

# Antibacterial Activity Testing on Acetyleugenol Against Staphylococcus aureus

*by Erwin Indriyanti*

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## Antibacterial Activity Testing on Acetylugenol Against *Staphylococcus aureus*

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<sup>1</sup> **Abstract:** The main contents of clove oil are phenolic compounds, namely eugenol, eugenol acetate and gallic acid, as well as 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 acetylugenol was sonicated for 30 minutes at a temperature of 70-80°C. The % yield of the synthesized compound is calculated and continued with testing which includes 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 starting at a temperature of 30°C and can dissolve in ethanol, methanol, chloroform and ether but does not dissolve 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. Acetylugenol at concentrations of 1.25%, 2.5%, 5%, and 10% was able to act antibacterial on *Staphylococcus aureus* cultures with a density of  $1.0 \times 10^8$  CFU/mL.

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

### INTRODUCTION

The clove plant (*Syzygium aromaticum*) is a spice that 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 and gallic acid, as well as 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).

<sup>1</sup> 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 is a reaction to form 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 has 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 pneumoniae*, *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, 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, sterile distilled water.

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

### Procedures

#### Synthesis of Acetylugenol

Take 5 mL of the Eugenol compound derived from pure clove oil and 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 taken and 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 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 that had been left overnight was immediately extracted with cold 5% sodium hydroxide twice, 10 mL each using a separating funnel, 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. After obtaining the synthesized compound, the compound obtained is weighed 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 aureus* 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 carried out to obtain a bacterial suspension concentration of 106 CFU/mL by pipetting 2.0 mL of the suspension (108 CFU/mL) added 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 has been 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 50 $\mu$ L and added with 50 $\mu$ L of DMSO as a 2.5% concentration, the 2.5% concentration was pipetted with 50 $\mu$ L and added with 50 $\mu$ L of DMSO as a 1.25% concentration. Each sample is made with 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 instilling a suspension of *S. aureus* test bacteria evenly 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 a clear 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 (-CH<sub>2</sub>-CH=CH<sub>2</sub>), phenol (-OH), and methoxy (-OCH<sub>3</sub>) and has the molecular formula C<sub>10</sub>H<sub>12</sub>O<sub>2</sub>. The use of a sodium hydroxide catalyst is added to speed up the reaction that occurs.

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 in order to accelerate molecular movement so that the frequency of collisions and their impact increases (Keenan et al., 1980). By increasing the frequency and impact of collisions, sufficient energy is produced 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, FTIR to see the suitability of the target compounds and the percentage purity of the compounds obtained.

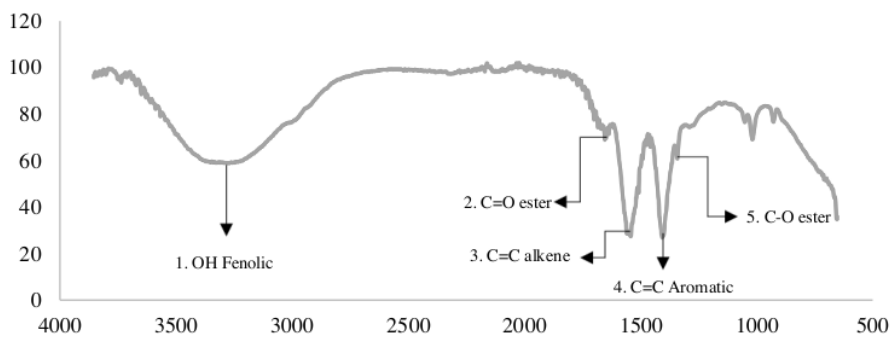
The acetyl eugenol synthesis process involves the esterification reaction of phenol compounds. Esterification of phenolic compounds can occur with a carboxylic acid or with a more reactive carboxylic acid derivative. Esterification with carboxylic acids generally has a small yield, so to increase the yield value more reactive derivative compounds are used, one of which is acetic acid anhydride (Griffin, 1969). This process occurs when the -OH group of eugenol is replaced with an OH group -COCH<sub>3</sub> from acetic acid anhydride becomes an ester derivative chemical compound using a sodium hydroxide catalyst. Sodium hydroxide is a strong base and is very reactive with acid compounds. The use of a sodium hydroxide catalyst aims to produce eugenolate ions which act as nucleophiles in substitution reactions so that the reactivity of eugenol increases and the reaction runs faster (Sarwono, 2010).



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 chloroform that is still dissolved 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 results of the synthesis of the acetyl eugenol compound were subjected to a solubility test to determine the solubility of the compound in several solvents such as aqueous, ethanol, methanol, chloroform and ether. The acetyl eugenol compound using the sonochemical method cannot be dissolved in distilled water but can be dissolved in ethanol, methanol, chloroform and ether. Solubility testing on compounds resulting from the synthesis of acetyl eugenol compounds is carried out to determine the solubility of the compound in various solvents so that it can be used as a reference solvent in 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 in accordance with 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 contained in the synthesized compound. The FTIR instrument uses the ATR system because the sample used for analysis is only a small amount and does not require complicated sample preparation so that the analysis process can take place at waves of 4000-400 cm<sup>-1</sup>.



**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 5 functional groups found in the synthesized compound. Acetylugenol. The -OH functional group is found at a wavelength of 3338 cm<sup>-1</sup>, C=C alkenes at a wavelength of 1636 cm<sup>-1</sup>, C=C aromatics at a wavelength of 1541 cm<sup>-1</sup>, C-O

esters at a wavelength of  $1288\text{ cm}^{-1}$ , and finally there is C=O ester at a wavelength of  $1790\text{ cm}^{-1}$ .

Infrared spectra of acetyl eugenol compounds synthesized using different manufacturing methods and sonication times show absorption that is not much different. The absorption that emerged was then compared with the absorption of the acetyl eugenol compound in the research of Dinurrosifa<sup>2</sup> and Indriyanti (2022) and Riswanto (2011). Band 1 shows the presence of the -OH group (free phenol) found in eugenol and is the functional group that differentiates eugenol and acetyl eugenol. In the synthesized compound, there is still absorption of the O-H group, which indicates that the synthesized compound still contains eugenol compounds. Eugenol is still formed due to less than optimal purification methods. This is also the same as Band 2 which indicates that there is still an alkene group (C=C).<sup>2</sup> and 3 indicates the absorption of aromatic compounds with C=C bonds. Band 4 shows the presence of a sharp and wide (C-O) ester group. This is because in<sup>2</sup> the synthesized compound there is an<sup>3</sup> overlap between the absorption produced by the C-O bond of the carboxylic acid<sup>2</sup> in the structure of the synthesized compound. Band 5 shows a vibration band which indicates the presence of 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 main 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, namely acetyl eugenol, was at the highest peak, namely the second peak with a retention time of 13.992 minutes.

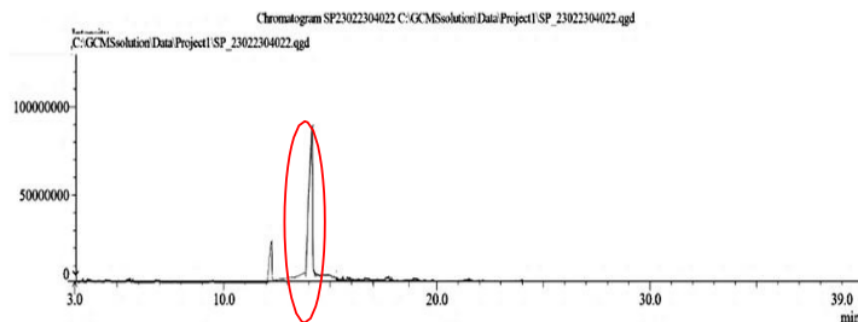


Figure 3. Chromatogram of Acetyl Eugenol Compound

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

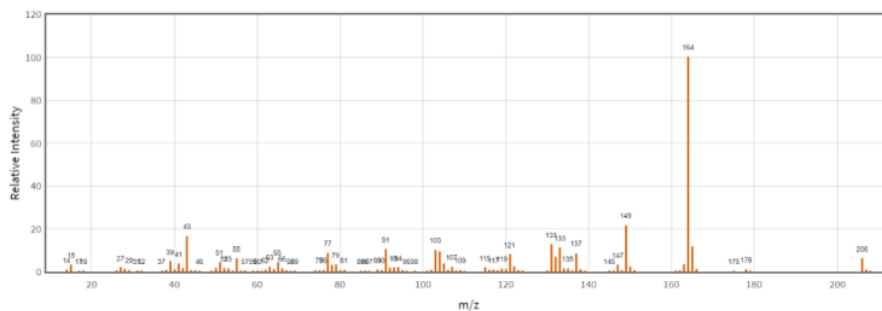


Figure 4. Mass Spectra of the Compound Acetyl Eugenol Library

In theory, the acetyl eugenol compound which has the molecular formula  $C_{12}H_{14}O_3$  has an Mr of 206.

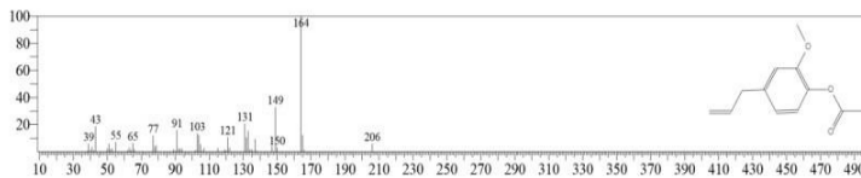


Figure 5. Mass Spectra of Acetyl Eugenol Compound

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

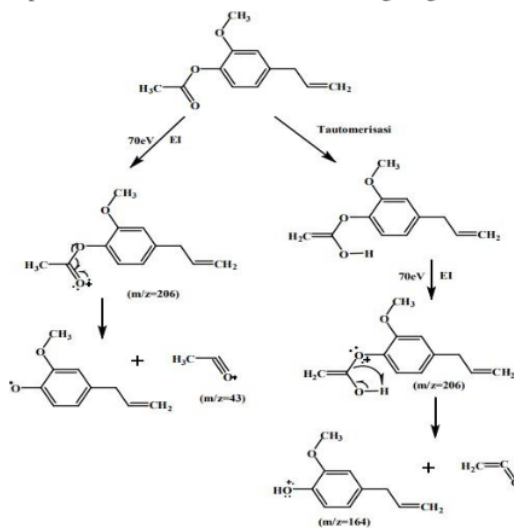


Figure 6. Fragmentation Pattern of Acetyl Eugenol Compound

Based on the fragmentation pattern in Figure 5, the acetyl eugenol compound experiences tautomerization in which the hydrogen atom undergoes displacement followed by the replacement of 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  $\text{CH}_2=\text{C}=\text{O}$  with an  $m/z$  value of 43.

Acetylugenol have various biological activities such as antibacterial. The results of the antibacterial activity test of acetylugenol compounds against *Staphylococcus aureus* bacteria can be seen in Figure 7 below.

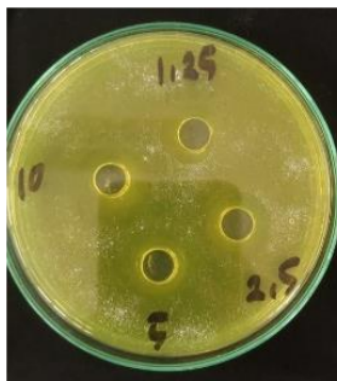


Figure 7. antibacterial activity test of acetylugenol compounds against *Staphylococcus aureus* bacteria

	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=0.526cm	1.418-0.802= 0.616cm	1.640-0.802= 0.838cm

Acetylugenol at concentrations of 1.25%, 2.5%, 5%, and 10% was able to act antibacterial on *S. aureus* cultures with a density of  $1.0 \times 10^8$  CFU/ml. This is in accordance with research by (R.T. Utami, 2019) which states that acetylugenol is able to 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 greater than the inhibition against Gram-negative 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 acetylugenol on the membranes of gram-positive and gram-negative bacteria has been studied. The results showed that acetylugenol induces cell lysis through leakage of proteins and lipids in the cell membrane. In addition, it appears that the timing of cell exposure to eugenol is also important. Because the cell walls and membranes of treated Gram-negative and Gram-positive bacteria were severely damaged after about 120 minutes of exposure (S. Mohammadi Nejad, 2017). It was also found that at certain concentrations of acetylugenol, the production of staphylococcal enterotoxin was significantly reduced. Therefore, it is suggested that acetylugenol 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

The acetylugenol compound can be synthesized using ultrasonic waves and produces a % yield of 3.50%. Acetylugenol at concentrations of 1.25%, 2.5%, 5%, and 10% was able to act antibacterial on *Staphylococcus aureus* cultures with a density of 1.0 X 10<sup>8</sup> CFU/MI. Acetylugenol with the concentration is 10% Most active between both compounds.

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