

Antioxidant activity, total phenolic and flavonoid content of several indigenous species of ferns in East Kalimantan, Indonesia

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Abstract. Nurhasnawati H, Sundu R, Sapri, Supriningrum R, Kuspradini H, Arung ET. 2019. Antioxidant activity, total phenolic and flavonoid content of several indigenous species of ferns in East Kalimantan, Indonesia. *Biodiversitas* 20: 576-580. This study aimed to determine the total phenolic and flavonoids content and antioxidant activity of ethanol extract of several indigenous species of ferns in East Kalimantan. Total phenolic content was determined by Folin-Ciocalteu method and flavonoid content was measured by colorimetric method. Antioxidant activity was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl) method. The result of photochemical screening indicated that the leaves of *Plagiogyria pycnophylla*, *Plagiogyria glauca*, and *Stenochlaena palustris* contained alkaloid, flavonoid, tannin, saponin, and steroid while *Acrostichum aureum* contained flavonoid, tannin, saponin and steroid. The result showed extract *Acrostichum aureum* had the highest total phenolic content (366.4573 ± 2.2117 mg GAE.g⁻¹), flavonoid content (228.6087 ± 2.2548 mg QE.g⁻¹), and very strong antioxidant activity with IC₅₀ value 29.5303 ppm. There is positive correlation between total phenolic content, flavonoid with antioxidant activity.

Keywords: ferns, antioxidant, DPPH, total phenolic, flavonoid

INTRODUCTION

Ferns are vascular plants that are widespread throughout Indonesia. There are approximately 12,000 species of ferns around the world and 1,300 of them are found in Indonesia (Wang 2017; Imaniar 2017). Local people use ferns for food, as ornamental plants and traditional medicine (Ridianingsih et al. 2017). *Plagiogyria pycnophylla* (Kunze) Mett. and *Plagiogyria glauca* (Blume) Mett. are species of ferns that can be found in West Kutai District in East Kalimantan. Empirically, the Dayak Benuaq ethnic group uses the leaves and rhizomes of those plants as anti-cancer, especially breast cancer and antitoxin. The rhizome of *Acrostichum aureum* L. is used to treat snake bites, wounds and pustules while the leaves are used to stop bleeding (Khan et al. 2013). Empirically, the Dayak people use the leaves of *Stenochlaena palustris* (Burm. F.) Bedd. for face powder.

A number of diseases are caused by excessive oxidation in the body which increases the amount of free radicals. Free radical is a substance or molecule having one or more unpaired electrons in its outer orbit (Phaniendra 2015). Free radicals are continuously produced endogenously by human body through normal metabolic processes, inflammation and malnutrition. Apart from those, free radical can also be derived exogenously through pollution, ultraviolet radiation and cigarette smoke.

Antioxidant is a substance that provides protection from oxidation reaction (Cross et al. 1994). The use of synthetic antioxidants as the source of exogenous antioxidants might

have side effects, so natural antioxidants are a necessary alternative. The choice and availability of natural antioxidant are still limited so that research on natural antioxidant becomes a trend among researchers (Sayuti and Yenrina 2015).

In this study, we determine the total phenolic, flavonoid content and the evaluation of antioxidant activity of several indigenous species of ferns in East Kalimantan as preliminary study considering the potential of ferns as source of medicine and cosmetics.

MATERIALS AND METHODS

Collection and ferns identification

Several species of ferns that have been used empirically for medicines and cosmetics were collected from West Kutai District and Samarinda City, East Kalimantan, Indonesia during October-December 2017. Identification of ferns was carried out at the Plant Anatomy and Systematics Laboratory of the Faculty of Mathematics and Natural Sciences in Mulawarman University, Samarinda, Indonesia. Species of ferns used in this study were: *Plagiogyria pycnophylla* (Kunze) Mett.; *Plagiogyria glauca* (Blume) Mett.; *Acrostichum aureum* L. and *Stenochlaena palustris* (Burm. F.) Bedd.

Chemical reagents

The reagents used were distilled water, acetic anhydride, gallic acid, aluminum chloride, hydrochloric

acid, sulfuric acid, amyl alcohol, iron (III) chloride, dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 70% ethanol, ethyl acetate, Folin-Ciocalteu, chloroform, quercetin, sodium hydroxide, sodium nitrite, *n*-hexane, Bouchard reagent, Dragendorf reagent, Mayer reagent, magnesium powder.

Extraction and phytochemical screening

Ferns were sorted, washed, drained, air-dried in the open air and protected from direct sunlight exposure due to in the humid condition, fungi will easily grow. Dried samples were ground using a blender and sieved with a 60 mesh sieve. Weighed as much as 250 g of simplicia soaked in ethanol solvent by maceration extraction method. Phytochemical screening was conducted using procedures described by Marjoni (2016) and Harborne (1987) to determine the presence of alkaloid, flavonoid, tannin, saponin, and steroid; that are described below.

Alkaloid. The sample was weighed as much as 0.5 grams, 1 ml of 2 N hydrochloric acid and 9 ml of distilled water were added, heated over water bath for 2 minutes, cooled and then filtered. The filtrate was used for the following test: 3 drops of filtrate were taken to add 2 drops of Mayer reagent (to produce white/yellow precipitate), Bouchardat reagent (dark brown precipitate), Dragendorf reagent (red brick precipitate). If two of the three tests above give a positive result, then the extract contains an alkaloid.

Flavonoids. A total of 0.5 grams of sample was extracted using 10 mL of distilled water, filtered, taken 5 ml then added 0.1 g of Mg powder, 1 mL of concentrated HCl and 2 mL of amyl alcohol, shaken and allowed to separate. Flavonoids are positive if there is red, yellow, orange in the amyl alcohol layer.

Tannin. A total of 0.5 grams of sample was extracted using 10 mL of distilled water. The extraction results are filtered then the filtrate obtained is diluted with distilled water until it is colorless. Taken as much as 2 mL, then added with 1-2 drops of FeCl₃ 1%. Blue or blackish green occurs indicating the presence of tannins.

Saponin. A total of 0.5 grams of sample is put into a test tube and added 10 mL of hot distilled water, cooled then shaken vigorously for 10 seconds, foam or foam is formed which for no less than 10 minutes is 1-10 cm high. 1 HCl 2 N was added, if the froth did not disappear indicating saponins.

Steroids/terpenoids. A sample of 1 gram is macerated with 20 mL *n*-hexane for 2 hours, then filtered. The filtrate is evaporated over water. At the residue, 2 drops of anhydrous acetic acid were added and 1 drop of concentrated sulfuric acid. A purple or red color indicates a terpenoid, then turns blue-green indicating steroids.

Determination of total phenolic

Total phenolic content in each extract was evaluated using Folin-Ciocalteu (FC) method, according to the method by Bajalan et al. (2017) and Adesegun et al. (2007) research with slight modifications. This method based on the inhibition power of phenolic hydroxyl group. Phenolic compounds react with Folin-Ciocalteu reagent. The

principle of Folin-Ciocalteu method was the formation of blue-color complex compound (Alfian and Susanti 2012).

Making a calibration curve

Gallic acid solution with a concentration of 200 mg/L (5 mg gallic acid was made in 25 ml of distilled water) was then made into a series of solutions with a concentration of 12.5; 25; 50; 100 and 200 mg/L (or ppm) of 10 ml each. Then 0.1 ml of each of the standard gallic acid series solution was taken and added 2 ml of Na₂CO₃, left for 5 minutes. Next, 1 ml of Folin Ciocalteu solution was diluted with distilled water (1:10) and incubated for 30 minutes. The absorbance is measured at a wavelength of 780 nm against reagent blank for each series of standard solutions so that a curve with a linear regression equation is obtained ($y = bx + a$).

Measurement of absorbance of the sample

A sample solution of 1000 mg/L was made (10 mg extract in 1 ml DMSO, the volume was sufficient to 10 ml with distilled water), then 0.1 ml was taken and added Na₂CO₃, left for 5 minutes. Then added 1 ml of Folin Ciocalteu solution and incubate for 30 minutes. The absorbance is measured at a wavelength of 780 nm against reagent blank.

Calculation of total phenolic levels

The sample absorbance value is entered as the *y* value in the linear regression equation, so the value of *x* can be determined. Total phenolic levels are calculated using the following formula: $TPC = (C \cdot V)/W$; where TPC = total phenolic content (mg.g⁻¹ extract); C = concentration of sample established from the calibration curve (mg/L); V = volume of sample solution (L); W = weight of ethanolic plant extract (g). Total phenolic content was expressed as mg gallic acid equivalent per gram extract (mg GAE.g⁻¹).

Determination of flavonoid content

The determination of flavonoid content in extract was done using colorimetric method, according to method by Chandra et al. (2014) and Bajalan et al. (2017).

Making a calibration curve

Quercetin solution with a concentration of 200 mg/L (5 mg in 1 ml DMSO, volume up to 25 ml with ethanol) was then made into a series of solutions with a concentration of 12.5; 25; 50; 100 and 200 mg/L (or ppm) of 10 ml each. Then 0.5 ml were taken from the standard quercetin series solution, added 0.15 ml 15% NaNO₂, left for 6 minutes. Then added 0.15 ml of AlCl₃ 10% and incubated for 60 minutes. Then added 2 ml of 4% NaOH, 2 ml of distilled water and left for 15 minutes. The absorbance is measured at a wavelength of 420 nm against reagent blank for each standard solution series to obtain a curve with a linear regression equation is obtained ($y = bx + a$).

Measurement of absorbance of the sample

A sample solution of 1000 mg/L was made (10 mg of extract in 1 ml of DMSO, sufficient volume of 10 ml with aquades), 0.5 ml was taken added 0.15 ml of 15% NaNO₂,

left for 6 minutes. Then added 0.15 ml of AlCl₃ 10% and incubated for 60 minutes. Then added 2 ml of 4% NaOH, 2 ml of distilled water and left for 15 minutes. The absorbance is measured at a wavelength of 420 nm against reagent blank.

Calculation of flavonoid levels

The sample absorbance value is entered as the y value in the straight line equation, so the value of x can be determined. Total phenolic levels are calculated using the formula: FC = (C · V)/W ; where FC = flavonoid content (mg/g extract); C = concentration of sample established from the calibration curve (mg/L); V= volume of sample solution (L); M = weight of ethanolic plant extract (g). Total flavonoid content was expressed as mg quercetin equivalent per gram extract (mg QE.g⁻¹).

Antioxidant assay

Antioxidant activity assay refers to Arung et al. (2006) with slight modifications. Antioxidant activity was determined by decolorization of DPPH solution and carried out using a UV/Vis spectrophotometer at room temperature. The range of concentration of the samples were 12,5; 25; 50; 100 and 200 mg/L (or ppm). One ml of the sample was added with 2 ml of DPPH 60 mg/L, and incubated at room temperature in the dark for 30 minutes. Absorbance was measured at 523 nm. Quercetin is used as a positive control with concentrations of 2, 4, 6, 8 and 10 mg/L. The percentage of inhibition was calculated using the following equation: % DPPH inhibition = [(Ab – As)/Ab]. 100 % ; where Ab = absorbance of blank sample, As = absorbance of sample.

The absorbance of each sample was measured and calculated to determine the value of inhibition percentage (reduction). Linear regression equation obtained from the scatter plot of extract concentration and inhibition percentage was used to calculate IC₅₀ value. The IC₅₀ value is the concentration of extract required to inhibit 50% of DPPH free radical. According to Miryanti et al. (2011) states that antioxidant activity is considered extremely high if the value of IC₅₀ is less than 50 ppm, high if the value of IC₅₀ is between 50 – 100 ppm, moderate if the value is between 100 – 150 ppm and low if the value is between 151 – 200 ppm.

Data analysis

The qualitative and quantitative data obtained were analyzed descriptively. Antioxidant activity was grouped according to IC₅₀ criteria, while total phenolic and flavonoids were determined as mean ± SD. All measurements were done in triplicate.

RESULTS AND DISCUSSION

Phytochemical screening

Phytochemical screening was carried out to determine the secondary metabolites in the sample. The result of the phytochemical screening assay can be seen in Table 1.

Phytochemical screening was carried out due to its simplicity, speed, minimum use of equipment and selectivity (Nohong 2009). The result of phytochemical screening shown in Table 1 indicated that *P. pycnophylla*, *P. glauca*, and *S. palustris* contain alkaloid, flavonoid, tannin, saponin, and steroid group, but alkaloid was absent in *A. Aureum*. The principle of the alkaloid test is the formation of sediment due to ligand replacement. Nitrogen atoms which are alkaloid lone pairs replace iodine ions in Dragendorf and Mayer reagents (Sangi et al. 2008).

All samples containing flavonoids were characterized by the formation of yellow/orange layers in the amyl alcohol layer. Flavonoids have the potential as antioxidants which can prevent the formation of free radicals.

Saponin contains glycosyltransferase acting as polar groups, while steroid and triterpene to function as nonpolar groups. All samples contain saponin. According to Saxena et al. (2013), many saponins are known to be antimicrobial, to inhibit mold, and to protect plants from insect attack.

Based on the results in Table 1, all samples contain condensed tannins. The addition of FeCl₃ causes the hydrolyzed tannin to turn into blue while the condensed tannin turns into green. Decolorization occurs because FeCl₃ reacts to one of the hydroxyl groups in the tannin (Sangi et al. 2008).

Steroid analysis was based on the ability of the compound to change color with concentrated H₂SO₄ in acetic anhydride (Sangi et al. 2008). The results show the formation of a greenish blue ring which means the extract contains steroids.

As far as we know, there is no report about phytochemical screening of *P. glauca* and *P. pycnophylla*. Study of Khan et al. (2013) showed that ethanol extract *A. aureum* contained alkaloids, glycosides, tannins, flavonoids, and terpenoids. The results of the study differ from Khan (2013) alleged because according to Salim et al. (2016) differences in the place of growth of a species affect the precursors of biosynthesis of secondary metabolites. While methanol extract contained proteins and amino acids, glycosides, steroids, triterpenes, saponins, and flavonoids (Raja and Ravindranadh 2014). Study of Chai (2012) showed that *S. palustris* contains polyphenols, flavonoids, cinnamic acid, and anthocyanins.

Table 1. Phytochemical detected in fern extracts collected from East Kalimantan, Indonesia

Species	Local name	Part	Alkaloid	Flavonoid	Tanin	Saponin	Steroid/Terpenoid
<i>Plagiogyria pycnophylla</i>	<i>Paku atai merah</i>	Leaf	+	+	+	+	+
<i>Plagiogyria pycnophylla</i>	<i>Paku atai merah</i>	Rhizome	+	+	+	+	+
<i>Plagiogyria glauca</i>	<i>Paku atai putih</i>	Leaf	+	+	+	+	+
<i>Acrostichum aureum</i>	<i>Paku laut</i>	Leaf	-	+	+	+	+
<i>Stenochlaena palustris</i>	<i>Kelakai</i>	Leaf	+	+	+	+	+

Note : (+) = presence; (-) = absence

Table 3. Total phenolic and flavonoid content in ferns ethanolic extract

Sample	Part	Total Phenolic Content (mg GAE.g ⁻¹ extract)	Flavonoid Content (mg QE.g ⁻¹ extract)
<i>P. pycnophylla</i>	Leaf	264.0565 ± 4.0365	114.8439 ± 1.6012
<i>P. pycnophylla</i>	Rhizome	286.9202 ± 9.4492	127.1995 ± 0.4558
<i>P. glauca</i>	Leaf	83.8114 ± 8.7162	81.1347 ± 0.8025
<i>A. aureum</i>	Leaf	366.4573 ± 2.2117	228.6087 ± 2.2548
<i>S. palustris</i>	Leaf	245.3011 ± 3.2382	166.1779 ± 4.1420

Note: The data are averaged ± SD, n=3

Table 2. IC₅₀ value and antioxidant activity of fern extracts collected from East Kalimantan

Sample	Part	IC ₅₀ (ppm)	Category
<i>P. pycnophylla</i>	Leaf	124.6806	Moderate
<i>P. pycnophylla</i>	Rhizome	106.5234	Moderate
<i>P. glauca</i>	Leaf	995.0497	No activity
<i>A. aureum</i>	Leaf	29.5303	Very strong
<i>S. palustris</i>	Leaf	140.7528	Moderate

Total phenolic

Phenolics, which are secondary metabolites of plants, exhibit remarkable bioactivities. A large body of epidemiological studies has proven the bioactivities of phenolics in both standard compounds and natural extracts: namely, anticancer, anti-inflammatory, and antibacterial activities as well as reducing diabetes, cardiovascular disease, and neurodegenerative disease. Phenolics also display anti-analgesic, anti-allergic, and anti-Alzheimer's properties (Shahidi and Yeo 2018). The results of total phenolic and flavonoid content were presented in Table 3.

Total phenolic has been known to have biological activity as free radical scavengers and antioxidants. Its antioxidant activity is mainly caused by redox properties which act as reducing agents, hydrogen donors and singlet oxygen quenchers (Chandra et al. 2014). Ethanol extract of *A. aureum* leaves showed the highest phenolic content (366.4573 ± 2.2117 mg GAE.g⁻¹) followed by *P. pycnophylla* rhizome (286.9202 ± 9.4492 mg GAE.g⁻¹), *P. pycnophylla* leaves (264.0565 ± 4.0365 mg GAE.g⁻¹), *S. palustris* (245.3011 ± 3.2382 mg GAE.g⁻¹) and *P. glauca* (83.8114 ± 8.7162 mg GAE.g⁻¹). The results of this study showed that total phenolic content was positively correlated with antioxidant activity. Based on Tables 2 and 3 it can be concluded that the higher the total content of phenol, the antioxidant activity is also getting stronger. Kumar et al. (2014) reported that phenolic compounds as the main contributors to antioxidant activity.

Flavonoid

Flavonoids are one of the largest natural phenolic compounds and are found in all plants, therefore it is certain that flavonoids are found in every plant extract (Markham 1988). The results in Table 3 showed that *A. aureum* had the highest flavonoid content (228.6087 ± 2.2548 mg QE.g⁻¹), followed by *S. palustris* (166.1779 ± 4.1420 mg QE.g⁻¹), *P. pycnophylla* rhizome (127.1995 ± 0.4558 mg QE.g⁻¹), *P. pycnophylla* leaves (114.8439 ±

1.6012 mg QE.g⁻¹), and *P. glauca* (81.1347 ± 0.8025 mg QE.g⁻¹). Generally, phenolics and flavonoids constitute a major group of compounds, which act as main antioxidant (Adesegun et al. 2007). Differences in flavonoid structure and group substitution affect the stability of phenoxyl radicals, thus affecting the antioxidant properties of flavonoids (Wojdyło et al. 2007).

Yao et al. (2010) showed a correlation between antioxidant activity and total flavonoid content and total phenolics in celery. This is in accordance with the results of this study on ferns where *A. aureum* extract contains the highest total phenolic, and flavonoids and antioxidant activity.

Antioxidant activity

Antioxidant activity of ethanol extract of several species of ferns was carried out using the DPPH method, and measured using a UV-Vis spectrophotometer. DPPH free radical scavenger method was chosen due to its simplicity, ease, speed, sensitivity and the little amount of samples needed (Marzuki et al. 2012). DPPH, is a stable free radical which possesses purple color will turn to yellow compound when it reacts with antioxidant compounds. In this reaction, antioxidants release electrons to DPPH.

The results in Table 2 show the IC₅₀ value of *A. aureum* extract is 29.5303 ppm which is categorized as a very strong antioxidant. According to Miryanti et al. (2011) IC₅₀ values < 50 ppm for antioxidant was very strong. The IC₅₀ value of quercetin as a positive control is 5.9866 ppm which was also categorized as very strong antioxidant activity. Based on the IC₅₀ value, the extracts of *P. pycnophylla* and *S. palustris* are categorized as moderate antioxidant activity, with IC₅₀ values ranging from 106.5234 - 140.7528, while *P. glauca* extract does not have antioxidant activity based on its IC₅₀ value (995.0497 ppm)

Antioxidant activity is influenced by total phenol levels and flavonoids. Phenol and flavonoid compounds have a linear contribution to antioxidant activity, so the higher the level the better the antioxidants (Ghasemzadeh and Ghasemzadeh 2011). High levels of total phenolic in the ethanol extract of *A. aureum* are thought to have an important role as antioxidants. Besides phenol and flavonoids, other phenolic components such as tannins, alkaloids and terpenoids (Saxena et al. 2013) also contribute as antioxidants.

Based on research, ethanol extract of *A. aureum* has the highest levels of total phenolic, flavonoids with very strong

antioxidant activity. There is a positive correlation between levels of total phenolic, flavonoids with antioxidant activity. Further research needs to be done to isolate active compounds of *A. aureum* as antioxidants.

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