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Antifungal Activity of Siamese Citrus (*Citrus nobilis L.*) Essential Oil against the Pathogen of *Blendok* (Diplodia) Disease by *Lasiodiplodia theobromae* Fungus

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Abstract: Siamese citrus (*C. nobilis L.*) is a type of citrus fruit that is most widely cultivated in Bangli Regency, Bali Province, Indonesia. The main disease that attacks citrus plants is blendok, which is caused by the fungus *L. theobromae*. Based on research Siamese citrus peel contains an essential oil that has potential as an antifungal. The aim of this study was to study the chemical content of the essential oil from Siamese citrus peel and its activity as an antifungal against *L. theobromae*. Essential oil was distilled using hydro-steam distillation and analyzed by means of GC-MS. Antifungal activity testing was carried out using the agar-well diffusion method with concentrations of 1%, 10%, 25%, 50%, 75%, and 100%. Dithane M45 6 g/l and DMSO 10% were used as positive and negative controls. The results showed that the Siamese citrus peel from Kintamani, Bangli Regency, Indonesia, contains an essential oil with the main chemical components D-limonene (57.26%), Beta-pinene (9.09%), and Beta-myrcene (4.03%), which has uses as an antifungal, additive, antitumor, asthma and allergy reliever, repellent, anti-inflammatory, antioxidant, anticancer, and antibacterial. An essential oil concentration of 25% to 100% can inhibit the growth of *L. theobromae*. The largest diameter of inhibition is shown at a concentration of 100% and the smallest diameter of inhibition is shown at a concentration of 25%.

Keywords: Antifungal, *C. nobilis L.*, essential oil, GC-MS, *Lasiodiplodia theobromae*,

1. Introduction

Orange (Citrus sp.) is an annual plant originating from Asia, especially China. Since hundreds of years ago, this plant has existed in Indonesia, both as a wild plant and as a plant in the yard (Pracaya, 2009). Of the various types

of oranges, the most dominant type and one that occupies an important position in the citrus world today is the Siamese citrus (*Citrus nobilis L.*). It is estimated that around 60% of the demand for citrus fruits is currently met by Siamese oranges. This orange has advantages and uniqueness, namely having a distinctive aroma, a sweet taste, and high productivity, as well as having high adaptability compared to other oranges.

Siamese citrus (*Citrus nobilis L.)* is a type of citrus fruit that is most widely cultivated in Kintamani, Bangli Regency, Bali, Indonesia. Siamese citrus production in Bangli Regency has continued to increase; in 2010 it was 668.286 tons, in 2011 it was 898.502 tons, in 2012 it was 1,096.55 tons, and it was 1,190.29 tons in 2013 (BPS Kabupaten Bangli, 2015). Siamese citrus productivity in Kintamani can reach 40-70 kg per tree per year (Supartha et al., 2015). Most people only use the flesh of the orange for consumption, while the skin is just thrown away without considering the properties contained therein. Siamese citrus peel contains an essential oil that has important values for human life, one of which is being antifungal (Rana et al., 2021).

Lasiodiplodia theobrome (*L. theobrome*) (synonym: *Botryodiplodia theobromae*) is a fungal pathogen that attacks various plantation, horticultural and food crop commodities in tropical and subtropical regions (Sandra et al., 2021). This pathogen is opportunistic in that it causes diseases by invading through wounds or necrotic tissue, especially in fleshy or woody plant organs. In citrus plants, the fungus *L. theobromae*can cause the Diplodia disease or commonly called *blendok* (Singarsa, 2015). A common control used to control the fungus is using fungicides. Considering that fungicides have a negative impact on the environment, an alternative control of diseases caused by this fungus is needed and that is by using an essential oil from Siamese citrus peel (*C. nobilis L*).

Based on the descriptions above, this research was carried out to determine the composition of compounds contained in the essential oil of Siamese citrus peel and its activity as an antifungal against *L. theobromae*. Despite the recognized value of Siamese citrus peel essential oil in traditional applications, systematic scientific investigations into its antifungal properties, specifically against *L. theobromae*, are limited. Previous studies have predominantly focused on the broad antibacterial and antifungal effects of citrus oils without isolating their specific impacts on the pathogens affecting citrus plants in tropical regions. Therefore, this study aims to fill this gap by detailing the chemical composition of Siamese citrus peel essential oil and quantifying its antifungal activity against *L. theobromae*, the causative agent of the blendok disease. By employing hydro-steam distillation and advanced GC-MS analysis, this research not only aims to validate traditional uses but also explores the potential of this oil as a sustainable, environmentally friendly alternative to synthetic fungicides in agricultural practices in Indonesia and globally. This approach represents a novel contribution to the field, potentially setting a precedent for future agricultural and pharmacological applications of citrus peel essential oils.

2. Methodology

2.1 Research Location

The main ingredient used was Siamese citrus peel (*C. nobilis L.*) obtained from Madam Suka Garden, Belancan Village, Kintamani District, Bangli Regency, Bali Province, Indonesia. Madam Suka Garden is a location that sells ornamental plants, cacti and succulents. Including terrariums, souvenirs, gifts and home decoration. The coordinates of Madam Suka Garden are at 8° 17’ 2.4” east longitude and 115° 18’ 4.3” south latitude. Figure 1 shows sample location (Madam Suka Garden).

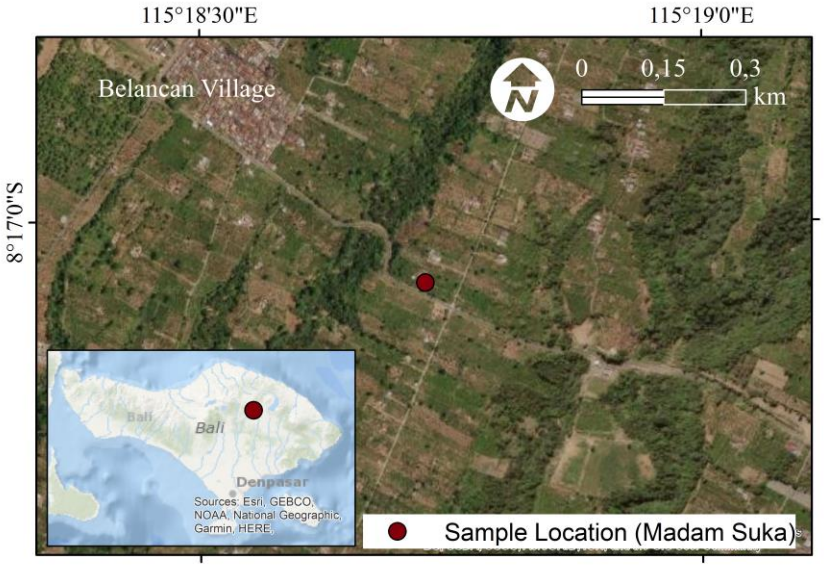

Figure 1 Sample Location

2.2 Material and Tools

L. theobromae fungus was isolated from citrus stems with Diplodia disease symptoms. The materials for this research included PDA (Potato Dextrose Agar) media, 70% alcohol, Dithane M45, Dimethylsulfoxide (DMSO), aluminum foil, and plastic wrap. PDA (Potato Dextrose Agar) is a common medium for growing fungi in the laboratory because it has a low pH (pH 4.5 to 5.6) so it inhibits the growth of bacteria that require a neutral environment with a pH of 7.0, and an optimum temperature for growth between 25-30 °C (Aisyah, et al.,, 2022). Dithane M45 is a contact (non-systemic) and protective fungicide that prevents diseases by inhibiting the development of fungal spores on plant parts (Ismail, et al., 2018). Dimethylsulfoxide (DMSO) is a chemical that dissolves many organic and inorganic substance. Higher concentrations of DMSO has been shown to inhibit the growth of several fungal species (Randhawa, 2006).

The tools used in this study included autoclaves, gas chromatography-mass spectrophotometry (GC-MS), laminar air flow cabinets, test tubes, petri dishes, Erlenmeyer flasks, oil and water separator flasks, jars, glass distillation pipes, Cimarec stirrers, statives and clamps, analytical balance, electric stove, beaker glass, measuring cup, sprayer, ball pipette, dropper pipette, micro pipette, tweezers, ose needle, cork borer, ruler, label paper, and camera.

2.3 Methodology

This research was conducted from March to December 2022 at the Bali-BRIN "Eka Karya" Botanical Garden Laboratory for the essential oil distillation, the Agricultural Biotechnology Laboratory, Faculty of Agriculture, Udayana University for the antifungal activity testing, and the Forensic Criminal Investigation Laboratory of the Polresta Denpasar (Denpasar City Police) for the analysis of essential oil chemical compounds. This research was conducted by laboratory experimental testing. The sample taken was Siamese citrus peel (*C. nobilis L.*) which was distilled using the hydro-distillation method for 3 hours. The essential oil obtained was then analyzed for its chemical compounds using the Gas Chromatography-Mass Spectrometry (GC-MS) method. Fungal isolates *L. theobromae* were isolated from citrus plant stems with Diplodia disease symptoms, then identified morphologically according to the identification key (Barnett & Hunter, 1988).

The essential oil was then tested for its antifungal activity against *L. theobromae* using the agar-well diffusion method. The samples used in testing the antifungal activity totaled 24 samples which were divided into 8 treatment groups, namely the control group (+) 6 g/L concentration of Dithane M45, the control (-) 10% concentration of DMSO, and Siamese citrus peel essential oil with concentration variations of 1%, 10% , 25%, 50%, 75%, and 100%. These were repeated in triplicate in the same way. After that, the petri dishes were incubated for 2 days at room temperature and then observations and measurements were made. The data from the inhibition zone measurements were averaged and categorized for their antifungal inhibition strength based on the classification by Davis & Stout (1971) and Ouchari (2019). Davis and Stout (1971) classified the inhibition zone diameter of antifungal responses for inhibitory activity are categorized into four categories; if the inhibition zone diameter is greater than 20 mm, the category is very strong; if it is between 11-20 mm, the category is strong; if it is between 5-10 mm, the category is medium; and if it is less than 5 mm, the category is weak (Nurshadrina et al., 2021).

3. Results

3.1 Isolation of Siamese Citrus Peel Essential Oil by Hydro Steam Distillation

Isolation of Siamese citrus peel essential oil by hydro-steam distillation method produced an essential oil which has a characteristic of a clear yellow color and a distinctive aroma of Siamese citrus orange peel. The results of the I to III distillation yields with a distillation time of 3 hours were 0.21%, 0.19%, and 0.08%, which were relatively small. Table 1 shows the results of hydro-steam distillation of siamese citrus peel (*C. nobilis L.*)

Table 1. Results of Hydro-steam Distillation of Siamese Citrus Peel (*C. nobilis L.*)

Distillation	Fruit weight (kg)	Peel weight (kg)	Distillation time (hours)	Oil volume (mm)	Yield (%)
I	5.6	1.3	3	2.8	0.21
II	10.4	2.5	3	4.85	0.19
III	6.4	2.1	3	1.8	0.08

Note: Each distillery uses different raw materials

3.2 Results of the Compounds in Siamese Citrus Peel Essential Oil Extract

The results of the GC-MS analysis of Siamese citrus peel essential oil (C. nobilis) identified 32 peaks in the chromatogram having a quality of ≥90 with different retention times for each peak. Each peak chromatogram

shows the compounds contained in the Siamese citrus peel. The compound data taken in this study were compound data that had a quality of ≥90. According to Sutar et al. (2013) a quality value of >90% indicates that the detected compound has a similar fragmentation pattern to the compound in the database. Of the 32 peaks, 48 types of compounds were identified and 41 of them have known uses, which can be seen in Figure 1 and Table 2.

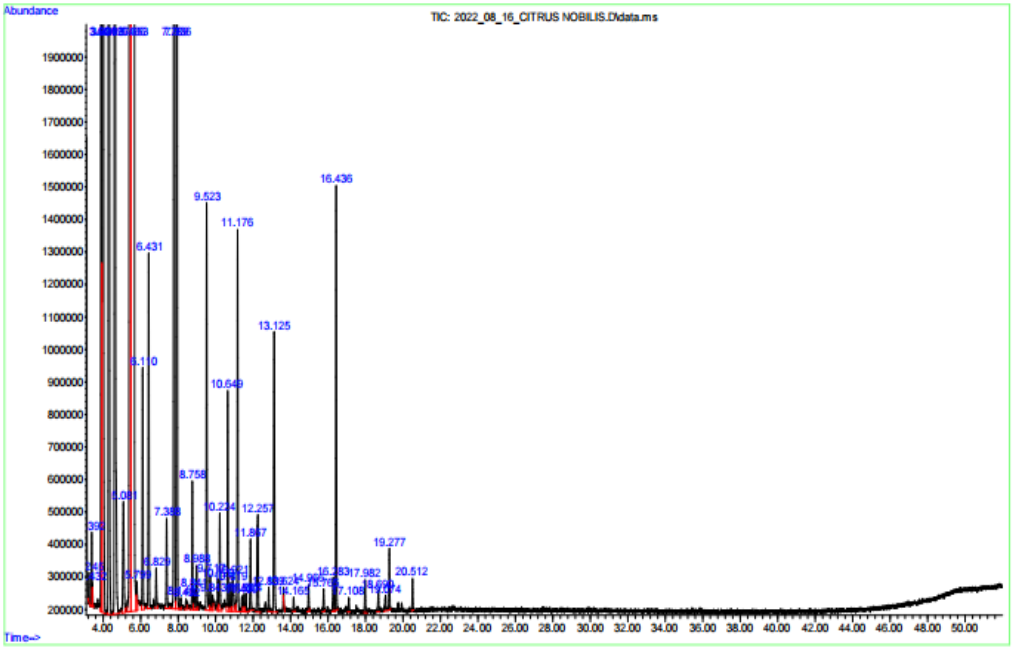

Figure 1. Siamese Citrus Peel (*C. nobilis L.*) GC-MS Chromatogram Results

Table 2. Uses of the Compounds of Siamese Citrus Peel Essential Oil

No.	Use	Compound Name
1.	Antioxidant	Beta-phellandrene; Beta-pinene; Octanal; Alpha-terpinene; D-limonene; Limonene; Gamma-terpinene; Terpinolene; Decanal; Citronellol; Cyclohexene, 1-methyl-4-(-methylethylidene)-; Beta-elemene; and Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-
2.	Anticancer	D-limonene; L-.alpha.-terpineol; Geranyl propionate; and Beta-elemene
3.	Insecticide	Chlorfenapyr; 1,4,7,- Cyclododecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-; and Germacrene D
4.	Repellent	Beta-pinene; Alpha-terpinene; Beta-ocimene; and Citronellal
5.	Larvacide	Terpine-4-ol; and Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1 methylethyl)-,(3R-trans)-
6.	Fumigant	D-carvone
7.	Attractant	1,3,6-octatriene,3,7-dimethyl-, Z; and 2,6-dimethyl-1,3,5,7-octatetraene
8.	Anti-inflammatory	Beta-mycrene; D-limonene; limonene; Gamma-terpinene; Terpinolene; D-carvone; and Cyclohexene, 1-methyl-4-(1-methylethylidene)-
9.	Antiviral	Limonene and Linalool
10.	Antitumor	D-limonene
11.	Asthma and allergy reliever	D-limonene

No.	Use	Compound Name
12.	Antimicrobial	Sabinene; Linalool; Terpine-4-ol; Decanal; and (-)- Perillaldehyde
13.	Antifungal	Beta-pinene; Alpha-terpinene; D-limonene; Limonene; Gamma-terpinene; Terpinolene; Linalool; Nonanal; Citronellal; Citronellol; Perillaldehyde; Cyclohexene, 1-methyl-4-(1-methylethylidene)-; and Beta-elemene
14.	Antibacterial	Sabinene; Beta-phellandrene; Octanal; D-limonene; Limonene; Beta-ocimene; Gamma-terpinene; Linalool; 1,3,8-p-Menthatriene; 1,3-dibromo-5,5-dimethylhydantoin; Citronellol; D-carvone; Perillaldehyde; Humulene; and Cyclohexane,1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-
15.	Pheromone	Germacrene A and Germacrene B
16.	Additive	Camphene; D-limonene; Limonene; (-)- carvone; and Beta-phellandrene
17.	Preservative	Limonene

3.3 Isolation and Identification of *L. theobromae* Fungus

Figure 2 shows the Fungus Morphology of *L. theobromae.* The results of macroscopic observations at 1-3 days after inoculation show that the isolates that grew had white *mycelium*, then turned white to gray in 5 days after inoculation and got darker with the increasing age of the isolates (Figure 2a). Microscopically, the hyphae were insulated and hyaline and then turned brown (Figure 2b). *Chlamydospores* formed at the tips of the isolate hyphae on PDA media (Figure 2c). Young conidia were oval in shape, not insulated and clustered (Figure 2d), while mature conidia were oval in shape, insulated and ungrouped (Figure 2e).

Based on the results of research conducted macroscopically and microscopically, it was shown that the fungus grown on PDA media was *L. theobromae*. According to research by Febbiyanti et al. (2017), colonies of *L. theobromae* are grayish white on the first day of culture and continue to turn black as the colony ages. Barnett & Hunter (1999) described the fungus *L.theobromae* as having a peculiarity which is marked by conidia that are hyaline and not insulated when young, while mature conidia were dark and had two cells.

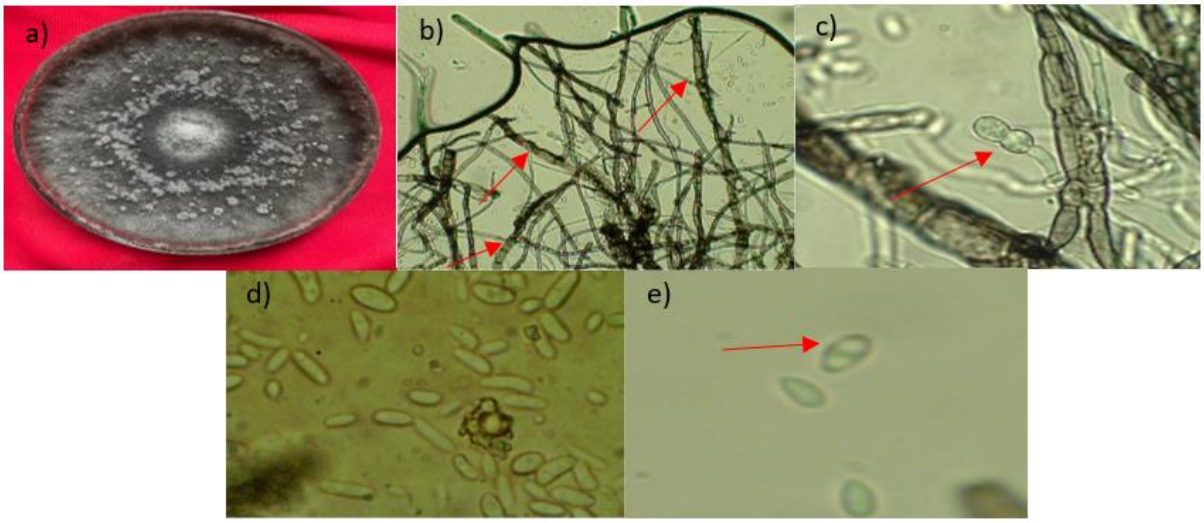

Figure 2. Fungus Morphology of *L. theobromae*

3.4 Antifungal Activity Testing of Siamese Citrus Peel Essential Oil Against *L. theobromae*

Based on the results of the research (Figure 4.), the essential oil of Siamese citrus peel (*C. nobilis L*) has been shown to have potential antifungal activity because it was able to inhibit the growth of the fungus *L. theobromae* in vitro on PDA media after being incubated at room temperature for 2 days. Inhibition zones in the essential oil group were formed starting from concentration variations of 25%, 50%, 75%, 100% with an average diameter of inhibition zone formed for each concentration, namely 1.16 mm, 2.08 mm, 2.67 mm, and 3.5mm. The highest inhibitory effect in the essential oil group was seen at a concentration of 100% (3.5 mm) compared to variations in other essential oil concentrations. The smallest concentration in the essential oil group that was able to inhibit the growth of *L. theobromae* was found at a concentration of 25% essential oil (1.16 mm).

Whereas the variations in essential oil concentrations of 1% and 10% did not provide inhibition because there was no visible clear zone. The treatment of the positive control group from Dithane M45 6g/L formed a radical

inhibition zone of 17.4 mm and the negative control from 10% DMSO had quite good results, in that it did not form an inhibition zone on research objects, which indicated that the control (-) used as a solvent had no effect on the antifungal activity test. This can be seen in Figure 3, Table 3 and Figure 4.

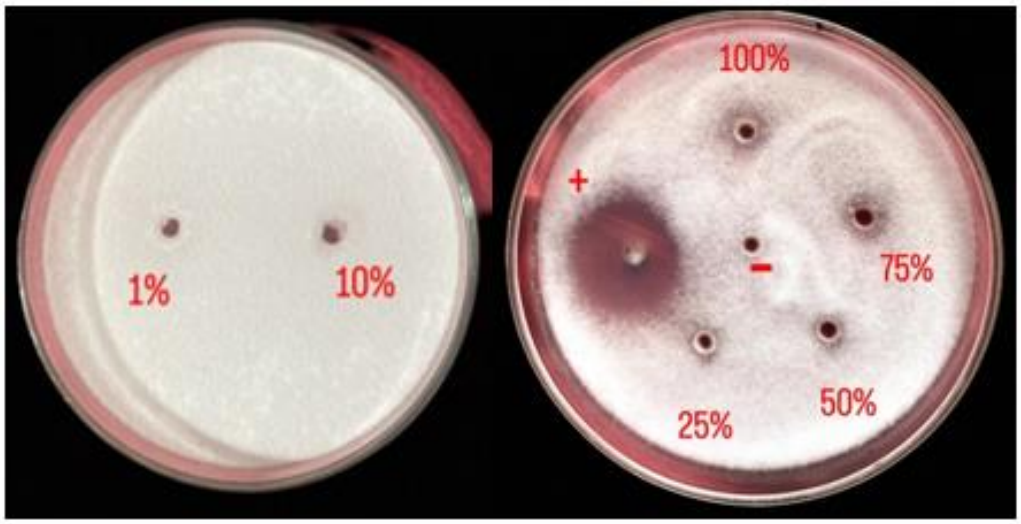

Figure 3. Antifungal Activity Test Results

Table 3. Data on Antifungal Activity Test Results

No.	Concentration	Inhibition Zone Diameter (Mm)			
		P1	P2	P3	Avg
1	1%	0	0	0	0
2	10%	0	0	0	0
3	25%	1	0.5	2	1.16
4	50%	2	1.5	2.75	2.08
5	75%	3	1.75	3.25	2.67
6	100%	4.5	1.75	4.25	3.5
7	Control (+) Dithane M45 6 g/l	20	19.75	12.5	17.41
8	Control (-) DMSO 10%	0	0	0	0

Note:
The diameter of the inhibition zone has been reduced by the diameter of the well (5 mm)
P1 = Repetition 1; P2 = Repetition 2; \P3 = Repetition 3

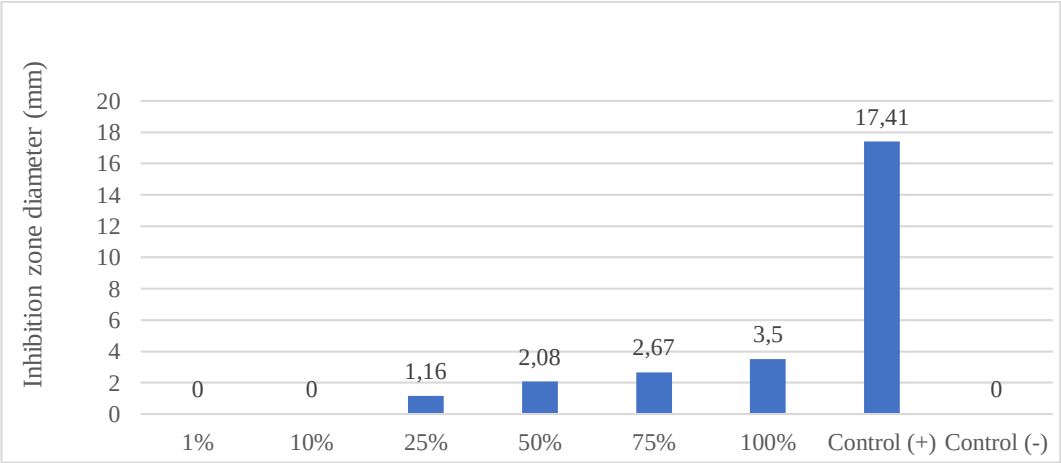

Figure 4. Average Diameter of Inhibition Zone from Table 3

4. Discussion

4.1 Isolation of Siamese Citrus Peel Essential Oil by Hydro Steam Distillation

The highest percentage of oil yield based on Figure 1 and Table 2 was produced in the first distillation (0.21%) compared to the second and third distillation of 0.19% and 0.08%. Yield is the ratio between the mass of oil produced and the mass of the raw material distilled. The difference in yield at each distillation can be affected by the contact between the solvent and the raw material.

The more the raw materials are used, the more oil content is obtained, but if the amount of materials exceeds the maximum capacity of a tool, the distillation results tend to decrease. This is because the solvent used is not able to diffuse and push the essential oil to the surface optimally, so that there is still much essential oil left in the raw material tissue (Cahyati, Kurniasih, and Khery, 2016). Refining time also affects the yield of oil. A distillation time of 3 hours was a relatively short time to distill. In a longer distillation, the contact between the water vapor and the material will be longer, and the amount of oil carried by the water vapor will also increase, so that the yield of oil obtained can be higher (Hidayati, 2012).

The third distillation obtained the lowest yield, namely 0.08%, compared to the first and second distillation of 0.21% and 0.19%. This might happen because the samples used in the third distillation were taken when it was raining, causing the orange peel to be wetter than the samples in the first and second distillations, which were taken when it was not raining, which affected the oil yield. Drier skin can produce a greater yield than wet skin, because the pores on dry skin are larger and of course it makes it easier for the oil stored under the surface of the orange peel to evaporate (Muhtadin et al., 2013).

4.2 Analysis of the Compounds in Siamese Citrus Peel Essential Oil Extract

Based on the results of the chromatogram that had been analyzed, the main component of the Siamese citrus peel essential oil is D-limonene (57.26%), followed by beta-pinene (9.09%), and beta-myrcene (4.03%). D-limonene with an Area Under Curve (AUC) value of 57.26% at a retention time of 5.653 can be used as a food additive, a beverage ingredient, and ingredients for fragrance and soap. In addition, it can also be used as an antitumor, asthma and allergy reliever, anti-inflammatory, antioxidant, anticancer, antifungal, and antibacterial (Anandakumar et al., 2021; D'allesio et al., 2013; Miler et al., 2011; Yu et al., 2022; Yao et al., 2017). At a retention time of 5.653, limonene was also found with an AUC of 57.26% which had uses as an anti-inflammatory, antibacterial, antiviral, antioxidant, fragrance, preservative, antifungal, and food additive (Fadilah et al., 2021; Hendrawan, 2018; Rana et al., 2021; Handayani, 2021). Furthermore, beta-pinene had a retention time of 3.991 and an AUC value of 9.09% which can be used as an antioxidant, antifungal, and repellent (Aprilia et al., 2021; Lely, 2019; Cahyati, Kurniasih, and Khery, 2016). Meanwhile, the beta-myrcene compound had a retention time of 4.309 and an AUC value of 4.03%, which is useful as an anti-inflammatory (Fadilah et al., 2021).

Based on Table 2, it can be seen in Figure 2 that, after grouping based on its use, the essential oil of Siamese citrus peel (C. nobilis) contains a lot of compounds that have a potential use as an antibacterial agent (as many as 15/41 compounds), followed the compounds that have a potential use as an antifungal agent (as many as 13/41 compounds), and 13/41 compounds have a potential use as antioxidants.

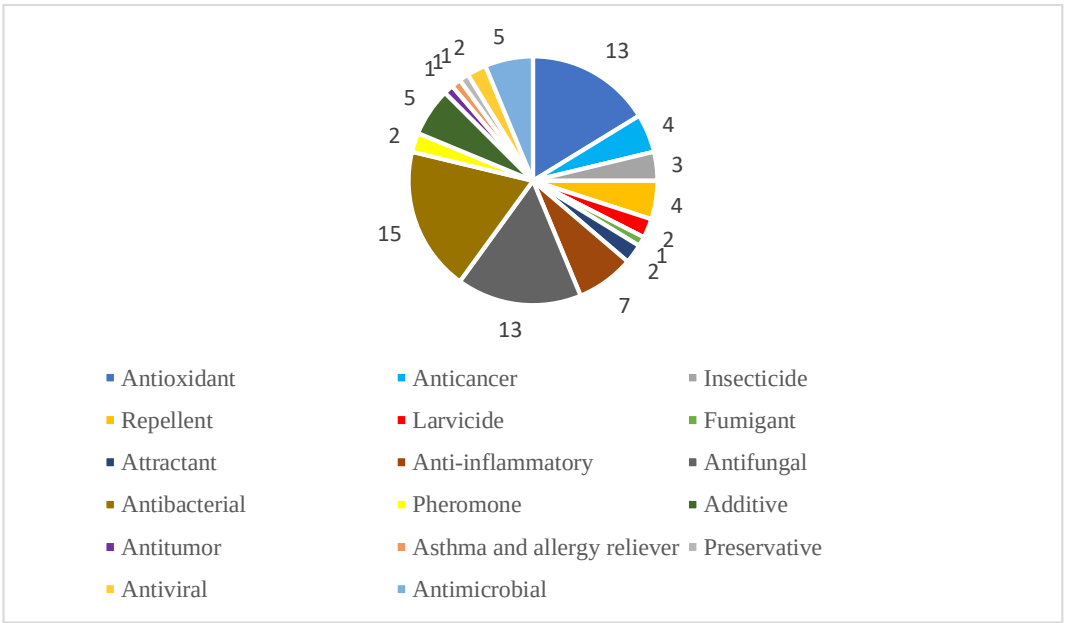

Figure 2. Uses of the Compounds of Peel Essential Oil

4.3 Analysis of Antifungal Activity Testing of Siamese Citrus Peel Essential Oil against *L. theobromae*

According to Davis & Stout (1971), the essential oil group with concentrations of 100%, 75%, 50%, and 25% was categorized as having weak antifungal activity (<5 mm), while the control group (+) Dithane M45 6g/mL was categorized as having strong antifungal activity (11-20 mm). These results indicate that the essential oil of Siamese citrus peel (C. nobilis) cannot be used as an effective botanical fungicide to control *L. theobromae*. The weak inhibition of Siamese citrus peel (C. nobilis) essential oil may be due to the volatile nature of the essential oil at room temperature (Ariyani, 2020; Wibawa et al., 2019), so that the content in the oil which had the potential as an antifungal is reduced. Reducing the concentration of the active antifungal compounds results in weak inhibition produced.

The antifungal activity possessed by Siamese citrus peel essential oil that could inhibit the growth of the fungus *L. theobromae* at concentrations of 25%, 50%, 75%, and 100% was suspected because in the essential oil of Siamese citrus peel (C. nobilis) from Kintamani, Bangli there are bioactive compounds that function as antifungals. Based on the results of the GC-MS analysis conducted, the essential oil of Siamese citrus peel (C. nobilis) from Kintamani, Bangli was dominated by terpenoid group compounds such as D-limonene (57.26%), beta-pinene (9.09%), and linalool (2.96%). Compounds in this group are fungistatic which can inhibit the action of certain enzymes resulting in disruption of the metabolism of fungal cells, so that the process of growth of fungal hyphae becomes inhibited, leading to disruption of the process of fragmentation of hyphae causing fungal cells to not be able to reproduce within a certain time. Terpenoid compounds could also reduce the mycelium so that shortening occurs at the ends of the hyphae. Branching also occurs a lot in an unusual way, so that in the end an abnormal mycelial growth is formed (Istikomah, Alami, and Purwani, 2015).

Yu Hao et al. (2022) in his study stated that D-limonene compounds could interfere with ion transfer and inhibit the formation of ATP in cell membranes caused by a decrease in cell potential, as well as an increase in cell permeability and integrity, thus disrupting fungal cell growth and eventually causing these cells to die. Beta-pinene (9.09%) is a compound that has antifungal properties. These compounds could affect changes in the colony and morphology of fungal cells by reducing their enzymatic activity and producing toxic effects on the structure of the fungal membrane. In fungal cells, beta-pinene can also disrupt cell integrity, inhibit the process of respiration and transport of H+ and K+ ions in fungal cells, and increase their permeability (Prabajati, Hernawan, and Hendarto, 2017). Furthermore, there were linalool (2.96%) and nonanal (1.25%) compounds which are also reported to inhibit the growth of fungi. Dias et al. (2017) reported that linalool from basil oil showed the best antifungal activity against C. tropicalis, followed by C. albicans and C. krusei. Linalool compounds inhibit fungi by interfering with cell wall biosynthesis and increasing the ionic permeability of fungal cell membranes. Meanwhile, nonanal can inhibit the growth of the fungus *Penicillium cyclopium* by disrupting the integrity of the fungal cell membrane, causing leakage of cell constituents and potassium ions, and triggering an increase in total lipid levels, pH, extracellular and membrane permeability (Zhang et al., 2017).

In conclusion, while this research has established the potent antifungal properties of the Citrus nobilis L. essential oil against *L. theobromae*, further studies are necessary to deepen our understanding of its full potential. Future research should focus on the in vivo application of the essential oil to assess its efficacy and safety on actual citrus crops affected by the blendok disease. Additionally, exploring the synergistic effects of this essential oil with other natural antifungals could yield more powerful and broad-spectrum biofungicides. Investigating the oil's mode of action at the molecular level would also provide valuable insights, potentially leading to the development of novel fungicides. Lastly, a comprehensive economic analysis would be crucial to evaluate the feasibility of large-scale production and integration of Siamese citrus essential oil into agricultural disease management practices.

5. Conclusions

Based on the results of the research that has been conducted, it can be concluded that the peel of the Siamese citrus (C. nobilis) from Kintamani, Bangli contains an essential oil with the main chemical components D-limonene (57.26%), beta-pinene (9.09%), and beta-myrcene (4.03%), which has uses as an antifungal, additive, antitumor, asthma and allergy reliever, repellent, anti-inflammatory, antioxidant, anticancer, and antibacterial. The essential oil of Siamese citrus peel (C. nobilis) from Kintamani, Bangli has relatively weak antifungal activity against L. theobromae at a concentration of 25 to 100%. The largest diameter of inhibition in the essential oil group was shown at a 100% concentration and the smallest diameter of inhibition at a 25% concentration.

Author Contributions

Conceptualization, I.G.P.W.; methodology, K.D.P, I.G.P.W., I.P.A.H.W.; validation, S.Y.H.; formal analysis, K.D.P, I.G.P.W., I.P.A.H.W., I.K.S., I.N.W., T.A.P..; data curation, K.D.P .; writing—original draft preparation, K.D.P .; writing—review and editing, K.D.P, I.G.P.W., I.P.A.H.W., I.K.S., I.N.W., T.A.P.; visualization, K.D.P.,

T.A.P.; supervision, I.G.P.W., I.N.W.. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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