CHEMICAL CONSTITUENTS AND ANTIBACTERIAL EFFECT OF ESSENTIAL OIL OF JAVANEESE PEPPER LEAVES (*PIPER RETROFRACTUM* VAHL.)

MEKANISME KIMIAWI DAN EFEK ANTIBAKTERI MINYAK ATSIRI DAUN CABE JAWA

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Abstrak

Daun cabe jawa (Piper retrofractum Vahl.) telah terbukti berkhasiat sebagai bahan antimikroba, akan tetapi belum ada informasi lebih lanjut tentang mekanisme aksi antibakteri dari minyak atsiri daun cabe jawa. Penelitian ini untuk mengetahui pengaruh minyak atsiri daun cabe jawa terhadap bakteri uji sensitif serta difokuskan untuk melihat kerusakan membran sel bakteri melalui analisis protein, asam nukleat dan ion-ion logam kalsium dan kalium. Distilasi uap minyak atsiri dari daun segar cabe jawa (Piper retrofractum Vahl.) menghasilkan 0.03% cairan kental kekuningan. Analisis dengan GC-MS mengidentifikasi 4 senyawa sebagai komponen utama, yaitu germakren D (24.20%), tetrametilsiklo[5.3.1.0(4.11)]-undek-8-ena (17.73%), ar-turmeron (11.55%) dan benzil benzoat (6.28%). Minyak atsiri cabe jawa terbukti aktif melawan beberapa bakteri patogen antara lain Staphylococcus aureus, Bacillus subtilis and Micrococcus luteus dengan diameter daya hambat berturut-turut 8.0; 9.7; 8.5 mm. Pengamatan lebih lanjut memperlihatkan nilai MIC dari minyak atsiri cabe jawa terhadap B. subtilis adalah 2% (v/v), dan juga merubah morfologi sel dari bakteri yang diobservasi menggunakan SEM.

Kata kunci: Piper retrofractum Vah., cabe jawa, minyak atsiri, aktivitas antibakteri

Abstract

The leaves of Javaneese pepper (Piper retrofractum Vahl.) has been proven efficacious as antimicrobial agent, however, there was no further information about action mechanism of antibacterial of Javaneese pepper leaves essential oil. This study was to determine the effect of essential oils of javaneese pepper leaves against bacterial sensitive test and is focused on investigating the damage of cell membranes of bacteria through the analysis of proteins, nucleic acids and metal ions, calcium and potassium. Steam distillation of essential oil from fresh leaves of Javaneese pepper (Piper retrofractum Vahl.) gave 0.03 % yellowish viscous liquid. Germacrene D (24.20 %), tetramethylcyclo[5.3.1.0(4.11)]-undec-8-ene (17.73 %), Ar-turmeron (11.55 %) and benzyl benzoate (6.28 %) were identified as major constituents analyzed by GC-MS. Javaneese pepper essential oil was active against pathogenic bacteria Staphylococcus aureus, Bacillus subtilis and Micrococcus luteus with diameter of clear zone of 8.0, 9.7 and 8.5 mm respectively. Further investigation showed MIC value of the oil against B. subtilis was 2 % (v/v), and was also alter the cell morphology of tested bacterium observed by SEM.

Key words: Piper retrofractum Vahl., Javeneese pepper, essential oil, antibacterial activity

Introduction

Traditionally, Javaneese pepper fruit (*Piper etrofractum* Vahl: Piperaceae) has long been known to treat upset stomach, aphrodisiac, carminative, expectorant, laxative, digestive, anti-amoebic, anti-asthma, anti-septic and also have activity against several infection bacterial diseases[1,2]. In several countries, Javaneese pepper fruit is commonly used to cure leprosy and tuberculosis[3].

Phytochemical investigated indicated that dried fruit of Javaneese pepper contain piper-

octadecalidine, piperine, pipernonaline, guineensine, methyl piperate, N-isobutyl-2E,4E,8Z-eicosatrienamide and β-sitosterol[4,5]. Pipernonaline has LC_{50} 0.35 mg/L against *Aedes aegypti* larva[6] and piperine has MIC value 12.5 mg/ml againsts *B. cereus* and *E. coli*[7]. Besides, the fruit of Javeneese pepper originated from India was reported to have 1% essential oil with three major components, β-caryophyllene (17%), pentadecane (17.8%) and β-bisabollene (11.16%)[8].

The leaves of Javaneese pepper could be used as traditional medicine, to cure stomach ache and mouthwash[9]. For that reason, the study has been done to find relatively safe alternative antibacterial medicine by utilizing essential oil of Javaneese pepper leaves (*P. retrofractum* Vahl) to determine the antibacterial activity and its inhibition mechanism. The bacterial used were *Staphylococcus epidermidis, S. aureus, Bacillus subtilis, Micrococcus luteus* and *Eschericia coli.*

Several literatures said that the leaves of Javaneese pepper also contain essential oil[10,11]. A little further, Amalia[10] reported that essential oil of Javaneese pepper leaves showed the broad antibacterial and anti-fungal activities. However, further investigation on chemical composition, especially potential antibiotic and essential oil effect of the leaves of Javaneese pepper on microbe morphology has never been reported at all yet. For that reason, this paper will report and discuss the property of Javaneese pepper leaves based on the chemical contents, antibacterial potent and the effects on bacterial morphology test.

Material and Method

Plant material and Strain bacterial

Experimental material consisted of fresh Javaneese pepper leaves (*P. retrofractum* Vahl.) collected from Balittro (Balai Penelitian Tanaman Obat dan Rempah), Cimanggu, Bogor in Juni 2009. Genus identification was done in Herbarium Bogoriense, Botani division. Research Center for Biology-LIPI, Cibinong. Bacterial test used in this study were *Bacillus subtilis* (NBRC 3134), *Staphylococcus aureus* (NBRC 14276), *Micrococcus luteus* (NBRC 14218) and *Escherichia coli* (NBRC 14237). Methanol was used as control negative.

Essential Oil Distillation

The fresh leaves of 6 kg Javeneese pepper washed out with water and distillated by steamed distillation for 6 hours. After that, the essential oil was separated from water layer followed by drying it with sodium sulfate anhydrate. The yield of the essential oil then calculated based on the weight of oil collected.

GC-MS Analysis

Amount of essential oil of Javaneese pepper leaves was diluted in diethyl ether and analyzed using ion trap Gas Chromatography- Mass Spectrometry (GC-MS, Varian Saturn 2000) which equipped with auto sampler. 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 230^0C and inter phase temperature 270^0C. Column temperature was programmed from 50^0C (3 minutes) to 150^0C with temperature increase speed 5^0C. Then, column temperature was increased again to 270^0C with 3^0C/minute. Inject volume was set 5µl 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 using for the test, the four bacterial tested were rejuvenated on nutrient agar (NA) at 37^0C for 24 hours. One ose of rejuvenated bacteria was then grown in 5 ml Mueller Hinton broth and was incubated at 37^0C with shaker at 100 rpm (reciprocal) for 18 hours. 100 µl bacterial suspension was then deployed on Mueller Hinton agar. After incubated for 15 minutes, place the paper disk with 10µl test solution with 50% concentration in 2% ethanol and 0.5% tween 80 (in distilled water). Furthermore, it was incubated for 24 hours at 37^0C, and antibacterial activity was marked with the formation of clear zone around the paper disk. The test was done duplo.

Determination of MIC Value

The determination of MIC value of essential oil of Javaneese pepper leaves was carried out using INT assay method. Bacterial suspension used to determine MIC value has density 5 x 10^5 cell/ml with plate count method. 100 µl of test solution with concentration 80%, 40%, 20%, 10%, 5%, 2.5%, 1.25% and 0.625% with each was added to 400 µl Mueller Hinton broth inoculated with 200 µl suspension of bacterial test cell, then was incubated on 37^0C with speed of stirring 150 rpm (reciprocal). After 24 hours, 100 µl suspension were moved to 96 well micro plate, then was added with 14 µl

iodinitrotetrazolium bromide solution (INT) with concentration 5 mg/ml. MIC value was determined based on the lowest concentration of essential oil of Javeneese pepper leaves without color change of INT. Alcohol and tween 80 at the same concentration with the treatment was used as control. The experiment was done 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 Javaneese pepper essential oil molecules. Ten ml of bacterial suspension grown for 18 hours at 37^0C, with 150 rpm was centrifuged for 20 minute with 3500 rpm at 4^0C. 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 in 1 MIC and 2 MIC solution, and phosphate buffer was again added to reach 10 ml of end volume. Then, the suspension was incubated on shaker incubation for 24 hours, then the suspension was centrifuged for 15 minute at 4^0C with speed of 3500 rpm, and then the supernatant and bacterial pellet were separated. The supernatant was used to determine the protein, nucleic acid content using UV/Vis Spectro-photometer (Shimadsu -1240) at 260 and 280 nm wavelength. The Ion K^+ and Ca^{2+} were determined using Atomic Absorbtion Spectrophotometer (AAS, Shimadzu AA-6800).

Morphological Change Analysis with SEM

Bacterial pellet from the treatment before, was then macerated in glutaraldehyde (2.5% in coccodilate buffer) for 4 hours at 4^0C, 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 consequtively with 50%, 70%, 80%, 95% ethanol absolute. Then, it was washed again twice with tert-butanol, and at last, pellet was being suspended in tert-butanol. After being suspended, apply a smear of bacterial cell on glass slip and then 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 was photographed using Scanning Microscope Electron (SEM, JSM-5310LV, Jeol).

Result

The steam distillation of Javaneese pepper yielded 0.03% essential oil with yellowish color and distinctive aroma of Javaneese pepper.

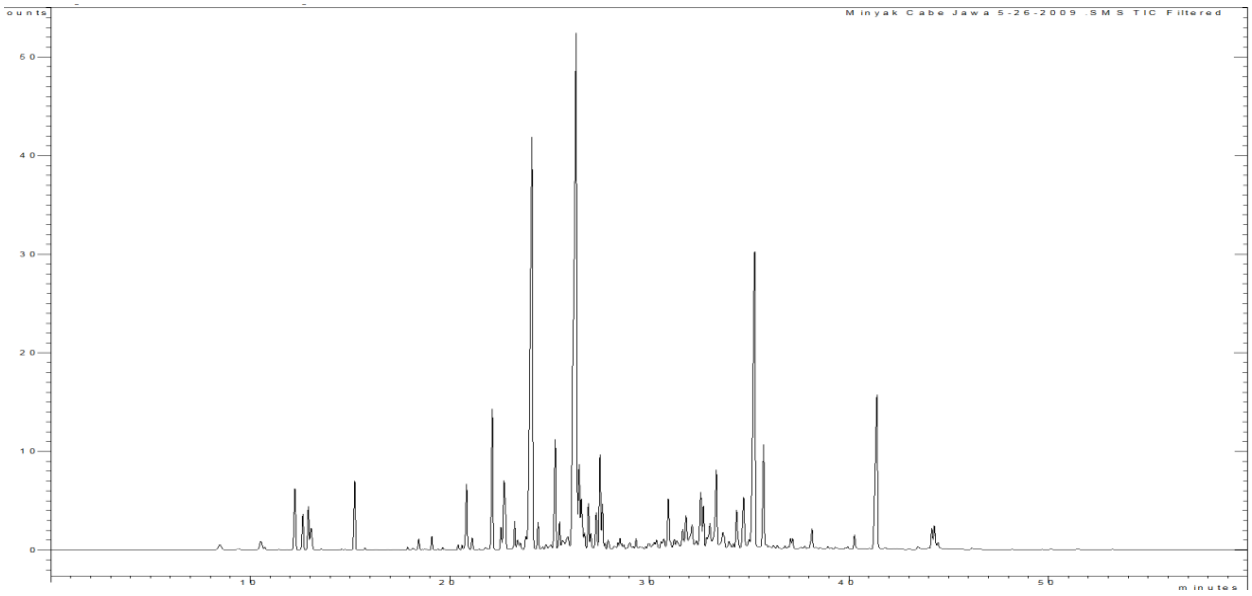

Figure 1. A GC-MS Chromatogram of Essential Oil of Javaneese Pepper

Table 1. Chemical Components of Essential Oil of Javaneese Pepper Leaves (*Piper retrofractum* Vahl.)

No. Peaks	Retention time	Components	Moleculer formula	Moleculer weight	Relative content (%)
1.	12,24	dl-limonene	$C_{10}H_{16}$	136	1,81
2.	12,64	(*E*)-3,7-dimethyl-1,3,6-octatriene	$C_{10}H_{16}$	136	0,92
3.	12,91	-3-carene	$C_{10}H_{16}$	136	0,75
4.	15,24	-phensyl acetate		196	1,83
5.	19,10	-terphynil acetate		154	0,27
6.	20,84	-elemene	$C_{15}H_{24}$	204	1,65
7.	22,13	-cubebene	$C_{15}H_{24}$	204	3,55
8.	22,57	-bourbonene	$C_{15}H_{24}$	204	0,52
9.	22,72	-cubebene	$C_{15}H_{24}$	204	2,59
10.	23,25	Pentadecane		212	0,52
11.	**24,12**	**tetramethyltricyclo[5.3.1.0(4,11)]undec-8-ene**	**$C_{15}H_{24}$**	**204**	**17,73**
12.	24,43	isokaryophyllene	$C_{15}H_{24}$	204	0,60
13.	25,29	-selinene	$C_{15}H_{24}$	204	2,93
14.	25,50	2-methoxy-4-(2-prophenyl)-phenol	$C_{10}H_{12}O_2$	164	0,50
15.	**26,33**	**germacrene D**	**$C_{15}H_{24}$**	**204**	**24,20**
16.	26,59	-selinene	$C_{15}H_{24}$	204	1,09
17.	26,95	-gurjunene	$C_{15}H_{24}$	204	0,89
18.	27,34	octahydro-7-methyl-4-methylene-1-(1-ethylmethyl)-benzene	$C_{15}H_{24}$	204	1,01
19.	27,53	-cadinene	$C_{15}H_{24}$	204	2,49
20.	27,66	selinen-3,7(11)-diene	$C_{15}H_{24}$	204	1,08
21.	30,96	Not identified	-	-	1,46
22.	31,84	torreyol	$C_{15}H_{26}O$	222	0,67
23.	32,57	-cadinene	$C_{15}H_{24}$	204	1,65
24.	32,71	-cadinol	$C_{15}H_{26}O$	222	0,96
25.	33,36	-muurolol	$C_{15}H_{26}O$	222	1,98
26.	34,37	turmerone	$C_{15}H_{22}O$	218	1,11
27.	34,73	Not identified	-	-	1,63
28.	**35,27**	**Ar-turmerone**	**$C_{15}H_{20}O$**	**216**	**11,55**
29.	35,73	curlone	$C_{15}H_{22}O$	218	2,67
30.	**41,40**	**benzyl benzoate**	**$C_{14}H_{12}O_2$**	**212**	**6,28**
31.	44,16	3,7,11,15-tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296	0,50
32.	44,29	phenylmethyl-2-hydroxibenzoate	$C_{14}H_{22}O_3$	228	0,56

Table 2. Chemical Constituents Classification in Javaneese Peper Leaves Essential Oil

No.	Groups	Relative content (%)
1	Monoterpene	3.48
2	Monoterpene alcohol	0.50
3	Sesquiterpene	63.44
4	Sesquiterpene alcohol	3.61
5	Other components	28.21

The GC-MS chromatogram (Figure 1) showed that essential oil of Javaneese pepper leaves contained 33 chemical components. There are 4 most dominant chemical constituents (relative content > 5%) in essential oil of Javaneese pepper leaves (Tabel 1), i.e: germacrene D (24.20%), tetramethylcyclo[5.3.1.0(4.11)] undec-8-ene (17.73%), Ar-turmerone and benzyl benzoate (6.28) respectively. While the other 3 sesquiterpene, α-cubebene (3.55%), β-cubebene (2.59%) and β-cadinene (2.49%) as well as sesquiterpene alcohol curlone (2.67%) and derivates of phenyl propanoate, 2-metoxy-4-(2-propenyl)-phenol (2.05%) showed the content between 2-4%. Whereas the content of other components were below than 2%.

Identification of each chemical component using NIST Library and Wiley showed that the essential oil of Javaneese pepper consisted of 3.48% monoterpene, 0.50% monoterpene alcohol, 63.44% sesquiterpene and 3.61% sesquiterpene alcohol and 28.21% other components. (Table 2)

Table 3. Sensitivity Test Result of Javaneese Pepper Leaves Essential Oil at 50% Concentration Against 5 Tested Bacterial

No	Bacterial tested	∑ GIA (mm)
1	*Staphylococcus aureus*	8.00
2	*Staphylococcus epidermidis*	-
3	*Bacillus subtilis*	9.5
4	*Micrococcus luteus*	8.50
5	*Eschericia coli*	-
6	Control methanol	-

The essential oil of Javaneese pepper was then tested for it sensitivity against five bacterial strains, *Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Micricoccus luteus* and *Eschericia coli* using paper disk method. Test result (Tabel 3) revealed that essential oil of Javaneese pepper was active against three tested bacteria, *B. subtilis, S. aureus* and *M. luteus* at 50% concentration with Growth Inhibition Area (GIA) 9.75, 8.00, and 8.50 mm consequently. While, there was no activity against *E. coli* at 50% concentration with no clear zone around the paper disk. From the result above, it showed that the essential oil of Javeneese pepper have the strongest biological activity against *B. subtilis* compared to the other bacterial test. Based on the test result, *B. subtilis* was used for further investigation. And from the determination of Minimum Inhibition Concentration (MIC) was known that the essential oil of Javaneese pepper leaves have MIC value 2% against *B. subtilis*.

Furthermore, the treatment of essential oil of Javaneese pepper leaves against *B. subtilis* at concentration 1 MIC indicated significant release of protein and nucleic acid from the cell to media (phosphate buffer). Measurement of media absorption at 260 nm (for nucleic acid) for tested bacteria with essential oil at concentration 1 MIC showed the increase of absorbance from 0.811 (control) to 2.270 and became triple (2.959) at concentration of 2 MIC. The same thing occurred to protein content measured at wave length 280 nm which increase from1.089 (control) to 3.436 at 1 MIC and became 3.913 with 2 MIC, as showed in Table4.

Table 4. The Level Of Cellular Metabolites Of *B. Subtilis* On Medium That Indicate Cells Leakage After Exposure With Javaneese Pepper Leaves Essential Oil.

Concentration of essential oil	Leakage level absorbtion of protein at 260 nm	Leakage level absorbtion of nucleic acid at 280 nm	Leakage percentage of K^+ ions (%)	Leakage percentage of Ca^{2+} ions (%)
Control (0 MIC)	0.811	1.089	0	0
1MIC	2.270	2.436	34.39	150.11
2MIC	2.959	3.913	61.73	165.3

1 MIC = 2% of essential oil of Javaneese pepper leaves

In addition to the leakage of protein, nucleic acid, K^+ and Ca^{2+} ions, observation with SEM also showed alteration on bacterial morphology of *B subtilis* when treated with Javaneese pepper leaves essential oil. The treatment with essential oil of Javaneese pepper leaves at concentration 1 MIC showed that elongation of *B. subtilis* cell followed by the change in cell surface become wavy or not smooth. At some parts of the cell surface seems to be concave that indicating the occurrence of cell wall leakage. As seen in Figure 3. Parts of *B. subtilis* cell wall which damage or leakage could be seen more clearly when treated with Javaneese pepper leaves essential oil at higher concentration, 2 MIC (Figure 3).

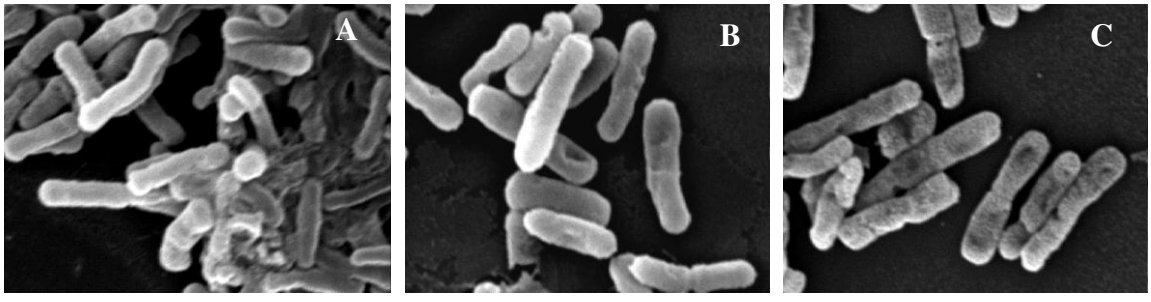

Figure 3. Cell Morpholofy of *B. Subtilis Treated* with Essential Oil of Javaneese Pepper Leaves at Several Concentration. A: Concentration 0 (control), B: 1 MIC, C: 2 MIC

Discussion

Essential oil chemical composition of Javaneese pepper leaves was different from the essential oil of the leaves (originated from India). The major chemical components of fruit essential oil are β-cayophyllene (17%), pentadecane (17.6%), and β-bisabolene (11.16%)[8]. From China was reported that essential oil of Javaneese pepper fruit contain the major components such as: β-caryophyllene (33.44%), 3-carene (7.58%), eugenol (7.39%), d-limonene (6.70%), zingiberene (6.68%), and cubenol (3.64%)[12].

From 33 identified components of GC-MS analysis, could be seen that the essential oil has high enough terpenoide content which consisted of 3.48% monoterpene, 0.50% monoterpene alcohol, 63.44% sesquiterpene, 3.61% sesquiterpene alcohol and the other 28.41% those were not included to terpene group (Table 3).

Based on the test of sensitivity against 5 kinds of tested bacteria using agar diffusion method, known that Javaneese pepper leaves essential oil was the sensitive against *B. subtilis* compared to *S. aureus* and *M. luteus*. Otherwise the essential oil Javaneese pepper leaves did not have antibacterial properties against *E. coli* and *S. epidermidis*. It was showed that with this reason, only *B. subtilis* would be used for further investigation / test.

The determination of MIC value from Javaneese pepper leaves essential oil against *B. subtilis* was carried out using micro broth dilution. While for result visualitation, 14 µl of iodintrotetrazolium was added as indicator for the presence or absence bacterial growth. The result of MIC value for *B. subtilis* was approximately 2% (v/v). The MIC value was determined based on minimum concentration which inhibit 100% bacterial growth.

Inhibition mechanism of javaneese pepper leaves essential oil at concentration 1 MIC and 2 MIC can cause cell leakage which was observed by cellular metabolite leakage (protein and nucleic acid), and ions leakage of metal (K^+ and Ca^{2+}) which could affect on morphological change of tested bacteria.

The provition of essential oil concentration 1 MIC and 2 MIC could lead to increase absorbance value of nucleic acid at 260 nm analogous with absorbance elevation of protein at 280 nm. This result was consistent with previously research conducted by Miksusanti[13], where the higher the essential oil dosage of temu kunci the higher the leakage occur. The increace of protein and nucleic acid out from bacterial cell indicated that there was damage of cell wall part and cytoplasmic membrane which was observed at morphological change of bacterial cell with Scanning Electron Microscope (SEM).

The same effect on the leakage of Ca^{2+} and K^+ which was increased at essential oil dosage of 2 MIC. Indication of damage to cytoplasmic membrane was the cytoplasmic content leakage and K^+ content released elevation was a sign of membrane permeability damage[14]. The same thing reported by Madigan *et al*., (2003) cited from[13], stability of bacterial cell membrane permeability was affected by K^+ concentration in cytoplasmic liquid. While bacterial membrane stability was stabilized by Ca^{2+} and the other ions.

The result from morphological observation using SEM showed that normal cell of *B. Subtilis* was rod shapes with surface tended to be smooth. Treatment with 1 MIC dosage of essential oil caused morphological change compared to control. The cell surface of *B. Subtilis* became more rough or seemed to be uneven, elongated and contracted. While with the give of 2 MIC dosage, seen that more severe damage occured signed by the formation of shape holes at the certain parts of cell surface. The formation of holes was suspected due to disruption on cell membrane and cell permeability change which ultimately led to the release of cell material out. It is supported by the results of the analysis of cell ion leakage of both protein and nucleic acid according to the data in Figure 2 and 3.

Antimicrobial activity was influenced by several factors such as chemical composition[15]. There was synergy between hydrophilic with hydrophobic component of the essential oil. Hydrophobic chemical components was interacted with hydrophobic peptidoglycan. This process corrupted the membrane permeability, making it easier for all essential oil chemical components to enter the cytoplasmic membrane. Further, the essential oil molecules accumulated and interfered the membrane stability.

According to Gauthier[16], antimicrobial compounds will inhibit cell wall synthesis, damage cell membrane and increase membrane permeability. The damage of membrane cell will make it easier for organic acids penetrated

cytoplasmic membrane and caused changes in the stability of the membrane that would eventually lead to the leakage of ions.

The oxygenated terpen content was suspected responsible for the antibacterial activity possesed by the essential oil of Javaneese pepper. Phenolic compounds at low concentration can inhibit bacterial growth as well as denaturated the protein and increased membrane permeability[17]. Moreover, several sesquiterpene group such as germacren-D also shown to play a role in inhibiting the *B. subtilis* and *E. coli* grow[18]. Organic acids and esters like benzyl benzoate was also known as an antimicrobial[19]. Other compounds thought to contribute to the role of the essential oil as antimicrobial was limonene. According to Vuuren[20], various enantiomer of limonene play a role against bacterial growth of *S. Aureus, P. Aeruginose* and *C. Neoformans.*

This study obtained more in-depth information especially of the effect on metabolite cellular leakage (protein and nucleic acid), metal ion leakage, as well as morphological change of bacterial cell test. Seen from the influence against tested bacteria, the essential oil of Javaneese pepper was potential to be developed as a natural source for infection control.

Conclusion

The fresh Javaneese pepper leaves contained 0.03% essential oil, consisted of 33 chemical components with 4 major components, germacrene-D, tetramethylcyclo[5.3.1.0 (4.11)]undec-8-ene, Ar-turmeron and benzyl benzoate. The essential oil of Javaneese pepper leaves was actived against bacterial phatogens, *S. aureus, B. subtilis,* and *M. luteus* with 2% MIC value against *B. subtilis*. At concentration 1 and 2 MIC, the essential oil of Javaneese pepper leaves could caused cell wall leaks of *B. subtilis* which was a partial mechanism and the action of the essential oil against bacteria phatogens.

Suggestion

Further research was needed especially on specific chemical compound that play the role in inhibiting the bacterial growth or changing the cell morphology. For that study, it is important to purify each compound of 4 major components of Javaneese pepper leaves essential oil followed by testing each compound against bacteria.

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References

1. Kritikar, KR. and BD. Basu. 1984. Piper longum Linn. Indian medicinal plants. Periodical expert Book Agency, New Delhi, India.
2. Warrier, PK., VPK. Nambiar and KC. Raman. 1995. Piper longum Linn.: Indian medicinal plants. Orient Longman Ltd. Madrs, India.
3. Srinivasa, RP., J. Kaiser, P. Madhusudhan, G. Anjani and B. Das. 2001. Antibacterial activity of isolates from Piper longum and Taxus bacc baccata. Pharm. Biol., 39, 236-238.
4. Ahn, JW., CO. Lee, EJ Kim, OP. Zee and HJ. Kim. 1992. Piperoctadecalidine, a new piperidine alkaloid from Piper retrofractum fruits. Bull. Korean Chem. Soc., 13 (4), 388-391.
5. Nakatani, N., R. Inatani, H. Ohta and A. Nishioka. 1986. Chemical constituents of pepper (Piper spp.) and application to food preservation: Naturally occurring antioxidative compounds. Environ. Health Prescriptives, 67, 135-142.
6. Yang, YC., SG. Lee, HK. Lee. MK. Kim, SH. Lee and HS. Lee. 2002. A piperidine amide extracted from Piper longum L. fruit shows activity against Aedes aegypti mosquito larvae. J. Agric. Food Chem., 50(13), 3765-3767.
7. Lokhande PD., KR. Gawai, KM. Kodam, BS. Kuchekar, AR. Chabukswar and SC. Jagdale. 2007. Antibacterial activity of extracts of Piper longum. J. Pharm. And Toxicol., 2 (6), 574-579.
8. Shankaracharya, NB., LJ. Rao and S. Nagalaksmi. 1997. Characterisation of chemical constituents of Indian long pepper (Piper longum L.). J. Food Sci. Technol., 34(1): 73-75.
9. Departemen Kesehatan RI, 1985. Daftar Tanaman Indonesia jilid I. Pusat Penelitian Farmasi, Badan Penelitian dan Pengembangan Kesehatan. Jakarta.
10. Amalia, L., A. Gana dan Elin Y. 1995. Uji aktivitas antibakteri dan antifungi minyak atsiri beberapa tanaman suku Piperaceae. Skripsi Dept. Farmasi ITB.
11. Purnomo dan R. Asmarayani. 2005. Hubungan kekerabatan antar spesies Piper berdasarkan sifat morfologi dan minyak atsiri daun di Yogyakarta. Biodiversitas, 6(1), 12-16.
12. Liu, L., G. Song and Y. Hu. 2007. GC-MS analysis of the essential oils of Piper nigrum L. and Piper longum L. Chromatographia, 66 (9-10), 785-790.
13. Miksusanti, B.S.R Jennie, B. Ponco, G. Trimulyadi.

2009. Antibacterial activity of temu kunci tuber (Kaempferia pandurata) essential oil against Bacillus cereus. Medical Journal Indonesia. Vol 18(1) 10-17.

14. Cox S.D., C.M.Mann, J.LMarkham, H.C. Bell, J.E. Gustafon, J.R. Warmington and S.G. Wyllie. 2000. The Mode of Antibacterial action of the essential oil of Melaleucca alternifolia Tea tree oil). Journal of Applied Microbiology. Vol 88 (1) 171-175.
15. Naidu, A.S. 2000. Natural Food Antimicrobial Systems. CRC Press. London.
16. Gauthier, Ernest. 2006. Minyak Atsiri jilid I, Penerjemah S, Ketaren. Penerbit: Universitas Indonesia Press. Jakarta.
17. Maillard J.J. 2002. Bacterial target sites for biocide action. J. of Applied Microbiology (92); 16-27.
18. Palazzo, M.C., H.L. Wright, B.R. Aigus, B.S. Wright, D.M. Moriarity, W.A. Haber and W.N. Setzer. 2009. Chemical Composition and Biological Activities of leaf essential oil of six species of Annonaceae from Montaverde, Costa Rica. Rec. Nat. Prod. Vol. 3(3): 153-160.
19. Price and Shirley. 1997. Aromateraphy for Health Professionals. Penerbit Buku Kedokteran EGC: Jakarta; 41-46.
20. Vuuren, S.F.v. and A.M, Viljoen. 2007. Antimicrobial Activity of Limonene enantiomers and 1,8-cineole and in combination. Flavour and Fragrance Journal, vol 22(6): 540-544.