

Antifungal activities of essential oils of *Syzygium aromaticum*, *Piper betle*, and *Ocimum sanctum* against clinical isolates of canine dermatophytes

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Received 21 Dec 2016

Accepted 12 Jul 2017

ABSTRACT: Current antifungal drugs for canine dermatophytosis are effective but may cause adverse effects or drug resistance. In this study, three essential oils from *Syzygium aromaticum* (L.) Merr. & Perry, *Piper betle*, and *Ocimum sanctum* were investigated for their antifungal activities. Fungal organisms were standard strains and clinical isolates of *Trichophyton mentagrophytes*, *Microsporum canis*, and *Microsporum gypseum*. Minimum inhibition concentration (MIC) and minimum fungicidal concentration (MFC) were evaluated. The composition of the essential oils was determined by GC/MS analysis. MIC and MFC tests indicated that the antifungal activities of these three essential oils were high (MIC range 0.2–1.6 $\mu\text{l/ml}$) and fungal samples from both sources had similar sensitivity to antifungal drug and essential oils. The results of essential oil analysis revealed that eugenol and its derivatives were the major components possessing antifungal activity. In conclusion, this study demonstrated the potential of all three essential oils as highly effective antifungal agents against canine dermatophytes.

KEYWORDS: antidermatophytic activity, volatile oil, dermatophytosis, antifungal agent

INTRODUCTION

Dermatophytosis is a common skin disease in both human and animals worldwide. The major pathogens are in the genera of *Epidermophyton*, *Trichophyton*, and *Microsporum*. The common dermatophytes in dogs are *Trichophyton mentagrophytes*, *Microsporum canis*, and *Microsporum gypseum*¹. The clinical symptoms are generally mild, but the significance of the disease is its zoonotic ability and costly and time-consuming therapy. As most families nowadays have pets in their houses and pet-human interaction is increasing, dermatophytosis transmitted via direct contact with dogs or cats is a common scenario. This zoonotic infection poses a greater risk for immunocompromised people².

Antidermatophytic drugs include several chemical groups, and the important drugs are griseofulvin, terbinafine, and azole drugs such as ketoconazole and itraconazole³. Even though they are effective, their adverse effects such as toxicities and drug resistance are of great concern. As dermatophytosis needs long-term therapy, the toxicities of antifungal drugs may be unavoidable. For example, ketoconazole

can inhibit adrenal steroid synthesis, and griseofulvin can cause gastrointestinal irritation and hepatotoxicity⁴. Chronic infection and re-occurrence are also a common nature of dermatophytosis, and a consequence is repetitive treatment which can cause the development of drug resistance. In addition, antifungal activities of azole agents, griseofulvin, and terbinafine are fungistatic which could contribute to the emergence of drug resistance⁵. These adverse effects are solid reasons for discovery and development of novel antifungal agents.

Essential oils are complex mixtures from plant origins which have been used for many purposes including use as pharmaceuticals. The constituents of essential oils differ based on their origins. Various biological activities have been reported including antimicrobial, anti-inflammatory, antimutagenic, antidiabetic and anticancer activities⁶. Recent studies have demonstrated the antidermatophytic activities of essential oils of several plant species, for example, *Artemisia* spp.⁷, *Cinnamomum* spp.⁸, *Allium* spp.⁹, *Nigella* spp.¹⁰, and *Matricaria* spp.¹¹. Furthermore, essential oils were able to produce synergistic effect when combined with syn-

thetic antifungal drugs^{9,12}.

Syzygium aromaticum (L.) Merr. & Perry (clove), *Piper betle* (betel vine), and *Ocimum sanctum* (holy basil) are common plants in Thailand, and their essential oils are available commercially. The essential oils of these plants have been studied extensively and demonstrated a variety of pharmacological activities. Previous studies showed that clove oil possesses antibacterial, antifungal, and antioxidant activities^{6,13}. Betel vine^{14,15} and holy basil essential oils^{12,16,17} have also shown similar activities in various studies. However, none of these reports studied their activities against canine dermatophytes. The present study evaluated antidermatophytic activities of essential oils from *S. aromaticum*, *P. betle*, and *O. sanctum* against clinical isolates and standard strains of canine dermatophytes.

MATERIALS AND METHODS

Essential oils

The essential oils of *S. aromaticum*, *P. betle*, and *O. sanctum* were purchased from Thai China Flavours & Fragrances Industry Co. (Thailand).

Dermatophytes

Clinical isolates and standard strains of canine dermatophytes were used in this study. The clinical isolates of *Trichophyton mentagrophytes*, *Microsporum canis*, and *Microsporum gypseum* were obtained from the Veterinary Teaching Hospital, Khon Kaen University, Thailand. The standard strains included *T. mentagrophytes* (DMST 19735), *M. canis* (DMST 29297), and *M. gypseum* (DMST 21146) which were obtained from the Department of Medical Sciences, Ministry of Public Health, Thailand. All fungal strains were maintained and subcultured on Sabouraud dextrose agar (SDA, Becton Dickenson) at 28 °C.

Determination of MIC and MFC

The minimal inhibitory concentration (MIC) of essential oils was determined by broth microdilution assay. Essential oils (50 µl) were serially diluted (50–0.024 µl/ml) with Sabouraud dextrose broth (SDB, Becton Dickenson) containing DMSO as a solubilizer in a microtitre plate. Ketoconazole (Sigma-Aldrich) in DMSO, as a control agent, was similarly diluted (250–0.122 µg/ml). An equal volume of the inoculum suspension (10⁶ CFU/ml) of each organism was added to each well. Growth and sterility controls were included for each assay and all tests were performed in triplicate. The microplates were

incubated at 28 °C for 7 days. Endpoint evaluation was performed visually based on comparison of the fungal growth in wells containing essential oil with that of the growth control. The MIC was defined as the lowest concentration at which no visible growth was observed.

To determine the minimal fungicidal concentration (MFC), 20 µl of samples from all wells with complete growth inhibition and from the last well showing growth were inoculated onto the surface of Sabouraud dextrose agar Petri dishes. The dishes were incubated at 28 °C for 3 days or until fungal growth was observed in the control samples. The MFC values were determined as the lowest concentration of essential oils giving no visible growth.

Determination of essential oil components using GC/MS

The chromatographic analyses were carried out with gas chromatography (Model US35120382, Agilent, China) coupled with a mass spectrometer (Model CN10402086, Agilent, USA). A DB-5ms capillary column (30 m × 0.25 mm i.d.) coated with 0.25 µm film 5% phenyl methylpolysiloxane was used for separation. The column temperature started with 70 °C for 5 min, subsequently increased to 120 °C at 3 °C/min then programmed at 5 °C/min to 270 °C for 3 min. Manual injection (1 µl) in a purged split mode (1:100) was conducted and high-purity helium was used as carrier gas at 1 ml/min flow rate. The scan range was 35–550 m/z and the scan rate was 1000 atomic mass unit per second (amu/s). Injected volume was 1 µl of 1% solution (diluted in hexane). The identification of the components was based on comparison of their mass spectra with those of a computer library (Wiley 7n MS Search library).

RESULTS

MIC and MFC

The MIC and MFC values are shown in Table 1. The three essential oils are effective against all dermatophyte strains at very low concentrations. The MICs ranged from 0.2–1.6 µl/ml, and the MFCs from 0.4–1.6 µl/ml. The MIC and MFC ranges of ketoconazole are 0.12–0.50 and 0.25–0.50 µg/ml, respectively. Among the three essential oils, antifungal activity of *S. aromaticum* is the highest, and *O. sanctum* is the lowest. In the view of dermatophytes, the sensitivities of clinical isolates and standard strains are not different. However, *M. gypseum* needed twice the drug concentration

Table 1 Antifungal activity (MIC and MFC) of the essential oils of *S. aromaticum*, *P. betle*, and *O. sanctum* against dermatophyte strains.

Dermatophytes	<i>S. aromaticum</i>		<i>P. betle</i>		<i>O. sanctum</i>		Ketoconazole	
	MIC ^a	MFC ^a	MIC ^a	MFC ^a	MIC ^a	MFC ^a	MIC ^b	MFC ^b
Clinical isolates [†]								
<i>T. mentagrophytes</i> (n = 5)	0.2	0.4	0.2–0.4	0.4	0.8	0.8	0.12	0.25
<i>M. canis</i> (n = 10)	0.2–0.4	0.4	0.2–0.4	0.4	0.4–0.8	0.8	0.12	0.25
<i>M. gypseum</i> (n = 5)	0.4	0.4	0.4–0.8	0.8	1.6	1.6	0.50	0.50
Standard strains								
<i>T. mentagrophytes</i> DMST 19735	0.2	0.4	0.2–0.4	0.4	0.8	0.8	0.12	0.25
<i>M. canis</i> DMST 29297	0.2	0.4	0.2	0.4	0.4	0.8	0.12	0.25
<i>M. gypseum</i> DMST 21146	0.4	0.4	0.8	0.8	1.6	1.6	0.50	0.50

^a MIC and MFC in µl/ml (v/v).^b MIC and MFC in µg/ml (w/v).[†] n = number of isolates.**Table 2** GC/MS analysis of essential oils of *S. aromaticum*, *P. betle*, and *O. sanctum*.

Components	Molecular formula	Retention time (min)	% of total
<i>S. aromaticum</i>			
eugenol	C ₁₀ H ₁₂ O ₂	23.37	98.9
trans-caryophyllene	C ₁₅ H ₂₄	26.35	1.1
<i>P. betle</i>			
<i>p</i> -chavicol	C ₁₁ H ₁₂ O ₂	22.71	4.9
eugenol	C ₁₀ H ₁₂ O ₂	24.12	38.7
trans-caryophyllene	C ₁₅ H ₂₄	26.35	4.0
α-amorphene	C ₁₅ H ₂₄	28.52	4.4
cubebene	C ₁₅ H ₂₄	28.71	3.1
δ-cadinene or δ-amorphene	C ₁₅ H ₂₄	30.00	2.5
eugenol acetate	C ₁₂ H ₁₄ O ₃	30.12	23.4
4-allyl-1,2- diacetoxybenzene	C ₁₃ H ₁₄ O ₄	33.36	19.1
<i>O. sanctum</i>			
eugenol	C ₁₀ H ₁₂ O ₂	23.23	8.7
β-elemene	C ₁₅ H ₂₄	25.09	4.5
methyl eugenol	C ₁₁ H ₁₄ O ₂	25.73	52.4
trans-caryophyllene	C ₁₅ H ₂₄	26.37	29.3
α-humulene	C ₁₅ H ₂₄	27.77	1.7
β-cubebene	C ₁₅ H ₂₄	28.71	1.8
β-elemene	C ₁₅ H ₂₄	29.61	1.6

compared to the others.

GC/MS analysis

Components of the three essential oils analysed by GC/MS are shown in Table 2. The compositions differed widely among the three oils. Two compounds were found in *S. aromaticum* essential oil of which eugenol (99%) was the principal component

followed by trans-caryophyllene (1%). The major components of *P. betle* essential oil was eugenol (39%), eugenol acetate (23%), and 4-allyl-1,2-diacetoxybenzene (19%). The major components of *O. sanctum* essential oil were methyl eugenol (52%), trans-caryophyllene (29%), eugenol (9%), and β-elemene (5%).

DISCUSSION

To be used as topical antifungal agent in veterinary medicine, essential oils possess several advantages in addition to their antimicrobial activities. Firstly, most of them demonstrate antioxidant activity that could help alleviate inflammation¹⁸. Secondly, essential oils are derived from plants and contain mixture of active components which may result in additive or synergistic effects or having action at multiple targets¹⁹. Thirdly, mechanisms of antifungal resistance are usually related to receptor-mediated action of antifungal drugs, therefore, essential oils that act hypothetically by their hydrophobicity and interfere with cell membrane structure may overcome the fungal resistance mechanism^{6,20}. Lastly, essential oils have less toxicity when used on skin, with the most common adverse effect being irritation⁶.

The results of this study initially revealed the nature of the two groups of tested dermatophyte samples. Standard strains and clinical isolates had the same susceptibility profiles for all species of dermatophytes as evidenced by similar MIC and MFC values of all essential oils and ketoconazole shown in Table 1. These dermatophytes were also considered to be non-resistant population as their responses to ketoconazole (MIC range 0.125–

0.5 µg/ml) were in the lower scale compared to the MIC range (0.125–16 µg/ml) reported by the others^{10,21,22}.

In view of fungal sensitivity, the MIC and MFC obtained in this study could be presented as *M. gypseum* > *M. canis* = *T. mentagrophytes* for all tested essential oils and ketoconazole. These findings are in agreement with other reports demonstrating more resistant characteristics of *M. gypseum* than the other two^{10,23}.

In view of the potency of essential oils, all these three essential oils possessed strong antifungal activities with small differences. The comparative effects were demonstrated by their MIC and MFC and could be presented as *S. aromaticum* > *P. betle* > *O. sanctum* for all dermatophytes tested. The differences in activities of these essential oils were also demonstrated by several reports. Our previous report showed equal activities of *S. aromaticum* and *P. betle* essential oils on *Malassezia pachydermatis*²⁴. A study that compared activity of essential oils on *Enterococcus faecalis* showed higher activity of *S. aromaticum* than *O. sanctum*²⁵.

The results of this study can also be compared to other essential oils that were studied on dermatophytes. A recent study demonstrated antifungal activities of twenty commercial essential oils and three of them were identified as highly active²⁶. These essential oils are of *Thymus serpyllum* (MIC range 0.25–2.5 µl/ml), *Origanum vulgare* (MIC range 0.25–5.0 µl/ml), and *Litsea cubeba* (MIC range 0.25–1.5 µl/ml). In comparison, our three tested essential oils could be also identified as highly active as they had a similar MIC range.

In relation to our findings, antifungal activities of these essential oils were strengthened in both efficacy and spectrum by other studies. *S. aromaticum* essential oil was able to inhibit growth of *Candida*^{23,27}, *Aspergillus*^{23,28}, *Fusarium*²⁸, *Epidermophyton*^{23,27}, *Trichophyton*^{23,27,28}, and *Microsporum*^{23,27,28} species. *P. betle* essential oil was also able to inhibit growth of *Candida*²⁹, *Malassezia*²⁴, *Aspergillus*¹⁴, *Penicillium*¹⁴, *Fusarium*¹⁴, and *Cladosporium*¹⁴ species. *O. sanctum* essential oil too was able to inhibit growth of *Candida*^{12,30}, *Aspergillus*^{16,31}, *Penicillium*^{16,31}, *Fusarium*¹⁶, and *Cladosporium*¹⁶ species. Even though these reports used different methods and fungal organisms, they support that antifungal activities of these essential oils are highly active and broad-spectrum.

Once these essential oils have been categorized as highly active, further investigations for clinical use are needed. The possibility is supported by

several recent reports showing effectiveness of other essential oils for treating animal dermatophytosis in vivo. In one study, 25% tea tree oil was able to treat 30 thoroughbred horses infected with *Trichophyton equinum*³². In other studies, 2% essential oil of *T. serpyllum* and 5% essential oil of *O. vulgare* were able to cure cats infected with *M. canis*³³, and sheep infected with *T. mentagrophytes*³⁴. A mixture of essential oils was also reported to effectively treat dogs with *Malassezia dermatitis* within 1 month³⁵. Based on these reports, our tested essential oils may be able to produce a favourable result in vivo if tested. The intended clinical application of our laboratory is to develop semi-solid preparations for topical use, and we are formulating two types of preparation: cream and ointment, for future studies.

Compositions of essential oils in plants are generally varied depending on many factors such as time of harvest, part of the plant, environment, or species variations. In addition, extraction method may change the composition of the resultant essential oil extract. These composition variations also affect their biological activities and may partly explain differences among publications. In this study, we analysed all essential oils using GC/MS analysis, and the major constituents of the essential oils are shown in Table 2. *S. aromaticum* essential oil contained mostly eugenol (99%) which is believed to be the active substance and is well known for its antimicrobial activity¹³. *P. betle* essential oil contained two major constituents, eugenol and eugenol acetate (total of 62%). As the antifungal activity of *P. betle* essential oil was similar to that of *S. aromaticum*, we may assume that both substances are active on dermatophytes. However, eugenol acetate was shown to have lower antimicrobial activity than eugenol³⁶. *O. sanctum* essential oil contains methyl eugenol as the major constituent (52%), followed by trans-caryophyllene (29%), and eugenol is only a minor constituent (9%). Methyl eugenol has been shown to have similar antimicrobial activity to eugenol³¹, while trans-caryophyllene has shown no antimicrobial activity³⁷. Thus methyl eugenol and eugenol (total of 61%) appear to be the major active substances. The difference in eugenol and eugenol derivatives may explain why *S. aromaticum* essential oil had more activity and *O. sanctum* had less activity. The mechanisms of eugenol antifungal activity have been previously proposed as disruption of cell membrane fluidity and permeability which then inhibits fungal growth and eventually causes cell death^{19,38}.

In conclusion, the essential oils of *S. aro-*

maticum, *P. betle*, and *O. sanctum* which are local and abundant in Thailand were demonstrated to be highly active antifungal agents against canine dermatophytes in vitro. The level of activity was comparable to those previously shown to be effective in treating dermatophytes in several species. The major active substances in these oils were eugenol and its derivatives. Further experiments in infected animals will prove their potential as alternative antifungal agents.

Acknowledgements: This study was supported by the Faculty of Veterinary Medicine, Khon Kaen University.

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