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Antibacterial Screening of Mangrove Extract Library: Accelerating Drug Discovery from Indonesian Biodiversity

Razethy Batubara[a], Julkipli Amsyir[a], Irmanida Batubara[b], and Kholis Abdurachim Audah[a*]

[a]*Department of Biomedical Engineering, Faculty of Life Sciences and Technology, Swiss German University, Tangerang 15143, Indonesia*
[b]*Biopharmaca Tropica Research Center, Bogor Agricultural University, Bogor 16128, Indonesia*

kholis.audah@sgu.ac.id

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Humans are at a continuous battle against different types of diseases, so that extraordinary effort to accelerate drug discovery has become a necessity. Indonesian biodiversity is abundant natural resources that can be utilized as potential drug sources. Mangroves are among potential plant medicine that grow nearly at all Indonesian coastlines. The aim of this study was to evaluate the potential of mangrove extracts (extract library) as antibacterial agents. In this study, eight mangroves species were used. There were 16 samples collected from different parts of the plants such as leaf, bark or root. Four types of solvents with different polarity were used producing 64 extracts. Disk diffusion method was used for antibacterial screening using five bacterial strains. There were 37 extracts showed antibacterial potential with the lowest and the highest recorded inhibition index were 0.0283 and 1.8983, respectively. The highest inhibition index was recorded for ethyl acetate extract of root of *Bruguiera gymnorrhiza* (77 Ea) screened against *Escherichia coli*. The second highest inhibition index was 0.7867 recorded for leaf of water extract of *Avicennia marina* (84 A) screened against *Staphylococcus aureus.* Phytochemical analysis of the extracts were also evaluated. The majority of samples showed saponin and tannin in considerable amount. This supported the data that mangrove extracts were potential as antibacterial agents.

Keywords: *Antimicrobial, drug discovery, drug resistant, extract library, Indonesian biodiversity*.

Drug discovery is a lengthy and expensive process. On the other hand, different types of diseases or drug resistant pathogens are increasing in numbers from time to time. The World Health Organization once reported that as many as 30 new diseases could emerge in 20 years period of time [1]. Therefore, finding alternatives for drug sources is urgently required. Drug discovery through screening process utilizing natural products can become a solution of the slow and expensive drug discovery process using conventional way.

Indonesia is well known as one of the richest country in the world in biodiversity [2]. The country possesses approximately 14000 islands, located between Indian and Pacific Oceans. According to Fauna and Flora International (FFI), Indonesia is home of approximately 11% or more than 30000 of the world's flowering plants and other biota both in land and marine with significant figures [3].

One of potential plants as medicinal sources and widely spread along Indonesian coastline is Mangrove. Mangrove and mangrove associates are very potential plants as medicinal sources [4]. Along roughly 90000 kilometer coastline, Indonesia is home of about 20 families with hundreds species of mangroves and their associates. Indonesia has the largest mangrove forest or about 23% of total world mangrove forests [5].

The aim of this study was to evaluate the potential of mangrove extracts (extracts library) as antibacterial agents. Since long time ago different parts of mangrove trees either roots, branches, leaves, flowers and the fruits had been utilized as food and medicinal sources.

Previous qualitative phytochemical studies showed that leaf extract of Rhizophora stylosa and Avicenna marina contain flavonoid, terpenoid, alkaloid, flavonoid and glycosidic phenolic [6]. Bioactivity of mangrove extracts against other types of diseases had also been reported.

Water content of simplisia (Tabel 1) showed that 15 out of 16 samples were kept below 10% which is a suitable percentage for simplisia analysis according to Indonesian Herbal Pharmacopeia [7]. Only one sample with sample code 83 which was originated from fruit of *Thespesia populnea* showed a slightly higher water content than 10% (10.06%).

Table 1: Water content of simplisia*.

Samples code	Water content (%)	Samples code	Water content (%)
72	8.60	81	7.56
73	2.30	82	7.54
74	7.36	83	10.06
75	5.34	84	9.43
76	3.57	85	8.55
77	8.23	86	8.98
79	4.78	87	9.45
80	4.85	88	7.23

*Water content of simplisia should be below 10% suitable for analysis.

The yield of extracts of the 16 samples were between 0.14% (highlighted green) to 26.23% (highlighted yellow) that belonged to *n*-hexane extract of root of *Rhizophora mucronata* and water extract of leaf of *Avicennia marina*, respectively as shown in Table 2. The data clearly showed that root extract using non polar solvent (*n*-hexane) resulted in lower yield percentage compared to more polar solvents (samples 74, 77, 81 and 85). This indicated that root sample contains less nonpolar constituents compared to other parts of plants.

Table 2: Yield of extracts (%)*

Samples code	Yield	Samples code	Yield	Samples code	Yield	Samples code	Yield
72H	1.28	76H	0.26	81H	0.14	85H	0.5
72Ea	3.22	76Ea	0.27	81Ea	0.15	85Ea	0.89
72Et	6.54	76Et	2.3	81Et	3.6	85Et	1.98
72A	16.2	76A	5.45	81A	2.53	85A	10.31

73H	0.42	77H	0.29	82H	2.46	86H	0.76
73Ea	0.43	77Ea	0.32	82Ea	2.73	86Ea	1.79
73Et	3.3	77Et	8.95	82Et	3.55	86Et	6.94
73A	1.84	77A	4.14	82A	15.1	86A	21.18
74H	0.25	79H	1.04	83H	3.08	87H	2.41
74Ea	0.25	79Ea	2.36	83Ea	3.64	87Ea	2.27
74Et	11.92	79Et	2.48	83Et	2.99	87Et	10.86
74A	6.31	79A	13.26	83A	13.41	87A	19.58
75H	2.6	80H	0.26	84H	1.7	88H	1.52
75Ea	1.34	80Ea	0.23	84Ea	1.4	88Ea	1.43
75Et	7.09	80Et	4.88	84Et	7.42	88Et	9.46
75A	20.32	80A	3.72	84A	26.23	88A	7.06

*Solvents abbreviation: H = *n*-hexane, Ea = ethyl acetate, Et = ethanol, A = water.

Antibacterial screening of mangrove extracts in this study were targeted against gram positive bacteria represented by *Staphylococcus aureus*, *Propionibacterium acnes* and *Rhodococcus equi* and against gram negative bacteria represented by *Pseudomonas mosselii* dan *Escherichia coli.* There were 37 out of 64 extracts that showed antibacterial activity as indicated by clear (inhibition) zone around the disk dropped with extract (Tabel 3). Only extracts that produced inhibition zone mentioned in the Table. Based upon inhibition index value obtained, root of *R. apiculata* (sample code 74) showed inhibition zone on agar media with gram positive bacteria *P. acnes* for all four solvents (74H, 74Ea, 74Et dan 74A) and extracts 74Ea and 74Et on media with *R. equi*. Sample 76Et also produced inhibition zone on agar media with *P. acnes* and *R. equi* bacterial strains.

Tabel 3. Bacterial inhibition zone (mm) and inhibiton index of extracts.

No.	Sample code	Bacterial strains	Disk diameter + inhibiton zone (mm)		Inhibition index	
			Positive control	Samples	Positive control	Samples
1	72H	*P acne*	15.95	7.54	1.6583	0.2567
		E coli	25.88	7.26	3.133	0.2100
2	72A	*S aureus*	17.32	7.11	1.8875	0.1850
3	72Et	*E coli*	25.88	6.94	3.3133	0.1567
4	73H	*R equi*	20.4	6.21	2.4	0.0350
5	73Ea	*R equi*	25.36	6.77	3.2275	0.1275
6	73Et	*P acne*	25.66	6.50	3.2767	0.0833
7	73A	*P acne*	25.66	6.99	3.2767	0.1650
8	74H	*P acne*	15.95	6.17	1.6583	0.0283
9	74Ea	*P acne*	11.25	6.29	0.8750	0.0483
		R equi	25.36	7.98	3.2275	0.3300
10	74Et	*P acne*	28.74	6.87	3.7892	0.1450
		R equi	23.48	7.91	2.9125	0.3183
11	74A	*P acne*	28.74	7.49	3.7892	0.2483
12	75Ea	*R equi*	25.36	6.44	3.2275	0.0733
13	75A	*S aureus*	16.90	6.43	1.8175	0.0717
14	76H	*P acne*	12.49	6.90	1.0817	0.1500
15	76Ea	*P acne*	17.88	6.89	1.9800	0.1483
		P mosselii	11.63	7.59	0.9383	0.2650
16	76Et	*P acne*	23.32	7.30	2.8858	0.2167
		R equi	25.41	6.52	3.2342	0.0867
17	77Ea	*S aureus*	31.6	7.74	4.2667	0.2900
		E coli	34.40	17.39	4.7325	1.8983
		P mosselii	11.63	7.89	0.9383	0.3150
18	77Et	*P acne*	31.6	7.02	4.2667	0.1700
19	77A	*P acne*	31.6	7.96	4.2667	0.3267
		E coli	34.40	7.71	4.7325	0.2850
20	79Ea	*R equi*	20.81	6.48	2.4683	0.0800
21	79A	*S aureus*	17.32	8.47	1.8875	0.4117
22	80H	*S aureus*	30.31	6.79	4.0517	0.1317
23	80Ea	*R equi*	20.81	7.25	2.4683	0.2083
24	81H	*R equi*	21.80	6.68	2.6325	0.1133
25	81Ea	*P mosselii*	11.88	6.25	0.9792	0.0417
26	82H	*P acne*	11.85	6.91	0.9750	0.1517
		R equi	21.80	7.13	2.6325	0.1883
27	83H	*E coli*	36.31	7.66	5.0517	0.2767
		P mosselii	10.46	7.60	0.7442	0.2667
		R equi	21.80	6.92	2.6325	0.1533
28	83Ea	*S aureus*	27.50	6.59	3.5825	0.0983
		E coli	36.31	6.85	5.0517	0.1417
29	83Et	*R equi*	23.40	7.80	2.8992	0.3000
30	84H	*P acne*	11.85	6.87	0.9750	0.1450
		R equi	21.80	6.80	2.6325	0.1333
31	84A	*S aureus*	16.90	10.72	1.8175	0.7867
32	85H	*P acne*	21.04	6.41	2.5067	0.0683
		R equi	19.37	7.78	2.2283	0.2967
33	86H	*R equi*	19.37	6.81	2.2283	0.1350
34	87H	*R equi*	19.37	6.49	2.2283	0.0817
35	88H	*R equi*	19.37	6.66	2.2283	0.1100
36	88Et	*P acne*	24.78	8.39	3.1292	0.3983
37	88A	*P acne*	24.78	7.58	3.1292	0.2633

Figure 1 showed an inhibition zone formed due to the addition of extract of root of *B. gymnorrhiza* (77Ea) on agar media with gram negative bacteria *E. coli*. The formed inhibition zone was the largest one with diameter 17.39 mm with inhibition index value of 1.8983. Extracts 77Ea also produced inhibition zone as large as 7.89 mm in diameter on agar media with *P. mosselii* and *S. aureus*. Extracts 76H, Ea and Et showed inhibition zone on agar media with *P. acnes*, *P. mosselii* and *R. equi*. The second highest inhibition index was 0.7867 recorded for leaf of water extract of *Avicennia marina* (84 A) screened against *Staphylococcus aureus.* Taken altogether, these data strongly suggested that mangrove extracts used in this study were potential as antibacterial agents with inhibition index value from the lowest to the highest were 0.0283 and 1.8983, respectively.

Previous studies reported that mangrove extracts had shown their activity against microbes or pathogen parasites in animals and plants [8,9] including HIV [10] and Hepatitis-B virus [11]. Phytochemical qualitative analysis showed that most if not all extracts contain saponin and tannin in considerable amount (Tabel 4). The two phytochemical constituents and flavonoid had shown their activity against some bacteria [12]. Samples collected contained flavonoid and steroid in less amount and no alkaloid detected in almost all samples.

It is important to note that chemical constituents and bioactivity of mangrove extracts and plants in general vary depend upon not only from species to species but also due to geographical conditions. This is also important to identify factors contributing to bioactivity, such as season, location and reproduction cycle stage [13]. Therefore, documentation of samples collection includes taxonomy, time and location, collector either individual or institution and species availability. This will be very helpful in tracing and sample monitoring during research process for accessibility purpose and benefit sharing and recollection.

Considering the very large area covered by mangroves in Indonesia and worldwide, mangrove research, particularly for the purpose of drug discovery is still very limited. This opens up opportunities for researches to start putting their efforts individually and collaboratively on mangrove research which also applies to mangrove's associates.

Tabel 4. Phytochemical qualtitative analysis of mangrove extracts.

Sample code	Alkaloid		Dragendof	Triter-penoid	Steroid	Quinon	Flavonoid	Saponin	Tanin
	Mayer	Wagner							
72	-	-	-	-	++	-	-	+	++
73	-	-	-	+	-	-	-	+++	++
74	-	-	-	-	-	-	+	+++	+++
75	-	-	-	-	++	-	+++	+++	+++
76	-	-	-	+	-	+	+	+++	++
77	-	-	-	-	-	-	-	+++	+++
79	-	-	-	-	++	-	+	+++	+++
80	-	-	-	-	-	+	-	+++	+++
81	-	-	-	-	-	+	+++	+++	++
82	-	-	-	-	+++	-	-	+++	+
83	-	-	-	-	+	-	-	-	+++
84	-	-	-	-	+	-	++	++	++
85	-	-	-	-	+	-	-	+	-
86	-	-	-	-	+	-	-	+++	+
87	+	+	+	-	+	-	+	+	++
88	-	-	-	-	+	-	++	-	+++

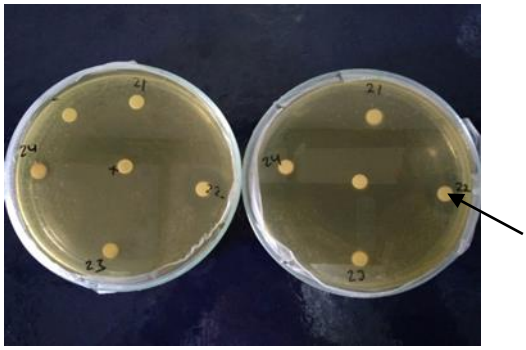

Figure 1: The largest inhibition zone formed due to the addition of extract of root of *B. gymnorrhiza* (77Ea) on agar media with gram negative bacteria *E. coli* with diameter 17.39 mm and inhibition index value of 1.8983 (as indicated by arrow).

Experimental

Plant materials: Mangroves plants were collected from the Eastern Coastline of Lampung Province, Indonesia in May 2017. There were eight species of mangroves used in this study with the total of 16 samples collected from different parts of plants such as leaves, barks and roots (Tabel 5). The eight species were identified for confirmation at the Herbarium of the Center for Biological Research of the Indonesian Institute of Sciences, Bogor, Indonesia.

Table 5: Mangroves species and part of the plants used in this study*.

Name of Plants	Part of Plants	Samples Code
Rhizophora apiculata	Leaf	72
	Bark	73
	Root	74
Bruguiera gymnorrhiza	Leaf	75
	Bark	76
	Root	77
Rhizophora mucronata	Leaf	79
	Bark	80
	Root	81
Thespesia populnea	Leaf	82
	Fruit	83
Avicennia marina	Leaf	84
	Root	85
Xylocarpus granatum	Leaf	86
Ceriops tagal	Leaf	87
Sonneratia alba	Leaf	88

*Part of plants collected were based upon the nature of the plants.

Solvents and chemicals: Distilled water, ethanol, *n*-hexane, ethyl acetate were obtained from Brata Chem. Concentrated HCl, *n*-amyl alcohol, acetone, dichloromethane, Liebermann-Burchard reagent, chloroform-ammonia, H_2SO_4 2 M, Mayer, Dragendorf and Wagner reagents, Magnesium powder, $FeCl_3$ 1%, NaOH 10% and DMSO 20% were obtained from Sigma-Aldrich.

Bacterial strains, media and antibiotic: *Escherichia coli*, *Staphylococcus aureus*, *Propionibacterium acnes*, *Pseudomonas mosselii* and *Rhodococcus equi* bacterial strains were all purchased from the Indonesia Culture Collection (InaCC), nutrient agar (NA), Tryptic Soy Broth (TSB), Whatmann filter (6.02 ± 0.02mm) and tetracycline.

Simplisia water content [14]**:** Empty porcelain dish was dried in the oven at 105 °C in 30 minutes and cooled in desiccator in 30 minutes and weighed. One gram of sample was weighed into the dish, heated in the oven at 105 °C for certain period of time. The steps were redone until constant weight was obtained. Water content was determined by using the following equation:

Water content = $\frac{B-A}{B} \times 100\%$; A = dry sample weight (g), B = sample weight (g)

Maceration extraction: Extraction method used was gradient maceration [7]. Four different solvents were used, which were *n*-hexane, ethyl acetate, ethanol and water. Ratio between solvent and simplisia was 5:1 with overnight maceration, 3:1 with maceration for 17 and 7 hours. Extracts separated from their residues was concentrated with rotary evaporator. The yields were then determined based on the ratio of concentrated extract weight with initial sample weight. Extractions were performed triplicate.

Antibacterial activity: Disk diffusion method was used to determine antibacterial activity of mangrove extracts [15]. Media used for *Escherichia coli* strain was Nutrient Agar (NA). Media used for *Staphylococcus aureus*, *Propionibacterium acnes*, *Pseudomonas mosselii* and *Rhodococcus equi* strains was Tryptone Soya Agar. All bacterial strains were subcultured in Triptic Soy Broth and incubated at 37 °C overnight. Sterilized agar media was prepared. Bacterial suspension was mixed with the agar and solidified for 5 minutes. Disk paper with the size of 6 mm in diameter was placed on the solid agar media with tweezers and was dropped with 20 µL of 1% extract in 20% DMSO solvent. Tetracycline was used as positive control. Bacterial cultures were incubated at 37 °C overnight. Inhibition zone diameter formed was measured in millimeter (mm). Bacterial inhibition index value was calculated by using the following equation:

$$\text{Inhibiton index} = \frac{\text{d inhibition zone} - \text{d disk}}{\text{d disk}}$$; d = diameter

Qualitative phytochemical analysis [16]: Alkaloids test was performed by mixing 4 ml of chloroform-ammonia mixture with 0.1 gram crude extract and was then filtered. Few drops of H_2SO_4 2 M was added into the filtrate and mixed until two layers formed. Transparent layer (acidic layer) was divided into 3 reaction tubes. Mayer, Wagner and Dragendorf reagents were added into each tubes. Positive alkaloids test results indicated by the formation of white, brown or red precipitation by addition of Mayer, Wagner or Dragendorf reagents, respectively.

Triterpenoid and steroid tests were performed by heating mixture of 0.1 gram crude extract with 5 ml ethanol at 50 °C and then filtered. The filtrate was then concentrated and dissolved with ether. The ether layer was dropped on a drop plate and air-dried. Few drops of Liebermann-Burchard reagent (concentrated H_2SO_4 and CH_3COOH anhydrate) was added onto the drop plate. Positive triterpenoid test result indicated by the formation of red color and positive steroid test results indicated by the formation of green or blue color.

Phenolic and flavonoid tests were performed by mixing 0.1 gram of crude extract with 5 ml of distilled water and then boiled for 2 minutes and filtered. NaoH 10% was added into 2 ml of filtrate for phenolic test. Red color indicates that phenolic compounds are present in the sample. The presence of flavonoid compounds can be detected by mixing 0.1 gram Magnesium powder, 1 ml of concentrated HCl and 1 ml of amyl alcohol with 2 ml of the filtrated. The formation of red, yellow or orange color indicate a positive result.

Saponin and tannin tests were performed by mixing 0.1 gram of crude extract with 5 ml distilled water and then boiled and filtered. Filtrate was divided into 2 reaction tubes. Saponin test was done by cooling the filtrated and mixed until foam formed. Positive result indicated by the formation of foam that lasts for about 10 minutes. Tannin test

was done by mixing the filtrate with $FeCl_3$ 10% solution. Positive result indicated by the formation of dark blue or blackish green color.

Typically this section should be divided into subsections, the contents of which varies according to the subject matter of the article. This must contain all the information to guarantee reproducibility. In an introductory paragraph, special equipment, etc. should be detailed. A precise workup containing all details, e.g., the amount of solvent used for extraction, details of chromatographic purifications and yields etc., should be given. Physical and spectroscopic data can be included in the experimental section or in tabular form.
Spectroscopic data should be stated in the order and format shown in following examples:

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