

***In vitro* antioxidant activity of Cocoa (*Theobroma cacao* L) peel**

Zubaydah, W.O.S., Sartinah, A., Nuralifah, Waelti, Sabarudin and *Yamin

*Faculty of Pharmacy, Universitas Halu Oleo, Indonesia***Article history:**

Received: 1 July 2021

Received in revised form: 9

August 2021

Accepted: 12 August 2021

Available Online: 5 August

2022

Keywords:Cocoa (*Theobroma cacao* L),

ABTS,

DPPH,

Antioxidant,

Phenolic content,

Flavonoid content

DOI:[https://doi.org/10.26656/fr.2017.6\(4\).477](https://doi.org/10.26656/fr.2017.6(4).477)**Abstract**

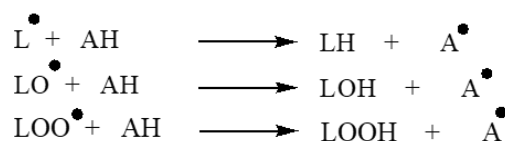
Free radicals are electron-deficient species that are very reactive to cause various diseases, such as diabetes, cholesterol, cardiovascular disease, cancer, hypertension, and others. For this reason, antioxidant compounds are needed that can inhibit the performance of free radicals. Cacao is a plant that contains many phenolic and flavonoid compounds that have potential as antioxidants. This study aimed to evaluate the total phenolic and flavonoid content as well as *in vitro* antioxidant assay. Cocoa fruit powder was macerated with methanol, and then fractionated with n-hexane, chloroform, and ethyl acetate as solvents. The antioxidant power was measured by DPPH and ABTS methods. The phenolic content was determined by the Folin-Ciocalteu method, and the flavonoid content was measured using the AlCl₃ colourimetric method. The ethyl acetate fraction showed the strongest antioxidant activity using both the DPPH and ABTS methods with IC₅₀ values of 5.61±0.032 mg/mL and 5.773±0.018 mg/mL, respectively. The total phenolic and flavonoid content of the ethyl acetate fraction showed high values, namely 22.767±0.1097 mg GAE/g sample and 90.337±0.552 mg QE/g sample, respectively. The cocoa peel can be developed as a natural antioxidant and can be used as a functional food.

1. Introduction

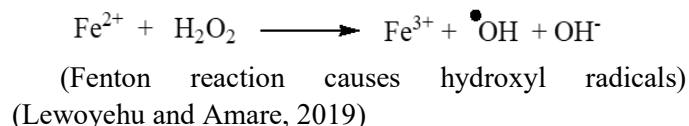
Free radicals are species or molecules that have unpaired electrons. These species are reactive oxygen species (ROS), such as hydroxide radicals (*OH), superoxide radicals (*O²⁻), and hydrogen peroxide H₂O₂. These radicals are produced by normal metabolic processes or as a result of exogenous agents and factors. As a result of the formation of ROS, it will cause oxidative damage to human cells. Several diseases caused by ROS include cancer, osteoporosis, cardiovascular, and other degenerative diseases (Kadriyani *et al.*, 2019).

Antioxidants are compounds or molecules that can overcome the problem of free radicals by donating their electrons or being reducing agents, thereby inhibiting the oxidation process or activating the oxidation reaction. Thus, it will inhibit the process of cell damage (Vifta *et al.*, 2019; Rachmawaty *et al.*, 2019). Antioxidants can stop chain reactions by blocking free radical intermediates and inhibiting other oxidative reactions. It does so by self-oxidizing. The mechanism of action of antioxidants is simply divided into two groups: primary or chain breaker antioxidants and secondary or preventive antioxidants, where the primary antioxidant

agent begins with the inhibition of radical initiation (reacting with lipid radicals) or propagation (reacting with alkoxy or peroxy radicals).



In addition, there are also secondary (preventive) antioxidants, which are compounds that can slow down the rate of oxidation, for example, transition metal ion chelators, which play a role in inhibiting Fenton-type reactions that form hydroxyl radicals:



In the last few decades, the use of antioxidants sourced from natural ingredients has attracted many people. The use of antioxidants from natural ingredients has been used as natural antioxidants, both as ingredients for pharmaceutical preparations, functional foods, and cosmetics, which act as substitutes for synthetic antioxidant materials that pose a risk to health due to their toxicity. Natural antioxidants in food, medicine, and nutrition are due to their potential as antitumor,

*Corresponding author.

Email: yamintaeri@uho.ac.id

antiallergic, and anti-inflammatory drugs (Liu *et al.*, 2018).

The use of antioxidants from natural ingredients is because natural ingredients contain many phenolic and polyphenolic compounds, such as flavonoids and proanthocyanidins. Antioxidant activity positively correlated with the content of flavonoids, polyphenols, and anthocyanidins (Liu *et al.*, 2018; Behera, 2019).

Cocoa (*Theobroma cacao* L) is traditionally a plant that is widely used in medicine. Several studies have shown that Cocoa is helpful as a good antioxidant, anti-atherogenic, anti-inflammatory agent, preventing cardiovascular disease, neurodegenerative disorders, lowering blood pressure, and improving insulin sensitivity through increasing the β -cell power (Bruna *et al.*, 2010; Oracz and Nebesny, 2016; Indiarito *et al.*, 2019). Cocoa beans contain a lot of phenolic compounds, especially flavonoids, including, anthocyanins, flavonols, phenolic acid, phenols, and hydroxylated stilbene derivatives (both in *trans*-resveratrol and *trans*-piceid) are known to have antioxidant abilities as free radical stabilizers, binding proteins, metal ion chelators, and inhibitory enzymes (Oracz and Nebesny, 2016; Mattia *et al.*, 2017). In addition, cocoa beans also contain alkaloids and terpenoids (Iflahah *et al.*, 2016). Therefore, this study aimed to evaluate the antioxidant activity of Cocoa peel using the DPPH and ABTS methods and their correlation with total phenolic and flavonoid content.

2. Materials and methods

2.1 Materials

Cocoa peels were obtained from Otole Village, Lasolo District, North Konawe Regency, Southeast Sulawesi Province, Indonesia, methanol, n-hexane, aluminium chloride, ethyl acetate, vitamin C, quercetin (Sigma-Aldrich®), gallic acid (Sigma-Aldrich®), potassium acetate, Folin Ciocalteu reagent (Sigma-Aldrich®), DPPH radical (Sigma-Aldrich®), potassium persulfate, ABTS (Sigma-Aldrich®).

2.2 Extraction

A total of 900 g of Cocoa peel powder was macerated with methanol every 24 hrs for 3 days. The filtrate obtained was concentrated using a rotary evaporator at 50°C to obtain the methanol extract. A total of 90 g of methanol extract was partitioned using the liquid-liquid method based on the polarity of the solvent, namely n-hexane, chloroform, and finally ethyl acetate. A rotary evaporator was used to obtain the n-hexane fraction, chloroform fraction, ethyl acetate fraction, and water fraction. The extraction process is shown in Figure 1.

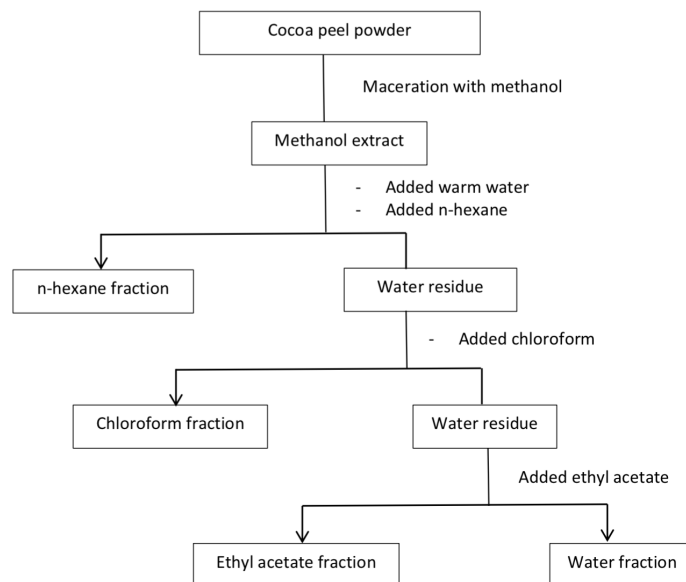


Figure 1. Schematic of extraction and fractionation of cocoa peel

2.3 Phytochemistry screening

Phytochemical screening of cocoa peel extract and skin fraction following the method of Ngibad (2014) and Zubaydah (2021).

2.4 Determination of total phenolic content

Total phenolic content of Cocoa peel was conducted according to the Folin-ciocalteu method by Noreen (2017) and Sabarudin *et al.* (2021). The Folin-Ciocalteu method is a test method based on electron transfer and shows the reduction capacity expressed as phenolic content. The total phenolic content obtained in the sample depends on the solvent used for extraction. External calibration was carried out using a series of gallic acid concentrations to obtain phenolic contents, namely 10 $\mu\text{g/mL}$, 20 $\mu\text{g/mL}$, 30 $\mu\text{g/mL}$, 40 $\mu\text{g/mL}$, and 50 $\mu\text{g/mL}$. To measure the phenolic content of the sample, 1 mL of the sample was placed in a test tube. Then 0.4 mL Folin-Ciocalteu reagent was added and allowed to stand for 5-8 mins. Then, 4 mL of Na_2CO_3 7% solution was added and shaken until homogeneous. It was allowed to stand for 30 mins at room temperature before the absorbance was measured at 750 nm. The total phenolic content was calculated as milligrams gallic acid equivalent (mg GAE/g sample) using a gallic acid calibration curve.

2.5 Determination of total flavonoid content

The total flavonoid content in the sample was measured by the aluminium chloride colourimetric method. The principle of measurement using the aluminium chloride method is that the flavonoids from the sample react to form a stable acid complex at the C-4 group and the hydroxyl group at C-3 or C-5 of the flavonoid structure. In addition, it can also form a labile

salt complex on the ortho-hydroxyl group on ring A and ring B of the flavonoid structure. Measurement of flavonoid content in the cocoa peel as Bag *et al.* (2015) and Sembiring *et al.* (2018), with slight modifications. External calibration using a series of quercetin concentrations, namely, 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL and 100 µg/mL. In summary, as much as 1 mL of sample was added to 3 mL of methanol p.a, followed by 0.2 mL of 10% AlCl₃ and 0.2 mL of 1 M potassium acetate. Then distilled water up to 10 mL was added. It was shaken until homogeneous and incubated at room temperature for 30 mins. Then the absorbance was measured at 417 nm. The total flavonoid content was calculated as milligrams of quercetin equivalent (mg QE/g sample) using a quercetin calibration curve.

2.6 Measurement of antioxidant activity using the DPPH method

The radical scavenging activity of cocoa peel against DPPH radicals was carried out following Yamin, Ruslin, Mistriyani *et al.* (2021) with a few modifications. In summary, 3 mL of 0.6 mM DPPH solution was mixed with 2 mL of sample solution (concentration series 1 µg/mL, 2 µg/mL, 3 µg/mL, 4 µg/mL and 5 µg/mL), then 3 mL of methanol was added. Then incubated for 30 mins, and the absorbance was measured at 517 nm. As a positive control, ascorbic acid was used. The radical scavenging activity was calculated according to the formula:

$$\% \text{ inhibition} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100\%$$

The IC₅₀ value is obtained by replacing y with 50 in the linear regression equation $y = bx + a$, and the value of x is obtained. The value of x is the IC₅₀ value of the sample.

2.7 Measurement of antioxidant activity using the ABTS method

Before determining the antioxidant properties of the sample using the ABTS method, the ABTS cation radical was prepared using the ABTS method by Mistriyani *et al.* (2018) and Olszowy and Dawidowicz (2018), 7 mM ABTS solution by dissolving 18 mg ABTS in 5 mL of solution, then adding 5 mL of potassium persulfate solution, incubating it in a dark room at 23°C for 16 hrs before use, producing ABTS with a dark blue colour. Measurement of the antioxidant capacity of samples with ABTS radicals was carried out by adding 3 mL of methanol to 1 mL of sample and 1 mL of ABTS cation radical solution. It was shaken until homogeneous and incubated in the dark for 10 mins at room temperature. The absorbance of the solution was measured at 745 nm.

2.8 Data analysis

All data were analyzed using SPSS 24 software. The data displayed were mean ± SD.

3. Results and discussion

The phytochemistry screening result showed that the extract and fraction of the cocoa peel contained flavonoids, saponins, alkaloids, terpenoids, and tannins as shown in Table 1.

Table 1. Phytochemistry cocoa peel

Sample	Test				
	Flavonoid	Saponin	Alkaloid	Terpenoid	Tannin
Methanol extract	+	+	+	+	+
n-hexane fraction	+	+	+	+	+
Chloroform fraction	+	+	+	+	+
Ethyl acetate fraction	+	+	+	+	+
Water fraction	+	+	+	+	+

3.1 Antioxidant activity

The interest in the benefits of natural ingredients as medicine has recently become a prevalent trend. This is because it can be afforded by the community. After all, the process does not have high costs. Besides that, the side effects are relatively small. The metabolites found in plants have an excellent therapeutic effect. Where the metabolites contained in plants are widely reported to have anti-cancer, anti-inflammatory, antimicrobial, antihypertensive, and antispasmodic activities (Smith *et al.*, 2018).

The DPPH method is widely used in evaluating antioxidant activity because of its simplicity. This method is based on the mechanism of DPPH radical reduction by antioxidant compounds through a hydrogen atom transfer mechanism. The process of hydrogen transport mechanism that occurs from antioxidant compounds in DPPH radicals as shown in Figure 2. The process of transferring hydrogen atoms to DPPH radicals is characterized by a change in the colour of the DPPH solution from purple to a stable yellow colour.

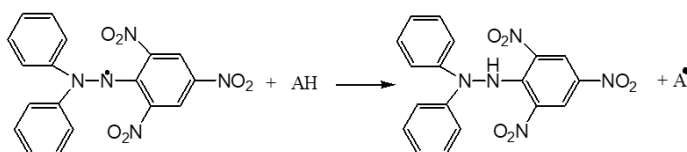


Figure 2. Mechanism of reaction between DPPH radicals and antioxidants

Presentation of this method also provides helpful information about the antioxidant capacity to scavenge hydrogen atoms, the ability of the reduction reaction, and the reaction mechanism between free radicals and antioxidant compounds (Sirivibulkovit *et al.*, 2018;

Enengedi *et al.*, 2019). Sharma and Bhat (2009) performed optimization that provides high sensitivity to dissolve DPPH, the results show that methanol solvent and methanol buffer provide high sensitivity compared to methanol solvent. Thus, in this study, methanol was used to dissolve DPPH. The results showed that the ability of cocoa peels to reduce DPPH radicals is indicated by the change in the colour of the DPPH after adding extracted and fraction cocoa peel. The colour change indicates bonds between DPPH radicals and protons from antioxidant compounds derived from samples of cocoa peel. The reaction between the DPPH radical (purple) and a proton forms DPPH-H (yellow) (2,2-diphenyl-1-picrylhydrazin) (Yamin, Sabarudin, Zubaydah *et al.*, 2021).

Table 2 shows the strength of the antiradical activity of DPPH extract and cocoa peel fraction as indicated by the IC₅₀ value. The IC₅₀ value in Table 2 shows the antiradical potential, which was prevalent in the strongest category due to the IC₅₀ value obtained at < 100 g/mL. According to Cane *et al.* (2020), if the IC₅₀ value is < 100 g/mL, it is classified as a very strong antiradical. From Table 2, the ethyl acetate fraction of Cocoa peel showed the strongest antiradical activity compared to methanol extract, n-hexane fraction, chloroform fraction and water fraction, with IC₅₀ values of 5.61±0.032 µg/mL, 6.10±0.024 µg/mL, 7.06±0.041 µg/mL, 8.23±0.029 µg/mL and 9.24±0.08 µg/mL, respectively, while ascorbic acid was used a positive control. This is in line with previous studies, whereby the ethyl acetate fraction of Raghur bark (Yamin *et al.*, 2020), ethyl acetate fraction of Aceh and Binjai rambutan peels (Rohman *et al.*, 2017), and ethyl acetate fraction of jackfruit seeds (Zubaydah *et al.*, 2021) equally displayed prevalent antiradical activity. This indicates that the semi-polar compounds contained in the sample have the strongest ability to stabilize free radicals (Vifta *et al.*, 2019).

Table 2. IC₅₀ Value extract and fraction of cocoa peel

Sample	IC ₅₀ value (µg/mL)
Ascorbic acid	4.142±0.026
Methanol extract	6.102±0.0302
n-hexane fraction	7.058±0.042
Chloroform fraction	8.225±0.028
Ethyl acetate fraction	5.611±0.031
Water fraction	9.243±0.067

In addition to the DPPH method to measure antioxidant capacity, the ABTS method is also widely used to measure the antioxidant activity of plants. The ABTS method test is based on the reaction of the ABTS radicals produced by ABTS oxidation with potassium persulfate. The radical 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt

(ABTS^{•+}) is widely used to screen plant antioxidant activity (Mistriyani *et al.*, 2018) due to the basis of ABTS/PP testing interaction that occurs between antioxidants and ABTS^{•+} cation radicals. Quantitative scavenging of ABTS^{•+} radicals is very easy to determine because their spectral characteristics occur at 414, 417, 645, 734, and 815 nm. The maximum characteristics were monitored at absorptions of 415 – 417 and 739 – 734 nm. However, the absorption of 730 – 734 is the recommended absorption to measure antioxidant activity using the ABTS^{•+} radical. This is likely due to interference from multiple samples. However, it is important to consider that solvents can shift the absorption band of ABTS^{•+}, such as methanol (744 – 745 nm), ethanol (753 nm), and 1-propanol (757 nm). This occurs due to the solvatochromic effect of the solvent (Ilyasov *et al.*, 2020). In this study, the ABTS^{•+} radical was dissolved in methanol, thus the absorption used was 745 nm.

The number of electrons exchanged by antioxidant compounds with ABTS^{•+} radicals depends on the number of OH groups attached to the aromatic ring or unsaturated carbon atoms. The oxidation of polyphenol compounds that occurs in the electron transfer process is depicted in Figure 3 (Abramovič *et al.*, 2018). Figure 4 shows the value of ABTS radical scavenging by antioxidant compounds measured at 745 nm and that the extract and fraction of cocoa peel have strong antioxidant properties because the IC₅₀ value is < 100 g/mL. The test results according to Figure 4 show that the ethyl acetate fraction has stronger antioxidant power than the methanol extract, n-hexane fraction, chloroform fraction, and water fraction with IC₅₀ values of 5.773±0.018 µg/mL, 6.337±0.0397 µg/mL, 6.564±0.0327 µg/mL, 8.827±0.0423 µg/mL, and 9.216±0.1608 µg/mL, respectively, while ascorbic acid was used as a positive control. This research was in agreement with previous studies whereby the ethyl acetate fraction of *Receptaculum nelumbinis* (Wu *et al.*, 2012), ethyl acetate fraction of kamena-mena leaf (Yamin, Sabarudin,

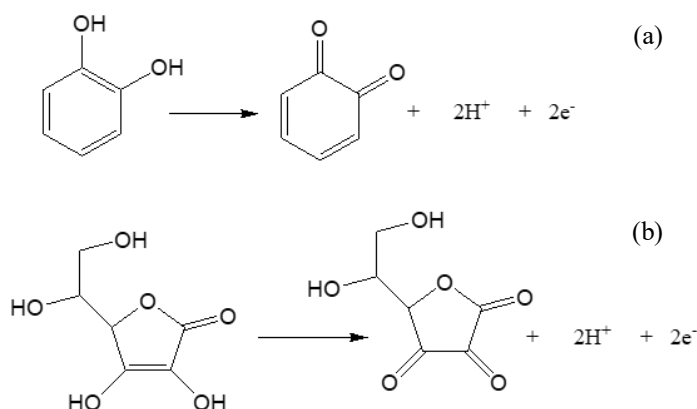


Figure 3. (a) oxidation of catechol compounds to quinones and (b) anediol (ascorbic acid to vicinal diketones) (Abramovič *et al.*, 2018)

Zubaydah et al., 2021), and the ethyl acetate fraction of rambutan peel (Mistriyani et al., 2018) had the highest antioxidant activity.

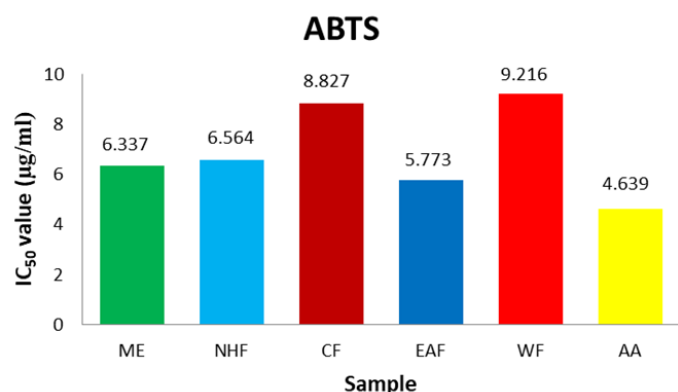


Figure 4. IC₅₀ value of Sample (ME = methanol extract, NHF = n-hexane fraction, CF = Chloroform fraction, EAF = ethyl acetate fraction, WF = water fraction, AA = ascorbic acid)

3.2 Determination of total phenolic and flavonoid contents

Many studies have been conducted *in vitro* and *in vivo* using natural flavonoid compounds to verify the correlation between flavonoid structure and activity as an antioxidant. The structure that typically exhibits antioxidant activity of flavonoid compounds is the hydroxyl group as shown in Figure 5, namely the hydroxyl group (section c), ortho-dihydroxy in ring B (section a), unsaturated bonds C2-C2 combined with carbonyl groups C-4 (section b), and O-methylation (Banjarnahor and Artanti, 2014).

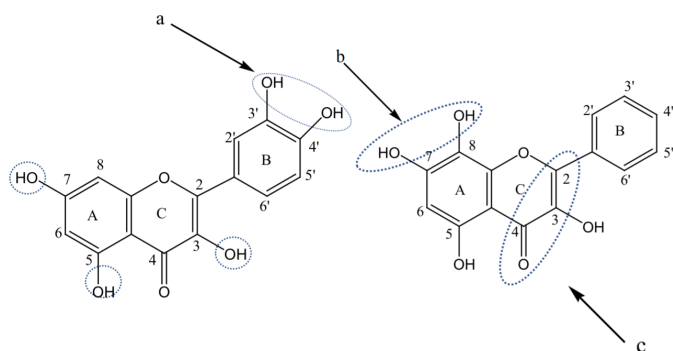


Figure 5. Summary of structure and antioxidant activity (Banjarnahor and Artanti, 2014)

The free hydroxyl groups found in phenolics and flavonoids will donate hydrogen to radical molecules. The radical molecule is stabilized when the flavonoid compound reacts to a flavonoid phenoxyl radical. Furthermore, the stable molecule reacts again with other radicals within the reaction. The number of hydroxyl groups contained in flavonoid and phenolic compounds will affect the antioxidant power of a sample (Pedrielli et al., 2001; Banjarnahor and Artanti, 2014).

Table 3 shows the total phenolic and flavonoid content of the extract and the cocoa peel fraction. Based on the data in Table 3, the ethyl acetate fraction has a higher total phenolic and flavonoid content than the methanol extract, n-hexane fraction, chloroform fraction and water fraction with values of 22.767±0.1097 µg/mL, 20.21±0.095 µg/mL, 18.23±0.085 µg/mL, 13.707±0.1002 µg/mL, and 10.463±0.0603 µg/mL, respectively for phenolic and 90.337±0.552 µg/mL, 71.123±0.381 µg/mL, 60.373±0.35010 µg/mL, 52.76±0.764 µg/mL, and 32,923±0.363 µg/mL, respectively for flavonoids.

Table 3. Total phenolic and flavonoid content of cocoa peel

Sample	Phenolic content (mg GAE/g sample)	Flavonoid content (mg QE/g sample)
Methanol extract	20.21±0.095	71.123±0.381
n-hexane fraction	18.23±0.085	60.973±0.3501
Chloroform fraction	13.707±0.1002	52.76±0.764
Ethyl acetate fraction	22.767±0.1097	90.337±0.552
Water fraction	10.463±0.0603	32.923±0.363

Table 3 shows that the phenolic and flavonoid compounds in the cocoa peel are semipolar phenolic and flavonoid groups. The strength of the ethyl acetate fraction as an antioxidant is due to the high content of phenolics and flavonoids in the ethyl acetate fraction. The higher the phenolic and flavonoid content, the stronger the antioxidant activity. The correlation between phenolic and flavonoid content in inhibiting free radicals is shown by the correlation value (R^2), as shown in Figure 6 and Figure 7. The correlation of phenolic

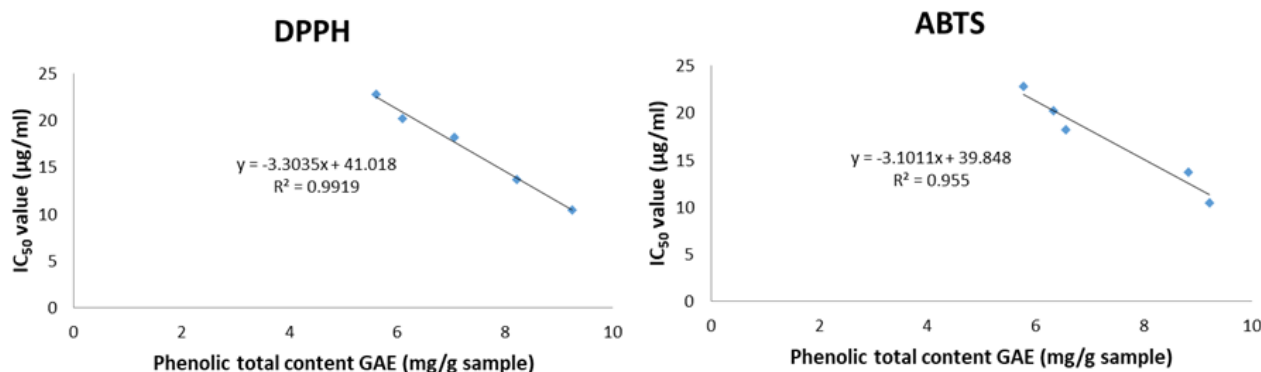


Figure 6. Correlation of phenolic content of cocoa peel versus IC₅₀ values of DPPH and ABTS radicals

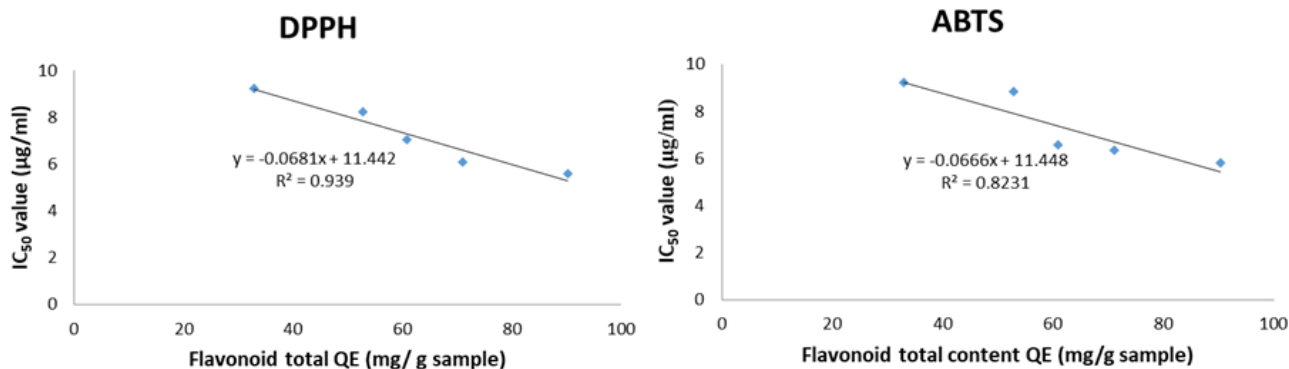


Figure 7. Correlation of flavonoid content of cocoa peel versus IC_{50} values of DPPH and ABTS radicals

content in the cocoa peel with the inhibition of DPPH radicals is $R^2 = 0.9919$, and for ABTS is $R^2 = 0.995$ (Figure 6). Meanwhile, the correlation between total flavonoid content and the inhibition of DPPH radicals was $R^2 = 0.939$, while for ABTS radicals, the correlation was $R^2 = 0.8231$ (Figure 7).

Figure 6 shows that each increase in 1 $\mu\text{g/mL}$ phenolic concentration will decrease the IC_{50} value by 0.0681 $\mu\text{g/mL}$ for the DPPH radical and decrease the IC_{50} value by 0.0666 $\mu\text{g/mL}$ ABTS radical. Meanwhile, Figure 7 shows that each increase in the flavonoid content of 1 $\mu\text{g/mL}$ will decrease the IC_{50} value by 0.0681 $\mu\text{g/mL}$ for DPPH radicals and will in turn decrease the IC_{50} value by 0.0666 $\mu\text{g/mL}$ for ABTS radicals.

4. Conclusion

To conclude, the extract and fractionated cocoa peel showed potentially strong antioxidant activity through the DPPH and ABTS radical methods. Among the samples evaluated, the ethyl acetate fraction of cocoa peel showed very strong antioxidant activity and very high phenolic and flavonoid content. Therefore, cocoa peel can be used as a source of natural antioxidants and as a functional food.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors would like to thank the Rector of Halu Oleo University who has provided a research grant via LPPM of Halu Oleo University.

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