Original Article

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Fresh Produce Antibacterial Rinse from Kaffir Lime Oil

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Abstract

Kaffir lime oil, a volatile oil from fruit peel of *Citrus hystrix* L., was analyzed for its constituents, using GC-MS. The major constituents were l-limonene, α-terpineol, 2-β-pinene, terpinene-4-ol, γ-terpinene, α-terpinene, and α–terpinolene. The minimum inhibitory concentration (MIC) against *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Salmonella typhimurium* ATCC 13311 were 0.1, 0.3, 0.4, and 0.6% v/v, respectively. The antibacterial rinse, formulated as an emulsion, consisted of 40% v/v Kaffir lime oil, 8% w/v gelatin, and 3% w/v lecithin. The emulsion was diluted with water into a soaking solution which contained 0.75% v/v of Kaffir lime oil. The soaking solution reduced the natural bacterial population on chinese cabbage, by means of aerobic plate count after the second water rinse, by 2.68, 3.30 and 4.27 log at 5, 10 and 15 min soaking time, respectively.

Key words: Kaffir lime oil, *Citrus hystrix*, Minimum inhibitory concentration, Antibacterial rinse, Sanitizer.

INTRODUCTION

Due to increased health consciousness among consumers, fresh fruits and vegetables have been largely consumed[1]. Fruits and vegetables can become contaminated with pathogenic microorganisms while growing in fields, orchards, or during harvesting, post-harvesting handling, processing, distribution and preparation in food service or home settings. Examples of pathogenic bacteria isolated from raw vegetables are *Salmonella*, *Aeromonas*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Vibrio cholerae*, *Staphylococcus*, *Campylobacter*, *Bacillus cereus*, *Shigella*, *Yersinia enterocolitica*, etc. Fresh fruits and vegetables that have been implicated in outbreaks of infections included cabbage, lettuce, carrot, tomato, mungbean sprout, etc.[2]

Several chemicals have been studied for their antibacterial activities.[3-13] The challenge is to attain the 5-log kill recommendation set by the Food and Drug Administration (FDA) for selected commodities[3]. The most commonly used commercial sanitizer for fresh produce is chlorinated water (50-200 μg/ml)[14,15]. The chlorinated water was capable of reducing only 2 log CFU/g on fruits and vegetables[3,16-19]. Higher bacterial reductions of 2 to 4 log CFU/g could be achieved with chlorine concentrations of 100-2,000 μg/ml[14]. In addition, chlorine may produce harmful by products, chloramines and trihalomethanes[20,21]. It has been repeatedly reported to react with trace amounts of organic materials on fresh produce to form various carcinogenic organochlorine compounds[22]. Chlorine dioxide does not produce harmful products[23], nor does it produce foul-smelling chlorophenols[24] . Aqueous chlorine dioxide, according to the FDA[25], could be employed to sanitize whole fresh fruits, whole vegetables, shelled beans,

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and peas with cuticles intact (not exceeding 5 µg/ml) as well as peeled potatoes (not exceeding 1 µg/ml). Gaseous chlorine dioxide, at 4.0 mg/L for 10 min has been reported to obtain a 5.5-log reduction of *L. monocytogenes* on the skin of apples[26] whereas in another study the concentration of 4.1 mg/L significantly reduced the population of pathogens by only 2 log CFU/g[27]. Other report showed only 1-log reduction[28]. The disadvantages of chlorine dioxide are that it is unstable and explosive when concentrated and decomposes at temperature greater than 80°C when exposed to light[29]. Ozone and hydrogen peroxide have the advantage that they do not produce any chemical residues[3]. FDA permits ozone for use in treating drinking water and recycled water in poultry plants at concentration that do not exceed 0.1 µg/ml[30]. Ozone (1.3mM, bubbled for 3 min) was capable of reducing mesophilic and psychrotrophic bacteria of lettuce by 1.4 and 1.8 log CFU/g, respectively, whereas following a longer exposure time (5-min) from a different batch, it could achieve 3.9 and 4.6 log CFU/g reduction in initial population, respectively[10]. Nevertheless, ozone treatment requires expensive generating equipment[3]. Hydrogen peroxide at ≤ 1% has been studied as a sanitizer, and in conjuction with acid or other disinfectants[31]. Hydrogen peroxide can achieve up to 3-log kill[12]. Its disadvantages were its lack of stability and effectiveness in solution over time[32]. Other sanitizers and their combinations were not able to reduce the bacterial population by 5 log at the recommended concentrations[11, 26,33-38]. As for sanitizers from natural products sources, one study showed that 35% white vinegar (1.9% acetic acid) was capable of reducing *E. coli* by 5 log after 5 min with agitation or 10 min without agitation; the latter caused changes in attributes[13]. Essential oils from coriander, mint, vanillin, parsley and citrus fruit peels were shown to have antimicrobial activities. Intense flavors from these natural chemicals may limit their use[3].

Kaffir lime (*Citrus hystrix* L., Rutaceae) has been used as flavoring ingredients in various traditional Thai dishes since the ancient time. Kaffir lime, especially the peel, exhibits strong but pleasant aroma as well. Moreover, it has also been described for its medicinal use in traditional Thai medicinal herbal remedies. Fruit peel provides steam-distilled oil, a volatile oil, that contains major constituents such as β-pinene (30.6%), limonene (29.2%), and sabinene (22.6%)[39]. Alcoholic extract of the fruit peel has been shown to exhibit the antibacterial activity against *Staphylococcus aureus*[40,41], *Bacillus cereus*[41], *Vibrio cholerae* Ogawa, and *Vibrio parahemolyticus*[40].

The objective of this study was to formulate a sanitizing product from natural source, a product known also as an antibacterial rinse, from Kaffir lime oil and evaluate for its effectiveness in reducing the natural bacterial population on a fresh vegetable, chinese cabbage.

MATERIALS AND METHODS

Screening of essential oils for antimicrobial activity

Essential oils. Six steam-distilled essential oils for preliminary screening were obtained from a local volatile oil plant (Thai China Flavours & Fragrances, Bangkok , Thailand). They were as follows: Lemongrass oil (*Cymbopogon citratus* (DC.) Stapf.), Kaffir lime oil from fruit peel (*Citrus hystrix* L.), Betel vine oil (*Piper betle* L.), Holy basil oil (*Ocimum tenuiflorum* L.), Galangal oil (*Alpinia galanga* (L.) Sw.), and Sweet basil oil (*Ocimum basilicum* L.). All samples were stored at 4°C throughout the study.

Preparation of pathogenic bacteria. Five strains of bacteria, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 9027, and *Salmonella typhimurium* ATCC 13311 were used for the determination. All the bacteria were obtained from Microbiological Research Center, Thailand Institute of Scientific and Technological Research, Bangkok, Thailand. Bacterial cultures were maintained at 4 °C on slants of Tryptic Soy Agar (TSA) (Difco Laboratories, Detroit, MI, U.S.A.) and propagated by culturing in Tryptic Soy Broth (TSB) (Difco) at 37 °C for 24 h for at least 2 consecutive days prior to being used in experiments. The bacteria were then each

inoculated in Muller Hinton Agar (MHA) (Difco) and incubated at 37 °C for 18-24 h. The bacterial colonies were diluted with 0.1% peptone until the turbidity was equivalent to the No. 0.5 McFarland Standard.

Disk Diffusion method. Plates containing 20 ml of MHA agar were prepared. Inoculums of 5 strains of bacteria were prepared from overnight culture, then suspended in broth to obtain the turbidity equivalent to the No. 0.5 McFarland Standard. A sterile cotton swab was dipped into the suspension, pressed firmly on the inside wall of the tube to remove excess inoculum from the swab. The swab was then swabbed over the entire surface of the agar in three different directions. Discs with absorbed volatile oils were placed on the surface of the inoculated agar with sterile forceps, gently pressed down onto the agar. The plates were incubated at 37°C for 24 h. The diameters of the zones of inhibition were measured.

Minimum inhibitory concentration determination by Agar Dilution method. MHA plates containing 0.25% (v/v) Tween 20 were prepared and allowed to cool at ambient temperature to 45°C. An appropriate volume of selected volatile oil was added to melted MHA to obtain the desired concentrations. The content was then mixed by inversion, poured into plate on a level surface, and allowed to solidify. For growth control, plates with no volatile oil were prepared. Each inoculum of the 5 strains of bacteria was prepared from overnight culture, suspended in broth to obtain turbidity that matched No. 0.5 McFarland standard. Three µl of each inoculum was delivered to the agar surface using micropipette, with sterile tips. The plates were incubated at 37°C for 24 to 48 h. The plates were observed for growth inhibition and the MICs of the oil were determined. MIC was the lowest concentration of the compound capable of inhibiting the growth of challenging organism. In this study, preparation of the plates was done by preparing different dilutions (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 (or more) %) of the oils in melted MHA (45°C) in screw-capped tubes, the total volume (melted MHA + oil) must not exceed 20 ml, mixed thoroughly before pouring onto plates and allowed to solidify.

Chemical analysis of the constituents of Kaffir lime oil

Kaffir lime oil was purchased from Thai China Flavours and Fragrances in a 1-litre aluminum bottle and stored at 4°C throughout the study. Kaffir lime oil was analyzed by gas chromatography/mass spectrometry on a Hewlett Packard 6890 instrument (Jeol JMS-DX 300), GC-MS: HP6890/5973 MSD. Samples (1µm) were injected on a capillary column (HP-INNOWAX; 60 m long, 0.25 mm internal diameter), well coated with cross linked PEG (0.25 µm). The carrier gas was helium (99.9995%), at the flow rate of 0.8 ml/min and an inlet split ratio of 1:100 was used. The injector and interface temperature was 250 °C. The temperature program adopted was 60 °C for 4 min, followed by an increase of 4 °C per min to 250 °C and finally held for 30 min. The experiments were run in the electron impact mode at 70 electron volts; the electron source temperature was 230 °C with the emission of 34.6. The scan mode was 30-450 with the EM volt of 1450. Compounds were identified by direct comparison to mass spectral and retention time data exhibited by reference compounds, and by similar data comparison with compounds catalogued in Wiley 275/version 6.0, NBS 45 K.

Formulation of the antibacterial preparation

The minimum inhibitory concentrations of Kaffir lime oil were taken into consideration to determine the minimal concentration of the essential oil needed in the formulation.

Wet methods were carried throughout as follows:

Gelatin, at 7, 8, 9, or 10% w/w, was sprinkled onto 60°C water. Water at room temperature was then added and the mixture was stirred until clear mucilage was obtained. Lecithin, at 1, 2, or 3% w/w for each concentration of gelatin, was dissolved in a small portion of Kaffir lime oil. The lecithin-containing Kaffir lime oil was then

gradually added to the water phase, with constant mixing, using an electric mixer on high speed. The remaining oil was added in the same manner; the total concentration of the oil in the formula was set at 40% w/w. The emulsification was continued for another 5 min. The volume was adjusted with water. The physical appearance of the emulsion was recorded.

Stability of prepared emulsions

Each prepared emulsion was divided into 2 portions and transferred to separate stoppered bottles. One bottle was stored at room temperature, the other at 4°C. The type of emulsion was tested by dropping a few drops of the prepared emulsion into 50 ml of water. Miscibility was recorded as oil-in-water type where immiscibility was recorded as water-in-oil type. The physical stability of the emulsion was observed and recorded as creaming (non-distinct separation which returned to its original state when mixed), cracking (permanent distinct separation of oil and water phases) and viscosity (ranging from low (+1) to high (+3)) every week for the period of 4 weeks. The most stable formulation was selected for further development into the antibacterial rinse for fresh produce. Inspections of creaming and cracking of the selected formula were then carried out, both at room temperature and at 4°C at the periods of 6 mo, 1, 1.5, and 2 yr.

Sensory evaluation test

Chinese cabbage leaves were cut into 3 cm X 3 cm pieces as in the antibacterial test and distributed into 3 separated containers. The emulsion was diluted with water to the same concentration as in the antibacterial test. The diluted solution was transferred into the 3 containers; the proportion of vegetable and soaking solution was 1:10. The vegetables were soaked for 5, 10, and 15 min, respectively. Vegetable pieces were rinsed for 1 min in the same amount of water twice. The vegetable pieces were then allowed to stand at room temperature for 1 h. Vegetable samples from 3 different soaking times were tested for difference, compared to unsoaked vegetable pieces in 3 separate sets by 10 trained panelists. The panelists were asked to identify the odd sample among the trio of each set, consisting of either 2 soaked samples and one unsoaked sample or 1 soaked sample and 2 unsoaked samples. The Difference test (the Triangle test)[42] was repeated in another 2 sessions. The results were recorded and interpreted.

Antibacterial activity of the prepared emulsions

The selected formulation was evaluated for their antibacterial activity against the representative bacteria, with controls, as follows:

Chinese cabbages were purchased from a local supermarket in Bangkok, Thailand. The bruised outer leaves were removed and discarded using aseptic technique, the leaves were then cut into 3 cm x 3 cm pieces.

Control: vegetable pieces were divided into 3 separate portions and transferred into 3 separate, sterilized, household poly-propylene bags (25 cm X 30 cm). Cool, boiled water was added to each bag; the proportion of vegetable and water was 1:10 by weight. The content in each bag was mixed using intermittent shaking. At time 0, one ml of water was pipetted from each bag for total viable count and regarded as the control sample for initial bacterial population for each bag of vegetables. The total viable count (TVC) was determined using Petrifilm™ APC (3M, St. Paul, Minnesota, U.S.A.). The vegetables were allowed to soak for 5, 10, and 15 min, respectively. Intermittent shaking by hands was applied to all 3 bags. After the designated times, the water was discarded. The vegetable pieces were then rinsed with the same proportion of water for 1 min, twice. The water from the second rinse was determined for its TVC representing the remaining bacterial population for each designated soaking times. All Petrifilm™ APC was incubated at 37 °C for 24-48 h. The number of bacteria grown on the media was recorded. The log reduction of each soaking time after the second rinse was calculated.

Emulsion: vegetable pieces were divided in the same manner into 3 separate

bags. Same proportion of water was added to each bag, intermittent shaking was applied for 1 min. Initial bacterial count was determined. Immediately, Kaffir lime oil emulsion was added to each bag in order to obtain the concentration of 0.75% v/v of Kaffir lime oil in the soaking solution. The vegetable pieces in the three bags were allowed to soak for 5, 10, and 15 min, respectively. The rinsing, sampling, plating, recording, and calculating were carried out in the same manner as the control samples.

RESULTS AND DISCUSSIONS

Screening of essential oils for antimicrobial activity

The antibacterial activity of 6 volatile oils was screened by Disk Diffusion method. The result was shown in Table 1. Zone of inhibition ranged from no zone to too large to measure. Apart from *Pseudomonas aeruginosa*, the essential oils exhibited antibacterial activity against the 5 strains of bacteria. Attribute of volatile oils (aroma/odor), prices (Table 2), and zone of inhibition were taken into consideration in selecting the volatile oil for further development. Sweet basil oil (with nauseating odor) and Galanga oil (with strong, piercing odor) were not selected due to their undesirable odors despite their strong antibacterial activity. Four remaining volatile oils which were selected for the minimum inhibitory concentration determination (Table 3) were Lemongrass oil (with strong antibacterial activity, and low price), Kaffir lime oil (with very pleasant aroma, and low price), Betel vine oil (with strong antibacterial activity, and mild aroma), Holy basil oil (with strong antibacterial activity and familiar aroma). Betel vine oil and Holy basil oil were subsequently excluded due to their high prices. Prices of Lemongrass oil and Kaffir lime oil were in low range. Although other oils showed stronger antibacterial activity, Kaffir lime oil was preferred and selected for further development because of its pleasant aroma and low price, and its MIC was low enough for the study.

Table 1. Zone of inhibition of 6 volatile oils by Disk Diffusion method

Bacteria	Zone of inhibition (mm)*					
	Lemongrass oil	Kaffir lime oil	Betel vine oil	Holy basil oil	Galanga oil	Sweet basil oil
Bacillus subtilis ATCC6633	α	15.0	15.5	17.5	24.0	α
Staphylococcus aureus ATCC25923	50.0	12.0	19.0	12.0	18.0	14.5
Escherichia coli ATCC25922	α	7.5	13.5	13.5	12.5	α
Salmonella typhimurium ATCC13311	14.0	8.5	13.0	9.0	14.5	10.0
Pseudomonas aeruginosa ATCC9027	7.5	6.0	7.0	6.0	7.5	6.0

*disk diameter = 6.0 mm, α = too large to measure

Table 2. Attributes and prices of 6 volatile oils

Attribute/price	Lemongrass oil	Kaffir lime oil	Betel vine oil	Holy basil oil	Galanga oil	Sweet basil oil
Aroma/odor	Strong, unpleasant odor	Pleasant aroma	Mild aroma	Mild, familiar aroma	Strong, piercing odor	Nauseating odor
Price/litre (Baht)*	2,400	2,200	22,000	10,000	26,000	3,500

*during the research period

Table 3. Minimum inhibitory concentration (MIC) of 4 volatile oils

Bacteria	Minimum inhibitory concentration (%v/v)			
	Lemongrass oil	Kaffir lime oil	Betel vine oil	Holy basil oil
Bacillus subtilis ATCC6633	0.06	0.1	0.02	0.03
Staphylococcus aureus ATCC25923	0.05	0.4	0.02	0.09
Escherichia coli ATCC25922	0.2	0.3	0.2	0.6
Salmonella typhimurium ATCC13311	0.4	0.6	0.4	1.6
Pseudomonas aeruginosa ATCC9027	>0.9	>1.0	>1.9	>1.8

Constituents of Kaffir lime oil

Kaffir lime oil, the volatile oil from the fruit peel, contained 54 constituents (Table 4) which were identified in the GC analysis by means of their retention times and mass spectral fragmentation patterns. The major constituents were 1-limonene, α-terpineol, 2-β-pinene, terpinene-4-ol, γ-terpinene, α-terpinene, and α-terpinolene. The analysis result agreed well with a previous study which showed high contents of β-pinene, and limonene[30].

Minimum inhibitory concentration of Kaffir lime oil

The minimum inhibitory concentration of Kaffir lime oil against 5 strains of the bacteria, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 13311, and *Pseudomonas aeruginosa* ATCC 9027 by agar dilution method was 0.1, 0.3, 0.4, 0.6, and more than 1.0% v/v, respectively. From the results, the MIC of 0.6% v/v was selected for further formulation since Kaffir lime oil at this concentration showed the antibacterial activity against both Gram positive and Gram negative bacteria except *P. aeruginosa*. This result agreed well with previous studies which showed that the alcoholic extract of Kaffir lime rind was effective in inhibition against *S. aureus*[40,41], *B. cereus*[41], *Staphylococcus flexneri*, and *Vibrio cholerae* Ogawa[40]. The resistance of *P. aeruginosa* was expected because of its ability to block foreign molecules from entering the cell wall. Kaffir lime oil, thus, proved to be a potential active ingredient in the antibacterial rinse. Since limonene was the major constituent (20.39%) of Kaffir lime oil; the compound was anticipated to contribute to the antimicrobial activity of the essential oil. Previous studies on limonene were inconclusive to whether limonene itself or the oxidized derivatives were responsible for the antimicrobial activity. Both D-limonene[43-45] and the oxidized D-limonene derivatives[7,46] were shown to exhibit the antimicrobial activity against selected strains of bacteria and/or yeasts. Furthermore, the inhibitory activity against bacteria might be strain dependent since the activity was selective[7].

Emulsion formulations, attributes, and stability

Since Kaffir lime oil was insoluble in water and the antibacterial rinse needed to be soluble, or at least miscible with water in order to be practical as an antibacterial rinse for fresh produce, oil-in-water emulsion seemed to be a justifiable choice of product. The emulsifying agents selected for the formulations of emulsion were of natural origins[47].

According to preliminary formulations, the highest concentration of Kaffir lime oil which could be formulated into emulsion form was 40% v/v; the large amount of oil phase did not favor the formation of an oil-in-water emulsion. Such high oil content normally tend to give large oil droplets which would subsequently join each other

Table 4. Constituents of Kaffir lime oil

Number	R_t (min)	Area (%)	Compound	Number	R_t (min)	Area (%)	Compound	Number	R_t (min)	Area (%)	Compound
1	6.98	2.46	α-Pinene	19	18.73	0.61	Citronellal	37	25.87	0.58	Germacrene-D
2	7.59	0.17	α-Fenchene	20	19.53	1.15	α-Ylangene	38	26.17	0.28	α-Muurolene
3	7.75	0.47	Camphene	21	20.48	0.47	Linalool	39	26.52	0.11	Bicyclogermacrene
4	8.64	10.62	2-β-Pinene	22	20.84	0.37	Germacrene-D	40	26.73	0.43	Geranyl acetate
5	8.82	2.12	Sabinene	23	21.38	2.00	(-)-Isopulegol	41	26.79	0.54	l-Citronellol
6	9.60	1.22	Myrcene	24	21.57	3.55	(-)-Isopulegol	42	27.09	1.92	γ-Cardinene
7	9.82	0.94	α-Phellandrene	25	21.74	0.82	D-Fenchyl alcohol	43	27.83	0.12	Cadina-1, 4-diene
8	10.21	5.92	α-Terpinene	26	22.48	7.69	Terpineneol-4	44	28.96	0.06	Geraniol
9	10.82	20.39	1-Limonene	27	22.72	0.77	Caryophyllene	45	34.86	0.07	α-Copaene; γ-Selinene
10	11.01	1.55	Sabinene	28	23.12	0.94	trans β-Terpineol	46	35.01	0.13	α-Ylangene
11	11.72	0.07	3,7-Dimethyl octa-1,4,6-trien-3-ol	29	24.13	0.46	Citronellyl acetate	47	35.16	0.38	Elemol
12	11.94	6.29	γ-Terpinene	30	24.26	0.08	Isoborneol	48	37.01	0.09	1H-Imidazole, 2- ethyl-4, 5-dihydro-2-methyl-4-methylenetetra hydropyra 2, 4-dimethyl-3, 6-dihydro-2H-pyran
13	12.45	0.58	Cyclohexene	31	24.32	0.09	Cyclofenchene	49	37.34	0.38	γ-Euesmol (Selinenol)
14	12.57	0.73	p-Cymene	32	24.42	0.11	δ-Cardinene	50	37.74	0.09	T-Muurolol
15	12.99	5.06	α-Terpinolene	33	24.59	0.20	cis- β-Terpineol	51	38.60	0.08	α-Eudesmol
16	13.16	0.45	α Terpinene	34	24.78	0.37	α-Humulene; β-Selinene	52	38.77	0.23	T-Muurolol
17	17.55	0.89	cis-Linalool oxide	35	25.15	12.52	α-Terpineol	53	48.49	1.16	Benzoic acid
18	18.40	0.54	Linalool oxide	36	25.48	0.10	Ledene	54	64.69	0.60	Octyl phenyl acetate

to become even larger, more unstable, and eventually leading to separations. It was widely known that mayonnaise was a semisolid oil-in-water food emulsion made from 65% edible vegetable oil, acidifying ingredients (vinegar), and egg yolk phosphatides as the emulsifying ingredient. Egg yolk, the only emulsifying agent permitted in mayonnaise in the United States, contained lipoproteins, lecithin and other phosphatides[48]. By rationale, lecithin was justified to be used in conjunction with other emulsifying agents in the formulations which consisted of high oil content of 40%. Gelatin was selected for its lack of flavor and odor after it was dissolved. It was anticipated to enhance the viscosity of the emulsion formed by lecithin.

The formulations which showed no oil separations at the time of preparations were all formulae with 10 and 9% w/v gelatin, and 8% w/v gelatin with 3% w/v lecithin. The details were as shown in Table 5. All formulae were oil-in-water type. When the emulsions were stored at room temperature (30°C) or at 4 °C for the period of 4 weeks, the stability results of the emulsions were as shown in Table 6.

Table 5. The attributes of the freshly-prepared Kaffir lime oil emulsions

Emulsifying agents (% w/v)		Attributes		
Gelatin	Lecithin	Viscosity	Flow	Separation
10	1	+2	Good	None
	2	+2	Good	None
	3	+2	Good	None
9	1	+2	Good	None
	2	+2	Good	None
	3	+2	Good	None
8	1	+2	Good	Partly
	2	+2	Good	Partly
	3	+2	Good	None
7	1	+1	Very good	Distinct
	2	+1	Very good	Distinct
	3	+1	Very good	Distinct

pH of all the formulae = 8, color of all the formulae = cream, +1 = low, +2 = medium

Gelatin was reported to be stable at a wide pH range of 4.5-9[47]. In this study, gelatin alone at 10% v/v could not retain the stability of the emulsion; cracking occurred after the first week both at room temperature and at 4 °C. Although the addition of lecithin at 1, 2, and 3% w/v could eliminate the cracking problem, the viscosity was still too high, both at room temperature and at 4 °C. The emulsion at both temperatures was totally set after the second week and unpourable. Reducing the gelatin concentration to 9% w/v, in combination with 1, 2, and 3% w/v of lecithin provided an emulsion of similar viscosity. The emulsion flowed slowly at room temperature but was totally set at 4 °C after the first week. The gelatin concentration of 7%, in combination with 1, 2, and 3% w/v lecithin, was unable to retain the stability and cracking occurred after the first week at both temperatures. At 8% w/v, in combination with 1, 2, and 3% w/v of lecithin, despite the medium viscosity, flowed well and no creaming and cracking occurred during the 4-week period at both temperatures. On the other

Table 6. Stability of Kaffir lime oil emulsion, stored at 4°C and 30°C for 4 weeks

Storage Temperature (°C)	Emulsifying agents (%w/v)		Stability (week)											
			Creaming				Cracking				Viscosity			
	Gelatin	Lecithin	1	2	3	4	1	2	3	4	1	2	3	4
4	10	1	-	-	-	-	-	-	-	-	Set	Set	Set	Set
		2	-	-	-	-	-	-	-	-	Set	Set	Set	Set
		3	-	-	-	-	-	-	-	-	Set	Set	Set	Set
	9	1	-	-	-	-	-	-	-	-	Set	Set	Set	Set
		2	-	-	-	-	-	-	-	-	Set	Set	Set	Set
		3	-	-	-	-	-	-	-	-	Set	Set	Set	Set
	8	1	-	-	-	-	-	-	-	-	+2	+2	+2	+2
		2	-	-	-	-	-	-	-	-	+2	+2	+2	+2
		3	-	-	-	-	-	-	-	-	+2	+2	+2	+2
	7	1	-	-	-	-	+2	+2	+2	+2	NA	NA	NA	NA
		2	-	-	-	-	+2	+2	+2	+2	NA	NA	NA	NA
		3	-	-	-	-	+2	+2	+2	+2	NA	NA	NA	NA
30	10	1	-	-	-	-	-	-	-	-	+3	Set	Set	Set
		2	-	-	-	-	-	-	-	-	+3	Set	Set	Set
		3	-	-	-	-	-	-	-	-	+3	Set	Set	Set
	9	1	-	-	-	-	-	-	-	-	+3	Set	Set	Set
		2	-	-	-	-	-	-	-	-	+3	Set	Set	Set
		3	-	-	-	-	-	-	-	-	+3	Set	Set	Set
	8	1	-	-	-	-	-	-	-	-	+2	+2	+2	+2
		2	-	-	-	-	-	-	-	-	+2	+2	+2	+2
		3	-	-	-	-	-	-	-	-	+2	+2	+2	+2
	7	1	-	-	-	-	+2	+2	+2	+2	NA	NA	NA	NA
		2	-	-	-	-	+2	+2	+2	+2	NA	NA	NA	NA
		3	-	-	-	-	+2	+2	+2	+2	NA	NA	NA	NA

- = none, +1 = low, +2 = medium, +3 = high, NA = not applicable

hand, at 1, and 2% w/v of lecithin, the emulsion did show medium oil separation during the preparation process. Thus 3% w/v lecithin seemed to be the appropriate level of choice to be used in conjunction with 8% w/v gelatin to obtain a stable emulsion. This formula was selected for further antibacterial test.

As for long-term stability of the emulsion at 4 °C, the selected formula showed no creaming or cracking at 6 mo, 1, 1.5, and 2 yr periods but was totally set into soft solid form. The set emulsion could be placed at room temperature and restored to its original state. While at room temperature, the selected formula started to show creaming at 1.5 and 2 yr periods; the emulsion could be shaken and restored to its original homogeneous state. The important property of gelatin was its ability to form soft gels that melted around body temperature. Gelatin gels melted on heating and set on cooling. The process was reversible[47]. According to this unique property, the formulated emulsion could be stored at lower temperature, for example, at 4 °C in the refrigerator, in order to retain

the highest stability of both the Kaffir lime oil and the emulsion. Both the oil and the emulsion would normally be more stable at cooler temperature than at high temperate temperature such as in Thailand. For later practical use, a small portion of emulsion could be placed at room temperature prior to its use; it should dissolve readily in water at room temperature.

Concentration of Kaffir lime oil in the rinse

For practical household use, minimal volume of the finished product should be used for vegetable soaking. Considering the MIC of Kaffir lime oil of 0.6% v/v, the emulsion should contain the highest concentration of the essential oil allowed in the formula, in order to retain its antibacterial activity when diluted with water for soaking. Kaffir lime oil of 0.75% v/v was selected as the final concentration of the oil in the soaking water for vegetables. At such concentration, the oil should be able to inhibit 4 strains of bacteria, *B. subtilis*, *E. coli*, *S. typhimurium*, and *S. aureus*. The bacteria, *P. aeruginosa* could not be inhibited at 0.75% v/v concentration. It can be predicted that the bacterial flora of the vegetables would be reduced considerably.

Water for vegetable soaking should be set at 4000 ml, the two-third capacity of a small household washing bowl normally used in Thailand, sufficient for a small head of vegetable soaking. Since the emulsion was set to contain 40% v/v of the oil, then 75 ml of the emulsion would be required to dissolve in 4000 ml of water to provide the effective antibacterial property.

Sensory evaluation

Attributes of soaked vegetable pieces and the results of Triangle test were as shown in Table 7. Vegetable pieces which were soaked for 5 min showed similar appearances to the unsoaked pieces. The 10-min soaked pieces were relatively softer and slightly wilted while the 15-min soaked ones were distinctly wilted. According to the Triangle test, the panelists correctly identified the different (odd) samples of the 10-min or the 15-min soaked pieces and the control (25 and 27 correct answers out of 30, respectively), but not the 5-min soaked pieces and the controls (18 correct answers out of 30). The characteristic Kaffir lime oil aroma had mostly disappeared during the 2 water rinses and the 1-hr stand. Thus safe vegetable pieces with similar physical appearance and tastes to unsoaked pieces could be obtained by soaking in the diluted Kaffir lime oil emulsion for 5 min.

Table 7. Attributes and Triangle test results of cabbage pieces after soaking time of 5, 10, 15 minutes

Soaking time (min)	Attributes	Number of correct answers
5	Similar to unsoaked pieces	18
10	Softer, slightly wilted	25*
15	Distinctly wilted	27*

n = 30, * $p<0.1$

Antibacterial activity of the selected emulsion

Chinese cabbage was used as a representative vegetable in the antibacterial test since it was often consumed uncooked along with several spicy Thai dishes. In addition, the wavy nature of the surface of the vegetable leaves was of concern as a possible harbour for natural pathogens. Rinsing procedure was set to imitate the normal household practice of vegetable washing and rinsing. The vegetable was soaked in the emulsion-dissolved water and then rinsed twice with water. It was found that the emulsion dissolved well in water and could be easily rinsed away with water, as well. The soaking time of 5, 10, and 15 min was set to determine the minimum time needed to inhibit the majority of the bacterial population on the vegetable. The result of

the bacterial populations, before and after soaking, the log number of bacterial population and the log reduction in bacterial number by water, and by diluted emulsion at different soaking time after the second rinse was shown in Table 8. Water alone could reduce the bacterial population by less than 2 log. There was no significant difference in log reduction among the 3 soaking times among the control group. With Kaffir lime oil emulsion, after 5-min soaking, the natural bacterial population was reduced by almost 3 log after the second rinse. When the soaking time was increased to 10 min, more than 3-log reduction was shown. After 15 min and 2 rinses of water, the natural bacterial load reduction was higher than 4 log. Statistically, the log reduction by soaking in diluted emulsion was significantly higher when the soaking time was longer ($p<0.05$). The log reductions by emulsion were significantly higher than controls at all 3 soaking times ($p<0.05$).

Table 8. Effect of Kaffir lime oil emulsion in soaking solution (0.75% v/v Kaffir lime oil) on the natural bacterial population on chinese cabbage

Control				Emulsion			
Initial bacterial population (log_{10} CFU/g)	Soaking time (min)	Remaining bacterial population* (log_{10} CFU/g)	Log reduction** (log_{10} CFU/g)	Initial bacterial population	Soaking time (min)	Remaining bacterial population* (log_{10} CFU/g)	Log reduction**
6.87 ± 0.27	5	5.21 ± 0.25	1.66 ±0.03 ef	6.91 ± 0.68	5	4.23 ± 0.24	2.68±0.57 d
6.97 ± 0.64	10	5.12 ± 0.32	1.85 ± 0.33 e	7.14 ± 0.27	10	3.83 ± 0.30	3.30 ± 0.53 bc
7.26 ± 0.76	15	5.45±0.4	1.79 ± 0.40 e	7.45 ± 0.69	15	3.18 ± 0.77	4.27±0.51 a

Values are means of values from three experiments with three replicates each.
*Population±SD, at the end of soaking time, after second water rinse.
**Mean values that are not followed by the same letter are significantly different ($p<0.05$).

Comparing the bacterial load reduction to some previous works on the antibacterial activities of other sanitizers, the Kaffir lime oil emulsion proved to be more superior than chlorine dioxide (1-log reduction, 1-5 ppm; 2-log reduction, 4.1 mg/L), chlorine (1-2-log reduction, 200 ppm; 2-4-log reduction, 100-2,000 µg/ml), ozone (<2-log reduction, 1-4 ppm)[3,14,27]. The emulsion produced, although not achieving the 5-log kill required by FDA[3], after 15 min soaking and 2 rinses, but was capable of eliminating more than 4 log of natural bacteria on the vegetable (at 4.27-log reduction); other sanitizers with over 4-log reduction were ozonated water (4.6-log reduction, 1.3 mM)[10], chlorine dioxide (5.5-log reduction, 4.0 mg/L)[26] and white vinegar (5-log reduction)[13]. The disadvantages of ozone and chlorine dioxide treatments are that the former requires expensive generating equipment[3] while the latter is unstable and explosive under some treatment conditions[29]. White vinegar was the only reported 5-log kill sanitizer and had the advantage of being the product from natural source. Nonetheless, white vinegar treatment had one drawback, the vegetable tasted noticebly sour after 10 minute soaking and was slightly wilted. One advantage of Kaffir lime oil emulsion over white vinegar, was that the Kaffir lime oil emulsion had a more pleasant, non-piercing aroma, compared to white vinegar. Furthermore, the aroma of the treated vegetables tended to fade away after they were left at room temperature for at least 1 h The antibacterial rinse from Kaffir lime oil might be considered as another effective alternative sanitizer from natural source.

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