Antibacterial activity assay of essential oils from limau kuit peel against *Staphylococcus aureus*

A Irwan¹*, N Humaida¹ and H S Nur²

¹Department of Chemistry, Faculty of Mathematics and Natural Sciences, University of Lambung Mangkurat, Indonesia
²Department of Biology, Faculty of Mathematics and Natural Sciences, University of Lambung Mangkurat, Indonesia

Email: airwan@ulm.ac.id

Abstract. This research aims to determine the antibacterial activity of essential oils from Limau Kuit (a local variety of limes of South Kalimantan) peel against *Staphylococcus aureus* ATCC25923, the minimum inhibitory concentration, and to identify compounds of essential oils contained. The essential oil has been isolated by hydro steam distillation and then was analyzed by using GC-MS to identify major compounds of the essential oils which were supposed as antibacterial agents. The result of this research showed the oils had antibacterial activity against *Staphylococcus aureus* ATCC25923 at concentrations 10%, 75%, 50%, and 25% with minimum inhibitory concentration 25% wet and dry sample. There was no significant difference in MIC between the two samples. At low concentrations, essential oils from wet samples had better inhibition power than dry samples up to a concentration of 75%. But at a concentration of 100%, their inhibitory power tended to decrease. Based on GC-MS data were demonstrated of 15 compounds in the wet sample and 16 compounds in the dry sample. Five major compounds of essential oil from Limau Kuit peel were limonene, γ-terpinene, β-pinene, α-pinene, and sabinene. Meanwhile, it was known that compounds limonene, α-pinene, γ-terpinene, β-pinene, α-terpineol, sabinene, and terpinen-4-ol had antibacterial activity.

1. Introduction

South Kalimantan possess local oranges are known as Limau Kuit. This orange has several peculiarities that can distinguish it from other types in terms of fruit aroma, fruit size, and what stands out is the surface morphology of wrinkled rind that is often associated with kaffir lime. The Banjar tribe community in South Kalimantan uses this orange as a chili sauce and food flavoring ingredient [1]. Part of the lime is the fruit peel usually only becomes waste, even though the orange peel consists of essential oils containing component compounds that can be utilized [2].

Orange peel contains essential oils consisting of various groups of compounds such as terpenes, sesquiterpenes, aldehydes, esters, and sterols. Orange peels have different compound contents depending on variety caused that the aroma is different [3]. Essential oils or known as essential oils, ethereal oils, or volatile substances are volatile compounds that do not dissolve in water [4]. Essential oils and extracts from various kinds of plants have been widely used in the health field [5].

Existing components in essential oils can be used to reduce the development and spread of bacteria [6]. The antibacterial activity of essential oils is supposed to be in the presence of phenol compounds, terpenes, and aldoacetone [7]. One of the bacteria due to some infections in humans and resistant to...
several types of drugs is *Staphylococcus aureus*. This bacterium is a common pathogen in humans frequently found as common biota on the skin [8,9].

A recent study on Limau Kuit is the use of ethanol extract from the peel against *S. aureus* [10] with concentrations of 100%, 75%, 50%, and 25%, resulting in inhibition zone diameter of 14 mm, 10.7 mm, 9.6 mm, and 0 mm, respectively. Based on the results of the study, it is urgent to determine the antibacterial activity of essential oils of the lime peel against *S. aureus* by taking into account the diameter of the inhibitory zone and the value of the minimum inhibitory concentration (MIC). Limau Kuit used were taken from the Astambul area, Kabupaten Banjar. The essential oils extracted by the steam-water distillation method then was analyzed by using GC-MS to identify the existing compounds.

2. Materials and methods

The Limau Kuit used in this research was taken from Astambul, Kabupaten Banjar, South Kalimantan, *S. aureus* ATCC 25923 bacterial culture was obtained from the The Institution of Research and Standardization of Industry Banjarbaru. The products were prepared by hydro-steam distillation with two sample preparations, wet/fresh and dry peel samples. Chemicals in the analytical grade were sodium sulfate anhydrous (Merck), H$_2$SO$_4$ (Merck), BaCl$_2$ (Merck), DMSO (Merck), and other materials were paper discs, chloramphenicol, NaCl (Oxoid), BHI Agar, BHI Broth, MH Agar (Oxoid), and distilled water.

2.1. Samples preparation

There were 2 variations of preliminary preparation, namely fresh fruit peel and dried fruit peel. There were 2 variations of preliminary preparation, namely fresh fruit peel and dried fruit peel. The fresh fruit rind was prepared by separating from the flesh of the fruit and then cut to the side size of ± 1 cm. Part of them then dried until withered for the dried sample.

2.2. Purification of extracted oils

The purification was conducted to remove the remaining water in oils. Anhydrous sodium sulfate was added as much as 1-3 g/L into oils in beaker glass, stirring it gently until the Na$_2$SO$_4$ anhydrous settles or agglomerates later, separated using filter paper. Purification results were stored again for antibacterial activity tests and composition analysis.

2.3. Antibacterial activity assay

Samples of oils from wet and dried peel were diluted to certain concentrations prior antibacterial assays. Dilution of essential oil was using DMSO (v/v) to make 75%, 50 %, 25% and 10% of concentration. Each concentration was then labeled as test tube II, III, IV, and V respectively. Test tube I was 100% of essential oils.

The BHI Agar media was prepared by weighing 0.94 g BHI Agar into an Erlenmeyer flask, adding 20 mL of distilled water, heated to boiling, and dissolved. The media was then sterilized using an autoclave at 121°C for fifteen minutes then poured into the petri dish aseptically. Media BHI Broth was made by weighing as much as 1.85 g of BHI Broth put into an Erlenmeyer flask adding 50 mL of dissolved distilled water. A total of 10 mL of media was put into each test tube. The media was then sterilized using an autoclave of 121°C for 15 minutes. Media MH Agar was made by weighing 3.8 g MH. To be put into the Erlenmeyer flask, 100 ml of distilled water added, heated to boiling, and dissolved. The media were sterilized using an autoclave at 121°C for 15 minutes. Then it was poured into the Petri dish aseptically.

2.4. Preparation of *S. aureus ATCC 25923* bacterial suspension

Bacterial culture obtained from the Research Institute and Industrial Standardization was taken as much as 1 ose and then inoculated into the BHI Agar medium and incubated in an incubator for 24 hours at ± 30°C. After incubating for 24 hours the rejuvenating bacteria were taken 1-2 ose then
inoculated into BHI Broth media and incubated in an incubator for 24 hours at ± 30°C. The suspension of the bacteria was equated to the standard McFarland I Solution (3x108 CFU.mL-1).

2.5. Antibacterial activity assay and determination of MIC values
The method used for the antibacterial activity test was the Antimicrobial Paper Discs Method using MH Agar media. *S. aureus* ATCC 25923 bacteria as much as 0.2 mL were spread into Petri dishes containing MH Agar media with sterile cotton swabs. Paper discs containing essential oils of lime peel concentration 100%, 75%, 50%, 25%, 10%, negative control, and positive control were placed on the test media incubated for 24 hours at ± 30°C, then the inhibition zone was measured using Calipers. The negative control used was DMSO, while chloramphenicol was used as a positive control. The formation of inhibitory zones around disc paper showed antibacterial activities.

Determination of the MIC (Minimum Inhibition Concentration) value aims to determine the lowest concentration of essential oils which can show antibacterial activity which is characterized by the formation of inhibitory zones. The method used to determine the MIC value was the same as the determination of the antibacterial activity of the essential oils of lime skin, namely the Paper Disc Plate method. The MIC value could be seen from the inhibition zone formed at the lowest concentration, while quantitatively determined using the Regression line by carotemplating the inhibitory diameter of essential oils in mm (Y axis) to the log concentration (X axis) the intersection of the equation obtained by linear regression $Y = aX + b$. The value of $Y$ was the dependent variable while $X$ was the independent variable. MIC value was obtained from the calculated $X$ value with $Y = 0$.

2.6. GCMS analysis
The components of the essential oils were analyzed by gas chromatography-mass spectrometry (GC-MS), using a Shimadzu QP2010S GC-MS Series. Samples were injected on a capillary column (RTX 5 MS). The carrier gas was Helium, at the flow rate of 0.55 ml/min. The inlet injection temperature was 300°C with the inlet split ratio of 139. The GC oven temperature was kept at 50°C for 3 min and programmed to 250°C at a rate of 3°C/min, held for 5 min and finally at 10°C/min and programmed to 300°C, held for 3 min. The GC/MS interface (Auxs) temperature was set at 300 °C. The Mass Spectrometer condition was as follows: EL Source temperature 250°C and El energy 70 eV. The MS library was Wiley 229 and NIST62.

3. Results and discussion
Essential oils of Limau Kuit peel resulting from steam-water distillation were separated between the water layer and the oil layer using a pipette. Essential oils were in the upper layer while the water in the lower layer. The essential oil was purified with anhydrous sodium sulfate to remove the remaining water in order to get the essential oil which was free of water then the yield was calculated. The essential oil yield showed the amount of oil produced from the distillation process in a certain amount expressed in percent (%). Wet samples produced yields of 0.5047% (w/w) and dried samples produced yields of 0.2739% (w/w).

The content of the essential oil compound Limau Kuit peel was analyzed using GC-MS Shimadzu QP2010S series. The results of GC-MS analysis of the components of wet and dried Limau Kuit peel essential oil obtained as chromatogram and mass spectra.

Based on the table 1, the five compounds which had relatively higher levels than other compounds making up the essential oils of Limau Kuit in wet samples are limonene (63.42%), β-pinene (18.50%), β-pinene (8.40%), α-pinene (1.51%), and sabine (1.35%) whereas for dry samples (table 2) are limonene (62.59%), γ-terpinene (16.94%), β-pinene (9.09%), α-pinena (1.75%), and sabine (1.30%).

The antibacterial activity test results of essential oils of Limau Kuit peel after incubated 1x24 hours at ± 30°C showed that essential oils were able to inhibit the growth of *S. aureus* ATCC 25923 bacteria, seen from the inhibition zone produced with variations in the concentration of 100%, 75%, 50%, and
25% while the 10% concentration there was no inhibition zone. The diameter of inhibitory zone of essential oils of lime skin could be seen from the presence of clear zone around the paper disk that showed no bacterial growth (figure 1). The measurement results of inhibition zone formed compared with the standards of the Clinical and Laboratory Standards Institute (CLSI) are shown in (table 3).

**Table 1.** The composition of Limau Kuit essential oils in wet samples.

| Peak | Retention Time (min) | Relative Concentration (%) | Formula | Compound | SI (Similarity index) (%) |
|------|----------------------|-----------------------------|---------|----------|-------------------------|
| 1    | 10.037               | 0.42                        | C\textsubscript{10}H\textsubscript{16}   | \(\alpha\)-thujene  | 93                      |
| 2    | 10.321               | 1.51                        | C\textsubscript{10}H\textsubscript{16}   | \(\alpha\)-pinene  | 94                      |
| 3    | 11.841               | 1.35                        | C\textsubscript{10}H\textsubscript{16}   | sabinene           | 94                      |
| 4    | 12.057               | 8.40                        | C\textsubscript{10}H\textsubscript{16}   | \(\beta\)-pinene  | 96                      |
| 5    | 12.463               | 1.27                        | C\textsubscript{10}H\textsubscript{16}   | Mircene            | 96                      |
| 6    | 13.002               | 0.25                        | C\textsubscript{6}H\textsubscript{16}O   | octanal            | 90                      |
| 7    | 13.507               | 0.39                        | C\textsubscript{10}H\textsubscript{16}   | \(\alpha\)-terpinene | 94                  |
| 8    | 13.833               | 1.23                        | C\textsubscript{10}H\textsubscript{14}   | cimene             | 93                      |
| 9    | 14.160               | 63.42                       | C\textsubscript{10}H\textsubscript{16}   | limonene           | 95                      |
| 10   | 15.118               | 18.50                       | C\textsubscript{10}H\textsubscript{16}   | \(\gamma\)-terpinene | 96                  |
| 11   | 15.997               | 1.00                        | C\textsubscript{10}H\textsubscript{16}   | \(\alpha\)-terpinolene | 95                  |
| 12   | 19.258               | 0.57                        | C\textsubscript{10}H\textsubscript{18}O  | terpinen-4-ol      | 95                      |
| 13   | 19.783               | 0.72                        | C\textsubscript{10}H\textsubscript{18}O  | \(\alpha\)-terpineol | 95                  |
| 14   | 20.001               | 0.33                        | C\textsubscript{12}H\textsubscript{24}O  | dodecanal          | 89                      |
| 15   | 28.136               | 0.63                        | C\textsubscript{15}H\textsubscript{24}   | germacrene         | 90                      |
| Total|                      |                             |         |          | 100                     |

**Table 2.** The composition of Limau Kuit essential oils in dried samples.

| Peak | Retention Time (min) | Relative Concentration (%) | Formula | Compound | SI (Similarity index) (%) |
|------|----------------------|-----------------------------|---------|----------|-------------------------|
| 1    | 10.040               | 0.44                        | C\textsubscript{10}H\textsubscript{16}   | \(\alpha\)-thujene  | 92                      |
| 2    | 10.324               | 1.75                        | C\textsubscript{10}H\textsubscript{16}   | \(\alpha\)-pinene  | 93                      |
| 3    | 11.843               | 1.30                        | C\textsubscript{10}H\textsubscript{16}   | sabinene           | 94                      |
| 4    | 12.063               | 9.09                        | C\textsubscript{10}H\textsubscript{16}   | \(\beta\)-pinene  | 96                      |
| 5    | 12.463               | 1.27                        | C\textsubscript{10}H\textsubscript{16}   | mircene            | 95                      |
| 6    | 13.004               | 0.49                        | C\textsubscript{6}H\textsubscript{16}O   | octanal            | 91                      |
| 7    | 13.508               | 0.34                        | C\textsubscript{10}H\textsubscript{16}   | \(\alpha\)-terpinene | 94                  |
| 8    | 13.833               | 1.15                        | C\textsubscript{10}H\textsubscript{14}   | cimene             | 94                      |
| 9    | 14.163               | 62.59                       | C\textsubscript{10}H\textsubscript{16}   | limonene           | 96                      |
| 10   | 15.118               | 16.94                       | C\textsubscript{10}H\textsubscript{16}   | \(\gamma\)-terpinene | 96                  |
| 11   | 15.467               | 0.30                        | C\textsubscript{10}H\textsubscript{18}O\textsubscript{2} | linalool oxide      | 91                      |
| 12   | 15.997               | 0.78                        | C\textsubscript{10}H\textsubscript{16}   | \(\alpha\)-terpinolene | 95                  |
| 13   | 19.280               | 1.11                        | C\textsubscript{12}H\textsubscript{24}O  | terpinen-4-ol      | 94                      |
| 14   | 19.729               | 1.23                        | C\textsubscript{10}H\textsubscript{18}O  | \(\alpha\)-terpineol | 96                  |
| 15   | 20.001               | 0.61                        | C\textsubscript{10}H\textsubscript{20}O  | decanal            | 91                      |
| 16   | 28.140               | 0.61                        | C\textsubscript{15}H\textsubscript{24}   | germacrene         | 90                      |
| Total|                      |                             |         |          | 100                     |
Figure 1. Diameter of inhibition zones of Limau Kuit essential oils against S. aureus ATCC 25923.

Table 3. The results of the measurement of inhibition zones of antibacterial activity of essential oils of Limau Kuit against S. aureus ATCC 29523.

| Concentration | Samples | Inhibition Zones (mm) | Sensitivity |
|---------------|---------|-----------------------|-------------|
|               |         | Oils                  | Positive Control (Chloramphenicol 30µg) | Negative Control (DMSO) |
| 100%          | Wet     | 19.5 (I)              | 30.5 (S)    | -           |
|               | Dry     | 26.8 (S)              |             |             |
| 75%           | Wet     | 38.3 (S)              | 28 (S)      | -           |
|               | Dry     | 39 (S)                |             |             |
| 50%           | Wet     | 27.9 (S)              | 29.8 (S)    | -           |
|               | Dry     | 26.3 (S)              |             |             |
| 25%           | Wet     | 32.9 (S)              | 31.7 (S)    | -           |
|               | Dry     | 10.8 (R)              |             |             |
| 10%           | Wet     | -                     | 31.8 (S)    | -           |
|               | Dry     | -                     |             |             |

R = resistant, S = Sensitive, I = Intermediate

Based on the table 3, the lowest concentration of essential oils of lime peel in inhibiting the growth of S. aureus ATCC 25923 was at a concentration of 25%. In this study, the inhibition zone diameter did not increase in proportion to the increase in concentration of essential oils. This could occur due to several factors including the volume of absorption of essential oils on paper discs, the thickness of agar media, and the diffusion ability of essential oils on agar media [5,11]. The mechanism of essential oil as an antibacterial based on microstructural observations was by increasing permeability and disrupting the integrity of cell membranes. The hydrophobic nature of essential oils allows essential oils to accumulate in cell membranes, disrupt structure, and increase membrane permeability. Damage from intracellular components and a decrease in the workings of enzymes and loss of cell contents in large numbers caused cell death [7,12,13,14,15,16,17,18]. Essential oil compounds that had antibacterial activity were limonene, α-pinene, β-pinene, γ-terpinene, α-terpineol, sabinene, and terpinen-4-ol.

4. Conclusion
Limau Kuit peel essential oils have antibacterial properties against S. aureus ATCC 25923 bacteria. The MIC value of citron peel essential oil was at a concentration of 25% of wet and dried samples...
with inhibition zone diameters of 32.9 mm and 10.8 mm. Dried and wet sample of Limau Kuit peel essential oils had 5 dominant components with relatively greater concentration values, namely limonene, γ-terpinene, β-pinene, α-pinene, and sabinene. Essential oil compounds that had antibacterial activity were limonene, α-pinene, β-pinene, γ-terpinene, α-terpineol, sabinene, and terpinene-4-ol.

References

[1] Kurniawati Y 2017 Pengaruh perlakuan pada daun limau kuit terhadap karakteristik minyak atsiri dengan metode ekstraksi soxhlet menggunakan pelarut n-heksana Skripsi (Banjarbaru: Universitas Lambung Mangkurat)

[2] Sugiantoro, Jayuska A and Alimuddin A H 2016 Biotransformasi limonena dari minyak atsiri kulit jeruk pontianak menggunakan jamur Rhizopus oligosporus dalam media air kelapa JKK. 5(3) 40-4

[3] Hidayati 2012 Distilasi minyak atsiri dari kulit jeruk pontianak dan pemanfaatanya dalam pembuatan sabun aromaterapi Biopropal Industri. 3(2) 39-49

[4] Kurniawen A, Kurniawan C, Indraswati N and Mudijjati 2008 Ekstraksi minyak kulit jeruk dengan metode distilasi, pengepresan dan learching Widya Teknik. 7(1) 15-24

[5] Sriskun V, Tribuddharat C, Nukoolkarn V, Bunyapraphatsara N, Chokephaibulkit K, Phoorniyom S, Chuanphung S and Srituengfung S 2012 Antibacterial activity of essential oils from Citrus hystrix (makrut lime) against respiratory tract pathogens Science Asia. 38 212-7

[6] Stefanakis M K, Touloupakis E, Anastasopoulos E, Ghanotakis D, Katerinopoulos H E and Makridis P 2013 Antibacterial activity of essential oils from plants of the genus Origanum Food Control. 34 539-46

[7] Fei Lv, Liang H, Yuan Q, Li C 2011 In vitro antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related microorganisms Food Research International. 44 3057-64

[8] Pradani N R 2012 Uji aktivitas antibakteri air perasan jeruk nipis (Citrus aurantiifolia, Swingle) terhadap pertumbuhan bakteri Staphylococcus aureus secara in vitro Skripsi. (Jember: Universitas Jember)

[9] Chouhan S, Sharma K and Guleria S 2017 Antimicrobial activity of some essential oils-present status and future perspectives Medicines. 4(58) 1-21

[10] Ariyani H, Nazemi M, Hamidah and Kurniati M 2018 Uji efektivitas antibakteri ekstrak kulit limau kuit (Citrus hystrix D.C) terhadap beberapa bakteri Journal of Current Pharmaceutical Sciences. 2(1) 136-41

[11] Dewi F K 2010 Aktivitas antibakteri ekstrak etanol buah mengkudu (Morinda citrifolia Linnaeus) terhadap bakteri pembusuk daging segar Skripsi. (Surakarta: Universitas Sebelas Maret)

[12] Cox S D, Mann C M, Marrkham J L, Gustafon J E, Warmington J R and Wyllie S G 2001 Determining the antimicrobial actions of tea tree oil Molecules. 6 87-91

[13] Burt S 2004 Essential oils: their antibacterial properties and potential applications in foods - a review International Journal of Food Microbiology. 94 223-53

[14] Oyedemi S O, Okoh A O, Mabinya L V, Pirochenva G and Afolayan A J 2009 The proposed mechanism of bactericidal action of eugenol, α-terpineol, γ-terpinene against Listeria monocytogenes, Streptococcus pyogenes, Proteus vulgaris and Escherichia coli African Journal of Biotechnology. 8(7) 1280-86

[15] Huang D F, Xu J G, Liu J X, Zhang H and Hu Q P 2014 Chemical constituents, antibacterial activity and mechanism of action of the essential oil from Cinnamomum cassia bark against four food-related bacteria Microbiology. 83(4) 357-65

[16] Raut J S and Karuppayil S M 2014 A status review on the medicinal properties of essential oils Industrial Crops and Products. 62 250-64
[17] Li Z H, Cai M, Liu Y S, Sun P L and Luo S L 2019 Antibacterial activity and mechanisms of essential oil from *Citrus medica* L. *var. sarcodactylis* *Molecules*. 24(1577) 2-10

[18] Guimaraes, A. C., L. M. Meireles, M. F. Lemos, M. C. C. Guimaraes, D. C. Endringer, M. Fronza, & R. Scherer. Antibacterial activity of terpenes and terpenoids present in essential oil *Molecules*. 24(2471) 1-12