Gas chromatography-mass spectrophotometry volatilomics for antibacterial activity of essential oils from temu kunci grown at various altitudes

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Summary

Volatile compounds of temu kunci (*Kaempheria pandurata*) essential oil were reported to inhibit various bacteria. However, their composition from plants grown at various altitudes varied, hence their antibacterial activity might vary. This study examined the volatile compounds of temu kunci essential oil using metabolomic approaches, their antibacterial activity toward *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella* Typhimurium, as well as the mechanism of inhibition. The study consisted of volatile compounds profiling through gas chromatography-mass spectrophotometry, multivariate data analysis, inhibition study using disk method and assessment of the inhibition mechanism. The study showed that the volatile compounds were affected by the altitude of plant growth and able to inhibit the tested pathogens significantly. The principal component analysis model had Q^2 value of 0.568 as a goodness of prediction and R^2X value of 0.829 as the X variable described by model, suggesting that the model's prediction was good. Using the partial least square model, the volatile chemical compounds assumed to affect the antibacterial activity toward *B. cereus* was β-bisabolene, *Staph. aureus* was myrtenol and *S.* Typhimurium was *cis*-β-ocimene. Bacterial exposure to temu kunci essential oil causes cell membrane damage, as significant increase in cell leakage occured along with the increased essential oil's concentration.

Keywords

antibacterial activity; essential oil; *Kaempheria pandurata*; metabolomic; volatile

Temu kunci (*Kaempheria pandurata* Ridl) is a spice used in Indonesia as a food ingredient, in herb drinks and for aroma therapy. The plant grows in various regions in Indonesia, the main producer being the district of Wonogiri, Central Java, with a production of 353 t per year [1]. The essential oil of temu kunci was reported to have antimicrobial activities, to inhibit the growth of *Bacillus cereus* with minimal inhibition concentration (*MIC*) of 1.2 $ml \cdot l^{-1}$ [2]. Additionally, the ethanol extract of temu kunci was also reported to inhibit the growth of *B. subtilis* and *Salmonella typhi* with *MIC* of 80–160 $ml \cdot l^{-1}$ and 40–80 $ml \cdot l^{-1}$, respectively [3].

Metabolomics is an approach to explore the profile of compounds contained in essential oils with certain functionalities. Essential oil which are secondary metabolites produced by plants and contained in spices are generally volatile, with a pungent taste and plant aroma [2]. Metabolomics involves comprehensive quantitative analysis and identification of metabolites having a 100 Da to

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1 000 Da molecular weight, which are produced in the cells, tissues or biological fluids [4]. The content of metabolites in plants may vary because of many factors, such as climate, soil conditions or altitude. It also depends on other factors, including plant genetics, seeding area, planting and maintenance conditions, harvest age and post-harvest processing [5]. Metabolomics targeting volatiles using gas chromatography-mass spectrophotometry (GC-MS) was used to evaluate balsamic [6] and eucalyptus essential oils [7]. Metabolomics based on essential oils show similarities and differences in the profiles of volatile compounds among samples of the same plant species grown at various altitudes. GC-MS has been proven to analyse volatile compound profiles with high sensitivity and resolution including essential oils [7].

Certain essential oils have an inhibitory activity against some food-borne pathogens. Among them, *Escherichia coli*, *B. cereus* and *Staphylococcus aureus* were the primary etiologic agents linked to the outbreaks in Indonesia [8]. While pathogenic *E. coli* and *Salmonella* spp. are Gram-negative (G^-) bacteria associated with fecal contamination and food-borne infection [9], *B. cereus* is a spore-forming Gram-positive (G^+) bacterium capable of causing food-borne infection and intoxication [10]. The ability to form spores allows the bacterium to survive in extreme conditions during heat processing [11]. Meanwhile, *Staph. aureus* commonly contaminates foods from workers.

The active compounds in temu kunci that were shown to contribute to the antimicrobial activity are hemiterpenes, monoterpenes and sesquiterpenes [2]. However, the volatile chemical profiles of temu kunci essential oils obtained from the plant grown at various altitudes were reported to vary [12]. Hence, their activity as antibacterial agents may also be different.

Essential oils usually contain compounds that can disturb the cell membrane's permeability [13]. Thus, temu kunci essential oil samples were also examined for the effects on bacterial cell leakage.

This study aimed to use a metabolomics approach with multivariate data analysis to examine volatile compound profiles of essential oils from temu kunci grown at four altitudes, their potential as antibacterial agents against *B. cereus*, *Staph. aureus* and *Salmonella* Typhimurium, together with examination of the mechanism of inhibition of the pathogens. We anticipated that the different volatile chemical compound profiles of temu kunci essential oils from various altitudes of plant growth would affect the antibacterial activity toward G^+ and G^- bacterial pathogens.

MATERIALS AND METHODS

Samples

The temu kunci essential oil (TKEO) samples were obtained from temu kunci planted in four altitudes in Central Java, Indonesia: TKEO A from Tirtomoyo (170 m above sea level), TKEO B from Pracimantoro (250 m), TKEO C from Jumapolo (450 m), and TKEO D from Karangtengah (600 m). Each sample was prepared in sextuplicate, hence the total samples were 24. All spices were harvested at ± 4 months of age, sliced (to 4–6 cm) and air-dried for 2–3 days. Each set of samples was steam-distilled for 6 h using steam distillation with capacity of 3 kg.

Microorganisms

The test bacteria were *B. cereus* ATCC 10876, *S.* Typhimurium ATCC 14028 and *S. aureus* ATCC 25923 obtained from SEAFAST Center IPB (Bogor, Indonesia). One loopful of 24 h cultures of *B. cereus*, *S.* Typhimurium and *Staph. aureus* was individually streaked onto slants of Nutrient agar (Oxoid, Basingstoke, United Kingdom) and incubated for 16–24 h at 37 ± 2 °C. Afterward, one loopful from each slant was used inoculate 5 ml of Mueller-Hinton broth (HiMedia, Mumbai, India). The cultures were incubated at 37 ± 2 °C with shaking (52.5 Hz) until the turbidity similar to 0.5 McFarland was achieved, which corresponds approximately to 10^8 CFU·ml^{-1} [14].

Analysis of volatile compounds

GC-MS on QP2020 NX (Shimadzu, Kyoto, Japan) with Stabilwax capillary column (60 m × 0.25 mm × 0.25 μm; Restek, Bellefonte, Philadelphia, USA) was used to analyse volatiles in temu kunci essential oil samples. Ultrahigh purity helium with a flow rate of 1.0 ml·min^{-1} was used as a carrier gas. The injection volume was 1.0 μl in split mode with 1 : 50 split ratio. The injection temperature was 250 °C. Separation was isothermal for 4 min at 70 °C, then the temperature was raised to 180 °C at 4 °C·min^{-1} and kept at this temperature for 10 min. The ion source temperature was 230 °C, the interface temperature was 250 °C and the solvent cut time was 1 min. The volatile compounds were identified using data from the database of National Institute of Standards and Technology (Gaithersburg, Maryland, USA) applying ion fragmentation similarity of more than 80 %. Relative quantification was achieved based on the peak areas [2].

Antimicrobial activity assay

Volumes of 0.3 ml of the bacterial cultures

of *B. cereus*, *S.* Typhimurium and *Staph. aureus* (10^8 CFU·ml^{-1}) were individually spread on Nutrient agar plates. Disks of 5 mm were prepared from Whatman filter paper 42 (Whatman, Maidstone, United Kingdom), dripped with 5 μl temu kunci essential oil and placed on the bacterial lawn on Nutrient agar. Incubation was done overnight at 37 °C. The same volume of sterile distilled water was used in a negative control and chloramphenicol 250 μg·l^{-1} as a positive control. The antimicrobial activity was categorized as active (> 11 mm), intermediate (6–11 mm) and inactive (< 6 mm) based on the clear zone diameter [14, 15].

Multivariate data analysis

Analysis of volatile component profiles and antibacterial activity was carried out using SIMCA Multivariate Data Analytics Solution version 16 (Sartorius, Göttingen, Germany). The multivariate data analysis used a principal component analysis (PCA) model with Pareto scaling and partial least square (PLS) with unit variance scaling. Both multivariate data analyses used an exponentially weighted moving average (EWMA) filter to filter the raw data. PCA was used to determine the grouping of the samples based on those volatile component profiles. Meanwhile, PLS was used to determine the correlation between volatile component profiles with the antibacterial activity, thus, the volatile compounds that may play a role as an antibacterial agent could be determined. The above analysis results were Q^2 value, R^2X value, and R^2Y. The Q^2 value is the goodness of the prediction value, the R^2X is a value showing how much the model can describe the X variable, while the R^2Y value is a value showing how much the model can describe the Y variable. A model is considered suitable for predicting the data when the Q^2 value is above 0.4 [16].

The correlation coefficients and variable influence on projection (*VIP*) value were used to determine the volatile compounds that may play a role as an antibacterial agent. The correlation coefficient value was used to determine the X variables, which may be crucial in affecting the Y variable. The higher value of correlation coefficients shows a more considerable influence, while the positive or negative sign shows whether the correlation has a positive correlation or negative correlation [16]. The *VIP* value is a parameter used to know the acceptance of each X variable to affect the Y variable in the model. The value of *VIP* to admit the effect in the model is more than 1 and if it is less than 0.5, then it is not relevant [17].

Minimal inhibition concentration assay

The temu kunci essential oil samples with the highest antibacterial activity based in the disk assay were subjected to *MIC* determination and cell leakage analysis. Bacterial cultures (approximately 10^6 CFU·ml^{-1}) were individually inoculated to Mueller-Hinton broth supplemented with 20 g·l^{-1} glucose and 5 ml·l^{-1} Tween 20 (Merck, Darmstadt, Germany) and individual samples of temu kunci essential oils were added at 0.31 ml·l^{-1}, 0.62 ml·l^{-1}, 1.25 ml·l^{-1}, 2.5 ml·l^{-1}, 5 ml·l^{-1}, 10 ml·l^{-1}, 20 ml·l^{-1} and 40 ml·l^{-1}. The cultures were then incubated for 16–20 h at 37 ± 2 °C with shaking (52.5 Hz). MIC was read as the lowest concentration at which no growth of bacteria appeared.

Analysis of antibacterial mechanism

Temu kunci essential oil samples at equivalent to 1 × MIC, 2 × MIC and 0 (control) were used to test the cell leakage [18]. Each bacterium was grown for 18–24 h in Nutrient broth (Oxoid) with shaking frequency 52.5 Hz and then centrifuged for 15 min at 1 500 ×*g*. Then, the sediment was washed with phosphate buffer (0.2 mol·l^{-1}, pH 7.4) twice and suspended in 10 ml phosphate buffer (0.2 mol·l^{-1}, pH 7.4) . Essential oil samples were added to the suspension and the mixture was incubated in a shaking incubator at 37 ± 2 °C for 24 h at 2.5 Hz. The suspension was centrifuged for 15 min at 1 500 ×*g* and the supernatant was withdrawn. Using a UV-Vis Spectrophotometer UV-2450 (Shimadzu), absorbance at 260 nm and 280 nm were measured. Based on this, nucleic acid and protein concentrations were calculated. The supernatants were also analysed by atomic absorption spectrophotometry using AA-6880 (Shimadzu) at 422.7 nm and 766.5 nm to determine the concentration of Ca^{2+} and K^+ ions, respectively. The electric current of hollow cathode lamp of 10 mA, 0.7 nm slit, Air-C_2H_2 flame type with flow of 2.0 l·min^{-1} and a burner 7.0 mm in height were used [19, 20].

RESULTS AND DISCUSSION

Volatile compound profiles

Samples of temu kunci essential oil from four plants growing at various altitudes was found to contain various chemical volatile compounds with similar profiles (Tab. 1). Fig. 1 presents the corresponding overlaid chromatogram. The volatile compounds detected can be divided into groups of terpenes, terpenoids and other hydrocarbons. Terpenes and terpenoids are the primary chemical compounds in temu kunci essential oil. Terpenes

Tab. 1. Relative content of volatile compounds in temu kunci essential oil samples.

Volatile Compound	Peak area [%]			
	A	B	C	D
Tricyclene	0.3	0.4	0.3	0.4
α-Pinene	2.2	4.7	4.5	2.4
α-Fenchene	0	0.1	0	0.1
Camphene	4.2	4.9	4.2	4.9
β-Pinene	0	0	0	0
cyclo-Fenchene	0	0	0	0
β-Myrcene	1.1	1	1.2	1.1
α-Phellandrene	0	0.1	0.1	0.1
3-Carene	0.1	0.1	0.1	0.1
D-Limonene	3	3.1	2.7	3.2
1,8-Cineole	9.2	10.2	10.1	10.3
β-Ocimene	3.2	0.8	1	3.5
cis-β-Ocimene	16.6	16.2	21.1	17.8
o-Cymene	0.2	0.1	0.1	0.2
4-Carene	0.4	0.4	0.3	0.4
α-Pinene oxide	0.3	0.1	0.1	0.2
Neo-allo-ocimene	0.1	0.1	0.2	0.1
α-Naginatene	0	0	0	0
Fenchone	0	0	0	0
trans-Linalool oxide	0.1	0.1	0	0.1
Carveol	0.1	0	0	0
Dihydro-*ar*-turmerone	0	0	0	0
Mono-methyl cinnamate	10	9.4	10	7
Z-β-*O*cimene epoxide	0	0	0	0
E-β-Ocimene epoxide	0.2	0.1	1.6	1.1
Geranyl-α-terpinene	0	0	0	0
Camphor	18.4	20.1	17.2	19.8
Linalool	1.1	1.8	2	1.2
trans-Z-α-bisabolene epoxide	0.1	0	0	0
Cinerone	0	0	0	0
Menthoglycol	0	0	0	0
Nerolidol	0.1	0	0	0
Fenchol	0.1	0.1	0.1	0.1
cis-β-Elemene	0	0	0	0
Camphene hydrate	2.1	2.2	1.4	2
γ-Muurolene	0	0	0	0
Hedycaryol	0	0	0	0
α-Camphorene	0.1	0	0	0
β-Caryophyllene	0.1	0	0.1	0
3-α-Bromolongifolene	0.2	0	0.1	0
Artemisia alcohol	0.1	0.1	0.2	0.2
trans-Ocimenol	0	0	0	0
Isoborneol	0.5	0.4	0.3	0.3
Humulene	0.1	0	0.1	0.1
Neral	0.1	0.1	0	0.1
α-Erpineol	1.7	1.5	1.4	1.7
endo-Borneol	1.4	1.7	1	1.2
Thujanol	0.1	0.2	0.1	0.2

Volatile Compound	Peak area [%]			
	A	B	C	D
cis-Sesquisabinene hydrate	0	0	0.2	0.1
α-Bergamotene	0	0	0.1	0.1
β-Bisabolene	0	0.1	0	0.1
Citral	0.4	0.2	0.1	0.3
α-Farnesene	0	0	0	0
Zerumbone	0.1	0	0	0
Piperitone	0	0	0	0
Geranylgeranyl acetate	0	0	0	0
Neryl acetate	0	0	0.1	0
Geranyl acetate	0.1	0.1	0	0
Linalool oxide pyranoid	0.1	0.1	0.1	0
Linalool oxide III	0.1	0	0	0
Linalool oxide I	0.1	0	0	0
Sesquiphellandrene	0	0.1	0.1	0
α-Curcumene	0.2	0.2	0	0.1
γ-Geraniol	0.2	0.1	0.1	0.1
Tumerone	0.1	0.2	0	0.3
cis-Myrtanol	0	0.1	0	0
Myrtenol	0.2	0.1	0.4	0.2
Isogeraniol	0	0	0	0
Neryl butyrate	0	0.1	0	0
Geranyl butyrate	0.2	0.2	0.3	0.3
Neryl propionate	0.1	0.1	0.1	0
Geranyl propionate	0	0	0	0.1
Neryl-2-methyl butanoate	0	0	0.1	0
Geraniol	17.4	16.5	15.4	17
Verbenol	0.1	0.1	0	0
Epicurzerenone	0	0.1	0	0
Curzerene	0	0	0	0
Curzerenone	0	0	0	0
Furanodienone	0	0	0.1	0
cis-Chrysanthenyl formate	0	0	0	0
p-Camphorene	0	0	0	0
Geranyl-α-terpinene	0	0	0	0
Myrtanyl acetate	0	0	0	0
Curlone	0.1	0.1	0	0.2
Linalyl acetate	0.1	0	0	0
7-Hydroxyfarnesen	0	0	0	0
ar-Turmerone	0.3	0.2	0.2	0.4
Caryophyllene oxide	0	0	0	0
Acetyl carene	0	0	0	0
Solanone	0	0	0	0
Nerolidyl acetate	0	0	0	0
Nerolidol	0	0	0	0
Humulene epoxide I	0	0	0	0
Geranyl geraniol	0	0	0	0
Other	0.5	0.5	0.3	0.4

A – temu kunci essential oil from Tirtomoyo (170 m above sea level), B – temu kunci essential oil from Pracimantoro (250 m above sea level), C – temu kunci essential oil from Jumapolo (450 m above sea level), D – temu kunci essential oil from Karangtengah (600 m above sea level).

are hydrocarbons compound of isoprene structure (hemiterpene), while terpenoids are their derivatives containing a functional group and an oxidized methyl group. The active biological functions of terpenoids are due to the variation of functional group structure. Several terpenoids are antimicrobial agents. The number of carbons is the basis for categorization of terpenes to monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20) etc. Terpenoids are categorized based on functional groups, such as alcohol, ester, ether or ketone. The other compounds detected in the temu kunci essential oil samples were alcohols, ketones, aldehydes, carboxylic acids, fatty acid esters and fatty alcohols. Terpenes and terpenoids are compounds with antimicrobial activity. Those compounds are contained in plants as secondary metabolites which support cell performance. Essential oils are active in protecting plants from herbivores and pathogens [21].

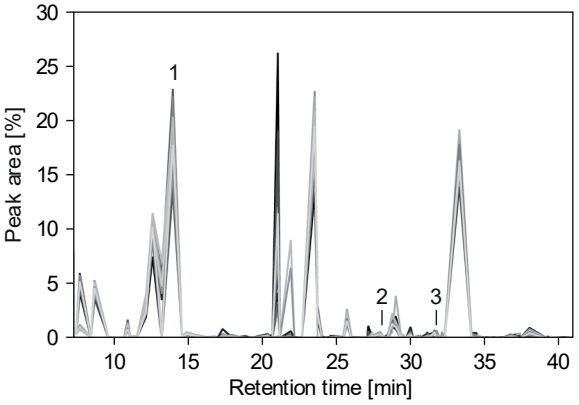

Fig. 1. Overlaid GC-MS chromatogram of temu kunci essential oil samples.

Peaks: 1 – *cis*-β-ocimene, 2 – β-bisabolene, 3 – myrtenol.

Antibacterial activity

The results of this study confirmed that temu kunci essential oils have antibacterial activity against G^+ and G^- bacteria although the degree of inhibition varies. They were found to inhibit *B. cereus* and *Staph. aureus* very well, but not *S.* Typhimurium (Fig. 2). The difference in the inhibitory activity was attributed to the cell wall composition of these bacteria. *B. cereus* and *Staph. aureus* are G^+ bacteria with a two-layer envelope in cell membrane structure, composed of carbohydrates and peptidoglycan [22]. The antimicrobial compounds of temu kunci essential oil are lipophilic and can easily pass the cell membrane of G^+ bacteria. These lipophilic compounds are known to change bacterial membrane structure by increasing its fluidity and permeability. In addition, they change the topology of protein in peptidoglycan, which causes distraction in all of the bacterial respiration processes [13]. Meanwhile, *S.* Typhimurium is G^- with three envelope layers in the cell membrane. They are the outer membrane, peptidoglycan cell wall, and inner membrane. The outer membrane contains outer membrane proteins and phospholipids bound with an inner sheet, and lipopolysaccharide bound with an outer sheet. The outer membrane is difficult to be passed by a range of compounds. This layer of cell membrane makes the G^- bacteria more resistant to some antibacterial agents [23].

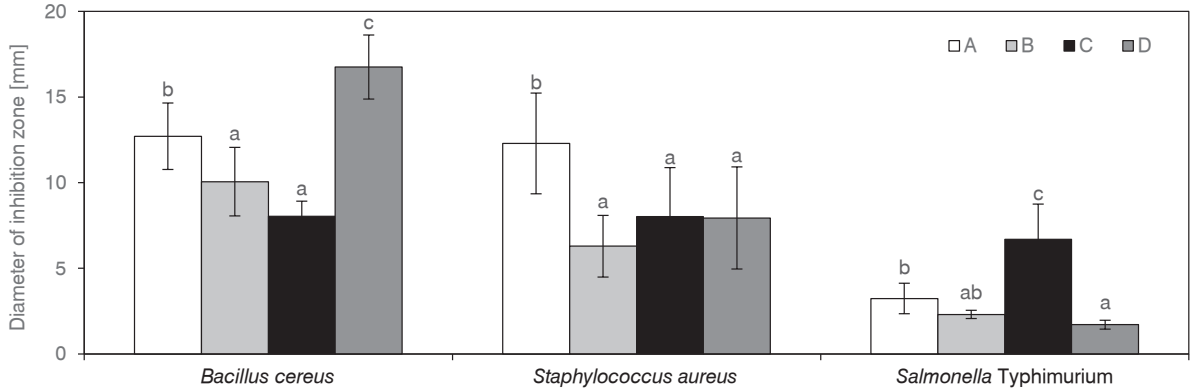

Fig. 2. Diameters of inhibition zones of temu kunci essential oil against three bacteria.

Values in the same column followed by the same letter do not differ markedly at the 5% test level (Duncan's multiple interval tests).

A – temu kunci essential oil from Tirtomoyo (170 m above sea level), B – temu kunci essential oil from Pracimantoro (250 m above sea level), C – temu kunci essential oil from Jumapolo (450 m above sea level), D – temu kunci essential oil from Karangtengah (600 m above sea level).

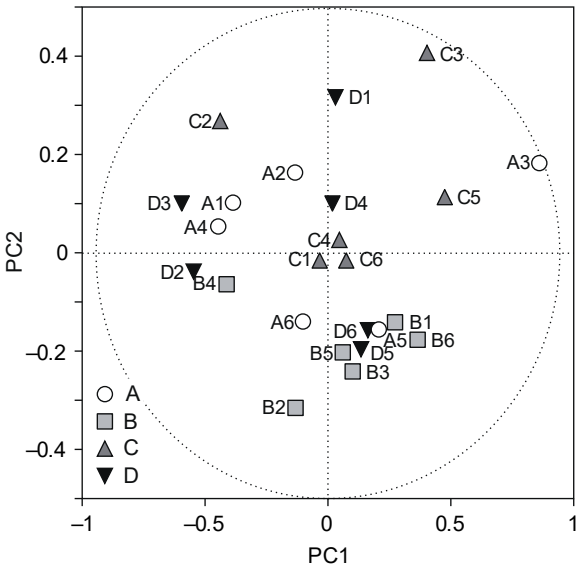

Fig. 3. Principal component analysis score plot of temu kunci essential oil volatile compounds composition.

A – temu kunci essential oil from Tirtomoyo (170 m above sea level), B – temu kunci essential oil from Pracimantoro (250 m above sea level), C – temu kunci essential oil from Jumapolo (450 m above sea level), D – temu kunci essential oil from Karangtengah (600 m above sea level).

All temu kunci essential oil samples were found to be capable of inhibiting *B. cereus*, similar to the previous report [2]. However, TKEO D and TKEO A inhibited *B. cereus* stronger (diameter of inhibition of 16.75 ± 1.87 mm and 12.71 ± 1.94 mm, respectively) than those TKEO B and TKEO C (data not shown). On the other hand, TKEO A inhibited *Staph. aureus* significantly by 12.29 ± 2.94 mm. At the same time, lower inhibitory activity was observed in three other temu kunci essential oil samples extracted from temu kunci grown in Pracimantoro, Tirtomoyo, and Karangtengah. These results also were in agreement with another study [24] that reported that temu kunci essential oils could inhibit *Staph. aureus* growth. In this study, we found that temu kunci essential oils had no antimicrobial activity toward *S.* Typhimurium and only TKEO C slightly inhibited this pathogen based on CLSI M07-A9 categorization. These results were similar to that of a previous study [3], which reported that temu kunci essential oil slightly inhibited the growth of *S.* Typhimurium. We conclude that temu kunci essential oils can inhibit G^+ bacteria but only slightly inhibit G^- bacteria. In addition, altitudes at which temu kunci grows significantly influence the antibacterial activity.

Data analysis of volatile compound profile

The multivariate data analysis with PCA resulted in four main components. The R^2X value was 0.829, suggesting that X variable could be explained by 82.9 %. The Q^2 value of this analysis was 0.568 suggesting that PCA was suitable for predicting the data based on established criteria [16]. The score plot of two main components in the PCA model (Fig. 3) showed that TKEO A, TKEO B and TKEO C were well separated and contained a different set of volatile compounds. However, the volatile compounds in TKEO D were spread in three sample areas meaning that this sample had no unique volatile compound profile and had the profile analogical to the other three samples.

Data analysis of antibacterial activity toward *Bacillus cereus*

The PLS model in multivariate data analysis of the antibacterial activity of essential oil samples from temu kunci grown in various altitudes toward *B. cereus* generated four main components. The Q^2 value was 0.554, the R^2X value was 0.462 and the R^2Y value was 0.966, suggesting that the data could be predicted well based on the Q^2 value. The score plot of two main components obtained here could show grouping of the samples by their activity. TKEO D with the highest activity against

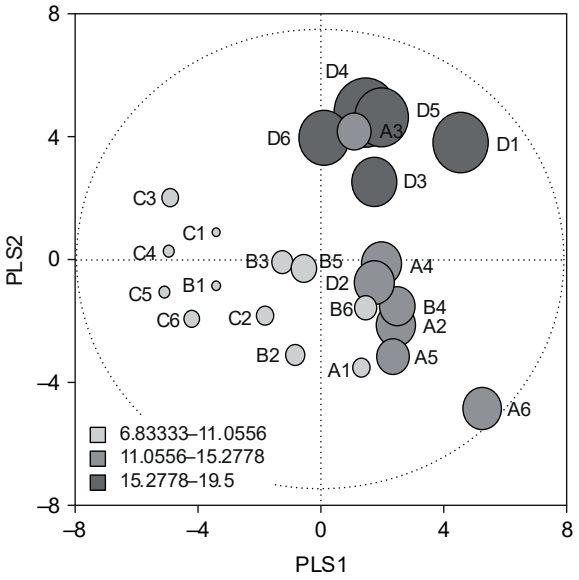

Fig. 4. Partial least square score plot of temu kunci essential oil activity against *Bacillus cereus*.

A – temu kunci essential oil from Tirtomoyo (170 m above sea level), B – temu kunci essential oil from Pracimantoro (250 m above sea level), C – temu kunci essential oil from Jumapolo (450 m above sea level), D – temu kunci essential oil from Karangtengah (600 m above sea level).

B. cereus was grouped separately from other samples in the right section. Meanwhile, the other three samples could be grouped into two groups in the left section (Fig. 4).

The volatile compounds that may play a role in inhibiting *B. cereus* based on this multivariate data analysis are shown in Tab. 2 [25–31]. The multivariate data analysis of the volatile compounds profile of temu kunci essential oil samples was proportional to the antibacterial activity toward *B. cereus*. Based on the criteria of the correlation coefficient and the *VIP* value, β-bisabolene, turmerone and curlone were the volatile compounds that potentially played a role as antibacterial agents. This prediction is also supported by the reference of these compounds as antibacterial agents, as stated in Tab. 2. The compound β-bisabolene was a minor component in the temu kunci essential oil samples because it had a low peak in the chromatogram as shown in Fig. 1 (marked with number 2).

Tab. 2. Volatile compounds that may affect the antibacterial activity in temu kunci essential oil.

Volatile compounds	Correlation coefficient	*VIP* value	Ref.
Antibacterial activity toward *Bacillus cereus*			
β-Bisabolene	0.12	1.80	[26]
Turmerone	0.12	1.68	[27]
Curlone	0.12	1.86	[28]
Antibacterial activity toward *Staphylococcus aureus*			
Myrtenol	7.48	1.27	[29]
ar-Turmerone	6.69	1.30	[27]
Verbenol	6.66	1.38	[30]
Antibacterial activity toward *Salmonella* Typhimurium			
cis-β-Ocimene	4.82	5.07	[31]
α-Pinene oxide	2.85	1.38	–
o-Cymene	2.64	2.66	[32]

VIP - variable influence on projection.

Data analysis of antibacterial activity toward *Staphylococcus aureus*

The multivariate data analysis with the PLS model of antibacterial activity of essential oil from temu kunci grown at various altitudes toward *Staph. aureus* generated six main components. The model was considered of good quality since the Q^2 value was 0.415. Based on the score plot of two main components that can be seen in Fig. 5, TKEO A was grouped separately from other samples in the right section, suggesting the highest antibacterial activities (marked with big size mark).

Considering the correlation coefficient and *VIP* value, the volatile compounds that may play a role as antibacterial agents toward *Staph. aureus* could be determined. As shown in Tab. 2, myrtenol, *ar*-turmerone and verbenol were the three volatile compounds with the highest correlation coefficient with a positive *VIP* value. In addition, the three volatile compounds were reported as having antibacterial activity toward *Staph. aureus* (Tab. 2). Myrtenol is a minor volatile compound in temu kunci essential oil samples as can be seen in Fig. 1 marked with number 3.

Data analysis of antibacterial activity toward *Salmonella* Typhimurium

The multivariate data analysis with the PLS model of antibacterial activity of essential oil from temu kunci grown at various altitudes toward *S*. Typhimurium generated four main components. The Q^2 value was 0.469, suggesting that the model could predict well based on the Q^2 value. Fig. 6 shows the score plot of two main components obtained from the analysis, which showed that TKEO C was grouped separately in the right section with a big size mark. TKEO C had the highest activity against *S.* Typhimurium.

The antibacterial activity of temu kunci essential oil samples toward *S.* Typhimurium was affected by the profile of volatile compounds. The

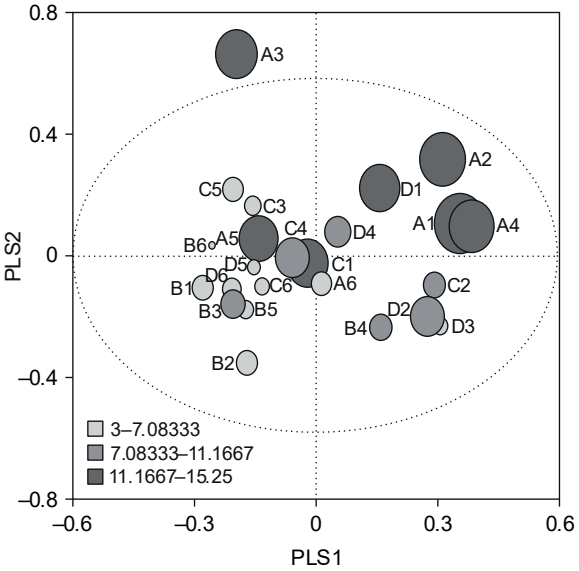

Fig. 5. Partial least square score plot of temu kunci essential oil activity against *Staphylococcus aureus*.

A – temu kunci essential oil from Tirtomoyo (170 m above sea level), B – temu kunci essential oil from Pracimantoro (250 m above sea level), C – temu kunci essential oil from Jumapolo (450 m above sea level), D – temu kunci essential oil from Karangtengah (600 m above sea level).

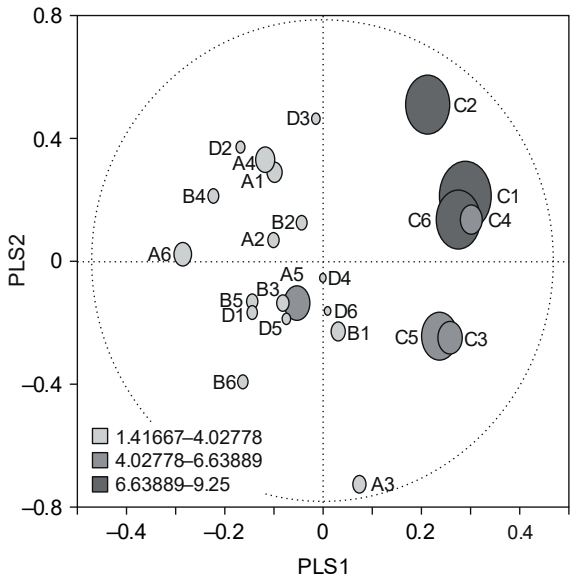

Fig. 6. Partial least square score plot of temu kunci essential oil activity against *Salmonella* Typhimurium..

A – temu kunci essential oil from Tirtomoyo (170 m above sea level), B – temu kunci essential oil from Pracimantoro (250 m above sea level), C – temu kunci essential oil from Jumapolo (450 m above sea level), D – temu kunci essential oil from Karangtengah (600 m above sea level).

volatile compounds with antibacterial activity history, good correlation coefficient and the *VIP* values identified in this study were cis-β-ocimene, *o*-cymene and β-caryophyllene (Tab. 2). The three volatile compounds were likely to act as antibacterial agents toward *S.* Typhimurium. The compound *cis*-β-ocimene was a major component of temu kunci essential oil as shown in the chromatogram (marked with number 1 in Fig. 1).

Minimum inhibitory concentrations

MIC of temu kunci essential oil samples against *B. cereus* and *Staph. aureus* were both 1.25 ml·l^{-1}, while that against *S.* Typhimurium was 2.5 ml·l^{-1}. The previous study reported that *MIC* of temu kunci essential oil from Bogor, Jawa Barat, Indonesia against *B. cereus* was 1.2 ml·l^{-1} [2]. In another study, *MIC* of 2.6 ml·l^{-1} was reported for temu kunci essential oil against *Staph. aureus* [32]. Meanwhile, *MIC* of temu kunci extract against *S*. Typhimurium was 4 ml·l^{-1} [3]. In comparison, results of the present study are in line with those of previous studies.

Mechanisms of antibacterial activity

Various mechanisms of antibacterial activity are known, such as inhibition of cell wall formation, disturbance of the cell membrane's permeability, inhibition of the enzymes or inhibition of the DNA function [21, 33]. In this study, we found that exposition of bacterial cells to higher concentrations of temu kunci essential oil resulted in a higher concentration of protein, nucleic acid, Ca^{2+}, and K^+ in the tested supernatant. Generally, the nucleic acid and protein concentrations of all tested bacteria were significantly different at different essential oil concentrations (Tab. 3). These results suggest that temu kunci essential oil can disturb the cell membrane allowing the cell material to leak out from the cells. A similar phenomenon was observed for Ca^{2+} and K^+ concentrations (Tab. 4). The mineral concentration in the supernatant significantly rose at higher concentrations of temu kunci essential oil samples.

Cell leakage in bacteria exposed to various concentrations of temu kunci essential oil (0, 1 × *MIC*, 2 × *MIC*) demonstrated the level of leakage due to membrane disruption. It was previously reported that the higher the essential oil concentration, the higher concentration of cell materials detected in the supernatant [34]. The results suggest that changes in the cell membrane permeability may have occurred causing the inner bacterial cell materials to leak out from the cells. Changes in the cell membrane permeability could lead to the cell's inability to maintain the integrity in keeping the cell materials. These conditions can reduce vitality of the cell. The lipophilic components of temu kunci essential oils were previously reported to disturb the cell membrane performance by increasing the cell membrane permeability [13].

The cell leakage patterns in the three bacteria exposed to temu kunci essential oil were different for G^+ and G^- bacteria. G^+ bacteria *B. cereus* and *Staph. aureus* leaked more cell material than the G^- bacterium *S.* Typhimurium. Carbohydrates and peptidoglycans are the main cell wall components of G^+ bacteria [22] and they make cells of these bacteria more vulnerable to lipophilic agents [13]. Meanwhile, G^- bacterial cells have an additional outer membrane, which makes them more resistant to lipophilic agents [23].

CONCLUSIONS

Samples of essential oil from temu kunci grown at various altitudes had different volatile compound profiles. Terpenes and terpenoids were the most abundant based on peak area of GC-MS analysis. These compounds are lipophilic that can disturb the cell membrane of bacteria, especially G^+ bacteria. It was proven that the inner cell material, namely, nucleic acids, proteins, Ca^{2+} and K^+ leaked out from cells, which means that the

Tab. 3. Nucleic acid and protein leakage from bacterial cells due to exposure to temu kunci essential oil.

TKEO concentration	Concentration [g·l-1]					
	Bacillus cereus D		*Staphylococcus aureus* A		*Salmonella* Typimurium C	
	Nucleic acids	Proteins	Nucleic acids	Proteins	Nucleic acids	Proteins
0	0.68 ± 0.01 a	0.63 ± 0.00 a	0.64 ± 0.01 a	0.60 ± 0.00 a	0.53 ± 0.01 a	0.48 ± 0.01 a
1 × *MIC*	0.99 ± 0.06 b	0.93 ± 0.06 b	1.06 ± 0.10 b	0.91 ± 0.10 b	0.88 ± 0.04 b	0.81 ± 0.02 b
2 × *MIC*	1.29 ± 0.00 c	1.30 ± 0.00 c	1.28 ± 0.11 b	1.23 ± 0.11 c	1.31 ± 0.05 c	1.28 ± 0.06 c

Values in the same column followed by the same lowercase letter in superscript do not differ markedly at the 5% test level (Duncan's multiple interval test).
MIC – minimal inhibitory concentration, D – using temu kunci essential oil from Karangtengah (600 m above sea level), A – using temu kunci essential oil from Tirtomoyo (170 m above sea level), C – using temu kunci essential oil from Jumapolo (450 m above sea level).

Tab. 4. Ca^{2+} and K^{+} leakage from bacterial cells due to exposure to temu kunci essential oil.

TKEO concentration	Minerals concentration [mg·l-1]					
	Bacillus cereus D		*Staphylococcus aureus* A		*Salmonella* Typimurium C	
	Ca^{2+}	K^{+}	Ca^{2+}	K^{+}	Ca^{2+}	K^{+}
0	5.39 ± 0.11 a	1 264.79 ± 19.25 a	5.04 ± 0.14 a	1 253.94 ± 26.46 a	5.23 ± 0.07 a	1 459.76 ± 45.27 a
1 × *MIC*	5.69 ± 0.03 b	1 507.87 ± 85.35 b	5.61 ± 0.05 b	1 679.23 ± 33.70 b	5.44 ± 0.08 b	1 538.29 ± 9.22 ab
2 × *MIC*	5.82 ± 0.04 b	1 550.86 ± 60.58 b	5.88 ± 0.17 b	1 812.68 ± 12.95 c	5.87 ± 0.02 c	1 559.83 ± 3.76 b

Values in the same column followed by the same lowercase letter in superscript do not differ markedly at the 5% test level (Duncan's multiple interval test).
MIC – minimal inhibitory concentration, D – using temu kunci essential oil from Karangtengah (600 m above sea level), A – using temu kunci essential oil from Tirtomoyo (170 m above sea level), C – using temu kunci essential oil from Jumapolo (450 m above sea level).

bacterial cell membranes were disturbed by temu kunci essential oil. The highest antibacterial activity toward *B. cereus* was manifested by TKEO D from Karangtengah (600 m above sea level), while toward *Staph. aureus* was TKEO A from Tirtomoyo (170 m above sea level). Nevertheless, temu kunci essential oil samples did not signigicantly inhibit *S.* Typhimurium, only TKEO C from Jumapolo (450 m above sea level) could inhibit it slightly. The multivariate data analysis with PCA and PLS models generated good models. The volatile compounds of TKEO A, TKEO B and TKEO C were grouped separately based on the PCA model's score plot, which means that these three temu kunci essential oil samples were each unique. Meanwhile, the volatile compounds of TKEO D manifested no uniqueness, as its profile was spread all over the three groups. The volatile compounds that may play a role as antibacterial agents toward the tested bacteria were different for individual bacteria – toward *B. cereus* they were β-bisabolene, turmerone and curlone, toward *Staph. aureus* they were myrtenol, *ar*-turmerone and verbenol, and toward *S.* Typhimurium they were *cis*-β-ocimene, *o*-cymene and β-caryophyllene.

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