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ENHANCED RECOVERY OF BIOACTIVE COMPOUNDS FROM COCOA BEAN SHELL WASTE USING ULTRASOUND-ASSISTED ETHANOL EXTRACTION: PROCESS OPTIMIZATION AND ANTIOXIDANT EVALUATION

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ABSTRACT
The study investigated the enhancement of ethanol-based ultrasonic extraction conditions to achieve maximum yield of bioactive substances, particularly phenolic compounds and flavonoids, from cocoa bean shell waste. Statistical modelling and optimisation were performed using a Box-Behnken experimental design, which incorporated three parameters (thermal conditions, extraction duration, and alcohol concentration) across three different levels, integrated with response surface analytical methods. The most effective extraction parameters were identified as 60°C for 60 minutes using 60% (v/v) ethanol in water solution. Under these conditions, the extract yielded a total phenolic content (TPC) of 244.45 ± 7.46 mg GAE/g DW and a total flavonoid content (TFC) of 170.20 ± 13.24 mg CE/g DW. Antioxidant activity was quantified through three well-established methodologies: DPPH (16.84 ± 1.25 mg TE/g DW), ABTS (382.57 ± 29.70 mg TE/g DW), and FRAP (642.32 ± 45.26 mg TE/g DW). These findings suggest that cocoa bean shells serve as an efficient, eco-friendly, and cost-effective source of naturally occurring antioxidants, with potential applications in functional food products, dietary supplements, and cosmetic formulations.
Keywords: Cocoa Bean Shells, Ultrasound-Assisted Extraction, Phenolic, Flavonoid, Antioxidant Capacity
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INTRODUCTION

Cocoa bean shell (CBS), which accounts for approximately 10–20% of the total cocoa bean mass, is a lignocellulosic by-product that remains largely underexploited despite being abundantly produced during the chocolate production process.[1,2] This biomass is notably rich in phytochemicals, especially active compounds like phenolic substances, encompassing flavonoid compounds such as catechin derivatives, epicatechin variants, proanthocyanidins, and purine alkaloids, including theobromine and caffeine.[3] These compounds contribute significantly to its strong antioxidant potential. Recent studies have reported TPC and TFC levels in CBS ranging from 6.04–197.40 mg GAE/g and 1.65–20.57 mg CE/g, respectively.[4-7] This highlights its potential as a natural antioxidant source with broad industrial applications. Harnessing the potential of CBS not only helps mitigate environmental concerns linked to agricultural waste but also offers economic value by recovering high-value phytochemicals for applications in food preservation, pharmaceuticals, and cosmetics. Moreover, integrating CBS utilisation into production systems supports circular economy initiatives by converting waste into valuable inputs, thereby promoting sustainability and reducing ecological impact.[8] Ultrasound-assisted extraction (UAE) is increasingly acknowledged as an advanced and environmentally sustainable technique for efficiently isolating bioactive constituents from cocoa bean shells.[9] This environmentally conscious approach utilises acoustic cavitation, which promotes improved mass transfer while maintaining the integrity of heat-sensitive phytochemicals, owing to its operation at lower temperatures and reduced processing time intervals when compared to traditional extraction techniques. The enhanced efficiency of the UAE can be attributed to the intense localised pressure and temperature generated during the formation and collapse of acoustic cavitation microbubbles, which significantly improve solvent diffusion and boost extraction performance.[10] Among optimisation tools, the Box-Behnken experimental design within the response surface methodology framework has

demonstrated excellent utility in optimising key extraction variables such as temperature, sonication time, and solvent concentration. This approach facilitates effective process optimisation with fewer experimental runs and robust statistical outcomes.[11] The present study seeks to establish and validate optimised UAE protocols aimed at maximising the yield efficiency of phenolic and flavonoid compounds of CBS using a comprehensive Box-Behnken design framework. The core objectives include: (i) systematic assessment of antioxidant activities of the extracted compounds and (ii) optimisation of extraction parameters to maximise yield and bioactivity. Progress in value-added CBS applications supporting both sustainable waste valorisation and the efficient utilisation of industrial resources.

EXPERIMENTAL

Sample Preparation

Cocoa bean shells used in this study were obtained from a local cocoa-processing community enterprise located in Surat Thani, Thailand. Raw samples were dehydrated at 45°C for two days, processed through fine grinding to obtain an 85 μm particle size, and preserved at 4°C until experimental procedures. The UAE was using an acoustic water bath (Elmasonic 300H, Germany; 37 kHz). In the individual extraction trials, 1 gram of powdered CBS was dissolved with 20 mL of ethanol and exposed to various extraction parameters encompassing temperature, duration, and ethanol concentration. Following completion of the extraction, the mixture underwent filtration and was adjusted to a volume of 25 mL with absolute ethanol. The extracts were kept at 4°C in light-free conditions before further analysis.

Optimisation of Ultrasonic-Assisted Extraction

The extraction solvent was ethanol for bioactive substances from cocoa shell residues. To explore the effects of critical process conditions, Box-Behnken experimental methodology involving three parameters at three levels was applied. The independent variables examined included extraction temperature (A, 30–70°C), extraction time (B, 20–60 min), and ethanol concentration (C, 30–90%). Individual parameters were assessed at three coded points: −1 (Bottom), 0 (centre), and +1 (Top), yielding 15 experimental combinations (Table-1). The correlation between process variables and TPC or TFC follows a quadratic polynomial equation, which is frequently employed in RSM for statistical modelling and process optimisation with multiple parameters, expressed as:[12]

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \beta_{12}AB + \beta_{13}AC + \beta_{23}BC \quad (1)$$

Where Y is the expected yield of TPC or TFC, β values are the regression coefficients, and A, B, and C are the independent variables. After determining the predicted optimal conditions, the model was validated by experimentally verifying the TPC and TFC and comparing these results with the model's predicted values. Response surface analytical methods were employed to optimise three crucial extraction conditions to maximise the extraction yield of functional substances from cocoa shell residues, using TPC and TFC as the primary response indicators. All analyses were conducted using spectrophotometric measurement with a 96-well plate reader (SPECTROstar® Nano, BMG LABTECH, Germany). Each analytical procedure was executed in three replicates (n = 3) to validate the accuracy and reproducibility of the obtained results. These experimental procedures utilised double-distilled water (DDW) as the solvent where applicable.

Table-1: Response of Actual and Predicted Values of TPC (mg GAE/g DW) and TFC (mg CE/g DW) in Cocoa Bean Shells Extracts Under Varying Conditions

Experimental Run	Independent variables			Response variables			
	Temp. (°C)	Time (min)	[EtOH] (%v/v)	TPC		TFC	
				Actual	Predicted	Actual	Predicted
1	30	20	60	155.85±4.12^{g}	173.02	100.58±11.03^{c}	119.54
2	70	20	60	201.31±7.68^{bc}	193.42	128.91±6.53^{b}	126.58
3	30	60	60	171.41±4.84^{f}	179.3	111.82±11.53^{c}	114.15
4	70	60	60	243.05±6.62^{a}	225.88	167.80±10.68^{a}	149.08
5	50	40	60	194.54±6.90^{dc}	199.01	137.25±20.28^{b}	142.46

6	30	40	90	12.20±1.03^{j}	1.67	38.60±5.05^{d}	27.39
7	70	40	90	183.32±3.71^{e}	193.85	107.05±10.06^{c}	118.26
8	30	40	30	182.32±4.21^{e}	167.8	103.79±4.77^{c}	93.95
9	70	40	30	28.07±0.17^{i}	42.59	35.22±3.60^{d}	45.06
10	50	40	60	196.61±5.57^{dc}	199.01	161.46±7.29^{a}	142.46
11	50	20	30	191.79±5.10^{d}	189.14	110.95±3.47^{c}	102.08
12	50	60	30	192.13±2.34^{d}	198.77	100.05±2.47^{c}	107.56
13	50	20	90	27.34±2.07^{i}	20.7	36.63±3.75^{d}	29.12
14	50	60	90	47.17±0.67^{h}	49.82	31.86±2.98^{d}	40.74
15	50	40	60	205.87±7.76^{b}	199.01	128.67±5.45^{b}	142.46

All experiments were performed in triplicate (n=3). The results were expressed as mean ± standard deviation.

Quantification of Total Phenolic Content

The TPC was analysed with a modified Folin–Ciocalteu assay.[13] Briefly, 60 µL of the extracts was combined with 300 µL of 10% Folin–Ciocalteu reagent. Following that, 225 µL of 7.5% Na_2CO_3 was introduced into the mixture, kept away from light at ambient temperature for one hour. The absorbance was measured with a microplate reader at 745 nm. The quantified TPC was reported as mg GAE/g DW.

Quantification of Total Flavonoid Content

The TFC of CBS extracts was measured through a modified aluminium chloride colour-based analysis adapted to a 96-well microplate.[14] For the experimental procedure, 100 µL of CBS extract was combined with 400 µL DDW and 30 µL of 5% $NaNO_2$. Then, 30 µL of 10% $AlCl_3$ was introduced. The solution was maintained at room temperature for 6 minutes. Subsequently, 200 µL of 1.0 M NaOH and 240 µL of DDW were added. Measurements of absorbance were performed at 510 nm, and the results were expressed as mg CE/g DW.

DPPH Radical Scavenging Assay

The CBS extract was assessed using the DPPH method with some adjustments.[15] The method consisted of combining the CBS extract 100 µL with 900 µL of 60 µM of the DPPH solution, kept away from light for 30 minutes.[16] The sample absorbance was quantified at 520 nm. DPPH values were reported as mg TE/g of dry weight.

ABTS Radical Scavenging Assay

The antioxidant capacity against ABTS radicals was determined with minor alterations.[17] To generate $ABTS^{\bullet+}$, 7.4 mM ABTS and 2.6 mM potassium persulfate were mixed and incubated in the dark for 12 hours at ambient temperature. An aliquot of 100 µL CBS extract was combined with 900 µL of $ABTS^{\bullet+}$ solution and incubated for 7 minutes at room temperature before absorbance was recorded at 734 nm. The Trolox standard curve ranged from 0.02 to 0.20 mM, and results were reported as mg TE/g DW.

Ferric Reducing Antioxidant Power (FRAP) Assay

The CBS extract antioxidant reducing power was analysed via the FRAP assay with some modifications.[18] A freshly prepared FRAP reagent was combined with 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3$ and 300 mM acetate buffer (pH 3.6), in a 1:1:10 volume ratio. Then, 900 µL of FRAP reagent was reacted with 50 µL of CBS extract and maintained at 37°C for 30 minutes. The calibration standard used was Trolox at concentrations ranging from 0.05 to 0.5 mM, with antioxidant activity expressed as mg TE/g dry matter.

Statistical Evaluation

Statistical evaluation was performed by one-way analysis of variance and Duncan's multiple range test using IBM SPSS Statistics 20 software. RSM and BBD were employed to enhance extraction techniques, including temperature parameters, extraction duration, and ethanol concentration levels, using Microsoft Excel 16 software.

RESULTS AND DISCUSSION

Optimisation of Ultrasound-Assisted Extraction

Ultrasonic-assisted extraction was utilised for cocoa bean shell processing because of its superior extraction performance, lower energy requirements, and abbreviated processing duration, providing particular benefits for recovering bioactive constituents. The experimental data obtained from 15 runs designed through the Box-Behnken design were subjected to statistical analysis using one-way ANOVA to establish regression coefficients associated with TPC and TFC, as presented in Equations 2 and 3, respectively. The role of extraction variables, including temperature application (A), processing period (B), and ethanol level (C), on TPC and TFC was assessed through second-order polynomial regression models, which are expressed as follows:

$$Y_{TPC\ (mg\ GAE/100g\ DM)} = 199.007 + 16.7463A + 9.68375B - 79.348C - 9.6158A^2 + 3.51417B^2 - 87.913C^2 + 6.545AB + 3.7175AC + 4.8725BC \quad (2)$$

$$Y_{TFC\ (mg\ CE/100g\ DM)} = 142.46 + 10.4938A + 4.27625B - 34.943C - 6.9163A^2 - 8.2063B^2 - 64.379C^2 + 6.9725AB - 1.66AC + 1.535BC \quad (3)$$

The estimated values of TPC and TFC derived from the regression models presented in Equations (2) and (3) are compiled in Table-1. These models served as tools to predict and interpret extraction yields across varying experimental conditions. According to the TPC model (Eq.-2), both temperature and extraction time contributed positively to phenolic compound recovery, whereas increasing ethanol concentration exerted a negative influence. Likewise, the TFC model (Eq.-3) indicated that higher temperature and longer extraction time enhanced flavonoid yield, while ethanol concentration negatively affected it. These observations emphasise the critical role of fine-tuning extraction parameters to attain maximum yield of functional compounds from cocoa shell waste. The experimentally obtained results showed good agreement with the expected values of both TPC and TFC. The high coefficient of determination (R^2) for the cocoa bean shell extracts reflects a strong relationship between actual values and the values forecast by the created model, as demonstrated in Fig.-1. These findings validate the capability of the BBD-designed model to accurately estimate the optimal conditions—specifically, extraction temperature, duration, and ethanol concentration—for enhancing both TPC and TFC in the UAE process.[19] Following the completion of experimental trials, RSM was utilised to identify the best extraction parameters for recovering bioactive compounds from cocoa bean shells. The 3-D surface plots illustrating the effects on TPC and TFC are displayed to visualise the interaction among variables in Fig.-2 and Fig.-3, respectively.

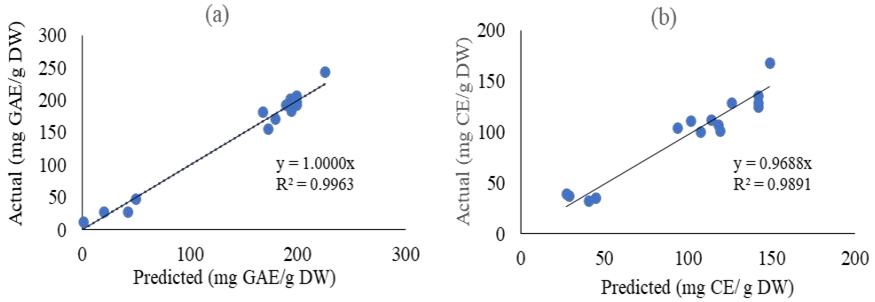

Fig.-1: The Pearson's Correlation Between Predicted and Experimental Value of TPC(a) and TFC(b)

Response surface methodology effectively determined the optimal ultrasound-assisted extraction parameters to maximise TPC and TFC from cocoa bean shell extracts. The three-dimensional surface plots and corresponding contour diagrams demonstrated clear synergistic interactions among the extraction variables. The optimal extraction conditions were established at 60°C, 60 minutes, and 60% (v/v) ethanol. The interaction between temperature and time contributed to enhanced phenolic recovery, primarily by improving solvent diffusion and promoting cell wall rupture. Moderate extraction temperatures ranging between 50°C and 90°C were found to be favourable, as they maintained a balance

between compound stability and extraction efficiency.[20] Ethanol concentrations within the 30–60% (v/v) range were the most effective, as aqueous ethanol mixtures facilitated the extraction of both hydrophilic and slightly hydrophobic phenolics more efficiently than pure solvents.[21] The alignment of optimal conditions for both TPC and TFC highlights the compatibility of their extraction behaviour under ultrasound-assisted extraction protocols.

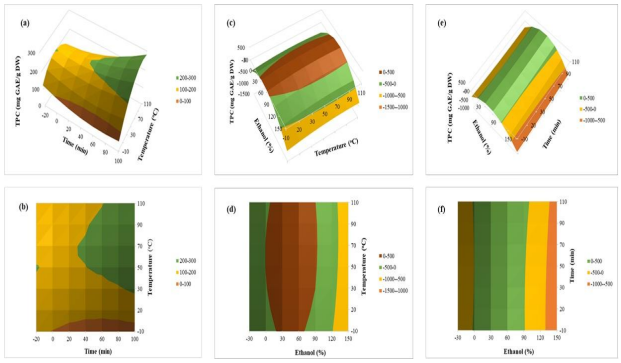

Fig.-2: The Response Surface and Contour Diagrams Illustrate How Extraction Temperature, Duration, and Ethanol Concentration Interact to Influence the TPC in CBS Extracts

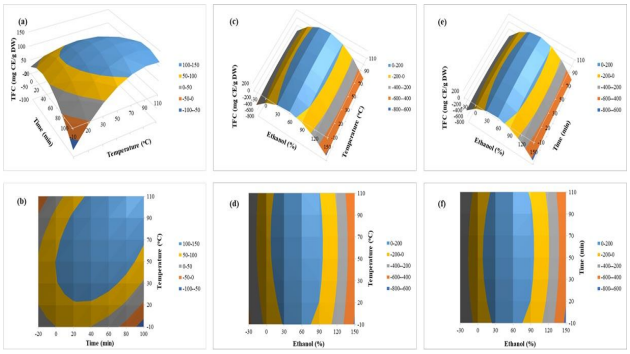

Fig.-3: The Surface and Contour Plots Provide a Visual Representation of how the Extraction Conditions Collectively Affect the Total Flavonoid Yield from Cocoa Bean Shell Extracts

Recovery of Bioactive Compounds

Ultrasound-assisted extraction (UAE) at a frequency of 37 kHz proved effective for recovering phenolic constituents from cocoa bean shell (CBS) extracts. The optimised conditions yielded a maximum TPC of 244.45 ± 7.46 mg GAE/g DW, as determined by the Folin–Ciocalteu assay. In parallel, the TFC reached 170.20 ± 13.24 mg CE/g DW, indicating the strong influence of extraction parameters on recovery efficiency. The interactive effects of temperature, extraction duration, and ethanol concentration demonstrated notable synergistic behaviour, improving overall extraction efficiency. Elevated temperatures improved phenolic compound recovery by increasing solubility and facilitating cell wall disruption, while prolonged extraction times enhanced the diffusion of target compounds. The UAE process benefits from ultrasonic cavitation, which generates microbubbles that collapse near the cellular structure, creating localised zones of high pressure. These effects disrupt plant cell walls and promote solvent penetration, thereby increasing extraction yields.[22] Furthermore, ethanol–water mixtures containing 50–70% (v/v) ethanol were found to provide optimal solvent polarity, enabling efficient solubilization of both hydrophilic and moderately lipophilic phenolics. These binary solvents demonstrated superior performance compared to pure ethanol or water, while also offering environmental advantages. An ethanol concentration range of 60–80% (v/v) was most favourable for flavonoid recovery; however, concentrations above 90% (v/v) led to reduced TFC, likely due to insufficient polarity for dissolving polar flavonoids.[23] Temperature optimisation also played a critical role in protecting thermosensitive compounds. Excessive heat can lead to oxidative degradation of key phytochemicals such as catechin, epicatechin, and p-coumaric acid, which are particularly susceptible to thermal instability.[24]

The study's findings indicate that the ideal extraction methodology represents a compromise between maximising the retrieval of bioactive phenolic and flavonoid components and preventing thermal-induced compound breakdown. These findings demonstrate how ultrasonic-assisted extraction methods successfully convert discarded cocoa husks into a valuable source of naturally occurring antioxidant substances.

Assessment of Antioxidant Properties

The antioxidant capability of CBS extracts was examined via three standard analytical procedures, such as DPPH, ABTS and FRAP. The DPPH radical inhibition potential recorded a value of 16.84 ± 1.25 mg TE/g DW, indicating notable antioxidant potential. This value significantly exceeds those reported by Sánchez (1.63–9.11 mg TE/g DW), likely due to the enhanced efficiency of UAE and solvent optimisation. However, the result is consistent with findings by Urbańska and Kowalska (10.5–25.2 mg TE/g DW), validating the method's effectiveness.[25,26] The ABTS assay revealed strong antioxidant activity at 382.57 ± 29.70 mg TE/g DW. ABTS is particularly well-suited for evaluating extracts containing both hydrophilic and lipophilic antioxidants due to its sensitivity and broad reactivity. Similarly, the FRAP assay yielded 642.32 ± 45.26 mg TE/g DW, reflecting strong reducing power via Fe^{3+} to Fe^{2+} conversion in the presence of TPTZ. The high FRAP values align with elevated TPC and TFC, underscoring the contribution of polyphenols to antioxidant capacity.[27] These findings confirm that the UAE significantly enhances the recovery of electron-rich phytochemicals. Ultrasonic cavitation promotes mass transfer and cell wall disruption, thereby facilitating improved solvent penetration.[28,29] The optimised conditions, moderate temperature, 60% ethanol, and appropriate extraction time, collectively improved both yield and antioxidant potential. Overall, the data reveal that systematic enhancement of UAE variables can successfully recover bioactive materials from CBS, substantiating its capacity as an eco-friendly antioxidant source.

CONCLUSION

This research successfully established the maximisation of bioactive substances from cocoa bean shells with ethanolic ultrasound-assisted extraction. The improved extraction performance was attributed to the combined effects of ultrasonic cavitation, appropriate solvent polarity, and precisely controlled temperature. A significant relationship was found between antioxidant capacity and the concentrations of phenolic and flavonoid compounds, confirming that polyphenols are the key contributors to the extract's radical scavenging capability. The results establish the promise of cocoa bean shells as an affordable and environmentally conscious source of natural antioxidants, suitable for use in food preservation, nutraceuticals, and cosmetic formulations. Further investigation should aim at process scale-up and industrial implementation to unlock the full commercial value of this green extraction strategy.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

The present work is related to *SDG-12: Responsible Consumption and Production*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID IDs, given below:

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