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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 substances was identified as 3,4,5-trihidroxybenzoic 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 Methicilin-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 Methicilin-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 Gunea 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*, *T. Sambesiaca* leaves inhibited the growth of *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Microsporum canis* and *Sporothrix schenkii*. Methanol extract of *T. trifolia* was active against *Hialohyphomycetes* (Muschietti *et al*., 2005) and the polar and semipolar

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extract from the root of *T. sericea* were active a gainstfungi *Candida albicans* and *Aspergillus niger* 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 acetic 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_{254} (Merck EM 5554) and 60RP-18 $F_{254}S$ (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. I 195013, vancomycin HCl (MP Biochem 195540) and Penicillin 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 dics on a spectrophotometer FT-IR JASCO 4200, ^{1}H and ^{13}C NMR spectra were recorded by spectrometer JEOL JNM ECA-500, operating at 500 MHz (^{1}H and ^{13}C), in CD_3OD, 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 acetat 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 coloumn 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 (EG), 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 sticked 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 HF_{254} 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 HF_{254} 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 pumpkin. This sediment was crystalized using chloroform-methanol (1:1), result to white-yellow powder labelled as EHJ isolate.

Table 1: Antibacterial activity of *T. muelleri* Benth. leaves extract

	Minimum inhibition concentration (μg mL^{-1})	
Extract	*E. coli*	*S. aureus*
n-hexane (H)	1000.00	1000.0
Ethylacetic (E)	0.01	0.1
Methanol (M)	1000.00	1000.0

Table 2: Antibacterial activity of fractions from ethylacetic extract

	Inhibition diameter (mm)	
Fraction (10 mg mL^{-1})	*E. coli*	*S. aureus*
EA	-	-
EB	-	-
EC	8.63±0.30	8.50±0.80
ED	-	8.45±0.25
EE	-	8.75±0.15
EF	-	8.60±0.20
EG	8.23±0.05	8.50±0.13
EH	8.80±0.40	18.80±1.18
EI	-	-

-: Not detected

Table 3: Antibacterial activity of EHJ isolate

	Inhibition diameter (mm)				
Concentration (μg mL^{-1})	*S. aureus*	MRSA	*B. subtilis*	*S. lutea*	*E. coli*
10.000	14.0±0.3	10.4±0.3	-	-	-
5.000	12.0±0.2	8.5±0.1	-	-	-
2.500	11.2±0.1	7.7±0.1	-	-	-
1.250	8.7±0.4	-	-	-	-
1.000	8.4±0.2	-	-	-	-
900	8.1±0.4	-	-	-	-
800	7.7±0.3	-	-	-	-
750	7.4±0.5	-	-	-	-
740	7.1±0.6	-	-	-	-
735	6.9±0.5	-	-	-	-
730	-	-	-	-	-

-: 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

Table 4: Mathematics equation of antibiotic activity to microbial tested

Antibiotics	*S. aureus*	MRSA
Penicillin G	$Y = 4.727x + 7.47$	
	$R^2 = 0.966$	-
Tetracycline	$Y = 7.379x+21.97$	$Y = 10.62x+14.8$
	$R^2 = 0.930$	$R^2 = 0.987$
Vancomycin	$Y = 4.887x+12.79$	$Y = 6.442x+9.75$
	$R^2 = 0.994$	$R^2 = 0.997$

Y: Inhibition diameter; X: Concentration logarithm; R^2: Correlation coefficient; -: Not tested

Table 5: The equivalency antibiotic activity of 1 mg EHJ isolate with reference antibiotic

Antibiotics (μg)	*S. aureus*	MRSA
Penicillin G	40.6035	-
Tetracycline	0.1396	0.6625
Vancomycin	2.9823	2.1213

-: Not tested

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 acetate→methanol. This fractionation aims to simplify the ethylacetic extract by deviding the extract chemical material into fraction group based on it's 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, EG, 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.

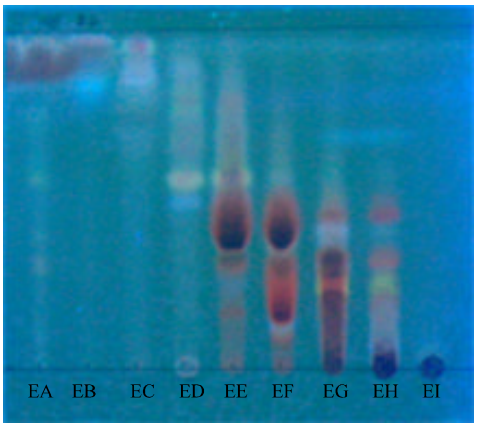

Fig. 1: TLC chromatogram of fraction from ethylacetic extract.TLC silica gel 60 HF_{254}; eluen: n-hexane-ethylacetic (4:6); spray reagents: sulfuric acid 10%

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-ethylacetat (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, anysaldehid for terpenoid, vanilin 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, H_2O-Methanol (9:1)→(8:2)→(7:3). Eluate is accomodated every 15 mL per-vial. Every multiples 5 vials, was monitored using TLC silicagel $60HF_{254}$, 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 rotaryvaporator, 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 HF_{254}, 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 R_f 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 λ_{maks} (MeOH) 271 nm. Maximum absorption on λ_{maks} 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 υ_{maks} 1612.2, 1538.9 and 1427.07 cm^{-1} for aromatic unit and strong absorption on υ_{maks} 3494.3 cm^{-1} for hidroxyl group presence. Other important information from the presence of strong absorption for hidroxyl group is hidroxyl 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 υ_{maks} 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 δ_H 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 ^{13}C 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 δ_C 110-160 ppm. At this range there is a signal in δ_C 122.03 dan 110.39 ppm which is a chemical shift characteristic of carbon atom that has double bound 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 δ_C 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 δ_C 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 δ_H 7.06 ppm and δ_C 110.39 ppm signal in HMQC spectrum shows that hydrogen atom is bound to the carbon atom which has double bound structures in aromatic group. The correlations between carbon atom and its hydrogen atom neighbour that is identified in HMBC spectrum shows that signal in δ_H 7.06 ppm were correlated to the signal in chemical movement of δ_C 110.39, 139.66, 146.45 and 170.49 ppm. This data shows that a hydrogen atom is neighboring to one carbon atom double-bounded in aromatic group (δ_C 110.39 ppm), two carbon atoms that bound hidroxy in aromatic group (δ_C 139.66 and 146.45 ppm) and one carbon atom in carbonyl group (δ_C 170.49 ppm). The correlation scheme between hydrogen and carbon atom in isolate EHJ shown in Fig. 3.

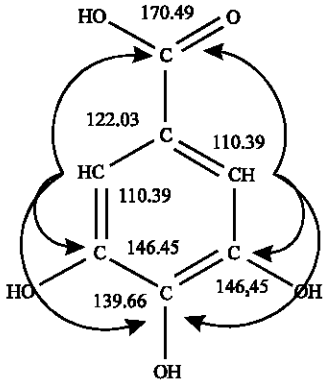

Fig. 3: The correlation between hydrogen atom and carbon of EHJ isolate

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; Panizzi *et al*., 2002; Penna *et al*., 2001; Shukla *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 bacterias, 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 vancomysin 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 penicyline 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 ethylacetic extract leaves and it was active against *S. aureus* (MIC = 735 μg mL^{-1}) and MRSA (2.500 μg mL^{-1}).

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