PHYTOCHEMICAL AND PROXIMATE COMPOSITION OF *Cymbopogon citratus* WITH REFERENCE TO SOLVENT-BASED VARIATION IN PHENOLIC CONSTITUENTS

BY

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DEPARTMENT OF MEDICAL LABORATORY SCIENCE,
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BEING A PROJECT SUBMITTED TO THE DEPARTMENT OF MEDICAL LABORATORY SCIENCE IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELORS DEGREE IN MEDICAL LABORATORY SCIENCE (BMLS) UNIVERSITY OF BENIN, BENIN CITY, NIGERIA.

SUPERVISED BY

DR. F. O. AMEGOR

SEPTEMBER, 2025

CERTIFICATION

This is to certify that this seminar work was carried out by **OGIESOBA, OSAMAKWE BENEDICT** with the matriculation number **BMS2005045** under the supervision of **DR. F.O. AMEGOR** in partial fulfillment for the award of Bachelor of Medical Laboratory Science (B.MLS) Degree.

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DR. F. O. AMEGOR **DATE**

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PROF. MATTHEW FOLARANMI OLANIYAN **DATE**

(External Examiner)

DEDICATION

I dedicate this project to God Almighty for his love, grace, wisdom and the knowledge he bestowed upon me throughout my time at the University of Benin.

ACKNOWLEDGEMENTS

I give thanks to Almighty God, my creator, who has granted me grace and strength to complete this project work within the limited time frame.

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I am especially grateful to my parents, MR. OGIESOBA and MRS. SAMSON, my brother, OGIESOBA VICTORY, as well as my entire family, whose prayers, love, understanding, and emotional and financial support kept me going throughout this journey.

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

ABSTRACT

Cymbopogon citratus (lemongrass) is an aromatic and medicinal grass widely used in food, cosmetics, and traditional medicine for its antimicrobial, antioxidant, and anti-inflammatory properties. These activities are linked to its diverse phytochemicals, including essential oils, phenolic acids, and flavonoids. Although the plant has been extensively studied, limited comparative data exists on how solvent polarity influences the extraction of its nutritional and bioactive constituents. This study therefore evaluated the proximate composition, phytochemical distribution, and phenolic profile of *C. citratus* using ethanol (polar) and diethyl ether (non-polar) as extraction solvents. Laboratory analyses employed various methods, including gravimetric techniques for proximate and phytochemical composition, spectrophotometric and acid titration methods for phytochemical determination, and GC-FID for detailed phenolic profiling. Fresh samples collected from the University of Benin were authenticated and subjected to standardized proximate and phytochemical analyses, supported by current literatures from PubMed, ScienceDirect, MDPI, and Google Scholar. Extraction yield was slightly higher with diethyl ether (1.72%) than with ethanol (1.56%), reflecting the solvent's efficiency in dissolving non-polar constituents. Proximate analysis revealed high carbohydrate content (71.820%) and crude fibre content (3.225%), moderate crude protein (6.133%), ash content (2.117%), and moisture content (16.720%), and very low-fat content (1.552%). Antinutritional factors such as oxalates, phytates, and cyanogenic glycosides were found only in trace amounts, confirming nutritional safety. Phytochemical screening detected alkaloids, flavonoids, tannins, saponins, steroids, terpenoids, cardiac glycosides, and phenolics in both extracts. Phenolic profiling showed diethyl ether enriched non-polar compounds including catechol (21.776ppm), hydroxyquinol (54.471ppm), and resorcinol (13.932ppm), while ethanol favored polar phenolics such as quinol (25.975ppm) and cinnamic acid (21.163ppm). The study showed that *C. citratus* is nutritionally safe and phytochemically rich, with solvent polarity playing a major role in determining extraction yield and phytochemical diversity. These findings reinforce its dual value as both a medicinal and dietary resource. Future investigations should incorporate broader sampling, employ advanced extraction technologies, and integrate bioactivity assays to directly link phenolic diversity with therapeutic potential.

CHAPTER ONE

INTRODUCTION

1.1 Background of Study

Cymbopogon citratus, commonly known as lemongrass, is a perennial aromatic grass from the Poaceae (Gramineae) family, native to tropical regions such as Asia, Africa, and the Americas, and is especially abundant in Southeast Asia, including the Philippines and Indonesia. Lemongrass is valued for its high content of essential oils, particularly citral, which gives it a distinctive lemon-like aroma and underpins its use in cosmetics, perfumes, soaps, and as a culinary flavoring (Oladeji *et al.,* 2019). Medicinally, *C. citratus* exhibits a wide range of pharmacological activities, including antioxidant, antimicrobial, anti-inflammatory, antidiarrheal, antifungal, and antimalarial effects, attributed largely to its essential oils (notably citral) and other phytoconstituents (Omotayo *et al.,* 2024). *C. citratus* is recognized for its nutritional and therapeutic significance, largely due to its rich content of bioactive compounds such as phenolic acids, flavonoids, and essential oils. These compounds contribute to strong antioxidant properties, which help combat oxidative stress and may support overall health by reducing the risk of chronic disease. Extracts have also demonstrated hypolipidemic and anti-obesity effects suggesting a potential role in managing obesity and related metabolic disorders by improving lipid profiles and modulating key metabolic hormones (Rahhal *et al.,* 2024). Ethanol extraction of *C. citratus* (lemongrass) yields a range of bioactive compounds, with the extract's polarity influencing the types and quantities of molecules obtained. Ethanol, being a moderately polar solvent, extracts both polar and some non-polar compounds, resulting in a rich profile of phenolics, flavonoids, and other secondary metabolites such as quercetin-3-O-rutinoside, caffeic acid, synapic acid, and benzoic acid, which contribute to the extract's antioxidant and antimicrobial activities. The polarity of ethanol allows it to extract a broader spectrum of compounds than water alone, including both hydrophilic and some lipophilic constituents, which is reflected in the higher total phenolic and flavonoid content and greater radical scavenging activity of ethanolic extracts (Hussain *et al.,* 2023). Diethyl ether is a relatively non-polar solvent commonly used in plant extraction to isolate specific classes of phytochemicals, particularly non-polar or moderately polar compounds such as aglycone flavonoids, terpene derivatives, and other lipophilic constituents (Anastasaki *et al.,* 2017). Several studies have highlighted the nutritional and therapeutic benefits of *C. citratus*, but there remains limited comparative data on the phenolic profiles of its extracts using solvents of different polarities. Establishing such comparative profiles will provide insight into the plant's bioactive potential, guide its application in food, pharmaceutical and strengthen its role in ethnomedicine. Therefore, this study aims to comparatively evaluate the proximate composition and phytochemical compositions of *C. citratus* with an emphasis on solvent-based variation in phenolic profiles, providing a better understanding of how solvent choice influences the extraction and characterization of its bioactive and nutritional components.

1.2 Statement of Problem

C. citratus (lemongrass) is well known for its nutritional and therapeutic value, particularly its antimicrobial, antioxidant, and anti-inflammatory properties (Rahhal *et al.,* 2024; Tazi *et al.,* 2024a). Most research has concentrated on aqueous or single-solvent extractions, while comparative studies involving the recovery of phenolic compounds using ethanol and diethyl ether remain scarce. This information gap restricts the optimization of extraction methods and the production of standardized plant-based formulations with reliable efficacy. Therefore, it is important to conduct a comparative assessment of the proximate composition and phytochemical profile of ethanol and diethyl ether extracts of *C. citratus* in order to enhance understanding of solvent influence, differences in phenolic constituents, improve its medicinal and nutritional applications, and support its wider use in food, pharmaceutical, and nutraceutical industries.

1.3 Justification of Study

This study is of great significance as it aims to assess the influence of different solvents on the extraction of phytochemicals, particularly phenolic compounds and proximate nutrients from *C. citratus*. Although the plant is well known for its nutritional and therapeutic value, limited research has compared how solvent polarity affects the yield and composition of its bioactive and phenolic constituents. Determining the most effective solvent will not only enhance its practical applications but also ensure the production of high-quality herbal preparations. In addition, the findings will generate dependable scientific evidence to support its broader use in the food, pharmaceutical, and nutraceutical industries.

1.4 Aim of Study

The aim of this study is to evaluate and compare the phytochemical and proximate composition of ethanol and diethyl ether extracts of *C. citratus* in order to determine the relative effectiveness of these solvents in extracting its bioactive and nutritional constituents.

1.5 Specific Objectives

The specific objectives of the study were to:

a. Determine the proximate composition of *C. citratus*
b. Determine the phytochemical components of *C. citratus*.

c. Compare the differences in the yield and phenolic composition between ethanol (polar) and diethyl ether (non-polar) extracts of *C. citratus*.

1.6 Research Questions

a. What is the proximate composition of *C. citratus*?

b. What phytochemicals are present in *C. citratus* independent of solvent extraction?

c. What phenolic constituents are found in the ethanol and diethyl ether extracts of *C. citratus*?

d. How do the yields and phenolic constituents differ between ethanol (polar) and diethyl ether (non-polar) extracts of *C. citratus*?

e. What do these findings reveal about the nutritional and medicinal relevance of *C. citratus*?

1.7 Research Hypothesis

1.7.1 Null Hypothesis

1. The proximate composition of *C. citratus* does not contain any significant nutrients.
2. There are no phytochemicals present in *C. citratus,* independent of solvent used for extraction.
3. There is no significant difference in the phenolic constituents between ethanol (polar) and diethyl ether (non-polar) solvent extracts of *C. citratus.*
4. There. is no significant difference in the yields and phenolic composition between the polar and non-polar extracts of *C. citratus.*

5. The findings do not reveal any meaningful nutritional or medicinal relevance of *C. citratus.*

1.7.2 Alternate Hypothesis

1. The proximate composition of *C. citratus* contains significant nutrients
2. There are phytochemicals present in *C. citratus,* independent of solvent used for extraction.
3. There is significant difference in the phenolic constituents between ethanol (polar) and diethyl ether (non-polar) solvent extracts of *C. citratus.*
4. There is significant difference in the yields and phenolic composition between the polar and non-polar extracts of *C. citratus.*
5. The findings reveal the meaningful nutritional and medicinal relevance of *C. citratus.*

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of *C. citratus*

C. citratus (lemongrass) is a perennial aromatic grass in the Poaceae family, native to tropical Asia but now widely cultivated in many warm regions globally. It has a long history of use in food, cosmetics, traditional medicine, and as a functional plant with both medicinal and nutritional value. Its uses range from flavoring in cuisine to treatments for infections, inflammation, hypertension, and mental health disturbances (Du *et al.,* 2023). Phytochemical profiling has repeatedly shown a rich variety of bioactive compounds, especially essential oils (with citral, β-myrcene, geranial, neral as major constituents), flavonoids, phenolic acids, and other semi-volatile terpenoids that underlie many of its pharmacological effects (Diop *et al.,* 2017; Nimenibo-Uadia and Nwosu, 2020; Manna *et al.,* 2024).

Nutritionally, *C. citratus* leaves and powders possess moderate levels of protein, carbohydrates, and fiber, along with low to moderate amounts of fat, moisture, and ash, indicating their potential use as a dietary supplement or functional food ingredient. (Yusuf *et al.,* 2023; Manna *et al.,* 2024). Mineral analyses also reveal appreciable concentrations of calcium, potassium, iron, and magnesium (Nimenibo-Uadia and Nwosu, 2020), reinforcing its value beyond purely medicinal uses.

2.1.1 Scientific classification of *C.citratus*

The scientific classification of Cymbopogon citratus, commonly known as lemongrass, is as follows:

Kingdom: Plantae (plants)

Division (Phylum): Tracheophyta (vascular plants)

Class: Liliopsida (monocotyledons)

Order: Poales

Family: Poaceae (Gramineae)

Genus: Cymbopogon

Species: Cymbopogon citratus (DC.) Stapf

(Gaba *et al.,* 2020)

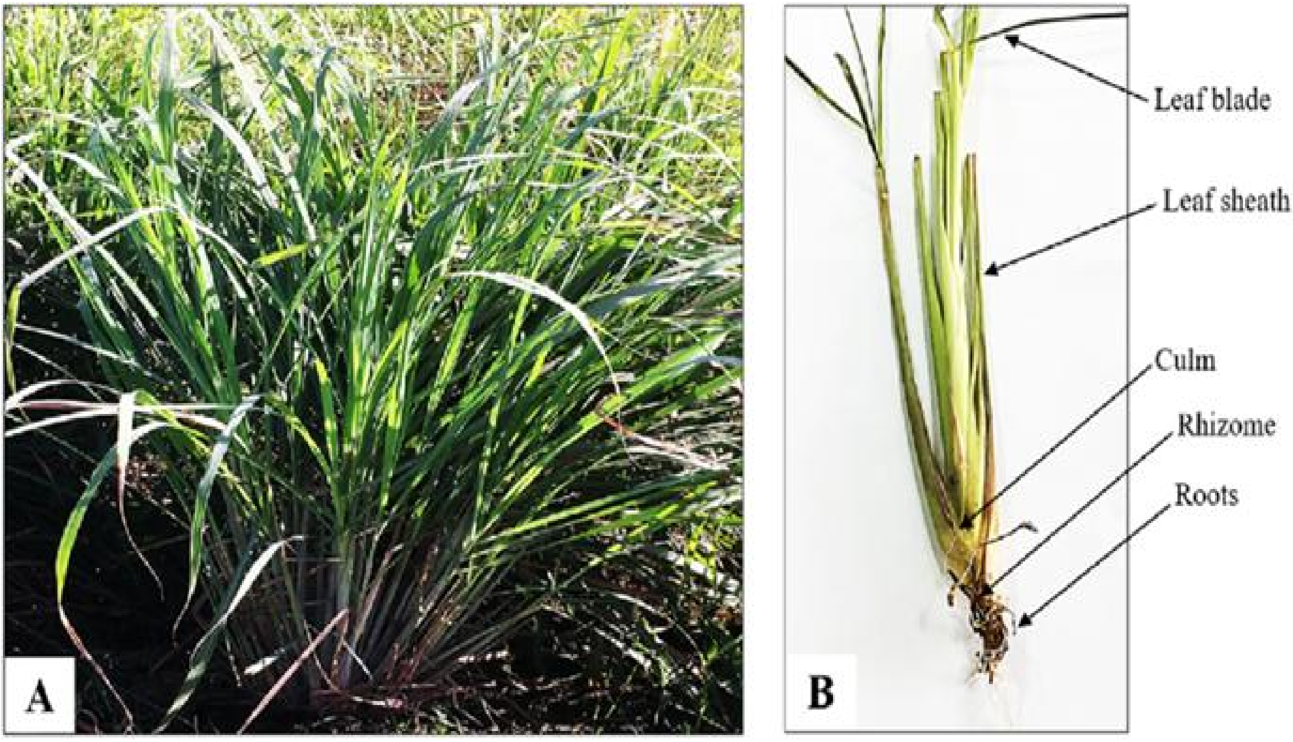

Figure 2.1: Showing photographs of *C. citratus*— (a) Whole grass in habitat; (b)Entire plant. (Madi *et al.,* 2022).

2.2 Nutritional significance of *C. citratus*

Across many cultures, *C. citratus* has been utilized in folk medicine for a variety of ailments. Traditional applications include using lemongrass tea or decoction for digestive upset, fever, stress, and to promote relaxation (Du *et al.,* 2023). In parts of West Africa, South America, and Asia, it is used as a remedy for hypertension, infections, headaches, and skin disorders (Yusuf *et al.,* 2023). The essential oil is also commonly used for insect repellence and aromatherapy, reflecting its strong aroma and volatile oil content.

2.3 Medicinal and Pharmacological properties of *C. citratus*

The pharmacological properties of *C. citratus* encompass a broad spectrum of therapeutic applications, which can be linked to its diverse phytochemical composition. Among these, antioxidant activity is one of the most consistently reported, as both methanolic and ethanolic extracts exhibit strong radical-scavenging effects due to their high levels of phenolic and flavonoid compounds. (Unuigbe *et al.,* 2019; Yusuf *et al.,* 2023). Mechanistic studies indicate that these extracts help regulate oxidative stress by boosting the activity of endogenous antioxidant enzymes, including catalase and superoxide dismutase, while decreasing lipid peroxidation markers, thus safeguarding cellular systems from oxidative damage. (Omam *et al.,* 2023). Antimicrobial activity is another prominent property. Antimicrobial activity is another notable attribute of *C. citratus*. Both its essential oils and solvent extracts exhibit inhibitory effects against various pathogenic bacteria and fungi, including *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. (Wahyuni *et al.,* 2024). The antimicrobial action is largely attributed to citral and other terpenoid constituents that disrupt microbial membranes and metabolic pathways. Other biological effects of *C. citratus* highlight its traditional uses. Decoctions have demonstrated anxiolytic and neuroprotective effects in murine models, where treated animals displayed reduced anxiety-like behaviors alongside improved oxidative stress biomarkers (Omam *et al.,* 2023). Cardiometabolic benefits have also been reported, with experimental evidence suggesting blood-pressure lowering and lipid-modulating activities that align with its folk use in managing hypertension and metabolic disorders (Du *et al.,* 2023). Collectively, these findings validate the ethnopharmacological applications of *C. citratus* while also indicating new therapeutic prospects that warrant further clinical exploration.

2.4 Nutritional and Proximate Composition

Beyond its medicinal value, *C. citratus* provides significant nutritional benefits, as reflected in its proximate composition. Analyses of leaf powders reveal appreciable amounts of carbohydrates, proteins, and dietary fibre, alongside moderate ash and low-fat content, giving the plant a profile consistent with functional food ingredients (Manna *et al.,* 2024; Nimenibo-Uadia and Nwosu, 2020). Carbohydrate content is particularly high, providing energy, while the notable crude fibre fraction supports digestive health and metabolic regulation. Protein values, though moderate, contribute additional nutritional value and complement the plant's dietary applications (Yusuf *et al.,* 2023). Mineral analysis reveals that *C. citratus* contains high levels of calcium, potassium, and magnesium, along with notable amounts of iron and phosphorus, all vital for various metabolic and physiological functions. (Nimenibo-Uadia and Nwosu, 2020; Yusuf *et al.,* 2023). The presence of vitamins A, C, and E further enhances its antioxidant capacity and underscores its nutritional significance (Yusuf *et al.,* 2023). Collectively, these proximate and micronutrient characteristics indicate that *C. citratus* serves not only as a medicinal resource but also as a valuable dietary component, supporting both preventive and restorative aspects of health.

2.5 Proximate Properties

2.5.1 Crude Fibre

Crude fibre is defined as the residue of plant food that remains after sequential extraction with dilute acid and then dilute alkali, a process originally developed in the early 19th century and known as the Weende method. This residue is intended to represent the least digestible fraction of plant material, primarily composed of cellulose and some hemicellulose, but it does not accurately reflect the total structural fibre content because much of the lignin and other components are lost during the extraction process (Jiang *et al.,* 2019).

2.5.2 Crude Protein

Crude Protein is an estimate of the total protein content of feeds and foods. It is not a direct measurement of true protein, but rather an estimate based on the nitrogen content of a sample, reflecting both protein and non-protein nitrogen sources (Greenhalgh *et al.,* 2020).

2.5.3 Ash Content

Ash content refers to the inorganic residue that remains after the complete combustion or oxidation of a sample, such as food, coal, or biomass. It represents the total mineral content and is determined by burning the sample at high temperatures (typically 500–600°C) until all organic matter is removed, leaving only the mineral ash (Ismail, 2017).

2.5.4 Crude Fat

Crude fat is defined as the total amount of fat that can be extracted from a sample using specific solvents, typically through methods such as Soxhlet or Soxtec extraction, and is determined gravimetrically (by weight) after extraction. This measurement is empirical, meaning the result depends on the exact conditions and solvents used in the procedure, and it includes not only true fats (triglycerides) but also other substances soluble in the extraction solvent, such as oils, waxes, and some pigments (Anderson, 2019).

2.5.5 Moisture Content

Moisture content represents the quantity of water contained in a material such as coal, biomass, food, or waste. It is commonly measured by heating the sample until a constant weight is obtained, during which water is removed through evaporation. The resulting weight loss reflects the moisture content, typically expressed as a percentage of the sample's initial mass (Thangaraj, 2015).

2.5.6 Carbohydrate

Carbohydrates refers to the portion of a food or sample that consists of sugars, starches, cellulose, and other polysaccharides, and is typically calculated by difference. This means that after measuring the moisture, ash (minerals), protein, and lipid (fat) contents, the remaining percentage is attributed to carbohydrates (Thangaraj, 2015).

2.6 Biochemical and Phytochemical Constituents

Phytochemical investigations have consistently identified a diverse suite of secondary metabolites in *C. citratus*. The essential oil fraction is particularly well characterized, dominated by citral (a mixture of the isomers geranial and neral), along with β-myrcene, geraniol, citronellal, and linalool (Diop *et al.,* 2024). These volatile constituents are primarily responsible for the plant's antimicrobial, anti-inflammatory, and aromatic properties. Non-volatile compounds such as flavonoids, phenolic acids, tannins, saponins, and alkaloids are also present, especially in polar solvent extracts, and contribute to its antioxidant, antimicrobial, and potential anticancer activities (Unuigbe *et al.,* 2019; Du *et al.,* 2023). Regional and methodological variations influence the phytochemical profile. For instance, oils obtained from fresh leaves differ in citral content compared to those distilled from dried material, while extraction with ethanol or methanol yields higher concentrations of phenolics and flavonoids than aqueous methods (Diop *et al.,* 2024). This variability underscores the need for standardized extraction protocols to ensure consistent therapeutic outcomes.

2.7 Phytochemical Components

2.7.1 Tannin

Tannins are a diverse class of high molecular weight, water-soluble polyphenolic compounds that occur naturally in many plants, including fruits, vegetables, cereals, and legumes (Smeriglio *et al.,* 2016).

2.7.2 Alkaloids

Alkaloids are a broad and diverse group of naturally occurring organic compounds that contain at least one nitrogen atom, usually within a heterocyclic ring. They are mainly found in plants but can also be produced by bacteria, fungi, and certain animals, and are typically derived from amino acids as secondary metabolites. Alkaloids are known for their complex chemical structures and significant biological activities, including roles as medicines, stimulants, and toxins, with well-known examples such as morphine, quinine, and codeine (Rajput *et al.,* 2021).

2.7.3 Oxalate

Oxalates are organic compounds, specifically dicarboxylic acids, that exist as divalent anions (oxalate ions) and are found widely in nature, especially in plant-based foods such as green leafy vegetables, spinach, rhubarb, tea, cacao, nuts, and beans. Oxalate is best known for its ability to form insoluble salts with minerals such as calcium and magnesium, which can lead to the development of calcium oxalate kidney stones and reduce the intestinal absorption of essential minerals like magnesium, zinc, iron, and calcium (Siener *et al.,* 2020)

2.7.4 Flavonoids

Flavonoids are a large group of natural polyphenolic compounds found abundantly in fruits, vegetables, grains, tea, and many other plant-based foods and beverages. Chemically, they are characterized by a basic structure consisting of two aromatic rings (C6) connected by a three-carbon bridge (C3), forming a C6-C3-C6 skeleton, and are subdivided into several classes such as flavanones, flavones, isoflavones, flavonols, flavanols, and anthocyanins based on variations in this structure (Panche *et al.,* 2016).

2.7.4 Saponin

Saponins are a diverse class of naturally occurring plant secondary metabolites distinguished by their amphiphilic structure, consisting of a hydrophilic (water-attracting) sugar unit attached to a lipophilic (fat-attracting) aglycone, usually a triterpenoid or steroid. This distinctive structure gives saponins surface-active properties, enabling them to form stable foams and function as natural emulsifiers and stabilizers in food and pharmaceutical applications (Timilsena *et al.,* 2023).

2.7.5 Phytate

Phytate, also known as phytic acid or myo-inositol hexakisphosphate (InsP6), is a phosphorus-containing compound that serves as the principal storage form of phosphorus in plants, particularly in seeds, whole grains, legumes, and oilseeds. Chemically, phytate is a polyanionic molecule formed by the stepwise phosphorylation of myo-inositol, resulting in a structure with six phosphate groups that can strongly bind (chelate) positively charged mineral ions such as calcium, iron, zinc, and magnesium (Suvarna *et al.,* 2017).

2.7.6 Phenolic Content

Phenolic content refers to the concentration of phenolic compounds within a sample, such as food, plant material, or extracts. These compounds are a broad group of plant secondary metabolites distinguished by one or more hydroxyl groups attached to aromatic rings. They include several classes, such as phenolic acids, flavonoids, tannins, lignans, and stilbenes. The phenolic content of a material serves as an important measure of its potential antioxidant properties and overall bioactivity (Shahidi and Ambigaipalan, 2015).

2.7.7 Cardiac Glycoside

Cardiac glycosides are a class of naturally occurring compounds, primarily derived from plants and some animals, that function as inhibitors of the Na+/K+-ATPase (sodium-potassium pump) in cells. This inhibition leads to increased intracellular calcium, which enhances the force of heart muscle contractions, making these compounds historically important in the treatment of heart failure and certain arrhythmias such as atrial fibrillation (Fender *et al.,* 2024).

2.7.8 Cyanogenic Glycoside

Cyanogenic glycosides are natural plant compounds composed of an α-hydroxynitrile (aglycone) linked to a sugar moiety, most commonly glucose. These compounds are found in over 2,000–3,000 plant species and serve as a chemical defense mechanism; when plant tissue is disrupted, cyanogenic glycosides are enzymatically hydrolyzed to release toxic hydrogen cyanide (HCN) (Sánchez-Pérez and Neilson, 2024).

2.7.9 Hydrogen Cyanide

Hydrogen cyanide (HCN) is a small and volatile compound best known for its toxic nature, yet it also has significant functions in plant physiology. In many plants, HCN is stored as cyanogenic glycosides, which decompose to release hydrogen cyanide when plant tissues are injured, providing a chemical defense mechanism against herbivores and pathogens (Brückner *et al.,* 2017).

2.7.10 Lignin

Lignin is a complex, high-molecular-weight phenolic polymer found primarily in the secondary cell walls of vascular plants, where it provides mechanical strength, rigidity, and hydrophobicity, enabling plants to grow tall and efficiently transport water and nutrients. Chemically, lignin is a polyaromatic macromolecule primarily formed through the oxidative polymerization of three monolignols: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. Other phenolic compounds may also be integrated into its structure, resulting in a highly complex and variable composition (Vanholme *et al.,* 2019).

2.7.11 Cellulose

Cellulose is the most abundant structural polysaccharide in plant cell walls. It consists of β-D-glucopyranose units linked by β(1–4)-glycosidic bonds, with the formula $(C_6H_{10}O_5)_n$, where the degree of polymerization ranges from hundreds to thousands of glucose units. Cellulose is insoluble in water and dilute solutions due to extensive hydrogen bonding, which also gives it resistance to hydrolysis and enzyme activity. It mainly occurs in crystalline forms that organize into microfibrils. The most common is polymorph I, subdivided into Iα (found in algae and bacteria) and Iβ (dominant in plants) (Frassoldati and Ranzi, 2019).

2.7.12 Hemicellulose

Hemicellulose is the second most abundant biopolymer in plant biomass after cellulose, but it differs significantly in structure. Unlike cellulose, which is composed solely of glucose, hemicellulose is a heteropolysaccharide made up of various monosaccharides, including both pentoses and hexoses such as glucose, xylose, mannose, galactose, arabinose, and uronic acids. Its polymer chains are relatively short, typically containing 50–200 sugar units, which makes it less rigid than cellulose. The composition and structure of hemicellulose depend on the plant source and are classified according to the types of sugar residues present (Machmudah *et al.,* 2017).

2.7.13 Terpenoid

Terpenoids represent the largest and most diverse group of phytochemicals, serving multiple roles such as light-harvesting pigments, plant hormones, phytoalexins, and semiochemicals. They are synthesized from basic five-carbon precursors that act as universal building units (Singh *et al.,* 2022).

2.7.14 Phenolic profile

Phenolic content refers to the concentration of phenolic compounds found in a sample, commonly in foods or plant materials. These compounds, often called phenolics or polyphenols, are a diverse group of naturally occurring substances distinguished by the presence of one or more hydroxyl groups attached to an aromatic six-carbon ring structure. These compounds are mainly found as secondary metabolites in plants and include simple phenols, phenolic acids, flavonoids, stilbenes, lignans, tannins, and more complex polymers (Delgado *et al.,* 2019).

2.7.15 Steroids

Steroids are a broad class of organic compounds characterized by a core structure of four fused carbon rings (cyclopentanoperhydrophenanthrene skeleton) and are widely distributed in plants as essential components and bioactive molecules. Plant steroids include phytosterols (plant sterols), which are important structural components of cell membranes and precursors for other bioactive compounds such as steroidal saponins, glycoalkaloids, phytoecdysteroids, and brassinosteroids (Batth *et al.,* 2020).

2.7.16 Carotenoids

Carotenoids are a class of natural pigments and antioxidants composed of long conjugated isoprenoid chains, typically with a C40 tetraterpene backbone, responsible for the yellow, orange, and red colors in many plants, algae, and some microorganisms. These lipophilic compounds are synthesized de novo in plant plastids and play essential roles in photosynthesis as accessory light-harvesting pigments, in photoprotection against excess light, and as precursors for important plant hormones such as abscisic acid and strigolactones (Maoka, 2019; Nisar *et al.,* 2015).

2.8 Extraction Methods and Solvent Effects

The yield and chemical profile of *C. citratus* extracts are closely linked to the extraction method and solvent polarity. Polar solvents such as ethanol and methanol generally recover higher amounts of phenolics, flavonoids, and tannins, resulting in extracts with stronger antioxidant and antimicrobial properties (Unuigbe *et al.,* 2019; Yusuf *et al.,* 2023). In contrast, hydrodistillation and steam distillation are optimized for recovering volatile essential oils rich in citral and myrcene, which underpin the plant's fragrance and antibacterial activity (Diop *et al.,* 2024). Pretreatment of plant material further affects outcomes. Drying has been shown to modify the ratio of citral isomers and overall essential oil yield, while leaf waste processing can still yield oils of pharmacological relevance. Emerging approaches such as ultrasound-assisted and microwave-assisted extraction, though less widely applied to *C. citratus*, hold promise for enhancing recovery of bioactive compounds while reducing solvent use and extraction time (Du *et al.,* 2023). These methodological considerations emphasize the importance of careful solvent and technique selection in optimizing the pharmacological potential of *C. citratus*.

2.9 Clinical and Experimental Evidence

Experimental studies provide substantial evidence supporting the pharmacological properties of *C. citratus*. In vivo models demonstrate anxiolytic activity, where decoctions significantly reduced anxiety-like behavior and improved oxidative stress markers in mice (Omam *et al.,* 2023). In vitro assays consistently confirm strong antioxidant and antimicrobial effects across solvent fractions and essential oils, aligning with traditional uses for infection control and oxidative stress mitigation (Wahyuni *et al.,* 2024). Variation in activity has been attributed to differences in solvent choice and phytochemical content. Ethanolic and methanolic extracts frequently show superior pharmacological profiles compared to aqueous extracts, reflecting their ability to solubilize a broader spectrum of bioactive compounds (Unuigbe *et al.,* 2019). Essential oils also vary geographically in citral concentration, influencing their potency in antimicrobial and antioxidant assays (Diop *et al.,* 2024). While preclinical evidence is robust, clinical investigations remain limited. The promising outcomes from animal and laboratory studies highlight the need for well-designed clinical trials to validate efficacy and safety in humans, particularly in areas such as metabolic health, anxiety disorders, and infection management.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Area of study

The study was carried out at the University of Benin, situated in Benin City, Edo State, Nigeria.

3.2 Study Location

The study was conducted at the University of Benin, located in Benin City, Edo State, Nigeria. Extraction procedures were carried out at the Department of Chemistry and the Department of Pharmacy (Specialty Pharmacognosy), both within the University of Benin, which are equipped with the necessary laboratory facilities for solvent extraction and related analyses. Subsequent proximate and phytochemical analyses were performed at Docchy Laboratories, Awka, Anambra State, Nigeria, a specialized laboratory facility furnished with modern equipment for biochemical and phytochemical investigations.

3.3 Materials

3.3.1 Chemical and Reagents

Ethanol (analytical grade) was obtained from Sigma-Aldrich, Germany, while Diethyl ether (LR grade, stabilized) was procured from Molychem, India. All solvents and reagents used were of high analytical grade.

3.3.2 Equipment

The major equipment used in this study included an electronic weighing balance (Model: TS200, OHAUS), rotary evaporator (Model: Julabo F10), UV-Visible spectrophotometer (Model: 752N, Hinotek, Genesys 10-S), hot air oven (Precision Electrothermal Model BNP 9052), water bath (Memmert WNB-10), muffle furnace (Model: HT-MF1400-6.75S/G), and gas chromatography–mass spectrometry (GC–MS) system (Agilent 7890 GC coupled with 5975 Mass Selective Detector).

3.4 Collection and Identification of *C. citratus*

Fresh samples of *C. citratus* were collected within the University of Benin premises around the Faculty of Basic Medical Sciences, Benin City, Edo State, Nigeria. The plant was identified and authenticated at the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, and its identity was confirmed under the voucher number UBH-C451 at the Herbarium Unit.

3.5 Preparation of Plant Extract

The plant extract was prepared at the Department of Chemistry and the Department of Pharmacy (Specialty Pharmacognosy), University of Benin. The whole plant was cleaned to remove debris, and thoroughly washed with clean tap water. They were air-dried at room temperature under laboratory conditions for two weeks and then pulverized into fine powder using a commercial blender. The total weight of the dried pulverized sample was 707.4g.

3.5.1 Polar Extraction

The extraction was carried out using a modified method of Onyeukwu *et al.,* (2024). A total of 250 g of the powdered leaf was macerated in 1.2 L of ethanol in a brown glass jar, properly sealed with aluminum foil, and left to stand for 72 hours at room temperature. The mixture was first filtered with cheesecloth to remove coarse debris and then passed through Whatman Grade 1 filter paper, yielding 565 ml of filtrate. The filtrate was concentrated using a rotary evaporator and further air-dried, producing 3.9 g of dry extract**.** The extract was subsequently stored at 4°C in a refrigerator until required for use.

3.5.2 Non-polar Extraction

The extraction was carried out using a modified method of Onyeukwu *et al.,* (2024). A total of 250 g of the powdered leaf was macerated in 1.2 L of diethyl ether in a brown glass jar, sealed with aluminum foil, and allowed to stand for 72 hours at room temperature. The mixture was filtered initially with cheesecloth and subsequently with Whatman Grade 1 filter paper, producing 520ml of filtrate. The filtrate was concentrated using a rotary evaporator and air-dried, yielding 4.3 g of dry extract. The extract was subsequently stored at 4°C in a refrigerator until required for use.

3.5.3 Percentage Yield

The percentage yield for each extraction was determined using the formula:

Yield (%) = (Dry weight of extract / Dry weight of plant material) ×100

Ethanol extraction: Yield = (3.9g / 250g) ×100 = 1.56 %

Diethyl ether extraction: Yield = (4.3g / 250g) ×100 = 1.72 %

The diethyl ether extraction gave a slightly higher yield (1.72%) compared to the ethanol extraction (1.56%). This difference may be attributed to the polarity of diethyl ether, which as a non-polar solvent, is more efficient in dissolving non-polar essential oils (citral and related terpenes) from *C. citratus* which it is naturally rich in. In contrast, ethanol, a non-polar solvent, primarily extracts polar constituents (phenolics, flavonoids), resulting in a relatively lower yield.

3.6 Biochemical Analysis

3.6.1 Proximate Analysis

A. Ash content

Method: Gravimetric method (Muffle Furnace Method) (AOAC, 1995)

Principle: The ash content of a food sample represents the inorganic residue that remains after all organic matter has been completely burned off. However, the composition of the resulting ash may not fully reflect the original mineral content, as some components can be lost through volatilization during the burning process (AOAC, 1995).

Procedures:

A clean, dry platinum crucible was weighed to record its initial mass. Approximately 2g of the sample was then placed in the crucible and incinerated in a muffle furnace at 550°C for 3 hours. After ashing, the crucible was cooled in a desiccator and reweighed to determine the ash content (AOAC, 1995).

Calculations:

$$\% \text{ Ash content} = \frac{W3 - W1}{W2 - W1} \times \frac{100}{1}$$

Where:

W1 = weight of empty platinum crucible

W2 = weight of platinum crucible and sample before burning

W3 = weight of platinum and ash (AOAC, 1995)

B. Moisture Content

Method: Oven Drying (AOAC, 1990)

Principle: The oven-drying method for moisture determination is based on the loss of water from a sample upon heating at a controlled temperature (usually 105°C) until a constant weight is achieved. The decrease in mass reflects the total moisture content of the sample, including free and some bound water, and is expressed as a percentage of the original sample weight (AOAC, 1990).

Procedure:

A clean petri dish was washed, dried in an oven, and weighed. Approximately 2 g of the sample was placed into the petri dish, and the combined weight was recorded before drying. The dish and sample were then heated in an oven at 105°C for 2 hours, after which the weight was recorded. Heating was continued for an additional hour and repeated until a constant weight was achieved, indicating complete removal of moisture (AOAC, 1990).

Calculations:

$$\%\text{ Moisture content} = \frac{W1-W2}{\text{Weight of sample}} \times 100$$

Where:

W1 = weight of petri dish and sample before drying

W2= weight of petri dish and sample after drying (AOAC, 1990).

C. Carbohydrate Determination

Method: Differential method (AOAC, 2016)

Calculation: 100 – (%Protein + %Moisture + %Ash + %Fat + %Fibre)

D. Crude Fibre

Method: Acid-Base Digestion (AOAC, 1990)

Principle: The crude fiber content of a sample is determined by sequentially treating the defatted sample with dilute acid and alkali to remove soluble substances, leaving behind the indigestible fibrous residue. The residue is then dried, weighed, and incinerated to remove ash. The difference in weight before and after incineration represents the crude fiber content, expressed as a percentage of the original sample weight (AOAC, 1990).

Procedure:

Approximately 2 g of the sample was defatted with petroleum ether if the fat content exceeded 10%. The defatted material was then boiled under reflux for 30 minutes with 200 mL of a solution containing 1.25 g of H_2SO_4 per 100 mL. The mixture was filtered through linen, and the residue was thoroughly washed with boiling water until the washings were neutral. The residue was then transferred to a beaker and boiled again for 30 minutes with 200 mL of a solution containing 1.25 g of carbonate-free NaOH per 100 mL. After boiling, the residue was filtered through a thin but tightly packed layer of washed and ignited asbestos in a Gooch crucible, dried in an electric oven, and weighed. It was then incinerated, cooled, and reweighed to determine the crude fiber content (AOAC, 1990).

Calculation:

The loss in weight after incineration x 100 is the percentage of crude fibre.

$$\% \text{ crude fibre} = \frac{\text{Weight of fibre}}{\text{Weight of sample}} \times 100$$

(AOAC, 1990).

E. Crude fat

Method: Soxhlet Fat Extraction (AOAC, 1990)

Principle: The Soxhlet extraction method for determining crude fat involves the continuous extraction of lipids from a sample using a non-polar organic solvent, typically petroleum ether. The solvent dissolves the fat, which is subsequently collected, evaporated, and weighed. The weight of the extracted fat, expressed as a percentage of the sample's initial mass, represents the crude fat content (AOAC, 1990).

Procedure:

Clean 250 mL boiling flasks were dried in an oven at 105–110 °C for approximately 30 minutes, then transferred to a desiccator to cool. Once cooled, the flasks were weighed and properly labeled. Each flask was filled with about 300 mL of petroleum ether (boiling range 40–60 °C). The extraction thimble was lightly plugged with cotton wool, and the Soxhlet apparatus was assembled and allowed to reflux for about 6 hours. After extraction, the thimble was carefully removed, and the petroleum ether in the upper chamber was collected and drained for reuse. When the flask was nearly free of solvent, it was dried in an oven at 105–110 °C for 1 hour, then transferred to a desiccator to cool before being reweighed (AOAC, 1990).

Calculation:

$$\% \text{ Fat} = \frac{\text{Wt of flask + oil - Wt of flask}}{\text{Wt of sample}} \times 100$$

(AOAC, 1990).

F. Crude Proteins

Method: Kjeldahl (AOAC, 1990)

Principle: This method involves digesting the sample with hot concentrated sulphuric acid in the presence of a metallic catalyst. During digestion, the organic nitrogen in the sample is converted to ammonia, which remains in the solution as ammonium sulphate. The solution is then made alkaline and distilled to release the ammonia, which is subsequently trapped in a dilute acid solution and quantified by titration (AOAC, 1990).

Procedures:

An accurately weighed 0.5 g portion of the sample was placed into a 30 mL Kjeldahl flask, ensuring that no material adhered to the neck or sides. The flask was stoppered and gently shaken. A 0.5 g portion of the Kjeldahl catalyst mixture was then added, followed by concentrated sulphuric acid. The flask was placed on a digestion rack and heated carefully until the digest became clear. After digestion, the flask was allowed to cool for 30 minutes, diluted with distilled water, and made up to 100 mL in a volumetric flask. A 5 mL aliquot of the digest was transferred into the Kjeldahl distillation apparatus, to which 5 mL of 40% sodium hydroxide solution was added. A 100 mL receiver flask containing 5 mL of 2% boric acid solution and an indicator mixture (5 drops of bromocresol blue and 1 drop of methylene blue) was placed beneath the condenser, with the delivery tube positioned about 20 cm below the liquid surface. Distillation was commenced immediately and continued until approximately 50 drops of distillate were collected in the receiver flask. The distillate was then titrated with 0.01 N hydrochloric acid until a pink endpoint was observed (AOAC, 1990).

Calculations:

% Nitrogen = Titre value x 0.01 x 14 x 4

% Protein = % Nitrogen x 6.25 (AOAC, 1990).

3.5.2 Qualitative Phytochemical

Qualitative analyses were carried out using the methods of Trease and Evans (1989) and

Harborne (1998) to ascertain the presences of the different phytochemicals in the leaves before quantitative analysis is carried out.

A. Test for Alkaloids

Reagent: 2% HCl

Methods: Acid Extraction (Harborne, 1998)

Procedure for preparation

A 1.0 mL portion of the extract was measured into a test tube, followed by the addition of 5.0 mL of 2% hydrochloric acid. The test tube was then placed in a water bath (Memmert) and heated for 10 minutes. After heating, the mixture was filtered through Whatman No. 1 filter paper, and the resulting filtrate was collected for further analysis.

a) Wagner's reagent test

Principle: Under acidic conditions and at room temperature, alkaloids react with a mixture of iodine and potassium iodide to produce a reddish-brown precipitate (Harborne, 1998).

Reagent: Wagner's reagent preparation: 2 g of iodine and 3 g of potassium iodide were weighed, mixed, and dissolved in 30 mL of distilled water. The solution was then diluted to a final volume of 100 mL with distilled water.

Procedure:

A 1.0 mL portion of the filtrate was transferred into a test tube, followed by the addition of 1.0 mL of Wagner's reagent. The contents were thoroughly mixed, and any resulting colour change was carefully observed (Harborne, 1998).

b) Meyer's reagent test

Principle: Under acidic conditions and at room temperature, alkaloids react with a mixture of mercuric chloride and potassium iodide to produce a cream-coloured precipitate (Trease and Evans, 1989).

Reagents: Dissolve Meyer's reagent: 1.4 of mercuric chloride in 60ml distilled water and

4.5g of potassium iodide in 20ml distilled water. The two solutions are mixed and diluted to a 100ml with distilled water.

Procedure:

A 1.0 mL aliquot of the filtrate was transferred into a test tube, followed by the addition of 1.0 mL of Mayer's reagent. The contents were thoroughly mixed, and any colour change was observed. The formation of a cream-coloured precipitate confirmed the presence of alkaloids. (Trease and Evans, 1989).

B. Test For Flavonoids:

a) Lead Acetate Test

Principle: At room temperature, flavonoids react with lead acetate to produce a yellow colour or a yellow precipitate. (Trease and Evans, 1989).

Reagent: 10% lead acetate solution**:** Weigh 10.0 g of lead acetate and dissolve it in distilled water. Make up the solution to a final volume of 100 mL with distilled water.

Procedure:

A 1.0 mL portion of the extract was transferred into a test tube, followed by the addition of 1.0 mL of 10% lead acetate solution. The contents were thoroughly mixed, and the appearance of a yellow colour or precipitate was observed, indicating the presence of flavonoids (Trease and Evans, 1989).

b) Sodium Hydroxide Test

Principle: At room temperature and under alkaline conditions, flavonoids form a visible precipitate (Trease and Evans, 1989).

Reagent: Dilute sodium hydroxide solution: Weigh 40 g of sodium hydroxide and dissolve it in distilled water. Make up the solution to a final volume of 1 litre with distilled water.

Procedure:

A 1.0 mL portion of the extract was transferred into a test tube, followed by the addition of 1.0 mL of dilute sodium hydroxide solution. The mixture was thoroughly mixed, and any colour change was noted. The appearance of a precipitate confirmed the presence of flavonoids (Trease and Evans, 1989).

C. Test for Tannins

a) Acid Test

Principle: Under acidic conditions, phlobatannins react with dilute hydrochloric acid to produce a red colour or a red precipitate (Harborne, 1998).

Reagent: 1% hydrochloric acid solution: Pipette 1.0 mL of concentrated hydrochloric acid and dilute it to a final volume of 100 mL with distilled water.

Procedure:

A 3.0 mL portion of the extract was mixed with 2.0 mL of 1% hydrochloric acid. The mixture was observed for the development of a red colour or the formation of a red precipitate, which indicated the presence of phlobatannins (Harborne, 1998).

b) Lead Acetate Test

Principle: At room temperature, phlobatannins react with lead acetate to produce a dark blue or black precipitate (Harborne, 1998).

Reagent: 5% lead acetate solution: Weigh 5.0 g of lead acetate and dissolve it in distilled water. Make up the solution to a final volume of 100 mL with distilled water.

Procedure:

A 2 mL portion of the extract was transferred into a test tube, and three drops of 5% lead acetate solution were added. The formation of a dark blue to black precipitate confirmed the presence of phlobatannins (Harborne, 1998).

D. Test for Saponins

Method: Frothing and Emulsion (Harborne, 1998)

Principle: Saponins are natural glycosides that reduce surface tension of water and produce stable froth when shaken. The addition of a small amount of oil forms an emulsion with the froth, confirming the presence of saponins (Harborne, 1998).

Procedure:

A 2 g portion of the powdered sample was weighed into a beaker, and 20 mL of distilled water was added. The mixture was heated in a water bath for a few minutes, then filtered to obtain the filtrate. A 10 mL portion of the filtrate was mixed with 5 mL of distilled water in a test tube and shaken vigorously. The appearance of a stable, persistent froth was noted. Three drops of olive oil were then added to the frothing solution, which was shaken again. The formation of an emulsion confirmed the presence of saponins (Harborne, 1998).

E. Test for Cardiac Glycosides

Method: Keller–Killiani (Harborne, 1998)

Principle: Cardiac glycosides contain deoxy sugars that react with strong acids and Fehling's solution to produce a characteristic brick-red precipitate, indicating their presence.

Procedure:

A 1 mL portion of the plant extract was transferred into a test tube, followed by the addition of 10 mL of 50% sulphuric acid (H_2SO_4). The mixture was heated in a boiling water bath for 5 minutes. After heating, 10 mL of Fehling's solution (prepared by combining 5 mL of solution A and 5 mL of solution B) was added, and the mixture was boiled. The appearance of a brick-red precipitate confirmed the presence of cardiac glycosides (Harborne, 1998).

F. Test for Steroids

Method: Salkowski Test (Harborne, 1998)

Principle: Steroids, when treated with concentrated sulphuric acid, produce a distinct colour at the interface between the layers. The development of a red or golden-yellow colour confirms the presence of steroids (Harborne, 1998).

Procedure:

0.5 mL of the chloroform extract was transferred into a clean test tube. Subsequently, 1 mL of concentrated sulphuric acid (H_2SO_4) was carefully introduced along the side of the test tube to form a distinct lower layer. The colour observed at the interface between the two layers was noted. The appearance of a characteristic colour indicated the presence of steroids (Harborne, 1998).

G. Test for Phenolic nucleus

Method: Ferric chloride test (Harborne, 1998)

Principle: At room temperature, the phenolic nucleus reacts with ferric chloride to produce a greenish-brown or black colour, or a precipitate, indicating the presence of phenolic compounds (Harborne, 1998).

Reagent: 10% Ferric chloride solution: Weigh 10.0 g of ferric chloride ($FeCl_2$) and dissolve it in 100 mL of distilled water. Mix thoroughly to ensure complete dissolution.

Procedure:

A 1.0 mL portion of the extract was measured into a clean test tube, followed by the addition of 1.0 mL of 10% ferric chloride solution. The mixture was thoroughly mixed, and any colour change was carefully observed. The appearance of a greenish-brown or black colour or precipitate confirmed the presence of a phenolic nucleus (Harborne, 1998).

H. Test for Resins

Method: Alcohol Precipitation (Harborne, 1998)

Principle: Resins are soluble in alcohol but insoluble in water. When an alcoholic extract of the sample is added to water, the resins precipitate, enabling their qualitative detection (Harborne, 1998).

Procedure:

A 0.2 g portion of the powdered sample was accurately weighed into a clean beaker, and 15 mL of 90% ethanol was added. The mixture was thoroughly shaken to extract the soluble components and then filtered to obtain the alcoholic extract. This extract was carefully poured into a separate beaker containing 20 mL of distilled water. The formation of a precipitate indicated the presence of resins (Harborne, 1998).

I. Test for Terpenoids

Method: Salkowski Test (Harborne, 1998)

Principle: In the presence of chloroform, terpenoids react with concentrated sulphuric acid to produce a reddish-brown colour at the interface, signalling the presence of terpenoid compounds (Harborne, 1998).

Procedure:

A 5 mL portion of each extract was transferred into a clean test tube, and 2 mL of chloroform (Numex, India) was added and gently mixed. Then, 3 mL of concentrated sulphuric acid (H_2SO_4) was carefully added along the side of the test tube to form a separate lower layer. The interface between the two layers was observed, and the appearance of a reddish-brown colour at the interface indicated the presence of terpenoids (Harborne, 1998).

3.5.3 Quantitative Phytochemical

A. Oxalate determination

This determination involves three major steps digestion, oxalate precipitation and permanganate titration

Method: Titration (Harborne, 1998)

Digestion

A 2 g portion of the sample was suspended in 190 mL of distilled water in a 250 mL volumetric flask. 10 mL of 6 M hydrochloric acid (HCl) was added, and the suspension was digested at 100 °C for 1 hour. After cooling, the mixture was diluted to the 250 mL mark with distilled water and then filtered (Harborne, 1998).

Oxalate precipitation

Two 125 mL portions of the filtrate were measured into separate clean beakers. Four drops of methyl red indicator were added to each, and ammonium hydroxide (NH_4OH) was gradually added until the solution shifted from salmon pink to a light yellow (pH 4–4.5). Each mixture was then heated to 90 °C, allowed to cool, and filtered to remove the precipitate containing ferrous ions. The clear filtrate was reheated to 90 °C, and 10 mL of 5% calcium chloride ($CaCl_2$) solution was added slowly with continuous stirring. After heating, the solution was cooled and left to stand overnight at 25 °C. The mixture was centrifuged at 2500 rpm for 5 minutes, the supernatant was removed, and the remaining precipitate was fully dissolved in 10 mL of 20% (v/v) sulphuric acid (H_2SO_4) (Harborne, 1998).

Permanganate titration

The total filtrate obtained from digesting 2 g of flour was diluted to 300 mL with distilled water. A 125 mL portion of this filtrate was transferred into a conical flask and heated almost to boiling. The hot solution was then titrated with 0.05 M standardized potassium permanganate ($KMnO_4$) solution until a faint pink colour persisted for 30 seconds, marking the endpoint (Harborne, 1998).

Calculation:

Oxalate (mg/100g) = $\underline{T \times (V_{me})(Df) \times 10^5}$

$(ME) \times Mf$

Where:

T = Titre of $KMn0_4$ (ml)

Vme = Volume-mass equivalent (i.e. 1ml of 0.05M $KMn0_4$ = 0.00225g anhydrous oxalic acid).

Df = Dilution factor (Vt/A) =2.4 (where Vt is the total volume of titrate (300ml) and A is the aliquot used (125ml))

ME = Molar equivalent of $KMn0_4$ in oxalate ($KMn0_4$ redox reaction)

Mf = Mass of sample used (g)   (Harborne, 1998).

B. Alkaloids Determination

Method: Gravimetric (Harborne, 1998; Obadoni and Ochuko, 2002)

Principle: Alkaloids are basic nitrogen-containing compounds that are soluble in acidic solutions such as dilute acetic acid. When the acidic extract is made alkaline with ammonium hydroxide, the alkaloids precipitate out. The precipitate is then collected, dried, and weighed, allowing for the gravimetric determination of alkaloid content in the sample.

Procedure:

A 5 g portion of the sample was placed into a 250 mL beaker, and 200 mL of 20% acetic acid in ethanol was added. The beaker was covered, and the mixture was left to stand for 4 hours at 25 °C. The mixture was then filtered through Whatman No. 42 filter paper, and the filtrate was collected. The filtrate was concentrated on a water bath (Memmert) until its volume was reduced to one-quarter of the original. Concentrated ammonium hydroxide was added dropwise to the concentrated extract until complete precipitation occurred. The mixture was allowed to stand to enable the precipitate to settle. The solution was filtered using pre-weighed filter paper to collect the precipitate, which was then washed with 1% dilute ammonium hydroxide (NH_4OH) solution. The filter paper containing the residue was dried in an oven (Precision Electrothermal Model BNP 9052, England) at 80 °C until a constant weight was reached. The final weight of the dried residue represented the alkaloid content, which was calculated and expressed as a percentage of the original sample weight (Harborne, 1998; Obadoni and Ochuko, 2002).

Calculation:

$$\%\text{weight of alkaloid} = \frac{\text{weight of filter paper with residue} - \text{weight of filter paper}}{\text{Weight of sample analyzed}} \times 100$$

(Harborne, 1998; Obadoni and Ochuko, 2002)

C. Flavonoids Determination

Method: Gravimetric (Boham and Kocipai, 1994)

Principle: Flavonoids, a class of polyphenolic compounds, can be extracted from plant materials using aqueous methanol. After extraction, the solvent is concentrated, and the residue is dried to a constant weight. The flavonoid content is then calculated by gravimetric analysis and expressed as a percentage of the original sample (Boham and Kocipai, 1994).

Procedure:

A 10 g portion of the plant sample was placed in a clean container and repeatedly extracted with 100 mL of 80% aqueous methanol at room temperature. The mixture was filtered through Whatman No. 42 filter paper (125 mm) to remove any solid residues. The resulting filtrate was transferred to a crucible and evaporated to dryness on a water bath. The dried extract was weighed, and heating and weighing were repeated until a constant weight was obtained, enabling the calculation of the flavonoid content (Boham and Kocipai, 1994).

Calculation:

$$\%\text{flavonoids} = \frac{\underline{(\text{weight of crucible + residue}) - (\text{weight of crucible})}}{\text{Weight of sample analyzed}} \times 100$$

(Boham and Kocipai, 1994)

D. Determination of Saponin

Method: Gravimetric (Obadoni and Ochuko, 2002)

Principle: Saponins are glycosidic compounds soluble in aqueous alcohol. They can be extracted from plant material using aqueous ethanol, and following acid-base treatment, they precipitate from the solution. The precipitate is then collected, dried, and weighed. The saponin content is determined gravimetrically by expressing the weight of the dried residue as a percentage of the original sample (Obadoni and Ochuko, 2002).

Procedure:

A 5 g portion of the sample was accurately weighed into a clean container and mixed with 20% acetic acid in ethanol. The mixture was placed in a water bath at 50°C and left to stand for 24 hours. After this period, the mixture was filtered to obtain the extract. The filtrate was concentrated over a water bath until its volume was reduced to one-quarter of the original. Concentrated ammonium hydroxide (NH_4OH) was added dropwise to the concentrated extract until complete precipitation occurred. The mixture was allowed to stand so the precipitate could separate fully, after which it was collected by filtration. The precipitate was dried and weighed carefully, and the saponin content was calculated as a percentage of the original sample weight (Obadoni and Ochuko, 2002).

Calculation:

$$\%\text{saponin content} = \frac{\underline{(\text{weight of filter paper + residue}) - (\text{weight of filter paper})}}{\text{Weight of sample analyzed}} \times 100$$

(Obadoni and Ochuko, 2002).

E. Cardiac Glycosides Determination

Method: Wang and Filled (as reported by Odeyemi *et al.,* 2023)

Principle: Cardiac glycosides are steroidal compounds that form insoluble precipitates when treated with specific reagents under alkaline conditions. The precipitate can be collected, dried, and weighed to determine the glycoside content in plant samples.

Procedure:

A 1 mL portion of the plant extract was transferred into a clean test tube, and 1 mL of 2% 3,5-dinitrosalicylic acid (DNS) solution prepared in methanol was added. This was followed by the addition of 1 mL of 5% aqueous sodium hydroxide (NaOH). The mixture was boiled for 2 minutes until a brick-red precipitate formed. The mixture was then filtered through pre-weighed filter paper, which was subsequently dried in an oven at 50°C until completely dry. The dried filter paper was weighed, and the weight of the residue was determined by calculating the difference between the final and initial weights of the filter paper (as reported by Odeyemi *et al.,* 2023).

Calculation:

$$\%\text{cardiac glycoside} = \frac{(\text{weight of filter paper + residue}) - (\text{weight of filter paper})}{\text{Weight of sample analyzed}} \times 100$$

(as reported by Odeyemi *et al.,* 2023)

F. Tannin Determination

Method: Follins-Dennis Titration (Pearson, 1974)

Principle: Tannins are phenolic compounds that form coloured complexes or precipitates when they react with alkali and specific reagents. The tannin content in a plant sample can be determined by titration with a standard base and expressed as a percentage of the sample's weight (Pearson, 1974).

Procedure:

A 20 g portion of the crushed sample was placed into a conical flask, and 100 mL of petroleum ether was added. The flask was covered and left to stand for 24 hours. The mixture was filtered, and the residue was allowed to sit for 15 minutes to ensure complete evaporation of the petroleum ether. The residue was then re-extracted by soaking in 100 mL of 10% acetic acid in ethanol for 4 hours, followed by filtration to collect the extract. To the filtrate, 25 mL of concentrated ammonium hydroxide (NH_4OH) was added to precipitate the alkaloids, and the solution was gently heated on an electric hot plate to remove any excess NH_4OH. The remaining solution volume was measured, yielding approximately 33 mL. A 5 mL portion of this concentrated solution was transferred into a titration flask, and 20 mL of ethanol was added. The solution was titrated with 0.1 M sodium hydroxide (NaOH) using phenolphthalein as an indicator until a pink colour persisted, indicating the end point. The tannin content was calculated using the formula $C_1V_1 = C_2V_2$ and expressed as a percentage of the original sample weight (Pearson, 1974).

Calculation:

Data

C_1 = conc. of Tannic Acid

C_2 = conc. Of Base

V_1 = Volume of Tannic acid

V_2= Volume of Base

Therefore $C_1 = \frac{C_2V_2}{V_1}$

% of tannic acid content = $\underline{C_1 \text{ x } 100}$

Weight of sample analyzed (Pearson, 1974)

G. Phytate Determination

Method: Ferric Chloride Titration (Young and Greaves, 1940; Lucas and Markakis, 1975)

Principle: The determination of phytate relies on the precipitation of phytate ions by ferric ions. During titration, ferric chloride ($FeCl_3$) reacts with the phytate in the sample until all phytate is bound. Any excess ferric ions then react with ammonium thiocyanate to form a brownish-yellow ferric-thiocyanate complex, which indicates the endpoint. The volume of ferric chloride used before reaching this endpoint corresponds to the phytate content of the sample (Young and Greaves, 1940; Lucas and Markakis, 1975).

Procedure:

A 0.2 g portion of each differently processed corn sample was weighed into separate 250 mL conical flasks. Each sample was treated with 100 mL of 2% HCl and left to soak for 3 hours with occasional shaking to aid extraction. The mixtures were then filtered to obtain clear filtrates. A 50 mL portion of each filtrate was transferred into a 250 mL beaker and diluted with 100 mL of distilled water. 10 mL of 0.3% NH_4SCN solution was added as an indicator, and the solutions were titrated with standard $FeCl_3$ solution containing 0.00195 g of iron per mL until a brownish-yellow colour appeared, indicating the formation of the ferric-thiocyanate complex. The volume of $FeCl_3$ used was recorded, and the phytate content of each sample was calculated (Young and Greaves, 1940; Lucas and Markakis, 1975).

Calculation:

$$\text{Phytic acid} = \frac{\text{Titre value x } 0.00195 \text{ x } 1.19}{\text{Weight of sample}} \text{ x } 100$$

(Young and Greaves, 1940; Lucas and Markakis, 1975)

H. Phenol Determination

Method: Spectrophotometric (AOAC, 1995)

Principle: The spectrophotometric determination of phenols relies on their reaction with ammonium hydroxide and amyl alcohol under alkaline conditions to form a coloured complex. The intensity of the resulting colour is directly proportional to the phenol concentration in the sample and is measured at 505 nm using a spectrophotometer (AOAC, 1995).

Procedure:

The plant sample was boiled with 50 mL of diethyl ether ($(CH_3CH_2)_2O$) for 15 minutes. A 5 mL portion of the boiled sample was transferred into a 50 mL volumetric flask, and 10 mL of distilled water was added and mixed thoroughly. To this mixture, 2 mL of ammonium hydroxide (NH_4OH) solution and 5 mL of concentrated amyl alcohol [$CH_3(CH_2)_3CH_2OH$] were added. The volume was then made up to the 50 mL mark with distilled water and mixed well. The solution was allowed to stand for 30 minutes to develop colour, and the absorbance was measured at 505 nm using a spectrophotometer to determine the phenol content (AOAC, 1995).

I. Hemagglutinin Determination

Method: Spectrophotometric Assay (AOAC, 1995)

Principle: The determination of hemagglutinin by spectrophotometric assay is based on the ability of hemagglutinin proteins (lectins) to agglutinate red blood cells. When extracted in saline solution and clarified by centrifugation and filtration, the crude agglutination extract can be quantified spectrophotometrically. The absorbance at 420 nm is directly related to the concentration of hemagglutinin present in the sample

Procedure:

A 2 g portion of each sample was weighed and placed into separate containers, and 20 mL of 0.9% sodium chloride (NaCl) solution was added to each. The suspensions were shaken vigorously for 1 minute to ensure thorough mixing and then left to stand for 1 hour to allow the supernatant to settle. The samples were centrifuged at 2000 rpm for 10 minutes, and the resulting suspensions were filtered to obtain clear solutions. The collected supernatants served as the crude agglutination extracts, and their absorbance was measured at 420 nm using a spectrophotometer (AOAC, 1995).

J. Cyanogenic Glycoside

Method: Acid Titration (AOAC, 1995)

Principle: The determination of cyanogenic glycosides by acid titration is based on the hydrolysis of cyanogenic compounds to release hydrogen cyanide (HCN). During distillation, HCN is liberated and absorbed in a known volume of standard silver nitrate ($AgNO_3$) solution. The unreacted $AgNO_3$ is then back-titrated with standard potassium cyanide (KCN), using ferric alum as an indicator. The amount of $AgNO_3$ neutralized by the HCN corresponds to the cyanogenic glycoside content, which is calculated stoichiometrically.

Procedure:

10 g of the ground sample, passed through a No. 20 sieve, were weighed into an 800 mL Kjeldahl flask. 100 mL of distilled water were added, and the mixture was macerated at room temperature for 2 hours. After maceration, an additional 100 mL of distilled water was added, and the mixture was subjected to steam distillation. The distillate was collected in 20 mL of

0.02 N $AgNO_3$ solution acidified with 1 mL of HNO_3, ensuring that the condenser tip was submerged below the liquid surface in the receiver. Distillation was continued until 150 mL of distillate had been collected. The distillate was filtered through a Gooch crucible, and both the receiver and crucible were rinsed with a small amount of distilled water to ensure complete transfer. The excess $AgNO_3$ in the combined filtrate and washings was titrated with 0.02 N KCN using ferric alum as an indicator. The HCN content was calculated using the factor: 1 mL of 0.02 N $AgNO_3$ = 0.54 mg HCN (AOAC, 1995).

K. Determination of Anthocyanins

Method: Gravimetric (Harborne, 1973)

Principle: The acid-hydrolysed sample was filtered and treated with ethyl acetate to extract anthocyanins. Amyl alcohol was then added to further extract the anthocyanins, and after drying, the anthocyanin content was determined gravimetrically as a percentage of the original sample weight (Harborne, 1973).

Procedure:

A 5.0g portion of the powdered sample was weighed and boiled in 100 mL of 2 M HCl for 30 minutes. The hydrolysate was filtered using Whatman filter paper to obtain a clear filtrate. The filtrate was transferred into a separating funnel, and an equal volume of ethyl acetate was added. The mixture was shaken thoroughly and allowed to separate into two layers. The ethyl acetate layer was collected, and the aqueous layer was discarded. The ethyl acetate extract was dried over a steam bath until completely dry. The dried extract was then treated with 10 mL of concentrated amyl alcohol to extract the anthocyanin. The mixture was filtered to obtain the alcohol extract, which was dried completely. Finally, the dried anthocyanin extract was weighed, and the percentage content was calculated based on the original sample weight (Harborne, 1973).

Calculation:

$$g\% = \frac{\text{Weight of Anthocyanin}}{\text{Weight of original sample}} \times 100$$

(Harborne, 1973)

L. Determination of Steroid content

Method: Spectrophotometric (Liebermann–Burchard reaction) (AOAC, 1995)

Principle: The steroid content was determined using the Liebermann–Burchard reaction, which involves the reaction of sterols with acetic anhydride in the presence of concentrated sulphuric acid to produce a characteristic green to blue-green colour. The intensity of the colour is directly proportional to the steroid concentration and is measured spectrophotometrically (AOAC, 1995).

Procedure:

A 1.0 g portion of the powdered sample was weighed and mixed with 100 mL of distilled water in a conical flask. The mixture was filtered to obtain a clear filtrate, which was then eluted with 0.1 N ammonium hydroxide solution. A 2 mL portion of the eluent was pipetted into a test tube and mixed with 2 mL of chloroform. To this mixture, 3 mL of ice-cold acetic anhydride was added and gently mixed. A standard sterol solution (200 mg/dL) was prepared and treated in the same manner as the test sample to serve as the blank. The absorbance of both the standard and the test sample was measured at 420 nm using a spectrophotometer, with the instrument zeroed using the blank (AOAC, 1995).

Calculation:

$$\text{Steroid (mg/100ml)} = \frac{\text{Absorbance of test}}{\text{Absorbance of std.}} \times \text{Conc of std}$$

(AOAC, 1995)

M. Phenolic Profile

Extraction method: Soxhlet Extraction (AOAC, 1995)

Principle: The Soxhlet extraction method is based on the continuous extraction of soluble phenolic compounds from plant material using a suitable solvent (ethanol). Heat applied to the solvent allows repeated washing of the sample, ensuring exhaustive extraction. The extract is then concentrated by solvent removal, leaving behind the phenolic compounds for analysis.

Procedure:

500 mL clean boiling flasks were dried in an oven at 105–110°C for approximately 30 minutes and then transferred into a desiccator to cool. 10 g of the sample was weighed and placed into a Soxhlet thimble, which was lightly plugged with cotton wool to aid filtration. The boiling flask was filled with approximately 300 mL of ethanol, and the Soxhlet apparatus was assembled. The extraction was carried out by refluxing for about 4 hours at 60°C. After reflux, the thimble was carefully removed, and the extract was poured into a volumetric flask to cool. The contents of the flask were then transferred to a rotary evaporator to remove the ethanol and obtain the concentrated extract (AOAC, 1995).

Extraction of phytochemicals

0.2 g of the extract was weighed and placed into a test tube. To this, 15 mL of ethanol and 10 mL of 50% m/v potassium hydroxide (KOH) solution were added. The test tube was heated in a water bath at 60°C for 3 hours to allow the reaction to proceed. After the reaction, the mixture was transferred into a separatory funnel. The test tube was rinsed sequentially with 20 mL of ethanol, 10 mL of cold water, 10 mL of hot water, and 3 mL of hexane, and each wash was added to the funnel. All washes were combined and the extracts were washed three times with 10 mL of 10% v/v aqueous ethanol solution. The ethanol solvent was then completely evaporated to obtain the residue. The dried residue was solubilised in 1000 µL of pyridine, and 200 µL of this solution was transferred into a vial for analysis (AOAC, 1995).

Quantification by GC-FID

The phytochemical analysis was carried out using an Agilent 6890 Gas Chromatography (GC) system fitted with a flame ionisation detector (FID). Separation was achieved using a RESTEK 15-meter MXT-1 column (15 m × 250 µm × 0.15 µm). The injector was maintained at 280°C, and 2 µL of sample was injected splitless, with a linear velocity of 30 cm/s. Helium (5.0 Pa·s) was used as the carrier gas at a flow rate of 40 mL/min. The oven temperature was initially set at 200°C, ramped to 330°C at 30°C/min, and held for 5 minutes. The detector temperature was 320°C. Phytochemical quantification was performed by calculating the ratio of the area of each compound to that of the internal standard, and concentrations were expressed in µg/g of sample. (See Appendix II and III)

N. Cellulose Content

Method: Gravimetric determination (Crampton and Mayrand, 1978)

Principle: The gravimetric method for cellulose determination is based on the selective removal of non-cellulosic materials (such as hemicellulose, lignin, proteins, and other soluble components) through acid digestion and solvent washing. The residue obtained after this treatment consists primarily of cellulose. By carefully weighing the crucible before digestion, after residue collection, and after ashing to remove all organic matter, the cellulose fraction was calculated by the difference in mass (Crampton and Mayrand, 1978).

Procedure:

0.3 g of the sample was weighed into a 50 mL glass centrifuge tube containing 50 mL of water. The mixture was centrifuged at 1500 rpm for 10 minutes, and the supernatant was decanted. The pellet was resuspended in 12.5 mL of glacial acetic acid and 2.5 mL of concentrated nitric acid. The mixture was digested in a boiling water bath for 20 minutes. After digestion, the supernatant was collected and transferred into a pre-weighed Gooch crucible (W1). The residue in the crucible was washed sequentially with hot alcohol, 10 mL of 90% benzene, and 60% ether solution. The crucible containing the residue was thoroughly dried and weighed (W3). Finally, the dried residue was ashed in a furnace to remove all organic matter, and the crucible was reweighed (W2) (Crampton and Mayrand, 1978).

Calculation:

$$\text{Cellulose content} = \frac{W3 - W2}{W1} \times 100$$

Where:

W3 = weight of dried sample

W2 = weight of ash content

W1 = weight of sample (Crampton and Mayrand, 1978)

O. Lignin content

Method: Acid-Insoluble Lignin (Klason Method, Gravimetric) (Sluiter *et al.,* 2012)

Principle: The Klason method involves hydrolysing the carbohydrates in the plant material using concentrated sulphuric acid, leaving an acid-insoluble residue. This residue mainly consists of lignin and some acid-insoluble ash. The lignin content is determined gravimetrically by weighing the residue after hydrolysis and subtracting the weight of the ash obtained after ashing (Sluiter *et al.,* 2012).

Procedure:

0.3 ± 0.01 g of the prepared sample was weighed to the nearest 0.1 mg and placed into a 16 × 100 mm test tube; this weight was recorded as W1. Each sample was run in duplicate at a minimum. Samples for total solids determination (LAP-001) were prepared and weighed simultaneously, and the average total solids value was recorded as Tfinal. To the test tube, 3.00 ± 0.01 mL (4.92 ± 0.01 g) of 72% H_2SO_4 was added and mixed with a glass stirring rod for 1 minute, or until the sample was fully wetted. The test tube was then placed in a water bath maintained at 30 ± 10 °C and hydrolysed for 2 hours. After hydrolysis, the test tube was cooled in a desiccator, and the weight W2 (weight of the test tube plus acid-insoluble lignin and acid-insoluble ash) was recorded to the nearest 0.1 mg (Sluiter *et al.,* 2012).

Calculation:

$$\%\ \text{acid} - \text{insoluble residue} = \frac{W_2 - W_3\ \text{X}\ \ 100}{W_1\ \text{x}\ \frac{\%\ \text{Tfinal}}{100}}$$

Where:

W_1 = initial weight of extracted sample

W_2= weight of crucible, acid- insoluble residue, acid – insoluble ash

W_3 = weight of crucible and acid- insoluble ash

% Tfinal = % total solids of the extracted sample determined at 105^0c as described by the standard method for the determination of total solids in biomass (Sluiter *et al.,* 2012).

P. **Hemicellulose Determination**

Method: Gravimetric (Crampton and Mayrand, 1978)

Principle: The gravimetric determination of hemicellulose involves hydrolysing the sample with concentrated sulphuric acid under reflux, which solubilises the hemicellulose into the hydrolysate. The remaining insoluble residue is collected, dried, and ashed to remove all carbonaceous matter. The hemicellulose content is then calculated from the difference in weight before and after ashing, expressed relative to the total solids of the original sample (Crampton and Mayrand, 1978).

Procedure:

1 g of the sample was weighed into a 20 × 150 mm test tube, and this weight was recorded as W1. Fifteen mL of 72% sulfuric acid (H_2SO_4) was added and stirred for 1 minute to ensure thorough wetting of the sample. The mixture was then transferred into a 1000 mL Erlenmeyer flask and diluted with deionised water to a total volume of 500 mL. The flask was placed on a heating manifold with a reflux condenser attached, and the solution was gently boiled and refluxed for 4 hours. After reflux, the condenser was rinsed with a small amount of deionised water, and the hydrolysed solution was transferred into crucibles, with the weight of the collected filtrate recorded. The crucibles with the residue were dried in an oven at 105 ± 3 °C for 2 hours, cooled in a desiccator, and weighed as W2. Subsequently, the crucibles were placed in a muffle furnace at 575 °C for a minimum of 3 hours to remove all carbonaceous material, cooled in a desiccator, and the final weight was recorded as W3. The hemicellulose content was then calculated based on the weight differences before and after ashing relative to the original sample (Crampton and Mayrand, 1978).

Note:

Total solids (%) = 100 – Moisture content (%)

Calculations

$$\% \text{ Hemicellulose} = \frac{W_2 - W_3}{W1 - \frac{\text{total solid}}{100}} \times 100$$

(Crampton and Mayrand, 1978)

Q. Total Carotenoids

Method: Spectrophotometric (Zakaria *et al.,* 1979).

Principle: The determination of total carotenoids involves extracting them into petroleum ether following saponification of the sample with alcoholic potassium hydroxide. Saponification eliminates chlorophylls and lipids that may interfere with the measurement. The carotenoids present in the petroleum ether layer are then quantified spectrophotometrically at 450 nm, where they show maximum absorbance. The measured absorbance is used to calculate the carotenoid concentration in the sample (Zakaria *et al.,* 1979).

Reagents:

1. Petroleum ether (40ºC - 60ºC)

2. Anhydrous sodium sulphate

3. Calcium carbonate

4. Alcoholic potassium hydroxide (12%)

Procedure:

The entire experiment was conducted in the dark to prevent photolysis of carotenoids. A 0.5 g portion of the homogenized sample was weighed and transferred into a suitable container, after which 2.5 mL of 12% alcoholic potassium hydroxide (KOH) was added. The mixture was saponified in a water bath at 60°C for 30 minutes. Following saponification, the sample was transferred to a separating funnel containing 10–15 mL of petroleum ether and mixed thoroughly. The mixture was allowed to separate into two layers: an upper petroleum ether layer containing carotenoids and a lower aqueous layer. The aqueous layer was removed to another separating funnel, and the upper petroleum ether layer was collected. This extraction was repeated until the aqueous layer became colourless. A small amount of anhydrous sodium sulphate was added to the final petroleum ether extract to remove residual moisture, and the final volume of the extract was recorded. The absorbance of the yellow petroleum ether solution was measured at 450 nm using a spectrophotometer (Genesys 10-S, USA), with petroleum ether serving as the blank. The concentration of total carotenoids was then calculated using the appropriate formula (Zakaria *et al.,* 1979).

Calculation:

$$\text{Amount of Total Carotenoids (mg/g)} = \frac{\text{A450} \times \text{Volume of the sample} \times 100 \times 4}{\text{Weight of the sample}}$$

Where:

A_{450} = Absorbance at 450 nm (for carotenoids)

Volume of sample = Final volume of the petroleum ether extract (mL)

Weight of sample = Weight of the original sample used (g)

(Zakaria *et al.,* 1979).

R. Hydrogen Cyanide

Method: Alkaline titration (AOAC, 1995).

Principle: The alkaline titration method for determining hydrogen cyanide (HCN) involves the release of HCN from cyanogenic glycosides under acidic conditions, followed by distillation. The distillate containing free HCN is treated with ammonia and potassium iodide (KI). During titration with standard silver nitrate ($AgNO_3$), the liberated cyanide ions react to form an insoluble silver cyanide precipitate. The endpoint is indicated by the appearance of a faint but persistent turbidity. The volume of $AgNO_3$ used is directly proportional to the HCN content in the sample (AOAC, 1995).

Procedure:

A 10 g portion of the ground sample was weighed and placed into a suitable container. To this, 200 cm³ of distilled water and 10 cm³ of orthophosphoric acid were added, and the mixture was left overnight to allow complete release of bound hydrocyanic acid (HCN). The mixture was then distilled, and 150 cm³ of the distillate was collected. A 20 cm³ portion of the distillate was transferred into a conical flask containing 40 cm³ of distilled water. 8 cm³ of 6 mol/dm³ aqueous ammonia was added, followed by 2 cm³ of 5% potassium iodide (KI) solution. The solution was titrated with 0.02 mol/dm³ silver nitrate ($AgNO_3$) until a faint but permanent turbidity appeared, indicating the end point (AOAC, 1995).

3.7 Statistical Analysis

All measurements were carried out in triplicate and expressed as mean ± standard deviation (SD). This approach was applied to proximate composition (Table 4.1), quantitative phytochemical composition (Table 4.3), and phenolic profiles from both ethanol and diethyl ether extracts (Table 4.4). Using mean ± SD provided a clear picture of central tendency and variability, with the low SD values indicating good precision and minimal experimental variation. No further inferential tests were performed, as the focus was on descriptive profiling and comparison of constituent levels between solvent extracts.

CHAPTER FOUR

RESULTS

Table 4.1 presents the proximate profile of the *C. citratus,* showing the percentage composition of nutritional parameters expressed as mean±SD. The moisture content was accounted to be 16.72 ± 0.1475%, the ash content was 2.117 ± 0.175%. The fats and oil content were determined to be 1.552 ± 0.2791%, while the fibre content was 3.225 ± 0.04158%. The protein content was found to be 6.133 ± 0.5252% and the carbohydrate was measured at 71.82 ± 1.357%.

Table 4.1: Proximate analysis of *C. citratus,* showing the proportions of nutritional parameters

Parameters	Content
Moisture (%)	16.72±0.1475
Ash (%)	2.117±0.175
Fat and Oil (%)	1.552±0.2791
Fibre (%)	3.225±0.04158
Protein (%)	6.133±0.5252
Carbohydrate (%)	71.82±1.357

Table presented in mean±SD

Table 4.2 presents the qualitative phytochemical screening of *C. citratus* extracts (ethanol and diethyl ether), with observations recorded as colour reactions, precipitate or emulsion formation. Both ethanol and diethyl ether extracts tested positive for flavonoids, tannins, saponins, terpenoids, and steroids, as indicated by characteristic color changes and reactions (++). Contrarily, resins, cardiac glycosides, and alkaloids were not present in either extract shown by the negative reactions (-).

Table 4.2: Qualitative phytochemical screening of *C. citratus* in ethanol and diethyl extracts

Test	Observation	Inference
Flavonoid		
Ethanol extract	Greenish brown coloration	(++)
Diethyl ether extract	Greenish brown coloration	(++)
Tannins		
Ethanol extract	Reddish coloration observed	(++)
Diethyl ether extract	Reddish coloration observed	(++)
Resins		
Ethanol extract	Precipitate formed	(-)
Diethyl ether extract	Precipitate formed	(-)
Saponins		
Ethanol extract	Emulsion observed	(++)
Diethyl ether extract	Emulsion observed	(++)
Terpenoids		
Ethanol extract	Reddish brown observed	(++)
Diethyl ether extract	Reddish brown observed	(++)
Steroids		
Ethanol extract	Colour change at interface (dark red)	(++)
Diethyl ether extract	Colour change at interface (dark red)	(++)
Cardiac glycosides		

Ethanol extract	Brick red observed	(-)
Diethyl ether extract	Brick red observed	(-)
Alkaloids		
Ethanol extract	Brick red	(-)
Diethyl ether extract	Brick red	(-)

Table 4.3 presents the phytochemical composition of *C. citratus*. Haemogluttin was present at a concentration of 0.9677 ± 0.001528 ppm, while cyanogenic glycosides were 4.698 ± 0.054 ppm. Steroids were detected at 8.642 ± 0.00755 ppm, and tannins were 2.097 ± 0.0515%. Oxalate and phytate concentrations were measured at 0.02533 ± 0.001155 ppm and 4.022 ± 0.07451%, respectively. Saponins accounted for 3.844 ± 0.002%, and alkaloids were measured at 5.605 ± 4.169%. Cardiac glycosides, cellulose, and lignin were present at 3.954 ± 0.07308%, 54.99 ± 0.34%, and 11.64 ± 4.423%, respectively. Terpenoids and carotenoids were recorded at 0.4947 ± 0.01498 ppm and 5.587 ± 0.1609 mg/ml, respectively. All results are expressed as mean ± standard deviation (SD).

Table 4.3: Quantitative phytochemical composition of *C. citratus*

Phytochemical parameters	Concentration
Haemogluttin (conc. ppm)	0.9677±0.001528
Cyanogenic glycoside (conc. ppm)	4.698±0.054
Steroids (conc. ppm)	8.642±0.00755
Tannin (conc. %)	2.097±0.0515
Oxalate (conc. ppm)	0.02533±0.001155
Phytate (conc. %)	4.022±0.07451
Saponin (%)	3.844±0.002
Alkaloids (%)	5.605±4.169
Cardiac Glycoside (%)	3.954±0.07308
Cellulose (%)	54.99±0.34
Lignin (%)	11.64±4.423
Terpenoid (ppm)	0.4947±0.01498
Carotenoid (conc. mg/ml)	5.587±0.1609

Table presented in mean±SD

Table 4.4 presents the phenolic profile of *C. citratus* of ethanol and diethyl ether extracts, expressed as concentrations in parts per million(ppm). Catechol measured 16.890 ppm in ethanol and 21.776 ppm in the diethyl ether extracts. Resorcinol was recorded at 5.414 ppm in ethanol extracts and 13.932 in the diethyl ether extracts, while quinol showed concentrations of 25.975 ppm and 3.261 ppm in ethanol and diethyl ether extracts respectively. Pyrogallol was 1.110 ppm in ethanol and 5.458 in diethyl ether, while hydroxyquinol was 33.190 ppm and 54.471 ppm respectively. Phloroglucinol was recorded at 3.358 ppm and 6.246 ppm in ethanol and diethyl ether respectively. p-cresol was found at concentrations of 1.458 ppm and 6.248 ppm in ethanol and diethyl ether respectively, whereas 3,5-hydroxytoluene was 0.977 ppm and 17.519 ppm. Phenetol was recorded at 0.593 ppm in ethanol and 19.100 ppm in diethyl ether, whereas alpha-naphtol was 0.533 ppm and

17.964 ppm respectively. Anisole was measured 0.731 ppm and 5.318 ppm in ethanol and diethyl ether respectively, picric acid had concentrations of 0.522 ppm and 4.607 ppm. 2-hydroxy phenol was 0.561 ppm in ethanonl and 1.218 pp in dierthyl ether extract. Flavonones was measured at 0.970 ppm and 0.644 ppm, xanthrone at 0.496 ppm and 1.240 ppm, (+)-pinocresol and 1.990 ppm and 1.398 ppm in ethanol and diethyl ether respectively. Lignans had concentrations of 0.790 ppm in ethanol and 0.928 ppm in diethyl ether, while assculetin was 1.020 ppm and 0.842 ppm respectively. Cinnamic acid measured 21.163 ppm and 0.463 ppm, resveratrol at 2.805 ppm and 3.178 ppm in ethanol and diethyl ether respectively. The percentage yield of extraction was 1.560% in the ethanol exrtract and 1.720% in the diethyl ether extract. The total concentration of phenolic compounds was 120.545 ppm in ethanol and 185.811 ppm in diethyl ether.

Table 4.4: Phenolic profile of *C. citratus* in ethanol and diethyl ether extracts

Phenol (conc ppm.)	Ethanol Extract (Concentration of phytochemicals)	Diethyl ether Extract (Concentration of phytochemicals)
Catechol	16.890	21.776
Resorcinol	5.414	13.932
Quinol	25.975	3.261
Pyrpgallol	1.110	5.458
Hydroxyquinol	33.190	54.471
Phloroglucinol	3.358	6.246
p-cresol	1.458	6.248
3,5-Hydroxytoluene	0.977	17.519
Phenetol	0.593	19.100
Alpha-Naphthol	0.533	17.964
Anisole	0.731	5.318
Picric acid	0.522	4.607
2-Hydroxy phenol	0.561	1.218
Flavonones	0.970	0.644

Xanthrone	0.496	1.240
(+)-pinoresinol	1.990	1.398
Lignans	0.790	0.928
Assculetin	1.020	0.842
Cinnamic acid	21.163	0.463
Resveratrol	2.805	3.178
% Yield	1.560	1.720
Total Concentration of the Phytochemicals	120.545	185.811

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

The moisture content of the sample (16.72 ± 0.1475%) lies within the range commonly reported for dried leafy materials and is higher than values reported for some studied wild leaves. For instance, *Senna siamea* leaves were reported at ~12% moisture in a recent proximate study, and *Heteromorpha arborescens* leaves were reported at ~8.4% moisture (Abifarin and Afolayan,2020; Ogunniran *et al.,* 2024). The relatively higher moisture in the present sample may reflect differences in drying protocol, residual water content at the time of analysis, or sample handling prior to proximate determination. Ash value (2.117 ± 0.175%) is lower than ash values reported for several nutrient-rich leafy species. For example, *H. arborescens* was reported with ash ~8.5%, some Moringa studies report ash values in the single-figures to higher single digits depending on genotype and soil and *C. citratus* from a different locale with a higher ash content of ~12% (Abifarin *et al.,* 2020; Yusuf *et al.,* 2023; Masitlha *et al.,* 2024), suggesting either lower mineral accumulation in the species sampled or environmental/soil differences at the collection site; methodological differences in ashing also affect absolute ash values and should be acknowledged when comparing studies (ashing temperature, completeness of combustion) also affect the measured value. Fat and Oil content (1.552 ± 0.2791%) is within the low range typically reported for leafy tissues and aligns with many herbaceous species that allocate little to storage lipids (Ogunniran *et al.,* 2024), literature on leafy medicinal species shows wide inter-species variability in ether-extractable fractions, but leaf tissues commonly present lipid values below ~3–5%. Crude fibre (3.225 ± 0.042%) is lower than values reported for several wild leafy vegetables such as *H. arborescens* (≈21.5%) (Abifarin and Afolayan, 2020). The fibre content is greater than *C. citratus* from a different locale with ~1% (Yusuf *et al.,* 2023). Lower fibre may indicate primarily younger leaf tissue in the sample or differences in the crude-fibre analytical procedure; because fibre is sensitive to plant maturity and which plant parts are included, differences in harvest stage or sampling composition are plausible explanations. Protein content (6.133 ± 0.525%) falls in a modest range relative to other leafy medicinal plants, it is lower than protein-rich species such as reported Moringa leaf figures (often substantially higher) and some wild leafy vegetables, yet comparable to many common edible herbs and underutilized greens (Peñalver *et al.,* 2022). Soil nitrogen availability, species genetics, and harvest timing can all protein content and likely account for part of the observed variation. Carbohydrate content (71.82 ± 1.357%) was the dominant proximate fraction and falls at the higher end of the range reported for medicinal leaves (40–80%). *S. siamea* has been reported around 70% carbohydrate, whereas *H. arborescens* showed a lower value (~41%) (Abifarin and Afolayan, 2020; Ogunniran *et al.,* 2024). The high carbohydrate proportion in this study likely results from the relatively lower contributions of protein, fibre, fat, and ash, since carbohydrate is often calculated by difference. Overall, the proximate profile of *C. citratus* indicates a carbohydrate rich, moderate protein, low lipid and fat composition compared to nutrient-dense species like *Moringa* or *H. arborescens.* Such variations exemplify the influence of specie differences, environmental conditions and analytical techniques.

Haemagglutinin/lectin-type proteins are often reported in plants as trace or low-level constituents and, in many studies, are described qualitatively or as "present/absent" rather than reported as consistent ppm standards. The measured haemagglutinin level (0.9677 ± 0.001528ppm) represents a measurable but low concentration that is consistent with reports that lectin-type proteins in non-legume leaves typically occur at low ppm levels (Tazi *et al.,* 2024b). Variation in haemagglutinin content between reports commonly reflects species differences, leaf age and extraction method (protein vs. crude extract) because lectin recovery depends strongly on buffer, salt and extraction conditions (Tazi *et al.,* 2024b). The cyanogenic glycoside level (4.698 ± 0.054ppm) is relatively low compared with strongly cyanogenic plants such as cassava, which can contain levels hundreds of times higher (Park *et al.,* 2024). Such a concentration suggests minimal toxicological concern but highlights environmental and genetic factors may influence cyanogenic glycoside accumulation. Factors such as growth conditions, season, and solvent extraction significantly contribute to variability between studies (Park *et al.,* 2024). Steroidal compounds are important secondary metabolites with structural and functional roles in plants. The concentration of 8.642 ± 0.00755 ppm observed here is modest, but comparable to levels found in leafy medicinal herbs (Evtyugin *et al.,* 2023). Steroid levels in plants are influenced by species, solvent polarity during extraction, and whether free sterols or bound glycosides are quantified. The tannin content of 2.097 ± 0.0515% lies within reported ranges for medicinal and leafy plants. For example, tannin levels in *Byttneria herbacea* leaves were reported between 1.8% and 3.5% depending on plant part (Sharma *et al.,* 2021). Similarly, tannins in underutilized legumes often fall between 1–4% (Ojo, 2022). Variations in tannin levels are attributed to plant maturity, tissue sampled, and whether condensed or hydrolysable tannins are measured. Oxalate levels in this study (0.02533 ± 0.001155 ppm) are extremely low compared to typical leafy vegetables, where oxalate values often range from 100–500 ppm or higher on a dry weight basis (Salgado *et al.,* 2023). Such low concentrations reduce dietary risks associated with calcium oxalate crystal formation and kidney stone development. Differences across studies stem from genotype, growing environment, and analytical extraction procedures. The phytate content of 4.022 ± 0.07451% is within the range observed in leafy plants and legumes, which often contain between 1–5% phytates depending on species (Pires *et al.,* 2023). Phytates are recognized as antinutrients due to their mineral-binding capacity, but they also provide antioxidant and anticancer benefits. Differences in phytate content may be linked to phosphorus availability in soils and plant developmental stage. Saponins at 3.844 ± 0.002% demonstrate a considerable concentration within the expected range for medicinal plants, where values typically span 1–5% (Liang *et al.,* 2024). Reported variability in saponin content arises from solvent polarity, genetic makeup, and maturity of plant tissue. The level observed here suggests potential bioactivity, including antimicrobial and cholesterol-lowering effects. The alkaloid content of 5.605 ± 4.169% is relatively high compared with many leafy vegetables, which generally contain <2% alkaloids (Daley and Cordell*,* 2021). This significant concentration highlights the pharmacological potential of the plant but also raises the need for toxicological consideration since alkaloids can be biologically active. High variability between replicates, as indicated by the large standard deviation, may be due to environmental stressors or uneven distribution in tissues. Cardiac glycosides at 3.954 ± 0.07308% are noteworthy, since these compounds are potent bioactives with both therapeutic and toxic potential. Although concentrations in classic cardiac glycoside plants like *Digitalis spp.* are higher, the measurable levels in *C. citratus* warrant attention. Reported variability across species is usually linked to genetic differences, environmental conditions, and solvent selectivity during extraction (Kumavath *et al.,* 2021). The structural components cellulose (54.99 ± 0.34%) and lignin (11.64 ± 4.423%) dominate the phytochemical profile. These values align with reports from fibrous grasses and leafy plants where cellulose typically comprises 40–60% of dry matter and lignin 5–20% (Ghouil *et al.,* 2024). Variation in lignocellulosic content is primarily influenced by plant age, tissue maturity, and extraction methodology. The terpenoid concentration of 0.4947 ± 0.01498 ppm is low in crude extracts but consistent with expectations since terpenoids are primarily concentrated in essential oils distilled from lemongrass leaves (Liu *et al.,* 2022). In crude solvent extracts, terpenoid values are typically reported at low ppm levels, while in essential oils, they dominate as the major constituents. Carotenoids at 5.587 ± 0.1609 mg/ml demonstrate a significant antioxidant fraction. Comparable values have been observed in leafy herbs like Moringa oleifera, which contain high carotenoid pools contributing to vitamin A precursors and oxidative stress protection (Pareek *et al.,* 2023). Differences in carotenoid levels arise from genetic variability, maturity at harvest, and sunlight exposure.

In this study, the phenolic profile of *C. citratus* revealed distinct variations between ethanol and diethyl ether extracts, with a total concentration of 120.545 ppm in the ethanol extract and 185.811 ppm in the diethyl ether extract. Hydroxyquinol was the most abundant compound in both solvents (33.190 ppm in ethanol; 54.471 ppm in diethyl ether), followed by quinol (25.975 ppm) in ethanol and catechol (21.776 ppm) in diethyl ether. These findings suggest that the solvent system strongly influences the recovery of phenolic metabolites, consistent with reports that polarity and solvent type alter the yield and diversity of phenolic compounds in *C. citratus* (De Oliveira Alencar *et al.,* 2022; Wahyuni *et al.,* 2024). The high recovery of hydroxyquinol and catechol indicates the predominance of simple phenolic derivatives in the extracts. Similar phenolic constituents have been detected in lemongrass extracts by HPLC and GC–MS analyses, which identified phenolic acids and flavonoids as major contributors to antioxidant capacity (Lara *et al.,* 2021; De Oliveira Alencar *et al.,* 2022). The variation observed between ethanol and diethyl ether extracts aligns with optimization studies showing that extraction parameters significantly affect phenolic composition and concentration (Lara *et al.,* 2021). Compounds such as pyrogallol (1.110 ppm in ethanol; 5.458 ppm in diethyl ether), phloroglucinol (3.358 ppm; 6.246 ppm), and p-cresol (1.458 ppm; 6.248 ppm) were present in lower amounts but are noteworthy for their bioactivities. Pyrogallol and phloroglucinol are recognized for antimicrobial and radical-scavenging properties, which may contribute synergistically to the bioactivity of lemongrass extracts (Wahyuni *et al.,* 2024). The presence of these phenolics corroborates earlier reports of bioactive secondary metabolites in *C. citratus* with roles in defense and pharmacological functions (Du *et al.,* 2023). Interestingly, diethyl ether extracts favored the enrichment of compounds such as phenetol (19.100 ppm), alpha-naphthol (17.964 ppm), and 3,5-hydroxytoluene (17.519 ppm), whereas ethanol extracts contained higher concentrations of cinnamic acid (21.163 ppm). This selective recovery highlights solvent-dependent solubility, as phenolic acids are better extracted with polar solvents, while non-polar solvents recover other aromatic (Sousa *et al.,* 2021). The detection of cinnamic acid is particularly relevant, as it is a hydroxycinnamic acid widely reported in lemongrass and linked to antioxidant and antimicrobial activities (Lara *et al.,* 2021). Resveratrol, a stilbene derivative, was also detected in both extracts (2.805 ppm in ethanol; 3.178 ppm in diethyl ether). Though typically associated with grapes and peanuts, its occurrence in lemongrass aligns with broader reports of stilbenes in medicinal plants (Lara *et al.,* 2021). The presence of (+)-pinoresinol and lignans further expands the phytochemical diversity observed, consistent with recent reviews highlighting lignans as common bioactive polyphenols in medicinal herbs (Sun and Shahrajabian, 2023). Overall, the results demonstrate that *C. citratus* possesses a rich and diverse phenolic profile comprising simple phenols, hydroxycinnamic acids, lignans, and stilbenes. The higher total concentration in the diethyl ether extract indicates greater recovery of less polar compounds, whereas ethanol more efficiently extracted polar phenolic acids such as cinnamic acid. These findings agree with recent literature emphasizing the critical role of extraction solvents in shaping phytochemical recovery and biological potential (Lara *et al.,* 2021; Wahyuni *et al.,* 2024).

5.2 Conclusion

This study showed that *C. citratus* contains moderate protein, tannins and moisture, low fat, fibre and ash, and very low antinutritional factors such as oxalate and cyanogenic glycosides. Cellulose and lignin dominated the plant matrix, while alkaloids, cardiac glycosides, saponins, steroids and carotenoids were present at bioactive levels. Diethyl ether yielded slightly more extract (1.72%) and enriched less polar phenolics such as catechol, resorcinol and hydroxyquinol, whereas ethanol (1.56%) concentrated polar compounds including quinol, cinnamic acid, flavonoids and pinoresinol. Hydroxyquinol was the dominant phenolic in both solvents. Overall, the results confirm *C. citratus* as a nutritionally safe, phenolic-rich plant whose phytochemical recovery depends strongly on solvent selection.

5.3 Recommendations

1. Studies on *C. citratus* should be conducted in different regions since soil composition, climate, and harvest stage likely influence proximate, antinutritional, and phytochemical composition.
2. Advanced extraction methods such as ultrasound-assisted, microwave-assisted, or supercritical fluid extraction should be explored to improve recovery of both polar and non-polar phenolics beyond what ethanol and diethyl ether achieve.
3. Nutraceutical and dietary applications should be further explored, given the plant's carbohydrate-rich, low-antinutrient profile, with a focus on incorporating lemongrass into functional food and herbal formulations.
4. Toxicological assessments are advisable, especially regarding the relatively high alkaloid and cardiac glycoside content, to establish safe dosage ranges for long-term consumption.

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APPENDIX I

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University of Benin

Department of Plant Biology and Biotechnology

Herbarium Unit

Faculty of Life Sciences

University of Benin, Benin City, Edo State

Plant Name: *Cymbopogon citratus* (DC.) Stapf.

Family: Poaceae

Local Name: West Indian Lemon grass, Lemon grass

Voucher Number: UBH-C451

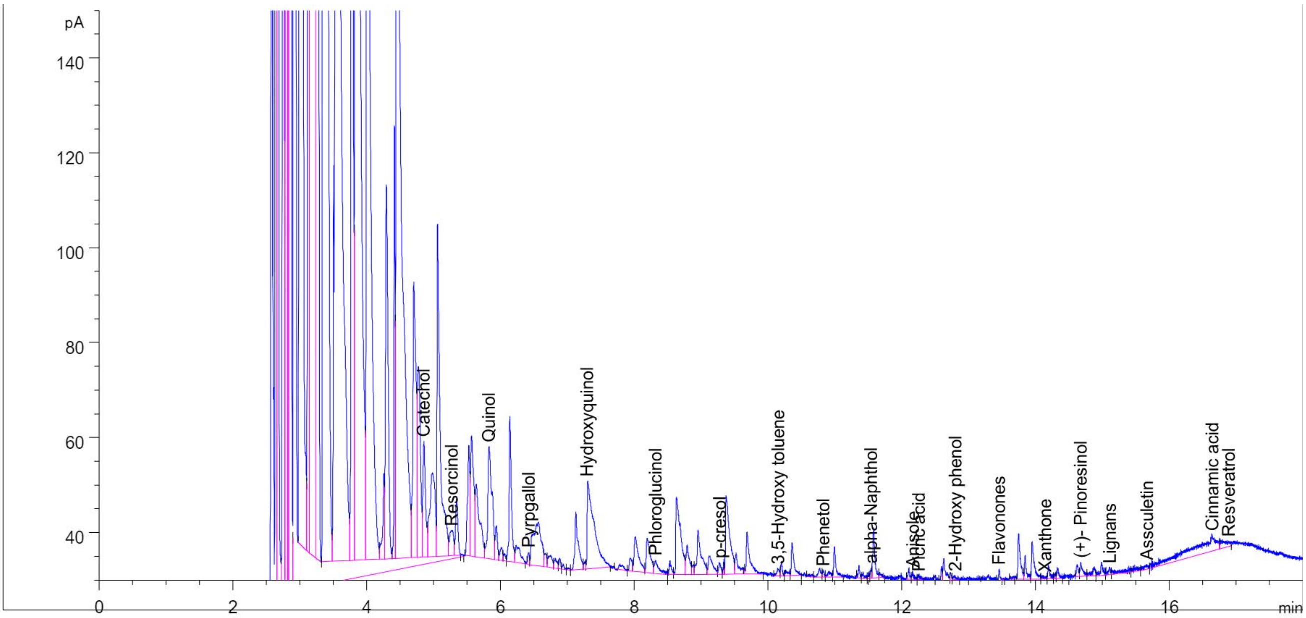
Student Name: Theophilus Illuebbey *et al.*

Plant Identification and Voucher Number Issued by:

28/05/2025

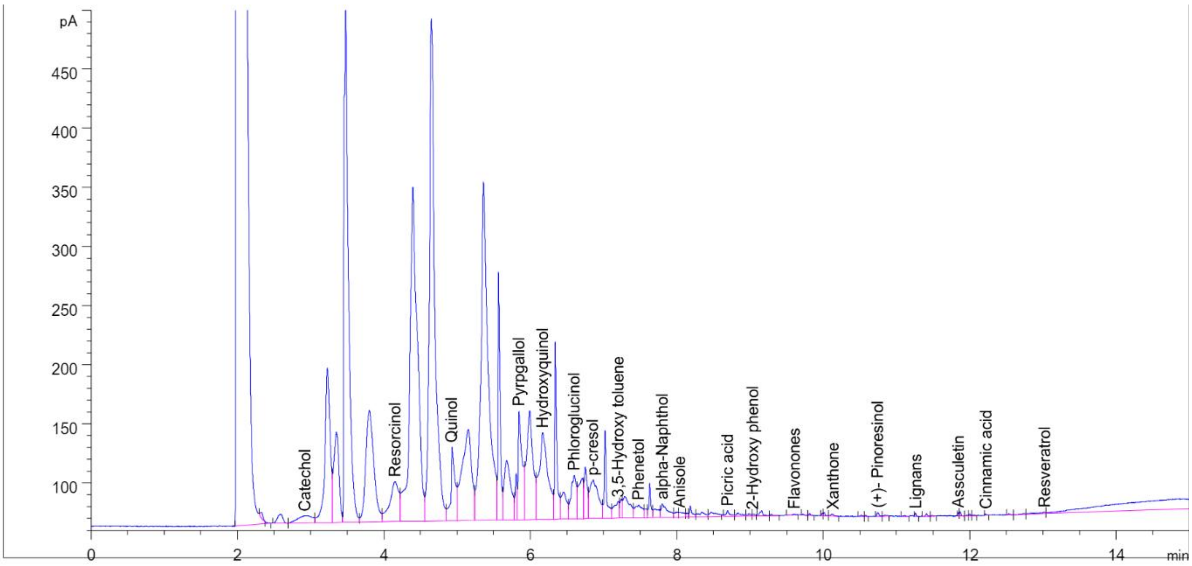
Prof. Akinnibosun Henry Adewale (FLS, MRSB; London, MECOSON, LMBOSON, MAEIAN; MFBAN Nigeria).

APPENDIX II



Phenolic profile of ethanol extract *C. citratus* using GC-FID

APPENDIX III



Phenolic profile of diethyl ether extract of *C. citratus* using GC-FID

APPENDIX IV

Weighing of pulverized *C. citratus*

APPENDIX V

Extraction using ethanol (polar) and diethyl ether (non-polar) in brown glass jars

APPENDIX VI

Filtration using Grade 1 Whatmann filter paper

APPENDIX VII

Concentration and solvent extraction using Rotary evaporator

APPENDIX VIII

Analysis using pulverized samples of *C. citratus*

APPENDIX IX

Gas Chromatography-Flame Ionization Detector