

A PRELIMINARY STUDY: IN-VITRO ASSAYS TO EVALUATE MALAYSIAN'S COCOA BEANS ANTIOXIDANT ACTIVITIES

Shahirah, A.* and Aznie Aida, A.

Cocoa Downstream Research Division, Cocoa Innovation and Technology Centre, Lot 12621 Kawasan Perindustrian Nilai, 71800 Nilai, Negeri Sembilan, Malaysia Second Author

*Corresponding author: shahirahaziz@koko.gov.my

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ABSTRACT – Cocoa beans are well-known for their rich content of polyphenols which contribute significantly to their antioxidant properties. These antioxidants protect against free radical agents and oxidative stress-related diseases. Investigating the antioxidant activity of cocoa beans is essential for understanding their potential health benefits and optimizing processing methods to retain these valuable compounds. This study was conducted to determine which assay is significant to evaluate the antioxidant activity in cocoa beans. Two in-vitro assays namely DPPH and ABTS were used to determine the antioxidant activities on cocoa beans from six different geolocations in Malaysia. Result shows that DPPH assay ($p < 0.05$; $F = 358.74$) and ABTS ($p < 0.05$; $F = 54.95$) was significantly different. The DPPH assay showing clear difference for all 6 samples ($p < 0.05$) with highest antioxidant activity by cocoa beans in Jeli (21.47 ± 0.73^a), Tawau (20.06 ± 0.04^b), Tenom (16.56 ± 0.18^c), Kinabatangan (11.91 ± 0.05^d), Hulu Perak (9.90 ± 0.69^e) and lowest was Ranau (6.67 ± 0.31^f). Meanwhile, ABTS assay shows that Tawau and Jeli cocoa beans has significantly highest antioxidant activity (43.90 ± 1.69^a) and (39.35 ± 0.78^a) respectively, followed by Ranau (30.30 ± 0.70^b) and Tenom (28.65 ± 4.17^b), Hulu Perak (22.20 ± 0.98^c) and lowest was Kinabatangan (16.05 ± 1.06^d). Two-tailed correlation analysis show that DPPH and ABTS assay was positively correlated at 0.679 and ($p = 0.015$; $p < 0.05$) indicated that the evaluated results was significantly comparable in all assays. Additionally, DPPH was more preferred due to its robustness and more practical with higher significant level for all different cocoa bean samples.

Keywords: Cocoa beans, DPPH, ABTS, antioxidant assay, polyphenols

INTRODUCTION

Cocoa is natural source of phenolic compounds, the substances that act as antioxidants and are capable of reducing or delaying oxidative damage from free radicals. The phenolic compounds are a vast class of secondary metabolites performing several roles for the defense and survival of plants. Three major groups of phenolic compounds in cocoa are (1) flavonoids, (2) terpenoids and (3) alkaloids (contained nitrogen or sulfur) (Vuolo *et al.*, 2019). According to LaličićPetronijević *et al.*, (2016) three types of flavonoids or polyphenol most dominant in cocoa beans are proanthocyanidins (~ 58%), catechins or flavan-3-ols (~ 37%) and anthocyanins (~ 4%). Notably, procyanidins and flavonoids especially the flavanols epicatechin and catechin, are among the most prominent compounds (Chow *et al.*, 2023). Fresh cocoa beans contain about 5–6% polyphenols, 1–2% alkaloid theobromine and less than 1% alkaloid caffeine (Kruszewski & Obiedziński, 2018).

Cocoa's high polyphenol content, along with its versatility to consumed in various type of food products, makes it beneficial as a food ingredient and health perspectives (Tsang *et al.*, 2019). Methylxanthines (such as theobromine and caffeine)

and the principal classes of polyphenols, including (+)epicatechin, (+)-catechin, anthocyanins, and proanthocyanidin dimers, are present in cocoa and cocoa-derived products and have been reported to play a significant role in the maintenance of human health (Batista *et al.*, 2016). As natural antioxidant sources, the content and composition of polyphenols in cocoa may differs depending on the genotype, origin, growth conditions, degree of ripeness of the cocoa fruit and the processing parameters (Urbańska & Kowalska, 2019). Previous study shows that Malaysia cocoa genotype KKM 22 was found to contain lower epicatechin, procyanidin B2 and procyanidin C1 compared to QH 1003 and MCBC 1 (Tee *et al.*, 2021). Whereas Oracz *et al.*, (2015) found that the Forastero group cocoa beans from Brazil exhibited the highest content of individual flavan-3-ols, anthocyanins, and flavanols, compared to Trinitario type from Papua New Guinea.

Therefore, the aim of this study was to compare the antioxidant activity of dried cocoa beans from different location in Malaysia and the ability cocoa beans extract from each location to inactivate free DPPH radicals and 2,2'-azinobis-(3 ethylbenzothiazoline-6sulfonic acid) (ABTS). The two assay was then compared for its reliability,

significance, and robustness to prepare for assays as routine analysis.

MATERIALS AND METHODS

Sample preparation and extraction

Initially 22 different samples of cocoa beans were sourced from different farmers and location in Malaysia. In each location, the farmers conducted fermentation of 25 kg wet cocoa beans in wooden box. The box was covered with banana leaves or jute bag to allow the fermentation process occurred appropriately for 4 to 5 days. Afterwards, fermented cocoa beans undergo sun drying on flat bed until moisture level by protimeter evaluation reach ~7.5%. The dried cocoa beans were then cleaned properly and good beans were selected from the batch. From 10 kg of dries & cleaned cocoa beans, quarterly sample was taken randomly for the antioxidant analysis. The 22 samples were then screened down to 6 samples by choosing samples within range of highest, intermediate and lowest ($p < 0.05$) significant antioxidant activities using preliminary testing by DPPH analysis to ensure meaningful comparison of assay and whether both methods used were correlated to each other. The remaining dried cocoa beans with similar or insignificant antioxidant activities were not selected for further analysis.

Continuously, selected dried cocoa bean samples were from Jeli, Tawau, Tenom, Kinabatangan, Hulu Perak and Ranau. All samples were defatted before the extraction. About 6 g of sample were treated with 15 mL petroleum ether in polypropylene tube and shake for 2 mins. The mixture then centrifuged at 12000 rpm for 5 minutes and the liquid was disposed carefully. The step was repeated twice and the sample are dried in fume hood for overnight. Defatted samples (0.05 g) were extracted with 5 mL of solvent (MeOH: H₂O: CH₃COOH) (70:28:2) in polypropylene tube and shake for 10 mins. Afterwards, the mixture was sonicated for 10 mins with ice and shake again for 10 mins. The extractant was centrifuge for 10 min at 12000 rpm at 4°C and was filtered using syringe filter (PDVF 2.0 µm). A volume of 100 µL from supernatant was taken and mixed with 900 µL of diluent (MeOH: H₂O: CH₃COOH) (70:28:2) in order to prepare the reacting sample.

Defatting dried cocoa beans

Cocoa beans (6g) were weighed in polypropylene tube and mixed with 15 mL of petroleum ether. The mixture was shaken using 1500 ShaQer (New Jersey, USA) for 2 minutes and centrifuge at 12,000 rpm for 5 minutes. The solvent then was discarded carefully to remove the fat and the steps were repeated with another 15 mL

petroleum ether. Afterwards, the cocoa beans sample was left overnight to air-dried in fume hood. The dried sample was then stored in humidity chamber (RH < 20 % and 20°C) prior to analysis.

Extraction of dried cocoa beans

Extraction was done by weighing 0.05 g of defatted sample and added with 5 mL of MeOH: H₂O:CH₃COOH with ratio of 70:28:2 and the mixture was sonicated for 10 mins at lower temperature (iced waterbath). After sonicated, the mixture was shaken for 10 mins and centrifuged for 10 mins, 12000 rpm at 4°C. The mixture was filtered using syringe filter PDVF 0.2 µm. The extract was stored at -4°C until further assay conducted.

Determination of DPPH radical scavenging assay

The DPPH radical scavenging assay for the samples was performed as described by (Brand-Williams et al., 1995) with some modifications. Briefly, 100 µL (1:10) of sample was added to 3.9 mL of the DPPH radical solution (0.06 mM) and stored in the dark for 2 h at room temperature. The free radical scavenging capacity was then evaluated by measuring the absorbance at 515 nm using spectrophotometer. A calibration curve in the range of 0.1–1.0 mmol Trolox/mL was used for the quantification of antioxidant activity. Results were expressed as percent of antioxidant activities (%) and analyses were performed in triplicate.

Determination of ABTS radical scavenging assay

The ABTS assay was performed according to (Re et al., 1999a) with minor modifications. Stock solutions of ABTS (7 mM) and potassium persulfate (140mM) were homogenized and kept at room temperature for 16 h in the dark. The ABTS radical solution was diluted with ethanol to obtain an absorbance of 0.70 (± 0.05) at 734 nm. Aliquots of 100 µL of samples were added to 3.0 mL of the ABTS radical solution, and after 6 min the absorbance readings were taken. A calibration curve was developed using a range of 0.1–1.0 mmol Trolox/mL. Results were expressed as percent of antioxidant activities (%) and analyses were performed in triplicate.

Statistical analysis

One way ANOVA was carried out to compare the difference in the mean values, whereby means were considered significant if $p < 0.05$. Pearson's correlation was carried out to investigate the correlation and robustness between DPPH and ABTS assay. All statistical analyses were carried out using SPSS statistical package version 22.0.

RESULTS AND DISCUSSIONS

In-vitro antioxidant analysis of DPPH and ABTS and standard calibration curve

The antioxidant analysis for DPPH and ABTS assay was conducted for all cocoa bean samples from different location.

The result was tabulated in Table 1 show statistical value highly significant at $p < 0.001$ with DPPH assay having higher F value ($F = 358.743$) suggesting a stronger discriminatory ability between sample groups and the differences between cocoa bean samples means are substantial and likely not due to random chance. The marked differences in both assays may be attributed to the varying levels of phenolic compounds, flavonoids, and other bioactive constituents known to influence antioxidant capacity (Prior *et al.*, 2005). While both assays offer significant antioxidant results, DPPH assay implies higher statistical sensitivity and suitable to be used to determine the antioxidant activities in cocoa beans compared to ABTS assay. The lower within-group mean squares for DPPH (0.193) compared to ABTS (3.916) indicate higher consistency and reproducibility within sample replicates. This further validates the robustness of the experimental design and the reliability of the antioxidant activity data by DPPH assay.

Table 1: ANOVA for antioxidant analysis

Analysis	Sum of	Mean Squares	Square	Sig.
	df		F	
DPPH Between Groups	345.769	5	69.154	358.743 <.001
Within Groups	1.157	6	0.193	
Total	346.925	11		
ABTS Between Groups	1075.908	5	215.182	54.952 <.001
Within Groups	23.495	6	3.916	
Total	1099.403	11		

The author conducts a standard calibration curve for both DPPH and FRAP essay using Trolox standard and results were as in Figure 1. Trolox is a water-soluble vitamin E analog commonly used as a standard reference compound. The results in Figure 1 demonstrate a strong and linear relationship between antioxidant activity and concentration measured by DPPH and ABTS assays. The regression equations

obtained for DPPH ($y=78.207x$; $r^2=0.999$) and ABTS ($y=76.504x$; $r^2=0.9974$) indicate that both assays exhibit high accuracy and reproducibility, with coefficients of determination approaching unity. This suggests that the antioxidant response using Trolox standard is highly concentration-dependent and both methods are suitable for evaluating radical scavenging capacity of the tested sample. Interestingly, the slope values of both assays are very similar, with DPPH showing a slightly higher response compared to ABTS. The similarity in scavenging capacity observed in this study was contributed by the Trolox chemical compounds that interact effectively with both radical systems.

DPPH assay operates on the principle of electron transfer, where antioxidants reduce the stable nitrogencentered of DPPH radical, leading to a measurable decrease in absorbance (Brand-Williams *et al.*, 1995). On the other hand, ABTS assay involves both electron and hydrogen atom transfer mechanisms, which enables it to detect the presence antioxidant compounds (Re *et al.*, 1999b). The comparable results obtained in this study indicate that Trolox standard exhibit both electron-donating and hydrogen atom transfer properties, allowing efficient scavenging of both DPPH and ABTS radicals.

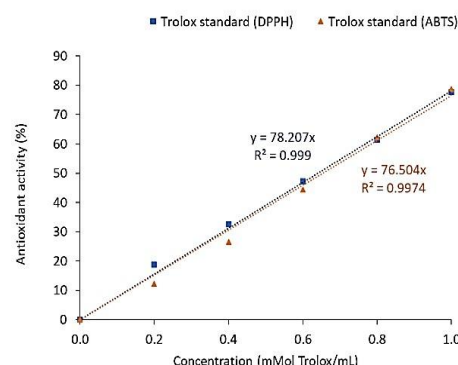


Figure 1: Calibration curve of antioxidant activities for DPPH and ABTS assay

However, this process might different when evaluating antioxidant in cocoa bean samples since the activity depend on amount and type of antioxidant presence in the cocoa beans. The antioxidant potential of cocoa has been extensively linked to its polyphenol composition. These compounds are capable of donating electrons or hydrogen atoms to neutralize free radicals, thus explaining the performance observed in antioxidant assays. Studies found that cocoa monomeric phenolics, particularly epicatechin and catechin exhibit potent antioxidant activity that contributes to healthpromoting benefits (Sorrenti *et al.*, 2020).

Statistical difference of ABTS assay and DPPH assay on different location cocoa beans

In analysis of variance (ANOVA), the Duncan test was used for post-hoc statistical test used to compare the means of multiple groups. From ABTS assay conducted and results tabulated in Table 2, there are 4 different groups from 6 cocoa beans sample analysed. The highest antioxidant analysis significant at $p < 0.05$ and was discovered from Tawau and Jeli ($43.91 \pm 1.73\%$; $39.35 \pm 0.72\%$), followed by Ranau and Tenom ($30.30 \pm 0.72\%$; $28.65 \pm 4.14\%$), Hulu Perak ($22.19 \pm 1.03\%$) and lowest was Kinabatangan ($16.01 \pm 1.05\%$).

Table 2: Statistical difference of ABTS assay on different location cocoa beans

Cocoa Beans				
Location	1	2	3	4
Kinabatangan	16.05			
Hulu Perak		22.20		
Tenom			28.65	
Ranau			30.30	
Jeli				39.35
Tawau				43.90

Means for groups in homogeneous subsets are displayed. Subset for alpha = 0.05

While for DPPH assay, results tabulated in Table 3 shows all six tested cocoa beans from different location give significantly different results correspond to 6 different geo-located cocoa bean samples. This indicted DPPH assay shows higher sensitivity due to a greater number of groups compared to ABTS.

Table 3: Statistical difference of DPPH assay on different location cocoa beans

Cocoa beans						
Location	1	2	3	4	5	6
Ranau	6.67					
Hulu Perak		9.90				
Kinabatangan			11.91			
Tenom				16.56		
Tawau					20.06	
Jeli						21.47

Means for groups in homogeneous subsets are displayed. Subset for alpha = 0.05

The pattern of antioxidant composition also almost similar with ABTS assay which were highest was Jeli ($21.47 \pm 0.71\%$) > Tawau ($20.06 \pm 0.0\%$) followed by Tenom ($16.56 \pm 0.21\%$) > Kinabatangan ($11.91 \pm 0.10\%$) > Hulu Perak ($9.90 \pm 0.71\%$) > Ranau ($6.67 \pm 0.35\%$). This similar pattern and level of antioxidant activities in both assay shows that the

antioxidant content in cocoa beans has positive relationship with the origin of the cocoa beans.

This variation may be attributed to differences in environmental factors such as soil composition, climate, altitude, and the presence of phenolic content of the respective samples, which are known to influence antioxidant activity. Notably, cocoa beans from Jeli and Tawau ($p < 0.05$) from both assays displayed the highest antioxidant activity, suggesting a richer presence of compounds such as flavonoids or polyphenols. Conversely, cocoa beans from Ranau (DPPH; $p < 0.05$) and Kinabatangan (ABTS; $p < 0.05$) exhibited significantly lower antioxidant activity compared to other cocoa beans probably due to a lower concentration of antioxidant compounds. Several studies have shown that cultivar, location, soil and climate conditions are significant factors influencing the content of the bioactive compounds. For instance, the effect of the geographical origin of cocoa beans was shown by Macedo *et al.* (2016) who demonstrated the activity of polyphenol oxidase in Brazil's cocoa beans were influenced by the cultivar, geographical origin and on the applied parameters of the fermentation process. Similar study by Carrillo *et al.* (2014) conducted at different cocoa-growing areas of Colombia results in significant differences ($p < 0.05$) in total polyphenol content (TPC), flavan-3-ol, epicatechin, catechin, caffeine and theobromine contents as well as the theobromine/caffeine ratio and the antioxidant capacity between some of the different sampled farms, showing a significant effect of the cocoa-producing region on antioxidant activity and compound presence.

Interestingly, the use of different location cocoa beans with higher polyphenol to make chocolates may results in product with higher antioxidant activities in the chocolate itself such discovered by Urbańska & Kowalska (2019). The researcher found that cocoa beans from 5 different location (Ghana, Venezuela, the Dominican Republic, Colombia and Ecuador) results in the highest total polyphenol content by cocoa beans originating from Colombia and in the chocolates obtained from them. These findings suggest that antioxidant activity of cocoa beans varies notably depending on the sample origin, likely due to environmental and biochemical factors. It is also found that the DPPH assay is sensitive and sufficient to measure the antioxidant activities of cocoa beans from different location in Malaysia.

Correlations analysis and Robust Test of Equality for antioxidant analysis

The Pearson correlation analysis in Table 4 was conducted to evaluate the relationship between antioxidant activities measured by DPPH and ABTS

assays and the number of samples tested. Results show that moderate and statistically significant positive correlation ($p < 0.05$) was found between DPPH and ABTS ($r = 0.679$, $p = 0.015$) indicating that these two assays promise to produce comparable antioxidant activity measurements across the tested samples. This finding aligns with previous research by Floegel *et al.* (2011) showing the significant correlation as assessed by these two assays suggests that free radical scavenging activity were attributed to similar antioxidant constituents or mechanisms present in the samples.

This result was also supported by the findings from Batista *et al.* (2016). In his study on antioxidant capacity of chocolate & cocoa beans using DPPH & ABBTS assays showed highly positive correlation. In another relationship study by Todorovic *et al.* (2015) also shows that the correlation on antioxidant assays using four different assays namely DPPH, ABTS, FRAP and ORAC results in statistical values for cocoa products were positively high ($r \sim 0.99$) and significant relationships among antioxidant assays ($p < 0.05$). This finding shows that the DPPH assay is a reliable method for confirming the antioxidant activity of cocoa beans.

Table 4: Correlations analysis for antioxidant analysis

Assay		DPPH	ABTS
DPPH	Pearson	1	0.679*
	Correlation Sig. (2-tailed)		0.015
ABTS	Pearson	0.679*	1
	Correlation Sig. (2-tailed)	0.015	

*Correlation is significant at the 0.05 level (2-tailed) where $N = 12$ for each DPPH, ABTS and Samples.

The strong association between DPPH and ABTS assays is particularly important for confirming the robustness and consistency of antioxidant capacity measurements. According to Thaipong *et al.*, (2006), using multiple assays enhances the reliability of antioxidant assessments due to the different mechanisms involved. In addition, cocoa bean extracts usually contain high pigments and turbidity. The turbidity can be corrected through filtering using PDVF syringe filter while background cocoa color pigments usually corrected by serial dilutions.

Theoretically DPPH relies on electron transfer and lipophilic antioxidants, while ABTS involves both electron and hydrogen atom transfer mechanisms. DPPH assay is suitable to be used in determining high fat and complex sample such as cocoa and chocolate

due to reaction to lipophilic polyphenols, efficient, ready-to-use commercially, rapid, simple to perform, and widely adopted due to its stability (Bibi Sadeer *et al.*, 2020). The results are reproducible and comparable to other radical scavenging methods with correlation reported on bioactive compounds (phenols, flavonoids) with a regression factor $R > 0.80$. Therefore, a significant positive correlation validates the samples' antioxidant efficacy across different reactive cocoa beans sample models. Additionally, ABTS is tolerant of both hydrophilic and lipophilic antioxidants, therefore it can also be performed on coca beans to provide a complementary measure for a comprehensive evaluation (Re *et al.*, 1999b). However, ABTS⁺ unstable radical always need to be prepared fresh, avoided from direct light exposure and requires longer running time. Prior to the reaction, ABTS stock and potassium persulfate solution mixture needs to be incubated for 12 – 16 hours at room temperature to sufficiently generate ABTS radical. This indicates ABTS required more time for preparation and time in conducting the assay compared to DPPH.

Robust tests of equality of means indicated significant differences among groups for both antioxidant assays. In cases where the assumption of homogeneity of variances violated, the use of robust tests of equality of means such as Welch's ANOVA and the Brown-Forsythe test is recommended, as these tests provide more reliable results than the classical ANOVA F-test (Brown & Forsythe, 1974; Welch, 1951). Robust procedures adjust the degrees of freedom to account for unequal variances, thereby maintaining the validity of statistical inferences (Tomarken & Serlin, 1986; Keselman *et al.*, 2004). Therefore, the use of this test complements and as a support to ANOVA test result as discussed in the beginning of this paper.

Table 5: Robust Test of Equality of Means analysis for antioxidant analysis

Assay	Test	Statistic ^a	df1	df2	Sig.
DPPH	Welch	2871.923	5	2.598	<.001
	Brown -Forsythe	358.743	5	2.507	<.001
ABTS	Welch	90.738	5	2.749	.003
	Brown-Forsythe	54.952	5	1.758	.027

^a asymptotically F distributed

In Table 5, shows the DPPH assay, both the Welch ($F = 2871.923$, $df = 5$, 2.598 , $p < 0.001$) and Brown-Forsythe ($F = 358.743$, $df = 5$, 2.507 , $p < 0.001$) tests revealed statistically significant differences in mean antioxidant activity across the tested groups. The lower p-values in DPPH assay indicate a highly significant result, strongly rejecting the null hypothesis of equal means. This suggests that at least one of the tested groups exhibits a significantly different DPPH

radical scavenging activity. Such variability may be attributed to differences in the phytochemical content or antioxidant profiles of the cocoa bean samples taken from different location which was consistent with findings in previous studies (Prior *et al.*, 2005). This statistic result suggest that the antioxidant potential varies significantly between samples from different locations and DPPH assays are sufficiently effective at detecting such differences. Costea et al. (2022) also using Welch ANOVA approach to see the antioxidant activity of five vegetal extracts with hepatoprotective potential. In her study shows that DPPH assay is able to maintain meaningful result on antioxidant determination while requiring comparatively simple assay preparation, reproducible and inexpensive therefore beneficial for routine and screening analysis.

CONCLUSIONS

In conclusion, this study shows that cocoa beans collected from Jeli and Tawau has displayed significantly highest antioxidant activity compared to cocoa beans originated from Ranau and Kinabatangan. Statistic shows that DPPH assays are sufficient, significant and simpler at determining the antioxidant activities from cocoa beans making it robust and much more practical for a routine analysis.

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