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

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Abstract: Cancer is still one of the leading death causes in the world. There will be an estimated 18.1 million new cancer cases and over 9 million cancer deaths in 2018. Furthermore, some studies reported that new types of cancer had been discovered. Therefore, it is very important to find different sources for anticancer agents. 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 anticancer 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. Brine Shrimp Lethality Test (BSLT) and MTT Assay were conducted for anticancer screening of mangrove extract library. There were 43 extracts showed anticancer potential with $LC_{50} < 1000$ ppm and 21 extracts with $LC_{50} > 1000$ ppm. Next, 16 out of 43 extracts which have $LC_{50} < 500$ ppm and some extracts with $LC_{50} > 1000$ ppm were selected to be evaluated in MTT Cytotoxicity assay. There were 3 extracts that highly inhibited HeLa and MCF-7 Cell line. The highest inhibition percentage was recorded for ethyl acetate extract of root of *Avicennia Marina* (85 Ea), although it was not toxic enough in BSLT due to the high LC_{50} value (>1000 ppm). The next highest inhibition percentage were recorded for water extract of leaves of *Sonneratia alba* (88 A) and ethyl acetate extract of leaves of *Xylocarpus granatum* (86 Ea). In addition, there were 2 extracts that only effective to inhibit HeLa cell line, namely ethanol extract of leaves *Bruguiera gymnorrhiza* (75 Et) and ethyl acetate extract of root of *Rhizophora apiculata* (74 Ea). Phytochemical analysis of the extracts was also evaluated. The majority of samples showed saponin and tannin in considerable amount. This supported the data that mangrove extracts were potential as anticancer agents.

Keywords: Anticancer, drug discovery, drug resistant, extract library, Indonesian biodiversity.

1. Introduction

Cancer is still one of the leading death causes in the world. There will be an estimated 18.1 million new cancer cases and over 9 million cancer deaths in 2018 (Bray *et al.*, 2018). Lung cancer is the most frequently diagnosed type of cancer and become the leading cause of cancer deaths in male and female. On the other hand, effective cancer therapy is still a great challenge for nowadays society due to some limitations and side-effects from the treatment. Therefore, finding alternatives for drug sources as anticancer agent is urgently required. Meanwhile, current anticancer research shows that herbal plants from tropical region such as mangrove have a potential cytotoxicity effects towards cancer cells (Kerry *et al.,* 2018). In addition, anticancer drug discovery is a lengthy and expensive process. Through screening process utilizing natural products, it can become a solution of the slow and expensive drug discovery process using conventional way. Indonesia is well known as one of the richest countries in the world in biodiversity where is a home of approximately 11% or more than 30,000 of the world's flowering plants and other biota both in land and marine with significant figures (Rintelen, Arida, & Häuser, 2017). 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 (Bandaranayake, 2002). Along roughly 90,000 kilometers coastline, Indonesia is home of about 20 families with hundreds of species of mangroves and their associates. Indonesia has the largest mangrove forest or about 23% of total world mangrove forests (Giri *et al.,* 2011). The aim of this study was to evaluate the potential of mangrove extracts (extracts library) as anticancer 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.

2. Research Methods

2.1. Material

The main raw material used in this study was various parts of mangroves plants such as leaves, stem and roots. There were eight different species of mangroves collected from the Eastern Coastline of Lampung Province, Indonesia in May 2017. The species identification was done at the Herbarium of the Center for Biological Research of the Indonesian Institute of Sciences, Bogor, Indonesia. Samples were collected from each parts of the plants in the total of 16 samples.

Table 1: Mangroves species and part of the plants used in this study*.

Name of Plants	Part of Plants	Samples Code
Rhizophora apiculata	Root	74
Bruguiera gymnorrhiza	Leaf	75
Rhizophora mucronata	Leaf	79
	Stem	80
Avicennia marina	Leaf	84
	Root	85
Xylocarpus granatum	Leaf	86
Ceriops tagal	Leaf	87
Sonneratia caseolaris	Leaf	88

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

The chemicals and solvents used in this study were obtained from Brata Chem including: Tween-80, Distilled water, ethanol, n-hexane, ethyl acetate 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% (Sigma-Aldrich). For cell culture medium, RPMI medium was used to culture HeLa and MCF-7 cell line supplemented with Fetal Bovine Serum (FBS) and antibiotic-antimycotic.

2.2. Equipment

Equipment used in this study were moisture balance (Mettler Toledo™), heating and drying Oven (Memmert), Thin-Layer-Chromatography Chamber, ELISA Microplate reader, Bio-Safety Cabinet level 2, cell culture CO_2 incubator, and serological pipette.

2.3. Analytical Procedure

2.3.1. Sample Preparation and Extraction

Preparation of sample and extraction were performed based on previous study (Audah *et al*., 2018). Sample preparation was carried out using an oven with a temperature of less than 50 ºC until the samples were dry. The time needed for leaves was approximately 3 days, while the roots and stems were about 6 days to dry. The dried samples were stored in plastic and the moisture was kept and observed. Extraction method used was gradient maceration (Farida *et al.,* 2014). Four different solvents were used, which were *n*-hexane, ethyl acetate, ethanol and water. yields were then determined based on the ratio of concentrated extract weight with initial sample weight. Extractions were performed triplicate.

2.3.2. Water Content Determination

The procedure for determining the water content of dried samples was carried out using a moisture balance. Five grams stored in an oven at 105℃ for 5 hours, then weighed. The difference of mass before and after heating is the value of the water content of sample (Sulasmi *et al*., 2016).

2.3.3. Qualitative Phytochemical Analysis

Phytochemical tests in this study include qualitative tests of alkaloid, phenolic, triterpenoid/steroid, and hydroquinone (Harborne, 1987).

2.3.4. Brine Shrimp Lethality Test (BSLT)

A tip of eggshell's *Artemia salina* (50–100 mg) was put into a container that had been filled previously with seawater and had been aerated using an aerator. The eggs were hatched at a temperature range of 25–30° C with light exposure until it was ready for use which was 48 hours old. Solution of extract for the test was made with various concentrations: 1000, 500, 100, and 50 ppm then dissolved in sea water with a little addition of Tween-80. Larvae were inserted into the vial (10 larvae/vial) and incubated for 24 hours. The number of dead larvae was quantified in 3 repetitions, then the obtained data were processed using Probit analysis on SPSS version 16 software to obtain LC_{50} value.

2.3.5. MTT Cytotoxicity Assay

MCF-7 and Hela cells line were cultured on 96-well plates in the media of Rosewell Park Memorial Institute (RPMI) with 100 µL medium per well containing approximately 5,000 cells per well. A total of 100 µL of active extract with a concentration of 500 ppm was added to the cultured cells, incubated for 48 hours in a 5% CO_2 incubator at 37° C. Next, 100 µL of tetrazolium monoculture salt (MTT) were added and incubated again for 4 hours in 5% CO_2 incubator. Cells that are still alive will react with MTT to form formazan which can be seen violet. The formed Formazan was dissolved in 96% ethanol. The absorbance reading was in a microplate reader ELISA spectrophotometer at $\lambda = 595$ nm. The tests were carried out using 1× RPMI media as a blank, MCF-7 breast cancer cells and HeLa cervical cancer cells as a negative control, and doxorubicin as a positive control.

3. Results

The yield of extracts of the 16 samples were between 0.25% (highlighted green) to 21.18% (highlighted yellow) that belonged to ethyl acetate extract of root of *Rhizophora apiculata* and water extract of leaf of *Xylocarpus granatum* respectively as shown in Table 2. The data clearly showed that root and leaf extract using non-polar solvent (*n*-hexane) resulted in lower yield percentage compared to more polar solvents (root samples 74 and 85, leaf samples 79 and 87) (Zahra *et al.,* 2017). This indicated that root sample contains less nonpolar constituents compared to other parts of plants.

Table 2: Yield of extracts (%)*

Sample Code	Yield (%)	Sample Code	Yields (%)	Sample Code	Yield (%)	Sample Code	Yields (%)
74Ea	**0.25**	79Et	2.48	85H	0.5	86Ea	1.79
75A	20.32	79H	1.04	85Ea	0.89	87A	19.58
75Et	7.09	80A	3.72	86A	21.18	87H	2.41
79Ea	2.36	84Et	7.42	86Et	6.94	88A	7.06

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

Water content of sample (Table 3) were kept below 10% which is a suitable percentage for simplisia analysis according to the Indonesian Herbal Pharmacopeia (Department of Health of the Republic of Indonesia., 2009). The largest shrinkage of drying is found in the leaves of all parts of the plant, while the smallest drying shrinkage is found in the stem.

Table 3: water content determination

Samples	Part	Shrinkage (%)	Water Content (%)	Samples	Part	Shrinkage (%)	Water Content (%)
R. apiculatae	Root	62	7.36	*A. marina*	Leaf	77	9.43
B. gymnorrhiza	Leaf	63	5.34		Root	71	-
S. caseolaris	Leaf	84	7.23	*X. granatum*	Leaf	80	8.98
R. mucronata	Leaf	71	4.78	*C. tagal*	Leaf	82	9.45
	Stem	47	4.85				

Each simplisia does not always produce a positive reaction to all tests. This shows that mangrove plants are quite rich in secondary metabolites and the composition is not the same one to another (Joel and Bhimba, 2013). Furthermore, the results of this test can be a simple assumption for continued anticancer screening. The following results of the dried sample phytochemical test were presented in Table 4.

Table 4: Phytochemical qualitative analysis of samples

Sample Code	Alkaloid			Phenolic					
	M	W	D	F	Sa	Ta	T	S	Q
74	-	-	-	+	+++	+++	-	-	-
75	-	-	-	+++	+++	+++	-	++	-
79	-	-	-	+	+++	+++	-	++	-
80	-	-	-	-	+++	+++	-	-	+
84	-	-	-	++	++	++	-	+	-
85	-	-	-	-	+	-	-	+	-
86	-	-	-	-	+++	+	-	+	-
87	+	+	+	+	+	++	-	+	-
88	-	-	-	++	-	+++	-	+	-

Description: M=Mayer, W=Wagner, D=Dragendorf, T=Triterpenoid, S=Steroid, Q=Quinon, F=Flavonoid, Sa= Saponin, Ta= Tanin

Brine Shrimp lethality Test (BSLT) is one of the initial methods to estimate the level of toxicity of a compound based on the lethal concentration value of 50% (LC_{50}). The shrimp larvae have thin skin and a sensitive body. If there are foreign compounds in the environment, the compound will be easily absorbed into the body of the larva diffusion. The toxic environment due to foreign compound will terminate the shrimp larvae. Chemical compounds are potentially bioactive if they have an LC_{50} of less than 1000 ppm. In this study, there were 43 extracts (table 5) which have the potential to have toxic compounds. After obtaining the LC_{50} value, some extracts were then selected which had LC_{50} below 500 ppm (green highlighted) and some extracts which had LC_{50} values more than 1000 ppm (yellow highlighted) for cytotoxicity testing. The lowest LC_{50} was 40.45 ppm (87 H).

Table 5: Brine Shrimp Lethality Test LC_{50}

Sample Code	LC_{50} (ppm)	Sample Code	LC_{50} (ppm)	Sample Code	LC_{50} (ppm)	Sample Code	LC_{50} (ppm)
74Ea	200.78	79Et	498.28	85H	499.23	86Ea	64.02
75A	>1000	79H	160.43	85Ea	>1000	87A	>1000
75Et	291.61	80A	>1000	86A	408.24	87H	40.45
79Ea	224.45	84Et	229.77	86Et	74.87	88A	488.93

The cytotoxicity of 16 extracts selected from the results of toxicity tests showed water extract of *Sonneratia caseolaris* leaf (88A), ethanol acetate extract of *Xylocarpus granatum* leaf (86Ea), and ethanol acetate extract of *Avicennia marina* root (85Ea) had a high percentage of inhibition in both cells. However, ethanol acetate extract of *Avicennia marina* root (85Ea) did not show bioactive potential that was toxic in BSLT because the obtained LC_{50} obtained was below 1000 ppm. Meanwhile, ethanol extract of *Bruguiera gymnorrhiza* leaf (75Et) and ethanol acetate extract of *Rhizophora apiculata* root (74Ea) have a high percentage of inhibition in HeLa cells line, although it was low in MCF-7 cells line.

Table 6: MTT Cytotoxicity Assay

Sample Code	% Inhibition		LC_{50} (ppm)	Sample Code	% Inhibition		LC_{50} (ppm)
	MCF-7 Cells	HeLa Cells			MCF-7 Cells	HeLa Cells	
74Ea	29.57	85.92	200.78	**85Ea**	98.06	94.04	>1000
75A	45.02	52.8	>1000	86A	44.4	64.8	408.24
75Et	16.2	90.79	291.61	86Et	74.87	85.92	351.52
79Ea	2.03	47.92	224.45	**86Ea**	96.65	92.96	64.02
79Et	18.09	51.71	498.28	87A	41.95	68.05	>1000
79H	-22.96	24.46	160.43	87H	52.53	29.06	40.45
80A	38.78	27.98	>1000	**88A**	97.4	92.42	488.93
84Et	48.37	68.5	229.77	Doxorubicin	94	95.76	-
85H	32.12	29.87	499.23	Control Cell	-	-	-

Description: Green= Highly potential, Yellow= slightly potential

The results of phytochemical analysis of *Avicennia marina* root (85Ea) indicated that there were only two active compounds, namely Saponin and Steroid which were detected in low-concentration. This result can explain the high value of LC_{50} (>1000 ppm) of the extract which was not toxic enough for *Artemia salina.* Extratcs of plants or animal are classified a cytotoxic when the LC_{50} value <1000 ppm. An extract is considered very toxic when it has a LC_{50} value < 30 ppm, and toxic if it has a LC_{50} value 30-1000 ppm and is considered non-toxic if LC_{50} > 1000 ppm. While pure compound was declared toxic if it has a value of LC_{50} <200 ppm (Meyer *et al.,* 1982). Meanwhile, other extracts that have more than two compounds were toxic for *Artemia salina.* For example, the extract of *Ceriops Tagal* leaf (87H) showed that there were seven compounds detected in the extract and had the lowest LC_{50} value of all (40.45 ppm).

Moreover, the high inhibition percentage of ethanol acetate extract of *Avicennia marina* root (85Ea) in both cells line indicated that there was a possibility for false positive result. This is due to the damaged mitochondria may be still able to reduce MTT to formazan crystals (Mosmann, 1983). Loveland et al. (1992) showed that cells with inactivated mitochondria were also able to produce formazan crystals as well as cells with active mitochondria. In addition, some ingredients of mangrove may have reducing activity such as the dehydrogenase activity of the cells that converts the MTT compound into the colored formazan. Therefore, the MTT assay may not be a suitable assay for mangrove extracts and it is important to conduct other cytotoxicity assay such as ATP assay, which gives more sensitive and reliable assay due to its interference-free feature (Karakas *et al.,* 2017).

Extracts of mangrove plant are considered to have potent cytotoxic activity against HeLa and MCF-7 cell lines. When compared between the cell lines HeLa cell lines are more suppressed than MCF-7 cell lines in general (Pradesh, 2018). The presence of different bio active compounds is the reason for its anticancer activity. Such compounds with antioxidant activity can inhibit mutation and cancer because they scavenge free radicals or induce antioxidant enzymes (Karami *et al.,* 2012). For example, saponins that contained in almost all extracts can inhibit the replication of cellular DNA and the proliferation of cancer cells can be prevented (Yildirim and Kutlu, 2015). In addition, Saponins may stimulate the disintegration of the microtubular network or actin filaments of cancer cells, which can lead to further non-apoptotic cell death (Chen *et al.,* 2009).

4. Conclusion

The methods and the results presented in this study suggested that developing extract library is a highly feasible and crucial for development of anticancer drugs discovery. Mangrove extracts are very potential as natural anticancer and antiproliferative agents. This report can be used as stepping stone towards acceleration anticancer drugs discovery.

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