

ANTIBACTERIAL ACTIVITY ASSAY OF MANGROVE EXTRACTS AGAINST SALMONELLA TYPHI AND LISTERIA MONOCYTOGENES

UJI AKTIVITAS ANTIBAKTERI EKSTRAK MANGROVE TERHADAP SALMONELLA TYPHI DAN LISTERIA MONOCYTOGENES

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ABSTRAK

Aktivitas antibakteri ekstrak tanaman mangrove, *Avicennia marina*, *Sonneratia caseolaris* (berasal dari Teluk Payo, Banyuasin, Sumatera Selatan), *Ceriops tagal*, *Rizhopora apiculata*, dan *Sonneratia alba* (berasal dari Sadai, Bangka Selatan) terhadap *Salmonella typhi* dan *Listeria monocytogenes* diteliti menggunakan uji disk difusi agar. Ekstraksi dilakukan menggunakan solven organik (metanol, etil asetat dan aseton secara berurutan). Sebagian besar dari ekstrak yang diuji menunjukkan potensi aktivitas antibakteri terhadap kedua patogen. Ekstrak metanol kulit batang *S. alba* dan buah *A. marina* menunjukkan zona hambat yang cukup besar (15 mm) terhadap *S. typhi*. Ekstrak aseton daun *S. alba* menunjukkan zona hambat terbesar (14 mm) ketika diuji terhadap *L. monocytogenes*. Purifikasi parsial lebih lanjut dari ekstrak terpilih yang menunjukkan daya hambat besar dilakukan menggunakan kromatografi kolom gel silika dengan berbagai komposisi eluen yang memiliki perbedaan polaritas. Fraksi ketiga dari ekstrak metanol daun *S. alba* yang dielusi dengan kloroform:metanol (1:5) menghasilkan zona hambat yang besar (23 mm) terhadap *S. typhi*. Fraksi ketiga dan ketujuh dari ekstrak aseton daun *S. alba* yang dielusi dengan etil asetat:metanol (7:3) menghasilkan zona hambat yang besar (15 mm) terhadap *L. monocytogenes*. Sedangkan fraksi keenam dari ekstrak metanol buah *A. marina* yang dielusi dengan kloroform:metanol (6:4) menghasilkan zona hambat terbesar (17 mm) terhadap *L. monocytogenes*. Hasil ini mengindikasikan bahwa ekstrak mangrove dapat dikembangkan menjadi biomaterial untuk industri biofarmasi maupun biopreservasi.

Kata kunci: aktivitas antibakteri, mangrove, kromatografi kolom

ABSTRACT

The antibacterial activities of mangrove species, *Avicennia marina*, *Sonneratia caseolaris* (collected from Teluk Payo, Banyuasin, South Sumatera), *Ceriops tagal*, *Rizhopora apiculata*, and *Sonneratia alba* (collected from Sadai, South Bangka) were screened against *Salmonella typhi* and *Listeria monocytogenes* by agar disk diffusion assays. Extractions were conducted using organic solvents (methanol, ethyl acetate, and acetone, subsequently). Most of the extracts tested showed potential antibacterial activity against both pathogens. The methanol extracts of the bark from *S. alba* and the fruit from *A. marina* showed particularly large inhibition zones (15 mm) against *S. typhi*. The acetone extract of *S. alba* leaves showed the largest inhibition zone (14 mm) when tested against *L. Monocytogenes*. Further partial purifications of selected extracts which showed strong inhibition were performed by silica gel column chromatography using various eluent compositions with different polarities. The third fraction of methanol extract from *S. alba* leaves eluted with chloroform:methanol (1:5) resulted in a remarkably large inhibition zone (23 mm) against *S. typhi*. The third and seventh fractions of acetone extract from *S. alba* leaves eluted with ethyl acetate:methanol (7:3) resulted in a large inhibition zones (15 mm) against *L. monocytogenes*. In addition, the sixth fraction of methanol extract from *A. marina* fruit eluted with chloroform : methanol (6:4) resulted in the largest inhibition zone (17 mm) against *L. monocytogenes*. These results indicated that mangrove extracts could be developed as potential biomaterials for biopharmaceutical as well as biopreservation industries.

Keywords: antibacterial activity, mangrove, column chromatography

I. INTRODUCTION

Health problems caused by bacterial pathogens derived from food spoilage remains to be a risk factor particularly in developing countries which have sanitation issues. Antibiotics and chemicals are commonly used as therapeutic medicines against bacterial infections. However, maltreatments of antibiotics and chemotherapeutic agents are heading to the new problem of drugs resistance. Thus, the discovery of new and safe antibacterial compounds is continuously needed to work against resistant pathogens.

The widespread use of plants for medication is already known for a long period of time. Plant extracts and phytochemicals have the potentials as alternative treatments against bacterial infections. Indonesia has been blessed with enormous tropical plant species that may represent a source of natural medicines. An interesting group of plants in this regard are mangroves. According to Kusmana (2014), there are approximately 3.2 million hectares of mangrove area in Indonesia with over 202 mangrove species. However, natural medicines derived from mangroves are still inadequately studied due to their limited distribution.

Mangroves are typical plants that live in littoral zone with harsh environmental conditions such as anaerobic soils, wide range of saline concentrations, tidal waves, winds and extreme temperatures. Mangrove plants often produce diverse groups of unique chemical components as secondary metabolites for protection and adaptation in such challenging conditions. Those compounds often have interesting antibacterial, antiviral and antifungal activities. Nevertheless, there are only limited investigations that have been conducted to identify the metabolite agents, which might be responsible for their bioactivities (Bandaranayake, 2002; Eldeen and Effendy, 2013).

In vitro screening for antibacterial activity could be one of the initial steps for the discovery of new antimicrobial agents

derived from mangrove extracts. The current study aims to explore the potentials of some selected mangrove species collected from Indonesian mangrove forests, with emphasized to screen their antibacterial activities against two representative foodborne pathogens- *Salmonella typhi* and *Listeria monocytogenes*.

S. typhi is a Gram-negative bacterium that belongs to the family *Enterobacteriaceae*. *S. typhi* infection could lead to the development of typhoid or enteric fever and remains as a threat for public health. Some risk factors such as the overuse, misuse and inappropriate antibiotics prescribing could be responsible for the increasing number of multi-drug resistance problem among *S. typhi* strains, including ineffective or sub-optimal therapy using ampicillin, chloramphenicol, trimethoprim-sulphamethoxazole, and fluoroquinolones such as ciprofloxacin, and ofloxacin (Zaki and Karande, 2011; Butler, 2011).

L. monocytogenes is a Gram-positive bacterium. *L. monocytogenes* is a causative agent of listeriosis, however, unlike any other foodborne pathogens, it has the ability to survive food-processing technologies, and could grow even in properly refrigerated food. People usually get infected through the consumption of contaminated food. Although it is a relatively rare infection, *L. Monocytogenes* is one of the most virulent foodborne pathogens since it could lead to severe infection with up to 30% fatality rates even with early and adequate antibiotics treatment. The most common clinical manifestation of listeriosis is meningitis, and usually being treated with ampicillin, penicillin or a combination of ampicillin and gentamycin. Other options are trimethoprim-sulfamethoxazole, erythromycin, vancomycin, fluoroquinolones (ciprofloxacin) and quinolones (levofloxacin and moxifloxacin). Some *L. monocytogenes* isolates were reported to have low resistance to these antibiotics (Allerberger and Wagner, 2010; Hernandez-Milian and Payeras-Cifre, 2014).

II. METHODS

2.1. Mangrove Plants Collection

The mangrove plants, *Avicennia marina* (Forsk.) Vierh., and *Sonneratia caseolaris* (L.) Engl., were collected from Teluk Payo, Banyuasin, South Sumatera, Indonesia. The locations extend from 02°26'08.6" to 02°26'12.45"S and 104°45'52" to 104°45'59.9"E. While *Ceriops tagal* (Perr.) C. B. Rob., *Rizhopora apiculata* Bl., and *Sonneratia alba* J.E. Smith, were collected from Sadai, South Bangka, Indonesia, with sampling locations extend from 03°00'02.1" to 03°00'15.2"S and 106°43'45.2" to 106°43'43.2"E. Information about the environmental conditions including salinity, temperature, pH and dissolve oxygen (DO) of the locations were recorded. The mangrove samples were taxonomically identified based on their morphological features at Marine Science Department, Faculty of Natural Science, Sriwijaya University, South Sumatera, Indonesia.

2.2. Mangrove Samples Extraction

The fresh samples were washed with water to remove dirt and then dried in the oven at 60°C for 3 days for leaf samples and up to 7 days for root, bark and fruit samples, respectively. The dried samples were then crushed and powdered into fine particles. Instead of the fresh samples, the dried mangrove plant materials were used since the differences in water content may affect the solubility. The dried samples were ground into fine particles to increase the surface areas, thus will increasing the extraction rates.

The extraction method aims to separate the bioactive compounds of the plant parts from the inactive components using selected solvents. The choice of the solvents for extraction had definite effects for the isolation of bioactive compounds since the solvents will diffuse into the solid plant materials and solubilise the biocompounds with similar polarity during extractions. The

longer the contact between solvents and materials the more the bioactive compounds will be extracted. Furthermore, the selected solvents should be less toxic and should not influence the antibacterial assay results (Ncube *et al.*, 2008). Based on the fact that most of the identified bioactive compounds which possess antibacterial activity are commonly less polar or not water soluble, organic solvents (methanol, ethyl acetate and acetone) were used in this study.

The powdered samples were extracted with a series of organic solvents at room temperature according to the standard methods (Joel and Bhimba, 2010; Sahoo *et al.*, 2012) with some modifications. Briefly, to prepare methanol extracts, 100 g of powdered samples were soaked into 250 mL 80% methanol and stirred. The solvent was substituted in every 24 hours for 3 days. The total extracts were then filtered through Whatman No.1 filter paper. The filtrate was concentrated using a rotary evaporator at 50°C to get crude methanol extracts. To prepare ethyl acetate extracts, methanol extract residues were soaked into 250 mL 80% ethyl acetate and stirred. The solvent was substituted in every 24 hours for 3 days. The total extracts were then filtered through Whatman No.1 filter paper. The filtrate was concentrated using a rotary evaporator at 40°C to get crude ethyl acetate extracts. To prepare acetone extracts, ethyl acetate extract residues were soaked into 250 mL 80% acetone and stirred. The solvent was substituted in every 24 hours for 3 days. The total extracts were then filtered through Whatman No.1 filter paper. The filtrate was concentrated using a rotary evaporator at 40°C to get crude acetone extracts. The extracts were stored at 4°C in air-tight glass vials prior to *in vitro* antibacterial screening.

2.3. Antibacterial Activity Assay

Initial screening of potential antibacterial activity or commonly known as antimicrobial susceptibility testing (AST) of the crude mangrove extracts was performed

against *Salmonella typhi* (P2KIM collection) and *Listeria monocytogenes* (BTCCB693) by Kirby-Bauer agar disk diffusion test method (Bauer *et al.*, 1966; CLSI, 2012). The AST method is formerly used as an essential method to determine the microbial resistance to antimicrobials. In this study, the AST method was used as preliminary screening to elucidate the efficacy of antimicrobial candidates derived from mangrove extracts against representative microbial pathogens. The agar diffusion based is the conventional reference method, however, the bactericidal and bacteriostatic effects could not be differentiated using this method.

Briefly, the obtained crude extracts dissolved in the respective solvents (20 µl) and positive controls (penicillin or chloramphenicol) were individually applied to the sterilized filter paper disks (Filtres Fioroni[®], France; 6 mm in diameter) and then placed on nutrient agar (NA-Oxoid[®], England) test plates inoculated with the overnight culture of pathogens which had been prepared from the suspension equivalent to 0.5 McFarland turbidity standard (10⁸ CFU/ml). Sterile forceps were used to assure complete contact between the paper disks and the surface of agar medium. The disks were arranged in a proper space to prevent the overlapping of the inhibition zones. The whole set up was first incubated at 4°C for 2 hours to give sufficient time for the samples to diffuse into the medium. Then the test plates were kept at 37°C for 16, 20, or 24 hours to allow the bacterial growth. The antibacterial activity assays were determined by measuring the diameter of inhibition zones formed around the paper disks.

2.4. Partial Purification of Bioactive Compound

Selected crude mangrove extracts which showed strong inhibition zone were applied into a column chromatography packed with silica gel (60-120 Mesh, Merck) based on the method of Mishra and Sree (2007) with some modifications. Each extract

was eluted with various selected eluent compositions initially tested on thin layer chromatography (TLC) according to the method described by Harborne (1984), for optimization of the gradient polarities i.e., chloroform: methanol (1:5); ethyl acetate : methanol (7:3); and chloroform : methanol (6:4). Individual fractions were then collected and tested for antibacterial activity screening by the same method as described above. Further TLC analysis using silica gel GF₂₅₄ (Merck) and chloroform: methanol (8:3) as eluent was carried out for the fraction with the strongest antibacterial activity. The TLC results were visualized after heat treatment and under UV light 254 nm.

III. RESULTS AND DISCUSSIONS

3.1. Mangrove Samples Collection and Extraction

Mangrove plants are generally undervalued and poorly managed. However, numerous mangrove plants had been used in folklore medicine as alternative treatment against human and animal diseases. In this regard, scientific evidence of their bioactivities are considerably needed. Mangroves are usually need warm condition for survival, thus most of their habitats are in tropical areas. In this study, five species of mangrove plants (i.e., *A. marina*, *C. tagal*, *R. apiculata*, *S. alba* and *S. caseolaris*) were collected from Indonesian mangrove habitats. Different plant parts of the mangrove species (roots, barks, leaves and fruits) were selected as the samples for the screening of antibacterial activity (Figure 1). In addition, the environmental factors such as salinity, temperature, pH, and dissolve oxygen that could affect the bioactive constituents of the samples and leading to the differences in their bioactivities, were recorded (Table 1).

3.2. Antibacterial Activity Assay

Food borne pathogens and drug resistance microbes such as *S. typhi* and *L. monocytogenes* remain to be problems

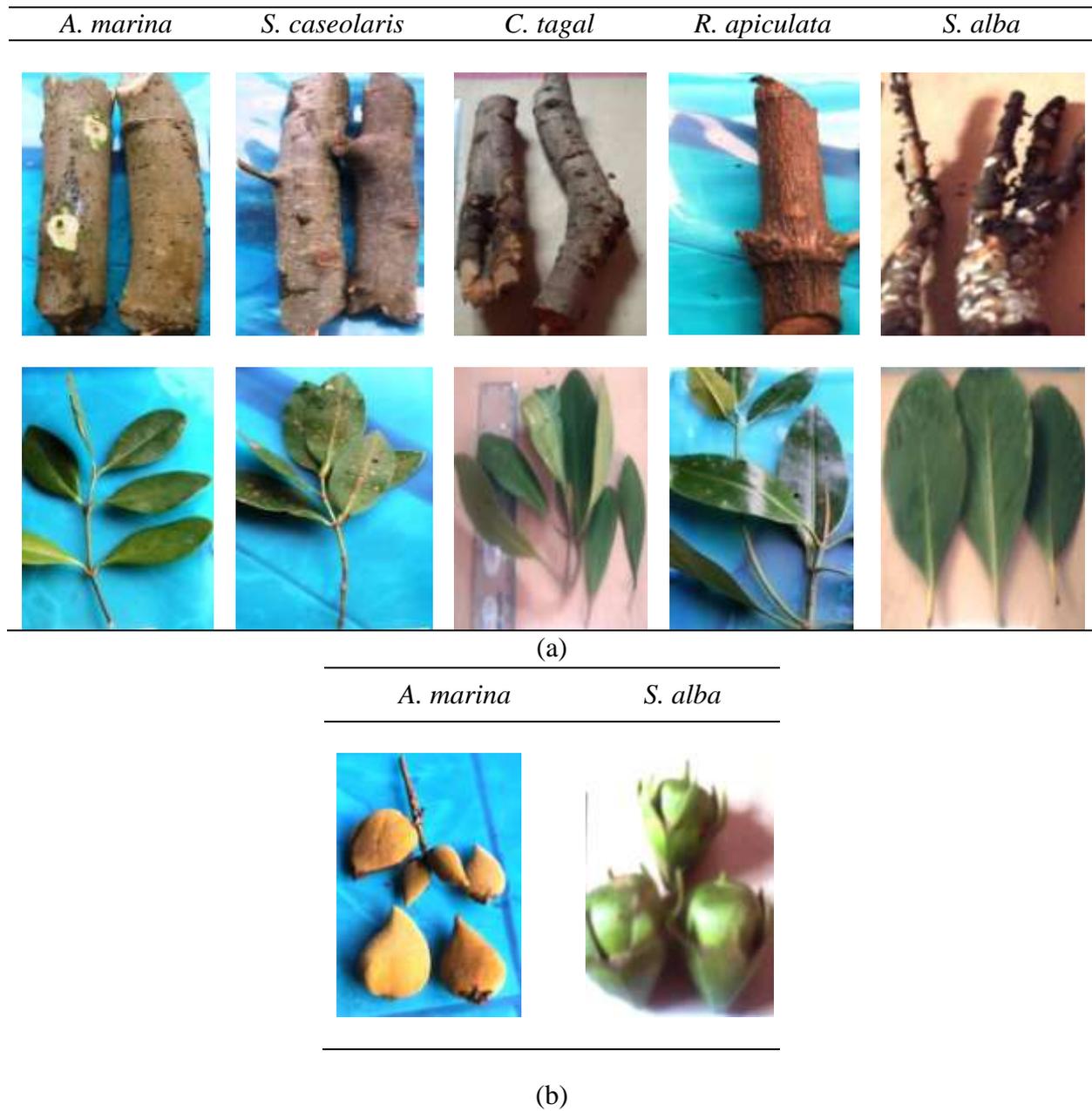


Figure 1. Photographs of mangrove plants collected in this study (personal collection, 1:10 in scale). Barks and leaves (a), Fruits (b).

Table 1. Environmental characteristics of mangrove habitat at Teluk Payo, Banyuasin, South Sumatera and Sadai, South Bangka.

No	Parameter	Teluk Payo			Sadai	
		<i>A. marina</i>	<i>S. caseolaris</i>	<i>C. tagal</i>	<i>R. apiculata</i>	<i>S. alba</i>
1	Salinity (‰)	14.00	14.00	25.00	25.00	25.00
2	Temperature (°C)	31.20	34.60	30.00	29.00	30.00
3	pH	7.70	7.60	7.80	7.90	7.70
4	DO (mg/L)	5.04	4.38	5.02	4.90	5.60

among food industries. In the meantime, people are questioning the safety of antibiotics and chemical preservatives. In this study, natural inhibitors for food borne pathogens derived from the extracts of selected mangrove plants were evaluated. This study revealed that the mangrove crude extracts derived from different plant parts and eluted with different solvents show positive antibacterial activities against both of *S. typhi* and *L. monocytogenes*.

Strong inhibition activities against *S. typhi* were shown by methanol extracts of the bark from *S. alba* and the fruit from *A. marina* (15 mm, respectively), compared to the positive control (10 mm), after 16 hours observations (Table 2). While acetone extract of the leaves from *S. alba* exhibited particularly large inhibition zone against *L. monocytogenes* (14 mm), although it was smaller compared to the positive control (18 mm), after 24 hours observations (Table 3). This variable results of inhibition zones could be due to the differences of bioactive compounds solubility among the crude extracts.

Previous research articles also provide informations about the biological activities of mangrove extracts. Abeysinghe (2010) reported that some mangroves species had antibacterial activities against antibiotic resistant *Staphylococcus aureus*. Dhayanithi *et al.* (2012) reported that methanol extract of the leaves from *A. marina* could inhibit the

growth of *S. aureus*. The silvernanoparticles derived from water extract of the leaves from *C. tagal* exhibited antimicrobial activity against pathogenic bacteria and fungi (Dhas *et al.*, 2013). According to Pimpliskar *et al.* (2012), ethanol extract of the stem from *R. apiculata* showed antibacterial as well as antifungal activities. It is reported that ethanol extract of the leaves from *S. alba* could inhibit the growth of *S. typhi* (Sahoo *et al.*, 2012), while methanol extract of the seeds from *S. caseolaris* exhibited growth inhibitory effect against *S. aureus* (Mahadlek *et al.*, 2012). The methanol extract of *S. caseolaris* also reported to have antibacterial activities against multi-drug resistant pathogens by Yompakdee *et al.* (2012).

3.3. Partial Purification of Bioactive Compound

Mangrove species are known as a rich source of bioactive compounds including steroids, triterpenes, phenolics, saponins, flavonoids, alkaloids and tannins (Bandaranayake, 2002). These phytochemicals have toxicological, pharmacological and ecological importance. A knowledge of the chemical constituents of the bioactive compounds derived from natural products is required since it can be great value in discovering new sources of economic phytochemicals particularly in regard of mangrove plants. The phytochemicals could be responsible for the

Table 2. Antibacterial activity of crude mangrove extracts against *S. typhi* after 16 hours observation.

Mangrove	Zone of inhibition (mm)											
	methanol extracts				ethyl acetate extracts				acetone extracts			
	root	bark	leaf	fruit	root	bark	leaf	fruit	root	bark	leaf	fruit
<i>A. marina</i>	7	7	8	15	8	9	11	-	-	-	-	-
<i>C. tagal</i>	9	11	9	-	10	14	11	-	-	-	12	-
<i>R. apiculata</i>	-	12	7	-	-	10	9	-	-	-	-	-
<i>S. alba</i>	-	15	11	-	-	13	13	-	-	-	13	-
<i>S. caseolaris</i>	-	-	12	-	-	-	11	-	-	-	-	-

Note: (-) : not determined.

Positive control (penicillin) showed 10 mm of inhibition zone.

Table 3. Antibacterial activity of crude mangrove extracts against *L. monocytogenes* after 24 hours observation.

Mangrove	Zone of inhibition (mm)											
	methanol extracts				ethyl acetate extracts				acetone extracts			
	root	bark	leaf	fruit	root	bark	leaf	fruit	root	bark	leaf	fruit
<i>A. marina</i>	-	-	9	12	-	-	10	-	-	-	-	-
<i>C. tagal</i>	10	11	-	-	10	12	10	-	-	-	13	-
<i>R. apiculata</i>	-	12	11	-	-	9	-	-	-	-	-	-
<i>S. alba</i>	-	13	-	-	-	12	12	-	-	-	14	-
<i>S. caseolaris</i>	-	-	12	-	-	-	13	-	-	-	-	-

Note: (-) : not determined.

Positive control (chloramphenicol) showed 18 mm of inhibition zone.

antibacterial activities as shown by the mangrove extracts tested in present study. It might be possible that each of the mangrove extracts contain multiple bioactive compounds, and they inhibit bacterial growth in a various ways. Bioactivity guided fractionation using column chromatography was performed as an initial approach to separate the targeting compounds from the mixture within the crude extracts (Joel and Bhimba, 2010).

Some of the obtained fractions showed stonger inhibition activities compared to the crude extracts. The third fraction of methanol extract of the leaves from *S. alba* eluted with chloroform: methanol (1:5) resulted in 23 mm of inhibition zone against *S. typhi* after 16 hours observation (Table 4 and Figure 2). While chloramphenicol as positive control also showed 23 mm of inhibition zone. The third and seventh fractions of acetone extract of the leaves from *S. alba* eluted with ethyl acetate: methanol (7:3) resulted in 15 mm of inhibition zones against *L. monocytogenes*. The sixth fraction of methanol extract of the fruit from *A. Marina* eluted with chloroform: methanol (6:4) resulted in 17 mm of inhibition zone against *L. monocytogenes* after 20 hours observation

(Table 5 and Figure 3). While chloramphenicol as positive control showed 20 mm of inhibition zone. In addition, thin layer chromatography (TLC) analysis of the fraction of *S. alba* which exhibited the largest inhibition zone was performed as initial detection of bioactive compound (Figure 4).

Table 4. Inhibition zone (mm) of column fractions against *S. typhi* after 16 hours observation.

Fraction eluted from column chromatography	Methanol extract of <i>S. alba</i> (leaves) chloroform : methanol (1:5)
1	7
2	8
3	23
4	15
5	8
6	7
7	9
8	8
9	8
10	9
Positive control	Chloramphenicol: 23

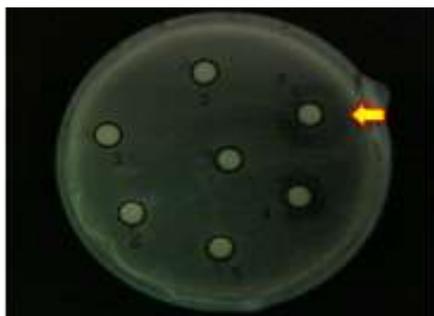


Figure 2. The arrow indicates the inhibition zone of the third fraction of methanol extract of the leaves from *S. alba* eluted with chloroform : methanol (1:5) against *S. typhi* after 16 hours observation.

Table 5. Inhibition zone (mm) of column fractions against *L. monocytogenes* after 20 hours observation.

Fraction eluted from column chromatography	Acetone extract of <i>S. alba</i> (leaves) ethyl acetate : methanol (7:3)	Methanol extract of <i>A. marina</i> (fruit) chloroform : methanol (6:4)
1	-	-
2	-	-
3	15	14
4	-	-
5	9	-
6	-	17
7	15	8
8	-	10
9	7	9
10	7	10
Positive control	Chloramphenicol: 20	Chloramphenicol: 20

Note: (-) : negative.

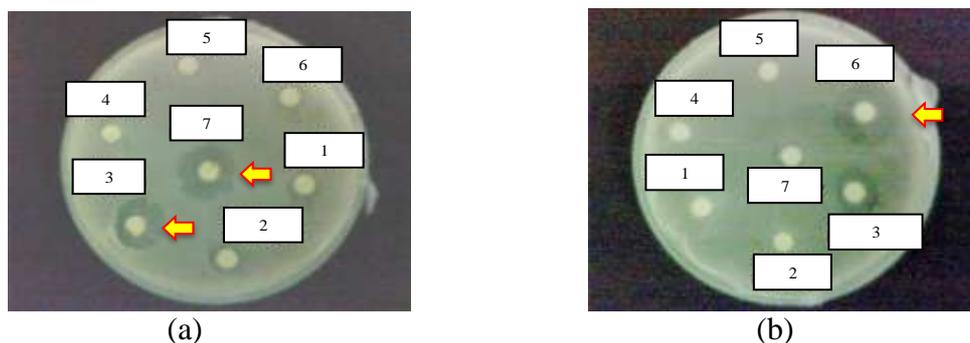


Figure 3. The arrows indicate the inhibition zone of :
 (a). The third and seventh fractions of acetone extract of the leaves from *S. alba* eluted with ethyl acetate : methanol (7:3) against *L. monocytogenes* after 20 hours observation.
 (b). The sixth fraction of methanol extract of the fruit from *A. Marina* eluted with chloroform : methanol (6:4) against *L. monocytogenes* after 20 hours observation.

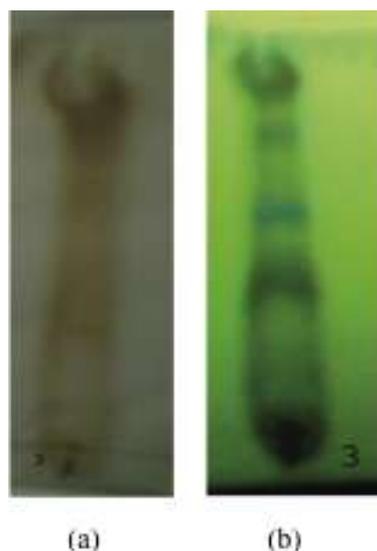


Figure 4. Thin layer chromatography (TLC) analysis of the third fraction of methanol extract of the leaves from *S. alba* eluted with chloroform : methanol (8:3). Detection after heat treatment (a), detection under UV light 254 nm (b).

IV. CONCLUSION

The crude extracts derived from different parts of the mangrove plants with different solvents exhibited various level of antibacterial activities. The results suggest that the column fractions of mangrove extracts particularly from *S. alba* showed similar antibacterial activity compared to the positive control. This promising findings suggest the presence of bioactive properties against bacterial pathogens as the alternative sources of antibacterial compounds. The mangrove plants, originated from Indonesian areas, have potential to be explored as valuable sources for pharmaceutical applications. However, further elucidation of the compounds responsible for the bioactivities including identification, sufficient isolation and purification, and also the analysis of antibacterial mode of action need to be performed for natural drug development in the future.

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