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Effect of Manuka essential oil on

*Staphylococcus pseudintermedius*

isolated from
canine skin and ears

g개의 피부와 귀에서 분리한 *Staphylococcus pseudintermedius*에 대한 Manuka essential oil의 효과

2013 년 2 월
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Abstract

*Staphylococcus pseudintermedius* is a primary pathogen of bacterial skin infections in dogs. Recently, the emergence of methicillin-resistant *S. pseudintermedius* (MRSP) has become a challenge because it shows resistance toward various kinds of antibiotics. Thus, there is growing interest in novel antimicrobial compounds and Manuka is one of these. The aim of this study was to
confirm the antimicrobial and biofilm formation inhibition activities of Manuka essential oil against clinically isolated *S. pseudintermedius*, including MRSP. Fifty *S. pseudintermedius* were isolated from clinical cases of canine pyoderma and otitis externa and show a high MRSP rate (78%). To confirm the antimicrobial activity of Manuka essential oil, the minimum inhibitory concentrations (MICs) of Manuka essential oil for *S.pseudintermedius* isolates were determined using the agar dilution method and the results were compared with cephalexin. The MICs of Manuka essential oil and cephalexin against 50 *S. pseudintermedius* isolates ranged from $2^{-6}$ to $2^{-9}$ (v/v) and 0.125 to 128 mg/ml, respectively. Only 41 isolates (82%) were susceptible to cephalexin; however, Manuka essential oil showed antimicrobial activity against all clinically isolated *S. pseudintermedius*, including MRSP ($2^{-7}$ to $2^{-9}$ v/v). Biofilm formation was assessed by colorimetric assay with crystal violet staining. Statistically significant (p<0.05) inhibition of biofilm formation was found in *S. pseudintermedius* exposed to 0.01%, 0.1%, and 1% (v/v) Manuka essential oil, dose-dependently. These results showed that Manuka essential oil has antimicrobial and antibiofilm formation activity on *S. pseudintermedius* and can be a potential treatment option for controlling *S. pseudintermedius* infections in dogs.

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**Keywords:** Manuka essential oil, *S.pseudintermedius*, Antimicrobial activity, Biofilm, dogs  
**Student Number:** 2011-21672
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**Introduction**

Bacterial skin infections involving canine pyoderma and otitis externa are some of the most common complaints from owners who visit veterinary clinics. Of the numerous bacteria that can cause canine bacterial skin infections, *Staphylococcus pseudintermedius*, formerly known as *Staphylococcus intermedius*, is the most common bacterial pathogen of canine skin and ear infections [3, 11, 23]. In veterinary practice, bacterial skin infections caused by *S. pseudintermedius* are usually treated with systemic antibiotics and a topical antimicrobial compound [24]. Recently, however, therapeutic options have become limited due to a considerable increase of methicillin-resistant *S. pseudintermedius* (MRSP) [14, 16, 20-22, 29]. Thus, there has been increasing interest in finding novel antimicrobial agents.

Essential oils obtained from plant materials, such as tea tree oil and rosemary oil, have been traditionally used for a variety of medicinal purposes because of their pharmacological effects, including antimicrobial, antifungal, and anti-inflammatory activity [10, 26]. Due to these characteristics, there has been a growing international interest in the use of essential oils as a substitution for synthetic antimicrobials. In previous studies, it was demonstrated that honey and varieties of the essential oil of Manuka, derived from the Manuka tree (*Leptospermum*
**Scoparium** in New Zealand, had a remarkable effect on wound healing, dental plaque and gingivitis, and dermatophytosis [1, 4, 7, 12, 28]. Recently, triketone-rich Manuka essential oil has become commercially important because of its antimicrobial activity against gram-positive bacteria, including antibiotic-resistant strains [6, 17].

To evaluate the potential use of Manuka essential oil as a natural antimicrobial compound in veterinary medicine, this study investigated the antimicrobial activity and biofilm formation inhibition activities of Manuka essential oil against *S. pseudintermedius* isolated from dogs with skin and ear infections.
Materials and Methods

1. Sample collection and *S. pseudintermedius* identification

A total of 50 clinical isolates were collected aseptically from dogs suffering from pyoderma (n=34) or otitis externa (n=16). After collection, isolates were cultured overnight at 37°C on blood agar plates. These isolates were presumptively identified as SIG (*S. intermedius* group) by standard biochemical tests and the Vitek 2 system (Biomerieux, Lyon, France). To differentiate *S. pseudintermedius* from the other SIG species, all the SIG isolates were examined using the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP). Staphylococcal genomic DNA was extracted using a DNase tissue kit (Qiagen, CA, USA) according to the manufacture’s recommendation. PCR amplification of the *pta* gene (primer F, 5’-AAA GAC AAA CTT TCA GGT AA-3’; primer R, 5’-GCA TAA ACA AGC ATT GTA CCG-3’) was conducted, and the 320 bp PCR products were digested with MboI restriction enzyme, into two restriction fragments of 213 bp and 107 bp only in *S. pseudintermedius*, as reported previously [2].
2. Detection of MRSP

For the identification of MRSP, determination of the MICs of oxacillin was performed according to CLSI guideline [15] and detection of the methicillin-resistance encoding gene \textit{mecA} was determined using a PCR assay. PCR primers were used to amplify the \textit{mecA} gene fragment of 310 bp (primer F, 5’-TGG CTA TCG TGT CAC AAT CG-3’; primer R, 5’-CTG GAA CTT GTT GAG CAG AG-3’) and the conditions were as described previously [31].
3. Manuka essential oil

Pure 100% Manuka essential oil was extracted using the steam distillation method provided by Honey Collection, Marlborough, New Zealand.
4. Determination of the MICs of Manuka essential oil on

*S. pseudintermedius*

Minimum inhibitory concentrations (MICs) of Manuka essential oil and cephalexin against *S. pseudintermedius* isolates were determined using the agar dilution method, according to the CLSI guideline [15]. Briefly, a series of twofold dilutions of manuka essential oils (ranging from 2⁻¹ % to 2⁻¹⁴ % v/v) and cephalexin (ranging from 256 to 0.125 mg/ml) was prepared in Brain Heart Infusion (BHI; BD Diagnostic systems, Sparks, MD, USA) agar plates. The agar plates were then inoculated with a final inoculum of 5 x 10⁷ CFU/ml of each isolate. Inoculated BHI plates were incubated at 37°C for 24 h. The MICs were determined as the lowest concentration of test agents inhibiting the visual growth of bacteria on the agar plate. All determinations were performed in duplicate.
5. Effect of Manuka essential oil on biofilm formation

The effect of different concentrations of Manuka essential oil (ranging from $2^{-1}$ to $2^{-14}$ v/v) on the biofilm-forming ability was tested with a colorimetric microtiter plate assay. All isolates were grown overnight at 37°C in tryptic soy broth (TSB). An aliquot of 100 μl from the bacterial culture was inoculated into each well of a 96-well flat-bottomed polystyrene microtiter plate (Corning, NY, USA) in the presence of 100 μl of Manuka essential oil. The wells containing only bacteria served as the control. After overnight incubation, media and non-adherent bacteria were removed by gently washing the plates with PBS. The remaining adherent bacteria were stained with crystal violet and the wells were washed with PBS. The quantitative analysis of biofilm production was performed by adding 200 μl of 95% ethanol to de-stain the wells. One hundred μl from each well was transferred to a new microtiter plate, and the stained bacteria and control wells were read at 540 nm using a microtiter plate reader (Bio-Rad, Munich, Germany). All experiments were performed in triplicate.
6. Statistical analysis

Student’s t-test was performed to compare the biofilm inhibition activity between each concentration of Manuka essential oil and the controls. \( P \) values of less than 0.05 were considered as statistically significant.
Results

All 50 clinical isolates from canine skin and ear infections were confirmed as *S. pseudintermedius* and 39 (78%) of the 50 isolates were identified as MRSP through the detection of the methicillin-resistant gene (*mecA*) and determination of the MICs of oxacillin.

1. MIC of Manuka essential oil

To confirm the antimicrobial activity of Manuka essential oil, the MICs of Manuka essential oil for *S. pseudintermedius* isolates were determined using the agar dilution method. These results were compared with those of cephalexin, a commonly used antibiotic for bacterial skin infections in dogs. Table 1 shows the ranges of MICs and the concentrations necessary to inhibit 50% (MIC$_{50}$) and 90% (MIC$_{90}$) of the isolates.

Table 1. Minimum inhibitory concentrations (MICs) of Manuka essential oil and cephalexin against *Staphylococcus pseudintermedius*

MRSP: methicillin-resistant *Staphylococcus pseudintermedius*; MSSP: methicillin-susceptible *S. pseudintermedius*
<table>
<thead>
<tr>
<th></th>
<th>Total isolates (n=50)</th>
<th>MRSP (n=39)</th>
<th>MSSP (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Manuka</td>
<td>cephalexin</td>
<td>Manuka</td>
</tr>
<tr>
<td>MIC</td>
<td>$2^9$ to $2^4$ (v/v)</td>
<td>0.125 to 128 mg/ml</td>
<td>$2^9$ to $2^4$ (v/v)</td>
</tr>
<tr>
<td>MIC$_{50}$</td>
<td>$2^8$ (v/v)</td>
<td>0.5 mg/ml</td>
<td>$2^8$ (v/v)</td>
</tr>
<tr>
<td>MIC$_{90}$</td>
<td>$2^7$ (v/v)</td>
<td>16 mg/ml</td>
<td>$2^7$ (v/v)</td>
</tr>
</tbody>
</table>

The MICs of Manuka essential oil and cephalexin against 50 *S. pseudintermedius* isolates ranged from $2^{-6}$ to $2^{-9}$ v/v and 0.125 to 128 mg/ml, respectively. All 50 *S. pseudintermedius* isolates were susceptible to Manuka essential oil, whereas 41 (82%) of the 50 isolates were susceptible to cephalexin. This study also confirmed the antimicrobial activity of Manuka essential oil against MRSP, as the MIC results were divided into MRSP and MSSP group. The distribution of MIC results for Manuka essential oil were similar between the MRSP ($2^{-7}$ to $2^{-9}$ v/v) and MSSP ($2^{-9}$ to $2^{-6}$ v/v) groups. In contrast, the MICs of cephalexin were different between the MRSP (0.125 to 128 mg/ml) and MSSP groups (0.125 to 1 mg/ml) (Figure 1). These results showed the antimicrobial activity of cephalexin against *S. pseudintermedius*, affected by presence of *meca* gene whereas,

Manuka essential oil has antimicrobial activity regardless of the presence of the *meca* gene.
Figure 1. Distribution of the MICs of Manuka essential oil and cephalaxin against MRSP and MSSP

MRSP: methicillin-resistant *Staphylococcus pseudintermedius*; MSSP: methicillin-susceptible *S. pseudintermedius*. 
2. Biofilm inhibition activity of Manuka essential oil

To assess the ability of Manuka essential oil to inhibit the formation of *S. pseudintermedius* biofilms, different concentrations (0.01%, 0.1%, and 1% v/v) of Manuka essential oil were tested against biofilm-grown *S. pseudintermedius* isolates. The biofilms produced by *S. pseudintermedius* declined with increasing concentrations of Manuka essential oil. The percentages of biofilm inhibition using 0.01%, 0.1%, and 1% (v/v) of Manuka essential oil were 50%, 22%, and 12% of the control containing only bacteria, respectively. There were significant differences with respect to concentrations of Manuka essential oil as compared with the control (*p* < 0.001) (Figure 2).

To confirm the biofilm formation inhibition activity of Manuka essential oil against MRSP, biofilm formation inhibition results were divided into two groups, MRSP and MSSP. The percentages of biofilm inhibition by 0.01%, 0.1%, and 1% (v/v) of Manuka essential oil were 48%, 22%, and 11% in the MRSP group, and 60%, 25%, and 21% in the MSSP group, respectively. The inhibition of biofilm formation by Manuka essential oil was significantly increased in both the MRSP and MSSP groups (*p* < 0.001 and *p* < 0.05, respectively) (Figure 2).
Figure 2. Results of colorimetric assay for quantification of *S. pseudintermedius* biofilm formation inhibition by Manuka essential oil

Absorbance was measured using a microtiter reader at optical density 540 nm.

Results are means ± standard error of triplicate assays. MRSP: methicillin resistant *S. pseudintermedius*; MSSP: methicillin resistant *S. pseudintermedius* (*p<0.05, ***p<0.001)
Discussion

*S. intermedius* has traditionally been identified as the primary pathogen of bacterial skin infections in dogs. Recently, the isolates formerly identified as *S. intermedius* were reclassified into SIG, including *S. intermedius*, *S. delphini*, and *S. pseudintermedius*, and based on 16s rRNA gene sequence analysis it was discovered that *S. pseudintermedius*, not *S. intermedius*, is the primary pathogen of canine pyoderma and otitis externa [3, 11]. In the past, *S. pseudintermedius* was generally susceptible to penicillin-stable β-lactam antibiotics and cephalosprins [16, 18, 19]. Recently, however, the increasing prevalence of MRSP has became a problem in veterinary practice because these strains are usually resistant to the various classes of antibiotics, and thus only limited treatment options remain [14, 16, 20-22, 29]. Because of this situation, interest in plants as sources of biological active compounds was growing, and Manuka essential oil from *Leptospermum scoparium* was considered as one of these biological active compounds. Previous studies reported that Manuka essential oil has antimicrobial activity, but there is a lack of knowledge on its effectiveness on *S. pseudintermedius* from dogs [6, 17]. In this study, we investigate the antimicrobial activity of Manuka essential oil against clinically isolated *S. pseudintermedius* from dogs with bacterial
skin infections.

All of the 50 isolates from dogs with pyoderma and otitis externa were identified as *S. pseudintermedius* and 39 of the 50 *S. pseudintermedius* (78%) were MRSP. The results of this study show a higher MRSP rate than previous studies in Japan, Canada, the US, and South Korea [13, 14, 22, 30].

To confirm the antimicrobial activity of Manuka essential oil, the agar dilution test was performed against clinically isolated *S. pseudintermedius* from dogs with pyoderma and otitis externa, including 39 MRSP. Then, the results were compared with those of cephalaxin, one of the most commonly used antibiotics for bacterial skin infections in dogs. The MICs of Manuka essential oil and cephalaxin against 50 isolated *S. pseudintermedius* ranged from $2^{-9}$ to $2^{-6}$ (v/v) and 0.125 mg/ml to 128 mg/ml (CLSI cephalaxin-susceptible breakpoint; MIC $\leq$ 8 mg/ml), respectively. All 50 *S. pseudintermedius* isolates were susceptible to Manuka essential oil at a concentration of $2^{-6}$ (v/v), whereas only 41 (82%) of the 50 isolates were susceptible to cephalaxin according to CLSI recommendation. For comparing antimicrobial activity, MIC$_{50}$ and MIC$_{90}$ of the Manuka essential oil and the cephalaxin were determined. MIC$_{50}$ and MIC$_{90}$ of cephalaxin against the MSSP group were the same at a concentration of 0.5 mg/ml; however, MIC$_{50}$ and MIC$_{90}$ against MRSP were 1 mg/ml and 128
mg/ml, respectively. In contrast, MIC$_{50}$ and MIC$_{90}$ of Manuka essential oil against MSSP and MRSP were the same at a concentration of $2^7$ (v/v). From these results, we can guess that cephalexin is less effective against MRSP isolates and treatment failure can occur when used on MRSP. On the other hand, Manuka essential oil inhibits the growth of all isolates in a narrow range of concentration, regardless of the existence of the $mecA$ gene. Therefore, this plant-delivered compound can be used effectively against cephalexin-resistant *S. pseudintermedius* and MRSP from dogs.

Biofilm formation is associated with chronic and acute bacterial skin infections. Biofilm can give rise to planktonic bacteria, and if the host immune mechanism cannot kill these individual bacteria, biofilm can act as the nidus of acute infection [8]. Chronic infection is also associated with biofilm, as bacteria uses the biofilm as a defensive shield against hostile environments and antibiotics. The defense mechanism is mediated by the down-regulated rates of cell division of the embedded bacteria [5]. This structure can prevent inward diffusion of some antibiotics [27] and offer defense shields from reactive oxidants produced by the host immune system [9]. Thus, the biofilm inhibition activity of Manuka essential oil has clinical implications in the treatment of persistent *S. pseudintermedius* infections. In this study, the biofilm inhibition activity of Manuka essential oil on MSSP and
MRSP was demonstrated with various different concentrations (0.01, 0.1, and 1% v/v), and Manuka essential oil significantly inhibits biofilm formation level dose-dependently, and statistically significant differences were found in comparison with the control group (p < 0.05). Based on these data, the antimicrobial and biofilm formation inhibition activities of Manuka essential oil against S. pseudintermedius are confirmed. Manuka essential oil was able to inhibit the growth of all S. pseudintermedius isolates, regardless of meca gene existence, at concentrations of 2⁻⁶ (v/v). Although the specific mechanisms of the antimicrobial activity of Manuka essential oil is poorly understood, the lipophilic character of the essential oil may contribute to this antimicrobial action [25]. Additionally, we found that Manuka essential oil can inhibit biofilm formation dose-dependently. The limitation of this study is that all experiments were performed only in vitro; therefore, if further in vivo studies definitely show the safety and efficacy of Manuka essential oil, this natural antimicrobial compound may represent a valuable new treatment option against MSSP and MRSP from dogs.
References


22.


국문 초록

개의 피부와 귀에서 분리한 Staphylococcus pseudintermedius에 대한 Manuka essential oil의 효과

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송 치 윤

_Staphylococcus pseudintermedius_는 개 피부감염증의 주 원인균이다. 최근에, methicillin-resistant _S.pseudintermedius_ (MRSP)가 다양한 환경에 저항성을 보이면서 수의분야에서 새로운 도전과제가 되었다. 따라서 새로운 환경물질에 대한 관심도가 증가하고 있으며 Manuka는 이러한 물질중에 하나이다. 본 연구의 목적은 MRSP를 포함한 _S.pseudintermedius_에 대한 Manuka essential oil의 항균력과 Biofilm 형성억제효과를 확인하는 것이다. 개 농장, 의료병원에서 50개의
*S. pseudintermedius*가 분리되었으며 높은 methicillin-resistant 비율을 나타냈다 (78%). Manuka essential oil의 항균력을 확인하기 위해서 Manuka essential oil의 minimum inhibitory concentrations (MICs)를 Agar dilution법을 이용하여 측정하였으며 그 결과를 Cephalexin의 MIC와 비교하였다. 50개의 *S. pseudintermedius*에 대한 Manuka essential oil과 Cephalexin의 MIC는 각각 2⁻⁶ ~ 2⁻⁹ (v/v)와 0.125 ~ 128 mg/ml 였다. 분리된 *S. pseudintermedius*중 41개의 분리균만 Cephalexin에 감수성을 보였으나 (82%) Manuka essential oil은 임상적으로 분리된 MRSP를 포함한 50개의 *S. pseudintermedius* 모두에 대해 항균력을 나타냈다.

Biofilm 형성억제능력은 Crystal violet염색을 이용한 Colarimetric 법을 이용하여 평가 하였다. 각각 0.01%, 0.1%, 1% 농도의 Manuka essential oil과 반응한 *S. pseudintermedius*는 통계적으로 유의적으로 Manuka essential oil ( p < 0.05 ) 농도와 비례하여 Biofilm 형성이 억제 되었으며 이러한 결과들을 통해 Manuka essential oil이 *S. pseudintermedius*에 대해 항균력과 Biofilm 형성억제효과가 있음을 확인하였고 개에서 *S. pseudintermedius* 감염을 치료할 수 있는 잠재적인 치료물질이 될 수 있을 것이라고 생각된다.

주요어: Manuka essential oil, *S. pseudintermedius*, Antimicrobial activity, Biofilm, dogs
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