Antioxidant and cytotoxic activity of ethyl asetat extracts of cocoa pod husk (Theobroma cacao L)

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Abstract. Isolation of ethyl acetate extract from cocoa pod husks (Theobroma cacao L) and testing of their cytotoxic as well as antioxidant activities has been done. The cytotoxic activities test has been carried out employing Brine Shrimp Lethality Test (BSLT) method, while the antioxidant activities test was employing 1,1-diphenyl-2-picrylhydrazyl (DPPH) and vitamin C as positive control. In the cytotoxic test, all fractions showed excellent cytotoxic activity with a range of LC50 = ∞ ppm to 107.15 ppm. The extract also showed antioxidant activity with IC50 = 163.35 ppm and vitamin C = 0.014 ppm. Isolation of the active components of ethyl acetate extract using silica gel as stationary phase and n-hexane:ethyl acetate (9:1) eluent, yielded a combination of 9 fractions (TCE1-TCE9). The separation of TCE 5 produced white needle crystals with a melting point of 128 - 131°C. Based on the stain pattern, the crystal belongs to a phenolic compound.

1. Introduction
Cocoa (Theobroma cacao L) is one of Indonesia's export products whose production growth reaches 3.72% per year and 639.14 thousand tons of dried beans. Indonesian cocoa production is the second highest after Ivory Coast and Ghana [1]. The island of Sumatra is the second largest cocoa producer in Indonesia after Sulawesi Island. The province that produces the most cocoa on Sumatra Island is that Aceh Province where Southeast Aceh Regency are the main producers. At present, the total cocoa production in Southeast Aceh on 20.130 hectares of land is 8,989 tons per year [2]. This high cocoa production produces considerable waste, namely cocoa pod husks (CPH). Every ton of cocoa beans produces 10 tons of CPH waste. The waste not only requires a serious disposal site [3][4], but also provides an opportunity for its assessment and use.

So far, the main utilization of CPH in Indonesia is only for animal feed (Aceh AIAT, 2015). Meanwhile, on a laboratory scale, several studies on CPH have been carried to explore their effectiveness as adsorbents for Rhodamine B [5]. Meanwhile, on a laboratory scale, several studies on CPH have been carried to explore their effectiveness as adsorbents for Rhodamine B [6], as sources of pyrolysis oil [7], as pectin sources [8][9][10][11], as mineral sources, as soap-making ingredients, as activated carbon [12], as anti-inflammatory [13][14], as anti-caries on teeth [15][16], as dietary ingredients [17] and as antioxidants [18][19][20][21].

Several studies have also been conducted to isolate useful chemical compounds from cocoa plantation waste [22][7][23]. Carmona (2017) reports that fresh CPH contains phenolic compounds, catechins, quercetin, (-) - epicatechin, gallic acid, coumarin and protocatechuic. Other research results show that

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polyphenol compounds and their derivatives contribute to antioxidant activity [24], cytotoxic activity [25] and are also good for synthesis of metal nanoparticles [26][27][28][29]. Although secondary metabolite compounds of CPH from ethanol extract have been previously reported to have antioxidant activity [24], but it is very possible that the isolated compound of the CPH investigated will be different from those that have been reported. This is due to the fact that the geographical location and the difference in season will affect the content of secondary metabolites in each plant [30][31].

Based on the background above, it is interesting to do further research on the isolation of ethyl acetate extract of cocoa pods taken from Southeast Aceh and performing the cytotoxic activity test using the Brine Shrimp Lethality Test (BSLT) method as well as antioxidant activity test using diphenyl picrylhydrazyl (DPPH). These methods are easy, inexpensive, and suitable for screening toxicity and antioxidants [32][33].

2. Experimental part

2.1. Sample extraction

Extraction of cocoa fruit peel was started by drying and mashing process. The cocoa pod husk powder as much as 15 kg was macerated with methanol for 24 h. Methanol filtrate was concentrated using a rotary evaporator to obtain methanol extract. It was then added with methanol and partitioned with n-hexane to obtain methanol and n-hexane extract. Methanol extract was extracted with ethyl acetate, the filtrate obtained was evaporated using rotary evaporator to obtain ethyl acetate extract. Each extract was tested for cytotoxic activity and antioxidant activity.

2.2. Isolation of active extracts of cocoa pod husk (CPH)

The active extract (30 g) was isolated by chromatography, the gravity column using silica gel 60 G as the stationary phase and the mobile phase (eluent) that had been determined using TLC. Each fraction was collected in a 25 mL test tube and monitored using TLC. Fractions with the same stain pattern were combined and tested for cytotoxic activity and antioxidant activity. The chromatographic combined fractions and the resulting isolates were recrystallized until pure compounds were obtained, it was then monitored using 2-dimensional TLC and determined their melting point.

2.3. Cytotoxic activity test (BSLT method)

Toxicity tests were carried out based on the method of Ginting et al. (2014) which was initiated by hatching Artemia salina Leach larvae for 48 h using sea water. After 48 h the shrimp eggs will hatch into small sea shrimps called naupii. They are ready to be used for research. The toxicity test was carried out by entering 10 of 48-hour Artemia salina Leach shrimp larvae into a bottle containing extract solution and sea water which had varied in concentrations of 500, 100 and 10 ppm. Each concentration was repeated three times (triplo), where sea water with absence of extract was employed as control. The trial bottle is stored under TL lamp lighting. Observations are made after 24 h. The number of dead shrimp larvae for each concentration was calculated and recorded. The standard criterion for measuring mortality of larvae based on their movement during observation. The results are compared with controls [34]. The LC50 value was obtained by using microsoft excel 2019 where the LC50 value was the concentration of the test substance that could kill shrimp larvae by 50%.

2.4. Antioxidant activity test (DPPH method)

The testing of antioxidant activity from the extract was carried out by DPPH method, namely by preparing a DPPH solution of 0.4 mM, Vitamin C solutions with concentration of 3, 6, 9, 12 and 15 ppm, and preparing the extract solution with concentration of 25, 50 and 100 ppm. 1 mL of the DPPH solution was added each of following solution: 250 µL of the 25 ppm extract solution, 500 µL of 50 ppm extract solution, and 1000 µL of 100 ppm extract solution. The volume each obtained mixtures are then added with methanol to reach 5 mL. Containers were then covered with aluminum foil. Mixtures were then homogenized using a vortex mixer and incubated for 30 min at 37°C. The absorbance of DPPH (violet) to
DPPH-H (yellow) were then recorded at 517 nm. Distilled water or solvent is used as a negative control and ascorbic acid or vitamin C as a positive control [35]. The sample concentration required to reduce 50% DPPH radical (IC50) was calculated from linear regression analysis. The extract is declared active if the IC50 value is less than 100 ppm (μg/mL) (Awe et al. 2013). DPPH radical activity from sample extracts is calculated as follows (equation(1)).

\[
DPPH \text{ Inhibition Ability (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100
\]  

3. Results and Discussion

3.1. Preparation of ethyl acetate extract of Cocoa Pod Husks (CPH)
The CPH was collected from Laweloning sub-district, in Southeast Aceh. Once collected, the wet CPH is removed from the fat and cut into small pieces. It was then dried at room temperature. After drying, 15 kg CPH is mashed into powder, then macerated with methanol solvent. The results of maceration of 6 kg dried CPH with methanol produced extracts of 36.78 g (44.88%). Methanol extract was partitioned with n-hexane and produced 68.8 g (40.43%) of n-hexane extract. Methanol extract which was partitioned with n-hexane was extracted with ethyl acetate solvent and produced 17.98 g (10.51%) of ethyl acetate extract. It was then tested for phytochemicals. The results of phytochemical tests showed that CPH contained alkaloid compounds, phenols, flavonoids, terpenoids, steroids and did not contain saponins. Furthermore, the isolation of the fraction of the compounds was done using gravity chromatography columns. The extracts and ethyl acetate fractions were then employed in cytotoxic and antioxidant activity tests.

3.2. Isolation of ethyl acetate extract of cocoa pod husks
The *Theobroma cacao* L. ethyl acetate extracts (TCE) as many as 38 g was separated by fraction using gravity chromatography column and produced nine combined fractions (TCE 1-TCE 9). Needle-shaped crystals (30 mg) were obtained in TCE 5 with a melting point of 128 - 131°C. Furthermore, the crystals were object for TLC on two different plates. One TLC plate was sprayed with vanillin sulfate and the other dipped with FeCl₃. The spot pattern was obtained as shown in Figure 1.

![Figure 1. a. TLC plate dipped in FeCl₃; b. TLC plate sprayed with vanillin-sulfate](image)

Based on the results of TLC and melting point, it is very possible that the obtained crystals contain the same compound due to the similarity of the spot pattern on TLC. On the other hand, the range of melting point is less than five degrees. Based on the spot pattern formed, it showed that the crystal contains compounds from the family of polyphenols due to blackish green color when sprayed with FeCl₃, and blackish purple when sprayed with vanillin sulfate [36].
3.3. Test of cytotoxic activity of the extract and TCE1-TCE9 fractions (BSLT method)
The extract and the combined fraction (TCE 1-TCE 9) obtained were tested for cytotoxic activity against Artemia salina Leach larvae. Cytotoxic test results are shown in Table 1.

Table 1. The results of the toxicity test of ethyl acetate extract and the combined fraction of TCE 1-TCE 9 using the BSLT method

| Samples | Concentrations (ppm) | LC50 (ppm) |
|---------|----------------------|------------|
|         | 10 100 500 |               |
| Extract | Living Larvae | Living Larvae | Living Larvae | Dead Larvae | Living Larvae | Living Larvae | Dead Larvae | Living Larvae | Living Larvae | Dead Larvae | Living Larvae | Living Larvae | Dead Larvae | LC50 |
| 4 10 6 22 8 5 5 10 20 10 3 3 10 16 14 47.86 |
| TCE 1 6 4 3 1 17 0 4 4 8 22 3 3 0 3 27 2.14 |
| TCE 2 8 7 10 25 5 5 7 4 16 14 3 4 3 10 20 107.15 |
| TCE 3 0 0 0 0 30 0 0 0 0 30 0 0 0 0 30 ∞ |
| TCE 4 4 4 0 8 22 2 3 3 8 22 2 2 3 7 23 2.09 |
| TCE 5 3 2 3 8 22 2 2 4 8 22 2 1 2 5 25 0.81 |
| TCE 6 4 4 5 13 17 4 4 4 12 18 2 0 2 4 26 3.39 |
| TCE 7 1 0 1 2 28 0 1 1 2 28 0 0 1 1 29 60.25 |
| TCE 8 4 1 4 9 21 2 3 1 6 24 1 0 0 1 29 100 |
| TCE 9 3 5 6 14 16 2 5 7 14 16 1 3 3 6 24 38.90 |
| Control 10 10 10 0 0 10 10 10 0 0 10 10 10 0 0 ∞ |

Description: the number of larvae in each concentration are 10, total of three repetitions are 30.

Based on Table 2, it can be concluded that the extract and all its fractions have very good cytotoxic activity. The best cytotoxic activity is the TCE 3 fraction because all shrimp larvae die at all concentrations. It is therefore recommended to carry out testing with a smaller concentration. The highest sequence of cytotoxic activities based on the highest to lowest LC50 was TCE3 (LC50 = ∞ ppm), TCE5 (LC50 = 0.81 ppm), TCE 4 (LC50 = 2.09 ppm); TCE1 (LC50 = 2.14 ppm), TCE6 (LC50 = 3.39 ppm), TCE9 (LC50 = 38.90 ppm), TCE extract (LC50 = 47.86 ppm), TCE7 (LC50 = 60.25 ppm), TCE8 (LC50 = 100 ppm) and TCE2 (LC50 = 107 ppm). The cytotoxic properties of ethyl acetate extract showed very good activity because the extract contained a group of polyphenols which still bind to the sugar group (glycosides), it is known that compounds containing OH groups are more active to kill Artemia salina Leach larvae [34].

3.4. Antioxidant activity test of the extract and TCE1-TCE9 fractions using DPPH method

Measurements of the antioxidant activity test were carried out using the instrument UV-Vis spectrophotometer at the maximum wavelength at λmax of 517 nm. The positive control used was vitamin C and the negative control was DPPH. The test results of antioxidant activity of the extract showed DPPH absorbance value of 0.927 ppm, while absorbance of vitamin c at 3, 9 and 12 ppm concentrations are 0.417, 0.050 and 0.036 respectively. This antioxidant activity test was carried out at variations in concentrations of 25, 50 and 100 ppm with 3 repetitions, so that the average values of absorbance of the extract and the combine fractions (TCE1-TCE9) were obtained and could be viewed in in Table 2. The table shows that the extract has an IC50 value of 163 ppm. Based on the literature about the strength of antioxidant activity [37][38], the extract and TCE1-TCE6 fraction have weak antioxidant activity due to IC50 above 150 ppm. While the TCE7-TCE9 fraction has antioxidant activity which is relatively strong because it has IC50 < 100 with a range of 62 - 75 ppm.
Table 2. The results of the antioxidant activity test of the extract and the combined fraction of TCE 1-TCE 9 using the DPPH method

| Samples  | DPPH Absorbance | Concentrations (ppm) | Sample Absorbance | Inhibition (%) | IC 50  |
|----------|-----------------|----------------------|-------------------|----------------|--------|
|          |                 | 25                   | 0.693             | 25.24272       |        |
|          |                 | 50                   | 0.682             | 26.42934       | 163.35 |
|          |                 | 100                  | 0.568             | 38.72708       |        |
| Extract  |                 | 25                   | 0.891             | 3.883495       |        |
|          |                 | 50                   | 0.840             | 9.385113       | 387.74 |
|          |                 | 100                  | 0.800             | 13.70011       |        |
| TCE 1    |                 | 25                   | 0.881             | 4.962244       |        |
|          |                 | 50                   | 0.819             | 11.65049       |        |
|          |                 | 100                  | 0.789             | 14.88673       | 380.24 |
| TCE 2    |                 | 25                   | 0.813             | 12.29773       |        |
|          |                 | 50                   | 0.78               | 15.85761       |        |
|          |                 | 100                  | 0.774             | 16.50485       | 762.04 |
| TCE 3    |                 | 25                   | 0.814             | 12.18986       |        |
|          |                 | 50                   | 0.778             | 16.07335       |        |
|          |                 | 100                  | 0.735             | 20.71197       | 362.83 |
| TCE 4    | 0.927           | 25                   | 0.782             | 15.64186       |        |
|          |                 | 50                   | 0.756             | 18.4466        |        |
|          |                 | 100                  | 0.735             | 20.71197       | 551.10 |
| TCE 5    |                 | 25                   | 0.748             | 19.3096        |        |
|          |                 | 50                   | 0.74               | 20.1726        |        |
|          |                 | 100                  | 0.717             | 22.65372       | 704.88 |
| TCE 6    |                 | 25                   | 0.716             | 22.7616        |        |
|          |                 | 50                   | 0.462             | 50.16181       |        |
|          |                 | 100                  | 0.275             | 70.33444       | 62.07  |
| TCE 7    |                 | 25                   | 0.356             | 61.59655       |        |
|          |                 | 50                   | 0.328             | 64.61704       |        |
|          |                 | 100                  | 0.063             | 93.20388       | 6.09   |
| TCE 8    |                 | 25                   | 0.706             | 23.84035       |        |
|          |                 | 50                   | 0.563             | 39.26645       |        |
|          |                 | 100                  | 0.356             | 61.59655       | 75.36  |

The most powerful antioxidant fraction was the TCE8 fraction with IC_{50} of 6.089 ppm. The small IC50 shows that only with a small concentration (6.089 ppm), the TCE8 fraction has been able to counteract 50% of free radicals. The antioxidant properties of these polyphenolic compounds derive from their ability to donate an electron to free radical compounds such as DPPH [39][40]. The correlation and the linearity between the extract- or fraction concentration and the inhibition ability are shown in Figure 2.
4. Conclusion
The conclusion of this study is that the extract of cocoa pod husks contains polyphenol compounds. The extract showed relatively weak antioxidant activity with IC$_{50}$ = 163.35 ppm, but it had strong cytotoxic activity with LC$_{50}$ = 47.86 ppm. The fraction that showed the highest antioxidant activity was the TCE8 fraction with IC$_{50}$ = 6,089 ppm. While the fraction that shows the highest cytotoxic activity is TCE3. Some reported literatures noted that polyphenol compounds can be used to synthesize metal nanoparticles or metal complexes to increase antioxidant and cytotoxic activity. The future research can be continued with the synthesis of metal nanoparticles or metal complexes using the extract.

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