TOTAL PHENOL AND FLAVONOID CONTENT OF DRIED *Gossypium* (COTTON) SEEDS

BY

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BMS1601925

DEPARTMENT OF MEDICAL BIOCHEMISTRY

SCHOOL OF MEDICAL SCIENCE

COLLEGE OF MEDICAL SCIENCE

UNIVERSITY OF BENIN

IN FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCES DEGREE IN MEDICAL BIOCHEMISTRY

NOVEMBER, 2025

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A PROJECT SUBMITTED TO THE DEPARTMENT OF MEDICAL BIOCHEMISTRY, SCHOOL OF BASIC MEDICAL SCIENCES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE, B.Sc. (HONS) MEDICAL BIOCHEMISTRY, OF THE UNIVERSITY OF BENIN, BENIN CITY

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CERTIFICATION

We the undersigned hereby certify that ONAGHISE ESEOSASERE IZIENGBE (BMS1601925) carried out this research in the Department of Medical Biochemistry, University of Benin, Benin city and thereby approve same as adequate in scope and quality for the award of Bachelor of Science Degree (B.Sc) in Medical Biochemistry.

Signed

....

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External Examiner **(Date)**

DEDICATION

This project is dedicated to Almighty God, the giver of life who has made it possible to complete my Bachelor of Science Degree (B.Sc) program in the Department of Medical Biochemistry and my entire family for their tender care and love for me.

ACKNOWLEDGEMENT

My gratitude goes for Almighty God for his grace in all my endeavors, unto him is all the glory. My sincere appreciation goes to my amiable supervisor Dr. N.B. Aguebor-Ogie who doubles as the Head of Department, alongside other lecturers in the department for their words of wisdom and encouragement.

ABSTRACT

Cotton (*Gossypium spp.*) seeds, a primary by-product of the global cotton industry, are often underutilized but represent a potential source of valuable bioactive compounds. This study was designed to quantify the total phenolic content (TPC) and total flavonoid content (TFC) of dried cotton seeds to evaluate their potential for value-added applications. An aqueous extract was prepared from dried, powdered seeds, and the presence of phenols and flavonoids was confirmed using standard qualitative phytochemical screening. Total phenolic content was determined spectrophotometrically using the Folin-Ciocalteu method with tannic acid as the standard, while total flavonoid content was measured via the aluminum chloride colorimetric assay using quercetin as the standard. The quantitative analysis revealed a substantial total phenolic content of 82.35 ± 0.40 g of tannic acid equivalents per kilogram (g TAE/kg) of the dried sample. The total flavonoid content was also significant, measured at 9.15 ± 1.24 g of quercetin equivalents per kilogram (g QE/kg). These findings demonstrate that dried cotton seed is a rich source of natural phenolic antioxidants, highlighting its potential for valorization as a value-added ingredient in functional food, nutraceutical, and animal feed industries and promoting a more sustainable circular economy in cotton production.

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CHAPTER ONE

1.0 INTRODUCTION

1. 1 Background of the Study

Cotton (*Gossypium* spp.) is one of the world's most important agricultural crops, primarily cultivated for its fiber, which is used in textile production. However, cotton cultivation also yields significant by-products, including cotton seeds, which constitute approximately 15-20% of the total cotton boll weight (Vitale *et al.,* 2024). These seeds are ovoid in shape, measuring 3.5-10 mm in length, and are densely covered with white or rusty, long, woolly hairs known as lint. Cottonseed is the seed of the cotton plant, formed inside the cotton boll. Cottonseed is the source of cottonseed oil and cottonseed meal (Zubair *et al.,* 2021). Cottonseed, like the rest of the cotton plant, contains high amounts of gossypol that can be toxic to humans (Wani and Nazir, 2024).

Nutritionally, cotton seeds are rich in protein (approximately 23% on a dry matter basis), oil (20%), and crude fiber (24%), making them a valuable resource for various applications. The seeds also contain minerals essential for human health and livestock feed, further enhancing their utility (Kebede, 2024; Shahrajabian *et al.,* 2020). Traditionally, cotton seeds are processed through crushing to extract oil for human consumption and meal for animal feed, including use in dairy and beef cattle diets where they serve as a high-energy and protein supplement (Zubair *et al.,* 2021). Additionally, cottonseed oil is noted for its favorable fatty acid composition, including linoleic, palmitic, and other acids, which contribute to its potential in skin health and cosmetics (Gutierrez and Komarnytsky, 2024).

Beyond their macronutrient profile, cotton seeds also contain bioactive phenols and flavonoids—secondary metabolites that provide antioxidant, defensive and stress-response functions in the plant and offer therapeutic benefits in humans (anti-inflammatory, antimicrobial, anticancer) by

scavenging free radicals and limiting oxidative damage; diets rich in these compounds are associated with reduced oxidative stress and improved health outcomes (Abiola *et al.,* 2024; Riaz *et al.,* 2023; Prakash *et al.,* 2023; Oluwole *et al.,* 2022).

Drying which is a routine post-harvest step, strongly influences phenolic and flavonoid retention: high temperatures and long drying times promote thermal degradation and oxidation, while gentle methods (e.g., freeze-drying) better preserve antioxidant activity. Reported total phenolic levels in cottonseed vary widely (e.g., ≈0.145 mg GAE/g in *G. herbaceum*), underscoring processing sensitivity and the potential to valorize dried seeds for nutraceutical applications (Tesema and Fetene, 2024; ElGamal *et al.,* 2023; Belwal *et al.,* 2022; Patel and Mishra, 2024).

1.2 Justification of the Study

This study holds substantial significance for advancing the utilization of cotton seeds, an underutilized agricultural by-product, in sectors such as nutraceuticals, pharmaceuticals, and animal feed enhancement (Egbuta *et al.,* 2017; Zaini *et al.,* 2022). By quantifying phenols and flavonoids in dried forms, the research can inform optimized processing techniques to maximize bioactive retention, thereby contributing to waste reduction and value addition in cotton production. The findings may promote the incorporation of dried cotton seed extracts into functional foods, leveraging their antioxidant properties to combat oxidative stress-related health issues. Scientifically, it fills gaps in data on processing effects, aiding future studies on plant bioactives and sustainable agriculture. Ultimately, this could enhance economic opportunities for cotton farmers through diversified product development.

1.3 Aim of Study

The primary aim of this study was to determine the total phenolic and total flavonoid content in dried *Gossypium* (cotton) seeds

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 OVERVIEW OF COTTON SEEDS

Cotton seeds are the ovular reproductive units of the cotton plant (*Gossypium* spp.) and are the principal by-product of commercial cotton fibre production. They develop inside the locule of the cotton boll and, once separated from the fibre by ginning, are available as whole (fuzzy) or delinted (slick) seed (Yue *et al.,* 2022). Cotton seed morphology, anatomy and chemical composition vary with species, cultivar and growing conditions; these variations influence both industrial uses (oil, meal, hulls, linters) and the seed's suitability as a source of bioactive compounds such as phenolics and flavonoids. Because cotton seed contains both nutritionally valuable components (oil and protein) and anti-nutritional/toxic constituents (notably gossypol produced in gland tissues), careful characterization of seed fractions and processing history is essential when studying phytochemical content (Rojo-Gutiérrez *et al.,* 2020).

Cotton seeds are available where cotton fibre is produced. Cottonseed production (both for oil and feed use) was 42 million tons in 2010. In 2009, 9.2 million tons of cotton seed were used for feed (Kebede, 2024). Cotton seeds are mainly used locally and only small amounts are exchanged (0.74 million exported and 0.73 million ton imported in 2009). The main producers are China, India, USA, Pakistan, Uzbekistan and Brazil, which represented 96% of cottonseed world production in 2010 (Zhang *et al.,* 2025).

2.1.1 Composition

Cottonseed typically contains ~20% oil, ~20% protein and ~3.5% starch, though values vary by variety and environment (Cheng *et al.,* 2020). Industrial fractionation commonly yields roughly 16% oil, 26% hulls, 45.5% meal and 8.5% linters of the whole seed (Cheng *et al.,* 2020). A

notable compositional feature is the polyphenol gossypol localized in pigment glands, which has important anti-nutritional and toxicological implications for food and feed use. (Jan *et al.,* 2022).

Figure 2.1: *Gossypium* Seeds

2.1.2 Scientific Classification of *G. herbaceum*

Table 2.1: Showing Scientific classification of *G. herbaceum*

Kingdom:	Plantae
Clade:	Tracheophytes
Clade:	Angiosperms
Clade:	Eudicots
Clade:	Rosids
Order:	Malvales
Family:	Malvaceae
Genus:	*Gossypium*
Species:	***G. herbaceum***
Binomial name	
Gossypium herbaceum	

2.1.3 Cottonseed Crushing Process

Cottonseed crushing extracts oil and produces by-products; processing conditions like temperature, solvent exposure and mechanical treatment, influence composition and product quality (Kaur *et al.,* 2022).

The crushing process generally involves several key stages:

1. **Cleaning:** Raw cottonseeds are first passed through revolving and vibrating screens to remove impurities such as leaves, stems, and dirt.

2. **Delinting:** Short fibers (linters) attached to the seed coat are mechanically removed using fine-toothed circular saws. The process yields first- and second-cut linters, which are classified based on fiber length and quality.

3. **Hull Removal:** The delinted seeds are dehulled using knives and shaker screens that separate the tough outer hull from the inner kernel. Hulls are often pelleted or blended with cottonseed meal to enhance handling and nutritional value.

4. **Kernel Conditioning and Flaking:** The seed meats (kernels) are conditioned to suitable temperature and moisture, then rolled into thin flakes (0.01–0.015 inches thick) for efficient oil extraction. Expanders are sometimes used to reduce free gossypol levels.

5. **Oil Extraction:** Oil is extracted from the flakes using hexane solvent, yielding crude cottonseed oil. The oil is refined to remove free fatty acids and used for edible products such as cooking oil, margarine, and shortening.

6. **Meal Formation:** The defatted flakes are desolventized, toasted, and ground to form cottonseed meal, which is further dried to 10–12% moisture and either used in powdered form or pelleted for livestock feed.

2.1.4 Storage

Cotton seeds can be stored for up to one year if kept dry and cool. To prevent mycotoxin development, maintain moisture below 10%, monitor relative humidity, and use air-cooling to avoid heating; overheated seeds should be cooled to about 15.6°C to prevent further deterioration (Fatima *et al.,* 2025).

2.1.5 Grinding

Coarse grinding of whole cotton seeds is preferred to fine grinding. Coarse grinding enhances seed digestibility while fine grinding results in fat release that may reduce shelf-life, and in breaking of gossypol glands, which releases free gossypol (Majumdar *et al.,* 2019).

2.1.6 Uses of Cottonseed (shortened)

Cottonseed is a valuable agricultural by-product used mainly for livestock feed, oil production, and industrial applications. Crushed and hulled seed yields high-protein cottonseed meal, produced by mechanical or solvent extraction, which is widely used alone or blended as animal feed and is especially marketed for dairy rations because of its lower starch content compared with corn (Wang *et al.,* 2021; Aslam *et al.,* 2024). Cottonseed hulls are a fibrous, cellulose-rich feed ingredient that mixes easily with other forages (Munir *et al.,* 2020).

Cottonseed oil, after refining to remove gossypol, is used for cooking, salad dressings, shortening and margarine and ranks among major global vegetable oils (Shahrajabian *et al.,* 2020; Liu *et al.,* 2022). Raw cottonseed and some products contain gossypol and must be processed before human consumption (Liu *et al.,* 2022).

The de-oiled meal also serves as an organic fertilizer and soil amendment due to its high protein and nutrient content (Shaji *et al.,* 2021). Refined cottonseed oil and its derivatives are additionally used in cosmetics and personal-care formulations (Riaz *et al.,* 2023).

2.1.7 Nutrient Composition

Whole cottonseed supplies both protein and energy, mainly from its 17.5% oil content, though its use is limited by high fat and gossypol toxicity. Cottonseed meal serves as a protein supplement, typically ≤15% of diet dry matter, and along with whole cottonseed, provides high phosphorus about twice the beef cattle requirement. Cottonseed hulls and gin trash are used as roughage in grain diets, but hulls are limited by low energy, while gin trash suits cattle with low nutrient needs. Cotton by-products are low in calcium and must be balanced with supplemental mineral and trace-salt mixes (Ninkuu *et al.,* 2024).

2.1.8 Gossypol

Gossypol is a polyphenolic pigment found in cotton (Gossypium spp.) and other Malvaceae plants, localized in pigment glands (Zhao *et al.,* 2020). It occurs as two stereoisomers, (+) and (−), with the (−) isomer generally more biologically harmful (Liu *et al.,* 2022). Isomers exist in bound and unbound forms; the unbound form is the most biologically active, while bound gossypol is largely unavailable though some activity may persist (Sharma *et al.,* 2025). Content varies by variety: whole cottonseed commonly contains ~1.5–2.0% gossypol (unbound), though levels can be as low as ~0.4% in some commercial types; Pima varieties tend to have higher total and a larger proportion of the (−) isomer than Upland types (Kong *et al.,* 2025).

Clinical signs of gossypol toxicity are non-specific but include dyspnea, reduced growth or feed intake, weakness, anorexia, gastroenteritis, abdominal distension, pulmonary oedema and, in

severe cases (notably in cattle), decreased milk yield, ruminal stasis, hemoglobinuria and sudden death (Risco and Chase, 2020; Sihag *et al.,* 2020; Kebede, 2024; Cope, 2025).

2.2 Secondary Metabolites

Secondary metabolites are non-primary organic compounds produced by plants, fungi and microbes that mediate ecological interactions (defence, pollinator attraction, stress adaptation) and are valuable for pharmaceuticals and nutraceuticals (Bocso and Butnariu, 2022). Among these, phenolic compounds are one of the largest and most structurally diverse groups (≈8,000 reported structures) produced mainly via the phenylpropanoid pathway; they contribute to pigmentation, UV protection, antioxidant activity and sensory properties in fruits, vegetables, seeds and other tissues (Hussein and El-Anssary, 2019).

Phenolics are broadly divided into flavonoids and non-flavonoids (Ahmad *et al.,* 2021). Non-flavonoid classes include simple phenols, phenolic acids (hydroxybenzoic and hydroxycinnamic acids), coumarins, lignans, stilbenes (e.g., resveratrol) and tannins (hydrolyzable and condensed). Polyphenols occur as monomers (phenolic acids, flavonoids, stilbenes, lignans) or polymeric tannins (gallotannins, ellagitannins, proanthocyanidins).

Flavonoids share a C6–C3–C6 skeleton (two aromatic rings linked by a three-carbon bridge) and are grouped into major subclasses—flavan-3-ols (catechin, epicatechin), flavanones (naringenin), flavones (apigenin), flavonols (quercetin, kaempferol), isoflavones (genistein) and anthocyanidins (cyanidin)—with additional minor types (chalcones, aurones, dihydroflavonols) (Ikikii and Kahindo, 2025; Kiriyama *et al.,* 2024; Guven, 2019; Patil and Murumkar, 2024). Many flavonoids occur as glycosides, which affect solubility, stability and bioavailability, underpinning their nutritional and pharmacological relevance.

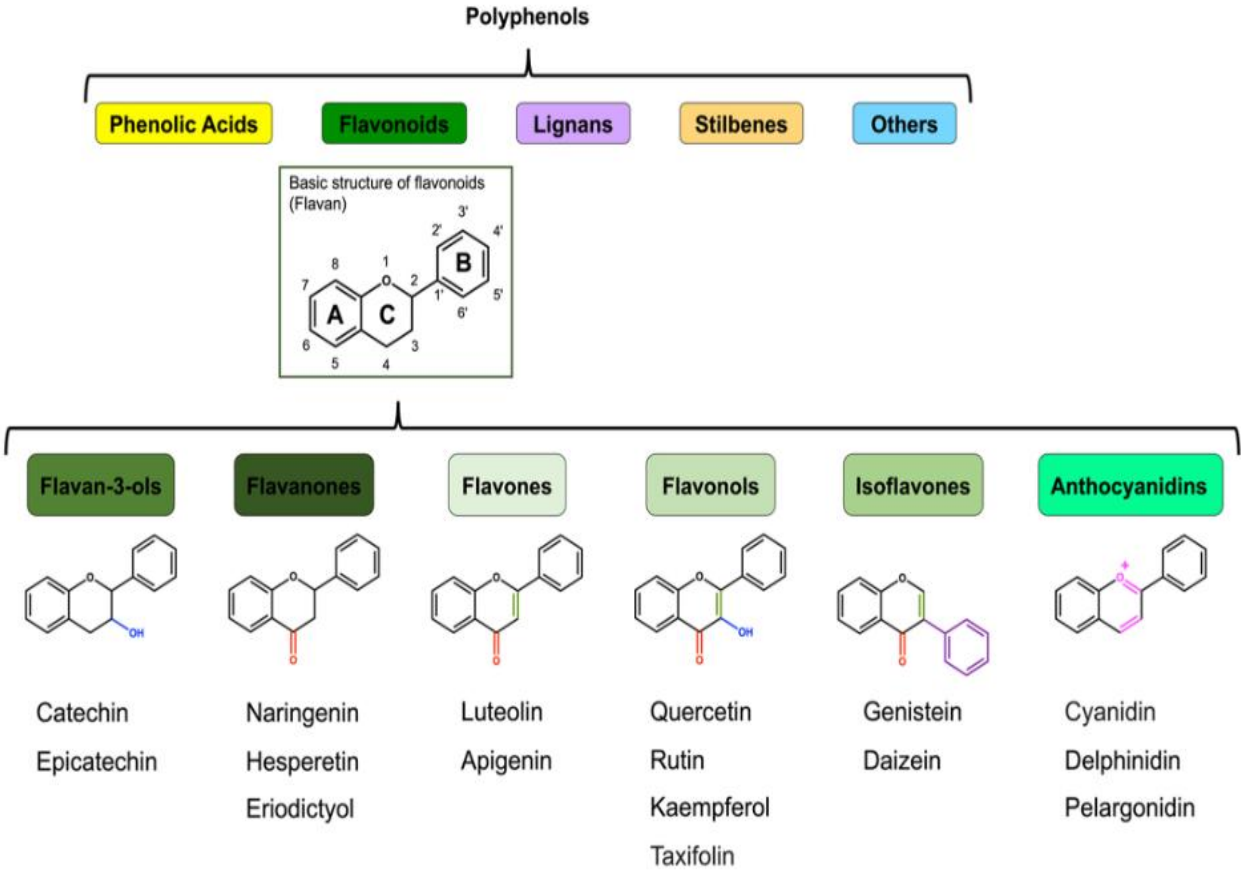

Figure 2.2 Classification of polyphenols and flavonoids. Polyphenols are classified into five classes, viz., phenolic acids, flavonoids, lignans, stilbenes, and other nonflavonoids. Flavonoids are divided into six major subclasses, including flavan-3-ols (3-OH-flavanones), flavanones, flavones, flavonols, isoflavones, and anthocyanins. The basic structure of flavonoids is a diphenylpropane skeleton(C6–C3–C6, flavan).

2.2.1 Biosynthesis Pathways in Plants

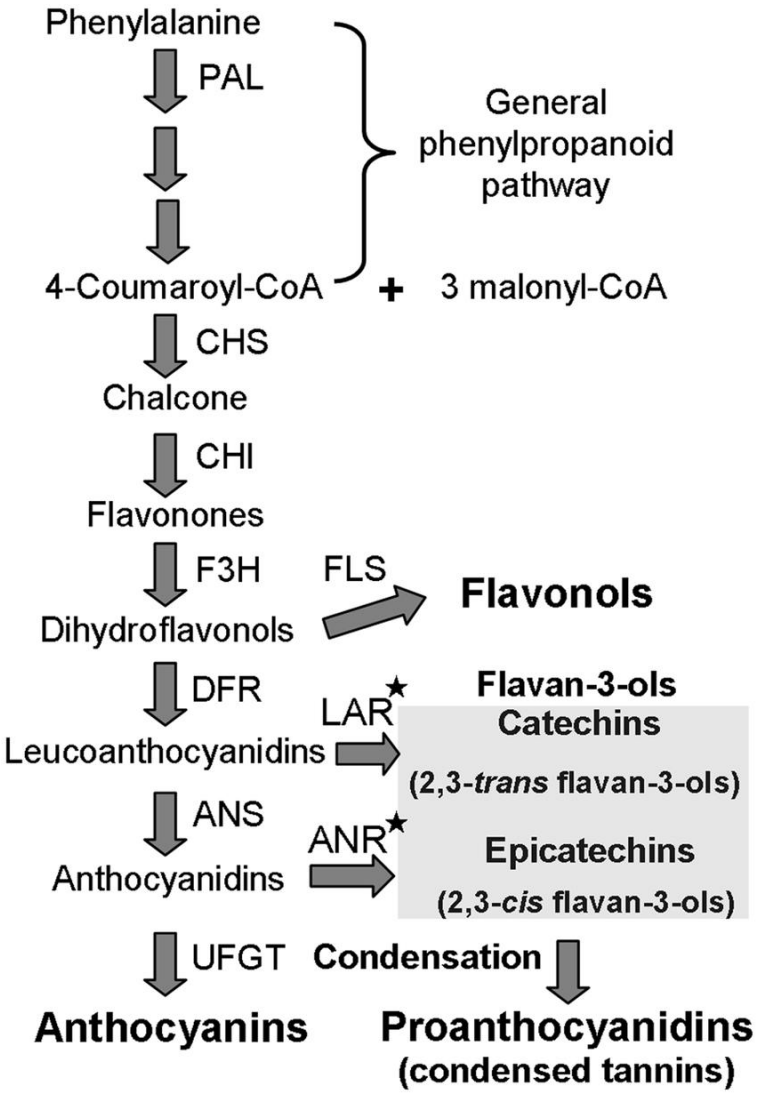

Figure 2.3. Schematic diagram of the flavonoid biosynthesis pathway, including main branches of anthocyanins, PAs, and flavonols. PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavonone 3-hydroxylase; DFR, dihydroflavonol reductase; FLS, flavonol synthase; ANS, anthocyanin synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; UFGT, UDP-glucose: flavonoid 3-O-glucosyltransferase. Asterisks indicate key structural enzymes in PA biosynthesis.

Phenols are synthesized via the phenylpropanoid pathway, starting from phenylalanine, which is converted to cinnamic acid by phenylalanine ammonia-lyase (PAL), followed by hydroxylation to form phenolic acids. Flavonoid biosynthesis branches from this pathway at chalcone synthase (CHS), which condenses p-coumaroyl-CoA with malonyl-CoA to form chalcones, then isomerized to flavanones by chalcone isomerase (CHI), with further modifications by enzymes like flavonoid 3'-hydroxylase (F3'H) and flavonol synthase (FLS). Regulation involves transcription factors like MYB and bHLH, responsive to light, temperature, and stress signals (Aluko *et al.,* 2025).

2.2. Roles in Plant Physiology and Defense

Phenols and flavonoids are multifunctional secondary metabolites that protect plants by scavenging reactive oxygen species and providing photoprotection (Ahlawat *et al.,* 2025). They deter herbivores and inhibit pathogens (acting as antimicrobial phytoalexins), with concentrations often rising after biotic attack. Flavonoids also act as signalling molecules in plant–microbe interactions, contribute to pigmentation (anthocyanins) for pollination and photoprotection, and modulate growth processes such as auxin transport and root development. Additionally, they enhance tolerance to abiotic stresses (drought, salinity, heavy metals) via metal chelation and membrane stabilization, and may exert allelopathic effects on neighbouring plants (Nwozo *et al.,* 2023).

2.3 Phenolic and Flavonoid Constituents of Cottonseed

Early investigations of flavonoids in *Gossypium hirsutum* focussed on the flavonoids from flowers. These studies aimed to identify those compounds responsible for floral colouration, determine phylogenetic relationships within *Gossypium*, and investigate the compounds

responsible for suppressing larval growth of the tobacco budworm *Heliothis virescens* (Khalid *et al.,* 2021).

Flavonoids and other phenolics function as important chemical defenses in cotton, inhibiting the growth and feeding of herbivorous insects and contributing to fungal resistance (Dixit *et al.,* 2020). Specific flavonoids (e.g., cyanidin-3-glucoside, isoquercitrin, quercetin glycosides) and condensed tannins have been shown to reduce larval growth or act as feeding deterrents against pests such as *Helicoverpa* and *Heliothis*; certain gossypetin glycosides are especially toxic compared with common quercetin derivatives (Tyagi *et al.,* 2022). Herbivory induces increases in total phenols (including catechin and phenolic acids), which can provoke detoxifying enzyme responses in insect guts. Higher levels of catechin and gallocatechin are also associated with increased resistance to fungal pathogens (e.g., *Rhizoctonia* and *Verticillium*), indicating a dual role for these compounds in defence against both insects and fungi (Kumar *et al.,* 2024).

2.3.1 Role of Flavonoids in Cotton Production

Flavonoids influence cotton fibre colour and quality: brown cotton shows significantly higher flavonoid accumulation and biosynthetic gene expression than white fibre, linking the flavonoid pathway to pigmentation. Flavonoid metabolism also affects fibre development during elongation, impacting fibre length and micronaire (Shi *et al.,* 2024). The flavanone naringenin is negatively associated with fibre growth as higher naringenin correlates with shorter fibres. Hence, targeting the genes controlling naringenin metabolism offers a potential route to improve fibre development (Adetunji *et al.,* 2023).

2.4 Flavonoids of *Gossypium hirsutum* and Their Distribution

Flavonoids are found throughout a variety of plant organs in *Gossypium hirsutum*, from the roots through to the bolls, with the flowers and leaves being richest in diversity (Table 2.3).

Table 2.3 showing flavonoids diversity in *Gossypium hirsutum*

	Plant Organ							
Flavonoid Class	**Leaves**	**Roots**	**Stem**	**Seed**	**Boll**	**Flower**	**Fibre**	**Total**
Isoflavones			1					**1**
Flavonols	11	2	1	10	2	29	2	**36**
Flavanones	2	2	1			2	2	**2**
Anthocyanidins	3		2		2	3		**5**
Flavanols	1	4	4	1	4	4		**4**
Flavanonol	2	2				2	2	**2**
Leucoanthocyanidins		2	2		2	2		**2**
Total	**19**	**12**	**11**	**11**	**10**	**42**	**6**	**52**

2.5 Medicinal Properties of Phenolics and Flavonoids

2.5.1 Antioxidant Properties

Oxidative stress drives many chronic conditions including cancer, neurodegeneration, cardiovascular disorders and ageing (Reddy, 2023; Oyovwi *et al.,* 2025). Antioxidants delay or inhibit oxidation even at low concentrations (Ali *et al.,* 2020). Most commercial antioxidants are synthetic and may pose safety concerns, increasing interest in plant-based alternatives (Gulcin, 2020). Phenolics and flavonoids demonstrate stronger antioxidant activity than vitamins C, E, and carotenoids (Mutha *et al.,* 2021), acting by scavenging ROS/RNS, chelating pro-oxidant

metals, inhibiting radical-generating enzymes, and upregulating endogenous antioxidant defenses (Shamsudin *et al.,* 2022).

2.5.2 Anticancer Properties

Cancer progression is influenced by genetic, metabolic and environmental factors. Diets rich in antioxidant-containing plants reduce cancer risk, and polyphenols exhibit anticancer effects across multiple cell lines with minimal toxicity (Agrawal and Nirmal, 2025). Their activity includes inhibition of tumor initiation, proliferation and metastasis.

2.5.3 Anti-inflammatory Properties

Phenolics and flavonoids suppress inflammation by inhibiting pro-inflammatory cytokines (TNF-α, IL-6), blocking COX and LOX enzymes, and downregulating NF-κB signaling (Al-Khayri *et al.,* 2022). Compounds such as quercetin and resveratrol reduce mast cell activation, histamine release and oxidative inflammation in chronic inflammatory conditions (Chaudhary *et al.,* 2025).

2.5.4 Cardioprotective Properties

These bioactives improve endothelial function, enhance nitric oxide availability, reduce blood pressure, and prevent LDL oxidation and platelet aggregation (Sharifi-Rad *et al.,* 2020). Flavonoids such as quercetin and anthocyanins support healthy lipid profiles by increasing HDL and reducing triglycerides, contributing to reduced risks of cardiovascular events (Parmenter *et al.,* 2020).

2.5.5 Antidiabetic Properties

Phenolics and flavonoids modulate glucose metabolism by inhibiting α-amylase/α-glucosidase, enhancing insulin sensitivity, activating AMPK pathways, and promoting GLUT4 translocation (Sok-Yen *et al.,* 2021). They also protect pancreatic β-cells and reduce diabetic complications through antioxidant and anti-inflammatory mechanisms, with evidence linking flavonoid-rich diets to improved glycemic control (Adu *et al.,* 2022).

2.6 Effects of Processing on Phenolic and Flavonoid Content in Plant Materials

The measured levels of phenols and flavonoids depend strongly on post-harvest handling and processing; steps such as drying, milling, defatting and extraction can markedly alter yield, composition and stability of these bioactives in complex oilseed matrices like cotton seed (Afzal *et al.,* 2020).

2.6.1 Drying Methods and Their Impact

Drying reduces water activity and slows microbial and enzymatic spoilage but can degrade thermolabile phenolics and flavonoids via heat-induced oxidation and decomposition (Alp and Bulantekin, 2021; Obasa *et al.,* 2023). Freeze-drying (lyophilisation) best preserves phytochemicals by avoiding high temperatures and oxidative loss, yet its high cost and long processing time limit industrial use (Santos *et al.,* 2025; Nwankwo *et al.,* 2023). In oilseeds, drying often lowers total phenolics and antioxidant activity; compounds such as gossypol in cotton seed are heat-sensitive (Liu *et al.,* 2022).

2.6.2 Extraction Techniques for Bioactives

Extraction efficiency depends on method, solvent, temperature, time and matrix properties. Conventional solvent extractions (maceration, Soxhlet) using hydroalcoholic solvents (methanol/ethanol) are common because their polarity captures a broad range of phenolics and

flavonoids, but the oil-rich matrix of cotton seed may require a hexane defatting step to improve access to polar compounds (Ghaffar and Perveen, 2025). Advanced techniques—ultrasound-, microwave-assisted and supercritical fluid extraction—reduce time and solvent use while often increasing yield and preserving bioactivity (Geow *et al.,* 2021). Choose the method that best matches the study objective: rapid screening versus compound-level recovery and structural characterisation.

2.7 Analytical Methods for Quantification of Phenols and Flavonoids

2.7.1 Spectrophotometric and Colorimetric Assays

Rapid, low-cost spectrophotometric assays are widely used for screening total phenolics and flavonoids. The Folin–Ciocalteu assay (results as mg GAE/g) measures reducing capacity but lacks specificity because it reacts with other reductants (Nikolaeva *et al.,* 2022; Cacique *et al.,* 2021). The aluminium chloride method estimates total flavonoids (e.g., mg QE/g) by complex formation with flavones/flavonols but under-responds for other flavonoid classes (Sultana *et al.,* 2024; Shraim *et al.,* 2021). These methods are suitable for high-throughput screening but can over- or under-estimate specific compounds.

2.7.2 Advanced Chromatographic and Spectroscopic Techniques

Chromatography and hyphenated techniques are required for separation, identification and accurate quantification of individual phenolics/flavonoids. HPLC-UV/DAD provides reliable profiling by retention time and spectra, while LC-MS or LC-MS/MS gives definitive molecular identification and structural information (Oladimeji and Valan, 2020; Chaudhary *et al.,* 2025). GC-MS can analyse volatile or derivatised phenolics but requires extra sample preparation.

NMR offers conclusive structural elucidation for purified compounds though it is not routine for quantification (Marcarino *et al.,* 2020).

Choice of method should match the objective: colorimetric assays for total-content screening, chromatographic/hyphenated methods for compound-level identification and quantification.

2.7.3 Antioxidant Activity Evaluation

Since the biological function of phenolic and flavonoid compounds is often attributed to their antioxidant properties, their quantification is frequently accompanied by antioxidant activity assays. These assays evaluate the ability of an extract to scavenge free radicals or reduce oxidising agents. Common assays like DPPH, ABTS, FRAP, and ORAC are used to measure this activity, which is often correlated with the phenolic content.

These assays operate via two main mechanisms: hydrogen atom transfer (HAT) and single electron transfer (SET).

- **DPPH (2,2-diphenyl-1-picrylhydrazyl) Assay:** A SET-based method where the antioxidant reduces the stable DPPH radical, causing a colour change from purple to yellow, which is measured spectrophotometrically (Yeo and Shahidi, 2019).
- **ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) Assay:** Also a SET-based method, it measures the reduction of the pre-generated ABTS radical cation. It is versatile as it can be used in both aqueous and organic media.
- **FRAP (Ferric Reducing Antioxidant Power) Assay:** This assay measures the ability of an extract to reduce a ferric complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}) at low pH (Gulcin and Alwasel, 2025).

- **ORAC (Oxygen Radical Absorbance Capacity) Assay:** This is a HAT-based method that measures the ability of an antioxidant to protect a fluorescent probe from damage by peroxyl radicals. It is considered to be more biologically relevant than SET-based assays (Munteanu and Apetrei, 2021).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Apparatus and Equipments

The apparatus or equipment used for this study were gotten from the Chemistry laboratory at the University of Benin,and were confirmed to be in good working condition before use. They include: Beakers, spectrophotometer (Jenway 6100,Dunmow,Essex,U.K), water bath(37°C), oven, analytical balance, filter paper (whatman No.1), mortar and pestle.

3.1.2 Chemicals and Reagents

All the chemicals and reagents used in this study were of analytical grade. They include:

Foli-ciocalteu reagent, Sodium carbonate, Vanillian reagent, Ethanol, Sulphuric acid, Aluminum chloride, Sodium acetate, Methanol, Quercetin, Folin-Denis reagent, Tannic acid solution, Acetic acid, Ammonium hydroxide (conc), Ammonium solution

3.2 Methods

3.2.1 Sample Collection and Preparation

A 1.0 g sample of dried, powdered cotton seed was weighed and dissolved in 50 mL of cool, boiled-out distilled water in a 100 mL beaker. The mixture was then quantitatively transferred to a 100 mL volumetric flask. The beaker was rinsed three times with approximately 10 mL of boiled-out distilled water, with the rinsings added to the volumetric flask to ensure complete transfer. The solution was then made up to the 100 mL mark with the same distilled water. The flask was stoppered and inverted four times to ensure thorough mixing. The resulting aqueous extract, with a final concentration of 10,000 µg/mL (10 mg/mL), was set aside for analysis.

3.3 Phytochemical Screening

Preliminary qualitative phytochemical screening was carried out on the cotton seed extract to confirm the presence of phenols and flavonoids using standard methods as described by Tiwari et al. (2001).

3.3.1 Test for Phenols

A 1.0 mL aliquot of the plant extract was treated with 4 drops of ferric chloride solution. The formation of a bluish-black color was taken as a positive indication for the presence of phenols.

3.3.2 Test for Flavonoids

The presence of flavonoids was determined using two separate tests:

1. **Alkaline Reagent Test:** A few drops of 2 mol/dm³ sodium hydroxide solution were added to the plant extract. The formation of an intense yellow color which became colorless upon the addition of dilute hydrochloric acid (2 mol/dm³) indicated the presence of flavonoids.
2. **Lead Acetate Test:** A few drops of lead acetate solution were added to the plant extract. The formation of a yellow precipitate was considered a positive test for flavonoids.

3.3.3 Quantitative Analysis

3.3.3.1 Determination of Total Phenolic Content (TPC)

The total amount of phenolic compounds in the extract was determined using the Folin-Ciocalteu reagent method as described by Singleton and Rossi (1965), with slight modification.

Briefly, 1.0 mL of the extract solution (prepared at a concentration of 250 µg/mL) was added to a test tube. To this, 1.0 mL of Folin-Ciocalteu reagent was added, and the contents were mixed

thoroughly. After 5 minutes, 15.0 mL of 20% sodium carbonate (Na_2CO_3) solution was added. The mixture was allowed to stand in the dark for 2 hours for color development. The absorbance was subsequently measured at 760 nm using a UV-Vis spectrophotometer. The total phenolic content was calculated from a standard calibration curve prepared using tannic acid and expressed as micrograms of tannic acid equivalents per milliliter (µg TAE/mL).

3.3.3.2 Determination of Total Flavonoid Content (TFC)

The total flavonoid content was determined using the aluminum chloride colorimetric method as described by Ilahy *et al.* (2011).

An aliquot of 30 µL of the sample extract was diluted with 90 µL of methanol. To this mixture, 6 µL of 10% Aluminum chloride ($AlCl_3$) and 6 µL of 1 mol/L Sodium acetate (CH_3CO_2Na) were added. Finally, the total volume was made up with an additional 170 µL of methanol. The mixture was incubated at room temperature for 30 minutes. The absorbance was read at 415 nm. Quercetin was used as a standard to create a calibration curve, and the total flavonoid content was calculated and expressed as milligrams of Quercetin equivalents per kilogram of the sample (mg QE/kg).

CHAPTER FOUR

RESULTS

The phytochemical analysis of the aqueous extract of dried cotton seeds. The results are presented in two parts: the preliminary qualitative screening to confirm the presence of key phytochemicals, followed by the quantitative determination of the total phenolic and flavonoid content.

4.1 Preliminary Phytochemical Screening

The aqueous extract of dried cotton seeds was subjected to qualitative chemical tests to identify the presence of various phytochemical classes. The results of these tests, based on visual observations such as color changes and precipitate formation, are summarized in Table 4.1.

Table 4.1: Qualitative Phytochemical Composition of Dried Cotton Seed Extract (*Gossypium*)

Phytochemicals	**Indication**
Phenols	Present (+)
Flavonoid	Present (+)

Key + = Present. – = not present

Table 4.1 displays the results of the qualitative tests, indicating the presence (+) of phenols and flavonoids based on specific color changes and precipitate formation after the addition of the respective reagents.

The screening confirmed the presence of both phenols and flavonoids in the extract, justifying the subsequent quantitative analysis.

4.2 Quantitative Phytochemical Analysis

Following the positive preliminary screening, the total phenolic content (TPC) and total flavonoid content (TFC) in the dried cotton seed extract were quantified. The experiments were performed in triplicate to ensure the reliability of the results. The findings are presented in Table 4.2.

TABLE 4.2 Quantitative Phytochemical Result

Phytochemicals	**Mean ± S.E.M**
Total phenolic content (g TAE/kg)	82.35 ± 0.40
Flavonoid content (g QE/kg)	9.15 ± 1.24

Table 4.2 presents the triplicate and mean (± Standard Error of the Mean) values for the total phenolic content (expressed as grams of Tannic Acid Equivalents per kilogram) and total flavonoid content (expressed as grams of Quercetin Equivalents per kilogram) of the sample.

The mean total phenolic content was determined to be 82.35 ± 0.40 g TAE/kg, while the mean total flavonoid content was 9.15 ± 1.24 g QE/kg. These values represent the concentration of phenols and flavonoids in the original dried cotton seed sample.

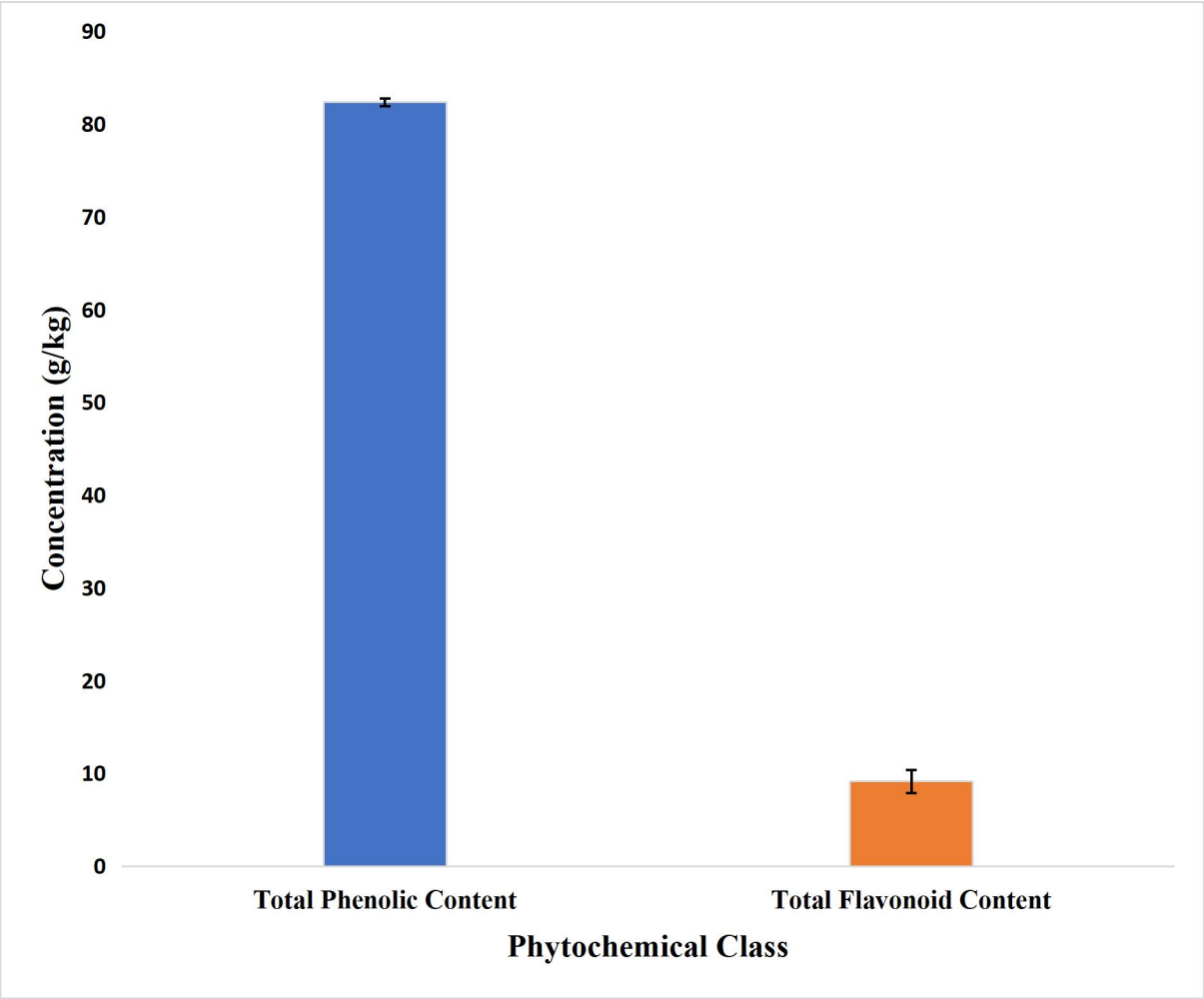

Figure 4.1: Total Phenolic and Flavonoid Content of Dried Cotton Seed Extract

Figure 4.1 showing the comparison of the total phenolic and flavonoid content in the aqueous extract of dried cotton seeds. Bars represent the mean concentration (g/kg) and the error bars indicate the Standard Error of the Mean (S.E.M.). All determinations were performed in triplicate (n=3).

CHAPTER FIVE

DISCUSSION

The preliminary qualitative screening (Table 4.1) confirmed the presence of phenols and flavonoids in the aqueous extract of dried cotton seeds. The positive result from the ferric chloride test (bluish-black color) is a classic indicator of phenolic compounds, while the intense yellow color from the alkaline reagent test and the yellow precipitate from the lead acetate test are strong confirmations of flavonoids. This initial screening was a crucial validation step, justifying the progression to more detailed quantitative analysis. These findings align with known literature, which establishes that cotton seeds are a known reservoir of bioactive secondary metabolites, including various phenolic classes (Hussein and El-Anssary, 2019; Zubair *et al.,* 2021).

The quantitative analysis revealed a mean total phenolic content of 82.35 ± 0.40 g TAE/kg (or 82.35 mg TAE/g) in the dried cotton seed extract. This value is remarkably high and suggests that dried cotton seeds are an exceptionally rich source of phenolic compounds.

When compared to existing literature, this finding stands out. For instance, a study by Patel and Mishra (2024) reporting TPC values as low as 0.145 mg GAE/g in *Gossypium herbaceum*. The value obtained in this study with a mean total phenolic content of 82.35 ± 0.40 g TAE/kg (or 82.35 mg TAE/g) in the dried cotton seed extract, is several orders of magnitude higher.

This significant discrepancy can be attributed to a combination of factors. Firstly, the specific species and cultivar used in this study may inherently possess a higher concentration of phenolics, as the literature encompasses a range of varieties including *G. hirsutum* and *G. herbaceum*. Furthermore, the focus on dried seeds is a critical consideration; the drying process can concentrate bioactive compounds by removing water, thereby increasing their content per unit of

dry weight (Belwal *et al.,* 2022). Another critical factor is the extraction solvent. This study utilized distilled water, and solvent polarity is a well-known determinant of extraction efficiency (Ghaffar and Perveen, 2025). Water is effective at extracting a broad range of polar phenolic compounds, which may be particularly abundant in cotton seeds. Finally, it is important to consider the specificity of the Folin-Ciocalteu assay. While it is a standard method, this assay reacts with any reducing substance, not exclusively phenols. The complex matrix of cotton seeds, which is rich in protein and other components (Kebede, 2024), may contain interfering compounds such as ascorbic acid, certain amino acids, or reducing sugars that could contribute to an overestimation of the true phenolic content (Nikolaeva *et al.,* 2022).

Despite the potential for overestimation, the result strongly indicates that dried cotton seeds are a potent source of phenolic compounds, far exceeding some previously reported values and underscoring their potential as a source of natural antioxidants.

As flavonoids are a subclass of polyphenols, it is expected that the TFC value would be lower than the TPC value. The results are consistent with this biochemical principle, with flavonoids constituting approximately 11.1% of the total phenolic content measured in this study (9.15 g/kg out of 82.35 g/kg).

This ratio suggests that while flavonoids are present in significant amounts, the majority of the phenolic compounds in the extract are likely non-flavonoid phenolics. This could include phenolic acids, tannins, and other polyphenolic structures like gossypol, which is described as a major polyphenolic compound localized in the pigment glands of cottonseed (Zhao *et al.,* 2020). The aluminum chloride assay used for TFC is most effective for specific flavonoid subclasses like flavones and flavonols (Shraim *et al.,* 2021), meaning the concentration of other subclasses

may not be fully represented, though the result still provides a valuable estimate of flavonoid presence.

The high concentrations of both phenols and flavonoids in dried cotton seeds have significant implications, reinforcing the justification for this study. Cotton seed is often treated as an underutilized by-product of the cotton fiber industry.

The rich phenolic profile suggests a strong potential for applications in nutraceuticals and functional foods, leveraging the well-documented antioxidant, anti-inflammatory, and cardioprotective properties of these compounds (Oluwole *et al.,* 2022; Al-Khayri *et al.,* 2022). The results provide a scientific basis for promoting dried cotton seed extracts as natural additives to enhance the nutritional and health value of food products or for use in animal feed to improve livestock health.

5.2 CONCLUSION

This study was conducted to quantify the total phenolic and flavonoid content in an aqueous extract of dried cotton seeds. The investigation successfully confirmed the presence of both phytochemical classes through preliminary screening. The subsequent quantitative analysis determined that the dried cotton seeds contain a very high total phenolic content and a significant total flavonoid content.

In conclusion, this research demonstrates that dried cotton seed, a major by-product of the cotton industry, is an exceptionally rich and largely untapped source of natural phenolic compounds, including flavonoids. The findings strongly support the potential for utilizing this raw material in the development of functional foods, nutraceuticals, and other value-added products, thereby contributing to a more sustainable and economically efficient agricultural system.

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