

3,3'-di-O-methylellagic acid, an Antioxidant Phenolics Compound from *Sonneratia alba* Bark

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ABSTRACT

Free radicals play an important role in some pathogenesis of serious diseases, such as neurodegenerative disorders, cancer, liver cirrhosis, cardiovascular diseases, atherosclerosis, cataracts, diabetes and inflammation. Compounds that can scavenge free radicals have great potential in ameliorating these diseases. It is reported that phenolic compounds in plants possess strong antioxidant activity and may help to protect cells against the oxidative damage caused by free radicals. Previous study revealed that mangrove trees, *Sonneratia alba* Bark showed strong antioxidant activity. Ethyl acetate fraction exhibited the best antioxidant performance. The antioxidant activity of this fraction was attributed to the presence of compounds of different polarities such as phenolics. Furthermore, the phenolic antioxidant of ethyl acetate fraction were purified and identified with UV, IR, MS, and 2D NMR spectrometry. 3,3'-di-O-methylellagic acid was found in brown amorphous powder. Antioxidant activity was evaluated and compared with L-(+)-ascorbic acid as standard by using DPPH assay. The compound has strong antioxidant activity than ascorbic acid standar with the value of IC₅₀ 11.35 and 17.64 µg/mL, respectively. The high value of antioxidant activity of compound indicate that *S. alba* is a potential source of natural antioxidant agent.

Keywords: DPPH, free radicals, mangrove, phenolics, scavenging activity

ABSTRAK

Radikal bebas berperan penting dalam berbagai penyebab penyakit berbahaya seperti kelainan *neurodegeneratif*, kanker, penyakit hati, kardiovaskuler, aterosklerosis, katarak, diabetes, dan inflamasi. Senyawa yang dapat menangkap radikal bebas memiliki potensi yang besar untuk mengatasi penyakit-penyakit tersebut. Telah dilaporkan bahwa senyawa fenolik dalam tumbuhan memiliki aktivitas antioksidan yang kuat dan dapat membantu untuk melindungi sel terhadap kerusakan oksidatif yang disebabkan oleh radikal bebas. Studi pendahuluan menunjukkan bahwa tumbuhan mangrove, *Sonneratia alba*, kulit batangnya memiliki aktivitas antioksidan yang kuat. Fraksi etil asetat menunjukkan aktivitas antioksidan yang paling kuat. Aktivitas fraksi ini menggambarkan adanya senyawa dengan polaritas yang berbeda seperti fenolik. Dalam penelitian ini diperoleh senyawa antioksidan fenolik dari fraksi etil asetat yang dipurifikasi dan diidentifikasi menggunakan spektrometri UV, IR, MS, dan NMR 2D. Asam 3,3'-di-O-metilellagat diperoleh dalam bentuk serbuk coklat. Aktivitas antioksidan dievaluasi dan dibandingkan dengan asam askorbat L-(+) sebagai standar dengan menggunakan metode DPPH. Senyawa fenolik tersebut memiliki aktivitas antioksidan yang kuat dibandingkan standar asam askorbat dengan nilai IC₅₀ berturut-turut sebesar 11,35 dan 17,64 µg/mL. Aktivitas antioksidan senyawa yang kuat tersebut menunjukkan bahwa *S. alba* merupakan sumber antioksidan alami yang potensial.

Kata Kunci: aktivitas tangkap, DPPH, fenolik, mangrove, radikal bebas

INTRODUCTION

There is an increasing interest in the role of free radical-mediated damage in the aetiology of human diseases. Free radicals formed during oxidation process occurring in various

products and biological systems are known to be responsible for oxidative deterioration, health damage and accelerated aging (Aruoma 2003). Consequently, antioxidants have become an essential part of preservation technology and contemporary health care. Synthetic antioxidants have been widely used in food and medicine. The most commonly used

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synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylated hydroxyquinone (TBHQ) (Lo liger 1991). However, BHA, and BHT were found to be anticarcinogenic as well as carcinogenic in experimental animals. Originally, BHA appeared to have tumor-initiating as well as tumor-promoting action. Recently, it has been established that tumor formation appears to involve only tumor promotion caused by BHA and BHT (Botterweck *et al.* 2000). The potential toxicity of some synthetic antioxidants, however, has intensified efforts to discover and utilise antioxidants from natural sources (Madsen & Bertelsen 1995; Nieto *et al.* 1993; Shahidi & Wanasundara 1992; Thamavit *et al.* 1985).

Sonneratia alba, one of mangrove species, has been used as preservative in production of alcoholic traditional beverage from palm trees. It has been reported that *S. alba* bark extract played an important role to prevent the formation of acetate acid generated from oxidation of ethanol (Firdaus & Sinda 2003). Furthermore, extract of this plant has been used as astringent, antiseptic, and to treat internal bleeding and disentry. We suggest that preservative property of this plant due to the presence of antioxidant or antibacterial compound (Bandarnayake 2002; Kumar *et al.* 2008). Therefore, this plant might be a good candidate for further development as a nutraceutical or for its antioxidant remedies. However, the potential compound of the bark extracts of *S. alba* as a source of natural antioxidant have not been studied in detail to date. Therefore, it is important to find out the compound that responsible for the extract activity.

The stable radical species 1,1-diphenyl-2-picrylhydrazyl (DPPH), has been widely used for antioxidant capacity screening and estimation due to its clear reaction mechanism, solvent compatibility and the technical simplicity of its assays which requires no special equipment (Chen *et al.* 1999; Cheng *et al.* 2006). The purple coloured of DPPH has a strong characteristic absorption at 515 nm and can undergo reactions with hydrogen donating antioxidant compounds to yield the stable yellow DPPH-H molecule easily monitored with Ultra Violet (UV) spectroscopy (Prior *et al.* 2005; Roginsky & Lissi 2005). In this study, several column chromatography (CC) were applied separate and purify the *S. alba* bark extracts; and their antioxidant activities were further evaluated by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay.

MATERIALS AND METHODS

Materials. All chemical and reagent were analytical grade purchased from Merck (Darmstadt-Germany). 1, 1-diphenyl-2-picryl hydrazyl (DPPH), L-(+)-ascorbic acid, hexane, chloroform, ethyl acetate, methanol, column Chromatography was carried out on Merck silica gel (200–400 mesh).

Sample preparing was carried out at Organic Chemistry laboratory of Hasanuddin University. The measuring of ¹H, ¹³C, EI-MS, UV, and IR spectra was done at LIPI Serpong Banten. Antioxidant activity was determined at Uji Biofarmaka Laboratory of LPPM IPB Bogor.

Extraction of Bark. Dried and powdered bark of *S. alba* (10 kg) were extracted with methanol. After filtration, the combined extracts were evaporated to dryness to produce the methanolic extract (183 g). The methanolic extract was dissolved in a mixture of methanol:water (2:3, v/v) at room temperature and partitioned successively with hexane, chloroform and ethyl acetate, produced hexane (23 g), chloroform (49 g) and ethyl acetate (65 g) extracts, respectively. The extracts were concentrated under vacuum. EtAOc showed the highest antioxidant activity, so only this fraction would be continued for further analysis (Figure 1).

The ethyl acetate extract was evaporated in vacuo to give 65 g residue. The residue was subjected to (CC) on silica gel column, eluted with hexane:ethyl acetate (2:10–5:10) to give five fractions. Antioxidant activity assay showed the IC₅₀ values of those fraction as E (15.12 µg/mL) > fraksi D (15.47 µg/mL) > fraksi C (16.65 µg/mL) > fraksi B (60.88 µg/mL) > fraksi A (113.95 µg/mL). Fraction C and D were rechromatography on silica gel column eluted with hexane:ethyl acetate (1:7–5:7) to give compound 1 (13.25 mg).

DPPH Radical Scavenging Assay. The reaction was performed in 3 mL of methanol containing freshly prepared 1,1-diphenylpicrylhydrazyl (DPPH) and 1 mL of fractions/compounds. Reaction mixtures were protected from light and incubated for 90 min at room temperature, after which the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The scavenging activity of compound was measured as the decrease in absorbance of the DPPH expressed as a percentage of the absorbance of a control DPPH solution without compound (Chen *et al.* 2000). All analyses were carried out in triplicate.

RESULT AND DISCUSSION

Isolation and Identification of Phenolic Antioxidants from the Bark of *S. alba*. The phenolic antioxidants of EtOAc fraction (Figure 1) were purified and identified by using IR, UV, MS, and 2D NMR spectrometry. Compound 1 was obtained as brown amorphous powder from ethyl acetate, decomposed at 325°C.

¹H-NMR spectrum shows three proton signal, first signal at ¹H 10.75 ppm which support the present of hydroxyl group, second signal is at ¹H 7.5 ppm identified as protons in the aromatic system and strengthen the structure of the phenolic substituted because it is the only aromatic protons, and the last signal is at ¹H 4.0 ppm correspond to the protons on the methoxy group.

MS spectrum shows some peak at m/z 279, 330, 331, dan 305 derived from some fragmentation pattern of acid 3,3'-di-O-metilelagat obtained as the incorporation of molecular compounds or fragments with sodium ions (Na), potassium (K), or molecules of H₂O, and CH₃OH. It is common in molecules with ester or ether groups, the ions are derived from the solvent used.

The results of ¹³C-NMR spectrometer (125 MHz, DMSO) analysis shows that there are 8 chemical shifts of carbon atoms in a compound with different environments at ¹³C 61.0; 111.7; 141.3; 140.3; 152.3; 111.5; 112.2; and

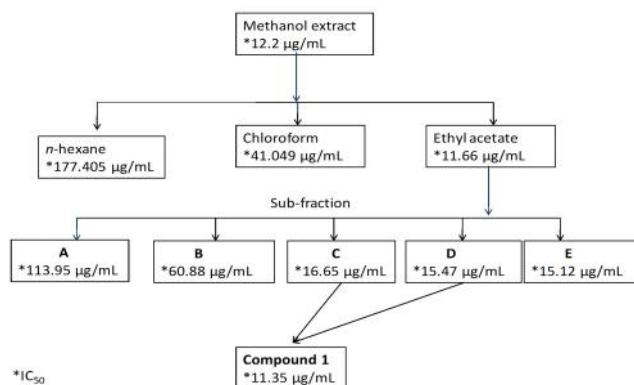


Figure 1 Extraction, fractionation and column chromatography separation of *S. alba* Bark

158.6 ppm (Table 1). Signals with chemical shift ¹³C 111.5; 111.7; and 112.2 ppm is double bond carbon signal in aromatic system. Based on those chemical shifts, it determined that the hydroxyl groups attached to the carbon at ¹³C 152.3 ppm, while the methoxy group attached to the carbon with ¹³C 141.3 ppm. The number of aromatic carbon atoms as 6 indicates a benzene unit in the compound. Chemical shift ¹³C 158.6 ppm predictable as carbonyl carbon in the cyclic ester or lactone group, where its chemical shift is lower than other types of carbonyl carbon. The data are consistent with the DEPT 135 spectrum showing the presence of the methyl carbon, a carbon metin, and six tertiary carbon. The presence of the unit benzene and lactone groups and fact that the number of carbon atoms only 8 leads to a symmetrical molecule structure.

Data from HMQC spectrum showed that the proton at ¹H 7.5 ppm attached to the carbon with ¹³C 111.5 ppm, while the protons at ¹H 4.0 ppm attached to the carbon with ¹³C 61.0 ppm (Figure 2). HMBC spectrum showed a correlation between the proton at ¹H 7.5 ppm with the carbon atom at ¹³C 112.2, 140.3, 152.3, and 158.6 ppm. Protons at ¹H 4.0 ppm correlated with a carbon atom at ¹³C 140.3 ppm.

Based on the value of the ¹³C-NMR spectra and DEPT 135, it was determined the position of the carbon atom and hydrogen as follows: ¹³C 61.0 ppm (2 x OCH₃), 111.6 ppm (C-1, C-1'), 141.2 ppm (C-2, C-2'), 140.2 ppm (C-3, C-3'), 152.2 ppm (C-4, C-4'), 111.4 ppm (C-5, C-5'), 112.1 ppm (C-6, C-6'), 158.5 ppm (2x C = O), for the hydrogen atom as follows: ¹H 10.75 ppm (Ar-OH), 7.5 ppm (H-5, H-5'), and 4, 0 ppm

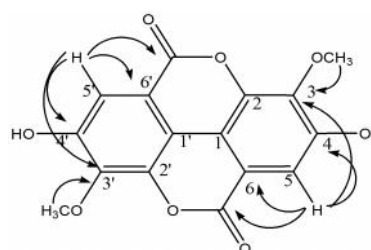


Figure 2 Long distance correlation between proton and carbon atom in 3,3'-di-O-methylellagic acid (HMBC spectra)

Table 1 Data of NMR. ¹H (500MHz) and ¹³C (125MHz) obtained compound and reference compound

¹³ C-NMR obtained compound	¹ H-NMR obtained compound	¹³ C-NMR reference compound (Tian <i>et al.</i> 2009)	¹ H-NMR Tian <i>et al.</i> (2009)
111.6	7.50 (2H,s)	111.5	7.50 (2H,s)
141.2		141.1	
140.2		140.2	
152.2		152.2	
111.4		111.4	
112.1		112.0	
158.5		158.4	
61.0	4.0 (6H,s,OCH ₃) 10.75 (OH)	60.9	4.04 (6H,s,OCH ₃) -

(2x OCH₃). Spectroscopic data analysis was agreed well with the data reported by Tian *et al.* (2009) (Table 2). Thus, compound 1 was identified as 3,3'-di-O-methylellagic acid (Figure 2).

IR spectrum of compound 1 showed an absorption in the region above 3000 cm⁻¹ (3232 cm⁻¹) derived from unsaturated CH bond. The presence of absorption at 1612 cm⁻¹ support that CH is derived from unsaturated aromatic group. Strong absorptions at 1730 cm⁻¹ indicates that the compound contains a carbonyl group (C = O), and the absorption is specific for lactone (cyclic ester). Broader absorption at 3277 cm⁻¹ indicates that the compound contained an OH group, this absorption is too low for the range of NH groups. The presence of the methyl group is characterized by the presence of absorption in the region near 1385 cm⁻¹ and also at 1354 cm⁻¹. The existence of more than one absorption in the region 1000-1300 cm⁻¹ states that there are many type C-O bonding. UV spectrum (methanol) shows a maximum absorption at 249 nm (methanol). This data is consistent with the molecular structure of obtained compounds that indicate the presence of substituted aromatic systems.

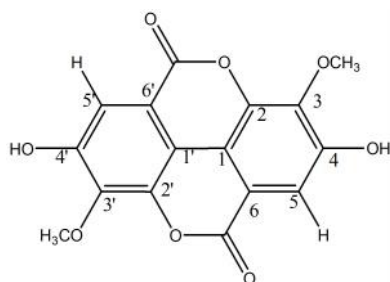


Figure 3 Phenolic compounds isolated from *S. alba* Bark 3,3'-di-O-methylellagic acid

3,3'-di-O-methylellagic acid is not a new compound. Tian *et al.* (2009) has reported several compounds from *S. caseolaris* involve 3,3'-di-O-methylellagic acid. It leads that this compound may attributed compound to the family of mangrove trees, Sonneraceae.

IC₅₀ values of extract, fractions, compound from *S. alba* Bark, and control were determined from linier regression as shown in Tabel 1. 3,3'-di-O- methylellagete acid, and L-(+)-ascorbic acid showed concentration dependence-activity until reach the maximum (Figure 3). Maximum activity is reached at concentration 34 µg/mL for 3,3'-di-O- methylellagic acid.

The DPPH assay, which measures the ability of compounds to transfer labile H-atoms to radicals, is the commonest method of antioxidant activity evaluations (Brand-Williams *et al.* 1995). The abstraction of hydrogen by this stable free radical is known to lead to the bleaching of the maximum absorption 517 nm and can easily be monitored spectrophotometrically. The synthetic nitrogen-centered DPPH radical is not biologically relevant but DPPH assay is often used to evaluate the ability of antioxidant to scavenge free radicals which are known to be a major factor

Table 2 The DPPH scavenging activities of methanolic extracts, fractions and isolated Compound from the Bark of *S. alba* against DPPH free radical

Specimens	IC ₅₀ (µg/mL)
Methanol extract	12.20
<i>n</i> -Hexane fraction	177.05
Chloroform fraction	41.09
Ethyl acetate fraction	11.66
3,3'-di-O-methylellagete acid	11.35
L-(+)-ascorbic acid	17.64

All the antioxidant measurements were performed in triplicate, and the data were expressed as average

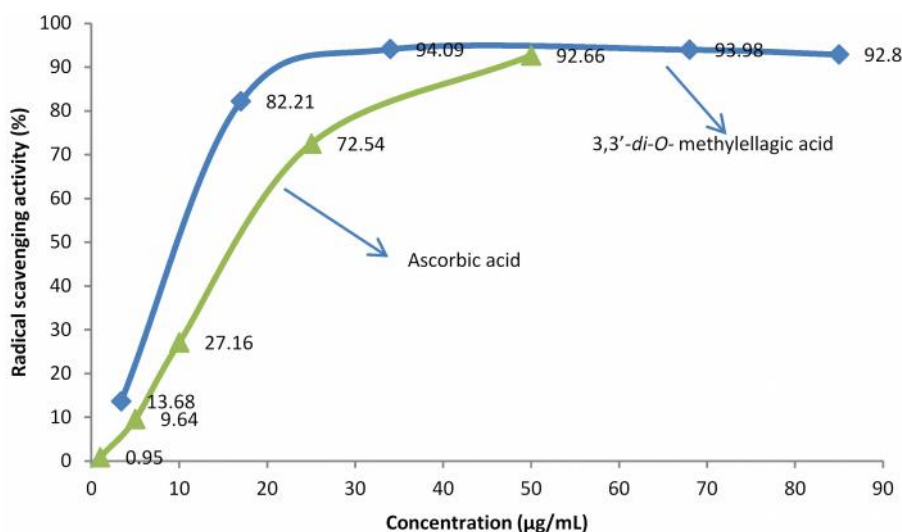


Figure 4 Free-radical scavenging activity of 3,3'-di-O-methylellagete acid and L-(+)-ascorbic acid measured by the DPPH

in biological damages caused by oxidative stress. This assay is known to give reliable information concerning the antioxidant ability of the tested compounds (Huang *et al.* 2005).

As shown in Table 2, compounds 3,3'-di-O-methylellagat acid showed significant free radical scavenging activity with IC₅₀ values of 11.35 µg/mL which was much more active than L-(+)-ascorbic acid as a positive control. Based on the results obtained from this established assay system, it appears that the compound showed a potent and concentration dependent free radical scavenging effect (Figure 4).

The results in this study reveal that phenolic compounds from *S. alba* Bark showed strong antioxidant. These findings may provide an explanation for some of its uses in folk medicine and preservative property.

CONCLUSION

The 3,3'-di-O-methylellagat acid is major phenolic antioxidant compound in *S. alba* Bark. It has a very strong antioxidant activity with IC₅₀ value of 11.35 µg/mL. The high antioxidant activity of that compound indicate that *S. alba* is potential source of natural antioxidant agent.

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