Antibacterial Agents of *Terminalia muelleri* Benth. Leaves

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**Abstract:** The objective of this study was to know the antibacterial compound from *T. muelleri*. Extraction of leaf of *T. muelleri* using solvents of increasing polarity, namely, n-hexane, ethyl acetate and methanol, respectively, yielded dry extracts. The extracts were tested for antibacterial activity. Ethyl acetate extract exhibited the strongest activity against standard strains of *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. The ethyl acetate extracts had been fractionated, yielded 9 fractions. The fraction EH exhibited the strongest activity against *S. aureus*. The separation of chemical contents of fraction EH was carried out by Sephadex LH-20 column chromatography, yielded a pure substance. The substance was identified as 3,4,5-trihydroxybenzoic acid by chromatographic and spectroscopic analysis. This substance is a well-known antibacterial activities. The activity of 1 mg 3,4,5-trihydroxybenzoic acid was equal to 0.1396 µg tetracycline HCl against *S. aureus* and 0.6455 µg against Methicillin-Resistant *Staphylococcus aureus*, also equals to 40.6035 µg penicillin G against *S. aureus* and equals to 2.9823 and 2.1213 µg vancomycin HCl against *S. aureus* and Methicillin-Resistant *Staphylococcus aureus*, respectively.

**Key words:** *Terminalia muelleri*, Combretaceae, antibacterial, gallic acid, *S. aureus*, MRSA

**INTRODUCTION**

*Terminalia muelleri* Benth. or *ketapang kencana* is classified in to genus *Terminalia*, family Combretaceae. This flowery plant came from Australia and is widely spread in tropical area including India, Indonesia, Malaysia, New Guinea and also can be found in North America (Lemmens and Wulijarni-Soetjipto, 1992). Genus *Terminalia* was reported containing cyclic triterpene and its derivatives, flavonoid, tannin and other aromatic agent. Some of the compounds were known to have antifungi, antibacterial, anticancer and hepatoprotector activity (Kandil and dan Nassar, 1998; Tang et al., 2006; Srivastava et al., 2001).

Studies to many kinds of *Terminalia* showed that this plant genus was potential as antimicrobial source, as reported by Masoko et al. (2005), in which acetone extract of *T. prunioides*, *T. brachystemma*, *T. sericea*, *T. gazensis*, *T. mollis*, and *T. Sambesiaca* leaves inhibited the growth of *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Microsporum canis* and *Sporothrix schenckii*. Methanol extract of *T. trifolia* was active against *Hyalohyphomycetes* (Muschiatti et al., 2005) and the polar and semipolar
extract from the root of *T. sericea* were active against fungi *Candida albicans* and *Aspergillus niger* as also against bacteria *Staphylococcus aureus*, *Escherichia coli* and *Bacillus anthracis* (Moshi and Mbwambo, 2005). Suganda et al. (2004) reported that ethanol extract from the falling leaves and fresh leaves of *T. catappa* were active against microbes. This study supported Goun’s et al. (2003) statement that methanol extract of *T. catappa* leaves from Bogor Botanical Garden had a high activity to fungi. Furthermore, Suganda et al. (2006) also reported that *T. muelleri* had the strongest activity as antimicrobes (*in vitro*) compared to others 11 kinds of *Terminalia* in Indonesia. Anam et al. (2009) completed the report and stated that ethyl acetate extract from *T. muelleri* leaves had stronger activity against *S. aureus*, *E. coli* and *C. albicans* compared to n-hexane and methanol extract.

In this opportunity, will be reported antibacterial agent from *T. muelleri* Benth. leaves which was directly isolated, guided by antibacterial test. Basically, the aim of this study was to elucidate the agent responsible to the antibacterial activity of *T. muelleri* leaves.

**MATERIALS AND METHODS**

**General**

Vacuum Liquid Chromatography (VLC) was performed on a Silica gel 60 H (Merck EM 7734) column, TLC analysis was carried out with silica gel 60 HF<sub>254</sub> (Merck EM 5554) and 60RP-18 F<sub>254</sub>S (Merck EM 5559) plates. Column Chromatography was performed on a Sephadex LH-20 column (Amersham Biosciences 17-0090-01). Spots were visualized under UV light (254 and 365 nm), by spraying with sulfuric acids 10% or iron(III) chloride (10%). The references of the antibiotics compounds were tetracycline HCl (ASEAN Reference substance, control no. 1195013, vancomycin HCl (MP Biochem 195540) and Pencillin G (Sigma P-7794)). Molecular Mass was recorded by LC-MS ESI Mariner Biospectrometry. UV-Vis absorption spectra were recorded in methanol on spectrophotometer UV-Vis Hewlett Packard HP 8452. IR spectra were recorded using KBr discs on a spectrophotometer FT-IR JASCO 4200, <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded by spectrometer JEOL JNM ECA-500, operating at 500 MHz (<sup>1</sup>H and <sup>13</sup>C), in CD<sub>3</sub>OD, solution with TMS as an internal reference.

**Plant Material**

*Terminalia muelleri* Benth. leaves were collected from Bogor Botanical Gardens, West Java, Indonesia, in March 2006. The plant was determined by The Center for Plant Conservation-Bogor Botanical Garden, Indonesian Institute of Sciences.

**Test Organisms Used**

*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis*, *Sarcina lutea* ATCC 9341 and Methicillin-Resistant *Staphylococcus aureus* (MRSA), were obtained from Microbiology Laboratory, PT Bio Farma, Bandung, Indonesia.

**Preparation of Extracts**

The dried milled leaves (2.176 kg) were successively extracted with n-hexane, ethyl acetate and methanol, at room temperature. The extracts were evaporated to dryness with a rotary evaporator to give 81.16, 252.69 and 486.795 g of n-hexane, ethyl acetate and methanol extract, respectively.

**Antibacterial Activity Test**

The extract, fraction and pure compound were tested to screen their antimicrobial activity against *S. aureus* and *E. coli* Antimicrobial activity was determined by disc diffusion
method (Chanwitheesuk et al., 2007). Discs with the solvents used for extraction were used as negative controls, while tetracycline was used as positive controls. Results of qualitative screening were recorded as the average diameter of the inhibition zone around the discs. The values were averages of three measurements disc⁻¹, taken at three different directions. The inhibition zones were expressed as the means of four separation experiments.

**Fractionation of the Pharmacologically Active Extract**

Ethylacetic extract was fractionated with Vacuum Liquid Chromatography (VLC). Silica Gel 60 H was used as stationary phase and n-hexane, dichloromethane, ethyl acetate and methanol as mobile phase gradiently. The extract used was of 70.56 g in weight and the silica was 8.5 cm in height in the column diameter of 6 cm and the mobile phase volume was 100 mL. The fractionation resulted in 31 fractions, these fractions from VLC were analysed by Thin Layer Chromatography (TLC), the mobile phase of n-hexane-ethylacetate (8:2) was used for fractions 1-21, n-hexane-ethylacetate (6:4) for fractions 22-27 and n-hexane-ethylacetate (2:8) for fractions 28-31. Fractions that had the same chromatogram profile were united, fractions 1-3 (EA), fractions 4-5 (EB), fractions 6-9 (EC), fractions 10 (ED), fractions 11 (EE), fractions 12-13 (EF), fractions 14-15 (ES), fractions 16-28 (EH) and fractions 29-31(EI). The obtained fractions were dried and the antibacterial activities were tested. The result of the antibacterial activity test is presented in Table 2.

**Bioautography Test**

Bioautography test of antibacterial activity was conducted by thin layer chromatography. Active fraction with concentration 1% was applied on the TLC plate, then developed with mobile phase which is suitable for compound separation in the pharmacologically active fraction. Chromatogram was stuck to agar medium which was filled with inoculum bacteria and the spots in the chromatogram were traced to the petri dish. The chromatogram was let to stick into the agar medium for 30 min so that, the active compound diffused into the agar medium. Petri dish with inoculum bacteria was incubated for 24 h at temperature of 37°C. After incubation, it can be noticed some transparency spots or inhibitory area appeared and it is considered as the location of the active compound (Betina, 1973; Tyler et al., 1988).

EH active fraction was bioautography tested against *E. coli* dan *S. aureus* bacteria. TLC Silica Gel 60 HF₂₅₄ plate was used as stationary phase and n-hexane-ethylacetate (8:2) as developer to separate the compounds in this fraction.

**Purification of Pharmacologically Active Isolate**

Purification of active compound in EH fraction was carried out by column chromatography method using stationary phase of sephadex LH-20. Four grams of EH fraction was used, with sephadex height 60 cm and column diameter 4 cm. Elution was conducted gradiently with decreasing polarity using mobile phase water-methanol (9:1), (8:2) and (7:3). The 1 L volume for each composition and fraction was held every 15 mL vials⁻¹.

The fractions obtained from the column were analyzed by thin layer chromatography using silica gel 60 HF₂₅₄ with developer ethylacetate-formic acid-water (18:1:1) and the spray reagent used was iron (III) chloride 10%. In vials number 87-98, phenolic compound was found. Solution of eluate in that vial was evaporated using vacuum evaporator, leaving sediment on the wall of evaporator pump. This sediment was crystalized using chloroform-methanol (1:1), result to white-yellow powder labelled as EHJ isolate. 199
Table 1: Antibacterial activity of *T. muelleri* Benth. leaves extract

<table>
<thead>
<tr>
<th>Extract</th>
<th>Minimum inhibition concentration (µg mL⁻¹)</th>
<th>E. coli</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-hexane (H)</td>
<td>1000.00</td>
<td>1000.0</td>
<td></td>
</tr>
<tr>
<td>Ethylacetic (E)</td>
<td>0.01</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Methanol (M)</td>
<td>1000.00</td>
<td></td>
<td>1000.0</td>
</tr>
</tbody>
</table>

Table 2: Antibacterial activity of fractions from ethylacetic extract

<table>
<thead>
<tr>
<th>Fraction (10 mg mL⁻¹)</th>
<th>Inhibition diameter (mm)</th>
<th>E. coli</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>EA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EB</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EC</td>
<td>8.63±0.30</td>
<td>8.00±0.80</td>
<td></td>
</tr>
<tr>
<td>ED</td>
<td>-</td>
<td>8.45±0.25</td>
<td></td>
</tr>
<tr>
<td>EE</td>
<td>-</td>
<td>8.75±0.15</td>
<td></td>
</tr>
<tr>
<td>EF</td>
<td>-</td>
<td>8.61±0.20</td>
<td></td>
</tr>
<tr>
<td>EG</td>
<td>8.23±0.05</td>
<td>8.50±0.13</td>
<td></td>
</tr>
<tr>
<td>EH</td>
<td>8.80±0.40</td>
<td>18.80±1.18</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*: Not detected

Table 3: Antibacterial activity of EHJ isolate

<table>
<thead>
<tr>
<th>Concentration (µg mL⁻¹)</th>
<th>S. aureus</th>
<th>MRSA</th>
<th>B. subtilis</th>
<th>S. haemolyticus</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000</td>
<td>14.0±0.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5,000</td>
<td>12.0±0.2</td>
<td>8.5±0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>2,500</td>
<td>11.2±0.1</td>
<td>7.7±0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1,250</td>
<td>8.7±0.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1,000</td>
<td>8.4±0.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>900</td>
<td>8.1±0.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>800</td>
<td>7.7±0.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>750</td>
<td>7.4±0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>740</td>
<td>7.1±0.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>735</td>
<td>6.9±0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>730</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

*: Not detected

Characterization of the Active Isolate

The EHJ isolate characterization was carried out by ultraviolet spectrophotometry, infrared spectrometry, mass spectrometry and nuclear magnetic resonance spectrometry.

Determination of Minimum Inhibitory Concentration (MIC)

MIC determination was conducted by agar diffusion method. The test material was diluted in to various concentrations and the activity against sample bacteria was tested. The smallest concentration of test material which was still be able to inhibit the growth of the bacteria was determined as Minimum Inhibit Concentration (MIC). The result of MIC determination is presented in Table 1 and 3.

Determination of the Antimicrobial Activity Equivalency with the Reference Antibiotic

The equivalency of extract antimicrobial activity was carried out to compare antibiotic tetracycline HCl, ketokonazole and vankomisin HCl. The method used was similar to the method of the antimicrobial activity test. The sample microbes inhibition diameter of the compared antibiotic was determined in some concentration. The result test is converted to mathematics equation between the inhibition diameter of bacteria growth to logarithm
comparison of antibiotic concentration (Table 4). Based on this mathematics equation, the
equality of EHJ isolate activity to antibiotic is determined by entering the inhibition diameter
value of bacteria or fungi growth which is added with the EHJ isolate in that mathematics
equation. The equivalency antibiotic activity is shown in Table 5.

RESULTS AND DISCUSSION

Antibacterial activity from the three extracts is determined by measuring the minimum
inhibition concentration using the agar diffusion method. Reagent used was DMSO and also
served as negative control, while tetracycline HCl were used as positive control. The
antibacterial activity result test of the extract showed that the three extracts were active
against the sample microbes (Table 1). Ethylacetic extract had the smallest MIC value
followed by n-hexane and methanol extracts, which means that ethylacetic extract is the most
active extract against the sample microbes.

Isolation of antibacterial agent in ethylacetic extract is done directly guided by
bioautography test developed by Betina (1973) and Tyler et al. (1988). Ethyl acetic extract is
fractionated by vacuum liquid chromatography silica gel 60 H with gradient eluent gradient
of increasing polarity, using n-hexane reagent—methylenechloride—ethyl aceta—methanol.
This fractionation aims to simplify the ethylacetic extract by deviding the extract chemical
material into fraction group based on its polarity and interaction with silica gel as stationary
phase in vacuum liquid chromatography. Based on this fractionation results, nine fractions,
which are: EA, EB, EC, ED, EE, EF, E3, EH and EI, were obtained (Fig. 1).

To determine the fraction which contains the active agent, the antibacterial activity of
the fractions is determined by agar diffusion method. Based on the result test as presented
in Table 2, could be stated that EH fraction has the strongest activity to the gram positive
bacteria, S. aureus by inhibition diameter of 18.8±1.18 mm in concentration of 10 mg mL⁻¹.
Thus, the antibacterial active agent isolation was focused on the EH fraction. Considering
the active subfraction still consists of some agents, then bioautography test was carried out
to examine the active agent. This method is very helpful to get the active agent because it
does not need to isolate and test bacterial activity of all agents in the fraction. Bioautography
was used to detect the antibiotic at the first time in 1946 by Goodal dan Levi. Few years later
the bioautography method had been used to detect hundreds of antibiotics that now is used
for medication. Working principal of bioautography test is the diffusion of active agent from
silica plate to the agar medium, so the agent will resist the surrounding microbes growth.
Figure 2 show bioautogram of EH fraction.
Fig. 1: TLC chromatogram of fraction from ethylacetic extract. TLC silica gel 60 HF_{254} eluent: n-hexane-ethylacetic (4:6), spray reagents: sulfuric acid 10%

Fig. 2: Bioautogram of EH fraction

Based on the bioautography result, it was shown that active agent in EH fraction is on the starting line of its TLC chromatogram. This TLC profile is made using n-hexane-ethylacetic (4:6) mobile phase in silica gel 60HF_{254}. To identify the chemical group of this active agent, 10% sulfuric acid was used as universal spray reagent to examine all agents that successfully separated by TLC. Spray reagents used were Iron(III)chloride 10% for phenolic compounds and cerium(IV)sulphate for compounds which have benzopirone core, anisaldehyde for terpenoid, vanillin 10% and HCl for flavonoid and ammonia vapour was also
used for flavonoid. Based on this spot, the active agent in EH subfraction is categorized as phenolic compounds.

Isolation of phenolic compounds in EH fraction was carried out by column chromatography method using stationary phase sephadex LH-20 and gradient elution, H₂O-Methanol (9:1)→(8:2)→(7:3). Eluate is accommodated every 15 mL per-vial. Every multiples 5 vials, was monitored using TLC silicagel 60 HF₅₂₅₄, ethylacetate-formic acid-water (18:1:1) and spraying reagent of iron(III)chloride 10%. In vials number 87-98 phenolic compounds were found. Eluate in that vial is evaporated using vacuum rotary evaporator, resulting in sediment on evaporator flask wall. The sediment was crystalized using chloroform-Methanol (1:1) and resulting in white-yellow solid powder labelled as EHJ isolate. The test using thin layer chromatography silicagel 60 HF₅₂₅₄ spray reagent iron(III)chloride 10% and eluent of ethylacetate-formic acid-water (18:1:1) showed that EHJ isolate is a single compounds, which had the Rₜ value of 0.76 and 0.42 on TLC plate silica gel RP-18 with eluent of Chloroform-Methanol (8:2).

The structure of EHJ isolate was elucidated based on the result of spectroscopy characterization. The molecular mass of EHJ isolate was 271.06 mz [M-H]⁺ which was determined by LC-MS ESI. Spectrum UV of this compounds presents maximum absorption on λₘₐₓ (MeOH) 271 nm. Maximum absorption on λₘₐₓ 271 nm indicated the presence of substituted benzene structure or substituted aromatic. This estimation is supported by spot test using spray reagent sulfuric acid 10% and iron (III) chloride 10% that the isolate is a phenolic compounds. Furthermore, this estimation also strengthened by spectrum IR data which shows the phenolic group by the presence of absorption on νₐₙₜ 1612.2, 1538.9 and 1427.07 cm⁻¹ for aromatic unit and strong absorption on νₙₐₜ 3494.3 cm⁻¹ for hydroxyl group presence. Other important information from the presence of strong absorption for hydroxyl group is hydroxyl group source, in addition to the phenol aromatic structure, it was also estimated from carboxyl group that is supported by the presence of strong absorption on νₙₐₜ 2665.14 cm⁻¹. Thus, it could be suggested that compound resulted from the isolation is a substituted carboxyl phenol group.

SIGNAL IN ¹H NMR spectrum also convinced the substitute phenol by the presence of singlet signal in δₜ 7.06 ppm that shows the presence of proton in aromatic group. That signal is the only proton signal from isolates EHJ, thus can be identified as proton in ortho position in aromatic group based on its chemical shift values. Signals in ¹³C NMR spectrum is completing the estimation shape of isolates EHJ chemical structure. The main frame in the form of aromatic group is shown by signal in range of δ₁ 110-160 ppm. At this range there is a signal in δ₁ 122.03 dan 110.39 ppm which is a chemical shift characteristic of carbon atom that has double bond structures in aromatic group. Carbon atom in this aromatic group is also known to bound hydroxyl group, this is detected by the presence of signal in δ₁ 146.44 and 139.66 ppm. Furthermore, isolate EHJ also known to have carbonyl group that its carbon atom was detected as a signal in δ₁ 170.49 ppm.

The correlations between hydrogen atom and carbon atom and also their atomic positions in isolate EHJ can be identified from HMQC and HMBC spectrum. The correlations between δ́ₜ 7.06 ppm and δ́ₖ 110.39 ppm signal in HMQC spectrum shows that hydrogen atom is bound to the carbon atom which has double bond structures in aromatic group. The correlations between carbon atom and its hydrogen atom neighbour that is identified in HMBC spectrum shows that signal in δ́ₜ 7.06 ppm were correlated to the signal in chemical movement of δ́ₖ 110.39, 139.66, 146.45 and 170.49 ppm. This data shows that a hydrogen atom is neighboring to one carbon atom double-bonded in aromatic group (δ́ₖ 110.39 ppm), two carbon atoms that bound hydroxy in aromatic group (δ́ₖ 139.66 and 146.45 ppm) and one carbon atom in carbonyl group (δ́ₖ 170.49 ppm). The correlation scheme between hydrogen and carbon atom in isolate EHJ shown in Fig. 3.
Fig. 3: The correlation between hydrogen atom and carbon of EHJ isolate

Isolate EHJ is a 3,4,5-trihydroxybenzoic acid compound or known as gallic acid based on this spectroscopy data analysis. The activity testing result shows that this compound is active against S. aureus. This is in accordance to the previous research which shows that this compound is reported as an anti-bacteria and anti-fungi (Akiyama et al., 2001; Panizza et al., 2002; Penna et al., 2001; Slukla et al., 1999) and also known to have antioxidant activity and anti-inflammation (Kroes et al., 1992) and anti-tumor (Kawada et al., 2001).

Work spectrum activity of antimicrobial isolate EHJ is given to gram positive and negative bacteria (Table 3). The isolate inhibit the growth of bacteria S. aureus (MIC 735 μg mL⁻¹) dan MRSA (MIC 2,500 μg mL⁻¹), but prohibit the growth of B. subtilis, S. lutea, dan E. coli. This shows that isolate EHJ is active against gram positive bacteria and also selective, means that it only active to certain gram positive bacteria such as S. aureus dan MRSA, but inactive to other gram positive bacteria, such as B. Subtilis. Isolate EHJ does not inhibit the growth of gram negative bacteria E. coli. The MIC value of EHJ isolate against S. aureus is lower than the MIC value (1,250 μg mL⁻¹) reported by Chanwitheesuk et al. (2007), which means that this result is better and more accurate than the previous result.

In order to compare antimicrobial isolate EHJ activity to antibiotic that has been used for therapy, their equality activities to tetracycline, penicillin G and vancomycin are determined first. The result test showed 1 mg EHJ isolate equal to 0.1396 μg tetracycline HCl to S. aureus and 0.6625 μg to MRSA; 40.6035 μg penicillin G to S. aureus also equal to 2.9823 and 2.1213 μg vancomycin HCl, respectively to S. aureus and MRSA.

Based on the result of the research, it was known that the EHJ isolate had an exceptional behavior towards bacteria. The effect of EHJ isolate towards S. aureus cell morphology was evaluated by electron microscope and the report was presented in Bandung International Conference on Medicinal Chemistry (6-8 August 2009) and in the process of publication. Another interesting finding in this research is that EHJ isolate could be used as the biomarker of the antibacterial activity of T. muelleri.

CONCLUSION

Based on this study can be concluded that T. muelleri leaves antibacterial activity is caused by the presence of gallic acid compound. This compound is isolated from ethylacetate extract leaves and it was active against S. aureus (MIC = 735 μg mL⁻¹) and MRSA (2,500 μg mL⁻¹).
REFERENCES


