Comparison of volatile compounds and antibacterial activity of *Citrus aurantifolia*, *Citrus latifolia*, and *Citrus hystrix* shell essential oils by pilot extraction

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Abstract. Citrus peel essential oil has considerable potential to be used as a direct agent against a wide range of Gram-positive and Gram-negative bacteria. The three citrus peel species used in this study included *Citrus aurantifolia*, *Citrus latifolia*, and *Citrus hystrix*. Essential oils were extracted on a pilot model and analyzed by gas chromatography-mass spectrometry (GC-MS). Gram-positive bacteria (*Bacillus subtilis*, *Listeria monocytogenes*, *Staphylococcus aureus*) and gram-negative (*Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*) were used to counteract the antibacterial effects of the samples. The yield of hydrodistillation distilled essential oil is *C. aurantifolia* (1.15%), *C. latifolia* (1.32%), and *C. hystrix* (3.18%). The essential oil contains the main ingredient D-Limonene. The highest antibacterial activity against *L. monocytogenes* is *Citrus latifolia* essential oil. The main chemical composition of essential oils are β-pinene, D-limonene, γ-terpinene, terpinolene, α-terpineol, ... and the antimicrobial activity of the essential oil is affected by the variation of D-limonene. and β-pinene in essential oils.

1. Introduction
The essential oil consists of many aromatic volatile compounds produced by secondary metabolism of the plant (mainly terpenes and triterpenes), with a characteristic odor depending on the source of the material. The chemical composition of essential oils includes terpenoids and oxygen-containing derivatives of terpenoids such as alcohols, aldehydes, ketones, esters, acids ... Although there are many constituents, there are usually only a few key constituents that have value. and create a distinctive fragrance for essential oils [1- 4]. Citrus essential oil has the main ingredient D-limonene (a substance with high antibacterial and antioxidant properties) and various compounds. The antimicrobial properties of essential oils have been recognized for centuries, with increasing demand from changes in consumption trends and increasing isolation of antibiotic-resistant pathogens, a need to find killer agents bacteria based on chemicals [5-8]. Commonly used traditional methods are water distillation, steam-enticing distillation, solvent extraction,
and cold pressing. Each method has its own advantages and disadvantages. New and modern extraction methods are increasingly being developed to improve the yield and quality of essential oils. These new methods include the supercritical extraction method, ultrasonic combined extraction, and microwave combined extraction [9-11]. Although there are many new methods of extracting essential oils, traditional extracts such as hydrodistillation are still widely used especially for commercial-scale production because of their simplicity, low cost. Essential oils, especially citrus essential oils, have long been used extensively in aromatherapy and as an alternative to partial relief of pain relievers. Under such conditions, this study aimed to study the effects of three essential oils of *Citrus aurantifolia*, *Citrus Hystrix*, and *Citrus latifolia* with the main ingredients in citrus essential oils as β-pinene, limonene, γ-terpinene, terpinolene, α-terpineol, and citral, are very likely to be responsible for good antimicrobial activity, especially gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, and *Staphylococcus cholermidis*) [12]. This group of essential oils can provide natural antimicrobials that the food industry requires to meet both its and consumer requirements. Vietnam with tropical natural conditions is very favorable for the formation and development of plants, in which essential oil-containing plants are confirmed to be abundant and unique. Currently, there are a number of establishments in Vietnam that have distilled citrus essential oil, and when analyzing some citrus essential oil samples on the market, it showed that the quality of the essential oil was not high, some of the main ingredients in the essential oil, have a low percentage and do not meet some essential oil standards. This greatly limits the ability to supply citrus essential oil on a large scale or as a raw material for establishments manufacturing cosmetics, pharmaceuticals, aromas.

Therefore, obtaining essential oils from the peels and finding suitable storage conditions to develop a variety of different products, and extracting essential oils from the peel is an effective solution to utilize secondary sources of citrus at low cost, at the same time creating products with high value. Contributing to improving the economic value of the local citrus tree, creating a stable market for potential raw materials of the province: from there, building a chain of production-consumption in a sustainable direction with stable output.

2. Experimental

2.1. Plant material

![A. Citrus aurantifolia](image1a.jpg)  ![B. Citrus hystrix](image1b.jpg)  ![C. Citrus latifolia](image1c.jpg)

*Figure 1. Image of (A) Citrus aurantifolia, (B) Citrus hystrix and (C) Citrus latifolia*

*C. latifolia* are collected directly from home gardens in popular growing areas in Hau Giang. The *C. aurantifolia* samples used for this study were collected in the Ben Tre province (Figure 1). *C. Hystrix* are harvested and selected from the border area of An Giang province in the southwest of Vietnam. The raw materials are transported to the laboratory when harvested. After that, conducting preliminary treatment, citrus are washed to remove crushed fruits and impurities. The pods are then separated and the meat is
removed. The shells after the treatment will be pureed to be approximately 0.5 mm in size and placed in the extraction device.

2.2. *Hydro-distillation pilot procedure*

Fruit after harvest is pre-treated, put into storage tanks, the amount of water is added to suit each experiment, and put into the distillation system at the rate of 1:6 material/water. The distillation temperature is 150°C (inside the 100°C unit) and the distillation time is calculated from the moment the first drop of liquid appears (after 50 minutes), until the quantity of the essential oil remains constant. After the extraction of essential oils, we obtain a mixture of water and essential oils after going through a condenser to recover the essential oil. The essential oil obtained with a little water should be anhydrous with Na2SO4 salt to remove the water in the oil. After the elimination process, we obtain pure essential oils.

2.3. *Gas Chromatography – Mass Spectrometry*

The chemical composition of essential oils was determined by GC-MS analysis using GC Agilent 6890 N instrument combined with inert HP5-MS and MS 5973 columns. The pressure of the head column is 9.3 psi. Essential oil is added with 1 ml of n-hexane and dehydrated with Na2SO4. Constant flow rate at 1 mL/min. Nozzle temperature is 250 °C and the dispensing rate is 30. Thermal program for samples: 50 °C held for 2 minutes increments of 2 °C / min to 80 °C, further increments of 5 °C / min to 150 °C, continue to increase 10 °C / min to 200 °C, increase 20 °C / min to 300 °C hold for 5 minutes.

2.4. *Antibacterial activity testing*

In this study, the antibacterial activity of citrus essential oils was tested against certain gram-positive bacteria (*Bacillus subtilis*, *Listeria monocytogenes* and *Staphylococcus aureus*) and gram-negative (*Escherichia coli*, *Salmonella typhimurium* and *Pseudomonas aeruginosa*). All laboratory equipment and culture media were prepared and autoclaved at 121°C for 15 minutes. The test strains were shaken in 5 ml of Mueller Hinton Broth medium (MHB) for 18-20 hours at 35 ± 2 °C. Then calibrated to Mc Farland 0.5 equal to 1 – 2.108 with 0.9% NaCl physiological saline. 100 μl of 10⁸ CFU / ml concentration of bacteria broth is pumped into MHA agar plates and spread evenly on the surface of the culture dish. In this experiment, the well diameter used is 6mm, which corresponds to 50 μl of essential oil sample.

3. **Results and Discussion**

3.1. *Essential oils extraction*

![Figure 2. The efficiency of essential oils at the pilot production scale](image-url)
The results of extracting essential oils from different types of citrus peel are shown in the graph in Figure 1. As shown in Figure 1, the efficiency is obtained from three types of *C. aurantifolia* peel (1.15%), *C. latifolia* (1.32%), and *C. hystrix* (3.18%). *C. hystrix* peel essential oil is twice as effective as *C. aurantifolia* and *C. latifolia* peel oil. The differences in the essential oil content between citrus depend on the individual characteristics of the ingredients, growing conditions, soil, and harvesting stage. The year 2020 Quyen et al. extracted *C. aurantifolia* peel essential oil at a laboratory scale with an efficiency of 2.1%. Meanwhile, Atti-Santos and his team used a Clevenger device to distill essential oils from *C. latifolia* peels in the laboratory, with the essential oil content being 5.45% [13]. The team of Hongratanaworakit and Buchbauer from Srinakharinwit University extracted *C. hystrix* essential oil from fresh skin by hydrodistillation method on Clevenger device for 2 hours, yield 1.5% w/w. On the other hand, Chanthaphon et al. use *C. hystrix* source grown in Songkhla (Thailand) from May to July 2005 to extract essential oils, with 2.56% content recovered through the hydrodistillation process [14]. However, each material will have different advantages and disadvantages in the extraction process. Distillation time depends on a number of factors such as raw material, temperature, ingredient/solvent ratio, sample size, etc. The longer the distillation time, the higher the amount of essential oil. The process of distillation with high temperature when extracting will denature volatile compounds in essential oils. However, up to a certain time, the amount of essential oils does not increase anymore, and if further distillation can affect the quality of the product, the product is likely to be denatured and consume an unnecessary amount of energy equipment leads to increased costs.

3.2. Chemical composition of lime peel essential oil

![Figure 3. Essential oil chromatogram: a) Citrus latifolia, b) C. aurantifolia, c) C. Hystrix](image)
Table 1. Chemical composition of essential oil

| Name            | C. latifolia | C. aurantifolia | C. hystrix |
|-----------------|--------------|-----------------|------------|
| α-Thujene       | 0.467        | 0.3             | 0.207      |
| α-Pinene        | 2.206        | 1.857           | 2.699      |
| Camphene        | 0.106        |                 | 0.172      |
| Sabinene        |              |                 | 13.003     |
| β-Pinene        | 10.581       | 4.225           | 28.221     |
| β-Myrcene       | 1.207        | 1.502           | 1.301      |
| α-Terpinene     | 0.608        | 0.59            |            |
| ρ-Cymene        | 1.138        | 0.869           | 1.999      |
| D-Limonene      | 56.076       | 70.52           | 20.121     |
| γ-Terpinene     | 16.108       | 15.418          | 1.831      |
| Terpinolene     | 0.957        | 1.078           |            |
| Linalool        | 0.341        |                 | 0.682      |
| Citronellal     |              |                 | 9.777      |
| Terpinen-4-ol   | 1.379        | 0.386           | 7.464      |
| α-Terpineol     | 2.586        | 0.573           | 2.551      |
| Citronellol     |              |                 | 3.125      |
| β-Citral        | 0.992        |                 |            |
| α-Citral        | 1.174        |                 |            |
| Nerol acetate   | 1.362        |                 |            |
| Caryophyllene   | 0.65         | 0.689           | 0.46       |
| trans-α-Bergamotene | 0.992   | 0.511           |            |
| α-Caryophyllene |              |                 | 0.168      |
| Germacrene D    |              |                 | 0.352      |
| β-Bisabolene    | 1.071        | 0.853           |            |
| δ-Cadinene      |              |                 | 0.894      |

The percentage content and the name of the volatile compound obtained from the essential oils of the three shells (Citrus latifolia, C. aurantifolia, C. Hystrix) are shown in Table 1, via GC-MS analysis. Based on Table 1 and Figure 2, it can be seen that the components that account for high concentrations of the three essential oils are hydrocarbon monoterpenes such as D-Limonene (56.076%, 70.52%, 20.121%), β-pinene (10.581%, 4.225%, 28.221%), γ-Terpinene (16.108%, 15.418%, 1.831%) respectively. However, C. Hystrix essential oil contains a number of specific ingredients that account for relatively high levels of Sabinene (13.003%), Citronellal (9.777%), Citronellol (3.125%) compared to the other two essential oils. On a laboratory scale, essential oils of C. aurantifolia (farm G. Corigliano, Bovalino, Italy), were extracted by hydrodistillation, analyzed by GC-MS by Spadaro et al. (2012) [12]. The main components limonene
(58.4%), β-pinene (15.4%), γ-terpinene (8.5%) and citral (4.4%) have been shown. Asnaashari and his team used essential oils from Barij-Essence, Iran, and GC-MS analysis of *C. aurantifolia* essential oil resulted in the identification and quantification of about 22 key compounds, accounting for 88, 85% of the total number of ingredients. Limonene (28.27%) is the main ingredient, followed by α-terpineol (19.61%), ρ-cymene (8.6%) and β-pinene (5.7%) [15]. In 2001, Pino and Rosado experimented on samples of *C. aurantifolia* Swingle and *C. latifolia* Tanaka collected in Cuba. The results showed differences found in odor and taste in both essential oils, γ-terpinene (9.5% and 11.8%), Limonene (40.4% and 55.6%), α -terpineol (12.7% and 6.6%) and terpinolene (8.7% and 5.2%) are the main ingredients, respectively [16].

With shell material *C. Hystrix* research group of Zuhra et al. extracted and analyzed essential oils obtained 30 compounds. Limonene (10.59%), β-pinene (23.03%), terpinene-4-ol (11.43%), sabine (13.37%), and citronella (10.41%) seem to be the main compounds of essential oils obtained from the hydrodistillation [17]. As can be seen, the difference between the composition of distilled oil in the pilot-scale and in the laboratory. However, GC-MS analysis results give data consistent with previous work on citrus essential oils distilled in the laboratory, with equivalent amounts of D-limonene, β-pinene, terpinolene, α -terpineol, citral.

3.3. Antimicrobial Activity of essential oils

Table 2. Antibacterial results of 3 types of citrus peel essential oils

| Tested Bacteria            | *Citrus aurantifolia* | *Citrus latifolia* | *Citrus hystrix* | Amoxicillin 0.25 mg/ml |
|----------------------------|-----------------------|--------------------|------------------|------------------------|
| *S. aureus* NRRL B-313     | 10±0                  | 14±0.57            | 12±0.57          | 41±1.66                |
| *L. monocytogenes* NRRL B-2354 | 18±0.6               | 18.6±0.6           | 17.3±0.33        | 39±0.33                |
| *B. cereus* ATCC 10876     | 13.3±0.88             | 14±0.88            | 12.6±0.33        | 11.6±0.66              |
| *S. typhimurium* YSI646    | -                     | -                  | -                | 27±0.57                |
| *E. coli* NRRL B-409       | -                     | -                  | -                | 15.3±0.33              |
| *P. aeruginosa* NRRL B-14781 | -                    | -                  | -                | 24.3±0.33              |

In this study, the antibacterial activity of citrus essential oils was tested against certain gram-positive bacteria (*Bacillus subtilis*, *Listeria monocytogenes*, *Staphylococcus aureus*) and gram-negative (*Escherichia coli*, *Samonella typhimurium*, *Pseudomonas aeruginosa*). Concentrations of essential oils compared with standard antibiotics (Amoxicillin), which show marked antibacterial activities, are manifested by their inhibitory regions (Figures 3. A, B, C).

However, according to the results, the following statements can be made: The antibacterial ability of most citrus essential oils on gram-positive microorganisms is better than that of gram-negative microorganisms. Experimental results on the diffusion of agar plates show resistance to some tested strains of microorganisms. The results of antibacterial ring diameter data are summarized in Table 3.3. Specifically, seedless citrus essential oil (14 ± 0.57, 18.6 ± 0.6, 14 ± 0.88), seeded citrus (10 ± 0, 18 ± 0.6, 13.3 ± 0.88) and citrus (12 ± 0.57, 17.3 ± 0.33, 12.6 ± 0.33) were resistant to 3 strains of gram-positive bacteria (*Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus subtilis*) respectively. Results showed that Citrus essential oil had broad-spectrum antibacterial activity on even gram-positive bacteria, probably because of its D-limonene component. All samples showed antibacterial activity against gram-positive bacteria with a variation of antimicrobial activity depending on the composition and content of D-Limonene, β-Pinene, and γ-terpeneol in essential oils [18].
Figure 4. Antibacterial activity of citrus oil properties a) C. aurantifolia, b) C. Hystrix, c) C. latifolia with six bacteria (A): Positive control - Amoxicillin; (B): Seedless citrus essential oil; (C): The control was H2O negative.

4. Conclusion
Through research results extracted from the bark (C. aurantifolia, C. Hystrix, C. latifolia) on a pilot scale, it shows that kumquat peel oil obtained from 3 types of citrus are light yellow, spicy, gills characteristic fragrance. The yield obtained was C. aurantifolia (1.15%), C. latifolia (1.32%), and C. Hystrix (3.18%). The chemical composition of citrus essential oil is mainly terpene hydrocarbons with main active ingredients D-Limonene, α-pinene, γ-terpineol, ρ-cymene, and β-pinene. According to the research, this is the chemical composition related to the ability of essential oils to show activity. Results of biological activity test of three essential oil samples obtained from different citrus all have antibacterial properties against bacteria strains Bacillus cereus, Listeria monocytogenes and Staphylococcus aureus, but essential oil samples obtained from essential oil C. Latifolia has a higher antibacterial activity.

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5. References

[1] Kim Ngan T T, Hien T T, Le X T, Anh T T, Quan P M, Cang M H, Le Ngoc T T, Danh V T, Yen Trung L N and Toan T Q 2019 *Asian J. Chem.* **31** 2855–8

[2] Hien T T, Nhan N P T, Trinh N D, Ho V T T and Bach L G 2018 *SSP* **279** 217–21

[3] Tran T H, Nguyen P T N, Ho V T T, Le T H N, Bach L G and Nguyen T D 2019 *IOP Conf. Ser.: Mater. Sci. Eng.* **479** 012015

[4] Tran T H, Nguyen P T N, Pham T N, Nguyen D C, Dao T P, Nguyen T D, Nguyen D H, Vo D V N, Le X T, Le N T H and Bach L G 2019 *IOP Conf. Ser.: Mater. Sci. Eng.* **479** 012002

[5] Sanei-Dehkordi A, Sedaghat M M, Vatandoost H and Abai M R 2016 *J Arthropod Borne Dis* **10** 577–85

[6] Costa R, Bisignano C, Filocamo A, Grasso E, Occhiuto F and Spadaro F 2014 *J Essent Oil Res* **26** 400–8

[7] Lemes R S, Alves C C F, Estevam E B B, Santiago M B, Martins C H G, Santos T C L D, Crotti A E M and Miranda M L D 2018 *An. Acad. Bras. Ciênc.* **90** 1285–92

[8] Ruiz-Pérez N J, González-Ávila M, Sánchez-Navarrete J, Toscano-Garibay J D, Moreno-Eutimio M A, Sandoval-Hernández T and Arriaga-Alba M 2016 *Sci. Rep.* **6** 25371

[9] Tan Q, Kieu X and Hong N 2012 *Emir. J. Food Agric* **24** 25

[10] Dongmo P M J, Tatsadjuie L N, Sonwa E T, Kuate J, Zollo P H A and Menut C 2009 *African J. Agric. Res.* **4** 354–358

[11] Atti-Santos A C, Rossato M, Serafini L A, Cassel E and Moyna P 2005 *Braz. arch. biol. technol.* **48** 155–60

[12] Spadaro F, Costa R, Circosta C and Occhiuto F 2012 *Nat. Prod. Commun.* **7** 1934578X1200701

[13] To Quyen N T, Ngoc Quyen N T, Kieu Linh H T, Le Ngoc T T, Tuan Anh H L, Khoi Nguyen N H, Tran T H, Thanh Tam H N and Cang M H 2020 *Asian J. Chem.* **32** 965–9

[14] Chanthaphon S, Chanthachum S and Hongpattarakere T 2008 *SJST* **30** 125-131.

[15] Asnaashari S, Delazar A, Habibi B, Vasfi R, Nahar L, Hamedeyazdan S and Sarker S D 2010 *Phytother. Res.* **24** 1893–7

[16] Pino J A and Rosado A 2001 *J. Essent. Oil Res.* **13** 179–80

[17] Zuhra C F, Lenny S and Nurtjahya K 2014 Proceedings of *International Conference on Natural and Environmental Science (ICONES)* **0** 67–72

[18] Aripin D, Julaeha E, Dardjan M and Cahyanto A 2015 *Padjadjaran Journal of Dentistry* **27**