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Original Research

Bactericidal activity of herbal volatile oil extracts against multidrug-resistant *Acinetobacter baumannii*

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ABSTRACT

Aim: The aim of the study is to investigate the antibacterial activity of 10 volatile oils extracted from medicinal plants, including galangal (*Alpinia galanga* Linn.), ginger (*Zingiber officinale*), plai (*Zingiber cassumunar* Roxb.), lime (*Citrus aurantifolia*), kaffir lime (*Citrus hystrix* DC.), sweet basil (*Ocimum basilicum* Linn.), tree basil (*Ocimum gratissimum*), lemongrass (*Cymbopogon citratus* DC.), clove (*Syzygium aromaticum*), and cinnamon (*Cinnamomum verum*) against four standard strains of *Staphylococcus aureus, Escherichia coli*, *Pseudomonas aeruginosa, Acinetobacter baumannii,* and 30 clinical isolates of multidrug-resistant *A. baumannii* (MDR-*A. baumannii*). **Materials and Methods:** Agar diffusion, minimum inhibitory concentration, and minimum bactericidal concentration (MBC) were employed for the determination of bactericidal activity of water distilled medicinal plants. Tea tree oil (*Melaleuca alternifolia*) was used as positive control in this study. **Results:** The results indicated the volatile oil extracted from cinnamon exhibited potent antibacterial activity against the most common human pathogens, *S. aureus*, *E. coli*, *P. aeruginosa,* and *A. baumannii*. Most of volatile oil extracts were less effective against non-fermentative bacteria, *P. aeruginosa*. In addition, volatile oil extracted from cinnamon, clove, and tree basil possessed potent bactericidal activity against MDR-*A. baumannii* with MBC_{90} of 0.5, 1, and 2 mg/mL, respectively. **Conclusions:** The volatile oil extracts would be useful as alternative natural product for the treatment of the most common human pathogens and MDR-*A. baumannii* infections.

KEY WORDS: Medicinal plant, multidrug-resistant-*Acinetobacter baumannii*, volatile oil

INTRODUCTION

Acinetobacter baumannii is one of the most important drug-resistant pathogens worldwide. Recently, the World Health Organization indicated that drug-resistant A. *baumannii* is defined as the first priority pathogen, in which researches and developments for new antibiotics are urgently needed [1]. The bacteria has been revealed to persist on dry surfaces for a month and presented several drug-resistant mechanisms including drug efflux pumps, drug-inactivating enzymes, and drug target mutations [2]. Infected patients have many serious diseases including septicemia, pneumonia, and urinary tract infections [2,3]. The number of global drug-resistant A. *baumannii* was vary in estimation [4]; therefore, the high prevalence accounted to be approximately 54% and 77% of A. *baumannii* isolates have been revealed in Italy and India, respectively [5,6]. In Thailand, surveillance in the 2010 period indicated the rate of multidrug-resistant (MDR)-A. *baumannii* collected from clinical specimens was approximately 59% [7]. Regarding the limit of antibiotic treatment, many studies have focused on the alternative drugs and phytomedicine. Several studies revealed the effectiveness of extracted herbs on drug-resistant pathogens including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, and MDR-A. *baumannii*, whereas the antimicrobial activity of volatile oils extracts was rarely reported [8,9]. Herein, 10 volatile oils extracted from various medicinal plants were determined for their inhibitory effect on the growth of the most common human pathogens and MDR-A. *baumannii*.

MATERIALS AND METHODS

Bacterial Strains

S. aureus ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and A. *baumannii* ATCC 19606 were purchased from the Department of Medical

Sciences, Ministry of Public Health, Thailand. 30 clinical isolates of MDR-*A. baumannii* were collected from the Diagnostic Laboratory, Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand, during February-April, 2012. Both biochemical tests followed by Constantiniu *et al*. [10] and molecular biology test using amplified ribosomal DNA-restriction enzyme analysis were performed for identification of *A. baumannii*. Primers used for 16S rDNA gene amplification were designed as followed by the previous report [11]. The antimicrobial susceptibility testing was performed using disk diffusion method following the Clinical and Laboratory Standards Institute (CLSI) guidelines [12,13]. The MDR was defined according to the unsusceptible of at least one in three agents of antimicrobial classes [14]. All 30 clinical isolates resisted to eight antibiotics in six antimicrobial classes consisting of amikacin, piperacillin/tazobactam, ciprofloxacin, cefoperazone/sulbactam, ceftazidime, trimethoprim/sulfamethoxazole, imipenem, and meropenem.

Volatile Oils Extraction

Volatile oils were extracted from 10 medicinal plants by water distillation. Galangal, ginger, plai, lime, kaffir lime, sweet basil, tree basil, lemongrass, clove, and cinnamon were selected in this study [Table 1]. The material was subjected to hydrodistillation using a Clevenger-type glass apparatus for 3-5 h [15]. Yields of the volatile oils obtained from the plants were calculated as the percent yield. All volatile oils were stored at 4°C until used.

Antibacterial Activity Testing

The antimicrobial activity testing was modified from Prabuseenivasan *et al*. [16]. Briefly, bacterial suspension was adjusted to McFarland standard No. 0.5 (1×10^8 CFU/mL) and spread over the Mueller-Hinton agar (MHA) plates using a sterile cotton swab. Each volatile oil was dissolved in 10% aqueous dimethyl sulfoxide (DMSO) with 0.5% v/v Tween 80 and sterilized by filtration. Sterilized disks (Whatman No. 5, 6 mm diameter) were impregnated with 20 μL of volatile oils and placed on the surface of MHA. The volatile dissolving buffer (10% aqueous DMSO, 0.5% v/v Tween 80) and tea tree oil were used as negative and positive control, respectively. After incubation at 37°C for 16-18 h, the inhibition zone was measured. All experiments were performed independently in triplicate and mean value was calculated.

Table 1: Medicinal plants used in this study

Common name	Botanical name	Families	Parts
Galangal	*Alpinia galanga* (Linn.) Swartz	Zingiberaceae	Rhizome
Ginger	*Zingiber officinale* Roscoe	Zingiberaceae	Rhizome
Plai	*Zingiber cassumunar* Roxb.	Zingiberaceae	Rhizome
Lime	*Citrus aurantifolia* Swingle	Rutaceae	Peel
Kaffir lime	*Citrus hystrix* DC.	Rutaceae	Peel
Sweet basil	*Ocimum basilicum* Linn.	Lamiaceae	Leaf/stem
Tree basil	*Ocimum gratissimum*	Lamiaceae	Leaf/stem
Lemongrass	*Cymbopogon citratus* DC. Stapf.	Poaceae	Leaf/stem
Clove	*Syzygium aromaticum* (L.) Merr. & Perry	Myrtaceae	Bud
Cinnamon	*Cinnamomum verum* J. Presl	Lauraceae	Bark

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Based on CLSI guidelines, MICs were determined by using broth microdilution method [17]. The preparation of water-insoluble volatile oils was slightly modified from the recommended CLSI guidelines. Each volatile oil was dissolved with 50% DMSO and serial 2-fold diluted in a 96-well microtiter plate ranging from 0.125 to 8 mg/mL. The bacterial suspension was diluted into approximately 1×10^6 CFU/mL, and 100 μL of bacterial suspension was applied to each well. The inoculum with 2.5% DMSO and media without inoculum were used as cell and media control, respectively. The microplates were incubated at 35°C for 20 h. Due to the turbidity of volatile oil suspensions, iodonitrotetrazolium chloride (INT) (BioChemica) was used as color indicator to visualize the bacterial growth [18]. The MIC was detected after added 50 μL of 0.2 mg/mL INT and further incubated at 35°C for 30 min. To determine the MBC, 10 μL of bacterial inoculums were taken aseptically from the wells with no color change and plated onto MHA plate and incubated at 35°C for 20-24 h. All experiments were separately performed in triplicate and calculated as mode, median, and 90th percentile. Median MIC value (MIC_{50}) represented the MIC value of one-half of the tested population. The 90th percentile (MIC_{90}) represented the MIC value of 90% of the tested population [19]. Likewise, MBC_{50} and MBC_{90} were the MBC values at which 50% or 90% of isolates in a tested population were killed, respectively.

Statistical Analysis

In this study, the inhibition zone of each volatile oil was compared with tea tree oil and statistically analyzed using independent Student's *t*-test (SPSS version 22). The MIC and MBC values in each of the tested volatile oils and tea tree oil were statistically analyzed by Mann–Whitney *U*-test (SPSS version 22).

RESULTS

The percent yields of the water-distilled volatile oils were calculated. The yields ranged from 0.1% to 4.3% w/w – ginger (0.1), lemongrass (0.2), tree basil (0.2), galangal (0.3), sweet basil (0.3), cinnamon (0.9), lime (1.0), plai (1.1), kaffir lime (2.1), and clove (4.3). A disk diffusion method was performed to preliminarily evaluate the antibacterial activity of the volatile oils against four reference bacterial strains (*S. aureus*, *E. coli*, *P. aeruginosa*, and *A. baumannii*). Except *P. aeruginosa*, the positive control tea tree oil represents antibacterial activity to the bacteria tested. No inhibition zone was observed in volatile dissolving buffer. The difference in the inhibition zones between tea tree oil and each volatile oil was analyzed using independent Student's *t*-test. The results indicated that cinnamon oil exhibited a high potency of antibacterial activity against all bacterial strains tested ($P < 0.01$). Sweet basil and lemon grass were highly active against *S. aureus* and *E. coli*; however, these volatile oils showed no significant activity when tested with both non-fermentative Gram-negative bacilli, *A. baumannii*, and *P. aeruginosa*. The volatile oils of clove, tree

basil, lime, and ginger were moderately active against some bacterial strains ($P < 0.05$). The antibacterial activity of plai and kaffir lime was rather inactive compared to tea tree oil. The inhibition zones of various volatile oils against *S. aureus*, *E. coli*, *P. aeruginosa*, and *A. baumannii* standard strains were shown in Figures 1-4, respectively. Many volatile oils showed an inhibitory effect against MDR-*A. baumannii* including tea tree oil [Figure 5]. However, the mean of the inhibition zones of the cinnamon and clove oils was significantly higher than tea tree oil ($P < 0.01$). Both standard strains of *A. baumannii* ATCC 19606 and MDR-*A. baumannii* isolates were determined for MIC and MBC by broth microdilution method. The MIC and MBC of the positive control tea tree oil against *A. baumannii* ATCC 19606 were 2 and 4 mg/mL, respectively. Cinnamon oil was highly active, with MIC and MBC values of 0.25 mg/mL. The MICs and MBCs of the volatile oils tested against *A. baumannii* ATCC 19606 were shown in Table 2. The MICs and MBCs of each volatile oil tested against MDR-*A. baumannii* isolates were statistically analyzed using Mann–Whitney *U*-test. The modes were equivalent to the medians. The tea tree oil exhibited anti-MDR-*A. baumannii* activity with MIC_{90} and MBC_{90} of 2 and 4 mg/mL, respectively. The mean MICs of four volatile oils, cinnamon, clove, tree basil, and kaffir lime were significantly lower than the positive control tea tree oil with the MIC_{90} of 0.25, 0.5, 1, and 1 mg/mL, respectively ($P < 0.05$). The MIC and MBC of the volatile oils against 30 clinical strains of MDR-*A. baumanni* were shown in Table 3.

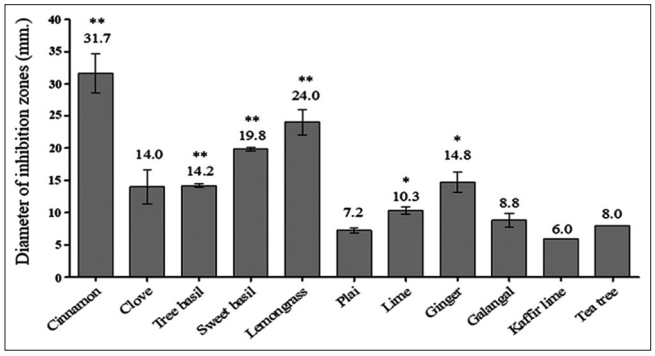

Figure 1: Inhibition zones of 11 volatile oils against *Staphylococcus aureus* ATCC 25923. *Indicated a significant difference at $P < 0.05$, and ** indicated a highly significant difference at $P < 0.01$

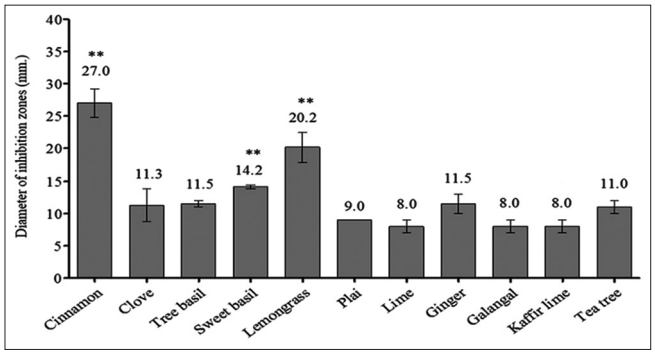

Figure 2: Inhibition zones of 11 volatile oils against *Escherichia coli* ATCC 25922. **Indicated a highly significant difference at $P < 0.01$

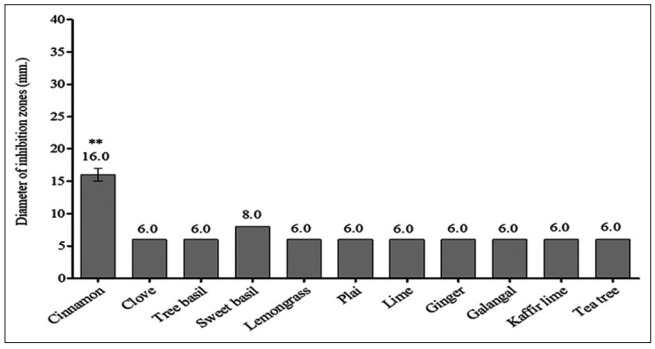

Figure 3: Inhibition zones of 11 volatile oils against *Pseudomonas aeruginosa* ATCC 27853. **Indicated a highly significant difference at $P < 0.01$

DISCUSSION

The most problematic of *A. baumannii* infections nowadays are the MDR and it becomes a serious issue, in which most antibiotics drug therapy are unable to cure the diseases. Finding new and effective antibacterial compounds against MDR-*A. baumannii* is urgent; volatile oils are one such compound worth screening. In this study, 10 volatile oils were determined for antibacterial activity against *S. aureus*, *E. coli*, *P. aeruginosa*, *A. baumannii*, and 30 isolates of MDR-*A. baumannii*. The antimicrobial activity of tea tree oil against aerobic bacteria has previously been

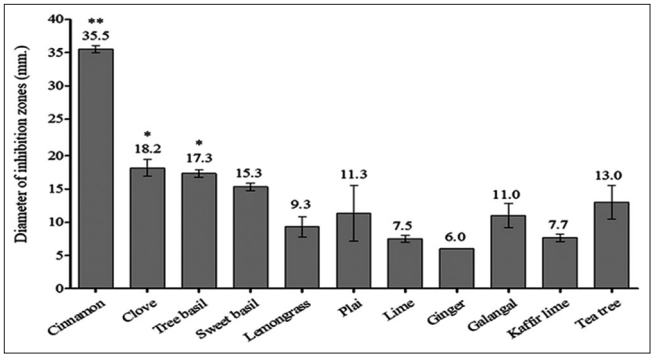

Figure 4: Inhibition zones of 11 volatile oils against *Acinetobacter baumannii* ATCC 19606. *Indicated a significant difference at $P < 0.05$, and **Indicated a highly significant difference at $P < 0.01$

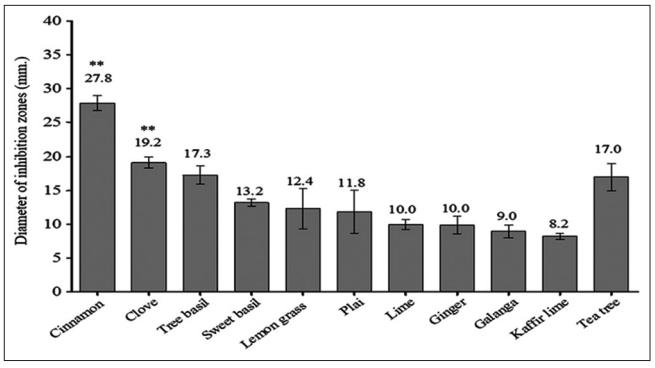

Figure 5: Average inhibition zone in diameter obtained from various volatile oils against 30 multidrug-resistant - *Acinetobacter baumannii* isolates. **Indicated a highly significant difference at $P < 0.01$

Table 2: MICs and MBCs of volatile oils against standard strain *A. baumannii* ATCC 19606

Volatile oils	MIC (mg/mL)	MBC (mg/mL)
Cinnamon	0.25	0.25
Clove	0.5	1
Tree basil	1	1
Sweet basil	1	8
Lemongrass	1	>8
Plai	2	>8
Lime	2	8
Ginger	2	4
Galangal	>8	>8
Kaffir lime	1	2
Tea tree	2	4

MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, *A. baumannii: Acinetobacter baumannii*

Table 3: The MICs and MBCs of volatile oils against MDR-*A. baumannii*

Volatile oils	MIC (mg/mL)		MBC (mg/mL)	
	MIC_{50}	MIC_{90}	MBC_{50}	MBC_{90}
Cinnamon[a,b]	0.25	0.25	0.5	0.5
Clove[a,b]	0.5	0.5	1	1
Tree basil[a,b]	1	1	2	2
Sweet basil	2	2	4	8
Lemongrass[b]	2	2	2	4
Plai	2	4	4	>8
Lime	2	4	4	>8
Ginger	2	4	4	4
Galangal	>8	>8	>8	>8
Kaffir lime[a,b]	1	1	2	4
Tea tree	1	2	4	4

[a]Indicated the volatile oil that had the mean of MIC significantly lower than tea tree oil ($P<0.05$). [b]Indicated the volatile oil that had the mean of MBC significantly lower than tea tree oil ($P<0.05$). MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, *A. baumannii: Acinetobacter baumannii,* MDR: Multidrug-resistant

published; the compounds involved in its antibacterial activity such as terpinen-4-ol, α-terpinene and γ-terpinene have been characterized [20]. Similarly to Carson and Riley's study, an inactive effect of tea tree oil against *P. aeruginosa* was observed in this study [20]. A. *baumannii* ATCC 19606 and MDR-A. *baumannii* isolates could be inhibited by tea tree oil with MIC_{90} and MBC_{90} concentrations of 2 and 4 mg/mL, respectively.

The extracted volatile oils were preliminarily screened for antibacterial activity by disc diffusion method. Among the medicinal plants tested, cinnamon oil exerted the highest activity to inhibit the growth of all bacteria while sweet basil and lemon grass strongly inhibited in some bacteria. Standard broth microdilution method was performed and revealed that the volatile oils of cinnamon, clove, tree basil, and kaffir lime showed strong antibacterial activity against MDR-A. *baumannii* isolates. The antimicrobial activity of cinnamon oil against *S. aureus*, *E. coli*, *Acinetobacter lwoffii*, and *P. aeruginosa* has previously been demonstrated [21]. Recently, Rath and Padhy indicated that the MIC and MBC of methanolic extract of both clove and cinnamon against MDR-A. *baumannii* were 1.51 and 3.41 mg/mL, respectively [22]. The inhibition zones of tree basil and tea tree oil were indifferent; the major constituents of tree basil volatile oil have previously been identified including thymol, γ-terpinene, eugenol, and ρ-cymene [23]. The mode of antibacterial action of thymol still unknown but it has been proposed to involve in outer and inner membrane disruption [24]. Cinnamon oil possessed the highest inhibition effect against all bacterial strains and MDR-A. *baumannii* isolates. Gas chromatography–mass spectrometry analysis was performed in this study to identify the active ingredients with antimicrobial activity. Thirteen peaks were observed and interpreted based on specific retention time compared to a reference database. The major ingredients in cinnamon oil were cinnamaldehyde (75.89%), *trans*-cinnamyl acetate (7.07%), hydrocinnamaldehyde (2.39%), and 1,8-cineole (2.17%) (data not shown). Cinnamaldehyde has previously been reported to inhibit in both Gram-positive and Gram-negative bacteria [25,26]. Noteworthy, aldehyde groups might be associated with the antimicrobial activity of cinnamon oil since these chemicals have an ability to covalently cross-link with the amine groups of DNA and proteins and interfere their functions in the cells. Although the mode of action of cinnamaldehyde is inconclusive [24], Gill and Holley demonstrated that cinnamaldehyde at a concentration of 30 mM could kill *L. monocytogenese* through its effect on the energy generation and membrane permeability of the bacteria [27,28]. In addition, the interaction of cinnamaldehyde with essential enzymes and bacterial cell wall damage at high concentration has been investigated [29]. Although cinnamaldehyde possesses potent antimicrobial activity against MDR pathogen, its cellular and *in vivo* cytotoxicity have been reported [30,31]. In addition, it has been reviewed to be a cause of allergic reaction in toothpaste [32]. Consequently, a dosage level at which no adverse effects is indispensable determined before use in the future application.

CONCLUSIONS

Our study indicated the antibacterial activity of volatile oils extracted from herbs against several bacteria, including MDR-A. *baumannii*. These plant extracts would be promising antimicrobial agents for further treating of human pathogens, including drug-resistant bacteria.

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