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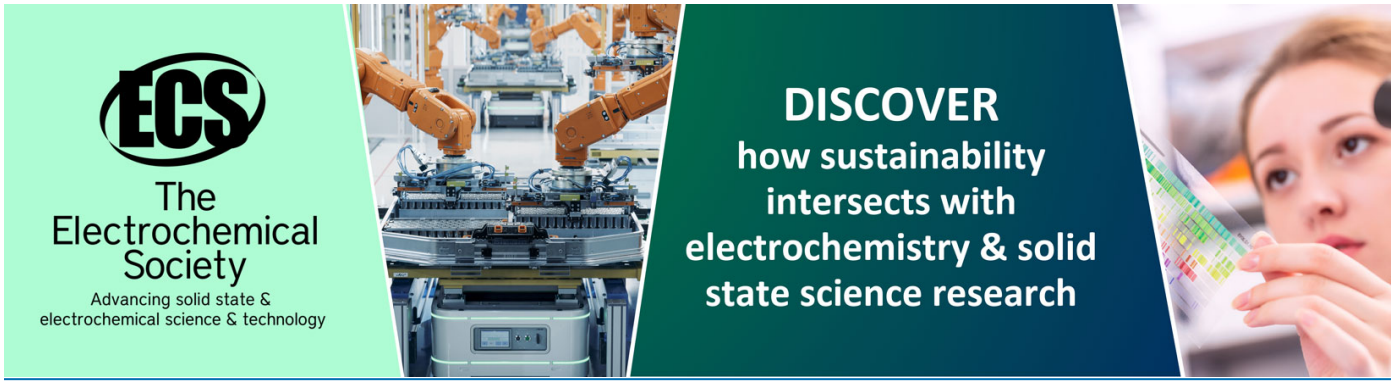

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Comparative study of antioxidant effect from extract and fraction of 'Paku Atai Merah' (*Angiopteris ferox* Coupel)

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Abstract. Paku Atai Merah tubers with the Latin name *Angiopteris ferox* Coupel have been used by the Dayak people as empirical treatment and have anticancer and antioxidant activity. The purpose of this study was to find out more the movement of extracts and fractions of Paku Atai Merah tubers in reducing free radicals using the total antioxidant capacity (TAC), CUPRAC, and Iron chelating methods. In this study, the sample used was the ethanol extract (EE) of *A.ferox* Copel and then partitioned using n-hexane (FHP), ethyl acetate (FEAP), and aqueous-ethanol (FEP) solvents. The effectiveness of the sample in reducing free radicals with various antioxidant assay methods. The results obtained from this study indicate that the extract and fraction of Paku Atai Merah tubers have good effectiveness as an antioxidant agent. The total antioxidant capacity (TAC) method showed that EAP had a total antioxidant capacity of 101.60 µM/mg QEAC compared to EE (44.62 µM/mg QEAC), FEAP (89.88 µM/mg QEAC), and FHP (81.21 µM/mg QEAC). In the CUPRAC method using gallic acid as a standard for comparison, the results obtained were EE (4.00 µM/mg GEAC), FHP (1.45 µM/mg GEAC), FEAP (8.66 µM/mg GEAC), and APA (8.72 µM/mg GEAC). The antioxidant activity using the chelating iron method in each sample of EE, HPA, EAP, and APA showed activity with IC_{50} values of 384 µg/mL, 387.41 µg/mL, 339.23 µg/mL, and 347.3 µg/mL. Based on the results obtained from this study, it can conclude that the extract and fraction of Paku Atai Merah tubers have an excellent antioxidant agent to develop as a food supplement.

Keywords: Antioxidant; Paku Atai Merah (*Angiopteris ferox* Copel); Iron Chelating; TAC; CUPRAC.

1. Introduction

Free radicals are molecules that do not have an electron pair in their outer orbital. Increased exposure to free radicals in the body is a significant factor in the increase in sufferers of degenerative diseases such as Alzheimer's, cancer, diabetes mellitus, Parkinson's, and several other degenerative diseases [1]. In general, exposure to minimal amounts of free radicals can be stabilized by endogenous antioxidants such as superoxide dismutase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate

dehydrogenase, and catalase. These endogenous antioxidants can stabilize free radicals through enzymatic reactions to convert reactive free radicals into unreactive radicals in the body [2–4]. However, excessive exposure to free radicals in the body requires additional antioxidants from exogenous sources derived from natural ingredients that can effectively reduce free radicals through direct interaction with reactive species to produce stable complexes [2].

Antioxidants are molecules that can prevent free radical damage by donating electrons from antioxidant sources to damaged cells and can convert free radicals into unreactive products that can be removed from the body. In general, the mechanism of action of antioxidants is through hydrogen atom transfer (HAT) and single electron transfer (SET) to free radicals to stabilize free radicals [3,5]. Antioxidant compounds can work enzymatically (endogenously) and non-enzymatically (exogenously). Enzymatic antioxidants have a function as an initial defense against free radicals such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and glutathione. Superoxide dismutase (SOD) is the first line of defense in neutralizing free radicals by catalyzing superoxide anion radical dismutase (O_2^{*-}) into hydrogen peroxide (H_2O_2) through a reduction reaction so that free radicals become non-reactive. The product form of SOD reduction produces H_2O_2 in the presence of the catalase enzyme, and glutathione peroxidase (GPx) will convert H_2O_2 through a reduction reaction into H_2O and O_2 [6]. The non-enzymatic antioxidants are generally divided into two parts, i.e., metabolic antioxidants and nutritional antioxidants. Metabolic antioxidants include endogenous antioxidants produced from the body's metabolic products such as lipoic acid, glutathione, L-arginine, coenzyme Q10, melatonin, bilirubin, uric acid, metal chelating proteins and several other metabolic antioxidants. Meanwhile, in nutritional or exogenous antioxidants, which are secondary metabolites derived from biosynthesis of natural ingredients, which are capable of acting as antioxidants, such as vitamin E, vitamin C, carotenoids, flavonoids, phenolics and several other natural antioxidants [6,7]

Natural ingredients plants have activity as exogenous antioxidants. One of which is Paku Atai Merah (*Angiopteris ferox* Copel) tuber. Paku Atai Merah tubers are ferns found in the Kalimantan Islands, precisely in West Kutai, and have been widely used by local people as traditional medicine [8]. The Paku Atai Merah tuber has been reported [9,10] phytochemical screening results, namely flavonoids, alkaloids, tannins, saponins, steroids, terpenoids, and phenolics. Angiopteroside is one of the new compounds that have been found in the species *A. ferox* Copel [8]. According to Nur *et al*.,2019, from the test results, the total flavonoid and phenolic content of the Paku Atai tubers has good flavonoid and phenolic levels [11]. Scientifically, it has been proven that the Paku Atai Merah tubers contain compounds that have activity as antibacterial [10], anticancer [12], and antioxidant [9,11]. Based on the research results [9], it has been reported that Paku Atai tuber has antioxidant activity from the extract. The fraction of Paku Atai Merah tubers has good antioxidant activity by using various antioxidant activity testing methods. The results showed that the ethyl acetate fraction and ethanol extract had intense activity using the NO and ABTS methods. However, it has antioxidant activity with a weak category in reducing lipid peroxide in the BCB method.

Based on the above background in this study, we would like to carry out further testing of extracts and fractions of Paku Atai Merah tubers in their activity in reducing cupric, chelating essential metal ions and total antioxidant capacity by using the TAC, CUPRAC, and iron-chelating methods which can support Paku Atai Merah tuber as a source of antioxidant agents.

2. Materials and Methods

2.1 Material

The materials used in this study were aluminum foil, Aquadest (One Med®), ammonium molybdate, gallic acid, sulfuric acid, CuCl2, 70% ethanol (One Med®), ethyl acetate (Marck, Germany), $FeSO_4$ (Sigma Aldrich, Germany), n-hexane (Marck, Germany), quercetin (Sigma Aldrich, Germany), sodium phosphate, neocuproine (Sigma Aldrich, Germany) and Ferrozine reagent kit (HACH).

2.2 Sample preparation

The samples of Paku Atai Merah tubers were obtained from Linggang Bigung District, West Kutai Regency, East Kalimantan. The criteria for the samples of the Paku Atai tubers used are approximately two years old. The samples of Paku Atai tubers were made simplicia with several series of processes ranging from wet sorting to removing dirt attached to the tubers, washing with running water, and reducing the size to facilitate the drying process until dry simplicia was obtained.

2.3 Extraction Process

Dry Simplicia powder has been powdered as much as 1.2 kg and then extracted by maceration with 5 liters of 96% ethanol as solvent. The sample was stored in a dark place to be protected from the sun for 3x 24 hours, stirring occasionally. After that, it was filtered to obtain the filtrate and residue. The residue was re-macerated using the same solvent (96% ethanol) until the liquid was clear, indicating that the extraction process had been maximized. The filtrate was collected and evaporated using a rotary evaporator until a thick extract was obtained (EE).

2.4 Fractionation

The thick extract of the Paku Atai Merah tubers was dissolved in a mixture of aqueous: ethanol (1:9). The mixture was put into a separating funnel, then 50 mL of n-hexane was added, shaken vigorously, and allowed to stand until completely separated. The n-hexane phase was then separated, and the aqueous: ethanol phase was fractionated again with n-hexane until the solution obtained was clear. The ethanol: water phase was then added with 50 mL of ethyl acetate solvent in a separating funnel. The mixture was shaken and allowed to form two layers: the ethyl acetate phase at the top and the aqueous: ethanol phase at the bottom. The two layers are separated. Aqueous: ethanol phase obtained was fractionated again using ethyl acetate until the solution obtained was clear. Each fractionated filtrate was evaporated to obtain hexane (FHP), ethyl acetate (FEAP), and aqueous: ethanol (FEP) fractions.

2.5 Total Antioxidant Capacity (TAC) Assay

Antioxidant activity testing using the Total Antioxidant Capacity (TAC) is a colorimetric method of testing antioxidant activity to determine the potential of compounds in a sample to reduce Mo (VI) to Mo (V) to form complex bonds under acidic conditions marked by a color change from blue to green. Each stock solution extract and the fraction of Paku Atai Merah tuber was taken 1 mL, then put into a vial and reacted with 4 mL of a reagent mixture containing (2 mL 0.6 M sulfuric acid, 1 mL sodium phosphate 28 mM, and 1 mL ammonium molybdate 1%). The mixture was incubated at 95^0C for 10 minutes. After incubation, cooled to room temperature, the absorbance was measured using UV-Vis spectrophotometry at a wavelength of 695 nm. In this test, the quercetin solution was used as a standard for making standard curves. The antioxidant activity of the sample was determined by the reducing power expressed in Quercetin Equivalent Antioxidant Capacity (%w/w QEAC).

2.6 Cupric Ion Power Reducing (CUPRAC) Assay

Testing the reduction activity of copper ions from extracts and fractions of Paku Atai Merah tubers with slight modifications [13]. Each extract and fraction of Paku Atai Merah tuber was made a stock solution with 1000 µg/mL concentration. Each volume of the stock solution was taken from the extract, and the fraction was then reacted with 1 mL of 10 mM CuCl2 reagent, 1 mL of 7.5 mM neocuproin, 1 mL of 1 M ammonium sulfate, and the volume was made up to 5 mL in a volumetric flask with distilled water. The absorbance of each mixture was measured using UV-Vis spectrophotometry at a wavelength of 450 nm. In this test, gallic acid was used as a standard solution with a concentration series. The antioxidant activity of the sample solution using the CUPRAC method was determined based on Gallic Acid Equivalent Antioxidant Capacity (%w/w GAEAC).

2.7 Iron Chelating Assay

Testing antioxidant activity using the iron-chelating assay method is one method of testing antioxidant activity to evaluate the potential of a sample to chelate iron ions. The antioxidant activity test based on

procedure [14] was slightly modified. Stock solution (1000 µg/mL) was prepared from each extract and fraction from Paku Atai Merah tubers. Then, a series of concentrations of each extract and fraction solution was made by taking a volume of solution added with 0.1 mL FeSO4 and 0.1 mL ferrozine kit (HACH). The volume of the mixture was then made up with distilled water up to 2 mL. The mixture was incubated at room temperature for 10 minutes. After the incubation period, the maximum absorption of each extract and fraction was measured at a wavelength of 563 nm by visible spectrophotometry. In this test, Na_2EDTA was used as a positive control and distilled water as a blank (without a test sample). The antioxidant activity of each sample was determined based on the IC50 value obtained from the standard curve equation plotted between the concentration of the sample solution and the percent inhibition. Percent inhibition (%) of each sample can be calculated based on equation 1.

$$\% = [(Ao-As)/Ao]_x\, 100\% \quad (1)$$

where AO is the negative control absorbance and As is the extract/standard absorbance.

3. Results and Discussion

Tests of antioxidant activity of extracts and fractions of Paku Atai Merah tubers have been carried out using various test methods. This test was conducted to evaluate the potential of ferns that can be developed as antioxidant candidates. The antioxidant activity testing carried out in this study used several test methods such as total antioxidant capacity (TAC), cupric ion reduction power capacity (CUPRAC), and iron-chelating assay method. The mechanism of free radical neutralization is based on hydrogen atom transfer (HAT) and single electron transfer (SET) so that both mechanisms can neutralize free radicals. The antioxidant capacity is determined by the 50% inhibition concentration (IC_{50}) parameter in the iron-chelating assay method. The IC_{50} value was obtained from the regression equation plotted between the concentration of the sample solution and the percentage of inhibition. The smaller the IC_{50} value obtained, the greater the antioxidant activity. The strength of the antioxidant activity of a compound is categorized based on the IC_{50} value, where the categories are strong (< 50 g/mL), strong (50-100 g/mL), moderate (100-200 g/mL), and weak (> 200 g/mL). In the CUPRAC and TAC assay methods, the antioxidant capacity was determined by GAEAC (Galic Acid Antioxidant Capacity) and QEAC (Quercetin Equivalent Antioxidant Capacity), respectively, to determine sample activity using gallic acid and quercetin standards. [2,9].

3.1 Total Antioxidant Capacity (TAC) Assay

The antioxidant capacity of the extract and fraction of Paku Atai Merah tubers using the TAC method was based on the potential of the sample to reduce phosphomolybdate Mo(VI) to Mo(V) under acidic conditions to form phosphomolybdenum. The presence of antioxidant activity can be shown visually by a color change from blue to green. The results obtained from this study are shown in Table 1. Table 1 shows that FEAP has a higher activity with a QEAC value of 10.16 µM/mg than EE (4.46 µM/mg) and FEP (8.98 µM/mg), and FHP (8.12 µM/mg). The results obtained can be influenced by the presence of compounds in each sample. According to Nur *et al.*,2019, it has been reported that the ethyl acetate fraction contains phenolic and flavonoid compounds that are better than the ethanol extract, water-ethanol fraction, and hexane fraction from Paku Atai Merah tubers [11]. The principle of this method is based on the free radical content or capacity that can be neutralized in the test sample solution. The advantage of this method is that it can provide an initial picture to determine the capacity of a sample that has activity in reducing an essential metal.

Table 1. Capacity reducing the power of extracts and fractions of Paku Atai Merah tubers by TAC and CUPRAC Assay.

Samples	Reduction Power (μM/g Sample ± SD)	
	TAC*	CUPRAC*
Ethanol Extract (EE)	44.62 ± 0.06	4.00 ± 0.01
Aqueous-Ethanol Fraction (FEP)	89.88 ± 0.58	8.72 ± 0.02
Ethyl Acetate Fraction (FEAP)	101.60 ± 0.01	8.66 ± 0.02
Hexane Fraction (FHP)	81.21 ± 0.75	1.45 ± 0.01

*The values were expressed as mean ± SD (n = 3). Quercetin was used as a positive control (PC) for the reduction power test in the TAC method and gallic acid in the CUPRAC method.

3.2 Cupric Ionic Reducing Antioxidant Capacity Test (CUPRAC)

The antioxidant capacity of extract and fraction of Paku Atai Merah tubers using the CUPRAC method is based on the ability of the sample to reduce Cu^{2+} to Cu^{+} as indicated by the color change that occurs from blue to yellow in the test reaction mixture solution. The results obtained from this study using the CUPRAC method (Table 2) showed that FEP and FEAP had better potency with GAEAC (Gallate Acid Equivalent Antioxidant Capacity) values of 8.72 μM/mg and 8.66 μM/mg compared to EE (4.00 μM/mg) and FHP (1.45 μM/mg). The results obtained can be affected along with the increasing content of polyphenol compounds from a sample so that the sample activity in reducing copper ions also increases. The copper ion (Cu) is present in all living organisms in the Cu^{2+} and Cu^{+} state, which functions in survival and as an essential catalytic cofactor in redox chemistry for proteins, which will carry out the fundamental biological functions required for growth and development. Cu intake varies significantly from individual to individual depending on food choices, beverages, and environmental factors [15]. The United States Environmental Protection Agency (2013) has set a maximum Cu intake or exposure of 1.3 mg/L or 1.3 μg/mL. This amount is based on health risks with a sufficient margin of safety to prevent potential health problems. However, excessive Cu exposure can modulate the formation of free radicals through a redox reaction process that triggers the formation of free radicals. Excess Cu can cause peroxidative damage to lipid membranes through the reaction of lipid radicals and oxygen to form peroxyl radicals. In addition, increased levels of Cu can modify oxidative low-density lipoprotein (LDL) and trigger atherogenesis by increasing the transformation of macrophages into foam cells by developing vasoconstrictive and prothrombotic properties and triggering the formation of hydroxyl radicals through the Haber-Weiss reaction [16]. However, in reducing agents in enzymatic reactions, both endogenous and exogenous antioxidants can reduce Cu^{2+} to $Cu^{+,}$ thus preventing the formation of free radicals through redox reactions in the body.

3.3 Iron chelating Assay

The extracts and fractions of Paku Atai Merah tubers were tested the antioxidant activity of using the iron-chelating assay method based on the ability of a sample to compete in the presence of a ferrozine ligand to bind ferrous Fe ions in the mixed solution. The antioxidant activity of an antioxidant compound can be characterized by a color change that occurs in the solution mixture to a fading purple color which indicates that there is no complex bonding of the Fe ion with Ferrozine. The results obtained from the study using the iron-chelating assay method (Figure 1) showed that the extract and fraction of the Paku Atai Merah tubers had activity in chelating Fe ions in the weak category with IC_{50} values obtained from EE, FEP, FEAP, and FHP >200 μg/mL. This result indicates that the extract and fraction of Paku Atai Merah tubers have weak activity in chelating Fe ions. Iron (Fe) is an essential metal that has many roles and functions in the body. However, excess iron levels in the body can cause several diseases such as atherosclerosis, neurodegenerative thalassemia, etc. [17,18]. Iron chelation is a treatment method to reduce excess iron in the body by binding metal ions to eliminate it. For example, iron chelation therapy

can use Na_2EDTA, which can treat poisoning caused by excess lead levels in the body, and also Na_2EDTA is one of the strong chelator groups [18].

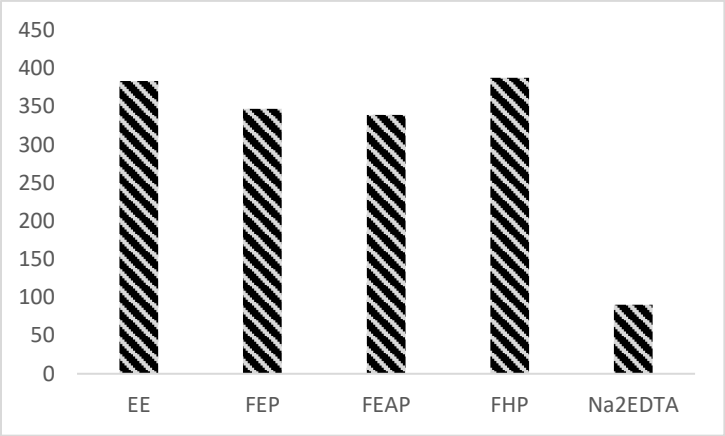

Figure 1. Graph of antioxidant activity using the iron-chelating assay method from ethanol extract (EE), the aqueous-ethanol fraction (FEP), ethyl acetate fraction (FEAP), and hexane fraction (FHP) of Paku Atai Merah tubers.

The antioxidant activity of natural plant material is influenced by the content of compounds contained in a sample, in this case, the secondary metabolites of a sample. Several secondary metabolites have antioxidant activity, such as carotenoids, vitamin E, vitamin C, flavonoids, and phenolics. The Paku Atai Merah tubers contain good phenolic and flavonoid content, so that they provide an antioxidant and anticancer activity that have been studied. Flavonoids are secondary metabolites derived from polyphenols, generally found in plants identified and classified into 4000 types of flavonoids that have many advantages on human health in preventing or delaying several chronic diseases and several other degenerative diseases [6,19].

4. Conclusion

The results of the research that have been carried out indicate differences in the antioxidant activity profile of each sample to the test method carried out. An antioxidant compound from each sample can influence the existence of differences in inactivity. However, among all methods, it was shown that the ethyl acetate fraction (FEAP) of Paku Atai Merah tubers had more potent antioxidant activity than the other samples.

Conflict of Interest

Based on this research, it stated that there is no conflict of interest

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