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Effect of Drying Temperature Variation on the Antioxidant Activity Ethanol Extract of Cocoa Bean (*Theobroma* cacao L.) with ABTS [*2,2-azino-bis(3-ethylbenzotiazolin-6-sulphonic acid*)] Method

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Abstract

Plants contain chemical compounds that are used as natural medicinal ingredients as antioxidants and have the potential to act as free radicals. Cocoa plants are rich in polyphenols such as flavanols, which have antioxidant and anti-inflammatory properties against degenerative diseases. The aim of the research is to determine whether variations in drying temperature of cocoa bean ethanol extract (*Theobroma cacao* L.) affect antioxidant activity. This research is in the form of experimental research carried out in a laboratory. The drying method uses an oven for 8 hours at temperatures of 40, 50, and 60ºC. The dried cocoa beans were extracted using the maceration method using ethanol solvent. Antioxidant analysis in this study used the ABTS [2,2-azino-bis(3-ethyl-benzothiazolin-6-sulphonic acid)] method with a microplate reader at a wavelength of 630 nm. The value used to determine antioxidant activity was the IC_{50} value. All samples BKS 40, BKS 50, and BKS 60 had air contents of 49.34%, 32.7%, and 24.32%. The results of this study prove the antioxidant activity of cocoa bean ethanol extract (BKS 40, 50, and 60) is included in the strong category with IC_{50} values 86, 88, and 71 µg/mL, respectively. The results prove that temperature does not affect antioxidant activity.

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INTRODUCTION

Along with the development of the times, most people have experienced lifestyle changes including consuming foods that contain chemicals and preservatives. With this development, more and more health-related issues are emerging, one of the most significant issues is free radicals (Pantria Saputri et al., 2020). Free radicals are compounds that have unpaired electrons that are unstable and can trigger the appearance of degenerative diseases such as cancer, diabetes, inflammation, and cardiovascular (Natsir Djide, 2022). Free radicals have an effect that can result in damage to natural components found in human body tissues, such as DNA, proteins, and fats (Ipandi et al., 2016). For this reason, antioxidants from outside the body are needed that can be used to ward off the occurrence of radicals in the cell, hydrogen bonding, electron bonding, and singlet oxygen. In addition, antioxidants can inhibit the effects of lipid peroxidation through the oxidative stress response mechanism of various reactions initiated by free radicals, so they are called preventive or preventative antioxidants (Natsir Djide, 2022).

Plants contain chemical compounds that are used as natural medicinal ingredients and have the potential as free radical reducers. These chemical compounds are dispersed in plant parts such as flowers, leaves, fruits, stems, bark, and roots (Najihah et al., 2018). One of the plants that

can be used as antioxidants is the cocoa plant (*Theobroma cocoa* L.) that grows in the tropics. Cocoa plants are rich in polyphenols such as flavanols, which have antioxidant and anti-inflammatory properties against degenerative diseases (Agustriana et al., 2023; Di Mattia et al., 2017). The cocoa plant is also widely consumed and is a rich source of biophenol for biological activity regarding its beneficial health effects in its high phenolic content, especially *Epicatechin* (EC), which is the most abundant flavonoid in cocoa beans. Cocoa beans can also protect the body from the effects of free radicals, reduce oxidative stress, and are excellent sources of nutrients such as protein, carbohydrates, polyphenols, and flavonoids(Ibrić & Ćavar, 2014; Priani et al., 2019).

In this study, antioxidant analysis used the ABTS method, because it can provide specific absorbance at the visible wavelength, has a faster reaction time, is more sensitive to all pH, and can detect compounds that are lipophilic or hydrophilic. The DPPH method is only sensitive to acidic pH and is a little difficult to analyze hydrophilic compounds. The function of ABTS is to measure antioxidants that react with ABTS cation radicals (Kurniawati & Sutoyo, 2021).

According to the Ministry of Health of the Republic of Indonesia, the temperature range used for drying is 30-90ºC, but the best temperature does not exceed 60ºC. Drying gotu gotu leaves using temperature variations of 50, 55, and 60ºC obtained the best results of antioxidant activity at 50ºC of 36,1 µg/mL (Yulianti, et al., 2020). Drying of telang flowers using drying temperatures of 50, 60, and 70ºC obtained the best results of antioxidant activity at 50ºC of 128.25 µg/mL. So the temperature and drying time greatly affect antioxidant activity, the higher the temperature and length of drying time used, can reduce antioxidant activity (Ayu Martini et al., 2020). Antioxidant activity of watermelon mesocarp extract with the ABTS method showed a change in color from blue-green, which slowly disappeared. This indicates the reaction between antioxidants and ABTS radicals, so that the study obtained very strong antioxidant results with an IC_{50} value of 31.42 µg/mL (Amin et al., 2021).

Based on the description above, the author aims to determine the effect of drying temperature variations on the antioxidant activity of ethanol extract of cocoa beans (*Theobroma cacao* L.) using the ABTS [2,2-azino-bis(3-ethyl-benzothiazolin-6-sulphonic acid)] method.

METHOD

Instrument and Material

This research uses a material, namely cocoa beans obtained from Tabanan Regency, Bali Province. The chemicals used in this study were ABTS powder [*2,2-azino-bis(3-ethyl-benzotiazolin-6-sulphonic acid*)] (sigma), ethanol 96%, potassium persulfate, Trolox (Sigma), aquadest, iron(III) chloride ($FeCl_3$), magnesium powder, HCl, 10% NaOH. The instrument used is analytical balance, waterbath, stove, filter paper, maserator, drip pipette, flannel cloth, *micropipette tip*, glass funnel (Pyrex®), test tube (Pyrex®), test tube rack, stirring rod (Pyrex®), spatula, measuring cup (Pyrex®), beaker cup (Pyrex®), measuring cup (Pyrex®), aluminum foil, oven (Memmert), extract cup, blender, porcelain cup (Pyrex®), *Rotary Vacuum Evaporator* (Buchi/Rotavapor R-11®), *Microplate 96 well flat*, and *Microplate Reader*.

Sample Preparation

Cocoa beans (*Theobroma cacao* L.) that have been removed from the fruit and cleaned of contaminants using clean water. Temperature variations ensue, including dry sorting in the oven for 8 hours at temperatures of 40ºC (BKS 40), 50 ºC (BKS 50) and 60ºC (BKS 60). To obtain cocoa beans without a pallet, the cocoa beans are peeled after drying. After that, the pallets are blended into cocoa bean powder and weighed.

Determination of the moisture content of simplicia

2 grams of simplicia are placed in a porcelain cup, heated to 105ºC for 30 minutes, then left to cool until the weight remains (Ifmalinda et al., 2023). Drying shrinkage is calculated by the following formula:

$$\text{Moisture content} = \frac{b-c}{b-a} \text{ x } 100\%$$

Information, a = cup weight (g)
b = cup weight + sample before drying (g)
C = cup weight + sample after drying (g)

Extract manufacturing

1000 grams of cocoa bean powder is macerated with 3000 mL of 96% ethanol solvent, then left covered for 24 hours at room temperature. It is then stirred for 5 minutes every 6 hours, filtered with filter paper and the pulp is macerated again 2 times until the solvent is colorless. The treatment results are collected and concentrated with *Rotary Vacuum Evaporator*.

Phytochemical Tests

a. Flavonoids

0,1 grams of the extract was incubated for 5 minutes using 5 mL of aquadest, the sample was divided into three test tubes and tested (Wibawa et al., 2024):

1. Wilstater Test

   1 mL filtrate is mixed with magnesium sulfate, then two drops of concentrated HCl are added and let sit. Orange indicates the presence of flavonoids, while dark red indicates the presence of flavonols.

2. Bate-Smith Test

   1 mL of filtrate is mixed with a few drops of concentrated HCl and then heated using a bath. The dark red to purple color indicates flavonoids with a type of anthocyanidin.

3. 10% NaOH Test

   1 mL of filtrate is mixed with 10% NaOH for a few minutes until a discoloration occurs. This color indicates the presence of phenols.

b. Phenolic

A total of 50 mg of the extract is dissolved with ethanol. Then two drops of 5% (b/v) $FeCl_3$ solution are added to ethanol. The green, blackish-green color produces a dark color, indicating the presence of phenolics (Anwar et al., 2022).

c. Tannins

A total of 50 mg of the extract is mixed with 100 mL of hot water and incubated for five minutes in an erlenmeyer before filtering. Then two drops of 5% $FeCl_3$ are added to 5 mL of filtrate. Results are shown in violet green/ brownish-green color (Wibawa, 2021).

Determination of antioxidant activity by ABTS method

Antioxidant activity with the ABTS method was carried out by ABTS radical cation decolorization test with several modifications (Tang et al., 2019). A total of 5 mL of ABTS 7 mmol/L solution was mixed with 88 µL of 140 mM potassium persulfate solution to make the ABTS test solution. The mixture is placed in a dark place at room temperature for 16 hours. Then, the prepared ABTS solution is diluted with 96% ethanol to obtain an initial absorption of 0.7 at 734 nm. Then, 10 µL of extract or standard is mixed with 290 µL of diluted ABTS

solution that has been prepared in a 96-well plate and incubated at room temperature for 6 minutes in a dark place. The next process after incubation, the test solution was measured at a wavelength of 734 nm with *Microplate Reader*. Antioxidant ability is given as a Trolox equivalence number (mg TEAC/g dw) of the sample using a calibration curve prepared for trolox, plotted at concentrations different from the standard range of 0–2000 µg/mL.

Data Processing

Determination of Antioxidant Activity by ABTS Method

a. Calculation of % inhibition

The antioxidant activity of the sample is determined by the amount of ABTS absorption resistance (% inhibition) calculated by the formula:

$$\% \text{ inhibition} = \text{x } 100\% \frac{Control\ Absorbance - Sample\ Absorbance}{Control\ Absorbance}$$

b. IC_{50} Value

The calibration curve was made by comparing the concentration (µg/mL) to the % inhibition, then the regression equation y = bx + a was obtained with the coordinates (y) being the value of the % antioxidant reduction and extract concentration (µg/mL).

Data Analysis

The data analysis techniques used in this study were carried out quantitatively and qualitatively. In quantitative testing, the data obtained from the results of antioxidant activity using the ABTS method are presented in the form of tables and curves, which are then described.

RESULTS AND DISCUSSION

Moisture Content of Simplicia

Moisture content testing is carried out on cocoa beans at temperatures of 40, 50, and 60ºC, namely to determine the water content in the cocoa beans. The results of moisture content can be seen in Table 1.

Table 1. Yield Moisture Content

Sample	Cup Weight (g)	Sample weight before drying + cup (g)	Sample weight after drying + cup (g)	Moisture Rate (%)
BKS 40	157,2	180,2	168,9	49,34
BKS 50	120,5	138,8	132,8	32,7
BKS 60	157,2	175,7	171,2	24,32

In the study, a test was carried out on the water content, because it is very important to know the quantity of water contained in cocoa beans. Moisture content at temperature variations of 40, 50, and 60ºC (Table 1) obtained mixed results which means that temperature and length of drying time greatly affect the moisture content. The higher the temperature and the longer the drying, the smaller the moisture content of a simplicia produced. The higher the temperature and the length of drying time given, can have a very large influence on the speed of water transfer, so that the water content in the material will be lower (Anwar Fauzi et al., 2022). However, the moisture content in the results obtained is not below 10% as a condition to produce simplicia with good quality, which is a good moisture content in cocoa beans which is 7.5% according to the Standar Nasional Indonesia (SNI) 23223-2008. The drying temperature and the length of drying time greatly affect antioxidant activity, the higher the

drying temperature used, the lower the antioxidant activity obtained and the length of drying time affects the moisture content of the simplicia (Ifmalinda et al., 2023).

Phytochemical Screening Results

Phytochemical screening testing was carried out on cocoa bean ethanol extract at temperatures of 40, 50, and 60ºC, namely to find out whether or not flavonoids, phenolic and tannin compounds are present. The results of phytochemical screening test, as showing in Table 2.

Table 2. Phytochemical Screening Results

Compound	Reagents	Sample			Information
		BKS 40	BKS 50	BKS 60	
Flavonoids	Mg+HCl (Wilstater)	++	++	++	Orange Color
	HCl + heat (Bate-Smith)	++	++	++	Dark red color
	NaOH 10%	++	++	++	Yellow
Phenolic	$FeCl_3$	++	++	++	Blackish green color
Tannins	$FeCl_3$	++	++	++	Blackish-brown color

(+) = positive/contain the compound tested
(++) = very strong positive/contains the compound tested with a more intense color intensity

Phytochemical screening testing of cocoa bean ethanol extract showed positive results as evidenced by flavonoid testing carried out 3 tests, where the wilstater test was carried out by adding Mg and HCl showed that cocoa bean extract contained flavonoids, which were characterized by the appearance of an orange color. The Bate-Smith test is carried out by adding a concentrated HCl solution and then heating. The addition of concentrated HCl aims to hydrolyze and break the flavonoid bonds into their glycones, namely by hydrolyzing O-glycosyl. O-glycosyl will be replaced by H^+ of acids, because they are electrophilic. The heating process is carried out to accelerate the hydrolysis reaction. The phytochemical results show that cocoa bean ethanol extract contains flavonoids such as anthocyanidine type, which are characterized by the appearance of a dark red color (Dwi Susiloningrum, 2020). The 10% NaOH test is done by adding a few drops of 10% NaOH and there will be a discoloration. The change that occurred was caused by the 10% NaOH reagent being a alkaline catalyst that caused the breakdown of crystalline compounds which are derivatives of flavone compounds into acetophenone molecules. Phytochemical results show that cocoa bean ethanol extract contains flavonoids which are indicated by a change in color (Dwi Susiloningrum, 2020).

Results of phytochemical screening testing of phenolic assays using $FeCl_3$ reagents 5% showed positive results by showing the presence of phenol groups characterized by the formation of a blackish-green color after the sample solution was added with a 5% $FeCl_3$ reagent. The formation of a blackish-green color is caused the phenol compounds contained in the extract sample form a complex with Fe^{3+} ions (Anwar et al., 2022). Tannin testing showed that cocoa bean ethanol extract produced a blackish-brown color that occurred due to the formation of complex compounds between tannins and $FeCl_3$ (Ikalinus et al., 2015). This is in accordance with research (Wibawa, 2021) that the formation of a blackish-brown color indicates that the sample contains tannin compounds.

Results of Antioxidant Activity Test Ethanol Extracts of Cocoa Bean

The results of the antioxidant activity test on ethanol extract of cocoa bean were determined using the ABTS [2,2-azino-bis(3-ethyl-benzothiazolin-6-sulphonic acid)] *method.* The results of the antioxidant activity test ethanol extract cocoa bean can be obtained using by microplate *reader* testing at a wavelength of 630 nm. The results of the antioxidant activity test of ethanol extract cocoa bean used 3 temperature variations 40, 50, and 60 ºC with IC_{50} values respectively 86 µg/mL (strength), 88 µg/mL (strength), and 71 µg/mL (strength). Meanwhile, Trolox has an IC_{50} value of 5 µg/mL (highly) (Figure 1).

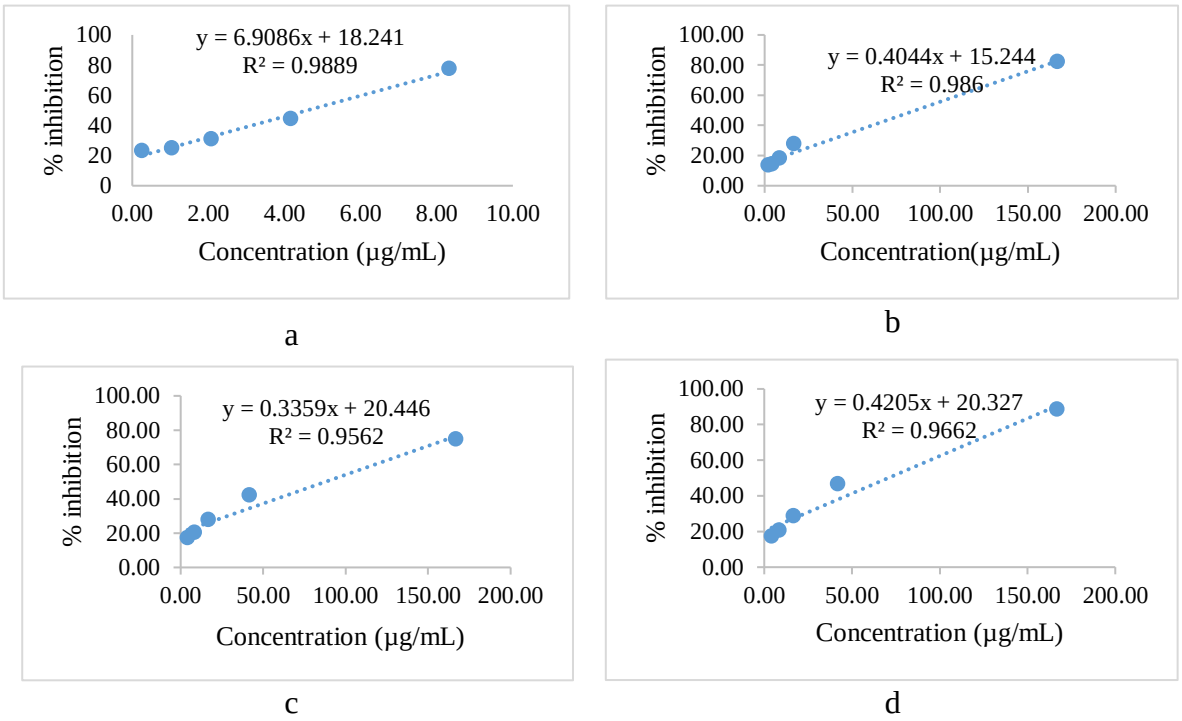

Figure 1. Calibration of Curve Antioxidant Activity in Standard Trolox(a); BKS 40(b), BKS 50(c), and BKS 60(d).

In the other research states that temperature and length of drying time can affect the content of active compounds in the material, where the lower the IC_{50} value, this means that the stronger the antioxidant activity power produced (Yulianti, et al., 2020). Antioxidant activity at temperatures of 40, 50, and 60 ºC due to low temperature and drying time can also cause IC_{50}. The higher it is caused by the water content that is still too high. The high water content in the sample easily damaged so that antioxidant activity cannot be seen. In addition, the fairly high moisture content in the sample can prompt some enzymes to change the chemical content of the material into other products that make the antioxidant activity read low in the test (Anwar Fauzi et al., 2022). Heating treatment including drying of materials can lead to the release of some phenolic compounds with low molecular weight such as flavonoids. The release of flavonoids after passing through the drying process causes the antioxidant power of an ingredient to be more readable during testing (Jeong et al., 2017). This statement is in accordance with the results of the research obtained where antioxidant activity gets stronger as the temperature increases to a certain limit will weaken again due to its bioactive components being degraded by too high temperatures.

Antioxidant capacity in oven Cocoa Beans (42,454 mg/mL) is higher than dried Cocoa Beans (27,730 mg/mL). If you refer to the results obtained, the simplicia drying method affects the antioxidants of cocoa beans. Drying at a stable temperature provides better antioxidant values and when using less stable heating, the ability of flavonoid compounds and tannins as antioxidants slowly decreases (Wibawa, 2021).

CONCLUSION

The results of this study prove that the effect of the drying temperature of the simplicia does not affect the antioxidants in cocoa beans as measured by the ABTS method of cocoa bean ethanol extract (BKS 40, 50, and 60) is included in the strong category with IC_{50} values 86, 88, and 71 µg/mL.

RECOMMENDATIONS

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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