



## Isolation and Antifungal Activity of Caryophyllene from Clove Leaf Oil (*Syzygium aromaticum* L.) on Mahogany Leaf Composites

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**Abstract:** Mahogany leaf composites mostly consist of cellulose which is easily weathered due to rotting fungi. One way to overcome this is to use anti-weathering chemicals. Chemical potential as a natural anti-weathering and abundant in Indonesia is caryophyllene compounds as a by-product in the eugenol industry which processes it from clove leaf oil. In this work, isolation and antifungal activity of caryophyllene from clove leaf oil on mahogany leaf composites was evaluated. Based on GC-MS analysis of clove leaf oil is known that eugenol and caryophyllene has yielded 42.83% and 23.87%, respectively. Selective extraction by using sodium hydroxide solution (2,5% NaOH, the ratio of oil to solution is 1:5) and fractional distillation under reduced pressure (Distillate fraction, DF P= -76 cmHg, R=5:1) and continued redistilled are needed to isolate of caryophyllene. Redistilled can improve the purity, then distillate collected while the temperature changed with a purity of 99.10%. Meanwhile, mahogany leaf composite has been successfully synthesized from mahogany leaves by using a polyurethane binder and glass fiber as reinforcement. Minimum inhibitory concentration (MIC) of caryophyllene was determined. The composite was coated with caryophyllene and evaluated for its antifungal activity against *Aspergillus fumigatus*. Observation using a CCD camera (Charge Couple Device) microscope with a magnification of 50x shows the growth of fungal spores on the composite surface. As a result, *A. fumigatus* can be inhibited by caryophyllene oil in a 1.2% concentration on mahogany leaf composite.

**Keywords:** antifungal, caryophyllene, composite, mahogany

### INTRODUCTION

The processing of organic waste has been studied for its handling and processing. One alternative method of processing leaf waste that can be done is to use it as a source of reinforcement in the composite field (Bourmaud et al., 2017). Masturi et al. (2020) have conducted a study on the manufacture of composite materials made from mahogany leaf waste with polyurethane as a binding material and fiberglass as a reinforcing material. The resulting composite material has good physical and mechanical properties, namely low density but high compressive strength. However, the composite material does not yet have good biological properties in the form of resistance to rotting fungi, so the resulting composite has low resistance.

*Aspergillus* is the most dominant genus of fungus in tree and soil tissues and is a type of wood rot fungus (Mukrimin et al., 2021). Wood rot fungi grow on the material characterized by mycelium's appearance on its surface. If not handled properly, the fungus will thrive, causing the material to lose weight. This weight loss causes the material to become brittle and easily damaged (Djarwanto et al., 2018). One way to strengthen the resistance of composite materials to the growth of wood rot fungi is to add a substance that has acted as an inhibitor of the growth of rotting fungi.

Previous studies have shown that essential oils have good antifungal properties (Oliveira et al., 2020). Clove oil is known to inhibit the growth of wood rot fungi (Alfauziah & Budiman, 2016), (Xie et al.,



2015). The main terpenoid constituents of clove oil are eugenol and caryophyllene (Plata-Rueda et al., 2018). Neta et al. (2017) showed that caryophyllene compounds had antifungal activity against fungi (*A. niger*, *A. flavus*, and *A. fumigatus*), but the research was limited to extraction antifungal testing of caryophyllene compounds, there has been no application to certain products. Therefore, research on caryophyllene compounds' application and activity test from clove oil on mahogany leaf composites needs to be done.

This research was conducted to determine the activity of antifungal compounds extracted from caryophyllene from clove leaf oil against *A. fumigatus* and its application as a coating on mahogany leaf composites. The use of clove oil in this study is for its antifungal properties and for increasing the potency and utilization of essential oils in Indonesia, especially clove oil. This research aims to obtain composite material from mahogany leaves, which have antifungal activity, making them stronger, longer-lasting, and distinctive aroma.

## METHODS

### Material

Leaves of (*Syzygium aromaticum* L.) was collected from Ungaran District, Central Java, Indonesia. This leaf was selected, dried, and prepared for one day. The mahogany (*Swietenia mahagoni*) leaves waste was collected from Universitas Negeri Semarang, Semarang, Indonesia. Mahogany leaves were cleaned with water, then dried at 50°C for 2 hours. Dried mahogany leaves are ground using a grinding machine and sieved to produce a fine powder. Commercial polyurethane polymers and glass fiber were purchased from Bratachem, Semarang, Indonesia. *A. fumigatus*, potato dextrose agar (PDA), potato dextrose broth (PDB). All the chemicals used in these studies were pure analytical reagents from Merck.

### Steam Distillation of clove leaf oil

The essential oils were collected by the steam distillation method. Leaves of *Syzygium aromaticum* L. were packed into the kettle sitting on a perforated plate above the boiling water. The essential oils were volatilized with boiling water at temperature 100°C for 8 hours or more. After the steam distillation process, the oil will be collected and separated used the separatory funnel which can be used to separate the immiscible liquids of two layers such as oil and water. The oils which have lighter density than water will be visible at the top of the separatory funnel. Wait to the water and oil separated completely and formed two layers and oil was collected. Solid of Na<sub>2</sub>SO<sub>4</sub> was used to adsorb the residual of water that fused with the oil.

### Isolation and purification of caryophyllene from clove leaf oil

Clove leaf oil mixed with 2,5% NaOH with a volume ratio of oil to NaOH sodium is 1:5. The mixture was then stirred using a magnetic stirrer for 3 hours and allowed to stand in a separating funnel until two layers formed. The top layer contains caryophyllene and continued fractional distillation under reduced pressure (Distillate fraction, DF P= -76 cmHg, R=5:1) and redistilled are needed to isolate of caryophyllene and the results were analyzed using GC-MS (Radiastuti et al., 2011; Wijaya et al., 2015).

### Determination of MIC (Minimum Inhibitory Concentration)

Preliminary screening of antifungal activity of caryophyllene isolated from clove leaf oil was done through agar well diffusion assay. Caryophyllene concentration was diluted using dimethyl sulfoxide (DMSO) to obtain a concentration of 0.1- 0.5%. Spore suspension of *A. fumigatus* isolate was prepared by taking a single fungal disc aseptically with a 5 mm cork borer from sporulating culture and mixed in 20 ml sterile distilled water to which 50 µl of Tween-80 was added. The total number of spores was determined by counting under microscope. Fungal spore count was adjusted to 1 x 10<sup>6</sup> spores/ml by making appropriate dilutions. Petriplates containing agar media were inoculated aseptically with 500 µl spore suspension through spread plate technique and kept in the laminar flow for 30 min. Wells of 5 mm diameter were punched in the center of the petriplates by using sterile cork borer. Into each well, 10 µl of the each diluted caryophyllene was dropped. After the samples were distributed on the agar media, the fungal colonies were then placed on the media and incubated at 37°C until one week. Ketoconazol at the concentration of 1 mg in 1 ml of DMSO was used as positive control. DMSO without caryophyllene was set as negative control.



The diameter of the zone of inhibition around each well was measured in mm. MIC was determined for *A. fumigatus* strains through agar dilution method. Plates were inoculated in the center with mycelial disc (4 mm) and incubated for 7 days followed by measurement of mean radial colony diameters. Three replicates were made for each treatment. The plate with lowest concentration of caryophyllene, showing no visible growth was regarded as the MIC (Lee et al., 2007; Choonharuangdej et al., 2021).

### Synthesis and coating of mahogany leaves composite

Mahogany leaf composite has been synthesized from mahogany leaves by using a polyurethane binder and glass fiber as reinforcement. 15 gram of mahogany leaves waste powder was mixed with 2.5 of polyurethane and 0.4 gram of fiberglass until evenly distributed. Composites are pressed using a hydraulic press with a pressure of 5000 Pa for 15 minutes. The dried composite was then coated with the dipping method for 30 seconds. Data from MIC of caryophyllene were used in composite coating. The concentration of oil used is the concentration that has been determined in the MIC test (Masturi et al., 2020).

### Antifungal assay of composite coated caryophyllene

Antifungal potential of composite coated caryophyllene was determined through agar well diffusion assay. Caryophyllene concentration based on data MIC was diluted using DMSO. Ketoconazol was used as positive control. DMSO without caryophyllene was set as negative control. *A. fumigatus* strains were placed on composites coated with essential oils and incubated at 37°C. The zone of inhibition was measured after 24h of incubation. Observation using a CCD camera (Charge Couple Device) microscope with a magnification of 50x shows the growth of fungal spores on the composite surface (Lee et al., 2007; Choonharuangdej et al., 2021).

### Statistical analysis

The experimental data was performed using one-way analysis of variance (ANOVA). Data were expressed as the mean ± standard deviation (n = 3). Homogenous groups and the least significant difference (LSD) were determined at the significance level of  $p \leq 0.05$ . All statistical analyses were performed using SPSS 24 software.

## RESULT AND DISCUSSION

### Clove oil and caryophyllene characterization

The term essential oil is used to refer to highly volatile substance isolated by steam distillation from an odoriferous plant of a single botanical species. This method is the favorite method for essential oil extraction process was steam distillation. In this study, the clove leaf oil from leaves of *Syzygium aromaticum* L. was extracted by the hot steam that applied in this method. The final of steam distillation process will be condensed and separated into the liquid form that was the mixture of oil and steam. The oils were more dense than water and could be separated using proper method and instruments.

The oil are yellow with aroma freshly distilled and spicy. This essential oil yielded varied from 0.976 to 0.989 and shown in **Table 1**. The physical properties of clove leaf oil shown in **Table 2**. They are only slightly soluble in water and dissolve fairly well in ethanol and mixed very well with vegetable oils, glycerol, and propylene glycol. The solubility of clove leaf oil shown in **Table 3**.

**Table 1. The yield of clove leaf oil (*Syzygium aromaticum* L.)**

Parameter	Clove leaf oil					Mean	S.D
	I	II	III	IV	V		
Sample Weight (kg)	10.00	10.20	10.20	10.00	10.01	10.01	0.05
Content of Oils (g)	97.60	99.45	99.90	98.90	99.03	98.98	2.17
Yield (%)	0.976	0.975	0.979	0.989	0.989	1.01	0.05

**Table 2. The physical properties of clove leaf oil (*Syzygium aromaticum* L.)**

Parameter	Clove leaf oil				
	I	II	III	IV	V
Appearance	Oily liquid				

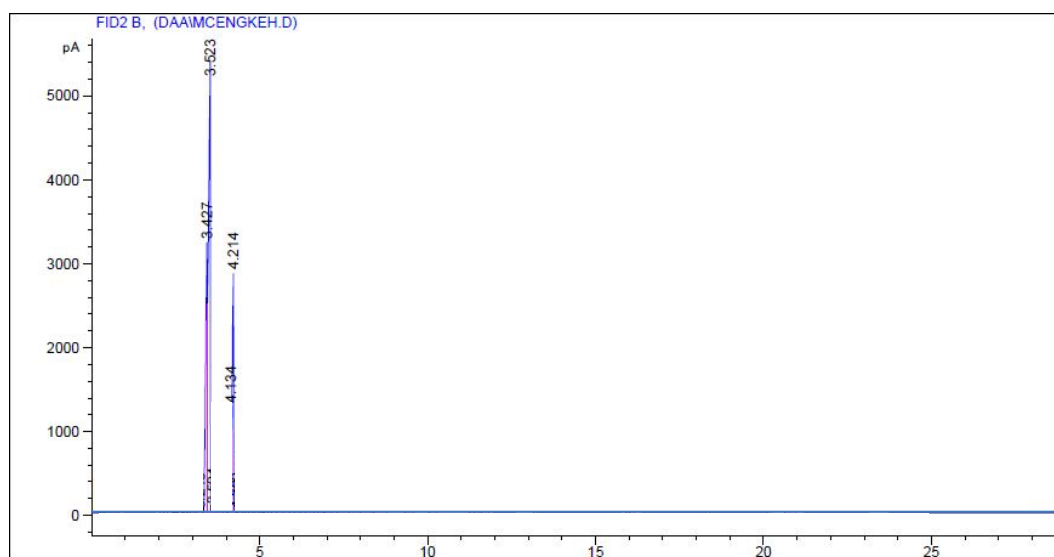


Color		Yellow			
Odor		Spicy, woody, warm, terpenic			
Refractive index at 20 °C	1.529	1.525	1.526	1.528	1.528
Specific gravity at 25 °C	1.027	1.030	1.026	1.025	1.024

**Table 3. The Solubility of clove leaf oil (*Syzygium aromaticum* L.)**

Solvent	Volume ratio (Oil : Solvent)	Java citronella oil				
		I	II	III	IV	V
Water	1 : 1			Immiscible		
	1 : 5			Cloudly		
Ethanol	1 : 1			Miscible		
Ethanol 70%	1 : 2			Miscible		
Chloroform	1 : 1			Miscible		
Ether	1 : 1			Miscible		
Petroleum ether	1 : 1			Miscible		
Vegetable oil	1 : 1			Miscible		
Glycerol	1 : 1			Miscible		
Propylene glycol	1 : 1			Miscible		

The combination of gas chromatography and mass spectrometry (GC-MS) allows rapid identification of essential oil components, provided that these compounds are already known and their mass spectra available in a library. According to the data of GC-MS analysis, we investigated essential oil in leaves of *Syzygium aromaticum* L. consist mostly of eugenol and caryophyllene compounds. The chromatogram of clove leaf oil shown in **Figure 1**.

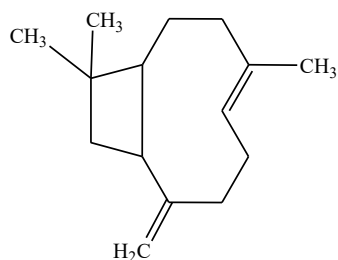


**Figure 1. Chromatogram of clove leaf oil**

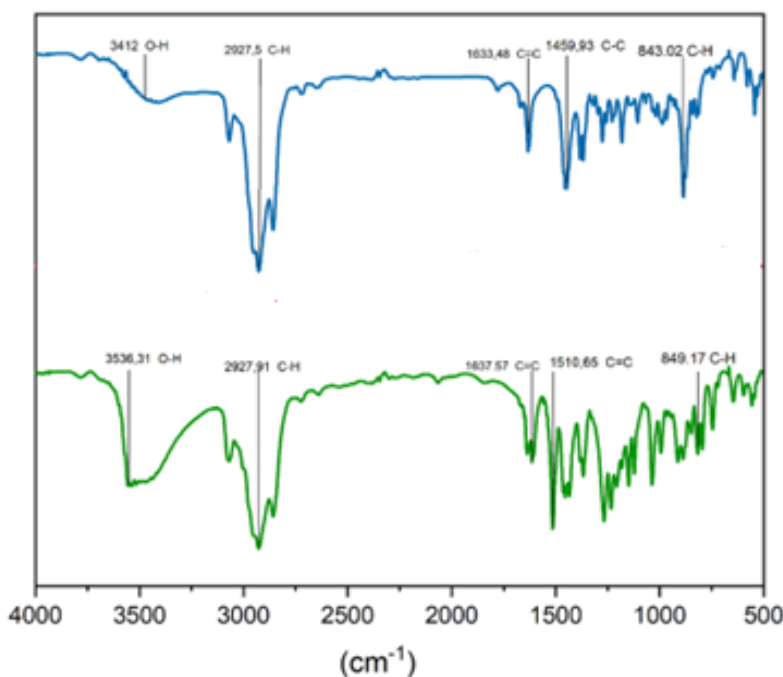
Based on **Figure 3**, clove leaf oil consist of eugenol and caryophyllene has yielded 42.83% and 23.87%, respectively. Clove leaf oil from the distillation is included in the category of bad oil because according to Indonesian standar (SNI) good clove leaf oil has a minimum eugenol content of 78% and a maximum caryophyllene content of 17% (Jayanudin, 2011), this is a natural thing because the material used is waste clove leaf, so it will definitely not reach SNI.

Caryophyllene is a by-product of eugenol isolation from clove leaf oil with a strong base and is a non-phenolic or tepenoid compound (Riyanto et al., 2016; Tahir et al., 2020). Caryophyllene was obtained by selective extraction using sodium hydroxide solution (2,5% NaOH, the ratio of oil to solution is 1:5) and fractional distillation under reduced pressure (Distillate fraction, DF P= -76 cmHg, R=5:1) and continued redistilled are needed to isolate of caryophyllene (Putra et al., 2014). Redistilled can improve the purity, then distillate collected while the temperature changed with a purity of 99.10%. Caryophyllene has the molecular

formula  $C_{15}H_{24}$  with the structure presented in **Figure 2** and confirmation of its structure is evidenced by the FTIR spectra shown in **Figure 3**. The result of caryophyllene purification is shown in **Figure 4**.



**Figure 2. Structure of Caryophyllene**



**Figure 2. Fourier Transform Infrared (FTIR) spectrum of ( — ) clove leaf oil dan ( — ) caryophyllene**

The transmittance spectra inform the hydroxyl group (-OH) in the wave number in the 3650-3100  $cm^{-1}$  region with absorption peaks in the 3536.31  $cm^{-1}$  region caused by stretching or stretching O-H (Nandiyanto et al., 2019; Valderrama et al., 2017) and the more sloping in the caryophyllene, which means that the -OH group decreases in the caryophyllene. Both spectra have sharp peaks in the spectral range of 2935-2915  $cm^{-1}$ . The absorption in this area was identified due to the asymmetric methylene C-H range which showed the presence of functional groups belonging to the methylene group. In addition, there is an absorption band in the area of 1680-1620  $cm^{-1}$  which indicates a stretching of the C=C alkene group. In the clove leaf oil spectra there is absorption in the 1510-1450  $cm^{-1}$  area which indicates the presence of an aromatic compound which is included in the C=C-C group part of the aromatic ring (aryl group), the aromatic compound is indicated by an absorption band of 1510.65  $cm^{-1}$  at clove leaf oil.

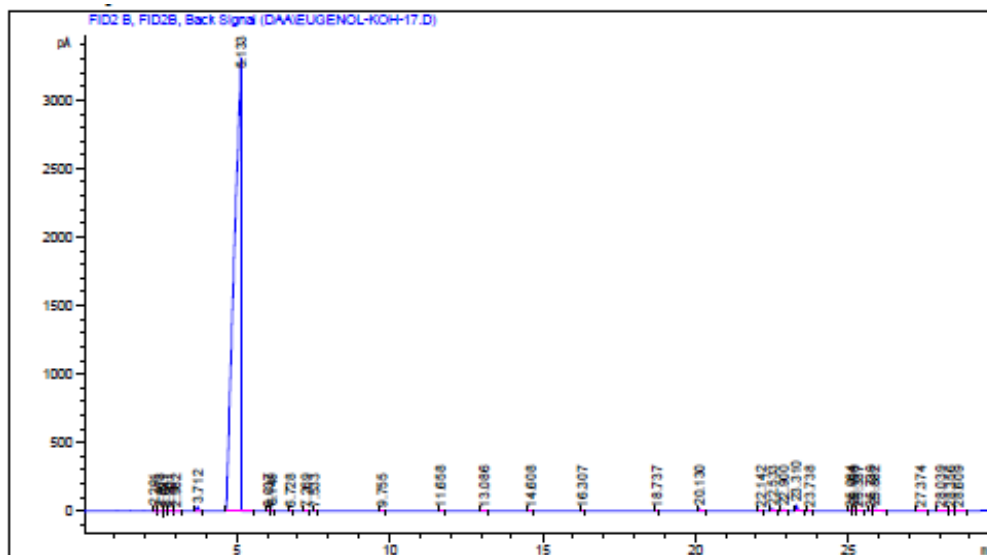


Figure 4. Chromatogram of caryophyllene

### 2. Characterization of mahogany leaves composite coated with caryophyllene

In making mahogany leaf composite using a simple mixing process, mahogany leaf powder was mixed with polyurethane and fiberglass. The dried composite was then coated with the dipping method for 30 seconds. Data from MIC of caryophyllene were used in composite coating. The concentration of oil used is the concentration that has been determined in the MIC test (Masturi et al., 2020). The results of the composite mahogany leaf are round, have denser pores, and have a smoother surface, with a diameter of  $\pm 5$  cm and a width of  $\pm 1.5$  cm, as shown in Figure 5.



Figure 4. mahogany leaves composite coated with caryophyllene

### 3. Antifungal assay on composite coating with caryophyllene

The process of coating caryophyllene on the composite has been completed. The concentration of caryophyllene oil used in the coating process was based on the MIC test concentration. Caryophyllene exhibited antifungal activity against fungal species *fumigatus*. Caryophyllene works by involving the fungal membrane structure that affects the growth of its mycelium. Table 1 data on the results of the MIC test of caryophyllene against the fungal species *A. fumigatus* in the concentration range of 0.2-1.2% for 24 hours of incubation.

**Table 4. Minimum Inhibitory Concentration (MIC) of caryophyllene against *Aspergillus fumigatus***

Caryophyllene oil concentration (%)	Fungal diameter colony (cm)
0,2	0,8
0,3	0,9
0,4	1,2
0,5	0,8
0,6	0,8
0,7	1,4
0,8	1,4
0,9	0,5
1,0	1,4
1,2	0,4

Based on the percentage of inhibition, it is known that caryophyllene can inhibit the fungus at a minimum concentration of 1.2%. The time of caryophyllene oil affects cell wall damage, which directly correlates with fungal cell death. The damage to the cell wall shown through the compound will damage the polar group (phosphate group) so that the phospholipid molecule breaks down into glycerol, carboxylic acid, and phosphoric acid. Therefore, the phospholipids cannot maintain the shape of the cell membrane, and the cell dies. Increasing the concentration of caryophyllene used, the lower the diameter of fungal growth, and the higher the concentration of an antifungal agent, the higher the antifungal activity. The results of the preliminary screening clearly indicate that caryophyllene has antifungal activity towards tested *A. fumigatus* fungal. Spore growth of *Aspergillus fumigatus*, which has been dripped on the composite surface, is observed in microscopy using a CCD camera microscope with a magnification of 50x. The activity of caryophyllene oil, which is expected to function as antifungal in mahogany leaf composites.

## CONCLUSION

Caryophyllene was obtained by using selective extraction with sodium hydroxide solution (2,5% NaOH, the ratio of oil to solution is 1:5) and fractional distillation under reduced pressure (Distillate fraction, DF P= -76 cmHg, R=5:1) and continued redistilled are needed to isolate of caryophyllene. Redistilled can improve the purity, then distillate collected while the temperature changed with a purity of 99.10%. The results of the clearly indicate that caryophyllene has antifungal activity towards tested *A. fumigatus* fungal at a concentration of 1.2%. Spore growth of *Aspergillus fumigatus*, which has been dripped on the composite surface, is observed in microscopy using a CCD camera microscope with a magnification of 50x. The activity of caryophyllene oil, which is expected to function as antifungal in mahogany leaf composites.

## CONFLICT OF INTEREST

There are no conflicts of interest.

## ACKNOWLEDGEMENT

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