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Research Article
Effects of Cocoa Bean (*Theobroma cacao* L.) Fermentation on Phenolic Content, Antioxidant Activity and Functional Group of Cocoa Bean Shell

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Abstract

Background and Objective: Cocoa bean shell (CBS) has phenolic content and potential as cheaper source of antioxidants. The objective of this study was to evaluate the effects of cocoa bean fermentation on total phenolic content and antioxidant activity of cocoa bean shell. **Materials and Methods:** Cocoa beans were placed in fermentation box following spontaneous fermentation for 24, 48, 60, 72, 96 and 120 h. The CBS was separated from the cocoa beans and extracted with acetone-water (70:30 v/v). Folin-Ciocalteu method, 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and Fourier Transform Infrared Spectroscopy (FTIR) analysis were used to determine total phenolic contents, antioxidant activity and functional group of the samples, respectively. **Results:** For total phenolic contents, absorbance of various samples concentrations was plotted against gallic acid standard curve and the value indicated that CBS from partially fermented cocoa beans (24 h) contained the highest amount of phenolic content. Various concentrations of samples were also used for antioxidant activity measurement. Highest scavenging activity of 88.67±1.12% was obtained by 100 μg mL^{-1} CBS from partially fermented cocoa beans (24 h), while the lowest of 44.60±2.48% was obtained by CBS from 120 h fermented cocoa beans. A high correlation was observed between phenolic content and antioxidant activity. The FTIR analysis shows that CBS from 24 h fermented cocoa beans had more functional group compare to other samples. **Conclusion:** Fermentation of cocoa bean can affect total phenolic content and antioxidant activity of the CBS. Fermentation also causes functional group shift of CBS. The results of the study showed that CBS is a good source of antioxidants and phenolic compounds. Further study on the structural elucidation of the CBS individual phenolic compounds and evaluation of their mechanisms of action is recommended.

Key words: Fermentation, cocoa bean shell, antioxidant activity, total phenolic contents, functional group, Folin-Ciocalteu, DPPH, FTIR

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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

Cocoa bean shell (CBS) is cocoa industry by-product with low economic value. The CBS is part of the cocoa bean that separated from the cotyledons. The CBS makes about 15% of the weight of the cocoa bean[1]. Indonesia processed 425.000 t of cocoa beans in 2014, generating approximately 63.750 t of shells[2].

Cocoa beans are reportedly contain high level of polyphenols with antioxidant activity, mainly flavan-3-ol (monomeric epicatechin and catechin, as well as their oligomers from dimers to decamers, the procyanidins), with small amounts of anthocyanin (mainly cyanidin glycosides) and flavonols (quercetin glycosides)[3,4]. Their polyphenolic structure has one or more aromatic ring and at least has two hydroxyl groups[5], with ability to inhibit free radicals[3]. The chemical structure of cocoa flavanols and procyanidins determine their antioxidant activity play as radical scavenging and metal chelating agent[6].

However, most of studies are mainly concentrated on cocoa bean with far less attention on the CBS, despite its potential as cheaper source of antioxidants. It has beneficial effects such as antioxidants[7-10], antimicrobial[11] and anti-caries[1,12,13]. Nsor-Atindana *et al.*[11] reported that total polyphenolic and total flavonoid content of CBS were 41.82 mg GAE g^{-1} and 5.49 mg CE g^{-1}, respectively. Its polyphenolic compounds are procyanidins, epicatechin, p-hydroxybenzoic acid, anthocyanin, proanthocyanin and clovamide[7,14,15].

Cocoa beans fermentation is a critical step in cocoa processing for the development of the chocolate flavour and taste due to aroma precursors generation and affect phenolic content of cocoa beans. Fermented cocoa beans had much lower epicatechin content than the partially fermented[16]. Moreover, polyphenol content of CBS from fermented cocoa beans is higher than CBS from partially fermented cocoa beans. Cocoa beans fermentation lead in death and cell permeability damaged of seed, resulting diffusion of polyphenol compounds from pigment cells to all parts of the cotyledons. During fermentation, the migration of epicatechin from cocoa beans into CBS has occurred[16,17]. Coinciding with the decreased of epicatechin in the bean, epicatechin in the shell increased, during the second and third day of fermentation. At the end of fermentation (6 days), epicatechin content of both cocoa beans and shells decreased[16]. Although, polyphenolic compounds usually accumulate in the outer parts of plants such as shells, skins, etc.[18], there is limited information about the effect of cocoa beans fermentation to the antioxidant compound of CBS. The objectives of this study were to evaluate the effects of cocoa beans fermentation on phenolic content, antioxidant activity and to determine the functional groups of CBS extract using FTIR analysis.

MATERIALS AND METHODS

Materials: The CBS used in the experiments were obtained from fermented cocoa bean (24, 48, 72, 96 and 120 h of fermentation) and CBS from unfermented cocoa bean used for comparison. Forastero cocoa was collected in June 2015 from a local farmer in Yogyakarta, Indonesia. Hexanes, methanol and gallic acid were purchased from Merck Millipore (Germany), acetone was purchased from Mallinckrodt (Ireland). The DPPH and Folin-Ciocalteu were purchased from Sigma-Aldrich (Germany). All other chemicals and solvents were of analytical grade.

Fermentation: Cocoa pods were sorted based on visual appearance and those with physical damage or disease symptoms were dismissed. Selected pods were manually opened and the beans were placed in boxes following spontaneous fermentation for 120 h. Cocoa mass was manually mixed every 48 h in a wooden box with approximately 100 kg of cocoa raw beans stored per unit. Nibs were manually sampled, daily and in the center of the fermentation mass, about 5 kg each sampling. Samples were washed to stop the fermentation, then dried for a week using combination of solar and cabinet drying. The end of drying was determined when cocoa beans moisture content was down to 7%.

Extraction procedure: The CBS was separated from the cocoa beans and subjected to the procedure described in previous method with some modifications[1,19]. Dried samples were ground in a blade grinder. Approximately 20 g of the ground CBS was defatted with 40 mL of hexane for 30 min in a magnetic stirrer and followed by centrifugation at 2.054 g for 20 min. This defatting procedure was repeated twice. Afterwards, defatted CBS material was extracted with a 400 mL of acetone-water (70:30), stirred for 4 h and centrifuged at 2.054 g for 30 min. This extraction procedure was repeated three times. Acetone was evaporated at 40°C and under vacuum. Concentrated extracts were dried with freeze dryer. Total phenolic compounds: Total phenolic content of the extracts was determined by a colorimetric Folin-Ciocalteu reagent assay[20]. Briefly, 0.1 mL of extract was added to test tube, followed by the addition of 0.5 mL of the Folin Ciocalteu reagent and 6 mL of distilled water. After mixing and resting

for 3 min, 20% sodium carbonate was added (w/v, 1.5 mL) and the test tube was adjusted with distilled water to 10 mL. After 2 h incubation at room temperature, absorbance was determined spectrophotometrically at 760 nm. Distilled water was used as a blank or control. Gallic acid (25-500 μM) was used as standard and the results were expressed as mg gallic acid equivalents per gram of dried weight (mg GAE g^{-1} DW). Analyses were carried out in triplicates.

DPPH radical scavenging activity: Scavenging free radical potentials were tested in the ethanolic solution of DPPH with a slight modification[21]. From aqueous extracts at different concentrations, solution was prepared by adding 1 mL of the extract and 2 mL of the DPPH solution. To determine the antioxidant activity, ethanol solution of 0.1 mM DPPH was prepared. The absorbance was measured at 517 nm. A reference sample was prepared with 1 mL methanol. The BHT was used as positive control. The antiradical activity was calculated as a percentage of DPPH decoloration using the following equation:

$$\text{Antiradical activity} = \frac{\text{1-Absorbance of sample}}{\text{Absorbance of reference}} \times 100$$

FTIR analysis: FTIR spectra of all samples were recorded between 4000 and 400 cm^{-1} using Shimadzu IR Prestige 21 spectrometer for all the samples. The discs were prepared by first mixing 1 mg of dried sample with 100 mg of potassium bromide in an agate mortar and then pressing the resulting mixture at 10 t cm^{-2}.

Statistical analysis: Data were reported as Means±Standard Deviation. Statistical comparisons were evaluated by analysis of variance (ANOVA) using SPSS (version 16). Duncan MRT Post-ANOVA test was used to compare treatment means. The difference between treatment means was judged at 5% significance level.

RESULTS AND DISCUSSION

Extraction yields and total phenolic content: The yield of extracted materials and their total phenolic contents are reported in Table 1. This study used 70% acetone (70:30 acetone:water) as the most effective solvent for CBS antioxidants extraction[10]. The yield varies from 14.22% (extract from 48 h of fermentation) to 23.11% (extract from 72 h of fermentation).

Table 1: Yields and total phenolic contents of CBS extracts

CBS extract from cocoa bean with fermentation (h)	Yields (%)	Total phenolics (mg GAE g^{-1} dry weight)
0	22.15±0.34^{b}	23.73±0.36^{bc}
24	16.95±0.05^{ab}	39.41±0.29^{e}
48	14.22±0.06^{a}	26.66±0.74^{d}
72	23.11±0.16^{b}	24.06±0.29^{c}
96	20.49±0.17^{ab}	27.51±0.40^{d}
120	14.53±0.31^{a}	19.40±1.17^{a}

The data given are Mean±Standard Deviation for three different values of each sample. GAE: Gallic acid equivalents. Means within the same column with different letters differ significantly ($p \leq 0.05$)

Result in Table 1 showed that total phenolic content of CBS extract from unfermented and fermented cocoa bean tested varied, ranging from 19-39 mg GAE g^{-1} dry weight. The CBS extract from partially fermented cocoa bean (24 h) was found to have the highest total phenolic content (39.41 mg GAE g^{-1} dry weight). There was an increase in total phenolic content at 24 h of fermentation, but decreased with longer fermentation. Increasing polyphenols content due to diffused of polyphenols out of the cells through the CBS. Diffusion continues until 72 h of fermentation, no phenol was detected in cocoa bean cells[22]. Polyphenols diffuse along with cell liquids from their storage cells, oxidized and forming into high molecular mass, such as insoluble tannins. Polyphenol oxidase converts polyphenols into quinones. Polyphenols and quinones then form a complex with other polyphenols, proteins and peptides[23]. This might contribute to the increase of total phenolics or phenolics like complexes that contributed to higher absorbance readings.

After 24 h of fermentation, total phenolics were decreased until the end of fermentation (120 h). This could be attributed to the degradation or polymerization products of CBS polyphenols during fermentation. Mariani[24] reported that total phenolic content of CBS from unfermented cocoa bean as 22.72 mg GAE g^{-1} dry weight and from fermented cocoa bean as 14.8 mg GAE g^{-1} dry weight. This were in line with other study reported that the total polyphenols in cocoa beans decreased by 10% during 6 days of fermentation[25]. During fermentation, polyphenol undergoes polymerization and form a complex with the protein[26]. In the cocoa bean, polyphenols content was reduced from 16.11% (unfermented) to a 6.01% in 6 days of fermentation[27].

DPPH radical scavenging activity: In this study, free radical (DPPH) scavenging method was used to measure the total antioxidant activity of CBS extracts. Discoloration of violet color indicates that DPPH free radicals were quenched or

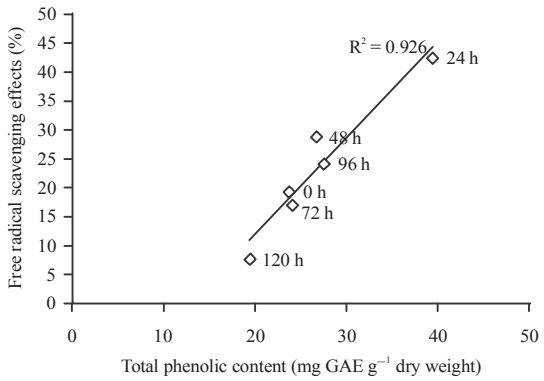

Fig. 1: Correlation between free radical scavenging activity and total phenolic content of CBS extract. The CBS extract from fermented cocoa bean (24, 48, 72, 96 and 120 h of fermentation) and unfermented (0 h) cocoa bean

Table 2: Antioxidant activity of CBS extracts at different concentration

CBS extract from cocoa bean with fermentation (h)	Scavenging effect (%)	
	10 μg mL^{-1}	100 μg mL^{-1}
0	19.38±2.67^{b}	61.56±3.43^{b}
24	42.43±1.12^{d}	88.67±5.92^{d}
48	28.77±0.09^{c}	84.97±0.46^{c}
72	17.02±2.08^{b}	64.65±1.23^{b}
96	24.22±1.71^{bc}	82.46±1.88^{c}
120	7.770±2.41^{a}	44.6±2.48^{a}

$^{a-d}$Each value is presented as Mean±SD (n = 3). Means within the same column with different letters differ significantly ($p \leq 0.05$)

reduced by the antioxidants of the sample. Total antioxidant activity shown in Table 2 was expressed as a percentage of inhibition.

Total antioxidant activity of CBS extracts were significantly affected by fermentation. In line with total phenolic content, CBS extract from partially fermented cocoa bean (24 h) was found to have the highest scavenging effect, while the lowest was found at 120 h of fermentation were consistent with the decreasing pattern of total phenolic. The order of total antioxidant activity obtained by different fermentation time was 24>48>96>72>0>120 h. Other study reported that antioxidant capacity (percentage of inhibition DPPH free radicals) of cocoa bean decrease from 96% in the first day of fermentation to 79% in the 6th day[27]. The results of CBS crude extract were still lower compare to those of BHT as pure compound. Total antioxidant activity of BHT at 10 and 100 μg mL^{-1} were 62.55 and 88.67%, respectively.

Antioxidant activity of phenolic as free radical scavenger associated with antioxidant capability such as a proton donor. Hydrogen protons donation is largely influenced by the number and position of the aromatic hydroxyl groups or hydroxyl from phenolics[28,29].

Correlation between antioxidant compounds and antioxidant activity: The coefficient of correlation between the phenolic compound and antioxidant activity of the CBS is shown in Fig. 1.

Antioxidant activity as free radical scavenging was significantly correlated ($p<0.05$) to total phenolic content with $R^2 = 0.926$ as shown in Fig. 1. Significant positive correlations indicated that free radical scavenging activity is mainly attributed to total phenolic content. In this study, total phenolic content was more likely to be responsible to scavenge most of free radicals in the studied. Therefore, it could be expected that phenolic compounds might have been the major contributor to antioxidant activity in the CBS.

Phenolic compounds activity largely depends on their molecular weight, structure and concentration of these compounds. The configuration and total number of hydroxyl groups in the flavonoid molecules influence their radical scavenging efficiency. The presence of the ortho-dihydroxy (catechol) structure in the B-ring, 2,3-double bond in conjugation with a 4-oxo function in the C-ring and hydroxyl groups at positions 3 and 5 enhances radical scavenging activity. Furthermore, the antioxidant activity of procyanidins also depends on their degree of polymerization[30]. Thus properties (hydrophilic or lipophilic) of certain compounds present in the cocoa samples may affect the results of antioxidant activity assays[31].

FTIR analysis: The FTIR analysis to determine functional group of CBS extracts was carried out at wave number 4000-400 cm^{-1} The FTIR spectrum of CBS crude extract can be seen in Table 3. Vibration will occur when infrared radiation is absorbed by the bond in a molecule. The amount of absorption of a bond depends on the type of bond vibration. Therefore, different types of bonds will absorb infrared radiation at different wavelengths.

Table 3 shows that functional groups area between 4000-1400 cm^{-1} had identical spectrum, suggested that CBS polyphenols, either from unfermented or fermented, had identical functional groups. Changes in the CBS spectra from unfermented and fermented cocoa bean occur due to shift in the functional group during fermentation process.

Spectra at 3410-3394 cm^{-1} shows the absorption intensity was very strong and wide, indicating an O-H stretch. In the polyphenol molecule, the O-H bond is the most

Table 3: FTIR spectrum band of CBS

		CBS extract from cocoa bean with fermentation (h)					
Functional group	Wave numbers range (cm^{-1})	0	24	48	72	96	120
C=O (carbonyl)	1820-1600	1612	1612	1612	1612	1612	1612
C=O (anhydride)	1810-1650	-	1720	-	-	-	-
O-H (acid)	3400-2400	3394	3402	3402	3402	3394	3410
C-H	3300–2900	2924	2924	2924	2924	2924	2924
C-H (aldehyde)	3000-2750	2854	2854	2854	-	-	2854
CH_2	about 1450	1442	1442	1442	-	-	-
CH_3	about 1375	1396	1373	1381	1404	1404	1404
C-O (ester)	1300-1000	1064	1064	1072	1072	1072	1072
C-O-C	1300-1000	-	1103	-	-	-	-

responsible of antioxidant activity as proton donor. Changes in the number of OH due to fermentation determined the changes of antioxidant activity, thus lower number of O-H lead to smaller antioxidative activity. The antioxidative activity of polyphenols is also determined by O-H position in the molecule. The O-H bond at C_3, C_4 and C_5 position in B-ring and O-H at C_3 in C-ring play an important role. Loss of OH other than those positions did not affect antioxidative activity[32].

The CBS obtained from 24 h of cocoa bean fermentation had more functional groups than others, thus had the highest antioxidant activity. Spectra at 1720 cm^{-1} showed a C=O stretch which came from carboxyl groups that are supported by very wide O-H absorption due to the bonding of hydrogen with its dimer. The CBS (24 h of fermentation) also has C-O-C symmetric (the glycoside bond) that are shown at 1103 cm^{-1}.

CONCLUSION AND FUTURE RECOMMENDATIONS

The results of the study showed that CBS is a good source of antioxidants and phenolic compounds. It revealed that fermentation of cocoa bean can affect total phenolic content and antioxidant activity of the CBS. Fermentation also causes functional group shift of CBS. Further study on the structural elucidation of the CBS individual phenolic compounds and evaluation of their mechanisms of action is recommended.

SIGNIFICANCE STATEMENTS

The CBS is cocoa industry by-product with low economic value and contain polyphenolic compounds for example procyanidins, epicatechin and anthocyanin. However, most of studies are mainly concentrated on cocoa bean with far less attention on the CBS. Cocoa beans fermentation is a critical step in cocoa processing for the development of the chocolate flavor that can affect phenolic content of cocoa. In this study we evaluated the effects of cocoa beans fermentation on phenolic content, antioxidant activity and to determine the functional groups of CBS extract using FTIR analysis. This research is useful to prove that CBS has potential as cheap source of antioxidants.

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REFERENCES

1. Kim, K.H., K.W. Lee, D.Y. Kim, H.H. Park, I.B. Kwon and H.J. Lee, 2004. Extraction and fractionation of glucosyltransferase inhibitors from cacao bean husk. Process Biochem., 39: 2043-2046.
2. MIRI., 2015. Industri kakao olahan serap investasi Rp 6 triliun. Ministry of Industry Republic of Indonesia (MIRI), September 21, 2015. http://www.beritasatu.com/ekonomi/253381-industri-kakao-olahan-serap-investasi-rp-6-triliun.html
3. Adamson, G.E., S.A. Lazarus, A.E. Mitchell, R.L. Prior and G. Cao *et al*., 1999. HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. J. Agric. Food Chem., 47: 4184-4188.
4. Hammerstone, J.F., S.A. Lazarus, A.E. Mitchell, R. Rucker and H.H. Schmitz, 1999. Identification of procyanidins in cocoa (*Theobroma cacao*) and chocolate using high-performance liquid chromatography/mass spectrometry. J. Agric. Food Chem., 47: 490-496.
5. Bernaert, H., I. Blondeel, L. Allegaert and T. Lohmueller, 2012. Industrial Treatment of Cocoa in Chocolate Production: Health Implications. In: Chocolate and Health, Conti, A., R. Paoletti, A. Poli and F. Visioli (Eds.). Springer, New York, USA., ISBN: 978-88-470-2037-5, pp: 17-31.

6. Kelm, M.A., J.C. Johnson, R.J. Robbins, J.F. Hammerstone and H.H. Schmitz, 2006. High-performance liquid chromatography separation and purification of cacao (*Theobroma cacao* L.) procyanidins according to degree of polymerization using a diol stationary phase. J. Agric. Food Chem., 54: 1571-1576.
7. Arlorio, M., J.D. Coisson, F. Travaglia, F. Varsaldi, G. Miglio, G. Lombardi and A. Martelli, 2005. Antioxidant and biological activity of phenolic pigments from *Theobroma cacao* hulls extracted with supercritical CO_2. Food Res. Int., 38: 1009-1014.
8. Lecumberri, E., R. Mateos, M. Izquierdo-Pulido, P. Ruperez, L. Goya and L. Bravo, 2007. Dietary fibre composition, antioxidant capacity and physico-chemical properties of a fibre-rich product from cocoa (*Theobroma cacao* L.). Food Chem., 104: 948-954.
9. Bruna, C., I. Eichholz, S. Rohn, L.W. Kroh and S. Huyskens-Keil, 2009. Bioactive compounds and antioxidant activity of cocoa hulls (*Theobroma cacao* L.) from different origins. J. Applied Bot. Food Qual., 83: 9-13.
10. Martinez, R., P. Torres, M.A. Meneses, J.G. Figueroa, J.A. Perez-Alvarez and M. Viuda-Martos, 2012. Chemical, technological and *in vitro* antioxidant properties of cocoa (*Theobroma cacao* L.) co-products. Food Res. Int., 49: 39-45.
11. Nsor-Atindana, J., F. Zhong, K.J. Mothibe, M.L. Bangoura and C. Lagnika, 2012. Quantification of total polyphenolic content and antimicrobial activity of cocoa (*Theobroma cacao* L.) bean shells. Pak. J. Nutr., 11: 672-677.
12. Osawa, K., K. Miyazaki, S. Shimura, J. Okuda, M. Matsumoto and T. Ooshima, 2001. Identification of cariostatic substances in the cacao bean husk: Their anti-glucosyltransferase and antibacterial activities. J. Dental Res., 80: 2000-2004.
13. Ito, K., Y. Nakamura, T. Tokunaga, D. Iijima and K. Fukushima, 2003. Anti-cariogenic properties of a water-soluble extract from cacao. Biosci. Biotechnol. Biochem., 67: 2567-2573.
14. Zou, T., S.S. Percival, Q. Cheng, Z. Li, C.A. Rowe and L. Gu, 2012. Preparation, characterization and induction of cell apoptosis of cocoa procyanidins-gelatin-chitosan nanoparticles. Eur. J. Pharmaceut. Biopharmaceut., 82: 36-42.
15. 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.
16. Kim, H. and P.G. Keeney, 1984. (-)-Epicatechin content in fermented and unfermented cocoa beans. J. Food Sci., 49: 1090-1092.
17. Roelofsen, P.A., 1958. Fermentation, drying and storage of cacao beans. Adv. Food Res., 8: 225-296.
18. Bravo, L., 1998. Polyphenols: Chemistry, dietary sources, metabolism and nutritional significance. Nutr. Rev., 56: 317-333.
19. Sanbongi, C., N. Osakabe, M. Natsume, T. Takizawa, S. Gomi and T. Osawa, 1998. Antioxidative polyphenols isolated from *Theobroma cacao*. J. Agric. Food Chem., 46: 454-457.
20. Singleton, V.L., R. Orthofer and R.M. Lamuela-Raventos, 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods Enzymol., 299: 152-178.
21. Burda, S. and W. Oleszek, 2001. Antioxidant and antiradical activities of flavonoids. J. Agric. Food Chem., 49: 2774-2779.
22. De Brito, E.S., N.H.P. Garcia, M.I. Gallao, A.L. Cortelazzo, P.S. Fevereiro and M.R. Braga, 2001. Structural and chemical changes in cocoa (*Theobroma cacao* L) during fermentation, drying and roasting. J. Sci. Food Agric., 81: 281-288.
23. Camu, N., T. de Winter, S.K. Addo, J.S. Takrama, H. Bernaert and L. de Vuyst, 2008. Fermentation of cocoa beans: Influence of microbial activities and polyphenol concentrations on the flavour of chocolate. J. Sci. Food Agric., 88: 2288-2297.
24. Mariani, L., 2011. Ekstraksi dan identifikasi senyawa polifenol dalam kulit biji kakao dan potensinya sebagai antioksidan. M.Sc. Thesis, Gadjah Mada University, Yogyakarta, Indonesia.
25. Afoakwa, E.O., J. Quao, F.S. Takrama, A.S. Budu and F.K. Saalia, 2012. Changes in total polyphenols, o-diphenols and anthocyanin concentrations during fermentation of pulp pre-conditioned cocoa (*Theobroma cacao*) beans. Int. Food Res. J., 19: 1071-1077.
26. Nazaruddin, R., L.K. Seng, O. Hassan and M. Said, 2006. Effect of pulp preconditioning on the content of polyphenols in cocoa beans (*Theobroma cacao*) during fermentation. Ind. Crops Prod., 24: 87-94.
27. Aikpokpodion, P.E. and L.N. Dongo, 2010. Effects of fermentation intensity on polyphenols and antioxidant capacity of cocoa beans. Int. J. Sustain. Crop Prod., 5: 66-70.
28. 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.
29. Su, Y.L., J.Z. Xu, C.H. Ng, L.K. Leung, Y. Huang and Z.Y. Chen, 2004. Antioxidant activity of tea theaflavins and methylated catechins in canola oil. J. Am. Oil Chem. Soc., 81: 269-274.
30. Lin, C.C., C.W. Li, Y.T. Shih and L.T. Chuang, 2014. Antioxidant and anti-inflammatory properties of lower-polymerized polyphenols in oolong tea. Int. J. Food Propert., 17: 752-764.
31. 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.
32. Shahidi, F., 1997. Natural Antioxidants: Chemistry, Health Effects and Applications. AOCS Press, Illinois, ISBN: 9780935315776, Pages: 414.