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Cocoa Beans Processing Chains on its Extractable Total Phenolic Contents and Free Radical Scavenging Capability

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Received: September 29, 2020; **Published:** October 31, 2020

Abstract

The polyphenols in cocoa beans are stored in the pigment cells of the cotyledons. Depending on the number of anthocyanins those pigment cells, also called polyphenol-storage cells, are white to deep purple. During fermentation, cocoa protein undergoes progressive alteration and polyphenols diffuse with cell liquids from their storage cells. Roasting of cocoa beans involve reactions among the partial hydrolysis of protein and sugar components, Maillard browning and oxidative and phenolic compounds, that are readily available as cocoa flavour precursors from the fermentation and drying processes; to develop further the chocolate flavour. Study showed that there were no significant differences in term of Total Phenolic Contents (TPC) between fresh cocoa beans and fermented dried cocoa beans. Nevertheless, a significant drop of TPC was observed in roasted cocoa beans. Similar profiles were observed in their capability to scavenge free radicals from a 0.06 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

Keywords: *Cocoa; Free Radicals Scavenging; Total Phenolic Contents*

Introduction

The processing of cocoa beans involve fermentation, drying and roasting. The main purpose of this processing chain is to develop the desire cocoa flavours.

Cocoa beans are extracted from the fresh harvested cocoa fruits. Fresh cocoa beans are then undergoing fermentation process for 5 - 6 days in a wooden fermentation box with turning for aeration at every two days interval. Well fermented cocoa beans are sun dried for another 6 - 7 days to reduce its moisture contents not more than 7.5% (wt/wt) [1] for storage besides to stop fermentation process. Lots of biochemicals reactions, which are not well understood, occur during these processes. Nevertheless, it is well understood that these processes are crucial to form the desire cocoa flavours precursors.

Roasting of the fermented cocoa beans is to remove the undesired compounds with low boiling points, such as acetic acid, and the formation of the typical desired cocoa flavours [2]. The cocoa roasting process is carried out at high temperatures (120 - 150°C). Roasting from unfermented beans fails to produce the desired cocoa flavours.

The cocoa beans kernels consisting of cotyledon and the embryo axis. In the parenchymatic tissue of the cotyledons, two types of storage cells have been detected, which contain either lipids and protein or polyphenols and alkaloids. The characteristic features of lipid-protein cells are multiple small vacuoles with lipids, proteins or storage granules. The polyphenol cells (14 - 20% dry beans weight), however, are characterized by a large central vacuole containing polyphenols, caffeine, theobromine and theophylline. Depending on the number of anthocyanins, those polyphenol-storage cells, also known as pigment cells, are range in colour from white to deep purple. For instances, *Criollo* cocoa beans which are only approximate two third of the polyphenols content of *Forestero* beans have rather whiter in colour compared to deep purple of *Forestero* beans [3].

Polyphenols have become an intense focus of research interest due to their physiological functions. Polyphenols are formed biogenetically from the shikimate and the acetic pathway. Antioxidant, antimutagenic and anti-tumour are among the health-beneficial effects offered by phenolic compounds [4].

Objective of the Study

The objectives of this paper are to report the effect of the cocoa beans processing chain towards its total phenolic contents (TPC) and its free radicals scavenging capability.

Materials and Methods

Fresh unfermented cocoa beans and well fermented dried cocoa beans were collected from MCB research centre located at Bagan Datuk, Perak, Malaysia. Roasting processes on the well fermented dried cocoa beans was carried out in the laboratory with the roasting parameters as in table 1.

Temperature (°C)	Duration (min)
190	5
180	5
160	5
150	15

Table 1: *Cocoa beans roasting parameters.*

Extraction of cocoa phenolics

One gram of de-shelled cocoa beans was added with 50 ml of extraction medium, ground with food processor in low speed for 3 seconds and extracting at various extraction methods, temperature and extraction duration as shown in table 2. The extract was then filtered with filter paper (Whatman no. 4). De-pulping processes were carried out for fresh unfermented cocoa beans and freeze dried prior to extraction. Each treatment was carried out in triplicates.

Total phenolic content (TPC) determination

The extract was diluted six folds with distilled water. One millilitre of diluted extract was added with 2.5 ml Folin-Ciocalteu reagent and 2.0 ml 7.5% sodium bicarbonate. The mixture was vortexed and incubated at 45°C for 15 minutes prior to measure with UV-visible

Extraction medium	Extraction method	Temperature (°C)	Duration (min)
Aqueous	Incubation at 150 rpm stirring rate	40, 60, 80	5, 10, 15
Aqueous	Sonication (sonicator probe with ½" hon at 20KHz)	40, 60, 80	5, 10, 15
Ethanol: water (30:70; 55:45; 80:20)	Incubation at 150 rpm stirring rate	40, 60, 80	5, 10, 15
Ethanol: water (30:70; 55:45; 80:20)	Sonication (sonicator probe with ½" hon at 20KHz)	40, 60, 80	5, 10, 15

Table 2: *Extraction parameters.*

spectrophotometer at 750 nm. A standard curve of gallic acid with a series concentration of 0 to 0.5 mg/ml was prepared. Total phenolic content, C in mg GAE/g sample, was calculated based on the equation below [5]:

C (GAE/g sample) = k x c x V/M {1}

Where

k = Sample dilution factor

c = Concentration determined from standard curve (mg/ml)

V = Volume of extraction medium (ml)

M = Sample weight used for extraction (g).

Free radicals scavenging capability

Free radicals scavenging capability of the extract were determined by drawing 0.5 ml sample added with 5.0 ml 0.06 mM DPPH solution, mixed well and incubated in dark for 30 minutes prior to measure with UV-Visible spectrophotometer at 520 nm. Percentage of free radicals scavenging capability was calculated as the equation below [5]:

RSC(%)= [(Abs(c)- Abs(S)/Abs(c)] x 100 {2}

where:

RCS = DPPH radical scavenging capability

Abs(c) = Abs for control

Abs(S) = Abs for sample.

Results and Discussions

Extractable Total Phenolic Content (TPC) of cocoa beans along the processing chains from fresh beans to roasting were monitored with various of extraction methods and its free radicals scavenging capability based on DPPH assay were conducted.

Extractable total phenolic contents (TPC)

Table 3 below is the results from principle component analysis of extractable TPC from different processing stages of cocoa beans (Fresh = fresh beans extracted from pod; Dry = Dried beans after sufficient fermentation and sun drying; Roasted = well roasted beans).

Principle component 1 (PC1) has considerable high loadings on plant materials showed that the processing chain of cocoa beans gave significant impact on its extractable TPC. Extraction methods, whether incubation or sonication; extraction temperature and effect of solvent (ethanol) were playing substantial roles in cocoa beans extractable TPC as shown in PC2, PC3, and PC4 respectively with the highest absolute loading values. PC5, PC6 and PC7 had an eigenvalue less than 1 and hence were not discussed.

Eigenvalue	2.6258	1.4325	1.1197	1.0000	0.4510	0.2727	0.0983
Proportion	0.375	0.205	0.160	0.143	0.064	0.039	0.014
Cumulative	0.375	0.580	0.740	0.883	0.947	0.986	1.000
Variable	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**
Fresh	0.496	0.451	0.017	0.000	0.052	0.205	0.711
Dry	0.470	-0.321	0.256	-0.000	-0.434	-0.644	0.086
Roasted	0.502	-0.239	-0.268	0.000	-0.389	0.596	-0.336
Temperature	0.214	-0.142	-0.809	0.154	0.372	-0.341	0.032
Period	0.295	-0.214	0.420	0.615	0.526	0.114	-0.151
Extraction	0.363	0.567	0.100	-0.341	0.227	-0.207	-0.571
Solvent	0.130	-0.500	0.143	-0.694	0.437	0.127	0.154

Table 3: *Principal component analysis: fresh, dry, roasted, temperature, period, extraction methods (Eigen analysis of the correlation matrix).*

Analysis of variance showed no significant differences in term of extractable TPC between fresh beans and dried beans. However, extractable TPC was significantly lower from the roasted beans (Figure 1).

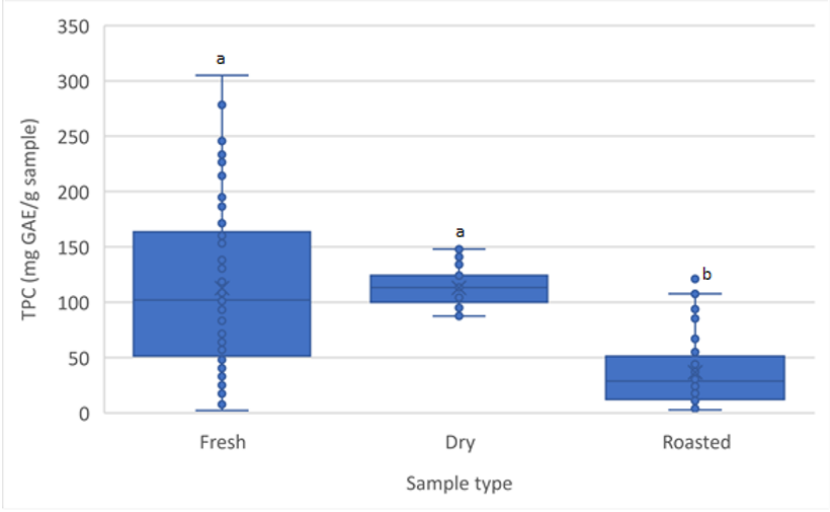

Figure 1: *Extractable total phenolic contents (TPC) in mg GAE/g sample from fresh cocoa beans, dried cocoa beans and roasted cocoa beans. Fresh cocoa beans had an average extractable TPC value of 102 mg GAE/g sample; dried cocoa beans had 113 mg GAE/ g sample; and roasted cocoa beans had 29 mg GAE/g sample.*
Note: Samples with same letter were not significantly different at $p > 0.05$.

High standard deviation observed on fresh beans samples were expected due to difference extraction methods. Polyphenols are stored in the 'polyphenols storage cells' of the cocoa cotyledons. Aqueous incubation methods based on passive diffusions gave lowest amount of extractable TPC compared to sonication methods which are able to penetrate through the cells and extracting from its within.

During fermentation, sugars in the pulp are transformed to acetic acid and lactic acid by environmental microorganisms and, acidification of the cocoa beans occur [6]. Proteolytic processes start after 1 - 3 days of fermentation when the beans are killed by opening of the shell (testa) at the micropyle caused by acidification of the pulp and increased temperature, thereby enabling penetration of the nib by acetic and lactic acids [3]. The extractable TPC results of the study showed no significant differences between fresh cocoa beans and well fermented dried cocoa beans suggested that during fermentation, no significant reaction on polyphenols is taking place. It is believed that during fermentation, polyphenols are just diffused out with cell liquids from their storage cells and stayed in within the matrix. It was further supported by the facts that dried beans samples were having small standard deviation (Figure 1), whereby, difference extraction methods were applied.

These polyphenols, together with the hydrophilic peptides and hydrophobic free amino acids from the progressive alteration of cocoa protein by aspartic endoprotease and carboxypeptides, are to form cocoa specific flavour precursors [7]. A significant drop of extractable TPC in roasted cocoa beans (Figure 1) indicates phenolic tanning or oxidation occurs in roasting processes. Cocoa beans are roasted to develop further the chocolate flavour, which should already exist in the form of precursors arising from the correct fermentation and drying of the original beans.

Sonication methods generally gave higher TPC value compared to incubation methods. Optimum extraction temperature and solvent strength were 80°C and 55 ethanol: 45 water respectively (Table 4).

	Means	Std deviation	P value
Extraction Methods			0.0000
Incubation	51.49	14.25	
Sonication	167.60	38.86	
Solvent effect (% ethanol)			0.0140
0%	107.12	34.98	
30%	116.52	53.70	
55%	146.05	73.14	
80%	68.62	43.74	
Incubation temperature (°C)			0.0356
40	97.80	47.80	
60	103.96	52.88	
80	127.00	48.16	
Extraction duration (min)			0.0860
5	85.24	45.82	
10	111.36	57.50	
15	132.13	61.61	

Table 4: *ANOVA table for extraction methods, solvent effect, incubation temperatures, and extraction duration on the extractable TPC (mg GAE/g sample) from fresh cocoa beans.*

Sonication uses ultrasonic sound energy to induces microstreaming effect on a liquid (extraction medium) containing crushed cocoa beans sample. These increases the permeability of the samples' cell walls and produces cavitation, and thus, results in the cell wall destruction, and consequently, provide better contact and interactions of extraction medium in and out of the plant materials [8,9]. Thus, increases of mass transfer in sonication treatment explained better extraction yield compares to incubation method.

Existence of solvent (ethanol) in the water will alter its overall polarity to less polar, and thus, gave better solubility towards polyphenols. Increasing of solvent content was able to improve extraction yield by giving higher extractable TPC value. Nevertheless, results showed that 55% of ethanol in water was having the best polarity in extracting polyphenols in cocoa beans.

Higher temperature provides higher energy in general. However, too high the extraction temperature may degrade the polyphenols. Extraction temperature below 40°C might not good enough because of low extractable TPC.

Free radicals scavenging capability

Free radicals scavenging test by DPPH assay showed that the extract from fresh cocoa beans, well fermented dried cocoa beans, and cocoa roasted beans were respectively able to inhibit 78.31%, 77.27% and 60.17% of the available free radicals in 0.06 mM of DPPH reagent (Table 5).

Sample	Means (%)
Fresh cocoa beans	78.31 ± 1.373[a]
Well fermented dried cocoa beans	77.27 ± 1.427[a]
Roasted cocoa beans	60.17 ± 0.297[b]

Table 5: *Free radicals scavenging capability of 0.5ml samples extract (0.02 g/ml) in 5.0 ml 0.06 mM DPPH*

Note: Means value with the same alphabet were not significantly different at p > 0.05 by Turkey comparison.

Statistically with Turkey comparison method showed that the extract from fresh cocoa beans and well fermented dried cocoa beans were having same capability in free radicals scavenge. These was in line with the results shown above that both were having similar amount of extractable TPC. A significant dropped of free radicals scavenging capability in roasted cocoa beans is expected as roasted cocoa beans had less extractable TPC.

The absolute half maximal inhibitory concentration (IC_{50}) is a quantitative measure on how much of the cocoa extract in inhibiting, *in vitro*, free radicals availability in the test medium by 50% [10]. Based on 5 ml of 0.06 mM DPPH assay, results showed IC_{50} for both fresh cocoa beans and well fermented cocoa dried beans were about 4 mg cocoa/ml extract whereas roasted cocoa beans needed higher concentration of about 18 mg cocoa/ml (Figure 2).

Conclusion

This study revealed that fresh cocoa beans and well fermented dried cocoa beans gave comparable amount of extractable total phenolic contents (TPC) indicated no substantial reactions on phenolic compounds during the phases of fermentation and drying of cocoa

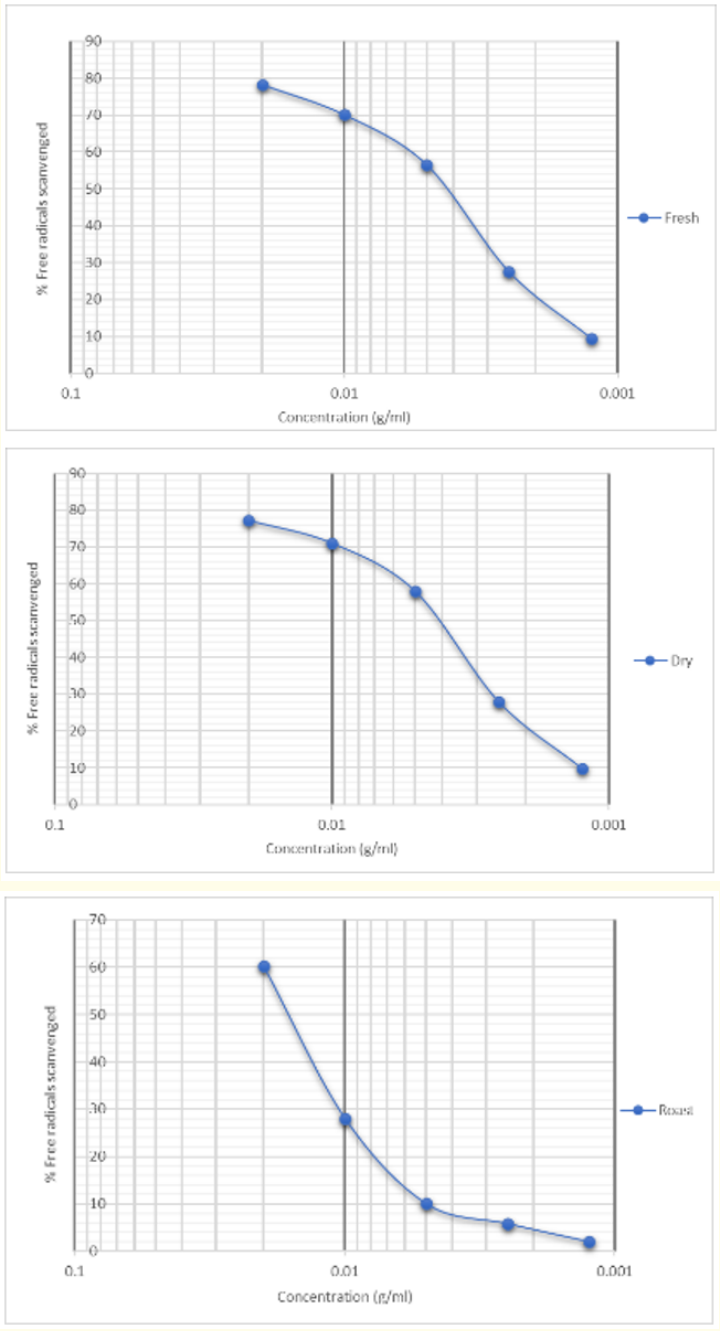

Figure 2: *Plot of % free radicals scavenged vs log (concentration) for fresh cocoa beans (Fresh); well fermented dried cocoa beans (Dry); and roasted cocoa beans (Roast).*

beans. A significant drop of extractable TPC on the roasted cocoa beans suggested that phenolic tanning or polyphenols oxidation occurs in roasting process. In term of its free radicals scavenging capability, both fresh cocoa beans and well fermented dried cocoa beans required lower concentration of extract compared to roasted cocoa beans to achieve its IC_{50}.

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Volume 15 Issue 11 November 2020