IMMUNOMODULATORY PROPERTIES OF *Terminalia brownii* Fresen. AND *Carissa edulis* (Forssk.) Vahl EXTRACTS

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Immunomodulatory Properties of *Terminalia brownii* Fresen and *Carissa edulis* (Forssk.) Vahl Extracts

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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DEDICATION

This work is dedicated to my late parents; Reuben Mbiri Kamanda and Esther Wangeci Wahome, late guardians; James Kamanda and Bertha Kamanda, and my sister Beth Mbiri for their immense sacrifice, guidance, and support towards my education.

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ABBREVIATIONS AND ACRONYMS

AIDs	Acquired Immune Deficiency Syndrome
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
AMPs	Antimicrobial Peptides
ANOVA	Analysis of Variance
APCs	Antigen Presenting Cells
APPs	Acute Phase Proteins
AST	Aspartate Transaminase
BA	Buehler Assay
BAFF	B cell–Activating Factor
BCR	B Cell Receptors
BTK	Bruton's Tyrosine Kinase
BW	Body Weight
CBA	Cytometric Bead Array
CD	*C. edulis* DCM extract
CD16	Cluster of Differentiation 16
CD_4	Cluster of Differentiation 4
CD40L	Costimulatory Signals
CD8	Cluster of Differentiation 8
Cl^-	Chloride Ions
CLRs	C-type Lectin Receptors

CM	*C. edulis* methanol extract
Cp	Ceruloplasmin
CRP	C-reactive Protein
CSF	Colony-stimulating Factor
CSMF	Colony-Stimulating Granulocyte-Macrophage Factor
CXCL8	C-X-C Motif Chemokine Ligand 8
DAMPs	Damage-Associated Molecular Patterns
DCM	Dichloromethane
DCM	Dichloromethane
DCs	Dendritic Cells
DMSO	Dimethyl Sulfoxide
DTH	Delayed-Type Hypersensitivity
ELISA	Enzyme-Linked Immunosorbent Assay
EMPs	Erythro-Myeloid Precursors
FACS	Fluorescence-Activated Cell Sorting
FcγR	Fcγ Receptors
FO	Follicular
GA	Glatiramer acetate
GCF	Granulocyte Colony-Stimulating Factor
GC-MS	Gas Chromatography-Mass Spectrometry
Hb	Haemoglobin
HCT	Haematocrit

HIV/AIDs	Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome
HMGB1	High Mobility Group Box Protein-1
HSC	Hematopoietic Stem Cells
IFN-γ	Interferon-gamma
IgE	Immunoglobulin E
IL	Interleukin
IL-1β	Interleukin-1 beta
IP	Intraperitoneal
K^+	Potassium Ions
KEMRI	Kenya Medical Research Institute
KU	Kenyatta University
LC-MS	Liquid Chromatography-Mass Spectrometry
LD_{50}	Median Lethal Dose
Lf	Lactoferrin
LLNA	Local Lymph Node Assay
LPA	Lymphocyte Proliferation Assay
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MHC	Major Histocompatibility Complex

MPS	Mononuclear Phagocyte System
MPV	Mean Platelet Volume
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
MZ	Marginal Zone
Na^+	Sodium Ions
NAG	N-acetylglucosamine
NAM	N-acetylmuramic Acid
NETs	Neutrophil Extracellular Traps
NF-κB	Nuclear Factor Kappa B
NK cells	Natural Killer cells
NLR	Nod-like Receptors
NO	Nitric Oxide
NOTCH2	Notch Receptor 2
NSIT	National Institute of Standards and Technology
OECD	Organization for Economic Co-operation and Development
Pam3Cys	S-(2,3-bispalmitoyloxypropyl)-N-palmitoylcysteine
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate-Buffered Saline
PCT	Plateletcrit
PFC	Plaque Forming Cell
PGN	Peptidoglycan
PLT	Platelets
PRRs	Pathogen Recognition Receptors

RAG1	Recombination-Activating Genes 1
RAG2	Recombination-Activating Genes 2
RBCs	Red Blood Cells
RDW	Red Cell Distribution Width
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute Medium
RT	Retention Time
SAA	Serum Amyloid A
SEM	Standard Error of the Mean
SLE	Systemic Lupus Erythematosus
SRBCs	Sheep Red Blood Cells
STAT	Signal transducer and activator of transcription
TCR	T cell Receptors
TD	*T. brownii* DCM extract
Tf	Transferrin
Tfh	Follicular helper T
Th	helper T cells
Th1	T-helper 1
TLR7	Toll-like receptor 7
TLRs	Toll-like receptors
TM	*T. brownii* methanol extract
TNFR1	Tumor Necrosis Factor receptor type 1
TNF-α	Tumor Necrosis Factor-alpha

WBC	White Blood Cells
WHO	World Health Organization

ABSTRACT

Microbial infections are currently on the rise and this significantly increases the rates of mortality and cases of disability worldwide. This surge is attributed to the emergence of antimicrobial resistance, rendering antimicrobial drugs ineffective. Therefore, there is a critical need to develop alternative therapeutic agents with a different mechanism of action, including immunomodulation. *Terminalia brownii* and *C. edulis* are traditionally used to manage infectious diseases including sexually transmitted infections, hepatitis, tuberculosis, pneumonia, among others. However, their impact on the immune system remains unclear. Therefore, the present study investigated the effect of these plants on the immune system. Methanol and dichloromethane were used for the extraction of plant materials. Female Swiss albino mice and Wistar rats were used in this study. To determine the plant extracts' effects on innate immune responses, leucocyte counts, and production of nitric oxide and tumour necrosis factor-alpha by murine macrophages were determined. The 3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide assay was conducted to assess the viability of Vero cells treated with the plant extracts. To establish the plant extracts' effects on cellular and humoral immune responses, levels of tumour necrosis factor-alpha, interferon-gamma, interleukin-2, interleukin-4, and interleukin-5 in mice serum were assessed, and the delayed-type hypersensitivity reaction and haemagglutination antibody titer assay were conducted. Phytochemical analysis was accomplished through gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry. In addition, the safety of the plant extracts was assessed through acute and sub-acute toxicity studies. Administration of the plant extracts significantly ($p < 0.05$) augmented total and differential leukocyte counts in pyrogallol-treated mice in contrast with the control. The extracts did not have an effect on Vero cells' viability at all tested concentrations. Furthermore, the extracts stimulated macrophages to produce significantly ($p < 0.05$) elevated levels of nitric oxide and tumour necrosis factor-alpha. The serum of extract-treated mice showed significantly ($p < 0.05$) increased levels of tumour necrosis factor-alpha, interferon-gamma, and interleukin-2 but significantly ($p < 0.05$) decreased levels of interleukin-4 and interleukin-5 compared to the serum of the control mice. The extracts were also found to significantly ($p < 0.05$) augment delayed-type hypersensitivity and antibody titers. Additionally, *T. brownii* and *C. edulis* exhibited synergism in both innate and adaptive immune responses. The extracts did not induce any acute or sub-acute toxicity in rats. The plant extracts were found to contain diverse compounds (phenols, triterpenoids, flavonoids, tannins, fatty acids, and steroids) with immunomodulatory properties. In conclusion, the modulatory effect on the immune system exhibited by the tested extracts was ascribed to the identified compounds. Additionally, the extracts were non-toxic. The findings of this study provide a crucial ethnopharmacological lead towards the development of novel immunomodulatory agents of herbal origin that can be used to manage microbial infections and immune-related disorders. *Terminalia brownii* and *C. edulis* methanol and dichloromethane extracts should therefore be considered as potential candidates for developing immunomodulatory herbal formulations.

CHAPTER ONE

INTRODUCTION

1.1 Background

At present, there are considerable efforts to fight human ailments and boost human health by modulating the immune system, the body's defense system (Iqbal Yatoo *et al.,* 2021). The immune system refers to an organization of chemicals, cells, and processes that serve to protect the body against infections (Marshall *et al.,* 2018). It is composed of two lines of defense, innate and adaptive immunity. Innate immunity, also known as natural immunity, is the first line of defense against intruding pathogens (McComb *et al.,* 2019). It is a non-specific/antigen-independent mechanism of defense that the host employs immediately or within a few hours of antigen encounter (McComb *et al.,* 2019). Innate immunity has no immunologic memory hence unable to notice the same pathogen when the body is re-exposed (Marshall *et al.,* 2018; McComb *et al.,* 2019). Adaptive immunity, also known as acquired immunity, is specific/antigen-dependent and it's the second line of defense (Delves *et al*., 2017). Adaptive immunity shows a lag time from the time of antigen exposure to the maximum response and it has an immunologic memory (Marshall *et al.,* 2018). Acquired immunity is divided into antibody-mediated and cell-mediated immunity (Marshall *et al.,* 2018).

The occurrence of microbial infections is currently on the rise (Zhao *et al.,* 2022). This is attributed to the development of antimicrobial resistance that renders antimicrobials ineffective (Dhingra *et al*., 2020; Zhao *et al.,* 2022). However, immunomodulation has been reported to have the potential to alleviate this challenge; when the host is targeted, rather than the microbe, the chances of the microbes developing antimicrobial resistance are reduced (Nijnik, 2013). Consequently, research on various agents with immunomodulatory potentials has escalated over recent times in search for more effective antimicrobial agents (Kraehenbuehl *et al.,* 2022).

Immunomodulation refers to any adjustment in the immune response including inhibition, expression, stimulation, or amplification of any stage or portion of the immune response (Abood *et al*., 2014). Immunomodulators are therefore elements exploited for their impacts on the immune system (Abood *et al*., 2014). On the basis of their effects, immunomodulators can be classified into immunosuppressors, immunostimulators, and immunoadjuvants (Behl *et al.*, 2021). Immunostimulants are non-specific and they enhance the resistance of the body to infection (Behl *et al.*, 2021). They act via both adaptive and innate immune responses (Billiau and Matthys, 2001). In healthy persons, the immunostimulants serve as the promoter and prophylactic agents because they increase the basic magnitude of an immune response (Subramani & Michael, 2017). In persons with impaired immune responses, they serve as immunotherapeutic agents (Billiau and Matthys, 2001; Subramani & Michael, 2017). On the other hand, immunosuppressants regulate immune responses and are a functionally and structurally heterogeneous category of drugs (Tönshoff, 2020). They are usually given in combination regimens to manage a number of autoimmune diseases and organ transplant rejections (Tönshoff, 2020).

Immunoadjuvants improve the efficacy of vaccines hence they are viewed as specific immune enhancers. They maintain the promise of being the immune responses' true immunomodulators (Kaur *et al.,* 2020). Immunoadjuvants have been proposed to be used as selectors between humoral and cellular helper T1 and helper T2 cells, immunodestructive and immunoprotective, and immunoglobin G versus reagenic types of immune response, that pose a great difficulty to vaccine development (Kaur *et al.,* 2020).

Immunomodulation can be achieved using various agents including microbial products, physiological products including cytokines, panchgavya (cow therapy), herbal products, and synthetic chemicals (Pant & Chauhan, 2020). However, among these, the use of herbal products for immunomodulation is more preferred due to their diverse biological activities, fewer side effects, easy availability, and affordability (Yatoo *et al*., 2018). Plant-derived immunomodulators have a huge potential for the production of novel pharmaceutical products (Carqueijeiro *et al.,* 2020).

Immunomodulatory properties of some *Terminalia* species have been documented. *Terminalia bellerica* has been reported to stimulate the phagocytic activity of macrophages and lymphocytes proliferation (Saraphanchotiwitthaya *et al.,* 2008; Gupta *et al.,* 2020). *Terminalia arjuna* extracts were found to reduce the levels of pro-inflammatory cytokines, including TNF-α, IL-6 and IL-18, in coronary artery disease patients (Kapoor *et al.*, 2015), The fruit extract of *Terminalia chebula* increased antibody titer levels and Delayed Hypersensitivity (DTH) reaction in mice (Shivaprasad *et al.,* 2006, 2016; Aher & Kumar 2010). Ethanolic extract of *Terminalia catappa* demonstrated immunosuppressive properties by inhibiting the production of TNF-α, IL-6, and IL-23 in rats and IL-1β and NO by macrophages (Abiodun *et al.,* 2016).

Carissa species have also been reported to have immunomodulatory properties. Carissone and scopoletin, isolated from *Carissa carandas,* have been reported to reduce the production of proinflammatory mediators including TNF-a, nitric oxide (NO) and IL-1b by J774A.1 cells (Galipalli *et al*., 2015). Ethanolic fruit extract of *C. carandas* and lanostane, a triterpenoid isolated from the extract was shown to increase the numbers of white blood cells (WBCs) in immunosuppressed mice *(*Arif *et al.,* 2013). *Carissa macrocarpa* extracts have been reported to reduce the production of a pro-inflammatory mediator, NO, in macrophages (Souilem *et al*., 2019). Other medicinal plants with immunomodulatory properties include *Trigonella foenum* (Martel *et al*., 2017), *Withania somnifera* (Singh *et al*., 2016), *Acacia catechu* (Ismail & Asad, 2009), *Picrorhiza kurroa* (Sharma *et al.,* 2017), *Achillea wilhelmsii* (Sharififar *et al*., 2009), *Caesalpinia bonducella* (Singh *et al*., 2016), *Picrorhiza scrophulariiflora* (Singh *et al*., 2016), and *Allium sativum* (Sharma *et al.,* 2017), among others.

Terminalia brownii is a member of the family Combretaceae (Machumi *et al.,* 2013). It is used traditionally as a remedy for diabetes, fungal infections, malaria, yellow fever, jaundice, dermatitis, hepatitis, wounds, syphilis, gonorrhea, urethral pain, leucorrhea, malaria, urinogenital problems, epilepsy, and as anthelminthic (Mbwambo *et al.,* 2007; Alema *et al.,* 2020). The biological activities of *T. brownii* include anticancer (Sintayehu *et al*., 2017), pre-neoplastic lesion prevention (Tesfaye

et *al.,* 2018), anti-inflammatory (Mbinda *et al*., 2016a), antimalarial (Biruk *et al.,* 2020), antioxidant (Sintayehu *et al*., 2017), anti-diabetic (Alema *et al.,* 2020), antinociceptive (Mbiri *et al*., 2016), antipyretic (Mbinda *et al*., 2016b) and antifungal (Machumi *et al.,* 2013).

Carissa edulis belongs to the family Apocynacea (Opande, 2022). It is traditionally used for the management of cancer, diabetes, mental illness, schistosomiasis, gonorrhoea, syphilis, malaria, women's infertility, abdominal pains, polio symptoms, chest pains, gastric ulcers, and toothache and as an expectorant (Mudimba and Nguta, 2019). *Carissa edulis* has several biological activities including antiviral (Tolo *et al.,* 2006), anti-tumor (Ya'u *et al*., 2016), anti-inflammatory (Hassan *et al*., 2010; Ya'u *et al.,* 2017), antimalarial (Kebenei *et al*., 2011), cardiovascular effects (Hanan and Hassan, 2010), antioxidant (Fanta *et al*., 2019), hepatoprotective effects (Al-Awthan, 2019), diuretic (Kebamo *et al*., 2015), anti-convulsant (Ya'u *et al.,* 2008), antimicrobial (Opande, 2022) and analgesic (Maina *et al*., 2015).

Terminalia brownii stem bark and *Carissa edulis* root bark decoctions are traditionally used as remedies for microbial infections including hepatitis, sexually transmitted infections, tuberculosis, pneumonia, among others (Alema *et al*., 2020; Fanta Yadang *et al*., 2019). However, their effect on the immune system has not been studied. This study was therefore devised to determine the immunomodulatory properties of *T. brownii* stem bark and *C. edulis* root bark extracts.

1.2 Statement of Research Problem

Microbial infections are currently on the rise and this has significantly increased mortality rates and disability cases worldwide (Zhao *et al.,* 2022). This increase is attributed to the emergence of antimicrobial resistance, rendering antimicrobial drugs ineffective (Dhingra *et al*., 2020; Zhao *et al.,* 2022). Antimicrobial resistance is a major global health threat; in the year 2019, antimicrobial resistance was reported to cause 1.2 million deaths worldwide (Tang *et al*., 2023). It estimated that by the year 2050, antimicrobial resistance could result in approximately 10 million deaths globally (Pulingam *et al*., 2022). Antimicrobial resistance is also a threat to the economic sector (Pulingam *et al*., 2022). Rising antibiotic resistance is likely to

exacerbate poverty levels, primarily driven by increased treatment expenses, prolonged hospital stays, and premature deaths, all of which directly impact overall productivity rates (Pulingam *et al*., 2022). Therefore, there is a critical need to develop alternative therapeutic agents with a different mechanism of action.

1.3 Justification of the study

Despite microbial infections being on the rise, antimicrobials have become less effective due to the development of antimicrobial resistance (Dhingra *et al*., 2020). There is therefore a need to develop novel drugs with new mechanisms of action, including immunomodulation. Immunomodulation promotes host resistance to microbial infections (Nijnik, 2013). Additionally, targeting the host instead of the microbe reduces the chances of the development of antimicrobial resistance by the microbes (Nijnik, 2013). The use of herbal products for immunomodulation is currently on the rise due to their few side effects, diverse biological activities, and easy availability and affordability (Yatoo *et al.*, 2018). *Terminalia brownii* and *C. edulis* have been traditionally used to manage microbial infections, including sexually transmitted infections (STIs), hepatitis, tuberculosis, pneumonia, among others (Alema *et al*., 2020). They should therefore be investigated to establish if they have immunomodulatory properties. The results this study provides a crucial ethno-pharmacological lead towards the development of novel immunomodulants for the management of infectious and immune-related diseases.

1.4. Hypothesis

Methanol and dichloromethane (DCM) crude extracts of *T. brownii* stem bark and *C. edulis* root bark do not have immunomodulatory effects.

1.5. Objectives of the Study

1.5.1 Broad Objective

To evaluate the immunomodulatory properties of *T. brownii* and *C. edulis* extracts.

1.5.2 Specific Objectives

1. To determine the effect of *T. brownii* stem bark and *C. edulis* root bark extracts on innate immune responses in Swiss albino mice, individually and synergistically.
2. To determine the effect of *T. brownii* stem bark and *C. edulis* root bark extracts on humoral and cellular immune responses in Swiss albino mice, individually and synergistically.
3. To establish acute and sub-acute toxicity levels of *T. brownii* stem bark and *C. edulis* root bark extracts in Wistar rats.
4. To identify and quantify the major phytochemical secondary metabolites in *T. brownii* stem bark and *C. edulis* root bark extracts through liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS).

1.6 Scope of the Study

The proposed study focused on the evaluation of the immunomodulatory properties of *T. brownii* stem bark and *C. edulis* root bark extracts. Plant samples were obtained from Kitui County, Kenya and the study was conducted for a period of 36 months. The study was restricted to determining the effect of *T. brownii* and *C. edulis* extracts on innate, humoral, and cellular immune responses, and the establishment of toxicity levels and the phytochemical profiles of the two plant species.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

This chapter provides a detailed review of the literature related to the objectives of the study. The reviewed content provides context in relation to the components of the immune system: adaptive and innate immunity, immunomodulatory agents: synthetic and natural agents as well as the role of plants as immunomodulatory agents. Besides, the safety of medicinal plants as therapeutic agents has been reviewed.

2.2 The Immune System

The immune system is a body defense powerhouse that comprises a large network of organs including the one marrow, Peyer's patches, lymph nodes, spleen, thymus) (Carrillo *et al.,* 2017). These organs are connected by specific flows of proteins, chemicals, and cells in blood vessels, extracellular fluids, and lymph vessels (Chowdhury *et al.,* 2020). The immune system is present in all forms of life to protect the body against a wide range of diseases, injuries, cell changes, and foreign substances or pathogens (Cohen & Efroni, 2019). It therefore plays a crucial role in identifying and neutralizing threats and enhancing immunological memory that maintains health (Chowdhury *et al.,* 2020).

Two kinds of responses are exhibited by the immune system: innate immune responses that provide a fast and unspecific response and adaptive immune responses that take over the body's defense from innate immunity and provide a slow though specific response (Diamond & Kanneganti, 2022). These two immune responses are complementary in their action. However, excessive immune responses can be extremely deleterious, and therefore tight regulation is essential (Wenzek *et al.,* 2022). Several types of immune cells can be allocated either to the innate or the adaptive immunity arm (Diamond & Kanneganti, 2022).

White blood cells (leukocytes) are the main players in the immune system (Gasteiger *et al.,* 2017). These cells travel throughout the body via the blood and lymphatic

vessels patrolling for foreign antigens (Chowdhury *et al.,* 2020). Leukocytes consist of cells originating from myeloid progenitor cells (neutrophils, eosinophils, basophils, monocytes) and lymphoid progenitor cells (T lymphocytes, B lymphocytes, and natural killer (NK) cells) (Michaud *et al.,* 2015; Gasteiger *et al.,* 2017). Both myeloid and lymphoid progenitors originate from hematopoietic stem cells (HSC) (Gasteiger *et al.,* 2017). hematopoietic stem cells, through a process of developmental signals, become epigenetically programmed to develop into the myeloid (myeloid-biased) or lymphoid lineages (lymphoid-biased) which can be programmed into their specific cellular fates (Michaud *et al.,* 2015; Gasteiger *et al.,* 2017).

2.2.1 Innate Immunity

Innate immunity also referred to as natural response is the first line of body defense that is fast, nonspecific, and does not retain the memory of previous responses (Gasteiger *et al.,* 2017). Elements of innate immunity can be found in all multicellular organisms (Sommerfeld, 2019). In mammals, it embraces nearly all tissues, particularly barrier surfaces for example conjunctiva, skin, and the mucosal surfaces of the gastrointestinal and respiratory tract (Carrillo *et al.,* 2017). In addition, anti-infective chemicals (alarmins, antimicrobial peptides (AMPs), complement factors, cytokines/chemokines) and enzymes (chitinases/chitinase-like proteins and proteases) present on these surfaces, impede access of potential pathogens to the epithelium and underlying tissues during early stages of the infection (Smith *et al.,* 2019; Woodell-May & Sommerfeld, 2019).

If a pathogen passes through the mechanical barriers, it is detected by various preserved types of pathogen recognition receptors (PRRs) on epithelial cells and resident cells of the innate immune system (Woodell-May & Sommerfeld, 2019). Pathogen recognition receptors recognize the pathogen-associated molecular patterns (PAMP) found in the pathogens and damage-associated molecular patterns (DAMPs) (Gasteiger *et al.,* 2017; Smith *et al.,* 2019). Damage-associated molecular patterns are molecules that are often released from necrotic cells damaged by invading pathogens (Gasteiger *et al.,* 2017; Smith *et al.,* 2019). There are many classes of

PRRs in humans that include cytoplasmic proteins (NOD-like receptors (NLR) and the Retinoic acid-inducible gene-I-like receptors), and transmembrane receptors (C-type lectin receptors (CLRs) and the Toll-like receptors (TLRs)) (Smith *et al.,* 2019; Woodell-May & Sommerfeld, 2019).

Recognition of the invading pathogens leads to blood vessel dilation and increased blood flow to the site of infection/damage (Gasteiger *et al.,* 2017; Smith *et al.,* 2019). This enables innate immune cells [neutrophils, eosinophils, basophils, and monocytes (that can transform into macrophages in the tissue), as well as mast cells and dendritic cells (found in tissue)] to reach the target site and thus participate in several responses depending on their cell subtype (Michaud *et al.,* 2015; Smith *et al.,* 2019). The responses include phagocytosis that subsequently destroys the pathogen, the release of inflammatory mediators, activation of complement system proteins, synthesis of acute phase proteins, cytokines, chemokines, and activation of the adaptive immune system (Carrillo *et al.,* 2017; Woodell-May & Sommerfeld, 2019). Various cellular components involved in innate immunity include:

2.2.1.1 Neutrophils

Neutrophils are the most abundant leukocytes in circulation and play a key role as the first cell line of defense for the innate immune response against invading pathogens (especially of bacterial and fungal origin) (Carrillo *et al.,* 2017; Gasteiger *et al.,* 2017). Neutrophils develop in the bone marrow for fourteen days in response to both extracellular stimuli and intracellular regulators (Gasteiger *et al.,* 2017). They undergo two stages: a mitotic stage and a maturation stage. The mitotic stage lasts for seven days and results in the formation of a myelocyte (Mortaz *et al.,* 2018; Ustyantseva *et al.,* 2019). The myelocyte enters the maturation stage producing a mature segmented neutrophil that is retained in the storage pools before entering the bloodstream (Gasteiger *et al.,* 2017). The release of neutrophils is controlled by several factors such as granulocyte colony-stimulating factor (GCF) and colony-stimulating granulocyte-macrophage factor (CSMF) (Mortaz *et al.,* 2018; Ustyantseva *et al.,* 2019).

After pathogens have evaded the epithelial barriers, neutrophils are recruited from the bloodstream to the site of infection (Ding & Xiang, 2018). Neutrophils cross the blood vessels and migrate to the infection site with help of chemotactic factors including leukotriene B, complementary peptide C5a, platelet-activating factor, and bacterial peptide formyl-methionyl-leuco-phenylalanine and cytokines including TNF and interleukin-8 (Mortaz *et al.*, 2018; Ustyantseva *et al.*, 2019). The chemotactic factors and cytokines are produced as inflammatory signals during tissue damage caused by the invading pathogens (Ding and Xiang, 2018). Activated neutrophils reach the infection site and initiate the phagocytosis process by generating Reactive Oxygen Species (ROS), and releasing granules packed with proteases and specific anti-microbial peptides (Ustyantseva *et al.*, 2019).

Likewise, neutrophils can generate neutrophil extracellular traps (NETs) formed by granule substances and nuclear components capable of cooling off virulence factors and destroying extracellular pathogens (Lehman & Segal, 2020). A large quantity of NETs is present in inflammatory sites, acting directly on microorganisms (Ding & Xiang, 2018). They also serve as a physical barrier that prevents pathogens from spreading (Ding & Xiang, 2018). Under normal conditions, neutrophils are cleared from the circulation and inflamed tissues by apoptosis (Mortaz *et al.*, 2018). Disturbances in apoptosis of these cells have been associated with several autoimmune conditions such as systemic lupus erythematosus (SLE) and severe sepsis (Ustyantseva *et al.*, 2019; Carrillo *et al.*, 2017).

2.2.1.2 Eosinophils

Eosinophils are multipurpose granulocytes that are involved in host defense against helminth infection, allergic reactions, and asthma (Aoki *et al.*, 2021). Eosinophils develop in the bone marrow under the influence of CSMF, interleukin-5, and 3 (Carrillo *et al.*, 2017). After maturation, they circulate through the bloodstream in small amounts and can be found in greater numbers in mucosal regions for example respiratory, gastrointestinal, and genitourinary tracts (Carrillo *et al.*, 2017; Aoki *et al.*, 2021). Eosinophils are recruited to sites of parasitic infections and allergic

reactions by adhesion molecules (β7, β2, and β1 integrins), cytokines (interleukine-5, 4 and 13), and chemokines (eotaxins) (Carrillo *et al.*, 2017).

Once activated, eosinophils induce inflammation through the production and release of eosinophilic cationic contents of granules (Carrillo *et al.*, 2017; Wechsler *et al.*, 2021). The main components of these granules are major basic protein, eosinophil cationic protein, eosinophil derived neurotoxin, and eosinophil peroxidase. These components have great potential cytotoxicity on parasites but also can cause tissue injury (Wechsler *et al.*, 2021). Eosinophil cationic protein and neurotoxin are ribonucleases with antiviral properties and stimulate mucus secretion in the airways (Aoki *et al.*, 2021). The major basic protein presents toxicity to parasites, induces degranulation of mast cells and basophils, and activates the synthesis of remodeling factors by epithelial cells (Wechsler *et al.*, 2021). Activated eosinophils die by eosinophil cytolysis with lytic degranulation. Elevated blood counts should prompt further evaluation for eosinophilic disease (Aoki *et al.*, 2021).

2.2.1.3 Basophils

Basophils develop from hematopoietic stem cells through stimulation by interleukin-3 and are released into the blood in a relatively small proportion (Carrillo *et al.*, 2017). The lifespan of basophils is thought to be relatively short approximately sixty hours (Chauhan *et al.*, 2022). Upon stimulation, either via IgE immune complex formation or by innate signals, basophils cytoplasmic granules can release an array of immune mediators such as histamine, granzyme B, or cytokines such as TNF-α (Carrillo *et al.*, 2017). These immune mediators play key roles in eliciting powerful effector functions in different allergic diseases and the manifestation of type 1 hypersensitivity (Carrillo *et al.*, 2017). Beyond allergies, basophils can be recruited to tissues in chronic and autoimmune inflammation, and respond to parasitic, bacterial, and viral infections. Basophils may be involved in antigen presenting via MHC class II to CD4+ T cells (Chauhan *et al.*, 2022).

2.2.1.4 Monocytes

Monocytes are 25 to 30 μm in size round cells with a kidney-shaped nucleus. Monocytes are categorized in the mononuclear phagocyte system (MPS) alongside dendritic cells (DCs) and macrophages (Teh *et al.,* 2019). Prenatally, monocytes are derived from erythro-myeloid precursors (EMPs) in the fetal liver which is important for populating the majority of tissue-resident macrophages (Teh *et al.,* 2019). After birth, monocytes arise from bone marrow-derived HSCs and are released into the circulation accounting for 5 to 10% of all blood immune cells (Teh *et al.,* 2019). Monocytes have a life span of about one to three days once they mature (Teh *et al.,* 2019). Mature monocytes survey peripheral tissues and maintain endothelial integrity (Teh *et al.,* 2019). Upon sensing pathogenic breaches or inflammatory stimuli, monocytes migrate into tissues where their plasticity allows them to differentiate into inflammatory macrophages or DCs according to the environmental niche (Teh *et al.,* 2019). Alternatively, they may also migrate into tissues in absence of inflammation and remain in an undifferentiated state where they perform homeostatic roles (Teh *et al.,* 2019).

2.2.1.5 Macrophages

Macrophages reside in every tissue of the body and exhibit great functional diversity. They play an essential role in tissue development, surveillance, and monitoring of tissue changes, as well as maintaining tissue homeostasis (Nakase *et al.,* 2018). Many tissue macrophages are of embryonic origin (fetal liver or yolk sac) and capable of self-renewal which are maintained independently from the bone marrow-derived monocytes (Austermann *et al.,* 2022). Tissue macrophages can switch their phenotype from a basal state (homeostatic functions) to a proinflammatory state that eliminates pathogens. They can also change to an anti-inflammatory/pro-resolving phenotype depending on the stage of the disease and signals they receive from the microenvironment (Nakase *et al.,* 2018).

Toll-like receptors (TLRs) present on the surface of pro-inflammatory macrophages detect and bind to various types of components derived from bacteria or viruses as ligands (Austermann *et al.,* 2022). For example, TLR4 is involved in the detection of

Gram-negative bacteria and their associated endotoxins such as lipopolysaccharide (LPS) (Austermann *et al.,* 2022). On the other hand, TLR2 recognizes Gram-positive bacteria's lipoteichoic acid (LTA) and peptidoglycan (PGN) (Austermann *et al.,* 2022; Lendeckel *et al.,* 2022). Toll-like receptors also can sense and bind to DAMPs such as DNA, calprotectin (S100A8/S100A9), high mobility group box protein-1 (HMGB1), uric acid, and heat-shock proteins (Dominguez-Andres and Netea, 2019). This triggers the kappa-light-chain-enhancer of activated B-cells (NF-κB) signaling pathway, which ultimately leads to the production and release of proinflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), interleukin-6, 12, and chemokine ligand 8 (CXCL8, IL-8) (Lendeckel *et al.,* 2022). These cytokines increase phagocytotic activity and pathogen killing (Dominguez-Andres & Netea, 2019; Austermann *et al.,* 2022).

Also, there is increased production of ROS and nitrogen oxide (NO) that enable the effective killing of phagocytosed pathogens and combat infection efficiently (Nakase *et al.,* 2018; Dominguez-Andres & Netea, 2019). Activated pro-inflammatory macrophages utilize large quantities of glucose to fuel glycolysis and oxidative phosphorylation pathway that are necessary for the production of pro-inflammatory cytokines and reactive oxygen species (Austermann *et al.,* 2022). In anti-inflammatory and resolution macrophages, ROS play a critical role in the clearance of apoptotic cells (Lendeckel *et al.,* 2022). Macrophages also function as antigen presenting cells (APCs). They present peptide antigens derived from digested bacteria within tissues on the major histocompatibility complex class II and stimulate acquired immunity by activating helper T cells (Austermann *et al.,* 2022; Lendeckel *et al.,* 2022).

2.2.1.6 Natural Killer (NK) Cells

Natural killer (NK) cells are large cytotoxic granular lymphocytes that mediate the death of tumor cells, virus-infected cells, and other intracellular pathogens (Lendeckel *et al.,* 2022). They differentiate and mature in the bone marrow, lymph node, spleen, tonsils, and thymus where they then enter into the circulation (Lendeckel *et al.,* 2022; Eeden *et al.,* 2020). These cells account for 10 to 15% of

blood lymphocytes and may increase significantly in number during viral infections (Brauning *et al.,* 2022). Interleukin-12 leads to the activation of killer NK (Eeden *et al.,* 2020). They release cytotoxins (perforin and granzyme) from their cytoplasmic granules and induce apoptosis of their targets (Lendeckel *et al.,* 2022). They also secrete cytokines like interferon-gamma and TNF-α which recruit additional immune cells to the site of infection or inflammation (Brauning *et al.,* 2022). Natural killer cells express CD16, which allows the detection of antibody-coated target cells, leading to NK cell antibody-dependent cell cytotoxicity (Eeden *et al.,* 2020).

2.2.2 Adaptive Immunity

The adaptive immunity, found in most vertebrates, is antigen-specific. It detects specific antigens that are foreign to the host (Woodell-May & Sommerfeld, 2019). It can form an immunologic memory, maintained by memory cells, allowing the immune system to mount an efficient and quick response upon secondary exposure to the same antigens (Woodell-May & Sommerfeld, 2019). The adaptive immune response is partially determined by genetic variants, but a large component of the response is modulated by lifetime exposures to infection and allergens (Woodell-May & Sommerfeld, 2019). The adaptive immune response is controlled by lymphocytes. The highly mobile lymphocytes comprise B cells and T cells (Woodell-May & Sommerfeld, 2019).

The two types of cells, B and T lymphocytes, arise in the bone marrow (Michaud *et al.,* 2015). The B cells mature in the bone marrow whereas T cells migrate to the thymus where they mature (Michaud *et al.,* 2015). After developing in the primary lymphoid organs (thymus and bone marrow), lymphocytes are trafficked to secondary lymphoid organs; lymph nodes, and the spleen. These lymphoid organs serve to capture circulating antigens from lymph and blood (Michaud *et al.,* 2015). The B cells produce antibody proteins that specifically bind antigens while T cells coordinate the elimination of pathogens (Woodell-May & Sommerfeld, 2019). The T cells can be broadly categorized into helper T cells (Th cells) (CD4+) and cytotoxic T cells (Tc cells) (CD8+) (Woodell-May & Sommerfeld, 2019). Helper T cells are generally divided into Th1, Th2, and Th17 cells (Woodell-May & Sommerfeld,

2019). They secrete cytokines to stimulate the proliferation and differentiation of cells involved in the immunologic response. On the other hand, cytotoxic T cells, also known as T killer cells, eliminate targeted cells and memory T cells (Woodell-May & Sommerfeld, 2019).

2.2.2.1 B Lymphocytes

B cells are programmed plasma cells that produce antibodies (Schultz, 2021). These antibodies permeate extracellular spaces, where they protect against infection (Schultz, 2021). The B cells make up about 20% of the total number of lymphocytes in the body at any given time (Doyon-Laliberté *et al.,* 2022). B cells pass through several distinct developmental stages that occur in different tissues and organs of the immune system (Doyon-Laliberté *et al.,* 2022). It is in the bone marrow that they undergo rearrangement of their B cell receptors' heavy chains (during the pro-B stage) and their light chains (during the pre-B stage) via the action of the recombination-activating genes 1 (*RAG1)* and *RAG2* (Cyster & Allen, 2019). Generally, during these recombination steps, B cells also undergo positive and negative selection to ensure that the new B cell receptors (BCR) do not bind to self-antigens (Doyon-Laliberté *et al.,* 2022). This entire developmental sequence occurs in the absence of any contact with an exogenous antigen (Doyon-Laliberté *et al.,* 2022). Thus, it is called antigen-independent B-cell development (Doyon-Laliberté *et al.,* 2022). The immature B-cells, which express the newly rearranged BCR of the IgM isotype on their surface are capable of recognizing a huge diversity of antigens (Cyster & Allen, 2019; Doyon-Laliberté *et al.,* 2022). The B-cells that exit the bone marrow and migrate to the spleen or other secondary lymphoid tissues are called transitional immature B-cell (TI) (Hoffman *et al.,* 2016).

The second phase of B-cell development called the antigen-dependent phase occurs in secondary (or peripheral) lymphoid tissues such as lymph nodes, tonsils, Peyer's patches, and spleen after encountering an antigen and becoming activated (Hoffman *et al.,* 2016). Various signals namely B cell–activating factor (BAFF), transcription factors, Notch Receptor 2 (NOTCH2), and Bruton's tyrosine kinase (BTK) will commit TI B-cells to either follicular (FO) or marginal zone (MZ) B-cell fates

(Cyster & Allen, 2019). Marginal zone B cells are retained in the spleen while FO B cells recirculate, populating various secondary lymphoid tissues (Hoffman *et al.,* 2016). Within the lymphoid tissues, B cells migrate non-directionally surveying for protein, glycolipid, and polysaccharide antigens which could be free soluble or displayed on the surface of subcapsular macrophages and follicular dendritic cells (Hoffman *et al.,* 2016; Cyster & Allen, 2019). Marginal zone B-cells are activated via a T-independent pathway by polysaccharide and glycolipid antigens which are poor activators of T cells and are derived from pathogens or damaged cells (Hoffman *et al.,* 2016). The MZ B-cells express TLRs that recognize the PAMPs and DAMPs leading to rapid differentiation into IgM or isotype-switched plasma cells or memory B cells (Cyster & Allen, 2019). MZ B cells also interact with other lymphocytes example natural killer T cells, neutrophils, and DCs, that provide cytokines (BAFF, a proliferation-inducing ligand, IL-21, IL-6, and IL-10) and costimulatory signals (CD40L) within the extrafollicular areas (Cyster & Allen, 2019). This promotes immunoglobulin isotype switching independent of T cells that provide adequate immunity before the recruitment of effective T-cell help (Hoffman *et al.,* 2016; Cyster & Allen, 2019).

Follicular B cells activation is initiated upon protein antigen recognition by B-cell surface immunoglobulin receptors (Cyster & Allen, 2019). These receptors signal the cell's interior to trigger essential gene expression programs that internalize the antigen and deliver it to endosomal compartments (Hoffman *et al.,* 2016). In the endosomal compartments, the antigen is degraded into peptides that are then bound to MHC-II molecules and recycled to the surface of the B lymphocyte to be presented to antigen-specific CD4 T-cells (Hoffman *et al.,* 2016). The activated CD4 T-cells in turn secrete costimulatory ligands (CD40L) and cytokines (IL-4, IL-21, and IFN-γ) (Hoffman *et al.,* 2016). B cells express similar co-stimulating molecules such as CD40, B7-1 (CD80), and B7-2 (CD86) (Cyster and Allen, 2019). These molecules stimulate B-cell proliferation and differentiation into short-lived plasma cells (Cyster & Allen, 2019). These cells secrete low-affinity antibodies without somatic mutation or enter a follicle to establish a germinal center where B cells can change from producing IgM and IgD to other isotypes, such as IgG, IgA, and IgE (Hoffman *et al.,* 2016; Cyster & Allen, 2019).

During an immune response, B-cells produce antibodies that fight against multiple antigens of different origins to protect the body against possible harm (Hoffman *et al.,* 2016). Some B cells acquire the ability to produce effector cytokines such as IFN-γ, and TNF-α (that have direct injurious effects on endothelial and epithelial cells, thus contributing to both allograft rejection and inflammatory renal disease) (Hoffman *et al.,* 2016). Some also produce IL-17 which stimulates cytokine and chemokine production by endothelia, epithelia, and fibroblasts that in turn drive neutrophil infiltration and inflammation (Hoffman et al., 2016).

2.2.2.2 T Lymphocytes

T cells (also called T lymphocytes) are major components of the adaptive immune system (Kumar *et al.,* 2018). T cells populate virtually every organ and tissue with the majority being in the skin and mucosal sites (Kumar *et al.,* 2018). Here, they act against microbe-infected cells and foreign tissues (Kumar *et al.,* 2018). They also regulate the activation and proliferation of other immune system cells such as macrophages, B cells, and other T cells (Kumar *et al.,* 2018). They are also implicated as major drivers of many inflammatory and autoimmune diseases. CD4+ T cells and CD8+ T cells make up the majority of T-lymphocytes (Kumar *et al.,* 2018). In the thymus, as T cells, T cell receptors (TCR) are rearranged to generate CD4+ CD8+ double-positive (DP) cells (Kumar *et al.,* 2018). The selection follows where DP thymocytes with receptors for self-antigens receive negative signals and are removed leaving behind CD4+ or CD8+ single-positive (SP) thymocytes (Kumar *et al.,* 2018). The thymocytes interact with thymic epithelial MHC class II or MHC class I molecules, respectively (Kumar *et al.,* 2018). These cells are afterward exported to the peripheral lymphoid organs as fully differentiated but antigen-naive T cells (Kumar *et al.,* 2018).

Naïve T lymphocytes are activated through the interaction of their TCRs with antigenic peptides complexed with MHC molecules (Ali *et al.,* 2020). Once the T lymphocytes recognize their specific antigens, they proliferate and differentiate into effector T lymphocyte subsets (Ali *et al.,* 2020). CD8+ T cells can interact with endogenous antigens encoded either in the host genome or by infecting viruses or

other pathogens replicating intracellularly on almost any cell expressing MHC class I molecules (HLA-A, HLA-B, and HLA-C) (Kumar *et al.,* 2018). These T cells kill their target cells primarily by releasing cytotoxic granules into the target cell (Ali *et al.,* 2020). In contrast, the TCRs of CD4+ T lymphocytes also known as T helper (Th) lymphocytes engage exogenous peptides bearing MHC class II molecule (HLA-DR, HLA-DQ, and HLA-DP) through phagocytosis or endocytosis (Kumar *et al.,* 2018). MHC class II molecules are presented on APCs. CD4+ T cells carry out various functions, ranging from activating other immune cells, releasing cytokines, and helping B cells to produce antibodies (Kumar *et al.,* 2018; Ali *et al.,* 2020).

CD4+ T cells are subdivided into two main subpopulations Th1 and Th2 cells, each group produces mutually exclusive panels of cytokines (Ali *et al.,* 2020). Th1 cells secrete cytokines such as IFN-γ, TNF-α, and IL-2. Th1 cell cytokines drive cell-mediated immune responses, activate mononuclear phagocytes, natural killer (NK) cells, and cytolytic T cells to kill intracellular microbes and protect the body against viruses introduced through infection or vaccines (Ali *et al.,* 2020). On the other hand, Th2 cells produce IL-9, IL-5, IL-25, IL-13, and IL-4 in response to helminthic, bacterial, and parasitic pathogens (Ali *et al.,* 2020). These pathogens include *Streptococcus pneumonia, Clostridium tetani, Pneumocystis carinii, Staphylococcus aureus, Schistosoma mansoni,* and *Trichinella spiralis* (Ali *et al.,* 2020). Th2 cell cytokines enhance antibody production. Other types of CD4+ T cells include Th17 cells secrete IL-17 important for resisting extracellular fungi and bacteria and contribute to allergic responses and autoimmune pathogenesis in diseases including multiple sclerosis, rheumatoid arthritis, psoriasis, and inflammatory bowel disease (Ali *et al.,* 2020). The follicular helper T (Tfh) cell is yet another sub-population that secretes IL-21 cytokines upon antigenic stimulation (Ali *et al.,* 2020). In addition, Tfh provides home to B cell follicles where differentiation of B cells into germinal center B cells and antibody-secreting plasma cells occur (Ali *et al.,* 2020).

2.2.2.3 Humoral Immune Responses

Humoral responses are mediated by macromolecules produced by cells and released into the extracellular fluids following infection by a pathogen (Smith *et al.,* 2019).

Some of the most studied humoral components include the complement system, lysozyme, antimicrobial peptides, and acute phase proteins (Smith *et al.,* 2019). These components have many different functions including the promotion of inflammation, phagocytosis, and direct bactericidal effects (Smith *et al.,* 2019).

The complement system is composed of approximately thirty plasma glycoproteins (Woodell-May & Sommerfeld, 2019). Upon activation, these proteins generate products that facilitate the recruitment of immune cells to the site of infection/damage to eliminate the pathogen through opsonization and phagocytosis (Carrillo *et al.,* 2017). Activation of the complement system occurs through three pathways; the classical, alternative and lectin pathways. The classical pathway is activated by antibody-antigen complexes (Woodell-May & Sommerfeld, 2019). The alternative pathway is activated directly by pathogens and is independent of antibodies (Woodell-May & Sommerfeld, 2019). Thirdly, the lectin pathway is activated by the binding of the mannose-binding lectin (MBL), or ficolin, to mannose (or other sugar) residues present on the pathogen surface (Carrillo *et al.,* 2017; Woodell-May & Sommerfeld, 2019).

These pathways induce activation of the multimolecular enzyme complex (C3 convertase), which cuts inactive C3 components to form soluble C3a and C3b (Carrillo *et al.,* 2017). The C3b fragment that is generated binds to the C3 convertase to form the C5 convertase, which in turn cleaves the C5 component to C5a and C5b (Woodell-May & Sommerfeld, 2019). Then, C5b recruits complement components C6, C7, C8, and C9 to form the membrane attack complex that osmotically lyses the pathogen' cell membranes (Carrillo *et al.,* 2017; Woodell-May & Sommerfeld, 2019). The complement system can recognize and eliminate a diverse number of pathogens using its multiple activation pathways (Woodell-May & Sommerfeld, 2019). In addition, it can eliminate apoptotic cells through neutrophil and macrophage phagocytic activities by depositing a low amount of C3b molecules (Carrillo *et al.,* 2017). Moreover, activation of the complement cascade can increase vascular permeability and vasodilation, increase the synthesis of leukotriene, and promote chemotaxis of neutrophils and monocytes (Carrillo *et al.,* 2017; Woodell-May & Sommerfeld, 2019).

Lysozymes (muramidase or N-acetylmuramic acid hydrolase) are lytic enzymes that are protein in nature (Ferraboschi *et al.,* 2021). Lysozymes are present in tissues and organs secretion (for example blood, placenta, sperm, leukocytes), milk, nasal discharge, saliva, and tears (Ferraboschi *et al.,* 2021). Lysozymes act on the peptidoglycan layer of bacterial cell walls by hydrolyzing the β-1, 4-glycosidic bonds between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) resulting in cell death (Ferraboschi *et al.,* 2021). The antibacterial action of lysozyme is particularly efficient against gram-positive bacteria but its effects against gram-negative bacteria are significantly weaker due to the presence of a protective lipopolysaccharides layer on the outer membrane (Ferraboschi *et al.,* 2021). Lysozymes are also involved in other defenses such as opsonization, phagocytosis, and activation of the complement system (Ferraboschi *et al.,* 2021). Lysozymes are used as an antibiotic alternative with application in various fields: in medicine it is used in wound healing, treating infectious diseases, and acts as anti-biofilm. In agriculture, it is used as feed, growth promoter, protects both plants and animals as well as preserves foods (Ferraboschi *et al.,* 2021).

Antimicrobial peptides (AMPs) also known as host defense peptides are oligopeptides with a varying number of amino acids that are mostly cationic or anionic (Zhang *et al.,* 2021). AMPs are present in animals, bacteria, plants, and fungi (Gan *et al*., 2021). Nowadays, several thousands of various AMPs are natural or synthetic (Moravej *et al.,* 2018). Natural AMPs are expressed by specific constitutive or inducible genes (Moravej *et al.,* 2018). Cationic AMPs have ten to a hundred amino acids with a net charge of + 2 to + 9 (Gan *et al*., 2021). Cationic AMPs neutralize the charge of the bacterial cell membranes thus penetrating through them and causing bacterial death (Zhang *et al*., 2021). They also reduce the possibility of bacterial drug resistance (Zhang *et al*., 2021). Anionic AMPs, on the other hand, have five to seventy amino acid residues with a net charge range of − 1 to − 8 (Gan *et al*., 2021).

Antibiotics have been employed for the treatment of bacterial infections but recently, due to the abuse of antibiotics in agriculture and medicine, bacteria have developed resistance to most conventional antibiotics (Moravej *et al.,* 2018). Antimicrobial

peptides that can be extracted from nature are looked upon as alternatives to these drugs because they are more efficient than traditional antibiotics with rapid germ-killing ability (Zhang *et al*., 2021). They have shown potent synergistic antimicrobial effects with traditional antibiotics in the neutralization of endotoxin (Moravej *et al.,* 2018). They have broad-spectrum properties against a wide variety of microorganisms (fungi, bacteria, protozoans, and viruses) with the least toxicity to the host (Zhang *et al*., 2021). They have lower levels of drug- resistance, good thermal stability, and water solubility (Zhang *et al*., 2021).

Antimicrobial peptides act on multiple targets on the plasma membrane and intracellular targets of pathogenic bacteria and have potent activity on drug-resistant ones (Gan *et al.,* 2021). Antimicrobial peptides also directly inhibit the activities of certain adenosine triphosphate (ATP)-dependent enzymes by interacting with ATP (Moravej *et al.,* 2018). Besides, they have been discovered to possess antitumor activity against cancerous cells (Zhang *et al*., 2021). They are also able to directly recruit antigen-presenting cells (e.g., monocytes and macrophages) to the site of infection and enhance phagocytosis or indirectly via the induction of chemokines (Zhang *et al*., 2021). Furthermore, they can suppress the expression of pro-inflammatory cytokines while inducing the expression of anti-inflammatory cytokines and promote wound healing (Moravej *et al.,* 2018). Antimicrobial peptides have immunomodulatory activities that are important in adaptive immunoregulation and inflammatory response (Zhang *et al*., 2021). They exhibit advantages over immunoglobulins in various ways. First, their synthesis by the host cells mainly phagocytic granulocytes and mast cells, requires less time and energy (Gan *et al.,* 2021). Secondly, they can reach the target faster because of their small size (Moravej *et al.,* 2018). Moreover, some organisms such as insects and other invertebrates lack lymphocytes and depend on the synthesis of a series of AMPs to remove invading microorganisms (Moravej *et al.,* 2018).

Most mammals mainly have two classes of AMPs termed cathelicidins and defensins (Lei *et al.,* 2019). Cathelicidins have a cathelin domain and distinct amino acid sequences, peptide lengths, and protein structures (Huan *et al.,* 2020). Most cathelicidins AMPs are amphiphilic α-helical in nature (Lei *et al.,* 2019; Huan *et al.,*

2020). They have biological activities against bacteria, viruses, and fungi (Lei *et al.,* 2019). Upon leukocyte activation, cathelicidins which are stored in the non-functional form in neutrophils or macrophage secretory granules are processed before they become activated (Huan *et al.,* 2020). The only cathelicidin that is found in humans, LL-37, consists of 37 amino acids with two leucine residues at its N-terminus (Lei *et al.,* 2019). On the other hand, defensins are small cationic peptides rich in conserved cysteine residues. (Lei *et al.,* 2019; Huan *et al.,* 2020). They are divided into three subtypes based on the arrangement of disulfide bonds: α-, β-, and θ-defensins (Lei *et al.,* 2019). To some extent, a disulfide bridge is necessary for its antimicrobial activity (Huan *et al.,* 2020). In humans, there are only α-defensins and β-defensins but no θ-defensins due to an early termination codon in their mRNA (Huan *et al.,* 2020). The α-defensins in mammals mainly exist in neutrophils, macrophages, and intestinal paneth cells (Lei *et al.,* 2019; Huan *et al.,* 2020). On the other hand, β-defensins exist more extensively in leukocytes and epithelial cells in the skin, the respiratory, digestive, and genitourinary tracts, as well as the blood and urine (Huan *et al.,* 2020).

Acute phase proteins (APPs) are plasma proteins primarily synthesized by the liver as part of the acute phase response (Charlie-Silva *et al.,* 2019). The acute phase response is a non-specific and complex group of physiological processes that occur soon after the onset of various stressors such as inflammation, infection, trauma, injury, and neoplasia (Khalil *et al.,* 2020). The process is highly coordinated with several changes in biochemical, physiological, behavioral, and nutritional signals which lead to isolation and destruction of the infectious agent(s) and restoration (Charlie-Silva *et al.,* 2019). During an injury, infection, and inflammation, recruited immune cells such as macrophages, neutrophils and monocytes secrete various cytokines into the bloodstream, which stimulate the liver to produce and release APPs (Khalil *et al.,* 2020).

Acute phase proteins are classified based on the direction of change (positive or negative) and the extent to which their concentrations change (major, intermediate and minor) (Charlie-Silva *et al.,* 2019). Major APPs represent those that increase ten to hundred-fold markedly within the first 24–48 h after the triggering event and often

exhibit a rapid decline due to their very short half-life (Charlie-Silva *et al.,* 2019). Moderate APPs increase two to tenfold, and minor proteins are characterized by only a slight increase (Charlie-Silva *et al.,* 2019). Moderate and minor APPs may both increase more slowly and persist for longer, depending on the triggering event (Charlie-Silva *et al.,* 2019). The two are observed more often during chronic inflammatory processes (Charlie-Silva *et al.,* 2019). During the acute phase response, an APP whose plasma concentration increases by at least 25% is a positive APP while negative AAP decrease in similar proportions (Charlie-Silva *et al.,* 2019). Positive APPs that increase during infection and help in the destruction or inhibition of microorganisms' growth include C-reactive protein, alpha-2-macroglobulin, ferritin, complement system factors, haptoglobin, ceruloplasmin, and haptoglobin are examples of. Negative APPs that decrease during inflammation include transferrin, albumin, transthyretin, antithrombin, and transcortin (Charlie-Silva *et al.,* 2019).

Alpha-1 antitrypsin is the major inhibitor of serine proteases such as neutrophil elastase and proteinase-3, limiting host tissue injury (Khalil *et al.,* 2020; Ehlting *et al.,* 2021). Haptoglobin (Hp) binds free hemoglobin released from erythrocytes and thereby inhibits its oxidative activity (Khalil *et al.,* 2020). In addition, the Hp-haemoglobin binding also reduces the availability of the haem residue for bacterial growth. Serum amyloid A (SAA) is mainly involved in the transportation of cholesterol from tissue to hepatocytes (Ehlting *et al.,* 2021). They are also involved in opsonization, platelet activation, and inhibition of phagocyte oxidative bursts (Ehlting *et al.,* 2021). Transferrin (Tf) transport iron from absorption centers in the duodenum and white blood cell macrophages to all tissues for growth purpose while Lactoferrin (Lf) function by binding to and transferring iron from $Fe3^{+}$ ions hence conferring bactericidal effects (Khalil *et al.,* 2020; Ehlting *et al.,* 2021). Fibrinogen provides a substrate for fibrin formation and is involved in homeostasis and tissue repair (Khalil *et al.,* 2020). Ceruloplasmin (Cp), a ferroxidase enzyme, is the major copper-carrying protein in the blood (Ehlting *et al.,* 2021). C-reactive protein (CRP) stimulates immune cells by binding to Fcγ receptors (FcγR) on leukocytes of myeloid lineage and increases the production of IgG, linking the innate and adaptive immune systems (Khalil *et al.,* 2020). CRP-FcγR binding also facilitates anti-inflammatory responses (Khalil *et al.,* 2020; Ehlting *et al.,* 2021).

2.2.2.4 Cytokines

Cytokines are small secreted signaling molecules that are proteins, peptides, or glycoproteins in nature with a molecular weight of about 5 - 20 kDa (Ferreira *et al.,* 2018). These molecules are produced throughout the body by a wide range of cells including T and B lymphocytes, granulocytes, macrophages, mast cells, fibroblasts, endothelial cells, and stromal cells (Gulati *et al.,* 2016). More than one type of cell may be involved in producing a single cytokine (Liu *et al.,* 2021). Cytokines may act through receptors either synergistically or antagonistically on cells that secrete them, on nearby cells, or on distant cells (Liu *et al.,* 2021). They regulate the immune system by striking a balance between adaptive and humoral immune responses; they also control immune cells' growth, maturation, and responsiveness (Gulati *et al.,* 2016). In addition, cytokines regulate inflammation (Ferreira *et al.,* 2018). They are important determinants of health and have been adopted as biomarkers in the accurate assessment of various diseases (Gulati *et al.,* 2016). Examples of classes of cytokines include interferons, chemokines, tumor necrosis factor, interleukins, and lymphokines (Gulati *et al.,* 2016; Liu *et al.,* 2021). Some of the prominent cytokines involved in immunomodulation include;

2.2.2.4.1 Interleukin 2 (IL-2)

Interleukin-2, previously known as the T cell growth factor, is a four α-helical bundle cytokine with a molecular weight of 15kDa (Arenas-Ramirez *et al*., 2015). It is mainly produced by antigen-activated T cells which consist of CD4+ and CD8+ T cells for autocrine use and is stimulated in a paracrine fashion in neighbouring cells that express its receptors (Zhou *et al.,* 2020). Moreover, it can be expressed by activated mast cells, dendritic cells, and natural killer cells (Arenas-Ramirez *et al*., 2015). They bind with high affinity to their receptors (IL-2R) on the plasma membrane. IL-2R is composed of three subunits IL-2Rα (CD25), IL-2Rβ (CD122), and γc (CD132), all necessary for the binding of IL-2 (Zhou *et al.,* 2020). On triggering IL-2R, signal transduction occurs via three major pathways: Janus kinase (JAK)–signal transducer and activator of transcription (STAT); phosphoinositide 3-kinase (PI3K)–AKT and mitogen-activated protein kinase (MAPK) (Arenas-Ramirez

et al., 2015). IL-2 promotes the development of T cells, acts as a B cell growth factor, stimulates antibody synthesis, and promotes the proliferation and differentiation of NK cells. IL-2 has been extensively used as an anti-cancer therapy (Zhou *et al.,* 2020).

2.2.2.4.2 Interleukin 4 (IL-4)

The IL-4 is a short four-helix bundle peptide pleiotropic cytokine that has a molecular mass of 12–20 kDa (Junttila, 2018). It is produced by the immune system cells including Th2 helper T cells, mast cells, basophils, and activated eosinophils in response to a receptor-mediated activation (Junttila, 2018). It has two receptors that are distributed throughout the human body (Junttila, 2018). These receptors are IL4-R type I, composed of CD124 (IL-4rα), and CD 132, Type II, which consists of IL-4Rα and IL-13Rα1 (Junttila, 2018). IL-4 is known to play several different roles such as the regulation of differentiation of naive TH2 helper T cells (Junttila, 2018). These T cells also produce IL-5, IL-9, and IL-13 beside IL- 4, which can all participate in the allergic inflammatory response (Junttila, 2018). IL-4 promotes B cell proliferation and B cell class switching to IgE and mast cells' expression of leukotriene C4 synthase (Junttila, 2018).

2.2.2.4.3 Interleukin 5 (IL-5)

IL-5 is pro-inflammatory cytokine with a molecular weight of 15 kDa (Pelaia *et al.,* 2019). It is produced by various cells in the immune system; T lymphocytes, mast cells, macrophages, and eosinophils (Massey & Suphioglu, 2022). IL-5 has an impact on eosinophil growth, maturation, and release from bone marrow and may trigger eosinophilia (Pelaia *et al.,* 2019). Moreover, IL-5 acts on mast cells causing them to release histamine which is involved in allergic reactions (Ye *et al.,* 2020). Additionally, this cytokine increases the IL-2Rα expression on B lymphocytes and enhances IgA production, as well as triggering the proliferation and differentiation of cytotoxic T lymphocytes (Ye *et al.,* 2020).

2.2.2.4.3 Tumor Necrosis Factor-alpha (TNF-α)

Tumor necrosis factor α also called cachectin or differentiation-inducing factor is a pro-inflammatory cytokine produced by various cells in the immune system, macrophages, T lymphocytes, and mast cells (Jang *et al.,* 2021). It is also produced by endothelial cells, fibroblasts, and smooth muscle cells (Laha *et al.,* 2021). It is functionally known to trigger the release of a series of inflammatory molecules including IL-1, IL-6, and chemokines (Laha *et al.,* 2021). It has two receptors, ubiquitously expressed and found in all cell type TNF receptor type 1 (TNFR1) and TNFR2 which is expressed mostly by immune cells (Jang *et al.,* 2021). The major role of TNF-α is the initiation of apoptosis, immune defense against pathogens as well as cell survival mechanism through TNFR1 (Laha *et al.,* 2021). Through TNFR2 activation, TNF-α can promote tumor growth and progression or enhance their immunosuppressive effect by boosting suppressive cells such as Myeloid-derived suppressors (MDSC) (Jang *et al.,* 2021). Inappropriate or excessive production of TNF-α can be harmful as it may lead to diseases such as psoriasis, rheumatoid arthritis, and inflammatory bowel disease. TNF- α also acts on the hypothalamus to produce fever (Laha *et al.,* 2021).

2.2.2.4.4 Interferon gamma (IFN-γ)

Interferon-γ (IFNγ) is the only member of the type II IFN family (Castro *et al.,* 2018). It is primarily produced by activated CD4 T helper type 1 (Th1) cells and CD8 cytotoxic T cells, γδ T cells, and natural killer cells (Kak *et al.,* 2018). It's also produced by natural killer T cells, B cells, and professional antigen-presenting cells (APCs) to a lesser extent (Castro *et al.,* 2018). Its expression is induced by mitogens and cytokines, such as IL-15, IL-18, IL-12, IFN-α, and IFN-β (Kak *et al.,* 2018). IFN-γ plays a role in the recognition and elimination of pathogens such as bacteria and viruses enabled through the activation of macrophages (Kak *et al.,* 2018). IFN-γ stimulates macrophages to mount effective immune response; enhanced antigen processing and presentation through upregulation of class II MHC, increased secretion of pro-inflammatory cytokines, and increased ROS and NOS production (Kak *et al.,* 2018). It also increases NK cells' tumoricidal activity after activating

them (Castro *et al.*, 2018). Moreover, it regulates antibody production to modulate B cell responses (Castro *et al.*, 2018). IFN-γ enables the growth and maturation of other cell types and leukocyte migration. Its hyperactivity leads to the pathogenesis of several autoimmune diseases (Kak *et al.*, 2018).

2.3 Immunomodulation

Immunomodulators refer to biological or synthetic agents capable of altering a phase or component of the immune system including the adaptive and/or innate branches of the immune system (Jantan *et al.*, 2015). These agents can either inhibit or amplify the components of the immune system (Catanzaro *et al.*, 2018). Immunomodulation involves a complex network of interaction between the immunomodulatory agent and components of the immune network; specific and nonspecific, and humoral and cellular responses (Ogila, 2012). Immunomodulators in clinical practice are categorized as immunostimulants and immunosuppressants (Sharma *et al*., 2017). Immunostimulants are envisaged as enhancers of immune defenses and resistance to infection (Sharma *et al*., 2017). They can act through innate as well as adaptive immune responses (Catanzaro *et al.*, 2018). Immunosuppressants are agents that suppress immune responses (Jantan *et al.*, 2015).

Immunomodulating agents are being considered attractive novel approaches for the treatment of various human ailments such as acquired immune deficiency syndrome (AIDs), cancer, organ transplantation rejection, neurological disorders, crohn's disease, rheumatoid arthritis, systemic lupus erythematosus and other autoimmune conditions (Catanzaro *et al.*, 2018; Varadé *et al.*, 2021; Carballido *et al.*, 2020), among others.

2.3.1 Immunomodulatory Effects of Plant Extracts

The use of plant products as agents of immune modulation has a historical standing in human health. Numerous plants used in traditional medicine, including some *Terminalia* and *Carissa* species, have been empirically confirmed to possess immunomodulatory efficacies. *T. bellerica* has been reported to stimulate the phagocytic activity of macrophages and lymphocytes proliferation

(Saraphanchotiwitthaya *et al.,* 2008; Gupta *et al.,* 2020). *Terminalia arjuna* extracts were found to reduce the levels of pro-inflammatory cytokines, including TNF-α, IL-6 and IL-18, in coronary artery disease patients (Kapoor *et al.,* 2015), The fruit extract of *Terminalia chebula* increased antibody titer levels and Delayed Hypersensitivity (DTH) reaction in mice (Shivaprasad *et al.,* 2006, 2016; Aher & Kumar 2010). Ethanolic extract of *Terminalia catappa* demonstrated immunosuppressive properties by inhibiting the production of TNF-α, IL-6, and IL-23 in rats and IL-1β and NO by macrophages (Abiodun *et al.,* 2016).

Carissone and scopoletin, isolated from *C. carandas*, have been reported to reduce the production of proinflammatory mediators including TNF-a, nitric oxide (NO) and IL-1b by J774A.1 cells (Galipalli *et al*., 2015). Ethanolic fruit extract of *C. carandas* and lanostane, a triterpenoid isolated from the extract was shown to increase the numbers of white blood cells (WBCs) in immunosuppressed mice *(*Arif *et al.,* 2013). *Carissa macrocarpa* extracts have been reported to reduce the production of a pro-inflammatory mediator, NO, in macrophages (Souilem *et al*., 2019).

Leaf extract of *Musanga cecropioides* has been reported to boost antibody production in cyclophosphamide-induced immunosuppressed mice (Ogbue *et al.,* 2022). *Caulerpa lentillifera* preparations have been revealed to enhance the production of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and antibodies in cytoxan-initiated immunosuppression in mice (Sun *et al.,* 2019).

Immunostimulatory potency of the aqueous extract of *Carica papaya* has been demonstrated in mice as confirmed by increased antibody percentage stimulation value, neutrophil migration, and phagocytic index (Briggs *et al.,* 2020). *Tinospora crispa* ethanol extract has been reported to improve macrophage phagocytic ability and nitric oxide production. Additionally, the extract enhanced DTH, and the production of antibodies (IgG and IgM) in mice (Ahmad *et al.,* 2016). Methanol and ethanol extract of *Helleborus purpurascens* exhibited immunostimulatory effect in dexamethasone immunosuppressed rats as evidenced by increased total leukocytes counts, lymphocyte and neutrophil (Grigore *et al.,* 2021).

Aqueous extract of *Pachyrizus erosus* fiber has been reported to enhance immune responses in mice by increasing macrophage phagocytic activity and nitric oxide production (Baroroh *et al.,* 2020). The extract also enhanced lymphocyte proliferation evidenced by high splenic index and IgG production. Besides the extract increased production of interleukin - 10 and tumor necrosis factor - α (Baroroh *et al.,* 2020). Moreover, a polyherbal formulation containing aqueous extracts of five plant materials; *Acacia polyacentha, Bauhinia rufescens, Senegal senegal, Adansonia digitata,* and *Allium sativum* has been indicated to boost the immune system by stimulating enhanced delayed-type hypersensitivity responses and expression of IL-2, IFN-γ, IL-4 and IL-6 in Cyclophosphamide immunosuppressed rats (Muhammad *et al.,* 2021).

Ethanol extract from *Annona squamosa* leaf has been shown to increase the phagocytic index in pyrogallol immunosuppressed mice (Bharathi *et al.,* 2022). This demonstrates the ability to evoke phagocytosis mediators in the immune system (Bharathi *et al.,* 2022). The immunostimulatory potential of *Hibiscus tiliaceus* leaf methanolic extract has been investigated in pyrogallol immunosuppressed mice (Rajeswari *et al.,* 2013). The extract enhanced delayed-type hypersensitivity and increase phagocytic activity of neutrophils as well as total WBC count (Rajeswari *et al.,* 2013). Besides, in the same study, the extract increased antibody production (Rajeswari *et al.,* 2013).

Petroleum ether and aqueous extracts of *Thaumatococcus daniellii* have been demonstrated to boost the immune system in pyrogallol-induced immunosuppression in rats (Adeyemo *et al.,* 2021). The extracts increased antibody production and phagocytic index. However, the extract did not increase neutrophil levels (Adeyemo *et al.,* 2021). *Anemarrhena asphodeloides* ethanol extract has been revealed to block the production of NO and pro-inflammatory cytokines; IL-1β, IL-6, and TNF-α in macrophages in vitro (Ji *et al.,* 2019).

Curcuma longa has been shown to reduce levels of total White Blood Cells (WBC), neutrophil, eosinophil, antibodies (IgE), interleukin-4 (IL-4) and nitric oxide in asthmatic rats while increasing levels of interferon-gamma (IFN-*γ*) (Boskabady *et*

al., 2021). Hydroalcoholic extracts of *Arctium lappa* and *Canavalia gladiata* have been revealed to possess immunomodulatory properties (Ji *et al.,* 2018). The extracts inhibited the expression of nitric oxide in lipopolysaccharide-stimulated macrophages. The extracts also stimulated the expression of T and B cells in mice. Besides, the extracts increased the production of immunoglobulin A and G (Ji *et al.,* 2018).

Ethanol extract of *Anemarrhena asphodeloides* has been demonstrated to inhibit the expression of IL-1β, IL-6, and TNF-α in LPS-stimulated macrophages (Ji *et al.,* 2019). Aqueous extracts of *Gelidium amansii* increased macrophage proliferation and activated the production of TNF-α, nitric oxide, interleukin 6, and 1β (Wang *et al.,* 2013). However, the ethanol extracts of *G. amansii* inhibited TNF-α, IL-6, and IL-1β production in LPS-treated macrophages (Wang *et al.,* 2013). *Seseli gummiferum* subsp. has been indicated to inhibit the production of NO and TNF-α in LPS-treated macrophages (Chun *et al.,* 2016).

Immunostimulatory effects of the aqueous extract of *Limoniastrum guyonianum* have been reported. The extracts stimulated macrophages to produce NO and promoted enhanced NK cell activity (Krifa *et al.,* 2013). Also, the methanol extract of *L. guyonianum* enhanced NO production by macrophages as well as the activity of NK cells (Krifa *et al.,* 2015). Ethanolic extract of *Terminalia catappa* has demonstrated immunosuppressive properties by inhibiting the expression of TNF-α, IL-6, and IL-23 in rats (Abiodun *et al.,* 2016). Also, the extract inhibited the production of IL-1β and NO macrophages (Abiodun *et al.,* 2016).

Hydromethanol *Olea europaeao* leaf extract has been revealed to inhibit the production of TNF-α, IL-1β, IL-6, and IL-8 in mice (Vezza *et al.,* 2017). Combined aqueous extract of *Curcuma longa* (Turmeric) root and *Piper nigrum* seeds was shown to reduce the expression of IL-6 and TNF-α in SARS-COV-2 infected Huh7 cells (Roshdy *et al.,* 2020). Aqueous *Phyllanthus niruri* extract was shown to promote the proliferation of peripheral blood mononuclear cells and macrophages harvested from pulmonary tuberculosis patients (Putri *et al.,* 2018). Also, the extract

enhanced the phagocytic efficacy of macrophages and NO production (Putri *et al.,* 2018).

Aqueous extract of *Momordica charantia* has been demonstrated to alleviate Head and neck squamous cell carcinoma in mice via decreasing infiltrating regulatory T and Th17 cells in the tumor microenvironment (Bhattacharya *et al.,* 2016). *Aqueous extract of Chlorella pyrenoidosa* and ethanol extract of *Lycium barbarum* promoted the proliferation of NK cells and enhanced their activity against colon cancer cells (Kwaśnik *et al.,* 2021). Hydro-alcohol extract of *Ocimum basilicum* leaf was shown to reduce levels of IL-4, and IgE but enhanced IFN-γ in asthmatic rats resulting in therapeutic effects (Eftekhar *et al.,* 2019).

Clinacanthus nutans ethanol extract was shown to reduce the production of IL-6 and TNF-α in macrophages (Nordin *et al.,* 2021). However, its aqueous extract promoted the expression of IL-6 but inhibited TNF-α production. Both extracts were shown to enhance the expression of IL-1β. It was postulated that these are the mechanisms through which *Clinacanthus nutans* inhibits the proliferation of MDA-MB-231 triple-negative cancer cell lines (Nordin *et al.,* 2021). Ethanol extract of *Pistascia lentiscus* and *Eucalyptus spp.* leaf revealed a reduction of IL-6 and TNF-α against polymorphonuclear cells (Qabaha *et al.,* 2016).

In a 30-day clinical trial, powdered ethanolic extract of *Withania somnifera* root markedly increased levels of antibodies (IgA, IgM, and IgG) as well as IFN-γ and IL4 in the study individuals. Also, the extract increased levels of natural killer cells, T and B lymphocytes (Tharakan *et al.,* 2021). In an 8 weeks clinical trial, a combination of *Arctium lappa* and *Canavalia gladiata* (1:4) hydroethanolic extract boosted the immune system of the study individuals by increasing the level of IL-10 and activity of natural killer cells in the test group (Lyu *et al.,* 2020). Daily administration of hydroalcoholic ethanol extract of *Berberis Integerrima* to Rheumatoid arthritis patients for 12 weeks was revealed to reduce the severity of the disease by increasing levels of IL-10 and decreasing IL-17 levels (Aryaeian *et al.,* 2021).

A 90-day treatment of healthy individuals with green coffee extract resulted in several immune boosting outcomes through enhanced phagocytosis, IgA, and IgG production (Narayanaperumal *et al.,* 2022). As well, the activity of NK cells following green coffee extract administration was augmented. On the other hand, inflammatory cytokines; IL-6, and TNF-alpha levels were significantly inhibited (Narayanaperumal *et al.,* 2022). During 8 weeks of a clinical trial, an aqueous extract of *Pleurotus ostreatus* up-regulated natural killer cells activity, IFN-g, and IL-12 in test subjects (Tanaka *et al.,* 2016). Six weeks of consumption of *Allium sativum* by obese adults resulted in a reduction of TNF-alpha and IL-6 (Xu *et al.,* 2018).

2.3.2 Phytochemicals and their Immunomodulatory Mechanisms

Phytochemicals including flavonoids, alkaloids, phenols, tannins, terpenoids, and essential oils, among others have been noted to have immunomodulatory properties (Mohan, 2019). They act by modulating adaptive and innate responses (Mohan, 2019). A detailed review of the immunomodulatory effect of various classes of phytochemicals is hereby outlined.

2.3.2.1 Phenols

This is a group of phytochemicals that comprise a broad range of plant compounds made up of an aromatic ring (Yahfoufi *et al.,* 2018). Majorly, they include flavonoids, phenolic acids, polyphenols, anthocyanins, and tannins (Yahfoufi *et al.,* 2018), among others. Most phenolic compounds are known to possess multiple medicinal properties; anti-cancer, anti-malaria, reduction of the risk of cardiac diseases, antioxidant, anti-rheumatoid arthritis, anti-obesity, anti-diabetes, neuroprotective, anti-inflammatory, immunomodulatory (Brindha, 2016; Kilani-Jaziri *et al.,* 2017; Yahfoufi *et al.,* 2018).

Phenols have been revealed to modulate the immune system by modifying immune cell populations and activity, cytokines, and antibody production through which they achieve therapeutic effects. Resveratrol, a polyphenol commonly found in multiple plants including nuts and red wine grapes, is known to induce cardio-protection via

inhibition of pro-inflammatory cytokines; TNF-α and IL-1β (Li *et al.,* 2022; Börzsei *et al.,* 2022).

Curcumin derived from *Curcuma longa,* has been shown to inhibit the expression of IL-1 and TNF alpha in human umbilical vein endothelial cells (Marchiani *et al.,* 2014). Curcumin stimulates the cytotoxic activity of NK cells to produce IFN-γ achieving antitumor efficacy against pancreatic cancer cell lines (Halder *et al.,* 2015). Curcumin has been reported to enhance tumor-infiltrating lymphocytes; $CD4^+$ and $CD8^+$, resulting in increased death of tumor cells in mice (Pal *et al.,* 2005; Bhattacharyya *et al.,* 2010). Curcumin has been revealed to inhibit sepsis-initiated liver, heart, and kidney damage by reducing the expression of IL-6, TNF-α, and IL-1β, while increasing the production of the anti-inflammatory cytokine IL-10 (Zhong *et al.,* 2016).

Quercetin and luteolin have been indicated to enhance the activity of NK cells revealed by increased IL-2 production. Also, quercetin has been demonstrated to inhibit the production of TNFα, IL-1β, IL-6, and IL-8 in human mast cells (Oo, 2022). Quercetin was shown to enhance T-cell cytotoxic activity on NCI-H460 and MDA-MB-231 cancer cells. In the same study, xenograft isolated from mice had increased levels of IFN-γ after quercetin treatment (Jing *et al.,* 2021). Luteolin has been revealed to inhibit the proliferation of human colon tumor cell lines; HCT116, SW48, and Caco2, via increased expression of interleukin-2 while reducing the production of interleukin6, interleukin 1β, interferon β, and tumor necrosis factor-alpha (Yao *et al.,* 2022).

The list of immunomodulatory phenols is growing, and the review hereby provides just but a few, to demonstrate the significance of these compounds in immune responses.

2.3.2.2 Alkaloids

Alkaloids are a major class of phytocompounds with significant therapeutic efficacies. The compounds contain nitrogen atoms alongside a network of carbon and oxygen atoms (Omojate *et al.,* 2014). Alkaloids have been observed to possess

various medicinal properties; anticancer (vinka alkaloids), antimicrobial (cepharanthine), and analgesic (morphine) (Moudi *et al.,* 2013; Brindha, 2016; Kim *et al.,* 2019), among others.

Alkaloids modulate the immune responses via multiple mechanisms. Piperine, an alkaloid derived from *Piper longum* has been shown to raise levels of total WBC count and antibody expression (Sunila & Kuttan, 2004). Piperine is known to stimulate mice thymocytes to produce IL-2, IFN-γ, and IL-4 (Kumar *et al.*, 2015). Murine splenocytes treated with piperine showed increased proliferation of B and T cells, production of Th-1 cytokines (IL-2 and IFN-γ), and augmented macrophage stimulation (Sharma *et al.,* 2014). This efficacy was shown to improve the therapeutic efficiency of piperine in immunocompromised tuberculosis (TB) patients. Besides, piperine derivatives have been indicted to induce antitumor efficacy in Ehrlich ascites tumor models in mice via increased levels of cytokines IL-1β, TNF-α, and IL-4 (Ferreira *et al.,* 2020).

Berberine isolated from *Hydrasti canadensis* has been revealed to reduce the expression of IFN-γ, TNF-α, and NO in mice (Li *et al.,* 2006). Berberine inhibits the progression of breast cancer in mice by enhancing the activity of NK cells, T cells (CD68 and CD209) subpopulations, and IL-6 (Ma *et al.,* 2020). Berberine has been shown to inhibit the production of IL-18 and IL-1β a mechanism through which it mediates antitumor effects on glioma cells (Tong *et al.,* 2019). It has also been reported to decrease the expression of IL-12 while enhancing the production of IFN-*γ* and promoting macrophage polarization; from M2 to M1 thereby inhibiting intestinal tumorigenesis in mice (Piao *et al.,* 2016).

Tetrandrine derived from *Stephania tetrandra* has been demonstrated to inhibit the production of NO, TNF-α, and IL-6 in LPS-stimulated microglial cells thereby revealing neuroprotective potential (Xue, *et al.,* 2008). Indeed, tetrandrine has been shown to block neuroinflammation by reducing the expression of IL-1β, IL-6, and TNFα, thus improving cognitive abilities in Alzheimer's Disease mice models (Ren *et al.,* 2021). Tetrandrine has been indicated to block the expression of IL-6, TNF-α,

and IL-1β in mice with Frozen shoulder disease, demonstrating the potential to treat the disease (Zhao *et al.*, 2021).

With continued research, the number of alkaloids with immunomodulatory efficacies continues to rise. The herein review provides just but a few to demonstrate their immunomodulatory potential.

2.3.2.3 Saponins

Saponins are commonly distributed in plants. Structurally, they contain carbon skeletons with sugar side chains organized into linear or branched patterns (Podolak *et al.,* 2022). Saponins have been demonstrated to possess multiple pharmacotherapeutic properties such as antitumor, immunomodulatory, and antimicrobial, among others (Sparg *et al.,* 2004; Tagousop *et al.,* 2018).

Asiaticoside, a saponin isolated from *Centella asiatica* has been shown to enhance the immune system by increasing total WBC levels and the phagocytic index of macrophages (Punturee *et al.,* 2005). Asiaticoside has been indicated to inhibit the production of TNF-α, IL-4, 8, and 1β by mast cells, hence demonstrating the potential to inhibit mast cell-mediated allergic attacks (Jiang *et al.*, 2017). It blocks TNF-α expression in a spinal cord injury in rat models thus mediating therapeutic effects (Fan *et al.,* 2020). Furthermore, asiaticoside has been indicated to reduce the production of IL-6 and TNF in the brains of rats treated with β-amyloid, thus revealing the potential for the treatment of Alzheimer's disease (Zhang *et al.,* 2017).

Glycyrrhizin isolated from *Glycyrrhiza glabra* has been revealed to inhibit transforming growth factor-β in diabetes-mediated cardiac atrophy in rats, thus unveiling cardioprotective potential (Thakur *et al.,* 2021). Also, gycyrrhizin has been reported to promote the production of IFN-γ and nitric oxide (Wang *et al.,* 2018) but inhibits the production of IL-1β, 6, 8, 10 and TNFα in macrophages (Huan *et al.,* 2017). Via inhibition of the production of IL-33, gycyrrhizin attenuates LPS-initiated lung injury, there showing potential for use in the treatment of acute respiratory distress syndrome (Fu *et al.,* 2016).

Platycodin D, derived from *Platycodi radix* has been indicated to possess several effects on the immune system (Wang *et al.,* 2018). Platycodin D activates splenic lymphocytes and enhances the production of IL-2, 4, and $CD8^+/CD4^+$ ratio by splenic lymphocytes (Wang *et al.,* 2018). Also, platycodin blocked the expression of IL-6, 1β, and TNF-α in LPS-stimulated rat microglia cells (Fu *et al.,* 2018). It has been reported that platycodin D reduced levels of IL-6, 1β, 17A, and TNF-α while enhancing the expression of IL-10 in mice with alloxan-induced liver injury. In the same study, platycodin D reduced Th17 cells and elevated levels of Treg cells in liver tissues. Through these effects, platycodin revealed hepatoprotective potential (Chen *et al.,* 2015). Through inhibition of IL-6 and TNF-*α,* platycodin D was shown to alleviate symptoms of rheumatoid arthritis in mice (Kwon *et al.,* 2014).

Taken together, these examples reveal the promising potential of saponins as immunomodulatory agents.

2.3.2.4 Terpenoids

Terpenoid is a class of many diverse naturally occurring organic compounds derived from isoprene units; a 5-carbon structure (Ata *et al.,* 2022). Multiple terpenoids are known to possess pharmacotherapeutic properties; antiarthritic, anticancer, antiphlogistic, and neuroprotective (Ludwiczuk *et al.,* 2017; Gallily *et al.,* 2018; Zeng *et al.,* 2019), among others. Some terpenoids have been shown to mediate their biological efficacies via immunological mechanisms.

Andrographolide isolated from *Andrographis paniculate* has been shown to block chikungunya viral infection by up-regulating the expression of IFN-gamma and reduced levels of TNF-α, thus showing potential as a promising therapeutic antiviral agent (Gupta *et al.,* 2018). Andrographolide inhibits the proliferation of *P. berghei* NK65 in mice by increasing the production of IL-10 and IL-4 whilst decreasing levels of IFN-gamma, thus demonstrating the potential for use in the management of malaria (Hassan *et al.,* 2019). Andrographolide is known to alleviate ulcerative colitis by reducing levels of IL-1β, IL-6 IL-17A, IL-23, TNF-α, and Th17 cells in a colitis mouse model (Zhu *et al.,* 2018).

Ursolic acid commonly found in several vegetables, fruits, and medicinal plants has been revealed to possess immunomodulatory activities (Pitaloka *et al.,* 2020). It has been shown to inhibit the proliferation of Mycobacterium avium by increasing the production of interleukin-1β and nitrite but decreasing expression of tumor necrosis factor-α and interleukin-6 by macrophages (Pitaloka *et al.,* 2020). Also, ursolic acid attenuates levels of IL-1β and IL-6 in TNF-alpha-treated chondrocytes demonstrating the potential to alleviate osteoarthritis (Wang *et al.,* 2020). Ursolic acid is known to exhibit antitumor effects against breast cancer by inhibiting the production of IL-10, and reducing levels of myeloid-derived suppressor cells and Tregs in tumor tissues developed in the 4T1 tumor mice model (Zhang *et al.,* 2020).

Collectively, the above literature demonstrates phytochemicals as immunomodulators and their promising potential as therapeutic agents via the immunomodulatory mechanism.

2.3.3 Pyrogallol

Pyrogallol is an immunosuppressant that mediates immunosuppression via the production of free oxidative radicals (Joharapurkar *et al.,* 2004). It is a potent producer of superoxide radicals categorized as reactive oxygen species (Joharapurkar *et al.,* 2004). Humoral and cellular components of the immune system are vulnerable to oxidative stress (Pangrazzi, 2019). Reactive oxygen species result in early immunosenescence with ultimate immune deficiencies (Cabrera, 2015; de Punder *et al.,* 2019). Also, pyrogallol could cause immunosuppression by weakening endogenous antioxidant mechanisms which result in deleterious effects on immune cells (Joharapurkar *et al.,* 2004).

2.3.4 Levamisole

Levamisole, an immunostimulatory anthelminthic drug, is used to treat hookworm and ascariasis infections (Moser *et al.,* 2017; Clarke *et al.,* 2019). It is being investigated for use as a chemotherapy adjuvant to treat various cancers (Qiao *et al*., 2020; Wang *et al*., 2020). Already, in combination with fluorouracil, levamisole is being administered for the treatment of colon cancer (Eswaradass and Namasivayam,

2020). Levamisole achieves its therapeutic efficacy via immunostimulatory mechanisms (Mohamed *et al*., 2016; Kuropka *et al*., 2022). It enhances immune function through the stimulation of antibody production, and dendritic cell activation, promoting T-cell and macrophage activity (Fu *et al.,* 2016; Mohamed *et al*., 2016; Al-Kuraishy *et al.,* 2021).

2.4 Toxicity

Development and application of various therapeutic drugs is largely derailed by toxicity (Fuchs & Hewitt, 2011). About 92% of all novel compounds discovered for therapeutic use, including immunomodulators, fail to achieve their market potential due to adverse undesirable effects observed in clinical trials (Fuchs & Hewitt, 2011). Indicators of toxicity include hepatic, renal, and blood indices. Blood tissue, hepatic and renal organs play major roles in drug metabolism: transportation, detoxification, and excretion. Thus, they are largely affected by drug-associated harmful complications (Fuchs & Hewitt, 2011).

2.4.1 Liver Biomarkers of Toxicity

The liver is highly susceptible to toxic effects of noxious drugs due to its role in drug metabolism and waste excretion (Dollah *et al.,* 2013). More than 27% of drugs are recalled from clinical use due to liver toxicity whereas about 40% of clinical trials withdrawal is attributed to hepatotoxicity (Chang *et al.,* 2011). Liver injury is commonly assessed with enzyme biomarkers: aspartate transaminase (AST), alkaline phosphatase (ALP), and alanine transaminase (ALT) (McGill, 2016). Also, liver function is assessed by determining the levels of total proteins, albumin, and bilirubin in the blood (Ge *et al.,* 2014).

Alanine transaminase catalyzes the conversion of alanine to pyruvate (McGill, 2016; Kumar & Gill, 2018). Besides in the liver, ALT is found in the muscle tissues, heart tissue, kidney, bone, placenta, and intestine, among others (Kumar and Gill, 2018). Alkaline phosphatase catalyzes dephosphorylation of phosphate monoesters at pH > 7 (Green & Sambrook, 2020). The enzyme comprises four isozymes categorized according to the site of expression; placental ALP, intestinal ALP, tissue nonspecific/

bone/ liver/kidney (B/L/K) ALT, and germ cell ALP (Zhang *et al.,* 2021). Marjory, serum ALP is released from the liver, kidney, and skeletal tissues, and damage to these tissues could increase ALP levels in the blood (Zhang *et al.,* 2021). Thus, AST and ALP are commonly assessed as clinical auxiliary indicative markers for liver, kidney, and skeletal diseases (McGill, 2016).

Aspartate aminotransferase (AST) converts alpha-ketoglutarate and aspartate to glutamate and oxaloacetate (Aulbach & Amuzie, 2017). It is extensively distributed in various tissues mainly cardiac, hepatic, kidney, and skeletal muscle. Its elevated levels in serum indicate disease or damage to these organs (Aulbach & Amuzie, 2017).

The ALT and ALP are more predominant in the liver compared to AST whose levels are equivalent in the kidney, heart, liver, brain, and muscle tissues (McGill, 2016; Maksymchuk *et al.,* 2017). Upon liver damage, ALT activity remains elevated in serum for a longer time than AST (McGill, 2016; Maksymchuk *et al.,* 2017). Indeed ALT/AST > 40U/L is an indicator of hepatic toxicity (Hartley *et al.,* 2018; Zhang *et al.,* 2020). Besides, serum levels of ALT 3 times higher than the normal concentrated indicate liver toxicity. The ALT/ALP ratio is essential for categorizing the type of liver injury. A ratio ≥ 5 shows hepatocellular damage and ≥ 2 indicates cholestatic liver assault; impaired bile delivery, itching, and jaundice (Moosvi *et al.,* 2020; Pieters *et al.,* 2021). Besides, elevated levels of ALP could signal hepatobiliary damage (Dollah *et al.,* 2013).

Bilirubin is produced during the breakdown of worn-out and aged erythrocytes in the reticulo-endothelial system (Memon *et al.,* 2016). During these events, heme is separated from heme-containing proteins including hemoglobin and metabolized to bilirubin then bound to glucuronic acid (Memon *et al.,* 2016). Subsequently, it's transported via the bile duct and excreted through the digestive tract (Memon *et al.,* 2016). Elevations of serum levels of bilirubin occur due to compromised hepatic clearance indicating bile ducts or liver disease; hepatobiliary injury, and cholestasis (Memon *et al.,* 2016), among others. Serum levels of bilirubin twice above normal ranges indicate liver injury (Shehu *et al.,* 2017).

Plasma proteins and albumin are synthesized by the liver; hence, their profiles are markers of liver integrity (Niu *et al.,* 2019; Ozougwu, 2017). These proteins are building units of all body tissues and cells (Ozougwu, 2017). In case of liver injury, total plasma protein levels as well as individual protein fractions can notably deviate from the normal ranges (Niu *et al.,* 2019). Clinically, total protein levels are monitored to diagnose various liver diseases: liver cirrhosis, and hepatitis (Chalasani *et al.,* 2018), among others.

Albumin is a non-glycoprotein, accounting for about 55-65 % of the total plasma protein (Levitt & Levitt, 2016; Moman *et al.,* 2017). Albumin plays a role in stabilizing the plasma colloidal osmotic pressure, and transportation and storage of various ligands (Belinskaia *et al.,* 2021). It also serves as a reservoir for amino acids (Moman *et al.,* 2017). Marjory, it attaches to toxic heavy metals in circulation and multiple drugs (Spinella *et al.,* 2016). Hypoalbuminemia which arises from among others, impaired production due to liver disease damage, is health-wise detrimental. Albumin levels are essential for monitoring liver functionality since it is synthesized in the liver (Gounden *et al.,* 2018; Soeters *et al.,* 2019).

2.4.2 Kidney Biomarkers of Toxicity

The kidney is made up of nephrons that filter approximately 150-180 liters of plasma daily after which the filtrate is processed to regulate electrolytes and acid-base homeostasis while excreting waste materials from the body (Zuk & Bonventre, 2016; Cabral *et al.,* 2021). Also, the kidney produces hormones involved in skeletal muscle, and cardiovascular and hematologic homeostasis (Powers *et al.,* 2018). Nephrotoxicity has been a common occurrence in drug screening and clinical use (Yin *et al.,* 2020). Drugs account for approximately 19 to 25 % of the total incidences of acute nephrotic damage in critically ill patients (Bonventre *et al.,* 2010; Rapando, 2021).

There are two canonical serum biomarkers for nephrotic toxicity: urea and creatinine (CRE) (Kuzu *et al.,* 2019). Urea is produced during the breakdown of amino acids in the liver (Wolfe *et al.,* 2022). Small amounts of urea are removed from the body in sweat and degraded by bacteria in the intestines (Getahun *et al.,* 2019). On the other

hand, the kidney glomeruli filters majority of urea out of blood (Higgins, 2016). Its levels in serum are directly correlated with kidney function, hence blood urea is associated with renal illnesses, injury, or failure (Dollah *et al.,* 2013; Higgins, 2016).

Besides urea, levels of creatinine in serum are used to assess renal function (Besseling *et al.,* 2021). Creatinine is generated from the catabolism of creatinine phosphate as a result of natural muscle activity (Andres *et al.,* 2017). Once formed, it is filtered out from circulation by the kidneys. The rate at which creatinine is generated is stably constant; 1 - 2% of creatine is transformed into creatinine daily (Kazak and Cohen, 2020). Serum creatinine levels above the standard intervals could indicate kidney malfunction (Besseling *et al.,* 2021).

2.4.3 Haematological Markers of Toxicity

Haematological indicators namely red blood cells, white blood cells, and platelets indices can be assessed as diagnostic indicators of undesirable toxic effects caused by drugs.

2.4.3.1 Red Blood Cells and Associated Indices

Red blood cells (RBCs) carry oxygen throughout the body (Koury & Blanc, 2022). The cells contain hemoglobin which binds oxygen from the lungs and transports it to the tissues. Also, it binds carbon dioxide and transports it from the tissues to the lungs for elimination from the body (Koury & Blanc, 2022). Damage of red blood cells results in diminished Hb levels, a condition referred to as anaemia (Bhadra & Deb, 2020), while high levels of Hb in serum are usually associated with haemolysis of RBCs (Ugwu *et al.,* 2013; Ścibior & Kurus, 2019).

Haematocrit (HCT) is an RBC-related index that indicates the percentage of RBC volume in total blood and is a predictor of anaemia (Ayyıldız & Tuncer, 2020). The HCT index is a determinant of the viscosity of blood where highly viscous blood leads to insulin resistance (Sun *et al.,* 2022). Another, RBC-related index is Mean corpuscular volume (MCV) which indicates the average size of RBCs. Normal MCV range indicates normocytic, low MCV denotes microcytic and high MCV reveals

macrocytic RBCs (Hennek *et al.,* 2016; Doig, & Zhang, 2017). Low values of MCV are linked to iron deficiency, microcytic anemia, and thalassemia whereas large MCV indexes are associated with prolonged alcoholism, vitamin B12, and folate deficiency (Hennek *et al.,* 2016; Doig, & Zhang, 2017). Mean corpuscular hemoglobin (MCH), another RBC-related index, indicates the average intracellular amounts of Hb. Mean corpuscular hemoglobin concentrations are derived to show the average intracellular concentration of hemoglobin. It is usually used for clinical diagnosis of iron deficiency (Namrata, 2013; Doig, & Zhang, 2017).

2.4.3.2 White Blood Cells and Associated Indices

White blood cells are a diverse group of nucleated cells found in circulation during their lifetime (Manik *et al.,* 2016). The cells are made in the bone marrow from pluripotent hematopoietic stem cells. Their usual levels in the blood vary from 4,000 to 10,000/µl of blood (Hawley *et al.,* 2006). The WBCs play a vital role in immune functions; they defend against infections via multiple mechanisms. They include macrophages, basophils, neutrophils, and monocytes which act via phagocytosis (Manik *et al.,* 2016). Lymphocytes help in defense against foreign invasion via the production of cytokines and antibodies (Manik *et al.,* 2016). Elevated levels of WBCs are indicators of infection or toxic reaction being fought by the body (Arika *et al.,* 2016).

2.4.3.3 Platelets

Platelets (PLT) are blood cells involved in coagulation and blood clotting (Budak *et al.,* 2016). Whenever PLT encounter injured sites they change their shape, enlarge their surface area and express biomolecules that mediate the process of blood clotting (Lopez *et al.,* 2015). Also, PLT stimulate wound healing, inflammatory responses, defence against microbes, angiogenesis, and tissue remodelling (Golebiewska & Poole, 2015).

Mean platelet volume (MPV) is a measure of platelet amounts in circulation (Budak *et al.,* 2016). Notably, the MPV index above the normal levels reveals an inflated platelet diameter, a marker for the rate at which platelets are generated and activated.

Platelet distribution width reveals volume variations in platelet size i.e., platelet anisocytosis (Budak *et al.,* 2016). Platelet distribution width reflects the heterogeneity in platelet morphological features (Budak *et al.,* 2016). Plateletcrit (PCT) indicates the volume of platelets in circulation (Zhang *et al.,* 2015).

2.4.4 Safety of Plants as Therapeutic Sources

Medicinal plants traditionally used as sources of therapeutic preparations are viewed to possess few or no adverse reactions as compared to conventional treatments (Khan & Ahmad, 2019; Poswal *et al.,* 2019). Besides, they are culturally adopted and easily available as well as broadly used as medication by about 80% of the global populace (Khan *et al*., 2020). The criteria for determining the safety of herbal preparation have been established by the World Health Organization with aim of halting adulterations and quality compromises (WHO, 2000; Doughari *et al.,* 2009). For instance, only subtle adverse reactions have been documented for herbal preparations containing resveratrol, curcumin, and polyphrnon E (Boocock *et al.,* 2007).

Numerous studies reported the safety of plant extracts as therapeutic agents. A wide array of studies has been conducted to evaluate the toxicity of different plant extracts. Some medicinal plants are toxic while others are non-toxic. *Lantana camara* is reported as hepatotoxic in several animal species (Sharma *et al*., 2007). *Momordica charantia* has been reported to cause deadly hypoglycaemia in children (Mensah *et al*., 2019). Leaf extracts of *Syzygium guineense* didn't show any toxicological signs in the body weight, gross pathology, and behavior in rats (Loha *et al*., 2019). Acute toxicity studies of aqueous extracts of the aerial parts of *Caralluma dalzielii* showed that this medicinal plant was non-toxic because the extracts showed no lethal effects in mice and rats (Ugwah-Oguejiofor *et al.,* 2019).

Liao *et al.* (2017) reported that few or no side effects have been noted in herbal preparation used in Chinese traditional medicine. Besides, various studies conducted on African herbal medicine have indicated that these formulations are safe for consumption (Awounfack *et al.,* 2016). However, despite the widely accepted safety herbal preparation WHO recommends their safety testing before use (WHO, 2000).

2.5 Profiles of the Studied Plants

2.5.1 *Carissa edulis*

2.5.1.1 Description

Carissa edulis belong to Apocynaceae family, genus Carissa (Guya, 2020). It is widely distributed in Elgon, Mau, Loita, Aberdares highlands, and Mt. Kenya Forest edges (Guya, 2020). Besides, it is found in Mumias, Kitale, Kisii, Baringo, Narok, Nanyuki, Machakos, Kitui, Kajiado, Embu, Kenyan Coastal lines, and shores of Lake Victoria. Its growth altitude is 1 to 2550 m. The local names of the plant include Legatetwo (Marakwet/Tugen), Mtanda-Mboo (Swahili), Ochuoga (Luo), Mukawa (Kikuyu, Kamba), and Olamuriaki (Maasai) (Guya, 2020).

Carissa edulis is a scrambling shrub that grows to about 14 meters in height (Mudimba & Nguta, 2019). It has oval-shaped leaves that are elliptic, rounded base, acute obtuse, and up to 1.5-4 cm. Its spines are simple, hardly forked, and about 0.5-6 cm long. Its leaves are hairless, about 4 cm long (Mudimba & Nguta, 2019). The plant produces white-colored flowers on the inside, red-pinkish on the outside with dense cymes, about 13 to 20 mm corolla tubes, and 4 to 9 cm long lobes. The fruit produced by *C. edulis* is black, and round, about 6 to 14 mm, occasionally having a sharp apex (Mudimba & Nguta, 2019).

Figure 2.1: *Carissa edulis*

2.5.1.2 Medicinal and Nutritional Uses of *Carissa edulis*

Fruits of *C. edulis* are consumed as food (Mudimba & Nguta, 2019). Occasionally, aqueous root decoction is taken to treat dysentery and as a painkiller (Mudimba & Nguta, 2019). Decoctions of the plant are used to manage indigestion and pain in the lower abdomen during pregnancy. Besides, root decoction is used to manage chest pains. Aqueous extract of *C. edulis* roots is used to alleviate polio and malaria symptoms (Mudimba & Nguta, 2019). In Ghana, root decoction is used to reinvigorate virility. Root decoction is also used for the management of gonorrhea, mental illnesses, and antigiardiasis in Kenya. In Egypt, its pulverized leaf is utilized to manage diabetes mellitus whereas in South Africa it is used to control schistosomiasis. Multiple compounds have been isolated from *C. edulis*; acetophenone, acetophenone-2- hydroxy benzenoid, carinol sesquiterpene, ortho-ydroxy benzenoid, among others (Mudimba & Nguta, 2019; Nguyen *et al.*, 2020).

2.5.2 *Terminalia brownii*

2.5.2.1 Description

Terminalia brownii belongs to the family Combretaceae, *Terminalia* genus (Zhang, *et al*., 2019). Ecologically it is distributed along Sothern Asia, Madagascar, the Himalayas, Australia, Nigeria, Ethiopia, Somalia, Sudan, and Kenya (Zhang *et al.*, 2019). In Kenya, it is widely localized among deciduous woodlands, bushlands, grasslands as well as river banks. It is also found in semi-arid and arid areas due to their ability to thrive in drought and termite-infested areas (Zhang, *et al*., 2019).

The species is deciduous with a maximum growth height of about 14 meters (Chafiamo *et al.*, 2018). Young *T. brownii* forms a huge shady canopy. Its bark is cracked and grey-colored. Its roots are superficial and tap-rooted disappearing beneath the ground as they age (Chafiamo *et al.*, 2018). Its evergreen leaves are positioned at the edge of its hairy shoots. Additionally, the leaves are simple, oval-shaped with piercing apex and connected veins. The lower part of the leaf is hairy and becomes red during the falling phase (Chafiamo *et al.*, 2018). Its flowers are

white with an irritating spiky smell. The species produces yellow fruits with angular-shaped dark red or black drupes (Chafiamo *et al.,* 2018).

Figure 2.2: *Terminalia brownii*

2.5.2.2 Medicinal and Nutritional Uses of *T. brownii*

Terminalia brownii has been indicated to have multiple biological activities: antimicrobial, antimalarial, antifungal, liver protection, anti-inflammatory, anticancer, analgesic, immune regulation, anti-diabetes, cardio-protective, antinociceptive and fastens the process of wound healing (Mbiri *et al.,* 2016; Chafiamo *et al.,* 2018). It is utilized in herbal medicine to treat fungal infections, diabetes, malaria, hepatitis, jaundice, yellow fever, wounds, abdominal pain, dermatitis, burns, dandruff, gynaecological illnesses, eye ailments such as conjunctivitis (Alema *et al.,* 2020), among others. *Terminalia brownii* is also used as a fungicide in traditional granaries, and to spray field crops, particularly in Northern Kenya and Sudan (Chafiamo *et al.,* 2018). Juice squeezed from its leaves is sprayed on wooden houses and furniture to protect them against decay induced by fungal infection predominantly among the pastoralist communities (Mariod *et al.,* 2014). Its fruits are edible, nutritious, and endowed with medicinal efficacy. Some of the classes of compounds noted in *T. brownii* and whose medicinal potency has been attributed to include flavonoids, tannins, and phenolics (Chafiamo *et al.,* 2018), among others.

2.6 Methods for Screening Immunomodulatory Agents

2.6.1 White Blood Cell Count

White blood cell (WBC) count refers to the number of basophils, eosinophils, monocytes, lymphocytes, neutrophils, and atypical cells present in one microliter of blood (Chabot-Richards & George, 2014). Increased levels of WBCs (leucocytosis) are observed in a range of conditions including malignant and benign conditions (George, 2012; Chabot-Richards & George, 2014). To determine the cause of the disease, an accurate morphological evaluation and differential count of the peripheral blood smear are required for elevated WBC levels. Decreased levels of WBCs (leukopenia) also require the same (Valent, 2012). Total and differential WBC counts are used to assess effects of test substances on innate immune responses (Obi *et al*., 2018).

2.6.2 Functional Tests for Macrophages and Monocytes

These include assessment of phagocytosis, NO, TNF-α, and ROS production by macrophages and monocytes (Lock *et al.,* 2019). The acid phosphatase assay has been conducted on peritoneal macrophages cultured with two polyethylene terephthalate test substances (Lock *et al.,* 2019). However, Cytoselect^{TM} 96 well phagocytosis assay is a more current technique that utilizes IgG-opsonized sheep red blood cells (Lock *et al.,* 2019). In addition, the carbon clearance test is used to study macrophages' phagocytic activity (Mao *et al.,* 2019). ROS, TNF-α, and NO production by immune cells treated with the test substances shows cellular activation (Lock *et al.,* 2019). These tests are used to assess effects of test substances on innate immunity (Chan-Zapata *et al*., 2018; Mao *et al.,* 2019).

2.6.3 Neutrophil Adhesion Test

This test is used to study innate immune responses whereby the cell adhesion properties of neutrophils are assessed (Baregama & Goyal, 2020). Neutrophils vasculature adhesion plays a crucial part in the inflammatory responses to pathogens and injury (Wilhelmsen *et al.,* 2013). The very first leukocytes to reach the site of

injury or pathogen infection, and attach to the endothelium are neutrophils (Wilhelmsen *et al.,* 2013). Neutrophils eliminate microbes directly, recruit other white blood cells to the site and, stimulate repair of the wound (Wilhelmsen *et al.,* 2013). Therefore, the recruitment and subsequent adherence of neutrophils to the endothelial wall is an important step in the inflammatory response to clear pathogens and heal the injured site (Wilhelmsen *et al.*, 2013).

2.6.4 Haemagglutination Antibody Titer Assay

Haemagglutination involves the binding of the produced anti-RBCs antibodies, specifically IgM, to the antigenic red blood cells (RBCs) during a second encounter with the antigen (Sunil *et al.*, 2019). Haemagglutination arises from rosette formation of the RBCs on the antibodies (Sunil *et al.*, 2019). Red blood cells are commonly used because they are readily available and agglutination can be observed via the naked eye. Haemagglutination is used for determining antibody titers, viral quantification, and blood grouping (Costabile, 2010).

2.6.5 Plaque Forming Cell (PFC) Assay

The PFC assay is also referred to as the Antibody Forming Cell (AFC) Assay. The PFC assay is used in many laboratories and it’s the most accepted endpoint in immunotoxicology (Ladics, 2007). The assay can be conducted *in vitro* entirely utilizing immunocompetent cells sourced from either naïve or treated animals (Ladics, 2007). This assay however doesn’t quantify the amount of antibody that is produced, it quantifies only the number of specific plasma cells that produce antibodies in a certain tissue including the spleen (Ladics, 2007). It doesn't account for antibodies produced in other sites including lymph nodes and bone marrow (Ladics, 2007).

2.6.6 Sheep Red Blood Cells (SRBC)-Specific IgM ELISA

This assay uses solubilized hemoglobin-free SRBC membranes (Ladics, 2007). It evaluates SRBC-specific antibodies in serum (Ladics, 2007). This reflects systemic

antibody-mediated immune response. For this assay, there are no commercial SRBC membranes available (Ladics, 2007).

2.6.7 Lymphocyte Proliferation Assay (LPA)

This assay is used to study cellular immune responses (Mahooti *et al.,* 2019). It is based on lymphocytes' ability to proliferate when cultured (Descotes, 2014). Mitogens that serve as polyclonal activators of T or B lymphocytes are used to enhance the proliferation of lymphocytes (Descotes, 2014).

2.6.8 Cyclophosphamide-Induced Neutropenia Assay

This assay is used to investigate innate immune responses (Nasim *et al.,* 2018). Neutropenia is a condition whereby there is a reduction in neutrophil count in blood (Newburger & Dale, 2023).

2.6.9 Delayed-Type Hypersensitivity (DTH)

The delayed or type IV hypersensitivity response (DTH) is a reaction that takes more than 12 hours to develop with a maximum reaction time occurring between 48 to 72 hours (Basu & Banik, 2018; Maker *et al.,* 2019). On the contrary other types of hypersensitive responses are considered immediate hypersensitivity responses since they occur within 12 hours (Maker *et al.,* 2019). DTH reaction is mediated by cells that cause inflammatory reactions against both endogenous and exogenous antigens (Basu & Banik, 2018). The major cells involved are T lymphocytes, monocytes, macrophages, eosinophils, and neutrophils (Maker *et al.,* 2019). The DTH reaction is used to study cellular immune responses (Nasim *et al.,* 2018).

2.6.10 Cytotoxic T Lymphocyte Assays

Co-culturing splenocytes obtained from control and treated animals with mitomycin C-inactivated histo-incompatible target cells to induce T lymphocyte cytotoxicity is used to study cellular immune responses *in vitro* (Descotes, 2014). To chromium-labelled target cells, splenocytes are added and from the radioactivity emitted into the

supernatant, cytotoxicity is measured (Descotes, 2014). A flow cytometer can also be used to measure cell-mediated cytotoxicity (Descotes, 2014).

2.6.11 Immune Cells Viability Tests

One way of determining cytotoxicity on immune cells in response to a test substance is by conducting viability tests (Lock *et al.,* 2019). The most common method used to assess cell viability involves visualizing the number and morphology of immune cells populating a biomaterial's surface (Lock *et al.,* 2019). Staining and microscopy protocols including calcein-acetoxymethyl live-dead (Schutte *et al.,* 2009), actin, and nuclei staining (Almeida *et al.,* 2014) have been used to directly image cells on a biomaterial. This offers easy and quick visualization of the response of immune cells to a particular material (Almeida *et al.,* 2014). Metabolic assays including the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide **(**MTT) assay, G6PD assay, and alamarBlue® are also used to evaluate the viability of innate immune response cells (Yahyouche *et al.,* 2011; Kou *et al.,* 2012). Metabolic assays are quick and affordable (Lock *et al.,* 2019). However, they only measure viable cells (Lock *et al.,* 2019).

2.7 Method for Acute Toxicity Analysis

2.7.1 Skin-Sensitization Methods

Buehler assay (BA) and pig maximization test are some of the dermal sensitization tests (Chan *et al*., 2014). In both tests, low doses of the test substance are intradermally injected or topically applied on shaved skin (Chan *et al*., 2014). Irritation denotes chemical sensitization (Chan *et al*., 2014). Mouse local lymph node assay (LLNA) is another skin sensitization method (Chan *et al*., 2014). Here, the test substance is applied to the dorsal part of the ear for three consecutive days (Chan *et al*., 2014). LLNA is the most preferred skin sensitization test because it reduces the number of experimental animals used, the animals experience less pain, is faster, and provides dose-dependent information (Chan *et al*., 2014).

2.7.2 Skin Irritation Method

Skin absorption of the test substance varies significantly throughout the skin (Chan *et al*., 2014). The substance to be tested is applied to a shaved skin patch on the back uniformly (Chan *et al*., 2014). The test area is then examined for oedema and erythema (Chan *et al*., 2014).

2.7.3 Eye Irritation Method

The test substance is normally introduced in the lower eyelid pouch, held in place for one second, and then released (Chan *et al*., 2014). The untreated eye acts as the control (Maheshwari & Shaikh, 2016).

2.7.4 Acute Systematic Toxicity Method

Acute toxicity refers to the deleterious effects that occur within twenty-four hours following ingestion of a test substances (Amos *et al.*, 2015). It is usually expressed as LD_{50} which is a statistical measure of an agent's acute lethality (Chan *et al*., 2014).

2.8 Method for Sub-Acute Toxicity Analysis

2.8.1. Oral Sub-Acute Toxicity Test

In this test, the test substance is orally administered daily in three dosages for 28 days. The animals are closely observed daily for any signs of toxicity and their body weights are taken weekly. On the 29^{th} day, blood is obtained for assessment of hematological and biochemical parameters. Several body organs are also harvested for histopathology analysis (OECD, 2008).

2.8.2. Inhalation Sub-Acute Toxicity Test

This test allows for toxicity evaluation of test substances in the form of solid aerosol, liquid aerosol, vapour or gas. Experimental animals are exposed to three concentration levels of the test substance for 6 hours a day, 5 days a week. The study duration is 28 days. On the 29^{th} day, the animals are sacrificed and the

bronchoalveolar lavage fluid (BALF) is assessed for toxicity. The left lung is used for histopathology while the right one is used for BALF analysis (OECD, 2018).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Introduction

Terminalia brownii stem bark and *C. edulis* root bark samples were obtained from Kitui County and bioassays were conducted at the animal breeding and experimentation facility at Kenyatta University (KU) and KEMRI. Methanol and dichloromethane (DCM) solvents were used for the extraction of the plant materials. Female Swiss albino mice were used in the study of the effects of the plant extracts on the immune system while female Wistar rats were used for toxicity studies. To evaluate the effects of the extracts on innate immune responses, total and differential leucocyte counts, and production of NO and TNF-α by macrophages were assessed. Effects of the extracts on cellular immune responses were assessed via the DTH reaction and production of Th1 and Th2 cytokines while hemagglutination antibody titer assay was used to study the effects of the extracts on humoral immune responses. Acute and sub-acute toxicity studies were used to determine the toxicity levels of the extracts in rats. Phytochemical profiling of the extracts was accomplished via GC-MS and LC-MS. GraphPad Prism 8 software was used for data analysis and values of $p \leq 0.05$ were taken as significantly different values.

3.2 Collection of Plant Material

Stem bark and root bark samples of *T. brownii* and *C. edulis*, respectively, were gathered from Kitui County (situated at 37.7558° E, 1.3099° S coordinates). The samples were collected on April 2021. An aboriginal herbalist guided the plant identification process. Plant samples were verified at the East African Herbarium and voucher specimens deposited there as JWM001 for *T. brownii* and JWM002 for *C. edulis*. The samples were taken to KU animal house for the bioassays. Samples were cleaned utilizing running tap water, chopped into smaller pieces, dried under shade until perfectly dried, and then milled following the protocol by Musila *et al.,* (2017).

3.3 Plant Material Extraction

Methanol and dichloromethane solvents were used for extraction. Three litres of methanol and DCM were poured into conical flasks containing one kilogram of sample powder separately. The admixture was stirred until all the powder was completely soaked and left to stand at room temperature for two days. Plant extracts were filtered using an aspirator pump and the extracts were concentrated using a rotary evaporator. The filtrates were refrigerated at 4°C as per the protocol by Adnan *et al*., (2019).

3.4 Experimental Animals

Effects of the plant extracts on the immune system were evaluated using Swiss albino female mice aged between 7 to 9 weeks and weighing between 25 and 30g. Toxicity studies of the extracts were conducted using female Wistar rats aged between 2 to 3 months and weighing between 140 to 160g. The animals were procured from the department of Zoology, Kenyatta University, animal house. They were provided with conventional pellets and water as often as necessary. The present study was approved (PKUA/005/005) by the Kenyatta University ethical review committee

3.5 Evaluation of the Effect of *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol and DCM Extracts on Innate Immune Responses

3.5.1 Effect of *T. brownii* and *C. edulis* Methanol and DCM Extracts on Total and Differential Leukocyte Counts

The effect of the extracts on leukocyte counts was assessed following the protocol described by Trivedi *et al*. (2017). Weights of the mice were taken and the animals were randomly categorized into six groups. The treatments administered were as outlined in Table 3.1. Category I mice, normal control, were given the vehicle (0.2ml of 2.5 % Dimethyl sulfoxide (DMSO) mixed in distilled water). Category II mice, negative control, received the immunosuppressant, pyrogallol, at 50mg/kg BW for seven days. Categories III, IV, V, and VI mice, received pyrogallol from day 0 to day

7 and then treated with levamisole (20mg/kg BW), 50mg/kg BW, 100mg/kg BW, and 150mg/kg BW plant extract, respectively, until day 14. The tested plant extracts included *T. brownii* methanol extract, *T. brownii* DCM extract, *C. edulis* methanol extract, *C. edulis* DCM extract and a combination of *T. brownii* and *C.edulis* methanol extracts. The treatments were given orally. Twenty-four hours later, blood was obtained from tail veins for the determination of leukocyte counts. Blood from the negative control mice was obtained on the 8^{th} day of the experiment.

Table 3.1: Treatments Administered for the Evaluation of Total and Differential Leukocyte Counts

Group	Treatment
Normal control	2.5% DMSO in distilled water
Negative control	50mg/kg Pyrogallol (7 days)
Positive control	50mg/kg Pyrogallol (7 days) + 20mg/kg levamisole (day 8 – day 14)
Plant Extract	50mg/kg Pyrogallol (7 days) + 50mg/kg extract (day 8 – day 14)
Plant Extract	50mg/kg Pyrogallol (7 days) + 100mg/kg extract (day 8 – day 14)
Plant Extract	50mg/kg Pyrogallol (7 days) + 150mg/kg extract (day 8 – day 14)

DMSO = dimethyl sulfoxide

3.5.2 Effect of *T. brownii* and *C. edulis* Methanol and DCM Extracts on Nitric Oxide Production by Murine Macrophages

3.5.2.1 Isolation of Peritoneal Macrophages

To prompt the production of murine macrophages, mice intraperitoneally received newborn calf serum (1ml). After three days, peritoneal exudates were drawn into Roswell Park Memorial Institute Medium (RPMI) 1640 culture medium, they were centrifuged for twenty minutes at 1,000 rotations per minute and erythrocytes were lysed via hypotonic lysis. Macrophage pellets were washed twice using RPMI 1640 medium and the cells suspended in culture media (RPMI 1640 medium) were boosted with 100u/ml penicillin, 0.04mg/ml gentamycin, newborn calf serum (10%), and 2mM glutamine. Methylene blue test was used to determine the viability of the

macrophages and the number of cells established using a hemocytometer (Djafoua *et al.,* 2015).

3.5.2.2 Effect of *T. brownii* and *C. edulis* Extracts on Vero Cells and Macrophage Viability

The viability of Vero cells and macrophages was assessed through the MTT assay using 96 well culture plates (Bolla *et al*., 2019). Vero cells were obtained from KEMRI. RPMI 1640 culture medium (100µl) enhanced with 10% FBS was introduced into all the wells. A hundred microliters of every extract (3mg/ml) were added into row H in triplicates segregated by blanks. Two-fold serial dilution of the various extracts was carried out from rows H to C. The surfeit solution, 100µl, was disposed of. Rows A and B (normal control wells) received no treatments. Macrophages were also cultured with 1µg/mL levamisole, positive control for measurement of NO and TNF-α levels. 100µl of Vero cells and macrophages (1×10^5 cells/mL) were added to all the wells but not into the blanks.

The culture plates were then incubated for 24h in a carbon dioxide incubator and supernatants were drawn for measurement of TNF-α and NO levels. Forty microliters of MTT (5mg/ml) were introduced into all the wells and plates were again incubated for four hours. Culture media plus MTT was removed from the wells, DMSO (100µL) was added and plates were incubated again for twenty minutes. Absorbance was read at 570nm using an ELISA plate reader. The following formula was applied to calculate percentage viability:

Percentage cell viability = (Sample OD / Control OD) * 100.

3.5.2.3 Determination of NO production

Nitric oxide levels in macrophage culture supernatants were determined using the Griess reagent system (Chan-Zapata *et al,* 2018). The assays were conducted in 96 well culture plates. Griess reagent (50µl) was added to the plates in triplicates followed by the addition of the supernatants (50µl). The two components were thoroughly mixed and the plates were incubated for ten minutes at room temperature.

Absorbance readings were obtained using an ELISA reader at 490nm. A nitrite standard reference curve was prepared to establish nitrite concentrations.

3.5.3 Effect of *T. brownii* and *C. edulis* Methanol and DCM Extracts on TNF-α by Macrophages

Mouse-specific Th1/Th2 cytokine Cytometric Bead Array kit was employed to measure TNF-α levels in macrophage culture supernatants (Lehmann *et al.,* 2017; Afolayan *et al.,* 2020). Fifty microliters of bead populations coupled with antibodies specific to TNF-α were pipetted into 50µl of every sample in triplicates and the micro-Fluorescence-activated cell sorting (FACS) tubes were incubated for ninety minutes at room temperature. Samples and the standards were washed to dislodge any loose matter. Detection antibodies (phycoerythrin-sheathed) were added to the samples and the tubes were incubated for an hour after which they were washed. BD FACS-Calibur flow cytometer was employed to measure the generated fluorescent signals and FCAP Array software was used to establish concentrations of the analyte from the standard curves.

3.6 Evaluation of the Effect of *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol and DCM Extracts on Cellular and Humoral Immune Responses

Effect of the extracts on cellular immune response was studied by evaluating serum levels of Th1 and Th2 cytokines and DTH reaction while the effects of the extracts on humoral immune responses was evaluated through the hemagglutination antibody titer assay.

3.6.1 Effect of *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol and DCM Extracts on Th1 and Th2 Cytokines

The experimental design described by Qi *et al.* (2018), with a few changes, was followed to evaluate the effects of the extracts on Th1 and Th2 cytokines. Th1 cytokines studied included IL-2, TNF-α, and IFN-γ while Th2 cytokines included IL-4 and IL-5. Mice were categorized into six groups randomly and the treatments administered were as outlined in Table 3.2. Category I mice, normal control, were

given 0.2ml of the vehicle (2.5 % DMSO contained in distilled water). Category II mice, the model control group, received 0.2ml of the pyrogallol from the 1st day to the 7th day. Categories III, IV, V, and VI experimental animals were immunosuppressed using 50mg/kg BW pyrogallol from the 1st day to the 7th day and then treated with levamisole (20mg/kg BW), 50mg/kg BW, 100mg/kg BW and 150mg/kg BW plant extract, respectively, upto day 10. Treatments were orally administered. The tested plant extracts included *T. brownii* methanol extract, *T. brownii* DCM extract, *C. edulis* methanol extract, *C. edulis* DCM extract and a combination of *T. brownii* and *C.edulis* methanol extracts. On the 11th day, blood was drawn from caudal veins and centrifuged for 10 minutes at 2000rpm to obtain serum for measurement of cytokine levels. Blood from the model control animals was drawn on the 8th day.

Mouse-specific Th1/Th2 cytokine Cytometric Bead Array (CBA) kit was used to assess Th1 and Th2 cytokine levels in mice serum (Lehmann *et al.,* 2017; Afolayan *et al.,* 2020). Fifty microliters of bead populations coupled with antibodies specific to the analytes were, in triplicates, pipetted into micro FACS tubes containing 50µl of every sample. The tubes were incubated for 90min at room temperature. Samples and the standards were washed to dislodge any loose matter. Detection antibodies (phycoerythrin-sheathed) were added to the samples and the tubes were incubated for an hour after which they were washed. BD FACS-Calibur flow cytometer was used to quantitate the generated fluorescent signals and FCAP Array software was used to establish concentrations of the analyte from the standard curves.

Table 3.2: Treatments Administered for the Determination of Th1 and Th2 Cytokine Levels in Mice Serum

Group	Treatment
Normal control	2.5% DMSO in distilled water
Negative control	50mg/kg Pyrogallol (7 days)
Positive control	50mg/kg Pyrogallol (7 days) + 20mg/kg levamisole (day 8 – day 10)
Plant Extract	50mg/kg Pyrogallol (7 days) + 50mg/kg extract (day 8 – day 10)
Plant Extract	50mg/kg Pyrogallol (7 days) + 100mg/kg extract (day 8 – day 10)
Plant Extract	50mg/kg Pyrogallol (7 days) + 150mg/kg extract (day 8 – day 10)

DMSO = dimethyl sulfoxide

3.6.2 Effect of *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol and DCM Extracts on Delayed-Type Hypersensitivity (DTH) Reaction

A delayed Type Hypersensitivity reaction test was carried out to study the effects of the extracts on cellular immune responses. It was conducted following the methodology outlined by Kumolosasi *et al.,* 2018 with a few adjustments. Mice were randomly arranged into six categories comprising five mice each and the treatments administered were as outlined in Table 3.3. Category I mice, normal control, orally received the vehicle, 2.5% DMSO. Category II mice, model control, orally received pyrogallol for seven days. Categories III, IV, V, and VI groups received pyrogallol for seven days then 20mg/kg levamisole, 50mg/kg extract, 100mg/kg extract, and 150mg/kg extract, respectively from day 8 to the 14^{th} day of the experiment. The tested plant extracts included *T. brownii* methanol extract, *T. brownii* DCM extract, *C. edulis* methanol extract, *C. edulis* DCM extract and a combination of *T. brownii* and *C.edulis* methanol extracts. Mice in all groups, except normal control, were intraperitoneally sensitized with 0.1ml SRBCs (20%) on the 8^{th} day, before administration of the treatments, and challenged with the same volume and concentration of SRBCs on the 16^{th} day, subcutaneously. 24h later, a Vanier caliper was used to measure footpad thickness. Values obtained in the experimental groups were compared to the values obtained in control mice.

Table 3.3: Treatments Administered for the Evaluation of DTH Reaction

Group	Treatment
Normal control	2.5% DMSO in distilled water
Negative control	50mg/kg Pyrogallol (7 days) + SRBCs (day 8-IP & day 16-SQ)
Positive control	50mg/kg Pyrogallol (7 days) + 20mg/kg levamisole (day 8 – day 14) + SRBCs (day 8-IP & day 16 - SQ)
Plant Extract	50mg/kg Pyrogallol (7 days) + 50mg/kg extract (day 8 - day14) + SRBCs (day 8-IP & day 16 - SQ)
Plant Extract	50mg/kg Pyrogallol (7 days) + 100mg/kg extract (day 8 - day14) + SRBCs (day 8-IP & day 16 - SQ)
Plant Extract	50mg/kg Pyrogallol (7 days) + 150mg/kg extract (day 8 – day 14) + SRBCs (day 8-IP & day 16 - SQ)

DMSO = Dimethyl sulfoxide; SRBC = Sheep Red Blood Cells; IP = intraperitoneal; SQ = subcutaneous

3.6.3 Effect of *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol and DCM Extracts on Antibody Titer Levels

Hemagglutination antibody titer assay was done to evaluate the effects of the extracts on humoral immune responses following the protocol described by Shruthi *et al*., (2018). The assay specifically evaluated the effects of the extracts on the production of anti-SRBCs antibodies. Mice were randomly sorted into six categories with five mice each and the treatments administered were as outlined in Table 3.4. Category I mice, normal control, orally received the vehicle, 2.5% DMSO. Category II mice, negative control, received pyrogallol orally for seven days. Categories III, IV, V, and VI groups received pyrogallol for seven days then 20mg/kg levamisole, 50mg/kg extract, 100mg/kg extract, and 150mg/kg extract, respectively from days 8 to day 14. Mice in the negative control group, positive control group, and the three experimental groups (50mg/kg, 100mg/kg, and 150mg/kg extract) were intraperitoneally immunized with 0.1ml SRBCs (20%) on the 8^{th} day of the experiment before administration of the treatments. The tested plant extracts included

T. brownii methanol extract, *T. brownii* DCM extract, *C. edulis* methanol extract, *C. edulis* DCM extract and a combination of *T. brownii* and *C.edulis* methanol extracts.

On the 15th day, blood (200µl) was collected from tail veins, and centrifuged for 10 minutes at 2000rpm to obtain serum. Serial dilutions, two-fold, of the serum samples, were done in V-bottomed 96 well hemagglutination plates having 100µl phosphate-buffered saline (PBS). Fifty microliters of SRBCs (1%) suspended in PBS were pipetted into every well and the plates were incubated for one hour at 37°C after which they were visually examined for hemagglutination. The highest dilution of the serum producing hemagglutination was considered the antibody titer. Antibody titers were expressed as ranks with the lowest dilution (1/2) ranked as 1 (Nimbalkar *et al.,* 2018).

Table 3.4: Treatments Administered for the Evaluation of Antibody Titer Levels

Group	Treatment
Normal control	2.5% DMSO in distilled water (day 0 to 14)
Negative control	50mg/kg Pyrogallol (7 days) + SRBC IP (day 8)
Positive control	Pyrogallol (7 days) + SRBC IP (day 8) + 20mg/kg levamisole (day 8 to 14)
Plant Extract	Pyrogallol (7 days) + SRBC IP (day 8) + 50mg/kg extract (day 8 to 14)
Plant Extract	Pyrogallol (7 days) + SRBC IP (day 8) +100mg/kg extract (day 8 to 14)
Plant Extract	Pyrogallol (7 days) + SRBC IP (day 8) + 150mg/kg extract (day 8 to 14)

DMSO = dimethyl sulfoxide; SRBC = Sheep Red Blood Cells; IP = intraperitoneal

3.7 Evaluation of the Synergistic Immunomodulatory properties of *T. brownii* Stem Bark and *C. edulis* Root Bark Extracts

The plant extracts with the highest immunomodulatory activity were combined in a ratio of 1:1 and their effects on innate and adaptive immune responses were evaluated (Parveen *et al*., 2021).

3.8 Evaluation of Acute and Sub-acute Toxicity Levels of *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol and DCM Extracts

3.8.1. Assessment of Acute Toxicity Levels of *T. brownii* and *C. edulis* Extracts in Female Wistar Rats

Acute toxicity tests were done following the Organization for Economic Co-operation and Development directives (OECD, 2022). Limit tests were done since the LD50 of *T. brownii* and *C. edulis* extracts has been reported to be above 2000mg/kg BW (Osseni *et al*., 2016; Biruk *et al.,* 2020). Rats were separated into five groups; normal control and four experimental groups containing five animals each. The four experimental groups orally received 0.5 ml of *T. brownii* methanol extract, *T. brownii* DCM extract, *C. edulis* methanol extract, and *C. edulis* DCM extract, respectively. The extracts were administered in a single dosage, 200mg/kg BW. Normal control rats orally received the vehicle, DMSO (2.5%) in distilled water.

Rats underwent fasting overnight before administration of the treatments. Weights of the animals were taken after fasting and the treatments given. Food was again withheld for four hours. Rats were observed for two weeks for death and toxicity signs. The body weights of the animals were again taken and recorded on the 7^{th} and 14^{th} days of the study. Observations included general appearance; changes in the fur, skin, mucous membranes, and eyes and incidences of salivation, diarrhea, tremors, convulsions, coma, lethargy, and sleep.

3.8.2. Assessment of Sub-acute Toxicity Levels of *T. brownii* and *C. edulis* Extracts in Female Wistar Rats

The Organization for Economic Co-operation and Development's recommendations were followed in the execution of sub-acute toxicity tests (OECD, 2008). Rats were put into four groups at random; one control group and three experimental groups for each extract. Normal control rats orally received 0.5ml of the vehicle and 2.5% DMSO in distilled water. The three experimental groups orally received extracts at dosages 300, 520, and 900mg/kg BW respectively. Treatments were given every day up to the 28^{th} day. Rats were observed daily for death and toxicity signs. The rats were weighed weekly. On day 29, the weights of the rats were taken, the rats were sacrificed through cervical dislocation, blood was collected through the cardiac puncture, and the weights of different organs were taken. Blood samples were centrifuged for 3000G per minute for ten minutes to obtain serum for biochemical analysis (Maimaiti *et al.,* 2021). Analyzed biochemical and hematological parameters are documented by OECD, (2008).

3.9 Identification and Quantification of Phytochemical Secondary Metabolites in *T. brownii* Stem bark and *C. edulis* root Bark Extracts

3.9.1 Phytochemical Profiling of *T. brownii* Stem Bark and *C. edulis* Root Bark Extracts Via Gas Chromatography-Mass Spectrometry (GC-MS)

Gas chromatography-mass spectrometry analysis of the extracts was conducted as outlined by Saad *et al*. (2021) with some modifications. The samples were weighed (100 mg) and dissolved in 1 ml DCM. The mixtures were vortexed for ten seconds, sonicated for ten minutes, and centrifuged at 14,000 rotations per minute for five minutes. Supernatants were filtered and diluted up to 100ng/µL. Phytochemical profiling was conducted in three replicates.

Gas chromatography-mass spectrometry instrumentation was composed of a TRACE GC Ultra Gas Chromatograph along with a Thermo MS detector. The system was equipped with a TR-5 MS column. A constant flow rate of helium was maintained at 1.25 mL/min. A split mode of injection was used. Temperatures of the oven were set

at thirty-five degrees centigrade for five minutes to two hundred and eighty degrees centigrade for ten and a half minutes at 10°C/min to two hundred and eighty-five degrees centigrade for 29.9 minutes at 50°C/min. Seventy minutes was the run time.

Serial dilutions (1-100ng/L) of the standard, undecane, were done and run in GC-MS to produce a linear calibration curve. The generated equation, y = 186096x + 1000000, was employed to calculate the concentrations of the identified compounds. Identification of the compounds was achieved using the National Institute of Standards and Technology (NSIT) based on retention times and mass spectrum interpretation. Detected unknown compounds were compared to standard mass spectra of known compounds in the NSIT database.

3.9.2 Phytochemical Profiling of *T. brownii* Stem Bark and *C. edulis* Root Bark Extracts Via Liquid Chromatography–Mass Spectrometry (LC-MS)

Liquid chromatography-mass spectrometry analysis of *T. brownii* and *C. edulis* extracts was done following the protocol described by Sinan *et al.,* (2021). One millilitre of methanol with 0.01% formic acid was used to dissolve 20 mg of each sample. The solutions were, sonicated for 10 minutes and centrifuged at 13,000 rpm for 15 minutes. The supernatants were filtered through a plug and transferred in to sterile vials.

The chromatographic separation was achieved on Agilent 1260 Infinity HPLC system (Agilent Technologies, Palo Alto, CA) coupled to an Agilent 6120 mass detector MS with a single quadrupole analyser (Agilent Technologies, Palo Alto, CA). A ZORBAX SB-C18, 4.6×250 mm, 3.5µm column, operated at 40 °C, was used. The mobile phase used was made of water (A) and methanol (B) each with 0.01% formic acid. The following gradient system was used: (0.01 min, 5% B; 5 min, 5% B; 10 min, 20% B; 15 min, 20% B; 20 min, 80% B; 25 min, 80% B; 30 min, 100% B; 35 min,100% B; 37 min, 5% B; 42 min, 5% B.

The flow rate was held constant at 0.5 mL/min and the injection volume was 1µL. The LC was interfaced to a quadruple mass spectrometer operated on either ESI positive or negative mode at a mass scan range of m/z 100–2000. The dwell time for

each ion was 50ms. Rutin hydrate standard (94%, Sigma–Aldrich (St Louis, MO) was serially diluted (1–100 ng/μl) to produce a linear curve. Peak areas were plotted against concentration producing the following equation. The equation served as an external quantitation foundation.

$$[y = 5578.4x - 39094 \ (R^2 = 0.9990)]$$

Identification of the detected unknown compounds was accomplished using the Adams and Chemical mass spectral databases.

3.10 Data Analysis

Data analysis was done using the GraphPad Prism 8 software. One-way analysis of variance was used to determine differences between the groups. For the separation of means, Tukey's post hoc test was used. Values of $p \leq 0.05$ were taken as significantly distinct values.

CHAPTER FOUR

RESULTS

4.1 Introduction

The present study evaluated the ability of methanol and DCM extracts of *T. brownii* stem bark and *C. edulis* root bark to modulate immune responses in pyrogallol-immunosuppressed mice. The effect of the various extracts on innate, cellular, and humoral immunity was investigated. Methanol and DCM extracts of *T. brownii* and *C. edulis* increased levels of total and differential leukocyte counts. Moreover, the tested extracts augmented the production of NO and TNF-α by mouse macrophages. *Terminalia brownii* and *C. edulis* methanol and DCM extracts elevated levels of IL-2, TNF-α, and IFN-γ. However, the extracts reduced levels of IL-4 and IL-5. In addition, the extracts augmented antibody titers and DTH reaction in mice.

Acute and sub-acute toxicity tests of *T. brownii* and *C. edulis* methanol and DCM extracts were also conducted in the present study. The extracts did not affect the body weights, relative organ weights, haematological parameters, biochemical parameters, behavioural patterns, and general appearance of the rats.

Phytochemical profiling of *T. brownii* and *C. edulis* methanol and DCM extracts was done and diverse compounds were identified in the extracts.

4.2 Effect of *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol and DCM Extracts on Innate Immune Responses

Terminalia brownii and *C. edulis* methanol and DCM extracts increased total and differential leukocyte counts in mice, NO, and TNF-α production by mouse macrophages.

4.2.1 Effect of *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol and DCM Extracts on Total and Differential Leucocyte Counts

All the tested extracts augmented the production of total and differential leukocyte counts in pyrogallol-immunosuppressed mice.

4.2.1.1 Effect of *T. brownii* Stem Bark Methanol Extract on Total and Differential Leucocyte Counts

Pyrogallol-treated mice produced significantly ($p < 0.05$) reduced total and differential leukocyte counts compared with the normal control mice. However, mice treated with *T. brownii* methanol extract produced significantly ($p < 0.05$) elevated WBCs, neutrophils, lymphocytes, monocytes, eosinophils, and basophil counts compared with negative control mice (Table 4.1). The extract at dosages 50, 100 and 150 mg/kg bw produced significantly ($p < 0.05$) different levels of WBC, neutrophils, lymphocytes, and eosinophils. However, there was no significant difference ($p > 0.05$) in monocyte counts produced by mice treated with the extract at 100 and 150 mg/kg bw (Table 4.1). On the other hand, mice administered with 50 and 100 mg/kg bw showed a comparable increase ($p > 0.05$) in basophils counts.

The extract at 150 mg/kg bw dosage and levamisole (positive control) led to the production of similar ($p> 0.05$) WBC, neutrophils, lymphocytes, and monocytes counts in mice (Table 4.1). However, the extract, at the tested dosages, resulted in significantly lower eosinophil and basophil counts compared with levamisole.

Table 4.1: Effect of *T. brownii* Stem Bark Methanol Extract on Total and Differential Leukocyte Counts

Treatment	Leukocyte Count (10^9/L)					
	WBCs	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
Normal Control	5.94±0.11[a]	2.50±0.07[a]	2.82±0.06[a]	0.35±0.02[a]	0.20±0.01[a]	0.07±0.00[a]
Negative Control	0.92±0.09[d]	0.40±0.05[d]	0.48±0.04[d]	0.01±0.00[e]	0.01±0.00[e]	0.01±0.00[d]
Levamisole	6.02±0.18[a]	2.62±0.11[a]	2.81±0.06[a]	0.31±0.02[ab]	0.20±0.01[a]	0.07±0.00[a]
TM (50mg/kg bw)	2.85±0.14[c]	1.21±0.07[c]	1.42±0.08[c]	0.13±0.00[d]	0.06±0.00[d]	0.03±0.00[c]
TM (100mg/kg bw)	3.95±0.16[b]	1.70±0.06[b]	1.90±0.12[b]	0.22±0.01[c]	0.10±0.01[c]	0.03±0.00[c]
TM (150mg/kg bw)	5.46±0.12[a]	2.39±0.05[a]	2.60±0.05[a]	0.26±0.03[bc]	0.15±0.01[b]	0.05±0.00[b]

Values were expressed as Mean ± SEM for the five mice in each group. Statistical comparison was done within a column and values with a similar superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p > 0.05$). TM = *T. brownii* methanol extract. Normal control group had received the vehicle; Negative control group had received the immunosuppressant; Levamisole group (positive control) had received the immunosuppressant and levamisole.

4.2.1.2 Effect of *T. brownii* Stem Bark DCM Extract on Total and Differential Leucocyte Counts

The study assessed the effect of *T. brownii* stem bark DCM extract on total and differential leukocyte counts in mice after pyrogallol-induced immunosuppression. Administration of pyrogallol significantly reduced ($p < 0.05$) total and differential leucocyte counts compared to the negative control. However, extract-treated mice demonstrated significantly higher ($p < 0.05$; Table 4.2) total and differential leucocyte counts compared with the negative control mice. Mice treated with the extract at all the tested dosages produced significantly lower ($p < 0.05$; Table 4.2) leucocyte counts compared with the levamisole-treated mice (positive control).

The extract, at 150mg/kg bw dose, produced significantly ($p < 0.05$) higher WBCs, lymphocytes, eosinophils, and basophils counts compared with the extract at dosages 50 and 100mg/kg bw. No significant difference ($p > 0.05$, Table 4.2) was observed in

WBCs, lymphocytes, eosinophils, and basophils counts in mice treated with the extract at 50 and 100mg/kg bw dosages. Additionally, there was no significant difference ($p > 0.05$) in monocyte counts in mice treated with the extract at 50, 100, and 150mg/kg bw. However, the extract increased neutrophil counts in a dose-dependent manner at the tested dosages (Table 4.2).

Table 4.2: Effect of *T. brownii* Stem Bark DCM Extract on Total and Differential Leukocyte Counts

Treatment	Leukocyte Count (10^9/L)					
	WBCs	**Neutrophils**	**Lymphocytes**	**Monocytes**	**Eosinophils**	**Basophils**
Normal Control	5.95±0.11[a]	2.50±0.07[a]	2.82±0.06[a]	0.35±0.02[a]	0.20±0.01[a]	0.07±0.00[a]
Negative Control	0.91±0.09[d]	0.40±0.05[e]	0.48±0.04[d]	0.01±0.00[d]	0.01±0.00[d]	0.01±0.00[d]
Levamisole	6.02±0.18[a]	2.62±0.11[a]	2.82±0.06[a]	0.31±0.02[b]	0.20±0.01[a]	0.07±0.00[a]
TD (50mg/kg bw)	2.70±0.07[c]	1.07±0.08[d]	1.42±0.05[c]	0.13±0.00[c]	0.06±0.00[c]	0.03±0.00[c]
TD (100mg/kg bw)	3.14±0.05[c]	1.43±0.06[c]	1.46±0.05[c]	0.16±0.00[c]	0.07±0.00[c]	0.03±0.00[c]
TD (150mg/kg bw)	4.63±0.09[b]	2.03±0.07[b]	2.28±0.04[b]	0.16±0.00[c]	0.12±0.01[b]	0.04±0.00[b]

Values were expressed as Mean ± SEM for the five mice in each group. Statistical comparison was done within a column and values with a similar superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p > 0.05$). TD = *T. brownii* DCM extract. Normal control group had received the vehicle; Negative control group had received the immunosuppressant; Levamisole group (positive control) had received the immunosuppressant and levamisole.

4.2.1.3 Effect of *C. edulis* Root Bark Methanol Extract on Total and Differential Leucocyte Counts

The effect of *C. edulis* methanol extract on total and differential leukocyte counts was also investigated. Pyrogallol-treated mice produced significantly reduced ($p < 0.05$) total and differential leukocyte counts compared with the normal control mice. The extract, at all the tested dosages, significantly increased ($p < 0.05$) total and differential leukocyte count in mice compared with the negative control (Table 4.3). The increase was found to be dose-dependent in WBCs, neutrophils, lymphocytes,

monocytes, and eosinophil counts. However, there was no significant difference ($p > 0.05$) in the effect of the extract at dosages 50 and 100mg/kg bw with regards to basophil counts (Table 4.3).

Mice treated with methanol extract of *C. edulis* at 150 mg/kg bw dosage and levamisole (positive control) produced similar ($p > 0.05$) neutrophils and lymphocytes counts. However, mice treated with the extract at 50 and 100 mg/kg bw produced significantly lower numbers of neutrophils and lymphocytes compared with levamisole (Table 4.3). At all the tested extract dosages, mice produced significantly lower ($p < 0.05$; Table 4.3) WBCs, monocytes, eosinophils, and basophils counts compared with levamisole.

Table 4.3: Effect of *C. edulis* Root Bark Methanol Extract on Total and Differential Leukocyte Counts

Treatment	**Leukocyte Count (10^9/L**					
	WBCs	**Neutrophils**	**Lymphocytes**	**Monocytes**	**Eosinophils**	**Basophils**
Normal Control	5.95 ± 0.11^{a}	2.50 ± 0.07^{a}	2.82 ± 0.06^{a}	0.35 ± 0.02^{a}	0.20 ± 0.01^{a}	0.07 ± 0.00^{a}
Negative Control	0.92 ± 0.09^{e}	0.40 ± 0.05^{d}	0.48 ± 0.04^{d}	0.01 ± 0.00^{f}	0.01 ± 0.00^{e}	0.01 ± 0.00^{d}
Levamisole	6.02 ± 0.18^{a}	2.62 ± 0.11^{a}	2.82 ± 0.06^{a}	0.31 ± 0.02^{b}	0.20 ± 0.01^{a}	0.07 ± 0.00^{a}
CM (50mg/kg bw)	2.83 ± 0.07^{d}	1.20 ± 0.02^{c}	1.41 ± 0.06^{c}	0.13 ± 0.00^{e}	0.06 ± 0.00^{d}	0.03 ± 0.00^{c}
CM (100mg/kg bw)	3.91 ± 0.18^{c}	1.70 ± 0.08^{b}	1.89 ± 0.10^{b}	0.19 ± 0.00^{d}	0.10 ± 0.01^{c}	0.03 ± 0.00^{c}
CM (150mg/kg bw)	5.38 ± 0.10^{b}	2.34 ± 0.06^{a}	2.59 ± 0.05^{a}	0.24 ± 0.00^{c}	0.15 ± 0.01^{b}	0.05 ± 0.00^{b}

Values were expressed as Mean ± SEM for the five mice in each group. Statistical comparison was done within a column and values with a similar superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p > 0.05$). CM = *C. edulis* methanol extract. Normal control group had received the vehicle; Negative control group had received the immunosuppressant; Levamisole group (positive control) had received the immunosuppressant and levamisole.

4.2.1.4 Effect of *C. edulis* Root Bark DCM Extract on Total and Differential Leucocyte Counts

Administration of pyrogallol significantly reduced ($p < 0.05$) total and differential leucocyte counts compared to the negative control. However, *C. edulis* DCM extract, at all the tested dosages, produced significantly higher ($p < 0.05$) numbers of total and differential leukocytes in mice compared with the negative control (Table 4.4). The extract, at 150mg/kg bw dose, produced significantly different ($p < 0.05$) WBCs, neutrophils, lymphocytes, eosinophils, and basophils counts compared with the 50 and 100mg/kg bw dosages. On the other hand, there was no significant ($p > 0.05$) difference in monocyte counts at the tested dosages of the extract. All the extract-treated mice showed significantly lower ($p < 0.05$) counts of total and differential leukocytes compared with levamisole-treated mice (positive control).

Table 4.4: Effect of *C. edulis* Root Bark DCM Extract on Total and Differential Leukocyte Counts

Treatment	**Leukocyte Count (10^9/L)**					
	WBCs	**Neutrophils**	**Lymphocytes**	**Monocytes**	**Eosinophils**	**Basophils**
Normal Control	5.95±0.11[a]	2.50±0.07[a]	2.83±0.06[a]	0.35±0.02[a]	0.20±0.01[a]	0.07±0.00[a]
Negative Control	0.91±0.09[d]	0.40±0.05[d]	0.48±0.04[d]	0.01±0.00[d]	0.01±0.00[d]	0.01±0.00[d]
Levamisole	6.02±0.18[a]	2.62±0.11[a]	2.82±0.06[a]	0.31±0.02[b]	0.20±0.01[a]	0.07±0.00[a]
CD (50mg/kg bw)	2.78±0.11[c]	1.09±0.08[c]	1.41±0.06[c]	0.13±0.00[c]	0.06±0.00[c]	0.03±0.00[c]
CD (100mg/kg bw)	3.12±0.07[c]	1.42±0.07[c]	1.45±0.07[c]	0.15±0.00[c]	0.06±0.01[c]	0.03±0.00[c]
CD (150mg/kg bw)	4.44±0.16[b]	2.03±0.09[b]	2.11±0.09[b]	0.16±0.00[c]	0.09±0.01[b]	0.04±0.00[b]

Values were expressed as Mean ± SEM for the five mice in each group. Statistical comparison was done within a column and values with a similar superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p > 0.05$). CD = *C. edulis* DCM extract. Normal control group had received the vehicle; Negative control group had received the immunosuppressant; Levamisole group (positive control) had received the immunosuppressant and levamisole.

4.2.1.5 Effect of the Combined *T. brownii* and *C. edulis* Methanol Extracts on Total and Differential Leucocyte Counts

Terminalia brownii and *C. edulis* methanol extracts were combined in a ratio of 1:1 and their effect on total and differential leukocyte counts was tested in pyrogallol-immunosuppressed mice. Negative control mice demonstrated significantly reduced ($p < 0.05$) total and differential leukocyte counts compared to the normal control mice. However, the combined extract significantly increased ($p < 0.05$; Table 4.5) total and differential leukocyte counts in mice compared with the negative control. The increase was found to be dose-dependent. At 150mg/kg bw dosage, the combined extract and levamisole (positive control) produced a comparable increase ($p > 0.05$; Table 4.5) in total and differential leukocyte counts.

Table 4.5: Effect of the Combined *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol Extract on Total and Differential Leukocyte Counts

Treatment	Leukocyte Count (10^9/L)					
	WBCs	**Neutrophils**	**Lymphocytes**	**Monocytes**	**Eosinophils**	**Basophils**
Normal Control	5.95±0.11^{a}	2.50±0.07^{a}	2.83±0.06^{a}	0.35±0.02^{a}	0.20±0.01^{a}	0.07±0.00^{a}
Negative Control	0.92±0.09^{d}	0.40±0.05^{d}	0.48±0.04^{d}	0.01±0.00^{d}	0.01±0.00^{d}	0.01±0.00^{d}
Levamisole	6.02±0.18^{a}	2.62±0.11^{a}	2.82±0.06^{a}	0.31±0.02^{ab}	0.20±0.01^{a}	0.07±0.00^{a}
TM + CM (50mg/kg bw)	3.10±0.20^{c}	1.40±0.12^{c}	1.48±0.08^{c}	0.13±0.00^{c}	0.06±0.00^{c}	0.03±0.00^{c}
TM + CM (100mg/kg bw)	4.74±0.10^{b}	1.20±0.04^{b}	2.32±0.08^{b}	0.25±0.02^{b}	0.14±0.00^{b}	0.05±0.00^{b}
TM + CM (150mg/kg bw)	6.30±0.18^{a}	2.71±0.09^{a}	2.92±0.07^{a}	0.31±0.03^{a}	0.20±0.01^{a}	0.08±0.00^{a}

Values were expressed as Mean ± SEM for the five mice in each group. Statistical comparison was done within a column and values with a similar superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p > 0.05$). TM = *T. brownii* methanol extract; CM = *C. edulis* methanol extract. Normal control group had received the vehicle; Negative control group had received the immunosuppressant; Levamisole group (positive control) had received the immunosuppressant and levamisole.

4.2.1.6 Comparison of the Effect of all the Extracts on Total and Differential Leukocyte Counts

The current study compared the effects of all the studied extracts on total and differential leukocyte counts at each tested dosage. Overall, the combined extract (S) showed the highest activity with regard to total and differential leukocyte counts increment in mice. This was followed by *T. brownii* methanol extract (TM), *C. edulis* methanol extract (CM), *T. brownii* DCM extract (TD), and lastly, *C. edulis* DCM extract (CD).

All the extracts, at 50 mg/kg bw, resulted in the production of similar ($p > 0.05$) total and differential leukocyte counts in mice.

Mice treated with *T. brownii* methanol extract (TM) and *T. brownii* DCM extract (TD), at 100 and 150mg/kg bw, produced significantly different ($p < 0.05$) WBCs, neutrophils, lymphocytes, monocytes, and eosinophils counts (Figure 4.1). However, there was no significant difference ($p > 0.05$) in basophil counts produced by mice treated with TM and TD extracts at 100 and 150mg/kg bw dosages (Figure 4.1).

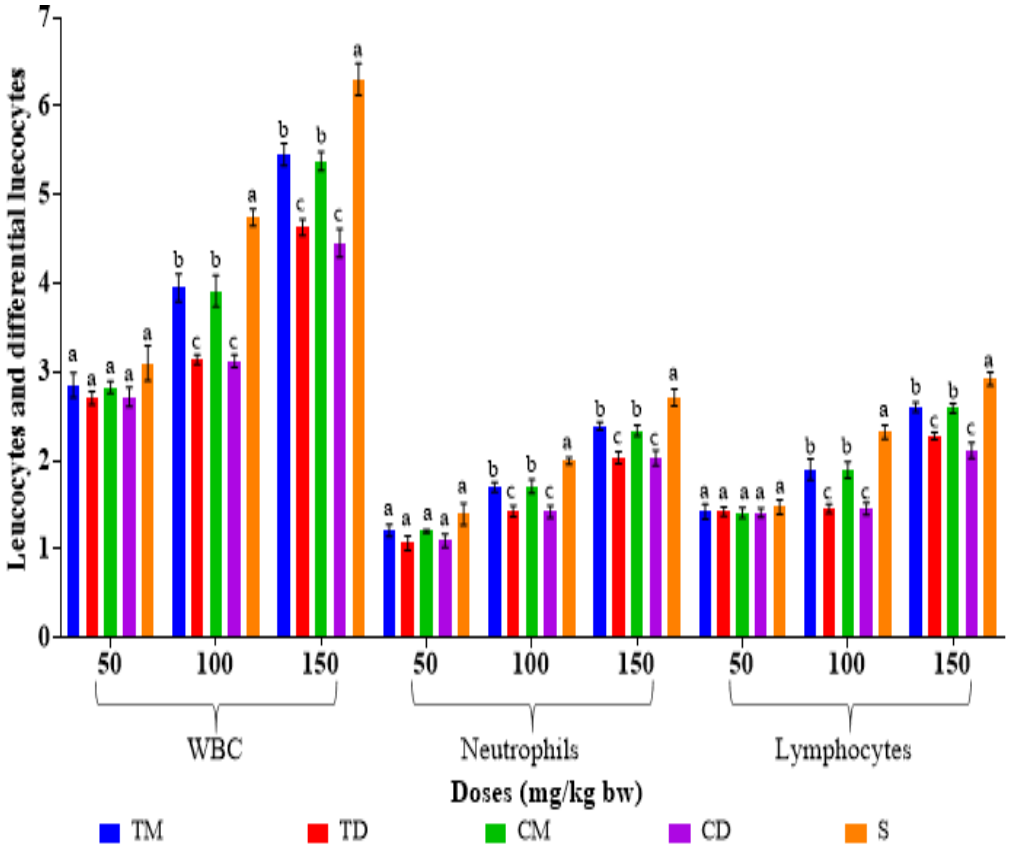
Similarly, at 100 and 150mg/kg bw, a significant difference ($p < 0.05$) was noted in the numbers of WBCs, neutrophils, lymphocytes, and eosinophils produced by mice treated with *C. edulis* methanol extract (CM) and *C. edulis* DCM extract (CD) (Figure 4.1). No significant difference ($p > 0.05$) was observed in monocyte counts produced by the mice treated with CM and CD extracts at 100mg/kg bw. However, there was a significant difference ($p < 0.05$) in monocyte numbers produced by CM and CD extracts-treated mice at 150mg/kg bw. With regards to basophils counts, CM and CD extracts had comparable ($p > 0.05$) effects at dosages 100 and 150mg/kg bw (Figure 4.1).

Mice administered with *C. edulis* methanol extract (CM) and *T. brownii* methanol extract (TM) at 100 and 150mg/kg bw showed similar ($p > 0.05$) WBCs, neutrophils, lymphocytes, monocytes, and eosinophils counts (Figure 4.1). However, there was no significant difference in basophil counts produced by mice treated with CM and TM at 100 and 150mg/kg bw

At 100 and 150mg/kg bw, *C. edulis* DCM extract (CD) and *T. brownii* DCM extract (TD) produced comparable ($p > 0.05$) WBCs, neutrophils, lymphocytes, monocytes, and eosinophils counts (Figure 4.1). However, there was no significant difference ($p > 0.05$) in basophil counts produced by mice treated with CD and TD extracts at 100 and 150mg/kg bw dosages (Figure 4.1).

Mice that received the combined extract (S), at 100 and 150mg/kg bw dosages, produced significantly higher ($p < 0.05$) total and differential leukocyte counts compared with mice treated with individual plant extracts at the two dosages (Figure 4.1).

A



B

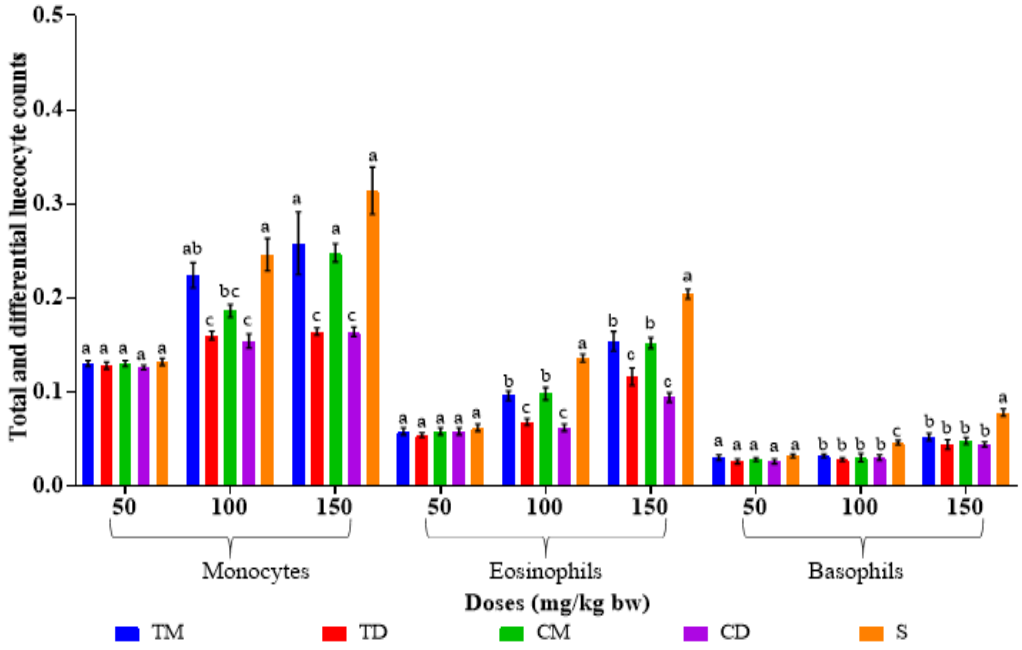


Figure 4.1A: Comparison of the Effect of all the Extracts on Wbcs, Neutrophils and Lymphocytes Counts in Mice. B: Comparison of the Effect of all the Extracts on Monocytes, Eosinophils and Basophils Counts in Mice. Bars with the same lowercase letter are not significantly different in each dose dosage ($p>0.05$). TM = T. brownii stem bark methanol extract; TD = T. brownii stem bark DCM extract; CM = C. edulis root bark methanol extract; CD = C. edulis root bark DCM extract; S = Synergy; combined methanol extracts of T. brownii stem bark and C. edulis methanol root bark.

4.2.2 Effect of *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol and DCM Extracts on Production of NO by Mouse Peritoneal Macrophages

Methanol and DCM extracts of *T. brownii* and *C. edulis* were investigated for their ability to elicit the production of NO by mouse macrophages. Prior to macrophages culture with the plant extracts, the effect of the extracts on Vero cells viability was investigated. The extracts were found not to affect the viability of Vero cells at all the tested concentrations. Overall, control cells produced low levels of NO. However, high levels of NO were noted in macrophages treated with the tested extracts and levamisole.

4.2.2.1 Effect of *T. brownii* Stem Bark Methanol and DCM Extracts on Vero Cells Viability

The present study investigated the effect of *T. brownii* methanol and DCM extracts on the viability of Vero cells. At all the tested concentrations, methanol and DCM extracts of *T. brownii* maintained above 50% viability of Vero cells (Table 4.6). The highest viability was observed at the lowest extracts' concentration (46.88µg/ml) whereas the lowest viability was noted at the highest concentration of the extracts (3000µg/ml) (Table 4.6). Increasing the concentration of the extracts reduced Vero cells viability (Table 4.6).

Significant difference ($p < 0.05$) was observed between the viability of extract-treated cells and control cells. Additionally, there was a significant difference ($p < 0.05$) in the viability of Vero cells between all the tested concentrations of *T. brownii* methanol and DCM extracts (Table 4.6). In comparison, the DCM extract of *T. brownii* resulted in significantly higher ($p < 0.05$) viability of Vero cells compared with the methanol extract at all the tested concentrations (Table 4.6).

Table 4.6: Effects of *T. brownii* Stem Bark Methanol and DCM Extracts on Vero Cells Viability

Group	% Viability	
	Methanol	DCM
Control cells	99.64 ± 0.11^{a}	99.64 ± 0.11^{a}
T. brownii (46.88µg/ml)	91.09 ± 0.32^{bA}	93.94 ± 0.26^{bB}
T. brownii (93.75µg/ml)	88.85 ± 0.30^{cA}	92.24 ± 0.32^{cB}
T. brownii (187.5µg/ml	85.70 ± 0.37^{dA}	88.61 ± 0.26^{dB}
T. brownii (375µg/ml)	80.06 ± 0.32^{eA}	83.27 ± 0.42^{eB}
T. brownii (750µg/ml)	68.61 ± 0.75^{fA}	72.35 ± 0.09^{fB}
T. brownii (1500µg/ml)	60.00 ± 0.56^{gA}	64.79 ± 0.26^{gB}
T. brownii (3000µg/ml)	54.85 ± 0.37^{hA}	60.18 ± 0.21^{hB}

Values were expressed as Mean ± SEM. Values with a similar lowercase superscript in each and similar uppercase superscript in each row are not significantly different by one-way ANOVA followed by Tukey's post hoc test and unpaired t-test, respectively ($p > 0.05$). The control cells had been treated with the vehicle.

4.2.2.2 Effect of *C. edulis* Root Bark Methanol and DCM Extracts on Vero Cells Viability

The findings of this study revealed that over 50% of Vero cell treated with DCM and methanol extracts of *C. edulis* root bark were viable at all the tested concentrations (Table 4.7). The highest viability was noted at the lowest extract concentration (46.88µg/ml) of the extracts after which it gradually reduced as the concentration increased.

The tested concentrations of these extracts resulted in significantly different ($p < 0.05$; Table 4.7) levels of cell viability. Besides, at all the tested concentrations, the viability of extracts-treated Vero cells was significantly different ($p < 0.05$; Table 4.7) from the control cells. *Carissa edulis* root bark DCM and methanol extracts showed comparable ($p > 0.05$) viability of Vero cells at all the tested concentrations (Table 4.7).

Table 4.7: Effects of *C. edulis* Root Bark Methanol and DCM Extracts on Vero Cells Viability

Group	**% Viability**	
	Methanol	**DCM**
Control cells	99.64 ± 0.11^{a}	99.64 ± 0.11^{a}
C. edulis (46.88µg/ml)	92.97 ± 0.37^{bA}	94.30 ± 0.37^{bA}
C. edulis (93.75µg/ml)	90.55 ± 0.28^{cA}	92.49 ± 0.42^{cA}
C. edulis (187.5µg/ml	87.94 ± 0.42^{dA}	88.52 ± 0.39^{dA}
C. edulis (375µg/ml)	81.64 ± 0.42^{eA}	83.39 ± 0.58^{eA}
C. edulis (750µg/ml)	73.33 ± 0.16^{fA}	73.03 ± 0.32^{fA}
C. edulis (1500µg/ml)	63.03 ± 0.16^{gA}	65.21 ± 0.68^{gA}
C. edulis (3000µg/ml)	59.70 ± 0.47^{hA}	59.82 ± 0.63^{hA}

Values were expressed as Mean ± SEM. Values with a similar lowercase superscript in each and similar uppercase superscript in each row are not significantly different by one-way ANOVA followed by Tukey's post hoc test and unpaired t-test, respectively ($p > 0.05$). The control cells had been treated with the vehicle.

4.2.2.3 Effect of the Combined Methanol Extracts *C. edulis* and *T. brownii* on Vero Cells Viability

At all the tested concentrations, Vero cells treated with the combined methanol extracts of *C. edulis* and *T. brownii* (1:1) showed over 50% viability. Cell viability was concentration-dependent whereby, the lowest viability was observed in cells treated with the highest concentration (3000µg/ml), and the highest viability was noted in cells treated with the lowest concentration (46.88µg/ml) of the combined extract.

The viability of Vero cells was significantly different ($p < 0.05$) between all the tested concentrations of the combined extract. Moreover, there was a significant difference ($p < 0.05$) between the viability of extract-treated cells and control cells (Table 4.8).

Table 4.8: Effect of the Combined Methanol Extracts of *T. brownii* Stem Bark and *C. edulis* Root Bark on Vero Cells Viability

Group	% Viability
Control cells	99.64 ± 0.11^{a}
TM + CM (46.88µg/ml)	91.39 ± 0.26^{b}
TM + CM (93.75µg/ml)	89.21 ± 0.42^{b}
TM + CM (187.5µg/ml	84.00 ± 0.46^{c}
TM + CM (375µg/ml)	81.88 ± 0.37^{c}
TM + CM (750µg/ml)	68.55 ± 1.00^{d}
TM + CM (1500µg/ml)	61.39 ± 0.98^{e}
TM + CM (3000µg/ml)	54.85 ± 0.53^{f}

Values were expressed as Mean ± SEM. Statistical comparison was done within a column and values with a similar superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p > 0.05$). TM = *T. brownii* methanol extract; CM = *C. edulis* methanol extract. The control cells had been treated with the vehicle.

4.2.2.4 Effect of *T. brownii* Methanol and DCM Extracts on Production of NO by Mouse Peritoneal Macrophages

Significantly different ($p < 0.05$) dose-dependent increment in NO levels was noted after macrophages were treated with methanol and DCM extracts of *T. brownii*. Additionally, all the extract-treated macrophages produced significantly higher ($p < 0.05$) levels of NO compared with the unstimulated cells (Table 4.9).

No significant difference ($p > 0.05$; Table 4.9) in NO levels was observed in cells treated with methanol extract of *T. brownii* at 3000 µg/ml concentration and levamisole-treated cells. On the other hand, macrophages treated with *T. brownii* stem bark DCM extract, at all the tested concentrations, produced significantly lower ($p < 0.05$) levels of NO compared with levamisole-treated macrophages (positive control) (Table 4.9).

Macrophages treated with *T. brownii* methanol extract produced significantly higher ($p < 0.05$) levels of NO in comparison with macrophages treated with *T. brownii* DCM extract at all tested concentrations (Table 4.9).

Table 4.9: Effect of Methanol and DCM Stem Bark Extracts of *T. brownii* on NO Production

Group	Nitrite Concentration (µM)	
	Methanol	**DCM**
Control cells	2.23 ± 0.24^{h}	2.23 ± 0.24^{g}
Levamisole (1µg/ml)	53.46 ± 0.54^{a}	53.46 ± 0.54^{a}
T. brownii (46.88µg/ml)	11.04 ± 0.24^{gA}	2.41 ± 0.21^{gB}
T. brownii (93.75µg/ml)	13.62 ± 0.17^{fA}	4.07 ± 0.47^{fgB}
T. brownii (187.5µg/ml)	18.83 ± 0.43^{eA}	5.75 ± 0.45^{fB}
T. brownii (375µg/ml)	24.65 ± 0.33^{dA}	10.01 ± 0.36^{eB}
T. brownii (750µg/ml)	31.78 ± 0.28^{cA}	15.55 ± 0.76^{dB}
T. brownii (1500µg/ml)	45.92 ± 0.36^{bA}	22.43 ± 0.76^{cB}
T. brownii (3000µg/ml)	54.73 ± 0.85^{aA}	37.72 ± 0.59^{bB}

Values were expressed as Mean ± SEM. Values with a similar lowercase superscript in each and similar uppercase superscript in each row are not significantly different

by one-way ANOVA followed by Tukey's post hoc test and unpaired t-test, respectively ($p > 0.05$). The control cells had been treated with the vehicle.

4.2.2.5 Effect of *C. edulis* Methanol and DCM Extracts on Production of NO by Mouse Peritoneal Macrophages

All the tested concentrations of methanol and DCM extracts of *C. edulis* root bark increased the production of NO by macrophages (Table 4.10). Macrophages treated with the two extracts, at all the tested concentrations except at 46.88µg/ml, showed a significant increase ($p < 0.05$; Table 4.10) in NO production compared to the control cells. There was no significant difference ($p > 0.05$) between NO levels produced by macrophages treated with the extracts at 46.88µg/ml concentration and the levels noted in control macrophages (Table 4.10).

Macrophages administered with DCM extracts of *C. edulis* generated a dose-dependent increase of NO. The increase was significantly different ($p < 0.05$) at all the tested concentrations apart from cells treated with 46.88 and 93.75µg/ml concentrations (Table 4.10). On the other hand, levels of NO produced by macrophages treated with the *C. edulis* methanol extract significantly increased ($p < 0.05$) progressively from the lowest to the highest concentration (Table 4.10). Macrophages treated with *C. edulis* methanol and DCM extracts produced significantly lower ($p < 0.05$) levels of NO in contrast to levamisole-treated macrophages (positive control).

Nitric Oxide levels produced by macrophages treated with *C. edulis* methanol extract were comparable ($p > 0.05$) with the levels produced by cells treated with DCM extracts of *C. edulis* at concentrations 46.88µg/ml and 93.75µg/ml. However, NO levels produced by macrophages treated with *C. edulis* methanol extract were significantly higher ($p < 0.05$) than the levels produced by cells treated with DCM extracts of *C. edulis* from concentration 187.5µg/ml to concentration 3000µg/ml (Table 4.10).

Table 4.10: Effect of *C. Edulis* Methanol and DCM Root Bark Extracts on NO Production by Macrophages

Group	Nitrite Concentration (µM)	
	Methanol	**DCM**
Control cells	2.23 ± 0.24^{h}	2.23 ± 0.24^{h}
Levamisole (1µg/ml)	53.46 ± 0.54^{a}	53.46 ± 0.54^{a}
C. edulis (46.88µg/ml)	3.71 ± 0.26^{hA}	2.97 ± 0.26^{ghA}
C. edulis (93.75µg/ml)	6.16 ± 0.45^{gA}	5.02 ± 0.31^{fgA}
C. edulis (187.5µg/ml	9.32 ± 0.60^{fA}	6.53 ± 0.47^{fB}
C. edulis (375µg/ml)	16.41 ± 0.50^{eA}	10.06 ± 0.43^{eB}
C. edulis (750µg/ml)	23.83 ± 0.43^{dA}	15.71 ± 0.50^{dB}
C. edulis (1500µg/ml)	39.69 ± 0.54^{cA}	22.82 ± 0.55^{cB}
C. edulis (3000µg/ml)	45.51 ± 0.50^{bA}	38.58 ± 0.57^{bB}

Values were expressed as Mean ± SEM. Values with a similar lowercase superscript in each and similar uppercase superscript in each row are not significantly different by one-way ANOVA followed by Tukey's post hoc test and unpaired t-test, respectively ($p > 0.05$). The control cells had been treated with the vehicle.

4.2.2.6 Effect of the Combined *T. brownii* and *C. edulis* Methanol Extracts on NO Production by Mouse Peritoneal Macrophages

Terminalia brownii and *C. edulis* methanol extracts were combined in a ratio of 1:1 and their effect on NO production by macrophages was investigated. Cells treated with the combined extract produced significantly higher ($p < 0.05$; Table 4.11) levels of NO compared with the control cells. Macrophages treated with the combined extract at the concentrations of 187.5, 375, 750, 1500, and 3000µg/ml produced significantly differing ($p < 0.05$) levels of NO. However, at the concentrations of 46.88 and 93.75µg/ml, the combined extract stimulated macrophages to produce comparable ($p > 0.05$) levels of NO.

The highest concentration (3000µg/ml) of the combined extract stimulated macrophages to produce significantly higher ($p < 0.05$) levels of NO than those

observed in the cells administered with levamisole (Table 4.11). Macrophages treated with 1500µg/ml concentration of the combined extract and levamisole produced comparable ($p > 0.05$) levels of NO. However, levamisole stimulated macrophages to produce significantly more ($p < 0.05$) NO than the combined extract at all the other tested concentrations (46.88, 93.75, 187.5, 375, and 750 µg/ml) (Table 4.11).

Table 4.11: Effect of the Combined Methanol Extracts of *T. brownii* Stem Bark and *C. edulis*

Group	Nitrite Concentration (µM)
Control cells	2.23 ± 0.24^{g}
Levamisole (1µg/ml)	53.46 ± 0.54^{b}
TM + CM (46.88µg/ml)	13.75 ± 0.33^{f}
TM + CM (93.75µg/ml)	15.80 ± 0.38^{f}
TM + CM (187.5µg/ml)	20.39 ± 0.43^{e}
TM + CM (375µg/ml)	28.09 ± 1.42^{d}
TM + CM (750µg/ml)	38.99 ± 0.76^{c}
TM + CM (1500µg/ml)	55.43 ± 0.83^{b}
TM + CM (3000µg/ml)	65.51 ± 1.59^{a}

Values were expressed as Mean ± SEM for the triplicates in each group. Statistical comparison was done within a column and values with a similar superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p > 0.05$). TM = *T. brownii* methanol extract; CM = *C. edulis* methanol extract. The control cells had been treated with the vehicle.

4.2.2.7 Comparison of the Effect of all the Extracts NO production by Mouse Macrophages

In the present study, the effect of all the tested plant extracts and the combined extract (S) on NO production by macrophages was compared. The combined extract (S) stimulated macrophages to produce significantly higher ($p < 0.05$) levels of NO compared with all the other extracts, at all the tested concentrations apart from

187.5µg/ml concentration (Figure 4.2). At 187.5µg/ml, combined extract (S) elicited macrophages to produce comparable ($p > 0.05$) NO levels to those produced by cells treated with *T. brownii* methanol extract (TM). However, NO levels produced by the combined extract at 187.5µg/ml concentration were significantly higher ($p < 0.05$) than NO generated by macrophages treated with all the other extracts at the same concentration.

Methanol extract of *T. brownii* (TM) showed the second highest activity with regards to stimulation of macrophages to produce NO. The extract evoked macrophages to generate significantly different ($p < 0.05$) NO amounts compared with the cells treated with all the other extracts, at all the tested concentrations except at 187.5µg/ml (Figure 4.2).

Macrophages treated with methanol extract of *C. edulis* (CM) produced the third highest levels of NO compared with all the other tested extracts. Nitric Oxide levels produced by macrophages treated with this extract were significantly different ($p < 0.05$) from the levels noted in cells treated with the other extracts at concentrations 187.5, 375, 750, 1500, and 3000µg/ml (Figure 4.2). However, at the concentrations of 46.88 and 93.75µg/ml, *C. edulis* methanol (CM) and DCM (CD) extracts produced comparable ($p > 0.05$) NO levels (Figure 4.2).

The fourth highest amount of NO was exhibited by macrophages treated with DCM extract of *C. edulis* (CD). Macrophages treated with this extract produced comparable ($p > 0.05$) NO levels with cells treated with DCM extracts of *T. brownii* (TD) at all the tested concentrations (Figure 4.2). The results indicated that macrophages treated with DCM extracts of *T. brownii* (TD) stem bark produced the lowest levels of NO at all the investigated extract concentrations.

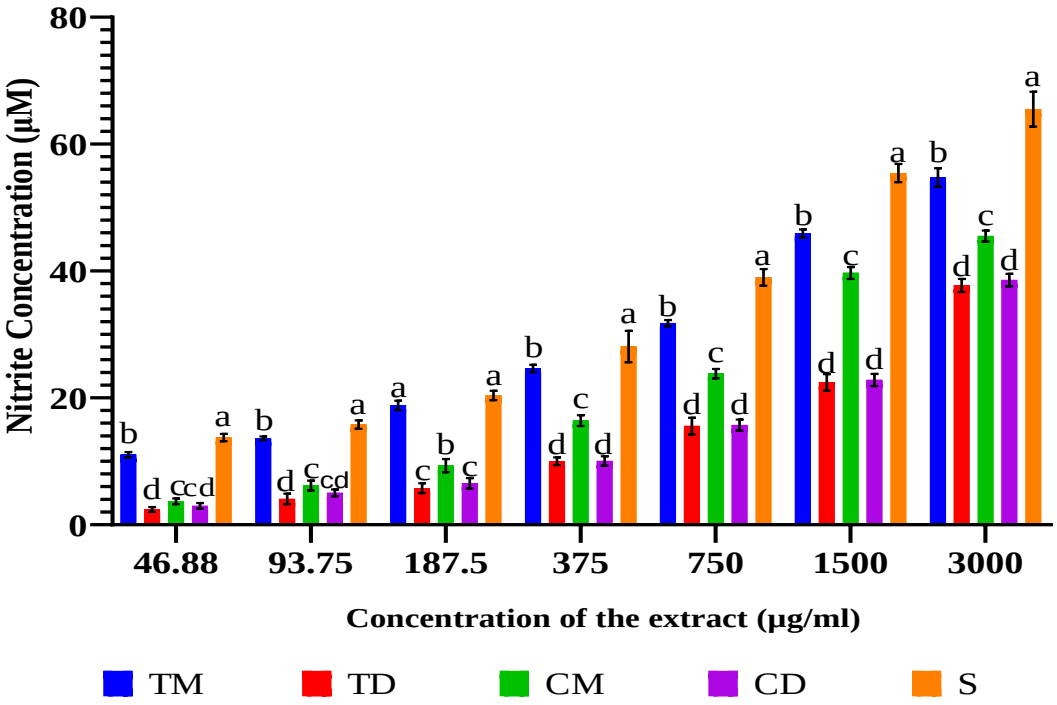


Figure 4.2: Comparison of Effects of all the Extracts on NO Production by Macrophages. Bars with the same lowercase letter are not significantly different in each concentration (p>0.05). TM = *T. brownii* methanol stem bark extract; TD = *T. brownii* DCM stem bark extract; CM = *C. edulis* methanol root bark extract; CD = *C. edulis* DCM root bark extract; S = Synergy; combined methanol extracts of *T. brownii* stem bark and *C. edulis* methanol root bark.

4.2.3 Effect of *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol and DCM Extracts on TNF-α Production by Mouse Peritoneal Macrophages

The ability of methanol and DCM extracts of *C. edulis* and *T. brownii* to elicit the production of TNF- α by macrophages was also investigated. The extracts, at all the tested concentrations, were found to augment the production of TNF-α by mouse macrophages.

4.2.3.1 Effect of *T. brownii* Methanol and DCM Extracts on TNF-α Production by Macrophages

Macrophages treated with methanol extract of *T. brownii*, at all the tested concentrations, significantly increased ($p < 0.05$) TNF-α levels compared with the control cells (Table 4.12). The increase was found to be dose-dependent. At the highest concentration, 3000µg/ml, methanol extract of *T. brownii* induced macrophages to produce comparable ($p > 0.05$) levels of TNF-α with levamisole (Table 4.12). However, at all the other tested concentrations, the extract produced significantly lower ($p < 0.05$) levels of TNF-α compared with levamisole (Table 4.12).

Macrophages treated with *T. brownii* DCM extract, at all the tested concentrations, produced significantly higher ($p < 0.05$) levels of TNF-α than the unstimulated macrophages (Table 4.12). *Terminalia brownii* DCM extract, from concentration 187.5 to 3000µg/ml, stimulated macrophages to produce significantly different ($p < 0.05$) levels of TNF-α (Table 4.12). However, there was no significant difference ($p > 0.05$) in the amounts of TNF-α produced by macrophages treated with the extract at concentrations 46.88 and 93.75µg/ml (Table 4.12). Cells treated with DCM extract of *T. brownii*, at all the tested concentrations, produced significantly lower TNF-α ($p < 0.05$) levels compared with levamisole-treated cells (Table 4.12).

Terminalia brownii methanol extract stimulated macrophages to produce significantly ($p < 0.05$) more TNF-α than *T. brownii* DCM extract at all the tested concentrations (Table 4.12).

Table 4.12: Effects of *T. brownii* Stem Bark Extract on TNF-α Production by Macrophages

Group	TNF-α Concentration (pg/ml)	
	Methanol	**DCM**
Control cells	97.3 ± 18.0^{h}	97.3 ± 18.0^{h}
Levamisole (1µg/ml)	2855.7 ± 23.8^{a}	2855.7 ± 23.8^{a}
T. brownii (46.88µg/ml)	1313.0 ± 31.6^{gA}	915.0 ± 33.4^{gB}
T. brownii (93.75µg/ml)	1553.0 ± 29.7^{fA}	1042.0 ± 23.4^{gB}
T. brownii (187.5µg/ml)	1737.3 ± 22.6^{eA}	1294.7 ± 20.5^{fB}
T. brownii (375µg/ml)	2114.7 ± 60.6^{dA}	1499.0 ± 9.45^{eB}
T. brownii (750µg/ml)	2364.0 ± 30.9^{cA}	1765.7 ± 36.4^{dB}
T. brownii (1500µg/ml)	2653.0 ± 34.1^{bA}	2006.7 ± 36.5^{cB}
T. brownii (3000µg/ml)	2853.7 ± 27.6^{aA}	2388.0 ± 35.2^{bB}

Values were expressed as Mean ± SEM. Values with a similar lowercase superscript in each and similar uppercase superscript in each row are not significantly different by one-way ANOVA followed by Tukey's post hoc test and unpaired t-test, respectively ($p > 0.05$). The control cells had been treated with the vehicle.

4.2.3.2 Effect of *C. edulis* Methanol and DCM Extracts on TNF-α Production by Macrophages

Herein, the effects of methanol and DCM extract of *C. edulis* on TNF-α production by macrophages are reported. The two extracts of *C. edulis* stimulated macrophages to produce significantly higher ($p < 0.05$) levels of TNF-α compared with the control cells. *Carissa edulis* methanol and DCM extracts, at all the tested concentrations, elicited macrophages to produce significantly lower ($p < 0.05$) levels of TNF-α compared with levamisole-treated cells.

Carissa edulis methanol extract resulted in a dose-dependent increase in TNF-α levels (Table 4.13). Also, *C. edulis* DCM extract stimulated macrophages to produce TNF-α in a dose-dependent manner. However, the activity significantly varied ($p < 0.05$) only from concentration 187.5 to 3000 µg/ml (Table 4.13).

In comparison, macrophages treated with *C. edulis* methanol extract produced significantly higher ($p < 0.05$) amounts of TNF-α compared with cells treated with DCM extract of *C. edulis* at all the tested concentrations (Table 4.13).

Table 4.13: Effect of *C. edulis* Methanol and DCM Root Extracts on TNF-α Production by Macrophages

Group	TNF-α Concentration (pg/ml)	
	Methanol	**DCM**
Control cells	97.3 ± 18.0^{i}	97.3 ± 18.0^{h}
Levamisole (1µg/ml)	2855.7 ± 23.8^{a}	2855.7 ± 23.8^{a}
C. edulis (46.88µg/ml)	1200.0 ± 27.5^{hA}	847.7 ± 27.2^{gB}
C. edulis (93.75µg/ml)	1328.3 ± 34.0^{gA}	905.7 ± 32.4^{fgB}
C. edulis (187.5µg/ml	1535.0 ± 28.0^{fA}	1040.0 ± 31.2^{fB}
C. edulis (375µg/ml)	1778.3 ± 9.56^{eA}	1298.3 ± 28.6^{eB}
C. edulis (750µg/ml)	2051.3 ± 24.7^{dA}	1540.3 ± 11.7^{dB}
C. edulis (1500µg/ml)	2391.0 ± 15.0^{cA}	1850.3 ± 36.3^{cB}
C. edulis (3000µg/ml)	2645.7 ± 31.2^{bA}	2177.7 ± 35.4^{bB}

Values were expressed as Mean ± SEM. Values with a similar lowercase superscript in each and similar uppercase superscript in each row are not significantly different by one-way ANOVA followed by Tukey's post hoc test and unpaired t-test, respectively ($p > 0.05$). The control cells had been treated with the vehicle.

4.2.3.3 Effect of the Combined *T. brownii* and *C. edulis* Methanol Extracts on TNF-α production by mouse macrophages

The effect of the combined *T. brownii* and *C. edulis* methanol extracts (1:1) on TNF-α production by macrophages was also investigated. The combined extract, at all the tested concentrations, significantly increased ($p < 0.05$) TNF-α production by macrophages compared with the control cells (Table 4.14). The increase was found to be dose-dependent. Macrophages treated with 1500µg/ml of the combined extract and levamisole produced comparable ($p > 0.05$; Table 4.14) levels of TNF-α. The highest concentration of the combined extract, 3000µg/ml, stimulated macrophages

to produce significantly higher ($p < 0.05$) levels of TNF-α compared with levamisole (Table 4.14). Macrophages treated with other concentrations of the combined extract generated significantly less ($p < 0.05$; Table 4.14) amounts of TNF-α compared with levamisole-treated cells.

Table 4.14: Effect of the Combined Methanol Extracts of *T. brownii* Stem Bark and *C. edulis* Root Bark on TNF-α by Macrophages

Group	TNF-α Concentration (pg/ml)
Control cells	97.3 ± 18.0^{h}
Levamisole (1µg/ml)	2855.7 ± 23.8^{b}
TM + CM (46.88µg/ml)	1752.0 ± 25.4^{g}
TM + CM (93.75µg/ml)	1956.7 ± 23.2^{f}
TM + CM (187.5µg/ml)	2253.0 ± 31.2^{e}
TM + CM (375µg/ml)	2458.3 ± 28.4^{d}
TM + CM (750µg/ml)	2646.0 ± 34.4^{c}
TM + CM (1500µg/ml)	2861.0 ± 27.8^{b}
TM + CM (3000µg/ml)	3062.3 ± 28.7^{a}

Values were expressed as Mean ± SEM for the triplicates in each group. Statistical comparison was done within a column and values with a similar superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p > 0.05$). TM = *T. brownii* methanol extract; CM = *C. edulis* methanol extract. The control cells had been treated with the vehicle.

4.2.3.4 Comparison of the Effect of all the Extracts on TNF-α Production by Mouse Macrophages

In the contemporary inquiry, the effect of all the extracts on the production of TNF-α by macrophages was compared (Figure 4.3). The combined methanol extracts of *T. brownii* and *C. edulis* (S) induced macrophages to produce the highest levels of TNF-α compared with all the other extracts at all the tested concentrations (Figure 4.3). This was followed by *T. brownii* methanol extract (TM), *C. edulis* methanol extract (CM), *T. brownii* DCM extract (TD), and lastly, *C. edulis* DCM extract (CD).

Macrophages treated with methanol extract of *T. brownii* (TM) produced significantly different ($p < 0.05$) levels of TNF-α compared with the cells treated with the other extracts at all the tested concentrations apart from the concentration of 46.88µg/ml (Figure 4.3). At this concentration (46.88µg/ml) cells treated with methanol extracts of *T. brownii* (TM) and *C. edulis* (CM) produced comparable ($p > 0.05$) amounts of TNF-α (Figure 4.3).

Methanol extract of *C. edulis* (CM) stimulated macrophages to produce TNF-α levels which significantly differed ($p < 0.05$) from the levels produced by cells treated with all the other extracts from the concentration of 93.75µg/ml to 3000µg/ml (Figure 4.3). At 46.88µg/ml, the extract-induced macrophages generated significantly higher ($p < 0.05$) TNF-α levels than the cells treated with DCM extracts of *C. edulis* (CD) and *T. brownii* extract (TD) (Figure 4.3).

Macrophages treated with DCM extract of *T. brownii* (TD) produced significantly different ($p < 0.05$) levels of TNF-α compared with the other tested extracts at all the concentrations except at the concentration of 46.88µg/ml (Figure 4.3). At 46.88µg/ml, macrophages that received DCM extracts of *T. brownii* (TD) and *C. edulis* (CD) generated similar ($p > 0.05$; Figure 4.3) levels of TNF-α. All the tested concentrations of DCM extract of *C. edulis* (CD) induced macrophages to produce significantly different ($p < 0.05$) TNF-α levels compared with the other tested extracts from the dosage of 93.75µg/ml to 3000µg/ml (Figure 4.3).

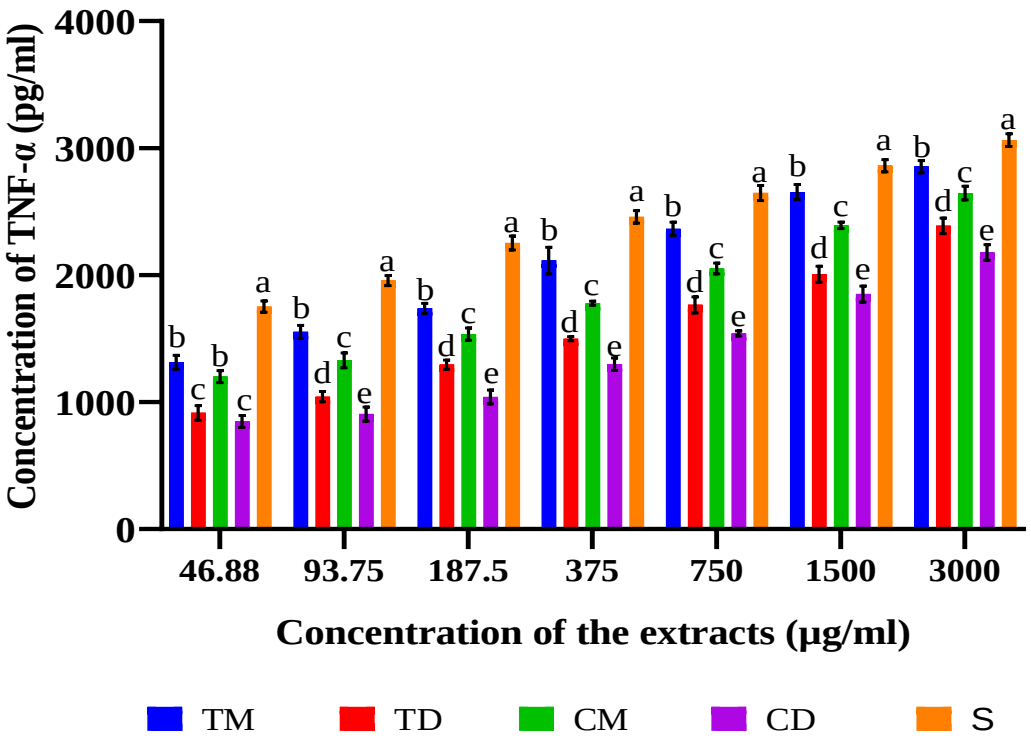


Figure 4.3: Comparison of Effects of all the Extracts on TNF-a Production by Macrophages. Bars with the same lowercase letter are not significantly different in each dose dosage ($p>0.05$). TM = T. brownii methanol stem bark extract; TD = T. brownii DCM stem bark extract; CM = C. edulis methanol root bark extract; CD = C. edulis DCM root bark extract; S = Synergy; Combined methanol extracts of T. brownii stem bark and C. edulis root bark.

4.3 Effect of *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol and DCM Extracts on Cellular and Humoral Immune Responses

The present study investigated the effects of *C. edulis* and *T. brownii* extracts on cellular and humoral immune responses. This was done through the assessment of the levels of Th1 cytokines

(IL-2, TNF-α, and IFN-γ) and Th2 cytokines (IL-4 and IL-5) in mice serum, DTH reaction, and antibody titer levels. The extracts increased IL-2, TNF-α, and IFN-γ,

however, they decreased IL-4 and IL-5. Additionally, the extracts augmented antibody titer levels and DTH reaction in pyrogallol-immunosuppressed mice.

4.3.1 Effect of *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol and DCM Extracts on Cellular Immune Responses

4.3.1.1 Effect of *T. brownii* Stem Bark Methanol Extract on IL-2, TNF-α, IFN-γ, IL-4, and IL-5

Overall, *T. brownii* methanol extract induced a stimulatory effect on IL-2, TNF-α, and IFN-γ and an inhibitory effect on IL-4 and IL-5 in pyrogallol-immunosuppressed mice. Pyrogallol significantly reduced ($p < 0.5$) levels of IL-2, TNF-α, and IFN-γ as shown by the mice in the negative control group compared with the normal control mice. However, this decline was reversed by the methanol extract of *T. brownii*. As such, at all the tested doses, mice that received *T. brownii* methanol extract showed significantly enhanced ($p < 0.5$) levels of IL-2, TNF-α, and IFN-γ compared with the negative control mice (Table 4.15). At all the administered doses of *T. brownii* methanol extract, mice produced significantly lower ($p < 0.5$) levels of IL-2, TNF-α, and IFN-γ compared with levels noted in the positive control mice (Table 4.15).

Methanol extract of *T. brownii* induced a dose-dependent increase of IL-2, TNF-α, and IFN-γ. The administered extract doses stimulated mice to produce significantly distinct ($p < 0.05$) levels of IL-2 and IFN-γ cytokines (Table 4.15). However, mice administered with 100 and 150 mg/kg bw of the extract produced comparable ($p > 0.05$) levels of TNF-α (Table 4.15).

Pyrogallol significantly elevated ($p < 0.05$) levels of IL-4 and IL-5 compared with the normal control mice (Table 4.15). However, *T. brownii* methanol extract significantly reduced ($p < 0.05$) levels of IL-4 and IL-5 compared with the negative control. Extract-treated animals showed significantly ($p < 0.05$) higher levels of IL-4 and IL-5 in comparison with levamisole-treated animals (positive control) (Table 4.15). A concentration-dependent decrease in IL-4 and IL-5 levels was noted in mice administered with methanol extract of *T. brownii* and the levels significantly varied ($p < 0.5$) between the tested doses.

Table 4.15: Effect of *T. brownii* Stem Bark Methanol Extract on Th1 and Th2 Cytokines

Groups	Concentration (pg/ml)				
	Th1 Cytokines			Th2 Cytokines	
	IL-2	TNF-α	IFN-γ	IL-4	IL-5
Normal control	571.80±4.53[a]	98.60±5.01[a]	394.00±7.80[a]	140.60±7.57[e]	104.00±7.26[e]
Negative control	318.20±4.47[e]	33.20±3.76[c]	191.60±5.89[e]	307.80±4.07[a]	219.60±3.83[a]
Levamisole (20 mg/kg bw)	561.20±4.16[a]	97.00±4.64[a]	387.80±4.41[ab]	145.60±5.81[e]	109.00±4.48[e]
TM (50mg/kg bw)	473.60±6.00[d]	33.40±6.06[c]	306.80±4.40[c]	224.40±4.53[b]	188.60±6.80[b]
TM (100mg/kg bw)	503.60±6.00[c]	60.80±9.91[b]	332.60±4.37[d]	197.40±4.03[c]	159.40±5.19[c]
TM (150 mg/kg bw)	536.20±7.66[b]	70.00±4.60[b]	366.00±4.46[b]	169.40±4.34[d]	133.40±4.27[d]

Values were expressed as Mean ± SEM for the five mice in each group. Statistical comparison was done within a column and values with a similar superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p > 0.05$). TM = *T. brownii* methanol extract. Normal control group had received the vehicle; Negative control had received the immunosuppressant.

4.3.1.2 Effect of *T. brownii* Stem Bark DCM Extract on IL-2, TNF-α, IFN-γ, IL-4, and IL-5

The immunosuppressant, pyrogallol, significantly reduced ($p < 0.05$) the production of IL-2, TNF-α, and IFN-γ compared with the normal control. However, mice administered with DCM extract of *T. brownii* stem bark, at all tested doses, produced significantly enhanced ($p < 0.05$) levels of IL-2, TNF-α, and IFN-γ compared with levels noted in the negative control mice. Levels of IL-2, TNF-α, and IFN-γ observed in extract-treated mice were significantly ($p < 0.05$) lower than those exhibited by positive control mice (Table 4.16).

Increasing extract dosage progressively elevated levels of IL-2, TNF-α, and IFN-γ. Levels of IL-2 and IFN-γ produced by mice administered with DCM extract of *T. brownii* were significantly different ($p < 0.05$) between the tested dosages. However, at all the tested doses, extract-treated mice produced comparable ($p > 0.05$) levels of TNF-α.

Levels of IL-4 and IL-5 were significantly elevated ($p < 0.05$) in negative control mice compared with the levels noted in the normal control mice. However, mice administered with DCM extract of *T. brownii*, at all the tested doses, exhibited significantly attenuated ($p < 0.05$) levels of IL-4 compared with the levels noted in the negative control mice. However, IL-5 levels were not significantly different ($p > 0.05$) between mice treated with the lowest dose of the extract (50mg/kg bw) and negative control mice (Table 4.16). On the other hand, 100 and 150 mg/kg bw of the extracts stimulated mice to produce significantly reduced ($p < 0.05$) levels of IL-5 compared with the levels produced by the negative control mice. Interleukin-4 and IL-5 levels noted in all extract-treated mice were significantly lower ($p < 0.05$) that the levels observed in positive control mice (Table 4.16).

Table 4.16: Effect of *T. brownii* Stem Bark DCM Extract on Th1 and Th2 Cytokines

Groups	Concentration (pg/ml)				
	Th1 Cytokines			Th2 Cytokines	
	IL-2	TNF-α	IFN-γ	IL-4	IL-5
Normal control	571.80±4.53^{a}	98.60±5.01^{a}	394.00±7.80^{a}	140.60±7.57^{e}	104.00±7.26^{d}
Negative control	318.20±4.47^{e}	33.20±1.91^{c}	191.60±5.89^{e}	307.80±4.07^{a}	219.60±3.83^{a}
Levamisole (20 mg/kg bw)	561.20±4.16^{a}	97.00±4.64^{a}	387.80±4.41^{a}	145.60±5.81^{e}	109.00±4.48^{d}
TD (50mg/kg bw)	440.80±3.60^{d}	33.00±3.16^{c}	262.80±3.88^{d}	267.00±4.66^{b}	206.00±3.67^{ab}
TD (100mg/kg bw)	474.20±4.19^{c}	44.00±1.58^{bc}	294.60±3.39^{c}	234.40±4.95^{c}	191.00±5.91^{b}
TD (150 mg/kg bw)	498.60±3.34^{b}	53.60±1.36^{b}	328.80±5.54^{b}	208.00±4.09^{d}	163.60±6.04^{c}

Values were expressed as Mean ± SEM for the five mice in each group. Statistical comparison was done within a column and values with a similar superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p > 0.05$). TD = *T. brownii* DCM extract. Normal control group had received the vehicle; Negative control had received the immunosuppressant.

4.3.1.3 Effect of *C. edulis* Root Bark Methanol Extract on IL-2, TNF-α, IFN-γ, IL-4, and IL-5

Levels of IL-2, TNF-α, and IFN-γ were significantly reduced ($p < 0.05$) in the negative control mice compared with the normal control mice. However, *C. edulis* methanol extract significantly increased ($p < 0.05$) levels of IL-2, TNF-α, and IFN-γ in extract-treated mice compared with the levels produced by the negative control mice. The increase was found to be dose-dependent for IL-2 and IFN-γ levels. However, mice administered with the extract at 100 and 150mg/kg bw showed comparable ($p > 0.05$) levels of TNF-α (Table 4.17). Mice treated with *C. edulis* methanol extract, at all the administered doses, produced significantly lower ($p < 0.05$) levels of IL-2, TNF-α, and IFN-γ compared with levamisole-treated mice (Table 4.17).

Administration of pyrogallol resulted in significantly elevated ($p < 0.05$) levels of IL-4 and IL-5 compared with the normal control. However, the methanol extract of *C. edulis* significantly reduced ($p < 0.05$) levels of IL-4 and IL-5 compared with the negative control (Table 4.17). The reduction was dose-dependent and there was a significant difference ($p < 0.05$) in IL-4 and IL-5 levels at all the tested doses of the extract. Levels of IL-4 and IL-5 produced by all extract-treated mice were significantly lower ($p < 0.05$) in contrast with levamisole-administered mice (Table 4.17).

Table 4.17: Effect of *C. edulis* Root Bark Methanol Extract on Th1 and Th2 Cytokines

Groups	Concentrations (pg/ml)				
	Th1 Cytokine			Th2 Cytokine	
	IL-2	TNF-α	IFN-γ	IL-4	IL-5
Normal control	571.80±4.53[a]	98.60±5.01[a]	394.00±7.80[a]	140.60±7.57[e]	104.00±7.26[e]
Negative control	318.20±4.47[e]	33.20±3.76[c]	191.60±5.89[e]	307.80±4.07[a]	219.60±3.83[a]
Levamisole (20 mg/kg bw)	561.20±4.16[a]	97.00±4.64[a]	387.80±4.41[a]	145.60±5.81[e]	109.00±4.48[e]
CM (50mg/kg bw)	462.60±6.00[d]	33.40±6.06[c]	300.60±4.25[d]	227.40±4.53[b]	192.60±6.80[b]
CM (100mg/kg bw)	491.40±5.89[c]	60.20±10.3[b]	327.80±4.19[c]	201.80±4.19[c]	165.40±5.19[c]
CM (150 mg/kg bw)	528.20±6.97[b]	68.00±4.60[b]	361.40±4.68[b]	176.20±4.39[d]	138.60±4.23[d]

Values were expressed as Mean ± SEM for the five mice in each group. Statistical comparison was done within a column and values with a similar superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p > 0.05$). CM = *C. edulis* methanol extract. Normal control group had received the vehicle; Negative control had received the immunosuppressant.

4.3.1.4 Effect of *C. edulis* Root Bark DCM Extract on IL-2, TNF-α, IFN-γ, IL-4, and IL-5

The levels of IL-2, TNF-α, and IFN-γ were significantly reduced ($p < 0.05$) in negative control mice compared with the normal control mice (Table 4.18). However, the *C. edulis* DCM extract significantly elevated ($p < 0.05$) levels of IL-2, TNF-α, and IFN-γ in immunosuppressed mice compared with the negative control mice (Table 4.18). Levels of IL-2, TNF-α, and IFN-γ produced by all extract-treated mice were significantly lower ($p < 0.05$) in contrast with positive control mice (Table 4.18).

As the results indicated, increasing extract concentration subsequently increased levels of IL-2, TNF-α, and IFN-γ in the immunocompromised mice. Levels of IL-2 and IFN-γ significantly varied ($p < 0.05$) in mice that received different doses of the extract. However, the extract, at all the tested doses, showed no significant difference ($p > 0.05$) in TNF-α levels (Table 4.18).

At 100 and 150 mg/kg bw, *C. edulis* DCM extract significantly reduced ($p > 0.05$) levels of IL-4 and IL-5 compared to the negative control. However, mice that received the extract at 50 mg/kg bw and negative control mice produced similar ($p < 0.05$) levels of IL-4 and IL-5 (Table 4.18). Mice that received *C. edulis* DCM extract at all the tested doses revealed significantly higher ($p < 0.05$) levels of IL-4 and IL-5 compared with levamisole-treated mice.

Carissa edulis DCM extract dose-dependently reduced IL-4 and IL-5 levels. However, mice administered with 50 and 100 mg/kg bw of the extract produced comparable ($p > 0.05$) levels of IL-5. Mice that received 150 mg/kg bw of the extract showed significantly higher ($p < 0.05$) levels of IL-5 compared with the levels exhibited by the other tested doses (Table 4.18).

Table 4.18: Effect of *C. edulis* Stem Bark DCM Extract on Th1 and Th2 Cytokines

Groups	Concentrations (pg/ml)				
	Th1 cytokines			Th2 cytokines	
	IL-2	TNF-α	IFN-γ	IL-4	IL-5
Normal control	571.80±4.53^{a}	98.60±5.01^{a}	394.00±7.80^{a}	140.60±7.57^{d}	104.00±7.26^{d}
Negative control	318.20±4.47^{e}	33.20±0.58^{c}	191.60±5.89^{e}	307.80±4.07^{a}	219.60±3.83^{a}
Levamisole (20 mg/kg bw)	561.20±4.16^{a}	97.00±4.64^{a}	387.80±4.41^{a}	145.60±5.81^{d}	109.00±4.48^{d}
CD (50mg/kg bw)	432.00±2.81^{d}	33.40±1.72^{c}	251.80±3.88^{d}	298.60±4.34^{a}	211.00±3.67^{ab}
CD (100mg/kg bw)	461.20±4.19^{c}	36.60±2.38^{bc}	283.20±3.51^{c}	246.40±3.75^{b}	196.00±5.91^{b}
CD (150 mg/kg bw)	490.80±1.36^{b}	49.40±2.38^{b}	317.80±5.30^{b}	214.00±3.18^{c}	167.60±5.54^{c}

Values were expressed as Mean ± SEM for the five mice in each group. Statistical comparison was done within a column and values with a similar superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p > 0.05$). CD = *C. edulis* DCM extract. Normal control group had received the vehicle; Negative control had received the immunosuppressant.

4.3.1.5 Effect of the combined *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol Extract on IL-2, TNF-α, IFN-γ, IL-4, and IL-5

Terminalia brownii and *C. edulis* methanol extracts were combined in a ratio of 1:1 and their effect on IL-2, TNF-α, IFN-γ, IL-4, and IL-5 was investigated. Negative control mice (pyrogallol-treated) produced significantly lower ($p < 0.05$) levels of IL-2, TNF-α, and IFN-γ compared with the normal control mice. However, mice treated with the combined extract (S) produced significantly higher ($p < 0.05$) levels of IL-2, TNF-α, and IFN-γ compared with the negative control mice. At the highest tested dosage, (150 mg/kg bw), the combined extract induced mice to produce comparable ($p > 0.05$) levels of IL-2, TNF-α, and IFN-γ with the positive control mice. However, at lower doses (50 and 100mg/kg bw), mice given the combined extract exhibited significantly less ($p < 0.05$) amount of IL-2, TNF-α, and IFN-γ compared with levamisole-administered mice. As the findings showed, the increase of IL-2, TNF-α, and IFN-γ was dose-dependent, whereby there was a significant ($p < 0.05$) gradual rise of the cytokines levels as the concentration of the combined extract increased (Table 4.19).

Administration of pyrogallol significantly elevated ($p < 0.05$) the production of IL-4 and IL-5 in negative control mice compared with the normal control mice. However, the combined extract significantly lowered ($p < 0.05$) the production of IL-4 and IL-5 compared with pyrogallol. The combined extract lowered IL-4 and IL-5 levels in a dose-dependent trend and there was a significant difference ($p < 0.05$) in IL-4 and IL-5 levels at all the tested doses of the extract (Table 4.19). No significant difference ($p > 0.05$) was observed in IL-4 and IL-5 levels produced by mice treated with the extract at 150mg/kg bw and levamisole (Table 4.19).

Table 4.19: Effect of Combined Methanol Extracts of *T. brownii* Stem Bark and *C. edulis* Root Bark on Th1 and Th2 Cytokines

Groups	Concentration (pg/ml)				
	Th1 Cytokines			Th2 Cytokines	
	IL-2	TNF-α	IFN-γ	IL-4	IL-5
Normal control	571.80±4.53[a]	98.60±5.01[a]	394.00±7.80[a]	140.60±7.57[d]	104.00±7.26[d]
Negative control	318.20±4.47[d]	33.20±3.76[d]	191.60±5.89[d]	307.80±4.07[a]	219.60±3.83[a]
Levamisole (20 mg/kg bw)	561.20±4.16[a]	97.00±4.64[a]	387.80±4.41[a]	145.60±5.81[d]	109.00±4.48[d]
TM + CM (50mg/kg bw)	505.40±5.03[c]	57.40±4.03[c]	332.40±4.15[c]	199.40±4.53[b]	154.60±4.83[b]
TM + CM (100mg/kg bw)	535.20±4.84[b]	77.00±4.39[b]	359.20±6.13[b]	172.60±5.02[c]	131.60±3.89[c]
TM + CM (150 mg/kg bw)	566.20±4.16[a]	97.20±3.81[a]	391.60±2.86[a]	143.40±6.65[d]	106.40±5.05[d]

Values were expressed as Mean ± SEM for the five mice in each group. Statistical comparison was done within a column and values with a similar superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p > 0.05$). TM = *T. brownii* methanol extract; CM = *C. edulis* methanol extract. Normal control group had received the vehicle; Negative control had received the immunosuppressant.

4.3.1.6 Comparison of the Effects of All Tested Extracts on Th1 Cytokines (IL-2, TNF-α, and IFN-γ)

This study compared the effects of all tested extracts on the production of IL-2, TNF-α, and IFN-γ in immunocompromised mice (Figure 4.4). The combined *T. brownii* and *C. edulis* methanol extracts (S) stimulated mice to produce significantly the highest ($p < 0.05$) levels of all IL-2 and IFN-γ cytokines compared with the other extracts at all the administered dosages. Mice given the combined extract showed the highest levels of TNF-α at all the tested doses, which significantly varied ($p < 0.05$) from the levels produced by mice given the other extracts at the doses of 50 and 150 mg/kg bw. However, at 100 mg/kg bw, mice that received the combined extract (S), *T. brownii* methanol stem bark extract (TM), and *C. edulis* methanol root bark (CM) extract produced comparable levels ($p > 0.05$) of TNF-α (Figure 4.4).

Mice that received methanol extract of *T. brownii* stem bark showed the 2nd highest levels of IL-2, IFN-γ, and TNF-α at all the investigated doses. However, levels of IL-2 and IFN-γ in mice administered with *T. brownii* methanol extract did not significantly differ ($p > 0.05$) from those produced by mice administered with the methanol extract of *C. edulis* root bark (CM) at all the investigated doses. Methanol extract of *T. brownii* stimulated mice to produce levels of IL-2 and IFN-γ that were not comparable ($p < 0.05$) with the amounts produced by mice given DCM extracts of *C. edulis* (CD) and *T. brownii* (TD), at all the probed doses (Figure 4.4).

Levels of TNF-α were comparable ($p > 0.05$) in mice treated with methanol extracts of *T. brownii* (TM) and *C. edulis* (CM) at all the tested dosages (Figure 4.4). At 50 and 100 mg/kg bw doses, mice administered with methanol extract of *T. brownii* (TM) and DCM extract of *C. edulis* (CD) produced alike ($p > 0.05$) levels of TNF-α. However, mice given TM and CD extracts at the dosage of 150 mg/kg bw exhibited significantly different ($p < 0.05$) TNF-α levels (Figure 4.4).

Among all the studied extracts, mice that were given *C. edulis* methanol (CM) extract exhibited the third-highest levels of IL-2, IFN-γ, and TNF-α at all the investigated doses (Figure 4.4). At the dosages of 50 and 150 mg/kg bw, mice administered with *C. edulis* methanol (CM) extract and DCM extracts of *T. brownii* and *C. edulis* produced significantly different ($p < 0.05$) levels of IL-2 (Figure 4.4). At 100 mg/kg bw, IFN-γ levels significantly varied ($p < 0.05$) between mice administered with *C. edulis* methanol extract (CM) and DCM extract of *T. brownii* (TD). Levels of IL-2 were similar ($p > 0.05$) between mice treated with 100 mg/kg bw of CM and TD (Figure 4.4). Levels of TNF-α were similar ($p > 0.05$) between mice administered with *C. edulis* methanol (CM) extract and DCM extract of *T. brownii* (TD) at 50, 100, and 150mg/kg bw.

The fourth highest levels of IL-2, IFN-γ, and TNF-α were noted in mice administered with DCM extract of *T. brownii* (TD) at all the investigated doses. The least amount of IL-2, IFN-γ, and TNF-α were seen in mice administered with *C. edulis* DCM extract (CD) (Figure 4.4). The TD and CD extracts evoked mice to generate similar ($p > 0.05$) amounts of IL-2, IFN-γ, and TNF-α at all the tested doses (Figure 4.4).

Methanol extracts of the studied plants (TM and CM) stimulated mice to produce significantly higher ($p < 0.05$) levels of IFN-γ compared with the levels observed in mice treated with DCM extracts of the studied plants (TD and CD) at all the tested doses. Levels of IL-2 were significantly higher ($p < 0.05$) in mice treated with methanol extracts of the tested plants (TM and CM) compared to the levels noted in mice treated with DCM extracts of the investigated plants (TD and CD) at the dose of 50 and 150 mg/kg bw (Figure 4.4).

At 50 and 100 mg/kg bw, levels of TNF-α were alike ($p > 0.05$) in mice treated with methanol and DCM extracts of the studied plants. However, at 150 mg/kg bw, methanol extracts of the investigated plants stimulated mice to produce significantly higher and alike ($p < 0.05$) levels of TNF-α compared with amounts observed in mice treated with DCM extracts (Figure 4.4).

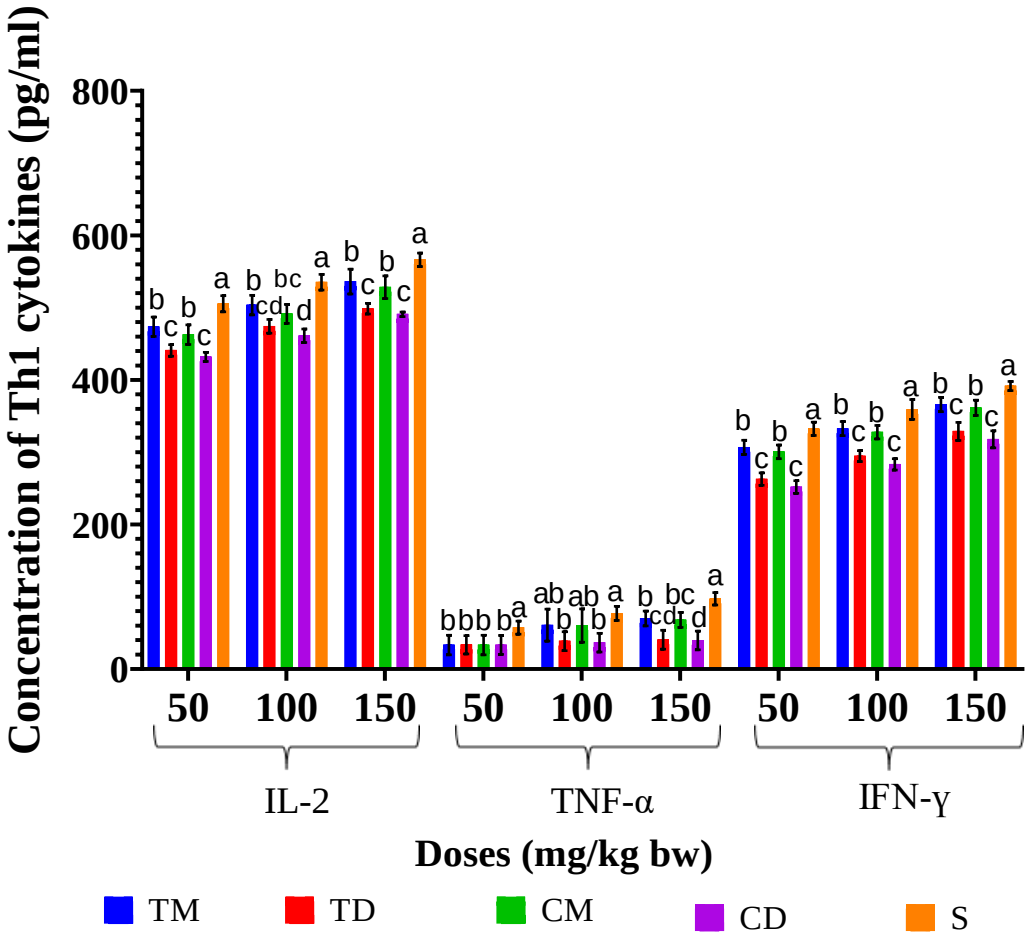


Figure 4.4: Comparison of Effects of all Studied Extracts on Levels of IL-2, TNF-α, and IFN-γ. Bars with the same lowercase letter are not significantly different in each dose dosage ($p>0.05$). TM = *T. brownii* methanol stem bark extract; TD = *T. brownii* DCM stem bark extract; CM = *C. edulis* methanol root bark extract; CD = *C. edulis* DCM root bark extract; S = synergy; combined methanol extracts of *T. brownii* stem bark and *C. edulis* methanol root bark.

4.3.1.7 Comparison of the Effects of all Tested Extracts on Th2 Cytokines (IL-4 and IL-5)

Subsequently, this study compared the effects of all the studied extracts on IL-4 and IL- 5 (Figure 4.5). The efficacy of the studied extracts on IL-4 and IL- 5 was inferred

by inverse proportionality in which the lowest levels of IL-4 and IL- 5 revealed the most active extract. Mice given the combined methanol extracts of *C. edulis* and *T. brownii* (S) produced the lowest levels of IL-4 and IL- 5. These levels significantly differed ($p < 0.05$) from the levels expressed by mice that received the other extracts, at all the investigated dosages (Figure 4.5). Thus, the combined extract demonstrated the optimal effect on IL-4 and IL-5 levels.

Mice administered with methanol extracts of *T. brownii* (TM) exhibited the second-lowest levels of IL-4 and IL- 5. The third lowest levels of IL-4 and IL- 5 were exhibited by mice treated with methanol extract of *C. edulis* (CM) at all the tested doses (Figure 4.5). Mice administered with DCM extract of *T. brownii* (TD) extract showed the fourth lowest levels of IL-4 and IL- 5 cytokines at all the tested doses (Figure 4.5). The highest levels of IL-4 and IL-5 were produced in mice administered with DCM extract of *C. edulis* (CD) at all the investigated doses (Figure 4.5).

Mice administered with the methanol extracts of *C. edulis* (CM) and *T. brownii* (TM), at all the investigated doses, exhibited similar ($p < 0.05$) levels of IL-4 and IL-5. At 100 and 150 mg/kg bw doses, mice treated with DCM extract of *C. edulis* (CD) and *T. brownii* (TD) produced comparable ($p > 0.05$) levels of IL-4 and IL-5 cytokines. However, at 50 mg/kg bw, mice administered with these extracts (CD and TD) produced significantly different ($p < 0.05$) levels of IL-4 (Figure 4.5). On the other hand, at 50 mg/kg bw, mice given individual extracts (TM, TD, CM, and CD) exhibited comparable ($p > 0.05$) levels of IL-5 (Figure 4.5).

Moreso, mice administered with methanol extracts (TM and CM) produced significantly lower ($p < 0.05$) levels of IL-4 compared with mice given DCM extracts (TD and CD) of the studied plants at all the investigated doses. Similarly, at 100 and 150 mg/kg bw doses, IL-5 levels were significantly lower ($p < 0.05$) in mice that received methanol extracts compared with the levels in mice administered with DCM extracts of the investigated plants (Figure 4.5).

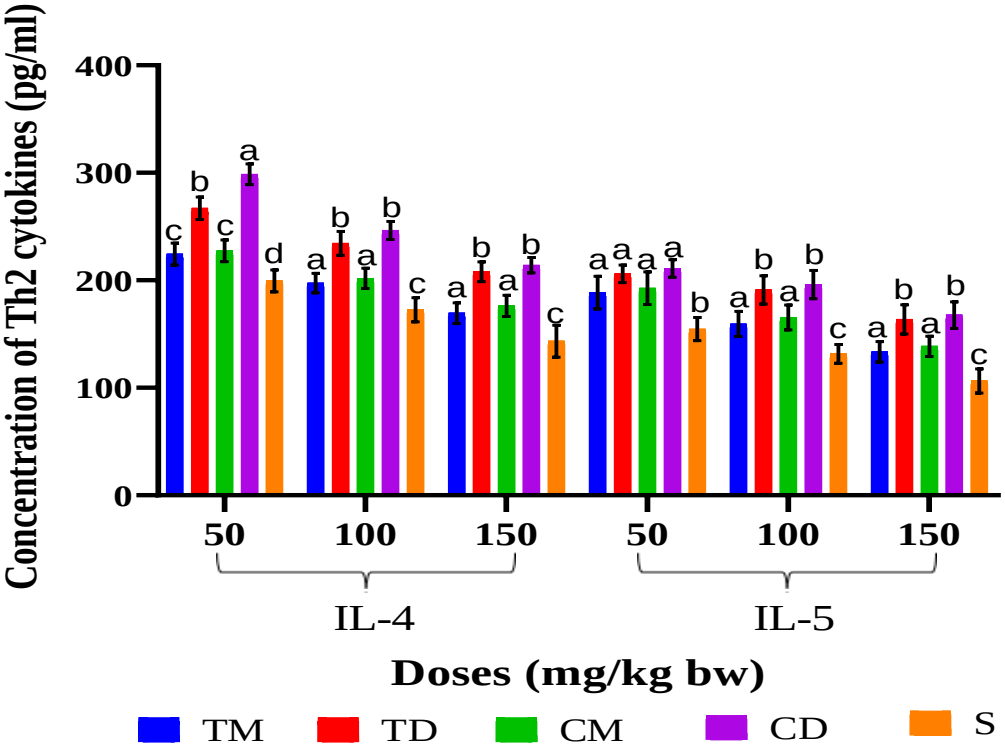


Figure 4.5: Comparison of Effects of all Studied Extracts On Levels of IL-4 and IL-5. Bars with the same lowercase letter are not significantly different in each dose dosage ($p>0.05$). TM = *T. brownii* methanol stem bark extract; TD = *T. brownii* DCM stem bark extract; CM = *C. edulis* methanol root bark extract; CD = *C. edulis* DCM root bark extract; S = synergy; combined methanol extracts of *T. brownii* stem bark and *C. edulis* methanol root bark.

4.3.1.8 Effects of *T. brownii* Stem Bark Methanol and DCM Extracts on Delayed-Type Hypersensitivity (DTH) Reaction

Negative control mice showed significantly increased ($p < 0.05$) paw sizes compared with the normal control mice (Table 4.20). At all the investigated doses, mice administered with methanol and DCM extracts of *T. brownii* showed significantly bigger ($p < 0.05$) paw sizes compared with the negative control mice (Table 4.20). Methanol and DCM extract of *T. brownii* increased paw sizes of the

immunocompromised mice in a dose-dependent trend. Thus, the levels of DTH reactions elevated as the extract's concentrations increased. Moreso, different concentrations of these extracts led to significantly different ($p < 0.05$; Table 4.20) paw sizes. Among the tested doses of the extracts, the maximum paw size was noted in mice administered with the highest concentration of the methanol and DCM extract of *T. brownii* (150 mg/kg bw; Table 4.20).

Mice treated with methanol extract of *T. brownii* at 150mg/kg dosage and levamisole produced comparable DTH reactions ($p > 0.05$; Table 4.20). However, there was a significant difference ($p < 0.05$) in the DTH reaction between mice treated with *T. brownii* DCM extract, at all tested doses, and the standard drug.

The methanol extract of *T. brownii* demonstrated a more stimulatory effect on the DTH reaction compared to the *T. brownii* DCM extract. This finding was confirmed by the significantly larger ($p < 0.05$) paw sizes in mice that received methanol extract of *T. brownii* compared with the paw sizes of mice which were administered with *T. brownii* DCM extract at all the investigated dosages (Table 4.20).

Table 4.20: Effect of Methanol and DCM Extract of *T. brownii* Stem Bark on DTH Response

Group	Paw edema (mm)	
	Methanol	**DCM**
Normal control	1.74 ± 0.03^{e}	1.74 ± 0.03^{f}
Negative control	2.11 ± 0.02^{d}	2.11 ± 0.02^{e}
Levamisole (20 mg/kg bw)	3.69 ± 0.03^{a}	3.69 ± 0.03^{a}
T. brownii (50mg/kg bw)	2.67 ± 0.36^{cA}	2.33 ± 0.02^{dB}
T. brownii (100mg/kg bw)	3.10 ± 0.04^{bA}	2.52 ± 0.04^{cB}
T. brownii (150 mg/kg bw)	3.54 ± 0.08^{aA}	2.74 ± 0.07^{bB}

Values were expressed as Mean ± SEM. Values with a similar lowercase superscript in each and similar uppercase superscript in each row are not significantly different by one-way ANOVA followed by Tukey's post hoc test and unpaired t-test,

respectively ($p > 0.05$). Normal control group had received the vehicle; Negative control had received the immunosuppressant.

4.3.1.9 Effects of C. edulis Root Bark Methanol and DCM Extracts on Delayed-Type Hypersensitivity (DTH) Reaction

Overall, methanol and DCM extracts of *C. edulis* stimulated the DTH reaction in mice. The paw edema in mice administered with methanol extracts of *C. edulis* at all the tested doses was significantly larger ($p < 0.05$) compared with the paw size of negative and normal control mice (Table 4.21). At 100 and 150 mg/kg bw, the DCM extract of *C. edulis* elicited mice to exhibit significantly larger ($p < 0.05$) paw edema compared with that observed in negative and normal control mice. However, at 50 mg/kg bw, mice given the DCM extract of *C. edulis* and negative control showed comparable ($p > 0.05$) DTH reactions. Methanol and DCM extracts of *C. edulis*, at all the probed doses, stimulated significantly lower ($p < 0.05$) DTH reactions compared to levamisole.

Methanol and DCM extracts of *C. edulis* increased DTH reactions dose-dependently. Mice administered with the highest (150 mg/kg bw) produced the optimal DTH responses as the animals in this group revealed the largest paw size. The least DTH reactions were observed in mice treated with the lowest concentrations (50mg/kg bw) of the extracts (Table 4.21).

Mice that received different doses of *C. edulis* methanol extract revealed significantly varying ($p < 0.05$) paw edema (Table 4.21). Thereby, different extract doses stimulated different levels of DTH responses. However, at 50 and 100 mg/kg bw, mice treated with *C. edulis* root bark DCM extract showed comparable ($p > 0.05$) paw sizes. At 150 mg/kg bw, the extract stimulated mice to develop significantly different ($p < 0.05$) paw edema compared with other extract-treated groups (Table 4.21).

Mice treated with methanol extract of *C. edulis* exhibited significantly larger ($p < 0.05$) paw edema compared with the mice that received DCM extract of *C. edulis* at all the administered doses (Table 4.21).

Table 4.21: Effects of Methanol and DCM Extracts of *C. edulis* Root Bark on DTH Response

Group	Paw edema (mm)	
	Methanol	**DCM**
Normal control	1.74 ± 0.03^{f}	1.74 ± 0.03^{e}
Negative control	2.11 ± 0.02^{e}	2.11 ± 0.02^{d}
Levamisole (20 mg/kg bw)	3.69 ± 0.03^{a}	3.69 ± 0.03^{a}
C. edulis (50mg/kg bw)	2.50 ± 0.05^{dA}	2.24 ± 0.08^{cdB}
C. edulis (100mg/kg bw)	2.96 ± 0.04^{cA}	2.42 ± 0.05^{cB}
C. edulis (150 mg/kg bw)	3.32 ± 0.08^{bA}	2.69 ± 0.07^{bB}

Values were expressed as Mean ± SEM. Values with a similar lowercase superscript in each and similar uppercase superscript in each row are not significantly different by one-way ANOVA followed by Tukey's post hoc test and unpaired t-test, respectively ($p > 0.05$). Normal control group had received the vehicle; Negative control had received the immunosuppressant.

4.3.1.10 Effect of the Combined *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol Extracts on Delayed-Type Hypersensitivity (DTH) Reaction

The present study tested the effect of the combined methanol extracts of *T. brownii* and *C. edulis* (ratio of 1:1) on DTH reaction in immunocompromised mice. At all the tested doses (50, 100 and 150 mg/kg bw), mice administered with the combined extract exhibited significantly bigger ($p < 0.05$) paw edema compared with the mice in the negative and normal control groups. The increase in the DTH reaction was dose-dependent and the extract, at all the tested doses, demonstrated significantly different ($p < 0.05$) paw sizes (Table 4.22).

Mice that received the combined extract at the dose of 50 mg/kg bw showed significantly smaller ($p < 0.05$) paw sizes compared with the paw sizes noted in levamisole-treated mice. On the other hand, at 100 mg/kg bw dosage, mice that received the combined extract and levamisole-treated mice exhibited comparable ($p > 0.05$) paw sizes. Mice treated with 150 mg/kg bw of the combined extract

stimulated significantly bigger ($p < 0.05$) paw edema compared with levamisole-treated mice (Table 4.22).

Table 4.22: Effect of Combined Methanol Extracts of *T. brownii* Stem Bark and *C. edulis* Root Bark Extracts on DTH Reaction

Group	Paw edema (mm)
Normal control	1.74 ± 0.03^{e}
Negative control	2.11 ± 0.02^{d}
Levamisole (20 mg/kg bw)	3.69 ± 0.03^{b}
TM + CM (50mg/kg bw)	3.31 ± 0.05^{c}
TM + CM (100mg/kg bw)	3.59 ± 0.08^{b}
TM + CM (150 mg/kg bw)	4.03 ± 0.04^{a}

Values were expressed as Mean ± SEM for the triplicates in each group. Statistical comparison was done within a column and values with a similar superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p > 0.05$). TM = *T. brownii* methanol extract; CM = *C. edulis* methanol extract. Normal control group had received the vehicle; Negative control had received the immunosuppressant.

4.3.1.11 Comparison of the Effect of All the Tested Extracts on Delayed-Type Hypersensitivity (DTH) Reaction

The present study compared the immunostimulatory effects of all the extracts on DTH reaction in mice (Figure 4.6). The combined methanol extracts of *C. edulis* root bark and *T. brownii* stem bark (S) stimulated the optimal DTH reactions compared to the other extract treatments at all the tested doses. This finding was confirmed by significantly larger ($p < 0.05$) paw edema in mice treated with the combined extract compared with the edema observed in mice given the other extracts at all the probed doses (Figure 4.6).

Mice that received methanol extract of *T. brownii* (TM), at all the investigated doses, showed the second biggest paw sizes compared with the mice treated with the other

extracts. Thus, TM demonstrated the second-highest efficacy to stimulate the DTH reaction. At all the administered doses, methanol extract of *C. edulis* (CM) stimulated mice to produce the third highest DTH response. DTH responses induced by methanol extract of *T. brownii* (TM) and *C. edulis* (CM) did not significantly differ ($p > 0.05$) at all the tested doses. This was revealed by comparable ($p > 0.05$) paw edema in the mice that received the two extracts (TM and CM).

Mice treated with DCM extract of *T. brownii* (TD) showed the fourth highest DTH responses whereas those treated with *C. edulis* DCM extract (CD) revealed the lowest DTH responses at all the tested doses. Mice treated with TD and CD showed similar ($p > 0.05$) paw sizes at all the tested doses (Figure 4.6).

At 100 and 150 mg/kg bw, mice that received methanol extracts of *C. edulis* (CM) and *T. brownii* (TM) revealed significantly higher ($p < 0.05$) paw edemas than the mice administered with DCM extracts of *T. brownii* (TD) and *C. edulis* (CD) (Figure 4.6). However, at 50 mg/kg bw, DCM extract of *T. brownii* (TD) stimulated mice to exhibit similar ($p > 0.05$) DTH reactions compared with mice treated with methanol extracts of *C. edulis* (CM) (Figure 4.6).

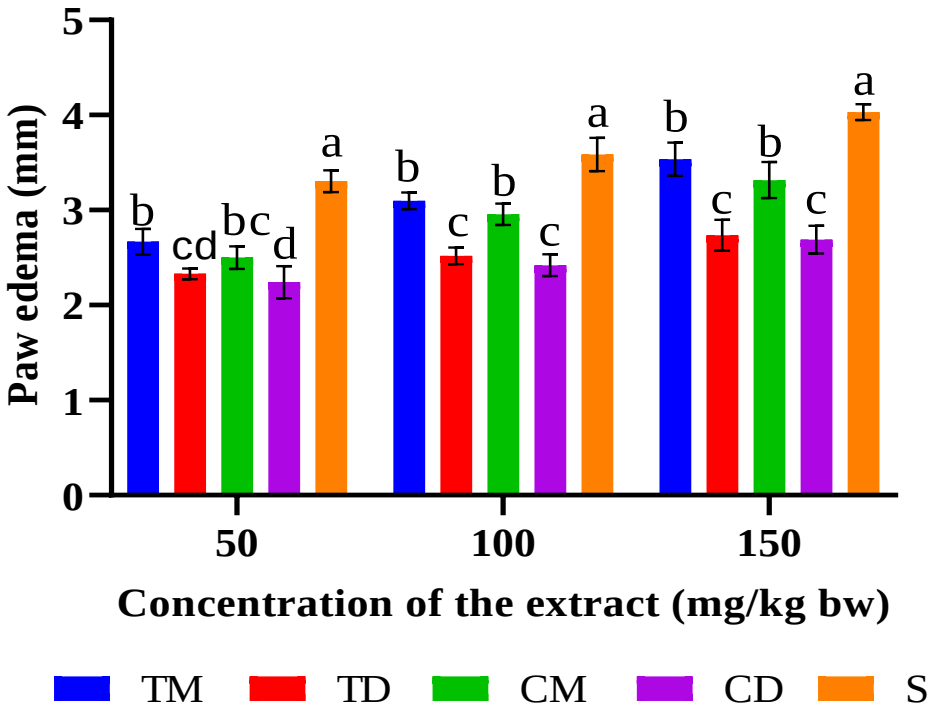


Figure 4.6: Comparison of the Effect of all the Extracts on DTH Reaction in Mice. Bars with the same lowercase letter are not significantly different in each dose dosage ($p>0.05$). TM = T. brownii stem bark methanol extract; TD = T. brownii stem bark DCM extract; CM = C. edulis root bark methanol extract; CD = C. edulis root bark DCM extract; S = synergy; combined methanol extracts of T. brownii stem bark and C. edulis methanol root bark.

4.3.2 Effect of *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol and DCM Extracts on Humoral Immune Responses

This study evaluated the effect of *T. brownii* and *C. edulis* extracts on humoral immune responses by assessing antibody titer levels in the serum of pyrogallol-immunosuppressed mice. Immunosuppression reduced antibody titer levels, however, *T. brownii* and *C. edulis* methanol and DCM extracts augmented the titer levels.

4.3.2.1 Effect of *T. brownii* Stem Bark Methanol and DCM Extracts on Antibody Titer Levels

Negative control mice showed significantly lower ($p < 0.05$) antibody titer levels compared to the normal control mice (Table 4.23). However, mice treated with methanol and DCM extracts of *T. brownii* exhibited significantly higher ($p < 0.05$) antibody titer levels compared with the negative control mice. The extracts increased antibody titer levels in a dose-dependent trend (Table 4.23).

At the highest dosage (150 mg/kg bw), *T. brownii* methanol extract demonstrated an antibody titer that was comparable ($p > 0.05$) with that noted in the positive control mice. However, mice treated with *T. brownii* DCM, at all the tested doses, showed significantly lower ($p < 0.05$) antibody titer levels compared with levamisole-treated mice.

A significant difference ($p < 0.05$) was noted in antibody titers exhibited by mice treated with the methanol extract of *T. brownii* at all the tested dosages. However, mice administered with 50 and 100mg/kg bw of the *T. brownii* DCM extract demonstrated comparable ($p > 0.05$) antibody titers (Table 4.23).

Mice administered with the methanol extract of *T. brownii* revealed significantly higher ($p < 0.05$) antibody titer levels compared with the mice given the DCM extract, at all the probed doses (Table 4.23).

Table 4.23: Effect of Methanol and DCM Extracts of *T. brownii* Stem Bark on Antibody Titer

Group	Antibody titer (Units/µl)	
	Methanol	**DCM**
Normal control	4.00 ± 0.00^{dA}	3.67 ± 0.33^{dA}
Negative control	1.67 ± 0.33^{e}	1.67 ± 0.33^{e}
Levamisole (20 mg/kg bw)	11.00 ± 0.58^{a}	11.00 ± 0.58^{a}
T. brownii (50mg/kg bw)	6.33 ± 0.33^{cA}	4.67 ± 0.33^{cdB}
T. brownii (100mg/kg bw)	8.33 ± 0.33^{bA}	5.67 ± 0.33^{cB}
T. brownii (150 mg/kg bw)	10.33 ± 0.33^{aA}	7.67 ± 0.33^{bB}

Values were expressed as Mean ± SEM. Values with a similar lowercase superscript in each and similar uppercase superscript in each row are not significantly different by one-way ANOVA followed by Tukey's post hoc test and unpaired t-test, respectively ($p > 0.05$). Normal control group had received the vehicle; Negative control had received the immunosuppressant.

4.3.2.2 Effect of *C. edulis* Root Bark Methanol and DCM Extracts on Antibody Titer Levels

Negative control mice showed significantly lower ($p < 0.05$) antibody titer levels compared to the normal control mice. However, mice administered with methanol and DCM extracts of *C. edulis,* at all the investigated doses, produced significantly higher ($p < 0.05$) antibody titer compared with negative control mice (Table 4.24). Mice administered with methanol extracts of *C. edulis* at 150 mg/kg bw and levamisole-treated mice produced comparable ($p > 0.05$) antibody titers (Table 4.24). However, mice given *C. edulis* DCM extract at all the investigated doses demonstrated significantly lower ($p < 0.05$) antibody titer in comparison with the positive control animals (Table 4.24).

The increase in antibody titer levels in mice administered with methanol and DCM extracts of *C. edulis* was dose-dependent (Table 4.24). The 50 and 100 mg/kg bw dosages of methanol and DCM extracts of *C. edulis* elicited mice to produce comparable ($p > 0.05$) antibody titer levels. Nonetheless, these titers were

significantly lower ($p < 0.05$) than those expressed by mice that received 150 mg/kg bw of the extracts (Table 4.24).

The antibody titer in mice administered with the methanol extract of *C. edulis* was significantly higher ($p < 0.05$) than the titer observed in the mice that received DCM extract of *C. edulis*, at all the probed doses (Table 4.24).

Table 4.24: Effects of Methanol and DCM Extracts of *C. edulis* Root Bark on Antibody Titer

Group	Antibody titer (Units/µl)	
	Methanol	**DCM**
Normal control	3.33 ± 0.33^{cA}	4.00 ± 0.00^{cA}
Negative control	1.00 ± 0.67^{dA}	1.00 ± 0.58^{dA}
Levamisole (20 mg/kg bw)	11.00 ± 0.58^{a}	11.00 ± 0.58^{a}
C. edulis (50mg/kg bw)	5.67 ± 0.33^{bA}	3.67 ± 0.33^{cB}
C. edulis (100mg/kg bw)	7.67 ± 0.33^{bA}	4.67 ± 0.33^{cB}
C. edulis (150 mg/kg bw)	10.00 ± 0.58^{aA}	7.33 ± 0.33^{bB}

Values were expressed as Mean ± SEM. Values with a similar lowercase superscript in each and similar uppercase superscript in each row are not significantly different by one-way ANOVA followed by Tukey's post hoc test and unpaired t-test, respectively ($p > 0.05$). Normal control group had received the vehicle; Negative control had received the immunosuppressant.

4.3.2.3 Effect of the Combined Methanol Extracts of *T. brownii* Stem Bark and *C. edulis* Root Bark on Antibody Titer Levels

Also, this study determined the effect of the combined methanol extracts of *T. brownii* and *C. edulis* (1:1) on antibody titer levels. Negative control mice showed significantly reduced ($p < 0.05$) levels of antibody titer compared with the normal control mice (Table 4.25). However, all the combined extract doses elicited mice to produce significantly higher ($p < 0.05$) antibody titers compared with the titer exhibited by the negative control mice (Table 4.25). Mice that received 100mg/kg

bw of the combined extract produced an antibody titer that was comparable ($p > 0.05$) with the titer generated by mice given levamisole.

Antibody titer increment induced by the combined extract was dose-dependent, whereby the highest dosage (150 mg/kg bw) triggered the highest antibody titer. These levels were significantly different ($p < 0.05$) from those observed in mice that received the other tested doses of the extract (50 and 100 mg/kg bw).

Table 4.25: Effects of Combined Methanol Extracts of *T. brownii* Stem Bark and *C. edulis* Root Bark on Antibody Titer

Group	Antibody titer (Units/µl)
Normal control	3.67 ± 0.33^{d}
Negative control	1.67 ± 0.33^{e}
Levamisole (20 mg/kg bw)	10.67 ± 0.33^{b}
TM + CM (50mg/kg bw)	8.00 ± 0.00^{c}
TM + CM (100mg/kg bw)	10.33 ± 0.33^{b}
TM + CM (150 mg/kg bw)	12.00 ± 0.33^{a}

Values were expressed as Mean ± SEM for the triplicates in each group. Statistical comparison was done within a column and values with a similar superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p > 0.05$). TM = *T. brownii* methanol extract; CM = *C. edulis* methanol extract. Normal control group had received the vehicle; Negative control had received the immunosuppressant.

4.3.2.4 Comparison of the Effect of all the Studied Extracts on Antibody Titer Levels

The present study compared the effects of all the extracts on antibody titer levels. At all the probed doses, mice treated with the combined extract (S) demonstrated the highest antibody titer. These titers significantly differed ($p < 0.05$) from those exhibited by mice that were treated with the other tested extracts at all the investigated doses.

The second highest antibody titer was observed in mice administered with *T. brownii* methanol extract (TM) followed by the titer in mice administered with *C. edulis* methanol (CM) extract. Mice given these extracts (TM and CM) produced comparable ($p > 0.05$) antibody titers at all the administered doses (Figure 4.7).

Next, *T. brownii* DCM extract (TD) stimulated mice to generate the 4th highest antibody titer. This was followed by *C. edulis* DCM extract (CD) which stimulated mice to produce the lowest titers. There was no significant difference ($p > 0.05$) between antibody titers in mice administered with TD and CD extracts.

The results revealed that methanol extracts of *T. brownii* and *C. edulis* (TM and CM), at 100 and 150 mg/kg bw, stimulated mice to produce significantly more ($p < 0.05$) antibody titers than mice given the DCM extracts (TD and CD) of the studied plants. However, at 50 mg/kg bw, mice treated with DCM extracts of *T. brownii* (TD) and methanol extract of *C. edulis* (CM) showed comparable ($p > 0.05$) antibody titers (Figure 4.7).

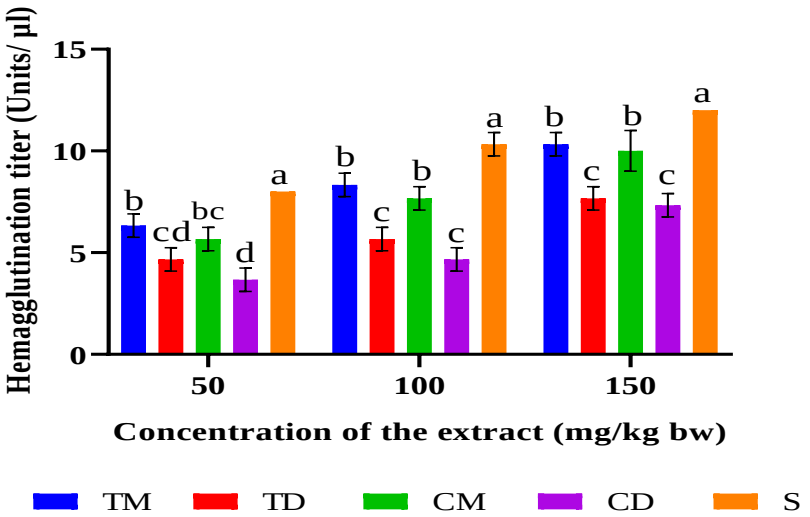


Figure 4.7: Comparison of the Effect of all the Extracts on Antibody Titer Levels. Bars with the same lowercase letter are not significantly different in each dose dosage ($p>0.05$). TM = *T. brownii* stem bark methanol extract; TD = *T. brownii* stem bark DCM extract; CM = *C. edulis* root bark methanol extract; CD = *C. edulis*

root bark DCM extract; S = synergy; combined methanol extracts of *T. brownii* stem bark and *C. edulis* methanol root bark.

4.4 Acute and Sub-Acute Toxicity Studies of *C. edulis* Root Bark and *T. brownii* Stem Bark Methanol and DCM Extracts in Female Albino Wistar Rats

4.4.1 Acute Toxicity Studies of *C. edulis* and *T. brownii* Methanol and DCM Extracts in Female Albino Wistar Rats

In acute toxicity studies, the extracts did not affect the behavioural patterns, general appearance, and body weights of the experimental animals.

4.4.1.1 Behavioural Patterns and General Appearance of Female Albino Wistar Rats Administered with *C. edulis* and *T. brownii* Methanol and DCM extracts in Acute Toxicity Study

All the tested extracts, administered as a single dose of 2000 mg/kg bw, did not cause mortality or any signs of acute toxicity (tremors, convulsions, salivation, and diarrhoea) in rats throughout the assessment period (Table 4.26). Also, rats administered with a single dose (2000 mg/kg bw) of methanol and DCM extracts of *T. brownii* stem bark (TM and TD) showed normal behavioural patterns and appearance (lethargy, sleep, coma, skin, fur, eyes, and mucous membranes). Likewise, the behavioural patterns and appearance of rats did not change upon administration of DCM and methanol extracts of *C. edulis* root bark (CD and CM) (Table 4.26).

Indeed, the behavioural patterns and appearance of the rats treated with all the stated extracts administered in a single dose of 2000 mg/kg bw were similar to those noted in the normal control rats (Table 4.26). Accordingly, the lethal dose (LD_{50}) of the studied extracts was above 2000 mg/kg during the acute toxicity study.

Table 4.26: Effects of Combined Methanol Extracts of *T. brownii* Stem Bark and *C. edulis* Root Bark on Antibody Titer

Behaviour and appearance	Observation				
	Normal Control	TM	TD	CM	CD
Tremors	Not observed	Not observed	Not observed	Not observed	Not observed
Convulsions	Not observed	Not observed	Not observed	Not observed	Not observed
Salivation	Not observed	Not observed	Not observed	Not observed	Not observed
Diarrhoea	Not observed	Not observed	Not observed	Not observed	Not observed
Lethargy	Not observed	Not observed	Not observed	Not observed	Not observed
Sleep	Normal	Normal	Normal	Normal	Normal
Coma	Not observed	Not observed	Not observed	Not observed	Not observed
Changes in the skin	No change	No change	No change	No change	No change
Changes in the fur	No change	No change	No change	No change	No change
Changes in the eyes	No change	No change	No change	No change	No change
Changes in the mucous membranes	No change	No change	No change	No change	No change

TM = *T. brownii* stem bark methanol extract; TD = *T. brownii* stem bark DCM extract; CM = *Carissa edulis* root bark methanol extract; CD = *Carissa edulis* root bark DCM extract. Normal control group had received the vehicle.

4.4.1.2 Body Weights of Rats Administered with *C. edulis* and *T. brownii* Methanol and DCM Extracts in Acute Toxicity Study

Upon administration of a single dose (2000 mg/kg bw) of the studies extracts, the weights of the rats were checked at three-time points seven days apart (days 0, 7, 14). On all the days of assessment, no significant difference ($p > 0.05$) was noted between the body weights of the normal control rats and the rats that received the single dose of *T. brownii* stem bark methanol and DCM extracts (TM and TD; Table 4.27). Also, rats treated with the single dose of *C. edulis* root bark methanol and DCM extracts (CM and CD) showed comparable ($p > 0.05$) body weights with that of normal control rats at the observation days: 0, 7, and 14 days (Table 4.27). On this account, the tested extracts of *T. brownii* and *C. edulis* did not alter the body weights of the experimental animals.

Table 4.27: Effect of *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol and DCM Extracts on Body Weights (G) of Female Albino Wistar Rats

Treatment	Day 0	Day 7	Day 14
Normal Control	208.40±2.69	216.60±2.96	222.40±2.73
TM	211.00±1.92	219.60±1.66	225.80±1.53
TD	205.40±3.19	214.00±2.86	219.00±4.09
CM	205.80±2.84	213.20±2.60	219.40±2.50
CD	209.80±2.71	217.40±2.79	223.40±2.89

Values were expressed as Mean ± SEM. Statistical comparison was done within a column; values were not significantly different by one-way ANOVA ($p > 0.05$). TM = *T. brownii* stem bark methanol extract; TD = *T. brownii* stem bark DCM extract; CM = *Carissa edulis* root bark methanol extract; CD = *Carissa edulis* root bark DCM extract. Normal control group had received the vehicle.

4.4.2 Sub-Acute Toxicity of *C. edulis* Root Bark and *T. brownii* Stem Bark Methanol and DCM Extracts in Female Albino Wistar Rats

In sub-acute toxicity studies, the tested extracts of both plants did not affect the body weights, relative organ weights, or haematological and biochemical parameters of the rats.

4.4.2.1 Body Weights (g) of Female Albino Wistar Rats Administered with T. brownii Stem Bark Methanol and DCM Extracts in Sub-Acute Toxicity Studies

Methanol and DCM extract of *T. brownii* stem bark at all the tested doses did not alter the body weights of Wistar rats during the sub-acute toxicity study. On all the observation days, the body weights of rats administered with *T. brownii* stem bark methanol and DCM extract at all doses were similar ($p > 0.05$) to the body weights of the normal control rats (Table 4.28).

Table 4.28: Effect of Methanol and DCM Extracts of *T. brownii* Stem Bark on Body Weights (G) of Female Albino Wistar Rats

Days	Treatment						
	Normal Control 2.5% DMS0	300mg/kg bw		520mg/kg bw		900mg/kg bw	
		TM	TD	TM	TD	TM	TD
0	207.40±2.23	205.60±3.28	207.60±2.42	209.40±2.42	210.40±3.74	207.00±1.64	208.60±3.22
7	215.40±2.36	215.60±2.99	218.60±2.25	219.00±2.47	221.40±3.14	218.20±1.53	220.00±2.81
14	221.20±2.96	223.00±2.70	226.80±2.89	226.60±2.68	230.40±2.91	225.80±1.59	228.80±2.48
21	225.60±3.26	228.80±2.76	232.20±2.63	230.80±1.46	236.00±2.81	231.40±6.00	234.40±3.41
28	229.00±3.16	231.60±2.84	235.60±2.48	234.40±3.31	239.40±2.91	234.60±1.57	238.40±3.28

Values were expressed as Mean ± SEM. Statistical comparison was done along the rows; values were not significantly different by one-way ANOVA ($p > 0.05$). TM = *T. brownii* stem bark methanol extract; TD = *T. brownii* stem bark DCM extract. Normal control group had received the vehicle.

4.4.2.2 Body Weights (g) of Female Albino Wistar Rats Administered with *C. edulis* Methanol and DCM Extracts in Sub-Acute Toxicity Studies

Rats treated with *C. edulis* root bark DCM and methanol extracts (CM and CD) at all the tested doses had comparable ($p > 0.05$) body weights with the normal control rats during the sub-acute toxicity assessment (Table 4.29).

Table 4.29: Effect of *C. edulis* Root Bark Methanol and DCM Extracts on Body Weights (G) Of Female Albino Wistar Rats

Days	Treatment						
	Normal Control 2.5% DMS0	300mg/kg bw		520mg/kg bw		900mg/kg bw	
		CM	CD	CM	CD	CM	CD
0	207.40±2.23	208.20±2.92	210.00±3.16	206.60±2.86	208.20±3.87	207.20±3.57	209.60±3.36
7	215.40±2.36	217.80±3.25	221.60±2.56	216.00±2.70	219.60±3.59	218.40±3.59	220.80±2.92
14	221.20±2.96	225.40±3.54	230.40±2.93	223.20±2.35	227.80±4.35	226.00±3.55	229.80±3.06
21	225.60±3.26	230.80±3.28	236.00±3.62	229.20±2.35	233.20±4.59	231.00±3.58	235.40±3.19
28	229.00±3.16	233.20±3.44	240.00±3.48	232.00±2.47	236.60±4.63	234.80±3.51	239.00±3.42

Values were expressed as Mean ± SEM. Statistical comparison was done along the rows; values were not significantly different by one-way ANOVA ($p > 0.05$). CM =

Carissa edulis root bark methanol extract; CD = *Carissa edulis* root bark DCM extract. Normal control group had received the vehicle.

4.4.2.3 Relative Organ Weights of Female Albino Wistar Rats Administered with *T. brownii* Stem Bark Methanol and DCM Extracts in Sub-Acute Toxicity Study

Methanol and DCM extracts of *T. brownii* stem bark (TM and TD) did not change the relative weights of brains, hearts, livers, kidneys, lungs, and spleens in Wistar rats (Table 4.30). No significant difference ($p > 0.05$) was noted between the relative weights of the brain, hearts, livers, kidneys, lungs, and spleens harvested from the rats that received DCM and methanol extracts of *T. brownii* stem bark at all the doses and the normal control rats (Table 4.30).

Table 4.30: Effect of *T. brownii* Stem Bark Methanol and DCM Extracts on Relative Organ Weights in Female Albino Wistar Rats

Organs	Treatment						
	Normal Control	300mg/kg bw		520mg/kg bw		900mg/kg bw	
	2.5% DMS0	TM	TD	TM	TD	TM	TD
Brain	0.73±0.04	0.75±0.02	0.73±0.02	0.72±0.03	0.71±0.03	0.72±0.02	0.75±0.03
Heart	0.40±0.01	0.39±0.02	0.37±0.02	0.40±0.02	0.39±0.01	0.37±0.02	0.37±0.02
Liver	3.53±0.11	3.49±0.13	3.49±0.19	3.39±0.16	3.41±0.15	3.40±0.10	3.38±0.16
Kidney	0.64±0.03	0.65±0.02	0.63±0.02	0.63±0.04	0.60±0.02	0.64±0.02	0.61±0.02
Lungs	0.59±0.03	0.56±0.02	0.58±0.03	0.58±0.02	0.58±0.04	0.59±0.03	0.57±0.03
Spleen	0.41±0.05	0.42±0.07	0.44±0.06	0.43±0.08	0.41±0.08	0.40±0.04	0.43±0.06

Values were expressed as Mean ± SEM. Statistical comparison was done along the rows; values were not significantly different by one-way ANOVA ($p > 0.05$). TM = *T. brownii* stem bark methanol extract; TD = *T. brownii* stem bark DCM extract. Normal control group had received the vehicle.

4.4.2.4 Relative Organ Weights of Female Albino Wistar Rats Administered with *C. edulis* Root Bark Methanol and DCM Extracts in Sub-Acute Toxicity Study

Relative weights of brain, hearts, livers, kidneys, lungs, and spleens in rats administered with DCM and methanol extracts of *C. edulis* root bark (CM and CD) at all the doses had no significant difference ($p > 0.05$) with the relative organ weights of normal rats (Table 4.31). Therefore, the tested extracts of *C. edulis* root bark did not affect the relative organ weights of the experimental animals.

Table 4.31: Effect of C. edulis Root Bark Methanol and DCM Extracts on Relative Organ Weights in Female Albino Wistar Rats

Organ	Treatment						
	Normal Control 2.5% DMS0	300mg/kg bw		520mg/kg bw		900mg/kg bw	
		CM	CD	CM	CD	CM	CD
Brain	0.73±0.04	0.73±0.03	0.70±0.03	0.75±0.02	0.72±0.02	0.72±0.03	0.73±0.04
Heart	0.40±0.01	0.37±0.02	0.37±0.02	0.39±0.02	0.39±0.02	0.38±0.02	0.36±0.02
Liver	3.45±0.11	3.37±0.12	3.33±0.11	3.48±0.12	3.32±0.19	3.34±0.16	3.41±0.18
Kidney	0.64±0.03	0.63±0.02	0.62±0.03	0.63±0.03	0.64±0.02	0.64±0.03	0.63±0.04
Lungs	0.59±0.03	0.58±0.02	0.57±0.03	0.57±0.03	0.57±0.03	0.58±0.03	0.57±0.02
Spleen	0.41±0.05	0.40±0.06	0.41±0.06	0.41±0.04	0.42±0.06	0.43±0.06	0.40±0.05

Values were expressed as Mean ± SEM. Statistical comparison was done along the rows; values were not significantly different by one-way ANOVA ($p > 0.05$). CM = *Carissa edulis* root bark methanol extract; CD = *Carissa edulis* root bark DCM extract. Normal control group had received the vehicle.

4.4.2.5 Haematological Parameters of Female Albino Wistar Rats Treated with *T. brownii* Stem Bark Methanol and DCM Extracts in Sub-Acute Toxicity Study

Levels of the assessed haematological parameters in rats treated with DCM and methanol extracts of *T. brownii* stem bark (TM and TD), at all the doses, were comparable ($p > 0.05$) with the levels noted in the normal control rats (Table 4.32).

Table 4.32: Effect of *T. brownii* Stem Bark DCM and Methanol Extracts on Haematological Parameters of Female Albino Wistar Rats

Parameters	Treatment						
	Normal control 2.5% DMS0	300mg/kg bw		520mg/kg bw		900mg/kg bw	
		TM	TD	TM	TD	TM	TD
WBCs (10^3/µl)	8.99±0.23	8.64±0.23	8.83±0.31	9.00±0.32	8.94±0.29	8.88±0.33	8.86±0.25
Neutrophils (10^3/µl)	1.68±0.06	1.58±0.06	1.67±0.05	1.64±0.07	1.65±0.05	1.60±0.05	1.62±0.05
Lymphocytes (10^3/µl)	6.37±0.11	6.13±0.22	6.24±0.22	6.34±0.25	6.35±0.16	6.28±0.24	6.32±0.19
Monocytes (10^3/µl)	0.85±0.03	0.84±0.07	0.83±0.05	0.93±0.08	0.84±0.03	0.92±0.16	0.83±0.04
Eosinophils (10^3/µl)	0.07±0.004	0.07±0.004	0.07±0.004	0.07±0.002	0.07±0.002	0.07±0.002	0.07±0.003
Basophils (10^3/µl)	0.02±0.004	0.02±0.003	0.02±0.002	0.02±0.002	0.03±0.002	0.01±0.002	0.02±0.003
RBC (10^6/µl)	7.72±0.26	7.82±0.31	7.69±0.26	7.68±0.26	7.81±0.17	7.93±0.23	7.48±0.34
HGB (g/dl)	13.79±0.62	13.96±0.45	13.44±1.01	13.82±0.71	13.68±0.79	13.46±0.82	13.74±0.71
HCT (%)	43.10±3.75	44.20±1.44	41.84±3.59	46.84±3.78	39.76±3.05	41.66±4.06	44.54±5.25
MCV (fl)	55.48±3.67	59.14±3.27	54.90±2.81	53.28±3.67	56.10±3.29	56.44±3.58	53.46±3.47
MCH (pg)	21.60±2.66	22.34±2.23	23.02±1.87	20.20±1.72	22.98±2.04	23.04±2.13	20.82±1.84
MCHC (g/dl)	33.86±3.33	31.44±1.28	36.76±3.39	35.30±3.07	33.72±3.08	37.36±3.18	36.00±3.09
RDW (%)	16.12±2.77	16.58±2.01	14.60±1.29	17.36±2.55	15.36±1.69	16.80±2.19	17.04±1.45
PLT (10^3/µl)	728.60±19.50	712.00±24.20	746.80±25.50	749.80±22.40	713.80±29.10	748.60±24.80	716.80±23.10
MPV (fl)	7.62±0.81	7.50±1.01	7.38±1.02	7.10±1.07	7.22±0.79	7.52±0.97	7.60±0.97

Values were expressed as Mean ± SEM. Statistical comparison was done across rows; values were not significantly different by ANOVA ($p > 0.05$). WBCs = White Blood Cells; RBCs = Red Blood Cells; HGB = Haemoglobin; HCT = Haematocrit; MCV = Mean Corpuscular Volume; MCH = Mean Corpuscular Haemoglobin; MCHC = Mean Corpuscular Haemoglobin Concentration; RDW = Red Cell Distribution Width; PLT = Platelets; MPV = Mean Platelet Volume. TM = *T. brownii* stem bark methanol extract; TD = *T. brownii* stem bark DCM extract. Normal control group had received the vehicle.

4.4.2.6 Haematological Parameters of Female Albino Wistar Rats Treated with *C. edulis* Root Bark Methanol and DCM Extracts in Sub-Acute Toxicity Study

Rats administered with DCM and methanol extracts of *C. edulis* root bark at all the doses and normal rats had comparable ($p > 0.05$) levels of haematological parameters

including neutrophils, lymphocytes, monocytes, eosinophils, basophils, WBCs, RBCs, HGB, HCT, MCV, MCH, MCHC, RDW, PLT, and MPV (Table 4.33).

Table 4.33: Effect of *C. edulis* Root Bark DCM and Methanol Extract on Haematological Parameters of Female Albino Wistar Rats

Parameters	Treatment						
	Normal control	300mg/kg bw		520mg/kg bw		900mg/kg bw	
	2.5% DMS0	CM	CD	CM	CD	CM	CD
WBCs (10^3/µl)	8.99±0.23	8.86±0.34	9.01±0.26	9.02±0.22	8.78±0.27	8.82±0.25	8.84±0.15
Neutrophils (10^3/µl)	1.68±0.06	1.60±0.04	1.69±0.06	1.68±0.05	1.63±0.08	1.63±0.06	1.68±0.05
Lymphocytes (10^3/µl)	6.36±0.11	6.32±0.18	6.38±0.10	6.37±0.21	6.25±0.18	6.28±0.25	6.24±0.19
Monocytes (10^3/µl)	0.86±0.03	0.85±0.05	0.85±0.06	0.87±0.06	0.81±0.07	0.82±0.05	0.83±0.04
Eosinophils (10^3/µl)	0.07±0.004	0.07±0.003	0.07±0.002	0.07±0.004	0.07±0.002	0.07±0.004	0.07±0.002
Basophils (10^3/µl)	0.02±0.004	0.02±0.004	0.02±0.002	0.03±0.002	0.02±0.003	0.02±0.004	0.02±0.002
RBC (10^6/µl)	7.72±0.26	7.67±0.24	7.58±0.26	7.79±0.19	7.73±0.26	7.32±0.38	7.57±0.38
HGB (g/dl)	13.79±0.62	13.31±0.79	14.02±0.40	13.46±0.71	13.85±0.38	13.52±0.61	13.94±0.72
HCT (%)	43.10±3.75	45.34±3.49	41.06±4.07	42.64±2.15	39.50±2.61	46.26±3.71	44.86±2.93
MCV (fl)	55.48±3.67	57.08±3.03	52.86±3.68	56.50±3.52	55.28±3.77	51.66±4.13	56.28±3.68
MCH (pg)	21.60±2.66	20.54±1.39	22.58±1.88	22.90±2.09	21.40±1.64	21.00±2.28	19.56±1.49
MCHC (g/dl)	33.86±3.33	37.40±3.27	35.52±3.15	38.08±3.77	34.50±2.38	36.16±2.66	30.20±2.37
RDW (%)	16.12±2.77	16.60±2.11	15.98±1.34	14.90±1.97	17.00±1.57	17.02±2.29	15.32±1.66
PLT (10^3/µl)	728.60±19.50	732.40±29.20	747.60±26.40	746.40±35.70	738.00±28.70	738.80±21.10	760.60±25.00
MPV (fl)	7.62±0.81	7.36±0.79	7.14±0.78	7.30±0.87	7.62±0.88	7.59±0.96	7.60±0.92

Values were expressed as Mean ± SEM. Statistical comparison was done across rows; values were not significantly different by ANOVA ($p > 0.05$). WBCs = White Blood Cells; RBCs = Red Blood Cells; HGB = Haemoglobin; HCT = Haematocrit; MCV = Mean Corpuscular Volume; MCH = Mean Corpuscular Haemoglobin; MCHC = Mean Corpuscular Haemoglobin Concentration; RDW = Red Cell Distribution Width; PLT = Platelets; MPV = Mean Platelet Volume. CM = *Carissa edulis* root bark methanol extract; CD = *Carissa edulis* root bark DCM extract. Normal control group had received the vehicle.

4.4.2.7 Effect of *T. brownii* Stem Bark Methanol and DCM Extracts on Biochemical Parameters of Female Albino Wistar Rats in Sub-Acute Toxicity Study

Rats treated with Methanol and DCM extracts of *T. brownii* stem bark (TM and TD) at all the tested doses and normal rats showed comparable ($p > 0.05$) concentrations of kidney function indices including creatine, urea, potassium, chloride, and sodium ions. Rats administered with methanol and DCM extracts of *T. brownii* stem bark at all the investigated doses and normal control rats showed comparable ($p > 0.05$) concentrations of liver function indicators (alkaline phosphatase, alanine transaminase, aspartate transaminase, albumin, bilirubin, and total protein) (Table 4.34).

Table 4.34: Effect of T. brownii Stem Bark Methanol and DCM Extracts on Biochemical Parameters of Female Albino Wistar Rats

Parameter	Treatment						
	Normal Control 2.5% DMSO	300mg/kg bw		520mg/kg bw		900mg/kg bw	
		TM	TD	TM	TD	TM	TD
K^+ (mmol/L)	4.72±0.31	4.66±0.29	4.90±0.15	4.60±0.23	4.67±0.18	4.88±0.27	4.72±0.20
Cl^- (mmol/L)	105.60±1.21	104.20±3.69	103.26±4.40	103.22±6.00	104.38±5.06	106.07±5.54	101.52±4.41
Na^+ (mmol/L)	141.80±3.48	143.36±6.19	142.90±4.62	141.27±5.99	143.60±3.57	139.43±6.17	138.24±3.67
Creatine (µmol/L)	51.66±2.39	51.93±3.42	50.63±2.23	53.78±3.72	48.84±2.31	56.82±3.24	51.03±2.58
Urea (mmol/L)	6.21±0.26	6.52±0.24	6.03±0.24	6.49±0.15	6.53±0.19	6.43±0.18	6.12±0.23
ALP (U/L)	132.60±6.74	134.09±7.16	144.00±7.36	136.80±3.92	135.20±4.89	133.40±3.93	139.80±3.35
ALT (U/L)	69.32±4.03	65.56±3.08	69.93±3.56	65.27±3.40	64.06±4.23	70.82±4.55	61.71±3.32
AST (U/L)	108.60±7.33	106.00±4.70	110.00±4.28	108.20±7.37	104.20±3.99	110.80±6.73	101.00±5.32
Albumin (g/L)	35.11±2.64	34.04±1.87	36.10±1.95	36.92±2.42	29.59±1.59	35.88±2.37	37.38±2.27
Bilirubin (µmol/L)	2.94±0.08	3.12±0.19	2.95±0.08	2.62±0.22	2.92±0.06	2.89±0.15	2.70±0.05
Total protein (g/L)	63.98±2.37	60.30±2.72	69.83±2.02	65.02±2.38	61.35±2.17	62.47±2.70	65.83±1.98

Values were expressed as Mean ± SEM. Statistical comparison was done across rows; values were not significantly different by one-way ANOVA ($p > 0.05$). K^+ = potassium ions; Cl^- = chloride ions; Na^+ = sodium ions; ALP = alkaline phosphatase; ALT = alanine transaminase; AST = aspartate transaminase. TM = *T. brownii* stem

bark methanol extract; TD = *T. brownii* stem bark DCM extract. Normal control group had received the vehicle.

4.4.2.8 Effect of *C. edulis* Root Bark Methanol and DCM Extracts on Biochemical Parameters of Female Albino Wistar Rats in Sub-Acute Toxicity Study

Rats treated with *C. edulis* root bark methanol and DCM extracts at all the tested doses and normal control rats showed comparable ($p > 0.05$) concentrations of kidney function indices including creatine, urea, potassium, chloride, and sodium ions (Table 4.35). No significant difference ($p > 0.05$) was observed between concentrations of liver function indicators (alkaline phosphatase, alanine transaminase, aspartate transaminase, albumin, bilirubin, and total protein) exhibited by rats administered with methanol and DCM extracts of *C. edulis* root bark at all the investigated doses and normal rats (Table 4.35).

Table 4.35: Effect of *C. edulis* Root Bark Methanol and DCM Extracts on Biochemical Parameters of Female Albino Wistar Rats

Parameter	Treatment						
	Normal control 2.5% DMSO	300mg/kg bw		520mg/kg bw		900mg/kg bw	
		CM	CD	CM	CD	CM	CD
K^+ (mmol/L)	4.72±0.31	4.81±0.31	5.05±0.15	4.52±0.21	4.76±0.12	4.93±0.14	4.82±0.31
Cl^- (mmol/L)	105.60±1.21	106.54±5.40	103.82±4.68	110.20±1.85	103.11±4.38	109.62±3.08	110.18±1.93
Na^+ (mmol/L)	141.80±3.48	138.28±5.46	153.20±3.89	144.77±3.56	145.52±3.19	137.05±3.81	142.40±2.94
Creatine (µmol/L)	51.66±2.39	55.60±2.75	49.86±2.21	52.75±3.27	55.62±2.75	50.55±2.46	53.94±2.28
Urea (mmol/L)	6.21±0.26	6.41±0.22	6.50±0.16	5.82±0.27	6.10±0.25	6.51±0.20	6.37±0.22
ALP (U/L)	132.60±6.74	129.60±4.32	138.40±3.97	131.20±5.33	145.20±6.59	134.60±6.59	127.00±6.06
ALT (U/L)	69.32±4.03	71.24±2.84	71.76±3.83	74.27±3.35	71.38±2.61	66.60±2.88	69.91±3.56
AST (U/L)	108.60±7.33	105.40±5.07	110.29±5.34	105.00±3.78	101.71±5.22	102.93±4.94	105.55±3.67
Albumin (g/L)	35.11±2.64	30.70±1.63	33.95±1.91	35.13±1.87	36.94±1.72	33.30±2.35	29.87±1.73
Bilirubin (µmol/L)	2.94±0.08	2.78±0.14	2.82±0.16	2.72±0.07	2.65±0.07	2.98±0.07	2.63±0.07
Total protein (g/L)	63.98±2.37	63.11±2.62	60.39±2.36	68.83±2.37	64.47±2.02	66.44±2.11	66.79±2.13

Values were expressed as Mean ± SEM. Statistical comparison was done across rows; values were not significantly different by ANOVA ($p > 0.05$). K^+ = potassium ions; Cl^- = chloride ions; Na^+ = sodium ions; ALP = alkaline phosphatase; ALT = alanine transaminase; AST = aspartate transaminase. CM = *Carissa edulis* root bark

methanol extract; CD = *Carissa edulis* root bark DCM extract. Normal control group had received the vehicle.

4.5 Phytochemical Profiles of *T. brownii* and *C. edulis* Methanol and DCM Extracts

4.5.1 Secondary Metabolites in *T. brownii* Stem Bark Methanol Extracts

Liquid chromatography–mass spectrometry (LC-MS) analysis of *T. brownii* stem bark methanol extract showed that the extract contained several compounds (Table 4.36). The extract contained triterpenoids including lupeol, betulinic acid, taraxasterol, ursolic acid and squalene, phenols including resveratrol, chlorogenic acid and scopoletin, flavonoids (naringenin and quercetin), steroids including β-sitosterol and campesterol, a fatty acid (octadecanoic acid), and corilagin which is a tannin. The most abundant compound in *T. brownii* stem bark methanol extract quantified with LC-MS was chlorogenic acid whereas the least abundant was betulic acid (Table 4.36; Figure 4.8).

Table 4.36: Phytochemical Profile of *T. brownii* Methanol Bark Extract Analyzed with LC-MS

RT (Min)	Compound	Class	Molecular formula	Conc. (µg/mg)
5.63	Naringenin	Flavonoid	$C_{15}H_{12}O_5$	0.73
5.78	Resveratrol	Phenol	$C_{20}H_{22}O_8$	0.49
16.31	Squalene	Triterpenoid	$C_{30}H_{50}$	0.84
16.31	Octadecanoic acid	Fatty acid	$C_{18}H_{36}O_2$	1.44
17.08	Chlorogenic acid	Phenol	$C_{16}H_{18}O_9$	1.81
26.16	β-sitosterol	Steroid	$C_{29}H_{50}O$	0.76
26.33	Campesterol	Steroid	$C_{28}H_{48}O$	0.57
26.69	Scopoletin	Phenol	$C_{10}H_8O_4$	0.55
26.84	Ursolic acid	Triterpenoid	$C_{30}H_{46}O_3$	0.47
27.46	Quercetin	Flavonoid	$C_{15}H_{10}O_7$	0.49
28.78	Taraxasterol	Triterpenoid	$C_{30}H_{50}O$	0.56
34.01	Corilagin	Tannin	$C_{27}H_{22}O_{18}$	1.02
35.20	Lupeol	Triterpenoid	$C_{30}H_{50}O$	0.41
36.20	Betulinic acid	Triterpenoid	$C_{30}H_{48}O_3$	0.29

Key: RT = retention time; Conc. = concentration

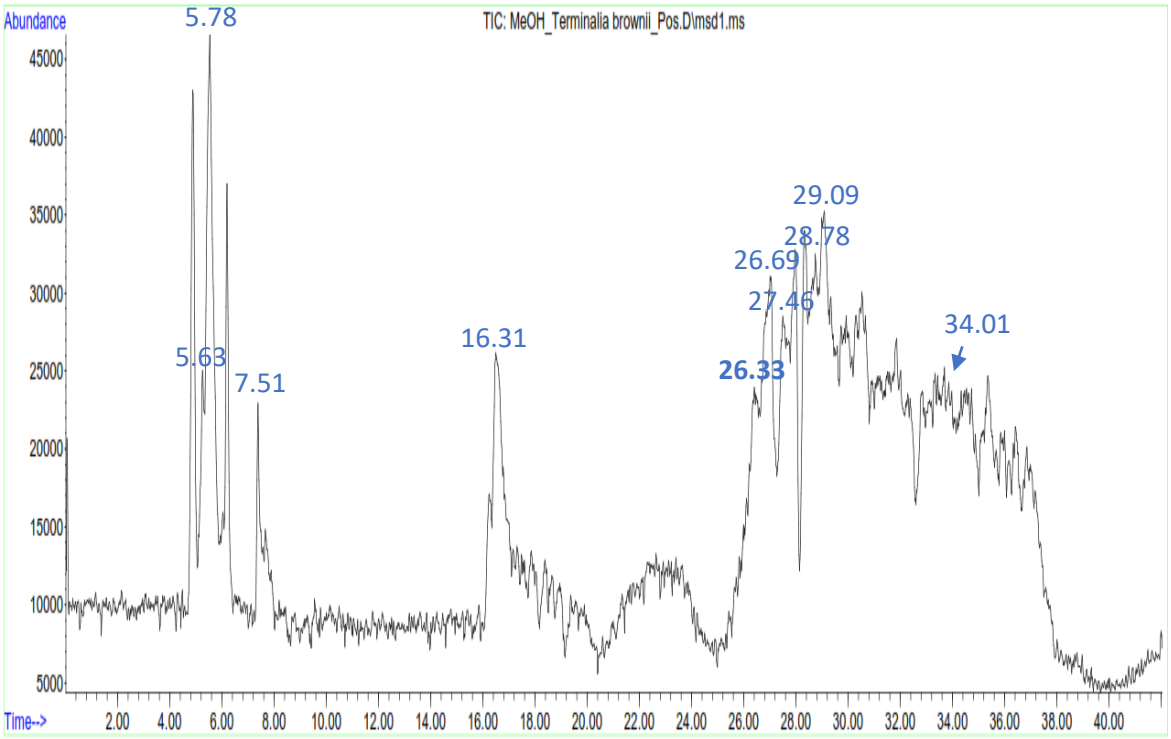


Figure 4.8: LC-MS Chromatogram of *T. brownii* Stem Bark Methanol Extract

4.5.2 Secondary Metabolites in *T. brownii* Stem Bark DCM Extracts

Phytochemical analysis of *T. brownii* stem bark DCM extract using GC-MS showed the presence of several compounds (Table 4.37). The extract contained fatty acids (linoleic acid, hexadecanoic acid, and stearic/octadecanoic acid), steroids (campesterol, stigmasterol, and beta-sitosterol), and squalene which is a triterpenoid. Among the compounds identified with GC-MS in *T. brownii* stem bark DCM extract, hexadecanoic acid was the most abundant at 30.11 % whereas stigmasterol (0.14 %) was the least abundant (Figure 4.9; Table 4.37).

Table 4.37: Phytochemical Profile of *T. brownii* Stem Bark DCM Extract Analyzed by GC-MS

RT (Min)	Compound name	Class	Molecular formula	Conc. (µg/g)	Abundance (%)
23.71	Hexadecanoic acid	Fatty acid	$C_{16}H_{32}O_2$	14.61	30.11
25.35	Linoleic acid	Fatty acid	$C_{18}H_{32}O_2$	5.01	10.32
25.56	Octadecanoic acid	Fatty acid	$C_{18}H_{36}O_2$	3.05	6.28
31.05	Squalene	Triterpenoid	$C_{30}H_{50}$	0.10	0.20
37.43	Campesterol	Steroid	$C_{28}H_{48}O$	0.30	0.61
37.58	Stigmasterol	Steroid	$C_{29}H_{48}O$	0.07	0.14

Key: RT = retention time; Conc. = concentration

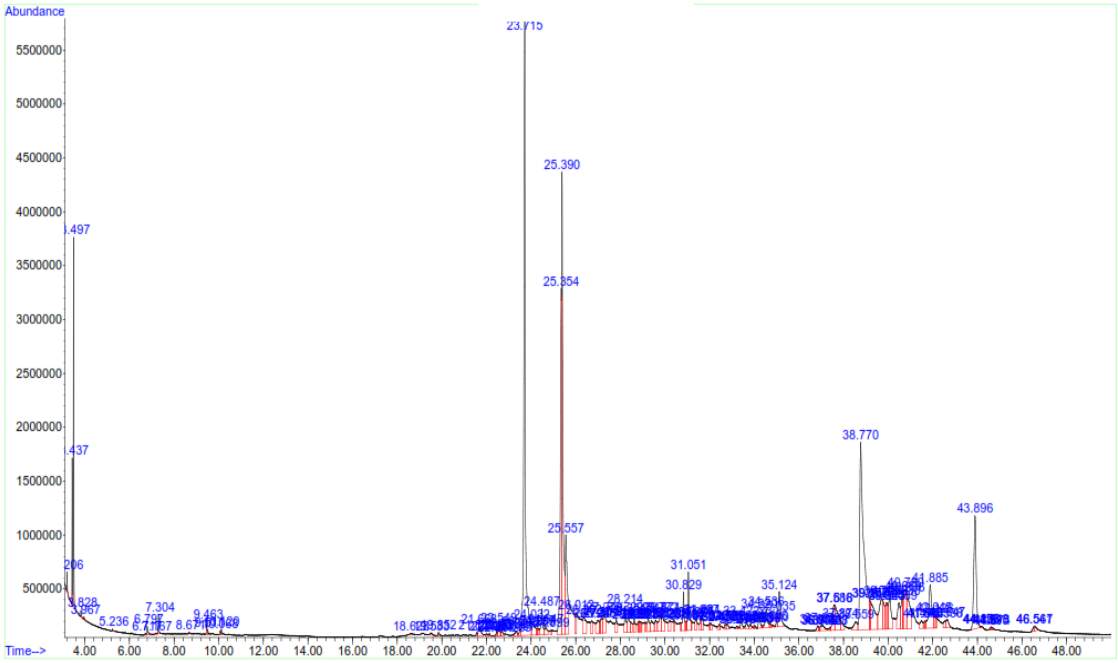


Figure 4.9: GC-MS Chromatogram of *T. brownii* Stem Bark DCM Extract

4.5.3 Secondary Metabolites in *C. edulis* Root Bark Methanol Extracts

Phytochemical screening of *C. edulis* root bark methanol extract with LC-MS showed the presence of triterpenoids including lupeol and ursolic acid, flavonoids (quercetin and luteolin), phenols including resveratrol, chlorogenic acid and oleuropein and β-sitosterol which is a steroid. Accordingly, the most abundant compound detected by LC-MS in *C. edulis* root bark methanol extract was chlorogenic acid whereas the least was resveratrol (Table 4.38; Figure 4.10).

Table 4.38: Phytochemical Profile of *C. edulis* Root Bark Methanol Extract Analyzed by LC-MS

RT (Min)	Compound	Class	Molecular formula	Conc. (µg/mg)
5.78	Resveratrol	Phenol	$C_{20}H_{22}O_8$	0.23
17.08	Chlorogenic acid	Phenol	$C_{16}H_{18}O_9$	2.53
26.16	β-sitosterol	Steroid	$C_{29}H_{50}O$	0.39
26.84	Ursolic acid	Triterpenoid	$C_{30}H_{46}O_3$	0.46
27.10	Oleuropein	Phenol	$C_{25}H_{32}O_{13}$	0.45
27.46	Quercetin	Flavonoid	$C_{15}H_{10}O_7$	0.45
35.20	Lupeol	Triterpenoid	$C_{30}H_{50}O$	0.65
36.73	Luteolin	Flavonoid	$C_{15}H_{10}O_6$	0.26

Key: RT = retention time; Conc. = concentration

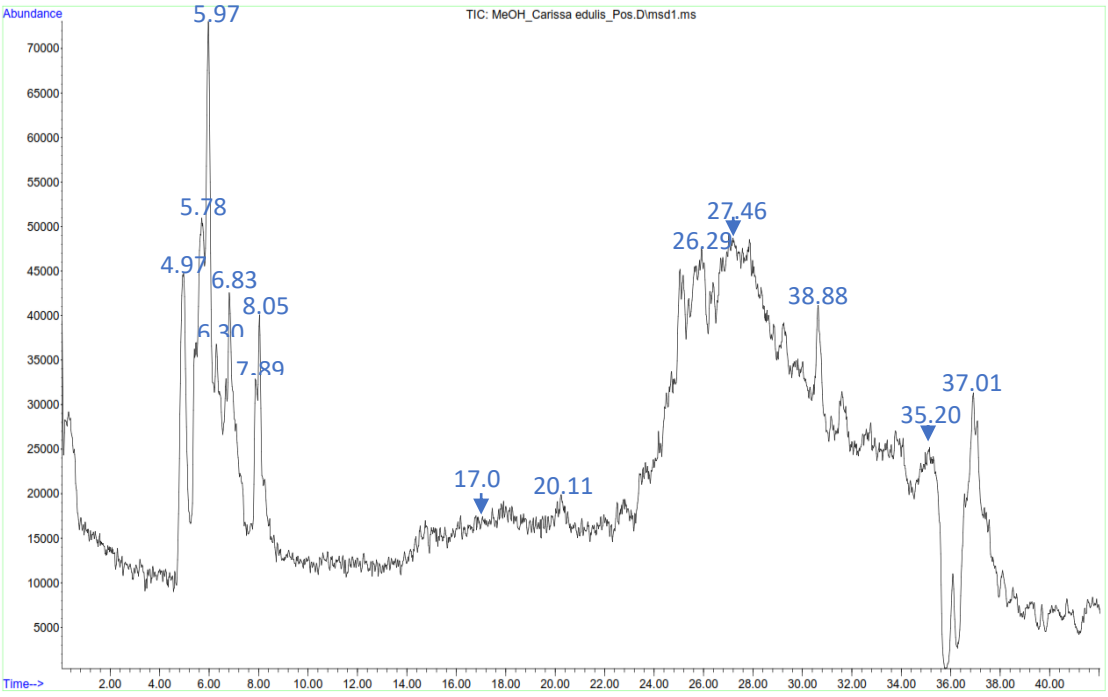


Figure 4.10: LC-MS Chromatogram of *C. edulis* Root Bark Methanol Extract

4.5.4 Secondary Metabolites in *C. edulis* Root Bark DCM Extracts

Analysis of *C. edulis* root bark DCM extract with GC-MS identified fatty acids (hexadecanoic acid, oleic acid, and octadecanoic acid), stigmasterol which is a steroid and triterpenoids including squalene and taraxasterol (Table 4.39).

Amidst the phytoconstituents detected with GC-MS in *C. edulis* root bark DCM extract, the most abundant was hexadecanoic acid (2.49 %). On the other hand, the analysis showed that the least abundant was stigmasterol at 0.36 percent (Table 4.39; Figure 4.11).

Table 4.39: Phytochemical Profile of *C. edulis* Root Bark DCM Extract Analyzed by GC-MS

RT (Min)	Compound name	Class	Molecular formula	Conc. (µg/g)	Abundance (%)
23.68	Hexadecanoic acid	Fatty acid	$C_{16}H_{32}O_2$	4.00	2.49
25.36	Oleic acid	Fatty acid	$C_{18}H_{34}O_2$	3.94	2.45
25.54	Octadecanoic acid	Fatty acid	$C_{18}H_{36}O_2$	0.86	0.53
31.05	Squalene	Triterpenoid	$C_{30}H_{50}$	1.37	0.85
37.51	Stigmasterol	Steroid	$C_{29}H_{48}O$	0.58	0.36
42.76	Taraxasterol	Triterpenoid	$C_{30}H_{50}O$	1.09	0.68

Key: RT = retention time; Conc. = concentration

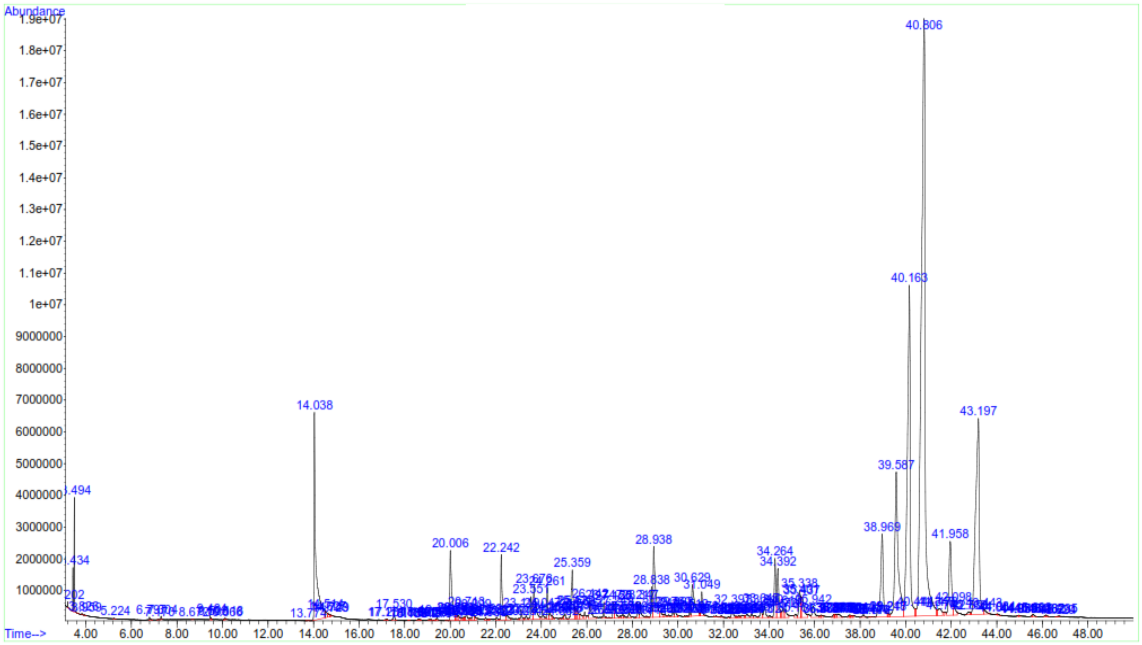


Figure 4.11: GC-MS Chromatogram of *C. edulis* Root Bark DCM Extract

CHAPTER FIVE

DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

5.1 Introduction

This chapter provides detailed discussion of the present study's results, conclusions and recommendations. The chapter is organized following the study objectives.

5.2 Discussion

5.2.1 Effect of *T. brownii* and *C. edulis* Methanol and DCM Extracts on Innate Immune Responses

The study herein reports the ability of methanol and DCM extracts of *T. brownii* stem bark and *C. edulis* root bark extract to stimulate innate immunity. The plants did so by stimulating the production of leukocytes (neutrophils, lymphocytes, monocytes, eosinophils, and basophils), NO, and TNF-α. The ability of the studied extracts to increase the production of total WBCs, NO, and TNF-α revealed the potential of the extracts to act as immunostimulants on innate immunity (Oladunmoye and Osho, 2007; Safriani, 2022).

Pyrogallol significantly impaired both innate and adaptive immune responses resulting in immunosuppressed mice models. This outcome has been corroborated by other studies using pyrogallol as a tool for immunomodulator screening. Pyrogallol was used to induce immunosuppression during the screening of immunomodulatory efficacy of *Rubia cordifolia* (Joharapurkar *et al.*, 2004), *Lagenaria vulgaris* (Mehta *et al.*, 2011), *Hibiscus tiliaceus* (Rajeswari *et al.*, 2013), water-soluble polysaccharides isolated from *Metroxylon sagu* (Pulla *et al.*, 2014), *Pithecellobium dulce* (Wagle *et al.*, 2015), biofield energy healing based herbomineral formulation (Trivedi *et al.*, 2017) and *Citrullus lanatus* (Apeh *et al.*, 2022).

Increased numbers of WBCs may have occurred due to the ability of the extract to stimulate the production of white blood cells by the bone marrow (Okokon *et al*., 2005; Neboh and Ufelle, 2015, Munir *et al.*, 2022). Also, the extract may have

enhanced the production of mediators of leucocytosis (Mohammed, 2016). Similar to *T. brownii* and *C. edulis,* other medicinal plants have revealed stimulatory activities on innate immune responses by enhancing the production of white blood cells. *Ocimum basilicum* has been demonstrated to increase total leucocyte counts in mice (Rapando, 2021). In the same study, *Ocimum basilicum* was shown to stimulate neutrophil production thereby enhancing components of innate immunity (Rapando, 2021). *Silene nocturna, Matricaria chamomilla,* and *Nigella sativa* have been revealed to raise total leukocyte counts in mice (Ghonime *et al.,* 2011). In addition, Obi *et al.* (2018) showed that *Moringa oleifera* increased total WBC counts as well as lymphocytes, eosinophils, monocytes, and neutrophils in mice. *Mahonia aquifolium* has been shown to increase lymphocyte numbers (Andreicuț *et al.,* (2019).

The ability of *T. brownii* stem bark and *C. edulis* root bark extract to enhance the production of leucocytes demonstrated its ability to boost the first line of defense in the body. Neutrophils constitute an indispensable component of the innate immune system (Mayadas *et al.,* 2014; Burn *et al.,* 2021). They act as effector antimicrobial cells especially offering defense against bacteria (Talla *et al.,* 2019), viral (Giraldo *et al.,* 2016), and fungi (Swidergall *et al.,* 2019) pathogens. Also, neutrophils have been indicated to combat malignancies such as colon cancer (Governa *et al.,* 2017) and breast cancer (Granot *et al.,* 2011). Furthermore, neutrophils have been shown to stimulate other cells of the immune system. Neutrophils promote dendritic cell (DC) recruitment, stimulating DC activity of antigen presentation and induction of T cell responses (Krishnamoorthy *et al.,* 2018; Souwer *et al.,* 2018). Also, neutrophils and their products have been shown to directly or indirectly regulate macrophages. For instance, neutrophils have been reported to prime alveolar macrophages to produce interleukin-1β (IL-1β) (Peiro´ *et al.,* 2018).

Carissa edulis and *T. brownii* increased lymphocyte numbers in immunocompromised mice. Lymphocytes involved in the innate immune system are regarded as innate lymphoid cells (ILCs) (Vivier *et al.,* 2018). Some of the most studied ILCs are natural killer cells (NK cells). NK cells offer antitumor immunity and mediate cytotoxicity on viral, fungal, and bacteria-infected cells in the body

(Mandal and Viswanathan, 2015; Vivier *et al.,* 2018). They also cells secrete interferon-γ (IFN-γ) and tumor necrosis factor α (TNF) which stimulate adaptive immune responses (Vivier *et al.,* 2011; Quintino-de-Carvalho *et al.,* 2022).

Eosinophils are known to protect the body against bacterial (Blanchard and Rothenberg, 2009), fungal (Ravin and Loy, 2016; Gaur *et al.,* 2022), metazoan parasitic invaders, including platyhelminths, and allergens infections (Furuta *et al.,* 2014). Also, eosinophils are involved in neoplasm surveillance, antitumor activities (Reichman *et al.,* 2016; Varricchi *et al.,* 2018), tissue repair (Chusid, 2018), tissue remodeling during puberty and pregnancy (Gouon-Evans *et al.,* 2000; Gouon-Evans and Pollard, 2001). Thus, it could be concluded that by the virtue of increasing numbers of eosinophils, *C. edulis* and *T. brownii* could induce similar physiological outcomes mediated by eosinophils. As corroborated elsewhere, *Viscum album* have been indicated to promote the antitumor role of eosinophils (Oei *et al.* 2019). *Carica papaya* has been revealed to enhance the antimicrobial efficacy of eosinophils (Gunde and Amnerkar, 2016).

Basophils are known to generate various effector molecules of the immune system including cytokines, chemokines, proteases, and histamine through which they mediate various immune reactions (Miyake *et al.,* 2021). Basophil-derived cytokines are known to stimulate other cells of the immune system including T and B cells, eosinophils, macrophages as well as innate lymphoid cells (Miyake and Karasuyama, 2017; Voehringer, 2017; Karasuyama *et al.,* 2018). Additionally, basophils have been demonstrated to offer resistance against ectoparasites such as ticks and scabies (Ito *et al.,* 2011). Basophils have been shown to activate B cells to secrete antibodies against bacteria pathogens thereby revealing indirect antimicrobial activity by priming humoral immune responses (Chen *et al.,* 2009). Notably, *T. brownii* and *C. edulis* demonstrated the potential to orchestrate these immune responses indirectly via basophil-mediated mechanisms as it revealed the ability to increase basophil production.

Terminalia brownii and *C. edulis* increased the numbers of monocytes. Monocytes have been reported to scavenge tumor material and stimulate NK cell activation and

recruitment in the tumor microenvironment (Hanna *et al.,* 2015; Plebanek *et al.,* 2017). They have also been reported to participate in antigen presentation (Brisen˜ o *et al.,* 2016) and tissue repair especially in myocardial infarction (Hilgendorf *et al.,* 2014; Graubardt *et al.,* 2017). Also, monocytes have been indicated to possess microbiocidal effects (De Trez *et al.,* 2009; Jenkins *et al.,* 2011). Additionally, monocytes protect skeletal muscle destruction against myotoxins by mediating myogenesis, fiber growth, and restoring muscle integrity (Arnold *et al.,* 2007). By increasing monocyte counts, *T. brownii* and *C. edulis* demonstrated the potential to induce similar outcomes via monocyte-mediated mechanisms. Yaseen *et al.* (2017) corroborate this observation by reporting that *Mandevilla veraguasensis, Byrsonima crassifolia* and *Verbesina oerstediana* bark exerted antimicrobial activity via increasing monocyte counts. Also, the antiviral activity of Sambucol, a blackberry natural product was associated with its ability to augment monocytes to express inflammatory cytokine (Barak *et al.,* 2001). *Mahonia aquifolium* has been demonstrated to augment the antitumor capacity of monocytes (Andreicuț *et al.,* 2019).

The observed increase in NO and TNF-α production by mouse macrophages treated with *T. brownii* and *C. edulis* extracts could be due to the ability of the extracts to classically activate macrophages (Maury *et al*., 2021). Classical activation of macrophages leads to the production of cytotoxic mediators, including NO, and pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) (Maury *et al*., 2021). Nitric Oxide has several biological functions including cellular signaling and an effector molecule mediating immune responses (Palmieri *et al.,* 2020). It is toxic to micro-organisms and plays a crucial role in inflammatory responses to infectious agents (Malone-Povolny *et al.,* 2019; Porrini *et al.,* 2020). In response to microbial stimuli, macrophages produce NO to kill the pathogens (Frawley *et al.,* 2018; Rong *et al.,* 2019). Nitric oxide also mediates the role of macrophages to kill tumor cells (Fauskanger *et al.,* 2018; Pan *et al.,* 2020). Thereby, it is conceivable that *T. brownii* and *C. edulis* could achieve antitumor and antimicrobial activities by stimulating the production of NO by macrophages. Indeed, other plants such as *Curcuma longa, Rosmarinus officinalis,* and *Thymus vulgaris* have demonstrated antimicrobial effects by activating macrophages to produce NO (Figueira *et al.,* 2020). *Nitraria retusa* has

been reported indicated to inhibit tumor growth via a mechanism involving NO produced by extract-activated macrophages (Boubaker *et al.,* 2018). The antitumor efficacy of a coumarin-rich fraction was ascribed to its ability to stimulate macrophages to produce NO which mediated cytotoxic effects (Stefanova *et al.,* 2007).

Terminalia. brownii and *C. edulis* stimulated immunosuppressed macrophages to produce significantly increased levels of TNF-α compared to the control cells. Tumor necrosis factor (TNF)-alpha is a mediator of innate immune responses. It is clinically approved for the management of malignancies such as soft tissue sarcoma (Hoving *et al.,* 2006; Josephs *et al.,* 2018). Also, TNF-alpha activates components of adaptive immunity such as T-effector cells. Tumor necrosis factor alpha mediates inflammation during infections leading to the elimination of pathogenic fungi, bacteria, and viruses, and contributes to tissue repair after the elimination of the pathogenic agents (Marino *et al.,* 1997; Rocha *et al.,* 2017; Pathakumari *et al.,* 2020). Consequently, by stimulating the expression of TNF-alpha, it is attributable that *T. brownii* and *C. edulis* could activate similar therapeutic outcomes mediated via the TNF-alpha pathway. Similar studies have corroborated this argument. *Melia azedarach* was shown to inhibit tumor growth via TNF-α mediated mechanisms (Nerome *et al.,* 2020). Microbicidal abilities of *Plantago* major leaf extract were linked to its ability to stimulate macrophages to express TNF alpha (Gomez-Flores *et al.,* 2000).

Other medicinal plants have demonstrated stimulatory effects on innate immunity by enhancing the production of NO and TNF-α by macrophages. *Morinda citrifolia* has been demonstrated to enhance the NO and TNF-α production capacity of macrophages (Hong *et al.,* 2019). *Melia azedarach* has been revealed to induce TNF-α release by macrophages (Nerome *et al.,* 2020). Additional plants revealed to have the potential to stimulate macrophages to increase the production of TNF-α include *Alchornea cordifolia* (Kouakou *et al.,* 2013) and *Citrus unshiu* (Shin *et al.,* 2018). Boubaker *et al.* (2018), demonstrated that *Nitraria retusa* augments macrophage capacity to produce NO. Other plants possessing efficacy to promote NO production

by macrophages include *Basella rebra* (Park, 2014), *Ixeris polycephala* (Luo *et al.,* 2018), and *Pterospartum tridentatum*, among others (Martins *et al.,* 2017).

5.2.2 Effects of *T. brownii* and *C. edulis* Methanol and DCM Extracts on Cellular Immune Responses

5.1.3.1 Effects of *T. brownii* and *C. edulis* Extracts on TNF-α, IL-2, IFN-γ, IL-4, and IL-5

As revealed by the negative control, pyrogallol significantly altered mediators of adaptive immunity namely; TNF-α, IL-2, IFN-γ, IL-4, and IL-5. Pyrogallol significantly reduced levels of TNF-α. Pyrogallol has been previously reported to inhibit the expression of TNF-α (Son *et al.,* 2019; Choi *et al.,* 2019). However, the methanol and DCM extracts of *T. brownii* and *C. edulis* reversed this decline. TNF-α plays a role in the functioning and homeostasis of host immune defence. It has been demonstrated to possess antitumor properties against some types of cancer by inducing tumour necrosis (van Horssen *et al.,* 2006). In Europe, TNF-alpha is approved for the treatment of metastatic melanomas (van Horssen *et al.,* 2006). Besides, TNF-alpha has been shown to enhance the activity of anticancer cytostatic drugs (van Horssen *et al.,* 2006). Increased levels of TNF-alpha have been shown to enhance the protective immunity of RTS, S/AS malaria vaccine in a clinical trial (Lumsden *et al.,* 2011). Absence of TNF-α aggravated viral myocarditis in mice. However, injection of TNF-α was shown to protect mice against viral myocarditis (Wada *et al.,* 2001). Further, TNF-α potentiates the differentiation of naïve $CD4^+$ T cells to Tumor-specific Th9 cells and enhances the survival and proliferation of T cells thereby achieving an antitumor effect in mice tumor model (Jiang *et al.*, 2019). Thus, *T. brownii* and *C. edulis* could potentially induce similar pharmacological efficacies via mechanisms involving increased levels of TNF-α.

These findings concur with the findings of previous studies. Other plants have been revealed to enhance cellular immune responses by boosting levels of TNF-α in vivo. Ethanol extract of *Terminalia chebula has been reported to increase serum levels of* TNF-α in cyclophosphamide-induced immunosuppression in rats (Aher and Wahi, 2011). Alcohol extracts of *Curcuma aeruginosa* have been demonstrated to possess

immunostimulatory activities by elevating levels of TNF-α in immunocompromised rats (Sulfianti *et al.,* 2019). Aqueous extracts of *Scoparia dulcis* and *Indigofera tinctoria* were shown to inhibit noise stress immunosuppression in rats by boasting levels of TNF-α (Madakkannu and Ravichandran, 2017). These studies, among others, present plants as promising stimulatory agents for cellular immune responses via enhancing *in vivo* production of TNF-α.

Pyrogallol was shown to significantly suppress amounts of IL-2. On the contrary, *T. brownii* and *C. edulis* extracts boosted levels of IL-2 in pyrogallol-treated mice. IL-2 promotes the proliferation of macrophages, NK cells, and lymphocytes (Hoyer *et al.,* 2008; Abbas *et al.,* 2018). Despite of IL-2 being a pro-inflammatory cytokine, its deficiency results in strong Th1- and Th2-dependent immune reactions that result in the development of autoimmune illnesses (Abbas *et al.,* 2018). Deficiency of IL-2 in mice leads to the development of systemic autoimmune disorders including colitis and autoimmune hemolytic anemia (Hoyer *et al.,* 2008). This has been associated with a lack of production of Treg cells which are maintained and controlled by IL-2. Tregs act by suppressing effector T-cell proliferation and cytokine expression as well as inhibiting dendritic cells. In absence of IL-2, maintenance and proliferation of Tregs is compromised which results in uncontrolled activity of Th1- and Th2 responses. This in turn is manifested in autoimmune disorders. This mechanism is attributed to the pathogenesis of multiple sclerosis, systemic lupus erythematosus, diabetes, and Crohn's disease (Crispin *et al.,* 2003; Erdman *et al.,* 2003; Ehrenstein *et al.,* 2004). By indirect mechanism, via increased levels of IL-2, *T. brownii* and *C. edulis* extracts offer a promising remedy to boost the activity of Tregs and inhibit the progression of the associated autoimmune conditions.

Interleukin-2 is currently used to treat metastatic renal carcinoma and melanoma (Jiang, 2016). Besides, it is also being explored in combined therapy for the treatment of other types of cancer (Jiang, 2016). More so, IL-2 deficient mice models have aberrant structural hippocampal modifications as well as learning and memory deficits recognized in Alzheimer's disease (Alves *et al.,* 2017). IL-2 has been shown to expand and activate regulatory T cells which inhibits neuroinflammation. Also, it activates and recruits astrocytes in the hippocampus thereby decreasing amyloid

plaque amounts. Concomitantly, it led to the reversal of memory deficits suggesting the anti-Alzheimer's disease effects of IL-2 (Alves *et al.,* 2017). By augmenting the production of IL-2, *T. brownii* and *C. edulis* extracts could potentially be applied to augment the antitumor and anti-Alzheimer's efficacy of IL-2.

Besides *T. brownii* and *C. edulis,* other plant extracts have demonstrated the potential to boost the production of IL-2. Hydro-extract of *Nyctanthes arbor-tristis* flowers has been demonstrated to enhance IL-2 production by splenocytes (Bharshiv *et al.,* 2016). Ethanol extract of *mango mistletoe* leaf was reported to increase IL-2 levels in aged mice (Handono *et al.,* 2021). Aqueous bark extracts of *Rhizophora mangle* were revealed to stimulate the production of IL-2 in Jurkat T cells (De Armas *et al.,* 2016). *Angelica Keiskei* leaf ethanol extract enhanced the release of IL-2 in mice (Sudira and Merdana 2017). Ethanol extract of *Moringa olifera* extract was shown to boost IL-2 in diabetic rats (Wadee *et al.,* 2018). These findings reveal the potential of plants as immunostimulatory agents via stimulating IL-2 production.

Pyrogallol significantly suppressed levels of IFN-γ. However, extracts of *T. brownii* and *C. edulis* reversed the pyrogallol-mediated decline of IFN-γ in mice. Interferon-gamma advances adaptive immunity by regulating T-cell homeostasis; activation, differentiation, expansion, and survival (Green *et al.,* 2017). IFN-γ activates NK cells, macrophages, neutrophils, and dendritic cells. In viral infections, IFN-γ activates the switching of IgG2a isotype in B cells. A deficiency of IFN-γ has been indicated to fuel autoimmune illnesses. Due to its immune versatility, IFN-gamma has been revealed to possess antiviral, antimicrobial, and antitumor activity (Miller *et al.,* 2009; Xia *et al.,* 2016). Currently, it is being explored for the treatment of various viral diseases and malignancies. It has been approved by the Food and Drug Administration to treat severe malignant congenital osteopetrosis and chronic granulomatous disease (Miller *et al.,* 2009). Thus, by enhancing levels of IFN-gamma, *T. brownii* and *C. edulis* showed the potential to boost cellular immunity to fight conditions such as cancer and viral infections.

Other plants have been revealed to enhance cellular immunity by increasing levels of IFN-γ. Sudira and Merdana (2017) reported that ethanol extract of *Angelica Keiskei*

leaf enhanced the production of IFN-γ in mice. Extracts of *Echinacea angustifolia, Echinacea pallida,* and *Echinacea purpurea* have been revealed to enhance levels of IFN-γ in mice (Zhai *et al.,* 2007). *Olea europaea* extract was shown to enhance the release of IFN-γ from peripheral blood mononuclear cells (Magrone *et al.,* 2018). Extracts of *Eriobotrya japonica* enhance IFN-γ expression in mouse spleen cells, CD3+ T cells, and NK cells (Matalka *et al.,* 2016).

Th1 cells produce IL-2, TNF-α, and IFN-γ cytokines (Broere & van Eden, 2019). *Terminalia. brownii* and *C. edulis* extracts probably enhanced the production of IL-2, TNF-α, and IFN-γ by activating Th1 cells which are responsible for cellular-mediated adaptive immunity (Broere & van Eden, 2019). These cytokines stimulate macrophages as well as other myeloid lineage immune cells thereby enhancing phagocytic-dependent protective responses (Gandhi *et al.,* 2018; Lacaille-Dubois, 2019). Mainly, Th1 cells develop in response to intracellular bacterial and viral infections (Gandhi *et al.,* 2018; Lacaille-Dubois, 2019). Thus, *T. brownii* and *C. edulis* showed the ability to enhance activities mediated by Th1 cells in cellular immunity.

The negative control showed significantly elevated levels of IL-4 and IL-5. However, *T. brownii* and *C. edulis* lowered the levels of IL-4 and IL-5, in pyrogallol-treated mice. Perpetuation of IL-4 and IL-5 has been implicated in numerous allergic diseases. Interleukin IL-4 and IL-5 induce allergic inflammatory responses aimed at the elimination of environmental allergens or pathogens from the body (Gandhi *et al.,* 2017). However, exaggeration of these inflammatory responses results in the development of multiple illnesses; allergic rhinitis, allergic asthma, and atopic dermatitis (Gandhi *et al.,* 2017) and others. Multiple agents are evaluated to manage excessive allergic inflammatory responses by inhibiting the expression of IL-4 and IL-5. *Terminalia brownii* and *C. edulis* showed promising potential for use in the management of excessive allergic inflammatory responses by attenuating levels of IL-4 and IL-5 in mice.

Similarly, other medicinal plants have been demonstrated to attenuate levels of IL-4 and IL-5. *Siraitia grosvenorii* extract exerts anti-asthmatic activity by inhibiting

ovalbumin (OVA)-stimulated production of IL-4 and IL-5 in lungs of mice (Sung *et al.,* 2019). *Carica papaya* reduces the severity of allergic asthma by decreasing the expression of IL-4 and IL-5 stimulated with OVA in muce (Inam *et al.,* 2017). *Pistacia integerrima* attenuates inflammatory airway by reducing levels of IL-4 and IL-5 in an ova-treated mouse model of allergic asthma (Rana *et al.,* 2016). Topically applied *Allium cepa* extract has been indicated to attenuate allergic rhinitis severity in mice allergic models by inhibiting the expression of OVA-stimulated IL-4 and IL-5 (Seo *et al.,* 2019). Thus, plants are promising agents for use in the management of allergic inflammation mediated via the IL-4 and IL-5 axis.

Reduction of IL-4 and IL-5 by *T. brownii* and *C. edulis* extracts revealed the ability of the plants to modulate cell-mediated immune responses orchestrated through Th2 cells. IL-4 and IL-5 are produced by Th2 cells (Nakayama *et al.,* 2017). Hence, the extracts can inhibit inflammatory responses induced by Th2 cells by inhibiting their ability to produce the effector cytokines: IL-4 and IL-5.

5.2.2.1 Effects of *T. brownii* and *Carissa edulis* Methanol and DCM Extracts on the DTH Reaction

Extracts of *T. brownii* and *C. edulis* enhanced DTH responses in SRBCs-sensitized mice. The DTH reaction is mediated by Th1 cells (Chan *et al*., 2007). The response is orchestrated in two phases; the sensitization phase in which Th1 cells become activated after interaction with an antigen. This is followed by clonal expansion of the activated Th1 cells. Their expansion is aided by antigen-presenting cells (dendritic cells, monocytes, and macrophages) expressing class II major histocompatibility complex molecules. Consequent antigenic encounter stimulates the effector phase of the DTH reaction. During the effector phase, Th1 cells express cytokines that mobilize and stimulate macrophages as well as non-specific inflammatory inducers (Vinegar *et al.,* 1987; Bafna *et al.,* 2004).

The lag time observed at the onset of the DTH reaction is attributed to the period taken by cytokines to potentiate the recruitment and activation of macrophages (Bafna *et al.,* 2004). The DTH reaction is directly correlated with cell-mediated adaptive immunity. Thus, by augmenting the DTH reaction, the extract revealed the

ability to enhance cellular immune responses. It is conceivable that *T. brownii* and *C. edulis* extracts enhanced the DTH reaction by stimulating committed Th1 cells to secrete cytokines and chemokines that drive the DTH reaction (Wang *et al*., 2017).

Previously plants have been revealed to enhance DTH reaction. Doshi and Une (2015) reported that extracts of *P. Iongifolia* leaf enhanced DTH response in rats. Extract of *Bombax ceiba* has been documented to enhance DTH reaction in mice (Wahab *et al.,* 2014). *Ocimum basilicum* has also been indicated to augment DTH reactions in mice (Rapando, 2021). Ogila (2013) reported that extracts of *Caesalpinia volkensii* and *Asparagus setaceous* enhanced DTH reaction in mice. *Psidium guajava* was shown to increase DTH reaction in cyclophosphamide-treated mice (Shabbir *et al.,* 2016). *Tinospora crispa* has demonstrated the ability to potentiate DTH in SRBC-sensitized mice. Extract of *Moringa oleifera* leaf was shown to elevate DTH reaction in mice sensitized with inactivated HSV-1 antigen. *Leptadenia pyrotechnica* extract augmented DTH response in SBRC-sensitized mice (Rasheed *et al.,* 2016). *Curcuma mangga* was shown to increase DTH responses in cyclophosphamide-treated rats (Yuandani *et al.,* 2021). Collectively, these examples reveal plants as promising agents to augment cellular immunity via stimulating DTH reaction.

5.2.3 Effects of *T. brownii* and *Carissa edulis* Methanol and DCM Extracts on Humoral Immune Responses

5.2.3.1 Effects of *T. brownii* and *Carissa edulis* Extracts on Antibody Titer Levels

Pyrogallol suppressed antibody production in mice. Nevertheless, extracts of *T. brownii* and *C. edulis* reversed the pyrogallol-induced inhibition of antibody production in mice. This was manifested by increased antibody production in extract-treated mice. Indeed, the findings confirmed the ability of the extract to enhance humoral immune responses. Antibodies are the cornerstone of the body's defense system. They attach to antigens thereby neutralizing them (Theofilou *et al.,* 2021; James, 2022). Besides, they cross-link with antigens forming a matrix broken down by phagocytic cells of the immune system (Theofilou *et al.,* 2021; James, 2022).

It is arguable that *T. brownii* and *C. edulis* enhanced antibody titers probably by stimulating B and T cells involved in antibody production. Plasma B lymphocytes interact with soluble antigens in circulation whereby they are stimulated by T cell-produced cytokines to produce antibodies (Doshi and Une, 2015). Also, long-lived memory B cells produce antibodies to previously encountered antigens (Doshi and Une, 2015). The findings of the present study suggest that the extract has the potential to stimulate and enhances B lymphocytes as well as subsets of T cells involved in the synthesis of antibodies. Thereby, the findings confirm the ability of the extracts to augment humoral immunity.

Multiple plants have been demonstrated to stimulate humoral immunity. Ogila (2013) reported that extracts of *Asparagus setaceous* and *Caesalpinia volkensii* enhance humoral immunity by stimulating antibody production in mice. Extracts of *E. pallida, E. purpurea,* and *E. angustifolia* were shown to enhance antibody production in mice models (Zhai *et al.,* 2007). Extracts of *Polyalthia Iongifolia* leaf do demonstrate abilities to enhance antibody production in rats (Doshi and Une, 2015). *Arisaema jacquemontii* extract has been reported to alleviate mitogen-mediated suppression of T and B cell proliferation with the ultimate increase of humoral response as indicated by high antibody titter mice (Sudan *et al.,* 2014). *Bombax ceiba* extract has been indicated to enhance antibody production in mice (Wahab *et al.,* 2014). Rapando (2021) indicated that *Ocimum basilicum* enhances antibody production in cyclophosphamide-immunosuppressed mice. Bharshiv *et al.* (2016) documented the ability of the extract of *Nyctanthes arbor-tristis* flowers to enhance antibody production in mice. Taken together, these findings demonstrated the ability of herbal plants to enhance humoral immunity.

5.2.4 Safety Profiles of *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol and DCM Extracts

Acute and sub-acute studies were conducted to establish toxicity levels of *T. brownii* and *C. edulis* methanol and DCM extracts. Safety studies of herbal remedies are crucial in ascertaining probable detrimental effects and establishing susceptibility

levels (Ugwah-Oguejiofor *et al*., 2019). Thus, safety studies aid in the selection of doses for pre-clinical studies (Kpemissi *et al.,* 2020).

Mortality, changes in body weights, and the general appearance and behavior of experimental animals are crucial indices in evaluating the initial toxicity signs of herbal products (Ezeja *et al*., 2014). In the acute toxicity studies, *T. brownii* and *C. edulis* methanol and DCM extracts, given orally as a single dose of 2,000 mg/kg, did not prompt death or affect the body weights, behavior, and appearance of the rats. The LD_{50} of the extracts was therefore deduced to be above 2,000 mg/kg (OECD, 2022). According to the OECD guidelines and the GSH system of classification, the extracts were considered to be under the Class 5 category of chemicals since they were not toxic (OECD, 2022).

Besides *T. brownii* and *C. edulis*, other medicinal plants have been shown to be non-toxic in acute toxicity studies. *Withania frutescens* extract did not affect mice's body weights or behavior up to 2,000mg/kg (El Moussaoui *et al*., 2020). The LD_{50} of *Vernonia mespilifolia* aqueous extract was demonstrated to be above 5,000mg/kg (Unuofin *et al.,* 2018). In addition, *Reinwardtia indica* leaves hydro-alcoholic extract was shown to be safe up to 5,000mg/kg (Upadhyay *et al*., 2019). *Artemisia annua* hydroethanolic extract, administered up to 5,000 mg/kg, did not trigger mortality nor cause any toxic signs (Nkuitchou-Chougouo *et al.,* 2022). *Caralluma dalzielii* aqueous extract did not cause mortality or toxicity in rats and mice up to a dosage of 2,000mg/kg (Ugwah-Oguejiofor *et al*., 2019).

Acute toxicity studies result in data that have minimal application in clinical studies. This is because even quite low dosages of herbal products can cause accumulative adverse effects (Al-Afifi *et al.,* 2018). Therefore, studies involving repeated administration of plant extracts are crucial in determining the safety of medicinal plants. Variations in body weights of experimental animals depict deleterious effects of test drugs or chemicals (Amenya *et al.,* 2014). Over 20% reduction in body weight is considered a humane end-point (OECD, 2000). In the sub-acute toxicity studies, all the tested extracts, at 300, 520 and 900mg/kg, did not affect the body weights of the rats throughout the test period; weight gain was normal from weeks one to four.

These results demonstrate that *T. brownii* and *C. edulis* methanol and DCM extracts were not toxic (Vysakh *et al*., 2020).

Similarly, several medicinal plants have been demonstrated not to cause changes in the body weights of laboratory animals in sub-acute toxicity studies. *Crassocephalum rabens* aerial parts ethanol extract, administered up to 1,666.7 mg/kg, showed no effect on the body weights of rats (Hsu *et al.,* 2022). Rats treated with the methanol extract of *Dracaena cinnabari* at 1,500 mg/kg showed comparable body weights with the normal control rats ((Al-Afifi *et al.,* 2018). *Croton blanchetianus* ethyl acetate fraction at 1,000mg/kg did not trigger changes in the body weights of mice (de Oliveira *et al.,* 2022). No significant difference was noted between the body weights of rats treated with *Crassocephalum rubens* aqueous extract up to 1,000mg/kg and the control rats over the four weeks of the study (Adewale *et al.,* 2016). Rats treated with *Nasturtium officinale* standardised extract at 1,000 mg/kg did not show significant changes in body weights compared with the control rats (Clemente *et al.,* 2019).

Changes in organ weight could symbolize toxicity, organ injury, physiological imbalance, or effects on various enzymes (Ugwah-Oguejiofor *et al*., 2019). *Terminalia brownii* and *C. edulis* methanol and DCM extracts, at the three tested doses, did not affect the weights of the various rat organs. These findings further indicate the non-toxic nature of *T. brownii* and *C. edulis* (Vysakh *et al*., 2020). These results are in agreement with those of previous studies. *Rapanea melanophloeos* stem bark chloroform extract was found not to affect organ weights up to 1,000mg/kg in sub-acute toxicity studies (Alkahtani *et al.,* 2022). Mice treated with *Rhizophora mucronata* extract at 1,000mg/kg exhibited comparable relative organ weights with the normal control rats (Chitra *et al*., 2020). No significant difference was observed in the relative organ weights between mice treated with *Artemisia afra* aqueous extract up to 1,800 mg/kg and the normal control rats (Mekonen *et al.,* 2020).

The haematological system is highly vulnerable to noxious compounds. It is, therefore, a crucial index for the pathological and physiological status of animals and humans (Ghosh *et al,* 2019). The principal transport medium throughout the body is

blood. This makes blood components the first to be exposed to high concentrations of toxins (Adewale *et al.,* 2016). Assessment of hematological parameters is consequently crucial while studying the safety of medicinal plants. *Terminalia brownii* and *C. edulis* methanol and DCM extracts, at the three tested doses, did not alter concentrations of the analysed hematological parameters. Therefore, the extracts can be considered non-toxic.

These findings are in agreement with the results of previous studies. The ethanol extracts of *Cassia fistula* (Abid & Mahmood, 2019), *Nectandra leucantha* (Ferreira *et al.,* 2021) and *Acacia catechu* seeds (Thangavelu *et al.,* 2020), *Crassocephalum crepidioides* leaves aqueous extract (Nguemfo *et al*., 2021) and *Lippia multiflora* leaves extract (Allo *et al*., 2020), among others, did not alter concentrations of the studied hematological parameters up to a 1,000 mg/kg dose.

One of the major functions of the liver is toxicants/drugs/chemicals metabolism, making the liver highly vulnerable to damage (Yusuf *et al.,* 2018). It is therefore crucial to assess biochemical parameters, including ALP, AST, ALT, total proteins, albumin, and bilirubin in safety studies of medicinal plants (Ugwah-Oguejiofor *et al*., 2019). Increased concentrations of these parameters in serum indicate liver toxicity, liver necrosis, or hepatitis (Mia *et al.,* 2022). Among the liver enzyme markers, ALT is the best marker to detect liver toxicity. This is because alterations in AST concentrations are also seen in various muscle and heart diseases (Ugwah-Oguejiofor *et al*., 2019). Additionally, ALP is also assessed in the diagnosis of biliary system obstruction (Ezeja *et al*., 2014; Adewale *et al.,* 2016). The kidneys are involved in the regulation of urea and creatinine excretion, and electrolytes re-absorption into the blood. Glomeruli damage results in the accumulation of these parameters in the blood (Shittu *et al*., 2015; Yusuf *et al.,* 2018). Therefore, electrolytes, creatinine, and urea are crucial parameters in the assessment of renal function (Abouelghar *et al*., 2020). *Terminalia brownii* and *C. edulis* methanol and DCM extracts, at the tested doses, did not alter concentrations of the examined biochemical parameters. These findings demonstrate *T. brownii* and *C. edulis* as non-toxic (Vysakh *et al*., 2020).

According to Vadakkan (2019), the aqueous root extract of *Solanum torvum* didn't alter concentrations of the assessed biochemical parameters. Other medicinal plants reported not to alter concentrations of biochemical parameters in sub-acute toxicity studies include *Brassica carinata* (Nakakaawa *et al*., 2023), *Solanum giganteum* (Shende & Kakadiya, 2022), *Triclisia gilletii* (Cimanga-Kanyanga *et al*., 2018) and *Combretum dolichopetalum* (Emelike *et al.,* 2020), among others.

5.2.5 Phytochemical Profiles of *T. brownii* Stem Bark and *C. edulis* Root Bark Methanol and DCM Extracts

Phytochemical profiling of *T. brownii* and *C. edulis* extracts revealed the presence of multiple compounds with immune-modulation efficacies. Lupeol, a triterpene, was noted in *T. brownii* methanol extract. Besides, lupeol has been identified in various medicinal plants including *Platonia insignis*, *Hylocereus polyrhizus*, among others (Souza *et al*., 2017; Wahdaningsih *et al.,* 2020). Lupeol has been shown to modulate both innate and adaptive immune responses. It was reported to enhance innate immunity via activating macrophages infected with *L. donovani* to produce a pro-inflammatory mediator, NO (Das *et al.,* 2017). Lupeol has also been demonstrated to stimulate cellular immune responses through augmentation of DTH reaction in immunosuppressed mice, the elevation of Th1 cytokines production, and reduction of Th2 cytokine levels in macrophages infected in *L. donovani* (Das *et al.,* 2017; Kaur *et al.,* 2019). Lupeol was shown to increase antibody titer in mice hence augmenting humoral immune responses (Maurya *et al*., 2012). Immunomodulatory properties of *P. insignis* and *H. polyrhizus* have been partly attributed to the presence of lupeol (Souza *et al*., 2017; Wahdaningsih *et al.,* 2020). Similarly, the immunomodulatory effects of *T. brownii* methanol extract could be associated with the presence of lupeol whose immune-modulating effects have been documented.

Betulinic acid, a lupine-type triterpene, was identified in *T. brownii* methanol extract. The compound has been detected in several medicinal plants including *Betula papyrifera, Euphorbia spinidens, Lycopus lucidus* amongst others. Immunomodulatory properties *of E. spinidens B. papyrifera* and *L. lucidus* have been ascribed to the presence of betulinic acid (Yun *et al*., 2003; Yi *et al*., 2010; Tiwari *et*

al., 2019). Betulinic acid has been reported to modulate innate immune responses. It has been shown to increase lymphocyte production in mice and activate mouse macrophages to produce a pro-inflammatory cytokine, TNF-α (Yi *et al*., 2010; Lis *et al.,* 2019). Therefore, the observed capacity of *T. brownii* extracts to modulate innate immune responses in the present study could be due to the presence of betulinic acid.

Resveratrol, a polyphenolic compound, was also detected in the stem bark methanol extract of *T. brownii.* The presence of resveratrol has been reported in various medicinal plants including *Veratrum grandiflorum, Polygonum cuspidatum, Vitis vinifera,* among others (Gambini *et al.,* 2015; Salehi, *et al* 2018). The immunomodulatory properties of resveratrol have been widely studied. Resveratrol restored WBC counts to normal levels in LPS-treated rats (Wang *et al.,* 2017). Cyclophosphamide-immunosuppressed mice produced enhanced levels of Th1 cytokines after treatment with resveratrol (Lai *et al.,* 2016). In addition, resveratrol was demonstrated to modulate adaptive immune responses by restoring Th1/Th2 cytokine levels in human T lymphocytes (Schwager *et al.,* 2021). Dietary supplementation using resveratrol augmented the DTH response in rats hence enhancing cellular immune responses (Yuan *et al*., 2012). The immunomodulatory activity of stem bark methanol extract of *T. brownii* could therefore be partly attributed to the resveratrol present in the extract.

In *T. brownii* methanol extract, the presence of a flavonoid, quercetin, was noted. Quercetin has been identified in other medicinal plants with immunomodulatory properties including *Illicium verum, Tamarindus indica, Allium cepa, Vitis vinifera,* among others (Liu *et al.,* 2020; Yi *et al.,* 2021). Quercetin has been reported to modulate both innate and adaptive immune responses. Administration of quercetin to immunotoxicated mice significantly increased total and differential leukocyte counts (Sallam *et al.,* 2022). In addition, quercetin restored WBC counts to normal levels in Adriamycin-immune dysregulated rats (Merzoug *et al.,* 2014). Lipopolysaccharide-stimulated RAW 264.7 cells were found to produce enhanced levels of NO compared to the control (Ilyas *et al*., 2021). Quercetin was also demonstrated to augment cellular immune responses via increasing DTH reaction in mice and stabilizing Th1/Th2 cytokine levels in splenic mononuclear cells (Farhadi *et al.,* 2014;

Ravikumar *et al*., 2020). The ability of *T. brownii* methanol extract to modulate both innate and adaptive immune responses could thus be ascribed to the presence of quercetin in the extract.

Naringenin, a flavanone, was identified in *T. brownii* methanol extract. Naringenin has also been identified in *Codonopsis clematidea, Ficus carica, Solanum lycopersicum*, among other medicinal plants (Kaur *et al.,* 2018; Soltana *et al.,* 2018; Salehi *et al*., 2019). Naringenin was found to enhance innate immune responses by augmenting the production of NO in *L. donovani*-infected mouse macrophages (Kaur *et al.,* 2018). Additionally, naringenin significantly enhanced DTH response and restored Th1/Th2 cytokine levels to normalcy in the serum of *L. donovani*-inflected mice hence stimulating cellular immune responses (Kaur *et al.,* 2018; Noori *et al.,* 2022). Immunomodulatory properties of *C. clematidea, F. carica,* and *S. lycopersicum* have been attributed to naringenin. In the same way, the observed immunomodulatory effect of *T. brownii* methanol extract in pyrogallol-immunosuppressed mice could be due to the presence of naringenin.

The presence of an ellagitannin, corilagin, was also noted in *T. brownii* methanol extract. Corilagin has been isolated from a variety of medicinal plants with immunomodulatory effects including *Phyllanthus niruri, Dimocarpus longan, Phyllanthus maderaspatensis,* among others (Moreira *et al.,* 2013; Zheng *et al.,* 2016; Kochumadhavan *et al.,* 2019). Corilagin has been reported to augment innate immune responses via activating *L. major*-infected RAW 264.7 cells to produce NO and TNF-a (Li *et al.,* 2018). The observed innate immunity-enhancing effect of *T. brownii* methanol extract could hence be ascribed to the presence of corilagin in the extract.

Methanol extract of *T. brownii* was also found to contain a phenol, scopoletin. Moreover, scopoletin has been reported in other medicinal plants with immunomodulatory properties including *Aegle marmelos, Morinda citrifolia, Urtica diocia, Argyreia speciosa* among others (Kashyap *et al*., 2020; Joshi *et al.,* 2021; Çakır *et al.,* 2022). Scopoletin has been shown to modulate humoral immune responses by lowering levels of Th2 cytokines produced by EL-4 T cells and

ovalbumin-sensitized mice (Cheng *et al.,* 2012; Aldi *et al.,* 2015). In the same way, *C. edulis* methanol extract was probably able to modulate humoral immune responses via reducing Th2 cytokine levels in mice serum due to the presence of scopoletin in the extract.

Palmitic acid, a saturated fatty acid, was detected in *T. brownii* DCM extract. In agreement with this finding, palmitic acid has also been reported in *Aesculus indica, Sisymbrium irio, and Labisia pumila* among other medicinal plants with immunomodulatory properties (Karimi *et al.,* 2015; Nengroo & Rauf *et al.,* 2019). Classical activation of mouse macrophages, hence stimulation of innate immune responses, by palmitic acid has been reported (de Lima *et al.,* 2006; de Lima-Salgado *et al.,* 2011; Korbecki & Bajdak-Rusinek 2019; Tsai *et al.,* 2021). Thereby, the presence of palmitic acid in *T. brownii* DCM extract could have contributed to the observed innate immunity-enhancing effects of the extract.

In *T. brownii* DCM extract, linoleic acid (fatty acid), was also noted. Linoleic acid has been also been detected in other medicinal plants with immunomodulatory properties including *Perilla frutescens, Aesculus indica, Sisymbrium irio,* among others (Nengroo & Rauf *et al.,* 2019; Quílez *et al.,* 2020). Linoleic acid was reported to augment innate immune responses via stimulating macrophages to produce NO, a key pro-inflammatory mediator (de Lima *et al.,* 2006). Dichloromethane extract of *T. brownii* could thus have stimulated innate immune responses partly due to the presence of linoleic acid.

Stearic acid, a fatty acid, was identified in *T. brownii* DCM extract. In addition, stearic acid has been identified in *Citrullus colocynthis, Mentha piperita, Salvia sclareae,* among other medicinal plants (Tulukcu, 2011; Berwal *et al.,* 2022). Stearic acid has been shown to augment innate immune responses. Low dosages (1 to10µM) of stearic acid stimulated the production of NO by J774 cells (de Lima *et al.,* 2006). Further, LPS-treated J774 cells produced enhanced levels of TNF-α upon treatment with stearic acid (de Lima-Salgado *et al.,* 2011). Therefore, the observed ability of *T. brownii* DCM extract to classically activate macrophages could be attributed to the presence of stearic acid.

Dichloromethane extract of *T. brownii* stem bark was also found to contain a triterpene, squalene. Various medicinal plants with immunomodulatory properties including *Amaranthus spinosus, Olea europaea, and Vitis vinifera,* among others, have been reported to contain squalene (Lozano-Grande *et al.,* 2018). Squalene was demonstrated to reverse 3-methylcholanthrene-induced immunotoxication by restoring WBC counts to normal levels thus augmenting innate immune responses (Suriyakalaa *et al*., 2018). Similarly, dichloromethane extract of *T. brownii* was probably able to reverse the reduced WBC counts in pyrogallol-immunosuppressed mice due to the presence of squalene.

Campesterol, a steroid, was detected in *T. brownii* DCM extract. Besides, campesterol has been identified in *Artemisia absinthium, Artemisia annua* among other medicinal plants. Innate immunity stimulatory effects of campesterol have been reported. Campesterol was found to activate mouse macrophages to produce a pro-inflammatory cytokine, TNF-α (Kurano *et al.,* 2011). The ability of *T. brownii* DCM extract to augment innate immune responses via stimulating murine macrophages to produce TNF-α could hence be ascribed to the presence of campesterol in the extract.

Terminalia brownii DCM extract was also found to contain stigmasterol, a steroid. Furthermore, stigmasterol has been identified in plants with immunomodulatory properties including *Pimpinella anisum, Carum carvi, Apium graveolens, Foeniculum vulgare*, among others (Saini *et al.,* 2021). Stigmasterol has been reported to augment both innate and adaptive immune responses. Treatment of RAW264.7 cells and splenocytes obtained from immunosuppressed mice with stigmasterol augmented the production of a pro-inflammatory mediator, NO, thus stimulating innate immune responses (Kurano *et al.,* 2011). Stigmasterol was also found to enhance the production of Th1 cytokines in the serum and spleen of cyclophosphamide-immunosuppressed mice thus augmenting cellular immune responses (Kurano *et al.,* 2011). The presence of stigmasterol in *T. brownii* DCM extract could consequently be attributed to the observed immunostimulatory activity of the extract.

Carissa edulis root bark methanol extract was found to contain a triterpenoid, ursolic acid. Ursolic acid has been identified in medicinal plants including *Punica granatum, Olea europaea, Calendula officinalis, Coffea arabica* among others (Sharifiyan *et al.,* 2019; Gudoityte *et al*., 2021). Immunomodulatory properties of ursolic acid have been reported. Ursolic acid-treated mice were found to produce elevated levels of WBC counts in comparison with the control mice, hence augmenting innate immunity (Raphael & Kuttan, 2003). In addition, ursolic acid-treated mice produced an enhanced DTH response compared with control mice thus enhancing cellular immune responses (Raphael & Kuttan, 2003). Ursolic acid enhanced humoral immune responses by increasing antibody titers in mice serum (Raphael & Kuttan, 2003). Therefore, the observed ability of *C. edulis* methanol extract to enhance both innate and adaptive immune responses could be associated with the presence of ursolic acid in the extract.

Carissa edulis root bark methanol extract was found to contain oleuropein, a phenolic compound. Oleuropein has also been identified in *Olea europaea, Syringa pubescens, Ligustrum lucidum, Fraxinus angustifolia,* among other medicinal plants (Hashmi *et al*., 2015; Hassen *et al*., 2015). Oleuropein has been shown to activate LPS-treated macrophages to produce a pro-inflammatory mediator, NO, thus augmenting innate immune responses (Omar, 2010). Methanol extract of *C. edulis* probably stimulated innate immune responses due to the presence of oleuropein.

Beta-Sitosterol, a steroid, was detected in *C. edulis* methanol extracts. Besides, β-sitosterol has been identified in *Punica granatum, Persea americana, Cucurbita pepo* among other medicinal plants (Nweze *et al*., 2019; Pizzorno & Murray, 2020). Immunomodulatory properties of β-sitosterol have been reported. Beta-sitosterol was found to enhance the production of TNF-α, a proinflammatory cytokine, by macrophages (Sabeva *et al.,* 2011; Plat *et al.,* 2019). In addition, β-sitosterol was demonstrated to augment innate immune responses via increasing differential leukocyte counts in mice (Paniagua-Pérez *et al*., 2008). Treatment of asthmatic mice with β-sitosterol reduced Th2 cytokine levels in bronchoalveolar lavage and lung tissues thus stabilizing humoral immune responses (Yuk *et al*., 2007). In addition, β-sitosterol was reported to stabilize humoral immune responses by reducing Th2

cytokine (IL-4 and IL-10) levels in mitogen-treated splenocytes (Le *et al.,* 2017). Similarly, the methanol extract of *C. edulis* was probably able to modulate immune responses in pyrogallol-immunosuppressed mice due to the presence of β-sitosterol in the extract.

Chlorogenic acid, a polyphenol, was identified in *C. edulis* methanol extract. Similarly, chlorogenic acid has been identified in other medicinal plants including *Coffea arabica, Rosmarinus officinalis, Lonicera japonica* among others (Moreira *et al.,* 2018; Chaowuttikul *et al*., 2020). Chlorogenic acid modulates cellular and humoral immune responses. Treatment of allergic rhinitis mice with chlorogenic acid reversed the imbalance in Th1 and Th2 cytokine levels induced by ovalbumin thus augmenting cellular immune responses and stabilizing humoral immunity (Dong *et al.,* 2020). Therefore, the observed ability of *C. edulis* methanol extract to modulate immune responses could be ascribed to the chlorogenic acid present in the extract.

Carissa edulis methanol extract was also found to contain a flavonoid, luteolin. Luteolin has also been identified in other medicinal plants including *Codariocalyx motorius, Chrysanthemum indicum, Artemisia asiatica,* among others (Aziz *et al*., 2018). Luteolin has been shown to restore leukocyte counts to normal levels in mercuric chloride-treated rats thus stabilizing innate immune responses (Yang *et al*., 2016). Additionally, luteolin-treated NK cells were found to produce increased levels of Th1 cytokines (Oo *et al*., 2022). Therefore, the observed ability of *C. edulis* methanol extract to enhance both innate and adaptive immune responses could be associated with the presence of luteolin in the extract.

Oleic acid, a fatty acid, was detected in *C. edulis* DCM extract. In agreement with this finding, oleic acid has been detected in *Citrullus colocynthis*, *Olea europaea, Sisymbrium irio* among other medicinal plants (Berwal *et al.,* 2022; Hernández *et al.,* 2021). Oleic acid has been reported to stabilize humoral immune responses by reducing the production of Th2 cytokines in bronchoalveolar lavage fluid obtained from ovalbumin-treated mice (Kim *et al*., 2021). Consequently, *C. edulis* DCM extract could have stabilized humoral immunity due to the presence of oleic acid in the extract.

Carissa edulis DCM extract was also found to contain a triterpenoid, taraxasterol. Taraxasterol has also been identified in other medicinal plants with immunomodulatory properties including *Mangifera indica, Olea europaea, Taraxacum officinale, Hypericum perforatum* among others. Asthmatic mice models treated with taraxasterol produced reduced levels of Th2 cytokines including IL-5, IL-4, and IL-13 in bronchoalveolar lavage fluid thus stabilizing humoral immune responses. The presence of taraxasterol in *C. edulis* DCM extract could thus be attributed to the observed modulatory activity of the extract on humoral immunity.

5.3 Conclusions, Limitations of the Study and Recommendations

5.3.1 Conclusions

i. Methanol and DCM extracts of *Terminalia brownii* and *C. edulis* enhanced innate immune responses by stimulating production of leukocytes (neutrophils, lymphocytes, monocytes, eosinophils and basophils) in immunosuppressed mice. The extract also stimulated macrophages to produce nitric oxide (NO) and tumor necrosis factor-α (TNF-α).
ii. *Terminalia brownii* and *C. edulis* methanol and DCM extracts modulated cellular immune responses by enhancing production of TNF-α, IFN- γ and IL-2 while reducing levels of IL-4 and IL-5 in immunocompromised mice. Besides, they augmented DTH responses in SRBCs sensitized mice.
iii. Methanol and DCM extracts of *T. brownii* and *C. edulis* augmented humoral immune responses as evidenced by increased antibody titer in immunosuppressed mice.
iv. *Terminalia brownii* and *C. edulis* exhibited synergism in both innate and adaptive immune responses.
v. *Terminalia brownii* and *C. edulis* methanol and DCM extracts did not cause acute and sub-acute toxicity in mice. This was confirmed by the normal body weight gain, behaviour and general appearance of mice, organ weights and concentrations of haematological and biochemical parameters.
vi. Methanol and DCM extracts of *T. brownii* and *C. edulis* contained phytochemicals with immunomodulatory properties. Thus, the study

concludes that the immunomodulatory properties exerted by the extracts of *C. edulis* and *T. brownii* could be attributed to the presence of those compounds.

5.3.2 Limitations of the Study

i. The study did not use an infection to determine the effects of the plant extracts on production of antibodies.

5.3.3 Recommendations

i. *Terminalia brownii* and *C. edulis* methanol and DCM extracts should be considered as promising candidates for developing immunostimulatory herbal formulations.
ii. Further comprehensive studies on the mechanism of immunomodulation by the extracts should be carried out.
iii. Isolation of immunomodulatory compounds from the extracts of *T. brownii* and *C. edulis* should be conducted.
iv. Study on the efficacy of *T. brownii* and *C. edulis* extracts on specific immune-related disorders should be done to establish potential of the extracts to treat such disorders.
v. To fully elucidate the effects of the extracts on antibody production, and hence humoral immune responses, a study employing an infection can be conducted.
vi. Toxicity tests of *T. brownii* and *C. edulis* extracts in higher organisms should be carried to confirm safety of the plant extracts.

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APPENDICES

Appendix I: NACOSTI Research Permit

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: 134085

Date of Issue: 31/January/2022

RESEARCH LICENSE

This is to Certify that Miss.. jane Wanja mbiri of Jomo Kenyatta University of Agriculture and Technology, has been licensed to conduct research in Nairobi on the topic: IMMUNOMODULATORY PROPERTIES OF Carissa edulis (Forssk.) Vahl AND Terminalia brownii Fresen. EXTRACTS for the period ending : 31/January/2023.

License No: NACOSTI/P/22/14916

134085

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

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Appendix II: Ethical Approval Letter

KENYATTA UNIVERSITY
ANIMAL CARE AN USE COMMITTEE

Fax: 8711242/8711575
Email: chairman.kuerc@ku.ac.ke

P. O. Box 43844,
Nairobi, 00100

Website: www.ku.ac.ke

Tel: 8710901/12

Our Ref: **KU/ACUC/APPROVAL/VOL.1/05**

Date: 4th October, 2022

Jane Wanja Mbiri
P.O BOX 43844-00100
Nairobi.

Dear Jane,

RE: IMMUNOMODULATORY PROPERTIES OF TERMINALIA BROWNII FRESEN AND CARISSA EDULIS (FORSSK) VAHL EXTRACTS

This is to inform you that ***KENYATTA UNIVERSITY ANIMAL CARE AND USE COMMITTEE*** has approved the study protocol together with the attached SOPs dated 30th August 2022. Your application approval number is ***PKUA/005/005.*** The approval period is from ***4th October 2022 to 4th October 2023.***

This approval is subject to compliance with the following requirements;

i. Only approved documents including SOPs will be used in the study
ii. All amendments, deviations are to be submitted using relevant forms for review and approval by the Animal Care and Use Committee
iii. Death and life threatening problems of the study animals and handlers and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to the Animal Care and Use Committee within 72 hours of notification.
iv. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study animals and handlers or affect the integrity of the research should be reported to the Animal Care and Use Committee.
v. Clearance for export of biological specimens must be obtained from relevant institutions.

vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report using relevant forms to support the renewal.

vii. Submission of an executive summary report within 90 days upon completion of the study to Animal Care and Use Committee.

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://research-portal.nacosti.go.ke and also obtain other clearances needed.

To serve you better, researchers are kindly requested to access and complete a customer feedback form and sent it back online as you continue with research and upon completion of data collection found on the following website link; ;(https://docs.google.com/forms/d/1ytWefDwvyz5h1oz_VIn0xbxg3uGdlDzMXFWNDsMrRPQ/edit?usp=sharing

Yours sincerely

KENYATTA UNIVERSITY
OFFICE OF THE DEAN
OCT 2022

Prof. Michael Gicheru
Chairman, Animal Care and Use Committee

Appendix III: Analysis of the Effect of *T. brownii* Methanol Extract on Total and Differential Leukocyte Counts

Descriptive Statistics: WBC (10^9/L)

Variable	Group	Mean	SE Mean	StDev
WBC (10^9/L)	100mg/kg bw	3.950	0.155	0.346
	150mg/kg bw	5.456	0.123	0.276
	50mg/kg bw	2.852	0.139	0.312
	Negative control	0.9200	0.0867	0.1938
	Normal Control	5.954	0.112	0.251
	Positive control	6.016	0.177	0.395

Descriptive Statistics: Neutrophils (10^9/L)

Variable	Group	Mean	SE Mean	StDev
Neutrophils (10^9/L)	100mg/kg bw	1.7000	0.0575	0.1286
	150mg/kg bw	2.3900	0.0466	0.1042
	50mg/kg bw	1.2100	0.0666	0.1488
	Negative control	0.4000	0.0518	0.1158
	Normal Control	2.5040	0.0737	0.1647
	Positive control	2.620	0.106	0.236

Descriptive Statistics: Lymphocytes(10^9/L)

Variable	Group	Mean	SE Mean	StDev
Lymphocytes(10^9/L)	100mg/kg bw	1.898	0.120	0.269
	150mg/kg bw	2.6020	0.0539	0.1205
	50mg/kg bw	1.4240	0.0820	0.1832
	Negative control	0.4840	0.0383	0.0856
	Normal Control	2.8260	0.0643	0.1438
	Positive control	2.8180	0.0566	0.1266

Descriptive Statistics: Monocytes (10^9/L)

Variable	Group	Mean	SE Mean	StDev
Monocytes (10^9/L)	100mg/kg bw	0.2240	0.0133	0.0297
	150mg/kg bw	0.2580	0.0334	0.0746
	50mg/kg bw	0.13000	0.00316	0.00707
	Negative control	0.010000	0.000000	0.000000
	Normal Control	0.3540	0.0163	0.0365
	Positive control	0.3080	0.0156	0.0349

Descriptive Statistics: Eosinophils (10^9/L)

Variable	Group	Mean	SE Mean	StDev
Eosinophils (10^9/L)	100mg/kg bw	0.09600	0.00510	0.01140
	150mg/kg bw	0.1540	0.0103	0.0230
	50mg/kg bw	0.05800	0.00374	0.00837
	Negative control	0.01400	0.00245	0.00548
	Normal Control	0.1980	0.0116	0.0259
	Positive control	0.20200	0.00860	0.01924

Descriptive Statistics: Basophils (10^9/L)

Variable	Group	Mean	SE Mean	StDev
Basophils (10^9/L)	100mg/kg bw	0.03200	0.00200	0.00447
	150mg/kg bw	0.05200	0.00374	0.00837
	50mg/kg bw	0.03000	0.00316	0.00707
	Negative control	0.01200	0.00200	0.00447
	Normal Control	0.07200	0.00374	0.00837
	Positive control	0.06800	0.00374	0.00837

One-way ANOVA: WBC (10^9/L) versus Group

Factor Information

Factor	Levels	Values
Group	6	100mg/kg bw, 150mg/kg bw, 50mg/kg bw, Negative control, Normal Control, Positive control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	5	102.947	20.5895	224.83	0.000
Error	24	2.198	0.0916		
Total	29	105.145			

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
Positive control	5	6.016	A
Normal Control	5	5.954	A
150mg/kg bw	5	5.456	A
100mg/kg bw	5	3.950	B
50mg/kg bw	5	2.852	C
Negative control	5	0.9200	D

Means that do not share a letter are significantly different.

O`ne-way ANOVA: Neutrophils (10^9/L) versus Group

Factor Information

Factor	Levels	Values
Group	6	100mg/kg bw, 150mg/kg bw, 50mg/kg bw, Negative control, Normal Control, Positive control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	5	19.1706	3.83412	157.75	0.000
Error	24	0.5833	0.02430		
Total	29	19.7539			

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
Positive control	5	2.620	A
Normal Control	5	2.5040	A
150mg/kg bw	5	2.3900	A
100mg/kg bw	5	1.7000	B
50mg/kg bw	5	1.2100	C
Negative control	5	0.4000	D

Means that do not share a letter are significantly different.

One-way ANOVA: Lymphocytes(10^9/L) versus Group

Factor Information

Factor	Levels	Values
Group	6	100mg/kg bw, 150mg/kg bw, 50mg/kg bw, Negative control, Normal Control, Positive control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	5	21.7689	4.35379	158.99	0.000
Error	24	0.6572	0.02738		
Total	29	22.4261			

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
Normal Control	5	2.8260	A
Positive control	5	2.8180	A
150mg/kg bw	5	2.6020	A
100mg/kg bw	5	1.898	B
50mg/kg bw	5	1.4240	C
Negative control	5	0.4840	D

Means that do not share a letter are significantly different.

One-way ANOVA: Monocytes (10^9/L) versus Group

Factor Information

Factor	Levels	Values
Group	6	100mg/kg bw, 150mg/kg bw, 50mg/kg bw, Negative control, Normal Control, Positive control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value

Group	5	0.39572	0.079144	52.47	0.000
Error	24	0.03620	0.001508		
Total	29	0.43192			

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
Normal Control	5	0.3540	A
Positive control	5	0.3080	A B
150mg/kg bw	5	0.2580	B C
100mg/kg bw	5	0.2240	C
50mg/kg bw	5	0.13000	D
Negative control	5	0.01000	E

Means that do not share a letter are significantly different.

One-way ANOVA: Eosinophils (10^9/L) versus Group

Factor Information

Factor	Levels	Values
Group	6	100mg/kg bw, 150mg/kg bw, 50mg/kg bw, Negative control, Normal Control, Positive control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	5	0.148097	0.029619	98.73	0.000
Error	24	0.007200	0.000300		
Total	29	0.155297			

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
Positive control	5	0.20200	A
Normal Control	5	0.1980	A
150mg/kg bw	5	0.1540	B
100mg/kg bw	5	0.09600	C
50mg/kg bw	5	0.05800	D
Negative control	5	0.01400	E

Means that do not share a letter are significantly different.

One-way ANOVA: Basophils (10^9/L) versus Group

Factor Information

Factor	Levels	Values
Group	6	100mg/kg bw, 150mg/kg bw, 50mg/kg bw, Negative control, Normal Control, Positive control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	5	0.013937	0.002787	55.75	0.000

Error 24 0.001200 0.000050
Total 29 0.015137

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
Normal Control	5	0.07200	A
Positive control	5	0.06800	A
150mg/kg bw	5	0.05200	B
100mg/kg bw	5	0.03200	C
50mg/kg bw	5	0.03000	C
Negative control	5	0.01200	D

Means that do not share a letter are significantly different.

Variable	Treatment	Mean	SE Mean	StDev
HA (units/µL)	100mg/kg bw	4.667	0.333	0.577
	150mg/kg bw	7.333	0.333	0.577
	50mg/kg bw	3.667	0.333	0.577
	Negative control	1.000	0.577	1.000
	Normal control	4.0000	0.000000	0.000000
	Positive control	11.000	0.577	1.000

Appendix IV: Analysis of the Effect of *C. edulis* Methanol Extract on NO Production by Mouse Macrophages.

Descriptive Statistics: CM

Variable	Treatment	Mean	SE Mean	StDev
CM	1500µg/ml	39.689	0.544	0.943
	187.5µg/ml	9.320	0.604	1.046
	3000µg/ml	45.508	0.497	0.861
	375µg/ml	16.410	0.497	0.861
	46.88µg/ml	3.705	0.260	0.451
	750µg/ml	23.828	0.426	0.738
	93.75µg/ml	6.164	0.450	0.779
	Negative control	2.216	0.238	0.413
	Positive control	53.459	0.544	0.943

One-way ANOVA: CM versus Treatment

Factor Information

Factor	Levels	Values
Treatment	9	1500µg/ml, 187.5µg/ml, 3000µg/ml, 375µg/ml, 46.88µg/ml, 750µg/ml, 93.75µg/ml, Negative control, Positive control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	8	9080.66	1135.08	1738.06	0.000
Error	18	11.76	0.65		

Total 26 9092.42

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Treatment	N	Mean	Grouping
Positive control	3	53.459	A
3000µg/ml	3	45.508	B
1500µg/ml	3	39.689	C
750µg/ml	3	23.828	D
375µg/ml	3	16.410	E
187.5µg/ml	3	9.320	F
93.75µg/ml	3	6.164	G
46.88µg/ml	3	3.705	H
Negative control	3	2.216	H

Means that do not share a letter are significantly different.

Appendix V: Analysis of The Effect of Combined Methanol Extracts of *T. brownii* Stem Bark and *C. edulis* Root Bark on IL-2, TNF-α, IFN-γ, IL-4, and IL-5

Descriptive Statistics: IL-2

Variable	Treatment	Mean	SE Mean	StDev
IL-2	100mg/kg bw	535.20	4.84	10.83
	150mg/kg bw	566.20	4.16	9.31
	50mg/kg bw	505.40	5.03	11.24
	Negative control	318.20	4.47	9.98
	Normal Control	571.80	4.53	10.13
	Positive control	561.20	4.16	9.31

Descriptive Statistics: TNF

Variable	Treatment	Mean	SE Mean	StDev
TNF	100mg/kg bw	77.00	4.39	9.82
	150mg/kg bw	97.20	3.81	8.53
	50mg/kg bw	57.40	4.03	9.02
	Negative control	33.20	3.76	8.41
	Normal Control	98.60	5.01	11.19
	Positive control	97.00	4.64	10.37

Descriptive Statistics: IFN-γ

Variable	Treatment	Mean	SE Mean	StDev
IFN-γ	100mg/kg bw	359.20	6.13	13.70
	150mg/kg bw	391.60	2.86	6.39
	50mg/kg bw	332.40	4.15	9.29
	Negative control	191.60	5.89	13.16
	Normal Control	394.00	7.80	17.45
	Positive control	387.80	4.41	9.86

Descriptive Statistics: IL-4

Variable	Treatment	Mean	SE Mean	StDev
IL-4	100mg/kg bw	172.60	5.02	11.22
	150mg/kg bw	143.40	6.65	14.88
	50mg/kg bw	199.40	4.53	10.14
	Negative control	307.80	4.07	9.09
	Normal Control	140.60	7.57	16.92
	Positive control	145.60	5.81	12.99

Descriptive Statistics: IL-5

Variable	Treatment	Mean	SE Mean	StDev
IL-5	100mg/kg bw	131.60	3.89	8.71
	150mg/kg bw	106.40	5.05	11.28

50mg/kg bw 154.60 4.83 10.81

Negative control 219.60 3.83 8.56

Normal Control 104.00 7.26 16.23

Positive control 109.00 4.48 10.02

One-way ANOVA: IL-2 versus Treatment

Factor Information

Factor Levels Values

Treatment 6 100mg/kg bw, 150mg/kg bw, 50mg/kg bw, Negative control, Normal Control, Positive control

Analysis of Variance

Source DF Adj SS Adj MS F-Value P-Value

Treatment 5 235209 47041.9 455.76 0.000

Error 24 2477 103.2

Total 29 237687

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Treatment N Mean Grouping

Normal Control 5 571.80 A

150mg/kg bw 5 566.20 A

Positive control 5 561.20 A

100mg/kg bw 5 535.20 B

50mg/kg bw 5 505.40 C

Negative control 5 318.20 D

Means that do not share a letter are significantly different.

One-way ANOVA: TNF versus Treatment

Factor Information

Factor Levels Values

Treatment 6 100mg/kg bw, 150mg/kg bw, 50mg/kg bw, Negative control, Normal Control, Positive control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	5	17884	3576.77	38.74	0.000
Error	24	2216	92.33		
Total	29	20100			

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Treatment	N	Mean	Grouping
Normal Control	5	98.60	A
150mg/kg bw	5	97.20	A
Positive control	5	97.00	A
100mg/kg bw	5	77.00	B
50mg/kg bw	5	57.40	C

Negative control 5 33.20 D

Means that do not share a letter are significantly different.

One-way ANOVA: IFN-γ versus Treatment

Factor Information

Factor Levels Values

Treatment 6 100mg/kg bw, 150mg/kg bw, 50mg/kg bw, Negative control, Normal Control, Positive control

Analysis of Variance

Source DF Adj SS Adj MS F-Value P-Value

Treatment 5 151332 30266.4 204.09 0.000

Error 24 3559 148.3

Total 29 154891

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Treatment N Mean Grouping

Normal Control 5 394.00 A

150mg/kg bw 5 391.60 A

Positive control 5 387.80 A

100mg/kg bw 5 359.20 B

50mg/kg bw 5 332.40 C

Negative control 5 191.60 D

Means that do not share a letter are significantly different.

One-way ANOVA: IL-4 versus Treatment

Factor Information

Factor Levels Values

Treatment 6 100mg/kg bw, 150mg/kg bw, 50mg/kg bw, Negative control, Normal Control, Positive control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	5	103476	20695.2	125.72	0.000
Error	24	3951	164.6		
Total	29	107427			

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Treatment	N	Mean	Grouping
Negative control	5	307.80	A
50mg/kg bw	5	199.40	B
100mg/kg bw	5	172.60	C
Positive control	5	145.60	D
150mg/kg bw	5	143.40	D
Normal Control	5	140.60	D

Means that do not share a letter are significantly different.

One-way ANOVA: IL-5 versus Treatment

Factor Information

Factor Levels Values

Treatment 6 100mg/kg bw, 150mg/kg bw, 50mg/kg bw, Negative control, Normal Control, Positive control

Analysis of Variance

Source DF Adj SS Adj MS F-Value P-Value

Treatment 5 49847 9969.3 79.00 0.000

Error 24 3029 126.2

Total 29 52875

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Treatment N Mean Grouping

Negative control 5 219.60 A

50mg/kg bw 5 154.60 B

100mg/kg bw 5 131.60 C

Positive control 5 109.00 D

150mg/kg bw 5 106.40 D

Normal Control 5 104.00 D

Means that do not share a letter are significantly different.

Appendix VI: Analysis of the Effect of *C. edulis* DCM Extract on DTH Reaction

Descriptive Statistics: FPT

Variable	Treatment	Mean	SE Mean	StDev
FPT	100mg/kg bw	2.4200	0.0510	0.1140
	150mg/kg bw	2.6900	0.0653	0.1461
	50mg/kg bw	2.2420	0.0759	0.1698
	Negative control	2.1120	0.0271	0.0606
	Normal control	1.7400	0.0319	0.0714
	Positive control	3.6920	0.0312	0.0698

One-way ANOVA: FPT versus Treatment

Factor Information

Factor	Levels	Values
Treatment	6	100mg/kg bw, 150mg/kg bw, 50mg/kg bw, Negative control, Normal control, Positive control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	5	11.2813	2.25627	176.25	0.000
Error	24	0.3072	0.01280		
Total	29	11.5886			

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Treatment	N	Mean	Grouping

Positive control 5 3.6920 A

150mg/kg bw 5 2.6900 B

100mg/kg bw 5 2.4200 C

50mg/kg bw 5 2.2420 C D

Negative control 5 2.1120 D

Normal control 5 1.7400 E

Means that do not share a letter are significantly different.

Appendix VII: Analysis of the Effect of *T. brownii* Methanol Extract on Antibody Titer Levels

Descriptive Statistics: HA (units/µL)

Variable	Treatment	Mean	SE Mean	StDev
HA (units/µL)	100mg/kg bw	8.333	0.333	0.577
	150mg/kg bw	10.333	0.333	0.577
	50mg/kg bw	6.333	0.333	0.577
	Negative control	1.667	0.333	0.577
	Normal control	4.0000	0.000000	0.000000
	Positive control	11.000	0.577	1.000

One-way ANOVA: HA (units/µL) versus Treatment

Factor Information

Factor	Levels	Values
Treatment	6	100mg/kg bw, 150mg/kg bw, 50mg/kg bw, Negative control, Normal control, Positive control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	5	200.278	40.0556	103.00	0.000
Error	12	4.667	0.3889		
Total	17	204.944			

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Treatment	N	Mean	Grouping
Positive control	3	11.000	A
150mg/kg bw	3	10.333	A
100mg/kg bw	3	8.333	B
50mg/kg bw	3	6.333	C
Normal control	3	4.000	D
Negative control	3	1.667	E

Means that do not share a letter are significantly different.

Appendix VIII: Published Papers

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Hindawi

Research Article

Terminalia brownii Fresen: Stem Bark Dichloromethane Extract Alleviates Pyrogallol-Induced Suppression of Innate Immune Responses in Swiss Albino Mice

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Terminalia brownii is widely used in folklore medicine and has diverse biological activities. However, its effect on the immune system is yet to be studied. Therefore, our study evaluated the immunomodulatory effect of *T. brownii* on nonspecific immunity. Innate immunity is the initial defence phase against pathogens or injuries. Dichloromethane plant extracts were tested on female Swiss albino mice and Wister rats. The effect of the extract on innate immunity was assessed via total and differential leukocyte counts, tumor necrosis factor-alpha, and nitric oxide production by mouse macrophages. The 3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide assay was employed for viability testing. Phytochemical profiling was carried out using gas chromatography-mass spectrometry, while toxicity studies were carried out following the Organization for Economic Co-operation and Development guidelines. Our results demonstrated that administration of *T. brownii* stem bark dichloromethane extract to pyrogallol-immuno compromised mice significantly ($p < 0.05$) increased total and differential leukocyte counts compared with the control. The extract showed no adverse effect on the viability of Vero cells and macrophages and significantly ($p < 0.05$) augmented tumor necrosis factor-alpha and nitric oxide production. Hexadecanoic acid, linoleic acid, octadecanoic acid, squalene, campesterol, stigmasterol, and β-sitosterol, all of which stimulate, were identified in the extract. The extract did not cause any death or toxic signs in rats. In conclusion, *T. brownii* dichloromethane extract has an immunoenhancing effect on innate immune responses and is not toxic. The observed immunoenhancing impact of the extract was attributed to the presence of the identified compounds. The results of this study provide crucial ethnopharmacological leads towards the development of novel immunomodulators for managing immune-related disorders.

1. Introduction

Innate immunity is the initial phase of defence against viral and bacterial infections and sterile inflammation [1]. It is a nonspecific defence mechanism that the host uses after an antigen encounter, instantly or within a few hours. However, innate immunity does not have immunologic memory. pH, and temperature), phagocytic and endocytic, and inflammatory barriers [2]. Nonspecific immunity comprises cellular and humoral components that identify, inactivate, and kill invading pathogens [3]. The cellular part includes phagocytes (neutrophils and macrophages), eosinophils, basophils, monocytes, mast cells, dendritic cells, innate lymphoid cells, and natural killer cells [2, 4]. However, the

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Original Article

Acute and Sub-acute Oral Toxicity Profile of Root Bark Methanol Extract of *Carissa Edulis* Vahl

Jane Wanja Mbiri[1,*], Kenneth Ogila[1], Patrick Kisangau[2], Michael Gicheru[3]

ABSTRACT

Background: *Carissa edulis* is widely used in traditional medicine to manage numerous ailments. However, few studies have assessed its toxicity. Therefore, this study aimed to determine acute and sub-toxicity levels of *C. edulis* methanol extract. **Methods:** In the acute toxicity probe, a limit test was conducted whereby the extract was given as a solo dose by gavage. The rats were observed for two weeks. The observations included mortality and changes in the general appearance and behavior of the experimental animals. The body weights of the rats were taken weekly. For the sub-acute toxicity probe, the rats received the extract daily at dosages 300, 520, and 900 mg/kg by gavage for 28 days. Body weights were also taken weekly. On day twenty-nine, the weights of the rats were taken, the rats were sacrificed, and blood was collected for biochemical and hematological analysis. Body organs were harvested, and their weights were taken. **Results:** The results of the acute toxicity probe showed that the extract didn't cause mortality or toxicity signs throughout the study duration. The LD_{50} of the extract was therefore deemed to be above 2,000 mg/kg. The sub-acute toxicity probe results demonstrated that the extract, at all the tested dosages, didn't cause mortality or affect the rats' organ weights, body weights, or hematological and biochemical parameters throughout the study duration. **Conclusions:** In conclusion, the methanol extract of *C. edulis* is not toxic since it didn't cause mortality or toxicity signs in both acute and sub-acute toxicity probes.

Key words: Biochemical parameters, Body weights, Hematological parameters, Organ weights.

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INTRODUCTION

Medicinal plants have been utilized to manage and treat various ailments since time immemorial.[1] The World Health Organization (WHO) reports that most people in the world (80%) depend on traditional herbal remedies for their primary healthcare needs.[1] The increased reliance on herbal medicine is due to the adverse side effects and high costs of conventional drugs.[2] Besides, herbal medicine has been reported to be safer, readily available, and more affordable than conventional drugs.[3]

Diverse biological activities of numerous medicinal plants have been validated scientifically. Medicinal plants are thus potential candidates for drug

pain, worm infestation, sickle cell anemia, hernia, fever, among other conditions.[8,9] Various biological activities of *C. edulis* including, antiplasmodial,[10] anti-inflammatory,[11] analgesic,[12] antipyretic,[8] antiviral,[13] anticonvulsant,[14] hypoglycemic,[15] diuretic,[16] antimicrobial,[17] among others, have been reported.

Despite the wide usage of *C. edulis* in traditional medicine, only minimal scientific toxicity data is available. Therefore, this study's objective was to investigate acute and sub-acute toxicity levels of *C. edulis* root bark methanol extract in albino Wistar rats. This study provides crucial information on the safety profile of *C. edulis* that can be leveraged in determining dosages for pre-clinical trials.

Appendix IX: Pictorials

Collection of *T. brownii* bark samples

Plant extract filtration

Culture media preparation

Obtaining absorbance readings

Oral plant extract administration

Increased foot pad thickness denoting DTH reaction