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Research Article

Effect of Ultrasound-Assisted Maceration on Yield Percentage, Chemical Compound and Antimicrobial Activity of *Citrus nobilis* Peel Essential Oil

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Yield

Abstract: Increasing concerns about the activity of antibacterial growth promoters have prompted the need to discover alternatives from natural materials that can be found in various types of plants and herbs. Siamese orange peels (*Citrus nobilis*) are one of the plentiful organic waste-containing essential oils known for their antibacterial, antioxidant, and other biological properties promising as the potential alternative to antibiotic growth promoters in livestock. This study aimed to evaluate the antimicrobial properties of orange peel essential oil between 2 different extraction methods and the effect of different solvent ratios on the yield of citrus essential oil, chemical compounds, and the antibacterial activity against *Escherichia coli* and *Lactobacillus casei*. The evaluated methods were ultrasonic-macerated citrus essential oil (UMCEO) and conventional-macerated citrus essential oil (CMCEO), while the solvent ratios were 1:2, 1:4, and 1:6. The result showed that the solvent ratio was found to significantly give effect ($P<0.05$) to the yield of citrus peel essential oil with a solvent ratio of 1:2 obtaining the highest yield. Pretreating the citrus peel with ultrasound before hydro-distillation did not give a significant effect ($P>0.05$) on the chemical compounds of the citrus essential oil as analyzed by GC-MS. Both citrus essential oils were predominantly composed of cycloheptane (CMCEO: 48.5%; UMCEO: 44.09%) and d-limonene (CMCEO: 26.76%; UMCEO: 36.02%). The CMCEO showed a higher inhibition zone against *Escherichia coli* and *Lactobacillus casei.* The conclusion is that CMCEO seems to produce citrus peel essential oil with a stronger antibacterial effect against *Escherichia coli* and *Lactobacillus casei,* in particular with solvent ratio 1:2.

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1. Introduction

Siamese orange *(Citrus nobilis*) is the fourth most produced fruit in Indonesia (Central Bureau of Statistics, 2023), with production of 2 551 999 tons in 2022. The abundance of Siamese orange production in Indonesia is not only due to the large demand from the public for beverage products but also because of the adaptive nature of citrus plants that can grow well in various altitudes in Indonesia.

It is important to pay attention to the processing of citrus peel waste as well as other fruit waste because it can cause serious pollution if it is not treated properly including careless disposal and burning. From the whole fruit, it is reported that 27% is fruit peel, comprised of 17% part of albedo and 10% of flavedo (Mahato et al., 2018). The potency of orange peel becomes 689 040 tons/year. This waste treatment is then essential and also to overcome environmental challenges with the new concept of circular economy.

Orange peel is a type of waste that has many useful contents in addition to its abundant availability. Orange peel generally consists of two layers, albedo and flavedo. The flavedo layer, which is characterized by green, orange, and yellow colors on the peel, has many oil glands which are then called essential oils. Essential oils contain about 10% of the total weight of citrus fruits (Malik et al., 2021).

Essential oils also commonly referred to as aromatic oil, volatile oil, etheric oil, or essence oil, are volatile, dissolve in organic solvents, and do not dissolve in water at room temperature. Essential oils are considered colorless lipoid mixtures and are in different structures compared to fixed oils. Essential oils are grouped in odorous principles, consisting of a mixture of esters, ketones, terpenes, and aldehydes (Tassou, 2012). Orange peel essential oil is widely used as an aromatic compound in food, cosmetics, or aromatherapy in biomedical applications. Recently, numerous studies have been conducted on the effect of adding orange peel essential oil, which has biological effects such as antibacterial, antioxidant (Gürsoy, 2010), and anti-inflammatory in farm animals. The active substances in citrus peel essential oil can improve the digestive system of poultry so that it has the potential to replace the usage of antibiotics as growth promoters in livestock that have been prohibited by the government because they cause resistance in livestock and residues in consumers.

Essential oil of Siamese orange peel contains 76.77% of limonene, 8.24% of γ-Terpinene, 3.65% of Methyl chavicol, 3.01% of linalool, 2.38% of myrcene, 1.08% of α-Pinene, and several others (Gursoy et al., 2010). Essential oils have antibacterial properties as done by Frassinetti (2011), and Li (2019) showed a significant reduction in the bacterial growth rate by causing cell wall lysis, leakage of intracellular material, and as a result, cell death occurred. Aydin and Alçiçek (2018) showed that the addition of orange peel essential oil can increase carcass yield, meat, carcass weight, breast, back, wings, heart weight, and abdominal fat significantly ($P<0.05$) as the dose of essential oil added to the feed increases.

Several methods can be used to extract the essential oil, by microwave-assisted supercritical fluid extraction (Rajput et al., 2023), sonication extraction (Sandhu et al., 2021), soxhlet extraction (Karne et al., 2023), steam-distillation extraction (Dao et al., 2021), cold-pressing extraction (Aydeniz-Guneser et al., 2020), microwave-assisted extraction (Martínez-Abad, 2020), and hydro-distillation (Bustamante et al., 2016). Hydro-distillation stands out as the most prevalent method for extracting essential oils due to its simplicity in equipment and ease of use. Ultrasonics is widely recognized as one of the most popular methods used to increase mass transfer phenomena. The growing interest in applying sonochemistry to product extraction comes from its advantages, including reduced extraction time, energy savings, increased yield, and more. One important reason is that ultrasonic macerated essential oil starts at a much earlier time than the conventional one, which is due to the acceleration effect of ultrasonic by the ability to break the cell wall (Zhang, 2019). Up to now, sonication has primarily been utilized for obtaining both lipophilic and hydrophilic plant extracts, thereby circumventing problems inherent in conventional extraction methods. These problems include low productivity, time-consuming processing, and the formation of by-products due to the decomposition of thermolabile or vulnerable to hydrolysis (Smigielski, 2014).

In this study, the method utilized for the extraction of orange peel essential oil is ultrasound-assisted maceration followed by hydro-distillation. The study aims to observe the optimum yield with different maceration techniques and solvent ratios, determine the antimicrobial activity against various bacteria, and analyze the chemical components of citrus essential oil.

2. Material and Methods

2.1. Experimental details and treatments

The method used in this study was a distillation experiment with different methods at the Laboratory of Animal Feed Industry, Faculty of Animal Science, Universitas Brawijaya. This study consisted of two parameters, different maceration methods and the solvent ratio. Each treatment was repeated six times, resulting in 36 experimental units. Each experiment used the same sample of 150 grams of *Citrus nobilis* peel in fresh condition. The distillation process used a hydro-distillator with a heating mantle as the heater. The amount of essential oil or yield produced was observed.

The treatments used were two factors, the first factor was the maceration methods, namely the conventional maceration method (maceration without ultrasonic for 20 minutes) (CMCEO) and maceration with sonication (maceration with 40kHz ultrasonic waves for 20 minutes) (UMCEO). The second factor was the orange peel to a solvent ratio (w v^{-1}) with the ratio of orange peel and solvent 1:2, 1:4, and 1:6. The best results of the fresh orange peel to solvent ratio in each maceration method were then tested for antibacterial activity against *Lactobacillus casei and Escherichia coli* and tested quantitatively for chemical contents by using GC-MS analysis.

2.2. Experimental material

Siamese orange peels were washed after being collected from the waste of orange juice from eateries or restaurants in Malang City, East Java. The peels are separated from the other waste of the orange juice such as orange segments, seeds, and grains. The flavedo was collected using a peeler to separate it from the albedo part then cut into smaller pieces using a knife. The pieces of orange peel were further reduced in size by using a blender with distilled water solvent. For the maceration experiment, orange peel (150 grams) was macerated with distilled water (aqua dest) in a ratio of 1:2, 1:4, and 1:6 (w v^{-1}). The blender used was a Miyako BL-301 PL Blender with a capacity of 1 liter for 2-5 minutes until the texture changes to a slightly rough slurry. This was done to reduce the particle size so that it would make it easier for essential oils to be lifted along with the vapor of distilled water to the condenser.

2.3. Maceration

The orange peel juice was macerated by two methods, using conventional maceration (left in a round bottom flask at room temperature) for 20 minutes, and maceration with ultrasonic waves with an EECOO Digital Ultrasonic Cleaner 120W sonicator of 3L capacity with a frequency of 40kHz for 20 minutes. In the previous study, the use of ultrasonic maceration for 20 minutes as an initial step before distillation of essential oil from the thyme leaves resulted in a significant rise in oil yield, reaching approximately 9% compared to oil obtained by conventional maceration (Kowalski et al., 2009).

2.4. Distillation

Distillation was carried out by the hydro-distillation method using a heating mantle as a heat source. The duration of distillation was 60 minutes as suggested by Zheljazkov et al. (2013), except for the solvent ratio 1:2 was modified and shortened for only 45 minutes. The orange peel solution was not filled more than half the capacity of the boiling flask so that there would be no burst of orange peel that could potentially contaminate the distillation product. The steam temperature is regularly monitored to verify that it remains within safe parameters. The optimal steam temperature for distillation is 96 ºC (Sikdar et al., 2016). The distillation results will form two layers, the essential oil layer above, and hydrosol below as they have different molecular weights so additional treatment is needed to collect essential oil out of hydrosol by using a separator funnel.

2.5. Yield percentage

The yield of essential oil is calculated using the formula:

$$yield\ (\%) = \frac{the\ essential\ oil\ obtained\ (ml)}{weight\ of\ fresh\ citrul\ peel\ (gram)} x\ 100 \quad (1)$$

2.6. GC-MS analysis

Gas chromatography-mass spectrometry (GC–MS) analyses were conducted in the Faculty of Agricultural Technology, Universitas Brawijaya, and performed using an Agilent 6890 with selective mass detector Agilent 5973. Gas chromatography-mass spectrometry (GC–MS) analyses instrument was performed by using QP-2020NX/Shimadzu with Helium as a carrier gas, adjusted column temperature at 70.0 °C and injection temperature 310.0 °C, using spit less injection mode and flow control mode 19.8 kPa pressure. The chemical constituents were identified by their percentages of area.

2.7. Inhibition zone test

The antibacterial assay of the essential oils was analyzed using the pour plate method in Healthy Animal Clinic. First, a bacterial suspension of 106 CFU ml^{-1} and 12 g of MacConkey Agar media were prepared, each dissolved in 180 ml of distilled water and then boiled and sterilized for 15 minutes. MacConkey Agar that has been diluted, put into a sterile Petri dish of as much as 15 ml and put *Escherichia coli* bacteria into a petri dish containing MacConkey Agar each 1 ml and let it sit until it solidified. The blank disk was smeared with essential oil and then put on to the solidified agar medium. The petri dish was then wrapped with plastic wrap tightly and incubated at 37 °C for 24 hours. Observations were made by looking at the clear zone around the treatment hole on the dish which showed the area of bacterial growth that was inhibited by the treatment. The vertical and horizontal clear zone diameters were measured using a caliper.

2.8. Statistical analysis

The data obtained in this study were analyzed using Analysis of Variance (ANOVA) on Randomized Group Design with the assistance of Microsoft Excel. Duncan's Multiple Range Test (DMRT) will be conducted if significant or highly significant data are obtained.

3. Results

3.1. Effect of maceration method and solvent ratio on yield of citrus peel essential oil

The results of the effect of the maceration method and solvent ratio on the yield of citrus peel essential oil are presented in Figure 1.

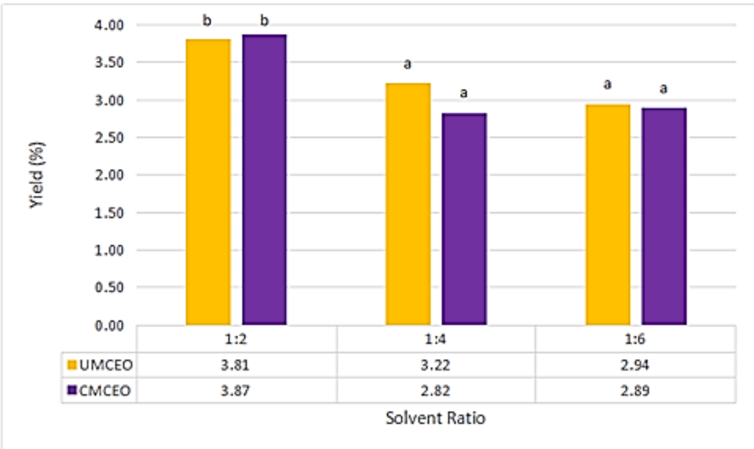

Figure 1. Effect of different maceration methods and solvent ratios on the yield of essential oil. Different letter markings indicate significant differences ($P < 0.05$).

The statistical analysis showed that the ultrasonic maceration method did not give a significant difference (P>0.05) effect on the yield of citrus peel essential oil. However, the yield of orange peel essential oil was greater when the ultrasonic maceration method was applied at solvent ratios of 1:4 and 1:6, but slightly lower for solvent ratio of 1:2. The statistical analysis showed that the solvent ratio had a highly significant effect (P<0.01) on the yield of citrus essential oil. The solvent ratio of 1:2 in UMCEO (3.81%) and CMCEO (3.87%) showed the highest yield as compared to other solvent ratios. The higher the solvent ratio, the lower the yield of both UMCEO and CMCEO. In CMCEO, the yields of essential oil in solvent ratios 1:4 were (2.82%) and 1:6 (2.89%), while UMCEO were 2.94 and 2.89, respectively.

3.2. Effect of maceration method on the chemical compound of citrus peel essential oil

The results of the effect of the maceration method on the chemical composition of citrus peel essential oil are presented in Table 1.

Table 1. Chemical composition of *Citrus nobilis* essential oil

No	Chemical Compound	% Area	
		CMCEO[1]	UMCEO[2]
1	Cycloheptene, 5-ethylidene-1-methyl-	48.50	44.09
2	D-limonene	26.76	36.02
3	Pentane, 2,3-dimethyl-	7.62	7.12
4	beta-Myrcene	3.17	2.46
5	Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-	2.72	2.07
6	Linalool	1.94	1.63
7	Pentacosane	0.71	0.16
8	Decanal	0.53	0.43
9	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-	0.45	0.31
10	2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-	0.43	0.24
11	Cyclohexanemethanol, 4-ethenyl-.alpha.,.alpha.,4-trimethyl-3-(1-methylethenyl)-	0.34	0.01
12	1-Cyclohexene-1-carboxaldehyde, 4-(1-methylethenyl)-	0.25	0.10
13	1-Octanol	0.18	0.15
14	Cyclohexene,4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-,(3R-trans)-	0.12	0.08
15	Terpinen-4-ol	0.09	0.06
16	Undecanal	0.09	0.06
17	Dodecanal	0.08	0.07
18	Copaene	0.07	0.03
19	Limonene oxide, trans-	0.06	0.05
20	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethylidene)-,(E,E)-	0.05	0.04
21	Heneicosane	0.03	0.05
22	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	3.14	-
23	Tetrapentacontane, 1,54-dibromo-	0.84	-
24	1-Dodecanol, 2-octyl-	0.61	-
25	alpha.-Terpineol	0.37	-
26	3-Methylhexacosane	0.32	-
27	Tetracosane	0.26	-
28	trans-Carveol	0.09	-
29	2,6-Dodecadien-1-al	0.09	-
30	Cyclohexene, 1-methyl-4-(1-methylethylidene)	0.06	-
31	Bicyclo[3.1.0]hexan-3-ol, 4-methyl-1-(1-methylethyl)-	0.01	-
32	alpha.-Pinene	-	1.70
33	1-Pentene, 2-methyl-	-	1.05
34	Tetracontane	-	0.28
35	L-.alpha.-Terpineol	-	0.27
36	Tetratetracontane	-	0.24
37	2,6-Dimethyl-1,3,5,7-octatetraene, E,E-	-	0.18
38	Germacrene D	-	0.10

Table 1. Chemical composition of *Citrus nobilis* essential oil (continued)

No	Chemical Compound	% Area	
		CMCEO[1]	UMCEO[2]
39	Pentatriacontane	-	0.08
40	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)-	-	0.08
41	6-Octenal, 3,7-dimethyl-, (R)-	-	0.06
42	Cyclohexene, 3-methyl-6-(1-methylethylidene)	-	0.06
43	2,6-Octadienal, 3,7-dimethyl-, (E)-	-	0.05
44	Neral	-	0.05
45	(-)-Carvone	-	0.05
46	Carveol	-	0.05
47	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethenyl)-,trans-	-	0.04
48	7-Tetradecenal, (Z)-	-	0.04
49	Humulene	-	0.04
50	1-Isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydronaphthalene	-	0.03
51	gamma.-Elemene	-	0.03
52	Octacosane, 2-methyl-	-	0.03
53	Geranyl acetate	-	0.03
54	2-Dodecenal, (E)-	-	0.03
55	.gamma.-Terpinene	-	0.02
56	Caryophyllene	-	0.02
57	2-Decenal, (E)-	-	0.02
58	(E)-4,8-Dimethylnona-1,3,7-triene	-	0.02
59	(1S,2E,6E,10R)-3,7,11,11-Tetramethylbicyclo[8.1.0]undeca-2.6-diene	-	0.02
60	Azulene,1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-	-	0.02
61	Heneicosane	-	0.02
62	(E)-Tetradec-2-enal	-	0.01
63	trans-Geranic acid methyl ester	-	0.01
64	Pentadecane	-	0.01
65	2,3-Diazabicyclo[2.2.1]hept-2-ene, 4-methyl-1-(pent-4-en-1-yl)-	-	0.01
66	Dotriacontane, 1-iodo-	-	0.01
67	6-Octen-1-ol, 3,7-dimethyl-, acetate	-	0.01
68	2-Hexenal, (E)-	-	0.01
69	alpha.-Guaiene	-	0.00
Total compounds		100	100

[1]CMCEO : conventional-macerated citrus essential oil; [2]UMCEO : ultrasonic-macerated citrus essential oil.

The composition of the essential oil from *Citrus nobilis* was analyzed using GC–MS. Across the two maceration methods, a total of 69 compounds were identified in the essential oil. Based on the result in Table 1. citrus essential oil pre-macerated with ultrasonication showed a higher variety of compounds contained than the one without ultrasonication. There were 21 common compounds were found in both UMCEO and CMCEO. The UMCEO actually contained 59 compounds, but 38 compounds were not found in CMCEO, meanwhile, CMCEO contained 31 compounds, but 10 compounds were different and not found in UMCEO. In addition, the Mann-Whitney test toward 21 common compounds found in both maceration methods showed no significant effect indicating that the quantities found were similar (Asymp. Sig. 0.263). The dominant compounds from both methods were also similar. For example, the highest compound is cycloheptene compounds found at 48.5% in CMCEO and 44.09% in UMCEO. The second highest proportion is D-limonene and found 26.76% in CMCEO and 36.02% in UMCEO.

3.3. Effect of maceration method on the antibacterial activity of citrus peel essential oil

The results of the effect of the maceration method on the antibacterial activity of citrus peel essential oil are presented in Figure 2.

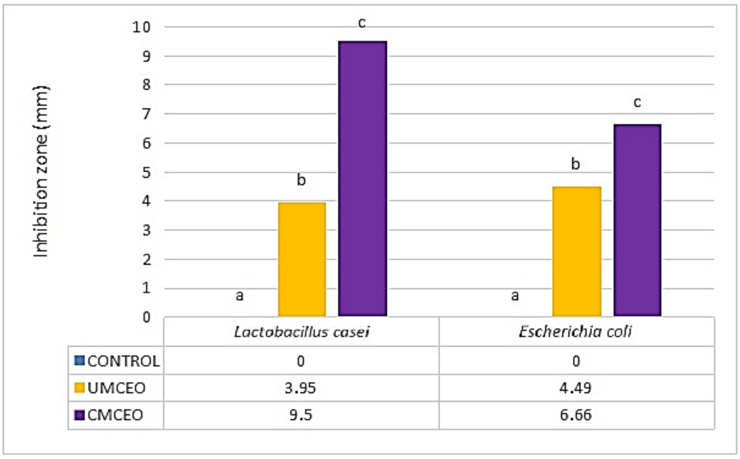

Figure 2. Effect of different maceration methods on the antibacterial activity of essential oil. Different letter markings indicate significant differences ($P < 0.01$).

The statistical analysis showed that the maceration method gave a highly significant difference effect on the inhibition zone diameter against gram-positive and gram-negative bacteria (P<0.01). CMCEO has the highest inhibition zone effect compared to the UMCEO and the control treatment. The inhibition zone diameter of CMCEO against *Lactobacillus casei* was 9.5 mm and was categorized as strong inhibition. Meanwhile, the diameter of the inhibition zone of CMCEO against *Escherichia coli* was 6.66 mm, UMCEO against *Lactobacillus casei* was 3.95 mm, and *Escherichia coli* was 4.49 mm. CMCEO has stronger antibacterial activity against *Lactobacillus casei* and *Escherichia coli* compared to UMCEO.

4. Discussion

4.1. The effect of solvent ratio

The distilled water at different proportions has been used as a medium for carrying the essential oil components as it is vaporized and condensed during the distillation process. The water proportion will impact the extraction rate, yield, and composition of the resulting essential oil. The essential oil yield tends to decrease with increased water addition, likely due to the solubility of polar components in the essential oil. Ngo (2022) showed that the quantity of water added significantly impacted the duration of the essential oil extraction process but had minimal influence on the yield. It was found in this experiment that using 200 ml of water gives a higher essential oil yield compared to 400 ml and 600 ml of solvent. The increase in solvent volume is not aligned with the increase in essential oil yield. The effectiveness of essential oil extraction depends on many factors including the material or sample and solvent ratio. Zhang (2019) discovered that the rose essential oil yielded optimally at a solvent-to-solid ratio of 1:5. Beyond this ratio, there was no notable change in the yield. This indicated that a solvent ratio of 1:2 mixed with citrus peel is sufficient for distilling essential oil from the plant-based material which proves that the ability of the solvent to carry the essential oil through distillation could reach the optimum ratio, and the yield will gradually decrease as the solvent volume is being increased. However, the optimal volume of water needed for hydro-distillation depends on several factors, the specific characteristics of the plant material, the method of the extraction, and the distillation apparatus used.

In this study, the three solvent ratio treatments were given at different times for the essential oil distillation process. In the solvent ratio of 1:2 conducted with a distillation time of only 45 minutes, due to the solvent was almost completely removed by evaporation. Meanwhile, in the solvent ratio of 1:4 and 1:6, the distilled water used 60 minutes to completely evaporate. When the volume of water surpasses a specific limit then the process of extracting essential oil becomes ineffective, time-consuming, and results in added energy expenses because more energy is needed for the heating process.

(Ngo, 2022). In addition, extending the extraction time over a certain limit may not further increase the yield of essential oil and could potentially affect the product quality (Tran, 2020). Therefore, it is necessary to determine the suitable solvent ratio for achieving the most appropriate extraction time and economical extraction technique.

4.2. The effect of maceration method

The yield of orange peel essential oil was higher when the ultrasonic maceration method was applied at solvent ratios of 1:4 and 1:6, but slightly lower at a solvent ratio of 1:2. Ultrasonic waves can break down the cell walls of the orange peel making it easier for the solvent to come into direct contact with the essential oil to be extracted. This aligns with Zhang (2019), who states that the mechanical action of ultrasonic waves expedites the release of essential oil by rupturing plant cell walls, improving mass transfer, and aiding solvent penetration into the cell contents. The increased extraction efficiency achieved through sonication is attributed to the particular action of ultrasound. As ultrasound spreads in the slurry, it generates pressure waves and cavitation, causing plant cell disruption and subsequent release of cell contents.

Moreover, as indicated by Smigielski (2014), who conducted essential oil extraction from waste carrot seeds using ultrasonic maceration, sonication speeds up water absorption, causes plant material to swell and consequently augments the cell walls porosity. This process facilitates mass transfer and enhances the extractive value (EV). Tiwari (2015) further emphasizes that ultrasonic extraction is based on the acoustic cavitation principle which can damage the cell walls of the plant matrix and thereby supports better results compared to the conventional maceration techniques. This phenomenon involves the formation, expansion, and implosive collapse of microbubbles in liquids subjected to ultrasound. A range of physical and chemical phenomena contribute to the ultrasonic effect including agitation, vibration, shock waves, pressure, micro jets, shear forces, compression and rarefaction, acoustic streaming, cavitation, and radical formation. Therefore, ultrasonic treatment plays a crucial role in not only increasing the yield but also accelerating the essential oil extraction process.

4.3. Chemical compound of citrus peel essential oil

The data indicated that ultrasonication could release a greater variety of compounds bound in the citrus peel. Ultrasonic waves create cavitation bubbles in the solvent, leading to the formation of micro jets and shock waves upon collapse so that they could release more compounds. This is in accordance with Ohl (2015) who stated that the agitation in ultrasonication enhances mass transfer, allowing for more efficient extraction of a wider variety of compounds from the plant material. The high-intensity ultrasonic waves might disrupt cell walls and membranes more effectively than conventional maceration, releasing a broader spectrum of compounds present in the plant material. Ultrasonic waves might help in the penetration of the solvent into the plant material, ensuring better contact between the solvent and substrate, which leads to more efficient extraction of various compounds (Shen, 2023).

The source of the oils allows them to be categorized among herbal medicines. However, owing to their elevated levels of active components, their impact is notably more potent compared to herbal extracts. The composition of these oils comprises a diverse range of chemical compounds, often reaching several hundred substances with varied structures and properties. The diversity of these substances is so high that, in certain instances, the precise composition of a specific oil cannot be determined even with the most advanced apparatus available (Kowalski, 2014).

In the previous studies, it was found that cycloheptane is the most dominant chemical compound in essential oil. Citrus is mostly dominated by d-limonene. Citrus peel compounds may undergo biodegradation in certain environmental contexts. Factors related to sample preparation, such as drying, grinding, and storage conditions of the citrus, might affect the composition of the essential oils extracted. The production of citrus oil from the fruits derived from citrus waste often involves the use of large quantities of the fruits. These fruits might undergo storage for a period of time before processing, leading to the potential inclusion of some rotten fruits alongside the ripe ones. It has been noted that storing limes with rotten fruits notably impacts the volatile compounds present in such fruits affecting the quality of their essential oils (Afolayan, 2008). Koprivnjak (2002) added that there is an enzymatic process involving hydroperoxide lyase, alcohol dehydrogenase, and alcohol acetyltransferase. It is possible for both the quantity and composition of volatile compounds to be changed, as well as the sensory properties of the oils. Thus, regulating the temperature and duration of the pre-distillation storage phase is necessary.

Cycloheptane is a cyclic hydrocarbon with a seven-membered ring structure. Cycloheptane appears as a colorless oily liquid, insoluble in water, and less dense than water. Cycloheptane is not commonly associated with citrus essential oils, which typically contain compounds such as limonene, citral, and linalool. Cycloheptane itself is a relatively simple hydrocarbon and is not known to possess significant biological properties on its own. It may have some indirect effects or uses in certain applications. Cycloheptane might interact with other compounds present in a mixture to enhance or modify their biological activities. In some cases, it could act as a solvent or carrier for bioactive compounds with antimicrobial or antioxidant properties, facilitating their delivery or enhancing their efficacy. Indeed, the effectiveness of medicinal plant extracts comes from the interaction of multiple compounds, which may exhibit synergistic, additive, or antagonistic effects (Vaou, 2022).

Cycloheptane is one of the irregular monoterpenes that occur in essential oils. Irregular monoterpenes are also present and can be classified into two categories. The first category encompasses troponoids or substituted cycloheptane monoterpenes. These compounds are thought to emerge from the expansion of the p-menthane skeleton, leading to a seven-membered ring structure, along with oxygenation of the side chain(s) (Carson, 2011).

4.4. Antibacterial activity of citrus peel essential oil

As shown in Table 1. CMCEO contains slightly higher levels of cycloheptane and resulted in stronger inhibition zones for both gram-positive and negative bacteria. Cycloheptane itself is not typically recognized for its antimicrobial properties. However, cycloheptane, a hydrocarbon might synergistically augment the antimicrobial effectiveness of citrus essential oils when combined with other compounds present in them. Scientific investigations into the antibacterial properties of constituents in essential oils have consistently indicated that aldehydes and phenolics generally exhibit a greater antibacterial efficacy compared to other types of constituents (Ultee, 2022), the nonphenolic alcohols often exhibit moderate antibacterial activity, while oxides and hydrocarbons tend to have the lowest antibacterial activity. These hydrocarbon compounds have been shown to permeabilize cellular membranes, leading to membrane swelling. This process inhibits respiratory enzymes, ultimately causing a partial disruption of both the pH gradient and electrical potential, which are essential components of the cellular energy system (Griffin, 1999).

Essential oils are complex mixtures containing numerous chemical compounds, and the combined effects of these compounds may surpass the cumulative impact of their individual components. Cycloheptane, when present alongside other antimicrobial compounds such as D-limonene, could enhance the overall effectiveness of the essential oil against bacteria. One might hypothesize that the effectiveness of extracted volatile oils correlates with the composition of their functional groups, the potential for synergistic interactions among constituents, the ratios in which these oils are present, and the chemical structure of their constituent components (Eslahi, 2017). In this framework, it has been proposed that alterations in the structures of essential oils result in varied activities. Incremental levels of cycloheptane within essential oils may enhance the solubility of other antimicrobial agents, thereby augmenting their bioavailability and efficacy against bacterial pathogens. Cycloheptane might function as a solvent or carrier for these agents, aiding their ingress into bacterial cells and eliciting more potent inhibitory actions. Indeed, the overall efficacy of medicinal plant extracts, arising from the concerted action of multiple compounds, may demonstrate synergistic, additive, or antagonistic effects (Vaou, 2022).

Cycloheptane does not possess well-documented biological activities such as antimicrobial or antioxidant properties. It is a simple hydrocarbon with a relatively inert nature categorized as cycloalkane. However, Abdallah (2018) stated that Numerous derivatives of cycloalkane and ring systems are recognized for their significance as antimetabolites in various biochemical processes.

Based on Figure 2. The result has shown that CMCEO could inhibit *Lactobacillus casei* Gram-positive bacteria exhibit greater resistance compared to *Escherichia coli* (a gram-negative bacterium), potentially attributable to the presence of an outer membrane enveloping the cell walls of gram-negative bacteria. This membrane restricts the diffusion of hydrophobic substances, such as oils, across the lipopolysaccharide layer of the wall.

The higher content of d-limonene contained in UMCEO compared to CMCEO showed a shorter diameter of inhibition zone in both types of bacteria. This is in accordance with Bozkurt (2017) The chemical composition and antibacterial properties of several citrus species were analyzed, and it was not within the scope of the current study to directly observe a proportional relationship between the quantity of d-limonene and its antimicrobial efficacy.

The essential oil contain several forms and derivatives of hexane and cyclohexane like bicyclo[3.1.0] hexane, 4-methylene-1-(1-methyl), cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-me, cyclohexanemethanol, 4-ethenyl-.alpha.,.alpha, 1-cyclohexene-1-carboxaldehyde, 4-(1-methyl, cyclohexene, 4-ethenyl-4-methyl-3-(1-methyle, cyclohexene, 1-methyl-4-(1-methylethylidene), bicyclo[3.1.0]hexan-3-ol, 4-methyl-1-(1-meth, cyclohexene, 3-methyl-6- (1-methylethylidene), and 2-cyclohexen-1-ol, 1-methyl-4-(1-methylethen). The results of the antibacterial test showed that the essential oil obtained from the upper parts of the plant during the fruiting phase, characterized by pentylcyclohexa-1,3-diene as the predominant compound, demonstrated the most significant efficacy against *Staphylococcus aureus*, *Enterococcus faecium*, and *Escherichia coli*, with minimum inhibitory concentration (MIC) values of 2.0 mg/ml, 8.0 mg/ml, and 4.0 mg/ml, respectively (Miran, 2018). Antibacterial or other biological properties are strongly influenced by the content of the overall compounds contained in it, including those with a small percentage of content in essential oil. No correlation was detected between the antibacterial efficacy of these citrus essential oils (CEO) and the concentration of limonene, suggesting that the antibacterial properties of both essential oils were probably influenced by the presence of minor compounds (Raspo, 2020).

Conclusion

This research explored various factors influencing the yield percentage and antimicrobial efficacy of citrus essential oil obtained through hydro-distillation, including solvent ratio, maceration techniques, and sample storage prior to distillation. It was determined that using a material-to-solvent ratio of 1:2 was the most effective method for citrus essential oil distillation, yielding the highest percentage. All essential oils, whether pre-treated with ultrasonic treatment or not, exhibited inhibitory effects against the two bacteria tested, with conventionally pre-macerated citrus essential oils demonstrating the highest effectiveness against *Escherichia coli* and *Lactobacillus casei*. The predominant chemical compounds in both citrus essential oils were cycloheptane (44.09% - 48.5%) and d-limonene (26.76% - 36.02%). Further investigation into the specific mechanisms underlying the antimicrobial properties of citrus essential oil, particularly the role of cycloheptane, would be beneficial.

Ethical Statement

Ethical approval is not required for this study because the experimental method used in this study was *in vitro* analysis by testing the microbial effect of extracted citrus peel essential oil without involving animal testing subjects.

Conflict of Interest

All authors declare no conflict of interest.

Author Contributions

Conception and design of the study: Fadillah and Dr. Widodo
Acquisition of data: Fadillah
Analysis and/or interpretation of data: Dr. Djunaidi and Dr. Widodo
Drafting the manuscript: Fadillah and Dr. Widodo
Critical review/revision: Dr. Djunaidi

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