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Mixtures of Essential Oils in an Air Conditioning Prototype to Reduce the Prevalence of Airborne Pathogenic Bacteria

Supayang Piyawan Voravuthikunchai [1, 2,*], Sukanda Minbutra [2], Lavanya Goodla [1], Jennifer Jefferies [3], Somboon Voravuthikunchai [4]

[1]Natural Products Research Center, Faculty of Science,
Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand.
[2]Department of Microbiology, Faculty of Science,
Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand.
[3] www.jenniferjefferies.com, Increasing your Health,
Wealth and Sanity, PO Box 867, North Lakes QLD 4509, Australia
[4] Department of Mechanical Engineering, Faculty of Engineering,
Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

Abstract: The rationale for this work was to test the possibility of creating a protective atmosphere by using natural compounds to minimize hazards from chemicals and to control the risk of common infections. The antimicrobial activities of essential oils of *Agronis fragrans*, *Cinnamomum zeylanicum*, *Lavandula angustifolia*, *Melaleuca alternifolia* (tea tree), *Melaleuca nesophila* (honey myrtle), *Pelargonium x asperum* (geranium), *Pogostemon cablin* (patchouli), *Thymus serpyllum*, and *Thymus vulgaris* were evaluated against an array of environmental-borne pathogenic bacteria. Gram-positive bacteria included *Bacillus subtilis* and *Staphylococcus aureus*; four Gram-negative bacteria covered *Acinetobacter baumanii*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. In addition, eleven isolates of methicillin-resistant *S. aureus* (MRSA) and a multidrug-resistant *A. baumanii* were incorporated. In agar disc diffusion tests, cinnamon had shown the strongest activity that could inhibit all pathogens, followed by lemon thyme, honey myrtle and lavender, while pachouli exhibited the weakest inhibition. Though fragonia possessed a broad range of activity against all pathogens, it did not inhibit some of the MRSA isolates. Cinnamon and lemon thyme demonstrated strong activity measured by minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations. In disk volatilization assay, thyme oil demonstrated the greatest inhibition, followed by lemon thyme, while no inhibition from patchouli was observed. Subsequently, the composition of the atmosphere generated by three different oil blends in our invented portable air-conditioning prototype was assessed. The results showed that mixture of oil blend No. 2 can produce good synergistic effect in reducing the prevalence of airborne pathogens, and thus preventing the spreading of infections.

Key words: *Acinetobacter baumanii*, Air conditioning system, Essential oil, *Staphylococcus aureus*, Multidrug-resistant pathogenic bacteria

Introduction

Droplet infections have long been one of the most deadly branches of infectious diseases. Airborne contamination continues as by far the

*Corresponding author (Supayang Piyawan Voravuthikunchai)
E-mail: < supayang.v@psu.ac.th >

most important route by which infectious diseases spread. In the 21st century, the infections are still a major public health issue for both developing and developed countries. For developing countries, poverty, lack of health infrastructure and sanitation, immigration, trade, globalization contributes to the spread of the diseases. For developed countries, infectious diseases are a threat on the horizon because of the problem of new and drug-resistant microorganisms migrating to the industrialized countries. Numbers of highly antibiotic resistant bacterial strains are increasing. Methicillin-resistant *Staphylococcus aureus* (MRSA) has become increasingly widespread as a cause of both nosocomial and community infections. In 2002, vancomycin-resistant *S. aureus* (VRSA) strains emerged in the United States [3], followed by reports of these isolates from other parts of the World [1,6]. More recently, multidrug-resistant (MDR) *Acinetobacter* spp. have emerged as major causes of nosocomial infections associated with significant morbidity and mortality rates [4,8,12,14].

Apparently concerns over the use of chemicals that could endanger people have been raised. A growing demand from consumers for safe products, desiring fewer synthetic substances together with their increased quality and safety, has resulted in extensive investigations from researchers to assess the feasibility of techniques and to improve the quality and safety of products, while maintaining efficacy. There is a worldwide trend to explore new alternatives to control infectious diseases, giving priority to methods that reduce disease incidence and avoid adverse effects on human health. During the past decades, much attention has been paid to the use of essential oils which are volatile oily liquids obtained from different plant parts. Essential oils from an estimated 3,000 plant species are known, of which about 300 are commercially produced, mainly for the flavours and fragrances market [2].

In spite of having been long recognized for their antibacterial, antifungal, antiviral, insecticidal and antioxidant properties [11,17], the recent interest in alternative natural substances has led to a new scientific awareness of the value of essential oils. In other context, these substances have been extensively studied for their antibacterial activities against a wide range of microorganisms [2,10,18]. Some authors have suggested the use of essential oils for prevention of the transmission of resistant and harmful pathogens including MRSA [16].

Selected essential oils commonly employed in aromatherapy were used in this study. These included *Agronis fragrans* J.R. Wheeler & N.G. Marchant (fragonia), *Cinnamomum zeylanicum* Blume Cheel (cinnamon), *Lavandula angustifolia* Mill. (Lavender), *Melaleuca alternifolia* (tea tree), *Melaleuca nesophila* (honey myrtle), *Pelargonium x asperum* (geranium), *Pogostemon cablin* (Blanco) Benth. (Patchouli), *Thymus serpyllum* L. (lemon thyme), and *Thymus vulgaris* L. (thyme). The work presented in this communication has three main aims: (i), to check the effectiveness of selected essential oils against different pathogenic bacteria in solid phase by means of the disk diffusion method; (ii), to evaluate their effectiveness in vapour phase; and (iii), to apply the atmosphere generated by the most effective essential oils in a portable air conditioning prototype.

Materials and methods

Essential oils

Essential oils were extracted by hydro distillation from the air dried plant material from cinnamon (*Cinnamomum zeylanicum*), fragonia (*Agonis fragrans*), lemon thyme (*Thymus serpyllum*), patchouli (*Pogostemon cablin* (Blanco) Benth.), geranium (*Pelargonium xasperum*), honey myrtle (*Melaleuca nesophila*), tea tree (*Melaleuca alternifolia*), lavender (*Lavandula angustifolia*), and thyme (*Thymus vulgaris*) using a Clevenger-type apparatus for 4 hours. The oils were extracted with $CHCl_3$ and then were dried over anhydrous Na_2SO_4 and stored under N2 atmosphere at 20°C in a sealed vial until use.

Preparation of essential oil mixtures

Oil blend No. 1	
Cinnamon	10%
Geranium	30%
Tea tree	10%

Thyme	50%
Oil blend No. 2	
Cinnamon	20%
Lemon thyme	30%
Patchouli	30%
Tea tree	20%
Oil blend No. 3	
Cinnamon	23%
Lavender	23%
Lemon thyme	39%
Thyme	15%

Tested bacterial strains

Reference guidelines for culturing and antibiotic sensitivity testing were used [5]. Pathogenic Gram-positive and Gram-negative bacteria commonly found in the environment including *Acinetobacter baumanii*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were tested for their sensitivity to the essential oils. In addition, eleven clinical isolates of MRSA and a clinical isolate MDR *A. baumanii* JVC1053 were included in our study. Clincally bacterial isolates were obtained from the Regional Medical Sciences Center, Songkla and Sonklagarind Hospital, Thailand. Each bacterial strain was suspended in Mueller Hinton broth (MHB, Difco, Detroit, USA) and incubated at 37°C for 18 h. Mueller Hinton agar (MHA, Difco) was used for antibacterial assay.

Paper disc agar diffusion method

Sterile filter paper discs (6 mm) were soaked with 10 ml of each undiluted essential oil. The disks were applied onto the surface of MHA plates seeded with 5-h broth culture of the tested bacteria. The plates were then incubated for 18 h at 35°C. The antibacterial activity was evaluated by measuring the diameter of inhibition zone. The experiments were performed in triplicate and the mean of the diameters of the inhibition zones were recorded. Antibiotic susceptibility discs including amikacin, ampicillin, gentamicin, kanamycin, and tetracyline (10-30 µg) were incorporated as control.

Vapour diffusion assay

Mueller Hinton agar was inoculated with 5 h broth culture of the tested bacteria. Each essential oil (100 µl) at its minimal concentration was added onto 10 mm sterile blank filter discs and subsequently placed on the medium-free cover of each Petri dish. The Petri dishes were placed with the lid upside down, sealed with sterile adhesive tape and incubated at 35°C for 18 h [13]. Mean growth measurements were calculated from triplicates of each bacterial species. The effectiveness of the essential oil was calculated by measuring the diameter (in mm) of the zone of microorganism growth inhibition above the disc. The size of the zone with visible growth reduction around the inhibition zone was also measured.

Determination of minimal inhibitory concentration (MIC) and Minimal bactericidal concentration (MBC)

A modified agar microdilution method [5] was used to determine the MIC of essential oils that produced inhibition zones. One microliter of an overnight culture of each bacterial strain, containing approximately 10^4 CFU, was applied onto MHA supplemented with the essential oils at concentrations ranging from undiluted oil to 1:128. The plates were incubated at 35°C for 18 h. Observations were performed in triplicate and the results were expressed as the lowest concentration of essential oils that produced a complete suppression of colony growth, MIC. Minimal bactericidal concentration using agar dilution method in petri dishes with Millipore filter [13] was performed with the essential oils that showed significant efficacy against each bacterial strain. Amikacin, ampicillin, gentamicin, kanamycin, and tetracyline (10-32 µg/ml) were included as reference standards.

Applications of oil blends in a portable air conditioning prototype

Oil blend was applied to the system as indicated in the diagram (Fig. 1). Each pathogen was grown in trypticase soy broth (TSB, Difco). An aliquot of 0.01 µl of diluted inoculum (approximately 10^4 CFU/ml) was then swabbed on trypticase soy agar (TSA, Difco). In a closed system, the portable air conditioning prototype which was applied with different oil blends was used to create an environment of each oil blend. The

petri-plates with each bacterial inoculum were exposed to the environment created due to the vapours of each oil blend for 8h with a time interval of 1 h each. The bacterial numbers were enumerated at time intervals after exposure to the oil blend of different formulas. The experiments were performed in duplicate and the mean of the colony counts were recorded after 24 h incubation period.

Results and discussion

In agar disc diffusion test (Table 1), cinnamon was the most potent essential oil that inhibited all pathogens, followed by lemon thyme and honey myrtle that could affect most organisms, except *Pseudomonas aeruginosa*. In Contrast, lavender, geranium and tea tree oils showed moderate inhibitory effect on most of the bacteria and patchouli exhibited the weakest inhibition. Though fragonia seemed to possess a broad range of activity against most pathogens, it did not inhibit some of MRSA isolates. It is to be noted that thyme oil produced large inhibition zones against most bacteria including all drug-resistant organisms except *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Cinnamon and lemon thyme demonstrated strong activity measured by minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (Table 2). A number of interesting compounds including (*E*)-cinnamaldehyde, alcohol and acetate, R-phellandrene, and citronellal were detected in cinnamon [13]. Cinnamaldehyde is well-known to have antimicrobial activity [7]. Antimicrobial properties of lemon thyme in vapour phase have also been reported by other workers [15]. Therefore, these two oils were selected as major components in our oil blends.

Comparison of antimicrobial activities in vapour phase is quite difficult since there is no standard method. Although there are many methods used by different authors, not all of them are suitably adapted for fast screening of large quantities of samples. One of the most promising methods for this purpose seems to be disk volatilization test. Hence, we decided to assess antimicrobial properties of each essential oil at its minimal concentration by this method. The results demonstrated that thyme oil possessed the best activity, followed by lemon thyme (Table 3). Patchouli did not produce inhibition zones in any of the pathogenic bacteria. Differences among the volatiles in the essential oils and their hydrophobicity may be responsible for the differences

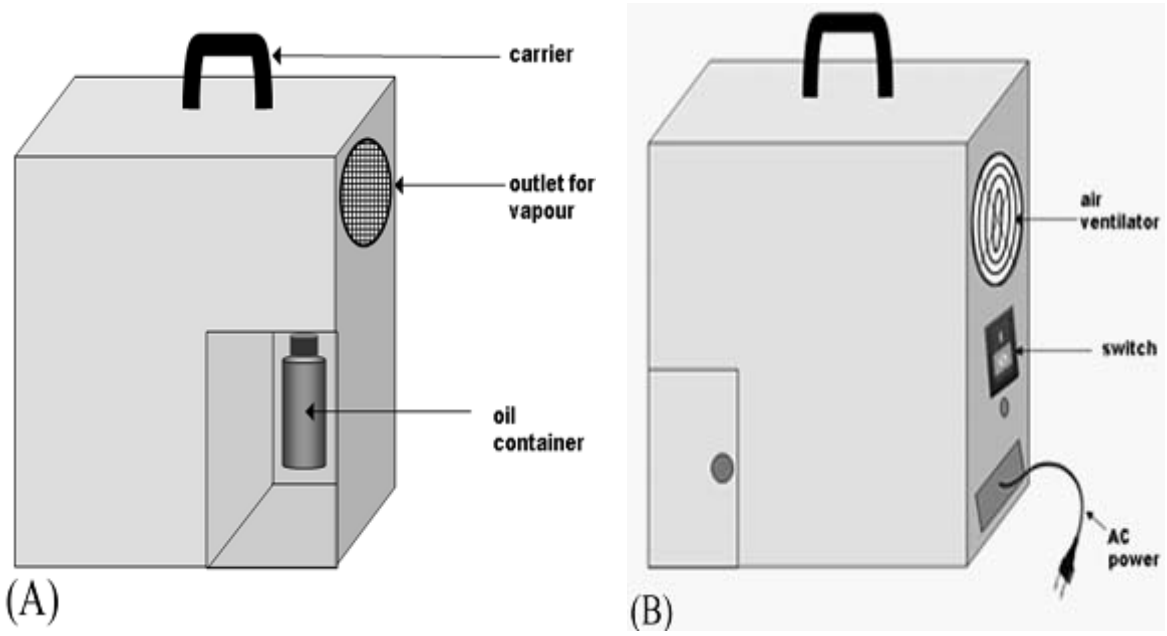

Fig. 1. Diagram of a Portable Air Conditioning Prototype **(A)** Front view and **(B)** Back view

Table 1. Antibacterial activity testing of essential oils against pathogenic bacteria by agar diffusion method

Bacterial strain	Mean values of inhibition zones ± standard errors (mm) produced by essential oils (10 ml/disc)								
	AF	**CZ**	**MA**	**MN**	**PA**	**PC**	**TS**	**TV**	**LA**
Gram-positive bacteria									
Bacillus subtilis PSSCMI0075	35±1	31±1	14±1	13±1	160	22±8	28±2	48±1	15±2
Staphylococcus aureus ATCC 25923	10±1	34±1	27±2	29±2	17±2	295	31±2	64±0	18±1
Methicillin-resistant *S. aureus* (11 isolates)	8±3*	33±0	24±2	32±2	22±3	10±4	352	58±1	12±1
Gram-Negative bacteria									
*Acinetobacter baumanii*ATCC 19606	14±2	30±3	15±2	16±2	9±2	-	23±1	34±0	14±2
Multidrug-resistant *A. baumanii* JVC 1053	18±2	29±3	17±0	14±2	11±4	-	23±2	18±0	-
Escherichia coli ATCC 25922	20±2	23±1	15±1	12±1	111	-	19±2	32±1	22±1
*Klebsiella pneumonia*PSSCMI 0031	12±1	24±2	-	10±1	-	-	14±1	-	12±1
*Pseudomonas aeruginosa*PSSCMI 0048	10±1	24±5	-	-	-	-	-	-	13±2

* = only 9 isolates demonstrated inhibition zones,
- = No inhibition zone; Values are mean ± S.E
AF: *Agonis fragrans* (Fragonia); CZ: *Cinnamomum zeylanicum* (Cinnamon; MA: *Melaleuca alternifolia* (Tea tree);
MN: *Melaleuca nesophila*(Honey myrtle); PA: *Pelargonium x asperum* (Geranium); PC: *Pogostemon cablin*(Patchouli);
TS: *Thymus serpyllum* (Lemon thyme); TV: *Thymus vulgaris* (Thyme); LA: *Lavandula angustifolia* (Lavender);

Table 2. Minimal Inhibition Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of Essential Oils on Pathogenic Bacteria

Bacterial strain	Determination of MIC/MBC (dilution)								
	AF	**CZ**	**MA**	**MN**	**PA**	**PC**	**TS**	**TV**	**LA**
Gram-positive bacteria									
Bacillus subtilis PSSCMI 0075	1:8/1:16	1:64/1:64	1:8/1:8	1:32/1:32	1:3/1:32	1:16/1:8	1:32/1:32	1:16/1:16	1:18/1:16
Staphylococcus aureus ATCC 25923	1:8/1:2	1:64/1:64	1:8/1:2	1:64/1:32	1:16/1:2	1:4/>U	1:32/1:32	1:16/1:4	1:16/1:8
Methicillin-resistant *S. aureus* NPRC 001	1:8/1:4	1:64/1:64	1:8/1:2	1:16/1:16	1:32/1:16	1:4/>U	1:32/1:32	1:16/1:4	1:8/1:8
Gram-Negative bacteria									
Acinetobacter baumanii ATCC 19606	1:1/1:8	1:64/1:64	1:8/1:8	1:16/1:16	1:8/1:4	NA	1:32/1:32	1:8/1:8	1:16/ 1:16
Multidrug-resistant *A. baumanii* JVC 1053	1:8/1:4	1:64/1:64	1:16/1:16	1:16/1:16	1:8/1:8	NA	1:32/1:32	1:8/1:8	1:8 / 1:4
Escherichia coli ATCC 25922	1:8/1:8	1:64/1:64	1:8/1:8	1:16/1:8	1:8/1:8	NA	1:32/1:32	1:8/1:8	1:16 / 1:16
Klebsiella pneumoniae PSSCMI 0031	1:4/1:2	1:64/1:32	1:8/1:8	1:8/1:8	NA	NA	1:16/1:16	1:8/1:8	1:8/1:8
Pseudomonas aeruginosa PSSCMI0048	U/>U	1:64/1:64	1:2/1:2	U/>U	NA	NA	1:16/1:8	1:2/1:2	1:4 / 1:4

NA = Not applicable, U = undiluted oil.

AF: *Agonis fragrans* (Fragonia); CZ: *Cinnamomum zeylanicum* (Cinnamon; MA: *Melaleuca alternifolia* (Tea tree);

MN: *Melaleuca nesophila*(Honey myrtle); PA: *Pelargonium x asperum* (Geranium); PC: *Pogostemon cablin*(Patchouli);

TS: *Thymus serpyllum* (Lemon thyme); TV: *Thymus vulgaris* (Thyme); LA: *Lavandula angustifolia* (Lavender);

Table 3. Antibacterial Activity Testing of Essential Oils against Pathogenic Bacteria in Vapour Contact

Bacterial strain	Mean values of inhibition zones ± standard errors (mm) produced by essential oilsat minimal inhibition concentration								
	AF	**CZ**	**MA**	**MN**	**PA**	**PC**	**TS**	**TV**	**LA**
Gram-positive bacteria									
Bacillus subtilis PSSCMI 0075	ND	43±1	18±1	ND	40±0	-	29±3	38±0	30±2
Staphylococcus aureus ATCC 25923	ND	-	-	ND	48±1	-	35±1	60±3	18±1
Methicillin-resistant *S. aureus* (11 isolates)	ND	-	-	ND	-	-	29±3	52±3	12±1
Gram-Negative bacteria									
Acinetobacter baumanii ATCC 19606	ND	30±0	89±1	ND	-	-	29±1	40±0	-
Multidrug-resistant *A. baumanii* JVC 1053	ND	19±1	-	ND	-	-	18±1	50±1	19±1
Escherichia coli ATCC 25922	ND	22±1	65±2	ND	-	-	29±1	85±2	-
Klebsiella pneumoniae PSSCMI 0031	ND	-	13±0	ND	-	-	-	33±3	20±6
Pseudomonas aeruginosa PSSCMI 0048	ND	-	90±0	ND	-	-	55±2	85±2	15±1

ND = Not determined; - = No inhibition zone; Values are mean ± S.E,
AF: *Agonis fragrans* (Fragonia); CZ: *Cinnamomum zeylanicum* (Cinnamon; MA: *Melaleuca alternifolia* (Tea tree);
MN: *Melaleuca nesophila*(Honey myrtle); PA: *Pelargonium x asperum* (Geranium); PC: *Pogostemon cablin*(Patchouli);
TS: *Thymus serpyllum* (Lemon thyme); TV: *Thymus vulgaris* (Thyme); LA: *Lavandula angustifolia* (Lavender);

in their antimicrobial patterns in two different types of contact test. Thyme oil at absolute concentration could not inhibit *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in agar diffusion test, but the activity is enhanced in vaporization assay, resulting in inhibition of all pathogenic bacteria tested, even at the MIC.

A synergistic effect on antimicrobial activity may be produced from combinations of substances. It has been recently demonstrated that combination of essential oils in vapour phase could wield a synergistic effect on the inhibition of *Listeria monogenes*, *Bacillus cereus*, and *Yersinia enterocolitica* [9]. Taken together the results from previous experiments, we decided to formulate oil blends. According to the results obtained in Table 4 and Table 5, oil blends No. 2 and 3 were further selected to incorporate in our portable air conditioning prototype. Antibacterial efficacy in the atmosphere generated by selected oil blends in our invented air conditioning system was assessed. Exposure of bacterial colonies to the system resulted in a marked decrease in counts, especially with the Gram-positive bacteria (Fig. 2). Both Gram-positive and Gram-negative bacteria demonstrated noticeably smaller size in their colony appearance towards the end of the first hour. This was due to cells being injured from vaporization. Oils in the vapour phase were shown to have significant antimicrobial activity. Thus, the vapour-phase approach appears to be promising as a control protocol and could be applied in the ventilation, creating a protective atmosphere with no risk effects from chemicals.

In conclusion, our results demonstrated that this air conditioning prototype, incorporated with oil blend No. 2 is of advantage in preventing spreading of infections.

Its application is useful, especially for use in risk environment such as hospital wards, theatres, conference rooms and other public spaces.

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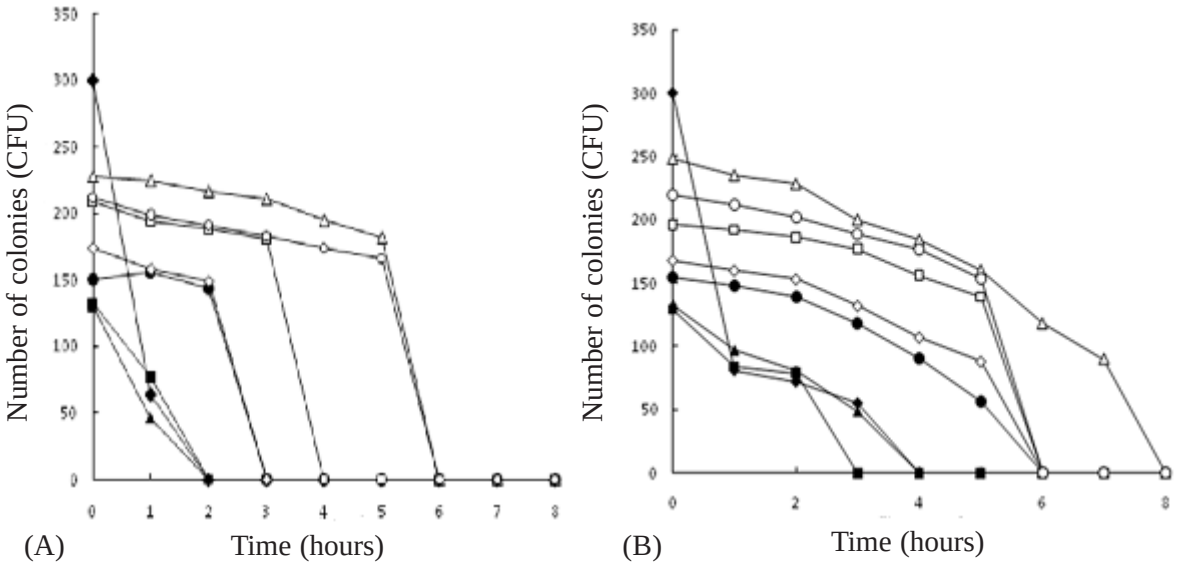

Fig. 2. Antibacterial efficacy of oil blends in air conditioning prototype. During the first hour, all bacteria demonstrated noticeably smaller size in their colony appearance. Reduction in numbers of colony counts: *Bacillus subtilis* (◆), *Staphylococcus aureus* (■), methicillin-resistant *S. aureus* (▲), *Acinetobacter baumanii* (●), multidrug-resistant *A. baumanii* (◆), *Escherichia coli* (□), *Klebsiella pneumoniae* (Δ), and *Pseudomonas aeruginosa* (O) after incorporation of oil blend No. 2 (cinnamon 20%, lemon thyme 30%, patchouli 30%, tea tree 20%) **(A)** and oil blend No. 3 (cinnamon 23%, lavender 23%, lemon thyme 39%, thyme 15%) **(B)**.

Table 4. Antibacterial activity testing of blended essential oils against pathogenic bacteria by agar diffusion method

Bacterial strain	**Mean values of inhibition zones ± standard errors (mm) produced by blended oils (10 ml/disc)**		
	Oil blend 1 (Cinnamon 10%, Geranium 30%, Tea Tree 10%, Thyme 50%)	**Oil blend 2 (Cinnamon 20%, Lemon thyme 30%, Patchouli 30%, Tea tree 20%)**	**Oil blend 3 (Cinnamon 23%, Lavender 23%, Lemon thyme 39%, Thyme 15%)**
Gram-positive bacteria			
Bacillus subtilis PSSCMI 0075	29±1	24±1	25±1
Staphylococcus aureus ATCC 25923	31±1	29±1	28±1
Methicillin-resistant *S. aureus* (11 isolates)	24±1	19±1	25±1
Gram-Negative bacteria			
Acinetobacter baumanii ATCC 19606	20±1	23±1	23±1
Multidrug-resistant *A. baumanii* JVC 1053	18±1	22±1	22±1
Escherichia coli ATCC 25922	16±1	16±1	20±1
Klebsiella pneumoniae PSSCMI 0031	11±1	15±1	-
Pseudomonas aeruginosa PSSCMI 0048	10±1	21±1	10±1

Values are mean ±S.E

Table 5. Minimal inhibition concentration (MIC) and minimal bactericidal concentration (MBC) of blended oils against pathogenic bacteria

Bacterial strain	**Determination of MIC/MBC (dilution)**		
	Oil blend 1 (Cinnamon 10%, Geranium 30%, Tea Tree 10%, Thyme 50%)	**Oil blend 2 (Cinnamon 20%, Lemon thyme 30%, Patchouli 30%, Tea tree 20%)**	**Oil blend 3 (Cinnamon 23%, Lavender 23%, Lemon thyme 39%, Thyme 15%)**
Gram-positive bacteria			
Bacillus subtilis PSSCMI 0075	1:16/1:16	1:32/1:32	1:32/1:16
Staphylococcus aureus ATCC 25923	1:32/1:8	1:64/1:32	1:16/1:16
Methicillin-resistant *S.aureus* NPRC 001	1:16/1:8	1:16/1:16	1:16/1:32
Gram-Negative bacteria			
Acinetobacter baumanii ATCC 19606	1:16/1:8	1:16/1:16	1:32/1:16
Multidrug-resistant *A. baumanii* JVC 1053	1:16/1:8	1:16/1:16	1:16/1:16
Escherichia coli ATCC 25922	1:8/1:8	1:16/1:8	1:16/1:16
Klebsiella pneumonia PSSCMI 0031	1:8/1:8	1:8/1:8	1:4/1:2
Pseudomonas aeruginosa PSSCMI 0048	1:4/>U	1:2/1:2	1:8/1:2

U = undiluted oil.

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