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Extraction of Phenolic Total Compound and Determination of Antioxidant Activity of Cocoa Leaves Extracted Using Various Solvents

Subekti Hartiningsih[1]✉, Supriyanto[2]

[1]Department of Agricultural Technology, Nahdlatul Ulama University, Yogyakarta, INDONESIA
[2]Department of Food Technology and Agricultural Products, Gadjah Mada University, Yogyakarta, INDONESIA

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✉Corresponding Author:
subektihartiningsih@unu-jogja.ac.id

ABSTRACT

In the cultivation of cocoa plants there are by-products, one of which is cocoa leaves. This by-product comes from periodic pruning of cocoa plants which has not been utilized optimally. Phenolic compounds contained in the cocoa leaves are known as bioactive compounds which also have a role as antioxidants. The content of bioactive compounds in cocoa leaves can be extracted from plant tissues by extraction method. The objective of this research was to achieve cocoa leaf extract with the highest antioxidant content using the maceration method. Extraction by maceration on old cocoa leaves with a variety of solvents, namely methanol, acetone, and ethyl acetate. The results showed that the extract of old cocoa leaves with methanol as a solvent showed the highest content of polyphenolic compounds. A yield of 7,129% was attained from old cocoa leaves with total phenolic content of 349,952 ± 0,051 mg EAG/g sample, EC 50 antioxidant value of 287,958 ± 0.144, and antioxidant activity of 86,928 % RSA. The yield of phenolic compounds and antioxidant activity contained in cocoa leaf extract (sequentially from the highest) were obtained with methanol solvent, followed by acetone and ethyl acetate solvents.

1. INTRODUCTION

Following Ghana and Ivory Coast, Indonesia is the third-largest producer of cocoa in the globe. Cocoa plantations in Indonesia receive intensive attention from the government because they have an important meaning for the welfare of farmers, approximately equivalent to 1.4 million people (Pancaningtyas, 2013). In cacao cultivation, there are still by-products that are currently not utilized optimally. One of them is cocoa leaves. Cocoa leaves are the result of crop pruning done to increase productivity and plant maintenance such as maintaining the economic life of plants. These by-products from cocoa plantations are usually only left to rot, and have not been used optimally or only as compost, animal feed, and so on. It is well known that cocoa leaves contain phenolic compounds, a class of

bioactive substances that also serves as an antioxidant (Supriyanto *et al*., 2014). Cocoa leaves also contain theobromine, caffeine, anthocyanins, leucoanthocyanins and catechols, the amount of which varies, influenced by leaf age and plant age (Supriyanto *et al*., 2014).

According to Othman *et al*. (2007) total polyphenols in old leaves showed a higher yield of 32.4%, while that of in young leaves was 27.3%. Another study, according to Osman *et al*. (2004) showed that the total polyphenols in the old leaves were 28.4%. While that of in the younger leaves were 19%. The total catechins in the old leaves was 5.25% and the young leaves were 9.75%. The content of antioxidants, flavonoids and other bioactive compounds found in cocoa leaves can be extracted from plant tissues by extraction method. The extraction method was chosen based on several factors such as the nature of the bioactive compound to be targeted and the sample of the material used.

Maceration is one of the commonly methods used in extraction because the procedures and equipment used are relatively simple. The maceration method is carried out by immersing the material in a solvent. The extraction process with solvents is based on the nature of the polarity of the substance in the solvent at the time of extraction. Only polar solvents, such as ethanol, methanol, butanol, and water, can dissolve polar substances. Similarly, non-polar solvents like ether, chloroform, and n-hexane are the only ones that can dissolve non-polar substances (Gritter *et al*., 1991) Polar solvents commonly used for flavonoid extraction are methanol, acetone, ethanol, water and isopropanol (Suryani *et al*., 2016). While organic solvents commonly used to produce concentrates, extracts, absolutes or essential oils from flowers, leaves, seeds, roots, and other parts of plants are ethyl acetate, acetone, and water (Mukhopadhyay, 2002). Therefore, this study aimed to determine which type of polar solvents is the most optimum (in term of the highest total phenolic content and antioxidant activity) for extracting the phenolic compounds from cocoa leaves.

2. MATERIALS AND METHODS

The experiment was carried out at the Laboratory of Process Engineering and Food Processing and Food Chemistry, Gadjah Mada University, Yogyakarta in January – July 2018. The tools used in making the leaves into cocoa powder were cabinet dryer, analytical balance, Philips blender, 30 mesh sieve shaker. While the tools used for extraction were hotplate with reverse cooling, magnetic stirrer size 2 cm, rotary vacuum evaporator, erlenmeyer. The tools used for analysis were water baths, centrifuge tubes, Genesys 10S UV/VIS spectrophotometer, PH meter. The material used in this study was the old leaves of the cacao plant (*Theobroma cacao* L.) namely leaf number 5 from the top of the leaf plus 3 lower leaves from the cocoa plant with the Lindak type obtained from Gunung Kidul. The second material used compared with cocoa leaf was dry cocoa beans. The materials used for the analysis of total phenol were Folin-Ciocalteu solution, 20% sodium carbonate, gallic acid, antioxidant activity analysis materials, namely DPPH, methanol (p.a.), aquadest, TCA 10%, K4(Fe (CN)6) 1%, FeCL3 0 ,1%, phosphate buffer.

2.1. Research Stages

2.1.1 Sampling

Old cacao (*Theobroma cacao* L.) leaves picked from Gunung Kidul were dark green leaves or the 5th leaf. Then after arriving in the laboratory the sample was prepared for the next stage.

2.1.2 Cocoa Leaf Powder Process

Cocoa leaves used are leaves that have dark green appearance specifications and have strong and firm (inelastic) leaf bones. The leaves that have been picked, were put into a container, and immediately steamed. The leaves that have been picked were weighed to determine the wet weight of the cocoa leaves. Next, it is steamed at a temperature > 100°C for 5-10 minutes. Furthermore, drying was carried out using a cabinet drier at a temperature of 50°C for 12 hours where the leaves were placed on an aluminum baking sheet. Then the dried leaves were powdered using a blender, and sieved to pass 30 mess size siever

2.1.3. Cocoa Leaves Extraction

The identification of bioactive compounds in cooca leaves and cocoa beans with extraction in Figure 1. Extraction was carried out by weighing 20 grams each of dried old leaf powder and dry cocoa beans powder then put into a 250 ml erlenmeyer, then added 100 ml of solvent, the ratio of the sample to the solvent was 1:5. The variety of solvents used were methanol, acetone and ethylacetate (Yang *et al*., 2011). Furthermore, extraction was carried out every 5 hours and continued with filtration using Whatman No.1 paper to remove the filtrate from the dregs or residues. The pulp was then re-extracted with the same solvent as before and carried out 2 times. Extraction was carried out using a hotplate with reverse cooling at 50°C and stirred using a magnetic stirrer. The extracted sample was then evaporated with a vacuum rotary evaporator at 40°C at 100 rpm. The thick extract obtained was weighed to calculate the yield of the extract then placed in a dark brown bottle, for further analysis.

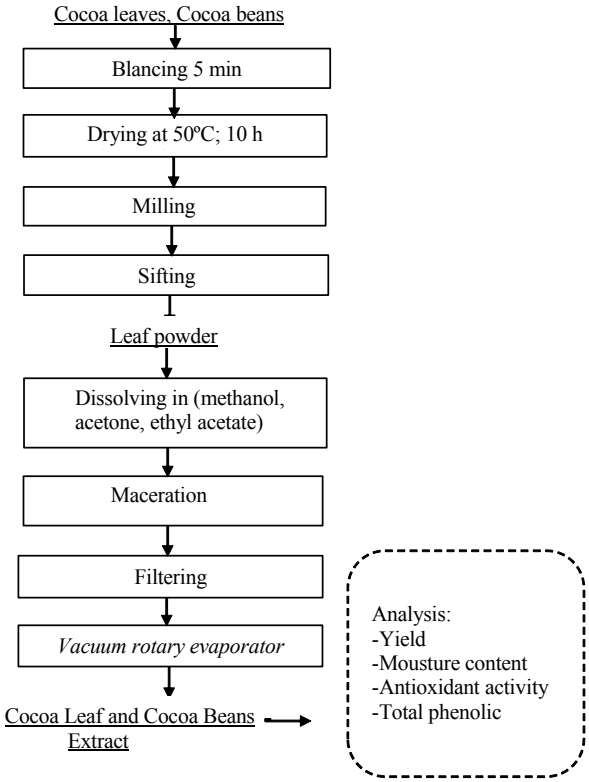

Figure 1. Flowchart of Identification of Bioactive Compounds in Cocoa Leaves

2.2. Analysis Antioxidant Activity using DPPH Method

Amount of 0.1 grams of cocoa leaf extract sample per weight of solid was weighed and then dissolved into a 25ml volumetric flask with the same solvent when used to extract up to the mark line. Then the solution was made to vary the concentration to 800 ppm, and 0 ppm. Each was added 3.9 ml of 40 M DPPH solution. Then incubation was carried out for 30 minutes and then the absorbance was measured. The absorbance of DPPH was measured by spectrometer with 515 nm (Zuhra *et al*., 2008). The following method was used to determine the DPPH solution's absorption value both before and after the addition of the extract:

$$\% \text{ RSA} = \frac{(A_{\text{blank}} - A_{\text{sample}})}{A_{blank}} x\ 100\% \quad (1)$$

where A_{blank} is absorbance of blank (without samples)

2.3. Antioxidant Activity Test

FRAP (Ferric Reducing Antioxidant Power) was used to measure the activity of antioxidant (Ozsoy *et al*., 2007). The solutions were made in series with concentrations of 800, 600, 400, 200, 0 ppm. A 10 ml test tube was used to mix 1 ml sample with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml solution of 1% $K_3(Fe(CN)_6)$. The mixture was incubated in a 50ºC water bath shaker for 20 min. After incubation, TCA solution of 2.5 ml of 10% was supplemented and centrifuged for 10 min. An amount of 2.5 ml of the top layer were thoroughly mixed with 0.5 milliliter of the 0.1% ferric chloride solution and 2.5 ml of distilled water. A spectrophotometer with a wavelength of 700 nm was used to determine the absorbance. The absorbance value of the sample at a wavelength of 700 nm was put into the linear equation of $y = ax + b$, which was derived from the gallic acid calibration curve, to determine the antioxidant reducing power. The standard curves were made with grade 0; 200; 600; 800 ppm. The value of EC 50 (Effective Concentration needed to inhibit the reduction of ferric ions to ferrous) is obtained from the value of x after replacing y with 50. The smaller the value of EC50 means the higher the antioxidant activity.

2.3. Total Phenolic Content

The goal of total phenolic content analysis is to count all compounds belonging to the phenolic group present in the sample. The polyphenol content in cocoa leaf extract was performed using the procedure of Folin-Ciocalteu (Cirillo, *et al*, 2012). The sample extract or standard of 0.5 ml were mixed with 0.5 ml of the Folin-Ciocalteu reagent, and then vortexed and incubated for 1 min. Sodium Carbonate 20% (w/v) of 1.5 ml was added and the mixture was vortexed. After that, 7.5 ml of distilled water was added to the mixture, and it was left at room temperature for 2 h. The absorbance measurement was performed at l 760 nm, and the concentration was then calculated. Total phenolic in the sample refers to the standard curve of gallic acid that has been prepared. Again, the absorbance of the sample at l = 700 nm was put into the linear equation of $y = ax + b$ derived from the gallic acid calibration curve, to determine phenol content. Results were expressed in mg EAG/gram, namely mg gallic acid equivalents per gram.

2.4. Experimental design

For each treatment, a completely randomized design (CRD) with three replications was performed. The data obtained were then analyzed using the Statistical Product and Service Solution software version 17 with the General Linear Model method at a significance level of 95% with a comparison of means using the Duncan method.

3. RESULTS AND DISCUSSION

3.1. Yield of Extraction

The yield of old cocoa leaf extract obtained by extraction with various solvents and cocoa bean extract with methanol solvent can be seen in Table 1. Based on Table 1, it can be seen that the highest yield was found in old cocoa leaves with methanol as solvent. The best yields of cocoa leaf extract with methanol as solvent this indicated that methanol can extraction optimally. This shows that the level of polarity in cocoa leaves with methanol solvent is close to the polarity of the extracted compound. According to Harborne (1987), solvent's capacity and nature for dissolving phenolic compounds was influenced by polarity degree of extracted compounds and the solvent. Additionally, in accordance with the polarization principle, a substance will only dissolve in a solvent having same polarity. A solvent's polarity can be determined based on the value of the dielectric constant. A solvent is considered to be more polar if its dielectric constant is higher. The value of dielectric constant of methanol (33.6) > acetone (21) > ethyl acetate (6.08) (Sudarmadji, 1989), therefore, methanol has the highest polarity compared to acetone and ethyl acetate solvents. Methanol is a universal solvent that all polar, semi-polar, and non-polar chemical elements present in natural plant products can be bound by it.

Table 1. Yield of cocoa leaf extract and cocoa beans

Sampel	Rendemen (%)±SD
Ethyl acetate solvent leaf extract	$4{,}846^{b}$±0,025
Acetone solvent leaf extract	$5{,}859^{c}$±0,051
Methanol solvent leaf extract	$7{,}129^{d}$±0,338
Methanol solvent cocoa bean extract	$2{,}909^{a}$ ±0,393

Superscript signs with different letters indicate significantly different values (α = 5%)

3.2. Moisture Content

Moisture content of cocoa leaf extract obtained by extraction with various solvents and methanol solvent cocoa bean extract can be seen in Table 2. From Table 2, it can be seen that cocoa leaf extract from various treatments had a moisture content in the range of 5.57-49.73%. The sample extract with the lowest moisture content was leaf extract with ethyl acetate as solvent. The best result based on water activity was cocoa leaf extract with ethyl acetate because the sample almost dry and given clearly analysis. Sample extract with ethyl acetate solvent in the form of dry paste so that the water content is relatively smaller than other solvents. The sample extract with acetone and methanol solvent was in the form of a wet paste so it tended to have a higher water content. Based on the degree of polarity, it is stated that methanol is the most polar than acetone and ethyl acetate, and acetone is more polar than ethyl acetate (Sudarmadji, 1989). Meanwhile, water is non-polar, ethyl acetate has a relatively low water content because based on its polarity, and ethyl acetate is the most non-polar compared to methanol and acetone. While the water is non-polar, this causes the water to be easily separated from the extract. So that the extract with ethyl acetate solvent tends to dry.

Table 2. Moisture content of cocoa leaf extract and cocoa beans (% wb)

Sample	Moisture content (%)±SD
Ethyl acetate solvent leaf extract	5,570[a] ± 0,141
Acetone solvent leaf extract	27,290[c] ±0,327
Methanol solvent leaf extract	13,633[b]± 0,167
Methanol solvent cocoa bean extract	49,730[d] ± 0,160

Superscript signs with unequal letters indicate significantly different values (α = 5%)

3.3. Total Phenolic

The total phenolic extract of old cocoa leaves obtained by extraction with various solvents and cocoa bean extract with methanol solvent can be seen in Table 3. Determination of phenolic content in this study was carried out using Folin reagent (Andarwulan *et al*., 1999). In this work, gallic acid was employed as a standard. Gallic acid is a phenolic compound and has strong antioxidant activity. The reducing ability of the phenolic hydroxy group served as the foundation for this research method. With the Folin-Ciocalteu reagent, all phenolic compounds, including simple phenols, can react (Huang *et al*., 2005). The presence of phenolic aromatic compounds can reduce phosphomolybdate phosphotungstate to blue molybdenum which can be measured by UV-Vis spectrophotometer. The number of milligrams of gallic acid in 1 gram of sample is used to describe the total phenolic content in plants as GAE (gallic acid equivalent) (Gheldof & Engeseth 2002).

Table 3. Total phenolic extract of cocoa leaves and cocoa beans (mg EAG/ g sample)

Sample	Average ± SD
Ethyl acetate solvent leaf extract	140,434 ± 0,091 [a]
Acetone solvent leaf extract	195,357 ± 0,184 [b]
Methanol solvent leaf extract	349,952 ± 0,051 [c]
Methanol solvent cocoa bean extract	391,402 ± 0,212 [d]

Different superscript letters indicate significantly different values at α = 5%.

According to the results, methanol had the greatest capacity in extracting total phenolic content from cocoa leaves. This is due to the fact that the polarity of the solvent and the extracted component affects the solvent's capacity and nature for dissolving phenolic compounds. According to the polarity law, a compound will dissolve in a solvent of the same polarity (Harborne, 1987). The total phenol and flavonoid content of cocoa leaf extract increased with increasing the polarity of the solvent used. Extraction process with methanol which is more polar than acetone and ethylacetate, which can produce extract with the highest total phenol and flavonoid content tall. Based on the degree of polarity, methanol is the most polar than acetone and ethyl acetate, while acetone is more polar than ethyl acetate (Sudarmadji, 1989). Phenolic compounds including flavonoids are polar compounds because they have a number of bound sugars, therefore flavonoids are more likely to soluble in polar solvents such as methanol. Measurement of phenolic compounds also are based on its reducing power. The reduction ability of the extract is positively correlated with the total content the phenol. The higher the total phenol content of the extract lead to higher abilities the reduction (Adaramola *et al*., 2016). Methanol extract which has the ability the highest

reduction was thought to be due to the extract of methanol contains the highest total phenol compared to acetone and ethyl acetate. It in line with other research, in the three extracts tested, namely methanol extract, ethanol and ethylacetate, the results showed that the extract methanol is an extract that has the ability to the highest reduction compared to extractethanol and ethylacetate (Cepeda *et al*., 2018). Based from this research indicated that methanol has the ability to the highest reduction compared to acetone and ethylacetate. Differences in the total phenol content of plant extracts depending on the nature of the polarity of the solvent is thought to be due to the difference solubility of phenolic compounds in solvents used. According to Zlotek *et al*. (2016), difference the structure of phenolic compounds will determine the solubility in different solvents polarity so that the number of phenolic compounds extracted depending on the type of solvent used. The high total phenolic in old cocoa leaf extract with methanol solvent explains that the characteristics of phenolic compounds in old cocoa leaf extract have the same polarity as methanol it can be measured with the value of the dielectric constant of a solvent, so that old cocoa leaf extract with methanol solvent produces the highest total phenolic compound content. Based on the value of the dielectric constant, a solvent can be distinguished by its polarity. The more polar a solvent is, the higher its dielectric constant value. The dielectric constant is in order of methanol (33.6) > acetone (21) > ethylacetate (6.08) (Sudarmadji, 1989). According to Firdaus (2011), phenol is a compound that has a hydroxy group and is capable of donating hydrogen so that it is stabilized by the resonance contained in the phenolic structure, so that this compound can function as an antioxidant.

3.4. Antioxidant activity by DPPH method (2,2-diphenyl-1-pycrilhydrazil)

The results of the antioxidant activity of old cocoa leaf extract obtained through extraction with various solvents and methanol-solvent cocoa bean extract can be seen in Table 4. According to the table, the highest antioxidant activity was obtained in the treatment using methanol solvent which was 86.99%. This means that old cocoa leaf extract with methanol as solvent contains bioactive compounds that can function as better antioxidants (Sultana *et al*., 2009). When compared to old cocoa leaf extract with other solvents, making it more effective in inhibiting DPPH free radicals.

Table 4. Antioxidant activity of cocoa leaf extract and cocoa beans

Sample	% RSA ±SD
Ethyl acetate solvent leaf extract	$39{,}774^{a}$ ±0,115
Acetone solvent leaf extract	$71{,}895^{b}$±0,630
Methanol solvent leaf extract	$86{,}928^{c}$±0,299
Methanol solvent cocoa bean extract	$93{,}486^{d}$±0,133

Superscript signs with unequal letters indicate significantly different values (α = 5%)

The presence of bioactive compounds in old cocoa leaf extract with methanol as a solvent indicates that the compound has relatively the same polarity as methanol. The other research, in the three extracts tested, namely methanol extract, ethanol and ethylacetate, the results showed that the extract methanol is an extract that has the ability to the highest reduction compared to extract ethanol and ethylacetate (Cepeda *et al*., 2018). It indicated that cocoa leaf extract with methanol has the ability the highest reduction compared the acetone and ethylacetate. Height leaf extract antioxidant activity with methanol solvent in line with the high extracted bioactive

components such as phenol, flavonoids, and tannins using a solvent methanol. Shahidi & Naczk (1995) suggested that compounds belonging to natural antioxidants from the class of phenolic compounds such as simple phenolic compounds, flavonoids, and tannins. In accordance with the principle of polarization, the acquisition of chemical compounds is based on the similarity of polarity to the solvent used (Harborne, 1987). When compared between methanol solvent leaf extract and cocoa bean extract, the amount of antioxidant activity in cocoa beans is greater. This indicates that the bioactive compounds present in cocoa beans have more polarity with methanol compared to leaves.

3.5. Antioxidant Activity using FRAP Methods

The results of the EC 50 antioxidant value of old cocoa leaf extract obtained through extraction with various solvents and methanol cocoa bean extract can be seen in Table 5. The test results from the FRAP method are expressed in the form of Effective Concentration (EC50) which is the concentration of antioxidant compounds from leaf extract needed to reduce ferric ions to ferrous ions. An antioxidant compound's potential is indicated by its reducing power. The capacity of an antioxidant to change Fe_3^+ into Fe_2^+ in this instance serves as a gauge for the reducing power (Cirillo & Iemma, 2012).

Table 5. EC 50 antioxidant value of cocoa leaves and cocoa beans

Sample	EC 50 ±SD (ppm)
Ethyl acetate solvent leaf extract	$2274{,}667^{d}$±0,577
Acetone solvent leaf extract	$698{,}380^{c}$±0,329
Methanol solvent leaf extract	$287{,}958^{b}$±0,144
Methanol solvent cocoa bean extract	$221{,}426^{a}$±0,262

Superscript signs with unequal letters in the same column and row indicate significantly different values (α = 5%)

Based on Table 5, it can be seen that the amount of antioxidants between solvents showed significantly different results. Antioxidant activity in leaf extract with methanol solvent showed the highest yield followed by acetone and then ethyl acetate. The amount of antioxidant activity in old cocoa leaf extract with which has the largest to the smallest results in a row is methanol, acetone, ethyl acetate solvent, which corresponds to the degree of polarity which indicates the polarity of the methanol solvent is higgest than acetone and ethyl acetate. The other reason, that cocoa leaf extract with methanol has the ability the highest reduction compared the aseton and ethylacetate Extracts that have the ability to reduce Fe+3 ions to Fe+2. indicates that the extract is an electron donor that can reduce metal ions which speed up the process oxidation so that it can function as an antioxidant secondary (Kasote *et al*., 2015). The higher the total antioxidant content in the extract lead to higher abilities the reduction. Methanol extract which has the ability the highest reduction was due to the extract methanol contains the highest phenolic compounds.

4. CONCLUSIONS

The best type of solvent to obtain leaf extract with total phenolic content, highest antioxidant activity, and yield of cocoa leaf extract was methanol solvent with total

phenolic content of 349.826 ± 0.386 mg EAG/g sample, antioxidant activity in % RSA was 86.928% ± 0.299. The EC 50 antioxidant value of old cocoa leaves was 287.958 ± 0.144 and the yield was 7.129 % ± 0.388. The amount of antioxidant activity and total phenolic of old cocoa leaf extract showed higgest results in methanol solvent. Phenolic compounds contained in cocoa leaf extract with methanol > acetone > ethyl acetate as solvent.

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