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Research

Nutritional Composition and Optimization of Extraction Conditions of Cocoa Pod Husk using Response Surface Methodology

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ABSTRACT

Cocoa pod husks (CPH) are usually disposed of from the farm, and this can lead to environmental problems, such as being a breeding ground for the cocoa pod borer. This study aimed to determine the nutritional composition and concentration of ultra-trace elements (As, Cd, Pb & Hg) in CPH. The optimization of the extraction conditions of CPH in response to the ferric-reducing antioxidant power (FRAP) by using response surface methodology (RSM) was also conducted. The findings show that the total carbohydrate and crude fibre content of CPH are high (35.75% & 35.47%, respectively) while having low levels of moisture, ash, crude protein, and fat (11.86%. 8.60%, 7.46% & 0.86, respectively). In addition, the results demonstrate that CPH has a low content of toxic metals As, Cd, Pb, and Hg (0.0046 mg/kg, 0.0028 mg/kg, 0.0011 mg/kg & 0.00003 mg/kg respectively) which is considered as a safe range. The optimized extraction conditions were a solvent concentration of 93.64%, a temperature of 38.18°C, and a time of 73.64 min. The actual value of the flavonoid content of CPH obtained was 1038.94 µmoL Fe^{2+}/L, which is acceptable compared to the predicted value of 1039.40 µmoL Fe^{2+}/L. The discovery from this research represents a significant contribution towards finding cocoa pod husk from a plentiful, affordable, and feasible source, which could potentially be used in various fields such as pharmaceutical, medical, and nutraceuticals.

Key words: Cocoa pod husk, flavonoids, heavy metals, nutritional composition, response surface methodology

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INTRODUCTION

The governments of numerous tropical nations have strongly emphasised their support for the expansion of the chocolate industry by convincing cocoa farmers to plant more trees as the world's production of *Theobroma* cocoa beans (*Malvaceae*) has dropped (Vriesmann *et al*., 2011). According to Uy *et al*. (2019), this effort has increased cocoa bean production by more than 50%. This caused a rise in output that led to the growth of undesired by-products on cocoa fields and plantations such as cocoa pod husk (CPH). This scenario has led to relatively new and emerging studies on the compounds found in CPH. The research on the properties of CPH has already made significant progress, highlighting its abundance in bioactive antioxidants, dietary fibre, lignin, and polyphenols, which are currently underutilized (Lu *et al*., 2018). The creation of texturing agents, gums, flavour compounds, and other ingredients are examples of possible uses of CPH in the food and beauty fields (Ouattara *et al*., 2021). Therefore, these studies show a great number of promising solutions and applications of CPH in various fields.

There are numerous implementations of CPH. For example, CPH's pectin has undergone significant study and extraction, and it is a valuable product in the food industry (Vriesmann & de Oliveira Petkowicz, 2017). Additionally, due

to its high level of organic components and minerals, CPH serves as a good substrate for biofertilizers (Atere *et al*., 2020). Moreover, CPH contains high levels of phenolic compounds, which may be extracted using both classic and subcritical or supercritical extraction methods (Muñoz-Almagro *et al*., 2019). This shows that CPH was economically beneficial to most of the industries in Malaysia, as well as, generating money for farmers while promoting overall economic growth (Lu *et al*., 2018).

Generally, to perform quantitative and qualitative analyses of the active components in herbs or plants, the solvent extraction method is crucial. Numerous parameters, especially extraction conditions such as method, solvent, duration, liquid-solid ratio, and temperature can influence the target products' extraction rate and the composition of the extraction products (Oludemi *et al*., 2018). A previous study by Abdul Karim and Abdullah (2021), reported that concentration, temperature, and time can be used to extract the polyphenols antioxidant compounds from cocoa pods. Therefore, these three factors: solvent concentration (X_1), temperature (X_2) and time (X_3) were implemented in this study. Box and Wilson (1992) introduced response surface methodology (RSM), an optimization technique involving comprehensive experimental design and mathematical modelling. RSM offers advantages like fewer trials, greater accuracy, and greater forecasting performance, making it a significant development in the field. Hence, to extract flavonoids and polyphenols antioxidants from plants or herbs, RSM has been frequently utilized. The present study aimed to determine the nutritional proximate composition, the concentration of heavy metal, and the optimal extraction condition of CPH.

MATERIALS AND METHODS

Plant materials

Discarded CPH were collected from the Malaysia Cocoa Board cocoa plantation research centre in Bagan Datuk, Perak. To get rid of the debris, water was used to wash the CPH. A mechanical fruit slicer was used to slice the husk into pieces for ease of drying using a drying cabinet. Dried CPH was ground into 1 mm particle sizes by using a grinder (IKA, Staufen, Germany).

Nutritional composition of cocoa pod husk

CPH was sent to the accredited MS ISO/ISE 17025 Analytical Services Laboratory, MCB, Cocoa Innovation and Technology Centre (CITC) for proximate analysis. The ash, crude protein, and crude fibre contents of CPH were analyzed according to the Association of Official Analytical Chemists (1984, 1975 & 2000, respectively). The ash was determined after the incineration of a 2 to 5 g sample in a muffle furnace (STM 1200, LabTech, US) at 600°C. Crude protein was measured using the combustion technique (rapid N exceed, Elementar, Germany) using 100 mg of sample. The crude fibre determination was estimated by digesting 2 g of sample. Moisture content (ISO8534:1996E) was determined by using an oven (Memmert, Germany), by drying a 5 g homogenised sample at 103°C for 16 hr. The fat content (IOCCC: Pg8a-E 1978) was measured by using a 4 to 5 g homogenized sample by extraction in a Soxhlet apparatus and using petroleum ether as a solvent. The carbohydrate quantity was calculated by deducting the combined percentage of ash, crude protein, crude fibre, moisture content and fat from the total by using the formula (McCleary & McLoughlin, 2021):

$$\% \text{ Carbohydrate} = 100-(\text{ash}+\text{crude protein}+\text{crude fibre}+\text{moisture}+\text{fat}) \quad \text{– Equation 1}$$

Heavy metal test of cocoa pod husk

The presence of As, Pb, Cd, and Hg were determined. The samples were digested using the digestion procedure (USEPA Method, 1993) for total metal concentration. The sample was weighed into a cleaned digestion vessel and mixed with 50 mL nitric acid (HNO_3). Next, the sample was placed into a microwave digestor. The elements were detected using standard mode Inductively Couple Plasma Mass Spectroscopy (ICP-MS, Perkin Elmer Nexion 2000, USA).

Ferric-Reducing Antioxidant Power (FRAP) assay

Othman *et al*. (2007) and Benzie and Strain (1996) techniques were used to determine the FRAP concentration in CPH extract. The FRAP reagent was used in a 10:1:1 ratio. At 37 °C, 300 mm acetate buffer (pH 3.6), 10 mM TPTZ and 20 mM $FeCl_3$. $6H_2O$ were combined and stored as a stock solution. The calibration standard or 100 µL of the sample was combined with 3 mL of the FRAP reagent from the stock solution. After that, the mixture was incubated for 10 min at 37 °C after the addition of 300 µL water. A UV-visible spectrophotometer (Cary 60, Agilent Technologies, Santa Clara, CA, USA) was used to detect absorbance at 593 nm.

Ultrasound-assisted Extraction (UAE)

One gram of CPH was weighed and combined with 20 mL of ethanol at different concentrations (26-94%) in 50 mL conical flasks. The extraction process involved the use of a sonication bath (Wiseclean, 289 W, Wongju-si, Korea), operating at a frequency of 40 kHz, with varying temperatures (38-72 °C) and

durations (6-74 min). Following the extraction, the CPH extract was filtered using Watman filter paper (Watman No. 4), and the solvent was evaporated using a rotary evaporator (IKA, Staufen, Germany). To eliminate water content from the extract, freeze-drying was conducted (Labconco, Kansas City, MO, USA) at -80 °C for 24 hr, resulting in the production of crude extract. This crude extract was then stored in sample bottles at -40 °C for subsequent analysis.

Central composite design (CCD) for extraction optimization

Evaluation of value and formation of CCD were performed by Minitab version 14 software (Minitab, United States). The central composite design (CCD) with three variables and five levels of point observation was chosen for the study The impacts of solvent (ethanol) concentration (X_1), temperature (X_2), and time (X_3) were examined on the outcome of ferric-reducing antioxidant power (FRAP) concentration. The extraction procedure was conducted at a different solvent (ethanol) concentration (X_1), between 26 to 94%, temperatures (X_2) between 38 to 72 °C, and time (X_3), between 6 to 74 min. Table 1 displays the codes and levels of independent variables for the composite central design (CCD). 20 experimental groups made up the CCD.

The value of FRAP was expressed mathematically using a second-order polynomial regression model which was produced by software on Minitab version 14. The significance test on the regression model and individual model coefficients was performed using ANOVA. The mathematical model is displayed as Equation 2.

$$Y = \beta 0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=j}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_{ij} \quad - \text{Equation 2}$$

In which Y is the output (FRAP concentration); β0 represents the coefficient that denotes the model's intercept; β_i, β_{ii}, and β_{ij} are the linear, quadratic, and interaction impact, respectively; X_i and X_j *stands* for the independent parameters.

Table 1. The levels of independent variables in the Composite Central Design (CCD)

Independent Variables	Symbol	Level		
		-1	0	1
Solvent percentage (%)	X_1	40	60	80
Temperature (◦C)	X_2	45	55	65
Time (min)	X_3	20	40	60

Statistical analysis

Data was presented as mean values, and each experiment was run in triplicate. The actual value of the experiment was compared with the predicted value of the optimized conditions and the significant difference was measured using a t-test. The relationship between the dependent variables was also investigated using Pearson correlation. When a correlation value's *p*-value is less than 0.05, it is considered significant.

RESULTS AND DISCUSSION

Nutritional composition of cocoa pod husk

Table 2 shows that CPH had high contents of crude fibre and carbohydrates (35.47% & 35.75% by dry weight, respectively). This study demonstrated a strong correlation between the amount of fibre and carbohydrate contents, considering that fibre is a constituent of carbohydrates. These findings are comparable with previous studies on red kidney bean (*Phaseolus vulgaris*) in which, the carbohydrate and fibre values were the highest among the other components (44.90 ± 2.62 & 24.83 ± 0.45 g/100 g, respectively) (Zaidan *et al*., 2019).

CPH was also found to have low contents of moisture, ash, crude protein, and fat (11.86%, 8.60%, 7.46% & 0.86% by dry weight, respectively). The moisture content of CPH that was found in this study was in range with the moisture content in cocoa bean shells (3.60-13.13%) (Rojo-Poveda *et al*., 2020). Kilama *et al*. (2019) reported that CPH has a lot of potential in feedstock production as it possesses a great caloric value (17.5 MJ/kg on a dry basis) at a moisture content of 14%, which is almost similar to commonly used feedstocks like bagasse and wood (18.6 kJ/kg on a dry basis).

Additionally, the contents of fat, crude protein, and crude fibre in CPH were lower than those in cocoa bean shells (2.3%, 20.9% & 55.1% by dry weight respectively). The carbohydrate and ash content in CPH was higher than that in cocoa bean shells (7.85% & 7.9% by dry weight, respectively) (Rojo-Poveda *et al*., 2019). Although CPH has lower fat content compared to cocoa beans, it is balanced out

by a significantly higher percentage of nutritional fibre. These findings demonstrated that the nutritional composition of various parts of cocoa differed remarkably and was directly related to the structural characteristics of the cocoa, with the CPH serving as a key barrier against insects and the negative physical, chemical, and microbial effects of the environment.

Table 2. Nutritional Composition of cocoa pod husk

Proximate composition	Dry Weight (%)
Crude fibre	35.47
Carbohydrate	35.75
Moisture	11.86
Ash	8.60
Crude protein	7.46
Fat	0.86

Heavy metal test

The concentration of several types of heavy metal in CPH was determined, and the findings are depicted in Table 3. The data shows that the CPH passed the heavy metal test as the concentrations of all heavy metals (Pb, As, Cd & Hg) were detected below the concentration limit in the cosmeceutical product.

Few studies have been conducted on CPH in the cosmeceutical field. For instance, Abdul Karim *et al*. (2016), reported that CPH was able to block the activity of various enzymes that cause skin ageing due to the high concentration of antioxidant compounds in cocoa pod extract. Moreover, there have been discoveries regarding the high demand for natural skin-care products, such as soap made from CPH potash (Gyedu-Akoto *et al*., 2015).

The results also revealed that CPH complied with the allowable limit established by the international standard (FAO/WHO, 1984) and the Malaysia Food Regulation 1985 (Food-Act 281, 1994) for Pb and Cd. The limits are 1.5 mg/kg and 2 mg/kg for Pb and 0.05 mg/kg, and 1.00 mg/kg for Cd, respectively. According to Ara and Usmani (2015), Pb does not serve any physiological purpose in the body, and even minimal levels of Pb can induce toxic effects. Pb inhibits enzymes from carrying out their typical functions. It interferes with the regular DNA transcription process and can impair bone function. Elevated Cd levels can disrupt calcium metabolism, result in the formation of kidney stones, cause bone softening, and interfere with the synthesis of pancreatic DNA (Assa *et al*., 2018).

Table 3. Concentration of heavy metals in cocoa pod husk

Metal contaminants	Concentration (mg/kg)	Concentration limit in cosmeceutical (mg/kg)	FAO/WHO (mg/kg)	Malaysia Food Regulation 1985 (mg/kg)
Lead (Pb)	0.00110	10	1.5	2
Cadmium (Cd)	0.00280	1	0.05	1
Arsenic (As)	0.00460	3		
Mercury (Hg)	0.00003	1		

Fitting the model

Table 4 shows the actual and predicted value for the FRAP assay. The highest FRAP concentration was obtained when the solvent concentration was set at 80%, the temperature at 40 °C, and the extraction time at 60 min. In contrast, the lowest FRAP concentration was observed under conditions of 40% solvent concentration, 65°C temperature, and 60 min of extraction time. By using a model-fitting approach, the predicted data were obtained and correlated adequately with the actual data. From the system suiting method, it was seen that the predicted data were adequately linked to the actual data. The complex interaction between the variables was analysed to determine the constant value of β as in Equation 3.

$$Y_{FRAP} = 24 - 2.94X_1 + 12.87X_2 + 14.46X_3 + 0.0117X_1X_2 + 0.1326X_1X_3 - 0.3817X_2X_3 - 0.00778X_1^2 - 0.0311X_2^2 - 0.00646X_3^2 \quad \text{– Equation 3}$$

Table 5 reveals that the *F*-value (105.6) for the model was significant (p<0.0001). There was simply a 0.01% possibility that an *F* value this high could happen because of the noise. The lower *p*-value for the parameters and their relationships showed that they had a bigger influence (significant) on the outcome (He *et al*., 2010) Besides, the coefficient of determination (R^2) of the model was 0.989,

which signifies the model's ability to accurately capture the interaction among the factors studied. The nearer the R^2 to 1, the greater the interaction and the greater the system estimated the outcome (Tepe & Dursun, 2014).

In addition, the large value of adjusted R^2 (0.980) and predicted R^2 (0.929) data possess great conformity among adjusted R^2 and predicted R^2 data. Consequently, the system certainly indicates that the system could be applied to evaluate the correlation between independent parameters and outcomes (Sodeifian *et al*., 2016; Maran *et al*., 2017).

The lack of fit test evaluates the inability of the system to signify value in the regression (Bezerra *et al*., 2008). The lack of fit for the *F*-value (3.610) was insignificant relative to pure error. There is a 9.3% (*p*-value, 0.093) chance that lack of fit *F*-value this large could occur due to noise. Therefore, it shows that the model sufficiently demonstrated the experimental value within the chosen intervals. Therefore, the model is suitable for FRAP analysis and prediction.

Table 4. Central Composite Design layout and outcome for the FRAP assay

Run	Solvent Concentration (X_1)	Temperature (X_2)	Time(X_3)	FRAP (μmoL Fe^{2+}/L)	
	(%)	(◦C)	(min)	Actual value	Predicted value
1	60	71.82	40	420.49	423.53
2	40	45	60	567.81	563.15
3	40	45	20	473.44	480.28
4	80	45	60	728.82	747.55
5	40	65	20	540.35	525.86
6	80	45	20	454.82	452.55
7	60	55	40	530.67	518.48
8	40	65	60	296.85	303.36
9	80	65	60	499.70	497.11
10	60	55	40	525.57	518.48
11	60	55	40	513.67	518.48
12	60	55	40	514.63	518.48
13	60	55	40	512.64	518.48
14	60	55	73.64	550.30	541.65
15	60	55	40	512.67	518.48
16	26.36	55	40	434.36	439.86
17	60	38.18	40	604.85	595.81
18	93.64	55	40	590.98	579.48
19	60	55	6.36	478.04	480.69
20	80	65	20	498.58	507.49

Table 5. Analysis of variance (ANOVA) of the central composite design layout for the FRAP assay

Source	Coefficient (β)	Sum of Square	Degree of freedom	Mean square	*F*-value	*p*-value
Model	24	133324	9	14813.8	105.63	<0.0001
Linear		63841	3	21280.4	151.74	<0.0001
X_1	-2.94	23530	1	23530.2	167.78	<0.0001
X_2	12.87	35825	1	35824.9	255.44	<0.0001
X_3	14.46	4486	1	4486.1	31.99	<0.0001
Cross product		3	69169	23056.4	164.40	<0.0001
X_{12}	0.0117	1	44	43.7	0.31	0.5890
X_{13}	0.1326	1	22499	22498.5	160.42	<0.0001
X_{23}	-0.3817	1	46627	46626.9	332.47	<0.0001
Quadratic		3	314	104.7	0.75	0.5480
X_1^2	-0.00778	1	140	139.8	1.00	0.3420
X_2^2	-0.0311	1	140	139.8	1.00	0.3420
X_3^2	0.00646	1	96	96.2	0.69	0.4270
Error		10	1402	140.2	-	-
Lack of fit		5	1098	219.6	3.61	0.093
Pure error		5	305	60.9	-	-
R^2= 0.989						
Adjusted R^2= 0.980						
Predicted R^2 = 0.929						

X_1: Solvent concentration (%); X_2: Temperature (◦C); X_3: time (min)

Response surface analysis of FRAP

Figure 1 depicts the correlation between solvent concentration and temperature. An increase in solvent concentration at higher temperatures led to higher FRAP concentration. Previous research has demonstrated that utilising a solvent during the extraction process results in greater extraction of antioxidant chemicals compared to water extraction (Hromádková *et al*., 2008). Moreover, a study by Zhang *et al*. (2019) described that flavonoid content can be extracted by using an ethanol solution with a medium-high concentration ranging from 70% to 90%. This finding is consistent with the research conducted by Md Yusof *et al*. (2019), which found similar concentrations effective for extracting antioxidant compounds from other parts of cocoa, such as cocoa shells.

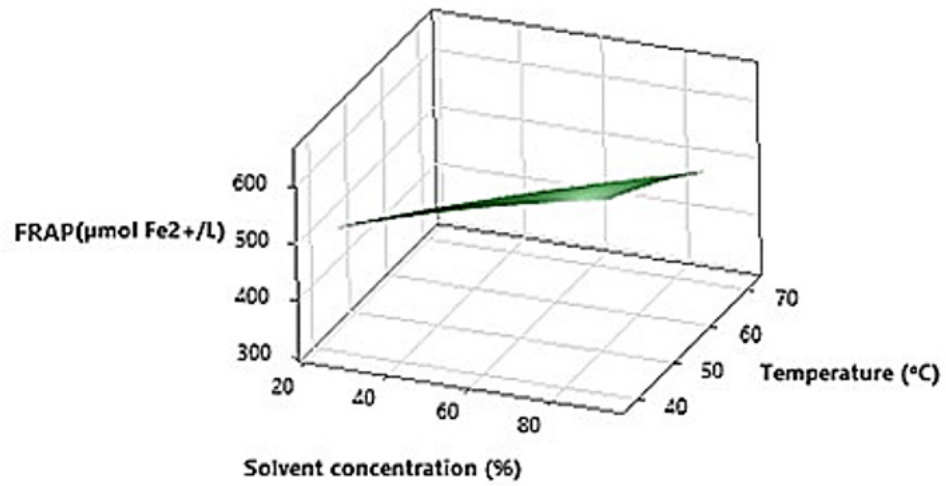

Fig. 1. Influence of solvent concentration (%) and temperature (°C) on FRAP.

Figure 2 illustrates the interaction between solvent concentration and time on the FRAP content. It shows that a longer extraction time results in relatively higher FRAP content. Previous studies have shown that the highest FRAP was achieved within a certain time range, such as 116–120 min for brewers' spent grain (BSG), as a longer time allows for greater extraction of flavonoids (Andres *et al*., 2020). A previous study by Berlim *et al*. (2018) reported that various parameters, including treatment technique, solvent type, temperature, pressure, natural material, and analysis method, can affect the yield of antioxidant molecules.

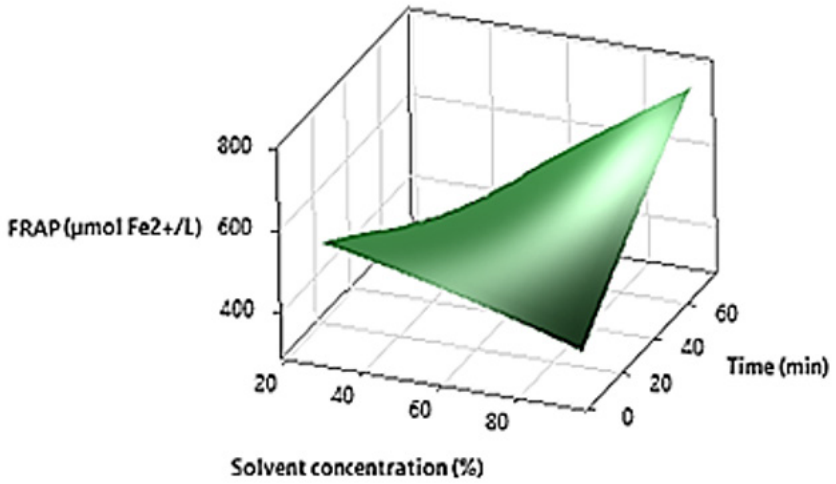

Fig. 2. Influence of solvent concentration (%) and time (min) on FRAP.

Figure 3 describes the effect of temperature and time. It can be observed that a higher FRAP content can be obtained by decreasing the temperature while increasing the time. Research by Abdul Karim *et al*. (2021), shows that the optimal extraction temperature for cocoa pod can be achieved at low temperature which is 35.29 °C. This is due to, flavonoid molecules can rather be denatured at higher temperatures (Sheng *et al*., 2013; Wang *et al*., 2017).

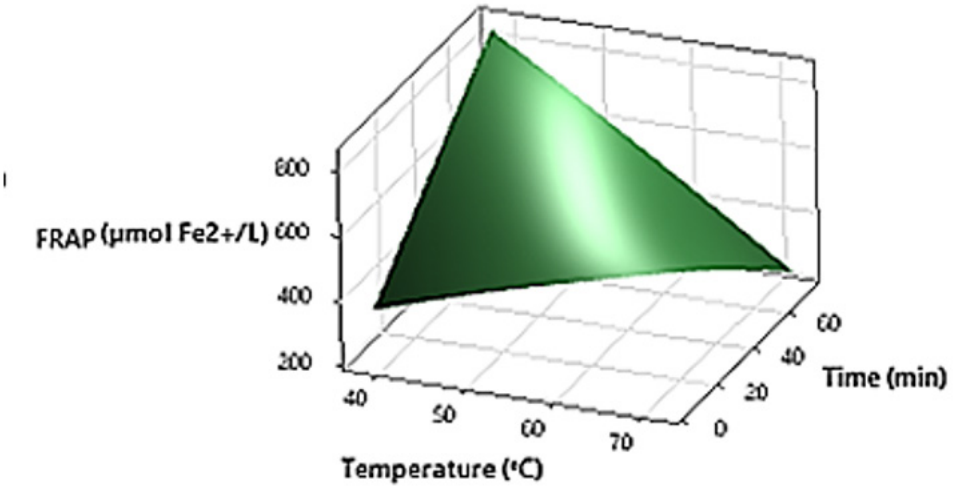

Fig. 3. Influence of temperature (°C) and time (min) on FRAP.

Reaction optimization and model validation

The FRAP concentration obtained under the optimum criteria was 1038.94 µmoL Fe^{2+}/L, which showed great concurrence with the predicted results. (1039.40 µmoL Fe^{2+}/L). This demonstrated that an empirical model built using RSM can be used to adequately clarify the relationship between the extraction conditions and the concentration of FRAP. The optimized extraction conditions were solvent concentration of 93.64%, temperature of 38.18 °C and, time of 73.64 min (Table 6). Table 7 shows the comparison of optimum extraction conditions and maximum FRAP concentration obtained from the previous study. The present study reported the highest solvent concentration (93.64%) and time (73.64 min) for optimum extraction conditions while exhibiting the lowest temperature (38.18 °C) among all the studies (Tabaraki & Nateghi, 2011; Md Yusof *et al*., 2019; Jafari *et al*.,2023).

Table 6. Experimental value and predicted value of FRAP at optimum extraction conditions

Optimum extraction conditions			FRAP (µmoL Fe^{2+}/L)		*P*-Value
Solvent concentration (%)	Temperature (°C)	Time (min)	Actual Value	Predicted Value	
93.64	38.18	73.64	1038.94	1039.40	0.264

Table 7. Optimum extraction conditions and maximum FRAP concentration compared with previous studies

Studies	Maximum FRAP value	Optimum extraction conditions		
		Solvent concentration (%)	Temperature (°C)	Time (min)
Present study	1038.94 µmoL Fe^{2+}/L	93.64	38.18	73.64
Jafari *et al*. (2023)	1197.68 mM Trolox/100g dry wt	60	55	60
Md Yusof *et al*. (2019)	1.41 µmoL/L	70	65	30
Tabaraki & Nateghi (2011)	54.94 µmoL Fe^{2+}/g dw	65-67	51-54	40-45

CONCLUSION

CPH exhibited high contents of total carbohydrate and crude fibre but low levels of moisture, ash, crude protein, and fat. The concentration of four heavy metals (Pb, Cd, As & Hg) was below the maximum critical levels. Additionally, the extraction conditions of CPH were successfully optimized using RSM. It was determined that 93.64% solvent concentration 38.18 °C, and 73.64 min were the optimum extraction conditions. Under these conditions, the experimental FRAP is 1038.94 µmoL Fe^{2+}/L and the predicted value is 1039.40 µmoL Fe^{2+}/L. Thus, CPH demonstrates significant potential as an innovative functional ingredient as an antioxidant that offers health benefits to consumers.

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ETHICAL STATEMENT
Not applicable.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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