GSC Biological and Pharmaceutical Sciences

eISSN: 2581-3250 CODEN (USA): GBPSC2

Cross Ref DOI: 10.30574/gscbps

Journal homepage: https://gsconlinepress.com/journals/gscbps/

(RESEARCH ARTICLE)

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Comparative analysis of total flavonoid, total phenolic content, and antioxidant activity of *Theobroma cacao* Pod Husk extract and its derived fractions

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GSC Biological and Pharmaceutical Sciences, 2023, 25(03), 159–166

Publication history: Received on 06 November 2023; revised on 18 December 2023; accepted on 21 December 2023

Article DOI: https://doi.org/10.30574/gscbps.2023.25.3.0525

Abstract

Secondary metabolites from plants are known to be promising sources of raw materials for drugs in terms of safety. This research focuses on cacao pod husk as a candidate for discovering natural antioxidant compounds. The stages in this study include the extraction of cacao pod husk using methanol as a solvent, followed by liquid-liquid fractionation to produce ethyl acetate and acetone fractions. Subsequently, total flavonoid and phenolic content, DPPH antioxidant activity tests, and GCMS metabolite analysis were conducted. The research results indicate that the ethyl acetate fraction has the highest total flavonoid and phenolic content compared to methanol extract and acetone fraction. The methanol extract has the highest antioxidant activity with an IC_{50} of 0.0736 mg/mL. The identified metabolites belong to the groups of polyphenols, flavonoids, steroids, and their derivatives, as well as some volatile compounds such as hexadecanoic acid methyl ester. Compounds with potential antioxidant properties include Eugenol, Myristicine, Piperine, and Theobromine.

Keywords: Cacao pod husk; DPPH; Antioxidant; Cacao

1. Introduction

Reactive Oxygen Species (ROS) are normal byproducts of cellular metabolism. These molecules possess unpaired free electrons, making them highly reactive as they seek to pair with electrons. Naturally, ROS can be stabilized by the presence of endogenous antioxidants produced by the body. However, an imbalance between ROS production and antioxidants can lead to conditions causing damage to macromolecules, including DNA and proteins. The accumulation of ROS can induce oxidative stress, which may trigger various diseases such as diabetes, cancer, and cardiovascular diseases [1]. When the body is unable to balance its antioxidant production, external sources of antioxidants become necessary, such as dietary intake, supplements, and pharmaceuticals with antioxidant properties. In recent decades, researchers have started exploring natural alternatives for antioxidants derived from plant-based natural products. Antioxidant compounds from these natural products are being developed as raw materials for pharmaceuticals. Several studies suggest that antioxidant compounds from plant extracts have the potential to prevent diseases [2, 3].

Theobroma cacao Linn. (*T. cacao*) is one of the agricultural commodities in the Lampung Province. Cacao production in Lampung Province reached 56,671 tons in 2021 [4]. Chocolate fruit is cultivated to yield cacao and chocolate products. Cacao pod husk is a byproduct of chocolate processing, and if not handled properly, it becomes an environmental waste. In recent decades, numerous studies have indicated that cacao pod husk possesses pharmacological capabilities as an anti-inflammatory [5], anticancer [6], antidiabetic, antioxidant, and antibacterial agent [7]. Karim et al [8] reported that the antioxidant activity of 80% ethanol extract ranged from 20.35 to 45.26 mg/mL. The antioxidant activity of fresh Cacao pod husk (CPH) ranged from 30.6 to 15.1 µM TEAC/g [9]. The antioxidant capability of CPH varies depending on its geographical origin, solvent type, and extraction method. Additionally, the antioxidant capacity is influenced by the

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total phenolic and flavonoid content in the CPH extract. There is currently no research comparing the crude extract and fractions of Cacao pod husk (CPH). Therefore, this study aims to compare the antioxidant activities of CPH from methanol extract, ethyl acetate fraction, and acetone fraction. Additionally, this research includes an analysis of metabolite compounds using GC-MS. The results of this study are expected to determine the optimal solvent for extracting antioxidant compounds, total flavonoid, and total phenolic content.

2. Material and methods

2.1. Material

2.1.1. Plant Material

Fresh cacao pod husk was obtained from East Lampung, Lampung Province, Indonesia

2.1.2. Chemicals and Reagents

2.2. Methods

2.2.1. Sample Preparation

Fresh cacao pod husks were sliced, washed, and dried under sunlight for 4 days. The dried samples were ground using a grinding mill and sieved to form a 100-mesh powder.

2.2.2. Sample Extraction

Cacao pod husk powder was extracted with methanol through maceration in a ratio of 1:10 for 3 days. The Supernanat was filtered to get methanol extract. This extract was partitioned by liquid-liquid extraction in a ratio of 1:1 with solvent acetone and ethyl acetate. The Obtained methanol extract and its fractions were concentrated using a rotary evaporator and yielded methanol extract, acetone, and ethyl acetate fractions.

2.2.3. Total Phenolic Content Determination

Total phenolic content (TPC) was determined by Folin-Ciocalteu's method. Weigh 0.50 mg of the sample carefully, and add 0.5 mL of Folin-Ciocalteu reagent and 7.5 mL of distilled water. Allow the mixture to stand for 10 minutes at room temperature, then add 1.5 mL of 20% sodium carbonate. Add distilled water to a final volume of 10 mL. Dilute as needed. Transfer to a cuvette, and maintain absorption at a wavelength of 760 nm by Spectrophotometer UV-Vis. Extrapolating from a Gallic acid calibration curve assessed the total flavonoid content. The determination of flavonoid compounds was conducted in triplicate. The total flavonoid content was reported as the mean ± standard deviation of Gallic Acid Equivalents (GAE/g of dried extract) [10].

2.2.4. Total Flavonoid Content Determination

Total flavonoid content (TFC) was determined by The $AlCl_3$ method. Take 50 mg of a solid sample, and add 0.3 mL of 5% sodium nitrite. After 5 minutes, add 0.6 mL of 10% aluminum chloride, wait 5 minutes, then add 2 ml of 1 M sodium hydroxide. Add distilled water to adjust the volume to 10 mL with a volumetric flask. Dilute as needed. Transfer to a cuvette, measure the absorbance at a wavelength of 510 nm by Spectrophotometer UV-Vis. Extrapolating from a Quercetin calibration curve assessed the total flavonoid content. The determination of flavonoid compounds was conducted in triplicate. The total flavonoid content was reported as the mean ± standard deviation of Quercetin Equivalents (QE/g of dried extract) [10].

2.2.5. Antioxidant Activity Determination

Antioxidant Activity was determined by the DPPH method. A total of 50 µL of test samples with various concentrations (concentrations providing IC values, i.e., 50 concentrations of extract/fraction providing 50% antioxidant activity compared to control through a linear regression equation) were combined with 1.0 mL of 0.4 mM DPPH and 3.950 mL of ethanol. The mixture was then vortexed and left for 30 minutes. Subsequently, the solution's absorbance was measured at a wavelength of 517 nm against a blank (composed of 50 µL of extract and 4.950 mL of ethanol). Absorbance control measurements were also conducted, consisting of 1.0 mL of DPPH and 4.0 mL of ethanol. Vitamin E and Vitamin C were used as reference standards.

2.2.6. Chemical Compound Identification

The Chemical compound of methanol extract, acetone fraction, and ethyl acetate fraction was determined by instrument Gas Chromatography-Mass Spectroscopy (GC-MS). A volume of 1 µL from each extract and fraction was injected into GC-MS (Agilent GC seri 7890 and Agilent MS seri 5975, USA). Injector and detector temperature 250 °C, coloum temperature 60 °C- 210 °C, coloum internal diameter 30 m x 0,25 mm with thickness 0,25 µm, Helium carrier gas with flow rate 0.6 $mL.min^{-1}$. The NIST MS standard determined the chemical structure.

2.2.7. Statistical Analysis

The data analysis was done by IBM SPSS statistic version 16.0. The information is conveyed as the average values along with the standard deviation (SD), derived from independent analyses conducted in triplicate. Statistical analysis was carried out using one-way analysis of variance (ANOVA).

3. Results and discussion

3.1. Total Phenolic Content

The total phenolic content in this study was determined using the Folin-Ciocalteu method. The results indicated 0.1% milligrams of Gallic Acid Equivalent (GAE) per 100 grams of extract, equivalent to the total phenolic content (Table 1). The highest total phenolic content was found in the ethyl acetate fraction (9.1000 ± 0.1414), followed by the acetone fraction (6.1300 ± 0.0707), and the lowest in the methanol extract (4.4950 ± 0.3046). Yahya *et al* [11] reported that the total phenolic content of the ethyl acetate fraction was 570.44 mg/g GAE. The total phenolic content of husk can vary between samples depending on soil conditions, the environment, solvents used, and extraction methods [12]. Soil, air, and environmental conditions can influence the physical and chemical content of the samples [11].

3.2. Total Flavonoid Content

The total flavonoid content of methanol extract, acetone fraction, and ethyl acetate fraction was determined using the Aluminum Chloride method. The results of the total flavonoid content indicated 0.1% milligrams of Quercetin Equivalent (QE) per 100 grams of the extract. The total flavonoid content of the extract and fractions of cacao pod husk is presented in Table 1. The ethyl acetate fraction exhibited the highest total flavonoid content (8.2450 ± 0.1212) compared to the methanol extract (4.6750 ± 0.0636) and acetone fraction (2.6750 ± 0.0070). Yahya e*t al.* [11] stated that the total flavonoid content of the ethyl acetate fraction was 4.34 mg/g QE. The significant difference in the total flavonoid content of the ethyl acetate fraction from previous studies could be attributed to the origin of the cacao pod husk samples used. The total flavonoid content of cacao pod husk is still underexplored, as some previous studies only determined the total phenolic content.

Table 1 Comparison of Total Flavonoid and Total Phenolic Content of Methanol Extract and Its Fractions

No	Sample	Flavonoid Content % (b/b)	Phenolic Content % (b/b)
1	Methanol Extract	4,6750±0,0636^{b}	4,4950±0,3046^{a}
2	Acetone Fraction	2,6750±0,0070 ^{a}	6,1300±0,0707^{b}
3	Ethyl Acetate Fraction	8,2450±0,1212^{c}	9,1000±0,1414^{c}

*Different letters in the same column indicate significant differences ($p < 0.05$) based on Duncan's post hoc test. The displayed values represent the mean ± SD from three replicates.

3.3. Antioxidant Activity

The antioxidant activity in this study was determined using the 2,2-diphenyl-2-picrylhydrazyl (DPPH) method. DPPH provides information about the extract or fraction's ability to prevent free radicals from attacking DNA, carbohydrates, proteins, fatty acids, and amino acids in biological systems [11]. DPPH is a widely used and more stable organic free radical for determining the scavenging activity of the antioxidants from plants. This method is easy, sensitive, and provides rapid results [11].

The antioxidant abilities of the extract and fractions are indicated by the IC_{50} values (µg/mL). The IC_{50} value represents the extract concentration that captures 50% of free radicals. A lower IC_{50} value indicates higher antioxidant activity [13,14]. Figure 1 shows the IC_{50} values for methanol extract, acetone fraction, and ethyl acetate fraction. The methanol

extract exhibited the highest antioxidant activity (IC_{50} 0.0736 µg/mL) compared to the acetone and ethyl acetate fractions. The study by Baharum et al. [6] reported that the methanol extract of cacao pod husk showed no antioxidant activity compared to other parts of the cocoa plant. Indrianingsih *et al.* [7] demonstrated that the methanol extract had antioxidant activity with an IC_{50} value of 44.5 µg/mL, while the ethyl acetate fraction had an IC_{50} value of 82.4 µg/mL. The antioxidant activity of the plant extract is highly affected by many factors including the composition of extracts, assay, and conditions [10]. The variation in antioxidant activity indicates differences in phytochemical content in the samples. This phytochemical content depends on the solvent, extraction technique, and plant part used [11].

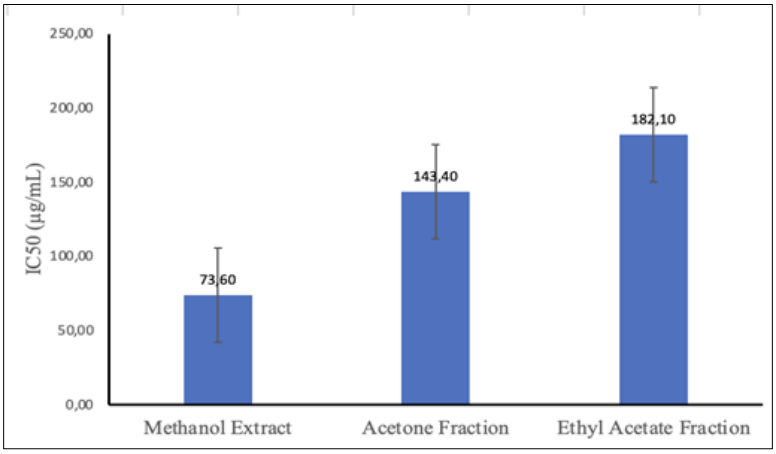

Figure 1 Antioxidant Activity of Methanol Extract, Acetone Fraction, and Ethyl Acetate Fraction

3.4. Correlation of Total Phenolic Content, Total Flavonoid Content, and Antioxidant Activity

Based on several previous studies, it is known that the content of phenols and flavonoids are phytochemical compounds that play a role in the antioxidant capacity of brown bark [11,7,15]. Therefore, this study analyzed the correlation between antioxidant activity, total phenolic, and flavonoid content (Table2). The results of Pearson correlation analysis indicate that the total phenolic and flavonoid contents are not correlated. In contrast, the total phenolic content positively correlates with antioxidant activity (correlation coefficient, $r = \sim 0.9$, $p < 0.01$). This suggests that a high phenolic content is a crucial factor in determining the antioxidant activity of pod husk extract. Some studies have revealed that the antioxidant activity of phenolic compounds may be attributed to their ability to act as reducing agents and hydrogen donors [7,16]

Table 2 Correlation Analysis of Antioxidant Activity, Total Flavonoid, and Total Phenolic Content

		Flavonoid	**Phenolic**	**Antioxidant**
Flavonoid	Pearson Correlation	1	0.751	0.498
	Sig. (2-tailed)	-	0.086	0.315
Phenolic	Pearson Correlation	0.751	1	0.944**
	Sig. (2-tailed)	0.086	-	0.005
Antioxidant	Pearson Correlation	0.498	0.944**	1
	Sig. (2-tailed)	0.315	0.005	-

** Correlation is significant at the 0.01 level (2-tailed).

3.5. Chemical Compounds Analysis

The analysis of metabolite content in the extract and fractions of cacao pod husk revealed differences in the quantity and types of compounds successfully identified. The identified metabolites belong to the groups of polyphenols, flavonoids, steroids, and their derivatives, as well as some volatile compounds such as hexadecanoic acid methyl ester. Recent literature confirms that cocoa skin contains several active compounds, including steroids [17], and some volatile compounds such as methyl esters of octadecanoic acid, methyl esters of hexadecanoic acid, and octyl esters of benzene dicarboxylic acid [18]. Metabolites identified in the ethyl acetate fraction amounted to 22 compounds, 21 in methanol extract, and 12 in the acetone fraction. These results are consistent with the total phenolic and flavonoid tests, where

the ethyl acetate fraction has the highest phenolic and flavonoid content. Most identified metabolites have biological activities such as anticancer, antiproliferative, and antioxidant properties. Metabolites highlighted in bold have anticancer, antiproliferative, and antioxidant activities based on the literature review (Table 3).

The ethyl acetate fraction contains more compounds known for their anticancer activity. Myristicin and Isoelemicin can inhibit cancer cell proliferation by stimulating the Caspase-3 pathway [19]. n-Hexadecanoic acid is known for its cytotoxic properties against cancer cells through interaction with topoisomerase 1 enzyme [20]. Ethyl P-Methoxycinnamate is known as an active Anti-Metastasis agent [21]. Additionally, based on several studies, 1,2-Propanediol, 3-chloro-, 1,2-Propanediol, 3-chloro-, N-Hydroxymethylacetamide, Eugenol, Phenol, 2,6-dimethoxy-4-(2-propenyl), 9,12-Octadecanoic acid, methyl ester, E-3,3-Dimethoxy-4,4-dihydroxystilbene, Benzene, 1,2-dimethoxy-4-(1-propenyl)-, and piperine is compounds with anticancer properties [22]. Theobromine is a distinctive compound found in cocoa plants, including the husk. This compound was only identified in the acetone fraction and is known for its anticancer activity [26]. The methanol fraction contains several potent antioxidants, such as Tetradecanoic acid, Oleic acid, Octadecanoic acid, Stigmasterol, and gamma-sitosterol. Most of the identified metabolites in the methanol extract are derivatives of steroid compounds, a group known for its antioxidant properties [27].

Table 3 Chemical Compounds of Cacao pod husk by GC-MS

Retention Time	Chemical Compound	%Area
Ethyl Acetate Fraction		
3.47	(S)-Methyl-2,3-dihydroxypropanoate	2.83
4.36	2H-Pyran-2,6 (3H)-dione	1.34
6.05	**1,2-Propanediol, 3-chloro-**	1.22
7.14	Dimethyl(S)-(-)-malate	4.46
7.82	**4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl**	6.78
12.77	**N-Hydroxymethylacetamide**	4.33
14.76	**Eugenol**	1.71
20.86	**Myristicine**	1.38
24.08	**Phenol,2,6-dimethoxy-4-(2-propenyl)**	1.65
28.99	**Ethyl p-methoxynnamate**	10.96
31.05	Hexadecanoic acid, methyl ester	14.35
31.56	**n-Hexadecanoic acid**	7.61
32.28	**9,12-Octadecanoic acid, methyl ester**	1.59
32.32	9-Octadecenoic acid (Z,Z)-methyl ester	3.46
32.65	**9-Octadecenoic acid (E)**	3.14
32.77	**Isoelemicin**	**1.10**
35.18	**E-3,3-Dimethoxy-4,4-dihydroxystilbene**	**1.75**
35.55	**2-Hydroxy-3,4,5-trimethoxychalcone**	1.04
35.63	**Benzene,1,2-dimethoxy-4-(1-propenyl)-**	2.79
36.30	**Benzene,1,2,3-trimethoxy-5-(2-propenyl)-**	2.72
36.63	**Piperine**	5.62
Acetone Fraction		
3.34	1-Amino-3-methoxypropan-1-ol	1.19

3.49	Ethanol,2-(vynyloxy)	1.52
3.56	2-Cyclopenten-1-one,2-hydroxy	6.42
3.67	(S)-Methyl-2,3-dihydroxypropanoate	1.65
4.52	2H-Pyran-2,6 (3H)-dione	1.15
6.16	1,2-Propanediol, 3-chloro-	1.10
7.88	**4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl**	1.13
11.01	5-Hydroxymethylfurfural	10.76
30.98	Theobromine	19.44
31.49	**n-Hexadecanoic acid**	2.57
32.32	**Neo-Inositol**	50.67
Methanol Extract		
7.73	**4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl**	3.00
11.13	5-Hydroxymethylfurfural	14.50
20.87	**Myristicine**	1.00
29.50	Tetradecanoic acid	2.72
31.06	Hexadecanoic acid, methyl ester	9.60
31.65	**n-Hexadecanoic acid**	9.60
32.28	9,12-Octadecadienoic acid (Z,Z)-methyl ester	2.32
32.32	9-Octadecanoic acid, methyl ester €	5.33
32.47	Methyl stearate	2.71

4. Conclusion

This study concluded that cacao pod husk can vary in total flavonoid, phenolic content, and antioxidant activity between methanol extract, acetone fraction, and ethyl acetate fraction. Methanol extract and ethyl acetate fraction could be considered potential candidates for anticancer purposes or as foundational elements for developing drugs with similar objectives.

Compliance with ethical standards

Acknowledgments

Gratitude is extended to Poltekkes Kemenkes Tanjungkarang for providing funding for this research.

Disclosure of conflict of interest

No conflict of interest is to be disclosed.

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