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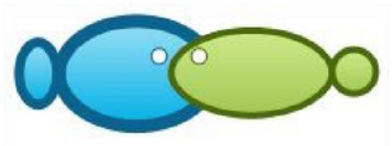

Antibacterial activity of mangrove *Avicennia marina* leaves extract against *Virgibacillus marismortui* and *Micrococcus luteus* bacteria

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Abstract. Sources of bioactive compounds are widely found in natural resources such as mangrove plants. *Avicennia marina* is one of the natural materials that contain antibacterial bioactive compound. The purpose of this research is to investigate the potency of *A. marina* leaves as a source of antibacterial agents against *Virgibacillus marismortui* and *Micrococcus luteus*. Leaves of *A. marina* were collected from mangrove ecosystem Tugurejo village, Semarang. Experimental laboratories method was used in this research, while data was analyzed descriptively. Sample was extracted by using methanol. Agar Diffusion method was utilized on anti bacterial test against *V. marismortui* and *M. luteus* with concentration of 1000 µg disc^{-1}, 500 µg disc^{-1} and 250 µg disc^{-1}. The result of the test showed that *A. marina* leaves extract of 1000 µg disc^{-1} could inhibit both bacteria, with 13.50±0.13 mm inhibition zone for *V. marismortui* and 13.46±0.32 mm for *M. luteus*, at concentration of 500 µg disc^{-1} to inhibit both bacteria with inhibition zone of 5.69±0.30 mm for *V. marismortui* and 6.56±0.51 mm for *M. luteus*, the concentration of 250 µg disc^{-1} did not inhibited bacteria. Antibiotic Amoxicillin as positive control showed smaller inhibition zone compared to inhibition zone that was formed by the extract of mangrove leaves. It was assumed because the high concentration of mangrove extracts, so that the bigger inhibition zone could be formed compared to the antibiotic treatment.

Key Words: bioactive compounds, turtle diseases, focal erosive dermatitis, antibacterial agent, inhibition.

Introduction. Conservation is an important policy in managing marine organism, particularly endangered species (Yusuf et al 2009; DeBoer et al 2014; Ambariyanto 2017). One of the ways to save the sea turtle population from extinction is by preparing turtle hatchlings in captivity before being release to the sea. However, the main challenge in hatchlings captivity is the mortality caused by diseases (Lutz & Musick 1996). One of the frequent diseases that infect sea turtle hatchlings is FED (Focal Erosive Dermatitis) bacterial infection. This disease is characterized by the progressive disintegration of cutaneous tissues and formation of shallow, erosive lesions which are sometimes covered with crusts of necrotic tissues (Leong et al 1989). Bacteria *Virgibacillus marismortui* and *Micrococcus luteus* are those that associated with FED disease (Ulmursida 2017).

Many medical efforts have been conducted to overcome the disease, one of them is using antibiotic and chemical substances, however the long term use of antibiotic can cause negative effect towards water environment and also pathogenic resistance (Sukenda et al 2008). Other alternatives must be done to heal FED disease, for example by using natural anti-bacterial substances. One of the natural resources that potentially having antibacterial characteristic is mangrove *Avicennia marina*. Some researches on antibacterial activity of mangrove in killing and inhibiting bacterial growth have been done (Abeysinghe & Wanigatunge 2006; Ravikumar et al 2010; Amirkaveei & Behbahani 2011; Prabhu & Guruvayoorappan 2012; Saptiani et al 2013).

Further research about potential of mangrove leaves *A. marina* in inhibiting the growth of bacteria that associated to FED disease has not been done. Big potential of *A.*

marina in the form of bioactive compound must be developed to be used as FED disease healer in turtle hatchlings in captivity.

The purpose of this research is to investigate the activity of the natural antibacterial extract from mangrove leaves *A. marina* to inhibit the growth of the bacteria *V. marismortui* and *M. luteus*.

Material and Method

Sampel extraction. Antibacterial activity test was conducted using *A. marina* leaves extract that was collected from mangrove ecosystem Tugurejo village, Semarang, Indonesia. The sample was finely cut with a knife, taken as many as 100 grams, and then separated into 3 erlenmeyer flasks. The leaves were immersed in 300 methanol solvent until completely submerged. After 24 hours, the solvent is evaporated using rotary evaporator (Trianto 2001).

Preparation of bacterial culture. Bacteria *V. marismortui* and *M. luteus* obtained from Tropical Marine Biotechnology Laboratory, Laboratorium Terpadu, Universitas Diponegoro, Semarang, Indonesia. Pure bacterial culture was inoculated to Zobell 2216E marine agar medium and incubated at 37°C for 24 hours (Asraf 2015). Some colonies of bacteria taken using an ose needle and these bacteria was inoculated into liquid Zobell 2216E marine agar medium, then homogenized with a vortex for ±1 minute to make a suspension and incubated at a temperature of 42°C for 24 hours. Turbidity of the solution was equated to McFarland 0.5 turbidity standard (Dwidjoseputro 1998).

Sensitivity test. The test to know the influence of mangrove leaves extract towards selected bacteria was conducted using agar diffusion method (Burnley 2000). Cultured bacteria inside liquid agar medium based on standard 0.5 McFarland were spread into Petri dish which contained solid Zobell 2216E marine agar medium until well-spread using sterilized spreader. Petri dish was divided into quadrants and paper disc (Ø 4.7mm) was placed, which contained extract of mangrove *A. marina* with concentrations of: 250, 500 and 1000 µg $disc^{-1}$, also amoxicillin for the positive control and methanol solution as the negative control was used, the volume that was poured to the paper disc was 10 µL for each concentration and control using micro pipette on every quadrant.

Medium was incubated at the temperature of 30°C for 24 hours and the inhibiting zone that appeared surrounding paper disc was measured using calipers. The activity of antibacterial substance towards certain bacteria was determined by the diameter of inhibiting zone which formed around paper disc. The inhibiting zone was measured from the distance of the zone edge to the other zone edge. The bigger diameter of inhibiting zone meant that the bigger potential of that antibacterial substance (mangrove) to kill or inhibit the growth of tested-bacteria (Mulyani et al 2013).

Inhibition zone measurement. If the sensitivity test shows a positive result of inhibition zone diameter formed is measured. The amount of inhibition zone is the diameter of inhibition zone minus diameter of paper disc (4.7 mm).

Results and Discussion. Antibacterial test results showed that the extract of mangrove leaves *A. marina* could inhibit the bacteria *V. marismortui* and *M. luteus* growth with the biggest inhibition zone for concentration of 500 µg $disc^{-1}$ recorded on the 36th hours cycle 3 as follows: 5.69±0.30 mm, bacteriostatic for *V. marismortui* and on the 36th hours cycle 2 was 6.56±0.51 mm, bacteriostatic for *M. luteus* (Table 1 & 2). Extract of *A. marina* with concentration of 1000 µg $disc^{-1}$ showed that the biggest inhibition zone with diameter of 13.50±0.13 mm was bactericidal on the 36th hours cycle 2 for *V. marismortui* and 13.46±0.32 mm was bactericidal on the 36th hours cycle 2 for *M. luteus*. Extract of mangrove leaves *A. marina* with concentration of 250 µg $disc^{-1}$ formed a very low inhibition zone in every cycle, it was assumed because the concentration of active compound contained in paper disc was too low.

Table 1

Inhibition zone (mm) resulted from antibacterial activity of *Avicennia marina* leaves extract towards bacteria *Virgibacillus marismortui*

Repetition	*Observations*	*Concentration (µg/disc)*	*A. marina leaves extract*		*Control (+)*		*Control (-)*	
					Amoxicillin		*Methanol*	
			Ø (mm)	*Activity*	*Ø (mm)*	*Activity*	*Ø (mm)*	*Activity*
1	12^{th} hours	250	0.00±0.00	-	2.54±0.29	Static	0.00±0.00	-
		500	1.38±0.16	Static				-
		1000	7.38±0.18	Cidal				-
	24^{th} hours	250	1.23±0.14	Static	5.39±0.15	Static	0.00±0.00	-
		500	3.68±0.36	Static				-
		1000	12.6±0.23	Cidal				-
	36^{th} hours	250	1.28±0.10	Static	5.61±0.27	Static	0.00±0.00	-
		500	3.69±0.36	Static				-
		1000	12.66±0.24	Cidal				-
2	12^{th} hours	250	0.00±0.00	-	1.34±0.11	Static	0.00±0.00	-
		500	0.00±0.00	-				-
		1000	8.69±0.23	Static				-
	24^{th} hours	250	0.93±0.16	Static	4.47±0.17	Static	0.00±0.00	-
		500	2.47±0.25	Static				-
		1000	13.47±0.13	Cidal				-
	36^{th} hours	250	0.97±0.20	Static	4.79±0.12	Static	0.00±0.00	-
		500	2.49±0.24	Static				-
		1000	13.50±0.13	Cidal				-

Repetition	Observations	Concentration (µg/disc)	A. marina leaves extract		Control (+) Amoxicillin		Control (-) Methanol	
			Ø (mm)	Activity	Ø (mm)	Activity	Ø (mm)	Activity
3	12th hours	250	0.00±0.00	-	0.86±0.13	Static	0.00±0.00	-
		500	1.73±0.11	Static				-
		1,000	7.81±0.18	Cidal				-
	24th hours	250	0.54±0.07	Static	3.48±0.23	Static	0.00±0.00	-
		500	5.66±0.29	Cidal				-
		1,000	12.78±0.13	Cidal				-
	36th hours	250	0.56±0.08	Static	3.53±0.19	Static	0.00±0.00	-
		500	5.69±0.30	Static				-
		1,000	12.83±0.12	Cidal				-

Note:
- Ø (mm) = area of inhibition zone;
- Ø (mm) 0.00 = no activity;
- Activity (-) = no activity; cidal = bactericidal; static = bacteriostatic;
- The data had been reduced by paper disc diameter of 4.7 mm;
- Data are the average result from 3 times repetition ± standard deviation.

Table 2

Inhibition zone (mm) resulted from antibacterial activity of *Avicennia marina* leaves extract towards bacteria *Micrococcus luteus*

Repetition	*Observations*	*Concentration (µg/disc)*	*A. marina leaves extract*		*Control (+)*		*Control (-)*	
					Amoxicillin		*Methanol*	
			Ø (mm)	*Activity*	*Ø (mm)*	*Activity*	*Ø (mm)*	*Activity*
1	12^{th} hours	250	0.00±0.00	-	3.63±0.51	Static	0.00±0.00	-
		500	0.96±0.22	Static				-
		1,000	2.87±0.26	Cidal				-
	24^{th} hours	250	1.81±0.12	Static	7.27±0.35	Static	0.00±0.00	-
		500	2.23±0.40	Static				-
		1000	3.49±0.18	Cidal				-
	36^{th} hours	250	1.93±0.22	Static	7.33±0.40	Static	0.00±0.00	-
		500	2.35±0.39	Static				-
		1,000	3.66±0.11	Cidal				-
2	12^{th} hours	250	0.31±0.11	Static	2.17±0.35	Static	0.00±0.00	-
		500	3.89±0.12	Static				-
		1,000	6.23±0.31	Cidal				-
	24^{th} hours	250	1.31±0.29	Static	5.83±0.55	Static	0.00±0.00	-
		500	6.47±0.49	Cidal				-
		1,000	13.31±0.37	Cidal				-
	36^{th} hours	250	1.35±0.23	Static	5.94±0.57	Static	0.00±0.00	-
		500	6.56±0.51	Static				-
		1,000	13.46±0.32	Cidal				-

Repetition	Observations	Concentration (µg/disc)	A. marina leaves extract		Control (+)		Control (-)	
					Amoxicillin		Methanol	
			Ø (mm)	Activity	Ø (mm)	Activity	Ø (mm)	Activity
3	12th hours	250	0.00±0.00	-	0.77±0.45	Static	0.00±0.00	-
		500	0.00±0.00	-				-
		1,000	4.85±0.21	Static				-
	24th hours	250	0.36±0.14	Static	1.37±0.42	Static	0.00±0.00	-
		500	0.87±0.30	Cidal				-
		1,000	10.33±0.23	Cidal				-
	36th hours	250	0.44±0.10	Static	1.61±0.30	Static	0.00±0.00	-
		500	0.91±0.32	Static				-
		1,000	10.41±0.21	Cidal				-

Note:

- Ø (mm) = area of inhibition zone;
- Ø (mm) 0.00 = no activity;
- Activity (-) = no activity; cidal = bactericidal; static = bacteriostatic;
- The data had been reduced by paper disc diameter of 4.7 mm;
- Data are the average result from 3 times repetition ± deviation standard.

The average inhibition zone that was formed from concentration of 250 µg $disc^{-1}$ showed diameter of inhibition zone <5 mm in every observation, which meant it had low antibacterial activity. The average inhibition zone that was formed from concentration of 500 µg $disc^{-1}$ showed diameter of inhibition zone <5 mm in the observation on 12^{th} hours and 5-10 mm in the 12^{th} hours and in the 36^{th}, which meant it was having antibacterial activity in low-medium category. Extract of mangrove with high concentration of 1000 µg $disc^{-1}$ in every tested extract showed the average inhibition zone diameter 5-10 mm, during the 12^{th} hours observation and 10-20 mm on the 24^{th} hours and on the 36^{th}, which meant it was having medium-high antibacterial activity, therefore it has a potential to inhibit bacterial growth.

The categorizing of the results was based on Davis & Stout (1971) who mentioned that the antibiotic/antibacterial inhibition zone with diameter >20 mm means very strong, inhibition zone with diameter 10-20 mm means strong, inhibition zone with diameter 5-10 mm means medium, and weak inhibition zone ranges around <5 mm. With the inhibition zone obtained, extract of mangrove leaves *A. marina* in high concentration had a strong antibacterial activity while the other extracts with low concentration showed weak antibacterial activity.

The test results on amoxicillin as positive control showed smaller inhibition zone compared to inhibition zone that was formed by the extract of mangrove leaves. It was assumed because the high concentration of mangrove extracts, so that the bigger inhibition zone could be formed than in the antibiotic treatment. As for negative control which used methanol solvent, there was no any antibacterial activity observed. This solvent did not contain any bioactive compound that could inhibit bacterial growth, so that the inhibition zone was not formed (negative). Extract of mangrove leaves contained equal antibacterial bioactive compounds in each concentration and the amount was not significantly different, so that the resulted inhibiting activity was not significantly different among concentrations. According to Herawati et al (2011), generally mangrove contains flavonoid, steroid, tannin, saponin compounds which were having antibacterial characteristic.

Flavonoid compound is potential as antibiotic and antibacterial. This compound is synthetized by plants as the defense system during its response towards infection caused by microorganism, so that this compound is effective as antimicrobial compound towards a number of microorganisms (Parubak 2013).

Flavonoid compounds as antibacterial substances which naturally can be found in mangrove, especially in its leaves, can be extracted using methanol solvent (CH_3OH). The use of this methanol solvent was because methanol is classified as polar solvent which could dissolve polar compounds such as alkaloid, quartener, phenolic components, carotenoid, flavonoid, and tannin (Wardhana et al 2005). According to Pavia et al (1985), methanol has no characteristic as antibacterial that could inhibit the growth of the bacteria. Methanol is also having low boiling point at 65°C, is relativity affordable, and easily accessed (Susanti et al 2012).

According to the measurement result, the high concentration on extract of mangrove has the ability to inhibit bacteria growth. Although the inhibition zone that formed was not quite different in each extract concentration, but the resulted inhibition ability was aligned to the increase of concentration. In this case, the higher was the concentrations the higher was the formed inhibition zone. This was assumed because every increase of concentration, the amount of bioactive compound increases so the ability to inhibit bacteria growth could also increase. According to Sucianti et al (2012), the higher concentration of extract of mangrove leaves, the more the amount of antibacterial material contains. The result from antibacterial test showed that extract of mangrove leaves (*A. marina*) could inhibit the growth of bacteria *V. marismortui* and *M. luteus*. The ability extract to inhibit the bacterial growth generally were found in the concentration of 500 µg $disc^{-1}$ and 1000 µg $disc^{-1}$ for 36 hours. Meanwhile the concentration of 250 µg/disc could not inhibit the bacterial growth of *V. marismortui* and *M. luteus*.

Conclusions. Extract of mangrove leaves *A. marina* has potential as antibacterial for bacteria *V. marismortui* and *M. luteus* with high concentration. *A. marina* leaves extract with 1000 µg disc^{-1} concentration could inhibit bacteria *V. marismortui* with inhibition of 13.50±0.13 mm and 13.46±0.32 mm for *M. luteus*, as for the concentration of 500 µg disc^{-1} could inhibit *V. marismortui* with inhibition zone of 5.69±0.30 mm and 6.56±0.51 mm for *M. luteus*, lastly the concentration of 250 µg disc^{-1} could not inhibit the subjected bacteria.

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