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In vitro effects of various essential oils on biofilm viability; their antibacterial and antibiofilm activities against clinical *Staphylococcus aureus* isolates

Çeşitli esansiyel yağların klinik *Staphylococcus aureus* izolatlarında biofilm canlılığına in vitro etkileri, antibakteriyal ve anti-biofilm aktiviteleri

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ABSTRACT

Objective: Antimicrobial resistance is a public health threat and is related to high mortality and morbidity. Because no development of new antibiotics can combat antibiotic resistance for pathogenic bacteria, the need for natural products has emerged. *Staphylococcus aureus* (*S. aureus*) is an important human pathogen responsible for community- and hospital-acquired infections. The goal of this study was to identify the in vitro effects of *Origanum onites*, *Lavandula stoechas*, *Salvia officinalis* and *Thymus vulgaris* essential oils (EOs) on biofilm viability as well as the antibacterial and anti-adherent properties on clinical *S. aureus* isolates from wound, biopsy and abscesses samples.

Methods: The antibacterial activities of the EOs were assessed on 71 clinical *S. aureus* isolates by broth microdilution to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Biofilm-forming isolates were determined, and EOs' ability to prevent biofilm formation in a dose-dependent manner was calculated.

ÖZET

Amaç: Antimikrobiyal direnç yüksek mortalite ve morbidite ile ilişkili bir halk sağlığı sorunudur. Yeni antibiyotikler de patojenik bakteriler için antibiyotik direnciyle mücadelede yeterli olmadığından doğal ürünlere olan ihtiyaç ortaya çıkmıştır. *Staphylococcus aureus* (*S. aureus*), toplum kaynaklı ve sağlık hizmeti ilişkili enfeksiyonlardan sorumlu önemli bir insan patojenidir. Bu çalışma *Origanum onites*, *Lavandula stoechas*, *Salvia officinalis* and *Thymus vulgaris* esansiyel yağlarının (EO) yara, biyopsi ve apse örneklerinden izole edilen klinik *S. aureus* izolatlarındaki antibakteriyel ve anti-adeziv aktivitesini ve biyofilm canlılığına olan etkisini *in vitro* olarak belirlemeyi amaçlamaktadır.

Yöntem: EO'ların antibakteriyel aktiviteleri, minimum inhibitör ve bakterisidal konsantrasyonlarını (sırasıyla MİK ve MBK) belirlemek üzere sıvı mikrodilüsyon yöntemi, 71 klinik *S. aureus* izolatı üzerinde değerlendirilmiştir. Biyofilm oluşturan izolatlar tespit edilmiştir ve EO'ların biyofilm oluşumunu doza bağlı olarak önleme yeteneği hesaplanmıştır. Minimum

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Minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication (MBEC) and the number of viable bacteria in sub-inhibitory doses of EOs were calculated by BioTimer-Assay (BTA).

Results: All of the tested isolates showed high sensitivity towards the *Thymus vulgaris* and *Origanum onites*; MICs ranging from below 0.039 to 0.625 µl/ml. While the highest MIC value was determined as 5 µl/ml for *Salvia officinalis*, it was calculated as greater than 10 µl/ml for *Lavandula stoechas*. Each tested oil was detected to prevent biofilm formation at a significant percentage.

Conclusion: The essential oils of *Origanum onites* and *Thymus vulgaris* showed bactericidal properties against clinical *S. aureus* isolates, including methicillin-resistant strains, which may become a promising alternative for multidrug-resistant pathogens. In addition, the number of viable bacteria in biofilm in sub-inhibitory doses of *Lavandula stoechas* and *Salvia officinalis* were found to increase when applied doses decreased.

Key Words: Antibacterial, antibiofilm, biofilm viability, biotimer assay, essential oils

biyofilm inhibitör ve eradikasyon konsantrasyonları (sırasıyla MBİK ve MBEK) ve EO'ların alt inhibitör dozlarındaki canlı bakteri sayısı BioTimer-Assay (BTA) ile hesaplanmıştır.

Bulgular: Test edilen izolatların tümünün, *Thymus vulgaris* ve *Origanum onites*'e karşı, MİK değerleri 0.039'dan küçük ve 0.625 µl/ml arasında değişmektedir, yüksek hassasiyet gösterdiği tespit edilmiştir. *Salvia officinalis* için en büyük MİK değeri 5 µl/ml olarak belirlenirken, *Lavandula stoechas* için bu değer 10 µl/ml'den büyük olarak hesaplanmıştır. Test edilen her yağın, biyofilm oluşumunu önemli bir oranda önlemediği belirlenmiştir.

Sonuç: *Origanum onites* ve *Thymus vulgaris*'in EO'ları metisiline dirençli suşlar da dahil olmak üzere klinik *S. aureus* izolatlarına karşı bakterisidal etki göstermiştir ve bu da söz konusu esansiyel yağların çok ilaca dirençli patojenlerle başetmek için umut verici bir aday olabileceğini göstermiştir. Ayrıca, *Lavandula stoechas* ve *Salvia officinalis*'in alt inhibitör dozlarında uygulanan dozlar azaldıkça biyofilmdeki canlı bakteri sayısının arttığı tespit edilmiştir.

Anahtar Kelimeler: Antibakteriyal, antibiyofilm, biyofilm canlılığı, biotimer assay, esansiyel yağlar

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is an important human pathogen responsible for community- and hospital-acquired infections. It is a leading cause of a wide range of infections, and skin and soft tissue infections are among the most common. *S. aureus* can easily acquire antibiotic resistance, and methicillin-resistant *S. aureus* (MRSA) is multidrug-resistant, leading to the need to use last resort antibiotics and increase mortality and morbidity (1). *S. aureus* is also a potent biofilm former on both implanted medical devices and host tissue. Biofilm protects bacteria from host immunity and antibiotics, complicating treatment of infections (2). Bacterial biofilms have a variety of potential antimicrobial resistance mechanisms (3) and as much as six-log difference in cell viability was reported on biofilm comparing to planktonic cells in the presence of antibiotics (4). In this view, there is a need for new natural substances having both antibacterial and antibiofilm activity against *S. aureus* strains.

Essential oils (EOs) are an aromatic mixture of terpenoid and phenolic compounds obtained from flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots (5,6). Thousands of EOs are known,

most of which have commercial importance and have comprehensive biological properties including antibacterial, antiviral, antifungal, and antiparasitic properties; interest in medicinal applications (7,8). Researches demonstrate antimicrobial and antibiofilm activities of EOs against various human pathogens, (9,10,11,12) making them excellent candidates for new natural drug discovery.

The main components of the *Origanum* and *Thymus* species are carvacrol and thymol (13). Carvacrol is a monoterpene and an important component of several essential oils because of its biological property. It demonstrates a wide spectrum of antimicrobial activity. Thymol is a p-cymene derivate known for its antiseptic and antimicrobial properties (14). Genus *Lavandula* and *Salvia* belong to the Lamiaceae family. The antibacterial, anti-inflammatory and antifungal activities of *Lavandula* come from its phenolic compounds such as phenolic acids and flavone glycosides (15). The main component of *Salvia officinalis* is terpenes such as manool, viridiflorol, eucalyptol, borneol, and thujone. In addition, its leaves contain carnosol, carnosic acid, rosmarinic acid, flavonoids, polysaccharides, tannic acid, oleic acid, ursonic acid, ursolic acid, fumaric acid, chlorogenic acid, caffeic acid, and estrogenic (16).

Thus, in this study, four essential oils, *Origanum onites* (oregano), *Thymus vulgaris* (thyme), *Lavandula stoechas* (lavender) and *Salvia officinalis* (clary sage), were evaluated in terms of antimicrobial and anti-adherent activities against clinical *S. aureus* isolates from wound, abscess and biopsy material. Besides, this research aims to determine the effects of EOs on biofilm viability.

MATERIAL and METHOD

This study was approved by Baskent University Institutional Review Board (Project No: KA21/73).

Bacterial Strains

Clinical isolates of *S. aureus* in the bacterial culture collection of the Medical Microbiology Laboratory of Baskent University, isolated from wound, abscess and biopsy materials between 2016-2020 were included in the study. All *S. aureus* isolates were routinely tested for methicillin resistance by cefoxitin screening test described in EUCAST standards, (17) and *S. aureus* ATCC 43300 and *S. aureus* ATCC 29213 were used as positive and negative control respectively.

Essential Oils

EOs of thyme, oregano, lavender, and clary sage EOs were provided by the manufacturer (Caliskan Agriculture, Turkey). All the EOs were obtained by the hydro-distillation method.

Determination of MIC and MBC

The broth microdilution test was performed to determine the minimum inhibitory concentrations (MICs) as recommended by the Clinical and Laboratory Standards Institute (CLSI) (18). The EOs in combination with 0.5% dimethyl sulfoxide (DMSO), were diluted twofold in Mueller Hinton Broth (MHB) (BD Difco^{tm}, France) and 100 µl of each dilution was transferred into wells. The bacterial suspension was inoculated in each well with $1.5x10^8$ CFU/ml. After 24h of incubation at 37°C, absorbance was measured at 570 nm (BioTek Instruments, ELX 800, USA). The lowest concentration of EOs that demonstrated no growth was evaluated as MIC. All studies were performed in triplicate. While thyme and oregano EO concentrations ranged from 0.039 to 10 µl/ml, lavender and sage EO concentrations ranged from 0.039 to 40 µl/ml. Minimum bactericidal concentration (MBC) was determined by subculturing the 10µl of broths used for MIC assay onto Tryptic Soy Agar (TSA) (Condalab, Spain) (19). MBC was considered the lowest concentration of the EOs that results in the killing of 99.9% of the bacteria after the incubation period at 37°C for 24h.

Biofilm Formation

For biofilm formation, the method described by Peeters et al. (20) was used. Briefly, flat-bottom polystyrene microplates containing 180 µl of tryptic soy broth (TSB) (BD Difco^{tm}, France) were inoculated

with 20 µl of bacterial culture adjusted to $1x10^6$ CFU/ml. After 24h of incubation, biofilm formation was detected by the crystal violet staining method. The absorbance was measured at 570 nm (21), and strains were evaluated as negative, weak, intermediate and strong biofilm former (22). *S. aureus* ATCC 6538 and *S. aureus* ATCC 29213 were used as positive and negative control of biofilm formation, respectively.

Calculation of Biofilm Formation Inhibition Percentage of EOs In a Dose-Dependent Manner

The effect of tested 4 EOs against biofilm-forming isolates and control strains was detected in 96-well plates in a dose-dependent manner. Briefly, 100 µl of bacterial suspension for each isolate was added to each well containing different doses of EOs ranging from 0.039 µl/ml to 10 µl/ml. After 24h of incubation, a biofilm staining procedure was applied, and OD values were quantified. Wells without EO was used as a control to calculate the percentage of biofilm inhibition as follows (23):

Percentage of inhibition = ((Control OD570 nm - Sample OD570 nm) / Control OD570 nm) x 100.

Effects of EOs on Biofilm Viability

- BioTimer Assay

BioTimer Assay (BTA) was employed to determine the EOs susceptibility of *S. aureus* clinical isolates without any manipulation. BTA enumerates viable bacteria in biofilm before and after the treatment with EOs. BioTimer medium with phenol red (BT-PR) was prepared as described by Pantanella et al. (24) Colour change from red to yellow was monitored every hour until 23 hours. The time required for the colour switch directly links to the initial concentration of bacteria. *S. aureus* ATCC 6538 was employed to draw a correlation line, and overnight cultures were prepared in 2 ml of BT-PR medium. Serial 10-fold dilutions were performed in test tubes. Bacterial suspension in each dilution was cultured on Mueller Hinton Agar (MHA) (Condalab, Spain) simultaneously, and after 24 hours of incubation, colonies were counted by colony-forming unit (CFU) method and $\log_{10}$CFU versus time of colour change were plotted.

- EOs Efficacy on Biofilm

To detect EOs susceptibility of *S. aureus* biofilm-forming strains, methods by Pantanella et al. (24) with slight modifications were applied. Biofilms were formed in 96-well plates with peg lids (Thermo Fisher Scientific, Nunc-TSP, Denmark) as described in 2.6.3., and 2-fold serial dilutions of EOs were prepared in BT-PR medium for each oil. Final oil concentrations ranged from 0.039 to 10 µl/ml. For lavender and clary sage oils highest concentration tested was adjusted to 40 µl/ml. After biofilm formation, wells were washed three times with phosphate-buffered saline (PBS) and filled with 2-fold serially diluted EOs in BT-PR medium. Peg lids were placed, and the time required for colour change was monitored every hour. After 24 hours of incubation, the lowest concentration of EOs that inhibits colour change in BT-PR medium (wells that remaining red) was recorded as minimum biofilm inhibitory concentration (MBIC). To determine minimum biofilm eradication concentration (MBEC), peg lids from the wells that colour change did not occur were transferred to the new BT-PR media without EO. Microplates were incubated at 37°C for 24 hours. The lowest concentration of EO that colour change was not observed determined as MBEC.

- Enumeration of Viable Bacteria in Biofilm in Sub-Inhibitory Doses of EOs

After the determination of MBIC, the sub-inhibitory doses of EOs were evaluated to demonstrate their quantitative effects on bacterial viability in preformed biofilm. Previously tested biofilm-forming strains' inoculation were prepared (1×10^6 CFU/mL), and strains were incubated in TSB at 37°C for 24 hours in 96-well plates with peg lids (Thermo Fisher Scientific, Nunc-TSP, Denmark). After 24 hours, wells were washed three times with PBS and filled with 200 µl of BT-PR medium containing different EO doses. Peg lids from previous microplates were placed and incubated at 37°C. The colour change was monitored

every hour and noted. The time required for colour change was employed to enumerate bacteria in biofilm according to the correlation line.

RESULTS

Among 182 clinical S.aureus isolates, 71 of them isolated from wound, abscess and biopsy materials were included in the study. Among them, 16 (23.6%) were MRSA, and 55 (76.4%) were MSSA.

MIC and MBC

All of the isolates were susceptible to thyme, sage and oregano EOs, whereas 88.7% (63/71) of the isolates were susceptible to lavender oil. MIC_{50} of lavender EO was calculated as 2.5 µl/ml, while MIC_{90} was 10 µl/ml. MIC_{50} of clary sage EO was calculated as 1.25 µl/ml, and MIC_{90} was five µl/ml. On the other hand, MIC_{50} for both oregano and thyme was <0.039 µl/ml, and MIC_{90} was 0.078 and 0.156 µl/ml for oregano and thyme, respectively. Lowest MIC and MBC ranges were obtained from oregano; for MIC <0.039 to 0.312 µl/ml and for MBC <0.039 to 0.625 µl/ml. Followed by thyme; MIC values were ranged from <0.039 to 0.625 µl/ml and MBC values were ranged from <0.039 to 1.25 µl/ml. MIC values for clary sage were between 0.078 to 10 µl/ml, while MBC were between 0.625 to 20 µl/ml. Lastly, the highest MIC and MBC ranges were obtained for lavender; MICs were between 0.039 to 20 µl/ml, and MBCs were 0.078 to 40 µl/ml.

Biofilm Formation

Biofilm formation assay showed that 28.2% (n=20; 8 MRSA and 12 MSSA) of the isolates were able to form biofilm, and 13 of them were considered low-grade biofilm former, and 7 were intermediate-grade biofilm former. 50.0% of MRSA isolates were biofilm-forming isolates, whereas 21.8% of MSSA isolates formed biofilm. S. *aureus* ATCC 6538 appeared as a strong biofilm former.

Calculation of Biofilm Formation Inhibition Percentage of EOs In Dose-Dependent Manner

Lavender EO showed biofilm formation inhibition activity on all tested isolates. Biofilm formation inhibition was detected in 30% of isolates at all lavender concentrations ranging from 0.039 to 10 µl/ml, and 50.0% of them were MRSA. While biofilm inhibition percentages at lavender concentration 10 µl/ml were ranged from 92.4% to 28.6%, at concentration 5 µl/ml, inhibition percentages were ranged from 88.5% to 5.4%. At lavender concentration below 2.5 µl/ml, there was no biofilm inhibition activity for 45.0% of isolates, and 37.5% of them were MRSA. At the oregano concentration ranging from 10 to 0.312 µl/ml, biofilm formation was inhibited in all of the isolates. Also, 45.0% of the isolates had biofilm inhibition activity in all concentrations of and 44.4% of them were MRSA. At the lowest concentration (0.039 µl/ml) of oregano, biofilm inhibitory percentages were ranged from 75.6% to 1.5%. Biofilm inhibition was detected in 25.0% of isolates at all concentrations of thyme, and 20% of them were MRSA. At the lowest concentration (0.039 µl/ml) of thyme, biofilm formation was inhibited at a percentage ranging from 71.7% to 12.8%. Biofilm formation inhibition was detected in 41.2% of isolates at all clary sage concentrations, and 28.6% of them were MRSA. In general, as the concentration of EOs decreased, so did the biofilm formation inhibition percentages (Fig. 1).

Effects of EOs on Biofilm Viability

- Biotimer Assay

A serial 10-fold dilution of S. *aureus* ATCC 6538 in BT-PR medium was employed to count the number of bacteria through a correlation line (Fig. 2). There was a linear correlation between time for complete colour change and $\log_{10}$CFU. Linear correlation was calculated as: y=-0.0073x + 10.2822 and r =0.9826.

- EOs Susceptibility Test on Biofilm

MBIC and MBEC values of each of the four EOs were demonstrated in (Table 1). MBIC and MBEC of tested EOs were higher than those MIC and MBC, with some exceptions.

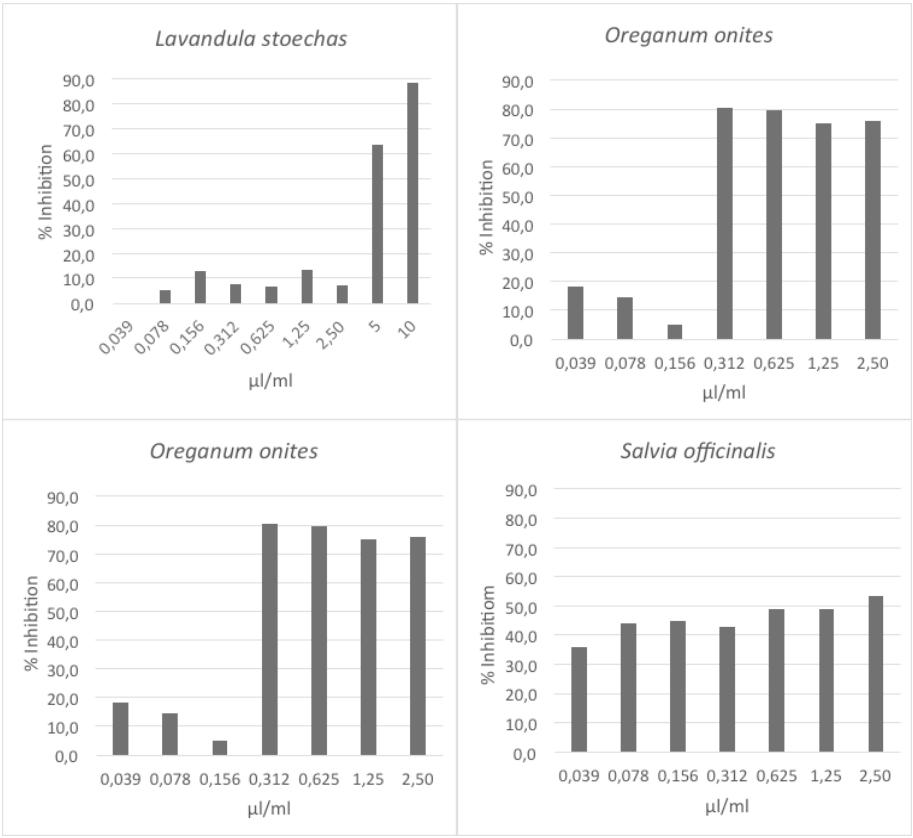

Figure 1. Concentrations versus biofilm inhibition percentages of each EO

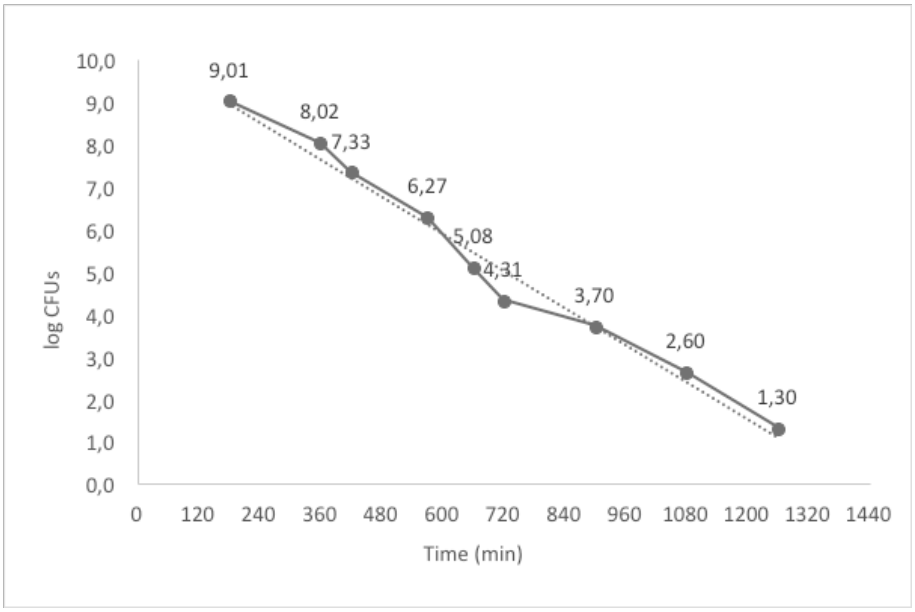

Figure 2. Correlation line of number of *S. aureus* ATCC 6538 and time of complete color change from red to yellow

Table 1. Lavender, thyme, oregano and clary sage Essential Oils MIC, MBIC, MBC and MBEC values of biofilm-forming isolates

Methicillin Sensitivity	Isolate Number	*Lavandula stoechas*				*Origanum onites*				*Thymus vulgaris*				*Salvia officinalis*			
		MIC (µl/ml)	MBIC (µl/ml)	MBC (µl/ml)	MBEC (µl/ml)	MIC (µl/ml)	MBIC (µl/ml)	MBC (µl/ml)	MBEC (µl/ml)	MIC (µl/ml)	MBIC (µl/ml)	MBC (µl/ml)	MBEC (µl/ml)	MIC (µl/ml)	MBIC (µl/ml)	MBC (µl/ml)	MBEC (µl/ml)
MRSA	8	5	2.5	20	20	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	0.312	1.25	10	20
MRSA	12	5	5	20	40	0.078	<0.039	0.312	<0.039	<0.039	<0.039	<0.039	<0.039	0.156	0.156	20	40
MRSA	14	1.25	5	20	40	<0.039	<0.039	<0.039	<0.039	0.156	<0.039	0.312	<0.039	1.25	5	20	40
MRSA	16	2.5	5	20	40	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	0.078	0.312	0.312	20	40
MRSA	21	5	5	20	40	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	1.25	5	20	40
MSSA	44	5	2.5	20	40	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	0.156	<0.039	5	10	10	20
MSSA	49	5	2.5	20	40	<0.039	<0.039	<0.039	<0.039	0.156	<0.039	0.625	<0.039	2.5	5	10	20
MSSA	50	5	5	20	20	<0.039	<0.039	<0.039	<0.039	0.625	<0.039	1.25	<0.039	1.25	5	10	10
MSSA	51	5	10	20	40	<0.039	<0.039	<0.039	<0.039	0.312	<0.039	0.625	<0.039	1.25	5	10	10
MSSA	52	5	2.5	20	40	<0.039	<0.039	0.039	<0.039	0.625	<0.039	1.25	0.039	0.312	0.625	10	10
MSSA	56	2.5	5	10	40	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	2.5	5	20	20
MSSA	71	2.5	5	20	40	<0.039	<0.039	<0.039	<0.039	0.156	<0.039	0.625	0.078	0.078	0.078	1.25	10
MSSA	72	2.5	5	20	40	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	0.312	0.312	5	10
MSSA	78	2.5	5	20	40	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	1.25	1.25	10	20
MSSA	82	5	2.5	20	40	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	0.625	1.25	10	20
MSSA	86	5	2.5	20	40	<0.039	<0.039	<0.039	<0.039	0.312	<0.039	0.625	<0.039	0.312	1.25	10	20
MRSA	163	1.25	5	20	20	0.078	<0.039	0.312	<0.039	0.156	<0.039	0.625	<0.039	0.625	0.625	20	20
MRSA	165	1.25	10	20	20	0.156	<0.039	0.625	<0.039	0.312	<0.039	1.25	<0.039	1.25	10	10	20
MRSA	166	1.25	5	20	20	0.078	<0.039	0.625	<0.039	0.625	<0.039	1.25	<0.039	1.25	5	10	40
MSSA	172	1.25	5	20	20	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	0.156	<0.039	1.25	5	10	40
MSSA ATCC	6538	1.25	5	1.25	20	<0.039	<0.039	0.156	<0.039	<0.039	<0.039	<0.039	<0.039	0.039	0.625	1.25	1.25
MSSA ATCC	25923	2.5	5	10	20	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	<0.039	0.312	0.312	2.5	10

- 3.4.3. Enumeration of Viable Bacteria in Biofilm in Sub-Inhibitory Doses of EOs

The number of viable bacteria in biofilm in sub-inhibitory doses of lavender and clary sage EOs was quantified with BTA. The number of *S. aureus* ATCC 6538 after treatment with a sub-MIC concentration from 1/2 to 1/64 was calculated as a percentage by correlation line (Table 2). As the concentration of oils decreases, the percentage of viable bacteria increases.

Table 2. Number of viable *S. aureus* ATCC 6538 after treatment with sub-inhibitory doses of lavender and clary sage essential oil as a percentage

Lavandula stoechas		*Salvia officinalis*	
MIC (µl/ml)	Percentage of viable bacteria (%)	MIC (µl/ml)	Percentage of viable bacteria (%)
1/2	70.44	1/2	87.49
1/4	72.89	1/4	89.68
1/8	87.44	1/8	ND
1/16	92.22	1/16	ND
1/32	94.78	1/32	94.06
1/64	99.67	1/64	ND

*ND: not detected.

DISCUSSION

Plants and derivatives such as EOs are well-known natural substances having broad-spectrum activity against pathogens. In this research, oregano, thyme, clary sage and lavender EOs antibacterial activities were evaluated, and the highest antibacterial activity was observed with thyme and oregano followed by clary sage. The lowest antimicrobial activity against 71 clinical *S. aureus* isolates was detected in lavender. While 85.9% of isolates were susceptible to oregano at a concentration below 0.039 µl/ml, 70.4% of isolates were susceptible to thyme at a concentration below 0.039 µl/ml. MBC for oregano was found to be between <0.039 to 0.625 µl/ml, while for thyme, MBC varies between <0.039 to 1.25 µl/ml. MIC and MBC values for lavender and clary sage were higher than those for thyme and oregano. MSSA showed greater susceptibility to EOs than MRSA. However, even at the lowest concentration of thyme and oregano, most of the MRSA isolates were detected to be susceptible to these EOs.

A study on the antibacterial property of thyme oil on *S. aureus* isolates demonstrated that MIC varied between 0.5 to 1 µl/ml (25). Even though these MIC values were relatively higher than the current study, Fabio et al., demonstrated that MIC for *Thymus vulgaris* against *S. aureus* was 0.0125 µl/ml (26). It is known that compositions of EOs play significant roles in the antimicrobial properties of

EOs. Several factors can affect EOs compositions, such as harvesting seasons and geographical sources (6), which may be the reason of the differences in efficacy of EOs. A previous study showed that clary sage EO was effective at concentrations between 3.75 and 5.25 µl/ml on 27 clinical *S. aureus* isolates, which is in concordance with our findings; clary sage EO's MIC_{50} was found 5 µl/ml for 71 clinical *S. aureus* isolates (27). Previous studies demonstrated that EOs of lavender and oregano are highly effective against a wide range of microorganisms including *Escherichia coli*, *S. aureus*, *P. aeruginosa* and *C. albicans* (28, 29, 30). These results highlighted that EOs and their components are promising alternatives. Therefore, further experiments including in vivo studies are required to evaluate EOs as a therapeutic agent in the medicinal application.

The antibiofilm activity of various EOs has been demonstrated on a variety of microorganisms and many EOs and their components were accepted as potent agents against biofilms. Different EOs mechanisms of action as antibiofilm agents were also reported for *S. aureus* however; there are not sufficient studies particularly related to multidrug-resistant clinical isolates such as MRSA (31). Besides, the lack of knowledge regarding effective doses for preventing biofilm formation is a pivotal limitation for EO studies. In this study, each of the 4 EOs was tested against their ability to prevent biofilm formation in a dose-dependent manner. Higher doses of EOs inhibited biofilm formation at higher percentages, as expected. While thyme and oregano were able to prevent biofilm formation at a significant percentage even in the lowest concentrations, lavender and clary sage were able to inhibit biofilm formation at higher doses. Even though isolates were more susceptible to clary sage than lavender, lavender was more successful in preventing biofilm formation. Based on this result, the components involved in the antibacterial property of EOs were different from those associated with biofilm formation. It was seen that in a biofilm-forming isolate, biofilm inhibition was significantly reduced as low as 0.156 µl/ml oregano concentration. At the concentration of 0.156 µl/ml, oregano showed nearly no activity for preventing biofilm formation (5%); this may be due to the fact that this concentration is the MIC concentration of this isolate. Also, this study is limited because, at the highest concentrations of thyme, oregano and clary sage, oil intensity was too high, which can confuse the calculation of inhibition percentage. Therefore, the first two highest concentration of thyme and clary sage (10 and 5 µl/ml), and the highest concentration of oregano was not included in the calculation of biofilm inhibition percentage (Fig. 1). Even though biofilm formation was inhibited in these concentrations, the percentages of biofilm formation inhibition for the highest concentrations can be misleading. Therefore, these EOs may require to work in a lower concentration.

EOs' efficacy in biofilm is a concern among microbiologists. There used to be no optimized method for counting the number of viable bacteria in the biofilm. Pantanella et al. (24) presented BTA, which is able to count the number of viable bacteria without manipulation. *S. aureus* ATCC 6538 was used to draw a genus-specific correlation line that correlates the time for colour change and the first concentration of bacteria. This method was also confirmed by the CFU method, and the results were in concordance. Besides, BTA allows to count of the number of viable bacteria in biofilm and provides MBIC and MBEC for each isolate. The number of viable bacteria in sub-inhibitory doses of lavender and clary sage was detected and indicated as a percentage. MIC 1/8, 1/16 and 1/64 were not detected for clary sage due to the rapid colour changes. The number of viable bacteria in the biofilm was found to increase as the concentration of EOs decreased. Thyme and oregano were not included in this study because even in sub-inhibitory doses, there are few viable bacteria, so the BT-PR medium remained red, which means that thyme and oregano EOs were highly effective even in sub-inhibitory doses.

In conclusion, this study employs BTA to determine actual bacteria count in biofilm, which has pivotal significance since EOs susceptibility is affected by bacterial concentration. It is known that BTA is the first method that enumerates viable bacteria in sub-MICs of antibiotics (24). To the best of our knowledge, this is the first study that enumerates viable bacteria in sub-MICs of EOs.

Results obtained from this research may provide the basis for further research for developing these natural substances as new therapeutics for bacterial infections, especially those caused by MSSA or MRSA. High efficacy of EOs, especially thyme and oregano in clinical *S. aureus* isolates, including MRSA, is promising and may become a new method for treating multidrug-resistant pathogens. The *in vitro* results of this study provide evidence that these EOs can be an alternative for anti-infectious agents. Usage of these EOs alone and in combination with either other EOs or antibiotics will provide significant information regarding the clinical values of these natural antimicrobials.

ETHICS COMMITTEE APPROVAL

* This study does not require Ethics Committee Approval.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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