

Fractionation, Purification, and Toxicity of Methanolic Cacao Pod Husk Extract (*Theobroma cacao* L.)

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ABSTRACT

Cacao (*Theobroma cacao*, L.) is a plantation product which has good prospects, but its processing produces a by-product in the form of cacao pod husks that have not been optimally utilized. The current research examines the toxicity of methanol extracts, fractions, and subfractions of cacao pod husks using the Brine shrimp lethality test (BSLT) assay, and to purify active compounds using gravity column chromatography and thin-layer chromatography (TLC). The fractionation process of the methanol extract produced 5 fractions and 4 subfractions. The findings from the toxicity assessment indicated that the TCM 2 fraction and the TCM 2.1 subfraction had the highest toxicity, with LC₅₀ values of 9.39 ppm and 5.02 ppm, respectively. Further purification process produced a single isolate identified as a terpenoid compound. The results of this study indicate that cacao pod husks have its applicability as a natural alternative source of toxicity and can be developed for use in the pharmaceutical field.

Introduction

Cacao (*Theobroma cacao*, L.) is a plantation product with good prospects. This is due to its relatively high price and important role in trade in Indonesia. Cacao plants grow in various regions in Indonesia, mostly on the islands of Sulawesi, Java, and Sumatra. In 2015, Aceh province had a cacao plantation area of 102,649 hectares, and production reached 30,661 tons [1]. Cacao production generates a byproduct, cacao pod husks. The utilization of these husks has been limited. Cacao pod husks are a major byproduct of the cacao industry, accounting for 70-80% of total cacao pod mass. Therefore, the main waste from cacao processing is the husks [2].

The cacao pod husk contains several secondary metabolites, including alkaloids, saponins, steroids, terpenoids, flavonoids, and phenolics. Previous studies reported that terpenoids and steroids were most found in nonpolar extracts [3], whereas alkaloids, saponins, flavonoids, and phenolics are more commonly found in polar and semi-polar

extracts [4]. These findings indicate the potential value of cacao pod husk beyond its role as an industry byproduct. Secondary metabolites from these different extracts exhibit diverse bioactivities. Toxicity assays conducted in earlier studies showed that fractions 7 and 9 from the n-hexane extract had substantial toxic effects on shrimp larvae (*Artemia salina* L.), yielding an LC₅₀ value of 0.29-0.45 ppm and the main compound identified was eucalyptol [3]. Additionally, the crude n-hexane extract inhibited the growth of *Staphylococcus aureus* [5]. Other extracts with semi polar solvent showed high antioxidant activity and inhibited the growth of breast cancer cells (MCF-7) and high toxicity activity [6,7]. Moreover, the ethanol (polar) and dichloromethane (semi-polar) extracts demonstrated antibacterial, antioxidant, and antidiabetic properties [8].

However, research concerning the methanol extract has thus far centered mainly on the quantification of phenolic and flavonoid compounds along with antioxidant activity [9]. Studies on toxicity activity have largely focused

on primary extracts, while the activity exhibited by both fractions and subfractions has not been widely reported. Therefore, Toxicological evaluation of fractions and subfractions requires further investigation.

To address this research gap, toxicity was assessed using the brine shrimp lethality test (BSLT). *Artemia salina* L. are utilized as test organisms in this in vitro method because of their ease of handling and effectiveness [10]. The BSLT method is widely used as an initial screening method, which can then serve as a basis for further exploration of pharmacological activity [11].

Methodology

Tools and materials

The equipment used included an analytical balance, grinder, maceration container, filter paper, gravity chromatography column, chamber, thin-layer chromatography (TLC) plates, 254 nm UV lamp, rotary evaporator, *Artemia salina* hatching container with an aerator, micropipettes, and other laboratory glassware.

The materials used were cacao pod (*Theobroma cacao* L.) husks obtained from Lawe Loning Village, Lawe Sigala-gala District, Southeast Aceh Regency, Indonesia; ethyl acetate, silica gel 60 F254, distilled water, seawater, *Artemia salina* L. eggs, 5% dimethyl sulfoxide (DMSO), n-hexane, Dragendorff's reagent, Mayer's reagent, (FeCl₃), Wagner's reagent, magnesium powder, concentrated HCl, Liebermann–Burchard reagent, and vanillin sulfate reagent, methanol,

Cacao Pod Husk Maceration

The cleaned cacao pod husk samples were processed into smaller fragments and air-dried at ambient temperature until the water content was reduced. The samples were then ground in a grinder until the particles were powder. A 4000 g of sample powder was macerated in methanol at a solvent-to-sample ratio of 1:10 (w/v) for 1x24 hours. Following filtration, the filtrate was subjected to concentration by rotary evaporator to obtain a thick methanol extract. Maceration was repeated until a clear filtrate was produced.

Phytochemical Analysis

The methanol extract of cacao pod husk was qualitatively analyzed for secondary metabolite content using the Harborne method [12].

Gravity Column Chromatography of Cacao Pod Husk

The methanol extract and its active fraction were fractionated using gravity column chromatography. A gradient solvent system consisting of n-hexane and ethyl acetate (100:0–0:100; 10% increment) was applied as the mobile phase and silica gel 60 F254 as the stationary phase. The eluates were collected in a test tube, and the stain pattern of each eluate was monitored using a TLC plate. The stains were observed under a UV 254 lamp and with vanillin sulfate staining reagent. Eluates with the same stain pattern were combined to obtain a combined fraction or subfraction group.

Brine shrimp lethality test (BSLT) assay

Hatching of *Artemia Salina* L. Larvae

The *Artemia salina* L. larvae hatching aquarium is designed into two compartments, a dark section and a light section. These two compartments are separated by a perforated partition. The *Artemia salina* L. eggs are then placed in the dark compartment containing seawater, which is aerated using an aerator. *Artemia salina* L. eggs are allowed to hatch for 48 hours, and the resulting larvae are transferred to the light compartment containing seawater. The resulting larvae will then be used as bioindicators for toxicity testing.

Toxicity Activity Test

The lethality evaluation using brine shrimp was performed following the previous research protocol [13]. Extract, fraction, and subfraction samples were tested for their toxicity against shrimp larvae. The test was conducted by varying the concentration of the solution dissolved in seawater to 10 ppm, 100 ppm, and 1000 ppm. 2–3 drops of 5% DMSO were added to facilitate solution homogenization. 5 mL of the test solution was added to 10 *Artemia salina* L. larvae, and the solution was then incubated at room temperature for 24 hours. After incubation, the larvae's movement was

observed. Larvae that did not move within 30 seconds were considered dead larvae. The percentage of larval mortality was then calculated, and the 50% lethal concentration (LC₅₀) was determined by regression in SPSS software version 21 for Windows. This analysis was performed in triplicate.

Compound Purification

Subfractions with the best activity were further purified using gravity column chromatography and TLC plates. The eluate from gravity column chromatography was spotted on a TLC plate and eluted with an appropriate eluent. The resulting single spot pattern was taken from the TLC plate and dissolved using methanol. The methanol-soluble compound was filtered through filter paper, and the collected filtrate was evaporated to yield a concentration. The compound was reanalyzed on a TLC plate using the same eluent. The spot pattern was observed under UV light at 254 nm and with vanillin sulfate reagent.

Results and Discussion

Secondary Metabolite Analysis

The determination of secondary metabolites was carried out qualitatively. A qualitative phytochemical assessment of the cacao pod husk methanol extract is provided in Table 1.

Based on phytochemical tests, the *Theobroma cacao* methanolic (TCM) extract contains flavonoids, phenolics, alkaloids, and saponins. This extract is negative for terpenoids and steroids. Previous research identified only terpenoid and steroid compounds present in the hexane extract of cacao pod husk [3]. These results indicate that terpenoids and steroids can generally be extracted using nonpolar solvents, such as hexane. Flavonoid and phenolic compounds are generally polar because they contain many hydroxyl groups. Ethanol extract of cacao pod husk is proven to contain phenolics

and flavonoids of $157,491 \pm 7,941$ and 2.22 ± 0.20 , respectively, which are categorized as high and medium content [14]. The methanol extract of cacao pod husk is also shown to contain 5-Hydroxymethylfurfural compounds, which are part of the phenolic compounds [15]. In this analysis, the alkaloids and saponins in the extract have polar groups, so they are extracted with methanol.

Table 1. Phytochemical Identification in Methanol Extract of Cacao Pod Husk

Secondary Metabolites	Analysis	Results
Terpenoids	Liebermann	-
	Burchard reagent	
Steroids	Liberman	-
	Burchard reagent	
Flavonoids	Mg + HCl	+
Phenolic	FeCl ₃	+
Alkaloids	Mayer reagent	+
	Wagner reagent	+
	Dragendorff reagent	+
Saponins	Shake with hot water	+

+ = positive, - = negative

Fractionation of methanol extract of cacao pod husk and its toxic activity

The TCM extract was fractionated using gravity column chromatography and produced 53 eluates. Each eluate was observed for its staining pattern under UV 254 light and produced 6 combined fractions (Figure 1.a). However, the results of TLC monitoring using vanillin sulfate staining reagent (Figure 1.b) showed that the 4th and 5th staining patterns showed the same pattern shape, so these two fractions were combined. The results of staining pattern monitoring showed that the extract was fractionated into 5 combined fractions (TCM 1-TCM5).

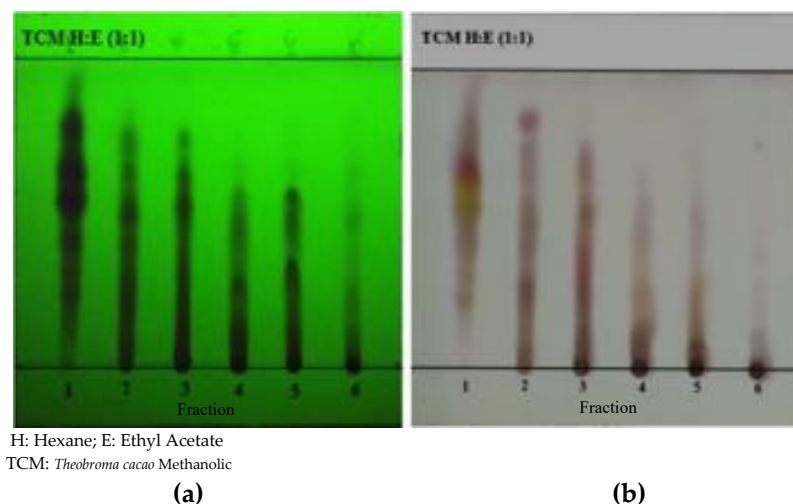


Figure 1. TLC chromatograms of TCM extract fractions visualized (a) under ultraviolet light and (b) following spraying with vanillin–sulfuric acid reagent

Table 2. Toxicity profiles of TCM and TCM fractions

Sample	Concentration (ppm)	LC ₅₀ (ppm)	95% Confidence Interval
TCM Extract	[10–1000]	139.94	81.09–252.62
TCM 1	[10–1000]	11.55	2.10–26.48
TCM 2	[10–1000]	9.39	2.11–19.81
TCM 3	[10–1000]	21.23	5.84–46.20
TCM 4	[10–1000]	50.91	Not estimated
TCM 5	[10–1000]	67.17	Not estimated

TCM: *Theobroma cacao* Methanolic

TCM Extract showed toxicity, with an LC₅₀ below 1000 ppm (Table 2) [16]. TCM 2 fraction showed the lowest LC₅₀ (9.39 ppm), indicating the strongest toxicity among the extract and other fractions. This difference in activity was due to TCM 2 fraction containing a more concentrated toxic compound. Other fractions were also categorized as having high toxicity, with LC₅₀ values of 11.55 ppm, 21.23 ppm, 50.91 ppm, and 67.17 ppm against TCM 1, TCM 3, TCM 4, and TCM 5, respectively. Overall, the toxicity of the fractions was higher than that of the extract. A similar trend was reported by our previous research, which showed that the toxicity of the hexane extract of cacao pod husk was lower than that of its fraction [3].

Fractionation of TCM 2 and Its Toxicity



C: Chloroform; E: Ethyl Acetate

Figure 2. TLC chromatograms of TCM 2 subfractions visualized after spraying with vanillin–sulfuric acid reagent

TCM 2 was the most active fraction in terms of its toxic activity. TCM 2 was further fractionated using gravity column

chromatography, and four subfractions were obtained, namely TCM 2.1 – TCM 2.4 (Figure 2). Based on the TLC staining pattern analysis, subfractions TCM 2.1 and TCM 2.2 showed purple and orange staining, while subfractions 3 and 4 showed staining that was not clearly visible. This difference indicates that the compounds in the TCM 2.1 and TCM 2.2 subfractions have begun to separate through gravity column chromatography. The purplish-blue stain in the TCM 2.1 and 2.2 subfractions suggest the presence of terpenoid compounds [17]. However, this observation is based solely on TLC analysis using vanillin–sulfuric acid reagent and should be considered preliminary. In contrast to the phytochemical analysis, which showed negative results for terpenoids, this condition likely occurred because the terpenoid

levels in the extract were very low and were not detected by the screening method. The fractionation process can concentrate secondary metabolites, allowing compounds that were initially undetectable in the crude extract to become observable through TLC analysis.

The results of the toxicity analysis (Table 3) showed that the TCM 2.1 subfraction was the most active, with an LC_{50} of 5.02 ppm, followed by TCM 2.3, TCM 2.2, and TCM 2.4. These four subfractions were classified as highly toxic samples because their LC_{50} values were less than 100 ppm [16]. Compared with the TCM 2 fraction, TCM 2.1 and TCM 2.3 subfractions showed higher toxicity. This is presumed because the active compounds in these subfractions are more concentrated.

Table 3. Toxicity profiles of TCM 2 subfractions

Sample	Concentration (ppm)	LC_{50} (ppm)	95% Confidence Interval
TCM 2.1	[10–1000]	5.02	0.41–10.43
TCM 2.2	[10–1000]	10.59	3.06–20.49
TCM 2.3	[10–1000]	7.67	1.75–14.76
TCM 2.4	[10–1000]	18.19	5.86–36.8415

TCM: *Theobroma cacao* Methanolic

TCM 2.1 Subfraction Purification

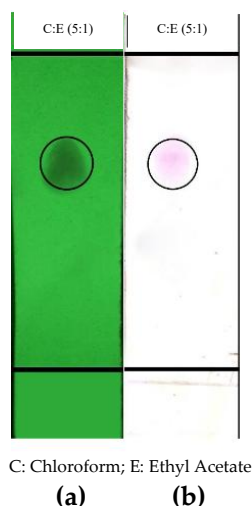


Figure 3. TLC chromatograms of isolate visualized under (a) UV light and (b) after spraying with vanillin–sulfuric acid reagent

Purification was carried out on TCM 2.1 subfraction because it has the highest toxicity with a good separation stain pattern. The

purification of the compound is shown in Figure 3. A single spot with a light purple color is suspected to be a terpenoid compound [17]. In our previous study, it was reported that the terpenoids patchouli alcohol, pinene, and limonene were the dominant compounds and contributed most to the toxic activity in the cacao pod husk subfraction whose extraction process used hexane solvent [17]. Terpenoid compounds have various biological activities, including cytotoxic effects in several cancer cell lines such as breast, liver, and pancreatic cancer cells [18]. In general, terpenoid compounds are active as anticancer agents through various mechanisms, namely, inducing cell cycle arrest, inhibiting cancer cell differentiation, inducing apoptosis, and inhibiting angiogenesis and metastasis [19]. This study used a preliminary toxicity screening method and can be an early indication of potential bioactivity that still requires further testing using a more specific method.

Conclusion

The methanol extract of cacao pod husk reveals the presence of secondary metabolite groups, including alkaloids, flavonoids, phenolics, and saponins, and is categorized as an extract with high toxicity activity. Fractionation of the methanol extract yielded 5 fractions, with the most active being TCM 2, which has an LC₅₀ of 9.39 ppm. Further purification of the TCM 2 fraction yielded 4 subfractions, with the TCM 2.1 subfraction exhibiting the strongest toxicity, with an LC₅₀ of 5.02 ppm. The isolation process from the methanol extract successfully obtained an isolate identified as a terpenoid compound and may be primarily responsible for the toxicity activity of cacao pod husk. Further research is recommended to fully elucidate the compound's structure and conduct more targeted biological activity testing.

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