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Effect of Extraction Solvents on Phenolic Compounds of Theobroma Cacao L. By-products Using Ultrasound-Assisted Extraction

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***Abstract*— Nowadays, recent studies focus on plant-derived food additives and plant by-products extract that possess health-promoting properties. Cocoa pod husk is the cocoa fruit's outer layer that is left unused after the cocoa bean processing in the cocoa industry. This by-product (cocoa pod husk) is rich in phenolic compounds due to its antioxidant level. Significant steps in separating the phytochemicals from the plant's cellular matrix are crucial, which is usually carried out by selecting the proper extraction method and choice of solvent. Phenolic compounds from cocoa pod husk were determined by ultrasound-assisted extraction using three types of solvents [aqueous ethanol (ethanol: water, 70:30 v/v), aqueous methanol (methanol: water, 70:30 v/v), and aqueous acetone (acetone: water, 70:30 v/v)]. Methanolic extracts contained significantly higher total phenolic content (34.88 mg GAE/g) and ferric reducing antioxidant power, FRAP, (521.73 µg/g TE) compared to other solvents. Meanwhile, ethanolic extracts exhibited higher flavonoid content (62.18 mg/g RE) and DPPH radical scavenging activity (73.67 µg/mL TE) compared to methanolic extracts, which indicated 33.30 mg/g RE and 65.00 µg/mL TE, respectively. Total flavonoid content had a strong positive correlation coefficient with DPPH scavenging activity (r= 0.951), while FRAP assay gave a strong negative correlation (r= -0.851). This finding provides an alternative method to reduce the accumulation of cocoa pod husk to be utilized as a potential antioxidant source.**

***Keywords*— Cocoa pod husk; extraction; total phenolics; total flavonoids; antioxidant activity.**

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I. INTRODUCTION

Cocoa pod husk is considered as wastage after separating cocoa beans from the cocoa fruit. Each cocoa fruit can produce nearly 52 to 76% of pod husk, which then creates tons of cocoa by-products that lead to environmental problems. The cocoa pod husk's high dietary fiber content makes it a good alternative to animal feed, as it is cheaper. Unfortunately, cocoa pod husk usage to animals has several problems as it contains high theobromine and fiber content that leads to adverse effects and digestibility [1]. Due to these restrictions, cocoa pod husk is left in the cocoa plantations, contributing to added cocoa plants' added problems and further challenges in waste disposal issue [2].

Cocoa pod husk contains a mixture of cellulose, hemicelluloses, lignin, pectin, and crude fiber. Cocoa pod husk can be valorized due to the composition of phenolics and antioxidant properties. Cocoa has phenolic compounds antioxidants like catechins, anthocyanidins, and proanthocyanidins. Phenolic compounds from plant-based products contribute to organoleptic properties such as aroma, flavor, and color. Phenolic compounds also acts as the precursor for antioxidant activity in cocoa beans. In the previous study, pod husk has been found to be an excellent source of phytochemicals such as polyphenol, tannins, alkaloids, and saponins [3].

Cocoa pods have a higher scavenging effect at low concentrations, which is further explained in the study of Umri *et al.* [4] that antioxidant properties are reflected by the available pigment such as chlorophyll carotenoids, and phenolics. Phenolic compounds can be found in pigment cells within cocoa beans. The composition differs on cocoa type, the origin of cocoa, growing condition, and maturity of cocoa fruit. A large number of phenolic compounds found in cocoa

are proanthocyanidins which rich in dietary fiber. In unfermented cocoa beans, flavan-3-ol, epicatechin, and procyanidins present widely give bitterness and astringency to the beans.

Valorization of phenolic compounds from cocoa pod husk can give benefits to human health, and strong antioxidant activity makes it suitable to apply into any other process. Green extraction concept is applied to meet the requirements in producing natural antioxidant using plant-based materials or by valorizing is important in gaining higher profitability [5]. Ultrasound-assisted extraction depends on the acoustic cavitation and mechanical effect to increase its efficiency.

The yield and resulting bioactive compounds depend on the extraction method and solvent involved. In this study, the aqueous solvents used are ethanol, methanol, and acetone which is the ratio of solvents: water (70: 30), respectively. Hereto this study was conducted to investigate the effects of using ultrasound-assisted extraction and various solvents on total phenolics, total flavonoids, and antioxidant (i.e., DPPH and FRAP assay) of *Theobroma cacao L.* by-products.

II. Materials and Method

3 kg of cocoa pod husk obtained from Cocoa Development and Research Centre, Jengka, Pahang, Malaysia. Dried pod husks were ground to fine particles using a laboratory mill and were sieved through a sieve 0.6 µm mesh. The extract was prepared by having a hundred milliliters of aqueous solution (70% of ethanol, 70% of methanol, and 70% of acetone) in a conical flask with five grams of cocoa pod powder. The mixture was homogenized using a shaker at 120 rpm, 50°C, for 2 hours [6]. Samples were then directly sonicated using ultrasound-assisted extraction for 30 minutes at 40 °C with 400 W ultrasound powers. The decanted portion was centrifuged at 10 000 g-forces for 10 minutes. The supernatant was obtained, evaporated to dryness, and kept at -20 °C until further analysis.

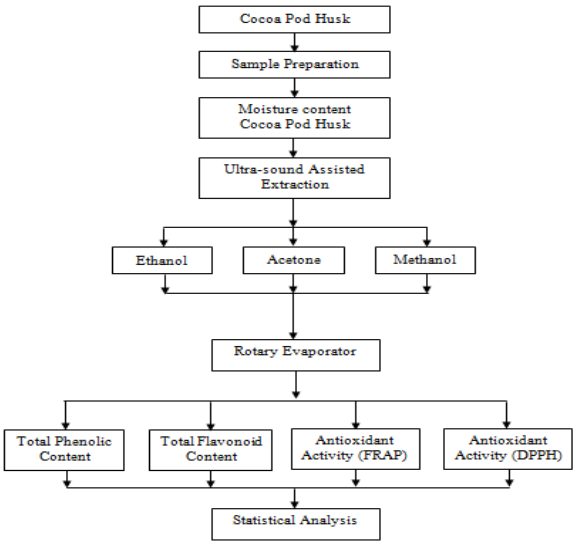

Fig. 1 Overall experimental design.

The experimental design was conducted with 3 independent replications for each extraction solvents used. Chemical analysis was performed on moisture content using the air oven method [7], where an aluminum dish was dried for four hours at 105 °C. Then, 1 g sample was placed in the aluminum dish and kept at 60 °C (drying oven) overnight. The drying process was repeated for triplicate measurement, and the percentage moisture (wt/wt) was calculated.

Phytochemicals of cocoa pod husk (total phenolic content) [8], was measured using the calibration curve. Gallic acid was used as standard. 0.5 mL extract was mixed with distilled water (4 mL) followed with Folin-Ciocalteu reagent (0.5 mL). Then, sodium carbonate (1.8 mL) was added and mixed. The solution was let stand for 1 hour at room temperature. Measurement was carried out at 765 nm using UV-Vis's spectrophotometer.

For flavonoids [9], extract (1 mL) was added with distilled water (4 mL), and 0.3 ml of 5% w/v sodium nitrite solution was transferred to 10 mL flask. Next, 10% w/v aluminum chloride solution (0.3 mL) and 1M sodium hydroxide (2 mL) were added after five and six minutes respectively. Distilled water was added into the flask and mixed well. Measurement was carried out at 510 nm. A standard calibration curve (10-90 mg/L) was established using Rutin, and results were expressed as mg Rutin equivalent per gram of extracts.

Antioxidant activity and antioxidant assay (ferric reducing power and DPPH radical scavenging assay) [10,11]. FRAP reagent was prepared by mixing 300 mM acetate buffer (10 mL, pH 3.6) to 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ, 1 mL) that was being dissolved in 40 mM hydrochloric acid and 20 mM $FeCl_3.6H_2O$ (1 mL) in 10:1:1 ratio. Then, FRAP reagent was placed in a water bath (37 °C, 10 min). Measurement was carried out using 3.9 mL FRAP reagent and 0.1mL extracts at 593 nm using UV-Vis spectrophotometer in triplicate. A standard curve was prepared at a concentration range between 200-1000 µM.

While DPPH, 0.1 mL extracts sample that varies in different concentrations (20 ppm to 100 ppm) was added with 3.9 mL of ethanolic solution of DPPH (10 mM). The tubes were vortex and kept away from light for 30 minutes. Then, absorbance was measured using a UV-Vis spectrophotometer at 515 nm. Trolox was used as an antioxidant standard. The concentration needed to reduce DPPH radical scavenging by 50% (EC50) was also calculated from the standard curve. Data were analyzed with analysis of variance, followed by the Duncan test at 5% level.

III. Result and Discussion

A. Moisture Content, Total Phenolic Content, and Total Flavonoid Content of Cocoa Pod Husk

The moisture content of dried cocoa pod husk was found to be 8.31%. This was expected since dried cocoa pod husk has been found by [12] was 8.50% which was reduced after subjecting the fresh cocoa pod husk to drying. According to [13] the moisture content of cocoa bean hull was 10.12% were from the result; it showed that the composition was different due to different parts of cocoas. The differences were related to the structural characteristics of the cocoa pod.

The moisture content of dried samples obtained in this study was below 15%, which was a significant factor in preserving plant materials [14]. The lower moisture contents of dried samples below 8.31% indicate that the dried samples were more shelf-stable than fresh samples. The moisture must be lower as the water present in samples. There will be a place

for phenolic compounds degradation; the degradation was due to enzymatic oxidation such as polyphenols-oxidase. The drying method using a cabinet drier was allowed to eliminate water and protect bioactive compounds [15].

The remaining cocoa pod is rich in phenolic acids, i.e., caffeic, chlorogenic, and coumaric. In this study, significantly higher phenolic content recorded was 34.88±0.29 mg GAE/g in methanolic extracts (Table 1). However, a significantly lower concentration of phenolic compounds for cocoa pod husk with ethanol and acetone extracts was 19.83±1.10 mg GAE/g and 17.62±0.46 mg GAE/g, respectively. The total phenolic content of cocoa pod husk extracts using ethanol and acetone extracts has been reported to be 49.92 mg GAE/g and 94.92 mg GAE/g [35]. The variation in phenolic compounds may be due to differences in the polarity of extracting solvent from results. The hydroxyl group's presence will cause polar phenol compounds to become polar and dissolve in polar solvents. The polarity index of acetone, ethanol, and methanol was 5.1, 5.2, and 6.6, respectively, which influence the extraction compound as the more polar will give higher yield.

Based on the polarity index, an extraction of corn silk resulted in the highest phenolic content obtained from methanol extracts compared to ethanol and acetone extract [16]. More polar solvents capable of dissolving phenol better than non-polar solvents. In this study, methanol has the highest polarity index, followed by ethanol and acetone. True to its polarity index, methanol resulted in extracting higher total phenolic compounds. The combined usage of water and solvent during extraction may enhance phenolic compounds' solubility to be extracted. Lower phenolic compounds found in ethanol and acetone extract may be attributed to less extraction process enhancement [17]. Table 1 shows that values are expressed as mean ± SD of triplicate measurement. Superscript with different letters is significantly different at $p<0.05$ within the different columns.

TABLE I
TOTAL PHENOLIC AND TOTAL FLAVONOID CONTENT OF COCOA POD HUSK EXTRACTS USING DIFFERENT SOLVENTS

Solvents	Total phenolic content (mg GAE/g)	Total flavonoid content (mg RE/g)
Acetone	$17.62±0.46^{c}$	$50.35±1.15^{b}$
Ethanol	$19.83±1.10^{b}$	$62.18±3.35^{a}$
Methanol	$34.88±0.29^{a}$	$33.30±2.45^{c}$

It was found total flavonoid content of *Theobroma cacao L.*of ethanolic extract was significantly higher (62.18 mg RE/g) compared to acetone and methanol (50.35 mg RE/g and 33.30 mg RE/g, respectively), as shown in Table 1. High flavonoid content in ethanolic extracts might be due to the water content increases the solubility of methoxylated and hydroxylated compounds, thus increase the extraction yield [18]. In the previous study [19], ethanol's least polar solvents yielded the highest flavonoid.

The result from this study was different from the previous study of eggplant by Rojo-Poveda *et al.,* [20], where acetone extract (18.52±0.07 mg QE/ g) has higher flavonoid content compared to methanolic and ethanolic extracts (16.26±0.26 mg QE/ g and 16.13±0.12 QE/g respectively). This might be due to different compounds extracted using a different solvent having different solubility. In general, the variation in this study between other studies could reflect the extraction method and solvent used. Previously, Fidrianny *et al.* [21] reported that the flavonoid content depends on the extraction method, extraction time, and temperature. The flavonoid value depends on the ultrasound condition, such as the amplitude, where the amplitude used in this study was low, leading to a higher total flavonoid extract in ethanol. This is proven in the previous study by Abdeltaif *et al*. [22] to obtain a high yield of the bioactive compound; the condition of ultrasound extraction used was 30% amplitude, 70% of ethanol solvents, and maceration for 24 hours.

B. The Antioxidant Content of Cocoa Pod Husk Ferric Reducing Antioxidant Power Assay

Ferric reducing power (FRAP) assay and DPPH radical scavenging activity assay were used to determine antioxidant activities of cocoa pod husk. The reducing power of FRAP was studied by observing the transformation of ferric to ferrous ion. The transformation reflects on the oxidation process and indicates antioxidant capability in the plant, which can be seen in yellow color changes to blue shades. Reducing power refers to the capability of the substance to donate electrons which can react with free radicals.

All extracts possessed some degrees of electron-donating capacity, but among the extracts, methanol extracts gave significantly higher reducing power between extracts (Table 2). The reducing power of methanol was obtained 521.73±1.89 µg TE/g followed by acetone and ethanol, which were obtained 506.11 µg TE/g and 332.49 µg TE/g respectively. Lanez *et al.* [23] found that ethanol extracts of cocoa pod husk have a reduced power of 390.94, which is slightly different from the value of ethanol extract obtained in this study.

Meanwhile, for DPPH assay, a free radical scavenging assay was used to determine the cocoa pod husk's antioxidant capability. The oxidation process in DPPH assay relies on color change (violet to yellow) [27]. Electron transfer coming from soluble phenolic fractions from the cocoa pod husk by-product determined as an antioxidant. The greater capability to search for free DPPH radicals and strong yellow color indicates stronger antioxidant potential. In this study, the effective concentration was determined by using the linear regression curve. Effective concentration (EC_{50}) was determined to decrease DPPH radicals by 50%.

The effective concentration of the cocoa pod husk was significantly different ($p<0.05$). Results indicated that methanol has significantly higher scavenging activity, 65.00 ug TE/ml compared to acetone and ethanol (72.67 ug TE/ml and 73.67 ug TE/ml, respectively). In contrast, according to Guenane *et al.,* [28], the EC_{50} of cocoa pod husk extracts in ethanol extracts obtained 26.10 ug/ml, and in [29] the antioxidant activity of cocoa shells in ethanol extracts in polyphenol effectiveness obtained was 66.56%. The difference might be due to the non-optimum conditions of extraction method of studies unable to produce the best phenolic and flavonoid levels, thus affecting the antioxidant activity. Table 2 shows that values expressed as mean ± standard deviation of triplicate measurement. Superscript with different letters is significantly different at $p<0.05$ within the different columns.

TABLE II
REDUCING POWER AND SCAVENGING ACTIVITY OF COCOA POD HUSK EXTRACTS USING DIFFERENT SOLVENTS

Extraction solvents	Reducing power (µg TE/g)	EC_{50} (µg TE/ml)
Acetone	506.11±2.71[b]	72.67±2.31[a]
Ethanol	332.49±10.04[c]	73.67±2.08[a]
Methanol	521.73±1.89[a]	65.00±2.65[b]

For FRAP assay, extraction solvents used in extracts influenced the reducing potential that can be measured by the amount of hydrogen atom donor that breaks the free radical chain [24]. Here, methanol was found as an effective solvent to extract antioxidant activity from cocoa pod husk. Similarly, in the DPPH assay, the sample tested with EC50 value has the lowest value of methanol 65.00 ug/ml. Methanol solvent was proven to scavenge free radicals [30] efficiently. This study's data indicated that cocoa pod husk could act as potential electron donor that can react with free radicals and convert them into stable compounds. Higher antioxidants in methanol extracts could be related to high phenolic compounds content. Lesser reducing power in acetone and ethanol extracts proved a secondary metabolite content due to the partitioning process between the solvents [31]. In agreement with Ye *et al.* [25], methanol was widely used to extract plant by product and extract antioxidant and phenolic compounds. Scavenging activities decreased from methanol to acetone and ethanol; thus, the polarity of solvents has an indirect function that could subsequently be related to increased ability to extract antioxidants [26].

C. Correlation Analysis

Pearson's correlation coefficient between antioxidant capacities was denoted by r, where it measures the relationship between several parameters. Pearson correlation coefficient of antioxidant capacity between total phenolic and total flavonoid contents were shown in Table 3.

TABLE III
CORRELATION ANALYSIS BETWEEN TOTAL PHENOLIC CONTENT AND TOTAL FLAVONOID CONTENT IN COCOA POD HUSK

	DPPH (EC_{50})	FRAP
Total phenolic content	-0.975	0.462
Total flavonoid content	0.951	-0.851

Data were analyzed using Pearson's correlation coefficient test.

The relationship between total phenolic and flavonoid content can be influenced by the mechanism involved in the oxidation and reduction reaction of ferrous ions. The addition and donating electrons exhibit the redox capacity and antioxidant capability in cocoa pod husk. This mechanism can be correlated with the redox properties of antioxidant compounds in the plant. There were several meaningful correlations in this study conducted.

The cocoa pod husk extract reacted with Folin-Ciocalteu reagent, and hence the total phenolic compounds can be calculated. Hence, measured the concentration of phenolic groups in the sample. In the total phenolic assay, the deep blue color of sample solution was formed indicates the sample contains high phenolic concentration, while the formation of the light blue color showed low phenolic content.

From the statistical analysis, the correlation relationship between total phenolic content and FRAP assay indicated a moderate positive relationship (r= 0.462) while a strong negative correlation was noted with total flavonoid content (r= -0.851). This showed the decreased amount of flavonoid content increased the reducing power. Thus, flavonoids have more power to inhibit radical reaction by donating its electron to free radicals and becoming more stable. However, contradictory findings by [32] reported that phenolic compounds from extracts are capable of converting free radicals into stable products.

DPPH assay had a strong positive relationship with total flavonoid content at (r= 0.951) while having a strong negative correlation coefficient with total phenolic content (r= -0.975). From the correlation, it indicated that total phenolic content and flavonoid content reflect the antioxidant capability. The properties and characteristics of these compounds influence the capability to act as free radical scavengers. Based on the study by Fitriansyah *et al.* [33], *the hydroxyl group and double bonds in flavonoids were* able to increase the antioxidant activity in extracts. The phenolics and flavonoids may act as the precursor for antioxidant activities, meaning they can counteract an imbalance in free radical species by converting into stable products such as unreactive oxyradicals [34].

IV. CONCLUSION

The type of solvents used in extraction will affect the extraction process of cocoa pod husk. The type of solvent used affected significantly ($p<0.05$) on total phenolic compounds, total flavonoid content, and antioxidant activity as measured using FRAP and DPPH assay. Among the extraction solvents, the extraction using methanol was more effective in extracting total phenolic content and revealed in effective concentration (EC_{50}). At the ratio of methanol: water extracts, total phenolic content (34.88 mg GAE/g), while reducing power obtained was 521.7 µg TE/g and scavenging activity of effective concentration 65.00 µg/ml. Total flavonoid content for ethanol extraction of *Theobroma cacao L.* was the highest, 62.18±3.35 mg RE/g followed by acetone and methanol extracts was 50.35 mg RE/g and 33.30 mg RE/g, respectively.

In the DPPH assay, total phenolic content and flavonoid content acts as a precursor to the antioxidant activity as both obtained a strong correlation coefficient which was r = -0.975 and r = 0.951, respectively. The correlation between total flavonoid content and FRAP indicated a strong negative correlation coefficient. In conclusion, methanol extraction yields higher antioxidants compared to ethanol and acetone extraction.

Consequently, this study has proven that the number of phenolic compounds and flavonoids contributing to antioxidant activities depends on the solvent being used and the extraction method. Further research exploration is needed in the phenolic compounds profiling or other bioactive compounds extracted from cocoa pod husk. Additionally, potential antimicrobial activity in cocoa pod husk may open to the utilization of natural preservatives in food industries.

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