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Original Article

Efficiency of clove essential oil against planktonic cells and biofilms of *Malassezia pachydermatis* isolated from canine dermatitis

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Abstract

This study investigated the ability of clove oil and eugenol to inhibit biofilm production and eradicate canine *Malassezia pachydermatis* in biofilm form. Biofilm formation was studied using a crystal violet assay and was quantified by optical density (OD) at 595 nm. Killing time was assessed by time-kill assay. All 17 isolates of canine *M. pachydermatis* were capable of biofilm formation, however, 6 isolates formed more extensive biofilms than the other isolates and were selected for susceptibility testing. Clove essential oil and eugenol showed effectiveness against both planktonic and biofilm cells with identical minimum planktonic inhibitory concentration ($MPIC_{50}$) and minimum planktonic fungicidal concentration ($MPFC_{50}$), 0.156 and 0.312 mg/ml, respectively. The minimum biofilm inhibitory concentration ($MBIC_{50}$) and the minimum biofilm eradication concentration ($MBEC_{50}$) were 0.312 and 0.625 mg/ml, respectively. These studies indicate that biofilms of *M. pachydermatis* are more resistant to clove essential oil and eugenol. In addition, eugenol is the component of clove essential oil responsible for its antifungal activity. The results of the time-kill study showed that the antimicrobial activity of clove essential oil was time and concentration-dependent.

Keywords: biofilm, essential oil, eugenol, *Malassezia pachydermatis*

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Introduction

Biofilms are a major problem in human health but they can also cause health problems in animals. Various infectious diseases of pets and livestock such as mastitis, pneumonitis, hepatitis, enteritis and wound infection are caused by biofilm forming microorganisms (Olson *et al*., 2002). Biofilms increase the resistance of microbes to antibiotic drugs and it is estimated that 10 - 1,000 times higher concentrations of antimicrobial agents are required to treat biofilms than for non-biofilm forming microbes (Mah and O'Toole, 2001). Moreover, the biofilm structure helps microbes to evade the host immune system (Abdullahi *et al*., 2016) causing chronic infections that are difficult to cure. These problems lead to high direct and indirect costs in medical care (Chakraborty *et al*., 2018). Diseases of the integumentary system are commonly found in pets (O'Neil *et al*., 2014). The most common causes of these diseases are ectoparasites, skin infections, tumour formation and skin allergies. Infectious dermatitis in dogs is caused by bacteria and fungi. The yeast *Malassezia pachydermatis* is a principal cause of dermatitis and otitis externa in dogs (Hill *et al*., 2006; Hnilica and Patterson, 2016). Figueredo *et al*., (2012) studied biofilm formation of *M. pachydermatis* isolated from dog skin. They found that 95.2% (n=59) of their isolated yeasts produced biofilms. Moreover, Bumroongthai *et al*., (2016) showed that *M. pachydermatis* and *Candida parapsilosis* isolated from dogs produced biofilms that exhibited resistance to the antifungal agent itraconazole and ketoconazole at concentrations 530 times higher than non-biofilm forming strains.

Clove essential oil is a herb extract with high anti-microbial activity. Previous studies have shown that clove essential oil can inhibit the growth of *M. pachydermatis* isolated from canine dermatitis and otitis externa (Asawapattanakul *et al*., 2013; Khosravi *et al*., 2016; Vaczi *et al*., 2018). Eugenol comprises 67-89% of clove oil (Cortes-Rojas *et al*., 2014; Rodriguez *et al*., 2014) and shows activity against yeasts, which suggests it might be the substance in clove oil responsible for its anti-microbial activity (Schmidt *et al*., 2013; Kunduhoglu, 2017). However, most previous studies of clove oil and eugenol antimicrobial activity have examined planktonic cells. Therefore, this study aimed to investigate the ability of clove essential oil and eugenol to inhibit pre-biofilm formation and eradicate *M. pachydermatis* in biofilms.

Materials and Methods

Yeast strains and culture conditions: *M. pachydermatis* isolates were obtained from clinical cases of canine *Malassezia* dermatitis. Isolates were identified from the Veterinary Clinical Diagnostic Laboratory Unit of the Animal Hospital of the Faculty of Veterinary Medicine, Khon Kaen University, Thailand. Samples were collected from skin lesions by sterile cotton swabs based on the methods of Markey *et al*., (2013) and Khosravi *et al*. (2016). Briefly, the cotton swabs were rolled firmly on the skin lesion then streaked onto Sabouraud dextrose agar (SDA, Becton Dickinson, France) plates supplemented with 0.5% chloramphenicol and cycloheximide (Sigma-Aldrich, Germany). All the plates were incubated at 37^0C for 48 h. The *Malassezia spp*. colonies were cream to light yellow in colour. Single colonies were stained with lactophenol cotton blue for microscopic observation. The microscopic appearance and biochemical tests were performed to identify species of the yeast. *M. pachydermatis* cells were oval-shaped and budding yeast cells were observed. It was a growth on Dixon agar at a temperature of 32, 37 and 40^0C and SDA at 32^0C. This yeast also displayed positive growth on 2% glucose agar and 1% peptone agar, was positive to catalase test and all Tweens utilization tests (Tween 20, 40, 60 and 80) (Gueho *et al*., 1996). Inocula were prepared by resuspending single colonies of *M. pachydermatis* isolates in Sabouraud dextrose broth (SDB, Becton Dickinson, France) and incubating at 37^0C for 48 h. The optical density of the inocula was measured by vis-spectrophotometer (Genesys 10 VIS, Thermo Scientific, USA) and cultures were diluted with SDB to give a final concentration of 10^7-10^8 CFU/mL (Usman *et al*., 2013).

Antifungal agents: The antifungal agents used in this study were clove essential oil (Thai-China Flavors and Fragrances Industry Co., Ltd., Thailand) and eugenol (Sigma-Aldrich, Germany). Ketoconazole (Sigma-Aldrich, Germany) was used as the antifungal control.

Quantification of biofilm formation: Quantification of biofilm formation was achieved by staining with crystal violet according to Takahashi *et al*., (2007) with modifications. Briefly, 50 µl aliquots of *M. pachydermatis* suspension (10^8 CFU/ml) were inoculated into wells of 96-well flat-bottomed cell culture plates (Corning Incorporated, USA) containing 50 µl of SDB in each well. The plates were incubated at $37\ ^0$C for 48 h. The culture medium containing planktonic cells was removed and the wells were washed with 200 µl of sterile distilled water. The adherent yeasts were stained with 50 µl of 0.1% crystal violet (Brightchem Sdn Bhd, Malaysia) for 15 mins at room temperature. After rinsing twice with 200 µl of sterile distilled water, the dye bound to the pre-biofilms was extracted with 200 µl of absolute ethanol for 20 mins. The extracted dye was quantified by measuring the OD at 595 nm (Figueredo *et al*., 2012; Jerzsele *et al*., 2014) with a microplate reader (EZ Read 400, Biochrom, UK). Each experiment was performed in triplicate.

Determination of planktonic inhibitory and fungicidal activity: The minimum planktonic inhibitory concentration (MPIC) and fungicidal concentration (MPFC) of clove essential oil, eugenol and ketoconazole were determined by the broth microdilution method according to the Clinical and laboratory standard institute (CLSI) (2008) with modifications. A serial dilution of tested substances (0.25-0.000488% w/v) with Saboroaud dextrose broth (SDB) was prepared in 96-well flat-bottomed microtiter plates (Costar®, Corning Incorporated) (50 µl per well). Fifty microliters of diluted organism suspension were added into each well to give a final concentration of 5 x 10^6 CFU/mL, confirmed by viable counts. The wells of all organisms cultured in the broth without tested

agents served as positive growth control wells and the mixture of broth and tested agents without organism served as negative growth control wells. The plates were incubated at 37 ^{0}C for 48 h. All tests were performed in triplicate. The MPIC was defined as the lowest concentration of antifungal agent that prevented visible growth after 48 h of incubation. Ten microliter samples from the wells with no visible growth were inoculated onto SDA plates and incubated at 37 ^{0}C for 48 h. MPFCs were determined from the lowest concentration of antifungal agents that showed no growth on SDA.

Determination of minimum biofilm inhibition concentrations (MBICs) and minimum biofilm eradication concentrations (MBECs): The protocol of MBICs and MBECs assay according to Takahashi *et al*., (2007) and Chamdit and Siripermpool (2012) with modifications was used. The aliquots of *M. pachydermatis* suspensions (10^8 CFU/ML) were inoculated in 96-well flat-bottomed microtiter plates (Costar®, Corning Incorporated) (50 µl per well). The plates were incubated at 37 ^{0}C for 48 h. The culture medium containing planktonic cells was removed and the wells were washed twice with 200 µl of sterile distilled water. The tested substances were added and serially diluted two-fold across the plate (final concentration 0.25-0.000488%). The plates were incubated at 37 ^{0}C for 48 h. Antifungal agent-free wells and biofilm-free wells were used as positive and negative biofilm-formed control wells. The minimum biofilm inhibitory concentration (MBIC) was defined as the lowest concentration of tested substances preventing visible growth and the minimum biofilm eradication concentration (MBEC) was determined from the lowest concentration of tested substances that inhibited growth on SDA.

Time-kill assay: The antifungal time-kill effect of clove essential oil on *M. pachydermatis* isolates was studied according to Chamdit and Siripermpool (2012). Briefly, a 100 µl aliquot of *M. pachydermatis* suspension was added to the appropriate amount of clove essential oil in the tube to give a final concentration of 0.5, 1, 5, 10 and 20 times of MBIC, then mixed by a vortex mixer for 1 min. After 0.5, 1, 3, 6 and 24 h of incubation at 37 ^{0}C, it was 10-fold diluted with 0.89% sodium chloride solution to stop the antimicrobial reaction of the substances. The whole 100 µl of dilution 10^{-1} to 10^{-4} was spread onto SDA plates. Each experiment was performed in triplicate.

Results

Biofilm formation: The 17 isolates of *M. pachydermatis* used in this study were collected from canine *Malassezia* dermatitis. After incubation at 37 ^{0}C for 48 h, it was found that all strains were able to produce biofilms with 6 strains showing more biofilm producing capacity than others. These 6 strains were isolated numbers 2, 7, 9, 14, 15 and 16 which had OD at 595 nm in the range of 0.522-0.663 (Figure 1). These isolates were selected for follow-up experiments.

Planktonic inhibitory and fungicidal activity: The susceptibility results of clove essential oil, eugenol and ketoconazole against *M. pachydermatis* demonstrated that the six selected strains were susceptible to all agents. Ketoconazole was used as a standard antifungal control and showed increased anti-fungal activity against all tested strains compared to clove oil and eugenol ($MPIC_{50}$ and $MPFC_{50}$ both $p<0.05$) while clove oil and eugenol showed identical $MPIC_{50}$ and $MPFC_{50}$ values. The $MPFC_{50}$ values of all tested agents were twice their respective $MPIC_{50}$ values (Table 1).

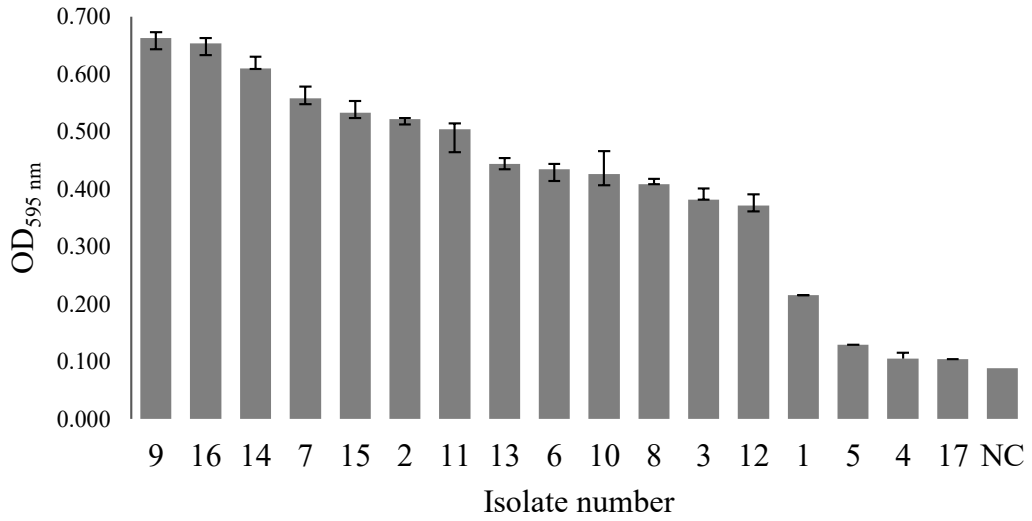

Figure 1 Biofilm formation by *M. pachydermatis* isolates. Biofilms were stained with crystal violet and OD was measured at 595 nm. NC = negative growth control. Values represent the means of triplicate with error bars (standard deviation).

Table 1 $MPIC_{50}$ and $MPFC_{50}$ for planktonic *M. pachydermatis* isolates (n=17)

Antifungal agent	$MPIC_{50}$ (mg/ml)	$MPFC_{50}$ (mg/ml)
Clove essential oil	0.156[a]	0.312[a]
Eugenol	0.156[a]	0.312[a]
Ketoconazole	0.019[b]	0.038[b]

$MPIC_{50}$ = minimum planktonic inhibitory concentration for 50% of tested isolates, MPFC50 = minimum planktonic fungicidal concentration for 50% of tested isolates. Values represent means of triplicate. Different superscript letters within a column indicate statistically significant differences between the means (p<0.05).

Biofilm inhibition and eradication: The inhibition and eradication results of clove essential oil, eugenol and ketoconazole against biofilms of *M. pachydermatis* are shown in Table 2. The $MBIC_{50}$ and $MBEC_{50}$ values are twice their respective $MPIC_{50}$ and $MPFC_{50}$ values, for all antifungal agents tested, indicating decreased susceptibility of biofilms to these antifungal agents. Ketoconazole showed higher anti-biofilm activity compared to clove oil and eugenol ($MBIC_{50}$ and $MBEC_{50}$ both p<0.05).

Time-kill assay: The fungicidal effects of clove essential oil against *M. pachydermatis* per unit time is shown in Figure 2. Clove essential oil at concentrations 10 and 20 times the MBIC (3.125 and 6.25 mg/ml) took 6 and 3 h, respectively, to decrease the number of biofilm *M. pachydermatis* by 90% or a 1 log reduction. The most effective was the 20 times concentration of MBIC that reduced the amount of yeast by 3 logs (99.9%) after 24 hours.

Table 2 $MBIC_{50}$ and $MBEC_{50}$ for biofilms of *M. pachydermatis* isolates (n=6)

Tested agents	$MBIC_{50}$ (mg/ml)	$MBEC_{50}$ (mg/ml)
Clove essential oil	0.312[a]	0.624[a]
Eugenol	0.312[a]	0.624[a]
Ketoconazole	0.038[b]	0.076[b]

$MBIC_{50}$ = minimum biofilm inhibitory concentration for 50% of tested isolates, MBEC50 = minimum biofilm eradication concentration for 50% of tested isolates Values represent means of triplicate. Different superscript letters within a column indicate statistically significant differences between the means (p<0.05).

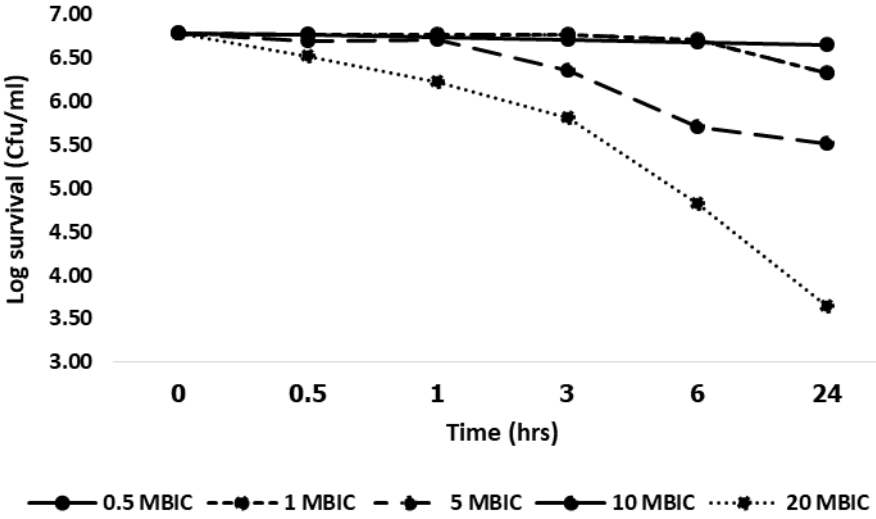

Figure 2 The time-kill assay of clove essential oil against *M. pachydermatis* biofilms (n=6). MBIC = minimum biofilm inhibitory concentration. 0.5 MBIC = 0.156 mg/ml, 1 MBIC = 0.312 mg/ml, 5 MBIC = 1.56 mg/ml, 10 MBIC = 3.12 mg/ml and 20 MBIC = 6.24 mg/ml. Values represent means of triplicate with error bars (standard deviation).

Discussion

The results of biofilm formation showed that all tested *M. pachydermatis* isolates were able to form biofilms, which is consistent with the previous studies of Figueredo *et al*. (2012), Jerzsele *et al*., (2014) and Bumroongthai *et al*., (2016) that demonstrated 95-100% of *M. pachydermatis* isolates were biofilm forming strains. Biofilm production by yeast is a similar process to bacteria, in which cell attachment to a surface is followed by the growth of microcolonies that eventually develop into hyphae. The next step is the detachment of cells in planktonic form from the biofilm structure. While the biofilm is formed, there are several genes expressed that are important in biofilm production. Each yeast strain expresses different levels of the genes, which leads to the difference in quantity

and quality of biofilms (Reynolds and Fink, 2001; Verstrepen and Klis, 2006; Harding *et al*., 2009; Modiri *et al*., 2019).

MPICs and MPFCs of clove essential oil and eugenol against planktonic *M. pachydermatis* were determined using the broth microdilution method. The results were in the range of 0.1-0.32 mg/ml and MPFCs were higher than MPICs, which is in agreement with previous studies that reported MPICs and MPFCs ranging from 0.03 to 1 mg/ml (Asawapattanakul *et al*., 2013). However, the slight differences between studies are likely to be due to differences in the microbial strains tested, the sources of herb extracts, extraction methods, storage conditions and culture conditions including type and source of culture media (Alma *et al*., 2007; Wenqiang *et al*., 2007).

MBICs and MBECs were higher than MPICs and MPFCs indicating that biofilms were more tolerant to clove essential oil, eugenol and ketoconazole. We found that yeast biofilms were 2 times more tolerant, which is much lower than the figure reported by Figueredo *et al*. (2013), in which their biofilms were 18-169 and 13-124 times more tolerant to itraconazole and ketoconazole, respectively. The ketoconazole was similar to clove and eugenol only in the ratio between MPICs and MBICs (2 times), which may have occurred due to the similarity of the resistance mechanisms to these drugs of tested yeast biofilms. These results indicate that the tested eugenol and ketoconazole had strong antifungal activity against both planktonic cells and biofilms. However, the results variation between reports may be due to the difference of tested organisms which differ in their biofilm-forming and drug- tolerant abilities. The increased tolerance of biofilms might be because the biofilm forming strains express several genes differently to planktonic cells, some of which impart resistance to antifungal agents such as the MDR1 and CDR1 genes that are related to drug efflux pump systems (Mateus *et al*., 2004). Moreover, mature biofilms have reduced amounts of ergosterol in their cell membranes, which is the main target of several antifungal agents. (Mukherjee *et al*., 2003).

There were no significant differences between clove essential oil and eugenol MPICs, MPFCs, MBICs and MBECs suggesting that eugenol was the major component of clove essential oil that inhibited our tested *M. pachydermatis* isolates. Our results correspond with the study of Shahi *et al*., (2015) who found that eugenol is the main component of clove essential oil and possessed high antifungal activity. Eugenol disturbs the yeast cell membrane and causes H^+, K^+ and Ca^{2+} electrolyte imbalance (Filgueiras and Venetti, 2006; Tippayatum and Chonhenchob, 2007). Moreover, eugenol inhibits the attachment of yeast cells to surfaces and polymer materials, which is important for the initiation process in biofilm formation by yeasts (de Paula *et al*., 2014). Our time kill study showed that clove essential oil, at 20 times concentration of MBIC, could eradicate 99.9% of yeast cells within 24 h. Lower concentrations also inhibited the *M. pachydermatis* isolates but at lower efficacy, taking more than 24 h. These results indicate that the antimicrobial activity of clove essential oil against tested yeast, *M. pachydermatis* was dependent on concentration and exposure time.

In conclusion, clove essential oil exhibited antifungal activity that could be used to develop topical formulations against *M. pachydermatis* in dogs. In addition, other dog skin pathogens should be examined for susceptibility to clove essential oil as a guideline to drug formulation development that covers more skin pathogens in canine.

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