Distillation Delayed Time on The Characteristics of Lemon Peel Oil and Activity against *Staphylococcus aureus*

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Abstract. Delay time effect (curing) in lemon peel essential oil extraction often occurs in factories when the number of distillers does not match the amount of available lemon peel. This delay time can reduce the quantity and the lemon peel oil quality due to degradation. This study aimed to determine the delay time period before distillation process that affected changes in the lemon peel oil aromatic compound quantity and quality based on the yield, limonene percentage, and the inhibitory effect of lemon peel oil on *Staphylococcus aureus* bacterial growth. The distillation method used the cohobation distillation technique with a delay time of 1, 3, 5, and 7 hours. Distillation process was carried out by steaming and boiling. The results showed that steam cohobation method was better than boiled cohobation method based on yield and limonene percentage. The 1-hour delay time in steam cohobation method was significantly different from other delay times in boiled cohobation method. Steam cohobation method produced 79.95% limonene. *S. aureus* antibacterial test at 100% oil concentration had the strongest antimicrobial activity. Therefore, the 1-hour delay time using the steam cohobation method obtained the highest yield of 0.325% and inhibition level of *S. aureus* at 21.375 mm.

1. Introduction
Over the past few years, lemons have been widely cultivated in Indonesia. Increased crop yields is followed by the increased lemon juice product processing industry growth. Therefore, more lemon peel waste is also produced during the juice production process.

The lemon peel disposal to the dump can cause waste accumulation that can increase carbon emissions, although lemon peels contain valuable elements or compounds, namely flavonoids, carotenoids, dietary fiber, polyphenols, essential oils, and ascorbic acid [1]. Based on various results of phytochemical analysis, lemon peel essential oil contains alkaloids, saponins, flavonoids, carbohydrates, glycosides, citric acid, and tannins, which can be useful as antibacterial and antioxidant [2,3]. Flavonoids in lemon peel can be efficacious as an antibacterial, including *Staphylococcus aureus* bacteria which causes food-poisoning [4]. Therefore, lemon peel oil can be used as a natural preservative. The lemon peel processing into valuable products can increase the added value of the lemon juice industry. To obtain a lemon peel oil containing the expected compounds, extraction can be carried out with different methods to produce oil containing different compounds.
Extraction methods that are popular around the industry are steam-water distillation, steam distillation, and cohabation methods. Cohabation method produces more oil quantity and quality, as the condensate water removed from the separator can automatically return to the vessel for the re-distillation process, which gains a higher yield [5] than the other distillation methods. Essential oil produced from the lemon peel [6] contains D-limonene (46.93%), -terpinene (16.89%), tri-cyclene (6.67%), 1-β-pinene (4.69 %), and 2-β-pinene (3.86%). Limonene in lemon peel oil is functioned as anti-inflammatory and anti-infective agent [7].

The essential oil distillation process at the factory encounters several obstacles, namely improper capacity and number of distillation equipment and lemon peels, which causes a delay in the distillation process. Delayed distillation can cause a degradation, which reduces the quantity and quality of the lemon peel oil produced.

Delayed distillation is commonly called as curing. Curing is a delayed process before distillation occurred in materials containing essential oils [8]. Delayed process also occurs in vanilla, bay leaf, kecombrang, and pandan leaf extractions, which causes a decreased volatile compound that exceeds a certain time limit and is different from each ingredient [8, 9, 10, 11]. Different curing condition mentioned in this study is an unintentional process due to the lack of sufficient tools. This is important because orange peels in large quantities will degrade if not being utilized immediately, resulting in an unobtained added value expected from the lemon peel oil.

[8] stated that the delayed ingredient process containing essential oils was influenced by temperature and time, which causes biosynthesis and flavor compound changes through transformation and degradation reactions [12]. Based on these conditions, this study aimed to identify the time of delay time before the lemon peel distillation process, which could obtain high yields, high limonene percentage, and good microbial activity.

2. Materials and Methods

2.1. Materials
Materials used in this study were lemon (Citrus lemon) peel, water, 70% alcohol, cotton swab, nutrient media (Na), antibiotic disk paper, physiological NaCl, Tween 80, aquadest, aluminum foil, cotton, and thread.

2.2. Equipment
The equipment used in this study was a water-steam distillation equipment with cohabation method, Beaker glass, Erlenmeyer glass, Bunsen lamp, laboratory tripod, Petri dish, autoclave, label paper, match, sterile cotton-bud, oven, pipette drop, knife, napkin, stirrer, volumetric flask, reaction tube, reaction tube shelf, incubator, filter paper, tissue, ruler, board marker, paper disk, scale, and Agilent Technologies 7890 GC-MS.

2.3. Location and Period
This study was performed in the Laboratory of Extraction and Laboratory of Microbiology, Center for Agrobase Industry, Bogor on April – December, 2020.

2.4. Methods
This study contained two steps, namely: Step 1. Distillation of lemon peel essential oil with delayed time using cohabation method. Step 2. Inhibitory activity of lemon peel essential oil on bacterial growth of S. aureus.
2.5. Procedures

2.5.1. Lemon Peel Extraction. Lemon was cleaned and cut in small pieces at 2-3 cm. Small-cut lemon was pressed, while the lemon peel was distilled with a cohobation method. Before being distilled, lemon peel was treated with a curing, namely extraction delay period of 1, 3, 5, and 7 hours. After delayed process was occurred, lemon peel was inserted in a stainless-steel glass filled with water following the steaming limit. Lemon peel oil distillation was performed until the oil was absent (4-5 hours). The lemon peel oil produced was calculated its yield [13] following the formula: \[\text{Yield} = \left(\frac{a}{b}\right) \times 100\]; whereas, \(a = \) essential oil, \(b = \) vegetal material.

2.5.2. Aromatic Compound Profiling with Gas Chromatography-Mass Spectrometry (GC-MS). Agilent Technologies 7890 Gas Chromatograph with Auto Sampler and 5975 Mass Selective Detector and Chem-station data system. Ionization mode: Electron impact. Electron energy: 70 eV. Column: Capillary Column: HP Ultra 2. length (m) 30 X 0.20 (mm), thickness I.D X 0.11 (mm), hold for 1 minute and finally rising 20°C/min to 280°C, hold for 26 minutes, Condition of chromatography, injector port temperature: 250°C, Ion Source Temperature 230°C, Quadrupole Temperature 140°C, Carrier gas Helium, Column mode: Constant Flow, Flow Column: 1.2 mL/minutes, Injection volume: 5 m. Split: 8:1.

2.5.3. Anti-bacterial Inhibitory Level Against S. aureus Bacteria. The lemon peel oil of good result as aromatic compound The anti-bacterial inhibitory level test against \(S.\ aureus\) was performed by preparing the NA media, rejuvenation, and preparing the microbial inoculum test, based on [14] with modification, microbial inoculum production, and lemon peel oil concentration production.

2.5.3.1. NA Media Preparation NA medium was measured at 9 g and inserted to 500 ml Erlenmeyer flask solved with 450 ml aquadest, and stirred until homogenous. The 7-8 ml NA media were inserted to each 2 reaction tubes for bacterial rejuvenation, and the medium residue was used for anti-bacterial test. All homogenous media were sterilized using an autoclave at 121°C for 15 minutes.

2.5.3.2. Microbial Inoculum Rejuvenation Bacterial stock production was produced to multiply the bacteria by taking 1-2 Ose of microbes (\(S.\ aureus\)), then streaked in slant NA media and incubated for 48 hours at 37°C, based on [14] with modification.

2.5.3.3. Microbial Inoculum Preparation The rejuvenated microbial dilution, namely: 1-2 Ose. The microbes were inserted in the reaction tube filled with 10 ml 0.85% NaCl until homogenous. Then, the NaCl solution filled with microbes was taken at 1 ml and inserted to a reaction tube filled with 9 ml 0.85% NaCl until homogenous. Dilution was performed at 10^{-1} and 10^{-3}. Microbes added with physiological salt was poured on a Petri dish and stood until the solution became solid. The 0.5 cm paper disk was placed on each medium, after soaking in lemon peel oil (100%, 75%, 50%, 25%) and negative solution (Tween oil) in solid medium surface. Petri dish was closed tightly and wrapped with a plastic wrap, then incubated for 24 hours at room temperature and measured the clear zone (inhibition zone), based on [15] with modification.

2.6. Statistical Analysis and Experimental Design

• Step 1: Delay time effect on yield and aromatic compounds produced. The experimental design used in step 1 was a factorial completely randomized design with two factors. The A factor was curing period effect containing 4 levels, such as A1=1 hour, A2=3 hours, A3=5 hours, and A4=7 hours. Meanwhile, factor B was cohobation method containing 2 levels, such as B1=steam cohobation and B2=boiled cohobation.
• Step 2: Cohobation extracted lemon peel oil inhibitory effect against \(S.\ aureus\) growth. The experimental design used in step 2 was a factorial completely randomized design with two
factors, namely (A) lemon peel oil concentration, containing A1=25%, A2=50%, A3=75%, and A4=100%, (B) Dilution factor, containing B1=10^{-1} and B2=10^{-3}.

3. Results and Discussions

3.1. Yield

Lemon peel aromatic compound extraction used steam and boiled cohabation methods with different delay time was performed to identify the delayed lemon peel process period before cohabation method, that still contained strong limonene compounds as the distinct characteristics of lemon peel aroma. The lemon peel oil yield based on the statistical analysis results is presented in table 1.

| Delay time | Steam  | Boiled | Average |
|------------|--------|--------|---------|
| 1          | 0.400  | 0.250  | 0.3250  |
| 3          | 0.315  | 0.190  | 0.2525  |
| 5          | 0.230  | 0.140  | 0.1850  |
| 7          | 0.175  | 0.090  | 0.1325  |
| Average    | 0.280 b | 0.168 a |         |

Note: Similar superscript letters show an insignificant difference at α=0.05.

In table 1 and figure 2, the effect of delay time at 5 and 7 hours was insignificantly different on yield (p>0.05). However, the 1- and 3-hour delay treatment obtained a significant different on yield. The 1-hour delay was significantly different from the 5-and 7-hour delay. The 3-hour delay was also significantly different from the 5- and 7-hour delay. The extraction process can be delayed for 1 hour, while delay time more than 1 hour will impact on yield reduction due to degradation reaction because of high water, carbohydrate, and acid contents [2]. In addition, the delay time occurred in kecombrang and bay leaves obtained reduced yield due to water and aromatic compound evaporation [9,10]. Figure 1 describes that the yield of each cohabation methods with delay time, as the steamed cohabation method is greater than the boiled cohabation method (figure 1).
Figure 3. Effect of cohobation methods on lemon peel oil yield

Figure 4. Effect of delay time on D-limonene percentage in lemon peel oil extracted with steam and boiled cohobation methods

In figure 3, steam and boiled cohobation methods obtained a significant different result (p< 0.05) on lemon peel oil yield concentration, as steam method produced a higher yield concentration than boiled method. In steam distillation process, the distilled material was not directly exposed to high temperatures (below 100°C). When boiling, the material is exposed to temperatures of 100°C or more [16].

3.2. Aromatic Compound Profiles

Delay time effect before the lemon peel oil extraction on aromatic compound compositions can be shown in table 2 and figure 4. The aromatic compound, namely limonene in lemon peel oil continued to decrease along with the increased delay time. However, other compounds remained relative stable, even though the number goes up and down, the changes are very slight. Decreased aromatic compounds due to delay time also occurred in aromatic compounds of bay leaves and torch kecombrang (9,10).

Table 2. Delay time effect on aromatic compounds in lemon peel essential oil.

| Delay time | RT  | Quality | Compound                                      | Content (%) |
|------------|-----|---------|-----------------------------------------------|-------------|
| Steam 1hr  | 10.087 | 96  | β-myrcene                                    | 1.24        |
|            | 11.763 | 99  | d-limonene                                   | 79.95       |
|            | 28.745 | 96  | linalool                                      | 1.25        |
|            | 36.03  | 96  | Neral                                         | 3.11        |
|            | 36.819 | 90  | 3-cyclohexene-1-methanol, α α-4-trimethyl-R   | 2.79        |
|            | 38.277 | 62  | 1,3-cyclohexadiene                           | 2.15        |
|            | 38.75  | 95  | 2,6-octadiene,3,7 dimethyl                   | 5.27        |
| Steam 3hr  | 10.092 | 97  | β- myrcene                                    | 1.08        |
|            | 11.742 | 99  | D-limonene                                    | 74.3        |
|            | 13.641 | 97  | β- o-cymene                                   | 1.06        |
|            | 28.756 | 97  | linalool                                      | 2.36        |
|            | 36051  | 96  | Neral                                         | 4.05        |
|            | 36829  | 90  | 3-cyclohexene-1-methanol α-4-trimethyl-R      | 1.65        |
|            | 38287  | 74  | p-mentha-1,5-dien-8-ol                        | 1.99        |
|            | 38.764 | 96  | Citral                                        | 6.46        |
| Steam 5 hr | 10.097 | 96  | β- myrcen                                     | 1.99        |
|            | 12.354 | 97  | D-lemonen                                    | 6.46        |
|            | 28.75  | 96  | Linalol                                       | 1.37        |
|            | 36.21  | 96  | Neral                                         | 72.55       |
|            | 36881  | 90  | 3-Cyclohexene-1-methanol, α,4 trimethyl,R     | 1.33        |
|            | 38282  | 90  | p-Mentha-1,5-dien-8-ol                        | 4.35        |
|            | 39,024 | 96  | Cytral                                        | 2.71        |
The extracted lemon peel oil in this study with steam and boiled cohabation methods produced 12 compounds (table 2). These compounds were classified into two groups, namely monoterpenes and aldehydes. [6] performed a hydro-distillation process on lemon peel and produced a lemon peel oil containing 4 groups, namely monoterpenes, sesquiterpenes, aldehydes, and esters. Monoterpene hydrocarbons are a group that dominates on 5 lemon varieties [6].

In table 2, are aroma compound of the lemon peel oil was identified to contain limonene as the main compound, compared to other compounds with smaller content. This result was similar to [17] that β-myrcenes (3.79%), octanols (0.84%), and α-pinenes (1.24%) were contained in smaller amount of lemon peel oil.
Figure 5. Dilution and concentration effect of lemon peel oil on inhibitory activity level of *S. aureus*.

More decreased D-limonene occurred from the steam and boiled cohabation methods, in addition to showing an insignificant difference (figure 4). [7] obtained d-limonene from water distillation method/boiling (hydro-distillation) at 46.93%. Meanwhile, [17] obtained limonene compound at 94.13%.

3.3. Inhibitory Level against *S. aureus*

Lemon peel essential oil concentration and dilution obtained a significant difference on bacterial inhibitory level diameter. Therefore, a continued statistical analysis test was performed to identify which treatment obtained the highest inhibitory level.

**Table 3.** Effect of lemon peel oil dilution and concentration against *S. aureus* bacteria.

| Concentration (A) | B1       | B2       | Average (A) |
|-------------------|----------|----------|-------------|
| A1 (25%)          | 11.4000b | 11.1050b | 11.2525b    |
| A2 (50%)          | 12.8800c | 15.4400d | 14.1600b    |
| A3 (75%)          | 13.7100d | 15.2050d | 14.4575e    |
| A4 (100%)         | 10.3100a | 21.3750e | 15.8425f    |
| Average (B)       | 12.0750x | 15.7812y | 13.9281     |

Note: Similar superscript letters show an insignificant difference at α=0.05.

In table 3, the effect of lemon peel oil at 50% and 75% obtained a similar inhibitory zone diameter, besides the 75% and 100% concentrations. However, the 10⁻¹ and 10⁻³ dilutions obtained a significant difference on the inhibitory zone diameter.

The continued test for 10⁻¹ and 10⁻³ dilution treatments obtained a significant difference. Meanwhile, interaction between dilution and concentration can be shown in the A4B2 treatment (100% + 10⁻³) by obtaining the strongest inhibitory level with the highest inhibitory zone diameter at 21.375 mm (figure 4). This study was similar to [2], as the inhibitory level of lemon peel methanolic extract against *S. aureus* at 100 µL concentration was 20.6 mm, while lemon extract through 50% ethanol extraction obtained smaller inhibitory zone at 14 mm than lemon peel oil at 16 mm [18]. This study was also supported by [19] that lemon peel ethanolic extract from 70% ethanol extraction obtained an inhibitory level at 30 mm.
4. Conclusions
The lemon peel extraction can be performed with delay time, which obtained the highest yield and aromatic compound productions at 1-hour compared to 3, 5 and 7 hours. The steam cohabation method produced higher yield than the boiled cohabation method. The main aromatic compound from lemon peel oil was limonene at 79.95%. Decreased D-limonene compound until 7-hour period only reached at 68%. The inhibitory level test against S. aureus bacteria with lemon peel oil produced from 1-hour delay time method obtained the strongest inhibitory capability at 100% concentration and 10⁻³ dilution level.

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