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Analysis Antioxidant of Fractions Cocoa Beans (*Theobroma Cacao* L.) as Potential Herbal Medicine

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Abstract
The cocoa seeds of the Gumbrih-Bali region are a natural commodity with great potential for development or processing by local communities. The purpose of this research is to provide information about the antioxidant potential contained in cocoa seeds. Testing the antioxidant capacity in this study uses the Uv-Vis spectrophotometry method at 517 nm wavelengths. Free radical scavanger is measured with control absorption and samples was analyzed with regression curve. The research results proved that the antioxidant (AAI) in the n-butanol fraction show result very strong ability of 4.64 compared to the 0.42 ethyl acetate fraction and 0.46 n-hexane fraction. These results prove that the potential chemical content of cocoa seeds is high in polar solvents (n-butanol fractions) such as flavonoid compounds. Furthermore, this research will be used as a basis for developing natural herbal ingredients such as cocoa which can be consumed in the community to prevent degenerative diseases.

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INTRODUCTION

Increased levels of oxidative stress in the cell the progression of disease toward higher disease severity damaging the cells and the response to the conventional drugs used in the treatment of chronic diseases (Albano et al.,2022). Reactive Oxygen Species (ROS) are sensitive biomolecules and stay in the body longer when antioxidant defenses are unable to effectively neutralize them. Overexposure to ROS can harm membrane lipids, proteins, and nucleic acids, impairing normal cellular activity (García-Sánchez et al., 2020). However, aging and chronic degenerative pathologies, such as Alzheimer's disease (AD) and Parkinson's disease (PD), cardiovascular disease (CVD), diabetes mellitus (DM), and chronic kidney disease (CKD), are caused by oxidative stress mechanisms. We also outline preventative actions that could be taken to fight chronic degenerative diseases and aging (Leyane et al., 2022).

All small molecule found rich in commodities of its natural resources. One of the uses of plant natural resources by humans is as a source of primary metabolites such as carbohydrates, proteins, fats, vitamins, and minerals as well as nutrients (Seca & Pinto, 2018; Kim et al., 2022). Cacao is one of the main sources of the basic ingredients of chocolate making. The main component of the cocoa fruit that is widely used in the chocolate industry is its seeds (Crozier et al., 2011). Cocoa seeds as one of the potential natural ingredients that has been known to have quite strong antioxidant content (Katz et al., 2011; Oracz & Nebesny, 2016). However,

the surrounding community is still not scientifically aware of the antioxidant ability of the cocoa seed fraction from the village of Gumbrih Jembrana-Bali that is beneficial to health.

Oxidative damage occurs as a result of low antioxidants in the body so that it cannot compensate for the reactivity of the oxidative compounds. One effort is to consume an antioxidant intake from the outside of both vitamins and medicines (synthetic and herbal) to reduce oxidative stress (Katz et al., 2011). Antioxidants have been known to be anti-free radicals that can prevent the occurrence of lipid oxidation. The mechanism of antioxidant action is that it can break radical/radical chain scavenging by donating hydrogen atoms to peroxyl radicals and converting them into more stable radical products (Jomova et al., 2023). In addition, the ability of an antioxidant compound to act as a metallic glitter (Lokesh et al., 2018) and as a xanthine oxidase inhibitor has also been (Mohamed Isa et al., 2018).

The flavonoid compound has been to be one of the antioxidant compounds that can reduce oxidative stress by suppressing the superoxide dismutase radicals in vivo and in vitro using the DPPH method (2,2 diphenyl-1-picrylhydrazyl) (Jadid et al., 2017). Flavonoid compounds are one of the most abundant polyphenol compound groups in nature. A study conducted by Yusuf et al., (2021) found that cocoa seeds have the highest polyphenol content of 236.28 mg of GAE/100 g compared to 159.61 mg of cranberry extract/100 g and 181 mg of butterfly GAE/100 g. In preliminary research, it is known that the total antioxidant capacity in the treatment variation of cocoa seeds oven is higher by 42.454 mg/mL GAEAC compared to the cocoa drying by 27.730 mg/mL GAEAC (Wibawa, 2021). The purpose of this research on the chemical ability of the compounds in particular of the cacao beans from the province of Bali, to date no one has the antioxidant capacities in detail. Researchers are interested in finding information about antioxidant capabilities quantitatively using spectrophotometry UV-vis.

METHOD

Instrument

The instruments used in this study are knives, blenders, analytical balances, glass cups, cranes, reaction tubes, erlenmeyer, split crane, drip pipettes, measuring glasses, mixer bars, filter paper, vial bottles, dryer, porcelain cup, spectrofotometer UV-vis (Shimadzu 1800), rotary evaporator (Buchi).

Material

The materials used in this study are: cocoa seeds (*Theobroma cacao* L.) obtained from the Gumbrih-Jembrana, methanol, ethanol 96%, n-hexane (C_6H_{14}), ethyl acetate ($C_4H_8O_2$), n-butanol ($C_4H_{10}O$), aquades, chloric acid (HCl), sodium hydroxide 10% (NaOH), ammonium hydrogen oxide (NH_4OH), magnesium powder (Mg), sulfate acid (H_2SO_4), $FeCl_3$ 1%, Meyer, Willstater, bate smith, Lieberman-Burchard, and ascorbic acid standard (Merck).

Sampel Preparation

5 kg of cocoa seed is washed with water until clean. Cocoa beans are dried in an oven (40°C) for 2 days. After the cocoa beans are dry, the next stage is carried out, namely the extraction stage.

Extraction

Maceration method of solid-liquid extraction. In this process, powdered solid material is placed in vessel. In this research, maceration method refers to Rasul, (2018). Extraction process was carried out using the maceration method with 96% ethanol solvent. The cocoa beans are soaked in solvent until they are completely submerged. Then the sample was left in the solvent for 24 hours and occasionally stirred consistently every 2 hours. The macerate obtained is separated

from the dregs by filtering. Then the dregs are macerated again with the same solvent for 24 hours and the filtrate is collected. Then, the collected filtrate is concentrated using a rotatory vacuum evaporator.

Fractionation

The production of n-hexane, ethyl acetate and n-butanol fractions from cocoa extracts is carried out by liquid fractioning method using a separating funnel. A total of 12.5 g of cocoa-seed thick extract is dissolved in 150 mL of aquadest. The solution is then inserted into a separate pot with a closed crane. A maximum of 50 mL of n-hexane is added to the extract solution and mounted until homogeneous. The n-hexane fraction is separated from the water fraction by opening the crane of the separated pipe and stored in the erlenmeyer. This treatment is repeated twice with the same solvent. The remaining water fraction of n-hexane is added with 50 mL of ethyl acetate solvent. The process is done in the same way as in the n-hexane fraction, thus obtaining the ethylacetate fraction and the water fraction. The resulting water fractions are added with n-butanol solvent in the amount of 50 mL. The resulting fraction is stored in a vial bottle and then applied with a rotary evaporator at a temperature of 40°C to obtain a concentrated fraction of n-hexane (HF), ethyl acetate (EAF) and n-butanol (BF).

Phytochemical Identification

Phytochemical tests are performed on the compounds resulting in determining the group of active compound. Phytochemical tests performed are alkaloids, flavonoids, terpenoids, saponins, and tannins. Here are the steps of phytochemical analysis (Pujiastuti & Andreana, 2022).

Alkaloid Test

Each 5 mL fraction (HF, EAF, and BF) adds HCl 2 N and then adds Meyer reaction. Mayer will provide positive results with formation of a white precipitate.

Flavonoid Test

There are three reagents used in flavonoid test.

a. Wilstater Reagen

   A total of 1 mL of the sample is inserted into the reaction tube, then added with magnesium powder (Mg) and 2-4 drops of concentrated chloric acid, then crushed and formed a thick colour that indicates the presence of flavonoids of the groups flavonols and flavanones.

b. Bate Smith Reagen

   As much as 1 mL of each fraction is inserted into the reaction tube, and then added with concentrated chloric acid a few drops. Then the mixture is heated for 15 minutes on top of the resin. The formation of the red color indicates the presence of the flavonoids of the anthocyanidins.

c. NaOH 10%

   A total of 1 mL of each fraction was put into a test tube, then added with a few drops of 10% Sodium Hydroxide solution, a color change occurred which indicated the presence of flavonoids because they are classified as phenolic compounds.

Saponin Test

Each fraction was added water and then coated strongly and a stable foam was formed that did not disappear when added acid indicates the presence of saponins. Stable foam for 10 minutes indicates saponins (Novilda & Marcellia, 2022; Rohsaita et al., 2024).

Triterpenoid/Steroid Test

Each fraction (HF, EAF, and BF) was added to the Liebermann-Burchard reaction and a positive test would be shown with the presence of a purple-red wama for the triterpenoids and green to blue for the steroids (Novilda & Marcellia, 2022; Rohsaita et al., 2024)

Tannin Test

Each fraction is added a few drops of FeCl3 1%. Positive tannin when it turns green.

Determination In Vitro of Antioxidant Activity using DPPH

An in vitro test of antioxidant activity in this study was conducted by determining the IC_{50} fraction of cocoa seeds. 10 mg cocoa fraction was dissolved with ethanol in a 10 mL, then matched and filtered to obtain a parent solution. The solution was then prepared from the parent solution at a concentration of 10; 25; 50; 75; and 100 µg/mL. Each test solution was piped at 1.0 mL and then inserted into the reaction tube and 1.0 ml DPPH (2,2-diphenyl-1-pycrylhydrazyl) 0.1 mM. The mixture was then divortexed and incubated at room temperature in dark conditions for 30 minutes. The absorption was measured with spectrophotometer UV-Vis at the maximum wavelength of 517 nm (Baliyan et al., 2022). The antioxidant activity in this study was measured as a decrease in the absorption of the DPPH solution caused by the addition of samples. The parameter used to measure antioxidant activity is IC_{50}. The IC_{50} value is the number that shows the concentration of extract that can inhibit the activity of a free radical by 50%. The smaller the IC_{50} value indicates the higher the antioxidative activity (Gulcin & Alwasel, 2023). The presentation of inhibition can be calculated by the formula:

$$\%\ inhibition = \frac{control\ absorbance\ -\ sample\ absorbance}{control\ absorbance}$$

RESULTS AND DISCUSSION

The crude extract obtained in this study was 38.2 grams. The amount of crude extract obtained was fractionated to separate the compound components based on their polarity level (Wibawa, 2016). The weight of n-hexane, ethyl acetate, and n-butanol fractions presented in Table 1.

Table 1. Weight of N-Hexane, Ethyl acetate and N-Butanol Fraction

Sample		
HF	**EAF**	**BF**
2,2270 g	1,0612 g	2,0291 g

Phytochemical Analysis of Fractionation

Phytochemical screening tests were carried out to identify compounds contained in the n-hexane, ethyl acetate, and n-butanol fractions. The following are the results of phytochemical screening tests from the n-hexane, ethyl acetate, and n-butanol Fractions (Table 2).

Table 2. Phytochemical Profiling of Sample Fraction

Secondary Metabolite	Reagent	Sample Fraction		
		HF	**EAF**	**BF**
Alkaloids	Meyer	-	-	-
Flavonoids	NaOH 0%	-	+	+
	Wilstater	-	+	+
	Bate Smith	-	+	+
Saponin	Water	+	+	+
Terpenoid	Lieberman-Burchard	+	-	-
Tannin	$FeCl_3$ 1%	+	+	+

After carrying out phytochemical screening tests, the results were obtained that the n-hexane fraction did not contain flavonoid compounds but only contained tannin compounds. The ethyl acetate fraction contains low-intensity flavonoid compounds, while the n-butanol fraction contains very strong flavonoid compounds, tannins, saponins, and terpenoids. The high flavonoid content is in line with its ability as a potentially powerful antioxidant (Hassanpour & Doroudi, 2023).

Antioxidant Activity Analysis

Analysis of Ascorbic Acid with DPPH

Testing of the antioxidant activity of ascorbic acid at concentrations of 1, 2, 3, 4, and 5 µg/mL as well as a DPPH solution with a concentration of 0.1 mM which acted as a control solution. The five solution series of sample concentrations and the DPPH solution were measured at a maximum wavelength of 517 nm (Kholifah et al., 2023). The percent inhibition value from measuring the standard ascorbic acid solution can be seen in Table 3.

Table 3. Ascorbic Acid Inhibition

Concentration (µg/mL)	Inhibition (%)
1	12,30
2	24,80
3	37,30
4	49,80
5	61,34

Based on the results of calculating the % inhibition of ascorbic acid, a relationship was then made between the concentration and the % inhibition to obtain a linear regression curve. The linear regression curve for ascorbic acid can be seen in Figure 1.

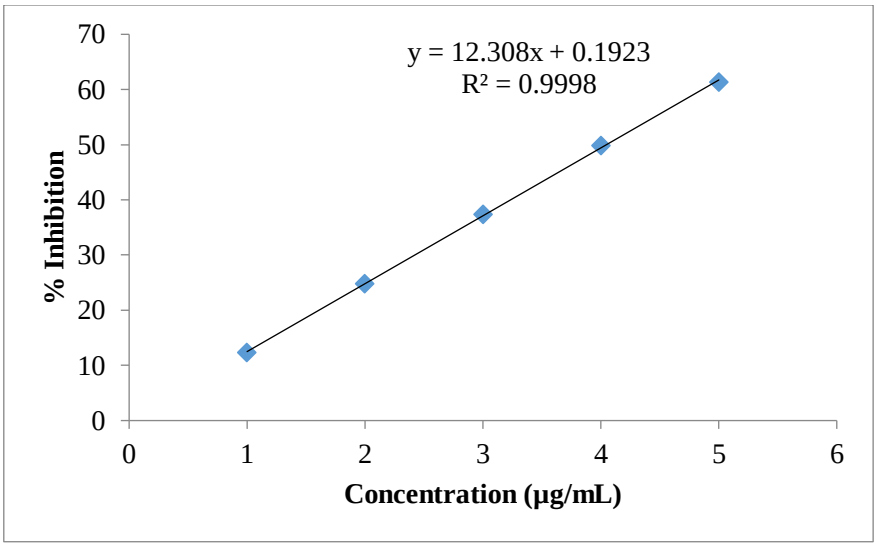


Figure 1. Ascorbic Acid Regression Curve

Based on the relationship between solution concentration and absorbance, a linear regression equation is obtained, namely y=12.30x+0.192, with R^2 = 0.999. From the linear regression equation, calculations can then be carried out to find the IC_{50} value (Jarantow et al., 2023). The IC_{50} value of ascorbic acid was found to be 4.05 µg/mL. One of the abilities of ascorbic acid (vitamin C) in human biological systems is collagen synthesis with the help of the enzymes procollagen-proline dioxygenase and pro-collagen-lysine dioxygenase (prolyl and lysyl hydroxylase). Chemically, ascorbic acid can donate hydrogen atoms to neutralize free radical activity (Njus *et al*., 2020).

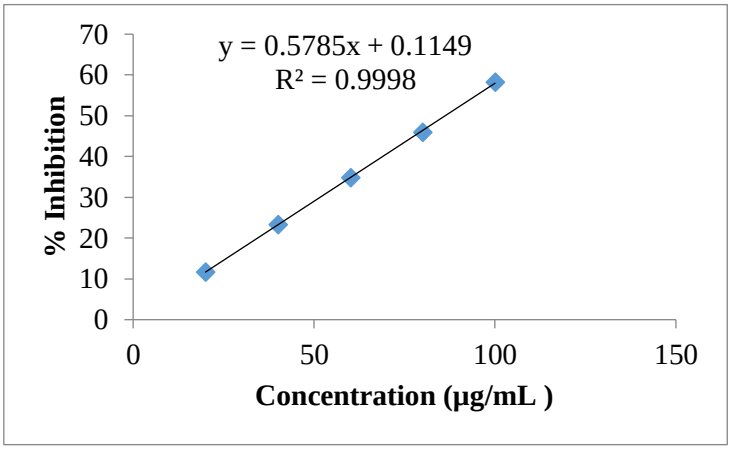
Antioxidant Activity of n-hexane, Ethyl acetate and n-butanol Fractions

The antioxidant activity test of the ethyl acetate fraction was made in five series of concentration solutions, namely 20, 40, 60, 80, and 100 µg/mL as well as a DPPH solution with a concentration of 0.1mM which acted as a control solution. The five solution series of sample concentrations and the DPPH solution were measured at a maximum wavelength of 517 nm. Then, calculations were carried out to find the % inhibition of each concentration series solution. The results of the % inhibition calculation can be seen in Table 4.

Table 4. Calculation Results of % Inhibition of n-Hexane, Ethyl Acetate and n-Butanol Fractions

Sample	Concentration (µg/mL)			% Inhibition		
	HF	EAF	BF	HF	EAF	BF
	20	20	2	11,68	14,20	11,61
	40	40	4	23,37	24,24	24,60
	60	60	6	34,86	33,71	36,22
	80	80	8	45,97	42,99	46,85
	100	100	10	58,23	51,13	56,88

Based on the results of calculating the % inhibition, a relationship was then made between the concentration and the % inhibition to obtain a linear regression curve. The linear regression curve of n-hexane, ethyl acetate, and n-butanol fractions can be seen in Figure 2.



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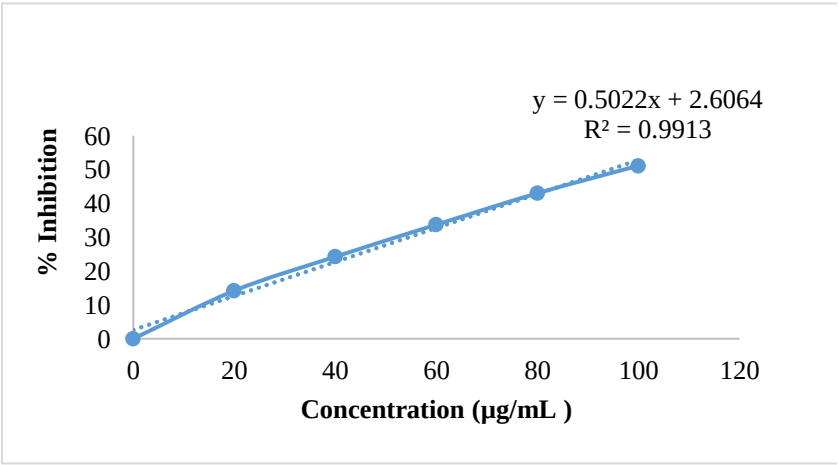

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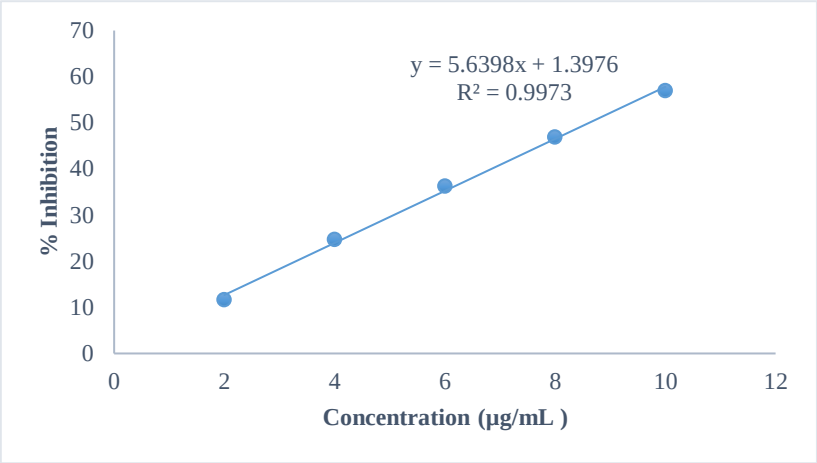

c

Figure 2. Regression Linier Curve; (a) n-Hexane Fraction, (b) Ethyl Acetate Fraction, (c) n-Butanol Fraction

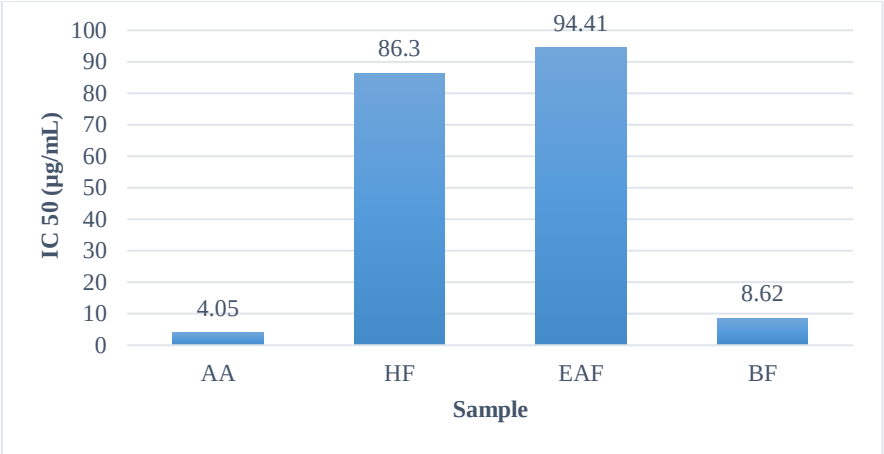

Figure 3. Diagram of IC_{50} Values in Ascorbic Acid (AA), N-Hexane (HF), Ethyl Acetate (EAF) and N-butanol fractions (BF) of Cocoa Beans

Based on Figure 3, the IC_{50} values for the n-hexane, ethyl acetate, and n-butanol fractions obtained respectively IC_{50} values of 86.30; 94.41; and 8.62 µg/mL The results of this study explain that the non-polar content found in cocoa beans has very weak antioxidant abilities. This is in line with research conducted by Akbar *et al.*, (2021) obtained the results that the n-hexane and ethyl acetate fractions had lower flavonoid and phenolic contents compared to the methanol fraction.

Based on the relationship between solution concentration and absorbance, a linear regression equation is obtained y= 0.502x+2.606 with R^2=0.9913. The IC_{50} value of the ethyl acetate fraction was found to be 94.41 µg/mL with an AAI (Antioxidant Activity Index) value of 0.42 (weak). This result indicates the presence of semi-polar compounds which can reduce the antioxidant activity of the sample. The high fatty acid content in cocoa bean extract has an important role as a natural antioxidant. The fatty acid (oleic acid) contained in cocoa is needed for health and metabolism in the human body (Manzano *et al*., 2017).

Based on the AAI value of the n-butanol fraction in the table above, it can be said that the n-butanol fraction is classified as a very strong antioxidant (Djebbari et al., 2015). The cause of the weak antioxidant activity of the ethyl acetate and n-hexane fractions is thought to be caused by the presence of disruptors such as fats, proteins, carbohydrates, and other compounds which

are still not separated properly, thereby reducing their ability as antioxidants. This is in accordance with the theory, that flavonoid compounds which are polyphenolic compounds are contained in many polar compounds (n-butanol fraction) (Oracz & Żyżelewicz, 2020). The presence of O-H, C-H, C=O, C=C, and C-O-C groups contained in cocoa bean extracts and fractions plays a very important role, as a DPPH radical reducer (Indiarto *et al*., 2019).

Table 5. AAI Values of Ascobric Acid, n-hexane, Ethyl Acetate, and n-butanol Fractions

No.	Sample	AAI value	Criteria
1	Ascorbic Acid	13,56	Very Strong
2	HF	0,46	Weak
3	EAF	0,42	Weak
4	BF	4,64	Very Strong

Index: AAI (*Antioxidant Activity Index*): Weak (0-0,5); Medium (0,5-1); Strong (1-2); Very Strong (>2)

CONCLUSION

This research proves that the n-butanol fraction in cocoa bean extract is able to provide higher antioxidant capacity compared to the n-hexane and ethyl acetate fractions. The antioxidant might be due to the synergistic actions of bioactive compounds present in them. Therefore, the plant has promising compounds to be tested as potential antioxidant drugs for treatment of diseases resulting from oxidative stress. The study will be helpful to understand cocoa in Jembrana as potential herbal medicine.

RECOMMENDATION

Future research needs to carry out further tests regarding the types of active ingredients, especially flavonoid compounds in the n-butanol fraction and this can be applied in pre-clinical tests with test animals.

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