การสกัดสารต้านอนุมูลอิสระจากเปลือกเมล็ดโกโก้

EXTRACTION OF ANTIOXIDANT FROM COCOA BEAN SHELL

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บทคัดย่อ

เปลือกเมล็ดโกโก้ (Cocoa Bean Shell, CBS) เป็นวัสดุที่ได้จากอุตสาหกรรมโกโก้ที่มีสารสำคัญหลากหลายชนิด อาทิเช่น โพลีฟีนอลชนิดต่าง ๆ ซึ่งสารเหล่านี้สามารถนำมาพัฒนาเป็นสารออกฤทธิ์ทางชีวภาพในผลิตภัณฑ์ต่าง ๆ ได้ การศึกษานี้ได้ทำการสกัดเปลือกเมล็ดโกโก้ด้วยตัวทำละลายที่มีขั้วต่างกัน ได้แก่ น้ำ เอทานอล ร้อยละ 95 (95% EtOH) และร้อยละ 50 (50% EtOH) และ โพรพิลีนไกลคอล ร้อยละ 100 (100% PG) และ ร้อยละ 50 (50% PG) โดยวิธีสกัดแบบแช่ย่า พบว่าปริมาณฟีนอลิกรวม (TPC) ผลปริมาณฟลาโวนอยด์ทั้งหมด (TFC) และฤทธิ์ในการกำจัดอนุมูลอิสระ (DPPH) และการรีดิวซ์เฟอร์ริก (FRAP) ของสารสกัด 50% EtOH และ 50% PG สูงกว่าการใช้สารละลายเดี่ยว แต่ไม่มีความแตกต่างกันอย่างมีนัยสำคัญ การหาปริมาณสารประกอบโดยใช้การวิเคราะห์ HPLC พบว่าสารสกัด 50% EtOH มีสารคาเฟอีน คาเทชิน และธีโอโบรมีนสูงที่สุดคือ 8.360 0.586 และ 47.606 มิลลิกรัมต่อกิโลกรัม ตามลำดับ และตามด้วยสารสกัด 50% PG คือ 7.992 0.395 และ 38.463 มิลลิกรัมต่อกิโลกรัม ตามลำดับ จากการทดสอบความคงตัวที่อุณหภูมิ 25 องศาเซลเซียส 4 องศาเซลเซียส และ 37 องศาเซลเซียส เป็นเวลา 28 วัน พบว่าสาสกัด 50% PG มีฤทธิ์ต้านอนุมูลอิสระคงตัวมากที่สุด และยังแสดงให้เห็นความสัมพันธ์ของความคงตัวของปริมาณ TPC และ TFC ต่อความคงตัวของฤทธิ์ต้านอนุมูลอิสระ การศึกษานี้จึงสรุปได้ว่า 50% EtOH และ 50% PG เป็นตัวเลือกของสารละลายในการสกัดเปลือกเมล็ดโกโก้ที่เหมาะสมแก่การนำไปศึกษาต่อไป

คำสำคัญ: เปลือกเมล็ดโกโก้ สารต้านอนุมูลอิสระ ความคงตัว โพลีฟีนอล ธีโอโบรมีน

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Received: 15 October 2024; Revised: 18 December 2024; Accepted: 19 December 2024
DOI: https://doi.org/10.14456/lsej.2024.38

Abstract

Cocoa bean shell (CBS) is a by-product from the cocoa industry that contains various phytochemicals that could be applied as functional ingredient development. This study investigated the use of various polarity solvents namely, aqueous, ethanol 95% (95% EtOH) and 50% (50% EtOH), and propylene glycol 100% (100% PG), and 50% (50% PG). CBS extracted by 50% EtOH and 50% PG showed significantly the highest total phenolic content (TPC), total flavonoid content (TFC), free radical scavenging activity (DPPH), and ferric reducing power (FRAP) but not significantly different from each other. While HPLC analysis showed that 50% EtOH extract possessed the highest caffeine, catechin, and theobromine resulting in 8.360, 0.586, and 47.606 mg/kg, respectively in 50% EtOH, followed by 50% PG which were 7.992, 0.395, and 38.463 mg/kg, respectively. The stability testing at 25°C, 4°C, and 37°C for 28 days found that the antioxidant activity of 50% EtOH and 50% PG extracts was highly stable. A strong correlation of antioxidant activities with TPC and TFC was found. This study can conclude that cocoa bean shell extracted with either 50% EtOH or 50% PG are suitable choices for further studies.

Keywords: cocoa bean shell; antioxidant; polyphenols; theobromine

Introduction

Cocoa (Theobroma cacao L.) product market of chocolate, cocoa drinks, and cocoa-derivative or functional foods is constantly growing by approximately USD 14.5 billion in 2022 and is expected to grow at a compound annual growth rate (CARG) of 4.7% between 2023 and 2028 to attain a value of USD 19.1 billion by 2028 (EMR, 2023). Most of them are obtained from cocoa beans after field and industrial processing. The cocoa bean is the common name for cocoa seeds, it consists of an outer shell surrounding two cotyledons and a small germ (Balentić et al., 2018). Cocoa bean shell or CBS the by-products from the cocoa or chocolate industry constitute 12-20% of the cocoa bean weight (Durdica et al., 2020). CBS contains many functional properties, it has high dietary fiber content also known as polysaccharides which are constituted by residues of plant cell walls such as protein, carbohydrates, etc. (Rojo-Poveda et al., 2021). Fat content such as oleic acid, palmitic acid etc. (Dayane et al., 2017). Phenolic compounds are obtained from the secondary metabolism of plants, it is interesting to have hydroxyl

groups as substituents in aromatic compounds which are characterized by their type. Many studies reported that the main compounds responsible for the bifunctional properties of cocoa by-products are flavonol compounds such as epicatechin, and catechin (Wollgast et al., 2000). Methylxanthine compounds such as theobromine and caffeine are also found in cocoa (Mellinas et al., 2020) which have the potential for human health such as antibacterial, antiviral, anticarcinogenic, antidiabetic, or neuroprotective activities, benefits for the cardiovascular system, or an anti-inflammatory and antioxidant (Rojo-Poveda et al., 2021). Some studies have analyzed the use of cocoa-derived phytochemicals as effective approaches for skincare, such as catechin protecting against negative effects by modulating antioxidant enzyme activity. Therefore, exploitation of the potential of cocoa bean shell byproducts by contributing the value should be considered in cocoa waste management.

This study presents a quantitative analysis of cocoa phenolic, flavonoid, and methylxanthines and their activities as well as their stability testing measured by antioxidant assays, in various solvents of CBS extracts. Compare their activity, stability, and correlation. Due to the low energy required, it can be considered an interesting alternative to conventional extraction techniques, showing higher extraction yields, high efficiency, eco-friendliness, low cost, and reduced processing times. It is considered that homogeneous tissue, such as dried powder is suitable for the method's quantitative validation (Zhang et al., 2018). A further objective is also recommended, the inhibition of skin aging associated enzymes, and their toxicity and adding value by using them as ingredients of cosmetic products which cocoa bean shell byproduct is the possible source and gain more interest by industry.

Methodology

Sample and Extraction of plant material

Fermented and sun-dried cocoa bean shell were obtained from a cocoa-producing farm in Chiang Rai. Sample. The extraction method was modified from Rojo-Poveda et al. (2021). Dry materials were ground using a blender until homogeneous particle size was obtained (Philips HR2056). Then transfer 10 g of cocoa bean shell powder into a volumetric flask, and add 100 mL of 6 different solvents separately consisting of DI water, 50% EtOH, 95% EtOH, 50% PG, and 100% PG. Cover the lid with

aluminum foil and shake with an orbital shaker at 125 rpm for 3 hours (WIGGENS orbital shaker WS-50D). Then filtered by using vacuum filters (WIGGENS GmbH, VE-11) and centrifuge at 8000 rpm for 30 minutes (Hanil Science Combi-408 Centrifuge).

Analysis of Caffeine, Catechin and Theobromine by High Performance Liquid Chromatography (HPLC)

HPLC-MS analyses were performed according to the previous method (Caprioli et al., 2020). A liquid chromatography system (Shimadzu, UHPLC Nexera X2) with ACE Excel C18 (2.1*100 mm*17 μm) was used. The binary mobile phase was a gradient consisting of 0.2% formic acid (Pump A) and acetonitrile (Pump B). Caffeine, catechin, and theobromine were quantified using their calibration curve.

Total Phenolic Content (TPC)

The total phenolic content was determined by the Folin-Ciocalteu method (Barbosa-Pereira et al., 2018) with some modifications. Gallic acid was used as a standard. The sample was diluted 10X with DI water, Folin reagent, and 7.5% of sodium carbonate were added respectively. Incubate for 30 minutes at room temperature and then measure at 765 nm using (Mapada, UV-1200). The results were expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g).

Total Flavonoid Content (TFC)

The total flavonoid content was determined by the aluminum chloride colorimetric method (Bhandari et al., 2014). Quercetin was used as a standard flavonoid. The sample was mixed with sodium nitrite (5%) and aluminum chloride (10%) and after 5 minutes, sodium hydroxide (4%) solution was added. Then measured at 510 nm in 8 minutes. The results were expressed as milligrams of quercetin equivalent per gram of extract (mg QE/g).

DPPH scavenging activity

The DPPH scavenging capacity was determined as per a previous report (Tawata et al., 2008). Trolox was used as a standard antioxidant. Incubate for 30 minutes in dark conditions at room temperature and then measure at 510 nm. The results were expressed as milligrams of Trolox equivalent antioxidant capacity per gram of extract (mg TE/g).

Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power was determined by the Ferric Chloride and 2,4,6-Tris-(2-pyridyl)-s-triazine) colorimetric method that was previously applied

(Zengin et al., 2015). For this article, prepared FRAP solution by TPTZ solution (10 mM), ferric chloride (20 mM), and acetate buffer (300 mM) pH 3.6, the ratio 1:1:10 (v/v), respectively. The reaction was incubated for 30 minutes, 37°C, and measured at 593 nm. The results were expressed in milligram equivalents of Trolox antioxidant capacity per g of extract (mg TE/g).

Stability testing

The stability testing was achieved at 3 storage temperatures of 25°C, 4°C and 37°C. TPC, TFC, DPPH, and FRAP assays were monitored on days 0, 3, 7, 14, 21, and 28, respectively.

Statistical analysis

Experiments were done in triplicate and the results were expressed as mean±standard deviation (SD). One-way analysis of variance across the mean of different groups. Paired sample t-tests measure the change at two different times. The Pearson correlation coefficient measures how strong the relationship between two groups of methods is. A $p < 0.05$ denoted statistically significant using IBM SPSS software.

Results

The extractability of six types of solvent against cocoa bean shells was determined and compared. The results in Table 1 showed that 50% PG and 50% EtOH had significantly higher levels of phenolic content, flavonoid content, and antioxidant activities superior to the uses of single aqueous, ethanol, or propylene glycol ($p<0.05$). The TPC values of 50% PG and 50% EtOH were 8.066±0.062 and 7.611±0.067 GAE/g, respectively. Where the TFC values were 28.439±0.517 and 27.223±0.769 mg QE/g, respectively.

Table 1 TPC, TFC, and antioxidant activity of cocoa bean shell extracts from different solvents.

Solvent	TPC (mg GAE/g)	TFC (mg QE/g)	DPPH (mg TE/g)	FRAP (mg TE/g)
DI water	4.669±0.018^{b}	16.508±0.650^{b}	8.172±0.380^{b}	2.151±0.015^{b}
100% PG	0.258±0.058^{c}	0.701±0.140^{d}	1.452±0.199^{c}	0.164±0.010^{c}
50% PG	8.066±0.062^{a}	28.439±0.517^{a}	21.141±1.697^{a}	3.456±0.291^{a}
95% EtOH	0.825±0.042^{c}	1.819±0.000^{c}	1.598±0.097^{c}	0.314±0.021^{c}
50% EtOH	7.611±0.067^{a}	27.223±0.769^{a}	21.041±0.558^{a}	3.496±0.173^{a}

Remark Values are the mean and ± standard deviation (n=3).

Antioxidant activities were determined using radical scavenging capacity assay (DPPH) and ferric reducing antioxidant power assay (FRAP). Extracts of 50% PG and 50% EtOH possessed non-significantly different scavenging capacities of 21.141±1.697 and 21.041±0.558 mg TE/g, respectively. Ferric reducing power activity was 3.456±0.291 and 3.456±0.291 mg TE/g, respectively. In summary 50% PG and 50% EtOH in aqueous provide higher bioactive compounds extractability and thus antioxidant activities.

Table 2 Caffeine, catechin, and theobromine contents in cocoa bean shell extracts by HPLC.

Sample	Caffeine (mg/kg)	Catechin (mg/kg)	Theobromine (mg/kg)
DI water	6.679	0.176	17.824
100% PG	0.515	0.055	17.376
50% PG	7.992	0.395	38.463
95% EtOH	2.087	0.089	11.903
50% EtOH	8.360	0.586	47.606

The extract of cocoa bean shell extracted by different solvents was determined by the quantitative analysis of caffeine, catechin, and theobromine using HPLC-MS as shown in Table 2. The highest caffeine, catechin, and theobromine were presented in 50% EtOH extract followed by 50% PG extract. The results show that mixing water with propylene glycol or ethanol facilitates the extraction of these 3 main compounds.

The high level of extractability of 50% EtOH and 50% PG CBS extracts was chosen to monitor the stability of TFC, TPC, DPPH, and FRAP through storage at 25°C, 4°C, and 37°C. Figure 1 shows the stability of TPC and TFC. Figure 1 a-b and Figure 1c-d shows the stability of TPC and TFC, respectively.

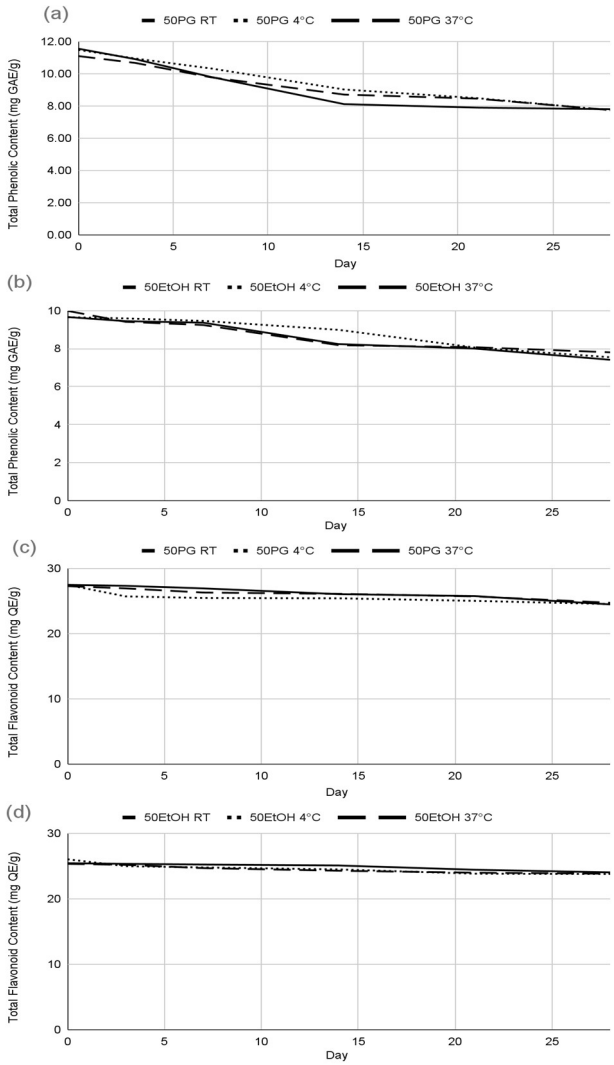

Figure 1 The stability of total phenolic content (TPC) of 50% PG extract (a) and 50% EtOH extract (b). Total flavonoid content (TFC) stability of 50% PG extract (c) and 50% EtOH extract (d).

The stability of antioxidant activities, DPPH, and FRAP are shown in Figure 2e-f and Figure 2g-h, respectively. DPPH scavenging activity and ferric-reducing capacity were shown to be affected by higher temperatures than TPC and TFC. The DPPH 50% PG extract (Figure 2e) showed a 2.03% reduction at 4°C and 9.13% at 37°C. The reducing capacity of 50% PG was decreased as 10.56% at 4°C and 26.33% at 374°C (Figure 2g).

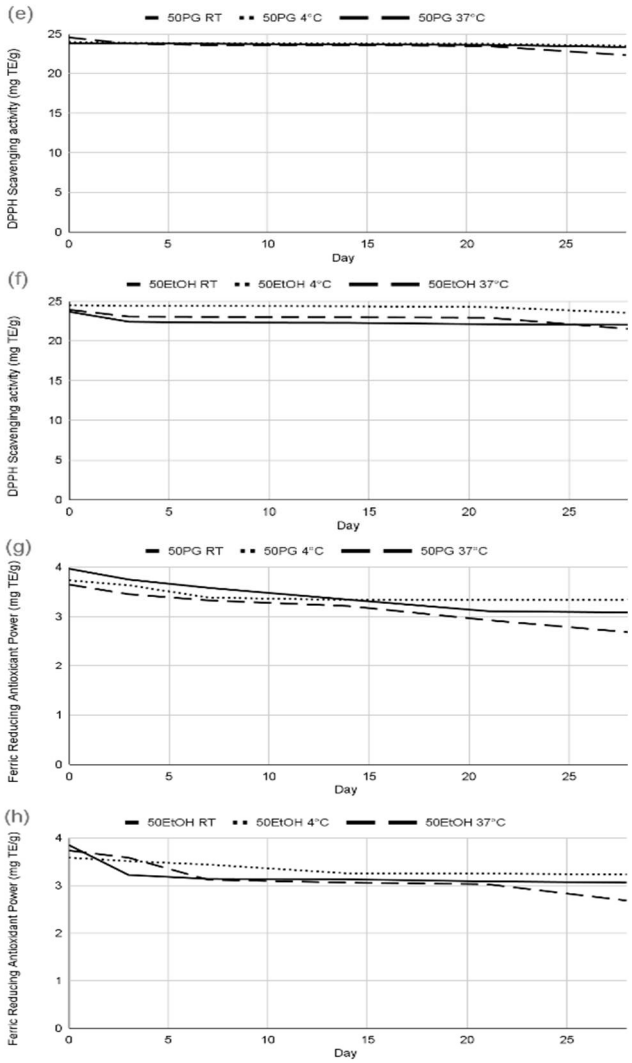

Figure 2 The stability of DPPH radical scavenging activity stability of 50%PG extract (e) and 50% EtOH extract (f). The stability of ferric reducing activity of 50% PG extract (g) and 50% EtOH extract (h).

Discussion

Previous study reported the compound content and antioxidant activity in range 3-43 mg GAE/g of TPC (Rojo-Poveda et al., 2021), 6.44-90.37 mg QE/g of TFC (Chen et al., 2022), 1.57-33.93 mg TE/g of DPPH and 0.67-4.69 mg TE/g of FRAP (Viuda-Martos et al., 2012). Since the main antioxidant components of cocoa bean shell are polyphenol, methylxanthines, and theobromine (Rojo-Poveda et al., 2021) their polarities are varied

and the extraction selectivity is different (Tiwari et al., 2021). So, it can be indicated that the cocoa bean shell extract of this present work mainly contained water-soluble polyphenol which has the relative ability of each other molecules to engage in strong interaction (Kumoro et al., 2021). The excellent result obtained from 50% EtOH, then 50% PG which are the result and suggestion condition extraction by using the combination of polar and nonpolar or lower polar solvent and increasing of solvent polarity, reducing of ethanol ratio by replacing with water can increase the extraction efficiency of phytochemicals with excellent antioxidant activity (Hossain et al., 2020; Nawaz et al., 2020; Thanesuan et al., 2022). Followed by the polar solvent also suggested to extraction of phytochemical compounds when compared to the non-polar solvent.

From HPLC analysis, the most found compound was theobromine followed by caffeine and catechin which is the same order as reported (Osorio et al., 2021). Theobromine and caffeine were detected by 50% EtOH in the highest concentration 47.60 mg/kg and 8.360 mg/kg compared to other reports using 50% EtOH 4.66-9.95 g/kg and 0.84-5.80 g/kg while catechin is 0.586 mg/kg and other was 5.04-48.31 mg/kg followed by 50% PG and hexane was the lowest amount of theobromine while caffeine and catechin were not found. The different amounts of content were found due to the different backgrounds and the preparation of the sample (Todorovic et al., 2017). Apart from those three compounds, cocoa bean shell is also widely reported to contain protocatechuic acid ranging from 12.07-113.08 mg/g, epicatechin range from 30.59-298.00 mg/g (Barbosa-Pereira et al., 2021). Moreover, geographic origin, variety, plant genotype, and even the harvest season (Rojo-Poveda et al., 2021).

Phenolic compound has been observed to degrade at high temperatures (Yi & Bi et al., 2021). Remarkably, another study has shown no significant degradation of phenolic compound was observed after pasteurization at 85°C and also found that the phenolic acid content increased during thermal treatment at temperatures from 25 to 55°C, which can be explained that the thermal treatment caused oxidation as well as hydrolysis of phenolic glycosidic and ester bonds, resulting in the release of phenolic acids. Also, the flavonoid content can tolerate the temperature to 75°C which destroys enzyme activity and blocks the synthesis pathway of flavonoids (Bai et al., 2021). In summary, different temperatures do not affect TPC and TFC. However, antioxidant

activities were significantly lower at high temperatures, which indicates that the stability of antioxidant activity is rather good in low temperatures, provided the structure of polyphenol is relatively suitable and the flavanol structure is much more stable (Zhang et al., 2019). These results suggest that the solvent used affects the extraction of the polyphenol and correlation coefficient, that is to say, 50% PG extract has a higher correlation of TPC and TFC on antioxidant activity than 50% EtOH extract with increasing storage time which is in agreement that polyphenol play an important role in antioxidant activity based on free radical scavenging and reducing power ability and these compound dependent on cocoa processing and its background as well as different in solvent extraction (Todorovic et al., 2017; Gul et al., 2017). Furthermore, 50% PG extraction showed high stability since it is a good choice for the polyphenol compound in short-term storage to provide an efficient way to improve antioxidant activity (Kao et al., 2021).

Conclusion

From this study, the antioxidant extraction of cocoa bean shell depends on the type of solvent. Combining aqueous with either ethanol or propylene glycol at 50% showed superior effects on TPC, TFC, and antioxidant activities. The difference of temperature does not affect TPC and TFC stability much but the lower temperature is rather good for antioxidant activities in both solvents. When considering the correlation between TPC and TFC on the antioxidant activities, 50% PG is the better choice to be the suitable solvent for Cocoa bean shell extraction. In addition to developing as a functional ingredient for the cosmetic industry, further study was recommended to investigate more types of extraction solvents and other bioactive molecules as well as more in vitro and in vivo model testing should be done.

Acknowledgements

Sarita Sangthong received funding supported by National Science, Research and Innovation Fund (NSRF), Thailand through Fundamental Fund 2023 (NRIIS number 178968). Authors would like to thank to the school of Cosmetic Science, Mae Fah Luang university for facilities support.

References

Bai W, Capanoglu E, Simal-Gandara J. Xiao J. Available technology on improve the stability of polyphenols in food processing: review. Food Frontiers 2021; 2:109-139.

Barbosa-Pereira L, Belviso S, Ferrocino I, Rojo-Poveda O, Zeppa G. Characterization and classification of cocoa bean shells from different regions of Venezuela using HPLC-PDA-MS/MS and spectrophotometric techniques coupled to chemometric analysis. Foods 2021;10(8):1791.

Bhandari L, Rajbhandari M. Isolation of quercetin from flower petals, estimation of total phenolic, total flavonoid and antioxidant activity of the different parts of Rhododendron arboreum smith. Scientific World 2014;12(12):34-40.

Caprioli G, Nzekoue Kamgang F, Angeloni S, Navarini L, Angeloni C, Freschi M, Hrelia S. et al. Coffee silverskin extracts: Quantification of 30 bioactive compounds by a new HPLC-MS/MS method and evaluation of their antioxidant and antibacterial activities. Food Research International 2020; 133:109128

Chen W, Cai S, Chan W, Chiang, E, Chee W. Antioxidant and sensory evaluation of cocoa (Theobroma cacao L.) tea formulated with cocoa bean hull of different origins. Future Foods 2022;5,100108

Dayane CG O, Sandra LB N, Christianne EC R. Cocoa shell and its compounds: Applications in the food industry. Trend in Food Science & Technology 2017:63;103-102.

Durdica A, Veronika B, Antu. J, Ivana F, Drago S, Jurislav B, Borislav M, Kristina. D. Difficulties with use of cocoa bean shell in food production and high voltage electrical discharge as a possible solution. Sustainability 2020;12(10), 3981.

Gul R, Jan SU, Faridullah S, Sherani S, Jahan N. Preliminary phytochemical screening, quantitative analysis of alkaloids, and antioxidant activity of crude plant extracts from Ephedra intermedia Indigenous to Balochistan. Scientific World Journal 2017:5873648. doi:10.1155/2017/5873648

Kao TT, Wang MC, Chen YH, Chung YT, Hwang PA. Propylene glycol improves stability of the anti-inflammatory compounds in Scutellaria baicalensis extract. Processes 2021;9(5):894.

Kumoro AC, Hasan M, Singh H. Effects of solvent properties on the Soxhlet extraction of diterpenoid lactones from Andrographis paniculata leaves. Science Asia 2009;35(1):306-309.

Mellinas AC, Jiménez A, Garrigós MC. Optimization of microwave-assisted extraction of cocoa bean shell waste and evaluation of its antioxidant, physicochemical and functional properties. LWT 2020; 127:109361.

Nawaz H, Shad MA, Rehman N, Andaleeb H, Ullah N. Effect of solvent polarity on extraction yield and antioxidant properties of phytochemicals from bean (Phaseolus vulgaris) seeds. Brazilian Journal of Pharmaceutical Sciences 2020;56: e17129.

Rojo-Poveda O, Barbosa-Pereira L, Zeppa G, Stévigny C. Cocoa bean shell-a by-product with nutritional properties and biofunctional potential. Nutrients 2020;12(4):1123.

Rojo-Poveda O, Zeppa G, Ferrocino I, Stévigny C, Barbosa-Pereira L. Chemometric classification of cocoa bean shells based on their polyphenolic profile determined by RP-HLC-PDA analysis and spectrophotometric assays. Antioxidants 2021;10(10):1533.

Zengin G, Sarikurkcu C, Uyar P, Aktumsek A, Uysal S, Kocak MS, Ceylan R. Crepis foetida L. subsp. rhoeadifolia (Bieb.) Celak. as a source of multifunctional agents: Cytotoxic and phytochemical evaluation. Journal of Functional Foods 2015;17:698-708.

Tawata S, Ao C, Li A, Elzaawely AA, Xuan TD. Evaluation of antioxidant and antibacterial activities of Ficus microcarpa L. fil. extract. Food Control 2018;19:940-948.

Todorovic V, Milenkovic M, Vidovic B, Todorovic Z, Sobajic S. Correlation between antimicrobial, antioxidant activity, and polyphenols of alkalized/nonalkalized cocoa powders. Journal of Food Science 2017;82(4):1020-1027.

Viuda-Martos M, Martíneza R, Torresa P, Menesesa MA, Figueroaa JG, Pérez-Álvarezb JA. Chemical, technological and in vitro antioxidant properties of cocoa (Theobroma cacao L.) co-products. Food Research International 2012;4939–4945

Wollgast J, Anklam E. Review on polyphenols in Theobroma cacao: changes in composition during the manufacture of chocolate and methodology for identification and quantification. Food Research International 2000;33(6):423-47.

Zhang QW, Lin LG, Ye WC. Techniques for extraction and isolation of natural products: A comprehensive review. Chinese medicine 2018;13:1-26.

Zhang X, Wang X, Wang M, Cao J, Xiao J, Wang Q. Effects of different pretreatments on flavonoids and antioxidant activity of Dryopteris erythrosora leave. PloS one 2019;14(1): e0200174.