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Introduction

Malassezia pachydermatis is a commensal yeast that commonly colonizes in the superficial layers of the epidermis, and it is the predominant organism in the skin mycobiota of dogs [18]. *M. pachydermatis* is classified as a lipophilic, non-lipid dependent, nonmycelial saprophytic yeast, which is commonly found on skin, in ear canals, on mucosal surfaces, lip, chin, rectum, in the anal sacs and vagina of normal dogs [9, 6, 29]. Favorable growth conditions in the local environment allow excessive multiplication of this organism, which may then function as an opportunistic secondary pathogen in dermatitis and otitis externa [1, 21]. The most common clinical sign of *Malassezia* dermatitis is moderate to intense pruritis, which may be only partially responsive to corticosteroids and antibiotics. Affected animals typically have an offensive odor, which some clinicians refers to as yeasty or rancid [34]. *Malassezia* dermatitis is manifested either as a generalized or localized dermatitis. It is common in dogs and usually occurs concurrently with other dermatoses [27]. *M. pachydermatis* related otitis externa and dermatitis is very difficult to control because *Malassezia* overgrowth usually associated with an underlying cause, such as allergies (atopy, food allergy, flea allegy, and contact allergy), keratinization disorders (seborrhea), bacterial skin diseases, endocrinopahties (hyperadrenocortisim, hypothyroidism, diabetes mellitus), metabolic disease (zinc-responsive dermatosis and superficial necrolytic dermatitis), and cutaneous or internal neoplasia [13, 4]. Moreover, a hypersensitivity response to the yeast itself is likely to occur in many dogs with allergies [14, 13, 30]. Canine atopic dermatitis tends to be most frequently diagnosed in dogs concurrently with *Malassezia*-related dermatitis [17, 5]. *Malassezia* hypersensitivity in dogs is thought

to manifest as a highly inflammatory and pruritic response mounted to relatively low numbers of yeast [38]. Current therapeutic options for canine *Malassezia* dermatitis and otitis are reported to include systemic therapy with ketoconazole or itraconazole and topical therapy with azole derivatives [33, 3]. Many antifungal products and actives are available in the market and include miconazole, clotrimazole, thiabendazole, posaconazole and nystatin [38]. The treatment of severe infections may involve systemic therapy, with prolonged high doses of antifungal drugs [7]. Well-known side effects of antifungal azole drugs are gastrointestinal problems and hepatotoxicity in human and veterinary medicine [28, 25]. Recently, recurrent *Malassezia* otitis has been reported more frequently [24]. Difficult-to-control yeast overgrowth is often attributed to 'resistance' to antifungal drugs. Published studies have reported isolated cases of resistance of *M. pachydermatis* to various azoles [8, 23, 24, 20]. Thus, there is an increased need for alternative antifungal agents.

Multiple botanicals have shown evidence for anti-malassezian activity *in vitro* including limonene, many essential oils, *propolis* extract [12, 38]. Especially, essential oils obtained from plant material, such as lavender, tea tree have traditionally been used for a variety of medicinal purposes because of their antifungal, antibacterial, antiviral, antioxidant properties [36, 12, 31]. Due to the characteristics, there has been increasing interest in the use of essential oils as substitutes for synthetic antimicrobial agents. Manuka essential oil is indigenous to New Zealand and has a long history of medical use [26]. Several studies have described its remarkable antimicrobial properties [20, 37]. Although the antimicrobial activity of manuka essential oil is associated with the fraction containing triketones [15], the mechanism of its activity against *M. pachydermatis* is not clearly understood. The aim of this study was to evaluate the use of manuka essential oil as an alternative to

the established antifungal agents in veterinary medicine. Therefore, this study investigated the minimum inhibitory concentrations (MICs) and the minimal fungicidal concentrations (MFCs) of manuka essential oil against *M. pachydermatis* isolates from dogs with and without skin and ear lesions.

Materials and Methods

1. *Malassezia pachydermatis* isolates

Thirty *M. pachydermatis* isolates were obtained from 23 dogs with or without skin lesions and otitis referred to the Veterinary Medical Teaching Hospital, Seoul National University (The Republic of Korea) in 2010 and 2011. The isolates were divided into those obtained from healthy animals and those from non-healthy animals, as determined by the dermatologist who evaluated the original swabbed site. The non-lesion group of 13 isolates from dogs in good general health, with no history of skin or ear diseases. The lesion group included 17 isolates taken from the ears of dogs with ear discharge and thickening of the ear canal or the skin with erythema, malodor, alopecia, seborrhea and lichenification. Animals that had diseased skin or ear lesions were put on an anti-fungal therapy for 4 weeks. Antifungal agents were oral medication of ketoconazole 5mg/kg PO BID or itraconazole 10mg/kg PO SID. All ear canals, ventral neck, interdigital region, nasal fold, perianal region, axillary region, internal elbow and inguinal region samples were obtained with sterile cotton swabs, moistened with sterile saline (0.9% NaCl) solution. All the isolates had been identified as *M. pachydermatis* in previous molecular study [19]. Table 1 shows the phenotypes and sampling sites of the isolates. The isolates were maintained in Sabouraud Dextrose Broth (SDB; BD Diagnostic systems, Sparks, MD, USA) containing 20% glycerol and stored at -80 °C. To perform the tests, the isolates were subcultured on the Sabourad Dextrose Agar (SDA; BD Diagnostic systems, Sparks,

MD, USA) and incubated 37 °C for 3-7 days.

Table 1. Population of isolates in accordance with phenotype and sampling site

	non lesion (n=13)	lesion (n=17)
<i>ITS-1</i> ^a	A ^b = 10	A = 14
Genetic type	B = 0 C = 3	B = 0 C = 3
Site	Ear canal = 3 Skin = 10 (inguinal = 2, interdigits = 5, nasal fold = 2, neck = 1)	Ear canal = 11 Skin = 6 (nasal fold = 1, neck = 3, elbow = 1, interdigits = 2)

^a : Genotype of *Malassezia pachydermatis* (Internal transcribed spacer-1)

^b : Subgenotypes

2. Manuka essential oil

Pure 100% manuka essential oil was purchased from Honey Collection (Marlborough, New Zealand). It was extracted using the steam distillation method.

The collecting location, year, and extracting institutions are shown in Table 2.

Table2. Detailed information of manuka essential oil

Leptospermum scoparium

Collecting location	East Cape New Zealand
Collecting year	2013
Extracting institution	Honey collection Marborough, New Zealand

1. *In vitro* susceptibility testing

Many studies have been conducted to determine the antifungal susceptibility of *M. pachydermatis*, but it has not yet to be established. These studies followed the procedure for susceptibility testing of yeasts established by the Clinical and Laboratory Standard Institute (CLSI) Subcommittee for Antifungal Susceptibility Testing [7, 35]. The CLSI M27-A3 is the gold standard protocol, which was established to provide a consensus on the evaluation of the susceptibility of yeasts against anti-fungal drugs [22]. However, the protocol includes invasive yeasts, such as *Candida spp.* and *Cryptococcus spp.*, and it cannot be applied in standard evaluations of *Malassezia spp.* due to their lipid-dependent properties [32, 7, 11, 8]. Thus, the modified M27-A3 method was developed to assess the anti-fungal susceptibility of *Malassezia spp.* This study used SDB (BD Diagnostic Systems), with 0.1% Tween 80 (Sigma-Aldrich, St. Louis, USA) as the lipid source. A stock solution of itraconazole (Sigma-Aldrich) was prepared in dimethyl sulphoxide (Noblechem™, Hwaseong, Korea), and itraconazole (0.016 – 16 µg/mL) was diluted in the SDB with 1 % Tween80. Two-fold dilutions of the drug solutions (ranging from 0.016 - 16 µg/ml) were dispensed into 96-well plates (Corning, NY, USA). A similar (albeit unvalidated) procedure was adopted for manuka essential oil from *Leptospermum scoparium* (100% oil extract). Briefly, a series of two-fold diluents of manuka oil (ranging from 2⁻¹ to 2⁻¹⁴ % v/v) were prepared on a 96-well plate.

The inoculum was incubated on a SDA plate (BD Diagnostic Systems), and the numbers of *M. pachydermatis* were counted. To avoid clustering, the yeasts were centrifuged at 3,000 g, 4 ° C for 1 minute to produce micropellet.

The final concentration of the *M. pachydermatis* inoculum in SDB was adjusted to an optical density of 0.5 using a turbidometer (DensiCHEK plus, BioMerieux, France), which was approximately 1×10^4 CFU/ml. A total of 50 μ L of the suspension was transferred into a 96-well plate. The plate was incubated at 37° C for 2 days. All the tests were performed in duplicate, and each series included a positive control (diluted inoculum working solution) and a negative control (itraconazole free) for growth. The 96-well plate was incubated at 37° C for 2 days.

4. Determination of MIC

After incubation, 1 μL of suspension from each well was subcultured onto the Sabouraud dextrose agar (SDA) at 37° C for 3 days. The MIC was the lowest concentration that completely inhibited fungal growth on the agar. The MIC was recorded after 3 days of incubation on the SDA plate.

5. Determination of MFC

After incubation, 1 μL of suspension from each well was subcultured onto the SDA at 37° C for 3 days. The MFC was the lowest concentration that completely inhibited fungal growth on the SDA plate.

Results

This study determined the susceptibility of 30 isolates phenotypically identified as *M. pachydermatis* to itraconazole and manuka essential oil. The study was divided into two groups of *M. pachydermatis* obtained from lesions and non-lesions.

1. MICs of manuka essential oil and itraconazole

To confirm the antifungal activity of the manuka essential oil, the MICs of the manuka essential oil against *M. pachydermatis* isolates from nonlesion and lesions were determined using the modified CLSI M27-A3 broth microdilution method. The results were compared with those of itraconazole, a commonly used antifungal drug for fungal skin disease in veterinary practice. Figure 1 shows the distribution of the MICs of the manuka essential oil and itraconazole against *M. pachydermatis* from the non-lesions and lesions. The MICs of the manuka essential oil and itraconazole against the 30 *M. pachydermatis* isolates ranged from 2^{-14} to 2^{-11} % v/v and 0.016 $\mu\text{g/mL}$ to $>16 \mu\text{g/mL}$, respectively. All 30 *M. pachydermatis* isolates were susceptible to the manuka essential oil, whereas itraconazole did not inhibit the growth of one isolate from the lesion group at the highest concentration prepared on the 96-well plate. The MIC₅₀ of the lesion and nonlesion groups exposed to the manuka essential oil and itraconazole was measured. MIC₅₀ is the minimum concentration of antifungal capable of inhibiting the growth of 50% of the isolates.

The results are shown in Table 3. The MIC₅₀ of itraconazole in the nonlesion group ($n=13$) and lesion group ($n=17$) was 0.128 and 0.5 $\mu\text{g/mL}$, respectively. The MIC₅₀

of the manuka essential oil in the nonlesion group ($n=13$) and lesion group ($n=17$) was 2⁻¹² and 2⁻¹³% v/v, respectively.

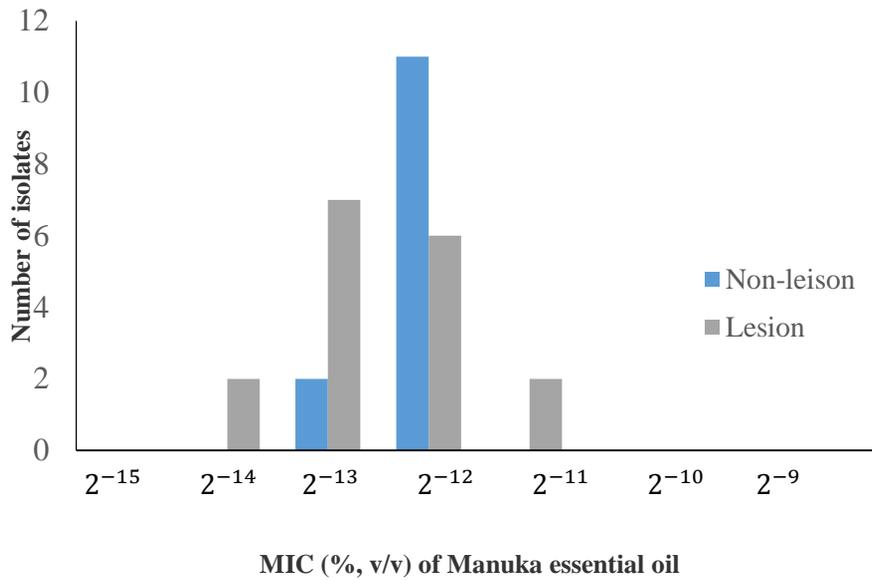
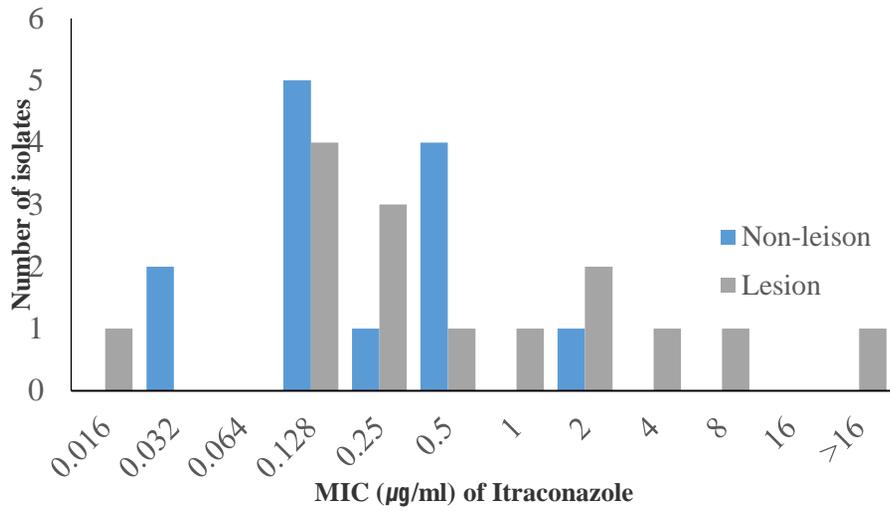


Figure 1. Distribution of the MICs of Manuka essential oil and itraconazole against *Malassezia pachydermatis*

Table 3. MIC₅₀ of itraconazole and Manuka essential oil

	Non-lesion group (n=13)	Lesion group (n=17)
Itraconazole	MIC ₅₀ = 0.128 $\mu\text{g/ml}$	MIC ₅₀ = 0.5 $\mu\text{g/ml}$
Manuka oil	MIC ₅₀ = 2 ⁻¹² %, v/v	MIC ₅₀ = 2 ⁻¹³ %, v/v

MIC₅₀, minimum concentration of antifungal capable of inhibiting the growth of 50% of the isolates

2. MFCs of manuka essential oil and itraconazole

The MFCs of the manuka essential oil against the *M. pachydermatis* isolates from the non-lesion and lesion groups were determined using the modified CLSI M27-A3 broth microdilution method. Figure 2 shows the distribution of the MFCs of the manuka essential oil and itraconazole against *M. pachydermatis* from the non-lesion and lesion groups. The MFCs of the manuka essential oil and itraconazole against the 30 *M. pachydermatis* isolates ranged from 2^{-13} to 2^{-10} % v/v and 0.016 $\mu\text{g/mL}$ to $>16 \mu\text{g/mL}$, respectively. The MFCs of the manuka oil were similar for the non-lesion and lesion groups, However, the MFCs of itraconazole varied in for the non-lesion and lesion groups such as result of the MICs. One of the 30 isolates in the lesion group that was exposed to the highest concentration of itraconazole continued to grow. Thus, this isolate may be resistant to itraconazole and suggests that the presence of *in vitro* resistance to antifungal drug. However, manuka essential oil has antifungal effect to this isolate without reference to resistance to itraconazole. This result suggests manuka essential oil does not have microorganism resistance, it is expected to be a potential new remedy a long with existing antifungal agents.

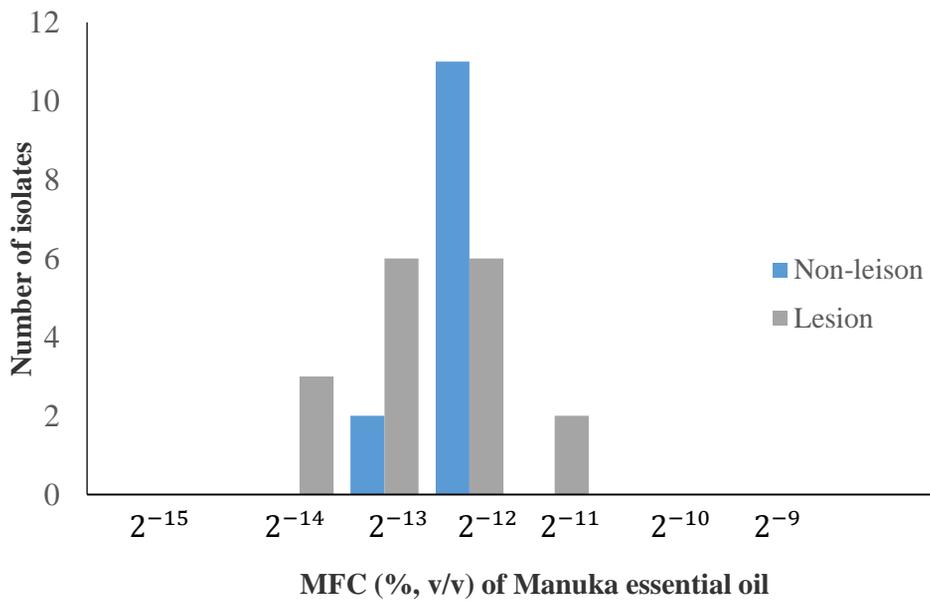
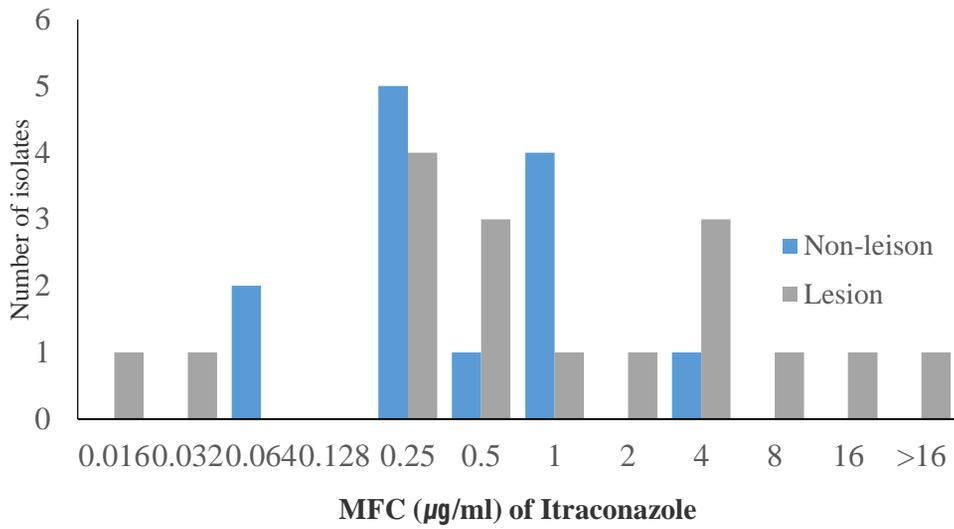


Figure 2. Distribution of the MFCs of Manuka essential oil and itraconazole against *Malassezia pachydermatis*

Discussion

M. pachydermatis is the predominant pathogen of fungal skin diseases in veterinary practice. It is a commensal yeast that colonizes in the skin and mucosal sites of healthy dogs, cats, and animal owners [2]. This yeast may become pathogenic under certain circumstances, causing dermatitis and otitis [2, 14]. Azole drugs are commonly to treat *M. pachydermatis* infection. However, in recent years, *in vitro* studies have reported anti-fungal resistant isolates [23, 24, 30]. Rare cases of resistance have also been reported in veterinary practice [24, 23]. Long-term use and high doses of azole drugs have also been reported to cause side effects, such as gastrointestinal disturbances and hepatotoxicity [28]. Thus, Increasing interests in azole-resistant *M. pachydermatis* and alternative antifungal material from natural products [20, 37]. Manuka essential oil from *Leptospermum scoparium* in New Zealand is one such natural product. Manuka essential oil has traditionally been used as topical therapy for skin diseases in New Zealand. Previous studies reported that manuka oil has antimicrobial, anti-inflammatory, and antiviral activity [15, 26, 37]. Although the antimicrobial activity of manuka essential oil is associated with the fraction containing triketones [15], the mechanism of activity of manuka essential oil against *M. pachydermatis* is not clearly understood. In this study, we investigated the antifungal activity of manuka essential oil against clinically isolated *M. pachydermatis* from the skin and ears of dogs.

Currently, there is no standard protocol to determine the antifungal susceptibility of *Malassezia* spp. A variety of susceptibility tests with limited inter-laboratory agreement has been proposed. The CLSI's M27-A3 broth microdilution protocol is

the most commonly employed test in published studies [7, 35, 22, 11]. However, this reference protocol to test the susceptibility of yeasts to antifungal agents is inapplicable to yeasts of the genus *Malassezia* because of their lipid-dependent properties.

In the present study, all the 30 isolates from the dogs with lesions and healthy skin were identified as *M. pachydermatis*. To confirm the antifungal effect of the manuka essential oil, we used the modified CLSI M27-A3 broth microdilution protocol and replaced the RPMI 1640 in the CLSI M27-A3 broth microdilution protocol with SDB and 1% Tween80.

This study then compared the results with those obtained with itraconazole, one of the most commonly used antifungal drugs for *Malassezia* infections in dogs. The MICs of the manuka essential oil and itraconazole against the 30 isolated *M. pachydermatis* ranged from 2^{-14} to 2^{-11} %, v/v and 0.016 $\mu\text{g/mL}$ to >16 $\mu\text{g/mL}$, respectively. The MICs of the manuka oil were similar for the non-lesion and lesion groups, ranging from 2^{-13} and 2^{-12} . The MICs of itraconazole varied in for the non-lesion and lesion groups, ranging from 0.032 to 2 $\mu\text{g/mL}$ and 0.016 to >16 $\mu\text{g/mL}$, respectively. One isolate was not inhibited by itraconazole at the highest concentration of the drug (>16 $\mu\text{g/mL}$). Thus, this isolate may be resistant to itraconazole and suggests that the presence of *in vitro* resistance to antifungal drug.

However, manuka essential oil has antifungal effect to this isolate without reference to resistance to itraconazole. This result suggests manuka essential oil does not have microorganism resistance, it is expected to be a potential new remedy along with existing antifungal agents.

To compare the antifungal activity of the manuka essential oil and itraconazole, their MICs were determined from non-lesion and lesion groups. The MIC₅₀ against the manuka essential non-lesion and lesions were 2⁻¹²% (v/v) and 2⁻¹³% (v/v), respectively. In contrast, the MIC₅₀ of itraconazole against the lesions (= 0.5 µg/mL) was higher than against the non-lesions (= 0.128 µg/mL). These results suggest that long-term antifungal therapy might induce antifungal resistant isolates.

Nakano *et al.* previously reported an increase in the *in vitro* MICs of nystatin, ketoconazole, and terbinafine (but not in the MIC of an antiseptic, β-thujaplicin) in 10 samples of *M. pachydermatis* exposed to the antifungals at subminimum inhibitory concentrations for 30 passages [31]. At the end of the passages, the MIC of β-thujaplicin was unchanged, whereas the MIC of nystatin, ketoconazole, and terbinafine increased two-fold, sixteen-fold, and eight-fold, respectively. Another study created fluconazole-resistant *Malassezia* isolates *in vitro* and examined the MICs of itraconazole, ketoconazole, voriconazole. In that study, the MICs of all the azole drugs increased [23]. The authors suggested that the acquisition of cross-resistance might be associated with the up-regulation of *candida* drug resistance (CDR) genes or with altered membrane sterol composition. These studies point to the ability of *M. pachydermatis* to acquire resistance to antifungal medication. Other studies have proposed that the formation of drug-resistant biofilms in *M. pachydermatis* might lead to the acquisition of resistance [10, 16]. In those studies, multiple isolates of *M. pachydermatis* formed biofilms on artificial substrates.

In this study, we confirmed the antifungal activity of manuka essential oil and its potential as a valuable new alternative treatment against *M. pachydermatis*. There are no *in vivo* investigations of the antifungal effect of manuka essential oil and no

data on clinical side effects of various concentrations. Thus, further *in vivo* studies are required to determine the potential of manuka essential oil in clinical veterinary practice.

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국문 초록

개의 피부와 귀에서 분리한 *Malassezia pachydermatis* 에 대한 Manuka essential oil 의 효 과

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Malassezia pachydermatis 는 개의 피부에 상재하는 정상 미생
물총으로, 환경이나 조건에 따라 병원성을 가지고 개의 피부나 귀
에서 병변을 야기한다. 개의 *Malassezia* 피부염 및 외이염의 치료
법은 주로 itraconazole 이나 ketoconazole 등의 azole 계열의 약

물들을 이용한 국소 및 전신요법이 일반적이다. 하지만, 최근들어 *in vitro* 상에서 이러한 약물들에 대한 내성균주가 확인되고 있으며, 실제 임상에서도 흔하지는 않지만 다른 기저질환들이 배제되었음에도 항진균제에 반응하지 않는 *Malassezia* 피부염 및 외이염이 보고되고 있다. 본 연구의 목적은 *M. pachydermatis* 에 대한 azole 계열의 대표적인 항진균제인 itraconazole 대비 Manuka essential oil의 항진균력을 확인함으로써 기존 치료제의 대체 치료물질로의 가능성을 확인하는 것이다. 2010년과 2011년에 서울대학교 동물병원에 내원한 개 *Malassezia* 피부염 및 외이염 환자에서 총 30개의 샘플을 실험에 사용하였으며, 이를 병변이 없는 그룹과 병변이 있는 그룹으로 분류하였다. Manuka essential oil의 항진균력을 확인하기 위하여 CLSI M27-A3 broth microdilution 방법을 수정한 실험법을 사용하여 minimum inhibitory concentrations (MICs)와 minimum fungicidal concentrations (MFCs)를 측정하였고, 그 결과를 itraconazole 과 비교하였다. 30개의 *M. pachydermatis* 균주에 대한 Manuka essential oil 과 itraconazole 의 MIC는 각각 2^{-14} to $2^{-11}\%$, v/v 와 $0.016 \mu\text{g/mL}$ to $>16 \mu\text{g/mL}$ 였으며, MFC는 각각 2^{-13} to $2^{-10}\%$, v/v 와 $0.016 \mu\text{g/mL}$ to $>16 \mu\text{g/mL}$ 였다. 또한, 병변그룹과 병변이 없는 그룹의 itraconazole 과 Manuka essential oil 의 MIC₅₀을 비교한 결과

itraconazole의 경우 병변이 없는 그룹의 0.128 $\mu\text{g}/\text{mL}$ 보다 항진균제 복용 경력이 있는 병변그룹에서 0.5 $\mu\text{g}/\text{mL}$ 으로 높은 수치가 확인되었다. 이는 노출 빈도가 높을수록 *Malassezia*의 저항성 획득 가능성이 높을 수 있음을 시사한다. 본 연구의 결과들은 Manuka essential oil 이 기존에 사용되던 항진균 약물들을 대체하여 개의 *Malassezia* 피부염 및 외이염의 잠재적인 치료물질이 될 수 있을 것임을 시사한다.

주요어 : Manuka essential oil, *Malassezia pachydermatis*, Antifungal effect, dogs

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