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Anti-biofilm activity of Thai herbal essential oils against *Staphylococcus pseudintermedius* causing canine pyoderma

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ABSTRACT: The aim of this study was to investigate the efficacy of 11 essential oils (EOs) against *Staphylococcus pseudintermedius* planktonic and biofilm forms. Twenty three bacterial samples were collected from dogs with superficial pyoderma and 9 samples were identified as *S. pseudintermedius* by polymerase chain reaction-restriction fragment length polymorphism method. The minimum planktonic inhibitory concentration (MPIC), minimum planktonic bactericidal concentration (MPBC) and minimum biofilm eradication concentration (MBEC) of EOs were determined by broth microdilution using a transferable solid phase. Among all, lemongrass EO was the most effective against both planktonic and biofilm forms of *S. pseudintermedius* with the MPIC, MPBC and MBEC of 0.02, 0.039 and 0.078% v/v, respectively. EOs of betel vine and citronella had the second most inhibitory effect (MPIC and MPBC: 0.039% v/v and MPBC: 0.156% v/v) followed by clove (MPIC and MPBC: 0.078% v/v) and sweet basil (MPIC and MPBC: 0.625% v/v). The EOs of ginger, plai, kaffir lime, turmeric, holy basil and galanga had the lowest inhibitory effect with MPICs and MPBCs ranging from 1.25% v/v to greater than 2.5% v/v. The results of this study indicated that the anti-planktonic and anti-biofilm effects of the tested EOs were concentration dependent, and higher concentrations were required for anti-biofilm activity. Lemongrass EO showed the most potential as a therapeutic antimicrobial agent for both planktonic and biofilm forms of *S. pseudintermedius*.

KEYWORDS: essential oils, *Staphylococcus pseudintermedius*, anti-biofilm activity

INTRODUCTION

Pyoderma in dogs is one of the most important small animal dermatologic diseases [1]. The animal with signs of the disease suffers from a skin infection, which leads to decreased skin immunity, increased skin disorders and complications with other microbial infections, resulting in a disease that is difficult to be diagnosed and treated. Infected animals show various symptoms such as pruritus, alopecia, follicular papules or pustules, erythema, epidermal collarettes and serous crusts [2]. The gram-positive bacteria *Staphylococcus pseudintermedius* is the most important causative pathogen of this disease, which is an opportunistic pathogen in dogs [3]. Moreover, it can also infect ears and skin of other animals such as cats, parrots and horses [4]. Systemic and topical antibiotics are currently used to treat superficial and deep canine staphylococcal pyoderma, but long-term treatments are required and most animals suffer from recurrent infections [5]. Today, the development of antibiotic resistance in the causative bacteria is an important problem in veterinary medicine and public health, especially methicillin-resistant *S. pseudintermedius* [6].

Biofilm formation of *Staphylococcus* spp. including *S. pseudintermedius* is well known as a factor which increases the severity of diseases [7]. Biofilms are groups of bacteria which are enclosed by the extracellular polymeric substances that help bacteria to survive in inappropriate environments longer than planktonic cells, which leads to decrease in antibiotic susceptibility [8, 9]. Because of an increasing problem of antibiotic resistance of the biofilm forming *S. pseudintermedius*, it is necessary to search for new agents that have anti-biofilm effect to control the growth of this bacteria.

Essential oils contain various chemical constituents such as monoterpenes, sesquiterpenes, diterpenes and other aromatic or aliphatic com-

pounds. Most EOs contain at least 1 active constituent that has antimicrobial activity [10]. The EOs of various plants have been used in traditional medicine for a long period of time, and their antimicrobial and anti-inflammatory activities have been confirmed. Many studies have reported that EOs have antimicrobial activity against planktonic cells of yeast, mold and bacteria [11, 12], but very few studies have examined *S. pseudintermedius* biofilm. Therefore, we studied the anti-planktonic and anti-biofilm activities of EOs from 11 Thai herbs namely: betel vine (*Piper betle*), citronella (*Cymbopogon nardus*), clove (*Syzygium aromaticum*), galanga (*Alpinia galanga*), ginger (*Zingiber officinale*), holy basil (*Ocimum tenuiflorum*), kaffir lime (*Citrus hystrix*), lemongrass (*Cymbopogon citrates*), plai (*Zingiber montanum*), sweet basil (*Ocimum basilicum*) and turmeric (*Curcuma longa*) against *S. pseudintermedius* isolated from dogs with superficial pyoderma.

MATERIALS AND METHODS

Essential oil preparation

EOs of betel vine, citronella, clove, galanga, ginger, holy basil, kaffir lime, lemongrass, plai, sweet basil and turmeric prepared by steam distillation were purchased from Thai-China flavours and fragrances industry Co., Ltd. Stock solutions of each EO were prepared by dilution with polyoxyethylene (20) sorbitan monooleate (Tween-80, Ajax Finechem Pty Ltd., Australia) in sterile distilled water.

Bacterial collection, identification and preparation

Bacterial samples were collected from skin lesions of dogs diagnosed as superficial pyoderma in the animal hospital of the Faculty of Veterinary Medicine, Khon Kaen University, Thailand. The bacteria produced pin-point colonies that showed beta haemolysis on blood agar and were gram positive and arranged in grape-like clusters of cocci. Bacteria that were catalase and coagulase positive, oxidase and hyaluronidase negative and produced anaerobic acid from mannitol, sucrose and trehalose were confirmed to be *S. pseudintermedius* by PCR-restriction fragment length polymorphism (PCR-RFLP) method, as described previously [13] with modifications. Briefly, DNA was extracted by BacterialXpress™ nucleic acid extraction kit (Chemicon, Germany). Then, 1 µl of DNA sample was mixed with 12.5 µl of 2xGoTaq® Green master mix (Promega, USA), 10.5 µl of nucleus free water and 0.2 µM of each primer: pta_f1 (5′-AAA GAC AAA CTT TCA GGTAA-3′) and pta_r1 (5′-GCA TAA ACA AGC ATT GTA CCG-3′) (Bio Basic Canada Inc., Canada). The mixture was placed into a thermocycling machine (T100™ Thermocycler, Bio Rad, USA), and the conditions were set as follows: 95 °C for 2 min, then 35 cycles of 95 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min, and finally 72 °C for 7 min. PCR products were digested with 2.5 units of *Mbo*I restriction enzyme (Promega, USA) for 4 h at 37 °C. The pre and post-digest PCR products were run on agarose gel electrophoresis at 100 V for 35 min. Bands of DNA fragment were detected using UV transilluminator (Gel Doc™ XR+, Bio Rad, USA). The length of the DNA fragment of the pre-digest PCR product of *S. pseudintermedius* was 320 bp, and the post-digest PCR products were 213 and 107 bp. *S. pseudintermedius* isolates were preserved in Mueller Hinton agar (MHA) (Becton Dickinson, France) at 4 °C until use. The selected, identified *S. pseudintermedius* isolates were subcultured into Mueller Hinton broth (MHB) (Becton Dickinson, France) and incubated at 37 °C for 24 h. Before use, 10^6–10^7 CFU/ml bacterial concentrations were prepared by measuring the optical density (OD) at 600 nm.

Determination of minimum planktonic inhibitory concentration (MPIC) and minimum planktonic bactericidal concentration (MPBC) using the transferable solid phase (TSP)

The MPICs of 11 EOs were determined using the TSP according to the protocols of Harrison et al [14] and Sadekuzzaman et al [15] with modifications. Briefly, bacterial suspension (10^6–10^7 CFU/ml) was added to all tested wells of a 96-well flat-bottomed microtiter plate (Thermo Scientific, USA). The TSP lid with 96-pins (Thermo Scientific, USA) was inserted into the wells of the microtiter plate, and they were incubated together for 24 h at 37 °C. The TSP lid was then transferred to a challenge 96-well flat-bottomed microtiter plate containing the serial 2-fold dilutions of the EOs in MHB. The challenge plate and TSP lid were incubated together for 24 h at 37 °C. The TSP lid was removed, and the OD_{595} of the wells in the challenge plate were measured by microplate reader (EZ Read 400, Biochrom, UK). The OD values of the challenge wells were adjusted by subtracting with the OD of the negative growth control well. The MPIC was defined as the lowest concentration of EO that inhibited growth of bacteria in the challenge plate (OD less than 0.1). All wells of the challenge plate that showed no growth

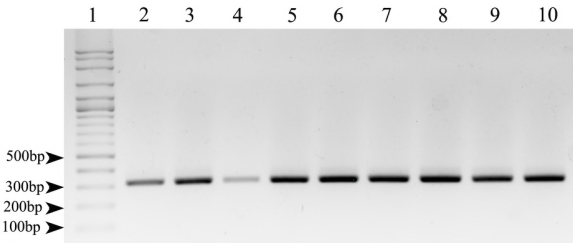

Fig. 1 The agarose gel electrophoresis of *pta* PCR products. Lane 1, 100-bp DNA ladder; lanes 2–10, *S. intermedius* group isolate number 1–9, respectively.

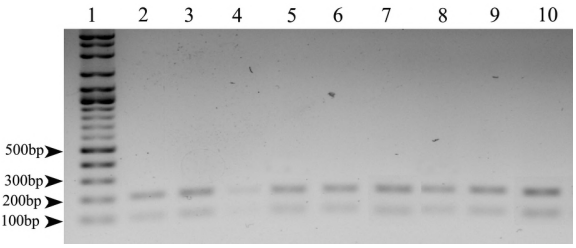

Fig. 2 The agarose gel electrophoresis of *Mbo*I restriction digested of *pta* PCR products. Lane 1, 100-bp DNA ladder; lanes 2–10, *S. pseudintermedius* isolate number 1–9, respectively.

were plated onto MHA, and the MPBC was determined from the lowest concentration of EO that showed no growth of bacteria after incubation for 24 h at 37 °C. All tests were performed in triplicate.

Determination of minimum biofilm eradication concentration (MBEC) by MBEC assay using the TSP

The MBECs of 11 EOs were determined by MBEC assay according to the process described above [14, 15]. Briefly, the TSP lids with biofilms on the pegs were removed from the EO challenge plate and submerged into sterile distilled water for 1 min, then submerged into 150 µl/well of recovery medium (MHB) in a flat-bottomed 96-well microtiter plate. The plate and TSP lid were sonicated for 5 min, then the TSP lid was removed. The recovery plate was incubated for 24 h at 37 °C. The OD_{595} of the wells in the recovery plate were measured and adjusted for the OD value in the negative growth control well. The MBEC was defined as the lowest concentration of EO that inhibited growth of bacteria in the recovery plate (OD less than 0.1). All tests were performed in triplicate.

RESULTS AND DISCUSSION

Planktonic inhibitory and bactericidal activity

The total of 9 isolates of *S. pseudintermedius* was identified by PCR-RFLP method. The pre-digest PCR product was 320 bp (Fig. 1), and *Mbo*I digested products were 213 and 107 bp (Fig. 2). The effectiveness of 11 kinds of EO against the planktonic cells of *S. pseudintermedius* isolates is shown in Table 1. Lemongrass EO had the highest inhibitory activity (MPIC = 0.02% v/v) against all tested bacterial isolates. Betel vine and citronella EOs had the same value for both MPIC and MPBC (0.039% v/v), twice that of lemongrass. The MPIC and MPBC values of clove and sweet basil EOs were 4 and 32 times higher than lemongrass (0.078 and 0.625% v/v), respectively. The MPICs of ginger, kaffir lime and plai EOs were 64 times higher than lemongrass (1.25% v/v). Holy basil and turmeric EOs were found to have less activity against the tested organisms with the MPICs 128 times higher than lemongrass (2.5% v/v), and galangal EO had the least inhibitory activity (MPIC > 2.5% v/v). No previous research has investigated the activity of these EOs against *S. pseudintermedius*, but some reports have shown antibacterial effects against other gram-positive bacteria. Aiemsaard et al [16] found that lemongrass had the highest inhibitory effect against *Staphylococcus aureus* and *Streptococcus agalactiae* isolated from cow milk with mastitis when compared to EOs of kaffir lime, holy basil, citronella, betel vine, sweet basil and turmeric. The lemongrass had an MIC value of 0.054% v/v, 4–32 times lower than other EOs. The report of Chamdit et al [17] showed that EOs of lemongrass and clove were

Table 1 MPIC, MPBC and MBEC for planktonic and biofilm forms of *S. pseudintermedius* isolates ($n = 9$).

Essential oil	MPIC (% v/v)	MPBC (% v/v)	MBEC (% v/v)
Betel vine	0.039	0.039	0.156
Citronella	0.039	0.039	0.156
Clove	0.078	0.078	0.156
Galanga	>2.500	>2.500	>2.500
Ginger	1.250	2.500	2.500
Holy basil	2.500	>2.500	>2.500
Kaffir lime	1.250	2.500	>2.500
Lemongrass	0.020	0.039	0.078
Plai	1.250	2.500	>2.500
Sweet basil	0.625	0.625	1.250
Turmeric	2.500	2.500	>2.500

Values represent the MPICs, MPBCs and MBECs collected from triplicate experiments.

highly effective in inhibiting planktonic *S. aureus* with MICs of 0.125–0.5 and 2–5% v/v, respectively. These findings are in accordance with our studies.

The different antibacterial effects of each EO depend on its chemical constituents [18]. EOs are hydrophobic, which enables them to destroy the lipid-containing membranes of bacterial cells such as cell membranes and mitochondrial membranes [19]. The major constituents of lemongrass are E-citral (geranial, 40–45%) and Z-citral (neral, 24–33%) [20–22]. In addition, citral is the main component of many other EOs; citronella contains 35.7% E-citral, 22.7% trans-citral and 14.2% cis-citral [23] and sweet basil contains 18.6% E-citral and 15.1% Z-citral [24]. Citral acts by reducing the concentration of the intracellular energy-carrying molecule-adenosine triphosphate (ATP), reducing the intracellular pH and inducing cell membrane hyperpolarization, resulting in bacterial cell membranes and cell structures losing their function [20, 25]. Citronellal, which is the main component of citronella EO (4.6–31%) [20, 23], and kaffir lime EO (15.66%) [26] and geraniol, which is the main component of citronella EO (19.5–35.7%) [20, 23], have the effect of breaking down and agglutinating bacterial cells and destroying the cell membrane to cause loss of structure or function [27]. Eugenol, which is the main component of EOs of betel vine (4.97–28%) [20, 28], clove (87%) [29] and sweet basil (25.3–51.5%) [30], and terpineol, which is the main component of galanga EO (8.95%) [31] and plai EO (21.85–29.96%) [20, 32], have similar effects to citronellal and geraniol [27].

Biofilm eradication activity

The minimal bactericidal eradication concentrations (MBECs) of 11 EOs against biofilms of *S. pseudintermedius* isolates are shown in Table 1. All tested EOs showed a higher concentration of EO was required against bacteria in biofilm than against planktonic cells. Among the tested EOs, lemongrass possesed the highest biofilm eradication effect; the MBEC (0.078% v/v) was 4 times higher than MPIC. The EOs of betel vine and citronella had the second most inhibitory effect; the MBEC (0.156% v/v) was 4 times higher than MPIC. The MBEC of clove EO (0.156% v/v) was 2 times higher than MPIC, which was the same as MBEC of sweet basil and ginger (1.25 and 2.5% v/v, respectively). For the galanga, holy basil, kaffir lime, plai and turmeric EOs, the MBECs were over the maximum tested concentration (> 2.5% v/v). The results of this study correspond to several studies, which have reported that the concentration of antimicrobial agents required to inhibit biofilms is 2–1000 times more than that required to inhibit planktonic cells [33]. Song et al [34] reported that biofilms of *S. pseudintermedius* isolated from dogs showed decreased susceptibility to manuka EO (*Leptospermum scoparium*). Manuka EO concentrations of 7.8–800 times the MIC (0.1% w/v) inhibited formation of biofilms by 76.2%. The study of Ferran et al [35] showed the inhibitory effect of antibiotics such as marbofloxacin (0.00005% w/v), clindamycin (0.001% w/v), doxycycline (0.001% w/v), amoxicillin (0.002% w/v) and cefalexin (0.005% w/v) against *S. pseudintermedius* biofilms was 1.2–1.4 times lower than that against planktonic cells.

The effect of these 11 tested EOs on the formation of *S. pseudintermedius* biofilms has not been reported. However, the anti-biofilm activity of some of these EOs against other animal pathogens has been studied. Chamdit et al [17] found that the concentration of clove and lemongrass EOs required to eradicate *S. aureus* biofilms was 2–4 times more than that for the planktonic cells. Aiemsaard et al [20] studied the ability of lemongrass EO and its chemical constituents to inhibit biofilm formation by *S. aureus* isolated from cow milk with mastitis. They found that a sub-concentration of the lemongrass EO MIC (0.025% v/v) could inhibit biofilm formation by 44.9–83.4% while citral concentration of 2.5–5 times MIC and geraniol concentration of 5 times MIC could inhibit biofilm formation by 37.6–77.2%.

Moreover, Taweechaisupapong et al [36] studied the ability of lemongrass and citral EOs to inhibit biofilm formation by *Candida albicans* and *C. krusei*. The results showed that lemongrass concentration of 4–8 times MIC (0.2–0.4% v/v) inhibited biofilm formation of both tested yeasts by more than 80% while citral concentration of 0.5 times MIC (0.025% v/v) inhibited biofilm formation by 61–90%. Many of the major constituents of EOs can interfere with the biofilm formation process and eradicate bacterial cells in the biofilms. Eugenol, carvone, carveol, thymol and cavacrol have been shown to prevent adhesion of bacterial cells to material surfaces and also to destroy bacterial cells and adhesion between bacterial cells, resulting in a decrease in the biomass of biofilms [37, 38]. In the process of bacterial biofilm formation, various genes are expressed, which are important for the formation of biofilms and related to antimicrobial resistance, resulting in bacterial biofilms being more resistant to various antimicro-

bial substances than planktonic cells [39]. Eugenol acts to inhibit the expression of the intercellular adhesion gene (icaD), *Staphylococcus* enterotoxin A gene (seA) and staphylococcal accessory regulator A gene (sarA), which are important in biofilm formation by staphylococcal bacteria [40].

In conclusion, the *in vitro* anti-biofilm activity of lemongrass EO against clinical *S. pseudintermedius* isolates shows the potential of lemongrass EO as a therapeutic antimicrobial agent for controlling superficial pyoderma in dogs. Further study is required to develop a suitable formulation and to determine the *in vivo* efficacy in experimental animals.

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