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Antioxidant Activity Test of Ethanol Extract of Ripe and Young Cocoa Pods (Theobroma Cacao L.)

Andriana & *Minarni R. Jura
Program Studi Pendidikan Kimia/FKIP – Universitas Tadulako, Palu – Indonesia 94119

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Abstract

Antioxidant activity test of ethanol extract of ripe and young cocoa pod skin (Theobroma Cacao L.) with DPPH (1,1-diphenyl-2-picrylhydrazyl) using UV-Vis spectrophotometer has been carried out. This study aimed to determine the IC_{50} value of the ethanol extract of ripe cocoa pods and young cocoa pods (Theobroma Cacao L.). The mature and young cocoa pods were extracted using absolute ethanol solvent and examined for their antioxidant activity using DPPH (1,1-diphenyl-2-picrylhydrazyl). The concentration variations used in the ethanol extract of ripe cocoa pods and young cocoa (Theobroma Cacao L.) were 20, 40, 60, and 80 ppm. The results showed that the IC_{50} values of the ethanol extract of ripe and young cocoa pods were 76.094 ppm and 91.884 ppm, respectively. Meanwhile, the IC_{50} value of vitamin C compared was 63.519 ppm. Based on the IC_{50} value data above, it can be seen that the antioxidant activity of the ethanol extract of young cocoa pods and ripe cocoa pods are potent antioxidants.

Keywords: Cacao pods skin, antioxidant, DPPH, UV-Vis spectrophotometer

Introduction

The chocolate plant, commonly called cocoa (Theobroma Cacao L.), comes from the Sterculiaceae family. Cocoa is a plantation crop with important economic significance in Indonesia as an export commodity. Farmers generally only harvest cocoa beans to be processed into chocolate at harvest time so that a large amount of cocoa pod waste is produced. The existence of this waste is often not used correctly and is only left to become agricultural waste (Mulyatni, 2012).

Figuera & Janick (1993) stated that cocoa pod waste consisted of fruit skin (76.6%), seed coat (21.74%), and placenta (2.59%). Cocoa pod skin is the outer shell covering the cocoa bean with a rough, thick, and hard texture. Cocoa fruit cultivars contain an active alkaloid compound, namely theobromine (3,7-dimethylxanthine). One of the effects of these substances is as a sedative so that it becomes a limiting factor in the use of cocoa pod waste as animal feed. Cocoa pods contain active compounds of flavonoids or condensed tannins such as anthocyanidins, catechins, and leucoanthocyanidin, which are heavily bound to glucose. These compounds have antibacterial properties (Matsumoto et al., 2004). The content of flavonoid compounds contained in the skin of cocoa pods has antioxidant activity. Flavonoids are one of the natural phenolic antioxidant compounds that are widely distributed and found in all plants. Flavonoids have a lot of OH groups, so they can be efficacious as antioxidants (Listyannisa, 2012).

Flavonoids are phenolic substances that can be isolated from various vascular plants, with more than 8000 well-known individual compounds. Flavonoids can act as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants, and light screening. Flavonoids work as antioxidants because of their ability to reduce the formation of free radicals (Karmila et al., 2018).

Antioxidants are compounds that are important in maintaining a healthy body. Antioxidants function to neutralize free radicals, protect cells from the toxic effects produced by free radicals that complement their orbitals, and play a role in disease prevention (Chanda & Dave, 2009). Based on the source, there are two kinds of antioxidants: natural antioxidants and synthetic antioxidants. Synthetic antioxidants such as BHA (Butylated hydroxytoluene) have been known to have significant side effects and cause liver damage. At the same time, natural antioxidants can be obtained from plants that have flavonoid compounds, vitamin C, and beta-carotene (Kikuzaki, 1993).

Antioxidants work by donating one electron to oxidant compounds so that the activity of these

*Correspondence:
Minarni R. Jura
e-mail: minarni.ramajura@gmail.com

oxidant compounds can be inhibited. In the presence of antioxidants, free radicals can be stabilized. Antioxidants will inhibit the chain reaction of the formation of free radicals (Winarsi, 2007). Antioxidants can neutralize free radicals by donating one proton atom to make free radicals stable and unreactive (Nurmiati et al., 2020).

Free radicals are a form of reactive compounds known as compounds that have unpaired electrons. Free radicals are formed when a molecule that has lost an electron becomes unstable. The presence of free radicals in the human body can cause inflammation and aging and trigger carcinogenic substances that cause cancer (Rizkayanti et al., 2017). Therefore, the body needs a defense to neutralize free radicals with antioxidant compounds.

The existence of free radicals in the body through two events, where free radicals are formed endogenously, result from metabolism, and can cause inflammation. Free radicals formed exogenously due to air pollution, solar radiation, X-rays, alcohol, and cigarettes (Buhang et al., 2019).

Cocoa pods used as antioxidants were tested using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method. This method measures antioxidants that are simple, fast, and do not require a lot of reagents. DPPH acts as a free radical, which is suppressed by antioxidants from the test material, where DPPH will react with these antioxidants to form 1-1-diphenyl-2-picryl hydrazine. This reaction will cause a color change from purple to yellow and can be measured using a visible spectrophotometer at a wavelength of 517 nm (Mariani et al., 2018).

Cocoa pods used in this study were ripe cocoa pods marked with yellow or red color and young cacao pods marked with green color. It was done to determine the difference in antioxidant activity of the two samples.

Methods

The equipment used is UV-Vis spectrophotometry PG instrument Ltd, blender, analytical balance, digital balance, 80 mesh sieve, baking sheet, a rotary vacuum evaporator, erlenmeyer, glass funnel, etc. cuvette, beaker, and glassware contained in the laboratory. The materials used were ripe and young cocoa pods, 96% absolute ethanol, vitamin C, aquades, and 1,1-diphenyl-2-picrylhydrazil (DPPH).

Sample preparation

Sample preparation carried out in this study was to prepare and clean the skin of ripe and young cocoa pods that had been separated from the seeds. Then cut into small pieces and dried by airing in the open air in the room to dry. After drying, the samples were mashed using a blender and sieved using an 80 mesh sieve to produce a fine powder of ripe cocoa pods and young cocoa.

Extraction with ethanol

Ripe cocoa pods and young cocoa pods were weighed as much as 30 grams each using a digital balance. Then each sample was macerated using 300 mL absolute ethanol for 2 × 24 hours. The extract was filtered after 2 × 24 hours to separate the filtrate and residue. Next, the residue is macerated again in the same way. The extraction results obtained from filtering are then concentrated using a rotary evaporator to get a thick and concentrated extract.

Antioxidant activity test

2.5 mg of DPPH was dissolved with ethanol in a 25 mL volumetric flask; then, the volume was made up with ethanol to the limit mark.

2.5 mL of DPPH solution was pipetted and then put into a 25 mL volumetric flask, and the volume was made up with ethanol to the mark, then allowed to stand for 30 minutes.

Each ethanol extract of ripe cocoa pods and young cocoa was weighed as much as 25 mg and then put into a 25 mL volumetric flask. Then the volume is made up of ethanol up to the mark.

A total of 25 mg of vitamin C was weighed, then sufficient distilled water in a 25 mL volumetric flask and made up to volume with ethanol to the mark.

The mother liquor was pipetted at 0.5, 1, 1.5, and 2 mL, after which 2.5 mL of DPPH solution was added to each solution. Then the volume is made up of ethanol up to the mark. So that obtained extract solutions with concentrations of 20, 40, 60, and 80 ppm.

The mother liquor of vitamin C as a comparison was taken as much as 0.5, 1, 1.5, and 2 mL of each solution; after that, each of the solutions was added 2.5 mL of DPPH solution, and then the volume was filled with ethanol to the limit mark. So that obtained extract solutions with concentrations of 20, 40, 60, and 80 ppm. Vitamin C is a comparison solution because vitamin C or ascorbic acid is a natural antioxidant.

Blank absorbance measurement

The blank solution was put into a cuvette, and its absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 517 nm.

Measurement of the absorbance of the test solution and comparison of vitamin C

Each test solution and comparison solution of vitamin C with concentrations of 20, 40, 60, and 80 ppm were put into a cuvette. The absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 517 nm. All work is carried out in a room that is protected from sunlight.

The amount of DPPH radical inhibition determines the antioxidant activity of the sample by calculating the percentage inhibition of DPPH absorption using the following formula (Zuhra et al., 2008):

$$Antioxidant\ power = \frac{absorb.(blank - sample)}{absorb.blanko}\ x\ 100\%$$

The IC_{50} value is determined by calculating a percentage used to calculate the probit value. The probit value obtained is then plotted with the log concentration value to get the regression equation y = ax + b. The regression equation is then used to find the IC_{50} value (x in the regression equation) by replacing the y value of 50 from the 50% probit price (Rinidar et al., 2013).

Results and Discussion

The absorbance data obtained from this study are presented in **Table 1**.

Table 1. Results of measurement of DPPH absorbance on an ethanol extract of ripe and young cocoa pods and Vitamin C.

Concentration (ppm)	Absorbance		
	Cooked Cocoa Fruit Peel	Young Cocoa Fruit Skin	Vitamin C
20	0.316	0.325	0.337
40	0.275	0.314	0.298
60	0.214	0.256	0.214
80	0.192	0.211	0.176

Absorbance blank = 0.386

The results of the antioxidant activity test of the ethanol extract of ripe and young cocoa pods and the comparison solution of vitamin C are presented in **Table 2**.

Table 2. Antioxidant activity test results

Sample	IC_{50} value (ppm)	Antioxidant power
Cooked Cocoa Fruit Peel	0.316	0.325
Young Cocoa Fruit Skin	0.275	0.314
Vitamin C	0.214	0.256

Antioxidant activity test

Extraction is the process of withdrawing secondary metabolites with the help of a solvent (Harborne, 1987). The extraction method used is the maceration method using absolute ethanol as a solvent. The principle of extraction using the maceration method is the diffusion of the filtered fluid into plant cells containing active compounds. This diffusion causes the osmotic pressure inside the cell to be different from that outside. Due to osmotic pressure outside the cell, the active compounds are then pushed out (Budilaksono et al., 2014).

Effective plant tissue extraction uses an appropriate solvent ten times the volume or weight of the sample (Harborne, 1987). Therefore, in this study, 300 mL of absolute ethanol was used for every 30 grams of ripe and young cocoa pods. The choice of total ethanol solvent is based on like dissolves like, which means that polar solvents will dissolve polar compounds, and non-polar solvents will dissolve non-polar compounds (Suryani et al., 2016). In addition, the choice of absolute ethanol solvent was adjusted to the method used. In this study, the antioxidant test method used DPPH, where this method was only used to test antioxidant compounds soluble in organic solvents, especially alcohol (Bahriul et al., 2014).

The first treatment in this study was sample preparation. The skin of ripe cocoa pods and young cocoa separated from the seeds are washed thoroughly and then cut into small pieces. It was then dried in a way to aerate the room. It aims to remove the moisture content of the cocoa pod skin and prevent the secondary metabolites contained in it from being damaged (Nur et al., 2016). After drying, the sample was blended until smooth, which aims to reduce the sample size so that the contact between the solids (sample) and the solution in the extraction process takes place ideally so that the sample and solvent diffusion process takes place optimally (Buhang et al., 2019).

The powder of ripe cocoa pods and young cacao pods were weighed as much as 30 grams each using a digital balance; then, each sample was put into a 500 mL beaker. The sample was then macerated with 300 mL absolute ethanol solvent for 2 × 24 hours and covered with aluminum foil so that the solvent did not evaporate. After 2 × 24 hours, the sample was filtered using a funnel and filter paper to separate the filtrate and residue. The residue obtained from each sample was then macerated again in the same way for 2 × 24 hours, after which the sample was filtered again to bring the filtrate. The filtrate obtained from this filtering is then combined with the filtrate from the first filtering and then concentrated with a rotary evaporator. The ethanol extract of ripe cocoa pods is reddish-brown, and young cocoa pods are dark green. The purpose of the concentration is to separate and evaporate the solvent and the extract so that a thick extract is obtained (Fadhli et al., 2018).

Antioxidant activity test

Antioxidant activity test of ethanol extract of ripe and young cocoa pods using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay method. This method is a quantitative test method to determine the activity of cocoa pod peel extract as an antioxidant. This method is because the DPPH method is a simple, straightforward method and uses a small sample and a short measurement time (Molyneux, 2004).

The principle of quantitative measurement of antioxidant activity using the DPPH method is that there is a change in the intensity of the purple color of the DPPH, which is proportional to the solution. DPH free radicals with unpaired electrons will give

a purple tint and turn yellow when the electrons are paired. This change in the intensity of the purple color occurs due to the reduction of free radicals. They were produced by the reaction of the DPPH molecule with the hydrogen atoms released by the compound molecules from the sample so that the diphenyl picryl hydrazine compound will form and cause the DPPH color to decay from purple to yellow (Rizkayanti et al., 2017).

Antioxidant activity testing by spectrophotometry was carried out at a wavelength of 517 nm, the maximum wavelength for DPPH. This maximum wavelength will absorb the test solution and provide the most incredible sensitivity. The DPPH test method is based on the decrease in absorbance due to changes in the color of the DPPH solution, where DPPH will react with hydrogen atoms from the DPPH solution.

Free radical scavenging compounds form DPPH-Hydrazine, which is more stable. DPPH reagent will change color from purple to yellow; the intensity of the color depends on the ability of antioxidants (Molyneux, 2004).

Observation of antioxidant activity of ethanol extract of ripe and young cocoa pods was carried out at different concentrations, namely 20 ppm, 40 ppm, 60 ppm, and 80 ppm. The concentration of the test solution was varied to determine the level of color immersion due to the presence of antioxidant compounds that could reduce the intensity of the purple color of DPPH (Bahriul et al., 2014). The next step is to measure the absorbance of each sample with a UV-Vis spectrophotometer at a wavelength of 517 nm, which is the maximum wavelength of DPPH. The antioxidant activity of free radical scavengers can be seen by decreasing uptake.

The relationship curve between the concentration and absorbance of the ethanol extract of ripe and young cocoa pods can be seen in **Figure 1**.

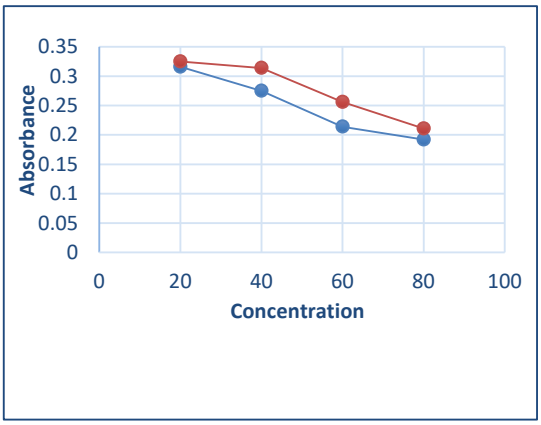

Figure 1. Relationship curve of concentration (ppm) of ethanol extract of ripe cocoa pods and young cocoa with absorbance value (A)

Based on the curve, it is known that the absorbance value of the ethanol extract of ripe and young cocoa pods will decrease with increasing concentration. It occurs due to the reduction of DPPH radicals by antioxidants. The higher the concentration of ethanol extract in ripe and young cocoa pods, the greater the antioxidant activity and causes the absorbance to decrease (Molyneux, 2004).

According to Sulaeha et al. (2017), a decrease in absorbance indicates a reduced concentration of free radicals from DPPH due to the reaction of antioxidant compounds where this reaction will cause the DPPH molecule to be reduced, followed by a decrease in the intensity of the purple color of the DPPH solution. When all the DPPH free radical electrons have been paired, the solution will change color from dark purple to bright yellow.

The IC_{50} value in the ethanol extract of ripe and young cocoa pods was determined using a linear regression equation from the curve of the relationship between the sample concentration and the percentage of inhibition presented in **Figure 2**.

Antioxidant activity was tested by determining the percentage of inhibition of the ethanol extract of ripe and young cocoa pods. The percentages of ripe cocoa pods inhibition at concentrations of 20, 40, 60 and 80 ppm were 18.1, 28.7, 44.5, and 50.2%, respectively. Meanwhile, the percentage of inhibition for the ethanol extract of young cocoa pods at concentrations of 20, 40, 60, and 80 ppm was 15.8, 18.6, 33.6, and 45.3%, respectively. Based on the curve above, it can be seen that the highest percentage inhibition value for the ethanol extract of ripe cocoa pods was 50.2%, and young cocoa was 45.3%. The percentage of free radical inhibition of ripe cocoa pod extract was greater than that of young cocoa pods. This result is due to differences in the concentration of secondary metabolites in the cocoa pod skin; the more secondary metabolites contained in the plant, the more particles of antioxidant compounds contained in the extract, so the stronger the antioxidant

activity and causes the absorbance to decrease (Mariani et al., 2018).

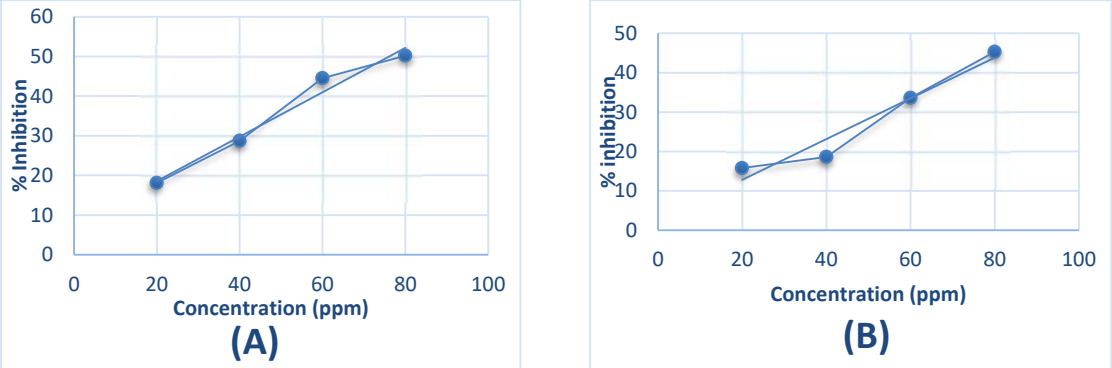

Figure 2. Concentration curve (ppm) of the ethanol extract of ripe cocoa pods (A) and young cocoa (B) with the percentage of inhibition (%).

The curve in **Figure 2**. obtained the value for linear regression of ethanol extract of ripe cocoa pods, namely y = 0.5605x + 7.35 and linear regression value for an ethanolic section of young cocoa pods y = 0.5175x + 2.45. The linear regression equation data obtained from the ethanol extract of ripe and young cocoa pods were used to calculate their antioxidant activity using the formula y = ax + b. The IC_{50} values for the ethanol extract of mature and young cocoa pods were calculated at 76.094 ppm, respectively, and 91.884 ppm. These results explain that the IC_{50} value of the ethanol extract of ripe cocoa pods is lower than that of young cocoa pods. The lower the IC_{50} value obtained, the more active the substance is so that the antioxidant activity is more potent. Therefore, the antioxidant activity of ripe cocoa pods was more potent than that of young cocoa pods. (Yuliantari et al., 2017).

The difference in IC_{50} values in ripe and young cocoa pods was caused by the number of antioxidants contained in the extract. The content of secondary metabolites in ripe cocoa pods is more than that of young cacao pods. It indicates that the level of fruit maturity affects the levels of secondary metabolites, which are antioxidants that can affect fruit growth and ripening (Nainggolan et al., 2019).

Jusmiati et al. (2015) stated that the level of ripeness of the fruit dramatically affects the content of secondary metabolites, which are antioxidants. The difference in secondary metabolite content at different maturity levels is due to differences in chemical content in the growth and ripening process of fruit, where ripe fruit contains many secondary metabolites such as flavonoids which will also impact the level of free radical inhibition.

The positive control used is vitamin C which is a water-soluble antioxidant. The use of positive control in the antioxidant activity test aims to determine how strong the antioxidant potential is in the ethanol fraction of ripe cocoa pod extract and young cocoa compared to vitamin C (Mulangsri et al., 2017). The concentration variations used are 20 ppm, 40 ppm, 60 ppm, and 80 ppm. Based on the data obtained, the greater the concentration of vitamin C, the greater the percentage value of DPPH free radical inhibition that occurs (Masrifah et al., 2017).

The curve of the relationship between concentration and absorbance of vitamin C can be seen in **Figure 3**. The absorbance value in the antioxidant activity test of vitamin C obtained the percentage of free radical inhibition of DPPH. **Figure 3** shows the relationship between vitamin C concentration and the percentage of free radical inhibition of DPPH. The greater the concentration of vitamin C, the stronger the vitamin C in reducing DPPH free radicals so that the absorbance value is getting smaller. The more significant the concentration of vitamin C, the more particles that can oxidize the existing DPPH free radicals (Molyneux, 2004).

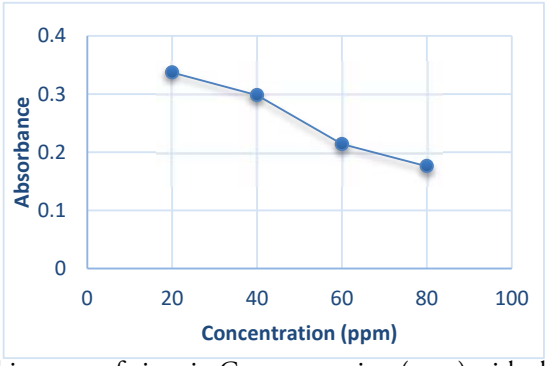

Figure 3. Relationship curve of vitamin C concentration (ppm) with absorbance value (A).

The IC_{50} value for vitamin C was determined using a linear regression equation from the relationship curve of the sample concentration to the percent inhibition presented in **Figure 4**.

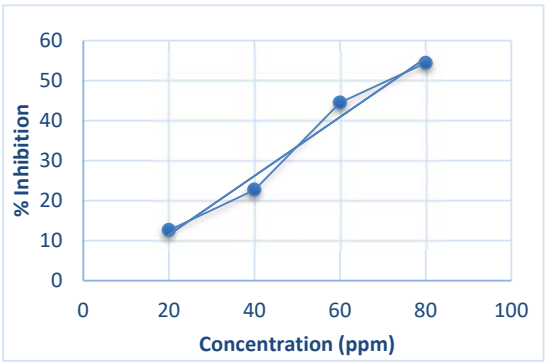

Figure 4. Concentration curve (ppm) of vitamin C (ascorbic acid) with the percentage of inhibition (%)

Based on the % inhibition curve of vitamin C above, the linear regression value can be obtained: y = 0.736x-3.25 with the highest inhibition value of 54.4%. The inhibitory percentage of vitamin C was more significant than the inhibitory percent of the ethanol extract of cocoa pods. The percent inhibitor, which was the main objective of the study, showed more minor results when compared to the percent inhibition of vitamin C as the positive control. The IC_{50} value of Vitamin C obtained is 63.519 ppm, which means that the antioxidant activity of vitamin C is greater than the ethanol extract of ripe and young cocoa pods. These results indicate that vitamin C as a comparison or positive control is classified as a potent antioxidant (Arif et al., 2018).

Vitamin C is an antioxidant that works as an oxygen scavenger, which binds oxygen, so it does not support oxidation reactions. Vitamin C will react with oxygen in the system to decrease the amount of oxygen. In addition, vitamin C has a free hydroxy group that acts as a free radical scavenger, and if it has a hydroxy group, it will increase antioxidant activity (Nadia et al., 2016).

The IC_{50} value obtained from vitamin C as a comparison or positive control shows that the antioxidant activity of vitamin C is stronger than the antioxidant activity of the ethanol extract of ripe and young cocoa pods. It can be seen from the IC_{50} value for vitamin C obtained, which is lower than the IC_{50} value for the ethanol extract of ripe and young cocoa pods. Based on the parameter IC_{50} value, the antioxidant activity of the ethanol extract of mature and young cocoa pods and vitamin C as a positive control were categorized as solid antioxidants because the IC_{50} value was less than 100 ppm.

According to Molyneux (2004), the antioxidant activity of a compound is classified based on the IC_{50} value obtained. Potent antioxidant if the IC_{50} value is between 50 ppm, powerful antioxidant if the IC_{50} value is between 50-100 ppm, moderate antioxidant if the IC_{50} value is between 100-150 ppm, weak antioxidant if the IC_{50} value is between 150-200 ppm. Meanwhile, if the IC_{50} value exceeds 200 ppm, the antioxidant activity is fragile.

According to Day & Underwood (1981), several things cause the data and research results to be poor: the lack of good preparation of the concentration series of the solution used. The UV-Vis spectrophotometer instrument was not calibrated correctly, and impurities in the cuvette were used as the initial solution that was not correctly calibrated cleaned.

Conclusions

The IC_{50} values of the ethanol extract of ripe cocoa pods and young cocoa (Theobroma Cacao L.) obtained are 76.094 ppm 91.884 ppm, respectively. So it is known that the rind of ripe cocoa pods has higher antioxidant activity than the ethanol extract of young cacao pods. These inhibitory concentrations were less than 100 ppm and were classified as potent antioxidants.

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