Potential of mangrove *Avicennia rumphiana* extract as an antioxidant agent using multilevel extraction

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Abstract. *Avicennia rumphiana* is one of abundant mangrove found in Indonesia. Multilevel extraction methods were simultaneously conducted to screen the antioxidant activity from mangrove. The leaves, fruits and barks were consequently extracted using n-hexane, ethyl acetate and ethanol. The presence of phenolic, flavonoids and tannins compounds were characterized by quantitative and qualitative phytochemical assay as well as the antioxidant activity was examined using DPPH-free radical scavenging assay. The phytochemical test revealed that all of the extracts showed positive result. The fruits extract exhibited the highest phenolic, flavonoid and tannin (23.86 mg/g, 13.77 mg/g and 74.63 mg/g), respectively. The extracts were further confirmed for antioxidant using IC₅₀ value and revealed that ethyl acetate extract has antioxidant activity better than n-hexane and ethyl acetate extract. Furthermore, this study indicated that mangrove *Avicennia rumphiana* could be subsequently explored for other biological activities due to their potential secondary metabolites.

1. Introduction

Natural plants are commonly used as primary medicine due to their pharmacological properties. The inquiry of the efficacy of plant-based drugs has been paid great attention because of their milder, safer, and has more availability compared to chemical medicine [1]. *Avicennia rumphiana*, also known as black mangrove, is the best mangrove species in stabilizing the soil habitat. It spreads in the first zone or often called as pioneer zone, which is directly faced to the sea. pioneer zone is a habitat for mangrove that has strong resistance to withstand wave blows, and they are able to assist in the process of sediment accumulation.

Free radicals are atoms or molecules that are unstable and highly reactive because they contain one or more unpaired electrons in their outermost orbital. Therefore, free radicals easily react with surrounding molecules to obtain electron pairs to achieve atom or molecular stability. excess Free radicals are known to cause degenerative diseases [2]. Free radical detection through the addition of antioxidant compounds is associated with the ability of antioxidants to donate hydrogen protons. Antioxidant compounds are able to counteract free radicals by inhibiting reaction rates at the propagation and initiation stage. Ordinarily, many synthetic antioxidants, such as butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), and butylated hydroxyanisol (BHA), have been applied to reduce oxidative damage in the human body. However, the use of synthetic antioxidants in humans might cause several side effects, such as carcinogenesis and liver damage [3].
Antioxidants are compounds that can inhibit or retard oxidation of lipids and other biomolecules. They prohibit the start of an oxidizing chain reaction or quench the propagation [4]. Natural antioxidants are found in plants on their stems, leaves, roots, and fruits [5]. Bioactive compounds that act as antioxidants can be obtained through the extraction process. Multi-stage extraction is used to obtain component with higher purity than a single-stage extraction. The DPPH method is one of the most frequently-used methods to assess the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate the antioxidant capacity of samples [6]. No biological activities have been reported for mangrove *Avicennia rumphiana* leaves, barks, and fruits extract. This study was aimed to screen the antioxidant activity of *Avicennia rumphiana* using DPPH free radical scavenging assay as a rapid screening tool for crude extracts. The sample was extracted using multilevel extraction to produce specific compounds based on solvents differences. Furthermore, the phytochemical contents were also determined quantitatively and qualitatively.

2. Methodology
2.1. Materials and chemical reagents
Mangrove *Avicennia rumphiana* was collected from Wonorejo, Surabaya, Indonesia. Ethanol, ethyl acetate, n-hexane, hydrochloric acid (HCl), magnesium (Mg), ferric chloride (FeCl₃), sulfuric acid (H₂SO₄), ascorbic acid, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and all other chemicals used in this study were of reagent grade.

2.2. Sample preparation
In order to prove the species of the mangrove, the fresh and young leaves, barks, and fruits were identified at Purwodadi Botanical Conservation Center, Indonesian Institute of Science (LIPI), Indonesia. The samples were dried for two weeks and then powdered using mechanical grinder. The powder (200 g) was then extracted using n-hexane, ethyl acetate, and ethanol, consecutively. The extraction using n-hexane was conducted for 24 h using ratio of 1:5 (w/v) and then the solution was filtered. The filtrate was collected as n-hexane extract. The residue was extracted further using ethyl acetate and was left for 24 h with ratio of 1:5 (w/v). The filtrate was collected as ethyl acetate extract. Subsequently, the residue was extracted using ethanol with ratio of 1:5 (w/v) for 24 h and the filtrate was collected as ethanol extract. All of the extraction was conducted using orbital shaker at 180 rpm. Extracts were concentrated under rotary evaporator and the crude extract kept at 4 °C for subsequent phytochemical analysis. The yield percentage was measured from the dry extract powder.

2.3. Phytochemical assay
In order to screen the presence of secondary metabolites content from each extracts, the analysis of phytochemicals was conducted according to the previous study by Harborne with slight modification. The crude extract (0.5 mg) was dissolved in 5 mL distilled water then heated. Two mL of the extract then was added with 0.2 mL 5 % FeCl₃. The presence of blue or violet color indicated the phenolic content. Next, 2 mL extract was added with 0.4 mL amyl alcohol containing 37 % HCl and 96 % ethanol, 0.5 mg magnesium, and 70 % alcohol. The presence of red or orange color indicated the flavonoid content. Subsequently, in order to identify the tannin content, 2 drops of 1 % FeCl₃ and H₂SO₄ was added in 2 mL extract. The presence of brown precipitate indicated the tannin content.

2.4. Antioxidant assay
The antioxidant activity of crude extract was measured by the free radical scavenging 1,1-diphenyl-2-picrylhydrazyl according to previous study by Sun et.al. with slight modification. The mixture solution was composed of DPPH and crude extract as the inhibitor. The ascorbic acid was used as positive control. All of the solution was incubated at 37 °C for 30 min. The solution was detected using a UV spectrophotometer at 517 nm. The antioxidant activity (ppm) was calculated according to the following equation:

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\text{Antioxidant activity (\%)} = (\Delta \text{A control} - \Delta \text{A inhibitor})/ (\Delta \text{A control})
\]
where $\Delta A$ inhibitor and $\Delta A$ control are absorption of sample with and without inhibitor, respectively. The $IC_{50}$ value was defined as the concentration of inhibitor which was able to inhibit 50% of DPPH activity.

2.5. Statistical analysis
The experiments were run in triplicate and data were expressed as the mean ± standard deviation. The data was analyzed using SPSS version 16.0 (SPSS, Inc., Chicago, IL). Differences among mean values were analyzed by Analysis of Variance (ANOVA) followed by Duncan’s multiple comparison test if the resulting p-value was lower than 0.05.

3. Results and discussion
3.1. The yield of crude extract
The yield of leaves, bark, and fruits extract of mangrove Avicennia rumphiana was obtained from percentage of weight of extract divided by the original weight of Avicennia rumphiana used. The resulting yield value was different for each extraction solvent. The efficacy of solvent extraction is affected by many factors such as the type of solvent, solvent concentration, time, temperature, pH, number of steps, liquid-to-solid ratio and particle size of the plant material [9]. In our study, we used the multilevel extraction method with n-hexane, ethyl acetate, and ethanol. The purpose of using n-hexane as the first solvent is due to the coated cell wall of phospholipid. Dissolution of phospholipid causes opening of the cell to make it easier for the compounds stored in cells to be extracted using the next solvent. The residue from the extraction process using n-hexane was dissolved in a semi-polar solvent (ethyl acetate). Ethyl acetate is capable of attracting hydrophilic and lipophilic compounds [10]. Extract from the ethyl acetate extraction was then dissolved in ethanol. The yield of n-hexane, ethyl acetate, and ethanol extracts from mangrove Avicennia rumphiana can be seen in table 1. According to Jacob et al., extraction of Avicennia marina leaves using methanol solvent resulted in 9.61% yield, while yield of ethyl acetate and n-hexane extraction were 1.28% and 0.62%, respectively [11].

| Sample       | Weight (g) | Weight of extract (g) | Yield (%) |
|--------------|------------|-----------------------|-----------|
|              | Bark       | Fruit                 | Leave     | Bark | Fruit | Leave |
| n-hexane     | 200        | 0.78                  | 6.31      | 6.28 | 0.39  | 3.15  | 3.14  |
| Ethyl acetate| 200        | 5.71                  | 7.33      | 2.76 | 2.85  | 3.66  | 1.38  |
| Ethanol      | 200        | 6.73                  | 10.55     | 12.48| 3.36  | 5.27  | 6.24  |

3.2. Screening of phytochemical contents from Avicennia rumphiana
In order to comprehensively screen the phytochemical contents of Avicennia rumphiana, various solvent was used to extract the diverse compounds. Typically, the leave, bark, and fruit of mangrove Avicennia rumphiana contain most of the phytochemicals like flavonoid, phenol, and tannin (table 2). Test solution turned greenish when added with $\text{FeCl}_3$ 5%. It showed that there were phenol compounds in the extract. When ethyl acetate and ethanol extracts were added with amyl alcohol and Mg, orange color appeared in amyl alcohol extraction. It shows there were flavonoid compounds in the extract, but in the n-hexane test the resulting color appeared vague. Results showed that there were flavonoid compounds in n-hexane extract although in small amount. Samples added with 1% $\text{FeCl}_3$ and $\text{H}_2\text{SO}_4$ solution showed brownish green color and brown precipitate, which indicated that there were tannin in the extract. The color changes in phytochemical test can be seen in figure 1. Phytochemicals, plant-derived non-nutritive compounds, are the results of the plant synthesis process and they play an important role in various functions in the human body [12]. According to the result above, all of the extracts contained phenol, flavonoids, and tannins. It showed that phenolic
compounds can be dissolved using non-polar, semi-polar, and polar solvents. Flavonoids are intermediates that act as lipophilic and hydrophilic antioxidants [13]. The red color produced in flavonoid test is due to the formation of the flavilium salt [14]. While tannin compounds have OH groups that affects polarity, so by having tannins, the extract was able to be dissolved in polar compounds.

Figure 1. Phytocemical test solution of *Avicennia rumphiana* (A) Result of phenolic assay (1) n-hexane extract (2) ethyl acetate extract (3) ethanol extract. (B). Result of flavonoid assay (1) n-hexane extract (2) ethyl acetate extract (3) ethanol extract. (C) Result of tannin assay (1) n-hexane extract (2) ethyl acetate extract (3) ethanol extract.

Table 2. Quantitative analysis of phytochemicals in *Avicennia rumphiana* crude extract.

| No. | Sample (crude extract) | Weight of sample (g) | Total of phenol (mg ekv. Gallic acid/gram extract) | Total of flavonoid (mg ekv. Quersetin/gram extract) | Total of tannin (mg ekv. Tannic acid/gram extract) |
|-----|------------------------|----------------------|---------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| 1   | Ethanol extract of bark | 0.1                  | 0.9072                                            | 4.8151                                          | 4.1008                                          |
| 2   | Ethyl acetate extract of bark | 0.1                | 1.2413                                            | 6.1796                                          | 5.7699                                          |
| 3   | n-hexane extract of bark | 0.05                 | 0.9654                                            | 2.0258                                          | 2.9869                                          |
| 4   | Ethanol extract of fruit | 0.1                  | 9.9428                                            | 13.1323                                         | 11.0055                                         |
| 5   | Ethyl acetate extract of fruit | 0.02                | 23.8643                                           | 13.7796                                         | 74.6339                                         |
| 6   | n-hexane extract of fruit | 0.1                  | 0.2881                                            | 1.4075                                          | 5.0033                                          |
| 7   | Ethanol extract of leave | 0.1                  | 0.8761                                            | 2.3043                                          | 2.3536                                          |
| 8   | Ethyl acetate extract of leave | 0.1                | 1.4360                                            | 13.8000                                         | 21.2940                                         |
3.3. Antioxidant activity
In order to determine whether the crude extract of different solvents had antioxidant activity, the IC_{50} value was calculated. From different solvent extraction, ethyl acetate exhibited lower IC_{50} compared with others (table 3). DPPH radical is scavenged by antioxidants through donation protons forming reduced DPPH [15].

Table 3. IC_{50} value of Avicennia rumphiana extracts.

| Sample   | IC_{50} value (ppm) | Strength of antioxidant activity |
|----------|---------------------|----------------------------------|
| Ascorbic acid | 9.06 ± 0.08        | Very strong                      |
| Leave    | n-hexane            | 1122.99 ± 1.30                  | Very weak                        |
|          | Ethyl acetate       | 251.98 ± 1.59                   | Very weak                        |
|          | Ethanol             | 492.22 ± 1.03                   | Very weak                        |
| Bark     | n-hexane            | 1717.10 ± 1.26                  | Very weak                        |
|          | Ethyl acetate       | 515.77 ± 1.95                   | Very weak                        |
|          | Ethanol             | 1088.95 ± 2.08                  | Very weak                        |
| Fruit    | n-hexane            | 1140 ± 2.32                     | Very weak                        |
|          | Ethyl acetate       | 611 ± 1.03                      | Very weak                        |
|          | Ethanol             | 884 ± 1.67                      | Very weak                        |

The results showed the low antioxidant activity of leaves extract from the mangrove Avicennia rumphiana. N-hexane, ethyl acetate, and ethanol extracts had different antioxidant activity that depended on to the type of antioxidants contained in each extract. Phenolic compounds commonly found in plant and flavonoids are a group of phenolic compounds that have antioxidant activity [16]. In our study, even though there were qualitative proofs of the existence of phenolic compounds in leaves of Avicennia rumphiana, the antioxidant activity was relatively low, which might be due to its low quantity. On the other hand, the n-hexane extract had a high yield, but the antioxidant activity value was low. Most of the compounds extracted by n-hexane were potentially non-antioxidant compounds. N-hexane is capable of extracting non-polar compounds such as fats and oils. Avicennia rumphiana leaves have high salt content, which is a mineral with polar property, thus they are able to dissolve in a polar solvent such as ethanol.

On the other hand, the high inhibition activity of ethyl acetate extract from the bark was due to the nature of ethyl acetate that is a polar solvent which can extract polar or nonpolar antioxidant active compounds even though the yield of ethyl acetate extraction was lower than in ethanol. Hardiningtyas et.al. stated that the highest antioxidant activity was present in ethyl acetate extract of Avicennia marina mangrove ethyl acetate is a semi polar solvent, thus it can extract both lipophilic and hydrophilic antioxidants. Antioxidants are categorized based on their solubility into lipophilic and hydrophilic antioxidants [18]. Hydrophilic and lipophilic antioxidants work synergistically to create a strong ability to inhibit the attack of free radicals. Tannin was also positively detected in n-hexane, ethyl acetate, and ethanol extracts from the bark. Tannins consist of a complex mixture of polyphenols compounds and they have many hydroxyl (OH) groups which are polar so that it can optimally be extracted with polar solvents. Tannins are very effective as electron donors as well as chelating agent on metals because of their hydroxyl group and conjugated double bonds, which allow the occurrence of electron delocalization [19]. It can be seen in table 2 that tannins are the most abundant compound found in Avicennia rumphiana bark. Nuraini reported that tannins are produced through polymerization of flavonoids and they are usually rich in wood plants that have the ability in the tannery leather. The presence of secondary metabolite compounds in Avicennia rumphiana bark is influenced by several factors such as genetics, age of the plant, climate, habitat, pests, and disease.
4. Conclusion
The current study has demonstrated that multilevel extraction (n-hexane, ethyl acetate and ethanol) can potentially be used to screen phytochemicals and antioxidant activities of *Avicennia rumphiana* mangrove. The crude extract of the mangrove contained phenolics, flavonoids, and tannins. The ethyl acetate extract had better antioxidant activity compared to n-hexane and ethyl acetate extracts in leaves, bark and fruits of *Avicennia rumphiana*, with IC\textsubscript{50} values of 152.42 ± 1.30 ppm, 182.32 ± 2.03 ppm, and 232.43 ± 1.43 ppm, respectively.

5. References
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