Antibacterial Activity of Essential Oil Extracted from *Citrus maxima* (Pomelo) against Methicillin-resistant *Staphylococcus aureus*

Apichai Sreepian, Ketsani Pasumee, Thitaree Patjakreng, Thanut Kuruwanaporn, Pimchanida Chinaau, Pruethipong Sampanporn, Kanyanan Kritsiriwuthinan and Preeyaporn M. Sreepian*

Faculty of Medical Technology, Rangsit University, Pathum Thani, Thailand
*Corresponding author: E-mail address: preeyaporn.m@rsu.ac.th

Abstract

Citrus maxima Merr. or Pomelo is a plant with potential antibacterial activity against a broad spectrum of bacterial pathogens that has the key active compound as limonene, which is a major constituent in the fruit peel. This study investigated the antibacterial activity of *C. maxima* essential oil (CMEO) against methicillin-resistant *Staphylococcus aureus* (MRSA). The antibacterial activity was determined by the agar disk diffusion and broth macro dilution methods against *S. aureus* ATCC 25923 (methicillin-susceptible *S. aureus*; MSSA), *S. aureus* ATCC 43300 (MRSA) and ten clinical isolates of MRSA. The bacteria-killing rate of 1×MIC CMEO at different exposure times was also evaluated by the time-kill assay. The inhibition zones of CMEO against *S. aureus* ATCC 25923, *S. aureus* ATCC 43300 and MRSA isolates were 10.0, 9.0 and 9.9 mm, respectively, but no zone was observed for DMSO control, thus indicating a weak activity of CMEO. The inhibition zone of CMEO against MRSA isolates was significantly different from that of limonene ($p < 0.05$), indicating a synergistic effect of limonene with other minor compounds in CMEO. The MIC value of CMEO to *S. aureus* ATCC 43300 was lower than that of *S. aureus* ATCC 25923 (4.0 vs 10.7 mg/mL; $p < 0.05$). The MIC value of CMEO against MRSA isolates was 8.1±4.8 mg/mL with MIC indexes less than 4. Besides, CMEO completely killed both MSSA and MRSA within 4 and 8 hours, respectively. These findings demonstrated the bactericidal effect of CMEO against MRSA; thus, it has the potential to be developed as a novel antibacterial agent.

Keywords: *Essential oil, Pomelo, Citrus maxima, Limonene, Antibacterial activity, MRSA*

1. Introduction

Staphylococcus aureus is one of the important human pathogens. The clinical manifestations are varied from mild skin and soft-tissue infections to life-threatening endocarditis, chronic osteomyelitis, pneumonia, or bacteremia (McGuinness, Malachowa, & DeLeo, 2017). Community-acquired and hospital-acquired infections of methicillin-resistant *Staphylococcus aureus* (MRSA) are among the major public health problem worldwide. They affect an increase in the morbidity and mortality rates which lead to a longer staying in the hospital and increasing in treatment expenses. In Thailand, the prevalence rate of MRSA was reported as high as 28% in 2006 and gradually decreased to 9.1% in 2019 (NARST, 2019). However, the resistance of MRSA to vancomycin, which is a current antibiotic drug for MRSA treatment, has been observed at the rates of 0.3-0.6% since 2016 (NARST, 2019).

The use of plant extracts as antibacterial agents has a great benefit in that they have lesser toxicity and adverse side effects compared to existing antimicrobial agents. In the past decade, previous studies demonstrated the broad antibacterial activities of essential oils extracted from plants in the family Rutaceae including *Citrus limon* L. (lemon) (Frassinetti, Caltavuturo, Cini, & Della Croce, 2011), *C. aurantium* (sour orange) (Frassinetti, Caltavuturo, Cini, & Della Croce, 2011; Torres-Alvarez et al., 2017), *C. reticulata* (mandarin orange) (Frassinetti, Caltavuturo, Cini, & Della Croce, 2011; Torres-Alvarez et al., 2017), *C. sinensis* (sweet orange) (Frassinetti, Caltavuturo, Cini, & Della Croce, 2011; Torres-Alvarez et al., 2017), *C. aurantifolia* (lime) (Lemes et al., 2018), *C. hystrix* (kaffir lime) (Wongsariya, Phanthong, Bunyapraphatsara, Srisukh, & Chomnawang,2014; Borusiewicz et al., 2017; Sreepian, Sreepian, Chanthong, Mingkhwancheep, & Prathit, 2019) and *C. maxima* (pomelo) (Tao & Liu, 2012; Prusty & Patro, 2014; Ou, Liu, Sun, & Chan, 2015; Saeb, Amin, Gooybari, & Aghel, 2016; Chen, Lin, Lin, & Jen, 2018). *Citrus maxima* (Burm.) Merr. or *C. grandis* (L.) Osbeck (Common names: Pomelo or Shaddock) is a

Published online: Copyright © 2016-2020 Rangsit University

tropical fruit that is widely cultivated in central, northern and southern parts of Thailand. Previous studies in different regions reported that the main constituent in *C. maxima* essential oil (CMEO) was limonene with various amounts ranging from 69.4 to 95.7% (Minh Tu, Thanh, Une, Ukeda, & Sawamura, 2002; Chen et al., 2018). CMEO extracted from the fruit peels contained a higher amount of limonene than that in flowers, but limonene was not found in the leaf-derived CMEO (Thavanapong et al., 2010; Tsai et al., 2017). The variation of chemical compositions of CMEO was mainly dependent on harvesting seasons, agro-climatic condition, stage of maturity, adaptive metabolism of plants and distillation conditions (Burt, 2004; Anwar, Ali, Hussain, & Shahid, 2009). CMEO from different countries including China, Taiwan, India, as well as Thailand, exhibited higher activity against Gram-positive bacteria, especially to *S. aureus*, than Gram-negative bacteria (Tao & Liu, 2012; Prusty & Patro, 2014; Ou et al., 2015; Chen et al., 2018). The production of antibacterial agents from the plant waste materials, such as the fruit peel, would commercially increase their values and reduce environmental waste. Up to now, there is no data on the antibacterial activity of CMEO against MRSA. Consequently, it is necessary to obtain preliminary data on the antibacterial activity in order to develop CMEO as antibacterial agents to fight MRSA in the future.

2. Objectives

To determine *in vitro* antibacterial activity of CMEO against reference strains and clinical isolates of MRSA.

3. Materials and Methods

3.1 Plant material

The fresh fruits of *Citrus maxima* (Burm.) Merr. cv. Khao Yai were collected from Chiang Rai Province, located in the northernmost region of Thailand, in November 2018. The peel was separated and then processed through steam distillation. The extracted *C. maxima* essential oil (CMEO) was stored at 4°C and protected from light until used. A stock solution of CMEO was prepared at a concentration of 400 mg/mL (w/v) in dimethyl sulphoxide (DMSO) before use.

3.2 Bacterial organisms

Bacterial strains used in this study included two strains of American Type Culture Collection (ATCC); *Staphylococcus aureus* ATCC 25923 (methicillin-susceptible *S. aureus*; MSSA), *S. aureus* ATCC 43300 (methicillin-resistant *S. aureus*; MRSA) and ten clinical isolates of MRSA. *S. aureus* ATCC 25923 and 10 clinical isolates of MRSA originated from the collection from Microbiology Laboratory, Faculty of Medical Technology, Rangsit University, Thailand; and *S. aureus* ATCC 43300 was obtained from the Faculty of Science, Rangsit University, Thailand. The bacteria were maintained at -20°C in Trypticase soy broth (TSB, Difco; Becton, Dickinson and Company) with 10% glycerol and cultivated on blood agar at 37°C for 18-24 hours.

3.3 Agar disk diffusion

Agar disk diffusion was performed to evaluate the susceptibility profiles of tested bacteria and to screen the *in vitro* antibacterial activity of CMEO as previously described (CLSI, 2019; Sabulal et al., 2006) with some modifications. *S. aureus* ATCC 25923, *S. aureus* ATCC 43300 and 10 clinical isolates of MRSA were included in the experiments. The turbidity of tested bacteria was adjusted to 0.5 McFarland units using the densitometer (DEN-1; Biosan, England). The bacterial suspension (approximately 1.5 x 10^8 CFU/mL) was spread on Mueller Hinton agar (MHA). The sterilized disk (6 mm) impregnated with 10 µL of CMEO (0.9 g/mL), 10 µL of limonene (97%) (Sigma-Aldrich, MO, USA) and 10 µL of DMSO (2.3% v/v) were placed on the surface of each plate. The plates were incubated at 37°C for 18-24 hours. The inhibition zone diameter (IZD) of CMEO was measured and interpreted using the following criteria: no activity, IZD = 6 mm; weak activity, 6 mm < IZD < 12 mm; moderate activity, 12 mm < IZD < 20 mm; and strong activity, IZD > 20 mm (Lv, Liang, Yuan, & Li, 2011). The susceptibility of 7 selected antibiotics namely; cefoxitin

Published online: Copyright © 2016-2020 Rangsit University

(30 µg), ampicillin (10 µg), amoxicillin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), erythromycin (5 µg) and clindamycin (2 µg) were also examined on MHA (CLSI, 2019).

3.4 Determination of MIC and MBC by broth macro dilution

The minimum inhibitory concentration (MIC) of CMEO was evaluated by broth macro dilution method following the Clinical and Laboratory Standards Institute (CLSI) document M07-A10 (CLSI, 2015) with some modifications. One milliliter of bacterial suspension (approximately 5×10^5 CFU/mL) was inoculated with 1 mL of CMEO; the ranged concentrations from 0.25 to 16 mg/mL. After incubating at 37°C for 18-20 hours, the MIC was determined by the lowest concentration that completely inhibits visible bacterial growth. Consequently, one loop of the MIC suspension that showed visually clear was cultivated on agar plates and incubated at 37°C for 18-24 hours. The minimum bactericidal concentration (MBC) was determined by the lowest concentration that completely inhibits bacterial growth on the agar plate. Each experiment was performed in triplicate. Broth control (CMEO with MHB), bacterial control (bacteria with MHB) and DMSO control (bacteria with 2.3% DMSO) were also included. The MIC index (MBC/MIC ratio) was calculated to classify the type of antimicrobial substances, as previously described by Gatsing et al. (2009).

3.5 Determination of the bacteria-killing rate

The bacteria-killing rate was determined by standard time-kill assay following previous methods by Chimnoi et al., (2018) with some modifications. Tubes containing MHB and CMEO at 1×MIC were inoculated with bacterial suspension (approximately 5×10^5 CFU/mL) and incubated at 37°C for 0, 1, 2, 4, 8, 12 and 24 hours. DMSO control was also used. At the end of each period, 10-fold serial dilutions were prepared with sterile normal saline. Subsequently, 100 µL of the samples were plated onto MHA plates in duplicate. Viable counts were determined after the plates were incubated at 37°C for 24 hours. The percentages of relative viability counts were plotted against exposure time. The percentages of relative viability counts of all samples were calculated using the following formula:

$$\text{Relative viability count (\%)} = \frac{\text{Number of living cells at the exposure time}}{\text{Number of living cells at the initial time}} \times 100$$

3.6 Statistical analysis

Descriptive statistic was performed using the IBM Statistical Package for Social Services (SPSS) version 21.0 (IBM, Armonk, NY). The results were expressed as mean ± standard deviation (SD) of MIC, MBC and IZD of triplicate experiments. The Mann-Whitney U test was used to compare the inhibitory effect between CMEO and limonene as well as that of methicillin-susceptible and methicillin-resistant *S. aureus*. P-value < 0.05 was considered statistically significant.

4. Results and Discussion

The antibiotic susceptibility profile of 7 antibiotics among two reference strains of *S. aureus* and ten clinical isolates of methicillin-resistant *S. aureus* (MRSA) is shown in Table 1. *S. aureus* ATCC 25923, which is a methicillin-susceptible strain, was highly sensitive to both cefoxitin and other antibiotics tested. In comparison, *S. aureus* ATCC 43300 which is a methicillin-resistant strain was resistant to cefoxitin, and some tested antibiotics except to amoxicillin, gentamicin and ciprofloxacin. The IZDs of *S. aureus* ATCC 25923 and *S. aureus* ATCC 43300 to cefoxitin were 36.0 mm and 18.0 mm, respectively. For clinical isolates of MRSA, all of them were resistant to cefoxitin with IZD ranging from 6.0 to 20.0 mm. However, the susceptibility profile to other antibiotics was different among the isolates with a resistant pattern to at least three antibiotics, indicating multidrug resistance was observed. Moreover, all isolates (10/10, 100%) were resistant to ampicillin and erythromycin; 5 isolates (5/10, 50%) were resistant to amoxicillin and ciprofloxacin; and only two isolates (2/10, 20%) were resistant to gentamicin. Among ten isolates, there were two isolates (2/10, 20%); MRSA001 and MRSA002, were found to be resistant to all tested antibiotics except to clindamycin (Table 1). The results demonstrated that all MRSA isolates used in this study were

Published online: Copyright © 2016-2020 Rangsit University

highly multidrug-resistant to currently used antibiotic drugs. These bacterial isolates were further used for the determination of *in vitro* antibacterial activity of *C. maxima* essential oil (CMEO).

Table 1 Antibiotic susceptibility pattern of tested bacteria

Bacteria	Inhibition zone diameter (IZD; mm)[1]						
	FOX[2]	AMP	AMC	CN	CIP	E	DA
S. aureus ATCC 25923	36.0±3.5 (S)	36.7±2.1 (S)	39.0±2.6 (S)	29.3±0.6 (S)	26.3±1.3 (S)	27.3±3.1 (S)	28.7±4.2 (S)
S. aureus ATCC 43300	18.0±0.0 (R)	14.3±1.5 (R)	24.3±2.1 (S)	27.3±2.1 (S)	26.0±1.4 (S)	6.0±0.0 (R)	6.0±0.0 (R)
MRSA001	7.0±1.0 (R)	10.7±0.6 (R)	12.3±0.6 (R)	11.7±2.9 (R)	6.0±0.0 (R)	6.0±0.0 (R)	24.0±1.0 (S)
MRSA002	6.3±0.6 (R)	10.3±0.6 (R)	12.7±1.5 (R)	10.3±0.6 (R)	6.0±0.0 (R)	6.0±0.0 (R)	22.3±0.6 (S)
MRSA003	7.3.±0.6 (R)	11.0.±0.0 (R)	13.0.±1.0 (R)	12.7±0.6 (I)	6.0±0.0 (R)	6.0±0.0 (R)	27.0±1.0 (S)
MRSA004	17.0±1.7 (R)	13.0±2.0 (R)	25.0±3.6 (S)	28.3±2.1 (S)	24.3±0.5 (S)	6.0±0.0 (R)	26.0±1.0 (S)
MRSA005	14.7±0.6 (R)	10.7±0.6 (R)	14.0±1.0 (R)	15.7±4.0 (S)	6.0±0.0 (R)	6.0±0.0 (R)	22.7±0.6 (S)
MRSA006	18.0±2.0 (R)	13.3±1.5 (R)	25.3±1.2 (S)	27.7±2.5 (S)	27.0±2.2 (S)	6.0±0.0 (R)	26.7±1.2 (S)
MRSA007	15.0±0.0 (R)	11.0±0.0 (R)	13.7±0.6 (R)	18.0±1.0 (S)	6.0±0.0 (R)	6.0±0.0 (R)	21.7±0.6 (S)
MRSA008	17.0±2.0 (R)	14.0±1.7 (R)	25.7±2.1 (S)	27.7±2.5 (S)	26.0±1.4 (S)	6.0±0.0 (R)	26.3±1.5 (S)
MRSA009	14.7±2.5 (R)	12.3±0.6 (R)	25.3±1.5 (S)	28.3±0.6 (S)	25.3±2.1 (S)	6.0±0.0 (R)	23.7±0.6 (S)
MRSA010	16.0±0.0 (R)	11.7±1.2 (R)	26.3±2.5 (S)	28.3±0.6 (S)	23.7±1.7 (S)	6.0±0.0 (R)	24.7±1.2 (S)
MRSA001-010							
No. of resistance (%)	10 (100%)	10 (100%)	5 (50%)	2 (20%)	5 (50%)	10 (100%)	0 (0%)
No. of intermediate (%)	0 (0%)	0 (0%)	0 (0%)	1 (1%)	0 (0%)	0 (0%)	0 (100%)
No. of sensitive (%)	0 (0%)	0 (0%)	5 (50%)	7 (70%)	5 (50%)	0 (0%)	10 (100%)

[1] Values are expressed as mean ± SD of triplicate experiments.
[2] Interpreted criteria for cefoxitin: IZD ≤ 21 mm is resistant (R) and IZD ≥ 22 mm is susceptible (S) (CLSI, 2019).
FOX; cefoxitin (30 µg), AMP; ampicillin (10 µg), AMC; amoxicillin (30 µg), CN; gentamicin (10 µg), CIP; ciprofloxacin (5 µg), E; erythromycin (5 µg) and DA; clindamycin (2 µg).
S; susceptible, I; intermediate and R; resistant.

Table 2 Antibacterial activity of the *Citrus maxima* essential oil and purified limonene against tested bacteria by agar disk diffusion

Bacteria	Inhibition zone diameter (IZD; mm)[1, 2]	
	CMEO	Limonene
S. aureus ATCC 25923	10.0±0.0 (W)	11.3±2.3 (W)
S. aureus ATCC 43300	9.0±1.0 (W)	10.0±1.0 (W)
MRSA001	10.7±1.2 (W)	9.0±0.0 (W)
MRSA002	10.0±0.0 (W)	9.3±1.2 (W)
MRSA003	9.3±0.6 (W)	9.7±0.6 (W)
MRSA004	10.0±0.0 (W)	10.0±1.0 (W)
MRSA005	10.0±0.0 (W)	8.0±2.0 (W)
MRSA006	9.0±1.0 (W)	9.0±1.0 (W)
MRSA007	11.3±1.5 (W)	8.7±2.3 (W)
MRSA008	9.7±0.6 (W)	8.0±1.0 (W)
MRSA009	9.3±1.2 (W)	8.3±1.5 (W)
MRSA010	9.7±2.1 (W)	7.0±1.7 (W)

[1] Values are expressed as mean ± SD of triplicate experiments.

Published online: Copyright © 2016-2020 Rangsit University

[2] Interpreted criteria of antibacterial activities: IZD = 6 mm is no activity (N), 6 mm < IZD ≤ 12 mm is weak activity (W), 12 mm < IZD < 20 mm is moderate activity (M), and IZD ≥ 20 mm is strong activity (S) (Lv, Liang, Yuan, & Li, 2011).

Antibacterial activities of CMEO and purified limonene against the reference strains of *S. aureus* and ten clinical isolates of MRSA by agar disk diffusion are shown in Table 2. The results revealed that CMEO displayed weak antibacterial activities against both of the reference strains; methicillin-susceptible *S. aureus* (ATCC 25923) and methicillin-resistant *S. aureus* (ATCC 43300) with IZDs of 10.0 mm and 9.0 mm, respectively (p = 0.200). For MRSA isolates, the IZD value of CMEO was significantly higher than that of its key compound; limonene (9.9 mm vs 8.7 mm, p = 0.02). Also, CMEO displayed weak antibacterial activity against all clinically isolated MRSA (10/10, 100%). The IZD value of gentamicin (10 µg), which is a positive control, was ranging from 10.0 to 30.0 mm, while that of negative control (2.3% DMSO) was not observed (data not shown). Previous studies proposed that the antibacterial activities of essential oil might be produced by a single major compound or by the synergistic or antagonistic effect of various minor compounds in EO (Nazzaro, Fratianni, De Martino, Coppola, & De Feo, 2013). In this study, the results of the agar disk diffusion method revealed that the inhibitory effect of limonene alone was lower than that of crude CMEO; therefore the synergistic effect of limonene with other minor components in CMEO is proposed to play a crucial role in the antibacterial activity. However, geographical and seasonal variations; such as climate, soil and nutrition, are mainly attributed to the content of chemical components in CMEO and consequently affect the level of antibacterial activity.

The tested bacteria that exhibited the IZD of CMEO > 6 mm by disk diffusion was further determined for the MIC and MBC by broth macro dilution. The MIC and MBC of CMEO against tested bacteria are shown in Table 3. Interestingly, the MIC value of CMEO to *S. aureus* ATCC 43300 was lower than that of *S. aureus* ATCC 25923 (p = 0.034), implying a significant inhibitory effect against MRSA. However, there was inconsistency in the inhibitory effect observed by the disk diffusion and broth macro dilution methods. Therefore, the larger number of clinically isolated MRSA, as well as MSSA, should be included in further investigation. For MRSA isolates, the MIC and MBC values were 8.1±4.8 mg/mL and 11.7±4.8 mg/mL, respectively (data not shown). A study in Taiwan by Tao and Liu (2012) reported the MIC of CMEO against *S. aureus* ATCC 25923 at 9.38 µL/mL. According to several variations in methods of antibacterial testing and tested bacterial strains, comparison to previous studies is not feasible. MIC indexes suggested that CMEO exerted a bactericidal effect toward all tested bacteria, including both MRSA and MSSA (12/12, 100%, MIC indexes ≤ 4). The standard time-kill assay revealed that at 1×MIC CMEO exposure, the relative viable count of methicillin-resistant *S. aureus* (ATCC 43300) decreased from 42.3% to 13.1% within 1 to 4 hours and completely removed within 8 hours after exposure. Methicillin-susceptible *S. aureus* (ATCC 25923) decreased from 42.6% to 16.6% within 1-2 hours and was completely removed within 4 hours after exposure (Figure 1).

Table 3 Antibacterial activity of the *Citrus maxima* essential oil against tested bacteria by broth macro dilution

Bacteria	**MIC[1] (mg/mL)**	**MBC[1] (mg/mL)**	**MIC index**
S. aureus ATCC 25923	10.7±4.6	8.0±6.9	0.8
S. aureus ATCC 43300	4.0±0.0	5.3±2.3	1.3
MRSA001	5.3±2.3	10.7±4.6	2.0
MRSA002	5.3±2.3	5.3±2.3	1.0
MRSA003	10.7±4.6	13.3±4.6	1.3
MRSA004	5.3±2.3	10.7±4.6	2.0
MRSA005	4.0±0.0	10.7±4.6	2.7
MRSA006	16.0±0.0	16.0±0.0	1.0
MRSA007	6.7±2.3	12.0±6.9	1.8

Published online: Copyright © 2016-2020 Rangsit University

Bacteria	MIC[1] (mg/mL)	MBC[1] (mg/mL)	MIC index
MRSA008	10.7±4.6	16.0±0.0	1.5
MRSA009	3.3±1.2	9.3±6.1	2.8
MRSA010	13.3±4.6	13.3±4.6	1.0

[1] Values are expressed as mean ± SD of triplicate experiments.

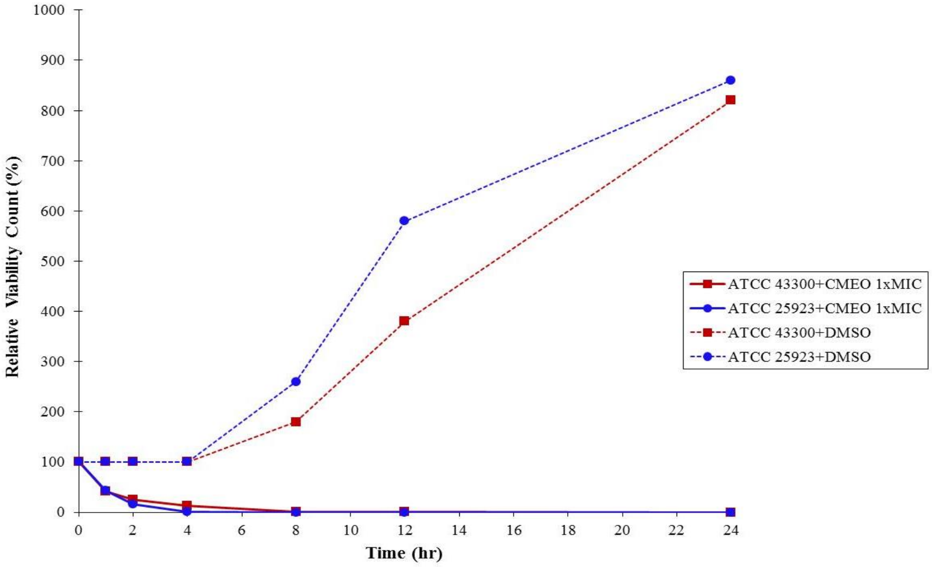

Figure 1 Time-kill curves of the *Citrus maxima* essential oil at 1×MIC and DMSO control to methicillin-resistant *S. aureus* (ATCC 43300) and methicillin-susceptible *S. aureus* (ATCC 25923) within 24 hours

Previous studies reported the antibacterial activity of CMEO against *S. aureus* and other bacterial strains but not for MRSA. Therefore, this study is the first attempt to highlight the antibacterial potential of CMEO against MRSA. The bactericidal effect of CMEO is attributed to the hydrophobic property of the EO and the majority component in CMEO, limonene that is a monoterpene hydrocarbon. Several targets of antibacterial action have been extensively reported in the EO, but only a few with limonene; these targets included the membrane fatty acids, cytoplasmic membrane proteins, intracellular- and extracellular-ATP/ATPases of bacteria (Nazzaro et al., 2013; Espina, Gelaw, de Lamo-Castellví, Pagán, & García-Gonzalo, 2013). The hydrophobic property of EO would enable them to partition in the lipids of bacterial cytoplasmic membrane and mitochondria (Burt, 2004; Chouhan, Sharma, & Guleria, 2017). EO would enter through the fatty acyl chains of membrane lipid bilayers, followed by disrupting the lipid composition and alter membrane fluidity and permeability (Wang et al., 2012). Increased cell permeability may lead to the leakage of cellular- and intracellular components resulting in depleting of intracellular potassium ion and interfering cell respiration (Swamy, Akhtar, & Sinniah, 2016). Finally, the further study of the synergistic effect of CMEO to other plant-derived essential oils and the chemical compositions by GC-MS analysis will be investigated.

Published online: Copyright © 2016-2020 Rangsit University

5. Conclusion

CMEO exhibited a bactericidal effect to methicillin-resistant *S. aureus* with a complete killing time of 8 hours. Further evaluations on the mode of actions of CMEO and synergistic effect with other plant-derived essential oil's as well as *in vivo* adverse effects are necessary before applying CMEO as new antibacterial agents.

6. Acknowledgements

The authors would like to thank the Faculty of Medical Technology, Rangsit University, Thailand for providing research facilities and Assist.Prof. Supaluk Popruk from the Faculty of Tropical Medicine, Mahidol University, Thailand, for providing plant sample. Also grateful thank to Assist.Prof. Acharawan Thongmee from the Microbiology Unit, Department of Medical Sciences, Faculty of Science Rangsit University, Thailand for providing S. aureus ATCC 43300 and Dr. Brian Andrew Vesely for review of the manuscript.

7. References

Anwar, F., Ali, M., Hussain, A. I., & Shahid, M. (2009). Antioxidant and antimicrobial activities of essential oil and extracts of Fennel (*Foeniculum vulgare* Mill.) seeds from Pakistan. *Flavour and Fragrance Journal*, *24*, 170-176.

Borusiewicz, M., Trojanowska, D., Paluchowska, P., Janeczko, Z., Petitjean, M. W., & Budak, A. (2017). Cytostatic, cytotoxic, and antibacterial activities of essential oil isolated from *Citrus hystrix*. *ScienceAsia*, *43*, 96-106.

Burt, S. (2004). Essentials oils: Their antibacterial properties and potential applications in foods: A review. *International Journal of Food Microbiology*, *94*, 223-253.

Chen, G. W., Lin, Y. H., Lin, C. H., & Jen, H. C. (2018). Antibacterial activity of emulsified pomelo (*Citrus grandis* Osbeck) peel oil and water-soluble chitosan on *Staphylococcus aureus* and *Escherichia coli*. *Molecules*, *23*, 840. doi:10.3390/molecules23040840

Chimnoi, N., Reuk-Ngam, N., Chuysinuan, P., Khlaychan, P., Khunnawutmanotham, N., Chokchaichamnankit, D., et al. (2018). Characterization of essential oil from *Ocimum gratissimum* leaves: Antibacterial and mode of action against selected gastroenteritis pathogens. *Microbial Pathogenesis*, *118*, 290-300.

Chouhan, S., Sharma, K., & Guleria, S. (2017). Review: Antimicrobial activity of some essential oils-present status and future perspectives. *Medicines*, *4*, 58. doi:10.3390/medicines4030058

Clinical and Laboratory Standards Institute (CLSI). (2015). *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard* (10th ed). CLSI document M07-A10. Wayne: PA.

Clinical and Laboratory Standards Institute (CLSI). (2019). *Performance standards for antimicrobial susceptibility testing* (29th ed). CLSI supplement M100S. Wayne: PA.

Espina, L., Gelaw, T. K., de Lamo-Castellví, S., Pagán, R., & García-Gonzalo, D. (2013). Mechanism of bacterial inactivation by (+)-limonene and its potential use in food preservation combined processes. *PloS one*, *8*(2), e56769.

Frassinetti, S., Caltavuturo, L., Cini, M., & Della Croce, C. M. (2011). Antibacterial and antioxidant activity of essential oils from *Citrus* spp. *Journal of Essential Oil Research*, *23*(1), 27-31.

Gatsing, D., Tchakoute, V., Ngamga, D., Kuiate, J. R., Tamokou, J. D. D., Nji, N. B., ... & Fodouop, S. P. (2009). *In vitro* antibacterial activity of *Crinum purpurascens* herb leaf extract against the *Salmonella* species causing typhoid fever and its toxicological evaluation. *Iranian Journal of Medical Sciences*, *34*, 126-136.

Published online: Copyright © 2016-2020 Rangsit University

Lemes, R. S., Alves, C. C., Estevam, E. B., Santiago, M. B., Martins, C. H., Santos, T. C. D., ... & Miranda, M. L. (2018). Chemical composition and antibacterial activity of essential oils from *Citrus aurantifolia* leaves and fruit peel against oral pathogenic bacteria. *Anais da Academia Brasileira de Ciências*, *90*(2), 1285-1292.

Lv, F., Liang, H., Yuan, Q., & Li, C. (2011). *In vitro* antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related microorganisms. *Food Research International*, *44*(9), 3057-3064.

McGuinness, W. A., Malachowa N., & DeLeo, F. R. (2017). Review: Vancomycin resistance in *Staphylococcus aureus*. *Yale Journal of Biology and Medicine*, *90*, 269-281.

Minh Tu, N. T., Thanh, L. X., Une, A., Ukeda, H., & Sawamura, M. (2002). Volatile constituents of Vietnamese pummelo, orange, tangerine and lime peel oils. *Flavour and Fragrance Journal*, *17*(3), 169-174.

National Antimicrobial Resistance Surveillance of Thailand (NARST). (2019). *Antimicrobial Resistance 2000-2019*. Retrieved March 2, 2020, from http://narst.dmsc.moph.go.th/data/AMR%202000-2019-06M.pdf

Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R., & De Feo, V. (2013). Effect of essential oils on pathogenic bacteria. *Pharmaceuticals*, *6*(12), 1451-1474.

Ou, M. C., Liu, Y. H., Sun, Y. W., & Chan, C. F. (2015). The composition, antioxidant and antibacterial activities of cold-pressed and distilled essential oils of *Citrus paradisi* and *Citrus grandis* (L.) Osbeck. *Evidence-Based Complementary and Alternative Medicine*, 2015:804091. doi: 10.1155/2015/804091

Prusty, A. K., & Patro, S. K. (2014). Study of *in vitro* antibacterial activity of leave extract of *Citrus maxima*. *Annals of Plant Sciences*, *3*(12), 899-904.

Sabulal, B., Dan, M., Pradeep, N. S., Valsamma, R. K., & George, V. (2006). Composition and antimicrobial activity of essential oil from *Amomum cannicarpum*. *Acta Pharmaceutica*, *56*, 473-480.

Saeb, S., Amin, M., Gooybari, R. S., & Aghel, N. (2016). Evaluation of antibacterial activities of *Citrus limon*, *Citrus reticulata*, and *Citrus grandis* against pathogenic bacteria. *International Journal of Enteric Pathogens*, *4*(4), 3-37103.

Sreepian, A., Sreepian, P. M., Chanthong, C., Mingkhwancheep, T., & Prathit, P. (2019). Antibacterial activity of essential oil extracted from *Citrus hystrix* (kaffir lime) peels: An *in vitro* study. *Tropical Biomedicine*, *36*(2), 531-541.

Swamy, M. K., Akhtar, M. S., & Sinniah, U. R. (2016). Antimicrobial properties of plant essential oils against human pathogens and their mode of action: An updated review. *Evidence-Based Complementary and Alternative Medicine*, 2016:3012462. doi: 10.1155/2016/3012462

Tao, N. G., & Liu, Y. J. (2012). Chemical composition and antimicrobial activity of the essential oil from the peel of Shatian pummelo (*Citrus grandis* Osbeck). *International Journal of Food Properties*, *15*, 709-716.

Thavanapong, N., Wetwitayaklung, P., & Charoenteeraboon, J. (2010). Comparison of essential oils compositions of *Citrus maxima* Merr. peel obtained by cold press and vacuum stream distillation methods and of its peel and flower extract obtained by supercritical carbon dioxide extraction method and their antimicrobial activity. *Journal of Essential Oil Research*, *22*(1), 71-77. doi: 10.1080/10412905.2010.9700268

Torres-Alvarez, C., Núñez González, A., Rodríguez, J., Castillo, S., Leos-Rivas, C., & Báez-González, J. G. (2017). Chemical composition, antimicrobial, and antioxidant activities of orange essential oil and its concentrated oils. *Journal of Food*, *15*(1), 129-135.

Published online: Copyright © 2016-2020 Rangsit University

Tsai, M. L., Lin, C. D., Khoo, K. A., Wang, M. Y., Kuan, T. K., Lin, W. C., ... & Wang, Y. Y. (2017). Composition and bioactivity of essential oil from *Citrus grandis* (L.) Osbeck 'Mato Peiyu'Leaf. *Molecules*, *22*(12), 2154.

Wang, Y. W., Zeng, W. C., Xu, P. Y., Lan, Y. J., Zhu, R. X., Zhong, K., ... & Gao, H. (2012). Chemical composition and antimicrobial activity of the essential oil of kumquat (*Fortunella crassifolia* Swingle) peel. *International Journal of Molecular Sciences*, *13*(3), 3382-3393.

Wongsariya, K., Phanthong, P., Bunyapraphatsara, N., Srisukh, V., & Chomnawang, M. T. (2014). Synergistic interaction and mode of action of *Citrus hystrix* essential oil against bacteria causing periodontal diseases. *Pharmaceutical Biology*, *52*(3), 273-280.

Published online: Copyright © 2016-2020 Rangsit University