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The Influence Of Leaf Age On Total Phenolic, Flavonoids, And Free Radical Scavenging Capacity Of *Aquilaria beccariana*

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ABSTRACT

Each leaf had a different level of exposure to environmental conditions and may affect the distribution of phytochemicals and antioxidant capacity in *Aquilaria beccariana*. The aims of this study were to compare total phenolic, flavonoids, and free radical scavenging of *A. beccariana* leaves at different stages of maturity based on its position in a twig. Leaves at different positions from apex to base in a twig represented gradient increase in the leaf age (young, mature, and old). The powder of dried *A. beccariana leaves* were macerated using 70% ethanol. Levels of total phenolic were determined by Folin-ciocalteau reagent, total flavonoids assay used $FeCl_3$ reagent, and the antioxidant activity was analyzed using 2,2-diphenyl-1-picrylhydrazyl (DPPH). The results showed that the level of phenolic and flavonoids in mature leaves of *A. beccariana* was higher than the young and old leaves. Level of phenolic in the young and old leaves was higher than the flavonoids, while the level of phenolic and flavonoids in mature leaves was not significantly different. The mature leaf of *A. beccariana* was the most potential as an antioxidant with an IC_{50} value of 72.25±0.72 ppm. This study revealed that leaf age influence the level of secondary metabolites and their antioxidant properties.

Keywords: *Aquilaria beccariana*; leaf position; flavonoids; phenolic; radical scavenging

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INTRODUCTION

Fungal disease infections attack on the agarwood-producing plant of Thymeleaceae family produce fragrant resin known as agarwood, aloeswood, Eaglewood, aloes, kalamabak or oudh depending on the region [1]. In Indonesia, this fragrant resin called gaharu. Products of gaharu has been known since the 3rd century for a religious ritual in China, perfume binder, cosmetic, aromatherapy, and medicine for human health. There are more than 26 species of agarwood-producing trees of the genus Aquilaria, Gyrinops, Aetoxylon, Wikstroemia [2]. *Aquilaria beccariana* is one plant that produce agarwood from Aquilaria genus.

The use of agarwood is not limited to the resin alone. Overall plant parts such as leaves, stem bark, fruit, seeds, and roots of aloes have potential medicinal properties [3]. Several studies have shown activity of this plant as antioxidant [2] [3], analgesic, antipyretic, anti-inflammatory [4], antihyperglycemic [5], and antimicrobial [3]. In the Province of Central Kalimantan, Indonesia, agarwood leaves empirically used to lower blood glucose levels. A phytochemical study revealed that agarwood leaf contained alkaloids, saponins, phenolic, flavonoid and terpenoids [2]. Phenolic and flavonoids secondary metabolites in plant are responsible for antioxidant activity [6] [7]. Phenolic characterized with presence of phenol group, while flavonoid is a largest group of phenolic compounds in nature with C_6-C_3-C_6 configuration [8].

Agarwood leaves commonly used by public without considering the age of leaves. Leaf buds of plant of tea are used because they contain the highest phenolic compounds. In most plants mature leaves were used because it contains more than 90% vacuole in cells. The vacuole is a place to store organic and inorganic materials. In the old leaves, the activities of leaf decrease because the aging process in leaves can reduce the content of secondary metabolites [9]. Based on this, supposedly there are variations in the levels of secondary metabolites on leaf based on its position in a twig. Variations can affect the levels of antioxidant activity, thus affecting the pharmacological effects.

Several studies have demonstrated an association between phenolic, flavonoids content and antioxidant activity. Content of phenols and flavonoids in three plants is directly proportional to the antioxidant activity [10]. This study was focused on the total phenolic, flavonoid content and antioxidant activity of *A. beccariana* leaves extract on different leaf position in a twig. The purpose of this study was to determine the levels of total flavonoids, total phenolic and antioxidant activity from young leaves, mature leaves and old leaves in *A. beccariana*.

MATERIALS AND METHODS

Plant materials:

Aquilaria beccariana leaves were obtained from a farm land located in Barabai, South Kalimantan, Indonesia in April 2016. The leaves were grouped [11] into three (Fig. 1) based on their position on the one twig that indicated the increasing age of the leaves (from apex to base): young leaves (first 3 leaves of apex), mature leaves (4th leaves from apex to 5 leaves before basal), and old leaves (4 last leaves of base).

Chemical materials:

Quercetin, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), Gallic acid (GA), and Na_2CO_3 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Folin–Ciocalteau's reagent, methanol, and acetic acid were purchased from E. Merck, Darmstadt, Germany. All other chemical reagents used were of analytical grade..

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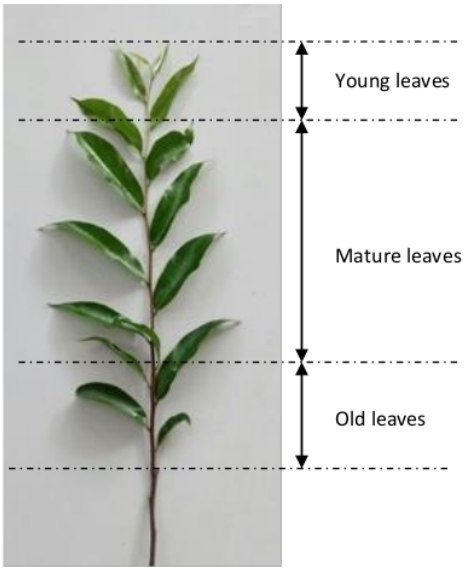

Figure 1. Sampling of leaves in three different age group from the apex to the base on one twig of *Aquilaria beccariana*

Methods:

Preparation of ethanolic extract:

Leaves of *A. beccariana* were air-dried without direct sun lighting for 2 days after washing with water. Before powdered using a blender, leaves were dried using the oven at 60° C for 2 hours. Ethanolic extract was obtained by macerating dried powder of *A. beccariana* leaves using ethanol 70% for 24 hours. Maceration was done two times. All filtrate was evaporated to dryness using vacuum rotary evaporator and water bath at 60°C [12].

Estimation of total phenolic content:

Determination of total phenolic content was done using the Folin-Ciocalteau reagent based on method [13]. A total of 0.5 ml sample was added with 0.75 ml of Folin-Ciocalteau reagent 10% and 2.0 mL Na_2CO_3 2%. The absorbance of the mixture was measured at 771 nm wavelength after 15 min incubation. The standard curve of gallic acid with a concentration series 0.010; 0.020; 0.030; 0.040; and 0.050 mg/ml was used as a standard. Total phenolic levels expressed in mg of Gallic acid equivalents (GAE)/g extract.

Estimation of total flavonoids content:

Total flavonoids content was determined by the method [14] [15]. A total of 1.0 ml of the sample in ethanol was added with 1.0 ml of $AlCl_3$ 2% and 8.0 ml of glacial acetic acid5 %. After incubation for 10 min, the absorbance was measured at 416 nm wavelength. The standard curve of quercetin with a concentration series 0040; 0.060; 0.080; 0.100; and 0.120 mg/ml was used as a standard. Levels of total flavonoids expressed in mg quercetin equivalent (QE)/g extract.

DPPH assay:

A. beccariana leaves extract was dissolved in methanol to obtained concentration series 0.020, 0.030, 0.040, 0.050, and 0.060 mg/ml. A total of 1.0 ml of the sample extract was added to 1.0 ml of DPPH (0.4 mM) and methanol 4.0 ml. Before absorbance was measured at a wavelength of 517 nm, the mixture incubated for

30 min at room temperature [16]. Standard quercetin used as a comparison with a concentration of 5; 7.5; 10; 1.5 and 15 mg/ml. Percent inhibition is calculated using the formula:

DPPH antioxidant activity (%) = [1- (Abs. sample / Abs. control] x 100

Data Analysis

Data were expressed as Mean ± SD. Statistical analysis was performed by SPSS 21.0 One-way analysis of variance (ANOVA) was utilized to evaluate differences.

RESULTS AND DISCUSSION

Results and Discussion

Total phenolic and flavonoids content

Total phenolic and flavonoids levels content of ethanolic extract of *A. beccariana* leaves was measured in the position I (young leaves), II (mature leaves), and III (old leaves) (Table 1). Folin-Ciocalteau reagent form a dark blue complex solution when treated with a solution containing phenolic compounds in alkaline conditions [17]. Phenolic expressed as Gallic acid equivalent (µg/mg). The mature leaf extract showed the highest total phenol content (60.69±0.44 µg/mg). Chemical structure of phenol compounds determined levels of total phenols. Phenol compounds with lot of hydroxyl functional groups or in the free state (aglycone) will produce high levels of total phenols [18]. Phenol compounds are generally different for each position (age) of leaves.

Determination of total flavonoid levels done with colorimetric methods that based on the formation of color complex from $AlCl_3$ with the group ortho dihydroxy and hydroxy ketones in flavonoids [19]. Levels of total flavonoids were expressed as quercetin equivalent (µg/mg). The results showed that the highest levels of flavonoids was in mature leaves (59.38±2.72 µg/mg).

Table 2. Result of total phenolics, flavonoids contents and antioxidant activities in leaves from different position in twig of *Aquilaria beccariana*

Leaf Position	Phenolics (µg/mg)	Flavonoids (µg/mg)	IC 50 (ppm)
I (young leaf)	3.03±0.19	9.7±0.26	381.1±23.27
II (mature leaf)	60.69±0.44	59.38±2.72	72.25±0.72
III (old leaf)	7.86±0.12	30.95±1.11	106.75±4.92

The results showed that phenolic levels in young and old leaves are higher than the levels of flavonoids. In mature leaves, phenolic and flavonoid levels were not significantly different. Phenolic and flavonoid levels increased rapidly from the position I (young leaves) to position II (mature leaves). However, from the position II (mature leaves) to position III (old leaves) levels of phenolic and flavonoid was reduced. Variation of phenolic and flavonoid content of the ethanolic extract of *A. beccariana* leaves were due to differing developmental age of leaves that occurs in the different leaves location [11]. Mature leaves have the optimum ability to produce secondary metabolites, so the levels of phenolic and flavonoids was large. At young leaf, secondary metabolites are still not produced in large quantities, while secondary metabolites content of the older leaves generally decreased. Beside this, seasonal, genetic, and agronomic factors also affect the level of phenolics and flavonoids content [20] [21].

DPPH free radical scavenging activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) method has the advantage of being simple, easy, fast and requires little sampel. The principle of this method is the presence of the hydrogen atoms donor of the sample to the DPPH radical, thereby becoming non radical compound [16]. Antioxidant compounds reduce the intensity of the purple color of the DPPH due to the reaction between diphenyl picrylhydrazyl radical

molecules with one hydrogen atom released by the sample. This led to the formation of diphenyl picrylhydrazine compounds, causing a color change from purple to yellow [16] [22].

In this study, the extract of *A. beccariana* mature leaves have the most powerful antioxidant activity (IC_{50} 72.25±0.72 ppm). Low IC_{50} value of extracts indicates the ability to inhibit 50% of free radicals in low extract concentrations. Antioxidant activity is proportional to phenol and flavonoid content level from previous results [1] [3] [11]. Mature leaves with the highest levels of total phenolic and flavonoids, have the most powerful antioxidants too. The young leaf that had lowest total phenols and flavonoids levels, it has the lowest antioxidant activity. Based on these results it can be stated that the phenolic and flavonoids compounds contained in ethanolic extracts of *A. beccariana* leaves contributing to the antioxidant activity..

CONCLUSIONS

Level of phenolic and flavonoids content in the mature leaves of *A. beccariana* was higher than young and old leaves. In the young and old leaves, total phenolic content was higher than the flavonoids, while the level of phenolic and flavonoids in mature leaves was not significantly different. The mature leaf of *A. beccariana* was the most potential as an antioxidant with an IC_{50} value of 72.25±0.72 ppm. From this study can be concluded that leaf age influence their antioxidant and phytochemical (phenols and flavonoids) properties.

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