
COMPARATIVE PHYTOCHEMICAL PROFILE AND ANTIOXIDANT ACTIVITY OF COCOA BEANS METHANOL EXTRACT AND ITS N-BUTANOL FRACTION OBTAINED FROM JEMBRANA, BALI, INDONESIA

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ABSTRACT

This study aims to evaluate the differences in phytochemical profiles and antioxidant activity between the methanol extract of cocoa beans and the n-butanol fraction obtained from Jembrana, Bali. Maceration technique was used in this research. The crude extract of cocoa beans was partitioned based on polarity to determine which fraction has the most potential as a source of bioactive compounds. The n-butanol fraction was evaporated to obtain a polar fraction from the methanol extract of cocoa beans. UV-Vis spectrophotometry was used to measure the total content of flavonoids and tannins in the methanol extract of cocoa beans and the n-butanol fraction. Research found that the methanol extract of Balinese cocoa beans had a total flavonoid content of 0.34 g/100 g and tannins of 0.37 g/100 g. However, the n-butanol fraction had a total flavonoid content of 15.34 g/100 g and tannin 20.16 g/100 g. The IC₅₀ value of the methanol extract was 29.66 µg/ml, while the n-butanol fraction was 2.63 µg/ml. The AAI of the methanol extract was 1.35 and the n-butanol fraction was 2.63, indicating that the n-butanol fraction had stronger antioxidant activity with higher flavonoid and tannin content than the methanol extract. The implication of this research is that the n-butanol fraction of cocoa bean methanol extract has the potential as a source of bioactive compounds that can be utilized in the development of high added value products, especially in the context of food pharmaceuticals or other health product.

Keywords: Cocoa Beans, Methanol Extract, N-Butanol Fraction, Flavonoid, Tannins, Antioxidant Activity Index.

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INTRODUCTION

Cocoa can be regarded as an important crop in the Province of Bali, Indonesia. Jembrana Regency currently is the main contributor to total cocoa production in Bali, producing approximately 2942 tons of cacao beans in 2019 (BPS, 2021). The cocoa industry in Indonesia faces many issues, one of which is a relatively lower quality of the beans than that of most other areas (Fibrianto et al., 2021). The COVID-19 pandemic poses additional problems by lowering the price of cocoa beans, at least in Jembrana, Bali (Darmada, 2020). COVID-19 eats away at Balinese cocoa farmers' income, further adding detrimental effects to the regional economy of Bali, which relies heavily on the tourism sector. To support the government of Indonesia in tackling the issues above in the cocoa industry, it is crucial to explore additional values of Balinese cocoa beans in Jembrana through scientific study.

Extraction, using various solvents, is one of the most widely deployed methods to investigate the phytochemical and pharmacological profile of natural products. Methanol compound is

relatively polar and possess a role as universal solvent capable of extracting non-polar components (Riyadi et al., 2023). Based on a study using spirulina extract, using methanol solvent can increase the antioxidant activity of the extract compared to other solvents with a lower polarity level. A group of phytoconstituents named as flavonoids are regarded as endogenous antioxidants. Flavonoids protect plants from a myriad of biotic and abiotic stressors, and act as unique UV filters (Panche et al., 2016). Flavonoids are also recognized to exert medicinal benefits to human health. Catechins are classified as flavonoids, also known as flavanols or flavan-3-ols. Catechin, gallic catechin, epicatechin (EC), epigallocatechin (EGC) are non-esterified catechins, whereas the esterified catechins are epigallocatechin gallate (EGCG), epicatechin gallate (ECG), gallic catechin gallate (GCG), and catechin gallate. Catechins are distributed in a variety of natural products such as cocoa (Rauf et al., 2019). Other important phytoconstituents that contribute to human health are tannins. Tannins are proteins-precipitating polyphenols reported to undergo biotransformation by human gut microbiota, which determine their bioavailability and pharmacological effects. Most of the health-promoting properties of natural products can be attributed to the presence of tannins (Sallam et al., 2021), thus it is important to reveal the availability of tannins in studied samples of natural product.

To harvest antioxidants (i.e, flavonoids and tannins) from plants, maceration and partition extraction are considered as relatively simple methods (Slamet et al., 2022). These phytoconstituents are soluble in polar solvent such as methanol (Ng et al., 2020) and n-butanol (Larit et al., 2019). The higher the polarity of the solvent, the higher the extract's antioxidant content (Guo et al., 2020). Up to date, scientific data regarding total flavonoids, tannins and antioxidant activity of Balinese cocoa beans obtained from Jembrana regency is non-existent. Therefore, we conducted a phytochemical profiling study on cacao bean extract using methanol solvent and its n-butanol fraction as a preliminary step in developing this bioproduct as an additional source of therapeutic compounds (i.e., as a pharma food). The results of this study are expected to partially contribute to the added value of Balinese cocoa beans from Jembrana in the future.

In a phytochemical profile study on Bali cocoa bean extract from Jembrana Regency using methanol solvent and its n-butanol fraction, the aim of the study was to evaluate the difference in phytochemical profile and antioxidant activity between cocoa bean methanol extract and its n-butanol fraction obtained from Jembrana, Bali. This research aims to contribute to the development of bioproducts as an additional source of therapeutic compounds, so as to increase the added value of Bali cocoa beans from Jembrana in the future. The benefit of this research is to provide a deeper understanding of the potential phytochemical compounds contained in cocoa beans from Jembrana, as well as provide useful information for the cocoa industry related to the development of products with high added value, such as pharmaceutical foods containing therapeutic compounds. Thus, this research can make a real contribution in increasing the added value of Bali cocoa beans from Jembrana and supporting the economic growth of cocoa farmers in the region.

METHOD

Sample Preparation

Cocoa beans were obtained from Gumbrih Village, Jembrana Regency, Bali Province, Republic of Indonesia. The cocoa beans separated from the fruit were washed using clean water to remove impurities. Cleansed cocoa beans were then air-dried at room temperature. After the cocoa beans were dry, they were peeled to obtain them without the epidermis (pallets). Next, the pallets were blended until a fine powder of cocoa beans was obtained.

Extraction

Two hundred grams of cocoa bean powder was put into a glass bottle, mixed with methanol solution, covered and left for 1 x 24 hours, protected from light, and stirred every 2 hours. After 24 hours, the dregs and filtrate were filtered. Subsequently, the dregs were re-macerated using methanol for 24 hours. All the macerates were combined and evaporated using a rotary vacuum evaporator until a thick methanol extract was obtained.

Partitioning

The thick methanol extract was partitioned based on its polarity to determine the fraction that has the potential to be a bioactive compound. The concentrated extract was dissolved in ethanol: water (3: 7) and then evaporated to produce cocoa bean extract in the water phase. The extract was then partitioned using n-butanol. The n-butanol fraction was quantitatively tested for flavonoid, tannin and antioxidant activity using UV-Vis spectrophotometry.

Total Flavonoid Content Analysis

Standard Solutions

Total flavonoid analysis was conducted according to the protocol published by (Cong-Hau et al., 2021). Standard quercetin solutions with concentrations of 2, 4, 8, 12, 16 and 20 µg/mL were pipetted with a volume of 1.00 mL. The next step was adding 0.30 mL of 5% w/v NaNO₂ (wait for 5 minutes). Then add 0.30 mL of 10% w/v AlCl₃, 2.00 mL NaOH 1 mol, and distilled water to 10.00 mL into the pipetted standard quercetin solutions. These mixtures were incubated for 30 minutes at room temperature, and the absorbances were measured using UV-Vis spectrophotometry with a wavelength of 510 nm.

Test Samples

One hundred milligrams of extract were weighed and dissolved in 10 ethanol. Pipette 1 mL of the extract, then mixed with 0.30 mL of 5% w/v NaNO₂ (wait for 5 minutes). Then add 0.30 mL of 10% w/v AlCl₃, 2.00 mL NaOH 1 mol, and distilled water to 10.00 mL into the pipetted standard quercetin solutions. These mixtures were incubated for 30 minutes at room temperature, and the absorbances were measured using UV-Vis spectrophotometry with a wavelength of 510 nm. The sample solution was made in three replications so that the flavonoid levels obtained were equivalent to quercetin.

Total Tannin Analysis

Standard Solutions

Analysis of total tannin content was conducted according to a method disseminated by (Ci, 2016). For each concentration of tannic acid (20, 40, 60, 80 and 10 µg/mL), 0.1 mL was pipetted, then 0.5 mL of Folin Ciocalteu reagent was added, shaken and left for 3 minutes. 1mL of 35% Na₂CO₃ solution was added, shaken until homogeneous, and left for 60 minutes at room temperature. The

absorbances were measured at a wavelength of 765 nm. The results were plotted into a calibration curve.

Test Samples

Weigh 10 mg of methanol extract and n-butanol fraction samples each, put them in a beaker, and dissolve them in 10 mL of 70% ethanol: distilled water (1:1). A volume of 0.1 mL of the extract solution was mixed with 0.5 mL of Folin-Ciocalteu reagent, homogenized then left for 3 minutes. Add 1.00 mL of 35% sodium carbonate (Na_2CO_3) solution to the mixture and let stand for 60 minutes at room temperature. The absorbance of the solution was measured with a UV-Vis spectrophotometer at the maximum wavelength.

RESULTS AND DISCUSSION

Taxonomically, Balinese cocoa is identified as *Theobroma cacao* L. (Letter Number: B-491/IPH.7/AP/VI/2019). Detailed taxonomic information on Balinese cacao is written as follows:

Table 1. Balinese cocoa taxonomy

Kingdom:	<i>Plantae</i>
Subkingdom:	<i>Tracheobionta</i>
Division:	<i>Magnoliophyta</i>
Superdivision:	<i>Spermatophyta</i>
Class:	<i>Magnoliopsida</i>
Subclass:	<i>Rosidae</i>
Order:	<i>Malvales</i>
Family:	<i>Malvaceae</i>
Genus:	<i>Theobroma</i>
Species:	<i>Theobroma cacao</i> L.

Comparative selected chemical analyses have been conducted on the methanolic extract of Balinese cocoa and its n-butanol fraction. Cocoa is globally known as the raw material for chocolate and derivative products such as chocolate beverages, bakeries and pastries. It is also developed into cosmetic products. In the current study, Balinese cocoa beans from Jembrana, Bali, Indonesia, were revealed to contain secondary metabolites and demonstrate potent antioxidant activity. These findings further validate the extended spectrum of Balinese cocoa beans utilization, at least in the form of methanol extract and its n-butanol fraction, as a pharmafood and potentially as cosmeceutical products. Pharmafood is endowed with medicinal benefits due to the existence of specific constituents, such as those with antioxidant activity (Mahendra & Pramatha, 2021).

Flavonoid Content Analysis

Total flavonoid levels were analysed using the UV-Vis spectrophotometric method with quercetin as a standard based on the study conducted by (Yahya et al., 2021). The measurement analysis results obtained on the quercetin standard showed the line equation $y=0.0297x+0.0066$ with an R^2 of 0.999. Through the linear equation values, the total flavonoid content in the methanol extract of cocoa beans was 0.3358 ± 0.002 g QE/100g. The analysis results of total flavonoid levels in the n-butanol fraction were higher than the methanol extract at 0.372 ± 0.011 g QE/100g (Table 1).

A study measured the total levels of flavonoids in the methanol extract of *C. Decapetala* wood at 3.93 ± 0.005 mg QE/g (Pawar & Surana, 2010). Other research on total flavonoid levels using *Enna*

singing plant extract revealed a total flavonoid level of 7.37 mg QE/g (Mwamatope et al., 2020). The ability of flavonoid compounds as antioxidants can be seen from the total flavonoid content. Through the mechanism of action of flavonoids, they can act as ROS scavengers by donating electrons to free radicals (Dias et al., 2021). Flavonoid compounds can play a role in preventing lipid peroxidation caused by ROS in vivo (Ebuehi et al., 2019). They may act as an organ protector (i.e. nephroprotective) against a constellation of pathophysiological events triggered by damage-associated molecular patterns (DAMP) such as uric acid (Mahendra et al., 2023; Mahendra & Dewi, 2020).

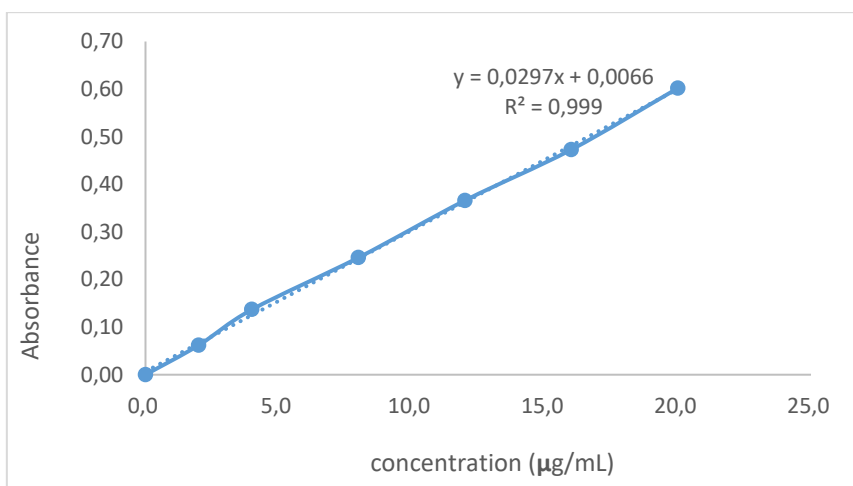


Figure 1. Calibration Curve of Total Flavonoid Content.

Tannin Content Analysis

Total tannin content was analysed using the UV-vis spectrophotometric method with tannic acid as a standard. The measurement analysis results obtained on the tannic acid standard showed the line equation $y=0.013x + 0.0099$ with R^2 of 0.998 (figure 2). The line equation values revealed that the total tannin content in the methanol extract was 15.3446 ± 1.34 g TAE/100g. Total flavonoid levels in the n-butanol fraction were higher compared to the methanol extract, amounting to 20.1565 ± 0.82 g TAE/100g. Complete data on total flavonoid and total tannin levels can be seen in Table 1.

Tannin compounds are secondary metabolites that have roles as antioxidants, UV and antibacterial agents (Guo et al., 2020). An example of a condensed tannin compound is proanthocyanidin (Rauf et al., 2019b). In another study using methanol extract of *Indigofera tinctoria*, tannin levels were 229 ± 0.001 mg TAE/g (Guo et al., 2020). This finding shows that cocoa beans are superior to this test bioproduct in terms of tannin content.

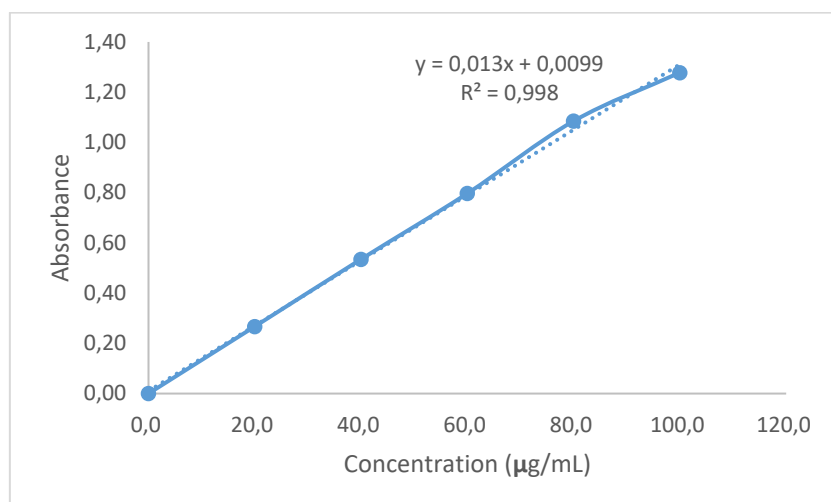


Figure 2. Calibration Curve of Tannin Total Content

Table 2. Flavonoid and Tannin Content of Cocoa Beans Methanol Extract vs. Its N-butanol Fraction

Sample	Replication	Flavonoid (g QE/100g)	Tannin (g TAE/100g)
Methanol Extract	I	0,33405	14,8871
	II	0,33491	16,8544
	III	0,33851	14,2923
N-butanol Fraction	I	0,38534	19,3696
	II	0,36846	20,0854
	III	0,36273	21,0147

Antioxidant Activity Index (AAI)

The DPPH method, with a concentration of 0.1 mM, was used to investigate the antioxidant activity of the test substance in this study. The results of antioxidant testing of the methanol extract of cocoa beans obtained an IC₅₀ value of 29.66 µg/mL and the n-butanol fraction with an IC₅₀ value of 15.23 µg. /mL. The antioxidant activity value can be seen from how big the IC₅₀ (Inhibitor Concentration) value is obtained from the linear regression equation of extract concentration on the free radical scavenger activity value as in Figures 3 and 4. The y coefficient in this regression equation is IC₅₀.

In contrast, the x coefficient in the equation is the fraction's concentration whose value will be sought, where the value of x obtained is the concentration required to reduce 50% of DPPH radical activity. From the equation, it can also be seen that the R² value shows the correlation between the percentage (%) of free radical scavenger activity and the sample concentration and sample immersion. The results provide a positive correlation, as seen from the R² value of the methanol extract of cocoa beans of 0.958. At the same time, the n-butanol fraction is 0.9898, and both R² values of the fractions are close to 1, illustrating that increasing extract concentration increases the free radical scavenger activity (Scherer & Godoy, 2009).

The IC₅₀ value is obtained from the regression equation, which is a reference for high and low antioxidant activity. IC₅₀ is a parameter for interpreting DPPH test results. The IC₅₀ value is the concentration of the test compound that can absorb free radicals by 50%, or IC₅₀ is a number that shows the concentration of the extract that can inhibit the oxidation process by 50%. The classification of antioxidants is divided into 5, namely < 50 ppm (very strong), 50-100 ppm (strong), 100-150 ppm (medium), 150-200 ppm (weak), and > 200 ppm is very weak (Scherer & Godoy, 2009).

From the test results obtained, the cocoa bean fraction has a strong potential as an antioxidant. Based on research conducted with the results of the ethyl acetate fraction, an IC_{50} value of 8.66 $\mu\text{g/mL}$ was obtained, and the n-butanol fraction had an IC_{50} value of 15.41 $\mu\text{g/mL}$. So, the ethyl acetate fraction and n-butanol fraction are classified as very strong antioxidants because they are at <50 ppm.

The AAI value serves to classify the extract's antioxidant properties, where the AAI is obtained by calculating the final concentration of the DPPH solution divided by the IC_{50} value of the antioxidant compound. Based on the AAI value, if the AAI value is less than 0.5, then it is said to have "weak" antioxidant activity; it is said to be "medium" if the AAI value is between 0.5 - 1, it is said to be "strong" if the AAI value is between 1 - 2. It is "very strong" if the AAI value exceeds 2 (Scherer & Godoy, 2009). Based on research that has been carried out, it was found that the AAI value for the methanol extract of cocoa beans was 1.35, and the n-butanol fraction was 2.63. Referring to the AAI values, cocoa beans' methanol extract and n-butanol fraction have very strong activity.

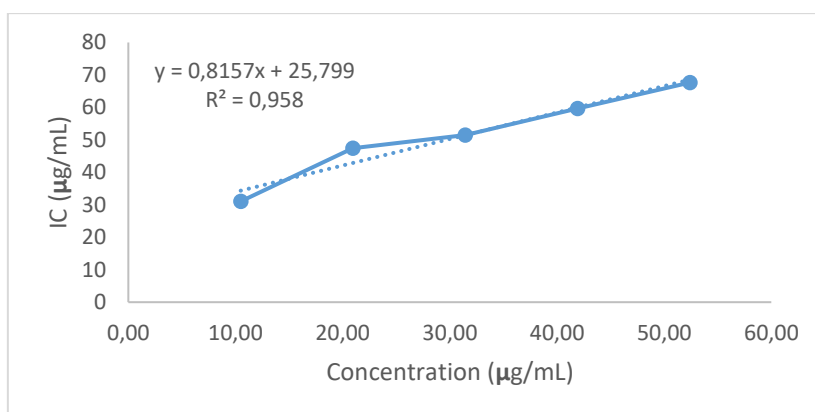


Figure 3. IC_{50} of Balinese cocoa beans methanol extract

Based on the curve results obtained, the line equation $y = 20.8157x + 25.799$ with an R^2 of 0.958 was obtained, so the IC_{50} value of the ethanol extract of cocoa beans was 29.66 $\mu\text{g/mL}$. Another study that carried out tests carried out by (Mzid et al., 2017) found that the reduction of free radicals in *Urtica urens* ethanol extract was 245.65 ± 10.2 $\mu\text{g/mL}$.

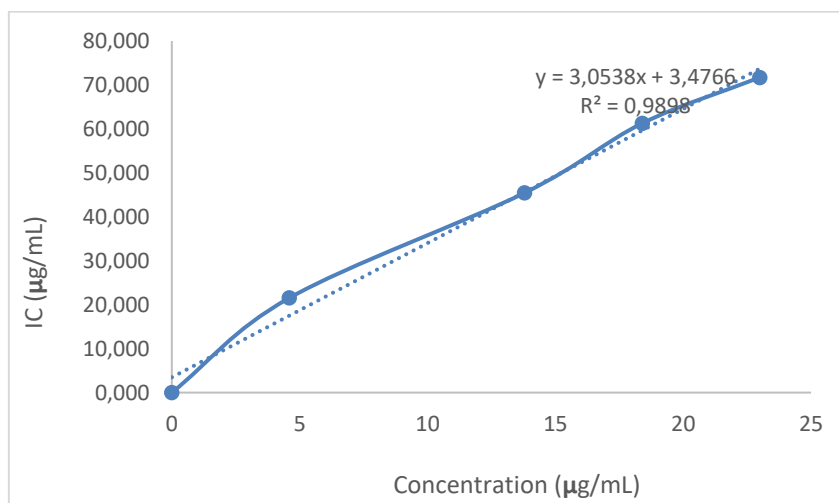


Figure 4. IC_{50} Value of n-butanol fraction

Table 3. IC₅₀ and AAI values of ethanol extract and n-butanol fraction

Sample	IC ₅₀	AAI
Methanol extract	29,66 µg.ml ⁻¹	1,35
N-butanol fraction	15,23 µg.ml ⁻¹	2,63

Medicinal properties of bioactive compounds in cocoa beans and cocoa-derived products are subjects of scientific pursuit. Specific bacterial-fungal alliance and the resulting metabolites associated with cocoa bean fermentation may also contribute to the medicinal effect of cocoa beans, which exert potential medicinal benefits on human health. The integrality of these microbiological interactions and the resulting metabolites is called cocobiota, a novel term coined by (Petyaev & Bashmakov, 2016). The microbial diversity of cocobiota of cocoa beans harvested from southern Brazil revealed the presence of *Hanseniaspora* spp., *Saccharomyces* spp., and *Pichia* spp. as the native yeast population. *Lactococcus* spp., *Staphylococcus* spp., *Acetobacter* spp. and *Lactobacilli* strains were demonstrated as the bacterial components of this cocobiota (Bastos et al., 2018). Cocobiota, thus, is an important factor that shall be considered in future studies to elucidate the medicinal properties of Balinese cocoa beans.

It is essential to examine further the bioactive compound armamentarium contained in Balinese cocoa beans using various solvents and pre-extraction treatments. Safety issues (i.e., acute and chronic toxicity profiling) and other relevant issue, such as cocobiota, shall be addressed. These studies will provide a sound basis for further investigations in developing Balinese cocoa beans as a source of bioactive compounds with medicinal properties.

CONCLUSION

The n-butanol fraction of Balinese cocoa (*Theobroma cacao* L.) beans exhibits higher total flavonoid and tannin content than the methanolic extract counterpart. The AAI value of the methanol extract is lower than its n-butanol fraction, but both test agents display very strong antioxidant activity. Further studies should be conducted to elucidate the complete picture of Balinese cocoa methanol extract's and its n-butanol fraction's phytochemical profile and antioxidant properties.

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