CAPABILITY OF COCOA BEANS EXTRACT IN FERRIC REDUCTION AND QUENCHING OF FREE RADICALS

Samuel Yap Kian Chee*, Arief Huzaimi, M.Y., Chin, H.H., Nur Azilah, A., Nor Haslita, I., Sarini, H.
Malaysian Cocoa Board, Lot 12621, Kawasan Perindustrian Nilai, 71800 Nilai
**Corresponding author: samuel@koko.gov.my*

Malaysian Cocoa J. 14: 163-167 (2022)

ABSTRACT - *The ability of cocoa beans extract in antioxidant were tested and compared between its ability to reduce ferric to ferrous and number of free radicals removed from organic chemical compound 2,2-diphenyl-1-picrylhydrazyl. This study revealed that freeze dried cocoa beans aqueous extract of 50ppm was able to reduce 1.8 µmol/L of ferric to ferrous. Concentration below 20 ppm generally was not ideal in reducing Fe(III) to Fe(II). Whereas, at concentration of 45ppm freeze dried cocoa beans aqueous extract was estimated able to remove 77% available free radicals from 0.0003mM DPPH.*

Key words: Cocoa, antioxidant, ferric reduction, free radical quencher

INTRODUCTION

Antioxidants can be categorized into two types: (i) non-enzymatic antioxidants such as vitamin C, vitamin E, plant polyphenols, carotenoids, and glutathione (GSH); and (ii) enzymatic antioxidants such as superoxide dismutase, glutathione peroxidase, and catalase (Gutpa, 2015). On the other hand, there are multiple free radical and oxidant sources [e.g., O_2•-, $^1O_2$, HO• , NO• , ONOO-, HOCl, RO(O)• , LO(O)•], and both oxidants and antioxidants have different chemical and physical characteristics (Brewer, M.S., 2011). Individual antioxidants may, in some cases, act by multiple mechanisms in a single system or by a different single mechanism depending on the reaction system. Furthermore, antioxidants may respond in a different manner to different radical or oxidant sources (Satish & Dilipkumar, 2015). For example, carotenoids are not particularly good quenchers of peroxyl radicals relative to phenolics but are exceptional in quenching singlet oxygen, at which most other phenolics are relatively ineffective. However, singlet oxygen is not a radical and does not react via radical mechanisms but reacts mostly by the addition to double bonds, forming endoperoxides that can be reduced to alkoxyl radicals that initiate radical chain reactions. Because multiple reaction characteristics and mechanisms as well as different phase localizations are usually involved, no single assay will accurately reflect all of the radical sources or all antioxidants in a mixed or complex system.

FRAP assay is conducted at acidic pH 3.6 to maintain iron solubility. Reaction at low pH decreases the ionization potential that drives electron transfer and increases the redox potential, causing a shift in the dominant reaction mechanism (Spiegel et al., 2020; Zhong & Shahidi, 2015). Often, FRAP values have a poor relationship to other antioxidant measures. It has been argued that the ability to reduce iron has little relationship to the radical quenching processes (H transfer) mediated by most antioxidants. However, oxidation or reduction of radicals to ions still stops radical chains, and reducing power reflects the ability of compounds to modulate redox tone in plasma and tissues. The FRAP mechanism is totally electron transfer. In addition, because reduced metals are active propagators of radical chains via hydroperoxide reduction to RO• , it would be interesting to evaluate whether high FRAP values correlate with the tendency of polyphenols to become pro-oxidants under some conditions. This has been shown for some flavones and flavanones, which also have high FRAP values. FRAP results can vary tremendously depending on the time scale of analysis. Fast-reacting phenols that bind the iron or break down to compounds with lower or different reactivity are best analyzed with short reaction times, for example, 4 min. However, some polyphenols react more slowly and require longer reaction times for detection, for example, 30 min. The order of reactivity of a series of antioxidants can vary tremendously and even invert, depending on the analysis time. FRAP actually measures only the reducing capability based upon the ferric ion, which is not relevant to antioxidant activity mechanistically and physiologically. However, in contrast to other tests of total antioxidant power, the FRAP assay is simple, speedy, inexpensive, and robust and does not require specialized equipment.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay is one of the few stable organic nitrogen radicals, which bears a deep purple color. It is commercially available. This assay is based on the measurement of the reducing ability of antioxidants toward DPPH•. Antioxidant assays are based on measurement of the loss of DPPH color at 520 nm after reaction with test compounds, and the reaction is monitored by a spectrometer. Nevertheless, DPPH has no similarity to the highly reactive and transient peroxyl radicals involved in lipid peroxidation. Many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH due to steric inaccessibility.

Studies showed that methods of extraction and extraction mediums affect greatly on the free radicals quenching power of the cocoa beans extract. A significant drop on the free radical scavenging capability was also observed from the fresh and dry beans compared to roasted beans (Samuel Yap & Arief Huzaimi, 2020). Major phenolic compositions of the fresh cocoa beans aqueous extract were catechin and epicatechin (Samuel Yap, 2018).

The objectives of this paper are to report the behaviors of cocoa beans aqueous extract towards FRAP and DPPH antioxidant assays that are commonly used in antioxidant study.

MATERIALS AND METHODS

Extraction of cocoa phenolics

One gram of cocoa beans was added with 50ml of distilled water, ground with a food processor in low speed for 3 seconds, incubated at 80°C in an incubator shaker with 150rpm orbital shaking mode for 15 minutes. The extract was then filtered with filter paper (Whatman no. 4). De-pulping processes were carried out for fresh unfermented cocoa beans and freeze dried prior to extraction.

Ferric Reducing Antioxidant Power (FRAP) assays

A series of sample dilutions from 1750ppm to 500ppm with 250ppm decreasing concentrations were prepared for FRAP analysis. Prior to FRAP tests, a standard curve of Fe_2SO_4 from 0.2µM to 2.0µM versus absorbance at 593nm was plotted.

Two hundred milliliters of buffer (3.2 ml of acetic acid mixed with 196.8 ml of distilled water and 0.62g of sodium acetate) (pH 3.6) was added with 20 ml of TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine) solution (0.063g of TPTZ in mixture of 78.8µl of hydrochloric acid) and with 20 ml of $FeCl_3$ (20 mmol/L). The mixture of buffer, TPTZ solution and ferric chloride in the ratio of 10:1:1 respectively, is known as FRAP assays (Noor Asna & Noriham, 2014). Samples/ standards (100µL) were added with 3.0ml of FRAP assays and incubated for 1 hour. Absorbance was then measured by using UV-Vis spectrophotometer at 593 nm against the blank. Its reducing power from ferric to ferrous was gauged in correlating to the standard curve respectively.

Free Radicals Scavenging Capability

A series of dilutions (500ppm, 250ppm, 125ppm, 62.5ppm, 31.25ppm, 15.63ppm, and 7.81ppm) were prepared from a stock solution of 1,000 ppm.

Free radicals scavenging capability of the extract were determined by drawing 0.5ml sample added with 5.0ml 0.06 mM DPPH solution, mixed well and incubated in dark for 30 minutes prior to measure with UV-Visible spectrophotometer at 520nm. Percentage of free radicals scavenging capability was calculated as the equation below (Samuel Yap, 2018):

$$RSC (\%) = [(Abs(b) - Abs(S)/Abs(b)] \times 100 \quad \{2\}$$

where:
RSC = DPPH radical scavenging capability
Abs(c) = Abs for blank
Abs(S) = Abs for sample

RESULTS AND DISCUSSIONS

Ferric Reducing Antioxidant Power (FRAP) assays

A ferrous standard curve from 0.2µmol/L to 2.0µmol/L versus absorbance value at 593nm was constructed as in Figure 1.

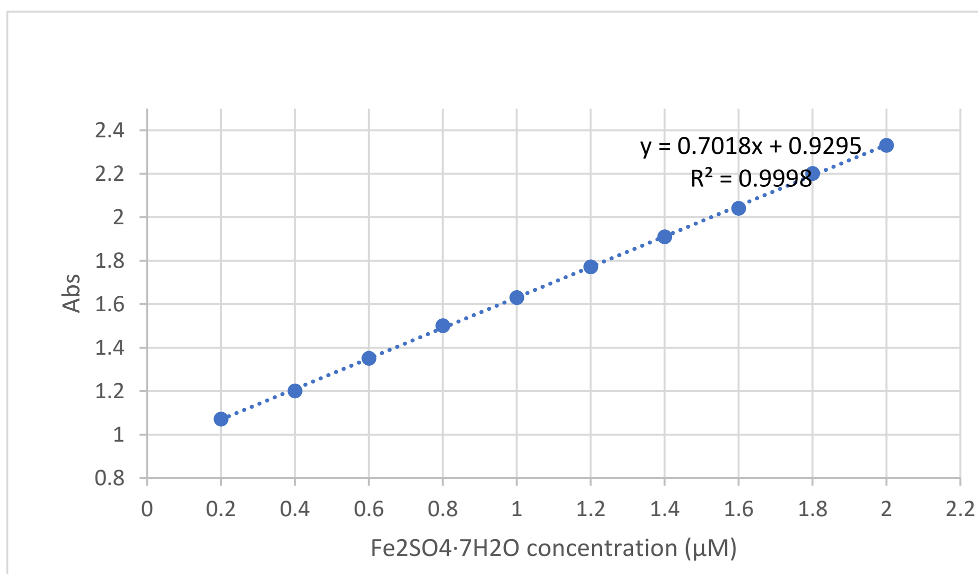

Figure 1. Standard curve of ferrous concentrations vs absorbance.

Based on the methods outlined above, the normalized concentration on each dilution introduced in the test assays were calculated as shown in Table 1. The reducing power of the cocoa beans aqueous extract and ascorbic acid to reduce ferric to ferrous were determined based on their absorbance value in correlating to the value in the standard curve above (Figure 1), and the results were tabulated in Table 1.

Table 1: Reducing power from ferric to ferrous by cocoa beans aqueous extract and ascorbic acid.

Series Concentration (ppm)		**Absorbance at 593nm (Average of 3 readings)**		**Fe(II) µmol/L**	
Original concentration	**Normalized concentration**	**Cocoa beans aqueous extract**	**Ascorbic acid**	**Cocoa beans aqueous extract**	**Ascorbic acid**
1750	56.45	2.220	OR	1.84	-
1500	48.39	2.192	OR	1.8	-
1250	40.32	2.199	OR	1.81	-
1000	32.26	1.834	2.454	1.29	2.17
875	28.23	1.722	2.216	1.13	1.83
750	24.19	1.603	1.929	0.96	1.42
625	20.16	1.274	1.641	0.49	1.01
500	16.13	0.931	1.353	0	0.60

Note: OR denoted “out of range” by the UV-vis spectrometer

It could be noted that cocoa beans aqueous extract had weaker reducing power than ascorbic acid in reducing ferric to ferrous from FRAP assays. At the normalized concentration below 20ppm, cocoa beans aqueous extract basically approaches zero reducing power.

Ability in quenching of free radicals

In this study, only 0.5ml from each series of dilutions were drawn and added into 5.0 ml assays, hence, a normalized concentration in the reaction was calculated as in Table 2 respectively. Average absorbance value for blank was 2.057. The power of the cocoa beans aqueous extract and ascorbic acid in quenching organic nitrogen radicals from DPPH assay in terms of percentage of free radicals scavenging capability were calculated and tabulated in Table 2.

Table 2: Percentage of free radicals scavenging capability from the series of concentrations for cocoa beans aqueous extract and ascorbic acid.

Series Concentration (ppm)		**Absorbance at 520nm (Average of 3 readings)**		**% scavenging**	
Original concentration	**Normalized concentration**	**Cocoa beans aqueous extract**	**Ascorbic acid**	**Cocoa beans aqueous extract**	**Ascorbic acid**
1000	90.9	0.388	0.175	81.1	91.5
500	45	0.476	0.186	76.9	91.0
250	22.5	0.987	0.195	52.0	90.5
125	11.25	1.393	0.248	32.3	87.9
62.5	5.63	1.752	0.273	14.8	86.7
31.25	2.81	1.896	0.739	7.8	64.1
15.63	1.41	2.074	1.086	-0.8	47.2
7.81	0.70	2.067	1.177	-0.5	42.8

Results showed the cocoa beans aqueous extract indeed had lower quenching power in removing nitrogen organic radicals compared to ascorbic acids. A useful normalized concentration of cocoa beans aqueous extract should be 22.5ppm and above where more than 50% of the available free radicals could be quenched. At normalized concentrations of lower than 2ppm, cocoa beans aqueous extract might lose its quenching ability.

CONCLUSIONS

Cocoa beans aqueous extract performed better in quenching the nitrogen organic radicals if compared to its ability to reduce ferric to ferrous. A normalized concentration of 22.5ppm and above was needed to quench more than 50% of the available free radicals in 0.0003mM DPPH solution and lost its quenching power at concentration lower than 2ppm; whereas at 20ppm and lower, cocoa beans aqueous extract basically was unable to reduce ferric into ferrous.

ACKNOWLEDGEMENTS

The authors would like to thanks the Government of Malaysia for funding this project via RMK11 Development Project.

REFERENCES

Brewer, M.S. (2011). Natural Antioxidants: Sources, Compounds, Mechanisms of Action, and Potential Applications. *Comprehensive Reviews in Food Science and Food Safety.* **10**: 221-247.

Gutpa, D. (2015). Methods for Determination of Antioxidant Capacity: A Review. *Int J Pharm Sci Res 2015*, **6(2)**: 546-66.

Noor Asna, A. & Noriham, A. (2014). Antioxidant Activity and Bioactive Components of Oxalidaceae Fruit Extracts. *The Malaysian Journal of Analytical Sciences,* **18(1)**: 116-126.

Samuel Yap, K.C. (2018). Free Radicals Scavenging Capability from Different Fractions of Cocoa Fresh Beans Aqueous Extract. *Food Sci Nutr Res,* **1(1):** 1-3.

Samuel Yap, K.C. & Arief Huzaimi, M.Y. (2020). Cocoa Beans Processing Chains on Its Extractable Total Phenolics Contents and Free Radical Scavenging Capability. *EC Nutrition*, **15.11:** 81-88.

Satish, B.N. & Dilipkumar, P. (2015). Free Radicals, Natural Antioxidants, and their Reaction Mechanism. *RSC Adv*, **5:** 27986-28006.

Spiegel, M., Kapusta, K., Kolodziejczyk, W., Saloni, J., Zbikowska, B., Hill, G.A., & Sroka, Z. (2020). Antioxidant Activity of Selected Phenolic Acids-Ferric Reducing Antioxidant Power Assay and QSAR Analysis of the Sructural Features. *Molecules***, 25**:3088.

Zhong, Y. & Shahidi, F. (2015). Chapter 12: Methods for the Assessment of Antioxidant Activity in Foods. **In:** *Handbook of Antioxidants for Food Preservation*. 287-333.