THE IMMUNOMODULATORY EFFECT OF A BIHERBAL FORMULATION CONSISTING OF *Bryophyllum pinnatum* AND *Ocimum gratissimum* ON WISTAR ALBINO RATS

BY

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LSC2007322

(PHYSIOLOGY AND PHARMACOLOGY TECHNIQUES)

DEPARTMENT OF SCIENCE LABORATORY TECHNOLOGY

FACULTY OF LIFE SCIENCES

UNIVERSITY OF BENIN,

BENIN CITY.

NOVEMBER, 2025

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A PROJECT WORK SUBMITTED TO THE DEPARTMENT OF SCIENCE LABORATORY TECHNOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY, IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF BACHELOR OF SCIENCE DEGREE (B.Sc) IN SCIENCE LABORATORY TECHNOLOGY (PHYSIOLOGY AND PHARMACOLOGY TECHNIQUES)

NOVEMBER, 2025

CERTIFICATION

This is to certify that this project work titled "**THE IMMUNOMODULATORY EFFECT OF A BIHERBAL FORMULATION CONSISTING OF *Bryophyllum pinnatum* AND *Ocimum gratissimum* ON WISTAR ALBINO RATS"** was carried out and submitted by **Miracle Ekene OCHEI-OSIDE** with matriculation number, **LSC2007322** in the Department of Science Laboratory Technology, Faculty of Life Sciences, University of Benin, Benin City.

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DEDICATION

I wholeheartedly dedicate this project to God Almighty, whose endless grace, wisdom, and strength carried me through every stage of this work. Without Him, none of this would have been possible. I also dedicate it to my beloved parents, Mr. Frank and Mrs. Uche Oside. Their boundless, unconditional love, the immeasurable sacrifices they silently made, and their unwavering encouragement have not merely inspired me; they have been the sacred, beating heart of this entire endeavor. Every success, every lesson, and every facet of the person I am today is a direct reflection of their tireless care and unending support. My gratitude is a debt of the heart I can never fully repay.

ACKNOWLEDGEMENTS

First and foremost, I sincerely thank Almighty God for His unending faithfulness, grace, and mercy throughout the course of my study. His divine guidance, strength, and provision have been my anchor in every challenge and success. I also want to appreciate my project supervisor, Dr. P. O. Obaro and his wife, Dr. (Mrs.) O. E. Obaro-Oneyezi, the Dean of Science Laboratory Technology and lecturers of Science Laboratory Technology. I am overflowing with gratitude for these incredible individuals; they are nothing short of a profound blessing: Aunty Onyeisi, Uncle Odiaka, Mr. & Mrs. Kevin Mordi, Mr. & Mrs. Emmanuel Mordi, Aunty Agi, Aunty Cordy, Mr. & Mrs. Chuks Ifeozo, Uncle Emeka Oside, Deac. Tessy Onyemuche, and my exceptional Grandma. Words feel inadequate to express the profound appreciation I hold for each of you. Your love was my strength, your encouragement my fuel, and your contributions were the essential anchors that guided this project to completion. I also wish to express my deep gratitude to my dear friend, Destiny Ehiomhen, for being such an amazing source of strength, motivation, and genuine friendship. Your support and belief in me meant more than words can express. To all my friends who stood by me with encouragement and laughter through every stage of this journey; thank you, my darlings. I carry immense joy knowing that I am supported by and uplifted by such wonderful and inspiring people. My life is richer because of them. God bless you all!

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ABSTRACT

The study investigated the immunomodulatory effect of a biherbal formulation consisting of *Bryophyllum pinnatum* and *Ocimum gratissimum* in Wistar albino rats. The plants were collected, air-dried, powdered, and extracted with methanol. Twenty-five healthy rats were divided into five groups: control, two extract-treated groups (50 mg/kg and 200 mg/kg), a positive control (Vitamin C 50 mg/kg), and a negative control (Cyclophosphamide 30 mg/kg). Treatments were administered orally for seven days prior to immunization with sheep red blood cells (SRBC). Immunomodulatory activity was assessed using delayed-type hypersensitivity (DTH), hemagglutination antibody titer, total and differential leukocyte counts, CD4 T-cell count, and organ weight indices. Results showed that the biherbal formulation significantly ($p < 0.05$) increased body weight, thymus and spleen indices, total white blood cells, lymphocytes, monocytes, and neutrophils compared to the control. Serum protein, hemoglobin, and albumin-globulin ratio were also elevated, while Cyclophosphamide markedly reduced these parameters. The extract enhanced $CD4^+$ T-cell and B-cell populations, indicating stimulation of both humoral and cellular immune responses. Additionally, the formulation reduced paw edema in DTH assay, demonstrating anti-inflammatory potential. The methanolic extract of *Bryophyllum pinnatum* and *Ocimum gratissimum* possesses potent immunomodulatory properties, acting as both an immunostimulant and an anti-inflammatory agent. These findings provide scientific evidence supporting its traditional use and suggest that the formulation may serve as a natural, affordable alternative for immune enhancement and regulation.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

The immune system is a complex network of organs, cells, and molecules that protects the body against infections, tumors, and abnormal cells while maintaining tolerance to self-antigens. A well-functioning immune system is central to survival, but its dysregulation is associated with numerous disorders. Immune suppression predisposes individuals to opportunistic infections and cancers, whereas excessive immune activation underlies autoimmune diseases, allergies, and chronic inflammation (Medzhitov and Janeway, 2000; Chaplin, 2010). Because of this, strategies that can modulate immune function either by stimulating or suppressing it are critical in disease management. Several synthetic immunomodulators such as corticosteroids, cyclosporine, and biologics have proven useful in clinical practice; however, their use is often limited by adverse effects, high costs, and limited accessibility in low-resource settings (Okoye and Uzor, 2014). This has generated increasing interest in medicinal plants as potential sources of safer, more affordable immunomodulatory agents. Medicinal plants are rich in secondary metabolites such as flavonoids, terpenoids, alkaloids, and glycosides, many of which exhibit immunomodulatory, anti-inflammatory, and antimicrobial effects (Newman and Cragg, 2020). Several modern drugs including artemisinin, paclitaxel, and morphine were originally derived from plants, highlighting the relevance of ethnomedicine in drug discovery. In Nigeria and other parts of Africa, medicinal plants play a particularly important role in managing common diseases due to their availability, affordability, and cultural acceptance (Sofowora *et al*., 2013). Among these, *Bryophyllum pinnatum* (commonly known as “life plant” or “miracle leaf”) and *Ocimum gratissimum* (“scent leaf” or “clove basil”) are widely used in traditional medicine. Although these plants are

extensively used in folk medicine, their immunomodulatory effects have not been fully validated scientifically. Given the global burden of immune-related diseases, the limitations of current synthetic immunomodulators, and the widespread traditional use of *B. pinnatum* and *O. gratissimum*, there is a strong rationale for investigating their potential as natural immunomodulators. Establishing such evidence may not only provide scientific justification for their ethnomedicinal uses but also contribute to the development of affordable plant-based therapies for managing immune-related conditions.

1.2 AIM OF STUDY

The aim of this study is to evaluate the Immunomodulatory effect of a biherbal formulation consisting of *Bryophyllum pinnatum* and *Ocimum gratissimum*.

1.3 OBJECTIVES OF STUDY

The specific objectives of this study are;

1. To determine the effect of biherbal formulation on key immunological parameters in the treated rats.
2. To compare the immunomodulatory effects of the formulation with standard control.
3. To evaluate any potential toxicological effects of the biherbal formulation by analyzing liver and kidney function biomarkers.

CHAPTER TWO

LITERATURE REVIEW

2.1 BIOLOGY OF *Bryophyllum pinnatum*

2.1.1 DESCRIPTION OF *Bryophyllum pinnatum*

Bryophyllum pinnatum (Crassulaceae) is native to tropical and subtropical regions and has become widespread across Africa, Asia, the Americas, and parts of India and China. In Igbo (southeastern Nigeria), it's called "Oda-opue". In Yoruba, it's referred to as "Abamoda" (Adebayo *et al*., 2021). It's also commonly known as the "miracle leaf," "air plant," or "life plant," reflecting its remarkable ability to vegetatively propagate. It is astringent, sour in taste, sweet in the post digestive effect and has hot potency (Kamboj and Saluja, 2009). All over the world, *Bryophyllum pinnatum* has been used locally to treat ulcer, fever hypertension, boils, cough, jaundice, small pox, arthritis, and diabetes (Ghasi *et al*., 2011; Elufioye *et al*., 2022).

2.1.2 TAXONOMIC CLASSIFICATION OF *Bryophyllum pinnatum*

Kingdom: Plantae

Phylum: Angiospermatophyta

Class: Eudicots

Order: Saxifrageles

Family: Crassulaceae

Genus: *Bryophyllum*

Species: *Bryophyllum pinnatum*

2.1.3 MORPHOLOGY OF *Bryophyllum pinnatum*

Botanical Description

The plant typically attains a height of 50 to 200 cm, bearing erect, hollow, and fleshy stems which may appear green or occasionally tinged with red. One of its most striking features is its ability to propagate vegetatively through adventitious buds formed along the margins of its leaves. These plantlets detach naturally, root easily upon contact with soil, and give rise to new plants, contributing to the species' high potential for invasiveness in favorable habitats.

Leaves

The leaves of *Bryophyllum pinnatum* are simple, fleshy, and arranged oppositely in a decussate fashion along the stem. They are ovate to elliptic in shape, with margins that are crenate or serrated. The upper surface is glossy and dark green, while the underside is slightly paler. Leaf size varies considerably, ranging from about 5 to 20 cm in length and 2 to 12 cm in width, depending on the growing conditions. A prominent midrib is visible, with pinnate venation extending to the margins.

Fruit

The fruit is a dry follicle, typical of members of the Crassulaceae family. It is composed of four slender and elongated follicles, each containing numerous minute brown seeds. Upon maturity, the follicles split open to release the seeds, which are light enough to be carried away by wind or water. This adaptation, in combination with vegetative propagation, allows the plant to spread effectively in its environment.

Flowers

Each flower is bisexual, tubular, and pendulous, with a calyx that is green to reddish and divided into four lobes, the lower part forming a tube. The corolla is also tubular, flaring into four free lobes that may range in color from pale green to pinkish-red or reddish-purple. There are eight stamens arranged in two whorls, attached to the inner wall of the corolla tube. The ovary is superior and composed of four distinct carpels, each with its own style and stigma. Flowering generally occurs in the dry season or during cooler months in tropical climates, and the blossoms are visited by insects, particularly bees and butterflies, which serve as pollinators.

Ecology

In its naturalized habitats, *Bryophyllum pinnatum* grows in a wide range of conditions but thrives best in well-drained sandy or rocky soils. It can tolerate nutrient-poor or degraded substrates and is remarkably drought-resistant due to its succulent tissues, which allows it to minimize water loss. The plant reproduces both sexually through seeds and vegetatively through leaf-borne plantlets, although the latter is the dominant mode in most environments. Seeds may be dispersed by wind and water, while the plantlets fall directly to the ground and readily take root. It is commonly found

in disturbed areas, along roadsides, and in abandoned fields, where it often outcompetes native vegetation and becomes invasive. Its flowers attract pollinating insects, adding some ecological value, but in many regions its aggressive spread makes it an environmental concern.

2.2 PHYTOCHEMICALS FOUND IN *Bryophyllum pinnatum*

The health benefits of *Bryophyllum pinnatum* are due to a variety of phytochemicals present in its leaves and stems.

2.2.1 Flavonoids

Flavonoids are one of the major groups of compoundfound in B. pinnatum. Studies have reported several kaempferol rhamnoside derivatives, which are known to fight microbes and protect the body against oxidative stress (Tatsimo *et al*., 2012). Another investigation showed that the leaf extract is rich in total flavonoids, and the higher the flavonoid content, the stronger its antioxidant and anticholinesterase activities (Elufioye *et al*., 2019).

2.2.2 Phenolic Compounds and Tannins

The plant also contains large amounts of phenolic compounds and tannins, both of which have antioxidant properties. (Elufioye *et al*., 2019) found a clear relationship between these compounds and the plant's ability to neutralize free radicals and inhibit cholinesterase enzymes. In simple terms, phenolics and tannins help reduce oxidative damage and support brain health.

2.2.3 Polyphenols in Leaves and Stems

Another study carried out on different extracts of the plant's leaves and stems showed that they are rich in polyphenols. However, compounds such as anthocyanins and anthraquinones were not detected (Daniel *et al*., 2020). These results further prove that B. pinnatum is a good source of antioxidants.

2.2.4 General Phytochemical Screening

When the stem bark and wood of the plant were tested using different solvents, they were found to contain reducing sugars, saponins, steroids, tannins, alkaloids, flavonoids, and phenols (Akacha *et al*., 2016). This shows that B. pinnatum is chemically diverse and has many bioactive compounds.

2.2.5 Organic and Volatile Compounds (GC–MS Analysis)

Advanced chemical analysis using GC–MS revealed that B. pinnatum leaves contain a wide variety of compounds such as sterols, fatty acids, simple sugars, alcohols, organic acids, vitamin E (tocopherol), and hydrocarbons (Faboro *et al*., 2016). These compounds play important roles in nutrition, cell protection, and antimicrobial defense.

2.3 PHARMACOLOGICAL PROPERTIES OF *Bryophyllum pinnatum*

2.3.1 Anti-inflammatory Activity

Bryophyllum pinnatum is well known for its strong ability to fight inflammation, mainly because of natural chemicals in the plant such as flavonoids and bufadienolides. Research on animals has shown that extracts from its leaves can greatly reduce swelling, tissue overgrowth, and harmful

substances that cause inflammation. A research found that the plant's hydro-ethanolic extract reduced both short-term and long-term inflammation in rats, working almost as effectively as indomethacin, a common anti-inflammatory drug (Onwubuya *et al*., 2021). Another research also shows that the extract blocks certain enzymes like COX-2 and reduces the production of inflammatory chemicals such as IL-1β, IL-6, and TNF-α (Oladejo *et al*., 2021). This explains why the plant has traditionally been used to treat swelling, wounds, and different inflammatory conditions.

2.3.2 Antioxidant Activity

Oxidative stress happens when harmful molecules in the body build up and damage cells, and it is linked to many long-term diseases. *Bryophyllum pinnatum* has been found to work as a strong antioxidant, meaning it helps the body fight this damage. An animal study showed that extracts from the plant's leaves helped restore the normal levels of protective enzymes in rats that had been exposed to chemicals causing oxidative damage (Alli-Smith *et al*., 2021). The treatment boosted the body's natural defense molecules like glutathione while reducing harmful substances such as malondialdehyde, which indicate cell damage. This suggests that B. pinnatum can protect important organs like the liver and heart, and may help prevent diseases caused by oxidative stress.

2.3.3 Antimicrobial and Antibacterial Properties

Researchers have tested the antimicrobial ability of *Bryophyllum pinnatum* against several pathogenic activities. A research was conducted where *Bryophyllum pinnatum* was compared with another medicinal plant, *Newbouldia laevis* and found that *B. pinnatum* was very effective at stopping the growth of bacteria such as *Staphylococcus aureus, Escherichia coli*, and *Klebsiella pneumoniae* (Ugwuoji *et al*., 2022). The strength of its action depended on the amount used, with

higher doses working better. Scientists believe this effect comes from natural chemicals in the plant, like flavonoids, tannins, and terpenoids, which can break down bacterial cell walls and block their growth. This explains why the plant has long been used in traditional medicine to treat wounds, ulcers, and infections.

2.3.4 Antidiabetic Properties

Researchers have studied *Bryophyllum pinnatum* to see if it can help manage diabetes. The leaves contain substances that can slow down the action of two important digestive enzymes (α-amylase and α-glucosidase) that normally break down starchy foods into sugar. By slowing down these enzymes, the plant helps prevent sharp increases in blood sugar after eating. Lab tests showed that the plant extract was very effective at this (Ogunmoyole *et al*., 2025). This means the plant has real promise as a natural support in controlling diabetes.

2.3.5 Anti-Cancer Properties

Scientists have found that *Bryophyllum pinnatum* may help fight cancer by slowing down the growth of cancer cells. In one study, its leaf extract was tested on cervical cancer cells. The whole extract reduced cancer growth by about 30%, but when the extract was separated into parts, one fraction (called F4) worked much better, stopping about 55% of the cancer cells at the same amount. The strongest effect was seen in the first 24 hours, although some cancer cells started growing again after 2–3 days. This shows that the plant has powerful natural chemicals that could be studied further for making anti-cancer medicines (Mahata *et al*., 2012).

2.4 BIOLOGY OF *Ocimum gratissimum*

2.4.1 DESCRIPTION OF *Ocimum gratissimum*

Ocimum gratissimum L., commonly known as African basil, clove basil, or "scent leaf," is a perennial aromatic herb that belongs to the family Lamiaceae. It is widely distributed in tropical and subtropical regions of Africa, Asia, and South America, where it is cultivated both as a culinary herb and as a medicinal plant. In Nigeria, it is locally called efinrin (Yoruba), nchanwu (Igbo), and daidoya (Hausa), and is valued as both food and medicine (Ugbogu *et al*., 2021).

Traditionally, the plant has been used in managing gastrointestinal disorders, respiratory infections, fever, skin diseases, and wounds. The fresh leaves are commonly consumed as vegetables, brewed into teas, or applied topically in poultices (Kpadonou *et al*., 2012)

2.4.2 TAXONOMIC CLASSIFICATION OF *Ocimum gratissimum*

Kingdom: Plantae

Phylum: Angiospermatophya

Class: Eudicots

Order: Lamiales

Family: Lamiaceae

Genus: *Ocimum*

Species: *Ocimum gratissimum*

2.4.3 MORPHOLOGY OF *Ocimum gratissimum*

Botanical Description

Ocimum gratissimum grows up to 1–2 meters in height with a strong woody base. The stems are quadrangular (four-angled), a typical feature of the mint family, and are green and slightly hairy when young, but turn woody and brown with age. The plant has a profuse branching system, giving it a bushy appearance.

Leaves

The leaves are simple, opposite, and ovate-lanceolate in shape with pointed tips (acuminate) and serrated (toothed) margins. They measure about 5–10 cm long and 2–5 cm wide. The surface of the leaves is covered with glandular hairs (trichomes) that secrete essential oils.

Fruits

The fruit of *O. gratissimum* is a schizocarp, which splits into four small nutlets at maturity. Each nutlet is ovoid, tiny, and brownish to black in color. The seeds are enclosed within these nutlets and become mucilaginous (slimy) when soaked in water.

Flowers

The flowers are small, white to purplish-white, and arranged in terminal spikes or panicles. They are bilabiate (two-lipped), as is typical of the mint family. Each flower has a tubular calyx and a bilobed corolla. The flowers are hermaphroditic, containing both stamens and carpels, which allows cross-pollination by insects, especially bees.

Ecology

Ocimum gratissimum thrives in tropical and subtropical climates and is commonly cultivated around homes and farms. It prefers well-drained soils and grows well under full sunlight. The plant is relatively hardy, drought-tolerant, and adapts well to disturbed habitats, but it flourishes best with moderate rainfall.

2.5 PHYTOCHEMICALS FOUND IN *Ocimum gratissimum*

2.5.1 Essential Oils (Aromatic Compounds)

Ocimum gratissimum leaves contain essential oils that give the plant its strong smell and medicinal power. Some plants are eugenol-rich (Joshi, 2017), giving a clove-like aroma with strong antimicrobial and antioxidant effects. Others are thymol-rich, giving more of a thyme-like smell with strong antiseptic properties (Kpadonou *et al*., 2012). The exact oil type depends on where the plant grows.

2.5.2 Phenolic Acids

Ocimum gratissimum leaves contain phenolic acids, which are natural plant chemicals that protect the body from damage caused by harmful molecules (free radicals). One of the main ones is rosmarinic acid, along with others like caffeic, ferulic, and p-coumaric acids (Venuprasad *et al*., 2014). These substances act like shields, stopping cell damage, protecting fats in the body, and keeping cell walls stable.

2.5.3 Flavonoids

The plant also has flavonoids such as quercetin, rutin, apigenin, luteolin, and nevadensin. These compounds support anti-inflammatory, heart-protective, and anti-cancer effects, and they boost antioxidant capacity (Ganguly *et al*., 2021)

2.5.4 Tannins, Saponins, Sterols, and Alkaloids

Beyond the oils and phenolics, the leaves contain Tannins, which help with wound healing; Saponins, beneficial for lowering cholesterol and boosting immunity; Sterols like β-sitosterol, known to support heart health; Alkaloids, which can have pain-relieving and antimicrobial effects. These contribute further to the plant's health-promoting properties (Igbinosa *et al*., 2013).

2.6 PHARMACOLOGICAL ACTIVITY OF *Ocimum gratissimum*

2.6.1 Antimicrobial Activity

Recent studies show that the essential oil from *Ocimum gratissimum* is good at stopping the growth of bacteria like *Staphylococcus aureus* and *E. coli*. One experiment encapsulated the oil into tiny particles (microparticles), which made it even more effective; able to kill bacteria at lower concentrations than plain oil (Grandos *et al*., 2024).

2.6.2 Antidiabetic Activity

A 2021 study showed that giving diabetic rats the aqueous extract of *O. gratissimum* improved their blood counts and helped their antioxidant systems bounce back, offering protection from high blood sugar damage (Oyem *et al*., 2021). Some studies also suggest that the extract may also improve lipid profiles (reducing total cholesterol and increasing HDL cholesterol) in experimental models of diabetes (Mohammed *et al*., 2007).

2.6.3 Anti-Inflammatory Activity

Flavonoid-rich extracts from *O. gratissimum* leaves were shown to reduce inflammation and improve behaviours like lethargy or pain in mice caused by bacterial toxins, suggesting calming effects on both immune and behavioral responses (Ajayi *et al*., 2017).

2.6.4 Pro-Fertility Activity

Studies suggest that *Ocimum gratissimum* may improve sperm quality, and increase testosterone levels. It could also influence the production of metabolites that impact testicular function and hormone regulation (Obruche and Andrew, 2025).

2.7 THE IMMUNE SYSTEM

The immune system is a complex defense network made up of different cells, tissues, and proteins. It is like a biological security force. It recognizes and eliminates invaders (like bacteria and viruses), regulates inflammation, and prevents harmful reactions against the body (Calder, 2013). The gut plays a big role too: the Gut-Associated Lymphoid Tissue (GALT) houses a huge share of immune cells, helping train the immune system to tolerate good microbes and food while responding to threats (Galls and Hooper, 2012). There are two main types of immunity and they are:

- Innate immunity: The Innate Immune System represents the body's first line of defense. It provides an immediate response that is non-specific, meaning it reacts to general threat patterns rather than unique antigens. The Innate Immune System is divided into two; Cellular Barriers and Molecular Components. Cellular Barriers involve Phagocytes (like Macrophages and Neutrophils) which engulf and destroy invaders and Natural Killer (NK) cells which detect and kill infectious or cancerous host cells. They act within minutes to

hours and set the stage for repair and clean-up. Molecular Components like Cytokines enhance phagocytosis and directly lyses microbes. Sleep, stress, diet and exercise directly affect these inflammatory set-points (Irwin, 2019).

- Adaptive immunity: Adaptive Immune System is characterized by specificity, diversity, and memory. This type of immunity is divided into two; Humoral Immunity and Cell-Mediated Immunity. Humoral Immunity is mediated by B Lymphocytes (B cells) which mature into Plasma Cells to secrete specific Antibodies. Antibodies circulate in the blood and neutralize pathogens or make them for destruction. Cell-Mediated Immunity is mediated by T Lymphocytes. T Lymphocytes are majorly of three types; Cytotoxic T-cells (T_C) which directly recognize or kill host cells that are internally infected or cancerous, Helper T Cells (T_H) are the master regulators, coordinating responses by releasing potent cytokines to activate B cells, T_C cells, and macrophages, and Regulatory T Cells (T_{reg})which actively suppress the function of other lymphocytes. They are essential for maintaining immunological self-tolerance and preventing auto-immune disease (Sakaguchi *et al*., 2010). Vaccination "trains" Adaptive Immunity to respond faster later. Diet quality and activity influence how robustly these cells expand and form memory (Qiu *et al*., 2017).

The Immune System can be strengthened by the following:

1. Good Nutrition: Adequate nutrition is critical. Both undernutrition and overnutrition impair your immune response. Immune cells need energy and

nutrients to function, especially during illness (Calder, 2013). Key nutrient players include:

- Vitamin C: Supports antioxidant defense and immune cell activity.
- Vitamin D: Essential for activating T cells.
- Vitamin E: Boosts immune cell function.
- Zinc: Crucial for white blood cell maturation and antiviral defense.
- Vitamin A: Supports mucosal defenses and immune regulation.

(Al Mahmud *et al*., 2023)

2. Gut Health: The gut microbiome communicates with the immune system constantly. Fiber (from fruits, vegetables, whole grains) is fermented by gut bacteria into Short-Chain Fatty Acids (SCFAs) that enhance immune responses and gut barrier protection (Holscher, 2017).
3. Adequate, Regular Sleep: Good sleep strengthens immune memory and reduces inflammation; poor sleep does the opposite (Irwin, 2015).

2.8 IMMUNOMODULATION

This refers broadly to any therapeutic intervention that adjusts or regulates the immune system, either by stimulating or suppressing it until desired immunological state has been achieved. This includes restoring balance in autoimmune diseases or enhancing responses against infections or cancers. It can be specific (targeting precise immune pathways) or non-specific and may modulate cellular functions like cytokine production, and lymphocyte activity (Bascones-Martinez *et al*., 2014). There are two types of immunomodulators:

Immunosuppressants:

These agents diminish immune system activity, typically by inhibiting immune cell activation, proliferation, or cytokine signaling. They are vital in preventing transplant rejection or treating autoimmune and inflammatory disorders (Kovarik, 2013). Immunosuppressants include:

- Calcineurin Inhibitors: They block calcineurin, preventing Interleukin-2 transcription and T-cell activation. These drugs are the mainstay medications used to prevent the body's rejection of a transplanted organ. They can also be used in some autoimmune conditions (Plich *et al*., 2021).
- JAK Inhibitors: These agents inhibit JAK-STAT signaling, which many cytokines require. These drugs are used to suppress overactive immune activity in conditions like, Rheumatoid Arthritis, Psoriatic Arthritis, and Inflammatory Bowel Disease (Harrington *et al*., 2023).
- mTOR Inhibitors: They block the mammalian Target of Rapamycin (mTOR) signaling pathway. mTOR is a protein kinase that regulates lymphocyte proliferation and metabolism in response to growth signals (Halloran, 2004).

Immunostimulants:

Immunostimulants are agents used to increase or amplify the immune response. They are primarily used in cancer therapy (immunotherapy), as vaccine adjuvants, and in treating certain immunodeficiency states. Immunostimulants include:

- Vitamin C: Vitamin C supports many front-line immune tasks; helping white blood cells move to infection sites, enhancing microbe killing, and protecting cells from oxidative damage, and supporting skin and mucosal barriers. People who are low in vitamin C are more susceptible to infections (Calder *et al*., 2020).
- Immune Checkpoint Imhibitors: These drugs disable the immune checkpoints that restrain T-cell activity so that, the T-cells can attack cancer vigorously (Postow *et al*., 2018).
- Pattern-Recognition Receptor Agonists: These drugs stimulate TLR7 receptors found on innate immune cells on the skin, resulting to the release of interferons and cytokines (Sun *et al*., 2022).

2.9 VITAMIN C

Vitamin C (ascorbic acid) is a water-soluble essential vitamin that humans must obtain from the diet because the body cannot synthesize it. The vitamin plays a vital role in collagen formation, wound healing, iron absorption, and maintenance of connective tissues, skin, and blood vessels. It also functions as a key antioxidant, protecting cells from oxidative damage and supporting immune defense. Deficiency results in scurvy, characterized by gum bleeding and poor wound healing (Carr and Maggini, 2017).

Pharmacodynamics: Vitamin C accumulates in the leukocytes and supports chemotaxis, phagocytosis, and oxidative burst regulation, helping immune cells function optimally. It acts as a potent antioxidant thereby, protecting biomolecules from oxidative damage (Ang *et al*., 2018).

Pharmacokinetics: IV Vitamin C follows first-order kinetics with a half-life of ~2 h, whereas oral clearance is slower due to renal reabsorption mechanisms (Chen *et al*., 2022).

CHAPTER THREE

MATERIALS AND METHODS

3.1 STUDY DESIGN

The study was a preclinical laboratory based experimental study. It was carried out at Science Laboratory Technology (SLT) laboratory, Faculty of Life Sciences, University of Benin.

3.2 COLLECTION OF PLANT MATERIAL: The *Bryophyllum pinnatum* leaves and *Ocimum gratissimum* leaves were collected from Ekosodin village and the University of Benin.

3.3 SAMPLE PREPARATION: The *Bryophyllum pinnatum* and *Ocimum gratissimum* leaves were washed with water, chopped into small pieces and air dried in a shade with further drying in an incubator for 3 days at 45^0C, blended into powder and stored in an air tight container.

3.4 EXTRACTION PROCEDURE

The powder was extracted with 250ml of methanol solvent using cold maceration method. 500g of the dried extract was poured in a glass jar and made up to ¾ of the glass jar. The mixture was vigorously shaken as often as possible and kept in a dark cupboard so that sun rays would not affect the phytochemicals. After 72 hours, the mixture was poured in a bowl and the concentrate was separated from the solution using a cheesecloth. The concentrate was then poured into crucibles and dried in a water bath at 40^0C for 3 hours after which it was dried completely in an oven at 40^0C for 3 hours. The resulting powder extract was stored in an airtight container and kept in the refrigerator (Ihedioha *et al*., 2021).

3.5 CONSTITUTION OF ALSEVER'S SOLUTION

Alsever's solution is composed of 2.05% dextrose, 0.8% sodium citrate, 0.055% citric acid and 0.42% sodium chloride. To constitute the solution, the reagents were weighed into a conical flask and dissolved with distilled water and made up to the 100ml mark. The solution was dispensed into sterile 10ml bottles and sterilized by autoclaving at 116^0C for 10 minutes. The solution was allowed to cool and the lid tightened with the bottle properly labeled. The solution was stored under refrigeration until it was needed.

3.6 ANTIGEN PREPARATION

Fresh blood was collected from a healthy sheep at a sheep abattoir in Aduwawa market, Benin City and mixed with sterile Alsever's solution at a 1:1 ratio (20 ml of blood in 20ml of alsever solution). The blood was centrifuged at 6400 revolution per min for 10mins to enable red blood cells to settle at the bottom of the test tube, the supernatant was discarded, leaving sheep red blood cell (SRBC) pellets that were washed three times with same Alsever's solution and then resuspended in normal saline to get a concentration of 0.1ml containing 1×10^8 cells. They were then kept under refrigeration for use in immunization and challenge (Gupta *et al*., 2024).

3.7 DRUGS

Cyclophosphamide was used as negative control and Vitamin C was used as positive control. All chemicals and reagents used were of analytical grade and were checked to ensure that they were not expired before the experimentation.

3.8 RESEARCH TOOLS

Cages (polyvinyl cages), plates for food and water, gavage (orogastric tube), sample bottles, EDTA bottles, Hypodermal needles, microtiter plates and vernier caliper.

3.9 LABORATORY ANIMALS

25 disease free Wistar albino rats aged between 6 and 8 weeks were randomized into five experimental groups. They were maintained under standard laboratory conditions and temperature ($25^0C \pm 1^0C$) and light and dark cycle (12h light and 12h dark cycle) in polyvinyl cages. They were fed pelletized feed and given clean water ad libitum. Male and female rats were kept in separate cages from their female counterpart before and during the time of study to avoid conception during the study time. The animals were acclimatized for 2 weeks before the experimental study was carried out. The study was approved by the Faculty of Science Laboratory Technology Research Ethical Committee with refernce number UNIBEN/FSLT/00025.

3.10 GROUPING AND ADMINISTRATION

Animals were classified into five groups and were administered based on their groupings.

GROUP 1: Control group received 10ml/kg of normal saline

GROUP 2: Received 50 mg/kg body weight of plant extract

GROUP3: Received 200 mg/kg body weight of plant extract

GROUP 4: Received 50 mg/kg of Vitamin C

GROUP5: Received 30 mg/kg of Cyclophosphamide

Animals received the reference drug and plant extract for a period of 7 days before immunization with sheep red blood cell.

3.11 DELAYED TYPE HYPERSENSITIVITY RESPONSE (DTH) SRBC CHALLENGE

DTH assay was carried out using Sheep Red Blood Cells (SRBC) antigenic challenge. Delayed Type Hypersensitivity response was determined in control, standard drug and *Bryophyllum pinnatum* and *Ocimum gratissimum* treated rats. Rats were divided into five groups each group comprised five rats. 0.02ml of SRBC suspension was injected into the right paw of Wistar albino rats to sensitize the rats. Prior to sensitization of the rats they were administered plant extracts for three days and continued for 7 days after sensitization. The rats were challenged on day 7 by subcutenous injection of 0.02ml of SRBC left hind foot pad. After the challenge paw thickness was measured using vernier caliper within 24, 48, 72 and 96 hrs (Gupta *et al*., 2024).

3.12 HEMAGLUTTINATION ANTIBODY TITRE

On day 14 after the immunization of the rat through intraperitoneal administration of SRBC, the rats were sacrificed using chloroform inhalation and blood collected via cardiac puncture. Blood was centrifuged at 1609.92× *g* to get serum. Serial two-fold dilutions of serum were made with normal saline in microtiter plates of 96-well capacity and SRBC (25 µL of 1% SRBC prepared in normal saline) added to each of these dilutions. The hemagglutination plates were then incubated at 37 °C for 1 h and then examined for hemagglutination. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the hemagglutination antibody titer (Balmer *et al*., 2007).

3.13 T CELL PROLIFERATION ASSAY

Wistar albino rats (either sex, body weight 250-300gm, n=5 per group) were sensitized with 10% SRBC (1 x 10^8 cells /ml), as described in 3.12 above. The immunized groups were given ethanol extracts of *Bryophyllum pinnatum* and *Ocimum gratissimum* at different doses of 50 and 100

mg/kg/day of body weight and standard immunomodulator drug (Vitamin C) and an Immunosuppressent drug Cyclophosphamide (30 mg/kg), while immunized control group received normal saline. The animals were fed standard rodent pellets and water ad libitum. On day 14, the animals were sacrificed and blood was collected from the rats and T-cells were enumerated. Thymus weight was determined immediately after the animals were euthanized. The weight was measured in milligrams, T-cell, B cell and CD4 count was performed at university of Benin teaching hospital (Adan *et al*., 2016)

3.14 DETERMINATION OF CD4 T CELL COUNT

1. Animals blood was collected into EDTA bottles.
2. 20 µl of the sample was added to 20 µl of phycoerythrin PE antibody in a rohren tube.
3. This was adequately mixed and incubated in the dark for 15 minutes at room temperature.
4. 800 ul of the CD4 no lyse buffer was added to the tube and mixed adequately.
5. The sample tube was plugged onto the sample port of the cyflow machine for counting of CD4+ T cells.
6. The result of the counting was displayed on the monitor and was recorded as the number of cell/µl of blood in the worksheet.

3.15 DETERMINATION OF COMPLETE BLOOD COUNT

A quantitative hematology analyser Couter HMX Hematology analyser was employed for complete blood count in University of Benin Teaching Hospital. Animal blood was collected into

EDTA bottles via venupuncture. The tube containing the blood was labeled and properly mixed and placed in the analyser were it is read out on the worksheet.

3.16 STATISTICAL ANALYSIS

Bryophyllum pinnatum and *Ocimum gratissimum* treatment groups were compared with the control group by employing one way ANOVA and Turkeys multiple comparison tests using GraphPad Prism 8 (GraphPad Software, Inc., CA, USA). Differences with a p-value of 0.05 or lower were considered to be statistically significant.

CHAPTER FOUR

RESULTS

Table 1: Effect of methanolic extract of *Bryophyllum pinnatum* and *Ocimum gratissimum* on the weight and organ weight of Wistar albino rats

Treatment mg/kg	Body weight Initial	Final	Liver	Thymus	Spleen
Control	182±5.15	214±4.3	3.24±0.09	0.39±0.02	1.28±0.1
50 MEPAOP	188±4.6	222±3.5	3.66±0.09	0.64±0.02**	1.52±0.01*
200 MEPAOP	183±4.6	227.2±2.4	3.52±0.06	0.45±0.02	1.38±0.02
50 Vitamin C	190±5.2	225±1.7	3.54±0.12	0.432±0.01	1.32±0.01
50 Cyclo.p.	203.6±3.8	190.8±3.5	29.5±0.14**	25.4±0.12	20.9±0.04**

The result is expressed as mean±SEM, n=5, $p<0.01$ (**) represents the significance level compared with the control.

Key : Cyclo.p**. =** Cyclophosphamide

MEPAOP = Methanol extract of *Bryophyllum pinnatum* and *Ocimum gratissimum*

Table 2: The effect of methanolic extract of *Bryophyllum pinnatum* and *Ocimum gratissimum* on hematological parameters

Group(G1-4) Treatment mg/kg	WBC	Lymphocyte	Monocyte	Neutrophil	Eosinophil
G1 control	1829±184.8	40.6±1.208	4.24±0.2	44.2±2.3	1.26±0.08
G2 50 MEPAOP	2531±31.4**	44.2±2.29	8±0.17****	49.6±1.1	1.5±0.07
G3 200 MEPAOP	2500±22.8**	45.8±0.86	6.54±0.19****	46.8±1.28	1.43±0.06
G4 50 Vit C	2750±22.7**	42.6±0.93	5.2±0.17*	46±1.3	1.52±0.0 9
Cyclo.P 50	1900±32.2	35.2±1.16	3.5±0.1844	41.60±1.1	0.92±0.09*

The result is expressed as mean±SEM, n=5, $p<0.01$ (**), $p<0.001$(****) represents the significance level compared with the control.

Key : Cyclo.p**. =** Cyclophosphamide

MEPAOP = Methanol extract of *Bryophyllum pinnatum* and *Ocimum gratissimum*

Table 3: The effect of methanolic extract of *Bryophyllum pinnatum* and *Ocimum gratissimum* on serological parameters

Groups Treatment mg/kg	**%Glucose (mg/dl)**	**%Hemoglobin (g/dl)**	**Serum protein (g/100ml)**	**Albumin globulin**
G1 control	80.2±1.66	5.84±0.27	7.22±0.4	7.4±0.19
G2 50	80.8±1.84	7.16±0.24*	7.6±0.18*	7.6±0.14
G3 200	86.6±1.36*	8.68±0.2*	9.9±0.13*	8.7±0.9*
G4 50 Vit.C	88.4±1.36**	7.08±0.39*	7.5±0.17	7.6±0.11
G5 50 Cyclo.P.	69.4±1.631**	4.7±0.21*	6.38±0.15*	6.1±0.12**

The result is expressed as mean±SEM, n=5, p<0.01 (**) represents the significance level compared with the control.

Key : Cyclo.p**. =** Cyclophosphamide

MEPAOP = Methanol extract of *Bryophyllum pinnatum* and *Ocimum gratissimum*

Table 4: The effect of methanolic extract of *Bryophyllum pinnatum* and *Ocimum gratissimum* on Delayed Type Hypersensitivity Reaction in Wistar Albino Rats

Groups (G1-5)	**Hypersensisitivity reaction(increase in paw volume (mm))**			
Treatment mg/kg	**Time (hours)**			
	0	**24**	**48**	**96**
G1 Control	3.5±1.2	3.6±0.2	3.4±0.9	3.8±0.3
G2 50	3.5±0.5	3.4±0.3	3.2±0.43	3.0±0.9*
G3 200	3.5±0.2	3.3±0.4	3.1±0.7	2.9±0.1**
G4 50 Vit. C	3.5±0.3	3.4±0.7	3.3±0.6	3.0±0.8*
G5 50 Cyclo.p.	3.5±0.5	3.7±0.1	3.8±0.3	4.2±0.7

The result is expressed as mean±SEM, n=5, $p<0.05$ (*), $p<0.01$ (**) represents the significance level compared with the control.

Table 5: The effect of methanolic extract of *Bryophyllum pinnatum* and *Ocimum gratissimum* on CD4 and B-Cells in Wistar Albino Rats

Groups **Treatment mg/kg**	**CD4 X10^9/L**	**T-CellsX10^9/L**	**B-Cells X10^9/L**
G1 Control	51.9±0.51	2.7±0.3	2.3±2.4
G2 50	54.3±0.66*	3.7±0.9*	4.0±1.7*
G3 200	57.3±0.54*	3.8±0.7*	4.4±3.0*
G4 50 Vit. C&E	54.3±1.2*	3.4±0.9*	4.3±2.1*
G5 50 Cyclo.p.	39±0.71**	1.5±0.07**	0.9±0.29**

The result is expressed as mean±SEM, n=5, $p<0.05$ (*), $p<0.01$(**), represents the significance level compared with the control.

Key : Cyclo.p**. =** Cyclophosphamide

MEPAOP = Methanol extract of *Bryophyllum pinnatum* and *Ocimum gratissimum*

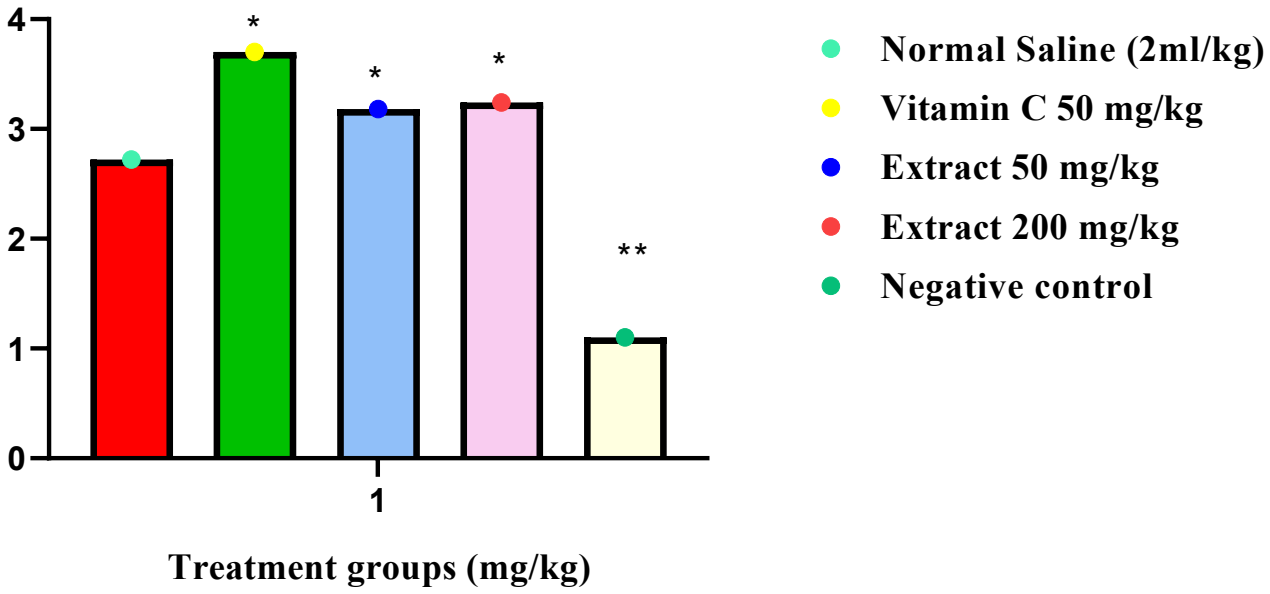

Figure 1: Effect of *Bryophyllum pinnatum* and *Ocimum gratissimum* on CD4 in Wistar Albino Rats

There was significant increase ($p<0.05$) , ($P<0.01$) and (0.001)of T cells at 50 and 200 mg/kg body weight of the plant extract, there was significant decrease ($p<0.001$) of T cells at mg/kg body weight with the negative control.

CHAPTER FIVE

DISCUSSION

5.1 THE EFFECT OF THE BIHERBAL FORMULATION OF *Bryophyllum pinnatum* AND *Ocimum gratissimum* ON THE WEIGHT AND ORGAN WEIGHT OF WISTAR ALBINO RATS

Treatment with the extract led to an increase in body weight as well as higher thymus and spleen indices compared with the control group. In contrast, cyclophosphamide, a standard immunosuppressive drug, caused marked reduction of these organs. The thymus is where T cells mature, while the spleen serves as a major site for immune responses and blood cell turnover. Enlargement of these organs in extract-treated rats suggests enhanced lymphocyte production and improved immune activity. This study shows that the extract promotes overall health and improved immune activity (Ebeye *et al*., 2014; Yemitan *et al*., 2018).

5.2 THE EFFECT OF THE BIHERBAL FORMULATION OF *Bryophyllum pinnatum* AND *Ocimum gratissimum* ON HEMATOLOGICAL PARAMETERS

The extract significantly increased the number of white blood cells (WBCs), lymphocytes, monocytes, and neutrophils. High WBC counts indicate that the extract stimulates the bone marrow to produce more immune cells, which strengthens the body's defense system. The increase in lymphocytes points to improved adaptive immunity, which is essential for long-term protection against infections. The rise in monocytes shows better support for antigen presentation, while the

increase in neutrophils demonstrates stronger innate immune readiness. Cyclophosphamide reduced all these cells, as expected, since it damages bone marrow. Interestingly, the biherbal formulation reversed this suppression, proving its restorative effect. This indicates that the extract has the ability to improve blood cell recovery (Ekpo *et al*., 2021).

5.3 THE EFFECT OF THE BIHERBAL FORMULATION OF *Bryophyllum pinnatum* AND *Ocimum gratissimum* ON SEROLOGICAL PARAMETERS

The extract also increased serum protein, hemoglobin, and the albumin-to-globulin ratio compared with control. Total serum protein is an indicator of the body's ability to produce important molecules such as antibodies. An increase in serum protein therefore points toward improved immune readiness and better nutritional status. The rise in hemoglobin suggests that the extract supported red blood cell production and protected them from oxidative damage, which is common with cyclophosphamide use. This study shows that the formulation has the ability to promote Red Blood Cell formation and protein synthesis(Bassey *et al*., 2021).

5.4 THE EFFECT OF THE BIHERBAL FORMULATION OF *Bryophyllum pinnatum* AND *Ocimum gratissimum* ON DELAYED TYPE HYPERSENSITIVITY REACTION

Paw swelling was reduced in the extract-treated groups compared with the control. This suggests that the extract suppressed cell-mediated immunity, which is mainly driven by T-helper 1 (Th1) cells and macrophages. A lower response indicates decreased recruitment of T cells and

macrophages to the site of antigen challenge and reduced local inflammation. Vitamin C, known immune booster, produced a similar effect, further supporting this interpretation. This shows that the extract also has anti-inflammatory properties (Onwubuya *et al*., 2021).

5.5 THE EFFECT OF THE BIHERBAL FORMULATION OF *Bryophyllum pinnatum* AND *Ocimum gratissimum* ON CD4 AND B-CELLS IN WISTAR ALBINO RATS

The analysis revealed that extract treatment led to significant increases in $CD4^+$ T cells, total T cells, and B cells compared with the control. $CD4^+$ T cells are central coordinators of the immune system, helping both in cellular and humoral immunity. The increase in these cells indicates that the extract enhances immune coordination and responsiveness. The rise in B cells shows that the extract has the potential to improve antibody production. The study indicates the extract has immunorestorative properties (Dobutur *et al*., 2025).

CONCLUSION

The biherbal formulation of *Bryophyllum pinnatum* and *Ocimum gratissimum* demonstrated both immune-enhancing and immune-regulating effects in Wistar rats. It improved body weight and immune organ indices, stimulated blood cell production, enhanced protein metabolism, and restored lymphocyte populations. At the same time, it suppressed delayed-type hypersensitivity responses, indicating anti-inflammatory activity. This highlights its potential as a natural immunomodulator with both stimulatory and regulatory functions.

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