In vitro antioxidant activity of *Sonneratia ovata* Backer extract

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Abstract
Sonneratia ovata Backer is a mangrove plant as well as folk medicine from Aru Islands, Maluku-Indonesia which is traditionally used for treatment of many diseases. The present research has been purposed at antioxidant activity from stem bark of S. ovata. The methanol extract showed the highest in vitro antioxidant activity with IC_{50} values of 4.73 µg/mL and 2.00 µg/mL for DPPH and ABTS free radical scavenging respectively. The isolation and identification of chemical constituents of S. ovata also have been performed and yielded β-sitosterol, stigmasterol and 3β-acetoxy-lup-20(29)-en-2α-ol.

Keywords: Antioxidant, mangrove, herbal supplement.

Introduction

A free radical is a chemical compound containing one or more unpaired reactive electrons that lead it to react to other molecules in order to defend its stability. Reactive oxygen species (ROS) are the free radicals produced from oxygen which can be ions, atoms, or molecules. ROS are produced normally, but in excessive amounts, become harmful if not eliminated, because they can initiate bimolecular oxidation *in vivo* and *in vitro*. The reaction can cause cell death and oxidative stress which in turn is the cause of many diseases such as Parkinson's, Alzheimer's, cardiovascular disease and even cancer.

Furthermore, DNA can be damaged by excessive ROS resulting in chromosome damage and mutation. Body cells have some mechanisms to fight excessive ROS naturally, but when those become unbalanced, the body needs other antioxidant sources to help fight ROS[1]. One kind of strong antioxidant agent is an herbal supplement from natural resources, which is traditionally known as 'the best medicine of all'.

Sonneratia is a group of mangrove plants from the Lythraceae family that grow widely in the area between land and sea in tropical and subtropical regions of the world[2]. Species in the genus of *Sonneratia* include *S. alba*, *S. apetala*, *S. caseolaris*, *S. griffithii*, *S. hainanensis*, *S. lanceolata* and *S. ovata*[3]. Due to the ability of these plants to adapt to extreme environments with high salinity, high temperature, strong winds and muddy anaerobic soils, *Sonneratia* has metabolites with unique biological activity rich in medical potential.

Sonneratia plants have been used in traditional medicine to treat diseases such as asthma, fever, ulcers, hepatitis, hemorrhoids (piles), sprain and hemorrhages[4]. In 1998, it was reported that *S. caseolaris* was successfully used to treat piles and hemorrhage, as an antiseptic, astringent and in sprain poultices as well as for dysentery[5,6]. Fruits of *S. alba* have traditionally been used for the treatment of hemorrhage, swelling, intestinal parasites and coughs[7,8]. From this wide range of traditional uses of *Sonneratia*, it is considered to have sufficient potential to warrant study of its chemical constituents and pharmacological activity.

S. griffithii has demonstrated very good activity as an antihyperglycemic[9] while *S. apetala* has produced positive results in antioxidant activity tests, enhanced insulin-releasing activity, insulin mimetic activity, modification of glucose utilization, stimulation of regeneration of islets of Langerhans in the pancreas and enhanced transport of blood glucose to the peripheral tissue[10-13]. *S. caseolaris* has shown strong antioxidant activity and anti-lipid peroxidation, intestinal α-glucosidase inhibitory activity, potentiation of pancreatic secretion of insulin, high glucose uptake from serum and low glucose absorption from gut[14-18].

S. alba exhibits antimicrobial activities against certain microorganisms and hypoglycemic activities, such as modifying glucose utilization, increasing the level of serum insulin and decreasing blood glucose level[14,19,20]. One species of *Sonneratia, S. ovata*, grows along the Indonesian coastline, especially on Aru Island, Maluku. Traditionally, people called the *S. ovata* used for medicinal purposes *Manggustang Pante*. Further investigation and research of *S. ovata's* chemical constituents and bioactivities such as antioxidants are needed.

Material and Methods

Extraction method: The dried and powdered 1.5 kg stem bark of *S. ovata* were extracted three times using seven litres of methanol within three days (3×24 hours) at room temperature. The solvent then was evaporated to give 153 grams of brown methanol extract.

Antioxidant assay method: The methods used in this research were adapted from the methods done by Hidayati et al.[21]

DPPH free radial scavenging method: DPPH free radical scavenging result can be monitored by the color changes from purple to yellow on a spectrophotometer. 1.182 mg of

DPPH was dissolved in 50 mL of methanol to make 6 × 10^{-5} M DPPH solution and 10 mg of sample was dissolved in 1 mL of methanol to make the sample solution as well. Furthermore, 33 µL of methanolic sample was mixed with 1 mL of 6 × 10^{-5} M DPPH solution, incubated for 20 minutes and was the absorbance measured at 517 nm. The inhibition rate was calculated by formula :

Inhibition (%) = $((A_0-A_1)/A_0)$ x 100%

where A_0 is the absorbance of gallic acid as the positive control and A_1 is the absorbance of the sample.

ABTS free radial scavenging method: ABTS solution preparation was done by dissolving 19.2 mg of powdered ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) in 5 mL of distilled water to make a 7 mM ABTS solution. Then, potassium peroxydisulfate buffer solution was prepared by dissolving 3.33 mg of powdered $K_2S_2O_8$ in 8.8 µL of distilled water. Those two solutions were mixed and incubated for 12-16 hours to give $ABTS^{\bullet+}$ working solution, then add ±272 mL of ethanol until the absorbance reached 0.7±0.02 at 734 nm. Sample preparation was examined by dissolving 10 mg of methanol extract of *S. ovata* in 1 mL of DMSO and mixed well using vortex. To calculate the inhibition rate, 10 µL of the sample solution was mixed with 1 mL of $ABTS^{\bullet+}$ working solution, incubated for 4 minutes and the absorbance was measured using UV-vis spectrometer at 734 nm with trolox as the positive control.

Isolation method: In the present research, three compounds of *S. ovata* have been isolated including sterol and lupeol groups; β-sitosterol (1a) along with stigmasterol (1b) and 3β-acetoxy-lup-20(29)-en-2α-ol (2) (figure 1). The isolation methods described below.

Isolation of compound 1a and 1b: 70 grams of *S. ovata* methanol extract were fractionated by vacuum column chromatography method with 100% *n*-hexane, 100% ethyl acetate and 100% methanol as the eluents and yielded five fractions. Fractions 2, 3 and 4 were combined and then fractionated by using silica gel column chromatography with increasing polarity of *n*-hexane:ethyl acetate (100:0; 98:2; 95:5; 93:7; 85:15; 65:35; 0:100) and 100% methanol as the final eluent. From the fractionation, five fractions were obtained as well, then fraction G2 was crystalized and gave 16,5 mg compound 1a and 1b which were then identified with NMR.

Isolation of compound 2: Crude methanol extract of *S. ovata* (70 grams) was fractionated by vacuum column chromatography as well as the first compounds obtained, until to the second step of it. Fraction G5 was then fractionated with silica gel column chromatography by increasing the polarity, with the eluent of *n*-hexane:ethyl acetate (100:0; 19:1; 9:1; 17:3; 13:7; 0:100) and yielded 5 more fractions, two of them were combined based on the TLC profile. The combined fraction (H2, H3) was then being separated three times and gave fractions I, J and K. The three fractions (I4, J2 and K2) were being fractionated with increasing polarity of eluent and gained 4 fractions of L. The fraction L2 then was crystalized and yielded 17 mg of compound 2 which was then identified with NMR.

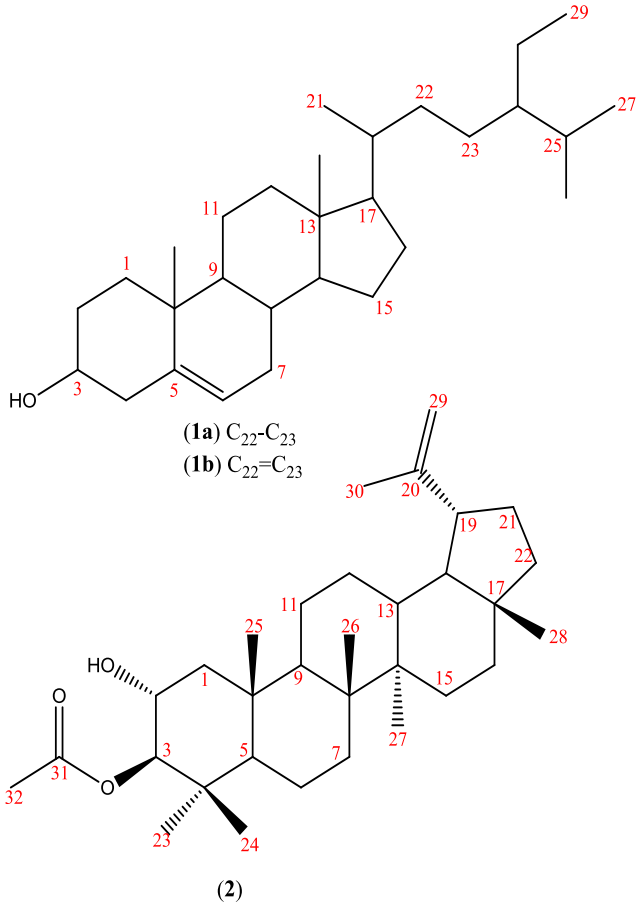

Figure 1: Chemical constituents obtained from *S. Ovata*

Results and Discussion

Antioxidant assay results: The antioxidant activities of a methanol extract of *S. ovata* measured using the DPPH and ABTS free-radical scavenging method in triplicate showed high levels.

The DPPH free-radical scavenging method was performed on *S. ovata* extracted using four solutions: methanol; ethyl acetate; dichloromethane and *n*-hexane. Methanol extract showed the highest inhibition rate of 88.345% followed by ethyl acetate with a rate of 81.697%. The other extracts had rates below 20% of inhibition as shown in figure 2. The IC_{50} value of methanol extract of *S. ovata* was 4.73µg/mL. Due to the DPPH free-radical scavenging results, we chose methanol as the solvent for extraction. In addition, from the ABTS free radical scavenging method, the IC_{50} value of the methanol extract of *S.ovata* is known to be 2.00 µg/mL which is four times better than the positive control, Trolox, which has an IC_{50} value of 7.95 µg/mL as shown in figures 3 and 4.

Despite some studies of the antioxidant activity of mangrove, especially *Sonneratia*, information about specific species activities is limited. From the activity data gained in this research, we consider that *S. ovata* has potential as an antioxidant agent due to its better antioxidant activity compared to the widely known antioxidant agent Trolox (table 1).

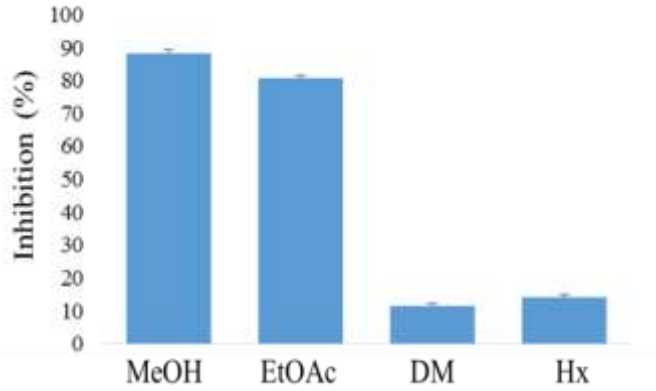

Figure 2: DPPH inhibition of *S. ovata* extracts at the concentration of 319.45 µg/mL; values are mean ± SD of three independent experiments in triplicate at each concentration. MeOH, methanol extract; EtOAc, ethyl acetate extract; DM, dichloromethane extract; Hx, *n*-hexane extract

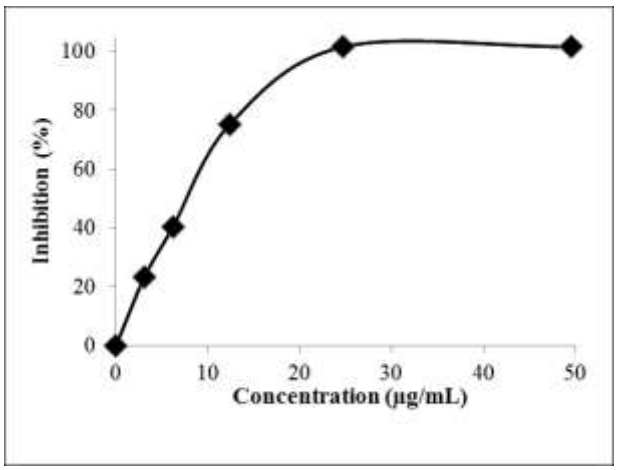

Figure 3: The inhibition rate of Trolox as the positif control using AB radical scavenging assay

Table 1
Antioxidant activity of *S. ovata* extract

Samples	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)
Methanol extract	4.73	2.00
Ethyl acetate extract	31.89	-
Trolox	-	7.95

Isolation result: This research used the stem bark of *S. ovata* Backer gathered from the Aru Islands, Indonesia, as the sample. The isolation process included extraction, separation by column chromatography and identification using NMR spectrometer. Extraction was conducted so as to obtain crude extract rich in the secondary metabolites contained in the stem bark of *S. ovata*. The solvent used in the extraction process was methanol because this extract showed the strongest activity with IC_{50} of 4.73 µg/mL in the antioxidant assay using DPPH free-radical scavenging. From the 153 g of brown crude methanolic extract of *S. ovata*, three chemical compounds were isolated: β-sitosterol (1a), stigmasterol (1b) and 3β-acetoxy-lup-20(29)-en-2α-ol (2). NMR chemical shift data are presented in table 2.

Table 2
NMR chemical shift data of compounds 1a, 1b and 2

Carbon position	δ_C compound (ppm)		
	1a	1b	2
1	37.391	37.391	49.4
2	31.791	31.791	68.6
3	71.954	71.954	79.1
4	42.352	42.429	38.5
5	140.878	140.878	56.4
6	121.870	121.870	18.4
7	32.040	32.040	32.2
8	32.040	32.040	39
9	50.262	50.262	55.4
10	36.646	36.646	37.3
11	21.227	21.227	25.64
12	39.910	39.818	27.5
13	42.429	42.429	37.1
14	56.902	57.003	40.8
15	24.451	24.512	28.1
16	28.400	29.078	34.4
17	56.184	56.080	42.5
18	12.007	12.193	50.6
19	19.549	19.549	47
20	36.294	40.660	150.5
21	18.925	21.227	30.6
22	34.078	138.467	38.8
23	26.186	129.395	29.8
24	45.964	51.384	16.2
25	29.273	32.040	16.1
26	19.974	21.366	15.4
27	19.173	19.126	14.8
28	23.199	25.564	18.4
29	12.129	12.410	109.8
30	-	-	19.5
31	-	-	179.8
32	-	-	20.9

The identification of compounds 1a and 1b was accomplished using a 125-MHz Agilent NMR Spectrometer with deuterated chloroform as the solvent. The NMR spectrum showed signals on the chemical shift 140.878, 138.467, 129.395 and 121.870 ppm as groups of alkene (C=C). Signal 71.954 ppm for –C–OH; 36.294 ppm for –CH–; 29.078 ppm for $–CH_2$; and 12.41 ppm for $–CH_3$. Comparison to previous research by De-Eknamkul clarified

that compound 1a and 1b were β-sitosterol and stigmasterol, respectively[23].

Compund 2 was identified using a 500-MHz Agilent NMR Spectrometer with deuterated chloroform as the solvent. The NMR spectrum showed 32 signals that represented the 32 carbons in compound 2. Signals on the chemical shift 68.6 and 79.1 ppm showed C=O bonding; 179.8 ppm for carbonyl (C=O); 109.4 and 150.8 ppm for C=C; there were 8 signals for the methyl group on chemical shift 14.8, 15.4, 16.1, 16.2, 18.4, 19.5, 20.9, 29.8 ppm; and 9 signals for the methylene group on chemical shift 18.4, 25.64, 27.5, 28.1, 30.6, 32.2, 34.4, 38.8 and 49.4 ppm.

Based on the number of carbons that appeared on the spectrum and the literature study, we hypothesized that compound 2 is a triterpene group. Comparison with the research of Rungsimakan and Rowan[24] suggested that compound 2 has a chemical shift number similar to those of most of the signals on the spectrum. Thus, we concluded that compound 2 is 3β-acetoxy-lup-20(29)-en-2α-ol.

The compounds found (1a, 1b and 2) were also tested for antioxidant activity using the DPPH free-radical scavenging assay. The compounds were less active in antioxidants with an inhibition rate for compounds below 30%. Compared to the methanolic extract of *S. ovata*, the inhibition rate of the compounds was significantly lower. We hypothesized that the high antioxidant activity of *S. ovata* is either due to the synergy of all compounds contained in the extract, or to other active compounds in the extract. Thus, in future research, we intend to isolate more compounds to clarify the chemical constituent of *S. ovata* extract that has antioxidant activity.

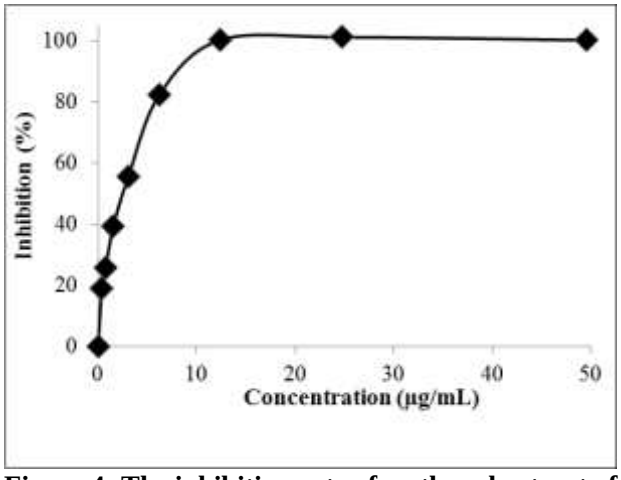

Figure 4: The inhibition rate of methanol extract of *S. ovata* using ABTS radical scavenging assay

Conclusion

The antioxidant activity of *S. ovata* Backer was determined. We confirmed the antioxidant activity extract of the stem bark of *S. ovata* and identified β-sitosterol, stigmasterol and 3β-acetoxy-lup-20(29)-en-2α-ol as three of the chemical constituents.

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