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Screening of antiradical activity from some central Sulawesi mangroves

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ABSTRACT

Antiradicals are substances with important functions for human health. Mangrove leaves are one of potential sources of natural antiradical. The objective of this research was to identify the type and fraction of mangrove leaves extract with the highest antiradical activity. The research procedure included sampling and extraction of mangrove leaves, assay of antiradical activity (DPPH), phytochemical assay and determination of IC_{50} for the fraction with the highest inhibition percentage. Mangrove leave samples (*Rhizophora* sp., *Avicennia* sp. and *Sonneratia* sp.) used in this study were collected from the Palu Bay coastal. Results showed the highest yield of extracts was from *Rhizophora* sp., followed by *Avicennia* sp. and *Sonneratia* sp. Inhibition percentage was higher from dried compared to fresh mangrove leaves. Additionally, the inhibition percentages of the ethanol fraction was higher than that of the ethyl acetate and n-hexane fractions, while *Sonneratia* sp. gave a higher inhibition percentage than *Avicennia* sp. and *Rhizophora* sp. The ethanol fraction IC_{50} was determined for *Sonneratia* sp. (46.05 ± 0.18 µg/mL), *Avicennia* sp. (88.41 ± 0.29 µg/mL) and *Rhizophora* sp. (103.95 ± 0.38 µg/mL). Phytochemical assays showed that the three ethanol fractions contained flavonoids, phenols (tannins) and steroids compounds. From the research, it can be concluded that the three leaves of mangrove potential as antiradicals.

Keywords: mangrove, antiradical, *Rhizophora* sp., *Avicennia* sp., *Sonneratia* sp.

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INTRODUCTION

Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (ROS) are unstable and highly reactive, damaging cells with chain reactions and causing oxidative damage to biomolecules, such as deoxyribonucleic acid (DNA), lipids, proteins, and carbohydrates (Krishnamoorthy *et al.*, 2011). ROS molecules are physiological metabolites formed as a result of oxygen metabolism. DNA, cell membranes and other cellular constituents are targets of ROS molecules through oxidation/degradation processes. These oxidation processes can cause or contribute to serious disease in humans, such as arterosclerosis, rheumatoid arthritis, cataracts, and cancer, as well as accelerated aging (Kovatcheva *et al*., 2001 and Ruberto *et al*., 2001).

Antioxidants (antiradical) can be defined as substances that can inhibit or prevent the oxidation of a substance in a chain reaction (Leong and Shui, 2002 and Halliwell and Whiteman, 2004). Antioxidants (antiradical) serve to protect cells from damage caused by free radicals, because antioxidant substances can give electrons, enabling free radical molecules to become stable. Antioxidant (antiradical) substances with ROS capture capabilities have great relevance in the prevention of oxidative stress. Antioxidant substances prevent cell damage from reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anions (O^{2-}) and hydroxyl free radicals (OH^-) (Devi *et al.*, 2011).

Currently, Antioxidants (antiradical) substances are considered important because of their functions for promoting human health. Antiradical (antioxidants) have become common in health-care, providing support in resisting or overcoming various kinds of cardiovascular disease and arterosclerosis, as therapeutic treatment in cancer therapy, and as agents inhibiting aging processes (Loo *et al*., 2007). Moskovitz *et al*., (2002) suggests many human diseases such as aging, cancer, inflammation, cardiovascular and neurodegenerative disorders are related to cell oxidation by free radicals. Thus, antioxidants (antiradical) are needed as prophylactic supplements to protect the body as well as to help heal disease (Gao and Xiao, 2012).

Bioactive substance produced by plants are a good source of medicinal ingredients and play an important role for human health (Jasuja *et al*., 2013). Recently researchers have focused on the development of antioxidant formulations from plants (Bairwa *et al*., 2011 and Jasuja *et al*., 2012b). The bioactive substances of plants, such as tannins, flavonoids, and terpenoids, play an important role as part of the defense system of the plant itself, but can also serve as natural products that are beneficial to human health (Wibowo *et al*., 2009; Jasuja *et al*., 2013; and Mitter, 2015).

Currently the food industry employs many synthetic antioxidants to prevent the oxidation of fats in food and to extend shelf life; these include butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ), L-ascorbic acid and butylated hydroxytoluene (BHT) (Li *et al.*, 2007). However, these synthetic antioxidants are limited in their use as they allegedly have carcinogenic and/or toxic properties and can affect human health (Dubey and Batra, 2009; and Jasuja *et al*., 2012a). Therefore, it is important to continue the search for antioxidants from natural sources that are safe to use as food additives, pharmaceutical preparations and able to counteract the negative effects of free radicals (Lee *et al*., 2004; Song *et al*., 2010; and Amelia and Tanod, 2016).

Mangrove leaves are one potential source of natural Antiradical (antioxidants) (Jacoeb *et al*., 2011; Elfita, 2013; and Hardiningtyas *et al*., 2014). Mangroves forests grow readily in tropical coastal environments, however they have not been utilized as bioactive substance sources. Mangrove trees are plants which provide many benefits for humans, ranging from ecosystem services to food and medicine (Nurjanah *et al*., 2015). One important reason for the study of the bioactive substances from mangroves is their ready availability combined with indications of their (as yet unrealized) potential for use in medical applications (Latief *et al.,* 2015).

Mangroves have an unusually high tolerance to fluctuations in salinity, enabling them to grow in river estuaries and the intertidal zones of tropical and subtropical coasts. One adaptation to these environmental stresses by mangroves is the development of an effective system for antioxidative defense, consisting of enzymatic and non-enzymatic antioxidants (Jithesh *et al*., 2006 and Agoramoorthy *et al*., 2008). Mangroves produce secondary metabolites in order to survive in extreme

habitat conditions. Bandaranayake (2002) reported bioactive substances found in mangroves belonging to the alkaloids, phenolics, steroids, and terpenoids. Brugine is an alkaloid isolated from *Bruguiera sexangula* (Loder and Russell, 1969). Secondary metabolite 2-nitro-4- (2'- nitroethenyl) phenol isolated from fruit *Sonneratia acida* (Bose *et al.*, 1992). Stigmasterol a steroid compound commonly found in plants and is found abundantly in *Acanthus illicifolius* (Huang *et al.*, 2014). A monoterpenoid xylomollin isolated from *Xylocarpus moluccensis* (Kubo *et al.*, 1976) and a triterpenoidal saponin compound isolated from *Acanthus illicifolius* (Minocha and Tiwari, 1981).

Central Sulawesi Province has around 26.536 Ha of mangrove forests, distributed across the districts of Donggala, Poso, Banggai, Buol, Toli-Toli, Morowali, Banggai Kepulauan, Banggai Laut, Tojo Una Una and Parigi Moutong (Jabir, 2014). The mangrove forests around the Palu Bay coastal are dominated by the genera *Sonneratia*, *Rhizophora* and *Avicennia*; extensive stands are found in the coastal villages of Kabonga Besar and Kabonga Kecil, Donggala District (Department of Fisheries and Marine, 2007 and Eyrika, 2011). This study examined the antiradical potential of leaf extracts from these three predominant mangrove genera in Palu Bay coastal. The research objective was to determine the type and fraction of mangrove leaf extract with the highest antiradical activity.

MATERIALS AND METHODS

Location and Time

Research was conducted between May and September 2017. Bioactive substances extraction and antiradical assay were conducted at the Fisheries Technology Production Laboratory of the Palu Fisheries and Marine Institute and the Chemical Research Laboratory of Tadulako University.

Materials and Equipment

Mangrove leaf samples (*Avicennia* sp., *Rhizophora* sp. and *Sonneratia* sp.) were collected in May 2017 from Kabonga Besar Village on the Palu Bay coastal of Donggala District, Central Sulawesi Province, Indonesia. Other materials used included ethanol (EtOH Merck), ethyl acetate (EtOAc Merck), n-hexane (Merck), crystalline 1,1-Diphenyl-2-picryl hydrazil (DPPH Merck), Wagner reagent, Meyer reagent, Dragendroff reagent, chloroform, anhydrous acetate, concentrated sulfuric acid, magnesium powder, amyl alcohol, hot water, HCl 2 N solution, ethanol 70%, and FeCl3 5% solution. Equipment used included digital scales, aluminum foil, Whatman filter paper, erlenmeyer flasks, volumetric pipettes, micro pipettes, a blender, rotary vacuum evaporator (EYELA N-1100), Finco oven, separator funnel, glass bottles, becker glasses and a spectrophotometer (UV-VIS T90 + PG Instruments Ltd).

Research Procedures

The research comprised several stages: sampling and mangrove leaf extraction, assay of antiradical activity, phytochemical assay, and determination of IC_{50} value from fraction showing the best inhibition percentage.

Mangrove leaf extraction

In this research, both fresh and dried mangroves leaves (dried at a temperature of 40-50 °C) were used. The mangrove leaves were finely mashed with a blender. The extraction method was modified from Cruz *et al*., (2015) and Wahyuni *et al*., (2015). Extraction was performed with 3 different solvent types: n-hexane, ethyl acetate and ethanol. Finely mashed mangrove leaves (100 g/sample) were macerated with n-hexane (1: 2 wt / v) for 48 hours, with occasional shaking. The resultant maceration product was filtered through filter paper, separating the filtrate and residue. The filtrate was evaporated, to obtain crude extract, and weighed. In a similar manner, the residue was further macerated with ethyl acetate and then with ethanol. These processes produced dried and fresh hexane extract; dried and fresh ethyl acetate extract; and dried and fresh ethanol extract. The mangrove leaf extraction procedure can be seen in Figure 1. The percentage yield of extracts obtained was calculated using equation 1:

$$\text{Mangrove leaf extract yield}(\%) = \frac{\text{Weight extract (g)}}{\text{Initial sample weight (g)}} \times 100\% \qquad (1)$$

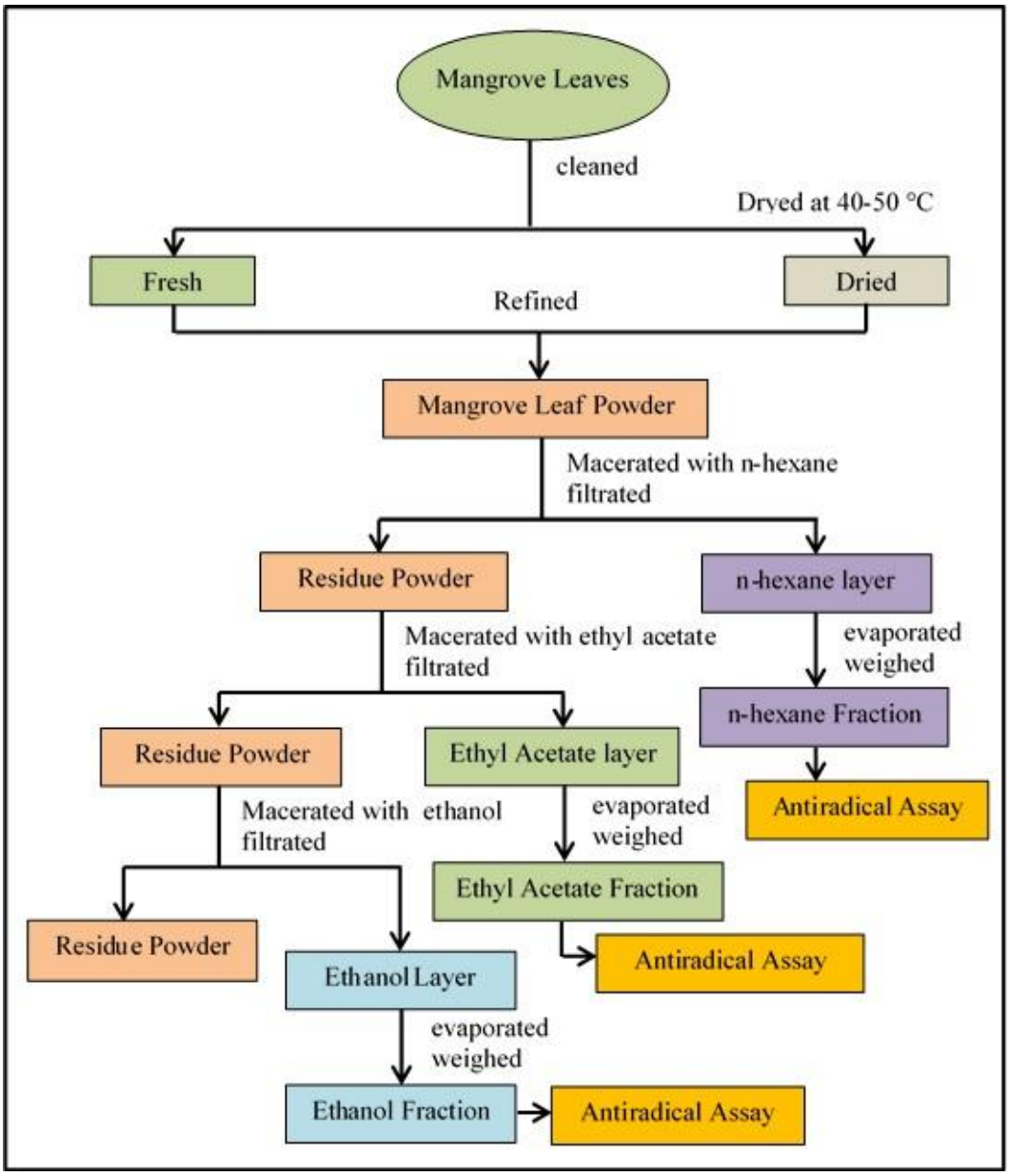

Figure 1. Flow Chart of mangrove leaves extraction

Antiradical assay

The antiradical activity of each extract was determined using DPPH free radical (Molyneux, 2004). A 25 mg was taken from each extract, and placed in a 25 mL flask. Ethanol solvent was then added to obtain an extract solution concentration of 1000 µg/mL and further diluted to obtain an extract solution concentration of 200 µg/mL. A 2 mL aliquot of this solution was added to 2 mL of 50 µM DPPH solution. The mixture was homogenized and left for 30 minutes in the dark before measuring the free radical absorption at 517 nm wavelength. The DPPH solution absorbance value was also measured. The sample inhibition percentage was calculated using equation 2:

$$\%\ \text{Inhibition} = \frac{\text{Blank Absorbance} - \text{Sample absorbance}}{\text{Blank Absorbance}} \times 100\% \qquad (2)$$

Thereafter, the IC_{50} of the fraction showing the best inhibition percentage was determined. The extract solution was diluted to produce a series of 10, 30, 50, 70, and 90 µg/mL. Extract solution aliquots of 2 mL were added to 2 mL of DPPH 50µM solution, homogenized and left for 30 minutes in the dark. Absorbance was then measured at a wavelength of 517 nm and plotted on the y axis (x axis being the concentration of the diluted extracts), to obtain a linear regression equation. The IC_{50} was determined as the extract solution concentration required to reduce DPPH free radicals by 50%.

Phytochemical Assay

The fractions that showed the highest antiradical activity were subjected to phytochemical screening assays to determine the presence of secondary metabolites (alkaloids, flavonoids, steroids, saponins and polyphenols) using conventional standard protocols as described by Harborne (1998).

Data Analysis

Data analysis was based on the completely randomized factorial design, with 3 treatments, 2 factors and 3 replicates. The analysis was continued using the LSD test to compare the antiradical power of the various treatments. The data were statistically analyzed using the software Microsoft Excel version 2010. Differences were considered significant at $p < 0.05$. IC_{50} analysis used linear regression.

RESULTS AND DISCUSSION

Based on the characteristics of leaves and roots, the mangrove samples used in this research, identified *Avicennia* sp., *Rhizophora* sp., and *Sonneratia* sp. Identification of the sample morphology mangrove done by observing the type of root, shape and tip of a leaf. Genus *Avicennia* generally has a horizontal root system and roots breathing; leaf shape obovate, rounded elongated, elliptical; and leaf tip tapered to round. Genus *Rhizophora* sp. generally have typical roots of penetration root and air roots that grow from the lower branches and has a narrow elliptical to widen leaf shape with the tapered leaf tip. Genus *Sonneratia* sp. generally have a cable-shaped root system as a cone-shaped vertical breathing root, leaf shape is oval and elongated round with the rounded leaf tip. The procedure of identification of mangrove samples follows the instructions of Noor *et al*., (2006). Figure 2 shows a selection of mangrove leaf samples.

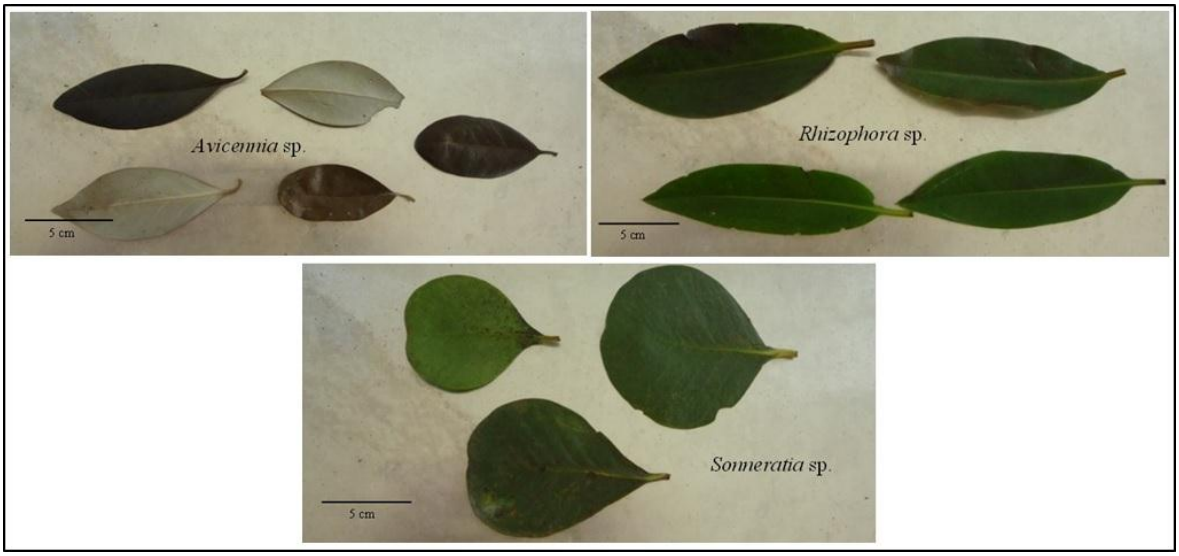

Figure 2. Mangrove leaf samples

The extract yield varied between mangrove genera and samples. The main factor influencing the yield was the solvent used (Hardiningtyas *et al*., 2014). In addition, the yield of the extract can also be

affected by habitat and location as a response to the environment (Nopiyanti and Agustriani, 2016). The percentage yield of mangrove leaf extracts obtained is presented in Figure 3.

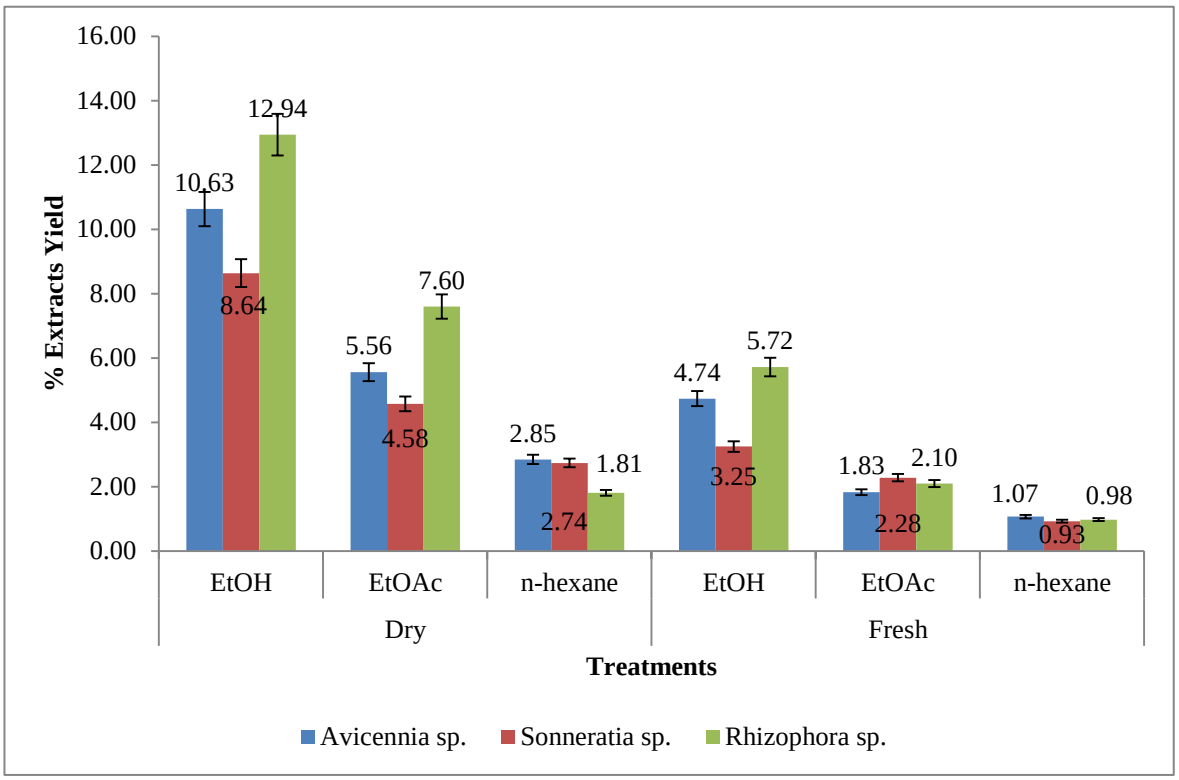

Figure 3. Mangrove leaf extract yield

Figure 3 showed that the drying treatment results in a higher extract yield than the extraction from fresh leaves. This were likely due to the reduction in leaf water content, enabling the solvent to access and extract the bioactive substance in the samples more readily (efficiently). Previous studies report varied levels of water content in leaves of the three mangrove genera used; *Avicennia marina* leaves can have a water content of 68.16% (Jacoeb *et al.*, 2011), *Rhizophora apiculata* a water content of 54.40% (Bunyapraphatsara *et al.*, 2002), and *Sonneratia alba* leaves water content could be as high as 80.12% (Sen and Rajpurohit, 2012). Therefore, it was reasonable that the highest extract yield should come from *Rhizophora* sp. leaves, followed by *Avicennia* sp. and *Sonneratia* sp.

The extraction process aims to separate bioactive substances from a complex material. To obtain a large amount of bioactive susceptibility, the sample needs to be reduced in size and dried (Sari, 2008). The yield of the extract was the ratio of the weight of the extract obtained from the initial weight of the sample used. The yield of the extract indicates the effectiveness of a solvent to separate and carry material during the extraction process, but does not indicate the activity of the extract (Putri *et al.*, 2016). The results of Hardiningtyas *et al*., (2014) report yields of *Avicennia marina* leaf extract as 9.61% (methanol), 1.28% (ethyl acetate) and 0.62% (n-hexane). Reported yield of *Sonneratia alba* leaf extract using methanol solvent was 7.9% (Putri, Hasanah and Kusimaningrum, 2016), while *Rhizophora mucronata* leaf extracted using methanol was 21.47% (Tarman, Purwaningsih and Negara, 2013). Suciati *et al*., (2012) reported extract yields from fresh *Rhizophora mucronata* leaves of 7.1 g (methanol); 10.8 g (ethyl acetate); and 0.6 g (n-hexane); and from dried leaves of 2.9 g (methanol); 2.4 g (ethyl acetate); and 1.7 g (n-hexane). Variation in the extract yield can be strongly influenced by water content and other components (Podungge *et al.*, 2015).

Figure 3 also showed higher extract yields from the polar and semi-polar fractions compared to the nonpolar fraction; this indicates that bioactive substances contained in mangrove leaves can be

dissolved in a polar or a semipolar solvent. Bioactive substances can only be extracted with a solvent polarity similar to the polarity of the bioactive substance itself (Jacoeb *et al.*, 2011). Sari (2008) stated that substances dissolved during the extraction process have the same polarity as that of the solvent used, thus solvent polarity will affect the yield of the resulting extract. The high ethanol extract yield was likely due to the presence of chlorophyll in the leaves, as chlorophyll can be extracted by such a polar solvent Jacoeb *et al*., (2011).

Antiradical activity was measured by counting the purple light intensity levels of DPPH, proportional to the reduction in the concentration of DPPH. This reduction was caused by the reaction of Diphenyl-2-picryl hydrazil molecules with hydrogen atoms released by the molecular components of the sample, thus forming diphenylpicryl hydrazine compounds, and causing DPPH to change color from purple to yellow (Huliselan *et al.*, 2015). Antiradical activity indicates the ability of a bioactive substance to inhibit the oxidation reaction, expressed as the inhibition percentage. In this research, the inhibition percentage was measured for each sample, under all treatments and for each mangrove leaf extract fraction, as can be seen in Figure 4.

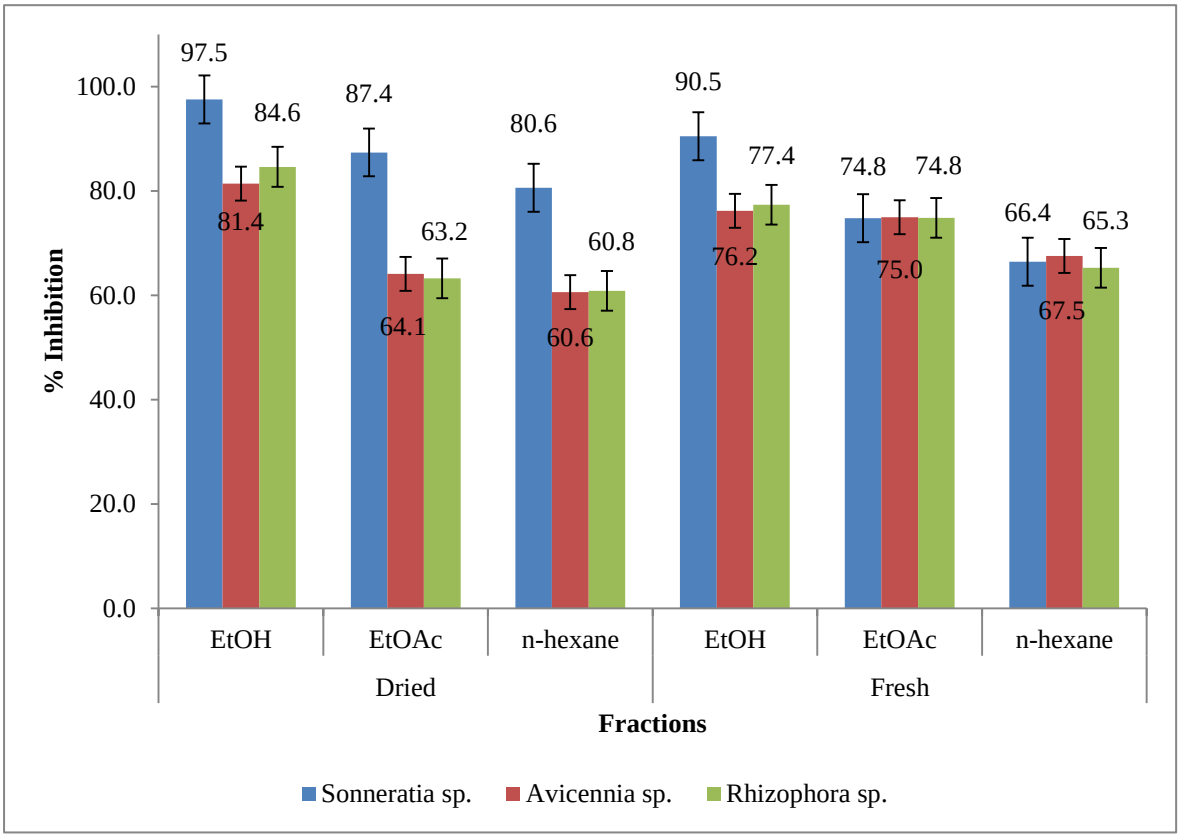

Figure 4. Mangrove Leaf Extract inhibition percentages

Figure 4 showed that dried mangrove leaf extracts have higher inhibition percentages than fresh mangrove leaves. In addition, the ethanol fraction gave higher percentages of inhibition than the ethyl acetate and n-hexane fractions. Looking at the three mangrove genera, *Sonneratia* sp. extracts produced higher inhibition percentages than *Avicennia* sp. or *Rhizophora* sp. The results of two-factor analysis of variance indicate that leaf treatment and solvent type significantly ($p < 0.05$) affect the inhibition percentage of mangrove leaf extracts. The results of the IC_{50} analysis for the three mangrove leaf ethanol fractions can be seen in Table I. Analysis of IC_{50} used regression linear.

The results of this research were consonant with those of Patra *et al*., (2015), who report antioxidant potential ranging from 128.63±4.06 to 271.52±3.8 mg/g dried weight for polar fraction extracts of *Sonneratia* leaves, with IC_{50} values from 39.90±0.47 to 41.92±0.98 µg/mL. Similarly,

Jacoeb *et al*., (2011) and Srikanth *et al*., (2015) obtained IC_{50} values from the polar fractions of *Avicennia* leaf extracts in a range of 143 - 257%, with DPPH capture percentage of 74.55% (Lincy *et al.*, 2013). Molaee *et al*., (2017) showed inhibition of 0.5 to 12 mg/mL from *Avicennia marina* leaf extracts. Khushi *et al*., (2016) also reported inhibitory potential above 90% at concentrations of 50-500 µg/mL using *Avicennia officinalis* leaf polar extracts. While for *Rhizophora* leaves, IC_{50} values obtained from 6.65±0.10% (Adhikari *et al.*, 2016) and 0.15±0.02 mg/mL (Cruz *et al.*, 2015).

Table I. IC_{50} Values from Antiradical Assays using DPPH radicals of mangrove leaf ethanol fractions

Mangrove	IC_{50} (µg/ml)
Sonneratia sp.	46.05 ± 0.18
Avicennia sp.	88.41 ± 0.29
Rhizophora sp.	103.95 ± 0.38

Blois (1958) stated that a compound to be a very powerful antioxidant (antiradical) if IC_{50}< 0.05 mg/ml (50 µg/ml), strong if IC_{50} is between 0.05 to 0.10 mg/ml (50 -100 µg/ml), moderate if the IC_{50} is between 0.10 and 0.15 mg/mL (100 - 150 µg/mL) and weak when the IC_{50} is in the range from 0.15 mg/mL - 0.20 mg/ml (150 µg/ml - 200 µg/mL). Table I showed that the ethanol fraction extracted from *Sonneratia* sp. leaves can classified as containing very powerful antiradical, while the *Avicennia* sp. extracts contained strong antiradical, while *Rhizophora* sp. produces moderate antiradical.

Mangrove extract can be obtained from percolation using different solvents by polarity. Cruz *et al.*, (2015) reported that extracts using ethanol showed higher antioxidant (antiradical) activity than those obtained using other solvents. *Avicennia marina* mangrove leaf extract showed powerful antioxidant (antiradical) properties, which may help to prevent the formation of free radicals that can reduce tissue damage (Mayuresh and Sunita, 2016). Sadhu *et al*., (2006) also reported that *Sonneratia caseolaris* ethanol fractions showed antioxidant (antiradical) potential of the flavonoid group. Haq (2012) also reported antioxidant (antiradical) potential from *Rhizophora mucronata* extracts with high phenolic content. The results of mangrove leaf extract phytochemical assays for the ethanol fractions can be seen in Table II.

Table II. Phytochemical assay results for mangrove leaf ethanol fractions

Phytochemical Assay	Mangrove Species			Standard
	***Avicennia* sp.**	***Rhizophora* sp.**	***Sonneratia* sp.**	
Saponins	-	+	-	Stable foam formed for 15 minutes
Phenols (tannins)	+	+	+	Brown precipitate formed
Steroids	+	+	+	Green or blue colour produced
Alkaloids	-	-	-	Orange precipitate formed
Flavonoids	+	+	+	Orange, pink or red colour produced

Based on the data in Table II, the three mangrove leaf ethanol fractions contained flavonoids, phenols (tannins) and steroid compounds. The active components detected in the polar fraction (ethanol) of *Rhizophora* sp. leaf extract are in accord with Tarman *et al*., (2013) and Nurdiani *et al.*, (2012) who report that crude extracts of the polar fraction of *Rhizophora mucronata* leaves contained active components comprised of tannins, saponins, phenols and flavonoids. Srikanth *et al*., (2015) report that polar (hydroalcoholic extract) fractions of *Avicennia marina* leaf extracts contained active components comprised of phytosterols, flavonoids, tannins and phenols. The polar fraction (methanol)

of *Avicennia officinalis* leaves was reported as containing active components comprised of steroids, tannins and flavonoids (Ganesh and Vennila, 2011). While active components detected in the polar fraction (ethanol) of *Sonneratia* sp. leaf extracts include flavonoids (Sadhu *et al*. 2006), steroids (Mitter, 2015) and phenols (Haq *et al.*, 2014). These bioactive compounds are thought to be produced by mangroves as a response to (stressful) interaction with the environment.

Flavonoid compounds are typically present in almost all parts of the tree (Kar *et al*. 2006 and Putu Sri Dia *et al*. 2015), contributing to ecofarmacological activity in mangrove plants (Sadhu *et al.*, 2006). Flavonoids act as antioxidants, anti-inflammatory agents and can have antibacterial properties. Flavonoids, as effective reducing compounds, can inhibit many oxidation reactions enzymatically or non-enzymatically. Flavonoid compounds can act as free-radical catchers, thus protecting lipid membranes from damaging reactions (Salamah *et al.,* 2018).

Tannins are polymeric organic components derived from glycoside found in many plants. Flavonoids and tannins both act as antioxidants because they have a -OH (hydroxyl) group attached to an aromatic ring. Tannins are effective in capturing free radicals, as electron donors and sources of atomic hydrogen, active in metal chelating because of the -OH group and the conjugated double bond that allow the formation of electron delocalacies. The phenol compounds may be flavonoids, simple monocyclic phenols, phenylpropanoids, or phenolic quinones. The phenol hydroquinone and its derivative compounds act as oxidative inhibitors which bind to free radicals and react with the Reactive Oxygen Species (ROS) compound to form more stable compounds (Harborne, 1998). Thus, such phenol compounds can help protect mangroves from solar radiation (Agati *et al.*, 2009).

Saponin is a glycoside commonly found in plants, characterized by foaming, and readily soluble in polar solvents (Sumarto *et al.*, 2011). Saponin compounds are antioxidants and free radical catchers; they form hydrogen peroxide (H_2O_2) as an intermediary compound, and can donate a hydrogen atom to compound DPPH radicals, thus terminating the chain reaction (Xiong *et al.*, 2012). Firdayani *et al*. (2015) placed saponin within the group of phenolic compounds suspected to inhibit free radicals.

Steroid compounds, common in and once considered limited to animals, have recently been found in plants also (Harborne, 1998). Steroids are commonly used as antibacterial agents. Steroids are bioactive components categorized as non-polar. However, in this research, steroid compounds showed positive results in polar solvents. This may be due to the dipole moment of polar compounds which induce non-polar molecules that do not have a dipole through electrostatic forces. This force can makes non-polar compounds at least partially soluble in polar solvents (Firdayani *et al.*, 2015).

Mangroves are plants of tidal habitats, experiencing daily variations in abiotic pressures that can adversely affect morphological, physiological, biochemical and molecular processes (Dasgupta *et al.*, 2012). These abiotic pressure variations can trigger the production of ROS. Enzyme antioxidants found in mangrove plants are thought to be responsible for stabilizing the ROS (Thatoi, Patra and Das, 2014). Thus, mangroves are a potential source of antioxidant compounds which can be extracted to obtain substances beneficial to human health.

CONCLUSION

From our results it can be concluded that the leaves of three mangroves (*Avicennia* sp., *Rhizophora* sp. and *Sonneratia* sp.) are potential sources of antiradicals. The ethanol fraction of *Sonneratia* sp. leaf extract showed the highest antiradicals activity. The ethanol fraction of these three types of mangrove should be purified and characterized in greater depth, in order to identify the most effective antiradical compounds.

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