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Antibacterial Effect of Component of *Terminalia muelleri* Benth. against *Staphylococcus aureus*

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Abstract: A component of ethylacetate extract of *Terminalia muelleri* Benth. leaves, was found to inhibit *Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* growth therefore, the aims of this study were to investigate the effect of the active components on morphology of bacteria cell which was observed by Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM). Tetracycline HCl, penicillin G and vancomycin HCl were used as reference antibiotic. The active component at twice of the MIC caused shrinkage and thinning of the cell wall. The cell damage pattern which is caused by the active compound was similar to the damage caused by vancomycin HCl. It was explained that the antibacterial target of action of the active compound was inhibit the synthesis of the cell wall

Key words: *Terminalia muelleri*, antibacterial, electron microscope, cell wall, mode of action

INTRODUCTION

Many of the drugs currently used to treat bacterial and other infections were isolated from natural sources including ethnomedicinal plant. Such plants may provide new sources of therapeutic agents against multi-drug resistant bacterial infections, such as Methicillin-resistant *Staphylococcus aureus* (Pasewu *et al.*, 2008). *Staphylococcus aureus*, a Gram-positive organism, is responsible for numerous infections ranging in severity from skin and soft tissue infections to endocarditis and septic arthritis *Staphylococcus aureus*, which can induce bacteremia (associated with 80% mortality in the preantibiotic era), proved to be susceptible to the earliest antimicrobial substance; however, as antibiotic use increased, staphylococcal resistance rapidly developed. Methicillin-resistant *Staphylococcus aureus* (MRSA), resistance of which was due to penicillin-binding protein (PBP) 2' production. MRSA is resistant to not only methicillin and other β -lactams but also many other antibacterial agents. Since, MRSA exhibits multidrug resistance, it has been emerging worldwide as one of the most important hospital and community pathogens. Therefore, new agents are needed to treat the MRSA (Kwon *et al.*, 2007; Leung *et al.*, 2009).

Terminalia muelleri is one of the flowering plant of genus *Terminalia* family Combretaceae. This plant is

known in Indonesia as "ketapang kencana". The distribution of the plant includes India, Indonesia and North America (Nanakorn, 1985; Lemmens and Wulijarmi-Soetjpto, 1992). The phytochemical content of *T. muelleri* has never been reported previously. Meanwhile, it is known that genus *Terminalia* extract have antifungi, anticancer, antioxidant activities and inhibit α -glucosidase (Masoko *et al.*, 2005; Muschietti *et al.*, 2005; Anam *et al.*, 2009a). The genus is known to contain cyclic triterpene and its derivatives, flavonoid, tannin and other aromatic compounds. Part of the compounds is known to have antifungi, antibacterial, anticancer and hepatoprotector activities (Kandil and dan Nassar, 1998; Tang *et al.*, 2006; Srivastava *et al.*, 2001).

The report of biological activity of *T. muelleri* demonstrated that the ethanolic extract of the leaves were used as antioxidants (Bajpai *et al.*, 2005) and inhibited the growth of *E. coli*, *S. aureus* bacteria and *C. albicans* fungi (Suganda *et al.*, 2006). Furthermore, Anam *et al.* (2009b) completed the report and stated that ethyl acetate extract from *T. muelleri* leaves had stronger activity against *S. aureus*, *E. coli* and *C. albicans*. Recently, the author reported that the EHJ compound in the ethyl acetate extract of *T. muelleri* was the agent responsible to the antibacterial activity of this plant (Anam *et al.*, 2010). In this opportunity, the effect of EHJ compound against morphology of *S. aureus* cell will be reported.

MATERIALS AND METHODS

Microbial strains: *Staphylococcus aureus* ATCC 25923 and Methicillin-resistant *Staphylococcus aureus* (MRSA) were obtained from PT Biopharma Bandung, Indonesia in April 2009.

Active principle of *Terminalia muelleri* Benth.: Compound EHJ was isolated as a pure substance from a ethyl acetate extract of *T. muelleri* Benth. Leaves previously described (Anam *et al.*, 2010). It is poorly active as an antibacterial agent against *S. aureus* (MIC 0.735 mg mL⁻¹) and MRSA (MIC 2.5 mg mL⁻¹).

Electron microscopy: The preparation of microbial tests was carried out using the standard procedures by Bozzola and Russell (1998). *Staphylococcus aureus* strain was grown overnight at 35°C in nutrient broth (Merck CM 10) and MRSA in tryptic soy broth (Sigma 22092) with or without the addition of compound EHJ (2xMIC). Tetracycline HCl (ASEAN Reference substance, control No. I 195013), Penicilline G (Sigma P 7794) and Vancomycin HCl (MP Biochem 195540) were used as reference compounds. Bacteria were harvested by centrifugation and washed once in phosphate-buffered saline, pH 7.4.

Cells were prepared for Transmission Electron Microscopy (TEM) by fixation in 1.5% glutaraldehyde (Sigma G5882) for at least 2 h at room temperature, post-fixation in osmium tetroxide (Sigma 75632), embedding in epoxy resin, sectioning and staining with uranyl acetate followed by Reynolds' lead citrate. The ultrathin sections were viewed and photographed using a JEOL JEM-1010 transmission electron microscope. Dimensions of cells and cell clusters were measured from photographs, the cell wall thickness from images on the screen.

For Scanning Electron Microscopy (SEM), bacterial cells fixed in glutaraldehyde were isolated on Millipore filters and post-fixed with osmium tetroxide (Sigma 75632). Cells were dehydrated by passage through graded acetone (Merck)/water mixtures and treatment with tetramethylsilane (Sigma T24007). The air-dried cells were coated with gold and examined using a JEOL JSM-6360LA scanning electron microscope.

RESULTS AND DISCUSSION

The antibacterial activity of compound EHJ against the tested bacteria *S. aureus* and MRSA is categorized as weak with MIC value of 0.735 mg mL⁻¹ and 2.5 mg mL⁻¹. Although the activity was weak, but the tested bacteria given the compound showed *S. aureus* and MRSA

structural cell changes, which was observed in the examination by SEM and TEM.

The observation by SEM (Fig. 1a, b) showed that *S. aureus* cell, which was in contact with compound EHJ (1.47 mg mL⁻¹) had an abnormal cell wall, which was indicated by the presence of pores and shrinkage in the cell wall. The pore in the cell surface and the shrinkage in the cell wall were hypothesized due to the imperfect synthesis of the cell wall. The damage in the cell wall ensure the cause of the cell death.

The effect of compound EHJ against *S. aureus* cell was further observed by TEM. In Fig. 2a and b, it was showed that *S. aureus* cell after contact with compound EHJ (2x MIC), the cell wall became thinner and lost its cell wall. Some part of the cell was known to appear normal, but the cell wall thickened to 74.5 nm compared to control, 52 nm. The thickening of the cell wall was a form of *Staphylococcus* response to defend itself against antibiotic (Gemmell and Lorian, 1996). This does not always occur, but has been reported both for compounds that inhibit the cell wall synthesis, such as penicillins (Paul *et al.*, 1995; Giesbrecht *et al.*, 1998) and those that have other mechanisms of action, e.g., pristinamycin, clindamycin, rifampicin, chloramphenicol (Giesbrecht *et al.*, 1998; Lorian *et al.*, 1994). However, the

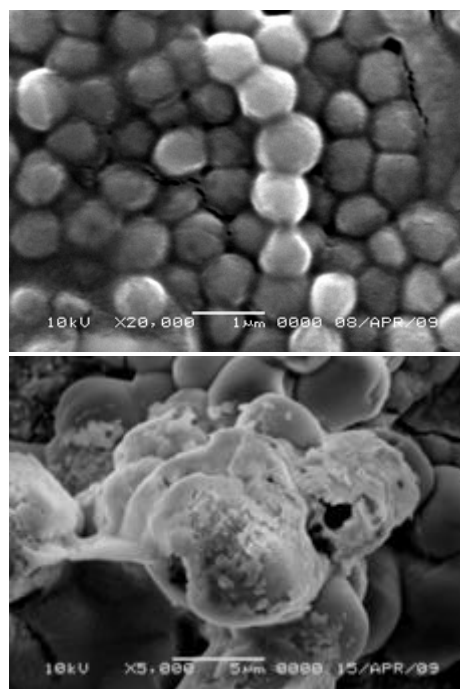


Fig. 1: SEM micrograph of *S. aureus* (a) control and (b) after growth in the presence of 1.47 mg mL⁻¹ of compound EHJ

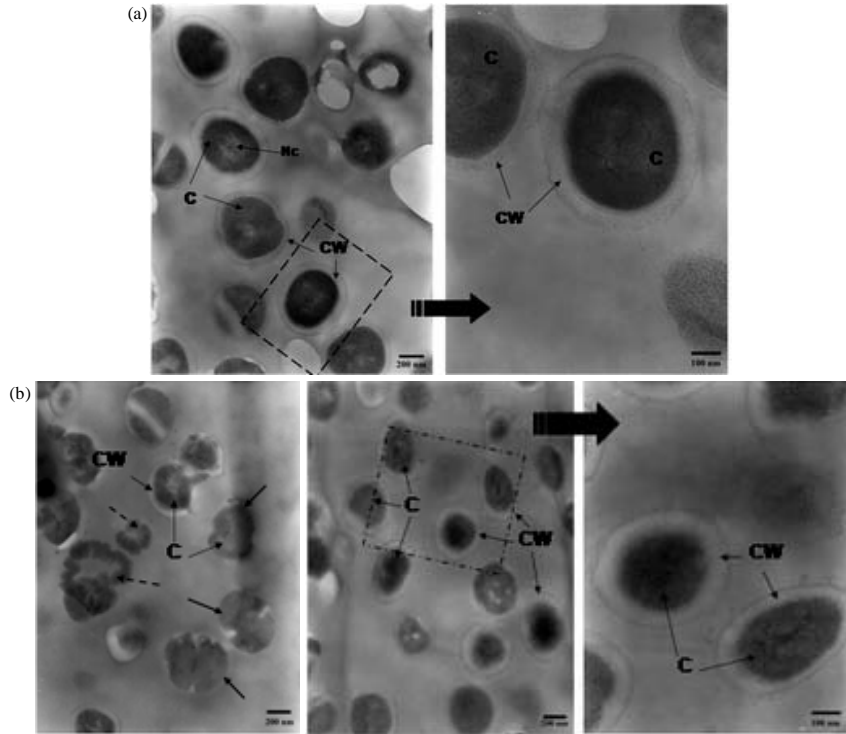


Fig. 2: TEM micrograph of *S. aureus*: (a) control and (b) after growth in the presence of 5 mg mL⁻¹ of compound EHJ. CW: Cell wall, C: Cytoplasm cell with thin cell wall: Cell without cell wall

thickened cell wall was more rigid and it caused the membrane permeability change so that the cell wall is easily broken. This emerges the hypothesis that compound EHJ also inhibit the cell wall synthesis.

In order to have further information of the antibacterial mode of action of the compound EHJ against *S. aureus* cell, therefore it was compared to the cell contacted with antibiotic. The antibiotics used as reference were tetracycline HCl, penicillin G and vancomycin HCl.

The SEM micrograph of *S. aureus* cell which was in contact with tetracycline HCl in concentration of 0.3 mg mL⁻¹ (Fig. 3a) showed that the morphology of *S. aureus* cell was relatively normal and similar to the *S. aureus* cell which acts as control (Fig. 1a). This normal cell morphology was related to the bacteriostatic properties of tetracycline against gram negative and positive bacteria. In concentration of 1 mg mL⁻¹, some part of the *S. aureus* cells shrank (Fig. 3b), while in concentration of 3 mg mL⁻¹ all cells were damaged (Fig. 3c). The result of the observation was in line with the literature study that in lower concentration, tetracycline is bacteriostatic and in high concentration, it is bacteriocidal. Tetracycline enters the microorganism through two ways, passive diffusion and active process which is

energy-dependent. When it is inside the cell, tetracycline bind itself reversibly to the subunit 30S from the bacteria ribosome and inhibit the bonding of tRNA-aminoacyl to the receptor sites in the ribosome mRNA complex. This inhibited the addition of the amino acid towards the peptide which is being formed (Katzung, 2007). Therefore, it could be stated that tetracycline works by inhibiting the protein synthesis and *S. aureus* cell surface which in contact with tetracycline will have abnormal morphology in high concentration when observed by SEM.

The influence of penicillin G against *S. aureus* cell was shown by cell wall was not formed (Fig. 4a). Penicillin G is a β -lactam antibiotic which inhibits the bacterial growth by inhibiting specific step in the bacterial cell wall synthesis. β -lactam antibiotic is a structural D-Ala-D-Ala natural analog which covalently bound by Penicillin Binding Protein (PBP) in active sites. When a β -lactam antibiotic was in contact with PBP, the transpeptidation reaction stops so that the last alanine could not be released in order to form cross linkage with the nearest peptide and caused inhibition of peptidoglycan synthesis which was shown by the bacterial cell wall was not formed (Katzung, 2007).

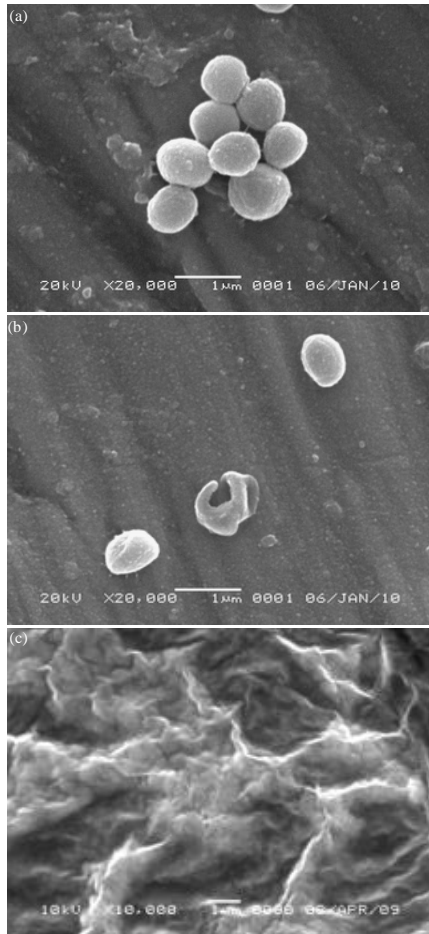


Fig. 3: SEM micrograph of *S. aureus* after growth in the presence of: (a) 0.3 mg mL⁻¹, (b) 1 mg mL⁻¹ and (c) 3 mg mL⁻¹ of tetracycline HCl

Figure 4b showed that *S. aureus* after in contact with vancomycin HCl, which had damage in the cell surface, the appearance of pores and shrinkage. The cell damage was caused by vancomycin which inhibit the cell wall synthesis by binding strongly the end of D-Ala-D-Ala from pentapeptide peptidoglycan which is newly formed. Vancomycin inhibits transglycosilation, therefore it inhibits the peptidoglycan becoming longer and also it inhibits cross linkage. The result is peptidoglycan weakens and the cell became vulnerable and easily lysed. The lysed cell caused the cell wall shrank. Besides that, the cell membrane also become damaged and it emerges the antibacterial effect (Katzung, 2007).

The SEM micrograph of MRSA (Fig. 5a, b) showed that the normal MRSA cell is circle and the surface is smooth. In the same manner, the TEM observation showed that the normal cell is circle and oval, cytoplasm and nucleotide appears normal and the cell wall thickness

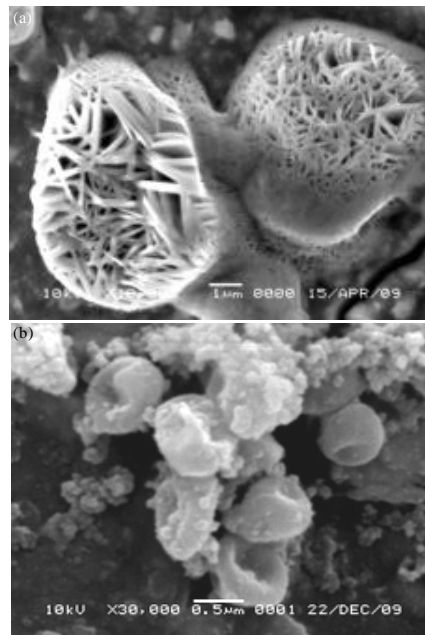


Fig. 4: SEM micrograph of *S. aureus* after growth in the presence of: (a) 1 mg mL⁻¹ of penicillin G and (b) 1 mg mL⁻¹ of vancomycin HCl

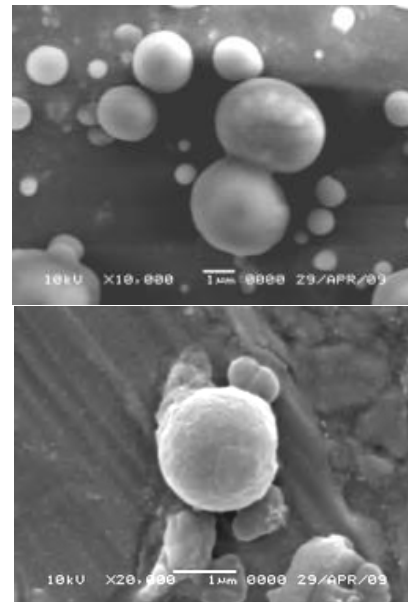


Fig. 5: SEM micrograph of MRSA (a) Control and (b) after growth in the presence of 5 mg mL⁻¹ of compound EHJ

is 34 nm (Fig. 6a). After in contact with compound EHJ (5 mg mL⁻¹), in the cell surface, pores appear and some other part of the cell the morphology became oval and it

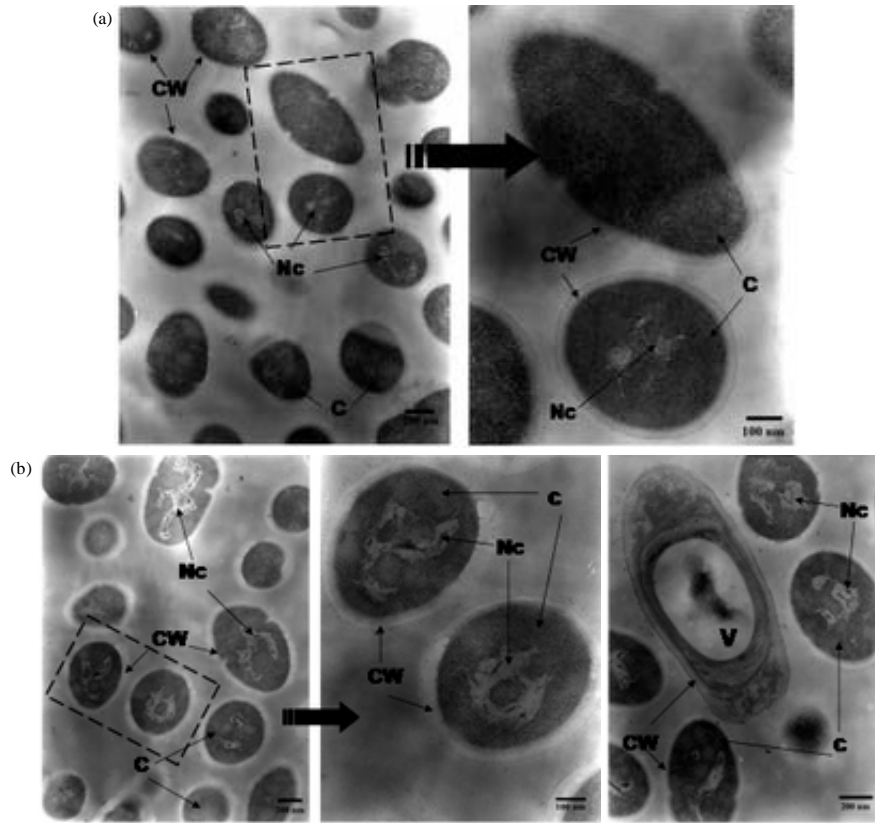


Fig. 6: TEM micrograph of MRSA: (a) control and (b) after growth in the presence of 5 mg mL⁻¹ of compound EHJ. CW: Cell wall, C: Cytoplasm, V: Vacuole, NC: Nucleotide



Fig. 7: SEM micrograph of MRSA after growth in the presence of: (a) 1 mg mL⁻¹ of tetracycline HCl, (b) 1 mg mL⁻¹ of penicillin G and (c) 1 mg mL⁻¹ of vancomycin HCl

shrank (Fig. 6b). The appearance of pores was hypothesized that the cell wall synthesis was imperfect, while the shrinkage was due to the cell lysis. TEM micrograph showed that the effect of the compound EHJ towards the MRSA cell morphology: cell nucleotide area became larger, the vacuole became bigger, the cell wall thickened (52.5 nm) from the normal MRSA normal and some part of the cells lost their walls. The description of the cell damage was the

information of the mode of action of compound EHJ in MRSA bacteria, that is in cell wall.

The antimicrobial mode of action of compound EHJ against MRSA bacteria was observed by comparing the description of the cell damage caused by compound EHJ with antibiotics of tetracycline HCl, penicillin G and vancomycin HCl. MRSA cell which was in contact with tetracycline HCl had a circle shape like normal cell, but a large part of the cell wall had holes (Fig. 7a). Similar to the

MRSA cell in contact with penicillin, the cell morphology remained normal and was not damaged (Fig. 7b). This in accordance with the MRSA properties which is resistant against meticillin antibiotics. On the other hand, the MRSA cell which is in contact with vancomycin HCl, the surface was not smooth and pores were found (Fig. 7c).

Based on the micrograph SEM and TEM analysis, it was known that the damage pattern of *S. aureus* and MRSA cell by compound EHJ was similar to the one caused by vancomycin. Therefore, it was explained that compound EHJ was inhibit the synthesis of the cell wall.

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