Study of the Temperature and Molarity Ratio Effects in Geraniol Esterification and Testing Its Antibacterial Activity

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Abstract: This study modifies the molarity ratio of geraniol to isobutyric acid (1:1, 1:1.1, and 1:1.3) and temperature (RT, 40°C, 60°C, and 80°C) in the synthesis of geranyl isobutyrate ester using 5% (w/w) NaOH as a base catalyst. The antimicrobial activity was tested against both gram-positive and gram-negative bacteria. Ester products were separated and purified using column chromatography, and identified using Fourier Transform Infrared Spectroscopy (FTIR) and Gas Chromatography-Mass Spectrometry (GCMS). The antimicrobial activity was assessed using the disk diffusion method. The results showed that the esterification product with a 1:1.1 molar ratio at 80°C had the best separation based on thin-layer chromatography (TLC) and antimicrobial properties. GCMS analysis of the purified product revealed five compound peaks with geranyl isobutyrate at R_T = 13.376 minutes (2.77%). FTIR confirmed the presence of C=O ester carbonyl groups at 1717.82 cm^{-1} and C-O groups at 1080.37 cm^{-1}. Antimicrobial tests showed inhibition zones on gram-positive bacteria of 18.33±2.62 mm for *Bacillus subtilis* and 15.67±0.47 mm for *Staphylococcus aureus*, and against gram-negative bacteria of 10.67±0.47 mm for *Pseudomonas aeruginosa* and 16.67±2.36 mm for *Escherichia coli.*

1 INTRODUCTION

The development of essential oils in Indonesia is progressing rapidly due to their diverse benefits in the pharmaceutical and medicinal fields. Essential oils, commonly derived from plants, include lemongrass oil, which is currently popular with global consumption reaching around 2,000-2,500 tons per year (Direktorat Jendral Perkebunan, 2020). The diverse benefits of lemongrass oil are its antiseptic properties and medicinal uses, make it a highly valuable commodity. Lemongrass oil is rich in beneficial compounds such as citronellal, citronellol, and geraniol.

In Indonesia, there is a significant demand for geraniol derived from lemongrass essential oil, especially in the pharmaceutical and perfume industries. Furthermore, geraniol exhibits a variety of beneficial medical properties, including antioxidant, anti-inflammatory, antimicrobial, antitumor, hepatoprotective, cardioprotective, and neuroprotective effects (Pavan et al., 2018). Some researchs have shown that geraniol has strong antimicrobial activity due to its lipophilic properties, which allow it to bind to the lipid membranes of microorganisms, demonstrating effectiveness against various bacteria, including *Candida* and *Staphylococcus*(Lira et al., 2020). Nevertheless, geraniol exports from Indonesia decreased from 11,789.3 million USD in 2019 to 8,251.1 million USD in 2020 (Badan Pusat Statsitik, 2021). Therefore, efforts are needed to increase the market value of geraniol by discovering its derivatives to enhance bioactivity and application potential through derivatization into geranyl esters.

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Numerous studies have shown that derivatives of geraniol, such as geranyl formate, geranyl acetate, geranyl butyrate, and geranyl isobutyrate, possess antibacterial properties. For instance, a study indicated that organophilic bentonite incorporated with geranyl acetate exhibited antibacterial activity against *Staphylococcus aureus* and *Salmonella typhimurium* (Capelezzo et al., 2023). Additionally, geraniol derivatives such as geranyl butanoate have shown potential as anticancer agents (Widiyarti et al., 2019). On the other hand, geranyl isobutyrate has been proven to have superior antimicrobial properties compared to other geraniol derivatives, as demonstrated in studies testing antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*. The antibacterial effect of geranyl isobutyrate against *E. coli* was the strongest among the 12 compounds tested (Zhaoshuang et al., 2016). However, further research is needed due to the limited studies examining the antimicrobial activity of geranyl isobutyrate, and most existing studies have not included *B. subtilis* as one of the bacteria. Moreover, additional research is required regarding the synthesis method of geranyl isobutyrate, as its quantity derived from plants is usually limited, such as in *Conyza incana*, which yields only about 2.5% (Zabin, 2018). The synthesis of geranyl isobutyrate generally involves an esterification reaction between alcohol and carboxylic acid compounds to form the desired ester.

Conceptually, the esterification reaction requires high temperatures or heating above 55°C (Tolvanen et al., 2014). Additionally, catalysts play a role in accelerating the reaction. Acid catalysts are generally used, but recent research has shown that the use of base catalysts can be an effective alternative. Base catalysts are known to increase reaction efficiency, be environmentally friendly, and reduce water formation (Khan et al., 2021). Although the use of base catalysts for esterification is still in the research phase, previous studies have successfully produced geraniol derivatives using a base catalyst of NaOH. These derivatives include geranyl butyrate, geranyl caproate, and geranyl caprylate, with yields of 66.94%, 76.72%, and 54.92%, respectively (Widiyarti et al., 2019). This finding indicates the potential use of heterogeneous base catalysts in the esterification process, which has not been widely reported.

Based on the above issues, this research aims to synthesize geranyl isobutyrate using sodium hydroxide (NaOH) as a base catalyst to enhance the antibacterial activity of geraniol derivatives. The geraniol esters will be identified using TLC, FTIR, and GCMS. The geranyl isobutyrate product is then antibacterial activity tested against *B. subtilis, S. aureus, E. coli, P. aeruginosa.* These four bacteria are bacterial models that are usually used for antibacterial activity tests for natural products screening and their derivative compounds that have the potential to be antibacterial.

2 RESEARCH METHODS

2.1 Synthesis of Geranyl Isobutyrate (GI)

The esterification of GI based on 1:1 molarity ratio is carried out by reacting 20 mmol (3.086 g) of geraniol, which is placed into a 250 mL round-bottom flask and mixed with NaOH catalyst at 5% of the weight of geraniol. After 4 hours, 20 mmol (1.762 g) of isobutyric acid is added. The solution mixture is reacted at room temperature (RT) for 24 hours. The same procedure is repeated for each molar variation of isobutyric acid for 1:1.1 (22 mmol, 1.939 g) and 1:1.3 (26 mmol, 2.290 g).

Next, the synthesis product is extracted using a 100 mL separatory funnel with a solvent solution of ethyl acetate and distilled water (1:1). The mixture is shaken and allowed to stand until two phases form, and the organic phase is collected. This process is performed in triplicate. The collected organic phase is evaporated using a rotary evaporator until crude GI ester product is obtained. The yield of the crude ester is calculated using the following formula:

$$\%\,Yield = \frac{molarity\ of\ GI}{molariy\ of\ geraniol\ (G)}\ x100\% \quad (1)$$

The product ester is then purified using column chromatography with eluent *n*-hexane, ethyl acetate, and methanol eluted gradually. The separated compound is identified using TLC, FTIR, and GCMS.

This procedure is repeated for each molar variation at different temperatures of 40^0C, 60°C and 80°C.

2.2 Purification and Characterization of GI Ester

The purification of GI crude ester is carried out using gradient column chromatography with *n*-hexane and ethyl acetate as solvents. The purified product is subsequently analyzed by GCMS Agilent

Tecnologies 7890C and FTIR Shimadzu prestige 21 using KBr pellets.

2.3 Antibacterial Test Using Disc Diffusion Method

The bacterial culture of *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 8739, and *P. aeruginosa* ATCC 9027 was prepared by swiped of 1 ose of bacteria culture from stock culture on the sterile Nutrient Agar (NA) agar slant. The culture is stored in an incubator at 37°C for 24 hours. Bacterial culture on rejuvenated agar slants was then added with 10 mL of 0.9% NaCl solution, shaken until all colonies on the surface are loose and suspended in NaCl solution 0.9%. The bacteria as much as 100 µl were put into the petri dish, and then an amount of 10 mL of NA is added, and allow it to solidify. Afterwards, the discs (approximately 6 mm in diameter) are placed on the NA. Finally, 5 µl of the sample at a desired concentration is added on top of each disc. For each sample, a triplicate test was carried out. The petri dishes are then incubated under suitable conditions at 37^0C for 18-24 hours, then the resulting diameters of inhibition zone was measured (Yusmaniar et al., 2017; Hossain, 2024).

3 RESULTS AND DISCUSSION

3.1 Synthesis of GI

Esters are produced through the reaction of carboxylic acids with alcohols, forming water as a by-product and replacing the hydroxyl group (-OH) with an alkoxy group (-OR) (Melvine et al., 2021). The esterification of geraniol with isobutyric acid, aided by the base catalyst NaOH, results in the formation of geranyl isobutyrate (GI). The presence of NaOH is used to increase the reaction rate without undergoing chemical change and to lower the activation energy (Ea) (Setyaningsih et al., 2017). The synthesis and mechanism reaction of GI as shown in Figure 1 and Figure 2.

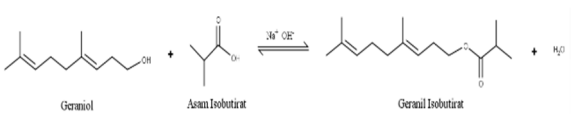

Figure 1: Synthesis Reaction of GI.

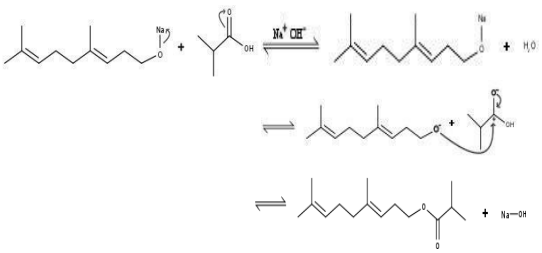

Figure 2: Esterification Reaction Mechanism of GI.

The hydroxyl group (-OH) reacts with the catalyst, resulting in proton abstraction and the formation of an alkoxide ion. This nucleophilic alkoxide ion attacks the carbon atom of the carbonyl group, leading to resonance stabilization and the formation of GI ester. The mechanism occurs because the alcohol acts as a good nucleophile, making the reaction with carboxylic acid more effective in forming the geranyl ester. This aligns with previous research, which states that heterolytic cleavage occurs in the presence of the catalyst, followed by the alcohol attacking the catalyst, enhancing its nucleophilicity and forming an intermediate. Subsequently, this intermediate decomposes, eliminating the catalyst and forming the ester (Kohsaka et al., 2018). However, this reaction may produce side products if the alkoxide ion reacts with other compounds in the mixture.

The reaction product exhibits a pH of 5-6, indicating that it is non-corrosive. Liquid-liquid extraction is then performed to separate the organic phase from the aqueous phase (Figure 3). Ethyl acetate (EtOAc) is used as the solvent to ensure the efficient distribution of the geranyl ester in the organic phase. EtOAc is soluble in various compounds and has relatively low viscosity, facilitating separation (Schneider et al., 2021).

Figure 3: Liquid-liquid extraction separation of geranyl ester.

Subsequently, the crude ester as a yellowish-brown substance was produced by evaporating the extracted material as shown in Figure 4.

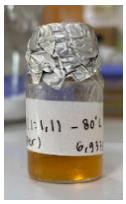

Figure 4: Crude GI product.

The percentage yields of GI crude esters were calculated based on the percentage molarity of GI divided by the molarity of starting material/reactan from geraniol.

The relatively high yield is attributed to the presence of impurities and geraniol that have not been completey synthesized into GI ester. The crude ester yield under various reaction conditions is presented in Table 1.

Table 1: Crude ester yield.

Sample	Weight (g)	Yield (%)
GI (1:1) – RT	3.480	77.28
GI (1:1,1) – RT	4.011	89.11
GI (1:1,3) – RT	4.208	93.53
GI (1:1) - 40°C	4.143	92.15
GI (1:1,1) - 40°C	3.472	77.10
GI (1:1,3) - 40°C	3.791	84.46
GI (1:1) - 60°C	3.433	76.43
GI (1:1,1) - 60°C	3.522	78.29
GI (1:1,3) - 60°C	4.035	89.46
GI (1:1) - 80°C	4.104	90.98
GI (1:1,1) - 80°C	4.248	94.78
GI (1:1,3) - 80°C	4.173	92.95

The highest yield of 94.78% was produced in the esterification of geraniol at a temperature of 80℃ and a reactant molarity ratio of 1:1.1.

Afterthat, esterification results were analyzed by TLC using silica gel plates (Kiesel gel 60F254 0.25 mm), with an eluent ratio of n-hexane to ethyl acetate (9:1) at λ = 254 nm. The results showed that at RT, the Retention factor (R_f) value for GI matches the R_f of geraniol of 0.73, indicating esterification has not occurred yet. However, there is a gradual decrease in the R_f value from 0.65 to 0.6 and finally to 0.55 at 80°C, suggesting that 80°C is the optimal temperature for forming the geranyl ester product.

Temperature is a crucial parameter that regulates the rate and extent of esterification. An increase in reaction temperature significantly impacts esterification, where higher temperatures lead to faster conversion. However, higher reaction temperatures risk increasing product darkness. It has been observed that even a small temperature difference of 10-20°C can significantly affect the reaction rate and product yield (Mazubert et al., 2014).

In the esterification process, the addition of excess reactants can drive the esterification reaction toward higher product yield, consistent with Le Chatelier's principle (Peris, 2021). Based on TLC also showed that (GI) with a concentration ratio of 1:1.1 has the best R_f value, while other concentrations show no significant change. Although the 1:1.3 concentration is higher than 1:1.1, the R_f value decreases because the 1:1.1 concentration achieves the optimal stoichiometric level. There is a threshold where further increases do not significantly enhance conversion and may even reduce it due to reaction mixture saturation and catalyst deactivation (Lade et al., 2014).

The rate of esterification is influenced by the structure of the carboxylic acid. Linear chain acids esterify more readily than branched acids due to steric hindrance. The presence of branched chains in the acid slows the reaction rate. Adding more chains to the acid structure further reduces the reaction rate. However, branched chain acids offer higher conversion rates than linear chain acids. Additionally, certain substituents can either accelerate or decelerate the reaction rate (Jin et al., 2012).

R_f value for synthesized compound of GI is 0.55, lower than R_f value of 0.73 from the starting material geraniol. This is allegedly because larger size and structure of GI than geraniol, less polar and causing more interaction with the stationary phase of silica gel.

Furthermore, antibacterial tests were conducted to determine the efficacy of the crude ester with the best results, as shown in Table 2.

Table 2: Antibacterial test results of crude GI extract.

Sample	Inhibition Zone			
	B. subtilus	*S. aureus*	*P. aeruginosa*	*E. coli*
GI(1:1,1) - 80°C	16.67 ±0.94	17.00 ±1.63	16.33 ±0.47	16.67± 1.25
GI(1:1) - 80°C	16.67 ±0.47	15.33±0 .47	16.33 ±1.70	16.00± 0.82
GI(1:1,3) - 80°C	16.33 ±0.47	17.00±0 .82	15.33 ±0.47	16.33± 1.25
GI(1:1,1) - RT	17.67 ±2.05	16.33±0 .94	16.00 ±0.82	15.33± 1.25
GI(1:1,1) - 40°C	16.33 ±0.94	16.67±0 .47	16.33 ±0.47	16.00± 1.63
GI(1:1,1) - 60°C	17.00 ±0.82	16.33±1 .25	16.67 ±1.70	16.00± 1.41
Geraniol	12.33 ±1.25	11.67±1 .25	11.33 ±0.47	10.00± 0.00

Antibacterial test results indicate that the crude ester with an isobutyric acid molar ratio of 1:1.1 at 80°C demonstrated the best antibacterial activity against both gram-positive and gram-negative bacteria. This condition proved to be more effective compared to other molarity and temperature variations. Therefore, the molar ratio of 1:1.1 at 80°C is selected as the optimal synthesis condition. This condition will be used for subsequent purification and characterization stages. This is in accordance with the yield produced, where the highest yield is produced under these conditions.

3.2 Purification and Characterization of GI by Using GCMS and FTIR

A total of 3500 mg of crude GI was purified using gradient column chromatography, yielding 19 fractions. This method utilized silica gel 60 (230-400 mesh) and a gradient solvent mixture of n-hexane and ethyl acetate. Fractions F6-F9 in *n*-hexane: ethyl acetate (9:1), and F10-13 in *n*-hexane ; ethyl acetate (8:2) were identified as geranyl isobutyrate, with yields of 24 mg (0.682%), and 28 mg (0.795%) respectively. The TLC of the purified ester as shown in Figure 5.

Figure 5: The TLC of purified GI.

Tailing was observed in the TLC of the purified product grom GI, likely due to the presence of impurities or other by-products in the sample. To confirm the presence of the compounds, further analysis was conducted using GCMS and FTIR.

GCMS analysis was performed to identify the components of the synthesis product based on mass spectra, area and molecular weight. GC chromatogram of pure GI showed 62 compound peaks. It is due to geraniol as starting material itself had 30 contaminant compounds with an area of 71.56% (Figure 6).

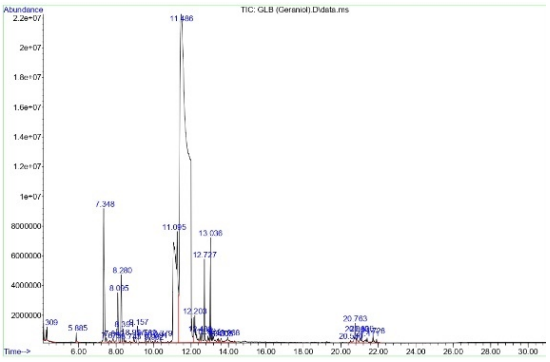

Figure 6: The chromatogram of geraniol

Table 3: Dominant peak in GCMS of GI ester.

No	Compound	R_t (minut1es)	Area (%)
1	3-Buten-2-ol	17.976	33.89
2	Cyclohexanecarboxylic Acid	18.177	5.75
3	3,7-dimethyl-2,6-octadienyl	13.376	2.77
4	Geraniol	11.409	4.50
5	Oxalic Acid	7.326	3.28

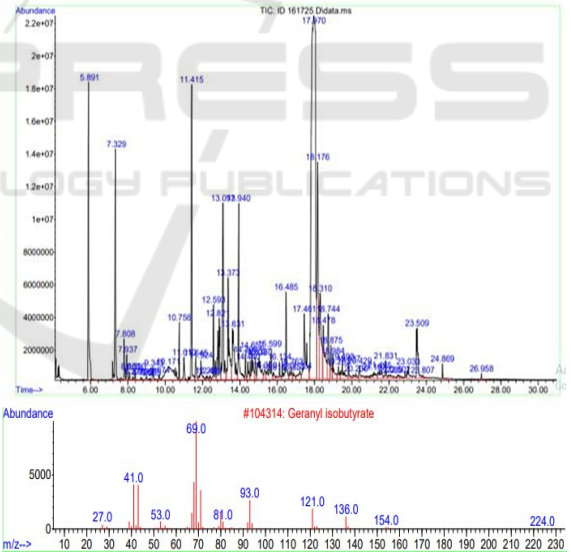

Figure 7: GC chromatogram of synthesis product.

The five dominant peaks of GI ester chromatogram presented in Table 3 and Figure 7. The retention time (R_T) of 13.376 minutes corresponds to 3,7-dimethyl-2,6-octadienyl or geranyl isobutyrate (GI) compound with the area percentage was 2.77%, as shown in Table 3 and Figure 7.

Based on the mass spectra in Figure 7, the geranyl isobutyrate compound has a molecular mass (M) of 224 with a base peak at m/z = 69. Other characteristic

peaks appear at m/z = 136 (M+-88), indicating the loss of isobutyric acid. Another important peak is at m/z = 93, formed from the loss of ethylene from m/z = 121, and the peak at m/z = 154 is suspected to be a fragment of the ester moiety.

FTIR analysis was conducted to determine the functional groups present in the ester product. The FTIR spectrum can be seen in Figure 8. The interpretation of the wavenumbers for GI is provided in Figure 8 and Table 4.

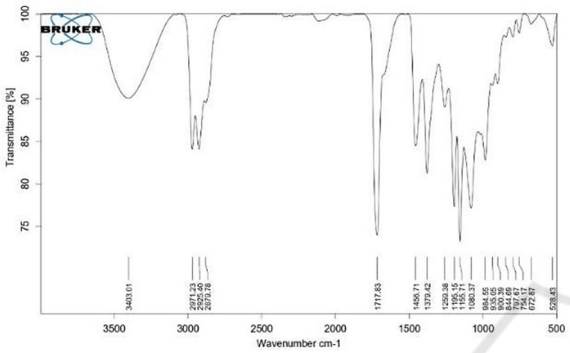

Figure 8: FTIR Spectra of GI.

Table 4: The IR Interpretation of GI.

Wavenumber (cm^{-1})	Functional Group
3403,01	O-H *stretch*
2925,40;2971,23;2879,78	C-H sp^3
1717,83	C=O
1456,707	-C=C- *bend*
1080,37	C-O

The FTIR results show several characteristic absorption bands. At a wavenumber of 1717.83 cm^{-1}, there is a significant absorption band corresponding to the carbonyl (C=O) stretching vibration of the ester. The presence of this group is further confirmed by the absorption at 1080.37 cm^{-1}, indicating the presence of the C-O group of the ester. Additionally, the absorption at 3403.01 cm^{-1} shows the presence of residu of unreacted geraniol. Wang et al. reported that geranyl esters have an IR peak around 1735.87 cm^{-1}, indicating the presence of a carbonyl (C=O) group, and a C- O absorption at 1175.32 cm^{-1} (Wang et al., 2019).

3.3 Antibacterial Test Using Diffusion Method

The diffusion method is carried out using the paper disk, with the bacterial count for sensitivity testing ranges from 10^5 to 10^8 CFU/mL. Paper disks containing antibiotics or samples are placed on a medium containing the microbes, then incubated and the results read based on the microbial inhibition around the disk (Yusmaniar et al., 2017). The basic principle of the method is the measurement of the diameter of the clear zone, which indicates the antibacterial compound's inhibition of bacterial growth in the test sample (Bhargav et al., 2016.).

GI is a monoterpenoid with good antimicrobial properties, and its results are optimized with proper incubation time. The incubation process is conducted for 18-24 hours as the bacteria are still in the exponential phase. Interaction with the hydrophobic structure of bacteria plays a key role in the antimicrobial effects of hydrocarbons. Bacteria are more sensitive during the exponential phase compared to the stationary phase. Several antimicrobial agents cause significant changes to the plasma membrane, resulting in total cell lysis. Although the activation of autolytic enzymes may be responsible for this effect, lysis can also be caused by the weakening of the cell wall and disruption of the cell membrane due to osmotic pressure, rather than specific action on the membrane (El Kolli et al., 2016).

The antibacterial test conducted on crude ester and pure GI produced at optimum esterification process at a 1:1.1 molarity ratio and 80°C temperature compared to reactant of geraniol (G). Tetracycline (TS), streptomycin (MS), and dimethyl sulfoxide (DMSO) were used as positive and negative controls, respectively, at a concentration of 16,000 ppm. This activity test was performed against gram-positive bacteria, namely *B. subtilis* and *S. aureus*, as well as gram-negative bacteria, *P. aeruginosa* and *E. coli.*

The results showed at Table 4. It can be seen that DMSO, as a negative control, exhibited no antimicrobial activity, with a consistent inhibition zone of 6 mm. This confirms that the observed antimicrobial activity is due to GI ester compound. Tetracycline and streptomycin were used as positive controls due to their broad-spectrum activity against various gram-positive and gram-negative bacteria and their use in both veterinary and human medical treatments (Araby et al., 2020). Comparatively, GI showed fairly good antibacterial activity, indicating potential as a therapeutic agent. Visualization of the inhibition zones in the antibacterial disk diffusion test is shown in Figure 9.

Table 5: The antibacterial activity of samples.

Sample	Inhibition Zone (mm)			
	B. subtilus	*S. aureus*	*P. aeruginosa*	*E. coli*
TS	23.67 ±0.47	30.00 ±0.00	18.00 ±0.00	20.00 ±0.00
SM	21.33 ±1.89	30.00 ±0.00	23.33 ±2.36	26.33 ±0.94
DMSO	6.00 ±0.00	6.00 ±0.00	6.00 ±0.00	6.00 ±0.00
Pure GI	18.33 ±2.62	15.67 ±0.47	10.67 ±0.47	16.67 ±2.36
Crude GI	16.67 ±0.94	17.00± 1.63	16.33 ±0.47	16.67 ±1.25
G	12.33 ±1.25	11.67± 1.25	11.33 ±0.47	10.00 ±0.00

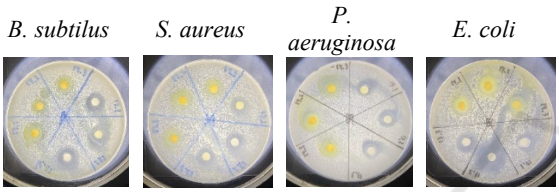

Figure 9: Inhibition zone of pure GI.

Additionally, Table 5 shows stronger antibacterial activity against gram-positive bacteria, with inhibition zones of 18.33 mm for *B. subtilis* and 15.67 mm for *S. aureus*. Based on these inhibition zones GI, is categorized as a strong antibacterial (Ullah & Ali, 2017). Zabin declared that essential oils with geranyl isobutyrate as the main product also exhibit good activity against gram-positive bacteria (Zabin, 2018). Previous studies have shown that the antibacterial effect of monoterpenoids is weaker against gram-negative bacteria due to their hydrophilic nature, which prevents the contact of hydrophobic monoterpenoid components with bacterial cells. In contrast, gram-positive bacteria can directly damage their cell membranes, leading to cell membrane rupture, inhibition of enzyme systems, and increased ion permeability (Lang, 2010). Pure GI showed a significant increase in inhibition zones compared to geraniol as starting material. However, there was a decrease in against *P. aeruginosa* and *S. aureus* when compared to crude GI (1:1.1). This may be due to the some compounds present in GI were separated during purification, which may contribute to antibacterial activity against *P. aeruginosa* and *S. aureus*. Additionally, *P. aeruginosa* is challenging to eradicate due to its biofilm-forming ability (Srivastava et al., 2021). Meanwhile, *S. aureus* is a major concern due to its high resistance levels (Zabin, 2018). Consequently, pure GI showed decreased antibacterial activity against these bacteria compared to crude GI, which contains more compounds with potential antibacterial activity against these bacteria.

5 CONCLUSIONS

The study indicate that both temperature and reactant molarity ratio significantly influence the efficiency of GI ester formation. The optimum reaction of esterification was at a 1:1.1 of molarity ratio and a temperature of 80°C which produces the largest crude GI of 4.248 g (94.78%), nevertheless the pure GI produced was only 52 mg (1,48%). One of the dominant peaks on the GCMS chromatogram revealed the presence of GI compound at R_T of 13.376 minutes with an area of 2.77% as well as the FTIR spectra displayed functional groups of carbonyl (C=O) at 1717.82 cm^{-1} from ester compound.

Antibacterial activity test demonstrated that GI exhibited significant antibacterial activity against gram-positive bacteria, with inhibition zones of 18.33±2.62 mm for *B. subtilis* and 15.67±0.47 mm for *S. aureus*, and against gram-negative bacteria, with inhibition zones for *P. aeruginosa* 10.67±0.47 mm and for *E. coli* 16.67±2.36 mm. Therefore GI is classified as an strong antibacterial agent against gram-positive bacteria.

Further purification is needed to increase the pure ester yield, as well as minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) analysis.

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