

**TOXICITY AND IMMUNOMODULATORY PROPERTIES OF METHANOL
EXTRACT OF THE STEM BARK OF *LANNEA ACIDA* A. RICH.
(ANACARDIACEAE) IN WISTAR RATS**

BY

JOSEPH OGIRIMA OVOSI

**DEPARTMENT OF PHARMACOLOGY & THERAPEUTICS
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA**

NOVEMBER 2017

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(ANACARDIACEAE) IN WISTAR RATS**

By

**Joseph Ogirima OVOSI, MBBS (ABU) 2004
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FACULTY OF PHARMACEUTICAL SCIENCES,
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA**

NOVEMBER, 2017

DECLARATION

I declare that the work in this dissertation entitled “Toxicity and immunomodulatory properties of methanol extract of the stem bark of *Lannea acida* A. Rich (Anacardiaceae) in Wistar rats” was carried out by me in the Department of Pharmacology & Therapeutics, Ahmadu Bello University, Zaria, Nigeria. The information derived from the literature has been acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Joseph Ogirima OVOSI

Name of student

Signature

Date

CERTIFICATION

This dissertation entitled “TOXICITY AND IMMUNOMODULATORY PROPERTIES OF METHANOL EXTRACT OF THE STEM BARK OF *LANNEA ACIDA* A. RICH (ANACARDIACEA) IN WISTAR RATS” by Joseph Ogirima OVOSI, meets the regulations governing the award of the degree of *Masters of Science* of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Professor A.U. Zezi Date
Chairman Supervisory Committee

Professor I. Abdu-Aguye Date
Member Supervisory Committee

Dr M.G. Magaji Date
Head of Department

Prof. Kabir Bala
Dean, School of Postgraduate Studies,
Ahmadu Bello University, Zaria.

Date

DEDICATION

This work is dedicated to my loving wife, Dr. (Mrs) Beatrice Ohunene Bello-Ovosi and my daughter, Alexandria Schatzi Onize Onyi-aname Ovosi for their unquantifiable supports in all my endeavors,

And to,

The memory of my friend and teacher, Late Dr. Tijani Yahaya of the Nigerian Institute of Pharmaceutical Research and Development (NIPRD) with whom I conceived and prepared for this work. May his gentle soul rest in perfect peace.

Amen!

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brother, Engr. Emmanuel Ovosi and family; you all have been a source of courage and comfort both at home and in the field. I thank you all for being there for me.

ABSTRACT

Lannea acida A. Rich. belongs to the family of *Anacardiaceae*; and the bark, leaves and roots have been reported to possess many medicinal properties, including immunomodulatory activity. Despite this widespread belief, data on the immunomodulatory properties and safety of this plant is still lacking. The aim of this work was to determine the toxicity and immunomodulatory properties of the stem bark of *Lannea acida* in Wistar rats. Methanol extract of *Lannea acida* A. Rich. was screened for phytochemical properties using standard methods. The acute and the subacute (28-day daily repeated dose) toxicity studies were performed using the OECD Fixed Dose Procedure guideline 420 and OECD guideline 407 respectively. To evaluate the immunomodulatory properties, 12 male and 12 female Wistar rats were pre-sensitised with 0.1ml sheep red blood cell intraperitoneally, and subsequently divided into 4 groups of 6 animals each (3 males, 3 females). The first group served as the control while the remaining three groups received daily oral doses of 250, 500 and 1,000 mg/kg body weight of methanol extract of *L. acida*, respectively for 14 days. The humoral response (IgM and IgG) to sheep red blood cells was determined by ELISA method and read by microplate reader. The cellular immune response was determined by estimation of the CD4⁺ and CD8⁺T-cell counts using flow cytometer, and measuring the delayed type hypersensitivity reaction from the paw thickness of rats given subcutaneous injection of SRBC. The methanol extract of the stem bark of *L. acida* was found to contain carbohydrate, reducing sugars, alkaloids, glycosides, anthraquinones, triterpenes, cardiac glycosides and tannins; and had an acute lethal dose that was > 3,000 mg/kg. The 28-day oral toxicity study showed a significant decrease ($p < 0.05$) in the relative organ weight percent of the spleen and kidneys of rats that received a dose of 1,000 mg/kg body weight

compared with the control; and a significant decrease ($p < 0.05$) in the serum potassium levels across all the doses compared with the control. There was also a statistically significant increase ($p < 0.05$) in serum alanine transaminase level at the dose of 500 mg/kg. Histological examination of the organs following 28-day daily oral administration showed hepatic sinusoidal vascular congestion and peri-portal inflammation; splenic red pulp hemorrhage; kidney glomerular necrosis and tubulo-interstitial inflammation; and thymocyte depletion in the experimental groups compared to the control. There was a significant decrease ($p < 0.05$) in the levels of serum immunoglobulin at the doses of 1,000 mg/kg for IgM and the doses of 500 and 1,000 mg/kg for IgG. In conclusion, methanol extract of the stem bark of *L. acida* has relatively low acute toxicity but when given as a daily dosing for 28 days, it has hepatotoxic, nephrotoxic and immunotoxic effects and hence, potential for multi-organ toxicity when employed for sub-acute and chronic uses.

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LIST OF ABBREVIATIONS

ABU	Ahmadu Bello University
ADCC	Antibody Dependent Cell-mediated Cytotoxicity
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
ANOVA	Analysis Of Variance
APC	Antigen Presenting Cell
AST	Aspartate Transaminase
BCG	Bacillus Calmette-Guerin
BDH	British Drug House
CBC	Complete Blood Count
CD	Cluster of Differentiation
CFA	Complete Freund's Adjuvant
C _p G	Cytosine-phosphate-Guanine
CTL	Cytotoxic T Lymphocyte
CYP	Cytochrome
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DSMO	DimethylSulfoxide
DTH	Delayed Type Hypersensitivity
EDTA	Ethylene Diamine Tetra-acetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Flourescent Activated Cell Scan
FDA	Food and Drug Administration
FKBP	FK-Binding Protein

fL	femtoliter
GHS	Global Harmonized System
GLP	Good Laboratory Practice
HILI	Herbal-Induced Liver Injury
HIV	Human Immune Virus
HLA	Human Leukocyte Antigen
HRP	Horseshoe Radish Peroxidase
IFA	Incomplete Freund's Adjuvant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
LPS	Lipopolysaccharide
MALT	Mucosa Associated Lymphoid Organ
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
MHC	Major Histocompatibility Complex
mTOR	Mammalian Target of Rapamycin
NFAT	Nuclear Factor for Activated T-cell
NIPRD	National Institute of Pharmaceutical Research and Development
NK	Natural Killer
NO	Nitric Oxide
OECD	Organization for Economic Co-operation and Development
OI	Organ Index

PALS	Periarteriolar Lymphoid Sheath
PBS	Phosphate Buffered Solution
pH	Hydrogen Potential
RBC	Red Blood Cell
RBC	Red Blood Cell
ROW	Relative Organ Weight
SEM	Standard Error of Mean
SHE	Syrian Hamster Embryo
SRBC	Sheep Red Blood Cell
TMB	3,3', 5,5' - Tetramethylbenzimidine
TGF	Transformed Growth Factor
T _H	T-Helper
TNF	Tumor Necrotic Factor
US	United States
WBC	White Blood Cell
WHA	World Health Assembly
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Preamble

The use of medicinal plants for the treatment of various ailments has been part of mankind since antiquity. Despite the advancement of orthodox and pharmaceutical medicine, herbal medicines are still popular in Europe and North America because they are believed to be natural and safe. In the African folklore, many ailments have been treated and continue to be treated with herbal medicines. Many of today's pharmaceutical products have their precursors in medicinal plants (Cowan, 1999; Newman *et al.*, 2000; Butler, 2004).

Lannea acida belongs to the family of *Anacardiaceae*, a major group of angiosperms (flowering plants). In the African traditional folklore, the bark, leaves and roots of *L. acida* are used for the management of various ailments such as: conjunctivitis, sores, diarrhoea, stomach aches, gonorrhoea, rheumatism, fever, malaria, skin diseases, cough and dysentery (Koné *et al.*, 2004; Ouattara *et al.*, 2011a). The plant has also been suggested to have antibacterial properties against drug-resistant gram-positive organisms (Koné *et al.*, 2004), as well as antidiarrhoeal (Etuk *et al.*, 2009), immunomodulatory and anti-*Mycobacterium* H37Rv activities (Ouattara *et al.*, 2011b). The stem bark also enhances the count, morphology and motility of sperm as well as the testosterone levels in male Wistar rats (Ahmed *et al.*, 2010).

1.2 Statement of Research Problems

Many traditional herbal preparations find their way for use in humans without the empirical knowledge of their pharmacological properties and safety. This inadvertently worsens the health of the individuals who receive them. In most cases, the preparations do not possess the pharmacological properties and the efficacies that

are attributed to them (Firenzouli and Gori, 2007). Preparations containing *Lannea acida* are used in humans for their immunomodulatory properties despite the paucity of data about their safety and efficacy. Therefore, there is the need to properly elucidate the safety and immunomodulatory properties of the plant *Lannea acida*.

1.3 Justification

Most African countries including Nigeria are saddled with high prevalence of diseases and the accessibility of drugs to combat these is still problematic. Pharmaceutical products are generally unaffordable and unavailable to the common people who suffer the greatest brunt of these diseases. The World Health Organization (WHO) estimates that about 80% of the population in developing countries still depends on traditional medicine for their primary health care needs (Bodeker *et al.*, 2005), because they are accessible, available and affordable by most consumers.

The immune system has been noted to play a key role in the pathogenesis of many human diseases such as arthritis, ulcerative colitis, allergy, asthma, infections including HIV; and parasitic infestations, cancers and other diseases such as prion disease (Hansson *et al.*, 2002; Opitz *et al.*, 2010; Fong, 2014). The use of conventional drug products that stimulate or suppress immune systems (immunomodulators) have played a crucial role in the treatment of most of these diseases, but they are limited by their accessibility, costs and side-effects which make the sufferers of these diseases to resort to the traditional herbal medicine for their healing. However, the herbal medicine systems are not devoid of problems. There are safety and efficacy issues associated with their use. These concerns made the World Health Assembly (WHA) in her resolution 42.43 of 1989 to state *inter alia* that “member states should make a comprehensive evaluation of their traditional systems

of medicine, to make a systematic inventory and assessment (pre-clinical and clinical) of the medicinal plants used by the traditional practice individuals and by the population; to introduce measures for the regulation and control of medicinal plant products and for the establishment and maintenance of suitable standards; and to identify those medicinal plants, or remedies derived from them, which have a satisfactory efficacy/side-effect ratio and which should be included in national formularies or pharmacopoeias.”(WHA, 1989).

Despite the report that *L. acida* may possess some immunostimulatory properties (Ouattara *et al.*, 2011b), and that preparations containing the herb extracts may enhance immunity in HIV patients (USP, 2010), its specific role in immune system has not been well elucidated. This study therefore attempts to determine the safety, and immunomodulatory properties of *L. acida*.

1.4 Aim and Objectives

1.4.1 Aim:

The aim of this study is to determine the toxicity and immunomodulatory properties of methanol extract of the stem bark of *L. acida* in Wistar rats.

1.4.2 Specific Objectives:

The specific objectives of this study are:

1. To determine the phytochemical constituents of methanol extract of the stem bark of *L. acida*
2. To determine the acute oral toxicity of the methanol extract of the stem bark of *L. acida* in Wistar rats.
3. To determine the subacute oral toxicity of methanol extract of *L. acida* in Wistar rats.

4. To evaluate the immunological parameters (IgM, IgG, CD4+ and CD8+T-cell Counts)of Wistar rats administered with methanol extract of *L. acida*.

1.5 Research Hypothesis

- Null Hypothesis:

Methanol stem bark extract of *L. acida* does not possess immunomodulatory properties and is not safe at oral dose of $\leq 3,000$ mg/Kg in Wistar rats.

- Alternative Hypothesis:

Methanol stem bark extract of *L. acida* possesses immunomodulatory property and is safe at oral dose of $\leq 3,000$ mg/Kg in Wistar rats.

1.6 Scope of the Work

This research involved the determination of the acute oral toxicity of *L. acida* using the fixed-dose method (OECD, 2002), and the sub-acute (28-day daily dose) oral toxicity (OECD, 2008).

The immunomodulatory properties of *L. acida* were determined by measuring the humoral (IgM and IgG) and cellular (CD4+ and CD8+T-lymphocyte) immune responses as well as the delayed type hypersensitivity response.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

Lannea is one of the genera of the group of plants belonging to the family *Anacardiaceae*, a major group of flowering plants (*Angiosperms*). There are over forty species of *Lannea* scattered all over the tropics from sub-Saharan Africa to Asian subcontinent. The group has been credited with various medicinal properties and is part of many ethnopharmacological preparations in tropical Africa (Kone *et al.*, 2004; Ahmed *et al.*, 2010; Ouattara *et al.*, 2011a). *L. acida* is one of the species used for its medicinal properties in Northern Nigeria and has formed integral part of most ethnopharmacological preparations in this region (Muhaisen, 2013).

2.2 Description of the Plant

Lannea acida (syn *Odina acida*) is commonly known as African grape, but in the local Hausa folklore, it is called faárú, and in the Fulani-fulfulde as faruhi (Gill, 1992).

The other synonyms of the plant are: hil gbur (Tiv), ekika (Yoruba) and Onoruwa (Ebira).

- Authority: Linn
- Kingdom: Plantae
- Phylum: Angiosperm
- Order: Sapindales
- Family: Anacardiaceae
- Genus: *Lannea*
- Species: *Lannea acida*

It is widely distributed in the areas covering the Sudan and Guinea Savannahs, and

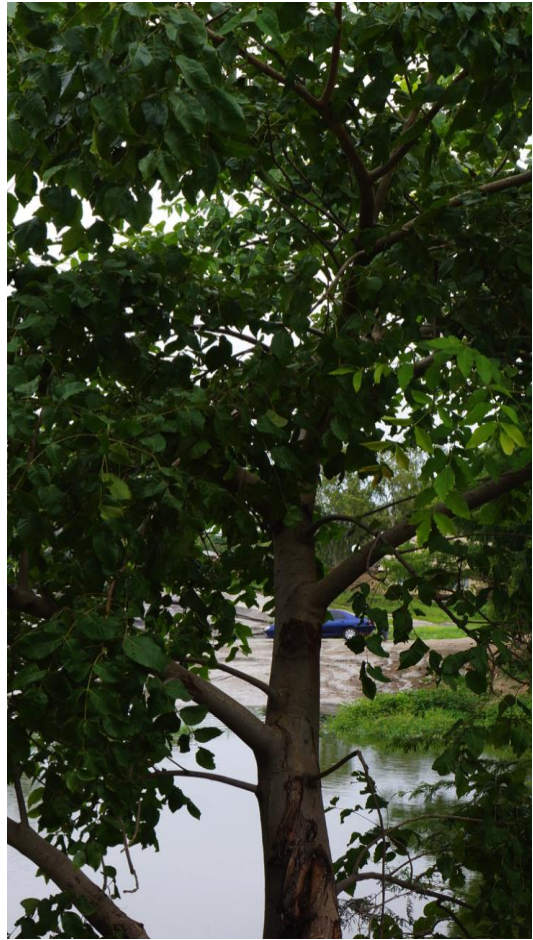
extending from Sudan in east Africa to Senegal, Nigeria, Ghana, Burkina Faso in West Africa (Burkill, 1995).

L. acida is a dioecious, androecious or polygamodioecious shrub or tree with inflorescences and young stems and leaves covered with stellate trichomes.

They grow on wooded savanna, often on gravelly (deep) soils, rocky places and hill, bare granite rock or bush far from villages; usually at an altitude of 600-1600 meters (Burkill, 1995). The shrub or tree may be 1.5-10 meters tall, but trees as high as 18 meters have been reported, and girth may be up to 2-3 meters, and growth requires precipitation at 635mm (Burkill, 1995).

The bark is fibrous, with a thick fissure, which help the tree to resist bush fires while the wood is whitish and soft and considered hard enough to make soft stools (Burkill, 1995).

Other species of *Lannea* includes: *Lannea alata*, *Lannea ambacensis*, *Lannea angolensis*, *Lannea antiscorbutica*, *Lannea barteri*, *Lannea coromandelica*, *Lannea discolor*, *Lannea edulis*, *Lannea fruticosa*, *Lannea fulva*, *Lannea gossweileri*, *Lannea humilis*, *Lannea rubra*, *Lannea schimperi*, *Lannea schweinfurthii*, *Lannea velutina*, *Lannea welwitschii* (Lannea, 2016).



Plates I and II: *L. acida* stem bark and tree in the wet woodland of Zaria

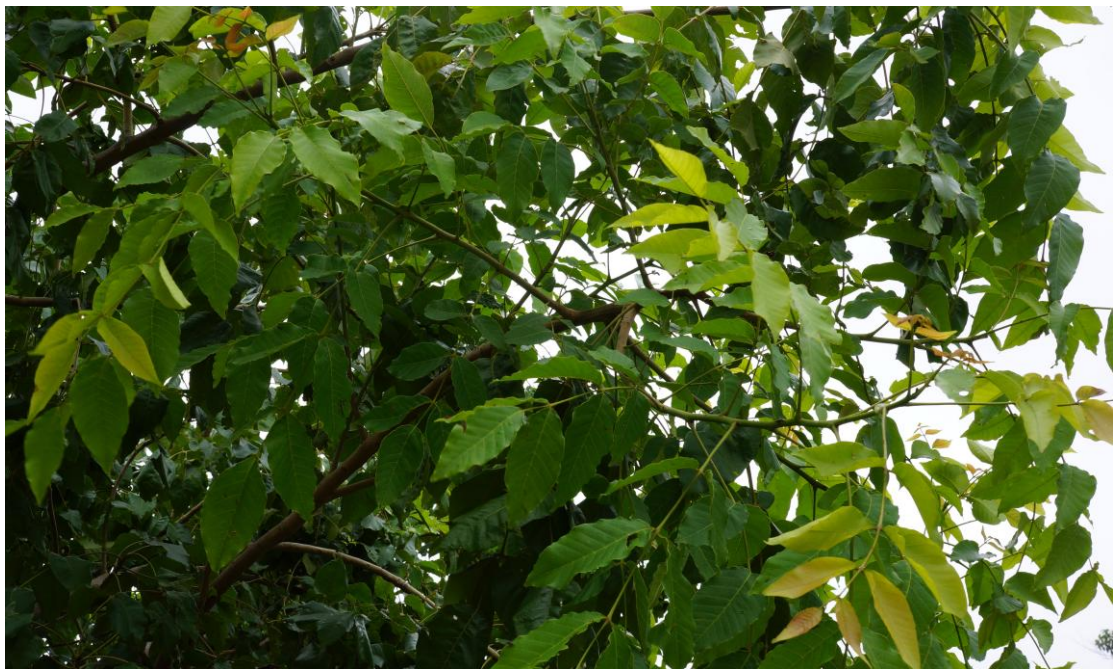


Plate III: Leaves of *L. acida*

2.3 Phytochemical Studies on the Stem Bark of *L. acida*

2.3.1 Chemical constituents of the stem bark of *L. acida*

A preliminary screening of *L. acida* revealed the presence of alkaloids and tannins in the bark (Etuk *et al.*, 2009). Other studies revealed high levels of phenolic compounds and flavonoids in the bark (Karou *et al.*, 2005, and Ouattara *et al.*, 2011a). Muhaisen characterized the four chemical compounds from the bark of *L. acida* (Muhaisen, 2013):

- (1). 6,7-(2'', 2''-dimethyl chromeno)-8- γ , γ -dimethyl allyl flavanone,
- (2). 3'4' dihydroxyl-7-8 (2'', 2''-dimethyl chromeno)-6- γ , γ dimethyl allyl flavanol
- (3). 7-methyltectorigenin
- (4). Irisolidone

2.3.2 Chemical constituents of phytomedicines with immunomodulatory properties

Phytochemical analysis of medicinal plants shows that glycosides, upon enzymatic or acid hydrolysis, yield one or two moieties that have immunomodulatory properties (Nagarathna *et al.*, 2013). Several types of flavonoids also exert immunomodulatory activities including isoflavonoids, flavones and anthocyanidins (Nagarathna *et al.*, 2013). Other plant constituents known to have immunomodulatory activities are saponin, terpene, tannic acid, carotenoids and polyphenols (Fulzele *et al.*, 2003).

2.4 Toxicology Studies of *L. Acida*

Etuk *et al.* (2009) in an ethnobotanical survey and preliminary evaluation of medicinal plants with antidiarrhoeal properties found that the oral administration of 3,000 mg/kg extract of *L. acida* did not produce any death in Wistar rats.

Though other toxicological tests for *L. acida* were scarce, a similar test in another species of *Lannea*, *Lannea kerstingii* Engl. also showed that the lethal dose for the

plants in this genus is generally above 5,000 mg/kg (Diallo *et al.*, 2010). In this same study, *L. kerstingii* showed a significant decrease in weight of experimental animals when given at a dose of 1,000 mg/kg for 28 days (Diallo *et al.*, 2010).

2.5 Uses of *L. Acida*

2.5.1 Medicinal uses

L. acida is used in the traditional treatment of many ailments. It is used solely or in combination with other phytomedicines in the treatment of the following diseases: conjunctivitis, sores, diarrhoea, stomach aches, gonorrhoea, rheumatism, fever, malaria, skin diseases, coughs, dysentery, malnutrition and debility; as well as possessing immune stimulation and anti-tuberculosis activity (Kone *et al.*, 2004 and Ouattara *etal.*, 2011b). The antibacterial and immunostimulatory properties of the bark of *L. acida* were also demonstrated in two separate works (Kone *et al.*, 2004 and Etuk *et al.*, 2009).

2.5.2 Other uses

The leaves could be eaten as vegetable and the bark may be pounded to make red dye for cloths. In certain parts of West Africa, the fruits may be made into a fermented drink or used as a resinous taste. The wood could be fabricated into cooking utensils, or used for making benches and bows; and could be used as a source of biofuel for domestic combustion. The leaves and fruits could also be used as a livestock fodder (Burkill, 2000; Neuwinger, 2000 and Arbonnier, 2004).

2.6 Overview of Herbal Toxicities

Toxicology is the study of chemical or physical agents that produce adverse effects in biological organisms. These agents are called poisons, and often denote

substances that have almost exclusively harmful effects. However, Paracelsus (1473-1541), in his famous write up extended this term when he wrote: *“What is there that is not poison? All things are poison and nothing without poison. Solely, the dose determines that a thing is not a poison”* (Pagel, 1982).

Herbal medicines are increasingly being used as part of Complementary and Alternative Medicines in the United States and throughout the world (Bent and Ko, 2004). Despite this widespread use, there is still growing concern about the safety and quality of the millions of herbal medicines that are in use globally (Nortier and Vanherweghem, 2007; Luyckx and Naicker, 2008).

2.6.1 Goals of toxicity testing of herbal medicines

The goals of toxicity testing are to identify and delineate the risks and benefits associated with the use of these medicinal products, and to determine the limits of exposure level at which this occur. Toxicity testing also helps in the detection of toxic plant extracts or compounds derived thereof in the early (pre-clinical) and late (clinical) stages of drug discovery and development from plant sources; and therefore, facilitates the identification of toxicants which can be discarded or modified during the process (Gamaliel, 2000). In some instances, dose reduction, chemical group or structural adjustment may improve the tolerability of compounds (Obidike and Salawu, 2013).

2.6.2 Preclinical toxicity testing

These are battery of tests conducted in non-human models. They are important because regulatory authorities require the demonstration of safety and/or efficacy in non-human experimental models before clinical trials in humans. Preclinical toxicity testing may be non-animal based or animal-based.

A. *Non-Animal-Based Study*

In vitro cells and tissue cultures have proven to be a powerful investigative tool in drug development. Unlike *in vivo* testing, which is time consuming, *in vitro* screening tests are fast, cheap, suitable for high throughput, can provide human data as well as mechanistic information (Hibernia, 2014).

The key *in vitro* toxicology tests performed during drug development are: cytotoxicity, mutagenicity, carcinogenesis, hepatotoxicity, organ specific toxicity and computer-based toxicity projection.

1. Cell-based cytotoxicity tests

Cultured cells are exposed to the test substance for a short-term to detect the effects of the test substance on basal or specialized cell functions. They are based on the basic principles of measuring cell viability and measuring cell release. Cells are grown to a semi-confluent stage in 96-well plates, the test substance is added and incubated to various time periods. Positive controls such as dimethylsulfoxide (DSMO) or triton are used to ensure that the assay is working. After the incubation period, the test substance and the medium are removed and the cells used for the assay (O'Brien and Haskings, 2006; Hibernia, 2014).

The cell viability and membrane integrity is measured by the uptake of certain dyes such as tryptan blue which stain cells blue if the membrane is damaged but does not enter intact cells; and the nuclear integrity is measured by the uptake of propidium iodide which binds to DNA and makes it fluoresce (Hibernia, 2014). In cell release assay, intracellular substances like lactate dehydrogenase that are only released if cells are damaged are measured using a coloured substrate (Hibernia, 2014). The other indicators of toxicity are ability of the test substance to inhibit cellular growth

and proliferation; detection of cell viability markers or morphologic and cellular differentiation markers (O'Brien and Haskings, 2006).

2. Mutagenicity tests

Mutagenicity studies look for the potential of a drug to damage DNA and alter the function of a cell. The Ames test is the best known and most used protocol for mutagenicity testing. It uses bacteria instead of cells, which makes it cheaper and easier to do (Tejs, 2008). The principle of the test involves the use of a strain of bacteria, *Salmonella enterica* sv *Typhimurium* that carry a mutation (His operon) involved in histidine synthesis, and therefore, require histidine for growth but cannot produce it. Exposure to a mutagenic agent cause reverse mutation in the His operon and allow the bacteria to undergo a reverse mutation that make it grow in histidine-free media (Mortelmans and Zeiger, 2000). The effect is tested on billions of bacteria. *In vitro* cytogenetic assays such as chromosomal aberration tests and sister chromatid exchange can also be used to test for mutagenicity. In chromosomal aberration test, cell cultures are exposed to the test substance both with and without metabolic activation. At predetermined intervals after exposure, they are treated with a metaphase-arresting substance (eg. Colcemid[®] or colchicine), harvested, stained and metaphase cells are analyzed microscopically for the presence of chromosome aberrations (OECD, 1997).

3. Carcinogenicity studies

In vitro assays can be used to identify carcinogenicity by studying the change in cell morphology. Syrian hamster embryo (SHE) cells have been used extensively to study the process of *in vitro* cell transformation and, hence assess the potential for carcinogenicity of test substances (Isfort *et al.*, 1996). The SHE cells when cultured in an environment of reduced bicarbonate (low PH), will morphologically transform at a

rate enough to allow for rigorous statistical evaluation (Mauthe *et al.*, 2001). SHE cells which can be cryopreserved, can maintain a competent metabolic system to activate a procarcinogenic agent to their carcinogenic form, which obviates the need for the addition of exogenous metabolic activation systems as typically required in other *in vitro* assays (Isfort *et al.*, 1996). The transformed phenotype of the SHE cell has been associated with neoplastic potential and the morphological endpoint can be determined in 7-9 days (LeBoeuf *et al.*, 1990). The advantages of SHE cell test is that it is of higher predictive value because it measures a transformed phenotype as the endpoint, and because cancer is a multistep process, it is a better predictor of potential for carcinogenesis than mutation or chromosomal aberration tests (Mauthe *et al.*, 2001).

The comet assay is a gel electrophoresis-based method that can be used to measure DNA damage in individual eukaryotic cells. It is versatile and sensitive to DNA damage of 50 breaks per diploid mammalian cell. Single cells are embedded in agarose, and an electric current is applied. Electrophoresis causes unwound or damaged DNA to leave the cells, and this produces a comet-like tail (Olive and Banath, 2006).

4. *In Vitro* metabolic screening

The liver is the site of metabolism of herbs and therefore, it is also very sensitive to herbal toxicity. Human liver cell lines can be used to screen test compounds. The advantage of using human liver cell lines is that these will naturally generate human metabolites and the effects of genetics on toxicity can be verified (Hibernia, 2014). Cytochrome P450 is a family of enzymes that play key roles in drug metabolism and herbal remedies can inhibit or induce activity of these enzymes (Wienkers and Heath, 2005). The effect of herb-drugs interaction that is also a key to

predicting toxicity is also affected by CYP450 enzyme system (Guengerich, 1997).

Metabolism studies are done using fluorescent-labeled reagents that are substrates of the CYP450 enzymes. Only the metabolite is fluorescent, and the fluorescence is proportional to enzyme activity. This can be used in high throughput assays and recombinant human cytochromes can be used to check for specific cytochromes (Hibernia, 2014).

5. Toxicogenomics

Herbal toxicogenomics combine different “-omic” tools such as DNA microarrays, proteomics, and metabolomics to measure the potential toxic outcomes of interaction of herbal extracts at sub-molecular, molecular, cellular, tissue and organ levels (Waring, 2002).

6. High throughput next generation sequencing

This involves the creation of large volume of DNA sequences and large genetic databases of plants from which potentially toxic plants can be identified (Schuster, 2008).

B. *Animal studies*

Animal models provide a more useful data on the behaviour of toxicants in human system. While cell-based studies and *in vitro* studies are predictive of toxicity, the animal studies incorporate toxicokinetics of the test substances when administered by route similar to its intended use (Obidike and Salawu, 2013). However, the proliferation of Animal Right groups and the increasing awareness of animal welfare issues have made the independent review of animal study protocol by an ethics committee mandatory. The following are some of the toxicity testing done in animals during preclinical research:

1. Acute toxicity

Acute toxicity measures the relative toxicological response of an experimental animal to a single or brief exposure to test substance (OECD, 2002). The Global Harmonized System (GHS) defined acute toxicity as “*those adverse effects that occur following the dermal administration of a single dose of a substance, or multiple doses given within 24 hours, or an inhalation exposure of 4 hours*” (OECD, 2002). Standardized tests are available for oral, inhalational and dermal exposures. The aim of acute toxicity test is to calculate the therapeutic index, which is the ratio of the median lethal dose to the median effective dose in the same species and strains of animals. Species of animals that may be used range from mice, rats, rabbits, guinea pigs. Following the administration of the test substance, animals are observed individually at least during the first 30 minutes, and periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days (OECD, 2002).

2. Sub-acute, sub-chronic and chronic toxicity studies

The GHS defined these studies as “*specific target organ/systemic toxicity arising from repeated exposure*” (OECD, 2008). In rats, these studies range in duration from 28-day (sub-acute) to 90-day (subchronic) and even 12-months (chronic) studies and consist of repeated doses in oral, inhalational and dermal administrations (OECD, 1998). The aim is to determine the adverse effects arising from repeated administration in lower dosages than used in acute toxicity studies and determine which dose to be used in human studies (OECD, 1998; OECD, 2008). Data generated include general parameters such as daily food consumption and water intake, and

body weight measurements, serum biochemistry and hematology, as well as gross and microscopic examination of the organs (OECD, 1998; OECD, 2008).

2.6.3 Reported specific toxicities of herbal medicines

a) *Hepatotoxicity*

The liver is a vital organ with a range of functions, which includes detoxification and protein synthesis. The liver is prone to toxicity from the barrage of chemicals including herbs and environmental toxins that pass through it daily, however, the identification of herb-induced liver injury could be very challenging because it may be difficult to differentiate from other liver specific diseases (Navarro, 2009). Herbs and herbal products contain dozens of various chemicals, rendering compound-specific causality difficult (Teschke *et al.*, 2013). Features of hepatotoxicity may vary from derangement in liver enzymes to overt clinical diseases like toxic hepatitis, acute liver failure in acute toxicity; and liver cirrhosis and hepatocellular carcinoma in chronic toxicity (Teschke *et al.*, 2012)

Herbal-induced liver injury (HILI) has been noted in many medicinal plants that were hitherto, thought to be safe. Kava, a herbal sedative prepared from South Pacific plant, *Piper methysticum*, has been reported to be associated with severe toxic hepatitis (Escher *et al.*, 2001). The main bioactive compounds in Kava are yangonin, desmethoxyyangonin, 11-methoxyyangonin, kavain and dihydroxykavain and the kava lactones can inhibit the CYP450 enzymes and also interact with other drugs or herbs metabolised by this enzyme system (Mathews *et al.*, 2002). Other medicinal plants with reported hepatotoxicity are: *Larrea tridentate* (Chaparral) used as an antioxidant and anticancer (Alderman *et al.*, 1994; Gordon *et al.*, 1995); Mistletoe used as

digestive aid, heart tonic and sedative(Laliberte andVilleneuve, 1996); Germander usedfor weight loss and as a general tonic(Stickel *et al.*, 2000).

b) *Nephrotoxicity*

The kidneys receive 25% of the cardiac output in humans. It functions to purify toxic metabolic waste products from the blood in several hundred thousand functionally independent units called nephrons (Kurts *et al.*, 2013).Other functions of the kidneys are regulation of electrolytes, fluid, and acid-base balance; stimulation of red blood cell production, regulation of blood pressure via the renin-angiotensin-aldosterone system; controlling reabsorption of water and maintaining intravascular volume; also reabsorption of glucose and amino acids; as well as hormonal functions via erythropoietin, calcitriol, and vitamin D activation(Kurts *et al.*, 2013).

The kidney is particularly vulnerable to injury because of its high blood flow rate, high endothelial surface area, high metabolic activity; and active uptake by the tubular cells and medullary interstitial concentration of substances (Singh and Prakash, 2011). Herbal medicines may damage the liver via various mechanisms such as: direct or immune-mediated nephrotoxicity, nephrolithiasis, rhabdomyolysis or hepatorenal syndrome (Nauffal and Gabardi, 2016).

Below are some of the examples of nephrotoxicity attributed to herbal medicine preparations:

i. *Chinese herb nephropathy*

The Chinese weight loss herbal remedy was reported to have caused nephrotoxicity in 100 young Belgian women who were taking slimming herbs, 70 of which required dialysis while 30 subsequently developed urothelial carcinoma (Vanherwehem *et al.*,1993).The culprit herbs were *Stephania tetrandra* and *Magnolia officinias*, which contains the substance Aristocholic acid (AA). AA is a nitrophenanthrene carboxylic

acid which forms DNA adducts in renal cells as well as tissues after metabolic activation. The DNA adducts result in the typical renal interstitial fibrosis and urothelial carcinoma (Vanherweghem *et al.*, 1996).

ii. *Balkan endemic nephropathy*

Balkan endemic nephropathy was reported amongst the Balkans who took plant products contaminated with the fungal mycotoxin ochratoxin A, which forms mutagenic DNA products in the kidney (Tatu *et al.*, 1998).

iii. *Djenkol bean poisoning*

Djenkol beans are a pungent smelling edible fruit from the hardwood tree *Pithecellobium labatum*. It contains djenkolic acid, a sulphur-rich cysteine thioacetal of formaldehyde. Djenkolic acid is thought to produce severe tubular necrosis, forms needle-like crystals in concentrated acidic urine in distal tubules (Wong *et al.*, 2007).

Other herbs with reported nephrotoxicity are: *Callilepis laureola*, mushroom containing nephrotoxin orellanine, *Glycyrrhiza glabra*, *Ephedra sinica*, *Averrhoa carambola*, *Salix daphnoides*, and *Fucus vesiculosus* (Singh and Prakash, 2011).

c) *Cardiotoxicity*

Though many herbal drugs are said to be cardiogenic and hence useful in heart disease, besides their use in cardiac insufficiency, some of these herbs have also been noted to be responsible for cardiotoxicity. These herbs include: *Aconitum carmichaeli*, *Aconitum kusnezoffii*, *Digitalis lanata*, *Urginea maritime*, *Glycyrrhiza glabra*, *Convallaria majalis* and *Ephedra sinica* (De Smet, 2002).

2.7 Overview of the Immune System

The immune system is a complex system that exists in vertebrate animals to defend them against foreign substances that may be pathogenic or non-pathogenic. In doing so, the system must be able to distinguish self from non-self. This role, though crucial to the survival and healthy living of the host, may also act as a double-edged sword. In immunodeficiency states, the host is susceptible to a barrage of invading organisms such as bacteria, viruses, protozoa, fungi and tumors; in another scenario, a hyperactive immune system may lead to a fatal sequelae such as anaphylaxis, while in some cases, the immune system may lose its capacity to distinguish self from non-self resulting in autoimmune disorders.

The immune system has been implicated in virtually all known human diseases ranging from inflammatory diseases of the skin, gut, respiratory tract, joints, visceral organs, HIV and other infectious diseases to several non-communicable diseases including malignancies (Hansson *et al.*, 2002; Opitz *et al.*, 2010; Fong, 2014).

Animal disease models have been used to study the role of immune system in the pathogenesis of diseases and also to explore the role of medicines (allopathic or traditional) in the prevention and treatment of immunological diseases.

2.7.1 The lymphoid system

The lymphoid system is an aggregation of lymphoid organs involved in the immune response. This could be primary (central) lymphoid organ, which help in the formation, proliferation and maturation of lymphocytes in the absence of antigen. The foetal liver and bone marrow (*Bursa of Fabricius* in birds) are the primary lymphoid organs responsible for B-lymphocyte maturation while the thymus is responsible for the T-cell maturation.

The secondary or peripheral lymphoid organs are spleen, lymph nodes, Mucosal Associated Lymphoid Tissues (MALT) that includes the tonsils and Peyer's patches of the gut.

A. *Thymus*

The thymus is a primary lymphoid organ and the initial site for the development of T-cell immunological function. It is morphologically similar across all species. The thymus is developed from the endoderm and mesenchyme of the 3rd and 4th pharyngeal pouches (Dijkstra and Veerma, 1990). It is a bilobed organ located in the thorax, and each lobe has trabeculae that produce lobules. The epithelial cells form an open framework containing cells predominantly T- lymphocytes, smaller population of B- lymphocytes and plasma cells and scattered neuroendocrine cells, arranged into morphologically distinct cortex and medulla separated by vascular corticomedullary zone (Pearse, 2006a). The medullary thymocytes express high levels of MHC class 1 and also contain Hassall's corpuscle where clonal deletion of lymphocyte takes place. Histologically, thymus is consistent across species (Haley, 2003). The darkly staining cortex contain densely-packed immature lymphocytes with sparse epithelial cell population but large mitotically active lymphoblasts which are round or oval with one or two prominent nucleoli and strongly basophilic cytoplasm may be found in subcapsular cortex (Pearse, 2006b).

The medulla is paler, less densely cellular and continuous with the adjacent lobules. It contains more mature T cells, prominent epithelial cells, Hassall's corpuscles, admixed macrophages, dendritic cells, B-lymphocytes and rarely myeloid cells (Pearse, 2006b).

The immune status of the thymus is reflected by thymic cellularity as reflected in the histological appearance or the relative organ weights. This is affected by factors such as the age, genetic background, nutrition and stress (Pearse, 2006b).

B. Spleen

The spleen is an encapsulated organ located in the abdomen directly beneath the diaphragm and developed from the splanchnic mesoderm plate (Mebius and Kraal, 2005). The splenic artery pierce the capsule and proceed as a tree of branching arterial vessels, in which the smaller arterioles end in venous sinusoidal system that drains back into the splenic vein. Morphologically, the spleen is divided into the red pulp, the white pulp and the marginal zone.

The red pulp is made of splenic cords and venous sinuses. The splenic cords are composed of reticular fibers, which play a role in splenic contraction; as well as reticular cells and macrophages (Saito *et al.*, 1988). Within the spaces between the cords are red blood cells, granulocytes and circulating mononuclear cells (Cesta, 2006). The red pulp macrophages are phagocytic and remove senescent RBCs, and blood borne particulate matter. Extramedullary hemopoiesis is common in rodent red pulp especial during the fetal and neonatal period (Cesta, 2006).

The white pulp is organised as lymphoid sheaths consisting of three zones: the periarteriolar lymphoid sheath (PALS), the follicles and the marginal zone. The PALS are composed of lymphocytes and concentric layers of reticular fibers and flattened reticular cells (Dijkstra and Veerman, 1990). The PALS are divided into the inner PALS, which are a T-cell dependent region and made up of small lymphocytes, and the outer PALS which contain predominantly CD4+ T cells, but

small numbers of CD8+T cells may also be present (Dijkstra and Veerman, 1990; Matsuno *et al.*, 1989).

The follicles are continuous with PALS and are found at the bifurcation site of the central arterioles with predominantly B-cells, few follicular dendritic cells and CD4+T cells, but typically do not contain CD8+T cells (Van Rees *et al.*, 1996). The white pulp act as storage site for B and T lymphocytes and is the site of their development following antigenic stimulation.

Marginal zone is situated at the interphase of the red pulp with PALS and follicles and it function to screen the systemic circulation for antigens and pathogens, and play an important role in antigen processing (Kuper *et al.*, 2002; Mebuis and Kraal, 2005).

C. *Lymph node*

Lymph nodes are round or kidney shaped, multiple lymphoid lobules surrounded by lymph-filled sinuses and enclosed by a capsule. They are located at the junction of lymphatic vessels. The lymph node consists of the cortex, paracortical areas and the medulla. The B- and T- cells are homed in separate areas within these compartments where they interact with antigen presenting cells (APC) and undergo clonal expansion (Willard-mack, 2006).

The lymphoid lobule is the basic anatomical and functional unit of the lymph node. Each lobule has a bulbous apex and a base, and is separated from the capsule by the subcapsular sinus. The outermost layer, the cortex contains predominantly B-lymphocytes, macrophages and follicle dendritic cells arranged in primary follicles. After antigenic challenge, the primary follicles enlarge into secondary follicles, each containing germinal center (Haley *et al.*, 2005). Beneath the cortex is the paracortex, which consists of predominantly T-lymphocytes, and interdigitating dendritic cells,

which express high level MHC class II. Lymphoid-lineage cells, many of which are plasma cells actively secreting antibody molecules, sparsely populate the innermost layer, the medulla.

D. Mucosa-associated lymphoid tissues (MALT)

The mucosa-associated lymphoid organs are a group of organized lymphoid tissues that line the mucous membranes of the digestive, respiratory and urogenital systems of the body. These tissues range from loose, or barely organized clusters of lymphoid cells in the lamina propria of intestinal villi to organized structures such as the tonsils, appendix, Peyer's patch. Peyer's patch is noted to be present in mouse as early as 15 day of development (Jung *et al.*, 2010).

Morphologically, the Peyer's patch and other MALT are organized into three domains- the follicular area, the interfollicular area and follicle-associated epithelium. The follicular and interfollicular areas consist of lymphoid follicles with germinal centers containing proliferating B-lymphocytes, follicular dendritic cells and macrophages (Neutra *et al.*, 2001). The follicle associated epithelium is characterized by large number of infiltrated B-cells, T-cells, macrophages and DCs, and it lacks the subepithelial myofibroblast and basal lamina propria is more porous compared to regular epithelium (Sierro *et al.*, 2000).

2.7.2 Types of immune system

The immune response of the body against non-self is composed of two types: the innate (or natural, non-specific) and the adaptive (acquired or specific) responses.

Both of these responses are carried out by the interplay of cellular and humoral factors (Patil *et al.*, 2012; Saroj *et al.*, 2012).

A. *Innate immune responses*

The innate immune response is phylogenetically ancient and developed to provide non-specific responses to pathogens that are encountered regularly but rarely cause diseases (Saroj *et al.*, 2012). Their responses are rapid, generic and leave no memory and they include barrier, cellular or humoral immune functions.

1. *Barrier functions*

- *The skin*

The skin is the largest organ in the body and presents an impenetrable barrier to microbes and other harmful agents. The skin also contains the sweat and sebaceous glands, which gives the skin a PH of 3-5, acidic enough to protect against most microorganisms.

- *Other external surfaces*

The digestive and the respiratory tracts represent other potential routes of entry of harmful agents into the body. The cell linings of the smaller bronchi and bronchioles secrete mucus, which trap organisms before they can reach the lungs. In addition, other layers have cilia, which continuously sweep the mucus towards the glottis for expectoration.

2. *Cellular non-specific immune response*

The cellular non-specific responses to harm are provided by the macrophages, neutrophils, and natural killer cells. The macrophages are large, irregularly shaped cells that kill microbes by ingesting them through phagocytosis. In the macrophages, the membrane-bound vacuole containing the bacterium fuses with the lysosome. This fusion activates large quantities of oxygen free radicals, which then kill the offending harmful agent.

The neutrophils are special types of macrophages that ingest and kill bacteria by phagocytosis. They also contain hydrolytic enzymes that kill bacteria. The natural killer cells are morphologically similar to small or medium-sized lymphocytes, which usually destroy tumor or virus-infected cells or any other target in the body that has antibody coating.

3. *Humoral non-specific immune response*

These include the complements, transferrin, interferons, lysozymes and properdin. The complement system consists of approximately 20 different proteins that circulate freely in blood plasma. They are important in opsonisation, which is the coating of the foreign agent with specific complement fragments that makes them recognizable by receptors present in the phagocytic cells. They also aggregate to form membrane attack complex that inserts itself into the foreign plasma membrane and thereby kills the invading organism. Transferrin is an iron-transporting protein that inhibits the growth of many bacteria. The iron-trapping of transferrin inhibits many bacteria that require iron for growth. The interferon gamma is released by T-lymphocytes when they are in contact with antigen. This helps in the activation of macrophages and induces the expression of HLA class II molecules on macrophages and other immune cells. They also activate macrophages and natural killer cells to express cytolytic functions.

B. Adaptive immune responses

The adaptive immunity differs from the innate response because it is specific, has element of memory and it is unique to vertebrates (Saroj *et al*, 2012). It has both cellular and humoral aspects. Both cellular and humoral specific immune responses are directed by two subset of lymphocytes-the T- and B-lymphocytes.

1. The cellular adaptive immunity

The T-lymphocytes originate in the bone marrow and migrates to the thymus where they develop to identify microorganisms by the antigens expressed in their cell surfaces. The T-lymphocytes also express a variety of non-polymorphic function – associated molecules of which CD4 and CD8 are themost important. CD4-molecules are expressed in about 60% of mature T-lymphocytes while the CD8 is expressed in about 30%. CD4 cells bind to theMHC class II expressed in antigen-presenting cells while CD8 binds to class I MHC. The CD4 and CD8 cells perform distinct but overlapping functions. The CD4 is the master regulator of immune system. It secretes soluble factors (cytokines) that help influence all other cells of the immune system, including other T-cells, B-cells, macrophages and natural killer cells. There are two functionally different population of CD4-helper cells viz: The T_H1 subset which synthesis and secrete IL-2 and IFN- γ ; and th T_H2 –helper cells which produce IL-4 and IL-5. The T_H1 is involved in facilitating delayed hypersensitivity; macrophage activation and synthesis of IgG2b antibodies while the T_H2 aid the synthesis of other classes of antibodies including IgE. The CD8+cells secretes cytokines but mediate their action by acting primarily as cytotoxic cells.

2. *Humoral adaptive immunity*

The B-lymphocytes constitute about 10-20% of the circulating peripheral lymphocytes. They are also present in the bone marrow, lymph nodes, spleen and the tonsils. On antigenic stimulation, B-cells form plasma cells that secrete immunoglobulins that help to mediate humoral immunity. Unlike the T-cells that bind to antigens coupled to MHC, the immunoglobulins bind to free, unprocessed antigens. The immunoglobulins have 4-polypeptide chain structure consisting of 2 Heavy polypeptide chains and 2 Light polypeptide chains linked together by disulfide bonds. The light chains can consist of either a kappa (κ) or lambda chain (λ). Each component Heavy chain consists of one variable (V) domain and 3 or 4 constant (C) domains while the light chains contain only one constant domain (Schroeder and Cavacini, 2010).

a) *IgM*

IgM is the first immunoglobulin expressed during B-cell development. Naïve B cells express monomeric IgM on their surface, but on maturation and antigenic stimulation, multimeric (usually pentameric and rarely hexameric) IgM in which single IgM units link to each other by disulfide bond; and the pentamer also contains a polypeptide chain, the J-chain which is bound to 2 of the monomers by disulfide bond (Schroeder and Cavacini, 2010). The J-chain facilitates secretion at the mucosal surfaces. The IgM antibodies are associated with primary immune response and are used to diagnose acute exposure to an immunogen or pathogen.

b) *IgD*

Circulating IgD is found at very low levels in the serum because of its short half-life. The function is unclear but has been postulated that membrane-bound IgD regulates B-cell at specific developmental stages (Gersberger *et al.*, 2006).

c) *IgG*

This is the predominant isotype in the body and has the longest serum half-life. Based on the structural, antigenic and functional differences in the constant (C) region of the heavy chains, 4 subclasses of IgG viz: IgG1, IgG2, IgG3 and IgG4 are identified and ranked in order of serum levels from IgG1 (highest) to IgG4 (lowest). IgG antibodies contribute directly to immune responses, including neutralization of toxins and viruses; and complement activation (Schroeder and Cavacini, 2010).

d) *IgA*

Serum IgA in humans appear higher than IgM but considerably lower than IgG. IgA levels are much higher than IgG at the mucosal surfaces and in secretions including saliva and breast milk (Woof and Mesky, 2005). IgA is critical in protecting mucosal surfaces from toxins, viruses and bacteria by means of direct neutralization or prevention of binding to the mucosal surfaces.

e) *IgE*

This immunoglobulin is present at the lowest serum concentration and has the shortest half-life. It is associated with hypersensitivity and allergic reactions as well as

response to parasitic worm infestations. IgE binds with very high affinity to Fc portion expressed on mast cells, basophils, Langerhan cells and eosinophils, and recently, anti-IgE antibodies have been developed as therapy for asthma and allergy (Stubbe *et al.*, 2000).

2.8 Mechanism of Immune Injury

2.8.1 Hypersensitivity reactions

This refers to antigen-specific immunological reactions that have adverse effects. Coombs and Gell (1963) first used the traditional classification system, and it was classified into four types viz: Type I (Ig E-mediated immediate-type hypersensitivity), Type II (IgG- or IgM-mediated antibody dependent cytotoxicity), Type III (IgG-mediated immune complex reactions) and type IV (lymphocyte mediated delayed type hypersensitivity reaction).

A. *Type I hypersensitivity reaction (anaphylactic type)*

This is defined as a rapidly developing immunologic reaction occurring within minutes after the combination of an antigen with an antibody bound to mast cells or basophils in individuals previously sensitized. There are two subtypes: systemic hypersensitivity (anaphylaxis) and local reaction (skin allergy, asthma, hives, allergic rhinitis, allergic conjunctivities) (Kay, 2001). Mast cells and basophils are key to the development of Type I hypersensitivity. The cross-linking of high affinity IgE Fc receptors activates these cells and complement components such as complement C3a and C5a (anaphylatoxins). The first step in the synthesis of IgE is the presentation of an allergen to precursor T_H2 cells by antigen presenting cells (APC). This leads to the

production of cytokines by T_H2 cells such as IL-3, IL-4, IL-5 and GM-CSF. IL-4 is essential for turning the IgE producing B cells while IL-3, IL-5 and GM-CSF promote the survival of eosinophils. IgE antibodies attach to mast cells and basophils, which have receptors for Fc portion of IgE, and when re-exposed to a specific allergen, triggers the release of inflammatory mediators that are responsible for Type I hypersensitivity (Baraniuk, 1997).

B. Type II hypersensitivity reaction

This is mediated by antibodies directed towards antigens present on the surface of cells or tissues. Hypersensitivity results from the binding of antibodies to normal or altered cell surface antigens. There are three different antibody-dependent mechanisms responsible for this viz:

1) Complement dependent reactions

IgM or IgG reacts with antigen on the surface of cells causing activation of complements and resulting in the assembly of membrane attack complex that disrupt the cell membrane integrity. In another method, susceptible cells are coated by complement C3b (an opsonin) and this makes them susceptible to phagocytosis. These reactions are seen in blood transfusion reactions, erythroblastosis fetalis, autoimmune hematological diseases, and pemphigus vulgaris.

2) Antibody-dependent cell-mediated cytotoxicity (ADCC)

This does not involve complement fixation but rather, the target cells are coated with low concentration of IgG and killed by a variety of non-sensitized cells. This is seen in phagocytosis of tumor cells and graft tissues.

3) *Antibody-mediated cellular dysfunction*

In this case, antibody directed against cell surface antigens impair or dysregulate functions without causing injury or inflammation. Example is seen in Graves' disease.

C. *Type III hypersensitivity (immune complex mediated)*

Antigen-antibody complexes produce tissue damages as a result of their capacity to activate complement system mediate this. Examples are seen in systemic lupus erythematosus, rheumatoid arthritis, glomerulonephritis etc

D. *Type IV hypersensitivity (cell mediated)*

This is initiated by specific sensitized T lymphocytes. There are two forms: the classic delayed- type hypersensitivity reactions initiated by CD4 T cells and the direct cell cytotoxicity mediated by CD8 T cells.

1) *Delayed type hypersensitivity (DTH)*

This is typically exemplified by tuberculin reaction. It begins with first exposure to the allergen (eg tubercule bacilli); naïve CD4 T cells recognized the peptides in the bacilli in association with MHC II on the surface of macrophages and dendritic cells. This encounter drives the differentiation of naïve CD4 T cells to T_H1 cells. Some of the CD4 T cells so sensitized enter the circulation and form the pool of memory cells. On intracutaneous injection of tuberculin, the memory T cells undergo blast transformation and proliferation, accompanied by the secretion of cytokines such as IL-12, IFN- γ , IL-2 and TNF- α (Abbas *et al.*, 1996).The combined effects of these cytokines are the reddening and induration at the site of injection of tuberculin within 8-12 hours. This peak at 24-72 hours and thereafter, slowly subsides.

2) *T cell-mediated cytotoxicity*

In this variant, sensitized CD8+ T cells, called cytotoxic T- lymphocytes (CTL) kill antigen-bearing cells. These CTLs directed against cell surface histocompatibility antigen class I plays important role in the mediation of graft versus host disease.

2.9 Immunomodulation

Immunomodulators are substances-synthetic or biological, which can stimulate, suppress or modulate any components of the immune system (Agarwal and Singh,1999). They have very useful application in therapeutics because of their ability to modify the natural and adaptive defense mechanisms for the benefit of the recipient (Alamagir and Uddin, 2010).

2.9.1 Classification of immunomodulators

Immunomodulators can be classified into three categories viz: Immunoadjuvants, immunostimulants and immunosuppressants.

1) *Immunoadjuvants*

These agents are specific type of immunostimulants but are used exclusively in enhancing the efficacy of vaccines (Nagarathna *et al.*, 2013).The pure recombinant and synthetic antigens used in modern vaccine development are less immunogenic and the quality of these agents can be augmented by adjuvants that stimulate the immune system to respond to vaccine more vigorously.

The adjuvants perform this task by mimicking specific sets of evolutionary conserved molecules such as lipopolysaccharide (LPS), components of bacterial cell wall, endocytosed nucleic acids and unmethylated CpG dinucleotide containing DNA (Mohan *et al.*, 2013). In addition to enhancing immunogenicity, adjuvants also provide antigen-dose sparing, accelerate immune response, reduce the need for booster immunizations, increase the duration of protection and improve efficacy in poor responder population (Mohan *et al.*, 2013).

Major adjuvant groups:

A. *Alum-based adjuvants*

Aluminum phosphates and hydroxide salts are the major adjuvants recommended by the Food and Drug Administration (FDA) for vaccine development. They are relatively weak and rarely induce cellular immune response but slow down the release of antigen, thereby prolonging the duration of their interaction with the immune system (Lindblad, 2004). Other minerals salts such as calcium, iron and zirconium have been tried as adjuvant but not as successful as the alum salts.

B. *Complete Freund's Adjuvant (CFA)*

These contain heat-killed mycobacteria, which are used to stimulate antibody production in laboratory animals and man. However, it has been shown to come with potential health and safety threats (Jackson and Fox, 1995). Complete Freund's adjuvant is effective in stimulating cellular immune response and also may potentiate IgA and IgG response (Bomford, 1980).

C. *Incomplete Freund's Adjuvant (IFA)*

This is water in oil emulsion prepared from non-metabolized oils. It induces TH2-biased response through the formation of depot at the injection site and stimulation of antibody producing plasma cells (Freund, 1956).

D. *Other adjuvants*

Other substances that have been known to have adjuvant properties are: Adjuvant 65, Montanide, MF59; Bacteria toxins such as cholera toxin, pertussigen, clostridium difficile toxin, shiga toxin and staphylococcal enterotoxin (Orozco-Morales *et al.*, 2012; Gupta *et al.*, 2011; Lee *et al.*, 2009; and Ghose *et al.*, 2007).

2) *Immunostimulants*

They are agents, biologic or synthetic that act in a non-specific way to enhance the body's immune function. These actions could be on the innate or adaptive immune system (Chandua *et al.*, 2011; Clement *et al.*, 2010). In healthy individuals, immunostimulants serve as immune potentiators by enhancing the basal level of immune response, and in those with impairment of the immune system, they serve as immunotherapeutic agents (Agarwal and Singh, 1999).

Drugs with immunostimulatory activities

A. *Levamisole*

This drug was originally synthesized as an antihelmimthic drug but has been noted to restore depressed immune function of B-lymphocytes, T-lymphocytes, monocytes and macrophages and is mainly used as an adjuvant therapy (Arutiunian and

Grigorian,2003). Levamisole has also been noted to enhance the immune response towards T_H1 development through the activation of dendritic or T cell aspects while suppression the T_H2 immune response (Chenet *et al.*,2008).

B. *Thalidomide*

Thalidomide was initially developed as an antiemetic agent but its use was limited due to the severe and life-threatening birth effect it caused to pregnant women (Lary *et al.*, 1999; Smithells and Newman, 1992). It has been shown to possess anti-inflammatory effect and useful in erythema nodosum leprosum; also has anti-angiogenic property, hence useful in multiple myeloma (Saroj *et al.*, 2012).Its use in rheumatoid arthritis is probably due to its anti-tumor necrotic factor (TNF) effect (Kay *et al.*,2009).

C. *Bacillus Calmette-Guérin (BCG)*

This is an attenuated, live culture of the bacillus of Calmette and Guèrin strain of *Mycobacterium bovis* that induces granulomatous reaction at the site of administration. By unknown mechanism, it is active against tumors and is used as a prophylaxis against carcinoma *insitu* of the urinary bladder and for the prophylaxis of primary and recurrent stage Ta and/or T1 papillary tumors following transurethral resection (Parterson and Patel, 1998).

D. *Recombinant cytokines*

i. Interferons

Interferons (alpha, beta and gamma) were initially identified by their antiviral activity

but have been shown to have immunomodulatory properties (Tilg and Kaser, 1999). They bind to specific cell-surface receptors that lead to: enzyme induction, inhibition of cell proliferation, and enhancement of immune activities, including increased phagocytosis by macrophages, and augmentation of specific cytotoxicity by T lymphocytes (Tompkins, 1999).

- *Interferon alpha-2b*

This is produced and secreted by cells in response to viral infections and other inducers. It is indicated in the treatment of variety of tumors such as hairy cell leukemia, malignant melanoma, follicular lymphoma and AIDS-related Kaposi's sarcoma (Sinkovics and Horvath, 2000).

- *Interferon gamma-1b*

This is a recombinant polypeptide that activates phagocytes and induces their generation of reactive oxygen metabolites that are toxic to microorganisms. It is useful in limiting the frequency and severity of infection associated with chronic granulomatous disease.

- *Interferon beta-1a*

Interferon beta-1a is a 166 amino acid recombinant glycoprotein that has both antiviral activity and immunomodulatory activity. It is an effective therapy against relapsing multiple sclerosis. It is also observed to induce changes in dendritic cell cytokine secretion and inhibit IL-17A, which is a cytokine that promotes inflammatory responses that are protective against certain bacterial infections but also implicated in the pathogenesis of many autoimmune diseases (Abbas and Andrew,

2009; Zhang *et al.*, 2009).

ii. Interleukin-2 (IL-2)

This is a human recombinant IL-2 produced by recombinant DNA technology in *E. coli* (Taniguchi and Minami, 1993). *In vivo* administration of IL-2 in animals produces multiple effects in a dose-dependent manner (Whittington and Faulds, 1993).

3) Immunosuppressants

These agents suppress the immune response to various antigens and to self, and hence are used widely in autoimmune diseases, graft rejection, graft versus host disease, hypersensitivity reaction and immune pathology associated with infections (Agarwal and Singh, 1999).

Drugs with immunosuppressive activities:

A. Calcineurin inhibitors

These are specific T-cell inhibitors. They act by inhibiting the lymphocyte signal to prevent immune cell activation and proliferation.

- *Cyclosporine*

This is a lipophilic cyclic polypeptide made of 11 amino acids derived from the fungus *Beauveria nivea*. It inhibits the antigen-triggered signal transduction in T-lymphocyte, blunting expression of many lymphokines including IL-2 and expression of antiapoptotic proteins. Cyclosporine forms a complex with a cytoplasmic receptor protein, cyclophilin. Cyclosporine-cyclophilin complex bind to calcineurin and this leads to the inhibition of calcium-dependent dephosphorylation of the cytosolic component of the nuclear factor for activated T-cells (NFAT). Cyclosporine also

increase expression of transformed growth factor- β (TGF- β) which is a potent inhibitor of IL-2 stimulated T-cell proliferation (Patil *et al.*, 2012).

Cyclosporine is used therapeutically in kidney, liver, heart and other organ transplantation; rheumatoid arthritis, psoriasis, Behcet's disease etc

- Tacrolimus

Tacrolimus is a macrolide antibiotic produced from the actinobacteria *Streptomyces tsukubaensis*. It binds to intracellular immunophilin, protein FK506-binding protein-12 (FKBP-12) that is structurally similar to cyclophilin. The complex tacrolimus-FKBP-12, binds to calcineurin in the presence of calcium and calmodulin to inhibit NFAT and T-cell activation (Saroj *et al.*, 2012).

B. Mammalian target of rapamycin (MTOR) inhibitors

- Sirolimus

This is the prototype of mTOR and is a macrocyclic lactone produced by *Streptomyces hygroscopicus*. It acts by inhibiting T-lymphocyte activation and proliferation by forming a complex with immunophilin, FKBP-12. However, the sirolimus-FKBP-12 does not bind to calcineurin, but binds and inhibits the protein kinase, mammalian target of rapamycin (mTOR), which is the key enzyme in cell cycle progression. This blocks the cell-cycle progression at the G₁ to S-phase transition (Patil *et al.*, 2012).

C. Cytotoxic agents

- Azathioprine

This is an antimetabolite belonging to the purine subclass. It is an imidazolyl

derivative of 6-mercaptopurine. It is selectively taken into cells where it undergoes intracellular conversion to 6-mercaptopurine and additional metabolites that inhibit *de novo* synthesis of purine. This leads to inhibition of cell proliferation. It is used in kidney transplantation and other graft rejections (Patil *et al.*, 2012; Saroj *et al.*, 2012).

- *Cyclophosphamide*

Cyclophosphamide is a widely used alkylating immunosuppressive agent in autoimmune diseases and in graft-verses host disease. It acts by introducing alkyl group into nucleophilic moieties, which leads to cross-linking between the strands of DNA and cell death (Patil *et al.*, 2012). It is noted to suppress B-lymphocyte proliferation but may enhance T-cell response (Patil *et al.*, 2012).

- Other cytotoxic agents that are useful in the treatment of many immune diseases are: mycophenolate mofetil, methotrexate, chlorambucil etc

D. Glucocorticoids

- *Prednisolone*

Prednisolone and other steroids are used to suppress acute rejection of solid allograft and in chronic graft verses host disease. They enter the cell and nucleus, binds with the glucocorticoid receptors and activate and regulate DNA transcription (Saroj *et al.*, 2012).

E. Cytokine inhibitor

Tumor necrotic factor (TNF- α), IL-1 and IL-2 are proinflammatory cytokines that

have implicated in the pathogenesis of many diseases including rheumatoid arthritis, crohn's disease and many other immunological diseases.

Activated TH1 cells and macrophages secrete TNF- α that bind TNF receptors in fibroblasts, neutrophils and endothelial cells which leads to the release of cytokines such as IL-1, IL-6, and adhesion molecules that enhance leukocyte activation and migration.

- *Etanercept*

It is a fusion protein made up of two soluble TNF_{p75} receptors moieties linked to F_c portion of human IgG₁. It provides artificial binding sites to TNF- α receptor and prevent the endogenous TNF- α from binding, thereby, cutting off the pro-inflammatory cascades (Patil *et al.*, 2012).

- *Infliximab*

This is a chimeric monoclonal antibody obtained from mice. It acts by cross-linking with membrane bound TNF- α receptors on the surface to inhibit T-cell activation and macrophage function.

2.9.2 Medicinal plants with immunomodulatory activities

A host of medicinal plants have been noted to have immunomodulatory activities and other workers have extensively researched their properties. Some of these are found in Table 2.1.

Table 2.1: Medical Plants with Immunomodulatory Properties

S/No	Plants	<i>In vitro</i> properties	<i>In vivo</i> properties	References
1	<i>Allium sativum</i>	Augment NK cells and macrophage activities	Augment NK cells, stimulate T cells and IL-2 production	Jain, 1994
2	<i>Aloe vera</i>	Enhance antibody production, stimulate IL-6, TNF- α and NO production	Improve wound healing, stimulates production of IL-1, TNF- α	Chithra <i>et al.</i> , 1998a, 1998b
3	<i>Andrographis paniculata</i>	Stimulates macrophage migration phagocytosis of radio-labelled <i>E.coli</i>	Induce antibody stimulation and DTH response to SRBC in mice	Chiou <i>et al.</i> , 1998
4	<i>Asparagus racemosus</i>		Induce lag in tumor development, prevent leucopenia produced by cyclophosphamide	Dhuley, 1997
5	<i>Azadirachia indica</i>	Stimulates IL-1, TNF-, IFN- and enhances proliferative response of spleen to Con A and tetanus toxoid	Enhances macrophage phagocytosis, expression of MHC II antigen, IgM, IgG, and DTH in psoriasis patients	Katiyar <i>et al.</i> , 1997 SaiRam <i>et al.</i> , 1997
6	<i>Curouma longa</i>	Increase mitogenic response of lymphocytes, inhibit NO	Shows anti-inflammatory and antitumor activities production	Rao <i>et al.</i> , 1995
7	<i>Nyctanthes arbor-tristis</i>	Stimulate macrophage migration	Stimulate humoral and DTH response to SRBC in mice	Khan <i>et al.</i> , 1995
8	<i>Ocimum sanctum</i>		Inhibits tumor development in mice, increase colony forming unit in spleen	Kim <i>et al.</i> , 1998; Xiaoguang <i>et al.</i> , 1998

2.10 Methods for Testing Immunomodulatory Activities

Phytomedicines contain a number of chemicals, which may be active or inactive. The ideal process of testing phytochemicals is to first extract the chemical entities that are most responsible for the activity under investigation and confirm the activity via *in vitro* or *in vivo* methods. The whole animal model is the classical pharmacological screening method, and it has the advantage over the *in vitro* methods because the researcher has the privilege of observing both the efficacy and toxicity of the test medicines (Vogel, 2002).

In immunotoxicology studies, toxicology alert is first noticed by the behavior of the experimental animals. Further test that may alert immunotoxicity are: changes in differential white blood counts, changes in clinical chemistry (serum albumin/globulin ratios), alteration in organ weights (thymus, spleen and/or histology of primary and secondary lymphoid organs (Hartung and Corsini, 2013). Table 2.2 highlights some of the methods for evaluating immunomodulatory activities.

2.10.1 *In vivo* methods

Animal studies still remain the gold standard for testing immunotoxicity *in vivo*. However, this is challenging and could be confusing especially when the immunologic effects are minimal-to-moderate (Germolec, 2004). In order to generate useful information, observations from the 28-day general toxicity test can be complemented by *in vitro* studies (Hartung and Corsini, 2013).

Immunotoxic alerts in 28-day repeat dose toxicity studies in animals include: changes in total and differential white blood cell counts (leukocytopenia/leukocytosis, granulocytopenia/granulocytosis or lymphopenia/lymphocytosis), changes in clinical chemistry (serum immunoglobulin levels or albumin/globulin ratios), alteration in

organ weights (thymus and spleen) and/or histology of primary and secondary lymphoid organs. Others are increased incidence of infections, increased occurrence of tumors or chemical retention in organs/cells of the immune system (Hartung and Corsini, 2013).

2.10.2 *In vitro* methods

In *in vitro* methods, hypersensitivity and immunosuppression are the primary focus of the assays, but in contrast with the *in vivo* methods, fewer assay methods exist for immunostimulation and autoimmunity (Hartung and Corsini 2013).

Other limitations of *in vitro* methods according to Hartung and Corsini are:

1. They are not suitable for materials that require biotransformation
2. Physicochemical characteristics of the test substances may interfere with *in vitro* systems
3. It is difficult to reproduce the integrity of the immune system *in vitro*
4. The *in vitro* system does not allow for the evaluation of induction of a memory response
5. The *in vitro* system does not account for the neuro-immuno-endocrine interaction

The table below is a summary of the *in vitro* and *in vivo* tests for evaluation of immunomodulation (Agarwal and Singh, 1999).

Table 2.2: Test Methods for Evaluating Immunomodulatory Activities

***In vitro* and *in vivo* tests**

Immunosuppressant activity:
In vitro: a) Inhibition of lymphocyte proliferation and cytokine production of either T_H1 (IL-2, IFN- α and TNF- β) or T_H2 (IL-4, IL-5 and IL-10 following stimulation with mitogen or specific antigen
b) Inhibition of antibody forming cells in Jerne's plaque assay anantibody production
In vivo: a) Suppression of antibody production by specific antigens
b) Suppression of DTH response to specific antigens
c) Suppression of clearance by reticuloendothelial cells

2 Immunostimulatory activity:
In vitro: a) Stimulation of lymphocyte proliferation and cytokine production of either T_H1 (IL-2,IFN- γ , and TNF- β) or T_H2 8IL-4, IL-5 and IL-10 type following stimulation withsuboptimal dose of mitogen or specific antigen
b) Quantification of the expression of cell activation markers on cell surface viz:CD25, CD69, CD70 and CD86 by fluorescent activatedcell scan (FACS)
In vivo: a) Stimulation of antibody titer to specific antigens
b) Stimulation of DTH to specific antigen

3 Immunoadjuvant activity: The agent is administered in combination with potential vaccine and the effect is estimated by:
a) Specific antibody profile and titre in immunized animals (*in vivo*)
b) DTH response to specific antigen (*in vivo*)
c) Jerne's plaque assay using splenocytes from immunized animals (*in vitro*)
d) Lymphocyte proliferation test using splenocytes from immunize animals against T-cell epitopes of the immunizing antigen (*in vitro*)
e) T_H1/T_H2 cytokine profile (*in vivo*)

4 Effector arm of the immune response:
a) Chemokine levels in the treated animal and *in vitro* assay ofchemotaxis
b) Inhibition/stimulation of phagocytosis (*in vitro/in vivo*)
c) Free radical production (*in vitro*)
d) Nitric oxide production (*in vitro*)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

The following materials were used:

3.1.1 Apparatus

- Pestle and mortar
- Mechanical shaker (no. 3017GBh, Germany)
- Whatman filter paper (No.1)
- Desiccator
- Organ bath
- Test tubes
- Test-tube rack
- Sterile EDTA bottles
- Pipettes
- Micropipettes
- Precision pippete and tips
- Vortex mixer
- Bucket centrifuge (Cole Medical Instrument, England)
- Electronic weighing scale
- Caliper
- Microplate incubator
- Microplate wells
- Microplate reader (Biorad Inc, USA),
- Automated CBC Analyser (Symmex KX-21TM, Kobe, Japan),
- Partec Flow Cytometer (Sysmex Partec, GmBH, Germany)

- Spectrophotometer 20D(Technel & Technel, USA).

3.1.2 Chemicals

- Absolute methanol (BDH Poole, England)
- Absolute ethanol (BDH Poole, England)
- Chloroform (Sigma Chemical Co. U.S.A),
- Sodium hydroxide (BDH Poole, England)
- Potassium hydroxide (BDH Poole, England)
- Hydrochloric acid (BDH Poole, England)
- Sulphuric acid solution (BDH Poole, England)
- 3, 5-dinitrobenzoic acid solution (BDH Poole, England)
- Ferric chloride (BDH Ltd Poole, England)
- Fehling solution A and B (BDH Ltd Poole, England)
- Meyer's Reagent (BDH Poole, England)
- Ammonium solution (BDH Ltd Poole, England)
- Anti-rat IgG-HRP (Life diagnostics, West Chester, PA, USA)
- Anti-rat IgM-HRP (Life diagnostics, West Chester, PA, USA)
- TMB reagent (Life diagnostics, West Chester, PA, USA)
- Stop solution (Life diagnostics, West Chester, PA, USA)
- Normal saline
- Phosphate buffer saline tablets.

All the chemicals were of analytical grade.

3.1.3 Animals

The Animal House of the Department of Pharmacology and Therapeutics, Ahmadu Bello University (ABU), Zaria, Nigeria provided all animals for the study. The animals were housed under standard environmental conditions and fed with rodent standard diet and water *ad libitum*. The animals were allowed to acclimatize for 2 weeks before the commencement of each study. Animal care and handling were in accordance to good laboratory practice (GLP) and OECD guidelines (OECD 2002, 2008).

I. Animals for acute toxicity studies

Adult female Wistar rats (n = 5) weighing between 100-200 grams were used for the study.

II. Animals for 28-day oral toxicity study

Twenty-four (24) healthy adult Wistar rats (12 males and 12 females) weighing between 100-200 grams were used for the study.

III. Animals for immunomodulatory studies

Twenty-four (12 males, 12 females) healthy adult Wistar rats were used for this study.

A total of fifty-three (53) Wistar rats were used.

3.2 Methods

The following methods were used in the study:

3.2.1 Preparation of plant extract

Fresh stem bark of *L. acidat* together with the other parts was collected from a fully-grown plant at a farm center in Zaria along Zaria-Kano Expressway. The plant was

identified and authenticated by Mr. Namadi Salihu of the Department of Biological Science, Ahmadu Bello University, Zaria; and the voucher specimen was deposited at Herbarium (Voucher No. 7341).

The stem bark was washed thoroughly in running water to remove soil and adhering materials, and air-dried under shade for 7 days. The material was then chopped into smaller pieces and then ground into coarse powder using pestle and mortar. The coarse powder was then cold macerated in methanol-water 70% (v/v) for 72 hours with occasional shaking and then filtered with Whatman size 1 filter paper. The filtrate was then evaporated in an organ bath at a temperature of 45⁰C to yield the methanol extract residue, which was kept in dried airtight container until needed.



Plate IV: Harvesting of the stem bark of *Lannea acidaat* the farm site in Zaria

3.2.2 Phytochemical screening

Phytochemical screening of the extract was carried out to test for the presence of glycosides, alkaloids, flavonoids, saponins, tannins, anthraquinones and reducing sugars using standard phytochemical methods (Evans, 2002).

1. *Tests for carbohydrates*

a) *Molisch test*

Two grams of the extract was transferred into a test tube and few drops of Molisch reagent was added and concentrated sulphuric acid was added down the side of the test tube to form a lower layer. A reddish coloured ring at the interphase was formed which indicated the presence of carbohydrates (Evans, 2002).

b) *Fehling's test*

A small portion of the extract was transferred into a test tube, and 5 ml of equal mixture of Fehling's A and B solution were added and boiled on water bath. A brick red precipitate was formed which indicated the presence of reducing sugars (Evans, 2002).

2. *Tests for alkaloids*

a) *Mayer's Test*

To a portion of the extract in a test tube, a few drops of Mayer's reagent were added. A cream precipitate was formed which indicated the presence of alkaloids (Evans, 2002).

3. *Tests for glycosides*

To a portion of the extract in a test tube, 5 ml of dilute sulphuric acid was added and boiled in water bath for 15 minutes. This was then cooled and neutralized with 20% KOH. Then, 5 ml of Fehling's solution A and B was added and the mixture boiled. A brick red precipitate was formed and this confirmed the release of reducing sugars due to the hydrolysis of glycosides (Evans, 2002).

4. *Test for anthraquinone (Bontrager's test)*

A portion of the extract was transferred into a test tube and 5 ml of chloroform was added and shaken for at least 5 minutes. This was then filtered and filtrate was then shaken with equal volume of 10% ammonium solution. A bright pink color was formed in the upper layer, which indicated the presence of anthraquinones (Evans, 2002).

5. *Tests for unsaturated steroid and triterpenes*

a) *Lieberman Buccharad Test*

A portion of the extract was transferred into a test tube, and equal volume of acetic acid anhydride was added and mixed gently. 1 ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer. A blue to green color was formed in the upper layer and then a reddish colour over a period of 1 hour, which indicated the presence of triterpene (Evans, 2002).

b) *Salkowski test for unsaturated sterols*

A small portion of the extract was transferred into a test tube, and 3 drops of concentrated sulphuric acid was added at the side of the test tube. A cherry red colour was formed over a period of 1 hour, which indicated the presence of sterols (Evans, 2002).

6. *Tests for cardiac glycosides*

a) *Keller-Kiliani test*

A portion of the extract was dissolved in 1 ml of glacial acetic acid containing traces of ferric chloride. This was then transferred into a dry test tube and 1 ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer at the bottom. A pale green color was formed at the upper acetic acid layer, which indicated the presence of cardiac glycosides (Evans, 2002).

b) *Kedde's test*

A portion of the extract was transferred into a test tube, and 1 ml of 2% solution of 3, 5-dinitrobenzoic acid in 95% alcohol was added. The solution was made alkaline by adding 5% sodium hydroxide. A purple-blue color was formed which indicated the presence of cardenolides (Evans, 2002).

7. *Test for saponin glycosides*

a) *Frothing test*

A portion of the extract was transferred into a test tube and 10 ml of distill water was added and the mixture shaken vigorously for 30 seconds. The tube was allowed to stand in a vertical position and was observed for 30 minutes. A honeycomb froth that persists for 15 minutes was formed which indicated the presence of saponins (Evans, 2002).

8. *Test for tannins*

a) *Ferric chloride test*

A portion of the extract was transferred into a test tube, and 4 drops of ferric chloride was added. A greenish-black precipitate was formed which indicated the presence of condensed tannins (Evans, 2002).

9. *Tests for flavonoids*

a) *Shinoda test*

A portion of the extract was transferred into a test tube, and 2 ml of 50% methanol was added and heated with metallic magnesium chip and few drops of concentrated hydrochloric acid. A red color was formed which indicated the presence of flavonoids (Evans, 2002).

b) *Sodium hydroxide test*

A portion of the extract was transferred into a test tube and few drops of 10% sodium hydroxide were added. A yellow coloration was formed which indicated the presence of flavonoids (Evans, 2002).

3.2.3 Preparation of doses of the extract

Three (3) grams of the cherry red extract of the dried methanol extract of the stem bark of *L. acida* was dissolved in 6 ml of distilled water to yield a thick chocolate paste of 500mg/ml of the stock solution. The respective doses used for the study were prepared in mg per kilogram body weight of the rats.

The dose of the extract to be administered to each rat was calculated as follows:

$$\text{Volume to be administered:} = \frac{\text{Dose} \times \text{Weight of rat (kg)}}{\text{Concentration of Stock solution (500mg/ml)}}$$

3.2.3 Preparation of the Sheep Red Blood Cells (SRBC)

The sheep red blood cell was prepared according to the protocol described by the manufacturer (Sigma-Aldrich[®], USA).

Protocol:

Using aseptic technique, we obtained 40 ml of sheep blood from the Dogarawa, Zaria Abattoir and transferred into a sterile EDTA bottle. This was centrifuged in sterile test-tube at 3000 revolutions per minute for 10 minutes. The supernatant was carefully removed with a sterile pipette and discarded. The sheep blood was then washed twice by suspending them in 0.9% normal saline and centrifuging at 3,000 revolutions per minute (rpm) and carefully removing the supernatant after each wash with a sterile pipette. The resultant cells were then washed again in phosphate buffered saline (PBS), (PH = 7) by suspending them (50 ml) and centrifuging and carefully removing the supernatant.

Two times the original volume of PBS was added to make 100 ml of SRBC suspension (suspension A).

The additional volume of PBS to be added to make a concentration of 10^9 cells/ml was determined as follows:

1. A 15-fold dilution of a suspension with 10^9 cells/ml has an absorbance of 0.7 at 541nm.
2. 0.5 ml of the washed SRBC suspension was added to 7.0 ml of distilled water and mixed to lyse the cells. The Absorbance at 541nm of the SRBC was read against the absorbance of distilled water (A_{541} vs. distilled water).
3. Additional dilution was made with a 3-fold dilution by adding 1ml of diluted cells to 2ml of distilled water until absorbance was ≤ 1 .
4. Volume of PBS (Volume B) to make 10^9 /ml of SRBC was thus:
$$V_B = \left(\frac{A_{541}}{0.7}\right) \times (ml \text{ of suspension A}) \times (Additional \text{ Factor})$$
5. Volume B – Volume A = ml of PBS to be added.

3.2.4 Acute toxicity testing

The extract was assessed for acute toxicity testing using the Fixed Dose Procedure, OECD guideline 420 (OECD, 2002). Five healthy female, nulliparous, non-pregnant Wistar rats weighing between 150-200 grams and labeled A1, A2, A3, A4 and A5 were used. They were maintained in the Animal House of the Department of Pharmacology, Ahmadu Bello University Zaria with food and water *ad libitum* and under standard laboratory conditions.

The result of previous acute toxicity test suggests that *L. acida* is non-lethal and safe when administered orally at a dose of 3,000 mg/kg (Etuk *et al.*, 2009); and similar specie, *L. kerstingii* was also non-lethal at a dose of 5,000 mg/kg (Diallo *et al.*, 2010); so a limit dose of 3,000 mg/kg was used.

Food but not water was withheld overnight before the test. A test dose of 3000 mg/kg was administered orally as a single dose, and at a sequential interval of 48 hours, to each of the 5 animals by gavage using a gastric tube. Food was withheld for further 3 hours after the dosing before feeding the animals. Each animal was observed immediately after the dosing for any sign of acute toxicity for at least 1 hour and then at least once daily for 14 days. Animals were weighed weekly until the end of the experiment. All the surviving test animals were subjected to gross necropsy and histological examinations of the organs.

3.2.5 Sub-acute toxicity test (28-day repeated dose study)

The repeated dose test was performed in accordance to the OECD guideline 407 (OECD, 2008). Wistar rats were divided into four groups each of 6 animals (3 males, 3 females). Group I served as the control group while group II, III, and IV served as the experimental group. The control group received 10 ml/kg body weight/day of

distilled water while the experimental groups II, III and IV received 250, 500 and 1000 mg/kg body weight per day of the extract of *L. acida*, served at the same time for 28 consecutive days. Toxic manifestations and mortality were monitored 12 hourly.

a) *Weekly body weight*

The body weights of the animals were taken using the sensitive weighing balance before the start of the experiment and subsequently, weekly and on the day of sacrifice (OECD, 2008).

b) *Relative organ weight percent*

On the 29th day, after an overnight fast, rats were euthanised with chloroform anaesthesia. Gross necropsy was performed on all animals and organ weights (heart, liver, kidney, thymus, spleen) were measured as a percentage of body weight of each rat.

The relative organ weight percent of each animal was calculated as follows:

$$\text{Relative organ weight percent} = \frac{\text{Absolute Organ Weight (g)}}{\text{Body Weight of Rat on Sacrifice day (g)}} \times 100$$

Therefore,

Relative organ weight percent, liver:

$$= \frac{\text{Absolute Weight of liver (g)}}{\text{Body Weight of Rat on Sacrifice day (g)}} \times 100$$

Relative organ weight percent, Spleen:

$$= \frac{\text{Absolute Weight of spleen (g)}}{\text{Body Weight of Rat on Sacrifice day (g)}} \times 100$$

Relative organ weight percent, Kidney

$$= \frac{\text{Average of the absolute Weight of 2 Kidneys (g)}}{\text{Body Weight of Rat on Sacrifice day (g)}} \times 100$$

Relative organ weight percent, Thymus

$$= \frac{\text{Absolute Weight of Thymus (g)}}{\text{Body Weight of Rat on Sacrifice day (g)}} \times 100$$

c) Histopathological studies

Histopathological examinations of the preserved organs were evaluated according to the method of Pieme *et al.* (2006). The organ pieces (3-5 µm thick) were fixed in 10% formalin for 24 hours and washed in running water for 24 hours. Samples were dehydrated in an autotechnicon and then cleared in benzene to remove absolute alcohol. Embedding was done by passing cleared samples through cups containing molten paraffin at 50⁰C and then in cubical block of paraffin made by “L” moulds. The samples were done cut with microtome and the slides were stained with hematoxylin-eosin. Two experienced scientists, a histopathologist and a histologist carried out detailed microscopic examination in those organs of both the control and treatment groups.

d) Hematological test

Blood samples were collected from the euthanized animals on the last day of the studies into a sterile EDTA coated bottle. Aliquots (≈10µL) of the sample was used for the quantification of total white blood cells (WBCs), lymphocytes, monocytes, red blood cells (RBCs), hemoglobin (Hb), hematocrit (HCT), platelets, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular hemoglobin (MCH) using Complete Blood Count (CBC) machine (Automated CBC Analyzer: Sysmex KX-21TM, Kobe, Japan).

e) Biochemical test

Blood samples were collected from euthanized animals on the last day of the studies into a plain bottle. Centrifuging blood at 3000 revolutions per minute for 10 minutes

separated the serum. The supernatant was transferred into an Eppendorf tube and immediately analyzed for liver enzymes such as alanine and aspartate transaminases, alkaline phosphatase, and serum proteins. Further test on the kidney function such as quantification of creatinine, urea, and serum electrolytes were also done. All tests were done using commercial kits from Reckon Diagnostic Ltd India and Spectrophotometer 20D (Technel & Technel, USA).

3.2.6 Tests for immunomodulatory activities

a) Experimental design

Twenty-four (24), 12 female and 12 male Wistar rats, aged between 8-12 weeks were used for this experiment. On day 0, all animals were sensitized with 0.1 ml of Sheep Red blood Cells (SRBC) administered intraperitoneally. The animals were then divided into four groups containing six animals (3 males, 3 females) each:

Group I: Control- received 10 ml/kg of normal saline

Group II: received 250 mg/kg body weight of methanol extract of stem bark of *L. acidaper* oral from 1 -14 day

Group III: received 500 mg/kg body weight of methanol extract of stem bark of *L. acidaper* oral from 1 -14 day

Group VI: received 1,000 mg/kg body weight of methanol extract of stem bark of *L. acidaper* oral from 1 -14 day.

b) Delayed type hypersensitivity test

On the 10th day, the left hind paw of each animal was measured using caliper and subsequently, each animal received a subcutaneous dose of 0.1 ml of solution containing 1×10^9 SRBC on the left hind paw. Left paw thickness was measured again at 24, 48, 72 and 96 hour. The difference in thickness between the measurements at day 10 and the subsequent measurements represented a measure of the delayed type hypersensitivity.

c) Humoral immunity test

The effect of the test substances on antibody production was determined using the enzyme-linked immunosorbent assay (ELISA) method as described by Life Diagnostics, Inc, USA.

On the 15th day, animals were euthanized and blood collected, centrifuged at 3,000 rpm and separated into serum. Aliquots of the test samples (2.5 μ L) were diluted and incubated in the anti-rat IgG or IgM coated microliter wells for 45 minutes along their respective standards (IgG or IgM standards). The microliter wells were subsequently washed, and anti-rat IgG-HRP conjugate (for IgG detection) or anti-rat IgM-HRP conjugate (for IgM detection) was added and the samples incubated for 45 minutes. The wells were then washed to remove the unbound HRP-labeled antibodies, and a solution of 3,3', 5,5'-Tetramethylbenzimidine (TMB) reagent was added and incubated for another 20 minutes at room temperature. A blue coloration was formed which indicated that the reaction was completed. Color development was stopped by the addition of Stop solution, which changed the color to yellow. The optical density was measured spectrophotometrically at 450nm using microplate reader (Biorad Inc, USA). The concentration of immunoglobulin was proportional to the optical density

of the test sample and was derived from a standard curve (Life Diagnostic Inc, USA; Catalogues:IGM-2 and IGG-2).

d) Estimation of T-lymphocyte subsets

On the 15th day, blood from the euthanized animals were collected in EDTA anticoagulant bottles and analyzed within 6 hours after the collection. 20 µL of whole blood in the EDTA anticoagulant bottle was added to Partec test tube. Another 20 µL of CD4 or CD8 monoclonal antibody-phycoerythrin was added and mixed gently and incubated for 15 minutes at room temperature and away from light. 800 µL of no lyse buffer was added and the mixture shaken and analysed in a Partec Flow cytometer (Sysmex Partec GmbH, Germany).

e) Immunohistological evaluation

Histological examination of the lymphoid organs (spleen, thymus and liver) of the rats in Groups I, II, III and IV as done from the organs harvested during the sub-acute toxicity test according to the methods described above (Pieme *et al.*, 2006).



Plate V: Quantification of the IgM and IgG of the Wistar rats at the Immunology Laboratory, ABUTH Shika

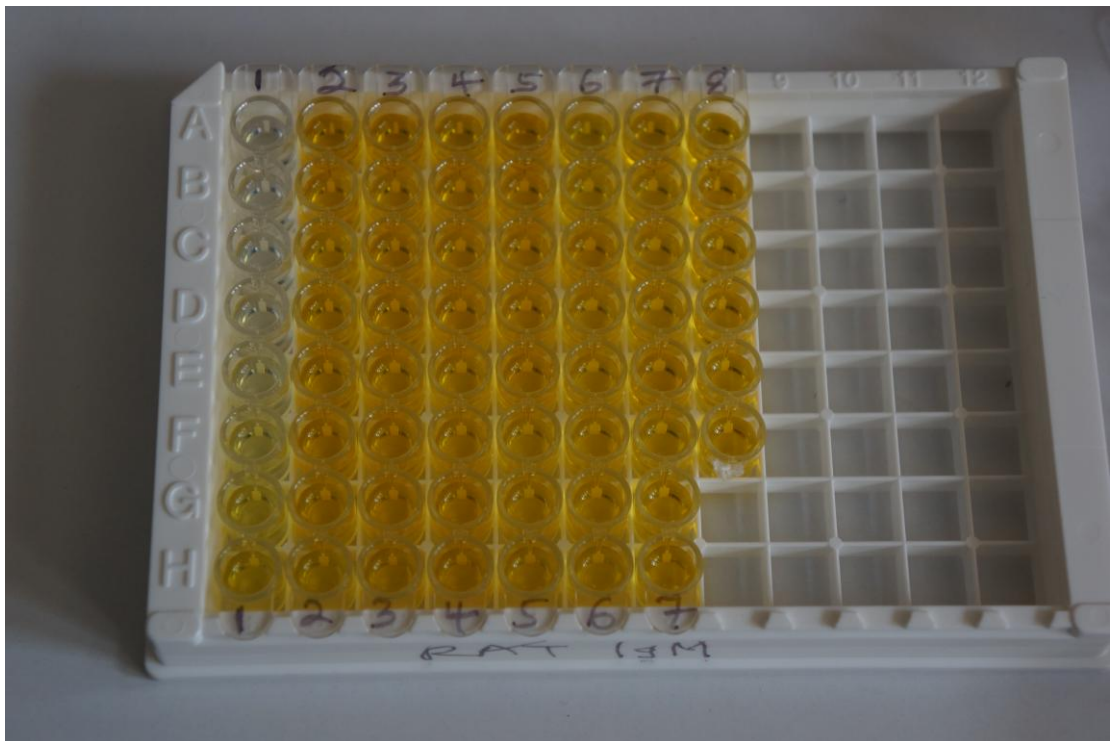


Plate VI: Incubation of the microtiter wells with prepared Rat IgM

3.3 Study Laboratories

The study was conducted in the Animal House of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria. The humoral immunity markers (IgM and IgG), biochemical parameters and hematological parameters were analysed at the Immunology, chemical pathology and hematology laboratories of Ahmadu Bello University Teaching Hospital, Shika, Zaria. The cellular immunity markers (CD4+ and CD8+ T-cell counts) were analysed at the microbiology laboratory of the Nigerian Institute of Pharmacology Research and Development (NIPRD), Idu, Abuja.

3.4 Ethics

The study protocol was subjected to the Animal Ethics Review Committee of the Department of Pharmacology & Therapeutics, Ahmadu Bello University, Zaria. Animals and materials were handled in accordance to Good Laboratory Practice (GLP).

3.5 Statistical Analysis

The data was checked for completeness and analyzed with Stata statistical software package (Stata[®], version 14.1, Stat Corp, Texas, USA). Numerical data were expressed as mean \pm standard error of the mean (SEM). Comparison was done between and within group using the one-way analysis of variance (ANOVA). In addition, *post hoc* Pairwise comparison was done using Bonferroni test; repeated measures ANOVA was used to compare changes in factors that were measured repeatedly. Statistical significance was when $p < 0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Phytochemical constituents

The coarse powder (1.5 g) of the coarse powder of the stem bark of *L.acida* yielded 202.33 g (13.5%) of methanol residue. The phytochemical screening of the methanol extract of the stem bark of *Lannea acida* showed the presence of carbohydrates and reducing sugars, alkaloids, glycosides, anthraquinones, triterpenes, cardiac glycosides and tannins. However, sterols, cardenolides, saponin glycosides and flavonoids were found to be absent (Table 4.1).

Table 4.1: Phytochemical constituents of the methanol extract of the stem bark of *Lannea acida*

Phytochemical constituent	Test method	Result
Carbohydrate	Molisch Test	Present
• Reducing sugar	Fehling Test	Present
Alkaloids	Mayer's Test	Present
Glycosides	Fehling A and B	Present
Anthraquinone	Bontrager's Test	Present
Unsaturated sterol and triterpene		
• Triterpenes	Lieberman Bouchard Test	Present
• Unsaturated Sterol	Salkowski Test	Absent
Cardiac glycosides	Keller-Kiliani Test	Present
Cardenolide	Kedde's Test	Absent
Saponin glycosides	Frothing Test	Absent
Tanins (condensed)	Ferric chloride Test	Present
Flavonoids	Sodium Hydroxide Test Shinoda	Absent Absent

4.2 Toxicity

4.2.1 Acute toxicity

(1) Signs of acute toxicity and mortality following the oral administration of a single dose of 3,000 mg/kg of the methanol extract of the stem bark of L. acida

The test dose of 3,000mg/kg of the methanol extract of the stem bark of *L. acida* did not cause mortality in the Wistar rats within the first 24 hours as well as during the follow-up for two weeks. The physical and behavioral observations of the Wistar rats during these periods showed no visible sign of toxicity.

(2) Effects of a single oral administration of 3,000 mg/kg of methanol extract of the stem bark of L. acida on the weight of animals and their relative organ weight percent

Each of the five experimental animals showed weight gain during the first week of the experiment. However, in animals A1, A3, A4 and A5, this peaked at day 7 and thereafter, there was steady decline up to the 14th day post-dosing; while A2 showed a continuous weight gain up to the 14th day of the experiment (Appendices I and II).

Overall, the mean \pm standard error of the mean (SEM) of the weights of the animals at the beginning of the study (Day 0) was 142.00 ± 2.00 g (95% CI = 136.40 – 147.60); this increased to 156.40 ± 2.90 g (95% CI = 148.30 – 164.50) at Day 7; and decreased to 149.20 ± 3.80 g (95% CI = 138.70-159.70) on day 14 (Table 4.2).

The mean \pm SEM of the relative organ weight percent of the liver, spleen, kidney and thymus were: 4.02 ± 0.24 , 0.48 ± 0.06 , 0.71 ± 0.03 and 0.32 ± 0.05 respectively

(Table 4.2, Appendix III).

Table 4.2: Effects of a single dose of 3,000 mg/kg of methanol extract of the stem bark of *Lanea acida* on the weight and the relative organpercent of Wistar rats

	Weight of animals (g)			Relative organ weight (%)			
	Day 0	Day 7	Day 14	Liver (%)	Spleen (%)	Kidney (%)	Thymus (%)
Mean ± SEM	142.00±2.00	156.40±2.90*	149.20±3.80	4.02±0.24	0.48±0.06	0.71±0.03	0.32±0.05
95% CI	136.40-147.60	148.30-164.50	138.70-159.70	3.40-4.70	0.33-0.64	0.63-0.80	0.18-0.46

n = 6, One-way repeated measure ANOVA for weight; $F(2,12) = 5.78, p < 0.05$; * Significant (*Bonferroni post hoc test*)

4.2.2 Repeated daily dose oral toxicity

*(1) Effects of repeated daily oral administration of the methanol extract of the stem bark of *Lannea acida* on the mean body weight of animals over 28 days*

The mean weight of the animals in the control and experimental groups experienced a change at each time interval. There was an increase in weight between Day 0 and 7 for both the control and the experimental group; and then a decline at Day 14, after which both control and experimental groups experienced weight gain up to the 28th Day. The effect of time for each treatment and the control was statistically significant, $F(4,12) = 17.87, p < 0.05$ and maximum at Day 28; (Table 4.3, Appendix IV).

However, comparing the overall mean body weight changes of the rats in the control verses the experimental groups, there was no statistically significant difference, $F(3, 20) = 2.35, p < 0.05$ during the 28-day period of observation (Table 4.3)

Table 4.3: Effects of 28-day repeated daily oral administration of methanol extract of stem bark of *Lanea acida* on mean body weight of Wistar rats

Treatment	Body weight				
	Day 0	Day 7	Day 14	Day 21	Day 28
	Mean ± SEM				
Control	138.80 ± 6.10	152.00 ± 8.20*	148.20 ± 7.20*	151.20 ± 8.00*	157.70 ± 8.30*
250 mg/kg LA	161.70 ± 8.70	171.80 ± 10.70*	172.50 ± 10.20*	178.00 ± 10.90*	181.20 ± 13.10*
500 mg/kg LA	153.20 ± 4.80	158.50 ± 5.10*	157.40 ± 8.20*	166.20 ± 10.00*	168.80 ± 11.50*
1000 mg/kg LA	167.50 ± 6.80	172.70 ± 8.00*	171.20 ± 4.90*	183.20 ± 7.90*	185.40 ± 11.80*

n =6, One-way repeated measure ANOVA (control vs. experimental groups), F (3, 20) = 2.35, $p > 0.05$. Time interaction, F (4,12) = 17.87, $p < 0.05$. * Significant (*post hoc* Bonferonni test); LA = *Lanea acida*

(2) *Effects of repeated daily oral administration of methanol extract of the stem bark of Lannea acida on the relative organ weight percent over 28 days.*

The mean \pm SEM of the relative organ weight percent of the spleen showed a non-remarkable increase in the experiment group at the doses of 250- and 500-mg/kg body weight. However, at the dose of 1,000 mg/kg, the mean \pm SEM of the relative organ weight percent of the spleen showed a statistically significant decrease, $F(3,18) = 4.61, p < 0.05$ (Table 4.4).

The mean \pm SEM of the relative organ weight percent of the kidney also showed a slight increase at the dose of 250 mg/kg; however, there was a decrease at a higher dose of 500 mg/kg, though, not statistically significant ($p > 0.05$). At the dose of 1,000 mg/kg, the animals experienced a remarkable decrease, $F(3, 18) = 3.58, p < 0.05$ in the mean \pm SEM of the relative organ weight percent of the kidneys (Table 4.4).

The mean \pm SEM of the relative organ weight percent of the liver and the thymus of the Wistar rats shows no statistically significant difference, $F(3, 18) = 0.45, p > 0.05$ and $F(3, 18) = 1.10, p > 0.05$ between the control and the experimental groups for liver and for thymus respectively (Table 4.4).

Table 4.4: Effects of 28-day daily oral administration of methanol extract of stem bark of *Lannea acida* on the relative organ weight percent of

Wistar rats

Treatment	Liver (%)	Spleen (%)	Thymus (%)	Kidney (%)
	Mean ± SEM			
Control	3.36 ± 0.3	0.57 ± 0.09	0.44 ± 0.13	0.75 ± 0.06
250 mg/kgLA	3.48 ± 0.28	0.58 ± 0.11	0.35 ± 0.10	0.76 ± 0.10
500 mg/kgLA	3.53 ± 0.41	0.60 ± 0.10	0.46 ± 0.10	0.74 ± 0.10
1000 mg/kgLA	3.34 ± 0.28	0.46 ± 0.04*	0.38 ± 0.08	0.63 ± 0.02*

n =6, One-way ANOVA (control vs. experimental groups), Spleen (%), F (3,18) = 4.61, $p < 0.05$; Kidney (%), F (3, 18) = 3.58, $p < 0.05$. * Significant *Post-hoc* Bonferonni test; LA = *Lannea acida*

(3) Effect of repeated daily oral administration of methanol extract of the stem bark of Lannea acida on haematological indices of Wistar rats over 28 days.

The effect of the methanol extract of the stem bark of *L. acida* on the haematological indices following a 28-day daily oral administration is shown in Table 4.5. There was no statistically significant difference between the experimental animals and the control group in the following haematological parameters: haemoglobin level (Hb), haematocrit, red blood cell (RBC) count, mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), white blood cell (WBC) count, lymphocyte percent, granulocyte percent and the platelet counts.

Table 4.5: Effects of 28-day daily oral administration of methanol extract of the stem bark of *Lanea acida* on the haematological parameters in Wistar rats

Treatment	Hb (g/dl)	Hct (%)	RBC (x10 ¹² /L)	MCV (fL)	MCHC (g/dL)	MCH (pg)	WBC (x10 ⁹ /L)	Lymph (%)	Gran (%)	Platelet (x10 ⁹ /L)
Mean ± SEM										
Control	13.3±0.6	38 ±2.9	6.83±0.4	55.7±3.4	35.1±1.2	19.5±0.7	10.2±3.5	80.5±4.2	10.1±3.4	512±57.2
250 mg/kg LA	14.4±0.9	40.4±2.5	7.4±0.5	54.6±1.1	35.6±0.4	19.5±0.4	9.1±3.3	75±4.9	16±2.9	466.7±83
500 mg/kg LA	13.8±1.5	39±4.5	7.1±0.9	54.6±2.3	35.5±0.4	19.4±0.7	13.8±5.2	83.5±8.3	9.3±5.2	484.4±213.9
1000 mg/kg LA	14.6±0.8	40.6±2.3	7.5±0.5	54.3±1.8	35.9±0.6	19.4±0.5	13.3±3.2	84.6±3.3	9±3.7	578.2±170.7

n = 6, One-way ANOVA, (Control vs. experimental group), $p > 0.05$; LA = *Lanea acida*, Hb = Haemoglobin, Hct = Haematocrit, RBC = Red Blood Cell count, MCV = Mean Corpuscular Volume, MCHC = Mean Corpuscular Haemoglobin Concentration, MCH = Mean Corpuscular Haemoglobin, WBC = White Blood Cell Count, Lymph = Lymphocytes, Gran = Granulocytes

(4)Effect of repeated daily oral administration of methanol extract of stem bark of *Lanne. acida* on hepatic indices of Wistar rats over 28 days.

The effects of the methanol extract of the stem bark of *L. acida* on hepatic indices following a 28-day daily oral administration is shown in Table 4.6. There was no statistically significant difference ($p > 0.05$) in the serum levels of aspartate transaminase (AST), alkaline phosphatase (ALP), protein and albumin between the experimental and control groups. However, there existed a difference, $F(3,18) = 3.54$, $p < 0.05$ in the serum alanine transaminase (ALT) level, between the experimental and the control groups. A *post hoc* Bonferonni test shows this difference to be at the dose of 500 mg/kg.

Table 4.6: Effects of 28-day daily oral administration of methanolextract of stem bark of *Lannea acida* on hepatic indices in Wistar rats

Treatment	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Protein (g/dL)	Albumin (g/dL)
Mean ± SEM					
Control	294.00±76.00	117.70±26.80	273.50±156.70	69.30±13.50	37.20±5.50
250 mg/kg LA	308.80±107.10	121.00±27.80	291.50±153.50	70.30±8.80	36.20±5.00
500 mg/kgLA	337.60±128.50	174.20±52.6*	273.40±58.00	78.80±8.00	39.20±5.00
1000 mg/kgLA	277.60±58.80	118.20±15.00	150.20±58.10	75.20±9.30	40.60±8.00

n = 6, One-way The ANOVA (control vs. experimental groups), ALT measurement, F (3,18) = 3.54, p< 0.05. * Significant *Post-hoc* Bonferonni test; LA = *Lannea acida*, AST = Asapartate Transaminase, ALT = Alanine Transaminase, ALP = Alkaline Phosphatase

(5) Effect of methanol extract of Lannea acida on the renal indices of Wistar rats following a 28-day daily administration

The serum levels of urea, creatinine, sodium, bicarbonate and chloride were not significantly different ($p > 0.05$) between the control and experimental groups. However, there was a statistically significant difference ($p < 0.05$) when serum potassium (K^+) level of the control was compared with the experimental groups. These differences were seen at doses 250, 500 and 1,000 mg/kg Table 4.7).

Table 4.7: Effects of 28-day daily oral administration of methanolextract of stem bark of *Lannea acida* on renal indices in Wistar rats

Treatment	Urea (mmol/L)	Creatinine (μ mol/L)	Na ⁺ (mmol/L)	K ⁺ (mmol/L)	HCO ₃ ⁻ (mmol/L)	Cl ⁻ (mmol/L)
Mean \pm SEM						
Control	3.30 \pm 1.00	53.00 \pm 19.10	138.80 \pm 3.70	5.50 \pm 0.50	20.40 \pm 5.10	102.00 \pm 3.20
250 mg/kg LA	2.90 \pm 0.40	42.30 \pm 9.40	138.50 \pm 2.30	3.20 \pm 0.90*	23.70 \pm 3.00	101.80 \pm 3.80
500 mg/kg LA	3.70 \pm 0.30	65.80 \pm 8.70	134.60 \pm 7.20	2.50 \pm 0.60*	21.00 \pm 7.10	97.40 \pm 8.30
1000 mg/kg LA	3.20 \pm 0.80	53.00 \pm 16.80	136.40 \pm 9.60	2.80 \pm 0.30*	23.80 \pm 6.30	101.00 \pm 10.40

n = 6, One-way ANOVA (control vs. experimental group), serum K⁺, F (3, 18) = 22.81, P < 0.05. * Significant *Post-hoc* Bonferonni test. LA = *Lannea acida*, Na⁺ = Sodium, K⁺ = Potassium, HCO₃⁻ = Bicarbonate, Cl⁻ = Chloride

(6) *Effect of daily oral administration of methanol extract of the stem bark of Lannea acida on the histology of the liver, spleen kidneys, and the thymus of Wistar rats over 28 days.*

a) Liver

i. Control group

The photomicrograph of the liver of the rat administered 10 ml/kg body weight of distilled water (control) shows a normal liver with cords of hepatocytes (H) arranged in 1-2 cells thick plates separated by hepatic sinusoids (HS). They have oval to round nuclei with even nuclear chromatin and abundant granular eosinophilic cytoplasm (Plate VII).

ii. 250 mg/kg group

The photomicrograph of the liver of the rat administered 250 mg/kg of methanol extract of *L. acida* showing numerous normal hepatocytes. The sinusoidal spaces show slight vascular congestion and are interspersed with hyperplastic Kupffer cells (Plate VIII).

iii. 500 mg/kg group

The photomicrograph of the liver of the rat administered 500 mg/kg of methanol extract of *L. acida* showing normal hepatocytes and mild-moderate vascular congestion of the hepatic sinusoids, and Kupffer cells hyperplasia (Plate IX).

iv. 1000 mg/kg group

The photomicrograph of the liver of the rat administered 1,000 mg/kg of methanol extract of *L. acida* showing hepatocytes with mild-moderate peri-portal inflammation and focal necrosis (Plate X).

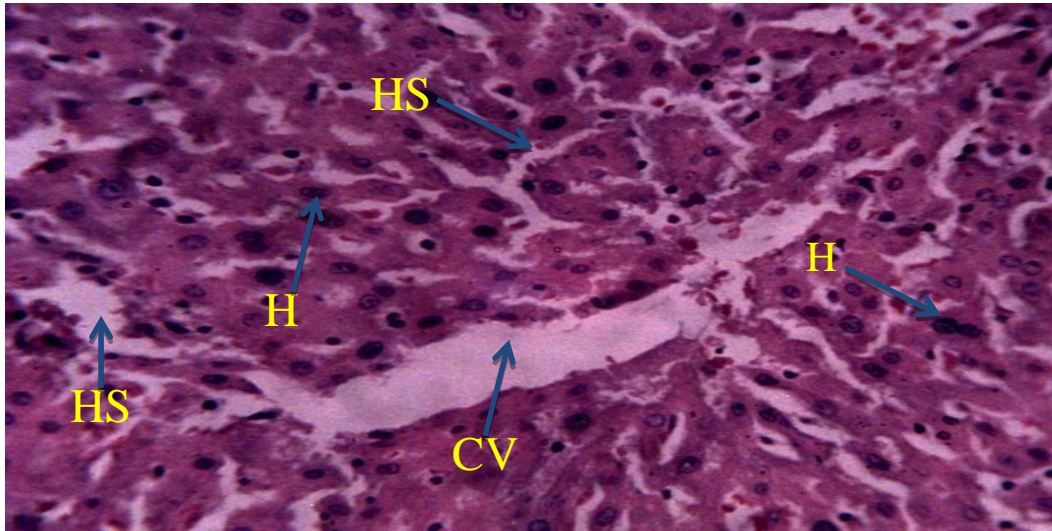


Plate VII: Photomicrograph of the liver of the rat administered with 10mls/kg of distilled water (control) showing normal hepatocytes (H), central vein (CV) and numerous hepatic sinusoids (HS). (Mag \times 40); H & E staining

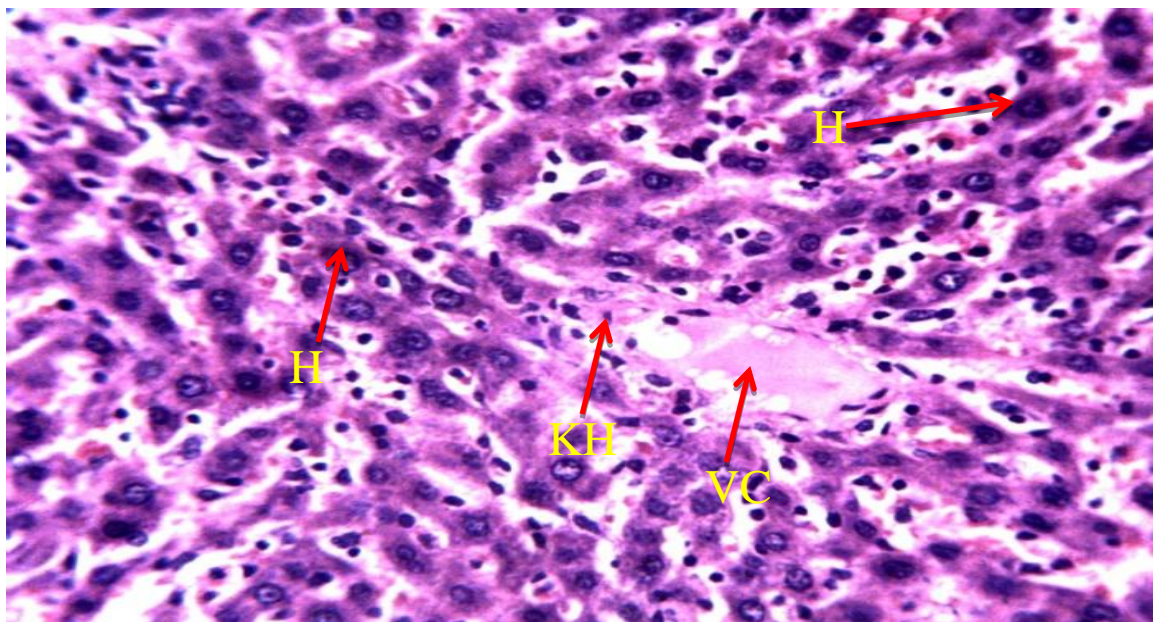


Plate VIII: Photomicrograph of the liver of the rat administered 250 mg/kg of methanol extract of *L. acida* showing normal hepatocytes (H), Kupffer cell hyperplasia (KH) and mild vascular congestion (VC). (Mag \times 40), H&E staining

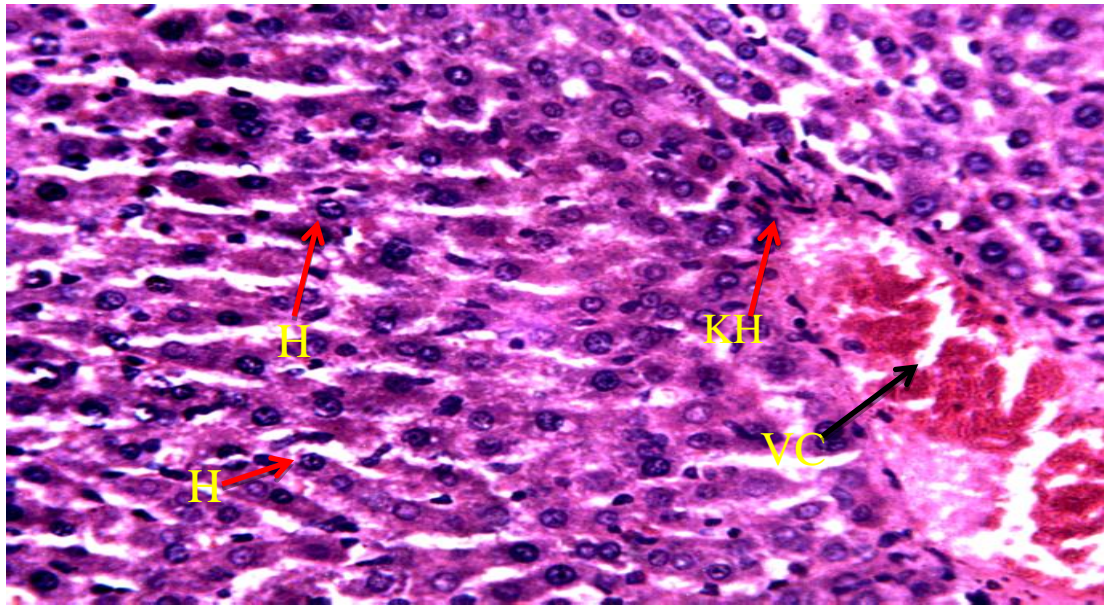


Plate IX:Photomicrograph of the liver of the rat administered 500 mg/kg of methanol extract of *L. acida* showing normal hepatocyte (H), Kupffer cell hyperplasia (KH) and mild-moderate sinusoidal vascular congestion (VC). (Mag \times 40), H&E staining

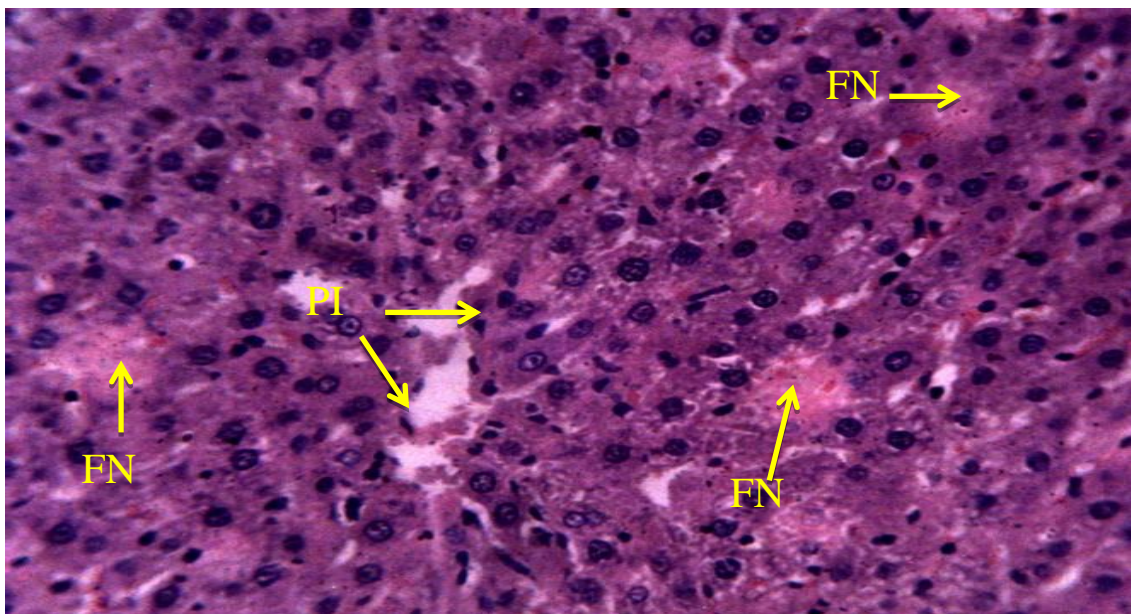


Plate X:Photomicrograph of the liver of the rat administered 1,000 mg/kg of methanol extract of *L. acida* showing areas of focal necrosis (FN) and periportal inflammation (PI). (Mag \times 40); H&E staining

b) Spleen

i. Control

The photomicrograph (Plate XI) of the spleen of the rat administered 10 ml/kg body weight of water (control) shows variably sized normal splenic white pulp composed of polymorph population of lymphoid cells separated by abundant vascularized red pulp. Numerous thin- and thick-walled vessels were seen.

ii. 250 mg/kg group

Section from the spleen of the rat administered with 250 mg/kg of methanol extract of *L. acida* (Plate XII) shows normal spleen with variably sized white pulp and few secondary follicles separated by highly vascularised red pulps.

iii. 500 mg/kg group

The photomicrograph of the section from the spleen of the rat administered with 500 mg/kg of methanol extract of *L. acida* (Plate XIII) shows normal spleen with variably sized white pulp separated by highly vascularised red pulps. There is an increase in size of white pulp and numerous secondary follicles.

iv. 1,000 mg/kg group

The photomicrograph of the section from the spleen of the rat administered 1,000 mg/kg of methanol extract of *L. acida* (Plate XIV) shows variably sized white pulp and a central artery separated by richly vascularised and haemorrhagic red pulps.

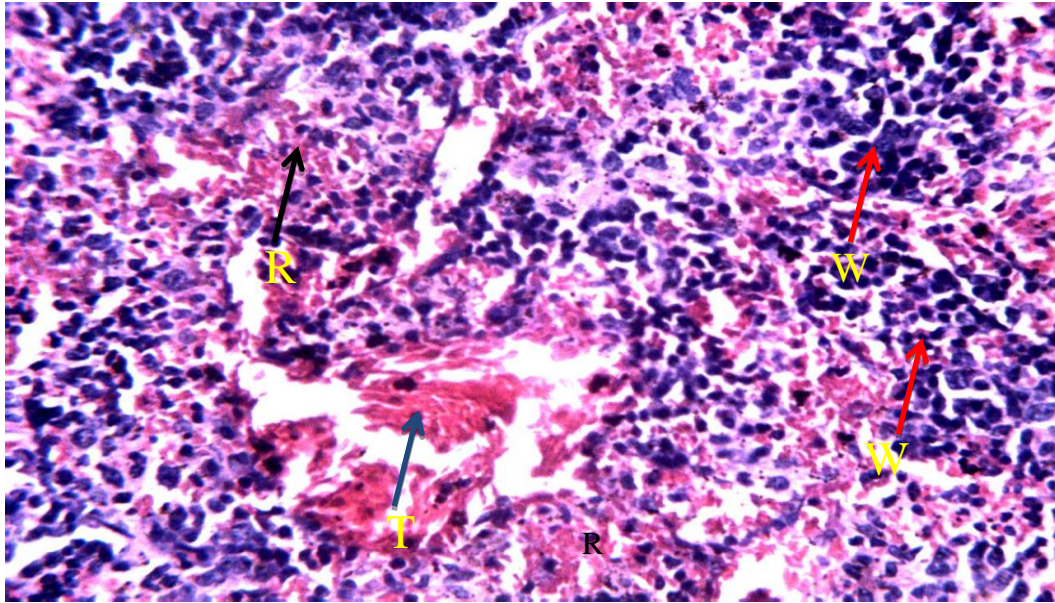


Plate XI: Photomicrograph of the spleen of the rat administered 10 ml/kg of water (Control) showing normal whitepulp (W), redpulp (R) and Trabaculae (T). (Mag ×40),H&E staining

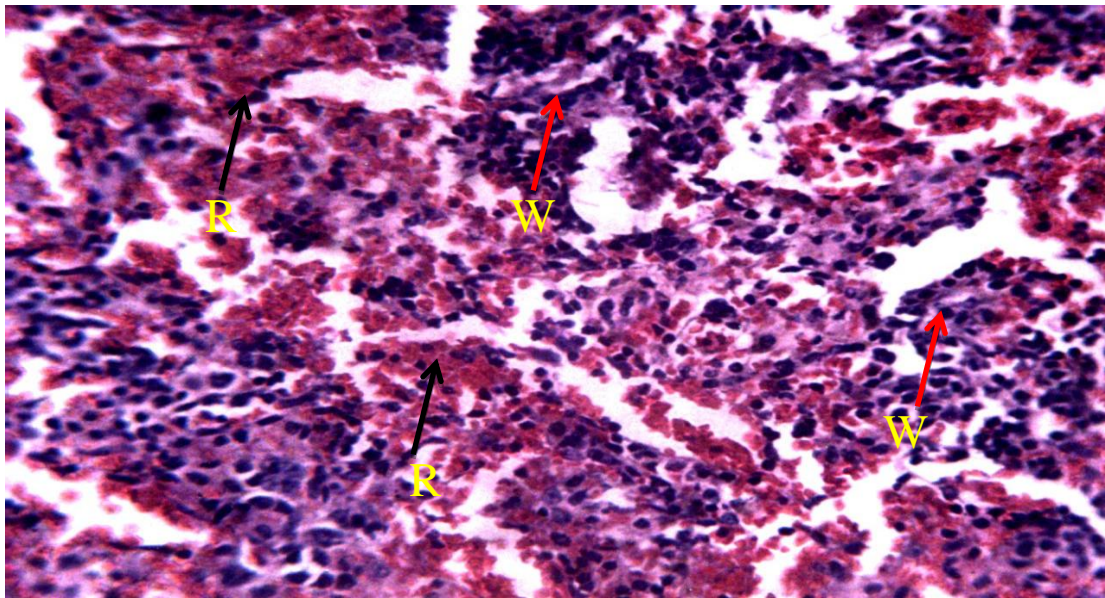


Plate XII: Photomicrograph of the spleen of the rat administered 250 mg/kg of methanol extract of *L. acida* showing variably sized white pulp (W) with secondary follicles, and vascularized red (R) pulps.

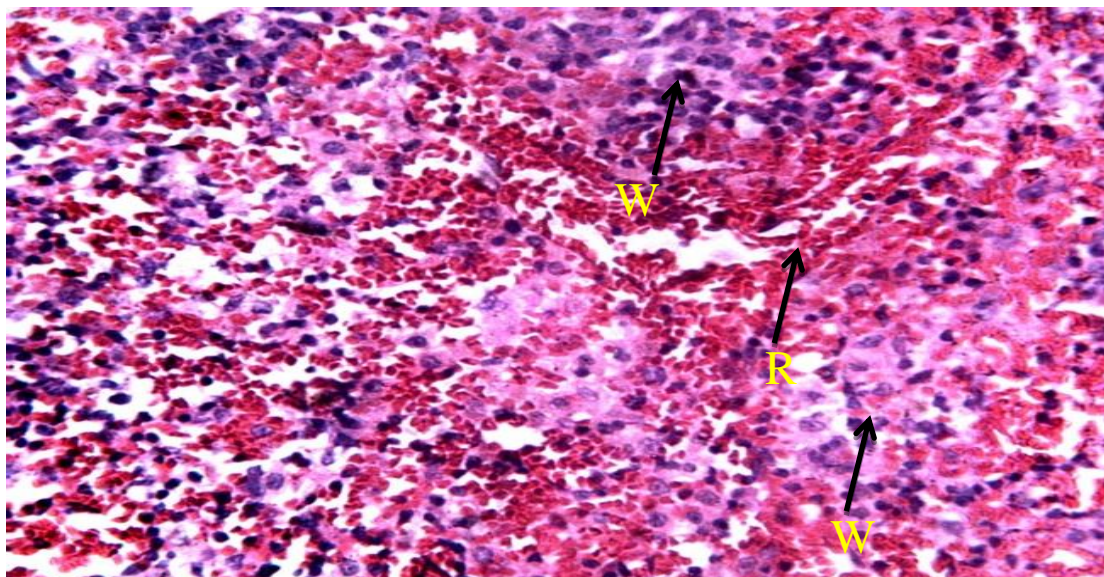


Plate XIII: Photomicrograph of the spleen of the rat administered 500 mg/kg of Methanolextract of *L. acida* showing numerous white (W) pulps with secondary follicles and highlyvascularized red (R) pulp. (Mag \times 40); H&E staining

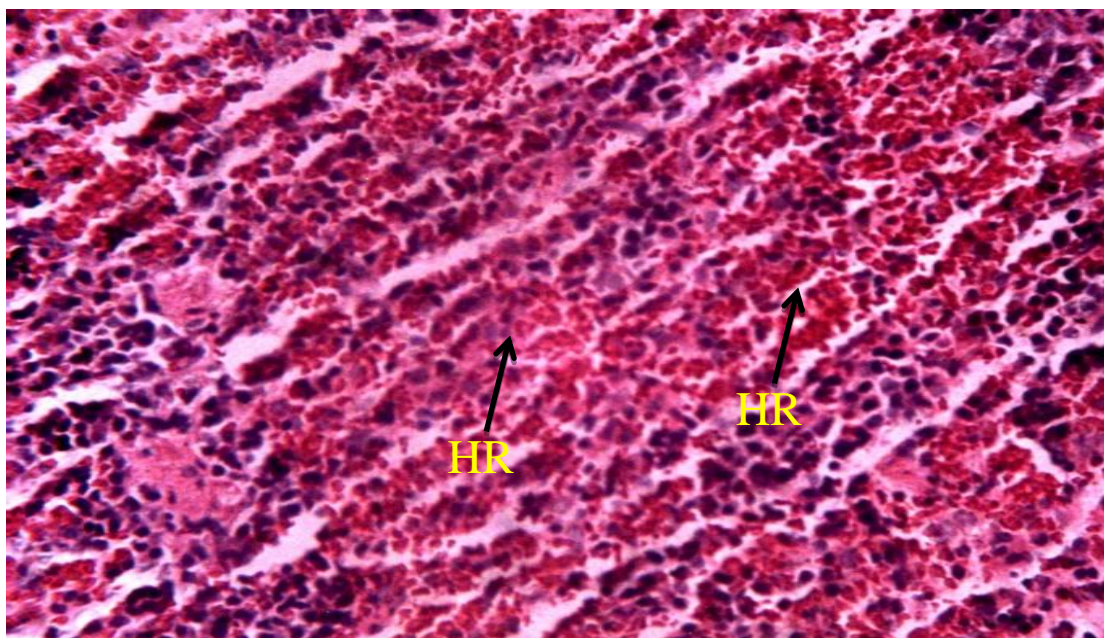


Plate XIV: Photomicrograph of the spleen of the rat administered 1,000 mg/kg of methanol extract of *L. acida* showing richly vascularized and hemorrhagic red pulp (HR).

c) Kidney

i. Control

Sections from the Kidney (Plate XV) of the rats administered 10 ml/kg body weight of water showing normal glomerular tufts and variably-sized normal renal tubules lined by cuboidal to low columnar cells having round nuclei and moderately eosinophilic cytoplasm.

ii. 250 mg/kg group

The photomicrograph (Plate (XVI) of the section from the kidney of the rat administered 250 mg/kg of the methanol extract shows slight glomerular necrosis and mild infiltration of the interstitium with inflammatory cells (polymorphs and lymphocytes)

iii. 500 mg/kg group

The photomicrograph (Plate XVII) of the section from the kidney of the rat administered 500 mg/kg of the methanol extract shows normal glomeruli and mild-moderate infiltration of the interstitium with inflammatory cells (polymorphs and lymphocytes).

iv. 1,000 mg/kg group

The photomicrograph of the section from the kidney of the rat administered 1,000 mg/kg of the methanol extract shows areas of focal glomerular necrosis and tubular necrosis (Plate XVIII).

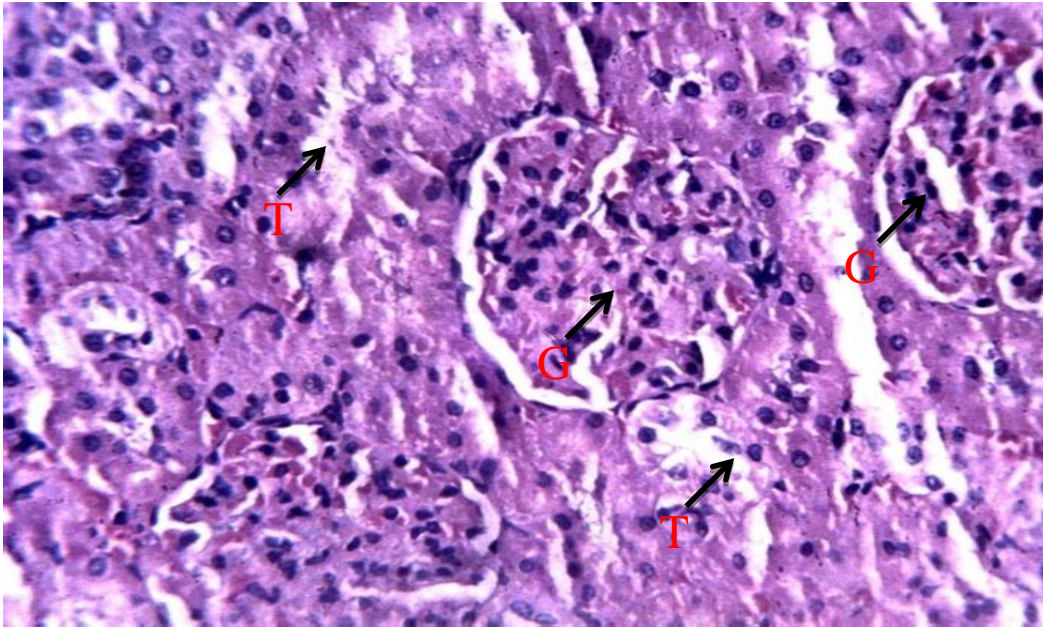


Plate XV: Photomicrograph of the kidney of the rat administered 10-ml/kg water (Control) showing normal glomeruli (G) and tubules (T). (Mag \times 40); H&E staining

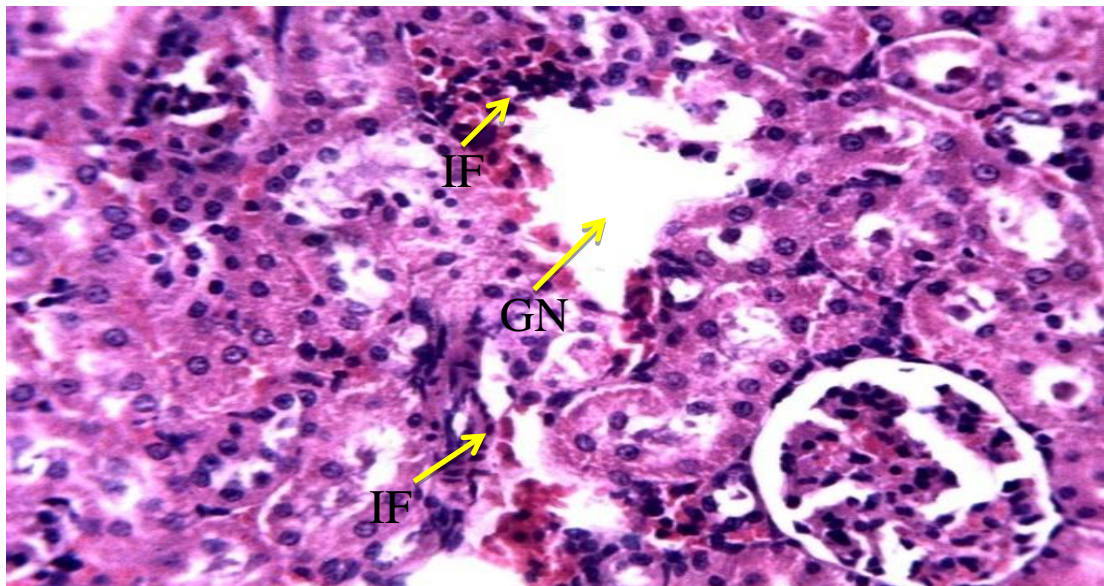


Plate XVI: Photomicrograph from the kidney of the rat administered with 250 mg/kg of methanol extract of *L. acida* showing slight glomerular necrosis (GN) and interstitial inflammation (IF). (Mag \times 40); H&E staining

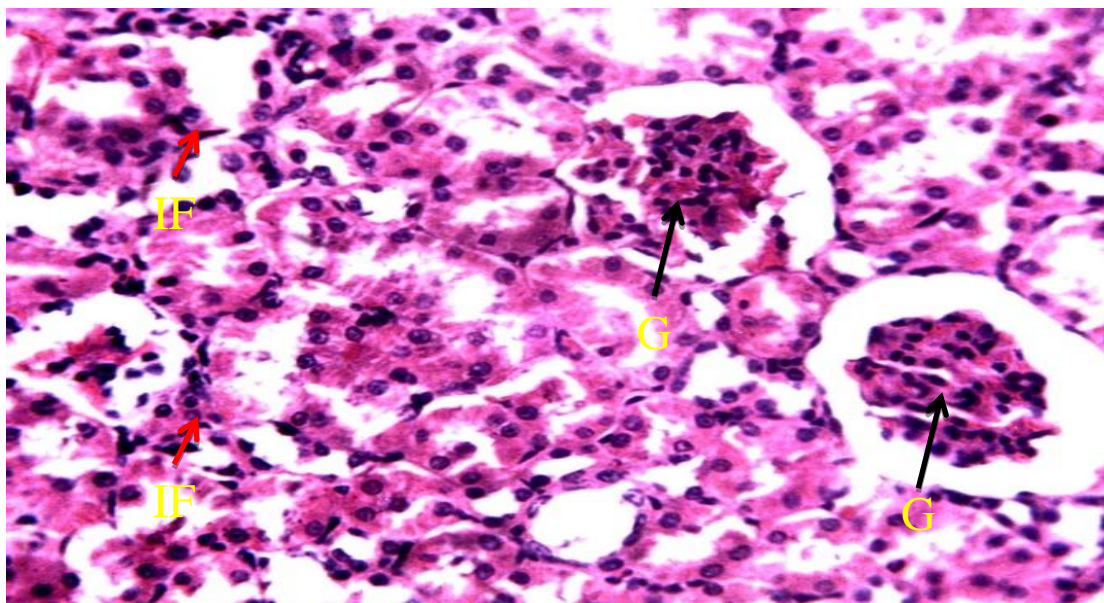


Plate XVII: Photomicrograph from the kidney of the rat administered 500 mg/kg of the methanol extract of *L. acida* showing normal glomerulus (G) and areas of mild-moderate interstitial inflammation (IF). (Mag \times 40); H&E staining

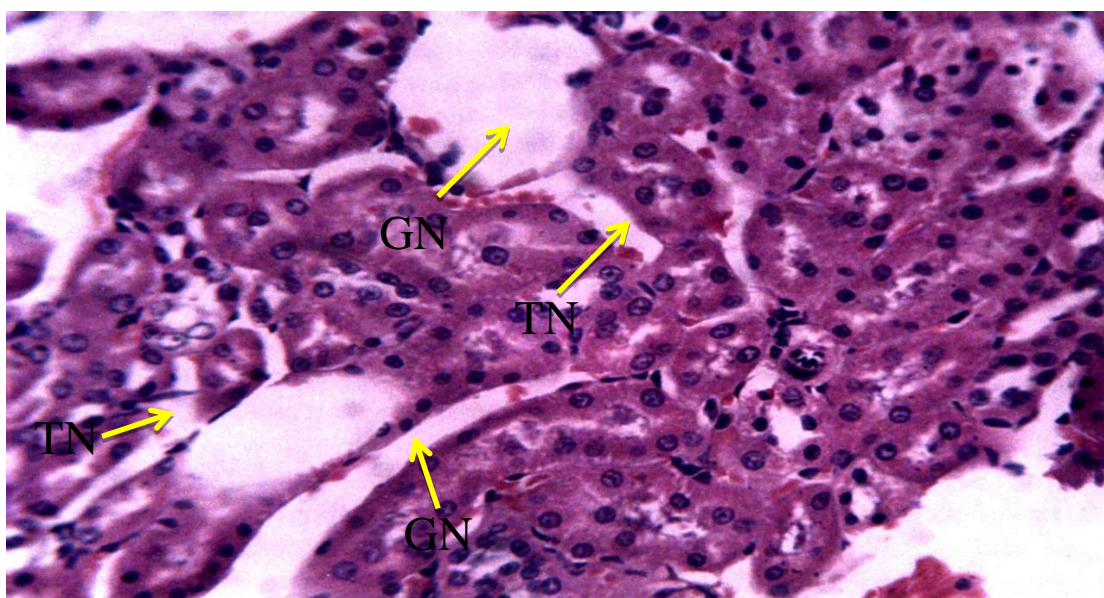


Plate XVIII: Photomicrograph from the kidney of the rat administered 1,000 mg/kg of the methanol extract of *L. acida* showing areas of focal glomerular (GN) and tubular necrosis (TN). (Mag \times 40); H&E staining

d) Thymus

i. Control

The photomicrograph (Plate XIX) of the thymus of the rat administered 10 ml/kg of water (control) shows numerous variably sized normal lymphoid follicles (arrows), most having germinal centers and composed of polymorphous population of lymphoid cells.

ii. 250 mg/kg group

The thymus (Plate XX) shows a degree of antigenic stimulation of lymphoid cells with few secondary follicles interspersed with many primary follicles and histiocytes. There are areas of slight necrosis of the thymocytes (arrows).

iii. 500 mg/kg group

The thymus shows a degree of antigenic stimulation of lymphoid cells with many secondary follicles. There are areas of thymocyte necrosis and depletion (Plate XXI).

iv. 1,000 mg/kg group

The thymus shows Hassall's corpuscle proliferation (HC) with areas of secondary lymphoid follicle separated by fibrous tissue septa. The lymphoid follicle is composed of polymorphous population of lymphoid cells. There are also focal areas of thymocyte depletion (Plate XXII).

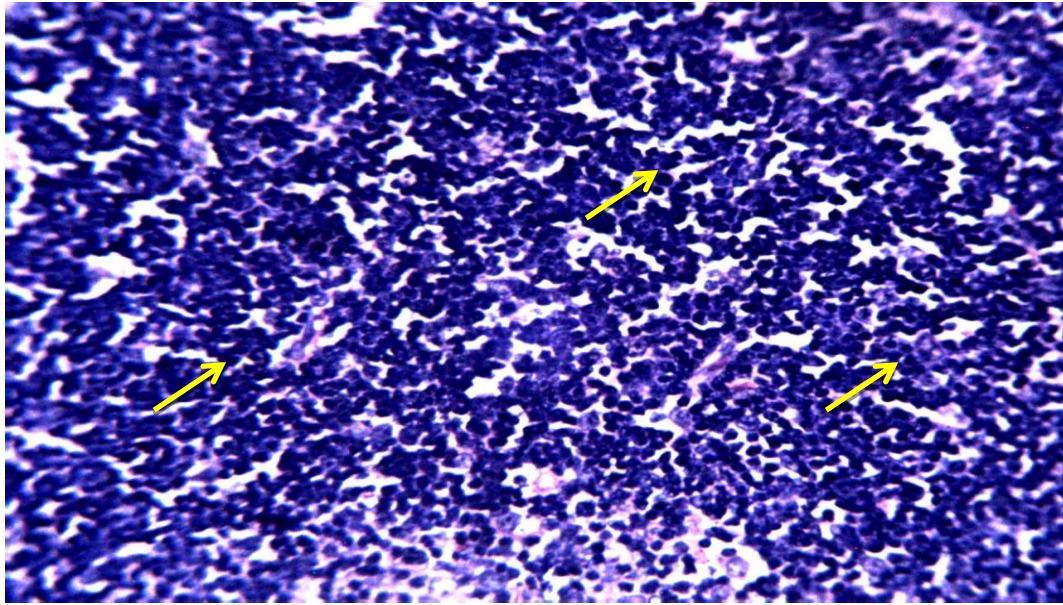


Plate XIX: Photomicrograph of the cortex of thymus of the rat administered 10 ml/kg of water (Control) showing numerous amount of lymphocyte producing thymocytes (arrows). (Mag \times 40); H&E staining

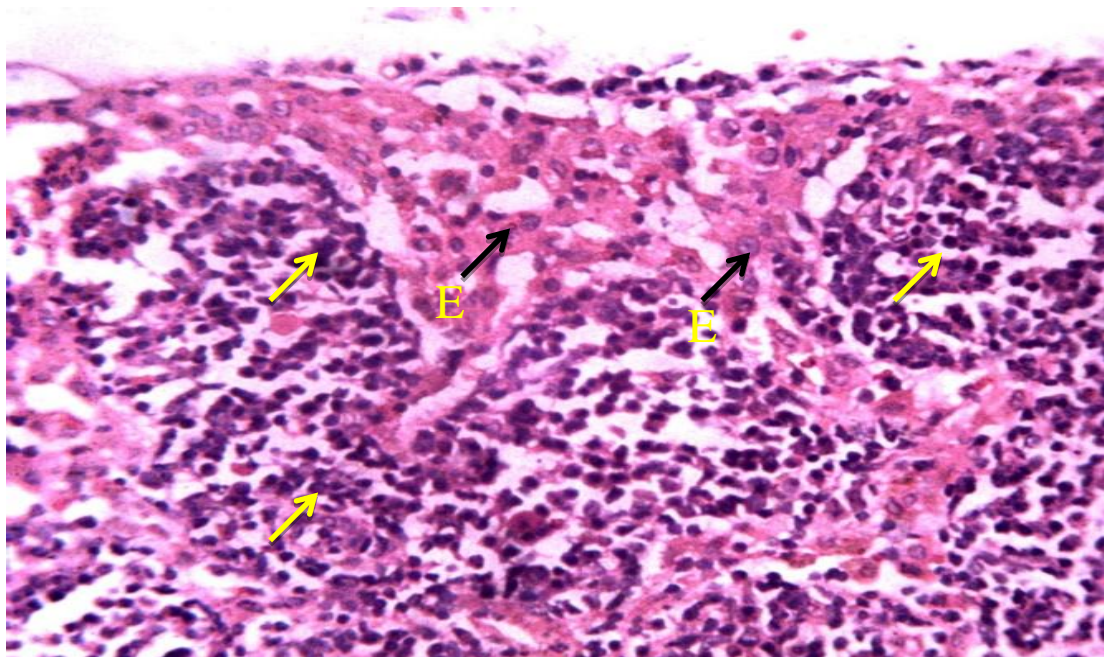


Plate XX: Photomicrograph of the medulla of thymus of the rat administered 250 mg/kg of methanol extract of *L. acida* showing area of thymocytes depletion (Arrow) and proliferation of the epithelioid cells (E). Mag \times 40; H&E staining

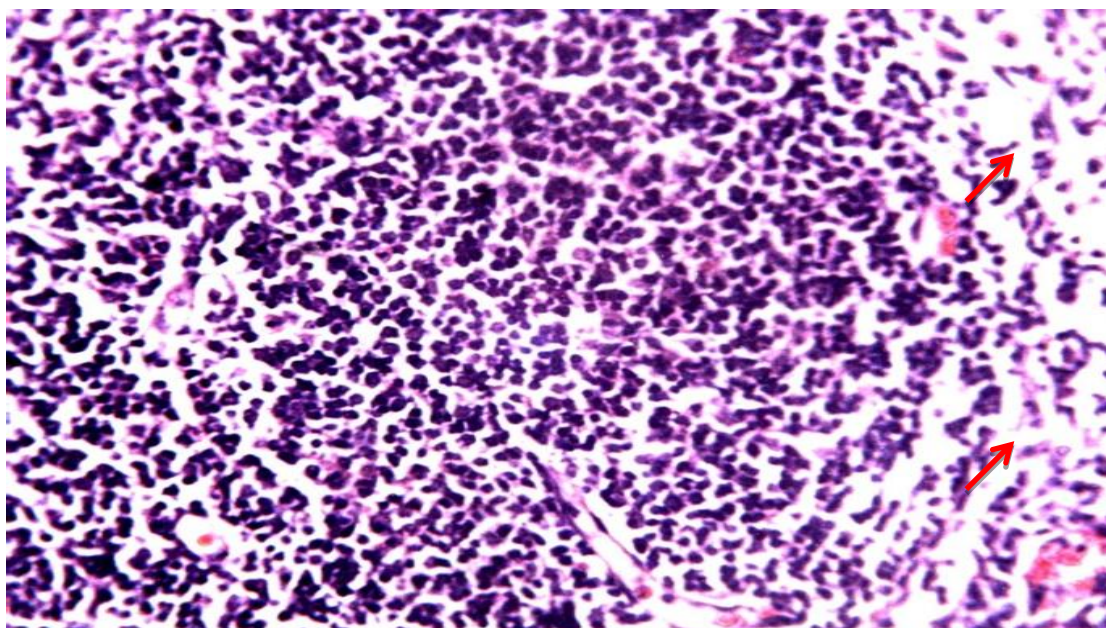


Plate XXI: Photomicrograph of the thymus of the rat administered with 500 mg/kg of methanol extract of *L. acida* showing area of necrosis and depletion of thymocytes (Arrow). Mag \times 40; H&E staining

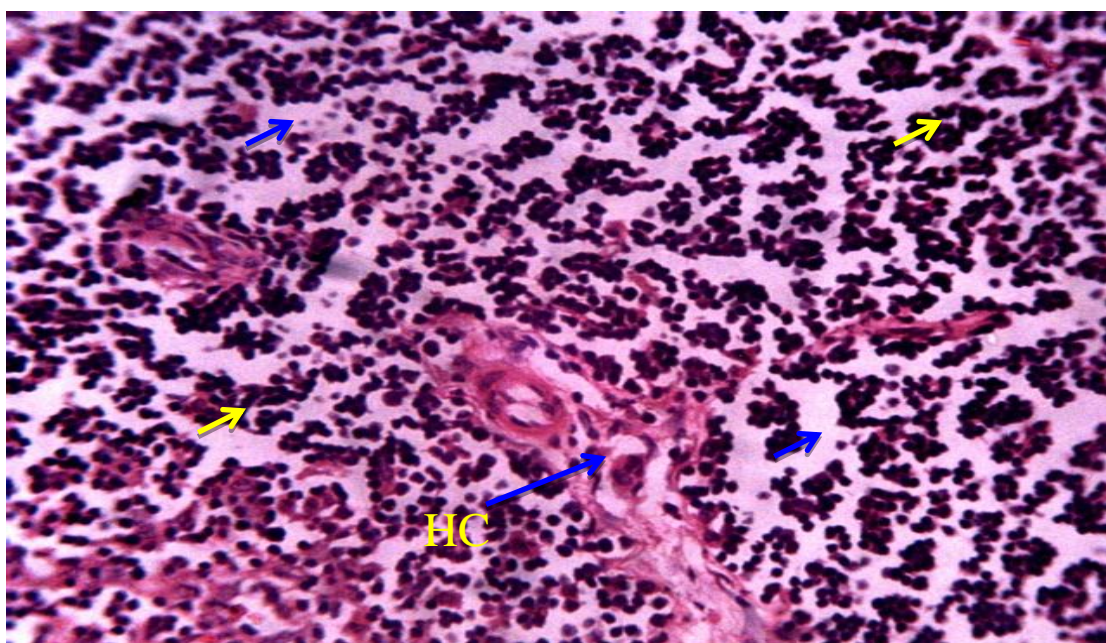


Plate XXII: Photomicrograph of the medulla of thymus of the rat administered 1,000 mg/kg of methanol extract of *L. acida* showing secondary lymphoid follicles (yellow arrow), proliferation of Hassall's corpuscle (HC) and areas of thymocyte depletion (blue arrow). Mag \times 40; H&E staining

4.3 Immunomodulatory Effects

4.3.1 Humoral immunity

Effects of the graded doses of methanol extract of the stem bark of L. acida on IgM and IgG levels of the Wistar rats treated orally for 14 days

The mean \pm SEM of the IgM of the Wistar rats showed a gradual decrease at the escalation of dose from 250 mg/kg to 1,000 mg/kg body weight. However, the decrease was statistically significant, $F(3,19) = 15.17, p < 0.05$ only at the dose of 1,000 mg/kg. A similar trend was found for the IgG. There was a decrease in the IgG across the dose ranges, which was statistically significant, $F(3,19) = 6.56, p < 0.05$ at the doses of 500 and 1,000 mg/kg (Table 4.8).

Table 4.8: Effects of methanol extract of the stem bark of *Lannea acida* on the IgM and IgG levels of Wistar rats treated orally for 14 days

Treatment	IgM ($\times 10^{-3}$ g/L)	IgG ($\times 10^{-2}$ g/L)
	Mean \pm SEM	
Control	1.92 \pm 0.03	3.58 \pm 0.02
250 mg/kg LA	1.91 \pm 0.04	3.54 \pm 0.02
500 mg/kg LA	1.90 \pm 0.02	3.34 \pm 0.07*
1000 mg/kg LA	1.50 \pm 0.01*	3.40 \pm 0.05*

n = 6, One-way ANOVA (control vs. experimental), IgM, F (3, 19) = 15.17, $p < 0.05$; IgG, F (3, 19) = 6.56, $p < 0.05$. * Significant *Post-hoc* Bonferonni test. LA = *Lannea acida*, IgM = Immunoglobulin M, IgG = Immunoglobulin G

4.3.2 Cellular immunity

*1) Effects of graded doses of the methanol extract of stem bark of *Lannea acida* on the CD4+ and CD8+T-lymphocytes of the Wistar rats treated orally for 14 days*

The effects of the methanol extract of the stem bark of *L. acida* on the cellular immunity of the Wistar rats is shown in Table 4.9. There was no statistically significant difference, $F(3, 19) = 0.34, p > 0.05$ and $F(3, 17) = 0.44, p > 0.05$ respectively in the mean levels of the serum CD4+ T-cells and serum CD8+T-cells counts, between the experimental and control groups.

Table 4.9: Effects of methanol extract of stem bark of *Lannea acida* on CD4+ and CD8+T-lymphocyte counts of Wistar rats treated orally for 14days

Treatment	CD4+T cell (/μL)	CD8+T cell (/μL)
	Mean ± SEM	
Control	16.7 ± 1.93	11.4 ± 5.70
250 m/kg LA	18.4 ± 1.12	15.8 ± 5.70
500 mg/kgLA	17.5 ± 1.65	13.0 ± 2.50
1000 mg/kgLA	18.7 ± 1.28	9.5 ± 1.28

n =6, One-way ANOVA (control vs. experimental group), CD4 + T- cells, F (3, 19) = 0.34, $p > 0.05$ and CD8+ T-cells, F (3, 17) = 0.44, $p > 0.05$ respectively. LA = *Lannea acida*, CD = Cluster of Differentiation

2) Effects of graded doses of methanol extract of the stem bark of Lannea acida on delayed type hypersensitivity of Wistar rats treated orally for 14 days

There was no statistically significant difference, $F(3,20) = 2.22, p > 0.05$ in the mean levels of the DTH reaction between the experimental and control groups (Table 4.10).

Table 4.10: Effects of methanol extract of the stem bark of *Lannea acida* on delayed type hypersensitivity reaction of Wistar rats treated orally for 14 days

Treatment	DTH0	DTH1	DTH2	DTH3	DTH4
	Mean ± SEM				
Control	2.03 ± 0.11	3.5 ± 0.36	2.8 ± 0.26	3.03 ± 0.42	2.54 ± 0.44
250 mg/kg LA	2.08 ± 0.06	3.32 ± 0.35	3.0 ± 0.31	2.80 ± 0.16	2.75 ± 0.20
500 mg/kg LA	2.14 ± 0.13	3.2 ± 0.08	2.8 ± 0.24	2.7 ± 0.05	2.4 ± 0.11
1000 mg/kg LA	2.1 ± 0.06	3.1 ± 0.17	3.1 ± 0.22	2.94 ± 0.28	2.7 ± 0.15

n = 6, One-way repeated measures ANOVA (control vs. experimental groups), F (3,20) = 2.22, $p < 0.05$. LA = *Lannea acida*, DTH = Delayed Type Hypersensitivity.

CHAPTER FIVE

5.0 DISCUSSION

The phytochemical screening of the methanol extract of the stem bark of *Lannea acida* showed the presence of sugars, alkaloids, glycosides, anthraquinones, triterpenes, cardiac glycosides and tannins; but sterols, cardenolides, saponin glycosides and flavonoids were absent. These findings are consistent with a similar study on the stem bark of *L. acida* in Sokoto where the presence of alkaloid, tannin but not flavonoids was reported (Etuk *et al.*, 2009). In other studies, Ouattara *et al.* (2011a) and Muhaisen (2013) demonstrated the presence of flavonoids in the stem bark of *L. acida*, which was absent in our preparation. The reasons for these differences may be due to geographical factors and the techniques used for the extraction. The study by Ouattara *et al.* (2011a) used stem bark of *L. acida* collected from Burkina faso which has different climatic conditions from Nigeria; while that by Muhaisen (2013) used successive extraction methods with sequential solvents such as acetone, petroleum ether, chloroform and ethylacetate and these may have helped in the yield of flavonoids.

The oral administration of a single dose of 3,000 mg/kg body weight of the methanol extract of the stem bark of *L. acida* did not cause death in the animals within the first 24 hours and during the two weeks of follow-up, neither was there any visible sign of toxicity during this period. This implies that the lethal dose of *L. acida* is greater than 3,000 mg/kg and according to the OECD guideline 420 (2002), *L. acida* is of relatively low acute toxicity and is of category 5 of the global harmonized classification system (GHS) of toxic substances. Similar study by Etuk *et al.* (2009) also found the acute lethal dose of *L. acida* to be greater than 3,000 mg/kg. Other studies by Diallo *et al.* (2010) and Olatokunboh *et al.* (2010) on other members of the

genus *Lannea* also showed that members of this genus have generally relatively low acute toxicity. Diallo *et al.* (2010) demonstrated that hydro-alcoholic extract of *L. kerstingii* is non toxic at a dose of greater than 5,000 mg/kg body weight in experimental animals while Olatokunboh *et al.* (2010) also corroborated this by demonstrating that the dose of 2,000 mg/kg body weight of *L. welwitschii* does not cause mortality in experimental animals when administered orally; but when given intraperitoneally, doses as low as 800 mg/kg and 1,600 mg/kg body weight of *L. welwitschii* caused visible signs of toxicity in experimental animals. However, in our study, since only the oral route was used, the effect of administration of methanol extract of the stem bark of *L. acida* cannot be deduced.

The effect of daily administration of graded oral doses of 250, 500 and 1,000 mg/kg of the methanol extract of *L. acida* over 28-day period on Wistar rats did not show any behavioral changes or deaths over the course of 28 days. It also showed no significant differential weight gain among the experimental animals and the control, but there were differential weight changes across all the time points in all the groups (experimental and control). Initially, there was a rise in body weight, which plateaued at Day 7, then declined at Day 14, after which the animals experienced continuous weight gain up to the end of the experiment at 29th day. Since there were no differences in weight gain between the control groups and the various doses of the experimental groups, it means that our extract did not significantly affect the appetite or the catabolic processes in the animals. However, the initial weight loss during the start of the experiment may represent the period of adaptation of the animals to the stress induced experimental conditioning while the significant weight gain from the 14th day up till the end of the experiment at 29th day may represent the natural growth of the animals following adaptation to the environment and the feeding condition.

Though, organ weight changes have long been accepted as a sensitive indicator of chemically induced changes to organs and occur long before morphological changes, comparison of organ weight between treated and untreated groups of animals have their own limitations in toxicological assessment in animal models (Michael *et al.*, 2007). They are usually limited by the differences in the body weight between groups, hence, relative organ weights of animals is a more useful measure of toxicity (Bailey *et al.*, 2004).

The comparison of the mean of the relative organ weight percent for the control and experimental groups in this study did not show any statistically significant differences ($p > 0.05$) for the liver and thymus, but there were significant differences ($p < 0.05$) between the control and experimental groups for the spleen and kidneys relative organ weight percent. These differences were marked at the dose level of 1,000 mg/kg dose of the extract and could signify toxicity (Souza *et al.*, 2010). However, relative organ weight percent for the thymus and the spleen are considered to be of limited value in toxicity studies because of their inherent variability from factors such as dissection techniques, and age-related involution, and stress-related effects and therefore, must be interpreted with caution (Michael *et al.*, 2007). On the other hand, a relative organ weight change for the kidney, as in our study, should be taken serious because it is considered sensitive in predicting toxicity and physiological perturbations and often correlates well with the histopathologic changes (Michael *et al.*, 2007).

The hematopoietic system is one of the most sensitive organs to toxic effects of phytomedicines and is an important index of physiological and pathological status in man and animals (Adeneye *et al.*, 2006; Diallo *et al.*, 2008). The result of our analysis of hematological parameters in our study showed no statistically significant difference between the control and the experimental groups. This suggests that *L.*

acida may be non-toxic to circulating white and red blood cells and platelets and neither does it interfere with the hematopoietic system.

The result of the hepatic indices also showed no significant difference ($p > 0.05$) between the control and experimental groups in all the parameters measured except in the serum alanine aminotransferase levels, which showed a significant rise ($p < 0.05$) at the dose of 500 mg/kg. This implies that the methanol extract of *L. acida* may be toxic to the liver and this is corroborated by other reports of hepatic toxicities by phytomedicines (Corns, 2003; Pitler and Ernst, 2003). However, this might be a spurious elevation since it is expected that 'real' increase in ALT would have also occurred at a higher dose of 1,000 mg/kg; and other indicators of hepatotoxicity such as AST, ALP and serum protein and albumin should also have been affected.

Renal indices such as creatinine and urea levels, as well as serum electrolyte levels are used as biochemical markers of renal function (Obidah *et al.*, 2009). In this study, the renal indices showed significant differences in serum potassium levels of the control versus the experimental groups. This was seen as a progressive decline in serum potassium from the dose of 250 mg/kg up to the dose of 1,000 mg/kg. When juxtaposed with the findings of significant decrease in the relative organ weight percent of the kidney, the finding of hypokalemia may suggest renal potassium loss induced by methanol extract of *L. acida* and may be an indication of nephrotoxicity.

Histological evaluation of the organs and tissues still represent one of the cornerstones of assessing general toxicity and immunotoxicity of chemicals (Kuper *et al.*, 2000). In our study, the histopathological changes in the liver following 28-day daily oral administration of different doses of methanol extract of *L. acida* to Wistar rats showed mild hepatic vascular congestion at the dose of 250 mg/kg body weight of animal. This becomes more pronounced at the dose of 500 mg/kg body weight and at

the dose of 1,000 mg/kg, there was mild-to-moderate periportal inflammation and focal necrosis of the hepatocytes. These findings are consistent with hepatotoxicity and the vascular congestion may signal its initiation (Singh *et al.*, 2011, Adebayo *et al.*, 2014).

In the spleen, the histological findings showed secondary follicles which increased in number as the administered dose increased from 250 mg/kg to 500 mg/kg, with richly vascularized red pulp that become hemorrhagic at the dose of 1000 mg/kg. According to Wagle *et al.* (2015), toxicity of the spleen may occur as a distortion and atrophy of the white pulp, or hemorrhage of the red pulp. In our study, there was hemorrhage of the red pulp at the dose of 1,000 mg/kg of methanol extract of *L. acida*.

The histology of the kidney in our study showed glomerular necrosis and interstitial inflammation, which increased in intensity as the dose increased from 250 mg/kg to 500 mg/kg and to 1,000 mg/kg of the methanol extract of *L. acida*. At the dose of 1,000 mg/kg, there was also tubular necrosis. These findings are consistent and are buttressed by the findings of decrease in the relative organ weight of the kidney and the hypokalemia observed in experimental animals and therefore, suggest that methanol extract of *L. acida* is nephrotoxic to experimental animals. The findings of interstitial inflammation, tubular necrosis and potassium wasting may also lend credence to tubule-interstitial nephritis as a putative mechanism of nephrotoxicity of this herb.

Altered cellularity and depletion of thymocytes have long been reported to be indicative of immunotoxicity in experimental animals (Krzystyniak *et al.*, 1995; Kuper *et al.*, 2000). The histological observation of the thymus in our study showed thymocyte depletion and areas of necrosis across all the doses of methanol extract of *L. acida* and this may suggest immunotoxicity of *L. acida*.

Immunoglobulins are products of stimulated B-lymphocytes and plasma cells in response to foreign substances entering the living system. Decrease in the levels of immunoglobulin may be due to various conditions such as agammaglobulinemia, lymphoproliferative disorders, lymphoid aplasia, IgG and IgA myeloma and chronic lymphoblastic leukemia. Chemicals and phytomedicines may also alter immunoglobulin levels, and thereby increase or decrease serum antibody levels (Vos *et al.*, 1990). Lymphoid atrophy/hypoplasia or depletion may also occur with drugs that have immunotoxic effects. In this study, there was a significant decrease ($p>0.05$) in serum IgM (at dose of 1,000mg/kg of extract) and IgG levels (at doses of 500mg/kg and 1,000 mg/kg), thus, further highlighting the immunotoxic potentials of the extract. Phytochemical constituents such as flavonoids, saponin, terpene, tannic acid and polyphenols are believed to have immunomodulatory activities (Fulzele *et al.*, 2003; Nagarathna *et al.*, 2013), and glycosides upon acid hydrolysis or enzymatic reaction, yield moieties that have immunomodulatory properties (Nagarathna *et al.*, 2013). In our study, *L. acida* was found to contain glycosides, triterpenes and tannin, which may be responsible for their immune-inhibiting effects.

The T-lymphocytes participate in cellular immune responses and are responsible for the protection of the body against intracellular pathogens such as *Mycobacterium tuberculosis* and viruses. The integrity of this response could be measured by direct measurement of CD4+ and CD8+ T-cell counts. In this study, there were no statistically significant differences ($p>0.05$) in the CD4+T cell counts and the CD8+T-cell counts as well as the DTH responses between the experimental animals and the control, implying that the methanol extract of *L. acida* does not have effect on the cell mediated immunity in Wistar rats.

CHAPTER SIX

6.0 SUMMARIES, CONCLUSION AND RECOMMENDATION

6.1 Summaries

The methanol stem bark of *L. acida* was found to contain the following bioactive substances: carbohydrate, reducing sugars, alkaloids, glycosides, anthraquinones, triterpenes, cardiac glycosides and tannins. The methanol extract of *L. acida* belong to category 5 of the global harmonized classification system of toxic substances with a lethal dose that is greater than 3,000 mg/kg.

The extract has shown significant decrease in the relative organ weights of the spleen and the kidneys at the dose of 1,000 mg/kg body weight after 28-day oral administration; and decrease in the serum potassium levels across the graded doses of the extract. It has also shown rise in the serum alanine transaminase level when given at the dose of 500 mg/kg for 28 days.

Histological examination of the organs following 28-day oral administration of the extract shows hepatic sinusoidal vascular congestion and periportal inflammation; splenic red pulp hemorrhage; kidney glomerular necrosis and interstitial inflammation; and thymocyte depletion of the Thymus.

No statistical difference was observed for the measures of the cellular immune response, but significant decrease in the levels of serum immunoglobulin at the doses of 500 mg/kg for IgM and; 500 and 1,000mg/kg for IgG, respectively.

6.2 Conclusion

In conclusion, methanol extract of the stem bark of *L. acida* is relatively safe but when given as a dailydose for 28 days, it has nephrotoxic, hepatotoxic and

immunotoxic effects and hence, potential for multi-organ toxicity when employed for sub-acute and sub-chronic uses.

6.3 Recommendation

1. The methanol extract of *L. acida* should not be used for long-term treatment of ailments because of its potential for multi-organ toxicity.
2. When used for any ailment, patients should be watched out for nephrotoxicity, hepatotoxicity and immunotoxicity.
3. More studies such as immunophenotyping/immunohistochemistry, chronic toxicity and mechanism of toxicity studies should be conducted to further evaluate the effects of this herb on organ system.

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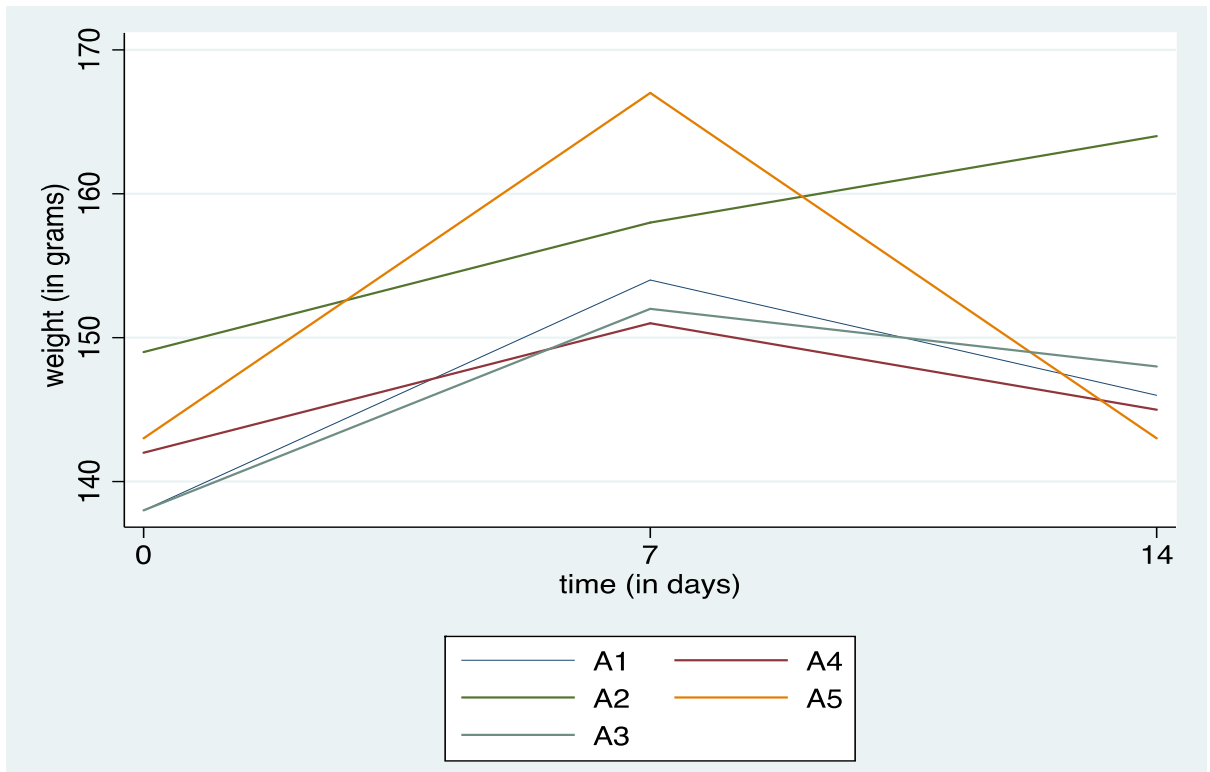
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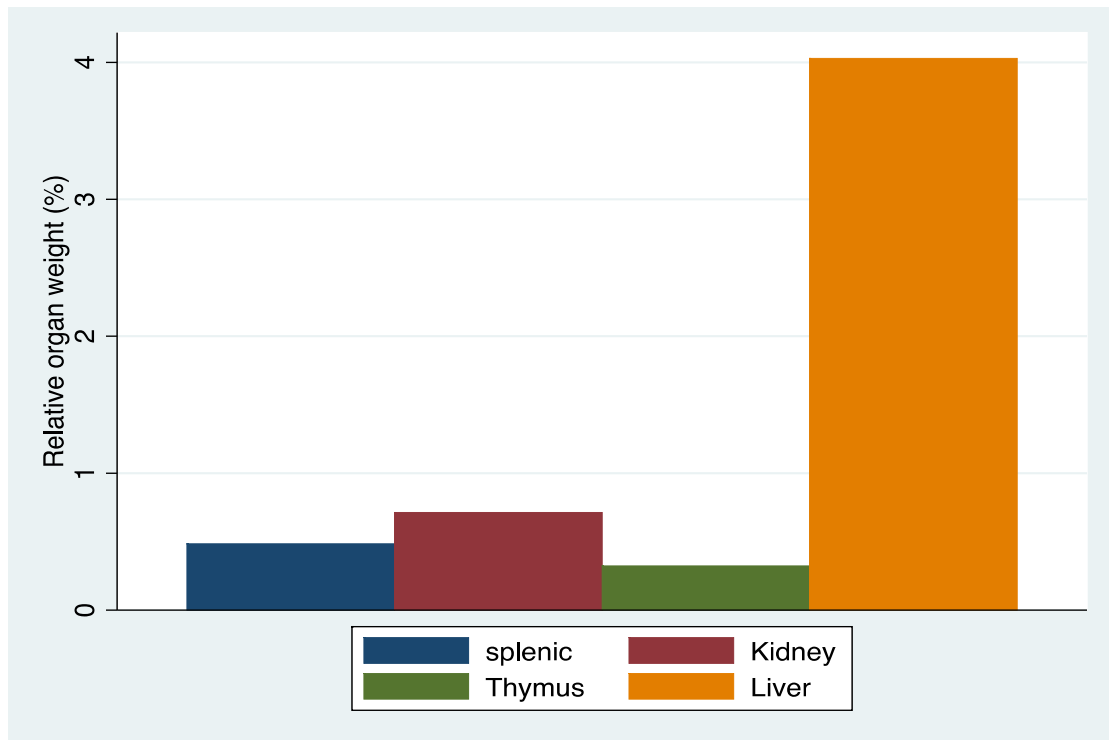
Appendix I: Effects of a single oral dose of 3000 mg/kg methanol extract of the stem bark of *Lannea acida* on the weight and relative organ weight percent of each animal over 14 days

Animal	Weight of animals (g)			Relative organ weight(%)			
	Day 0	Day 7	Day 14	Liver (%)	Spleen (%)	Kidney (%)	Thymus (%)
A1	138	154	146	3.5	0.68	0.75	0.27
A2	149	158	164	3.7	0.43	0.61	0.24
A3	142	151	145	4.6	0.48	0.76	0.41
A4	143	167	143	3.8	0.35	0.77	0.21
A5	138	152	148	4.7	0.47	0.68	0.47

Appendix II: Graph showing weight changes of each of the animals administered a single oral dose of 3000 mg/kg methanol extract of stem bark of *Lannea acida* over 14 days



Appendix III: Bar chart of the relative organ weight percent of the animals administered a single dose of 3000 mg/kg of methanol extract of stem bark of *Lannea acida* at the end of 14 days.



Appendix IV: Graph of the mean weight changes of the Wistar rats administered methanol extract of the stem bark of *Lannea acida* compared with the control over 28 days.

