OPTIMIZATION AND PHYSICOCHEMICAL CHARACTERIZATION OF ULTRASOUND-ASSISTED EXTRACTION OF COCOA BEAN SHELL POWDER

By

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KELAPPAJI COLLEGE OF AGRICULTURAL ENGINEERING AND FOOD

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TAVANUR-679573, MALAPPURAM

KERALA, INDIA

2024-2025

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PROJECT REPORT

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DEPARTMENT OF PROCESSING AND FOOD ENGINEERING

KELAPPAJI COLLEGE OF AGRICULTURAL ENGINEERING AND FOOD TECHNOLOGY, TAVANUR, MALAPPURAM-679573

KERALA, INDIA

2024-2025

DECLARATION

We hereby declare that this project report entitled **"OPTIMIZATION AND PHYSICOCHEMICAL CHARACTERIZATION OF ULTRASOUND-ASSISTED EXTRACTION OF COCOA BEAN SHELL POWDER"** is a bonafide record of research work done by us during the course of research and that the report has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Place: Tavanur

Date: 17-01-2025

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CERTIFICATE

Certified that this project entitled **"OPTIMIZATION AND PHYSICOCHEMICAL CHARACTERIZATION OF ULTRASOUND-ASSISTED EXTRACTION OF COCOA BEAN SHELL POWDER"** is a bonafide record of project work jointly done by NAZRIN H (2021-06-007) , HIBA P K (2021-06-009), NAVYA G (2021-06-010), ARDRA V R (2021-06-011) and ABHINAND RAMESH (2021-06-012) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to them.

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TABLE OF CONTENTS

LIST OF TABLES

`

LIST OF FIGURES

LIST OF PLATES

LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
&	:	And
/	:	Per
+	:	Plus
=	:	Equal to
±	:	Plus or minus
°C	:	Degree Celsius
3D	:	Three-Dimensional
a*	:	Greenness or redness
ANOVA	:	Analysis of variance
aw	:	Water activity
b*	:	Blueness or yellowness
cm	:	Centimetre
DPPH	:	2, 2-Diphenyl-1-picrylhydrazyl
et al.	:	And others
etc	:	Etcetera
Fig	:	Figure
g	:	Gram
GAE/g	:	Milligrams of gallic acid equivalents per gram
min	:	Minutes
i.e	:	That is
KAU	:	Kerala Agricultural University
KCAEFT	:	Kelappaji College of Agricultural Engineering and Food Technology

W	:	Watt
L*	:	Lightness or darkness
N	:	Normality
rpm	:	Rotation per minute
RSM	:	Response surface methodology
viz.,	:	Namely
mm	:	Millimetre
ml	:	Millilitre
m^3	:	Meter cube
MHz	:	Mega hertz
KHz	:	Kilo hertz
cm^3	:	centimetre cube
µg	:	Microgram
H_2SO_4	:	Sulphuric acid
$CUSO_4$	:	Copper sulphate
K_2SO_4	:	Potassium sulphate
UAE	:	Ultrasound assisted extraction
CBS	:	Cocoa bean shell
RSM	:	Response surface methodology
SLE	:	Solid-liquid extraction

CS	:	Cocoa shoot
GT	:	Green tea
EC	:	Epicatechin
BHA	:	Butylated hydroxyanisole
BA	:	Biogenic amines
MAE	:	Microwave assisted extraction
EAE	:	Enzyme assisted extraction
SFE	:	Super critical fluid extraction
AC	:	Acceralated solvent extraction
SXE	:	Soxhlet extraction
MOSFET	:	Metal oxide-semiconductor field effect transistor
TPC	:	Total phenol content
AA	:	Antioxidant activity
SE	:	Solvent extraction
ROS	:	Reactive oxygen species
RSA	:	Radical scavenging activity
VAC	:	Volts alternating current

CHAPTER I

INTRODUCTION

Bioactive compounds are naturally occurring chemical substances that have biological activity and can influence health and physiological functions .When consumed in sufficient quantities, they offer significant health benefits, including the prevention and treatment of diseases such as cardiovascular conditions, obesity, diabetes and cancer (Patil *et al*., 2009). This growing prevalence of dietary-related diseases has prioritized the development of functional foods within the food industry, incorporating bioactive compounds to enhance health outcomes. Functional foods, rich in these bioactive compounds, have proven effective in promoting health and mitigating disease risks through innovative incorporation into daily diets.

Plant bioactives, derived from agri-food waste, are valuable compounds with health-promoting properties used in pharmaceuticals, nutraceuticals and food industries. Utilizing agricultural by-products to extract these bioactives supports waste valorization, reduces environmental impact and promotes sustainable production.

The cocoa bean shell (CBS), a major by-product of chocolate production, contains bioactive compounds such as polyphenols, methylxanthines, flavonoids, alkaloids and fatty acids. Despite its nutrient-rich composition, CBS is often discarded or used in low-value applications like animal feed or fertilizers. However, there is increasing interest in transforming CBS into bioactive rich value-added products for the food, nutraceutical and pharmaceutical applications.

Extraction is a major challenge, particularly when isolating bioactive compounds from natural sources, due to factors such as the complexity of raw materials, the need for efficient recovery methods, and concerns over environmental and health impacts. Conventional solid-liquid extraction (SLE) methods, such as maceration, infusion, and Soxhlet extraction, are time-consuming and require large amounts of solvents. Both chlorinated (e.g., chloroform, carbon tetrachloride) and non-chlorinated solvents (e.g., acetone, methanol) are used based on the target compound's properties. However, concerns over safety, toxicity, solvent residues, and low yields have driven interest in green extraction technologies that reduce or eliminate organic solvents.

Green extraction technologies are reshaping the recovery of valuable compounds from natural resources by minimizing environmental impact and enhancing process sustainability. Ultrasound-assisted extraction (UAE) is one of the green extraction method and is an

interesting process to obtain high valuable bioactive compounds from plant source etc. The main benefits will be a more effective extraction, thus saving energy, and also the use of moderate temperatures, which is beneficial for heat-sensitive compounds. For a successful application of the UAE, it is necessary to consider the influence of several process variables, the main ones being the applied ultrasonic power, the frequency, the extraction temperature, the reactor characteristics, and the solvent–sample interaction. To optimize the process, rate equations and unambiguous process characterization are needed, aspects that have often been lacking.(Esclapez *et al*.,2011). Therefore, optimizing the extraction process is crucial to reduce time and chemical usage effectively.

Response surface methodology (RSM) has been reported as a useful tool for process optimization when the independent variables had non-linear and interactive effects on the desired response. RSM is presently being applied in process optimization in the chemical, pharmaceutical, and food industries . Several elements affecting the extraction can be optimized in this procedure, primarily to minimize the process's energy costs. Nowadays, RSM successfully models, improves and optimizes extraction processes. RSM can be used for the optimization of UAE from plant source. Therefore this study was aimed to optimize the process parameters for UAE of CBS for the production of bioactives from CBS.

The main objectives of this research are

1. To characterize the physico-chemical properties of cocoa bean shell powder (CBS)
2. Process optimization of ultrasound extraction of cocoa bean by Response surface methodology (RSM)
3. Evaluation of storage stability of cocoa bean shell extract based on antioxidant activity

CHAPTER II
REVIEW OF LITERATURE

This chapter presents a comprehensive overview of the existing research related to cocoa and its by-products, particularly cocoa bean shell (CBS), with a focus onits nutritional value, bioactive compounds, and extraction methods. Cocoa, derived from Theobroma cacao, plays a vital role in the global agricultural economy, while CBS, often treated as waste, is emerging as a potential source of valuable bioactives such as polyphenols and dietary fiber. The review highlights conventional and green extraction techniques, emphasizing the advantages of ultrasound-assisted extraction (UAE) for its efficiency and sustainability. Additionally, the role of response surface methodology (RSM) as a powerful statistical tool for optimizing extraction parameters is discussed. The chapter concludes by identifying a clear research gap in the optimized use of UAE for CBS extraction, which this study aims to address.

2.1 COCOA

Cocoa, derived from the seeds of the cacao tree (*Theobroma cacao*), is a vital agricultural commodity with extensive applications in the food, pharmaceutical, and nutraceutical industries. Beg *et al*. (2017) report that cocoa demand is projected to increase by 30% by 2020, driven by rising consumer consumption of cocoa-based products. The global cocoa market is growing at a rate of 3% annually, with India experiencing a higher growth rate of 18–20%. Cocoa farming provides livelihood to 40–50 million people, with 5 to 6 million farmers directly engaged in its cultivation. Annually, about 3.5 million tonnes of cocoa are produced, contributing $11.8 billion to the global economy. Wessel and Quist-Wessel (2015) study examines cocoa production in West Africa, accounting for 70% of global output. Côte d'Ivoire and Ghana are the largest producers, with growth from 2 million tons to 3 million tons by 2010. However, high yields require improved management, pest control, and higher cocoa prices. The authors suggest that climate change and increasing land demand may threaten the region's cocoa dominance in the long run. They recommend significant structural changes in the cocoa sector, including improving economic viability, managing land use, and addressing ecological impacts. Schroth *et al*. (2016) provide a comprehensive analysis of cocoa's vulnerability to climate change in West Africa, highlighting how increasing maximum dry season temperatures, rather than just water availability, may become a significant limiting factor for cocoa production. The authors stress the importance of using shade trees in cocoa farms to mitigate the effects of rising temperatures, reversing the trend of shade reduction currently seen in the region. Van Vliet and Giller (2017) discuss the mineral nutrition of cocoa, highlighting its significant role in global agriculture. They highlight nutrient limitations,

nutrient cycling, and fertilizer responses, highlighting soil depletion and the need for further research to improve farm-level nutrient management.

Aprotosoaie *et al.* (2016) explores the flavor chemistry of cocoa and cocoa products. The study reveals that cocoa's value and quality are largely influenced by its unique flavor characteristics, which vary between cocoa types. The aroma of cocoa is derived from over 600 odor-active compounds, and its formation is influenced by factors like postharvest processing, genotype, seed composition, and environmental conditions. Kongor *et al*. (2016) provide a detailed review of the factors that influence the flavour profile of cocoa beans, which is a key determinant of the quality and acceptability of cocoa products such as chocolate. They highlight the importance of cocoa genotype, soil chemical composition, the age of the cocoa tree, postharvest treatments (including fermentation and drying), and roasting in shaping the flavour of cocoa beans.

Handojo *et al*. (2019) explore the potential of cocoa bean shell, a by product of the cocoa industry, as a raw material for dietary fibre powder. The shell, which is often discarded as waste, can be processed into alkalized fibre powder, offering a sustainable solution for waste management in cocoa production. Okiyama, Navarro, and Rodrigues (2017) explore the potential applications of cocoa shell, a by-product of the cocoa industry, as a dietary fibre source, antioxidant additive, and flavouring agent in the food industry. Badrie, Bekele, Sikora, and Badrie (2015) provide a comprehensive review of cocoa, examining its history, classification, and industry developments. They discuss its health benefits, antioxidant properties, and the impact of milk on polyphenol bioavailability. Ramiro-Puig and Castell (2009) study explores the antioxidant properties of cocoa, primarily due to flavonoids like (−)-epicatechin, catechin, and procyanidins. They also discuss cocoa's role in immune function, modulating inflammatory mediator secretion and affecting intestinal and systemic immune functions. The study emphasizes the need for further research into redox-sensitive pathways through cocoa flavonoids. Panak Balentić *et al*. (2018) review the potential of cocoa shell, a by-product from the chocolate industry, which is rich in protein, dietary fibre, ash, and bioactive compounds like methylxanthines and phenolics. Due to its nutritional and bioactive properties, cocoa shell could serve as a valuable raw material in the production of functional foods, pharmaceuticals, cosmetics, and even biofuels. The paper highlights the importance of utilizing such waste products efficiently, emphasizing cocoa shell’s potential for wide application across various industries

Andújar *et al*. (2012) provide an in-depth review of the health benefits associated with cocoa polyphenols, particularly in relation to cardiovascular health, inflammation, metabolic

disorders, and cancer prevention. The authors emphasize the significant antioxidant properties of cocoa polyphenols, which play a crucial role in protecting against oxidative stress.Osman, Nasarudin, and Lee (2004) investigate the antioxidative potential of cocoa shoot (CS) and young leaves (CL), comparing them to green tea (GT) leaves. The study found that the polyphenol content in cocoa leaves (28.4 mg/100 mg for CL) was higher than in green tea (17.3 mg/100 mg). The main polyphenols in both cocoa leaves included epicatechin (EC) and epigallocatechin gallate (EGCG), with cocoa leaves showing significantly higher EC concentrations than green tea. Antioxidant tests revealed that cocoa leaf extracts exhibited comparable or superior antioxidative properties to green tea and the synthetic antioxidant BHA, suggesting their potential to replace synthetic antioxidants in food applications.

Katz *et al*. (2011) highlight the health benefits of cocoa, highlighting its high phenolic antioxidant content, particularly flavonoids like catechin, epicatechin, and procyanidins. These compounds scavenge reactive oxygen species, chelate metals, and upregulate antioxidant defenses. Cocoa's effects on vascular health, insulin resistance, inflammation, neuroprotection, skin protection, and mood-enhancing are linked to its influence on nitric oxide production and NF-κB activity. Corti *et al*. (2009) highlight the cardiovascular benefits of cocoa, including positive effects on blood pressure, insulin resistance, and vascular function. They differentiate between natural and processed chocolate, emphasizing the potential for improved heart health and reduced coronary heart disease risk .Ellam and Williamson (2013) review the health effects of cocoa, which contains bioactive compounds like flavonoids (epicatechin and procyanidins), theobromine, and magnesium. While epicatechin and theobromine are efficiently absorbed in the small intestine, procyanidins are poorly absorbed until transformed by gut microbiota. Over 70 human intervention studies have examined cocoa's impact on cardiovascular health, particularly endothelial function, blood pressure, and cholesterol levels. The review highlights that epicatechin influences nitric oxide synthesis and breakdown, suggesting cocoa's potential cardiovascular benefits, though the high calorie and sugar content of chocolate should be considered in intervention studies. Desch *et al*. (2010) conducted a systematic review and meta-analysis to evaluate the effect of cocoa products on blood pressure (BP). The study found that cocoa products, particularly dark chocolate and cocoa beverages, may lower BP due to their high flavanol content. These flavonoids, derived from plants, are believed to contribute to the BP-lowering effects. The review highlights the potential benefits of cocoa consumption in managing hypertension.

Thorold (1975) treatise on cocoa diseases provides an in-depth analysis of pathogens affecting cocoa crops worldwide. With approximately 1800 references, it details various cocoa

parasites, fungi, bacteria, and pests. The book's encyclopedic nature may detract from clarity, as it overshadows major pathogens that significantly impact cocoa yield. Critics argue for a more selective focus and a stronger emphasis on integrated ecological pest and disease management. Despite these criticisms, the book remains a critical resource for cocoa industry professionals and plant pathologists, offering substantial information on fungal, viral, and insect-associated diseases and nutritional disorders.

Fig.2.1. Processing of cocoa bean shell powder

2.2. NUTRITIONAL AND CHEMICAL COMPOSITION OF CBS

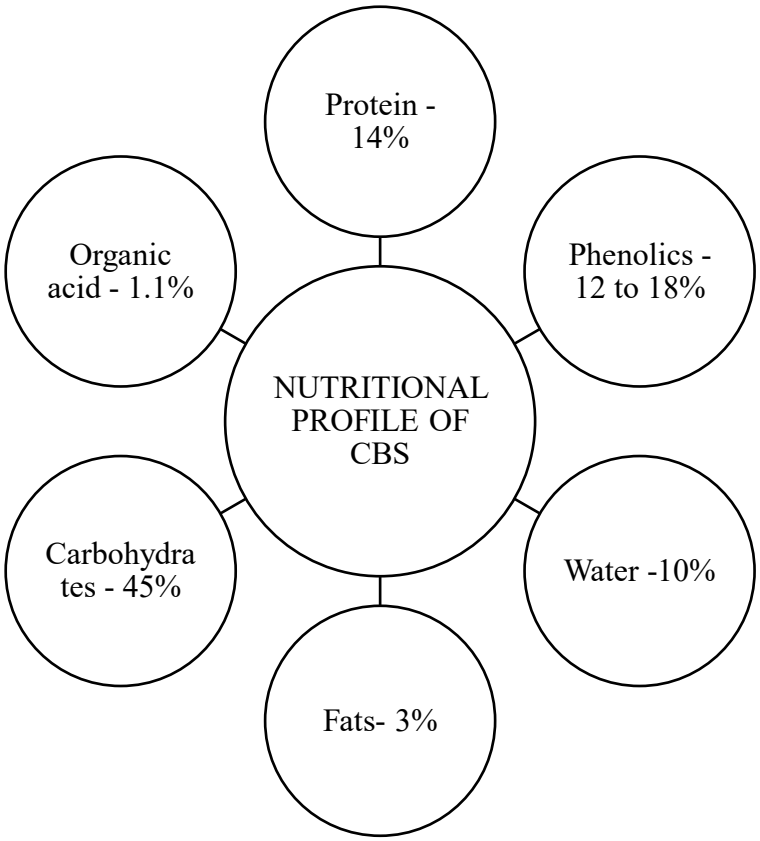

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Fig.2.2. Nutritional profile of CBS

The proximate composition of CBS has been reported by several authors is summarized in Table 2.1 (Rojo poveda *et al*., 2020). CBS proximate composition comprises proteins, fats, sugars, moisture, and ashes. Composition similar to cocoa beans, but CBS has less percentage of fats and higher number of fibres. CBS also have a higher content of proteins, fats, and carbohydrates compared to other cocoa by-products, such as cocoa pods (Pérez *et al.,* 2021). Proximate composition of CBS depending several variable factors, such as the climatic conditions of the farming area, the cocoa variety, processing conditions (fermentation, drying, roasting temperature)

Table 2.1 Nutritional and chemical composition of cocoa bean shells.

Parameter	Amount [a]
Energy (kcal/100 g)	122.00
Moisture (%)	3.60 – 13.13
Ash (g/100 g)	5.96 – 11.42
Proteins (g/100 g)	10.30 – 27.40
Fats (g/100 g)	1.50 – 8.49

Carbohydrates (g/100 g)	7.85 – 70.25
Starch (g/100 g)	0 – 2.80
Soluble sugars (g/100 g)	0.16 – 1.66
Dietary fiber (g/100 g)	39.25 – 66.33
Soluble fiber (g/100 g)	7.03 – 16.91
Insoluble fiber (g/100 g)	28.34 – 50.42
Pectin (g/100 g)	7.62 – 15.59
Minerals	
Calcium (g/100 g)	0.23 – 0.44
Phosphorus (g/100 g)	0.58 – 1.00
Magnesium (g/100 g)	0.48 – 1.29
Potassium (g/100 g)	1.25–1.82
Sodium (mg/100 g)	16.00 – 192.20
Iron (mg/100 g)	27.60 – 80.50
Manganese (mg/100 g)	4.53
Copper (mg/100 g)	2.35 – 6.62
Selenium (mg/100 g)	0.21
Cobalt (mg/100 g)	0.10
Zinc (mg/100 g)	2.75 – 19.00
Chromium (mg/100 g)	0.67 - 4.86
Vitamins	
B1 (µg/g)	0.70 – 3.10
B2 (µg/g)	0.90 – 3.10
B6 (µg/g)	Tr
D (µg/g)	Tr – 0.5
E (µg total tocopherols/g CBS fat)	1.02
Polyphenol content	
Total phenolic content [b]	3.12 – 94.95
Total flavonoid content [c]	1.65 – 40.72
Total tannin content [c]	1.70 – 25.30
Flavanols	
Epicatechin (mg/g)	0.21 – 34.97

Catechin (mg/g)	0.18 – 4.50
Procyanidin B1 (mg/g)	0.55 – 0.83
Procyanidin B2 (mg/g)	0.23 – 1.38
Methylxanthines	
Theobromine (g/100 g)	0.39 – 1.83
Caffeine (g/100 g)	0.04 – 0.42
Volatile organic compounds (aromatics; µg/g)	4.92 – 16.10

[a] Data are referred to a CBS dry weight basis unless indicated differently. Intervals have been created, comprising all the values from the cited literature).

2.3 COCOA BIOACTIVE COMPOUNDS

Rojas *et al*., (2022) analysed the bioactive compounds in native cocoa (*Theobroma cacao L.*), emphasizing its status as a superfood due to its complex chemical composition, influenced by genotype, origin, maturity, and post-harvest practices. These compounds include flavonoids, alkaloids, phenolic acids, and other polyphenolic compounds, which have been extensively studied for their antioxidant, anti-inflammatory, cardioprotective, and neuroprotective properties. Soares and Oliveira (2022) highlight that 10% of annual cocoa production is used as cocoa beans, with the rest being by-products like shells, pulp, and husk. These residues contain valuable nutrients and bioactive compounds, offering potential applications in the food industry.

Flavonoids, particularly flavanols, are among the most prominent bioactive compounds found in cocoa. These compounds, including epicatechin, catechin, and procyanidins, are known for their antioxidant properties and their positive effects on cardiovascular and cognitive health.Engler and Engler (2004) found flavonoid-rich cocoa and chocolate have vasculoprotective effects, including antioxidant activity, improved endothelial function, blood pressure reduction, and immune modulation, but called for larger clinical trials. Bauer *et al.* (2011) reviewed the effects of cocoa flavonoids on cardiovascular health, finding that high cocoa intake lowers blood pressure, improves endothelial function, and potentially enhances insulin sensitivity. However, no significant effects were observed on blood lipids or body weight. The review highlighted the need for further research on the optimal dose and long-term clinical outcomes of cocoa flavonoids. Martin and Ramos (2021) reviewed human studies on cocoa flavanols, highlighting their beneficial effects on health, particularly in reducing the risk of chronic diseases such as cardiovascular conditions and metabolic disorders. However, they

noted that further controlled trials and studies are needed to clarify mechanisms and determine appropriate dosages.

Rojas *et al*., (2022) highlighted that cocoa beans are rich in alkaloids, such as theobromine and caffeine, which stimulate the central nervous system, as well as phenolic compounds, particularly flavonoids, known for their antioxidant and anti-inflammatory properties. They noted that moderate chocolate consumption is associated with reduced risks of coronary heart disease, strokes, and peripheral vascular disease, although excessive intake can be harmful. Additionally, the mood-enhancing effects of cocoa are attributed to its sensory properties and bioactive compounds, including flavonoids, theobromine, caffeine, and salsolinol.

Cocoa is rich in polyphenolic compounds, particularly phenolic acids like ferulic acid and cinnamic acid, which contribute to its potent antioxidant activity. These antioxidants help protect cells from oxidative damage, which is a significant factor in aging and various chronic diseases. Sies *et al*., (2005) found that cocoa polyphenols, particularly flavan-3-ols, can improve cardiovascular health and inflammation. They found that cocoa polyphenols inhibit 5-lipoxygenase, reduce leukotriene synthesis, and improve endothelial function. Additionally, flavonoids suppress oxidative stress, reducing proinflammatory and proatherogenic effects, thus benefiting cardiovascular health.

Cocoa processing, including fermentation, drying, and roasting, can affect the levels of bioactive compounds present in the final product. The processing techniques play a significant role in determining the nutritional and therapeutic potential of cocoa. Melo *et al*., (2021) evaluated the fermentation process of cocoa beans and its effect on bioactive compound content and antioxidant activity. They found that after 48 hours of fermentation, there was a significant reduction in slate seeds, the appearance of partially fermented beans, and an increase in acidity and temperature. During this period, the content of bioactive compounds with antioxidant activity was highest. The study suggested that a blend of beans fermented for 48 hours and fully fermented beans could be used to produce functional chocolates with enhanced health benefits. Herrera-Rocha *et al*., (2024) studied the impact of cocoa post-harvesting processes on the metabolic composition of cocoa liquor. Using untargeted metabolomics and lipidomics, the research identified 22 bioactive and 41 flavour compounds in fine-flavour cocoa liquors. They found that drying had a similar effect on cocoa biochemistry as fermentation and roasting. The study traced changes in both volatile and non-volatile compounds during processing, highlighting their influence on flavour development and nutraceutical properties. These

findings provide a foundation for future efforts to optimize cocoa post-harvesting techniques to improve both flavour and health benefits.

Dang and Nguyen (2019) study found that cocoa beans' bioactive compounds, including proanthocyanidins, caffeine, theobromine, and antioxidant capacity, increased with maturity. However, prolonged fermentation, with or without the enzyme Pectinex® Ultra SP-L, significantly reduced these compounds, particularly proanthocyanidin content and antioxidant capacity. The study highlights the significant influence of maturity, fermentation method, and duration on bioactive compound levels. Tušek *et al*., (2024) explored the bioactive compounds in cocoa beans, including polyphenolics, which have potential health benefits. These compounds, including antioxidant, anti-carcinogenic, and anti-inflammatory effects, could contribute to overall health. They stressed the importance of efficient extraction techniques. Jiménez-Rodríguez *et al*., (2024) studied the effects of different drying temperatures (50°C, 60°C, and 70°C) on the bioactive composition, energy consumption, and vibrational properties of cocoa beans. Hurst *et al*. (2011) studied the effects of fermentation, drying, roasting, and Dutch processing on flavan-3-ol stereochemistry in cacao beans and cocoa ingredients. They found that fermentation leads to significant loss of (-)-epicatechin and (+)-catechin, while roasting decreases and increases (-)-catechin at higher temperatures. Dutch processing also results in loss of both, with heat-induced epimerization contributing to the increase. Delgado-Ospina *et al.,* (2020) studied the impact of fermentation, drying, and roasting on biogenic amines, polyphenols, anthocyanins, flavanols, and antioxidant properties in Colombian Criollo cocoa beans and shells. They found that fermentation and drying affected microbiota and biogenic amines content, while thermal processing increased BAs content. The study also highlighted serotonin degradation during roasting. Principal component analysis showed that polyphenols and BAs negatively influenced antioxidant properties, while anthocyanins, catechin, and epicatechin positively influenced antioxidant capacity.

2.4 EXTRACTION METHODS: CONVENTIONAL AND GREEN EXTRACTION

Extraction is a crucial step in isolating bioactive compounds from their natural sources. The purpose of extraction is to obtain concentrated bioactive substances that can be further analysed, characterized, and used in pharmaceutical, nutraceutical, and food industries. There are several methods for extracting bioactive compounds, and each is suited to specific compounds and materials.

The purpose of all extraction is to separate the soluble plant metabolites, leaving behind the insoluble cellular marc (residue). The initial crude extracts using these methods contain complex mixture of many plant metabolites, such as alkaloids, glycosides, phenolics,

terpenoids and flavonoids. Some of the initially obtained extracts may be ready for use as medicinal agents in the form of tinctures and fluid extracts but some need further processing. Several of the commonly used extraction methods are discussed below.

2.4.1 Conventional Extraction Methods

Extraction and recovery processes have surged in recent years due to trends in healthier lifestyles and the increasing use of antioxidants in diets. As a result, several extraction methods have been developed to enhance the yield of bioactive compounds. Techniques such as maceration, percolation, hydro-distillation, boiling, reflux, soaking, and Soxhlet have been proposed (Alara *et al.,* 2018). Soxhlet extraction is particularly popular due to its simplicity, low cost, ease of maintenance, and lower solvent usage compared to methods like soaking, boiling, or maceration (Sharma and Janmeda, 2017). Various solvents, including ethanol, methanol, benzene, chloroform, and ethyl acetate, have been tested in this method to assess their impact on extraction yields.

In general, liquid–liquid and solid–liquid extractions are the most commonly employed techniques for extracting bioactive compounds. Despite being traditional methods, maceration and water infusion are still in use today (Cujic *et al.*, 2016). These techniques have expanded to incorporate solvents like ethanol, methanol, and acetone, in addition to water, to extract bioactive compounds (Albuquerque *et al.,* 2018). Conventional extraction methods often require large volumes of solvent, have lower yields, and take more time compared to newer techniques. It is also noted that when heat is applied during extraction, it can cause degradation of the extracted compounds, reducing their bioactivity. Factors such as time, particle size, solvent type, mass-to-volume ratio, and temperature have been studied in the context of traditional extraction methods (Bergeron *et al*., 2005). The choice of solvent plays a significant role in determining which compounds are extracted and their biological activity. Ethanol and methanol are the most frequently used solvents due to their ability to achieve higher yields of bioactive compounds (Yu *et al.,* 2019).

2.4.2 Green Extraction

Green extraction refers to sustainable and eco-friendly methods of extracting valuable compounds from natural sources, aligning with the principles of green chemistry. These techniques aim to minimize the use of hazardous chemicals, reduce energy consumption, and lower environmental impact while maintaining efficiency and quality. Green extraction approaches include the use of renewable solvents, such as water, ethanol, or supercritical fluids like carbon dioxide, and innovative technologies such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and enzymatic extraction. These methods improve

extraction yields, reduce processing time, and eliminate or limit the generation of harmful waste. Additionally, green extraction emphasizes the valorization of by-products and biomass, promoting a circular economy. Advances in green extraction are increasingly applied in fields such as food, pharmaceuticals, and agriculture, enabling the isolation of bioactive compounds like polyphenols, essential oils, and pigments.

2.4.3 Microwave assisted extraction (MAE)

MAE utilizes microwave energy to facilitate partition of analytes from the sample matrix into the solvent . Microwave radiation interacts with dipoles of polar and polarizable materials (e.g. solvents and sample) causes heating near the surface of the materials and heat is transferred by conduction. Dipole rotation of the molecules induced by microwave electromagnetic disrupts hydrogen bonding; enhancing the migration of dissolved ions and promotes solvent penetration into the matrix .In non-polar solvents, poor heating occurs as the energy is transferred by dielectric absorption only (Handa *et al.,*2008)

MAE can be considered as selective methods that favour polar molecules and solvents with high dielectric constant. Tannins and anthocyanins may not be suitable for MAE as they were potentially subjected to degradation at high temperature.

2.4.4. Enzyme assisted extraction

Enzyme-assisted extraction (EAE) is a process that uses specific enzymes to break down the cell walls and other structural components of plant or other biological materials, facilitating the release of bioactive compounds.It offers several benefits compared to traditional methods. It involves milder reaction conditions, operating at lower temperatures and for shorter durations. This method also allows for the use of whole plant material, requires fewer processing steps, and provides high substrate specificity. As a result, EAE can extract a wide range of bioactive compounds, including those typically hidden within cellular organelles such as vacuoles and plant cell walls, which are otherwise difficult to access. These compounds also exhibit high bioavailability and quality with minimal residue levels. Additionally, EAE has the potential to reduce production costs by eliminating the need for multiple installations required in conventional extraction processes (Gligor *et al*.,2019).

2.4.5. Supercritical fluid extraction (SFE)

Supercritical fluid (SF) or also called as dense-gas is a substance that shares the physical properties of both gas and liquid at its critical point. Factors such as temperature and pressure are the determinants that push a substance into its critical region. SF behaves more like a gas but have the solvating characteristic of a liquid. An example of SF is CO_2 that become SF at

above 31.1°C and 7380 kPa. Interest in Supercritical-CO_2 (SC-CO_2) extraction due to excellent solvent for nonpolar analytes and CO_2 is readily available at low cost and has low toxicity. Even though SC-CO_2 has poor solubility for polar compounds, modification such as adding small amount of ethanol and methanol enable it to extract polar compounds. SC-CO_2 also produces analytes at concentrate form as CO_2 vaporizes at ambient temperature. SC-solvents strength can be easily altered by changing the temperature, pressure or by adding modifiers that lead to reduce extraction time. Optimization of SC-CO_2. A major drawback of this method is the initial cost of the equipment is very high (Naudé *et al*.,1998)

2.4.6. Accelerated solvent extraction (ASE)

ASE is an efficient form of liquid solvent extraction compared to maceration and Soxhlet extraction as the method use minimal amount of solvent. Sample is packed with inert material such as sand in the stainless steel extraction cell to prevent sample from aggregating and block the system tubing (Rahmalia *et al*.,2015)

Packed ASE cell includes layers of sand-sample mixture in between cellulose filter paper and sand layers. This automated extraction technology is able to control temperature and pressure for each individual samples and requires less than an hour for extraction. Similar to other solvent technique, ASE also critically depend on the solvent types.

2.5. ULTRASOUND ASSISTED EXTRACTION (UAE)

Ultrasound waves are akin to sound waves, but they possess a frequency exceeding 16 kHz, making them inaudible to the human ear (Fellows, 2000). The uses of ultrasonic technology are generally divided into two main categories: low-intensity ultrasonics and high-intensity ultrasonics (Fu *et al*., 2020). Low-intensity ultrasonics are characterized by their high frequency, which ranges from 5 to 10 MHz, and their relatively low power output of less than 1 W/cm^2. This type of ultrasonic technology is non-destructive and is ideal for testing and characterizing various materials, which is why it is often referred to as diagnostic ultrasonics (Carreira *et al*., 2021). High intensity ultrasonic technology, commonly known as power ultrasonics, functions at low frequencies between 20 and 100 kHz and operates at significant power levels ranging from 10 to 1000 W/cm^2. In contrast to low intensity ultrasonics, high intensity ultrasonics are noted for their destructive properties. This method is highly effective in speeding up chemical reactions through the process of cavitation. The collapse of cavitation bubbles generates energy that can produce a variety of effects, including extraction, crushing, and emulsification (Arvanitoyannis *et al*., 2017).

Ultrasound-assisted extraction is a non-thermal technique that utilizes acoustic energy to enhance the rates of release and diffusion of target substances through the cavitation of the solvent (Cui *et al*., 2021). It primarily operates on the principle of cavitation, which leads to the compression and expansion of the matrix, resulting in the permeabilization of the cell wall and an increased extraction yield of the desired compounds (Iqbal *et al*., 2021). In contrast to electromagnetic waves, ultrasonic waves are mechanical and can travel through solid, liquid, and gaseous mediums (Shen *et al*., 2023).

UAE is a part of power ultrasonic technology. Power ultrasonic is a field of ultrasonology dedicated to utilizing ultrasonic energy for matter processing. UAE employs thermal, mechanical, and cavitation effects to extract bioactive substances. The ultrasonic action disrupts cell walls, facilitating the release and diffusion of components contained within the cells (Chemat *et al*., 2017). The thermal impact of ultrasonic waves involves the absorption of their vibrational energy by the medium, which is then transformed into heat. As a result, the temperature of the medium rises correspondingly. The amount of heat produced is influenced by various factors, including the characteristics of the medium, the power of the ultrasonic waves, and the duration of exposure (Qiu *et al*., 2020). The mechanical effect of ultrasonic waves refers to the situation where applying ultrasonics to a medium causes the particles within the medium to vibrate in alignment with the mechanical wave. This leads to an enhancement in particle motion, thereby speeding up the mass transfer process (Wen *et al*., 2018). Among the various ultrasonic effects, cavitation is recognized as the most significant. Ultrasonic cavitation refers to the process where tiny bubbles, known as cavitation nuclei, within a liquid vibrate, expand, and accumulate energy from the acoustic field under the influence of ultrasound. When this accumulated energy exceeds a certain threshold, the cavitation bubbles collapse and close abruptly. Cavitation bubbles are generally categorized into two types: transient and stable. Transient cavitation bubbles have a very short lifespan, whereas stable cavitation bubbles persist for a longer duration (Shen *et al.,* 2023). During cavitation, intense hydrodynamic shear forces are generated, leading to the formation of free radicals and a rise in both pressure and temperature (Constantino and Garcia-Rojas, 2020).

UAE utilizes the thermal, mechanical, and cavitation effects generated by ultrasound in the extraction process. As illustrated in Fig. 2, ultrasound enhances the penetration of solvents into cells, thereby improving mass transfer. Furthermore, ultrasound disrupts cell walls, promoting the release of cellular contents. Consequently, efficient cell disruption and enhanced mass transfer are regarded as the two key factors contributing to the increased production achieved with UAE (Shen *et al*., 2023).

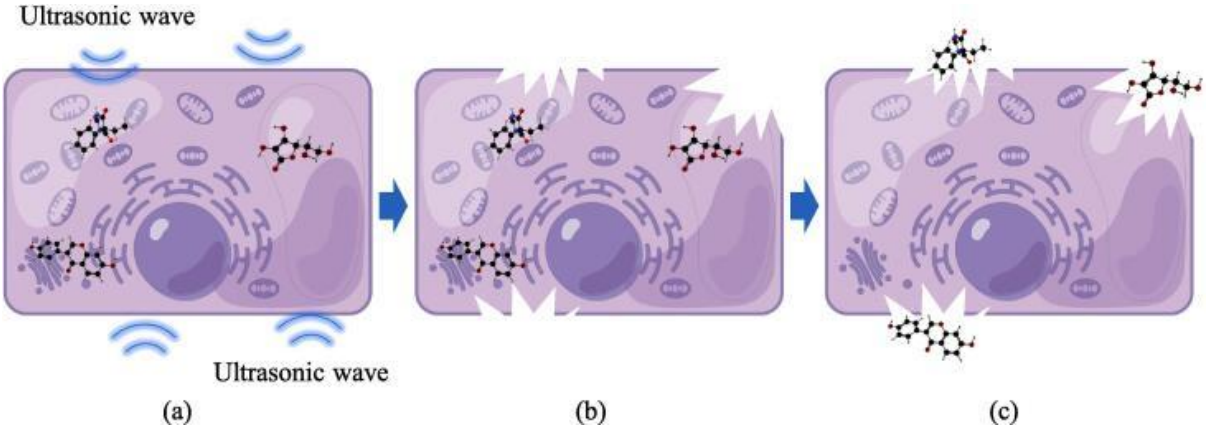

Fig.2.3. The mechanism of ultrasonic assisted extraction (a) indicates that ultrasonic will act on intact cell. (b) indicates cell rupture after ultrasonic treatment. (c) indicates that the bioactive components are released.

The influence of UAE on various bioactive components varies depending on its application in the extraction process. The effect of UAE can be either beneficial or detrimental, based on the specific conditions and parameters utilized. The selection of extraction parameters plays a crucial role in determining the outcomes of the extraction. Additionally, these parameters can affect the physicochemical properties of bioactive components, highlighting the importance of careful optimization to achieve the desired results (Shen *et al*., 2023).

The ultrasound-assisted extraction technique was shown to be very efficient in the extraction of oil from grape seeds. Compared with Soxhlet, ultrasound-assisted extraction carried out at 20 kHz, 150 W for 30 min produced similar oil recovery. The fatty acid compositions of the oil was not affected significantly by the application of ultrasound (Di Stefano *et al*. 2021).Samaram *et al.* (2013) evaluate the suitability of ultrasound-assisted extraction (UAE) for the recovery of oil from papaya seed as compared to conventional extraction techniques (*i.e*., Soxhlet extraction (SXE) and solvent extraction (SE)). Results indicated that both solvent extraction and ultrasound-assisted extraction (UAE) methods recovered relatively high yields.

Ma *et al.* (2009) investigated the effects of ultrasonic variables including extraction time, temperature, and ultrasonic power on the yields of seven phenolic acids. Results showed that the yields of phenolic compounds increased with both ultrasonic time and temperature increased, whereas the opposite occurred with increasing time at higher temperature to some certain. A positive effect on the yields of phenolic compounds was observed with increase of ultrasonic power. When ultrasonic power increased from 3.2 to 30 W, the yields of most phenolic compounds were significantly increased ($p < 0.05$), and then slowly after 30 W.

When appropriately applied with adequate power, ultrasound-assisted extraction offers several advantages over traditional heating methods. These benefits include improved extraction efficiency, higher yields, and faster processing times. Additionally, ultrasound-assisted extraction is generally safer, consumes less energy, and requires smaller amounts of solvent. However, one limitation is the lack of uniformity in the process, as the intensity of ultrasound waves diminishes with increasing distance from the emitter (Cui *et al*., 2021).

2.6. UAE OF PLANT EXTRACTS

Wen *et al.* (2018) highlight that plant bioactive components, such as terpenes, flavonoids, and alkaloids, play a vital role in physiological processes, making their efficient extraction a focus for various industries. Belwal *et al.* (2018) emphasize that extraction serves as a cornerstone for industries like pharmaceuticals and food processing, where precise methods directly impact production efficiency and product quality. I. Calinescu *et al.* (2021) point out that traditional extraction methods face significant drawbacks, including long durations, high energy costs, and degradation of heat-sensitive components, which necessitate innovative solutions. Wei *et al.* (2015) advocate for green extraction technologies that not only address these challenges but also align with environmental sustainability by reducing waste, energy usage, and solvent reliance.

Chemat *et al.* (2017) explain that UAE leverages ultrasonic cavitation to enhance extraction rates, reduce processing time, and produce high-purity extracts, making it a leading green extraction method.Sun *et al*. (2020) stress that optimized UAE conditions ensure the preservation of biological activities, which is critical for functional applications in bioengineering, cosmetics, and nutraceuticals. Kadam *et al.* (2013) argue that UAE not only meets industrial demands for cost-effectiveness and high yields but also supports environmentally friendly practices, making it a transformative technology. They emphasize the need for further studies on UAE's integration with other technologies, such as microwave or enzymatic methods, to unlock its full potential and expand its applications across diverse sectors.

Xu *et al.,* (2018) found that low-frequency ultrasonic treatments (20, 28, and 35 kHz) have a more pronounced effect on the secondary structure of proteins compared to high-frequency treatments (40 and 50 kHz). Jin *et al*.,(2016) demonstrated that ultrasonic treatment conditions significantly alter the secondary structure of proteins, impacting functionality. Huang *et al*.,(2019) observed that prolonged ultrasonic treatment induces distinct structural changes, such as increasing α-helix content in soy protein isolate. Xu *et al.,* (2018) emphasized

the importance of optimizing ultrasonic parameters, including sound pressure and sound field uniformity, for efficient protein extraction. Jin et al. highlighted that the specific ultrasonic frequency and treatment duration must be tailored to the protein type to maximize bioactive functionality. Huang *et al*.(2019),concluded that understanding protein-specific responses to ultrasonic waves is critical for leveraging UAE in bioactive component extraction.

2.7. RESPONSE SURFACE METHODOLOGY

The response surface methodology (RSM) is a commonly used mathematical and statistical approach for modelling and analysing processes where the response of interest is influenced by multiple variables. The main goal of RSM is to optimize this response. The variables influencing the process are referred to as independent variables, while the outcomes are termed dependent variables (Aydar, 2018). In general, the UAE process is influenced by several factors, including ultrasonic power and frequency, temperature, ultrasonication time, solvent properties and composition, particle size, and the solid-to-solvent ratio. Optimizing the extraction process is essential to obtain antioxidant compounds with high bioactivity. Numerous researchers have applied RSM to optimize the extraction of bioactive compounds from various biomass sources, such as olive tree leaves (Martínez-Patiño *et al*., 2019).

RSM is a powerful statistical tool widely employed in engineering applications to develop accurate models for optimization design (Aghbashlo *et al*., 2012). It is primarily used for enhancing and optimizing process parameters. RSM is extensively applied across various stages, including experimental design selection, generation of response surfaces, creation of contour plots, prediction and validation of model equations, identification of multi-response parameters and their optimal levels, and determination of optimal conditions. This methodology allows process optimization to be achieved with minimal cost and time.Hossain *et al*., (2012) optimized ultrasound assisted extraction (UAE) conditions to maximize antioxidant activity, total phenol content, and polyphenol content of marjoram extracts. They identified optimal conditions using response surface methodology, and found that optimal UAE yields were significantly higher than solid/liquid extracts.

Tomsik *et al*.,(2016) investigated ultrasound-assisted extraction for obtaining bioactive compounds from *Allium ursinum*. Using a three-level, four-variable face-centered cubic design combined with response surface methodology (RSM), the effects of temperature (40–80 °C), ethanol concentration (30–70%), extraction time (40–80 min), and ultrasonic power (19.2–38.4 W/L) were optimized. The second-order polynomial model indicated optimal conditions at 80 °C, 70% ethanol, 79.8 min, and 20.06 W/L. The experimental data closely aligned with

predicted values, validating the model.Ghasemzadeh *et al*., (2014) used response surface methodology to optimize ultrasound-assisted extraction of catechin, myricetin, and quercetin from curry leaves. The optimal conditions yielded these compounds with enhanced antioxidant activity (83%), significant anticancer effects against HeLa cells (67.2 µg/mL), and no toxicity to normal cells.

Carrillo *et al*., (2017) developed a method to quantify cocoa methylxanthines, flavanol monomers, and major procyanidins using ultrasound-assisted extraction. The method achieved optimal yields with a 20-minute extraction time and improved separation using a sub-2-µm particle column. This method demonstrated 20% higher extraction efficiency compared to the AOAC method for polyphenol analysis in cocoa-based matrices.Md Yusof *et al.*, (2019) optimized the ultrasound-assisted extraction of flavonoids from Malaysian cocoa shell extracts using response surface methodology. The optimal conditions for maximum total flavonoid content were 80% ethanol, 55°C, and 45 minutes, yielding 7.47 mg RE/g DW. Validation experiments confirmed the model's accuracy, with a TFC of 7.23 ± 0.15 mg RE/g DW under refined conditions.

2.8. RATIONALE AND RELEVANCE OF THE WORK

Cocoa bean shell (CBS), a by product of cocoa processing, is often discarded or underutilized despite its rich composition of bioactive compounds, including polyphenols, dietary fibers, and antioxidants. The valorization of CBS not only aligns with the principles of sustainability and waste management but also offers opportunities to develop functional ingredients for various industries, such as food, pharmaceuticals, and neutraceuticals. Ultrasound-assisted extraction (UAE) has emerged as an efficient, green technology for extracting valuable compounds, offering advantages such as reduced extraction time, lower solvent consumption, and improved yield compared to conventional methods.To maximize the efficiency of this process, response surface methodology (RSM) is employed as a robust statistical tool to optimize the extraction parameters, enabling precise control over variable interactions and ensuring reproducibility. This study, therefore, aims to harness UAE for the physicochemical characterization and optimization of CBS extraction, contributing to sustainable processing technologies and enhancing the economic value of cocoa industry by products.

The " optimization and physicochemical characterization of ultrasound-assisted extraction of cocoa bean shell powder" project aims to transform waste into valuable bioactive compounds from discarded cocoa bean shells (CBS), promoting sustainable practices in the cocoa industry. The project aligns with global goals of reducing food waste and enhancing

resource efficiency, contributing to a circular economy. The use of ultrasound-aided extraction (UAE) minimizes solvent usage, reduces extraction time, and improves yield and quality. RSM is a powerful statistical tool for optimizing processes, reducing experimental costs and improving reproducibility. The physicochemical analysis of CBS extracts is crucial for understanding their functional and nutritional potential, which can guide their application in food, pharmaceutical, and cosmetic industries. CBS extracts may contain bioactive compounds with antioxidant, antimicrobial, and health-promoting properties, which can be used in functional food and beverage development, nutraceuticals, natural preservatives, and cosmetic formulations. The findings can contribute to academic research and provide a framework for industrial scalability.

2.9. RESEARCH GAP

Despite growing interest in ultrasound-assisted extraction (UAE) for extracting valuable bioactive compounds from cocoa bean shell powder, there is still a lack of in-depth studies that examine all the physico-chemical factors affecting extraction efficiency. While UAE has been optimized for various plant materials, cocoa bean shells, which are often discarded as a byproduct in the cocoa industry, have received much less attention. Furthermore, we don't yet fully understand how different ultrasound setting temperature, substrate ratio and extraction time interact with solvent properties to affect both the yield and quality of the extracted compounds. Many studies also overlook how these factors influence the physicochemical properties of the extracted material and the overall sustainability of the process. There is a clear gap in applying Response Surface Methodology (RSM) to fine-tune and better understand how these variables work together during the UAE of cocoa bean shell powder.

This gap gives the opportunity to investigate how ultrasound conditions can not only optimize extraction efficiency but also enhance the quality and properties of the final product. By using methods like RSM, we could gain valuable insights into how these factors interact, leading to more effective and sustainable extraction processes.

CHAPTER III

MATERIALS AND METHODS

This chapter describes the ultrasound assisted extraction from cocoa bean shell. The materials used for the extraction and analysis of product was explained. The optimization of process parameters for ultrasound assisted extraction was carried out in terms of maximum yield, antioxidant activities, and phenolics.

3.1 MATERIAL AND SAMPLE PREPARATION

The cocoa waste (Cocoa bean shell) was collected from the Cocoa Research Station (Kerala Agricultural University, Thrissur). Sample was prepared by cleaning, washing, drying and pulverizing the cocoa bean shell into fine powder. Ground cocoa bean shell was stored in a glass container in dark and dry conditions to avoid components oxidation.

Plate 3.1. Cocoa bean shell powder

3.2 PHYSICO-CHEMICAL PROPERTIES OF CBS

Prior to the extraction from CBS, the physiochemical properties of CBS were studied. Physico-chemical properties such as moisture content, bulk density, porosity and colour were determined by standard methods as explained in the following sections.

3.2.1 BULK DENSITY

The bulk density of a powder is the ratio of the mass of an untapped powder sample and its volume including the contribution of the inter-particulate void volume. The bulk density is expressed in kilogram per cubic meter, because the measurements are made using cylinders. The bulk density of cocoa bean shell powder is measured in 10 ml cylinder. The powder was filled in cylinder and the filled cocoa bean shell powder is weighted.

$$\text{Bulk density (g/cm3)} = \frac{weight\ of\ cocoa\ bean\ shell\ powder,(kg)}{volume\ of\ beaker,(m3)} \quad (1)$$

3.2.2 True Density

Known weight of cocoa bean shell was transferred into a measuring cylinder. Slowly add toluene into the measuring to fill the voids. Measure the amount of toluene added. True density of cocoa bean shell was determined using the following equation 2.

$$\text{True density} = \frac{weight\ of\ sample,(kg)}{(bulk\ volume - volume\ of\ toluene),m3} \quad (2)$$

3.2.3 Porosity

Porosity of the cocoa bean shell was computed from the bulk and true density using a formula as explained . The reported values are means of 10 replications.

$$\text{Porosity} = \frac{true\ density - bulk\ density}{true\ density} \quad (3)$$

3.2.4 Moisture Content

The moisture content of cocoa bean shell powder was determined using an infrared moisture analyser. This device can measure the moisture content of various materials and operates based on the thermogravimetric principle. While measuring the sample's weight, the analyser uses infrared heating elements and water evaporation channels to dry the sample quickly. During the drying process, the instrument continuously measures and instantly displays the moisture loss (%) of the sample in real time. Once drying is complete, the final moisture content is locked. By pressing the display button, additional data such as moisture value, initial weight, starting value, and measurement time can be viewed.

3.2.5 Colour Measurement

The Lovibond colorimeter is a precision instrument used to measure the colour of cocoa bean shell powder by analysing its surface reflectance. It is calibrated against standard reference materials, such as white and black glass, to ensure accuracy and consistency. The device provides colour measurements in the CIELAB colour space, including L* (lightness), a* (red-green spectrum), and b* (yellow-blue spectrum) values. Additionally, it calculates chroma (C*), which indicates the saturation or intensity of colour, and hue angle (h°), representing the type of colour perceived, derived from the a* and b* coordinates. These parameters offer a comprehensive understanding of the colour characteristics, making the Lovibond colorimeter a valuable tool in assessing quality attributes like uniformity and appearance of cocoa bean shell powder.

Plate 3.2. Colourimeter

3.2.6 Water Activity

Water activity (a_w) refers to the amount of free moisture available in a sample for chemical and biological reactions. A water activity meter operates by measuring the relative humidity in the headspace when the liquid phase water in the sample reaches equilibrium with the vapor phase water in the headspace. The water activity of cocoa nibs was determined using a water activity meter (Model: Aqua Lab, Decagon Devices Inc., Pullman, USA), as illustrated in Plate 3.5. The sample was placed in a cup provided with the meter. To begin the measurement, the drawer knob was turned to the OPEN position, and the sample port was opened by pulling the handle. The sample was then inserted into the port, the chamber was sealed, and the knob was turned to the READ position. The water activity of the sample, adjusted to the atmospheric temperature, was displayed on the screen (Kha *et al*., 2010).

Plate 3.3. Water activity meter

3.2.7. FAT CONTENT

Fat content was determined using a Soxhlet apparatus (Model SCS 06 AS DLS TS SOCS PLUS). The Soxhlet apparatus works on the principle of Randall's Soxhlet chemistry. Two sets of about 1g of cocoa mass was taken in a thimble made from thick filter paper. The thimble then loaded in the extraction tube of Soxhlet apparatus containing extraction solvent (hexane). The fat present in the cocoa mass was extracted through siphoning of hexane through the apparatus and fat will settle at the bottom. This was transferred to a pre-weighed

beaker and kept in a cabinet dryer for the hexane to evaporate. The cream-coloured substances left behind after evaporation of solvent was the fat which is weighed and expressed as percentage.

Fat content of the sample is obtained by following equation,

$$\text{Fat Content (\%)} = \frac{w1-w2}{w} \quad (4)$$

where, w - weight of sample taken, g

w1- initial weight of beakers, g

w2 - final weight of beaker, g

3.2.8. **CARBOHYDRATE**

Total carbohydrate present in cocoa nibs was determined by anthrone reagent. Principle of the analysis is hydrolyzation of carbohydrates into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxyl methyl furfural. This compound forms green coloured product with anthrone reagent with an absorption maximum at 639 nm. The sample (100 mg) was taken in a boiling tube. It was hydrolyzed by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N hydro chloric acid and cool to room temperature. Neutralize with solid sodium carbonate until the effervescence ceases. Made up volume to 100 ml and centrifuged. Supernatant was collected and 0.5 and 1 ml aliquots were taken for analysis. Standard glucose (stock) solution. was prepared by dissolving 100 mg in 100 ml distilled water. The working standard was made by diluting 10 ml stock in 100 ml distilled water. After adding few drops of toluene stored at refrigerated condition. Standards were prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard ("0" serves as blank).

By adding distilled water, volume was made up to 1 ml in all the tubes including the sample tubes. Then 4 ml of anthrone reagent (200 mg antrone in 100 ml ice cold 95 per cent sulphuric acid) was added and is heated for 8 minutes in a boiling water bath. Cooled rapidly and read green to dark green colour at 630 nm using spectrometer. Concentration of the standard versus, absorbance graph was plotted. From the graph, amount of carbohydrate present in the sample was calculated by the equation given below

$$\text{Carbohydrate present in 100 mg of sample} = \frac{mg\ of\ glucoce}{volume\ of\ test\ sample} \times 100 \quad (5)$$

3.2.9. **PROTEIN**

The crude protein content in the cocoa nibs was determined using Kjeldahl method (AOAC, 2005). The experiment was conducted using a protein analyzer (M/s. Pelican Equipments, model: KEL PLUS). The sample of 0.5 g was taken into the digestion tube. The

digestion mixture was prepared by mixing 2.7 g of potassium sulphate (K2SO4) and 0.3 g of copper sulphate (CuSO4). Add 0.5 g to the digestion mixture and add 10 ml of concentrated sulphuric acid (H2SO4) to the sample. The sample was digested in the digestion unit (400°C for 1-2 h) till it became colourless. After completion of digestion, the tubes were cooled and transferred into distillation unit. 40 per cent NaOH solution was allowed in to the tube. Liberated ammonium was absorbed in boric acid (4 per cent) solution containing mixed indicator (10 ml bromocresol green and 7 ml of methyl red). The colour of boric acid (pink) solution was turned to green colour in the distillation unit and the obtained solution was titrated against 0.1 N hydrochloric acid (HCL) until pink colour will obtained.

$$\text{Protein (\%)} = \frac{14 \times (normality\ of\ acid) \times (titrant\ value\ burette\ reading)}{sample\ weight \times 1000} \times 6.25 \quad (6)$$

3.2.10. TOTAL ASH

The total mineral content or crude ash of cocoa nibs was determined by muffle furnace method described in AOAC, 2005 (Method No.930.30). Platinum crucible was heated to 600°C in muffle furnace for 1 h. Cooled in desiccator and weighed. 2 gram of accurately weighed sample was taken in crucible. Crucibles along with weighed samples were loaded into muffle furnace and heated about 5 h at 550°C to greyish white ash. After cooling in desiccators take the weight. Difference in weights taken as the total ash content and is expressed in percentage.

$$\text{Total ash (\%)} = \frac{weight\ of\ ash\ (g)}{weight\ of\ sample(g)} \times 100 \quad (7)$$

3.3 EXPERIMENTAL PROCEDURE

3.3.1 Ultrasound assisted extraction (UAE)

The sample was mixed with the suitable substrate (80% ethanol) at fixed ratio. The ultrasound assisted extraction of CBS was carried out in an ultrasonicator (Athena technology, 33 kHz, 250 W, 250 VAC) at the predetermined ratios of temperature, and time. The extract was filtered using Whatman No. 1 filter paper and the filtrate was evaporated using a rotary vacuum evaporator (70ºC, superfit digital bath). It was followed by open evaporation for the complete removal of ethanol. The of extraction was obtained as follows;

$$Yield = Weight\ of\ the\ flask\ after\ evaporation - Empty\ weight\ of\ the\ flask \quad (8)$$

The extract was scraped off the flask with the aid of ethanol and was made up into 10 mL and stored in a freezer (-10^{o}C) for further analysis.

3.3.1.1 Ultrasound water bath

Ultrasound refers to oscillating sound waves with frequencies exceeding the human hearing threshold (>20 kHz) and is a promising technology in the food industry. High-power ultrasound (US) at lower frequencies (20–100 kHz) can generate cavitation, which disrupts microbial cell membranes, leading to microbial inactivation. US technology preserves the nutritional and sensory qualities of food, ensures greater homogeneity, and achieves significant energy savings. To enhance inactivation efficiency, US is often combined with treatments such as pressure, heat, and antimicrobials.

The ultrasound production system consists of three main components: a generator, a transducer, and an application system. The generator produces electrical or mechanical energy, which the transducer converts into ultrasound waves at suitable frequencies.

Ultrasound treatment was conducted in an ultrasound bath with a chiller (Athena Technology, Mumbai, Model ATSC–10) operating at 33 kHz frequency and 250W output power. The ultrasound bath (depicted in Plate 3.8) is constructed from stainless steel (SS 304) with dimensions of 445 mm × 420 mm × 545 mm and a capacity of 10 liters. It features an advanced MOSFET-based SMPS generator and five piezoelectric sandwich-type (PZT) transducers attached to the tank's base. These transducers convert high-frequency electrical energy into ultrasound waves, creating microscopic vacuum bubbles that grow and collapse rapidly—a phenomenon known as cavitation. Intense cavitation ensures effective microbial inactivation. Digital tuning of the transducers with the generator prevents frequency shifting during operation.The ultrasound bath also includes a compact, built-in cooling system to maintain temperatures between 10°C and 30°C. Copper cooling coils surrounding the tank circulate refrigerant and are connected to a condenser and compressor, which regulate the bath temperature. A PT–100 simplicon sensor is used for precise temperature measurement. Additionally, a digital temperature controller (adjustable between 10°C and 30°C) and a timer (adjustable from 0 to 99 minutes) enable precise control over the sonication process.

Plate 3.4. Ultrasonicator bath

3.3.1.2 Rotary Vacuum Evaporator

The Rotary Vacuum Evaporator (Superfit Digital Bath) is a laboratory device used for efficient and gentle evaporation of solvents under reduced pressure. It operates by rotating a sample flask in a heated water bath, which facilitates the evaporation process by increasing the surface area of the liquid and reducing the boiling point of solvents. The system is equipped with a digital control interface that allows precise adjustment of the bath temperature, rotation speed, and vacuum pressure, ensuring optimal evaporation conditions. The evaporator is typically used for applications such as concentrating solutions, removing solvents, and performing distillations. It features a high-quality glassware setup and a vacuum pump that maintains low pressure for effective solvent removal. The Superfit model is designed for ease of use, offering consistent results and increased process efficiency in laboratory-scale applications.

Plate 3.5. Rotary evaporator

3.3.2 Anti-oxidant activity of CBS extract

The radical scavenging activity (RSA) of CBS extracts on DPPH radical was determined according to Kumar *et al*. (2020) with minor modifications. In DPPH assay, antioxidants donate hydrogen, and DPPH, being a stable free radical, accepts an electron or

hydrogen radical to become a stable molecule (Chan *et al*. 2014). An aliquot of 1 mL of freshly prepared 2 mM methanolic DPPH was added to test tubes with 1 mL of CBS extracts. The reaction mixture was mixed thoroughly and incubated in the dark for 30 min at room temperature. The absorbance was measured at 517 nm with UV–Vis spectrophotometer against blank (methanol). An equal amount of methanol and DPPH served as control. All measurements were performed in duplicates. The radical scavenging activity was calculated as follows;

$$RSA\ (\%) = \frac{C-S}{C} \times 100 \quad (9)$$

Where C is the absorbance of control solution and S is the absorbance of the sample.

3.3.3 Total phenolic content (TPC)

TPC of CBS extracts was determined as described by Kumar *et al*. (2020) with minor modifications. Briefly, 0.5 mL and 0.8 mL aliquot samples made up to 1 mL using distilled water was mixed with 5 mL of Folin-Ciocalteu reagent (10% in distilled water) in separate test tubes. After 5 min, 4 mL of sodium carbonate (7.5% in distilled water) was added to each test tube. The test tubes were cap screwed and vortexed. The samples were incubated at room temperature for 2 h under dark conditions. Absorbance was measured at 750 nm using a UV–Vis spectrophotometer (Shimadzu, Japan). Gallic acid was used as standard with a concentration range of 0–0.1 mg/mL. All experiments were performed in duplicates. Results were expressed as mg gallic acid equivalent per gram of extract (mg GAE/g).

$$Total\ phenolics\ \left(mg\frac{GAE}{g}\right) = \frac{Concentraion\ of\ sample\ calculated\ using\ standard\ curve\ \times volume\ made\ up}{weight\ of\ sample\ taken\ \times volume\ of\ aliquot\ taken\ for\ esstimation} \quad (10)$$

3.3.4 OPTIMISATION OF EXTRACTION PARAMETERS

Optimisation of extraction parameters was done using Response surface methodology (RSM).The process parameters which would influence the yield, antioxidant activity and phenolics were chosen as independent variables. Yield, antioxidant activity and phenolics were taken as dependent variables.

Independent Variables:

1.Temperature (°C): 30, 20, 10

2.Substrate ratio: 50, 30, 10

3.Time (min): 60, 40, 20

Dependent Variables:

1. Yield

2.Antioxidant activity

3.Phenolics

Response surface methodology was made use of for optimising the process variables. Nine dependent variables (responses) were employed for the purpose of optimisation. The formula N = 2 k (k - 1) + Co (where k is the number of factors and Co is the number of central points) gives the number of experiments or runs in the Box-Behnken design, N. There were 13 experiments with three components and five central points in this study. Design-Expert (version 12.0.0) was used as the statistical analysis software.

Table 3.1. Experimental design with the actual values of process variables for the extraction of CBS

RUN	INDEPENDENT VARIABLES		
	Temperature (°C)	Substrate ratio	Time (min)
1	30	30	60
2	30	10	40
3	20	30	40
4	30	30	20
5	20	10	60
6	20	10	20
7	10	30	60
8	30	50	40
9	20	50	20
10	10	10	40
11	20	50	60
12	10	50	40
13	10	30	20

3.3.5 EXTRACTION USING OPTIMIZED CONDITION AND STORAGE STABILITY OF UAE OF CBS BY ANTIOXIDANT ASSAY

The storage stability of cocoa bean shell (CBS) extract obtained via ultrasound-assisted extraction was evaluated by monitoring its antioxidant activity over a three-week period using the DPPH assay. The CBS extract, rich in bioactive compounds like polyphenols, was stored in microtubes under controlled ambient conditions to simulate practical storage scenarios. The DPPH assay, a widely used method for assessing radical scavenging activity, was conducted at the end of the first and second weeks to measure the extract's ability to neutralize free radicals.

During the first week, the antioxidant activity of the extract was measured to establish a baseline. This involved mixing the CBS extract with the DPPH reagent and measuring the reduction in absorbance at 517 nm, which indicates the level of free radical scavenging. The same procedure was repeated at the end of the second week to determine any changes in the antioxidant potential over time.

CHAPTER IV

RESULTS AND DISCUSSION

This chapter outlines the results obtained from various experiments conducted to determine some engineering properties of cocoa bean shell powder. Ultrasound assisted extraction from cocoa bean shell have been evaluated along with the optimization of process parameters for maximum yield, antioxidant, phenol and maximum absorbance value. Also, the ultrasound extraction process is compared with conventional extraction process.

4.1. PHYSIOCHEMICAL PROPERTIES OF COCOA BEAN SHELL POWDER

4.1.1. Physical properties of CBS

The average values of various physical properties of cocoa shell powder are presented in Table 4.1. The average moisture content of cocoa bean shell powder was 10.62 percent (wb). The true density and bulk density were 1333.33 and 480 kg/m^3, respectively. The porosity was 63.99 percent. Water activity of cocoa bean shell powder found as 0.474 Aw. The values found for the moisture of CBS range from 3.60% to 13.13%, which highly depends on whether the CBS are roasted or not (Rojo-Poveda, 2020). The particle density and porosity of category B cocoa beans determined by Bart-Plange and Baryeh, (2003) increased from 946 to 991 kg/m^3 and 20.58 to 31.59% respectively while the bulk density decreased from 560 to 505 kg/m^3.

Table 4.1. Physical properties of CBS

Sl. No.	PHYSIOCHEMICAL PROPERTIES	VALUE
1	Moisture content %	10.62
2	True density, Kg/m^3	1333.33
3	Bulk density, Kg/m^3	480
4	Porosity, %	63.99
5	Water activity, Aw	0.474

4.1.2 Color parameters of CBS

The colour values of cocoa bean shell powder were measured in terms of lovibond L*, a*, b*,c*,h°. The results showed that the L*, a*, b*, C*, and h* values were 32.1, 8.55, 10, 13.2, and 49.45°, respectively. The low L* value indicates that the powder is relatively dark, which is typical for cocoa-based materials. The positive a* and b* values suggest the presence

of both red and yellow tones, contributing to an overall reddish-yellow. This is further supported by the hue angle (h*) of 49.45°, which falls in the orange-yellow region of the color spectrum. The chroma (C*) value of 13.2 reflects a moderate color saturation, indicating that the CBS powder has a noticeable but not overly vivid coloration. These color attributes are important as they can influence consumer perception and may also serve as indicators of compositional or processing-related changes.

In a study conducted by Lembong *et al.,* (2021), cocoa bean shell powder had an L* value between 42.50 and 55.33. The cocoa bean shell's a* value ranged from 8.51 to 11.65, whereas the powdered cocoa bean shell's b* values ranged from 18.97 to 16.04. The powdered cocoa bean shell had a color value between 57.64 and 62.05. The polyphenolic content in cocoa is what gives it its dark hue. According to Lembong *et al*., (2021), the processing parameters like fermentation, soaking and drying affect the final colour values of cocoa bean shell.The studies of Fakhlaei *et al*. (2020) suggest that roasting temperature has a strong influence on the overall color profile of cocoa shell powder. The color transformation is attributed to thermal processes such as Maillard reactions, polyphenol oxidation, and structural breakdown.

Table 4.2. Optical properties of CBS

Sl. No.	OPTICAL PROPERTIES	VALUE
1	L*	32.1
2	a*	8.55
3	b*	10
4	c*	13.2
5	h*	49.45

4.1.3. Chemical properties of CBS

Protein, carbohydrate, total fat, total ash, g/100g were 7.24%, 35.78%, 7.78% ,8%.The values found for the moisture of CBS range from 3.60% to 13.13%, which highly depends on whether the CBS are roasted or not. The fat content accounts for 1.50%–8.49% of dried CBS and is therefore considered a minor component of the by-product when compared to the approximate 50% fat content in cocoa beans. Carbohydrates constitute 7.85%–70.25% of the CBS dry weight) (Rojo-Poveda, 2020). According to Younes *et al*. (2023), cocoa bean shell (CBS) powder contains crude protein in the range of 6.3–10.6% or 7.0–29.0%, crude fat

between 5.46–13.93% or 6.0–17.3%, and ash content ranging from 1.41% to 11.67% (w/w, dry basis). Our results fall within these ranges, confirming the reliability of our data. Additionally, Agus, Mohamad, and Hussain (2018) emphasized that the chemical composition of CBS is highly variable and dependent on several production factors such as genotype, soil characteristics, climate, harvest conditions, and bean quality. Processing conditions including fermentation, roasting, and winnowing also play a significant role. For instance, the carbohydrate content in cocoa beans has been reported to vary from 3.62% to 55.85% w/w, depending on these processing methods, which are critical to flavor development in cocoa products.

Table 4.3. Chemical properties of CBS

PARAMETERS	VALUE
Protein,%	7.24
Carbohydrate,%	35.78
Total fat,%	7.78
Total ash,%	8

4.2. EFFECT OF PROCESS PARAMETERS PERFORMANCE OF ULTRASOUND ASSISTED EXTRACTION OF CBS

The effect of process parameters (Temperature, Substrate ratio,Time) on the performance of ultrasound assisted extraction of CBS were analysed and discussed in the following section.

Table 4.4. Experimental observations of yield,antioxidant activity and phenolics

SL.NO	Temperature (°C)	Substrate ratio	Time (min)	YIELD(mg)	RSA (%)	Phenolics mg(GAE/g)
1	30	30	60	300	71.63	3.345
2	30	10	40	200	76.8	6.120
3	20	30	40	200	76.67	8.706
4	30	30	20	100	65.05	12.484
5	20	10	60	100	68.85	13.380
6	20	10	20	100	77.34	16.448
7	10	30	60	200	73.41	9.794
8	30	50	40	200	71.75	6.844
9	20	50	20	200	72.64	6.672
10	10	10	40	100	82.09	10.552
11	20	50	60	300	73.52	4.977
12	10	50	40	200	76.58	9.724
13	10	30	20	200	66.08	8.932

4.2.1. YIELD

The effects of process parameters on yield of CBS are presented in Table 4.5 and the respective ANOVA tables are presented in Table 4.5. The 3D graphs representing the response surface generated by the model (Equation. 11) are depicted in Fig 7.

The optimization of the yield from cocoa bean shell extraction was evaluated using Response Surface Methodology (RSM). The ANOVA results (Table) confirm that the quadratic model employed is statistically significant, with a high F-value of 14.96 and a p-value of 0.0009. The coefficient of determination (R^2 = 0.9506) demonstrates that 95.06% of the variability in the response can be attributed to the selected model. Furthermore, the adjusted R^2 of 0.8870 signifies good predictive capability, confirming the reliability of the model for optimization purposes. The yield varied from 100 mg to 300 mg.

The effects of ultrasound temperature (A), substrate ratio (B), and extraction time (C), along with their interactions, were studied. Increasing the temperature showed a positive impact on yield up to an optimal point. Beyond this, degradation effects might occur, as seen in the response surface graphs. Substrate ratio exhibits a direct linear effect on yield. An increase in substrate ratio (up to the optimal level) enhanced the extraction efficiency, likely due to better solvent accessibility. Prolonged extraction time improved the yield but showed diminishing returns at higher values, suggesting saturation of extraction under certain conditions.

A second-order quadratic equation was fitted between independent variables and yield using the experimental values. Following regression model is obtained to predict the yield of CBS extract.

$$\text{Yield} = 200 + 12.50A + 25.00B + 37.50C - 25.00AB + 50.00AC + 25.00BC + 25.00A^2 - 50.00B^2 - 25.00C^2 \quad (R^2 = 0.9506) \quad (11)$$

Where,

A= ultrasound temperature (°C)

B= substrate ratio

C= Time (min)

Table 4.5. Analysis of variance (ANOVA) for the response surface quadratic model for yield

Model	48088.24	9	5343.14	14.96	0.0009	significant
A-Ultrasound temperature	1250.00	1	1250.00	3.50	0.1036	
B-Substrate ratio	5000.00	1	5000.00	14.00	0.0072	
C-Time	11250.00	1	11250.00	31.50	0.0008	
AB	2500.00	1	2500.00	7.00	0.0331	
AC	10000.00	1	10000.00	28.00	0.0011	
BC	2500.00	1	2500.00	7.00	0.0331	
A^2	2631.58	1	2631.58	7.37	0.0300	
B^2	10526.32	1	10526.32	29.47	0.0010	
C^2	2631.58	1	2631.58	7.37	0.0300	
Residual	2500.00	7	357.14			
Lack of Fit	2500.00	3	833.33			
Pure Error	0.0000	4	0.0000			
Cor Total	50588.24	16				

The study showed that increasing ultrasound (US) temperature and time had a positive impact on the yield of CBS extract. Specifically, when the temperature was raised from 10°C to 30°C, the yield increased dramatically from 100 mg to 300 mg. This improvement can be attributed to the effects of acoustic cavitation during ultrasound-assisted extraction. Cavitation creates localized forces like intense shear, shock waves, and enhanced mass transfer, which break down the structure of the cocoa shell. This damage allows water to penetrate the shell more easily, helping to release bioactive compounds and boost the extraction yield.

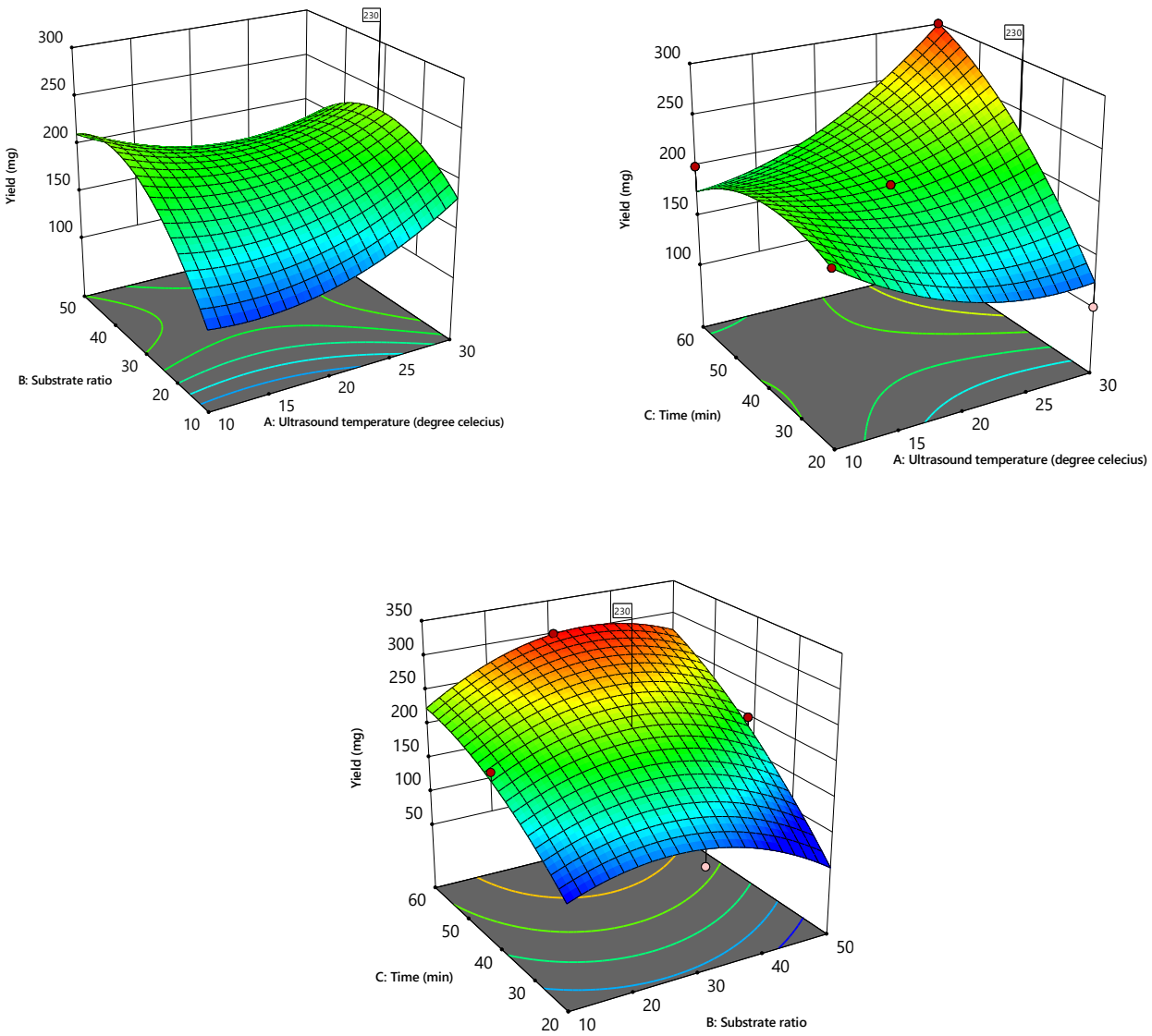

Fig.4.1. Effect of process parameters on yield

Higher temperatures also help by lowering the viscosity of the solvent, allowing it to flow more freely into the cocoa shell and improving the overall efficiency of the extraction process. Additionally, the increased temperature likely softens the shell, making it more vulnerable to the mechanical effects of cavitation. Combined with a longer sonication time, which sustains these effects over a longer duration, this leads to even better compound diffusion and higher yields. The extraction yield was significantly impacted by the intended parameters (time, temperature, and liquid to material ratio) ($P < 0.05$) upon the ultrasound assisted extraction of Myristica fragrans conducted by Poorhashemi *et al*., (2020). The maximum

extraction yield was achieved by increasing the solubility of plant components by high extraction duration and temperature.

Cavitation and thermal effects play a crucial role in enhancing extraction yield. Ultrasound induces cavitation bubbles that collapse with high intensity, disrupting cell structures and aiding in extraction. The associated thermal effect causes tissue swelling and loosening, improving solvent diffusion, matrix penetration, and polysaccharide solubility. Initially, ultrasound promotes material swelling and pore enlargement, facilitating solvent entry and polysaccharide release. An increased liquid-to-solid ratio enhances yield up to a certain point, after which further increases show minimal benefit (Moorthy *et al.,* 2017).

These findings are supported by a study from Nguyen and Nguyen (2018), who found that treating mulberry mash with ultrasound at various temperatures resulted in extraction yields ranging from 83.62% to 92.07%. Their research emphasizes how important sonication temperature is for improving mass transfer and releasing compounds from plant materials. By carefully adjusting parameters like temperature and time, it's possible to maximize yield without compromising the quality of bioactive compound.

4.2.2. EFFECT OF PROCESS PARAMETERS ON TOTAL PHENOLIC CONTENT

The total phenolic content (TPC) at different operating conditions are depicted in Fig. 8 shows the 3D surface plot for the total phenolic content at different processing conditions.

The response surface methodology (RSM) analysis was conducted to optimize the ultrasound-assisted extraction (UAE) conditions for phenolic compounds from cocoa bean shell. The statistical summary of the regression model indicates that the model is significant ($p = 0.0308$), suggesting that the extraction parameters have a substantial impact on the phenolic yield. However, the coefficient of determination ($R^2 = 0.4835$) and the adjusted R^2 (0.3643) indicate moderate model predictability, highlighting possible room for improving the model fit. A lower predicted R^2 (-0.0787) compared to adjusted R^2 emphasizes potential overfitting or unexplained variability.

Table 4.6. Analysis of variance (ANOVA) for the response surface quadratic model for phenols

Model	76.07	3	25.36	4.06	0.0308	significant
A-Ultrasound temperature	13.03	1	13.03	2.08	0.1725	
B-Substrate ratio	41.78	1	41.78	6.69	0.0226	
C-Time	21.26	1	21.26	3.40	0.0881	
Residual	81.25	13	6.25			
Lack of Fit	81.25	9	9.03			
Pure Error	0.0000	4	0.0000			
Cor Total	157.32	16				

All three parameters negatively influence the phenolic yield, with substrate ratio (B) exerting the most significant effect ($p = 0.0226$), followed by extraction time (C) ($p = 0.0881$). Ultrasound temperature (A) showed the least significant impact ($p = 0.1725$), although it contributed to the overall model. Increasing the substrate-to-solvent ratio negatively impacts phenolic extraction, likely due to inefficient diffusion of phenolics at higher substrate concentrations. Prolonged sonication time decreases phenolic yield, possibly due to phenolic degradation at extended exposure. The temperature effect is less pronounced but still indicates a decline in phenolic yield, potentially due to thermal degradation of heat-sensitive compounds.

The response surface plots illustrate the interactive effects of various parameters on phenolic yield. The plots suggest that phenolic yield decreases significantly at higher substrate ratios across all tested temperatures, with minimal impact from temperature variations within the examined range. Phenolic yield also declines with increasing substrate ratio, regardless of sonication time, and longer sonication times exacerbate this decline. Furthermore, phenolic content decreases over time at all temperatures, with the steepest decline observed at higher temperatures, indicating a combined effect of thermal and time-dependent degradation

The experimental values were used to fit a linear equation between independent variables and TPC. The TPC of CBS extract was predicted using the regression model that follows:

TPC =8.99-1.28A-2.29B-1.63C R^2 =0.4835

Where,

A= US temperature, °C

B= Substrate ratio

C= Time ,min

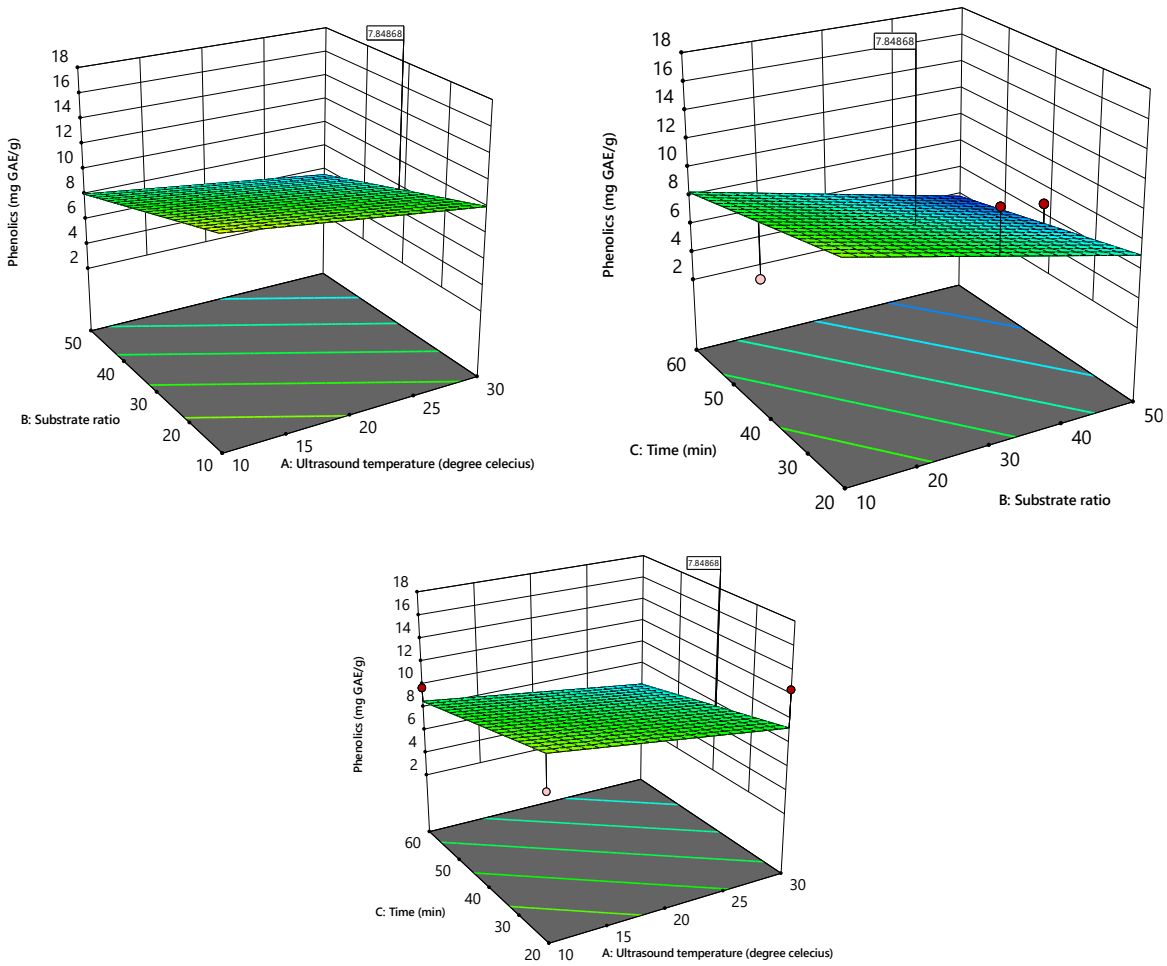

Fig 4.2 Effect of process parameters on total phenol content

Phenolic compounds are secondary metabolites derived from the plant extract. For the significant reduction of numerous physiological and degenerative disorders in the human body, phenolic compounds are highly significant and advantageous to human health (Aadil *et al*., 2013). The total phenolic content of the CBS varied from 3.345 to 16.448 GAE/g, respectively. The effects observed on the total phenolic content (TPC) during ultrasound-assisted extraction (UAE) from cocoa bean shells (CBS) can be understood by considering how the different

extraction parameters interact with each other. When the substrate-to-solvent ratio (B) is increased, the phenolic yield decreases because higher substrate concentrations hinder the efficient diffusion of phenolic compounds into the solvent. As the ratio rises, the solvent becomes saturated, making it harder for phenolics to be extracted effectively. Similarly, longer sonication times (C) result in a lower phenolic yield, as extended exposure to ultrasound can cause degradation of phenolic compounds due to excessive cavitation, heat, and the formation of free radicals. While ultrasound temperature (A) initially improves the extraction process by enhancing the solubility of phenolic compounds, higher temperatures can break down heat-sensitive compounds, leading to a reduction in yield. The combined effects of higher substrate ratios, prolonged sonication times, and elevated temperatures worsen this decline, as seen in the response surface plots, where the greatest reduction in TPC occurs under these conditions.

Excessive heat can lead to the degradation of phenolic substances, which lowers antioxidant activity. Due to chemical and enzymatic degradation brought on by the high temperature used during extraction, polyphenols become less stable and lose their antioxidative properties (Jafari *et al*., 2023). This is further supported by literature findings that ethanol–water mixtures can optimize phenolic extraction, where moderate ethanol concentrations (around 60%) significantly improve phenolic yield, while higher concentrations (80–95%) result in a sharp decline due to co-extraction of non-phenolic compounds like lipids, which inhibit phenolic extraction (Yu et al., 2002; Nepote, Grosso, & Guzmán, 2005). Despite variability in experimental responses (C.V. = 27.81%), the study emphasizes the substrate ratio as the critical factor influencing phenolic yield. Optimization of this parameter, with moderate extraction time and controlled temperature, is essential for maximizing phenolic recovery from cocoa bean shells.

Cavitation effects, which enhance cell wall disruption and phenolic release, are hindered at higher temperatures due to increased vapor pressure, reducing extraction efficiency. These findings corroborate our observation that while mild temperature may aid in extraction, higher temperatures contribute to phenolic degradation (Sengkhamparn, N. and Phonkerd, N, 2019). Higher temperatures generally enhance recovery in solid–liquid extractions, they also accelerate oxidative and enzymatic degradation of phenolics, especially above 30–40°C, resulting in net loss. Therefore, evaluating the full working temperature range is crucial to balance recovery and degradation. Moreover, prolonged extraction time has been shown to plateau phenolic recovery beyond certain durations.In the studies there is no

significant gain in phenolic yield beyond 6 minutes, and anthocyanin content, a subset of total phenolics actually declined at 15 minutes due to degradation. This supports our findings where extended sonication time led to reduced phenolic content likely due to structural breakdown under continued cavitation (Carrera *et al*., 2012).

4.2.3. EFFECT OF PROCESS PARAMETERS ON ANTIOXIDANT ACTIVITY

Antioxidant activity (AA) at different operating conditions is illustrated in Table 4.7 .The same are depicted in 3D graphs representing the response surface generated and are shown in Fig 8.

From the analysis of variance Table 4.7, it was concluded that the three independent process parameters viz., US temperature, substrate ratio and time had no significant effect ($p < 0.05$) on antioxidant activity. The model's R^2 and adjusted R^2 values were 0.7463 and 0.4201, respectively.

The antioxidant activity of CBS extract varied from 65.05% to 82.09%, respectively. The first-order model showed the best fit for the antioxidant activity with various operating conditions. The experimental values were used to fit a linear equation between independent variables and antioxidant activity. The antioxidant activity of CBS extract was predicted using the regression model that follows:

Antioxidant activity= 76.67-1.62A-1.32B+0.7875C $R^2 = 0.7463$ (12)

Where,

A= US temperature, °C

B= Substrate ratio

C= Time ,min

Table 4.7. Analysis of variance (ANOVA) for the response surface quadratic model for antioxidant activity

Model	230.71	9	25.63	2.29	0.1440	not significant
A-Ultrasound temperature	20.90	1	20.90	1.87	0.2143	
B-Substrate ratio	14.02	1	14.02	1.25	0.3002	
C-Time	4.96	1	4.96	0.4428	0.5271	
AB	0.0529	1	0.0529	0.0047	0.9471	
AC	0.1406	1	0.1406	0.0126	0.9139	
BC	21.95	1	21.95	1.96	0.2043	
A^2	16.09	1	16.09	1.44	0.2697	
B^2	18.39	1	18.39	1.64	0.2409	
C^2	135.48	1	135.48	12.09	0.0103	
Residual	78.43	7	11.20			
Lack of Fit	78.43	3	26.14			
Pure Error	0.0000	4	0.0000			
Cor Total	309.13	16				

Awarikabey *et al*.(2014) conducted the DPPH radical scavenging and total antioxidant capacity assays to determine the antioxidant potency of the extracts and the effect of temperature or the processing methods on these activities. The antioxidant activities of extracts are attributed to their phenolic content and other constituents. In biological systems, reactive oxygen species (ROS) formed by free radicals processes are involved in both initiation and promotion of carcinogenesis by causing heritable DNA damage. The ability of an extract to scavenge the DPPH radical suggests that it is capable of preventing cancer, inflammatory and neurodegenerative diseases in humans.

The Table 4.7 shows that the antioxidant activity of all the extracts exceeds a value of 60% which indicates a good value. Hence the model is biologically significant owing to the health benefits rendered upon consumption despite of its statistical insignificance.

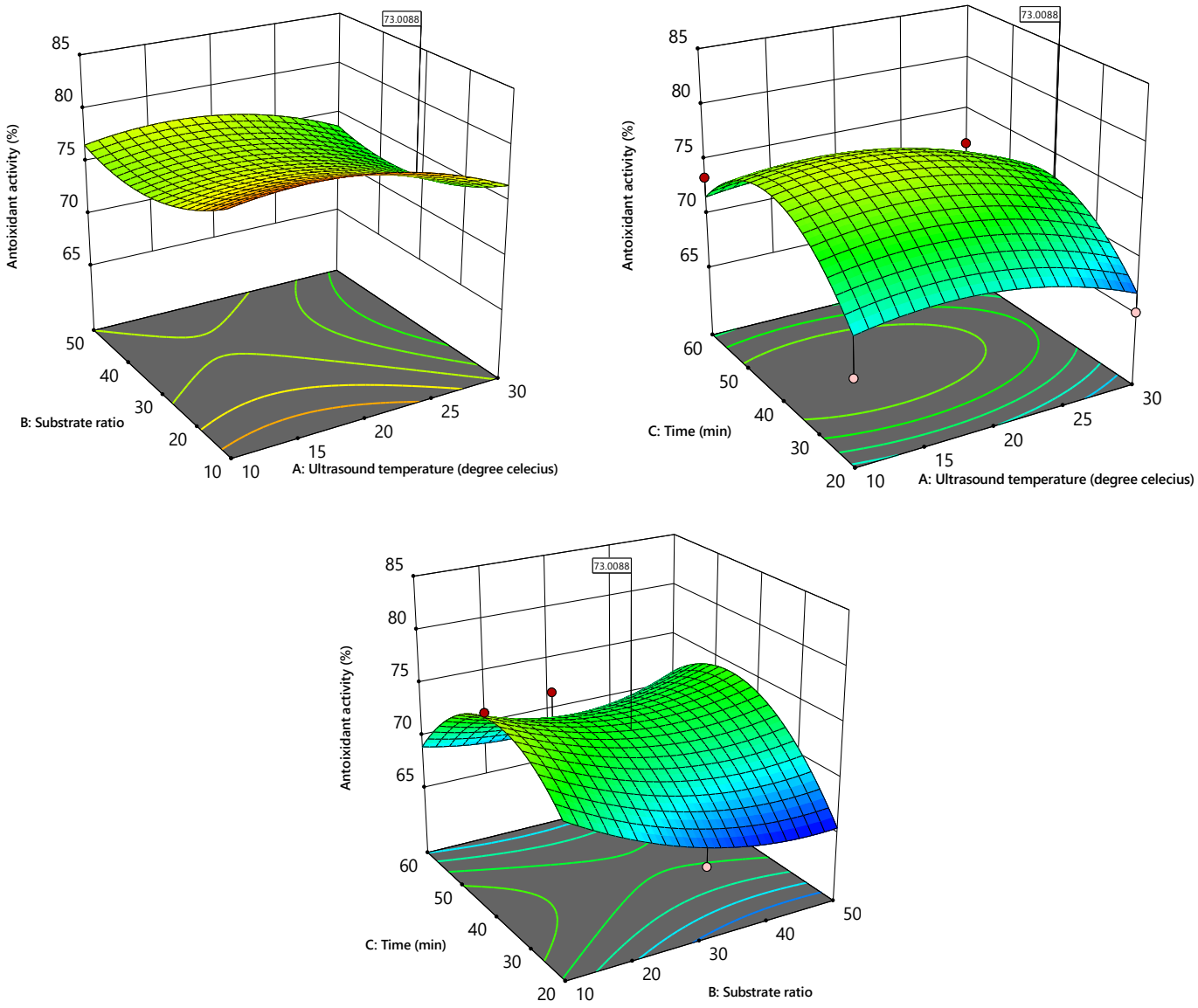

Fig 4.3. Effect of process parameters on antioxidant activity

Antioxidants derived from dietary sources may reduce the progression of atherosclerosis, and observational data collected in humans suggest that the ingestion of antioxidants is associated with preventing cardiovascular disease. (Yoshihara et al., 2010)

Awarikabey *et al.*(2014) conducted the DPPH radical scavenging and total antioxidant capacity assays to determine the antioxidant potency of the extracts and the effect of

temperature or the processing methods on these activities. The antioxidant activities of extracts are attributed to their phenolic content and other constituents. In biological systems, reactive oxygen species (ROS) formed by free radicals processes are involved in both initiation and promotion of carcinogenesis by causing heritable DNA damage. The ability of an extract to scavenge the DPPH radical suggests that it is capable of preventing cancer, inflammatory and neurodegenerative diseases in humans.

The Table 4.7 shows that the antioxidant activity of all the extracts exceeds a value of 60% which indicates that the model is biologically significant owing to the health benefits rendered upon consumption despite of its statistical insignificant.

Solvent is one of the most important factors in an extraction process and the extraction efficiency depends on the solubility of the analytes in the extraction solvent(Li *et al,*2009).Since the yield of antioxidant compounds from plant materials is mainly influenced by the conditions under which the process is carried out, it is necessary to optimise the extraction process in order to have an efficient process(Routray and Orsat .,2012).

4.3. STORAGE STABILITY OF UAE OF CBS BY ANTIOXIDANT ASSAY

The results showed that the antioxidant activity of the CBS extract experienced minimal degradation, indicating good stability of the bioactive compounds under the given storage conditions. This stability is essential for the practical application of CBS extract in food, cosmetics, and nutraceutical industries, as it ensures the extract retains its functional properties during storage. The study underscores the effectiveness of ultrasound-assisted extraction in producing high-quality extracts with sustained antioxidant activity. The optimized extraction conditions, determined using response surface methodology (RSM), were a temperature of 20°C, a substrate-to-solvent ratio of 50, and an extraction time of 20 minutes.The DPPH radical scavenging activity was recorded as 73% in the first week, 72.7% in the second week, and 71.5% in the third week.

CHAPTER V

SUMMARY AND CONCLUSION

Cocoa, derived from *Theobroma cacao*, plays a vital role in global agriculture, supporting millions of livelihoods and significantly contributing to the economy, especially in tropical regions like West Africa, which accounts for 70% of global production. While cocoa beans are the primary focus of the industry, cocoa bean shells (CBS), often treated as waste, possess a rich nutritional and bioactive profile. They are abundant in dietary fiber, polyphenols, methylxanthines (such as theobromine and caffeine), proteins, and essential minerals. These compounds have demonstrated antioxidant, anti-inflammatory, cardioprotective, and neuroprotective properties, making CBS a valuable resource for functional food ingredients, dietary supplements, natural antioxidants, and cosmetic applications. The literature emphasizes the presence of key bioactives like flavonoids (epicatechin, catechin) and phenolic acids (ferulic and cinnamic acid), which justify the need to explore and optimize sustainable extraction methods to harness CBS's full potential across multiple industries.

The research work entitled *"*Optimization and Physicochemical Characterization of Ultrasound-Assisted Extraction of Cocoa Bean Shell Powder*"* was undertaken with the aim of exploring the potential of cocoa bean shell (CBS) as a source of valuable bioactive compounds. The main objectives of the study were: (1) to characterize the physico-chemical properties of cocoa bean shell powder (CBS); (2) to optimize the ultrasound-assisted extraction process of CBS using Response Surface Methodology (RSM); and (3) to evaluate the storage stability of the cocoa bean shell extract based on its antioxidant activity.

The study employed ultrasound-assisted extraction (UAE) to recover valuable bioactive compounds from cocoa bean shell (CBS). The CBS was collected from the Cocoa Research Station (KAU, Thrissur), cleaned, dried, and ground into a fine powder. The physico-chemical properties of the powder were first analyzed using standard methods. These included bulk density, true density, and porosity calculations; moisture content using an infrared analyzer; color measurement using a Lovibond colorimeter (parameters L*, a*, b*, C*, h°); water activity using an Aqua Lab meter; fat content by Soxhlet extraction (hexane solvent); carbohydrates using the anthrone reagent method; protein using the Kjeldahl method with a KEL PLUS analyzer; and total ash content using a muffle furnace at 550°C. These analyses established baseline quality parameters necessary for optimizing extraction.

The ultrasound-assisted extraction was carried out in a bath sonicator (33 kHz, 250 W, Athena Technology), using 80% ethanol as the solvent. Extraction parameters such as

temperature (10–30°C), substrate ratio (10–50 mL/g), and time (20–60 min) were varied according to a Box–Behnken design (13 runs) using Response Surface Methodology (RSM). The extraction yield was calculated by measuring the difference in flask weight before and after solvent evaporation using a rotary vacuum evaporator. The antioxidant activity was assessed using the DPPH radical scavenging assay, and total phenolic content (TPC) was measured using the Folin–Ciocalteu method, expressed as mg gallic acid equivalent (GAE)/g. Optimization was aimed at maximizing yield, antioxidant activity, and phenolic content. Additionally, storage stability of the CBS extract was tested over two weeks, with antioxidant activity monitored using the DPPH assay. Results showed that the antioxidant potential was retained well over time, confirming the stability of the ultrasound-extracted CBS under ambient storage conditions.

The ultrasound-assisted extraction (UAE) of cocoa bean shell (CBS), focusing on the influence of various process parameters such as temperature, substrate-to-solvent ratio, and time on yield, total phenolic content (TPC), and antioxidant activity. The physico-chemical characterization of CBS showed a moisture content of 10.62%, true and bulk densities of 1333.33 kg/m³ and 480 kg/m³ respectively, porosity of 63.99%, and water activity of 0.474. Color parameters indicated a dark reddish-yellow powder with L* = 32.1, a* = 8.55, b* = 10, chroma (C*) = 13.2, and hue angle (h°) = 49.45. Chemically, CBS was found to contain 7.24% protein, 35.78% carbohydrate, 7.78% fat, and 8% total ash, values well within the ranges reported in previous studies, confirming the robustness of the data and the bioactive potential of CBS.

The UAE process was optimized using Response Surface Methodology (RSM) with 13 experimental runs. The yield of extract ranged from 100 mg to 300 mg, with the highest yield obtained at 30°C, 30 mL/g substrate ratio, and 60 minutes. The regression model for yield had a high R^2 value of 0.9506, indicating excellent model fit. The phenolic content of the extract ranged from 3.345 to 16.448 mg GAE/g, with the highest TPC observed at 20°C, 10 mL/g, and 20 minutes. However, the R^2 value for the TPC model was moderate (0.4835), suggesting some variability not captured by the model. TPC decreased with increased substrate ratio, time, and temperature, likely due to phenolic degradation under prolonged exposure and high heat. Antioxidant activity ranged from 65.05% to 82.09%, with the highest activity observed at 10°C, 10 mL/g, and 40 minutes. Though the statistical model for antioxidant activity was not significant ($p > 0.05$), all extracts showed activity above 60%, indicating strong biological relevance and functional potential despite model limitations.

In terms of storage stability, the antioxidant activity of CBS extract obtained under optimized conditions (20°C, substrate ratio 50, time 20 minutes) was monitored over three weeks using the DPPH assay. The scavenging activity was recorded as 73.0% in week 1, 72.7% in week 2, and 71.5% in week 3, showing only a 1.5% reduction over 21 days. This minimal decline indicates strong stability of the bioactive compounds, making CBS extract suitable for practical applications in food preservation, cosmetics, and nutraceuticals. The retention of antioxidant activity over time reinforces the efficiency of UAE in producing stable, high-quality extracts. Overall, this study confirmed that optimized UAE conditions can maximize yield and maintain bioactive integrity, offering a sustainable method for valorizing cocoa processing waste.

Key conclusions:

- Successful valorization of cocoa bean shell as a rich source of bioactive compounds.
- Ultrasound-assisted extraction (UAE) significantly enhances extraction efficiency and sustainability.
- Optimization through RSM ensures maximized yield and quality of extracts.
- Extracted bioactives demonstrate potent antioxidant activity with multiple health benefits.
- Supports sustainable waste management and circular economy by converting waste into wealth.
- Potential applications in food, pharmaceutical, and nutraceutical industries are promising.

Future Scopes:

- Advanced Optimization: Expand the use of Response Surface Methodology (RSM) by incorporating additional extraction parameters and broader variable ranges to fine-tune the ultrasound-assisted extraction process. This will help in achieving even higher extraction efficiencies with better precision.
- Scale-up and Industrial Application: Investigate the feasibility of scaling up the optimized UAE process for commercial production. This includes evaluating cost-effectiveness, energy consumption, and environmental impact at industrial scales to facilitate adoption by food and pharmaceutical industries.

- Application to Other By-products: Extend the UAE and RSM optimization approach to other agricultural and food processing residues to explore their potential as sources of valuable bioactives, thus promoting wider sustainable utilization of agro-industrial wastes.
- Bioavailability and Functional Studies: Conduct in-depth studies on the bioavailability, stability, and efficacy of CBS-derived bioactive compounds in real food systems and biological models. This will help validate their health benefits and functional properties for consumer applications.
- Product Development: Explore the incorporation of CBS extracts into novel functional foods, nutraceuticals, and pharmaceutical formulations. Develop strategies to maintain bioactive stability during processing and storage.
- Environmental and Economic Impact Assessment: Perform lifecycle assessments and economic analyses to quantify the environmental benefits and profitability of CBS valorization via UAE, supporting the adoption of green extraction methods.

By pursuing these future directions, the full potential of cocoa bean shell as a sustainable source of health-enhancing bioactives can be realized, contributing significantly to eco-friendly food innovation such as nutraceutical applications and green chemistry goals.

CHAPTER VI

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OPTIMIZATION AND PHYSICOCHEMICAL CHARACTERIZATION OF ULTRASOUND-ASSISTED EXTRACTION OF COCOA BEAN SHELL POWDER

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ABSTRACT OF PROJECT REPORT

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ABSTRACT

This study aimed to valorize cocoa bean shell (CBS), a nutrient-rich by-product of the chocolate industry, by extracting its bioactive compounds using ultrasound-assisted extraction (UAE) optimized via Response Surface Methodology (RSM). CBS was characterized as a low-moisture material (10.62%) with good storage stability (water activity: 0.474) and high porosity (63.99%). It is rich in fiber, protein, minerals, and bioactives like polyphenols, flavonoids, and methylxanthines, indicating potential as a functional food ingredient. UAE using 80% ethanol was optimized through a Box-Behnken design. The highest yield (300 mg) was obtained at 30°C, 30 mL/g, 60 min; the greatest phenolic content (16.448 mg GAE/g) at 20°C, 10 mL/g, 20 min; and peak antioxidant activity (82.09%) at 10°C, 10 mL/g, 40 min. Antioxidant activity remained relatively stable over three weeks (from 73% to 71.5%). UAE proved superior to conventional methods by reducing solvent use, extraction time, and preserving heat-sensitive compounds. RSM effectively optimized the process with minimal experimentation. CBS shows strong potential as a sustainable source of bioactives for food, nutraceutical, cosmetic, and pharmaceutical applications. Future work should focus on process scale-up, bioactivity studies (e.g., antimicrobial and anti-inflammatory properties), and exploring hybrid green extraction methods.