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# Antibacterial Activity Testing on Acetyleugenol Against *Staphylococcus aereus*

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***Abstract:*** *The main contents of clove oil are phenolic compounds, namely eugenol, eugenol acetate, gallic acid, and flavonoids. One of the derivatives of the eugenol compound is acetyl eugenol, which was developed by the esterification reaction between eugenol and acetic acid anhydride using the sonochemical method. Synthesis of acetyl eugenol was sonicated for 30 minutes at a temperature of 70-80°C. The % yield of the synthesized compound is calculated and continued with testing, including solubility, melting point, FTIR, and GC-MS tests, which are then tested for antimicrobial activity. The research results show that eugenol and acetic acid anhydride compounds with a NaOH catalyst can be synthesized using ultrasonic waves and produce a % yield of 3.50%. The resulting synthesis can melt at 30°C and dissolve in ethanol, methanol, chloroform, and ether but not in distilled water. FTIR testing on the acetyl eugenol compound showed the presence of -OH phenolic groups, C=C alkenes, C=C aromatics, C-O esters, and C=O esters. Acetyleugenol at concentrations of 1.25%, 2.5%, 5%, and 10% acted antibacterial on Staphylococcus aureus cultures with a 1.0 X 108 CFU/mL density.*

***Keywords****: Acetyleugenol, Antibacterial, Sonochemical, Staphylococcus aureus*

## INTRODUCTION

The clove plant (Syzygium aromaticum) is often used in various fields. This plant is very famous for its clove oil. The main contents of clove oil are phenolic compounds, namely eugenol, eugenol acetate, gallic acid, and flavonoids (Safitri & Purnamawati, 2021). Eugenol or which has another name, namely 4-allyl-2-methoxyphenol, has pharmacological activity as an analgesic, anti-inflammatory, antimicrobial, antiviral, antifungal, antiseptic, antispasmodic, antiemetic, stimulant, local anesthetic so that this compound is widely used in the pharmaceutical industry. Apart from that, another use of eugenol is that it can be used as an antioxidant (Towaha, 2012).

One derivative of the eugenol compound is acetyl eugenol. Acetyl eugenol, which has the chemical name 4-allyl-2-methoxyphenyl acetate, was developed from the eugenol compound by an esterification reaction. The esterification reaction forms ester compounds by direct reaction between a carboxylic acid and an alcohol. In the synthesis of acetyl eugenol, an esterification reaction is formed in the eugenol compound, which has a phenolic -OH group, and the acylating agent used is acetic acid anhydride, which is a carboxylic acid derivative.

The essential oil contained in the roots, stems, leaves, and flowers of cloves have several active ingredients in the form of eugenol, tannins, saponins, flavonoids, and alkaloids. Several studies have shown that clove flowers can inhibit bacterial growth

*Escherichia coli, Proteus mirabilis, Klebsiella pneumonia, Staphylococcus aureus,* and Methicillin-Resistant *Staphylococcus aureus* (MRSA) (Panuluh, 2019). The ability of acetyl eugenol as an antibacterial is because it has a mechanism that causes changes in the macromolecular components of bacteria, such as damaging cell membranes and inactive membrane proteins irreversibly and causing damage to nucleic acids. (Azizah et al., 2018). Based on this point of view, this research was conducted to determine the bioactivity of the acetyl eugenol compound against *Staphylococcus aureus* bacteria.

## RESEARCH METHODS

### Materials and Tools

The tools used in this research were glassware in the laboratory, Autoclave, laminar flow, test tubes, Erlenmeyer, Petri dishes, sterile tubes, tweezers, sterile, Eppendorf pipettes, measuring cups, spirit lamps, 300C incubators, nutrient agar, paper discs, and sterile distilled water.

The materials used in this research are Acetyl Eugenol, DMSO solvent, distilled water, and Staphylococcus aureus microbes.

### Procedures

#### *Synthesis of Acetyleugenol*

Take 5 mL of the Eugenol compound derived from pure clove oil, put it in an Erlenmeyer flask, and add 10% sodium hydroxide to 13 mL in a sonicator for 15 minutes at 60°C. Then, 9.2 mL of acetic acid anhydride was added to the mixture for 60 minutes at a temperature of 70-80°C. The compound formed was then extracted liquid-liquid with chloroform twice, 20 mL each, using a separating funnel, and the chloroform phase was taken. The chloroform phase is stored in the refrigerator until the temperature is less than 10° C for one night.

The synthesized compound left overnight was immediately extracted with cold 5% sodium hydroxide twice, 10 mL each, using a separating funnel, and then the chloroform phase was taken. The chloroform phase was heated over a water bath at 100°C for 60 minutes. Chloroform is allowed to evaporate, and then the synthesized compound is obtained. The compound obtained is weighed after obtaining the synthesized compound, and the yield obtained is calculated. The synthesized compound is then subjected to compound identification, including organoleptic tests, melting point, and FTIR analysis.

#### *Preparation of Inoculum*

From the culture stock of Staphylococcus aerus bacteria that have grown, the culture is taken using a sterile tube needle and then suspended in a tube containing 10 mL of sterile distilled water until turbidity is obtained that matches the Mc. Farland standard turbidity, the concentration of the bacterial suspension is around 108 CFU/mL. After that, dilution was performed to obtain a bacterial suspension concentration of 106 CFU/mL by pipetting 2.0 mL of the suspension (108 CFU/mL) into a tube containing 8 mL of sterile distilled water. This suspension will be used for anti-microbial testing.

#### *Making Test Solutions with Various Concentrations*

The Eugenol derivative compound used is Acetyl eugenol. Acetyl eugenol was obtained from synthesis in our laboratory, and the compound was identified using FTIR spectroscopy. All the samples above were made to a concentration of 10% by weighing 10 mg, each dissolved in 100µL DMSO. Then the 10% concentration was pipetted into 50µL and added with 50µL of DMSO as a 5% concentration, the 5% concentration was

pipetted with 50µL and added with 50µL of DMSO as a 2.5% concentration, the 2.5% concentration was pipetted with 50µL and added with 50µL of DMSO as a 1.25% concentration. Each sample has concentrations of 1.25%, 2.5%, 5%, and 10%, which will be used for anti-microbial.

***In Vitro Anti-Microbial Testing***

The anti-microbial activity test was carried out by evenly instilling a suspension of S. aureus test bacteria onto an agar plate. A paper disc with a diameter of 6 mm was inserted into the agar plate with the Acetyl Eugenol sample, then incubated at a temperature of 300C for 18-24 hours and then seen for an apparent area/inhibitory effect on bacterial growth in the area around the paper disc.

## RESULTS AND DISCUSSION

The acetyl eugenol compound can be synthesized through the esterification reaction of phenol compounds. Research conducted by Riswanto (2011) stated that the compound acetyl eugenol can be synthesized from eugenol and acetic anhydride with sodium hydroxide catalyst accompanied by a stirring process at a temperature of 70-80°C. This research was carried out using sonochemical methods to shorten the mixing time. *Eugenol* is a compound that has functional groups, namely allyl (-CH2-CH=CH2), phenol (-OH), and methoxy (-OCH3), and has the molecular formula C10H12O2. A sodium hydroxide catalyst is added to speed up the reaction.

The sonication process was carried out for 60 minutes at a temperature of 70-80°C. The choice of temperature is based on the optimum temperature in the synthesis process in the esterification reaction in a synthesis to accelerate molecular movement so that the frequency of collisions and their impact increases (Keenan et al., 1980). Increasing the frequency and impact of collisions produces sufficient energy to exceed the activation energy so that the reaction rate increases. (Silberberg, 2006). The compounds obtained were then subjected to organoleptic tests, solubility tests, melting point tests, and FTIR to see the suitability of the target compounds and the percentage purity of the compounds obtained.

The process of synthesizing acetyl eugenol involves the esterification reaction of phenol compounds. Esterification of phenolic compounds can occur with a carboxylic acid or a more reactive carboxylic acid derivative. Esterification with carboxylic acids usually results in a low yield; to increase the yield, more reactive derivative compounds are used, such as acetic acid anhydride (Griffin, 1969). In this process, the -OH group of eugenol is replaced with an -COCH3 group from acetic acid anhydride, forming an ester derivative chemical compound using a sodium hydroxide catalyst. Sodium hydroxide is a strong base and reacts readily with acid compounds. The use of a sodium hydroxide catalyst is aimed at producing eugenol ions, which act as nucleophiles in substitution reactions, enhancing the reactivity of eugenol and accelerating the reaction (Sarwono, 2010).

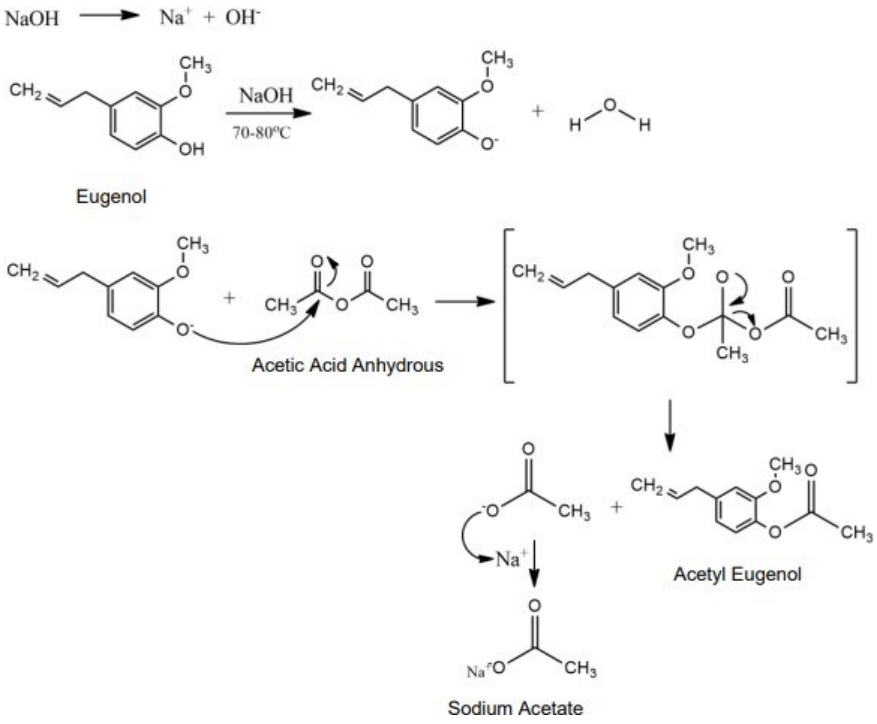


**Figure 1.** Acetyl Eugenol Formation Reaction (Dinurrosifa dan Indriyanti, 2022)

The sonochemical method is used because it can shorten the reaction time when ultrasonic waves are passed through a liquid, which causes the molecules to be pulled away from each other, resulting in the release of energy which causes bonds to break so it can help speed up the reaction process of the acetyl eugenol compound. The sonochemical method is environmentally friendly because it can reduce dangerous substances (Mitarlis et al., 2016). The longer the sonication time, the more cavitation bubbles and shock waves are formed, making collisions between particles move faster and affect the resulting crystals.

The resulting sonication mixture was extracted with chloroform, and the chloroform phase was taken. This extraction is based on the solubility of the acetyl eugenol compound, which is more soluble in nonpolar solvents. At the same time, the remaining sodium hydroxide and sodium acetate formed will separate because they are more soluble in polar solvents. This separation stage forms two layers: the top layer is water, and the bottom layer is chloroform because the specific gravity of chloroform is more significant than water. The solution that has been separated is then left overnight in the refrigerator. The chloroform phase left overnight was then purified by an extraction method using cold 5% sodium hydroxide (Ntamila & Hassanali, 1976). The resulting compound was extracted with 5% sodium hydroxide 10 mL twice and separated between the water and sodium hydroxide phases. Temperature regulation and the use of low concentrations of sodium hydroxide are necessary to prevent the hydrolysis reaction of the synthesized acetyl eugenol from occurring into eugenol and to prevent the breakdown of acetyl eugenol from forming sodium eugenol and sodium acetate (Griffin, 1969).

The sodium hydroxide phase was heated at a temperature of 100°C. This is done because chloroform can evaporate at a temperature of 61.2°C, so it is hoped that the still-dissolved chloroform can evaporate quickly. Heating is carried out until the solution forms white crystals. The acetyl eugenol compound was subjected to organoleptic tests, which included shape, color, and odor, which aimed to determine the nature of the

synthesized compound. The results obtained using the sonochemical method took 60 minutes to produce white crystals with an aromatic odor like eugenol.

The synthesis results of the acetyl eugenol compound were subjected to a solubility test to determine the compound's solubility in several solvents, such as aqueous, ethanol, methanol, chloroform, and ether. In the sonochemical method, the acetyl eugenol compound cannot be dissolved in distilled water but in ethanol, methanol, chloroform, and ether. Solubility testing on compounds resulting from the synthesis of acetyl eugenol compounds is carried out to determine the compound's solubility in various solvents to be used as a reference solvent when selecting solvents in subsequent tests. The synthesized compound was tested for melting point using a melting point apparatus. Melting point testing was carried out on the results of the synthesis of the acetyl eugenol compound using the sonochemical method in 60 minutes due to its white crystalline solid form and the aromatic aroma of cloves, with the result being a melting temperature of 30.8°C.

The results of the melting point test are based on literature from the National Library of Medicine, which states that the acetyl eugenol compound can melt at a temperature of 30-31°C. The acetyl eugenol compound was then analyzed using an FTIR-ATR spectrophotometer instrument to determine the functional groups in the synthesized compound. The FTIR instrument uses the ATR system because the sample used for analysis is tiny and does not require complicated sample preparation. The analysis process can take place at waves of 4000-400 cm-1.

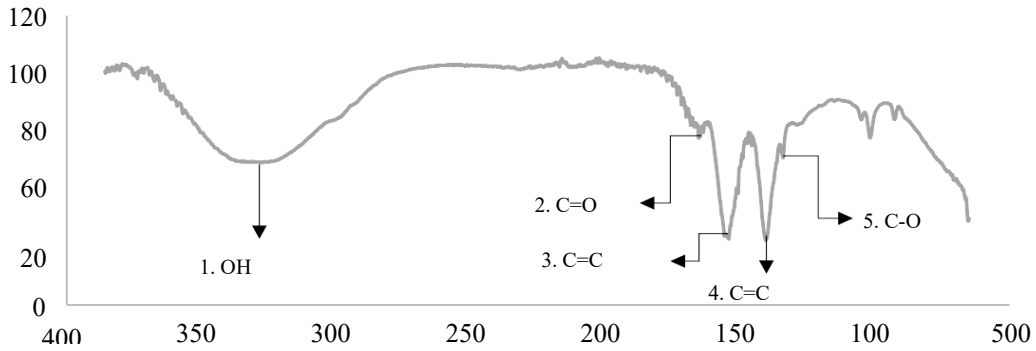


**Figure 2.** FTIR Spectra of Acetyl Eugenol Compound with 60 Minute Sonication Method

The compound synthesized using the 60-minute sonication method presented in Figure 1 produces five functional groups found in the synthesized compound. Acetyleugenol. The -OH functional group is found at a wavelength of 3338 cm-1, C=C alkenes at a wavelength of 1636 cm-1, C=C aromatics at a wavelength of 1541 cm-1, C-O esters at 1288 cm-1. Finally, there is C =O ester at a wavelength of 1790 cm-1.

Infrared spectra of acetyl eugenol compounds synthesized using different manufacturing methods and sonication times show similar absorption. The absorption that emerged was then compared with the absorption of the acetyl eugenol compound in the research of Dinurrosifa and Indriyanti (2022) and Riswanto (2011). Band 1 shows the -OH group (free phenol) found in eugenol and is the functional group differentiating eugenol and acetyl eugenol. In the synthesized compound, the O-H group absorption is still present, indicating that the compound still contains eugenol compounds. Eugenol is still formed due to purification methods that could be more optimal. This is also the same as Band 2, which indicates that there is still an alkene group (C=C). Band 3 indicates the

absorption of aromatic compounds with C=C bonds. Band 4 shows the presence of a sharp and broad (C-O) ester group. This is because, in the synthesized compound, there is an overlap between the absorption produced by the C-O bond of the carboxylic acid ester and the structure of the synthesized compound. Band 5 shows a vibration band, indicating a C=O bond in the ester group.

The results of the infrared spectra obtained can be concluded that the presence of these five functional groups indicates that the functional groups of the compound are still similar to the standard, namely eugenol. Theoretically, the phenolic OH group only belongs to the central compound molecule, namely eugenol, and the C=O ester group only belongs to the acetyl eugenol molecule.

The synthesis results were analyzed using GC-MS—the results of the synthetic compound produced two peaks, as shown in Figure 3. The target compound, acetyl eugenol, was at the highest peak, the second peak, with a retention time of 13.992 minutes.

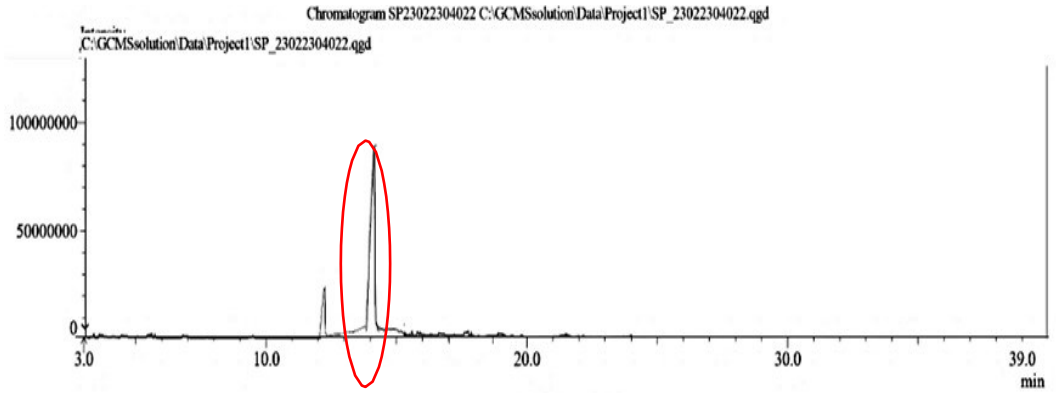


**Figure 3.** Chromatogram of Acetyl Eugenol Compound

The following analysis is tested using MS or mass spectroscopy. Mass spectroscopy is used to determine the chemical structure of organic molecules by calculating the molecule's mass and fragmentation pattern. The mass spectra pattern with g/mol molecular weight and base peak with m/z values with relative abundance show similarities to the Wiley Library in Figure 4.

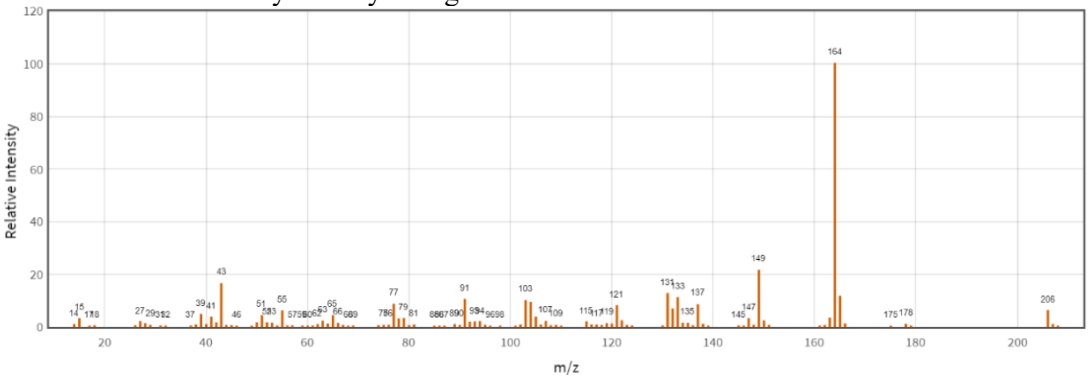


**Figure 4.** Mass Spectra of the Compound Acetyl Eugenol Library

In theory, the acetyl eugenol compound which has the molecular formula C12H14O3 has an Mr of 206.

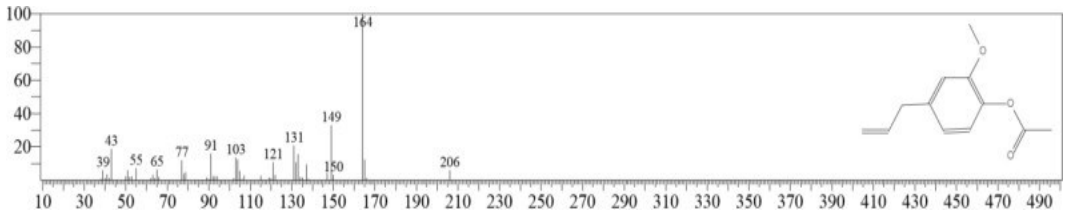


**Figure 5.** Mass Spectra of Acetyl Eugenol Compound

The mass spectrum in Figure 13 shows that the synthesized compound has a molecular ion with m/z = 206. This follows the theory that the molecular weight of acetyl eugenol is 206 g/mol. The analysis results from mass spectroscopy are presented as the following fragmentation pattern.

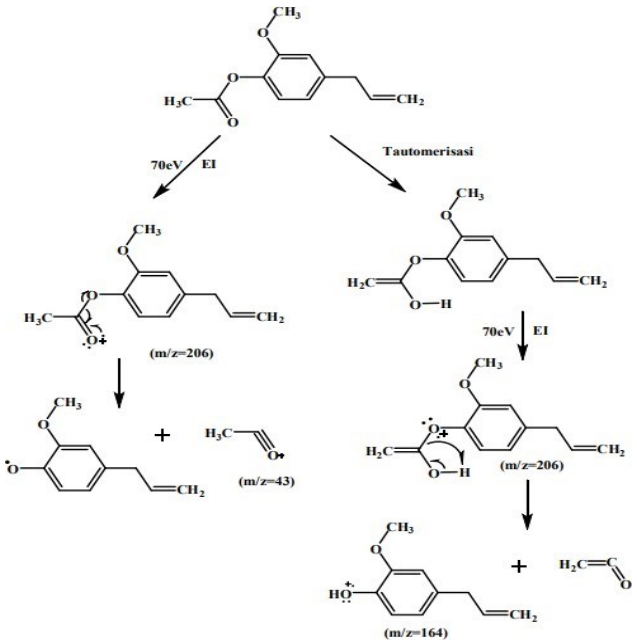


**Figure 6.** Fragmentation Pattern of Acetyl Eugenol Compound

Based on the fragmentation pattern in Figure 5, the acetyl eugenol compound experiences tautomerization in which the hydrogen atom undergoes displacement, followed by replacing the single bond with the double bond next to it. The molecular ions release the compound, producing a peak with an m/z value of 206 and leaving the compound CH2=C=O with an m/z value of 43.

Acetyleugenol has various biological activities, such as being antibacterial. The results of the antibacterial activity test of acetyl eugenol compounds against Staphylococcus aureus bacteria can be seen in Figure 7 below.

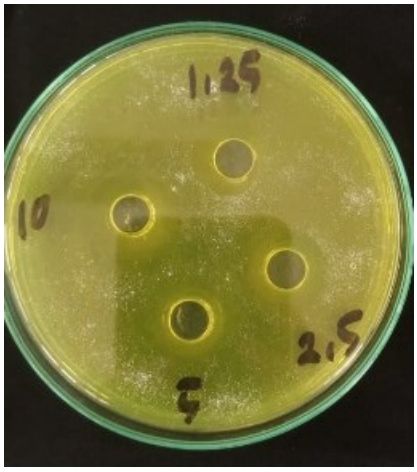


**Figure 7.** antibacterial activity test of acetyleugenol compounds against Staphilococcus aureus bacteria

| | **1,25%** | **2,5%** | **5%** | **10%** |
|---|---|---|---|---|
| **Rep 1** | 1.102-0.802= 0.300cm | 1.322-0.802= 0.52cm | 1.402-0.818= 0.584cm | 1.648-0.776= 0.872cm |
| **Rep 2** | 1.088- 0.764=0.324cm | 1.318-0.798= 0.52cm | 1.22-0.810 =0.612cm | 1.634-0.806= 0.828cm |
| **Rep 3** | 1,106-0,788= 0,318cm | 1.330- 0.804=0526cm | 1.418-0.802= 0.616cm | 1.640-0.802= 0.838cm |

Acetyleugenol at concentrations of 1.25%, 2.5%, 5%, and 10% acted anti-bacterial on S. aureus cultures with a 1.0 X 108 CFU/ml density. This follows research by (R.T. Utami, 2019), which states that acetylguenol can inhibit gram-positive bacteria because of its hydrophobic nature; the compound will damage cell structures by entering the lipopolysaccharide in the cell membrane. Meanwhile, according to (M.J. Kalalo, 2020), Clove oil has a broad anti-bacterial spectrum. The MIC value against Gram-positive and Gram-negative bacteria shows good inhibitory power. Cloves show killing power against several bacteria, but the inhibition against Gram-positive bacteria is more significant than the inhibition against Gram-positive bacteria.

According to Y.D. Safitri and Purnamawati (2021), eugenol has very effective antibacterial capabilities against gram-positive and gram-negative bacteria. The antibacterial mechanism that eugenol has against Staphylococcus aureus is through changes in fatty acids in bacterial membranes, which can cause changes in cell membrane permeability and lead to bacterial cell death. The eugenol compound can also increase the production of intracellular Reactive Oxygen Species (ROS), which results in the death of Staphylococcus aureus bacterial cells.

The mechanism of action of acetyl eugenol on the membranes of gram-positive and gram-negative bacteria has been studied. The results showed that acetyl eugenol induces cell lysis through leakage of proteins and lipids in the cell membrane. In addition, the timing of cell exposure to eugenol is also essential. Because the cell walls and membranes of treated Gram-negative and Gram-positive bacteria were severely damaged after about 120 minutes of exposure (S. et al., 2017). It was also found that at specific concentrations of acetyl eugenol, the production of staphylococcal enterotoxin was significantly reduced. Therefore, it is suggested that acetyl eugenol can be used as a food

additive because of its inhibitory effect on bacterial growth and its suppressive effect on S. aureus exotoxin production.

## CONCLUSIONS

Acetyl eugenol was synthesized using ultrasonic waves, yielding 3.50%. When tested at concentrations of 1.25%, 2.5%, 5%, and 10%, it exhibited antibacterial activity against Staphylococcus aureus cultures with a density of 1.0 × 10^8 CFU/mL. Notably, the highest activity was observed at a concentration of 10% acetyl eugenol compared to lower concentrations.

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