PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

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Pakistan Journal of Nutrition 11 (7): 672-677, 2012
ISSN 1680-5194

Quantification of Total Polyphenolic Content and Antimicrobial Activity of Cocoa *(Theobroma cacao L.)* Bean Shells

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Abstract: This study evaluated the total Phenolic Compound Content (PC) and biological activity of Cocoa Bean Shell (CBS). Proximate compositions of the dry matter (CBS) were investigated, while the antimicrobial activity was of CBS phenolic extracts in 80% acetone, ethanol, methanol and water performed by paper disk diffusion and micro broth dilution methods against 4 bacterial strains. The results showed that CBS consisted of mainly dietary fiber (60%), followed by protein (16.93%), fat (6.87%), polyphenols (4.85%) and moisture (3.73%). The extracts inhibition zones diameter against the tested strains ranged from 16.1 to 9.19 (mm) and were all significantly higher ($p<0.05$) than the negative control. The minimum inhibitory concentration ranged from 0.78 TPmg/mL to 2.58 TPmg /mL. The relatively high levels of dietary fiber coupled with associated phenolics implies that this by-product might be of interest to the food industry, considering its potential application as a functional ingredient in confectionery, bakery or in the preparation of low-fat, high-fibre dietetic products.

Key words: Cocoa shells, phenolic content, antimicrobial activity, proximate

INTRODUCTION

Bioactive compounds from plants' sources such as phenolics have gained substantial interest in recent years owing to their unique functions and nutritional values including antioxidant, antimicrobial, antimutagenic and antitumor activities. Antioxidants are bioactive micro-components present in food, having the ability to retard or impede lipid oxidation. Free radicals have been implicated for aging as well as food and chemical deterioration. Substantial literatures have been published demonstrating, that plant polyphenolic compounds scavenge free radicals, thereby protecting humans against oxidative damage and free radical related diseases (Beecher, 1999; Cicerale *et al*., 2009; Okawa *et al*., 2001). Recently, the use of synthetic additives as antioxidants in food has come under heavy criticisms due to their undesirable consequences on human health (Goli *et al*., 2005). In order to minimize or eliminate the use of these artificial antioxidants in food, essential oils or plant extracts as natural antioxidants have been used as agents against the proliferation of microorganisms or protect food from oxidation (Bubonja-Sonje *et al*., 2011).

Cocoa beans and their related products have been extensively investigated in relation to their antioxidant and health promoting activities (Abbe-Maleyki and Ismail, 2010; Othman *et al*., 2007). The raw cocoa bean total polyphenols is reported to contain 60% of both flavanol monomers (epicatechin and catechin) and procyanidin oligomers (dimmer to decamer). These compounds are implicated as potential candidates to combat free radical (Adamson *et al*., 1999). However, most of these studies have concentrated on cocoa powder and its related products such as chocolate, with far less attention on the cocoa processing by-products which could be a cheap source of antioxidants. Moreover, conflicting reports and observations exist on the influence of the antimicrobial activities of cocoa polyphenols (Percival *et al*., 2006) probably due to insufficient studies involving cocoa phenolics in the area. Meanwhile, accumulating evidence in relation to ability the ability of polyphenolics from other plants' sources to inhibit microbial growth has been reported (Njume *et al*., 2011; Oonsivilai *et al*., 2008) Considering, its readily availability coupled with reports that CBS, as by-product, several tones of it are disposed as waste every year (Redgwell *et al*., 2003), it stimulate the thinking CBS could be a cheap source of polyphenolic compounds for food fortification. The extraction and quantification of these phenolic compounds are there necessary to allow assessment and eventual value added utilization of these cocoa processing waste products.

Few published reports are available regarding the antimicrobial power of cocoa and its' related materials polyphenolic extract, especially CBS. Consequently, the study was initiated to investigate the proximate composition of cocoa bean shells as well some

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properties related to its nutritional quality, such as its polyphenolic content and antimicrobial properties of the polyphenolic extracts using different solvents (water, ethanol, methanol and acetone).

MATERIALS AND METHODS

Cocoa Bean Shells (CBS) and chemicals: The CBS was obtained from Jiangsu Linzhi Shanyan Group Co. Ltd, (Wuxi, China). The samples were air-dried, comminuted by laboratory grinder (DFY 200, Wenling LINDA Machinery Co., Ltd, China) and passed through a 60 mesh. The powdered CBS was packed in polyethylene bags and kept in a desiccator till further use. Catechin, gallic acid and Folin-Ciocalteu reagent were purchased from Sigma Adrich Chemical Co (St Louis, MO, USA). All other chemicals and standards used were of analytical grade.

Proximate analysis: The total nitrogen content of the CBS was determined by Kjedahl method (AOAC, 1995).The crude protein was computed by multiplying the nitrogen content by a factor of 6.25. The moisture, dietary fiber and ash contents were determined based on previous method (AOAC, 1995). Soxhlet method using petroleum ethers (30°C-60°C) was used to estimate the fat content (for 8 h). Dietary fiber (soluble and insoluble dietary fibers) was estimated in powdered Cocoa Bean Shells (CBS) (AOAC, 1995). The total dietary fiber was calculated as: the sum of soluble dietary and insoluble dietary fiber.

Sample preparations and extraction: The powdered sample prior to extraction was defatted twice with petroleum ethers (b.p.30°C-60°C) and n-hexane successively using soxhlets apparatus for 12 h and the solvent removed by air drying for 3 h. The pulverized, defatted CBS mass was extracted in 250 mL (50 ml solvent: 2 g CBS) beaker with acetone, ethanol, methanol [80% (v/v)] and de-ionized water using a laboratory scale microwave extraction apparatus (WP800SL 23-2 MZG 1500S-Microwave). The microwave power was set to 500 W for 5 min and the temperature was controlled (<50°C). The phenolic extracts were separated from the solid mass by filtration (Watman's No. 1). Each filtrate obtained was collected into a graduated cylinder and the volume adjusted up to 50 mL with the same extracting solvent in order to estimate the total polyphenol, total flavanoid contents and other minor constituents as well as assayed for antioxidant activity test. For the antimicrobial studies, the solvent of each extract was evaporated to dryness to yield 252.21 mg, 221.42 mg, 238.86 mg and 208.24 mg dry extracts for acetone, ethanol, methanol and water and respectively. 200 mg of each dry crude extracts was re-dissolved in 2 mL Dimethyl Sulfoxide (DMSO) and each kept at -20°C until antimicrobial tests were performed.

Total polyphenolic compound content determination (TP): The TP content was calorimetrically estimated using Folin Ciocalteu reagent according to (Sultana *et al*., 2009) with minimal adjustments. Briefly, 0.04 mL of each crude extract was mixed with 0.2 mL Folin-Ciocalteu reagent in 10 mL test tube. The mixture was allowed to rest for 5 min before addition of 0.5 mL sodium carbonate (20%). The volume was adjusted to 5 mL with distilled water and vortexed for 10 sec. The mixture was incubated at 45°C for 30 min and cooled to room temperature before reading the absorbance at 750 nm using a UV spectrophotometer (2802 UV). The TP contents were quantified and expressed as Gallic Acid Equivalent (GAE) mg/g CBS dry weight basis (dw) CBS. The range of a calibration curve was from 0.01-0.1 mg/mL with R^2 = 0.9990.

Determination of Total Flavonoids content (TF): The TF content was determined by the aluminum chloride colorimetric assay (Sultana *et al*., 2009). An aliquot of the extract (60 µL) was added to 10 mL test tube already containing 2 mL of distilled water. 5% $NaNO_2$ (0.15 mL was added to each mixture and rested for 5 min before addition of 0.75 mL $AlCl_3$ (10%). The resulting mixture and allowed to stay for 6 min before adding 2 mL 1.0M NaOH and votexed for 10 s. The absorbance was measured against prepared reagent blank at 510 nm and the TF content expressed as Catechin Equivalent (CE) mg/g CBS, on dw) based on a calibration curve ranging from 50-125 µL) with R^2 = 0.9995.

Other compositional analysis of CBS polyphenolic extracts: The Total Condensed Tannins (TCT), Total Protein (TPC) and Total Carbohydrates (TC) of the polyphenolic extracts of the plant material were performed by calorimetrically using vanillin-HCl assay, Bradford reagent and anthrone assay, respectively. Catechin, Bovine Serum Albumin (BSA) and glucose were the respective standards used for the calibration curves and results expressed as mg of the corresponding standard equivalence/mL extract and standard equivalence/g CBS on dw.

Antimicrobial investigations

Bacterial strains and preparation of the standardized bacterial suspension: All the bacterial strains used in this study were from, Jiangnan University, (Wuxi, Jiangsu, China) microbiology culture collection. The organisms included the following; *E. coli*, *Staphyloccocus aureus*, *Salmonella* and *Bacillus cereus*. General culture medium, Lysogeny Broth (LB) and cultivation condition (37°C aerobic) were used. The bacteria were maintained and tested on Nutrient agar and broth, all from Oxoid (Basingstoke, Hamsphire, England). For experimental use, the organisms were sub-cultured in liquid LB at 37°C for 2 to 8 h after which

the optical density of the bacterial suspensions was estimated using a spectrophotometer at 600 nm. They were then streaked on solid medium in plates and incubated at 37°C for 24 h and the number of bacterial cells extrapolated by counting the colony units formed. Microbial suspensions (10^6) were made in 9% saline for the test. Starting inoculums for all experiments were approximately 10^6 CFU/mL.

Sample preparation for antibacterial activity: Two methods (paper disk diffusion and micro broth dilutions) were employed to investigate the antimicrobial activity studies. Each crude extract previously prepared in Dimethyl Sulfoxide (DMSO) and stored in under -20°C were filtrated through 0.22µ millipore filter to prevent possible contamination.

Disk diffusion method: The disk diffusion method was based on the method of Vander and Vlietinck (1991) with slight changes. Sterile Petri dishes containing solid media were impregnated with each microbial suspension ($1x10^6$). Sterile paper disks (6mm, diameter) made from filter paper (Whatman No. 5) previously soaked in each extract having equal concentrations, (100 mg dried extract/mL) were placed on the agar surface. Disks impregnated with cefradine (0.1 mg/mL) and salt solutions (9%) were used as positive and negative controls respectively. This was followed by incubation at 37°C for 24 h and the diameter of any resulting zone of growth inhibition measured.

Broth microdilution method: The broth microdilution technique was employed to determine the Minimum Inhibitory Concentration (MIC) of the individual extracts against the four bacterial strains used in the study by a Spectrophotometric Microdilution Method (SMM). The extracts (30 mg dried extract/mL in DMSO) were serially diluted with sterile liquid LB in 8 folds to 0.117 mg/ml in 96-well ELISA trays (100 µL in each well). A 100 µL of exponentially growing cultures ($1x10^6$ cfu/mL) were added to each well. The absorbance of each well was determined using an automatic ELISA tray reader adjusted at 600 nm (Softmax Pro 5, SMP 500-16295-0BVN). The plates were incubated at 37°C for 24 h, agitated and the absorbance read again. The differences between the absorbance before and after 24 h incubation were computed. This procedure eliminated the interference of the tested substance. The MICs value for each extract against individual bacterium was expressed as the lowest concentration that inhibits the bacterial growth.

Statistical treatment: All analyses were performed at least in triplicate. The SPSS version 17.0 software package was used for statistical analysis.
Results were analyzed statistically using a 2 sample t-test, assuming equal variances and one-way Analysis of Variance (NOVA).The difference was considered significant if $p<0.05$. Statistical events were performed at a sign.

Table 1: Proximal composition of the cocoa bean shells (% dry matter)

Proximate	%
Moisture	3.73±0.46[a]
Ash	5.96±0.26[b]
Crude fat	6.87±0.52[c]
Crude protein	16.93±0.59[e]
Soluble dietary fiber	11.08±0.05[d]
Insoluble dietary fiber	48.94±1.21[h]
Polyphenols	4.85±0.81[f]

Values within the same column with different superscripts are significantly different at the level of $p<0.05$

RESULTS AND DISCUSSION

Proximate analysis: Proximate analysis is an important index for identification and classification of the nutritional value of a food material. The proximate analyses of CBS are presented on Table 1. Total dietary fiber (soluble and insoluble fibers) was identified as the chief component of CBS accounting over 60% of the dry matter, conforming many previous studies which also reported dietary fiber as the dominate constituent of CBS (Alvarado *et al*., 1983; Bonvehi and Beneria, 1998; Chung *et al*., 2003; Lecumberri *et al*., 2007). These investigators also identified protein as the second dominate compound. The fat content of the present study was lower than that reported by Lecumberri *et al*. (2007) This may be partly linked to the fat acquired from the un-separated cocoa nib adhered to the shell at the period of it separation The content of polyphenols (<5%), which together with the low fat content represent a nutritional advantage to these by-products, with a reduced caloric value and potential bioactivity. The fact that the total dietary fiber content of this the CBS was extremely high, over 60% of the dry matter, implied that this product might be of interest to food industry, considering its potential application as a functional ingredient in confectionery, bakery or in the preparation of low-fat, high-fibre dietetic products.

Total polyphenol, total flavanoid contents and other compositional analysis of extracts: Polyphenolic extract yields and resulting biological activity of plant matter are greatly influenced by the nature of the extracting solvent owing to the presence of different bioactive compounds of different chemical properties and polarities that may or may not be soluble in a particular solvent. Thus differences in polarity coupled with safety reasons informed the choice the four solvents used for the study. Table 2 shows the total polyphenolic and flavonoic contents as well as other constituents of the phenolic

Table 2: TP, TF, TCT, TPC and TC contents of four different solvents' extracts of CBS

	Composition/Quantity				
Extract	TP (mg GAE/g)	TF (mg CE/g)	TCT (mg CE/g)	TPC (mg BSAE/g)	TC (mg GE/g)
Acetone	41.82±1.11^{d}	5.49±0.03^{c}	12.90±0.12^{d}	3.58±0.23^{a}	1.41±0.02^{a}
Ethanol	23.36±1.59^{b}	2.23±0.90^{b}	5.63±0.04^{cb}	2.38±0.01^{b}	1.55±0.04^{a}
Methanol	25.15±0.79^{c}	2.50±0.02^{b}	7.85±0.01^{c}	2.46±0.31^{b}	1.45±0.07^{a}
Water	17.21±0.80^{a}	1.65±0.14^{a}	2.36±0.01^{a}	1.08±0.01^{a}	2.84±0.05^{b}

Values within the same column with different superscripts are significantly different at the level of p<0.05. Values are expressed in terms of mg of the respective standards equivalents per 1 gram of CBS on dry weight basis

Table 3: Antimicrobial activity of different solvents extract of CBS expressed as inhibition zone diameter. Cefradine and saline solution were used as positive and negative controls respectively

	Microorganism/zone of inhibition diameter (mm)			
Solvent	*E. coli*	*Staphyloccocus aureus*	*Salmonella*	*Bacillus cereus*
Acetone	16.10±0.34^{a}	13.11±0.42^{ab}	12.41±0.12^{bc}	15.22±1.28^{ca}
Ethanol	11.23±21^{b}	10.98±0.31^{ac}	11.34±0.53^{bc}	12.21±0.34^{cb}
Methanol	11.15±10^{b}	10.87±0.13^{ac}	11.45±0.16^{bc}	12.04±0.11^{cb}
Water	9.21±0.34^{c}	9.87±0.17^{ad}	9.19±1.21^{bd}	9.54±0.19^{cd}
Cephadex (PC)	35.50	35.00	34.80	36.00
SSNC	7.20±0.13^{d}	6.99±0.09^{af}	7.21±0.26^{bf}	7.07±0.32^{cf}

Values are of triplicate experiments mean ± standard deviations. Results with different upper case letters within columns are significantly different (p<0.05). PC = Positive control, SSNC = Salt solution, negative control

extracts determined in acetone, ethanol, methanol and water extracts respectively. While the least polar solvent (acetone) yielded significantly (p<0.05) higher amount of phenolics (TP = 41.82 mg GAE/g, TF = 5.49 mg CE/g and TCT = 12.90 mgCE/g) than all the other solvents extract tested, the most polar one (water) recorded the least total phenolic contents. The TP was observed to be the major compound and incomparable to TF and TCT as well as the other components in all the four extracts. All solvents were more effective in extracting phenolics than protein and carbohydrate suggesting that the abilities of the solvents to extract these compounds (protein and carbohydrate) were poor.

The differences observed in yields could be related to the polarity of solvent used in the extraction. Since the least polar solvent (acetone) yielded the highest contents of phenolics and that of the most polar solvent (water) recorded the lowest content of the phenolics, it appeared that higher amount of low polar phenolic compounds existed in CBS. Higher extraction yields of phenolics were obtained with a decrease in polarity of the solvent in the extraction of phenolic compounds from barley where it was noted that the yields of phenolics were in the order of acetone>methanol> ethanol>water (Zhao *et al*., 2006). With regard to the TP contents, the current study also demonstrated that CBS contained high amount TP comparable to that of cocoa powder, where Crozier *et al*. (2011) reported the TP content of cocoa powder to be 48.2 mg GAE/g. However, The TF content of the present study was too low and incomparable to the data obtained by Crozier *et al*. (2011) in the cocoa powder (30.1 mg CE/g).

Polyphenols are also well known bioactive compounds that have shown to be protective against diseases like coronary heart disease, cancer, neurodegenerative disorders, etc., mostly through their antioxidant and free radical scavenging capacities (Bravo and Calixto, 1998; Wan *et al*., 2001).

Generally, extracts with high total phenolic contents would be expected to exhibit high antioxidant and antimicrobial activity and contribute significantly to bioactivity of food components (Maisuthisakul *et al*., 2007; Sun and Ho, 2005).

Antimicrobial activities on the basis of the diameter of zone of inhibition: The *in vitro* antimicrobial activity of CBS extracts against the four strains employed in the present study was evaluated by the presence or absence of inhibition zone. Table 3 displays the antimicrobial activity of different extracts of CBS against four bacterial strains expressed as inhibition diameter (mm). As can be seen in Table 3, all the four extracts of CBS exhibited a weaker antimicrobial power against all the tested strains compared to the positive control (Cefradine). While the acetone extract exhibited the highest antimicrobial with a significant difference (p<0.05) potential in terms of the diameter of the zone of inhibition, (12.41 to 16.10 mm) against all tested organisms that of aqueous extract showed the least inhibition effect (9.19 to 9.87 mm). Ethanol and methanol extracts on the other hand, did not vary significantly (p>0.05) from each other in relation to their inhibition potential against the tested microbes. These differences in inhibitory powers observed in the present study could

Table 4: Minimum Inhibitory Concentration (MIC) of polyphenol extracts of CBS against four bacterial strains

	Organism/MIC values (mg of extract/mL)			
Sample	*E-coli* Acetone	*Staphyloccocus*	*Salmonella*	*Bacillus cereus*
Acetone	0.468[a]	0.937[b]	0.937[b]	0.468[a]
Ethanol	1.875[c]	1.875[c]	1.875[c]	1.875[c]
Methanol	1.875[c]	1.875[c]	1.875[c]	1.875[c]
Bacillus cereus	1.875[c]	3.750[d]	3.750[d]	3.750[d]

The values represent means ± standard deviation of triplicates for all tested bacterial strains. Values with different superscripts are significantly different ($p<0.05$)

be attributed to the type and concentration of phenolic present in the individual extracts. This observation is consistent with Njume *et al*. (2011) who noticed that acetone extract offered the highest antimicrobial activity than ethanol, methanol and waters extracts of *Combretum molle*. Bubonja-Sonje *et al*. (2011) observed no antimicrobial activities (effect of inhibition) of cocoa powder phenolic extract by disk diffusion method. However, the data of the present study showed that CBS polyphenolic extracts possess antimicrobial activity based on the same method. This could be due to the differences in concentrations, where the former used very low concentration (0.2 to 6.4 mg/mL) compared to the high concentration used in the latter 100mg/mL). Other factors including the amount of phenolic extract absorbed by the paper disk, the diffusion range in agar and evaporation may also influence the diameter of the inhibition zone which determines antimicrobial capacity.

Minimum Inhibitory Concentration (MIC) of extracts: The MIC of the extracts were assessed by spectrophotometric method defined as the concentration at which there was a sharp decline in the absorbance value are shown in Table 4. The MICs values were visually and spectrophotometrically obtained in order to minimize errors. MIC of acetone extract in most cases was found to be 0.937 mg extract/mL. In the case of methanol and ethanol extracts, MIC values were the same (1.875 mg extract/ml) and that of water extract was 3.750 mg extract/ml (Table 4). Broth micro dilution method was the best approach used for the detection of inhibitory activity detection because it provides accurate results of inhibition at very limited concentration (Ncube *et al*., 2008) in contrast with paper disk where high concentrations are needed.

Conclusion: The results of the present study showed that CBS is of high nutritional value and therefore can be a cheap potential source of many nutrients, especially dietary fiber, protein and polyphenols. It was also revealed that the acetone effectively extract polyphenols better than the other there solvents. However, it was noted that there was no distinct correlation between phenolic levels and antimicrobial powers. Therefore further study to establish this as well as to identify the bioactive compounds responsible for the biological activity will be useful.

ACKNOWLEDGEMENT

This research was supported by a grant from the National Science Foundation of China (number 30871744), the Foundation Research Funds for the central universities (JUSRP11015) and 111 Project-B07029 - PCSIRT0627.

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