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Research Article

Antimicrobial activities of some Thai essential oils against *Escherichia coli* and *Salmonella typhimurium* in microbial medium and on fresh vegetables

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Abstract

The antibacterial action of black pepper (*Piper nigrum* L.), kaffir lime (*Citrus hystrix*), lemongrass (*Cymbogon citratus*) and turmeric (*Curcuma longa*) was investigated against two microbial pathogens, *Escherichia coli* and *Salmonella typhimurium*, in microbiological medium and on fresh vegetables, including tomatoes, cabbages, chinese leaves and lettuces. In a broth dilution method, the minimum bactericidal concentration (MBC) of lemongrass oil against *E. coli* and *S. typhimurium* was 3.0 and 33.3% (v/v), respectively. Lemongrass and kaffir lime oil could significantly inhibit the growth of the two studied pathogens using an agar diffusion method, while black pepper oil could only control the growth of *E. coli*. Reduction in the microbial number of *E. coli* in the range of 1.28 to 4.23 log cfu/g could be achieved by lemongrass and kaffir lime oils in the washing solution to decontaminate the fresh vegetables.

Keywords: *Cymbogon citrates, Citrus hystrix,* decontamination, MBC, Thailand, Indonesia.

Introduction

Essential oils are recognized as odours, volatile products of an aromatic plant's secondary metabolism that are biosynthesized in glandular structures of a plant cell [1, 2]. The oils or the corresponding plant parts, such as flowers, buds, seeds, leaves, herbs, fruit and roots, have been used as perfume, flavouring and preservative agents since ancient times [3]. The capability of the essential oils to control and/or kill microorganisms has revived interest in having more application of essential oils in food products. The utilization of the oils that are generally recognized as safe (GRAS) substances [1] holds greater appeal for customers, particularly those who find objectionable the application of synthetic food additives.

Several researchers have reported on the effectiveness of a wide range of essential oils, both from herbs, spices and medicinal plants, against pathogenic microorganisms [4, 5, 6]. However, there is still a low number of published results regarding the application of these essential oils to food, particularly to control the presence of pathogens on a wide variety of fresh vegetables. Karagözlü *et al.* [7] applied mint and basil essential oils to control *Escherichia coli* 0157:H7 and *Salmonella*

typhimurium on fresh-cut lettuce and purslane, while Gündüz *et al*. [8] only used oregano oil to inactivate *S. typhimurium* on lettuce. Another work of Gündüz *et al*. [9] utilized sumac extract and oregano oil to decontaminate *S. typhimurium* on tomatoes. On the other hand, Uyttendaele *et al.* [10] worked with thyme essential oil to regulate the growth of *Aeromonas* spp. on carrots, lettuce and bell peppers. In this research paper, four Thai herb essential oils, including black pepper, kaffir lime, lemongrass and turmeric, were selected to be studied in and on microbiological media and the corresponding results were directly applied to inactivate pathogens on different fresh vegetables. The aim of the project was to have a better understanding about the effectiveness of the selected herb essential oils to control the presence of *E. coli* and *S. typhimurium* on tomatoes, cabbages, lettuces and chinese leaves.

Materials and Methods

Essential oils

Four types of essential oils, including black pepper, lemongrass, kaffir lime and turmeric were purchased from Thai-China Flavour and Fragrance Industry, Thailand. The purchased oils were recognized to have 100% concentration.

Microbial preparation

E. coli TISTR 117 and *S. typhimurium* TISTR 292 were obtained from Thailand Institute of Scientific and Technological Research (TISTR), Pathumthanee, Thailand. Both pathogens were maintained as stock cultures on Tryptic Soy Agar (TSA) (Criterion, Hardy Diagnostics, USA) slant at 4ºC. When fresh cultures of the pathogens were needed, a loop of the microbial culture from the slant was inoculated into 10 ml of Tryptic Soy Broth (TSB) (Criterion, Hardy Diagnostics, USA) and incubated at 37ºC for 18 h. The growth of the overnight culture would reach an average microbial number of 10^8 cfu/ml. This microbial population would be further diluted into 10^4-10^5 cfu/ml using 9 ml of sterile 0.1% peptone water (Himedia, Mumbai, India).

Minimum Inhibitory Concentration (MIC) in a broth dilution method

The MIC was carried out following the method of Oonmetta-aree et al. [11] and Nanasombat and Lohasupthawee [12] with some modification. Briefly, herb essential oils were serially diluted in 0.5% Tween 80 into a ratio of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256 (that is equivalent with 50.0, 33.3, 20.0, 11.1, 5.9, 3.0, 1.5, 0.8 and 0.4% (v/v) oil, respectively). An amount of 40 µl of the oil was loaded onto a sterile filter paper disc (∅ 6 mm, Whatman, England) twice with air-drying in between. The MIC of the essential oil was carried out in a microtiter plate (Corning, USA). Into a well of the plate, 100 µl of fresh TSB was added with 100 µl of inoculum suspension, containing approximately 10^5 cfu/ml. The infiltrated paper disc (80 µl) was then submerged into the well. The negative and positive controls were determined using sterile distilled water supplemented with 0.5% Tween 80 and Penicillin G (Oxoid, England) containing 10 units, respectively. Triplicate wells were done for each essential oil concentration. The plate was incubated at 37ºC for 18-24 h. The MIC of the oil was recognized as the lowest concentration of oils that did not permit for any turbidity of the tested microorganisms [11].

Minimum Bactericidal Concentration (MBC) in a broth dilution method

The MBC was examined using the microtiter plate of the MIC. All the wells of the plate that did not display any turbidity of the tested microorganisms and the last well with turbidity were determined for MBC. A sample of 100 µl of the suspension was spread onto TSA and incubated at 37ºC for 18-24 h. The MBC was defined as the lowest concentration of the oil in which the initial inoculum was killed for 99% or more [11].

Agar diffusion method

The antimicrobial efficacy of the essential oils was also studied using an agar diffusion method following a modification of the procedure by Hussain *et al*. [13]. Briefly, an amount of 100 µl of the tested microorganisms, containing approximately 10^5 cfu/ml of the bacteria cell was spread on TSA agar. A filter disc was loaded with 15 µl of the essential oil and placed on the agar plates, which had previously been inoculated with *E. coli* or *S. typhimurium*. A disc with sterile distilled water was used as a negative control, while Penicillin G (10 unit) was utilised as a positive reference for the tested bacteria. The petri dish was kept at 4ºC for 2 h and then incubated at 37ºC for 18-24 h. The antimicrobial activity of the oil was assessed by measuring the diameter of the growth inhibition zones in mm (including the disc diameter of 6 mm) for the tested organisms. The determination of inhibition zones was done from three separate replicates.

Antimicrobial activity of essential oils against E. coli and S. typhimurium on fresh vegetables

Fresh vegetables, including tomatoes, cabbages, chinese leaves and lettuces were bought from the local market (Mae-Hia fresh market, Chiang Mai, Thailand) and directly transferred to the laboratory room. Except for tomatoes, the outer leaves of leafy vegetables and the core of the cabbage were separated using a sharp knife. All the utensils used for preparing the fresh-cut pieces were sanitised by autoclaving them at 121ºC for 15 min or wiping them using 90% alcohol to reduce contamination on the vegetables. The inner vegetable leaves were cut into small pieces, approximately 3 x 3 cm^2 before being used in any experiment. To remove the initial microbial load on the vegetables, all of the vegetables were sanitised with 200 ppm chlorinated water (Union Science, Thailand) for 5 min at room temperature. The treatment was carried out with some agitation [14]. The sanitised vegetables were air-dried for 1 h in a bio safety cabinet (Heal Force, China). Some of the washed vegetables were also analysed for their microbial load to check the effectiveness of the chlorine water. The washed vegetable was then inoculated with either *E. coli* or *S. typhimurium*. To do this, 18 to 24 h microbial cultures was added into 1 l of 0.1% peptone water to produce a microbial number of approximately 10^4 cfu/ml. The cleaned vegetables were contaminated by immersing them into the culture solution for 1 min with a soft rotating motion using a sterile glass stem [15] and air-dried for 1 h in a bio-safety cabinet. Some of the contaminated vegetables were analyzed for the initial number of the contaminated pathogens on the fresh vegetables. To decontaminate the pathogens, the contaminated vegetables were soaked in sterile distilled water containing the essential oil for 1 min at room temperature with some rotating motion using a sterile glass stem to ensure complete coverage and contact of surfaces with the essential oil solution [16]. A sterile 0.5% of Tween 80 was also added to the decontaminated solution [17]. The decontaminated vegetables were left to be air-dried for 1 h in the bio-safety cabinet to remove the soaked solution [16] before being kept in sterile plastic bags at refrigerated temperature for analyses. Each decontamination treatment was done in triplicate. As a control treatment, the contaminated vegetables were washed with sterile distilled water with 0.5% Tween 80.

Microbiological analysis

To analyse the microbial number on vegetable samples, 10 g of samples were aseptically transferred into 90 ml of 0.1% sterile peptone water and homogenised for 60 s. A serial decimal reduction was then carried out for the homogenized sample using the same diluent. The microbial number was determined by a pour plate method using 1 ml of the appropriate diluted sample on TSA at 37ºC for 24-48 h [18]. The results were expressed in log cfu/g [16].

Statistical analysis

All experiments were performed in triplicate. The recorded data were expressed as mean $\pm$ standard deviation for the measurements of inhibition zones of the agar diffusion method and reduction of bacterial number on the fresh vegetables. A statistical package (SPSS, version 10.0 for Windows, SPSS Inc.) was utilised for the data processing. Differences were considered significant at $p < 0.05$.

Results and Discussion

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of essential oils

The MIC and MBC results of four Thai herb oils are displayed in Table 1. The data showed that lemongrass oil had the strongest activity against the tested pathogens, *E. coli* and *S. typhimurium*, in the broth dilution method. This antimicrobial activity could be due to the presence of active ingredients in the oil, including geranial, neral and limonene [1]. The MBC of the oil against *S. typhimurium* was higher than that of the MIC value. A similar finding had been reported by Naik *et al.* [19] for the antibacterial activity of lemongrass against several pathogenic bacteria. However, these researchers [19] reported a MBC value of 0.12% for the lemongrass oil against *E. coli*, which was much lower than the concentration found in this study. Differences in the finding could be affected by plant culture condition (climate, location, temperature, fertility, diseases and pest exposure), plant species, part of plant material, essential oil extraction method and type of solvent and strains of target microorganisms [4, 20, 21]. The other essential oil that could inhibit the target microorganisms was kaffir lime. The oil was effective against *E. coli*, but it could not control *S. typhimurium*. Chanthaphon *et al.* [22] also observed that only the fresh kaffir lime peel could inhibit the growth of *E. coli* O157:H7, whereas the oil extract from a dried peel did not show any antibacterial activity. The capability of kaffir lime oil to control *E. coli* could be attributed to the presence of limonene, β-pinene, terpinene-4-ol, α-terpineol, γ-terpinene, α-terpinene and terpinolene in the oil [23].

Table 1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Thai herb oils against *Escherichia coli* and *Salmonella typhimurium*.

Microorganisms	Types of herb oil	MIC (%)	MBC (%)
Escherichia coli	Black pepper	50.0	50.0
	Kaffir lime	11.1	11.1
	Lemongrass	3.0	3.0
	Turmeric	50.0	+[*)]
	Penicillin G (10 unit)	–[**)]	–
	Sterile distilled water	+	+
Salmonella typhimurium	Black pepper	+	+
	Kaffir lime	+	+
	Lemongrass	20.0	33.3
	Turmeric	+	+
	Penicillin G (10 unit)	–	–
	Sterile distilled water	+	+

[*)] +: The bacteria grew in all studied concentrations.
[**)] –: No bacterial growth was observed.

The antimicrobial activity of essential oils on agar diffusion method

Findings from the agar diffusion method supported the results of the broth dilution method that only lemongrass and kaffir lime oils had significant inhibition on the 2 tested pathogens (Table 2). However, a significant inhibition of black pepper oil against *E. coli* was also found on the second method. Differences found between the two methods were affected by several factors, such as differences in microbial growth, exposure of microorganisms to the essential oil, the solubility of oil or oil component and the use and quantity of an emulsifier [4]. Work by Wannissorn *et al.* [5] also revealed that the leaf of *Citrus hystrix* DC (kaffir lime) and the leaf/stem of *Cymbopogon citratus* Stapf. (lemongrass) had antibacterial activities against *S. typhimurium* TISTR 292 and *E. coli* TISTR 292, while no inhibition zone was observed for the pathogens by the fruit of *Piper nigrum* L. (black pepper). Although the strain of salmonellae in the previous report was similar with the one used in this study, differences in the inoculum preparation, plant condition or extraction methods

could still affect the study results. Nanasombat and Lohasupthawee [12] also reported that there was not any antimicrobial activity of black pepper and turmeric against different *Salmonella* spp. and species of enterobacteria.

Table 2. Diameter inhibition (mm) of microbial growth in the presence of Thai herb oils on agar diffusion method.

Types of herb oil	*Escherichia coli*	*Salmonella typhimurium*
Black pepper	7.77 ± 1.21^{c}	6.00 ± 0.00^{d}
Kaffir lime	16.81 ± 7.33^{b}	9.86 ± 0.25^{b}
Lemongrass	49.31 ± 3.54^{a}	8.48 ± 0.33^{c}
Turmeric	6.00 ± 0.00^{d}	6.00 ± 0.00^{d}
Penicillin G (10 unit)	13.56 ± 2.22^{b}	12.20 ± 0.58^{a}
Sterile distilled water	6.00 ± 0.00^{d}	6.00 ± 0.00^{d}

[a-d] Different letters within a column indicated significantly different at a level of $p \leq 0.05$.

The antimicrobial activity of lemongrass and kaffir lime oils on fresh vegetables

The antimicrobial activity of lemongrass and kaffir lime oils against *E. coli* and *S. typhimurium* were further investigated on fresh vegetables, mainly tomatoes, cabbage, chinese leaves and lettuces. The level of the oils used in this part was the intermediate concentration between the MBC values of the 2 tested pathogens, since the *S. typhimurium* had a higher resistance to the oil antibacterial activities. The results in Table 3 significantly exhibited that *E. coli* was generally more sensitive to lemongrass and kaffir lime oils compared to *S. typhimurium*, confirming the results in and on the microbiological media. The highest inhibition of *E. coli* of 4.23 ± 0.03 log cfu/g was achieved by kaffir lime oil on tomatoes, while the lemongrass oil showed the highest antibacterial action against *E. coli* on lettuce. These results were similar to the findings of Karagözlü *et al*. [7] for the inactivation of *E. coli* O157:H7 on lettuce with 0.08 and 0.032 ml/l mint oil solution for 10 and 15 min washing time, respectively. Although the previous study used lower concentrations of the essential oil, the study applied longer washing time. This indicated that an interaction between oil levels and washing period could have a significant effect on the oil activity against a microorganism.

Table 3. Reduction number of *Escherichia coli* and *Salmonella typhimurium* (log cfu/g) on fresh vegetables by lemongrass and kaffir lime oils.

Essential oils	Vegetables	*Escherichia coli*	*Salmonella typhimurium*	Sterile distilled water
33.3% (v/v) kaffir lime	Tomatoes	4.23 ± 0.03^{c}	0.32 ± 0.05^{b}	-0.11 ± 0.06^{a}
	Cabbage	2.73 ± 0.03^{c}	-0.33 ± 0.01^{a}	$0.19 + 0.06^{b}$
	Chinese leaves	$3.49 + 0.10^{c}$	-0.79 ± 0.05^{a}	$0.17 + 0.02^{b}$
	Lettuce	$1.28 + 0.13^{b}$	1.83 ± 0.08^{c}	$0.34 + 0.06^{a}$
11.1% (v/v) lemongrass	Tomatoes	3.44 ± 0.02^{c}	$0.50 + 0.04^{b}$	$-0.11 + 0.02^{a}$
	Cabbage	2.93 ± 0.10^{c}	$-0.73 + 0.05^{a}$	$-0.11 + 0.02^{b}$
	Chinese leaves	3.19 ± 0.07^{c}	$-0.21 + 0.02^{b}$	$-1.01 + 0.02^{a}$
	Lettuce	3.92 ± 0.10^{c}	$1.06 + 0.06^{b}$	$0.33 + 0.05^{a}$

[a-c] Different letters within a row indicated significantly different at a level of $p \leq 0.05$.

The effectiveness of the lemongrass and kaffir lime essential oils against *S. typhimurium* was only found on lettuce and tomatoes. This suggested that the types of vegetable also played a role in the decontamination process by an essential oil. Several factors that could protect pathogens during sanitising or washing procedures were irregularities of leaf surface, the presence of pathogens in protected sites (within stomata, at cut surfaces or the presence of biofilms or bacterial aggregates) and the hydrophobicity of the leaf surface [24, 25]. Research by Gündüz *et al*. [8] also showed that the effectiveness of oregano oil at levels of 25 to 75 ppm to decontaminate *S. typhimurium* on

shredded iceberg lettuce was within 1 log cfu/g after washing for 5 min in the oil solution. However, the effectiveness of lemongrass and kaffir lime oils to control *S. typhimurium* on tomatoes was slightly weak in this study compared to the previous work of Gündüz *et al*. [9]. In this work, the researchers demonstrated that the population of *S. typhimurium* could be reduced by 0.95 to 3.7 log cfu/tomato by applying 25 to 100 ppm oregano oil in the washing solution (washing time of 5 min). Discrepancies in these findings could be affected by different strains of *S. typhimurium* and the media used to enumerate the pathogen.

Conclusion

Findings in this study clearly displayed that some of the Thai essential oils were effective to control microbial pathogens in the microbiological media and on fresh vegetables. The application of 11.1% (v/v) lemongrass and 33.3% (v/v) kaffir lime oils could significantly decrease the presence of *E. coli* on the vegetable surfaces and had the potential to be used in controlling the pathogen on fresh vegetables.

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