

**EFFECT OF AQUEOUS ETHANOL LEAF EXTRACT OF *COMBRETUM  
MICRANTHUM* G. DON (COMBRETACEAE) ON SOME INDUCED SYSTEMIC  
INFLAMMATORY IMMUNE RESPONSE SYNDROMES IN MICE AND RATS**

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**NIGERIA.**

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AND RATS**

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**A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES,  
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**DEPARTMENT OF PHARMACOLOGY AND THERAPEUTICS,  
FACULTY OF PHARMACEUTICAL SCIENCES  
AHMADU BELLO UNIVERSITY, ZARIA  
NIGERIA**

**DECEMBER, 2014**

## DECLARATION

I declare that the work in this thesis entitled “EFFECT OF AQUEOUS ETHANOL LEAF EXTRACT OF *COMBRETUM MICRANTHUM* G. DON (COMBRETACEAE) ON SOME INDUCED SYSTEMIC INFLAMMATORY IMMUNE RESPONSE SYNDROMES IN MICE AND RATS” was carried out by me in the Department of Pharmacology and Therapeutics under the supervision of Dr. J.I. Ejiofor and Dr. (Mrs.) B.B. Maiha. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for the award of a degree or diploma at this or any other Institution.

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Michael Abel ZEKERI

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Date

## CERTIFICATION

This thesis entitled “EFFECT OF AQUEOUS ETHANOL LEAF EXTRACT OF *COMBRETUM MICRANTHUM* G. DON (COMBRETACEAE) ON SOME INDUCED SYSTEMIC INFLAMMATORY IMMUNE RESPONSE SYNDROMES IN MICE AND RATS” by Michael Abel ZEKERI meets the regulations governing the award of the degree of Master of Science in Pharmacology of the Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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## Abstract

The cellular immunity is often suppressed by infection, disease conditions and drug medication that are in continuous association with mankind. *Combretum micranthum* is a plant known for its antioxidant, antimicrobial, anti-inflammatory and various other properties of immune boosting activity. The Plant extract was assessed for its ability to protect from tissue destruction. The aqueous ethanol leaf extract was screened for previously reported phytoconstituents; Lorke's method of 1983 was used to determine the LD<sub>50</sub> in mice and rats using both *i.p.* and oral routes. Effect of the extract on haematological parameters were assessed pre- Cyclophosphamide (200 mg/kg *i.p.*) induction on the 10<sup>th</sup> day and three days post induction according to Thatte *et al.* (1987) in four groups (n=5) of wistar rats. Group I received (1 ml/kg distilled water) while group II, III and IV received (50, 100 and 200 mg/kg of extract) respectively. The protective effect of the extract was also performed according to Pallable *et al.* (1998) by *Escherichia. coli* (1 ml of  $2.5 \times 10^8$  Cfu) induction *i.p.* after 15<sup>th</sup> day pretreatment with the extract. Effect of the extract on Sheep red blood cell (SRBC) induced delayed type hypersensitivity reaction was evaluated according to Chang *et al.* (1998) on five groups (n=5) wistar rats. Group I received (1 ml/kg distilled water), group II, III and IV received 50, 100 and 200 mg/kg of extract while group V received 0.2 mg/kg dexamethasone. All the rats were immunized on the 7<sup>th</sup> day with 0.25 ml SRBC *i.p.* and the treatment continued to the 14<sup>th</sup> after which the challenging dose of 0.25 ml SRBC was administered via the plantar side of the right hind foot paw. Measurement were taken before the challenging dose (0 hr reading) and repeated at 3 and 24 hours later. Agar plate dilution method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2011 was used to test the susceptibility of *Escherichia. coli*, *Staphylococcus. aureus*, *Klebsiella. pneumoniae*, *Salmonella. typhi*

and *Proteus, mirabilis*. The zone of inhibition compared with standard antibiotics was interpreted using interpretation criteria of EUCAST, 2011. The acute toxicity test showed an LD<sub>50</sub> of 3807.9 mg/kg *p.o.* and 2154.1 mg/kg *i.p.* in mice; and > 5,000 mg/kg *p.o.* and 3807.9 mg/kg *i.p.* in rats. The phytochemical constituents revealed the presence of alkaloids, anthraquinones, carbohydrates, cardiac glycosides, flavonoids, saponins, steroids, tannins and terpenoids. The study showed dose dependent increase in WBC and neutrophil of the extract pretreated groups compared with the control group. Cyclophosphamide significantly reduced the WBC, RBC, HCT, HGB and ANC both in control and treated group. The extract protected all the pretreated mice up to 72 hr and beyond. The oedema at 24 hours was significantly higher in extract pretreated groups with 100 mg/kg having the highest response ( $p < 0.001$ ) that increased progressively to 24 hours. The extract was susceptible to all the tested organisms. Consequent upon these activities above, it can then be concluded that aqueous ethanol leaf extract of *C. micranthum* contains biological potent active principle which may be beneficial in cellular mediated immune disease conditions.

## TABLE OF CONTENTS

<b>CONTENT</b>	<b>Page</b>
Title Page - - - - -	i
Declaration - - - - -	ii
Certification - - - - -	iii
Acknowledgement - - - - -	iv
Abstract - - - - -	-v
Table of Contents - - - - -	vii
List of Tables - - - - -	xii
List of Plates- - - - -	xiii
List of Appendices - - - - -	xiv
Abbreviations - - - - -	xv

## CHAPTER ONE

### 1.0 INTRODUCTION

<b>1.1 Systemic Responses in Immunosuppression from Microbial Infections, Drug Therapy and Disease Conditions</b>	<b>1</b>
<b>1.2 Statement of Research Problem</b>	<b>5</b>
<b>1.3 Justification of the Study</b>	<b>6</b>
<b>1.4 Aim and Objective of the Study</b>	<b>7</b>
<b>1.5 Research Hypothesis</b>	<b>8</b>

## CHAPTER TWO

<b>2.0</b>	<b>LITERATURE REVIEW</b>	-	-	-	-	-	-	<b>9</b>
<b>2.1</b>	<b>History and description of <i>Combretum micranthum</i> plant</b>	-	-	-	-	-	-	<b>9</b>
2.1.2	The plant taxonomy	-	-	-	-	-	-	10
2.1.3	Chemical constituents	-	-	-	-	-	-	13
2.1.4	Ethnomedicinal and other uses of <i>Combretum micranthum</i>	-	-	-	-	-	-	13
2.1.5	Previous studies reported <i>Combretum micranthum</i> plant	-	-	-	-	-	-	14
<b>2.2</b>	<b>The Immune System</b>	-	-	-	-	-	-	<b>15</b>
2.2.1	Immunity	-	-	-	-	-	-	15
2.2.2	Acquired immunity	-	-	-	-	-	-	16
2.2.3	Cells involved in acquired immune response	-	-	-	-	-	-	17
2.2.4	Humoral response type of acquired immunity	-	-	-	-	-	-	19
2.2.5	Cell mediated response type of acquired immunity involving the T lymphocytes	-	-	-	-	-	-	21
2.2.6	White blood cells (WBCs, Leukocytes or Leucocytes)	-	-	-	-	-	-	23
2.2.7	Vascular tissue response in immune regulation	-	-	-	-	-	-	26
2.2.8	Leucopenia	-	-	-	-	-	-	28
<b>2.3</b>	<b>Inflammation</b>	-	-	-	-	-	-	<b>30</b>
2.3.1	Acute and chronic inflammation	-	-	-	-	-	-	31
2.3.2	Acute inflammation	-	-	-	-	-	-	31

2.3.3	Symptoms of acute inflammation	-	-	-	-	-	32
2.3.4	Process of acute inflammation/ cell derived mediators	-	-	-	-	-	32
2.3.5	Chronic inflammation -	-	-	-	-	-	33
2.3.6	Systemic inflammatory immune response syndromes	-	-	-	-	-	34
2.3.7	Hypersensitivity reactions	-	-	-	-	-	37
2.3.8	Forms of hypersensitivity reactions	-	-	-	-	-	38
<b>2.4</b>	<b>The Models used for this Study</b>	-	-	-	-	-	<b>41</b>
2.4.1	Cyclophosphamide induced neutropenia in rats	-	-	-	-	-	41
2.4.2	<i>Escherichia coli</i> induced abdominal sepsis	-	-	-	-	-	42
2.4.3	Antibody response to sheep red blood cells	-	-	-	-	-	42

### **CHAPTER THREE**

<b>3.0</b>	<b>MATERIALS AND METHODS</b>	-	-	-	-	-	<b>43</b>
<b>3.1</b>	<b>Materials</b>	-	-	-	-	-	<b>43</b>
3.1.1	Collection and Identification of Plant Materials	-	-	-	-	-	43
3.1.2	Experimental Animals	-	-	-	-	-	43
3.1.3	Microorganisms	-	-	-	-	-	43
3.1.4	Sheep red blood cells	-	-	-	-	-	44
3.1.5	Equipments	-	-	-	-	-	44
3.1.6	Drugs, Chemicals and Solvents	-	-	-	-	-	44

<b>3.2</b>	<b>Methods</b>	-	-	-	-	-	-	-	-	<b>45</b>
3.2.1	Preparation of plant extract	-	-	-	-	-	-	-	-	45
3.2.2	Validation of phytochemical components	-	-	-	-	-	-	-	-	45
3.2.3	Acute toxicity study	-	-	-	-	-	-	-	-	49
3.2.4	Cyclophosphamide - induced neutropenia	-	-	-	-	-	-	-	-	49
3.2.5	Effect of the extract on <i>E. coli</i> induced abdominal sepsis study in mice	-	-	-	-	-	-	-	-	50
3.2.6	Effect of the extract on Sheep red blood cell (SRBC) induced delayed hypersensitivity reaction in rat-	-	-	-	-	-	-	-	-	51
3.2.7	Inhibitory effect of the extract on bacterial growth:	-	-	-	-	-	-	-	-	52
3.2.8	Statistical analysis	-	-	-	-	-	-	-	-	53
<b>CHAPTER FOUR</b>										
<b>4.0</b>	<b>RESULTS</b>	-	-	-	-	-	-	-	-	<b>54</b>
<b>4.1</b>	<b>Yield of the Plant Extract</b>	-	-	-	-	-	-	-	-	<b>54</b>
<b>4.2</b>	<b>Acute Toxicity Study</b>	-	-	-	-	-	-	-	-	<b>54</b>
<b>4.3</b>	<b>Preliminary Phytochemical Constituents of the Leaf Extract of <i>C. micranthum</i></b>	-	-	-	-	-	-	-	-	<b>55</b>
<b>4.4</b>	<b>Effect of <i>C. micranthum</i> Extract on Cyclophosphamide Induced Neutropenia</b>	-	-	-	-	-	-	-	-	<b>56</b>
<b>4.5</b>	<b>Effect of Extract on Sheep Red Blood Cell (SRBC) Induced Delayed Hypersensitivity Reaction</b>	-	-	-	-	-	-	-	-	<b>58</b>

<b>4.6</b>	<b>Effect of Extract on <i>E. coli</i> Induced Abdominal Sepsis in mice</b>	-	<b>60</b>
<b>4.7</b>	<b>Inhibitory effect of the Extract on Bacterial Growth</b>	- -	<b>63</b>

**CHAPTER FIVE**

<b>5.0</b>	<b>DISCUSSION</b>	- - - - -	<b>68</b>
------------	-------------------	-----------	-----------

**CHAPTER SIX**

<b>6.0</b>	<b>SUMMARY, CONCLUSION AND RECOMMENDATION</b>	-	<b>72</b>
<b>6.1</b>	<b>Summary</b>	- - - - -	<b>72</b>
<b>6.2</b>	<b>Conclusion</b>	- - - - -	<b>73</b>
<b>6.3</b>	<b>Recommendation</b>	- - - - -	<b>73</b>
	<b>REFERENCES</b>	- - - - -	<b>74</b>
	<b>APPENDICES-</b>	- - - - -	<b>83</b>

## LIST OF TABLES

<b>I.</b>	<b>Phytochemical Constituents of Aqueous Ethanol Leaf Extract of <i>C. micranthum</i></b> - - - - -	<b>55</b>
<b>II.</b>	<b>The Effect of Oral <i>C. micranthum</i> Pretreatment on Haematological Parameters in Rats before and after Cyclophosphamide Treatment</b> - -	<b>57</b>
<b>III.</b>	<b>Sheep Red Blood Cells (SRBC)-Induced Delayed Type Hypersensitivity (DTH) on Rats Pretreated with <i>C. micranthum</i> Leaf Extract</b> - - - - -	<b>59</b>
<b>IV.</b>	<b>Protective Effect of <i>C. micranthum</i> Leaf Extract on <i>Escherichia Coli</i>- Induced Abdominal Sepsis in 15 Days Pretreated Mice-</b> - - - - -	<b>60</b>
<b>V.</b>	<b>Growth Inhibition Zones of Two Extract Doses and Four Standard Antibiotics Specific for <i>Escherichia. coli</i></b> -	<b>63</b>
<b>VI.</b>	<b>Growth Inhibition Zones of Two Extract Doses and Four Standard Antibiotics Specific for <i>Staphylococcus. aureus</i></b> - - - - -	<b>64</b>
<b>VII.</b>	<b>Growth Inhibition Zones of Two Extract Doses and Four Standard Antibiotics Specific for <i>Klebsiella. pneumoneae</i></b> - - - - -	<b>65</b>
<b>VIII.</b>	<b>Growth Inhibition Zones of Two Extract Doses and Four Standard Antibiotics Specific for <i>Salmonella. typhi</i></b> -	<b>66</b>
<b>IX.</b>	<b>Growth Inhibition Zones of Two Extract Doses and Four Standard Antibiotics Specific for <i>Proteus. mirabilis</i></b> -	<b>67</b>

## LIST OF PLATES

<b>I.</b>	<b>The <i>plant Combretum micranthum</i> in its natural environment</b>	<b>- - 11</b>
<b>II.</b>	<b>The <i>plant Combretum micranthum</i> showing its leaves and flower-</b>	<b>12</b>
<b>III.</b>	<b>Photomicrographs of the stomach tissues from control and <i>C. micranthum</i> extract-pretreated mice (H and E staining, × 250)</b>	<b>61</b>
<b>IV.</b>	<b>Photomicrographs of the intestinal tissues from control and <i>C. micranthum</i> extract-pretreated mice (H and E staining, × 250)</b>	<b>62</b>

## **LIST OF APPENDICES**

- i.** European Committee on Antimicrobial Susceptibility Testing  
Interpretation Table for Zone diameter Growth Inhibition - - 83

## ABBREVIATIONS

Ab -	Antibody
ABU-	Ahmadu Bello University
Ag -	Antigen
AIDS-	Acquired Immune Deficiency Syndrome
ANC-	Absolute Neutrophil Count
APC -	Antigen Presenting Cell
ATCC-	American Type Culture Collection
BCR -	B-Cell Receptor
CD <sub>4</sub> -	Cluster of Differentiation type four
CD <sub>8</sub> -	Cluster of Differentiation type eight
CD <sub>25</sub> -	Cluster of Differentiation type twenty five
CfU -	Colony forming Unit
DLC -	Diferencial Leucocyte Count
DTH -	Delayed Type Hypersensitivity
EDTA-	EthyleneDiamineTetraacetic Acid
EUCAST-	European Committee on Antimicrobial Susceptibility Testing
HCT -	Haematocrit

HGB -	Haemoglobin
HIV -	Human ImmunoDeficiency Virus
i.p-	Intraperitonially
IFN- $\gamma$ -	Interferon gamma
Ig -	Immunoglobulin
IgA -	Immunoglobulin A
IgD -	Immunoglobulin D
IgE -	Immunoglobulin E
IgG -	Immunoglobulin G
IgM -	Immunoglobulin M
MAC-	Membrane Attack Complex
MCHC-	Mean Corpuscular Haemoglobin Concentration
MHCI	Major Histocompatibility Complex one
MHCII -	Major Histocompatibility Complex two
MIC-A-	Major Histocompatibility Complex Class I Polypeptide Sequence A
NAPRI -	National Animal Production Research Institute
NK -	Natural killer cell
$^{\circ}$ C-	Degree Celcius
$^{\circ}$ F -	Degree Farenheight

p.o -	Per oral
PAMP-	Pathogen-Associated Molecular Pattern
PPD-	Purified Protein Derivatives
PRR -	Pattern Recognition Receptor
RBC -	Red Blood Cell
SIRS -	Systemic Inflammatory Immune Response Syndromes
SLE-	Systemic Lupus Erythematosus
SRBC-	Sheep Red Blood Cell
TCR -	T-Cell Receptor
TH <sub>1</sub> -	T Helper one
TH <sub>2</sub> -	T Helper two
TLC -	Total Lymphocyte Count
UK -	United Kingdom
UNAID-	United Nations Programme on HIV/AIDS
UTI -	Urinary Tract Infection
WBC-	White Blood Cell
WHO-	World Health Organization

## CHAPTER ONE

### 1.0 INTRODUCTION

#### 1.1 Systemic Responses from Microbial Infections, Drug Therapy and Disease Related Immunosuppression

Generally, the human environment is full of various types of microbial organisms like bacteria, fungi and viruses. Infectious diseases caused by these microbes are major causes of death (Bataniila *et al.*, 2005). The World Health Organization (WHO, 2002) and United Nations Programme on HIV/AIDS (UNAID, 2007) reported that between 14 and 17 million people consequently die of infectious diseases each year. The economic importance of microbial infections can never be over-emphasized with the fact that malaria, HIV/AIDS, hepatitis, urinary tract infections (UTIs) and so forth are all serious global health problems of high morbidity and mortality, and the complications that arise from these, such as anaemia in both malaria and HIV infections also call for concern (WHO, 2004). Many of these microbial diseases are scourges of developing nations including Africa, India, Southeast Asia and South America and since most of these infections interfere with cellular immune functions, protection against them will depend on cellular immunity.

The immune system is involved in the etiology as well as pathophysiologic mechanisms of many diseases and agents that boost the immune system can provide supportive therapy to conventional chemotherapy (Fulzele *et al.*, 2003). The immune system comprises mostly of the blood components and majorly the white blood cells, and thus, when suppressed may lead to reduction in blood cell counts (immunodeficiency) and susceptibility to infections (Baumann and Preiss, 2001).

Some drug therapy also causes immunosuppression and in fact acquired neutropenia results from symptoms of medication overdose or toxicity (Suffredini *et al.*, 1999). One severe consequence of neutropenia is that it can increase the risk of infection and over the years, the incidence of idiosyncratic drug-induced agranulocytosis (acute neutropenia) has remained stable at 2.4 – 15.4 cases per million despite the emergence of new causative drugs: antibiotics (beta-lactam and cotrimoxazole), antiplatelet agents (ticlopidine), antithyroid drugs, sulfasalazine, neuroleptics (clozapine), antiepileptic agents (carbamazepine), nonsteroidal anti-inflammatory agents and dipyron (Andres *et al.*, 2006). Drug-induced agranulocytosis remains a serious adverse event due to the occurrence of severe sepsis that gives rise to severe deep infections of pneumonia, septicemia and septic shock. Chemotherapy-induced febrile neutropenia especially of cytotoxic drugs is of common occurrence for a solid tumour or non-Hodgkin,s lymphoma (Gudrun *et al.*, 2008). Cyclophosphamide an anticancer agent targeted to the tumour cells, but which at high dose in addition acts on the blood forming system (haematopoietic cells) to reduce the number of blood cells. Disease conditions like AIDs, diabetes, cancer and so forth also weakens the immune system to suppress immunity (Andres *et al.*, 2010).

The immune system as with other physiologic systems is carefully regulated in its function of maintaining homeostasis by generating protective responses that ensure good health. Disturbances in these regulatory mechanisms as may be caused by infections, disease states or drug-induced toxicities may have disastrous consequences. The WBCs are cells of the immune system most actively involved in immune regulation and are found in their various types throughout the body, particularly the blood and lymphatic system whereby some act as phagocytes - ingesting bacteria, viruses and

cellular debris, and others releasing enzymatic granules that damage pathogenic invaders (May and Machesky, 2001). Normally, the vascular tissues' response to remove harmful stimuli or initiate a healing process is via inflammation and such developed inflammatory responses are usually maintained by inflammatory mediators released by Leucocytes (Serhan, 2008).

Inflammation is part of the complex biological response of vascular tissues to harmful stimuli of pathogens, damaged cells, or irritants (Ferrero-Miliani *et al.*, 2007). It is a protective response by the organism to remove the injurious stimuli and to initiate a healing process and it is often considered a mechanism of innate immunity because of its stereotypic nature (Abbas and Lichtman, 2003). In its acute phase, the vascular tissues floods plasma and leukocytes (especially granulocytes) from the blood into the site of the stimulus and which produce classical signs as pain, heat, redness, swelling, and loss of function (Kawai and Akira, 2006). If the offending stimulus is not contained or destroyed by the actions of such acute phase-inflammatory-mediators, it gains access to the lymphatic system to infect the lymph vessels (lymphangitis) and lymph node (lymphadenitis). Such infection may spread further through lymphatic-bloodstream drainage into the circulatory system to involve the local vascular system and the immune system resulting in prolonged or chronic inflammation (Eming *et al.*, 2007).

The propagation of inflammatory responses of this type is often characterized by simultaneous progressive destruction of tissue cells, which when overwhelms the host defense, results in systemic inflammatory immune response syndromes. This condition of chronic inflammation is characterized by severe sepsis and changes in the quality and quantity of leucocytes and examples include septic bacterial infections including

peritonitis; hypersensitivity reactions especially of the delayed type and so forth (Carol, 2007).

The increase in the number of immature white blood cells (leucocytosis) or reduction in leucocyte counts (leucopenia) in circulation is commonly of reactive to infection which often triggers inflammation (Parakrama and Clive, 2005). Inflammation that occur in the absence of infection (such as in atherosclerosis) are usually described as maladaptive (Allison *et al.*, 2013). Leucopenia may be due to decreased production by the bone marrow or increased removal particularly of neutrophils that are not able to renew their used up lysosomes in digesting microbes and so often die after each activity (Bruce *et al.*, 2002).

Sepsis or septic bacterial infections is a potentially dangerous or life threatening medical condition in which there is the presence of harmful toxins of bacteria or other organisms in tissue wounds (Pavlidis, 2003). Usually it is the chemicals released into the blood to fight the septic infection that cause inflammation over the entire body, causing formation of tiny blood clots that block oxygen from vital organs and resulting in septic shock, organ failure and threatening drop in blood pressure (Tsiotou *et al.*, 2005). The risk of sepsis is more in conditions of suppressed immune system. Introduction of bacterial organisms into an otherwise sterile peritoneal environment is most times associated with severe septic shock (Bruce *et al.*, 2002; Tsiotou *et al.*, 2005). Infections of the peritoneum can be generalized (peritonitis) or localized (intra-abdominal abscess). There are primary, secondary and tertiary stages of peritonitis, but the primary form is that occurring in immunocompromised conditions as against the secondary relating to the

pathologic process in the visceral organ; and the tertiary from recurrent cases (Morrell, 2009).

Delayed (Type IV) hypersensitivity reaction is an exaggerated immune reaction to an invading pathogen (mycobacteria, fungi, virus and other parasites) that is also protective in nature and often initiated by immune cells called mononuclear leukocytes. Following an exposure to an antigen, there is a delay (48 to 72 hours) response whereby T cells that recognize the antigen can do one of two things: (1) directly destroy the antigen (killer T cells) or (2) help trigger infiltration of mononuclear leukocytes to destroy the antigen (helper T cells) (Ariza *et al.*, 2014).

## **1.2 Statement of Research Problem**

Mankind especially those residing in the tropical parts of the African continent of which Nigeria is one, is constantly exposed to various types of microbial parasites like bacteria, fungi and viruses; and which are associated with infectious diseases of high morbidity and mortality (Bert *et al.*, 2005). The effects of those microbial organisms include HIV/AIDS, hepatitis, urinary tract infections (UTIs), meningitis to mention but a few, and these are all serious global health problems.

Neutrophils usually make up 50-70% of the circulating white blood cells and serves as the primary defense against infections by destroying bacteria in the blood (Thomas *et al.*, 2003). Reduced neutrophils is an immunodeficiency condition associated with greater risk of infection and a variety of signs and symptoms of many diseases including most feverish conditions of body temperature above 38.5°C/101.3°F are often related to neutropenia (Bert *et al.*, 2005).

Millions of deaths around the world are due to infection-triggered chronic inflammation with its associated septic shock. The risk of sepsis is more in conditions of weakened immune system and inflammation-associated sepsis affect millions of people around the world and kill more than 1 in 4 people who contract it (Dellinger, 2003). Septic bacterial infection is a potentially life threatening systemic inflammatory response syndrome of high mortality rate, which according to Dellinger (2003) accounts to 60-70% death per year in the developing countries.

The primary form of peritonitis which most at times is associated with severe septic shock occurs in immunocompromised conditions and is known to be a complication of cirrhosis and ascites which has world prevalence as high as 18% (Bruce *et al.*, 2002).

### **1.3 Justification of the Study**

The immune system is involved in the etiology as well as pathophysiologic mechanisms of many diseases and since most infections interfere with cellular immune functions, protection against them will depend on cellular immunity and thus, agents that boost the immune system can provide supportive therapy to conventional chemotherapy (Fulzele *et al.*, 2003).

The need to look out for available natural plants that boosts the immune system is pertinent since immunosuppression is a frequently encountered phenomenon via disease conditions, drug therapy as well as microbial infections which are in continuous association with mankind.

According to World Health Organization (2002), 80% of population in Africa still rely on medicinal plants for their daily health care needs and Africa has an impressive natural vegetation with many plants that can be used as remedies for various ailments of both man and animals (Ojewole, 2008).

*Combretum micranthum* is a plant known for its antioxidant, antimicrobial, anti-inflammatory and various other properties of immune-related effects. Thus, it seemed worthwhile to undertake studies directed towards finding the extent of its effect on some systemic inflammatory response syndromes induced in experimental animal models.

#### **1.4 Aim and Objectives of the Study**

The aim of the study is to ascertain the effect of the crude aqueous ethanol leaf extract of *C. micranthum* on some systemic inflammatory immune responses induced in mice and rats.

The specific objectives are as follows:

- 1 To validate the phytochemical components
- 2 To evaluate the antibacterial potential of the extract
- 3 To assess the effect of the extract on:
  - I. Cyclophosphamide-induced neutropenia in rat
  - II. *E. coli*-induced abdominal sepsis in mice
  - III. Sheep red blood cells-induced delayed hypersensitivity reaction in rat

## **1.5 Hypothesis**

The antimicrobial, antioxidant and anti-inflammatory properties of *Combretum micranthum* plant are all factors that boost cellular immunity to protect from or reduce the occurrence of immunosuppression, tissue damage from prolonged or chronic inflammation, and/or death.

## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### 2.1 History and Description of Plant

The Combretaceae is a family of about 20 genera and has as many as 600 species, some of which are trees commonly seen on woodlands with a stem to 10 cm in diameter, while some are shrubs mostly on rocky hills. The most commonly occurring genus of the genera is Combretum which has almost 250 species including *C. aculeatum*, *C. albidum*, *C. apiculatum*, *C. bracteosum*, *C. coccineum*, *C. erythrophylum*, *C. extensum*, *C. flagrocarpum*, *C. imberbe*, *C. kraussii*, *C. leprosum*, *C. molle*, *C. micranthum*, *C. microphyllum*, *C. nigricans*, *C. platypetalum*, *C. zeyheri* and so forth (Burkill, 1985). The species, *C. micranthum* is commonly found in the tropical and sub-tropical countries including those of Africa like Senegal, Gabon, Gambia, Mali, Nigeria, Niger and Ghana where they are widely used in traditional medicine (Abdullahi, 2008).

The flowers are usually bisexual and are borne as auxiliary cluster on scaly stalks. The fruits are small and scaly, while the leaves are dorsiventral or more rarely centric with short stalk, the leaves turn from green to orange over time and are very useful in herbal medicine (Watson *et al.*, 2000). The plant is a very drought and fire-resistant. It is a browse plant for cattle and is of some importance as forage in the dry season in Northern Nigeria.

##### 2.1.2 *Combretum micranthum*

This plant is of the:

Division: Spermatophyta

Class: Dicotyledoneae

Family: Combretaceae

Genus: Combretum

Species: Micranthum

Scientific Name: *Combretum micranthum* G. Don

Common names according to Burkill (1985) are: Hausa: *Farar Geezaa*; Yoruba: *Okan*;

Igbo: *Nza Otego*; Fulanis in Sokoto: *Gumumi*



Plate I. The plant *C. micranthum* in its natural environment



Plate II.        The plant *C. micranthum* in its natural environment bearing fruit

### **2.1.3 Chemical constituents**

The reported chemical constituents in the aqueous ethanolic root extract of *C. micranthum* include flavonoids, saponins, carbohydrates, anthraquinones, tannins and cardiac glycosides, while from the aqueous leaf extract, alkaloids, sterols and terpenes were found in addition to the above (Abdullahi, 2008; Welch, 2010).

The chemical compositions from the ethyl-acetate and n-butanol fractions of the aqueous-ethanolic leaf extract as reported by Welch, (2010) include many polyphenolic compounds such as catechins, glycosylflavones, flavans and galloylated-C-glycosylflavone and epicatechin as well as gallic acid, mallic acid, betaine, choline, combretine, vitexin and isovitexin (C-glycosylflavone), m-inositol, sorbitol, myricetin-3-O-glucoside and myricetin-3-O-rutinoside.

### **2.1.4 Ethnomedicinal and other uses of *C. micranthum***

The leaf of *C. micranthum* is used in West Africa as herbal infusion in the treatment of biliary fever, kidney infections, naso-pharyngeal infection, colic and vomiting and has been found to possess antimalaria, diuretics, anti-inflammatory and antimicrobial properties against both gram positive and negative organisms (Yaouba *et al.*, 2012). The leaves decoction or fresh leaves have been used to treat coughs, bronchitis, hepatobiliary diseases, hepatitis and malaria (Ancolio *et al.*, 2002); and in combination with the other plant parts it is used in the treatment of beri-beri, haemorrhage, leprosy, enuresis, blenorrhagia, infantile and adult diarrhoea (Burkill, 1985, Kola and Benjamin, 2002).

A root decoction is vermifugal and is said to have been used for sore washes as antiseptic for washing open wounds and also drunk for treatment of guinea worm

infestations (Yaouba *et al.*, 2012). The dried powdered root and fruit when mixed with palm oil is used in the treatment of sprain, bruises, suppurating swellings and abscesses whether of syphilitic or other origin (Burkill, 1985).

Other traditional uses of the plant are as forage in the dry season for animals in Northern Nigeria; in making large baskets (Kwando) which are used to store grain or to hold fowls; as building materials and as fuel for firewoods (Burkill, 1985).

#### **2.1.5 Previous studies reported on *C. micranthum* plant**

The aqueous ethanol root extract had been reported to possess dose-dependent anticonvulsant activity in both electroshock and chemically induced convulsions (Danmalam *et al.*, 2011). The aqueous ethanol leaf and stem-bark extracts were also reported to have shown analgesic, anti-inflammatory and antidiarrhoeal properties (Abdullahi, 2008); while the aqueous leaf extract: showed antipyretic, analgesic and anti-inflammatory activity (Olajide *et al.*, 2003); as well as significant antidiabetic property for type II diabetes mellitus (Aminu and Oricha, 2010). The aqueous extract from the fresh leaves also showed antibacterial activity (Uduma *et al.*, 2012) and the methanol leaf extract showed inhibitory effect on herpes virus types I and II (Ferrea *et al.*, 1993).

## **2.2 The Immune System**

The immune system is a collection of organs, cells and tissues that work synergistically to protect the body from diseases caused mostly by pathogenic organisms (bacteria,

viruses, parasites and fungi) as to maintain homeostatic balance between the body and the environment (Finlay and McFadden, 2006). In its normal functions, the immune system maintains homeostasis by generating protective responses that ensure good health, but when tuned abnormally that the immune system becomes unable to distinguish between the normal body cells and foreign cells, conditions like autoimmunity, cancer, diabetes or chronic inflammations which are very difficult to treat may occur. As with other physiologic systems, the immune system is carefully regulated in its function and an important form of regulation concerns the prevention of immune responses against self antigens. Disturbances in these regulatory mechanisms as may be caused by conditions such as congenital defects, hormonal imbalance, or infection may have disastrous consequences. For instance, AIDs is associated with a viral infection of the T-lymphocytes that participates in regulating the immune response. The T-lymphocyte infested with human immunodeficiency virus (HIV) produces AIDs - a decrease in occurrence and function of one vital sub-population of T-cells, resulting in immunologic deficiency of inability to resist infections that are ordinarily benign (Mackay, 2001).

### **2.2.1 Immunity**

Immunity is the ability to withstand and resist a subsequent attack or exposure to the same offending agent - a mechanism used by the body for protection against foreign agents; and the component weapons for this could be surface or internal elements (cellular, non-cellular or chemical). There are Innate and Acquired immunity. Innate immunity is the use of inborn elements which are always present and available at very short notices to mount protection or immune response to foreign invaders; while acquired immunity uses specific invader contact elements that supplements the innate

elements against such invader; and an acquired immunity is present only in vertebrates. The innate and acquired immune systems play an integrated role in the prevention of and recovery from infectious diseases and are essential to the survival of an individual (Beck and Gail, 1996).

### **2.2.2 Acquired immunity**

An initial contact with the foreign agent (immunization) triggers a chain of events that leads to the activation of series of protein synthesis, some of which show specific reactivity against the invader (Mackay and Rosen, 2000). The goal of acquired immunity is to eliminate invading foreign substance, but in the process, some tissue damage may occur as the result of synthesized immune components with nonspecific effects. However, this damage is temporary, as soon as the invader is eliminated; the situation at that site reverts to normal.

The acquired immunity of eliminating antigen (Ag) is of humoral and cellular forms that collaborate to achieve an aim. The humoral is mediated mainly by B-cells and circulating antibodies (Abs) (serum Abs are proteins secreted by B-cells), while the cellular or cell mediated immunity is mediated by T-cells and the release of cytokines that affect other cells. The characteristics of acquired immune response include:

- 1 **Specificity:** response only to unique molecular entities (discriminatory ability)
- 2 **Adaptiveness:** ability to respond to previously unseen molecules that may have never existed; in other words, this type of immune response is not static, but grows and changes with exposure to and/or experience with foreign agents.
- 3 **Discrimination between self and non-self:** ability to recognize and respond to molecules that are foreign (non-self) and to avoid making a response to those

molecules that are self. This distinction between Ags and self is conferred by specialized cells –lymphocytes that bear on their surface Ag-specific receptors

- 4 **Memory:** ability to recall previous contact with a foreign molecule and respond to it in a learned manner (Kawai and Akira, 2006).

### **2.2.3 Cells involved in acquired immune response**

The three main cell types involved in acquired immunity and whose complex interactions are required for the expression of the full blown range of immune response are T-cells, B-cells and APCs (antigen presenting cells). The T- and B-cells are from a common lymphoid precursor cells, but differentiate along different developmental lines. The line that matures in the thymus yields the T-cells and the line that matures in the bone marrow yields the B-cells. The T- and B- cells differ in their functions, but the two shares the property of specificity to the Ag. Thus, the major recognition and reaction functions of the immune response are contained within the lymphocytes and which are the major cellular players in immune response (Harty *et al.*, 2000).

The T- and B- lymphocytes of myriad specificities exist and the lymphocytes have surface Ag-specific receptors to which the Ags bind for activation and onward release of various products. For B-lymphocytes, the receptors are Ab molecules with same specificity with the activated Ags, while for T-cells, the receptors are T-cell receptor (TCR) protein molecules (cytokines that regulates many participatory cells needed to mount up an effective response and/or Ag elimination process). It is also noteworthy that antigen receptors are expressed as trans-membrane molecules on B and T lymphocytes (Romero *et al.*, 2006).

The APCs (macrophages, dendritic cells) do not have Ag-specific receptors as do the lymphocytes and thus, their function is to process and present the Ags to their specific T-cell receptors on the lymphocytes (Kovasc *et al.*, 2000). They utilize two special surface molecules to do this (major histocompatibility complex – MHC class I and class II) which are encoded by a set of genes. The processed Ag is one that has been non-covalently bound to MHC class I or class II molecules (or both). Ag presented on MHC class I molecules is presented to one T-cell sub-population (cytotoxic T-cells) and participates in the activation of this T-cell. Ag presented on APCs MHC class II molecules activates another T-cell sub-population (the helper T-cell) (Mackay, 2001).

The other cell types that participate in immune responses (both innate and acquired) are neutrophils and mast cells which are primarily involved in the effector phase of the response. They have no specific Ag recognition properties, but are activated by various substances called cytokines released by various cells (including activated Ag-specific lymphocytes) (Eming *et al.*, 2007).

#### **2.2.4 Humoral response type of acquired immunity involving B-cell**

##### **immunoglobulins (Igs) antibodies**

The binding of Ags to B-cells specific membrane immunoglobulin (Ig) surface receptor molecules (BCRs) activates the B-cells to secret Abs (Agerberth and Gudmundsson, 2006). Each B-cell expresses approximately  $10^5$  BCRs of exactly same specificity.

Antibodies are heterogeneous mixture of serum globulins all of which have the ability to bind specific Ags. All serum globulins with Ab activity are referred to as immunoglobulins (Igs). All Ig molecules have common features of both specific recognizing-binding of an Ag and performing common biologic function after combining with the Ag. Each Ig molecule has two identical light (L) chains and two identical heavy (H) chains linked together by disulfide bridges. The portion of the Ig molecule that binds the Ag is composed of the amino terminal region of both the H and L chains. Thus, each Ig is symmetric and is capable of binding to identical epitopes on the same Ag or on different ones (Girardi, 2006).

There are 5 different Igs of H-chains (G, M, A, E, D) corresponding to ( $\gamma$ ,  $\mu$ ,  $\alpha$ ,  $\epsilon$ ,  $\delta$ ) and these have unique biologic effector properties as follows:

- i. IgA antibodies - are those that mainly protect exposed body surfaces such as nose, respiratory and digestive tracts, ears, eyes, vagina and so forth from foreign invasion on the outside. This type of antibody is also found in saliva and tears. About 10% to 15% of the antibodies in the body are of the IgA type and a small percentage of people do not make IgA antibodies.
- ii. IgG antibodies - are found in all body fluids. They are the smallest but most abundant of the antibodies, normally comprising about 75% to 80% of all the antibodies in the body. IgG antibodies are considered the most important antibodies in fighting bacterial and viral infections efficiently and are the only type of antibody that can cross the placenta to help protect the foetus.

- iii. IgM antibodies - are the largest type in blood and lymph fluid and are usually produced first in response to an infection. IgM antibodies normally comprise about 5% to 10% of all the antibodies in the body.
- iv. IgD antibodies - are found in small amounts in the tissues that line the abdominal and chest cavity of the body. They appear to play a role in allergic reactions to some substances such as milk, some medications, and some poisons. IgD remains attached to B cells and play a key role in initiating early B cells response.
- v. IgE. IgE antibodies are found in the lungs, skin, and mucous membranes. They cause the body to react against foreign substances such as pollen, fungus spores, and so forth. IgE antibody levels are often high in people with allergies (Schroeder and Cavacini, 2010).

The reaction between Ag and Ab activates the complement system (series of serum enzymes) and may result in either lyses of the target Ag or enhanced phagocytosis (phagocytic cells-Ag ingestion) as the activation also recruits highly phagocytic polymorphonuclear cells being part of the innate immune system that maximize the effective response made by this humoral arm of immunity against invading agents (Agerberth and Gudmundsson, 2006).

### **2.2.5 Cell mediated response type of acquired immunity involving the T-lymphocytes**

The B- cells produce soluble Ab that circulates to bind its specific Ags, but each T-cell bear many identical Ag receptors (TCRs) of approximately  $10^5$ /cell and they tend to

circulate directly to the site of Ag expressed on APCs and to interact with the APCs on a cognate (cell to cell) fashion (Holtmeier and Kabelitz, 2005). Each sub-population of T-cell may be phenotypically distinct in functions, but may have same specificity for an antigenic determinant (epitope), just as the different classes of immunoglobulin molecules may have identical specificity, but different biological functions. The T-cell cytokines are soluble mediators and they exert their effect on other cells to become activated (to grow).

The two major classes of T-cells are the helper T-cells ( $T_H$  cells) expressing CD4 molecules on their surface and the cytotoxic T-cells ( $T_C$  cells) expressing CD8 molecules (Von and Mackay, 2000).

Functions of T cells:

- i. They cooperate with B-cells to enhance production of Abs – (releasing cytokines which provide various activation signals for the B-cells). Cytokines are soluble substances / mediators released by cells (eg: lymphokines for lymphocytes). The released cytokines defines the functions of helper T-cells subsets ( $TH_1$ ,  $TH_2$ ). A group of low molecular weight cytokines has been given the name chemokines and these play a role in inflammatory responses.
- ii. Inflammatory effect: Activation of certain T-cell subpopulation ( $T_{DTH}$ ) releases cytokines which activate more monocytes and macrophages to migrate and this leads to delayed type hypersensitivity inflammatory reactions.
- iii. Cytotoxic effects: Another subset subpopulation of T-cells (T-cytotoxic =  $T_C$  cells) delivers a lethal hit on their target Ags (cytotoxic killer cells). This group expresses  $CD_8$  on their membranes and thus, can also be  $CD_{8+}$  cells.

- iv. Regulatory effects: The released cytokines defines the regulatory functions of helper T-cells subsets ( regulatory or suppressor T-cells). However, the TH<sub>1</sub> can negatively cross regulate TH<sub>2</sub> cells and vice versa; another regulatory or suppressor T-cells subset can co-express CD<sub>4</sub> and CD<sub>25</sub> (interleukin 2 receptor  $\alpha$ -chain). CD<sub>4+</sub> and CD<sub>25+</sub> play regulatory role and suppress autoimmunity.
- v. Cytokines effects: The many different cytokines released by the T-cells communicate and collaborate with many cell types of the immune system (Von and Mackay, 2000).

In all, the response to any particular pathogen is a complex interaction of innate, humoral and cellular immunity component arms of the immune system that ensure both the survival and protection of host from immune response against self. In general, the cellular component elements of immunity are majorly the white blood cells (Agerberth and Gudmundsson, 2006).

#### **2.2.6 White blood cells (WBCs, leukocytes or leucocytes)**

Leukocyte is a Greek word (leuco = white, kytos = hollow vessel and cytes = cell). The many WBCs are distinct in form and function, but are majorly distinguished by the presence or absence of granules (granulocytes or agranulocytes) (Handin *et al.*, 2003).

Granulocytes (polymorphonuclear leucocytes) are leucocytes whose cytoplasm in addition to lysosomes, contain different staining granules (lysozyme) – a membrane

bound enzyme that digests endocytosed particles. The three types of granulocytes include neutrophils, basophils and eosinophils named according to their staining properties (Handin *et al.*, 2003).

The WBCs are cells of the immune system that are found throughout the body including the blood and lymphatic system and they live for about 3-4 days in an average human body. Some act as phagocytes, ingesting bacteria, viruses and cellular debris, while others release enzymatic granules that damage pathogenic invaders (Bruce *et al.*, 2002). The number of leucocytes in the blood is often an indicator of disease, but normally it is approximately 7000/ $\mu\text{L}$  of blood – making up approximately 1% of the total blood volume in a healthy adult. The changes in the physical properties (volume, conductivity, granularity and so forth) of leucocytes are often due to activation, presence of immature cells or of malignancy as in leukemia. WBCs are the thin typical white layer of nucleated cells settled in the buffy coat and seen between the sedimented red blood cells and the blood plasma following centrifugation of whole blood. The buffy coat may sometimes be green if there are large amounts of neutrophils in the sample, due to its haem-containing enzyme (myeloperoxidase) (Handin *et al.*, 2003).

Agranulocytes (mononuclear leucocytes) are leucocytes whose cytoplasm do not contain granules, but have granule-like lysosomes. The three types of agranulocytes are lymphocytes, monocytes and macrophages (Thomas *et al.*, 2003).

Neutrophils: usually appear in large numbers at sites of bacterial and fungal infections, and their phagocytic activity and death forms pus. Thus, they are seen in large numbers in the pus of wounds. These cells are not able to renew their lysosomes used in

digesting microbes and so they often die after each activity. They make up 60-70% of total leucocytes count in human blood (Thomas *et al.*, 2003). The life span of a circulating human neutrophil is about 5.4 days. They have multi-lobed nucleus and fine pale lilac granules. In general, a healthy blood contains about 1,500 – 7000 neutrophils per mm<sup>3</sup> (1.5 – 7.0 x 10<sup>9</sup>/L). Reduction in this level of neutrophil (neutropenia) leads to greater risk of susceptibility to microbial infections especially of bacterial (Buttarelo and Plebani, 2008).

Eosinophil granulocytes: Their granules are pink-orange in colour with eosin stain and their nucleus bi-lobed. They deal primarily with parasitic infections and are the predominant inflammatory cells in allergic reactions (Abbas and Lichtman, 2003).

Basophil granulocytes: The granules are coarse, large and blue; and the nucleus bi or tri-lobed. These are chiefly responsible for allergic and antigen response release of histamine that cause vasodilation (Abbas and Lichtman, 2003).

Lymphocytes: are much more common in the lymphatic system than in blood and include:

1. B-cells: which make antibodies that can bind to pathogens, block pathogen invasion, activate the complement system, and enhance pathogen destruction
2. T-cells: They include:
  - i. CD4+ helper T cells: these are T cells that display co-receptor of T-cell receptors and CD4 molecules that in combination bind antigenic peptides. Helper T-cells make cytokines and perform other functions that help coordinate the immune response and they are of  $\alpha\beta$ -TCR.

- ii. CD8<sup>+</sup> cytotoxic T-cells: these are T-cells that display co-receptor CD8 both of which bind virus-infected or tumour cells and kill them. They are also of  $\alpha\beta$ -TCR.
- iii.  $\gamma\delta$  T cells are commonly found in tissues than in blood. They have some characteristics of the other T-cells (helper T cells and cytotoxic T cells as well as of natural killer cells).
- iv. Natural killer cells: are those that kill body cells that do not either display MHC class I molecules or their stress markers like MHC class I polypeptide-related sequence A (MIC-A). Cancerous cells and cells infected by a virus show or express low MHC class I, and high MIC-A (Abbas and Lichtman, 2003).

Monocytes: share the phagocytic function of neutrophils and because of its extra role of presenting pieces of pathogens to T- cells to be recognized again and killed by mounting Ab, it is often regarded as the vacuum cleaner. Unlike neutrophils, monocytes are able to replace their lysosomal contents and are thought to have a much longer active life. Monocytes eventually leave the bloodstream and become tissue macrophages, which remove dead cell debris as well as attacking microorganisms (Thomas *et al.*, 2003).

Macrophages: - Once monocytes move from the bloodstream out into the body tissues, they undergo changes (differentiation) allowing phagocytosis and are then known as macrophages. Very often other leukocytes migrate from blood into the tissues of the body to take up a permanent residence, and these cells most times have specific names depending upon which tissue they settle in; for instance, the fixed macrophages in the liver is known as kupffer cells and other such cells include histocytes, dendritic cells

(that often migrate to local lymph nodes upon ingesting antigen), mast cells, and microglia cells (Thomas *et al.*, 2003).

### **2.2.7 Vascular tissues response in immune regulation**

Normally, the vascular tissues' response to remove harmful stimuli or initiate a healing process is via inflammation and such developed inflammatory responses are usually maintained by inflammatory mediators released by Leukocytes. Leukocytes normally reside in blood, but tend to move into the inflamed tissue via extravasations to aid in inflammation (Ferrero-Miliani *et al.*, 2007). Due to the central role of leukocytes in the development and propagation of inflammation, defects in leucocyte function often result in a decreased capacity for inflammatory defense with subsequent vulnerability to infections. Dysfunctional leucocytes may be unable to correctly bind to blood vessels due to surface receptor mutations (causing increase in bacterial infection) due to inability to digest bacteria (chediak-Higashi syndrome), or produce microbodies (granulomatous disease). In addition, diseases affecting the bone marrow may result in abnormal or few leucocytes. Infection-triggered inflammation often affects the number of leucocytes present in the body resulting in either increase (Leucocytosis) especially of immature cells or decrease (Leucopenia) depending on the types of infection. Bacterial infection usually results in an increase of neutrophils creating neutrophilia, whereas diseases such as asthma, hay fever, and parasite infestation result in an increase in eosinophils (eosinophilia). Viral infection, rickettsia infection, some protozoa, tuberculosis, and some cancers can induce leucopenia (Ferrero-Miliani *et al.*, 2007).

The Qualitative disorders of white blood cells are those, in which the number of white blood cells is normal, but the cells do not function normally, but there are proliferative

conditions and leucopenia which are quantitative. In the proliferative disorders, there is an increase in the number (leucocytosis) of white blood cells in circulation, which is commonly of reactive especially to infection (due to infection); but may also be of cancerous, while in leucopenia, there is a decrease in the number of white blood cells (Carol, 2007). Neutropenia is an immunodeficiency condition of abnormally reduced number of neutrophils. Leucocytosis is most commonly caused by inflammation as a result of increased production in bone marrow, increased release from storage, decreased attachment to veins and arteries or decreased uptake by tissues. Leucocytosis may affect one or more cell lines and can be neutrophilic, eosinophilic, basophilic, monocytosis or lymphocytosis (Handin *et al.*, 2003).

Neutrophilia is an increase in the absolute neutrophil count in the peripheral circulation and may result from bacterial infection. Normal blood values vary by age. Neutrophilia can be caused by a direct problem with blood cells (primary disease) or as a consequence of an underlying disease (secondary). Most cases of neutrophilia are secondary to inflammation and neutrophils are the most common cell types seen in the early stages of acute inflammation (Handin *et al.*, 2003).

Eosinophilia: - A normal eosinophil count is considered to be less than  $0.65 \times 10^9/L$ . Eosinophils are higher in newborns and vary with age, time (lower in the morning and higher at night), exercise, environment, and exposure to allergens. Eosinophilia is never a common laboratory finding, but the most important causes of eosinophilia include allergens such as asthma, hay fever, and hives of parasitic infections (Handin *et al.*, 2003).

### 2.2.8 Leucopenia

A range of disorders can cause decrease in the WBCs usually of the neutrophil. In this case, the decrease may be called neutropenia or granulocytopenia (decrease in the absolute neutrophil count - ANC). Less commonly, a decrease in lymphocytes (called lymphocytopenia or lymphopenia) may be seen. Neutropenia is an immunodeficiency condition often resulting in susceptibility to bacterial infections and / or sepsis. Neutropenia occurs if neutrophil count falls below 1,000 cells per  $\mu\text{L}$  of blood and it can be acquired or intrinsic. A decreased level of neutrophils is often due to either decreased production by the bone marrow or increased removal from the blood and which may be as a result of medication (chemotherapy with certain drugs), toxins (like alcohol, benzenes), immune dysfunction (like disorders of collagen, AIDS, rheumatoid arthritis), blood cell dysfunction (marrow failure, acute leukemia, megaloblastic anaemia), any major infection, starvation or hypersplenism. The ANC varies widely in healthy individuals according to exercise, emotional state, and time of day, but it is normally greater than  $1.5 \times 10^9/\text{L}$ . The absolute neutrophil count (ANC) determines the stage or severity of neutropenia. ANC count ranges from 1.0 to  $1.5 \times 10^9/\text{L}$  in mild neutropenia; 0.5 to  $1.0 \times 10^9/\text{L}$  in moderate neutropenia and is  $0.5 \times 10^9/\text{L}$  in severe neutropenia (Algwaiz and Babay, 2007). Mean neutrophil levels are the same for healthy children and adults, but an increased count of neutrophils (neutrophilia) greater than  $10 \times 10^9/\text{L}$  is frequent at birth, and decreases in the first few days of life to the range of 2.0 to  $7.0 \times 10^9/\text{L}$  to become normal by 5 years of age. Neutropenia can also be classified into acute and chronic depending on the duration of illness, but prompt medical attention most times reverses the acute (less than 3 months) type, but if left unattended, the chronic condition of neutropenic sepsis sets in. Neutrophils play an important role in protecting the oral mucosa from bacterial infections, and thus, in

chronic neutropenia, gingivitis and mouth ulcerations are common problems of substantial risk of bacterial infections. Usually the bacterial organisms of infection are those of endogenous flora, the resident bacteria of the mouth, oropharynx, gastrointestinal tract and skin. Thus, in neutropenic conditions, symptoms like otitis media, tonsillitis, sore throat, mouth ulcers, gum infections and skin abscesses are common presentations and indeed most feverish conditions of body temperature above 38.5°C/101.3°F are often related to low neutrophil count. The common bacteremia organisms are *Escherichia. coli*, *Klebsiella pneumoniae*, and *Staphylococcus. aureus* (Bert *et al.*, 2005).

Chemotherapy-induced neutropenia is the most common cause of acquired neutropenia resulting from symptoms of medication overdose or toxicity. Chemotherapy-induced febrile neutropenia especially of cytotoxic drugs is of common occurrence. One severe consequence of neutropenia is that it can increase the risk of infection. Over the years, the incidence of idiosyncratic drug-induced agranulocytosis or acute neutropenia has remained stable at 2.4 – 15.4 cases per million (Andres *et al.*, 2006). Drug-induced agranulocytosis remains a serious adverse event due to the occurrence of severe sepsis with severe deep infections such as pneumonia, septicemia and septic shock (Bruce *et al.*, 2002).

### **2.3 Inflammation**

Inflammation is part of the complex biological response of the vascular tissues to harmful stimuli (pathogens, damaged cells, irritants and foreign bodies) (Ferrero-Miliani *et al.*, 2007). It is a normal body response in attempt to protect against or remove injurious stimuli or more commonly to initiate a healing process as to maintain

the well-being of body cells. Thus, inflammation is a key biochemical process and a mechanism of innate immunity (inborn protective process) of the immune system. Indeed, we live in such an inflammatory environment (from pollution, germs, diet and other sources) that it is tough to keep the inflammation process in balance. Inflammation that are not well controlled and which overwhelm the immune system often becomes chronic resulting in deadly or fatal conditions and this is often the underlying cause of many human diseases (Carol, 2007). However, abnormality in the inflammatory processes devoid of immune system involvement also occurs and these mediate some peculiar types of disease conditions such as cancer, atherosclerosis, ischaemic heart disease and so forth, and are often regarded as non-immune or maladaptive responses (Allison *et al.*, 2013).

Some immune system disorders also result in inflammatory disorders, for instance allergic reactions like hay fever, results from an inappropriate immune response-triggering inflammation. Hay fever is a hypersensitive response by nose mast cells to allergens in which pre-sensitized mast cells respond by degranulating and releasing vasoactive chemicals such as histamine which propagate excessive inflammatory response that may mature into a systemic response known as anaphylaxis. The other types of hypersensitivity reactions (Types II and III) are mediated by antibody reactions and induce inflammation by attracting leukocytes that damage surrounding tissues. Again inflammatory myopathies are caused by the immune system inappropriately attacking components of muscle and leading to signs of muscle inflammation and this may occur in conjunction with other immune disorders like systemic sclerosis (Ariza *et al.*, 2014).

### **2.3.1 Acute and chronic inflammations**

Inflammation can be classified as either acute or chronic. In its acute form, certain body tissues are momentarily destroyed with offending agents, but which gets back to normal after a while. However, progressive inflammation that gets chronic can lead to a host of diseases. In general, acute inflammation is mediated by granulocytes, where as chronic inflammation is mediated by mononuclear cells such as monocytes and lymphocytes (Eming *et al.*, 2007).

### **2.3.2 Acute inflammation**

Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. The causative agents include bacterial and other pathogens, injured tissues and so forth. The major cells involved in acute inflammation are neutrophils (primarily), basophils and eosinophils (in response to helminth worms and parasites). The primary mediators are mostly the vasoactive amines and eicosanoids; and the onset is immediate with few days of effect. Acute inflammation that does not result in the resolution of the injurious harm may result in abscess formation and/or chronic inflammation. Since the acute inflammation requires constant stimulation to be sustained; and its mediators have short half lives that quickly get degraded in the tissue, the process ceases as soon as the stimulus is removed (Serhan, 2008).

### **2.3.3 Symptoms of acute inflammation**

Acute inflammation is a short term process, usually appearing within a few minutes or hours and ceasing upon the removal of the injurious stimuli. The five classic signs and

symptoms of acute inflammation are redness, swelling, heat, pain and loss of function. The first and second signs are due to increased blood flow to the inflamed site, swelling is caused by accumulation of fluid; pain is due to release of chemicals that stimulate nerve endings, and happens only where the appropriate sensory nerve endings exist in the inflamed area; if there is no pain-sensitive nerve endings, pain would not occur as seen with the acute inflammation of the lung (pneumonia) which cause pain only when the parietal pleura is involved. Loss of function is not unique to inflammation and has multiple causes (Kawai and Akira, 2006).

#### **2.3.4 Process of acute inflammation/cell-derived mediators**

The cells in tissues (resident macrophages, dendritic cells, hepatocytes, kupffer cells, mastocytes) initiates the process of acute inflammation because they bear certain receptors (pattern recognition receptors PRRs) on their surfaces which recognize Ag/pathogen molecules and which are not of the host (referred to as pathogen-associated molecular patterns - PAMPs). In the event of infection, burn or other injuries, these cells are activated to release inflammatory mediators which bring up these clinical signs of inflammation. Vasodilation with its increased blood flow causes the redness and heat; increased permeability of the blood vessels with exudation/passage of plasma proteins and fluid into the site (edema or swelling); some released mediators like bradykinin increase the sensitivity to pain (hyperalgesia). The leukocytes especially neutrophils also migrate from blood vessels into the site (extravasation). In addition to cell-derived mediators, several acellular biochemical events occur to propagate the inflammatory response. In case of bacterial infection, the complement system is activated, while if it is burns or trauma, necrosis activate the coagulation and fibrinolysis systems (Daniel and Russian, 2012).

### **2.3.5 Chronic inflammation**

This results when acute inflammation is prolonged and it is due to a progressive shift in the cell types that infiltrate the site of inflammation and which in addition to healing attempts also destroy tissues. Thus, persistent acute inflammation due to non-degradable pathogens, viral infections, persistent foreign bodies or autoimmune reactions causes chronic inflammation (Ferrero-Miliani *et al.*, 2007). The major cells involved in chronic inflammation are mononuclear cells (monocytes, macrophages, lymphocytes, plasma cells and fibroblasts). The primary mediators of the chronic inflammation are the Interferon gamma (IFN- $\gamma$ ) and other cytokines, growth factors, reactive oxygen species and hydrolytic enzymes and the onset is delayed with effect from days, months or years and often results in tissue destruction, fibrosis and necrosis. When inflammation overwhelms the host, systemic inflammatory response syndrome is diagnosed (Tsiotou *et al.*, 2005).

### **2.3.6 Systemic inflammatory Immune response syndromes**

In addition to the quantitative changes in the number of leucocytes, septic bacterial infections, peritonitis, hypersensitivity reactions especially of the delayed type etc. are all systemic inflammatory immune response syndromes resulting from chronic inflammation. The increase in the number of white blood cells (leucocytosis) in circulation is commonly of reactive especially to infection; and in most cases is secondary to inflammation. However, neutropenia which is an immunodeficiency condition often seen in septic bacterial infections could be due either to decreased

production by the bone marrow or increased removal, being that neutrophils are not able to renew their lysosomes used in digesting microbes and so often die after each activity (Carol, 2007).

#### 2.3.6.1 Sepsis

Most times, septic conditions are variously described as bacteremia or septicaemia (blood poisoning). Severe sepsis denotes organ dysfunction distant from the site of infection (renal, cardiac, respiratory or brain) or hypotension (systolic < 90 mmHg or mean BP < 70) and is characterized by elevated heart rate (tachycardia greater than 90 beats/ min at rest), abnormal body temperature of either high (>100.4°F or 38°C) or low (< 96.8°F or 36°C), abnormal white blood cells count of >12000 cells/ $\mu$ L ( $\text{mm}^3$ ) or <4000 cells/ $\mu$ L or <10% immature (band) forms; and increased respiratory rate of >20 breath/min (Pavlidis, 2003). Complications of sepsis include – disturbed blood clotting, injury to the walls of blood vessels or endothelial system, excessive factors of infections such as tumour necrositic factor in blood, excessive cell death (apoptosis) that also contributes to low count of lymphocytes and endothelial cells, excessive activity of neutrophils, lack of blood sugar control and low levels of steroid hormones (Forceville *et al.*, 2014). Usually it is the chemicals released into the blood to fight the septic infection that cause inflammation over the entire body. Infection-triggered inflammation is termed sepsis, with the terms bacteremia being applied specifically for bacterial sepsis, septicaemia, if it is of blood and viremia specifically to viral sepsis. Such inflammatory conditions cause formation of tiny blood clots and which block oxygen from vital organs (Bruce *et al.*, 2002; Tsiotou *et al.*, 2005). Vasodilatation, organ dysfunction and tissue damages are serious problems associated with widespread infection-triggered chronic inflammations that often lead to septic shock and death

(Habashy *et al.*, 2005). The risk of sepsis is more in conditions of weakened immune system. Inflammation-associated sepsis affect millions of people around the world and kill more than 1 in 4 people who contract it; thus it is a leading cause of death in the developing countries of the world because of its high mortality rate which accounts for 60-70% death per year (Dellinger, 2003).

#### 2.3.6.2 *Peritonitis*

Peritonitis is an inflammatory response to peritoneal injury, most often caused by introduction of an infection by foreign bodies like irritants or bacterial organisms into an otherwise sterile peritoneal environment or by a pathological process in the visceral, such as an organ perforation of a hollow viscus or other abdominal pathology; and most times, it is associated with severe septic shock (Bruce *et al.*, 2002). Injury to the peritoneum could result from an influx of protein rich fluid, activation of the complement cascade, up-regulation of peritoneal mesothelial cell activity and/or invasion of the peritoneum with polymorphonuclear neutrophils and macrophages in an attempt to stimulate cytokine and chemokine production (Gupta and Kaushik, 2006). Peritonitis is traditionally classified as primary, secondary and tertiary. Primary peritonitis results from spontaneous bacterial infection of the peritoneum, while secondary peritonitis relates to a pathologic process in a visceral organ, such as perforated appendicitis, gastric or duodenal ulcer and so forth whereas tertiary peritonitis is a secondary peritonitis that fails to resolve despite what appears to be appropriate measures and it often results to multi-organ failure (Forceville *et al.*, 2014). The form most commonly encountered is the secondary peritonitis resulting from a pathological process in the visceral, such as organ perforation of a hollow viscus or other abdominal pathology. The pathogenesis of intra-abdominal infections stems from

bacterial contamination, the stimulus of which leads to an activation response that triggers the mesothelial cells to react and intersperse among peritoneal macrophages, endothelial cells and extra- and intra- vascular leukocytes (Pavlidis, 2003). The local consequences of this activation are the transmigration of granulocytes from peritoneal capillaries to the mesothelial surface to cause dilatation of peritoneal blood vessels for enhanced permeability, peritoneal edema and formation of protein-rich peritoneal exudates. The acute inflammatory process within the abdomen results in sympathetic activation, and suppression of intestinal peristalsis, or ileus, impaired fluid absorption through the walls of the bowel, and sequestration of tissue fluid within the gut lumen associated with hypovolemia. Reduced intestinal peristalsis promotes microbial overgrowth, translocation of bacteria and their products from the gut lumen into regional nodes, the peritoneal cavity, and portal circulation (Marshall, 2004). Such inflammatory conditions cause formation of tiny blood clots and which block oxygen from vital organs and lead to septic shock, organ failure and threatening drop in blood pressure. Septic shock is sepsis with hypotension unresponsive to fluid administration and requiring pressor agents (Bruce *et al.*, 2002).

### **2.3.7. Hypersensitivity reactions**

There are instances in which the power of the immune response, although directed against innocuous foreign substances (some medications, inhaled pollen particles, or substances of insect bites) produces a response that may result in severe pathologic consequences and even death. These responses are known collectively as hypersensitivity reactions (Rajan, 2003).

Hypersensitivity reactions are categorized in accordance with the effectors involved. One category is antibody-mediated and may be passively transferred to another individual by the appropriate numbers and type of antibody in serum. This group is in turn divided into three classes depending on the specific underlying mechanisms involved that could be: mast cells, complement or neutrophils. These reactions have in common a rapidity of response that can range from minutes to a few hours following the exposure to antigen and are therefore generally called the immediate type hypersensitivity reactions (Kindt *et al.*, 2006).

The second major category of hypersensitivity reactions is mediated largely by T-cells with consequent involvement of monocytes and is appropriately termed T-cell mediated immunity, but because the responses from this are much more delayed in appearance, generally taking 18-24 hours to reach their full expression, they have been traditionally referred to as delayed type hypersensitivity (DTH). Unlike antibody-mediated hypersensitivity, which can be transferred from a sensitive individual to a nonsensitive individual via serum, DTH may only be transferred by T- cells (Kindt *et al.*, 2006).

### **2.3.8. Forms of hypersensitivity reactions**

2.3.8.1. *Type I hypersensitivity reactions (immediate, allergy):* Mediated by IgE and IgG. This is characterized by fast responses which occur in minutes when Ags cross-links the IgE on mast cells and basophils such as hives, eczema, red and itching eyes, rhinitis, asthma, anaphylaxis, atopy. Skin test for specific IgE detects type I hypersensitivity reaction.

2.3.8.2. *Type II (cytotoxic, antibody dependent) hypersensitivity:* Mediated by IgM or IgG, Complement proteins and Membrane attack complex (MAC). This

occurs when IgM or IgG binds on a host cell that is perceived by the immune system as foreign (Ag) leading to cellular destruction via the MAC activation. Some drug reactions, non-matched blood transfusions; Myasthenia gravis, membranous nephropathy, erythroblastosis foetalis, thrombocytopenia, hemolytic anaemia and so forth are all type II hypersensitivity reaction. Direct and indirect Coombs test is use for detection.

**2.3.8.3.**        *Type III (immune complex disease) hypersensitivity:* Mediated by IgG, Complement and neutrophils. The binding of IgG to soluble antigen forms a circulating immune complex that is deposited in the vessel walls of the joints and kidneys to initiate a local inflammatory reaction. Rheumatoid arthritis, lupus nephritis, and systemic lupus erythematosus (SLE), serum sickness, post streptococcal glomerulonephritis and so forth are all forms of type III hypersensitivity reaction.

**2.3.8.4.**        *Type IV (Delayed Type hypersensitivity –DTH) hypersensitivity:* Cell mediated immune memory response, antibody independent) reaction: Common delayed hypersensitivity reactions include : Many skin rashes following exposure to poison oak or ivy, heavy metals in jewelry or cosmetics products as well as red indurations after exposure to purified protein derivative (PPD) especially during tuberculosis skin test, contact dermatitis, multiple sclerosis, chronic transplant rejection and so forth are all mediated by T-cells which on detecting an Ag, activates macrophages.

Delayed-type hypersensitivity (DTH = T-cell mediated or type IV hypersensitivity) is a type of an immune response whereby the antigen that triggers the response is specific for T-cells. When a responding T-cell is activated by its antigen, large amounts of

cytokines are released and which in turn may attract and activate other mononuclear cells (monocytes and macrophages) that are not antigen-specific and thus causing other reactions including deleterious reactions. The antigens may be an allograft, parasites, soluble proteins or chemicals that couple to body proteins. The clinical feature of DTH depends on the antigen and the route of its entrance. The major events that lead to DTH include:

- i. Activation of antigen specific inflammatory TH1 cells in a previously sensitized host.
- ii. Elaboration of pro-inflammatory cytokines by the antigen-specific TH1 cells.
- iii. Recruitment and activation of antigen-nonspecific inflammatory leucocytes.

These events occur over a period of several days (24-72 hr) hence the term delayed type hypersensitivity. This time course characteristically distinguishes DTH from antibody-mediated reactions which appear much more quickly (Lawrence and Ali, 2014).

**Mechanisms of DTH:** previous exposure (sensitization stage) to the antigen is required to generate DTH and which activates and expands the number of antigen-specific memory TH1 cells such that subsequent challenge with same antigen (elicitation stage) produces a DTH reaction. The sensitization stage typically occurs over a 1-2 wks period during which activation of T cells occurs. The elicitation stage takes approximately 24-72 hr from the time of antigen challenge to recruit and activate these cells – a period that culminates in the histological and clinical DTH (Lawrence and Ali, 2014).

The clinical manifestations of DTH can last for several weeks and in some cases can be chronic. The antigen-challenged TH1 cells produce several cytokines during the

elicitation stage, most notably, chemokines and IFN- $\gamma$  which causes chemotaxis and activation of macrophages. Another cytokine produced by these cells is interleukin -12 (IL-12). This suppresses the TH2 subpopulation and promotes the expansion of the TH1 subpopulation, driving the response to produce more TH1 synthesized cytokines that activate macrophages. DTH reactions also involve CD8+Tcells which are first activated and expanded during the sensitization stage. These cells can damage tissues by cell-mediated cytotoxicity (Lawrence and Ali, 2014).

In the most favourable circumstances, DTH results in destruction of an infectious organism that may have elicited the response in the first place, typically by macrophages ingestion. However, macrophage activation by IFN- $\gamma$  and its degradation by lysosomal enzymes are associated with the release toxins such as the by-products of respiratory activity (peroxide and superoxide radicals) which as well also results in tissue destruction (Lawrence and Ali, 2014).

2.3.8.5. *Type V (autoimmune disease, receptor mediated) reaction:* This as with Type II is mediated by IgM or IgG and complement; but unlike Type II in which the antibodies bind to cell surface components, here, they bind to the cell surface receptors to prevent the binding of the normal ligands of such receptors and in mimicking the effects of the ligands they impair the cell signaling. The disorders include Graves' disease and Myasthenia gravis. The use of Type V is rare as it is often included in Type II (Abbas and Lichtman, 2003).

## **2.4 The Models Used in this Study**

### **2.4.1 Cyclophosphamide induced neutropenia in rats**

Cyclophosphamide induced neutropenia in experimental animal models is an in-vivo method often used to check the effects of drugs on the blood forming (haematopoetic) system (Diwanay *et al.*, 2004). Cyclophosphamide is a prodrug metabolized into active alkylating species by hepatic cytochrome p-450 enzymes and the produced alkylating species then bind to DNA (Deoxyribonucleic Acid) to form cross-link that induce strand breakage and thus kill actively replicating cells. Thus, at high doses it is a potent suppressor of the immune function that results in sustained decrease in both the number and function of T and B cells (Cupps *et al.*, 1982; Diwanay *et al.*, 2004). A decrease in white blood cells due to Cyclophosphamide can be reduced or inhibited by agents that boost the immune system and so is often used to evaluate the immunomodulatory activity of drugs (Thatte *et al.*, 1987).

#### **2.4.2 *Escherichia coli*-Induced abdominal sepsis**

*Escherichia coli* is one of the major pathogens involved in septic conditions and *E.coli* peritonitis is a life threatening disease. *E.coli* often attacks the linings of the peritoneum causing tissue degradation, damage and release of protease enzymes which often activates the leucocytes to produce inflammatory host responses. *E.coli* strength of  $2.5 \times 10^8$  cells/ml had been reported to induce 100% mortality in mice due to sepsis (Pallable *et al.*, 1998). Agents that have the ability to enhance the production of antibodies usually counter the pathogenic effects due to *E.coli* infections by producing adequate host defense (Bruce *et al.*, 2002).

#### **2.4.3 Antigen-antibody response effect** (Antibody response to sheep red blood cell model): This model is widely used to evaluate delayed hypersensitivity reactions (an exaggerated immune reaction to pathogens). An immediate hypersensitivity reaction

appears in the form of wheel and swelling (erythema) within few minutes of pathogenic invasion by interacting with cell fixed antibodies usually IgE; while the delayed hypersensitivity reactions occurs in form of necrosis within 18 to 24 hr resulting from the interaction of circulating complement fixing antibodies (IgG or IgM). Delayed hypersensitivity reactions are usually maximal at 48 to 72 hr of pathogenic invasion. Usually, lymphocytes infiltration occurs within 4-6 hrs following pathogenic invasion and leading to the production of cytokines (Doherty, 1981).

## CHAPTER THREE

### 3.0 MATERIALS AND METHODS

#### 3.1 Materials

##### 3.1.1 Collection and Identification of Plant Material

Fresh leaves of *Combretum micranthum* were collected in September, 2011 from Malumfashi LGA of Katsina State and were identified and authenticated on 21<sup>st</sup> September 2011 by Mallam Umar Shehu Gallah, of the Herbarium unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria with an existing voucher number of 900257.

##### 3.1.2 Experimental Animals

Adult albino mice (18-25 g) and Wistar rats (110-250 g) of both sexes were used throughout the study. The animals were obtained from the animal house of the Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. The animals were allowed to acclimatize to laboratory conditions for two weeks with food and water provided *ad-libitum*.

**3.1.3 Microorganisms:** standard isolates of *Escherichia coli* (ATCC 11775) and *Staphylococcus aureus* ATCC 021001 and clinical isolates of *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus mirabilis* were obtained from the Department of Pharmaceutics and Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria.

**3.1.4 Sheep red blood cells:** Red blood cells were harvested from the sheep of Uda breed from the National Animal Production Research Institute (NAPRI), Ahmadu Bello University, Zaria.

### **3.1.5 Equipments**

Weighing balance (Wet. Avery Ltd, Birmingham, England)

Swelab machine (Boule Medical AB, XE-12613 Stockholm, Sweden)

Heamatocrit centrifuge (Denley, BS400, UK)

Digital calipers (United Precision Machine - UPR<sup>®</sup> Inc., China)

Mortar and Pestle, refrigerator spatula, animal cages, animal feeds, needles and syringes, cotton wool, cannular, scissors, evacuated (EDTA) tubes and other sample bottles, capillary tubes, marker and masking tape were used during the investigation.

### **3.1.6 Drugs, Chemicals and Solvents**

Cyclophosphamide injection 500 mg (Neon laboratories Ltd India). Batch Number-29206, Manufacturing Date- DEC-2012, Expiry Date-NOV-2013

Dexamethasone 1mg/tablet (Jianasu Pengyao Pharmaceutical Inc. China). Batch Number-0401, Manufacturing Date-04/2012, Expiry Date-04/2015

Ethanol (BDH Chemical Ltd Poole England)

Distilled water

Normal saline (0.9 g NaCl in 100 ml of Distilled Water)

Nutrient Broth (Oxoid Ltd England)

## **3.2 Methods**

### **3.2.1 Preparation of plant extract**

The leaves of *Combretum micranthum* were dried under shade for a period of 4 weeks until a constant weight was obtained and then crushed into powder form. The powdered material (500 g) obtained was packed into a filter thimble and soaked with 70% aqueous ethanol for 72 hours after which the mixture was filtered to obtain liquid extract. The filtrate was then evaporated to dryness using a rotary evaporator set at 55°C. The obtained dark-green ethanol extract was then stored in an airtight container until needed.

### **3.2.2 Validation of the phytochemical constituents**

The method of Evans (2002) was used to screen the extract for the previously reported constituents including carbohydrates, alkaloids, cardiac and steroidal glycosides, anthraquinones, steroids, saponins, flavonoids and tannins as outline below:

#### *3.2.2.1 Carbohydrates (Molisch'reagent test)*

Few drops of molisch reagent were added to 0.2g of extract dissolved in 5 ml of water in a test tube and concentrated H<sub>2</sub>SO<sub>4</sub> (1 ml) was added down the test tube side. Formation of a purple ring coloured layer interphase beneath the aqueous layer confirms the presence of carbohydrates (Evans, 2002).

#### *3.2.2.2 Alkaloids*

The extract 0.5g dissolved in 5 ml of water were mixed with equal volume of 1% aqueous hydrochloric acid (HCl), stirred over water bath for 3 minutes and filtered; 1ml of the filtrate was mixed with 1ml of Mayer's reagent; and another 1ml of the filtrate was mixed with Wagner's reagent; and yet another 1ml treated with Drangendorff's reagent. The mixtures were checked for a white precipitate, brown precipitate and rose red precipitate respectively that indicate the presence of alkaloids (Evans, 2002).

#### 3.2.2.3 *Cardiac glycosides*

**Kella-killiani Test:** The extract (0.5 g) was dissolved in 2 ml of glacial acetic acid, 1ml of ferric chloride solution was added; and 1ml of concentrated H<sub>2</sub>SO<sub>4</sub> was also added at an angle of 45 degree.

**Kedde's Test:** To 1ml of 2% 3, 5-dinitrobenzoic acid in 95% alcohol was added a little portion of extract and 5% sodium hydroxide and mixed. Both tests were checked for a purple ring interphase and a purple blue colour respectively that indicate presence of cardiac glycosides (Evans, 2002).

#### 3.2.2.4 *Test for Steroid*

The extract 0.5g was dissolved in 2 ml chloroform and filtered. To the filtrate, concentrated H<sub>2</sub>SO<sub>4</sub> acid (1 ml) in a pipette was added at the bottom of the test tube for Salkowsky reaction. Separation of the two liquids by a reddish brown ring colour indicates the presence of steroids (Evans, 2002).

**Liebermann-Burchard's Test:** 0.5g of the extract was dissolved in 2 ml of chloroform, 2 ml of acetic acid anhydride was added and mixed gently. 1 ml of concentrated sulphuric

acid was added down the side of the test tube. Blue-green colour in the upper layer indicates the presence of triterpens (Evans, 2002).

#### 3.2.2.5 *Anthraquinones*

Borntreger's Test: The extract (3 g) was dissolved with 10 ml of benzene and filtered to obtain the filtrate which was mixed with 5 ml of 10% ammonia solution. The mixture was shaken and the presence of a red colour in the ammonia (lower) phase indicates the presence of free hydroxyl anthraquinones (Evans, 2002).

For combined anthraquinones, 1 g of the extract was boiled with 5 ml of 10% hydrochloric acid for 3 minutes and filtered hot and then allowed to cool. The filtrate was mixed with 5 ml of benzene and pipetted off and half its volume was mixed with 10% ammonia solution and shaken gently. A red or pink colour in the ammonia (lower layer) phase indicates the presence of combined anthraquinones (Evans, 2002).

#### 3.2.2.5 *Saponins* (frothing test)

The extract (0.5g) was dissolved in 10 ml of distilled water and shaken vigorously for 30 seconds and allowed to stand for 30 minutes. A honey comb froth that persisted for 15 minutes indicates the presence of saponin (Evans, 2002).

#### 3.2.2.6 *Flavonoids*

Shinoda Test: The extract (0.2 g) dissolved in 2 ml of methanol and warmed over water bath was filtered. Magnesium metals (4-5 pieces) and few drops of concentrated hydrochloric acid (HCl) were added to the filtrate and observed for the pink colour of flavonoids (Evans, 2002).

Sodium Hydroxide Test: Few drops of aqueous sodium hydroxide added to 0.2g of the extract was also observed for a yellow colour of flavonoids (Evans, 2002).

#### *3.2.2.7 Tannins*

Lead sub-acetate Test: the extract (3 g) boiled with 10 ml of water was cooled and filtered. Addition of 3 drops of lead sub-acetate solution to 1ml of the filtrate was used to check for formation of a white precipitate of tannin (Evans, 2002).

Ferric chloride Test: A drop of 1% FeCl<sub>3</sub> solution added to 1 ml of the filtrate was also checked for blue-brownish precipitate of hydrolysable tannin (Evans, 2002).

### **3.2.3 Acute toxicity study**

Lorke's method of 1983 was used for the acute toxicity test. Three groups consisting of 3 per group were set up for both intraperitoneally (*i.p.*) and per-oral (*p.o.*) acute toxicity tests in mice and also repeated in rat. In the initial phase of the experiment, the extract at doses of 10, 100, 1000 mg/kg body weight was administered and observed for 24 hours for signs of changes in the behavioral pattern and/or death. The second phase was carried out based on the results of the first phase and the extract at doses of 1600, 2900, 5000 mg/kg body weight was administered through same routes to two sets of three groups consisting of one mouse in each group. The same procedure was repeated for rats; and the median lethal doses for both routes were calculated as the geometric mean of the highest non-lethal and the lowest lethal doses for both animal species.

#### **3.2.4 Cylophosphamide-induced neutropenia**

The method of Thatte, *et al.* (1987) was employed in this study. Adult Wistar rats in four groups of  $n = 5$  were treated with distilled water (1 ml/kg) and extract doses of (50, 100 and 200 mg/kg) respectively for 10 days. After the 10<sup>th</sup> day drug administration, blood samples were collected from all the animals via the tail tip in each group after which the rats were then given a neutropenic dose of 200 mg/kg cyclophosphamide subcutaneously, and kept for 3 days. Another blood sample was collected after the 3 days and the total leukocyte count (TLC) and the differential leukocyte count (DLC) where the values of White Blood Cells, Red Blood Cells, Haemoglobin, Haematocrit (%), Lymphocytes (%), Neutrophils (%), Absolute Neutrophil Count, Mean Corpuscular Haemoglobin Concentration and Platelet were obtained for both samples.

#### **3.2.5 Effect of the extract on *Escherichia coli*-induced abdominal sepsis in mice**

The experiment was conducted according to Pallable *et al.*, (1998), *Escherichia coli* strength of  $2.5 \times 10^8$  colony forming units/ml induces abdominal sepsis (localized peritonitis) that produces 100% mortality in mice. An overnight culture of a standard isolate of *Escherichia coli* was obtained whereby a medium of nutrient broth prepared by dissolving 13 g of the powder in 1 litre of distilled water was heated to boiling point for complete dissolution and then sterilized by placing it in an autoclave at 121°C for 15 minutes. Then 5 ml of the cold prepared broth was taken into a sample bottle and a loopful of the standard isolate of *Escherichia coli* (ATCC 11775) was inoculated onto it and incubated for 24 hours. Seven-fold serial dilution of this culture was made with normal saline from which the last dilution of 2.5 ml of broth and 7.5 ml of normal saline was the required *E. coli* strength of  $2.5 \times 10^8$  colony forming unit/ml for the study.

Twenty mice of both sexes were divided into 4 groups of 5 mice per group and pretreated with 10 ml/kg distilled water, 50, 100 and 200 mg/kg extract respectively for 15 days. Three hours after the 15<sup>th</sup> day pretreatment, 1 ml of the prepared *E. coli* strength was injected *i.p.* to all the groups to check for their protection from death. After 72 hr observation, the percentage mortality was calculated for all the groups. Post mortem histological examination of the *E. coli*-infected abdomen (stomach and intestinal mucosa) of one dead mouse of the control group and that of one mouse sacrificed from the extract pretreated groups (50 mg/kg) was performed to see the damages that may have occurred. The tissues from both mice were removed and fixed in 10% formalin and taken for analysis.

### **3.2.6 Effect of the extract on sheep red blood cell (SRBC)-induced delayed**

### **hypersensitivity reaction in rat**

The method of Schulten *et al.*, (2007) was used to prepare 5% v/v sheep red blood cell (SRBC) whereby whole blood collected from a sheep was centrifuged to obtain pure red blood cells. Five percent v/v SRBC was made by transferring 5 ml of the red blood cell into a beaker containing 95 ml of normal saline. The experiment was conducted according to Chang *et al.*, (1998) for induction of delayed hypersensitivity reaction. A fourteen day drug pretreatment was performed. Five (5) groups of 5 rats per group were treated orally with distilled water (1 ml/kg), extract doses of 50, 100 and 200 mg/kg and 0.2 mg/kg dexamethasone respectively. On the 7<sup>th</sup> day of pretreatment, the rats in all the groups were immunized with the SRBC (0.25 ml) *i.p* for sensitization and the treatment continued to the 14<sup>th</sup> day. Three hours after the 14<sup>th</sup> day treatment, the right hind foot-paw sizes of all the rats in all groups were measured with a vernier caliper (0 hour) and recorded as control paw volumes. The SRBC (0.25 ml) was again injected, but now into the right hind foot paw of all the rats and the time noted for each rat such that paw measurement is repeated 3 hours and 24 hours later to assess the degree of oedema.

### **3.2.7 Inhibitory effect of the extract on bacterial growth**

Agar plate dilution method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2011 was used for the antimicrobial studies of the extract on two standard organisms, *Escherichia coli* (ATCC 11775) and *Staphylococcus aureus* (ATCC 021001) and three clinical isolates, *Klebsiella pneumoniae*, *Salmonella typhi* and *Proteus mirabilis*. The Mc-Farland turbidity standard scale method for assessing microbial strength was used to obtain the strength of the organisms used for this study whereby 9.95 ml of 1% H<sub>2</sub>SO<sub>4</sub> and 0.05 ml of 1% BaCl<sub>2</sub> were mixed in a sample bottle to produce the turbid solution for cross-matching those of the organisms for similar strength. Nutrient broth (20 ml) was poured into each of 5 sterile petri-dishes and allowed to solidify, after which a loop-streak of each organism was made onto the solidified broth surface and labeled for the five organisms, being sure to sterilize the loop over bunsen burner after streak of each organism. The dishes were incubated at 37°C for 24 hours to obtain the overnight cultures of the organisms. A sterile loop was used to pick from the overnight culture of each organism and continuously stirred into a 10 ml normal saline until a similar turbid concentration as with that of the Mc-Farland turbid solution was obtained. Another 5 petri-dishes for each of the organisms were again prepared by pouring 20 ml of the previously prepared nutrient broth into each of the petri-dishes together with the 10 ml turbid solution obtained for each organism and allowed to solidify. A cork borer was used to bore 2 holes of 10 mm in each of the solidified petri-dishes and 0.1 ml extract concentrations of 100 and 200 mg/ml were dropped into the bored holes. Four standard antibiotic (Gentamycin, Ciprofloxacin, Ceftriaxone and Meropenem for *E. coli*) discs specific for each organism were also placed in the dishes which were then allowed for 2 hours at room temperature for diffusion before incubation at 37°C for 24 hours. The diameters of the zones of inhibition of the organisms were then measured and those of the antibiotic

standards compared with those of the two extract doses using the interpretation criteria of EUCAST, (2011).

### **3.2.8 Statistical analysis**

The research results obtained were expressed as mean and standard error of mean (SEM). Differences between control and treated mean values were analyzed using one way analysis of variance (ANOVA), with appropriate post hoc test for multiple comparisons and the levels of significance obtained were stated at  $P \leq 0.05$ . The results were presented in tabular form.

## **CHAPTER FOUR**

## **4.0 RESULTS**

### **4.1 Yield of the Plant Extract**

The weight of the powdered material obtained was 500g, while the weight of the concentrate was 64.6g. The calculated Percentage yield of the extract was 12.92%. This was sufficient for the entire study.

### **4.2 Acute Toxicity Study**

The LD<sub>50</sub> obtained for the aqueous ethanol leaf extract of *Combretum micranthum* were 3807.9 mg/kg per oral and 2154.1 mg/kg intraperitoneal (*i.p.*) administrations in mice, while that in rat was found to be greater than 5000 mg/kg per oral and 3807.9 mg/kg *i.p.* No observable behavioural changes were seen.

### **4.3 Preliminary Phytochemical Constituents**

The phytochemical screening of the extract of *C. micranthum* was found to contain chemical constituents as presented in Table 4.1.

**Table 4.1: Phytochemical Constituents of the Leaf Extract of *C. micranthum***

<b>Constituents</b>		<b>Remark</b>
Alkaloid	Mayer,s reagent test	Present
	Wagner,s reagent test	Present
	Drangendorff test	Present
Anthraquinone	Bornstrager,s test	Present
Carbohydrates	Molisch reagent test	Present
Cardiac glycosides	Kella-Killiani test	Present
	Kedde,s test	Present
Flavonoids	Shinoda test	Present
	Sodium Hydroxide test	Present
Saponins	Frothing test	Present
Steroids	Salkowsky test	Present
Tannins	Lead-subacetate test	Present
	Ferric Chloride test	Present
Terpenoids	Liebermann-Burchard test	Present

#### **4.4 Effect of *C. micranthum* Extract on Cyclophosphamide-Induced Neutropenia**

The extract pretreatment prior to cyclophosphamide injection showed slight, but insignificant increase in WBC count compared to the control group as presented in Table 4.2. In this study, a pronounced neutropenic effect of cyclophosphamide on haematological parameters was observed and the effect was in such a manner that the pre-count values were significantly reduced ( $P < 0.05$ ) for some (WBC, RBC, HCT, HGB and ANC), but not significantly for MCHC; while for percent (%) lymphocytes, the precount values decreased for both the control and the lowest extract-dose groups, but increased for the two higher extract-dose groups. The precount percent (%) neutrophil also increased for the control group and the first two extract-doses, but reduced for the highest dose of extract (200 mg/kg). Although the platelet also significantly decreased from their precount values, the precount value of platelet for the 200 mg/kg was quite reduced in relation to the other groups.

**Table 4.2: The Effect of Oral *C. micranthum* Pretreatment on Haematological Parameters in Rats before (Upper values) and after (Lower values) Cyclophosphamide Treatment**

Haematological Indices	Mean $\pm$ SEM of the Haematological Indices			
	Distilled H <sub>2</sub> O (1ml/ kg)	Ext. 50 mg/ kg	Ext. 100 mg /kg	Ext. 200 mg / kg
WBC ( $\times 10^3$ / $\mu$ L)	9.74 $\pm$ 1.34	11.82 $\pm$ 1.32	13.70 $\pm$ 2.28	14.20 $\pm$ 1.98
	1.92 $\pm$ 0.40***	1.78 $\pm$ 0.60***	2.88 $\pm$ 1.25**	3.20 $\pm$ 0.42***
RBC ( $\times 10^6$ / $\mu$ L)	7.65 $\pm$ 0.18	7.28 $\pm$ 0.23	7.86 $\pm$ 0.12	7.69 $\pm$ 0.21
	6.19 $\pm$ 0.14***	5.91 $\pm$ 0.39*	6.21 $\pm$ 0.29**	6.54 $\pm$ 0.18**
HCT (%)	46.10 $\pm$ 1.01	43.30 $\pm$ 1.54	47.66 $\pm$ 1.02	45.58 $\pm$ 1.21
	36.18 $\pm$ 1.14**	35.46 $\pm$ 2.25**	38.08 $\pm$ 2.06**	38.66 $\pm$ 0.89*
HGB (g / dl)	14.24 $\pm$ 0.18	13.62 $\pm$ 0.35	14.80 $\pm$ 0.17	14.26 $\pm$ 0.34
	11.26 $\pm$ 0.30**	10.82 $\pm$ 0.67**	11.54 $\pm$ 0.51**	11.66 $\pm$ 0.27**
% lymphocytes	71.76 $\pm$ 3.07	71.46 $\pm$ 2.14	72.54 $\pm$ 1.99	69.34 $\pm$ 4.07
	50.96 $\pm$ 3.70	52.42 $\pm$ 2.10	74.18 $\pm$ 3.24	76.26 $\pm$ 3.82
% neutrophils	24.44 $\pm$ 4.41	22.58 $\pm$ 1.71	25.52 $\pm$ 1.53	30.66 $\pm$ 4.07
	28.28 $\pm$ 4.41	27.58 $\pm$ 1.53	28.84 $\pm$ 3.22	23.74 $\pm$ 3.81
ANC ( $\times 10^3$ / $\mu$ L)	2.20 $\pm$ 0.80	2.60 $\pm$ 0.20	3.50 $\pm$ 0.07	4.14 $\pm$ 3.10
	0.56 $\pm$ 0.15**	0.46 $\pm$ 0.14***	0.70 $\pm$ 0.14***	0.82 $\pm$ 0.65***
MCHC (g /dL)	30.92 $\pm$ 0.36	31.50 $\pm$ 0.42	31.08 $\pm$ 0.46	31.30 $\pm$ 0.60
	30.30 $\pm$ 0.47	30.52 $\pm$ 0.17	30.36 $\pm$ 0.37	30.16 $\pm$ 0.40
Platelet	7.00 $\pm$ 0.33	7.20 $\pm$ 0.11	7.34 $\pm$ 0.65	6.85 $\pm$ 0.53
	4.85 $\pm$ 0.44**	4.69 $\pm$ 0.22**	4.01 $\pm$ 0.45**	5.27 $\pm$ 0.34

n = 5; \*p< 0.05 \*\*p< 0.01 \*\*\*p< 0.001 (One Way ANOVA; Turkey's Post hoc Test); (WBC: White Blood Cell; RBC: Red Blood Cell; HCT: Haematocrit; HGB: Haemoglobin; ANC: Absolute Neutrophil Count; MCHC: Mean Corpuscular Haemoglobin Concentration)

#### 4.5 Effect of the Extract on Sheep Red Blood Cell (SRBC)-Induced Delayed Hypersensitivity Reaction in Rats

The inflammation produced by the injection of the SRBC was maintained to various degrees in all the groups as can be seen in the 3 and 24 hours post induction depicting the immediate and delayed type hypersensitivity reactions respectively. It was observed that the oedema values at the phase inflammation (immediate type hypersensitivity effect) were higher than those of the delayed type in all the groups of both the controls and test doses with the exception of the 100 mg/kg group, the value of which was higher at 24 hr. However, at this 3 hours, there was no significant differences ( $P>0.05$ ) in oedema within the groups and the mean paw volumes of rats in the two control groups were almost similar, indicating that there is also no significant difference between the dexamethasone pretreated group and that of the normal saline negative control group. Also the mean paw volumes of the three extract dose groups were found to be higher than that of the negative control group. On the other hand, the oedema after 24 hr induction was significantly higher in the extract pretreated groups compared to the controls, with the 100 mg/kg having the highest response that increased steadily (Table 4.3).

**Table 4.3: Sheep Red Blood Cells (SRBC)-Induced Delayed Type Hypersensitivity (DTH) Reaction in Rats Pretreated Orally for 14 days with Aqueous Ethanol Leaf Extract of *C. micranthum***

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**MEAN  $\pm$  SEM of Paw Size (mm) at:**

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<b>Treatment Groups (mg/kg)</b>	<b>0 hour</b>	<b>3 hours</b>	<b>24 hours</b>
Control (1 ml/kg)	2.81 ± 0.95	4.37 ± .23	3.70 ± 0.11
CM (50)	2.70 ± 0.81	5.06 ± 0.29	4.37 ± 0.10*
CM (100)	2.69 ± 0.06	4.68 ± 0.24	5.59 ± 0.84***
CM (200)	2.47 ± 0.35	5.56 ± 0.15	4.52 ± 0.18**
Dexamethasone (0.2)	2.31 ± 0.47	4.14 ± 0.23	3.00 ± 0.16*

n = 5; \* = P < 0.05, \*\* = P < 0.01, \*\*\* = P < 0.001 (One Way ANOVA and Bonferroni post hoc test), CM = *C. micranthum*

#### **4.6 Effect of the Extract on *E. coli*-Induced Abdominal Sepsis in Mice**

The septic bacteremic effect of *E. coli* was very pronounced as seen from the control group in which all the mice died at intervals within 24 hours. The three extract

pretreated groups of mice (50, 100 and 200 mg/kg) produced 100% protection whereby none of the mice in any of these groups died up to the 72 hours of observation and even beyond. The post mortem histological analysis of one control dead mouse' abdomen (stomach and intestinal mucosa) and that of one from the 50 mg/kg extract pretreated group (Plate III) showed that only the stomach of the control mouse was grossly eroded with ulceration, while the intestines of both mice (Plate IV) retained their normal features (Table 4.4).

**Table 4.4: Protective Effect of *C. micranthum* Leaf Extract on *E. coli*-Induced Abdominal Sepsis in 15 Days orally Pretreated Mice**

Pretreatment Groups	Percentage (%) Protection After <i>E. coli</i> -induced Abdominal Sepsis at :				
	30 mins	1 hour	2 hours	24 hours	72 hours
Distl H <sub>2</sub> O (10 ml/kg)	80	60	40	0	0
CME 50 (mg/kg)	100	100	100	100	100
CME 100 (mg/kg)	100	100	100	100	100
CME 200 (mg/kg)	100	100	100	100	100

(n = 5); CME: *C. micranthum* aqueous ethanol leave extract

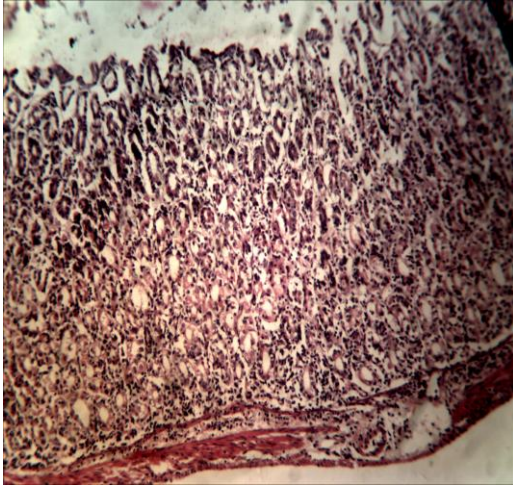


Plate IIIa

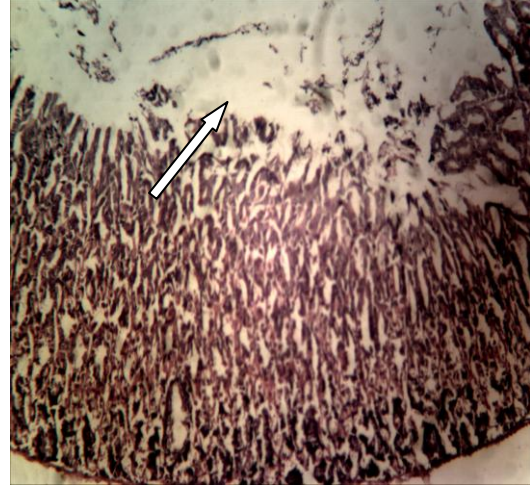


Plate IIIb

Plate III: Stomach mucosa of sacrificed mouse pretreated with *C. micranthum* (a) with no alteration versus that of the post mortem distilled water pretreated control mouse showing ulceration (b) (H and E staining,  $\times 250$ )

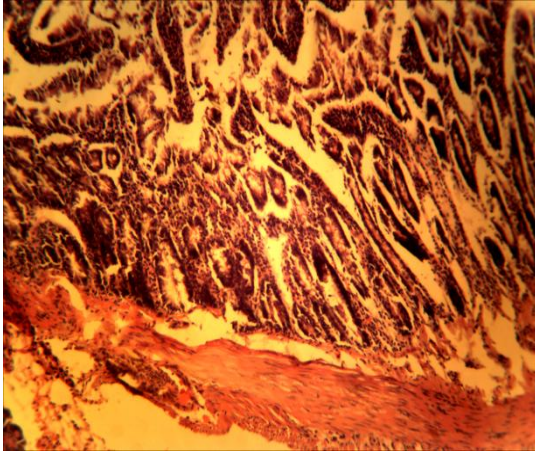


Plate IVa

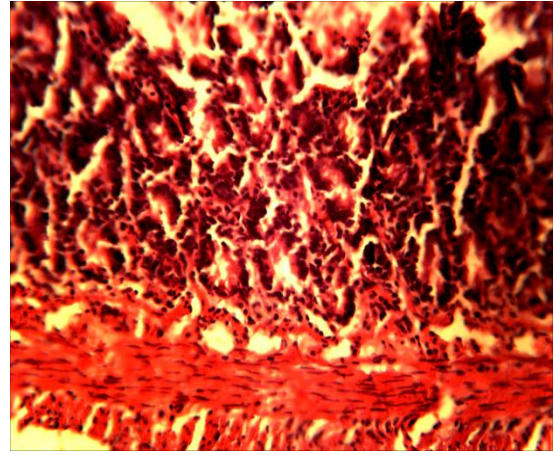


Plate IVb

Plate IV: Intestines of sacrificed mouse pretreated with *C. micranthum* (a) versus that of the post mortem distilled water pretreated control mouse (b) both of which showing not altered (H and E staining,  $\times 250$ ) .

#### 4.7 Inhibitory Effect of the Extract on Bacterial Growth

The extract at a concentration of 100 mg/ml did not inhibit the growth of *Escherichia coli*, but at 200 mg/ml, it had a zone diameter of 14.00 mm similar to that of gentamycin. According to European Committee on Antimicrobial susceptibility Testing Interpretation (Appendix I). *Escherichia coli* were susceptible to meropenem (23.00 mm), intermediate to gentamycin (14.00 mm) and ceftriaxone (22.00 mm) but resistant to ciprofloxacin (17.50 mm) (Table 4.5).

**Table 4.5: Growth Inhibition Zones of Two Extract Doses and Four Standard Antibiotics Specific for *Escherichia coli***

Organism	Agent	Zone of Inhibition	Interpretation
		Mean $\pm$ SEM	
<i>E. coli</i>	CME 100	Nil	R
	CME 200	14.00 $\pm$ 0	I
	Gentamycin	14.00 $\pm$ 0	I
	Ciprofloxacin	17.50 $\pm$ 0.5	R
	Ceftriaxone	22.00 $\pm$ 0	I
	Meropenem	23.00 $\pm$ 1.0	S

n=2, CME 100 (*C. micranthum* 100 mg/ml), CME 200 (*C. micranthum* 200 mg/ml), R = Resistant, I = Intermediate, S = Susceptibility.

The 100 and 200 mg/ml of the extract inhibit the growth of *Staphylococcus. aureus* with the zone diameter of 16.00 and 20.00 mm respectively. *Staphylococcus. aureus* was susceptible to both azithromycin (26.00 mm) and tetracycline (16.50 mm), while vancomycin and augmentin did not show any growth zone inhibition (resistant) (Table 4.6).

**Table 4.6: Growth Inhibition Zones of Two Extract Doses and Four Standard Antibiotics Specific for *Staphylococcus. aureus***

<b>Organism</b>	<b>Agent</b>	<b>Zone of Inhibition Mean <math>\pm</math> SEM</b>	<b>Interpretation</b>
<i>S. aureus</i>	CME100	16.00 $\pm$ 0	S
	CME200	20.00 $\pm$ 0	S
	Augmentin	Nil	R
	Vancomycin	Nil	R
	Tetracycline	16.50 $\pm$ 0	S
	Azithromycin	26.00 $\pm$ 0	S

n=2, CME 100 (*C. micranthum* 100 mg/ml), CME 200 (*C. micranthum* 200 mg/ml), R = Resistant, I = Intermediate, S = Susceptibility.

Both the 100 and 200 mg/ml of the extract had the same zone diameter of (20.00 mm). *Klebsiella pneumoniae* was susceptible to nitrofurantoin (21.00 mm), intermediate to

ofloxacin (20.50 mm), but resistant to both augmentine (11.00 mm) and amoxicillin (11.00 mm) (Table 4.7).

**Table 4.7 Growth Inhibition Zones of Two Extract Doses and Four Standard Antibiotics Specific for *Klebsiella. pneumoneae***

Organism	Agent	Zone of Inhibition	Interpretation
		Mean $\pm$ SEM	
<i>K. pneumoneae</i>	CME 100	20.00 $\pm$ 0	I
	CME 200	20.00 $\pm$ 0	I
	Augmentin	11.00 $\pm$ 1.0	R
	Amoxicillin	11.00 $\pm$ 1.0	R
	Ofloxacin	20.5 $\pm$ 0.5	I
	Nitrofurantoin	21.00 $\pm$ 1.0	S

n=2, CME 100 (*C. micranthum* 100 mg/ml), CME 200 (*C. micranthum* 200 mg/ml), R = Resistant, I = Intermediate, S = Susceptibility.

The extract at the tested doses (100 and 200 mg/ml) had similar zone of inhibition ( 20.00 mm). *Salmonella typhi* was susceptible to chloramphenicol (35.00 mm), cephalexin (31.00 mm), nalidixic acid (19.00 mm) and resistant to cefuroxime (15.00 mm) (Table 4.8).

**Table 4.8 Growth Inhibition Zones of Two Extract Doses and Four Standard Antibiotics Specific for *Salmonella. typhi***

Organism	Agent	Zone of Inhibition	Interpretation
		Mean $\pm$ SEM	
<i>S. typhi</i>	CME 100	20.00 $\pm$ 0	S
	CME 200	20.00 $\pm$ 0	S
	Cefuroxime	15.00 $\pm$ 1.0	R
	Nalidixic acid	19.00 $\pm$ 1.0	S
	Cephalexin	31.00 $\pm$ 1.0	S
	Chloramphenicol	35.50 $\pm$ 0.5	S

n=2, CME 100 (*C. micranthum* 100 mg/ml), CME 200 (*C. micranthum* 200 mg/ml), R = Resistant, I = Intermediate, S = Susceptibility.

*Proteus mirabilis* was susceptible to ceftriaxone (35.00 mm), cephalexin (17.00 mm) and nalidixic acid (24.00 mm), but resistant to ceftazidime (8.00 mm). Both extract doses, had 18.00 mm zone diameter (Table 4.9).

**Table 4.9 Growth Inhibition Zones of Two Extract Doses and Four Standard Antibiotics Specific for *Proteus. mirabilis***

Organism	Agent	Zone of Inhibition	Interpretation
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		Mean ± SEM	
<i>P. mirabilis</i>	CME 100	18.00 ± 2.0	S
	CME 200	18.00 ± 0	S
	Ceftazidime	8.00 ± 2.0	R
	Cephalexin	17.00 ± 0	S
	Nalidixic acid	24.00 ± 0	S
	Ceftriaxone	35.00 ± 1.0	S

n=2, CME 100 (*C. micranthum* 100 mg/ml), CME 200 (*C. micranthum* 200 mg/ml), R = Resistant, I = Intermediate, S = Susceptibility.

## CHAPTER FIVE

### 5.0 DISCUSSION

Acute toxicity study is often used to describe the harmful effects including death which appear promptly or within 24 hours of exposure to a single or more doses of chemical substances and it is an important initial step taken to assess the safety of drugs in biological systems (Mukinda and Syce, 2007). The acute effect is usually of the general

observation in behavioural changes, while the amount of the drug that kills 50% of the test animals is described as the median lethal dose ( $LD_{50}$ ) and which is often taken as the end effect to acute studies. Agaie *et al.* (2007) reported that substance with an *i.p.*  $LD_{50} > 1,000$  mg/kg as non-toxic and substance with *p.o.*  $LD_{50} > 5,000$  mg/kg are regarded as practically non-toxic. Thus, the  $LD_{50}$  of *C. micranthum* leaf extract was found to be relatively non-toxic as the values obtained were 3807.9 *p.o.* and 2154.1 *i.p.* mg/kg in mice; while in rats, it was  $> 5000$  *p.o.* mg/kg, but was 3807.9 for *i.p.* which agrees with Abdullahi (2008). The percentage yield of the extract (12.92%) was appreciable considering that water used for the extraction is a polar solvent in addition to ethanol which also absorbed non polar constituents of the extract.

From the preliminary screening carried out prior to this study, all the constituents reported by Welch (2010) and Abdullahi (2008), such as alkaloids, anthraquinone, carbohydrates, flavonoids, saponins, sterols, tannins and terpenoids were found to be present in the aqueous ethanol leaf extract of *C. micranthum* plant, which was collected from Malumfashi in Katsina State of Northern Nigeria in September, 2011. However, Osanwa *et al.* (2012) and Uduma *et al.* (2012) reported absence of alkaloids and anthraquinones from the ethanolic leaf extract collected in August, 2012 from Afaha Oku and Ikot Edebe both in Akwa-ibom State, Southern Nigeria. Generally, it has been reported that the type and quantity of secondary metabolites in plants depend on the nature of the soil, climatic condition and geographical location to which the plant is exposed (Osanwa *et al.*, 2012), thus, the location and time of collection of plants will affect the plant constituents as well as with the different plant parts and choice of solvent for extraction. Some of the chemical constituents found present in plants are responsible for their pharmacological activities and the side effects associated with their

use (Vivek *et al.*, 2013). Antibacterial potentials of the ethanol extract of the stem bark of *Combretum micranthum* (Osonwa *et al.*, 2012) reported the presence of all the constituents except alkaloid in agreement with (Danmallam *et al.*, 2011).

Chemotherapy-induced febrile neutropenia especially of cytotoxic drugs is of common occurrence (Gudrun *et al.*, 2008). Cyclophosphamide an anticancer agent which also acts on haematopoietic cells to reduce the number of blood cells. At high doses, it is a potent suppressor of immune functions resulting in a sustained decrease in both the number and functions of T and B cells (Diwanay *et al.*, 2004) - an effect often employed in inducing neutropenia in laboratory animals. The significant decrease in the absolute neutrophil count (ANC) obtained with cyclophosphamide in this study is a typical demonstration of its neutropenic suppressive activity since this parameter is often used as a marker to determine the severity of neutropenia (Eming *et al.*, 2007). The increase in the number of white blood cells in circulation (leucocytosis) commonly of reactive to infection and its associated inflammation is a normal phenomenon (Neil and Melton, 2000). It is likely that the 200 mg/kg extract caused some sort of untoward effect on the walls of the blood vessels or endothelial system that not only reduced the number of neutrophils, but also the platelet, because such changes often result in the decrease in production or increase in the degradation of blood cells and it is a known fact that reduction in the level of blood clotting cells causes bleeding (Carol, 2007).

Following *E. coli*-induced abdominal sepsis, all the mice in the control group died within 24 hours. However, the plant extract at all dose levels used in this study protected the mice from death confirming a high antiseptic ability of this plant extract. The post mortem histological analysis of one control dead mouse' abdomen (stomach

and intestinal mucosa) that was compared with that of one 50 mg/kg extract pretreated mouse which showed that the intestines of both mice retained their normal features confirmed the fact that abdominal sepsis is a localized type of peritonitis as reported by (Pavlidis, 2003).

In the assessment of the effect of *C. micranthum* extract on T-cell-mediated immunity, following 24 hours of oedematogen (sheep red blood cell) injection, the significant increased swellings of paws in all the extract treated groups indicated a beneficial effect of this plant. The extract seemed to have sustained the inflammatory mediators in their protection against the invading stimulus and this effect for the 100 mg/kg dose increased progressively from 3 hours to the 24<sup>th</sup> hour of observation. Usually, when the delayed hypersensitivity reactions does not occur or it is deficient, there will be high risk of susceptibility to opportunistic infections as seen in certain conditions like AIDS in which the helper T-cells are impaired *vis a vis* the delayed hypersensitivity reactions (Jacysyn *et al.*, 2001).

The *C. micranthum* extract inhibited *in-vitro* growth of all the test organisms of this study which are *E. coli*, *Proteus mirabilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Salmonella typhi*. This result in agreement with reports that many *Combretum* species have medical applications against many bacterial infections such as gonorrhoea, syphilis and diarrhoea (Fyhrquist *et al.*, 2002) thereby justifies its folkloric use against many bacterial infections such as gonorrhoea, syphilis and diarrhoea as reported by Burkill (1985), Kola and Benjamin (2002) and Uduma *et al.* (2012). This might be partly why this plant was reported to possess immunomodulatory activity; as

bacterial infections and/or risk of sepsis are highly implicated in immunosuppression or in conditions of weakened immune system. Both the pathogenesis of intra-abdominal infections and neutropenic conditions stems mostly from bacterial contaminations; and the growth of *E. coli*, *Klebsiella pneumoniae* and *Staph. aureus* reported to be highly involved in bacteremia were seen to be inhibited to a reasonable extent by this plant extract. In recent times in which the clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug resistant pathogens (Bandow *et al.*, 2003); the discovery of plants such as this will by no doubt be of much value.

## **CHAPTER SIX**

### **6.0 SUMMARY, CONCLUSION AND RECOMMENDATION**

#### **6.1 Summary**

This study showed the *Combretum micranthum* leave extract to be relatively non-toxic in both mice and rats. The location and time of collection of this plant leave did not affect its previously reported constituents in literature. The extract pretreatment prior to cyclophosphamide injection showed a dose dependent count increase in WBC and

neutrophils that although not significant compared to the control group, suggested an immune boosting effect to some extent. The extract demonstrated high antiseptic activity on *E. coli*-induced abdominal sepsis as it protected all pretreated mice up to 72 hours and beyond, while all the control mice died within 24 hours. From post mortem histological analysis, only the stomach of the control mouse was grossly eroded with ulceration, with no structural alteration in its intestines. The study on the antigen-antibody activity showed the mean paw volumes of the three extract dose groups at the 3<sup>rd</sup> hour to be slightly higher than that of the negative control group. On the other hand, the oedema at the interval of 24 hours was significantly higher in the extract pretreated groups compared to the controls, with the 100 mg/kg having the highest response ( $P < 0.001$ ) that increased progressively over 24 hours. The antibacterial potential evaluation showed that the two extract doses (100 and 200 mg/ml) used exhibited growth inhibition of the test organisms (*Escherichia. coli*, *Staphylococcus. aureus*, *Klebsiella. pneumoniae*, *Salmonella. typhi* and *Proteus. mirabilis*) in a concentration dependent manner.

## **6.2 Conclusion**

From the results of the various assays which was carried out on the aqueous ethanol leaf extract of *Combretum micranthum*, it could be concluded that the plant maintain a sustained oedema at sites of injection/infection which is an indication of usefulness in ameliorating T cell-dependent immune/inflammatory diseases. The plant also reduce damages to blood cells especially the white blood cells (WBCs) which are the cellular component elements of immunity – a vital action that can prevent disease complications of chronic inflammation; from the fact that defects in leukocyte function and/or their

reduction in number often results in a decreased capacity for inflammatory defense with subsequent vulnerability to infections. The extract Protect from septic bacterial infections of the abdomen and peritoneum that may result in ulcers, abscesses or other unhealthy conditions; as it inhibits growth of bacterial organisms.

### 6.3 Recommendations

The recommendations for further research on this study include:

- i. The sub-acute and chronic toxicity studies of the crude leaf extract that would document its comprehensive toxicity and/or safety profile.
- ii. The possible mechanism of its immune protective activity.

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Appendix I: European Committee on Antimicrobial Susceptibility Testing Interpretation Table for Zone diameter Growth Inhibition

Organisms	Antibiotics	Disc content (µg)	Zone of diameter (mm)		
			Intermediate	Susceptibility	Resistant
<i>E. coli</i>	Gentamycin	10	14-16	≥17	≤14
	Ciprofloxacin	5	19-21	≥22	≤19
	Ceftriaxone	30	20-22	≥23	≤20
	Meropenem	10	16-21	≥22	≤16
<i>S. aureus</i>	Augmentin	10		≥17	≤17
	Vancomycin	30	15-16	≥17	≤14
	Tetracycline	30	12-14	≥15	≤11
	Azithromycin	15	14-17	≥18	≤13
<i>Klebsiella pneumoniae</i>	Amoxicillin	10		≥14	≤13
	Ofloxacin	5	20-21	≥22	≤19

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	Nitrofurantoin	300	15-16	$\geq 17$	$\leq 14$
<i>S. typhi</i>	Cefuroxime	30		$\geq 18$	$\leq 18$
	Nalidixic acid	30	14-18	$\geq 19$	$\leq 13$
	Cephalexin	30		$\geq 12$	$\leq 12$
	Chloramphenicol	30		$\geq 17$	$\leq 17$
<i>P. mirabilis</i>	Ceftazidime	10	19-21	$\geq 22$	$\leq 19$
	Cephalexin	30		$\geq 12$	$\leq 12$

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