

DPPH-based antioxidant activity of dichloromethane extract from cocoa pod husk (*Theobroma cacao* L.)



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Abstract. *Theobroma cacao* L. is a plant that is reported to have strong antioxidant potential. A study was carried out to determine the antioxidant activity of dichloromethane extract of cocoa pod husk using 1,1-diphenyl-2-picrylhydrazyl (DPPH). The cocoa pod husk was macerated using *n*-hexane, ammonia-dichloromethane, and methanol. The TCD extract of cocoa pod husk showed the lowest IC₅₀ value of 14.59 ± 0.54 ppm based on the DPPH inhibition assay. The dichloromethane extract was isolated by gravity column chromatography with Silica gel G60 as the stationary phase and *n*-hexane: ethyl acetate as the mobile phase (gradient elution system). Fractions with identical staining patterns were combined into six fractions (TCD 1-TCD 6). The TCD 6 showed the lowest IC₅₀ value of 127.05 ± 0.54 ppm according to the DPPH inhibition assay. Based on the TLC results, TCD 4 was further isolated and four fractions (TCD 4.1 to TCD 4.4) were obtained. The TCD 4.4 had the lowest IC₅₀ value (38.66 ± 0.96 ppm). The TCD extract showed the presence of the dominant compound, γ -sitosterol, as determined by GC-MS analysis.

Keywords: antioxidant, cocoa pod husk, dichloromethane extract, DPPH, *Theobroma cacao* L.

INTRODUCTION

Theobroma cacao L. is a plant that is cultivated in many regions of Indonesia. In 2020, the average cocoa plantation area in Indonesia reached 1.5 million hectares, with cocoa production around 706,500 tonnes [1]. Cocoa production produces several wastes obtained from the process of removing cocoa beans [2]. Approximately 70-75% of the *T. cacao* fruit is considered as waste material. Producing cocoa waste releases carbon dioxide and methane, causing environmental damage [3]. Cocoa is a significant export commodity, contributing 12.7% to global cocoa production. Indonesia's cocoa production has averaged 683 kilotonnes over the past decade [4]. Cocoa pod husk waste is currently underutilized. Some parts of cocoa are reported to contain chemical compounds such as theobromine, luteolin, naringenin, quercetin, quercetin 3-O-arabinose, isoquercitrin, and quercetin 3-O-galactoside, and apigenin [5]. The high content of polyphenols such as arachatechin, anthocyanins, and proanthocyanidins in cocoa may have potential as antioxidants [6].

Previous studies have reported that the ethyl acetate extract of cocoa pod husk had an IC₅₀ value of 163.35 ppm and 137.51 ppm showing weak antioxidant activity [7, 8]. In another study, the ethyl acetate extract of cocoa pod husk obtained the highest IC₅₀ value of 8.75 ppm, indicating that the ethyl acetate extract had strong antioxidant activity [9]. The water extract of *T. cacao* exhibits antioxidant properties, as evidenced by an IC₅₀ value of 97.69 ± 0.46 ppm [10]. The chloroform extract was also reported to have an IC₅₀ of 4.56 ppm and its fractions showed an IC₅₀ ranging from 37.91 to 284.48 ppm [11]. Previous studies have provided evidence for the antioxidant properties of *T. cacao* pod husk extracted using *n*-hexane, water, ethyl acetate, and chloroform solvents. However, the extraction using the multistage maceration method and the DPPH-based antioxidant activity of dichloromethane extract of *T. cacao* pod husk has not been investigated. Thus, it is essential to investigate the potential of the dichloromethane extract of *T. cacao* pod husk as an antioxidant.



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METHODOLOGY

Materials

The samples were collected from Lawe Sigala-gala, Southeast Aceh Regency (3°21'10.49"N and 97°54'47.6" E). Chemicals used in this study included dichloromethane, *n*-hexane, methanol, ammonia, hydrochloric acid, Mg metal, distilled water, FeCl₃, Liebermann-Burchard reagent, Wagner reagent, Mayer reagent, Dragendorff reagent, silica gel G60, ascorbic acid, and 2,2-diphenyl-1-picrylhydrazyl (DPPH). The chemicals were analytical grade and procured from Merck.

Cocoa Pods Husk Extraction

The cocoa pod husk, which is brownish-yellow in color, was crushed using a pulp grinder. Then washed with water and air-dried at room temperature. The cocoa pod husk powder (3 kg) was macerated using *n*-hexane for 72 hours and then concentrated using a rotary evaporator to obtain the *T. cacao* *n*-hexane (TCH) extract. The residue obtained was treated using ammonia 10% and left for 12 hours. After that, the residue was macerated using dichloromethane. The filtrate was concentrated using a rotary evaporator to obtain *T. cacao* dichloromethane (TCD) extract. The residue was macerated using methanol to obtain *T. cacao* methanolic (TCM) extract [12].

Isolation of Dichloromethane Extract

The dichloromethane extract (3.31 g) was isolated by gravity column chromatography. Silica G60 was used as the stationary phase and *n*-hexane: ethyl acetate (8:2 v/v) as the mobile phase. A gradient elution system was used in which the elution was started with 100% *n*-hexane and ended with 100% ethyl acetate. The eluate was collected every 50 mL in a test tube. The staining patterns of fractions were observed under UV light at 254 nm and 366 nm. Then the fractions with identical staining patterns were combined to form a combined fraction (TCD 1-TCD 6). The resulting fraction was then assayed for DPPH inhibition assay [13].

DPPH Inhibition Assay

The antioxidant activity of the samples was evaluated using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. DPPH powder (7.9 mg) was dissolved in methanol in a 50 ml volumetric flask and homogenized to prepare the DPPH solution. The flask was then covered with aluminum foil and stored in a dark room. *T. cacao* dichloromethane (TCD) extract and fractions were varied in concentrations of 100, 50, 25, 12.5, and 6.25 ppm. 2.5 mg of extracts and fractions were dissolved in methanol p.a. Ascorbic acid standards at concentrations of 3, 6, 9, 12, and 15 ppm were used as positive control. DPPH 0.4 mM (1 mL) was added to each solution and homogenized by a vortex mixer then incubated at 37°C for 30 minutes. The absorbances of the sample were measured at 517 nm using a UV-Vis spectrometer. The IC₅₀ value was determined using a linear curve equation where the x-axis exhibits the concentration of the sample, and the y-axis represents the percentage of inhibition [10].

RESULTS AND DISCUSSION

Extraction and Phytochemical Analysis

The *T. cacao* dichloromethane (TCD) extract was obtained as much as 4.67 g (0.47%) and the *T. cacao* methanol (TCM) extract was obtained at 14.64 g (4.88%). The highest yield was found in TCM extract (4.88%). The difference in extract yields obtained may be caused by differences in the polarity of the compounds against the solvent used [14]. Qualitative screening of TCD extract showed positive results for terpenoids, alkaloids, and steroids. Moreover, following a reaction with Mg, TCD extract exhibits a positive result for phenolic compounds after reacting with FeCl₃. Besides, the TCD extract demonstrated a negative result for saponins. The TCM analysis demonstrated the positive result of terpenoids, alkaloids, flavonoids, steroids, saponins, phenolics, and tannins. A previous study [9] found alkaloids, phenolics, saponins, and flavonoids in the methanol extract of cocoa pod husk.

DPPH Antioxidant Activity

The extracts obtained from the maceration process were evaluated for antioxidant activity using DPPH. The smaller the IC₅₀ value obtained, the greater the antioxidant activity possessed by a sample [14]. The DPPH antioxidant activity of cocoa pod husk extracts is shown in Table 1.

Table 1 demonstrates that the higher the concentration of the samples, the higher the percentage inhibition value. The higher the percentage inhibition value, indicating the potential to act as an antioxidant. The IC₅₀ value was determined using the equation derived from the percent inhibition and sample concentration. The TCD extract exhibited the highest antioxidant activity as suggested by the DPPH inhibition assay, with an IC₅₀ of 14.59 ± 0.54 ppm, while the TCM and TCH extracts exhibited IC₅₀ values of 280.22 ± 0.32 ppm and 155.76 ± 1.10 ppm, respectively (Table 1). As the IC₅₀ value of the TCD extract is less than 50 ppm, it is classified as a particularly very strong antioxidant. The TCD extract is categorized as a very strong antioxidant with an IC₅₀ value below 50 ppm. Antioxidants are categorized as very strong if they have a value below 50 ppm, strong if IC₅₀ at 50-100 ppm, moderate if IC₅₀ at 100-150 ppm, and weak if IC₅₀ is above 150 ppm [15].

Nonetheless, the observed DPPH antioxidant activity of the TCH and TCM extracts was graded as weak. This finding corroborates prior experiments that classified the TCH extract as a weak antioxidant, as reflected in its IC₅₀ values of 172.68 ppm and 116.70 ± 0.86 ppm [9,10]. The TCD extract exhibits the highest IC₅₀ value of 14.59 ± 0.54 ppm as suggested by the DPPH inhibition assay. Therefore, column chromatography was conducted to isolate the active compound of the TCD extract.

Isolation of Dichloromethane Extract

The TCD extract (3.31 g) was isolated and impregnated using silica. The Elution process was performed using nonpolar to polar solvent (gradient elution system). Six fractions (TCD 1-TCD 6) were obtained by column

Table 1. DPPH antioxidant activity of *T. cacao* pod husk extracts and fractions (TCD 1-TCD 6)

Sample	Inhibition (%)			IC ₅₀ (ppm)
	100 ppm	50 ppm	25 ppm	
TCH	19.32 ± 0.08	12.60 ± 0.15	6.30 ± 0.08	280.22 ± 0.32
TCD	65.98 ± 0.07	54.40 ± 0.14	53.17 ± 0.32	14.59 ± 0.54
TCM	38.59 ± 0.01	24.68 ± 0.04	22.85 ± 0.15	155.76 ± 1.10
TCD 1	31.89 ± 0.02	23.32 ± 0.16	21.93 ± 0.07	233.68 ± 0.35
TCD 2	21.00 ± 0.07	17.85 ± 0.29	15.84 ± 0.07	525.09 ± 1.11
TCD 3	30.86 ± 0.15	25.23 ± 0.07	22.96 ± 0.22	282.41 ± 0.92
TCD 4	29.93 ± 0.01	26.01 ± 0.06	24.77 ± 0.00	387.82 ± 0.65
TCD 5	44.48 ± 0.01	37.82 ± 0.22	30.24 ± 0.15	127.05 ± 0.54
TCD 6	44.18 ± 1.57	40.79 ± 6.21	35.77 ± 0.15	130.54 ± 0.71

DPPH antioxidant activity was expressed as mean ± SD. TCH, TCD, and TCM refer to *n*-hexane, dichloromethane, and methanol extract from cocoa pod husk.

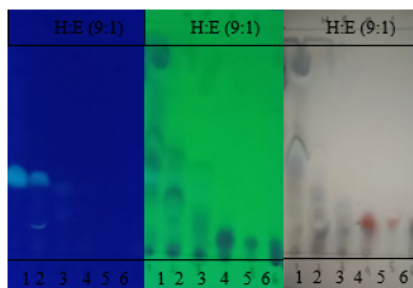


Figure 1. The TLC chromatogram of fractions TCD 1-TCD 6 using UV lamp 366 nm (a) UV lamp 254 nm (b) and spraying with vanillin sulfate stain reagent (c)

Table 2. DPPH antioxidant activity of subfractions (TCD 4.1- TCD 4.4)

Sample	Inhibition (%)			IC ₅₀ (ppm)
	100 ppm	50 ppm	25 ppm	
TCD 4.1	21.77 ± 0.16	11.48 ± 0.77	10.99 ± 0.31	289.44 ± 0.76
TCD 4.2	32.28 ± 0.08	19.61 ± 0.16	11.31 ± 0.01	163.28 ± 0.51
TCD 4.3	38.90 ± 0.00	32.92 ± 0.27	28.61 ± 0.08	180.99 ± 0.65
TCD 4.4	76.45 ± 0.23	65.20 ± 0.47	36.91 ± 0.53	38.66 ± 0.96

chromatographic elution. The thin-layer chromatography results of TCD 1 to TCD 6 are shown in Figure 1.

Figure 1 shows that the staining pattern with a low R_f value indicates that the compound is more polar and is retained longer in the column by interaction with silica, which acts as a stationary phase. The TLC results, as indicated by the change in color after spraying the staining reagent, show different secondary metabolite compounds in each fraction. The reddish-purple staining pattern suggests the presence of terpenoid compounds while the green staining pattern indicates the presence of steroids. The staining patterns of TCD 4 and TCD 5 showed a purple color, showing the presence of terpenoid compounds.

The antioxidant activity of the fractions (TCD 1 to TCD 6) was evaluated using the DPPH inhibition assay (Table 1). The TCD 1 fraction shows the highest percent inhibition at a concentration of 100 ppm, while the percent inhibition decreases at concentrations of 50 ppm and 25 ppm, showing that higher concentrations lead to a higher percentage of inhibition. The TCD 5 fraction gives the lowest IC₅₀ value of 127.05 ± 0.54 ppm and the TCD 2 fraction gives the highest IC₅₀ value of 525.09 ± 1.11 ppm. The DPPH antioxidant activity of TCD 5 and TCD 6 fractions, with IC₅₀ values of 127.05 ± 0.54 ppm

and 130.54 ± 0.71 ppm respectively, are considered moderate, as their IC₅₀ values are between 100 and 150 ppm, whereas TCD 1, TCD 2, TCD 3, and TCD 4, with IC₅₀ values of 233.68 ± 0.35 ppm, 525.09 ± 1.11 ppm, 282.41 ± 0.92 ppm, and 387.82 ± 0.65 ppm respectively, have weak antioxidant activity, as their IC₅₀ values are above 150 ppm. TCD 4 was further isolated using *n*-hexane: ethyl acetate (9:1) to obtain purer compounds, and 4 subfractions (TCD 4.1-TCD 4.4) were obtained. The DPPH antioxidant activity of the subfractions (TCD 4.1-TCD 4.4) is presented in Table 2.

Table 2 shows that TCD 4.4 has the highest IC₅₀ value of 38.66 ± 0.96 ppm based on the DPPH inhibition assay. In contrast, TCD 4.1, TCD 4.2, and TCD 4.3 exhibit weak antioxidant activity, with an IC₅₀ of 289.44 ± 0.76 ppm, 163.28 ± 0.51 ppm, and 180.99 ± 0.65, respectively.

Gas Chromatography-Mass Spectrometry Analysis

The TCD extract was analyzed using GC-MS Shimadzu QP2000A to evaluate the content of secondary metabolite compounds. The GC chromatogram of the TCD extract is shown in Figure 2.

Figure 2 illustrates that the TCD extract contained active compounds. Some compounds contained in the TCD extract are listed in Table 3.

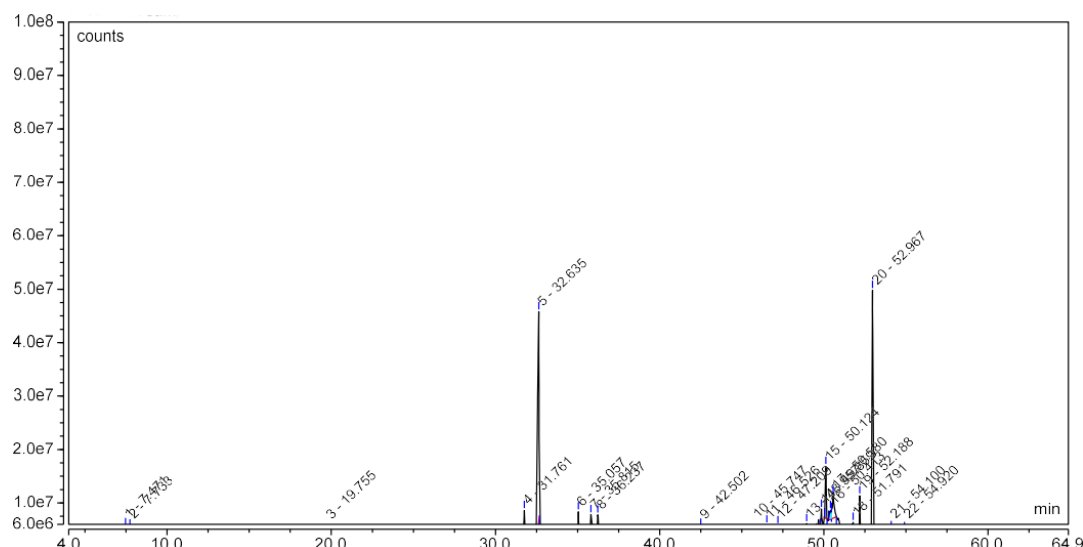


Figure 2. GC Chromatogram of TCD Extract

Table 3. Some compounds from the TCD extract

Compounds	Retention (Minutes)	Area (%)	Formula	Molecular Weight	Similarity Index (SI)
Hexadecanoic acid, methyl ester	31.76	3.37	C ₁₇ H ₃₄ O ₂	270	86.2
<i>n</i> -Hexadecanoic acid	32.64	31.97	C ₁₆ H ₃₂ O ₂	256	89.2
6-Octadecenoic acid, methyl ester, (Z)-	35.06	2.44	C ₁₉ H ₃₆ O ₂	296	85.4
cis-Vaccenic acid	35.82	7.52	C ₁₈ H ₃₄ O ₂	282	85.0
Octadecanoic acid	36.24	3.01	C ₁₈ H ₃₆ O ₂	284	80.5
Stigmasterol	52.18	3.52	C ₂₉ H ₄₈ O	412	84.4
γ -Sitosterol	52.97	21.04	C ₂₉ H ₅₀ O	415	89.2

The TCD extract obtained from the cocoa pod husk contained some steroid compounds, namely γ -sitosterol (21.04%) and stigmasterol (3.52%). A previous study reported the presence of stigmasterol, campesterol, and γ -sitosterol in the aqueous extract of cocoa pod husk [10]. Sitosterol, a steroid compound, possesses antioxidant and cytotoxic activity against lung cancer [16, 17].

CONCLUSION

The antioxidant activity of *T. cacao* pod husk extracts showed that the *T. cacao* dichloromethane (TCD) extract had the lowest IC₅₀ value of 14.59 ± 0.54 ppm as suggested by the DPPH inhibition assay. The dichloromethane extract was isolated using column chromatography, resulting in six fractions. Among these fractions, TCD 6 exhibited the lowest IC₅₀ value (127.05 ± 0.54 ppm). Further isolation of TCD 4 resulted in four subfractions, with TCD 4.4 exhibiting the lowest IC₅₀ value (38.66 ± 0.96 ppm). The TCD extract contained the bioactive compounds γ -sitosterol and stigmasterol, as determined by GC-MS analysis. It may be necessary to continue to isolate the purest compounds of the highest antioxidant activity.

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