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ISOLATION AND ACTIVITY ANTIOXIDANT TEST OF COCOA POD HUSK ETHYL ASETAT EXTRACTS (*Theobroma cacao* L)

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Abstract. Isolation and testing of antioxidant activity with*1,1-difenil-2-pikril hidrazil* (DPPH) from ethyl acetate extract ofcocoa pod husk(*Theobroma cacao* L) has been carried out.*Theobroma cacao*extract (TCE) showed highly strong antioxidant activity with IC_{50} = 8.75 ppm and vitamine C = 6.07 ppm as positive control. Isolation of the active components of TCE by column chromatography using silica gel absorbent 60 mesh ASTM (Merck 774) and the eluent *n*-hexane: ethyl acetate (9:1), obtained 7 fraction combined (TCE 1 to TCE 7). There are 6 combined fractions having the potential as antioxidants, namely TCE 2 to TCE 7 with a range of IC_{50} (6.46 – 91.8 ppm). TCE 2 fraction has a very strong antioxidant activity with IC_{50} = 6.46 ppm. Separation of TCE 2 fraction on silica column chromatography obtained 4 combined fractions (TCE 2.1 to TCE 2.4). The test results of antioxidant activity showed that TCE 2.4 had very strong activity with IC_{50} = 42.7 ppm. For the TCE 2.2 fraction, preparative TLC was carried out using eluent *n*-hexane: ethyl acetate (9.5: 0.5) obtained by TCE 2.2.4 isolate with a melting point of 114-120 °C and was a steroid class.

Keyword: Cacao pod husk (*Theobroma cacao* L), Ethyl acetate extract, Antioxidant Activity and*1,1-diphenyl-2-Pycrilhidrazil* (DPPH).

I INTODUCTION

Indonesia is a third cocoa producing country (*Theobroma cacao* L) in the world after Ivory Coast and Ghana with production that continues to grow every year [1]. Cocoa is one of the plants that already being expanded in order to increase the source of foreign exchange from the non-oil and gas sector. This plant can flower and bear fruit throughout the year, so it can be a daily or weekly income for the community [2]. Based on statistical data in 2015, it is state that Southeast Aceh is the largest cocoa producing district in Indonesia after Makassar. Until now, the area of cocoa plantations in Southeast Aceh Regency has reached 20.130 Ha with a total production of 8.9 tons/year [3]. The part of cocoa used is seeds that are used to produce cocoa powder andcocoa butter.Cocoa powder is used as the basic ingredient in making instant chocolate drinks, cake, candies, and ice cream while cocoa fat is used as an ingredient in making soap and various cosmetics [4]. Until now, the use of cocoa is still limited to the seeds, while fruit skins are thrown away. Cocoa fruit skin is the largest part of the cocoa fruit component with a percentage of 75% and 25% seeds [5].

The study of cocoa beans has been widely reported, Misnawi *et al*. stateds that cocoa beans contain polyphenol compounds that have antioxidant activities that are beneficial to human health [6]. Corti*et al*. reported that cocoa beans contain polyphenol compounds that are higher than other food ingredients such as tea and wine [7]. Cocoa beans have many benefits for human health, including as a natural antioxidant that has the ability to improve the immune system, prevent coronary heart disease and cancer [8]. Panganiban *et al*. reported that cocoa is efficacious as an antiseptic, antidiuretic, eclectic, emmenagogue (lowers blood pressure; diabetes, inflammation of the intestine and Alzheimer's [10], insulin resistance, glucose tolerance, and platelet reactivity [11]. Compounds that can contribute to antioxidant activity in general are polyphenols and flavonoids, but other secondary metabolites can also contribute to antioxidant activities such as steroids and terpenoids. Yumas, reported that extracts of the bark from cocoa beans contained steroid and terpenoid compounds [12]. Jusmiati stated that the ethyl acetate extract of cocoa pod husk contains polyphenol compounds andflavonoids which has a strong antioxidant activity with IC_{50} = 0.9 ppm, but the separation of the active

antioxidant compound ethyl acetate extract has not been done. Therefore it is interesting to isolate the ethyl acetate extract of cocoa pod husk and test its antioxidant activity with DPPH [13]. The initial stage of ethyl acetate extract is done by obtaining ethyl acetate extract. The compound was then carried out further separation using gravity column chromatography with silica gel stationary phase 60 mesh ASTM (Merck 774) and the mobile phase to be determined by thin layer chromatography (TLC). The combined fraction which has strong antioxidant activity is then chromatographed until pure isolates are obtained. The pure isolates obtained were then identified using the Gass Chromatography-Mass Spectra technique (GC-MS).

II METHODOLOGY

The equipment used in this study included equipment for extraction and isolation namely Erlenmeyer, measuring cup, beaker, separating funnel, glass funnel, magnetic stirrer, analytical scales (ME104t / 00), rotary evaporator (Buchi R-100), oven, column chromatography gravity (Pyrex 250 mm with diameter 20.5 mm) and other supporting tools. Structural control was carried out using TLC (Silica gel 60 F_{254}) and spectrophotometer instruments namely Ultraviolet (AE-S60-2UP UV), and mass spectroscopy (Shimadzu MS-TQ8040). The materials used in this study are methanol, *n*-hexane and ethyl acetate solvents, the solvents used for extraction are technical solvents. Liebermann-Burchard reagent (glacial acetic acid -H_2SO_4), Mayer reagent (potassium tetraiodo mercuric), Dragendorff reagent ($Bi(NO_3)_3$) and Wagnerreagent (I_2 in KI). The ingrediente used for the antioxidant test are metanolp.a, DPPH powder and vitamin C.

Plant material

The sample used in this study was the not too old or young brown pod husk (The obroma cacao L), which was obtained from Lawe Loning Village, Lawe Sigala-gala sub-district, Southeast Aceh district.

Cocoa Pod Husk Extraction

Extraction of cocoa pod husk is dried and mashed to powder. Samples (4 kg) of powder were macerated with methanol for 24 h and repeated macerated until the clear filtrate was obtained, filtered and obtained filtrate. The methanol filtrate was then evaporated with a rotatory evaporator to obtain methanol extract. The methanol extract was partitioned with n-hexane and then separated from the methanol layer and the *n*-hexane layer. The methanol layer is evaporated to obtain concentrated methanol extract (*n*-hexane free). The methanol extract was then extracted with ethyl acetate to obtain a soluble layer and insoluble layer. The insoluble layer is then evaporated to obtain 9.99 g ethyl acetate extract. Then it tested for antioxidant activity and further isolation.

Antioxidant Test

Making DPPH solution of 0.4 mM

DPPH powder (BM 394.32 g/mol) was weighed 7.9 mg, and then dissolved with methanol in a 50 mL measuring flask, closed then homogenized. Then the solution is stored in a dark bottle.

Making variety solutions of cocoa pod husk extract and vitamin C.

The variation of the methanol extract concentration of cocoa fruit skin was previously made 500 ppm mother solution by dissolving each extract as much as 5 mg into ethanol until the volume reaches 10 mL. Furthermore, from the mother liquor, variations in solution concentrations of 25 ppm, 50 ppm, and 100 ppm were made. Variations in the test concentrations of antioxidant activity of extracts of cocoa fruit peel extract were carried out with 3 concentration variations, namely 25, 50 and 100 ppm. As a comparison, the antioxidant activity of vitamin C was tested, because vitamin C is a compound that has very high antioxidant activity. The main vitamin C solution is made by dissolving 3 mg of vitamin C with methanol until the volume is exactly 5 mL. Then diluted to 3, 6, 9, 12, and 15 ppm. Then it was homogenized with a vortex mixer and was incubated for 30 min at 37°C, then measured for absorption at a wavelength of 517 nm.

Preparaing a blank solution

As much as 1 mL of DPPH solution of 0.4 mM and the volume was adjusted to 5 mL with methanol in a test tube then homogenized with vortex mixer and incubated for 30 min at 37°C. Then the absorption was measured at a wavelength λ = 517 nm using a UV-Vis instrument.

Antioxidant activity test of cocoa pod husk extract

Antioxidant tests in research follow the procedures reported by Ginting *et al*. [14]. Cocoa pod husk extract at a concentration of 25 ppm as much as 250 µL, a concentration of 50 ppm as much as 500 µL and a concentration of 100 ppm as much as 1000 µL, each added with a DPPH solution of 0,4 mM as much as 1 mL and the volume then 5 mL with methanol and

the container closed with aluminum foil. Then it was homogenized using a vortex mixer and incubated for 30 min at 37 ° C, and its absorption was read at λ = 517 nm [15].

Calculation IC_{50}

IC_{50} value is an antioxidant concentration in ppm (µg/mL) which can inhibit as much as 50% of free radicals. The IC_{50} value is obtained from the intersection of a line between 50% of the inhibition and the axis of concentration, then entered in the equation $Y = a + bX$ which $Y = 50$ and the value of X indicates IC_{50}. The percentage of inhibition is calculated using the following formula at Eq. (1).

$$\%\,Inhibition = \frac{blank\ adsorption - sample\ absorption}{blank\ absorption} \times 100 \quad (1)$$

The extract is declared active if the IC_{50} value is less than 100 ppm (µg / mL) [16].

III RESULTS AND DISCUSSION

Cocoa Pod Husk Extraction

Cocoa bark extract obtained in the form of Theobroma cacao methanol (TCM) extract Theobroma cacao ethyl acetate (TCE) and Theobroma cacao *n*-hexane (TCH) with the highest percentage of yields 5.08; 1.6 and 1.46% respectively. Methanol extract has the highest percent yield, this is in accordance with what was reported by Dwiningsih, that the methanol extract of cocoa peels obtained the maximum yield [17]. It is estimated that most of the bioactive compounds contained in the cocoa extract are more polar in nature. Methanol solvent is a polar solvent, so the yield of TCM extract is more extracted when compared with TCE and TCH extracts which are semipolar and non-polar.

Phytochemical test

The phytochemical test of a sample is carried out to determine the content of secondary metabolites contained in a sample so that it can be an important guideline in isolating secondary metabolites. The phytochemical content in a sample is seen based on the reaction which shows positive results with certain reagents. Phytochemical tests carried out include alkaloids, flavonoids, terpenoids, steroids, saponins, and phenolics. The phytochemical test results of cocoa peel extract showed that TCM extract contained alkaloid compounds, detected both with Mayer, Wagner and dragendorff reagents. TCM extract also contains flavonoids, saponins, and phenolics, but does not contain terpenoids and steroids. TCE extract showed the presence of alkaloid compounds with all three reagents. Alkaloids can be attracted to methanol and ethyl acetate solvents because alkaloid compounds are polar, and ethyl acetate is semi-polar. According to Sastrohamidjojo, alkaloid compounds are secondary polar metabolites [17]. Other secondary metabolites found in TCE are flavonoids, terpenoids, steroids, and phenolics, but do not contain saponins. The TCH extract only contains terpenoids and steroids, whereas alkaloids, flavonoids, saponins, and phenolics are not detected because TCH extracts are non-polar.

Phytochemical analysis was carried out to qualitatively determine the secondary metabolites contained in fruit skin. Identified compounds include alkaloids, flavonoids, terpenoids, steroids, saponins and phenolics, which were tested against the three extracts, namely methanol, ethyl acetate and *n*-hexane extract. The phytochemical test results stated that TCM and TCE extracts contained alkaloid compounds, detected both with mayer, Wagner and dragendorff reagents, while TCH did not contain alkaloids. These results are in accordance with those reported by Azizahstating that, methanol extract of cocoa pods contains alkaloids [19]. According to Sastrohamidjojo, alkaloid compounds are secondary polar metabolites, so they can be extracted using semi-polar to polar solvents such as ethyl acetate and methanol.

TCM and TCE extracts also contain flavonoids and phenolic compounds. These results are in accordance with those reported by Rahmawaty, stating that the cocoa peel extracted with ethanol and acetone contains flavonoids and phenolic compounds [20]. So that it can be said that TCH does not contain flavonoids and phenolic compounds because TCH is a non-polar compound. TCM extract was not identified as a class of terpenoids and steroids, but was identified in TCE extract and TCH. These results are in accordance with the reports of Azizah *et al*. stated that, the skin of cocoa fruit extracted with ethanol contained terpenoids and steroids. Naufalin *et al*. states that the steroid and terpenoid secondary metabolites are non-polar, because they belong to the lipid group [21]. Saponins were not identified in the TCE extract, but were identified in the TCE extract and TCH. Saponin test is done using hot water. According to Harbone the test for the presence of saponins will cause stable foam when shaken with water [22]. TCM extract showed a group of saponin compounds with stable foam

formation for 30 minutes, whereas TCH and TCE extracts did not contain saponin compounds. Based on the results of this study, it can be concluded that the TCE extract contains the most secondary metabolites, namely alkaloids, flavonoids, saponins and phenolics, while the TCH extract contains secondary metabolites containing only two compounds namely terpenoids and steroids.

Activity antioxidant extract test

Based on the test results of the antioxidant activity, TCE has the strongest antioxidant activity with IC_{50} = 8.75 ppm and vitamin C = 6.07 ppm. This is in accordance with Jusmiati's report stating that the cocoa peel extract which has the strongest antioxidant activity is found in ethyl acetate extract which is 0.9 ppm then followed by methanol extract of 44.4 ppm and *n*-hexane extract of 126.2 ppm. Antioxidant activity according to Molyneux, is categorized as very strong, strong, medium and weak depending on the range of IC_{50} values [23]. DPPH radical capture ability by an antioxidant is expressed by the percent inhibition value. A high percentage of inhibition indicates that the compound sample used has the potential as an antioxidant. The 50% inhibition was obtained from the curve between% inhibitions of the sample concentration from the linear regression equation (Figure 1).

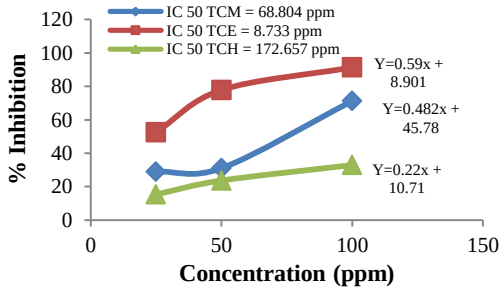

Figure 1 Correlation curve of % inhibition with cocoa pod husk extract concentration.

Cocoa pod husk extracts which have the highest linearity are TCE, TCM and TCH. It can be seen that TCE has the smallest IC_{50} value of 8.75 ppm. The TCE extract has a very strong antioxidant activity, this is because the IC_{50} value is below 50%. TCM extract has a strong antioxidant activity, because the IC_{50} value is below 100%, while the TCH extract is said to have no antioxidant activity, because the IC_{50} value is far above 200%. Based on pytochemical results TCE has flavonoids, terpenoids, steroids and phenolic compounds that are able to contribute to the antioxidant activity.This was obtained based on the report of Molyneux which categorizes the activity of an antioxidant.

Isolation of Cocoa pod husk Ethyl Acetate Extract

TCE extract (9.9 g) was impregnated with silica gel and put in a column filled with silica gel as much as 150 g which functioned as a stationary phase. The extract that has been put into the next column is eluted using the mobile phase of *n*-hexane: ethyl acetate (1:1) and (1:1,5) to obtain 90 fractions. Each fraction was monitored using thin layer chromatography (TLC) with an elution gradient system and obtained 7 combined fractions (TCE 2.1 through TCE 7). TCE 2 extract has the greatest weight, which is 4,6 g with the strongest antioxidant activity (IC_{50} = 6.46 ppm). This IC_{50} value is close to the IC_{50} value of positive control of vitamin C (IC_{50} = 6.2 ppm). The combined fraction of TCE 3 to TCE 7 is categorized as having strong antioxidant activity, because it has an activity of IC_{50} values below 100 ppm. Correlation curve% inhibition of TCE 1 concentrations up to TCE 7 (Figure 2).

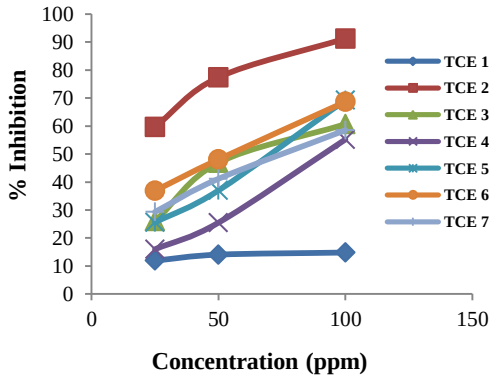

Figure 2 Colleration curve % inhibition to TCE 1-7 concentrations.

The combined fraction with the highest linearity ie TCE 2 fraction, the colleration of concentration and% inhibition showed the value of IC_{50} (Table 1). The regression equation will be used as an equation to determine the IC_{50} value of each combined fraction. Subsequent purification was carried out on TCE 2 (2.3 mg) fractions chromatographically. TCE 2 columnchromatography results were obtained as many as 60 fractions, then the demanded by TLC. The same stain pattern is combined to get 4 combined fractions. The test results of the antioxidant activity of the TCE 2.2.4 isolate can be determined by making a correlation curve of the % inhibition to the concentration of the combined fraction of TCE 2.2 (Figure 3).

Table 1 TCE (1-7) Combined Fraction Regression Equation.

Extract	Regression Equation	IC_{50} (ppm)
TCE 1	Y = 0.044x + 0.477	1104.7
TCE 2	Y = 0.432x + 52.79	6.6460
TCE 3	Y = 0.542x + 43.95	70.130
TCE 4	Y = 0.622x + 71.62	91.828
TCE 5	Y = 0.519x + 29.43	64.432
TCE 6	Y = 0.421x + 26.59	54.515
TCE 7	Y = 0.383x + 20.62	76.223

Note: TCE : *Theobroma cacao* Ethyl acetate
IC_{50}: *Inhibition Concentration* 50%

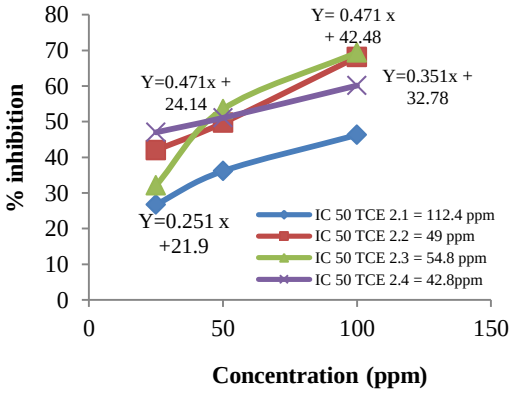

Figure 3 Correlation curve % inhibition to the concentration of the combined fraction of TCE2.2.1-2.2.4

Based on Figure 3 the regression equation is determined from each isolate so that IC_{50} values can be determined. The four isolates obtained had very strong antioxidant activity with IC_{50} values 42.8 – 49 ppm and vitamin C IC_{50} = 6.2 ppm.

CONCLUSION

Ethyl acetate extract has a very strong antioxidant activity with an IC_{50} value of 8.54 ppm. TCE 2.4 has the strongest antioxidant activity with an IC_{50} value of 42.8 ppm and is a steroid class.

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