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Cocoa Fresh Beans Aqueous Extract as Free Radical Quencher and Ferric Reducer

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Abstract

The presence of free radicals and oxidants in our body is either produced by our body through normal cell metabolisms or from environment surrounding us such as radiation, medication, pollutants or others. The free radicals and oxidants can be harmful to our body when their accumulation posing oxidative stress in the body. Cocoa beans are rich in polyphenols, which are functioning as antioxidant. However, each antioxidant has difference response towards either free radicals or other oxidants. The purpose of this research was to study the ability of cocoa fresh beans aqueous extract functions as antioxidant between its ability to reduce ferric to ferrous and number of free radicals removed from organic chemical compound of 2,2-diphenyl-1-picrylhydrazyl (DPPH). A series concentration of cocoa fresh beans aqueous were prepared. Its ability as ferric reducer was determined based on ferric reducing antioxidant power (FRAP) assays and its ability as free radicals scavenger was determined using DPPH assays. This study revealed that freeze dried cocoa fresh beans aqueous extract of 50 ppm was able to reduce 3.2 x 10^{15} of ferric molecules to ferrous. Concentration below 20 ppm generally was not able to reduce Fe(III) to Fe(II). Whereas, at concentration of 45 ppm freeze dried cocoa fresh beans aqueous extract was estimated able to remove 1.4 x 10^{17} organic nitrogen free radicals from DPPH assay. This study suggests that freeze dried cocoa fresh beans aqueous extract has better performance in quenching free radicals compared to reducing ferric into ferrous.

Keywords: cocoa, polyphenols, free radical quencher, ferric

INTRODUCTION

Free radicals and oxidants are produced either from normal cell metabolisms in situ or from external sources such as radiation, medication and pollution. These free radicals and oxidants are mostly destroyed by our body system naturally. Nevertheless, when too much of these free radicals and oxidants present in our body and they cannot gradually be destroyed, it can be harmful to the body when their accumulation reaches the threshold of can lead into the development of chronic and degenerative illness such as cancer, autoimmune disorders, aging, cataract, rheumatoid arthritis, cardiovascular and neurodegenerative diseases (Lien Ai *et al*., 2008). The purpose of taking anti- oxidants is to boast our body mechanisms to counteract this oxidative stress and consequently to maintain healthy.

Generally, there are two types of antioxidants, namely, non-enzymatic antioxidants

are categorized as non-enzymatic antioxidants.

There are multiple sources of free radicals and oxidants. This include, but not limited to superoxide anion radical, singlet oxygen, hydroxyl radical, nitric oxide, peroxynitrite, hypochlorous acid, peroxyl radical, and lipid peroxyl radical. Both oxidants and antioxi- dants are exhibiting differently in terms of their chemical and physical characteristics (Brewer, 2011). Hence, each antioxidant, in general, is responding in a different manner towards different sources of radical or oxidant. For instant, carotenoids are not good in quenching peroxyl radicals relative to phenolics but are exceptional good as singlet oxygen quencher compared to most of the phenolics, which are relatively ineffective (Satish & Dilipkumar,
2015).

Ability to reduce iron in ferric reducing antioxidant power (FRAP) test has little relationship to the radical quenching processes (H transfer) as mediated by most antioxidants. Nevertheless, reduced metals are active propa- gators of radical chains through formation of alkoxyl radical by hydroperoxide reduction mechanism. Hence, it is interesting to evaluate whether high FRAP values is correlating with the tendency of polyphenols to become pro-oxidants under some conditions.

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is one of the few stable organic nitrogen radicals, which is deep purple in color. This assay is based on the measurement of the reducing ability of antioxidants toward the organic nitrogen radicals of DPPH. The de- gree of antioxidant capability is based on measurement of the loss of DPPH color at the free radicals quenching power of the cocoa beans extract. A significant drop on the free radical scavenging capability is observed from the extract of fresh and dry beans compared to roasted beans (Samuel Yap & Arief Huzaimi,
2020). Major phenolic compositions of the fresh cocoa beans aqueous extract are catechin and epicatechin (Samuel Yap, 2018). The objective of this paper is to report the behaviors of cocoa fresh beans aqueous extract towards FRAP and DPPH

MATERIALS AND METHODS

The cocoa variety used in this study was a commercial cocoa clone by MCB. The supply of the cocoa materials were from the cocoa research study plot at Bagan Datuk, Perak of Malaysia.

Cocoa Phenolics Extraction

One gram of cocoa beans was added with
50 mL of distilled water, ground with food processor in low speed for 3 seconds, incubated at 80°C in an incubator shaker with 150 rpm 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

FRAP Assay

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

Two hundred milliliters of buffer (3.2 mL of acetic acid mixed with 196.8 mL of

water and 0.62 g of sodium acetate pH 3.6) was added with 20 mL of TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine) solution (0.063 g of TPTZ in mixture of 78.8 µL of hydrochloric acid) and with 20 mL of $FeCl_3$ (L^{-1}). The mixture of buffer, TPTZ solution and ferric chloride in the ratio of 10:1:1 respectively, is known as FRAP assays. Samples/ standards (100 µL) were added with 3.0 mL of FRAP assays and incubated for 30 minutes. Absorbance was then measured by using UV-Vis spectrophotometer at 593 nm against the blank. Its reducing power from ferric to

Free Radicals Scavenging Capability

A series concentration of cocoa fresh beans aqueous extract (500 ppm, 250 ppm, 125 ppm, 62.5 ppm, 31.25 ppm, 15.63 ppm, and 7.81 ppm) were prepared from a stock solution of 1,000 ppm.

Free radicals scavenging capability of the extract were determined by drawing 0.5 mL sample added with 5.0 mL 0.06 mM DPPH solution and tris-buffer (pH 7.6), mixed well and incubated in dark for 30 minutes prior to measure with UV-Visible spectrophotometer at 520 nm. Number of free radicals

$$N_{rad} = \frac{RSC(\%) \times N\,(DPPH)}{100} \quad \text{..... (1)}$$

where,

RSC (%) = percent of DPPH radicals quenched
= [(Abs(b) - Abs(S)/Abs(b)] x 100
N(DPPH) = available mole of DPPH in test solution x NA
Abs(b) = Absorbance for blank
Abs(S) = Absorbance for sample
NA = Avogadro constant = 6.022×10^{23} mol^{-1}.

RESULTS AND DISCUSSION

Antioxidant capability of the cocoa beans extract is contributed by the presence of phenolic contents in the cocoa study reported that cocoa fresh beans aqueous extract from the commercial clone by MCB contains mainly flavanol monomer of epicatechin and catechin (Samuel Yap, 2018). The anti- oxidant capability of the cocoa fresh beans aqueous extract was

FRAP Assay

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

Based on the methods outlined above, the normalized concentration on each dilution introduced in the test assays were calculated based on equation (2) and

$$(V_{sample} \times OC)/(V_{sample} + V_{assays}) \text{ ..... (2)}$$

Where:

V_{sample} = amount of sample used in the test, mL
OC = original concentration of the sample from the dilution series, ppm
V_{assay} = total amount of test assays solution, mL

The reducing power of the cocoa beans aqueous extract 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.

Percentage of the ferric molecules reduced to ferrous by the cocoa fresh beans aqueous extract (CFBAE) was determined based on the equation (3) and results were

$$FR\,(\%) = \frac{[Fe(II)]}{[Fe(III)]} \times 100 \quad \text{..... (3)}$$

Where,

FR(%) = percent of ferric reduced to ferrous
[Fe(II)] = concentration of Fe(II) as determined in correlation with Figure 1
[Fe(III)] = available concentration of Fe(III) in the test solution (3 mL FRAP assay)
$= \frac{0.005 \text{ mmol}}{3 \text{ mL}}$
= 1.67 mM

Number of ferric molecules reduced to ferrous (Fe(III) → Fe(II)) was

$$\text{Fe(III)} \quad \text{Fe(II)} = \frac{\frac{\text{FR (\%) x available mol of Fe(III) in test solution x NA}}{100}}{\frac{\text{FR (\%) x 0.005 mmol x NA}}{100}} \quad \ldots\ldots\ldots\ldots\ldots\ldots$$

It could be noted from Table 1 that at the normalized concentration below 20 ppm, cocoa fresh beans aqueous extract

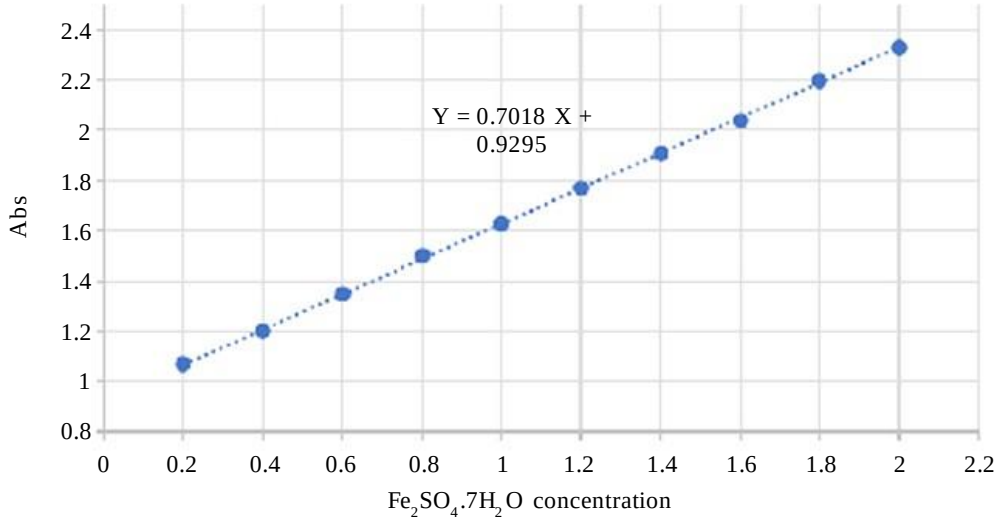

Figure 1. Standard curve of ferrous concentrations vs absorbance at 593 nm

Table 1. Reducing power from ferric to ferrous by cocoa fresh beans aqueous extract (CFBAE)

Series concentration of CFBAE (ppm)		Absorbance at 593 nm	Fe(II) (µM) (Average of 3 readings)	No. of ferric molecules reduced to ferrous
Original	Normalized			
1750	56.45	2.220 ± 0.002	1.84	3.32×10^{15}
1500	48.39	2.192 ± 0.007	1.80	3.25×10^{15}
1250	40.32	2.199 ± 0.002	1.81	3.26×10^{15}
1000	32.26	1.834 ± 0.008	1.29	2.33×10^{15}
875	28.23	1.722 ± 0.004	1.13	2.04×10^{15}
750	24.19	1.603 ± 0.007	0.96	1.73×10^{15}
625	20.16	1.274 ± 0.005	0.49	8.83×10^{14}
500	16.13	0.931 ± 0.006	0	0

Free Radicals Quenching

In this study, only 0.5 mL from each series of dilutions were drawn and added into 5.0 mL assays, hence, based on the equation (2), 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 fresh beans aqueous extract in quenching organic nitrogen radicals from DPPH assay in term of percentage of free radicals scavenging capability were calculated based on equation (1) and tabulated in Table 2.

Results showed a useful normalized concentration of cocoa beans aqueous extract was 22.5 ppm and above where more than
50% of the available free radicals were quenched. At normalized concentrations of lower than 2 ppm, cocoa beans aqueous extract might lose its quenching ability. A similar profiles were reported by Azizah *et al.* (2007) but with higher cocoa beans extract concentration, perhaps due to the direct uses of cocoa beans extract without freeze dried as mentioned in their methodology of the study. Azizah *et al.* (2007) reported that no significant free

DPPH versus FRAP

Figure 2 shows that cocoa fresh beans aqueous extract has better performance in organic nitrogen free radicals scavenging capability compared to ferric reduction to ferrous. Ferric reducing antioxidant power (FRAP) assay is conducted at acidic environ- ment of pH 3.6 to maintain its iron solubility. Reaction at low pH decreases the ionization potential and consequently, initiates electron transfer and increases the redox potential, causing a shift in the dominant reaction mechanism (Spiegel *et al*., 2020; Zhong & Shahidi, 2015). However, at lower pH condi- tion, the antioxidant activity of the phenols decreases due to the decreasing of its electron- donating ability upon deprotonation (Arzu *et al*., 2016; Pekal & Pyrzynska, 2015).

The other reasons maybe due to the higher steric inaccessibility by the phenolic compounds, mainly catechin and epicatechin, found in cocoa fresh beans aqueous extract (Samuel Yap, 2018). In TPTZ solution, ferric ion is trapped in between the tripyridylniazine ligands of the ferric triphyridylniazine complex, causing the

Table 2. Free radicals scavenging capability from the series of concentrations for cocoa fresh beans aqueous extract (CFBAE)

Series concentration of CFBAE (ppm)		Absorbance at 520 nm (Average of 3 readings)	% Scavenging	No. of free radicals quenched
Original	Normalized			
1000	90.9	0.388 ± 0.002	81.1	1.47×10^{17}
500	45	0.476 ± 0.004	76.9	1.39×10^{17}
250	22.5	0.987 ± 0.004	52.0	9.39×10^{16}
125	11.25	1.393 ± 0.002	32.3	5.84×10^{16}
62.5	5.63	1.752 ± 0.004	14.8	2.67×10^{16}
31.25	2.81	1.896 ± 0.006	7.8	1.41×10^{16}
15.63	1.41	2.074 ± 0.007	-0.8	-
7.81	0.70	2.067 ± 0.005	-0.5	-

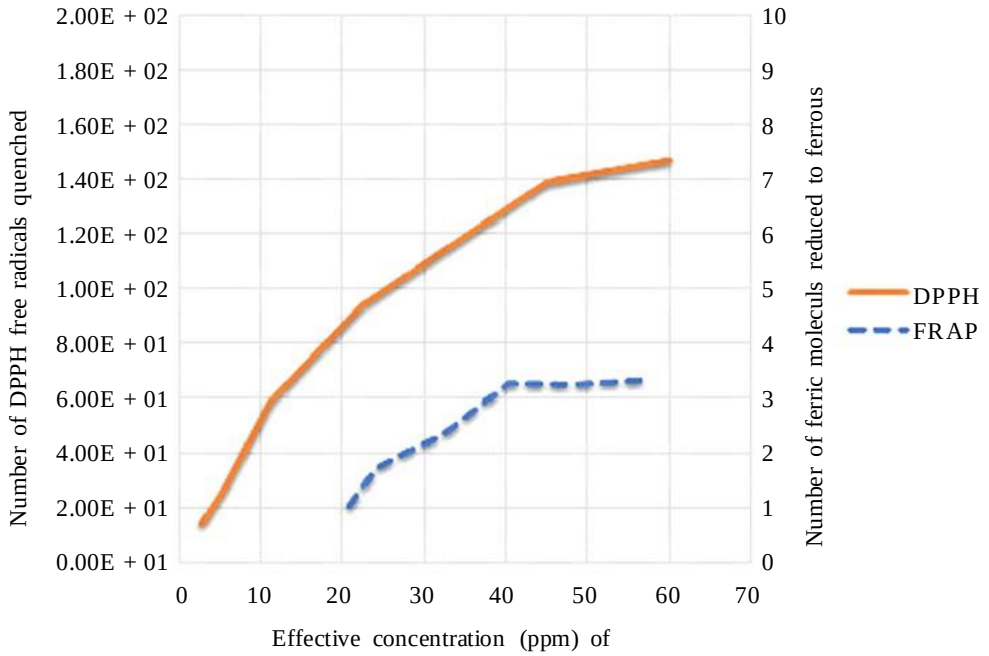

Figure 2. Relationship between effective concentrations of CFBAE and number of DPPH free radicals quenched and ferric molecules reduced to ferrous as revealed by DPPH and FRAP assays

its organic nitrogen free radicals by the phenolic
compounds contain in the cocoa fresh

basically was unable to reduce ferric into ferrous

CONCLUSIONS

Cocoa fresh beans aqueous extract (CFBAE) performed better in quenching the nitrogen organic radicals from DPPH assay if compared to its ability to reduce ferric to ferrous from FRAP assay. A normalized CFBAE concen- tration of 22.5 ppm and above was needed to quench more than 50% of the available free radicals in a 0.0003 mM DPPH solution and lost its quenching power at concentration lower than 2 ppm. However, to function as ferric reducer, higher concentration was needed as at 20

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