Esterification of Acetic Acid Anhydride with Eugenol and Its Activity as Antifungal

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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. The aim of this research is to synthesize the acetyl eugenol compound from the esterification reaction between eugenol and acetic acid anhydride using sonochemical methods, to determine the sonication time that can produce the acetyl eugenol compound with the largest yield percentage, and to determine its antifungal activity against the Candida albicans fungus. Synthesis of acetyleugenol was sonicated for 90 minutes at a temperature of eighty degrees celsius. The % yield of the synthesized compound is calculated and continued with testing which includes solubility, melting point, FTIR, GC-MS. NMR tests, which are then tested for antifungal 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 3.49%. The resulting synthesis can melt starting at a temperature of thirty degrees celcius and can dissolve in ethanol, methanol, chloroform and ether but does not dissolve in distilled water. H-NMR FTIR testing on the acetyl eugenol compound showed the presence of 0,866 (s, 3H)- 6,62 (d,1H)- 6,47 (d, 1H)- 1,875 (t,2H)- 4,983 (s, 2H)- 1,255 (d, 2H)- 7,061 (d,1H)- 3,84 (s, 3H). Acetyleugenol with concentrations of 5% and 10% it shows the ability to act as an antifungal for Candida albicans.

Keywords: Acetyleugenol; Antifungal; Candida albicans; Esterification; Sonochemical

1 Introduction

Eugenol has a fungicidal antifungal effect, namely killing fungi by destroying the lipid bilayer membrane so that cells lose their structure and function, resulting in cell lysis [1]. Indonesia is a country that produces spices and traditional medicines. To date, the International Organization for Standardization (ISO) lists 112 types of plants classified as herbs and spices. This plant is believed to be able to provide health effects for the body. This is caused by the content of bioactive compounds in herbal plants which are very useful for maintaining health.

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One of the spice plants with medicinal properties that has the potential to be researched is cloves (Syzygiumaromaticum). 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 [2]. Eugenol or which has another name, namely 4-allyl-2-methoxyphenol, has pharmacological activity as anti-inflammatory, antimicrobial, antiviral, antifungal, antiseptic, antispasmodic, antiemetic, stimulant and analgesic in dental and oral medicine [3].

Indonesia, as a tropical country, is fertile ground for the growth of fungi, especially Candida fungi. Candida species are microorganisms that cause oropharyngeal, vulvovaginal and skin infections. The Candida albicans fungus is considered to be the main cause of candidiasis. Candida albicans is an opportunistic fungus that causes mouth ulcers, skin lesions, vulvavaginitis, candida in the urine (candiduria), gastrointestinal candidiasis which can cause gastric ulcers, or can even be a complication of cancer [4]

The existence of resistance to the main antifungal drugs urges research to find attractive natural alternatives to antifungals. Most essential oils obtained from many tropical and subtropical plants contain eugenol as the main antifungal [5]. Eugenol alone had weaker antifungal activity than clove leaf essential oil against Candida albicans, also depending on major and other minor constituents of some samples with partially known anticandidal effects, such as methyl eugenol and linalool. This suggests that contents other than eugenol in essential oils have a synergistic and/or synergistic role in providing antifungal effects [6].

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 [7]. 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 [8].

Acetyl eugenol's ability as an antibacterial is due to its essential oil containing eugenol, tannins, saponins, flavonoids, alkoloids and phenol. Antibacterial mechanisms cause changes in the macromolecular components of bacteria such as damaging cell membranes, irreversibly inactivating membrane proteins and causing damage to nucleic acids [9]. Apart from that, it also has a fungicidal antifungal effect, namely killing fungi by destroying the lipid bilayer membrane so that cells lose their structure and function, resulting in cell lysis [10]. Based on this point of view, this research was conducted to determine the bioactivity of the acetyl eugenol compound against the fungus Candida albicans.

2 Method

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 were Acetyl Eugenol, DMSO solvent, distilled water, Candida albicans.

2.1 Synthesis of Acetyl Eugenol and Analysis of Compounds Resulting from Synthesis

Take 5 mL of the Eugenol compound derived from pure clove oil and put it in an Erlenmeyer flask and add 13 mL of 10% sodium hydroxide. Each mixture was placed in a magnetic stirrer at a speed of 450 rpm for 15 minutes at a temperature of 60°C. Then 9.2 mL of acetic acid anhydride was added and sonicated for 90 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 which 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, FTIR, GCMS and H NMR analysis.

2.2 Preparation of Inoculum

From the culture stock of the Candida albicans fungus that has 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.

2.3 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, GCMS and NMR spectroscopy tools. 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 is made with concentrations of 1.25%, 2.5%, 5% and 10% which will be used for anti- microbial testing.

2.4 In Vitro Anti-Microbial Testing

The anti-microbial activity test was carried out by instilling a suspension of the test fungus Candida albicans 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.

3 Result And Discussion

The acetyl eugenol compound can be synthesized through the esterification reaction of phenol compounds. Research conducted by Riswanto [11] stated that the compound acetyl eugenol can be synthesized from eugenol and acetic anhydride with the help of a 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. 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 speed up the movement of molecules so that the frequency of collisions and their impact increases [12] [13]. By increasing the frequency and impact of collisions, sufficient energy is produced to exceed the activation energy so that the reaction rate increases [14], [15]. The compounds obtained were then subjected to organoleptic tests, solubility tests, melting point tests, FTIR, GC-MS and HNMR 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 [16], [17].

This process occurs when the -OH group of eugenol is replaced with an OH group-$COCH_3$ 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 [18], [19]

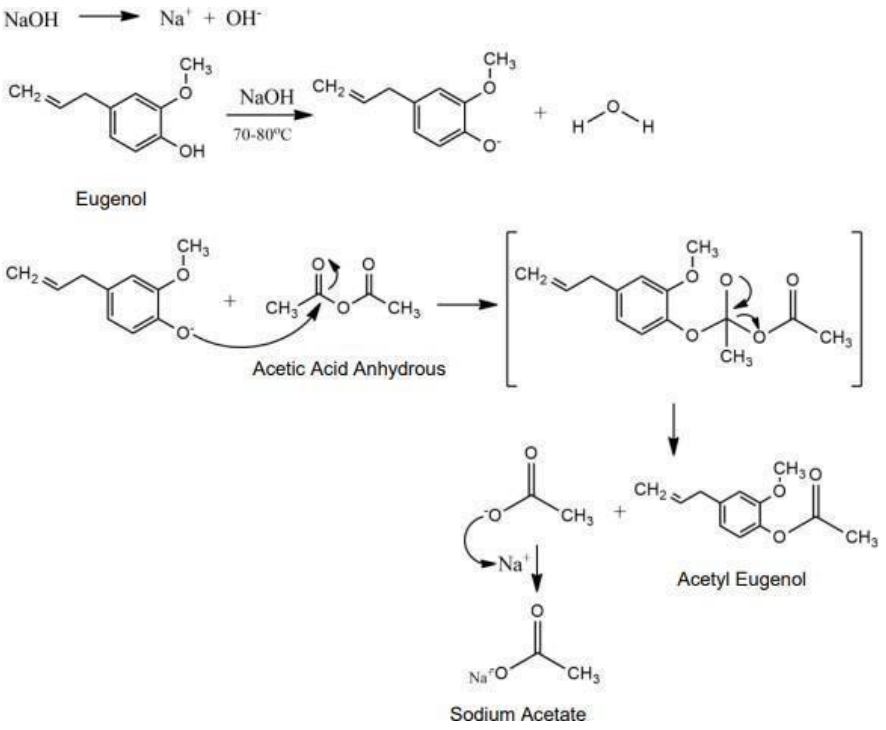

Fig. 1. Acetyl Eugenol Formation Reaction [7]

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. Apart from that, the sonochemical method is an environmentally friendly method because it can reduce dangerous substances [20], [21]. The longer the sonication time, the more cavitation bubbles and shock waves are formed, making collisions between particles move faster and will 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, while 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 chlorform because the specific gravity of chloroform is greater than water. The solution that has been separated is then left overnight in the refrigerator. The chloroform phase that had been left overnight was then purified by an extraction method using cold 5% sodium hydroxide. The resulting compound was extracted with 5% sodium hydroxide 10 mL twice and separated between the water phase and the sodium hydroxide phase. 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 to form sodium eugenolate and sodium acetate [21].

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 90 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 90 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^{-1} [22].

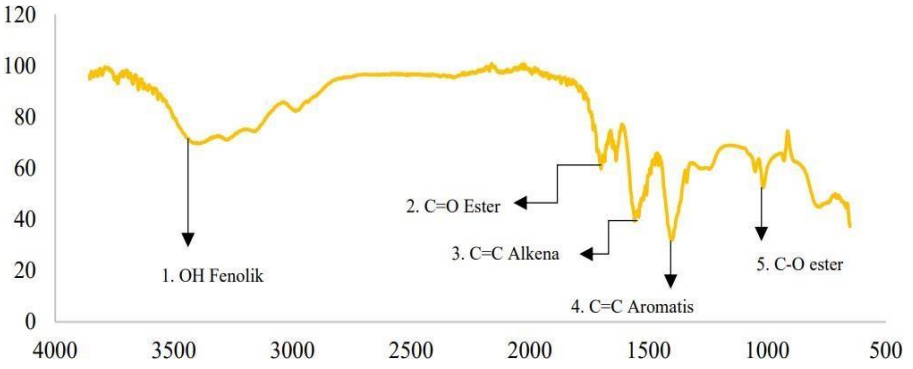

Fig. 2. FTIR Spectra of Acetyl Eugenol Compound with 90 Minute Sonication Method

The compound synthesized using the 90 minute sonication method presented in Fig. 2 produces 5 functional groups found in the synthesized compound. Acetyl eugenol. 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 a wavelength of 1288 cm-1, and finally there is C =O ester at a wavelength of 1790 cm-1.

The infrared spectra of the acetyl eugenol compound synthesized using different preparation methods and sonication times show that the absorption is not much different. The absorption that emerged was then compared with the absorption of the acetyl eugenol compound in the research of Dinurrosifa and Indriyanti [7] and Riswanto [11]. 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). Band 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 the synthesized compound there is an overlap between the absorption produced by the C-O bond of the carboxylic acid ester 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 compound was then tested using a GC-MS instrument.

The results of the synthetic compound using the sonochemical method produced ten peaks as shown in Fig. 3. The target compound, namely acetyl eugenol, was at the peak circled in red, namely peak eight with a retention time of 18,802 minutes.

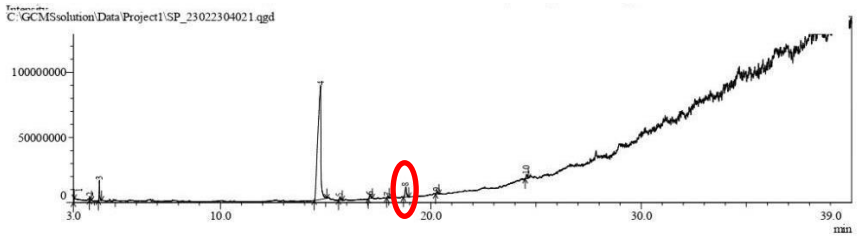

Fig. 3. Chromatogram of Acetyl Eugenol Compound Sonochemical Method Time 90 Minutes

GC-MS results show that the synthesis of acetyl eugenol using the sonochemical method has a % abundance of the target compound of 3.49%. This result was obtained

from calculating the weight of the compound resulting from the synthesis of acetyl eugenol as 3.3372 grams with a GC% of 3.62. 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 Fig. 4.

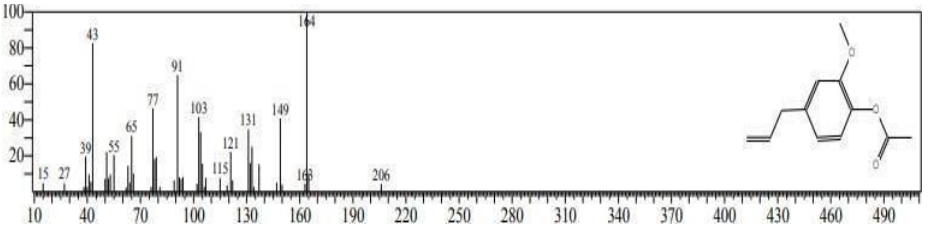

Fig. 4. Mass Spectra of Acetyl Eugenol Compound Sonochemical Method Time 90 Minutes

The mass spectrum in Fig. 4 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 patterns.

Based on the fragmentation pattern in Fig. 4, 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 CH2=C=O with an m/z value of 43 (Fig. 5).

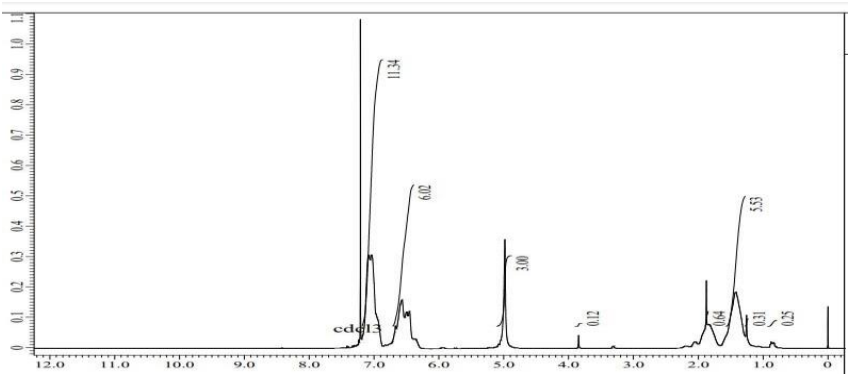

Fig. 5. The H-NMR spectra of the synthesized compound

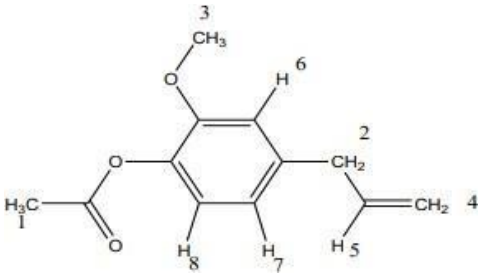

Fig. 6. Acetyl eugenol numbering

Peak analysis in 1H-NMR was carried out from the peak with the smallest to the largest chemical shift (δ). The 3- integral G singlet peak at δ 2.2 ppm is produced by three type 1 protons. The protons are bound to C which is sp3 hybridized, so they tend to have relatively small δ values compared to protons bound to C sp2 and sp. Resonance in carbonyls only has a small induction effect on this type of proton because the presence of O can donate electrons to the partially positive carbonyl C. Therefore, this proton tends to be shielded and creates an upper peak (upfield) compared to all other types of protons, but the δ value for type 1 protons is not smaller than 2 due to the carbonyl induction effect. The 2-integral F doublet peak at δ 3.5 ppm is produced by two type 2 protons.

This proton is bound to C sp3 which binds the ethene group and benzene ring. The existence of this bond causes an inductive influence of the ethene group and benzene ring which attracts electrons, so that type 2 protons have a relatively larger δ value than uninduced alkane protons. The effect of increasing δ on type 2 protons is greater than on type 1 so that the peak is located further downfield than on type 1 protons. The 3-integral E singlet peak at δ 3.8 ppm is produced by three type 3 protons. These protons are bound to C sp3 which binds the O substituent of the benzene chain. The bond with O which is a benzene substituent results in a large anisotropic effect and the δ value becomes larger, because the proton becomes less shielded by the electronegativity of the O atom. The 2-integral D doublet peak at δ 5.1 ppm is produced by type 4 and 9 protons. Type 9 protons 4 is directly bound to C which has a phi bond so it is subject to a strong anisotropic effect. Proton type 9 is a proton that is directly bound as –OH phenolic to the benzene ring. This causes the anisotropic effect it experiences to be quite strong. Peak D is thought to be a mixture of two peaks from different proton types (type 4 peak and type 9 peak) characterized by a wide and unnatural peak splitting pattern.

The 1-integral multiplet C peak at δ 5.9 ppm is produced by type 5 protons. Type 5 protons are protons bound to C sp2, but the anisotropic effect in this type is only carried by 1 proton, so the effect is greater than type 4 protons. So, the δ value of this proton falls down relative to type 4. The 2-integral multiplet B peak is produced at a value of δ 6.9 produced by type 6 and 7 protons. Type 6 and 7 protons are protons that are bound to the benzene ring, so they experience the effect very large and almost equal anisotropic. Therefore, the peaks that appear from two types of protons can be seen as a single peak that is integral, not one. The 1-integral doublet A peak at δ 7.2 ppm is

produced by type 8 protons. This proton is a proton that is bound to C sp 2 on the benzene ring, so the anisotropic effect is very large. In contrast to type 6 and 7 protons, type 8 protons are bound to C which is neighboring the C-O ester atom. At resonance, the O ester will donate its charge to the C carbonyl, so that the C in the C-O ester will be induced by the electronegativity of the O atom. This induction effect causes type 8 protons to be less shielded than type 6 and 7 protons. This causes the peak to appear lower relative to other types of protons.

From the 1H-NMR spectra obtained and the analysis results, it can be estimated that the synthesized compound is a mixture of eugenol and acetyl eugenol. This is characterized by the appearance of peaks that reflect the proton types of the two compounds in the spectrum of the synthesized compound, namely peak G which shows proton type 1 in the acetyl group of the acetyl eugenol compound, and peak D which shows proton type 9 in the –OH group of the phenolic compound. eugenol. However, overall, the 1HNMR spectra are similar to the 1H-NMR description of acetyl eugenol [23]. Apart from that, there is a clear difference between the 1HNMR spectrum of the synthesized compound and the 1H-NMR spectrum of eugenol at the δ value of 2.2 ppm which is the chemical shift value for the type 1 proton peak.

Eugenol which does not have this type of proton looks empty at the chemical shift value the Acetyleugenol have various biological activities such as antifungal. The results of the antifungal activity test of acetyleugenol compounds against Candida albicans can be seen in Fig. 7 below.

Fig. 7. The result of antifungal activity test

Table 1. The result of antifungal activity test

	1,25%	2,5%	5%	10%
Rep 1	-	-	1,202-0,888= 0,314cm	1,608-0,778=0,83cm

Rep 2	-	-	1,224-0,802= 0,422cm	1,626-0,846= 0,780cm
Rep 3	-	-	1,200-0,784= 0,416cm	1,642-0,800= 0,842cm

Eugenol at concentrations of 1.25% and 2.5% was not able to inhibit the growth of Candida albicans with a microbial density of 1.0. The antimicrobial ability of a compound is directly influenced by the concentration of the test microbe. The higher the concentration of the test microbe, the greater the compounds needed to inhibit or kill the test microbe. This affects the levels of antimicrobial compounds that enter the test microbial cells. The greater the amount or level of antimicrobial compounds that enter the cells, the greater the mechanism of action of the antimicrobial agents in destroying the components of the test microbial cells, so that microbial cell death will occur more quickly. Eugenol concentrations of 0.5%, 1.0% v/v produced greater inhibition of the growth of Candida albicans with a concentration of 10-6 cells/ml [24], compared to this study which had a Candida albicans concentration of 10-8 Cells/ ml. The greater the concentration of the extract given, the greater the killing power that is formed, because the greater the concentration of bioactive components contained in the extract. The effectiveness of an antimicrobial agent is influenced by the concentration of the substance administered. Increasing the extract concentration results in a higher content of active ingredients which function as antimicrobials so that the ability to kill microbial growth is also greater.

Eugenol can bind to Candida albicans membranes and reduce ergosterol biosynthesis, resulting in damage to cell walls and membranes. In addition, eugenol not only reduces tube formation in cell walls, which reduces nutrient absorption from host tissues, but also increases levels of lipid peroxidation and reactive oxygen species, which induce oxidative stress and cause high permeability of fungal cell membranes[25] Eugenol inhibits the adhesion capacity of Candida cells; In addition, eugenol also inhibits biofilm formation and eliminates Candida biofilms that already exist on various host mucosal surfaces. In addition, by disrupting the integrity of Candida cells, eugenol can increase the entry of antifungal drugs into Candida cells, thereby increasing the efficacy of treatment (forming a synergistic effect with other antifungal drugs). Therefore, eugenol can be used in the clinical management of various candidiasis symptoms, especially mucocutaneous symptoms such as oral and vulvovaginal infections. Clove oil can inhibit the growth of the fungus C. albicans due to the presence of phenolic compounds and organic acids. The mechanism of phenol compounds as antifungals is by denaturing protein bonds in the cell membrane, so that the cell membrane becomes lysed and the phenol compound can enter the cell nucleus. This can inhibit the growth of fungus and even the fungus will die [26]

4 Conclusion

The acetyleugenol compound can be synthesized using ultrasonic waves and produces a % yield of 3.49%. Acetyleugenol at concentrations of 5% and 10% is capable of antifungal properties on Candida albicans cultures with a density of 1.0 X 108 CFU/ml.

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