Investigation of the Antibiofilm Effects of *Mentha longifolia* Essential Oil on Titanium and Stainless Steel Orthopedic Implant Surfaces

Ozhan Pazarci[1], Ugur Tutar[2], Seyran Kilinc[1]

ABSTRACT

Objective: This study aimed to determine the antibiofilm activity of *Mentha longifolia* essential oil (EO) against biofilms forming on in-vitro implant surfaces.

Materials and Methods: *Enterococcus faecalis, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Candida albicans* biofilms were used. Stainless steel and titanium samples were grouped as control, water diluted, no EO addition, and reducing amounts of EO doses. The six microorganisms included in the study were investigated to examine if there were differences between the doses on the implant surfaces. The eradication effect of the EO in samples investigated with electron microscope was classified as 0: none, 1: mild, 2: moderate, and 3: severe. The chemical composition of the EO was determined with gas chromatography.

Results: In terms of biofilm formation, no difference was observed between implant surfaces. While *S. aureus* and *C. albicans* were observed to be the most susceptible, *P. aeruginosa* was identified as the most resistant. According to gas chromatography, *M. longifolia* EO comprised 61.40% carvacrol and 0.28% thymol.

Conclusion: In vitro, *M. longifolia* EO was shown to be effective against gram negative/positive and fungal biofilms forming on the surface of stainless steel and titanium implants.

Keywords: Biofilms, *Mentha Longifolia,* essential oil, implant surfaces

Introduction

Infection is one of the major complications after surgical treatment. In orthopedic surgery, titanium and stainless steel implants are commonly used for fracture fixation [1, 2]. Bacteria may stick to the surfaces of these implants and cause infection. Bacterial biofilms are a well-known problem in orthopedics. When bacteria find an appropriate environment, they pass from planktonic to biofilm form. By creating biofilm forms, they become more resistant to environmental stresses and antibiotics [3]. Unfortunately, the discovery and increasingly widespread use of antibiotics have led to the rapid appearance of antibiotic-resistant strains; more and more infections are caused by microorganisms that fail to respond to conventional treatments [4]. This situation is a global health problem. Infections forming on the surfaces of orthopedic implants may result in removal of the implant, suboptimal treatment results, and increased treatment costs.

Among infections observed after orthopedic surgery, *Staphylococcus aureus* was clearly at the forefront. In addition to this, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Candida albicans* were isolated from infected patients. Increased antibiotic resistance regarding all implant-associated infectious agents has been reported in the literature [5].

In the struggle against biofilms, researchers have investigated topics like implant surface choices and biofilm structures. Antibiotics affecting biofilms and chemicals are also research topics. The antibacterial and antifungal efficacy of essential oils (EOs) have been confirmed in the literature [6]. These effects are mainly shown by carvacrol and thymol within the composition of EO. These two materials are effective because they increase cell permeability [7].

This study aimed to determine the antibiofilm activity of *Mentha longifolia* EO against biofilms forming on in-vitro implant surfaces. Titanium and stainless steel implant surfaces were used in

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our study. Our study investigated gram positive, gram negative, and fungal microorganisms causing orthopedic infections. Our study is the first in the literature to investigate the efficacy of EO for orthopedic implant surface infections.

Materials and Methods

The whole method is schematized in Figure 1.

Plant Material and Extraction of EO

The aerial parts of *M. longifolia* were harvested in the Western Anatolia Region of Turkey. The samples were dried in the dark at room temperature in the laboratory. At the end of the drying process, the leaves and flowers were ground before the EO was extracted with the hydrodistillation method using the Clevenger apparatus (Ildam; Ankara, Turkey).

Analysis and Identification of EO

The chemical composition of the EO was determined according to the method of Aksit et al. The gas chromatography (GC) apparatus used was a PerkinElmer Clarus 500 Series GC system equipped with a flame ionization detector and BPX-5 apolar capillary column (30 m×0.25 mm, 0.25 m i.d.) connected to a mass spectrometer (PerkinElmer; Kağıthane, İstanbul). The EO was sterilized using the filtration method (Millex-FG syringe tip filter, 0.20 µm).

Implants

In this study, stainless steel Kirschner wire (316L) and titanium elastic nail, which are frequently used in orthopedic surgery practice, were used. Implant surfaces were smooth. The samples with dimensions of 2 mm × 3 mm were prepared by cutting.

Microorganisms

En. faecalis (ATCC29212), *Es. coli* (ATCC 11229), *S. aureus* (ATCC 25923), *P. aeruginosa* (ATCC 27853), and *K. pneumoniae* (ATCC 10031) bacteria and *C. albicans* (ATCC 10231) fungus biofilms were used.

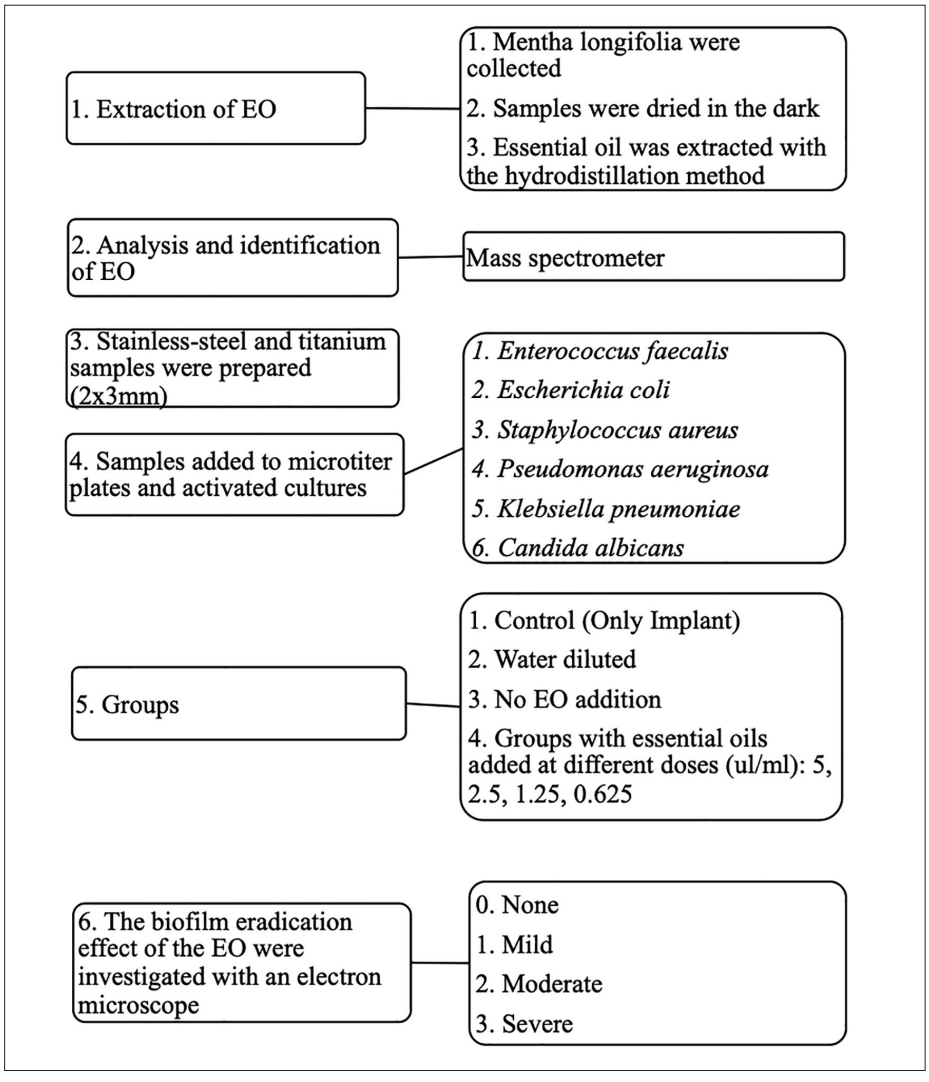

Figure 1. The schematic diagram of the method steps is shown

Determinations of Antibiofilm Activity

As cultures of titanium and stainless steel implants were to be used, standard sections of 2 mm×3 mm appropriate for microtiter plates were obtained. During the procedure, 84 samples, 14 for each microorganism including control groups, were prepared (Figure 2). After samples were prepared, the procedure continued as follows.

Activated cultures had 1% (w/v) glucose added within tryptic soy broth at 37°C and left for 24 hours incubation. Then the density of the twice-activated cultures was set to 0.5 McFarland standard (1.10^8 CFU/mL). After setting the density, 200 µL of cultures were taken and applied to the samples in microtiter plates. The plates were left for incubation at 37°C. Then medium in the wells was poured off, and they were washed with sterile PBS solution to remove planktonic cells. They were left to dry at room temperature. After a two-time dilution process for bacterial biofilms formed on the titanium and steel samples in the wells, EO concentrations prepared from 5-0.625 µl/mL were added and left for 24 hours incubation at 37°C. For controls, bacterial biofilms without EO added were used. At the end of the incubation duration, samples were investigated with an electron microscope, and the eradication effect of the EO on the biofilm structures was compared with the control group.

Electron Microscope Imaging

Electron microscope investigation was performed at our university's Advanced Technology Application and Research Center. A Tescan Mira 3 XMU brand field emission gun scanning electron microscope (Tescan; Brno-Kohoutovice, Czech Republic) equipped with secondary and backscattered electron modes was used for topographic and elemental distribution analysis respectively. The eradication effect of the EOs in samples investigated with the electron microscope was classified according to Walker et al. [8] as 0: none, 1: mild, 2: moderate, and 3: severe. This classification is based on a comparison of biofilm eradication through SEM images after application of EO.

Statistical Analysis

Stainless steel and titanium samples were grouped as control, water diluted, no addition, and reducing amounts of EO doses. The six microorganisms included in the study were investigated to examine if there were differences between the doses on the stainless steel and titanium implant surfaces. Data were analyzed with the SPSS program ver. 22 (IBM Corp.; Armonk, NY, USA). The chi-square test was used to examine whether there were differences between doses and between groups for titanium

Table 1. Stainless steel (S) and titanium (Ti) samples were grouped as control (only implant), water diluted, no essential oil (EO) addition, and reducing amounts of EO doses. The eradication effect of the EOs in samples investigated with the electron microscope was classified as 0: none, 1: mild, 2: moderate, and 3: severe

		En. faecalis	*Es. coli*	*S. aureus*	*P. aeruginosa*	*K. pneumoniae*	*C. albicans*	p[1]	p[1] (S vs Ti)
Gram		+	-	+	-	-	+		
Control	S	0	0	0	0	0	0	*N/A*[3]	*N/A*[2]
	Ti	0	0	0	0	0	0	*N/A*[3]	
Water diluted	S	3	3	3	3	3	3	n/a[3]	n/a[2]
	Ti	3	3	3	3	3	3	*N/A*[3]	
No EO addition	S	3	3	3	3	3	3	n/a[3]	n/a[2]
	Ti	3	3	3	3	3	3	n/a[3]	
5 ul/mL	S	0	0	0	1	0	0	*0.306*[3]	*0.067*[2]
	Ti	0	0	0	1	0	0	*0.306*[3]	
2.5 ul/mL	S	0	1	1	2	1	1	*0.285*[3]	*0.173*[2]
	Ti	1	1	1	2	0	1	*0.285*[3]	
1.25 ul/mL	S	0	2	1	3	2	1	*0.263*[3]	*0.112*[2]
	Ti	1	1	1	3	1	1	*0.306*[3]	
0.625 ul/mL	S	2	2	1	3	2	1	*0.285*[3]	*0.323*[2]
	Ti	3	2	1	3	2	1	*0.285*[3]	

[1]Significance level p≤0.05
[2]P values according to Pearson chi-square test (comparison of stainless steel and titanium surfaces)
[3]P values according to Pearson chi-square test (comparison of microorganisms with surfaces)
N/A: no available test because of constant values
EO: Essential oil; S: Stainless steel; T: Titanium

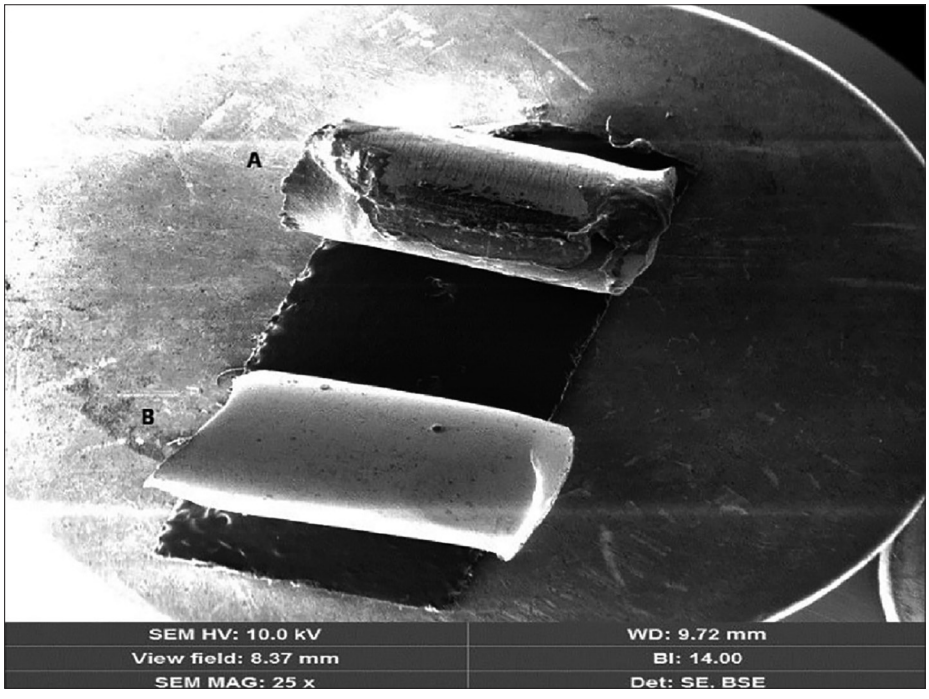

Figure 2. a, b. The SEM images of control and no essential oil addition group. a) The biofilm on the stainless steel implant surface* is shown. b) Biofilm-free implant surface (control groups) is shown. (SEM: Scanning electron microscope) (*Implant: Stainless steel 316L smooth surface)

and steel implants. In addition, one-way repeated measure ANOVA test was used to analyze the change in biofilm eradication on stainless steel and titanium surfaces after addition of different doses of EO.

Results

In terms of biofilm formation, no difference was observed between titanium and stainless steel implant surfaces. In terms of biofilm eradication with the same doses of EOs, no difference was observed between the two implant surfaces (p<0.005). But according to our results, biofilm eradication statistically significantly differed between different EO concentrations for stainless steel surfaces and titanium surfaces (p=0.012 and p=0.008, respectively). Biofilm eradication also statistically significantly differed among microorganisms between different EO concentration on titanium surfaces (p=0.040). However, there was no statistically significant difference among microorganisms between different EO concentrations on stainless steel surfaces (p=0.676) (Table 1).

Accordingly, at the highest applied dose of EO, very good biofilm eradication was observed. Only *P. aeruginosa* was observed to be mild: 1 (Figure 3). While *S. aureus* and *C. albicans* were observed to be most susceptible, *P. aeruginosa* was identified as the most resistant. At the lowest concentration, *S. aureus* and *C. albicans* were observed on samples under the electron microscope (mild: 1).

The chemical composition of *M. longifolia* EO according to GC is given in Table 2. Accordingly, there was 61.40% carvacrol and 0.28% thymol identified in the composition of *M. longifolia*.

Discussion

Each year, fracture fixation devices are implanted into a growing number of patients [9]. One

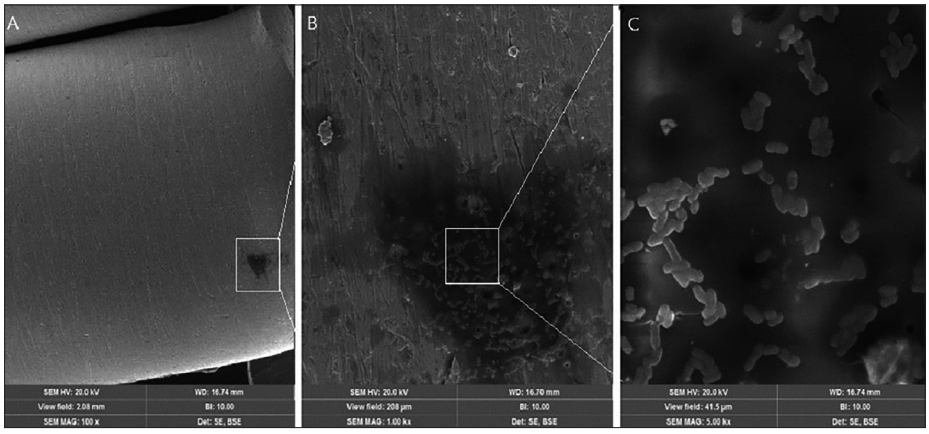

Figure 3. a-c. The SEM images of *Pseudomonas aeruginosa*. a) *Pseudomonas aeruginosa* eradication on the Ti implant surface* (1: mild) is followed by application of essential oil at a dose of 5 µl/ml. b) and c) The SEM images of P. *aeruginosa* at larger magnification. (SEM: Scanning electron microscope, Ti: Titanium) (*Implant: Titanium elastic nail smooth surface)

Table 2. The chemical composition of *Mentha longifolia*

No	%	Name
1	1.00	β-Thujene
2	0.72	α-Pinene
3	0.20	Camphene
4	0.62	1-Octen-3-ol
5	0.21	β-Pinene
6	1.02	β-Pinene
7	0.28	3-Octanol
8	0.15	α-Phellandrene
9	1.32	α-Terpinene
10	17.38	o-Cymol
11	8.27	γ-Terpinene
12	0.90	Endo-Borneol
13	0.80	4-Terpineol
14	0.17	α-Terpineol
15	0.29	α-Terpineol
16	1.04	Pulegone
17	0.28	Thymol
18	0.23	*unknown
19	61.40	Carvacrol
20	0.28	Propionaldehyde
21	1.65	Caryophyllene
22	0.25	Spathulenol
23	1.42	Caryophyllene oxide
24	0.13	Naphthalene

of the major complications of musculoskeletal trauma surgery is implant-related infection [1, 10]. While the infection rate for closed fractures is low, this rate increases up to 30% for open fractures [9]. As a result, in our study we investigated gram positive (*S. Aureus*, *En. faecalis*), gram negative (*Es. coli*, *P. aeruginosa*, *K. pneumoniae*) and fungus (*C. albicans*) causing infections after orthopedic surgery.

Our study presents a different approach to fighting biofilms on orthopedic implant surfaces from available antibiotics. In the struggle against infection, the discovery of antibiotics was a revolution in medicine. Meanwhile, the discovery and development of antibiotics have been rapidly declining over the past several decades; for instance, only five and two new antibiotics were approved during the years 2003-2007 and 2008-2012, respectively. In addition to the reduction in new antibiotic discovery, there is increasing resistance to major antibiotics [4]. Currently, the most notorious antibiotic-resistant bacteria are *S. aureus*, *En. faecalis*, *K. pneumoniae*, and *P. aeruginosa* [4]. Though bacterial biofilms are well defined, fungal biofilms are less well known [11]. Among these, *C. albicans* comes to the forefront in causing hospital-related infections [11]. Due to increasing resistance to antifungal medications and side effects, developing a new treatment modality against *C. albicans* is necessary [12]. Alternatives to antibiotics may reduce the use of antibiotics [4]. The antibacterial efficacy of EOs has been confirmed in the literature [6, 13, 14]. The *M. longifolia* EO included in our study was observed to have different levels of effect against six microorganism biofilms (Table 1).

Within the chemical composition of EOs, mainly carvacrol and thymol show antibiofilm activity [13]. These two materials are effective as they increase cell permeability [7]. Our study showed that the EO contained 60% carvacrol and 0.28% thymol (Table 2). Based on the content of this material in the EO, antibiofilm effects vary [7]. Different studies investigating the antibacterial effects of EOs have shown the antibacterial effect of *M. longifolia* EO [6, 15]. A study investigating the antibacterial efficacy of 52 EOs observed the most resistant microorganism was *P. aeruginosa* with the most susceptible *S. aureus* and *C. albicans* [6].

This article supports the results of our study (Table 1). Although *P. aeruginosa* was the most resistant microorganism in this study, it was observed that increasing doses of EO had antibiofilm effect even for *P. aeruginosa* (Table 1). Antibacterial activity of *M. longifolia* against *P. aeruginosa* has been reported in the literature [16].

According to our results, biofilm eradication statistically significantly differed between different EO concentrations for stainless steel surfaces and titanium surfaces ($p=0.012$ and $p=0.008$, respectively). At the highest dose of EO, applied at varying doses to biofilms on the implant surfaces, biofilm structures were observed to be removed by electron microscope investigation in all samples. Only *P. aeruginosa* was observed at this dose classed as 1: mild (Figure 2).

To determine the antibiofilm activity of EOs, there are assessment methods such as microdilution assay or electron microscopy [14, 17]. In our study, we assessed the biofilm formation with electron microscope imaging as in the study by Walker et al. [8]. Accordingly, biofilm eradication effects after the application of EO are based on the comparison of SEM images. In the literature, it is emphasized that SEM is a good method to investigate biofilm formation [18, 19].

Biofilm formation and structure is becoming better known through the years [20]. However, in spite of all infection prevention strategies, these complications are still encountered. Development of anti-infection implant surfaces is a strategy in the fight against infection [3]. Stainless steel and titanium are implant surfaces used in orthopedic surgery. In the literature, there are publications defending the slight superiority of titanium among these implant surfaces, while there are also publications showing no difference [1, 2]. In our study, no difference was observed between titanium and stainless steel implant surfaces in terms of biofilm formation. But biofilm eradication also statistically significantly differed among microorganisms between different EO concentration on titanium surfaces ($p=0.040$).

In addition to observing that EO had good antibiofilm activity in the study, the authors are aware of some limitations. The first is the difficulty in

directly extrapolating the results of experimental studies to the clinical setting. The main difficulty is the lack of in-vivo studies. However, in-vivo study is planned in the second stage. Accordingly, there will be an in-vivo comparison performed on the experimental groups after formation of biofilm on the implants. It was also planned to investigate the cytotoxic effect of EO on L929 mouse fibroblast cells. Additionally, the antibiofilm effect of EO was not compared with that of antibiotics.

In conclusion, antibiotic resistance and bacterial biofilms are one of the most significant health problems. In vitro, *M. longifolia* EO was shown to be effective against gram negative/positive and fungal biofilms forming on the surface of steel and titanium implants. While *P. aeruginosa* was least affected, the most susceptible were *S. aureus* and *C. albicans* biofilms. It was shown that *M. longifolia* is a beneficial agent in the treatment of implant-related infections. Our study will guide future studies of implant-related infection in experimental animal models.

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Conflict of Interest: The authors have no conflict of interest to declare.

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