

Full Length Research Paper

Epicatechin content and antioxidant capacity of cocoa beans from four different countries

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Natural antioxidant has received more attention to be part of daily diet. Cocoa beans is one of the main sources of polyphenols especially epicatechin. This study was conducted to investigate the relationship between antioxidant potential and epicatechin content of raw cocoa beans from different countries, namely Malaysia, Ghana, Cote d'Ivoire and Sulawesi (Indonesia). Antioxidant potential was determined using trolox-equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP) assays. Reversed-phase high performance liquid chromatography (HPLC) was used to quantify the amount of epicatechin. The epicatechin content of raw cocoa beans was in the range of 270 - 1235 mg/100 g cocoa beans. Based on the two assays, Sulawesi beans exhibited the highest antioxidant capacity followed by Malaysian, Ghanaian and Cote d'Ivoirian beans for both extracts. Both ethanolic ($r = 0.92$) and water ($r = 0.90$) extracts of cocoa beans showed a significant positive and high correlation between epicatechin and TEAC value. Similarly, FRAP assay also showed a positive and high correlation with epicatechin for both ethanolic ($r = 0.84$) and water ($r = 0.79$) extracts. Results indicated that antioxidant capacity using two different antioxidant assays exhibited a positive and high correlation with epicatechin content in cocoa beans. Thus, epicatechin content in cocoa beans could be responsible for the antioxidant capacity.

Key words: Epicatechin, cocoa beans, antioxidant activity.

INTRODUCTION

An antioxidant can be defined as any chemical substance which, when present at a relatively low concentration in the body, can significantly delay or prevent oxidation of substrates (Halliwell and Gutteridge, 2000). There are common dietary antioxidants such as β -carotene, vitamin E, vitamin C and selenium. Besides these components, polyphenols, which can be found naturally in fruits (Allaith, 2008), vegetables (Amin et al., 2004), legumes and beverage (Dreosti, 2000), have also been reported to have antioxidant properties. Currently, natural antioxidants

have received more attention by nutrition scientists and biochemists for research compared to synthetic ones. Cook and Samman (1996) reported that many of the polyphenols exhibit a wide range of biological effects including antibacterial, antiviral, anti-inflammatory, anti-allergic, antithrombotic and vasodilatory actions. Flavonoid is one of main classes of polyphenols, which are powerful antioxidants.

Cocoa beans are one of the main sources of polyphenols especially (-)-epicatechin. Epicatechin have also been reported to have antioxidant capacity (Lee et al., 2003). Dreosti (2000) reported that 60% of the total phenolic compounds in raw cocoa beans is flavanol monomers (epicatechin and catechin) and procyanidins oligomers (dimer to decamer). Catechin and epicatechin

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were reported to be a potential candidate to scavenge free radicals present in our body and food systems (Sanbogi et al., 1998). *In vitro* studies demonstrated that these compounds have several biological activities such as the ability to scavenge superoxide anion and hydroxyl radicals, to reduce lipid peroxy radicals and inhibit lipid peroxidation (Salah et al., 1995). In addition, it has been reported that polyphenols are incorporated into low density lipoprotein (LDL) particles and thereby decrease their ability for being oxidised (Aviram and Fuhrman, 2003).

Cocoa (*Theobroma cacao* L.) is one of the important crops in the economics of several countries such as Ghana, Cote d'Ivoire, Nigeria and Indonesia. These countries are the main producers of raw cocoa in the world market. Most of cocoa beans produced from Southeast Asia are sold at a lower price compared to the West African and Ghanaian beans due to some weaknesses in its quality in terms of low cocoa aroma, high astringent and bitter taste. One of the factors, which could cause this, is believed to be due to the high amount of the phenolic substances. A study done by Natsume et al. (2000), reported that phenolic content in cocoa liquor varied depending on the countries of origins. Several studies showed correlations between phenolic content and antioxidant capacity (Velioglu et al., 1998). Low and moderate correlations were found between phenolic content and antioxidant capacity (Allaith, 2008). In addition, studies on the contribution of individual phenolic compounds in foods towards antioxidant capacity are still limited. It is interesting to know which type of cocoa polyphenols correlate high to antioxidant capacity. Therefore, this present study was aimed at determining the epicatechin content and antioxidant capacity of cocoa beans from different countries of origins. The contribution of this compound on antioxidant capacity was also determined.

MATERIALS AND METHODS

Samples

Dried cocoa beans were collected from Malaysia, Ghana, Cote d'Ivoire and Sulawesi (Indonesia), purchased from KL Kepong (Cocoa Products) Sdn. Bhd, Selangor, Malaysia. Cocoa beans from Malaysia, Ghana and Cote d'Ivoire are fully fermented while beans from Sulawesi are under-fermented beans.

Preparation of extracts

Cocoa beans for antioxidant assay were extracted according to the method of Velioglu et al. (1998). Ground cocoa cotyledons were extracted with distilled water or 70% aqueous ethanol (25 ml) for 120 min at 50°C using an orbital shaker (Unimax 1010, Heidolph, Germany).

The ratio between samples to extraction medium was 1 to 25 (w/v). The mixture was then filtered through a filter paper (Whatman No. 1) using a Buchner funnel. The filtrate was used for antioxidant assay. To prepare the sample for the determination of epicatechin, ground cocoa cotyledons (1 g) were extracted with 100 ml of acetone/water (75/25, v/v) for 120 min at room temperature using a

shaker. Following filtration, the filtrate was saturated with sodium chloride and allowed to separate into two liquid phases. The lower layer was discarded. The upper layer, which is acetone phase, was transferred into a round bottom flask and then evaporated to dryness using a rotary evaporator (Unimax 1010, Heidolph, Germany). The mixture was dissolved again with 15 ml of distilled water and washed four times with chloroform. The lower layer, which is chloroform phase, was discarded. The aqueous phase was filtered through a 0.45 µm nylon filter and then the filtrates were made up to 25 ml with methanol (HPLC grade) before HPLC analysis.

Trolox-equivalent antioxidant capacity (TEAC) assay

The TEAC assay was determined based on the reduction of the 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical cation by antioxidants (Re et al., 1999). ABTS was dissolved in distilled water to a 7 mM concentration (stock solution). The ABTS radical cation (ABTS^{•+}) was prepared by mixing ABTS stock solution with 2.45 mM potassium persulphate (K₂S₂O₈). This mixture has to remain for 12 - 24 h in the dark at room temperature before use. For measurements, the ABTS^{•+} solution was diluted with distilled water for water extracts and with ethanol for the ethanolic extracts to an absorbance of 0.7 ± 0.05 at 734 nm. Then, 2 ml of the ABTS^{•+} solution was pipetted into test tube. A total of 200 µl of cocoa extracts or Trolox (standard) was then added to the same test tube. The mixture was mixed for 45 s and measured immediately after 1 min. Absorbance was read at 734 nm using a spectrophotometer. The percentage of antioxidant activity of the cocoa extracts and standard was calculated by determining the decrease in absorbance based on the following equation:

$$\text{Percent (\%)} \text{ antioxidant activity} = \frac{(A_{(\text{ABTS}^{\bullet+})} - A_{(\text{Sample or Standard})})}{A_{(\text{ABTS}^{\bullet+})}} \times 100$$

Where $A_{(\text{ABTS}^{\bullet+})}$ = Absorbance (734 nm) radical cation without sample or standard, and $A_{(\text{Sample or Standard})}$ = Absorbance (734 nm) radical cation with sample or standard.

To calculate the TEAC value, the antioxidant activity (%) of cocoa extracts was compared to that of the antioxidant activity (%) of Trolox (standard). Results were expressed as µmol Trolox equivalents antioxidant capacity per 100g sample. The concentration of Trolox used was in the range of 1.5 - 4.5 µM.

Ferric reducing/ antioxidant power (FRAP) assay

FRAP assay was determined based on the reduction of Fe³⁺-TPTZ to a blue coloured Fe²⁺-TPTZ. The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ and 20 mM FeCl₃·6H₂O in a ratio of 10:1:1, and heated to 37°C. FRAP reagent (3 ml) was pipetted into test tubes. A total of 100 µl of sample and 300 µl of distilled water was then added to the same test tubes and incubated at 37°C for 4 min. Each sample was run in triplicates. Absorbance was read using a spectrophotometer at 593 nm. The FRAP value was calculated using the equation described by Benzie and Strain (1996). In the FRAP assay, the antioxidant potential of sample was determined based on a standard curve plotted using a FeSO₄·7H₂O at the concentration range between 200 - 1000 µM. Results were expressed as µmol Fe²⁺/100 g samples.

Determination of epicatechin content

The amount of epicatechin content was determined according to the method of Serra and Ventura (1997). A Hewlett Packard HPLC

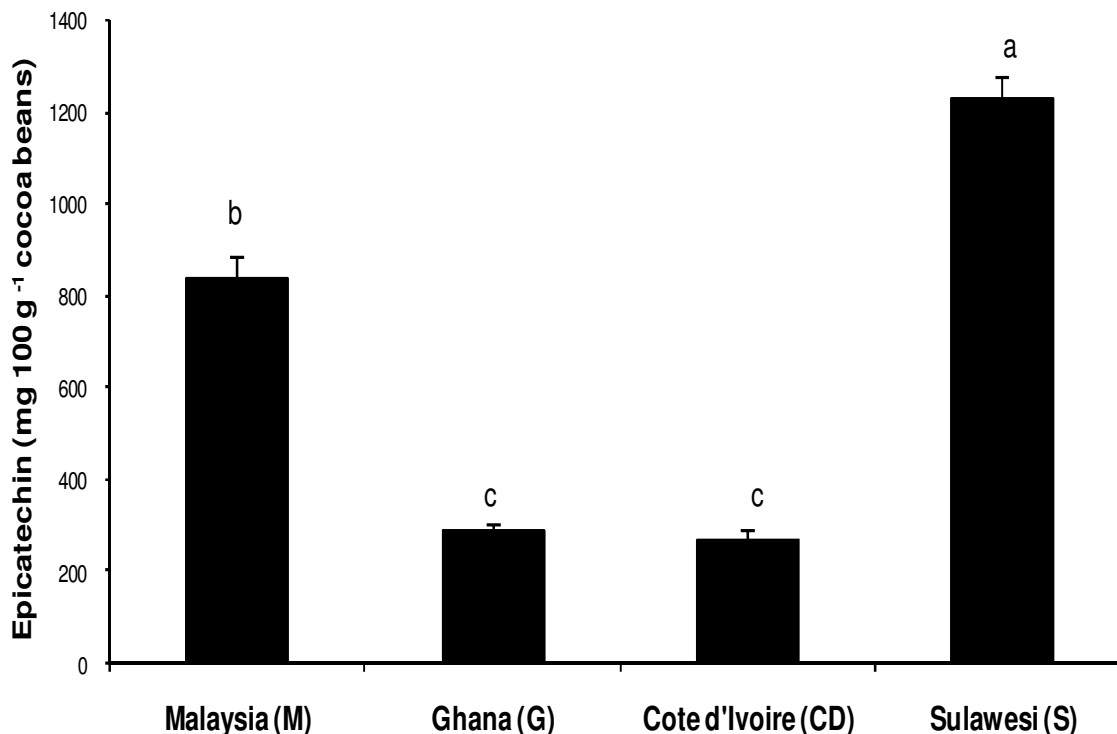


Figure 1. Epicatechin content of cocoa bean from different countries.

Series 1100, USA equipped with degasser, quaternary pump, auto-sampler and diode array detector (278 - 282 nm) was used. A Nucleosil C₁₈, 5 µm particle size and a 250 mm length x 4.6 mm I.D stainless steel column (Hewlett Packard, USA) was used. The parameters of HPLC condition for separation and identification of epicatechin were mobile phase (A) bidistilled water pH 2.6 (with H₃PO₄) and (B) methanol, flow rate (1 ml/min; 0 to 50% linear gradient of B for 16 min) and injection volume of 20 µl. The peak of epicatechin was identified with comparing the retention time of samples with that of standard epicatechin (Sigma, Co. Chemical, St. Louis, USA).

Statistical analysis

All data were expressed as mean ± standard deviation. Data were analyzed by one-way ANOVA using SPSS 11.5. Duncan's multiple-range test was used to assess differences between groups. A simple linear regression test was used to assess relationship between epicatechin content and antioxidant capacity. A significant difference was considered at the level of $p < 0.05$.

RESULTS

Epicatechin content

Epicatechin content of cocoa bean from different countries is presented in Figure 1. The chromatogram of epicatechin was identified by comparing the retention time with that of standard (Figure 2). The epicatechin content was in the range of 270 to 1235 mg/100 g cocoa beans. Sulawesi beans had exhibited the highest amount of epicatechin followed by Malaysian, Ghanaian and Cote d'Ivoirian beans. There was a significant difference ($p < 0.05$) between the epicatechin content of Sulawesi beans and other cocoa beans. ANOVA analysis showed a significant difference

($p < 0.05$) between Malaysian and Ghanaian beans. However, no significant difference between Cote d'Ivoirian and Ghanaian beans.

Trolox-equivalent antioxidant capacity (TEAC)

Antioxidant capacity test, two different extraction medium were used, namely water and ethanol. Table 1 shows the values of TEAC of cocoa beans extracts. The TEAC value ranged from 34.9 to 43.9 µmol TEAC/100 g cocoa beans for ethanolic extracts, while for water extracts the range was from 13.9 to 21.9 µmol TEAC/100g cocoa beans. The means of TEAC value for both extracts were in order of Sulawesi > Malaysia ≈ Ghana ≈ Cote d'Ivoire (44, 38, 36, 35% and 22, 17, 15, 14% for water and ethanolic extracts, respectively). In ethanolic extracts, Sulawesi beans exhibited the highest antioxidant activity followed by Malaysian, Ghanaian and Cote d'Ivoirian cocoa. Malaysian beans exhibited no significant difference with Ghanaian beans. In water extracts, Sulawesi beans showed significant ($p < 0.05$) highest antioxidant activity followed by Malaysian, Ghanaian and Cote d'Ivoirian ones.

Ferric reducing activity based on FRAP assay

Table 2 shows the ferric reducing activity of cocoa beans extracts based on FRAP assay. The reducing activity of ethanolic extracts followed the order of Sulawesi ≈ Malaysia > Ghana > Cote d'Ivoire. Ethanolic extract of Sulawesi beans exhibited the highest antioxidant potential among the extracts. However, there was no significant difference compared to Malaysian beans. A significant difference was revealed ($p < 0.05$) when Malaysian and Sulawesi cocoa beans were compared with Ghanaian and Cote d'Ivoirian beans. For water extracts, Sulawesi beans also exhibited the highest antioxidant potential followed by Ghanaian, Malaysian and Cote d'Ivoirian. The antioxidant potential of water extracts was in

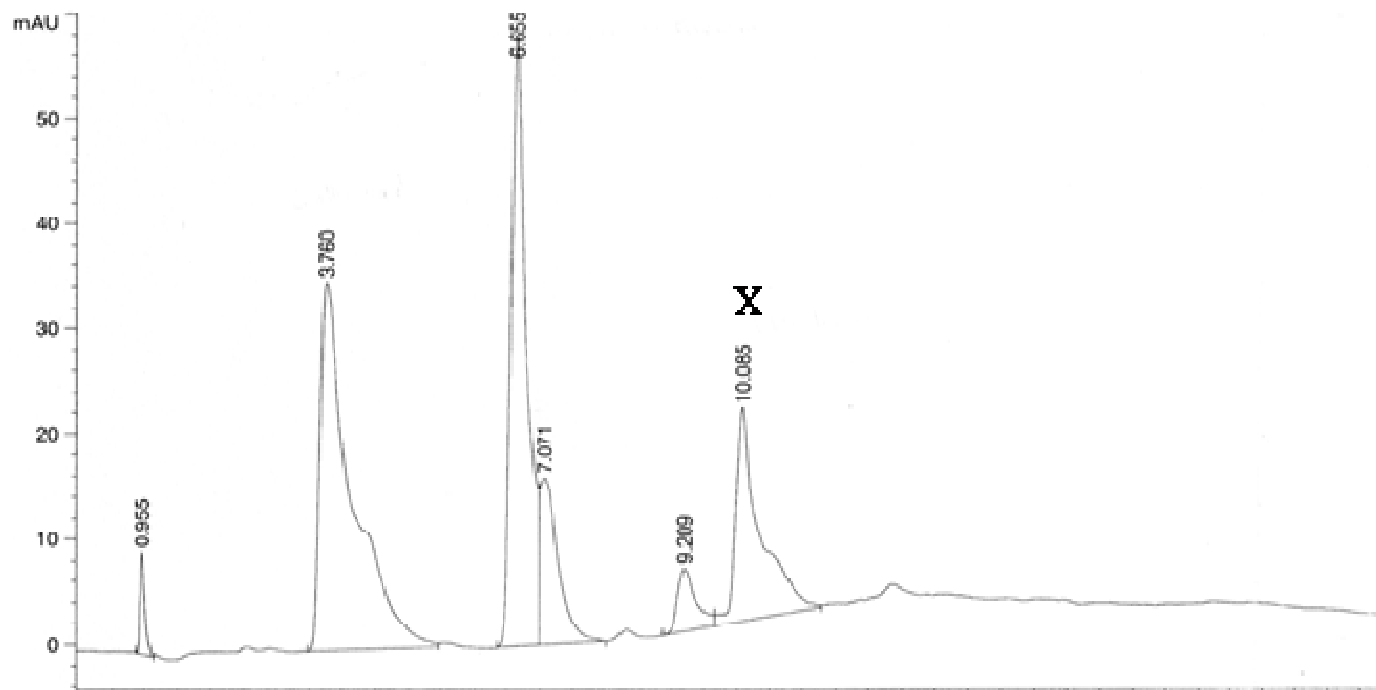


Figure 2. The chromatogram of epicatechin (X).

Table 1. Trolox equivalent antioxidant capacity (TEAC) in cocoa beans extracts.

| Countries of origin | TEAC (μmol Trolox equivalents/100 g cocoa beans extract) | |
|---------------------|--|-----------------------|
| | Ethanollic extracts | Water extracts |
| Malaysia | 38.10 ± 0.46^b | 16.73 ± 0.16^e |
| Ghana | 36.54 ± 1.95^{bc} | 15.35 ± 0.08^{ef} |
| Cote d'Ivoire | 34.98 ± 0.14^c | 13.98 ± 0.81^f |
| Sulawesi | 43.96 ± 0.05^a | 21.85 ± 2.49^d |

Concentration of sample was at 10 mg/ml. Values are expressed as means \pm standard deviation for $n = 3$. Means with different letters are significantly different (Duncan's multiple-range post hoc test, $p < 0.05$). Relative standard deviation was less than 11%.

Table 2. Antioxidant potentials of cocoa beans assayed by FRAP method.

| Countries of origin | FRAP (μmol Fe^{2+} equivalent/100 g cocoa beans extract) | |
|---------------------|---|--------------------|
| | Ethanollic extracts | Water extracts |
| Malaysia | 138.65 ± 6.19^a | 66.47 ± 0.54^d |
| Ghana | 113.64 ± 5.99^b | 68.84 ± 2.09^d |
| Cote d'Ivoire | 77.47 ± 0.82^c | 54.22 ± 0.95^e |
| Sulawesi | 143.37 ± 2.20^a | 80.21 ± 0.52^c |

Concentration of sample was at 10 mg/ml. Values are expressed as mean \pm standard deviation for $n = 3$. Means with different letters are significantly different (Duncan's multiple-range post hoc test, $p < 0.05$). Relative standard deviation was less than 5%.

the range of 66 to 80 μmol Fe^{2+} /100 g cocoa beans. All water extract of cocoa beans exhibited significant difference at $p < 0.05$ except between Ghanaian and Malaysian beans. ANOVA analysis

showed that ethanollic extracts of cocoa beans had significantly stronger ($p < 0.05$) reducing power than water extracts. This could be due to the high amount of reducing agents in ethanollic extracts

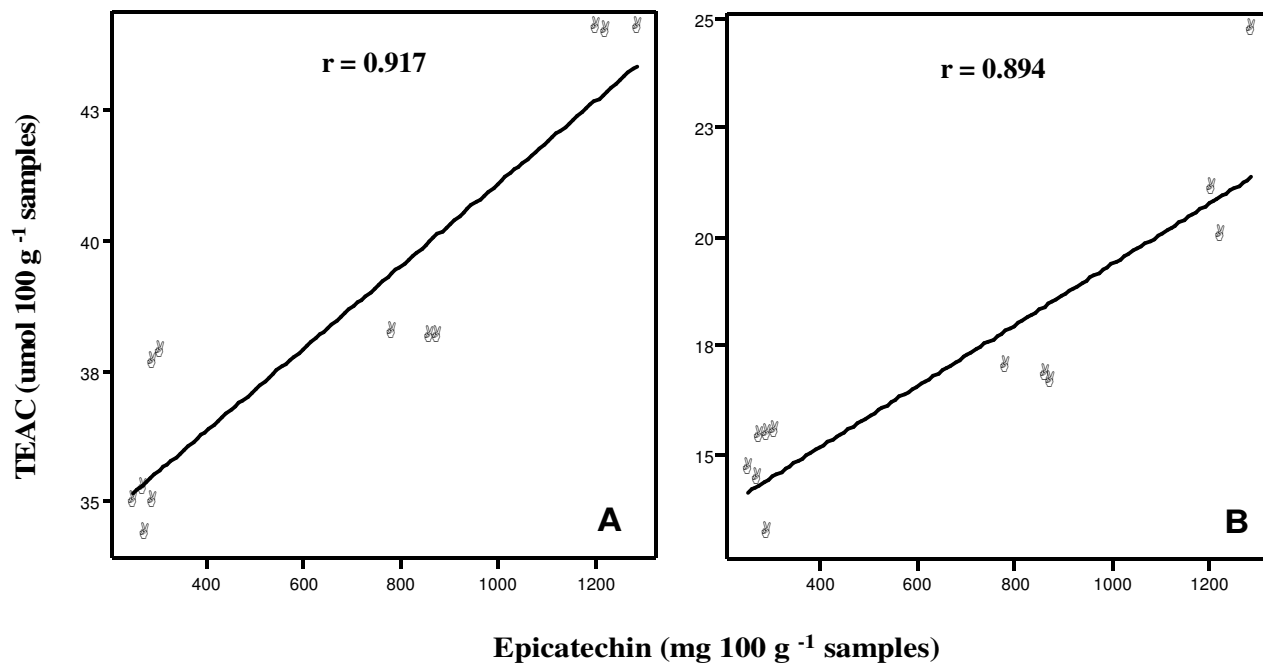


Figure 3. Correlation between TEAC and epicatechin content.

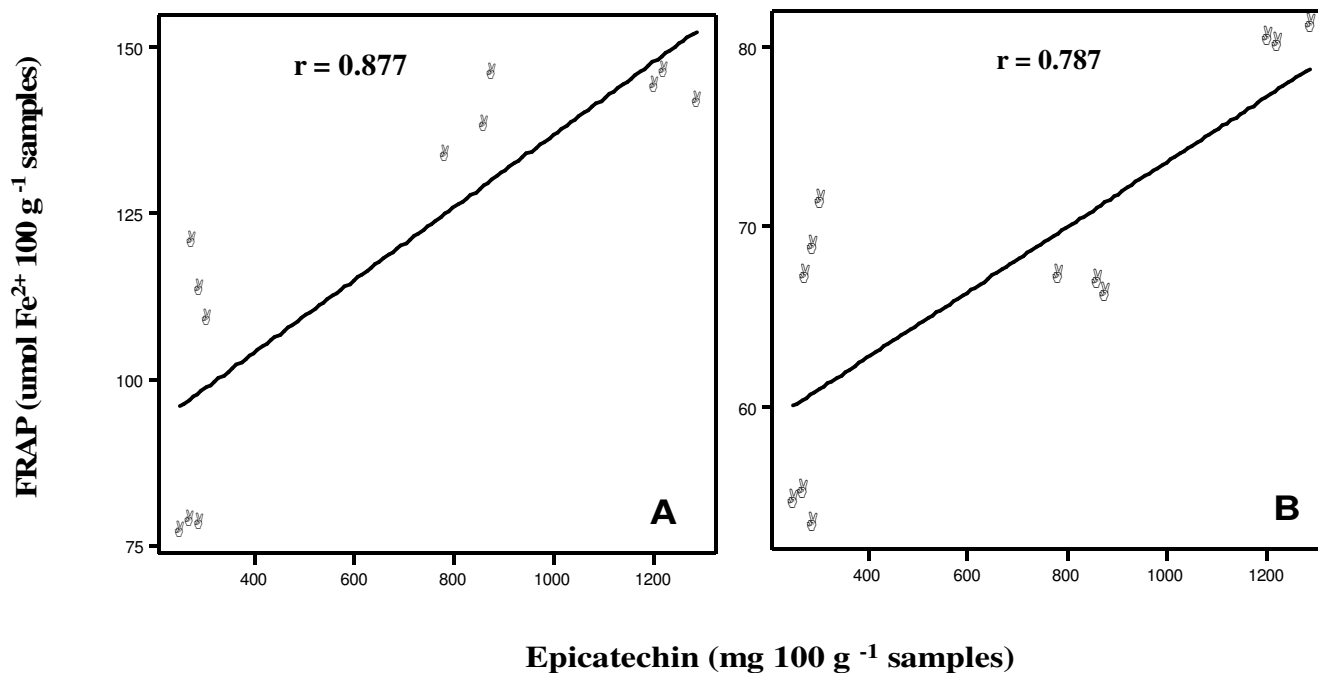


Figure 4. Correlation between FRAP and epicatechin content.

compared to water extracts.

Correlation between antioxidant capacity and epicatechin content

Ethanollic extracts of cocoa beans showed a positive and very high

correlation ($r = 0.918$) between epicatechin and TEAC value with a significant difference. Also, a positive and high correlation ($r = 0.895$) with a significant relationship was also found for the water extract (Figure 3). The present study showed a positive and high correlation between epicatechin content and antioxidant potential based on FRAP assay with a significant relationship for both ethanolic ($r = 0.837$) and water extracts ($r = 0.789$) (Figure 4).

DISCUSSION

Epicatechin content

Epicatechin is a major component of the polyphenols in cocoa beans and it is a monomer of procyanidins. It comprises approximately 35% of the total phenolic content in unfermented cocoa beans (Niemnak et al., 2006). Dreosti (2000) reported that 60% of the total phenolic in raw cocoa beans is flavanol monomers (epicatechin and catechin) and pro-cyanidins oligomers (dimer to decamer). According to Osakabe et al. (1998), the concentration of epicatechin in the cocoa beans extracts was about seven and ten-fold compared with cocoa liquor. Abbe and Amin, (2008) showed that cocoa beverages had the highest bound phenolic content compared to tea and coffee. In this study, Malaysian, Ghanaian and Cote d'Ivoirian beans contained lower epicatechin than Sulawesi beans. This could be due to the degree of fermentation as all the studied cocoa beans are fermented except for Sulawesi beans. Under-fermentation refers to the beans that are sun-dried without fermentation or fermented for only 1 - 2 days (Misnawi et al., 2002a). Fermentation process will reduce the polyphenol content through oxidation and exudation (Misnawi et al., 2002b). Polyphenol changes during cocoa fermentation and their impacts on astringency and bitterness (Wollgast and Anklam, 2000). A study done by Nazaruddin et al. (2006) revealed that unfermented Malaysian cocoa beans contained 1187 mg/100 g epicatechin, whereas, fermented cocoa beans contained only 985 mg/100 g. About 6 - 17% of epicatechin is degraded during fermentation process. However, fermentation is an important process for the development of the desirable colour and flavour in cocoa. A study has shown that unfermented beans do not develop any chocolate flavour when roasted, are excessively astringent and bitter. Our results showed Ghana and Cote d'Ivoire beans known to be good quality beans to contain much lower epicatechin content compared to Malaysia and Sulawesi.

Antioxidant capacity

In the present study, water and ethanol were used as extraction medium for extracting the antioxidant compounds from cocoa beans, in order to evaluate the antioxidant capacity using TEAC and FRAP assays. Sun and Ho (2005) showed the degree of antioxidant capacity and phenolics content were influenced by extracting solvents.

In this study, TEAC assay measures the ability of reducing cations by antioxidant compound and determines the decrease in absorbance. The net loss of cations is related to that from a standard antioxidant (Trolox). The result was expressed as TEAC $\mu\text{mol}/100\text{ g}$ cocoa beans. Reduction of $\text{ABTS}^{\cdot+}$ by a radical species

(R) ($\text{ABTS}^{\cdot+} + \text{RO}^{\cdot} \rightarrow \text{ABTS-H}$) or by an antioxidant (A) ($\text{ABTS}^{\cdot+} + \text{A} \rightarrow \text{ABTS-H} + \text{AO}^{\cdot}$) which causes a loss of absorbance at 734 nm (Cai et al., 2004). Water extracts exhibited lower antioxidant activity compared to ethanolic extracts. In this assay, two different solvent systems were used to dissolve the radical. For hydrophilic, the radical was dissolved in distilled water, whereas for lipophilic the radical was dissolved in ethanol. As a result, different antioxidant activity was obtained from the cocoa beans. This is an agreement with a study done by Schlesier et al. (2002), which found different antioxidant activity for TEAC by using different solvents for black currant, apple and tomato juice. Regardless of different extraction mediums, Sulawesi cocoa beans exhibited the highest TEAC compared to other studied beans. The results indicated that antioxidant compounds present in water and ethanolic extracts of Sulawesi beans, which strongly contributed to the antioxidant capacity, could be phenolics.

FRAP assay measures the reducing potential of the antioxidant to react with a ferric tripyridyltriazine (Fe^{3+} -TPTZ) complex to produce a coloured Fe^{2+} -TPTZ form at 593 nm at low pH (Benzie and Strain, 1996). The reducing properties are generally associated with the presence of reducing agents, which have been shown to exert antioxidant action by breaking the free radical chain through donating a hydrogen atom (Gordon, 1990). Some antioxidants such as glutathione (GSH) are not able to reduce Fe^{3+} . Therefore, this assay does not account for the SH-group of antioxidants, which is involved in quinone tanning process (Prior and Cao, 1999). Tannin is a group of polyphenolic phytochemicals, which is believed to be responsible for dietary astringency. Tannin is widely distributed in cocoa.

The reducing power of the studied extracts based on this assay supported the results obtained from TEAC assay. Sulawesi and Malaysian beans are well known to have a low cotyledon pH, while the Ghanaian has medium pH and Cote d'Ivoire has high pH (Jinap et al., 1995). Results shows that cocoa beans with a low pH which is Sulawesi beans exhibited the highest antioxidant potential based on FRAP assay. It indicated that pH of cotyledon may be influenced by the extraction medium. However, the pH values of cocoa beans extract showed were not so different. The pH of cocoa extract was in the range of 5.56 to 6.43. Thus, it showed that Sulawesi beans had the highest FRAP value not because of its acidity (low pH) cotyledon. Thus, it can be assumed that Sulawesi had the highest FRAP value may be due to fermentation factor. Sulawesi cocoa beans are under-fermented, while, other beans are fermented. The differences in phenolics content could be due to variety (Allaith, 2008), degree of fermentation (Misnawi et al., 2002a) and processing parameters (Adamson et al., 1999).

A number of studies have shown that the antioxidant capacity of fruits and vegetables is strongly correlated to

the phenolics content. Prior et al. (1998) found a high correlation between total phenolic concentration and antioxidant activity (ORAC) of different cultivars of *Vaccinium* species. Several studies found there is high correlation between total phenolic and antioxidant activity for cereals (Sun and Ho, 2005; Gorinstein et al., 2008). On the other hand, there are low or moderate correlations between total phenolic and antioxidant activity, whereby other compounds have been responsible for the antioxidant activity (Maillard and Berset, 1995; Bocco et al., 1998; Allaith, 2008). The antioxidant property of phenolics is mainly because of their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Rice-Evans et al., 1997). In this study, epicatechin content was tested for correlation with antioxidant capacity because, 60% of total phenolics in raw cocoa bean are flavanol monomers (epicatechin and catechin) and procyanidins oligomers (dimmer to decamer). The study found high correlation between the studied cocoa beans epicatechin and antioxidant capacity for both extracts. Skerget et al. (2005) showed that free forms of phenolic compounds (flavonoid aglycones) have potent antioxidant activity than their corresponding glycosides.

Conclusions

Antioxidant capacity based on two assays exhibited a positive and high correlation with epicatechin content of the studied commercial cocoa beans. Thus, epicatechin content in cocoa beans could be one of the candidates responsible to the antioxidant capacity.

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