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Antibacterial activity of essential oils from *Citrus hystrix* (makrut lime) against respiratory tract pathogens

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ABSTRACT: Many essential oils have antibacterial activity with a potential use in medicine. *Citrus hystrix* DC, or makrut lime, contains two essential oils, makrut leaf oil and makrut (fruit peel) oil, of which we determined the inhibitory effect against respiratory pathogens and evaluated their active components. Gas chromatography-mass spectrometry was used to analyse the chemical composition of the essential oils. The antibacterial activities were tested by disc-diffusion and broth microdilution methods against 411 isolates of groups A, B, C, F, G streptococci, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus* (methicillin-resistant and -sensitive *S. aureus*) and *Acinetobacter baumannii*, obtained from patients with respiratory tract infections. Makrut leaf oil and makrut oil were both effective against all the pathogens with minimal inhibitory concentration (MIC) ranges of 0.06–68 mg/ml and 0.03–17.40 mg/ml, respectively. Citronellal was found to be the major component (80.04%) in makrut leaf oil and had the lowest MIC. In contrast, makrut oil consisted of several components (limonene 40.65%, terpinene-4-ol 13.71%, α-terpineol 13.20%), and the most active component was α-terpineol, followed by terpinene-4-ol, and limonene. These results suggest that makrut leaf oil, makrut oil, and their components (citronellal, α-terpineol, terpinene-4-ol) may be alternative natural source medicine to prevent and treat many bacterial diseases.

KEYWORDS: kaffir lime, α-terpineol

INTRODUCTION

Respiratory tract infection is a public health concern in global scale. Controlling the number and growth of pathogens by effective natural products have been the prime targets of research. Essential oils and extracts from a wide variety of plants have long been used for medicinal purposes. They are potential sources of novel drugs especially against bacterial pathogens[1–3]. *Citrus hystrix* DC, commonly known as makrut lime, is a common tropical herb in the family Rutaceae found everywhere in Southeast Asia[4]. Makrut lime is a thorny bush with aromatic leaves and dark green fruits with irregular bumpy surface (Fig. 1). The valued parts of makrut lime are the leaves and fruit peel. Makrut lime is a key ingredient in many Thai, Cambodian, Indonesian, Laotian, Malaysian, and Philippine cuisines. There are two essential oils that can be extracted from makrut lime, the makrut lime leaf oil and makrut lime fruit peel oil (in short, makrut oil). The essential oils have been used as flavour and fragrance agents, as well as in perfumery and medicinal preparation[4].

Essential oils extracted from plants may have antibacterial properties[5] with synergistic interactions among them[6]. Essential oils usually consist of a large number of components and it is likely that their mode of action involves many targets in bacterial cells. A number of essential oil components have been identified as antibacterials such as carvacrol[6–8], citral[7], eugenol[6,7], geraniol[7], perillaldehyde[6,7], and thymol[8]. In addition, essential oils have antioxidant[2], repellent, insecticidal[9], antifungal[10], antiviral[11], and antiparasitic activities[12]. Makrut lime oil was reported

Fig. 1 Fruits and leaves of makrut lime (*Citrus hystrix*).

to be effective against 20 serotypes of *Salmonella* and 5 species of other enterobacteria[13]. This study aimed to evaluate the inhibitory effect of makrut lime essential oils against bacterial respiratory pathogens and to determine the active components responsible for the inhibitory activity.

MATERIALS AND METHODS

The makrut leaf oil (batch no. 5209234-1/1009; density = 0.86 g/ml) and makrut oil (batch no. 5209234/1009; density = 0.87 g/ml), were obtained from Thai-China Flavours and Fragrances Co., Ltd. The products were prepared by steam distillation. The components of the essential oils were analysed by gas chromatography-mass spectrometry (GC-MS). Samples of makrut leaf oil and makrut oil were diluted to 100 ppm prior to the analysis. The diluted oils were then analysed by GC-MS[14], using a Hewlett-Packard HP 6890 Series GC System and Hewlett-Packard HP 5973 Mass selective detector. Samples were injected on a capillary column (HP-INNOWAX; 60 m $\times$ 0.25 mm $\times$ 0.25 μm) well coated with cross linked PEG. The carrier gas was Helium (99.99%), at the flow rate of 0.80 ml/min. The inlet injection temperature was 200 °C with the inlet split ratio of 5:1. The GC oven temperature was kept at 70 °C for 2 min and programmed to 220 °C at a rate of 4 °C/min, held for 5 min and finally at 10 °C/min and programmed to 300 °C, held for 3 min. The GC/MS interface (Auxs) temperature was set at 250 °C. The Mass Spectrometer condition was as follows: EL Source temperature 250 °C, Emission 34.6, Ele energy 70 eV, Qoadrole temperature 150 °C, Scan mode: 303450; EM Volt 1450. The MS library was Wiley 275/version 6.0, NBS 45 K. The pure compounds in analytical grade of citronellal (density = 0.86 g/ml), limonene (density = 0.84 g/ml), terpinene-4-ol (density = 0.93 g/ml), α-terpineol (density = 0.94 g/ml) used in the testings were purchased from Sigma Chemical Co., USA.

A total of 411 clinical isolates used in the inhibitory testing were *Acinetobacter baumannii* (50 isolates), Groups A (61 isolates), B (27 isolates), C (4 isolates), F (3 isolates), G streptococci (11 isolates), *Haemophilus influenzae* (52 isolates), *Moraxella catarrhalis* (52 isolates), methicillin-resistant *Staphylococcus aureus* (MRSA; 50 isolates), methicillin-sensitive *S. aureus* (MSSA; 50 isolates) and *Streptococcus pneumoniae* (51 isolates). These isolates were from respiratory tract specimens (throat swab, pus in tonsils and adenoid tissues, sputum, bronchial wash, bronchoalveolar lavage) or blood collected from patients who had respiratory symptoms at Siriraj Hospital, a tertiary care centre in Bangkok, during January 2008–December 2010. Sputum was considered acceptable for culture if it contained more than 25 polymorphonuclear cells and less than 25 epithelial cells per low-powered field. Bacterial identification, disc-diffusion, and broth microdilution methods were performed by standard microbiological techniques[15,16]. Identification of the Lancefield groups of β-haemolytic streptococci was done by using a rapid latex agglutination test kit, Remel streptex (Remel Co., USA) according to the manufacturer's guideline.

The antibacterial activity of the essential oils was tested by disc-diffusion and broth microdilution methods. In the disc-diffusion method, a sterile Whatman disc (6 mm) saturated with 10 μl of essential oil[17] was put on a lawn of a bacterial inoculum which has a turbidity in 1% (w/v) tryptone water equated to a McFarland No 0.5 standard (approximately 10^8 CFU/ml). All values of inhibition zones were expressed as mean $\pm$ standard deviation. *S. pneumoniae* ATCC 49619, *H. influenzae* ATCC 49247, and *S. aureus* ATCC 25923 were used as quality controls. For broth

Table 1 Minimal inhibitory concentrations (MIC) and Minimal bactericidal concentrations (MBC).

Pathogens	Makrut lime leaf oil (mg/ml)				Makrut lime oil (mg/ml)			
	MIC range	MIC_{50}	MIC_{90}	MBC range	MIC range	MIC_{50}	MIC_{90}	MBC range
A. baumannii	2.10–17	4.30	8.50	4.30–68	1.10–4.40	1.10	4.40	1.10–8.70
Group A strep	0.30–8.50	1.10	4.30	0.30–8.50	0.30–4.40	2.20	2.20	0.30–4.40
Group B strep	0.30–8.50	0.50	1.10	0.30–8.50	0.30–8.70	1.10	2.20	0.30–17.40
Group C strep	0.30–4.30	0.50	4.30	0.30–4.30	0.30–4.40	0.50	4.40	0.30–4.40
Group F strep	0.30–4.30	2.10	4.30	0.30–4.30	0.50–4.40	1.10	4.40	0.50–4.40
Group G strep	0.30–4.30	0.30	2.10	0.30–4.30	0.30–4.40	1.10	4.40	0.50–4.40
H. influenzae	0.06–0.50	0.30	0.30	0.06–1.10	0.06–0.50	0.10	0.30	0.06–1.10
M. catarrhalis	0.03–0.25	0.03	0.03	0.03–0.50	0.03–0.25	0.03	0.03	0.03–0.50
MSSA	1.10–34	8.50	17	8.50–68	1.10–8.70	2.20	4.40	1.10–8.70
MRSA	2.10–68	8.50	34	8.50–68	1.1–17.40	2.20	4.40	2.20–17.40
S. pneumoniae	0.30–4.30	0.50	2.10	0.30–8.50	0.30–4.40	0.50	1.10	0.30–4.40

MIC_{50} and MIC_{90} are the minimal inhibitory concentrations required to inhibit the growth of 50% and 90% of bacteria, respectively.

microdilution method, the broth was supplemented with 0.25% (v/v) tween-20 (Sigma Chemical Co., USA). Two-fold serial dilutions of essential oils were prepared in a microtitre plate. The minimal inhibitory concentration (MIC) was lowest concentration of essential oil inhibiting visible bacterial growth after incubation for 20–24 h at 35 °C. Cell suspensions (1 loop) from the wells showing no growth were subcultured on sheep blood agar (or chocolate agar in case of *H. influenzae*) to determine if the inhibition was reversible or permanent. Minimal bactericidal concentration (MBC) was determined as the highest dilution (i.e., lowest concentration) at which no growth occurred on the agar plates.

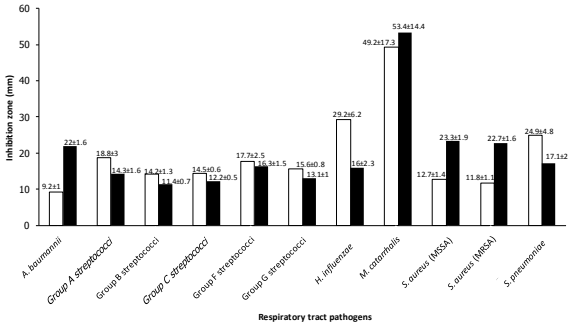

Fig. 2 Inhibition zones of makrut lime leaf oil (□) and makrut lime oil (■) by disc-diffusion method. Values were means ± standard deviation.

RESULTS

The results of gas chromatography analysis found that the most predominate component in makrut leaf oil was citronellal (80.04%) whereas in makrut oil were limonene (40.65%), terpinene-4-ol (13.71%) and α-terpineol (13.20%). Other components were presence in trace.

Both makrut leaf oil and makrut oil exhibited antibacterial properties for all bacteria tested by the disc-diffusion method, especially against *M. catarrhalis* (Fig. 2). The MIC and MBC of makrut leaf oil and makrut oil were lowest against *M. catarrhalis* and *H. influenzae* (indicating best activity), followed by *S. pneumoniae*, *Streptococcus* spp., *A. baumannii*, MSSA, and MRSA, respectively (Table 1). Taken together, the MIC of makrut leaf oil was in the range of 0.06–68 mg/ml and that for makrut oil was 0.03–17.40 mg/ml. Although the MIC and MBC varied among tested bacteria, the MIC in most cases was equivalent to MBC indicating a bactericidal action of oil.

In the active component testing citronellal, the major component in makrut leaf oil, was found to be more active than the whole makrut leaf oil against *A. baumannii*, *Streptococcus* spp., MSSA, and MRSA (Table 2). Likewise, α-terpineol, followed by terpinene-4-ol, were more active against *A. baumannii*, *Streptococcus* spp., and *H. influenzae* ATCC 49766 than makrut lime oil (Table 3). However, limonene, the most predominate component of makrut oil, had a much less antibacterial activity.

The antibacterial activity of makrut leaf oil and makrut lime oil was found to be stable upon storage at room temperature up to 4 months. The MIC and MBC values at 4 months were not different from those at 1, 2, and 3 months of storage.

Table 2 Effect of citronellal in makrut lime leaf oil on pathogens.

Pathogens	Makrut lime leaf oil			Major component Citronellal		
	Inhibition zone (mm)	MIC (mg/ml)	MBC (mg/ml)	Inhibition zone (mm)	MIC (mg/ml)	MBC (mg/ml)
A. baumannii	10	8.5	8.5	13	1.1	4.3
Group A streptococci	16	4.3	4.3	15	0.5	1.1
Group B streptococci	11	4.3	4.3	10	1.1	1.1
Group C streptococci	15	4.3	4.3	15	0.3	0.3
Group F streptococci	17	2.1	8.5	17	1.1	1.1
Group G streptococci	16	4.3	4.3	15	1.1	1.1
H. influenzae	21	0.3	0.3	20	0.5	0.5
H. influenzae ATCC 49766	22	0.5	0.5	20	0.3	0.5
S. aureus ATCC 25923	19	1.1	1.1	22	1.1	1.1
S. aureus (MSSA)	14	4.3	34	14	1.1	8.6
S. aureus (MRSA)	15	8.5	34	15	1.1	8.6
S. pneumoniae	24	0.3	0.3	24	0.5	0.5
*S. pneumonia*e ATCC 49619	23	0.5	0.5	23	0.5	0.5

MIC = Minimal inhibitory concentration; MBC = Minimal bactericidal concentration.

Table 3 Effect of limonene, terpinen-4-ol, and α-terpineol in makrut lime oil on pathogens.

Pathogens	Makrut lime oil			Various components						
				Limonene			Terpinene-4-ol		α-terpineol	
	Inh. zone (mm)	MIC (mg/ml)	MBC (mg/ml)	Inh. zone (mm)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
A. baumannii	22	1.10	1.10	6	> 134	> 134	5	5	0.07	0.10
Group A streptococci	14	2.20	2.20	6	> 134	> 134	1.25	2.50	0.60	0.60
Group B streptococci	11	4.40	4.40	7	> 134	> 134	0.30	0.60	0.60	0.60
Group C streptococci	12	4.40	4.40	6	> 134	> 134	0.15	0.15	0.60	0.60
Group F streptococci	15	1.10	1.10	6	> 134	> 134	1.25	2.50	1.20	1.20
Group G streptococci	13	4.40	4.40	6	> 134	> 134	0.15	0.30	1.20	1.20
H. influenzae	17	0.30	0.30	12	67	67	0.15	0.30	0.70	0.70
H. influenzae ATCC 49766	17	0.10	0.10	12	67	67	5	5	0.07	0.07
S. aureus ATCC 25923	26	1.10	1.10	11	> 134	> 134	10	20	0.30	1.20
S. aureus (MRSA)	20	2.20	2.20	6	> 134	> 134	2.50	5	2.40	2.40
S. aureus (MSSA)	21	4.40	4.40	6	> 134	> 134	10	20	2.40	2.40
S. pneumoniae	20	0.30	0.30	11	33	67	0.15	0.15	0.30	0.30
S. pneumoniae ATCC 49619	16	0.50	0.50	8	67	> 134	2.50	2.50	0.60	0.60

MIC = Minimal inhibitory concentration; MBC = Minimal bactericidal concentration.

DISCUSSION

Our study revealed the antibacterial effect of makrut leaf oil and makrut oil against respiratory bacterial pathogens. A preliminary study of antibacterial activities on medicinal herbs of Thai food ingredients against food-borne pathogens such as *Bacillus cereus*, *S. aureus*, and *Salmonella* Typhi was reported[18]. Our results are in agreement with a previous study that found makrut oil and ethanol extract of makrut fruit peels had a greater antibacterial effect than the extracts of makrut leaves[13]. The hydrophobicity of essential oils might enable them to partition in the lipid component of bacterial cell membrane, rendering them permeable and leading to leakage of bacterial cell contents[6].

This study showed excellent activity of makrut leaf oil and makrut oil against many respiratory bacteria at the various activity levels. The results of disc diffusion were not highly correlated with MIC and MBC. Disc diffusion is a screening method usually used as a preliminary check for antibacterial

activity prior to the more detailed study in liquid medium to determine MIC and MBC. There are many factors that may affect the results of disc-diffusion test, e.g., volume of essential oil placed on paper discs, thickness of agar, diffusion ability of oil in agar, and variation of essential oil concentration. Therefore, disc-diffusion method is not suitable for comparison the efficacy of essential oil. The broth microdilution method measures the strength of antibacterial activity and better be used for comparison.

The increasing incidence of multi-drug resistant *A. baumannii* and MRSA underscored the urgent need for effective alternative drug. The extraction of essential oil from plant is a crucial step for biosynthesis and may lead to the discovery of new drug for infectious diseases. This study revealed that all the multi-drug resistant bacteria were highly sensitive to makrut oil and makrut leaf oil. More than 80% of *A. baumannii* used in our study were resistant to all drugs tested in our routine clinical laboratory such as aminoglycosides, ampicillin, cephalosporins, carbapenem, fluoroquinolones; and only sensitive to colistin which is a drug with high nephro- and neurotoxic potential. All MRSA in our study were hospital-acquired and resistant to most drugs available.

The pure major lipid components of the essential oils, citronellal, α-terpineol, terpinene-4-ol, and limonene, were chosen to test for the active components responsible for the antibacterial effect. In a previous study the pure lipid compounds in essential oils of *Cinnamosma fragrans*, linalool and 1,8-cineole, were reported to be the active antimicrobial components[19]. To our knowledge, this is the first report of activity of the active components of makrut essential oils. The most interesting result was that α-terpineol was the most active component. Although this component is present only 13.20% in makrut oil, bioengineering of its synthesis in plant would be possible to provide greater yield. Our results were in agreement with previous studies that α-terpineol[6,20] and terpinene-4-ol[20] had antibacterial properties against foodborne pathogens, but limonene was not[7].

The results from this study could be applied to clinical use. For example, we found group A streptococci, the most common pathogenic bacteria causing sore throat in human, was very sensitive to makrut lime essential oils. Development of these essential oils throat spray may lead to the prevention or treatment of streptococcal pharyngotonsillitis. Likewise, an essential oil throat spray may be able to prevent nosocomial acquired pneumonia from resistant bacteria in hospitalized patients with respiratory conditions. Further studies are needed and may lead to clinical use of alternative medicine from natural sources.

In conclusion, makrut lime leaf oil and makrut lime oil had excellent antibacterial activities against various respiratory pathogens including the multi-resistant bacteria. These essential oils may be important for drug development for prevention and treatment of many bacterial diseases.

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