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Title: Investigation of the Antibiofilm Effects of Mentha longifolia Essential Oil on Titanium and Stainless-Steel Orthopaedic Implant Surfaces

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Abstract

Objective: In this study, the aim was to determine the antibiofilm activity of Mentha longifolia essential oil (EO) against biofilms forming on in vitro implant surfaces.

Materials and methods: E. faecalis, E. coli, S. aureus, P. aeruginosa, and K. pneumoniae and C. 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 see 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: There was no difference observed between implant surfaces in terms of biofilm formation. While S. aureus and C. albicans were observed to be most susceptible, the most resistant was identified as P. aeruginosa. The chemical composition of M. longifolia essential oil according to gas chromatography comprised 61.40% carvacrol and 0.23% thymol.

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

Key words: Biofilms, Mentha Longifolia, Essential oil, Implant Surfaces.

1. Introduction

Infection is one of the major complications after surgical treatment. Titanium and stainless-steel implants are commonly used for fracture fixation in orthopaedic surgery [1, 2]. Bacteria may stick to the surfaces of these implants and cause infection. Bacterial biofilms are a well-known problem in orthopaedics. 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 wide-spread use of antibiotics have led to the rapid appearance of antibiotic-resistant strains today; 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 orthopaedic implants may result in removal of the implant, suboptimal treatment results and increased treatment costs.

Among infections observed after orthopaedic surgery, S. aureus is clearly at the forefront. In addition to this, E. fecalis, E. coli, P. aeruginosa, K. pneumonia and C. albicans are microorganisms isolated from infected patients. Increased antibiotic resistance has been reported in the literature regarding all implant-associated infectious agents [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 (EO) 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 as they increase cell permeability [7].

In this study the aim was to determine the antibiofilm activity of Mentha longifolia essential oil against biofilms forming on in vitro implant surfaces. Titanium and stainless-steel implant surfaces were used in our study. In our study gram (+), gram (-) and fungal microorganisms causing orthopaedic infections were investigated. Our study is the first in the literature to investigate the efficacy of essential oil for orthopaedic implant surface infections.

2. Methods

The whole method is schematized in Table 1.

2.1. Plant material and extraction of essential oil

The aerial parts of Mentha longifolia were harvested in the Western Anatolia Region of Turkey. The samples were dried in the dark in the laboratory at room temperature. At the end of the drying process, the leaves and flowers were ground before the essential oil was extracted with the hydro distillation method using the Clevenger apparatus (Ildam/Turkey).

2.2. Analysis and identification of EO

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

2.3. Implants

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

2.4. Microorganisms

Enterococcus faecalis (ATCC29212), Escherichia coli (ATCC 11229), Staphylococcus aureus (ATCC 25923), Pseudomonas aeruginosa (ATCC 27853), and Klebsiella pneumoniae (ATCC 10031) bacteria and Candida albicans (ATCC 10231) fungus biofilms were used.

2.5. Determinations of anti-biofilm activity:

As cultures of titanium and stainless-steel implants were to be used, standard sections of 2 x 3 mm appropriate for microtiter plates were obtained. During the procedure, a total of

84 samples, 14 for each microorganism including control groups, were prepared (Figure 1). After samples were prepared, the procedure continued as follows:

Activated cultures had 1% (w/v) glucose added within tryptic soy broth (TSB) at 37 °C and left for 24 hours incubation. Then the density of the twice-activated cultures was set to 0.5 McFarland standard (1.108 CFU/ml). After setting the density, 200 µl of cultures were taken and applied to the samples contained 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, essential oil (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 essential oil on the biofilm structures was compared with the control group.

2.6. 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 equipped with secondary and backscattered electron modes was used for topographic and elemental distribution analysis respectively. The eradication effect of the essential oils in samples investigated with the electron

microscope was classified according to Walker et al. as 0: none, 1: mild, 2: moderate and 3: severe [8]. This classification is based on a comparison of biofilm eradication through SEM images after application of essential oil.

2.7. Statistical analysis:

Stainless-steel and titanium samples were grouped as control, water diluted, no addition and reducing amounts of essential oil doses. The 6 microorganisms included in the study were investigated to see if there were differences between the doses on the stainless-steel and titanium implant surfaces. Data were analyzed with the SPSS program (ver. 22). The Chi-square test was used to examine whether there were differences between doses and between groups for titanium and steel implants. In addition, one-way repeated measure ANOVA test was used to analyse the change in biofilm eradication on stainless steel and titanium surfaces after addition of different doses of EO.

3. Results

There was no difference observed between titanium and stainless-steel implant surfaces in terms of biofilm formation. There was no difference between the two implant surfaces in terms of biofilm eradication with the same doses of essential oils ($p<0.005$). 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 2).

Accordingly, at the highest applied dose of essential oil, the biofilm eradication was observed to be very good. Only P. aeruginosa was observed to be mild: 1 (Figure 2). While S. aureus and C. albicans were observed to be most susceptible, the most resistant was identified as P. aeruginosa. 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 essential oil according to gas chromatography is observed in table 3. Accordingly, there was 61.40% carvacrol and 0.23% thymol identified in the composition of Mentha longifolia.

4. Discussion

Fracture fixation devices are implanted into a growing number of patients each year [9]. One 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+ (S. Aureus, E. faecalis), gram- (E. coli, P. aeruginosa, K. pneumonia) and fungus (C. albicans) causing infections after orthopaedic surgery.

Our study presents a different approach to fighting biofilms on orthopaedic 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 declining rapidly over the past several decades; for instance, only 5 and 2 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, E. 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, it is necessary to develop a new treatment modality against C. albicans [12]. Alternatives to antibiotics may reduce the use of antibiotics [4]. The antibacterial efficacy of essential oils has been confirmed in the literature [6,13,14]. The M. longifolia essential oil included in our study was observed to have different levels of effect against six microorganism biofilms (Table 2). Within the chemical composition of essential oils, mainly carvacrol and thymol show antibiofilm activity [13]. These two materials are effective by increasing cell permeability [7]. In our study, it was shown that the essential oil contained 60% carvacrol and 0.28% thymol (Table 3). Antibiofilm effects vary based on the content of this material in the essential oil [7]. Different studies investigating the antibacterial effects of essential oils have shown the antibacterial effect of M. longifolia essential oil [6,15]. A study

investigating the antibacterial efficacy of 52 essential oils 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 2). Although P. aeruginosa was the most resistant microorganism in this study, it was observed that even for P. aeruginosa, increasing doses of essential oil had antibiofilm effect (Table 2). In the literature, antibacterial activity of M. longifolia against P. aeruginosa has been reported [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 essential oil, 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 1).

To determine the antibiofilm activity of essential oils, 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 essential oil 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 orthopaedic 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, there was no difference 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 essential oil 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 essential oil on L929 mouse fibroblast cells. Additionally, the antibiofilm effect of essential oil was not compared with that of antibiotics.

In conclusion; currently, antibiotic resistance and bacterial biofilms are one of the most significant health problems. In vitro, M. longifolia essential oil was shown to be effective against gram - / + 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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Figure Legends

Figure 1. 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)

Figure 2. 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) SEM images of Pseudomonas aeruginosa at larger magnification. (SEM: Scanning electron microscope, Ti: Titanium) (* Implant: Titanium elastic nail smooth surface)

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

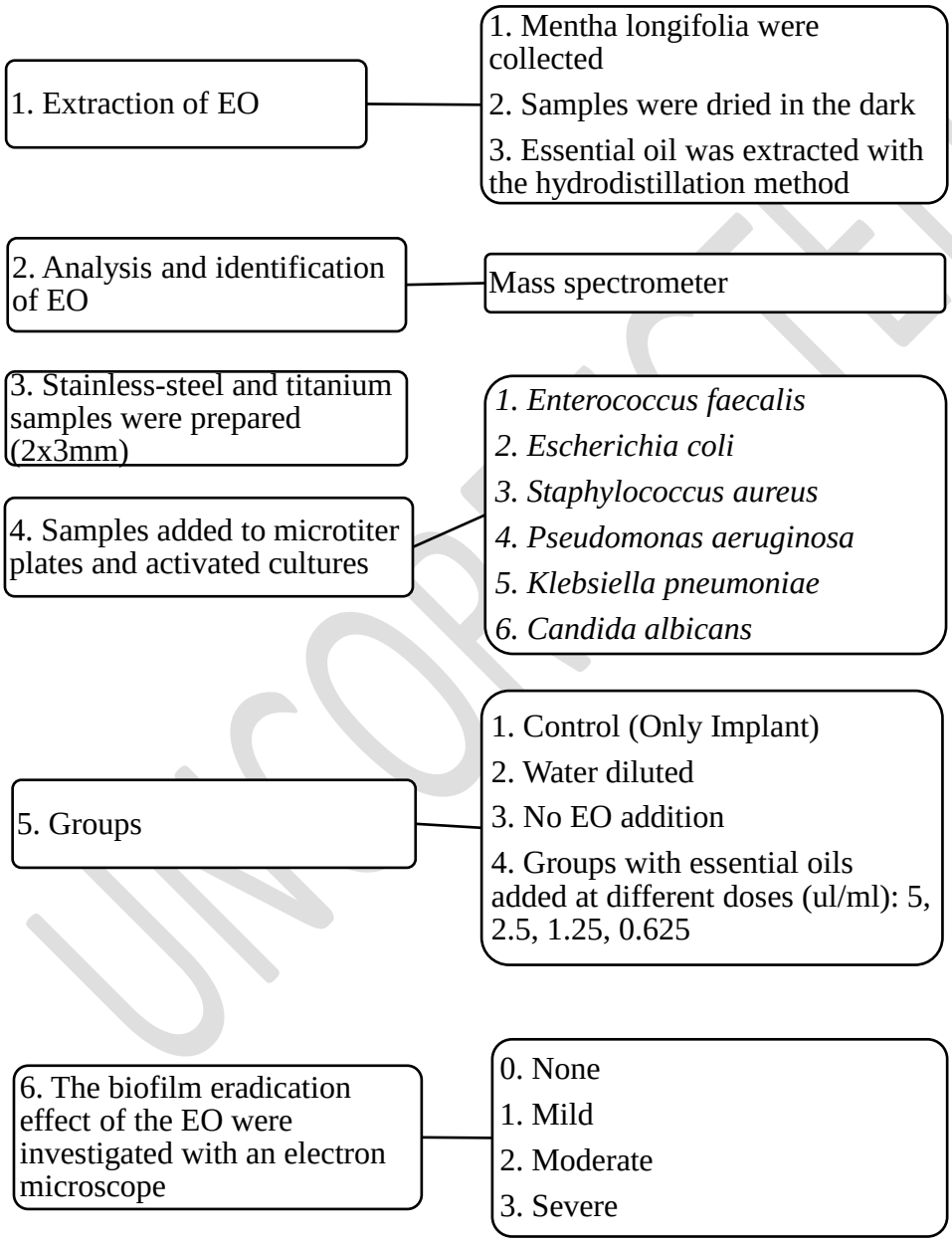

Table 2. Stainless-steel (S) and titanium (Ti) samples were grouped as control (only implant) , water diluted, no essential oil (EO) addition, and reducing amounts of essential oil doses. The eradication effect of the essential oils in samples investigated with the electron microscope was classified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

		E. fecalis	**E. coli**	**S. aureus**	**P. auroginosa**	**K. pneumonia**	**C. albicans**	**P[1] value**	**P[1] value (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]	

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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

Table 3

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

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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

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