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Methods Development for Mangrove Extraction towards Standardization of Indonesian Extract Library

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a r t i c l e i n f o

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a b s t r a c t

Natural resources, plants in particular, have been utilized by human for different purposes including as medicines. Drug discovery processes can be accelerated through a large collection of extracts in numbers, quality and quantity. By virtue of its vast numbers of biodiversity, developing a large collection of extracts (extract library) for drug discovery in Indonesia is very plausible thing to do. Due to the variations of chemical constituents of a plant, a reproducible method of extraction and profiling should be developed. The aim of this study is to develop some methods for extraction and profiling of mangrove plants. The methods will be used as the basis for development for Indonesian extract library especially for drug discovery and cosmetics. In this study, mangrove plants had been chosen due to their availability and potential as medicines. Several factors that could possibly determine chemical constituents were taken into consideration. These include but not limited to location, date and time, packaging, storage, species identification, solvents, extraction method and fingerprint profiling. Stability testing should also be conducted for mid and long-term storage. Extraction methods used in this study were maceration and ultrasonic assisted extraction by employing various solvents representing different polarity such as water, alcohol, ethyl acetate and hexane. Thin layer chromatography method was used for fingerprint profiling. In the future, implementation of barcoding system and integration of all data into a comprehensive information system is necessary. This can be utilized as a window of the Indonesian extract library as a whole.

1. Introduction

Nature has been known for centuries as invaluable sources for medicines. Traditional medicines originated from plants in particular have been practiced in countries such as China, India and Indonesia (Mittermeier et al., 2005). Indonesia for example possesses approximately 30,000 vascular plants which comprises about 10 percent of world's plants (Royal Botanical Garden Report, 2017). According to a report from 1980 until 2014, about 50 percent of drugs recognized by the Food and Drug Administration (FDA) of the United States of America and other similar organizations were originated from natural products or their derivatives (Newman and Cragg, 2016). In fact, for certain disease such as cancer, 75% of the drugs were originated from natural products (Newman and Cragg, 2016). By virtue of the richness in biodiversity, drug discovery process in Indonesia can be accelerated by having a large collection of extracts of natural products in large numbers (designated as extract library). This work was aimed to develop some methods to establish a protocol for preparation of extract library that should meet some criteria.

Adequate information of extracts should be obtained, so that extracts can be collected and stored in extract library repository for further usage such as drug screening purpose. Several important aspects that might contribute to the quality and quantity of chemical constituents of extracts were taken into consideration. These aspects include but not limited to geographical location, date and time, sample packaging, sample storage, method of extraction, solvents and fingerprint profiling. The effect of geographical location to chemical constituents of plants had been reported in different studies (Khattak, KF and Rahman, TR, 2015; Liu, W. *et. al.*, 2016; Margraf, T. *et. al.*, 2016). Stability testing should also be conducted for mid and long term storage. Additional information such as bioactivity of the extracts against certain disease(s) will be very useful. While crude extracts can be stored in any laboratories, all information accompanying the extracts eventually should be stored in a well-developed and maintained databases and can be easily accessed for future references.

In this study, mangrove plants were utilized as samples for methods development to establish the protocols for Indonesian extract library. This is because mangroves and their associates are very potential medicinal plants (Bandaranayake, 2002). Indonesia is home of about 20 family of mangroves and hundred species of their associates which comprises of approximately 23% of total world mangrove forests (Giri *et al.*, 2011).

2. Materials and Methods

In order to standardize the protocols for extract library repository, particular information need to be obtained from a biological material. Particular information such as geographical location, date and time, extraction, fingerprint profiling, and species identification are some of important aspects to be taken into consideration. Methods of choice will also vary depend upon biological materials to be extracted and or analyzed. For example, material handling for plant originated from marine will be different from land (Mccloud, 2010). This include but not

limited to harvesting, packaging or storage.

2.1 Solvents and chemicals

Distilled water (dH_2O), ethanol (EtOH), *n*-hexane (Hex), and ethyl acetate (EtOAc) 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.

2.2 Plant materials and experimental conditions

There were eight species of mangroves used in this study which were obtained from the Pasir Sakti Village in the Eastern Coastline of Lampung Province, Indonesia. The samples were collected from different parts of the plants such as leaves, barks or roots. The samples were designated as listed in Table 1.

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

Name of Plants	Part of Plants	Samples Code
Rhizophora apiculate	Leaf	72 (1)
	Bark	73 (2)
	Root	74 (3)
Bruguiera gymnorrhiza	Leaf	75 (4)
	Bark	76 (5)
	Root	77 (6)
Rhizophora mucronata	Leaf	79 (7)
	Bark	80 (8)
	Root	81 (9)
Thespesia populnea	Leaf	82 (10)
	Fruit	83 (11)
Avicennia marina	Leaf	84 (12)
	Root	85 (13)
Xylocarpus granatum	Leaf	86 (14)
Ceriops tagal	Leaf	87 (15)
Sonneratia alba	Leaf	88 (16)

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

2.3 Sample packaging, storage and transport

After harvesting at the collection site, the samples were first wrapped with paper and placed in plastic bags (Harcourt, 2015). The samples were then put in a cardboard box. This will protect the samples from heat to prevent moist loss during transport. The samples were then transported to the Tropical Biopharmaca Research Center LPPM IPB, Bogor. Upon arrival, the samples were stored in a freezer at -20°C.

2.4 Species identification

The eight species were identified for confirmation at the Herbarium of the Center for Biological Research of the Indonesian Institute of Sciences, Bogor, Indonesia. A combination of at least two parts of a plant should be provided for minimum requirement for plant identification. The combinations could consist of bark, flower, fruit, leaf, root, seed or stem.

2.5 Sample preparation

Preparation of sample was carried out using an oven with a temperature of less than 50 °C 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. Unless otherwise stated, the protocols for preparation in this study refer to the Indonesian Herbal Pharmacopeia (Department of Health of the Republic of Indonesia., 2009).

2.6 Water content determination

The procedure for determining the water content of dried samples was carried out using a moisture balance. Hexa grams stored in an oven at 105°C 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).

2.7 Qualitative Phytochemical analysis

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

2.8 Extraction

Extraction method used was maceration according to the Indonesian Herbal Pharmacopeia (Department of Health of the Republic of Indonesia., 2009). Four different solvents were used, which were *n*-hexane, ethyl acetate, ethanol and water. Ratio between solvent and dried sample was 5:1 with overnight maceration, 3:1 with maceration for 17 and 7 hours. Extracts separated from their residues and 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.

2.9 Thin Layer Chromatography-Bioautography

Thin layer chromatography (TLC)-bioautography was performed to obtain additional information regarding bioactivity of the fractions of an extract. TLC-bioautography was used to determine active bands as antibacterial before fractionation using column chromatography. The results of the TLC-bioautography can be used as a guide to recognize active fractions that have antibacterial activity. This method is relatively fast, easy to perform, and economical in using extracts. TLC-bioautography was carried out by attaching the TLC plates which had been eluted over the bacterial suspension medium. TLC was left for 24 hours to diffuse chemical components into the media (Yulianty *et. al.*, 2011). The activity of bacterial growth inhibition was characterized by the formation of specific clear zones on certain bands. The Rf values in the band was used as a reference for the isolation of antibacterial active compounds.

3. Results

3.1 Sampling and Preparation

The extraction results not only depend upon the types of biota such as species of plants or any other sources of natural products, but also depend upon other factors such as location and time. These conditions will determine on how plants or other natural sources generate secondary metabolites. The production of secondary metabolites is strongly influenced by seasons, environmental conditions, age and type of organ (Sampaio *et al.*, 2016). Thus, it is very important to record all necessary information related to those factors mentioned above. This is also to ensure that uniformity of samples for future need is well maintained. Species identification in particular, is one of key factors that needs to be performed. The time between sampling, temporary samples storing, samples storage in the freezer and simplisia preparation must be kept to minimum. The duration from sampling to simplisia preparation will influence the results of the extracts which related to the intercellular enzyme activity of the sample itself and the activity of microbes (bacteria and fungi attached to the sample). The recording of sampling details until the storage of simplisia needs to be recorded properly so that it can be used as a reference if re-collection is needed.

The use of extraction methods was determined by several things, namely cost efficiency, time, and extraction results. It is necessary to optimize the extraction so that the extraction yield can reach optimum results in terms of quantity and quality and to achieve an adequate amount of extract. It is also necessary to develop a method for storage of the extract which is related to the effectiveness of screening. An ideal storage must include a long shelf life and consistent extract quality so it can produce more representative results from many screening processes for various types of diseases. All samples that have been successfully collected are summarized in Table 2. All samples are coded and dried. The number of samples obtained varies according to the availability of samples at the sampling location. 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 2: sample supply used in this study

Samples	Part	Shrinkage (%)	Used Amount (g)	Water Content (%)
R. apiculate	Leaf	78	50.01 50.03 50.01	8.60
	Stem	47	50.10 50.02 50.06	2.30
	Root	62	50.01 50.01 50.05	7.36
B. gymnorrhiza	Leaf	63	50.02 50.03 50.02	5.34
	Stem	51	50.00 50.00 50.01	3.57
	Root	60	50.01 50.01	8.23

			50.00	
			50.01	
	Leaf	71	50.01	4.78
R. mucronata			50.01	
	Stem	47	-	4.85
	Root	60	-	7.56
			50.01	
T. populnea	Leaf	71	50.01	7.54
			50.00	
			50.01	
	Leaf	77	50.01	9.43
A. marina			50.01	
			50.02	
	Root	71	50.04	-
			50.02	
			50.01	
X. granatum	Leaf	80	50.01	8.98
			50.01	
			50.01	
C. tagal	Leaf	82	50.01	9.45
			50.05	
			50.00	
S. caseolaris	Leaf	84	50.01	7.23
			50.01	

3.2 Water content determination

Water content of sample (Table 2) 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).

3.3 Qualitative Phytochemical analysis

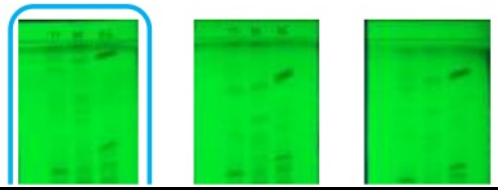
Each simplisia does not always produce a positive reaction to all tests. This shows that mangrove plants are quite rich in secondary metabolites and the composition is not the same one to another (Joel and Bhimba, 2013). Furthermore, the results of this test can be a simple assumption for continued antibacterial and antitumor screening. The following results of the dried sample phytochemical test were presented in Table 3.

Table 3. Phytochemical qualitative analysis of samples

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

3.4 Extraction

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 4. 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 (Harcourt, 2015). Different method such as ultrasound-assisted extraction can also be used for extracting mangrove samples (Audah *et.al*, 2018). However, due to the limitation of the equipment, maceration extraction was still favorable as the method of choice in this study.



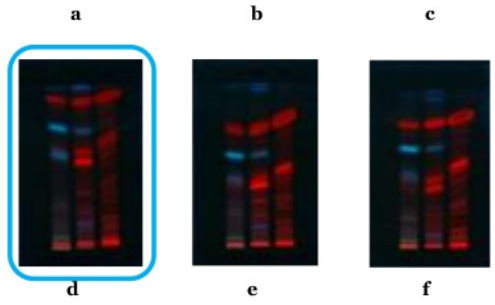


Figure 1. Chromatogram of ethyl acetate fraction of Xylocarpus granatum, Xylocarpus granatum leaves, and Avicennia marina. Mobile phase = Chloroform: Dichloromethane (v / v) by comparison (a,d) 9: 1, (b,e) 8: 2, and (c,f) 7: 3. UV 254 nm (top) and UV 366 nm (bottom).

Table 4. Yield of extracts (%)*

Sample code	Yield	Sample code	Yield	Sample code	Yield	Sample 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
72W	16.2	76W	5.45	81W	2.53	85W	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
73W	1.84	77W	4.14	82W	15.1	86W	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
74W	6.31	79W	13.26	83W	13.4	87W	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
75W	20.3	80W	3.72	84W	26.2	88W	7.06

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

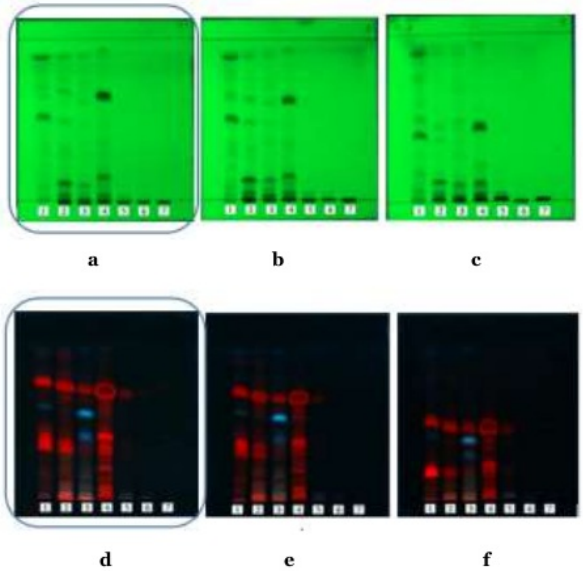
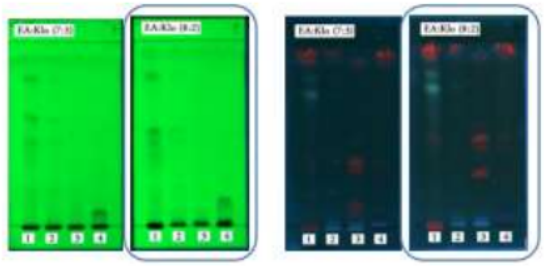


Figure 2. Chromatogram of (1) 74 H, (2) 74 EA, (3) 77 EA, (4) 86 EA, (5) 74 ET, (6) 74 W, (7) 88 W. Mobile phase: Chloroform: Dichloromethane (v/v) by comparison (a,d) 9:1, (b,e) 8:2, and (c,f) 7:3. UV 254 nm (top) and UV 366 nm (bottom).



a b

Figure 3. Chromatogram of (1) 74Et, (2) 74A, (3) 88A, (4) 84W. Mobile phase: yl acetate: chloroform (v/v) with 7:3 and 8:2 comparisons. (a) UV 254 nm, (b) UV 366 nm.

3.5 Thin Layer Chromatography-Bioautography

It is important to note that the elution results may vary from species to species as well as location and time. Therefore, whatever the results will be, recording all the information become critical for validity of the extract library stored in data repository. The presence of inhibitory activity against bacteria was characterized by the appearance of zones or specific clear areas on the band (R_f). The bioautogram showed that there was at least one inhibition zone detected. The inhibition zone was found at the R_f 0.18 (Figure 4). The R_f value in the band is used as a reference for the isolation of antibacterial active compounds (Patra *et al.*, 2012). The isolation process is going to be carried out by fractionation using column chromatography.

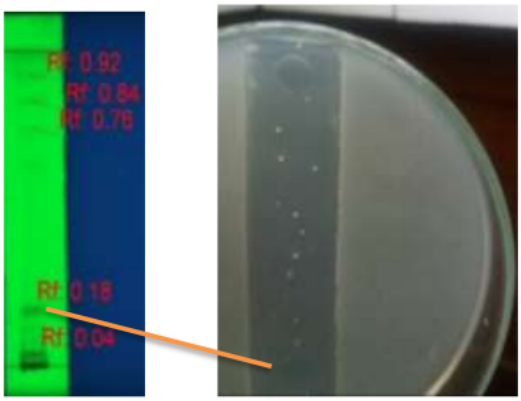


Figure 4. TLC-Bioautogram contacts Xylocarpus granatum leaves ethyl acetate fraction against *E. Coli* and with information on the R_f value under UV light 254 nm.

4. Discussion

The advancement of sciences and technology does not always positively correlate with the ability of human to overcome healthcare issues such as the availability of medicines for particular diseases. As a matter of fact, new diseases either communicable or non-communicable ones, emerge even before drugs for the existing diseases discovered. The World Health Organization once reported that approximately 30 new diseases emerge in every 20 years (World Health Report, 1996). Healthcare issues become more challenging by the increasing number of antibiotics resistant and re-emerging of microbial pathogens. Recently, the WHO issued a list of 12 bacteria for which new antibiotics are urgently needed as shown in table 5 (WHO, 2017). Antimicrobial resistance is a on the rise in Indonesia. For example, epidemiological data in 2001 on *E. Coli* from rectal samples showed remarkably high resistance to several antibiotics (Parathon, 2017). In 2010, there was some evidences from several patients in Semarang, Indonesia that showed a resistance of antibiotics against *S. pneumonia* (Farida et al., 2014). Misuse and overuse of antibiotics in humans, livestock and aquaculture are believed to be the main causes of resistance in the country. Taken altogether, these could give a tremendous burden and threat not only to an estimated population of more than 260 million of Indonesian people, but also to the world population as a whole.

Table 5. WHO priority pathogens list for R&D of new antibiotics

Type of Priority	Pathogens	Description
Critical	*A. baumannii*	carbapenem-resistant
	P. aeruginosa	carbapenem-resistant
	Enterobacteriaceae	carbapenem-resistant, ESBL-producing
High	*E. faecium*	vancomycin-resistant
	S. aureus	methicillin-resistant, vancomycin-intermediate and resistant
	H. pylori	clarithromycin-resistant
	Campylobacter spp.	fluoroquinolone-resistant
	Salmonellae	fluoroquinolone-resistant
	N. gonorrhoeae	cephalosporin-resistant, fluoroquinolone-resistant
	S. pneumoniae	penicillin-non-susceptible
Medium	*H. influenzae*	ampicillin-resistant
	Shigella spp.	fluoroquinolone-resistant

To overcome these challenges, researchers should come up with a solution on how to find drugs in most effective and efficient ways in terms of time and cost. Because conventional drug discovery is a lengthy and expensive processes. It can cost as much as USD 1 billion and can take as long as 12-15 years period of time to discover for one drug only (Hughes, J.P. *et. al.*, 2011).

Nature has been known almost as long as human civilization as a priceless sources for all human needs including medicine. Therefore, the exploration of biodiversity could be an answer to current healthcare issue in providing medicines for different types of diseases. This approach is very suitable with Indonesian condition which is well known as one of the richest country in the world in terms of biodiversity (Royal Botanical Garden, 2017). One of the developments to discover novel therapeutic drugs is through screening process of natural products. This work is to propose a legitimate approach based upon the Indonesian biodiversity which is to develop what so called the Indonesian Extract Library. The main purpose of the Indonesian Extract Library is to synergize and integrate all efforts in searching of medicines from Indonesian biodiversity. Eventually, all information related to collected extracts will be integrated into a comprehensive information system. Similar works had been done in some countries like Australia through the Department of Defe (DSTO, 2008) and the United States of America through the National Institute of Health under the National Center for Complementary and Integrative Health (NCCIH).

In this study, several methods have been tested to formulize a definitive protocols for preparation of extract library. This can be used as initial steps towards standardization of the Indonesian Extract Library. By establishing a protocol for preparation of extract library, it is hoped that drugs discovery in the future can be more effective and efficient. In addition, it can also reduce the production cost during drugs discovery and development. Mangroves had been chosen as plant materials of interest due to their potential as medicines. Mangroves and mangrove's associates are very potential medicinal plants (Bandaranayake, 2002). Besides, mangroves are also very easily found along approximately 90,000 kilometers of Indonesian coastline. The availability of the materials is one of important aspects for continuity and sustainability of drugs research and development. Although for commercial purpose, synthetic biology will be more plausible in ensuring continuity of the materials for the long run. The eight mangroves species selected in this study were based on traditional uses by local people (local wisdom) and their availability in the area.

During sampling, several aspects such as seasons, environmental conditions, age, and type of organ should be taken into consideration. This step is crucial due to the fact that extract library is closely related to how plants or other natural sources generate secondary

metabolites. The secondary metabolites and also therapeutic efficacy by medicinal plants can be influenced by those factors (Sampaio *et al.*, 2016; Ahmad *et al.*, 2011; Szakiel and Henry, 2011).

Samples packaging either during transportation or storage prior to extraction should be handled properly. This is to minimize the moisture loss and or oxidation of the sample during the shipping and or storage. Moisture loss and or oxidation can lead to damage or change in chemical composition of the samples especially volatile compounds or compounds function as antioxidants or microbial quality of samples (Ajayi *et. al.*, 2015; Bakan and Eksi, 2014). Mangroves are plants which are rich in antioxidants as shown in table 3. These findings were similar to previous studies that mangroves had high antioxidant contents (Audah *et. al.*, 2018; Rohaeti *et al.*, 2010). Some enzyme activities might affect the chemicals or metabolites composition of samples during storage (Sharma *et al.,* 2016). Therefore, it is important that the samples should be stored in a freezer at -20 °C upon arrival.

In preparation of simplisia, water content also can determine the quality of simplisia (Table 2). The water content should be less than 10% with the characteristics of simplisia is easy to break, not mouldy and smells like fresh materials in order to have high quality simplisia (Setiafianti, *et. al.*, 2017). Methods of extraction including solvent of choices will vary depend upon several factors that include but not limited to type of samples, compounds to be obtain and availability of the instruments. The most important thing of all is to record whatever methods used. Because eventually, the determining factor will be the elution steps which is required to obtain the best separation of compounds to be fractinated. Bioactivity of extracts or fractions is very useful information to have in the extract library repository. This information will help other researchers to select certain extracts or fractions for further studies such as for drug screening. Large collection of extract allows high throughput screening method to be applied. The concept of extract library was firstly introduced when pharmaceutical companies firstly implemented high throughput screening concept (Pereira dan Williams, 2007).

Mangroves as many of other plants contain various chemical compounds as shown in table 3. The success for fractionation will depend on the suitable solvents with the best combination of mixture. However, the application of the fractions themselves will depend on the right match between the compounds against particular diseases. In other words, all fractions or compounds either polar or non-polar should not be discarded. The results of this work had shown that some extracts or fractions were effective as antibacterial as shown in Figure 4 and our previous works (Audah *et. al.*, 2018). While some others were active as anticancer (unpublished results).

In conclusions, the methods and the results presented in this work suggested that developing extract library is a very plausible and important thing to do. A large collection of extracts or fractions or even single compounds can be easily obtain by exploring potential medicinal plants such as mangrove, mangrove associate and other forms or natural biological sources. This report can be used as stepping stone towards standardization for the development of Indonesian extract library from Indonesian biodiversity. Eventually, implementation of barcoding system and integration of all data and information into a comprehensive information system is necessary and can be utilized as a window of the Indonesian extract library as a whole.

Conflicts of interest

The authors declare that there is no conflict of interest on this research.

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