EFFECTS OF THREE NIGERIAN PLANTS ON THE HISTOPATHOLOGY OF CUTANEOUS LEISHMANIASIS (*Leishmania major*) AND IMMUNOTHERAPY OF LEISHMANIASIS USING DNA/PROTEIN VACCINE IN BALB/c MICE

By

JUDITH NKIRUKA ALAWA

A DISSERTATION SUBMITTED TO THE POSTGRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN HUMAN ANATOMY

DEPARTMENT OF HUMAN ANATOMY
FACULTY OF MEDICINE
AHMADU BELLO UNIVERSITY
ZARIA
NIGERIA.

JUNE, 2008

DECLARATION

I declare that the work in this dissertation entitled 'Effects of three Nigerian plants on the histopathology of cutaneous leishmaniasis (*Leishmania major*) and immunotherapy of leishmaniasis using DNA/protein vaccine in BALB/c mice, has been performed by me in the Department of Human Anatomy under the supervision of Professor (Mrs) Helen O. Kwanashie, Professor Andrew J. Nok, Dr. Samuel S. Adebisi and Dr (Mrs) Christine K. Carter.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation has been presented in any previous application for a higher degree or diploma.

Judith Nkiruka Alawa _____ Date

CERTIFICATION

This dissertation entitled “EFFECTS OF THREE NIGERIAN PLANTS ON THE HISTOPATHOLOGY OF CUTANEOUS LEISHMANIASIS (*LEISHMANIA MAJOR*) AND IMMUNOTHERAPY OF LEISHMANIASIS USING DNA/PROTEIN VACCINE IN BALB/C MICE” by Judith Nkiruka Alawa meets the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University, Zaria and is approved for its contribution to knowlwdge and literary presentation.

_____ _____

Professor (Mrs) H.O. Kwanashie — Date
Chairman, Supervisory Committee

_____ _____

Professor A.J. Nok — Date
Member, Supervisory Committee

_____ _____

Dr S.S. Adebisi — Date
Member, Supervisory Committee

_____ _____

Dr A.Y. Nadabo — Date
Head, Human Anatomy Department

_____ _____

Professor S.A. Nkom — Date
Dean, Postgraduate School

DEDICATION

This dissertation is dedicated to my perfect gift of love from the Almighty, my darling husband whose tremendous support and friendship has brought me thus far and to our bundles of joy, who are God's inspiration and blessings to us.

ACKNOWLEDGEMENTS

Firstly, I wish to acknowledge with profound gratitude, the guidance and contributions of my supervisors Prof (Mrs) Helen O. Kwanashie, Prof. Andrew J. Nok and Dr Samuel S. Adebisi. Your thoroughput criticisms and corrections brought out the best in me.

Secondly, my thanks are due to Dr Christine K. Carter, who supervised my work at the Institute for Biomedical Research, Strathclyde, Glasgow. Thank you for giving me a space in your lab and exposing me to cutting edge technology in vaccine production. I am grateful for all the love you gave even in the midst of disagreements. Also, thanks to the numerous student-friends especially Emma, as well as staff of the institute who made my stay comfortable through their kind gestures. I specially thank Dr Carol Clements for all her efforts and tutelage on the extraction of the plant materials, to Dr Sandy Gray for allowing me space in his lab at such short notice and at no cost to purify the plant extracts, as well as to Dr John Igoli for his assistance in putting me through the procedures.

I thank all my collegues in the department of Human Anatomy for the gift of friendship and all their care. Thanks to my Heads of department, immediate past and present for all the support I received while at this work.

I sincerely acknowledge my parents, sisters, brother, nieces and in-laws for all their prayers and encouragements while in pursuit of excellence in this work. I pay tribute and honour to my late brother Chuka Udobi whose memory remains evergreen. Continue to rest in perfect peace in the Lord.

I acknowledge the grants I received from Carnegie/ABU Fellowship for six months and from MacArthur Foundaion for the dissertation write-up and completion.These have gone a long way in improving me and thus impacting on others.

Sincerely, special and unreserved gratitude goes to the joy of my life, my dear husband who has showm me that to love is to sacrifice and you did all that in order that I achieve great heights in life. Your believe in me has given me the confidence and strength to pull through. I cannot thank you enough my love but I pray God will continue to prosper you and above all bless and keep you. Thank you.

Finally, I wish to acknowledge the greatness and faithfulness of Almighty God for the completion of this study. His divine intervention has brought this work to a perfect end against all odds.

ABSTRACT

Three Nigerian plants (*Annona senegalensis, Azadirachta indica* and *Vernonia amygdalina*) were evaluated for antileishmanial effects *in vitro* and *in vivo* using the *Leishmania major* parasites. It involved the preliminary testing of the hexane, methanol and aqueous extracts of these plants on infected murine macrophages and other cell lines. This was to determine the leishmanicidal activity and cytotoxicity on normal cell lines (L929 fibroblasts and macrophages), cancer cell lines (Jurkat and SH-5YSY) and *Trypanosome brucei brucei*. The leishmanicidal activity was determined using the microscopic counting method while the cytotoxicity involved the Alamar Blue colorimetric technique. Furthermore, dose-response sensitivity tests were carried out on bone marrow macrophages infected with *Leishmania major* parasites and only extracts with positive indications from the preliminary tests were used. Also, the efficacy of the extracts which gave positive indications was tested *in vivo* in BALB/c mice treated orally for five days at a dose of 50 mg/kg per day. The aqueous extract of *Vernonia amygdalina* significantly ($p<0.001$) suppressed infection rate of intracellular amastigotes (>60%) and significantly ($p<0.001$) reduced parasite levels to almost zero in the infected macrophages. In the dose-response sensitivity test, the methanol extract of *Vernonia amygdalina* suppressed infection rates (>50%) at a lower concentration (50 μg/ml) than that which caused toxicity to the macrophages. It also significantly reduced the lesion sizes by about 50% as well as minimised histopathological changes in the skin of infected mice. The hexane extract of *Annona senegalensis*, showed good trypanocidal effect against *Trypanosome brucei brucei* at a concentration of 3.13 μg/ml. This was over 15 fold more potent than the reference drug, cylemersan used at 50 μg/ml.

Also, the potentials of a DNA based vaccine and its recombinant protein were evaluated as vaccine candidates. *Leishmania donovani* gene encoding gamma glutamylcysteine synthetase and its soluble recombinant γGCS antigen in the presence of a non-ionic vesicular surfactant (NIVS) as an adjuvant were tested. Susceptible BALB/c mice were immunised with the plasmid encoding the full sequence for γGCS (pVAXγGCS), recombinant protein in presence of adjuvant (rγGCS-NIV) or plasmid alone (pVAX control) prior to challenge with a high dose of *Leishmania major* promastigotes. Mice immunised with the recombinant protein (rγGCS-NIV), demonstrated an enhanced production of specific IgG1 and IgG2a antibodies indicating that it was immunogenic. Also, it resulted in significantly lower lesion sizes ($p<0.05$) as compared to the controls. This partial protection corresponded to a significant increase ($p<0.05$) in gamma interferon production suggesting an enhanced Th1 response. These results showed that the protein vaccine from *Leishmania donovani* induced a strong immunoprotection against cutaneous leishmaniasis, suggesting that it represents a very good candidate for use as a vaccine against several leishmania species.

TABLE OF CONTENTS

Title page---------- i

Declaration----------ii

Certification----------iii

Dedication----------iv

Acknowledgement----------v

Abstract----------vii

Table of contents----------ix

List of tables----------xv

List of figures----------xvi

List of plates----------xvii

Appendix----------xviii

Abbreviations----------xix

CHAPTER ONE

1.0 Introduction----------1

CHAPTER TWO

2.0 Literature Review----------6

2.1 Leishmaniasis----------6

2.1.1 Types of Leishmaniasis----------7

2.1.2 Prevalence and distribution of Leishmaniasis----------8

2.1.3 Transmission and Life cycle of Leishmaniasis----------9

2.2 Cutaneous Leishmaniasis ----------12

2.3 Cutaneous Leishmaniasis in *L. major infection*----------14

2.4 Immune responses to *L. major* infection----------14

2.4.1 Role of T-cells----------14

2.4.2 Role of B-cells----------18

2.5 Diagnosis of Cutaneous Leishmaniasis----------19
2.6 Histopathology of cutaneous Leishmaniasis----------20
Skin----------20
Spleen----------21
Lymph node----------21
Liver----------22
2.7 Treatment of Leishmaniasis----------24
2.7.1 Chemotherapy----------24
2.7.2 Alternative Chemotherapy----------26
2.7.3 Immunotherapy----------27
Vaccines----------27
Attenuated Vaccines----------28
Protein/Subunit Vaccines----------29
DNA vaccines----------31
2.7.4 Phytotherapy----------39
Plants used as antileishmanials----------41
Quinones----------41
Alkaloids----------42
Terpenes----------43
Phenolic Derivatives----------44
Other Metabolites----------45
2.7.5 *Azadirachta indica*----------48
2.7.6 *Annona senegalensis*----------53
2.7.8 *Vernonia amygdalina*----------56

CHAPTER THREE

3.0 Materials and Method----------60

PLANT EXPERIMENTS

3.1 Materials----------60

3.1.1 Animals and Parasites----------60

3.1.2 Cell lines----------60

3.1.3 Chemicals, reagents and media----------60

3.1.4 Drugs----------61

3.1.5 Equipment----------61

3.2 Methods----------62

3.2.1 Plant Collection----------62

3.2.2 Preparation of plant extracts----------64

3.2.2.1 Hexane and Methanol Extraction----------64

3.2.2.2 Aqueous Extraction----------64

3.2.2.3 Purification and Isolation of crude extract of *V. amygdalina*----------65

3.3 *In Vitro* Studies----------66

3.3.1 Macrophage Sensitivity Test ----------66

3.3.1.1 Isolation of Macrophages from bone marrow ----------66

3.3.1.2 Infection of Macrophages----------67

3.3.2 Assessment of drug toxicity----------68

3.3.3 Antitrypanosomal Activity *(Trypanosome brucei brucei)*----------68

3.4 *In Vivo* Antileishmanial Test----------69

3.4.1 Parasites and Infection----------69

3.4.2 Drug Treatment----------69

3.4.3 Tissue Processing----------70

IMMUNOTHERAPY EXPERIMENT

3.5 Materials----------72

3.5.1 Animals and Parasites----------72

3.5.2 Chemicals and Reagents----------72

3.5.3 Drugs----------73

3.5.4 Bacteria and Protein----------73

3.5.5 Equipment----------73

3.6 Methods----------74

3.6.1 Plasmid Vaccine Construction and Purification----------74

3.6.2 Cloning of γGCS for Protein Expression----------75

3.6.3 Expression and Purification of Recombinant Protein----------76

3.6.4 Analysis of Protein----------77

3.6.4.1 Gel Electrophoresis (SDS-PAGE)----------77

3.6.4.2 Western Blot Analysis----------77

3.6.5 Immunization and Infection of mice----------78

3.6.6 T-Cell Proliferation Assay----------79

3.6.6.1 Cytokine Determination----------80

3.6.6.2 Nitrite Assay----------81

3.6.7 Antibody Production----------81

3.7 Statistical Analysis----------82

CHAPTER FOUR

4.0 RESULTS----------83

PLANT EXPERIMENTS

4.1 Extraction of plants----------83

4.1.1 Isolation of Compounds from *Vernonia amygdalina*----------85

4.2 *In Vitro* Experiments----------92

4.2.1 Macrophage Sensitivity Tests (Preliminary investigation)----------92

4.2.2 Drug Sensitivity Assay on Macrophages----------96

4.2.3 Cytotoxicity studies----------100

4.2.4 Antitrypanosomal Activity----------102

4.3 *In Vivo* Antileishmanial Activity----------105

IMMUNOTHERAPY EXPERIMENT

4.4 Expression of Recombinant protein----------110

4.5 Efficacy of DNA and Protein Vaccinations----------113

4.6 Humoral Responses to DNA and Protein Vaccinations----------117

4.7 Cytokine and Nitric oxide Production----------120

CHAPTER FIVE

5.0 DISCUSSION----------125

PLANT EXPERIMENTS

5.1 Plants Extraction----------125

5.2 *In Vitro* Experiments----------125

5.2.1 Macrophage Sensitivity Tests----------125

5.2.2 Cytotoxicity Tests----------128

5.2.3 Antitrypanosomal Activity----------131

5.3 *In Vivo* Experiment----------132

IMMUNOTHERAPY EXPERIMENTS

5.4 Efficacy of DNA and Protein Vaccines----------134

5.5 Humoral Responses to DNA and Protein Vaccines----------136

5.6 Cytokine and Nitric oxide production in Vaccine groups----------137

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS----------141

6.1 Significant and original contribution to scientific knowledge----------143

References----------144

Appendices----------165

LIST OF TABLES

Table 3.1 Plants and Ethnobotanical Information----------63

Table 4.1 Yields and Appearances of plant extracts----------84

Table 4.2 Yields and NMR results of fractions of *V. amygdalina*----------86

Table 4.3 Efficacies of extracts on intracellular survival of *L. major*----------95

Table 4.4 Effects of Extracts and (Sb V) on Mean Infection rate----------97

Table 4.5 Effects of Extracts and (Sb V) on Parasite number----------98

Table 4.6 Cytotoxic activity of extracts on cell lines----------101

Table 4.7 Antitrypanosomal activity of Extracts on *T. brucei brucei*----------104

LIST OF FIGURES

Figure 2.1 Life cycle of Leishmania----------11

Figure 4.1 TLC analysis of fractions off flash column----------87

Figure 4.2 TLC analysis of fractions at end of flash column----------87

Figure 4.3 Proton NMR spectrum (full) for VAHE 2----------88

Figure 4.4 Proton NMR spectrum (full) for VAHE 3----------89

Figure 4.5 Proton NMR spectrum (full) for VAHE 8----------90

Figure 4.6 Proton NMR spectrum (full) for VAHE 10----------91

Figure 4.7 Leishmanicidal effects of extracts and (SB V)----------99

Figure 4.8 Sample plate of trypanomicidal effects of extracts----------103

Figure 4.9 Effect of extracts on lesion growth----------106

Figure 4.10 Gamma GCS gene sequence in Agarose gel----------111

Figure 4.11a Gamma GCS recombinant protein in SDS PAGE----------111

Figure 4.11b Gamma GCS recombinant protein in Western blot----------112

Figure4.11c Quantitative aalysis of protein on Bio-rad assay----------112

Figure 4.12 Cross-protective effects of Vaccinations----------115

Figure 4.13 Effect of Vaccination on spleen to body weight ratio----------116

Figure 4.14 Effect of Vaccination on specific antibody responses----------119

Figure 4.15a Effect of Vaccination on cytokine production----------121

Figure 4.15b Effect of Vaccination on cytokine production----------122

Figure 4.16 Effect of Vaccination on nitrite production----------124

LIST OF PLATES

Plate 2.1a *Azadirachta indica* (Voucher specimen)----------50

Plate 21b *Azadirachta indica* (neem) plant----------51

Plate 2.2a *Annona senegalensis* (Voucher specimen)----------54

Plate 2.2b *Annona senegalensis* stem bark (Voucher specimen)----------54

Plate 2.2c *Annona senegalensis* growing in natural habitat----------55

Plate 2.3a *Vernonia amygdalina* (Voucher specimen)----------57

Plate 2.3b *Vernonia amygdalina* growing in natural habitat----------57

Plate 4.1 Untreated Macrophages----------93

Plate 4.2 Treated Macrophages----------93

Plate 4.3 Cross Section of Skin from control mouse (x250)----------106

Plate 4.4 Cross Sections of Skin from control mouse (A&B)----------106

Plate 4.5 Cross Section of Skin from AS treated mouse (x250)----------107

Plate 4.6 Cross Section of Skin from AS treated mouse (x400)----------107

Plate 4.7 Cross Section of Skin from VA treated mouse (x250)----------108

LIST OF APPENDICES

I. Composition of ELISA Buffers----------165

II. Composition of SDS PAGE Buffers----------165

III. Composition of DNA Extraction Buffers----------165

IV. Composition of Boyle's solution----------166

V. Gel Composition of SDS PAGE----------166

VI. Cell Media----------166

VII. Anisaldehyde-Sulphuric acid Reagent----------166

ABBREVATIONS

DMSO	Dimethyl Sulphoxide
PCR	Polymerase Chain Reaction
IL	Interleukin
IFN-γ	Interferon gamma
Th	T-helper cell
IgG	Immunoglobulin G
H&E	Haematoxylin and Eosin stain
AI	Azadirachta indica
AS	Annona senegalensis
VA	Vernonia amygdalina
MeOH	Methanol
Hex	Hexane
Aq	Aqueous
WHO	World Health Organisation
US	United States
NO	Nitric oxide
FBS/FCS	Foetal bovine or calf serum
DMEM	Dulbelco's modification of Eagle's medium
RPMI	Roswell Park Memorial Institute medium
PBS	Phosphate Buffered Saline
LPS	Lipopolysaccaride
SDS	Sodium Dodecyl sulphate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
NMR	Nuclear magnetic resonance imaging
Con A	Concanavalin A

CHAPTER ONE

1.0 INTRODUCTION

Leishmaniasis is a parasitic disease caused by haemoflagellate protozoan of the genus *Leishmania.* This protozoan was first described in 1903 by Leishman and Donovan who worked separately (Herwaldt, 1999). These protozoan parasites are members of the family Trypanosomatidae (order Kinetoplastida), which comprises unicellular organisms, characterised by the presence of a single flagellum and a DNA-rich mitochondria-like organelle called kinetoplast (Descoteux and Turco 2002). It is a spectral disease with high clinical diversity, which depends on the genetic potential, acute predisposition of the host's immune system (Kenner, 2007) and virulence of the *leishmania* specie involved as there are over 20 species and at least 16 species are pathogenic for mammals (Acha and Szyfres, 2003). It is transmitted by the bite of the female sandfly, which is a small hairy mosquito-like insect. It is zoonotic as wild and domestic animals such as dogs, rodents, foxes, wolves, jackals and other mammals serve as reservoir hosts.

Leishmaniasis has been present in the Americas and Africa for several centuries. It occurs from the tropical to Mediterranean regions and affects approximately 12 million people in 88 countries throughout Asia, Africa, Europe as well as North and South America but mostly in developing countries. Also, over 350 million people are at risk of infection in these areas. According to the World Health organisation (WHO) there is an annual incidence of 1.5 to 2 million new cases. The majority of cases are in the developing countries where approximately 90% of all cases occur (WHO, 2005). The global incidence of this infectious disease has increased in recent years because of increased international leisure,

missionary work and military-related travel, human alteration of vector habitats, and concomitant factors that increase susceptibility, such as HIV infection and malnutrition. In Africa, most cases are found in Sudan and Somalia while in Nigeria, cases of cutaneous leismaniasis have been documented since the mid 20th century by a number of workers (McCulloch, 1930; Elmes and Hall, 1944; Jelliffe, 1955; McMillan, 1957). It has been observed in Keana village in Awe, Plateau state in the north central part of Nigeria (Agwale *et al.,* 1993).

The clinical presentations are multifaceted but the leishmania species produce three types of leishmaniasis, which are cutaneous, mucocutaneous and visceral. Cutaneous leishmaniasis, caused by *Leishmania major (L. major)* is the focus of this study. It is the most frequently occuring epidemics and is characterised by clinical polymorphism (Masmoudi *et al.*, 2007). It affects the skin, mucous membrane or both tissues and is characterised by lesions, which start as small red erythematous lesions at the site where the promastigotes are inoculated into the skin by the bite of the sandfly. Microscopic examination of these lesions in human tissue reveals the cytology of the parasites that present as amastigotes which are ovoid or round shaped cells. These cells have relatively large nucleus, thin cell membranes and rod-shaped kinetoplast. The amastigotes are found within the macrophages, multiply in them and they serve as important models for the understanding of the regulation of immune (Th cell) responses as well as the immunomodulation of leishmaniasis (Alexander *et al.*, 1999). Leishmaniasis represents an emergent illness with high mortality and morbidity in the tropics and subtropics (Dilvani *et al.,* 2008). It is responsible for an estimated more than 80,000 deaths annually. Poor nutrition, infection, mass migration and inaccessibility to regular healthcare in the developing world predispose people to

the disease and increase the mortality rate. The social burden of the disease is also of great importance as it can cause severe deformities and disfigurements which leads to social isolation (Renee, 2006). Treatment has proven to be difficult due to the intramacrophagic location of the infectious forms, the amastigotes and its control has largely been limited to the use of chemotherapeutic agents such as the pentavalent antimonials. The efficacy of these antimonials has become increasingly compromised due to the emergence of resistance within the parasite population and the associated toxicity of the drugs. As a result of this, the search for substances with antileishmanial activity, without toxic effects, and able to overcome the emergence of drug resistant strains still remains the current goal and research on this has intensified. This has led to the renewed interest in the use of phytomedicine involving natural products and immunotherapy using cytokines and vaccines. These have become areas of intense study in the search for new treatments for this disease. The urgent need for alternative treatments has led to a program to screen natural products for potential use in the therapy of leishmaniasis and to develop a vaccine. Due to the limited availability of effective pharmaceutical products, most people in areas where leishmaniasis is endemic depend largely on popular treatments like cauterization procedures using copper sulphate or battery acid and on traditional medical practices which depend heavily on native plants to alleviate the symptoms (Carvalho and Ferreira, 2001) but many of these have not been documented. Thus, as part of the search for new drugs of high availability and less toxicity, the Tropical Disease Programme of the World Health Organisation has considered the screening of these plants used in tradomedicine for treatment of leishmaniasis as esential and of high priority. The leads obtained from the screening of plants for antileishmanial activity resulted in

the discovery of Miltefosine, the first oral drug shown to be efficient and safe in several clinical trials (Carvalho *et al.,* 2000).

Nigeria, as in most cultures in the world has great potentials in terms of biodiversity. In Nigeria, there are many local claims about medicinal plants and treatment of common diseases (Raul *et al*., 1990). However, little is known about indigenous plants with leishmanicidal activity hence, as part of the systematic search programme for antileishmanial agents of plant-origin, the present study assesses the leishmanicidal potentials of three Nigerian plants of medicinal value used against other protozoan diseases. These are *Azadirachta indica* (neem plant), *Annona senegalensis* (custard tree) and *Vernonia amygdalina* (bitter leaf). *Azadirachta indica* is reputed to be of great medicinal value and products made from it showed anthelmintic, antifungal, antidiabetic, antibacterial, antiviral and anti-infertility activities and particularly prescribed for skin diseases (Puri, 1999). *Annona senegalensis* has been used as an anti-trypanosomal (Igweh and Onabanjo, 1989), anti-leishmanial (Sahpaz, *et al*., 1994) and antisplasmodic (Langason *et al*., 1994). It is also used in the treatment of cancer (Graham *et al.*, 2000), malaria (Balansard and Timon-David, 1985), diarrhoea, dysentery (Kudi and Myint, 1999), intestinal worms (Alawa *et al*., 2003), snakebites (Adzu *et al.*, 2005) and veneral diseases (Tabuti *et al.*, 2003). *V. amygdalina*, is widely used in Nigeria for human consumption, for ethnomedical practices like antiplasmodial, anti-schistosomal (Jisaka *et al.*, 1992a), anti-tumoral and anti-microbial actions (Jisaka *et al.*, 1993). It is also used for ethnoveterinary practices like anti-tick in Ethiopia (Regassa, 2000) and as an anthelmintic in ruminants (Alawa *et al.,* 2003). *V. amygdalina* has been reported to be eaten by wild chimpanzees possibly as self medication against endo-parasites (Huffman *et al*., 1993).

In the light of the above, the first part of this research work is intended to determine any ethnopharmacological use of the three selected traditionally administered plants in the north central part of Nigeria. These are plants employed in the treatment of protozoan diseases such as trypanosomiasis and malaria. Hence, we wish to extend use to another protozoan, *Leishmania*. Ethnobotanical information and previous research works on these plants serve as a basis for the selection of the plants, which are *Azadirachta indica, Annona senegalensis* and *Vernonia amygdalina.* Thus, the aim of this study is to determine if each of these plants possesses any leishmanicidal activity with the following objectives:

- To fractionate and purify the crude extract of the plants in order to determine the constituent compounds
- To investigate the *in vitro* effects of the crude extracts on the amastigote form of *L. major* as well as the cytotoxicity of these plant extracts on various cell lines i.e. normal (L929) and cancerous (Jurkat and SH-SY5Y)
- To investigate the *in vivo* effects of the plant extracts and their effect on the histopathology of the organs (skin, liver and spleen) in BALB/c mice

The aim of the second part of this work is to investigate the immunocompetence of the DNA/Protein based vaccines with the following objective:

- To determine if the DNA encoding γGCS (pVAXγGCS) and the recombinant protein (rγGCS) from *L. donovani* species used to immunize BALB/c mice against *L. major* infection would confer cross protection as well as stimulate any immunological responses in the mice.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Leishmaniasis

Leishmaniasis occurs as a group of diseases which are related to environmental changes such as deforestation, building of dams, new irrigation schemes, urbanization and migration of people to endemic regions (WHO, 2007). They adversely affect the populace especially the impoverished ones and seriously hamper their socioeconomic progress. It is one of the most important infectious diseases worldwide and regarded as a major public health problem (WHO, 2005), causing significant morbidity and mortality in Africa, Asia and Latin America**.** The public health impact of the disease has been grossly underestimated due to lack of awareness. This has led to further spreading of the disease from endemic areas and a sharp increase in the number of recorded cases. This has worsened since the advent of HIV/AIDS. These two infections coexist in a deadly synergy and AIDS has transformed leishmaniasis from a sporadic to an epidemic threat. In persons infected with HIV, leishmaniasis accelerates the onset of AIDS by cumulative immunosuppression and by stimulating replication of the virus (WHO, 2007). Conversely, the epidemiological significance of the asymptomatic carrirers of the leishmania parasite has been amplified by the advent of AIDS as co-infection rapidly activates disease in these carriers. Sharing of needles by intravenous drug users contributes to the spread of leishmaniasis and HIV.

2.1.1 Types of leishmaniasis

The clinical presentations are multifaceted but the leishmania species basically produce three types:

- Cutaneous leishmaniasis (CL) also called oriental sore and caused by *L. tropica* complex, *L. major* and *L. aethiopica*. It is relatively a mild localized disease. It presents as nodular and oriental or topical sores, infiltrating ulcers and multiple popular lesions, which pose cosmetic problems. It starts as an itching papule surrounded by induration then changes slowly into an expanding ulcer with irregular borders (Titus and Ribeiro, 1988)
- Mucocutaneous Leishmaniasis (MCL) is also known as espundia. It is caused by *L. brazilensis* found only in the New World hence it is a predominantly New World disease (Magill, 2000). It may not manifest clinically until years after localised cutaneous disease has healed. It affects the mucous membrane of the nose, tongue, mouth and larynx. It presents as ulcerating or non-ulcerating lesion, which develop in the larynx and at mucocutaneous junctions of the nasal septum, anus and vulva. If untreated, may lead to cutaneous leishmaniasis and other severe secondary infection like extensive midfacial destruction and occasionally death (Kenner, 2007).
- Visceral leishmaniasis (VL) is also known as kala azar (Hindi for black fever). It is the most severe form and is caused by *L. donovani* complex and *L. chagasi* complex. It attacks the immune system and presents with high fever, weight loss and enlarged spleen and liver in the infected person. If it is left untreated it may become fatal. Death usually results from opportunistic infections as a result of immunosuppression (Davidson, 1999).

2.1.2 Prevalence and Distribution of Leishmaniasis

The incidence of leishmaniasis has been on the increase to the extent that it has gained a significant position worldwide among the causes of death by infectious diseases (Murray, 2005). Its incidence is greater in the Old World than in the New World. In the Old World, leishmaniasis is distributed in the East and West Africa, the Middle East especially in the Jordan valley, Iran, Iraq and Saudi Arabia, around the Mediterranean sea, as well as in Afganistan, India, China in Asia and several former Soviet Union Republics. It is responsible for cutaneous or visceral disease caused by *Leishmania* species, which include *Leishmania major*, *Leishmania tropica* and *Leishmania infantum*. New World leishmaniasis is distributed from the southern part of America to the northern parts of Argentina and Paraguay. It is caused by *Leishmania* speceis found in Central America and South America and produces cutaneous, mucocutaeous, and visceral disease. The species include *Leishmania amazonensis*, *Leishmania mexicana, Leishmania chagasi* and *Leishmania brazilensis.* Incidence of cutaneous leishmaniasis cuts across the Mediterranean and non-mediterranean including West Africa. In Africa, it is mostly found in Sudan and Somalia but the disease is spreading to new areas as a result of environmental changes and forced migration due to war, famine etc. Today, the number of detected cases has increased sharply and in Southern Sudan, the on-going war has contributed to one of the largest kala-azar epidemics ever recorded [Medecines Sans Frontieres (MSF), 2000]. Incidence of visceral leishmaniasis has changed since the advent of HIV/AIDS. In Mediterranean countries where it is endemic, it is considered as an AIDS related opportunistic disease as studies have shown that between 1.5 to 9% of HIV-positive patients develop leishmaniasis and that latent forms of the infection are reactivated in these patients due to immunosuppression and the

replication of the virus (Davidson, 1999). Co-infection with leishmaniasis has been documented in 34 countries in Africa, Asia, Europe and South America and up to 70% of adult cases of visceral leishmaniasis are associated with HIV infection. Users of injectable drugs are the most seriously affected group (WHO, 2005).

2.1.3 Transmission and Life cycle of Leishmaniasis

Leishmaniasis is a zoonosis because wild and domestic animals such as dogs, rodents, foxes, wolves, jackals and other mammals serve as reservoir hosts. Infected humans may also serve as occasional reservoir hosts. It is transmitted by the bite of female sandfly of the genus *Phlebotomus* in the Old world and *Lutzomia* in the New World. The sandfly is a small hairy mosquito-like insect 1.5 – 4 mm in length. Their small size enables them to pass through ordinary mesh screens and mosquito nets. Sandflies are found throughout the tropics and subtropics in rain forests and highlands.

The life cycle of leishmania involves two forms: Promastigotes and Amastigotes. The sandflies become infected when biting or taking blood from an infected reservoir host which includes man and other wild or domestic animals, macrophages harbouring the amastigotes are ingested. These amastigotes differentiate into promastigotes which multiply within the digestive tract of the sandfly and migrate to the pharynx where they are poised for transmission through the bite of the sandfly. The promastigotes exist as motile, elongated and flagellated parasites in the gut of the sandfly. These extracellular promastigotes (10 to 20 micrometers) are injected into the dermis of the skin during a blood meal along with the sandfly's saliva which potentiates parasite infectivity (Titus and Ribeiro, 1988). The promastigotes are rapidly taken up by the mononuclear

phagocytes mostly macrophages in their parasitophorous vacuoles. These fuse with the lysosomes and the acidity within the phagolysosome induces the promastigotes to transform into ovoid amastigotes. These are non-motile, obligate, unflagellated and contain only a single DNA-containing specialised mitochondrion called kinetoplast. They are intracellular and specially adapted to the elevated temperatures within the mammalian host (Solbach and Laskay, 2000). Massive multiplication and proliferation of the amastigotes occur within the macrophages and this leads to disruption of the host cell or macrophage hence the dying macrophages release these parasites which can infect additional macrophages thus cause disease in humans and affect cellular immunity. Resting macrophages support parasite multiplication and thus the spread of infection; while activation of macrophages signalled by the release of the cytokines such as interferon gamma (IFN-y) supports death of the parasite and thus healing or cure. Eventually, a sandfly will pick up the amastigotes while feeding and these will develop into the promastigotes form in the insect setting up a new cycle of transmission (Markle and Makhoul, 2004).

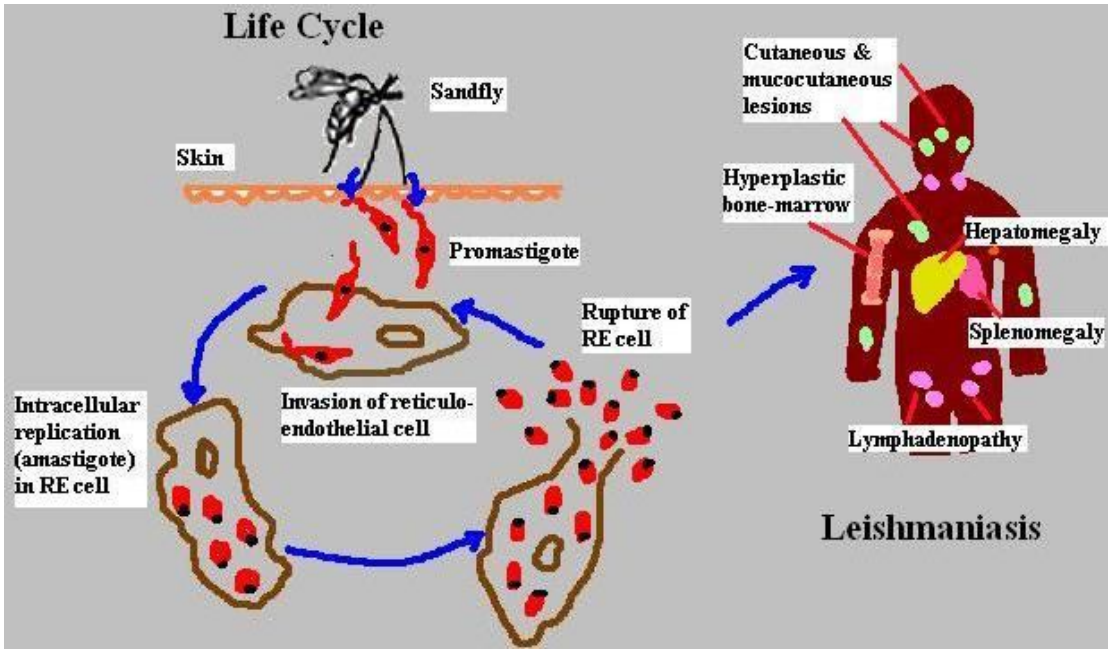


Fig 2.1: Life cycle of leishmania (Culled from Indian Journal of Dermatology, Venereology and Leprology, 15:23-24).

2.2 Cutaneous Leishmaniasis

Cutaneous leishmaniasis (Oriental button) is the most common form of leishmaniasis as ninety percent of all leishmania infections are restricted to the skin. It represents 50% to 75% of all new leismania cases. It is endemic to many parts of the world and the distribution is very tightly linked to geography as villages which are 15 miles apart could have different rates of infection (Wikipedia, 2007). The incidence is greater in the Old world than in the New world. Old World disease is found in parts of Asia, Africa and Europe and in the Middle East. The causative organism is the *Leishmania tropica (L. tropica)* complex (Hepburn *et al.,* 1993). The New World disease is also called chiclero ulcer, uta or bay sore and is found in the North and South America as well as Central America. It is caused by the *Leishmania mexicana* (*L. mexicana)* complex in these regions. There are several species in each complex and they are associated with different geographical regions. Species in the *Leishmania tropica* (*L. tropica)* complex include *L. tropica, Leishmania major (L. major)*, *Leishmania aethiopica* (*L. aethiopica)* and *Leishmania infantum (L. infantum)* which are prevalent in the Old world while species in the *L. mexicana* complex include *L. mexicana*, *Leishmania brazilensis* (*L. brazilensis)* and *Leishmania amazonensis* (*L. amazonensis)* which are prevalent in the New world (Grimaldi and Tesh, 1993). The reservoir host for cutaneous leishmaniasis is human for the species *L. tropica* while other species are zoonotic as they are traditionally associated with animal species (largely canines and rodents) but are becoming primarily human diseases (Vergel *et al.*, 2006).

The broad spectrum of clinical manifestations of cutaneous leishmaniasis is often compared with that of leprosy and it occurs in three forms which are localised,

diffuse and recidivans. Another form which is called the post-kala azar dermal leishmaniasis is the visceralising form.

- Localized cutaneous leishmaniasis (LCL): Characterised by crusted papules or ulcers which occur several weeks or months after the inoculation of the parasite via the bite of a sandfly. Lesions are solitary and usually heal spontaneously.
- Diffuse cutaneous leishmaniasis (DCL): Analogous to lepromatous leprosy. It is characterised by multiple widespread papules and nodules which begins as a single skin nodule. The lesion resembles keloids and they do not ulcerate but contain vast aggregate of foamy macrophages stuffed with leishmania parasites. Patients are usually immunologically unresponsive as they cannot mount a cell-mediated immune response to the leishmania parasite or other skin antigens. They often respond poorly to treatment (Berman, 1997).
- Recidivans cutaneous leishmaniasis (RCL): This is relatively uncommon and presents as a recurrence of lesions at the site of apparently healed disease years after the original infection. It is characterised by enlarging papules, plaque or coalescence of papules that heal with central scaring. It typically occurs on the face and relentless expansion at the periphery may cause significant facial destruction.
- Post-kala azar dermal leishmaniasis (PKADL): This is endemic to India and Sudan and it develops after visceral leishmaniasis infection. Cutaneous lesions demonstrate great variability ranging from hypopigmented macules to erythematous papules. Lesions may be numerous and persist for decades (Herwaldt, 1999).

2.3 Cutaneous Leishmaniasis in *Leishmania major* Infection

Cutaneous Leishmaniasis caused by *Leishmania major* is the most frequent and it is characterised by clinical polymorphism (Masmoudi *et al.*, 2007). These clinical forms include ulcerated and crusted form, lupoid form, sporotrichoid form and other atypical forms. This disease phenotype of *L. major* has been attributed to the expression of the PFP1 cysteine peptidase gene and independent of *Lsh* gene. These genes are absent in *L. mexicana* infection, which is the New world form and they contribute to the survival of the *L. major* parasite and the disease tropism which distinguishes it (Mock *et al.*, 1985; Sylvain *et al*., 2007).

2.4 Immune Responses to *Leishmania major* infection

2.4.1 Role of T-cells

In human and experimental cutaneous leishmaniasis, development of protective immunity is dependent on the capability of the host cell to generate antigen specific T-cell dependent response. Animal models mimicking human cutaneous leishmaniasis have been established through the induction of the disease by infecting mice subcutaneously with *L. major.* Different strains of in-bred mice have been used to study immunological responses and these have been confirmed in humans (Von Stebut, 2007). These experimental *L. major* infections are widely used to explore the control of T-helper cell differentiation and elucidate mechanisms underlying the susceptibility or resistance to intracellular microbial infections (Sacks and Noben-Trauth, 2002; Locksley and Scott, 1991). The most frequent experimental models used in cutaneous leishmaniasis (CL) is the BALB/c mice which develop severe pathology manifested by progressive lesion development, necrosis and death (Reiner and Locksley, 1995) and the C57BL/6 mice which control infection with transient lesion development and this

recapitulates the major hallmark of human infection. These mice as well as humans develop self-healing lesions that become prominent several weeks after postinfection and resolve within months. Thus, the former are known as 'Non-Healer' mice and the later 'Healer' mice. Recently, numerous studies have clearly established the immunological mechanisms governing the growth of *L. major* in experimental cutaneous leishmaniasis (Liew and O'Donnell, 1993). The control of *L. major* lesion growth in mice and humans has been associated with type-1 immune response characterised by the expansion of the T-helper 1 (Th-1) lymphocyte subset of the $CD4^+$ T-cell population and the production of the cytokines gamma interferon (IFN-γ), interleukin-12 (IL-12) and interleukin-2 (IL-2) (Heinzel *et al.*, 1991; Scott *et al.*, 1988). These activate the macrophages to kill the parasites through toxic metabolites of oxygen and nitric oxide. Conversely, the non-healing responses or disease exacerbation in susceptible mice have been related to the type-2 immune response characterised by the expansion of the T-helper 2 (Th-2) lymphocyte subset and production of the cytokines interleukin 4, 5,9,10 and 13 (IL-4, IL-5, IL-9, IL-10 and IL-13) (Locksley *et al.*, 1991). These prevent the killing of parasites by inhibiting activation of macrophages. Leishmania manipulate the innate host defences in order to facilitate their entry and survival in the host macrophages (Sacks and Sheer, 2002). They do this through the production of two abundant surface glycoconjugates by the promastigotes, which are important for their virulence (Beverly and Twico, 1995). These are:

i) Lipophosphoglycans: This forms a dense glycocalyx that both activates complement (leading to the deposition of C3b on the parasite surface) and inhibits complement action (by preventing membrane attack complex insertion into the parasite membrane). Thus the parasite becomes coated

with C3 but avoids destruction by membrane attack complex. The C3b on the surface of the parasite then binds to Mac-1 and CR 1 on macrophages which target the promastigotes for phagocytosis. Once inside the cell, the lipophosphoglycan protects the parasites within the phagolysosome by scavenging oxygen free radicals and by inhibiting lysosomal enzymes.

ii) Glycoprotein Gp 63: This is a zinc dependent protinase that cleaves the complement and some lysosomal antimicrobial enzymes. Gp63 also binds to fibronectin receptors on the macrophages and promotes promastigotes adhesion to macrophages.

Apart from the promastigotes surface glycoconjugates, the amastigotes also produce molecules that facilitate their survival and replication within the macrophages. They normally reproduce in macrophages, which have a pH of 4.5. However, they are also protected from the hostile environment by a proton-transporting ATPase, which maintains the intracellular parasite pH at 6.5. The immune responses evoked during the antileishmanial activity can be separated into three distinct phases based on the cell types that are activated. These phases include:

First or Silent phase

This is characterised by invasion of the resident macrophages or phagocytes in the skin. These are the cells of the innate immune system and the first to be in contact with the invading leishmanial parasites. This occurs when the sandfly comes in contact with the skin and inoculates the promastigotes by biting. The macrophages engulf the parasites and this triggers the activation of the components of the complement system. This results in opsonization of the parasites surface epitopes with C3bi and C3b. The opsonized promastigotes then undergo immune adherence reaction and bind to complement receptor 3 (CR3)

which is the main receptor responsible for leishmanial entry into the macrophage (Reiner and Locksley, 1995). On entry into the macrophages they transform into amastigote form which are capable of surviving in the phagosomes. This stage lasts 4-5 weeks and is characterised by absence of lesion and establishment of pack load of parasites in the dermis. The amastigotes replicate within the macrophages causing them to lyse thus reinfecting naïve macrophages (Belkaid *et al*., 2000).

Second phase

This involves the infiltration of inflammatory cells (neutrophils, eosinophils and macrophages) into the dermis (Belkaid *et al*., 2000). The macrophages act as the primary host cell and the major parasite reservoir *in vivo.* In *L. major* infection there is selective inhibition of the macrophages to produce IL-12 as the synthesis pathway is blocked (Carrera *et al*., 1996; Belkaid *et al*., 1998). However they are capable of producing proinflammatory cytokines such as transforming growth factor alpha (TGF-α) at the site of infection while the neutrophils, eosinophils and mast cells are involved in granuloma formation which is aimed at clearing or restricting the growth of parasites at the site of infection (Von Stebut *et al*., 2005). The major role of the macrophages is the killing of parasites through the induction of a respiratory burst. This is through the activation of the macrophage plasma membrane NADPH-oxidase and results in the production of reactive oxygen intermediates (ROI) such as superoxide, hydrogen peroxide and hydroxyl radicals at supraphysiological concentrations. When this exceeds the cell's antioxidant capacity, oxidative stress results and this leads to damage to the parasite. Also activation of the macrophages by cytokines induces the upregulation of the cytokine-inducible nitric oxide synthase (iNOS). This enzyme converts arginine into citrulline and then nitric oxide (NO), which is important in many biological

functions. The NO interacts with the oxygen intermediates to form highly cytotoxic peroxynitrite radicals, which directly kill the parasites. Another mechanism through which the macrophages mediate parasite killing is by the production of hydrolytic enzymes within the phagolysosomes. These enzymes function to degrade intracellular parasites. Thus macrophages are crucial effector cells in the induction of protective cutaneous responses against *L. major.* This stage lasts 5-6 weeks postinfection and is characterised by development of lesions.

Third phase

This is the time point when dendritic cells (DC) appear in the lesions of tissues. These are the most potent type of antigen presenting cells (APC) as they are the only cells capable of processing leishmania antigen in both the major histocompactibility complex (MHC) class I and II pathways. They are responsible for the initiation of immune responses and are an important source of IL-12 as their appearance coincides with the detection of IL-12 producing cells which are required for protective immunity. Hence this phase is characterised by parasite killing and lesion involution (Belkaid *et al.*, 2000). After this phase, is the chronic phase which lasts for the life of the animal during which a low number of parasites persists in the skin in the absence of overt pathology.

2.4.2 Role of B-cells

B cells are another type of antigen presenting cells in the host. They do not appear to accumulate in skin infected with intracellular pathogens such as *L. major* but play important roles for resulting adaptive immune responses through their antibodies (Woelbing *et al.*, 2006). These antibodies include IgG (both antigen-specific and natural), which are responsible for the opsonization of the pathogens so that the components of the innate immune system will recognise

them as foreign. In mice without B-cells or the Fcy receptor relevant for IgG binding, there was disease susceptibility which was directly attributed to the failure of dendritic cells to prime T-cells efficiently (Ochsenbein and Zinkernage, 2000).

2.5 Diagnosis of Cutaneous Leishmaniasis

Cutaneous leishmaniasis affects the skin, mucous membrane or both tissues. It is characterised by lesions, which start as small erythematous lesions at the site where the promastigotes were inoculated into the skin by the bit of the sandfly. These then develop within weeks into papule that enlarge, become crusted and break down to form ulcers that are painless, shallow with raised borders. Microscopic examination of these lesions in human tissue reveal, a cytology of the parasites which present as amastigotes which are ovoid or round shaped cells and 1.5 - 3μm in diameter. Cells have relatively large nucleus, thin cell membranes and rod-shaped kinetoplast. These amastigotes are found within the macrophages and multiply in them. Histologically these amastigotes stain well with Haematoxylin and Eosin or with Brown – Hopps modified tissue gram stain which makes them readily observable in the macrophages. The later stain is better as this best accentuates the kinetoplast and produces minimal background staining and maximum contrast. This is then observed on a light microscope at x1000 magnification which is mandatory inorder to visualize the cells. Other methods of visualizing the cells are through biopsy, tissue aspiration and *in vitro* culture which involve touch preparations and direct visualization of the parasites. These are used in clinically suspected cases and are complementary to tissue sectioning. Cells or smears are stained with Giemsa or Leishman stains and then viewed microscopically. These methods help in the confirmation of diagnosis e.g.

polyparasitic syndromes found during the early stages and in diffuse distribution are easily confirmed with this but oligoparasitic syndromes in which the parasites are few, occur during the late or healing stages are difficult to confirm hence multiple tissue sectioning are required (Berman, 2003).

2.6 Histopathology of Cutaneous Leishmaniasis

In cutaneous leishmaniasis, certain organs are involved and their histopathological findings have been documented. These include the skin, liver, spleen and lymph nodes.

2.6.1 Skin

This is one of the largest and most complex organs in the human body due to its great surface area. It is the target site and primary point of infection in cutaneous leishmniasis. Histologically, the skin consists of an epidermis, which is an epithelium and superficially located, dermis or corium, which is an elaborate connective tissue layer and hypodermis or subcutaneous tissue. The epidermis and dermis vary considerably in thickness in different parts of the body. In thin skin, only three layers are easily distinguished (stratum corneum and strata spinosum and germinativum or Malphigian layer) while in the thick skin five layers can be distinguished (strata corneum, lucidum, granulosum, spinosum and germinativum). The dermis consists of two regions: the papillary layer and reticular layer. The papillary layer consists of minute papillae or ridges which project into the epidermis. These papillae are vascular and contain a network of fine elastic fibres that support blood vessels, nerves and various cells of the immune system. The reticular layer is dense irregular connective tissue, composed of a network of collagen bundles interspersed with elastic fibres giving

the skin its elasticity. The hypodermis consists of loose connective tissue containing fat cells.

In the histopathology of cutaneous leishmaniasis, the skin is the target site as it is the part that is in contact with the sandfly. The epidermis in localised cutaneous leishmaniasis is characterised by single lesions which are hyperpigmented with raised edges and may or maynot be ulcerated. In the dermis, there is a dense infiltration of mixed inflammatory cells particularly the plasma cells, lymphocytes and histiocytes. Also small granulomata which are non-caseating replace the infiltrate as the lesions get older and the delayed–type immunity is up-regulated (Kenner, 2007). However in the diffuse type, the epidermis is characterised by multiple and widespread lesions which resemble lepromatous leprosy. The dermis contains sheets of macrophages with large numbers of amastigotes but few plasma cells and lymphocytes.

2.6.2 Spleen

This is one of the sites of secondary infection and visceralization of *L. major* infection. Histopathologically, it is characterised by follicular hyperplasia of the white pulp with increased number and size of the lymphatic nodules (Fabiola *et al.*, 2002). Parasites are found in the marginal zone (MZ) of the white pulp. The resident macrophages border the lymphoid follicles and phagocytose the amastigotes (Gorak *et al.,* 1998). However when these macrophages are infected they migrate to the red pulp area. The germinal centres are easily detectable and are surrounded by a large ring of small lymphocytes. The red pulp is infiltrated with vacuolated histiocytes or macrophages as well as small granulomata.

2.6.3 Lymph node

These are the routes through which infections spread. There is follicular hyperplasia and the germinal centres are larger, more numerous and are characterised by the presence of pale staining lymphocytes and numerous apoptotic bodies. In late infection there is marked hyperplasia of the sinusoids with infiltration of plasma cells as well as massive infiltration of infected macrophages with destruction of the germinal center and rupture of the capsule. Parasites are mainly found in the cortex and few in the medulla and sinus (Fabiola *et al.,* 2002).

2.6.4 Liver

This is characterised by inflammation and infiltration of the portal tract with infected macrophages. Kupffer cells are the resident macrophages in the liver and they are the initial hosts of the amastigotes. Resolution of the disease correlates with granuloma formation, which is a chronic delayed-type hypersensitivity reaction formed around the Kupffer cells. This leads to the formation of an inflammatory focus comprising of effector cells (T cells) and cytokines (IL-12, IL-2 and IFN-γ) and collaborates with parasite killing (Murray, 2000). Also infiltration of kupffer cells with parasites occurs in late infection and sinusoids are dilated (Fabiola *et al.,* 2002).

The histopathological spectrum of cutaneous leishmaniasis (CL) is essential in resolving the immunological mechanisms involved in the disease and to produce a classification related to the prognosis (Ridley, 1979). The observation of the histopathology of the disease before and after treatment is necessary to establish an accurate criterion for evaluating clinical cure in comparison to histopathological cure. This is because most clinicians adopt the clinical criteria based on the

complete healing and closure of the lesions but this may not necessarily be sufficient evidence for a complete cure (D'ultra e Silva, 1915) as studies have shown that leishmaniasis was still present in lesions that had healed over eight ears previously (Botelho *et al.,* 1998; Schubach *et al*., 1987). Also in another study involving 147 patients with no history of leishmaniasis, intradermal skin test was positive for 21 (8%) of them despite not finding any parasite in the nasal mucous membrane. This was indicative of subclinical infection (Guerra *et al.*, 1985). In another study, findings showed that some patients were still infected despite a clinical diagnosis declaring them cured (Marsden *et al.*, 1986). All these findings underline the importance of histopathological studies of organs in leishmaniasis, not only as an aid in diagnosis but also in the establishment of criteria to determine the success of the therapy. Thus, in spite of the search for the most effective drugs or therapy for leishmaniasis, it is necessary to establish accurate criterion for evaluating its cure. This has been used in several countries and several studies have characterized the different patterns that define the histopathology in cutaneous leishmaniasis.

Histopathological studies of leishmaniasis started in Brazil when the disease was first identified in the country and since then several authors have worked towards establishing the characteristic morphological patterns of the disease. The first histopathological classification was done by evaluating the American cutaneous and mucocutaneous leishmaniasis (Azulay*,* 1960) from the New world. Studies were then conducted on the diffuse cutaneous leishmaniasis if the Old World and this was classified into six histopathological groupings (Bryceson, 1969). Another study involving the Old world leishmaniasis proposed a simplified scheme with three histopathological phases (Nicolis *et al.*, 1978). In a related study utilizing

sixty biopsies from the prognosis of the lesions, a classification of five groups were made and later sub-groups were added but this made the scheme difficult to use and was abandoned (Ridley *et al.*, 1980). In a study using 487 skin fragments of patients from Tres Bracos, in the state of Bahia, Brazil, only three classifications were proposed while taking into account previous classifications. However, further studies were carried out using 378 skin biopsies and classification was into five groups of histopathological patterns which were type I-exudative-cellular reaction without granulomas, type II-exudative-necrotic reaction without granulomas, type III-exudative and necrotic-granulomatous reaction (i.e. unorganized granulomas), type IV-exudative granulomatous reaction without necrosis and type V- exudative tuberculoid reaction(ie organized granulomas) (Magalhaes *et al.*, 1986). Finally based on all the classifications made, a simplified classification of just three patterns was proposed by Bittencourt and Barral (1991). This has been used in the classification of histopathological patterns in several countries with slight modifications. In a study conducted in Mexico, 73 biopsies were taken and based on the patterns proposed by Bittencourt and Barral, the most common pattern was characterized by the presence of unorganized granulomas without necrosis (Andrade-Narvaez *et al.*, 2005).

2.7 Treatment of Leishmaniasis

2.7.1 Chemotherapy

The clinically used chemotherapeutics are based on the antimonials, which were developed before 1959. These are derived from the heavy metal antimony (Sb). These antimony-containing drugs have become the front line treatment of choice in many countries. They were first introduced as trivalent (Sb^{III}) forms but their

toxic side effects led to the development of the pentavalent antimonials (Sb^V). These pentavalent forms have been used for over 50 years and are still the mainstay of systemic therapy as they are 10-fold less toxic to mammals then the trivalent forms and thus have a higher therapeutic index (Roberts *et al.*, 1995). The two main pentavalent antimonials used are sodium stibogluconate (SSG) commercially available as Pentosam® and meglumine antimoniate available as Glucantimine®. However, they require extended courses of parenteral administration (Intramuscular and Intravenous) and have toxic side effects such as abdominal symptoms, liver enzyme elevation, pancreatitis bone marrow suppression, neuropathy, cardiac toxicity and sudden death (Herwaldt and Berman, 1992). In addition, there has been a decrease in their effectiveness due to parasite's increasing resistance to the drug hence higher doses are administered over long durations resulting in an increase in toxicity (Sundar, 2001). More recently is the use of combinatorial therapy of these antimonial drugs with other forms such as non-ionic surfactant vesicular formulations (SSG-NIV) (Carter *et al.,* 2001) or with cytokines such as interferon gamma and granulocyte macrophage colony stimulating factors or other adjuvants as a strategy to combat resistance. These have been shown to be more effective than the free pentavalent antimonials.

Studies on the pentavalent antimonials have shown that it is stage specific since it is more active on the amastigotes than the promastigotes as amastigotes are 73 to 271 fold more susceptible (Roberts *et al.,* 1995). Studies have also demonstrated that combinatorial therapy using these pentavalent antimonials with cytokines as adjuvant or other carrier systems resulted in greater antileishmanial efficacies. Examples of such studies showed that synergism of IL-2 stimulated

splenocytes and Pentosam® enhanced the killing of *L. donovani* parasites *in vitro* (Zohreh *et al.,* 1996). In another study, the use of a nonionic surfactant vesicular formulation of SSG (SSG-NIV) markedly improved the therapeutic efficacy of the pentavalent SSG (Carter *et al.,* 2001). However, the efficacy of SSG has become increasingly compromised due to the emergence of resistance within the parasite population and the associated toxicity. As a result of this, research studies have focused on the search for new, cheaper and less toxic drugs. Amphotericin B (AmB), one of the new drugs is an antibiotic used as an antifungal agent (Guerin *et al.*, 2001). It has been shown to be effective against leishmania species and superior to antimony containing drugs hence in many cases has become the first line of treatment (Mbonogo et al., 1998). Studies on its mode of action demonstrate that it is a form of programmed cell death (Lee *et al.*, 2001) as it binds to the sterols hence impairs the osmotic balance of the membrane which causes pores to form in the membrane and thus the leakage of small cations such as potassium and magnesium from the cell (Lamothe, 2001) resulting in the death of the cell. Studies have also shown that it is an effective antileishmanial drug but it is still toxic at high doses. To overcome its toxicity, new lipid formulations have been developed which are better tolerated but rather expensive (Guerin *et al.*, 2001).

2.7.2 Alternative Chemotherapy

Alternative treatments in chemotherapy have evolved as a result of the search for cheaper and less toxic drugs. These include highly effective lipid formulations such as amphotericin B, which is an antibiotic, used as parental antifungal agent (Guerin *et al*., 2001) and paromomycin, a topical antibiotic. Others are the oral azoles such as fluconazole, itrconazole and ketoconazole. These have to be given in higher dose regimen to overcome resistance (Shahbaz *et al.*, 2005). The

most recent development in the search for alternative drugs for treatment led to the discovery of mitelfosine. Mitelfosine (Hexadecylphosphocholine) was originally developed as a treatment against breast cancer but today it is the first effective oral therapy to be used in the treatment of leishmaniasis. It is a membrane-active alkyl phospholipid which inhibits the parasite's membrane signalling pathway (Paterson, 2002). Another new and promising agent is imiquimod (Aldara), an immunomodulator (Soto *et al.*, 2001). However, these options are very expensive and cannot be afforded in the third world countries where the disease is endemic as such further alternative treatments are strongly needed and particularly targeted at being cheap, less toxic and accessible to the people living in the rural endemic areas where lack of infrastructure does not allow for adequate chemotherapy. These alternatives lie in the use of naturally occurring plant-derived compounds and vaccines.

2.7.3 Immunotherapy

Vaccines

Historically, cutaneous leishmaniasis has been the focus of vaccination attempts. This may be due to the fact that it has been known since antiquity that individuals who had healed their skin lesions were protected from further infections. Thus, the impetus for the development of vaccine came from the solid immunity, which followed recovery from cutaneous leishmaniasis. This life long memory was most likely maintained by persisting organisms in a process called concomitant immunity (Aebischer *et al.,* 1993). Such practices to acquire this immunity was seen among the bedouin or some kurdistani tribal societies who traditionally exposed their babies' buttocks to sand fly bites in order to protect them from facial lesions. Also in the Middle East, another ancient technique used, was to transfer infectious material from lesions to uninfected individuals using a thorn (Handman,

2001). However, with the establishment of culture conditions necessary to maintain the growth of promastigotes by Nicolle and Manceau in 1908, life organisms were used for vaccination and to control infections (Nicolle, 1908). This was utilised on a large scale in the then Soviet Union and Israel (Greenblatt, 1980; Kellina, 1981) with a high percentage of successful lesion development but the success of the strategy depended on the viability and infectivity of the infected organisms as organisms which have lost virulence were shown to induce delayed-type hypersensitivity but did not protect from subsequent natural infection (Kellina, 1965). The use of these life vaccines or persistence of parasites had its disadvantages which included the development of large uncontrolled lesions, exacerbation of skin diseases and psoriasis, immunosuppression and reactivation of the disease in immunocompromised persons (Modabber, 1995). This led to the discontinuation of the use of life virulent organisms for vaccination and in the early 1990s the focus shifted to the use of other forms of vaccines such as killed forms and attenuated forms and recently molecular or protein and DNA vaccines. However, the use of life vaccines led to the conclusion on the properties of vaccines. These properties include: that a vaccine must be molecularly defined, induce long term memory in the absence of the persistent life organisms and should not induce deleterious immune responses which are dominated by the cytokine interleukin-4 (IL-4) or TH-2 type responses as this will lead to severe pathology (Locksley and Louis, 1992).

I. Attenuated Vaccines

This vaccine mimics natural infection without the side effects or long-term persistence of infective organisms. It was initiated by the isolation of avirulent clones of the leismania parasite but the lack of knowledge of the mutations which led to the loss of virulence and the risk of virulent revertants made it unacceptable

(Handman and McConville, 1990). This led to the production of attenuated forms such as radioattenuated parasites or biochemically-altered parasites that lack the dihydrofolate reductase or thymidilate synthetase gene but could invade macrophages and persist for several days hence conferring very good protection. Hence, the development of a safer attenuated vaccine is now on clinical trials in humans (Titus *et al.,* 1995). However, due to the complicated and costly mode of production and the logistics of delivery on a large-scale it was abandoned and other forms of vaccine adopted.

II. Protein/Subunit Vaccines

The use of peptide vaccines particularly those considered to be T-cell epitopes was common in the 1980s.These are molecularly defined vaccine candidates such as the surface protease gp63, the surface antigen gp46/M2 and the related parasite antigen 2 (PSA-2) (Xu *et al.,* 1995; McMahon-Pratt *et al.,* 1993; Handman *et al.,* 1995). These antigens or proteins induced significant protection in animal models when delivered with adjuvants. Other delivery systems include infectious vectors such as Salmonella, Bacillus (BCG) or Vaccinia. These vaccines have focused primarily on protein antigens because they are easy to identify, isolate, clone and study. The disadvantage of protein vaccines is that there maybe non-responders to a single antigen vaccines in the genetically diverse human population but this maybe overcome by the use of cocktail vaccines which contain several antigens and multiple epitopes of each antigen (Rafati *et al.*, 2001).

Several peptide antigens such as the gp63 which is a membrane protease present in promastigotes in all species have been studied. Studies have shown

that both the recombinant and the native proteins seem to protect against infection with *L. amazonensis* in animal models (Jardin *et al.*, 1991; Spitzer *et al.*, 1999). A second protein antigen tested in animal models is the membrane antigen gp46/M2 or parasite surface antigen 2 (PSA-2). It demonstrated protective effects in mice against infection with *L. major* or *L. mexicana* when administered as a purified protein or expressed in vaccinia virus (Champsi and McMahon-Pratt, 1988). Also a protein derived from the yeast *Pichia pastoris* provided good protection against infection with *L. donovani* (Handman *et al.*, 2001). The leishmanial eukaryotic ribosomal protein cIF4A is being considered as a vaccine candidate based on its ability to induce Th1-type cytokines in humans (Skeiky *et al.*, 1998). A similar protein antigen studied, is the *Leishmania* homologue of the receptor activated C kinase (LACK) which is expressed by both promastigotes and amastigotes. It has been shown to protect mice from infection particularly when administered with IL-12 as an adjuvant (Gurunathan *et al.*, 1997). However, today focus seems to have moved to the use of naked DNA vaccines or recombinant DNA. This is because several considerations make the peptide antigen less attractive such as the magnitude of the T-cell memory induced, the inability of all individuals in the population to respond to the peptide and the economics of production.

III. Nonprotein antigen vaccines

Studies have demonstrated that not all potential vaccine molecules are proteins as lipophosphoglycans (LPG), which play a major role in infection have also been used as vaccine to protect mice from infection with *leismania major* (Handman and Mitchell, 1985). Studies on vaccine development have shown that glycolipids such as Leishmania lipophosphoglycans (LPG) provided excellent protection (Russell and Alexander, 1988). However, the form in which these antigens occur

and the integrity of its molecules are essential as the water soluble form of LPG which lacks the glycoslyphosphatidylinositol anchor was not protective and resulted in exacerbation of the disease (Mitchell and Handman, 1986). Today, it is accepted that many novel microbial antigens including mycobacterial glycolipids can be recognised by T-cells hence are potential vaccine candidates (Moody *et al.*, 2000).

IV. DNA vaccines

The discovery that the direct injection of plasmid DNA encoding foreign proteins could lead to endogenous protein biosynthesis and a specific immune response against it opened new perspectives in vaccine development. This is a relatively recent and new development in vaccine methodology. It is also called nucleic acid vaccination and it involves the administration of genetic material (usually plasmid DNA) rather than protein or glycolipid antigen. The gene encoding the vaccine candidate is cloned in a mmammalian expression vector and the DNA is injected directly into muscle or skin (Ulmer *et al.*, 1996; Donnelly *et al.*, 1997; Walker *et al.*, 1998). The plasmid DNA is taken up by cells and translocated to the nucleus where it is transcribed into RNA and then translated in the cytoplasm of the cell into protein or antigen. The efficiency of uptake and the expression of the plasmid DNA maybe low but enough to provoke immune responses in both T and B cells (Hasan *et al.*, 1999; Gurunathan *et al.*, 2000). The antigen is then presented to the immune system via either the MHC class I or class II pathways (Wahren, 1996). This leads to the provocation of immune responses in both T and B lymphocytes. DNA vaccines induce a complete immune response against the encoded antigen but exact mechanisms involved in this process are still poorly understood, and particularly the type of $CD4^+$ and $CD8^+$ effector and memory cells activated (Liu *et al.*, 2006). Apart from their immunogenicity and efficacy,

there are several features of DNA vaccines that make them very advantageous against tropical diseases. Firstly, the possibility of a long-lived 'trickle' production of the antigen similar to the situation in infection which may give a Th1–cell mediated response and better immunological memory and no need for an adjuvant. Secondly, they are extremely safe as they do not contain any pathogenic organism that may revert in virulence. Furthermore, there are major concerns of genomic integration of the plasmid DNA or insertional mutagenesis (Wahren, 1996) but these have also been extensively studied in safety studies and found to be rather unlikely (Sheets *et al.*, 2006) Additional safety issues such as anti-DNA antibodies or induction of autoimmune disorders have also been addressed in a growing number of preclinical and clinical studies (Sheets *et al.*, 2006; Handman, 1997), which have confirmed the high safety of these vaccines. Thirdly, with respect to manufacturing, storage, and distribution, they present major benefits in that the production process is the same for any DNA vaccine, which is not the case for other types of biological and vaccines, for which a specific protocol has to be developed for each. This makes production easy and costs will likely go down as this type of vaccines become mainstream and future technological improvements are implemented. Fourthly, plasmid DNA is a very stable molecule, especially compared to recombinant or live attenuated vaccines, and this would greatly facilitate storage and distribution of DNA vaccine in tropical settings which have limited health infrastructure thus cutting down on the huge costs associated with the cold chain. Fifthly, administration is easy, as simple intramuscular or intradermal injections can be sufficient. Also, multiple plasmids can be combined for the elaboration of multivalent vaccines (Liu *et al.*, 2006). Overall, DNA vaccines may thus represent an ideally affordable alternative for disease control especially in the third world countries. This explains in part the

growing interest in their development for the control of tropical parasitic diseases such as malaria, leishmaniasis, or chagas disease.

Types of DNA vaccine

Over the past 15 years, DNA vaccines have gone from a scientific curiosity to one of the most dynamic fields of research and may offer new alternatives for the control of infectious diseases (Liu *et al.*, 2006). The first two DNA vaccines licensed in recent years were to protect horses from West Nile virus and salmons from infectious hematopoietic necrosis virus thus confirming the usefulness of this biotechnology. Leishmaniasis is caused by at least 20 different species of parasites with diverse relationships with the host and mechanisms of pathogenesis (Alexander and Bryson, 2005) as such several types of DNA vaccine have evolved and different strategies in their use are being studied. These include

Single antigen DNA vaccines

The earliest single antigen DNA vaccine experiments against Leishmania used the *L. major* Gp63 antigen, and this has been extensively used as a recombinant or protein vaccine. Immunization with a plasmid encoding Gp63 was able to induce a Th1-type cytokine profile and a significant reduction of lesion size after challenge of the immunized mice with *L. major* (Xu and Liew, 1995; Walker, 1997; Walker *et al.*, 1998). Subsequent studies investigated DNA vaccines encoding a large variety of Leishmania proteins and showed that many different DNA vaccines were able to induce a Th1 immune response, and confer variable degrees of protection as assessed by reduction in skin lesion size and/or parasite burdens in mouse models. Also, it is clear from studies comparing different DNA vaccines that the nature of the antigen encoded by the DNA is a key parameter for efficacy. Also, a few studies provided interesting comparisons of the same

antigens administered as recombinant protein or DNA vaccines and showed that the latter were more effective than their recombinant protein counterparts. Results of these studies showed that DNA vaccines were able to induce a stronger Th1 bias in the immune response, a longer-lasting immunity, and/or a better protection against disease progression (Gurunathan *et al.*, 1998; Iborra *et al.*, 2003; Rafati *et al.*, 2006). However, most of these studies used a rather artificial infectious challenge which was based on the injection of high parasite doses via unnatural routes. In other related studies the superior efficacy of DNA vaccines was also observed using a low-dose intradermal challenge in the ear, which was proposed to more closely mimick natural infection (Mendez *et al.*, 2001). In these studies, both DNA and protein vaccination were able to induce very similar level of short-term (2 weeks post vaccination) protection against infection with *L. major,* but DNA vaccine was able to induce long-term (12 weeks postvaccination) protection (Mendez *et al.*, 2001). These results not only confirmed the strong potential of DNA vaccines against *Leishmania*, but also indicated that in most cases only partial protection was achieved. Hence, the prime-boost immunization protocols were developed and have been tested with various antigens to increase vaccine potency. They are based on priming the immune response with a DNA vaccine and boosting with the corresponding recombinant vaccine based on recombinant virus or protein. In some studies, such immunization protocol resulted in increased immunogenicity of the vaccines and better protection levels (Ganzalo *et al.*, 2002; Tapia *et al.*, 2003) , but in others, DNA only remained the best formulation for optimum efficacy (Rafati *et al.*, 2001). Nonetheless, a major drawback of such vaccine formulation remains its complexity, which may limit their practical use.

Multiple antigen DNA vaccines

An alternative way to broaden vaccine immunogenicity and increase its efficacy has been to use combination of plasmids encoding various antigens. For example, a study demonstrated that cysteine proteinase a and b (CPa and CPb) DNA vaccines are not protective when used individually, but immunization with a combination of both plasmids induced long-term protective immunity (Rafati *et al.,* 2001). Alternatively, gene fusion has also been successively used to achieve expression of an antigenic fusion protein from a single plasmid construct (Campos-Neto *et al.*, 2002) and then this is used to immunize the mice. Overall, expression of several antigens mostly resulted in increased efficacy, but this also depended on the antigen combination (Dumonteil *et al.*, 2003; Iborra *et al.*, 2004; Rosado-Vallado *et al.*, 2005). Most authors thus argue that a successful Leishmania vaccine is likely to be based on multiple antigens. In all the above studies the DNA vaccine has been used as a prophylactic, however other studies have shown that it could be used as a therapeutic vaccine.

Therapeutic DNA vaccines

An additional advantage of DNA vaccines is their potential as therapeutic vaccines, aimed at reinforcing or redirecting the immune response of an infected host to control disease progression (Autran *et al.*, 2004). The major advantage of this strategy in addition to its efficacy is that it relies on short treatment regimens, and it is thus an attractive alternative to chemotherapy, particularly in the case of Leishmania with so few chemotherapeutic options. Thus, administration of as little as two doses of a DNA vaccine encoding PSA-2 has the ability to control an ongoing infection with *L. major* in mice (Handman *et al.*, 2000). The therapeutic effect is due to a shift of the immune response towards a Th1 immune response (Handman *et al.*, 2000). Similarly, a DNA vaccine encoding *L. donovani*

nucleoside hydrolase NH36 has therapeutic activity against murine visceral leishmaniasis caused by *L. chagasi* (Gamba-Leon *et al.*, 2006). The simplicity of such treatment makes them very advantageous compared to chemotherapy. In addition, the fact that the same DNA vaccine can be effective for both the prophylaxis (Aguilar-Be *et al.*, 2005) and the therapy of leishmania infection is thus very promising as this would provide a versatile tool for the control of this parasite.

Non-Leishmania antigens as vaccines

While all the above DNA vaccines were based on *Leishmania* antigens, an alternative approach used antigens derived from sand-fly's saliva. Indeed, it has been shown that sand-fly's saliva can exacerbate leishmania infection (Melby *et al.*, 2001), and preexposure of mice to saliva components may be sufficient to induce protection against infection (Kanhawi *et al.,* 2000). Thus, a number of salivary antigens have been tested as vaccines against *Leishmania*. Maxadilan is a potent vasodilator from sand-fly's saliva and was found to be responsible of most of the exacerbatory effects of whole saliva on leishmania infection (Morris *et al*., 2001). Immunization with this antigen (as a recombinant vaccine) protected mice against *L. major* infection. Other salivary components, such as *Phlebotomus papatasi* SP15, have been tested as DNA vaccines and found to protect mice against *L. major* and while the vaccine induced both humoral and DTH responses, protection seemed to be mostly accounted for by the latter, as B-cell deficient mice remain protected (Valnzuela *et al*., 2004). Thus, characterization of sand-fly's salivary proteins may lead to the identification of new vaccine candidates (Valnzuela *et al.*, 2004; Anderson *et al.*, 2006). However, as for Leishmania antigens, salivary protein polymorphism remains an important issue

and may limit the usefulness of such antigens as vaccine candidates (Milleron *et al.*, 2004; Elnaiem *et al.*, 2005).

Cross-protection against multiple Leishmania species

In view of the aim of this study which is on investigating the cross-protective effect of *L. donovani* γGCS vaccine against *L. major* infected mice, many studies have previously been conducted to study cross protective effects. This is necessitated because one of the challenges facing the development of a leishmanial vaccine is the large number of species, as well as the high variability within species. Early studies of cross-protection between *Leishmania* species clearly showed that it is a complex problem, with infection by one specie protecting or not protecting from subsequent infection by another specie. This is dependent on the species and the order of infections. Thus studies have focused on the polymorphism of leading antigens such as Gp63 and stuies have revealed that it is very polymorphic (Espinoza *et al.*, 1995; Guerbouj *et al.*, 2001). Such polymorphism has important implication for vaccine development as it may limit their efficacy against variant strains of parasites or novel escape mutants, and thus restrict vaccine protection to a single species (Alvarez-Valin *et al.*, 2000; Guerbouj *et al.*, 2000). Antigen polymorphism between multiple strains and species is today becoming a major issue in many vaccine development studies (Fitzpatrick *et al.*, 2005; Kumkhaek *et al.*, 2005). In the case of leishmania, few DNA vaccines have been tested against multiple species. Example is the LACK antigen, initially identified in *L. major* and was found to be very conserved between Leishmania species, hence could protect mice against *L. major* (Gurunathan *et al.*, 2000) and *L. amazonensis* (Pinto *et al.*, 2004), but not against *L. mexicana* (Dumonteil *et al.,* 2003), *L. donovani (*Melby *et al.,* 2001), or *L. chagasi* (Marqus-da-Silva *et al.,* 2005). On the contrary, *L. amazonensis* nuclease protein P4 could protect against both *L.*

amazonensis and *L. major,* but cross-protection required a different formulation (with IL-12 or HSP70 as adjuvant) respectitvely (Campbell *et al.*, 2003)]. In another related study, antigens from one specie were used to induce protection against other species (Iborra *et al.*, 2003), but the extent of cross-protection against the various species was not investigated. More recently, a single formulation of *L. donovani* NH36 DNA vaccine was found to induce a very good protection against both *L. chagasi* and *L. mexicana,* suggesting that this DNA vaccine may be able to provide broad protection against various Leishmania species (Aguilar-Be *et al.,* 2005). Although, most vaccine studies have been focusing on homologous protection, a single vaccine able to protect against all pathogenic species would be very ideal.

DNA vaccines against nonmurine leishmaniasis

Based on the success of many of these DNA vaccine studies in mice, a few vaccine candidates have been tested in additional animal models, possibly more relevant for the development of a veterinary or human vaccine. PFR-2 and KMP11 antigens were tested as DNA vaccines in hamsters, a highly susceptible animal model. PFR-2 was tested as protein, DNA, or DNA-protein immunization, and protection levels against *L. mexicana* varied greatly depending on vaccine formulation, route of immunization, and sex of the animals Saravia *et al.*, 2005). Also, contrary to mouse studies, protein vaccination seemed more protective than DNA only vaccination. However, in mouse studies, heterologous prime-boost vaccination with DNA and protein seemed better than DNA only (Saravia *et al.*, 2005). Another DNA vaccine encoding PapLe22 was found to be immunogenic in hamsters and decreased parasitemia after infection with *L. infantum,* but further assessment of disease was not performed (Fragaki *et al.*, 2001). Immunization with KPM11 DNA induced a mixed Th1/Th2 response, but was able to protect

hamsters against visceral leishmaniasis caused by *L. donovani* (Basu *et al.*, 2005). In dogs, while several protein vaccines have been tested and a purified protein vaccine has now been licenced for veterinary use (Saravia *et al.*, 2006), very few DNA vaccine studies have been performed. A heterologous prime-boost strategy using CPa and CPb DNA and protein was reported as immunogenic and protective (Rafati *et al.*, 2005), but the study was of limited power given the reduced number of animals. In another study, dogs were immunized with a mixture of DNA vaccines encoding 10 different antigens previously tested in mouse models, and this immunization induced a very good immune response, with a high production of IFNγ. However, evaluation of protection was limited to an acute in vitro assay (Saldarriaga *et al.*, 2006) and further studies will be required to assess the potential of this vaccine in dogs. In spite of their limitations, these studies clearly showed that several DNA vaccines can induce a potent immune response in nonmurine animal models, and it is likely that a good level of protection can be achieved in these as well, provided the correct antigens and vaccine formulation are used.

2.7.4 Phytotherapy

Natural products are not only the basis for traditional or ethnic medicine but recently have proved effective in providing highly successful drugs such as Artemisinin used in the treatment of malaria (Kayser *et al.*, 2003). Though the development of synthetic drugs decreased the use of these natural products in the past, there is presently a worldwide movement towards the use of these products and an increasing demand for herbal preparations (Ketzis, 1999). In the US, regulatory requirements are a barrier to the development of phytotherapeutics hence the US pharmaceutical industry prefers the use of

synthetic or semisynthetic compounds (Drummond and Waigh, 2000). On the other hand, globally, there is a patient-driven trend towards natural remedies as it is estimated that two-third of the world population rely on these traditional remedies due to limited availability and affordability of pharmaceutical products (Tagboto and Townson, 2001). Even in the US, there has been a 20% increase per year since the 1990s in the use of natural medicines as well as the sale of non-prescribed herbal medicines has been estimated at four billion dollars per year. However, in Europe and Asia were the regulations are less restricting; the number of natural product patents has surged in recent years (Drummond and Waigh, 2000). In developing countries, parasitic diseases of which leishmaniasis is one, remain public health issues and due to the poor economic environment particularly in the endemic regions, the standard antileishmanial chemotherapeutics are unaffordable hence the use of these ethnomedicinal preparations. Sequel to this, WHO has advocated the use of ethnomedicine where appropriate health services are inaccessible as well as sponsors programmes or research works to screen natural products for potential use in the therapy of leishmaniasis (Weniger *et al.*, 2001). Many new natural product groups have revealed antileishmanial properties of surprising efficacy and selectivity. These groups include the plant-derived alkaloids, terpenes and phenolics (Rocha *et al.,* 2005). Pharmaceutical companies the world over have now become more interested in finding new substances of natural origin that are effective but with low toxicity to man for their products in the treatment of these diseases. This could be achieved through the leads obtained from screening and study of indigenous plants (Carvalho *et al.*, 2000). However, some of the difficulties associated with the use of ethnomedicinal preparations include, severe chemo-physical drawbacks such as poor solubility, lack of proper documentation and

validation, difficulty in standardization and unavailability of plant parts all year round (Kayser *et al.*, 2003). In addition, because ethnomedicinal practice is not regulated, it can be prone to abuse and quackery.

Plants used as antileishmanials

Literature has revealed that limited success has been achieved despite enormous efforts by several workers in the field of chemotherapy to discover or develop an ideal antileishamnial drug. This has prompted the urgent need for alternative drugs to combat the disease. This is of high priority to the World Health Organization (WHO) and through its tropical disease programme, it leads the course in the investigation of plant products used in traditional medicine for the treatment of leishmaniasis. This has stimulated several research works to evaluate tropical plants for antileishmanial properties and results have shown that some of these plants possess leishmanicidal activity especially when used *in vitro.* Plants which have shown antileishmanial activity can be categorized based on the presence of metabolites such as quinones, alkaloids, terpenes and phenolic derivatives.

Quinones

In Bolivia, studies have shown that plumbagin a naphtoquinone derivative extracted from the bark of the plant *Pera benenisis* (Euphoribiaceae), commonly used in the treatment of cutaneous leishmaniasis, had both in vitro and in vivo effects against different species of leishmania and was not toxic to normal macrophages. Its mechanism of action is based on the generation of free oxygen radicals which the parasites are unable to defend (Fournet *et al.*, 1992). Also a quinone derivative, jacaranone isolated from the leaves of *Jacaranda copaia* (Bignoniaceae) showed antileishmanial activity when used *in vitro* but had a weak

in vivo activity when administered subcutaneously to mice with cutaneous leishmaniasis (Sauvian *et al.*, 1993).

The anthraquinone-2-carbaldehyde obtained from the roots of *Morinda lucida* (Rubiaceae) a plant which grows in Malaysia showed selective activity against *Leishmania major* specie. This activity is attributed to the presence of functional groups (Camacho *et al.*, 2000.).

Alkaloids

Studies have shown that the alkaloid Berberine has high leishmanicidal activity and it is the main constituent in various folkoric remedies used in the treatment of cutaneous leishmaniasis, malaria and amoebiasis (Phillipson and Wright, 1991). It is an isoquinolinic alkaloid found in number of plant families such as Annonaceae, Berberidaceae and Menispermaceae. It has been used in the treatment of leishmaniasis for over 50 years in Asia and it has demonstrated significant activity both *in vitro* and *in vivo* against several leishmania species. Studies have shown that when used at concentrations of less than 10 μg/ml, it effectively eliminates *L. major* parasites in peritoneal macrophages but shows minimal activity when applied topically on mice cutaneous lesions caused by *L. major* (Wright and Philipson, 1990). In another study, indole alkaloids from the bark of *Corynanthe pachycerus* (Rubiaceae), a plant used in the middle East, showed marked activity against promastigotes of *L. major* and did not show any significant cytotoxicity against normal cell lines such as KB and KB-Vero cell lines. This indicates an important selectivity in their activity with the mechanism of action based on the inhibition of the respiratory chain of the parasites (Staerk *et al.*, 2000).

Terpenes

Picroliv is standardized mixture of iridoid glycosides obtained from the roots and rhizomes of picrorhiza kurroa. Studies have reported its use in the induction of a

high degree of protection against leishmania and plasmodium infections and its ability to induce non-specific immune responses in hamsters (Puri *et al.*, 1992).It has also been shown to prevent liver damage in laboratory animals as a result of toxicity associated with the use of sodium stibogluconate (Pentosam) in the treatment of leishmaniasis (Mittal *et al.*, 1998). On this basis it has been proposed to be used as an adjuvant to increase the efficacy of leishmanicidal drugs used in chemotherapy. Espintanol, a monoterpenoid isolated from the bark of O*xandra espintana* (Annonaceae) showed significant activity against promastigotes of twelve species of leishmania including *L. mexicana* which is responsible for cutaneous leishamniasis in the New World (Hocquemiller *et al.*, 1991). *Vernonia brachycalyx* is a specie of Asteraecea family and it contains terpenes. Its major metabolite is sesquiterpene dilactone showed some activity (IC_{50}=17 μg/ml) against the promastigotes forms of *L. major*. However, it inhibited the proliferation of human lymphocytes suggesting that its activity is due to general toxicity and its administration could suppress the immune mechanism in humans (Oketch-Rabah *et al.,* 1997a). Hederin (α and β) and hederagenin are saponins obtained from the leaves of *Hedera helix* (Ardiaceae). Results of studies show that only hederagenin had significant activity against the amastigote forms of leishmania species (Majester-Savornin *et al.,* 1991). In another study, hederin (αandβ) showed some strong activity against promastigotes of the *Leishmania* species but also showed a strong antiproliferative activity on human monocytes. They inhibit the growth of the promastigotes by acting on the membrane of the parasite thereby eliciting a drop in membrane potential (Delmas *et al.*, 2000). Muzanzagewnin is a sapogenin obtained from the roots of Asparagus Africanus (Liliaceae) showed activity against promastigotes of *L. major* but also inhibited the

proliferation of human lymphocytes. This indicates that it does not possess a selective toxicity against the leishamania parasite (Oketch-Rabah *et al.*, 1997[b]).

Phenolic Derivatives

Some of the phenolic derivatives with antileishmanial activity include chalones and flavanoids which occur in a variety of plant species. Studies have shown that that the oxygenated chalone, Llicochalone A from the roots of the Chinese licorice plant (Fabaceae) inhibited the *in vitro* growth of promastigotes of *L. major* and *L. donovani.* It exhibited a remarkable capability to eliminate amastigotes of *L. major* in human peripheral blood monocyte-derived macrophages and U937 cells. It also prevented the development of lesions in BALB/c mice infected with *L. major* when treated intraperitoneally. Furthermore, it reduced the parasite load in the liver and spleen of hamsters infected with *L. donovani* when treated orally and intraperitoneally (Chen *et al.*, 1993; Chen *et al.*, 1994). The proposed mechanism of action involves the alteration of the ultrastructure and function of the mitochondria thus exerting its effect on the parasite respiratory chain without damaging the organelles of the macrophages or their phagocytic functions (Zhai *et al.*, 1995). However, results of the *in vitro* assessment on the proliferation of human lymphocytes showed an inhibitory effect thus its use for the treatment of leishmaniasis in human may have a suppressive effect on the immune system as undesirable side effect (Neilsen *et al.*, 1998). Also, desmethoxycurcumin, a curcumin which are phenolic diketones, isolated from the rhizomes of *Curcuma longa* exhibited leishmanicidal activity when tested against promastigotes of *L. major*. However, they inhibited the proliferation of human lymphocytes, thus they do not possess selective antiprotozoal activity (Rasmussen *et al.*, 2000).

Another group of metabolites which are biosynthetically related to the chalones are the aurones. Research has demonstrated their antileishmanial activity against

promastigotes forms of *L. major* and other species. Some have shown toxicity towards bone-marrow derived macrophages (Kayser *et al.*, 1999). Minquartynoic acid is a fatty acid derivative isolated from the cortex of *minquartia guianansis,* a plant from Malaysia. Studies have shown it has moderate activity against *L. major* in vitro and this has been attributed to its general cytotoxicity (Rasmussen *et al.*, 2000). Other metabolites which also have shown antileishmanial activities include the acetogenins such as senegalene, squamocine, asimicine and molvizarine isolated from the seeds of Annona senegalensis (Annonaceae). Results have shown their activity against promastigotes of L. major and cytotocity against KB and VERO cell lines (Shapaz *et al.*, 1994)). Although a clear structure-activity relationship has not been established for acetogenin, their leishmanicidal activity has been attributed to the presence of the hyroxy groups or tetrahydrofuran ring in their structure (Eshiet and Akinsanya, 1971; Majekodunmi *et al.*, 1996).

Other Metabolites

In addition to these classified metabolites, several screenings of medicinal plants used for the treatment of leishmaniasis have been carried out in regions such as Spain, Iran, Sudan, Peru, Ethiopia and Guinea Bissau. These are regions endemic for this disease. Studies have confirmed the importance of many plant species as potential sources for the isolation of novel metabolites with leishmanicidal or immunostimulatory activities. Such studies carried out in Iran, showed that the herbal extract designated Z-HE, which is a mixture of Iranian medicinal plants was more effective when applied topically, in alleviating the symptoms of cutaneous leishmaniasis (74.4%) than the regular meglumine antimoniate (27.1%) (Zerehsaz *et al.*, 1999). Also the methanolic extracts of Artemisia aucheri and Camalli sinensis were used on *L. major in vitro* and results demonstrated that the extract of *A. aucheri* was strikingly potent against *L. major*

at 750μg/ml as it inhibited the growth of promastigotes after 72 hours (Sharif *et al*., 2006). In Peru, extracts of seven medicinal plants used specifically in the treatment of cutaneous leishmaniasis were investigated *in vitro* for leishmanicidal effects against promastigotes and axenic amastigotes forms of *leishmania amazonensis* which is responsible for the New World form of the disease. Results showed that one of the plants, *Himatanthus succuba* (Apocynceae) had some interesting antileishmanial activity with IC_{50} of 5 μg/ml in amastigotes and this validated its traditional use in the treatment of cutaneous leishmaniasis in Peru (Castillo *et al.,* 2007). Another study in Vietnam showed that the methanolic extract of the leaves of *Maesae balansae*, which is a Vietnamese medicinal plant, used in the treatment of leishmaniasis had potent *in vitro* and *in vivo* activities against *L. infantum* responsible for visceral leishamniasis. Bioassay purification of the extract identified the triterpenoid saponins called maesabalides I-IV. No cytotoxicity was present in the human fibroblast (MEC-5) cell line. *In vivo* evaluation in BALB/c mice showed a reduction in the parasite burden in the liver after treatment with a single cutaneous dose of 0.2-0.4mg/kg bw (Nils *et al.,* 2005). In Brazil, research results show that the aqueous extract of *Kalanchoe pinnata* leaves which is popularly used for the treatment of topical wounds, delayed the onset of cutaneous leishmaniasis in BALB/c mice infected with *L. amazonensis* when given orally (Da Silva *et al.,* 1995).

In Africa, several research works have been conducted in many countries where the disease is endemic. Such studies include the *in vitro* assessment of the crude methanolic extract of some Sudanese plants for antileishmanial activity. Results showed that only thirteen species had considerable *in vitro* activity on *L. major* parasites and these were *Azadirachta indica, Alum sativa* and *Acacia nilotica* with

LC_{50} of 10.2, 4.9 and 89.4 μg/ml respectively (Khalid *et al.*, 2005). In another study in Sudan, eight medicinal plants were investigated for leishmanicidal activity and only three were found to possess considerable *in vitro* activity on *L. major* promastigotes at concentrations less than 0.5 μg/ml. These were *Azadirachta indica, Maytenus senegalensis* and *Eucalyptus globulus* (El Tahir *et al.*, 1998). In Cameroon, eight extracts from seven selected medicinal plants indigenous to Cameroon and traditionally used in the treatment of malaria and other protozoan diseases were tested *in vitro* for antileishmanial activity against *L. donovani*. Results demonstrated that only five out of the eight extracts were active against the *L. donovani* parasites with IC_{50} values below 5 μg/ml and the methanolic seed extract of *Harungana madagascarensis* showing the greatest activity (Ndjakou *et al.*, 2006). In Ethiopia, the antileishmanial activity of the chloroform and methanol extracts of *Vernonia amygdalina (V. amygdalina)* which is a plant widely used for the treatment of parasitic infections was investigated *in vitro*. Results showed that amastigotes were more sensitive to *V. amygdalina* than the promastigotes and the chloroform extract had a stronger parasiticidal activity than the methanol extract. This indicated that *V. amygdalina* displays potent antileishmanial activity (Tadesse *et al*., 1993).

In Nigeria, scanty literature exists on the systematic evaluation of indigenous plants for the treatment of leishmaniasis. Iwu *et al*., (1992) investigated the antileishmanial effects of eleven Nigerian plant extracts using the radio-respirometric microtechnique (RAM) which is based on the ability of the plant extract being evaluated to inhibit the catabolism of different ^{14}C-labelled substrates such as amino acids, sugars or fatty acids. Results obtained showed that of the eleven plants evaluated only five inhibited the catabolism of two or

more substrate in the parasites. However, the testing of medicinal plants for antileishmanial properties and their eventual recommendation will require elaborate systematic in vitro and in vivo studies. *In vitro* studies have been done using mostly the promastigotes form of the parasite, as it is easier to maintain under *in vitro* conditions. However, this is not the infective form of the parasite in vertebrate hosts, hence studies must be complemented with evaluations on the amastigotes forms as well as possible cytotoxicity on macrophages in order to establish if the activity of the extract is due to its general toxicity or it possesses a selective activity against leishmania parasite (Sauvain *et al*., 1993).

2.7.5 *Azadirachta indica*

Azadirachta indica also known as Neem is a tree in the mahogany family Maliacea. It is one of two species in the genus *Azadirachta* and is native to Nigeria, India, Burma and Pakistan. It grows in tropical and semi-tropical regions. Its vernacular names include “DogonYaro” in Hausa, “Agbo” in Yoruba and “Acha” in Ibo in three major Nigerian languages. Neem is a fast-growing tree that can reach a height of 15-20 m, rarely to 35-40 m. It is evergreen but under severe drought it may shed most or nearly all of its leaves. The branches are wide spread. The fairly dense crown is roundish or oval and may reach the diameter of 15-20 m in old, free-standing specimens. The trunk is relatively short, straight and may reach a diameter of 1.2 m. The bark is hard, fissured or scaly, and whitish-grey to reddish-brown. The leaves are alternate, pinnate and are 20-40 cm long, with 20-31 medium to dark green leaflets. The terminal leaflet is often missing. The young leaves are reddish to purplish in colour. The flowers are white and fragrant and are arranged axillary, normally more-or-less drooping panicles which are up to 25 cm long. The inflorescences branch up to the third degree and bear

150-250 flowers. An individual flower is 5-6 mm long and 8-11 mm wide. Protandrous, bisexual flowers and male flowers exist on the same individual (polygamous).The fruit is a glabrous olive-like drupe which varies in shape from elongate oval to nearly roundish, and when ripe is 1.4-2.8 x 1.0-1.5 cm. The fruit skin (exocarp) is thin and the bitter-sweet pulp (mesocarp) is yellowish-white and very fibrous. The mesocarp is 0.3-0.5 cm thick. The white hard inner shell (endocarp) of the fruit encloses one, rarely two or three; elongated seeds (kernels) which have brown seed coats. It is a typical tropical/subtropical tree and is noted for its drought resistance. Normally it thrives in areas with sub-arid to sub-humid conditions, with an annual rainfall between 400 and 1200 mm. It can grow in regions with an annual rainfall below 400 mm but in such cases it depends largely on the ground water levels. Neem can grow in many different types of soil but it thrives best on well drained deep and sandy soils.



Plate 2.1a *Azadirachta indica* (Voucher specimen no. 900151)



Plate 2.1b *Azadirachta indica* (neem) plant

Neem is reputed to be of great medicinal value and products made from it have proven to have anthelmintic, antifungal, antidiabetic, antibacterial, antiviral and anti-infertility. It is considered a major component in Ayurveda and is particularly prescribed for skin disease (Puri, 1999). All parts of the tree (seeds, leaves, flowers and bark) are used for preparing numerous medicinal compounds. Neem twigs are used for brushing teeth in different parts of the world. This practice is perhaps one of the earliest and most effective forms of dental care. Neem oil is used for preparing cosmetics (soap, shampoo, balms and creams) and is useful for skin care such as acne, and keeping skin elasticity. Neem gum is used as a bulking agent and for the preparation of special purpose food (those for diabetics) and a rich source of protein. Neem is a source of environment-friendly biopesticides. Among the isolated neem constituents, limonoids (such as Azadirachtin) are effective in insect growth-regulating activity. The unique feature of neem products is that they do not directly kill the pests, but alters the life-processing behavior in such a manner that the insect can no longer feed, breed or undergo metamorphosis (Wikipedia, 2007). Neem is very effective in the treatment of scabies although only preliminary scientific proof exists which still has to be corroborated. There is also anecdotal evidence of its effectiveness in treating infestations of head lice in humans. It is also very good for treating worms (i.e. the branches and leaves are soaked in lukewarm water and then drunk). The oil is also used in sprays against fleas in cats and dogs (Ganguli, 2002).It also has culinary uses as the tender shoots and flowers of are eaten as a vegetable in India. Neem flowers are very popular for their use in Ugadi (soup-like pickle) which is made on Ugadi day in South India and a soup like dish called *Veppampoo Rasam* (translated as 'juice of neem flower') in India. It is also eaten in the form of a local salad (zogale) in the northern part of Nigeria and eaten with

groundnut powder. The active principles of the plant were extracted and three bitter compounds identified from neem oil, which are nimbin, nimbinin, and nimbidin (Siddiqui, 1942).

2.7.6 *Annona senegalensis*

Annona senegalensis (Annonaceae) is native to Nigeria and most of tropical Africa. Propagation is by seeds, coppicing or root suckers. It is known as "Gwandar daji" in Hausa, "Uburu ocha" in Ibo and "Abo" in Yoruba (Majekodunmi *et al.*, 1996). It has aromatic flowers which are used as flavours in food. The fruit is yellow in colour when ripe and has a pleasant smell as well as a sweet edible jelly. It is eaten in the western and northern parts of Nigeria. Its shoots are used as signposts and for building houses due to its resistance to termite infestations. Its use in the field of ethnomedicine has been well documented. It is used in the treatment of coughs, chestpain, anaemia, urinary tract infections (Burkill, 1985; Muanza *et al.*, 1994) It is also used in the treatment of cancer (Graham *et al.*, 2000), malaria (Balansard and Timon-David, 1985), diarrhoea, dysentery (Kudi and Myint, 1999), intestinal worms (Alawa *et al*., 2003), snakebites (Adzu *et al.*, 2005) and veneral diseases (Tabuti *et al.*, 2003). It has shown to be effective as an antiulcer/antacid and as a smooth muscle relaxant (Langason *et al.*, 1994) It has also been used as a pesticide where it significantly reduced the population of post flowering insect pests of cowpea (Ameh *et al.*, 2000). *Annona senegalensis* has four bioactive ent-kaurene diterpenoids namely; ent-β-Hydroxykaur-16-ene, ent-kaur-16-ene 19-oic, ent-16, 1, 7, diacetoxy karan-16-oic acid and ent-19-CaromethoxyKauran-19-oic acid (Majekodunmi *et al.,* 1996). It also contains acetogenins such as Annonasenegalin and Annonagalene as well as an

aporphine alkaloid compound Roemerin. In addition it contains antrquinones (Ekpendu *et al.*, 1998), sterols, glycosides and flavanoids (Sahpaz *et al.*, 1996).



Plate 2.2a *Annona senegalensis* (Voucher specimen no. 900167a)



Plate 2.2b *Annona senegalensis* stem bark (Voucher specimen no. 900167b)



Plate 2.2c *Annona senegalensis* growing in natural habitat

2.7.7 *Vernonia amygdalina*

Vernonia amygdalina (Asteraceae) commonly referred to as bitter leaf is a native to Nigeria. It is known as “Ewuro” (Yoruba), “Shuwaka” (Hausa), “Onugbu” (Ibo) and “Etidot” (Calabar) in major Nigerian languages and “Ndole” in Cameroun. It is a shrub or small tree of 2-5 metres in height with petiolate leaves of approximately 6 millimetre and elliptical in shape. The leaves are green with a characteristic odour and a bitter taste which can be abated by boiling or soaking in several changes of clean water (Burkill, 1985). It grows over a range of ecological zones in Africa. It is a perennial plant which produces a mass of forage and it is drought resistant (Hutchinson and Dalziel, 1963 cited by Bonsi *et al.*, 1995). There are about 1000 species of Vernonia and these are found in South America, Africa Asia and North America.

V. amygdalina is widely used across Africa for food and medicinal purposes especially in most West and Central African countries. The leaves are used for human consumption in the form of soups and vegetables (Aletor *et al.*, 2001). It is eaten by goats in the central zone of Delta state, Nigeria (Aregheore *et al.*, 1998) and free living chimpanzees and gorillas in the wild eat the leaves for self medication against parasites (Huffman, 2003). The stem and root divested of the bark are used as chewing sticks and for the production of beer instead of hops in Nigeria. The stems are also used as fence posts and pot-herbs in villages.



Plate 2.3a *Vernonia amygdalina* (Voucher specimen no. 675)



Plate 2.3b *Vernonia amygdalina* growing in natural habitat

Nutritionally, *V. amygdalina* is used mainly in soups and as an appetizer and febrifuge in the tropics (Ijeh *et al.*, 1996; Iwu, 1996). It has been proven to be a successful supplement in weaning foods. Due to its availability almost all year round, it is a readily available source of proteins, vitamins, minerals and essential amino acids (Okafor, 1983). Its importance in animal nutrition in Nigeria has also been well documented (Onwuka *et al.*, 1989; Aregheore *et al.*, 1998).

Pharmacologically, all parts of the plant are useful. The leaves and roots are used in ethnomedicine to treat fever, hiccups, kidney problems and stomach discomfort (Burkill, 1985; Hamowia and Saffaf, 1994). The root and twigs are used for abdominal and gastrointestinal problems in humans while the decoction from the leaves is used as antimalarial in Guinea and as cough remedy in Ghana (Akinpelu, 1999; Masaba, 2000). The roots are also used for gingivitis and toothache due to its proven antimicrobial activity (Anonymous, 2005). The leaf is used as purgative and believed to be abortifacient (Awe *et al.*, 1999). It is also used for the treatment of sexually transmitted diseases in Zimbabwe (Kambizi and Afolayan, 2001). Recently, *V. amygdalina* has acquired special relevance as it has been proven in human medicine to possess potent antimalarial and anthelminthic properties (Abosi and Raseroka, 2003) as well as antitumoregenic properties (Izevbigie *et al.*, 2004). It has also been shown to have blood sugar lowering effect in experimental rabbit model that suggests possible beneficial effects as an anti-diabetic (Akah and Okafor, 1992).

V. amygdalina contains several bioactive principles notably among them are the sesquiterpene lactones – Vernodalin, Vernolide, Hydroxyvernolide, Vernomydin and Vernodal (Kupcham, *et al.*, 1969). It also contains some novel sigmastane-

type steroid glygosides, which are Vernonioside A1, A2, B1, B2 and B3 (Jisaka *et al*., 1992b). Almost all the pharmacological actions so far observed has been attributed to these bioactive compounds. Also the bitter taste has been attributed to the presence of compounds such as alkaloids, saponins, tannins and glycosides (Buttler and Bailey, 1973; Ologunde *et al.*, 1992 cited by Bonsi *et al.*, 1995). The chemical composition of *V. amygdalina* consists of a high proportion of ash (13.4%) and nitrogen content of 25 g/kg DM (El Hassan *et al.*, 2000).

CHAPTER THREE

3.0 MATERIALS AND METHOD

PHYTOTHERAPY EXPERIMENTS

3.1 Materials

3.1.1 Animals and Parasites:

Fifteen BALB/c male mice (20-25 g), which were specific pathogen free and obtained from the breeding stock maintained at the Strathclyde Institute for Biomedical Sciences, Glasgow and *L. major* promastigotes (MRHO/SU/598/P) strain which is a standard laboratory strain was used in this study. All animal experiments were carried out in accordance with UK Home office regulations.

3.1.2 Cell lines

L929 (mouse skeletal fibroblasts), Jurkat and SH-SY5Y cells (human cancer cells) were obtained from the European Collection of Animal Cell Culture (ECACC) Porton Down, UK. All cells were routinely subcultured in Dulbecco's medium (DMEM) supplemented with 10% v/v foetal bovine serum (FBS), 1% w/v penicillin (50 IU/ml) /streptomycin (50 μg/ml) and L-glutamine (2 mM) at 37oC in humidified atmosphere of 5% CO2,/95% air. All the reagents were obtained from Sigma-Aldrich UK. Trypanosome brucei brucei was obtained from the American Type Culture Collection (ATCC). Cells were cultured in HMI-9 medium and supplemented as above.

3.1.3 Chemicals, Reagents and Media

Chloroform, Dimethyl sulphoxide (DMSO), DPX Mountant, Ethanol, Histoclear, Neutral Buffered formalin (10%) and N-pentane were all purchased from Sigma-Aldrich. Ethylacetate, hexane and methanol were obtained from BDH.

Haematoxylin and Eosin and Polywax pellets (Fischer Scientific) while Dulbecco's modified medium and RPMI-1640 medium were obtained from Sigma Aldrich.

3.1.4 Drugs

Cymelarsan was obtained from Roche, UK while Sodium Stibogluconate (SSG) [31.1% (wt/wt) Sb (V)] was provided by Glaxo Wellcome Ltd (Ware, UK).

3.1.5 Equipment

I. Glasswares

Condensers (Pyrex, UK), Coverslips and Frosted glass slides (Thermo-shandon, UK), Glass soxhlet (Quick-fit, UK), Glass bottles (10 ml and long), Round bottomed flasks (250 and 500 ml) (Quick-fit UK), and Silica gel columns (60-120 mesh) (Pyrex, UK)

II. Plasticwares and Paper

Bijoux, sterile universals, Falcon tubes, syringes, histosettes were all obtained from Greiner Bio-one Ltd, UK,

Culture plates: 96-well (TechnoPlastic Products AG, Switzerland) and 24-well (Costar, UK)

Filter: sterile units (0.22 μm pore size) (Schleider and Schuell, Germany),

Whatman paper filters (Nos. 54, 1 and 0.5) (Whatman International Ltd, England)

Pippetes: Single channel of various sizes (Gilson) and 5 ml multichannel (Fischer Supplies UK).

Petri dishes (Fischer supplies, UK)

Tips: 100, 200, 500, 1000 μl (Fischer Supplies UK).

TLC aluminium sheets (Merck, Germany)

III. General

Centrifuge (Biofuge), Electromantle heater, Embedding machine (Citadel), Fluorometer (Spectramax), Freeze dryer, Haemocytometer (Haubaucher), Incubator (Sanyo and WTC binder), Light Microscope (Ziess Axiolab), Microtome (Leitz Wetzlar), Nuclear Magnetic Resonance Spectrophotometer, Roller and Rocking stirrers, Rotary evaporator, Tissue Processor (Thermo-Shandon Citadel, 1000), Vortex mixer (Fischer Supplies), Waring Blender, Water bath (Raymond A. Lamb, Waxes and General laboratory supplies, Alperton, Middlesex).

3.2 Methods

3.2.1 Plant Collection

All plants were collected from Zaria, Kaduna state in northern Nigeria in February 2007. The plants were identified by Mr Martins Musa, the taxonomist in charge of the herbarium in the Department of Biological Sciences (Botany division) in the Faculty of Sciences at the Ahmadu Bello University, Zaria and the following voucher numbers were assigned: *Annona senegalensis* – 900167, *Azadirachta indica* – 900151 and *Vernonia amygdalina* - 675. Specimens were deposited at the herbarium. The plant parts used in this study were leaves of *Azadirachta indica* (neem) and *Vernonia amygdalina* (bitter leaf) and stem bark of *Annona senegalensis* (custard plant). The ethnobotanical data of the three plants are summarised in table 3.1 below.

Table 3.1:
Plants and ethnobotanical information

Plant Name	Family	Common Name	Vernacular Names	Parts used
Azadirachta indica (AI)	Meliaceae	Neem plant	H-Dogon Yaro I-Acha Y-Agbo	Leaves
Annona senegalensis (AS)	Annonaceae	Custard plant	H-Gwanda daji I-Uburu ocha Y-Abo	Stem bark
Vernonia amygdalina (VA)	Asteraceae	Bitter leaf	H-Shuwaka I-Onugbu Y-Ewuro	Leaves

() - code, H-Hausa, I- Igbo and Y- Yoruba

3.2.2 Preparation of Plant Extracts

One kilogram each of fresh leaves of neem (AI) and bitter leaf (VA) as well as one kilogram of fresh stem bark of custard tree (AS) were air dried for two weeks, pounded in a mortar and pestle to coarse portions and milled into powder in a Waring commercial blender before being extracted in different solvents based on their polarity. In this study, hexane (non-polar) was used to defat extracts, methanol (semi polar) and water (polar) were used for extraction of more polar compounds.

3.2.2.1 Hexane and Methanol Extraction

The dried powder of neem (38 g), bitter leaf (29 g) and custard plant (32 g) were placed in seperate paper thimbles and then placed inside their respective glass sohxlets. These were each attached to a condenser above and to a pre-weighed 500 ml round bottomed flask below. Each flask contained 400 ml of the solvents and each apparatus was placed on electromantle heater at medium heat and left to extract for 24 hours or until the solvent in the soxhlet ran clear. After extraction the solvents were seperately filtered on Whatman filter papers (No. 54) and then evaporated in vacuum using a rotary evaporator at $40^{o}C$ leaving the extracts in the flasks. The flasks were reweighed to determine the weight of each extract. This process was carried out successively with hexane and methanol. The final residue was dissolved in 1% dimethyl sulfoxide (DMSO) to achieve a concentration of 10 mg/ml.

3.2.2.2 Aqueous Extraction

Dried powders (25 g) of each extract were poured into seperate paper thimbles and placed in respective glass sohxlet tubes as above. These were attached above to condensers and to preweighed flat-bottomed flasks below. The flasks contained 400 ml of distilled water and few anti-bumping granules. Each apparatus was placed on the electromantle burners at medium heat and left to extract for 24 hours or until solvent in sohxlet ran clear. After extraction the solvent was filtered and poured back into the flask and then frozen for 5 hours before freeze drying at a temperature of -4^{o}C and pressure of 1450 mmHg. Flasks were then reweighed to determine the weight of each dried extract. Final residues were dissolved in phosphate buffered saline (PBS) to make a stock solution of 10 mg/ml concentration.

3.2.2.3 Purification and Isolation of crude extract

The hexane extract of *Vernonia amygdalina* (VA) was evaporated on a rotary evaporator and weighed. It was reconstituted with 1000 ml of chloroform and adsorbed ointo 100 g of coarse silica gel. This was left in the fume cupboard to dry. Then it was passed through a silica gel column using different solvents with ascending polarity as eluent. The solvents used were a mixture of ethylacetate and hexane in different concentrations placed in an overhead solvent tank. 10 ml fractions were collected in glass bottles and these were subjected to thin layer chromatography (TLC) using silica-gel coated aluminium plates. The fractions were spotted on these plates with standardized capillary tubes and left in a closed chromatography tank containing the eluent solvents. These were allowed to stand until the adsorption of the solvent had reached the required marks on the TLC plates. The developed chromatograms were first inspected under UV-254 and

UV-365 nm light and then sprayed with 10 ml of newly constituted anisaldehyde-sulphuric acid reagent (appendix 7) in a fume cupboard and heated with a heat gun at 100^{o}C for 5 minutes or until colours developed. They were dried with a hair dryer and viewed under short and long ultraviolet lights again. The UV-254 nm, which is the shorter wavelength ultraviolet light was used to detect substances that quench fluorescence while the UV-365 nm was used to detect substances that fluoresce in long wave UV light. Dark or coloured spots visible in the respective lights were marked. Observations from the plates were documented and similar fractions from chromatograms were pooled together. These were left in the fume cupboard to evaporate to dryness in pre-weighed glass bottles. Residue in the bottle was weighed and this served as the yield of the purified compound. The colour and structure of the purified compound was documented. The purified compound was identified in the following manner: First it was washed with *n*-pentane and filtered on Whatman filter paper No. 0.5. The filtrate was allowed to dry and 10 mg was then dissolved in chloroform specific for nuclear magnetic resonance spectra (NMR). This was put in tubes which were inserted into the nuclear magnetic resonance machine to obtain the NMR spectra of the compound. The full proton NMR spectrum of each compound was obtained and this was used to determine the molecular formula of the compound.

3.3 *In vitro* Studies

3.3.1 Macrophage sensitivity tests

3.3.1.1 Isolation of macrophage from bone marrow

Bone marrow cells were harvested from the femur of individual healthy mice by injecting 10 ml of bone marrow medium into the isolated bones in a petri dish. The extracted cell suspension was incubated at 37^{o}C in 5% CO_2 / 95% air for 7-10

days to enable growth, differentiation and adherence of macrophages. The macrophages were then harvested from the petri dishes by dislodging with a cell scraper after refrigerating for 15 minutes. Cell suspension was pooled and pelleted by centrifugation (3000 rpm) for 5 minutes. The cells were then washed twice with 10 mls of incomplete medium and finally resuspended in 10 mls complete medium. Determination of viable cells was done by mixing 15 μl of cell suspension with trypan blue dye at a ratio of 1:1. This was loaded onto a haemocytometer and viable cells were counted. Cells were then adjusted to $1x10^5$ cells in appropriate volume of complete medium. Sterile 13 mm glass coverslips (round) were placed in each well of a 24-well tissue culture plate and the cell suspension was plated into each well. Plates were incubated at 37^oC in 5% CO_2 / 95% air for a minimum of 1 hour to permit adherence of macrophages. All experiments were carried out under aseptic conditions and repeated three times (i.e. n=4).

3.3.1.2 Infection of Macrophages and drug susceptibility assay

This was to assess the intracellular antileishmanial activity. Macrophage culture medium was removed from each well and replenished with fresh complete medium (uninfected controls) or fresh complete medium containing *L. major* promastigotes. Parasites were used at an infection ratio of 1:20 (macrophage to parasite). Plates were then incubated as before for 24 hours. Contents of the wells were removed and replenished with 990 μl of complete medium in drug wells and 1000 μl in control wells. Cells (n=4wells/treatment) were cultured with 10 μl of solvent used in dissolving extract or 10 μl of various concentrations of plant extracts in drug wells only. Plates were incubated for 72 hours as before and contents of wells were removed and the cells fixed in methanol and stained

with 10% Giemsa stain. Coverslips were removed and mounted with DPX mountant. Percentage of infected cells was microscopically determined at 1000 magnification and results expressed as percentage and percentage reduction of parasite burden as compared to untreated control wells (i.e. %infection and %suppression respectively).

3.3.2 Assessment of drug toxicity

Cytotoxicity of the plant extracts was determined using the cell line L-929 (mouse skeletal fibroblasts) and cancer cell lines (Jurkat and SH-SY5Y). 130 μl of cells were seeded into 96-well microtitre plates at appropraite concentration of 10^4 cells/well (L929) and $3x10^4$ (Jurkat and SH-SY5Y) in Dulbecco's modification of Eagle's minimun essential medium (DMEM) supplemeted with 10% foetal bovine serum, 2mM l-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin. 50 μl of extract was added into each well of the first row and a double-fold serial dilution ranging from 100 μl to 0.19 μl was done down the plate. Plates were incubated for 72 hours at 37^{o}C and 5% CO_2 in a humidified incubator and appropriate solvent controls were used for comparison. The percentage inhibition of cell growth relative to the control was evaluated colorimetrically using Alamar Blue and plates were read using a spectramax fluorometer at an excitation wavelength of 560 nm and emission length of 590 nm.

3.3.3 Antitrypanosomal activity *(Trypanosome brucei brucei)*

The assays were performed by pipetting 200 μl of the diluted extracts into the second column of a 96-well microtitre plate in duplicates. 100 μl of HMI-9 culture medium was added to the remaining wells and one to one serial dilutions were carried out to give a test concentration range of 100 μg/ml to 0.19 μg/ml.

Cymelarsan at a final concentration of 50 μg/ml was included as a positive control. The bloodstream form of trypanosomes from axenic culture of *trypanosome brucei brucei* were added to each well at a concentration of $2x10^3$/100 μl and incubated for 48 hours in a humidified atmosphere at 37^0C in 5% CO_2/95% air. 50 μl of 10 mg/ml extract was added to wells A2-H11 (to give final concentration of 100 μg/ml). Then twenty microlitres of Alamar blue was then added to each well and the plates incubated for a further 24 hours. Plates were read on a microplate flurometer at an excitation wavelength of 560 nm and emission wavelength of 590 nm. Fluorescence development was measured and expressed as percentage of the control.

3.4 *In vivo* Antileishmanial Test

3.4.1 Parasites and Infection

The virulent *L. major* promastigotes (MRHO/Su/598/P-strain) were cultured in complete medium. Parasites were harvested in the stationary phase after 8-10 days, then centrifuged and washed three times in incomplete medium. This was cooled and used for the innoculation of the mice. Mice were infected subcutaneously into the shaven rump with 1.6 x 10^6 stationary phase promastigotes of *L. major* in a final volume of 26 ml (in RPMI 1640 medium).

3.4.2 Drug Treatment

Fifteen (15) male BALB/c mice inbred in the Strathclyde Institute for Biomedical Sciences were used for the experiment. Infected mice were divided into three treatment groups (n=5 per group). Group 1 served as the control and was treated with a daily dose of 0.2 ml of 5% DMSO/PBS, group II was treated with a daily dose of 0.2 ml of 1 mg/ml VA in 5% DMSO/PBS (50 mg/kg BW) and group III was

treated with a daily dose of 0.2 ml of 1 mg/ml AS in 5% DMSO/PBS (50 mg/kg BW). Treatment commenced on day 9 postinfection and was given for 5 days in all groups via intragastric gavage. Mice were regularly observed for cutaneous lesions and ulcers. These were measured weekly using a Mitutoyo micrometer calliper. Mice were sacrificed on day 21 by carbondioxide inhalation and tissues were weighed after dissection. Skin lesions and samples of the spleen and liver were taken and processed for histological studies.

3.4.3 Tissue Processing

Skin lesions, spleen and liver tissues were fixed in neutral buffered formalin (10%) for 24 hours and then washed in two grades of ethanol (50% and 70%) for 12 hours each. Tissues were placed in histossettes and processed in the tissue processor by running it through changing grades of ethanol i.e. 70% (6 hours), 90% (30 mins), 100% (30 mins), 100% (30 mins), 50% histoclear/ethanol (30 mins), 100% histoclear (30 mins), 100% histoclear (30 mins), wax (30 mins) and finally left in hot liquid wax until it was ready for embedding. Tissues were embedded in the embedding machine using metallic moulds and left to cool on the freezing platform for one hour. They were removed from the moulds and left at room temperature overnight. Tissue blocks were shaven to remove excess wax and expose tissue using the microtome with dial set at 20μm. Then sections of tissues were first placed on ice to chill before being cut at 6 μm and placed in a water bath at 40^{o}C to float and spread out. Tissue sections were picked using pre-labelled frosted histological slides. These were left on the tissue dryer for an hour and then placed in the electric oven at 70^{o}C for wax to melt off. Slides were stained with haematoxylin and eosin in order to study the micro-architecture by light microscopy. This was done by rehydrating the tissues in several changes of

solvents i.e. 100% histoclear (5 mins), 100% histoclear (5 mins), 50% histoclear/ethanol (5 mins), 100% ethanol (5 mins), 100% ethanol (5 mins), 90% ethanol (2 mins), 70% ethanol (2mins), 50% ethanol (2mins), distilled water (2mins), stained in haematoxylin (1 min), then rinsed in running water for 1 min or until water turned clear, then in eosin (1min), and finally rinsed in running tap water until water turned clear. Tissues were then dehydrated in the reverse of the rehydrating procedure and square cover slips were mounted over the slides using DPX mountant and left to dry on a rack before observing on a light microscope. Images from slides were captured in a Zeiss Axiolab light microscope connected to a digital camera and a laptop.

IMMUNOTHERAPY EXPERIMENT

3.5 Materials

3.5.1 Animals and Parasites

Twenty-eight (28) female in house-bred BALB/c mice (20-25g) obtained from the breeding stock maintained at the Strathclyde Institute for Biomedical sciences, Glasgow were used in this study. Standard laboratory strain of *L. major* (MRHO/SU/598/P) and *L. donovani* (200016 or MHOM/ET/67/L82) strain were used for this study. The parasites were kept in a virulent state by continuous passage in BALB/c mice and golden Syrian hamsters respectively. All animal experiments were carried out in accordance with UK Home office regulations.

3.5.2 Chemicals and Reagents

Capture and detection anti IFN-γ, IL-4, IL-5 and IL-10 antibodies, standards and Streptavidin-alkaline Conjugate were obtained from PharMingen and supplied by Insight Biotechnology (Wembly, UK). Goat anti-mouse IgG_1 and IgG_{2a} were obtained from Southern Biotechnology Associates Inc. USA, Titrated thymidine (Amersham, UK). Heat-inactivated foetal calf serum (Sigma, UK), Qiagene Endofree® Plasmid mini and maxi kits and Gel extraction kits (Qiagen Ltd, Crawley, UK). Nitrocellulose membrane (Amersham Biosciences, UK), Novex ® Blot Module (Invitrogen, UK), Trizol reagent (Invitrogen, Paisley, UK), Superscript III reverse transcriptase (Invitrogen) and ReddyMix PCR Master Mix (Abgene, Epsom, UK). Dulbecco's medium and RPMI 1640 were purchased from Gibco BRL and supplied by invitrogen. Concanavalin A, Ammonium persulphate, N,N,N',N'–Tetra methylethylenediamine (TEMED), Ponceau S and *p*–nitrophenyl phosphate (PNP) were obtained from Sigma-Aldrich (poole, UK). Calf intestinal alkaline phosphatase (CIAP, Promega, Southampton, UK). Prosieve 50 gel

solution and Broth LB were from Cambrex Bioscience Rockland Inc. (Rockland, USA).

3.5.3 Drugs

Ampicillin, Chloramphenicol, kanamycin and Tetracycline were obtained from Sigma-Aldrich. Penicillin, streptomycin and L-glutamine were purchased from Gibco BRL.

3.5.4 Bacteria and Protein

Bacteria – *Escherica coli (*Rossette Blue strain) purchased from Public Health Laboratory Services (Porton Down, UK)

Protein/antigen:Gamma Glutamine cysteine synthetase (γGCS) genes isolated from genomic DNA of *L. donovani* promastigotes

Plasmid/vectors –pVAX 1 (Invitrogen) and pMAL2cE (New England Biolabs, Hitchin, UK)

Restriction enzymes: *Bam*HI and *Kpn*I (New England Biolabs, Hitchin,UK)

3.5.5 Equipment

I. Plastics

Centrifuge tubes (Fischer supplies), Columns: Gravity Flow (His Gravitrap), Bio-Gel columns (Greiner Bio-one), Dialysis tube (Sigma UK), Gel cassettes and Combs (Sure-lock), petri dishes (Fischer supplies), pipettes and tips (Gilson), universals, eppendorfs and bijoux (Grenier Bio-one).

II. Glasswares

Conical flasks (2 liters, 1 liter and 500 ml) (Pyrex) and Glass bottles (Sigma, UK)

III. General

Centrifuges: Microcentrifuge (Biofuge Pico, Haraeus), small centrifuge (Multifuge, Model 3S-R, Haraeus), Large centrifuge (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Du Pont, Connecticut, USA), French Cell Press (Sim Aminco, Spectronic Instruments, Rochester, USA), Haemocytometer (Haubaucher), Incubator shakers: Model S150 (Stuart Scientific, UK) and Model C25 (New Brunswick Scientific Co. Inc. NJ, USA), Magnetic stirrer, Microwave (Hinari), Polymerase Chain Reaction (PCR) Machine, Shakers: Platform and rocker, Sure –Lock Mini Cell (Sure–Lock), Water bath, GeneQuant pro spectrophotometer (Biochrom Ltd, Cambridge, UK)

3.6 Methods

3.6.1 Plasmid (pvaxγgcs) Vaccine Construct and Purification

Complete open reading frames of gamma GCS genes were isolated from the genomic DNA of stationary phase of *L. donovani* promastigotes (200016 or MHOM/ET/67/L82) strain. Briefly, promastigotes were subjected to an aqueous extraction, followed by heat inactivation and centrifugation. The aqueous supernatant was lyophilized and fractionated by gel filtration on Bio-Gel P-10 column, yielding the gamma Glutamyl-cysteine synthetase (γGCS). This was inserted into the EcoRI sites of the eukaryotic expression vector pVAX1 by growing in a petri dish inoculated with ampicillin and kanamycin. This resulted in a construct pVAX-γGCS. Insert size and orientation were checked by polymerase chain reaction. The plasmid DNA was purified from transformed *E. coli* (*Rossette Blue* strain) cultures using the Qiagen Endofree Plasmid maxi kit according to the manufacturer's instructions. This removes the lipopolysaccharide (LPS)

contamination. Plasmid concentration and quality were evaluated by agarose gel electrophoresis.

3.6.2 Cloning of γGCS for Protein Expression

PCR primers were designed to amplify the full length γGCS and introduce appropriate restriction enzyme sites to facilate cloning into pMAL2cE vector. The following primers were designed for the polymerase chain reaction (PCR): γGCSforward (GGAGATCCGC**G**GTACCAATGGGGCTCTTGA) containing *kpn*I site and γGCSrev (GGGAA**A**GAGGATCCACTTGGTGCTCTC) containing a *Bam*HI site. The γGCS gene was amplified fronm L. donovani strain 200016. Both the pMAL2ce plasmid and the γGCS PCR product were digestedsequentially with *Bam*HI and *Kpn*I. The vector was dephosphorylated for 1 hour at 37^{o}C with CIAP (Calf intestinal alkaline phosphatase). Cleanup of both the plasmid and the gene insert fragment was performed using the Qiagen PCR cleanup kit following the manufacturer's instructions. Ligation reactions using T4 DNA ligase were performed overnight at 4^{o}C in a final volume of 10 μl. Ligation products were used transform competent E. coli DH5α (14) and then plated out onto LB Agar containg 100 μg/ml ampicillin and incubated at 37^{o}C. A number of single colonies were picked for analysis and plasmid DNA prepared from 4-5 ml of a 10 ml overniht culture using the Qiagene miniprep kit. Clones were analysed by restriction enzyme nanlysis and by PCR to confirm the presence of the insert of the correct size.

3.6.3 Expression and Purification of Recombinant Protein

The plasmid construct corresponding to pMAL-γGCS was produced in fusion with an *N*-terminal histidine (His-6-tag). *Rosetta blue* an *E. coli* strain was transformed with the plasmid-DNA and the mixture was grown at 37^{o}C in 2 litres LB supplemented with 100 μg/ml ampicillin, 84 μg/ml chloramphenicol and 91 μg/ml tetracycline. The culture was induced with 1 mM isopropyl-1-thio-β-D-galactoside (IPTG) when the bacterial culture gave an OD 600 of 0.3-0.5, the bacterial were further grown for up to 15 hours or overnight at 30^{o}C. Cells were pelleted at velocity of 8 for 20 minutes on a refrigerated superspeed centrifuge and frozen overnight at -70^{o}C. The bacteria pellets were resuspended in extraction buffer and then sonicated on ice inorder to shear the DNA. Soluble protein was released by homogenization tgrough a French Pressure cell press twice at pressure of 1000 psi using the 40K cell. The suspension was then centrifuged at 13,000 rpm for 30 minutes at 4^{o}C. The resulting supernatant was removed (i.e. soluble fraction) and the pellet resuspended in binding buffer containing 6M urea and incubated on ice for one hour to completely solubilize the target protein. The suspension was centrifuged at 13,000 rpm for 30 minutes to remove insoluble material and the resulting supernatant (insoluble fraction) was applied to a gravity flow (His gravitrap®) column after the addition of 0.05M imidazole. The columns were washed extensively with buffer following the manufacturer's instruction. Elution buffer was used to elute the recombinant protein and this was dialyzed against PBS for 24 hours. The protein concentration was determined using the Bio-Rad protein assay. Also gel electrophoresis (SDS-PAGE) and western blot analysis were used to confirm the presence of recombinant protein.

3.6.4 Analysis of Protein

3.6.4.1 Gel Electrophoresis (SDS-PAGE)

10 ml of 12% resolving gel was constituted and poured into the cassettes upto ¾ full and then overlaid with water until gel polymerised. Stacking gel was poured into the cassette after decanting water. Combs were inserted and stacking gel left to solidify. Protein samples combined in 2X denaturing sample buffer in an eppendorf, were subjected to heat denaturation by boiling for a period of 5 minutes, and then placed on ice and centrifuged. The cassettes were placed into the mini-cell after marking wells and removing comb and tape at the bottom. Running buffer was poured into buffer chamber (space between two cassettes) ensuring that there was no leakage and then added to back and front parts of the chamber. Gels were loaded with 15 μl/well of the protein samples or 5 μl of the standard molecular weight protein marker. Proteins were separated at 200 V for approximately 60 minutes until the dye reaches the bottom of the resolving gel.

Following electrophoresis, one cassette was opened and gel removed and fixed in fixative for 2 hours with mild agitation on a platform shaker and then stained in Colloidal Coomassie blue stain overnight to form and visualize coloured bands.

Western Blot Analysis

The resolved proteins were transferred onto a nitrocellulose membrane in transfer buffer at 70V for 2 hours in the blot module. After electrophoresis, the transfer membrane was removed from the module and washed with 1x PBS on a platform shaker for 10 minutes. Membrane was left to dry at room temperature and stained with Ponceau S solution for 5 minutes to check and visualise the transferred proteins. The site of the protein standard along with the tops and bottoms of each lane were marked with ink to make them easy to locate and then washed in

distilled water. Non-specific binding sites on the nitrocellulose membranes were blocked by incubating with 10% FCS in PBS pH 7.4 for 1 hour with shaking and sealed with cling film. Membrane was washed three times in PBS for 10 minutes with gentle agitation. It was then incubated for 2 hours with the primary antibody in PBS (1:2000 dilutions) on shaker and sealed. This was followed by washing three times for 10 minutes each in PBS and then incubated with alkaline phosphatase conjugated secondary antibody for 1 hour on a shaker (1:3000 dilutions). Membrane was then washed as before then incubated for 15 minutes with two substrate components at 1:1 ratio and left for colour to develop. The membrane was then drained of excess substrate covered in clear plastic wrap and exposed to photographic film.

3.6.5 Immunization and Infection of mice

Five groups of seven female Balb/c mice (n=7 mice per group) were injected intramuscularly in the flank with 100 μg of plasmid DNA in 100 μl of phosphate buffered saline (PBS). Group I served as the infected control and was injected with phosphate buffered solution (PBS), group II was injected with plasmid alone (pVAX), group III injected with plasmid DNA (pVAX-γGCS), group IV injected with vesicular recombinant protein (NIV-rγGCS) and group V served as the uninfected control and was not injected at all. Mice were boosted two weeks later with the same treatments. At 2 weeks after the booster injection, mice were challenged with 0.5 ml subcutaneous injection of $1x10^7$ stationary phase promastigotes of *L. major* in the base of tail after shaving except group V. This was designated as day 0 (infection day). The time course of infection was monitored weekly by measuring the lesion size with a vernier calliper for up to 35 days post-infection. Mice were sacrificed 35 days post-infection spleens removed, weighed and used

for assessment of parasite burdens with Giemsa-stain. Relative spleen-to-body weight ratio was assessed in all animals and compared to that of the uninfected controls. Also sera were collected 14 days after the last immunisation, on the day of infection (day 0) as well as 7 and 35 days post-infection and analysed for antibody levels.

3.6.6 T-Cell Proliferation Assay

In proliferation assay, immediately before challenge and thirty-five (35) days after infection, three and four mice were sacrificed respectively and their spleens removed and placed in petri dishes containing 5 ml of complete medium. The spleens were teased apart with forceps to form a cell suspension. This was pippetted into Falcon tubes and centrifuged at 3000x g for 5 minutes to harvest the cells. Supernatant was decanted and cells resuspended in 3 ml of sterilized Boyles solution to remove erythrocytes and centrifuged as above. The supernatant which was deeply coloured was decanted and the pellets washed twice in incomplete medium. Finally cells were resuspended in 3 ml of complete medium and the number of viable cells was estimated by trypan blue exclusion. Cell count was adjusted to 5 x 10^6 by adding appropriate volumes. 100 μl of medium containing the spleen cells at 5x10^6 cell/ml was added to each well of 96 well flat bottomed tissue culture plates to which 100 ul of medium alone (unstimulated controls n=3), 5 μg/ml concanavalin A (stimulated control n=3) and 25 μg/ml γGCS lysate (antigen n=3). Plates were set up in duplicate and final volume was 200 μl/well. Cells were incubated for 60 hours and each well pulsed with 0.25 μCi of titrated thymidine in 20 μl of complete medium and further incubated for 12 hours. Plates were stored at -20^{o}C until nitrite and cytokine determination were performed.

3.6.6.1 Cytokine determination

Cytokine levels of gamma interferon (IFN-γ), IL-4, IL-5 and IL-10 in cell supernatants were determined using sandwich enzyme-linked immunosorbent assay (ELISA). Cell supernatants from the proliferation assay were harvested at 72 hours onto wells of 96-well microtitred plates and used for the cytokine assay.

50 μl of appropriate purified rat antimouse anti-cytokine antibodies for each cytokine was diluted to 2 μg/ml in coating buffer and added to respective plates. Plates were incubated overnight at 4^{o}C and then washed three times in wash buffer. 150 μl of 10% foetal calf serum (FCS) in phosphate buffered saline (PBS) was added into each well to block unbound antigen and was incubated at 37^{o}C for 1 hour. Plates were washed as above and then 30 μl of cell supernatants (samples) or cytokine standards (10 ng/ml in blocking buffer) was added to respective wells and plates were incubated for 2 hours at 37^{o}C and then washed. 100 μl of biotinylated anti-mouse anti-cytokine antibody (1 μg/ml in blocking buffer) was added to the wells of respective cytokine plates and incubated at 37^{o}C for 1 hour and then washed. For the detection of the bound biotinylated rat antibody, 100 μl of streptavidine-alkaline phosphatase conjugate (1:4000 dilution) was added to each well and plates incubated for 1 hour at 37^{o}C and washed. Binding was visualized by adding 100 μl per well of p-nitrophenyl phosphate substrate (PNPP at 1 mg/ml in glycine buffer) and incubated in the dark at room temperature for 20-60 minutes to allow colour (yellow) develop. Absorbance of the solutions in the wells was measured at 405 nM on a Titertek Multiskan plate reader and the concentration of each cytokine present (ng/ml) in the cell supernatants was determined from their respective standard curves. In each case

the linear regression analysis using the standards was carried out using the software provided and gave correlation coefficients of >0.97.

3.6.6.2 Nitrite Assay

50 μl of the test sample in triplicate (cell supernatant) was added to the appropriate wells of a 96-well plate. 100 μM of nitrite solution was prepared by dissolving 10 μl of sodium nitrite ($NaNO_2$) 100 mM stock solution in 1 ml of distilled water. 50 μl of this was added into appropriate wells of an ELISA plate in duplicates as standards and serially diluted in double starting at 100uM down to 0.1uM. Then 50 μl of Griess reagent was added to standard wells and plate was left on the bench at room temperature for 10 minutes to allow pink colour develop showing that the reagents are active. The Griess reagent was added to the wells of the test samples and left on the bench for colour to develop and absorbance of samples read at 540 nM on a Titertek Multiscan plate reader (Flow laboratories). Linear regression analysis using the nitrite standards was carried out using the standard curves and gave a correlation coefficient of >0.97.

3.6.7 Antibody Production

Serum samples were obtained from mice before and after infection by allowing blood to clot in the fridge for 3 hours after bleeding. All samples were stored at -20^{o}C before analysing for specific antibody contents. Gamma GCS-specific immunoglobulin G_1 (IgG_1) and immunoglobulin G_{2a} (IgG_{2a}) in sera were determined by ELISA. 100 μl of γGCS antigen (0.1 μg/ml in coating buffer) was used to precoat 96-well plates overnight at 4^{o}C.Plates were blocked after washing with Marvel milk (4% in PBS) at 37^{o}C for 1 hour to prevent non-specific binding. Sample sera were added after washing in 1:500 serial triplicate dilutions at 50 μl/well and incubated for 1 hour at 37^{o}C. Goat anti-mouse IgG_1 and IgG_{2a}

horseradish peroxidase–conjugated antibodies were added at 1:4000 dilutions in blocking buffer and incubated for 1 hour at 37^{o}C. After washing, the reaction was developed with tetramethylbenzidine (TMB) peroxidase substrate for 15 minutes in the dark for colour (blue) to develop. Substrate action was stopped by adding 10% sulphuric acid. Absorbance was read at 450 nM on a Titretek Multiskan plate reader.

3.7 Statistical Analysis

Experimental data obtained from the different experiments were pooled and expressed as means (±SEM). Statistical comparisons of the differences betweem controls and other treatment groups were performed with GraphPad Prism Software (version 4.03, GraphPad Software, San Diego, California, USA) using one-way analysis of variance (ANOVA; 95% confidence interval), followed by Tukey-kramer multiple comparison test for data on phytotherapy while data on cytokine, antibody and nitrite levels were subjected to non parametric Mann Whitney test. Values of $p \leq 0.05$ were taken to be statistically significant.

CHAPTER FOUR

4.0 RESULTS

PHYTOTHERAPY EXPERIMENTS

4.1 Extraction of Plants

Three medicinal plants which grow in Nigeria were extracted sequentially in hexane, methanol and water. A total of nine extracts were obtained from the three plants and the typical yields and appearances of each of the extracts following evaporation to dryness under reduced pressure are shown in table 4.1. Results showed that the methanol extracts gave better yields (>25%) than the hexane (<2%) and water (<15%) extracts. Also the apperarances of the extracts varied in constistency. Hexane extracts were oily, methanolic extracts were caramelised while the aqueous extracts were pelleted. Colour differences were observed with the deep colours seen in the extracts of *Vernonia amygdalina* and *Azadirachta indica* while those of *Anonna senegalensis* were lightly coloured.

Table 4.1:

Yields and Appearances of plant extracts.

Plant	Extract	%Yield	Appearance
Azadirachta indica	Hexane	1.57	Yellow smear
	Methanol	25.06	Dark green caramel
	Aqueous	13.30	Light brown pellets
Annona senegalensis	Hexane	1.63	Yellowish brown smear
	Methanol	25.56	Deep brown caramel
	Aqueous	14.30	Golden brown pellets
Vernonia amygdalina	Hexane	1.54	Greenish black smear
	Methanol	28.69	Deep green caramel
	Aqueous	15.03	Blackish brown pellets

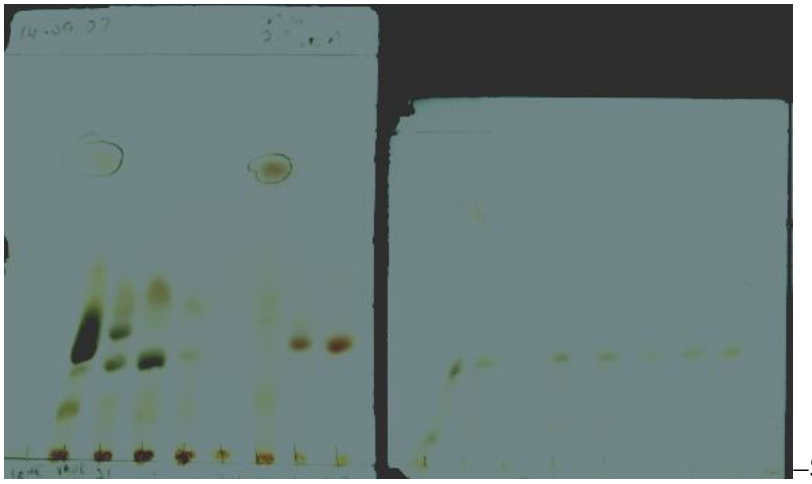
4.1.1 Isolation of Compounds from *Vernonia amygdalina*

Crude hexane extracts of *Vernonia amygdalina* was subjected to column chromatography using silica gel and the fractions were collected in 10 ml volumes. Bands in different colours were formed in the column indicating different compounds in the crude extract. Fractions of the extract were eluted in different colours greenish black, light green, yellow, yellowish–orange, red and cream with a 100% hexane gradient. However, the red band separated further into deep yellow, light yellow and then red while the cream colour separated into light yellow and cream with an increasing gradient of hexane-ethylacetate: 95:5 to 50:50. Fractions were then subjected to thin layer chromatography (TLC) on aluminium sheets inorder to separate and monitor the compounds at UV detection of 254 and 265 nm. Based on the UV chromatogram, similar fractions in colour and RF levels on the plates were pooled together as in figures 4.1 and 4.2. From a total of 221 fractions, 33-pooled fractions were obtained and their yields, TLC appearance and ^{1}H NMR data are listed in table 4.2. The proton ^{1}H nuclear magnetic resonance (NMR) data showed that VAHE 1 and 2 fractions were obtained as white amorphous powder, contained long chain fatty acid between 1.0 and 2.0 ppm and the molecular formular established as $C_{15}H_{30}COOH$ in comparison to previously characterised compounds. Fractions 8 to 10 showed the presence of flavanoids between 7.0 and 8.0 ppm. The structure of these compounds could not be determined from the mass spectrophotometer but efforts are presently directed at the establishment of the structure-activity relationship inorder to identify and isolate the active compound. The yields, TLC and NMR results are summarized in table 4.2 while figures 4.1 and 4.2 show samples of the chromatograms and figures 4.3 – 4.6 show the NMR results.

Table 4.2:

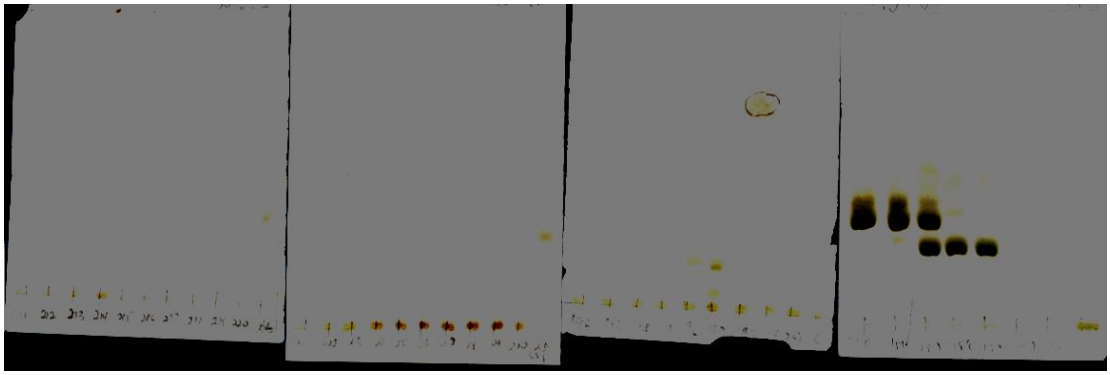
Yield and results of fractions of *V.amygdalina*

Code	Fractions	Yields (mg)	TLC result	NMR remark
VAHE 1	1 to 3	44 mg	1 spot	Fatty acid (FA)
VAHE 2	4 to 5	53 mg	1 spot	Alkane /FA
VAHE 3	64 to 69	50 mg	5 spots	Fatty alcohol
VAHE 4	61 to 63	49 mg	4 spots	Fatty alcohol
VAHE 5	33 to 35	143 mg	5 spots	FA ester
VAHE 6	77 to 80	25 mg	3 spots	Fatty acid
VAHE 7	39 to 42	51 mg	1 spot	Fatty acid
VAHE 8	36 to 38	169 mg	6 spots	FA/Flavanoids
VAHE 9	84 to 92	19 mg	2 spots	Flavanoids
VAHE 10	81 to 83	15 mg	2 spots	Flavanoids
VAHE 11	101 to 109	20 mg	1 spot	Not determined
VAHE 12	93 to 100	8 mg	1 spot	Not determined
VAHE 13	57 to 60	12 mg	No spot	Not determined
VAHE 14	70 to 76	51 mg	3 spots	Not determined
VAHE 15	6 to 11	3 mg	No spot	Not determined
VAHE 16	127 to 136	14 mg	2 spots	Not determined
VAHE 17	118 to 126	12 mg	1 spot	Not determined
VAHE 18	110 to 117	10 mg	1 spot	Not determined
VAHE 19	138 to 140	8 mg	1 spot	Not determined
VAHE 20	185 to 186	10 mg	7 spots	Not determined
VAHE 21	187	13 mg	8 spots	Not determined
VAHE 22	188 to 189	25 mg	8 spots	Not determined
VAHE 23	190 to 191	12 mg	5 spots	Not determined
VAHE 24	179 to 182	9 mg	No spot	Not determined
VAHE 25	183 to 184	16 mg	6 spots	Not determined
VAHE 26	141 to 148	11 mg	2 spots	Not determined
VAHE 27	149 to 160	28 mg	1 spot	Not determined
VAHE 28	194 to 197	14 mg	5 spots	Not determined
VAHE 29	161 to 170	18 mg	No spot	Not determined
VAHE 30	171 to 178	15 mg	2 spots	Not determined
VAHE 31	192 to 193	12 mg	3 spots	Not determined
VAHE 32	194 to 197	10 mg	4 spots	Not determined
VAHE 33	198 to 200	5 mg	2 spots	Not determined



1 2

Fig 4.1: Thin layer chromatography (TLC) analysis of fractions off flash column. Mobile phase is 98:2 Hexane to EtOAC. Fractions with spots at the same level (e.g. 93 to 100 on plate 1) are combined to form codes as above (e.g. VAHE 12), then evaporated to dryness and the NMR spectra obtained.



1 2 3 4

Fig 4.2: Thin layer chromatography (TLC) analysis of fractions at end of flash column showing fading of spots. Fractions 185 to 189 showed purer spots (plate 4) while plates 1 to 3 are fractions which contained no crude extract rather only eluent hence absence of spots.

Fig 4.3: Proton NMR Spectrum (full) for VAHE 2 showing long chain fatty acid.

Fig 4.4: Proton NMR spectrum (full) for VAHE 3 showing long chain fatty acid and presence of alkane between 2.0-4.0 ppm.

Fig 4.5: Proton NMR spectrum (full) for VAHE 8 showing presence of flavanoids (little) between 7.0-8.0 ppm.

Fig 4.6: Proton NMR Spectrum (full) for VAHE 10 showing the presence of flavanoids between 7.0-8.0 ppm.

4.2 *In vitro* EXPERIMENTS

4.2.1 Macrophage Sensitivity tests (Preliminary investigation)

All the extracts were screened preliminarily *in vitro* for antileishmanial activity against intracellular *L. major* amastigotes at a concentration of 100 μg/ml. From a total of nine extracts tested only two extracts exhibited good antileishmanial activity (based on the reduction in the percentage of infected cells to <50%). The most active extract was the aqueous extract of *Vernonia amygdalina* which demonstrated the highest percentage suppression of infected cells or chemosuppression (65.1±6.9). Two extracts were toxic to the macrophages at this concentration hence their effects could not be determined. These were methanolic extracts of *Vernonia amygdalina* and *Azadirachta indica.* Table 4.3 presents a summary of the result for the mean percentage of infected cells and chemosuppression percentages after treatment with the extracts. Infection rate of untreated macrophages or control was 80 to 90% which was consistent within each experiment. Also the solvents used to dissolve the extracts were tested (DMSO for hexane and methanolic extracts and PBS for aqueous extracts) and did not show any significant effect on the percentage of infected cells as compared to the control. The infection rate of untreated macrophages was normalized to 100% for further analysis of the results. Furthermore, the effect of the extracts on the mean number of parasites per host cell showed that all the extracts suppressed the parasite number (>50%) except for the two which were toxic to the macrophages. Aqueous extract of *Vernonia amygdalina* gave the greatest suppression (85.3% ± 0.7). Mean parasite burden per host cell in untreated macrophages was 12 to 20 amastigotes and was consistent within each experiment. The results demonstrate that the aqueous extract of *Vernonia amygdalina* exhibited pronounced leishmanicidal effect against the intracellular

form of *L. major* amastigotes. It showed both a decrease in the rate of infection and direct effect on the amastigotes by reducing the number of parasites per host cell without toxicity to the macrophages at this concentration. Plates 4.1 and 4.2 show micrographs of infected macrophages from control and treated groups respectively. Results of mean parasite burden per host cell (for 20 randomly chosen cells) and percentage suppression of parasites are shown in table 4.3.

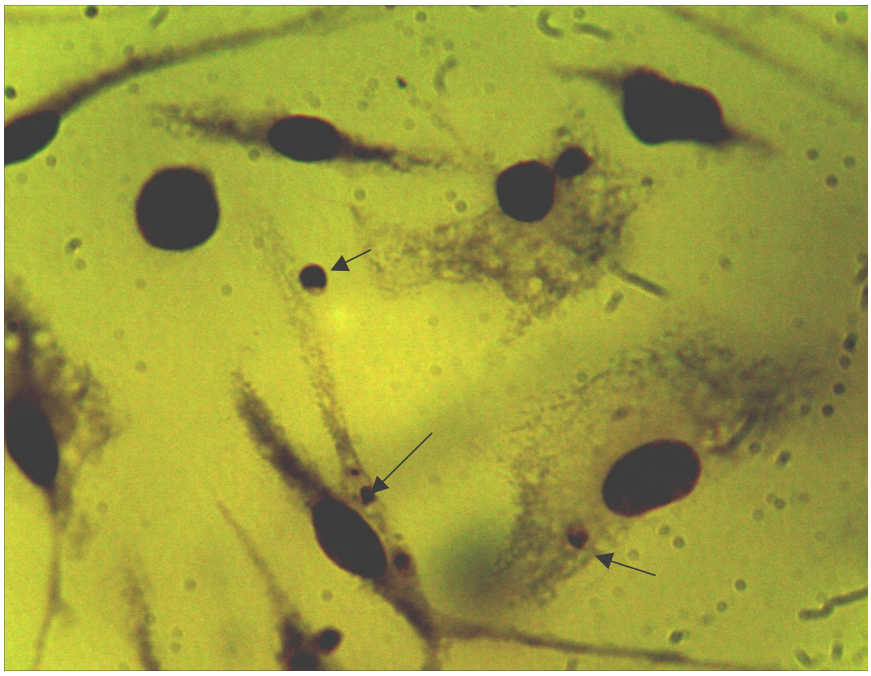


Plate 4.1: Untreated macrophages with amastigotes occupying the parasitophorous vacuoles (black arrows) in the cytoplasm. Macrophages are seen with their large nuclei, which are eccentrically placed while amastigotes are small round or ovoid structures. (x1000 Giemsa stain)

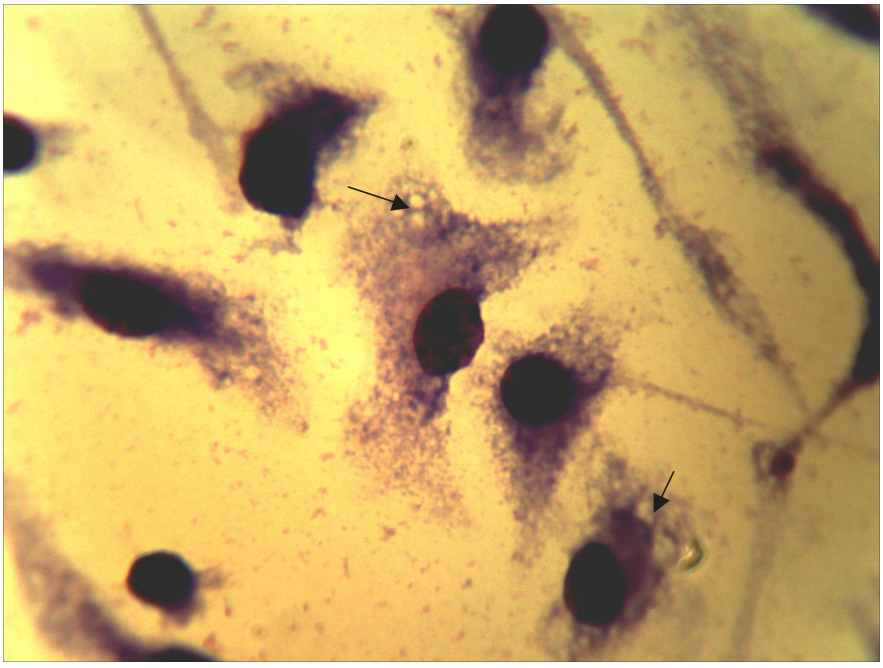


Plate 4.2: Treated macrophages with parasitophorus vacuoles (black arrows) not occupied by amastigotes. Macrophages are viable with cytoplasm and pseudopods. (x1000 Giemsa stain)

Table 4.3:

Efficacies of nine extracts (100 μg/ml) of three plants on the intracellular survival of *L. major* strain

Treatment	% infection of cells	% chemosuppression	No. of parasites per host cell	% suppression of parasites
Control	93.3 ± 2.5		14.3 ± 1.9	
DMSO	91.9 ± 2.5	1.6 ±1.0	13.9 ± 1.9	3.7 ± 2.5
PBS	92.9 ± 2.0	0.4 ±1.2	16.5 ± 5.5	0 ± 0
AI Hexane	84.4 ±1.9	11.6 ± 1.6	5.5 + 0.9⋆	59.9 ± 8.0
AI Methanol	ND	ND	ND	ND
AI Aqueous	82.1 ± 2.1	8.6 ±1.7	4.4 ± 0.4⋆	70.5 + 4.2
AS Hexane	73.9 ± 4.3	22.7 ± 4.9	5.2 ± 0.1	63.1 ± 2.4
AS Methanol	47.0 ± 1.4▪	48.4 ± 2.5	3.2 ± 0.6✶	74.6 ± 4.9
AS Aqueous	84.4 ± 7.0	9.1 ± 4.6	7.0 ± 4.0▪	59.1 ± 11.9
VA Hexane	74.1 ± 8.4	21.2 ± 7.3	5.4 ± 0.4⋆	61.3 ± 2.4
VA Methanol	ND	ND	ND	ND
VA Aqueous	31.2 ± 4.4▪	65.1 ± 6.9	2.3 ± 0.4✶	85.3 ± 0.7

ND indicates not determined
± indicates SE
Symbols ⋆ indicates $p<0.01$, ▪ indicates $p<0.05$ and ✶indicates $p<0.001$ as compared to the controls.
AI, AS and VA stand for *Azadirachta indica*, *Annona senegalensis* and *Vernonia amygdalina* respectively

4.2.2 Drug Sensitivity Assay on Macrophages

The results of the preliminary tests above informed the analysis of the activities of four selected extracts against intracellular *L. major* amastigotes. These were aqueous and methanolic extracts of *Vernonia amygdalina* as well as methanolic extracts of *Azadirachta indica* and *Annona senegalensis*. To this end, freshly isolated bone marrow macrophages were infected with *L. major* for 24 hours and then treated with increasing concentrations of the extracts for 72 hours. Results showed a dose dependent antileishmanial effect (based on the reduction in the percentage of cells infected and the mean number of amastigotes per host cell as seen in tables 4.4 and 4.5). Methanolic extract of *Vernonia amygdalinna* demonstrated the greatest suppression in the percentage of infected cells at much lower concentrations. This was followed by methanolic extract of *Annona senegalensis.* Aqueous extract of *Vernonia amygdalina* showed effects at higher concentrations while the methanolic extract of *Azadirachta indica* still affected macrophage survival at the concentrations used hence its effect could not be determined (ND). Treatment with dimethyl sulphoxide (DMSO) and phosphate buffered saline (PBS) which were the solvents used to dissolve the extracts showed no significant effect on the percentage of infected cells or on the number of amastigotes per host cell in the preliminary tests hence were not included in this assay. Data on the results of the sensitivity assay of the selected compounds is summarized in tables 4.4 and 4.5 below.

Table 4.4:

Effects of the different concentrations of the extracts and reference drug (Sb V) on the mean infection rate of bone marrow macrophages

Treatment	Concentrations (μg/ml)					
	Control	100	50	25	12.5	6.25
Sb (V)	75.10±4.1	ND	43.23±5.2	48.31±3.1	60.20±3.7	62.12±3.2
VAMe	72.25±2.3	ND	35.20±3.9	41.25±2.7	43.75±6.2	54.25±2.5
ASMe	76.21±4.3	46.32±1.1	47.43±1.2	48.31±4.2	64.61±3.6	68.50±2.4
VAAq	72.25±2.3	35.25±2.1	47.35±0.9	ND	35.75±2.2	38.25±0.9

Bone marrow macrophages from BALB/c mice were infected with *L. major* promastigotes at a parasite/cell ratio of 20:1. Cells were incubated for 72 hours; the mean percentage of cells infected was determined. All values are means ±SEMs.

ND indicates not determined due to toxicity, VAMe stands for *Vernonia aygdalina* methanolic extract, ASMe stands for *Annona senegalensis* methanolic extract, VAAq stands for *Vernonia amygdalina* aqueous extract.

Table 4.5:

Effect of the different concentrations of extracts and reference drug (Sb V) on the mean number of parasites per host cell

Treatment	Concentrations (μg/ml)					
	Control	100	50	25	12.5	6.25
Sb (V)	2.61±0.4	ND	1.92±0.2	2.50±0.2	2.43±0.2	2.51±0.2
VAMe	4.88 ± 0.3	ND	2.03±0.3	2.71±0.2	2.82±0.2	3.98±0.1
ASMe	10.41±0.3	2.35±0.2	2.51±0.3	2.82±0.3	4.42±0.5	4.81±0.2
VAAq	4.55±0.1	2.86±0.2	3.35±0.1	ND	2.71±0.2	2.98±0.3

Bone marrow macrophages from BALB/c mice were infected with *L. major* promastigotes at a parasite/cell ratio of 20:1. Cells were incubated for 72hours; the mean numbers of parasites per host cell was determined. All values are means ±SEM

ND indicates not determined due to toxicity, VAMe stands for *Vernonia aygdalina* methanolic extract, ASMe stands for *Annona senegalensis* methanolic extract, VAAq stands for *Vernonia amygdalina* aqueous extract.

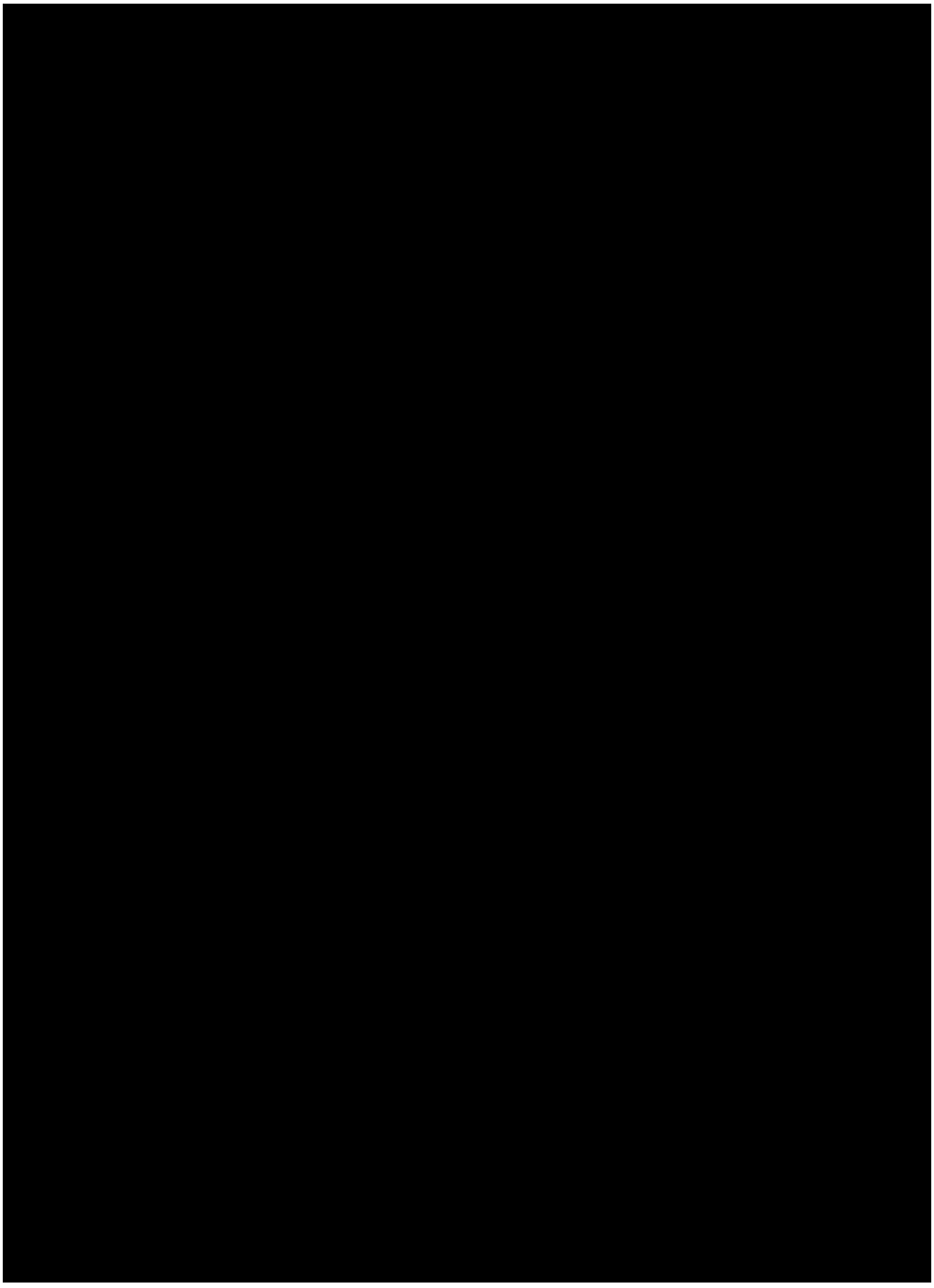


Fig 4.7: Effects of extracts and reference drug (Sb V) on the mean suppression in A, infection rate (chemosuppression) and on B, parasite per host cell (parasitaemia). The mean suppression in infection rate (A) and parasite per host cell (B) were calculated using the mean control for each treatment. Points indicate Means ± SEM.

4.2.3 Cytotoxicity studies

The cytotoxicity of the nine extracts were evaluated using the Alamar blue assay on L929 fibroblasts, Jurkat and SH-SY5Y cells at a concentration of 100 μg/ml. Results showed that the methanolic extracts of the three plants and hexane extract of *Annona senegalensis* were toxic to the L929 fibroblasts as they distorted the cells and there was colour interference due to the strong colour of some of the extracts. However, the hexane extract of *Azadirachta indica* (AI) and *Vernonia amygdalina* (VA) as well as all the aqueous extracts were not toxic to these cells at this concentration. Cytotoxicity on the macrophages was indirectly determined using the mean amastigote number per host cell. Where toxicity occurred this could not be determined. Results showed that only two of the extracts (methanolic extracts of AI and VA) were cytotoxic to macrophages at this concentration. However, there was no toxicity from the nine extracts on the Jurkat and SH-SY5Y cancer cell lines at this concentration. Table 4.6 shows the cytotoxic effects of the nine extracts on the different cell lines.

Table 4.6:

Cytotoxic activity of plant extracts (100 μg/ml) on different cell lines

Extract (100 μg/ml)	L929	Macrophages	Jurkat	SH-SY5Y
AI Hexane	-ve	-ve	-ve	-ve
AS Hexane	+ve	-ve	-ve	-ve
VA Hexane	-ve	-ve	-ve	-ve
AI Methanol	+ve	+ve	-ve	-ve
AS Methanol	+ve	-ve	-ve	-ve
VA Methanol	+ve	+ve	-ve	-ve
AI Aqueous	-ve	-ve	-ve	-ve
AS Aqueous	-ve	-ve	-ve	-ve
VA Aqueous	-ve	-ve	-ve	-ve

-ve indicates not toxic
+ve indicates toxic

4.2.4 Trypanocidal Activity

Nine extracts were analyzed for their *in vitro* trypanomicidal activity against *Trypanosome brucei brucei* at increasing concentrations. All the hexane and methanol extracts of the plants showed anti-trypanosomal activity, which was comparable to the reference drug cymelarsan used. The reference drug was used at 50 μg/ml and the six extracts showed trypanocidal effects similar to, or better than cymelarsan at concentrations of <50 μg/ml. The most effective was the hexane extract of *Annona senegalensis* with effective concentration or MIC of approximately 3 μg/ml. The aqueous extract of *Vernonia amygdalina* showed mild trypanocidal effect at 50 μg/ml while other aqueous extracts had no effect on the parasites and Fig 4.8 shows a sample of plate used to determine the colorimetric end-point. Results of the MIC of the nine extracts are shown in table 4.7.

Concentration of plant extracts (μg/ml)

G	100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0.19	S

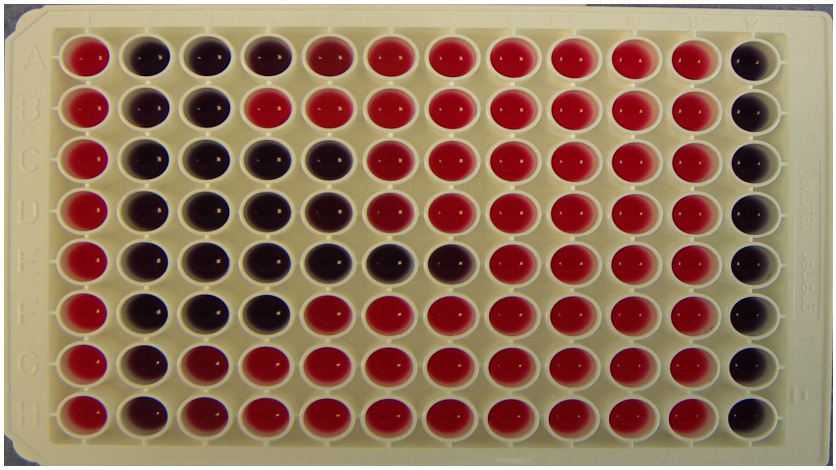


Fig 4.8: Sample plate showing results of trypanocidal activity of plant extracts obtained using the colorimetric endpoint determination method. G indicates growth control, S indicates sterility control, Rows A to F (from top to bottom) indicate plant extracts: AI MeOH, AS MeOH, VA MeOH, AI Hexane, As Hexane VA Hexane, Row G indicates DMSO and Row H indicates Cymelarsan (reference drug)

Table 4.7:
Inhibitory activity of plant extracts against *Trypanosome brucei brucei*

Code	*Extract*	*MIC (μg/ml)*
AI (*Azadirachta indica*)	Hexane	6.25
	Methanol	12.5
	Aqueous	>100
AS (*Annona senegalensis*)	Hexane	3.12
	Methanol	50
	Aqueous	>100
VA (*Vernonia amygdalina*)	Hexane	25
	Methanol	6.25
	Aqueous	50
Cymelarsan		50
DMSO		100
PBS		>100

MIC indicates the minimum inhibitory concentration as determined by colorimetric endpoint method. It is interpreted as the lowest sample concentration that remained blue (indicating no growth) or first concentration that changed from blue to slightly purple. This result is representative of two tests.

4.3 *In vivo* antileishmanial Activity

Histopathological studies were performed at day -21 post infection on mice treated with methanol extracts of *Vernonia amygdalina* and *Annona senegalensis* at a dose of 250 mg/kg bw. Macroscopic studies showed ulceration of some lesions at the site of infection. This was most pronounced in the control group. Lesion sizes were monitored weekly and the results showed that mice treated with methanolic extract of *Vernonia amygdalina* had significantly smaller lesion sizes ($p<0.05$) as compared to the control. Those treated with methanolic extract of *Annona senegalensis* did not show any significant difference in the size of the lesions as compared with control. Results of lesion sizes are shown in figure 4.9 below. Microscopic studies of the skin showed hyperplasia of the epidermis and hyperkeratosis that was pronounced in the control group. The keratinocytes are rounded up and swollen (balloon cells) with their nuclei being pleomorphic and hyperchromatic. Also dermal infiltration of inflammatory cells consisting of lymphocytes, plasma cells and other ovoid cells was present and this was marked in the control and *Annona senegalensis* treated groups. Plates 4.3 to 4.7 show micrographs of the histopathology of the skin. There were no significant histopathological changes in the liver and spleen. These results demonstrate that at 21 days post-infection, the methanolic extract of *Vernonia amygdalina* suppressed the rate of lesion growth and rate of histopathological changes in the skin of BALB/c mice at a dose of 250 mg/kg bw.



Fig 4.9: Effect of plant extracts on lesion growth following oral administration. BALB/c mice (n=5) were infected subcutaneously in the shaven rump with $1.6x0^6$ *L. major* promastigotes. Animals were treated with 1 mg of VA MeOH (▲), 1 mg of AS MeOH (▼) and untreated (■). Treatment lasted for 5 days and weekly measurements of the lesion sizes were taken at indicated times using a vernier calliper (Mean ± SEM). • symbol means $p<0.05$ indicating statistical significance.

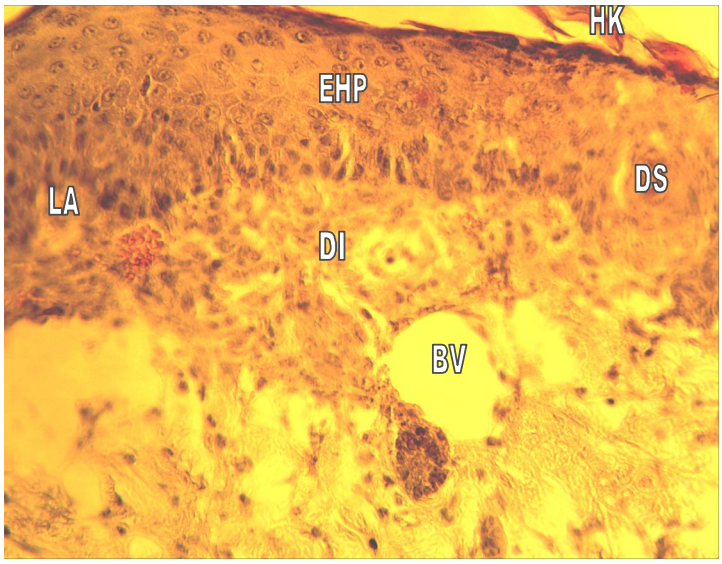


Plate 4.3: Cross section of skin from infected control mouse showing qualitative epidermal hyperplasia (EHP), desquamation (DS) and hyperkeratosis (HK). Dermal infiltration (DI) by numerous ovoid cells inflammatory cells with centrally located nuclei and lymphocyte aggregation (LA) at the dermal-epidermal junction. Distortion of the collagen fibres in the superficial dermis with blood vessel (BV). (H&E x250)

A) B)

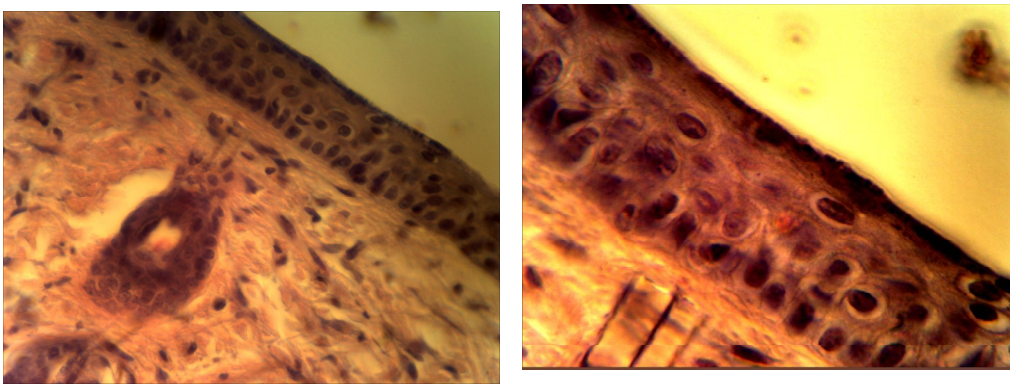


Plate 4.4: Cross section of skin from infected control mouse showing swollen keratinocytes (balloon cells) and inflammatory cells. A) Chronic inflammatory infiltration with perivascular infiltration in superficial dermis B) Desquamation of keratinocytes in upper epidermis leading to parakeratosis. A. (H&E x250) B. (H&E x1000,oil immersion)

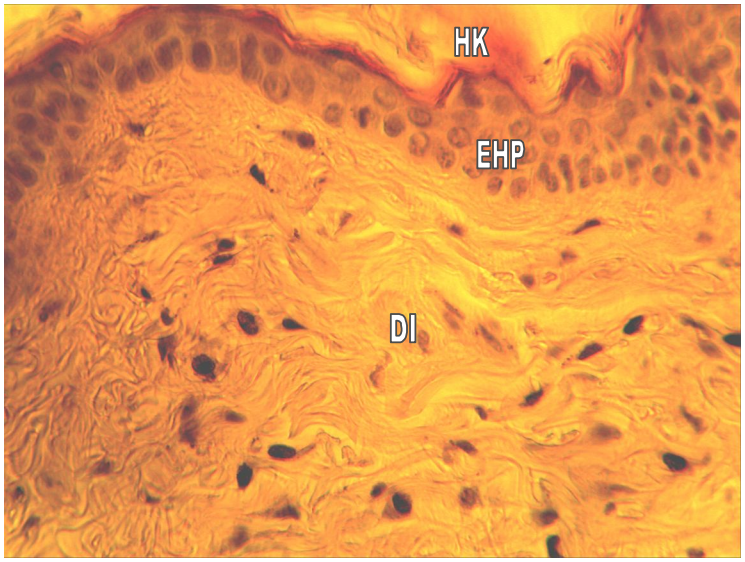


Plate 4.5: Cross section of skin from AS treated mouse showing epidermal hyperplasia (EHP), hyperkeratosis (HK) and dermal infiltration by ovoid cells with centrally located nuclei and macrophages (DI). No distortion of collagen fibres in superficial dermis. (H&E x250)



Plate 4.6: Cross section of skin from AS treated mouse showing epidermal hyperplasia with abscess formation in the nodules (arrows). Chronic infiltration of inflammatory cells in superficial dermis indicating chronic inflammation. Separation of dermal-epidermal junction (DEDJ) is artifactual (H&E x400).

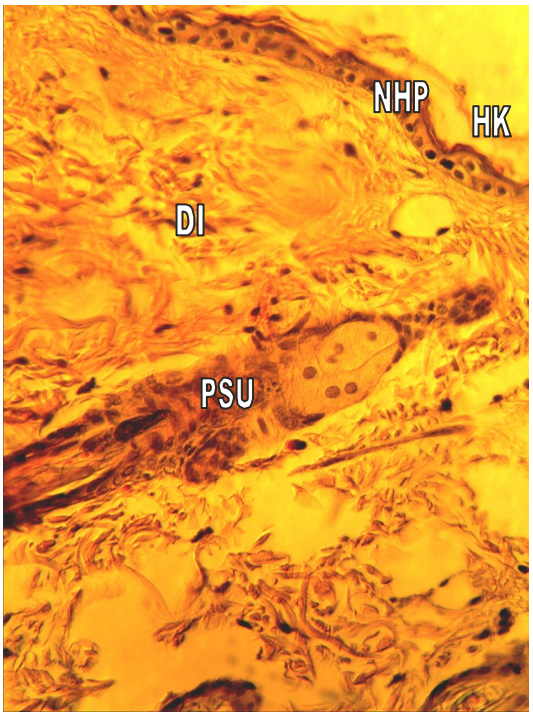


Plate 4.7: Cross section of skin from VA treated mouse showing non-hyperplasia (NHP) or thinner epidermis and hyperkeratosis (HK). Dermis is sparsely infiltrated (DI) with inflammatory cells suggesting milder inflammation. Prominent pilosebaceeous unit (PSU). (H&E x250)

IMMUNOTHERAPY EXPERIMENT

4.4 Expression of recombinant protein

The γGCS gene sequence from *L. donovani* was successfully cloned into the adapted pVAX I expression vector to create pVAX- γGCS. Typically the yield was between 0.8 and 1.2 mg per litre/batch. The construct sequence was observed using the agarose gel electrophoresis. Result is shown in figure 4.10 below.

The γGCS fused gene was amplified from the pVAX I and then subcloned into pMAL2cE vector for expression in *E.coli* DH5α cells. Expression of the recombinant protein was induced with 1 mM IPTG and the bacterial lysate analysed by SDS-PAGE. After staining the gel with Commassie Blue, a band at 76 kDa (Fig. 4.11a lane 3) was observed and this was not detected in the bacterial lysate before induction (fig. 4.11a lane 2). The intensity of the band decreased after purification and dialysis (fig. 4.11a lanes 8 and 9). Figure 4.11b shows that similar bands were produced at 76 kDa, on the coomassie blue gel and ponceau stained nitrocellulose. This indicates that the transfer process was efficient. As expected this was not visible on the protein marker. Some additional weak bands were seen below the protein of interest in the lysates but these appear to be non-specific reactions as they are also visible on the protein marker. The yield of the recombinant protein was 0.440 mg/ml after analysis using the Bio-rad protein assay (fig 4.11c). The protein was used in all the ELISA assays since it gave positive titres when used at low concentration of 0.1 μg/ml.

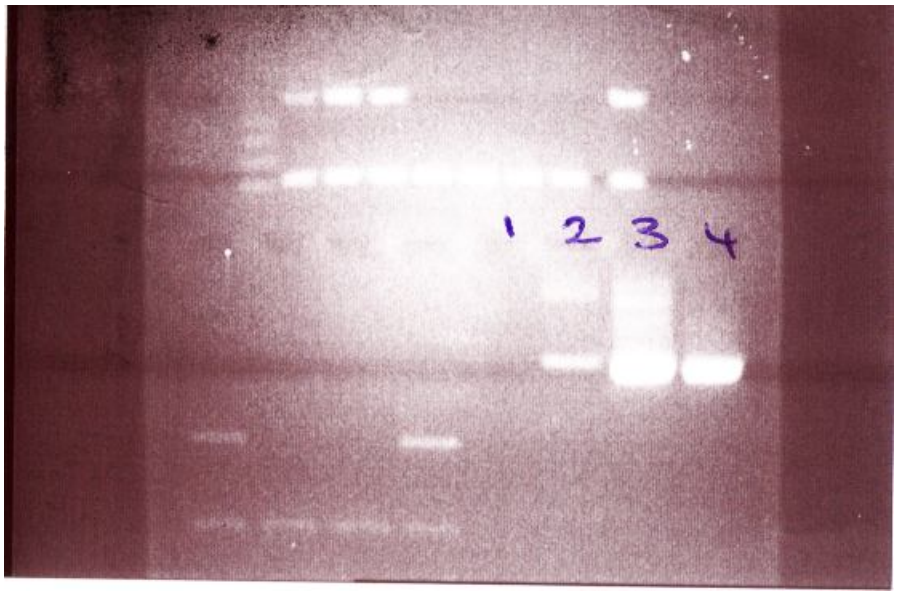


Fig 4.10: Expression of the γGCS gene sequence into the expression vector pVAX I to yield pVAX-γGCS. The construct sequence was analysed by Agarose gel electrophoresis followed by Coomassie staining. Lane 1: Molecular grade water, Lane 2: pVAX I plasmid, Lane 3: pVAX-γGCS construct, Lane 4: γGCS gene.

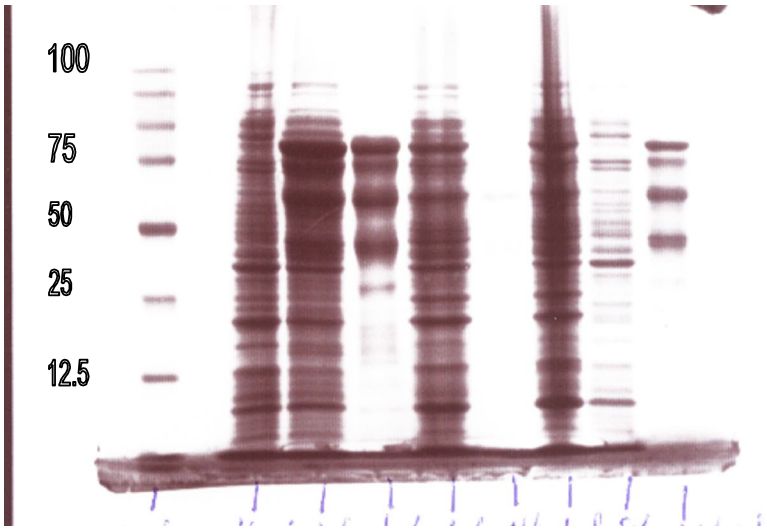


Fig 4.11a: Expression of *L. donovani* γGCS recombinant protein (rγGCS) in *Escherichia coli* B21 bacteria. Expression ws induced with 1 mM IPTG in B21 pMAL-γGCS bacteria strain transformed with a pVAX I expression plasmid. Total bacterial protein was analysed by SDS-PAGE followed by staining with Coomassie Blue. Lane 1: Molecular weight protein marker, Lane 2: total bacterial lysate before induction, Lane 3: Total bacterial lysate after 24 hours induction, Lane 4: Insoluble protein, Lane 8: Soluble protein Lane 9: Protein obtained after dialysis to remove urea.

Lane: 1 2 3 4 5 6

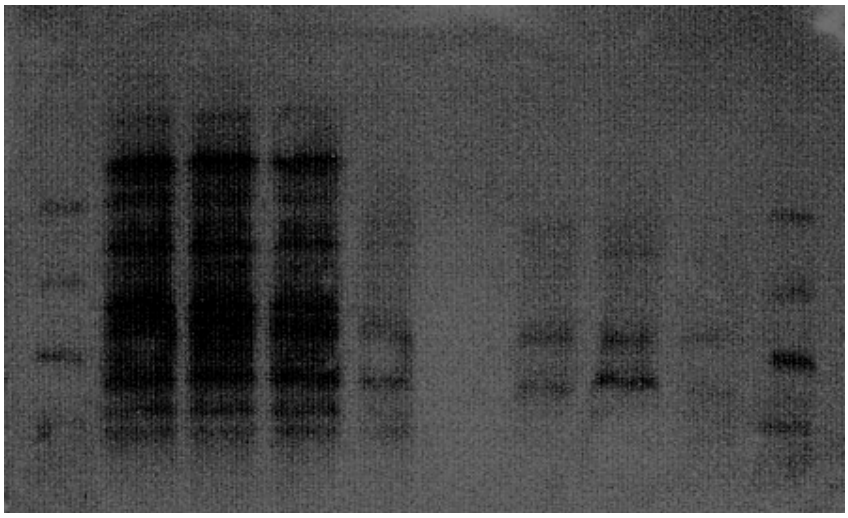


Fig 4.11b: Protein analysis by Western blotting of expressed *L. donovani* γGCS recombinant protein in total bacterial lysate by His antibody and stained with Ponceau. Lane 1: protein marker, Lane 2 and 3: show IPTG induced bacterial lysate, Lane 4: Insoluble protein, Lane 5: soluble protein and lane 6: dialysed protein

Sample	Wells	Values	R	Result	MeanResult	Std.Dev.	CV%
Un01	D7	1.126		0.425	0.440	0.021	4.8
	E7	1.169		0.455			

R - Outside standard range

Fig 4.11c: Expression of recombinant protein was evaluated using the Bio-rad protein assay. Mean result of quantity of protein in duplicate wells was obtained on an ELISA reader at 520 nm.

4.5 Efficacy of DNA and Protein Vaccinations

The efficacy of DNA vaccination with pVAX-γGCS and protein vaccination with rγGCS-NIV was evaluated by their capacity to confer protection against infection with *L. major* promastigotes in BALB/c mice cutaneous model. Time course lesion sizes of four different groups of mice (n=7 per group) were measured after challenge with $1x10^7$ highly infective promastigotes (figure 4.12). Immunization with pVAX-γGCS (group III) and rγGCS-NIV (group IV) showed a significant delay in the growth of lesions as compared to the control group (group I) treated with PBS for upto 14 and 21 days post infection respectively. However, these effects were not long lasting and by 35 days post-infection, the protective effect disappeared and lesion sizes in the mice immunized with pVAX-γGCS (group III) reached comparable sizes to that of the control group while that of the mice immunized with the recombinant protein in the presence of NIV (rγGCS-NIV) remained significantly smaller as compared to the control group. Immunization of mice with pVAX alone (group II) had no significant effect on lesion sizes since the lesions grew to similar sizes as in those of the control. These results show that at 35 days post-infection, no statistical differences could be observed between groups I, II and III. Also, that the best protection was achieved in mice which were immunized with the recombinant protein, as there was partial protection 21 days after infection and significantly smaller lesion sizes at termination of the experiment suggesting the control of lesion development.

Spleen-to-body weight ratio (normalised to normal or uninfected mice) was also measured after infection with *L. major* as an indicator of disease progression. Control mice which were infected and treated with PBS showed splenomegaly as compared to the uninfected or normal mice. No significant reduction in splenomegaly was observed in the mice immunized with pVAX (group II) or pVAX-γGCS (group III). In contrast, mice immunized with rγGCS or the recombinant protein showed smaller weights, suggesting milder inflammatory responses ($P<0.05$, Fig. 4.13).



Fig. 4.12: The cross protective effect of immunisation with pVAX (plasmid alone), pVAXγGCS (DNA plasmid), γGCS-NIV (recombinant protein) from *L. donovani* or unvaccinated (PBS) against subsequent infection with *L. major* promastigotes. BALB/c mice were immunised intramuscularly with 100 μg of the relevant plasmid preparation and received a booster 14 days later. Then, 14 days after the second immunisation, the animals were infected with *L. major* promastigotes in the shaven rump. Lesion sizes were measured weekly. • symbol indicates a significant difference ($p<0.05$) in the protein group as compared to the control group.

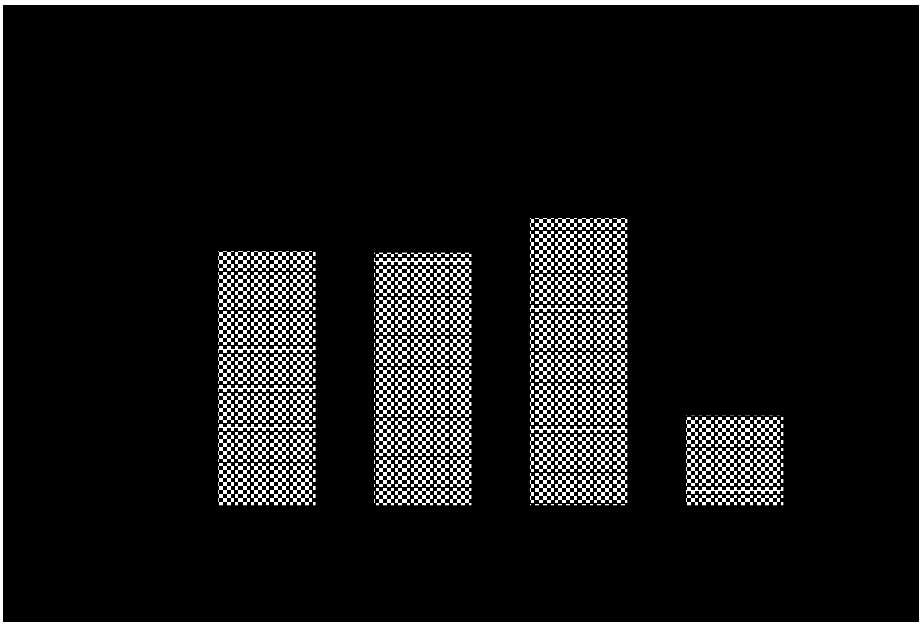


Fig 4.13: Protective effect of immunised mice against infection. Protection was assessed by measuring the normalised spleen to body weight ratios in mice at 35 days postinfection. Data are presented as means ± SEM of four mice per group. • symbol indicates a significant difference ($p<0.05$) in the protein (rγGCS-NIV) group as compared to the control group.

4.6 Humoral Responses to DNA and Protein Vaccinations

To monitor antigen-specific immune responses primed by immunization with pVAX, pVAX-γGCS and γGCS-NIV, the humoral responses were monitored in the immunized mice. The specific serum IgG isotypes were evaluated in response to the γGCS protein by direct ELISA. The titres of specific IgG1 and IgG2a were determined in sera taken from mice over a time course from before infection (day -15) to 5 weeks (day 35) after the infection. Naïve mice sera were also included for comparison. Results from Figure 4.14, showed that on day -15, 1 day after the second immunisation no specific IgG1 antibody titres were present as similar titres were present in all the groups except for the group vaccinated with the recombinant protein (rγGCS-NIV). This produced titres that were higher than the control groups but did not reach significant levels. However there was no significant difference in the titres for IgG2a for all the groups. By day 0, 14 days after the second immunisation and just before challenge specific IgG1 levels increased significantly in the group treated with the recombinant protein as compared to the control groups ($p< 0.05$). Also there was a slight increase in the control group which could be attributed to variations as there was no change in the pVAX and pVAX-γGCS groups. The titres for the IgG2a increased in all the groups but still were not significantly different. On day 7, 1 week postinfection, the levels of IgG1 and IgG2a in the group treated with the recombinant protein showed significant increase compared to the control ($p<0.05$). However in the group treated with the pVAX-γGCS, there was a significant decrease in the level of IgG1 ($p<0.05$) as compared to the control. It was also similar to that of the uninfected group while that of IgG2a also decreased slightly. However, it was the only group that showed higher IgG2a level compared to IgG1 after challenge suggesting some Th1 bias at this point. By day 35, which was the termination

day, the levels of IgG1 had dropped in all the groups except the group immunised with pVAX-GCS as compared to day 7. This group showed a significant increase in IgG1 titre compared to the control group and uninfected group ($p<0.05$). Also the group treated with the recombinant protein still showed significantly higher IgG1 titres compared to the control values. However the IgG2a values decreased in all the groups as compared to day 7 and their titre values were not significantly different although the group immunised with the recombinant protein showed the highest titre values. In summary, this result showed that the recombinant protein vaccine (rγGCS) was the most immunogenic as it produced high antibody levels especially IgG1 while the pVAX-GCS (DNA vaccine) appeared to be the least immunogenic as it induced low antibody levels. Also all the groups of mice developed an antibody isotype profile indicative of mixed Th1/Th2 responses after immunisation with the different vaccines

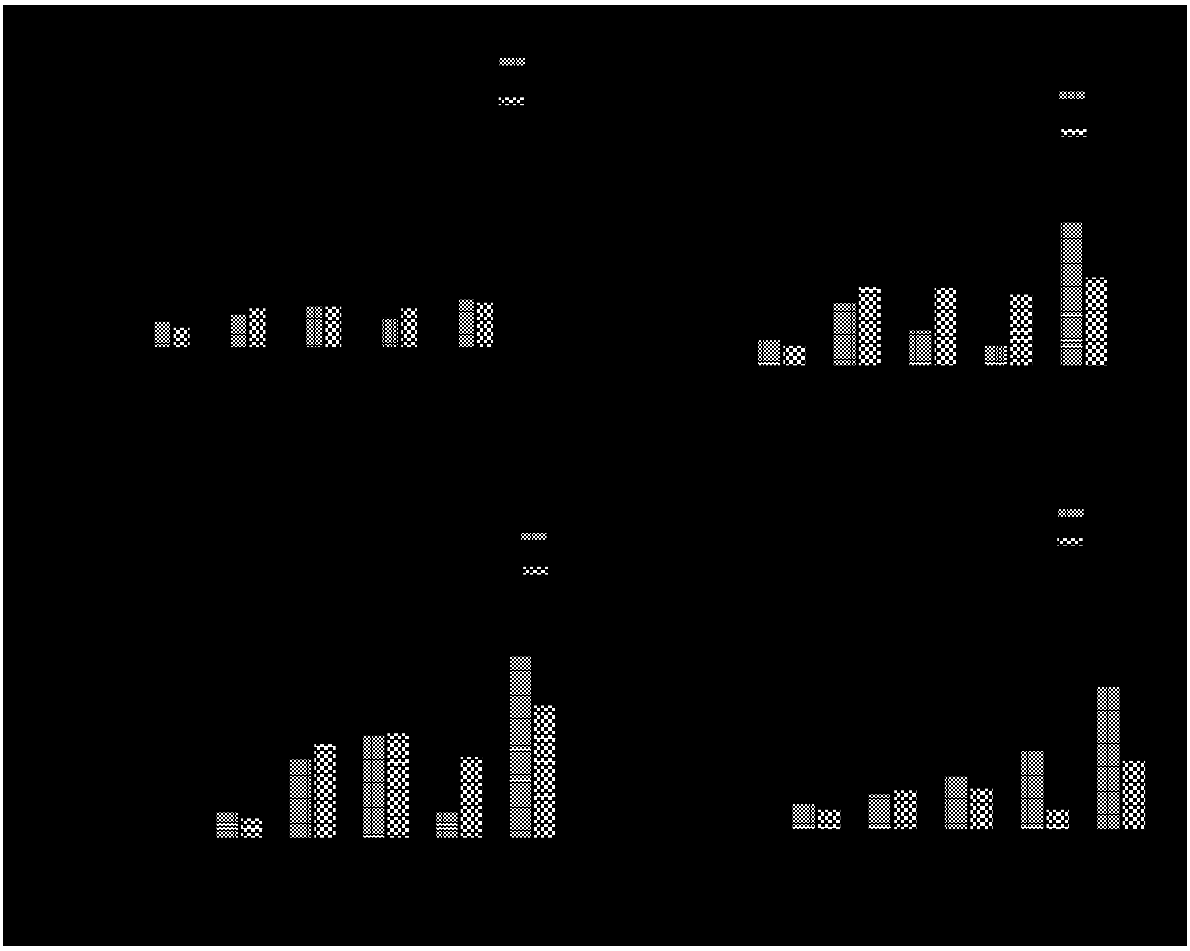


Fig 4.14: The effect of vaccination on the specific antibody response of immunised mice. Mice were immunised intramuscularly with 100 mg of pVAX alone (plasmid) or *L. donovani* pVAXγGCS (DNA plasmid) or γGCS-NIV (protein with NIV) on days -15, 0, 7 and 35. Specific IgG1 and IgG2a titres to the recombinant γGCS protein in normal, unvaccinated and vaccinated mice were determined on (a) day -15, (1day before second immunisation); (b) day 0 (day of infection); (c) day 7 (7 days postinfection) and (d) day 35 (35 days postinfection.

4.7 Cytokine and Nitric oxide Production in Vaccine Groups

For further investigation into the phenotype (Th1 or Th2) of the immune response elicited during control of *L. major* infection by immunization with DNA and protein vaccines, suspensions of splenocytes from the immunised mice were restimulated in vitro with rγGCS (25μg/ml and Con A (5μg/ml) as mitogen 14 days after second immunisation or immediately before challenge (day 0) and 35 days after challenge. The supernatants were measured for the production of the following cytokines: IFN-γ, IL-4, IL-5 and IL-10. From results shown in Fig 4.15, splenocytes produced significantly high level of IFN-γ ($p<0.05$) and low level of IL-5 when stimulated with the γGCS lysate, in the group immunised with recombinant protein (rγGCS-NIV) as compared to the control group. There was no significant difference in the other three groups. In fact the group immunised with pVAX-γGCS (DNA vaccine) showed relatively low production of IFN-γ but was not significant compared to the control. IL-4 and IL-10 were produced in little or no quantities. From fig 4.15, results show that by 35 days post infection, only IFNγ and IL-5 were detected in the supernatants of the cells stimulated with γGCS lysate although there was no significant difference. Negligible or background levels of IL-4 and IL-10 were produced in stimulated splenocytes while no cytokine was detected in the unstimulated (medium) splenocytes.

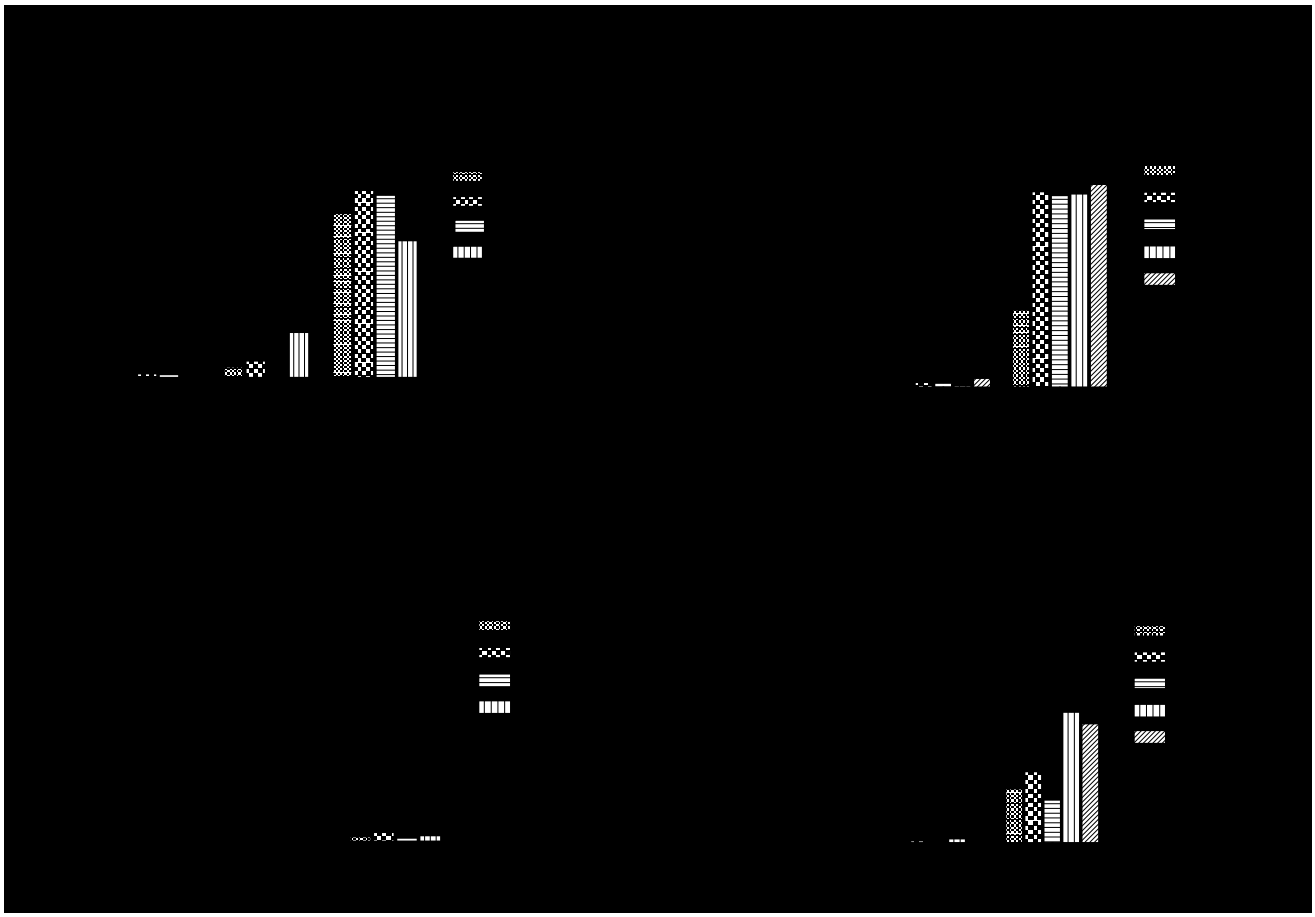


Fig 4.15a: Mean cytokine (IFN-γ and IL-4) production by splenocytes from mice in Fig. 4.14 before infection (A and B) and at week 7 after challenge (E and F) with *L. major*. Each group was incubated with medium alone (unstimulated), γGCS protein (25 μg/ml) or Con A (5 μg/ml).

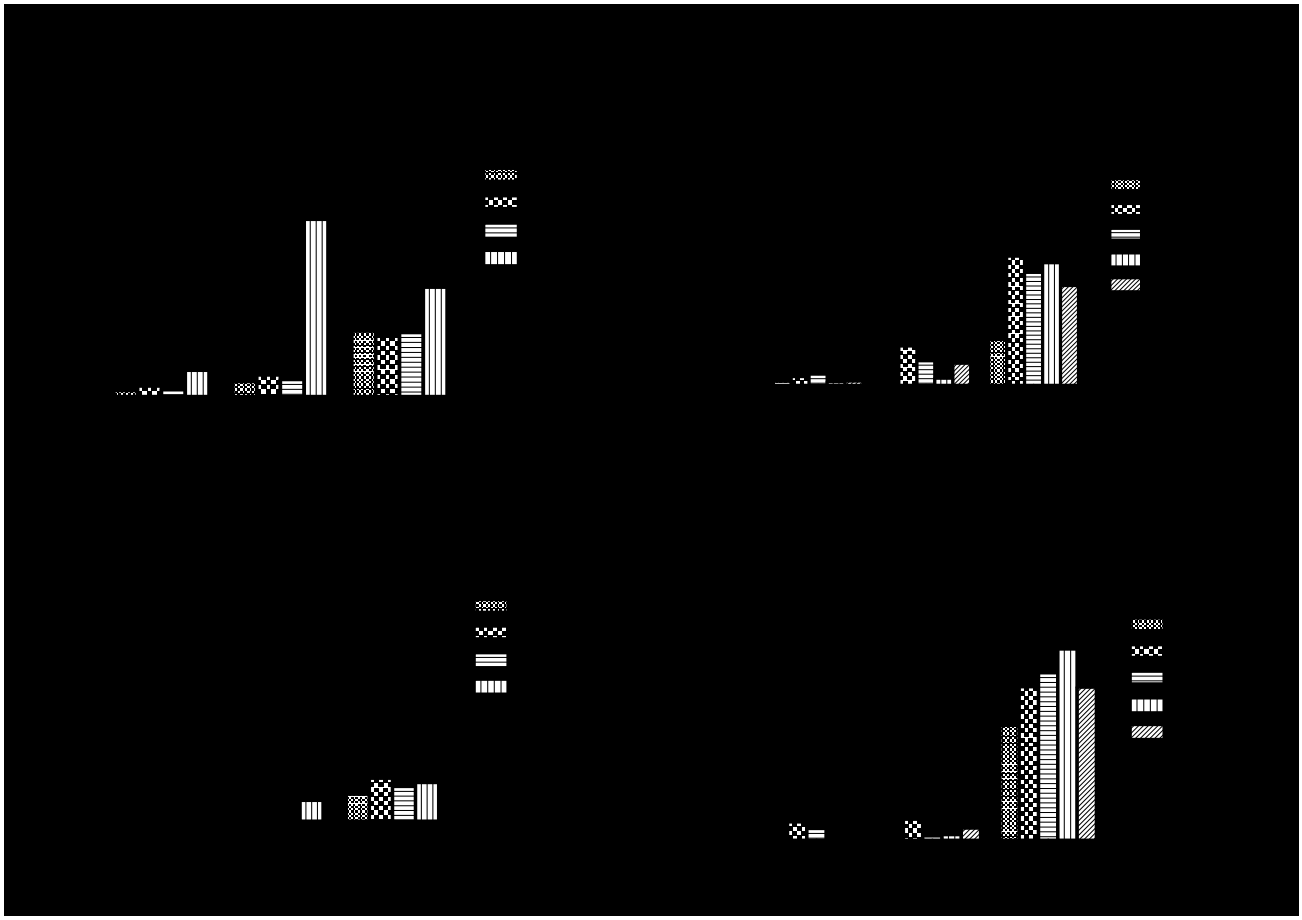


Fig 4.15b: Mean cytokine (IL-5 and IL-10) production by splenocytes from mice in Fig. 4.14 before infection (C and D) and at week 7 after challenge (G and H) with *L. major*. Each group was incubated with medium alone (unstimulated), γGCS protein (25 μg/ml) or Con A (5 μg/ml).

There was no significant difference in the nitric oxide production on day 35 among the immunised groups as compared to the control when stimulated with the medium or the rγGCS protein. This shows that stimulation with the protein did not result in a higher production in nitrite compared to the unstimulated for all the groups of mice (Fig 4.16). However stimulation with the Con A resulted in a further increase in nitrite production in all the groups but there was no significant difference between the groups (Fig 4.16). The least production of nitrite was in the protein group while the highest was in the DNA-vaccine group although this did not reach significant differences. This result is inversely proportional to the production of IFN-γ in the Con A stimulated splenocytes.

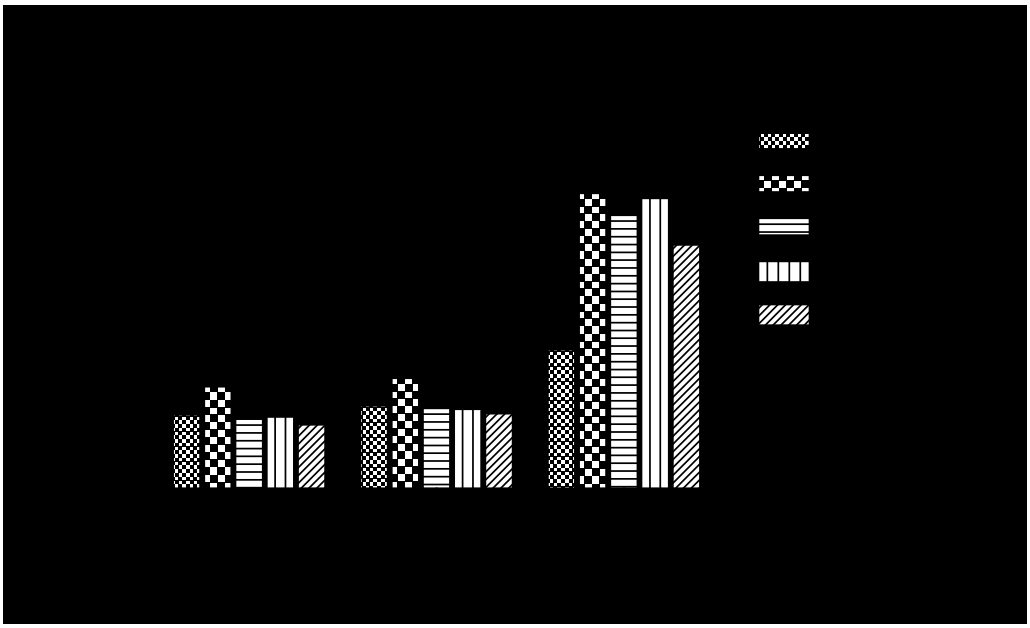


Fig 4.16: Mean nitrite production by splenocytes from vaccinated and unvaccinated groups. Mice were immunised and challenged as in Fig 4.22 and at 35 days postinfection spleen cells from the different groups were collected and incubated with medium only (unstimulated), recombinant protein (γGCS) 25 μg/ml or Con A (5 μg/ml).

CHAPTER FIVE

6.0 DISCUSSION

PHYTOTHERAPY EXPERIMENTS

5.1 Plants Extraction

Results demonstrated that the methanol extracts gave the highest yields while the hexane extracts had the lowest yields. This is in line with past works in which methanolic extracts are preferred due to their high yield in extraction processes. Water extraction naturally sets some limitations to the amount of compounds that can be extracted due to its polarity (Eloff, 1998). Observations of the structure and forms showed that the methanol extracts were in form of caramel as they were gluey. This is because of the sugar content extracted by methanol. The hexane extracts which gave the least yield were oily while the aqueous extracts were dry and pelleted. Colours of the extracts were green, yellow and brown. Extracts from the leaves gave deeper colours than that of the stem bark. *Vernonia amygdalina* extracts had the deepest colours and this was responsible for some colour interference in the cytotoxicity study, followed by that of *Azadirachta indica* while that of *Annona senegalensis* were light coloured.

5.2 I*n vitro* Experiments

5.2.1 Macrophage Sensitivity Tests

This study was carried out to investigate the leishmanicidal effects of three plants found in Nigeria. From the preliminary study in which a total of nine extracts were tested at 100 μg/ml, only two markedly inhibited the infection rate of the *in vitro* forms of *Leishmania major (L. major)* amastigotes without toxicity to the macrophages. However, seven of the nine extracts showed direct effect on the amastigotes as they significantly reduced the number of amastigotes per host cell

when compared to the control. Aqueous extract of *Vernonia amygdalina* exhibited the highest leishmanicidal activity as it suppressed the infection rate of amastigotes significantly with an inhibition percentage of 61.9. It also reduced parasite levels upto 85.3% of the infected control. This leishmanicidal activity was associated with lack of toxicity to the macrophages suggesting that it possesses a selective activity against leishmania parasites. This demonstrates that the aqueous extract of *Vernonia amygdalina* has promising anti-leishmanial activity when used *in vitro* at this concentration and this could serve as advancement in the search for new anti-leishmnial drugs.

Futhermore, results of this study showed that the methanolic extract of *Vernonia amygdalina* significantly suppressed the infection rate of the intracellular amastigotes and reduced parasite levels too. However, it showed toxicity at the highest concentration of 100 μg/ml but graded concentration/response tests showed that at concentration of ≤50 μg/ml, the extract was effective in suppressing the infection rate of amastigotes by >50% without toxicity to the macrophages. This shows that *V. amygdalina* was leishmanicidal at concentrations below that which caused toxicity to the macrophages. In addition, the effective concentration of this extract was similar to the concentration of sodium stibogluconate needed to decrease the infection rate by 50%, thus demonstrating that despite the higher toxicity of the methanolic extract against bone marrow macrophages, its efficacy against *L. major* mastigotes is similar to the reference drug used. This conforms to past studies in Ethiopia, in which the chloroform and methanol extracts of *V. amygdalina* showed antileishmanial activity against *L. aethiopica*. Also, the results showed that the amastigotes were more sensitive to *V. amygdalina* than the promastigotes (Tadesse *et al.*, 1993).

Annona senegalensis and *Azadirachta indica* also reduced parasite levels in the macrophages and these are in line with previous studies in Sudan, which showed that seeds of *Annona senegalensis* (Sahpaz *et al.,* 1994) and the leaves of *Azadirachta indica* (El Tahir *et al.,* 1998; Khalid *et al.,* 2005) had potent antileishmanial activity *in vitro* on *L. major* parasites. These results show that these extracts had direct effect on the multiplication of the amastigotes, which are the mammalian forms responsible for the clinical manifestation of leishmaniasis in humans and differ in cellular, molecular and biochemical characteristics from the promastigotes which are the insectal forms (Gupta *et al.*, 2001). Thus, the preferred use of the amastigotes to the promastigotes in this study is paramount in order to establish the predictive value for antileishmanial activity in the plants. This will also help to validate the results of the study as any chemotherapeutic potentials of drugs for leishmaniasis must be validated using the intracellular-amastigotes forms of the parasites as these mimic the response in humans (Gupta *et al.*, 2001). Furthermore, the two methods used in the assessment of antileishmanial activity by determining the proportion of macrophages free from the parasites (i.e. rate of infectivity or chemosupression) and the mean amastigote number per macrophage or host cell (i.e. number of parasite) are in line with the new concept adopted by Neal and Croft (1984) for the *in vitro* method to assess the susceptibility of leismania parasites to experimental agents. Today, microscopic counting has become the gold standard in leishmania parasite susceptibility assays, as it is simpler (Habtemariam, 2003).

The three plants have been previously evaluated against other protozoan infections caused by *plasmodium spp* and *trypanosome spp*. They have also been used against agents of cutaneous leishmaniasis in African countries such as Sudan, Ethiopia and Cameroon. The parasites used were *L. major, Leishmania aethiopica (L. aethiopica) and Leishmania mexicana (L. mexicana)* respectively. In Nigeria, there are no published scientific data available on the antileishmanial use of these plants. Past works have concentrated on the effects of these plants on other phylogenetically related parasites such as *plasmodium spp* (Abosi and Raseroka, 2003; Ajaiyeoba *et al.*, 2006) *trypanosome spp* (Igweh and Onabanjo, 1989; Atawodi *et al.*, 2003) and on *L. donovani* (Iwu *et al.*, 1992). To the best of our knowledge, this is the first study in Nigeria, undertaken to evaluate the *in vitro* effects of the crude extracts of these three plants on *L. major* amastigotes, which is the etiologic specie for cutaneous leishmaniasis in this country. This will serve as a pioneer study in the plant screening exercise for the discovery of cheaper, less toxic and readily accessible leads to new antileishmanial drugs especially in developing countries like Nigeria.

5.2.2 Cytotoxicity Tests

The cytotoxicity activities of the plant extracts were evaluated on normal mammalian cell lines (L929 cells), on cancer cell lines (Jurkat and SH-SY5Y cells) and indirectly on mammalian macrophages which are the important host cells of leishmania. Results showed that the methanolic extracts of *Vernonia amygdalina* and *Azadirachta indica* were toxic at a concentration of 100 μg/ml to the macrophages and to the L929 cells, which are the human fibroblast cell line. This result is in conformity to a previous study in which the methanolic extract of *Vernonia amygdalina* was cytotoxic to the human monocytic THP-1 cells with

LD_{50} of 243.4 μg/ml as determined by the methyl tetrazolium assay (Tadesse *et al.*, 1993). These results also elucidates the fact that plants which are used for ethnomedicinal purposes are usually assumed to be safe but may be potentially toxic especially to cells of the immune system. This may result in compromising the immune system or immunosuppression thereby exposing the body to the risk of infections. Hence, cytotoxicity studies are very essential in establishing the safety of these herbal preparations before they are declared fit for human consumption especially in the developing countries like Nigeria where a huge percentage of the populace depends on herbal preparations (Ketzis, 1999). *Vernonia amygdalina* is well known as a medicinal plant with several uses attributed to it. It is used in the treatment of gastrointestinal problems in humans and a decoction of the leaves is used as an antimalarial in Guinea and as cough remedy in Ghana (Akinpelu, 1999; Masaba, 2000). It has also been used in the treatment of diabetis, fever reduction and most recently, acquired relevance as it has been proven to posses potent antimalarial properties hence it is used as a non-pharmaceutical solution to persistent fever, headache and joint pain associated with AIDS (Abosi and Raseroka, 2003; Wikipedia, 2007). Despite these varied uses of the plant, there is dearth of information on its toxicological potentials in the animal system. Reports have shown that it contains some toxic phytochemicals (Areghore *et al.,* 1997; Bonsi *et al*., 1995a). Thus, its toxicity to normal cells maybe as a result of these toxic substances. This elucidates the need for a thoroughput investigation on this plant and other plants used in herbal preparations before they are declared safe for use. This will warrant further studies to evalute its toxicological effects especially in the liver and organs of the immune system. Furthermore, the results of the extracts on the cancer cell lines showed that they were not cytotoxic to the cells at the concentrations used. This

conforms to previous work to determine the effect of *Vernonia amygdalina* and *Annona senegalensis* on LPS/H_2O_2 stressed Jurkat cells. Results showed that the extracts had cytoprotective effect on the cells as they showed antioxidant activity (Alawa *et al.*, 2003). It is possible that these extracts may contain substances that are potential antioxidants such as vitamin C, E and carotenoids (Cher *et al.*, 1990). Another study also showed that the methanolic extract of *A. senegalensis* had low toxicity on human A2780 ovarian cancer cells with an IC_{50} value of 28.8 μg/ml (Ajaiyeoba *et al.*, 2006). On the other hand, the results of this experiment may not be conclusive as most cytotoxicity screens have used plant extracts without taking into account the fact that the naturally occurring compounds may not in themselves be cytotoxic but may be transformed into active substances by metabolic systems in the body hence serving as an explanation to their traditional or folkloric uses. These metabolic processes may occur in the gut e.g. the laxative drug senna in which the glycosides are hydrolysed by intestinal flora to release aglycones which are the compounds responsible for the alterations in the permeability of the gut wall to water hence causing an increase in peristalsis (Houghton *et al.*, 2007). It may also occur in the liver after absorption of compounds from the gut as in the case of the synthetic anticancer drug cyclophosphamide which is converted to cytotoxic DNA-alkylating phosphoramide mustard by cytochrome P450 enzymes (Chang *et al.*, 1993). Therefore it is not unreasonable to suppose that metabolic systems such as these may produce cytotoxic compounds from non-cytotoxics present in the plant or even aid in the detoxification or metabolism of compounds which may be cytotoxic to cells *in vitro* hence making them non-cytotoxic *in vivo*. Thus, it is recommended that plant extracts or derived compounds be further subjected to cell-based methods for cytotoxicity as well as extracts or compounds pre-incubated with systems

modelling or mimicking metabolic processes in the body before being declared cytotoxic or non-cytotoxic. This is very valid for extracts that do not display much direct cytotoxic activity and are often abandoned or neglected. Example of this is shown in the cytotocxicity study of the methanolic extract of *Iris tectorum*, which appeared to be enhanced after incubation with the metabolic enzymes (Houghton *et al.*, 2007). There will also be a need for *in vivo* trials in animal models using these plant extracts.

5.2.3 Antitrypanosomal Activity

Results of the *in vitro* trypanocidal effect of the nine extracts on *Trypanosome brucei brucei (T. brucei brucei)* showed that all the hexane and methanol extracts had antitrypanosomal activity, which was comparable to the reference drug cymerlarsan. The aqueous extracts did not show any significant activity. The most effective was the hexane extract of *Annona senegalensis*. Several studies on the antitrypanosomal effects of plant extracts and their derivatives have been conducted (Asuzu and Chineme, 1990; Nok *et al.*, 1993; Sepulveda-Boza *et al.*, 1995). Previous reports have shown that the methanol extract was not effective against *Trypanosome brucei brucei* when used in *in vitro* studies (Freibrughas *et al*., 1996; Atawodi *et al,* 2003). This does not conform to the results of this study as the methanolic extract showed good trypanocidal activity although in this study, the stem bark was used rather than the root bark as in the aforementioned work. This underscores the need to study all parts of a plant before any generalization is made on the pharmacological and therapeuticcal potentials of the plant. However, in another study using the methanolic extract of the seeds of *Annona senegalensis,* some activity against *Trypanosoma brucei brucei* was demostrated (Sahpaz *et al.,* 1994). This result showed that the hexane fraction

had the best trypanocidal activity and this conforms to results obtained from past works, which clearly indicated that different solvent extracts of the same plants might exhibit different trypanocidal activity just as different parts of the same plant (Freibrughas *et al.*, 1996). The high trypanocidal activity of the hexane extract may be attributed to the presence of oils which are better extracted in this solvent. Also in this study, the aqueous extract of *Annona senegalensis* was not effective against *T. brucei brucei in vitro* but in another study done *in vivo*, results showed that it was therapeutically effective against *T. brucei bruce*i in mice (Igwe and Onabanjo, 1989). It is worthy of note that the three extracts of *Vernonia amygdalina* showed antitrypanosomal activity with the methanolic extract having the greatest activity. This is in line with earlier reports on *in vitro* studies of *Vernonia amygdalina* showing broad antiprotozoan activity (Phillipson *et al.,* 1995). These activities were confirmed on *Plasmodium spp* (Abosi and Raseroka, 2003) and *Leishmania spp* (Tadesse *et al.,* 1993). This study confirms activity on *Trypanosome spp* as these are all phylogenetically related parasites.

5.3 *In vivo* Experiment

The result of this study demonstrated that the methanolic extract of *Vernonia amygdalina* was effective in controlling progressive infection with *L. major* in BALB/c mice. The efficacy of the treatment was confirmed by a reduction in lesion size as well as minimal distortion of the normal tissue structures in the skin evidenced by histopathology. This finding agrees with previous studies on the treatment of cutaneous leishmaniasis using plant extracts. These studies showed that these plant extracts suppressed histopathological changes in the skin, controlled the expansion of lesions or delayed the onset of the disease. Artemesin extracted from the Chinese herb *Artemisia annua* protected against

murine cutaneous leishmaniasis when administered orally (Yang and Liew, 1993). In that work, daily oral administration of 4 mg of purified artemisinin was effective in controlling *L. major* lesion growth. In another study, the aqueous extract of leaves of *kalanchoe pinnata* was shown to significantly delay the onset of the disease when given orally to BALB/c mice infected with *L. amazonensis* (Da silva *et al.*, 1995). In 1984, a plant-screening program for potential leishmanicides was initiated in French Guiana. This was based on the ethnomedicinal knowledge of the local population and several plant extracts were evaluated *in vivo* using cutaneous *L. amazonensis*. Results of this study showed that *Faramea guianensis* had the best activity against *L. amazonensis* infection in murine models as it prevented the growth of lesions (Sauvain *et al.*, 1994). Even though in literature many crude extracts of plants have been evaluated *in vitro* and shown potent antileishmanial activity, most of them still need to be screened *in vivo* as extracts which show both i*n vitro* and *in vivo* leishmanicidal properties are good leads or potential sources for the isolation and development of novel synthetic compounds to treat leishmaniasis. *Vernonia amygdalina* is widely used in Nigeria for human consumption as well as for ethnomedicinal and ethnoveterinary practices. The aqueous and alchoholic extracts of the stem, bark, roots and leaves are reported to be extensively used as purgatives, antimalarial and in the treatment of eczema (Kupcham, 1971). Recently, the plant has acquired special relevance as it has been proven in human medicine to possess potent antimalarial and anthelminthic properties (Abosi and Raseroka, 2003) as well as antitumorigenic properties (Izevbigie *et al.*, 2004). It has also been reported to have antiparasitic efficacy in zoopharmacognosy as it is eaten by wild chimpanzees possibly as self-medication or deparatisation against endo-parasites (Huffman, 2003). The leishmanicidal activity of *Vernonia amygdalina* both *in vitro*

and *in vivo* when given orally in comparatively low doses of the crude extract (lower than cytotoxic concentration) may provide some rational explanations and justifications for the ethnomedicinal uses of the plant in the folkloric treatment of other protozoan diseases. Although, the exact mechanism of its leishmanicidal activity could not be established in this study, its efficacy could be mediated through mechanisms that directly kill the parasites or have an indirect effect on the immune system through the induction of nitric oxide in macrophages. Studies have shown that flavanoids, titerpenoids, saponins, tannins and other plant secondary metabolites possess antiparasitic properties in various experimental animal models (Chan-Bacab and Pena-Rodriguez, 2001). As such, *Vernonia amygdalina* is known to contain numerous flavanoids and glycosides, hence, it will not be improper to speculate that the presence of these compounds may be responsible for its antileishmanial activity. This will require further studies, which is on-going.

IMMUNOTHERAPY EXPERIMENTS

5.4 Efficacy of DNA and Protein Vaccines

In this study, *L. major* infection through high dose subcutaneous inoculation in susceptible BALB/c mice has been used to evaluate the potency or efficacy and immunogenecity of DNA- and protein- based vaccines. The results of this study demonstrated that vaccination with *L. donovani* γGCS delivered either as recombinant protein (with NIV as adjuvant) or as DNA vaccine did not confer any cross protection against *L. major* challenge in BALB/c mice which are highly susceptible to infection (Bradley, 1977). However, the protein-based vaccine conferred partial protection which is evident in the lack of progression of the disease hence a significant delay in lesion size expansion. This conforms to

previous study which showed that recombinant CPA/B protein vaccine can partially protect the mice against *L. major* infection (Azita *et al.*, 2004). In this case, the protein vaccine was homologous to the infective parasite while in our study the vaccine used was heterologous. Also, our results demonstrated that the DNA-based vaccine partially protected the mice for about two weeks after which the effect wore off and allowed the progression of disease and subsequent expansion of the lesion sizes to comparable levels to that of the control. This does not agree with earlier studies using the same DNA-based vaccine to immunize mice challenged with homologous *L. donovani* amastigote parasites as this conferred protection to the BALB/c mice (Carter *et al.,* 2007). However, in another study two opposing results were obtained when similar DNA-based vaccines were used in homologous infections in murine models. In these studies, *L. major* LACK (p24) antigen, delivered as protein or DNA vaccine was highly effective in protecting against cutaneous *L. major* challenge while in contrast *L. donovani* LACK (p36) DNA vaccine did not confer protection against visceral *L. donovani* challenge (Melby *et al.*, 2001). This result indicates that the protein-based vaccine (with NIV adjuvant) was more potent than the DNA-based vaccine in conferring cross- protection. This is a deviation from normal speculations which show that DNA vaccines may have greater potentials than protein vaccines to protect against leismania infections, an observation repeatedly made in vaccine studies comparing various recombinant proteins and their DNA counterparts (Mendez *et al.*, 2001; Dumonteil *et al.*, 2003). However, these were based on the vaccines being homologous to the infective parasites. On the other hand, the lower efficacy of the DNA-based vaccine may not necessarily reflect a difference in potency but could be attributed to certain factors such as the vaccine schedule which provided two instead of three immunisations or the parasite strain. Also the

route of administration could play a role in its efficacy as there is evidence to suggest that the route plays a critical role in determining the type of response induced. This was confirmed in a study demonstrating that administration of the DNA vaccine using a gene gun failed to protect while the subcutaneous administration conferred protection (Gurunathan e*t al.*, 1997). Also another report showed that vaccinating BALB/c mice with interleukin 12 (IL-12) plasmid with SLA intradermally completely protected the mice from challenge with *L. major* while vaccination intramuscularly could not control the infection (Kazuo *et al*., 2001).

5.5 Humoral Responses to DNA and Protein Vaccines

Vaccination with the DNA and recombinant protein vaccines before and after infection (day 0 and 35) was associated with the induction of specific IgG1 and IgG2a antibodies against the γGCS protein even at a low concentration of 1 μg/ml of the antigen. The relative production of these isotypes can be used as a marker for the induction of Th1 or Th2 immune responses respectively. This indicates that the vaccines induced specific Th1 and Th2 immune responses. This showed a mixed response and although resistance to *L. major* infection is associated with the preferential induction of a Th1 response, Th2 responses have also been implicated in mediating protection (Alexander and Bryson, 2005). Also analysis of the sera from the immunised mice showed that the recombinant protein induced higher levels of antibodies throughout the time course while the DNA vaccine elicited low levels. This result showed that the recombinant protein was more immunogenic than the DNA vaccine. Such low humoral responses are common for DNA vaccines, which are biased towards cellular responses (Gurunathan *et al.*, 2000; Dumonteil *et al.*, 2003). The immunogenecity of the protein vaccine is indicative of its ability to induce humoral responses against a heterologous

antigen i.e. the protein can induce a strong cross reactivity with other species of leismania. Furthermore, the mixed immune response in both protein and DNA group was able to offer only partial and not complete protection. The difference in the two groups is that the protein partially protected the mice throughout the experiment hence preventing the rapid expansion of the lesions while the DNA could only protect for the first two weeks after infection, after which the protection waned off and the lesion sizes grew to comparable levels to that of the control. Although at this point, there is no clear explanation for these results there is need for further investigation and several reasons may be proffered. These are that the high level of specific IFN-γ and little or no level of IL-4/IL-5 induced by the recombinant protein before infection may have sustained the partial protection in the protein group as previous studies have shown that the cytokine milieu present at the initiation of infection plays an important role in the subsequent development of either a protective or non-curative response (Scott *et al.*, 1996). Also recent studies have shown that the mouse IgG1 antibody isotype comprise of two functionally distinct types: one type of IgG1 has anaphylactic activity and its synthesis is IL-4 dependent, whereas the other type lacks activity and its synthesis is stimulated by IL-12 or IFN-γ. It has also been shown that adjuvants can modulate the levels of anaphylactic type IgG1 antibodies produced in response to a particular antigen. Thus, in the protein group the induction of significant levels of IFN-γ before challenge may indicate the presence of non-anaphylactic type of IgG1 thus accounting for the partial protection.

5.6 Cytokine and Nitric oxide production in Vaccine groups

It is well established that the immunological control of *L. major* infections depends on the production of IFN-γ, which activates macrophages to kill the parasite via

induction of nitric oxide (NO) production (Green *et al.*, 1990; Kemp *et al.*, 1999). The results of this study showed that the ability of the recombinant protein to elicit a robust γGCS-specific IFN-γ before infection may have contributed to the effective control of the lesion growth. It should be noted, that the level of IFN-γ detected in the protein group after challenge was lower than that detected before challenge but the response was still sustained till the end of the experiment. This is in line with past works in which results showed that vaccination with the L-OprI lipoprotein from *Pseudomonas aeruginosa* protected BALB/c mice against *L. major* infection for at least 19 weeks. This protection was associated with the enhancement of antigen-specific IFN-γ production which was higher before than after challenge (Cote-Sierra *et al.*, 2001). In another study, similar results were obtained although the DNA vaccine rather than the protein vaccine was used. Results demonstrated that upon vaccination with DNA encoding LACK parasite antigen the vaccine induced a potent TH-1 type response with preferential induction of specific IFN- before infection and this response was sustained for at least six weeks after infection (Gurunathan *et al.,* 1997). The lowest production of IFN-γ was seen in the DNA-vaccinated group and this could explain the low immunogenecity in this group and its failure to control lesion development hence lesion sizes reached comparable levels to that of the control by termination of experiment. This is in conformity to previous study using this same DNA vaccine (pVAX-γGCS) in the control of *L. donovani i*nfection. Results showed that the vaccine did not induce any specific IFN-γ as there was no significant difference in the IFN-γ production by control or stimulated cells for all the groups of mice (Carter *et al.*, 2007). Also from the result of this study, the vaccination with the protein resulted in the increase in induction of specific IgG2a (consistent with increased IFN-γ) and IgG1 indicating a Th-2 response. This was not as a result of

the production of IL-4 as would be expected as IL-4 was not detected in any of the groups. Rather IL-5 was the Th-2 cytokine detected in appreciable quantities. IL-5 has also been implicated in the progression of disease in cutaneous leishmaniasis like IL-4. Thus the presence of both cytokines (IFN-γ and IL-5) may be responsible for the mixed immune response seen in the protein group. This is an unexpected result as previous studies have shown that IL-4 plays a crucial role in the pathogenesis of *L. major* infection in BALB/c mice (Chatelaine *et al.,* 1992). Studies have demonstrated that infection of BALB/c mice with parasites of *L. major* is followed by an early burst of IL-4. Thus the distinct susceptibility of BALB/c mice has been suggested to involve an impaired ability to down regulate IL-4 production (Reiner *et al.*, 1994; Wenner *et al.*, 1996). Overall, the cytokine profile herein is indicated by IFN-γ producing the TH-1 response and IL-5 producing the TH-2 response hence a mixed immune response. This corresponds to previous reports in which a mixed TH-1/TH-2 response was implicated for the partial protection elicited in an *L. major* vaccine study (Sjolander *et al.*, 1998). Also the results of this study showed that other immunised groups did not induce the production of specific IFN-γ as there was no significant difference as compared to the contro group. However, there was an increase in the induction of the production of IL-5 after challenge in these groups while the reverse was the case in the protein group. This may have resulted in the lack of protection in these groups and the ability to control lesion development in the protein group.

One of the mechanisms by which activated macrophages control the intracellular parasite load is via the generation of reactive oxygen and nitrogen intermediates, such as nitric oxide (NO) from INOS (Stenger *et al.*, 1994; Wilson *et al.*, 2005) Immunisation with the protein or DNA vaccines did not enhance the ability of the splenocytes to produce nitric oxide as there was no significant difference in nitrite

production which is an indirect measure of nitric oxide production. This conforms to previous work using this same vaccine in which specific stimulation with the fusion protein (MBPγGCS) did not increase nitrite levels compared with the unstimulated control group (Carter *et al.*, 2007). In another study, in which BALB/c mice were infected with *L. major,* results showed that substantial INOS transcription was not detected (Stenger *et al.*, 1994). The inability to generate nitric oxide in this study may suggest that the partial protection elicited by the protein vaccine may not be reliant on nitric oxide production or may be related to the low concentration of protein used to stimulate the cells (25 μg/ml) rather than an absolute inability to respond.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATION

In conclusion, the present study shows the potent antileishmanial activity of *Vernonia amygdalina*. The crude extracts (methanol and aqueous) were potent as they significantly suppressed the infective rate of intracellular amastigotes and reduced parasite levels to almost zero in *L. major* infected macrophages. Its efficacy was demonstrated when administration in comparatively low doses of the crude methanol extract *in vivo* controlled the expansion of lesions and histopathological changes in the skin of BALB/c mice. These findings lend pharmacological support to the suggested folkloric uses of *Vernonia amygdalina* as an antiparasitic. It also suggests that it contains an active compound, which needs to be identified. Thus, further work is recommended to subject the extract to biologically guided fractionation inorder to isolate and identify the active compound responsible for the antileishmanial property. Upon identification of the compound, structure-activity relationship of the compound and its synthetic analogs will be evaluated in an attempt to simplify and increase the yield and purity of this compound through derivatization in the laboratory. This may lead to the development of a new cheaper and safer pharmacophore in the treatment of leishmaniasis especially in the developing country like Nigeria.

Furthermore, the results of this study showed that at concentrations higher than that for leishmanicidal activity, *Vernonia amygdalina* was toxic to mammalian cells tested especially the immune cells like the macrophages but not toxic to the cancer cells. This suggests that care must be taken when administering this drug as well as further studies on the cytotoxicity of this extract using newer approaches such as the sulphorhodamine B enzyme assays (SRB) in which the

extracts are pre-incubated with systems or enzymes modelling metabolic processes in the body before being tested on the cells.

It also demonstrated the trypanocidal activity of the hexane extract of *Annona senegalensis* against *Trypanosome brucei brucei*, which is the causative organism for trypanosomoses. This warrants further study and development of the active principle.

This study demonstrated that *L. donovani* gamma-glutamylcysteine synthetase (γGCS) as a protein vaccine was highly immunogenic and conferred partial cross-protection on *L. major* infected BALB/c mice while the DNA vaccine did not confer any protection whatsoever. This suggests that in spite of the major differences in the mechanism of the pathogenesis between the different leishmania species the development of a vaccine with broad species specificity may be feasible. Thus, *L. donovani* γGCS which is essential to the survival *of L. donovani* may be a leading antigen for vaccine development despite its intracellular location. However, it is surprising to note that in this study, the protein vaccine had greater potential than the DNA vaccine to protect against leishmania, which is in contrast to observations made in previous vaccine studies which compared various recombinant protein and DNA vaccines. Thus, further studies is recommended in which several other approaches could be exploited such as

1) altering the dose or dosing regimen of the vaccines or changing the vector
2) using homologous vaccines i.e. cloning the antigen from the *L. major* parasite
3) using the vaccines with other adjuvants

However, further study is on-going to determine if the effect of the protein vaccine was solely due to the adjuvant (NIV) or due to the protein alone or as a result of synergism of both.

6.1 SIGNIFICANT AND ORIGINAL CONTRIBUTIONS TO SCIENTIFIC KNOWLEDGE

- *Vernonia amygdalina*, commonly eaten in Nigeria has good potentials as an antileishmanial drug as it was effective against *L. major* parasites both *in vitro* and *in vivo.*
- Hexane extract of *Annona senegalensis* is trypanocidal against *Trypanosome brucei brucei*
- Protein vaccines could be more effective than DNA vaccines and could confer cross protection against multiple leishmanial species.

REFERENCES

Abosi, A.O. and Raseroka, B.H. (2003): *In vivo* antimalarial activity of *Vernonia amygdalina*. *British Journal of Biomedical Science.* 60(2): 89-91

Acha, P.N. nd Szyfres, B. (2003): Zoonoses and communicable diseases common to man and animals. Scientific and Technical Publication of Pan American Health Organisation [PAHO]. 3 (580): 86-95

Adzu, B., Abubakar, M.S., Izebe, K.S., Akumka, D.D. and Gamaniel, K.S. (2005): Effect of *Annona senegalensis* rootbark extracts on *Naja nigricotlis nigricotlis* venom in rats. *Journal of Ethnopharmacology*. 96(3): 507-513

Aebischer T., Moody, S.F. and Handman, E. (1993): Persistence of virulent *Leishmania major* in murine cutaneous leishmaniasis: a possible hazard for the host. *Infection and Immunity*. 61:220-226

Aguilar-Be, I., da Silva Zardo, R., Paraguai de Souza, E., Borja-Cabrera, G.P., Rosado-Vallado, M., Mut-Martin, M., del Rosario Garcia-Miss, M., Palatnik de Sousa, C.B. and Dumonteil, E. (2005): Cross-protective efficacy of a prophylactic *Leishmania donovan*i DNA vaccine against visceral and cutaneous murine leishmaniasis. *Infection and Immunity*. 73(2):812–819.

Agwale, S.M., Dondji, B., Okolo, C.J. and Duhlinska, D.D. (1993): Clinical and parasitological prevalence of leishmaniasis in school children in Keana, Awe L.G.C of Plateau State, Nigeria. *Memoirs Institute Oswaldo Cruz, Rio de Janeiro, Brasil.* 93 (1): 23-27

Ajaiyeoba, E., Mofolusho, F., Ogbole, O., Okpako, L. and Akinboye, D. (2006): *In vivo* antimalarial and cytotoxic properties of *Annona senegalensis* extract. *African Journal of Traditional, Complementary and Alternative Medicines*. 3(1): 137-141

Akah, P.A. and Okafor, C.L. (1992): Blood sugar lowering effect of *Vernonia amygdalina* Del in an experimental rabbit model. *Phytotherapy Research.* 6(3): 171-173

Akinpelu, D.A. (1999): Antimicrobial activity of *Vernonia amygdalina* leaves. *Fitotherapia-The Journal for the study of Medicinal Plants.* 70(4): 432

Alawa, C.B.I., Adamu, A.M., Gefu, J.O., Ajanusi, P.A., Abdu, P.A., Chiezey, N.P., Alawa, J.N. and Bowman, D.D. (2003): *In vitro* screening of two Nigerian medicinal plants (*Vernonia amygdalina* and *Annona senegalensis*) for antihelmintic activity. *Veterinary Parasitology.* 113: 73-81

Aletor, O., Ogodi, A.A. and Ipinmoroti, K. (2001): Chemical composition of common leafy vegetables and functional properties of their leaf protein concentrates. *Food Chemistry*. 78 (1): 63-68.

Alexander, J. and Bryson, K. (2005): Th1 and TH2: A leishmania paradox rather than paradigm. *Journal of Immunology* 99: 17-23

Alexander, J., Satoskar, A.R. and Russell, D.G. (1999): Leishmania species: models of intracellular parasitism. *Journal of Cell Science*. 112 (*18*): 2993-3002

Alvarez-Valin, F., Tort, J.F. and Bernardi G. (2000): Nonrandom spatial distribution of synonymous substitutions in the GP63 gene from *Leishmania. Genetics*. 155(4):1683–1692.

Ameh, S.A., Ogunwole, E.O, Dike, M.C., Ajayi, O., Okunade, S.O., Okorokwo, N.O. and Abba, A.A. (2000): Comparative effectiveness of aqueous plant extracts and lambda cyhalothrin in controlling post flowering insect pests of cowpea in the Southern Guinea savanna of Nigeria. In: Entomology in Nation Building: The Nigerian Experience (Dike, M.C., Ajayi, O., Okunade, S.O., Okorokwo, N.O. eds). Proceedings of the Entomological Society of Nigeria. 30th Conference, Kano Nigeria. 4-7th October, 1999. 175-180.

Anderson, J.M., Oliveira, F., Kamhawi, S., (2006): Comparative salivary gland transcriptomics of sandfly vectors of visceral leishmaniasis. *BMC Genomics*. 7:52.

Andrade-Narvaez, F.J., Medina-Peralta, S., Vargas-Gonzalez, A., Canto-Lara, S.B. and Estrada-Parra, S. (2005): The Histopathology of cutaneuous leishamniasis due to *Leishmania mexicana* in the Yucatan Peninsula, Mexico. *Review of Journal of Tropical Medicine Sao Paulo.* 47(4): 191-194

Anonymous, (2007): *Vernonia amygdalina* [online]: http://www.chimie.uni-bonn.de/oc/ak_br/ANALYTICAL/nigeria/vernonia/vein_inf.html

Aregheore, E.M., Makkar, H.P.S. and Becker, K. (1998): Feed value of some browse plants from the central zone of Delta State of Nigeria. *Tropical science* 38(2): 97-104

Asuzu, I.U. and Chineme, C.N. (1990): Effects of *Morinda lucida* leaf extracts on *Trypanosoma brucei bruce*i infection in mice. J*ournal of Ethnopharmacology.* 30: 307-313

Atawodi, S.E., Bulus, T., Ibrahim, S., Ameh, D.A., Nok, A.J., Mamman, M. and Galadima, M. (2003): *In vitro* trypanocidal effect of methanolic extract of some Nigerian Savannah plants. *African Journal of Biotechnology.* 2(9): 317-321

Autran, B., Carcelain, G., Combadiere, B. and Debre, P. (2004): Therapeutic vaccines for chronic infections. *Science.* 305 (5681): 205-208

Awe, S.O., Makinde, J.M. and Olajide, O.A. (1999): Cathartic effect of the leaf extract of *Vernonia amygdalina*. *Fitotherapia-The Journal for the study of Medicinal Plants.* 70(4):161-165

Azita, Z., Tahere, T., Yasaman, T., Fatemah, D., Salmanian, A. and Rafati, S. (2004): Immunization with hybrid protein vaccine, consisting of *Leishmania major* cysteine proteinases Type 1(CPB) and Type II (CPA), partially protects against leishmaniasis. *Vaccine.* 22(15-16): 1930-1940

Azulay, R.D. (1960): Histopathology of cutaneous leishmaniasis. *Dermatological Journal of Latin-America.* 2: 7-15

Balansard, G. and Timon-David, P. (1985): Pharmaceuticals containing quinine or cinchona bark extracts and tannins. *Patent-Frodemande* 2(550): 16

Basu, R., Bhaumik, S., Basu, J.M., Naskar, K., De, T. and Roy, S. (2005): Kinetoplastid membrane protein-11 DNA vaccination induces complete protection against both pentavalent antimonial-sensitive and -resistant strains of *Leishmania donovani* that correlates with inducible nitric oxide synthase activity and IL-4 generation: evidence for mixed Th1- and Th2-like responses in visceral leishmaniasis. *The Journal of Immunology.* 174(11):7160–7171.

Belkaid, Y., Butcher, B. and Sacks, D.L. (1998): Analysis of cytokine production by inflammatory mouse macrophages at the single-cell level: selective impairment of IL-12 induction in Leishmania–infected cells. *European Journal of Immunology.* 28: 1389-1400

Belkaid, Y., Mendez, S., Lira, R., Kadambi, N., Milon, G. and Sacks, D. (2000): A natural model of *Leishmania major* infection reveals a prolonged 'silent' phase of parasite amplification in the skin before the onset of lesion formation and immunity. *Journal of Immunology*. 165: 969-977

Berman, J.D. (1997): Human leishmaniasis: clinical, diagnostic and chemotherapeutic developments in the last ten years. *Clinical and Infectious Diseases.* 24: 684-703

Berman, J. (2003): Current treatment approaches to leishmaniasis. *Current Opinion in Infectious Diseases.* 16(5): 397-401

Bervely, S.M. and Twico, S.J. (1995): Identification of genes modulating lipophosphoglycan biosynthesis by functional complementation of *Leishmania donovani* mutants. *Annals of Tropical Medicine (Parasitology)*. 89(1): 11

Bittencourt, A.L. and Barral, A. (1991): Evaluation of histopathological classifications of American cutaneous and mucocutaneous Leishmaniasis. *Memoirs do Instituto Oswaldo Cruz.* 86:51-56

Bonsi, M.L.K., Osuji, P.O., Tuah, A.K. and Umunna, N.N. (1995a): *Vernonia amygdalina* as a supplement to teff straw (*Eragrostis tef*) fed to Ethiopian Menz sheep. *Agroforestry Systems*. 31(3): 229-241

Botelho, A.C.C., Tafuri, W.L., Genara, O. and Mayrink, W. (1998): Histopathology of Human American cutaneous leishmaniasis before and after treatment. *Journal of the Brasilian Society for Tropical Medicine.* 31(1):11-18

Bradley, D.J. (1977): Regulation of leishmania population within the host. II: Genetic control of acute susuceptibility of mice to *Leishmania donovani* infection. *Clinical and Experimantal Immunology*. 30: 130-146

Bryceson, A.D.M. (1969): Diffuse cutaneous leishmaniasis in Ethiopia. The clinical and histopathological features of the disease. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* 63: 708-737

Burkill, H.M. (1985): The useful plants of West Africa. Royal Botanical Gardens, kew 103-105

Buttler, G.W. and Bailey, R.W. (1973): Chemistry and Biochemistry of herbage. Academic Press, London and New York. Volume 1

Camacho, M., Mata, R., Castenda, P., Kirby, G.C., Warhurst, S.C., Croft, S.L. and Phillipson, J.D. (2000): Bioactive compounds from *Celaenodrelon mexicanum. Planta Medica* 66: 462-468

Campbell, M., Diao, H., Ji, J.X. and Soong, L. (2003): DNA immunisation with the gene encoding P4 nuclease of *Leishamnia amazonensis* protects mice against cutaneous leishmaniasis. *Infection and Immunity*. 71:6270-6278

Campos-Neto, A., Webb, J.R., Greeson, K., Colar, R.N., Skeiky, Y.A. and Reed, S. G. (2002): Vaccination with plasmid DNA encoding TSA/LmSTII leishmanial fusion proteins confers protection against *Leishmania major* infection in susceptible BALB/c mice. *Infection and Immunology*. 70:2828-2836

Carrera, L., Gazzinelli, R.T., Badolato, R., Heiny, S., Muller, W., Kuhn, R. and Sacks, D.L. (1996): Leishmania promastigotes selectively inhibit interleukin 12 induction in bone marrow-derived macrophages from susceptible and resistance mice. *Journal of Experimental Medicine*. 183: 515-526

Carter, K.C., Mullen, A.B., Sundar, S. and Kenny, R.T. (2001): The efficacies of vesicular and free sodium stibogluconate formulations against clinical isolates of *Leishmania donovani*. *Antimicrobial agents and Chemotherapy.* 45: 3555-3559.

Carter, K.C., Henriquez, F.C., Campbell, S.A., Roberts, C.W., Nok, A., Mullen, A.B. and MacFarlene, E. (2007): DNA vaccination against the parasite enzyme gamma glutamylcysteine synthetase confers protection against *Leishmania donovani* infection. *Vaccine*. 25(22): 4502-4509.

Carvalho, P.B. and Ferreira, E.I. (2001): Leishmaniasis Phytotherapy. Nature's leadership against an ancient disease. Review. *Fitoterapia* 72: 599-618

Carvalho, P.B., Arribus, M.A.G. and Ferreira, E.I. (2000): Leishmaniasis: What do we know about the chemotherapy? *Review of Brasilian Ci. Farm.* 31(1): 69-96

Castillo, D., Arevalo, J., Herrera, F., Ruiz, C., Rojas, R., Rengifo, E., Vaisberg, A., Lock, O., Lemesre, J-L., Gornitzka, H. and Sauvain, M. (2007): Spirolactone iridoids might be responsible for the antileishmanial activity of a Peruvian traditional remedy made with *Himatanthus sucuuba* (Apocynaceae). Accepted manuscript in *Journal of Ethnopharmacology*.

Champsi, J. and McMahon-Pratt, D. (1988): Membrane glycoprotein M-2 protects against *Leishmania amazonensis* infection. *Infection and Immunity*. 56: 3272-3279

Chan-Bacab, M.J. and Pena-Rodriquez, L.M. (2001): Plant natural products with leishmanicidal activity. *Natural Product Research*. 18: 674 – 688

Chang, T.K., Weber, G.F., Crespi, C.L. and Waxman, D.J. (1993): *Cancer Research.* 53: 5629-5637

Chatelain, R., Varkila, K. and Coffman R.L (1992): IL-4 induces a Th2 response in *Leishmania major* infected mice. *Immunolgy.* 148: 1182 – 1190

Chen, M., Christensen, S. B., Blom, J., Lemmich, E., Nadelmann, K., Fich, K., Theander, T.G. and Kharazmi, A. (1993): Licochalone, a novel antiparasitic agent with potent activity against human pathogenic protozoan species of leishmania. *Antimicrobial Agents and Chemotherapy.* 37: 2550-2556

Chen, M., Christensen, S.B., Theander, T.G. and Kharazmi, A. (1994): Antileishmanial activity of Licochalone A in mice infected with *Leishmaia* major and in hamsters infected with *Leishmania donovani*. *Antimicrobial Agents and Chemotherapy.* 38: 1339-1344

Cher, Y., Jiang, Z. and Qin, W. (1990): Chemical composition and characteristics of Seabucthorn fruit and its oil. *Chemistry and Industry of Forest Products*.10: 163-175.

Cote-Sierra, J., Bredan, A., Toldos, C.M., Stijlemans, B., Brys, L., Cornelis, P., Segovia, M., de Baetselier, P. and Revets, H. (2001): Bacterial lipoprotein-based vaccines induce tumor necrosis factor–dependent type 1 protective immunity against *Leishmania major*. *Infection and Immunity.* 70(1): 240-248

Da Silva, S.A.G., Costa, S.S., Mendorica, C.S.F., Silva, E.M., Moraes, V.L.G. and Rossi-Bergmann, B. (1995): Therapeutic effect of oral *Kalanchoe pinnata* leaf extract in murine Leishmaniasis. *Acta Tropica* 60: 201-210

Davidson, R.N. (1999): Visceral Leishmaniasis in clinical practice. *Journal of Infectious Diseases.* 39:112-116

Delmas, F., Giorgio, C.D., Elias, R., Gesquet, M., Azas, V., Mahvildadze, F., Dekanosidze, E., Kemertelidze, E. and Timon-David, P. (2000): Antileishmanial effects of hederecolchiside, a saponin from *Hedera colchica* on *Leishmania infantum. Planta Medica* 66:343

Descoteux, A. and Turco, S.J. (2002): Functional aspects of the *Leishmania donovani* lipophosphoglycan during macrophage infection. *Forum in Immunology*. 4:975-981

Dilvani, O.S., Carlos, E.R.C., Maria, F.M., Boltimo, C.G., Vieira, R.F., Samara B.N., Bernerdino, A. And Castro H.C. (2008): Leishmaniasis treatment-a challenge that remains. A Review In: *Parasitology Research* 103(1): 1432-1955

Donnelly, J.J., Ulmer, J.B., Shiver, J.W. and Liu, M.A. (1997): DNA vaccines. *Annual Reviews in Immunology*. 15: 617-648

Drummond, A.J. and Waigh, R.D. (2000): Recent research developments in phytochemistry. *Phytochemistry*. 4:143-152

D'ultra de Silva, O. (1915): Sebre a leishmanoise Tegumentar e seutritamento. *Memoirs do Instituito Oswaldo Cruz.* 7:213-248

Dumonteil, E., Jesus, R.S.M., Javier, E.O. and Del Rosario, G.M. (2003): DNA vaccines induce partial protection against *Leishmania mexicana. Vaccine* 21: 2161-2168

Ekpendu. T.O.E., Obande, O.D., Anyogo, P.O. and Attah, A.D. (1998): Nigerian ethnomedicine and medicinal plant flora-the Benue experience part 1. *Journal of Pharmaceutical Research and Development.* 3: 37-46

El Tahir, A., Ibrahim, A.M., Satti, G.M.H., Theander, T.G., Kharazmi, A. and Khalid, S.A. (1998): The potential antileishmanial activity of some Sudanese medicinal plants. *Phytotherapy Research.* 12(8): 576-579.

Elmes, B.G. and Hall, R.N. (1944): Cutaneous leishmaniasis in Nigeria. *Transactions of the Royal society of Tropical Medicine and Hygiene*. 37: 437-439

Elnaiem, D.E.A., Meneses, C., Slotman, M., Lanzaro, G.C. (2005): Genetic variation in the sand fly salivary protein, SP-15, a potential vaccine candidate against *Leishmania major*. *Insect Molecular Biology.* 14(2):145–150.

Eloff, J.N. (1998): Which extract should be used for screening and isolation of antimicrobial components from plants? *Journal of Ethnopharmacology*. 60: 1-8.

Eshiet, I.T.U. and Akinsanya, A. (1971): Diterpene from *Annona senegalensis*. *Phytotherapy* 10: 3294-3295

Espinoza, J.R., Skinner, A.C. and Davies, C.R. (1995): Extensive polymorphism at the Gp63 locus in field isolates of *Leishmania peruvian*. *Molecular and Biochemical Parasitology.* 72(1-2):203–213.

Fabiola, A.T., Marie-Alexandra, L., Jon, D.L., Marjan, V.M., Robert, K., Jean-Christophe, N. and Yves, C. (2002): Parasitic load and histopathology of cutaneous lesions, lymph node, spleen and liver from BALB/c and C57BL/6 mice infected with *Leishmania mexicana. American Journal of Tropical Medicine and Hygiene.* 66(3): 273-279

Fitzpatrick, D.A., Creevey, C.J., McInerney, J.O. (2005): Evidence of positive Darwinian selection in putative meningococcal vaccine antigens. *Journal of Molecular Evolution.* 61(1):90–98.

Fournet, A., Angelo, A., Munoz, V., Hocquemiller, R. and Cave, A. (1992): Effect of natural naphthoquinones in BALB/c mice infected with *Leishmania amazonensis* and *Leishmania venezuelensis*. *Journal of Tropical Medicine (Parasitology)*. 43:219-222

Fragaki, K., Suffia, I., Ferrua, B., Rousseau, D., Le Fichoux, Y., Kubar, J. (2001) Immunisation with DNA encoding *Leishmania infantum* protein papLe22 decreases the frequency of parasitemic episodes in infected hamsters. *Vaccine.* 19(13-14):1701–1709.

Freiburghaus, F., Kaminsky, R., Nkuna, M.H.N. and Brun, R. (1996): Evaluation of African medicinal plants for their *in vivo* trypanocidal activity. *Journal of Ethnopharmacology.* 55: 1-11

Gamboa-Leon, R., Paraguai de Souza, E., Borja-Cabrera, G.P., Santos, F.N., Myashiro, L.M. and Pinheiro, R.O. (2006): Immunotherapy against visceral leishmaniasis with nucleoside hydrolase DNA vaccine of *Leishmania donovani. Vaccine* 24: 4863-4873

Ganguli, S. (2002): Neem: A therapeutic for all seasons. *Current Science.* 82(11): 1304

Gonzalo, R.M., del Real, G. and Rodriguez, J.R., (2002): A heterologous prime-boost regime using DNA and recombinant vaccinia virus expressing the *Leishmania infantum* P36/LACK antigen protects BALB/c mice from cutaneous leishmaniasis. *Vaccine*. 20(7-8):1226–1231.

Gorak, P.M.A., Engwerda, C.R. and Kaye, P.M. (1998): Dendritic cells, but not macrophages produce IL-12 immediately following *Leishmania donovani* infection. *European Journal of Immunology*. 28: 687-695

Graham, J.G., Quinn, M.L., Fabricant, D.S. and Farnsworth, N.R. (2000): Plants used against cancer- an extension of the work of Jonathan Hartwell. *Journal of Ethnopharmacology.* 73: 343-377

Green, S.J., Mellouk, S., Hoffman, S.L., Meltzer, S.M. and Nacy, C.A. (1990): Cellular mechanisms of non-specific immunity to intracellular infection: cytokine-induced synthesis of toxic nitrogen oxides from L-arginine by macrophages and hepatocytes. *Immunology.* 25:15-19

Greenblatt, C.L. (1980): The present and future of vaccination for cutaneous leishmaniasis. *Clinical Biology Research.* 47: 259-285.

Grimaldi, G.Jr., and Tesh, R.B. (1993): Leishmaniasis of the New World: Current concepts and implications for future research. *Clinical Microbiology Review*. 6(3): 230-250

Guerbouj, S., Guizani, I., Victoir, K., Le Ray, D. and Dujardin, J.C. (2000): Parasite candidate vaccines: A warning from polymorphic Leishmania populations. *Parasitology Today.* 16(6): 265–266.

Guerbouj, S., Victor, K. and Guizani, I. (2001): Gp63 gene polymorphism and population structure of *Leishmania donovani* complex: influence of the host selection pressure. *Parasitology.* 122(1): 25–35.

Guerin, P., Olliaro, P., Sunder, S., Booelaert, M., Croft, S., Desjeux, P., Wasunna, K.M., Legros, D. and Bryceson, A. (2001): Drugs for the treatment of Visceral Leishmaniasis: Current status, Needs and a Proposal. R and D Agenda MSF/DND Working Group: 25-28.

Guerra, M.O.P., Furtado, T., Barros, G.C., Sessa, P.A. and Carias, V.R.D. (1985): Subclinical Infections of American cutaneous leishmaniasis. *Journal of Brasilian Dermatologists.* 60: 365-369

Gupta, N., Goyal, N. and Rastogi, A.K. (2001): *In vitro* cultivation and characterization of axenic amastigotes of *Leishmania*. *Trends in Parasitology*. 17: 150-153

Gurunathan, S., Prussin, C., Sacks, D.L. and Seder, R.A. (1998): Vaccine requirements for sustained cellular immunity to an intracellular parasitic infection. *Nature Medicine.* 4(12):1409–1415.

Gurunathan, S., Sacks, D.L., Brown, D.R., Reiner, S.L., Charest, H., Glaichenhaus, N. and Seder, R.A. (1997): Vaccination with DNA encoding the immunodominant LACK parasite antigen confers protective immunity to mice infected with *Leishmania major*. *Journal of Experimental Medicine.* 186:1137-1147.

Gurunathan, S., Wu, C., Freidag, B.L. and Seder, R.A. (2000): DNA vaccines: a key for inducing long-term cellular immunity. *Current Opinion in Immunology.* 12: 442-447.

Habtemariam, S. (2003): *In vitro* antileishmanial effects of antibacterial diterpenes from two Ethiopian Premna species, *P. schimperi* and *P.oligotricha*. *BMC Pharmacology.* 3: 6

Hamowia, A.M. and Saffafm A.M. (1994): Pharmacological studies on *Vernonia amygdalina* (Del) and *Tithonia diversifolia* (Gray). *Veterinary Medical Journal Giza.* 2:91-97.

Handman, E. (1997): Leishmania vaccines : Old and New. *Parasitology Today*. 13:236

Handman, E. (2001): Leishmaniasis: Current status of Vaccine development. *Clinical Microbiology Reviews.* 14:229-243

Handman, E. and McConville, M.J. (1990): In New generation vaccines. Marcel Dekker 545-564.

Handman, E. and Mitchell, G.F. (1985): Immunisation with Leishmania receptor for macrophages protects mice against cutaneous leishmaniasis. *Procedures of the National Academy of Sciences U.S.A* 82: 5910-5914

Handman, E., Noormohammadi, A.H., Curtis, J.M., Baldwin, T., Sjölander, A. (2000): Therapy of murine cutaneous leishmaniasis by DNA vaccination. *Vaccine.*18(26):3011–3017.

Handman, E., Symons, F.M., Baldwin, M.T., Curtis, J.M. and Schearlinck, J.P. (1995): Protective vaccination with promastigote surface antigen 2 from *Leishmania major* is mediated by a Th1 type of immune response. *Infection and Immunity*. 63: 4261-4267

Hasan, U.A., Abai, A.M., Harper, D.R., Wren, B.W. and Morrow, W.J. (1999): Nucleic acid immunisation: concepts and techniques associated with third generation vaccines. *Journal of Immunological Methods*. 229:1-22

Heinzel, F.P., Sadick, M.D., Mutha, S.S. and Locksley, R.M. (1991): Production of Interferon gamma, interleukin 2, interleukin 4 and interleukin 10 by CD4+ lymphocytes in vivo during healing and progressive murine leishmaniasis. *Procedures of National Academy of Sciences.* 88: 7011-7015

Hepburn, N.C. (2003): Cutaneous leishmaniasis: an overview. *Journal of postgraduate Medicine*. 49(1): 50-54

Hepburn, N.C., Tidman, M.J. and Hunter, J.A.A. (1993): Cutaneous Leishmaniasis in British troops from Belize. *British Journal of Dermatology*. 128:63-68

Herwaldt B.L (1999): Leishmaniasis. *Lancet Infectious Diseases.* 354:1191-1199

Herwaldt, B.L. and Berman, J.D. (1992): Recommendations for treating leishmaniasis with sodium stibogluconate (Pentosam) and review of pertinent clinical studies. *American Journal of Tropical Medicine and Hygiene.* 46(3):296-306

Hocquemiller, R., Cortes, D., Arango, J., Myint, S.H., Cave, A., Angelo, A., Munoz, V. and Fournet, A. (1991): Isolation and Synthesis of Espintanol, a novel antiparasitic monoterpene. *Natural Products* 54: 443-452

Houghton, P., Fang, R., Techatanawatt, I., Steventon, G., Hylands, P.J. and Lee, C.C. (2007): The salphorhodamine (SRB) assay and other approaches to testing plant extracts and derived compounds for activities related to reputed anticancer activity. *Methods.* 40: 377-387

Huffman, M.A. (2003): Animal self medication and ethno-medicine exploitation of the medicinal properties of plants. *In: Proceedings of the Nutritional society.* 62(2): 371-381.

Huffman, M.A., Gotoh, S., Izutsu, D., Koshimizu, K. and Kalunde, M.S. (1993): Further observations on the use of *Vernonia amygdalina* by a wild chimpanzee, its possible effect on parasite load, and its phytochemistry. African Study Monographs. 14 (4): 227-240.

Hutchinson, J. and Dalziel, J.M. (1963): Flora of West Tropical Africa. 2nd Edition. Revised by Keay R.W.J, Crown Agents for Oversea Goverments and Administrator; London. 1(1): 484-493

Iborra, S., Soto, M., Carrión, J., Alonso, C. and Requena, J.M. (2004): Vaccination with a plasmid DNA cocktail encoding the nucleosomal histones of *Leishmania* confers protection against murine cutaneous leishmaniosis. *Vaccine.* 22(29-30):3865–3876.

Iborra, S., Soto, M. and Carrión, J.,(2003): The *Leishmania infantum* acidic ribosomal protein P0 administered as a DNA vaccine confers protective immunity to *Leishmania major* infection in BALB/c mice. *Infection and Immunity.* 71(11):6562–6572.

Igweh, A.C. and Onabanjo, A.O. (1989): Chemotherapeutic effects of *Annona senegalensis* in *Trypanosoma brucei bruce*i. *Annals of Tropical Medicine (Parasitology)*. 83: 527-534

Ijeh, I., Nwugo, V.O. and Obidoa, O. (1996): Comparative studies on the nutritive, phytochemical and antimicrobial properties of two varieties of *Vernonia amygdalina*. *In: Plant Proceedings in Research and Communication.*1: 71-75

Iwu, M. M., Jackson, J.E., Tally, J.D. and Klayman, D.L. (1992): Evaluation of plant extracts for antileishmanial activity using a mechanism-based radiorespirometric microtechnique (RAM). *Planta Medica* 58: 436-441

Iwu, M.M., Okunji, E., Akah, P.A., Tempesta, M.S. and Carley, D.G. (1996): Dioscoretine: The hypoglycaemic principles of *Dioscorea dumentorium. Planta Medica.* 56:119-126.

Izevbigie, E.B., Bryant, J.L. and Walker, A. (2004): A novel natural inhibitor of extracellular signal-regulated kinases and human breast cancer cell growth. *Experimental Biological Medicine (Maywood).* 229(2): 163-169.

Jardin, A., Alexander, J., The, H.S., Ou, D.W. and Olafson, R.W. (1991): Immunoprotective *Leishmania major* synthetic T cell epitopes. *Journal of Experimental Medicine*. 172: 645 – 648.

Jellife, R.S (1955): Cutaneous leishmaniasis in Nigeria and the Western Sudan. *West African Medical Journal*. 4: 92-94

Jisaka, M., Kawanaka, M., Sugiyama, H., Takegawa, K., Huffman, M.A., Ohigashi, H. and Koshimizu, K. (1992a). Antischistosomal activities of Sesquiterpene lactones and steroid glucosides from *Vernonia amygdalina,* possibly used by wild chimpanzees against parasite related diseases. *Bioscience Biotechnology and Biochemistry*. 56(5): 845-846.

Jisaka, M., Ohigashi, H. Takagaki, T., Nozaki, H. Tada, T., Hirota, M., Irie, R., Huffman, M.A, Nishida, T., Kaji, M. and Koshimizu, K. (1992b): Bitter steroid glucosides , Vernoniosides A_1, A_2, A_3 and related B1 from a possible medicinal plant, *Vernonia amygdalina* used by wild chimpanzees. *Tetrahedron* 48 (4): 625-632.

Jisaka, M., Ohigashi, H., Takegawa, K., Huffman, M.A., and Koshimizu, K. (1993): Antitumoral and antimicrobial activities of bitter Sesquiterpene lactones of *Vernonia amygdalina*, a possible medicinal plant used by wild chimpanzees. *Bioscience Biotechnology and Biochemistry.* 57(5): 833-834.

Kambizi, L. and Afolayan, A.J. (2001). An ethnobotanical study of plants used for the treatment of sexually transmitted diseases (njovhera) in Guruve district of Zimbabwe. *Journal of Ethnopharmacology*. 77: 5-9.

Kamhawi, S., Belkaid, Y., Modi, G., Rowton, E. and Sacks, D. (2000): Protection against cutaneous leishmaniasis resulting from bites of uninfected sand flies. *Science.* 290(5495):1351–1354.

Kayser, O., Kiderlen, A.F., Folkens, U. and Kolodziej, H. (1999): *In vitro* leishmanicidal activity of aurones. *Planta Medica* 65: 316-319

Kayser, O., Kinderlen, A.F. and Croft, S.L. (2003): Natural product as antiparasitic drugs. *Parasitology Research.* 90 (2): S55-S62

Kazuo, Y., Shinkichi, A., Masaki, S., Yoshio, N., Jyun-Ichi, M. and Takushi, T. (2001): A single intradermal administration of soluble leishmanial antigen and plasmid expressing interleukin-12 protects BALB/C mice from *Leishmania major* infection*. Parasitology International.* 50(2): 81-91

Kellina, O.I. (1965): Changes in the virulence of strains of *Leishmania tropica major*. *Medical Prasitology*. 6: 701-708

Kellina, O.I. (1981): Problems and current lines in investigations on the epidemiology of leishmaniasis and its control in the USSR. *Bulletin of the Society of Pathologists Exot.* 74:306-318

Kemp, M., Kurtzhals, J.A.I., BendtzenK, J., Poulsen, I.K., Hansen, M.B., Koech, D.K., Kharazmi, A. and Theander, T.G. (1999): *Leishmania donovani* reactive Th1 and Th2-like T-cell clones from individuals who have recovered from visceral leishmaniasis. *Infection and Immunity*.61: 1069-1073

Kenner, J. (2007): Leishmaniasis. Article in *Parasitic Infections*

Ketzis, J.K.L., (1999): The anthelmintic potential of *Chenopodium ambrosioides* in goats. Unpublished PhD Thesis. Cornell University, Ithaca, NY.

Khalid, F.A., Abdallah, M.N., Mohomed, H.E., Toum, A.M., Magzoub, M.M.A. and Ali, S.M. (2005): *In vitro* assessment of anti-cutaneous leishmaniasis activity of some Sudanese plants. *Turkiye Parazitoloji Deraisi* 29(1):3-6

Kudi, A.C. and Myint, S.H. (1999): Antiviral activity of some Nigerian medicinal plants extracts. *Journal of Ethnopharmacology*. 68: 289-294.

Kumkhaek, C., Phra-Ek, K. and Rénia, L. (2005): Are extensive T cell epitope polymorphisms in the *Plasmodium falciparum* circumsporozoite antigen, a leading sporozoite vaccine candidate, selected by immune pressure? *Journal of Immunology.* 175(6):3935–3939.

Kupcham, S.M. (1971): Drugs from natural products. Plant source in drugs discovery, science and development. *Journal of American Chemical Soiciety*. 6: 311-318

Kupcham, S.M., Hernichway, R.J. and Wermer, D. (1969): Vernodalin and Vernomygdin. Two new sesquiterpene lactones from *Vernonia amygdalina del. Journal of Organic Chemistry*. 3: 3908-3909.

Lamothe, J. (2001): Activity of Amphotericin B in lipid emulsion in the initial treatment of canine leishmaniasis. *Journal of Small Animal practice*. 42: 170-175

Langason, R.B.F., Akunyili, D.N. and Akubue, P.I. (1994): A prelimnary study of gastrointestinal effects of some Nigerian medicinal plants. *Fitoterapia* 65(3): 235-240.

Lee, N., Bertholet, S., Debrabant, A., Muller, J., Duncan, R. and Nakhasi, H.L. (2001): Programmmed cell death in the unicellular protozoan parasite *Leishmania. Cell Death and Differentiation.* 9: 53-64

Liew, F.Y. and O'Donnell, C.A. (1993): Immunology of leishmaniasis. *Advances in Parasitology*. 32: 161-258

Liu, M.A., Wahren, B. and Hedestam, G.B.K. (2006): DNA vaccines: recent developments and future possibilities. *Human Gene Therapy.* 17(11):1051–1061.

Locksley, R.M. and Louis, J.A. (1992): Immunology of leishmaniasis. *Current Opinions in Immunology.* 4: 413-418

Locksley, R.M. and Scott, P. (1991): Helper T-cell subset in mouse leishmaniasis: Induction, expansion and effector function. *Immunology Today*. 12: A58-A61.

Locksley, R.M., Heinzel, F.P., Holaday, B.J., Muthu, S.S., Reiner, S.L. and Sadick, M.D. (1991): Induction of a Th1 and Th2 CD4 subsets during murine *Leishmania major* infection. *Research in Immunology*. 142:28

Magalhaes, A.V., Moraes, M.A.P., Raick, A.N., Llanos-Cuentas, A., Costa, J.M.L., Cuba, C.C. and Marsden, P.D. (1986[b]): Histopathology of Cutaneous Leishmaniasis from *Leishmania brazilensis.* Review, *Institute of Tropical Medicine Sao Paulo.* 28:253-262

Magalhaes, R.N.M., Bilba, M.N., Neto, M., Williams, E.H., Michealic, M.S., Costa, C.A., Mayrink, W. and Taviva, P.T.C. (1987): Isoenzyme characterization of leishmania isolated from human hosts in the Rio Doce Valley - MG. *Memoirs de Instituto Oswaldo Cruz.* 83: 96-112

Magill, A.J. (2000): Leishmaniasis: In: Stickland, G.T. (Ed) *Hunter's Tropical Medicine.* pp 1224-1234

Majekodunmi, O.F., Audu, O.T., Takeda, Y., Zeng, L., Shi, G., Shimada, H. and McLaughlin, J.L. (1996). Bioactive ent-Kaurene Diterpenoids from *Annona senegalensis*. *Journal of Natural Products.* 59: 301-303

Majester-Savorinin, B., Elias, R., Diuaz-Lanza, M., Balansard, G., Delmas, F. and Gasquet, M. (1991): Saponins of the ivy plant, *Hedera helix* and their leishmanicidic activity. *Planta Medica* 57:260-262

Markle, W.H. and Makhoul, K. (2004): Cutaneous leishmaniasis: recognition and treatment*. American Family Physician.* 69(6):1455-1460

Marques-da-Silva, E.A., Cotelho, E.A.F. and Gomes, D.C.O. (2005): Intramuscular immunization with p36(LACK) DNA vaccine induces IFN-*γ* production but does not protect BALB/c mice against *Leishmania chagasi* intravenous challenge. *Parasitology Research.* 98(1):67–74.

Marsden, P.D., Notto, E.M., Badaro, R., Cuba, C.A.C., Costa, J.M. and Barrato, A.C. (1986): Cutaneous leishmsniasis. Correspondence In: *The American Journal of Tropical Medicine and Hygiene.* 35:449

Masaba, S.C. (2000): The antimalarial activity of *Vernonia amygdalina Del* (compositae). *Transcations of Royal Society of Tropical Medicine and Hygiene* 94(6): 694-695

Masmoudi, A., Ayadi, N., Boudaya, S., Meziou, T.J., Mseddi S., Marrekchi D., Bouassida S., Turki, H. and Zahaf, A. (2007): Clinical polymorphism of cutaneous leishmaniasis in central and south of Tunisia. *Bulletin of Society of Pathologists.* 100(1): 36-40

Mbonogo, N., Loiseau, P.M., Billion, M.A. and Robert-Gero, M. (1998): Mechanism of Amphotericin B Resistance in *Leishmania donovani* promastigotes. *Antimicrobial Agents and Chemotherapy* . 42: 352-357

McMahon Pratt, D., Rodriquez, D., Rodriquez, J.R., Zhang, Y., Manson, K., Bergman, C., Rivas, L. and Lohmanlick, I. (1993): Recombinant vaccina viruses expresssing GP46/M-2 protect against leishmania infection. *Infection and Immunity*. 61: 3351-3359

McMIllan, B. (1957): An unusual case of cutaneous leishmaniasis diagnosed in Kano, Northern Nigeria. *West African Medical Journal.* 6: 190-191

McCulloch, W.E. (1930): Annual report on the pathological laboratory, Kaduna. Annual Medical Sanitation Report, Nigeria, (1929), Appendix, pp 65

Medecines Sans Frontieres (MSF) (2000): Current status of Cutaneous leishmaniasis.

Melby, P. C., Yang, J., Zhoa, W., Perez, L.E. and Cheng, J. (2001): *Leishmania donovan*i p36 (LACK) DNA vaccine is highly immunogenic but not protective against experimental visceral leishmaniasis. *Journal of Infection and Immunology.* 69: 4719-4725.

Mendez, S., Gurunathan, S., Kanhawi, S., Belkaid, Y., Moga, M. A. and Skeiky, Y.A. (2001): The potency and durability of DNA-based and Protein-based Vaccines against *Leishmania Major* evaluated using low dose, intradermal challenge. *Journal of Immunology* 166: 5122-5128.

Milleron, R.S., Mutebi, J.P. and Valle, S. (2004): Antigenic diversity in maxadilan, a salivary protein from the sand fly vector of American visceral leishmaniasis. American *Journal of Tropical Medicine and Hygiene.* 70(3):286–293.

Mitchell, G.F. and Handman, E. (1986): The glycoconjugate derived from a *Leishmania major* receptor for macrophages is a suppresogenic, disease-promoting antigen in murine cutaneous leishmaniasis. *Parasite Immunology*. 8:255 – 263.

Mittal, N., Gupta, N., Saksena, S., Goyal, N., Roy, U. and Rostogi, A.K. (1998): Protective effect of picroliv from *Picrorhiza kurroa* against *Leishmania donovani* infections in *Mesocricetus auratus. Life Sciences.* 63: 1823-1834.

Mock, B.A., Fortier, A.H., Potter, M., Blackwell, J.M. and Nacy, C.A. (1985): Genetic control of systemic *Leishmania major* infection; identification of sublime differences for susceptibility to disease. *Current Topics in Microbiology and Immunology.* 122:115-121

Modabber, F. (1995): Vaccines against leishmaniasis. *Annuals of Tropical Medicine (Parasitology).* 89: 83-88

Moody, D.B, Ulrichs, T., Muhlecker, W., Young, D.C., Gurcha, S.S., Grant, E., Rosat, J.P., Brenner, M.B., Costello, C. E., Besra, G.S. and Porcelli, S.A. (2000): CD1c-mediated T-cell recognition of isoprenoid glycolipids in *Mycobacterium tuberculosis* infection. *Nature*. 404:884 – 888

Morris, R.V., Shoemaker, C.B., David, J.R., Lanzaro, G.C., Titus, R.G. (2001): Sandfly *maxadilan* exacerbates infection with *Leishmania major* and vaccinating against it protects against *Leishmania major* infection. *Immunology.* 167(9):5226–5230.

Muanza, D.N., Kim, B.W., Euler, K.L. and Williams, L. (1994): Antibacterial and antifungal activities of nine medicinal plants from Zaire. *International Journal of Pharmacognosy.* 32:337-345

Murray, H.W. (2000): Mononuclear cell recruitrent, granuloma assembly and response to treatment in experimental visceral leishmaniasis. *Infection and Immunity* 68: 6294-6299

Ndjakou, B.L., Vonthron-Senecheaub, C., Fongang, S.C.R., Tantagmod, F., Ngouelad, S., Kaisere, M., Tsamod, E., Antonb, R. and Wenigerb, B. (2007): *In vitro* antiprotozoal activities and cytotoxicity of some selected Cameroonian medicinal plants. *Journal of Ethnopharmacology* .111(1): 8-12

Neal, R.A. and Croft, S.L. (1984): An *in vitro* system for determining the activity of compounds against the intracellular amastigote form of *Leishmania donovani*. *Journal of Antimicrobial agents and Chemotherapy.* 14:463-475.

Nicolle, C.H. (1908): Cultures des corps de Leishman isoles de la rate dans trios cas d'anemic sptenique infantile. *Bulletin of Society of Pathologists Exotics*.1:121

Nielsen, S.F., Christensen, S.B., Crruciani, G., Kharazmi, A. and Liljefors, T. (1998): *In vitro* antileishmanial and cytotoxic effects of licochalone. *Journal of Medicinal Chemistry*. 41: 4819

Nils, G., Louis, M., Luc, V.P., Mai, V.T., Duong, A.T. and Nobert De, K. (2005): *In vitro* and i*n vivo* antileishmanial activity of triterpenoid saponins isolated from *Maesa balansae* and some chemical derivatives. *Journal of Medicinal Chemistry*. 48: 32-37

Nok, A.J., Esievo, K.A.N., Lingdet, I., Arowosafe, S., Onyenekwe, P.C., Gimba, C.E. and Kagbu, J.A. (1993): *In vitro* activity of leaf extracts against *Trypanosoma brucei brucei*. *Journal of Clinical Biochemistry and Nutrition* 15:113-118

Ochsenbien, A.F. and Zinkernagel, R.M. (2000): Natural antibodies and complement link in innate and acquired immunity. *Immunology Today.* 21: 624-630

Okafor, J.C. (1983): Horticulturally promising indigenois plants species of the Nigerian forest zone. *Acta Horticulture* 123:165-176

Oketch-Rabah, A., Lemmich, E., Dossaji, S.F., Theander, T.G., Olsen, C.E., Cornett, C., Kharamzi, A. and Christensen, S.B. (1997a): Two new antiprotozoal 5-methylcoumarins from *Vernonia brachycalyx*. *Journal of Natural Products*. 60: 458-461

Oketch-Rabah, A., Lemmich, E., Dossaji, S.F., Theander, T.G., Olsen, C.E., Cornett, C., Kharamzi, A and Christensen, S.B. (1997b): Antiprotozoal compounds of *Asparagus Africans*. *Journal of Natural Products*. 60: 1017-1023

Ologunde, M.O., Ayorinde, F.O., Shepard, R.K., Afolabi, O.A. and Oke, O.L. (1992): Sterols of seed oils of *Vernonia galenesis, Aaranthus cruentus, Amaranthus caudatus, amaranthus Hybrids* and *Amaranthus hypochondriachus* growth in the humid tropics. *Journal of Food and Agriculture.* 58: 221-225

Onwuka, C.F.I., Akinsoyimu A. O. and Tewe, O.O. (1989): Feed value of some Nigerian browse plants: Chemical composition and *in vitro* digestibility. *East African Agriculture and Forestry Journal.* 54: 157-163

Paterson, R. (2002): Mitelfosine presents a new hope for leishmaniasis patients. *Lancet Infectious Diseases* 2:452

Phillipson, J.D. and Wright, C.W. (1991): Antileishmanial activity of Beberine. *Transactions of the Royal society of Tropical Medicine and Hygiene*. 85: 18

Phillipson, J.D., Wright, C.W., Kirby, G.C., Warhurst, D.C., Hostettmann, K., Marston, A., Manilla, M. and Hamburger, M. (1995): Phytochemistry of plants used in traditional medicine. 95-135. Oxford University Press. Oxford, U.k.

Pinto, E.F., Pinheiro, R.O., Rayol, A., Larraga, V. and Rossi-Bergmann, B. (2004): Intranasal vaccination against cutaneous leishmaniasis with a particulated leishmanial antigen or DNA encoding LACK. *Infection and Immunity.* 72(8):4521–4527.

Puri, A., Saxena, R.P., Sumanti, P., Guru, V., Kalshreshtha, D.K., Saxena, K.C. and Dhawan, B.W. (1992): Immuostimulant and antileishmanial activity of Picroliv. *Planta Medica* 58: 528

Puri A (1999): Neem the wonder tree. Retrieved from wikipedia on July 19, 2007. http://en.wikipedia.org/wiki/Neem.

Rafati S, Nakhaee A and Taheri T (2005): Protective vaccination against experimental canine visceral leishmaniasis using a combination of DNA and protein immunization with cysteine proteinases type I and II of *L. infantum* . *Vaccine.* 23(28):3716–3725.

Rafati, S., Salmanian, A.H., Taheri, T., Vafa, M. and Fasel, N. (2001): A protective cocktail vaccine against murine cutaneous leishmniasis with DNA encoding cysteine proteases of *Leishmania major*. *Vaccine* 19: 3369-3375

Rafati, S., Zahedifard, F. and Nazgouee, F. (2006): Prime-boost vaccination using cysteine proteinases type I and II of *Leishmania infantum* confers protective immunity in murine visceral leishmaniasis. *Vaccine.* 24(12):2169–2175.

Rasmussen, H. B., Christensen, S. B., Kvist, L.P., Kharazmi, A. and Gonzales, A. (2000): Absolute configuration and antiprotozoal activity of Minquartynoic acid. *Natural Products*. 63: 1295-1296

Raul, P., Pedrazo, M. and Manuela, P. (1990): Animal health care in India In: *Information Centre for low external input and sustainable agriculture (ILEIA*) Newsletter 8(3): 22-33

Regassa, A. (2000). The use of herbal preparations for tick control in western Ethiopia. *Journal of South African Veterinary Association*. 71 (4): 240-243.

Reiner, S.L. and Locksley, R.M. (1995): The regulation of immunity to *Leishmania major*. *Annual Review of Immunology* 13: 151-177

Reiner, S.L., Zheng, S., Wang, Z.E., Stowring, L. and Locksley, R.M. (1994): Leishmania promastigotes evade interleukin 12 (IL-12) induction by macrophages and stimulate a broad range of cytokines and CD4+ T cells during initiation of infection. *Journal of Experimental Medicine* 179: 447-456

Renee, H. (2006): Leishmaniasis. emedicine [online]. http://www.emedicine.com/emerg/topic296.htm

Ridley, D.S. (1979): The pathogenesis of cutaneous leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* 73: 150-160

Ridley, D.S., Marsden P.D., Cuba C.C. and Barreto, A.C. (1980): A histological clasification of mucocutaneous leishmaniasis in Brazil and its clinical evaluation. *Transactions of the Royal society of Tropical Medicine and Hygiene.* 74: 508-514

Roberts, W., Berman, J.D. and Rainey, P.M. (1995): *In vitro* antileishmanial properties of Tri and Pentavalent antimonials Preparations. *Antimicrobial Agents and Chemotherapy.* 39:1234-1239.

Rocha, L.G., Almeida, J.R.G.S., Macedo, R.O. and Barbosa-Filho, J.M. (2005): A review of natural products with antileishmanial activity. *Phytomedicine.* 12(6-7): 514-535

Rosado-Vallado, M., Mut-Martin, M., García-Miss, M.R., Dumonteil, E. (2005): Aluminium phosphate potentiates the efficacy of DNA vaccines against *Leishmania mexicana* . *Vaccine*. 3(46-47):5372–5379.

Russell, D.G. and Alexander, J. (1988): Effective immunization against cutaneous leishmaniasis with defined membrane antigens reconstituted into liposomes. *Journal of Immunology*. 140: 1274 – 1279.

Sacks, D. and Sher, A. (2002): Evasion of innate immunity by parasitic protozoa. *Nature Immunology*. 3: 1041.

Sacks, D. and Noben-Trauth, N. (2002): The immunology of susceptibility and resistance to *Leishmania major* in mice. *Nature Review of Immunology.* 2: 845-858

Saldarriaga, O.A., Travi, B.L., Park, W., Perez, L.E. and Melby, P.C. (2006): Immunogenicity of a multicomponent DNA vaccine against visceral leishmaniasis in dogs. *Vaccine.* 2006;24(11):1928–1940.

Saraiva, N.G., Hazbón, M.H., Osorio, Y., (2005): Protective immunogenicity of the paraflagellar rod protein 2 of *Leishmania mexicana* . *Vaccine.* 2005;23(8):984–995.

Saraiva E.M., de Figueiredo, Barbosa A. and Santos F.N. (2006): The FML-vaccine (Leishmune®) against canine visceral leishmaniasis: a transmission blocking vaccine. *Vaccine*. 24(13):2423-2431

Sauvain, M., Dedet, J., Kunesch, N., Poisson, J., Gantier, J., Gayra, l.P. and Kunesch, G. (1993): *In vitro* and *in vivo* leishmanicidal activities of natural and syntetic quinoids. *Phytotherapy Research*. 7:161-171

Sauvian, M., Dedet, J.P., Kunesch, G. and Poisson, J. (1994): Isolation of flavans from the Amazonian shrub *Faramea guianensis*. *Journal of Natural Products*. 57:403-406

Schubach, A., Marzochi, M., Aruajo, M.L. and Conceicao, N.F. (1987): Healed lesion of cutaneous leishmaniasis- a positive culture for *Leishmania* species in the scar tissue years after cure. *Memoirs do Instituto Oswaldo Cruz.* 82(suppl 1):64

Scott, P., Eaton, A., Gause, W.C., Di Zhou, X. and Hondowicz, B. (1996): Early IL-4 production does not predict susceptibility to *Leishmania major*. *Experimental Parasitology* 84: 178-187.

Scott, P., Natovitz, P., Coffman, R.L., Pearce, E. and Sher, A. (1988): Immunoregulation of cutaneous leishmaniasis: T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. *Journal of Experimental Medicine.* 168:1675

Sepulveda-Boza, S. and Cassels, B.K. (1996): Plant metabolites active against *Trypanosoma cruzi*. *Planta Medica*. 62:98-105

Shahbaz, A., Janjua, M.D., Kjetil, K., Guldbakke, M.D. and Amor, K.M.D. (2005): What caused this crusted plaque? *Skin and Aging*. 13: 82-86

Shapaz, S., Bories, C., Loiseau, P.M., Cortes, D., Hocquemiller, R., Laurens, A. and Cave, A. (1994): Cytotoxic and antiparasitic activity from Annona senegalensis seeds. *Planta Medica* 60 (6): 538-540

Shapaz, S., Gonzalez, M.C., Hocquemiller, R., Zafra-Polo, M.C. and Cortes, D. (1996): Annosenegalin and Annogalene: two cytotoxic monotetrahydrofuran acetogenins from *Annona senegalensis* and *Annona cherimolla*. *Phytochemistry.* 42*:* 106-107

Sharif, M., Ziaei, H., Azadbakht, M., Daryani, A., Ebadattalab, A. and Rostami, M. (2006): Effect of Methanolic extracts of *Artemisia aucheri* and *Camellia sinensis* on *Leishmania major* (*in vitro*). *Turkish Journal of Medical* Sciences*.* 36(6): 365-369

Sheets, R.L., Stein, J. and Manetz, T.S., (2006): Biodistribution of DNA plasmid vaccines against HIV-1, Ebola, Severe Acute Respiratory Syndrome, or West Nile virus is similar, without integration, despite differing plasmid backbones or gene inserts. *Toxicological Sciences*. 91(2):610–619.

Siddiqui, S. (1942): Neem: The wonder tree. *Current Science.* 11:278-279

Sjolander, A., Baldwin, T.M., Curtis, J.M. and Handman, E. (1998): Induction of a Th1 immune response and simultaneous lack of activation of a Th2 response is required for generation of immunity to leishmaniasis. *Immunology.* 160:3949-3957.

Skeiky, Y.A., Kennedy, M., Kaufman, D., Borges, M.M., Guderian, J.A., Scholler, J.K., Ovendale, P.J., Picha, K.S. and Campos-Neto, A. (1998): LeIF: a recombinant leishmania protein that induces an IL-12 mediated Th 1 cytokine profile. *Journal of Immunology.* 161: 6171 – 6179.

Slyvain, C.P.E., Graham, H.C. and Jeremy, O. (2007): PFP1-like genes are expressed in *Leishmania major* pseudogenes in other *Lesihmania* species. *FEMS Microbiology Letter*. 260(1): 47-54

Solbach, W. and Laskay, T. (2000): The host response to leishmania infection. *Advances in Immunology.* 74: 275-317

Soto, J., Toledo, J., Gutierrez, P., Nicholls, R.S., Padila, J., Engel, J., Fischer, C., Voss, A. and Berman, J. (2001): Treatment of American cutaneous leishmaniasis with Miltefosine, an oral agent. *Clinical and Infectious Diseases.* 33:57-61

Spitzer, N., Jardim, A., Lippert, D. and Olafson, R.W. (1999): Long-term protection of mice against *Leishmania major* with a synthetic peptide vaccine. *Vaccine*. 17: 1298 – 1300

Staerk, D., lwemmich, E., Christensen, J., Kharazmi, A., Olsen, C.E. and Jaroszewski, N. (2000): Leishmanicidal, antiplasmodial and cytotoxic acytivity of indole alkaloids of *Corynanthe pachyteras*. *Planta Medica* 66: 531-536

Stenger, S., Thuring, H., Rollinghoff, M. and Bogdan, C. (1994): Tissue expression of inducible nitric oxide synthase is closely associated with resistance to *Leishmania major*. *Journal of Experimental Medicine.* 80:783-793

Sundar, S. (2001): Drug resistance in Indian visceral leishmaniasis. *Tropical Medicine and Internal Health.* 6: 849-854

Tabuti, J.R.S., Lye, K.A. and Dhillion, S.S. (2003): Traditional herbal drugs of Bulamogi, Uganda: Plants use and administration. *Journal of Ethnopharmacology.* 88:19-44

Tadesse, A., Gebre-Hiwot, A., Asres, K., Djote, M. and Frommel, D. (1993). The *in vitro* activity of *Vernonia amygdalina* on *Leishmania aethiopica*. *Ethiopian Medical Journal.* 31 (3): 183-189

Tagboto, S. and Townson, S. (2001): Antiparasitic properties of medicinal plants and other natural occurring products. *Advances in Parasitology*. 50: 199-295.

Tapia, E., Pérez-Jiménez, E., López-Fuertes, L., Gonzalo, R., Gherard, M.M. and Esteban, M. (2003): The combination of DNA vectors expressing IL-12 + IL-18 elicits high protective immune response against cutaneous leishmaniasis after priming with DNA-p36/LACK and the cytokines, followed by a booster with a vaccinia virus recombinant expressing p36/LACK. *Microbes and Infection.* 5(2):73–84.

Titus, R.G., Gueiro-Filho, F.J., de Freitas, L.A. and Beverly, S.M. (1995): Development of a safe life Leishmania vaccine line by gene replacement. *Procedures of the National Academy of Sciences U.S.A*. 92: 10267-10271

Titus, R.J. and Ribeiro, J.M. (1988): Salivary gland lysates from the sandfly *Lutzomyia Longipulpis* enhance leishmania infection. Science 239:1306

Ulmer, J.B., Sadoff, J.C. and Liu, M.A. (1996): DNA vaccines. *Current Opinions in Immunology.* 8:531-536.

Valenzuela, J.G., Garfield, M., Rowton, E.D. and Pham, V.M. (2004): Identification of the most abundant secreted proteins from the salivary glands of the sand fly *Lutzomyia longipalpis,* vector of *Leishmania chagasi*. *The Journal of Experimental Biology.* 207(21): 3717–3729.

Vergel, C., Palacios, R. and Cadena, H. (2006): Evidence for *Leishmania (Viannia)* parasites in the skin and blood of patients before and after treatment. *Journal of infectious Diseases.* 194: 503-511

Von Stebut, E. (2007): Immunology of cutaneous leishmaniasis: the role of mast cells, phagocytes and dendritic cells for protective immunity. *European Journal of Dermatology.* 17(2): 115-122

Von Stebut, E., Metz, M., Milon, G., Knop, J. and Maurer, M. (2003): Early macrophage influx in sites of cutaneous granulomas formation is dependent on MIP-1alpha/beta released from neutrophils recruited by mast cell-derived TNF alpha. *Blood* 101: 210-215

Wahren, B. (1996): Gene vaccines. *Immunotechnology.* 2: 77-81

Walker, P.S. (1997): Direct targeting of skin with DNA vaccines for genetic immunization against Leishmania in a murine model. American Academy of Dermatology 1997 Awards for Young Investigators in Dermatology. *Journal of the American Academy of Dermatology.* 37(5, part 1):776–777.

Walker, P.S., Scharton-Kersten, T., and Rowton, E.D., (1998): T cell type 1 immune response and protection in a murine model of leishmaniasis. *Human Gene Therapy.* 9(13):1899–1907.

Weniger, B., Robledo, S., Arango, G.J., Deharo, E., Aragon, R., Munoz, V., Callapa, J. Lobstein, A. and Anton, R. (2001): Antiprotozoal activities of Colombian plants. *Journal of Ethnopharmacology.* 78:193-200.

Wenner, C., Gutar, M., Macatonia, S., O'garra, A. and Murphy, K. (1996): Roles of IFN-gamma and IFN-alpha in IL-12 induced T helper cell-1 development. *Journal of Immunology*. 156: 1442-1447

Wikipedia (Free encyclopedia): http://en.wikipedia.org/wiki/Cutaneous_leishmaniasis

Wilson, M.E., Jeronimo, S.M. and Pearson, R.D. (2005): Immunopathogenesis of infection with the visceralizing Leishmania species. *Microbiology and Pathogenesis.* 38: 147-160

Woelbing F, Kostka S.L, Moelle K, Belkaid Y, Sunderkoetter C, Verbeek S, Waisman a, Nigg A.P, Knop J, Udey M.C and von Stebut E (2006): Uptake of Leishmania majorby dendritic cells is mediated by Fcgamma receptors and facilitates acquisition of protective immunity. J*ournal of Experimantal Medicine.* 203:177-188World Health Organisation (2002): Programme for the Survelliance and Control of leishmaniasis *World Health Organisation Bulletin*, Series 881, Geneva.

World Health Organisation (2005): Advances in the battle against leishmaniasis. *Tropical Diseases Research News* 57:2

World Health Organisation (2007): AIDS, leishmaniasis dangers of clash highlighted. *Tropical Disease Research News* 36 (1): 11

Wright, C.W. and Phillipson J.D. (1990): Beberine is effective against cutaneous leishmanisis caused by *Leishmania panamensis* in rats. *Phytotherapy Research* 4:127

Xu, D. and Liew, F.Y. (1995): Protection against leishmaniasis by injection of DNA encoding a major surface glycoprotein gp63 of *Leishmania major. Journal of Immunology.* 84: 173-176

Xu, D., McSorley, S.J., Chatfield, S.W., Dougan, G. and Liew, F.Y. (1995): Protection against *Leishmania major* infection in genetically attenuated *Salmonella typhimurium. Immunology* 85: 1-7

Yang, D.M. and Liew, F.Y. (1993): Effects of quinghaosu (artemisinin) and its derivatives on experimantal cutaneous leishmaniasis. *Parasitology*. 106: 7-11

Zerehsaz, F., Salmanpour, R., Handjani, F., Ardehali, S., Panjehshahin, M.R., Tabei, S.Z. and Tabatabace, H.R. (1999): The herbal extract (Z-HE) alleviates cutaneous leishmaniasis when applied topically. *International Journal of Dermatology.* 38: 610

Zhai, L., Blom, J., Chen, M., Christensen, S.B. and Kharazmi, A. (1995): Antileishmanial activity of Licochalone A in mice infected with *Leishmania major* and in hamsters infected with *Leishmania donovani*. *Antimicrobial Agents and Chemotherapy.* 38: 1339-1344

Zohreh, E., Denis, G. and Tanner, C.E. (1996): Synergism of IL-12 stimulated splenocytes and Pentosan enhances the killing of *Leishmania donovani in vitro*. *Clinical Immunology and Immunopathology*. 81(1): 74-81

APPENDIX

1 Composition of ELISA Buffers

PBS 10x

NaCl	80g	BDH Ltd, Poole, UK
KCl	2g	BDH Ltd, Poole, UK
Na_2HPO_4	11.5g	BDH Ltd, Poole, UK
KH_2PO_4	2g	BDH Ltd, Poole, UK

Make up to 1 litre with distilled water at pH 7

Coating Buffer

PBS (1X) at pH 9

Wash Buffer

PBS (1x) + 50µl of Tween 20 (Sigma, Irvine, UK) per 100mls

Glycine Buffer

Glycine	7.51g	BDH Ltd, Poole, UK
MgCl2	0.203g	BDH Ltd, Poole, UK
ZnCl	0.136g	BDH Ltd, Poole, UK

Make up to 980 mls with distilled water, then pH to 10 and make up to 1litre.

Blocking Buffer

10% FCS in PBS (1x)

II Composition of SDS PAGE buffers

Transfer buffer—12mM Tris-His, 96mM Glycine, 20% [v/v] methanol in DI water
Running (electrode) buffer—29 g Tris base, 144 g Glycine, 10 g SDS in upto 1 litre deionised water to make 10times concentrated solution

III Composition of DNA extraction buffers

Elution buffer (0.2M KCl, 0.05M Tris,(pH 7.4), 6M Urea and 500mM Imidazole)
Binding buffer (0.2M KCl, 0.05M Tris,(pH 7.4) 6M Urea and 50mM Imidazole)
Wash Buffer (0.2M Kcl, 0.05M Tris pH 7.4)

IV Composition of Boyles Solution

Tris 0.17M + Ammonium Chloride 0.16M

Tris (0.17M) is made by dissolving 1.03g in 500ml of distilled water

NH_4Cl (0.16M) is made by dissolving 4.15g in 500ml of distilled water

Mix both in 1:9 ratio and sterilize through 0.22μm sterile filter before use.

V Gel Components for SDS-PAGE

12 % Resolving Gel		**5% Stacking Gel**	
Deionised Water	4.9 ml	Deionised Water	2.25 ml
Prosieve ®	2.4 ml	Prosieve ®	300 μl
Tris HCl, pH 8.8	2.5 ml	Tris HCl, pH 8.8	390 μl
10% SDS	100 μl	10% SDS	30 μl
10% APS *	100 μl	10% APS *	30 μl
TEMED	4 μl	TEMED	3 μl

∗Solid APS granules were used

VI Cell Media

Complete Parasite Medium: 20% foetal calf serum, 1% penicillin/streptomycin, 1% L-glutamine made upto required volume with RPMI

Incomplete parasite medium: As above without foetal calf serum

Tissue culture medium: As above but with 10% foetal calf serum

Macrophage medium: 10% foetal calf serum, 30% L-cell, 1% Penicillin/streptomycin, 1% l-glutamine made upto required volume with Dulbecco's Modified Eagle medium (DMEM)

VII Anisaldehyde-Sulphuric acid Reagent: 0.5 ml anisaldehyde is mixed with 10 ml glacial acetic acid, followed by 85 ml methanol and 5 ml concentrated sulphuric acid in this order in a fume cupboard.