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ANTIMICROBIAL ACTIVITY OF MAHKOTA DEWA [*Phaleria macrocarpa* (*Scheff). Boerl.*] LEAF EXTRACT AGAINST *Pseudomonas aeruginosa* BY AGAR DILUTION AND SCANNING ELECTRON MICROSCOPY

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ABSTRACT

Pseudomonas aeruginosa can develop multiple resistances to some antimicrobial agents. The research conducted to found new antimicrobial substances from natural product which cheap, effective and safe. Mahkota dewa (Phaleria macrocarpa) are plants originally grown in Papua, Indonesia. Phytochemical screening of Phaleria macrocarpa leaf showed the existence of flavonoids, polyphenols, saponins and tannins. These substances from another plant are known to have antimicrobial activity. However, the antimicrobial activity of Phaleria macrocarpa leaf extract against P. aeruginosa has not been known. The aim of this research was to determine the antimicrobial activity, the minimum inhibitory concentration (MIC) and the effect of antimicrobial activity of Phaleria macrocarpa leaf extract to the P. aeruginosa cells. This experiment used the post test only control group design. Three steps research were conducted, the first was ethanol extraction of Phaleria macrocarpa leaf by maceration. The second was agar dilution antimicrobial testing against P. aeruginosa ATCC 27853. The final step was Scanning Electron Microscopy (SEM) observation. P. aeruginosa were grew on media which contain Phaleria macrocarpa leaf extract on concentration 0-3 x 10^4 µg/ml, but not grew on concentration 4 x 10^4 µg/ml and 5 x 10^4 µg/ml for all replication, therefore the MIC was 4 x 10^4 µg/ml. The SEM observation demonstrated division inhibition and elongation of P. aeruginosa's cell. In conclusion, we found the MIC of Phaleria macrocarpa leaf extract against P. aeruginosa was 4 x 10^4µg/ml. It was very high and reflected weak antimicrobial potency, so it is not suggested to use it as antimicrobial agent against P. aeruginosa.

Keywords: *Antimicrobial activity, MIC, Phaleria macrocarpa leaf, Pseudomonas aeruginosa, SEM*

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INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) can cause disease especially in immunocompromised human, acts as agent of respiratory tract infection, urinary tract infection, otitis, pneumonia, osteomyelitis, endocarditis, meningitis and bacteriemia (Hill et al 2007). *P. aeruginosa* infection increase the morbidity and mortality because its ability to produce toxin and invasive substance. This bacterium develops multiple resistant to some antimicrobial agents (Multiple Drug Resistant/MDR), therefore causes therapy become difficult and expensive (Brooks et al 2007). Research to found antimicrobial substances that cheap, safe and effective against *P. aeruginosa* must be conducted.

Mahkota dewa (*Phaleria macrocarpa* (Scheff). Boerl.) Plants are originally grown in Papua, Indonesia. Leaf and fruits of *Phaleria macrocarpa* traditionally have been used by Indonesian society for the therapy of skin disease, liver, cancer, high blood pressure and diabetes (Winarto 2007). The *Phaleria macrocarpa* leaf is containing of flavonoids, polyphenols, saponins, tanins, and steroids compounds (Juita 2004). Flavonoids, polyphenols, saponins and tanins which obtained from the other plants known have antimicrobial activity (Harborne et al 1999).

Based on the chemical compounds of *Phaleria macrocarpa* leaf above, it may have antimicrobial activity. Antimicrobial activity of *Phaleria macrocarpa* leaf extract against *P. aeruginosa* not yet been known. The quantitative antimicrobial activity to a certain bacterium is expressed as Minimum Inhibitory Concentration (MIC). MIC is the lowest concentration (in weight/volume) of the substance which can inhibit the growth of a certain bacterium. It is important to know the MIC of a substance as reference in its development as antimicrobial agent. There is no research of antimicrobial activity of *Phaleria macrocarpa* leaf extract against *P. aeruginosa* have been done to determine the MIC of its substances. The effects of antimicrobial activity of *Phaleria macrocarpa* leaf extract to the *P. aeruginosa* cells are also unknown. An antimicrobial substance can only inhibiting the growth of bacterium cell without causing bacterium cell

lysis, but another antimicrobial substance can causing bacterium cell lysis (Boyd 1995). To see the effect of antimicrobial activity to the bacteria cells can be obtained by Scanning Electron Microscopy (SEM) observation, specifically observation on morphology, division and amount of the bacteria cells. Along the fact above, an in vitro antimicrobial test research is required to obtain antimicrobial activity and to determine the MIC of *Phaleria macrocarpa* leaf extracts against *P. aeruginosa*, followed by SEM observation to know the effect of antimicrobial activity to the *P. aeruginosa* cells.

MATERIALS AND METHODS

Fresh *Phaleria macrocarpa* leaf obtained from Kebonsari Village, Jember Regency, and East Java Province, Indonesia. The plant determination conducted in Herbarium Jemberiense, Biology department of Science Faculty of Jember University. The solution of ethanol 96% used as solvent in leaf extraction by maceration method. *Pseudomonas aeruginosa* ATCC (American Type Culture Collection) 27853 obtained from Balai Besar Laboratorium Kesehatan (BLK) Surabaya. The Muller Hinton Agar (MHA) media (Oxoid) in petri dish Ø 35 mm used as media of bacteria *P. aeruginosa* for the MIC determination. The Muller Hinton Broth (MHB) media (Becton Dickinson) used as media of bacteria *P. aeruginosa* for the SEM observation. Materials of SEM observation: glutaraldehide, phosphate buffer, osmium tetroxide, aquadest, ethanol, amyl acetate, and gold for specimen coating.

The instruments of leaf extraction: huller, macerator, paper filter, glass funnel, rotary vacuum evaporator (rotavapor), Becker glass. The instruments of antimicrobial test: analytic balance, measurement glass, Becker glass, Erlenmeyer tube, electrics stove, Bunsen fire, calibrated loop 10 µl, volume pipette, micro pipette, micro pipette tip, petri dish Ø 35 mm, mixer, pH meter, refrigerator, autoclave and incubator. The instruments of SEM observation: centrifuge, Pasteur's pipette, reaction tube, critical point dryer, vacuum evaporator, Scanning Electron Microscope (JEOL JSM-T100).

Design of this experimental laboratory research was "The Posttest Only Control Group" (Notoatmodjo 2002). The samples of *Pseudomonas aeruginosa* ATCC (American Type Culture Collection) 27853 bacteria were divided in two groups: the control group, *P. aeruginosa* which not expose by *Phaleria macrocarpa* leaf extract (0 µg/ml), and the treatment groups, *P. aeruginosa* which exposed by *Phaleria macrocarpa* leaf extract 5 x 10^4 µg/ml; 4 x 10^4 µg/ml; 3 x 10^4 µg/ml; 2 x 10^4 µg/ml; and 1 x 10^4 µg/ml. These doses obtained from preliminary test. The antimicrobial test repeated 5 times, from the formula: (t-1) x (r-1) = 15, hence r = 4 (Hanafiah 2005). Research steps: (1). Ethanol extraction of *Phaleria macrocarpa* leaf by maceration; (2). Antimicrobial testing of *Phaleria macrocarpa* leaf extract against *P. aeruginosa* by agar dilution; (3). *P. aeruginosa* cell observation by Scanning Electron Microscopy (SEM).

Phaleria macrocarpa leaf air dried and then milled to become powder. The powder macerated for 20 hours within ethanol 96% solution, then filtered and dissociated the macerate from the residue. The ethanol in macerate evaporated by the rotavapor (Harborne 2006), resulted condensed extract of *Phaleria macrocarpa* leaf.

P. aeruginosa suspension made in physiological salt solution. The turbidity was equal to ½ Mc Farland (1,5 x 10^8 CFU/ml). Then the suspension thinned (1:150), so that became 1 x 106 CFU/ml, from this suspension as much 10µl (1 x 10^4 CFU) inoculated at MHA media that contain of extract (Forbes et al 2002). The MHA media was made by following the factory instruction. The MHA media which contain of *Phaleria macrocarpa* leaf extract made in various concentrations. In Erlenmeyer glass, 3 g of *Phaleria macrocarpa* leaf extract added, then media MHA which still melt added until the volume became 60 ml (3 g/60 ml = 5 x 10^4 µg /ml). Series of suspension dilution on several concentration of *Phaleria macrocarpa* leaf extract in MHA media in Erlenmeyer tagged as D1, D2, D3, D4, D5 that contained 5 x 10^4 µg/ml; 4 x 10^4 µg/ml; 3 x 10^4 µg/ml; 2 x 10^4 µg/ml; and 1 x 10^4 µg/ml respectively. Erlenmeyer KD as negative control only contained the MHA without extract (0 µg/ml).

From each Erlenmeyer divided into 5 petri dish (Ø 35 mm), each contain 3 ml (3 mm media thick). The rest of MHA media that contain the *Phaleria macrocarpa* leaf extract in each Erlenmeyer checked its acidity (pH) by the pH meter. MHA in the Petri dish left until become solid. Solid MHA media incubated overnight for sterility test. If media were sterile without contamination, 1 x 10^4 CFU of bacteria *P. aeruginosa* inoculated to each surface of MHA media in petri dish (Forbes et al 2002), using 10 µl calibrated loop, then incubated for 20 hours at 35°C (Forbes et al 2002). The growth of bacteria in each petri dish observed. The Minimum Inhibitory Concentration (MIC) of *Phaleria macrocarpa* leaf extract against *P. aeruginosa* determined. The means of MIC determined from 5 replications.

Scanning Electron Microscopy (SEM) observation procedure

The Muller Hinton Broth (MHB) media made for *P. aeruginosa* that will be exposed to the *Phaleria macrocarpa* leaf extract for SEM observation. Prepared tube "A" which contain the MHB media, *P. aeruginosa* suspension by concentration 1 x 10^4 CFU/ml without *Phaleria macrocarpa* leaf extract (0 µg/ml), used as negative control; and tube "B" contain the MHB media, *P. aeruginosa* suspension which concentration 1 x 10^4 CFU/ml, and *Phaleria macrocarpa* leaf extract that concentration equal with MIC means. The tube A and B incubated at 35°C for 20 hours, then the bacteria suspension taken from tube A and B used for specimen of SEM observation. The steps of specimen preparation were: Fixation by glutaraldehide in the buffer phosphate solution (BPS) at 0-4°C for 1-2 hours, rinsed with the BPS 3 times, 15 minute each. The second fixation by the osmium tetroxide in the BPS for 3-12 hours, then rinsed with the BPS for 15 minutes; Dehydration use the ethanol 25%, ethanol 50%, ethanol 70%, ethanol 80%, ethanol 90%, 15 minutes for each solution, then ethanol 100% for 15 minutes, twice, then dried by critical point dryer; Coating process, the preparation was coated by gold. The vapour gold would be condensed in vacuum chamber then fell coating the specimen (Hoediasmoro et al 1984).

RESULT

An amount of 50 g air-dried *Phaleria macrocarpa* leaf was milled to yield 45 g powder, then used for ethanol extraction by maceration, producing the condensed extract (without ethanol anymore) of *Phaleria macrocarpa* leaf as much 9.28 g. The acidity of MHA media which contain *Phaleria macrocarpa* leaf extract in various concentrations was measured by pH meter. The pH of MHA media that contain *Phaleria macrocarpa* leaf extract in various concentrations was 5.25–6.40.

Table 1. Material and result of extraction

Material	Weight	Solvent	Volume	Result	Rendemen
P. macrocarpa leaf powder	45 g	Ethanol 96%	500 ml	9.28 g	20,6 %

Table 2. Acidity of MHA media which contain *Phaleria macrocarpa* leaf extract.

Concentration of *Phaleria macrocarpa* leaf extract in MHA media	Acidity (pH)
5x10^4 µg/ml	5.25
4x10^4 µg/ml	5.43
3x10^4 µg/ml	5.72
2x10^4 µg/ml	6.04
1x10^4 µg/ml	6.40

The colonies of *P. aeruginosa* were grew on the surface of MHA media which contain *Phaleria macrocarpa* leaf extract on concentration 0 µg/ml-3 x 10^4 µg/ml, but not grow on concentration 4 x 10^4 µg/ml and 5 x 10^4 µg/ml (Tables 3 and Figure 1) for all replication (I-V), so the means of MIC was 4 x 10^4 µg/ml.

The SEM observation of *P. aeruginosa* which exposed to *Phaleria macrocarpa* leaf extract concentration 0 µg/ml (control, from tube A) demonstrated the cells still divided, lived without morphology damage, 0.8 µm bacterium cell length and lot of the bacteria cells (Figure 2). The *P. aeruginosa* bacteria which exposed to *Phaleria macrocarpa* leaf extract concentration 4 x 10^4 µg/ml (MIC, from tube B) shown the cells were not damage, the length of the cells increase up to 1.6 µm (cells elongation), and the cells still could replicate, but amount of cells were decrease (Figure 3).

Table 3. Colony of *P. aeruginosa* on MHA media which contain *Phaleria macrocarpa* leaf extract in various concentrations.

Replication	The colony of *P. aeruginosa* on petri dish					
	D1 (5x10^4µg/ml	D2 (4x10^4µg/ml)	D3 (3x10^4µg/ml)	D4 (2x10^4µg/ml)	D5 (1x10^4µg/ml)	KD (0µg/ml)
1	-	-	+	+	+	+
2	-	-	+	+	+	+
3	-	-	+	+	+	+
4	-	-	+	+	+	+
5	-	-	+	+	+	+

+ = the colony were exist;-= the colony were not exist.

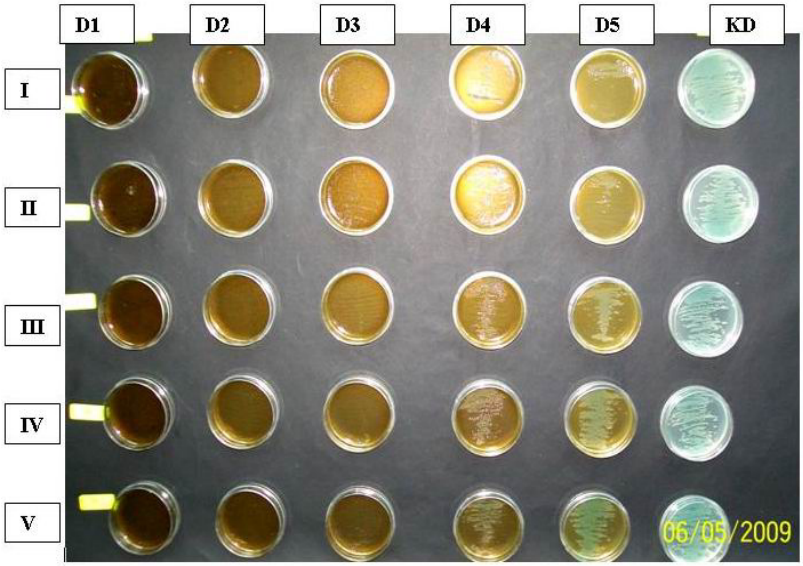

Figure 1. Result of antimicrobial testing of *Phaleria macrocarpa* leaf extract against *P. aeruginosa* (row I-V were replications, columns left to right were concentration of *Phaleria macrocarpa* leaf extract in MHA media, D1: 5 x 10^4 µg/ml, D2: 4 x 10^4 µg/ml, D3: 3 x 10^4 µg/ml, D4: 2 x 10^4 µg/ml, D5: 1 x 10^4 µg/ml and KD: 0 µg/ml).

DISCUSSION

Maceration methods had been used in this research to got extract of *Phaleria macrocarpa* leaf. Based on literature, both of the extraction methods, maceration and percolation were applicable to obtain plants extract that have antimicrobial substances (Dey 1991). Ethanol selected as extraction solvent in this research because most of plants compounds which have antimicrobial activity represent the aromatic compounds or dissolve in organic compounds which can be dissolved by ethanol or methanol (Cowan 1999).

Phaleria macrocarpa leaf extraction yielded the condensed ethanol extract of *Phaleria macrocarpa* leaf, its rendemen 20.6 % (weight of condensed extract/weight of powder x 100%). This extraction result indicated the extraction method that performed in this research yield the condensed extract as same as Oshimi's research, whom obtained rendemen of methanol extract of *Phaleria macrocarpa* fruits equal to 24% (Oshimi et al 2008). The *Phaleria macrocarpa* leaf extract mixed with MHA media and then tested for its sterility before inoculation of *P. aeruginosa*. The result of sterility test shown the MHA media that contain ethanol extract of *Phaleria macrocarpa* leaf were sterile, so that ascertained there was nothing bacteria from extract substance.

Acidity (pH) of extract and MHA media that contain of extract in various concentrations were checked before the antimicrobial testing conducted. Acidity measurement was needed to ensure appropriate environment for bacterial growth because the bacterium might not grew when the extract extremely acid or alkaline (Dey 1991). The MHA media which contained *Phaleria macrocarpa* leaf extract concentration 5 x 10^4 µg/ml-1 x 10^4 µg/ml had pH 5.25–6.40. These pH values were almost appropriate to environmental acidity for growth of *P. aeruginosa*, pH 5.6-8.0 (Todar 2008). The absence of bacteria growth on MHA media which contain *Phaleria macrocarpa* leaf extract on concentration 4 x 10^4 µg/ml and 5 x 10^4 µg/ml were not caused by the acidity, but rather caused by the addition of *Phaleria macrocarpa* leaf extract that have antimicrobial activity against *P. aeruginosa*. Antimicrobial testing of *Phaleria macrocarpa* leaf extract against *P. aeruginosa* conducted by agar dilution method in MHA media. Agar dilution represent the correct method for antimicrobial testing of plants extract, because the aerobic organisms cannot grow under the solid medium and even they contaminated on the surface will be easily recognized (Dey 1991). MHA media contain casein hydrolisat, beef infusion form, extract and the electrolyte ion that suitable for the *P. aeruginosa* growth (Forbes et al 2002).

Based on the result of antimicrobial testing, the *Phaleria macrocarpa* leaf extract could inhibit the growth of bacterium starting from concentration 4 x 10^4 µg/ml for all replications; therefore the MIC of *Phaleria macrocarpa* leaf extract against *P. aeruginosa* bacteria was 4 x 10^4 µg/ml. The MIC of *Phaleria macrocarpa* leaf extract was very high and reflected weak antimicrobial potency. Furthermore, this MIC value was over the standard to determine whether the extract can be developed and used as antimicrobial agent or not. A substance from natural product extract can be developed as antimicrobial agents if inhibit the bacterial growth on concentration 10-500 µg/ml (Nascimento et al 2000).

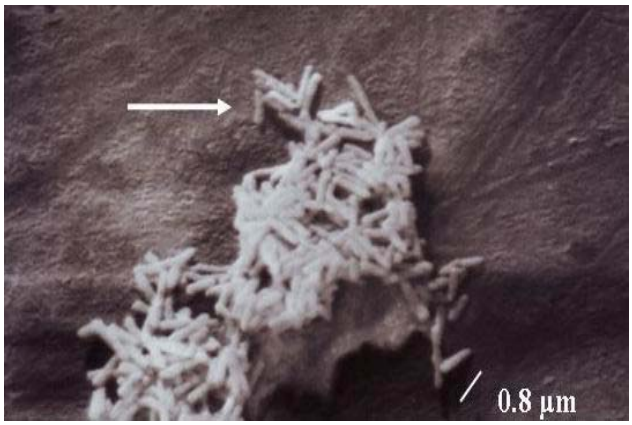

Figure 2. Scanning electron micrograph of *P. aeruginosa* exposed to 0 µg/ml *Phaleria macrocarpa* leaf extract (negative control). The arrow refers to the cell division and the line refers to the bacterium cell length. 7500 x.

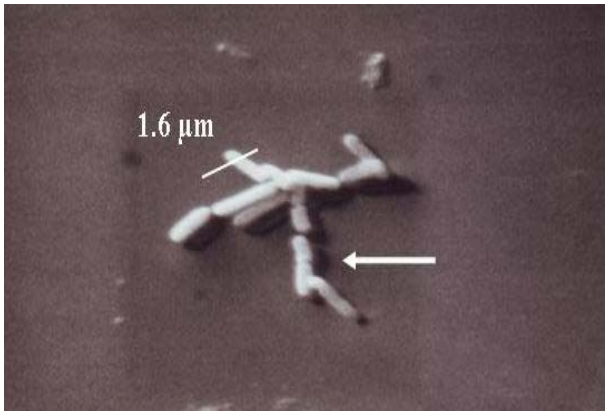

Figure 3. Scanning electron micrograph of *P. aeruginosa* exposed to *Phaleria macrocarpa* leaf extract concentration 4 x 10^4 µg/ml (MIC). The arrow refers to cell division and the line refers to the bacterium cell length. 7500 x.

Micrograph of scanning electron shown the *Phaleria macrocarpa* leaf extract on concentration 4 x 10^4 µg/ml (=MIC) was able inhibit the growth of *P. aeruginosa* which shown by the decrease of the amount of bacteria cells (Figure 3). In this research, the amount of *P. aeruginosa* which inoculated into each tube A and B were equal (1 x 10^4 CFU/ml) hence after incubation at same duration and temperature, the bacteria would increase with same speed and yielded same population, but the SEM observation demonstrated decrease of *P. aeruginosa* population when the concentration of *Phaleria macrocarpa* leaf extract increased. If amount of initial inoculated bacteria were equal but after its incubation the amount of bacterium differ (decrease) and also there were no damage or lysis of bacteria cell, hence that decrease possibly caused by the bacterial cell division inhibition (Brooks et al 2007).

The scanning electron micrograph demonstrated decrease of *P. aeruginosa* cells amount and cells elongation when exposed to *Phaleria macrocarpa* leaf extract on concentration 4 x 10^4 µg/ml (Figure 3). The presence of *P. aeruginosa* cells elongation at this research was similar to Aonuma's research that mentioned *P. aeruginosa* bacteria became longer when exposed to Sulbenicillin (Aonuma et al 1987). In Aonuma's research, cells elongation was observed by Transmission Electron Microscope (TEM) and demonstrated peptidoglican layer damage without outer membrane destruction, hence concluded that the mechanism of Sulbenicillin was destroy peptidoglican layer of *P. aeruginosa*'s cell wall (Aonuma et al 1987). The similarity of *P. aeruginosa* cells elongation which exposed to *Phaleria macrocarpa* leaf extract in the research with that exposed to Sulbenicillin in Aonuma's research may caused by same mechanism, so that gave suggestion the mechanism of active compound in *Phaleria macrocarpa* leaf extract was destroying the peptidoglican layer of *P. aeruginosa*'s cells wall.

Based on the result of in vitro agar dilution antimicrobial testing that MIC of ethanol extract of *Phaleria macrocarpa* leaf was 4 x 10^4 µg/ml show the potency of *Phaleria macrocarpa* leaf extract was very weak against *P. aeruginosa*. Antimicrobial activity that is found in this research may be caused by the active compounds in the *Phaleria macrocarpa* leaf extract: flavonoids, saponins, polyphenols and tanins. Purification, identification and quantification of active compounds that own antimicrobial activity from the *Phaleria macrocarpa* leaf extract were not conducted in this research. Another researcher got from other plants, the flavonoids compounds inhibited Klebsiella pneumoniae bacteria yielding Extended-Spectrum ß Lactamase (Ozcelik et al 2008), while Chrysin (5,7-dihydroxyflavone) inhibited *P. aeruginosa* and *E. coli*

(Bylka 2004). Meanwhile, saponins inhibit the Gram positive bacteria (S.aureus), but it is unable to inhibit the Gram negative bacteria (*E. coli*) (Soetan 2006). Another research proved the polyphenols from plants family Geraniaceae and Rosaceae have bacteriostatic or bactericide activity to an opportunistic bacteria: B.cereus, *E. coli*, *P. aeruginosa* and S.aureus (Nikitina et al 2007), while tanins inhibit the proliferation of bacteria by inhibiting the metabolism enzymes of bacteria cells (Geidam et al 2007).

The *Phaleria macrocarpa* leaf extract potency was very weak against *P. aeruginosa*, therefore if used as therapeutic agent of *P. aeruginosa* infection it will need a high dose to inhibit the growth *P. aeruginosa* bacteria. An effective antimicrobial substance that is used as therapeutic agents must have low MIC (obtained from in vitro testing) and also must reach the serum level above the MIC (Koneman et al 1997). The serum level of antimicrobial agent was influenced by the concentration and route of entering substance, for example Cefalexin 1000 mg by oral dose needed to reach the serum level 10.6 µg/ml, while Gentamicin 80 mg intravenous is required to reach the serum level 6.3 µg/ml (Koneman et al 1997).

The *Phaleria macrocarpa* leaf extract which has MIC 4 x 10^4 µg/ml against *P. aeruginosa* when used for therapy of systemic *P. aeruginosa* infection must have serum level at least 4 x 10^4 µg/ml. The blood volume of adult human is about 5000 ml (Guyton 1994), so the *Phaleria macrocarpa* leaf extract as much 4 x 10^4 µg/ml x 5000 ml = 2 x 10^8 µg (2 x 10^5 mg = 2 x 10^2 g = 0,2 kg) is needed to reach the serum level 4 x 10^4 µg/ml by intravenous dose. When given orally, it is required more extract because it would be influenced by gastrointestinal tract absorption.

Former research about influence of butanol extract of *Phaleria macrocarpa* fruit to mice kidney (single intraperitoneal injection then after 3 week perceived its effect to kidney tissue), dose 80 mg/kg BW generate the tubular epithelial cell disintegration, dose 170 mg/kg BW generate minimal necrosis and tubules proximal leakage although it does not cause renal function failure (Soeksmanto 2006). It may infer that very high dose of *Phaleria macrocarpa* leaf extract will lead to dangerous side effects for its consumer.

The factors that caused *P. aeruginosa* relatively resistant to antimicrobials substances were, first, Alginate layer around of *P. aeruginosa* cell (slime layer) which could fasten the antimicrobial cation and decrease diffusion into the cell (Lambert 2002). The second factor was *P. aeruginosa* cell wall structure, especially hydrophobic outer membrane that became antimicrobials barrier, so it causes the decrease of cell wall permeability to antimicrobials substances (Lambert 2002). The third factor was the efflux pump at *P. aeruginosa* bacteria (mexAB-oprM, mexCD-oprJ, mexEF-oprN and mexXY-oprM) could pump out the antimicrobials molecules and cause the disaccumulation of antimicrobials molecules in the bacteria cells (Lambert 2002).

CONCLUSION

The *Phaleria macrocarpa* leaf extract has antimicrobial activity against *P. aeruginosa* beginning from the concentration 4 x 10^4 µg/ml (MIC = 4 x 10^4 µg/ml). It is also cause division inhibition and elongation of *P. aeruginosa* cells. Antimicrobial activity of *Phaleria macrocarpa* leaf extract against *P. aeruginosa* is very weak, so it is not suggested to use it as antimicrobial agent against *P. aeruginosa*.

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