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Various antioxidant assays of agarwood extracts (*Gyrinops versteegii*) from West Lombok, West Nusa Tenggara, Indonesia

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Abstract. *Prihantini AI, Rizqiani K. 2019. Various antioxidant assays of agarwood extracts (*Gyrinops versteegii*) from West Lombok, West Nusa Tenggara, Indonesia. Asian J Agric 3: 1-5.* Agarwood extracts (Gyrinops versteegii) have not been widely explored as a source of natural products in particular antioxidant agents, which protect cells from damage caused by free radicals. The present study was aimed to evaluate antioxidant activities of agarwood extracts from West Nusa Tenggara using various antioxidant assays. The antioxidant activity of leaf, fruit and fruit bark extracts was investigated based on DPPH radicals scavenging activity, reducing power, and β-carotene bleaching assays. The total phenolic content was also investigated. The result showed that leaf extract revealed the strongest antioxidant activity on all assays performed such as DPPH radicals scavenging activity (IC_{50} 22.13±0.71 μg/mL); reducing power (251.85±0.03 mg QE/g dry extract); and β-carotene bleaching activity (IC_{50} 24.23±2.60 μg/mL). The total phenolic content (TPC) in the leaf was higher (184.90±0.76 mg GAE/g dry extract) than fruit bark and bark extracts. The high content of phenolic compounds in *G. versteegii* leaves indicated that these compounds might contribute to the antioxidant activities. In conclusion, these findings showed that *G. versteegii* leaves are potential for development as an antioxidant source.

Keywords: Agarwood, antioxidant, *Gyrinops versteegii*, West Nusa Tenggara

INTRODUCTION

Free radicals have an important role in our health. Reactive Oxygen Species (ROS) which are generated from oxygen, can lead to oxidative stress with the affection of various biological functions and structural changes during metabolism or other activities if it is increased and imbalanced between producing radical and antioxidant (Nimse and Pal 2015; Ramamoorthy and Bono 2007; Zima et al. 2001). This oxidative stress can cause a disturbance in numerous physiological processes and development to many degenerative disorders, such as cancer, cardiovascular, Alzheimer disease, neurodegenerative disease, asthma, autoimmune and gastrointestinal diseases, and the aging process (Siti et al. 2015; Arituluk et al. 2016; Wojtunik-Kulesza 2016).

The radicals can be overcome by antioxidants. Antioxidants are compounds that can inhibit ROS, reactive nitrogen species and other free radicals to prevent damage in normal cells, proteins and fats which ultimately prevent degenerative diseases (Arif et al. 2014). Presently, synthetic antioxidants commonly used such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) might affect genotoxic, carcinogenic effects, and hemorrhaging (Babbar et al. 2010; Salamah et al. 2011; Stankovic 2014). Due to this reason, natural antioxidant sources might be found to substitute synthetic antioxidants.

Agarwood is considered precious plant in the world, particularly in Asia, due to its high value of resin impregnated-heartwood from the family of *Thymelaeaceae* (Ismail et al. 2015). In Indonesia, agarwood is generally grown in Borneo (12 species), Sumatra (10 species), Nusa Tenggara (3 species), Papua (2 species), Sulawesi (2 species), Java (2 species), and Moluccas (1 species). The common main genus of agarwood in Indonesia are *Aquilaria malaccensis*, *A. microcarpa*, *A. beccariana*, *A. hirta*, *A. filaria*, *A. cumingiana* and *Gyrinops* (Hadi et al. 2011; Santoso et al. 2014). *Gyrinops versteegii* (Gilg.) Domke is an endemic agarwood that is in Lombok, West Nusa Tenggara Province. Most people in this area use it for resin production. The inoculation of fungi into the agarwood is conducted at the optimal age of the tree to produce optimal yield of resin. During waiting for the harvest of agarwood, certain communities use the agarwood leaves as a herbal tea which is believed that it is effective to cure various diseases including malaria, tumor, fatigue fever and flu symptoms (Nuringtyas et al. 2018). Mega and Swastini (2010) proposed that agarwood leaf extract contains secondary metabolites such as flavonoids, terpenoids and phenol compounds. These secondary metabolites were suspected to have antioxidant activities. Therefore, the aim of this study is to evaluate the antioxidant activities from leaves, fruits, and fruit barks of *G. versteegii* using various antioxidant assays.

MATERIALS AND METHODS

Extract preparation

Fresh and healthy leaves, fruits, and fruit barks of *G. versteegii* were dried at room temperature and blended. Approximately 20 g of the dried materials were extracted with methanol. The methanol extracts were then filtered and concentrated under a vacuum rotary evaporator and dried at room temperature prior to antioxidant assays.

DPPH radical scavenging activity assay

DPPH free radicals scavenging activity was evaluated according to Prihantini et al. (2014). Various concentrations of each sample were mixed with 0.5 mL of the 1 mM DPPH radical solution in methanol. For the control, a similar solution with the absence of sample was used. Samples were then incubated at room temperature under dark conditions for about 30 min. Absorbance (A) was measured with a spectrophotometer at 517 nm. The percentage of scavenging activity was determined by the following equation:

$$\text{Scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100\%$$

β-carotene bleaching assay

The β-carotene bleaching assay was performed according to Prihantini et al. (2014). Approximately 0.2 mg of β-carotene, 20 mg of linoleic acid and 200 mg of Tween40 were mixed in 0.5 mL chloroform, which was then removed using a rotary evaporator at 40°C. The resulting mixture was diluted with distilled water and mixed vigorously. The mixtures were made up to 50 mL and aliquots (4.8 mL) were added to test tubes containing various concentrations of each sample and methanol (control). A similar mixture without β-carotene was used for the background of the samples. The tubes were incubated at 50°C for 2 h. The absorbance at 470 nm was measured at 0 min either for the control (A_{c0}) or samples (A_{s0}). The absorbance of both the control (A_{c120}) and samples (A_{s120}) was measured after 120 min incubation. Furthermore, the antioxidant activity was evaluated with the following equation:

$$\text{Antioxidant activity (\%)} = 100[1-(A_{c0}-A_{s0})/(A_{c120}- A_{s120})]$$

Reducing power assay

The reducing power assay was performed according to Prihantini et al. (2014). Approximately 0.5 mL of various concentrations of samples in methanol was mixed with phosphate buffer (2.5mL, 0.2 M, pH 6.6) and potassium ferry-cyanide (2.5 mL, 1%). Trichloroacetic acid (2.5 mL, 10%) was added to the mixture after 20 min incubation at 50°C. Then, the mixture was centrifuged at 3000 rpm for 10 min. The upper layer solution (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride solution (0.5 mL, 0.1%). Absorbance was measured with a spectrophotometer at 700 nm. The reducing power assay was measured as quercetin equivalent.

Total phenolic content

The total phenolic content (TPC) of plant extracts was determined using Folin-Ciocalteu reagent (Prihantini et al. 2014). Approximately 500 μL of the extracts (1.0 mg mL^{-1}) was added with distilled water, made up to 8 mL and then mixed with 500 μL of 2 N Folin-Ciocalteu reagents. The mixture was allowed to stand for 8 min and 1.5 mL of 20% sodium carbonate was then added. The reaction mixture was incubated at room temperature for 2 h. Absorbance was measured at 765 nm and the phenolic content was determined using a calibration curve obtained from concentration of gallic acid.

Statistical analysis

All assays were performed in triplicate in independent three experiments. The data were expressed as the Mean+S.D value and analyzed by SPSS for windows followed by Tukey's post hoc test. Values with $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

There are increasing focuses on products extracted from nature to treat many human diseases. Antioxidant helps our body in prevention and healing process of oxidative stress caused by free radicals. A natural antioxidant can be extracted from plants (Chirag et al. 2013; Krishnaiah et al. 2011; Prihantini et al. 2015). However, due to the complex nature of phytochemicals, various antioxidant assays are necessary to evaluate antioxidant activity. Furthermore, total phenolic contents assay is also commonly used to complement antioxidant assays.

DPPH radicals scavenging activity

DPPH radicals scavenging assay is a common method to evaluate antioxidant activity of nature. The DPPH assay reflects a scavenging reaction between DPPH and the related samples. DPPH is a stable free radical, which turns from purple to yellow when it is scavenged by antioxidants as hydrogen donors. The delocalization of the DPPH radicals determines the occurrence of a purple color. When DPPH radical (2,2-diphenyl-1-picrylhydrazyl) reacts with a hydrogen donor, the stable molecule (2,2-diphenyl-1-hydrazine) is generated resulting in the discoloration of the purple color (Pisoschi and Negulescu 2011). The degree of discoloration indicates the scavenging potential hydrogen donating ability of the related samples.

The study revealed that leaf extract of *G. versteegii* showed the highest activity than others with IC_{50} 22.13±0.71 μg/ml followed by fruit bark (76.87±3.64 μg/ml) and bark extracts (119.49±3.84 μg/ml), and it is significantly different activities ($p<0.05$) among the extracts (Table 1). The result indicates that leaf extract has the most potential ability on hydrogen donation. The higher activity of leaf extract was also reported by Mahdi-Pour et al. (2012).

Table 1. DPPH radicals scavenging activity and β-carotene bleaching activity of *G. versteegii*

Extracts	IC_{50} on DPPH radicals scavenging activity (µg/ml)	IC_{50} on β-Carotene bleaching activity (µg/mL)
Leaf	22.13±0.71[b]	24.23±2.60[a]
Bark	119.49±3.84[d]	104.97±0.1[c]
Fruit bark	76.87±3.64[c]	35.18±1.27[b]
Quercetin*	7.4±0.1[a]	n.a

Note: Data is expressed as Mean±S.D values, different letters in the same column indicate significant differences ($p<0.05$) at Tukey's post hoc test, n.a: Not available, *: Prihantini et al. (2014)

Table 2. Reducing power of *G. versteegii*

Extracts	Reducing power (mg QE/g dry extract)
Leaf	233.89±14.14[c]
Bark	99.44±5.50[a]
Fruit bark	122.78±7.38[b]

Note: Data is expressed as Mean±S.D values, different letters in the same column indicate significant differences ($p<0.05$) at Tukey's post hoc test, QE: Quercetin equivalent

However, in some cases, the assay gives incorrect results and recommendations. Some complications could be caused by partial ionization of the related samples, which affect the rate of their reaction with DPPH radicals (Tirzitis and Bartosz 2010). Therefore, other antioxidant assays are necessary to confirm and consider the recommendations.

β-carotene bleaching activity

β-carotene bleaching assay is another assay to evaluate antioxidant activity of nature. It performs discoloration in the absence of an antioxidant compound. Therefore, the presences of antioxidant molecules inhibit the extent of β-carotene bleaching caused by linoleic-free radicals. The radicals may attack the double bonds of β-carotene and cause discoloration. Antioxidants reduce the discoloration of β-carotene by stabilizing the linoleic-free radical and other radicals formed in the system (Jayaprakasha et al. 2001).

β-carotene bleaching activity of leaf, bark, and fruit bark extracts of *G. versteegii* is shown in Table 1. As DPPH radical scavenging activity, the leaf extract had the highest activity (IC_{50} 24.23±2.60 µg/mL) compared to other extracts. The result showed that the activity was statistically significantly different with fruit bark and bark at IC_{50} 35.18±1.27 and 104.97±0.1 µg/mL, respectively. It indicates that leaf extract compounds had high capability of neutralizing the free radicals generated in the system. It is known that β-carotene assay has a different mechanism involved in the method compared with DPPH assay (Prihantini and Tachibana 2017). However, the present study results in a similar order of the antioxidant activity. Therefore, the result of β-carotene bleaching activity supports the potential order of antioxidant activity as leaf > fruit bark > bark as the result of DPPH radicals scavenging activity.

Reducing power

Reducing power may serve as a significant indicator of the potential antioxidant activity. Compounds with reducing power indicate that they are electron donors. The presence of antioxidants causes the conversion of ferricyanide (Fe^{3+}) complex in the system to ferrocyanide (Fe^{2+}) by donating their electrons. The ferrocyanide then reacts with ferric chloride to form ferric ferrous complex (Pearl's Prussian blue). By measuring the formation of Pearl's Prussian blue at 700 nm, it is possible to determine the concentration of Fe^{3+} ion electrons (Jayanthi and Lalitha 2011). The result of reducing power of *G. versteegii* is shown in Table 2. The reducing power activity is shown as quercetin equivalent (QE). The higher quercetin equivalent indicates the higher reducing power of the extract. Extracts showing high reducing power activity indicate an enhanced capability to donate electrons.

The reducing power assay revealed leaf extract with the highest value, followed by fruit bark, and bark extracts (233.89±14.14; 122.78±7.38; 99.44±5.50 mg QE/g dry extract, respectively). A similar trend as DPPH radical scavenging activity and β-carotene bleaching activity, resulted in statistically significantly different at $P < 0.05$ among the extracts. Therefore, the result suggested that leaf extract of *G. versteegii* had high capability to donate electrons. Furthermore, the reducing ability was found to be concentration-dependent in which the reducing power increased with the increasing of the concentrations as shown in Figure 1. Higher absorbance of the reaction mixture indicates higher reduction ability (Jayanthi and Lalitha 2011).

Total phenolic content

Most phenolic compounds consist in the plant. Phenolic compounds are considered to play a role against a wide range of diseases (Ibrahim et al. 2012). The total phenolic content of *G. versteegii* is shown in Table 3. The results were expressed in gallic acid equivalent.

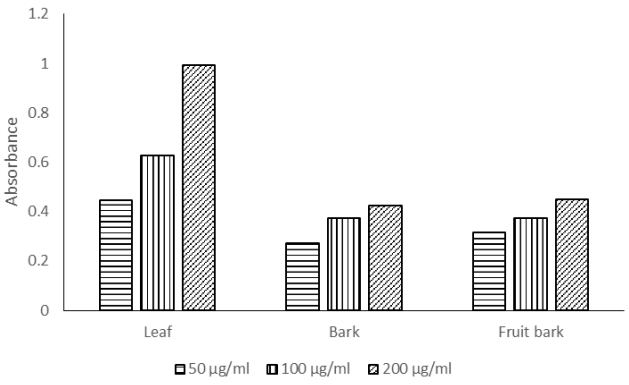

Figure 1. Reducing power assay of *G. versteegii* at different concentrations

Table 3. Total phenolic content of *G. versteegii*

Extracts	Total phenolic content (mg GAE/g dry extract)
Leaf	184.90±0.76[c]
Bark	67.65±0.23[a]
Fruit bark	113.02±0.38[b]

Note: Data is expressed as Mean±S.D values, different letters in the same column indicate significant differences ($p<0.05$) at Tukey's post hoc test, GAE: Gallic acid equivalent

The results showed that leaf extract had the highest total phenolic content, followed by fruit bark and bark extracts (184.90±0.76; 113.02±0.38; 67.65±0.23 mg GAE/g dry extract, respectively). It was statistically significant difference among the extracts at $P < 0.05$. The highest total phenolic content of leaf extract might be assumed that phenolic compounds present in the extract, gave contribution to the antioxidant activity in DPPH radicals scavenging assay, reducing power, and β-carotene bleaching assay. Phenolics can donate their electrons to stabilize radicals (Chanda and Dave, 2009; Seal, 2012). Furthermore, the donation of a hydrogen atom that stabilizes the free radicals is considered from phenolic hydroxyl groups (Prihantini et al. 2015). Other studies also reported that the phenolic hydroxyl group is excellent to scavenge free radicals (Jeong et al. 2007; Moalin et al. 2011). The electron donation is without resulting themselves reactive radicals, therefore suggesting that phenolics are considered as a good antioxidant. Furthermore, revealing a similar order of extract having the potencies in all assayed performed, the result is consistent with the findings of several studies in reporting correlation between antioxidant activity and total phenolic content. In conclusion, all the antioxidant assays investigated in the study revealed that leaf extract had the highest antioxidant activity. This indicates that *G. versteegii* leaves have potential to be developed as natural antioxidant sources.

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