PHYTOCHEMICAL EXAMINATION, ELEMENTAL ANALYSIS, VITAMIN PROFILE, ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF *OCIMUM GRATISSIMUM* (SCENT LEAVES) STEM EXTRACT

BY

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A THESIS SUBMITTED TO THE DEPARTMENT OF CHEMISTRY, FACULTY OF PHYSICAL SCIENCES, UNIVERSITY OF BENIN, BENIN CITY, EDO STATE, NIGERIA.

SEPTEMBER, 2025

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A THESIS SUBMITTED TO THE DEPARTMENT OF CHEMISTRY, FACULTY OF PHYSICAL SCIENCES, UNIVERSITY OF BENIN, BENIN CITY, IN PARTIAL FULFILMENT FOR THE AWARD OF BACHELOR OF SCIENCE (B.SC) DEGREE IN INDUSTRIAL CHEMISTRY

SEPTEMBER, 2025

CERTIFICATION

This is to certify that this project work was carried out by EHIOSU IRABOR, Department of Chemistry, Faculty of Physical Sciences, University of Benin, Benin City, in partial fulfillment for the award of B.Sc in Industrial Chemistry

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DEDICATION

This work is dedicated to God almighty for his abundant grace and faithfulness, for strengthening me and to also my parents who always stand by me.

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With a grateful heart, I give thanks to God for the grace and strength He gave me to complete this project.

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ABSTRACT

Ocimum gratissimum (Scent leaves) stem barks, a widely used medicinal herb, was investigated for its phytochemical composition, elemental analysis, vitamin profile, antioxidant activities, and anti-inflammatory activities. The stem bark of the plant was successfully extracted with ethyl acetate using maceration techniques. The phytochemical examination, elemental analysis, vitamin profile, antioxidant activities and anti-inflammatory activities extract of *Ocimum gratissimum* were determined using established method. Phytochemical screening of the water extract of the stem bark revealed the presence of alkaloids, flavonoids, saponins, glycosides, phenolic compounds, terpenoid, eugenols, protein and reducing sugar, indicating the plant's rich bioactive profile. Elemental analysis demonstrated significant levels of essential minerals, including calcium (21.70 mg/kg), sodium (14.20 mg/kg), potassium (13.60 mg/kg), magnessium (4.68 mg/kg), and trace elements vital for human health with calcium being the most abundant. Calcium plays a vital role in the body by strengthening bones and teeth. Vitamin profiling showed appreciable concentrations of both water-soluble vitamins (B and C) and fat-soluble vitamins (A, D, E). The concentration of vitamin B which includes B_1 (0.27 mg/L), B_2 (0.27 mg/L), B_3 (0.60 mg/L), B_6 (1.30 mg/L), and B_{12} (31.06 mg/L) were present in moderate amounts except vitamin B_{12}, which showed a slightly higher concentration. The other vitamins (A (77.84 mg/L), C (91.62 mg/L), D (66.33 mg/L), and E (104.48 mg/L)) were found in high concentrations, with vitamin E exhibiting the highest level, highlighting its nutritional relevance. The antioxidant potential were examined using FRAP (Ferric reducing antioxidant power) and Superoxide assays. In the FRAP assay, the extract shows increasing antioxidant power with %inhibition ranging from 36.08% to 66.60% at concentration of 40-160 mg/ml confirming its electron-donating capacity. In the superoxide radical scavenging assay, percentage scavenging ranged from 25.23% to 74.00% at concentration of 40-160 mg/ml, indicating concentration-dependent activity, while in anti-inflammatory studies, the extract effectively inhibited heat-induced haemolysis, with percentage inhibition increasing from 47.78% to 74.24% (40–80 mg/mL), and albumin denaturation, ranging from 55.40% to 64.40% at similar concentrations. These results indicate potent membrane stabilization and protein protection abilities of *O. gratissimum* stem bark extract. The findings demonstrate that *O. gratissimum* stem bark is rich in bioactive compounds, essential minerals, and antioxidant vitamins, which synergistically confer strong antioxidant and anti-inflammatory properties. The significant superoxide scavenging and ferric reducing power, along with inhibition of haemolysis and protein denaturation, validate its traditional use in treating oxidative stress-related disorders and inflammation. Thus, *O. gratissimum* may serve as a promising source of natural therapeutic agents for pharmaceutical and nutraceutical applications.

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1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Medicinal plants are traditionally used in folk medicine as natural healing remedies with therapeutic effects such as prevention of cardiovascular diseases, inflammation disorders, or reducing the risk of cancer (Sona *et al,* 2012). In addition, medicinal plants are a valuable source of a wide variety of chemical molecules having different structures and functionalities that exhibit important biological activities and are linked to a multitude of beneficial properties, such as antimicrobial, anticancer, antiviral, antioxidant and enzyme inhibitory, anti-aging, anti-inflammatory, anti-hypertensive, neuroprotective and anticoagulant effect (Alina *et al,* 2021). For the large proportions of world's population, medicinal plants continue to show a dominant role in the healthcare system and this is mainly true in developing countries, where herbal medicine has continuous history of long use (Refaz *et al,* 2017).

The widely used of modern synthetic drugs are associated with some undesirable side effects which may lead to other patho-physiological complications. As a result, traditional herbal medicine seem to have an important role which has minimal or no known side effects as compared with modern drugs (Priti Maji *et al,* 2020). The most important aspect for the continuous use of the traditional medicines are its ready accessibility, cheapness and sociocultural reason (Farhat *et al,* 2014).

Medicinal plants remain a vital source of therapeutic support for human illnesses. The quest for good health, longevity and remedies to ease pain and discomfort led early humans to explore their immediate environment, resulting in the use of various plants, animal products and minerals etc (Ighodaro and Ogbeide, 2020) . It is important to note, however, that certain medicinal plant contains harmful substances and for this reason, they should be used with caution despite their therapeutic value (Ana carolina and Daniela. 2018). Consequently, many of these plants have been scientifically studied and applied in the treatment of various illnesses. These medicinal herbs are utilized in botanical medicine to promote public health and therapeutic benefits, and they also serve as fundamental sources for the development of effective drugs (Ogbeide *et al,* 2024).

1.1.1 Importance of Medicinal Plants

Medicinal plants are invaluable reservoirs of bioactive compounds and despite modern advances in pharmaceuticals and drug development, they continue to serve as a primary source of medicine for a significant portion of the global population (Ali *et al,* 2014).

The use of medicinal plants extends beyond disease treatment to include the role in promoting overall health and well-being. Globally, nearly two-thirds of the population relies on herbal medicine as a primary source of healthcare, largely due to its cultural acceptability, compatibility with the human body and relatively fewer side effects (Oladeji, 2016). Historical records indicate that many conventional drugs are deprived from plant extracts, some of which contain active bioactive compounds. Recent research has shown that numerous plant-based drugs were developed from studies of traditional remedies, curative practices and particularly the folk knowledge of indigenous communities-wisdom that remains invaluable despite advances in modern science and technology (Oladeji, 2016).

Medicinal plants are often used as raw materials for extracting active compounds that serves as the basis of synthesizing various drugs. For example, many laxatives, blood thinners, antibiotics and anti-malaria medications are derived from plant-based ingredients (Singh R. 2015). medicinal plants are employed in the treatment of cardiovascular diseases, which result from disorders of the heart and blood vessels and include conditions such as heart attack cerebrovascular diseases, hypertension and heart failure (Raha *et al,* 2021). In addition to their therapeutic role, medicinal plants are also widely used as flavoring agents and natural preservatives in food.

1.1.2 BACKGROUND OF STUDY

1.1.2.1 ANTIOXIDANT ACTIVITY

Unfavorable conditions for plants such as extreme temperatures, drought, heavy metal exposure, nutrient deficiencies and high salinity lead to the accumulation of reactive oxygen species (ROS), which trigger oxidative stress. To counter this, plant cells rely on a sophisticated antioxidant system composed of both enzymatic and non-enzymatic components. The non-enzymatic molecules operate through diverse mechanisms, including inhibiting enzymes, chelating trace elements involved in free radical generation,

scavenging reactive species and enhancing protection by activating or strengthening other antioxidant defenses (Natividad *et al,* 2020).Among these molecules, secondary metabolites particularly phenolic compounds play a key role in protecting against oxidative stress (Pang *et al,* 2018). Their antioxidant action stems not only from their ability to donate hydrogen atoms or electrons but also from their stability as radical intermediates. In addition, when plants containing these compounds are consumed, phenolics exert protective effects in humans (Niciforovic *et al,* 2010). Generally, according to (Duthie *et al*, 2000), (Li A *et al*, 2014) and (Balmus *et al,* 2016), phenolic antioxidants are highly effective even at low concentrations, and their presence in the human diet has been linked to the prevention of cardiovascular diseases and certain cancers . Therefore, evaluating the antioxidant activity of extracts from various plant species is crucial for determining their potential as sources of novel antioxidant compounds (Miliauskas *et al, 2004), (*Gouthamchandra *et al,* 2010).

The first step in measuring the antioxidant activity of a plant extract is selecting an appropriate method (Abramovic *et al,* 2017). Numerous assays exist for this purpose (Apak *et al,* 2008), but the variability of experimental conditions across studies complicates both method selection and the comparison of results among researchers. This variability makes it difficult to rank plant species according to the antioxidant capacity of their extracts. To address this issue, data obtained using different methods and species should be analyzed with descriptive multivariate statistical techniques, which can help identify the most suitable method for comparing and ranking antioxidant activity.

Assays used to quantify antioxidant activity are typically classified according to their mechanism of action in halting chain-breaking reactions. These fall into two main categories: hydrogen atom transfer (HAT) methods, which measure the ability of antioxidants to donate hydrogen atoms, and single electron transfer (SET) methods, which assess reduction reactions through electron donation (Prior *et al,* 2005), (Perez, 2007). Commonly used SET assays include the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, ferric reducing antioxidant power (FRAP) assay, Trolox equivalent antioxidant capacity (TEAC or ABTS) assay, copper reduction (CUPRAC) assay, and the reducing power (RP) assay. Examples of HAT-based assays include the crocin bleaching assay, the total peroxyl radical-trapping antioxidant parameter (TRAP) assay, the total

oxyradical scavenging capacity (TOSC) assay, and the oxygen radical absorbance capacity (ORAC) assay (Prior *et al,* 2005).

1.1.2.2 ANTI-INFLAMMATORY ACTIVITY

Inflammation is the body's natural defense mechanism against harmful stimuli such as allergens or tissue injury. However, when this response becomes uncontrolled, it contributes to a wide range of disorders, including allergies, cardiovascular diseases, metabolic syndrome, cancer, and autoimmune conditions, thereby creating a significant economic burden on individuals and society (Bagad *et al,* 2013). Various medications—such as steroids, nonsteroidal anti-inflammatory drugs, and immunosuppressants—are commonly used to manage inflammation, but their use is often limited by adverse effects. The therapeutic goal is to achieve maximum efficacy at the lowest effective dose with minimal side effects. To this end, incorporating natural anti-inflammatory agents into treatment strategies may enhance pharmacological outcomes while reducing unwanted effects (Bagad *et al,* 2013), (Ghasemian and Owlia, 2015).

Herbal medicines represent a promising avenue in this context, though greater knowledge and scientific validation are still required. While complementary, alternative, and traditional medicine provide valuable insights into the use of herbal remedies, modern medicine must confirm their efficacy and safety through rigorous scientific investigation. This review aims to evaluate medicinal plants and the strongest clinical evidence supporting their anti-inflammatory properties.

1.1.3 STATEMENT OF PROBLEM

One of the main challenges associated with medicinal plants is the limited scientific evidence regarding their safety, effectiveness, and quality, which can result in adverse effects from unpredictable drug interactions or contamination. Additional concerns include biodiversity loss caused by over-harvesting and habitat destruction, the gradual disappearance of traditional knowledge due to insufficient documentation, and the urgent need for stronger regulation and quality control to ensure their safe and sustainable use.

1.1.4 JUSTIFICATION OF STUDY

Ocimum gratissimum, commonly known as African basil, is a medicinal plant widely used in traditional medicine for managing various ailments, including inflammation, infections, and conditions linked to oxidative stress. Despite its long history of ethnomedicinal use, there is still limited comprehensive evaluation of its phytochemical composition, vitamin content, elemental profile, and potential health-promoting activities.

Studying the stem bark of *O. gratissimum* is significant because it may serve as a rich source of natural compounds with antioxidant and anti-inflammatory properties. These activities are particularly important since oxidative stress and inflammation are strongly implicated in the progression of chronic and degenerative diseases. Assessing its phytochemical constituents alongside its nutritional and elemental profile will generate valuable information that supports its therapeutic applications, validates its traditional uses, and broadens its scope of utilization.

Moreover, providing scientific evidence on the medicinal and nutritional potential of *O. gratissimum* can contribute to natural product–based drug development, promote its safe use in healthcare, and enhance its economic value as a medicinal and nutraceutical resource.

1.1.5 SCOPE OF WORK

This study is limited to collection of *Ocimum gratissimum* stem bark sample, air dry and pulverized into fine powder, extract with ethyl acetate, identify the phytochemical, element, vitamin present and to study the anti-inflammatory and antioxidant activity.

1.1.6 AIM AND OBJECTIVES

The aim of this study is to examine the phytochemical screening, elemental analysis, vitamin profile, and to study the antioxidant activities and anti-inflammatory activities of the stem bark of *Ocimum gratissimum* in order to provide scientific evidence supporting its medicinal and nutritional applications.

OBJECTIVES

The objectives of this study are;

i. Identification of the phytochemicals constituents present in the stem bark of *O. gratissimum.*
ii. To identify and quantify the element present in *O. gratissimum* stem bark.
iii. To determine the vitamin profile of *O. gratissimum* stem bark.
iv. To determine the antioxidant potential of the stem bark of *O. gratissimum.*
v. To determine the anti-inflammatory activities of *O. gratissimum* stem bark.

1.2 LITERATURE REVIEW

Ocimum gratissimum, commonly referred to as scent leaf, is a well-recognized medicinal plant with promising potential as an alternative therapy for various diseases and as a source of novel drug candidates. It is a widely distributed, commercially valuable perennial herbaceous plant characterized by its strong aromatic fragrance. Belonging to the family Lamiaceae, it is native to Africa, Asia, and South America (Tanko *et al*., 2008; Akara et al., 2021). Beyond its medicinal value, the plant is commonly utilized as a natural flavoring agent, condiment, and vegetable in the preparation of soups, stews, meats, and fish. Traditionally, it has been employed in the management of several ailments including cough, pneumonia, fever, inflammation, anemia, diarrhea, pain, and infections caused by bacteria and fungi (Akara *et al.*, 2021).

1.2.1.1 TAXONOMICAL HIERARCHY AND DESCRIPTION OF *OCIMUM GRATISSIMUM*

Fig 1; Diagram of *Ocimum gratissimum*

Kingdom	**Plantae**
Division	Tracheophyta
Class	Magnoliopida
Order	Lamiales
Family	Lamiaceae
Genus	Ocimum
species	Ocimum gratissimum

COMMON NAME: Scent leaf

LOCAL NAMES OF *OCIMUM GRATISSIMUM* IN NIGERIA

Ocimum gratissimum is called Ebe-amwonkho in Edo, Nchanwu in Igbo, and Efinrin in Yoruba.

1.2.1.2 DESCRIPTION OF *OCIMUM GRATISSIMUM*

Ocimum gratissimum is a perennial herb widely distributed across the tropical regions of Africa and Asia. It belongs to the family Lamiaceae, under the genus *Ocimum* and the species *gratissimum*. The plant has a woody base and usually grows to a height of 1–3 meters. Its leaves are green, broad, and narrowly ovate, typically measuring 5–13 cm in length and 3–9 cm in width, with a slightly rough surface and serrated margins. The flowers are whitish to purplish and borne in terminal spikes, giving the plant both ornamental and medicinal value (Mohamed and Euloge 2016).

1.2.1.3 USES OF *OCIMUM GRATISSIMUM*

Ocimum gratissimum is widely accessible to local communities and frequently cited in ethnopharmacological surveys for its use in managing a broad spectrum of diseases (Ositadinma, 2021). This perennial, aromatic plant is now distributed across all continents and is well recognized for its medicinal properties. In Africa, its therapeutic applications are remarkably diverse and vary from one country to another (Kpoviessi *et al*., 2014). For instance, in Cameroon, infusions are regarded as tonic and pectoral, while the leaf juice is traditionally used to relieve dizziness, headaches, colds, and coughs. In Côte d'Ivoire, different preparations of the plant are employed against ear infections, dermatoses, and ophthalmias (Kpoviessi *et al.*, 2014). In Nigeria, it is prescribed for the treatment of diarrhoea (Kpoviessi *et al*., 2014), and [Sofowora (1970)] recommended its use for respiratory disorders and as an anthelmintic. Additionally, it has been applied in the management of headaches, fevers, ophthalmic and skin conditions, as well as pneumonitis. In Togo, infusions serve as antitussives, whereas the fresh leaf juice is used for its antidiarrhoeal and antidysenteric effects. Its aqueous maceration is further applied in cases of haematuria and purulent urethritis (Kpoviessi *et al*., 2014). In Benin Republic, aqueous extracts of the pulp or aerial parts are employed for conditions such as dystocia, pelvic pain, colic, candidiasis, digestive dysmenorrhoea, emesis, haemorrhoids, and diarrhoea. A decoction of the stem is traditionally used to manage hepatitis, cough, asthma, and wound infections (Chah *et al*., 2006, Kpoviessi *et a*l., 2014]). The leaf juice is also applied for angina, cephalgia, fever, and malnutrition, while its inflorescences are commonly used as flavoring agents in various dishes.

African basil leaves are eaten as vegetables and used as food flavoring. They are valued not only for their nutritional content—being rich in vitamins, minerals, and antioxidants—but also for their traditional medicinal uses in alleviating headaches, fevers, and other health conditions (Nweze, 2009).

1.2.2.1 PHYTOCHEMISTRY

Phytochemicals are naturally occurring plant-derived compounds that help protect plants from harm. They are present in grains, fruits, vegetables, nuts, and herbs, with over a thousand types already identified. Major groups of phytochemicals include carotenoids, polyphenols, isoprenoids, phytosterols, saponins, fibers, and polysaccharides. These bioactive compounds possess diverse pharmacological properties such as antimicrobial, antidiarrheal, anthelmintic, antiallergic, antispasmodic, and antiviral effects. In addition, they regulate gene transcription, boost immunity, and protect against cancers. Advances in translational research have further expanded their applications in functional foods.

Phytochemicals are particularly important in the development of functional foods and nutraceuticals once extracted from plant sources. However, these compounds vary in their solubility, solvent affinity, and tolerance to heat, making the choice of extraction method essential for preserving their quality and functional properties. Solvents generally fall into two groups: green solvents (e.g., water, ethanol, glycerol, fatty acids/oils, acetic acid, ionic liquids, carbon dioxide, and natural deep eutectic solvents) and conventional solvents (e.g., acetone, chloroform, butanol, methanol, ethyl acetate, methyl acetate, benzene, hexane, and cyclohexane) (Shikov *et al.*, 2022). The use of inappropriate solvents or high temperatures may result in the loss of bioactivity. Additionally, extraction efficiency depends on several factors related to the plant matrix, including type, structure, pre-treatment, particle size, and solid–liquid ratios, all of which can influence phytochemical recovery (Carreira-Casais *et al*., 2021).

To ensure high-quality phytochemical products, extraction must preserve their natural structure and functionality. This requires selecting suitable extraction methods. Conventional approaches such as maceration, percolation, decoction, reflux extraction, and Soxhlet extraction are widely used. In contrast, newer and more advanced techniques include pressurized liquid extraction (PLE), high hydrostatic pressure extraction (HHP),

microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), pulsed electric field extraction (PEF), vibro-cavitation extraction, and vacuum–oscillating boiling extraction..

New extraction approaches have also been developed, including milling, rotary-pulsation extraction (RPE), liquid gas extraction (LGS), enzyme-assisted extraction (EAE), supercritical fluid extraction (SFE), and natural deep eutectic solvent extraction (NADES) (Shikov *et al*., 2022). These methods are evaluated based on their suitability for different phytochemical extractions under varying conditions, such as substrate type, plant matrix, solvent, and temperature. Considering these factors allows researchers to make informed choices about the most effective extraction technique. This ensures that phytochemicals are recovered efficiently while maintaining their natural properties, ultimately supporting the development of functional foods and nutraceuticals of superior quality.

1.2.2.2. TYPES OF PHYTOCHEMICALS

Phytochemicals are generally grouped into primary and secondary metabolites. Primary metabolites include carbohydrates, amino acids, proteins, lipids, and chlorophylls, while secondary metabolites consist of alkaloids, saponins, steroids, flavonoids, tannins, and phenols (Bhardwaj and Dubey, 2019).

1.2.2.2.1 Primary metabolite

Primary metabolites are vital compounds directly involved in an organism's metabolic pathways, supporting growth, development, and reproduction. These metabolites are closely linked to physiological functions and are produced during the growth phase, often referred to as the trophophase. Their production is largely influenced by the availability of essential nutrients in the environment (Bhardwaj and Dubey, 2019).

They are widely distributed across cells and play fundamental roles in metabolism, acting as substrates or catalysts. While some are common to all organisms, others are specific to certain species or cell types. Unlike secondary metabolites, primary metabolites generally do not exhibit pharmacological effects. However, they are produced in large amounts due to the body's constant demand for them. They can be easily obtained through simple extraction processes and are broadly categorized into primary essential metabolites and primary metabolic end products (Bhardwaj and Dubey, 2019).

Examples include proteins and carbohydrates, which contribute to structural and physiological functions within organisms. Compounds like lactic acid and ethanol, which are byproducts of metabolic pathways, are considered primary metabolic end products. Overall, primary metabolites are regarded as the essential building blocks of life (Bhardwaj and Dubey, 2019).

1.2.2.2.2 Classification of primary metabolite

1.2.2.2.2.1 Alcohols

Primary metabolites are compounds that are directly involved in the growth, development, and reproduction of living organisms. Alcohols, particularly ethanol, serve as a classic example. During the exponential phase of microbial growth, many organisms, especially yeasts such as Saccharomyces cerevisiae, convert sugars (like glucose) into ethanol and carbon dioxide through the process of fermentation.

This production is growth-associated, meaning ethanol is synthesized simultaneously with cell multiplication. Because of this close relationship with essential metabolic activity, ethanol is classified as a primary metabolite, unlike secondary metabolites which are usually produced in the stationary phase and are not required for growth.

Ethanol has extensive industrial applications. It is the basis of the alcoholic beverage industry, where fermentation by yeast has been harnessed for centuries. Beyond beverages, ethanol is used as a biofuel, a solvent in laboratories and industry, and as a disinfectant in healthcare. The large-scale production of ethanol relies on fermentation technology, making it one of the most significant microbial primary metabolites from an economic and social standpoint.

1.2.2.2.2.2 Enzymes

Enzymes are biological catalysts, usually proteins (and sometimes RNA molecules), that speed up chemical reactions in living systems without being consumed in the process. They function by lowering the activation energy required for a reaction, thereby making biochemical processes occur rapidly under mild conditions of temperature and pH.

Each enzyme is highly specific to its substrate, binding at the active site to form an enzyme–substrate complex and converting it into product(s). Enzyme activity can be influenced by factors such as temperature, pH, substrate concentration, and the presence of inhibitors or activators.

Enzymes play central roles in metabolism, including processes such as digestion, energy production, DNA replication, and signal transduction. Clinically, enzymes are important as diagnostic markers (e.g., liver enzymes) and therapeutic agents (e.g., streptokinase in clot dissolution). Industrially, they are used in food processing, biotechnology, and pharmaceuticals.

1.2.2.2.2.3 Carbohydrates

Carbohydrates are important primary metabolites because they are directly involved in the growth, development, and energy metabolism of living organisms. As products of primary metabolism, they are synthesized during the active growth phase and are essential for cell survival and reproduction.

In microorganisms and plants, carbohydrates such as glucose, fructose, and sucrose serve as immediate sources of energy through pathways like glycolysis and the tricarboxylic acid (TCA) cycle. They also function as precursors for the biosynthesis of amino acids, nucleotides, lipids, and various structural components of the cell. Polysaccharides like starch, glycogen, and cellulose act as storage or structural carbohydrates, ensuring energy availability and maintaining cellular integrity.

Because of their fundamental role in metabolism, carbohydrates are classified as primary metabolites, in contrast to secondary metabolites that are not essential for growth. Moreover, carbohydrates have extensive industrial applications, including their use in fermentation processes (e.g., glucose as a substrate for ethanol production), in food industries as sweeteners and thickeners, and in pharmaceuticals as excipients or stabilizers.

1.2.2.2.2.4 Proteins

Protein is increasingly viewed positively by consumers, leading to rising demand for both plant- and animal-derived sources (Henchion *et al,* 2017). Clinical research, particularly in

older adults, also highlights the health benefits of protein intake at or above current dietary recommendations. Plant-based proteins are especially valuable, as they supply essential nutrients along with fiber and antioxidants that contribute to overall health (Henchion *et al,* 2017).

Demand for plant protein-based products is currently strong and projected to rise significantly over the next decade. Several factors are driving this trend;

(1) The potential health advantages linked to greater consumption of plant-based diets,

(2) Concerns about negative health outcomes from diets high in animal protein, such as elevated saturated fat intake,

(3) Growing awareness of the importance of environmentally sustainable food systems,

(4) Ethical considerations related to animal welfare, and

(5) The widespread consumer perception of protein as a beneficial nutrient, where "more is better."

Although diets rich in plant protein offer health and functional benefits, plant proteins may be nutritionally inferior to animal proteins in certain respects (Steven *et al,* 2020).

1.2.2.2.2.5 Vitamins

Vitamins are essential nutrients that play a crucial role in regulating cell division, growth, and metabolism. Since the human body can naturally synthesize only a limited number of vitamins, most must be obtained through the diet. Fruits and vegetables are rich sources of a wide variety of vitamins. These vitamins are not only vital for human health but are also required for the normal functioning of plant cells. Consequently, analyzing plant-derived vitamins is important for both plant biology and nutrition research.

The main categories of vitamins identified in plants include vitamin A, the vitamin B-complex (B1, B2, B3, B5, B6, B7, and B9), vitamin C, vitamin E, and vitamin K. Based on solubility, vitamins are further classified into two groups: fat-soluble vitamins (A, E, and K) and water-soluble vitamins (B-complex and C).

1.2.2.2.2.6 Lipids

Plant lipids are diverse and play critical roles in cellular function. They maintain the structural integrity of cells and organelles by forming hydrophobic membrane barriers.

Seeds store lipids as chemical energy, while lipids also serve as signaling molecules that regulate cellular metabolism (Ohlrogge J., Browse J, 1995, Li-Beisson *et al,* 2013).

The predominant plant lipids are glycerolipids, formed when the carboxyl group of fatty acids esterifies with the hydroxyl group of glycerol. Lipid biosynthesis involves multiple organelles: fatty acids are synthesized in chloroplasts and can combine with glycerol to produce galactolipids—major components of chloroplast membranes. Fatty acids are also exported to the cytoplasm, where they bind with glycerol in the endoplasmic reticulum (ER) to form membrane phospholipids (Chapman K.D., Ohlrogge J.B, 2012). Within the ER of seed cells, triacylglycerols (TAGs) are synthesized and stored in oil bodies (.Chapman *et al,* 2019). In epidermal cells, fatty acids are converted in the ER into cutin and wax—lipid components of the cuticle that reduce water loss (.Yeats T.H., Rose J.K, 2013).

Genetic studies using Arabidopsis mutants have revealed pathways for fatty acid, glycerolipid, and TAG biosynthesis (.Wallis J.G., Browse J, 2002). Current research is focused on identifying transcription factors that regulate lipid metabolism (Lee *et al,* 2018), exploring the role of galactolipids in photosynthesis (Li H.-M., Yu C.-W, 2018), investigating cuticle lipid synthesis and transport (Fich *et al,* 2016, Lee *et al,* 2015), and understanding lipid remodeling during development and stress (Yang Y., Benning C, 2018). To enhance plant oil quality and yield, ongoing studies aim to modify fatty acid composition and increase oil content (Lee *ei al,* 2015, Vanhercke *et al,* 2019).

1.2.2.2.2.7 Amino acids

Over the past decades, the critical role of amino acids in plant growth and stress responses has become increasingly recognized, drawing significant attention in both basic and applied plant science. This review highlights recent advances in amino acid research and provides an updated overview of their functions in plant physiology. Beyond serving as the fundamental building blocks of proteins, many amino acids—including some not directly involved in protein synthesis—have been shown to actively contribute to plant development and to the response against environmental stresses.

Amino acids also act as precursors for numerous primary and secondary metabolites and are essential in human nutrition, functioning as sources of nutraceutical compounds and

indispensable dietary components. Of the twenty-one proteinogenic amino acids, nine cannot be synthesized by animals, including humans, while several others are produced only in insufficient amounts to meet metabolic demands. These nutritionally essential amino acids must therefore be obtained from the diet, with plants being the primary source (Galili *et al*., 2016; Hou and Wu, 2018). Unlike humans and animals, plants possess the ability to synthesize all twenty-one proteinogenic amino acids

1.2.2.2.3 Secondary metabolites

Secondary metabolites are organic compounds produced by organisms through modifications of primary metabolic pathways. Unlike primary metabolites, which are essential for growth, development, and reproduction, secondary metabolites are generally synthesized at the end or near the stationary phase of growth. Although not directly involved in basic cellular functions, they play key ecological roles, particularly in defense, acting as antibiotics or pigments.

Several secondary metabolites are significant in industrial microbiology. For instance, atropine, obtained from various plants, is clinically important as a competitive antagonist of muscarinic acetylcholine receptors and is commonly used in the treatment of bradycardia. Antibiotics such as erythromycin and bacitracin are also secondary metabolites. Erythromycin, produced by Saccharopolyspora erythraea, is a widely used, orally administered antibiotic with a broad antimicrobial spectrum. Bacitracin, derived from Bacillus subtilis, is another clinically relevant example, typically employed as a topical antibiotic. In nature, bacitracin is synthesized by nonribosomal peptide synthetases, but in medicine, it is utilized for its antibacterial properties.

1.2.2.2.4 Classification of Secondary Metabolites

1.2.2.2.4.1 Carotenoids

Carotenoids are a class of isoprenoid metabolites that are essential for life. They are synthesized by all photosynthetic organisms, including plants, algae, and cyanobacteria, where they function as indispensable pigments. In plants, carotenoids are crucial for both photosynthesis and photoprotection, serving as light-harvesting pigments and as structural components of photosystems. They also act as precursors in the biosynthesis of the phytohormones abscisic acid (ABA) and strigolactones (SLs). Moreover, carotenoid-

derived molecules can function as signaling compounds in response to environmental and developmental signals or regulate plant growth. The high accumulation of carotenoids in flowers, fruits, and roots is responsible for their vivid yellow, orange, and red pigmentation, which carries significant ecological and agricultural importance.

Beyond their central roles in plants, carotenoids are also vital to human health and nutrition. Provitamin A carotenoids, such as β-carotene and α-carotene, are dietary precursors of vitamin A, an essential nutrient for vision and immune function. Vitamin A deficiency remains a global health concern, leading to severe outcomes such as blindness and even death, and affects nearly one-third of preschool children worldwide.

Dietary carotenoids function as antioxidants, helping to reduce the risk of chronic diseases such as cancer and cardiovascular disorders (Eggersdorfer and Wyss, 2018). In particular, lutein and zeaxanthin act as macular pigments that play an important role in lowering the risk of age-related eye diseases (Sauer *et al*., 2019). Considerable efforts have been devoted to enhancing carotenoid accumulation in food crops to improve their nutritional value and associated health benefits (Wurtzel *et al*., 2012, Giuliano, 2017, Zheng *et al*., 2020).

Given their importance to both plants and humans, carotenoid metabolism in plants has been extensively studied (Nisar *et a*l., 2015; Rodriguez-Concepcion *et al*., 2018; Sun *et al*., 2018; Wurtzel, 2019). The carotenoid biosynthetic pathway is now well characterized across diverse plant species, and recent research has increasingly focused on the regulatory mechanisms controlling carotenoid metabolism (Stanley and Yuan, 2019; Luan et al., 2020; Sun and Li, 2020; Liang *et al*., 2021). Alongside biosynthesis, carotenoid degradation and stable storage have emerged as key research areas, since the final carotenoid content in crops reflects the balance of synthesis, turnover, and storage (Cazzonelli and Pogson, 2010; Yuan *et a*l., 2015b; Sun *et al*., 2018; Hermanns *et al*., 2020; Liang *et al*., 2021; Torres-Montilla and Rodriguez-Concepcion, 2021).

While carotenoid-derived phytohormones such as ABA and SLs have been well studied (Finkelstein, 2013; Chen *et al*., 2020), increasing attention is being directed toward other apocarotenoids, which are emerging as important signaling molecules regulating plant

growth and development (Hou *et al*., 2016; D'Alessandro and Havaux, 2019; Felemban *et al*., 2019; Moreno et al., 2021).

In this review, we summarize the current understanding of carotenoid metabolism, highlighting recent advances in its intrinsic regulation at multiple levels. Furthermore, we explore the functional evolution of carotenoids, their agricultural and horticultural applications, and the future opportunities for deepening our knowledge of carotenoid metabolism and its diverse roles in plants

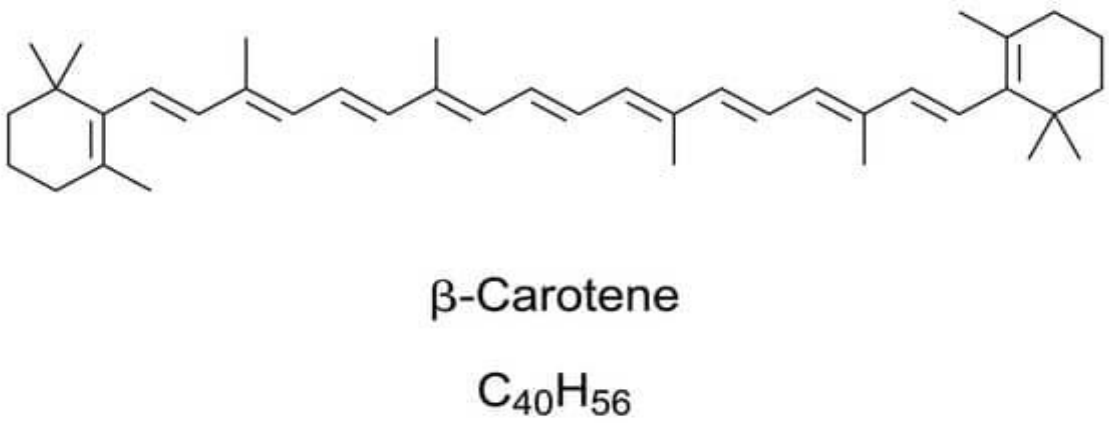

Fig 1.2.2.2.4.1 Carotenoids

1.2.2.2.4.2 Polyphenols

Polyphenols, also referred to as phenolic compounds (PCs), are among the most abundant secondary metabolites synthesized in all plant cells (Li Y *et al,* 2020, Qaderi *et al,* 2023). Considerable progress has been made in elucidating their structures and chemical properties (Šamec *et al,* 2021, Shen *et al,* 2022). The biosynthetic pathways of PCs are well studied, and the key enzymes involved have been identified (Šamec *et al*, 2021, Cheynier *et al,* 2013, Marchiosi *et al,* 2020). Advances in transcriptome research have further revealed genes responsible for the cellular capacity to produce these compounds (Cao *et al* 2020, .Pratyusha D.S., Sarada D.V.L., 2022). Additionally, numerous molecular markers have been identified, offering valuable tools for distinguishing phenol-producing plants (Zhan *et al,* 2022).

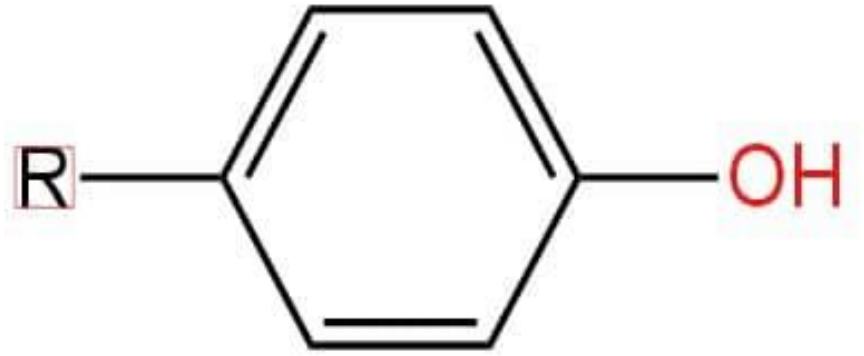

Fig 1.2.2.2.4.2 Polyphenol where R is an alkyl group

1.2.2.2.4.3 Isoprenoids

Isoprenoids constitute the most structurally and functionally diverse class of metabolites, with more than 50,000 distinct molecules identified in extant organisms (Thulasiram et al., 2007). The majority of these compounds occur in plants, where they function both as primary and secondary metabolites. As primary metabolites, isoprenoids are involved in essential processes such as photosynthesis, respiration, membrane fluidity, and the regulation of growth and development. In their role as secondary metabolites, they contribute to allelopathic interactions and plant–pathogen defense. Beyond their biological significance, plant isoprenoids hold substantial economic and practical value as components of essential oils, pharmaceuticals, rubber polymers, flavors, pigments, and agrochemicals. Consequently, deciphering the biochemical and molecular regulation of the isoprenoid biosynthetic pathway is of major importance for both scientific research and industrial applications.

The activity of a metabolic pathway can be assessed directly through metabolic flux, defined as the rate of molecular flow within a metabolic network (Ratcliffe and Shachar-Hill, 2006). Initially, the regulation of isoprenoid pathway flux in plants was thought to be straightforward, controlled by a single environmental or developmental signal that activated a specific branch of the pathway through one or a few rate-limiting enzymes. As a result, research focused heavily on identifying and characterizing these rate-limiting enzymes. However, with advancing knowledge, this view has shifted. It is now clear that regulation occurs at multiple levels: most, if not all, enzymes in the pathway are subject to control, and environmental or developmental cues can influence not only specific branches

but also the entire isoprenoid network. Furthermore, pathways of primary metabolism that supply precursors to the isoprenoid network are often co-regulated with isoprenoid biosynthesis.

To gain a complete understanding of isoprenoid regulation, it is necessary to study the complexity of the metabolic network and its interconnected pathways. Once the structural framework of the network is established, dynamic features can be analyzed at the level of enzyme activity and gene expression. Since Ruzicka's introduction of the biogenetic isoprene rule in the 1930s—which defined isoprenoids as compounds derived from isoprene precursors (Croteau *et a*l., 2000)—substantial progress has been made in characterizing the steps of the isoprenoid pathway. The advent of molecular biology techniques, and more recently functional genomics, has greatly advanced our understanding of the enzymes and metabolites involved, providing an increasingly comprehensive view of the isoprenoid pathway network.

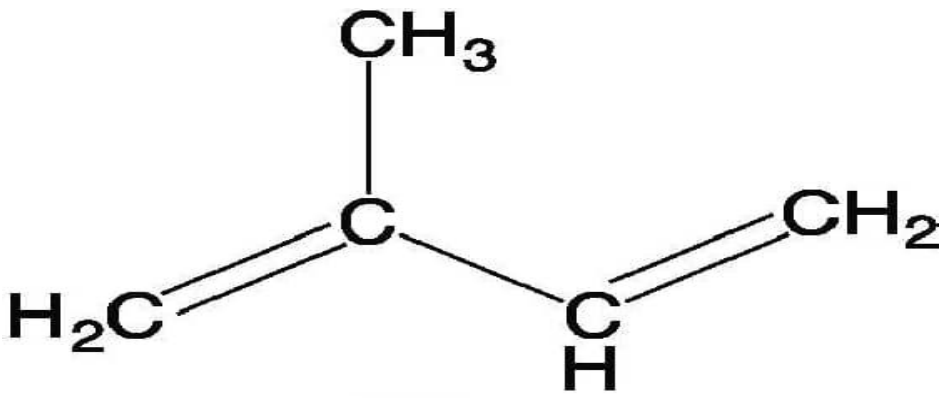

Fig 1.2.2.2.4.3 Isoprenoid

1.2.2.2.4.4 Phytosterols

Phytosterols (PSs) are plant-derived lipid compounds belonging to the steroid family and represent the major fraction of the unsaponifiable matter in plant lipids (Piironen et al., 2003). In contrast, cholesterol is the predominant sterol in animals. Structurally, PSs consist of a steroid backbone with a saturated bond between C-5 and C-6 of the sterol ring, an aliphatic side chain attached to the C-17 atom, and a hydroxyl group at the C-3 position (Figure 1). Both cholesterol and plant sterols naturally occur in free (non-esterified) and bound forms, such as esters of cinnamic or fatty acids and as glycosides. The bound forms are usually hydrolyzed in the small intestine by pancreatic enzymes. In humans, absorption

of free dietary cholesterol can reach up to 50%, with efficiency influenced by the structural variations and molecular weight of PSs (Fassbender *et al*., 2008).

Each plant species exhibits a unique phytosterol profile (Ogbe *et al*., 2015), and more than 250 PSs have been identified to date. Although phytosterols are present in virtually all plant-derived foods, unrefined plant oils—such as sesame, safflower, soybean, olive, macadamia, and almond oils—are especially rich sources. Nuts, seeds, whole grains, and legumes also contribute significantly to dietary intake (Sharifi-Rad *et al*., 2018; Piironen *et al*., 2003; Wadikar *et a*l., 2017).

Phytosterols are divided into sterols (unsaturated) and stanols (saturated) (Piironen *et al*., 2000). Among sterols, β-sitosterol, campesterol, and stigmasterol (Figure 2) are the most abundant in the human diet. While they share a cholesterol-like backbone, their side chains differ: β-sitosterol and stigmasterol both carry an ethyl group at C-24, whereas campesterol has a methyl group at the same position. Stigmasterol is derived from sitosterol through the activity of sterol C-22 desaturases. Minor sterols include brassicasterol and Δ-7-avenasterol. Stanols are also present in plants but account for only about 10% of total dietary PSs (Jones and AbuMweis, 2009).

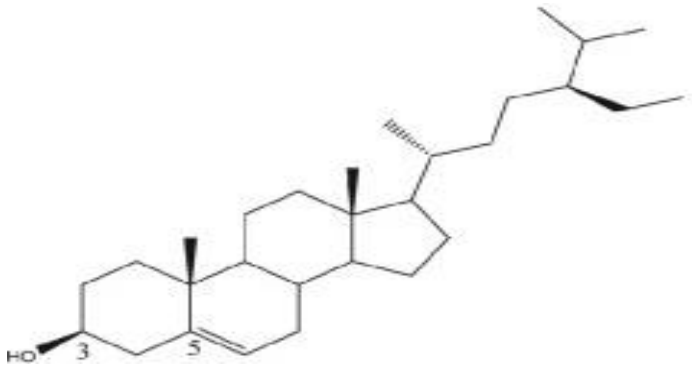

Fig 1.2.2.2.4.4a Phytosterol

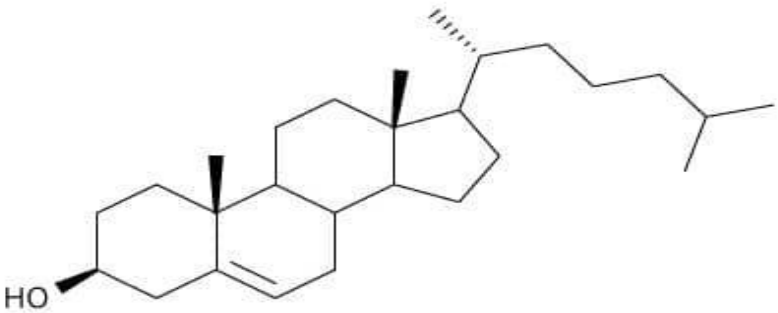

Fig 1.2.2.2.4.4b Cholesterol

1.2.2.2.4.5 Saponins

Saponins are a structurally diverse group of naturally occurring plant secondary metabolites found in a wide variety of foods, including grains, legumes, leafy greens, and even certain marine organisms. They are composed of a hydrophilic sugar moiety attached to a lipophilic aglycone, giving them an amphiphilic character and distinctive functional properties. This amphiphilic nature imparts surface-active behavior, enabling saponins to form stable foams and molecular complexes.

In food applications, saponins serve as natural emulsifiers, foaming agents, and stabilizers, thereby enhancing texture and product stability. Beyond their functional roles, they also offer health-promoting benefits such as cholesterol reduction and anticancer potential. Their broader bioactivities—anti-inflammatory, antimicrobial, antiviral, and antiparasitic—make them valuable candidates in the pharmaceutical sector. Notably, saponins exhibit cytotoxic effects against cancer cell lines and function as adjuvants by boosting immune responses to vaccines. Their ability to form stable complexes with drugs further highlights their promise in drug delivery systems.

Despite these advantages, challenges remain, including their inherent bitterness, cytotoxicity, and instability under certain processing conditions, which can limit their practical use in foods and related applications. This paper reviews the chemistry, functionality, and applications of saponins from diverse plant sources, with special attention to the regulatory aspects surrounding quillaja saponins in food. It also highlights future research directions aimed at fully harnessing the potential of saponins to improve food quality and human health. The review is intended as a valuable resource for researchers across food, feed, pharmaceutical, and material science fields (Kregie *et al,* 2017, Netala *et al,*2015).

In recent years, the rising consumer preference for natural products, combined with the unique physicochemical properties of saponins (notably their surfactant activity) and growing evidence of their biological functions—such as anticancer, hemolytic, and hypocholesterolemic effects—has positioned saponins as commercially valuable compounds with expanding applications across the food, cosmetics, and pharmaceutical industries (Mohan *et al,* 2016, Güçlü-Üstündağ Ö., Mazza G, 2007).

Despite their health-promoting potential and functional versatility, saponins also present certain challenges. A key limitation lies in their tendency to interact with other food components, forming complexes with proteins, lipids, and minerals such as iron, zinc, and calcium. The formation of these insoluble saponin–mineral complexes can reduce the bioavailability of essential nutrients (Milgate J., Roberts D.C.K, 1995). Additionally, some saponins exhibit poor water solubility, which complicates their incorporation and uniform distribution in food systems. This reduced solubility may impair their performance as emulsifiers or stabilizers in certain food applications (Schreiner *et al,* 2022).

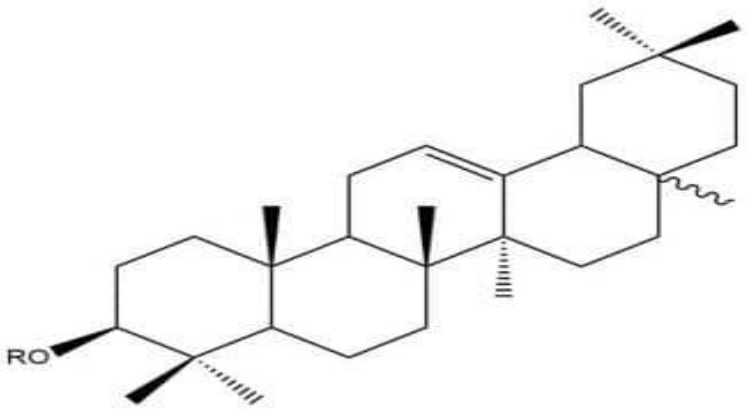

Fig 1.2.2.2.4.5 Saponin

1.2.2.2.4.6 Tannins

Tannins are a class of polyphenols, often referred to as plant polyphenols (Haslam E,1989). Historically, however, the term tannin was first applied to plant extracts exhibiting astringency, long before their chemical structures were understood. What differentiates tannins from other polyphenols is their characteristic ability to bind with proteins, alkaloids, pigments, high–molecular weight compounds, and metal ions, along with their antioxidant properties. These unique features also account for the distinct analytical approaches required for tannins compared with other polyphenols. Unlike general polyphenol quantification, tannin analysis relies on their binding activity. For instance, the classical hide powder method measures binding to animal skin proteins, while RA (relative astringency) and RMB (relative affinity to methylene blue) assays evaluate binding to blood proteins and methylene blue, respectively, under controlled pH conditions (Okuda, 1985).

The structural basis for these properties lies in the presence of two or three phenolic hydroxyl groups on a phenyl ring within moderately large molecules. Traditionally, tannins were divided into two categories: pyrogallol-type and catechol (or catechin-type) tannins, based on their phenolic group composition. Advances in tannin chemistry, however, led to their reclassification as hydrolyzable tannins and condensed tannins (Haslam E,1989, Okuda T, 2005, .Okuda T, 1999). Other types, such as caffetannins, labiataetannins, and phlorotannins, have also been classified as tannins (Okuda T, 2005, .Okuda T, 1999, Grassmann *et al,* 1958).

The discovery of bioactive stilbenoids, such as piceatannol (identified as the compound responsible for tannin activity in spruce bark) (Nakayama *et al,* 1989), along with resveratrol oligomers and phlorotannins from brown algae like monomeric eckol (Okuda *et al* 2009), has further broadened the scope of tannins and related polyphenols in recent decades.

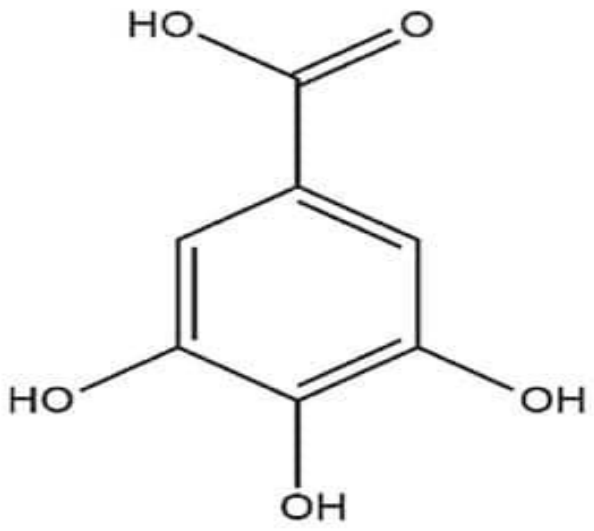

Fig 1.2.2.2.4.6 Tannins

1.2.2.2.4.7 Alkaloids

Alkaloids are naturally occurring specialized metabolites characterized by the presence of nitrogen in their chemical structures. Their wide-ranging biological activities arise from the diverse arrangements of atoms within these structures. Across different kingdoms, alkaloids appear in varied chemical forms and with distinct functional groups, contributing to their extensive biological significance. In marine organisms such as shellfish and

sponges, alkaloids including pinnatoxins, pinnamine, and halochlorine have been identified and are known for their potential in treating both cardiac and non-cardiac inflammatory conditions (Kuramoto *et al,* 2004). In terrestrial ecosystems, many ladybird beetles employ alkaloids as chemical defenses, releasing hemolymph rich in bitter alkaloids when threatened. Similarly, certain ant species produce alkaloids such as cis- and trans-2-methyl-6-alkylpiperidines, combined with proteinaceous compounds in their venom, which serve both protective and predatory functions (Braekman *et al,* 1998).

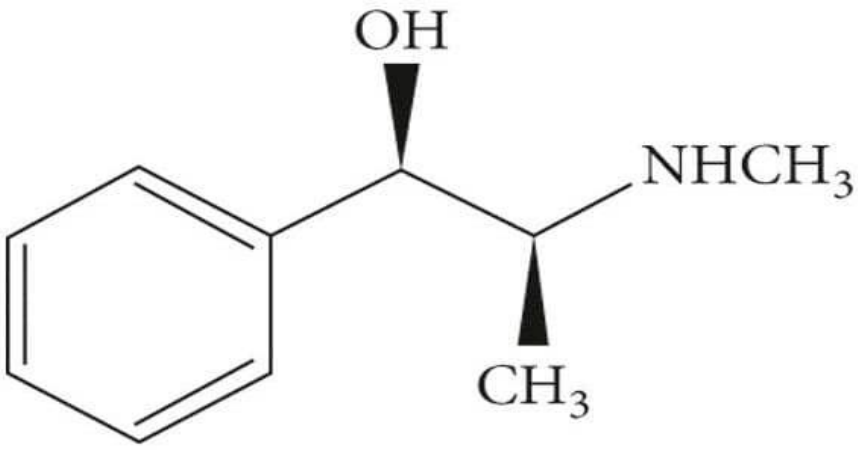

Fig 1.2.2.2.4.7 Alkaloids

1.2.2.3 PHYTOCHEMICAL SCREENING

Phytochemical screening is a cost-effective analytical technique used to identify the classes of phytochemicals—naturally occurring plant compounds—present in a plant extract. The process involves subjecting extracts to various chemical tests that produce observable changes, such as color shifts or the formation of precipitates, which indicate the presence of compounds like phenols, flavonoids, alkaloids, saponins, and terpenoids. This screening is valuable for detecting bioactive constituents with potential pharmaceutical or nutritional applications.

1.2.2.4 PHYTOCHEMICAL EXTRACTION METHODS

A wide range of extraction methods has been developed to obtain secondary metabolites from plant parts in high yield for potential use as drugs against various diseases (Mariamma, 2012). Plant extraction refers to the process of isolating active plant constituents, including secondary metabolites and pharmacologically active compounds,

through the use of suitable solvents and standardized procedures. Common extraction methods include digestion, maceration, infusion, percolation, decoction, supercritical fluid extraction, and Soxhlet extraction, among others (Abdullahi and Mainul, 2020). For purification of secondary metabolites from these extracts, techniques such as high-performance thin-layer chromatography (HPTLC), thin-layer chromatography (TLC), gas chromatography (GC), and paper chromatography (PC) are employed (Abdullahi & Mainul, 2020)

1.2.2.4.1 Traditional methods of extraction

1.2.2.4.1.1 Maceration

Maceration is a conventional extraction method in which coarsely powdered plant material is soaked in a suitable solvent at room temperature or under mild heating. This process enables the active constituents to diffuse into the solvent, thereby achieving extraction. It is particularly suitable for natural products that are sensitive to heat and rich in starch, mucilage, gums, or pectins (Velarde *et al,* 2023, Milic *et al,* 2020, Zaina *et al,* 2022, Chaudhry *et al,* 2022). The efficiency of this method depends on several factors, including the soaking time, solvent type, material-to-solvent ratio, and the particle size of the raw material (Cujic *et al,* 2016, Jovanovic *et al,* 2017).

Di *et al*, 2021 compared maceration with ultrasonic and high-power ultrasound methods for extracting phenolic compounds from hazelnut shells and found that phenolics obtained through maceration demonstrated stronger antioxidant activity. Despite its simplicity and effectiveness, maceration is generally time-consuming and yields relatively low extract volumes. For this reason, it is often combined with ultrasound to enhance efficiency. For instance, ultrasound-assisted maceration of olive leaves significantly increased the total phenolic content of olive oil while reducing extraction time compared with conventional maceration (Achat *et al,* 2012). Similarly, studies on citrus peel extraction showed that microwave- and ultrasound-assisted maceration greatly improved the efficiency of volatile compound recovery (Chanive *et al,* 2021).

1.2.2.4.1.2 Percolation

Percolation is a dynamic extraction method in which crushed plant material is placed in a percolation cylinder, and solvent is continuously added from the top, allowing it to

percolate through the material. As the solvent flows downward, it permeates the plant matrix, dissolving and leaching out the active constituents. This method ensures efficient solvent utilization, thorough extraction of bioactive compounds, and direct collection of the leachate. Percolation is typically carried out at room temperature, making it suitable for heat-sensitive substances. However, it is not appropriate for plant materials that swell excessively or lack organized structures. The major drawbacks of this method are its high solvent consumption and the relatively long extraction time (Wang *et al* ,2020, Wang *et al,* 2023).

Several factors influence the efficiency of percolation, including particle size, solvent composition, extraction time, percolation rate, and solvent volume (Avram *et al,* 2014, Shejawale *et al,* 2022, Kassing *et al,* 2012). Ethanol at varying concentrations, and in some cases white wine, is commonly used as the extraction solvent; therefore, measures should be taken to minimize solvent loss due to volatility. Wilson et al. (Wilson *et al,* 2022) compared maceration and percolation for extracting cannabidiol from Cannabis, reporting that percolation was more efficient. Likewise, in the extraction of phenolic compounds from Allium sativum, both extraction and recovery rates were higher with percolation than with maceration (Ahmad *et al,* 2020). In another study comparing methods for extracting volatile compounds from grapeseed oil, Soxhlet extraction yielded 67 volatile constituents, whereas percolation yielded 60 (Sevindik *et al,* 2022).

1.2.2.4.1.3 Decoction

Decoction is a traditional extraction technique in which the crude powdered plant material is boiled in water for a defined period, allowing the active constituents to be leached into the solvent. Water is the only solvent employed in this method. Although effective, decoction is not suitable for materials rich in starch, mucilage, volatile compounds, or constituents that readily degrade under heat. The method is most widely practiced in traditional Chinese medicine, where multiple herbs are often combined into formulas that are boiled and concentrated for therapeutic use over thousands of years.

Experimental evidence supports the pharmacological potential of decoction. For instance, Yang *et al,* 2022 reported that the Xuefu Zhuyu decoction exhibited neuroprotective effects in a controlled cortical impact (CCI) rat model, improving learning and memory and

suggesting its potential in treating traumatic brain injury. Other studies have demonstrated that decoction extracts are rich in phenolic compounds, contributing to notable antioxidant and antifungal activities (Silva *et al,* 2019, Martins *et al,* 2015). Martins *et al.* (2015, 2014) compared extracts obtained by decoction, infusion, and hydroalcoholic methods, finding that decoction yielded the highest flavonoid content and exhibited the strongest antioxidant and antimicrobial activities.

Despite its benefits, decoction has clear limitations. It can only extract water-soluble compounds, while heat-sensitive and volatile constituents may be lost or degraded. For example, Reynoso-Camacho *et al*, 2021, analyzed citrus decoctions and reported that flavanones and phytosterols were retained in the residual dregs but absent from the broth, while certain carotenoids degraded under high-temperature boiling. Similarly, Zan *et al.* (Zan *et al,* 2022) identified 85 volatile compounds from the Yinchenzhufu decoction and characterized its pharmacokinetic properties, providing insights into the therapeutic role of volatile components in disease treatment.

1.2.2.4.1.4 Reflux Extraction

Reflux Extraction is a conventional technique that employs volatile organic solvents, such as ethanol, to extract active compounds from plant materials. In this method, the extraction solvent is heated and distilled, while the vapor condenses and returns to the extraction vessel, repeatedly soaking the raw material. This continuous cycling enhances extraction efficiency and reduces overall solvent consumption. However, because the process requires prolonged heating, it is unsuitable for heat-sensitive compounds that may degrade under such conditions. Key factors influencing reflux extraction include the material-to-solvent ratio, extraction duration, and solvent concentration (Hu *et al,* 2018, Moldova *et al,* 2019).

Ma *et al*, 2022 optimized the reflux extraction of phenolic compounds from Pleioblastus amarus shells using ethanol as the solvent, identifying ideal conditions of 75% ethanol concentration, a 20:1 liquid–solid ratio, and an extraction time of 2.1 hours through response surface methodology. Similarly, studies have shown that pretreating raw materials by autoclaving prior to reflux extraction can significantly improve yield, as demonstrated in the extraction of polysaccharides and triterpenoids from Chaenomeles fruits, where autoclaving time markedly affected extraction efficiency (Shang *et al,*2021).

Despite its effectiveness, reflux extraction generally yields lower efficiency compared to modern methods such as ultrasound-assisted extraction and microwave-assisted extraction (Wei *et al,* 2016). For example, Yang *et al,* 2018, compared several methods for extracting apigenin from Scutellaria barbata D. Don, finding that ultrasound-assisted supercritical CO_2 extraction produced higher yields in less time than heat-reflux extraction. Similarly, in the extraction of pectin, microwave treatment was reported to affect both structure and quality, highlighting the sensitivity of some compounds to processing conditions (Zhang *et al,* 2018). Jiang *et al,* 2011, further compared steam distillation, reflux extraction, and ultrasound-assisted extraction for essential oils, noting differences in both yield and chemical composition. These findings emphasize the importance of selecting an appropriate extraction method based on the physicochemical nature of the target compounds.

1.2.2.4.1.5 Soxhlet Extraction

Soxhlet Extraction, also referred to as the continuous reflux extraction method, utilizes the siphon principle and solvent reflux to repeatedly wash solid material with fresh solvent. This design enhances extraction efficiency and reduces solvent consumption compared to conventional reflux extraction, which is limited by high solvent usage and repeated reflux cycles (Luque *et al,*2010). However, Soxhlet extraction is unsuitable for thermolabile compounds, as the process involves prolonged heating. The method is commonly applied for the extraction of phenolic compounds, essential oils, and other moderately soluble plant constituents (Saini *et al,* 2021, Teixeira *et al,* 2018, Da *et al,* 2013).

Alara *et al,* 2018, optimized Soxhlet extraction of phenolic compounds from Vernonia cinerea leaves, evaluating the influence of extraction time, feed-to-solvent ratio, and ethanol concentration on yield, total polyphenol, and flavonoid content. Their findings indicated that an extraction time of 2 hours, a feed-to-solvent ratio of 1:20 g/mL, and an ethanol concentration of 60% v/v provided the highest yield and bioactive compound recovery.

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1.2.2.4.1.6 Steam Distillation

Steam Distillation is a widely used method for extracting volatile constituents, particularly essential oils, from raw materials that are water-insoluble, volatile, and stable under steam

treatment (Haro *et al,* 2021, Abd *et al,* 2022, Pateiro *et al,* 2018). The process involves crushing and soaking the plant material, followed by passing steam through it to release volatile components, which are then condensed and collected as separate layers. Steam distillation is favored for its relatively simple equipment and operation. However, exposure to high temperatures during the process may degrade thermolabile constituents in essential oils. Additionally, incomplete separation of water and oil and the tendency of volatile oils to emulsify can reduce recovery efficiency.

Several factors influence the yield and quality of volatile oils, including herb-related parameters (e.g., moisture content, particle size, and plant origin) and extraction conditions (e.g., material-to-liquid ratio and distillation time) (kaya *et al,* 2020, Zheljazkov *et al,* 2013). Comparative studies have shown that hydrodistillation often achieves higher yields than steam distillation, highlighting the importance of method selection for optimizing extraction outcomes (Tadic *et al,* 2023, Peng *et al,* 2019).

1.2.2.4.2 Modern Extraction Method

1.2.2.4.2.1 Ultrasound-Assisted Extraction

The principle of ultrasound-assisted extraction (UAE) relies on the mechanical and cavitation effects generated by ultrasonic waves, which facilitate the release of bioactive compounds from plant matrices. The mechanical effect refers to the propagation of ultrasonic waves through a medium, causing particle vibration within its spatial range and thereby enhancing diffusion and mass transfer. The cavitation effect, in contrast, involves the formation, growth, and collapse of microbubbles in the medium due to ultrasound. When the applied sound pressure exceeds a critical threshold, bubbles expand by directional diffusion, form resonant cavities, and eventually collapse, releasing localized energy that disrupts cell walls and promotes solvent penetration (Cravotto and Cintas, 2006, Chemat *et al,* 2017, Wen *et al,* 2018).

Compared to conventional extraction techniques, UAE is considered a green, cost-effective, and sustainable technology. It offers reduced extraction time, improved efficiency, and lower solvent consumption, thereby contributing to energy conservation and reduced environmental impact (Song *et al,* 2023).

Experimental studies have demonstrated the advantages of UAE. For instance, Lin *et al,* 2023, reported that polysaccharides extracted from Shatian pomelo peel by ultrasound showed higher yields and stronger antioxidant activity compared with other methods. The main factors influencing extraction efficiency include solid–liquid ratio, ultrasonic power, sonication time, and extraction temperature. Optimization strategies, such as response surface methodology, are commonly applied to fine-tune these parameters. Achat *et al,* 2012, extracted phenolic compounds from olive leaves via ultrasonic maceration to enhance the nutritional value of olive oil, achieving optimal conditions at 60 W power, 16 °C temperature, and 45 min sonication. Similarly, Nurkhasanah *et al,* 2023 optimized anthocyanin extraction from pigmented corn, with optimal parameters of 36% methanol, pH 7, 73% ultrasonic power, 10 min extraction time, and 70 °C temperature.

1.2.2.4.2.2 Microwave-Assisted Extraction

Microwave-assisted extraction (MAE) utilizes microwave energy to disrupt plant cell walls and membranes, thereby releasing intracellular bioactive compounds. In addition, microwaves rapidly and uniformly heat the extraction system, significantly reducing extraction time and minimizing solvent consumption (Eskilsson and Bjorklind, 2000, Bagade and Patil 2021, Manzoor *et al,* 2021). The main factors influencing MAE efficiency include extraction time, extraction temperature, material–liquid ratio, and microwave power.

Hu *et al*, 2019 extracted polysaccharides from Camptotheca acuminata fruits using MAE and optimized conditions through response surface methodology. The optimal parameters were a material–liquid ratio of 1:40, microwave power of 600 W, extraction time of 14 min, and extraction temperature of 70 °C. Compared with traditional techniques, MAE consistently demonstrates higher efficiency and better yields. For example, Ding *et al,* 2014 compared ultrasound-assisted extraction, MAE, and reflux extraction with ionic liquids as solvents, and found that MAE achieved the highest efficiency. Similarly, Fernandez-Pastor *et al,* 2017 reported that microwave-assisted extraction of triterpene acids from olive skins was faster and yielded higher concentrations than Soxhlet extraction.

Beyond yield, MAE can also enhance the bioactivity of extracts. In a study on lavender, microwave-assisted extraction produced essential oils with stronger antibacterial activity

compared to oils obtained by hydrodistillation (Liu *et al,* 2018). These findings emphasize that MAE is a highly efficient, time-saving, and versatile method, making it a promising alternative to conventional extraction processes.

1.2.2.4.2.3 Supercritical Fluid Extraction

A supercritical fluid is formed when a substance is at its critical point (critical temperature, pressure, and density), where it exhibits unique dual properties of both liquids and gases. At this state, the fluid combines the density and solubility of a liquid with the viscosity and diffusion capacity of a gas, the latter being up to 100 times higher than that of a liquid (Rozzi and Singh, 2002).

Supercritical fluid extraction (SFE) is a modern green extraction technology that uses supercritical fluids as solvents, with carbon dioxide (CO_2) being the most widely applied (Diaz *et al,* 2006). CO_2 has several advantages as an extraction medium:

1. Its critical temperature is close to room temperature, making it suitable for extracting heat-sensitive compounds.
2. Its critical pressure is moderate, allowing safe and convenient operation.
3. Its density is highly responsive to changes in temperature and pressure, enabling fine-tuned control of solubility.
4. It is non-toxic, non-flammable, chemically stable, inexpensive, readily available, and environmentally friendly (Padrela *et al* 2018, Pradhan *et al,* 2015, Tarbernero and Cardela, 2020, Wrona *et al,* 2017).

Compared with conventional methods, SFE offers shorter extraction times, higher efficiency, and improved yields (Donelian *et al,* 2009). For instance, Zhang *et al,* 2022 compared steam distillation and supercritical CO_2 extraction of camphor tree essential oil, and found that SFE not only produced a higher yield but also yielded oils with a more diverse chemical profile, as confirmed by GC/MS analysis.

The efficiency of SFE is primarily influenced by extraction temperature and pressure, and conditions are often optimized using response surface methodology. Zermane *et al,* 2014 optimized the supercritical CO_2 extraction of essential oil from Myrtus communis L. leaves, reporting the highest yield at 313 K and 30 MPa.

1.2.2.4.2.4 Pressurized Liquid Extraction

Pressurized liquid extraction (PLE) is an automated extraction technique that employs organic solvents under elevated temperatures (50–200 °C) and high pressures (1000–3000 psi) (Mustafa and Turner, 2011). Elevated temperature weakens intermolecular forces—such as van der Waals interactions, hydrogen bonding, and dipole attractions—between target molecules and the sample matrix, thereby enhancing extraction efficiency (Giergielewicz *et al,* 2001). At the same time, high pressure maintains the solvent in a liquid state above its normal boiling point, improving solubility and penetration.

Compared with conventional solvent extraction, PLE offers several advantages, including reduced solvent consumption, faster extraction times, high recovery rates, and good reproducibility (Carabias *et al,* 2005, Conte *et al,* 2016). For example, Cam *et al,* 2010 [compared conventional organic solvent extraction with PLE for polyphenols from pomegranate peels, demonstrating that PLE is an efficient and sustainable alternative, supporting its classification as a green extraction method.

Key factors influencing PLE include solvent polarity, liquid–solid ratio, and extraction temperature (Cea *et al,* 2019, Jacque *et al,* 2008, Dobroslavic *et al,* 2022). Supasatyankul *et al,* 2022, optimized the extraction of phenolic and flavonoid compounds from mung bean (Vigna radiata L.) seed coat using response surface methodology, identifying optimal conditions at 160 °C, 1300 psi, and 50% ethanol. Similarly, in the extraction of volatile compounds from coffee beans, temperature was identified as the most significant factor (Cheong *et al,* 2013).

1.2.2.4.2.5 Enzyme-Assisted Extraction

Enzyme-assisted extraction (EAE) utilizes the ability of enzymes to degrade plant cell walls, thereby releasing intracellular compounds more efficiently. By selecting specific enzymes, certain low-polarity lipophilic components can be converted into water-soluble glycoside-like derivatives, improving extraction efficiency. Enzymes can also degrade or remove interfering substances such as starch, proteins, and pectin, enhancing the clarity and quality of the extract (Puri *et al*, 2012).

Giahi *et al*, 2021demonstrated that pretreating licorice roots with cellulase, hemicellulase, and pectinase significantly increased the yield of glycyrrhizic acid. Moreover, residues left after conventional extraction can be re-treated with enzymes to recover non-extractable active compounds, thus improving overall utilization (Dominguez *et al*, 2021).

The main factors influencing enzymatic extraction include the type of enzyme (optimum pH and temperature), enzyme concentration, and substrate concentration (Das *et al*, 2021). Since enzymatic extraction is typically carried out at mild temperatures, it is particularly suitable for isolating heat-sensitive natural products (Sowbhagya and Chitra, 2010).

EAE is often combined with other modern extraction technologies to enhance efficiency. Examples include ultrasound-assisted enzymatic extraction (Lin *et al,* 2023), enzyme-assisted supercritical fluid extraction (Mushtaq *et al,* 2017), and enzyme-assisted microwave extraction (Khruengsai *et al,*2023). Such synergistic approaches have been shown to significantly improve extraction yield and reduce processing time.

1.2.2.4.2.6 Ionic Liquid Extraction

onic liquids (ILs) are salts composed of organic cations and organic or inorganic anions that remain in the liquid state at room temperature. They are typically characterized by high viscosity and density (Khruengsai *et al,* 2023). Compared with conventional organic solvents, ILs offer several advantages, including low vapor pressure, non-volatility at room temperature, chemical and thermal stability, non-flammability, good electrical and thermal conductivity, a broad solubility range, and structural tunability (Endres 2002, Hallet and Welton, 2011, Kondrat *et al,* 2023, Tang *et al,* 2012).

The extraction mechanism of ILs lies in their ability to interact with cellulose in plant cell walls, breaking hydrogen bonds between cellulose molecules and facilitating cellulose dissolution. This process enhances the release of intracellular bioactive compounds, thereby improving the extraction yield. By tailoring the structural components of ILs, specific intermolecular interactions such as electrostatic forces, dispersion forces, and hydrogen bonding can be optimized, enabling highly selective and efficient extraction of target compounds.

Modern extraction approaches often integrate ILs with microwave or ultrasound-assisted techniques to further improve efficiency and selectivity (Rout *et al,* 2011, Gu *et al,* 2016, Liang *et al,* 2017, Tan *et al,* 2016). Ding *et al,* 2014 designed and synthesized 11 ILs for extracting Praeruptorin A from Radix peucedani, reporting that soluble guanidinium ILs achieved significantly higher yields compared with insoluble variants. Among them, [TMG]$CH_2CH(OH)COOH$ demonstrated an extraction efficiency comparable to methanol. Similarly, Wang *et al* 2015, applied ultrasonic-assisted IL extraction to isolate alkaloids from Phellodendron amurense Rupr. They found that shorter alkyl chain lengths in ILs correlated with higher alkaloid yields, due to increased solubility, with optimal extraction conditions being 100 W ultrasonic power, 75 min extraction time, and a material–liquid ratio of 1:14.

In addition to non-volatile compounds, ILs have also been employed to extract volatile components. For example, in the microwave-assisted extraction of essential oils from Schisandra chinensis using 1-lauryl-3-methylimidazolium bromide, ILs not only enhanced extraction efficiency but also significantly reduced extraction time (Liang *et al,* 2019).

1.2.3 ELEMENTAL ANALYSIS

Elemental analysis involves both the qualitative identification and quantitative measurement of chemical elements (atoms or ions) within a sample. Qualitative detection is based on observing analytical signals such as the formation of a precipitate or characteristic crystals, color changes, release of gases, appearance of distinct spectral lines, luminescence, and similar phenomena. Quantitative determination, on the other hand, requires measuring the magnitude of an analytical signal, which may include the mass of a precipitate, the intensity of an electric current, solution absorbance, spectral line intensity, luminescence, radioactivity, or reaction rate.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 SOLVENTS AND REAGENTS

- Ethyl acetate
- Water
- Distilled water
- Glacial acetic acid
- Ferric chloride solution
- Concentrated H_2SO_4
- Picric acid
- Wagner`s reagent
- Acetic acid
- Dilute H_2SO_4
- Chloroform
- 90% ethanol
- 10% FeCl
- 5% KOH solution
- Dilute HCl
- 10% lead acetate
- Ferric chloride ($FeCl_3$) solution
- Fehling`s solution
- Nitric acid
- Hydrichloric acid
- 0.6M H_2SO_4
- 28mM sodium phosphate
- 4mM ammonium molybdate
- Sodium nitroposside
- Phosphate buffer saline (PBS)
- Breiss reagent
- EDTA (Ethyl)
- MBT (Nitro blue tetrazolium)
- Riboflavin

- 10% red blood cell (RBC) suspension
- Saline
- Aspirin
- 1% aqueous solution of bovine albumin
- 1 N HCl

2.1.2 APPARATUS

- 250ml beaker
- Conical flask
- Maceration jar
- Stirrer rod
- Spatula
- Reagent bottle
- Sample bottle
- Volumetric flask
- Funnel
- Measuring cylinder
- Test tube

2.1.3 EQUIPMENT

- Rotary evaporator
- Heating mantle
- Oven
- Analytical balance
- Water bath
- PH meter
- Refrigerator
- UV/VIS spectrometer
- AAS
- Fume cupboard

2.1.4 OTHERS

- Whatman filterpaper

- Tissue
- Facemask
- Fuel paper
- Hand gloves

2.2 METHOD

2.2.1 SAMPLE COLLECTION

Fresh leaves and stem bark of *Ocimum gratissimum* were obtained from Sapele Road, Benin City, Edo State, NIgeria. The plant was then identified and authenticated by Prof. Henry Adewale Akinnibosun of the department of Plant Biology and Biotechnology, Faculty of Life Science, University of Benin with a voucher specimen number UBH-0333.

2.2.2 PREPARATION OF EXTRACT

Plant obtained i.e the stembark were separated from the leaves of *Ocimum gratissimum* and was air dried at room temperature for 2 weeks. The sample was pulverized to fine powder using a mechanical grinder according to the method of Wokocha and Okereke, (2005) with a net wet of 250g. The powdered stem bark was stored in an air-tight container for further analysis. An exhausive cold maceration was carried out by taking 180g of the dry stem bark sample of *O. gratissimum* and poured into a glass jar and was mixed with 800ml of ethyl acetate of which was left for 72hrs, then filtered. It was then concentrated using rotary evaporator. It was then exposed at room temperature to enable the solvent to evaporate from the extract and the percentage yield was calculated to be 6.39%. The extract was then stored in refrigerator at 4 ^{o}C.

Percentage Yield (%) =A/B
A= amount of extract recovered from solvent
B= amount of plant sample

2.2.3 PHYTOCHEMICAL SCRENENING

Approximately 0.5g of the crude powdered sample of *Ocimum gratissimum* was mixed with 10ml of distilled water. It was shakened and filtered with whatmann filterpaper. The filtrate obtained was used to carry out the following test.

2.2.3.1 TEST FOR ALKALOIDS (PICRIC ACID/ WAGNER`S REAGENT)

1 ml of the plant extract was transferred into two different test tube labeled A and B

TO PORTION A

2 ml of wagner`s reagent was added into it. The presence of reddish-brown precipitate indicate a positive test.

TO PORTIN B

2 ml of picric acid was added into it. The presence of alkaloids is indicated by a yellow precipitate.

2.2.3.2 TEST FOR GLYCOSIDE (GENERAL TEST)

About 1 ml of the extract was dissolved in 1 ml of glacial acetic acid containing one drop of ferric chloride solution. This was under-layered with 1 ml of concentrated H_2SO_4. A brown ring obtained indicated the presence of glycoside.

2.2.3.3 TEST FOR SAPONINS (FROTHING TEST)

About 0.5 ml of the extract was shaken vigorously with 10 ml of distilled water for one minute. The presence of saponin is indicated by a stable foam.

2.2.3.4 TEST FOR STEROIDS (ACETIC ANHYDRIDE/ H_2SO_4)

About 2 ml of acetic anhydride was added to 0.5 ml of the plant extract in 2 ml of dilute H_2SO_4. A colour change from violet to blue or green is required for the presence of steroids.

2.2.3.5 TEST FOR TERPENOIDS (SALKOWSKI TEST)

5 ml of the filtrate was mixed in 2 ml of chloroform and 3 ml of concentrated H_2SO_4 was carefully added down the side of the inner wall of the test tube to form a layer. The presence of terpenoids is indicated by a reddish brown colouration of the inter-phase.

2.2.3.6 TEST FOR PHENOLIC (ETHANOL/ $Fecl_3$)

5 ml of 90% ethanol was added to 1 ml of the plant extract. 1 drop of 10% FeCl was added into it. A pale yellow colouration indicates the presence of phenolic.

2.2.3.7 TEST FOR EUGENOLS (KOH/HCl)

2 ml of the filtrate was mixed with 5 ml of 5% KOH solution. The aqueous layer was separated and filtered. Few drops of dilute HCl were added to the filtrate. A pale yellow is indicative of a positive test.

2.2.3.8 TEST FOR FLAVONOIDS (LEAD ACETATE TEST)

About 2 ml of the extract was boiled in 10 ml of distilled water and filtered. A few drops of 10% lead acetate solution was added to 5 ml of the filtrate. The presence of flavonoids is indicated by a yellow precipitate.

2.2.3.9 TEST FOR PROTEIN (NITRIC ACID)

Few drops of nitric acid was added to 1 ml of the filtrate. The presence of protein is indicated by a bluish-violet colour.

2.2.3.10 TEST FOR TANNINS (FERRIC CHLORIDE)

10 ml of distilled water was added to about 2 ml of the extract and was boiled for 3 minutes and then filtered into halves. Ferric chloride ($FeCl_3$) solution was added to about 2 drops of the filtrate. The formation of a bluish precipitate indicates a positive test.

2.2.3.11 TEST FOR REDUCING SUGAR (FEHLING`S TEST)

Equal volume of fehling`s solution A and B was boiled for 1 minute and a volume of the plant extract was added and boiled for 5 minutes. A bricked-red precipitated is indicative of a positive test.

2.2.4 ELEMENTAL ANALYSIS

1g of the stem bark sample was taken into a 100 ml flask and 13 ml of mixed acid was added, i.e, Nitric acid (HNO_3) and Hydrochloric acid (HCl) in a ratio of 3:1. The sample was boiled in acid solution in fume cupboard with hot plate till the digestion was completed which was indicated by a cleared form. It was then allowed to cool. Then this digested sample was then transferred in 100 ml volumetric flask and the volume was made up to a 100 ml mark with distilled water. It was transferred into a sample bottle. The solution was analysed with the element of interest using Atomic Absorption Spectrometer (AAS).

2.2.5 VITAMIN PROFILE ASSAY

2.2.5.1 VITAMIN A

Vitamin A was estimated by the method of Bayfield and Cole (1980).The assay was based on the spectrophotometeric estimation of the colour produced by vitamin A acetate or palmitate with TCA (Trichloroacetic acid). All procedures were carried out in the dark to avoid the interference of light. One gram (1g) of sample was mixed with 1.0 ml of saponification mixture and refluxed for 20 minutes at 60 ^{O}C in the dark. The tubes were cooled and 20 ml of water was added and mixed well. Vitamin A was extracted twice with 10ml of (40-60^{O}C) petroleum ether. The two samples were pooled and washed thoroughly with water. Anhydrous sodium sulphate was added to remove excess moisture. An aliquot of the sample (1.0 ml) was taken and evaporated to dryness at 60^{O}C. The residue was dissolved in 1.0ml chloroform. Standards (vitamin A palmitate) of concentrations ranging from 0-7.5g were pipetted out into a series of test tubes.

The volume in all the tubes was made up to 1.0 ml with chloroform. TCA (Trichloroacetic acid) reagent (2.0 ml) was added rapidly, mixed and the absorbance was read immediately at 620 nm in a spectrophotometer (Genesys 10 UV). The same procedure was repeated for the sample tubes also.

A calibration curve was plotted and the concentration of the sample was extrapolated

The equation from the graph

Y = 0.0257x + 0.0806

Where:

Y = Absorbance

X = Concentration (mg/L)

2.2.5.2 VITAMIN B1 AND B_2

One gram (1 g) of sample was weighed into a conical flask and was dissolved with 100 ml of deionized water. It was shaken thoroughly and heated for 5 minutes and allowed to cool and filtered.The filtrate was poured into cuvette and their respective wavelength for the vitamins set to read the* absorbance using spectrophotometer.

Vitamin B1 = 261 nm

Vitamin B2 = 242 nm

Calculations:

$$\text{Concentration (mg\%)} = \frac{A \times D.F \times \text{volume of cuvette (3ml)}}{E}$$

$$D.F = \frac{\text{Final volume after dilution}}{\text{Volume of sample taken}}$$

Where A = absorbance

E = extinction coefficient = 25 for B1 and B

DF = dilution factor =1

2.2.5.3 VITAMIN B_3

5 g of sample was dissolved in 20 ml of anhydrous glacial acetic acid and warmed slightly. 5 ml of acetic anhydride was added and mixed. 2 -3 drops of crystal violet solution was added as indicator. It was titrated with 0.1 M perchloric acid to a greenish blue colour.

Calculation:

$$\text{VitaminB3} = \frac{\text{titre value} \times 0.0122}{0.1}$$

Titre value = Absorbance

2.2.5.4 VITAMIN B_6

5 g of sample was dissolved in a mixture of 5ml of anhydrous glacial acetic acid and 6 ml of 0.1 m mercury II acetate solution.2 drops of crystal violet was added as indicator.It was titrated with 0.1 m perchloric acid to a green colour end point.`

Calculation: each meal of 0.1 M perchloric acid is equivalent to 0.02056 g of C8H11NO3HCL (Perodixine hydrochloride or Vitamin B_6)

2.2.5.5 VITAMIN B_{12}

Sample preparation: 0.1 ml of equivalent sample was weighed and taken into separator. In separator, 5 ml of water was added, it was mixed well and extracted with 5 ml chloroform. The water layer was discarded and the chloroform was taken in dry 50 ml volumetric flask by passing it through anhydrous sodium sulphate and made up to 50 ml with chloroform.

Procedure: 2 ml sample and blank solution was taken into test tube. In each test tube, 2 ml of 0.2% sol ution of phenyl hydrazine (in hydrochloric acid and alcohol in ratio of 1:5 v/v) was added and mixed well. After that it was heated on water bath until it was almost dryed and was cool at room temperature. 2 ml solution mixture (ammonia and alcohol in ratio of 1:1) was added in each test tube and 1 ml pyridine was. It was absorbance recorded at 635 nm against blank. Standard cobalamine was also analyzed and treated same as sample.

Calibration curve was plotted and the concentration of sample extrapolated.

Equation from the graph:

$Y = 0.0477x + 0.0294$

Where:

Y = Absorbance

X = Concentration (mg/L)

2.2.5.6 VITAMIN C

Ascorbate was extracted from 1 g of the sample using 4% TCA (Trichloroacetic acid) and the volume was made up to 10 ml with the same. The supernatant obtained after centrifuging at 2000 rpm for 10 minutes was treated with a pinch of activated charcoal, shaken vigorously using a cyclomixer and kept for 5 minutes. The charcoal particles were removed by centrifugation and aliquots were used for the estimation.

Procedure

Standard ascorbate ranging between 0.2 – 1.0 ml and 0.5 ml and 1.0 ml of the supernatant (A clear liquid above it after it had been allowed to settled) were taken. The volume was made up to 2.0 ml with 4% TCA (Trichloroacetic acid). DNPH (2, 4 -dinitrophenyl hydazine) reagent (0.5 ml) was added to all the tubes, followed by 2 drops of 10% thiourea solution. The contents were mixed and incubated at 370^{o}C for 3 hours resulting in the formation of osazone crystals. The crystals were dissolved in 2.5 ml of 85% sulphuric acid, in cold. To the blank alon, DNPH reagent and thiourea were added after the addition of sulphuric acid. The tubes were cooled in ice and the absorbance was read at 540 nm in a spectrophotometer.

A standard graph was constructed using an electronic calculator set to the linear regression mode. The concentration of ascorbate in the sample were calculated .

Equation from the graph :

$y = 0.0135x + 0.0062$

Where:

Y = Absorbance

X = Concentration (mg/L)

2.2.5.7 VITAMIN D

Vitamin D was assayed according to the method of Brockmann *et al.* (1974) and the principle is base on the formation of a yellow color by reaction of the vitamin with a chloroform solution of trichloroacetic acid,

For the standard preparation, 25 mg of vitamin D3 was weighed accurately and its working standard was taken at 25 ml.

volumetric flask with solution mixture (chloroform and methanol in ratio 1:9) was dissolved and diluted with solution mixture and make up to the mark and it was mixed thoroughly.

For the sample preparation , 0.1 ml equivalent sample was weighed accurately and 25 ml volumetric flask with solution mixture (chloroform and methanol in ratio 1:9) was taken and the sample was dissolved and diluted with solution mixture and make up to the mark and was mixed thoroughly. Then 1.6 ml of 0.25N HCL, 0.5 ml of 15.0%trichloroacetic acid (TCA) and 0.5 ml of 0.375% of thiobarbituric acid (TBA) was added It was absorbance recorded at 464 nm against blank.

Equation from the graph:

$y = 0.03x + 0.0048$

Where:

Y = Absorbance

X = Concentration (mg/L)

2.2.5.8 VITAMIN E

The sample (2.5 g) was homogenized in 50ml of 0.1 N sulphuric acid and allowed to stand overnight. The contents of the flask were shaken vigorously and filtered through Whatman No.1 filter paper. Aliquots of the filtrate were used for the estimation.

Into 3 stoppered centrifuge tubes, 1.5ml of sample sample, 1.5 ml of the standard and 1.5 ml of water were pipetted out separately. To all the tubes, 1.5 ml of ethanol and 1.5 ml of xylene were added, mixed well and centrifuged. Xylene (1.0 ml) layer was transferred into another stoppered tube. To each tube, 1.0 ml of dipyridyl reagent was added and mixed well. The mixture (1.5 ml) was pipetted out into a cuvette and the extinction was read at 460 nm.

Equation from the graph:

$y = 0.0204x + 0\ 0296$

Where:

Y = Absorbance

X = Concentration (mg/L)

2.2.6 ANTIOXIDANTS STUDY

2.2.6.1 FERRIC REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

The principle of the assay is the quantification of ferric degradation product, by its condensation with the extract. The reducing power of the extracts was determined as described by Oyaizu (1986) methods.

0.25 ml of the extracts was mixed with 0.25 ml of 200 mMSodium phosphate buffer pH 6.6 and 0.25 ml of 1% Potassium ferricyanide. The mixture was incubated at 50 ^{o}C for 20 min, thereafter 0.25 ml of 10% trichloroacetic acid was added and incubated at 37 ^{o}C for 10 min, 1 ml of the upper layer was mixed with 1 ml of distilled water and 0.2 ml of ferric chloride and the absorbance was measured at 700 nm.

2.2.6.2 SUPEROXIDE SCAVENGING ACTIVITY

The superoxide scavenging ability of the sample was assessed by the method of Winterbourn *et al,* (1975) and the assay was based on the inhibition of the production of nitroblue tetrezolium formazon of the superoxide ion by the sample and was measured spectrophotometrically at 560nm.

Superoxide anions were generated in samples that contained in 3.0 ml, 0.02 ml, of the stem samples (20 mg), 0.2 ml EDTA (Ethylene diamine tetraacetic acid), 0.1ml of NBT (Nitro Blue Tetrazolium), 0.05 ml of riboflavin and 2.64ml of phosphate buffer. The control tubes were also set up where DMSO (Dimethyl Sulphoxide) was added instead of the sample. All the tubes were vortexed and the initial optical density was measured at 560nm in spectrophotometer (Genesys, 10-S, USA). The tubes were illustrated using fluorescent lamp for 30 minutes. The absorbance was measured again at 560 nm. The difference before and after illumination was indicative of superoxide anion scavenging activity.

2.2.7 ANTI-INFLAMMATORY EXAMINATION

2.2.7.1 HEAT INDUCED HAEMOLYSIS (Sakat *et al,* 2010)

The reaction mixture (2 ml) consisted of 1 ml test sample of different concentrations (100 - 500 µg/ml) and 1 ml of 10% RBCs suspension, instead of test sample only saline was

added to the control test tube. Aspirin was used as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56 ºC for 30min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates for all the test samples. The Percentage inhibition of Haemolysis was calculated as follows: Percentage inhibition = (Abs control – Abs sample) X 100/ Abs control

2.2.7.2 INHIBITION OF ALBUMIN DENATURE

The anti-inflammatory activity of *Ocimum gratissimum* was studied by using inhibition of albumin denaturation technique which was studied according to Mizushima *et al* [1968] and Sakat *et a*l [2010] followed with minor modifications. The reaction mixture was consisted of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount of 1 N HCl. The sample extracts were incubated at 37 ºC for 20 min and then heated to 51 ºC for 20 min, after cooling the samples the turbidity was measured at 660nm.(UVVisible Spectrophotometer Model 371, Elico India Ltd) The experiment was performed in triplicate. The Percentage inhibition of protein denaturation was calculated as follows: Percentage inhibition = (Abs Control –Abs Sample) X 100/ Abs control.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 RESULTS

3.1.0 ORGANOLEPTIC PROPERTIES OF POWDERED *OCIMUM GRATISSIMUM*

The organoleptic properies of powdered stem of *Ocimum gratissimum* is reported below.

- Colour……Brown
- State……..Solid
- Texture……..Grirry powder

3.1.1 PERCENTAGE YIELD OF THE EXTRACT

The percentage yield of the stem bark of *Ocimum gratissimum* was 6.39%

3.1.2 PHYTOCHEMICAL SCREENING

Table 3.1.2: phytochemical constituents of water extract of *Ocimum gratissimum* stem bark

Phytochemicals	Observation
Alkaloids	+
Glycosides	+
Saponins	+
Steroids	_
Terpenoids	_
Phenolics	+
Eugenols	+
Flavonoids	+
Protein	+
Tannins	_
Reducing sugar	+

+=Present =Absent

Table 3.1.2 shows the qualitative phytochemical component of water extract of the stem bark of *Ocimum gratissimum*

The result obtained for the preliminary qualitative phytochemicals screening of *O, gratissimum* stem bark using water as extracts indicate the presence of therapeutically active components such as alkaloids, glycosides, saponins, phenolic, eugenols, flavonoids, protein and reducing sugar. This outcome agrees with the existing literature by Nweze and Eze, 2009. This finding confirms the rich phytochemical composition of the plant.

According to research, alkaloids are widely known for their medicinal roles and one of the most biological properties of alkaloids is their toxicity against cells of foreign organisms. These activities have been widely studied for their potential use in the elimination and reduction of human cancer cell lines. (Chidinma *et al* 2023). In the treatment of illnesses like hyperglycemia and hypercholesterolemia, saponins are of great importance and also serves as antioxidants and anti-inflammatory agents. (Ogbeide *et al*, 2022). Tannins have also been known to be effective in wound healing and anti-parasitic activity. (Ogbeide *et al*, 2024). Phenolics are Strong antioxidants compounds that protect against heart disease, aging, and inflammation (Balasundram *et al*., 2006). Also Glycosides Shows cardiotonic, laxative, and anti-inflammatory activities and acts as pro-drugs releasing active molecules (Okwu, 2004).

Therefore, the result of this study confirms that *Ocimum gratissimum* stem bark contains important bioactive compounds responsible for its various medicinal uses, including antimicrobial, antioxidant, anti-inflammatory, and cardioprotective activities.

3.1.3 ELEMENTAL ANALYSIS

Table 3.1.3: result for elemental contents of the stem of *Ocimum gratissimum*

Element	Ca	Na	K	Mg	Fe	Zn	Mn	Cu
Concentration (mg/Kg)	21.70	14.20	13.60	4.68	1.40	0.69	0.90	0.17

Table 3.1.3 shows the quantitative results for elemental analysis of the stem bark of *Ocimum gratissimum.*

The elemental analysis of the stem of *Ocimum gratissimum* revealed the presence of calcium (Ca), sodium (Na), potassium (K), magnesium (Mg), iron (Fe), zinc (Zn), manganese (Mn), and copper (Cu). Calcium (21.7 mg/kg) was the most abundant element, followed by sodium (14.2 mg/kg) and potassium (13.6 mg/kg).

Calcium plays a vital role in strengthening plant cell walls and regulating membrane permeability (Rengel, 2015., Marschner, 2012). In humans, it supports bone and teeth formation, muscle contraction, and nerve signaling (FAO/WHO, 2004). The high calcium level in this study agrees with Idris *et al*. (2011), who also reported high calcium in the leaves and stems of *O. gratissimum,* indicating the plant's potential as a calcium source.

The concentrations of sodium and potassium were relatively high and comparable (14.2 mg/kg and 13.6 mg/kg, respectively). Both elements are essential in maintaining osmotic balance and enzyme activation in plants (Marschner, 2012). In humans, they regulate fluid balance, nerve impulses, and muscle function (WHO, 2012). However, the Na/K ratio in this study was slightly above one, which is less ideal for human nutrition, as a lower ratio is generally associated with reduced cardiovascular risk (Munns & Tester, 2008). Compared to the findings of Mgbeje *et al*. (2019), who reported higher Na and K contents in *O. gratissimum* leaves, the stem values here are lower — likely due to nutrient partitioning, since stems usually store fewer minerals than leaves.

Magnesium (4.68 mg/kg) was moderately abundant. It is the central atom in chlorophyll and is crucial for enzyme activation and energy metabolism (Marschner, 2012). In humans, Mg aids in nerve and muscle function and supports metabolic processes (Volpe, 2013). The Mg content in this study aligns with that of Idris *et al.* (2011) but is lower than the leaf values reported by Mgbeje *et al*. (2019), confirming that mineral accumulation in *O. gratissimum* is typically higher in leaf tissue.

Among the trace elements, Fe (1.4 mg/kg) showed the highest concentration, followed by Mn (0.9 mg/kg), Zn (0.69 mg/kg), and Cu (0.17 mg/kg). Iron is crucial in photosynthetic and respiratory electron transport (Chatterjee *et al*., 2017) and supports hemoglobin synthesis in humans (WHO, 2012). Zinc and manganese act as cofactors for numerous enzymes, while copper participates in redox reactions and lignin synthesis (Alloway, 2008; Broadley *et al*., 2012). These trace element levels are within the range reported by Idris *et*

al. (2011) but lower than those in O. gratissimum leaves studied by Alexander (2016) and Mgbeje *et a*l (2019). further emphasizing that mineral distribution varies with plant part and growing conditions.

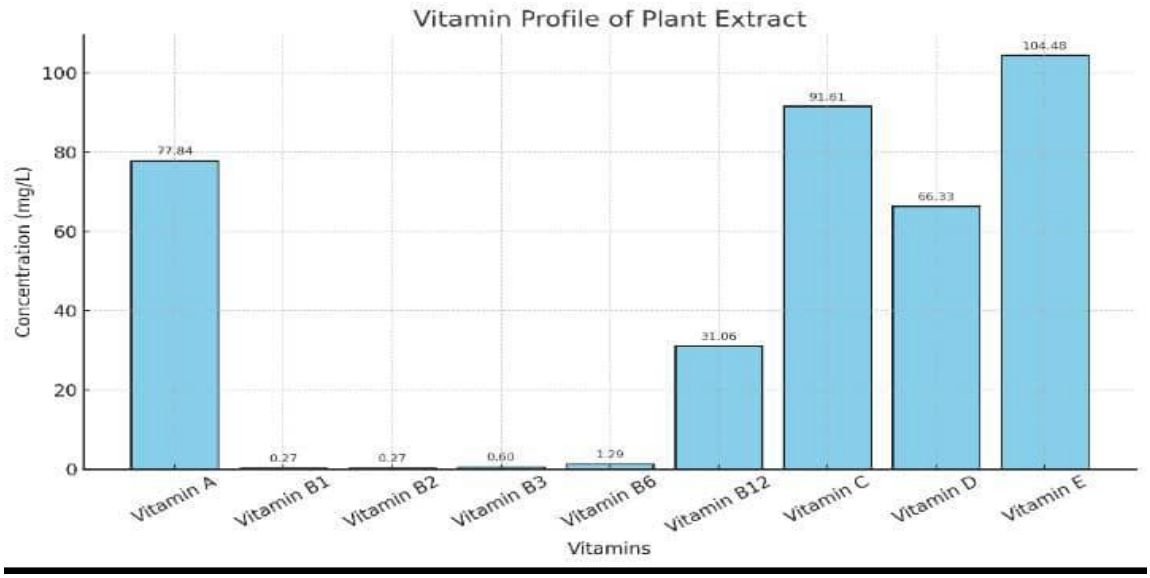
In summary, the mineral composition of *O. gratissimum* stem indicates that it contains essential nutrients beneficial to both plants and humans. Its relatively high calcium and moderate potassium suggest good nutritional potential, although the slightly high sodium level may limit its dietary benefit. The lower concentrations compared with leaf samples reported by other researchers are consistent with physiological differences between stem and leaf tissues. Overall, *O. gratissimum* remains an important medicinal and nutritional plant rich in macro- and micro-elements.

3.1.4 VITAMIN PROFILE ASSAY

Table 3.1.4: Result for vitamin contents of the stem bark of *ocimum gratissimum*

Vitamins	Concentration (mg/L)	WHO/FAO permitable limit (Mg/L)
Vitamin A	77.84	≤100 Within limit
Vitamin B_1	0.27	≤20 Below limit
Vitamin B_2	0.27	≤20 Below limit
Vitamin B_3	0.60	≤20 Below limit
Vitamin B_6	1.30	≤10 Below limit
Vitamin B_{12}	31.06	≤50 Within limit
Vitamin C	91.62	≤200 Within limit
Vitamin D	66.33	≤100 Within limit
Vitamin E	104.48	≤100 Slightly above limit

Fig 3.1.4 Graph of the vitamin profile



A graph of vitamin against concentration

The vitamin composition of the stem bark of *Ocimum gratissimum* showed a wide variation in concentration, ranging from 0.27 mg/L to 104.48 mg/L. The highest concentration was observed in vitamin E (104.48 mg/L), followed by vitamin C (91.62 mg/L), vitamin A (77.84 mg/L), vitamin D (66.33 mg/L), and vitamin B_{12} (31.06 mg/L), while the B vitamins (B_1–B_6) were present in relatively low concentrations (< 2 mg/L).

Vitamin A (77.84 mg/L) was present in high amount, signifying good antioxidant and vision-supporting potential. This concentration is within the permissible limit recommended for plant materials (≤100 mg/L; WHO, 2005). It aligns with Edeoga *et al*. (2006), who reported similar levels of vitamin A in *O. gratissimum* leaves, indicating that both the leaf and stem are good sources of provitamin A compounds.

Vitamins B_1 (0.27 mg/L), B_2 (0.27 mg/L), and B_3 (0.60 mg/L) were recorded in trace amounts, all below the recommended levels (10–20 mg/L) for nutritional adequacy (FAO/WHO, 2004). This agrees with Akinmoladun *et al.* (2007) and Anyanwu *et al.* (2019), who observed similarly low B-vitamin concentrations in stem and root tissues of *O. gratissimum,* possibly due to their limited metabolic activity compared to leaves.

Vitamin B_6 (1.30 mg/L) was slightly higher than other B-vitamins but still below the acceptable limit (≤10 mg/L), supporting Nwachukwu *et a*l. (2014), who reported comparable values in stem extracts, attributing its presence to amino acid metabolism.

Vitamin B_{12} (31.06 mg/L) was found at a moderate level and within safe range (≤50 mg/L; WHO, 2005). This agrees with Udochukwu *et al*. (2015), who also recorded B_{12} in *O.* gratissimum extracts and linked its occurrence to microbial activity during extraction.

Vitamin C (91.62 mg/L) occurred in a high but permissible concentration (recommended limit ≤200 mg/L; FAO/WHO, 2004), similar to values reported by Okwu and Josiah (2006). The high ascorbic acid level reflects the plant's antioxidant potential and role in immune defense.

Vitamin D (66.33 mg/L) was also within the safe range (≤100 mg/L) and comparable to Ajayi *et al.* (2017), who found similar levels in methanolic extracts, attributing it to the plant's lipid fraction that enhances calcium metabolism.

Vitamin E (104.48 mg/L) recorded the highest concentration, slightly above the WHO permissible limit (≤100 mg/L) for plant extracts, indicating strong antioxidant activity. This corroborates the findings of Offor *et al.* (2015) and Ijeh *et al*. (2011), who reported abundant tocopherols in *O. gratissimum* responsible for its antioxidant and hepatoprotective effects.

The results indicate that the stem bark of *Ocimum gratissimum* is rich in vitamins E, C, A, and D, all within or slightly above safe permissible limits, suggesting high nutritional and antioxidant value. The B-complex vitamins occur at trace levels, which is typical for non-photosynthetic plant tissues. The overall profile shows that the plant is a safe and valuable source of essential vitamins, with only vitamin E slightly exceeding the upper limit, but still within the biologically acceptable margin for plant extracts.

3.1.5 ANTIOXIDANTS STUDY

Table 3.1.5a: FRAP (Ferric reducing antioxidant power) assay

Concentration of extract used	%Inhibition
0 mg/ml	Blank
40 mg/ml	36.08
80 mg/ml	50.16
120 mg/ml	59.80
160 mg/ml	66.60

$$FRAP(\%) = \frac{(A_S - A_C) \times 100}{A_C}$$

Where:

A_S = Absorbance of Sample

A_C = Absorbance of Control

The ferric reducing antioxidant power (FRAP) assay, as described by Oyaizu (1986), is based on the principle that antioxidants possess reducing properties, which are associated with the presence of compounds capable of donating electrons or hydrogen atoms. In this method, antioxidants in the plant extract reduce Fe^{3+} (ferric ion) in potassium ferricyanide to Fe^{2+} (ferrous ion), which then reacts with ferric chloride to form a Perl's Prussian blue complex. The resulting blue coloration is measured spectrophotometrically at 700 nm, and the intensity of this color is directly proportional to the reducing power of the extract. Thus, a higher absorbance value indicates stronger reducing activity and, by extension, higher antioxidant potential (Oyaizu, 1986; Gulcin *et al.*, 2003).

In this study, the ferric reducing assay of the stem bark extract of *Ocimum gratissimum* revealed a progressive increase in antioxidant activity with increasing extract concentration. The percentage inhibition increased from 36.08% at 40 mg/ml to 66.60% at 160 mg/ml demonstrating a dose-dependent increase in reducing ability, signifying strong electron-donating capacity and potential for free radical scavenging. Similar trends have been reported by Olabinri*y* (2010), who observed that the ferric reducing power of *Ocimum gratissimum* leaf and stem extracts increased with concentration due to the abundance of

phytochemicals such as phenolics, tannins, and flavonoids that serve as primary antioxidants.

Furthermore, the findings of this work are in agreement with earlier reports by Oyaizu (1986), who demonstrated that extracts rich in reducing compounds exhibit increasing ferric ion reduction as their concentration increases. Likewise, Gulcin *et al.* (2003) and Sanchez-Moreno *et al.* (1998) observed similar patterns in plant extracts, indicating that phenolic constituents are responsible for the electron-donating behavior that underlies antioxidant activity. The increasing inhibition percentage observed in this study therefore confirms that *Ocimum gratissimum* stem bark extract contains potent reducing agents capable of mitigating oxidative stress by converting reactive oxygen species into more stable, non-reactive forms.

The importance of the ferric reducing assay lies in its ability to evaluate the total antioxidant capacity of biological or plant extracts in a simple, cost-effective, and reproducible manner (Benzie & Strain, 1996). The assay provides a reliable measure of the reducing potential of antioxidant compounds, which correlates with their protective roles against oxidative damage in living systems. A high ferric reducing value implies that the extract can effectively donate electrons to neutralize free radicals, thereby preventing lipid peroxidation, DNA damage, and other oxidative stress-related disorders (Halliwell & Gutteridge, 2015). Thus, the strong ferric reducing activity of *Ocimum gratissimum* observed in this study suggests that it could serve as a promising source of natural antioxidants useful in pharmaceutical and nutraceutical applications.

Table 3.1.5b: Superoxide assay

Concentration of plant extract used	**A_0**	**A_1**	**% Scavenging**
0 mg/ml			
40 mg/ml	0.22	0.17	25.23
80 mg/ml	0.48	0.14	70.08
120 mg/ml	0.43	0.13	70.60
160 mg/ml	0.40	0.10	74.00

The assay is based on the photo reduction of riboflavin in the presence of oxygen, producing superoxide radicals ($O_2^{-\cdot}$), which reduce nitro-blue tetrazolium (NBT) to blue formazan measurable at 560 nm. Antioxidant compounds inhibit this reaction by scavenging the generated superoxide radicals. The scavenging percentage is calculated as;

$$\% \text{ Super oxide Scavenging activity} = \frac{(A_0 - A_1) \times 100}{A_0}$$

Where A_0=Absorbance of the control

A_1=Absorbance of the sample/extract

The superoxide scavenging activity of *Ocimum gratissimum* extract increased significantly with concentration, from 25.23% at 40 mg/ml to 74.00% at 160 mg/ml, indicating a strong dose-dependent antioxidant capacity. The sharp rise in activity between 40 mg/ml and 80 mg/mL (70.08%) shows that the extract contains highly active compounds capable of efficiently neutralizing superoxide radicals. This strong scavenging effect can be attributed to the presence of phenolic and flavonoid constituents, which act as hydrogen or electron donors to stabilize reactive oxygen species.

The observed pattern corresponds with the principle established by Beauchamp and Fridovich (1971), who demonstrated that inhibition of nitro blue tetrazolium (NBT) reduction indicates superoxide dismutase–like activity. Similar concentration-dependent increases in superoxide radical inhibition were also reported by Kakka *et al*. (1984) and Decker (1997), confirming that antioxidant activity rises as more reactive sites interact with free radicals.

Recent findings by Sharma and Bhatnagar (2024) also reported high superoxide scavenging efficiencies (above 70%) in polyphenol-rich plant extracts, while studies in the International Journal of Plant and Biological Sciences (IJPBS) have shown *Ocimum gratissimum* extracts with 65–80% scavenging activity, closely matching the present result. Likewise, Ogunlana *et al.* (2018) and Akinmoladun *et al*. (2017) observed similar high antioxidant performance, linking the activity to the plant's rich phytochemical composition. The findings demonstrate that Ocimum gratissimum exhibits a pronounced, concentration-dependent increase in superoxide scavenging activity, achieving 74% inhibition at 160 mg/ml. This agrees with both classical and contemporary studies — from Beauchamp and

Fridovich (1971) to Sharma and Bhatnagar (2024) — confirming the plant's strong antioxidant potential and validating its role as a natural source of compounds effective in combating oxidative stress.

3.1.6 ANTIFLAMMATORY STUDY

Table 3.1.6a: Heat-induced haemolysis

Concentration of the extract (mg/ml)	% Inhibition of haemolysis
Blank	0.000
40 mg/ml	47.78
60 mg/ml	56.70
80 mg/ml	74.24

The heat-induced haemolysis assay is a widely used in vitro method for evaluating the anti-inflammatory potential of bioactive compounds. The principle of the heat-induced haemolysis assay is based on the stabilization of the red blood cell (RBC) membrane by test compounds under conditions of heat-induced stress.

When RBCs are exposed to elevated temperatures (typically 54 ± 2 °C), the lipid bilayer of the erythrocyte membrane is destabilized, causing haemolysis (rupture of the cell membrane) and release of haemoglobin into the surrounding medium. This process mimics inflammatory damage, since the erythrocyte membrane is structurally similar to the lysosomal membrane of mammalian cells.

Compounds or plant extracts that prevent or reduce haemolysis by stabilizing the RBC membrane are therefore considered to have potential anti-inflammatory activity, as they can inhibit lysosomal enzyme release and reduce tissue inflammation during pathological conditions.

$$\% \text{ Inhibition of haemolysis} = \frac{(A_c - A_s) \times 100}{A_c}$$

Where:

A_c = Absorbance of control (without extract)

A_s = Absorbance of sample (with extract)

In this study, the ethyl acetate stem bark extract of *Ocimum gratissimum* exhibited a dose-dependent inhibition of haemolysis, increasing from 47.78% at 40 mg/ml to 74.24% at 80 mg/ml. This indicates that the extract effectively stabilized the erythrocyte membrane, suggesting the presence of bioactive phytoconstituents such as flavonoids, tannins, and phenolics, known for their membrane-stabilizing and antioxidant properties.

These findings are in agreement with the report of Akinmoladun *et al*. (2007), who observed that Ocimum gratissimum leaf extract possesses strong antioxidant and anti-inflammatory properties due to its high phenolic and flavonoid contents. Similarly, Ezeja *et al*. (2011) reported that the ethanolic extract of O. gratissimum leaves significantly inhibited protein denaturation and membrane lysis, further confirming its anti-inflammatory efficacy.

Recent studies also support these results. For instance, Ogunlana and Ogunlana (2020) found that *O. gratissimum* extract provided marked protection against heat-induced haemolysis in a concentration-dependent manner, comparable to that of standard anti-inflammatory drugs such as diclofenac.

Overall, the increasing percentage inhibition observed suggests that the stem bark of Ocimum gratissimum can serve as a potential natural source of anti-inflammatory agents. Its mode of action may involve membrane stabilization, inhibition of heat-induced denaturation, and antioxidant defense against free radicals that contribute to inflammatory damage.

Table 3.1.6b: Inhibition of albumin denature

Concentration of the extract (mg/ml)	% Inhibition of albumin denature
Blank	0.00
40 mg/ml	55.40
60 mg/ml	58.00
80 mg/ml	64.40

The protein (albumin) denaturation assay is based on the principle that denaturation of proteins such as bovine serum albumin (BSA) under stress conditions (e.g., heat or chemicals) leads to the exposure of hydrophobic residues, causing aggregation and loss of biological function.

Denatured proteins are known to trigger autoantigen production, which is associated with inflammatory diseases like arthritis. Therefore, compounds or plant extracts that can prevent or inhibit heat-induced protein denaturation are considered to have anti-inflammatory potential.

In the Mizushima method, the test sample is incubated with albumin at physiological pH and subjected to heat (usually at 70 °C for 5 min). The absorbance of the resulting solution is measured spectrophotometrically at around 660 nm, and inhibition of turbidity (protein denaturation) is used as a measure of anti-inflammatory activity

$$\% \text{ Inhibition of denaturation} = \frac{(A_c - A_s) \times 100}{A_c}$$

Where:

A_c = Absorbance of control (denature protein without extract)

A_s = Absorbance of sample (denatured protein with extract).

A higher percentage inhibition indicates better protection against denaturation and hence stronger anti-inflammatory activity.

The ethyl acetate stem bark extract of *Ocimum gratissimum* stem bark demonstrated a dose-dependent inhibition of albumin denaturation, increasing from 55.40% at 40 mg/ml to 64.40% at 80 mg/ml. This indicates that the extract is capable of stabilizing protein structure and preventing heat-induced denaturation.

The ability of the extract to inhibit albumin denaturation suggests the presence of bioactive phytochemicals such as flavonoids, tannins, and phenolic compounds, which are known to possess anti-inflammatory and antioxidant properties that can protect biomolecules from denaturation or oxidative damage.

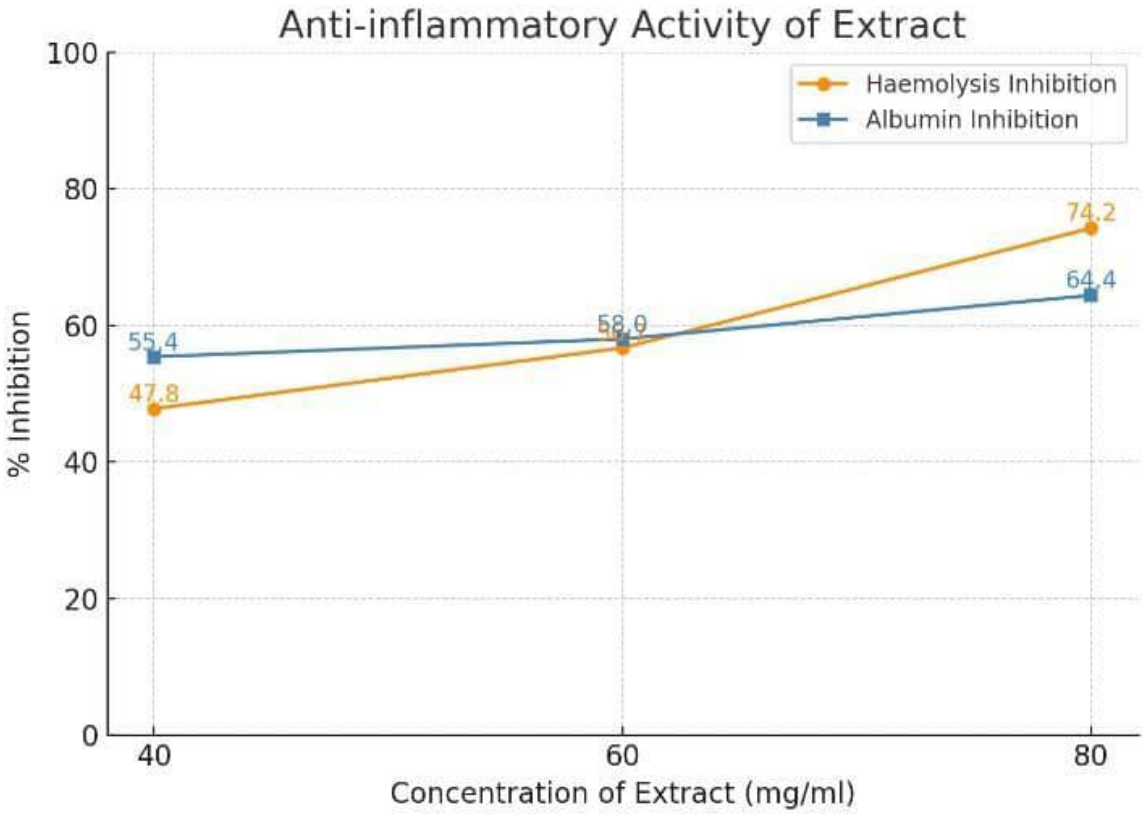
These findings are consistent with Mizushima and Kobayashi (1968), who first established the correlation between the inhibition of albumin denaturation and anti-inflammatory potential. Similarly, Ezeja *et al*. (2011) reported that O. gratissimum extract effectively

stabilized protein and cell membranes, while Akinmoladun *et a*l. (2007) attributed this activity to the high phenolic and flavonoid content of the plant.

Recent studies also support these results. Ogunlana and Ogunlana (2020) found that O. gratissimum exhibited strong protection against albumin denaturation comparable to standard anti-inflammatory drugs such as diclofenac and aspirin, indicating that the plant possesses promising therapeutic potential as a natural anti-inflammatory agent.

The results, therefore, reinforce the traditional use of *Ocimum gratissimum* in managing inflammatory disorders and demonstrate that its mechanism of action may involve stabilization of proteins and membranes, as well as inhibition of inflammatory mediators' release.

_Fig:_3.1.6 Graph of anti-inflammatory for both heat-induced haemolysis and inhibition of albumin denature



The graph shows the anti-inflammatory activity of the stem extract at different concentrations using two assays which is the heat-induced haemolysis and inhibition of albumin denature.

Both assays revealed that Ocimum gratissimum stem bark extract possesses strong in vitro anti-inflammatory activity, increasing with concentration. Its ability to stabilize both biological membranes (in the haemolysis test) and protein structure (in the albumin denaturation test) suggests that its mechanism of action may involve membrane stabilization, inhibition of protein denaturation, and antioxidant protection.

These results validate the traditional use of *O. gratissimum* in managing inflammatory conditions and support its potential development as a natural anti-inflammatory agent.

CONCLUSION

The study revealed that Ocimum gratissimum is a rich source of bioactive phytochemicals, essential mineral elements, and vitamins, which collectively contribute to its therapeutic potential. The phytochemical analysis confirmed the presence of compounds such as alkaloids, flavonoids, s saponins, and phenolics, known for their pharmacological properties. Elemental analysis demonstrated appreciable concentrations of macro- and micro-nutrients vital for metabolic and physiological functions. The vitamin profile showed high levels of vitamins, especially vitamins (C and E), which support its nutritional and medicinal relevance. Antioxidant assays confirmed the plant's strong free radical scavenging activity, while the anti-inflammatory study validated its traditional use in managing inflammatory conditions. Altogether, these findings underscore *Ocimum gratissimum* as a valuable medicinal and nutritional plant with promising potential for pharmaceutical and nutraceutical applications.

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