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RESEARCH ARTICLE

Effect of Oregano Essential Oil on Biofilms Formed By Staphylococci and *Escherichia coli*

Nebahat BİLGE ORAL * Leyla VATANSEVER * Berna DUMAN AYDIN *
Çiğdem SEZER * Abamüslüm GÜVEN * Murat GÜLMEZ *
Kemal Hüsnü Can BAŞER ** Mine KÜRKÇÜOĞLU **

* University of Kafkas, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, TR-36100 Kars - TÜRKİYE
** Anadolu University, Farmacy Faculty, Department of Pharmacognosy, TR-26470 Eskişehir - TÜRKİYE

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Summary

In the present study, it was aimed to investigate the effect of oregano *(Origanum onites)* essential oil (EO) on biofilm formation and established biofilm. *Staphylococcus aureus* (n=6), *Staphylococcus lugdunensis* (n=1), *Staphylococcus haemolyticus* (n=1), *Staphylococcus sciuri* (n=1) and *Escherichia coli* (n=1) were used as the test organisms. The crystal violet assay was used for assessing the growth of the biofilm on 96-well polystyrene microtitre plates. The minimum inhibitory concentration (MIC) was determined by broth dilution method as 0.05% (v/v) for staphylococci with the exception of *Staph. sciuri* (0.8%, v/v) and 0.1% (v/v) for *E. coli.* Oregano EO inhibited biofilm formation and eradicated established biofilm at MIC level. Subinhibitory concentrations of the EO reduced the level of biofilm formation of the test strains. Further investigations should be examined whether these observations extend to biofilms formed on other surfaces, particularly those found in food processing plants. The ability of biofilm-embedded microorganisms to resist clearance by antimicrobial agents points to the importance of a continuous search for novel agents. The use of oregano EO as a natural antimicrobial agent can be an effective alternative or a supplement for the control of microorganisms.

Keywords: *Biofilm, Staphylococci, Escherichia coli, Oregano, Essential oil*

Kekik Uçucu Yağının Stafilokoklar ve *Escherichia coli* Tarafından Oluşturulan Biyofilmler Üzerine Etkisi

Özet

Bu çalışmada, kekik *(Origanum onites)* uçucu yağının, biyofilm oluşumu ve oluşmuş biyofilm üzerine etkisini belirlemek amaçlanmıştır. Test mikroorganizmaları olarak *Staphylococcus aureus* (n=6), *Staphylococcus lugdunensis* (n=1), *Staphylococcus haemolyticus* (n=1), *Staphylococcus sciuri* (n=1) ve *Escherichia coli* (n=1) suşlarından yararlanılmıştır. Biyofilm gelişiminin 96 kuyucuklu polistiren plaklarda görüntülenebilmesi için, Kristal Viyole deneyi uygulanmıştır. Broth Dilusyon Yöntemi kullanılarak değerlendirilen minimum inhibisyon konsantrasyonları (MİK), *Staph. sciuri* (%0.8 v/v) dışındaki stafilokok suşları için %0.05 (v/v), *E. coli* için ise %0.1 (v/v) olarak belirlenmiştir. Kekik uçucu yağı, MİK düzeyinde kullanıldığında biyofilm oluşumunu inhibe etmiş, oluşmuş biyofilmi eradike etmiştir. İnhibitörik düzeyin altındaki konsantrasyonlarda ise test suşları tarafından biyofilm oluşturma düzeyini düşürmüştür. Başta gıda işleme yerlerinde bulunanlar olmak üzere, diğer yüzeylerde de benzer etkilerin meydana gelip gelmeyeceğinin belirlenmesi amacıyla, yeni çalışmaların yapılmasının yerinde olacağı kanaatine varılmıştır. Biyofilm içinde yer alan mikroorganizmaların, antimikrobiyel ajanlarla temizliğe direnç göstermesi, yeni ajanların bulunması yönündeki çalışmaların önemine işaret etmektedir. Bu anlamda kekik uçucu yağının, doğal bir antimikrobiyel madde olarak, mikroorganizmaların kontrol altına alınmasında etkili bir alternatif olabileceği ya da bu doğrultuda katkıda bulunabileceği görülmektedir. .

Anahtar sözcükler: *Biyofilm, Stafilokok, Escherichia coli, Kekik, Uçucu yağ*

İletişim (Correspondence)
☎ +90 474 2426807/1178
✉ nebahatbilgeoral@hotmail.com

INTRODUCTION

A biofilm is a multicellular layer of adherent bacteria surrounded by a matrix of extracellular polysaccharides [1]. Typically, anywhere that there is a flow of water, organisms and a solid surface, a biofilm can be formed. The solid surfaces that can harbour biofilms in food plants include stainless steel, aluminium, glass, nylon materials, Buna-N and Teflon seals. Surfaces that are pitted, scratched or cracked provide an excellent opportunity to trap food particles and bacteria, which begins the formation of a biofilm. Corrosion patches and dead ends are also areas where biofilms can grow [2].

In nature, biofilms or adhesion of microorganisms may be composed of a single species or represent a consortium of numerous species [3]. They can cause significant problems in many areas, both in medical settings (e.g. persistent and recurrent infections, device-related infections) and in non-medical (industrial) settings (e.g. biofouling in drinking water distribution systems and food processing environments [4,5]. The attachment of bacteria with subsequent development of biofilms in food processing environments is a potential source of contamination that may lead to food spoilage or transmission of disease [6].

It is well established that bacteria contained within biofilms exhibit increased resistance to antimicrobial treatments compared to individual cells grown in suspension. As the biofilm matures, resistance against various disinfectants is greater than with younger (less than 24 h) biofilms [2]. To control these problems, it has been recognized that a greater understanding of the interaction between microorganisms and food processing surfaces is required [3]. The best way of controlling biofilms is to prevent their development [2].

The control of food borne pathogens such as Staphylococci and *Escherichia coli* has received a great deal of attention because these organisms can form resilient biofilms on a range of surfaces [7,8]. Several genes and mechanisms are involved in biofilm production, but the regulatory mechanisms are poorly understood. The finding that biofilm formation may be promoted at conditions in the food industry indicates that the food producers should be aware that controlling biofilm formation by *S. aureus* may be of importance [7]. *E. coli* can behave as a commensal, intestinal diarrhoeagenic, and extraintestinal pathogenic microorganism. It has been shown that *E coli* strains causing prostatitis produce biofilms *in vitro* more frequently than those causing urinary tract infections, and that they are more likely to be haemolysin producers. In addition, biofilm-forming strains show significantly greater haemolysin and type 1 fimbriae expression [9].

Eradication usually requires the use of alkaline or acidic detergents and/or iodophores. Though efficacious, issues such as corrosion, product contamination, and toxicity limit the use of these compounds [10]. The ability of biofilm-embedded microorganisms to resist clearance by antimicrobial agents points to the importance of a continuous search for novel agents that are effective against bacteria in this mode of growth or work in synergy with the currently available myriad of anti-microbials [11]. The use of natural antimicrobial agents can be an effective alternative or supplement for the control of microorganisms [10]. One approach may be the use of essential oils that have been shown to be potential agents in the treatment of infections, and are safe in terms of human and animal health. In this context, oregano oil and its major phenolic components, carvacrol and thymol are known for their wide spectrum of antimicrobial activity, which has been the subject of several investigations *in vitro* and *in vivo* [12]. The objective of this study was to evaluate the activity of oregano oil *(Origanum onites)* on biofilm-grown Staphylococci and *E. coli* strains, as well as the effect of oil on biofilm formation.

MATERIAL and METHODS

Essential Oil

The oregano essential oil *(Origanum onites)* was provided by Türer Tarım Ltd. Şti. (Türer Tarım ve Orman Ürünleri İthalat İhracat Sanayii ve Ticaret Limited Şirketi, Kavaklıdere Köyü, Bornova, İzmir, Türkiye). The oil was analyzed by capillary GC and GC/MS using an Agilent GC-MSD system. The GC/MS analysis was carried out with an Agilent 5975 GC-MSD system. Innowax FSC column (60 m x 0.25 mm, 0.25 μm film thickness) was used with helium as carrier gas (0.8 ml/min). GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, and kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. Split ratio was adjusted 40:1. The injector temperature was at 250°C. MS were taken at 70 eV. Mass range was from m/z 35 to 450. The GC analysis was carried out using an Agilent 6890N GC system. In order to obtain same elution order with GC/MS, simultaneous injection was done by using same column and appropriate operational conditions. FID temperature was 300°C. The components of essential oils were identified by comparison of their mass spectra with those in the Baser Library of Essential Oil Constituents, Wiley GC/MS Library, Adams Library, Mass

Finder Library and confirmed by comparison of their retention indices. Alkanes were used as reference points in the calculation of relative retention indices (RRI). Relative percentage amounts of the separated compounds were calculated from FID chromatograms. The results of analysis are shown in *Table 1*.

Bacterial Strains

The bacteria used were *Staphylococcus aureus* (n=5) (Sa1, Sa2, Sa3, Sa4, Sa5), *S. lugdunensis* (n=1) (SL), *S. haemolyticus* (n=1) (Sh), *S. sciuri* (n=1) (Ss), isolated from restaurant workers and milk samples, belonging to our private collection, and the reference strains *S. aureus* NCTC 8325 (n=1) (Sa), *Escherichia coli* (Strain no: 97010) (n=1) (Ec) provided by Refik Saydam Hygiene Center Presidency National Type Refik Saydam Culture Collection Laboratory, Ankara, Turkey. Each isolate was characterized for biofilm related properties as reported previously [13]. The isolates were capable of forming biofilms with an OD_{570} ranging from 0.305 to 0.521.

Efficacy of Oregano Oil on Planktonic Cells

The minimum inhibitory concentrations (MIC) of oregano essential oil (EO) on planktonic cells were determined in Tryptic Soy Broth (TSB, Difco 211822) using the broth dilution method according to Nostro et al.[14]. An overnight bacterial culture was inoculated to TSB with EO at the level of 1%. The final concentrations of EO in the medium ranged from 0.25% to 3%. Tween 80 was used at concentration of 0.1% to enhance EO solubility in medium. The growth of test strains in TSB including EO was evaluated by plating on Tryptic Soy Agar (TSA, Difco 236950) after incubation at 37°C, 24 h. The MIC was defined as the lowest concentration of the oregano oil inhibiting the growth of each strain. All determinations were performed in duplicate and two growth controls consisting of TSB medium and TSB with 0.1% (v/v) Tween 80 included.

Effect on Biofilm Formation

The effect of different concentrations of oil on biofilm forming ability was tested on polystyrene flat-bottomed microtitre plates as described Hammer et al.[13] and Nostro et al.[12] with some modifications. As a treatment solution, the concentrations of oregano oil were prepared in TSB with 0.25% glucose and 0.1% Tween 80 (TSBG) at the level of 0.25 MIC, 0.50 MIC and MIC. Then, cultures were grown overnight in TSBG and 10 µl were dispensed into each well of microtitre plate containing 90 µl of treatment solutions. For the negative controls, 10 µl of TSBG were dispensed into each well consisting 90 µl of treatment solutions. The positive control group was also obtained by inoculating 10 µl of cultures to 90 µl of TSBG. After incubation at 37°C for 24 h, the contents of each well were removed and the wells were washed three times with sterile physiological saline (0.85% NaCl). Trays were shaken vigorously to remove non-adherent bacteria. Adherent bacteria were fixed by adding 99% methanol to wells and leaving for 15 min at room temperature. The wells were then emptied and left to dry. Biofilm was stained by adding 200 µl of 2% crystal violet stain for 5 min. The trays were then rinsed with water. After drying, stain was resolubilised by adding 160 µl of 33% glacial acetic acid to each well and agitating gently, and then OD_{570} was measured by spectrophotometer using an ELISA reader. Each assay was performed in triplicate. As a measure of efficacy, OD_{570} of negative control was subtracted from corresponding absorbance reading and compared to that of positive control.

Effect on Established Biofilms

The effect on established biofilms was verified as described by Nostro et al.[12] with some modifications. All isolates were grown as biofilms in wells of polystyrene flat-bottomed microtitre plates for treatment and positive control groups. Five wells for each isolate were not inoculated (Negative controls). After 24 h of incubation at 37°C, the planktonic-phase cells were gently removed and the wells were washed three times with physiological saline and filled with 200 µl twofold dilutions of the EO, ranging from MIC to 8 MIC. For the negative controls, not inoculated wells were also filled with those EO dilutions. The positive control group was also obtained by adding 200 µl of TSBG. The plates were incubated for 24 h at 37°C. The OD_{570} was measured at time 0 and after incubation for 24 h. The biofilm inhibitory concentration (BIC) was determined as the lowest concentration where no growth occurred in the supernatant fluid, confirmed by no increase in optical density compared with the initial reading. Samples of biofilms from the bottom of these wells were scarified by a metal loop, spread over the surface of TSA and incubated for 72 h at 37°C. The biofilm eradication concentration (BEC) was determined as the lowest concentration at which no bacterial growth occurred on the TSA plates. Data from four replicates were evaluated.

Statistical Analysis

The data were initially tested for normal distribution by one-sample Kolmogrov-Smirnov test. Following the confirmation of normal distribution ($P>0.001$), differences for individual parameters between control and treated groups were tested by paired-sample t-test using SPSS Version 9.05 for Windows. Differences were considered significant if the P value was less than 0.001.

RESULTS

Composition of Oregano Oil

GC/MS results indicated that the two phenols, carvacrol and thymol were the major components of oregano essential oil *(Table 1)*. Some of the researchers reported that compounds are mainly responsible for its antimicrobial activity [15,16].

Table 1. *The composition of Origanum onites oil*
Tablo 1. *Origanum onites yağının bileşimi*

RRI	Main Compounds	%
1280	*p*-cymene	7.0
1553	Linalool	3.6
1611	Terpinen-4-ol	1.7
1719	Borneol	1.2
1741	β-bisabolene	1.9
2198	Thymol	7.4
2239	Carvacrol	70.2
	Others	7

Table 2. *Minimum inhibitory concentration (MIC) of oregano EO on test strains*
Tablo 2. *Kekik uçucu yağının test suşlarına karşı minimum inhibisyon kansantrasyonu*

MIC (%, v/v, EO Concentration in Distilled Water)	Strain (Number)
0.05 %	*S. aureus* (n=6)
0.05 %	*S. lugdunensis* (n=1)
0.05 %	*S. haemolyticus* (n=1)
0.8 %	*S. sciuri* (n=1)
0.1 %	*E. coli* (n=1)

Efficacy of Oregano Oil on Planktonic Cells

A total of 10 isolates was tested for their susceptibility to oregano EO. The MICs of oregano EO for each organism are given in *Table 2*. The values ranged from 0.1% to 0.8%.

Efficacy of Oregano Oil on Biofilm Formation

Despite a different inhibitory effect among the strains, a reduced level of biofilm formation in the presence of subinhibitory concentrations of oregano EO was observed *(Table 3)*. Doses of MIC and .05 MIC showed a greater influence than that of 0.25 MIC.

Efficacy of Oregano Oil on Established Biofilm

A statistically significant reduction was noted in biofilms that were treated with oregano EO even at MIC level. The findings indicating biofilm eradication effect of EO are given in *Table 4.*

Table 3. *Effect of oregano oil on biofilm formation (means± standard deviation)*
Tablo 3. *Kekik yağının biyofilm oluşumu üzerine etkisi (ortalama±standart sapma)*

Strain	Biofilm Formation			
	MIC	0.5 MIC	0.25 MIC	Positive Control
Sa1	0.14±0.020 ^{a}	0.19±0.010 ^{a}	0.40±0.050 ^{b}	0.50±0.010 ^{b}
Sa2	0.07±0.050 ^{a}	0.23±0.020 ^{b}	0.36±0.020 ^{b,c}	0.46±0.010 ^{c}
Sa3	0.10±0.006 ^{a}	0.23±0.030 ^{b}	0.37±0.010 ^{c}	0.49±0.006 ^{d}
Sa4	0.08±0.007 ^{a}	0.13±0.010 ^{a,b}	0.24±0.030 ^{b,c}	0.32±0.010 ^{c}
Sa5	0.10±0.002 ^{a}	0.20±0.010 ^{b}	0.30±0.006 ^{c}	0.39±0.010 ^{d}
SL	0.08±0.004 ^{a}	0.18±0.010 ^{a}	0.34±0.030 ^{b}	0.47±0.010 ^{c}
Sh	0.09±0.004 ^{a}	0.14±0.003 ^{b}	0.22±0.003 ^{c}	0.40±0.006 ^{d}
Ss	0.10±0.008 ^{a}	0.23±0.020 ^{b}	0.36±0.020 ^{c}	0.46±0.010 ^{c}
Sa	0.10±0.003 ^{a}	0.24±0.020 ^{b}	0.38±0.010 ^{c}	0.50±0.010 ^{d}
Ec	0.08±0.005 ^{a}	0.13±0.004 ^{b}	0.23±0.010 ^{c}	0.41±0.010 ^{d}

Means with different letters (a, b) in the same row for each EO concentration are significantly different (P≤0.001)

DISCUSSION

Bacteria in biofilm are known to be much more resistant to antimicrobial agents than free-living cells and may act as continuous sources of spoilage and pathogenic bacteria that contaminate food. This increased resistance is not often considered during disinfection, and many studies dealing with the effect of disinfectant are carried out in broth cultures. The current interest in natural antimicrobial compounds has increased due to changes in consumer attitudes toward the use of synthetic preservative agents in food, surface detergents and disinfectants that have a negative impact on the environment [17]. Selected natural products that originate in plants can influence microbial biofilm formation through different mechanisms. Many plant essential oils, which are mixtures of numerous organic chemicals, contain compounds that inhibit microbial growth [18]. In our study, oregano essential oil showed antibacterial activity against all the test organisms. Moreover, it also had effect on biofilm formation and established biofilm even at MIC level. Nostro et al.[12] also reported that *Origanum vulgare* L. essential oil inhibited growth of preformed biofilm and interfered with biofilm formation during planktonic growth. It was documented in their article that carvacrol and thymol which are the principal phenolic components of oregano oil may be responsible for the effects observed on biofilm formation. They could diffuse through the polysaccharide matrix of the biofilm and destabilize it due to their strong intrinsic antimicrobial properties, the researchers continued. In this study, carvacrol (70.2%), thymol

Table 4. *Effect of oregano oil on established biofilm formation (means±standard deviation)*
Tablo 4. *Kekik yağının oluşmuş biyofilm üzerine etkisi (ortalama±standart sapma)*

Strain	Biofilm Formation				
	MIC	2MIC	4MIC	8MIC	Control
Sa1 (BI)	0.081±0.002	0.098±0.008	0.099±0.002	0.093±0.002	0.097±0.003 ^{k}
Sa1 (AI)	0.096±0.003 ^{a}	0.097±0.002 ^{a}	0.100±0.002 ^{a}	0.097±0.002 ^{a}	0.559±0.076 ^{b,l}
Sa2 (BI)	0.081±0.006	0.100±0.002	0.090±0.001	0.090±0.002	0.116±0.009 ^{k}
Sa2 (AI)	0.096±0.003 ^{a}	0.096±0.004 ^{a}	0.097±0.002 ^{a}	0.098±0.001 ^{a}	0.397±0.013 ^{b,l}
Sa3 (BI)	0.100±0.004	0.102±0.003	0.097±0.001	0.100±0.004	0.100±0.013 ^{k}
Sa3 (AI)	0.095±0.004 ^{a}	0.102±0.004 ^{a}	0.100±0.004 ^{a}	0.101±0.005 ^{a}	0.617±0.017 ^{b,l}
Sa4 (BI)	0.083±0.007 ^{a}	0.089±0.003 ^{a,b}	0.094±0.003 ^{a,b}	0.097±0.002 ^{b}	0.091±0.003 ^{a,b,k}
Sa4 (AI)	0.088±0.007 ^{a}	0.100±0.002 ^{a}	0.100±0.002 ^{a}	0.100±0.002 ^{a}	0.664±0.005 ^{b,l}
Sa5 (BI)	0.081±0.006 ^{a}	0.086±0.007 ^{a,b}	0.096±0.003 ^{a,b}	0.098±0.003 ^{b}	0.100±0.004 ^{b,k}
Sa5 (AI)	0.088±0.044 ^{a}	0.093±0.003 ^{a}	0.096±0.002 ^{a}	0.098±0.002 ^{a}	0.609±0.014 ^{b,l}
SL (BI)	0.101±0.003	0.098±0.001	0.100±0.003	0.093±0.006	0.099±0.003 ^{k}
SL (AI)	0.103±0.005 ^{a}	0.104±0.003 ^{a}	0.103±0.003 ^{a}	0.105±0.004 ^{a}	0.568±0.061 ^{b,l}
Sh (BI)	0.079±0.003	0.073±0.005	0.076±0.002	0.079±0.003	0.083±0.005 ^{k}
Sh (AI)	0.080±0.002 ^{a}	0.075±0.005 ^{a}	0.074±0.002 ^{a}	0.078±0.003 ^{a}	0.581±0.015 ^{b,l}
Ss (BI)	0.068±0.002	0.070±0.002	0.075±0.003	0.077±0.004	0.069±0.002 ^{k}
Ss (AI)	0.068±0.002 ^{a}	0.069±0.002 ^{a}	0.072±0.002 ^{a}	0.074±0.002 ^{a}	0.695±0.059 ^{b,l}
Sa (BI)	0.067±0.003	0.070±0.002	0.075±0.004	0.076±0.003	0.069±0.002 ^{k}
Sa (AI)	0.068±0.002 ^{a}	0.070±0.002 ^{a}	0.074±0.002 ^{a}	0.076±0.003 ^{a}	0.511±0.019 ^{b,l}
Ec (BI)	0.080±0.003 ^{a}	0.093±0.003 ^{a,b}	0.094±0.004 ^{a,b}	0.090±0.003 ^{a,b}	0.116±0.009 ^{c,k}
Ec (AI)	0.096±0.002 ^{a}	0.096±0.00 ^{a}	0.097±0.003 ^{a}	0.098±0.003 ^{a}	0.418±0.012 ^{b,l}

BI: *Before incubation,* ***AI:*** *After incubation*
Means with different letters (a, b) in the same row for each EO concentration are significantly different (P≤0.001)
Means with different letters (k, l) in the same column for each strain are significantly different (P≤0.001)

(7.4%) and *p*-cymene (7%) were the main components of oregano essential oil that we used.

Microbial cell surface interaction in leakage of intracellular constituents has been suggested as the biocidal mechanism for carvacrol-mediated antimicrobial activity [19]. Exposure of *S. aureus* to carvacrol during the early stages of biofilm development led to potent inhibition of matrix formation, with shedding of proteinaceous mass after each antimicrobial pulse. Rapid killing of *S. aureus* by carvacrol also led to disruption of the proteinaceous matrix of the film. However, the shedding of such proteinaceous mass did not coincide with viability reductions of staphylococci in the biofilm, possibly due to continuous exfoliation of the matrix. Lysostaphin is an agent with a mode of action similar to that of carvacrol [10] and in a study conducted by Wu et al.[20]; the researchers demonstrated that, *in vitro,* lysostaphin disrupted *S. aureus* biofilms on polystyrene, polycarbonate, and glass surfaces. Their findings also showed this antimicrobial eradicated both sessile *S. aureus* cells of the biofilm and the extracellular matrices.

Thymol is the one of the main compounds of oregano EO. Lebert et al. [17] reported that thymol did not kill any bacteria including *E. coli* and *S. aureus* in biofilm, while Satureja thymbra oil, containing thymol (41%), γ-terpinene (22.2%) and p-cymene (11.8%) as previously informed by Chorianopoulos et al.[21], reduced the population of *S. aureus* and *E. coli* grown in biofilm. Burt [16] suggested that the differences observed between the effects of pure thymol and the essential oil may be due to a combined effect of thymol and other molecules, such as terpinene and cymene, which can have a synergic or additive effect. In this study, oregano oil showed strong effect against *E. coli* and *Staphylococci strains* including *S. aureus.* This data supports the suggestion of Burt [16]. Similar results were reached by Quave et al.[22] for methicillin-resistant *S. aureus* biofilm using *Lonicera alpigena, Castanea sativa, Juglans regia, Ballota nigra, Rosmarinus officinalis, Leopoldia comosa, Malva sylvestris, Cyclamen hederifolium, Rosa canina var. canina* and *Rubus ulmifolius* extracts and also by Kuźma et al.[23] for antibiotic resistant staphylococci biofilm using *Salvia sclarea* L. extract.

According to our findings, oregano EO was effective on biofilm formed by test strains. It was reached that results using Crystal violet (CV) staining technique and it

was observed that CV assay suitable for determining the effect of essential oil on biofilm formation. In a study conducted by Niu and Gilbert [18], the researchers reported that Cinnamomum cassia essential oil reduced the extent of biofilm formation by *E. coli*. They also used this assay and informed CV staining has been widely adopted by microbiologists to investigate mutants with respect to adhesion or biofilm formation, attachment to diverse surfaces, and to compare biofilm development in different pathogens. Its greatest features are that it is inexpensive, relatively quick, and adaptable for use high-throughput screening with microtitre plates.

In this study, a reduced level of biofilm formation by Staphylococci and *E. coli* in the presence of subinhibitory concentrations of oregano EO was observed. Doses of MIC and 0.5 MIC showed a greater influence than that of 0.25 MIC. A statistically significant reduction was observed in biofilms that were treated with oregano EO even at MIC level. The MIC was 0.05% for *S. aureus* strains and 0.1% for *E. coli*. In contrast to our results Özkan et al.[24] noted higher concentrations (0.2-1%) of marjoram *(Origanum majorana),* oregano *(Origanum vulgare L.),* black thyme *(Thymbra spicata L.)* and thyme *(Thymbra sintenesi)* essential oils which have similar composition with *Origanum onites* EO to inhibit the growth of *S. aureus* and *E. coli*. This difference may be attributed to the paper disc diffusion method they used to detect the antibacterial activity of essential oils. Diffusion assays, in which the agent is applied to a well or paper disc in the centre of an agar plate seeded with the test microorganism, are unsuited to essential oil testing because the oil components are partitioned through the agar according to their affinity with water [25]. Broth and agar dilution methods are widely used to determine MIC of essential oils. In addition, when testing non-water-soluble antimicrobials such as essential oils, it is necessary to incorporate an emulsifier or solvent into the test medium to ensure contact between the test organism and the agent for the duration of the experiment. Tween 80 (polysorbate 80) is one of the most commonly used agents [26]. In this study, we used broth dilution method in TSB containing Tween 80 at the level of 0.1% (v/v).

In summary, we have shown that oregano EO exhibited antibacterial action on planktonic *S. aureus, S. lugdunensis, S. haemolyticus, S. sciuri, E. coli* and was able to prevent or at least interfere with biofilm formation on polystyrene surfaces. It also eradicated established biofilm even at MIC level. Further investigations should be examined whether these observations extend to biofilms formed on other surfaces, particularly those found in food processing plants.

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