PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

OPEN ACCESS Pakistan Journal of Nutrition

ISSN 1680-5194
DOI: 10.3923/pjn.2019.278.287

Research Article
Evaluation of Physicochemical Properties and Antioxidant Activity of Polyphenol-Rich Cacao Bean Extract Through Water Blanching

[1,2]Rossi Indiarto, [2]Yudi Pranoto, [2]Umar Santoso and [2]Supriyanto

[1]Department of Food Industrial Technology, Faculty of Agro-Industrial Technology, Universitas Padjadjaran, Jl. Raya Bandung-Sumedang Km. 21, Jatinangor, Sumedang 40600, Indonesia
[2]Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Jl. Flora No. 1 Bulaksumur, Yogyakarta 55281, Indonesia

Abstract

Background and Objective: The activity of the polyphenol oxidase enzyme during fermentation and drying causes a decrease in the polyphenol and flavonoid content of cacao beans. Blanching is important to inactivate the enzyme. This study aimed to evaluate the physicochemical properties and antioxidant activity of cacao bean extract in order to obtain a polyphenol-rich cacao extract. **Materials and Methods:** Unfermented and fermented cacao beans were blanched using water at 95°C ±2°C for 1, 3, 5 and 7 min. The process was then followed with drying and defatting using n-hexane and completed with extraction of polyphenol compounds using 80% ethanol. The blanched cacao beans were analyzed in terms of polyphenol oxidase enzyme activity and color, while the extracts of dried cacao beans were analyzed in terms of the relative activity of polyphenol oxidase, color, total polyphenol content, total flavonoid content, radical scavenging activity using DPPH, ferrous ion (Fe^{2+}) chelating activity and the presence of functional groups using fourier transform infrared (FTIR). **Results:** Blanching unfermented cacao beans at 95°C for 5 min reduced the relative activity of polyphenol oxidase by >99%, maintained the purple color of the unfermented cacao beans and produced the highest content of polyphenols and flavonoids. Five minute blanching also increased antioxidant activity compared to the activity of unblanched cacao beans. The process of fermentation and hot air drying had a significant effect ($p<0.05$) on the decrease of polyphenols and the associated DPPH antioxidant activity in cacao beans. However, there was no effect ($p>0.05$) on Fe^{2+} chelating activity in unblanched cacao beans. Total polyphenol and flavonoid contents were strongly correlated with DPPH antioxidant activity but were not correlated with Fe^{2+} chelating activity. **Conclusion:** Blanching unfermented cacao beans with hot water for 5 min at 95°C was shown to increase the free radical scavenging activity by deactivating the polyphenol oxidase enzyme and thereby increasing the total polyphenol content.

Key words: Antioxidant rich food, cacao beans, flavonoids compound, polyphenols, water blanching

Received: September 22, 2018 Accepted: December 11, 2018 Published: February 15, 2019

Citation: Rossi Indiarto, Yudi Pranoto, Umar Santoso and Supriyanto 2019. Evaluation of physicochemical properties and antioxidant activity of polyphenol-rich cacao bean extract through water blanching. Pak. J. Nutr., 18: 278-287.

Corresponding Author: Supriyanto, Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Jl. Flora No.1 Bulaksumur, Yogyakarta 55281, Indonesia Tel: +62-274-549650 Fax: +62-274-549650

Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Cacao products are known as one of the antioxidant-rich foodstuffs. The antioxidant potential of cacao is even greater than that of tea products and some fruits, which are also known as natural sources of antioxidants[1]. Flavonoids are a group of polyphenols that are widely found in cacao beans. The polyphenol content of cacao beans and cacao products is believed to make important contributions to the maintenance of health, since polyphenols can act as a source of antioxidants[2] and can have anticancer[3], antidiabetic[4], antihypertension[5], anti-inflammatory[3] and stress relief[6] effects, as well as strengthen resistance to hemolysis[7], nourish the heart[2] and act as an aphrodisiac[8].

Flavan-3-ols are flavonoid compounds found in cacao that consist of catechin and epicatechin monomers and the procyanidin dimer B2[3]. The content of these compounds is important since most studies report that the bioavailability of polyphenols in cacao is closely related to molecular size; generally polyphenols that are smaller in size are more beneficial. The compounds with low molecular weight are found in high concentrations in the blood and have a better ability to reach the target organs in the body[9]. Therefore, the higher the amount of monomers and dimers in cacao products, the greater the health benefits that can be obtained from these products.

During cacao processing steps, such as fermentation, drying and roasting, degradation of polyphenols occurs, especially of flavan-3-ol enantiomers, namely, epicatechin compounds. Epicatechin concentrations ranging from 36.4-43.2 mg are found in fresh cacao beans dried by freeze drying. However, during cacao processing, there is a significant degradation of epicatechin and catechin compounds[10]. This degradation is due to the presence of the polyphenol oxidase enzyme in cacao beans and its chemical byproducts are the precursors to further enzymatic browning reactions. In the aerobic fermentation process of cacao beans, epicatechin, catechin and anthocyanidin are oxidized and polymerized by the polyphenol oxidase enzyme[11].

Blanching of cacao beans can reduce the activity of the polyphenol oxidase enzyme, which plays a role in helping to recover the total polyphenol content of cacao beans after drying. Time is a critical factor in the blanching process. The proper blanching period results in processed products that are of good quality. During the blanching process, hot water is used since it does not react with the cacao beans and it deactivates the polyphenol oxidase enzyme. Several studies have focused on efforts to reduce the polymerization activity of the polyphenol oxidase (PPO) enzyme. For some agricultural commodities, study results show that blanching, in addition to reducing the enzyme's activity, can also increase the commodities' total polyphenol content and antioxidant activity, as is the case in white turmeric[12]. Tomas-Barberan *et al.*[13] and Menon *et al.*[14] blanched cacao beans and determined that the optimal conditions where the lowest enzymatic browning of cacao beans was observed were 5 min of blanching at 90-95°C. Nurhayati *et al.*[15] also reported that the use of 600 W microwaves for 180 sec can inactivate the PPO enzymes in cacao beans. Thus, the polyphenol content, especially flavanol content, in cacao beans, is very dependent on the handling and processing of the cacao beans. Therefore, efforts to prevent the degradation of polyphenols in cacao beans need to be made by using water blanching for the appropriate length of time.

This study aimed to evaluate the effect of water blanching on the physicochemical properties and antioxidant activity of cacao beans so that the optimal conditions are obtained to produce cacao bean extract that is rich in polyphenols and has the highest possible antioxidant activity.

MATERIALS AND METHODS

Raw materials: Unfermented (UFCB) and fermented (FCB) cacao beans from combined clones were obtained from cacao plantations "Sari Mulyo" in Gunungkidul, Yogyakarta, Indonesia. Fermentation was carried out spontaneously using a stratified fermentation box for 5 days. The cacao beans were obtained from pods that were ripe and optimally marked by yellow or reddish-yellow color.

Chemicals and reagents: Folin-Ciocalteu reagent, DPPH (2,2-diphenyl-1-picrylhydrazyl), ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p, p -disulfonic acid monosodium salt hydrate), EDTA (ethylenediaminetetraacetic acid), ascorbic acid, (+)-catechin hydrate, catechol, gallic acid, iron (II) sulfate heptahydrate, 98% iron (III) chloride hexahydrate, hydrochloric acid, aluminum chloride hexahydrate, sodium hydroxide, ethanol and n-hexane were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals were of analytical grade.

Sample preparation: One kg of cacao beans was depulped and then blanched at 95°C±2°C. Blanched cacao beans were placed immediately in 4°C cold water for 10 min and then drained and peeled. The peeled cacao beans were dried (hot air and freeze-drying), crushed and sieved at size 40 mesh (0.420 mm). Freeze drying (FD), using an Alpha 1-2 LD^{plus}, Martin Christ Gefriertrocknungsanlagen GmbH, Germany was conducted at a temperature of -45°C for 48 h, 0.0025 bar. Hot air drying (HD) was carried out using a cabinet dryer at 50°C for 36 h. The dry cacao powder was used for extraction.

Extraction of cacao beans: The dry cacao powder was defatted with hexane (1:5) 3 times, the supernatant was removed and the cacao solids were dried with nitrogen. Sample extraction was carried out according to methods previously described by Ioannone *et al.*[16] with several modifications. A total of ±50 g of defatted dried cacao powder was extracted using 250 mL of 80% ethanol (v/v), stirred with a magnetic stirrer for 1 h and followed by ultrasonication using ice water as medium for 15 min. The extraction process was repeated 3 times. The mixture was filtered to separate the solids from the solvent containing the extracts. The solvent was evaporated using a rotary evaporator at 40°C (RV06-ML, IKA®-Werke GmbH and Co. KG, Germany). The obtained concentrate was dried with a freeze dryer and the dried extract was stored at -18°C for subsequent chemical analysis.

Enzyme extraction and determination of polyphenol oxidase enzyme activity: The polyphenol oxidase (PPO) enzyme of cacao beans was extracted based on the methods described by Tribst *et al.*[17] with some modifications. Forty mL of phosphate buffer solution (0.1 M, pH 6.5, 4°C) was added to 20 g of cacao beans. The mixture was homogenized for 3 min with an homogenizer (Ultra Turrax® T 50 basic homogenizer, IKA®-Werke GmbH and Co. KG, Germany). The mixture was centrifuged at 15000 g for 20 min. All steps were carried out at a temperature of 4°C. The supernatant was collected in a dark bottle and stored in a refrigerator at 4°C as a crude enzyme extract for later analysis of the PPO enzyme activity. The PPO activity was determined using a UV-Vis spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific, USA) at a wavelength of 410 nm. The phosphate buffer (0.1 M, pH 6.5) and catechol (0.2 M) solutions were incubated (room temperature) for 10 min before use. Then, 1.5 mL of the phosphate buffer and 1 mL of the catechol solution were combined and 0.5 mL of the crude PPO extract was added to the reaction mixture. The phosphate buffer solution was used as a blank for enzyme extracts. The absorbance was measured every 30 sec for 3 min. One unit of enzyme activity (U) was expressed as the number of enzymes causing an increase in the absorbance of 0.001 per minute according to the test conditions and is based on the following equation[18]:

$$\text{Enzyme activity (U)} = \frac{\text{Slope of line} \times \text{dilution factor}}{0.001} \quad (1)$$

A control sample (unblanched) was used for comparison. Relative enzyme activity (REA) was calculated as a percent by dividing the activity of the blanched samples by the activity of the control and multiplying the quotient by 100 and is based on the following equation[17]:

$$\text{REA (\%)} = \frac{\text{Enzyme activity of blanched sample}}{\text{Enzyme activity control}} \times 100 \quad (2)$$

Color analysis: The color of the cacao beans before and after blanching was determined using a Chroma Meter (CR-400, Konika Minolta, Japan) according to the methods described by Rawson *et al.*[19] with slight modification. Color is given by the Hunter Lab unit L* (lightness), a* and b*. In addition, the chroma and total color difference (TCD) were calculated using the following equations[17]:

$$\text{Chroma} = \sqrt{a^{*2} + b^{*2}} \quad (3)$$

$$\text{TCD} = \sqrt{(L^* - L_0)^2 + (a^* - a_0)^2 + (b^* - b_0)^2} \quad (4)$$

where, L_0, a_0 and b_0 are the control values (unblanched). TCD shows the value of the color difference between the blanched sample and the unblanched sample. Color differences were classified as very different (TCD>3), different (1.5<TCD<3) and small difference (TCD<1.5)[20]. Importantly, the combination of color parameters was more effective for the evaluation of the total color difference induced by processing than were the individual parameters of L*, a*, b*.

Total polyphenol content: The total polyphenol content (TPC) was determined according to the Folin-Ciocalteu colorimetric method as described in Tamaroh *et al.*[21] with several modifications. Briefly, 0.75 mL of the Folin-Ciocalteu's reagent (10%) was added to 1 mL of the cacao extract and allowed to stand for 5 min at room temperature. Then, 0.75 mL of the sodium carbonate solution (6%) was added to the solution mixture and allowed to stand for 90 min. The absorbance was measured at a wavelength of 725 nm using a UV-Vis spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific, USA). The absorbance of the sample was compared with the standard curve of the gallic acid concentration. The results were expressed in milligrams of gallic acid equivalent (GAE) per gram of dried extract of cacao beans.

Total flavonoid content: The total flavonoid content (TFC) was determined based on the spectrophotometric method[22]. Briefly, 0.3 mL of $NaNO_2$ (5%) was added to 1 mL of the cacao extract. After 5 min, 0.3 mL of $AlCl_3$ (10%) was added to the mixture and allowed to stand for 6 min at room temperature.

Finally, the mixture was added to 2 mL NaOH (1 M) and brought up to 10 mL with ion-free water. The sample was left in a dark room for 15 min. The absorbance of the sample was measured at a wavelength of 510 nm using a UV-Vis spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific, USA). The results were expressed in milligrams of catechin equivalent (CE) per gram of dried extract of cacao beans.

DPPH free radical scavenging activity: The DPPH radical scavenging of samples was tested using methanol solvents based on methods described by Utami *et al.*[23] with minor modifications. A total of 1 mL of the cacao extract solution in methanol was added to 2 mL of the DPPH solution (0.01 mM). The solution mixture was homogenized and allowed to stand for 30 min in a dark room. The absorbance was measured at a wavelength of 517 nm using a UV-Vis spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific, USA). A control sample was prepared with 1 mL of methanol. Ascorbic acid and BHT were used as positive controls. Antioxidant activity was calculated as the percentage of DPPH color removal using the following equation[24]:

$$\text{Scavenging activity DPPH}(\%)=\frac{1-\text{absorbance of sample at 517 nm}}{\text{Absorbance of control at 517 nm}}\times 100 \quad (5)$$

The IC_{50} value was determined from a graph plotting the concentration of cacao bean extract on the x-axis and % of DPPH radical scavenging activity on the y-axis. IC_{50} is defined as the total antioxidant needed to reduce the radical concentration to 50% DPPH. The measurement was performed three times and the effect of DPPH radical scavenging activity was calculated based on the percentage of radicals caught by DPPH.

Fe^{2+} ion chelating activity: The Fe^{2+} ion chelating activity was determined by the methods used by Chew *et al.*[25] with slight modification. Cacao bean extract (1 mL) was mixed with 1 mL of $FeCl_2.4H_2O$ (0.1 mM) and 1 mL of ferrozine (0.25 mM). The mixture was incubated for 10 min before absorbance was measured at 562 nm using a UV-Vis spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific, USA). The absorption of the control was measured by replacing the sample with methanol. EDTA was used as a standard compound. The inhibition percentage of ferrozine-Fe^{2+} complex formation in the cacao extract was calculated using the following equation[26]:

$$Fe^{2+}\text{ chelating activity}(\%)=\frac{1-\text{absorbance of sample at 562 nm}}{\text{Absorbance of control at 562 nm}}\times 100 \quad (6)$$

FTIR analysis: The fourier transform infrared spectroscopy (FTIR) analysis of the cacao bean extracts was based on the methods described by Sugiyanti *et al.*[27] with little modification. The FTIR spectra of the cacao bean extracts were analyzed using an FTIR spectrophotometer (Nicolet iS10 FTIR spectrometer, Thermo Fisher Scientific, USA), which was furnished with additional attenuated total reactance (ATR) equipment, including the ZnSe reflection crystal. The FTIR analysis was carried out at room temperature with 32 scans/samples in the wavenumber range of 4000-400 cm^{-1} with 8 cm^{-1} resolution.

Statistical analysis: The data were presented as the mean values±standard deviations and was analyzed using one-way ANOVA at a 95% confidence level. Further differences between the means were analyzed using Tukey's test. Correlation testing between variables was conducted using Pearson's correlation coefficient (r).

RESULTS AND DISCUSSION

Effect of blanching time on the REA: The REA of PPO in UFCB following various blanching durations is shown in Table 1. Increasing the blanching time had a significant effect ($p<0.05$) on the relative activity of PPO (Table 1). PPO activity decreased with increasing blanching time. The activity of PPO decreased sharply from 41.34-0.78% in the UFCB that were blanched for 1-5 min. The PPO activities of UFCB that were blanched for 5 and 7 min were not significantly different ($p<0.05$) and blanching in both cases reduced enzyme activity >99%. Menon *et al.*[14] and Tomas-Barberan *et al.*[13] studies showed that the optimum conditions for PPO inactivation in cacao beans were blanching at 90-95°C for 5 min. Blanching at high temperatures (more than 90°C) was assumed to cause thermal degradation of polyphenol compounds.

Effect of blanching time on color attributes of cacao beans: The color attributes consisting of L*, chroma and TCD of UFCB that were both unblanched and blanched for 1, 3, 5 and 7 min were studied (Table 1). Increasing the blanching time had a significant effect ($p<0.05$) on the values of L*, chroma and TCD. The L* value in the UFCB ranged from 22.42-34.00. The UFCB blanched for 5 and 7 min had the highest L* value among other treatments but those blanched for 5 min had a

Table 1: The effect of blanching time on the REA of PPO, L*, chroma and TCD in UFCB

Blanching time (min.)	REA (%)	L*	Chroma	TCD
0	100.00	22.42±0.83^{d}	11.50±0.68^{b}	-
1	41.34±0.87^{a}	26.99±0.62^{c}	13.95±0.92^{a}	5.83±0.61^{c}
3	3.43±0.04^{b}	28.76±1.05^{bc}	13.97±0.86^{a}	11.90±1.94^{b}
5	0.78±0.15^{c}	30.90±2.27^{ab}	14.02±0.18^{a}	14.04±1.78^{ab}
7	0.55±0.09^{c}	34.00±1.49^{a}	13.77±0.28^{a}	16.22±0.85^{a}

Values followed by different lowercase letters in the same column show significant differences ($p<0.05$). Values are presented as an average±standard deviation from triplicate replications. REA: Relative enzyme activity, L*: Lightness, TCD: Total color difference, UFCB: Unfermented cacao beans, PPO: Polyphenol oxidase

slightly lower L* value than those blanched for 7 min. Meanwhile, the UFCB blanched for 3 min had slightly lower L* values than those blanched for 5 min but slightly higher values than those blanched for 1 min. The unblanched UFCB showed the lowest L* value compared to that of the other treatments. Blanching can increase the lightness level of beans by 1.2-1.5 times compared to the level of those not blanched.

Chroma describes the intensity or saturation of a color. Chroma was calculated from the color parameters a* and b* according to the given equation (3). Chroma in UFCB ranged from 11.50-14.02. The unblanched UFCB had the lowest chroma value. Blanching the UFCB significantly increased the chroma value ($p<0.05$) compared to that of the unblanched UFCB. Chroma values between blanching treatments did not differ significantly, ranging from 13.95-14.02.

Increasing the blanching time significantly increased the TCD in the UFCB. The TCD was a function of 3 coordinates of CIE Lab L*, a* and b*, which were calculated based on the given equation (4). The TCD in the UFCB blanched from 1-7 min ranged from 5.83-16.22, which indicated a very noticeable color difference after blanching. Choi *et al.*[28] reported that if the TCD value was more than 2, then the color difference could be observed visually between the two samples. The color characteristic of the UFCB blanched for 1 min was comparable to that of beans found in chocolate. Meanwhile, 3 min blanching produced a color that characteristic of the combination of purple and brown. The UFCB blanched for 5 and 7 min produced full purple colors with slightly different lightness levels. The TCD in the UFCB was associated with the relative activity of the PPO enzyme. The brown color of the cacao beans was triggered by an oxidation reaction catalyzed by the PPO enzyme. The PPO enzyme has two copper atoms with two catalytic active sides, monophenolase and diphenolase. Monophenolase activity catalyzes the hydroxylation of monophenol to o-diphenol and diphenolase activity is specific in the oxidation of o-diphenol to form o-quinone. Furthermore, these reaction products can react with phenol or vice versa, polymerizing to form melanin, which is responsible for browning reactions[29].

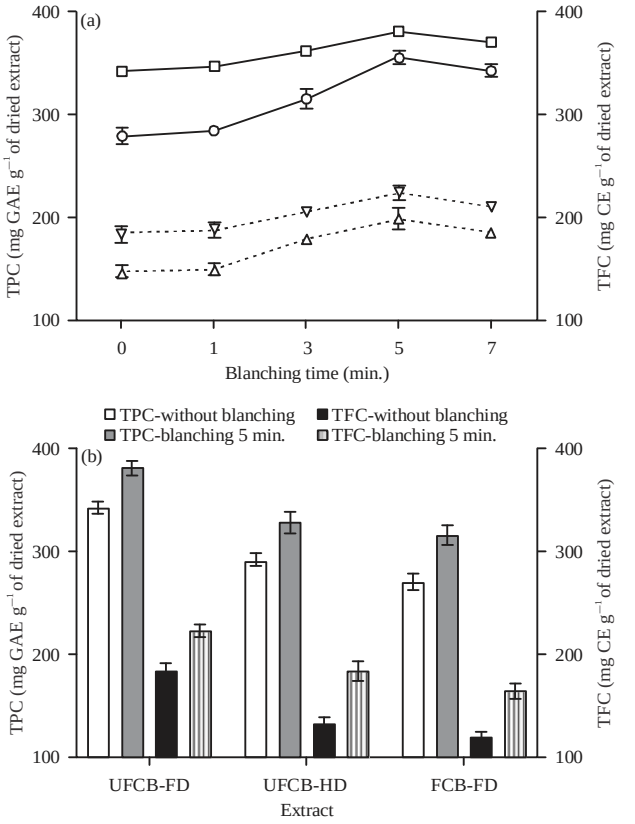

Fig. 1(a-b): The effect of blanching time on the (a) TPC (—) and TFC (- - -) of UFCB-FD, undefatted (● and ▲), defatted (■ and ▼) extracts, (b) UFCB-FD, UFCB-HD and FCB-FD defatted extracts

UFCB-FD: Unfermented cacao beans-freeze dried, UFCB-HD: unfermented cacao beans-hot air dried, FCB-FD: fermented cacao beans-freeze dried, TPC: Total polyphenol content, TFC: Total flavonoid content

The effect of blanching time on the TPC and TFC: The TPC and TFC of the unfermented-freeze dried (UFCB-FD), unfermented-hot air dried (UFCB-HD) and fermented-freeze dried (FCB) cacao bean extract at various blanching times is shown in Fig. 1a. Increasing the blanching time had a significant effect ($p<0.05$) on the TPC and TFC of the UFCB-FD extract (Fig. 1a). The TPC and TFC increased with increasing blanching time for up to 5 min. The TPC of the UFCB-FD

extract ranged from 279.96-382.04 mg GAE g^{-1} of dried extract and the TFC ranged from 148.14-223.96 mg CE g^{-1} of dried extract. (Fig. 1a). Defatting the UFCB-FD increased the TPC and TFC. This is because polyphenols and flavonoids are mainly found in nonfat cacao solids[30]. In addition, there was a strong correlation between the flavanol contents and the amount of the nonfat cacao solids in the cacao products[31] and the procyanidin content was strongly correlated with the total nonfat cacao solid content[32]. The UFCB-FD defatted extract, following 5 min of blanching, showed the highest TPC and TFC, namely, 382.04 mg GAE g^{-1} of dried extract and 223.96 mg CE g^{-1} of dried extract, respectively. Meanwhile, the unbalanced and blanched (1 min) UFCB-FD extracts showed the lowest TPC and TFC. The decrease in polyphenol and flavonoid content in the cacao beans was due to the oxidation of polyphenol compounds, followed by polymerization and the formation of compounds with insoluble high molecular weights[33]. Five minute blanching of the cacao beans resulted in PPO enzyme inactivation by >99% (Table 1). Tomas-Barberan *et al.*[13] reported that a blanching temperature of 95°C held for 5 min was optimal and resulted in the lowest rate of browning reaction in cacao beans. Moreover, blanching cacao beans for >5 min presumably resulted in the decomposition of polyphenols, which caused the TPC and TFC to decrease due to the excessive use of heat. Results from this study were in accord with those from Bamidele *et al.*[34], who reported that blanching vegetables at 90°C for the longer period of 10-15 min caused a significant decrease in the content of polyphenol compounds. This decrease was caused by polyphenol compounds leaching out from the vegetable tissues into the blanching water.

Figure 1b shows the effects of fermentation and hot air drying on cacao beans on the TPC and TFC. Fermentation and hot air drying of cacao beans had a significant effect on the degradation of polyphenols and flavonoids in the cacao bean extract. The reduction percentage in the TPC and TFC of the FCB-FD extract was greater than that of the UFCB-HD extract. In other words, the effect of fermentation was greater than that of hot air drying in the degradation of polyphenols and flavonoids in the cacao beans. This was due to the oxidation of polyphenol compounds during fermentation, followed by polymerization and the formation of compounds with insoluble high molecular weights[33]. Moreover, the diffusion of polyphenols through sweating during fermentation also contributed to the degradation of polyphenols[7], while drying had a minimal effect on changes in epicatechin and catechin levels[35]. Cacao beans after blanching had higher TPC and TFC than those not blanched in all treatments. It is suspected that the degradation of complex polyphenol compounds into simpler polyphenols occurs during blanching. Blanching also prevents polyphenol compounds from undergoing enzymatic oxidation, so their amount does not decrease. Turkmen *et al.*[36] stated that blanching by boiling chili beans for 5 min could significantly increase the total phenol content compared to that of unbalanced beans. In addition, the blanching process is thought to cause flavonoids, in the form of glycosides, to be degraded to aglycones and sugars, thereby increasing the total antioxidant activity[12].

Effect of blanching time on DPPH radical scavenging activity: The DPPH radical scavenging activity in the UFCB-FD, UFCB-HD and FCB-FD cacao beans' defatted extract is shown in Table 2. The antioxidant activity of the unfermented and fermented cacao bean extracts was measured for its ability in scavenging free radicals using the DPPH method and given as the IC_{50} value. The IC_{50} value indicated the concentration of the extract that could inhibit free radicals by 50%. A lower IC_{50} value indicated greater extract ability in scavenging DPPH free radicals. The IC_{50} values of cacao bean extract ranged from 9.07-20.36 μg mL^{-1}. The UFCB-FD extract following blanching showed the lowest IC_{50}, which was slightly higher than that of ascorbic acid, which had an IC_{50} of 7.38 μg mL^{-1}. The UFCB-HD and FCB-FD extracts that were not blanched showed the lowest IC_{50} values, ranging from 19.89-20.36 μg mL^{-1}. This value range was slightly higher than that of the BHT standard which had an IC_{50} of 17.03 μg mL^{-1}. The antioxidant activity of polyphenol compounds as free radical scavengers is conferred by the polyphenol's ability to serve as a hydrogen donor. The

Table 2: The effect of blanching time on the antioxidant activity of UFCB-FD, UFCB-HD and FCB-FD defatted extracts

	DPPH radical scavenging activity, IC_{50} (μg mL^{-1})*		Fe^{2+} chelating activity, IC_{50} (μg mL^{-1})**	
Cacao bean extract	Without blanching	After blanching (5 min.)	Without blanching	After blanching (5 min.)
UFCB-FD	11.65±0.47^{b}	9.07±0.09^{c}	809.07±9.37^{a}	763.33±4.70^{a}
UFCB-HD	19.89±0.45^{a}	17.20±0.36^{b}	843.90±20.20^{a}	781.73±14.77^{a}
FCB-FD	20.36±0.28^{a}	18.34±0.15^{a}	851.03±56.75^{a}	627.97±22.76^{b}

Values followed by different lowercase letters in the same column show significant differences ($p<0.05$). Values are presented as an average±standard deviation from triplicate replications. BHT and ascorbic acid were used as standards* with IC_{50} (μg mL^{-1}) values of 17.03±0.81 and 7.38±0.15, respectively; EDTA was used as a standard** with the IC^{50} (μg mL^{-1}) value of 17.74±0.13. UFCB-FD: Unfermented cacao beans-freeze dried, UFCB-HD: Unfermented cacao beans-hot air dried, FCB-FD: Fermented cacao beans-freeze dried

amount of hydrogen protons that can be donated is influenced by the number and position of the aromatic hydroxyl groups or the number of hydroxyls present in the phenolic component[37]. The polyphenols grouped within flavonoids contain more O-H groups than the synthetic antioxidants, such as BHT, which only contain one O-H group[7]. Overall, the IC_{50} of the cacao bean extracts after blanching was higher than that of the unblanched extracts. This means that 5 min blanching on cacao beans had an effect on the increase of DPPH radical scavenging activity. According to Pujimulyani *et al.*[38], the increase in antioxidant activity is assumed to be due to blanching, which can cause antioxidant components to be extracted more easily so that the amount of antioxidants extracted also increases. Furthermore, it is suspected that during blanching, there is hydrolysis of glycosides into aglycones and sugar. DPPH (IC_{50}) radical scavenging activity had a very strong and negative Pearson correlation with polyphenols (r) = -0.92 and flavonoids (r) = -0.88. The higher the polyphenol and flavonoid contents are, the smaller the IC_{50} values are, which means that DPPH radical scavenging activity is greater. Polyphenol compounds have strong antioxidant properties, so there is a correlation between the two[39]. Abbe Maleyki and Ismail[22] reported that flavonoids significantly contributed to DPPH radical scavenging (r) = 0.73 in cacao powder. Hu *et al.*[30] also reported that polyphenols and flavonoids were strongly correlated with the antioxidant capacity of cacao beans with r values of 0.81 and 0.98, respectively.

Effect of blanching time on ferrous ion chelating activity (Fe^{2+}): The ferrous ion chelating activity in the UFCB-FD, UFCB-HD and FCB-FD cacao beans' defatted extract is shown in Table 2. The ferrous ion chelating activity is expressed as IC_{50} values. The IC_{50} value of the ferrous ion chelating activity ranged from 627.97- 851.03 μg mL^{-1}. The smaller the IC_{50} value is, the stronger the activity is. The FCB-FD extract after blanching showed the highest ferrous ion chelating activity (IC_{50} of 627.97 μg mL^{-1}) compared to that of all other treatments. EDTA was used as a standard and had an IC_{50} value 35 times lower than that of the FCB-FD extract (17.74 μg mL^{-1}). Treatments did not have a significant effect on the ferrous ion chelating activity of the unblanched cacao beans, which had IC_{50} values ranging from 809.07-851.03 μg mL^{-1}. Cacao beans after blanching yielded smaller IC_{50} values than those without blanching for all treatments. This means that the ferrous ion chelating activity in cacao beans after blanching was higher than that in unblanched cacao beans. Polyphenol compounds in cacao beans include procyanidins, flavanol and quercetin monomers, all of which have ionizing chelating activity[40]. Procyanidin polymerization during fermentation causes an increase in the ionizing chelating activity because the ability of chelating ions and binding proteins depends on the structure and molecular weight distribution[40]. The binding efficiency of metal ions is closely related to the spatial conformation of the existing compounds, as well as the position and number of electron contributing groups (ligand)[41]. The ferrous ion chelating activity was not correlated with the polyphenol (r) = -0.29 and flavonoid (r) = -0.32 content in cacao bean extract. This is consistent with the study of Ebrahimzadeh *et al.*[42], who stated that the polyphenols and flavonoids in various medicinal plants were not correlated with ferrous ion chelating.

Effect of blanching time on FTIR spectra: The FTIR spectra in UFCB-FD defatted extract was used to determine functional groups that played a role in the antioxidant activity of the cacao bean extract at wave numbers of 4000-400 cm^{-1} (Fig. 2). The UFCB-FD extracts with and without blanching demonstrated very strong and wide intensities in the absorption of the O-H groups at wavenumbers of 3218-3233 cm^{-1}. In polyphenol molecules, the O-H groups are most responsible for antioxidant activity as proton donors. Changes in the numbers of O-H groups due to blanching determine changes in antioxidant activity, so higher intensities due to O-H groups lead to greater antioxidant activity. The antioxidant activity of phenolic components depends on the position and number of hydroxyl groups that can act as reducing agents, hydrogen donors and singlet oxygen absorbers[43]. The position of O-H in the molecule determines

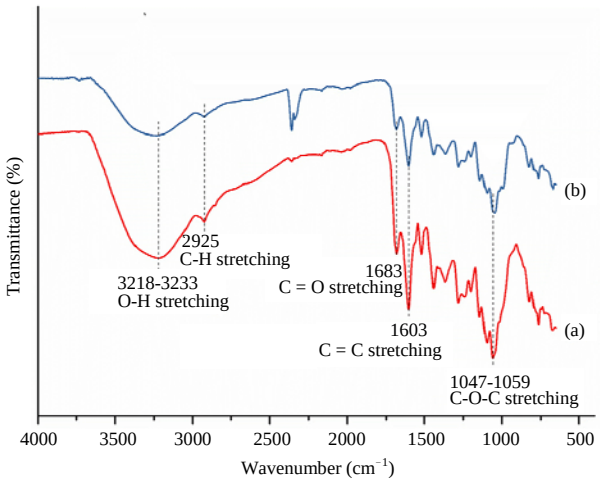

Fig. 2(a-b): FTIR spectra of UFCB-FD (a) without blanching and (b) after blanching (5 min) defatted extract
UFCB-FD: Unfermented cacao beans-freeze dried

the antioxidant activity of the compound. The O-H groups bound to positions C3, C4 and C5 in the B and O-H rings on C3 in the C-ring play a role in antioxidant activity. The loss of an O-H group not located at one of these positions does not significantly affect the antioxidant activity of the compound[44]. The number of O-H groups influences antioxidant activity and fewer O-H groups result in lower antioxidant activity.

Changes in the spectra of UFCB-FD extracts with and without blanching occur due to the shifting of functional groups during the blanching process. The amount of bond absorption depends on the type of bonding vibration. Therefore, various types of bonds will absorb infrared radiation at different wavelengths. The hydroxyl (O-H) group has a specific role in antioxidant activity. The UFCB-FD extracts that were unblanched and blanched for 5 min had similar absorptions of differing intensities. Five minute blanching increased the intensity of all functional groups compared to that of the unblanched cacao beans. The spectra of the wavenumber 1683 cm^{-1} showed stretching C = O from the carboxyl group, which was supported by a very wide O-H absorption due to the hydrogen bond with the dimer. The UFCB-FD extract also had a symmetrical C-O-C (glycoside bond), which was shown in the wavenumbers 1060-1282 cm^{-1}. In addition, the results of this study also identified C-H group absorption at the wavenumber 2925 cm^{-1}, C-H group absorption at the wavenumbers 1366-1441 cm^{-1}, = C-H group absorption at the wavenumbers 726-994 cm^{-1}, C-F group absorption at the wavenumbers 1200-1282 cm^{-1}, C-Cl group absorption at the wavenumbers 669-764 cm^{-1}, C = C group absorption at the wavenumber 2163 cm^{-1}, C-N group absorption at the wavenumbers 1096-1282 cm^{-1}, C = C group absorption at the wavenumbers 1440-1519 cm^{-1}, C-N group absorption at the wavenumbers 1060-1282 cm^{-1} and absorption of N-O groups at wavenumbers 1519 cm^{-1} and 1366-1368 cm^{-1}.

The results of wavenumber absorption analysis indicated that the predicted compounds in cacao bean extract were dominated by flavonoids. This is based on the content of aromatic functional groups and hydroxyl groups, which are one of the characteristics of flavonoid compounds. Changes in functional groups during blanching caused differences in antioxidant activity.

CONCLUSION

Blanching unfermented cacao beans with hot water for 5 min at 95°C was shown to increase the free radical scavenging activity by deactivating the polyphenol oxidase enzyme and thereby increasing the total polyphenol content. A correlation between total polyphenols and total flavonoids with DPPH radical scavenging activity was identified but these were not correlated with ferrous ion chelating activity. Future research is recommended to evaluate the components of flavonoid compounds that can increase the antioxidant activity in cacao bean extract.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial support of Doctoral Dissertation Research Grant for fiscal year 2018 from the Ministry of Research, Technology and Higher Education of the Republic of Indonesia.

REFERENCES

1. Wilkinson, S.L., 1999. Take two cups of coffee and call me tomorrow. Coffee and chocolate contain antioksidans that may promote health. Chem. Eng. News, 77: 47-50.
2. Vinson, J.A., J. Proch, P. Bose, S. Muchler and P. Taffera *et al*., 2006. Chocolate is a powerful *ex vivo* and *in vivo* antioxidant, an antiatherosclerotic agent in an animal model and a significant contributor to antioxidants in the European and American diets. J. Agric. Food Chem., 54: 8071-8076.
3. Lamuela-Raventos, R.M., A.I. Romero-Perez, C. Andres-Lacueva and A. Tornero, 2005. Review: Health effects of cocoa flavonoids. Food Sci. Technol. Int., 11: 159-176.
4. Grassi, D., C. Lippi, S. Necozione, G. Desideri and C. Ferri, 2005. Short-term administration of dark chocolate is followed by a significant increase in insulin sensitivity and a decrease in blood pressure in healthy persons. Am. J. Clin. Nutr., 81: 611-614.
5. Fisher, N.D.L. and N.K. Hollenberg, 2005. Flavanols for cardiovascular health: The science behind the sweetness. J. Hypertens., 23: 1453-1459.
6. Jalil, A. and A. Ismail, 2008. Polyphenols in cocoa and cocoa products: Is there a link between antioxidant properties and health? Molecules, 13: 2190-2219.
7. Wollgast, J. and E.E. Anklam, 2000. Review on polyphenols in theobroma cacao: Changes in composition during the manufacture of chocolate and methodology for identification and quantification. Food Res. Int., 33: 423-447.
8. Afoakwa, E., 2008. Cocoa and chocolate consumption-Are there aphrodisiac and other benefits for human health? S. Afr. J. Clin. Nutr., 21: 107-113.
9. Cooper, K.A., J.L. Donovan, A.L. Waterhouse and G. Williamson, 2008. Cocoa and health: A decade of research. Br. J. Nutr., 99: 1-11.

10. Kim, H. and P.G. Keeney, 1984. (-)-Epicatechin content in fermented and unfermented cocoa beans. J. Food Sci., 49: 1090-1092.
11. Schinella, G., S. Mosca, S.E. Cienfuegos-Jovellanos, M.A. Pasamar, B. Muguerza, D. Ramon and J.L. Rios, 2010. Antioxidant properties of polyphenol-rich cocoa products industrially processed. Food Res. Int., 43: 1614-1623.
12. Pujimulyani, D., S. Raharjo, Y. Marsono and U. Santoso, 2010. [The antioxidant activity and phenolic content of fresh and blanched white saffron (*Curcuma mangga* Val.)]. Agritech, 30: 68-74.
13. Tomas-Barberan, F.A., E. Cienfuegos-Jovellanos, A. Marin, B. Muguerza and A. Gil-Izquierdo *et al*., 2007. A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. J. Agric. Food Chem., 55: 3926-3935.
14. Menon, A., C. Hii, C. Law, S. Suzannah and M. Djaenali, 2015. Effects of water blanching on total polyphenol contents of dried cocoa beans. Proceedings of the 8th Asia-Pacific Drying Conference, August 10-12, 2015, Kuala Lumpur, Malaysia.
15. Nurhayati, S.F. Setyabudi, D.W. Marseno and Supriyanto, 2017. Inactivation of *Polyphenol oxidase* with microwave and its influence on total polyphenol content and antioxidant activity of cocoa beans (*Theobroma cacao* L.). Int. J. Sci. Technol., 5: 52-57.
16. Ioannone, F., C.D. di Mattia, M. de Gregorio, M. Sergi, M. Serafini and G. Sacchetti, 2015. Flavanols, proanthocyanidins and antioxidant activity changes during cocoa (*Theobroma cacao* L.) roasting as affected by temperature and time of processing. Food Chem., 174: 256-262.
17. Tribst, A.A.L., B.R.D.C. Leite Junior, M.M. de Oliveira and M. Cristianini, 2016. High pressure processing of cocoyam, Peruvian carrot and sweet potato: Effect on oxidative enzymes and impact in the tuber color. Innov. Food Sci. Emerg. Technol., 34: 302-309.
18. Altunkaya, A., 2014. Partial purification and characterization of polyphenoloxidase from Turkish tea leaf (*Camellia sinensis* L.). Int. J. Food Prop., 17: 1490-1497.
19. Rawson, A., B.K. Tiwari, M.G. Tuohy, C.P. O'Donnell and N. Brunton, 2011. Effect of ultrasound and blanching pretreatments on polyacetylene and carotenoid content of hot air and freeze dried carrot discs. Ultrasonics Sonochem., 18: 1172-1179.
20. Tiwari, B.K., K. Muthukumarappan, C.P. O'Donnell and P.J. Cullen, 2008. Effects of sonication on the kinetics of orange juice quality parameters. J. Agric. Food Chem., 56: 2423-2428.
21. Tamaroh, S., S. Raharjo, A. Murdiati and S. Anggrahini, 2018. Total phenolic content and antioxidant activity of anthocyanin extract from purple yam (*Dioscorea alata* L.) flour using different solvents. Pak. J. Nutr., 17: 260-267.
22. Abbe Maleyki, M.J. and A. Ismail, 2010. Antioxidant properties of cocoa powder. J. Food Biochem., 34: 111-128.
23. Utami, R.R., R. Armunanto, S. Rahardjo and Supriyanto, 2016. Effects of cocoa bean (*Theobroma cacao* L.) fermentation on phenolic content, antioxidant activity and functional group of cocoa bean shell. Pak. J. Nutr., 15: 948-953.
24. Muhammad, D.R.A., A.D. Saputro, H. Rottiers, D. Van de Walle and K. Dewettinck, 2018. Physicochemical properties and antioxidant activities of chocolates enriched with engineered cinnamon nanoparticles. Eur. Food Res. Technol., 244: 1185-1202.
25. Chew, Y.L., J.K. Goh and Y.Y. Lim, 2009. Assessment of *in vitro* antioxidant capacity and polyphenolic composition of selected medicinal herbs from Leguminosae family in Peninsular Malaysia. Food Chem., 116: 13-18.
26. Adjimani, J.P. and P. Asare, 2015. Antioxidant and free radical scavenging activity of iron chelators. Toxicol. Rep., 2: 721-728.
27. Sugiyanti, D., P. Darmadji, S. Anggrahini, C. Anwar and U. Santoso, 2018. Preparation and characterization of chitosan from Indonesian tambak lorok shrimp shell waste and crab shell waste Pak. J. Nutr., 17: 446-453.
28. Choi, M.H., G.H. Kim and H.S. Lee, 2002. Effects of ascorbic acid retention on juice color and pigment stability in blood orange (*Citrus sinensis*) juice during refrigerated storage. Food Res. Int., 35: 753-759.
29. Siegbahn, P.E., 2004. The catalytic cycle of catechol oxidase. J. Biol. Inorg. Chem., 9: 577-590.
30. Hu, S.J., B.Y. Kim and M.Y. Baik, 2016. Physicochemical properties and antioxidant capacity of raw, roasted and puffed cacao beans. Food Chem., 194: 1089-1094.
31. Miller, K.B., W.J. Hurst, N. Flannigan, B. Ou, C.Y. Lee, N. Smith and D.A. Stuart, 2009. Survey of commercially available chocolate-and cocoa-containing products in the United States. 2. Comparison of flavan-3-ol content with nonfat cocoa solids, total polyphenols and percent cacao. J. Agric. Food Chem., 57: 9169-9180.
32. Gu, L., S.E. House, X. Wu, B. Ou and R.L. Prior, 2006. Procyanidin and catechin contents and antioxidant capacity of cocoa and chocolate products. J. Agric. Food Chem., 54: 4057-4061.
33. Irina, I. and G. Mohamed, 2012. Biological Activities and Effects of Food Processing on Flavonoids as Phenolic Antioxidants. In: Advances in Applied Biotechnology, Petre, M. (Ed.). InTech Publisher, Rijeka, Croatia, ISBN: 978-953-307-820-5, pp: 101-124.
34. Bamidele, O.P., M.B. Fasogbon, O.J. Adebowale and A.A. Adeyanju, 2017. Effect of blanching time on total phenolic, antioxidant activities and mineral content of selected green leafy vegetables. Curr. J. Applied Sci. Technol., 24: 1-8.
35. Payne, M.J., W.J. Hurst, K.B. Miller, C. Rank and D.A. Stuart, 2010. Impact of fermentation, drying, roasting and Dutch processing on epicatechin and catechin content of cacao beans and cocoa ingredients. J. Agric. Food Chem., 58: 10518-10527.

36. Turkmen, N., F. Sari and Y.S. Velioglu, 2005. The effect of cooking methods on total phenolics and antioxidant activity of selected green vegetables. Food Chem., 93: 713-718.
37. Lai, L.S., S.T. Chou and W.W. Chao, 2001. Studies on the antioxidative activities of Hsian-tsao (*Mesona procumbens* Hemsl) leaf gum. J. Agric. Food Chem., 49: 963-968.
38. Pujimulyani, D., S. Raharjo, Y. Marsono and U. Santoso, 2012. The effect of blanching on antioxidant activity and glycosides of white saffron (*Curcuma mangga* Val.). Int. Food Res. J., 19: 617-621.
39. Caillet, S., S. Salmieri and M. Lacroix, 2006. Evaluation of free radical-scavenging properties of commercial grape phenol extracts by a fast colorimetric method. Food Chem., 95: 1-8.
40. Ojwang, L.O., L. Yang, L. Dykes and J. Awika, 2013. Proanthocyanidin profile of cowpea (*Vigna unguiculata*) reveals catechin-O-glucoside as the dominant compound. Food Chem., 139: 35-43.
41. Khokhar, S. and R.K.O. Apenten, 2003. Iron binding characteristics of phenolic compounds: Some tentative structure-activity relations. Food Chem., 81: 133-140.
42. Ebrahimzadeh, M.A., F.Pourmorad and A.R. Bekhradnia, 2008. Iron chelating activity, phenol and flavonoid content of some medicinal plants from Iran. Afr. J. Biotechnol., 7: 3188-3192.
43. Rice-Evans, C.A., N. Miller and G. Paganga, 1997. Antioxidant properties of phenolic compounds. Trends Plant Sci., 2: 152-159.
44. Shahidi, F. and M. Naczk, 2004. Phenolics in Food and Nutraceuticals. CRC Press, USA.