Antibacterial activity of temu kunci tuber (*kaempheria pandurata*) essential oil against *Bacillus cereus*

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Abstrak

Tujuan *Mekanisme khasiat antibakteri minyak atsiri rimpang temu kunci belum pernah dilaporkan. Telah dilakukan analisis mekanisme aktivitas antibakteri minyak atsiri rimpang temu kunci yang berasal dari Yogyakarta terhadap Bacillus cereus. Aktivitas yang diamati meliputi kemampuan minyak atsiri temu kunci dalam mengganggu permeabilitas membran sel sehingga menyebabkan kebocoran sel dan perubahan morfologi sel. Kebocoran sel diamati dengan keluarnya ion Ca^{+2}, K^{+}, protein dan asam nukleat. Kebocoran ion diukur dengan metoda spektrometri serapan atom. Kebocoran protein diamati dengan alat spektrofotometer UV pada panjang gelombang 280 nm, sedangkan asam nukleat pada 260 nm. Perubahan morfologi sel diamati dengan alat scanning electron microscopy.*

Hasil *Nilai minimum inhibitory concentration (MIC) dari minyak atsiri temu kunci adalah 0,12 % (v/v). Perlakuan B.cereus dengan minyak atsiri 1 MIC dan 2 MIC memberi pengaruh yang signifikan terhadap kebocoran sel dibanding kontrol ($P<0.05$). Ion K^{+} yang terlepas dari sel adalah 10.32-35.57%, dan ion Ca^{+2} adalah 15.05-41.54%. Protein yang teramati pada 280 nm menunjukkan absorbansi antara 0.6330-0.8670, sedangkan asam nukleat 0.4320-0.8307, dan semuanya berbeda bermakna dibanding kontrol ($P<0.05$). Pada pemberian 1 MIC minyak atsiri temu kunci sel B.cereus berubah menjadi lebih tebal, dengan lekukan-lekukan yang signifikan di seluruh sel. Pemberian 2 MIC minyak atsiri menyebabkan sel berubah menjadi transparan, kosong dan berpenampilan seperti ghost cell.*

Kesimpulan *Minyak atsiri kaempheria pandurata menyebabkan kebocoran dan perubahan morfologi bakteri.* **(Med J Indones 2009; 18: 10-7)**

Abstract

Aim The mechanism of temu kunci tuber essential oil potential as antimicrobial agent has not been reported. To analyze the mechanism of antibacterial activity of temu kunci tuber essential oil from Yogyakarta on B.cereus. Antibacterial activity of essential oil were analyzed for its ability to disrupt bacterial cell membrane, that caused cell leakage and altered the morphology of the bacteria. Leakage was measured by analyzing the Ca^{+2}, K^{+} ion outflow using an atomic adsorption spectrometry (AAS), and protein and nucleic acid using an ultraviolet spectrophotometer (UVS) on 280 nm and 260 nm respectively. Alterations in morphology were assessed using scanning electron microscopy (SEM).

Results Minimum inhibitory concentration (MIC) of temu kunci essential oil on B.cereus was 0.12% (v/v). Treatment of B. cereus using 1MIC and 2MIC showed significant leakage compared to control ($P<0.05$). The K^{+} and Ca^{+2} ion leakage from the bacterial cells were between 10.32-35.57% and 15.05-41.54% respectively and showed significant difference compared to control ($P<0.05$). The absorbance observed by UVS for protein and nucleic acid leakage were 0.6330-0.8670 at 280 nm and 0.4320-0.8307 at 260 nm, respectively, and were significantly different compared to control ($P<0.05$). Exposure of 1 MIC temu kunci essential oil on B.cereus caused thickening as well as irregularities on the cell wall. At 2 MIC cells seemed transparent, empty looking and showed a ghost-like appearance.

Conclusion Kaempheria pondurata essential oil could cause leakage and alter the morphology of the bacteria. **(Med J Indones 2009; 18: 10-7)**

Keywords: leakage, morphology

It has been estimated that as many as 30% of people in industrialized countries suffer from a food borne disease each year; and in the year 2000, according to the World Health Organization, at least two million people died from diarrhoea worldwide.[1]

One of foodborne pathogens is *Bacillus cereus*. *Bacillus cereus* is a Gram-positive, motile rod, that forms endospores in the middle of the cells. *Bacillus cereus* is the causative agent of two distinct forms of gastroenteritic disease connected to food poisoning. Due to changing lifestyle and eating habits *B.cereus* is responsible for an increasing number of food borne diseases in the industrial world. This pathogen causes two distinct types of toxin-mediated foodborne illnesses known as diarrheal and emetic syndromes. Diarrheal syndrome has been linked to three different enterotoxins: two protein complexes, hemolysin BL (HBL) and nonhemolytic enterotoxin (NHE) and an enterotoxic protein, cytotoxin K (cytK). Emetic syndrome is related to cereulide, a toxin encoded by the ces gen.[2,3]

The increasing incidence of food borne diseases, coupled with the resultant social and economic implications, means that there should be a constant striving to produce safer food and to develop new natural antimicrobial agent. Natural antimicrobial agents have been used years ago, to eliminate pathogens, but there are many resources that have not been exploited yet. Indonesia as a tropical country has many plants that can be used as antimicrobial agent. Some of those have not been revealed yet. One of the potential natural antimicrobial agent are essential oils. Essential oils are mixtures of compounds characterized by their capacity to generate flavor or aroma and are generally obtained from spices, aromatic herbs, fruits and flowers. Analysis of essential oils shows that of the different constituents, terpenoids are the most abundant and are present as either hemiterpenes, monoterpenes, sesquiterpen, or their derivatives.

The antimicrobial attributes of essential oils have been recognized long ago but only recently they have been established scientifically. One of the essential oil that has a potential as antimicrobial agent is essential oil from temu kunci tuber. The mechanism of this essential oil has not been reported before.

In this study, we reported the antimicrobial effect of temu kunci tuber essential oil on *B.cereus*. For a better understanding of their mechanisms of action, the capability of the temu kunci essential oil to damage biomembrane was evaluated by monitoring the release of ions, protein and nucleic acid. Morphological changes caused by this oil were also monitored under scanning electron microscopy (SEM).

METHODS

Extraction of the essential oil

Air-drying of temu kunci tuber was performed in a shady place at room temperature for 10 days. The tuber were used for the analysis of essential oil composition. A portion (2 kg) of temu kunci tuber was submitted for 8 h to hot water-distillation, using a Clevenger-type apparatus. The obtained essential oil (EO) was dried over anhydrous calcium sulphate and 2 µL was used for gas chromatography-mass-spectrometry (GC–MS) measurements.

Analysis of the essential oil

The analyses of the volatile compounds were carried out on a Hewlett-Packard GC-MS system (GC 5890 Series II;MSD 5971A). The fused-silica HP-15 M polyethylene glycol column (50m x0.2 mm i.d., 0.2 µm film thickness) was directly coupled to the mass spectrometer. The carrier gas was helium (1mL/min) and the program used was 4 min isothermal at 70^{o}C, followed by 70-180^{o}C at a rate of 4^{o}C/min, then held at 180^{o}C for 10 min; the injection port temperature was 250^{o}C. Ionization of the sample components was performed in the E.I. mode (70 eV). Individual constituents were identified by referring to compounds known in the literature data, and also by comparing their mass spectra with either known compounds or with the Wiley 229 and The National Institute of Standard and Technology 62 (NIST 62) mass spectral database.[4]

Bacterial strain and growth condition

B.cereus FNCC 057 strain (obtained from centre of university of Gajah Mada/UGM) was used in all experiments. Cells were grown at 30^{o}C in nutrient broth supplemented with 0.5% (w/vol) glucose (initial pH 6.7). Cultured cells were stored at -80^{o}C in 15% glycerol as a cryoprotectant.

The strain was propagated overnight at 30^{o}C in nutrient broth containing supplemented glucose. Cells were pelleted by centrifugation (5,000x g for 20 min., 4^{o}C), washed twice in 10 mL of 0.1% peptone water, and resuspended in 5 mL of 0.1% peptone water. The A_{660} was measured, and each suspension was diluted as necessary to obtain approximately certain cell densities. To confirm the exact cell strain, plating is also done after incubation time. All *B.cereus* that had been used were in log fase.

Determination of minimal inhibitory concentration (MIC)

The MIC of tested essential oils were determined using the agar dilution methods. Agar plates containing various concentrations of essential oil were innoculated with *B.cereus*. The working culture ($2x10^7$ colony forming unit [CFU]/mL) was diluted to obtain 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions in 0.85% NaCl solution, and 1 mL of the various dilutions were spread on the surface of the solidified agar plates. The positive control agars were inoculated with the same diluted culture without essential oil. Test and control plates were then incubated at room temperature. Plates were evaluated for the presence or absence of colonies after 24 h of innoculation. For each treatment, the absence of colonies on all plates tested were considered as an inhibitory effect. The lowest concentration of essential oil required to reduce growth of bacteria ≥90% from initial growth (CFU/mL) was confirmed as MIC.[5,6]

Leakage of cellular metabolites

Bacterial culture (10 mL) at the exponential growth stage were transferred into sterile centrifuge tubes and were centrifuged at 4,800 x g for 15 min. After the supernatant was discarded, the pellet was resuspended in 10 mL of nutrient broth (NB), pH 7. The suspension was centrifuged and resuspended twice in NB. Bacterial suspension of the same species in all centrifuge tubes were pooled, and OD 540 values and viable counts were determined. Then 10 mL aliquots were dispensed into each of six sterile flask (50 mL). Temu kunci essential oil were added at 1 MIC and 2 MIC concentrations. Flask containing only bacterial cultures served as controls. After the flask were incubated at 30°C with shaking at 200 rpm for 1 h., the suspensions were filtered through 0.45-µm filters and the filtrate were used to determine the A260 and A280 absorbance values. Differences in A260 and A280 absorbance values between control and test groups were used to estimate the release of metabolites. The experiments were repeated three times.[6,7] Mean values for each treatment were calculated and compared to the means of the corresponding untreated samples by using the two-tailed Student t test.

Leakage of potassium and calcium ions

All experiment steps were the same with the experiment steps in leakage of cellular metabolites. Potassium and calcium ion concentrations in cell suspensions were measured using atomic absorption spectrometry. The concentrations of total free potassium and calcium for *B.cereus* suspensions were measured after wet destruction of the cell with H_2SO_4 and H_2O_2. The mean of percentages of ion loss from treated suspensions were compared to the corresponding mean for the untreated control by two-tailed Student t test. Differences between means were considered significant when $P<0.05$. This experiment was conducted three times using three plates for each concentration of essential oils.

Scanning electron microscopy (SEM) of temu kunci oil (TK) -treated bacteria

Suspension of *B.cereus* in log phase of growth were prepared by inoculating and incubating 30 mL of NB. Organisms were harvested by centrifugation at 1.500 x g for 10 min., and the pellet was resuspended in fresh NB. Suspensions of *B.cereus* were treated with 1 MIC and 2 MIC of temu kunci oils for 10 min. Control in NB was stood for 10 min. After centrifugation at 1.500x g for 5 min., the pellets were fixed overnight in 2.5% glutaraldehid in 0.1 M cocodylate buffer at room temperature. Fixed microbial pellet were processed in graded alcohols, and ready to be examined. The bacteria were examined with a JEOL JSM-6390 A scanning electron microscopy at an accelerating voltage of 20 kV.

RESULTS

Essential oil composition of temu kunci tuber

Overall 34 compounds were characterized in the oil. The mayor components (>1%) were identified as hydrocarbon monoterpenes (myrcene 1.42%, ocimene 20.18%, and camphene 4.58%), and oxygenated monoterpenes (β-linalool 2.42%, camphor 20.39%, borneol 1.07%, geraniol 22.28%, cineole 14.97%, terpineol 1.52%, and cinamic acid methyl ester 6.6%) (Fig 1), (compounds that were less than 1% were not, mentioned). The diagram below shows the concentration of the components of the essential oil in relative percentage.

Ocimene, geraniol and camphor were found as the main components in temu kunci tuber (*Kaempheria pandurata*) oil, but other species and types that grow in different area/land (cemotypes) may contain mainly cyclic alcohol and ocimene.[7]

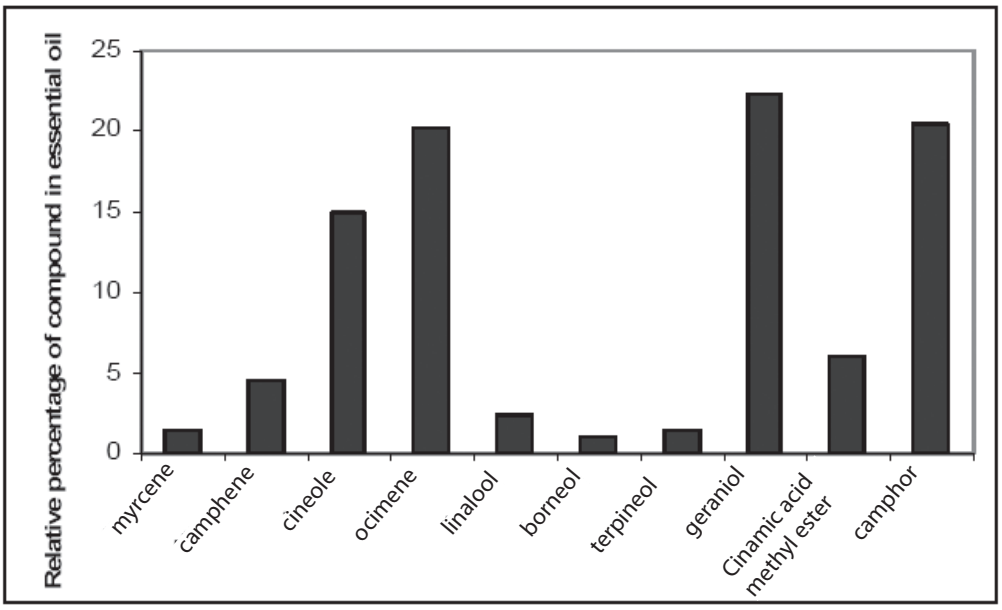

Figure 1. Main component of temu kunci tuber (Kaempheria pandurata) essential oil

Determination of MIC

Antibacterial activity of temu kunci oil was investigated in terms of minimum inhibitory concentration (MIC) after 20 hours of incubation. Figure 2 shows the effect of serial concentrations of temu kunci oil on the viability of *B.cereus*. Activity of temu kunci essential oil on *B.cereus* presented in Figure 2 showed that addition of temu kunci essential oil at concentration 0.12 % gave significant log CFU/mL decrease of bacteria from 6.0792 (initial) to 5.3685 *(P≤ 0.05)*. Therefore, minimum inhibitory concentration (MIC) of temu kunci essential oil to inhibit *B.cereus* is 0.12 % (vol/vol).

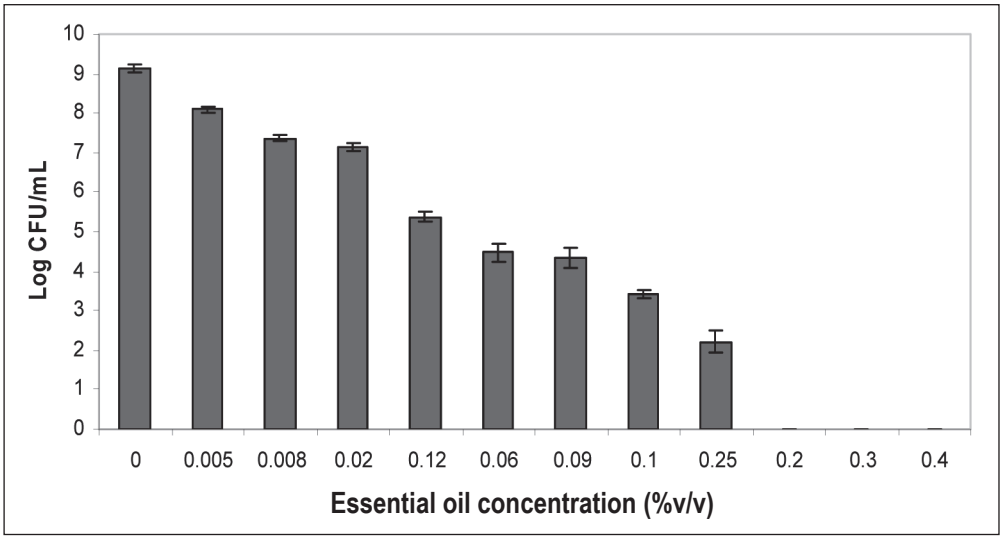

Figure 2. Curve to determine the MIC of temu kunci tuber essential oil

Leakage of cellular metabolites

The absorption at 260 nm and 280 nm of treatment and control filtrates were significantly different. Figure 3 shows the presence of 260-nm and 280-nm-absorbing materials in the filtrates of *B.cereus* in control and treatment groups. Significant increases in the absorption at 260 and 280 nm occurred after treatment with 1 MIC, and 2 MIC of temu kunci essential oil *(P<0.05)*. Control filtrates showed 280 nm and 260 nm absorbance of 0.0142 and 0.0129 respectively. Filtrates from treatment at 1 MIC gave absorbance of 0.8130 and 0.8301, and at 2 MIC, 280 nm absorbance range was 0.6330-0.8670 and that of 260 nm was 0.4320 to 0.8307.

Leakage of potassium and calcium ions

Temu kunci oil at 1 MIC, and 2 MIC induced leakage of potassium and calcium ions from *B.cereus* (Figure 4). The data, which were representatives of triplicate experiments that gave similar results, showed that leakage from *B.cereus* cells commenced immediately upon addition of temu kunci oil i.e. after 18 hours.

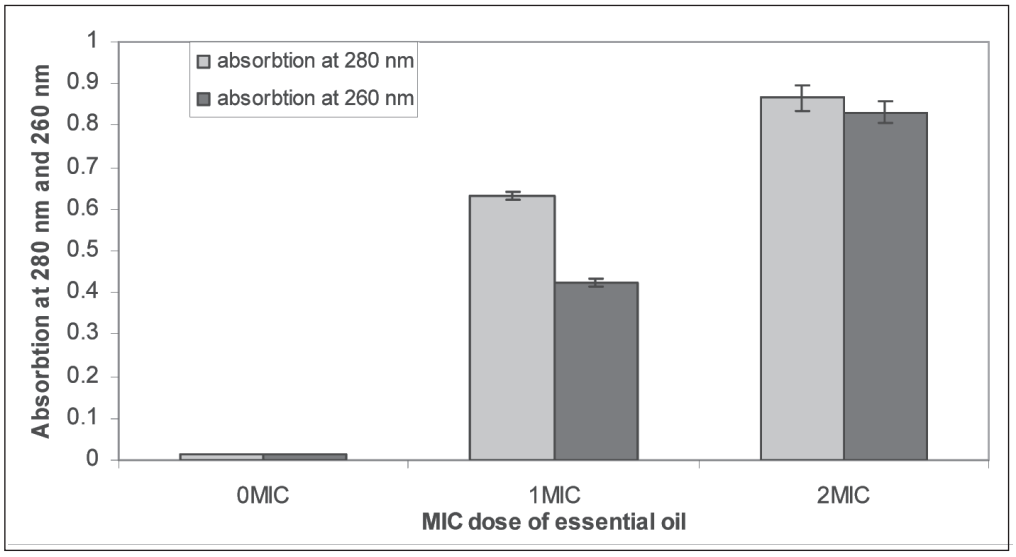

Figure 3. Protein and nucleic acid leakage

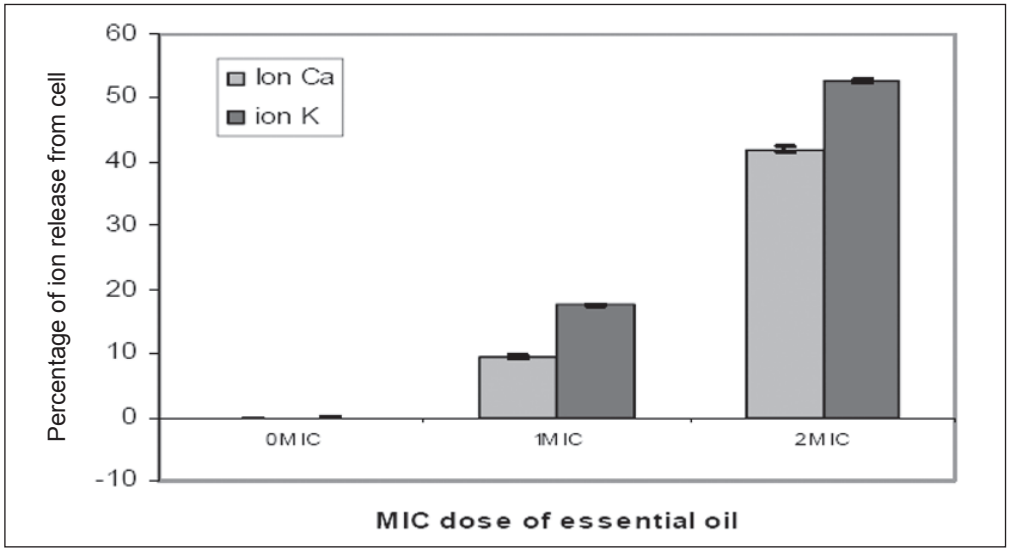

Figure 4. Potassium and calcium leakage

Increases in the temu kunci oil concentration in bacterial suspension caused increases in cellular leakage of potassium and calcium ions. Treatment with 1 MIC and 2 MIC of essential oil significantly increased the potassium and calcium leakage compared to control *(P<0.05)* i.e. 0.005 % for K^+ and 0.003 % for Ca^{+2} at 1 MIC and 10.32 % to 35.57% for K^+ and 15.05 % to 41.54 % for Ca^{+2} at 2 MIC.

Scanning electron microscopy of temu kunci oil-treated bacteria

To gain insight into the direct effect of temu kunci essential oil on the morphology of *B.cereus*, we used SEM at sublethal concentration.

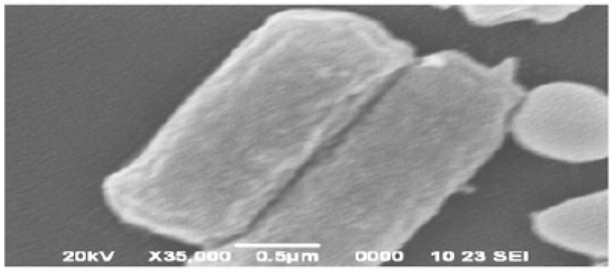

Figure 5a. B.cereus control (untreated)

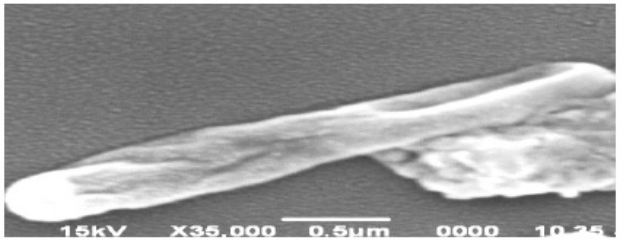

Figure 5b. B.cereus exposed to 1 MIC of temu kunci essential oil

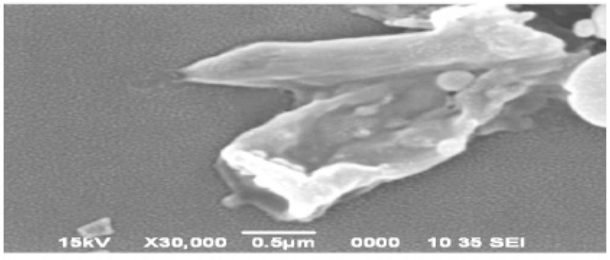

Figure 5c. B.cereus exposed to 2 MIC of temu kunci essential oil

The exposure of bacteria to 2 MIC caused remarkable modification of their cell shape, as shown by SEM. Untreated bacteria display a rough bright surface, typical of *B.cereus* strain, with no apparent cellular debris (figure 5a). In contrast, 1 MIC of essential oil exposed cells exhibited a wide range of significant abnormalities. At 2 MIC exposure, there was deep roughening of the cell surface. Temu kunci essential oil treated *B.cereus* cells showed different morphology (Figure 5c) that were not seen in untreated cells (Figure 5a). These included the collapse of the cell structure and a ghost-like appearance in which the cells seemed transparent, empty looking and flat, with cell debris arising from them (Figure 5c).

DISCUSSION

Although *B.cereus* is not a competitive microorganism, it grows after cooking and cooling (<48°C). Heat treatment causes spore germination, and in the absence of competing flora, *B.cereus* grows well. *Bacillus cereus* is a common soil saprophyte and easily spreads to many types of food, especially those of plant origin (rice and pasta), but it is also frequently isolated from meat, eggs, and dairy products. Increased numbers of *Bacilus cereus* strain that is resistant to physical environment (psychrotolerant strains), specifically in the dairy industry, have led to greater surveillance of *B.cereus* in recent years.[8]

Major components of temu kunci essential oil were found to be ocimene, geraniol, camphor cineol, camphene, cinamic acid methyl ester, linalool, borneol, terpineol and myrcene. These compounds belong to the group hydrocarbon and oxygenated monoterpenes.

Several previous reports were focused on the anti-microbial properties of essential oils and the main monoterpenes found in them. In fact, many terpenes are known to be active against a wide variety of microorganisms, including gram-positive and gram-negative bacteria and fungi.[9] Toxic effects on membrane structure and function have been generally used to explain the antimicrobial action of essential oil and their monoterpenes component. In fact, as a result of their lipophilic character, monoterpenes will be prefentially expelled from the aqueous phase to join the membrane structure.[10]

Considering the large numbers of different groups of chemical compounds present in temu kunci essential

oil, it is most likely that their antibacterial activity is not attributable to one specific mechanism but that there are several targets in the cell. The locations on target cell can be the cell wall, cytoplasm, membrane proteins (in cytoplasmic membrane) and proton motive force. Not all these mechanisms are separate targets; some are effected as a consequence of another mechanism being targeted.[11,12]

In this study MIC of temu kunci essential oil is much lower than those of other antibacterial essential oils such as those of *Rosemary*, *Tumeric*, *Lippia spp* that are 0.2% (v/v), 0.2% (v/v), and 5% (v/v) respectively.[6]

This study showed that temu kunci essential oil can cause leakage of ions, protein and nucleic acid. Although a certain amount of leakage from bacterial cell may be tolerated without loss of viability, extensive loss of cell contents or the exit of critical molecules and ions will lead to death. There are some evidence from this study with temu kunci essential oil and *B.cereus*, that cell death may occur before lyses (microscopic analyses).

From the results of this study we propose that geraniol, linalool, and cinamic acid methyl ester that have weak acid property can act on cell membrane permeability. These components can interact with the cell membrane, where they dissolve in the phospholipids bilayer and is assumed to align between the fatty acid chains.[13] These processes may cause expansion and destabilization of the membrane, increasing membrane fluidity and alter membrane permeability. The change of permeability may cause leakage of ion, protein and nucleic acid. This phenomena can bee seen in Figure 3 and 4. In this study, essential oil of temu kunci caused leakage of calcium (Ca^{+2}) and potassium (K^{+}) ions. Leakage of protein and nucleic acid was also observed after treatment of *B.cereus* with essential oil of temu kunci tuber.

It seems that the mechanism of action of having weak acid property components in temu kunci essential oil would therefore be similar to other known weak acid group such as carvacrol. Carvacrol can disturb the outer membrane, hence the ions that are attachéd to lipopolysaccharides (LPS) are released. Component of this essential oil can also disturb the cytoplasmic membrane. Undissociated molecules may diffuse through the cytoplasmic membrane towards the cytoplasm and dissociate by releasing their protons to the cytoplasm. It may then return to be undissociated by carrying a potassium ion (or other cation) from the cytoplasm which is transported through the cytoplasmic membrane to the external environment. A proton is taken up, and the compound in the protonated form diffuses again through the cytoplasmic membrane and dissociated by releasing a proton to the cytoplasm.[14,15] This phenomena may also be associated with *B.cereus* (which is also a gram positive bacteria) treated with essential oil from temu kunci. In this study potassium ion and calcium ion leakage from *B.cereus* treated with temu kunci essential oil were observed (Figure 4 and 5).

Many cyclic hydrocarbons e.g. aromatic, cycloalkanes and terpenes are toxic to microorganisms. Due to the hydrophobic character of cyclic hydrocarbons, the primary site of their toxicity is the outer membrane.[16,17] Hydrocarbons accumulate in the lipid bilayer according to a partition coefficient that is specific for the compound. Accumulation of compounds in the membrane may lead to alteration of the membrane structure and function. An important change is the apparent increase in surface area of the membrane upon accumulation of lipophilic compounds. The expansion observed with hydrocarbons was more than two times higher than the expansion by alcohols. This variation is probably due to differences in type of hydrophobic interaction and part of the membrane where lipophilic compound reside.[18]

An important characteristics of essential oil and their components is their hydrophobicity, which enables them to be introduced in the lipids of the bacterial cell membrane, disturbing the structure. At 1 MIC the temu kunci essential oil can alter the membrane structure without killing the bacteria. Our result supports the hypothesis that the main target of temu kunci essential oil is the bacterial outer and inner membrane, since the essential oil clearly causes appreciable membrane permeabilization, but not total disruption of cell. At higher concentration of essential oil (2 MIC), the membrane becomes leaky to cytoplasmic components (ion, protein and nucleic acid), which is concomitant with death of the bacteria. Therefore, it is reasonable to speculate that the actual mechanism of bacterial killing differs at high and low concentration of essential oil. At high concentration, rapid killing occurs owing to serious loss of membrane integrity (Figure 5c). The cell wall exhibited budding scars and underwent degenerative changes showing splitting of the wall layers. This budding scar manifestation has been reported by Harrison et al.[18] in yeast cells as a result of pulse electric fields (PEF). Maisner-Patin and Richard[19] reported that exposure of *L.inocua* to nisin concentration of 500

and 400 IU/mL induced cell wall irregularities. In this study, as the oil concentration increased, the cell wall of *B.cereus* lost smoothness and uniformity.

Nevertheless, at low concentrations, membrane perturbation would not the lethal. Under this condition, a small percentage of *B.cereus* might be killed due to other additional events. This essential oil might be suitable for facilitating the ingress of impermeable drugs, such as most of conventional antibiotics, into microbial targets. Therefore, this study suggests that temu kunci essential oil may have good potentials as anti *B.cereus* agent either in drug or food preservations.

In conclusion, the results of this study confirmed the possibility of using the temu kunci tuber essential oil or some of their components as antiseptic agent or as natural food preservatives, because the oil possess strong antibacterial activity. However, further research is needed in order to obtain informations regarding the practical effectiveness of the essential oil to prevent the growth of food borne and spoiling microbes under specific condition.

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