Chemical Compositions and Antibacterial Effect of Essential Oil of Key Lime Leaves (*Citrus aurantifolia* Swingle: Rutaceae)

Yuliasri Jamal, Aris Medta Pariwidjayanti, and Andria Agusta*

Research Center for Biology, Indonesia Institute of Sciences (LIPI), Indonesia

Abstract

Steam distillation of essential oil from fresh leaves of Key lime (*Citrus aurantifolia* Swingle) gave 0.4% yellow essential oil with distinctive fragrance. Five out of 40 components of the essential oil were identified as the major components with content above 5%, they were geranial (10.3%), limonene (10.2%), neral (8.94%), caryophyllene (5.72%), and citronellal (5.41%). The essential oil was active against four bacterial test, *Bacillus subtilis* NBRC 3134, *Staphylococcus aureus* NBRC 14276, *Micrococcus luteus* NBRC 14218, and *Escherichia coli* NBRC 14237. *B. subtilis* was the most sensitive bacterium with the widest inhibition area at 50% concentration of essential oil. Minimum Inhibition Concentration (MIC) value of essential oil against *B. subtilis* was 0.125%.

Keywords: essential oil, antibacterial, key lime, *Citrus aurantifolia* Swingle, MIC

Introduction

Key lime (*Citrus aurantifolia* Swingle) is a small lime, ripening to yellow, although often used when green. It tends to be more aromatic in flavor and scent than other limes. Key lime fruit have medicinal efficacy to overcome diseases such as whoopingcough, sorethroat, toothache, ringworm, tinea versicolor, and acne. Moreover, the fruit is also belief to remove the blockageof vitalenergy, mucolytics, and diuretic (Sarwono, 2001).

Research done by Chisholm *et al.* (2003) reported about the extracted and distilled oil from the peel of key lime. The two kinds of chemical oil (extracted and distilled oil) were identified using Gas Chromatography-Olfactometry (GC-O) and Gas Chrmatography-Mass spectrometry (GC-MS). From extracted oil, over 50 active components of volatile oil were detected, while from the distilled oil there were over 60 active components of volatile oil detected. Both oils were dominated by three chemical compounds, geranial, neral, and linolool. 7-Methoxycoumarin was found to be one of the more intense odorants in the extracted oil, whereas caryophyllene oxide and humulene oxide were found to be major odorants in the distilled oil.

*Corresponding author:
Cibinong Science Center, Jl. Raya Bogor Km. 46,
Cibinong 16911, Indonesia
Phone/Fax. 021-87907604/87654321
E-mail: bislunatin@yahoo.com

Based on research done by Pertiwi (1992), essential oil of key lime leaves had antibacterial activity against *Staphylococcus aureus* and *Eschericia coli* growth. The ability of the essential oil had also been proven in inhibiting fungi growth. According to Dongmo (2009), essential oil of key lime leaves could inhibit the growth of *Phaeoramularia angolensis*. Neirotti *et al.* (1996) also found that limonene compound in key lime essential oil had ability to inhibit several bacterial growth (*Azotobacter* sp., *Staphylococcus* sp., *Enterobacter* sp., *B. subtilis*, *Streptococcus faecalis,* and *Pseudomonas aeroginosa*) as well as some fungi (*Mucor* sp., *Penicillium* sp., *Aspergillus* sp., and *Trichoderma* sp.).

According to Ultee *et al.* (1999), active compounds that play a role as antibacterial had different inhibition mechanism, such as reacted with cell membrane, disrupted the stability of cytoplasmic membrane, increased membrane permeability, inhibited extracellular enzyme, and influenced on bacterial metabolism.

Up to now, there is no further research yet about inhibition mechanism of essential oil of key lime leaves against cell bacteria. So, the present research will learn antibacterial activity and inhibition mechanism of essential oil of key lime leaves against bacteria by conducting various determination including essential oil MIC test, cell leakage, and change of cell morphology.

Materials and Methods

Plant Material and Test Bacterial Strain. Material research, fresh leaves of key lime (*C. aurantifolia* Swingle.) was obtained from Balittro, Cimanggu, Bogor in June 2009. Genus identification was conducted at Herbarium Bogoriense, Botany Division, Research Center for Biology, Indonesia Institute of Sciences (LIPI), Cibinong, Indonesia. The test bacteria used in this study were *Bacillus subtilis* NBRC 3134, *Staphylococcus aureus* NBRC 14276, *Micrococcus luteus* NBRC 14218, and *Escherichia coli* NBRC 14237.

Essential Oil Distillation. The fresh leaves of 6 kg key lime were washed out with water and distillated by steam distillation for 6 hours. After that, the essential oil was separated from the water layer followed by drying it with addition of sodium sulfate anhydrate. The rendemen of the essential oil then calculated based on the weight of oil collected.

GC-MS Analysis. Amount of essential oil of key lime leaves was diluted in diethyl ether and analysed using ion trap Gas Chromatography-Mass Spectrometry (GC-MS, Varian Saturn 2000) which was equipped with autosampler. Analysis was conducted using Factor Four Capillary Column VF-17 (Varian, USA) with inner diameter 0.25 mm and 30 meter long. The analysis condition was arranged in such a way with injector temperature 230°C and interface temperature 270°C. Column temperature was programmed from 50°C (3 minutes) to 150°C with temperature increase speed 5°C. Then, column temperature was increased again to 270°C with 3°C/minute. Inject volume was set 5 µL and scan MS m/z 50 to 450. Chemical component identification was done by comparing mass spectrum of the sample with NITS Library and Wiley.

Antibacterial Activities. Before used for the test, the four bacterial tested were rejuvenated on nutrient agar (NA) at 37°C for 24 hours. One loop of rejuvenated bacteria was then grown in 5 mL Mueller Hinton broth and was incubated at 37°C with shaker at 100 rpm (reciprocal) for 18 hours. Hundred µL bacterial suspension was then spreaded on Mueller Hinton agar. After incubated for 15 minutes, place the paper disk with 10 µL test solution with 50% concentration in 2% ethanol and 0.5% tween 80 (in aquadest). Furthermore, it was incubated for 24 hours at 37°C, and antibacterial activity was marked with the formation of clear zone around the paper disk. The test was carried out duplo.

Determination of MIC Value. The determination of MIC value of essential oil of key lime leaves was carried out using INT assay method. Bacterial suspension used to determine MIC value has density $5'10^5$ cell/mL with plate count method. The test solution concentration were of 80%, 40%, 20%, 10%, 5%, 2.5%, 1.25% and 0.625%. Hundred µL of each solution was added to 400 µL Mueller Hinton broth inoculated with 200 µL suspension of test bacterial cell. Then it was incubated at 37°C with speed of stirring 150 rpm (reciprocal). After 24 hours, 100 µL suspensions were moved to 96 well micro plate, then 14 µL iodinitrotetrazolium bromide solution (INT) with concentration 5 mg/mL was added. MIC value was determined based on the lowest concentration of essential oil of key lime leaves without color change of INT. Alcohol and tween 80 at the concentration equal to the treatment were used as control. The experiment was carried out duplo.

Membrane Cell Leakage Analysis. The increase of protein, nucleic acid, ion K^+ dan ion Ca^{2+} on media was used as indication of membrane cell leakage of tested bacterial as exposed effect of the key lime essential oil molecules. Ten mL of bacterial suspension was grown for 18 hours at 37°C, with 150 rpm was centrifuged for 20 min with 3,500 rpm at 4°C. After discarded the filtrate, bacterial pellet was suspended with phosphate buffer (pH 7.4). After being added with amount of the essential oil solution to reach the end concentration of essential oil of 1 MIC and 2 MIC solution, and phosphate buffer was again added to reach 10 ml of the end volume. The suspension was incubated in shaker incubator for 24 hours, centrifuged for 15 min at 4°C with speed of 3,500 rpm, and after that, the supernatant and bacterial pellet was separated. The supernatant was used to determine the protein, nucleic acid content using UV/Vis Spectrophotometer (Shimadsu 1240) at 260 and 280 nm wavelength. The ion K^+ and Ca^{2+} were determined using Atomic Absorption Spectrophotometer (AAS, Shimadzu AA-6800).

Morphological Change Analysis with SEM. Bacterial pellet from the previous treatment was then macerated in glutaraldehyde (2.5% in coccodilate buffer) for 4 hours at 4°C, centrifuged and decantated. Then the bacterial pellet cell was again macerated in 1% tannic acid (in coccodilate buffer)

for 12 hours, centrifuged and decantated and was macerated again in 2% osmium tetraoxide solution for 2–4 hours. After that, the pellet was washed again with coccodilate buffer followed by washing it with 50% cold ethanol. Furthermore, the bacterial pellet was washed again consequently with 50%, 70%, 80%, 95% ethanol absolute. Then, it was washed again twice with tert-butanol, and finally, pellet was suspended in tert-butanol. After that, apply a smear of bacterial cell on glass slip and then it was dried using freeze dryer (Eyela, FDU-1200). The smear of bacterial cell that dry on glass slip was then coated by gold for 1 hour at vacuum condition and photographed using Scanning Microscope Electron (SEM, JSM-5310LV, Jeol).

Results and Discussion

The essential oil of key lime leaves produced from the steam distillation was yellow with a distinctive aroma. The amount of essential oil obtained was 0.4% (v/w) based on the wet weight of the plant part.

GC-MS analysis result showed that the essential oil of key lime leaves had 40 chemical components as shown in Table 1 with 5 major components above 5%. They are geranial (10.39%), limonene (10.20%), neral (8.94%), caryophyllene (5.72%), and citronellal (5.41%). The forty chemical components were then classified into 5 groups of chemical compounds, *i. e.*, monoterpene, monoterpene alcohol, sesquiterpene, sesquiterpene alcohol and some other compounds, as shown in the Table 2.

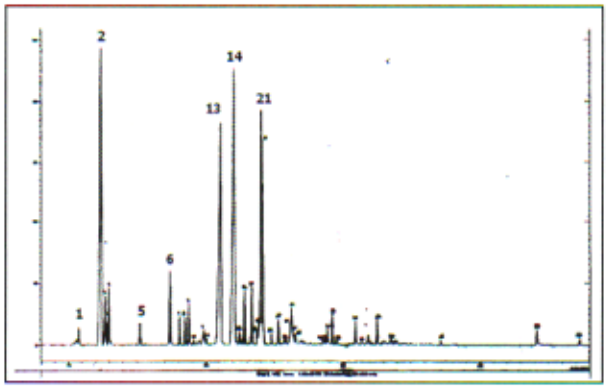

Figure 1. GC profile of essential oil of key lime leaves (Citrus aurantifolia Swingle)

Table 1. Chemical components of essential oil of key lime leaves (*Citrus aurantifolia* Swingle)

No.	Ret. time	Chem. compounds	MF	MW	Relative content
1.	10,76	2-β-Pinene	C10H16	136	1,27
2.	12,43	dl-Limonene	C10H16	136	10,20
3.	12,74	Linalyl acetate	C12H20O2	196	3,64
4.	12,97	1 ,3,6-0ctatriene,3, 7 - dimethyl	C10H16	136	3,16
5.	15,24	Bicyclo (2,2,1) Heptan-2-o1, 1,3,3-trimethyl-acetate	C12H20O2	196	1,74
6.	17,41	Citronellal	C10H18O	154	5,41
7.	18,09	Bicyclo (3,1,1) Hep-2-en-2-o1, 4,6,6-trimethyl-[1 S- (l-α, 2 β, 5 α)]	C12H16O	152	1,52
8.	18,50	Isopulegol	C10H18O	154	1,47
9.	18,77	Cis-Limonene oxide	C12H16O	152	2,44
10.	19,14	(1- Terpinenyl acetate	C12H20O2	196	1,26
11.	19,86	u-Phensyl acetate	C12H20O2	196	1,63
12.	20,06	Benzene Isosianomethyl	C9H7N	117	1,06
13.	21,12	Neral	C10H16O	152	8,94
14.	22,11	Geranial	C10H16O	152	10,39
15.	22,46	Iso-Methyl acetate	C12H22O2	198	1,91
16.	22,86	Elemene	C15H24	204	3,17
17.	23,40	Isopulegyl acetate	C12H20O2	196	3,16
18.	23,79	o-Bergamotene	C15H24	204	0,84
19.	23,90	T etradecanal		212	1,40
20.	24,10	Caryophyllene	C15H24	204	5,72
21.	24,16	Not identified	-	-	2,32
22.	24,80	Phenol, 4-etenyl-2-methoxy	C9H10O2	150	1,84
23.	25,30	o-Humulene	C15H24	204	1,66
24.	25,87	(z,z)- a - Phamesene	C15H24	204	0,64
25.	26,24	2,4A,8,8- Tetramethyl- 1, IA,4,4A,5,6, 7,8-oktahydro-cyclopropa Naphtalene	C15H24	204	3,20
26.	26,30	c ~-Selinene	C15H24	204	1,01
27.	26,40	2-Isoprenyl-4a, 8-dimethyl-1,2,3,4,4a,5,6,8a-octahydronaphtalene	C15H24	204	1,49
28.	26,54	a-Selinene	C15H24	204	2,40
29.	28,54	Veridiflorol	C15H26O	222	0,94
30.	28,64	Not identified	-	-	0,87
31.	28,85	5- a-Hydroxy-4a,8,10,11- (2-prophenyl)	C15H24O	220	0,85
36.	32,55	Spatulenol	C15H24O	220	1,64
37.	33,53	Cubenol	C15H26O	222	0,70
38.	37,16	Phenol, 5-(1,5-dimethyl-4-hexenyl)-2 methyl	C15H22O	218	1,09
39.	44,17	3,7,11,15- Tetramehyl-2-		296	1,19
					100,00

B. subtilis is normally rod-shaped with smooth surface as shown in Figure 2a. Provition of essential oil of key lime leaves at 1 MIC lead to morphology change of *B. subtilis*, *i.e.*, cells undergo shrinkage, stretching and cell surface become rough (Figure 2b). The addition of essential oil with higher concentration like 2 MIC, caused the formation of clear holes on cell surface of *B. subtilis* (Figure 2c).

Key lime is one of the traditional medicines and has been widely used by people to treat various diseases such as cough, sore throat, toothache, acne, ringworm, and skin fungus (Sarwono, 2001). This study has been done on the isolation, component identification, antibacterial activity test, and inhibition mechanism of key lime leaves in the form of essential oil for alternative antibacterial.

Tabel 2. Classivication of Components of Essential Oil

No.	Group of compounds	Relative content (%)
1.	Monoterpene	14,63
2.	Monoterpene Alcohol	2,99
3.	Monoterpene Aldehyde	24,74
4.	Sesquiterpene	22,04
5.	Sesquiterpene Alcohol	5,22
6.	Others	24,56
7.	Not identified	5,82
	Total	100,00

The leaf of key lime used in this study was collected from Balitro, Bogor and has been determined at Herbarium Bogoriense, Botany Division, Research Center for Biology, Indonesian Institute of Sciences (LIPI). The isolation process of key lime leaves using steam distillation was yielded 0.4% (v/w) yellow essential oil with distinctive aroma. Chemical components identification of GC-MS chromatogram (Figure 1) was conducted by comparing fragmentation pattern of mass spectrum of high sensitifity that was used for testing with dillution method and the next stage of the research.

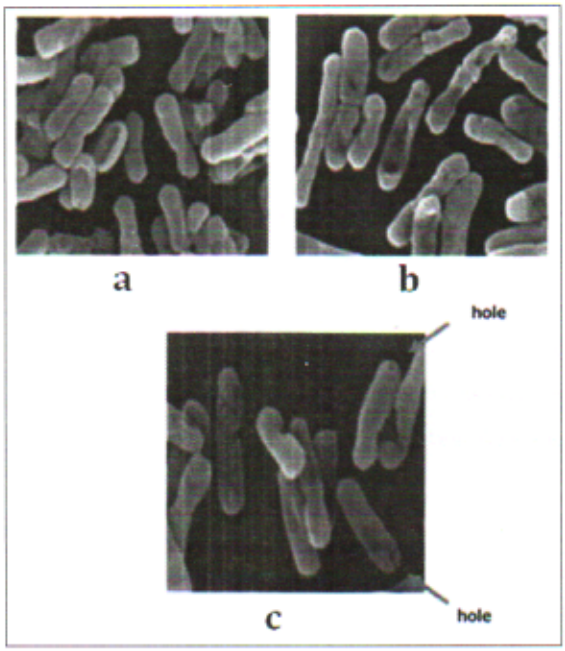

Figure 2. Cell morphology of *B. subtilis* treated with essential oil of key lime leaves at several concentration. A: 0 (control) B: 1 MIC, C: 2 MIC

Test result with diffusion method showed that the essential oil has activity against four bacterial tested with different inhibition areas (Figure 6). *B. subtilis* was the most sensitive bacteria to the essential oil of key lime leaves as it formed the widest inhibition area compared with other bacteria at 50% concentration and for that reason, this bacterium will be used for the future research. The essential oil of key lime leaves was effective in growth inhibition of *B. subtilis* and this result was supported by MIC test. At determination with tube dillution method was obtained MIC value of essential oil 0.125%. It was indicated by the absence of color change to red at 0.125% test solution after adding 14 µL of iodonitritetrazolium indicator (Figure 5).

Inhibition mechanism of essential oil of key lime leaves was through mechanism that caused cell leakage such as the leak of cellular metabolite (nucleic acid and protein) and metal ions (Ca^{2+} and K^{+}) that affected the morphological change of bacteria. The effect can be detected by the increasing of absorbance value at 260 nm for nucleic acid and 280 nm for protein (Miksusanti *et al.*, 2008). The damage of cell membrane or membrane permeability change can cause the release of cellular metabolites and metal ions.

Increase of absorbance value in nucleic acid or protein was appropriate with MIC concentration contacted to bacteria. The higher MIC concentration gave the higher leakage of cellular metabolite for both protein and nucleic acid. The release of metal ions from bacterial cell was a sign of antibacterial activity which caused membrane damage of bacteria cytoplasm. An increase of the release of ion K^{+} of bacteria was an indication of membrane permeability damage (Cox *et al.*, 2001). Ion Ca^{2+} was served to maintain bacterial membrane, therefore, with the present of ion leakage, the membrane stability will be disrupted that caused the death of bacteria (Suliantari, 2009).

Antibacterial activity of essential oil of key lime leaves can be influenced by chemical content of the essential oil. Based on the chemical structure, the major component of the essential oil was hydrocarbon monoterpene (limonene), oxigenated monoterpene (geranial, neral, citronelal) and sesquiterpene (caryophyllene). Terpenes have been reported to have antimicrobial activity, against

gram positive and gram negative bacteria as well as fungi (Trombetta *et al.*, 2005).

Limonene was a non-polar cyclic monoterpene (hydrophobic) whereas geranial, neral and citronellal were semi polar oxygenatic monoterpene (hydrophylic). Those were because they contained aldehyde group. The aldehyde group in essential oil was semi polar which was hydophylic (Miksusanti *et al.*, 2008).

Geranial and neral belong to weak acid group that plays a role in membrane permeability. Those components can interact with cell membrane, where the components were dissolved in phospholipid layer and bound between the fatty acid chains. This process can lead to membrane instability, increase membrane fluidity and alter membrane permeability (Miksusanti *et al.*, 2009). The alteration of membrane permeability can induce ion leakage, protein and nucleic acid. The essential oil key lime leaves generated Ca^{2+} and K^{+} ion leakage (Figure 4). The protein and nucleic acid leakage also occured after giving the essential oil toward *B. subtilis* (Figure 3).

Many cyclic hydrocarbon were toxic to microorganism (Sikkema *et al.*, 1994). This compound can lead to disruption on peptidoglycan part of cell wall so that the polar compounds can enter through the cell wall. This is due to the non polar character similarity (hydrophobic) between cyclic hydrocarbon and peptidoglycan of cell wall.

Essential oil characteristic was able to bind to lipid of bacterial cell membrane and influence on cell structure and membrane permeability (Prabuseenivasan *et al.*, 2006). From SEM result it can be seen that normal cell of *B. subtilis* (control) was rod-shaped with smooth surface. Treatment with 1 MIC essential oil led to cell membrane change compared to cell control. *B. subtilis* cell was elongated, cell surface was shrinkage and rough. By treatment with 2 MIC it caused more damage of the cell such as hole formation on the cell surface so that components in cytoplasm (such as protein, nucleic acid and metal ions) will be out of the cells. That leakage can bring death to cell bacteria. This was supported by analysis result of cell leakage of ions, protein, and nucleic acid as well.

Synergistic process occured between hydrophobic and hydrophylic essential oil components. The hydrophobic component interacted with hydrophobic peptidoglycan, while hydrophylic components interacted with phospholipid on cytoplasmic membrane. Furthermore, hydrophobic essential oil component interacted with fatty acid of hydrophobic phospholipid. This process will interfere membrane permeability making it easier for all essential oil components to enter the cytoplasmic membrane. The accumulation will induce membrane permeability change, so the components inside the cytoplasm will be released and followed by the death of cell bacteria.

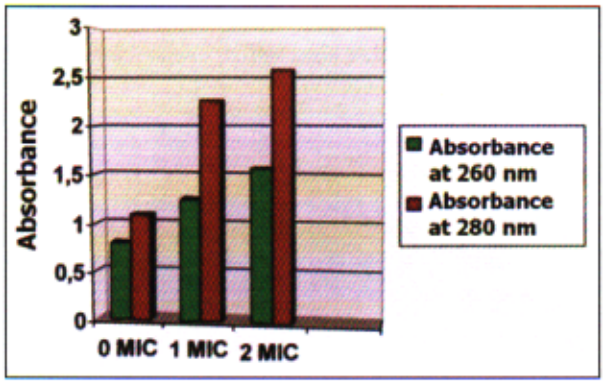

Figure 3. Level of protein and nucleic acid leakage from *S. aureus* in several essential oil concentration of key lime leaves

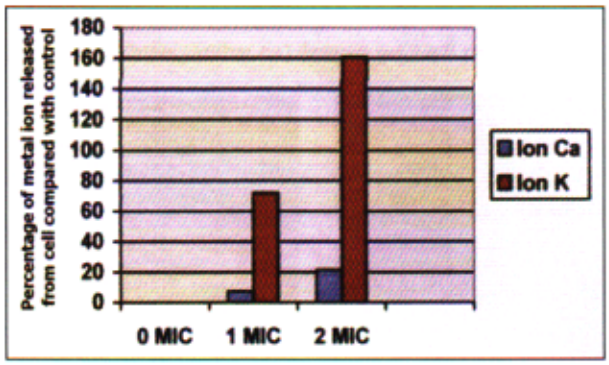

Figure 4. Level of ion leakage of *S. aureus* at several essential oil concentration of key lime leaves

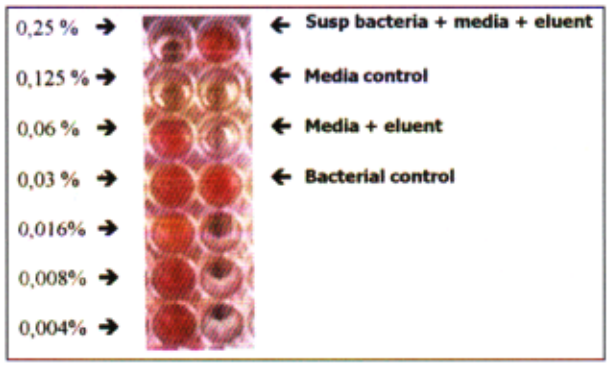

Figure 5. MIC results of key lime leaves essential oil

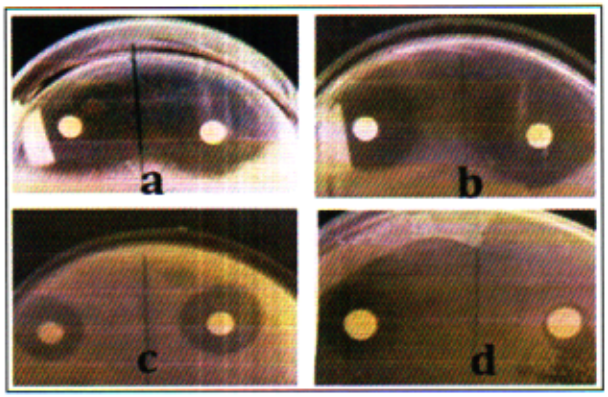

Figure 6. Antibacterial test results of key lime leaves essential oil against (a) *B. subtilis*, (b) *S. aureus*, (c) *M.Luteus*, and (d) *E. coli*.

Conclusions

The leaves of key lime (*C. aurantifolia* Swingle) contained approximately 0.4% essential oil consisted of 40 chemical components with 5 major components, geranial (10.39%), limonene (10,2%), neral (8.94%), caryophillene (5.72%), and citronelal (5.41%). The essential oil was active against several bacteria such as *S. aureus,S. Epidermidis, B. subtilis, M. luteus* and *E. coli*. MIC value of essential oil against *B. subtilis* was 0.125% (v/v).

Inhibition mechanism of essential oil of key lime leaves against *B. subtilis* occur through thedestruction of bacterial cell membrane that induced to cell leakage which could be observed in the presence of cellular metabolite leak.

References

Chisholm, M. G., M. A.Wilson & G. M. Gaskey. 2003. Characterization of aroma volatiles in Key lime essential oils (*Citrus aurantifolia* Swingle). *Flavour and Frag. J.*, 18 (2): 106–105.

Cox, S. D., C. M. Mann, J. L. Markham, E. Gustafson, J. R. Warmington & S. G. Wyllie. 2001. Determining the antimicrobial actions of Tea Tree Oil. *Molecules*, 6: 87–91.

Dongmo, P. M., L. N. Jazet, E. Tatsadjieu, E. T. Sonwa, J. Kuate, P. H. A. Zollo & C. Menut. 2009. Essential oils of *Citrus aurantifolia* from cameroon and their antifungal activityagainst *Phaeoramularia angolensis*. *AJAR,* 4: 354–358.

Ratih, D. P. 1992. Uji daya antibakteri dan identifikasi minyak atsiri dari daun jeruk nipis (*Citrus aurantifolia* Swingle). Fakultas Farmasi UGM, Yogyakarta.

Miksusanti, B. S., R. Laksmi, B. Syarief, Ponco & G. T. Mulyadi. 2008. Kerusakan dinding sel *Eschericiacoli* K1.1 oleh minyak atsiri temu kunci (*Kaempferia pandurata*). *Berita Biologi*, 9: 1–8.

Miksusanti, B. S., R. Laksmi, B. Syarief, Ponco & G. T. Mulyadi. 2009. Antibacterial activity of temu kunci tuber (*Kaempferia pandurata*) essential oil against *Bacillus cereus*. *Medicinal Journal Indonesia,* 18: 10–17.

Neirotti E., M. Moscatelli & S. Tiscornia. 1996. *Antimicrobial* activity of the limonene. *Arquivos* de. *Biologia e Tecnologia,* 39 (2): 233–237.

Prabuseenivasan, S., M. Jayakumar & S. Ignacimuthu. 2006. *In vitro* antibacterial activity of some plants essential oils. *BMC Complimentary and Alternative Medicine*, 6: 39.

Sarwono, B. 2001. *Khasiat dan manfaat jeruk nipis*. Jakarta: Agromedia Pustaka, 11 (19): 24–27.

Sikkema J., A. M. Jan, Bont de & B. Poolman. 1994. Interactions of cyclic hydrocarbons with biological membranes. *J. of Biol. Chem.*, 269: 8022–8028.

Suliantari. 2009. Aktivitas antibakteri dan mekanismepenghambatan ekstrak sirih hijau (Piper bettle Linn) terhadap bakteri patogen pangan. *Disertasi*. Sekolah Pasca sarjana IPB Bogor.

Ultee, A., E. P. W. Kets & E. J. Smid. 1999. Mechanism of action carvacrol on the food borne pathogen *Bacillus subtilis*. *AEM J.,* 65 (10): 4606–4646.