Fractionation of metabolite compound from *Medinilla speciosa* and their antioxidant activities using ABTS$^{\cdot+}$ radical cation assay

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Abstract. Phenolic compounds are one of the main parts in secondary metabolites. Parijoto fruit is a family of Melastomataceae which contains flavonoids as phenolic compounds which are known to have antioxidant potential. Flavonoids have an-OH groups that play an active role as free radical scavengers. The study was conducted as initial screening in testing the antioxidant activity of extracts and fractions of the Parijoto fruit (*Medinilla speciosa*). The study began with the phytochemical screening process using the Thin Layer Chromatography method to ensure the presence of flavonoid content in extracts and fractions of parijoto fruit, then proceed with the measurement of antioxidant power. Testing the antioxidant activity using the ABTS method (2,2 azinobis (3-ethylbenzothiazolin) -6-sulfonic acid) which is specific, simple and easy to apply. Parijoto fruit extracts and fractions were made in a series of concentrations, measured antioxidant activity, and each IC_{50} value was determined. Phytochemical screening test results showed the presence of flavonoids in extracts and fractions of parijoto fruit. The results of measurements of antioxidant power in extracts and fractions of parijoto fruit gave IC_{50} values of 6,520 ppm in ethanol extracts with very strong antioxidant categories. The n-hexane fraction produced an IC_{50} value of 118,424 ppm with a moderate antioxidant category, while the IC_{50} value of ethyl acetate and ethanol fractions was 4,246 ppm and 3,874 ppm, respectively, with a very strong antioxidant category. The activity and value of IC_{50} produced by extracts and fractions of Parijoto fruits showed potential as a candidate of antioxidant.

Keywords: ABTS, Antioxidant, Flavonoids, Medinilla speciosa, Phenolic

1. Introduction

Antioxidants are substances that play a role in preventing oxidation reactions. An antioxidant can contribute an electron to a free radical, so free radical formation can be inhibited [1]. Free radicals can have adverse effects such as aging, DNA damage, cancer, stroke, and other cardiovascular [2-3]. Free radicals can also reduce endogenous antioxidant activity in the body, thus requiring the presence of exogenous antioxidants [4].

Endogenous antioxidants are synthesized through metabolic processes and play a role in repairing body cell damage caused by free radicals, while exogenous antioxidants are antioxidants obtained from external sources such as food [5]. One of the exogenous antioxidants that play a role in the process of inhibiting free radicals comes from natural ingredients. Some uses of natural ingredients as exogenous antioxidants are due to economic factors, available in large quantities, as well as smaller side effects than synthetic antioxidants [6].

Medinilla speciosa or better known as Parijoto Fruit, contains metabolite compounds such as

flavonoids, saponins, tannins, and glycosides [7]. Parijoto fruit has traditionally been widely used by the public especially pregnant women in maintaining the health of the fetus. The content of phenolic compounds such as flavonoids in parijoto fruit has the potential as an antidote to free radicals. Phenolic compounds derived from natural substances have antioxidant activity by inhibiting free radicals and other reactive oxygen species [8].

Antioxidant activity testing can be done through the DPPH, ABTS, FRAP, ORAC, and FIC methods. Maesaroh et al., [9] compared the DPPH, FRAP, and FIC antioxidant test methods and obtained the result that the FIC method was less sensitive than the other two methods. Another study by Floegel et al., [10] states that the antioxidant activity of fruits, vegetables, and several other foodstuffs measured using the ABTS method results in higher significance values than measurements using the DPPH method. The pigments and hydrophilic content of the antioxidant compounds are more sentimental on the measurement using ABTS compared to DPPH.

ABTS (2.2 azinobis (3-ethylbenzothiazolin) -6-sulfonic acid) is one method of antioxidant testing that is easily applied, simple, and flexible in measuring the antioxidant activity that is hydrophilic or lipophilic in food extracts and liquids. Spirulina platensis produces higher antioxidant and anti-radical activity compared to DPPH [11]. The ABTS method can give an idea of the deterrence of free radicals by an antioxidant. The mechanism of ABTS begins with the formation of radicals between ABTS salts and potassium permanganate or potassium persulfate oxidants. BTS absorbance was measured at a specific wavelength of 734 nm [12].

This research will further examine the potential of Parijoto fruit as a natural antioxidant. Parijoto fruit extraction and fractionation were carried out using solvents with different levels of polarity. Testing the antioxidant activity using the ABTS method (2.2 azinobis (3-ethylbenzothiazolin) -6-sulfonic acid). Research is expected to provide an overview and further study regarding the use of natural materials in the field of phytopharmaca.

2. Materials and Methods

2.1. Materials

The main ingredient used is parijoto fruit powder derived from purplish parijoto fruit obtained from Colo Village, Kudus District. Test reagents include ABTS (2.2 azinobis (3-ethylbenzothiazolin)-6-sulfonic acid), $K_2S_2O_8$ from Sigma Aldrich, Methanol pro analysis from Merck, Aquadest, Ethanol 96% pro analysis Brataco, ethyl acetate, n-hexane, acid $K_2S_2O_8$ from Sigma Aldrich, Methanol pro analysis from Merck, Aquadest, Ethanol 96% pro analysis Brataco, ethyl acetate, n-hexane, acid glacial acetate, n-butanol from CV. Bratachem, and ammonia vapor.

Parijoto fruit powder is extracted by maceration method, then fractionated with three solvents (n-hexane, ethyl acetate, and ethanol). The active compound was identified by thin layer chromatography method. Parijoto fruit extracts and fractions were measured for their antioxidant activity by the ABTS method and IC_{50} values were determined.

2.2. Methods

2.2.1. Extraction and Fractionation

Parijoto fruit extraction was carried out by maceration method using two different solvents, namely 70% ethanol and 96%. The purpose of using solvent variations is to find out the best solvent that produces the most optimal yield. Extracts with optimal yield are then fractionated in stages using n-hexane, ethyl acetate, and ethanol solvents.

2.2.2. Flavonoid screening using TLC

Flavonoids were identified using thin layer chromatography with a modified procedure of Kumar et al., [13]. Silent silica phase GF254 and mobile phase used were n-butanol: glacial acetic acid: aquades. Flavonoid spotting viewers use ammonia vapor.

2.2.3. Antioxidant Activity Test with ABTS (Shalaby and Shanab, 2013)

Antioxidant measurement begins with determining the maximum wavelength of ABTS solution in the range of 600-800 nm and proceeds with determining the operational time or operating time. The antioxidant activity that continued by measuring each sample extract and fraction of the Parijoto fruit according to the flow diagram in Figure 1.

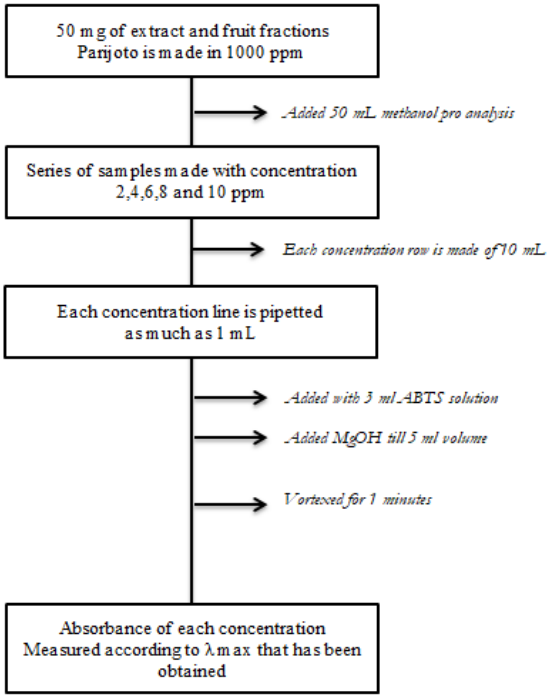

Figure 1. Mechanism of radical scavenger using $ABTS^{\cdot+}$ assay

3. Results and discussion

3.1. Extraction and Fractionation

Parijoto fruit extraction was carried out using ethanol solvents with different concentrations. Extraction with 70% ethanol solvent produced as much as 46 grams of thick extract from 300 grams of parijoto simplicia with a yield of 15.3%. Parijoto fruit extraction with 96% ethanol solvent resulted in a thick extract of 55.7 grams with a yield of 18.5% according to the results shown in Table 1. 96% ethanol solvent produced a greater yield than 70% ethanol solvent.

Table 1. Rendement of parijoto fruit exctract using etanol 70% and 96% solvent

Solvent	Weight of simplicia (gram)	Weight of exctract (gram)	Rendement (%)
70%	300	46,00	15,3
96%	300	55,70	18.5

The difference in yield in the two extraction processes is influenced by the difference in polarity between the two solvents and their interaction with the active compound contained in the fruit of Parijoto. The ideal solvent for finding simplicia fruit, leaves, fruit flesh is a mixture of alcohol-water with a concentration of 96% [14]. According to Senja et al., [15], ethanol 96% is capable of producing optimum yield and absorption in most fruit simplicia.

Fractionation of parijoto fruit is carried out in stages using three solvents with different polarity levels. Maceration extracted liquid-liquid fractionated with n-hexane, ethyl acetate and ethanol as a solvent. The use of different solvents allows the interest of active compounds in parijoto fruit and optimizes the process of withdrawal. The n-hexane fraction produced the greatest weight fraction compared to the ethyl acetate and ethanol fractions as shown in Table 2.

Table 2. Fractination result of parijoto fruit within n-hexane, ethyl acetate, and etanol solvent

Fraction	Weight of exctract (grams)	Weight of fraction (grams)
n-heksan	10,00	7,45
Etil asetat	10,00	2,01
Etanol	10,00	7,11

Luís et al., [16] and Gullón et al., [17] reported that fractionation allows the separation of active compounds according to their polarity with the principle of like dissolved like. Fractionation with n-hexane solvent is able to attract non-polar compounds in parijoto fruit, while ethyl acetate attracts semi-polar compounds. Fractionation with ethanol solvents is expected to increase the activity of polar active compounds in extracts of Parijoto Fruit (*Medinilla speciosa*), one of which is phenolic and flavonoid compounds which have antioxidant activity.

3.2. Flavonoids with TLC

Screening of the antioxidant activity of extracts and fractions of parijoto begins with the determination and identification of its active compounds. Determination shows that the test plants used are *Medinilla* species of the genus Melatomaceae. The identification results in Table 3 shown that both extracts and fractions of positive Parijoto fruit contain flavonoid compounds. Flavonoid compounds constitute most of the phenolic groups in natural materials which act as antidotes to free radicals.

Table 3. Identification of metabolite secondary compound of parijoto fruit using TLC

Sample	UV_{254} light	Rf	Amoniac vapor
Rutin	Brown spot	0.98	Yellowish green
Exctract	Brown spot	0.88	Yellowish green
FH	Brown spot	0.42	Yellowish green
FEA	Brown spot	0.91	Yellowish green
FE	Brown spot	0.98	Yellowish green

Keterangan :
FH : n-heksan fraction
FEA : ethyl acetate fraction
FE : ethanolic fraction

Thin layer chromatography (TLC) can be used effectively in the process of screening and evaluating the content of metabolites in a natural material. The chromatogram pattern produced at the time of separation with TLC provides a clear picture of the stability of natural materials. Other advantages of using TLC as a separation method include simple, applicable, and high speed separation method [18].

Flavonoid compounds in extracts and fractions of Parijoto fruit were identified by the mobile phase of n-butanol: glacial acetic acid: aquades (3: 1: 1) with high polarity properties. The choice of solvent is related to the nature of the compound and the stationary phase used. The solvent is not carcinogenic and does not react with the sample at the time of testing [13]. The stationary phase used is silica GF254 (Merck®) which has been widely used to separate phenolic compounds, alkaloids, fatty acids, sterols, and terpenoids.

Chromatographic results on extracts and fractions showed the presence of flavonoid compounds which were marked with brown spots when exposed to UV_{254} light and yellowish green spots with

ammonia vapor. The existence of flavonoids in natural materials, one of which is characterized by the presence of yellow fluorosis [19]. Flavonoids include phenolic compounds that have activity as exogenous antioxidants. Flavonoids are able to donate hydrogen atoms to free radicals, so that free radicals become unreactive.

3.3. Radical scavenger activity of parijoto fruit extract

Antioxidant activity begins with determining the maximum wavelength obtained in the 752 nm region. Determination of the maximum wavelength is done to measure the absorbance of compounds in the visible area, so that maximum absorption is obtained. The blank used in the test was methanol pro analysis. Antioxidant activity is determined by observing changes in color intensity in the ABTS solution used. The results of antioxidant activity tests on parijoto fruit extracts are shown in Table 4. Antioxidant activity is shown by an increase in the percentage of antioxidant activity as the extract concentration increases. IC_{50} value on parijoto fruit extract was 6,520 ppm with a very strong antioxidant category.

Table 4. Antioxidant activity of parijoto fruit exctract

Concentration (ppm)	Abs.	% Inhibition	IC_{50} (ppm)	Category
2	0.388	14.46		
4	0.301	33.77		
6	0.181	60.06	6.520	Very strong
8	0.166	63.51		
10	0.173	61.97		

The antioxidant activity of extracts and fractions of parijoto fruit was tested by the ABTS method (2.2 azinobis (3-ethylbenzothiazolin)-6-sulfonic acid). $ABTS^{\cdot+}$ is a radical source obtained from the activation process through the addition of potassium persulfate. Free radical reduction mechanism is characterized by a decrease in absorbance and a decrease in the color intensity of the test solution. The higher the antioxidant activity, the greater the intensity of lossing color produced during the testing process.

Antioxidant test on ethanol extract of parijoto fruit showed that the greater the concentration of the extract, the decreased intensity of absorbance was stronger as shown in Figure 2. The correlation between inhibitory activity (radical scavenger) with concentration, then used to determine the linear regression equation. The linear regression equation determines the IC_{50} value in ethanoicl extract of parijoto fruit.

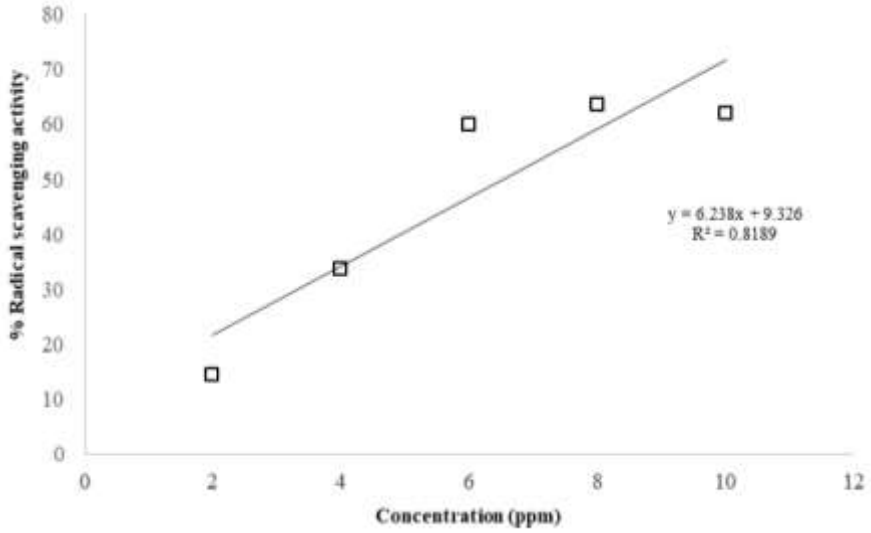

Figure 2. Correlation between radical scavenging activity and concentration of parijoto exctract

3.4. Radical scavenger activity of parijoto fruit fractions

Antioxidant testing was continued at the ethanol, ethyl acetate, and n-hexane fractions using the same method. In this test, $ABTS^{\cdot+}$ acts as a source of free radicals, while the Parijoto fractions act as a source of antioxidants that will reduce the presence of these radicals. The ethanolic fraction has an IC_{50} value of 3,874 ppm with a very strong antioxidant category as shown in Table 5.

Tabel 5. Antioxidant activity of parjoto ethanolic fraction

Concentration (ppm)	Abs	% Inhibition	IC_{50} (ppm)	Category
2	0.379	45.72		
4	0.357	46.12		
6	0.307	53.58	3.874	Very strong
8	0.141	78.65		
10	0.098	85.2		

The antioxidant activity of the parijoto ethyl acetate fraction in Table 6 shows that the ethyl acetate fraction can inhibit radicals with a stronger effectiveness than the n-hexane fraction. The ethyl acetate fraction produced antioxidant activity with IC_{50} values of 4,246 ppm and the antioxidant category was very strong. The difference in antioxidant activity produced by the two fractions is possible due to the influence of the polarity of the active compound on the fruit of Parijoto.

Tabel 6. Antioxidant activity of parijoto ethyl acetate fraction

Concentration (ppm)	Abs	% Inhibition	IC_{50} (ppm)	Category
2	0.343	34.17		
4	0.273	47.60		
6	0.184	64.62	4.246	Very strong
8	0.127	75.56		
10	0.083	84.13		

Antioxidant activity have correlation to the amount and composition of active compounds in a natural substance. The content and properties of active compounds in a natural material also affect its bioactivity. The antioxidant test of the parijoto n-hexane fraction shown in Table 7 gives lower results compared to the extract and the other two fractions. IC_{50} value of n-hexane fraction was 118.424 ppm with moderate antioxidant category.

Tabel 7. Antioxidant activity of parijoto n-hexane fraction

Concentration (ppm)	Abs	% Inhibition	IC_{50} (ppm)	Category
2	0.41	33.71		
4	0.39	36.89		
6	0.386	37.49	118.424	Medium
8	0.384	36.46		
10	0.401	35.17		

IC_{50} is an antioxidant concentration that can reduce 50% of free radicals. IC_{50} values in the parijoto fractions were determined in the same way through a linear regression equation. IC_{50}values of the ethanol and ethyl acetate fractions are included in the category of very strong antioxidants, meaning that at very small concentrations the ethanol and ethyl acetate fractions are able to inhibit the formation of free radicals. The graph of the relationship between the value of inhibition (free radical scavenger) and the concentrations of ethanol and ethyl acetate of parijoto fruit is shown in Figure 3 and 4. The increase in concentration in both fractions is in line with the increase in its radical inhibitory activity.

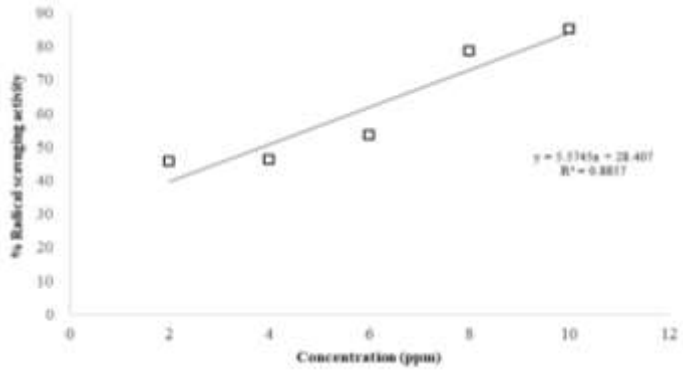

Figure 3. Correlation between radical scavenging activity and concentration of parijoto ethanolic fraction

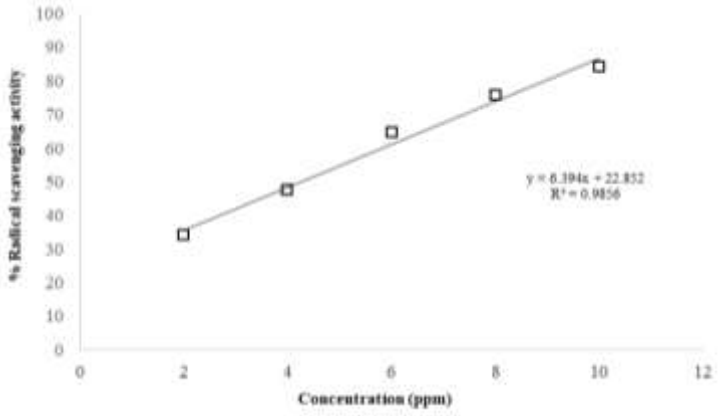

Figure 4. Correlation between radical scavenging activity and concentration of parijoto ethyl acetate fraction

Different results are shown by the antioxidant activity of the parijoto n-hexane fraction in Figure 5. The IC_{50} value produced by the n-hexane fraction is weaker than the ethyl acetate and ethanol fractions of the parijoto fruit. Active compounds that are attracted to n-hexane solvents tend to be non-polar. Polarity of the compound influences the antioxidant activity produced. The compounds that are thought to have antioxidant effects on parijoto are flavonoids, tannins, anthocyanins, and glycosides [7]. Most of these compounds are polar to semi-polar. The n-hexane fraction produces less optimal inhibitory activity because the active antioxidant potential of parijoto fruit is not completely attracted through non-polar solvents.

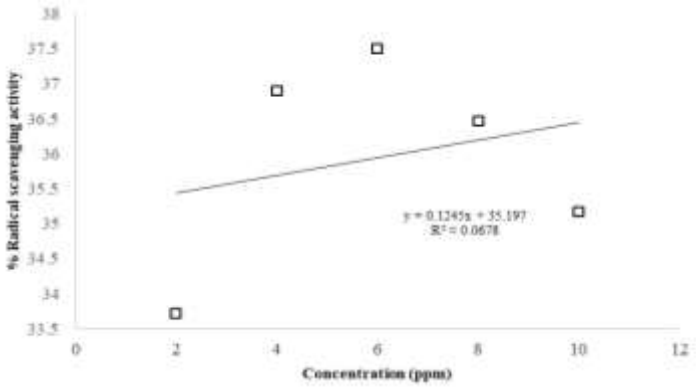

Figure 5. Correlation between radical scavenger activity and concentration of parijoto n-hexane fraction

Determining of antioxidant activity using ABTS method is very effective, simple, flexible, and easily repeated. ABTS is a radical with a nitrogen center with greenish-blue characteristics and when reduced by antioxidants to a colorless, non-radical form. The color change is due to the donation of hydrogen atoms from antioxidants to ABTS radicals [12, 20]. The principle of testing with this method is to measure the relative ability of antioxidants to reduce ABTS free radicals [19].

The secondary metabolite compounds in a natural material determine its bioactivity. Flavonoids are one of the phenolic compounds in natural materials that play an important role as neutralizers. Flavonoids have the potential as antioxidants and chelating metals. The antioxidant effect on flavonoids is closely related to its activities as anti-inflammatory, antiviral, anti-carcinogenic, hypo-

allergenic, and hepatoprotective [21]. The flavonoids contained in parijoto fruit have an excellent radical antidote activity, so they can be further developed as candidates for natural antioxidants.

4. Conclusion

Ethanol 96% solvent yields a more optimal yield with a yield of 18.5%. Testing the antioxidant activity of ethanol extract and parijoto fractions shows that the extract and fractions of parijoto fruit have antioxidant activity. The ethanol fraction of Parijoto has the best antioxidant activity compared to the extract and the two other fractions. IC50 value of ethanol fraction of 3,874 ppm with a very strong category of antioxidants.

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