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Mode of Action Temu Kunci (*Kaempferia pandurata*) Essential Oil on *E. coli* K1.1 Cell Determined by Leakage of Material Cell and Salt Tolerance Assays

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The essential oil of *Kaempferia pandurata* consist of terpen and oxygenated terpen that exhibits broad-spectrum antimicrobial activity. It's mode of action against the gram-negative bacterium *E. coli* K1.1 has been investigated using a range of treatments. The mode action of the essential oil were analyzed by it's ability to leakage *E. coli* K1.1 cell, to change permeability of the cell, and to alter salt tolerance of the cell. Ion leakage from the cell were analyzed by atomic absorption spectrophotometer. Salt tolerance assays was conducted by investigating the ability of *E. coli* K1.1 treated with temu kunci essential oil to grow on NA supplemented with NaCl. Protein and acid nucleic leakage were analyzed by UV spectrophotometer. There were inorganic compound leakage (potassium, calcium ion) and organic compound leakage (nucleic acid, protein) from cytoplasmic membrane, after exposing this organism to essential oil of *Kaempferia pandurata.* The more concentration of oil added, the more leakage was observed due to the loss of absorbing material such as nucleic acid (260 nm) and protein (280 nm), the loss of potassium and calcium ion, and loss of the salt tolerance of *E. coli* K1.1.

Key words: essential oil, *E. coli* K1.1, leakage, salt tolerance assay

INTRODUCTION

Escherichia coli strains that cause diarrhoea include enterotoxigenic, enterophatogenic, enteroinvasive, and enterohaemorrhagic strains. Recently entero aggregative *E. coli* (EAggEC) has also been found to be a diarrheogenic strains. Among these pathogenic strains, enterotoxigenic *E. coli* (ETEC) is the major strain which may cause human diarrhoea. ETEC strains resemble vibrio cholera in that they adhere to the small intestinal mucosa and cause diarrhoea not by invading the mucosa but by elaborating toxins that act on mucosa cells. The disease caused by ETEC is similar to cholera in many ways, although the diarrhoea can be fatal, especially for infant and young children. ETEC strains may produce one or two types of enterotoxin. This enterotoxin are divided into heat labile toxins (LT) and heat-stable toxins (ST). A special serotype of this group that has been characterize as pathogen is *Escherichia coli* K1.1. This strains had been isolated from young children diarrhoea (Robinson *et al*. 2000).

The increasing incidence of food borne diseases, doubled with the resultant social and economic implication, means there are constant striving to produce safer food and to develop new natural antimicrobial agents. Therefore new methods of reducing or eliminating food borne pathogens such as *E.coli* K1.1, possibly in combination with existing methods still needed. Thus, the food industry at present day uses chemical preservatives to prevent the growth of food borne and spoiling microbes. It has been suggested that some synthetic preservatives convert some of the ingested material into toxic substances or carcinogens by increasing the activity of microsomal enzymes (Sacchetti *et al*. 2005).

In recent years there has been a considerable pressure from consumers to reduce or eliminate chemically synthesized additives in their foods. Most plants produce antimicrobial as secondary metabolites, either as part of their normal program of growth and development or in response to pathogens attack or stress. A novel way to reduce the proliferation of microorganisms is the use of essential oils. The oils are natural products extracted from plant materials, which because of their antibacterial, antifungal, antioxidant, and anticarcinogenic properties, it can be used as natural additives in many foods. Essential oils are currently attracting a lot of attention because they showed activity against antibiotic-resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA).

Essential oils are the mixtures of compounds characterized by their capacity to generate flavors or aroma and which generally obtained from spices, aromatic herbs, fruits, and

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flowers. Analysis of essential oils shows that of different constituent compound, terpenoids are the most abundant and are present as their hemiterpenes, monoterpenes or sesquiterpen and their derivatives. Essential oils have been proven to be inhibitory against a wide range of food spoiling microbes, dependent upon their concentration, methods of testing, and active constituents present (Smith-Palmer *et al*. 2001).

Indonesia has a lot variety of plants that consist of essential oil. One of the herb which contain the essential oil is *Kaempferia pandurata* (*Zingiberaceae*) known as temu kunci in Indonesia or krachai in Thailand. It consists a largely amount of monoterpenes and which of it approximately ± 50% are oxygenated and ± 50% are hydrocarbon. Although the in vitro antimicrobial activity of *Kaempferia pandurata* has been reported, there are no information about its action mechanism against pathogenic bacteria. Since this will have implications for its spectrum of activity, selective toxicity, and the development of resistance, we examined the action mode of *Kaempferia pandurata* essential oil against *E. coli* K1.1.

MATERIALS AND METHODS

Distillation of the Essential Oils. Air-drying of the plant was performed in a shady place at room temperature for 10 days. Plant bulbs were used for the analysis of essential oil composition. A portion (2 kg) of *Kaempferia pandurata* tuber was submitted for 3 h to hot water-distillation, using a Clevenger-type apparatus. The obtained essential oil (EO) was dried over anhydrous sodium disulphate and 2 ìl essential oil was used for GC -MS measurements.

GC-MS Analysis Conditions. The analyses of chemical components of the essential oil were carried out on a Hewlett-Packard GC-MS system (GC 5890 Series II; MSD 5971A). The fused-silica HP-20 M polyethylene glycol column (50 m x 0.2 mm d: 0.2 ìm film thickness) was directly coupled to the mass spectrometer. The carrier gas was helium (1 ml/min) and the program used was 4 min isothermal at 70 °C, followed by 70-180 °C at a rate of 4 °C/min, then held at 180 °C for 10 min; the injection port temperature was 250 °C. Ionization of the sample components was performed in the E.I, mode (70 eV). The linear retention indices for all the compounds were determined by co-injection of the sample with solution containing the homologous series of C8-C22 *n*-alkenes.

Leakage of Cellular Metabolites. Bacterial culture (10 ml in nutrient broth) at the exponential growth stage were transferred into several sterile centrifuge tubes and were centrifuge 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 centrifuge and resuspended twice in 10 ml NB. Bacterial suspension of the species in all centrifuge tubes above were pooled, OD540 values and viable counts of bacteria were determined. Then 10 ml aliquots were dispensed into each of six sterile flask (50 ml). *Kaempferia pandurata* oil were added 0.11%, 0.22%, and control (without essential oil). Flask containing only bacterial cultures served as controls. After the flask were incubated at 37 °C with shaking at 200 rpm for 1 h, the suspension were filtered through 0.45-ìm sterile filters and the filtrate were used for determination nucleic acid at A260 nm and protein at A280 nm. The differences of absorption value at A260 and A280 nm between controls and test groups were used to estimate the release of metabolites. The experiments were repeated three times.

Leakage of Potassium and Calcium Ions. Bacterial cells that has been incubated for 24 h were harvested by centrifugation at 10,000 x g for 15 min from 500 ml cultures in nutrient broth and washed once under the same condition above with cold deionized water. Then they were suspended in 100 ml of deionized water. Samples (15 ml) of cell suspension were placed in 50 ml glass vials and essential oil with concentration 0.11 and 0.22% were added and incubated for 24 h. Control were conducted without essential oil. pH values were monitored throughout the incubation period with. Sample (3 x 10 ml) were taken for dry weight determination and digestion in a HNO_3/H_2O_2 solution of 6 mol/l at 100 °C for 24 h and then centrifuged rapidly in an Eppendrof microfuge. The supernatant fluids were assayed for total mineral content of cells (K and Ca) by atomic absorption spectrophotometer (Perkin –Elmer model 2380, Norwalk,CT,U.S.A).

Loss of Salt Tolerance. The ability of *E. coli* K1.1 cells treated with temu kunci essential oil or it's components to grow on nutrient agar supplemented with NaCl (Merck Pty.Ltd) was investigated. In preliminary experiments, control suspensions of *E. coli* K1.1 were plated onto nutrient agar (NA) without NaCl (control) and NA containing NaCl at 10 to 100 g/l (NA-NaCl) than incubated, and the resulting colonies were counted. Concentrations of NaCl that modestly compromised the colony- forming abilities of untreated organisms were selected. These were 50 and 75 g/l. Suspensions of bacteria were prepared as described above and treated with *Kaempferia pandurata* essential oil at concentration essential oil 0.06, 0.11, and 0.22%. After 30 min, samples were removed, serially diluted with 0.85% NaCL, and inoculated onto NA and the selective medium NA-NaCl. After incubation, the numbers of CFU per milliliter on each NA-NaCl plate were compared to those on the NA plate, and the result was expressed as a percentage. The mean proportions of survivors from treated suspensions were compared to the corresponding means for the untreated controls.

RESULTS

Composition of the Essential Oils. Individual constituents of temu kunci essential oil were identified by referring to the known compounds in the literature data Wiley mass spectral database. These datum are shown in Table 1, which compound that contained less than 0.1% were not mentioned. The mayor chemical components of crude essential oils of *Kaempferia pandurata* are shown in Table 1. Terpen and oxygenated terpen were the most abundant volatiles detected in *Kaempferia pandurata oil*. *Kaempferia pandurata* oil has a relatively narrow chemical profile and consisted in approximately equal volumes of terpen and oxygenated terpen. Geraniol, cineole, linalool, borneol, and cinamic acid are oxygenated terpen. Myrcene, camphor, and ocimene are non oxygenated terpen.

Table 1. The major components of *Kaempferia pandurata* essential oils (% relative) by GC-MS

Phytochemicals	RI ± SD	% relative	Molecular weight
Myrcene	7.592 ± 0.03	4.58	134
Camphor	8.475 ± 0.02	1.42	146
Cineole	9.567 ± 0.01	14.97	155
Ocimene	9.975 ± 0.11	20.18	152
Linalool	11.150 ± 0.14	2.42	123
Borneol	12.675 ± 0.05	1.07	156
Terpineol	13.142 ± 0.05	1.52	134
Geraniol	14.408 ± 0.01	22.28	154
Cinamic acid	16.925 ± 0.03	6.06	148

RI: retention indices, DB-15M column; SD: standar deviation of yields oils range.

Ocimene, Geraniol, and Cineole are found as the major component in *Kaempferia pandurata oil* but, some species and chemo types may contain mainly camphor and ocimene (Friedman *et al.* 2004).

Loss of 260 and 280 nm Absorbing Material. The absorption of material cell at 260 and 280 nm in spectrophotometer UV from filtrates sample and control suspension were significantly different. The absorption value indicate that there are material cell that leakage from the cell. Material that absorb at 260 nm are nucleic acids and at 280 nm are proteins. The more absorption value, the more material cell that loss from the cell *E. coli* K1.1.

Significant increases in the absorption at 260 nm occurred after treatment with 1 MICs (0.11%), 2 MICs (0.22%), essential oil in ethanol ($P < 0.005$, F value = 249.58). From Duncan analysis we get differentiation category 0.22% = A, 0.11% = B, 0 = C. Statistic analysis for 280 nm treatment, also gave the same phenomena.

Leakage of Potassium and Calcium Ions. Temu kunci oil at 2 MIC (0.22%), 1 MIC (0.11%) and control induced leakage of potassium ions from *E. coli* K1.1 (Figure 2). The data, which was representatives of triplicate experiments that gave similar results, shows that leakage from *E. coli* K1.1 cells commenced immediately upon addition of *Kaempferia pandurata* oil.

Increases in the *Kaempferia pandurata* oil concentration in bacterial suspension caused increases in celluler leakage of potassium and calcium ion. All treatment gave significantly difference in the leakage of potassium and calcium ion ($P < 0.005$, F value = 6007.61) Duncan analysis showed that each concentration of ion leakage were in certain group that have significantly difference [A = Concentration ion leakage because of 0.22% oil (2MIC); B = Concentration ion leakage because of 0.11% oil (1MIC); C= Concentration ion leakage because of no oil (0%)].

Loss of Salt Tolerance. The results tests of salt tolerance loss are shown in Figure 3. The addition of NaCl to NA reduced the colony forming of untreated *E. coli* K1.1 cells to 81.704% (n = 15) with NaCl at 50 g/liter and 53.494% (n = 15) with NaCl at 75 g/l. Treatment with *Kaempferia pandurata* essential oil, at concentration 0.06%, the survivors of colonies on NA-NaCl was not significantly reduced at all NaCl concentration. At concentration 0.11% the proportion of survivors able to form colonies on NA-NaCl was significantly reduced when NaCl was used at 50 g/l and when NaCl was used at 75g/l.

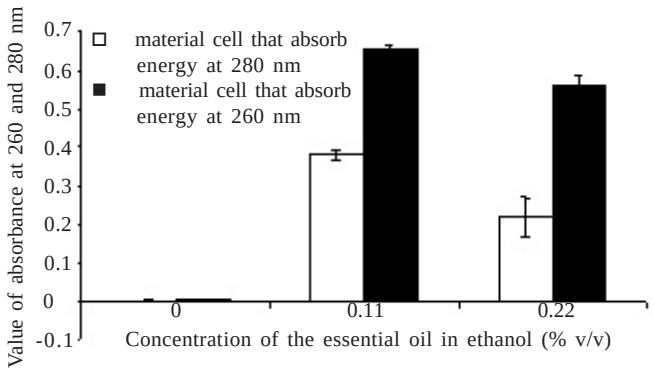

Figure 1. Appearance of 260 and 280 nm absorbing material in the filtrates of *E. coli* K1.1 control suspension and after treatment with the serial concentration of *Kaempferia pandurata* oil.

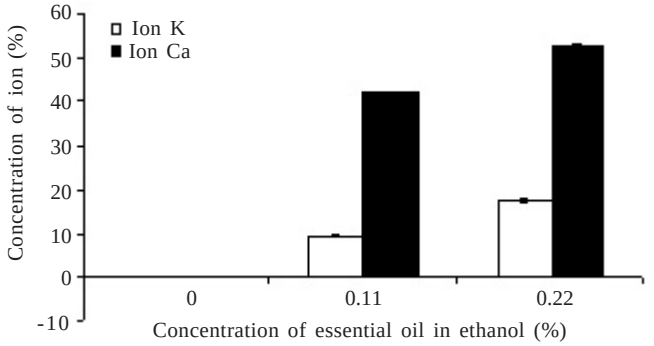

Figure 2. Effect of *Kaempferia pandurata* oil on potassium leakage and calcium leakage of *E. coli* K1.1.

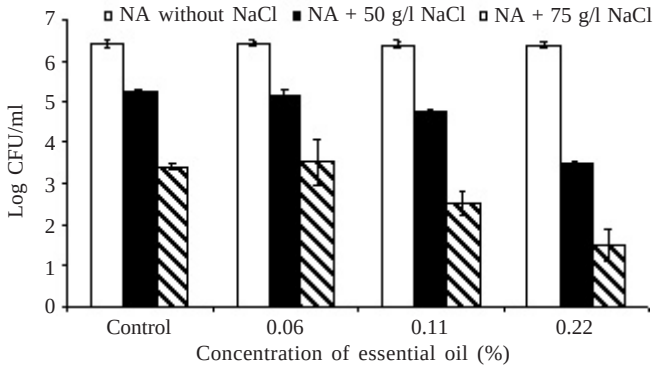

Figure 3. Effect of *Kaempferia pandurata* oil on loss of tolerance of *E. coli* K1.1 on NA-NaCl.

At concentration of essential oil 0.22%, significantly reduced of the ability survivors to form colonies on NA-NaCl (with NaCl at 50 or 75 g/l), with only 13% or less of the survivors able to form colonies.

DISCUSSION

The present study shows that the antimicrobial compound of *Kaempferia pandurata* oil, can inhibit and kill bacterial at low concentration. Our findings also revealed that minimum inhibitory levels of *Kaempferia pandurata* oil altered cell

membrane structure. Increase leakage of metabolite celluler, to which the cell membrane is normally impermeable, was observed. Also leakage of potassium ions commenced immediately upon adding *Kaempferia pandurata* oil to suspension containing *E. coli* K1.1.

The cytoplasmic membranes of bacteria provide barrier to the passage of small ions such as H^+, K^+, Na^+, Ca^{+2} and allow cells and organelles to control the entry and exit of different compounds. This permeability barrier role of cell membranes is integration to many cellular functions, including the maintenance of the energy status of the cell, other membrane-coupled energy–transuding processes, solute transport, regulation of metabolism and control of turgor pressure (Cox *et al*. 2000). Marked leakage of cytoplasmic material is considered indicative of gross and irreversible damaged to the cytoplasmic membrane. Many antimicrobial compounds that act on the bacterial cytoplasmic membrane induce the loss of 260 nm-absorbing material (nucleic acid) and 280 nm-absorbing material (protein), including chlorohexidine, hexachlorophene, phenetyl alcohol, tetracycline, polymixin, á-pinene, and lemongrass oil (Carson e*t al*. 2002). *E. coli* K1.1 suspension treated with *Kaempferia pandurata* essential oil, lost significant 260 and 280 nm-absorbing material, suggesting that nucleic acids and certain protein were lost through a damaged cytoplasmic membrane.

Toxic effects on membrane structure and function has been generally used to explain the antimicrobial action of essential oils and their monoterpene components (Sikkema *et al*. 1994) shows that as a result of their liphopilic character, cyclic monoterpenes will prefentially partition from an aqueous phase into membrane structures. This resulted in membrane expansion, increase membrane fluidity and inhibition of a membrane-embedded enzyme.

Helander *et al*. (1998) have described the effect of different essential oil components on outer membrane permeability in gram-negative bacteria. The fact that *Kaempferia pandurata* oil-induced damage to cell membrane structure accompanied by the decline in viability for all three micro-organisms included in this study has confirms it as the most likely cause of cell death.

According to Trombetta *et al*. (2002), besides depending on the bacterial strain employed, the antimicrobial effect of the oxygenated terpen might be the result of more processes. First, the antimicrobial directly elicit a gross perturbation of the lipid fraction of the bacterial plasmic membrane, resulting in alterations of intracellular material. Second, an enhanced membrane permeability resulted from the action of the oxygenated terpen, coupled with alterations in other membrane functions. Finally, the oxygenated monoterpenes may cross the cell membranes thus it can penetrate into the interior of the cell and interact with intracellular sites which is critical for antibacterial activity. On the other hand, the damage membrane itself may enhance the penetration of the drug into the cell.

Sublethal injury of microbial cell membranes may alter their permeability and affect the membrane's ability to osmoregulate the cell adequately or to exclude toxic materials. Consequently, the loss of tolerance to salts or other potentially toxic compounds may be exploited to reveal membrane damage in sublethally injured bacteria. Treatment *E. coli* K1.1 with *Kaempferia pandurata* oil reduced the ability of the survivors to form colonies on media containing NaCl. These result correlated well with *Kaempferia pandurata* at the MICs induced the loss of salt tolerance and 260-nm absorbing material. Treatment with these agents at one-half their MICs may have killed the most susceptible cells, leaving the more salt-tolerance cells (Carson *et al*. 2002).

For the conclusion, our observations confirm that the antimicrobial activity of *Kaempferia pandurata* oil results from it's ability to disrupted the permeability barrier of microbial membrane structures. This mode of action is similar to that of other broad-spectrum, membrane-active disinfectants and preservatives, such as phenol derivatives, chlorohexidine and para benzoic acid derivatives (Cox *et al*. 2000). This research support the observation that has been examined by other researcher about the action mechanism of liphopilic biocides effects on the cytoplasmic membrane and enzymes embedded in it (Uribe *et al.* 1991).

This present results lead us to speculate that the antimicrobial of mayor component of *Kaempferia pandurata* essential oil may be the result at least partially of the perturbation of the lipid fraction of the bacterial plasma membranes, due to the polarity of the component with polarity of plasma membranes. This process alterates permeability of the membrane and leakage the intracellular material, the component of *Kaempferia pandurata* essential oil might cross the cell membrane, penetrating the interior of the cell and interacting with intracellular sites critical for antibacterial activity. The potential of *Kaempferia pandurata* essential oil to inhibit *E. coli* K1.1 related to physicochemical characteristic of the each component in the essential oil, such as lipophilicity, water solubility of the component. Those physicochemical characteristic of essential oil may be match with lipid composition and net surface charge of the bacterial membranes *E. coli* K1.1, so the component of essential oil can penetrate and leakage the membrane. This process can alterates the morphology of the bacterial cell. At concentration 0.11% essential oil the cell size still the same with untreated cell, only slightly change in the shape, this is because of the ability of the cell to survive with low concentration of essential oil (data were not shown). Treated the cell with higher concentration of *Kaempferia pandurata* essential oil (0.22%) can alterates the cell become ghost cell (empty cell), this indicate that with height concentration of essential oil, the bacteria can not survive (Trombetta *et al*. 2005). Further experiments need to be conducted to investigate the effect on other organelles of *E. coli* K1.1. Further work is required to understand the fully mechanisms involved.

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