorganisms, some of which are the causative agents of mastitis, was determined using agar disc diffusion methods. The essential oil of *O. onites* had 32 mm inhibition zone against Methicillin-resistant *Staphylococcus aureus* (MRSA), while the antibiotic used as a standard had no effect. The essential oil of *T. polium* showed the smallest inhibition zone (3 mm) against MRSA and the biggest inhibition zone (20.5 mm) against *Pseudomonas aeruginosa*. Extracts of *V. agnus-castus* formed zones of inhibition against *Bacillus cereus CCM99* and *Staphylococcus aureus* ATCC 6538 of 23.5 and 36 mm, respectively. In addition, comparing the data of this study with literature data, it is suggested that β-farnesene in T. polium, carvacrol and o-cymene in *O. onites*, and 1,8-cineole in *V. agnus-cactus L*. may be more effective in antibacterial activity than other essential oil constituents. This study has shown that the essential oils of these plants could be important components of drugs that can be employed against multidrug-resistant pathogens that cause disease in humans and animals, many of which are foodborne.

Keywords: Essential oil, mastitis, *Origanum onites, Teucrium polium, Vitex agnus-castus*

Aromatik Bitkilerden Elde Edilen Uçucu Yağların Hayvanlarda ve Hayvansal Ürünlerde Patojen ve Antibiyotiklere Dirençli Olan Mikroorganizmalara Karşı Antibakteriyel Aktivitesinin Belirlenmesi

Öz: *Sığırlarda mastitis, çoğunlukla Staphylococcus aureus enfeksiyonlarından kaynaklanan nispeten yaygın bulaşıcı bir hastalıktır. Tüm dünyada süt üreticileri için önemli ekonomik kayıplara neden olmaktadır. İlaçların yanlış kullanımı nedeniyle ilaca dirençli patojenler ortaya çıkmaya başlamıştır. Bu patojenler yaygın olarak kullanılan tedavilere yanıt vermediği için ekonomik kayıp her geçen yıl artmaktadır. Bu çalışmada, Aydın ve çevresinden toplanan üç farklı bitkinin (Origanum onites, Teucrium polium ve Vitex agnus-castus L.), bazıları mastitis etkeni ve antibiyotik dirençli olan altı farklı mikroorganizma üzerindeki antimikrobiyal aktivitesi agar disk difüzyon yöntemleri kullanılarak belirlenmiştir. O. onites uçucu yağı Metisilin-dirençli Staphylococcus aureus (MRSA)'ya karşı 32 mm inhibisyon zonu oluştururken, standart olarak kullanılan antibiyotiğin hiçbir etkisi olmamıştır. T. polium uçucu yağı MRSA'ya karşı en küçük (3 mm) ve Pseudomonas aeruginosa'ya karşı en büyük (20,5 mm) inhibisyon zonunu göstermiştir. V. agnus-castus ekstraktları Bacillus cereus CCM99 ve Staphylococcus aureus ATCC 6538'e karşı sırasıyla 23,5 ve 36 mm'lik inhibisyon bölgeleri oluşturmuştur. Ayrıca, bu çalışmanın verileri literatür verileri ile karşılaştırıldığında, T. polium'daki β-farnesen, O. onites'deki karvakrol ile V. agnus-cactus L.'deki 1,8-sineolün antibakteriyel aktivitede diğer uçucu yağ bileşenlerine göre daha etkili olabileceği düşünülmektedir. Bu çalışma, bu bitkilerin uçucu yağlarının, insanlarda ve hayvanlarda hastalığa neden olan ve çoğu gıda kaynaklı olan çoklu ilaç direncine sahip patojenlere karşı kullanılabilecek ilaçların önemli bileşenleri olabileceğini göstermiştir.*

Anahtar kelimeler: *Esansiyel yağ, mastitis, Origanum onites, Teucrium polium, Vitex agnus-castus.*

INTRODUCTION

Intramuscular or intravenous injection of antibiotics, such as beta-lactams, is the primary therapy for mastitis (Tepeli, 2020). However, their careless usage has resulted in the growth of bacteria resistant to these antibiotics. An estimated 35,000 fatalities in the US are attributed to antibiotic-resistant illnesses each year. An approximate 94,000 invasive infections caused by Methicillin-resistant *Staphylococcus aureus* (MRSA) and 18,500 fatalities were reported in the US in 2005 (Hirschmann, 2009). One of the top antibiotic-resistant infection agents, MRSA is considered a priority by the Public Health Agency of Canada (PHAC). Because of its stubborn behavior against the antibiotics and other drugs that are most frequently used to treat both large and small infections, MRSA became known as a superbug. Currently, only a few expensive medications are effective in treating these illnesses. It is capable of continuously evolving and emerging. According to WHO studies, those infected with MRSA are 64% more likely to die than those infected with other diseases (Nandhini et al., 2022). Initially, MRSA infections were limited to health facilities. However, more

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and more cases are being acquired from the community. Moreover, it's still uncertain whether MRSA will broaden its range of resistance, leading to more serious infections and more problems for the medical community (Waness, 2010). With an infectious dosage as low as a few hundred cells, *E. coli serotype O157:H7* is one of the most well-known foodborne pathogens (Karmali, 2004). Worldwide, hemolytic uremic syndrome (HUS) and hemorrhagic colitis in humans have been linked to enterohemorrhagic *Escherichia coli 0157:H7* (Griffin, 1995). According to Mustafa and İnanç (2018), the *E. coli O157:H7* antibiotic sensitivity test revealed that the isolates were resistant to the antibiotics such as ampicillin, amoxicillin, chloramphenicol, ciprofloxacin, doxycycline, streptomycin, and tetracycline.

Gram-negative *Pseudomonas aeruginosa* (*Pseudomonadaceae*) is a common bacterium that can thrive in a range of conditions (Pang et al., 2019). Antibiotics frequently exhibit low efficiency because of *P. aeruginosa's* adaptability and high level of antibiotic resistance, which increases mortality (Thi et al., 2020).

The use of plants for therapeutic purposes has a long history in both developed and developing countries in Eastern and Western civilizations. Plants have long been used as a source of ingredients in traditional medicines in many parts of the world, and there is growing interest in using plants to treat infectious microbial diseases (Chariandy et al., 1999). The surprising prevalence of antibiotic resistance in medically important microorganisms means that there is a constant need for novel and efficacious therapeutic agents (Monroe and Polk, 2000). The antibacterial action of plant oils and extracts that suppress bacterial growth may have different processes than commonly used antibiotics. As a result, plant-based antimicrobials can be useful in treating resistant bacteria strains in clinical settings.

The antimicrobial, antioxidant, and medicinal uses of *Origanum onites, Teucrium polium*, and *Vitex agnus-castus* have been investigated by many researchers (Tepe et al., 2005; Özcan and Chalchat, 2008; Badawy and Abdelgaleil, 2014; Sevindik et al., 2016; Taşkın et al., 2017). However, differences observed in the antibacterial activity of these plant extracts are thought to be due to the variation in the extraction method, the time of collection, and the geographical location of the plant. Therefore, there is a need for further investigation of their antimicrobial activity to better understand the therapeutic potential of *O. onites, T. polium*, and *V. agnus castus* fruits water distillation against a wider range of microorganisms, including multidrug-resistant strains. In the present study, the antimicrobial activities of the above-ground parts of *T. polium, O. onites* plants, and *V. agnus-castus* fruits collected from Aydın and its surroundings were investigated against four different gram-positive pathogenic microorganisms (*S. aureus* ATCC 6538, methicillin-resistant *Staphylococcus aureus* (MRSA), *Bacillus cereus CCM 99*, and *Enterococcus faecium DSM* 13590) and two different gram-negative pathogenic microorganisms (*Escherichia coli Q157:H7* and *Pseudomonas aeruginosa*).

MATERIAL and METHODS

Collection of Plant Materials and Extraction of Essential Oil

During their flowering phases in July-August, the aerial portions of *T. polium*, *O. onites* plants, and *V. agnus-castus* fruits in September were gathered as study materials from Aydın and its surrounds. The samples that were gathered were sealed in cloth bags and stored in a dark room. 100 g or more of plants and 150 g of fruit samples were used in the process of extracting essential oils. Using water distillation by Clevenger apparatus, the extraction process was carried out.

Bacteria Strains

In this study, six bacterial strains were used: methicillin-resistant *Staphylococcus aureus* (MRSA), *S. aureus ATCC 6538, Bacillus cereus CCM 99, Enterococcus faecium DSM 13590, Pseudomonas aeruginosa,* and *Escherichia coli Q157:H7,* obtained from our own laboratory.

Antimicrobial Activity Using Agar Disc Diffusion Method

Three plant extracts were tested for their antibacterial properties against two different gram-negative microorganisms (*Escherichia coli Q157:H7* and *Pseudomonas aeruginosa*) and four different gram-positive microorganisms (*S. aureus ATCC 6538*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Bacillus cereus CCM 99*, and *Enterococcus faecium DSM 13590*) using the disc diffusion method, which was modified from Benali et al. (2020) and Rota et al. (2004). Liquid solutions of microorganism strains prepared according to the 0.5 McFarland turbidity standard from the fresh cultures of the strains obtained after incubation in Nutrient Agar at 37 ºC for 24 hours were used for antibiograms. Samples taken from the liquid solution tubes of each isolate with swabs were inoculated on Müeller Hinton Agar (MHA; Merck, USA) by smear inoculation method. After inoculation, sterile disks, with a diameter of 6 mm, containing 5 µL of pure essential oil and antibiotic disks were placed on the Müeller Hinton Agar surface at appropriate intervals, and the petri dishes were incubated at 37 ºC for 24 hours. The diameters of the inhibition zones around the disks used in the antibiogram were measured. The trials were carried out with two repetitions.

RESULTS and DISCUSSION

Research from all over the world confirms that the incidence of MRSA infections is on the rise. Both nations with substantial health resources and those with limited ones exhibit this pattern of increasing resistance. According to one Mexican study, the yearly rate of methicillin resistance increased from 37% in 2000 to 49% in 2007 (Alvarez et al., 2009). A method utilized to overcome these resistance mechanisms involves the administration of pharmacological combinations. Hemaiswarya et al. (2008) suggest that plants are a useful source of secondary metabolites for combination treatment. Epigallocatechin gallate and the ampicillin/sulbactam combination may be useful in treating MRSA infections, as demonstrated by Hu et al. (2001). In our research, it was found that *O. onites* essential oil alone was quite effective against MRSA. It was discovered that *O. onites* essential oil may be a natural extract that can be used instead of conventional treatments in MRSA infections (the MRSA inhibitory zone measured 32 mm) (Table 1).

Table 1. Antimicrobial activity (inhibition zones) of the essential oils from *O. onites* plants

Microorganisms	Inhibition zone (mm)*	
	O. onites	**Antibiotic (Tobramycin)**
Methicillin-resistant *Staphylococcus aureus* (MRSA)	32±0	0±0
Staphylococcus aureus ATCC 6538	42±2.8	22±2.8
Pseudomonas aeruginosa	24±0	12±0
Escherichia coli Q157:H7	23±1.4	20.5±4.9
Bacillus cereus CCM99	34±5.6	19±1.4
Enterococcus faecium DSM 13590	29±4.2	12±2.8

*Results are expressed as mean; ± standard deviation (SD).

Essential oil composition affects the antimicrobial activity of plants. It was reported that the principal constituents of the essential oil of *O. onites* collected from the Aegean and Mediterranean regions are carvacrol (30.01-71.96%), p-cymene (3.90-10.07%), thymol (1.53-38.25%), and γ-terpinene (2.25-5.07%) have a strong antimicrobial effect on *E. coli O157:H7* (Tekin 2013). Carvacrol (82.34%) was detected as the major component in the essential oil of *O. onites* collected from Çanakkale Kazdağ. In addition, the presence of glyceryl monostearate (5.64%) in the essential oil content was reported. 50 µl of ethanol extract of *O. onites* collected from Kazdağ generated an inhibition zone of 10 mm in *E. coli*, 18 mm in *Pseudomonas aeruginosa*, 20 mm in *Staphylococcus aureus* ATCC 25923, and 29 mm in *Staphylococcus aureus* (MRSA + MDR) (Canli et al. 2023). In our study, only 5 µl of the extract obtained from *O. onites* by the water distillation method caused an inhibition zone of 23 mm in *Escherichia coli Q157:H7*, 24 mm in *Pseudomonas aeruginosa*, 42 mm in *Staphylococcus aureus* ATCC 6538, and 32 mm in Methicillin-resistant *Staphylococcus aureus* (MRSA) (Table 1). The *O. onites* plant used in our study was collected from Aydın. Similarly, Sevindik et al. (2019) reported 37.08% carvacrol, 10.97% o-cymene, and 7.10% γ-terpinene in the essential oil content of the *O. onites* plant collected from Aydın. Although the carvacrol content was relatively low in the plant samples collected from Aydın, it was observed that the antimicrobial activity was high. In another study, the antibacterial activity of p-cymene and carvacrol was examined regarding their potential use as preservatives against *V. cholerae*, a foodborne microorganism. Carvacrol demonstrated a significant inhibitory effect on *V. cholerae*, whereas p-cymene did not exhibit this effect. Nevertheless, it was demonstrated that the combined use of carvacrol and p-cymene resulted in an enhanced inhibitory effect (Rattanachaikunsopon and Phumkhachorn 2010).

In the study by Sevindik et al. (2017), MRSA, *S. aureus* ATCC 6538, *E. faecium DSM 13590, E. coli Q157: H7, P. aeruginosa*, and *B. cereus CCM 99* were all tested against essential oil derived from the *Mentha pulegium plant*. *E. faecium* and *E. coli Q157:H7* produced the smallest zone of inhibition (9 mm), while *B. cereus CCM99* produced the biggest zone of inhibition (23 mm). The identical microorganisms were used in our investigation to test the antimicrobial activity of essential oils of *O. onites. O. onites* essential oil was found to have an inhibitory effect on *B. cereus CCM99* and *Enterococcus faecium DSM 13590*, with the zone of inhibition measured as 34 mm and 29 mm, respectively. In the study by Taşkın et al. 2017, antimicrobial test findings showed that methanol extracts of all Origanum species have a high potential for antibacterial activity against *S. aureus*. In our investigation, *O. onites* was very efficient against *S. aureus* ATCC 6538, with an inhibition zone of 42 mm (Table 1).

The essential oil of *T. polium* grown in Aydın ecological conditions was effective to varying degrees against MRSA, *S. aureus ATCC 6538, E. coli Q157:H7, P. aeruginosa, E. faecium DSM 13590*, and *B. cereus CCM 99*. The essential oil produced the smallest inhibition zone (3 mm) against MRSA and the largest inhibition zone (20.5 mm) against *P. aeruginosa* (Table 2)..

Table 2. Antimicrobial activity (inhibition zones) of the essential oils from *T. polium* plants

Microorganisms	**Inhibition zone (mm)***	
	T.polium	**Antibiotic (Tobramycin)**
Methicillin-resistant *Staphylococcus aureus* (MRSA)	3±4.2	0±0
Staphylococcus aureus ATCC 6538	7±1.4	22±2.8
Pseudomonas aeruginosa	20.5±4.9	12±0
Escherichia coli Q157:H7	8±2.8	20.5±4.9
Bacillus cereus CCM99	13±1.4	19±1.4
Enterococcus faecium DSM 13590	8±0	12±2.8

*Results are expressed as mean; ± standard deviation (SD).

Sevindik et al. (2016) examined the antimicrobial effect of T. polium collected from around Ardahan Çıldır Lake on MRSA and found the zone of inhibition to be 15 mm. In our study, it was determined that *T. polium* extract collected from around Aydın was not as effective on MRSA as *T. polium* extract collected from around Ardahan Çıldır Lake. The differences observed in the antibacterial activity of *T. polium* extract are thought to be due to the variation in the extraction method, the time of collection, and the geographical location of the plant. In addition, Sevindik et al. (2016) reported that β-farnesene (15.49%) was among the main components of the essential oil of *T. polium* collected from Ardahan. Similarly, Raei et al. (2013) reported that the essential oil *T. polium* collected from Tehran contains 13% β-farnesene, and *T. polium* essential oil is effective on *Klebsiella pneumoniae* with multidrug resistance. Kurtoğlu and Tin (2017) determined that the β-farnesene ratio in the essential oil of *T. polium* from Aydın and its surroundings was 1.32. The results indicated a correlation between the variation in the β-farnesene ratio in the essential oil according to geographical conditions and the antibacterial effect of *T. polium*. In a study on the *Tripleurospermum disciforme plant*, it was stated that the main component of the extracted essential oil during the flowering period was β-farnesene (22.46%) and had a strong antibacterial activity (Chehregani et al. 2010).

In another study, *T. polium* essential oil in which β-farnesene has not been detected had poor antibacterial activity on *P. aeruginosa ATCC27853*, but this activity against *E. coli ATCC 25922*, *S. aureus ATCC 25923*, and *S. sonnei* was significant, while *S. enterica ssp. Arizona CIP 81* was found resistant (Boukhebti et al., 2019).

The methanolic extract of *T. polium* growing in Iran demonstrated inhibitory activity against the growth of some bacteria, with different minimal inhibitory concentrations (MICs). While it inhibited the growth of *Staphylococcus aureus* and *Salmonella typhimurium* with a MIC of 40 mg/mL, the MIC value for *Bacillus anthracis* and *Bordetella bronchiseptica* was 10 mg/mL. *Salmonella typhimurium* was mostly well affected by *T. polium* hydroalcoholic extract (Darabpour et al., 2010). This study also showed the effect of geographical conditions on the antibacterial properties of the essential oil of *T. polium*.

In a different investigation in Algeria, the essential oil of the methanolic extract of *T. polium* did not affect *E. coli MC 4100* and *P. diminitus* (Zerroug et al., 2011). However, in another study, aqueous distillation of *T. polium* was found to be efficient against *P. aeruginosa* and *E. coli* in our investigation. Aqueous extracts of *T. polium* have been demonstrated in earlier research to be effective against bacteria and fungus, among other microbes (Jaradat, 2015). Thus, the extraction method for essential oils was also demonstrated to be effective in terms of antibacterial activity.

The antimicrobial properties of essential oils of some Labiatae family species from Şırnak–Silopi region was studied by Oğuz et al. (2008). In their study, also, the antimicrobial potecy of *T. polium* was tested on microorganisms, including *E. coli ATCC 29998, S. aureus ATCC 25923*, and *B. Cereus ATCC 1777*. The study showed that the inhibition zone diameters of *S. aureus, E. coli*, and *B. cereus* were 15 mm, 8 mm, and 19 mm, respectively (Oğuz et al., 2008). According to these results, it was seen that *T. polium* extract was more efficient on gram-positive bacteria but less effective on gram-negative bacteria like *E. coli*. However, based on the findings of our investigation, it is not possible to draw such a broad conclusion.

In this study, the antibacterial activity of the essential oil of *V. agnus-castus* fruits growing in Aydın ecological circumstances was detected by the agar disk diffusion method. The essential oils of *V. agnus-castus* fruits were effective to varying degrees against MRSA*, S. aureus ATCC 6538, P. aeruginosa, E. faecium DSM 13590, E. coli Q157:H7*, and *B. cereus CCM 99*. The largest inhibition zone (36 mm) was observed against *Staphylococcus aureus ATCC 6538*, while the smallest inhibition zone (3.5 mm) was obtained against MRSA (Table 3).

Table 3. The antimicrobial activity (inhibition zones) of essential oils from *V. agnus-castus* fruits

Microorganisms	**Inhibition zone (mm)***	
	***V. agnus-castus* fruits**	**Antibiotic (Tobramycin)**
Methicillin-resistant *Staphylococcus aureus* (MRSA)	3.5±4.9	0±0
Staphylococcus aureus ATCC 6538	36±2.8	22±2.8
Pseudomonas aeruginosa	11±0	11±1.4
Escherichia coli Q157:H7	5±7.0	21.5±6.3
Bacillus cereus CCM99	23,5±0.7	19±1.4
Enterococcus faecium DSM 13590	19±1.4	12±2.8

*Results are expressed as mean; ± standard deviation (SD).

Kavaz et al. (2022) showed that 312µg/mL ethanolic extract of *Vitex agnus-castus L*. seeds caused the formation of an inhibition zone of 11.0 mm on *S. aureus*, an inhibition zone of 9.0 mm on *S. typhimurium*, and an inhibition zone of 8.0 mm on *E. coli*. Balpınar et al. (2019) reported that *V. agnus-castus L*. extracts have an antibacterial effect on *S. aureus* and Coagulase-Negative Staphylococci (CNS) strains causing mastitis and that the extracts obtained from this plant can be utilized for mastitis treatment. These outcomes are in parallel with the results obtained in our study.

Different researchers determined that the major component of *V. agnus-cactus L* essential oil is 1,8-cineole (Tin et al. 2017). Therefore, unlike the essential oil content of *O. onites* and *T. polium*, it is thought that the essential oil of *V. agnus-cactus L* is more effective, especially on Gram-positive bacteria, and this activity may be due to the high 1,8-cineole compound in its content. Additionally, Şimsek and Duman (2017) reported in their study that 1,8-cineole enhanced the antimicrobial efficacy of chlorhexidine gluconate against all tested microbial strains, except for *Pseudomonas aeruginosa.*

CONCLUSION

This study highlights the remarkable antibacterial potency of the essential oils of *V. agnus-castus* fruits, *T. polium*, and *O. onites* against several important pathogenic bacteria, both Gram-positive and Gram-negative, including methicillin-resistant *Staphylococcus aureus*. In this context, the essential oils obtained from *V. agnus-castus* fruits, *T. polium*, and *O. onites* look promising as ingredients in antibacterial products for human, veterinary, and food use because *Escherichia coli Q157:H7* and *Bacillus cereus* are causative agents of food poisoning, *Staphylococcus aureus* causes mastitis and other diseases, and the other microorganisms used in the study are opportunistic pathogens. Methicillin-resistant *Staphylococcus aureus* is known to be resistant to many antibiotics. MRSA isolates pose a serious problem for both veterinarians and dairy cattle producers. The strong effect of *O. onites* on MRSA is very important data. Further studies are ongoing to identify the active chemical constituents of the essential oils of *O. onites*, *T. polium*, and *V. agnus-castus* fruits and to determine the antimicrobial mechanism.

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