Effect of Different Solvents on Phytosterols and Antioxidant Activity of Cocoa Beans

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***Abstract*—Cocoa beans are rich in a number of beneficial bioactive compounds that are good for health. However, they have not been exploited to their fullest to produce high quality extract containing stable bioactive compound. The main objective of this study was to determine the effectiveness of the different solvent used for extraction on yield of cocoa butter extracts, oxidative value, phytosterols content and antioxidant content of Malaysia cocoa beans. The cocoa beans were subjected to different types of solvents; Hexane (HE), Petroleum ether (PE), 2-propanol (PR), Ethanol (ET). Cocoa butter extracted from HE has significantly ($p<0.05$) high yield of extract; 43.24% and lower oxidative content; PV (1.46 meqO_2/kg) and AV (1.57 mg KOH/g oil). Whereas, the phytosterols and antioxidant value was significantly ($p<0.05$) higher in ET (4974 mg/100 g of extract, DPPH; 64.87% and TPC; 22.38 mg GAE/100 g of extract). This study is important to enhance the quality of cocoa butter extracts.**

***Index Terms*—cocoa beans, hexane, petroleum ether, 2-propanol, ethanol, phytosterols, antioxidant assay**

I. Introduction

Similar to palm kernel, groundnut, sesame seed, or any other oilseeds, cocoa beans are also natural oilseeds. The processing involve in order to obtain the oils from cocoa beans is different from other oilseed [1]. Many researchers described that processing method of cocoa beans was greatly determined the physicochemical constitution and the characterization of cocoa matrix [2]-[7]. The major portion of cocoa beans is cocoa butter which is about 50-57% [8].

Cocoa butter is natural and highly valued oil which contribute to unique textural characteristics for chocholate and contain bioactive compounds, such as tocopherols and phytosterols [9]-[11]. These natural compounds such as phytosterols and antioxidant show increasing attention due to their capabilities towards health benefits today. Phytosterols has been able to block cholesterol absorption sites in the human intestine, thus it can help to reduce cholesterol in humans [12]. About 51.48% of beta-sitosterol, 39.45% of stigmasterol, 5.77% of campesterols and 3.29% of cholesterol were present in the cocoa butter from Egypt [13]. Whereas, abundant antioxidants such as phenolics compound in cocoa beans are able to secure organisms from negative free radicals and other reactive forms of oxygen. These compounds are able to enhance the consistency of storage lipids and thereby determine the nutritive values of processing products by inhibiting oxidation of fatty acids [14], [15].

The extraction of oil using different type of solvents may produce oils with improved quality characteristics. The goal of this research was to investigate the effectiveness of the different polarity of solvents used during extraction towards yield of cocoa butter extracts, oxidative value, phytosterols content and antioxidant value of Malaysia cocoa bean.

II. Materials and Methods

The cocoa beans were purchased from Lembaga Koko Jengka, Pahang, Malaysia. Cocoa shells were removed manually from cocoa beans and the nibs were analyzed after smashing with a mortar and pestle. The nibs were stored in a dark closed container to prevent from high humidity and light.

A. Extraction Procedure

Soxhlet extraction was performed using a 120ml round bottom flask. Approximately 5g of cocoa nibs was weighted into the thimble. Then, 150ml of solvent (Hexane, Petroleum ether, 2-propanol and Ethanol) was added and refluxed for 6h using Soxhlet apparatus. The extracts were filtered and concentrated under reduced pressure until dried [16].

B. Oxidative Analysis

Iodine value (IV), Peroxide value (PV) and Acid value (AV) were determined according to AOCS method [17]-[19].

C. Phytosterols

The phytosterols composition of the cocoa butter was determined according to Careri *et al.* [20]. About 200mg

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of cocoa butter was added into 50ml round bottomed flask followed by the addition of 250µl of 5-α cholestane (1000µg/ml) in methanol as internal standard and 2ml of 2M KOH in methanol. The mixture was heated under reflux at 90 ℃ for 1h. After that, 4 ml of water and ethyl acetate were added to the mixture. The aqueous phase was washed three times with diethyl ether. Finally, the diethyl ether solution was dried with sodium sulfate anhydrous, filtered and then dried with a nitrogen stream.

Solid phase extraction (SPE): SPE was applied to perform the cleanup procedure. Silica gel (packing 500mg/6ml, Silicycle) SPE tubes were used and the mixture was dissolved in 5 ml hexane-ethyl acetate (95:5, v/v) and 6ml of hexane-ethyl acetate (60:40) v/v) to perform washing step. The eluate was dried under a nitrogen stream and resolved in 1ml of hexane. The extracts obtained were analyzed by HPLC with UV detection (HPLC-UV). HPLC Waters system consisted of an autosampler and a spectrophotometric UV-Vis variable-wavelength detector. Separation was carried out using C_{18} column (150×2.1mm, 5µm) (Silicycle) under isocratic condition with a mixture of acetonitrile-water (86:14, v/v). The operative wavelength was set at 200.4nm. The injection volume was 1µl. The samples were injected triplicate.

D. Antioxidant Content

The determination of antioxidant content in cocoa butter extract was described as follows to determine the content of antioxidant by DPPH assay and Total Phenolic Content (TPC). Approximately 10ml Erlenmeyer flask was filled with 1±0.01 g of cocoa butter and a solution of methanol in water (90:10). Then the solution was placed in an ultrasonic bath for 5 min. Subsequently, the sample was centrifuged for 10 min at 4,000 rotations per minute. The extraction procedure was carried out three times. All methanol extracts were combined, filtered and treated by rotary evaporator to dryness. The extracts were kept at -20 ℃, and just before spectrophotometric determination, they were mixed with 2.5mL of a methanol-in-water solution (10:90) and centrifuged as previously. The analysis was done in triplicate [21].

DPPH assays*:* For the DPPH antioxidant assay, 3.2mg of DPPH powder was diluted in 80% methanol. 3.9ml of methanol DPPH was added to 0.1ml of cocoa butter extract. The sample was kept in dark for 2h. The absorbance was measured at 517nm.

Total Phenolic Content (TPC): For TPC analysis, 500µL Folin-Ciocalteu and 1.5ml sodium carbonate (20%) were added in 100µL of cocoa butter extract. The sample was incubated in darkness at room temperature for 90min. The absorbance was measured at 730nm. A calibration curve was obtained against standard Gallic acid. The results were expressed as mg of Gallic acid per 100g of oil.

III. Results and Discussion

A. Yield Determination

Yield percentage was determined to measure which solvent and technique producing high content of cocoa butter extract. Yield of cocoa butter extracted using HE solvent was insignificantly different ($p>0.05$) from PE, 43.24% and 39.31%, respectively, but higher than PR (15.05%) and ET (14.25%) (Table I). This shows that HE and PE were able to extract more cocoa butter compared to others. The variation in the oil content was due to the use of solvents of different polarities. Nonpolar solvents like HE and PE gave higher oil yields compared to polar solvent such as PR and ET. HE and PE showed significantly ($p<0.05$) higher oil yields (43.24% and 39.31%) while PR and ET significantly ($p<0.05$) showed lower oil yields (15.05% and 14.25%). HE is considered the most suitable solvent for oil extraction and has been used extensively by many researchers for cocoa butter extraction [22], [23]. It is also the most widely used to extract edible oils from plant sources since it is an excellent solvent in terms of oil solubility and ease of recovery [24]. In contrast, ethanol has long been used for vegetable oil extraction as it has a GRAS (Generally Recognized as Safe) status. However, ethanol also extracts carbohydrate along with the oil [25].

TABLE I. Yield Percentage and Oxidative Analysis of Different Types of Solvents

Solvent	Yield (%)	IV (gI_2/100g)	PV (meq O_2/kg)	AV (mg KOH/g oil)
HE	43.24 ± 0.86^a	33.31 ± 0.53^a	1.46 ± 0.10^c	1.57 ± 0.01^b
PE	39.31 ± 0.53^a	33.49 ± 1.23^a	1.83 ± 0.04^b	1.58 ± 0.01^b
PR	15.05 ± 0.93^b	33.90 ± 1.32^a	1.99 ± 0.09^b	1.62 ± 0.01^a
ET	14.25 ± 0.30^b	34.10 ± 1.39^a	2.25 ± 0.09^a	1.63 ± 0.02^a

B. Oxidative Analysis

Table I shows the IV of the extracted cocoa beans from different types of solvent which range from 33.31 to 34.10g I_2/100g. The IV was not significantly different ($p>0.05$) and unaffected by the polarity of the extraction solvents. The initial PV of the extracted cocoa butter showed a significant increase ($p<0.05$) from 1.46 to 2.25meqO_2/kg with the increase in polarity of the extraction solvents (Table I).

The initial PV of the cocoa butter extracted with polar solvents was lower than that of non-polar extracts. This could be due to the presence of higher amounts of natural antioxidants in polar solvent extracts [26]. However, they also suggests that the presence of Maillard reaction products in polar solvent extracts which subsequently form melano-phospholipids have the ability to inactivate hydroperoxides formed during oxidation and contribute to the lower initial PV of polar solvent extracts [27], [28]. The AV of the extracted cocoa butter (1.57-1.63mg KOH/g oil) showed a significant increase ($p<0.05$) with the increase in polarity of the extraction solvent (Table I). The AV content of cocoa butter extracted with polar solvents was higher than that of oil extracted by non-polar solvents because AV is more soluble in polar solvents [26]. Moreover, the polar solvents possibly more efficient to hydrolyze the bonds of the free fatty acid from parent molecule [26].

C. Phytosterols

The total phytosterols significantly increased ($p<0.05$) with the polarity of the extraction solvents (Table II). The

cocoa butter extracted with polar solvents showed significantly higher ($p<0.05$) proportions of this bioactive compound as compared to the cocoa butter extracted by non-polar solvents. The total phytosterols ranged from 4503mg/100g of extract in HE to 4974mg/100g of extract in ET with the other type of extracts fell within this range. [26] reported that hexane usually extracts the non-polar lipids and very few polar lipids. As phytosterols acts as polar lipids, the ET was a selective solvent that extract phytosterols higher compared to others.

TABLE II. PHYTOSTEROLS CONTENT OF DIFFERENT TYPES OF SOLVENTS

Solvent	Phytosterol composition (mg/100g of extract) ± SD			
	Campesterol	Stigmasterol	Beta sitosterol	Total
HE	1600 ±1.06^{d}	1302 ±0.14^{b}	1600 ±0.28^{b}	4503 ±1.48^{d}
PE	1677 ±0.35^{c}	1358 ±0.28^{b}	1473 ±0.21^{d}	4508 ±0.85^{c}
PP	1687 ±0.07^{b}	1368 ±0.07^{b}	1483 ±0.14^{c}	4528 ±0.28^{b}
ET	1925 ±0.06^{a}	1404 ±0.06 ^{a}	1645 ±0.06 ^{a}	4974 ±0.18^{a}

D. Antioxidant Content

The study by [29] shows the value of antioxidant assay (AA) from ethanol extraction was 67.6%. The ET method of extraction used in this study showed similar AA in the cocoa extract 64.87% (Fig. 1). Whereas, significantly the lowest AA ($p<0.05$) was found in PE (48.62%). Previous study reported that antioxidant activity and the yield of phenolic content were influenced by different extracting solvents [30]. For example, a water extract of *Terminalia chebuta* showed good antioxidant activity, compared to methanolic extracts of *Lycopersicon esculentum* [31]. Moreover, from a toxicological point of view, ethanol and water are safer than acetone, methanol and other organic solvents [32]. The recovery of polyphenols from plant materials is influenced by the solubility of the phenolic compounds in the solvent used in the extraction process [33].

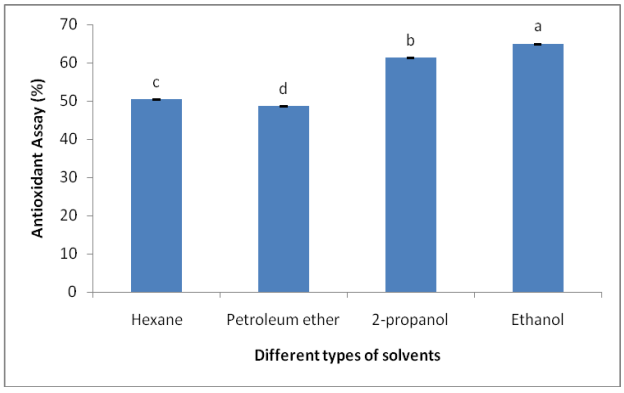

Figure 1. DPPH assay values of different types of solvents

Solvent polarity plays a key role in increasing phenolic solubility [34]. Obtained results showed that TPC generally increased by increasing polarity of solvents (ET>PR>PE>HE). The highest TPC in cocoa beans was extracted using ET (22.38mg GAE/100g of extract) (Fig. II). Ethanol and water mixtures are commonly used for the extraction of phenols from plant materials [35], [36]. [33] studied the Soxhlet extraction, where the highest TPC was found in *H. Sendtneri* (*Boiss.*) extracted using 96% ethanol, which agrees with cocoa beans results. This is due to the wide range of phenols able to dissolve in aqueous ethanol mixtures. Furthermore, ethanolic mixtures have been well accepted for human consumption [36].

The contrary results can be found in literature. Fresh leaves of *C. Siliqua* extracts presented the best TPC with solvents hexane and ethyl acetate [37]. Results showed that solvent significantly ($p<0.05$) influence DPPH assay, but extraction methods do not have significant ($p>0.05$) influence. Literature data showed that the DPPH assay differs depending on used solvent and food matrix. Anti-radical activity of cocoa beans differed significantly (64.87%) depending on the solvents used and the highest activity was determined in ET (Fig. 2).

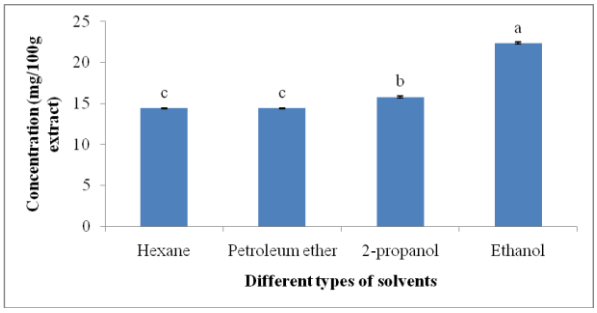

Figure 2. TPC values of different types of solvents

IV. CONCLUSION

The yield of cocoa bean extract, oxidative values, phytosterols composition and antioxidant content of Malaysia cocoa beans was affected by using different type of solvent. ET was able to extract significant high yield of phytosterols (4974mg/100g of extract), TPC (64.8mg GAE/100g of extract) and AA (22.38%) compared to other solvents. However, the cocoa butter extract and oxidative values result showed that HE was more effective to produce high amount of cocoa butter (43.24%) with lower oxidation values (PV, 1.46 meqO_2/kg; AV, 1.57mg KOH/g oil). The utilization of ET has proven to be more effective than HE, PE and PR in obtaining high phytosterols and antioxidant activity from plants. Furthermore, ET was widely used in extraction due to its GRAS status and selected due to its ability to dissolve a wide range of phenols compounds in the cocoa butter extract. ET showed better results compared to other solvents in terms of extracting higher amount of phytosterols and antioxidant content.

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