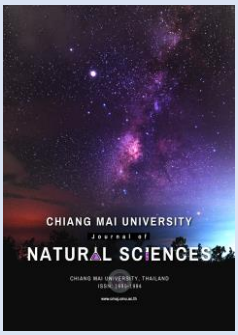

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Research article

The Effect of Some Essential Oils Against Subclinical Mastitis Bacteria Isolated from Dairy Goats

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Abstract The bacteria causing mastitis in goats reduce the quality of milk products resulting in economic losses and the use of antiseptic agents leave residues in the milk. Therefore, this study investigated the antibacterial activity of lemongrass, kaffir lime and holy basil essential oils against subclinical mastitis bacterial isolates from dairy goats. A total of 47 bacteria were isolated, consisting of 40 staphylococci (34 coagulase-negative staphylococci (CNS), 5 *S. aureus* and 1 *S. epidermidis*), 4 *α-Streptococcus* spp., 2 *Bacillus* spp. and 1 *Alcaligenes faecalis.* The main components of essential oil of lemongrass were geranial, neral, myrcene and geraniol (45.32-4.12%), kaffir lime consisted of β-pinene, DL-limonene, terpinene-4-ol and α-terpineol (25.58-12.24%) and holy basil consisted of methyl eugenol, caryophyllene and eugenol (52.43-8.68%). Lemongrass essential oil showed the highest antibacterial activity against CNS and *S. aureus* with average inhibition zone diameters of 25.00±1.75 and 31±6.61 mm, respectively, and minimum inhibitory concentration for 90% of tested samples (MIC_{90}) and minimum bactericidal concentration for 90% of tested samples (MBC_{90}) values at least eight fold less than kaffir lime and holy basil essential oils. The time-kill assay demonstrated that lemongrass essential oil at 5-10 times MIC_{90} reduced the survival of *S. aureus* by 99.9 and 99.99% respectively within 30 min, a greater reduction than seen for 0.54% w/v iodine solution. The results of this study show that lemongrass essential oil has the potential to be developed as an antibacterial agent or teat dip formula for control of subclinical mastitis in goats.

Keywords: Antibacterial activity, Essential oil, Mastitis

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INTRODUCTION

Goat milk and goat milk products appeal to consumers because they are highly nutritious and have health benefits. However, the problem of subclinical mastitis due to bacterial infection in dairy goats, which causes microbial contamination of the milk resulting in reduced quality of milk products and economic loss (Conteras et al., 2003; Ali et al., 2010; Sharma et al., 2010), is increasing (McDougall et al., 2002; Persson and Olofsson, 2011; Islam et al., 2012). Moreover, consumption of pathogens in contaminated milk can cause disease in humans (Wakwoya et al., 2006; Gamboa, 2009). The main pathogens that cause subclinical mastitis in dairy goats are coagulase-negative staphylococci (CNS) and *Staphylococcus aureus* (Taufik et al., 2008; Aydin et al., 2009; Virdis et al., 2010; Mishra et al., 2018).

Several antibiotics are currently used to treat mastitis in goats, but their use can increase the incidence of drug-resistant bacteria and also cause antibiotic residue problems in milk products (Adwan, 2006; Virdis et al., 2010). Therefore, the prevention of external infection to the teat by using pre- and post-milking teat dipping antiseptic is very important (Singh et al., 2018). However, the antiseptics commonly used are synthetic chemicals, which can leave residues in milk that affect the health of consumers and the quality of milk products (Dahl et al., 2003; Hebert et al., 2003). Because of these problems, antimicrobial agents from herbs are interesting alternatives to synthetic antimicrobial drugs or antiseptics, especially when used for pre- or post-milking teat dipping, as they can be very safe when used in suitable forms and concentrations.

Many natural substances have been shown to have antibacterial activity such as propolis, tannin and essential oils (Min et al., 2008; Santos Neto et al., 2009). Essential oils contain various chemical constituents such as monoterpenes, sesquiterpenes, diterpenes, and other aromatic or aliphatic compounds (Dhifi et al., 2016) that have therapeutic properties such as antibacterial, antifungal, anti-inflammatory and antioxidation activities. Also, they are safe to use, easy to prepare, inexpensive, and there has been no report of resistance among mastitis pathogens (Burt et al., 2004). There is one report showing that the essential oils of lemongrass, kaffir lime and holy basil are effective against the bacteria that cause mastitis in dairy cows (Aiemsaard et al., 2010), but there is no previous report about their efficacy in goats. Therefore, we studied the antibacterial activity of essential oils from three Thai herbs namely: lemongrass *(Cymbopogon citratus)*, kaffir lime *(Citrus hystrix),* and holy basil *(Ocimum sanctum*), against subclinical mastitis bacteria isolated from dairy goat.

MATERIALS AND METHODS

Bacterial isolation and culture conditions

Raw milk samples were collected from dairy goats that had not received any antibiotics at least 14 days before collection and had been diagnosed with subclinical mastitis by a farm veterinarian. Samples were collected from affected quarters of the udder. The udder was cleaned with 50 ppm chlorine solution and the nipples were wiped with rubbing alcohol. The first 10-15 ml of milk was discarded before 3-5 ml milk samples were collected in sterile test tubes and stored at 4°C in a temperature-controlled container. Upon reaching the laboratory, the milk samples were cultured on blood agar and MacConkey agar (Becton Dickinson, USA) and incubated at 37°C for 24-48 h (Markey et al., 2013; Mohammed 2014). The bacteria growing on culture media were identified by gram staining and routine biochemical testing at the Veterinary Diagnostic Laboratory, Animal Hospital Khon Kaen University, Thailand. The biochemistry tests including catalase test, oxidase test, motility test, coagulase test, urease tests, oxidation-fermentation test, carbohydrate fermentation test, aesculin hydrolysis test, indole test, methyl red test, citrate test and Voges–Proskauer test.

Before testing, the bacteria were transferred to Mueller Hinton broth (MHB, Becton Dickinson, France) and incubated at 37°C for 24 h. The optical density (OD) at 600 nm of bacterial suspensions was measured by Vis-spectrophotometer (Genesys 10 VIS,

Thermo Scientific, USA) and the bacterial concentration was adjusted to 10^6 CFU/ml, which was confirmed by viable counts (Usman et al., 2013).

Determination of chemical composition by gas chromatography-mass spectrometry (GC-MS) analysis

The essential oils of lemongrass, kaffir lime and holy basil were purchased from Thai-China Flavours and Fragrances Industry Co., Ltd., Thailand. The GC-MS analysis was performed according to the method previously described by Aiemsaard et al. (2010) and Amelia et al. (2017) with modifications for the Agilent CN10402086 gas chromatograph interfaced with the Agilent US35120381 mass spectrometer. Each essential oil was diluted with hexane (Merck, Germany) before injection into the GC-MS using a DB-5ms fused silica capillary column (30 m x 25 mm, film thickness 0.25 µm). The carrier gas was helium with a flow rate of 1 ml/min. The start temperature was 70°C for 5 min. The temperature was increased at a rate of 3°C/min to 120°C and held for 2 min, then from 120 to 270°C at a rate of 5°C/min and held for 17 min. The chemical constituents of the essential oils were identified by comparing with reference retention times.

Determination of antibacterial activity by disc diffusion method

The inhibition zones for lemongrass, kaffir lime and holy basil essential oils against CNS (n = 34) and *S. aureus* (n = 5) subclinical mastitis isolates were determined by the disc diffusion method according to the Clinical and Laboratory Standard Institute (CLSI) recommendations (2013) with modifications. Briefly, bacterial suspensions at a concentration of 10^6 CFU/ml were inoculated onto Mueller Hinton agar plates (MHA, Becton Dickinson, France) by streak plate technique. Then, sterile Whatman filter paper no. 1 discs (6 mm diameter) with 10% v/v essential oil diluted in dimethyl sulfoxide (DMSO, V.S. Chem House, Thailand) (20 µl/disc) were placed on the inoculated surface. The plates were incubated at 37°C for 24 h. The inhibition zones were measured using a ruler. All tests were performed in triplicate. All data are reported as the mean ± standard deviation.

Determination of antibacterial activity by broth microdilution method

The MICs and MBCs of lemongrass, kaffir lime and holy basil essential oils against CNS (n = 34) and *S. aureus* (n = 5) subclinical mastitis isolates were determined by the broth microdilution method according to CLSI recommendations (2008) with modifications. Briefly, serial dilutions of tested substances (2.5-0.00488% v/v) were prepared with MHB in 96-well flat-bottomed microtiter plates (Costar®, Corning Incorporated). Then, a 10^6 CFU/ml bacterial suspension was added into each well. Wells containing bacteria cultured without tested agents served as positive growth control wells and wells containing tested agents without bacteria served as negative growth control wells. The plates were incubated at 37°C for 24 h. All tests were performed in triplicate. The MIC was defined as the lowest concentration of essential oil that prevented visible growth after 24 h of incubation. Ten microliter samples from the wells with no visible growth were inoculated onto MHA and incubated at 37°C for 24 h. The MBC was determined from the lowest concentration of essential oil that showed no growth on MHA. The MIC_{90} and MBC_{90} were defined as the concentrations of essential oils that inhibited growth or killed bacteria in 90% of tested isolates, respectively.

Determination of time-kill kinetics of lemongrass essential oil against a *S. aureus* subclinical mastitis isolate

The essential oil which showed the highest antibacterial activity against both CNS and *S. aureus* in the broth microdilution test was determined the time-kill kinetics against a *S. aureus* subclinical mastitis isolate by time-kill test. *S. aureus* was selected to test in this study since it is the major identified pathogen and has virulent more than others. The study was performed according to the method of Chamdit and Siripermpool (2012) with modifications. Briefly, a 100 µl aliquot of bacterial suspension (10^6 CFU/ml) was added to tubes containing the essential oil at final concentrations of 1, 5 and 10 times MIC_{90}, then mixed on a vortex mixer for 1 min. After 0.5, 1, 2, 3, 4, 5 and 6 h of

incubation at 37°C, the mixtures were diluted 10-fold with 0.89% sodium chloride solution to stop the antimicrobial effects of the essential oil. A 100 µl aliquot of the 10^{-1} to 10^{-4} dilutions of each tube was spread onto MHA plates, which were incubated at 37°C for 24 h. The colonies of visible growth of tested microorganisms were counted and recorded. A 0.54% w/v iodine solution (Masodine®, Evans Vanodine International, UK), which is a widely used pre- and post-milking teat disinfectant, was used for the internal standard antibacterial control. The experiment was performed in triplicate. All data are reported as the mean ± standard deviation.

Statistical analysis

Each experiment was performed in triplicate. One-way ANOVA was used to compare means ($\alpha = 0.05$). All analyses were performed using SPSS for windows version 19.0 (SPSS Inc., USA), KKU license.

RESULTS

Dairy goat subclinical mastitis bacterial isolates

A total of 47 bacteria were isolated from 21 affected quarters of dairy goat udders with subclinical mastitis (Table 1). Staphylococci were the most commonly isolated bacteria with 40 isolates (85.10%), followed by *α-Streptococcus* spp. (4 isolates, 8.51%), *Bacillus* spp. (2 isolates, 4.26%) and *Alcaligenes faecalis* (1 isolate, 2.13%). The staphylococci bacteria could be further divided into 34 CNS (72.34%) consisting of 26 unidentified *Staphylococcus* spp., 5 *S. xylosus*, and 1 each of *S. cohini, S. capitis* and *S. caprae*, and 6 coagulase-positive staphylococci consisting of 5 *S. aureus* (10.63%) and 1 *S. intermedius*.

Table 1. The bacteria isolated from dairy goats with subclinical mastitis.

Bacterial species	Number of isolates
Staphylococci	
Coagulase-positive staphylococci	
S. aureus	5 (10.63%)
S. intermedius	1 (2.13%)
Coagulase-negative staphylococci	
Staphylococcus spp.	26 (55.32%)
S. xylosus	5 (10.63%)
S. cohini	1 (2.13%)
S. capitis	1 (2.13%)
S. caprae	1 (2.13%)
α-Streptococcus spp.	4 (8.51%)
Bacillus spp.	2 (4.26%)
Alcaligenes faecalis	1 (2.13%)
Total	**47 (100%)**

Essential oils chemical composition by gas chromatography-mass spectrometry (GC-MS) analysis

Table 2 shows the results of the GC-MS analysis of lemongrass, kaffir lime and holy basil essential oils. The results demonstrated that each of the tested essential oils did not share any main constituents. Lemongrass essential oil consisted of 4 main constituents: geranial (45.32%), neral (35.43%), myrcene (7.88%) and geraniol (4.12%). Kaffir lime essential oil consisted of 4 different main constituents: α-pinene (25.58%), DL-limonene (19.38%), terpinene-4-ol (14.12%) and β-terpineol (12.24%), and holy basil essential oil consisted of another 3 main constituents: methyl eugenol, caryophyllene and eugenol at concentrations of 52.43, 29.29 and 8.68%, respectively.

Table 2. The chemical composition of lemongrass, kaffir lime and holy basil essential oils.

Essential oil	Retention time (min)	Component	Area (%)
Lemongrass	7.15	Myrcene	7.88
	18.11	β-citral (neral)	35.43
	18.65	Geraniol	4.12
	19.48	α-citral (geranial)	45.32
Kaffir lime	6.62	β -pinene	25.58
	8.74	DL-limonene	19.38
	15.43	Terpinene-4-ol	14.12
	16.11	α-terpineol	12.24
Holy basil	23.23	Eugenol	8.68
	25.73	Methyl eugenol	52.43
	26.37	Caryophyllene	29.29

Antibacterial activity of essential oils by disc diffusion method

The study of the antibacterial activity of tested essential oils against 34 isolates of CNS and 5 isolates of *S. aureus* by disc diffusion method showed that 10% v/v lemongrass, kaffir lime and holy basil essential oils produced inhibition zones with average diameters ranging from 7 to 31 mm (Table 3). The DMSO diluent control did not show any antibacterial activity (inhibition zone ≥ 6 mm). Kaffir lime and holy basil essential oils had small average inhibition zone diameters for CNS (7.00 ± 0.83 and 9.00 ± 1.35 mm, respectively) and *S. aureus* (9.00 ± 0.83 and 9.00 ± 5.26 mm, respectively). The lemongrass essential oil had much larger average inhibition zone diameters than the kaffir lime and holy basil essential oils, 25.00 ± 1.75 mm for CNS and 31.00±6.61 mm for *S. aureus* (P <0.05).

Table 3. The average inhibition zone diameters of essential oils against staphylococcal bacteria isolated from dairy goats with subclinical mastitis.

Essential oil(Concentration of 10% v/v)	Inhibition zone (mm)	
	CNS (n = 34)	*S. aureus* (n = 5)
Lemongrass	25.00 ± 1.75[a]	31.00 ± 6.61[a]
Kaffir lime	7.00 ± 0.83[b]	9.00 ± 0.83[b]
Holy basil	9.00 ± 1.35[c]	9.00 ± 5.26[b]

Note: Values represent the means of triplicate experiments ± SD. Superscript letters within a column indicate statistically significant differences between the means (P <0.05).

Antibacterial activity of essential oils by broth microdilution method

The results of the broth microdilution tests are shown in Table 4. Lemongrass essential oil had the highest antibacterial effect against both CNS and *S. aureus*, the MIC_{90} and MBC_{90} were 0.30 and 0.15-0.30% v/v, respectively. The MIC_{90} values of kaffir lime and holy basil essential oil were 8-16 times higher than lemongrass essential oil and had the same MIC_{90} or MBC_{90} (2.50 and > 2.50% v/v, respectively).

Table 4. Minimum inhibitory concentration for 90% of tested samples (MIC_{90}) and minimum bactericidal concentration for 90% of tested samples (MBC_{90}) of lemongrass, kaffir lime and holy basil essential oils against staphylococcal bacteria isolated from dairy goats with subclinical mastitis.

Essential oil	CNS (n = 34)		*S. aureus* (n = 5)	
	MIC_{90} (% v/v)	MBC_{90} (% v/v)	MIC_{90} (% v/v)	MBC_{90} (% v/v)
Lemongrass	0.30	0.30	0.15	0.30
Kaffir lime	2.50	> 2.50	2.50	> 2.50
Holy basil	2.50	> 2.50	2.50	> 2.50

Note: Values represent the mode of experiments of 34 or 5 isolates.

Time-kill kinetics of lemongrass essential oil against a *S. aureus* subclinical mastitis isolate

Lemongrass essential oil was selected for the time-kill study as it had the highest antibacterial activity in disc diffusion and broth microdilution tests. The time-kill kinetics of lemongrass essential oil against one *S. aureus* subclinical mastitis isolate are presented in Figure 1. The lemongrass essential oil concentration of 10 times MIC_{90} (1.5% v/v) had the eradication effect higher than 5 times MIC_{90} (0.75% v/v) and 1 time MIC_{90} (0.15% v/v) lemongrass essential oil and 0.54% w/v iodine solution. It was eradicated the tested bacteria about 99.99% (4-log reduction) within 30 min and more than 99.9999% (6-log reduction) within 1 h. While the lemongrass essential oil concentration of 5 times MIC_{90} was given 99.9% (3-log reduction), 99.999% (5-log reduction) and more than 99.9999% reduction of the inoculum at 30 min, 1 h and 2 h, respectively. For the 1 times MIC_{90} lemongrass essential oil, it had eradication effect similar to 0.54% w/v iodine solution, which reduced the number of viable bacteria by 90% (1-log reduction), 99.9%, 99.999% and more than 99.9999% at 30 min, 1 h, 2 h and 3 h, respectively.

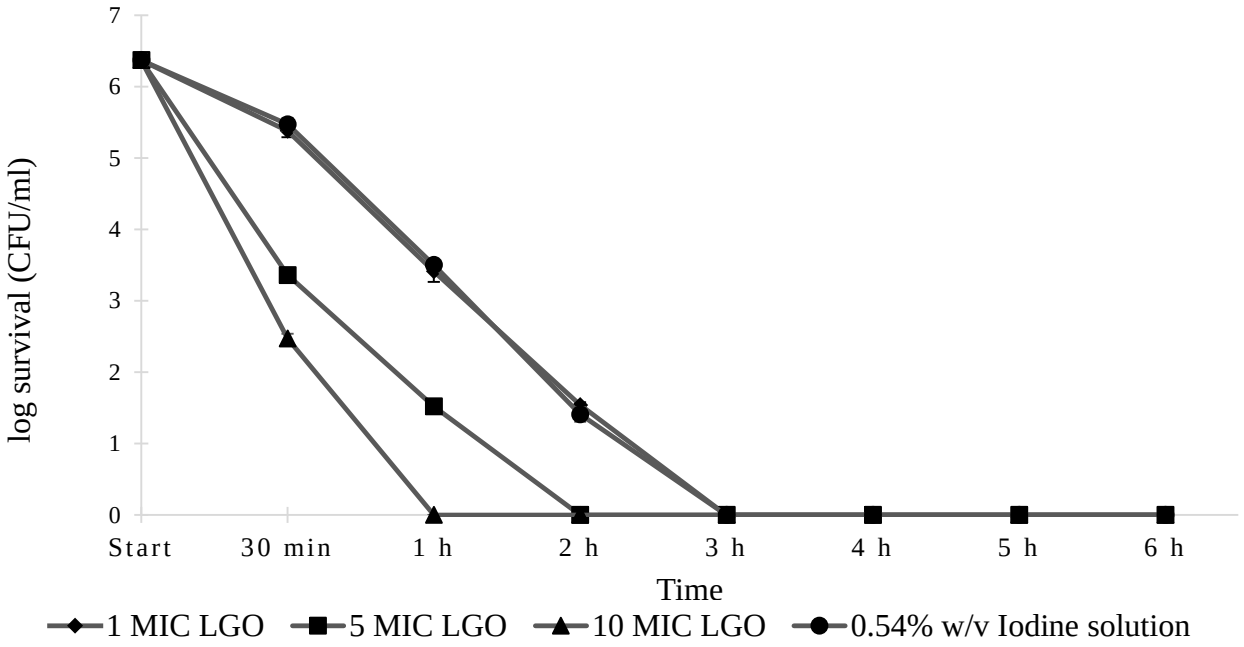

Figure 1. Time-kill assay of lemongrass essential oil (LGO) and iodine solution against *S. aureus* isolate. Values represent the means of triplicate experiments with error bars (SD). The MIC refer to MIC_{90}; 1 MIC = 0.15% v/v, 5 MICs = 0.75% v/v, 10 MICs = 1.5% v/v.

DISCUSSION

The bacteria most commonly collected from subclinical mastitis in dairy goats in this study were CNS (72.34%) and *S. aureus* (10.63%). This result is consistent with previous studies that have reported CNS and *S. aureus* were found in 50-90% and 5-37% of dairy goats with subclinical mastitis, respectively (da Silva et al., 2004, Taufik et al., 2008, Aydin et al., 2009, Santos Neto et al., 2009, and Virdis et al., 2010).

The GC-MS results showing the main chemical constituents of the three tested essential oils are also in agreement with previous studies. Citral has previously been reported to be the main constituent of lemongrass essential oil (75-82%) (Masamba et al., 2003; Aiemsaard et al., 2010), while the main constituents of kaffir lime are limonene (20-40%), terpinene-4-ol (10-14%) and α-terpineol (about 13%) (Aiemsaard et al., 2010; Srisukh et al., 2012). Aiemsaard et al. (2010) reported that methyl eugenol, caryophyllene and eugenol were the main constituents of holy basil essential oil (38, 26 and 16%, respectively), which was different to the studies of Kumar et al. (2010) and Kumari and Agrawal (2011) who found only two main constituents; eugenol 61-75% and caryophyllene 11-12%. The slight differences in composition between studies are likely to be due to differences in the source of the herbs, the harvest season and the parts of the plant used. Moreover, the conditions and method of extraction can influence the proportions of each constituent (Nurdjannah and Bermawie, 2012; Hemalatha et al., 2016).

The disc diffusion and broth microdilution tests demonstrated that lemongrass essential oil was a better antibacterial agent against the tested bacteria compared to kaffir lime and holy basil essential oil. Although no previous study has reported activity of essential oils against bacteria isolated from mastitis in dairy goats, some studies have reported the antibacterial effects of the essential oil against *S. aureus* isolated from mastitis in dairy cows. The report of Choi et al. (2012) showed that 10% lemongrass essential oil gave a 24 mm inhibition zone in a disc diffusion assay, which was 3 times higher than the same concentration of oregano essential oil (*Oreganum vulgare*). Also, Aiemsaard et al. (2011) reported MIC and MBC values for lemongrass essential oil against *S. aureus* isolated from cows with subclinical mastitis were 5.4% v/v, much higher than the MIC/MBC values seen in this study, which may be due to differences in bacterial strains, test conditions or source of essential oil. The results in the current study also showed that *S. aureus* was slightly more susceptible to lemongrass essential oil than CNS, which is consistent with multiple other reports of increased resistance to antimicrobial agents in CNS (da Silva et al., 2004, Aydin et al., 2009, Santos Neto et al., 2009, and Virdis et al., 2010). Moreover, Okmen and Turkey (2013) studied the effect of methanolic extract of *Elaegnus angustifolia* against CNS and *S. aureus* isolated from mastitis in cows by disc diffusion and broth microdilution methods, and showed that CNS was more resistant than *S. aureus*, similar to the study of Gulten (2013) with ethanolic extract of *Anthemis chia*. Researchers have proposed that this decreased susceptibility in CNS may be due to the use of antibiotics in dairy farms causing resistance, especially in CNS bacteria, which is common skin flora and opportunistic pathogen.

Lemongrass essential oil at concentrations of 0.75-1.5% v/v reduced survival of bacteria in the time-kill assay by 99.90-99.99% within 30 min, a higher reduction than 0.54% w/v iodine solution. Also, when the concentration of essential oil and contact time were increased, the antibacterial effect also increased. This is consistent with the report of Chamdit and Siripermpool (2012), who studied the effect of lemongrass and clove essential oils against *S. aureus* ATCC43300, and the report of Christensen and Anderson et al. (2017), who studied the effect of lemongrass essential oil and citral against *S. aureus* USA300.

The antibacterial activities of lemongrass essential oil are presumably due to the main components citral and geraniol. These two compounds affect the integrity of the bacterial cell wall leading to loss of transmembrane balance and cell death (Aiemsaard et al., 2011; Shi et al., 2016). In addition, citral reduces intracellular pH value and adenosine triphosphate (ATP) levels by increasing ATP hydrolysis and outflow of ATP hrough the injured cell membrane, which affects biological processes of the cells (Shi et al., 2016). Moreover, citral also reduces expression of the extracellular fibrinogen-

binding protein C (*efbC*) gene, an important virulence factor of *S. aureus* (Christensen and Anderson, 2017).

CONCLUSION

Lemongrass, kaffir lime and holy basil essential oils had different antibacterial effects against CNS and *S. aureus* isolated from dairy goats with subclinical mastitis. Lemongrass essential oil showed the highest antibacterial activity and has potential to be developed as an antibacterial agent or teat dip formula to control subclinical mastitis in goats. Further studies are required to develop a suitable formulation and to determine the *in vivo* efficacy in experimental animals.

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