RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2013.39.472

ScienceAsia 39 (2013): **472–476**

Antibacterial activity of essential oils and their active components from Thai spices against foodborne pathogens

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Received 15 Nov 2012
Accepted 25 Jun 2013

ABSTRACT: As the popularity of organic food especially fresh vegetables is increasing, it is a common practice to replace chemical fertilizers by manure which leads to high bacterial contamination. Some essential oils such as *Thymus vulgaris* (thyme) and *Ocimum basilicum* (basil) oils reduce spoilage flora and foodborne pathogens when used in washing water. This information prompted us to search for effective essential oils from Thai spices for vegetable washing products. Seven out of nine essential oils; fingerroot (*Boesenbergia pandurata* (Roxb.) Schltr.), galanga (*Alpinia galanga* (L.) Willd.), holy basil (*Ocimum tenuiflorum* L.), makrut leaf, makrut peel (*Citrus hystrix* DC.), sweet basil (*O. basilicum* L.), and turmeric (*Curcuma longa* L.) oils showed antibacterial activity. The active components were identified by thin layer chromatography (TLC) bioautography, preparative TLC, and gas chromatography-mass spectrometry. The results indicated that the active components were major components of the oils. The essential oils exhibited higher potency than their active components suggesting that the whole essential oils were more suitable than the pure compounds for product development.

KEYWORDS: TLC bioautography, GC-MS, active compounds, citral, biological guided separation

INTRODUCTION

Diarrhoea, a disease which causes high morbidity and mortality, caused at least two million deaths worldwide in year 2000. Besides, each year 30% of people in industrialized countries suffer from this foodborne disease[1]. The bacteria which are foodborne pathogens including *Escherichia coli*, *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Typhi, *Shigella flexneri*, *Bacillus cereus*, and *Staphylococcus aureus*, often contaminate food particularly fresh vegetable[2]. At present, the popularity of organic foods, especially fresh vegetables is increasing and it is a common practice to use manure instead of chemical fertilizers which leads to high bacterial contamination. Hence to control bacterial contamination, an effective and safe vegetable washing product is needed. Currently, vegetable washing products available in the Thai market are used to remove pesticides, therefore it is necessary to have the vegetable washing products with antibacterial agent in order to decrease the contamination of the foodborne pathogens.

Some essential oils such as thyme and basil oils are effective against spoilage flora and foodborne pathogens when used in washing water[3,4]. This information prompted us to search for the active compounds of essential oils from Thai spices. Previous studies indicated that some essential oils of Thai spices such as lemongrass, holy basil, turmeric, and galanga oils had antibacterial properties[5–7]. However, an extensive study to identify the active components has only been done on lemongrass oil, in which citral was identified as the active compound[5,8].

The aims of this study were to determine the efficacy of nine commercially available essential oils of Thai spices as antimicrobial against foodborne pathogens (*E. coli*, *S.* Enteritidis, *S.* Typhimurium, *S.* Typhi, *S. flexneri*, *B. cereus*, and *S. aureus*), and to identify the active components of each oil. Biological guided separation was used along with gas chromatography-mass spectrometry (GC-MS) to identify active components of the tested oils. Since

the essential oils selected for this study were obtained from edible plants and were volatile, it is likely that they will be safe and easy to remove. With proper research and development, essential oil products may be an alternative for cleaning fresh vegetables to eliminate microbial contamination.

MATERIALS AND METHODS

Essential oils

Nine essential oils of Thai spices; black pepper fruit (*Piper nigrum* L.), fingerroot rhizome (*Boesenbergia pandurata* (Roxb.) Schltr.), galanga rhizome (*Alpinia galanga* (L.) Willd.), holy basil leaf (*Ocimum tenuiflorum* L.), lemongrass stem (*Cymbopogon citratus* Stapf), makrut leaf and peel (*Citrus hystrix* DC.), sweet basil leaf (*O. basilicum* L.), and turmeric rhizome (*Curcuma longa* L.) oils were obtained from Thai-China flavour & fragrances industry Co., Ltd. (Bangkok, Thailand).

Reference compounds

The major compounds of essential oils; (+)-camphor, (−)-*trans*-caryophyllene, 1,8-cineole, citral, citronellal, citronellol, eugenol, geraniol, α-humulene, D-limonene, (±)-linalool, methyl chavicol, (−)-α-terpineol, and terpinen-4-ol were obtained from Sigma-Aldrich Chemical Co. Inc. (St. Louis, MO). Camphene was obtained from Carl Roth Co. (Schoemperlenstr, Karlsruhe). Methyl cinnamate and methyl eugenol were obtained from Tokyo Chemical Industry (Tokyo).

Bacterial cultures

Bacterial strains used in this study, *E. coli* (ATCC 25922), *S.* Enteritidis (DMST 15676), *S.* Typhimurium (ATCC 13311), *B. cereus* (ATCC 11778), *S. aureus* (ATCC 25923), *S. flexneri*, and *S.* Typhi were inoculated and maintained on tryptic soy agar (TSA). All bacteria were obtained from the Microbiology Laboratory Culture Collection, Department of Microbiology, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand.

Identification of the components in the essential oil

The components of the essential oils were identified by GC-MS on GCMS-QP 2010 GC-MS (Shimadzu) using a DB-5MS bonded phase fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA). Purified helium was used as carrier gas at constant flow rate of 0.68 ml/min. The oven temperature program for essential oil was modified from method of previous studies[9–12]. The injector temperature and ion source temperature were 200 °C and 250 °C, respectively. Electron impact mass spectra were obtained at 70 eV by operating in the full scan acquisition mode in the range of m/z 40–400. The identification of the active compounds were performed by comparing the obtained mass spectra with those from the Wiley and NIST spectral library and their retention time and mass spectral with those of the reference compounds.

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

The broth macrodilution method was used to determine the MICs of oils and reference compounds. Stock solutions of oils were initially dissolved in tween 80 and 95% ethanol then a series of two-fold dilutions of each oil (ranging 200 μg/ml to 1.5625 μg/ml) was prepared in tryptic soy broth (TSB). Freshly grown bacterial suspensions in TSB were adjusted to approximately 10^6 CFU/ml. For MIC determination, a set of 10 assay tubes in duplicate was employed per sample. The first test tube contained 1.8 ml of the seeded broth and the remaining tubes had 1 ml each. The solution of the test agent (0.2 ml) was added to the first tube to bring the volume to 2 ml. One ml of this was transferred to the next tube and the process was repeated giving a two-fold serial dilution. The tubes were incubated at 37 °C for 24 h. The first assay tube with no apparent growth of the microorganism containing the lowest dilution of the test essential oil represented the MIC. The bactericidal effect was assessed by MBC determination. Samples were removed from tubes that showed no turbidity and were dropped onto TSA plate. After incubation at 37 °C for 24 h the minimum concentration without visible growth was reported as MBC[13].

Determination of the active components by thin layer chromatography (TLC) bioautography

The TLC bioautographic method was used to detect the active components. After application of oils and reference standards on three silica gel G60 F_{254} aluminium plates (A, B, C), the plates were developed using toluene-ethyl acetate (93:7) as the solvent. Plate A was visualized under UV light at 254 nm and sprayed with anisaldehyde sulphuric acid. Then, it was heated at 110 °C for 5 min to detect the separated components and used as reference chromatogram. Plate B was prepared for the isolation of active compounds of essential oil.

The TLC bioautography method was modified from the method of Chomnawang et al[14]. The TLC plate (C) was developed as described above. The TLC plate (C) was placed on the top of agar base

and inocula of bacterial strains in TSA media (agar seed) was distributed over TLC plate (C). The plate (C) was incubated at 37 °C for 24 h. The active compounds were identified by the spots within the inhibition zones. Rf of the corresponding spots were compared with Rf of reference standard on plate A. Plate B was compared with plates A and C. The areas on the TLC plate which corresponded to the inhibition zones were scraped from the plate and the substances were eluted from the adsorbent with hexane. Eluted samples were filtered with filter paper (Whatman No 1), concentrated by removing the solvent under vacuum. Then, the active components were confirmed by gas chromatography-mass spectrometry (GC-MS).

RESULTS AND DISCUSSION

GC analysis of the components of the essential oils from Thai spices revealed that the major components of black pepper, fingerroot, galanga, holy basil, lemongrass, makrut leaf and peel, sweet basil, and turmeric oils, were δ-3-carene, *trans*-β-ocimene, 1,8-cineole, eugenol, *E*-citral, β-citronellal, limonene, methyl chavicol, and ar-tumerone, respectively (Table 1). The antibacterial testing against foodborne pathogens showed that seven essential oils of Thai spices exhibited the activity with MIC lower than 125 μg/ml (Table 1). In this study, the cut off limit was set up as recommended by UNIDO for commercially potential agents, which were 125 μg/ml and 10 μg/ml for crude extract and pure compound [13], respectively. Since lemongrass oil has been well known to contain citral as active component, it was used as a positive control in this study. As a result of the activity against the tested bacteria, none of the tested oils were comparable to lemongrass oil. However, among seven essential oils, holy basil and sweet basil oils showed the widest spectrum (6 strains) followed by makrut leaf and fingerroot oils (4 strains), galanga, makrut peel, and turmeric oils (1 strain), in that order (Table 1). The holy basil, makrut leaf, and sweet basil oils also showed the highest potency (25 μg/ml). The results suggest that besides lemongrass oil, sweet basil oil showed the highest potential for commercial products followed by holy basil and makrut leaf oils. Some of our results were in agreement with previous reports [6, 7].

Preliminary identification by TLC autobiography found positive bands on TLC chromatogram of each oil (Table 1). However, the identification by TLC was limited. The large amount of the oil required to produce antibacterial activity resulted in overlapping of the adjacent components and prevented a precise identification. Hence the identification was confirmed by GC-MS. Most of the active components were found to be the major constituents of essential oils. The major active components of fingerroot, galanga, holy basil, makrut leaf, makrut peel, sweet basil, and turmeric oils were 1,8-cineole/camphor, 1,8-cineole, eugenol, citronellal, terpinen-4-ol/α-terpineol, methyl chavicol, and ar-tumerone, respectively. All other active components are shown in Table 1. The attempt to isolate active components by preparative TLC was not successful due to the instability of each component. Hence all the commercially available active compounds were used and subjected to antibacterial testing and all of the compounds showed the MICs over 50 μg/ml which is over the cut-off limit recommended by UNIDO [13]. Thus the oils are recommended for product development rather than the active components.

Some of the antibacterial components found in Thai spices were reported to be active including *trans*-α-bergamotene, camphor, *trans*-caryophyllene, 1,8-cineole, citronellal, eugenol, α-humulene, (±)-linalool, methyl eugenol, methyl chavicol, neral, α-terpineol, and terpinen-4-ol [15–21]. In this study, new active components against foodborne pathogen, citronellol, ar-curcumene, β-farnesene, *cis*-farnesol, *trans*-farnesol, and turmerone, were found. None of these active compounds showed a potency comparable to that of citral (5 μg/ml). They exhibited the activity with MICs over the UNIDO cut off limit (10 μg/ml) [13]. Based on the results, the oils were more appropriate than active components for product development.

The potential role of essential oil as food preservatives has been recognized but the concentration necessary to inhibit foodborne pathogens is high which leads to the undesirable organoleptic effect [22]. Only few articles reported on the use of essential oil in the washing solution for decontamination of fresh vegetable [3, 4, 15]. Based on the antibacterial activity and the flavour of the essential oils, lemongrass, sweet basil, holy basil, and makrut leaf oils were suggested to be used in washing solution for fresh vegetables.

In this study, sweet basil oil contained methyl chavicol (estragol) as active components against *S. flexneri*, *S.* Typhi, and *S. aureus* which was in agreement with previous study [15]. Methyl chavicol has been reported to exhibit weaker activity against *Shigella* spp. than carvacrol and thymol which is probably due to the substitution of phenolic hydroxyl group with methyl group [15]. However, Thai sweet basil oil which contained 90% methyl chavicol showed the inhibitory activity against *E. coli* with the MIC of 25 μg/ml. Hence it was considered to be potential for the vegetable washing products.

Table 1 The constituents, minimum inhibitory concentration (MIC), maximum bactericidal concentration (MBC), and active components of essential oils from Thai spices identified by TLC-bioautography and GC-MS.

Essential oil	Constituents	MIC (μg/ml)	MBC (μg/ml)	Bacterial strains[a]	Active band	Identification by TLC and GC-MS
Black pepper	δ-3-carene (37%), limonene (32%), β-pinene (17%), caryophyllene (8%)	> 200	> 200	All 7 bacteria	ND	ND
Fingerroot	*trans*-β-ocimene (27%), camphor (24%),	100	200	*B. cereus*	3	geraniol, α-terpineol, 1,8-cineole, camphor, neral, and unidentified[b]
	1,8-cineole (17%), geraniol (11%),	100	100	*S.* Typhi	5	geraniol, α-terpineol, (±)-linalool, 1,8-cineole, camphor, neral, and unidentified[b]
	camphene (8%),	100	> 200	*S. flexneri*	3	geraniol, α-terpineol, (±)-linalool, and unidentified[b]
	cis-ocimene (3%), methyl-*cis*-cinnamate (3%)	100	> 200	*E. coli*	2	geraniol, α-terpineol, and unidentified[b]
Galanga	1,8-cineole (34%) β-farnesene (15%), *trans*-caryophyllene (12%), zingiberene (4%)	50	50	*B. cereus*	6	1,8-cineole,tau-muurolol, and unidentified[b]
Holy basil	eugenol (42%),	25	50	*B. cereus*	2	eugenol, methyl eugenol, and unidentified[b]
	caryophyllene (26%),	50	> 200	*S. aureus*	3	eugenol, methyl eugenol, and unidentified[b]
	methyl eugenol (15%),	50	100	*S.* Typhi	1	eugenol, methyl eugenol
	(−)-β-elememe (12%)	100	> 200	*S.* Typhimurium	2	eugenol, methyl eugenol, and unidentified[b]
		100	200	*S. flexneri*	2	eugenol, methyl eugenol, and unidentified[b]
		100	200	*E. coli*	2	eugenol, methyl eugenol, and unidentified[b]
Makrut leaf	β-citronellal (78%), citronellyl acetate (6%),	25	25	*B. cereus*	5	citronellol, *cis*-farnesol, *trans*-farnesol, citronellal, and unidentified[b]
	β-citronellol (5%)	50	50	*S.* Typhi	4	citronellol, *cis*-farnesol, citronellal, and unidentified[b]
		50	> 200	*S.* Typhimurium	1	unidentified[b]
		50	> 200	*S. flexneri*	5	citronellol, *cis*-farnesol, citronellal, and unidentified[b]
Makrut peel	limonene (46%), α-terpineol (19%), β-pinene (18%), terpinen-4-ol (17%)	100	100	*S.* Typhi	3	α-terpineol, terpinen-4-ol, and unidentified[b]
Sweet basil	methyl chavicol (90%),	50	100	*B. cereus*	2	2 unidentified[b]
	α-bergamotene (3%)	100	> 200	*S. aureus*	4	methyl chavicol and 3 unidentified[b]
		50	50	*S.* Typhi	3	methyl chavicol and 2 unidentified[b]
		50	> 200	*S.* Typhimurium	1	unidentified[b]
		25	50	*S. flexneri*	3	methyl chavicol and 2 unidentified[b]
		25	50	*E. coli*	2	2 unidentified[b]
Turmeric	Ar-tumerone (45%), curlone (14%), tumerone (12%), α-curcumene (7%), β-sesquiphellandrene (6%)	50	> 200	*S.* Typhi	2	tumerone, Ar-curcumene, and *trans*-caryophyllene
Lemongrass	*E*-citral (53%),	25	25	*S. aureus*	1	citral
	Z-citral (38%),	12.5	12.5	*B. cereus*	1	citral
	geraniol (4%)	100	100	*S.* Typhi	1	citral
	**Positive control*	100	100	*S.* Typhimurium	1	citral
		50	50	*S. flexneri*	1	citral
		100	100	*S.* Enteritidis	1	citral
		50	50	*E. coli*	1	citral

[a] The bacterial strains not present in this table exhibited MIC and MBC of oils higher than 200 μg/ml.
[b] Lack of reference compound.
ND: Not determined.

Holy basil, one of the active oils contained eugenol (42%) and methyl eugenol (15%) which was found to be active against *S.* Typhimurium, *S.* Typhi, *S. flexneri*, *E. coli*, *S. aureus*, and *B. cereus*. The results from this study supported previous report which indicated that eugenol was active. Eugenol exerted the activity not only at the membrane but also inhibited the production of amylase and protease by organisms[21,23]. However, the unpleasant flavour of eugenol may not be accepted by consumers. Another potential oil, makrut leaf oil contained different active compounds. The major active components were citronellal (78%) and citronellol (5%). The activities of citronellal and citronellol were due to aldehyde and alcohol functional groups, respectively[22].

CONCLUSIONS

In conclusion, four essential oils of Thai spices, lemongrass, holy basil, makrut leaf, and sweet basil oils showed the inhibitory activity against foodborne pathogens with the MICs 25–100 μg/ml. The major active components were identified along with other

components using TLC-bioautography and GC-MS techniques. Since most of the components are less active than the whole oils, it is recommended to use the whole oil for vegetable washing solution. This study also identified the new antibacterial agents against foodborne pathogens including citronellol, arcurcumene, and turmerone.

Acknowledgements: The authors are grateful to the Coordinating Centre for Research and Development to increase value of the Plants Indigenous to Thailand, Mahidol University and the Thailand Research Fund for financial support.

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