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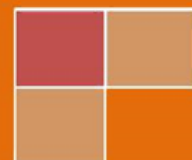
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Gum Benzoin (*Styrax benzoin*) as Antibacterial against *Staphylococcus aureus*

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Abstract: Gum benzoin (*Styrax benzoin*) is one of nontimber forest product classified into the resin group. The aim of the study was to determine the component from gum benzoin as an antibacterial against *Staphylococcus aureus*. Indonesian gum benzoin was obtained from North Sumatra and has antibacterial activity against *S. aureus* with the MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) values of its ethyl acetic extract of 1.00 mg/mL and 2.00 mg/mL, respectively. Thin layer chromatography (TLC) was used in purification of ethyl acetate extract of the gum benzoin. The active component was detected by TLC contact bioautography. The bands with *R_f* of 0.21, 0.77, and 0.87 had growth inhibition activity to the *S. aureus*. The active antibacterial band with *R_f* of 0.21 was isolated and it could be an alkaloid component.

Keywords: antibacterial, gum benzoin, *Staphylococcus aureus*, TLC, TLC-bioautography

1. INTRODUCTION

Benzoin tree is the only tree that produces gum containing balsamic acid. This compound is widely used in cosmetic and perfume industries as a fixative agent. Several studies have been conducted on some species of benzoin and they indicated that the benzoin was potential as a medicinal plant Castel et al. (2006).

Some reports related to the potency as medicinal plants had been reported. Acetone extract of *Styrax formosanum* has activity as an antioxidant by DPPH method with 50% inhibitory concentration value (IC₅₀) of 31.5 µg/mL Hou et al. (2003). Extract from *Styrax japonica* and its nonpolar fraction shows insecticidal activity against larvae of *Culex pipiens* Yamaguchi et al. (1950) and its essential oil has activity as an inhibitor of *Bacillus cereus*, *Salmonella typhimurium*, and *Staphylococcus aureus* growth Kim et al. (2004). The Essential oil of *Styrax tonkinensis* wood has bacteriostatic activity with minimum inhibitory concentration (MIC) of 0.78 mg/mL against *Aspergillus flavus* and *Aspergillus niger* (Shin, 2003), while the essential oil of *Styrax ferrugineus* leaves has activity as an antifungal against *Candida albicans* and *Cladosporium sphaerospermum* with MIC value 200

µg/mL and has activity as antibacterial against *Staphylococcus aureus* with MIC value 800 µg /mL Pauletti et al. (2000).

In Indonesia, the province of North Sumatra is one of the largest region that produces gum benzoin. According to Jayusman (1997) there are two types of benzoin in North Sumatra those are Toba Benzoin (*Styrax sumatrana*) and Durame Benzoin (*Styrax benzoin*). Both types of them belong to the order of *Ebenales*, a family of *Styraceae* and genus of *Styrax*. Sumatra gum benzoin contains benzaldehyde, styrene, cinnamic acid and the derivated of triterpenoids such as siaresinolic acid and sumaresinolic acid Castel et al. (2006). The cinnamic acid in the gum benzoin (*Styrax benzoin var Hiliferum*) reported that has biological activity as antibacterial, anesthetic, anti-inflammatory, antispasmodic, antimutagenic, fungicide, herbicide, tyrosinase inhibitor, and low-density lipoproteins (LDL) synthesis inhibitor (Kiswando et al. 2016; Teissedre and Waterhouse, 2000). In addition, the fraction obtained from the balsamic acid resin of *S. benzoin* shows immune stimulant activity in endoplasmic reticulum system inoculated with *Escherichia coli* Delaveau et al. (1980). Lots of activities from gum benzoin had been reported including as antibacterial, the active component from Sumatera gum benzoin is still less studied. It is necessary to conduct research on the activity of the Sumatera gum benzoin as an antibacterial and to identify its active component.

2. METHOD

Extraction

The gum benzoin used in this study was collected from North Sumatra. The gum was extracted by maceration technique using *n*-hexane as the solvent, with 2:1 ratio, for 24 hours. Subsequently, the resulting residue was extracted with 24 hours of ethyl acetate solvent. The filtrate was dried with a rotary evaporator at a temperature of 50 °C to get ethyl acetate extract.

Phytochemical Analysis

The method for phytochemical analysis was performed according to Harbone (1987). The methods are a qualitative method to identify alkaloid, phenol, flavonoid, saponin, tannin, steroid, and triterpenoid.

TLC Contact Bioautography

This method according to Yulianty et al. (2011) with some modification. About 10 µL ethyl acetate extract 2% was applied on TLC G₅₀F₂₅₄ plate (Merck, Darmstadt, Germany) using CAMAG Linomat 5 (CAMAG, Muttenz, Switzerland). The TLC plate was eluted for 15 minutes in the chamber with dichloromethane: diethyl ether: *n*-hexane (9:1:1) as the mobile phase. The chromatograms were documented by a Reprostar 3 documentation device that integrates winCATS firewalls. Then the TLC plate was attached to the growing bacteria suspension on agar medium (TSA) for several hours then the medium was recirculated at 37 °C for 24 hours. Furthermore, the medium was observed and the presence of clear white zone on the medium indicated the presence of antibacterial activity. The area

of inhibition of the chromatogram was compared with a previously detected chromatogram with UV light at λ 254 nm to determine the value of R_f having inhibitory activity.

Fractionation

Fractionation of ethyl acetate extract was done by using 16.00 g of silica gel to separate 1.00 g extract using column diameter 1.5 cm and height of column 47 cm. The ethyl acetate extract of the gum benzoin was separated by an isocratic system of elution. Fractions have high antibacterial activity and weight are further separated using preparative thin layer chromatography to obtain the most active single-stained fraction.

Antibacterial Assay

This method according to Batubara et al. (2009) with some modification. The antibacterial activity detection in the sample was done by microdilution method with a liquid medium (TSB) on the microplate. A sample of 100 μ L with a particular concentration is added to each well of a 96-well plate. Each well was added 100 μ L TSB medium and 20 μ L of inoculum. Then the mixture was incubated in the medium for 24 hours. Extract concentration at which there was no visually detectable bacteria growth was described as the minimum inhibitory concentration (MIC). Then, 100 μ L of each medium with no visually detectable bacteria growth was inoculated in 100 μ L of fresh medium. The concentration at which there was no bacteria growth after the second inoculation was described as the minimum bactericidal concentration (MBC). The negative control used was DMSO The positive control used was tetracycline and the negative control used was DMSO 20%.

Characterization by FTIR

This method according to Hidayat (2011). The most active fraction of 2 mg was added pure KBr powder 200 mg then stirred until blended. The mixture was placed in a mold and pressed using a mechanical suppressor. The KBr pellet that has been formed is placed in the sample spot on the FTIR spectrophotometer.

3. RESULTS AND DISCUSSIONS

Gum benzoin is produced when the bark of benzoin tree is injured. During the production, the gum benzoin produces biological activity to prevent the gum from pathogen attack including from bacteria (Pauletti et al. 2000). The ethyl acetate extract of gum benzoin was chosen for further analyzed because it had been considerable yield compared with *n*-hexane extract. It defined that the semipolar component was large amount compared with the nonpolar component in the Sumatera gum benzoin. The ethyl acetate extract of gum benzoin contained an alkaloid, flavonoid, and phenolic component (Table 1).

Table 1. Phytochemical of ethyl acetate extract of the gum benzoin

| Secondary Metabolite | Result | |
|----------------------|--------|--|
| Alkaloid | + | Note: +: detected and -: is not detected |
| Flavonoid | + | |
| Phenolic | + | |
| Saponin | - | |
| Steroid | - | |
| Triterpenoid | - | |

Detection of antibacterial activity on ethyl acetate extract of gum benzoin was applied to *S. aureus* and *E. coli* bacteria using microdilution method. Microdilution method is a useful method to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values of the sample against antibacterial activity (Choma and Grzelak, 2011). The MIC is the lowest concentration that can inhibit bacterial growth (Jorgensen and Ferraro, 2009). Meanwhile, the MBC is the lowest concentration that can kill 99.9% bacteria inoculated Balouiri et al. (2016).

The ethyl acetate extract of the gum benzoin had ability to inhibit the growth of *S. aureus* whereas in *E. coli* was not. The results of antibacterial activity on *S. aureus* showed that it could be as bacteriostatic and bactericidal with MIC and MBC values of 1.00 mg/mL and 2.00 mg/mL, respectively and not as good as tetracycline as a positive control (Table 2). However, antibacterial activity against *E. coli* requires MIC and MBC values higher than 4.00 mg/mL. This suggests that *S. aureus* (gram-positive) bacteria was more susceptible to ethyl acetate extract of gum benzoin compared with *E. coli* (gram-negative) bacteria. For the next, *S. aureus* bacteria were selected for further analysis of antibacterial detection.

Table 2. Extraction yield and antibacterial activity of gum benzoin

| Sample | Yield (%) | MIC (mg/mL) | | | MBC (mg/mL) |
|--------------------------|-----------|------------------|----------------|--|------------------|
| | | <i>S. aureus</i> | <i>E. coli</i> | | <i>S. aureus</i> |
| <i>n</i> -Hexane extract | 1.44 | >4.0 | >4.0 | | >4.0 |
| Ethyl acetate extract | 73.21 | 1.0 | >4.0 | | 2.0 |
| Fraction 6 | 4.47 | 1.0 | - | | 4.0 |
| Isolated component | 0.63 | 1.0 | - | | >4.0 |
| Tetracycline | 0 | 0.00125 | - | | 0.01 |

-: the test did not perform

The component profile of ethyl acetate extract by thin layer chromatography was shown in Figure 1. The targeted component based on the TLC profile was a spot with R_f 0.21, 0.77, and 0.87. To collect the spot, open column chromatography using the silica gel as the stationary phase was used.

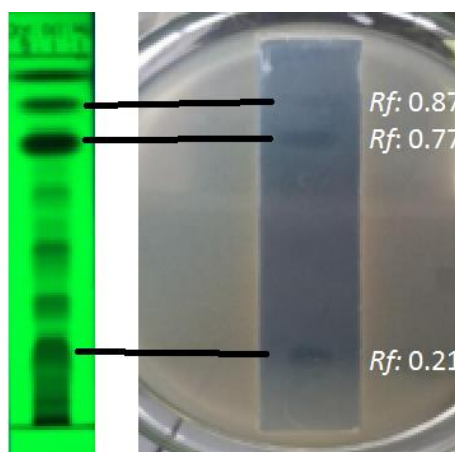


Figure 1. TLC Chromatogram ethyl acetate extract of the gum benzoin by mobile phase dichloromethane:diethyl ether:*n*-hexane 9:1:1 (v/v/v) with R_f information visualization on UV 254 nm (a) and (b) bioautography contact against *S.aureus*

The fractionation by column chromatography yielded 7 fractions. The chromatogram pattern of the 7 fractions was shown in Figure 2 and the weight of the fraction as well as the resulting yields could be seen in Table 3.

Table 3. The yield of the fractions of gum benzoin ethyl acetate extract

| No Fractio n | R_f of bands | | Weight (g) | Yield (% w/w) |
|--------------------|---|---|---------------|---------------------|
| | 254 nm | 366 nm | | |
| 1 | 0.10, 0.77, 0.90 | 0.43, 0.51, 0.62, 0.77, 0.93 | 0.0248 | 2.3952 |
| 2 | 0.10, 0.65, 0.77, 0.90 | 0.43, 0.63, 0.74, 0.83, 0.93 | 0.0286 | 2.7622 |
| 3 | 0.12, 0.43, 0.53, 0.65, 0.77, 0.92 | 0.40, 0.52, 0.63, 0.77, 0.93 | 0.0201 | 1.9413 |
| 4 | 0.28, 0.43, 0.53, 0.59, 0.65, 0.77, 0.92 | 0.02, 0.40, 0.52, 0.62, 0.67, 0.74, 0.84, 0.93 | 0.0208 | 2.0089 |
| 5 | 0.28, 0.43, 0.65, 0.92 | 0.40, 0.62 | 0.0228 | 2.2020 |
| 6 | 0.28, 0.43, 0.65, 0.77, 0.92 | 0.40, 0.62, 0.93 | 0.0463 | 4.4717 |
| 7 | 0.12, 0.30, 0.59, 0.65, 0.92 | 0.40, 0.63, 0.93 | 0.0226 | 2.1827 |

Each fraction showed the difference in the color of the band at UV 366 nm. Different colors of bands indicated different compounds. According to Markham (1998), in UV 366 nm, the presence of blue color indicates the presence of flavon, flavonon, or flavonol compounds, red anthocyanidin compounds, and green auron and flavon compounds. The fraction resulted from column chromatography separation had a different value of inhibitory zones (Figure 2). Fraction 1 and 7 had one clear white zone, and Fraction 2 to 6 had 2 clear white zones. Fraction 1 had an inhibitory zone of the band with a value of R_f 0.77, Fraction 2 had inhibitory zones in the band with values of R_f 0.10 and 0.63, Fraction 3 to 6 had inhibitory zones in the band with R_f values of 0.28 and 0.65, and fraction 7 had an inhibitory zone at band with the value R_f 0.12.

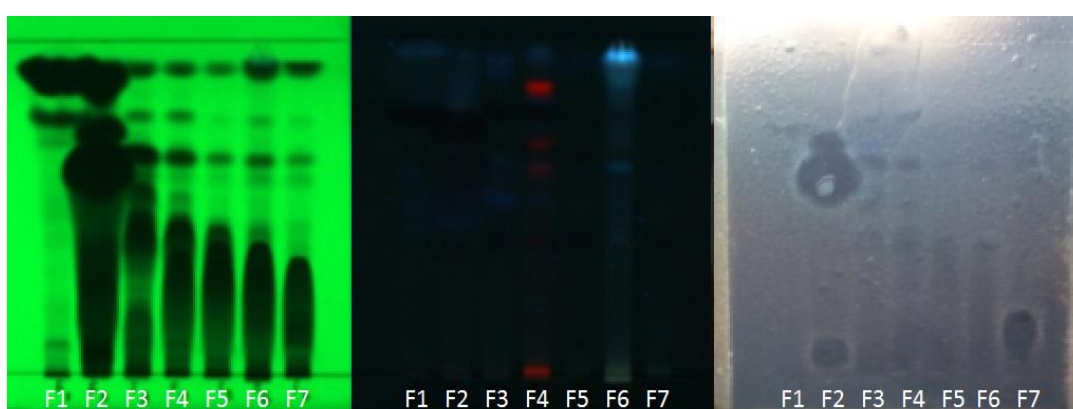


Figure 2. TLC Chromatogram of Fraction 1-7. Visualization on (a) UV 254 nm, (b) UV 366 nm, and (c) bioautogram

According to the result of detection and the yield of the fractions, Fraction 6 was selected to separate more with TLC preparative. The result of TLC preparative on fraction 6 yielded 4 bands that active based on the R_f value (R_f of 0.12, 0.28, 0.63, and 0.77) (Figure 3). Fraction 6.1 was collected from the band with R_f of 0.12. Based on the TLC chromatogram of Fraction 6.1, this fraction is not a single component because it has more than 1 spot (Figure 4).

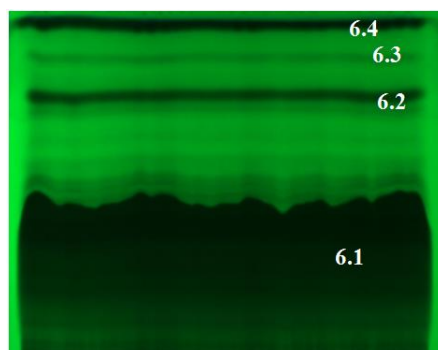


Figure 3. TLC preparative chromatogram of fraction 6 with visualization UV 254 nm

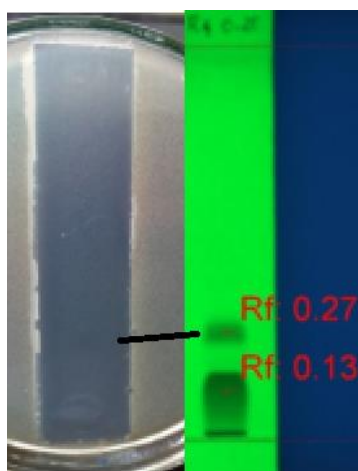


Figure 4. Chromatogram of isolated component (Fraction 6.1) with visualization on bioautogram contact against *S. aureus* (left) and on UV 254 nm (right)

Bioautogram Fraction 6.1 against *S. aureus* result indicated that the presence of antibacterial activity was characterized by the appearance of the clear white zone on the band with R_f 0.27 (Figure 4). This result was compared with contact bioautogram of ethyl acetate extract (Figure 1). The result showed that the clear white zone produced by its R_f value was almost the same between the contact bioautogram of ethyl acetate extract and the bioautogram of the 1st band. This indicated that the compound which has been isolated had antibacterial activity.

The MIC and MBC values which were obtained from the isolated component were 1.00 mg/mL and more than 4.00 mg/mL. Antibacterial activity on isolated component was lower than ethyl acetate extract and Fraction 6 (Table 1). The decrease in antibacterial activity on isolated component due to the separation of Fraction 6 into simpler fractions that could cause no synergistic effect. The value of MIC and MBC in the ethyl acetate extract, Fraction 6, and also isolated component were higher than tetracycline, which means not as strong as tetracycline as an antibacterial agent.

The isolated component which was obtained from TLC preparative was analyzed using UV-visible spectrometer and an infrared spectrometer. The result showed that the isolated component had a maximum absorbance at 270 nm wavelength (Figure 5a). These results indicated that transition π - π^* was a typical chromophore for conjugated double bonds system or aromatic ring. The absorption at wavelength 230-270 nm was uptake for benzene (Fessenden and Fessenden, 1982).

Isolated component (Fraction 6.1) which was obtained from TLC preparative was analyzed using an infrared spectrophotometer (Figure 5b). The analysis using infrared spectrophotometer gave information on the presence of aromatic C=C strain shown by uptake at wave numbers 1493 cm^{-1} and 1631 cm^{-1} and strained C-H at 3024 cm^{-1} . This was supported by UV-visible spectrum data showing the occurrence of π - π^* transitions at a maximum wavelength of 270 nm. The

wavelength was a typical chromophore for a conjugated double bond system or in an aromatic ring (Fessenden and Fessenden, 1982). Based on the existing functional group, it could be presumed that the antibacterial active isolated compound was an aromatic alkaloid group. This was supported by uptake at 3394 cm^{-1} which were the uptake of the N-H amine strain. Another possibility was the C-N bond between aliphatic N and C aliphatic was suspected from $2924\text{-}2853\text{ cm}^{-1}$ absorption detected as a C-H alkane bond. In addition, the presence of a C-N strain of amine at wave numbers $1231\text{-}1205\text{ cm}^{-1}$

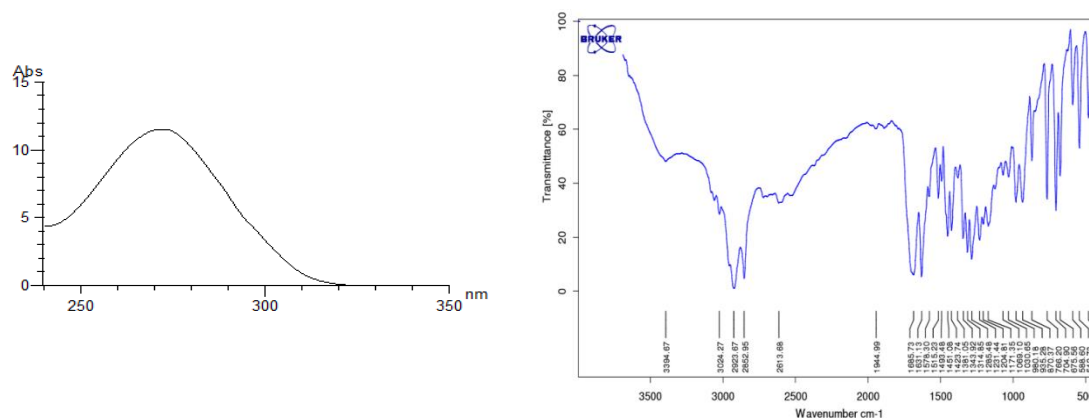


Figure 5. UV-Vis spectrum (a) and IR Spectrum (b) of the active antibacterial isolated compound from gum benzoin

4. CONCLUSION AND SUGGESTION

The ethyl acetate extract of gum benzoin has antibacterial activity against *S. aureus* based on TLC-contact bioautography (R_f 0.21, 0.77, and 0.87 is active) and microdilution test results (MIC and MBC values of 1.00 mg/mL and 2.00 mg/mL). The Fraction 6 has MIC and MBC value of 1.00 mg/mL and 4.00 mg/mL, while the isolated component has MIC and MBC of 1.00 mg/mL and higher than 4.00 mg/mL. The results of UV-visible identification and FTIR are suspected that the antibacterial active isolated compound of gum benzoin is an alkaloid group.

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