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Research Article
In vitro Antioxidant Activity and Profile of Polyphenol Compounds Extracts and their Fractions on Cacao Beans

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Abstract

Background and Objective: The content of polyphenols in cacao beans can be modified during the processing of cacao. This study aimed to obtain the fraction of cacao bean extract polyphenols with the highest antioxidant activity and bioactive compounds profile of extracts and their fractions on cacao beans. **Materials and Methods:** The cacao beans (fermented for 5 days and unfermented) were blanched (5 min; 95°C), followed with defatted, freeze-dried and extracted uses 80% ethanol solvent. The extract obtained was then fractionated using n-hexane, chloroform, ethyl acetate, n-butanol and aqueous. Extracts and fractions obtained are calculated for yield, total polyphenol uses Folin-ciocalteu reagent, total flavonoid uses $AlCl_3$, antioxidant activity uses DPPH and FRAP methods, functional group uses fourier transform infrared spectroscopy (FTIR) and polyphenol compound profiles uses UHPLC-MS/MS. **Results:** The results showed that the aqueous fraction had the highest yield but lowest chemical content and antioxidant activity. The unfermented cacao beans extract undergoing fractionation using ethyl acetate showed polyphenol content, flavonoids, DPPH free radical scavenging activity and highest ferric reducing activity. The FTIR analysis showed that the cacao bean extract and its fractions had O-H, C-H, C=O, C=C and C-O-C functional groups. Cacao beans extracts and ethyl acetate fractions were dominated by procyanidin compounds, especially dimer B2. Cacao bean fermentation caused a decrease in procyanidin compounds (monomer to nonamer) and alkaloids (theobromine and caffeine). **Conclusion:** Unfermented cacao bean extraction is then followed by fractionation with ethyl acetate solvent, obtained the fraction with highest chemical and antioxidant activity.

Key words: Cacao beans, antioxidant activity, procyanidin compounds, ethyl acetate solvent, unfermented, polyphenol, UHPLC-MS/MS

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cacao is called *Theobroma cacao* L. in Latin. It means food for the gods since it offers some medicinal benefits. A number of studies showed that consumption of foods containing cacao is beneficial to health as it is a good source of antioxidants[1] has anti-inflammation agent[2], offers anti-atherogenic effect[3], lowers blood pressure, prevents cardiovascular diseases[4] and repairs sensitivity to insulin through improvement of β-cell[5]. These health benefits are related to the phytochemical content found in cacao beans.

The flavonoid polyphenols are the dominant compound in cacao beans. The most common group of polyphenols in cacao is procyanidin (flavan-3-ol). Procyanidin has various molecular weights ranging from monomers such as epicatechin catechins, gallocatechins and epigallocatechins to long chain polymers[6]. Procyanidin is responsible for the antioxidant activity of cacao beans. This activity can be related to its degree of polymerization[7]. Polyphenol antioxidant activity depends on the position and number of hydroxyl groups which can act as reducing agents, hydrogen donors and singlet oxygen quenchers[8]. Cacao polyphenol compounds are very complex and have other phenolic groups which can be beneficial for health too.

Polyphenol content in cacao beans varies greatly and depends on the variety and the way it is processed[9]. The fermentation, drying and roasting are the main causes of degradation of cacao bean polyphenols during processing. During epicatechin fermentation process, 10-20% dissolved polyphenol content is slowly reduced. This is not only due to the oxidation process but also because of the diffusion of polyphenols in fermented pulp liquid[10]. Blanching on cacao beans can reduce the enzyme activity of polyphenol oxidase. According to Menon *et al.*[11] and Tomas-Barberan *et al.*[12] optimal conditions where the lowest enzymatic browning on cacao beans occurs after blanching 90-95°C for 5 min. Besides, blanching can also increase antioxidant activity. According to Pujimulyani *et al.*[13] blanching using a combination of hot water and citric acid from white turmeric causes the hydrolysis of quercetin-3-rutinoside into quercetin with high antioxidant activity.

Antioxidant activity in cacao beans can be determined using several methods. However, every method differs in principle. The antioxidant activity analysis using DPPH method is based on the ability of compounds to act as free radical scavenging or hydrogen donors[14] whereas FRAP is based on the ability of compounds to reduce ferric ions[15]. Identification and quantification of procyanidin in cacao using different chromatographic techniques has been conducted[16-18]. But among the analysis methods, a combination of liquid chromatography (LC) coupled with mass spectrometry (MS) can be used to identify procyanidin in cacao products[18]. While the information related to the determination of polyphenols in cacao bean extract and its fraction using ultrahigh-pressure liquid chromatography coupled with tandem mass spectrometer (UHPLC-MS/MS) was still limited. This study focused on obtaining the fraction from cacao bean extract with functional compounds and highest antioxidant activity, as well as bioactive compounds profile which can be used as ingredients in food industry without changing the organoleptic properties of the added product.

MATERIALS AND METHODS

Location and total time duration of research: This experiment was conducted at Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta, Indonesia during February-November, 2018.

Materials: Fermented cacao beans and unfermented cacao beans of various clones are obtained from the cacao plantation "Sari Mulyo", Gunung Kidul, Yogyakarta, Indonesia. Fermentation of cacao beans is conducted spontaneously in a 5 day in a tiered box so that the comparison can be done. Chemicals are (grade analysis) obtained from Sigma Aldrich, Merck KGaA, Germany: (+)-catechin hydrate, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH),3,5-di- tert-butyl-4-hydroxytoluene (BHT), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), Folin-Ciocalteu reagent, iron (II) sulfate heptahydrate, hydrochloric acid, aluminum chloride hexahydrate, sodium hydroxide, sodium nitrate, ethanol, n-hexane, chloroform, ethyl acetate, n-butanol and deionized water.

Sample extraction: Cacao bean blanched at 95°C for 5 min, freeze-dried, defatted three times by extracting with of hexane (1:5) and each time, the supernatant was charged and the lipid-free solids were dried under nitrogen flux.

Sample extraction was carried out according to the procedure described by Adamson *et al.*[19]. About 50 g of defatted material was extracted with 250 mL (ethanol: water) of 80:20 (v/v) by stirring for 60 min, followed with ultra sonication process at 20°C for 10 min. The mixture is filtered to separate solids and solvents containing extracts. The solvent is evaporated using a rotary evaporator (RV06-ML, IKA®-Werke GmbH and Co. KG, Germany) 40°C and total solids dried with freeze dryer. The dried extract was used for

chemicals analysis and solvent fractionation. Fermented and unfermented cacao beans are blanched 95°C for 5 min, freeze-dried and defatted were designated as FCB and UFCB, respectively.

Solvent fractionation of cacao beans extract: The fractionation was carried out according to the procedure described by Abu *et al.*[20]. The UFCB extract is dissolved in water with a ratio of 1:20 (w/v) and fractionated in stages using a separating funnel with the following solvents: n-hexane, chloroform, ethyl acetate, n-butanol (water-saturated) and deionized water 1:1 (v/v), respectively. The extract fractionation is evaporated from the solvent using a rotary evaporator at (RV06-ML, IKA®-Werke GmbH and Co. KG, Germany) 40°C. The obtained concentrate is freeze-dried (Alpha 1-2 LD_{plus}, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) and cold room stored at -18°C. The dried fraction to be chemically analyzed later. Extract and fraction yield are calculated using equation:

$$\text{Extraction yield (\%)} = \frac{\text{Weight of dry soluble solid (g)}}{\text{Weight sample (g)}} \times 100$$

Determination of total polyphenol content: Total polyphenol content (TPC) of cacao extracts was determined spectrophotometrically according to the Folin-ciocalteu method[21] with slight modification. Briefly, 1 mL of samples, 0.75 mL of folin-ciocalteureagent (10%) and 0.75 mL of Na_2CO_3 (6% w/v) were homogenized and kept in the dark for 90 min. The absorbance of the samples was measured at 725 nm. The TPC concentrations were obtained from a standard curve of gallic acid (ranging from 10-100 μg mL^{-1}) and the results were expressed as milligrams of gallic acid equivalent (GAE) per g of dried extract or fraction of cacao beans (mg GAE g^{-1}).

Determination of total flavonoid content: Total flavonoid content (TFC) was determined based on the spectrophotometric method[22]. Briefly,1 mL of sample was added 0.3 mL of $NaNO_2$ (5%). After 5 min, 0.3 mL of $AlCl_3$ was added (10%) and kept for 6 min at room temperature. Finally, the mixture was added with 2 mL NaOH (1 M) and made up to 10 mL with deionized water. The sample kept for 15 min in a dark room. Absorbance was read at 510 nm using a UV-Vis spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific, USA). Catechin in the range of 10-100 μg mL^{-1} was used as the standard. The results were expressed as milligrams of catechin equivalent (CE) per g of dried extract or fraction of cacao beans (mg CE g^{-1}).

Determination of DPPH free radical scavenging activity: The radical scavenging activity of the cacao samples was estimated according to the method of Soussi *et al.*[23] with several modification. Briefly, 1 mL of sample added 2 mL of DPPH radical solvent (0.1 mM) dissolved in methanol and kept in a dark environment for 20 min at a room temperature. Methanol is used as a control instead of extract. Ascorbic acid and BHT are used as antioxidant references. The free radical scavenging activity measures its absorbance (A) at a wavelength of 517 nm using spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific, USA) according to the equation:

$$\text{Scavenging activity DPPH (\%)} = \frac{1 - A_{517}\ \text{in sample}}{A_{517}\ \text{in control}} \times 100$$

Antioxidant activity as free radical scavenging is expressed as IC_{50} showing the concentration of the extract which can provide inhibition of free radicals by 50%. IC_{50} is expressed in μg mL^{-1}.

Determination of ferric reducing activity: FRAP (ferric reducing antioxidant power) determination refers to the research by Di Mattia *et al.*[24] with several modifications. Briefly, 1 mL of extract is mixed with 0.5 mL of FRAP reagent and then 1.5 mL of deionized water is added into it and incubated at 37°C for 10 min. FRAP reagent is prepared by mixing 300 mM of acetate buffer (pH 3.6), 10 mM of TPTZ in 40 mM of HCl and 20 mM of $FeCl_3.6H_2O$ in 10:1:1 ratio. The sample absorbance was determined at 593 nm wavelength using spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific, USA). Antioxidant activity is calculated using standard curve of $FeSO_4.7H_2O$ and stated in mmol Fe^{2+} g^{-1} of dried extract or fraction of cacao bean (mmol Fe^{2+} g^{-1}).

Functional group analysis using FTIR-ATR: Fourier transform infrared spectroscopy (FTIR) analysis of cacao bean extract and its fraction refers to the research by Batista *et al.*[21] with several modifications. FTIR spectrum of cacao beans extract is analyzed using FTIR spectrophotometer (Nicolet iS10 FTIR spectrometer, ThermoFisher Scientific, USA) combined with tools from attenuated total reactance (ATR) and ZnSe reflection crystal. The FTIR analysis is done at a room temperature with 32 scans/sample at a wave number of 4000-400 cm^{-1} with 8 cm^{-1} resolutions.

Evaluation of polyphenol compounds of cacao bean using UHPLC-MS/MS: Identification of cacao bean polyphenol compound profile is conducted based on the research by

Table 1: Optimized SRM condition by UHPLC-MS/MS

Ion molecules	Charge	Q1 (m/z)	Q3 (m/z)
ESI$^-$			
Procyanidins			
(−)-Catechin	$[M-H]^-$	289	205
(+)-Epicatechin	$[M-H]^-$	289	179
(-)-Epicatechin-3-gallate	$[M-H]^-$	441	169
Dimer B2	$[M-H]^-$	577	289
Trimer	$[M-H]^-$	865	577
Tetramer	$[M-H]^-$	1153	865
Pentamer	$[M-H]^-$	1441	1028
Hexamer	$[M-H]^-$	1729	863
Heptamer	$[M-2H]^{-2/2}$	1008	865
Octamer	$[M-2H]^{-2/2}$	1152	875
Nonamer	$[M-2H]^{-2/2}$	1296	577
Flavones			
Quercetin-glucoside	$[M-H]^-$	463	300
ESI+			
Alkaloids			
Theobromine	$[M+H]^+$	181	163
Caffeine	$[M+H]^+$	195	138

Q1: Parent mass, Q3: Product mass

Table 2: Extraction yields of UFCB extract and its fractions

Extract or fractions	Yield (%)
Crude extract (ethanol 80%)	12.94
n-Hexane fraction	0.09
Chloroform fraction	2.20
Ethyl acetate fraction	19.48
n-Butanol fraction	23.73
Aqueous fraction	54.49

Ortega *et al.*[18] and Oracz *et al.*[25] with several modifications. Sample dissolved in methanol with a concentration of 400 ppm. The results of quantification of procyanidins and flavones expressed as milligrams of epicatechin equivalent (ECE) per gram of dried extract or fraction of cacao beans (mg ECE g^{-1}). Separation of phenolic compounds is done using UHPLC (Accela type 1250, Thermo Fisher Scientific, USA) equipped with tandem mass spectrometer (TSQ Quantum™ Access MAX Triple Quadrupole Mass Spectrometer, Thermo Fisher Scientific, USA) with an electrospray ionization source (ESI) to obtain MS/MS data. Data is controlled by TSQ Tune software. The MS is operated in negative mode to analyze flavonoid group compounds. While, the positive mode is used for the analysis of alkaloid group compounds (caffeine and theobromine). Data were obtained in selected reaction monitoring (SRM). Optimization of SRM conditions for analysis of procyanidins and alkaloids was shown in Table 1. Hypersil Gold column (50×2.1 mm×1.9 μm) is used for analysis. Column temperature is maintained at 30°C. Gradient elution is done through a mobile phase consisting of 0.1% formic acid solution (solvent A) and acetonitrile (solvent B) with a flow rate set to 0.3 mL min^{-1}. Linear gradient profile is applied as follows for solvents B a) negative electrospray ionization (ESI−): 0.0-0.6 min 5% B, 0.6-1.0 min 10% B, 4.0-5.5 min 25% B, 5.5-6.0 min 75% B, 7.5-9.5 min 70% B and 10.5-12.5 min 5% B and b) positive electrospray ionization (ESI+): 0.0-1.0 min 5% B, 1.5-2.0 min 10% B, 4.0-4.5 min 75% B, 5.0-6.5 min 5% B. Negative and positive electrospray ionization is applied to the analysis of capillary stress 2.5 and 3.0 kV, evaporation temperature 250°C; capillary temperature 300°C; nitrogen (40 psi) as gas pressure sheath and 10 psi of aux gas pressure with argon.

Statistical analysis: All data were expressed as mean±standard deviations. Data are analyzed using one-way ANOVA in Minitab 18. The Tukey's test is used to determine the difference between means. Pearson correlation (r) test is used to determine the correlation between averages. Significant differences are used at 95% confidence level.

RESULTS

Extract yield and fraction of cacao beans: The extraction yield of cacao beans and their fractions can be shown in Table 2. The UFCB extract obtained a yield of 12.94%. The multilevel fractionation using solvents on various polarities (n-hexane, chloroform, ethyl acetate fraction, n-butanol and aqueous) showed the highest yield was aqueous fraction (54.49%), followed by n-butanol fraction with yields of (23.73%) and n-ethyl acetate fractions with yields of (19.48%). However, when compared to the three fractions, the chloroform fraction showed a much lower yield (2.20%). Whereas the n-hexane fraction showed the lowest yield of 0.09%, thus the fraction was excluded from the next analysis.

TPC and TFC of cacao beans extract: The TPC and TFC of FCB and UFCB extract were shown in Fig. 1a. Cacao beans extract and their fractions had TPC in the range of 207.14-721.83 of mg GAE g^{-1} and TFC ranged from 82.54-447.57of mg CE g^{-1}. The fermentation process in cacao beans significantly ($p<0.05$) reduced TPC (316.87 mg GAE g^{-1}) and TFC (162.48 mg CE g^{-1}) by 17.06 and 27.45%, respectively, compared to that of the unfermented ones TPC (382.04 mg GAE g^{-1}) and TFC (223.96 mg CE g^{-1}). These results indicated that flavonoid compounds were more sensitive to degradation during the fermentation process than polyphenols.

The fractionation process had a significant effect on TPC and TFC ($p<0.05$). Fractionation results using solvents with different polarity from UFCB extract show that ethyl acetate fraction result in the highest, while the aqueous fraction showed the lowest TPC and TFC. The order of TPC and TFC

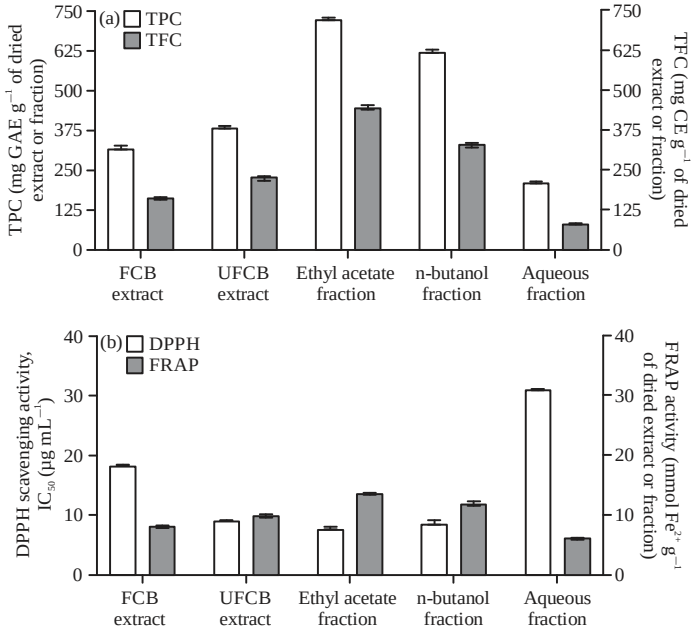

Fig. 1(a-b): (a) TPC and (b) TFC, DPPH free radical scavenging activity and FRAP antioxidant activity of FCB, UFCB extracts and its fractions

starts from the highest: Ethyl acetate fraction>n-butanol fraction>UFCB extract>FCB extract>aqueous fraction with TPC (mg GAE g^{-1}) as follows, respectively 721.83; 622.82; 382.04; 316.87; 207.15 and TFC (mg CE g^{-1}) as follows, respectively 447.57; 327.49; 223.96; 162.48; 82.54. It is suspected that most of the phenolic components in cacao beans are semi polar which are soluble in ethyl acetate. But on the contrary the aqueous fraction has the most polar solvent properties thus the phenolic component of the cacao bean extract can be slightly dissolved in the aqueous fraction.

Antioxidant activity of cacao bean extract and its fraction using DPPH and FRAP methods: DPPH free radical scavenging activity of UFCB extract and its fraction using ethyl acetate, n-butanol and aqueous solvent are shown in Fig. 1b. Radical scavenging activity varied with IC_{50} ranged from 7.74-30.94 µg mL^{-1} depending on the treatment of sample and used solvent (Fig. 1b). DPPH radical scavenging activity in ethyl acetate fraction>n-butanol fraction>UFCB extract>FCB extract>aqueous fraction with IC_{50} value (µg mL^{-1}) as follows, respectively: 7.74; 8.62; 9.07; 18.34; 30.94. IC_{50} in ethyl acetate fraction has slightly lower value than ascorbic acid (IC_{50}) 7.38 µg mL^{-1}. Meanwhile, BHT (IC50)17.03 µg mL^{-1} has slightly lower IC_{50} value than FCB extract. Figure 1 showed high polyphenol and flavonoid content tending to indicate strong DPPH radical scavenging activity. Total polyphenol and flavonoid content correlates strongly with DPPH radical scavenging activity with r correlation values, respectively, of -0.82 and -0.84 ($p<0.01$).

The fractionation of UFCB extract had a significant effect on FRAP antioxidant activity ($p<0.05$). The antioxidant activity of FRAP in cacao beans ranges from 6.09-13.59 mmol Fe^{2+} g^{-1}. The ethyl acetate fraction shows the highest antioxidant activity (13.59 mmol Fe^{2+} g^{-1}), followed n-butanol fraction (11.92 mmol Fe^{2+} g^{-1}) while the aqueous fraction shows the lowest activity 6.09 mmol Fe^{2+} g^{-1}. The FCB extract (8.09 mmol Fe^{2+} g^{-1}) can significantly reduce FRAP antioxidant activity ($p<0.05$) compared to UFCB extract (9.84 mmol Fe^{2+} g^{-1}). Total polyphenol and flavonoid content are strongly positively correlated to antioxidant activity of FRAP, with r correlation values of 0.98 and 0.99, respectively ($p<0.01$).

FTIR-ATR spectra of cacao beans: FTIR spectrum of FCB extract, UFCB extract and its fraction can be seen in Fig. 2. Figure 2 showed the functional group at the wave number range of 4000-400 cm^{-1} having an identical spectrum, which means that each samples has identical functional group. O-H stretches on cacao beans extract and fractions occur at 3155-3283 cm^{-1} wave number which shows very strong and wide absorption intensity. The FTIR spectrum showed that fermentation causes reduced functional groups. Fermentation

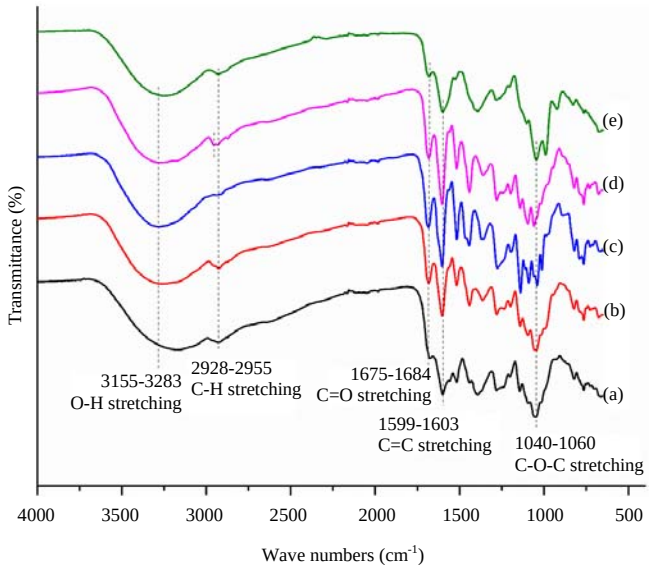

Fig. 2(a-e): Spectra of FTIR in (a) FCB extract, (b) UFCB extract, (c) Ethyl acetate fraction, (d) n-butanol fraction and (e) Aqueous fraction

(transmittance (%) of 73.82) reduces the amount of O-H, while it does not happen in unfermented cacao beans (transmittance (%) 72.58). The lower the transmittance (%), the higher the absorbance. The concentration of the O-H group is directly proportional to the absorbance. This data supports that fermented cacao bean extract is able to donate more hydrogen than fermented cacao bean extract. Thus, the DPPH free radical scavenging activity and ferric ion reduction activity in unfermented cacao bean extract is higher than in the fermented one. The aqueous fraction has fewer functional groups than others, thus it has low antioxidant activity.

The fermentation process shows the functional group shifts especially in the O-H functional group. Spectra at 1599-1603 cm^{-1} shows stretching of C=O from the carboxyl group which is supported by a very wide O-H absorption due to hydrogen bonding with the dimer. Unfermented cacao bean extract has the most O-H group and fermented cacao bean extract has the most C=O groups. This shows that alcohol oxidation O-H becomes aldehyde and carboxylic acid C=O during fermentation. The cacao bean extract and fraction also have the C-O-C group at 1040-1060 cm^{-1} wave number.

Evaluation of polyphenol compounds of cacao beans using UHPLC-MS/MS: Evaluation of polyphenols compounds of FCB, UFCB extracts and its fractions are shown in Fig. 3a. The UHPLC-MS/MS is used to evaluate the profile of polyphenol compounds on cacao beans extract and its fractions. Negative ionization mode is used to detect groups of procyanidin and flavonoid compounds (Fig. 3a), while ionization mode positive is used to detect alkaloid group compounds (Fig. 3b).

FCB and UFCB extracts indicate 11 procyanidin compounds, whereas ethyl acetate fraction indicates 9 groups of procyanidin compounds. Procyanidin in cacao beans extract and its fraction show results of monomers until nonamer. Procyanidin monomers and oligomers in FCB, UFCB extracts and ethyl acetate fractions are in the ranges of 0.11-10.96; 0.10-53.40 and 0.12-266.80 mg ECE g^{-1}, respectively. Dimer B2 showed $[M-H]^-$ ions at m/z 577 is the largest content in FCB extract, UFCB extract and ethyl acetate fractions, in the amount of 10.96, 53.40 and 266.80 mg ECE g^{-1}. Monomers such as epicatechin and catechins showed in the negative mode the same deprotonated molecular ion $[M-H]^-$ at m/z 289. These monomers detected in FCB, UFCB and ethyl acetate fractions. However, epicatechin-3-gallate showed $[M-H]^-$ ions at m/z 441 is only detected in FCB and UFCB extracts. Whereas oligomers, from dimer to nonamer are detected in both FCB and UFCB extracts and ethyl acetate fraction. However, pentamers is not detected in the ethyl acetate fraction. The concentration of dimer B2, trimer, tetramer, hexamer and quercetin-glucoside in UFCB extract is greater significantly than FCB extract ($p<0.05$). While the concentration of catechin, epicatechin, dimer B2, tetramer, nonamer and quercetin-glucoside in ethyl acetate fraction is greater significantly than UFCB extract ($p<0.05$). Quercetin-glucoside is a group of flavone compounds detected in cacao beans extract and its fraction. Quercetin-glucoside content in ethyl acetate fraction>FCB extract>UFCB extract.

The alkaloid groups detected in cacao beans include theobromine and caffeine compounds (Fig. 3b). In the extracts, theobromine and caffeine were identified by UHPLC-MS/MS in the positive mode (theobromine $[M + H]^+$m/z 181 and caffeine $[M + H]^+$ m/z 195). FCB extract, UFCB extract and ethyl acetate fraction contain theobromine and caffeine in the ranges of 2.88-4.68 and 0.32-0.91%, respectively. Theobromine and caffeine content of UFCB extract>FCB extract>ethyl acetate fraction. The ethyl acetate fraction has lower theobromine and caffeine contents compared to the crude extract. It is suspected that alkaloid compounds are polar, hence the semi polarity of the part of the compound dissolved in ethyl acetate solvent. The FCB extract decreases theobromine content of 31.87% and caffeine content of 34.36% from the UFCB extract.

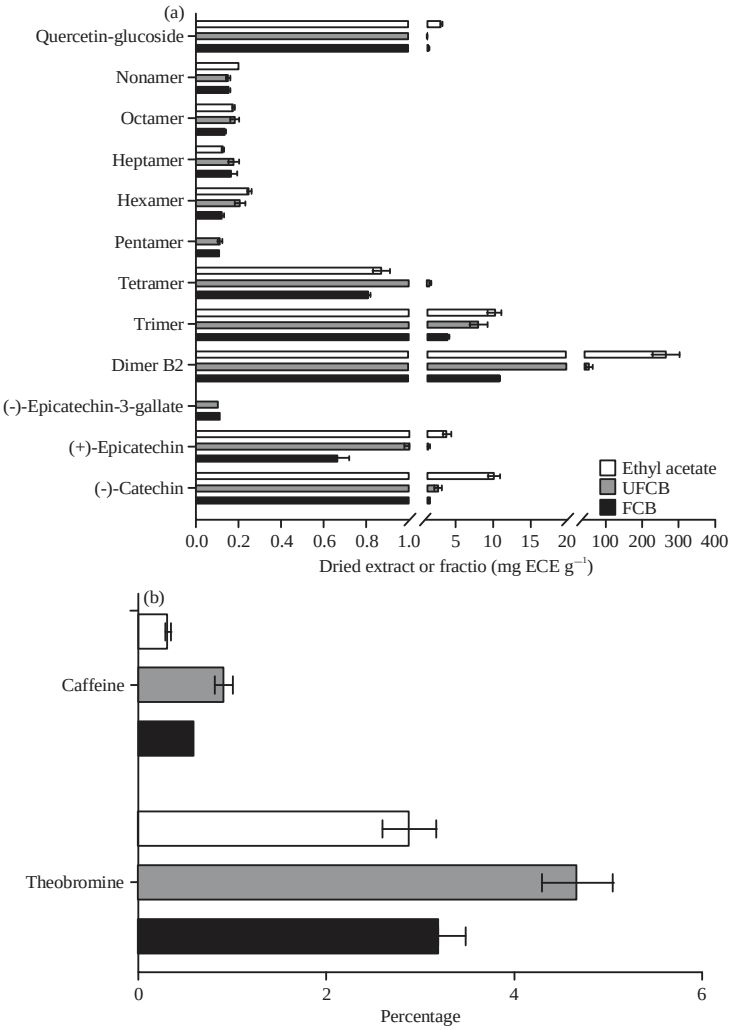

Fig. 3(a-b): Analysis of UHPLC-MS/MS (a) ESI− and (b) ESI+ in FCB extract, UFCB extract and its fraction (ethyl acetate fraction)

DISCUSSION

In this study, five fractions (n-hexane, chloroform, ethyl acetate, n-butanol and aqueous) were partitioned from 80% ethanol extract to evaluate the extraction yield of cacao beans (*Theobroma cacao* L.). The yield obtained in the study was higher than the yield of the extract of Sasa quelpaertensis Nakai 9.26% extracted with ethanol 80% in a research by Nakamura *et al.*[26]. This showed that most cacao beans extracts have semi polar and polar compounds. Another research is done by Nakamura *et al.*[26] on extract fractionation of Sasa quelpaertensis Nakai, showing hexane fractionation with the third highest yield in aqueous fraction and n-butanol of 24.20%. Aqueous has the highest polarity compared to n-butanol and ethyl acetate. The solvent polarity provides an important role in increasing the solubility of polyphenol compounds[27]. According to Stalikas[28], extraction results are significantly not only influenced by solvent polarity but also by other factors such as plant type and parts, storage temperature and duration, extraction methods and characteristics of compounds accumulated in plant tissues.

Blanching of the cacao beans aims to inactivate the polyphenol oxidase enzyme in the cacao beans. The activity of polyphenol oxidase is suspected to cause polyphenol degradation in cacao beans. According to Tomas-Barberan *et al.*[12], blanching using hot water at 95°C for 5 min is an optimal condition to inactivate PPO enzyme of cacao beans. Meanwhile, according to Menon *et al.*[11], cacao beans indicated optimal polyphenol recovery 119 mg GAE g^{-1} when blanched at 90°C for 5 min. Blanching is suspected to cause flavonoids in the form of glycosides to degrade into aglycones and sugars, thereby increasing antioxidant activity[13].

Polyphenol compounds in cacao beans are mostly dominated by flavonoid groups, consisting of ±58% proanthocyanidin group, ±37% flavan-3-ol/flavanol, ±4% anthocyanidin and ±1% flavonol glycoside[29]. Moreover, the monomeric flavan-3-ol compounds (+)-catechin, (-)-epicatechin and (–)-epicatechin gallate were quantified; they have antioxidant, anti-inflammatory, hypocholesterolemic and vasodilatory actions[30]. In general, polyphenol compounds in cacao beans after fermentation have decreased. According to De Brito *et al.*[31], the decrease in the content of polyphenols during fermentation is due to the oxidation by polyphenol oxidase enzymes, diffusion of polyphenols from cotyledons to the skin layer and polymerization of polyphenol compounds, especially epicatechin and proanthocyanidin to form tannin compounds and the formation of complexes with proteins and polysaccharides. According to Afoakwa *et al.*[32], polyphenol polymerization reaction and the formation of complexes with other compounds increase the solubility of polyphenols that are carried by the pulp fluid. Catechin, epicatechin and procyanidin trimer in cacao beans decrease during fermentation[33]. Procyanidin antioxidant activity is known to be higher than that of catechins and epicatechins because the number of O-H groups in procyanidin is greater than in the monomer[34]. Furthermore, the content of theobromine and caffeine also decreased after fermentation. These results are in accordance with Pelaez *et al.*[35] research which showed that during fermentation, the theobromine content in cacao is reduced between 21.33 and 29% and so is caffeine in the range 26-40.24%. The alkaloid content is generally higher on the first day of fermentation and gradually decreases as the result of fermentation[35]. Reduction in the content of theobromine may result in less bitter taste of cacao beans[36]. Alkaloids do not undergo chemical transformation during fermentation, but about 30% of alkaloids are lost due to diffusion and migration to the outer part of the beans during fermentation[37].

In this study defatting the cacao beans is done to reduce the fat in the cacao beans so that the extraction of polyphenols in cacao beans can become more optimal. Hu *et al.*[38] reported that defatted cacao beans indicates higher total polyphenols and flavonoids than the undefatted. This is because polyphenols and flavonoids are mainly located in non-fat cacao solids.

Solvent fractionation of cacao bean extract was carried out to separate polyphenol compounds in cacao beans based on their polarity. Most of the cacao bean polyphenols which have functional properties and high antioxidant activity dissolved in ethyl acetate solvents. This result is supported by the research by Hyun *et al.*[39] which showed that ethyl acetate fraction, crude extract and n-butanol fraction are more effective for obtaining phenolic compounds. Generally, lower polarity molecules tend to dissolve more effectively with lower polarity solvents[26]. It is possible for cacao beans with very low molecule polarity, while those of high molecule polarity may result in less.

The DPPH radical scavenging activity is stated as IC_{50} (μg mL^{-1}). The IC_{50} value is determined by the chart plotted for DPPH radical scavenging activity on the concentration of extract or fraction of cacao beans. IC_{50} value is defined as total antioxidant needed to lower initial concentration of DPPH radical for 50%. The smaller the IC_{50} value, the higher DPPH free radical scavenging activity. Kim and Lim[40] examined the antioxidant ability of all samples, mainly depending on the amount of total polyphenol content in each extract. According to Yokozawa *et al.*[41] reported the ability of scavenging of phenolic compounds for DPPH radicals to be closely related to their chemical structure. The presence of a hydroxyl group at position C-3 greatly influences the radical rinsing activity that is opposite to the C-5 position. The fraction that exhibits a strong DPPH antidote radical activity may contain great phenolic compounds, which are effective for capturing DPPH radicals. Antioxidant activity analysis using FRAP method is done to measure the antioxidant ability to react with the ferro-tripyridyltriazine complex (Fe^{3+}-TPTZ) to produce blue ferric-tripyridyltriazine (Fe^{2+}-TPTZ) which occurs at a low pH[42]. The higher the FRAP value, the greater the ability of reducing ferric ions which means that the antioxidant activity is higher. This result is in accordance with the research by Othman *et al.*[43], who reported the amount of cacao bean polyphenols in organic extracts was higher than water extracts. During the fermentation process, polyphenol compounds degradation occurs through oxidation by polyphenol oxidase enzymes, diffusion of polyphenols from cotyledons to the skin layer and polymerization of polyphenol compounds, especially epicatechin and proanthocyanidin to form tannin compounds and the formation of complexes with proteins and polysaccharides. The antioxidant activity of FRAP has the mechanism as a primary antioxidant. Primary antioxidants means the ability of compounds to donate hydrogen to stabilize radicals and the ability to reduce ferric ions by donating electrons to inhibit chain initiation and cut the chain propagation. Total polyphenol and flavonoid content are strongly correlated to antioxidant activity of DPPH and FRAP. In other words, the higher the total polyphenol and flavonoid content, the greater the antioxidant activity. According to Rice-Evans *et al.*[8], polyphenol compounds have oxidation and reduction (redox) properties which can function as reducing agents, hydrogen donors and quenching oxygen singlets which play an important role in determining antioxidant capacity.

FTIR-ATR analysis is used to determine functional group of FCB, UFCB extracts and its fraction, is done at a wave number of 4000-400 cm^{-1}. Vibration will occur when infrared radiation is absorbed by the bond in the molecule. This type of bond will absorb infrared radiation at different wave number. The hydroxyl group has a role in antioxidant activity. Antioxidant activity of polyphenols depends on the position and number of hydroxyl groups that can act as reducing agents, hydrogen donors and quenching oxygen singlets[8]. The position of O-H in the molecule determines the antioxidant activity of the compound. The O-H group bound to the position of C-3, C-4 and C-5 in B ring and O-H in the position of C-3 in C ring contributes to antioxidant activity. The loss of the O-H group in addition to this position does not significantly affect the antioxidant activity of the compound[44]. Changes in the number of O-H due to fermentation determined the changes of antioxidant activity, thus lower number of O-H lead to smaller antioxidative activity[45].

In food processing and storage, the polyphenols are unstable under various conditions such as presence of oxidative enzymes, high temperature conditions, pH, moisture, presence of light and oxygen[46]. The future research, encapsulation is an alternative process to increase the stability of susceptible compounds, protecting them from adverse environmental conditions.

CONCLUSION

This study focuses on obtaining the fraction of cacao beans extract with the highest chemical properties and antioxidant activity. Thus, the process modification needs to be done, with the unfermentation of cocoa beans followed by blanching process at 95°C for 5 minutes, freeze drying and defatting. Extraction using 80% ethanol is then followed by fractionation with ethyl acetate solvent and it results in the fraction with highest chemical and antioxidant activity. Polyphenol profiles in cacao beans extract and ethyl acetate fraction on ESI^- is dominated by groups of procyanidin compounds with dimer molecules and ESI+ is noted for the theobromine compounds.

SIGNIFICANCE STATEMENT

Evaluation cacao bean extract and its fractions as a source of natural antioxidants was carried out in this study. There have been many studies evaluating polyphenol compounds for human health, but effort to maintain polyphenols in cacao beans still gains less attention. Remembering the polyphenol content of cacao beans can be modified during processing, this study evaluated the chemical properties and antioxidant activity to produce fractions that have the highest antioxidant activity. The results showed that extraction of unfermented cacao beans followed by fractionation using ethyl acetate solvents produced the highest content of polyphenols, flavonoids and antioxidant activity. Most of the content of polyphenol compounds in cacao bean extract and ethyl acetate fraction are procyanidin dimer B2. Polyphenols are used as additives in food industry, food supplement and cosmetic.

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