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Antibacterial activity of essential oil extracted from *Citrus hystrix* (Kaffir Lime) peels: An *in vitro* study

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Abstract. The exploration of antimicrobial activities from various herbal plants such as *Citrus* species might be a solution to reduce the emergency of antimicrobial resistance. This study was conducted to determine the chemical composition of *Citrus hystrix* essential oil (CHEO) and its antibacterial activity against a broad range of Gram positive and Gram negative bacteria. CHEO was extracted from the peels of kaffir lime by steam distillation. The chemical composition was analyzed by gas chromatography-mass spectrometry (GC-MS). *In vitro* antibacterial activity was determined by the agar disk diffusion and broth macrodilution methods against 6 standard bacterial strains as well as 39 clinical bacterial isolates. GC-MS revealed twenty-seven compounds in CHEO with most predominant compounds like; D-limonene, followed by β-pinene and sabinene. CHEO had inhibitory effects on all tested bacterial isolates except for *Klebsiella pneumoniae*, *Salmonella paratyphi* A, *Salmonella enteritidis*, *Edwardsiella tarda* and *Pseudomonas aeruginosa*. Gram positive bacteria were generally more susceptible than Gram negative bacteria (ranged MIC; 1.0-8.0 mg/mL vs. 8.0 to >16.0 mg/mL) with *Staphylococcus aureus* and *Elizabethkingia meningoseptica* being the most susceptible. These findings demonstrated that CHEO has a potential to be developed as an antibacterial agent to combat the emerging antimicrobial resistant bacteria.

INTRODUCTION

Antimicrobial resistance (AMR) is a major global health problem. The Centers for Disease Control and Prevention (CDC) estimated that more than 2 million people are affected to serious complications from antibiotic-resistant pathogen, resulting in at least 23 000 deaths annually in the United States (CDC, 2013). In Thailand, it is estimated that AMR is resulting in 87 751 cases and 38 481 deaths, annually (Pumart *et al*., 2012). The antibiotic resistance has been commonly reported in *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Pumart *et al*., 2012). To control the emergence of AMR, one of the strategies is to reduce antimicrobial use in human by 2021 as mentioned in the National Strategic Plan on Antimicrobial Resistance (NSP-AMR), Ministry of Public Health, Thailand (AMR-CIC, 2016). One of the possible ways to support this strategy is the use of herbal medication.

Several studies have demonstrated that antibacterial activities of essential oils extracted from the plants in the family Rutaceae including *Citrus limon* (lemon), *C. aurantium* (sour orange), *C. reticulata* (mandarin orange), *C. sinensis* (sweet orange), *C. aurantifolia* (lime) and *C. hystrix* (kaffir lime) (Frassinetti *et al*., 2011; Madhuri *et al*., 2014; Wongsariya *et al*., 2014; Dadashi *et al*., 2015; Md Othman *et al*., 2016; Otang & Afolayan, 2016; Saeb *et al*., 2016; Borusiewicz *et al*., 2017; Geraci *et al*., 2017;

Intorasoot *et al*., 2017; Torres-Alvarez *et al*., 2017; Lemes *et al*., 2018). *Citrus hystrix* DC. (common names: kaffir lime and makrut lime) is a tropical fruit that is commonly found in Southeast Asia, including Thailand. It is a small tree that reaches up to 2 meters high. The leaves are simple, spiral and estipulate. The fruits are bumpy, green, and strongly aromatic (Wiart, 2006). The peel and leaf are commonly used for the extraction of essential oils (Srisukh *et al*., 2012; Md Othman *et al*., 2016). The proportion of chemical compositions of *C. hystrix* essential oil (CHEO) were varied, mainly depending on harvesting seasons, agro climatic condition, stage of maturity, adaptive metabolism of plants and distillation conditions (Anwar *et al*., 2009; Swamy *et al*., 2016). Major compounds of steam distilled-CHEO from the peels collected from Selangor, Malaysia, were sabinene (35.2%), limonene (19.8%), β-pinene (16.8%), citronellal (7.8%) and α-pinene (3.1%). However, those collected from northwestern Thailand were limonene (34.32%), β-pinene (17.4%), terpinen-4-ol (10.20%), α-terpineol (8.76%), α-pinene (3.59%) and sabinene (1.59%) (Kasuan *et al*., 2013; Borusiewicz *et al*., 2017). On the other hand, hydrodistilled-CHEO from the peels collected from southern Thailand were β-pinene (30.48%), sabinene (22.75%), citronellal (15.67%), limonene (8.13%), 4-terpineol (6.61%), α-pinene (3.05%) and citronellol (3.24%) (Chanthaphon *et al*., 2008). In the past decade, several studies revealed that CHEO contains a potential in antibacterial activity (Chanthaphon *et al*., 2008; Srisukh *et al*., 2012; Borusiewicz *et al*., 2017; Intorasoot *et al*., 2017; Soffian *et al*., 2017). The variation of activity is mainly depends on genetic and environmental factors (Swamy *et al*., 2016). To date, there is a limited data of antibacterial activity of CHEO against broad ranged pathogenic bacteria. Therefore, the present study aimed to determine antibacterial activity of essential oil extracted from the peels of *C. hystrix* fruits against Gram positive and Gram negative bacteria. In addition, the chemical composition of CHEO was also investigated by gas chromatography-mass spectrometry (GC-MS).

MATERIALS AND METHODS

Plant material

The fresh fruits of *Citrus hystrix* (kaffir lime) were collected from Chiang Rai Province, located in northernmost Thailand, in July 2016. The plant sample was identified and voucher specimen (BCU No. 015826) was housed at the Herbarium of the Department of Botany, Faculty of Science, Chulalongkorn University, Thailand. The peel was separated and then processed through a steam distillation. In this study, the percentage yield of extracted *C. hystrix* essential oil (CHEO) was 2.5% (w/v). An extracted CHEO was stored at 4°C and protected from light until used. A stock solution of CHEO was prepared at concentration of 400 mg/mL (v/v) in dimethyl sulphoxide (DMSO) before used.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The separation and identification of volatile components of CHEO were carried out by gas chromatography-mass spectrometry (GC-MS) (GC 7890A/MS 5975C-MSD; Agilent Technologies, CA, USA). The capillary column Mega-5MS (30 m × 0.25 mm × 0.25 µm) was used. The GC conditions were programmed as the injection temperature 230°C; with oven temperature initially set at 60°C for 1 min, and then gradually increasing at the rate of 3°C/min up to 240°C and held for 5 min. Helium was used as the carrier gas with a constant flow rate of 1.0 mL/min. The volume of injection was 1 µL of ethanol solution in a split mode (1: 20). The MS transfer line temperature was set at 250°C with electron ionization (EI) mode at 70 eV ionization potential. The mass-to-charge (m/z) range was from 40 to 650 m/z. Compounds were further identified by matching their mass spectra fragmentation pattern and retention time with standard reference compounds and compared their MS results with NIST

2011 (National Institute of Standards and Technology) library stored in GC/MS database for confirmation.

Bacterial organisms

The bacterial organisms determined in this study contained 6 American Type Culture Collection (ATCC) bacterial strains and 39 different clinical isolates. The ATCC bacterial strains were composed of *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, *Klebsiella pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa* ATCC 27853. These bacteria were obtained from Faculty of Medical Technology, Rangsit University, Thailand. The bacteria were cultivated on blood agar at 37°C for 18-24 hrs.

Agar disk diffusion

Agar disk diffusion was performed to screen the *in vitro* antibacterial activity as previously described (Sabulal *et al.*, 2016) with some modifications. The turbidity of tested bacteria was adjusted to 0.5 McFarland units using the densitometer (DEN-1; Biosan, England). Bacterial suspension was spread on either Mueller Hinton Agar (MHA) or Mueller sheep Blood Agar (MBA), depending on the type of bacteria. Sterilized disk (6 mm) impregnated with 10 µL of CHEO (0.9 g/mL) was placed on the surface of each plate. In addition, gentamicin disk (10 µg or 120 µg) was also included. The plates were incubated at 37°C for 18-24 hrs. The inhibition zone diameter (IZD) of CHEO 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 *et al.*, 2011).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC of CHEO was evaluated by broth macrodilution method following the Clinical and Laboratory Standards Institute (CLSI) document M07-A10 (CLSI, 2015) with some modifications. The ranged concentrations of CHEO were 0.125 to 16.0 mg/mL. The MIC was defined 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 hrs. The MBC was defined by the lowest concentration that completely inhibits bacterial growth on the agar plate.

Each experiment was performed in triplicate. Broth control (CHEO with MHB), bacterial control (bacteria with MHB) and DMSO control (bacteria with 2.2% DMSO) were also included. The MIC index (MBC/MIC ratio) was calculated to classify the type of antimicrobial substances according to previously described by Gatsing *et al.* (2009).

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.

RESULTS

Chemical composition of CHEO

The chemical composition of *C. hystrix* essential oil (CHEO) obtained by GC-MS analysis is presented in Table 1 and Figure 1. Retention time is the time at which the compound elutes from the column in GC. Twenty-seven compounds were identified, accounting for 89.98% of the total essential oil. CHEO consisted mainly of monoterpene hydrocarbons (65.98%) followed by oxygenated monoterpenes (20.68%) and sesquiterpene hydrocarbons (3.32%). D-limonene (25.28%), β-pinene (21.10%) and sabinene (14.99%) were the major components of monoterpene hydrocarbons, while citronellal (7.63%) and terpinen-4-ol (5.06%) were the major components of oxygenated monoterpenes. The compositions of remaining 22 compounds ranged from 0.11 to 2.82%.

Table 1. Chemical compositions of the *Citrus hystrix* essential oil

No.	Compounds	Retention time (min)	Retention index*	Composition (%)	Quality
1	α-Thujene	8.047	925	0.13	91
2	α-Pinene	8.375	933	2.22	97
3	Camphene	9.062	950	0.13	97
4	Sabinene	10.053	974	14.99	94
5	β-Pinene	10.324	981	21.10	97
6	β-Myrcene	10.652	989	1.02	91
7	α-Terpinene	11.922	1018	tr	95
8	Cymene	12.330	1027	0.96	95
9	D-Limonene	12.617	1033	25.28	99
10	γ-Terpinene	13.832	1059	0.15	97
11	Terpinolene	15.086	1086	tr	96
12	Linalool	15.829	1102	1.62	96
13	Isopulegol	18.194	1152	0.35	99
14	Citronellal	18.418	1156	7.63	96
15	endo-Borneol	19.361	1176	tr	97
16	Terpinen-4-ol	19.760	1185	5.06	97
17	α-Terpineol	20.455	1199	2.82	83
18	Citronellol	21.846	1230	2.50	98
19	Geraniol	22.900	1253	0.22	94
20	Citronellol acetate	27.278	1350	0.48	94
21	α-Copaene	28.373	1375	1.12	99
22	β-Cubebene	28.900	1387	0.85	98
23	β-Caryophyllene	30.267	1420	0.40	99
24	α-Caryophyllene	31.777	1456	0.22	98
25	D-Germacrene	32.831	1482	0.11	95
26	α-Muurolene	33.534	1498	0.11	99
27	(+)-δ-Cadinene	34.309	1518	0.51	97

tr- trace (<0.1%)
* Retention index relative to n-alkanes (C8-C40) on Mega-5MS column.

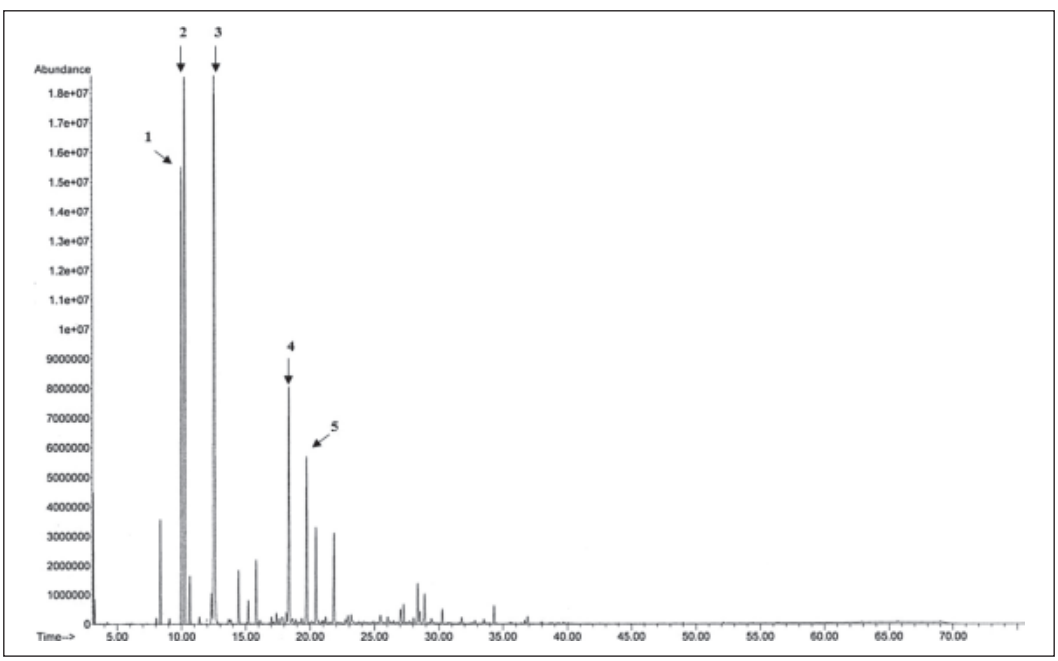

Figure 1. Representative GC-MS chromatogram of *Citrus hystrix* essential oil. Major compound peaks marked: sabinene (1), β-pinene (2), D-limonene (3), citronellal (4) and terpinen-4-ol (5).

Screening antibacterial activity of CHEO by agar disk diffusion

Antibacterial activity of CHEO against the ATCC bacterial strains, clinically isolated Gram positive and Gram negative bacteria are shown in Table 2-4. The screening of antibacterial activity was carried out by agar disk diffusion. The results demonstrated various antibacterial activities of CHEO against bacteria determined in this study. CHEO displayed antibacterial activities against 4 ATCC bacterial strains except for *K. pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 27853 (4/6, 66.7%) with a ranging IZD of 8.0-14.7 mm. The moderate antibacterial activity of CHEO was observed in *P. vulgaris* ATCC 13315 and *S. aureus* ATCC 25923. In addition, the weak antibacterial activity was observed in *E. faecalis* ATCC 29212 and *E. coli* ATCC 25922 (Table 2).

CHEO displayed antibacterial activity against most clinically isolated bacteria (34/39, 87.2%) with IZD ranging from 6.3-32.7 mm. The weak activity of CHEO was observed against all Gram positive bacteria (7/8, 87.5%) except for *S. aureus* in which moderate activity was observed (Table 3). On the other hand, a various degree of activity was observed among clinical isolates of Gram negative bacteria; from strong to weak activities (26/31, 83.9%, respectively) (Table 4). However, antibacterial activity of CHEO was not observed

Table 2. Antibacterial activity of the *Citrus hystrix* essential oil against the ATCC bacterial strains

Organisms	Agar disk diffusion; IZD (mm)		Broth macrodilution		
	CHEO[a]	CN	MIC (mg/mL)	MBC (mg/mL)	MIC index
S. aureus (ATCC 25923)	14.0±1.7 (M)	26.3±0.6	8.0±0.0	8.0±0.0	1.0
E. faecalis (ATCC 29212)	8.0±0.0 (W)	24.0±0.0	8.0±3.5	8.0±0.0	1.0
E. coli (ATCC 25922)	8.7±1.2 (W)	21.7±0.6	13.3±4.6	13.3±4.6	1.0
P. vulgaris (ATCC 13315)	14.7±1.2 (M)	26.0±0.0	8.0±0.0	8.0±0.0	1.0
K. pneumoniae (ATCC 700603)	6.0±0.0 (N)	14.0±0.0	ND[b]	ND[b]	ND[b]
P. aeruginosa (ATCC 27853)	6.0±0.0 (N)	19.3±0.6	ND[b]	ND[b]	ND[b]

Values are expressed as mean±SD of triplicate experiments.
CN – Gentamicin at a concentration of 10 µg/disk except for *E. faecalis* ATCC 29212 (120 µg/disk).
[a] 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 *et al.*, 2011).
[b] ND – not determined. MICs, MBCs and MIC index were not determined when inhibition zone was not presented (IZD = 6 mm).

Table 3. Antibacterial activity of the *Citrus hystrix* essential oil against the clinically isolated Gram positive bacteria

Organisms	Agar disk diffusion; IZD (mm)		Broth macrodilution		
	CHEO[a]	CN	MIC (mg/mL)	MBC (mg/mL)	MIC index
S. aureus	16.3±1.5 (M)	30.0±0.0	2.0±0.0	2.7±1.2	1.3
S. epidermidis	12.0±0.0 (W)	32.0±2.0	2.0±0.0	2.7±1.2	1.3
S. saprophyticus	10.3±1.5 (W)	36.0±2.0	5.3±2.3	6.7±2.3	1.3
S. pneumoniae	9.7±0.6 (W)	21.3±2.3	1.7±0.6	1.7±0.6	1.0
S. pyogenes	8.0±1.7 (W)	28.0±2.0	4.0±0.0	4.0±0.0	1.0
S. viridans	10.3±1.5 (W)	25.7±2.1	1.3±0.6	2.0±0.0	1.5
S. agalactiae	9.7±0.6 (W)	23.7±0.6	2.7±1.2	2.7±1.2	1.0
L. monocytogenes	8.0±0.0 (W)	33.3±1.2	3.3±1.2	4.0±0.0	1.2

Values are expressed as mean±SD of triplicate experiments.
CN – Gentamicin at a concentration of 10 µg/disk.
[a] 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 *et al.*, 2011).

Table 4. Antibacterial activity of the *Citrus hystrix* essential oil against the clinically isolated Gram negative bacteria

Organisms	Agar disk diffusion; IZD (mm)		Broth macrodilution		
	CHEO[a]	CN	MIC (mg/mL)	MBC (mg/mL)	MIC index
E. coli	9.3±1.2 (W)	20.7±0.6	13.3±4.6	13.3±4.6	1.0
K. pneumoniae	6.0±0.0 (N)	21.7±2.1	ND[b]	ND[b]	ND[b]
P. vulgaris	8.7±1.5 (W)	23.0±1.0	10.7±4.6	10.7±4.6	1.0
P. mirabilis	8.3±2.1 (W)	22.0±0.0	16.0±0.0	16.0±0.0	1.0
S. typhi	6.3±0.6 (W)	27.3±2.5	10.7±4.6	10.7±4.6	1.0
S. paratyphi A	6.0±0.0 (N)	23.0±1.7	ND[b]	ND[b]	ND[b]
S. enteritidis	6.0±0.0 (N)	20.3±0.6	ND[b]	ND[b]	ND[b]
S. arizonae	6.7±0.6 (W)	19.3±1.5	>16	>16	ND[b]
S. marcescens	6.3±0.6 (W)	19.3±0.6	>16	>16	ND[b]
S. rubidaea	7.7±1.5 (W)	26.0±1.0	>16	>16	ND[b]
Y. enterocolitica	9.7±1.5 (W)	26.3±0.6	6.7±2.3	8.0±0.0	1.2
E. tarda	6.0±0.0 (N)	19.3±1.2	ND[b]	ND[b]	ND[b]
C. freundii	7.3±0.6 (W)	20.3±0.6	>16	>16	ND[b]
S. flexneri	7.3±1.2 (W)	19.3±2.3	5.3±2.3	8.0±6.9	1.5
S. dysenteriae	9.0±0.0 (W)	18.0±0.0	10.7±4.6	10.7±4.6	1.0
S. sonnei	8.7±1.2 (W)	19.3±1.2	10.7±4.6	10.7±4.6	1.0
S. boydii	9.3±1.2 (W)	22.7±3.8	10.7±4.6	10.7±4.6	1.0
P. rettgeri	7.3±0.6 (W)	17.0±1.0	10.7±4.6	10.7±4.6	1.0
P. stuartii	6.7±1.2 (W)	19.0±1.0	13.3±4.6	13.3±4.6	1.0
P. agglomerans	6.7±0.6 (W)	21.3±1.2	6.7±2.3	16.0±0.0	2.4
E. cloacae	9.7±1.5 (W)	24.0±2.0	16.0±0.0	16.0±0.0	1.0
M. morganii	9.3±1.2 (W)	22.7±2.3	10.7±4.6	10.7±4.6	1.0
E. meningoseptica	32.7±4.2 (S)	42.0±0.0	1.2±0.8	1.2±0.8	1.0
S. maltophilia	11.3±0.6(W)	24.0±1.0	2.0±0.0	2.0±0.0	1.0
P. aeruginosa	6.0±0.0 (N)	22.0±1.0	ND[b]	ND[b]	ND[b]
A. baumannii	7.7±0.6 (W)	10.7±0.6	16.0±0.0	16.0±0.0	1.0
A. lwoffii	14.7±1.2 (M)	25.3±2.3	4.0±0.0	5.3±2.3	1.3
V. cholera	10.7±1.2 (W)	24.7±1.5	2.0±0.0	2.0±0.0	1.0
V. parahaemolyticus	10.7±2.1 (W)	18.7±0.6	1.7±0.6	2.0±0.0	1.2
V. vulnificus	10.0±1.0 (W)	19.0±1.7	1.7±0.6	1.7±0.6	1.0
P. shigelloides	15.3±1.2 (M)	17.7±3.8	1.2±0.8	1.2±0.8	1.0

Values are expressed as mean±SD of triplicate experiments.
CN – Gentamicin at a concentration of 10 µg/disk.
[a] 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 *et al.*, 2011).
[b] ND – not determined. MICs, MBCs and MIC index were not determined when inhibition zone was not presented (IZD = 6 mm).

in clinical isolates of *K. pneumoniae*, *S. paratyphi* A, *S. enteritidis*, *E. tarda* and *P. aeruginosa* (5/31, 16.1%) (Table 4). In this study, IZD of gentamicin at 10 µg was recorded in all tested bacteria except for *E. faecalis* ATCC 29212, the concentration of 120 µg was used to determine the susceptibility. All of them exhibited IZD of gentamicin ranging from 10.7 to 42.0 mm (45/45, 100%) (Table 2-4).

Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of CHEO

The tested bacteria those exhibited IZD > 6 mm by disk diffusion were further determined for the MICs by broth macrodilution. The MIC and MBC of CHEO against ATCC bacterial strains are shown in Table 2. The MIC values of ATCC bacterial strains ranged from 8.0-13.3 mg/mL which are equal

to their MBC values. For clinical isolates of Gram positive bacteria, the MIC and MBC ranged from 1.3-5.3 mg/mL and 1.7-6.7 mg/mL, respectively. The most susceptible Gram positive bacteria was *S. viridans* with MIC of 1.3 mg/mL (Table 3). Moreover, the ranged MIC and MBC of clinical isolates of Gram negative bacteria were equal (1.2 to greater than16 mg/mL). Regarding to clinical isolates of Gram negative bacteria, the most susceptible organisms were *E. meningoseptica* and *P. shigelloides* with equal MIC values of 1.2 mg/mL. The MIC and MBC of CHEO against 4 clinical isolates of Gram negative bacteria; *S. arizonae*, *S. marcescens*, *S. rubidaea* and *C. freundii* were more than 16 mg/mL, although their inhibition zones were presented (ranged IZD: 6.3-7.7 mm) (Table 4). MIC indexes suggested that CHEO exerted a bactericidal effect toward most tested bacterial organisms (34/45, 75.6%, MIC indexes $\leq$ 4).

DISCUSSION

The emergence of drug resistant microorganisms is one of the growing public health concerns worldwide and the searching for new antimicrobial compounds should be continued. The antimicrobial agents from natural sources might be one of the solutions. Therefore, the purpose of our research is to evaluate antibacterial activity of essential oil extracted from a various plants in the *Citrus* spp. against the important human pathogens. *Citrus hystrix* DC. is a traditional plant that commonly grows in the various parts of Thailand. Previous studies have revealed that it contains several biological activities including anti-oxidant (Abirami *et al.*, 2014; Ali *et al.*, 2015), anti-cancer (Tunjung *et al.*, 2015) as well as antimicrobial activities (Chanthaphon *et al.*, 2008; Srisukh *et al.*, 2012; Borusiewicz *et al.*, 2017; Intorasoot *et al.*, 2017; Soffian *et al.*, 2017). The present study evaluated antibacterial activity of CHEO by the agar disk diffusion and MIC against various pathogenic bacteria. These are responsible for the infections of urinary tract, gastrointestinal tract and respiratory tract and are among the great concern of emergence of AMR. The most majority chemical composition of CHEO found in this study was limonene (34.32%). This finding is in agreement to previous studies by Borusiewicz *et al.* (2017) and Srisukh *et al.* (2012) those reported that CHEO consisted of limonene at 26.33% and 40.65%, respectively. However, a study by Chanthaphon *et al.* (2008) reported that β-pinene was the most predominant compound. Study on the antibacterial activity of a singular compound of the terpenes; such as limonene, α-pinene, β-pinene, sabinene and α-terpinene, showed a low activity when it used alone (Nazzaro *et al.*, 2013), therefore, the antibacterial activity of CHEO demonstrated by this study seems to be via a synergistic effect of limonene, which is the most majority compound, and other minor components in the EO.

In the present study, CHEO was more active against Gram positive (MIC, 1.3–5.3 mg/mL) compared to Gram negative bacteria (MIC, 1.2 to greater than 16.0 mg/mL) which is similar finding to other studies (Huang *et al.*, 2014; Azhdarzadeh & Hojjati, 2016; Chimnoi *et al.*, 2018). This could be explained by the fact that the cell wall of Gram negative bacteria contains high contents of phospholipids and lipopolysaccharides, which is more complex compared to Gram positive bacteria (Nazzaro *et al.*, 2013). Lipopolysaccharide layer could limit the permeability of EO through outer membrane of Gram negative bacteria (Chimnoi *et al.*, 2018). On the other hand, Gram positive bacteria contains high content of peptidoglycan in their cell wall, thereafter allowing hydrophobic molecules to penetrate into the bacterial cell (Nazzaro *et al.*, 2013).

The present study demonstrated that CHEO acts as a bactericidal agent against a broad range of pathogenic bacteria in the family Micrococcaceae, Streptococcaceae, Listeriaceae, Morganellaceae, Vibrionaceae, Flavobacteriaceae, Xantho-

monadaceae and Moraxellaceae. Although clinical isolates of *S. arizonae*, *Serratia* spp. and *C. freundii* seem to be resistant to CHEO since their MIC values were high (>16 mg/mL). This finding indicated a weak activity of CHEO against these organisms which is evident from the inhibition zones. However, antibacterial activity at higher concentration of CHEO was not determined in the present study since CHEO cannot be dissolved in these concentrations. A study by Srisukh *et al.* (2012) demonstrated the antibacterial activity of CHEO against group A, B, C, F and G streptococci, *S. pneumoniae*, *H. influenzae*, *S. aureus* and *A. baumannii* with the ranged MIC of 0.03-17.40 mg/mL. Similarly, Intorasoot *et al.* (2017) demonstrated a weak activity of CHEO against *S. aureus*, *E. coli* and *A. baumannii* but not against *P. aeruginosa*. Since there are several variations in methods of extraction and tested bacterial strains, comparison to previous studies is not feasible. In this study, the bactericidal effect of CHEO is mostly due to the hydrophobic properties of CHEO and the most majority component in CHEO; limonene. The modes of action of EO have been extensively reported with several cellular targets including the membrane fatty acids, cytoplasmic membrane proteins, intracellular- and extracellular-ATP/ATPases of bacteria (Nazzaro *et al.*, 2013; Espina *et al.*, 2013). Limonene was considered to accumulate in the plasma membrane and consequently increased outer membrane permeability and altered β-sheet proteins in the outer membrane of Gram negative bacteria (Espina *et al.*, 2013). Similarly, the lipophilic property of the EO would enable them to partition in the lipids of bacterial cytoplasmic membrane and mitochondria (Burt, 2004; Chouhan *et al.*, 2017). It could enter through the fatty acyl chains of membrane lipid bilayers, followed by disrupt the lipid composition and alter membrane fluidity and permeability (Wang *et al.*, 2012). Consequently, increased cell permeability may lead to the leakage of cellular- and intracellular- components resulting in losing of intracellular K^+ ion and interfering cell respiration (Swamy *et al.*, 2016). In addition, Chimnoi *et al.* (2018) demonstrated that the EO affects to the bacterial cell membrane leading to release of the intracellular components and proteins, and increase in the permeability of the cytoplasmic membrane. However, study on the specific cellular targets of CHEO should be performed to elucidate the precise mechanisms of action.

In our study, CHEO displayed antibacterial activity to all tested Gram positive bacteria. However, a variation of activity was found against Gram negative bacteria of the family Enterobacteriaceae in which the inhibitory activity was observed only in *E. coli*, *S. typhi*, *S. arizonae*, *Shigella* spp., *Serratia* spp., *Y. enterocolitica*, *Citrobacter* spp., *Providencia* spp., *Enterobacter* spp., *M. morganii* and *P. shigelloides*. The inhibitory effects to *S. typhi* and *S. arizonae* but not to *S. paratyphi* A and *S. enteritidis* implied the serotype dependent activity of CHEO. Moreover, there was no activity observed against *K. pneumoniae*, *E. tarda* and *P. aeruginosa*. The protection from antibacterial action could be attributed to the biofilm formation of these organisms. In addition, it is possibly due to a combination of a very restrictive outer membrane barrier of *P. aeruginosa* and the polysaccharide based capsule surrounding the cell of *K. pneumoniae*. The capsule of *K. pneumoniae* plays a pivotal role in the evasion of phagocytosis and complement-mediated lysis by host cells as well as protection to antibacterial peptides (Doorduijn *et al.*, 2016).

In conclusion, our findings demonstrate that CHEO exerted an antibacterial agent toward a broad range of bacterial organisms. It implied that CHEO has a potential to be developed as an alternative antibacterial agent. The use of CHEO, which is lesser toxicity to human than the existing antibiotics, would reduce the antibiotic usage and ultimately reduce the emergence of antibiotic resistance. Further evaluations on mode of action of CHEO and synergistic effect with essential oil from other herbal

plants as well as *in vivo* adverse effects are needed to develop CHEO as alternative antibacterial agents.

Conflict of interest statement
We declare that we have no conflict of interest.

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REFERENCES

Abirami, A., Nagarani, G. & Siddhuraju, P. (2014). *In vitro* antioxidant, antidiabetic, cholinesterase and tyrosinase inhibitory potential of fresh juice from *Citrus hystrix* and *C. maxima* fruits. *Food Science and Human Wellness* **3**: 16-25.

Ali, M., Akhter, R., Narjish, S.N., Shahriar, M. & Bhuiyan, M.A. (2015). Studies of preliminary phytochemical screening, membrane stabilizing activity, thrombolytic activity and in-vitro antioxidant activity of leaf extract of *Citrus hystrix*. *International Journal of Pharmaceutical Sciences and Research* **6**: 2367-2374.

AMR Coordination and Integration Committee (AMR-CIC). (2016). National strategic plan on antimicrobial resistance 2017-2021, Thailand. http://www.fda.moph.go.th/sites/drug/Shared%20Documents/AMR/05.pdf (8 March 2018).

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.

Azhdarzadeh, F. & Hojjati, M. (2016). Chemical composition and antimicrobial activity of leaf, ripe and unripe peel of bitter orange (*Citrus aurantium*) essential oils. *Nutrition and Food Sciences Research* **3**: 43-50.

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.

Centers for Disease Control and Prevention (CDC). (2013). Antibiotic resistance threats in the United States. https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf (31 May 2018).

Chanthaphon, S., Chanthachum, S. & Hongpattarakere, T. (2008). Antimicrobial activities of essential oils and crude extracts from tropical *Citrus* spp. against food-related microorganisms. *Songklanakarin Journal of Science and Technology* **30**: 125-131.

Chimnoi, N., Reuk-Ngam, N., Chuysinuan, P., Khlaychan, P., Khunnawutmanotham, N., Chokchaichamnankit, D., Thamniyom, W., Klayraung, S., Mahidol, C. & Techasakul, S. (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 edition. CLSI document M07-A10, Wayne, PA.

Dadashi, M., Eslami, G., Goudarzi, H., Hashemi, A., Fallah, F., Dabiri, H., Taheri, S. & Ardeshiri, N. (2015). Antibacterial effects of *Citrus aurantium* on bacteria isolated from urinary tract infection. *Research in Molecular Medicine* **3**: 47-50.

Doorduijn, D.J., Rooijakkers, S.H., van Schaik, W. & Bardoel, B.W. (2016). Complement resistance mechanisms of *Klebsiella pneumoniae*. *Immunobiology* **221**: 1102-1109.

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**: 27-31.

Gatsing, D., Tchakoute, V., Ngamga, D., Kuiate, J.R., Tamokou, J.D.D., Nji-Nkah, B.F., Tchouanguep, F.M. & Fodouop, S.C. (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.

Geraci, A., Stefano, V.D., Martino, E.D., Schillaci, D. & Schicchi, R. (2017). Essential oil components of orange peels and antimicrobial activity. *Natural Product Research* **31**: 653-659.

Huang, D.F., Xu, J.G., Liu, J.X., Zhang, H. & Hu, Q.P. (2014). Chemical constituents, antibacterial activity and mechanism of action of the essential oil from *Cinnamomum cassia* bark against four food related bacteria. *Microbiology* **83**: 357-365.

Intorasoot, A., Chornchoem, P., Sookkhee, S. & Intorasoot, S. (2017). Bactericidal activity of herbal volatile oil extracts against multidrug-resistant *Acinetobacter baumannii*. *Journal of Intercultural Ethnopharmacology* **6**: 218-222.

Kasuan, N., Muhammad, Z., Yusoff, Z., Rahiman, M.H.F., Taib, M.N. & Haiyee, Z.A. (2013). Extraction of *Citrus hystrix* D.C. (kaffir Lime) essential oil using automated steam distillation process: Analysis of volatile compounds. *Malaysian Journal of Analytical Sciences* **17**: 359-369.

Lemes, R.S., Alves, C.C.F., Estevam, E.B.B., Santiago, M.B., Martins, C.H.G., Santos, T.C.L.D., Crotti, A.E.M. & Miranda, M.L.D. (2018). Chemical composition and antibacterial activity of essential oils from *Citrus aurantifolia* leaves and fruit peel against oral pathogenic bacteria. *Annals of the Brazilian Academy of Sciences* **90**: 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**: 3057-3064.

Md Othman, S.N.A., Hassan, M.A., Nahar, L., Basar, N., Jamil, S. & Sarker, S.D. (2016). Review: Essential oils from the Malaysian *Citrus* (Rutaceae) medicinal plants. *Medicines (Basel)* **3**(2). doi: 10.3390/medicines3020013

Madhuri, S., Ashwini, U.H., Srilakshmi, N.S. & Prashith Kekuda, T.R. (2014). Antimicrobial activity of *Citrus sinensis* and *Citrus aurantium* peel extracts. *Journal of Pharmaceutical and Scientific Innovation* **3**: 366-368.

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

Otang, W.M. & Afolayan, A.J. (2016). Antimicrobial and antioxidant efficacy of *Citrus limon* L. peel extracts used for skin diseases by Xhosa tribe of Amathole District, Eastern Cape, South Africa. *South African Journal of Botany* **102**: 46-49.

Pumart, P., Phodha, T., Thamlikitkul, V., Riewpaiboon, A., Prakongsai, P. & Limwattananon, S. (2012). Health and economic impacts of antimicrobial resistance in Thailand. *Journal of Health Systems Research* **6**: 352-360.

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): e37103.

Soffian, M.S., Mohamad, I., Mohamed, Z. & Salim, R. (2017). Antifungal effect of kaffir lime leaf extract on selected fungal species of pathogenic otomycosis in *in vitro* culture medium. *Journal of Young Pharmacists* **9**: 468-474.

Srisukh, V., Tribuddharat, C., Nukoolkarn, V., Bunyapraphatsara, N., Chokephaibulkit, K., Phoomniyom, S., Chuanphung, S. & Srifuengfung, S. (2012). Antibacterial activity of essential oils from *Citrus hystrix* (makrut lime) against respiratory tract pathogens. *Science Asia* **38**: 212-217.

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**. doi: 10.1155/2016/3012462

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**: 129-135.

Tunjung, W.A.S., Cinatl, J. Jr., Michaelis, M. & Smales, C.M. (2015). Anti-cancer effect of kaffir lime (*Citrus hystrix* DC) leaf extract in cervical cancer and neuroblastoma cell lines. *Procedia Chemistry* **14**: 465-468.

Wang, Y.W., Zeng, W.C., Xu, P.Y., Lan, Y.J., Zhu, R.X., Zhong, K., Huang, Y.N. & Gao, H. (2012). Chemical composition and antimicrobial activity of the essential oil of kumquat (*Fortunella crassifolia* Swingle) peel. *International Journal of Molecular Sciences* **13**: 3382-3393. doi:10.3390/ijms13033382

Wiart, C. (2006). Medicinal plants of Asia and the Pacific. In: Medical plants classified in the family *Rutaceae*. CRC Press, FL, pp. 211-217.

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**: 273-280.