The Effect of Fermentation Process, Extraction Methods and Solvents on Yield, Total Polyphenol, and Antioxidant Levels of Cocoa Beans

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Abstract. Processed cacao foods are widely consumed in the world and besides the distinctive taste, it also contains polyphenols, which are beneficial for health. The method used to extract natural compounds from cocoa beans is a critical process for obtaining a product of high-quality polyphenol, mainly to protect its nutritional value. This study aims to determine the effect of fermentation, extraction method, and different solvents, as well as their interactions on yield, total polyphenols, and antioxidant levels. The polyphenol compounds were obtained by using a different fermentation process (fermented and unfermented cocoa beans), extraction methods (maceration and ultrasound), and solvents (Methanol, Ethanol, and Acetone). The result showed that the polyphenol yield was determined by the interaction between the extraction process and solvents, while the content was determined by variations in solvents utilization. Antioxidant levels are not affected by variations in fermented/unfermented cocoa, extraction methods, and solvents, including their interactions. The ultrasound method has contributed to better polyphenol extraction more effectively than the maceration. While methanol and ethanol are helpful solvents for polyphenol extraction, the ethanol classified as GRAS is preferred because of its food application.

Keywords: polyphenol, cocoa, ultrasound, and extraction.

1. Introduction
Consumers are more concerned with healthy food and they still accept standard taste given that the product provides health benefits [1]. One type of compound that has a positive relationship to health is polyphenols as antioxidants [2,3] which are naturally found in many vegetables, fruits, cereals, and beverages. They are produced from secondary plant metabolites and are associated with plant defenses against ultraviolet radiation or pathogens [4]. In food, they contribute to bitterness, astringency, color, taste, smell, and oxidative stability [5]. Long-term consumption of foods rich in polyphenols protects against cancer development, diabetes, osteoporosis, cardiovascular and neurodegenerative diseases [6,7].

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Processed cocoa products are consumed globally, and besides the distinctive taste, it also contains health beneficial compounds [8]. It is known as a primary dietary source for antioxidants [9] by the strong phenolic content (procyanidins and flavanols) which is higher than in blueberries, cranberries, and pomegranates [10–12].

Polyphenol extraction from cocoa beans depends on pre-treatment [13] extraction method [14], temperature extraction [15], solvent type [14,16] and storage condition [15]. It has been reported that the blanching process increased polyphenol content in cocoa beans [17]. Traditionally, maceration and thermal processes such as heating, boiling, and reflux or combinations of them have been used to remove antioxidant compounds. Nevertheless, these affect the polyphenols’ stability and antioxidant capacity due to temperature effects and longer extraction times [18]. Alternative technologies, such as ultrasound which has positive effects, have been applied for polyphenols extraction in kinnow (Citrus reticulate L.) peel [19].

There is currently limited report on the interaction of the fermentation, extraction method, and solvent on yield, total polyphenol, and antioxidant levels in cocoa beans. Therefore, this study aims to determine the impact of the fermentation, extraction, solvents, and their interactions on yield, total polyphenols, and antioxidant levels.

2. Materials and methods
This study was conducted at Integrated Laboratory of Indonesian Industrial and Beverage Crops Research Institute (IIBCRI), Sukabumi, from March to December 2019.

2.1 Material
The cocoa beans were harvested from the Pakuwon Experimental Garden, IIBCRI, Sukabumi. The 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, gallic acid, sodium carbonate, acetone, ethanol, and methanol used were acquired from Merck.

2.2 Preparation of extracts
Unfermented and fermented cocoa beans were blanched for 5 minutes at 95°C, then immediately cooled. The shells were peeled, and the beans were dried at 50°C in a cabinet dryer for 36 hours until about 7% water content, followed by processing into powder that was soaked in hexane to remove their fat (defattting). The defatted powder was then macerated or subjected to ultrasound with solvents’ addition according to the treatment (methanol, ethanol, and acetone) with a ratio of 1:5. Extraction by maceration was carried out for 24 hours with a closed container and stirred for 6 hours using a magnetic stirrer, while the ultrasound process was conducted by mixing for 60 minutes and continued with ultrasound for 15 minutes. Then, the power was filtered to obtain filtrate, which was evaporated using the Buchi rotary evaporator at 40°C to get a thick extract.

2.3 Yield (%)
The cocoa extract percentage yield was calculated by dividing the extract weight with the sample weight and multiplying by 100 as expressed below [19].

\[
\text{Yield (\%)} = \frac{\text{weight of cocoa beans extract (g)}}{\text{weight of sample (g)}} \times 100\%
\]

2.4 Total polyphenol content
The total polyphenol content was determined using the method defined by Singleton and Rossi [20]. Briefly, 0.50 mL of sample was added with 0.5 ml Folin-Ciocalteu reagent, then mixed and incubated for 1 minute. Afterwards, 1.5 ml saturated sodium carbonate solution (about 20% w/v) and 7.5ml aquadest were added into the reaction mixture, followed by incubation at room temperature for two hours. The results were shown as gallic acid (reference standard) equivalent in milligram (GAE mg) / g
of dry sample weight. UV-VIS Spectrophotometer (Genesys 10S UV-Vis, USA) was used for measurement at 765 nm.

2.5 Antioxidant activity
The cocoa extracts antioxidant activity was determined by the DPPH assay following the methodology described by Cirillo et al. [21]. A 0.1 ml sample was briefly mixed with DPPH solution (3.9 ml, 0.004 g/ml) in methanol and incubated for 20 minutes. The mixture absorbance was measured at a wavelength of 517 nm. Based on % RSA (Radical Scavenging Activity), DPPH value was calculated using the formula below:

\[
\text{% RSA} = \frac{\text{absorbance of control} - \text{absorbance of the sample}}{\text{absorbance of control}} \times 100\% \quad (1)
\]

2.6 Study Design and Data Analysis
This study used a 3-factor completely randomized design (CRD) with two replications. The first factor was the difference in the raw ingredients (fermented and non-fermented materials), the second was the difference in the extraction method (Maceration and Ultrasound), and the third was the difference in solvents utilization (Methanol, Ethanol, and Acetone). The variables observed were yield, total polyphenols, and antioxidant levels. The collected data were analyzed for variance (ANOVA) at a 1% significance level. When the F test results were real, it was followed by the Tukey test at 5% significance level.

3. Results and Discussion
Extraction is the primary process to isolate certain phytochemical compounds from a plant. In this study, extraction was carried out in two fermentation processes (fermented and non-fermented), two extraction types (maceration and ultrasound), and three solvents (methanol, ethanol, and acetone). The ANOVA results were used to determine the treatment’s significant effect on the F 5% test. Analysis of variance was carried out on three variables of extraction effectiveness, namely yield, total polyphenols, and antioxidant capacity.

According to table 1, unfermented and fermented cocoa beans had no significant difference. Contrarily, there was a significant difference between the method and solvent type. Furthermore, the cocoa yield was influenced by the interaction between the extraction method and solvent used.

Table 1 shows total polyphenols were only affected by differences in the solvents used. In contrast, they were not affected by the differences in the fermentation process, extraction method, and interaction between treatments.

There was no significant effect on the differences in the fermentation process, extraction methods, solvents, or the interaction between treatments in the antioxidant level variable. This means that the extraction conditions or procedures did not change the overall effectiveness of the antioxidant extraction. Moreover, various methods have been used to calculate and compare antioxidants activity, such as DPPH, FRAP, ORAC, ABTS, and TEAC [22,23].

Table 1. The probability value of the ANOVA for yield, total polyphenols, and antioxidant capacity

| Treatment                      | Yields (%) | total polyphenols (mg GAE/g sample) | antioxidant capacity (% RSA) |
|--------------------------------|------------|------------------------------------|-----------------------------|
| Fermentation process (F)       | 0.532      | 0.062                              | 0.769                       |
| Extraction Method (M)          | 0.000 **   | 0.979                              | 0.100                       |
| Extraction Solvent (P)         | 0.000 **   | 0.000 **                           | 0.831                       |
| F x M                          | 0.224      | 0.300                              | 0.594                       |
| F x P                          | 0.109      | 0.329                              | 0.939                       |
| M x P                          | 0.000 **   | 0.253                              | 0.914                       |
| F x M x P                      | 0.497      | 0.418                              | 0.881                       |

** Significantly different (p<0.01).
3.1 Yield
The extraction method provided a maximum output of the crude extract containing polyphenol of the highest yield. The variance analysis (Table 2) showed that the ultrasound extraction method obtained a significantly higher yield than maceration. Ethanol and methanol utilization produced significantly higher yields than acetone solvents. However, considering the interaction between treatments, the ultrasound method with ethanol or methanol was used to obtain a high yield.

Table 2. Effect of different methods, types of solvents, and their interactions on yield

| Treatment                      | Yields (%) |
|--------------------------------|------------|
| Extraction Method (M):         |            |
| ● Maceration                   | 3.40±1.71 b|
| ● Ultrasound                   | 8.37±4.71 a|
| Extraction Solvent (P):        |            |
| ● Methanol                     | 7.39±4.28 a|
| ● Ethanol                      | 7.92±3.33 a|
| ● Acetone                      | 2.34±0.75 b|
| Interaction (MxP):             |            |
| ● Maceration x Methanol        | 3.35±1.13 b|
| ● Maceration x Ethanol         | 4.92±1.52 b|
| ● Maceration x Acetone         | 1.92±0.81 b|
| ● Ultrasound x Methanol        | 11.42±1.66 a|
| ● Ultrasound x Ethanol         | 10.91±1.38 a|
| ● Ultrasound x Acetone         | 2.76±0.36 b|

The values followed by a different letter are significantly different (p<0.05).

3.2 Total polyphenols
The DPPH method was used to calculate the total polyphenols. Normally, DPPH is a stable free organic radical that loses its absorption range at 515-528 nm by accepting an electron or a free radical element [24]. Based on Table 3, total polyphenol varied from various solvents to ethanolic extracts that had the highest amount. In contrast, the lower total polyphenol was contained in acetone extract. For complex food matrices, the suitable solvent choice is essential since the form and volume of polyphenols extracted are evaluated. Polyphenol content variations depend on solvents polarity, where aqueous alcohols like methanol and ethanol are used in compounds derived from plant materials[25]. Due to its application to food systems, ethanol classified as GRAS (Generally Recognized as Safe) is preferred [26].

Table 3. Effect of solvent type

| Solvent type | total polyphenols (mg GAE/g sample) |
|--------------|-------------------------------------|
| Methanol     | 333.25±48.68 a                      |
| Ethanol      | 306.63±31.42 a                      |
| Acetone      | 189.13±55.67 b                      |

The values followed by a different letter are significantly different (p<0.05).

3.3 Antioxidant capacity
According to Table 4, there was no significant difference between unfermented and fermented cocoa beans even though Albertini et al.[27] stated that the fermentation process reduces antioxidant capacity. Polyphenol degraded due to several factors, such as fermentation and sweating, as well as enzyme and non-enzyme oxidation. Polyphenols, particularly epicatechin, is more abundant in fresh cocoa beans [29]. Based on the average, the fermented cocoa beans antioxidant capacity value (87.89 %RSA) was smaller than that of unfermented (88.30 %RSA).
Table 4. Treatments effect on antioxidant capacity

| Fermentation process          | Solvent | Method (%RSA) |
|------------------------------|---------|---------------|
|                              |         | Maceration    | Ultrasound   |
| Fermented cocoa beans        | Methanol| 89.80±0.07 a  | 87.81±2.45 a |
| Ethanol                      | 88.78±0.68 a | 86.15±4.97 a  |
| Acetone                      | 89.87±0.00 a | 84.91±4.77 a  |
| Unfermented cocoa beans      | Methanol| 89.46±0.00 a  | 87.62±1.91 a |
| Ethanol                      | 89.19±0.27 a | 87.49±2.48 a  |
| Acetone                      | 88.78±0.41 a | 87.24±1.87 a  |

4. Conclusion
The cocoa extraction yield was influenced by the interaction between the methods and solvents used, while the polyphenol content was influenced by solvent type. Antioxidant levels were not affected by differences in raw ingredients, extraction methods, solvents, and interactions. The ultrasound method contributed to better extraction of polyphenol and is more effective than maceration. While methanol and ethanol are better solvents for this process, ethanol is classified as GRAS preferred because of its food application.

Authors Contribution
A. Aunillah (Main contributor); E. H. Purwanto (Main contributor); E. Wardiana (Member contributor); T. Iflah (Member contributor)

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