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# SELECTION AND EVALUATION OF TEN MEDICINAL PLANTS USED, IN THE VHEMBE DISTRICT, FOR LIFE-THREATENING INFECTIONS

By

**SIGIDI MUENDI TSHILILELWA**

A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS OF PHILOSOPHIAE DOCTOR (PHD) OF SCIENCE DEGREE IN MICROBIOLOGY

to the

**DEPARTMENT OF MICROBIOLOGY**
**SCHOOL OF MATHEMATICAL AND NATURAL SCIENCES**
**UNIVERSITY OF VENDA**
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February 2017

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# DECLARATION

I, Muendi Tshililelwa Sigidi (Student number: 11607014), declare that this dissertation for the award of PhD degree in Microbiology at the University of Venda is my original work and has not been submitted for any degree at this University or any other Institution. The thesis does not contain any other persons' writing unless specifically acknowledged and referenced accordingly.

Signature

2017/09/08

Date

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# ARTICLES PUBLISHED

- **Sigidi M.**, Traore A.N., Tshisikhawe P.M. and Potgieter N. (2017) Ethnobotanical review of selected medicinal plants used in treatment of several diseases around Venda region. *Indian Journal of Traditional Knowledge (*Accepted January 2017).
- C.P. Anokwuru, **M.T Sigidi**, T. Zininga, M.P. Tshisikhawe, A. Shonhai, I.D.I. Ramaite, A.N. Traoré, N. Potgieter (2016) Phenolic contents, antioxidant activity and spectroscopic characteristics of *Pterocarpus angolensis* DC stem bark fractions. *Indian Journal of Traditional Knowledge (*Accepted January 2017).
- **Sigidi M.T.**, Traore A.N., Boukandou M.M., Tshisikhawe P.M. and Potgieter N. (2016) Anti-HIV, pro-inflammatory and cytotoxicity properties of selected Venda plants. *Indian Journal of Traditional Knowledge (*Accepted January 2017).
- **M.T Sigidi**, C.P. Anokwuru, T. Zininga, M.P. Tshisikhawe, A. Shonhai, I.D.I. Ramaite, A.N. Traoré, N. Potgieter (2016) Comparative *in vitro* cytotoxic, anti-inflammatory and anti-microbiological activities of two indigenous Venda medicinal plants. *Translational Medicine Communications; 1:9,* DOI 10.1186/s41231-016-0009-x.


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# PRESENTATIONS AT CONFERENCES

1. **MT Sigidi**, AN Traoré, MMM Boukandou, PM Tshisikhawe, S Ntuli and N Potgieter. (2016) Comparative in vitro cytotoxic, anti-inflammatory and anti-microbiological activities of two indigenous Venda medicinal plants. 6th Annual Conference and Exhibition in Traditional and Alternate Medicine, The Hyatt Place hotel; Amsterdam (The Netherlands): 14th – 16th September.

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4. **MT Sigidi**, AN Traoré, MMM Boukandou, PM Tshisikhawe, S Ntuli and N Potgieter. (2016) Anti-HIV, pro-inflammatory and cytotoxicity properties of selected Venda plants. 2nd Univen-WSU Research Conference, The Ranch; Polokwane (South Africa): 5th – 7th October.


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# DEDICATION

This thesis is dedicated to my children; Wanga, Khangwelo and Wamashudu Sigidi

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took action. My father in law - Mr Sigidi MA -, never gave up motivating me, thus, it made it difficult to even give up, no matter how tough it was. Some days I had to work overtime, and with young kids I needed constant backup support for me to see my work through in time. For that reason I acknowledge the assistance from my mother- in-law - Sigidi ME and Vho-Silly. I always knew my kids were in safe care and that kept me going. My uncle -Tshisikule TB - and brother -Phadagi MM -; your words never went in vain, your support was amazing.

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# ABSTRACT

## Selection and evaluation of ten medicinal plants used, in the Vhembe District, for life-threatening infections

**Sigidi MT, Traoré AN, Tshisikhawe MP and Potgieter N**

**Background**

The Vhembe District of the Limpopo Province has a rich tradition in the use of medicinal plants. The use of these plant species, among rural communities has impacted on the development of various traditional medicinal systems. Such development has led to the exploration of different plant species in order to correlate traditional and scientific practices. In the Vhembe District, the vast majority of traditional medicine is indigenous knowledge-based and the pharmacological activities of plants are solely understood by traditional healers. This study investigated the medicinal activities of *Elaedendron transvaalense, Cassia abbreviata*, *Ornithogalum ornithogaloides*, *Ochna holstii*, *Lannea edulis*, *Elephantorrhiza elephantina*, *Coccinia rehmannii*, *Jatropha zeyheri, Ziziphus mucronata and Pterocarpus angolensis*.

**Objectives:** The general objective was to investigate the anecdotal claims by traditional healers to treat HIV/AIDS by evaluating the *in-vitro* cytotoxicity, anti-inflammatory, antimicrobial and immunomodulatory potential of the selected medicinal plants.

**Methods:** Water and ethyl acetate were used as the extraction solvents and the extracts were subjected to phytochemical screening. Determination of radical-scavenging activity of these plants' extracts was carried out using the DPPH assay and reducing-power assay. Different cell lines (RAW 264.7, U937, MeWo, Vero and PBMCs) were treated with various concentrations (50, 100, 125, 250 µg/mL) of *Elaedendron transvaalense, Cassia abbreviata*, *Ornithogalum ornithogaloides*, *Ochna holstii*, *Lannea edulis*, *Elephantorrhiza elephantina*, *Coccinia rehmannii*, *Jatropha zeyheri, Ziziphus mucronata and Pterocarpus angolensis* for anti-inflammation and cytotoxicity testing. The Nitric Oxide Assay was used to determine the anti-inflammatory potential of the plant extracts and the cytotoxicity of the extracts was tested using MTT and XTT assays. The immunomodulatory effects were determined by quantification of cytokine levels (IL-2, IL-6 and TNF-α) using commercial ELISA kits. Susceptibility testing was done using the microbroth dilution and INT assay. Reverse Transcriptase (RT) assay and HIV-p24 ELISA were used for the anti-HIV activity of the



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plants. The Alamar Blue Assay was used to test for the anti-mycobacterial activity of *P. angolensis* and *Z. mucronata*, which are the two most used plants from the region.

**Results:** Cytotoxicity of the aqueous extracts was cell specific. The plant extracts exhibited selective toxicity towards the cancer line (U937 and MeWo). *Ornithogalum ornithogaloides*, *E. transvaalense*, *Z. mucronata* and *P. angolensis* exhibited a higher degree of toxicity in U937 cells with a reduction of cell viability by 60%. Furthermore, the adverse toxicity of *O. ornithogaloides* and *E. transvaalense* was observed in Vero cells with reduction of cell viability by 80%. Similar toxic effects were observed in MeWo cells for the same extracts. The ethyl acetate extracts of *C. abbreviata* and *E. elephantina* were the most toxic at 50 and 100 µg/ml with detectable viable cells below 20%. All the extracts proved to possess immunomodulatory activities. The non-infected cells expressed higher levels of IL-2 and *Z. mucronata* stimulated production of IL-2 in HIV-infected cells. IL-6 expression in non-infected cells by *C. rehmannii* was the highest (590 pg/ml) and in HIV- infected cells the expression was suppressed. Cytokine TNF-α was relatively lower in non-infected cells since this cytokine is known to be produced in all stages of HIV. Even though all the plant extracts exhibited no activity against bacterial pathogens, 2 of the extracts (*Z. mucronata* and *P. angolensis*) exhibited strong anti-TB activity. Moreover, the extracts exhibited RT activity with percentage inhibition of 40 and 55% for *C. rehmannii* and *P. angolensis,* respectively. P24 antigen levels were also decreased in the presence of all the tested extracts. *L. edulis* exhibited a decrease of 23 pg/ml in P24 antigen levels.

**Conclusion:** The current work highlighted the biological effects of the 10 medicinal plants. The claims presented by the traditional healers on the efficacy of the selected plants in treatment of HIV/AIDS might be accounted for by the inhibitory activities observed in the reverse transcriptase assay, p24 antigen and also the strong anti-TB effects that possibly combated the opportunistic pathogen. In addition, the plant extracts also possess immunomodulatory effects that possibly boost the immune system to fight off the infections.

**Keywords:** Cytotoxicity. Anti-inflammation, Medicinal plants, Anti-HIV

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# LIST OF ABBREVIATIONS (ACRONYMS)

| | |
|---|---|
| **µg** | Microgram |
| **µl** | Micro liter |
| **mM** | Mini molar |
| **mg** | Milligram |
| **ml** | Millilitre |
| **nm** | Nanometer |
| **pg** | Pictogram |
| **AIDS** | Acquired immune deficiency syndrome |
| **ALT** | Alanine aminotransferase |
| **ANOVA** | One-way analysis of Variance |
| **ApoE** | Apolipoprotein E |
| **ART** | Antiretroviral therapy |
| **ATTC** | American Type Culture Collection |
| **ATP** | Adenosine triphosphate |
| **CAM** | Camptothecin |
| **CCK** | Cell counting kit |
| **CCR-2** | C-C chemokine receptor type 2 |
| **CD** | Cluster of Differentiation |
| **CPT** | Camptothecin |
| **COX** | Cyclooxygen |
| **CRP** | C-reactive protein |
| **CT** | Computed tomography |
| **CTLs** | Cytotoxic T Lymphocytes |
| **DMEM** | Dulbecco's Modified Eagle Medium |
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **DPPH** | 2,2-Diphenyl-1-Picrylhyazyl |
| **EC** | Electrical conductivity |
| **EC** | Epicatchin |
| **EGC** | Epigallocatchin |
| **ELISA** | Enzyme linked Immunoabsorbent assay |
| **Env** | HIV Envelope protein |


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| | |
|---|---|
| **$FeCl_3$** | Iron (III) chloride |
| **GAE** | Gallic acid equivalent |
| **Gag** | HIV Group-specific Antigen |
| **HCl** | Hydrochloric acid |
| **HIV** | Human Immunodeficiency Virus |
| **HRP** | Horse radish peroxidase |
| **$IC_{50}$** | Concentration of an inhibitor where the response is reduced by half |
| **Ig** | Immunoglobulin |
| **IL** | Interleukin |
| **IN** | Integrase |
| **INT** | 2–(4–iodophenyl)–3–(4–nitrophenyl)–5–phenyltetrazolium chloride |
| **LC** | Liquid chromatography |
| **$LC_{50}$** | Lethal dose at which 50% of the population if killed in a given period of time |
| **LDH** | Lactate dehydrogenase |
| **LPS** | Lipopolysaccharide |
| **mRNA** | Messenger ribonucleic acid |
| **MAEC** | Mouse aortic endothelial cells |
| **MCF** | Human breast adenocarcinoma cell line |
| **MDA** | Methylenedioxyamphetamine |
| **MeWo** | Human Skin Tumor Cells |
| **MS** | Mass Spectrometry |
| **MTT** | 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide |
| **$NaCO_3$** | Sodium carbonate |
| **NAD/NADH** | Nicotinamide adenine dinucleotide |
| **NaOH** | Sodium Hydroxide |
| **NF-κβ** | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| **NO** | Nitric oxide |
| **NR** | Neutral Red |
| **PAI-1** | Plasminogen activator inhibitor-1 |
| **PBMCs** | Peripheral blood mononuclear cells |
| **PCA** | Principal component analysis |
| **$PGE_2$** | Prostaglandin E2 |
| **Ph** | Potential of Hydrogen |
| **PHA** | Phytohemagglutanin |
| **PMS** | Phenazinium methyl sulfate |
| **PR** | HIV protease |

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| | |
|---|---|
| **RAW cells** | Macrophage-like, Abelson leukemia virus transformed cell line derived from BALB/c mice |
| **ROS** | Reactive Oxygen Species |
| **RPMI** | Roosevelt Park Memorial Institute |
| **RNA** | Ribonucleic acid |
| **SD** | Standard deviation |
| **SRB** | Sulforhodamine B |
| **SSA** | Statistics SA |
| **TCM** | Traditional Chinese Medecine |
| **Th** | Helper T cells |
| **TMB** | 3,3',5,5'-Tetramethylbenzidine |
| **TNF** | Tumor Necrosis Factor |
| **IUCN** | International Union for Conservation of Nature |
| **UNAIDS** | The Joint United Nations Programme on HIV/AIDS |
| **URL** | *Uniform Resource Locator* |
| **UV** | Ultra Violet |
| **VCMA** | Condensate Removal Pumps |
| **WHO** | World Health Organization |
| **WST** | water-soluble tetrazolium salt |
| **XTT** | 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide |



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# Chapter 1

# GENERAL INTRODUCTION

## 1.1 BACKGROUND

South Africa has a rich diversity of more than 24 000 indigenous plant species, which represents approximately 10% of all higher plants on Earth (National Reference Centre for African Traditional Medicines, 2004). The people of South Africa have a long tradition of medicinal-plant use with an estimated 70% of the population harvesting one, if not more, from the approximated 3000 species of plants used as traditional medicines (van Wyk and Gericke, 2000; National Reference Centre for African Traditional Medicines, 2004; Scott and Springfield, 2004). The country's vast variety of indigenous floral species together with their diverse medicinal uses indicate a great potential for uncovering of new bioactive chemicals (van Wyk and Gericke, 2000; Louw *et al.*, 2002; van Wyk and Wink, 2004).

Medicinal plants are widely used in many developing countries for their benefits in treating a wide range of ailments (Van Wyk and Wink, 2004). Several natural products and their derivatives constitute over 50% of all drugs with known antioxidant, antiviral, antibacterial and antifungal activities (Gurib-Fakim, 2006; Hasplova *et al.*, 2011). The selection of plants based on their ethno-medicinal usage increases the likelihood of finding an effective therapeutic agent as opposed to a random selection (Farnsworth, 1994; Fabricant and Farnsworth, 2001).

The World Health Organization (WHO) has reported that logical alliance of treatments with other related viral infections (such as hepatitis B) and closely linked disease symptoms (lymphadenopathy, Kaposi's sarcoma, genital ulcers) could increase the prospects of finding new plant leads for potential anti-HIV agents (WHO, 1989a; WHO, 1989b; Cardellina and Boyd, 1995).

## 1.2 RESEARCH PROBLEM / MOTIVATION

The shortage of scientific evidence on medicinal plants of South Africa makes them relatively unexploited potential sources of novel antiretroviral compounds (Mills *et al.*, 2005). Only recently, have South Africans begun assessing the anti-HIV properties of locally-available traditional medicinal plants mainly focusing on the immune and



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antiretroviral benefits of commonly-used plants such as *Sutherlandia frutescens* (Gericke *et al.,* 2001; Harnett *et al.,* 2005) and the sterols found in *Hypoxis hemerocallidea* (Bouic *et al.,* 1996; Bouic and Lamprecht, 1999; Breytenbach *et al.,* 2001). In addition, around the Vhembe District, several traditional healers are claiming power in curing several life-threatening diseases including HIV/AIDS by using a mixture of specific indigeneous plants (Own observation).

Thus, a need arose to engage with the community to properly investigate and identify these medicinal plants, to test for their various properties, their safety and partially identify their phytochemical composition. The information obtained will be of great importance in backing up the traditional healers' claims and advance the pursuit for new drug discovery.

Furtheremore, this study was motivated by the high rate of HIV/AIDS infections – an alarming 36.7 million people were living with HIV in 2015, as reported by the UNAIDS. A total of 2.1 million new infections were recorded in 2015 of which 150 000 were children under the age of 15. About 1.1 million AIDS-related deaths were recorded in 2015 (UNAIDS, 2016). Of the global HIV infections, Sub-Saharan Africa accounts for 25.6 million confirmed infections, from which 6.19 million are living in South Africa (SSA, 2015). HIV/AIDS does not kill; however, it is the opportunistic infections that take advantage of the suppressed immune system. The secret to curing or lengthening the lives of infected individuals might occured via the boosting of the immune system to keep the body healthy and fit to fight off any infections, effectively (Khanna *et al.*, 2017).

## 1.3 RESEARCH QUESTIONS

### 1.3.1 How safe are these plant extracts? Do they possess any toxic effects?

The physiological effects of plants' secondary metabolites have shown that some plants possess potential anti-cancerous ability due to their direct cytotoxicity on cancer cell lines or modulation of tumour development and eventually tumour hindrance (Ahmad *et al.*, 2017). The extent of their cytotoxicity have been tested *in vitro* using different human parts (such as, prostate, stomach and liver.) and animals



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(such as, monkey kidney cells) cell lines (Sigidi *et al.*, 2016). Although some of these plants' cytotoxicity might have promising therapeutic effects especially in HIV patients with opportunistic infection, the same compounds could be harmful to other human cell lines, leading to the development of complications resulting from this adverse toxicity (WHO, 2014-2023).

### 1.3.2 Are these plants active against opportunistic pathogens?

Certain medicinal plants are believed to combat microbial pathogen infections by either directly killing the organism or neutralizing the lethal effects of the chemicals produced. The use of synthetic plant-derived compounds targeting cellular proteins involved in HIV replication has paved a way for new research into the management of HIV/AIDS. A variety of compounds isolated have been investigated *in vitro* for any potential effects on HIV-1 replication, the reducing of the viral load and prolonging of lives (Esposito *et al.*, 2016). With the alarming HIV/AIDS statistics in South Africa, the potential of such compounds to alleviate the burden of the disease would be most welcome (SSA, 2015).

### 1.3.3 Are they capable of boosting the immune system?

Immunomodulators attained from medicinal plants are being considered extensively as alternative and/or complementary approaches to conventional treatment against various diseases (Khanna *et al.*, 2017). Extracts or molecules derived from plants or by chemical synthesis based on leads obtained from natural products, are opening new prospects for modern therapeutics to positively modulate the immune system against infections. In view of the relatively high cost and considerable side effects of many of the western medicines, researchers are now focusing on re-examining the traditional formulations used in different parts of the world that may alleviate the consequences of microbial infections.

An escalating area of research is the discovery of immunomodulatory agents from traditional medicinal plants that are free from toxic side effects and which could be used for a long period (Fulzele *et al.*, 2002).



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### 1.3.4 Which phytochemical compounds constitute the crude extracts?

Plants produce a variety of biologically active compounds as a form of defence against either bacterial or viral pathogens that infect them. The search for these active agents is of great scientific interest for the production of new and more effective chemotherapeutics.

Many useful drugs have been developed from medicinal plants, including the clinical drugs that are currently in use, such as, aspirin, atropine, digoxin, morphine, quinine, respine and tubocurarine (Esposito *et al.*, 2016). These drugs were identified through the study of ethnobotany, and most of the isolated compounds were then utilized in the manufacturing of new drugs.

## 1.4 HYPOTHESIS OF THE STUDY

The hypothesis of this study was that: the selected medicinal plants are active against opportunistic pathogens and boost the immune system, thus, increase the body's response against infections as these plants possess both antimicrobial and immunomodulatory effects.

## 1.5 OBJECTIVES OF THE STUDY

### 1.5.1 General objective

The general objective was to investigate the anecdotal claims by traditional healers to treat HIV/AIDS by evaluating the *in-vitro* cytotoxicity, anti-inflammatory, antimicrobial and immunomodulatory potential of the selected medicinal plants.

### 1.5.2 Specific objectives

(i) To conduct an ethno-botanical survey on the selected plants;

(ii) To collect and prepare plant extracts using aqueous and ethyl acetate solvents;

(iii) To analyse the chemical compounds in the plant extracts using the test-tube reactions method;



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(iv) To identify the constituents in the plant extracts using LC-MS;

(v) To determine the free-radical scavenging activity of the extracts using DPPH and reducing power assays;

(vi) To assess the pro-inflammatory potential of the extracts using cell cuture and ELISA;

(vii) To assess cell viability in order to confirm the absence of toxicity from the extracts, using MTT;

(viii) To cultivate and prepare pathogenic bacterial strains (*E. faecalis, E. coli, K. pneumoniae, Salmonella enterica, S. agalactiae, M. tuberculosis*) using standardized culture methods;

(ix) To evaluate the anti-bacterial activity of the plant extracts using the Microbroth dilution and INT assays;

(x) To evaluate the anti-mycobacterial activity of the plant extracts using microplate Alamar blue assay and

(xi) To evaluate the anti-HIV activity of plant extracts looking at viral protein replication and acitivity by using the P24 antigen Assay and the RT Assay.

## 1.6 SIGNIFICANCE OF THE STUDY

Over the past few years, various research groups, from different disciplines have investigated the ethnobotanical, chemical and health characteristics of medicinal plants in the Vhembe district (Bessong *et al.*, 2004; Bessong *et al.*, 2005; Samie *et al.*, 2005). People's traditional knowledge about plants and animals, in relation to their use within the Vhembe district ought to be documented in order for the information to be passed from one generation to the next (Mabogo, 1990). A number of these surveys (Brendt and Muller, 1995; Madzibane and Potgieter, 1999; Samie *et al.*, 2005; Tshikalange *et al.*, 2005; Mulaudzi *et al.*, 2011) highlight the fact that rural people rely heavily on traditional medicine for the treatment of many ailments. The use of such plants could be attributed to their attainability, affordability (Steenkamp, 2003) and cultural beliefs (Yirga, 2010). For some villagers, their dependency on medicinal plants is due to the lack of access to modern medical facilities or the lack of effective services.

Currently, it is vital to understand some of the defence mechanisms of the immune system during infection with life-threatening infections like HIV/AIDS, so as to help



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improve or boost these mechanisms' responses. Few studies have been conducted in the Vhembe region to assess the effects of medicinal plants on the immune system of those diagnosed with HIV and its life-threatening opportunistic infections. A study conducted by Bessong *et al.* (2004), evaluated the biological activities of *Terminalia sericea, Bridelia micrantha* and *Combretum molle* against HIV-1 (reverse transcriptase (RT) and RnaseH); another study was conducted in 2006 to evaluate *Bridelia micrantha* activity against HIV-1 RT and integrase enzyme (Bessong *et al.*, 2006). Additional efforts have also been made to investigate the immunomodulatory effects of *Combretum hereroense* and *Canthium mundianum* (Samie *et al.*, 2005).

The current study aimed at verifying the claims made by traditional healers on the healing properties, of the selected plants, against several life-threatening infections. The plants were selected on the basis of their exhaustive use, by healers when treating patients with HIV; as they claim that these plants have the possibility of boosting the immune system, cleansing the blood and controling viral replication.

This study screened 10 selected medicinal plants from the Vhembe region for their immunomodulatory effects, antimicrobial, anti-HIV and anti-cancerous activities. This was achieved by establishing viral antigen levels in cells pre-treated with medicinal plants in comparison to un-treated cells. The active compounds from these plants were, therefore, identified as they may be of importance in the discovery of new therapeutic agents to help reduce the burden associated with certain human disease; hopefully, the results obtained may lead to the manufacturing of drugs derived from plants.

## 1.7 THESIS LAYOUT

This thesis is divided into seven chapters and is structured as follow:

Chapter 1 incorporated the background, rationale, and objectives of the study; Chapter 2 deals with the ethno-botanical aspects of the study. Chapter 3 covers the phytochemical analysis; antioxidant and anti-inflammatory activities as well as LC-MS. Contents in Chapter 4 include a narration on the cytotoxicity of the selected plant extracts. This is then followed by Chapter 5 which presents the immune-modulatory effects of the plant extracts. Chapter 6 elaborates on the effects that the plant extracts possess against microbial pathogens. Lastly, Chapter 7 presents the



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overall concluding remarks and recommendations for further investigations. For publication purpose, the plant extracts were divided into two groups, experiments conducted for the two groups are similar, however, for *Ziziphus mucronata* and *Pterocarpus angolensis* additional tests were conducted.



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# Chapter 2

# PART I: ETHNOBOTANICAL ASPECTS OF THE SELECTED MEDICINAL PLANTS

## 2.1 BACKGROUND

The pharmacological therapy of diseases using herbs has been observed long ago (Schulz *et al.*, 2001). Plants have been used as basic traditional healing cures throughout the world for centuries. Herbal medicines are in great demand as a source of primary health care, in both developed and developing countries. This might be attributed to the biological and medicinal effects they possess, perceived safety of natural products, ease of accessibility, traditional beliefs and their affordability.

According to the World Health Organization (WHO) statistics, an estimated 70% of the world's population still rely on plants for primary health care (WHO, 2013). About 35,000 to 70,000 plant species are used in treatment of different diseases; this figure corresponds to 14 to 28% of the 250,000 plant species estimated to exist in the world (Farnsworth and Soejarto, 1991; Akerele, 1992; Padulosi *et al.*, 2002). Furthermore, in the international market there are more than 50 crucial drugs which originated from tropical plants (De Padula *et al.*, 1999).

The use of herbal molecules is believed to be the route to conquer microbial resistance; this action could be attributed to the interactive or synergistic effects from all compounds of plants (Lai and Roy, 2004; Tapsell *et al.*, 2006).

### 2.1.1 Traditional Chinese medicine

The use of traditional medicine by Chinese people dates back to ancient times and although animal and mineral substances were also used, the primary source of therapy remained botanical. Five hundred of the 12 000 prepared concoctions made by traditional healers are for routine medical use, as reported in the literature (Li, 2000). In clinical practice, traditional diagnosis may be followed by prescription of existing concoctions or individual therapies from plant origin.



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More than half of the Chinese population use TCM, with much frequent use in the rural areas. An estimated 5 000 traditional remedies are available in China; these accounts form one fifth of the entire Chinese pharmaceutical market (Li, 2000). Some of the plants used in the Chinese traditional system include, *Angelica polymorpha* var *sinesis*, *Artemisa annua*, *Ephedra sinka*, *Panax ginseng* and *Rheum palmatum* (Bhat, 1995; Berger, 2001).

### 2.1.2 Japanese traditional medicine

Many herbal remedies have found their way to the Japanese system of traditional healing, from China. Herbs indigenous to Japan were categorized in the first pharmacopoeia of Japanese traditional medicine, centuries ago (Saito, 2000). The Japanese traditional medicine has been used in the society for several decades and is subdivided into 'folk medicine' and 'Chinese medicine' (referred to as 'Kampo medicine'). Kampo medicine is so popular that its utilization in Japan represents the highest, worldwide. A total of 146 Kampo remedies are registered as drugs by the Ministry of Health and Welfare and are also enrolled as therapeutic drugs in the Japanese National Health Insurance.

The use of Kampo drugs was accepted without clinical validation studies, although about 80% of physicians were reportedly found to have being prescribing these medicines since 1989 (Scheid *et al.*, 2010). Japanese physicians predominantly recognize the use of traditional medicine as a supplement to modern medicine; thus these drugs are viewed in Japanese society as being practically safe (Zhang, 1998; Eguchi *et al.*, 2000; Saito, 2000).

### 2.1.3 Indian traditional medicine

Ayurveda is a traditional medical system fundamentally used in India and was discovered 5000 years ago (Sharma *et al.*, 2001). It integrates diet and herbal remedies, while accentuating the body, mind and spirit in disease prevention and treatment (Morgan, 2002). India has a great diversity of medicinal herbs and spices, comprising of over 2 000 species and has an extensive geographical area allowing greater chances of use of Ayurvedic practices (Sandhu and Heinrich, 2005).



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Few, however, of these plant species have been studied chemically and pharmacologically for their potential medicinal value (Gupta *et al.*, 2005; Sandhu and Heinrich, 2005). Some of the plants used in Ayuverda are *Azadirachta indica*, *Centella asiatica*, *Cinnamomum camphora*, *Terminalia* sp, *Withania somnifera*, *Rauwolfia serpentine* (Gurib-Fakim, 2006).

### 2.1.4 Traditional medicine in Europe and North America

In Europe and North America, herbal products have been integrated into alternative medical systems. Over time, the increasing interest in self-prescribed medication has led to an extensive growth in popularity of traditional healing practices (Tyler, 2000, Sammons *et al.*, 2016).

Most of the consumers have positive attitudes towards these herbal products because they believe them to be natural products rather than the chemically-synthesized drugs. It is believed that such products are more likely to be much safer than drugs although they might not be free from adverse effects (Ernst, 2002).

### 2.1.5 Traditional medicine in Central and South America

In Central and South America, information gathered by naturalists has contributed significantly to the growing knowledge of South American biodiversity and has produced significant advances in understanding of the continent's natural history (Brandao *et al.*, 2008). Some of the plants harvested for their medicinal purposes include *Cinchona pubescens*, *Erythroxylon coca*, *Ilax paraguariensis*, *Paullinia cupona* and *Spilanthes acmella* (Gurib-Fakim, 2006).

### 2.1.6 Traditional medicine in Africa

In Africa, traditional medicine is a part of the people's culture even though their use not as well organized or documented like in other countries. Traditional practitioners include herbalists, bone setters, village midwives or traditional birth attendants, traditional psychiatrists, herb sellers, and other specialists (Mahomoodally, 2013). Traditional healers and their plant-derived remedies play an important role in the



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health of millions of people in Africa and the sale of herbal plants is visible in commercial markets, as shown in Figure 2.1.




**Figure 2.1:** Medicinal plants in commercial markets. (www.gettyimages.com).

The ratios of traditional practitioners and qualified doctors, in comparison to the populations in African countries, are uneven. As reported in Ghana, there are 220 people for every traditional practitioner, compared to nearly 20 000 people for one medical doctor (Tabi and Frimpong, 2003). The demand by most of the people in developing countries for medicinal plants has been met by the extensive harvesting of medicinal flora, including those in the rain forests. As a result, many plant species have become extinct and some endangered (IUCN, 2007). Parts of the harvested medicinal plants are sold in markets, in urban centres in Africa.

### 2.1.7 Traditional medicine in South Africa

Traditional medicine features in the daily lives of thousands of people in South Africa and are better known by the native name "muti". The trade in muti has grown into a large and fast growing industry, with millions of consumers and the trade contributing about R2.9 billion to the national economy (Mander *et al.*, 2007). The use of traditional medicine is desirable and mandatory among many local communities for the treatment of a range of health problems (Figure 2.2). Seventy two percent of the



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black African population is estimated to rely on traditional medicine (Mander *et al.*, 2007).



**Figure 2.2:** Harvesting a rhizome of J. zeyheri for muti sales (Sigidi Muendi, 2014)

There are 2 main types of traditional health-practitioners among the VhaVenda people of the Vhembe District Municipality (Limpopo Province, South Africa); the herbalist (Tshivenda – *nanga*) and the diviner (Tshivenda – *sangoma*) (Sobiecki, 2012). The diviners are believed to throw bones and communicate with the ancestors in order to diagnose the patient and then give a prescription. The herbalist treat patients by prescribing medicine according to the symptoms of the ailment.

## 2.2 METHODOLOGY

### 2.2.1 Ethical clearance

Ethical approval was obtained from the Health, Safety and Research Ethics Committee of the University of Venda (SMNS/14/MBY/30/1210). The background of the study was verbally explained to the traditional healers and the Chiefs of the different villages. They all signed the informed consent forms before participation to show agreement and understanding to participate in the research.



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### 2.2.2 Interview with Traditionnal healers

A total of 15 traditional healers were interviewed on one-on-one basis using a semi-structured questionnaire. Voucher specimens of medicinal plant species recorded were collected and identified to their botanical names and families in the Department of Botany at the University of Venda. Voucher specimens were kept in the Department of Botany herbarium.

### 2.2.3 Selection and collection of medicinal plants

Medicinal plants were selected on the basis of their extensive use for the treatment of different ailments and also a literature search was conducted to corroborate the scientific findings with traditional findings.

Ten medicinal plants (as shown in Table 2.1) were chosen and collected from within the geographic locations of the Vhembe District Municipality of Limpopo Province. These plants were identified by their vernacular names and confirmed using taxonomic keys by an ethnobotanical specialist at the University of Venda (Limpopo Province; South Africa).

The plants were collected from March 2014 to December 2015. The different plant parts (bark, rhizome) were naturally dried (24°C ± 2°C) in the laboratory for 10-14 days. The dried parts were then milled to fine powder using an industrial grinder (Buchi Labortechnik AG, Flawil, Switzerland) then stored in clean packets in a cool dark place till further use.

### 2.2.4 Extraction procedure

Dried and milled plant materials were extracted by the mixing with hot water or ethyl acetate. The extraction procedures are detailed below.

#### *2.2.4.1 Aqueous extraction*

A total of 50 g of the ground plant material was soaked in 1000 ml of boiled distilled water for 24 hours. The water was filtrated and an additional 400 ml of water was added to the residue to extract the remaining compounds. The solution was filtered again using Whatman filter paper. The total collected filtrate (600 ml) was



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concentrated to dryness (FTS systems, Stone Ridge, NY) under reduced pressure as described by Huie (2002). The powder was stored in a cool, dry place till further use.

#### *2.2.4.2 Ethyl acetate extraction*

Extraction of the plant material was done using ethyl acetate as described by Ghani (2005) with slight mofifications. For each plant sample, the powdered materials (20g) were macerated in ethyl acetate for 24 hours and filtered through Whatman No1 filter paper. The filtrates contained the crude drug extract of ethyl acetate, the solvent was evaporated at 40°C using a rotar vapour (Buchi, Flawil, Switzerland) under reduced pressure. Concentrated crude drug were obtained, weighed and then stored in the refrigerator till further use.

### 2.2.5 Determination of plant yield

The crude extract yield after rotary evaporation was obtained by weighing final dried powder. The formula used, $W_f$-$W_e$. Where $W_f$ is the weight of the extract and the container, $W_e$ is the weight of the container only.

## 2.3 RESULTS

### 2.3.1 Report of the interview with Traditionnal Healers

The interviews conducted with fifteen traditional healers produced a list of thirty nine medicinal plant species used in treatment of different diseases. The medicinal plants recorded were distributed amongst 29 plant families. Fabaceae (n=4) was found to be dominant followed by Anacardiaceae (n=3), Apocynaceae (n=2), Capparaceae (n=2), Euphorbiaceae (n=2), and Poaceae (n=2). Other families contributed one species each. Most scholars have reported Fabaceae family to be a dominant family in medicinal plants used in treatment of diseases in many parts of the world including Venda area of South Africa (Yeneayehu *et al.* 2017; Masevhe *et al.* 2015; Sulaiman *et al.* 2015; Semenya and Maroyi 2012; Mushtaq *et al.* 2009).



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**Table 2.1:** Medicinal plants recorded to be used in treatment of diseases in Vhembe District Municipality.

| Family | Botanical name | Common English (E) and Venda (V) names | Parts used | Condition treated | Frequency |
|---|---|---|---|---|---|
| Anacardiaceae | *Lannea edulis* Engl. | Mutshutshungu (V), Wild grape (E) | Roots | Blood cleansing | 1 |
| Anacardiaceae | *Lannea schweinfurthii* Engl. | Mulivhadza (V), False marula (E) | Roots bark | Diarrhoea | 1 |
| Anacardiaceae | *Ozoroa paniculosa* (Sond.)R.Fern & A.Fern | Tshinungumafhi (V), Common resin tree (E) | Roots | Chest pains | 1 |
| Apocynaceae | *Carisa edulis* Vahl | Mutungulu (V), Simple-spined num-num (E) | Roots | Wounds | 1 |
| Apocynaceae | *Rauvolfia caffra* Sond. | Munadzi (V), Quinine tree (E) | Bark | Pneumonia | 1 |
| Asphodelaceae | *Aloe marlothii* A.Berger | Tshikhopha (V) | Leaves | Stomach problems | 4 |
| Asteraceae | *Bidens pilosa* L. | Mushidzhi (V), Black jack (E) | Whole plant | Open wounds | 1 |
| Burseraceae | *Commiphora tenuipetiolata* Engl. | Mutahadzi (V), Satin-bark corkwood (E) | Bark | Stomach ailments | 1 |
| Cactaceae | *Opuntia ficus-indica* | Mudoro (V), Pricklypear (E) | Leaves | Cough | 1 |
| Canellaceae | *Warburgia salutaris* (G.Bertol.) Chiov. | Mulanga (V) | Bark | Wounds | 4 |

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**Table 2.1:** Medicinal plants recorded to be used in treatment of diseases in Vhembe District Municipality... ***(Continued)***

| Family | Botanical name | Common English (E) and Venda (V) names | Parts used | Condition treated | Frequency |
|---|---|---|---|---|---|
| Capparaceae | *Maerua angolensis* | Mutambanamme (V), Bead-bean (E) | Bark | Stomach troubles | 1 |
| Capparaceae | *Capparis tomentosa* | Muobadali (V), Wooly caper-bush (E) | Roots | Blood cleansing | 1 |
| Celastraceae | *Elaeodendron transvaalense* (Burtt Davy)R.H.Archer. | Mukuvhazwivhi (V), Bushveld saffron (E) | Bark | Blood cleansing | 5 |
| Chrysobalanaceae | *Parinari curatelifolia* Planch. ex Benth. | Muvhula (V), Mabola-plum (E) | Bark | Blood cleansing | 1 |
| Cucurbitaceae | *Coccinia rehmannii* Cogn. | Galange (V), Wild cucumber (E) | Tuber | Blood cleansing | 1 |
| Euphorbiaceae | *Jatropha zeyheri* Sond. | Thundamali (V), Verfbol | Tuber | Blood cleansing | 1 |
| Euphorbiaceae | *Euphorbia ingens* E.Mey. ex Boiss. | Mukonde (V), Naboom (E) | Roots | Warts and cancer | 1 |
| Fabaceae | *Senna didymobotrya* | Tshinduhwana (V) | Whole plant | STDs | 1 |
| Fabaceae | *Cassia abbreviata* Oliv. | Mumboma (V), Sjambok tree (E) | Bark | Blood cleansing | 2 |



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**Table 2.1:** Medicinal plants recorded to be used in treatment of diseases in Vhembe District Municipality... ***(Continued)***

| Family | Botanical name | Common English (E) and Venda (V) names | Parts used | Condition treated | Frequency |
|---|---|---|---|---|---|
| Fabaceae | *Elephantorriza elephantina* (Burch.) Skeels | Gumululo (V), Elephant's root (E) | Tuber | Blood cleansing | 7 |
| Fabaceae | *Pterocarpus angolensis* D.C | Mutondo (V), Bloodwood (E) | Bark | Blood cleansing | 3 |
| Leguminosae | *Schotia brachypetala* Sond. | Mulubi (V), Weeping schotia (E) | Bark | Purify blood | 2 |
| Liliaceae | *Ornithogalum ornithogaloides* (Kunth) Oberm. | Tshiganame (V), Vlei chinkerinchee (E) | Bulb | Blood cleansing | 1 |
| Malvaceae | *Grewia occidentalis* L. | Mulembu (V), Crossberry (E) | Roots | Treat wounds | 1 |
| Meliaceae | *Melia azedarach* L. | Muserenga (V), Syringa (E) | Roots | Sores | 3 |
| Moringaceae | *Moringa oleifera* Lam. | Muringa (V), Moringa (E) | Leaves | Immune booster | 1 |
| Musaceae | *Musa paradisiaca* Colla | Muomva (V), Banana (E) | Leaves | Blood cleansing | 1 |
| Myrtaceae | *Syzygium cordatum* Hochst. ex Krauss | Mutu (V), Waterberry (E) | Fruits | Stomach ache | 1 |



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**Table 2.1:** Medicinal plants recorded to be used in treatment of diseases in Vhembe District Municipality... ***(Continued)***

| Family | Botanical name | Common English (E) and Venda (V) names | Parts used | Condition treated | Frequency |
|---|---|---|---|---|---|
| Ochnaceae | *Ochna holstii* Engl. | Tshipfure (V), Red Ironwood (E) | Tuber | Blood cleansing | 1 |
| Olacaceae | *Ximenia americana* L. | Mutanzwa (V), Tallow wood (E) | Bark | Fevers and colds | 1 |
| Phyllanthaceae | *Antidesma venosum* E Mey. ex Tul. | Mutshelakhwali (V), Tasselberry (E) | Bark | Stomach complaints | 1 |
| Piperaceae | *Piper capense* L.f | Mulilwe (V), Wild Pepper (E) | Bark | Diabetes mellitus | 1 |
| Poaceae | *Phragmites australis* (Cav.) Trin. ex Steud. | Lutanga (V), Common reed (E) | Stem | Gonorrhoea | 1 |
| Poaceae | *Hyperrhenia filipendula* (Hochst.) Stapf | Fundi (V), Thatching grass (E) | Whole plant | Syphilis | 1 |
| Rhamnaceae | *Ziziphus mucronata* Willd. | Mukhalu (V), Buffalo thorn (E) | Bark | Blood cleansing | 1 |
| Rosaceae | *Prunus persica* (L.) Batsch | Muberegisi (V), Peach (E) | Leaves | Mouth odors | 1 |

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**Table 2.1:** Medicinal plants recorded to be used in treatment of diseases in Vhembe District Municipality... ***(Continued)***

| Family | Botanical name | Common English (E) and Venda (V) names | Parts used | Condition treated | Frequency |
|---|---|---|---|---|---|
| Rutaceae | *Zanthoxylum capense* (Thunb.) Harv. | Mununguwane (V), Small knobwood (E) | Roots | Stomach ache | 4 |
| Santalaceae | *Osyris lanceolate* Hochst. & Steud. | Mpeta (V), Rock tannin-bush (E) | Roots | Stomach problems | 1 |
| Vitaceae | *Rhoicissus tridentata* (L.f.) Wild & R.B.Drumm. | Murumbulambudzana (V), Bushmans grape (E) | Tuber | Diarrhea | 1 |

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### 2.3.2 Overview of the selected medicinal plants

The 10 listed plant species (Table 2.2) were chosen based on their extensive use and prescription in treating life threatening infections around the Vhembe District Municipality, by the interviewed healers. For some of the plants, however, there is no documented information of their use in treating human ailments.

These plants are found to be used for the treatment of blood related diseases through blood cleansing were located and collected with the advice and supervision of a local traditional healer.

#### *2.3.2.1 Elaeodendron transvaalense*

*Elaeodendron transvaalense is* commonly known among the Vhavenda people as *Mukuvhazwivhi* (Figure 2.3). The bark discharges a red sticky sap, which is reduced to fine powder during grinding. The powder can be boiled in beer and the resulting concoction is ingested in a warm state for relief from stomach aches and fevers. The powdered dry sap can also be mixed with dried roots of *Ozoroa paniculosa* and is ingested for relief from menstrual pains and control of high blood pressure (Motlhanka *et al.*, 2008).



**Figure 2.3:** Leaves of *Elaedendron transvaalense* (www.plantzafrica.com/plantefg/elaedentrans.htm)



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The bark has been reported for treating piles, venereal diseases, diarrhoea, kidney and bladder infections, among other illnesses. Its durable wood is also used in making crafts, such as wooden utensils. The root extracts of *Mukuvhazwivhi* are rich in free radical scavenging compounds (Motlhanka *et al.*, 2008) and its bark shows activity against *Candida albicans* ATCC strain but not the clinical strains (Steenkamp *et al.*, 2007).

**Table 2.2:** Indigenous Venda medicinal plants selected for the study

| Family, Scientific names, Local Venda names | Common names | Voucher numbers | Availability | Crude methodology |
|---|---|---|---|---|
| Anacardiaceae *Lannea edulis* Engl. *Mutshutshungu* (V) | Wild grape | MPT00120 | Moderate | Half a cup of dried root decoction taken daily |
| Celastraceae *Elaeodendron transvaalense* (Burtt Davvy) R.H.Archer *Mukuvhazwivhi* (V) | Bushveld saffron | MPT00041 | Moderate | Half a cup of dried bark decoction taken daily |
| Cucurbitaceae *Coccinia rehmannii* Cogn. *Galange* | Wild cucumber | MPT00116 | Moderate | Half a cup of dried roots decoction taken daily |
| Euphorbiaceae *Jatropha zeyheri* Sond. *Thundamali* (V) | Verfbol | MPT00115 | Moderate | Half a cup of dried roots decoction taken daily |
| Fabaceae *Cassia abbreviata* Oliv. *Mumboma* (V) | Sjambok tree | MPT00122 | High | Half a cup of dried bark decoction taken daily |
| Fabaceae *Elephantorrhiza elephantina* (Burch.) Skeels *Gumululo* (V) | Elephant-root | MPT00117 | Moderate | Half a cup of dried roots decoction taken daily |
| Fabaceae *Pterocarpus angolensis* DC. Mutondo (V) | Bleedwood tree | MPT00118 | High | Half a cup of dried bark decoction taken daily |
| Liliaceae *Ornithogalum ornithogaloides* (Kunth) Oberm. *Tshiganame* (V) | Vlei chinkerinchee | MPT00121 | Low | Half a cup of dried roots decoction taken daily |
| Ochnaceae *Ochna holstii* Engl. *Tshipfure* (V) | Red Ironwood | MPT00119 | Moderate | Half a cup of dried roots decoction taken daily |
| Rhamnaceae *Ziziphus mucronata* Willd. *Mukhalu* (V) | Buffalo thorn | MPT00123 | High | Half a cup of dried bark decoction taken daily |



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### 2.3.2.2 *Cassia abbreviata*

*Cassia abbreviata* (Long tail Cassia or *Sjambok* tree) loses its leaves in the winter season and is easily identified through its' unusually long tail-like seed pods which has a brownish colour. The common name for this plant in Afrikaans is *Sambokpeul*, and the VhaVenda people call it, *Mulumanama/Mumboma. Cassia abbreviata* is an average-sized tree; the trunk is generally uniform and the bark is deeply and regularly cracked. The veins on the leaves are parallel and dark green in colour. The flowering season lasts only 4 to 6 weeks and it occurs as new leaves sprout. The seed pods or fruits of *C. abbreviata* are long (Figure 2.4), thin and cylindrically shaped; they mature after a year. The whip-like shape of the pods led to its common name - *Sjambok* tree- as it resembles the *sjambok* - a short whip which is used, quite often, for disciplining children in South Africa (own observation).



**Figure 2.4:** Seedpods of *Cassia abbreviata* (http://www.mozambiqueflora.com/speciesdata)

Traditional healers harvest both the root and the bark of *C. abbreviata* for the treatment of bilharzia and Blackwater fever. Other ailments treated with this tree extract, include backache, costiveness, diarrhoea, skin infections, and headache (Van Wyk *et al.*, 1997). Some of the biological effects it possess include, the treatment of diarrhoea and gonorrhoea (Maroyi, 2011), antimicrobial properties (Connely *et al.*, 1996; Leteane *et al.*, 2012; Madureira *et al.*, 2012).



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### 2.3.2.3 *Ornithogalum ornithogaloides*

*Ornithogalum ornithogaloides*, also known as the *Vlei chinkerinchee* and *Tshiganame* (Tshivenda) is a bulbous plant of only about 40 cm high. The bulb is small, at about 2 cm in diameter (Figure 2.5), white and enclosed in thin, membranous bulb-scales. Each bulb usually produces two inflorescences consecutively during the season. The flowers produced are small, white and star shaped. The fruit is 3-chambered, with 1 -3 seeds in each chamber (Yin *et al.*, 2016).



**Figure 2.5:** The Sliced bulbs of *Ornithogalum ornithogaloides* (Sigidi Muendi, 2014)

There are approximately over a hundred *O. ornithogaloides* species in Southern Africa and a large proportion of these have been found to be poisonous to grazing animals. Cattle are often blinded and even if they recover from the poisoning they remain blind for life (Vahrmeijer, 1981). No research has been published on *O. ornithogaloides* being utilized for treatment of any human ailments, so, for the first time in the literature this study reports on the use of *Tshiganame* as one of the concoctions used in the eradication of HIV/AIDS infection burden.



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### 2.3.2.4 *Ochna holstii*

*Ochna holstii* trees are averaged sized, bushy with rough stems, small branches with grey-brown stems and branches with tossed elevated dots. *O. holistii* is commonly known as *Tshipfure* in Tshivenda. The leaves alternate with mid vein and branched veins eminent from above and indented margins. Its flowers are yellow and densely clustered on side shoots (Figure 2.6). The flowering season is between September–November and the fruiting time is between November and January (Iniaghe *et al.*, 2015).



**Figure 2.6:** Leaves and flowers of *Ochna holstii* (www.prota4u.org)

The fruits of *Tshipfure* are pink to purple. The plant is used mostly in treating wounds topically, especially on domestic animals, by the people in the Vhembe District (Luseba and Tshisikhawe, 2013). The local traditional healers make use of this extract also as part of a concoction in hindering replication of HIV and boosting the immune system (information obtained from the interviews).



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### 2.3.2.5 *Lannea edulis*

*Lannea edulis* is a small shrublet with short branches arising from its underground tubers. The leaves are divided into 2-4 pairs of leaflets and its small yellow flowers are borne in erect clumps followed by purplish berries (Figure 2.7). It is known as *Mutshutshungu* in Tshivenda and is used in treating ailments such as diarrhoea (Hutchings *et al.*, 1996; Fawole *et al.*, 2008; Deutschlander *et al.*, 2009). In some communities, leaf infusions of this plant are applied topically on sore eyes and boils (Van Wyk *et al.*, 2009).



**Figure 2.7:** Rhizome of *Lannea edulis* cut into pieces for drying (Sigidi Muendi, 2014)

A root decoction is ingested to treat sterility and also to cleanse the blood of any foreign organisms (interviews' comments). Several uses have been recorded in Zimbabwe (Maroyi, 2011), including, for the treatment of convulsions, whooping cough, female sterility and bloody diarrhoea. The active ingredients within the bark of *Lannea edulis* are alkylphenols (cardonol 7 and cardonol 13) as well as 3 dihydroalkylhexenones (Queiroz *et al.*, 2003).



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### 2.3.2.6 *Jatropha zeyheri*

It is a climbing herb with a deep-rooted rhizome and slightly-branched leafy stems (Figure 2.8). It is mainly found in grasslands and in sandy soils. The genus comprise approximately 172 species, all with significance with regards to healing various ailments (Bhagat and Kulkarni, 2010). A decoction (prepare by boiling) of the tuber is known to be used in the treatment of inconsistent periods, period pain and to ensure a healthy and strong foetus before birth (van Wyk and Gericke 2007). It may also be used topically in wound treatment (Luseba *et al.*, 2007).



**Figure 2.8:** The rhizome of *Jatropha zeyheri* as shown by a local traditional healer (Sigidi Muendi, 2014)

### 2.3.2.7 *Elephantorrhiza elephantina*

Commonly known as *Gumululo* among the Vhavenda, *E. elephantina* has several unbranched, annual stems growing from an enormous underground rhizome (Figure 2.9). The leaves have numerous small, narrow leaflets and clusters of small, cream flowers. This plant is used as a traditional remedy for a wide range of ailments, including diarrhoea, dysentery, stomach disorders, haemorrhoids and peptic ulcers (Watt *et al.*, 1962; Pujol, 1990).



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*Elephantorrhiza* sp rhizomes are rich in tannins and are also known to contain 4-O-ethylgallic acid (Mehta *et al.*, 1988). The activity of the extract is not clear; however tannins are known as antidiarrheal and antiseptics and to be effective in the treatment of infectious diarrhoea (Bruneton, 1995). As an antibacterial and antifungal ointments, they are useful in the treatment of dermatitis and enhances tissue regeneration by forming a protective layer on the skin and mucosa (Bruneton, 1995).

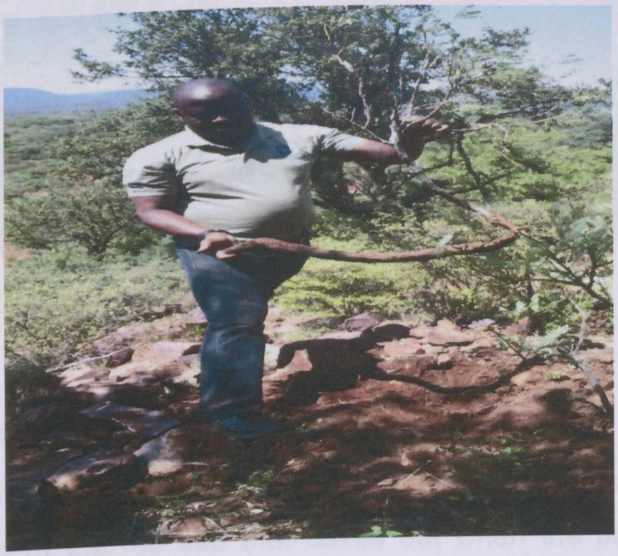


**Figure 2.9:** Rhizome of *Elephantorrhiza elephantina* (Sigidi Muendi, 2014)

### 2.3.2.8 *Coccinia rehmannii*

*Coccinia rehmannii* is a non-woody, climbing or scrambling herb, producing stems up to 5 metres long from a large rootstock (Figure 2.10). The slender stems scramble over the ground or clamber into surrounding vegetation, attaching themselves by means of tendrils. The plant is sometimes harvested from the wild for its edible roots and fruit, which are eaten locally. Local traditional healers also prepare concoctions from the bulbs/roots for treatment of different ailments (information from interviews).



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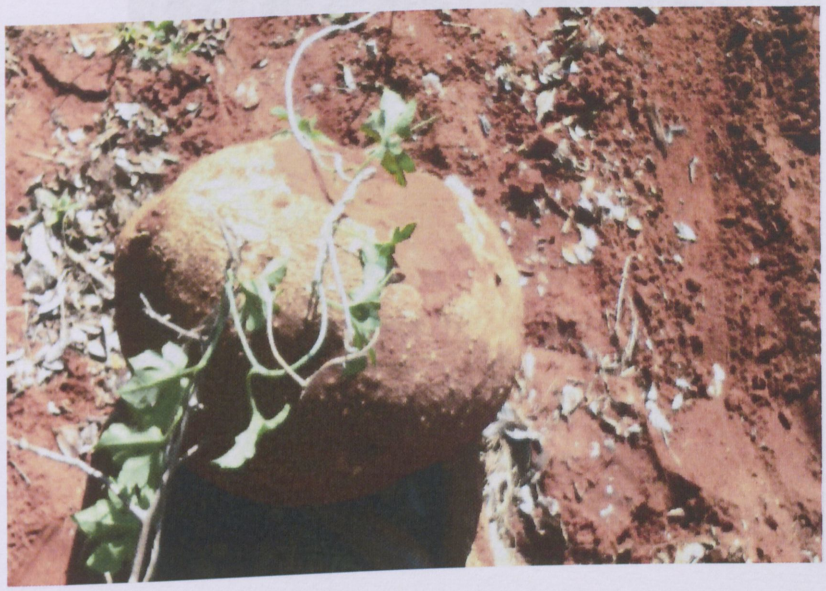


**Figure 2.10:** Bulb of *Coccinia rehmannii* (Sigidi Muendi, 2014)

### 2.3.2.9 *Pterocarpus angolensis*

*Pterocarpus angolensis* is a sweetly-scented tree, also known by the name 'bleed wood tree' because of the red sap that oozes out when the bark is cut (Figure 2.11). Its flowers blossom in spring, while in autumn the long-dangling leaves adopt a dark yellow colour (Palgrave, 2002; Takawira-Nyenya, 2005). It is commonly known as *Mutondo* in Tshivenda. The tree has a flattened crown consisting of large leaves that are separated in a feathery fashion. The stem has good quality wood, with varying colour from light brown to red or coppery-brown and is fissured (Palgrave, 2002). The seed pods of *Mutondo* are circular in shape surrounded by brownish wings which are borne in hanging clusters (Palgrave, 2002).



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**Figure 2.11:** Red sap oozing from bark of *Pterocarpus angolensis (egyptwn.com)*

The red-sticky sap from the tree is used in the manufacturing of dyes and has several medicinal properties (Van der Reit *et al.*, 1998). A matured tree has many uses and is of great value in the African continent at large. Its timber is used for furniture, implements, curios and canoe designs (Shackleton, 2002; Takawira-Nyenya, 2005). The red sap is mixed with animal fat to beautify women's faces and bodies. It is also believed to have supernatural properties for cleansing blood. Several ailments have been reported as being treated by the ingestion of a concotion of *Mutondo,* such as, fungal infections, eye problems, fever and stomach cramps (from interviews).

### *2.3.2.10 Ziziphus mucronata*

*Ziziphus mucronata* is a widely used medicinal plant, commonly known as *Mukhalu* in Tshivenda. It is an average-sized tree and its branchlets are widely spread. The bark is rough, brown and bears sharp thorns on its twigs (Figure 2.12). Its leaves have an intense green colour and are shiny with a characteristic yellowish-green colour borne in clusters (Hutchings *et al.*, 1996; Foden and Potter, 2005; Raimondo *et al.*, 2009).



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*Z. mucronata* fruits are small, rounded berries which become reddish-brown when ripe. Warm bark decoctions are used in the treatment of cough and chest problems and root decoctions are used in treatment of diarrhoea and dysentery (Rood, 2008).

A combination of root and leaves decoction can be applied topically to treat boils, sores and glandular swellings. The decoction is also taken orally to alleviate inflammation (Hutchings *et al.*, 1996; Rood, 2008).



**Figure 2.12:** Sharp thorns of *Ziziphus mucronata* on slender branches (www.ispot.org.za)

### 2.3.3 Yield of plants

The results in Figure 2.13 shows the extraction yield after rotary evaporation. The 10 plants yielded varying grams with *P. angolensis* yielding the highest followed by *E. transvaalense* and *C. abbreviatta*.



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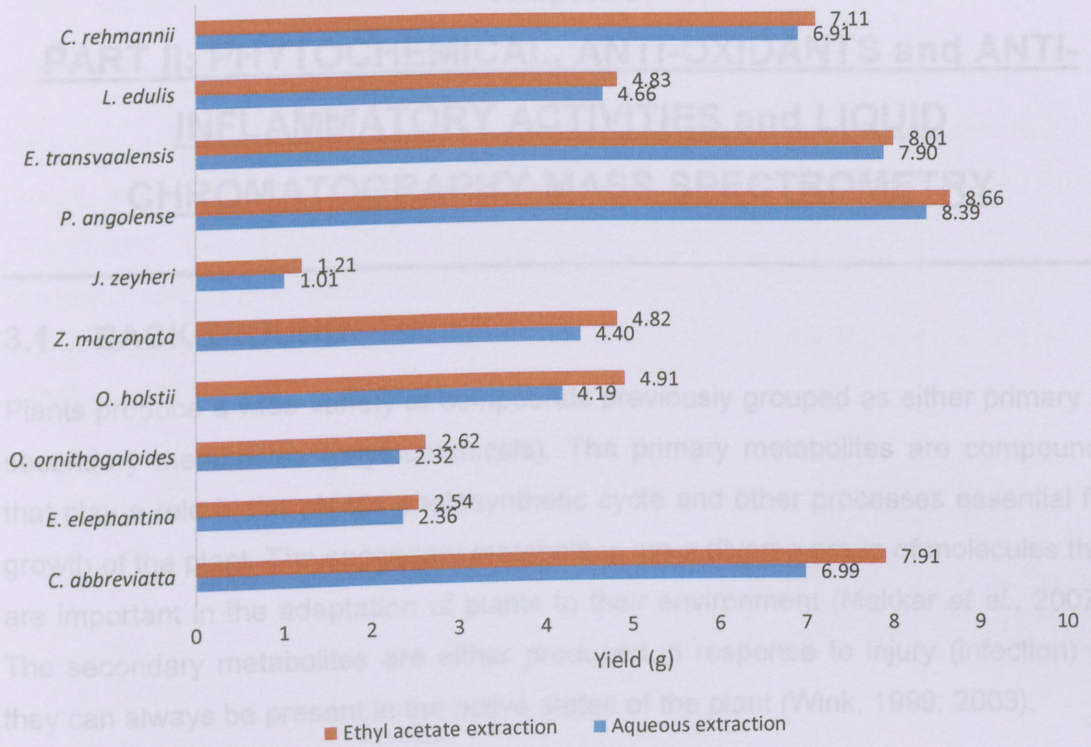



**Figure 2.13:** Crude plant yield as measured in grams

## 2.4 CONCLUSION

The knowledge for use of Venda medicinal plants still requires extensive research. The diversity of medicinal plant species that grows particularly in the North Eastern of SA has proven to be extensive. Traditional healers of the Vhembe District are knowledgeable with regards to indigenous Venda medicinal plants and treatment of various infections. The healers acknowledged inheriting the healing practise from their grandparents. From the conducted interviews, the recorded plant species were from 29 plant families, some with no frequent use in treating human ailments thus raising questions on their effectiveness. The 10 selected plants were associated with blood cleansing since the interest was based on investigating plants capable of healing HIV/AIDS and/or its symptoms.



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# Chapter 3

# PART II: PHYTOCHEMICAL, ANTI-OXIDANTS and ANTI-INFLAMMATORY ACTIVITIES and LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

## 3.1 BACKGROUND

Plants produce a wide variety of compounds previously grouped as either primary or secondary metabolites (phytochemicals). The primary metabolites are compounds that play a role in the plant's photosynthetic cycle and other processes essential for growth of the plant. The secondary metabolites are a diverse group of molecules that are important in the adaptation of plants to their environment (Makkar *et al.*, 2007). The secondary metabolites are either produced in response to injury (infection) or they can always be present in the active states of the plant (Wink, 1999; 2003).

Plants have over the years remodelled secondary biochemical pathways to manufacture a range of chemicals, often responding to specific environmental stimuli. Examples of such stimuli are, herbivore-induced damages, attacks by microbial pathogens, or nutrient deficiencies (Kennedy and Wightman, 2011). The most frequent and essential phytochemical groups include the terpenes, alkaloids, and phenolic compounds (Kennedy and Wightman, 2011).

Phytochemicals in traditional medicine have diverse biological activities. They are also used in the production of valuable synthetic compounds and are viewed as potential sources of new natural drugs like antibiotics, insecticides and herbicides (Bourgaud *et al.*, 2001; Crozier *et al.*, 2006; Shahidi, 2004; Shahidi *et al.*, 2008). Ingestion of some plant extracts (antioxidants) have been reported to reduce risks of chronic diseases, such as cancer, heart attack and diabetes (Zhu *et al.*, 2009).



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## 3.2 DESCRIPTION OF THE METABOLITES

### 3.2.1 Saponins

Saponins are abundantly available in higher plants and are mostly prevalent in roots, tubers, leaves and seeds. Based on their carbon skeletons, saponins are usually categorized into triterpenes and steroids. Their glycone parts are mainly oligosaccharides arranged either in a sequential manner or branched fashion (Figure 3.1) and adhered to it is a hydroxyl group through an acetal linkage (Sparg *et al.*, 2004).

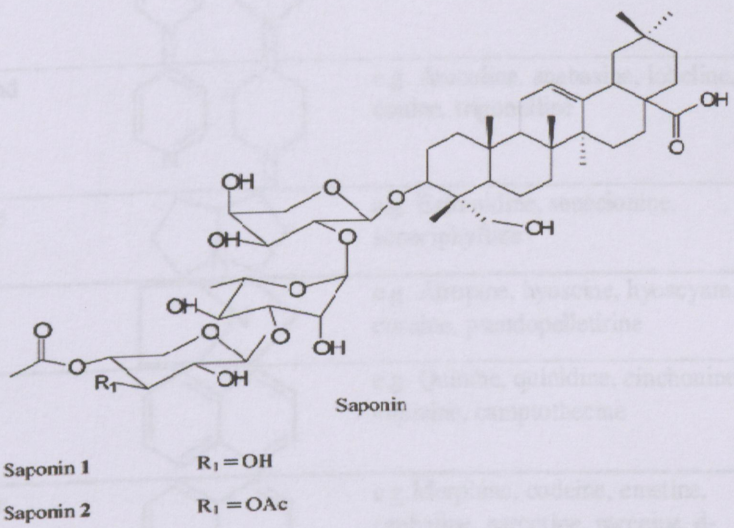



**Figure 3.1:** Chemical structure of saponins (www.scielo.br/scielo.php?script)

These compounds dissolve in water to form an evenly suspended mixture that foams upon shaking. Saponins are in great demand in the pharmaceutical industry because some of them form the basis for artificially manufactured drugs. Several have pharmacological effects and are used in phototherapy and in the cosmetic industries. They are believed to form the main element for several plant drugs and herbal remedies and are reported to possess many pharmacological effects (Estrada *et al.*, 2000).

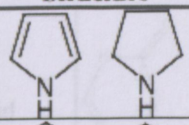
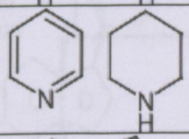
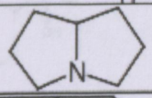
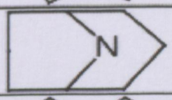
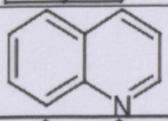
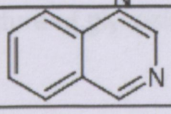


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### 3.2.2 Alkaloids

Alkaloids are a group of extremely diverse compounds which contain a ring structure and a nitrogen atom (Table 3.1). The location of the nitrogen atom is within the heterocyclic ring structure (Lu *et al.*, 2011). Alkaloids' presences are diverse in the plant kingdom and are much more abundant in plants with relatively complex characteristics (Lu *et al.*, 2011).

**Table 3.1:** Classification of alkaloids (https://image.slidesharecdn.com/alkaloidsars)

| Type | Structure | Examples |
|---|---|---|
| Pyrrole and pyrrolidine |  | e.g. Hygrine, coca species |
| Pyiridine and piperidine |  | e.g. Arecoline, anabasine, lobeline, conine, trigonelline |
| Pyrrolizdine |  | e.g. Echimidine, senecionine, seneciphylline |
| Tropane |  | e.g. Atropine, hyoscine, hyoscyamine, cocaine, pseudopelletirine |
| Quinoline |  | e.g. Quinine, quinidine, cinchonine, cupreine, camptothecine |
| Isoquinoline |  | e.g.Morphine, codeine, emetine, cephaline, narcotine, narceine, d-tubocurarine |

Furthermore, several alkaloids have shown some biological activities, such as the alleviation of asthma symptoms as well as pain-relieving and anticancer effects (Lu *et al.*, 2011). Alkaloids are amongst the most remarkable active constituents in natural remedies, and some of these compounds have already been developed into commercial drugs, such as camptothecin (CPT) a topoisomerase I inhibitor (Huang *et al.*, 2007).



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### 3.2.3 Glycoside

Glycosides are compounds that produce one or more sugars when hydrolysed. They are established from a simple sugar and another compound by substituting the hydroxyl group within the water molecule (Heinrich *et al.*, 2012). Thus glycoside comprises of two parts (Figure 3.2): the sugar moiety and the aglycone which may be any natural product (terpenes, and flavonoid).

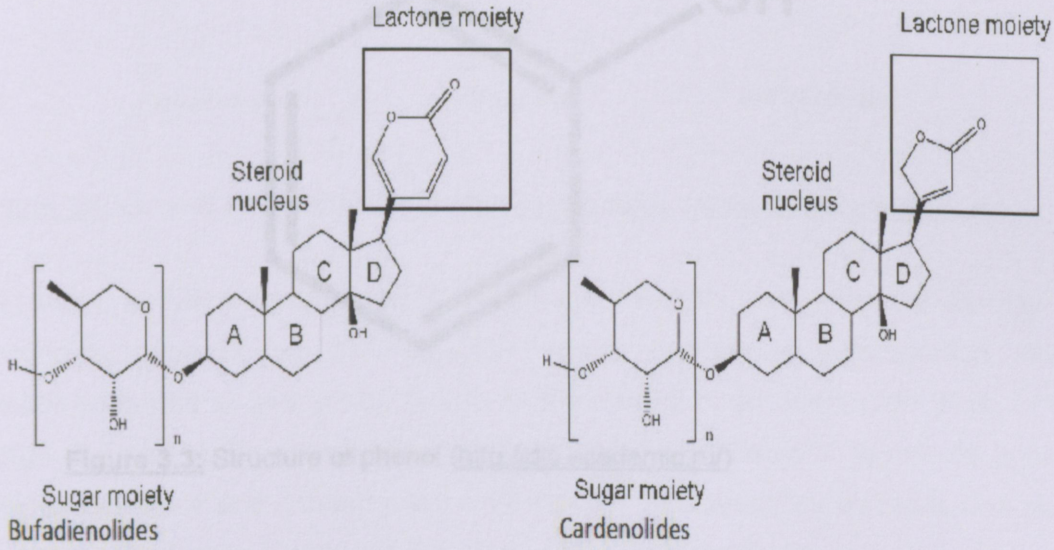



**Figure 3.2:** Chemical structure of glycosides (www.intechopen.com)

### 3.2.4 Phenols

One of the most significant groups of all these metabolites is the phenolic compounds. They are distinguished by the presence of at least one aromatic ring (Figure 3.3) with one or more hydroxyl groups. They are mostly synthesized from cinnamic acid, which is formed by the action of enzyme L-phenyloalanine ammonia-lyase PAL (EC 4.3.1.5).

Phenols can be placed into several groups, depending on the constitutive number of carbon atoms in combination with the structure of the basic phenolic skeleton (Rice-Evans *et al.*, 1997; Solecka, 1997). Phenolics have diverse functions in plants and the antidiabetic effects of flavonoids and phenolic acids have been described by several studies (Chaudiere and Ferrari-Iliou, 1999).



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Phenolics have been demonstrated to possess multiple biological activities. Examples include, the treatment of ulcers, reduction of inflammation, inhibition of oxidation and the formation of tumorous growth, fight against malaria and also the reduction of depression symptoms (Araujo and Leon, 2001; Murakami *et al.*, 2004; Ghasemzadeh and Jaafar, 2010).

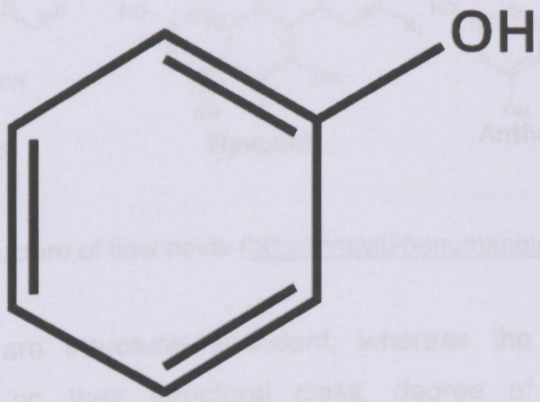



**Figure 3.3:** Structure of phenol (http://dic.academic.ru/)

### 3.2.5 Flavonoids

Flavonoids are primarily benzo-Á-pyrone (phenylchromone) derivatives. They have an enormous group (Figure 3.4) of polyphenolic compounds (Mahomoodally *et al.*, 2005; Pandey, 2007). These antioxidants comprise over 4,000 chemically eccentric and distinct moieties that are found in almost all plants. The largest group mostly comprises of distinct classes such as flavonols, flavans, flavanones and others. Flavonoids are hydroxylated phenolic substances and are known to be synthesized by plants as a defence mechanism in response to microbial infection.



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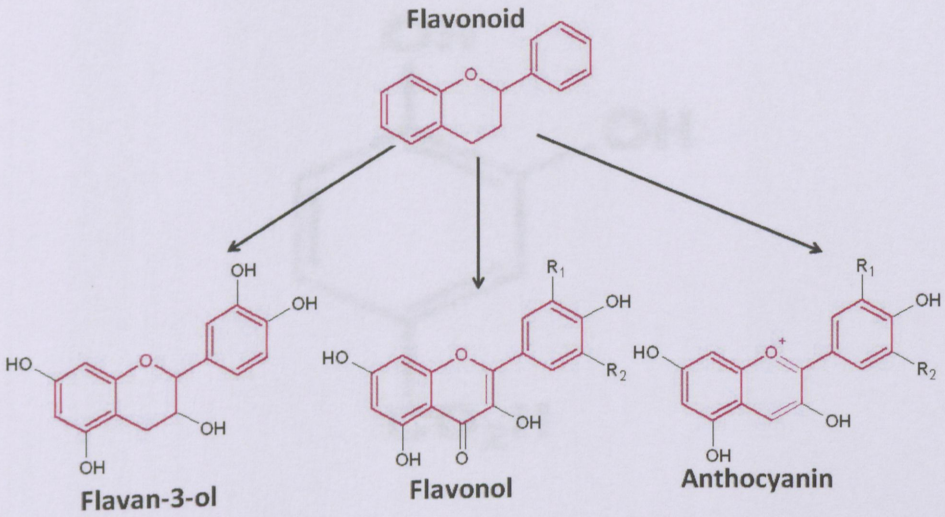



**Figure 3.4:** Generic structure of flavonoids (http://enoviti-hanumangirl.blogspot.co.za/)

Flavonoid activities are structure-dependent, whereas the chemical identity of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and combinations as well as the degree of polymerization (Kelly *et al.*, 2002). Several studies have highlighted the defensive effects of flavonoids against various infections and chronic diseases, such as, cardiovascular diseases and other age-related diseases (Cook and Samman, 1996; Pandey 2007).

### 3.2.6 Protocatechuic acid

It is a dihydrobenzoic acid present in many foods especially plant-derived products (Figure 3.5), hence, has been referred to in the literature as a compound that constitutes a dietary polyphenol (Guglielmi *et al.*, 2003). Several researches have shown the beneficial effects of protocatechuic acid (PCA) which is a major metabolite within the polyphenol group (Table 3.2).



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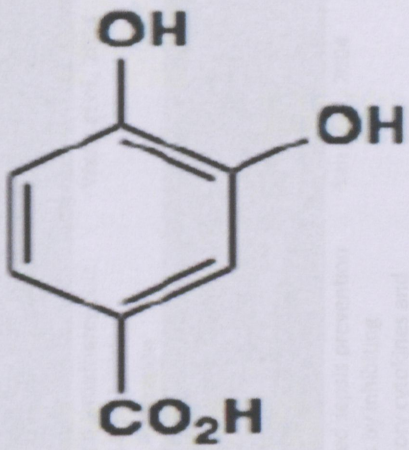



**Figure 3.5:** Chemical structure of protocatechuic acid (https://www.researchgate.net)

### 3.2.7 Caffeic acid

Caffeic acid is an organic compound that falls within the hydroxycinnamic acid (polyphenolic compound) (Figure 3.6). It is present in all plants since it plays a crucial role as an intermediate in the biosynthesis of lignin. Caffeic acid is known to possess both biological and pharmacological properties (Karthjikesan *et al.*, 2007). These include antiviral, antioxidant, anti-inflammatory, anti-carcinogenic and immune-modulatory effects (Kang *et al.*, 2009; Touaibia *et al.*, 2011; Khan *et al.*, 2012; Kim *et al.*, 2014).

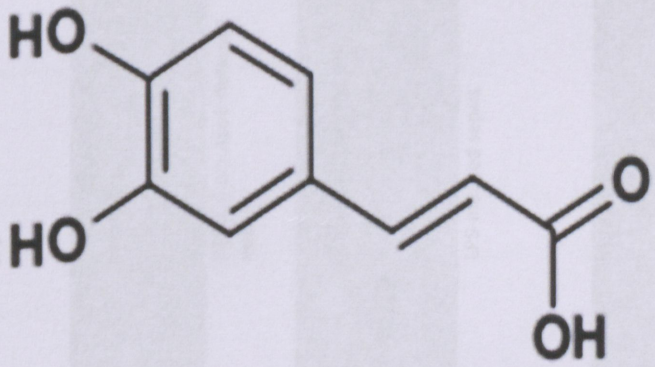



**Figure 3.6:** Structure of caffeic acid (http://www.google.st/patents)



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**Table 3.2:** Summary of the beneficial effects of PCA

| Model | Method | [PCA] | Outcomes | Interpretation | Reference |
|---|---|---|---|---|---|
| **Biochemical assay** | DPPH assay | 0.05 and 0.10 mg/ml | PCA increased % scavenging of DPPH | PCA exerted antioxidant activity | Sroka and Cisowski, 2003 |
| **Raw 264.7 cells** | LPS | 1, 2, 5 and 25µM | PCA decreased TNFα and IL-1, NO and $PGE_2$<br>PCA inhibited iNOS and COX-2 expression | PCA showed anti-inflammatory effects by regulating NF-Kβ and MAPk activation | Min *et al.*, 2010 |
| **Mouse aortic endothelial cells (MAEC)** | TNFα induced cellular damage | 0.05, 0.5, 5.0, 10, 20 and 40µmol/L | PCA inhibited adhesion of HL-60 cells to MAEC<br>PCA suppressed VCMA-1 and CAM-1 mRNA expression<br>PCA reduced NF-Kβ activation | PCA had an anti-inflammatory effect by inhibiting monocyte adhesion molecule | Wang *et al.*, 2010 |
| **Cell culture** | PBMC from ApoE-deficient mice | 0.125, 0.25 and 0.5µmol/L | PCA decreased CCR2 protein and mRNA expression<br>PCA inhibited mouse PBMC migration | PCA exerted antiatherogenic properties by inhibiting monocyte infiltration | Wang *et al.*, 2011 |
| **Mice** | Carrageenan induced inflammation in mice | 5 and 25mg/kg per 24h | PCA reduced exudate<br>PCA decreased protein content<br>PCA reduced leukocyte NO<br>PCa inhibited TNFα, IL-1β and $PGE_2$ level<br>PCA inhibited COX2 and NF-Kβ expression | PCA exerted anti-inflammatory effects by inhibiting NF-Kβ activation | Min *et al.*, 2010 |
| **Mice** | LPS-induced sepsis | 50mg/kg single dose | PCA reduced lethality<br>PCA suppressed TNFα and IL-10<br>PCA decreased plasma ALT levels<br>PCA decreased nitrate/nitrite levels<br>PCA decreased hepatic MDA levels | PCA exerted sepsis prevention properties by inhibiting inflammatory cytokines and antioxidant activity | Yan *et al.*, 2004 |
| **Mice** | STZ-induced DM (50mg/kg/iv) | 1%, 2% and 4% in diet for 8 weeks | PCA lowered plasma PAI-1 levels<br>PCA increased APIII levels<br>PCA increased protein C levels<br>PCA lowered plasma CRP levels<br>PCA lowered plasma van Willebrand factor<br>PCA reduced IL-6, TNFα and MCP-1 levels in heart and kidney | PCA exerted anti-coagulatory and anti-inflammatory effects by lowering inflammatory cytokines | Li *et al.*, 1993 |

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A study done by Li *et al* (2015) indicated that low levels of caffeic acid act as a hormesis stimuli in the improvement of cell viability as well as a protection against DNA damage inflicted by hydrogen peroxide ($H_2O_2$).

Similar results reported by Karthikesan *et al* (2007) and Kim *et al* (2014) suggest that caffeic acid has antioxidant properties and tremendous health benefits.

### 3.2.8 Gallic acid

It is an organic trihydroxybenzoic acid (Figure 3.7) found in most foods and several herbal remedies. Gallic acid is widely used as an antioxidant to prevent fats and oil spoilage (Yen *et al.*, 2002). Several studies have reported on gallic acid as exhibiting free-radical scavenging activity (Polewski *et al.*, 2002), and an anti-proliferative effect on cancer cells (Lapidot *et al.*, 2002).

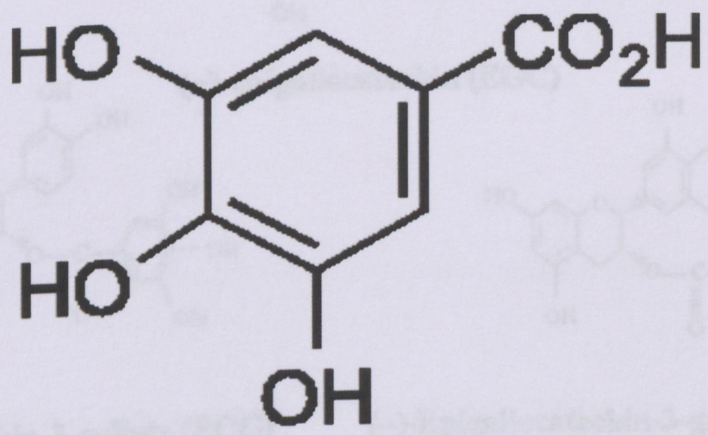



**Figure 3.7:** Structure of gallic acid (www.sigmaaldrich.com)

A research conducted by Rasool *et al.* (2010), showed that gallic acid also possess hepatoprotective effects in paracetamol-intoxicated mice. This could be attributed to its antioxidant activity.



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### 3.2.9 Catechins

Catechins are a type of antioxidant compound found especially in green tea. It is a secondary plant metabolite belonging to the flavonoid group (Figure 3.8). There are several types of catechins - epicatechin (EC), epigallocatechin (EGC) epicatechin gallate (ECG) and epigallocatechin gallate (EGCG). All these catechins have high free-radical scavenging activity, with ECG being the most active.

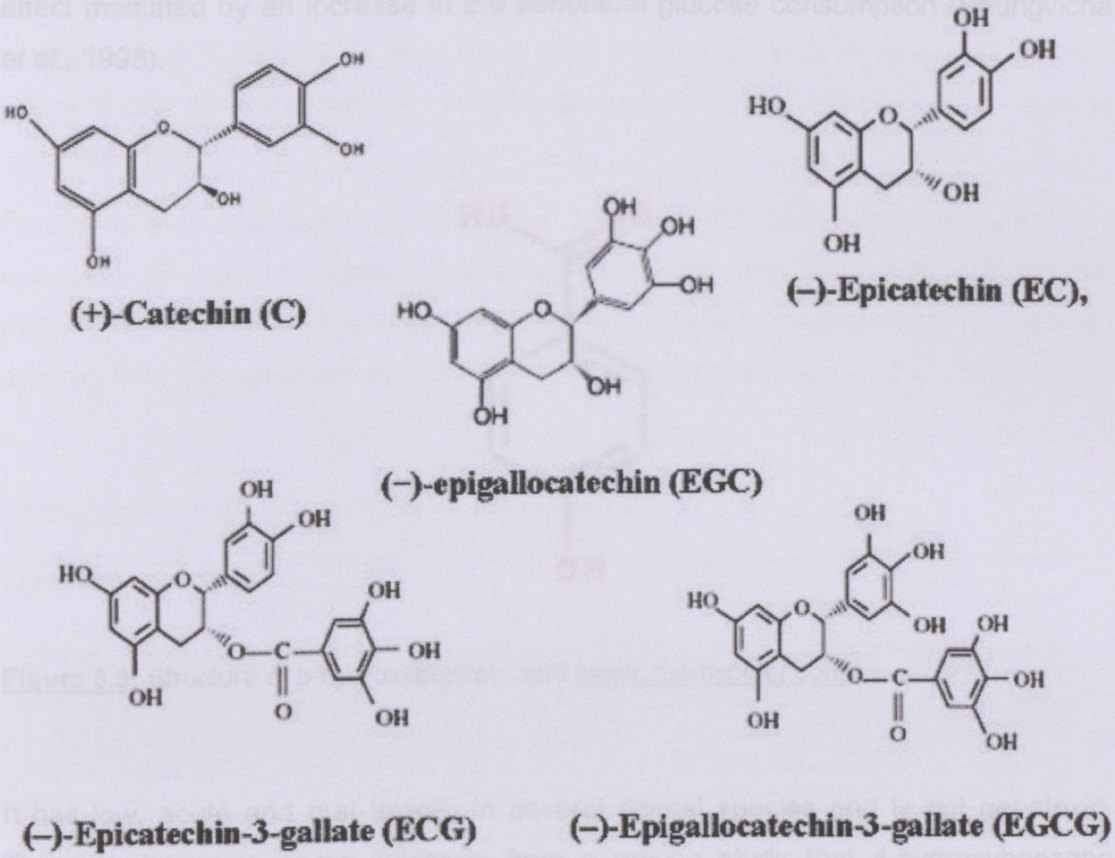



**Figure 3.8:** Chemical structure of different catechin molecules (www.researchgate.net)

Catechins have been shown to possess cytotoxic effects since they inhibited cancer (HCT116) cell growth (Du *et al.*, 2012), to be effective in suppressing hepatocyte growth-factor receptor activation in human colon cancer cells (Larsen *et al.*, 2009), they also inhibit proliferation of leukemic cells, and is an effective cytotoxic and apoptosis-inducing agent (Azam *et al.*, 2004) and those found in tea have been reported to scavenge a variety of free radicals *in vitro* like ROS, although *in vivo* the antioxidant capacity is equivocal.



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### 3.2.10 p-hydroxybenzoic acid

p-hydroxybenzoic acid (Figure 3.9), also known as 4-hydroxybenzoic acid is a widely used industrial chemical. It is used as an intermediate in the production of dyes, pesticides, cosmetics and drugs. P-hydrobenzoic acid (pHBA) is a solid compound with a low vapour pressure, a high melting point and low solubility in water. A study conducted has concluded that 4-hydroxybenzoic acid produces a hypoglycaemic effect mediated by an increase in the peripheral glucose consumption (Peungvicha *et al.*, 1998).

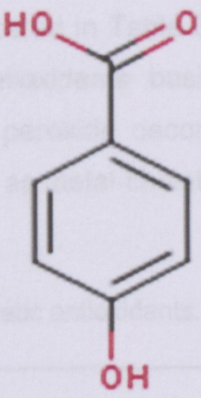



**Figure 3.9:** Structure of p-hydroxybenzoic acid (www.chemspider.com).

It has low, acute and oral toxicity in several animal species and is not genotoxic. There is, however, some evidence from a mouse study that 4-hydroxybenzene demonstrated a mild estrogenic response (Lemini *et al.*, 1997).

## 3.3 ANTI-OXIDANT PROPERTIES OF PLANTS

The term 'anti-oxidant' was initially used when referring to a specific chemical that prevented the consumption of oxygen (Matill, 1947). Antioxidants are considered as crucial nutraceuticals with regards to the role they play on many health issues (Droge, 2002, Lee *et al.*, 2004). They are fairly simple chemicals that interact and neutralize free radicals.



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These chemicals are also known as 'free-radical scavengers'. The human body produces some of the antioxidants it uses for neutralizing free radicals naturally; such antioxidants are referred to as 'endogenous anti-oxidants'. The same human body, however requires an intake of exogenous sources (dietary antioxidants) as well (Diplock *et al.*, 1998).

The sources of such external antioxidants include certain fruits, vegetables and grains (Diplock *et al.*, 1998; Bouayed *et al.*, 2010). Anti-oxidants are known to be stable enough to donate an electron to a free radical in order to neutralize it, thus, reducing any potential damage. These chemicals, in a way, inhibit cellular damage (Halliwell, 1995).

Examples of anti-oxidants as indicated in Table 3.3 are: glutathione, ubiquinol, and uric acid (Shi *et al.*, 1999). Antioxidants basically act as radical scavengers, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist as well as metal-chelating agents (Frie *et al.*, 1988).

**Table 3.3:** Enzymatic and non-enzymatic antioxidants.

| | |
|---|---|
| Enzymatic antioxidants | |
| **Superoxide dismutase** | Catalyzes breakdown of superoxide anion into oxygen and hydrogen peroxide (Banniste *et al.*, 1987; Zelko *et al.*, 2002). |
| **Catalase** | Catalyze the decomposition of hydrogen peroxide to water and oxygen (Chelikani *et al.*, 2004). |
| **Glutathione system** | Catalyze the breakdown of hydrogen peroxide and organic hydroperoxides. |
| Non-enzymatic antioxidants | |
| **Ascorbic acid (Vit C)** | Neutralizes ROS (Padayatty *et al.*, 2003). |
| **Glutathione** | thiol group can be reversibly oxidized and reduced |
| **Melatonin** | Terminal antioxidant that forms stable end products upon reacting with free radicals (Tax *et al.*, 2000). |
| **Tocopherols and tocotrienols (Vitamin E)** | Protects membranes from oxidation by reacting with lipid radicals (Traber and Atkinson, 2007). |
| **Uric acid** | Accounts for half the antioxidant ability of plasma (Jaeschke *et al.*, 2002). |



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### 3.3.1 Free radicals

Free radicals are molecular species that are highly reactive and with a great potential of damaging cells (Table 3.4). These radicals are created when an atom or molecule either gains or loses an electron. The presence or loss of an electron is a common property shared by most radicals. These molecules can either behave as oxidants or reductants by donating and accepting electrons from other molecules (Valko *et al.*, 2007).

The free radicals are formed naturally in the human body and are important in various cellular processes (Diplock *et al.*, 1998; Valko *et al.*, 2007). At very high concentrations, free radicals pose a serious threat to the body and may damage all major cell components including DNA, proteins and cell membranes, leading to possible development of cancer and other chronic health conditions (Diplock *et al.*, 1998; Valko *et al.*, 2007).

Attack of macromolecules by the free radicals can lead to homeostatic disruption. Free radicals, as well as other ROS are derived from normal metabolic processes in the human body or may be from external sources (Losada-Barreiro and Bravo-Diaz, 2017)

**Table 3.4:** Sources of free radicals

| Internally generated sources of free radicals | Externally generated sources of free radicals |
|---|---|
| **Xanthine oxidase** | Environmental pollutants |
| **Peroxisomes** | Radiation |
| **Inflammation** | Certain drugs, pesticides |
| **Phagocytosis** | Industrial solvents |
| **Arachidonate pathways** | Ozone |
| **Exercise** | |
| **Ischemia/reperfusion injury** | |

Cell damage due to free radicals appears to be significant in the aging process and they have also been implicated in the pathogenesis of over 50 diseases. Oxidative stress is the condition that results when the balance between free radical generation and antioxidant defences becomes unfavourable (Fu *et al.*, 2017).



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This imbalance affects different molecular species including lipids, proteins as well as nucleic acids (Rock *et al.*, 1996; Mc Cord, 2000). Oxidative stress has been implicated in conditions like atherosclerosis, inflammatory diseases (arthritis, vasculitis) and different cancers, acquired immunodeficiency virus (AIDS), neurological disorders (Alzheimers' disease, Parkinson's disease) to name but a few (Stefanis *et al.*, 1997).

Antioxidants can reduce oxidative stress-induced carcinogenesis by either the scavenging of ROS or by inhibiting cell proliferation. Research has shown that some of the nutritional antioxidants retards the aging process and prevents diseases (Cantuti-Castelvetri *et al.*, 2000).

## 3.4 ROLE OF PLANT EXTRACTS IN INFLAMMATION

Inflammation, the immune response of body tissues to injury or infection, is an important component of innate immunity. The inflammatory process involves a complex biological cascade of molecular and cellular signals that alter physiological responses, ultimately resulting in the familiar clinical symptoms of pain, swelling, heat, and redness of the affected area (Calder, 2006; Libby, 2007).

At the site of the injury, cells release molecular signals that cause a number of changes in the affected area: vasodilation, increased blood flow, increased vascular permeability, exudation of fluids containing proteins like antibodies and invasion by several types of leukocytes, including granulocytes, monocytes, and lymphocytes (Figure 3.10) (Scott *et al.*, 2004).

Inflammation is either acute or chronic. Acute inflammation may be an initial response of the body to harmful stimuli while in chronic inflammation, the inflammatory response is out of proportion resulting in damage to the body.

Cyclooxygenase (COX) is the key enzymes in the synthesis of prostaglandins, prostacyclin's and thromboxane's which are involved in inflammation, pain and platelet aggregation (Pilotto *et al.*, 2010). Inflammatory diseases are a major worldwide problem.



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Neutrophils are the first leukocytes to appear at the site of injury. These cells phagocytize and kill invading microorganisms through the release of non-specific toxins, such as superoxide radicals, hypochlorite, and hydroxyl radicals; these reactive oxygen species (ROS) kill pathogens as well as adjacent cells, as well as, sick and healthy cells in a similar manner.

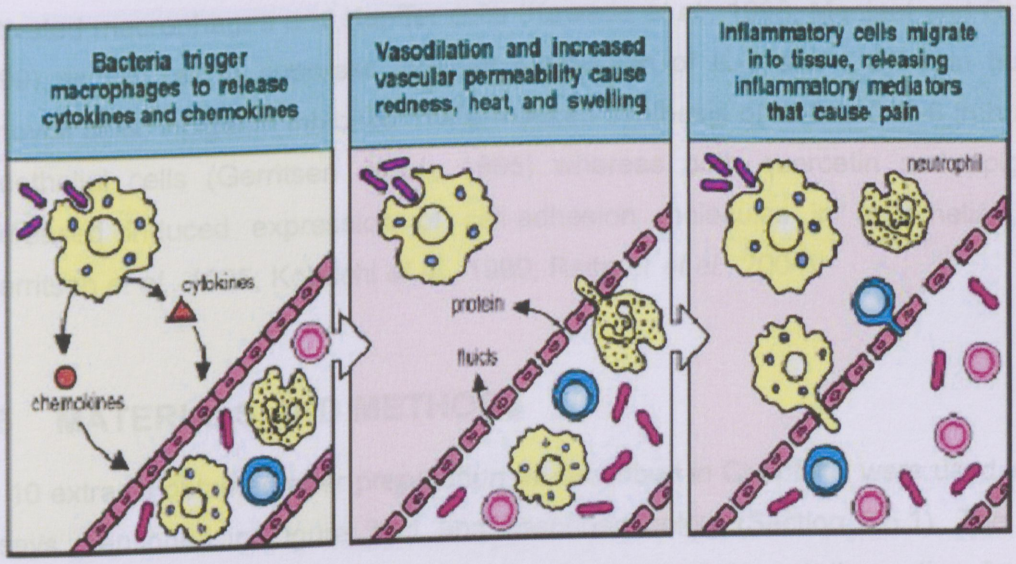



**Figure 3.10:** Immune response to foreign particles (intranet.tdmu.edu.ua)

Neutrophils also cause additional killing activities by releasing antimicrobial peptides and proteins. Neutrophils also release cytokines, including interleukin (IL)-1, IL-6, tumour necrosis factor (TNF)-α, γ interferon (INF-γ), and others (Feghali and Wright, 1997; Scott *et al.*, 2004). These pro-inflammatory cytokines in turn induce the liver to synthesize various acute phase reactant proteins and also induce systemic inflammatory responses (Feghali and Wright, 1997).

Preliminary results regarding the anti-inflammatory potential of plant extracts and plant-derived compounds are usually acquired from *in vitro* studies utilizing cultures of cells involved in inflammatory processes such as cells of monocyte origin, models of endothelial cells and hepatocytes. This kind of research comprises experiments that determine the abilities of the compounds to limit the synthesis of common



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mediators of inflammation exemplified by pro-inflammatory cytokines, chemokines, cell adhesion molecules or nitric oxide (Kris-Etherton *et al.*, 2004).

Several dietary plants-derived compounds showing anti-inflammatory activities have already been identified. Among the best identified are flavonoids, quercetin and apigenin, belonging to a large family of polyphenols.

Quercetin was shown to significantly inhibit TNFα and nitric oxide synthesis in LPS-activated macrophages and Kupffer cells (Kawada *et al.*, 1998; Manjeet and Ghosh, 1999) as well as to suppress induced expression of IL-8 and MCP-1 in human synovial cells. Apigenin inhibited TNFα-induced synthesis of IL-6 and IL-8 in human endothelial cells (Gerritsen *et al.*, 1995) whereas both quercetin and apigenin decreased induced expression of cell-adhesion molecules in endothelial cells (Gerritsen *et al.*, 1995; Kobuchi *et al.*, 1999; Reiterer *et al.*, 2004).

## 3.5 MATERIALS AND METHODS

All 10 extracts obtained after preparation as described in Chapter 2 were used in the assays mentioned in Figure 3.11 and described below (Section 3.5.1). The best extract commonly used was fractioned and re-tested to get the active fraction (Section 3.5.2).

### 3.5.1 Assays on Crude plant extracts

#### *3.5.1.1 Phytochemical analysis*

The powdered plant extracts (*C. abbreviata, E. elephantine, L. edulis, C. rehmannii, E. transvaalense, O. ornithogaloides, J. zeyheri* and *O. holstii*) were subjected to different kinds of chemical tests in order to analyse the presence of specific secondary metabolites. These tests were performed using standard procedures as described blow (Brain and Turner, 1975; Harborne, 1992; Evans, 1996).

***Test for Phenols:*** One mililitre of each plant extract was mixed with 2 ml of distilled water followed by a few drops of 10% aqueous ferric chloride solution. Formation of blue or green colour indicated the presence of phenols.



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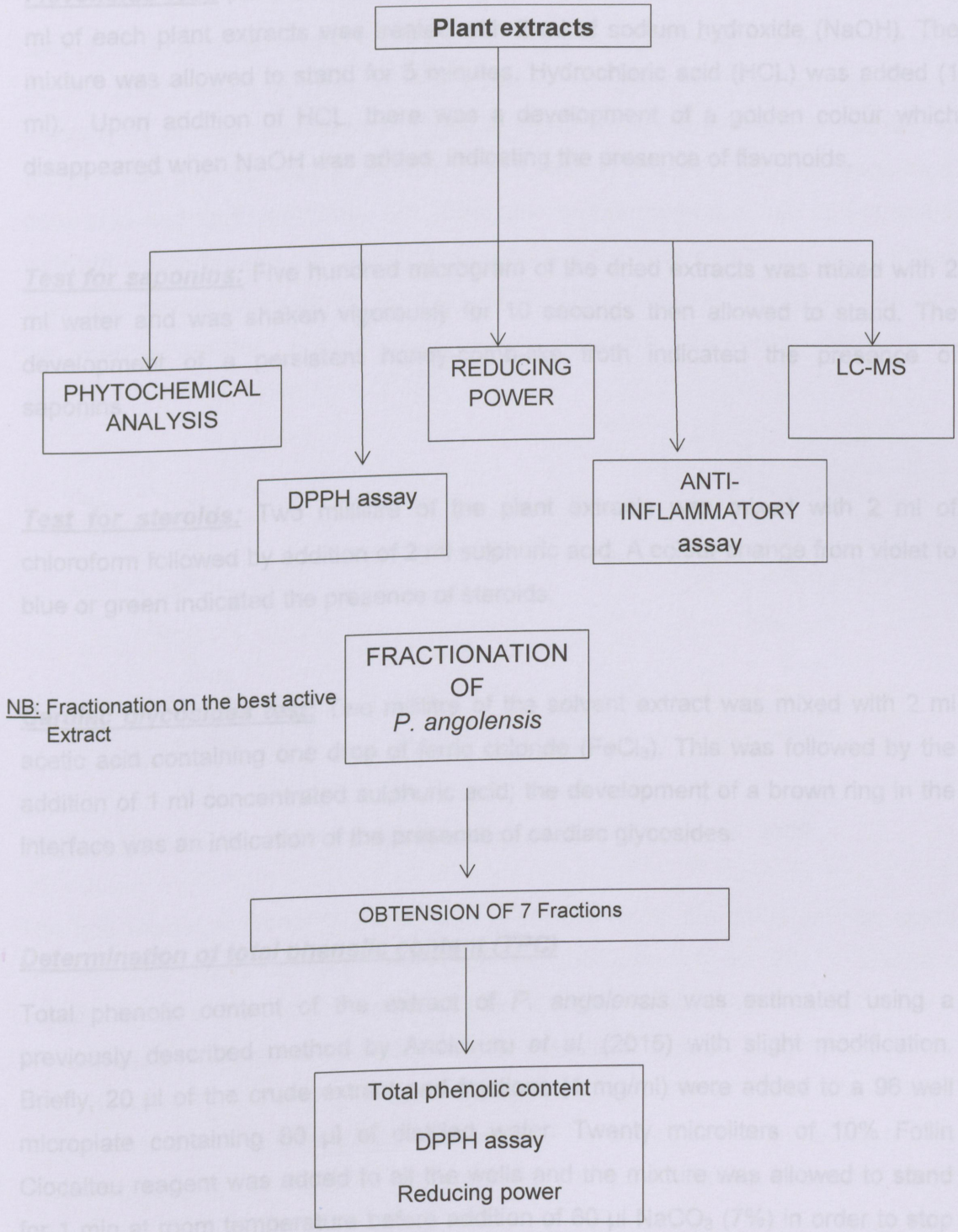



**Figure 3.11:** Flow chart of methods carried out in Chapter 3



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***Flavonoids test:*** plant extracts were diluted with solvent to colourless state, then 1 ml of each plant extracts was treated with 2 ml of sodium hydroxide (NaOH). The mixture was allowed to stand for 5 minutes. Hydrochloric acid (HCL) was added (1 ml). Upon addition of HCL, there was a development of a golden colour which disappeared when NaOH was added, indicating the presence of flavonoids.

***Test for saponins:*** Five hundred microgram of the dried extracts was mixed with 2 ml water and was shaken vigorously for 10 seconds then allowed to stand. The development of a persistent honey-comb-like froth indicated the presence of saponins.

***Test for steroids:*** Two millilitre of the plant extracts was mixed with 2 ml of chloroform followed by addition of 2 ml sulphuric acid. A colour change from violet to blue or green indicated the presence of steroids.

***Cardiac glycosides test:*** Two mililitre of the solvent extract was mixed with 2 ml acetic acid containing one drop of ferric chloride ($FeCl_3$). This was followed by the addition of 1 ml concentrated sulphuric acid; the development of a brown ring in the interface was an indication of the presence of cardiac glycosides.

***Determination of total phenolic content (TPC)***

Total phenolic content of the extract of *P. angolensis* was estimated using a previously described method by Anokwuru *et al.* (2015) with slight modification. Briefly, 20 µl of the crude extract and fractions (1 mg/ml) were added to a 96 well microplate containing 80 µl of distilled water. Twenty microliters of 10% Follin Ciocalteu reagent was added to all the wells and the mixture was allowed to stand for 1 min at room temperature before addition of 60 µl $NaCO_3$ (7%) in order to stop the reaction. Furthermore, 120 µl distilled water was added and immediately incubated for 30 mins before reading at 760 nm. Gallic acid was used as a standard for the calibration curve and the total phenolic content was expressed in mg Gallic Acid Equivalent (GAE)/ g of the extract (Anokwuru *et al.*, 2015).



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### 3.5.1.2 DPPH assay

DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) is a rapid, simple, inexpensive and widely used method to measure the ability of compounds to act as free radical scavengers or hydrogen donors. It is also used to evaluate antioxidant activity of foods. It can also be used to quantify antioxidants in complex biological systems in both solid and liquid samples. The advantage of this method is that DPPH reacts with the whole sample and sufficient incubation time allows DPPH to react slowly even with weaker antioxidants (Prakash, 2001). The principle of the method depends on electron-transfer (Figure 3.12) that produces a yellow solution in ethanol/methanol (Huang *et al.*, 2005).

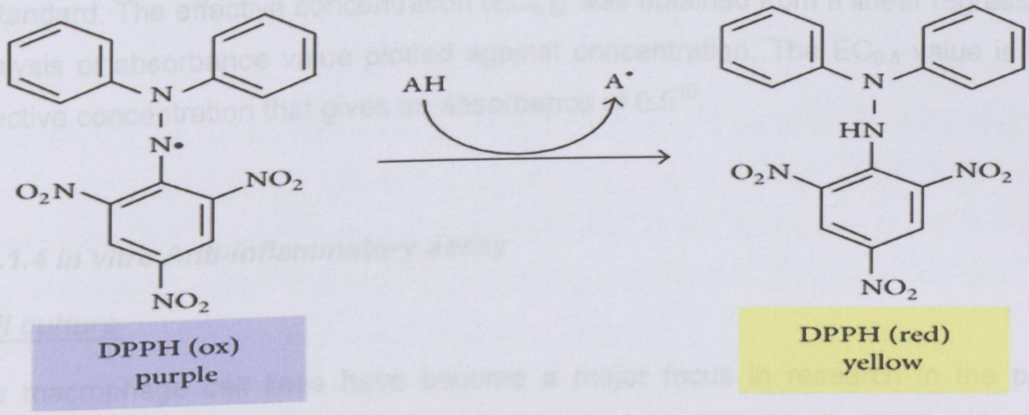



**Figure 3.12:** Principle of DPPH radical scavenging activity (Huang *et al.*, 2005)

The free radical scavenging activity was evaluated for all the crude extracts using DPPH assay as previously described by Anokwuru et al (2017). Different concentartions of the extracts were exposed to DPPH radicals (0,3 mM; 200 µl) in a 96 well plate. The mixture was then allowed to stand at room temperature for 30 mins. Absorbance was read at 517 nm using a microplate reader (Vera max, China). All the extracts were tested in triplicates and gallic acid was used as a standard. The radical scavenging activity (RSA) was calculated using the following equation:

$$\% \text{ RSA} = [(A_{DPPH} - A_s)/A_{DPPH}] \text{ X } 100$$

Where ADPPH is the absorbance of the DPPH solution and As is the absorbance of the samples and DPPH. The ability of the crude plant extracts to inhibit 50% of the free radical (IC50) was extrapolated from a graph of %RSA against concentration.



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### 3.5.1.3 Reducing power

The capacity of the 10 crude plant extracts to reduce $Fe^{3+}$ to $Fe^{2+}$ was assayed by a slightly modified method previously described by Pereira et al (2013). Different plant extract concentrations (0.03-0.5 mg/ml; 50 µl) were mixed with sodium phosphate buffer (0,2 M, pH 6.2; 50 µl) and 50 µl 1% potassium hexacyanoferrate [$K_3Fe(CN)_6$] aqueous solution. The reaction was incubated at 45°C for 20 mins after which 50 µl of 10% trichloroacetic acid was added. From each reaction well, 80 µl was transferred to a new 96 well plate containing an equal volume of deionized water and FeCl3 (0,1%, w/v; 16 µl). Absorbance was read at 690 nm using a microplate reader (Versa max, China). All the extracts were tested in triplicates; gallic acid was used as a standard. The effective concentration ($EC_{0.5}$) was obtained from a linear regression analysis of absorbance value plotted against concentration. The $EC_{0.5}$ value is the effective concentration that gives an absorbance of 0.5[10].

### 3.5.1.4 In vitro Anti-inflammatory assay

***Cell culture***

The macrophage cell lines have become a major focus in research in the past decade some of which are derived from tumours or tumour-like cells. Various macrophage hybridomas have been developed by fusion of various kinds of immortalized cells with normal macrophages. In this study, the RAW 264.7 cell line was used; this is a secondary cell culture established from ascites of a tumour induced in a male mouse by intraperitoneal injection of Abselon Leukemia Virus (Sigma-Aldrich Product Technical Report, 2016). The RAW cells are known to express adenosine receptors and play a critical role in the immune system and inflammatory response, and when activated they secret nitrogen intermediates and pro-inflammatory cytokines (Masek and Hunter, 2013).

Thus the RAW cell line is a great model for a study of the inflammatory process and their stimulation can be achieved using several agents such as lipopolysaccharide (Figure 3.13).



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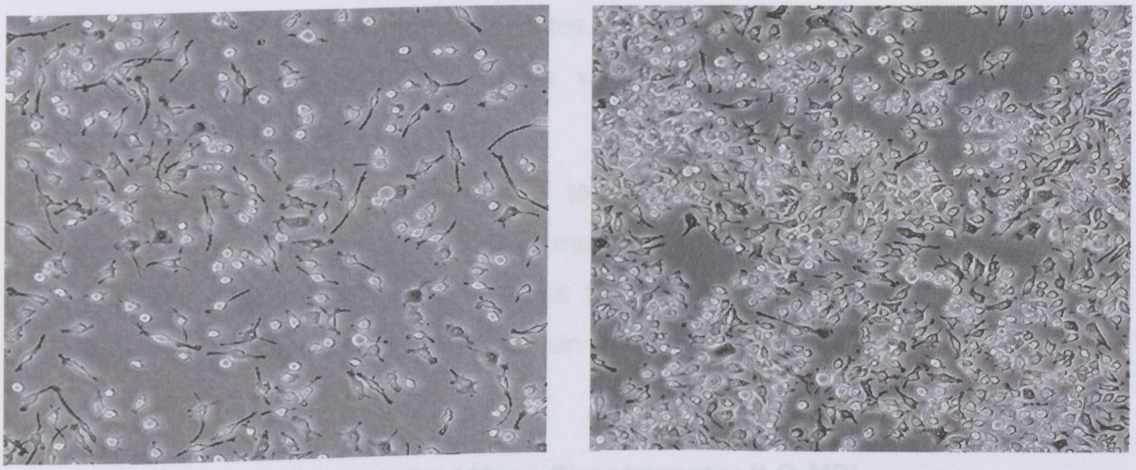


**Figure 3.13:** Microscopic pictures of RAW 264.7 cell line in low and high density (confluent culture)

RAW 264.7 cells were maintained in DMEM (Dulbecco's modified Eagle's Medium plus 10% fetal bovine serum), supplemented by penicillin (100 U/mL) and streptomycin (100 µg/mL), under a temperature of 37 °C, and atmosphere of 5% of $CO_2$ and 90% humidity. The cells were seeded at a density of 25 000 cells per well of the microtiter plates and allowed to attach overnight.

***Dosage of Nitric Oxide***

Nitrite ($NO_2^-$), a stable product from nitric oxide (NO), was used as an indicator of NO production in the culture medium. Nitrite released in the culture medium was measured according to the Griess reaction.

Briefly, RAW cells 264.7 ($1.0\times10^6$ cells/dish) were plated in a 24-well plate overnight. Cells were pre-treated with plant extracts diluted in complete culture DMEM medium at final concentrations of 50 and 100 µg/ml for 1 h, and incubated at 37 °C and 5% $CO_2$. Next, the cells were stimulated with LPS (50 µl/well) for 24 h. A well-known inhibitor of nitric oxide (Aminoguanidine, AG) served as a positive control. For negative control (pro-inflammatory activity), 50 µl of complete medium without LPS was added to the corresponding wells. The plates were further incubated for an hour. Fifty (50) µl of the spent culture medium was transferred to new 96 well plates and an equal amount of Griess reagent added and the plates were incubated for 10 min



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at room temperature. The microtitre plates were subjected to absorbance reading measured at 510 nm and the results were expressed in comparison to the appropriate untreated wells.

Cell viability was assessed using MTT to confirm the absence of toxicity as a contributory factor. Briefly, after LPS-stimulation for 24 h at 37 °C and 5% $CO_2$, 20 µL of MTT was added to the various treatment conditions. Following the 5 h incubation, absorbance was read at 540 nm through ELISA reader.

### *3.5.1.5 Liquid Chromatography / Mass Spectrometry (LC-MS)*

**Description**

LC/MS is a combination of liquid chromatographic separation with mass spectrometric detection. Such a combination gives the chemical analyst the ability to analyse virtually any molecular species. These includes, thermally labile, non-volatile, and high molecular-weight species. Mass spectrometry is capable of providing structure, molecular weight, empirical formula, and quantitative information about a specific analyte (Pitt, 2009).

In general, liquid chromatography (LC) separates the sample components based on differences in their affinity or retention strength for the stationary or mobile phases. Further detection separates components using ultra violet (UV), fluorescence, or electrical conductivity (EC) of the sample properties (Kushnir *et al.*, 2003).

Chromatography offers great resolution, as well as accurately qualifying and quantitating substances that can be difficult if multiple components elute around the same time. In contrast, mass spectrometry (MS) offers a highly sensitive detection technique that ionizes the sample components then separates the resulting ions in vacuum, based on their mass-to-charge ratios and simultaneously measures the intensity of each ion (Taylor *et al.*, 2002).

**Technique**

The presence of PCA, catechin, epicatechin, gallic acid, protocatechuic acid, taxifolin and other componds was determined in the 10 plant extracts using a UPLC-QToF-MS method developed by the Central Analytical Facilities (CAF), University of



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Stellenbosh, South Africa. The method was followed as described in one of our latest published article (Zininga *et al.*, 2017).

### 3.5.2 Fractionation of *P. angolensis*

The crude extract of the most active plant extract (according to in-house results) was subjected to bio-guided fractionation by solubilisation in water and sequential partition hexane and acetyl acetate.

#### *3.5.2.1 Solvent partitioning*

About 500 g of powdered bark extract of *P.angolensis* (prepared in Chapter 2) was macerated in 10 L dichloromethane: methanol (1:1) at 25°C for 24 h. Then, the extract was filtered with Whatman filter No 1 and evaporated with a rotary vapour at 40ºC resulting in 102 g of crude extract. Ninety five gram of the dried extract was subjected to silica gel 60 column chromatography and was eluted with hexane and increasing polarity with ethyl acetate and methanol to obtain 7 fractions as previously described (Anokwuru *et al.,* 2017). Fraction 1 (PaF1) was obtained from 50% hexane, 50% ethyl acetate (1:1), fraction 2 and 3 (PaF2a and 2b) were obtained from 900 ml ethyl acetate, 100 ml methanol (9:1), fractions 4 - 6 (PaF3a, PaF3b, and PaF3c) were obtained from 700 ml ethyl acetate, 100 ml methanol (7:3) while fraction 7 (PaF4) was obtained from 100 ml ethyl acetate, 900 ml methanol (1:9).

#### *3.5.2.2 Tests conducted with the PaF samples*

**Total phenolic content**

As described earlier in Section 3.5.1.1.

**DPPH assay**

As described earlier in Section 3.5.1.2.

**Reducing power**

As described earlier in Section 3.5.1.3.



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### 3.5.3 Data analysis

Results are expressed as mean ± Standard error (SE). The absorbance between experimental conditions was compared by One-Way Analysis Of Variance (ANOVA) while Pearson correlation was used for correlation analysis. All data analysis were performed using SPSS version 23.0.

## 3.6 RESULTS AND DISCUSSION

The extensive use of traditional medicine has highlighted the fact that rural people heavily depend on traditional remedies for treatment of many ailments. The use of such plants could be attributed to their accessibility, affordability (Steenkamp, 2003) and cultural beliefs (Yirga, 2010). For certain villagers, they depend on the medicinal plants because of lack of access to modern medical facilities or lack of effective services within the facilities, if present.

For the continuous discovery of new therapeutic drugs, different plants need to be evaluated for their phytochemical and pharmacological activities (Foye *et al.*, 2008). Different plant components possess active phytochemical constituents and the presence of such constituents could either aid the human body to fight off infections or alleviate the inflammation inflicted on any part of the body.

With the increase in knowledge of the occurrence of free radicals and antioxidants and the necessity for their balance in the human body, this has generated an area of interest for the discovery of effective, non-toxic natural compounds with anti-oxidative activities. The potentially harmful effect posed by an indispensable element of life (oxygen) requires an additional element to neutralize its effects (Bagchi and Puri, 1998; Mohammed and Ibrahim, 2004).

In this part, we are reporting on the results obtained and then discussed them in detail.



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### 3.6.1 Column chromatography for P. angolensis

*Column chromatography of P. angolensis on silica gel yielded 7 fractions (PaF1-4).* PaF3b obtained from 70% ethyl acetate yielded 22.5 g fraction followed by PaF2a obtained from 90% ethyl acetate with 12.8 g. The other fractions yielded less than 7g.

In the current research, fractionation of *P. angolensis* resulted in Paf3b having the highest yield. This result could be attributed to the solubility of more compounds in 70% ethyl acetate 30% methanol. The increased methanol ratio 30% during the column chromatography increased the solubility of more compounds in the crude extract and consequently increasing the yield of fractions. The high extraction yield of PaF3b, however, did not translate to a high phenolic content compared to the other fractions as shown in Table 3.6.

### 3.6.2 Phytochemical analysis

The result of the phytochemical screening of the different plant extracts is presented in Table 3.5. The aqueous and ethyl acetate extracts constituted of similar secondary metabolites with an exception of the lack of saponins in the ethyl acetate extracts.

***Total phenolic content for P. angolensis***

The phenolic contents of the crude extract and subsequent fractions are shown in Table 3.6. The total phenolic content of the crude extract (PaCE) was comparable to those of the fractions except PaF2a which was the lowest in relation to the other fractions.



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**Table 3.5:** Phytochemical screening of the plant extracts

| Constituents | *Cassia abbreviata* | | *Elaeodendron transvaalensis* | | *Ornithogalum ornithogaloides* | | *Ochna holstii* | | *Lannea edulis* | | *Elephantorrhiza elephantina* | | *Coccinia rehmannii* | | *Jatropha zeyheri* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **aq** | **EtAc** | aq | EtAc | aq | EtAc | aq | EtAc | aq | EtAc | aq | EtAc | aq | EtAc | aq | EtAc |
| Phenols | ++ | ++ | + | + | + | + | + | + | ++ | ++ | +++ | +++ | +++ | +++ | ++ | ++ |
| Flavonoids | +++ | ++ | ++ | ++ | +++ | +++ | ++ | ++ | + | + | + | + | +++ | +++ | + | + |
| Saponins | +++ | - | +++ | - | ++ | - | ++ | - | + | - | ++ | - | +++ | - | +++ | - |
| Steroids | +++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | + | ++ | ++ | ++ | ++ | +++ | +++ |
| Cardiac glycoside | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | ++ | ++ | +++ | +++ | +++ | +++ |

+ = low concentration; ++ = moderate concentration; +++ = high concentration; - = absence of phytochemicals

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**Table 3.6:** Total Phenolic Content of the crude extract and fractions of *P. angolensis*

| Extract/ fraction | TPC |
|---|---|
| **PaCE** | 6,74 ±0.01 |
| **PaF1** | 6.53 ±0.03 |
| **PaF2a** | 2.64 ± 0.1 |
| **PaF2b** | 6.64 ±0.01 |
| **PaF3a** | 6.4 ±0.04 |
| **PaF3b** | 6.64 ± 0.1 |
| **PaF3c** | 6.53 ± 0.02 |
| **PaF4** | 6.66 ±0.03 |
| **Gallic acid** | 2.77 ± 0.25 |

Results were recorded as a mean of three replicates

The present study has revealed the presence of active phytochemical constituents as mentioned in Table 3.5. The study revealed that both ethyl acetate and aqueous extracts of the selected medicinal plants contained phenols, flavonoids, steroids and cardiac glycosides. However, saponins were detected only in the aqueous extracts. The extracts showed the presence of rich variety of secondary metabolites with high degree of precipitation (+++) in aqueous extracts as compared to ethyl acetate. The preliminary qualitative phytochemical analysis revealed the presence of secondary metabolites that have previously been reported to have many biological as well as therapeutic properties (Vishnu *et al.*, 2013; Benedee *et al.*, 2013). The presence of such metabolites possibly explains some of the biological effects observed in this study. The plant phenolic compounds have been reported to have several medicinal properties, these includes antimicrobial, anti-inflammatory, anticarcinogenic and antiviral activities (Cushnie and Lamb, 2005; Kandaswami *et al.*, 2005). Flavonoids are known to have activity against cyclooxygenases, lipoxygenases and HIV-1 reverse transcriptase enzyme (Harborne and Williams, 2000; Mathee et al., 1999). The presence of these phytochemicals could explain the HIV-1 RT inhibition, antimycobacterial and anti-inflammatory activities observed with the selected plant



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extracts as shown in Chapter 2. The inability of ethyl acetate extract saponins from the crude material could be that the organic solvent failed to dissolve this metabolite maybe due to its polarity level and the nature of the plant species.

Several other phytochemical constituents were revealed by the LC-MS results as shown in Table 3.11. Protocatechuic acid was detected in all the plant extracts except for *O. ornithogaloides* and *O. holstii*. *Lannea edulis* exhibited the highest levels of PCA. Catechin was present in high concentrations of 29.94 in Z. mucronata and in other extracts it was present, however, in minute quantities. Furthermore, gallic acid was detected in high content in *E. elephantina*. P-hydrobenzoic acid was present at low concentration and epicatechin was detected in *C. abbreviata*, *Z. mucronata* at a higher concentration compared to other extracts. Moreover, the procyanin tetramer, trimer and dimer were only detected in the extract of *L. edulis*. Several studies have reported on gallic acid's possessing of anti-cancer activities against several cancer cell lines like leukemia (Sakaguchi *et al.*, 1998; Inoue *et al.*, 2000). Protocatechuic acid has been shown to possess anti-inflammatory effects which are in agreement with the results observed in this study (Figures 3.15 and 3.16). The presence of catechins might account for the cytotoxic effects on U937 and MeWo cancer cell lines also observed in this study (Figure 4.11).

The diversity of the phytochemicals in the plant extracts accounts for the varying effects they exert, either directly on the pathogens, or indirectly. Research has shown that saponins are well known to possess detergent properties and they exhibit anti-inflammatory properties (Abubakar *et al.*, 2008). Furthermore, cardiac glycosides have been reported to increase the concentration of $Ca^{2+}$ ions by inhibiting the $Na^{+}/K^{+}$ pump. The increase of such ions controls the contraction of the heart muscles and has been employed in the treatment of congestive heart failure (Ngbede *et al.*, 2008).

Moreover, flavonoids have been reported to be highly-potent antioxidant compounds that aid in the reduction of cancer and heart failure (Ghasemzadeh and Ghasemzadeh, 2011). The presence of these metabolites could be attributed to the stimulation of the macrophage cell line to produce nitric oxide, in response to foreign agents in the human body (Figures 3.13 and 3.14).



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### 3.6.3 DPPH assay

The radical-scavenging activity results for the 8 aqueous extracts are exhibited in Table 3.7. The data on DPPH radical scavenging activity with the best known natural and synthetic antioxidant standard (gallic acid) are presented in Table 3.7. The mean IC50 value of *O. ornithogaloides* (51.58 ±4.35) was lower which showed that the radical scavenging activity was shown to be effective. Further, the $IC_{50}$ value of L. edulis showed significant activity (53.25±3.98). Among all the extracts. *C. rehmannii* had the highest $IC_{50}$ value of 101.29±0.28 which indicates its poor scavenging activity. The crude extract of *P. angolensis* (PACE) showed less scavenging activity as compared to the fraction PaF2a. The fraction with the highest $IC_{50}$ value was PaF4 (32.86±0.17) which shows the least scavenging activity amongst the 7 fractions. The concentrations of antioxidant compounds are essential and they decrease the DPPH radicals by $IC_{50}$, this is a parameter used to estimate the antioxidant activity (Krishna *et al.*, 2013). The present study revealed that some extracts had significant scavenging effects on the DPPH radical. The observed high radical scavenging activity could be due to the presence of high flavonoid and phenolic content which is the most required metabolite for scavenging activity.

**Table 3.7:** Radical scavenging activity of the 8 aqueous extracts

| Plant extract | Radical scavenging activity |
|---|---|
| *C. abbreviata* | 54.22 ± 2.31 |
| *E. elephantina* | 70.15 ± 2.62 |
| *O. ornithogaloides* | 51.58 ± 4.35 |
| *O. holstii* | 56.43 ± 0.11 |
| *J. zeyheri* | 70.09 ± 0.86 |
| *E. transvaalense* | 54.35 ± 3.28 |
| *L. edulis* | 53.25 ± 3.98 |
| *C. rehmanni* | 101.29 ± 0.28 |
| Ascorbic acid | 38.87 ± 1.54 |
| Gallic acid | 2.77 ± 0.25 |

Results were recorded as a mean of three replicates



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***Radical scavenging activity of P. angolensis***

Amongst the 7 fractions from *P. angolensis,* PaF2a exhibited the highest radical-scavenging activity while PaF4 showed the lowest activity. Results of the DPPH assay are presented in Table 3.8.

**Table 3.8:** Radical scavenging activity of the crude extract and fractions of *P. angolensis*

| Extract/ fraction | Radical scavenging activity |
|---|---|
| PaCE | 28.82 ± 0.63 |
| PaF2a | 18.70 ± 0.87 |
| PaF2b | 23.79 ± 1.14 |
| PaF3a | 21.71 ± 0.46 |
| PaF3b | 20.06 ± 0.36 |
| PaF3c | 24.31 ± 0.44 |
| PaF4 | 32.86 ± 0.17 |
| Ascorbic acid | 38.87 ± 1.54 |
| Gallic acid | 2.77 ± 0.25 |

Results were recorded as a mean of three replicates

All the extracts presented different levels of DPPH radical scavenging and reducing-power activity. The crude extract of *P. angolensis* exhibited the highest scavenging activity (Table 3.7). The fractionated extracts, however, exhibited much greater activity, compared to the crude, except for Paf4.

*Cocccinia rehmannii* exhibited the least radical-scavenging activity and *L. edulis* showed the highest scavenging activity (Table 3.8). The reducing power assay for the fractions revealed that PaF2b exhibited the highest reducing power and PaF2a with the least activity (Table 3.9). Aqueous extracts of O. holstii exhibited the highest reducing power and J. zeyheri had the least activity (Table 3.10). The ethyl acetate extracts of O. ornithogaloides and C. rehmannii showed the highest and lowest reducing-power activities, respectively. The ethyl acetate extracts of *O.*



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*ornithogaloides* and *O. holstii* showed stronger reducing-power activities of 2.31±0.01 and 2.43±0.32, respectively. Comparison of aqueous and ethyl acetate extracts' reducing-power activities, it can be concluded that the ethyl acetate extracts have better anti-oxidant activities. The varying degree of activities could be attributed to the extraction solvents used, also the diverse phytochemical quantities present in the extracts.

### 3.6.4 Reducing power

The IC50 values are calculated against the gallic acid standard curve (Figure 3.14). Reducing-power activity for the 8 aqueous and ethyl acetate extracts are exhibited in Table 3.9 (reported as $IC_{50}$ values).

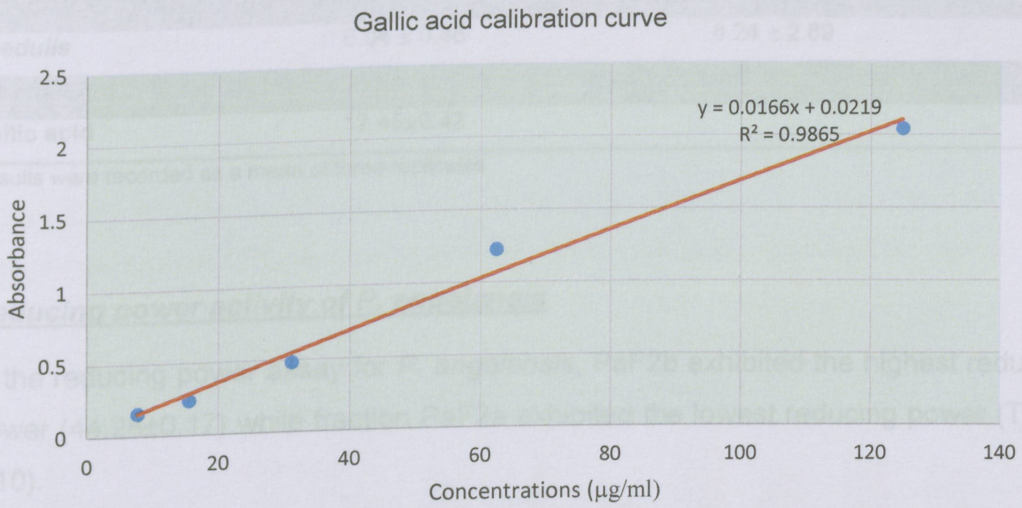



**Figure 3.14:** Calibration curve using Gallic acid

The reducing capacity of *L. edulis* was the highest (6.04±0.46), this activity is comparable to that of the standard. The least reducing power activity was observed in the aqueous extract of *J. zeyheri* (967.57±4.40). The reducing power might serve as a significant indicator of its potential antioxidant activity (Olivera *et al.*, 2008). In this study, yellow colour of the test solution was observed changing to various shades of green and blue. The change shows the presence of reductones which are known to impart antioxidant action by donating a hydrogen atom. This study



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presumed that the crude extract of *L. edulis*, *O. holstii*, *O. ornithogaloides* and *C. abbreviata* may have high amount of reductones hence the antioxidant capability observed.

**Table 3.9:** Reducing-power activity of the 8 aqueous and ethyl acetate extracts

| Plant extract | Reducing power | |
|---|---|---|
| | **Aqueous extracts** | **EtAc extracts** |
| ***C.abbreviata*** | 244 ± 3,6 | 392.17 ± 4.64 |
| ***E. elephantina*** | 643.8 ± 1.75 | 539.04 ± 4.31 |
| ***O. ornithogaloides*** | 18.8 ± 3.78 | 2.31 ± 0.01 |
| ***O. holstii*** | 10.54 ± 1.32 | 2.43 ± 0.32 |
| ***J. zeyheri*** | 967.57 ± 4.40 | 256.37 ± 3.32 |
| ***E. transvaalense*** | 703,33 ± 4,86 | 275.6 ± 4.53 |
| ***L. edulis*** | 6.04 ± 0.46 | 8.24 ± 2.89 |
| ***C. rehmanni*** | 161.52 ± 2.11 | 600 ± 1 |
| **Gallic acid** | 12.45±0.42 | |

Results were recorded as a mean of three replicates

## *Reducing power activity of P. angolensis*

In the reducing power assay for *P. angolensis*, PaF2b exhibited the highest reducing power (44.28±0.17) while fraction PaF2a exhibited the lowest reducing power (Table 3.10).

**Table 3.10:** Reducing power activity of the crude extract and fractions of *P. angolensis*

| Extract/ fraction | Reducing power |
|---|---|
| **PaCE** | 79.73 ± 2.59 |
| **PaF2a** | 94.43 ± 1.17 |
| **PaF2b** | 44.28 ± 0.17 |
| **PaF3a** | 72.01 ± 0.69 |
| **PaF3b** | 84.47 ± 1.3 |
| **PaF3c** | 80.5 ± 0.64 |
| **PaF4** | 81.37 ± 2.8 |
| **Gallic acid** | 12.45 ± 0.42 |

Results were recorded as a mean of three replicates



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### 3.6.5 Anti-inflammatory assay

The anti-inflammatory potential of the extracts was evaluated on RAW 264.7 cells. Figure 3.15 illustrates that 7 plant extracts significanty boosted the production of Nitic Oxide above that of the LPS control at 50 and 100 µg/ml.

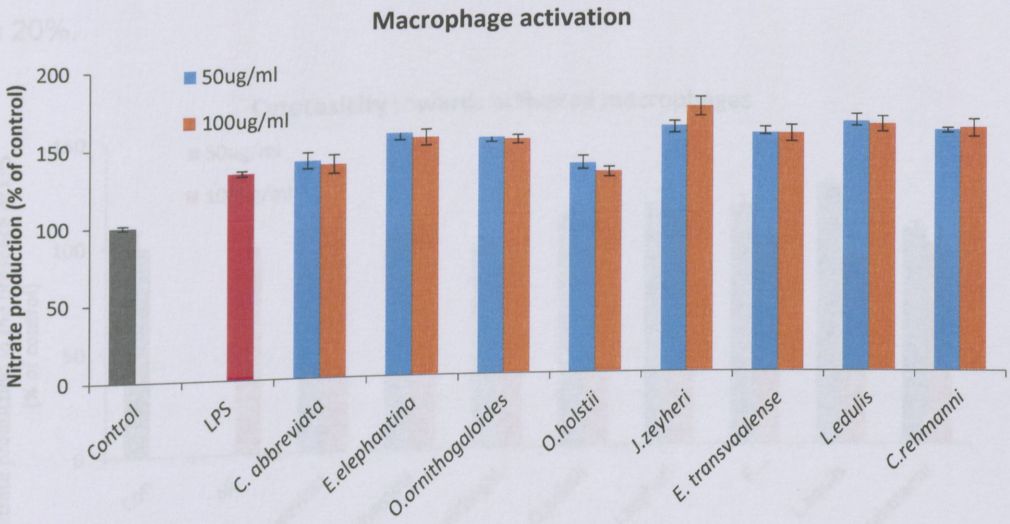



**Figure 3.15:** Effect of the 8 aqueous extracts on nitrate production in unstimulated RAW macrophages. *E.coli* LPS, an inducer of iNOS activity was used to demonstrate macrophage activation capacity.

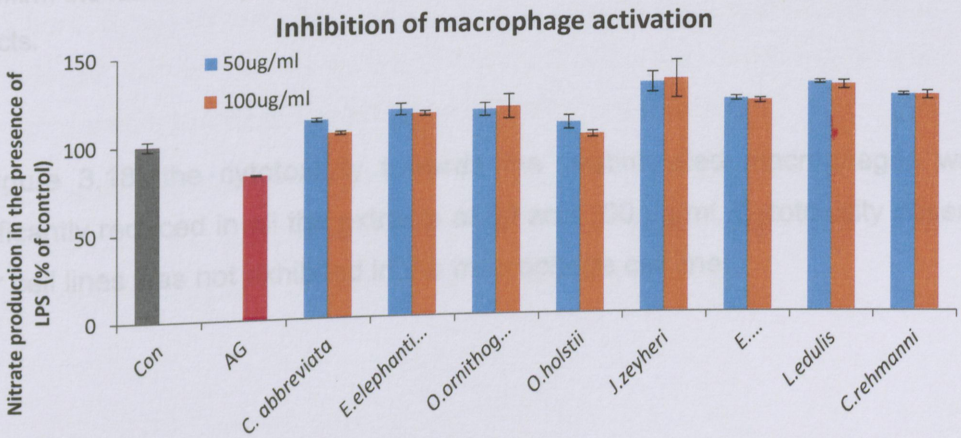



**Figure 3.16:** Effect of plant extracts on nitrate production in LPS –stimulated RAW macrophages. Aminoguanidine, an inhibitor of iNOS expression served as a positive control to confirm the functionality of the assay.



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In Figure 3.16, even in the absence of LPS stimulation, there appeared to be an increase in nitrate levels as compared to the controls. The extracts alone are capable of inducing nitrate production. In Figure 3.18, the viability assay demonstrated that the growth of the macrophage cells was not affected by the extracts. The percentage of viable cells was slightly reduced at 100 µg/ml by less than 20%.

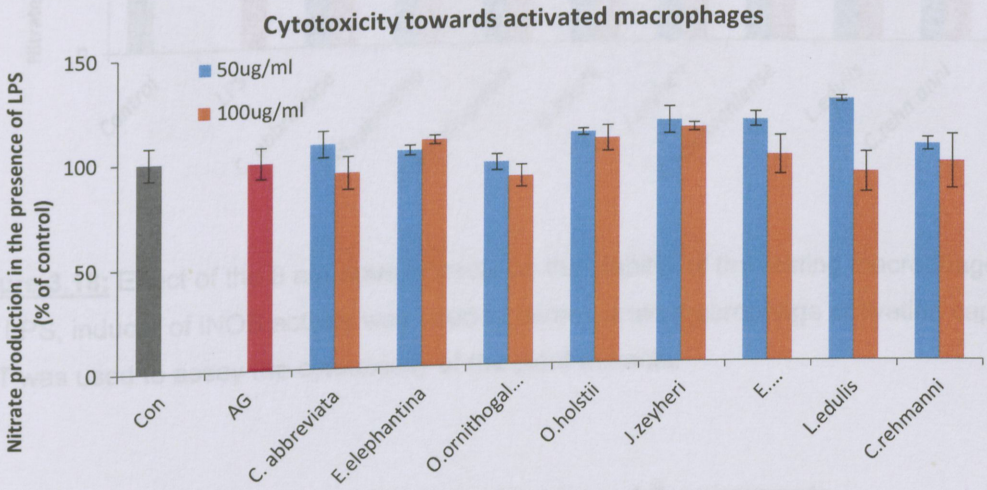



**Figure 3.17:** Effect of the 8 aqueous extracts on the cell viability of the activated macrophages. Aminoguanidine, an inhibitor of iNOS expression served as a positive control to confirm the functionality of the assay. MTT was used to assay the cytotoxicity of the plant extracts.

In Figure 3.18, the cytotoxicity towards the unstimulated macrophages was not significantly reduced in all the extracts at 50 and 100 µg/ml. Cytotoxicity observed in other cell lines was not exhibited in the macrophage cell line. .



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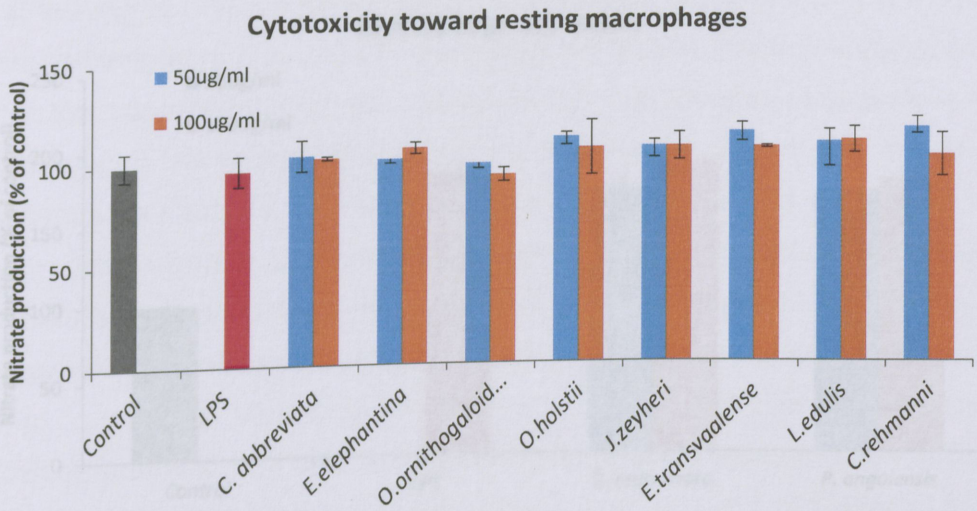



**Figure 3.18:** Effect of the 8 aqueous extracts on the viability of the resting macrophages. E. coli LPS, inducer of iNOS activity was used to demonstrate macrophage activation capacity. MTT was used to assay the cytotoxicity of the plant extracts.

## *Anti-inflammatory potential of P. angolensis and Z. mucronata*

Figure 3.19 illustrates the degree of inhibition in LPS-stimulated nitric oxide production. It can be seen that neither *Z. mucronata* nor *P. angolensis* could reduce nitrate levels below the untreated LPS-stimulated cells. Nitrate production levels were comparable to that of the LPS control for both extracts.

Even in the absence of stimulation, *Z. mucronata* and *P. angolensis* extracts induced the production of NO (Figure 3.20). The amount of NO produced by the unstimulated macrophages is comparable to the level of the stimulated cells.



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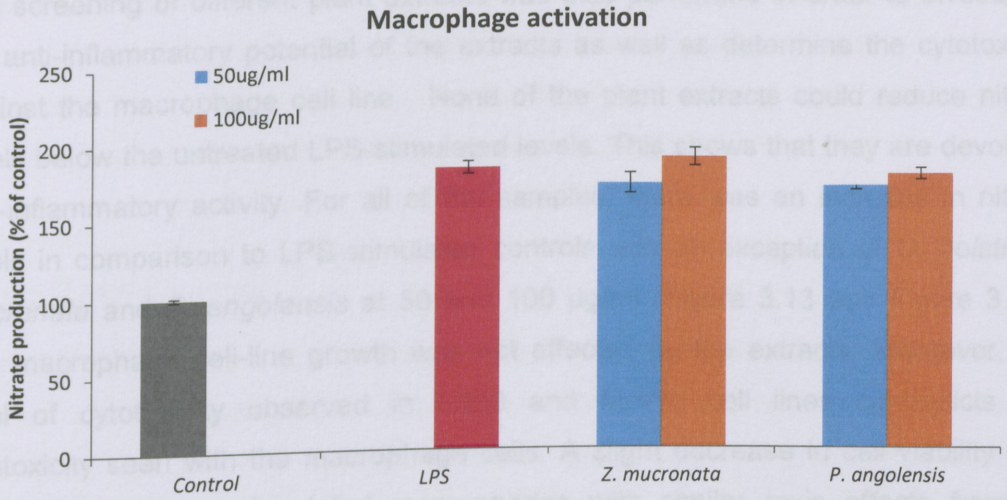



**Figure 3.19:** Stimulatory effects of *Z. mucronata* and *P angolensis* on nitrate production in unstimulated macrophages. *E. coli* LPS, inducer of iNOS activity was used to demonstrate macrophage activation capacity.

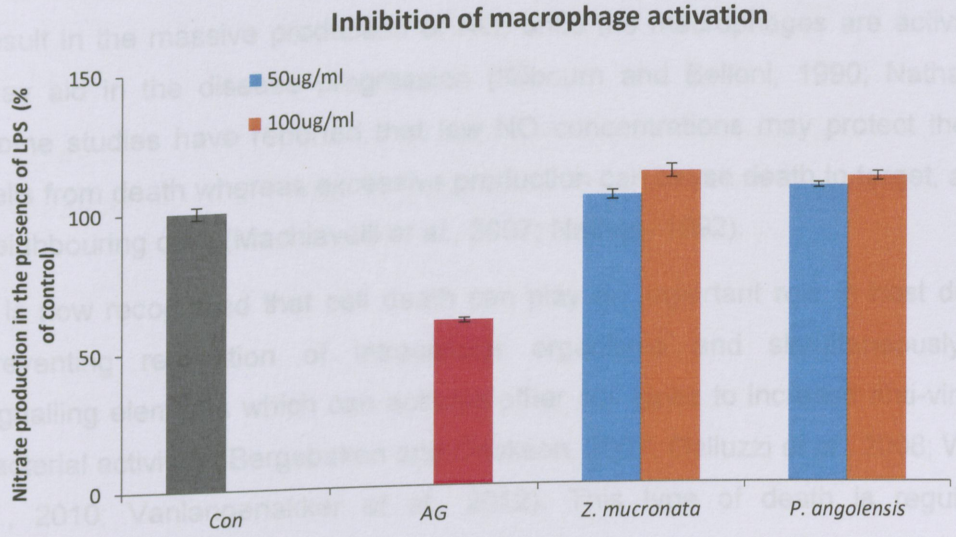



**Figure 3.20:** Stimulatory effects of *Z. mucronata* and *P. angolensis* on nitrate production in LPS stimulated macrophages. Aminoguanidine, an inhibitor of iNOS expression served as a positive control to confirm the functionality of the assay.



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The screening of different plant extracts was also performed in order to investigate the anti-inflammatory potential of the extracts as well as determine the cytotoxicity against the macrophage cell line. None of the plant extracts could reduce nitrate levels below the untreated LPS-stimulated levels. This shows that they are devoid of anti-inflammatory activity. For all of the samples, there was an increase in nitrate levels in comparison to LPS-stimulated controls with an exception of *O. holstii*, *Z. mucronata* and *P. angolensis* at 50 and 100 µg/ml (Figure 3.13 and Figure 3.17). The macrophage cell-line growth was not affected by the extracts. Moreover, the level of cytotoxicity observed in U938 and MeWo cell lines contradicts the cytotoxicity seen with the macrophage cells. A slight decrease in cell viability was observed in the un-stimulated macrophages with similar toxic effects from *O. ornithogaloides* extract as seen in activated macrophages (Figures 3.15 and 3.16).

Nitric Oxide (NO) is an intracellular-mediator free radical that can be produced in various mammalian cells. It is a crucial and versatile molecule involved in neurotransmission, acute and chronic inflammation and hosts defense mechanisms (Michael and Feron, 1997). For *in vitro* or *in vivo* experiments, a variety of stimuli can result in the massive production of NO, once the macrophages are activated. This may aid in the disease progression (Kilbourn and Belloni, 1990; Nathan, 1992). Some studies have reported that low NO concentrations may protect the immune cells from death whereas excessive production can cause death to target, as well as, neighbouring cells (Machiavelli *et al.*, 2007; Nathan, 1992).

It is now recognized that cell death can play an important role in host defence by preventing replication of intracellular organisms and simultaneously release signalling elements which can activate other cell types to increase anti-viral or anti-bacterial activities (Bergsbaken and Cookson, 2007; Galluzzi *et al.*, 2008; Webster *et al.*, 2010; Vanlangenakker *et al.*, 2012). This type of death is regulated and programmed, with the immunological outcome determined by downstream pathways which may be induced.

Further analysis in the current chapter confirmed that even in the absence of stimulation, there was significant increase in NO production (Figure 3.14 and Figure 3.18); this may indicate that the extracts have pro-inflammatory properties. The nature of the extracts, with regard to their polysaccharide content, is known to have immunomodulatory activity. We concluded that the extracts prepared from



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succulents or bulbs have greater chances of producing pro-inflammatory effect. To rule out or confirm such activities, further confirmatory tests, however, need to be conducted. To the best of our knowledge, this is the first report demonstrating the pro-inflammatory properties of the chosen medicinal plants to date.

### 3.6.6 LC-MS analysis

Table 3.11 shows the secondary metabolites contained in the crude extracts of the different plants. *Cassia abbreviata* contained the highest amount of epicatechin followed by *Z. mucronata* whereas *L. edulis* contained the highest protocatechuic acid. The other compounds are present, however, in minute quantities. The profiling of the plant crude extract using LC/MS analysis have shown the presence of gallic acid, epicatechin, catechin, protocatechuic acid, p-hydrobenzoic acid and rutin in varying levels and in some of the extracts. The various metabolites were detected at different retention times together with some of the unidentified peaks of unknown metabolites. The presence of gallic acid might account for the anti-inflammatory. Anti-carcinogenic and anti-mycobacterial effects observed in Chapter 6 (Chaubey *et al.*, 2015)

In Figure 3.21, an LC-MS chromatogram depicting the retention of PCA along with other unidentified secondary metabolites.



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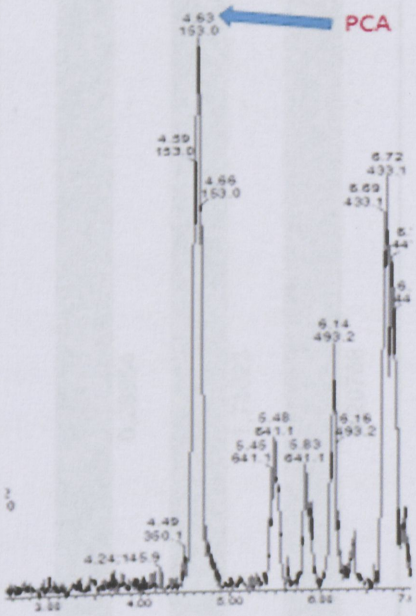



**Figure 3.20:** Chromatogram of *L. edulis* protocatechuic acid from the crude aqueous extract.

## 3.7 SUMMARY

Most of the evaluated plant extracts showed high radical scavenging activity possibly due to the flavonoid and phenolic content, the 2 metabolites required for the scavenging activity. Furthermore, the reducing power capability observed showed that the plant extracts have high reductone levels. It is believed that some of these plant extracts can serve as natural antioxidants which can protect biomolecules from degradation.



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**Table 3.11:** Summary of the phytochemicals obtained from the 8 aqueous extracts using LC-MS

| | PCA | Catechin | Gallic acid | p-hydroxybenzoic acid | epicatechin | Procyanin tetramer, trimer, dimer |
|---|---|---|---|---|---|---|
| ***C. abbreviatta*** | 8.99 | 14.82 | 4.12 | - | 162,18613 | - |
| ***E. elephantina*** | 7.87 | 3.35 | 88.8 | - | 6,46187 | - |
| ***O. ornithogaloides*** | - | - | - | - | 0,11225 | - |
| ***O. holstii*** | - | - | - | 0.58 | | - |
| ***Z. mucronata*** | 5.46 | 29.94 | 3.6 | - | 34,43708 | - |
| ***J. zeyheri*** | | 0.38 | - | 1.84 | 0,28954 | - |
| ***P. angolensis*** | 35.9 | - | 14.47 | 0.48 | 1,74292 | - |
| ***E. transvaalensis*** | 6.67 | 0.84 | - | 9.47 | 1,71023 | - |
| ***L. edulis*** | 120.49 | 2.7 | 31.2 | 4.54 | 17,04578 | 22 |
| ***C. rehmanni*** | - | - | | 1.3 | 0,20708 | - |

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# Chapter 4

# PART III: CYTOTOXICITY OF PLANTS

## 4.1 BACKGROUND

Cell cytotoxicity refers to the ability of certain chemicals (substances) or mediator cells to kill living cells. By being exposed to a cytotoxic compound, healthy living cells can either be urged to undergo accidental cell death (necrosis) or programmed cell death (apoptosis) as shown in Figure 4.1.

Measuring cell cytotoxicity has also proven to be critical in the process of discovering new therapeutic anti-cancer drugs. Through assessment of cytotoxicity levels on cancer cells, anti-cancer medications can stop the proliferation of target cells either by interfering with their genetic materials or by blocking entry of nutrients that the cells needs for survival (De Araujo Jnr *et al.*, 2012).

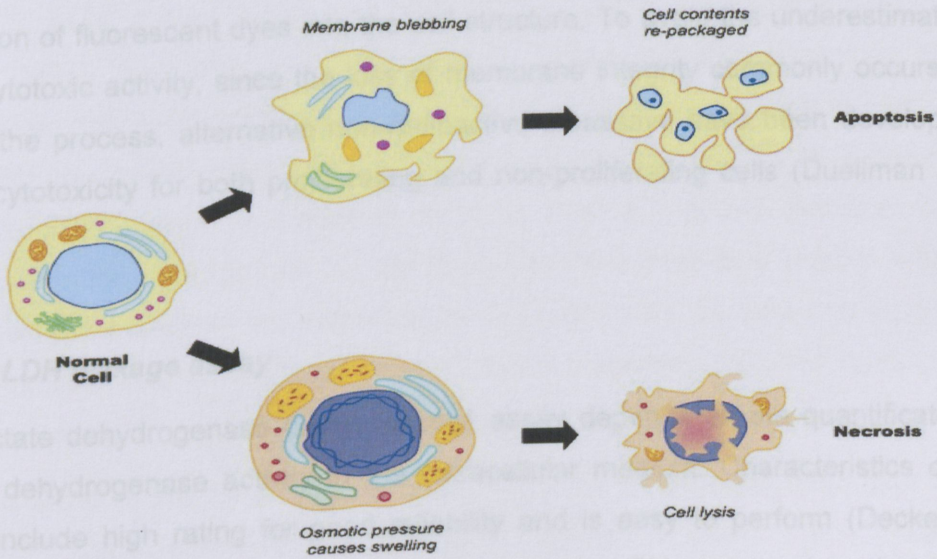



**Figure 4.1:** Apoptotic and necrotic induced cell death (www.vce.bioninja.com.au)

Cell viability and cytotoxicity assays are used for screening drugs and determining cytotoxicity levels of the various compounds. There are various methods used for detecting cell viability based on various cell functions and characteristics, such as



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enzyme activity, porosity of cell membrane, cell adhesiveness, ATP production, co-enzyme production, and nucleotide uptake activity. Cell viability can be evaluated through the use of vital dyes (formazan dyes), protease biomarkers or by measuring ATP content (Lundin *et al.*, 1986).

The formazan dyes produce coloured pigments (chromogenic products) formed through the reduction of tetrazolium salts by the action of dehydrogenases and reductases. These enzymes are released at cell death. Cell cytotoxicity can be examined using the SRB and WST-1 assays both deemed suitable for high throughput screening. A widely used technique for measuring viability is trypan blue which stains dead cells thus unstained cells can be counted through the microscope (Tran *et al.*, 2011).

### 4.1.1 Cytotoxicity assays

Cytotoxicity assays operate on the principle that dying cells have highly weakened cellular membranes which allows leakage of cytoplasmic components or the infiltration of fluorescent dyes into the cell structure. To avoid the underestimation of plant cytotoxic activity, since the loss of membrane integrity commonly occurs fairly late in the process, alternative non-radioactive bioassays have been developed to assay cytotoxicity for both proliferating and non-proliferating cells (Duellman *et al.*, 2015).

#### *4.1.1.1 LDH leakage assay*

The lactate dehydrogenase (LDH) leakage assay depends on the quantification of lactate dehydrogenase activity in the extracellular medium. Characteristics of this assay include high rating for good reliability and is easy to perform (Decker and Lohmann-Matthes, 1988). The loss of intracellular LDH and its detection in the culture medium is a signal for irremediable cell death due to the damage on the cell membrane. Lactate dehydrogenase is an oxidoreductase enzyme found in a wide-range of organisms. It speeds up the interconversion of pyruvate and lactate with associated interconversion of NADH and NAD. The high stability of the enzyme has increased its use to assess any damage and toxicity of tissue and cells. In the



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commercial LDH assay, the enzyme reduces NAD to NADH, which then interacts with a specific probe to generate a colour (Figure 4.2).

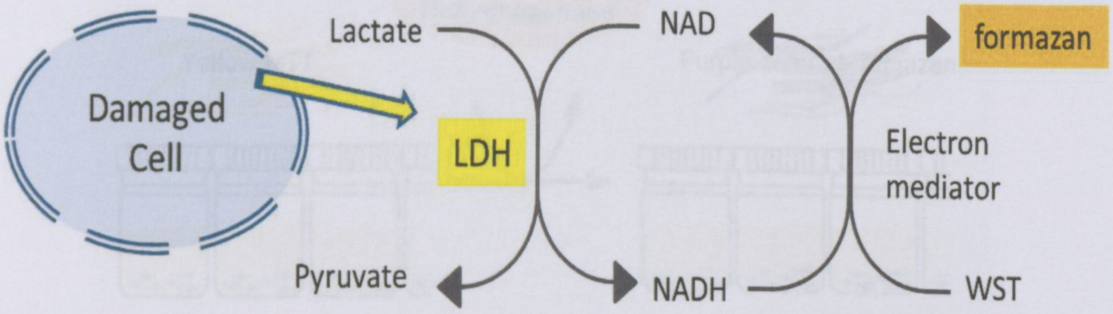



**Figure 4.2:** Principle of lactate dehydrogenase assay (www.researchgate.net)

### *4.1.1.2 MTT assay*

The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) is a cell viability assay, frequently used to determine cytotoxicity after subjection to toxic substances. The MTT assay has been tested to establish its validity in various cell lines and has been established by Mossman (1983). MTT is a water-soluble tetrazolium salt, which is transformed to an insoluble purple formazan by the breakdown of the tetrazolium ring by succinate dehydrogenase (Figure 4.3). The cleavage happens within the mitochondria. MTT infiltrates the cells and progresses to the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product.

The cells are then solubilised with an organic solvent, for example, isopropanol, thus releasing the solubilised formazan reagent which is measured spectrophotometrically. The reduction of MTT is known to enter in metabolically active cells, thus, the level of its activity is deduced as a measure of viable cells. A more recent evidence suggests that the reduction of MTT can also be mediated by NADH or NADPH within the cells and out of mitochondria (Berridge and Tan, 1992).



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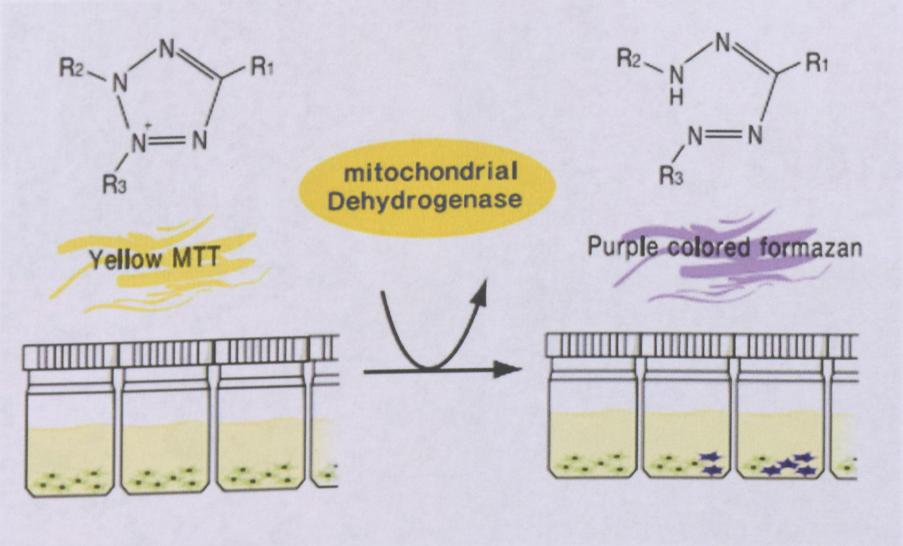



**Figure 4.3:** Principle of MTT assay (www.ncbi.nlm.nih.gov)

### *4.1.1.3 Neutral red assay*

The neutral red (NR) assay is a cell viability assay that is dependent on the capability of viable cells to assimilate and bind neutral red within the lysosomes. It is commonly conducted using adherent cell lines. NR is a weak cationic dye that effortlessly infiltrates the cell membrane and assembles intracellularly in lysosomes where it binds with the anionic sites of the lysosomal matrix. If there are any changes in the cell surface or the lysosomal membrane, it leads to lysosomal weakness and other changes that eventually become irreversible (Repetto *et al.*, 2008)

Such modifications brought about by the action of xenobiotics lead to a reduction in the uptake and binding of NR. It is thus feasible to discern between viable, damaged, or dead cells. This is a basic principle of this assay. The amount of the dye infiltrated (Figure 4.4) into the cells is measured spectrophotometrically and is directly proportional to the number of cells with an undamaged membrane (Repetto *et al.*, 2008). The assay can be used to evaluate cytotoxicity by the determination of the IC50 (50% inhibiting concentration).



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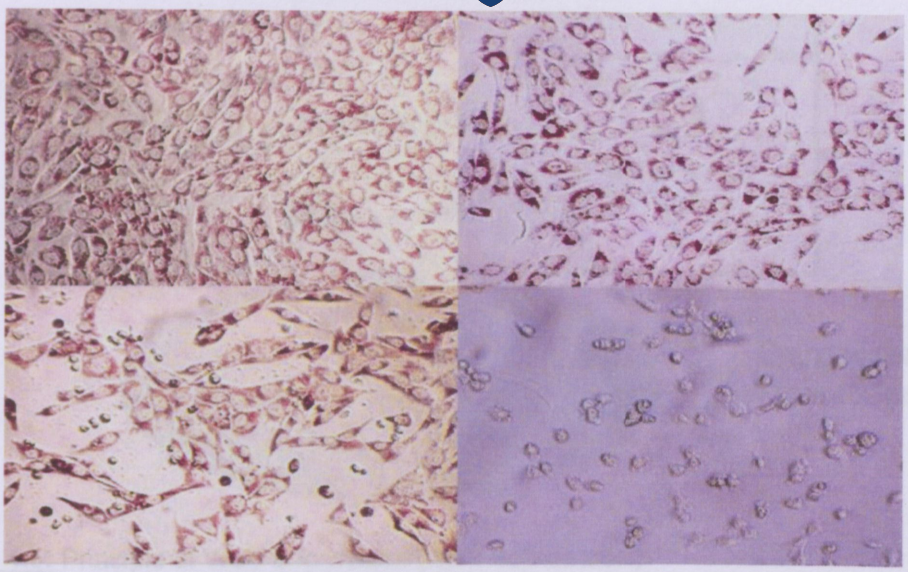


**Figure 4.4:** Microscopic pictures of neutral red stained cells (blogs.ubc.ca)

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#### *4.1.1.4 Water soluble tetrazolium salts*

Dojindo (Dojindo laboratories, Tabaru, Kumamoto Japan) has developed an immensely water-soluble tetrazolium salt called, WSTs. These WSTs produce water-soluble formazans and are applicable to cell proliferation and cytotoxicity assays. WSTs accept two electrons from viable cells to produce a yellow, orange, or purple formazan dye (Figure 4.5). WST-8 (an extremely stable WST) is effectively used in cell counting Kit-8 (CCK-8). The electron mediator used in this kit (1-Methoxy PMS) is also extremely stable. The advantage of using WST-8 and WST-8 formazan is that they are not cytotoxic in the cell-culture media, therefore additional experiments may be carried out using the cells from the previous assay (Ishiyama *et al.*, 1993).

The CCK-8 assay requires almost all of the dehydrogenase available in a cell while MTT only involves mitochondrial dehydrogenase, therefore, the MTT assay is dependent on mitochondrial activity and not the cell itself. Furthermore, CCK-8 is far more sensitive than the MTT assay. Since WST-8 formazan is water-soluble, it does not form crystals like MTT (Takenouchi and Munekata, 1995).



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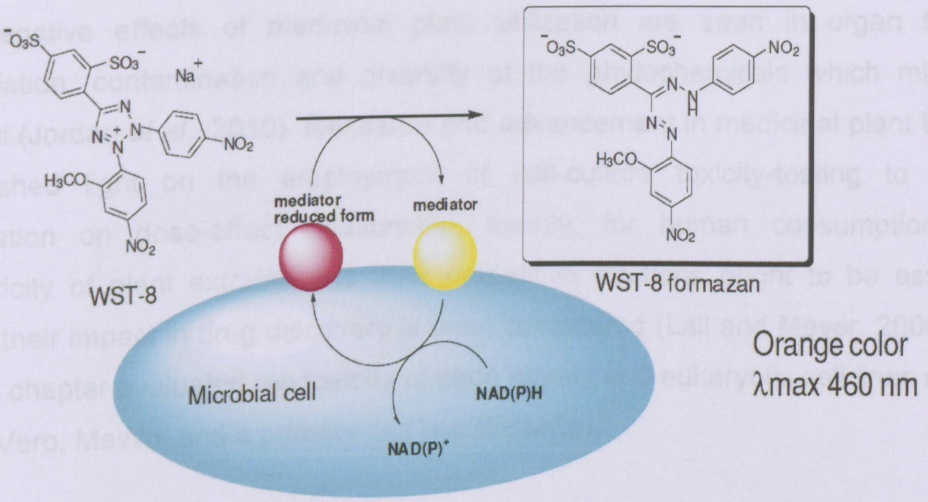



**Figure 4.5:** Principle of WST-8 formazan (www.dojindo.com)

## 4.2 PLANTS AND THEIR CYTOTOXIC EFFECTS

Plants have been all-important sources of natural products for easing pain caused by different microorganisms, since ancient days (Graham *et al.*, 2000). Their secondary metabolites have been studied and reported to possess numerous biological activities which also act as protective agents to counter the effects of pathogens. Different plant species have been used for centuries and employed in cancer therapy (Jo *et al.*, 2005). Such species are included in a list of over 3 000 species reported by Cragg and Newman (2005). The majority of these plant extracts and their specific fractions have been tested *in vitro,* for cytotoxicity, on human cell lines like, prostate, liver and colon, as well as animal cell lines, such as, monkey kidney cells (Al-Fatimi *et al.*, 2005; Lamidi *et al.*, 2005; Jo *et al.*, 2005; Don *et al.*, 2006).

Medicinal plants are presumed safe or non-toxic, for human consumption due to their natural origin and long usage. These concoctions are taken with the hope of improving health and or boosting the immune system to fight infections such as inflammation, cancer and diabetes. Scientific research, however, has revealed that most of these plants are potentially noxious, mutagenic and carcinogenic (Schimmer *et al.*, 1988; Higashimoto *et al.*, 1993; Schimmer *et al.*,1994; Kassie *et al.*, 1996).



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The negative effects of medicinal plant utilization are seen in organ toxicity, degradation, contamination and diversity of the phytochemicals which might be harmful (Jordan *et al.*, 2010). Research and advancement in medicinal plant therapy have shed light on the employment of cell-culture toxicity-testing to furnish information on dose-effect relationship, mainly, for human consumption. The cytotoxicity of plant extracts and their respective fractions ought to be assessed before their impact in drug discovery is even considered (Lall and Meyer, 2000). The current chapter evaluated the toxicity of each extract in 3 eukaryotic cell lines namely U937, Vero, MeWo, and a primary cell line (PBMCs).

## 4.3 MATERIALS AND METHODS

Ethical approval was obtained from the Health, Safety and Research Ethics Committee of the University of Venda (SMNS/14/MBY/30/1210; refer to Appendices). Signed informed consent forms were obtained to show agreement to participate in the research, from all volunteers, before collection of blood specimens. Confidentiality of volunteers was maintained throughout by assigning them numerical codes.

### 4.3.1 Plant materials and preparation of extracts

All 10 extracts obtained after preparation as described in Chapter 2 were used in the assays described in section 4.3.4. The dried plant extracts were reconstituted in DMSO to a final stock concentration of 50 000 µg/ml. Samples were then stored at 4°C for subsequent uses in assays.

### 4.3.2 Characteristics of cell lines

#### *4.3.2.1 Characteristics of U937 cells*

This cell line was originally isolated from a patient with histiocytic lymphoma. They are distinguished by the presence of lysozyme with no surface immunoglobulin (Ig) or Ig secretion. They are oval shaped with huge and non-uniformly-shaped nuclei (Sundstrom *et al.*, 1976). U937 monocytes have been utilized as an *in vitro* model of



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macrophage function, since human-primary-tissue macrophages cannot be duplicated. When this cell line is transformed to a macrophage lineage, they resemble the human macrophages (Figure 4.6).

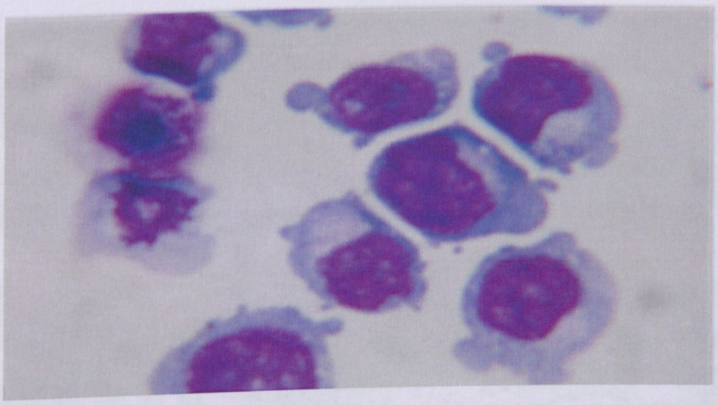


**Figure 4.6:** Morphology of U937 cells as viewed under microscope 1000X (www.intechopen.com)

### 4.3.2.2 Characteristics of Vero cells

Vero cells are of monkey kidney-cells origin (Figure 4.7). They were isolated from a disease free adult African green monkey in 1963 by Yasumura and Kawakita. Vero cell lines have been utilized as early as the late 1960s worldwide especially in virology laboratories. The cells are not transformed and they still maintain their original shape. Upon reaching confluency, they halt growing and die out. Therefore, it is crucial to monitor them during experimentation and to subculture them regularly as they form confluent monolayers. Actively growing Vero cell cultures double approximately every 24 hours (Nahapetian *et al.* 1986).

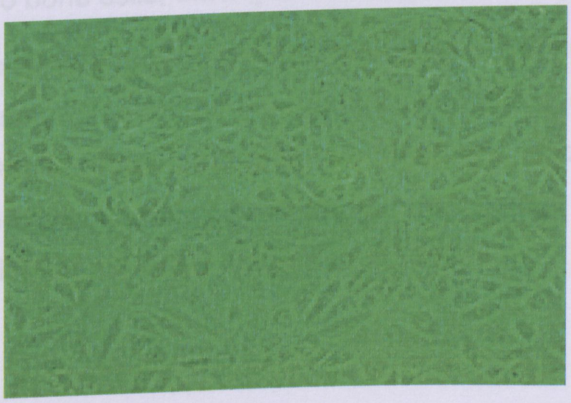


**Figure 4.7:** Morphology of Vero Cells (www.wikimedia.org)



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### 4.3.2.3 Characteristics of MeWo cells

The MeWo cell line was originally isolated from a metastatic tumour harbouring lymph node. Their morphology is well distinguished from other cells (Figure 4.8).

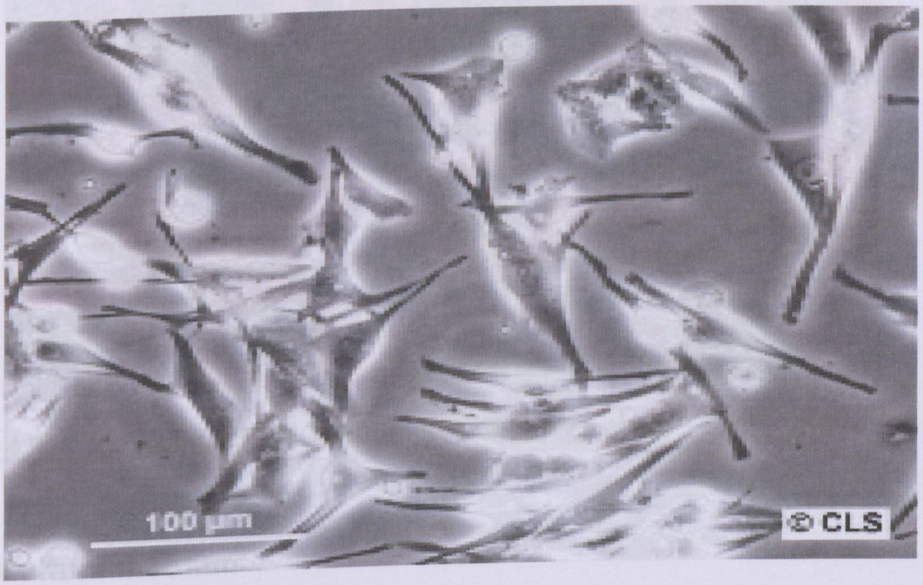



**Figure 4.8:** Morphology of MeWo cells as viewed under microscope (cellbank.nibiohn.go.jp)

### 4.3.2.4 Characteristics of PBMC cells

Peripheral blood mononuclear cells (PBMCs) are a basis of the cellular therapy field since they constitute a source of well-defined progenitor-cell populations. PBMCs are prized for their capability to undergo transformation in response to incubation conditions. They are round in structure and appear reddish under the microscope (Figure 4.9). They can be easily induced to differentiate from blood cells, epithelial cells, and also bone cells, among other cell types (Shang *et al.*, 2009).

PBMCs are an important source of lymphocytes and monocytes, and they are highly used in a variety of immunological assays, in order to contrast immune responses between healthy donors and patients. They are also utilized when looking for genotypic and phenotypic differences between normal and diseased immune cell populations (Bull *et al.*, 2007; Sambor *et al.*, 2014).



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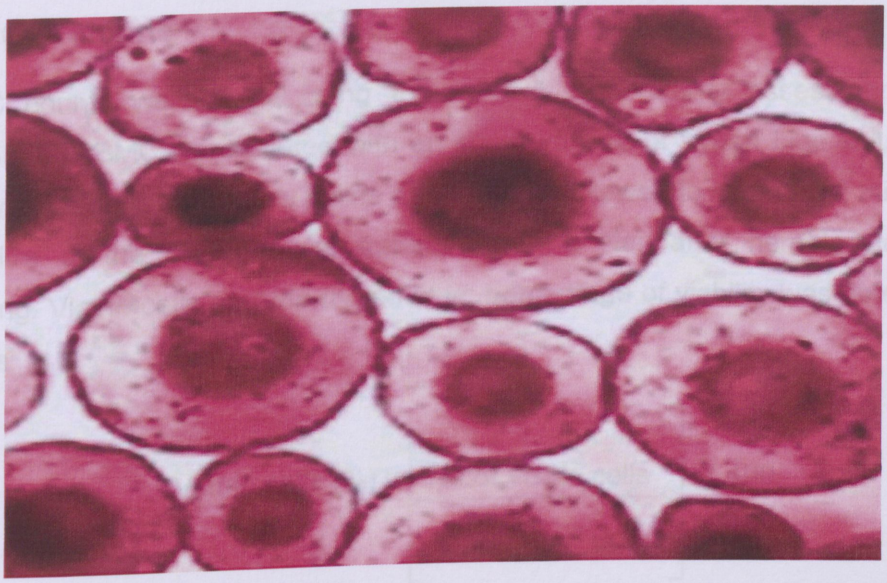


**Figure 4.9:** Morphology of PBMC cells (purecellresearch.com)

### 4.3.3 Culture medium

Cells were maintained and cultured in complete medium DMEM or RPMI 1640 containing 2mM L-glutamine (Sigma, Missouri, USA), heat-inactivated foetal-calf serum (10% v/v); and gentamycin sulphate (1ng/ml).

### 4.3.4 Isolation of Peripheral blood mononuclear cells

Peripheral venous blood was collected from 3 healthy individuals and 3 HIV infected patients. Blood was collected through the anticubital vein using sterile needle and syringe for each donor. About 5ml was collected into a BD vacutainer and transferred to the laboratory then mixed by inverting the tube several times. PBMCs were isolated by a density-gradient centrifugation using Ficoll-hypaque (Sigma, St Louis, MO). Briefly, the blood was diluted with RPMI media on a 1:1 ratio; after dilution, the blood was layered on top of an equal volume of ficoll hypaque followed by centrifugation for 30 min at 3000 rpm. The resulting layer of cells or buffy coat was carefully removed and washed twice with RPMI media then the cells were re-suspended in complete media containing 2 µg/ml PHA for stimulation and allowed to grow overnight in a 5% $CO_2$ humidified incubator (EcoTherm, Hartkirchen, Austria).



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### 4.3.5 Cell counts

PBMC counts were done using trypan blue dye viability assessment method. Aliquots of PBMC were diluted with an equal volume of trypan blue solution (0.4% w/v trypan blue in 0.85% w/v saline solution) and allowed to stand for 2 minutes. Ten microlitre of the solution was transferred to a Neubauer hematocytometer for counting. Viable cells exclude trypan blue; percentage of viability was determined as follows:

$$\text{Viable cells (\%)} = \left[ \frac{\text{No of viable cells}}{\text{Total No. of cells}} \right] \text{X } 100$$

### 4.3.6 In vitro cytotoxicity ativity

#### *4.3.6.1 Cytotoxicity on continuous cell lines*

The four different cell lines, described in section 4.3.2, were employed in this part. The continuous cell lines (U937, Mewo and Vero) were grown in RPMI (U937) and DMEM (MeWo and Vero), respectively. Both media were supplemented with 10% foetal bovine serum. The cells were seeded at a density of 10 000 cells/well (U937) or 6 000 cells/well (MeWo/Vero) using a volume of 100 µl per well and incubated for 24hours at a temperature of 37°C in a 5% $CO_2$ incubator and 100% relative humidity (EcoTherm, Hartkirchen, Austria).

Each extract was tested in 2 dilutions, 125 µg/ml and 250 µg/ml for U937 cells and 50 µg/ml and 100 µg/ml for the MeWo and Vero cells. Aliquots (100 µl) of each of the different extract dilutions were added to the appropriate well containing 100 µl of medium/cells. The plates were further incubated for 48hrs. To validate the cytotoxicity experiments conducted, 2 sets of controls were included, namely cells in media only (Baseline control) and cells in Melphalan (Positive control).

At the end of the incubation period, the medium was removed from the adherent plate (MeWo/Vero) and replaced with fresh DMEM containing MTT at a final concentration of 0.5 mg/ml. The plates were again incubated for a further 3 hours to



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allow formation of MTT crystals. For the suspension culture cell line (U937), 20 µl of MTT was added to each well and the plate incubated a further 4 hours. The MTT crystals were solubilized using acidified Isopropanol (acidified in 40mM HCl). Thereafter absorbance was read at 560 nm using a multiwell scanning spectrophotometer (Multiscan MS, Labsystems, Vantaa, Finland).

#### *4.3.6.2 Cytotoxicity on primary cells PBMCs*

Overnight stimulated PBMCs (as described in 4.3.4) were plated at a density of 500 000 cells/well and treated with a series dilutions of plant extracts ranging from 3.9 to 500 µg/ml. Dilution of stock solutions was made in culture medium yielding final extracts concentrations with a final DMSO concentration of 0.1%. This concentration of DMSO did not affect cell viability. Controls included cells in media only (Baseline control), cells in PHA (Negative control) and cells in DMSO (Positive control). All concentrations of plants extracts were tested in triplicates on the same cell batch.

### 4.3.7 Statistical analysis

All data were representative of three independent experiments performed in triplicate and presented as a mean ± standard deviation (SD). The Mann–Whitney test and one-way analysis of variance (ANOVA) were used for the evaluation of any statistical differences between the results and p values; $< 0.05$ were considered significant.

## 4.4 RESULTS AND DISCUSSION

In order for medicinal plants to be rendered important in clinical applications, the prepared extracts must be selectively toxic to the target disease causing pathogen or must, at least, interfere directly with a particular reaction pathway with minimal toxicity to the host cells. Medicinal plants are assumed safe to consume because they are of natural origin and have been used for centuries in treating different ailments (Fennel *et al.*, 2004). A study conducted by Till *et al.* (2003) has, however, revealed that some of the medicinal plants can have long term effects on the human body and one of such effect is DNA damage.



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The results from this research show high level of selective inhibitory effects from the crude plant extracts. The results also demonstrate toxicity effects on cancerous cell lines. The results obtained are described and discussed below.

### 4.4.1 Cytotoxicity on U-937 cell line

In Fig 4.10a, Extracts from *O. ornithogaloides* ($P< 0.0005$) and *E. transvaalense* ($P<0.0001$) showed highly significant toxicity followed by *Lannea edulis* ($P<0.0005$). This is observed by the decrease in concentration of live cells (relative to the absorbance reading). For *O. ornithogaloides* and *E. transvaalense*, cell viability percentage decreased from 100% to below 50% and greater cytotoxicity was observed with the 250 µg/ml concentration in both extracts. *Lannea edulis* extract's toxic effects were also observed especially at the same concentration range with the cell viability reduced by 50%. The rest of the extracts led to cell viability at over 60%.

The cytotoxicity of *Z. mucronata* and *P. angolensis* cytotoxicity was more pronounced in U937 cells as shown in Figure 4.10b. At both tested concentrations,125 and 250 µg/ml, cell viability was reduced by over 50% with a statistical significance of $P<0.0001$. The decrease of cell viability was found to be at the same level as melphalan, a known toxic chemical.

### 4.4.2 Cytotoxicity on Vero cell line

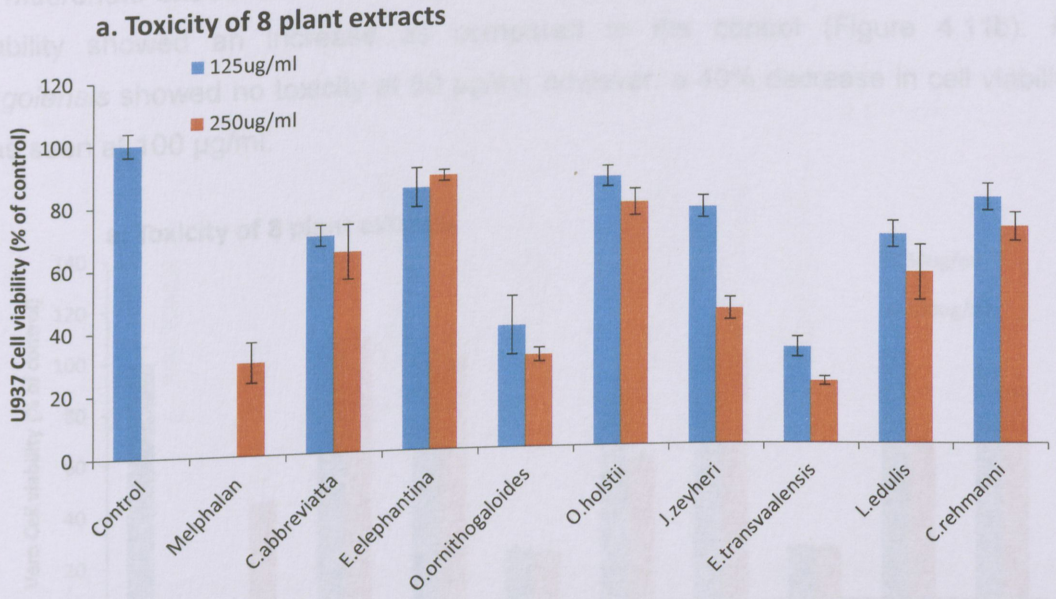
The trend of the cytotoxic effects of the 10 aqueous extracts on the Vero cell line (Figure 4.11) was similar to those observed in U937 cells. The degree of toxicity was much greater in *O. ornithogaloides* ($P<0.0001$) and *E. transvaalense* ($P<0.0001$). Both extracts decreased cell viability from 100% to 20%. *Lannea edulis* exhibited greater toxicity at 100 µg/ml seen as a 40% decrease in viability. *Ochna holstii* at 50 µg/ml decreased vitality by 20% ($P<0.01$) and at 100 µg/ml it decreased by over 40% ($P<0.0001$). The remaining samples showed minimal cytotoxic levels towards Vero cells.

The ethyl acetate extracts of *C. abbreviata* and *E. elephantina* led to death of Vero cells (over 80% cell death) as observed in Figure 4.12. In comparison, the cytotoxicity exerted by ethyl acetate extracts was more pronounced.



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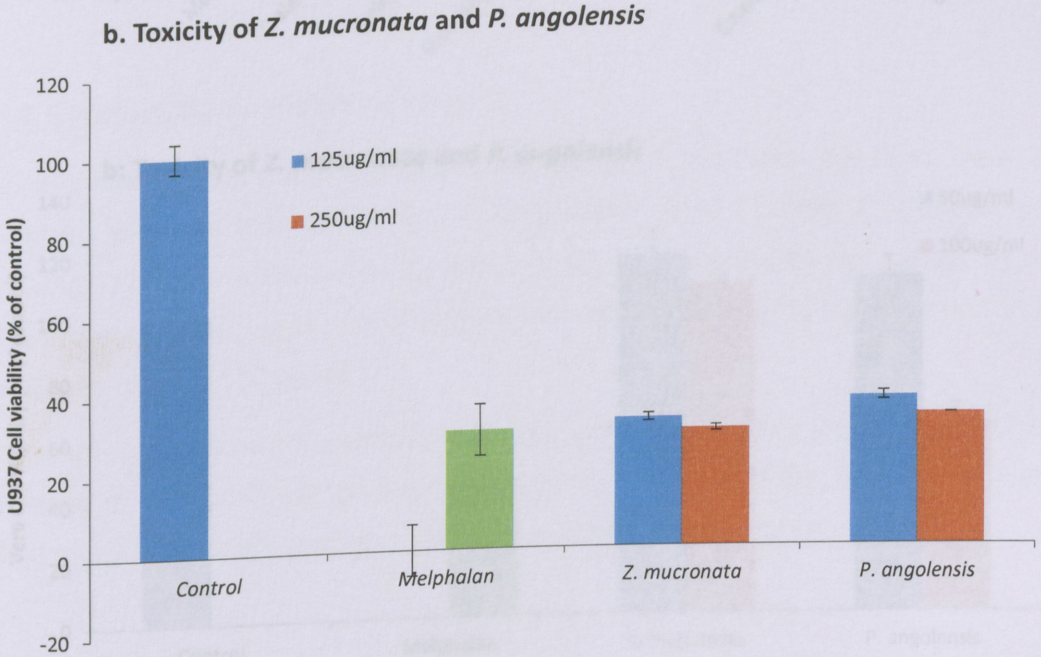
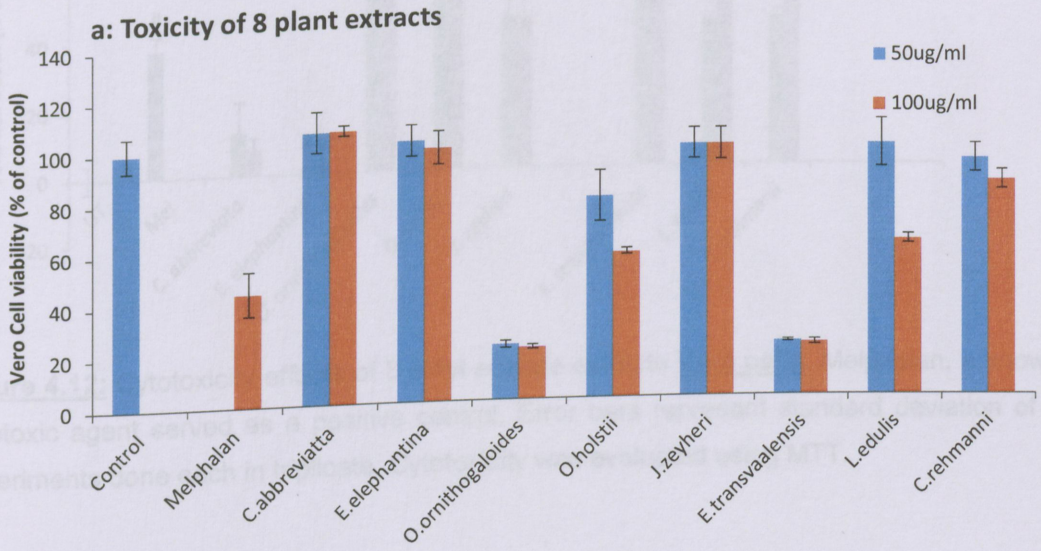




**Figure 4.10:** Cytotoxicity effects of 8 aqueous extracts (a) and *Z. mucronata* and *P. angolensis* (b) on **U937 cells**. Melphalan, a known cytotoxic agent served as a positive control. Error bars represent standard deviation of 3 experiments done each in triplicate. Cytotoxicity was evaluated using MTT.



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*Z. mucronata* extract showed no sign of toxicity towards the Vero cells, instead cell viability showed an increase as compared to the control (Figure 4.11b). *P. angolensis* showed no toxicity at 50 µg/ml, however, a 40% decrease in cell viability was seen at 100 µg/ml.



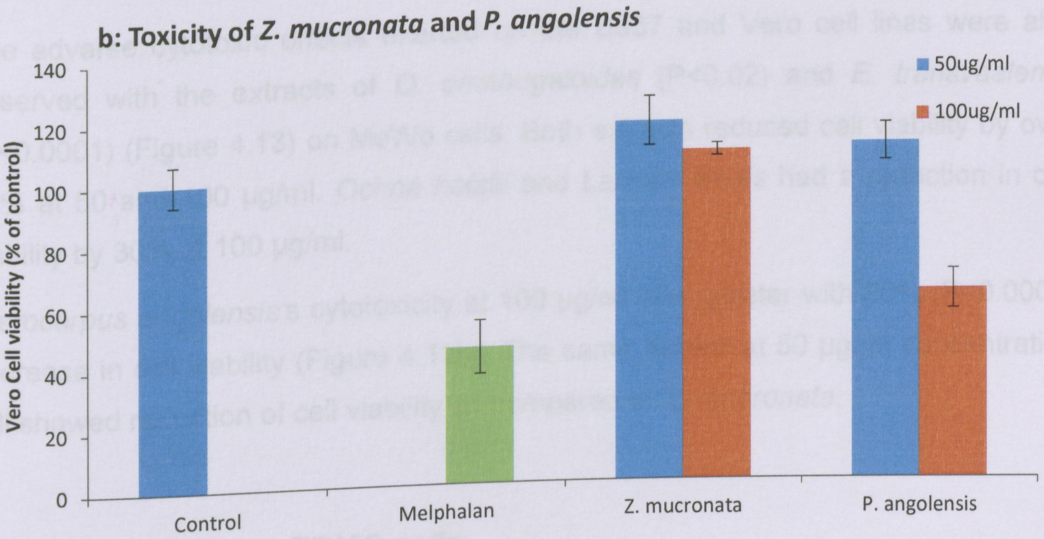




**Figure 4.11**: Cytotoxicity effects of 8 aqueous extracts (a) and *Z. mucronata* and *P. angolensis* (b) on **Vero cells**. Melphalan, a known cytotoxic agent served as a positive control. Error bars represent standard deviation of 3 experiments done each in triplicate. Cytotoxicity was evaluated using MTT.



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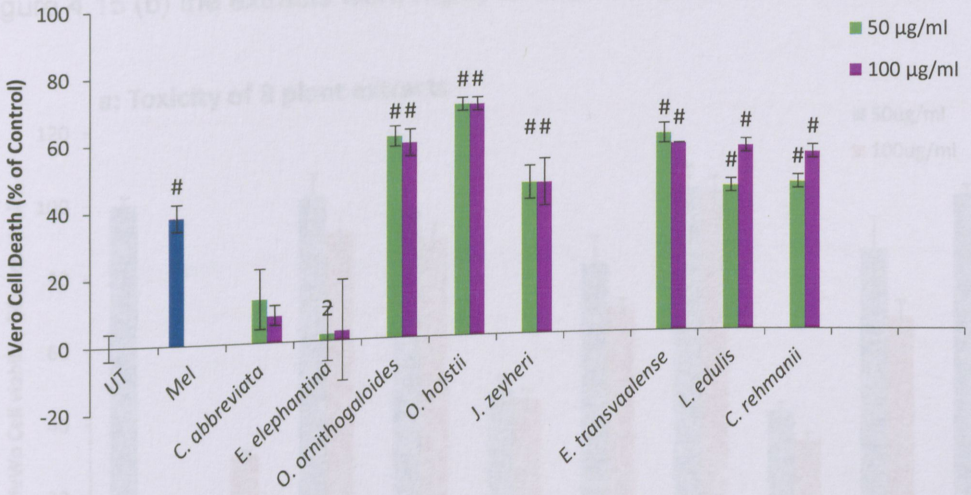



**Figure 4.12:** Cytotoxicity effects of 8 ethyl acetate extracts **Vero cells**. Melphalan, a known cytotoxic agent served as a positive control. Error bars represent standard deviation of 3 experiments done each in triplicate. Cytotoxicity was evaluated using MTT.

### 4.4.3 Cytotoxicity on MeWo cell line

The adverse cytotoxic effects exerted on the U937 and Vero cell lines were also observed with the extracts of *O. ornithogaloides* ($P<0.02$) and *E. transvaalense* ($P<0.0001$) (Figure 4.13) on MeWo cells. Both extracts reduced cell viability by over 50% at 50 and 100 µg/ml. *Ochna holstii* and *Lannea edulis* had a reduction in cell viability by 30% at 100 µg/ml.

*Pterocarpus angolensis*'s cytotoxicity at 100 µg/ml was greater with 50% ($P<0.0001$) decrease in cell viability (Figure 4.13b). The same extract at 50 µg/ml concentration still showed reduction of cell viability as compared to *Z. mucronata*.

### 4.4.4 Cytotoxicity on PBMC cells

Plant extract-induced cell death was observed to be dependent of the concentration of crude extract. This was observed with all 8 aqeous extracts (*C. abbreviata*, *E. elephantina*, *O. ornithogaloides*, *O. holstii*, *J. zeyheri*, *E. transvaalensis*, *L. edulis* and *C. rehmannii*). They all inhibited cell growth at high concentration (500 µg/ml). In Figure 4.14 (a), the 2 plant extracts exhibited a slight inhibitory effect at 62.5 µg/ml



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depicted by a rise in cells' growth as compared to higher concentrations and in Figure 4.15 (b) the extracts were highly toxic as compared to control cells.

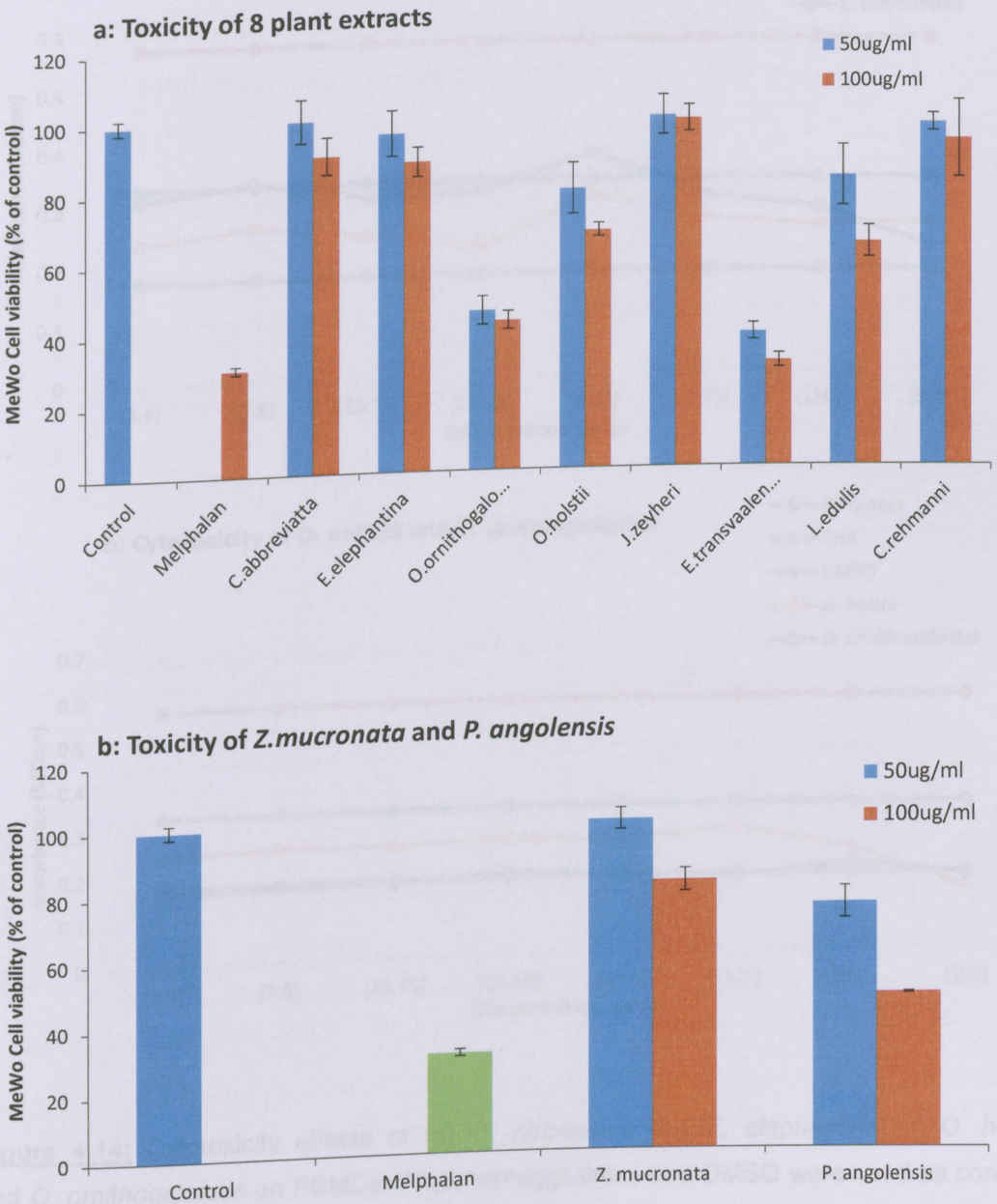
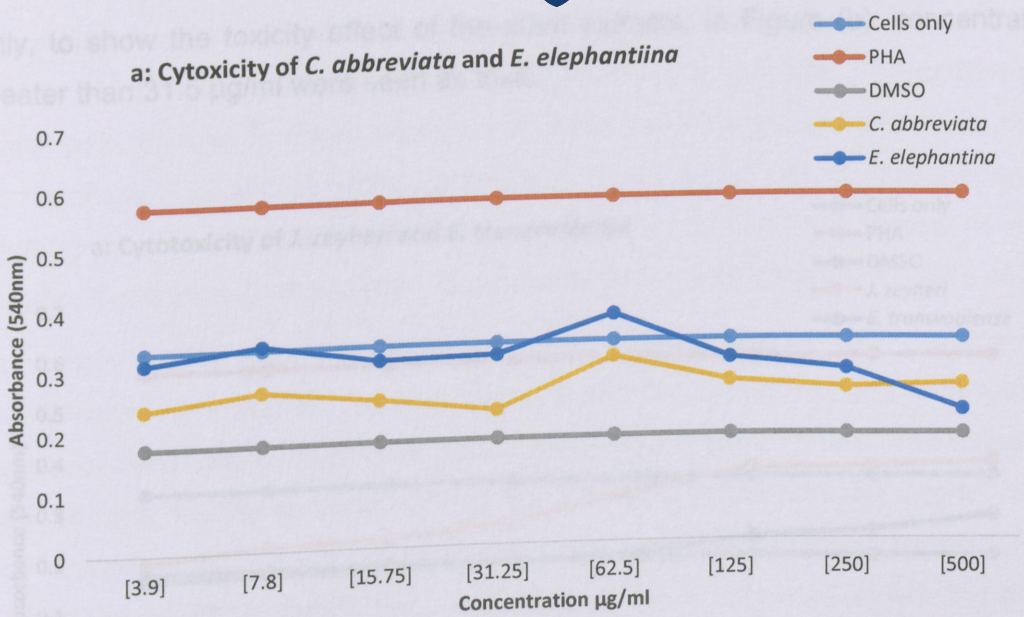



**Figure 4.13:** Cytotoxic effect of the 8 aqueous extracts (a) and *Z.mucronata* and *P. angolensis* (b) on **MeWo cells**. Melphalan, a known cytotoxic agent served as a positive control. Error bars represent standard deviation of 3 experiments done each in triplicate. Cytotoxicity was evaluated using MTT.



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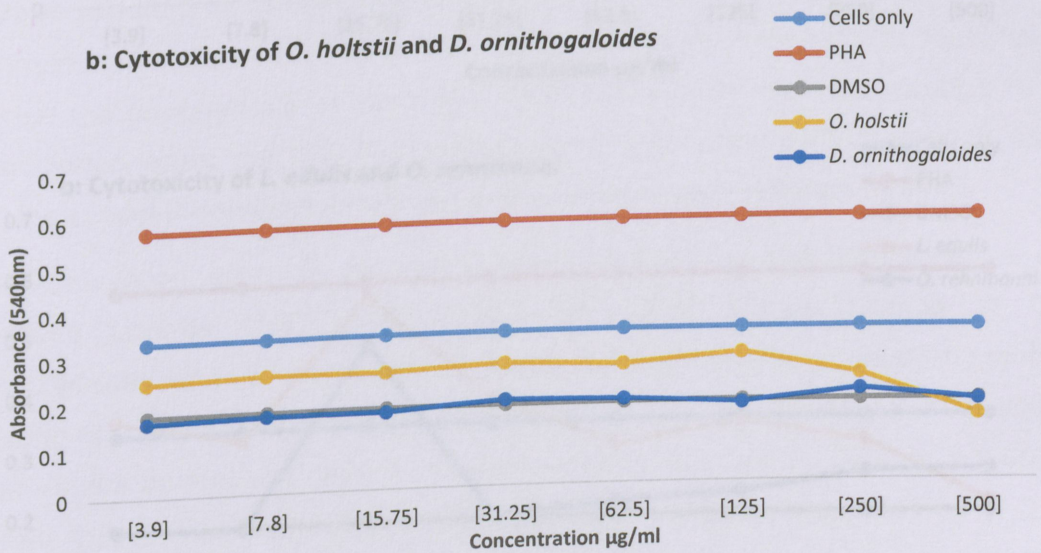




**Figure 4.14:** Cytotoxicity effects of (a) *C. abbreviata* and *E. elephantina* (b) *O. holstii* and *O. ornithogaloides* on PBMCs. Phytohemagglutinins and DMSO were used as controls to confirm the functionality of the assay. The data represents the average of 3 experiments done in triplicate. Cytotoxicity was evaluated using MTT.

In Figure 4.15, (a) the extracts of *J. zeyheri* and *E. transvaalense* decreased cell growth at high concentration. Cell viability was found to be below that of control cells-



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only, to show the toxicity effect of the plant extracts. In Figure (b), concentrations greater than 31.5 µg/ml were seen as toxic.

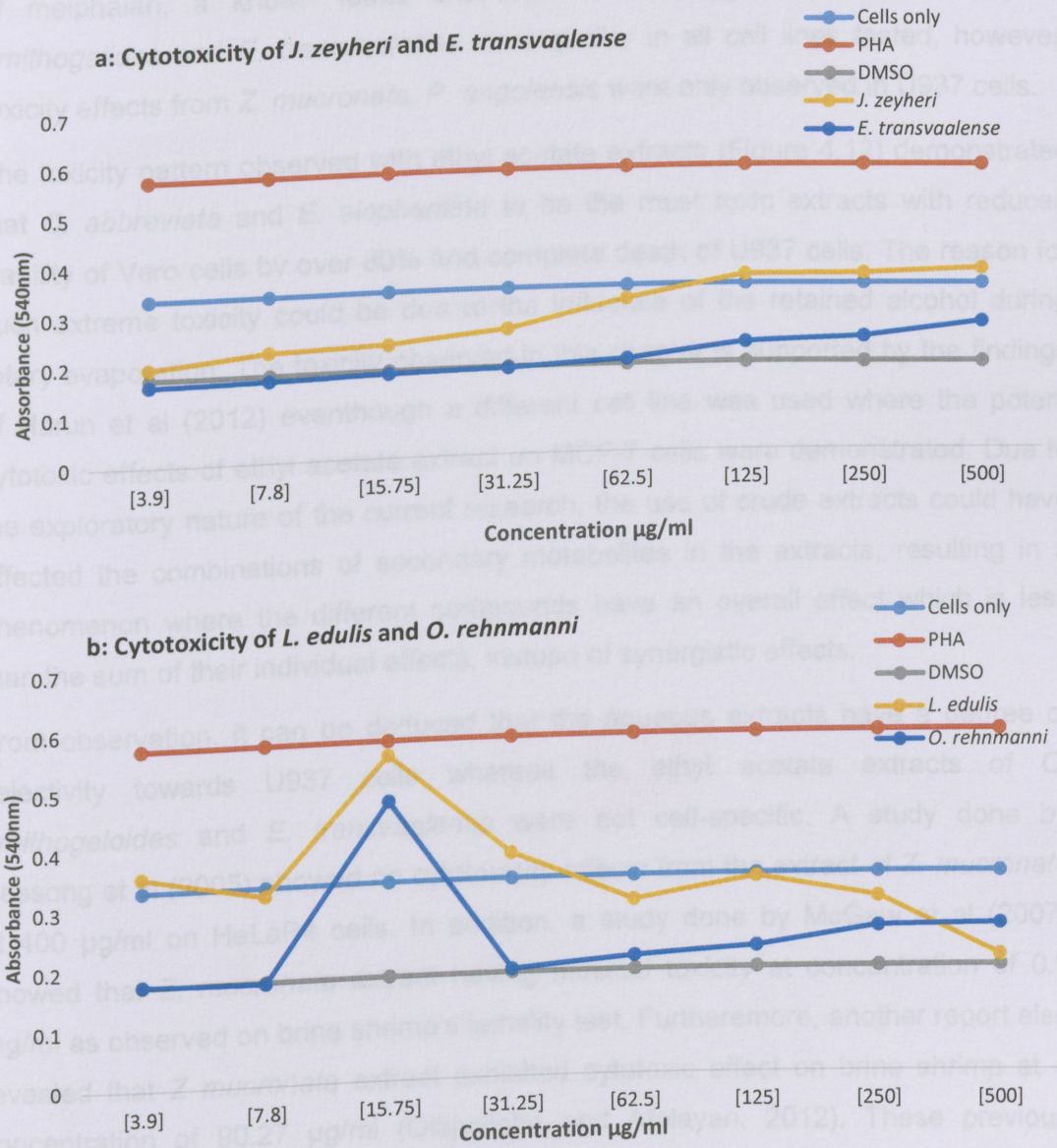



**Figure 4.15:** Cytotoxicity effects of (a) *J. zeyheri* and *E. transvaalense* (b) *L. edulis* and *C. rehmanii* on PBMCs. Phytohemagglutinins and DMSO were used as controls to confirm the functionality of the assay. The data represents the average of 3 experiments done in triplicate. Cytotoxicity was evaluated using MTT.



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The overall toxicity was more pronounced in the Vero cells (Figure 4.11) with a steep decline in cell viability by almost 70 – 80% from *Z. mucronata*, *P. angolensis*, *O. ornithogaloides* and *E. transvaalense*. The degree of toxicity was comparable to that of melphalan, a known lethal chemical. The toxicity effects exerted by *O. ornithogalides* and *E. transvaalense* were similar in all cell lines tested, however, toxicity effects from *Z. mucronata*, *P. angolensis* were only observed in U937 cells.

The toxicity pattern observed with ethyl acetate extracts (Figure 4.12) demonstrated that *C. abbreviata* and *E. elephantina* to be the most toxic extracts with reduced viability of Vero cells by over 80% and complete death of U937 cells. The reason for such extreme toxicity could be due to the influence of the retained alcohol during rotary evaporation. The toxicity observed in this chapter is supported by the findings of Harun et al (2012) eventhough a different cell line was used where the potent cytotoxic effects of ethyl acetate extract on MCF-7 cells were demonstrated. Due to the exploratory nature of the current research, the use of crude extracts could have affected the combinations of secondary metabolites in the extracts, resulting in a phenomenon where the different compounds have an overall effect which is less than the sum of their individual effects, instead of synergistic effects.

From observation, it can be deduced that the aqueous extracts have a degree of selectivity towards U937 cells whereas the ethyl acetate extracts of *O. ornithogaloides* and *E. transvaalense* were not cell-specific. A study done by Bessong et al (2005) showed no cytotoxicity effects from the extract of *Z. mucronata* at 400 µg/ml on HeLaP4 cells. In addition, a study done by McGaw et al (2007) showed that *Z. mucronata* extract having minimal toxicity at concentration of 0.9 mg/ml as observed on brine shrimp's lethality test. Furtheremore, another report also revealed that *Z mucronata* extract exhibited cytotoxic effect on brine shrimp at a concentration of 90.27 µg/ml (Olajuyigbe and Afolayan, 2012). These previous research findings contradict the results obtained in this chapter, however, the difference could be due to the different extraction solvents used in each study.

Over 50% of the aqueous plant extracts exhibited some extent of inhibitory effects with some statistical significance in both Vero and MeWo cell lines. A study done on *C. abbreviata* showed that methanolic extracts displayed elevated toxicity as observed in brine shrimp lethality test with $LC_{50}$ of 12.7 µg/mL (Mongalo and Mafoko, 2013). The ethanol extracts from the same plant were, however, less toxic with an



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$IC_{50}$ of 39,6. According to another study, the methanolic extract of *E. elephantina* exhibited an $LC_{50}$ of 1.8 which is considered toxic (Mpofu *et al.*, 2014). However, the individual effects of chloroform, ethyl acetate and methanol fractions of *E. elephantina* exhibited lower toxicities, with $LC_{50}$ of 10, 7.9, and 15, respectively.

## 4.5 SUMMARY

The cytotoxicity of medicinal plants was evaluated on different cell lines. The level of significance observed for the 4 extracts on U937 (cancer cell line) might render the extracts as great potentials for anti-cancer therapy. However, toxicity exerted on Vero and PBMCs might be of great concern. The toxicity data generated in this study can potentially be used to assess a possible human topical risk exposure to these extracts, however, further studies should focus on assessing the proliferation of cells over long term exposure.



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# PART IV: IMMUNO-MODULATORY EFFECTS OF PLANTS

## 5.1 BACKGROUND

An immunomodulator is any substance which is capable of suppressing or modulating any of the immune system components, including the innate and adaptive responses (Figure 5.1). The prospective uses of immunomodulators include reconstitution of immune deficiency and also the suppression of certain immune functions.

Immune-system modulation, thus, signifies any change in immune response that might involve induction, expression, amplification or inhibition of any parts of the system (Baxter, 2007). Immunomodulators are generally categorized into immunoadjuvants (those that enhance the efficacy of vaccine), immunostimulants (those that activate or induce the mediators of immune system) and immunosuppresants (those that suppress or reduce the strength of the body's immune system) (Mukherjee *et al.*, 2014).

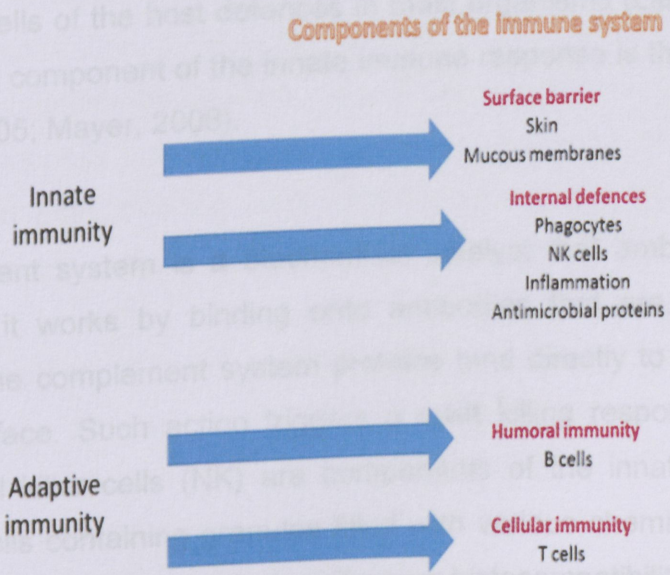



**Figure 5.1:** Flow chart showing components of the immune system



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The basic function of the immune system is to protect the body against foreign infectious agents. The factors that trigger the immune response include previous infections, immunizations and various stimuli that might be of external sources. Upon recognition of the foreign particle, the collective and coordinated response of mediators and specific cells, against the foreign particle constitutes the immune response (Baxter, 2007). Such action is achieved through, either innate or adaptive immunological mechanisms (Roshan and Savitri, 2013). The cell types that comprise the immune system have specialized functions; some can engulf bacteria while others kill parasites and also virus-infected cells. The action of these cells depends on the T-helper subset for activation signals in the form of cytokines, lymphokines and/or interleukins (Janeway Jr, 2001).

### 5.1.1 The innate immune system

Foreign particles that successfully enter an organism encounters the cells and mechanisms of the innate immune system which are triggered when these microbes are identified by pattern-recognition receptors (Medzhitov, 2007). The innate immune defences are non-specific (Alberts *et al.*, 2002); they do not give long-lasting immunity against pathogens previously encountered, although they are the authoritative cells of the host defences in most organisms (Litman *et al.*, 2005). The major humoral component of the innate immune response is the complement system (Rus *et al.*, 2005; Mayer, 2006).

The complement system is a biochemical catalyst that ambushes the surface of foreign cells; it works by binding onto antibodies that are, in turn, attached to microbes or the complement system proteins bind directly to carbohydrates on the microbes' surface. Such action triggers a swift killing response (Liszewski *et al.*, 1996). Natural killer cells (NK) are components of the innate immunity; they are lethal white cells containing granules filled with various chemicals (Figure 5.2). The NK cells recognizes cells that lack self-major histocompatibility complex molecules; they then destroy compromised host cells, including tumour and virus-infected cells (Janeway *et al.*, 2005).


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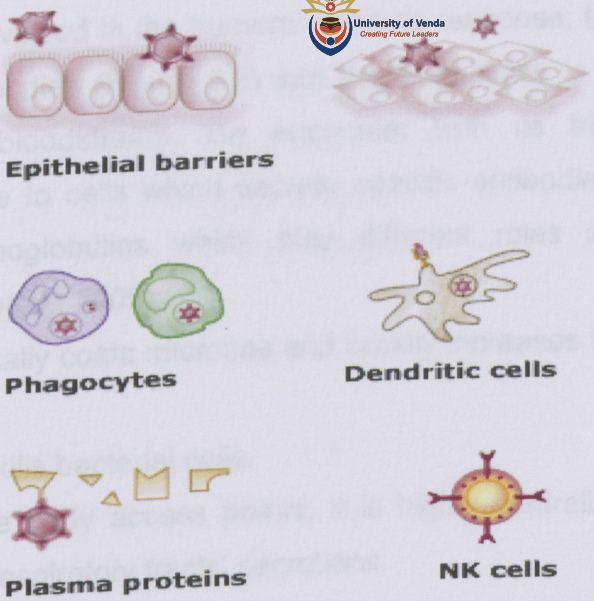



**Figure 5.2:** Components of the innate immune system (Gasqueet al., 2000)

Phagocytes are large white cells that are capable of swallowing and digesting microbes. Monocytes are types of phagocytes that flow freely in the bloodstream; upon reaching the tissues, they develop into macrophages. These macrophages rid the body of worn-out cells and other debris and the action is stimulated by production of monokines. Granulocytes contain granules filled with various substances which allow for the annihilation of microbes; Histamine is one of such chemicals. Neutrophil is a type of granulocyte which uses pre-packaged chemicals to break down ingested microbes. Eosinophils and basophils are granulocytes that degranulate by spraying their chemicals onto invading cells; blood platelets also contain substances that activate some of the immune defences (Brinkmann *et al.*, 2004).

### 5.1.2 The adaptive immune system

Adaptive immune system permits a much stronger immune response as well as immunological memory (Pancer and Cooper, 2006). The immune response is antigen-specific and it entails recognition of particular non-self-antigen through antigen presentation. The cells of the adaptive immune system are leukocytes also known as lymphocytes - B and T cells. These major types are acquired from hematopoietic stem cells in the bone marrow (Janeway *et al.*, 2005).


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B lymphocytes are involved in the humoral immune response; they function through the secretion of antibodies (Figure 5.3) into the body fluids to attack antigens that flow freely in the bloodstream. On encounter with its triggering antigen, B lymphocytes give rise to cells which secrete specific antibodies. These antibodies are basically immunoglobulins which play different roles in immune defence strategies (Janeway *et al.*, 2005).

- ❖ IgG systematically coats microbes and rapidly increases their uptake by other immune cells.
- ❖ IgM efficiently kills bacterial cells.
- ❖ IgA shields the body access points; it is highly centralized in tears, saliva, digestive and respiratory tracts' secretions.
- ❖ IgE shields against parasitic infections.
- ❖ IgD remains attached to B cells and plays a key role in initiating early B-cell response.

T lymphocytes are involved in cell-mediated immune response; their surfaces contain specialized antibody-like receptors that recognize small antigens' particles on the surface of infected or cancerous cells. These types of cells either directly regulate immune response or kill infected cells. Helper T cells (Th) coordinate immune responses by interacting with other cells. They enrich nearby B-cells with microbe-gobbling cells. Killer T cells (CTLs) or cytotoxic T lymphocytes directly attack other cells carrying foreign molecules. This type of T cells attacks mostly viruses since they recognise smaller fragments of viruses exposed on the cell membranes (Alberts *et al.*, 2002).

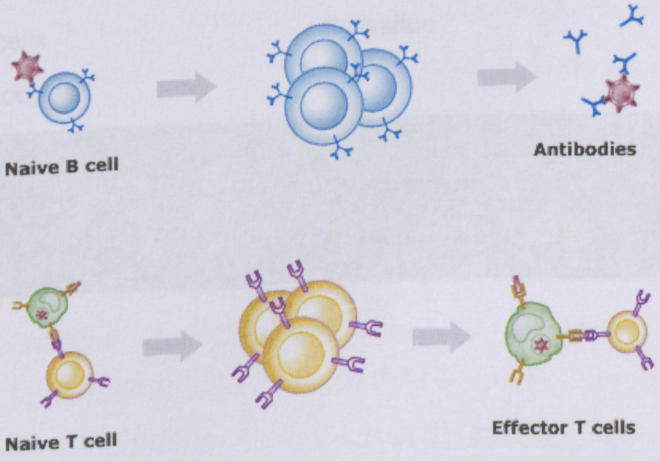



**Figure 5.3:** Cells of the adaptive immune response (Raulet, 2004)


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### 5.1.2.1 Effector cytokines

Components of the immune system interact with one another by exchanging cytokines (chemical messengers). These proteins are secreted by cells and act on other cells to induce a suitable immune response (Table 5.1). The cytokine types include interleukins, interferons and growth factors. Some of the cytokine are able to switch certain immune cell types, on and off.

Interleukin 2 (IL 2) prompts the immune system to manufacture T cells; its immunity-boosting abilities have triggered a promising treatment for several illnesses. Different cytokine families are classified with respect to their receptor type and also with respect to their structural and functional aspects (Sun *et al.*, 2016).

**Table 5.1:** Features of the selected cytokines (www.microbiologybook.org)

| Cytokine | Cell source | Primary effects | Cell target |
|---|---|---|---|
| TNF-α | Monocytes<br>Fibroblasts<br>Epithelial<br>Endothelial cells<br>Astrocytes<br>macrophages | Activates inflammation<br>Fever<br>Co-stimulatory<br>Acute phase reactant | T cells, B cells, Liver, Hypothalamus, endothelial cells |
| IL-2 | T cells,<br>NK cells<br>Monocytes | Growth<br>Activation | T cells, B cells, monocytes |
| IL-6 | T-cells<br>Fibroblasts<br>Macrophages | Growth<br>Co-stimulatory<br>Acute phase reactant | T cells, Liver, mature B cells |


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Cytokines IL2, IL 4, IL 7, IL 9 and IL 15 share a common receptor and function. They have a G chain receptor which is associated with the activation of the T lymphocytes growth. Cytokines IL-3, IL-5, and GM-CSF share the receptor of the B chain which is associated with the escalation and distinction of the hematopoietic precursors (Mui and Muyajima, 1994).

Cytokines are generally known as hormones of the immune system. They are involved in the innate immunity response since they induce macrophage and natural killer-cells activation, generating inflammatory and chemotaxis processes. They also play a role in the adaptive-immune response when they act on B and T lymphocytes. Within the cytokines are chemokines, interferons, interleukins, lymphokines and tumour necrosis factor (Paul and Seder, 1994). These cytokines are produced by a broad range of cells, including macrophages, B lymphocytes, T lymphocytes, mast cells, endothelial cells, fibroblasts and stromal cells. They act through receptors and modulate the balance between humoral and cell-based response (Silverstein, 2003).

## 5.2 MATERIALS AND METHODS

Cytokines are low-molecular-mass glycoproteins produced by immune cells in response to various activation stimuli. They are implicated in normal homeostasis and defence against many diseases and may contribute to the pathogenesis of a variety of disorders (Kantor *et al.*, 1992). Peripheral-blood mononuclear cells (PBMCs) serve as a convenient vehicle for cytokine assays mainly because of their accessibility. The evaluation of cytokine production was done using ELISA kits following the manufacturer's instructions (BD, Franklin lakes, NJ). Standard curves were prepared using recombinant cytokines provided in each kit.

PBMCs isolated (as described in Section 4.3.4) from both HIV-infected and non-infected individuals were stimulated in RPMI supplemented with 10% foetal bovine serum and PHA overnight. The cells were seeded at a density of 500 000 cells/well in a volume of 1 ml per well and exposed to 1 ml of plant extracts (50 µg/ml and 100 µg/ml) followed by an incubation for 72 hours in a 5% $CO_2$ incubator and 100% relative humidity (EcoTherm, Hartkirchen, Austria) at 37°C. Each treatment condition was tested in triplicate.


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At the end of the incubation period, 600 µl of media was removed from the bio-assay plate and stored for further testing. The remaining cultures were mixed and 200 µl were transferred to 96-well plates and 50 µl of XTT viability assay (Roche) was added to the wells and the plates were incubated for 5 hours. Thereafter absorbance was read at 475 nm and 660 nm using an ELISA spectrophotometer reader (Multi scan MS, Thermo Labsystems, Waltham, MA). The specific absorbance of the sample is expressed mathematically as follows:

Specific Absorbance = $A_{475nm}$(Test) – $A_{475nm}$(Blank) - $A660_{nm}$(Test)

Preserved supernatants from the bio-assay plates were used to assess cytokine production by the ELISA technique as per manufacturer's instruction. In brief, 50µl of the biotinylated antibody reagent was added to specific wells containing equal volume of sample and covered for incubation at room temperature for 2 hours. The washing step was repeated about 3 times before the addition of streptavidin HRP solution followed by another 30 minutes incubation. Prior to the addition of 100 µl TMB substrate, the plates were washed again and then developed for 30 minutes in darkness. To halt the reaction, 50 µl of the stop solution was added and then the absorbance was read at 450 nm.

All data, as described in Section 4.3.7, were representative of three independent experiments performed in triplicate and presented as mean ± standard deviation (SD). The mann–Whitney test and one-way analysis of variance (ANOVA) was used for the evaluation of statistical differences between the results and p values; $< 0.05$ were considered significant.


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## 5.3 RESULTS AND DISCUSSION

The therapeutic effects of medicinal plants might occur via their influence on the immune response. It has been shown that some plants can affect the immune responses through their anti and/or pro inflammatory actions. Cytokines are diverse molecules which can be significant in a negative or positive way. They are responsible for the survival or death of cells, cell replication, differentiation and transformation (Hirano *et al.*, 2000). Many of the cytokines possess stimulatory or inhibitory effects, hence, they are able to work synergistically with each other (Matsumoto and Kanmatsuse, 2000). The effects of the chosen plant extracts on the production of IL-2, IL-6 and TNF-α by PBMCs were studied because of the putative roles these cytokines play in health and disease progression.

### 5.3.1 Viability of cells (non-infected and HIV-infected cells)

Viability of both the non-infected and infected cells (Figure 5.4) was confirmed using XTT. The viability of the healthy cells grown in presence of *O. holstii* at both concentrations was low.

The effects of plant extracts on the viability of non-infected cells showed a decrease in the number of viable cells compared to the cells-only control at 100 and 50 µg/ml (Figure 5.4-a). The effects of *O. holstii* at both concentrations resulted in the least number of viable cells in comparison to other extracts. *Cassia abbreviata*, *E. elephantina*, *O. ornithogaloides*, *J. zeyheri*, *E. transvaalense* and *L. edulis* at 100 µg/ml had more surviving cells as compared to the cells-only control. For all the extracts, survival was more pronounced at 100 µg/ml.

Survival of the HIV-infected PBMCs in the presence of plant extracts was lower compared to the non-infected cells (Figure 5.4-b). Viable cells after exposure to *C. abbreviata* and *O. ornithogaloides* were 18 and 13%, respectively, at 100 µg/ml concentration. The survival of cells exposed to *C. rehmannii* plant extract showed the least percentage as compared to other extracts. The extracts of *O. holstii*, *Z. mucronata*, *P. angolesis*, *E. transvaalense*, *L. edulis*, *and J. zeyheri* viable cells were below 10% with an exception of *E. elephantina* (11%). As observed in the non-


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infected PBMCs, HIV-infected cells treated with 100 µg/ml survived better than those treated with 50 µg/ml.

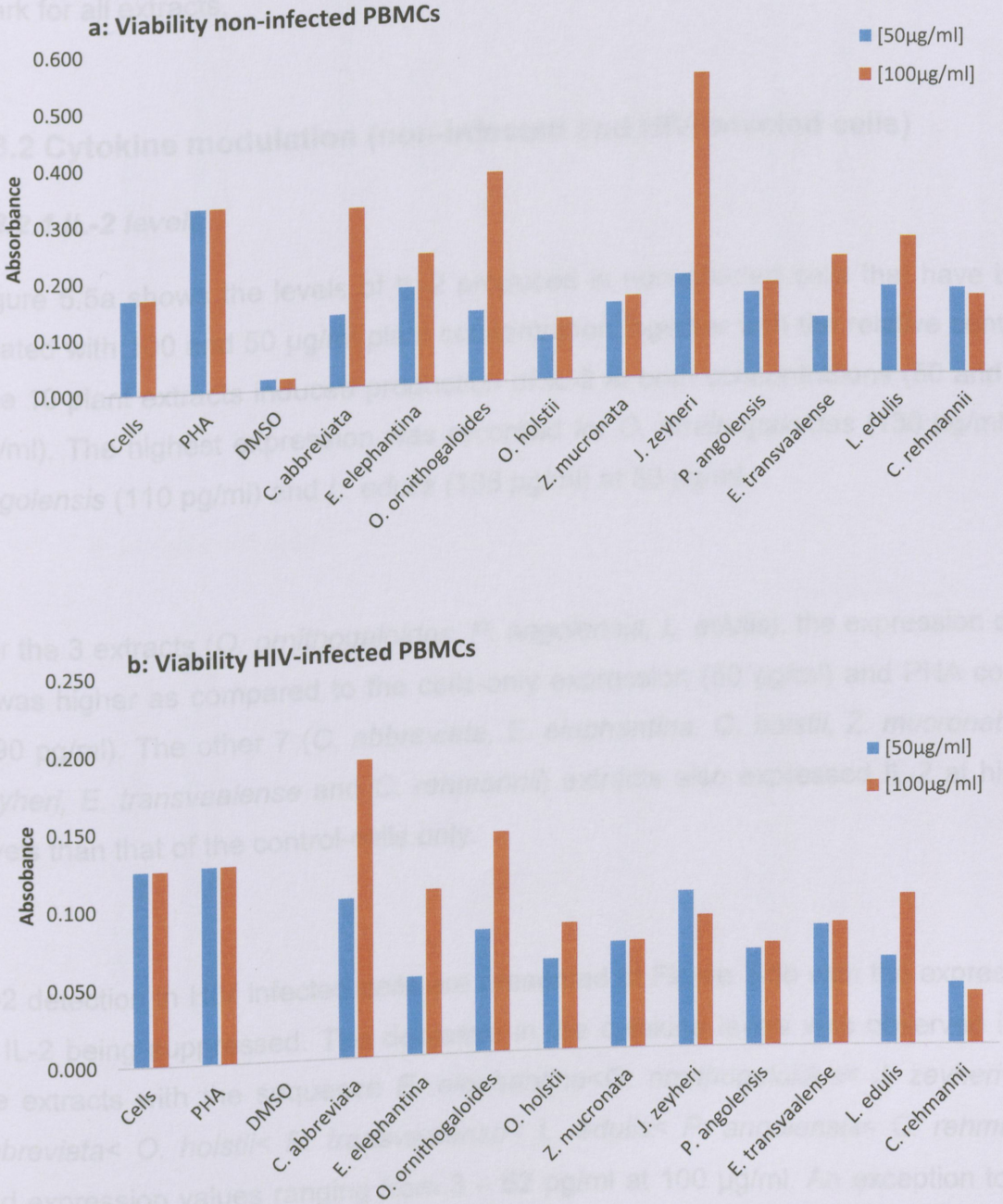



**Figure 5.4:** Effects of aqueous extracts on the viability of non-infected (a) and HIV-infected (b) PBMCs. Phytohemagglutinins and DMSO were used as controls to confirm the functionality of the assay. The data represents the average of three experiments done in triplicate.


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The toxicity observed might be due to the presence of toxic compounds in higher concentrations. The length of incubation time might also account for the decrease in viability. As for the HIV-infected cells, the expression or replication of the viral particles played a major role in the excessive cell death observed below the 20% mark for all extracts.

## 5.3.2 Cytokine modulation (non-infected and HIV-infected cells)

### *5.3.2.1 IL-2 levels*

Figure 5.5a shows the levels of IL-2 produced in non-infected cells that have been treated with 100 and 50 µg/ml plant concentration together with the relative controls. The 10 plant extracts induced production of IL-2 at both concentrations (50 and 100 µg/ml). The highest expression was recorded for *O. ornithogaloides* (130 pg/ml), *P. angolensis* (110 pg/ml) and *L. edulis* (105 pg/ml) at 50 µg/ml.

For the 3 extracts (*O. ornithogaloides, P. angolensis, L. edulis*), the expression of IL-2 was higher as compared to the cells-only expression (50 pg/ml) and PHA control (±90 pg/ml). The other 7 (*C. abbreviata, E. elephantina, O. holstii, Z. mucronata, J. zeyheri, E. transvaalense* and *C. rehmannii*) extracts also expressed IL-2 at higher levels than that of the control-cells only.

IL-2 detection in HIV infected cells are presented in Figure 5.5b with the expression of IL-2 being suppressed. The decrease in the cytokine levels was observed in all the extracts with the sequence *E. elephantina*<*O. ornithogaloides*< *J. zeyheri*< *C. abbreviata*< *O. holstii*< *E. transvaalense*< *L. edulis*< *P. angolensis*< *C. rehmannii* and expression values ranging from 3 – 62 pg/ml at 100 µg/ml. An exception to the decreasing trend was noted for *Z. mucronata* at lower concentration expressing 75 pg/ml, which is 10 pg/ml more than that of the control cells.


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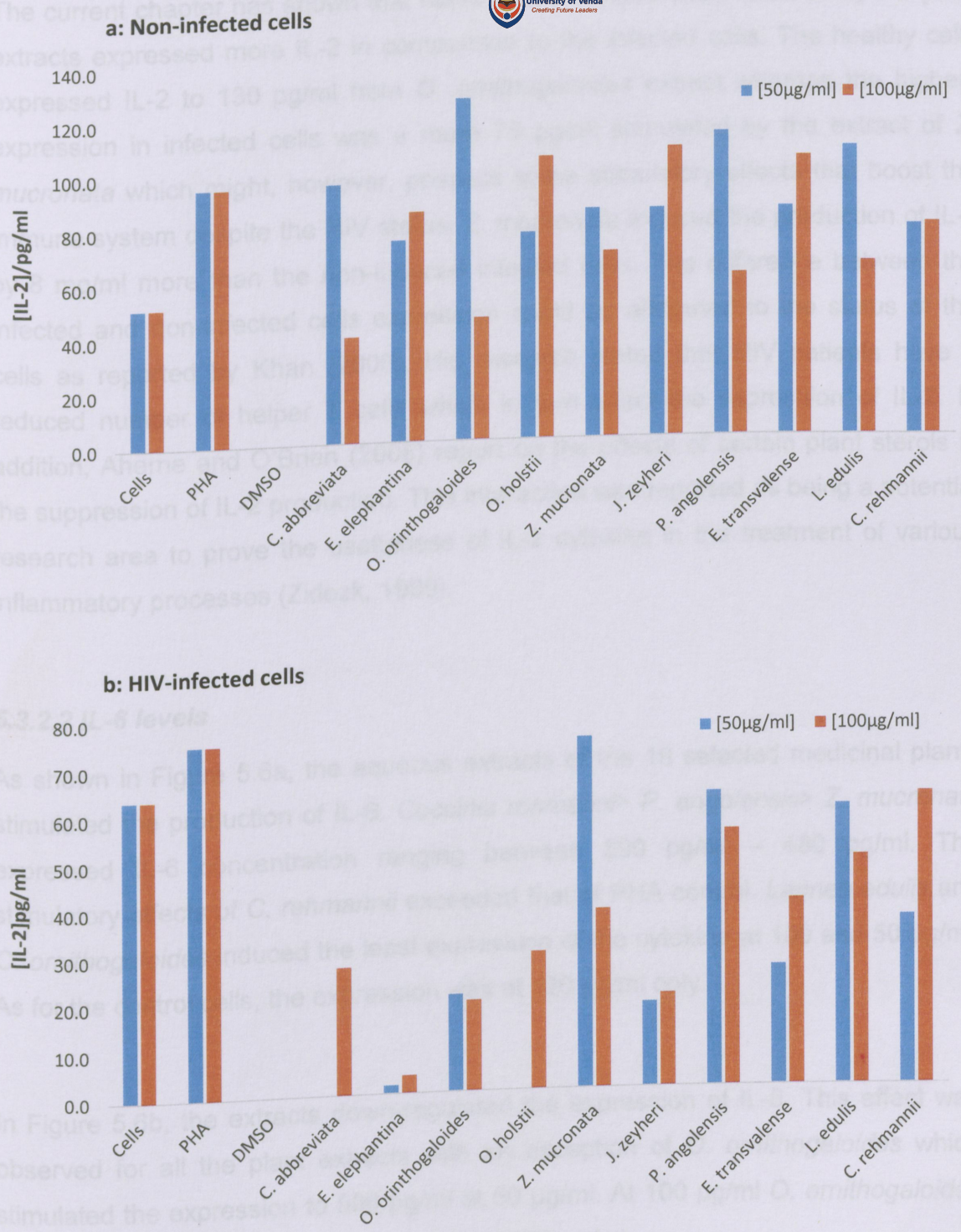



**Figure 5.5:** Effects of aqueous extracts on the production of IL-2 in non-infected (a) and HIV-infected (b) PBMCs. Phytohemagglutinins and DMSO were used controls to confirm the functionality of the assay. The data represent the average of three experiments done in triplicate.


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The current chapter has shown that non-infected PBMCs when induced by the plant extracts expressed more IL-2 in comparison to the infected cells. The healthy cells expressed IL-2 to 130 pg/ml from *O. ornithogaloides* extract whereas the highest expression in infected cells was a mere 75 pg/ml stimulated by the extract of *Z. mucronata* which might, however, possess some stimulatory effects that boost the immune system despite the HIV status. *Z. mucronata* induced the production of IL-2 by 8 mg/ml more than the non-induced infected cells. The difference between the infected and non-infected cells expression could be attributed to the status of the cells as reported by Khan (2008). His research stated that HIV patients have a reduced number of helper T cells which in turn affect the expression of IL-2. In addition, Aherne and O'Brien (2008) report on the effects of certain plant sterols in the suppression of IL-2 production. This interaction was reported as being a potential research area to prove the usefulness of IL-2 cytokine in the treatment of various inflammatory processes (Zidezk, 1999).

### *5.3.2.2 IL-6 levels*

As shown in Figure 5.6a, the aqueous extracts of the 10 selected medicinal plants stimulated the production of IL-6. *Coccinia rehmanni*> *P. angolensis*> *Z. mucronata* expressed IL-6 concentration ranging between 590 pg/ml – 480 pg/ml. The stimulatory effects of *C. rehmannii* exceeded that of PHA control. *Lannea edulis* and *O. ornithogaloides* induced the least expression of the cytokine at 100 and 50 µg/ml. As for the control cells, the expression was at 120 pg/ml only.

In Figure 5.6b, the extracts down-regulated the expression of IL-6. This effect was observed for all the plant extracts with an exception of *O. ornithogaloides* which stimulated the expression to 500 pg/ml at 50 µg/ml. At 100 µg/ml *O. ornithogaloides* extract also induced an expression of about 350 pg/ml.


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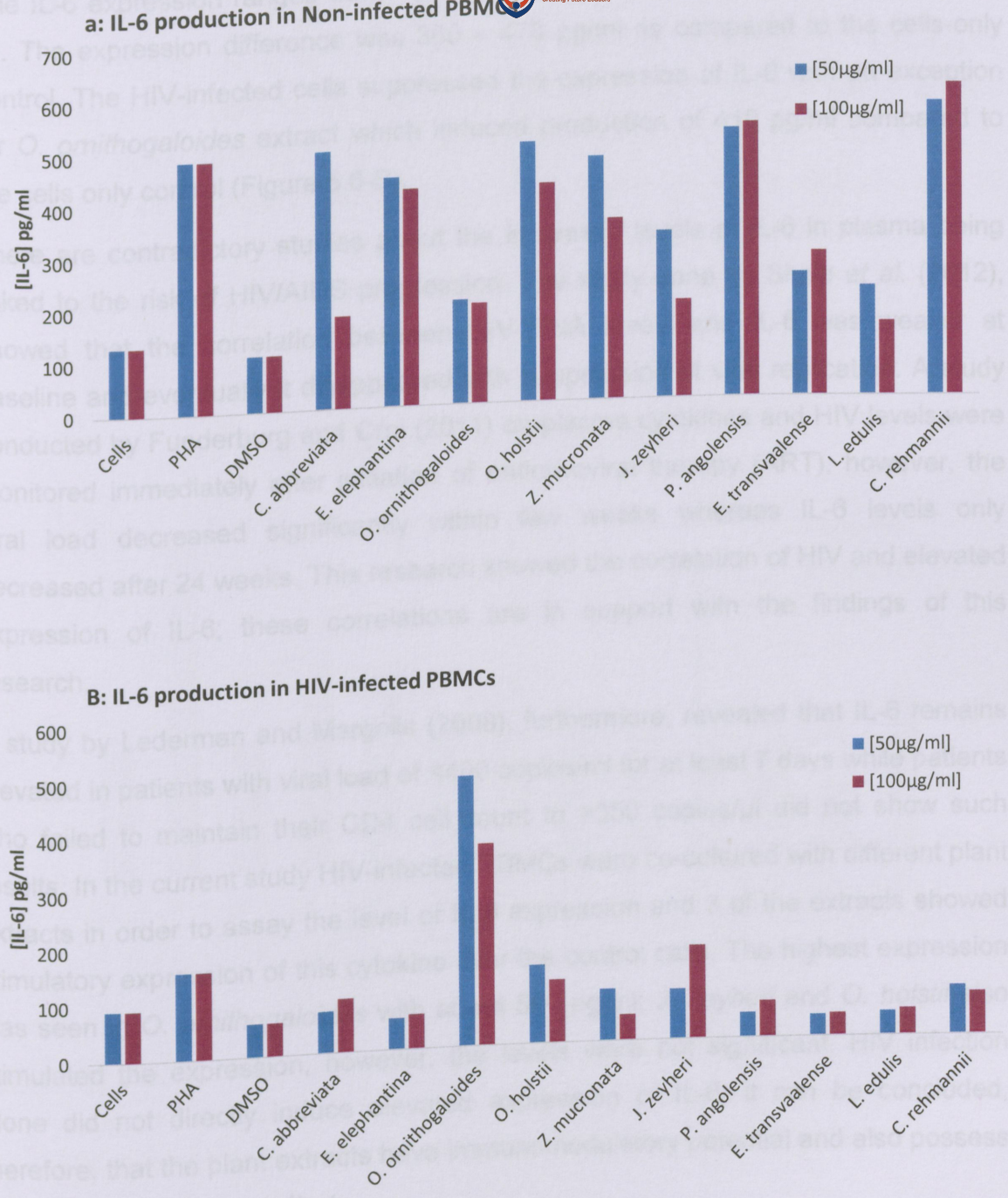



**Figure 5.6:** Effects of aqueous extracts on the production of IL-6 in non-infected (a) and HIV-infected (b) PBMCs. Phytohemaglutinins and DMSO were used as controls to confirm the functionality of the assay. The data represents the average of three experiments done in triplicate.


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The IL-6 expression ranged from 480 – 590 pg/ml in non-infected cells (Figure 5.6 A). The expression difference was 360 – 470 pg/ml as compared to the cells-only control. The HIV-infected cells suppressed the expression of IL-6 with an exception for *O. ornithogaloides* extract which induced production of 410 pg/ml compared to the cells only control (Figure 5.6-B).

There are contradictory studies about the increased levels of IL-6 in plasma being linked to the risk of HIV/AIDS progression. The study done by Shive *et al.* (2012), showed that the correlation between HIV RNA levels and IL-6 was weaker at baseline and eventually it disappeared with suppression of viral replication. A study conducted by Funderburg and Croi (2011) on plasma cytokines and HIV levels were monitored immediately after initiation of antiretroviral therapy (ART), however, the viral load decreased significantly within few weeks whereas IL-6 levels only decreased after 24 weeks. This research showed the correlation of HIV and elevated expression of IL-6; these correlations are in support with the findings of this research.

A study by Lederman and Margolis (2008), furthermore, revealed that IL-6 remains elevated in patients with viral load of <400 copies/ml for at least 7 days while patients who failed to maintain their CD4 cell count to >350 copies/µl did not show such results. In the current study HIV-infected PBMCs were co-cultured with different plant extracts in order to assay the level of IL-6 expression and 3 of the extracts showed stimulatory expression of this cytokine over the control cells. The highest expression was seen in *O. ornithogaloides* with about 500 pg/ml; *J. zeyheri* and *O. holstii* also stimulated the expression, however, the levels were not significant. HIV infection alone did not directly induce elevated expression of IL-6, it can be concluded, therefore, that the plant extracts have immunomodulatory potential and also possess some pro-inflammatory effects.

Previous studies have suggested that dysregulation of IL-6 expression contributes greatly to the pathogenesis of chronic inflammatory diseases. Elevated IL-6 levels have been linked to some adverse clinical outcomes in HIV-infected individuals (Borges *et al.*, 2014).


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### 5.3.2.3 TNF-α levels

The production of TNF-α by non-infected cells is shown in Figure 5.7. At 50 µg/ml, the extract of *P. angolensis* induced production of 36 pg/ml. The production of TNF-α at 100 µg/ml recorded above that of cells-only was for *C. abbreviata*, *E. elephantina*, *C. rehmannii* and *O. holstii* at 26 pg/ml, 21 pg/ml, 18 pg/ml and 23 pg/ml, respectively. For the other plant extracts, their stimulatory effects yielded < 18 pg/ml which is comparable to the yield by cells-only.

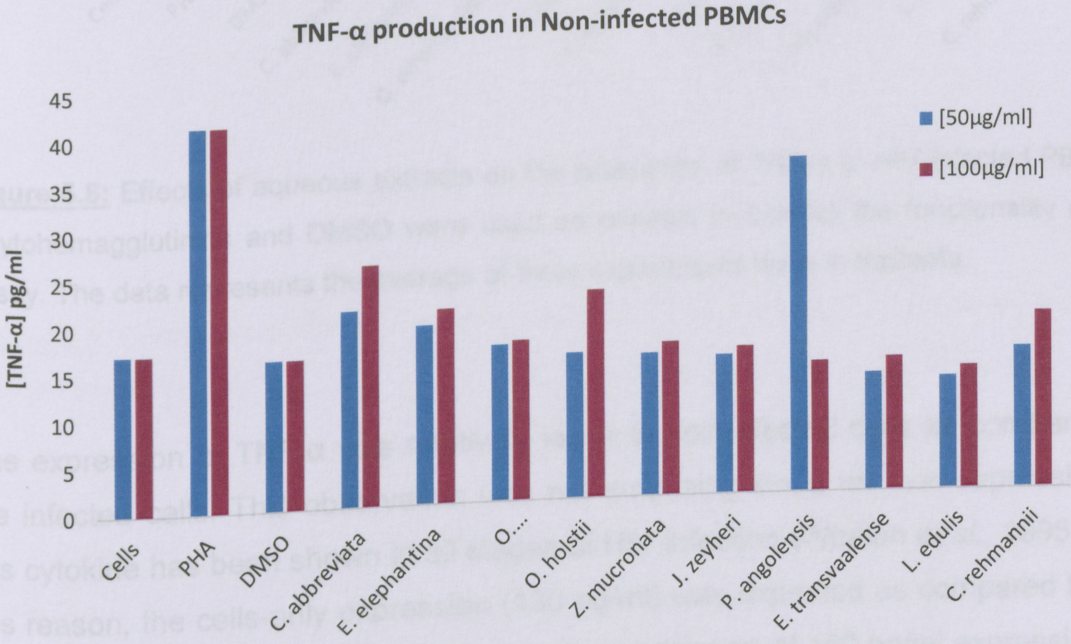



**Figure 5.7:** Effects of aqueous extracts on the production of TNF-α in non-infected PBMCs. Phytohemagglutinins and DMSO were used as controls to confirm the functionality of the assay. The data represents the average of three experiments done in triplicate.

The levels of TNF-α in infected cells were higher (Figure 5.8). The cells-only control yielded 130 pg/ml while *C. abbreviata* at 100 µg/ml stimulated production of about 150 pg/ml. The extracts of *Z. mucronata* and *O. ornithogaloides* expressed 130 pg/ml. For all the extracts, expression of TNF-α was upregulated. The least expression was observed in *C. rehmannii* at 99 pg/ml at 100 µg/ml.


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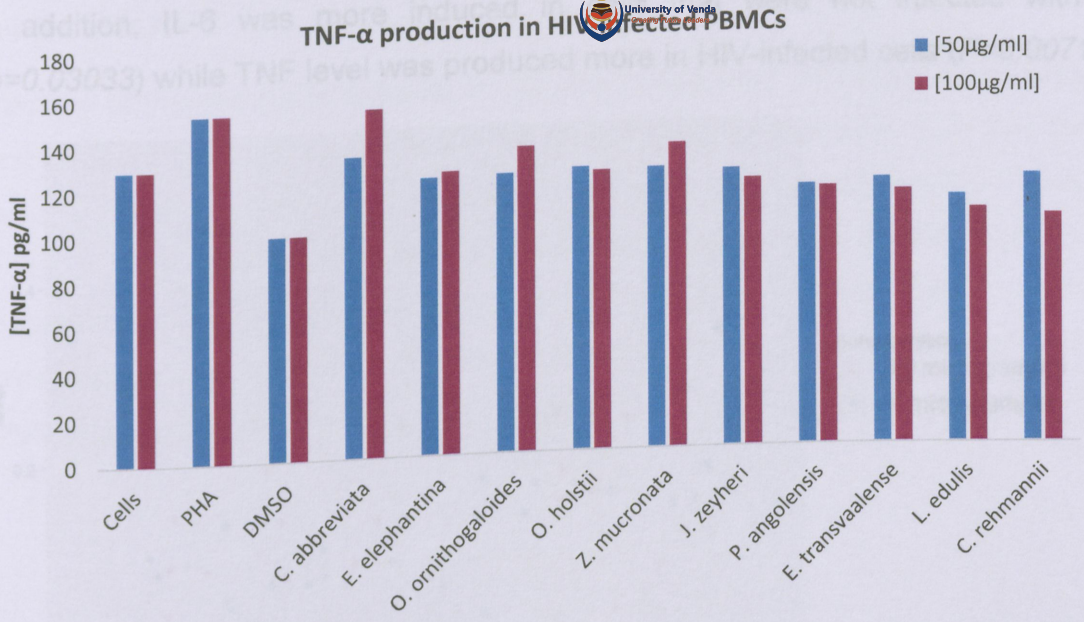



**Figure 5.8:** Effects of aqueous extracts on the production of TNF-α in HIV-infected PBMCs. Phytohemagglutinins and DMSO were used as controls to confirm the functionality of the assay. The data represents the average of three experiments done in triplicate.

The expression of TNF-α was relatively lower in non-infected cells as compared to the infected cells. This observation was not surprising since an over-expression of this cytokine has been shown in all stages of HIV infection (Whalen *et al.*, 1995). For this reason, the cells-only expression (130 pg/ml) was expected as compared to the 17 pg/ml in non-infected control cells and the modulation of 150 pg/ml expression by *C. abbreviata* shows this extract is a good stimulant of TNF-α. *Cassia abbreviata* extract when assayed in both the infected and non-infected cells showed elevated expression in infected cells and decreases expression in healthy cells.

### 5.3.3 Significance of results

The difference observed in viability between non infected and HIV-infected PBMCs was not statistically significant ($p=0.21025$) (Figure 5.9). There was a statistical significance in the different responses to HIV infection for the two independent variables, HIV status and the three cytokines (TNF, IL-6 and IL-2) measured (Figure 5.10). TNF and IL-6 responses were more pronounced than that of IL-2 ($p=0.02723$).


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In addition, IL-6 was more induced in cells that were not infected with HIV (*p=0.03033*) while TNF level was produced more in HIV-infected cells (*P=0.00716*).




**Figure 5.9:** Comparative effects of aqueous extracts on the viability of non-infected and HIV-infected PBMCs. PHA.

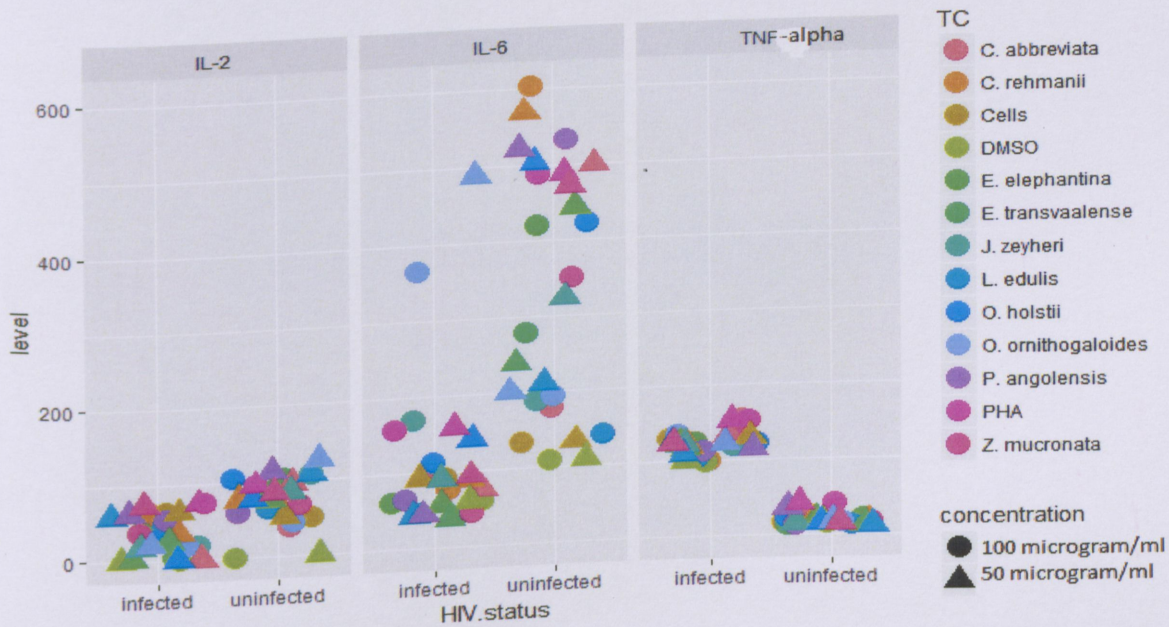



**Figure 5.10:** Comparative effects of aqueous extracts on the production of cytokines in non-infected and HIV-infected PBMCs.


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## 5.4 SUMMARY

The use of various plant extracts in specific doses may be helpful in inducing the host to produce molecules that will be able to protect the cells from any form of damage. This study showed that the plant extracts induced healthy PBMCs to produce more IL-2 as compared to HIV-infected cells. Instead the infected cells suppressed the expression of IL-6 which is believed to be a marker for HIV/AIDS disease progression. Cassia abbreviata proved to be a good stimulant of TNF-α, a cytokine proven to be produced in all stages of HIV infection.


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# Part V: PLANTS EFFECTS ON PATHOGENS

## 6.1 BACKGROUND

Long ago in the history of humankind, there has been a notion that certain plants had healing potential (Kelly, 2009). Such plants were believed to contain what we would characterize as 'antimicrobial principles' in the current times. Since ancient times, people have used plants to treat ordinary infectious diseases. Some of these traditional medicines are still being used in the treatment of various infections on regular basis (van Wyk and Gericke, 2000).

An antimicrobial agent is primarily known as an agent that kills or inhibits the multiplication of microorganisms (Figure 6.1). Classification of antimicrobial agents can be based on the basic function of the agents that are either microbicidal or biostatic (Heine *et al.*, 2007).

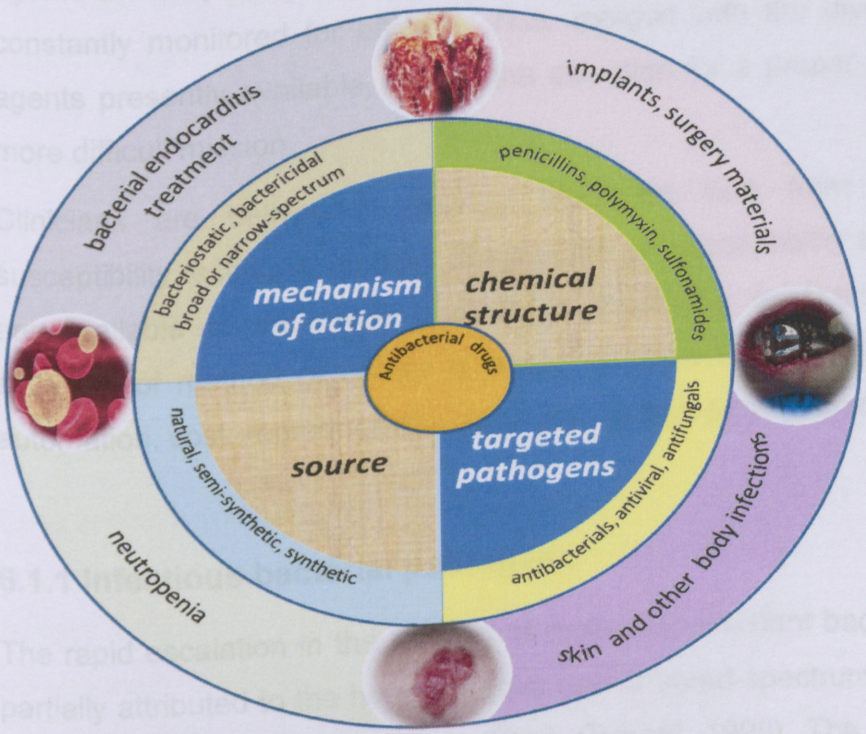



**Figure 6.1:** Different sources of antimicrobial drugs and the targeted pathogens (Wainwright, 1998).



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Antimicrobial susceptibility testing can be conducted with phenotypic or genotypic methods. Phenotypic methods are solely based on the minimum inhibitory concentration (MIC). Clinical MIC breakpoints are essential in determining whether the organism is classified as either susceptible, intermediate or resistant to the tested agent. Due to several reasons, the circulation of antimicrobial-resistant pathogenic bacteria has been recognized by the World Organization for Animal Health (OIE), the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) as a life-threatening universal health problem (Walsh, 2003; 2006).

The evolution of bacterial antimicrobial resistance is neither an unforeseen nor a new incident, however, it is a progressively distressing condition since the frequency for new emerging resistance phenotypes is occurring in almost all bacterial pathogens and even in commensal organisms. Numerous infections have been treated efficiently based on the clinician's past clinical experience, however, this kind of practice is becoming more of an exception than the rule (Walker, 2007). Resistance has been perceived to be a critical aspect and it is essential that the antimicrobial agents currently approved for use against human and animal diseases needs to be constantly monitored for efficacy. This, merged with the diversity of antimicrobial agents presently available, makes the selection for a proper agent an increasingly more difficult mission.

Clinicians are belived to depend more on data from *in-vitro* antimicrobial susceptibility testing. A number of antimicrobial susceptibility testing (AST) methods are available to determine bacterial susceptibility to antimicrobial agents. The selection of method of choice is based on factors such as practicality, flexibility, automation, cost, reproducibility, accuracy, as well as individual preference.

### 6.1.1 Infectious bacterial pathogens

The rapid escalation in the number of multi-drug resistant bacterial strains might be partially attributed to the haphazardous use of broad-spectrum antibiotics (Dean and Burchard, 1996; Gonzalez *et al.*, 1996; Graybill, 1998). The chosen plant extracts were tested for potential activity against selected bacterial pathogens (*Enterococcus*


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*faecalis, Escherichia coli, Klebsiella pneumoniae, Salmonella enterica, Streptococcus agalactiae*).

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#### 6.1.1.1 *Enterococcus faecalis*

Enterococci form part of the human/ animal normal intestinal flora, although, they have been long identified as one of the important human pathogens. They are increasingly becoming serious human pathogens (de Perio *et al., 2006*). The genus *Enterococcus* comprises of over 17 species of which not many cause clinical infections in humans. Since the dawn of the antibiotic era, major therapeutic challenges have emerged (Rice, 2001). These include the need for synergistic integration of antibiotics to efficiently treat enterococcal infective endocarditis (IE) (Hindler *et al.*, 2015). *Enterococcus* species are facultative anaerobes which are capable of surviving at 60°C, although, only for short periods of time (Courvalin, 2006; de Perio *et al.*, 2006; Deshpande *et al.*, 2007).

Previously they were classified as group D streptococci until they were allocated their own genus (Courvalin, 2006; De Perio *et al.*, 2006; Deshpande *et al.*, 2007). *Enterococcus faecalis* and *Enterococcus faecium* are the most prevailing species cultured from human samples. They account for over 90% of clinical isolates. Other enterococcal species known to cause human infection include *Enterococcus avium, E. gallinarum, E. casseliflavus, E. durans, E. raffinosus,* and *E. mundtii (De Perio et al., 2006). E. faecium* accounts for almost all vancomycin-resistant enterococci (VRE) infections.

Enterococci have both intrinsic and acquired resistance to antibiotics, making them important nosocomial pathogens. Intrinsically, Enterococci endure or resist beta-lactam antibiotics since they contain penicillin-binding proteins (PBPs). Such components enables them to continue synthesizing some cell-wall components. They use folic acid in the cell to assist them to bypass the inhibition of folate synthesis. This in turn results in resistance to trimethoprim-sulfamethoxazole (Hindler *et al.*, 2015).



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### 6.1.1.2 *Escherichia coli*

*Escherichia coli (E. coli)* is a bacterium that thrives in the digestive tracts of humans and animals. It is a peritrichous flagellated Gram negative rod, although some strains are non-motile. *Escherichia coli* is one of the most common causes of almost all bacterial infections such as cholecystitis, bacteremia, cholangitis, urinary tract infection (UTI), and traveller's diarrhoea (Voravuthikunchai and Limsuwan, 2006). It also causes other clinical infections such as neonatal meningitis and pneumonia. The vast majority of neonatal meningitis cases that are caused by *E. coli* and group B streptococcal infections are at 28.5% and 34.1%, respectively (Harrington *et al.*, 2006; Pitout and Laupland, 2008).

Pregnant women are at more risk of colonization with the K1 capsular antigen strain of *E. coli*; this strain is prevalent in neonatal sepsis and it carries a mortality rate of 8% (Samoilova *et al.*, 2014). In adults, *E. coli* meningitis is scarce yet may occur following neurosurgical trauma or complications of *Strongyloides stercoralis* hyper-infection involving the central nervous system (Gold, 1983; Agustin *et al.*, 1994).

### 6.1.1.3 *Klebsiella pneumoniae*

The genus *Klebsiella* belongs to the family Enterobacteriaceae. *Klebsiella* are basically non-motile, rod-shaped, Gram-negative bacteria with an eminent polysaccharide capsule that encapsulates the entire cell surface. The capsule accounts for the large morphological appearance of the organism when Gram-stained and gives resistance towards many host-defence mechanisms (Sharma *et al.*, 2009). Members of the genus *Klebsiella* commonly produce 2 types of antigens on their cell surface. The first is a lipopolysaccharide (O antigen) and the other is a capsular polysaccharide (K antigen). Pathogenicity of the organisms is attributed to existence of both of these antigens. The degree of virulence of all the serotypes appears to be indistinguishable (Mandell, 2009).

Three species in the genus *Klebsiella* are known for causing human illnesses. These include *Klebsiella pneumoniae, Klebsiella oxytoca,* and *Klebsiella granulomatis.*


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Organisms previously known as *Klebsiella ozaenae* and *Klebsiella rhinoscleromatis* are considered non-fermenting subspecies of *K pneumoniae* that show characteristic clinical presentations. With those exceptions, strains within this genus ferment lactose, and almost all secrete very mucoidy colonies on agar plates. This is attributed to the production of a rich polysaccharide capsule. All species within the genus are non-motile (Mandel, 2009). In recent years, *Klebsiella* has become an important pathogen in nosocomial infections (Nordmann *et al.*, 2009).

### 6.1.1.4 *Salmonella enterica*

Salmonellae are Gram-negative motile bacilli. The genus *Salmonella* was named after Daniel E. Salmon, an American veterinarian who discovered *Salmonella choleraesuis* from pigs with hog cholera in 1884. The genus belongs to the family Enterobacteriaceae (Crum-Cianflone, 2008).

There are over 2500 serovars of *S. enterica* which have been discovered (Andrews-Polymenis *et al.*, 2010). Out of the 2500, *S. enterica* is a major cause of foodborne illnesses, especially, in third world countries. This organism is known to cause periodic outbreaks of Salmonellosis in the developed world. The transmission of salmonellae commonly occurs through consumption of foods contaminated with the organism which can cause 4 different clinical manifestations (Ryan *et al.*, 2004).

The most common food sources is beef, poultry, and eggs. Research has shown that there are about 1.3 billion cases of non-typhoidal salmonellosis each year (Bronze and Greenfield, 2005). Consumption of contaminated egg shell fragments accounted for approximately 182,060 cases of enteritis in the United States in the year 2000 (Bronze and Greenfield, 2005; Connor and Schwartz, 2005). Some transmission patterns could be through improperly prepared fruit salads, vegetables, dairy products, and shellfish. Outbreaks have implicated these transmission patterns as sources of *Salmonella infections* (Crum-Cianflone, 2008).


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Powdered baby formula has been implicated in two large outbreaks of *S. enterica* serotype Agona among infants in France (Brouard *et al.*, 2007). In addition, human exposure to infected amphibians and reptiles is associated with approximately 74,000 *Salmonella* infections each year in the United States.

Salmonellosis outbreaks have also been associated with handling of chicks, ducklings, kittens, and hedgehogs (Woodward *et al.*, 1997; Mermin *et al.*, 2005).

#### 6.1.1.5 *Streptococcus agalactiae*

Streptococci are Gram-positive, non-motile; non-spore-forming organisms. *Streptococcus agalactiae*, is a well-known causative agent of postpartum infection and a leading cause of neonatal sepsis. Group B streptococcal infection is extremely scarce in healthy adults but is prevalent in young and middle-aged women. Infections by *S. agalactiae* in most cases are always associated with other underlying conditions such as diabetes (Huang *et al.*, 2006).

These organisms colonize the vaginal area, gastro-intestinal tract as well as the upper respiratory tract of healthy humans. *S agalactiae* is a Gram-positive coccus that forms glistering greyish colonies surrounded by a beta hemolysis when grown on sheep blood agar. It is an encapsulated organism which usually takes advantage of immunocompromised hosts. Development of infection in the absence of underlying medical conditions with Group B streptococcal is rare. The virulence of *S agalactiae* is associated with the polysaccharide toxin it produces. Immunity against it is serotype specific and is mediated by antibodies to the capsular polysaccharide (Marasini *et al.*, 2015). The portal of entry is not evident, yet the suspected possible areas include the skin, genital tract, urinary tract, and respiratory tract (Stevens *et al.*, 1989).

### 6.1.2 *Mycobacterium tuberculosis*

Tuberculosis (TB) is a disease caused by *M. tuberculosis*. This bacteria is airborne and can spread from person to person through aerosols. It commonly affects the lungs, but it can also affect other parts of the body, such as the brain, the kidneys or


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the spine (Bloom, 1994; Friedman, 2001). In almost all cases, TB is treatable and curable; however, people with TB can die if they do not get adequate treatment (Grange, 1996; Nwanga et al, 2005; Nguta *et al.*, 2015). Tuberculosis is one of the major life-threatening infections recorded in South Africa. It still remains one of the top killers amongst the different infectious diseases. Statistically, it is recorded that TB epidemic claims up to two million people each year worldwide. Furthermore statistics have recorded that an alarming 9.6 million new cases occured in 2014 (WHO, 2014).

The daunting task is the inadequate identification of people who are carriers of latent TB infection because they stand a greater chance of developing the disease (Getahun *et al.*, 2015). There are still major challenges around the disease, such as, in poor identification and diagnosis, inappropriate management of the infectious cases and issue of long term treatment especially for cases involving the multidrug resistant (MDR) TB strains (Monedero, 2014).

### 6.1.3 Human Immunodeficiency Virus (HIV)/AIDS

In many countries, the use of anti-HIV medicinal remedies and other natural products as part of HIV/AIDS regimen is an extremely delicate and controversial issue (Chinsembu, 2009). Many of the HIV-infected people in South Africa have access to antiretroviral drugs, but those who believe in natural products take the concoctions simultaneously. Recently, medicinal plants have become a paramount source of discovering pharmacologically active compounds (Rates, 2001).

The traditional remedies have been reported to be effective in the treatment of different ailments including cancer, diabetes, HIV/AIDS and other chronic as well as minor infections (from Interviews). The research focus usually targets the enzymes that constitute the polymerase gene; that is the protease (PR), integrase (IN) and reverse transcriptase (RT). Reverse transcriptase was first discovered by Howard Temin and David Baltimore (Coffin and Fan, 2016). This enzyme is present in reverse transcribing viruses, such as the human immunodeficiency virus (HIV) and


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the hepatitis B virus (Zhu *et al.*, 2001). The frequently-used RTs are the AMV reverse transcriptase from the avian myeloblastosis virus and the M-MuLV reverse transcriptase from the Moloney murine leukemia virus (Roth *et al.*, 1985). Reverse transcription is initiated when a viral particle enters the cytoplasm of a target cell.

The viral RNA genome enters the cytoplasm compacted in an uncharacterized nucleoprotein complex (Figure 6.2). The process of reverse transcription produces a linear DNA duplex generated via a series of steps. The generated DNA is co-linear with its RNA template, and it contains terminal duplications known as the long terminal repeats (LTRs). It is proposed that the LTRs are generated through strand-transfer reactions (Czernilofsky *et al.*, 1980).

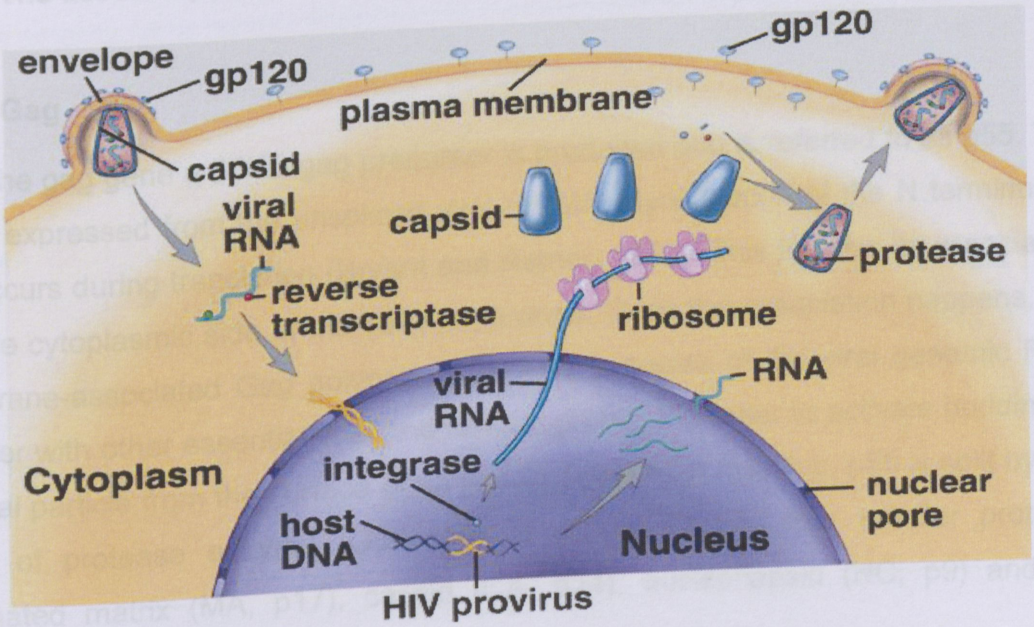



**Figure 6.2:** Reverse transcription of HIV-1 viral RNA (Roberts et al., 1988).

The current research focused on studying the efficacy of the selected plant extracts in inhibiting the action of RT enzyme and also employed the use of p24 ELISA kit (Helvetica Health Care, Geneva, Switzerland) to detect viral coat protein. The RT Assay is designed to quantitate RT activity in cell culture and other biological samples. The assay is used to determine the propagation of retroviruses in


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retrovirus-infected mammalian cells in culture. The assay is also used for *in vitro* screening for RT inhibitors.

### 6.1.3.1 Viral proteins and their functions

Human immunodeficiency virus type 1 in its compacted form is known as a provirus. This provirus is roughly about 9.8 kb in length (Muesing *et al.*, 1985). The 2 ends of the provirus are flanked by the long terminal repeats (LTRs) sequences. HIV-1 genes are positioned towards the central region of the proviral DNA. They encode about nine proteins which are divided in 3 major classes.

a. The major structural proteins, Gag, Pol, and Env
b. The regulatory proteins, Tat and Rev
c. The accessory proteins, Vpu, Vpr, Vif, and Nef

#### i. Gag

From the gag gene a 55KD gag precursor is produced and is referred to as p55. The p55 is expressed from the unspliced viral mRNA. Myristolation of the N terminus of p55 occurs during translation (Bryant and Ratner, 1990). This triggers its association with the cytoplasmic side of the cell membranes. Once the association happens, the membrane-associated Gag polyprotein recruits 2 copies of the viral genomic RNA together with other essential viral and cellular proteins in order to activate budding of the viral particle from the surface of an infected cell. After budding, p55 is split by the action of protease enzyme (Gottlinger *et al.*, 1989) into four smaller proteins designated matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p9) and p6 (Gottlinger *et al.*, 1989).

Most MA molecules remain attached to the virion lipid bilayer thereby stabilizing the viral particle. Part of MA infiltrates deeper layers of the virion where it forms part of a complex which guides the viral DNA to the nucleus (Gallay *et al.*, 1995). The CA protein forms the conical core of viral particles. Cyclophilin A has been shown to interact with the CA protein leading to its incorporation into HIV particles (Franke *et al.*, 1994; Thali *et al.*, 1994). Such an interaction is crucial since its disruption by cyclosporine A inhibits viral replication (Franke and Luban, 1996).


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The NC protein is accountable for recognizing the packaging signal of HIV which comprises of 4 stem loop structures at the 5' end of the viral RNA (Harrison and Lever, 1992). The packaging signal effects the incorporation of a heterologous RNA into HIV-1 virions (Poznansky *et al.*, 1991). Binding of NC to the packaging signal is mediated by 2 zinc-finger motifs. NC also facilitates the process of viral reverse transcription (Lapadat-Tapolsky *et al.*, 1993). Interactions between p55 and the accessory protein Vpr is mediated by polypeptide p6. This interaction facilitates the incorporation of Vpr into the assembling virions (Paxton *et al.*, 1993).

### ii. Protease (PR)

The polymerase gene (pol gene) comprises of 3 major regions that encode for 3 crucial enzymes. HIV-1 protease is an aspartyl protease enzyme (Ashorn *et al.*, 1990) that acts as a dimer. This enzyme is required for the splitting of the Gag and Gag-pol polyprotein precursors during virion maturation.

### iii. Integrase (IN)

The IN protein facilitates the insertion of the newly synthesized HIV proviral DNA into the host genomic DNA. The IN protein is responsible for 3 distinguished functions in a host cell (Bushman *et al.*, 1990). The first function is the trimming of 2 nucleotides from each 3' end of the viral DNA by an exonuclease activity. Then, an endonuclease activity cleaves the double stranded host DNA at the point of integration. Finally, a ligase enzyme catalyses the formation of single covalent linkage at each end of the proviral DNA after which the cellular enzyme then repairs the integration site (Pryciak and Varmix, 1992; Pruss *et al.*, 1994; Bushman, 1994; Wiskerchen and Muesing, 1995).

### iv. Reverse transcriptase (RT)

The pol gene also encodes reverse transcriptase. In the process of reverse transcription, the polymerase synthesizes a double-stranded DNA copy of the dimer of single-stranded genomic RNA. For the synthesis of the complementary DNA strand, RNAse H initially removes the original RNA template from the first DNA



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strand, allowing synthesis of the complementary DNA (Zack *et al.*, 1990). Since the polymerase enzyme lacks a proof-reading activity, replication is prone to mismatch errors and this institutes various point mutations into each new copy of the viral genome (Harrich *et al.*, 1996).

**v. Envelope (Env)**

The 160kD Env protein (gp160) is expressed from an mRNA that has been spliced once. The Env protein is firstly synthesized in the endoplasmic reticulum then it moves to the golgi complex for glycosylation. An additional 25 – 30 complex N-linked carbohydrate side chains are added at the asparagine residues. The process of glycosylation is crucial for viral infectivity (Capan and Ward, 1991). A protease enzyme cleaves gp160 to produce gp41 and gp120. Env exits as a trimer onto the host cell surface and virions (Bernstein *et al.*, 1995).

## 6.2 MATERIALS AND METHODS

### 6.2.1 Antibacterial susceptibility assay

#### *6.2.1.1 Selection of microorganisms*

The 5 ATCC strains were chosen because they are common causes of bacterial infections and are also important human pathogens. *Enterococcus faecalis, E. coli, K. pneumoniae, Salmonella enterica* and *S. agalactiae* were grown in Mueller-Hinton (MH) broth. For the growth of *S. agalactiae* the media was supplemented with 10% horse serum to provide nutrients required for bacterial growth. The strains were inoculated in the broth (20 ml) and allowed to grow overnight, for 16 hours at 37°C at 150 rpm (Ecotherm, HartKirchen, Austria).

#### 6.2.1.2 Control drugs

Gentamicin sulphate and vancomycin hydrochloride were used as positive controls against both Gram positive and negative bacterial species and the control drugs were essential for verifying the effectiveness of the antimicrobial activity. To prepare the working concentration of each antibiotic, they were dissolved in MH broth at 2


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mg/ml stock concentration and filter sterilized. Due to *Salmonella* resistance to gentamicin sulphate, imipinem dehydrate, penicillin G, chloramphenicol and ampicillin were tested as possible antibiotics.

### 6.2.1.3 Preparation of plant extracts

The plant extracts were dissolved in DMSO at a stock concentration of 50 mg/ml. From the stock solutions, working concentration (4 mg/ml) was prepared in MH broth and filter sterilized.

### 6.2.1.4 Microbroth dilution and INT assay

The assay was done as previously reported (Kobayashi *et al.*, 2004). Microtiter plates were used for the screening protocol. Fifty microliters of MH broth was added to all test wells except for the control wells wherein 100 µl of the working concentrations were added. Serial dilutions of the plant extracts were prepared from 2 mg/ml – 125 µg/ml and the antibiotics dilutions were 0.25 - 64 µg/ml. The bacterial cultures were standardized to 0.5 McFarland standard from which 50 µl was added to each test well.

The different controls were prepared as follows: antibiotic/medium control, plant extract for colour control, DMSO control and microorganism's control. The plates were sealed and incubated at 37°C for 24 hours (Ecotherm, HartKirchen, Austria). After the 24 hours incubation period, absorbance was read at 600nm (Multiscan MS, Thermo Labsystems, Waltham, MA). 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT, Sigma Aldrich, St Louis, MI) was prepared at a working concentration of 0.2 mg/ml in distilled water and filter sterilized. Twenty microliters of INT was added to each well and the plates further incubated for an additional 60 minutes until there was an observable colour change. The absorbance was read at 600nm (Multiscan MS, Thermo Labsystems, Waltham, WA).


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### 6.2.2 Anti-mycobacterial activity

The *Mycobacterium tuberculosis* H37 strain was obtained from the National Health Laboratory services (NHLS; Port Elizabeth; South Africa) and was grown in a culture broth containing Difco™ Middlebrook 7H9 broth, glycerol, Tween®80 and Middlebrook albumin-dextrose-catalase growth supplement at 37°C for 10 d (Ecotherm, HartKirchen, Austria). Rifampicin (1mg/ml) and ethambutol dihydrochloride (2 mg/ml) were dissolved at stock concentration of 1 mg/ml and 2 mg/ml, respectively, and they served as positive controls.

The working concentrations were prepared at 16 µg/ml and 1 mg/ml concentrations in culture broth without Tween®80 and then filter sterilized. The plant extracts were dissolved at working concentrations of 4 mg/ml prepared in culture broth without Tween®80. The assay was performed as described by Franzblau et al (1998) with slight modifications. Alamar blue reagent was used as an indicator of cellular viability.

Briefly, 200 µl of sterile deionised water was added to all the outer-perimeter wells of the 96 well plate to minimize evaporation. The wells from rows B to G were filled with100 µl of 7H9GC broth, 100 µl of plant extract solution was added to the wells in columns and serially diluted using a multichannel till column 11. Final concentrations across the microtitre plates were as follows: 2000 to 15,625 µg/ml. *Mycobacterium* inoculum was added (100 µl) to all the wells excluding the non- inoculum control wells. Plate setup is shown in Figure 6.3.

| Sterile $H_2O$ | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Sterile $H_2O$ | Control 1 | Control 2 | 2000 | 1000 | 500 | 250 | 125 | 62,5 | 31,25 | 15,625 |
| | Control 1 | Control 3 | 2000 | 1000 | 500 | 250 | 125 | 62,5 | 31,25 | 15,625 |
| | Sterile $H_2O$ | | | | | | | | | Sterile $H_2O$ |

**Figure 6.3:** Plate layout for the Alamar blue assay. Controls included: (i) antibiotic/medium control (ii) sample/medium control (iii) 4% DMSO control (iv) microorganism' growth control.

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The plates were sealed with parafilm and incubated at 37°C for 5 days (Ecotherm, HartKirchen, Austria). Fifty microliters of a freshly prepared 1:1 mixture of 10 X Alamar blue reagents and 10% tween 80 was added to one of extract/drug-free well and incubated a further 24hours. If the Alamar blue-test well turned pink then the reagent mixture was added to all the wells in the plate. The microplates were incubated for an additional 24hours. Fluorescence emission was read at 560 and 590 nm (Multiscan MS, Thermo Labsystems, Waltham, WA).

### 6.2.3 Anti-HIV testing

#### *6.2.3.1 Reverse transcriptase activity protocol*

Reverse transcriptase enzyme is a potential therapeutic target against retrovirus infection since it plays a vital role in the conversion of viral RNA to cDNA (Zhu *et al.*, 2001). The assay is based on the incorporation of dioxigenin and biotin labelled nucleotides into the new DNA synthesis. A commercially available kit was used and the assay was conducted as per the manufacturer's instructions (Roche Diagnostics; Risch-Rotkreuz, Switzerland). The detection and measurement of the synthesized DNA as a variable for RT activity follows an ELISA protocol: labelled DNA strands bind to the surface of streptavidin-coated microplate modules.

Then, an antibody to digoxigenin, conjugated to peroxidase was added and bound to the digoxigenin-labelled nucleotides. The peroxidase substrate ABTS was added to catalyse the cleavage of the substrate and produce a coloured reaction product. The absorbance of the samples was determined at a range of 405nm using a microplate reader (Multiscan MS, Thermo Labsystems; Waltham, WA), which is directly comparable to the level of RT activity in the sample.

#### *6.2.3.2 p24 assay*

The HIV-1 p24 antigen ELISA kit 2.0 is an enzyme linked immunosorbent assay. It detects HIV-1 p24 present in culture media. With the advantage of p24 amino acid sequences that are highly conserved, this assay allows detection of the p24 from HIV-1 clades A-F. The microtiter wells are pre-coated with a monoclonal antibody specific for the p24 gag gene product. The HIV-1 p24 produced into the culture


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media (supertants preserved from the previous cytotoxicity assays in chapter 4) binds to the immobilized antibody conjugated to horseradish peroxidase (HRP). Following addition of the substrate and incubation, a reactant colour develops due to reaction of the HRP enzyme. The measured optical density is proportional to the amount of HIV-1 p24 antigen present in the sample. A summary of the methodology is presented in the flow chart below (Figure 6.4).

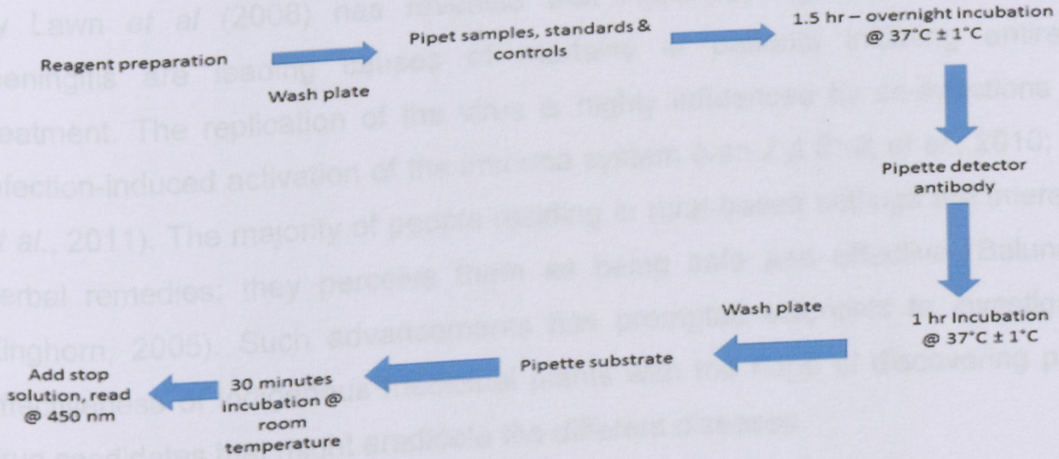



**Figure 6.4:** p24 procedural flow chart (Helevetica Health Care)

### 6.2.4 Statistical analysis

All data were representative of three independent experiments performed in triplicate and presented as mean ± standard deviation (SD). The Mann–Whitney test and one-way analysis of variance (ANOVA) were used for the evaluation of statistical differences between the results and p values $< 0.05$ were considered significant.

## 6.3 RESULTS AND DISCUSSION

The efficacy of the different plant extracts on microbial pathogens was analysed. With the current pandemic of HIV/AIDS, infected individuals are prone to various infections which can be life threatening given the status of their immune systems. Furthermore, the increasing trend of drug resistant mutations is raising great health concerns. The type and level of biological activities which can be exhibited by any plant extracts depends on many factors. These includes the plants' part used,

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geographical location of the plant, plant drying methods and storage conditions. The efficacy of the extracts can also be affected by elevated temperature during extraction (Wendakoon, 2012).

The burden of HIV/AIDS and opportunistic infections associated with the suppressed immune system continue to threaten the health of thousands in South Africa. A study by Lawn *et al* (2008) has revealed that AIDS-defining illnesses like TB and meningitis are leading causes of mortality in patients initiating antiretroviral treatment. The replication of the virus is highly influenced by co-infections due to infection-induced activation of the immune system (van Zyl Smit *et al.*, 2010; Ehrlich *et al.*, 2011). The majority of people residing in rural-based settings are interested in herbal remedies; they perceive them as being safe and effective (Balunas and Kinghorn, 2005). Such advancements has prompted scientists to investigate the effectiveness of indigenous medicinal plants with the hope of discovering potential drug candidates that might eradicate the different diseases.

### 6.3.1 Anti-bacterial activity of plant extracts

The absorbance readings of the 96 well plates was done at 600 nm wavelength before and after addition of INT. Viable cells reduce the colour of the dye to a pink/purplish colour and no colour change indicates inhibition of bacterial growth. The microtiter plates had a pinkish colour change, meaning growth of microorganisms being tested was not hindered by the presence of the extracts. From observation, it is apparent that the 10 plant extracts do not possess any form of antibacterial activity against the panel of microorganisms or the bacterial species were resistant to the extracts. The results of the antibacterial activity and MIC for the positive controls are shown in Table 6.1.

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**Table 6.1:** Antibacterial activity of the selected Venda indigenous medicinal plants

| Organism | Positive control | MIC* (µg/ml) | *E. transvaalense* | *C. abbreviata* | *O. ornithogaloides* | *O. holstii* | *L. edulis* | *E. elephantina* | *C. rehmannii* | *J. zeyheri* | *Z. mucronata* | *P. angolensis* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Enterococcus faecalis*** | Vancomycin hydrochloride | 2 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| ***Escherichia coli*** | Gentamicin sulfate | 8 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| ***Klebsiella pneumonia*** | Gentamicin sulfate | 2 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| ***Salmonella*** | Gentamicin sulfate/ imipenem dehydrate/ amicillin/ penicillin G/ chloramphenicol | Resis-tant** | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| ***Streptococcus agalactiae*** | Vancomycin hydrochloride | 2 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |

*MIC for positive controls; **In house Lab value of 10; NA=Not applicable

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The results of the antibacterial activity of the 10 selected plant extracts demonstrated no activity against the clinical bacterial strains (Table 6.1 above). The percentage for plant extract inhibition could not be calculated as there was no observable microbial growth when comparing the test and control wells. The results observed in this study contradict the findings by Olajuyigbe and Afolayan (2012). The bacterial isolates *E. faecalis, E. coli, K. pneumoniae* were shown to exhibit varied degree of susceptibility with zones of inhibition ranging between 17 and 27 ± 1.0 mm. The difference in results can be attributed to the extraction solvent they used. A study published by Samie et al (2009) showed that ethanolic extracted compounds from *P. angolensis* inhibited growth of *Staphylococcus aureus* with median inhibitory concentration of 25 µg/ml. The results obtained in this study are not surprising because previous reports have shown that some aqueous water extracts do not possess any biological activity (Luseba *et al.*, 2007).

### 6.3.2 Anti-mycobacterial activity of plant extracts

The result of the anti-microbial activity of a colour change in CellTiter-Blue® reagent was not clear for the higher plant extract concentrations (Figure 6.5). This might be due to the extracts colouration.

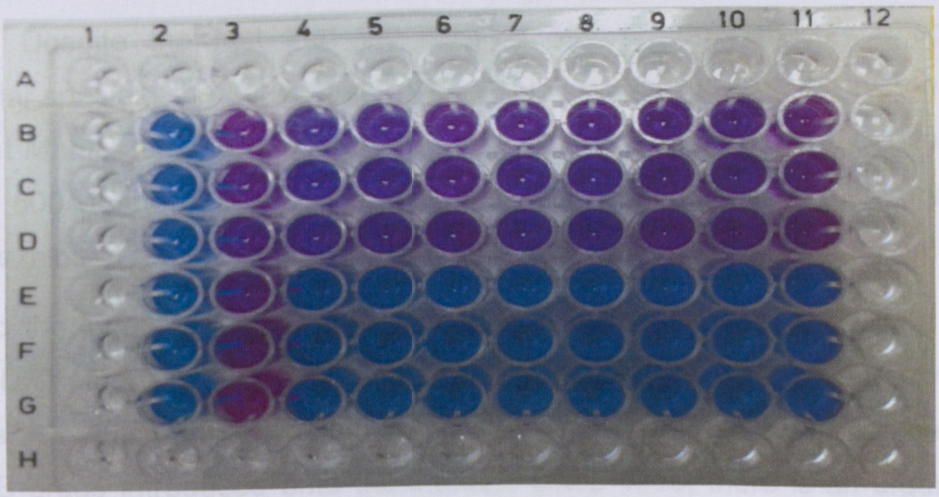



**Figure 6.5:** Visual evaluation of *P. angolensis* and *Z. mucronata* plant extracts as well as antibiotics' MIC values against *M. tuberculosis*.

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Positive controls' and plant extract MIC values are summarized in Table 6.2. Ethambutol dihydrochloride has shown no anti-tuberculosis activity against the *M. tuberculosis* H37 strain. This specific strain of *M. tuberculosis* might be resistant to ethambutol dihydrochloride. Anti-tuberculosis activity was shown by the extracts of *Z. mucronata* and *P. angolensis* only at concentrations >500 µg/ml.

**Table 6.2:** MIC values of antibiotics and aqueous extracts of *Z. mucronata* and *P. angolensis*

| Microorganism | Positive control | MIC (µg/ml) | *Z. mucronata* MIC(µg/ml) | *P. angolensis* MIC(µg/ml) |
|---|---|---|---|---|
| ***Mycobacterium tuberculosis*** | Ethambutol dihydrochloride | Not determined* | >500 µg/ml | >500 µg/ml |
| | Rifampicin | 0.5 | | |

* Could not be determined: *M. tuberculosis* strain might be resistant.

Tuberculosis is a global pandemic. With the emergence of drug resistant strains the use of first line and second line anti-tubercular treatment is of great concern (CDC, 2003; WHO, 2009). TB is regarded as one of the major health problem especially in developing countries and is listed as a leading cause of death worldwide. An estimation of about 80% individuals are diagnosed yearly (Dye *et al.*, 1999; Dye, 2006). Despite the fact that the extracts showed no inhibitory effects against bacterial species, the same plant extracts (*P. angolensis* and *Z. mucronata*) tested against *M. tuberculosis* H37 demonstrated strong anti-TB activities at concentrations above 500 µg/ml. A study by Ibekwe *et al.* (2014) showed that *Pterocarpus osun* Craib exhibited an *MIC* value of 1225 µg/ml against *M. tuberculosis* strain H37.

Due to the increase in multi-drug resistance (MDR) and extensive drug resistance TB (XDR) strains of *M. tuberculosis,* the need to discover new anti-mycobacterial agents to eradicate the infections has increased. Moreover, there has been no new anti-TB drugs introduced onto the markets since 1950; there is an urgent need for new therapeutic drugs in the pharmaceutical industry (WHO, 2007).

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## 6.3.3 Anti-HIV activity

### *6.3.3.1 Inhibition of RT activity by the plant extracts*

With the exception of *J. zeyheri*, all extracts induced inhibition of RT activity (Figure 6.6a). *Coccinia rehmannii* significantly inhibited RT activity ($P<0.0001$) by over 40% followed by *E. elephantorrhiza* ($P<0.0002$) and *E. transvaalensis* ($P<0.0008$). Overall, 3 of the plant extracts showed inhibition below 20% at low concentration, 4 of the extracts had inhibition between 20 – 40% and *J. zeyheri* had no observable activity.

Figure 6.6b illustrates the results of 2 additional plant extracts activity on HIV replication. Both extracts revealed inhibition of RT activity (higher than 20%). *Pterocarpus angolensis* yielded the strongest inhibition of 55% at 100 µg/ml. This inhibition is 30% less than the activity of niverapine.

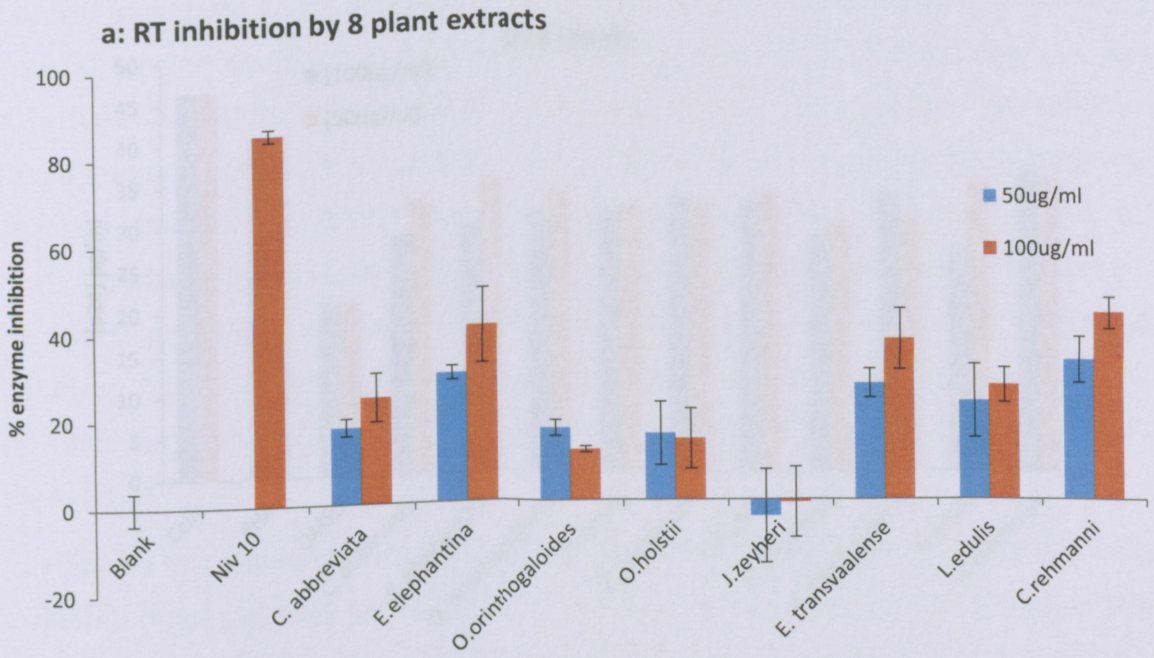
### *6.3.3.2 Inhibition of HIV-p24 levels by the plant extracts*

*Lannea edulis* exhibited decrease in levels of HIV-1 p24 antigen with greater activity recorded at the highest tested concentration. This extract was able to reduce the p24 concentration by 23 pg/ml (Figure 6.7) compared to the unstimulated cells. *Pterocarpus angolensis* reduced the antigen level by 16pg/ml and 17 pg/ml at 50 and 100 µg/ml concentrations, respectively. There was a statistical significance on the activity of the 9 plant extracts at both concentrations as summarized in Table 6.3. Only *O. holstii* did not lead to significant inhibition of p24 levels at both concentrations.

Overall there was a significant inhibition of p24 levels ($p<0.0001$) exerted by the plant extracts ranging from 12 to 23 pg/ml. The least activity was observed for the lower concentration *of E. elephantine* (Table 6.3).

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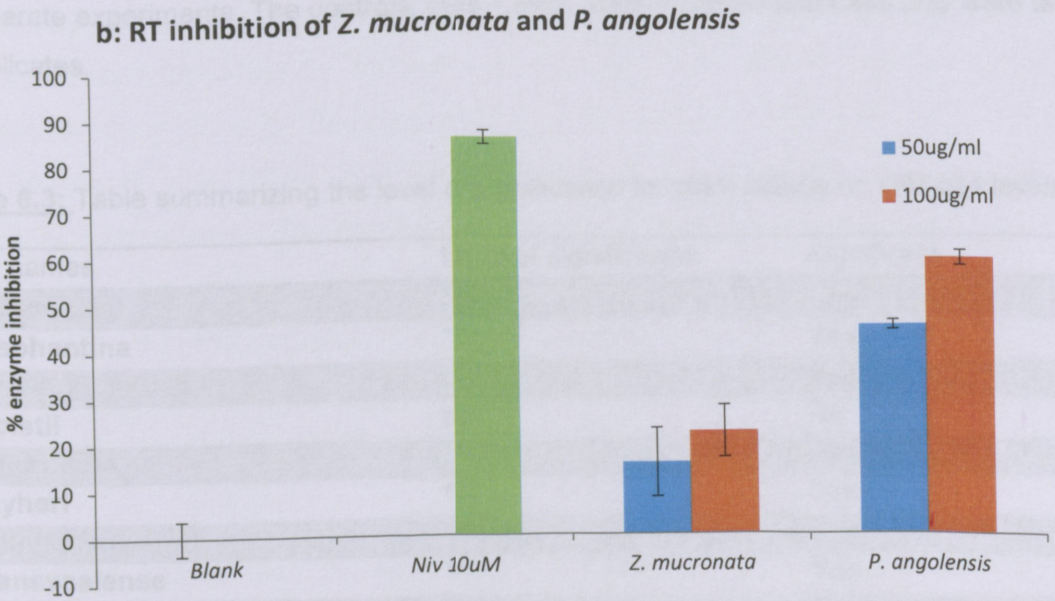




**Figure 6.6:** Reverse transcriptase inhibition by the 8 aqueous plant extracts (a) and *Z. mucronata* and *P. angolensis* (b). Nevirapine (10 µM) was used as positive control. Error bars represent standard deviation, the experiments were done in triplicates.

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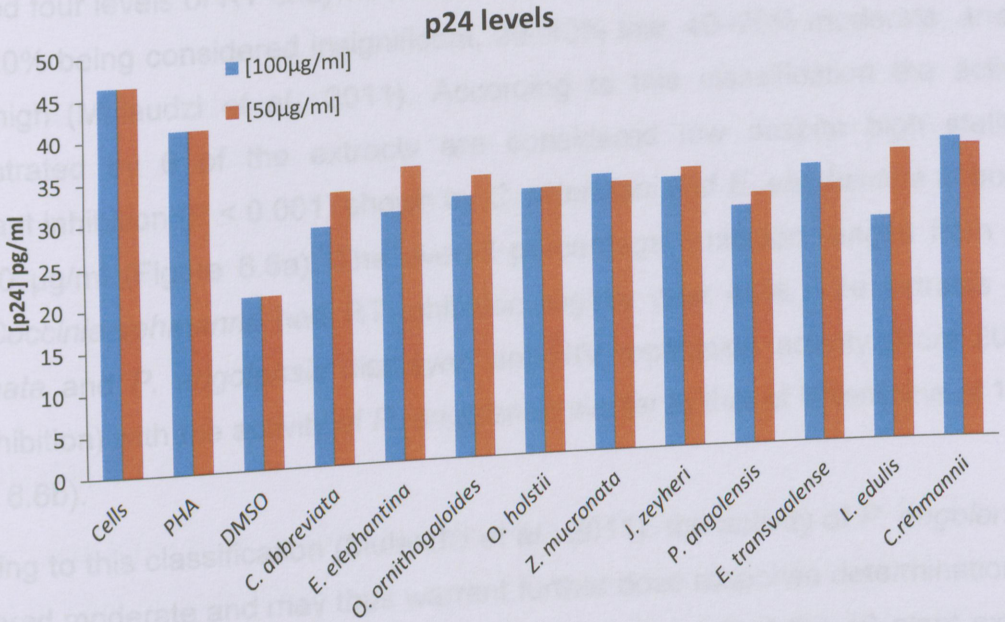



**Figure 6.7:** Inhibition of HIV-1 p24 antigen in culture supernatants by 10 medicinal plants used by Venda people in treatment of life threatening infections. All results are an average of 3 separate experiments. The controls, cells + PHA, cells + DMSO and Cells only were tested in triplicates.

**Table 6.3:** Table summarizing the level of significance for plant activity on HIV-p24 levels

| Plant names | Level of significance | Significant |
|---|---|---|
| **C. abbreviata** | **** | Yes |
| **E. elephantina** | **** | Yes |
| **O. ornithogaloides** | **** | Yes |
| **O. holstii** | ns | No |
| **Z. mucronata** | **** | Yes |
| **J. zeyheri** | **** | Yes |
| **P. angolensis** | **** | Yes |
| **E. transvaalense** | **** | Yes |
| **L. edulis** | **** | Yes |
| **C. rehmannii** | **** | Yes |


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The 10 aqueous extracts demonstrated anti-HIV activities. A previous study has classified four levels of RT enzyme inhibition for medicinal plant extracts; with activity below 20% being considered insignificant, 20–40% low, 40–70% moderate, and 70–100% high (Mulaudzi *et al.*, 2011). According to this classification the activities demonstrated by 6 of the extracts are considered low despite high statistical significant inhibition ($P < 0.001$) shown by *C. rehmanni* and *E. elephantina* at both 50 and 100 µg/ml (Figure 6.6a). The overall percentage inhibition ranged from 11 – 43%. *Coccinia rehmannii* had RT inhibition slightly over 40%. The extracts of *Z. mucronata* and *P. angolensis* displayed anti-HIV replication activity (from 20% to 60% inhibition) with the activity of *P. angolensis* similar to that of Niverapine at 10 µM (Figure 6.6b).

According to this classification (Mulaudzi *et al.*, 2011), the activity of *P. angolensis* is considered moderate and may thus warrant further dose response determination and calculation of an IC50 value. This study has also shown that the 10 plant extracts have anti-HIV activity on inhibiting the p24 antigen especially *L. edulis* which lowered the antigen concentration by 23 pg/ml (Figure 6.6b). The least observed activities exerted by the extracts revealed inhibition of 16 pg/ml. The results obtained are in line with research done by Mohanraj *et al.* (2010) on the extracts of *Calotropis procera*. A study done by Bessong et al (2011) showed RDDP $IC_{50}$ (77.5 and 81.5) and RNase H $IC_{50}$ (>100 and 75) activities of *Z. mucronata* for aqueous and methanol, respectively. The plant extracts used, however, were prepared from roots and leaves. The ethanolic extracts of *C. abbreviata* have been shown to display a direct inhibitory activity against HIV-1c p24 antigen production (Leteane *et al.*, 2012). The extracts were shown to retain their inhibitory effect against HIV even after tannin removal (94.5±1 %) ($P < 0.05$) and 98.1±3.1 % ($P < 0.05$) inhibition before tannin removal.

A study had shown that the aqueous root extract of *E. transvaalense* displayed greater inhibitory effect against HIV-1 RDDP than the methanolic extract (Bessong *et al.*, 2005). It was also revealed that the chloroform and ethyl acetate extracts of *E. transvaalense* showed good inhibitory activity of 64% and 76% at 1 µg/mL in the NF-kβ assay (Tshikalange *et al.*, 2008). In the same study, acetone extract from the same plant displayed no activity.

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The present study has demonstrated the ability of tested extracts to display inhibitory effects in agreement with a study conducted to investigate the inhibitory effects of some ayuverdic medicinal plants against HIV. This suggests that most of the tested plants extracts exerted anti-HIV activity via multiple mechanism of action (Rege *et al.*, 2010). With the findings of this study, the extracts might have no activity on bacterial pathogens, however they showed direct anti-HIV inhibition of the enzyme reverse transcriptase and p24 antigen. The activities shown by the extracts against HIV suggest that they could serve as good sources of anti-HIV agents.

## 6.4 SUMMARY

Plant extracts have been shown to possess a variety of phytochemicals which are believed to fight off pathogens. The selected plant extracts demonstrated no activity against the clinical bacterial strains. However, 2 of the extracts demonstrated strong anti-TB activities at concentrations above 500 µg/ml. Furthermore, the extracts demonstrated anti-HIV activities against HIV-1 RT enzyme with inhibition percentage ranging from 11-43%. The same plant extracts also inhibited the p24 antigen, it can be concluded that they can serve as good sources of anti-HIV agents.

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# Chapter 7

# CONCLUSION AND RECOMMENDATIONS

## 7.1 CONCLUSION

The general objective of this study was to evaluate the *in vitro* cytotoxicity, anti-inflammatory, antimicrobial and immunomodulatory potential of ten (10) medicinal plants. This was achieved by diving the study into 5 parts summed up as:

- Ethnobotanical study of plants
- Plant profiling and antioxidant activities of plants
- Cytotoxicity effects of plants
- Immunomodulatory effects of plants
- Anti-pathogenic effects of the plant extracts

The first objective was to conduct an ethnobotanical survey on the selected medicinal plants. The objective was achieved by (1) interviewing traditional healers from different regions within the Vhembe District and also by (2) using a questionnaire to document information on the different plant species they use in treating HIV/AIDS. The medicinal plants reported were distributed amongst 29 plant families. From the total of 39 species, only ten were selected because of their use in cleansing blood thereby treating blood pathogens.

The second objective was to collect and prepare extracts. The location of the selected plants was made possible with assistance from the traditional healers and community leaders in the respective villages. The plant specimens were collected and extracted using hot water and ethyl acetate yielding different grams of the plant crude extracts.

Objective three was aimed at identifying the different compounds using test tube reaction method. The 2 extraction solvents yielded similar phytochemical constituents, however, saponins were not detected in all the ethyl acetate extracts.

The fourth objective was to identify the constituents in the crude extracts using LC-MS. Epicatechin, p-hydrobenzoic acid and protocatechuic acid were detected in

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almost all the plant extracts. The presence of some of the detected could be associated with the observed biological activities.

Objective five was to determine the free radical scavenging activity of the extracts using DPPH and reducing power assays. The ethyl acetate extracts showed better reducing power capabilities in particular the extracts of *O. orithogaloides* and *O. holstii*. The crude drug of *P. angolensis* showed the highest scavenging activity.

The sixth objective was to assess the pro-inflammatory potential of the extracts using cell culture and ELISA. All the aqueous extracts showed pro-inflammatory activities, they induced the production of nitric oxide (a free radical mediator) in unstimulated cells. The immunomodulatory potential of the extracts on non-infected and HIV-infected PBMCs was achieved by measuring IL-2, IL-6 and TNF-α levels. The non-infected PBMCs expressed more IL-2 with the highest expression stimulated by *O. ornithogaloides*, however, the extract of *Z. mucronata* stimulated production of IL-2 expressing same quantity as the PHA-treated cells. IL-6 expression varied in non-infected cells with 590 pg/ml (*C. rehmannii*) being the highest expression value. HIV-infected cells suppressed the expression with the exception of *O. ornithogaloides* (490 pg/ml). The expression of TNF-α was relatively lower in non-infected cells since this cytokine is well known to be produced in all stages of HIV. *Coccinia rehmannii* suppressed the production by decreasing the expression by 37 pg/ml.

The seventh objective was to assess the cell viability to confirm the absence of toxicity exerted by the plant extrtacts. Adverse toxicity was observed from the extracts of *O.ornithogaloides*, *E. transvaalense*, *Z. mucronata* and *P. angolensis* against Vero cells. The ethyl acetate extracts of *O. holstii*, *O. ornithogaloides*, *E. transvaalense*, *L. edulis* and *C. rehmannii* were highly toxic against the Vero cells. The observed toxicity was marked higher than that of melphalan.

Objective and eight and nine were set to cultivate, prepare pathogenic bacterial strains and evaluate the anti-bacterial activity using microbroth dilution and INT assays. The strains were grown overnight in Mueller-Hinton broth to allow multiplication of bacterial cells. None of the plant extracts exhibited activity against the chosen clinical bacterial pathogens.

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Objective ten was to evaluate the antimycobacterial activity of the plant extracts. *Pterocarpus angolensis* and *Z. mucronata* exhibited strong anti-TB activity at concentration higher than 500 µg/ml.

Lastly objective eleven evaluated the anti-HIV activity of plant extracts by assessing viral protein replication using p24 antigen and RT assay. All the extracts induced inhibition of RT activity (statistical significant). *Coccinia rehmannii* and *P. angolensis* exhibited inhibition percentage of 40 and 55%, respectively. The P24 antigen levels were reduced by all the extracts. *Lannea edulis* exhibited a decrease of 23 pg/ml.

At the start of this study, several questions regarding safety and claims by the traditional healers needed to be clearly answered. The first question was how safe the plants were or whether they possess any toxic effects. The results obtained showed that the aqueous extracts in the concentrations tested are safe and less toxic to cells with an exception of *Tshiganame* and *Mukuvhazwivhi*.

The second question to be addressed was the activity of plants on opportunistic infections. The results showed that the plant extracts do possess inhibitory activities against *Mycobacterium tuberculosis* and HIV-1 reverse transcriptase and p24 antigen.

The third question was the boosting of the immune system by the selected plants. The ability of the plant extracts to induce production of IL-2 and also suppress the inflammatory cytokines showed that they are capable of boosting the immune system.

The fourth question to be addressed was the phytochemical constitution of the plant extracts. The results show a variety of phytochemicals ranging from saponins, flavonoids, phenols to name a few.

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In conclusion, this study has demonstrated that plant extracts are indeed an alternate source of therapy against some life-threatening infectious agents; these extracts can also work indirectly against the pathogens by boosting the immune system.

## 7.2 SHORTCOMINGS OF THE STUDY

### 7.2.1 Plant collection

- Endangered species - although the chosen plant species seems to be of great importance in healing a variety of organisms in local communities, their availability is of great concern. Some of them are already listed as extinct species because of their scarcity.
- Over-harvesting – the lack of proper knowledge on how to collect plant parts without killing the population of medicinal plants has led the majority of traditional healers to over-harvest the plant organs leaving the plant exposed to microbial infections. Some of the plants were completely cut down.

### 7.2.2 Bioassay preparation

- Donor variability – in each experiment, new sets of blood were drawn thus each of the results set varied.
- Donor's state of health – despite having criteria for selecting HIV-seronegative volunteers, some other underlying health effects could influence the inflammatory reactivity.
- Blood sample processing – the blood samples were drawn and processed immediately, however, the timeframe between collection and processing might account for some variability in the results.

## 7.3 RECOMMENDATIONS

The current study has provided the *in vitro* cytotoxicity, anti-inflammatory, antimicrobial and immunomodulatory potentials for 10 medicinal plant.

Therefore the following recommendations are necessary for further studies and research:

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- **Characterizations of each plant extract fractions.**

Medicinal plants contain thousands of phytochemicals with different polarities. It is of great essence to identify and characterize each bioactive compound within the extract. Fractionating the crude extract will yield pure single compounds which will then be assessed to determine the structure and biological activities. Since the extracts occur in multi-component mixtures, several chromatographic techniques inclusive of other purification methods are to be used.

- **Dose-dependent studies for each extract and subsequent fractions.**

The pharmacological response of every drug or plant extract is thought to be directly correlated at the specific site of action. This correlation is known to be dependent on various processes. These includes distribution of the extract, biotransformation of the extract to minimize liver damage, absorption of the extract and finally the excretion of the by-products. Dose-dependent studies have been reported in pre-clinical studies. Traditionally, it is assumed that the increase in dose levels is proportionally equated to safety margins of the extracts.

- **Assessment of the extracts' anti-cancer effect against malignant cells.**

Cancer patients are in need of drugs with improved efficacy. Drugs that will not kill cancerous cells at lower concentration while affecting healthy cells with same magnitude or drugs that will act on specific targets that play a role in proliferation and survival of cancerous cells. What is needed are drugs that will kill the malignant tumors at concentrations that will not significantly affect other healthy cells in the body. The plant extracts (*O. ornithogaloides*, *E. transvaalense*, *Z. mucronata* and *P. angolensis*) should be assayed for potential anti-cancer effects.

- **Assessment for anti-HIV activity against protease (PR) and integrase (IN) enzymes.**

The introduction of Highly Active Antiretroviral Treatment (HAART) has to a certain extent, significantly reduced AIDS-related morbidity and mortality. With the decline of HIV mortality, however, has emerged challenges including maintenance of long term adherence, drug resistant mutations, toxicities or side effects of the drugs. Such challenges can lead to virologic and immunological failure as well as clinical progressions of AIDS. It is of great importance to discover new, safe and efficient

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drugs with minimal side effects. Investigating natural products might just be the research gateway to establishing this goal.

- **Investigation of the plant extracts' toxicity, anti-inflammatory, immunomodulatory in vivo individually and in combinations.**

*In vivo* studies are crucial for the development of novel therapies. With *in vivo* testings', important data can be generated for function validation, clinical trials and FDA applications. The animal *in vivo* studies are often regarded as a gold standard for assessing oral dosage forms. Medical fields benefits from animal models to test the safety of natural products before they are used for clinical trials. It helps validate the in vitro findings in vertebrates, closest to humans.

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## APPENDIX A: CONSET FORM

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### Consent to be in research

**Study Title:** Biological of effects on selected medicinal plants

In this study, the researchers are collecting blood samples to learn more about effects of medicinal plants' extracts in modulating the immune system either by suppression or inducing production of cytokines. From the collected whole blood, we will isolate PBMCs. A total of 3 healthy donors and 3 HIV positive blood donors are to be recruited.

**What will happen if I take part in this study?**

If you agree to be in this study, you will have to answer a few questions then will go to University clinic and give blood sample. The blood will be drawn by putting a needle into a vein in your arm. Three small tubes of blood will be taken. This process does not take much time. The collected will be marked with respective codes for confidentiality.

**Are there risks?**

The needle stick may hurt. There is a small risk of bruising, and a rare risk of infection since there's a needle change for every individual.

**Are there benefits?**

There is no benefit to you. The blood will be used only for research.

If you wish to be in this study, please sign below.

_____ _____

Date Participant's Signature for Consent

_____ _____

Date Person Obtaining Consent

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# Appendix B: Questionnaire for blood collection

| | | | |
|---|---|---|---|
| 1 | Are you feeling well today? | Yes | No |
| 2 | In the last 4 hours have you had something to eat and/or drink? | Yes | No |
| 3 | In the past 3 days, have you been to the dentist? | Yes | No |
| 4 | Have you taken any painkillers, anti-inflammatories or aspirin? | Yes | No |
| 5 | In the past 7 days; have you had a cold, flu, sore throat, fever, infection or allergies? | Yes | No |
| 6 | In the past 30 days, have you had diarrhoea or vomiting? | Yes | No |
| 7 | Have you taken any drugs lately? | Yes | No |
| 8 | In the past 3 months, have you taken other medication (including traditional medication), injections or tablets? | Yes | N o |

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# APPENDIX C

## Ethnomedicinal survey data

**Questionnaire:**

**Informants' consent for the participation in the study:**

I..... (name of informant) hereby give my full consent consciously to participate in this study and declare that to the best of my knowledge the information that I have provided are true, accurate and complete.

Date..... (Signature/Thumb impression of Informant)

| |
|---|
| **Informants' details:** |
| Name: |
| Gender: |
| Age: |
| Occupation: |
| Education: |
| Location/Residence: |
| **Data about medicinal plant and its use:** |
| Plant (Local name)..... |
| Habit (Tree/ Herb/ Shrub/Climber/.....) |
| Plant part used..... |
| Cultivated/ Wild..... |
| If cultivated, cultivated for..... |
| If wild, availability in natural resources (easy/ difficulty/ very difficult)<br>Conservation needs:<br>Conservation efforts made by Government and local residents: |
| Method of collection and storage: |
| Name of disease(s) treated: |
| Method of crude drug preparation: |
| Mode of administration: |
| Dosage: |
| Other uses (if any)..... |

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# APPENDIX D

## List of reagents

| Product | Company | Headquarters |
|---|---|---|
| | **Culture media, solutions, cells** | |
| DMSO | Sigma Aldrich | St Louis, MI, USA |
| LPS | Sigma Aldrich | St Louis, MI, USA |
| U937 cells (ATCC® CRL1593.2) | Anatech | Fairhope,Alabama, USa |
| Vero cells (ATTC® CCL 81) | Anatech | Fairhope,Alabama, USA |
| MeWo cells (ATCC® HTB 65) | Anatech | Fairhope,Alabama, USA |
| Raw 264.7 cells (ATTC® TIB 71) | Anatech | Fairhope,Alabama, USA |
| DMEM | Sigma Aldrich | St Louis, MI, USA |
| RPMI | Thermofischer | WA, MA, USA |
| Foetal bovine serum | Sigma Aldrich | St Louis, MI, USA |
| MTT | Sigma Aldrich | St Louis, MI, USA |
| XTT | Sigma Aldrich | St Louis, MI, USA |
| Cell titre blue | Promega | Madison, Wisconsin, USA |
| MH broth | Sigma Aldrich | St Louis, MI, USA |
| Horse serum | Thermofischer | WA, MA, USA |
| Gentamicin sulphate | Sigma Aldrich | St Louis, MI, USA |
| Vancomycin hydrochloride | Sigma Aldrich | St Louis, MI, USA |
| Imipenem dehydrate | Sigma Aldrich | St Louis, MI, USA |
| Penicillin G | Sigma Aldrich | St Louis, MI, USA |
| Chloramphenicol | Sigma Aldrich | St Louis, MI, USA |
| Ampicillin | Sigma Aldrich | St Louis, MI, USA |
| Melphalan | Glaxo-Smithkline | Paris, France |
| INT | Sigma Aldrich | St Louis, MI, USA |
| Aminoguanidine | Sigma Aldrich | St Louis, MI, USA |

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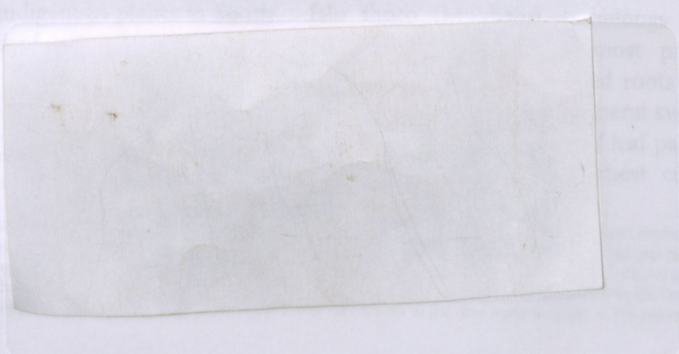
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| | | |
|---|---|---|
| Difco middlebrook 7H9 broth | Difco | Franklin lakes, New Jersey, USA |
| Glycerol | Sigma Aldrich | St Louis, MI, USA |
| Tween 80 | Sigma Aldrich | St Louis, MI, USA |
| Middle brook albumin-dextrose catalase growth supplement | Sigma Aldrich | St Louis, MI, USA |
| Alamar blue | Thermofischer | WA, MA, USA |
| Ethambutol dihydrochloride | Sigma Aldrich | St Louis, MI, USA |
| Griess reagent | Roche Diagnostics | Risch-Rotkreuz, Switzerland |
| **Chemicals** | | |
| Ethyl acetate | Sigma Aldrich | St Louis, MI, USA |
| Dichloromethane | Sigma Aldrich | St Louis, MI, USA |
| Methanol | Sigma Aldrich | St Louis, MI, USA |
| Hexane | Sigma Aldrich | St Louis, MI, USA |
| Ferric chloride | Sigma Aldrich | St Louis, MI, USA |
| Sodium hydroxide | Sigma Aldrich | St Louis, MI, USA |
| Hydrochloric acid | Sigma Aldrich | St Louis, MI, USA |
| Chloroform | Sigma Aldrich | St Louis, MI, USA |
| Acetic acid | Sigma Aldrich | St Louis, MI, USA |
| Follin ciocalteu reagent | Sigma Aldrich | St Louis, MI, USA |
| DPPH | Sigma Aldrich | St Louis, MI, USA |
| Gallic acid | Sigma Aldrich | St Louis, MI, USA |
| Sodium carbonate | Sigma Aldrich | St Louis, MI, USA |
| Potassium ferric cyanide | Sigma Aldrich | St Louis, MI, USA |
| Trichloroacetic acid | Sigma Aldrich | St Louis, MI, USA |
| Ascorbic acid | Sigma Aldrich | St Louis, MI, USA |
| **Kits** | | |
| IL-2 ELISA | BD | Franklin lakes, New Jersey, USA |
| IL-6 ELISA | BD | Franklin lakes, New Jersey, USA |

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| | | |
|---|---|---|
| TNF-α | BD | Franklin lakes, New Jersey, USA |
| P24 | Helvetica health Care | Geneva, Switzerland |
| RT ELIZA | Roche Diagnostics | Risch-Rotkreuz, Switzerland |
| **Consumables** | | |
| 96 well plates | Nunc | Rochester, NY, USA |
| 24 well plates | Nunc | Rochester, NY, USA |
| Filterpaper | Sigma Aldrich | St Louis, MI, USA |
| | | |



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RESEARCH

Open Access

# Comparative *in vitro* cytotoxic, anti-inflammatory and anti-microbiological activities of two indigenous Venda medicinal plants

M. T. Sigidi[1*], C. P. Anokwuru[2], T. Zininga[3], M. P. Tshisikhawe[4], A. Shonhai[3], I. D. I. Ramaite[2], A. N. Traoré[1] and N. Potgieter[1,5]

## Abstract

**Background:** The Vhembe region of the Limpopo province has a rich tradition of medicinal plants use. Traditionally, boiled roots of *Ziziphus mucronata* are used in the treatment of boils, general swelling and other skin infections. A combination of leaf paste and root infusion treats measles, dysentery, chest complains, and gland swelling. *Pterocarpus angolensis* is famous for the treatment of menorrhagia, infertility in women, wounds and pain management. The purpose of the present study was to compare the cytotoxicity, anti-inflammatory potential and anti-microbial activities of *Ziziphus mucronata* and *Pterocarpus angolensis* from the Vhembe region.

**Method:** U937, MeWo, Vero and RAW 264.7 cells were treated to various concentrations (50, 100, or 125 or 250 μg/ml depending on assays) of *Ziziphus mucronata* and *Pterocarpus angolensis*. Cytotoxicity assay was done using MTT; Anti-inflammatory activity was assessed using NO production; Anti-bacterial activity was done using the Micro-Broth dilution method and Anti-mycobacteria activity was determined using the Alamar Blue Method while RT activity was measured by ELISA.

**Results:** Cytotoxicity results showed that *Pterocarpus* was more toxic than *Ziziphus* as observed in the Vero and MeWo cells; however both displayed toxicity towards a Human cancer cell line. Both extracts did not inhibit nitrate production but induced significant increase in macrophage activation. The plant extracts have shown anti-tuberculosis activity at concentrations >500 μg/ml and there was moderation inhibition of HIV replication.

**Conclusions:** The results obtained indicated that the extracts have pro-inflammatory properties, and the observed toxicity on malignant cell lines must be investigated further for promising anti-cancer drug therapy.

**Keywords:** Medicinal plants, *Ziziphus mucronata*, *Pterocarpus angolensis*, Cytotoxicity, Cancer

## Background

The use of medicinal plants has been recorded for centuries with consistency regarding their uses as well as effectiveness in the treatment of diseases [2]. The use of traditional medicines by traditional healers has played an important role in the health care of millions of people. The diversity of about 24,000 indigenous plants in South Africa contributes an estimate of 10% of higher plants on globally [24]. The majority of people in South Africa rely heavily on the traditional use of medicinal plants with an estimated 70% of the population using one or more of the approximate 3000 species of plants used as traditional medicines [24, 33, 35].

*Ziziphus mucronata* Willd is commonly known as buffalo thorn (Mukhalu), it belongs to the Rhamnaceae family and is found in most parts of the country [10, 29]. Traditionally, boiled roots concotions are used in the treatment of boils, general swelling and other skin infections. A combination of leaf paste and root infusion treats measles, dysentery, chest complains, and gland

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# Ethnobotanical evaluation of selected medicinal plants used in treatment of diseases around Venda region: A literature review

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The importance of traditional medicine has been recorded throughout the world. It has also been practiced throughout Africa by more than 80 % of rural communities. Traditional medicine has since been imbedded in the culture of many rural communities. People of the world continues to use traditional medicine because of their accessibility and affordability. The use of medicinal plants around the Venda region is vast, yet the published data on the various plants is scanty. The review aims to bring forth the knowledge of the most widely used plants among the *Vhavenda* population. The data was compiled from peer reviewed and non-peer reviewed journals, textbooks, thesis and also by interviews conducted with the traditional healers. The review revealed that there are a number of traditional medicines that are being preferred by traditional healers in their practice, the use of the selected plants in literature has never reported to be used in curing HIV/AIDS and its related illnesses. Most of the plants recorded were found to have active compounds in most of their organs. Plants are great sources for the discovery of new medicines. Natural products and their synthetics can be utilized in the development of highly useful drugs through chemical procedures and isolation followed by analog synthesis through modern medicinal mechanisms. The knowledge and understanding of each utilized medicinal plant is of great importance for it is through the discovery of newer potential drugs that the emerging life threatening infections can be effectively combated. With the increasing rate of people migrating from either rural – urban or vice versa, the natural habitats for such herbal plants is in the process of being completely destroyed without preservation of the naturally important plant species. The fear is of losing such important plant from the local communities thus losing valuable and future vital drugs.

**Keywords:** Traditional medicine, Rural communities, *Vhavenda* population, Traditional healers, Natural products.
**IPC Int. Cl.**[8]: A61K 36/00

Since the dawn of human advancement, humans have found alternative cure within their natural territory and have acquired different remedial strategies depending upon climatic, phytogeogeographic, sociocultural, floral, and fauna characteristics. The worldwide significance of traditional medicine (TM) and its benefaction to human healthcare cannot be accentuated. It is estimated that approximately 80 % of people living in the rural parts of Africa are heavily dependent on local medicinal plants for their primary healthcare needs, though it is not fully investigated or documented[1]. The use of such un-refined traditional medicines have long been in practice and continue to be the foundation of many traditional medicines worldwide. In Asia, these remedies there are different types, these includes Japanese Chinese medicine (*Kampo*), traditional Chinese medicine (TCM), *Ayurvedic* medicine (India), and Korean Chinese medicine, *Jamu* (Indonesia). In Europe, phytotherapy and homeopathy have found diverse medicinal uses. In America, the combination of traditional medicine with conventional western medicine is termed ''integrative medicine''[2,3].

The people of Tarai region of Kumaun are reported to use medicinal plants on the trial and error basis, with the great lack of scientific research to confirm effectiveness of various plants they employ in treating illnesses[4]. With the diversity of plant species in jungles worldwide, it is highly recommended to investigate the chemical components and identifying the active compounds thereof before concluding on the tremendous effectiveness of any plant[5]. Although the global society comprise of different cultures and traditional beliefs, there are similarities in certain aspects of medicinal plant uses which can serve as a validation for the effectiveness of certain medicinal

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# Phenolic contents, antioxidant activity and spectroscopic characteristics of *Pterocarpus angolensis* DC. stem bark fractions

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Oxidative stress has been implicated in the damage of biological molecules resulting in aging and diseases such as Alzheimer, cancer, diabetes, cardiovascular disorders. The study aimed at determining the phenolic contents and antioxidant activities of *Pterocarpus angolensis* crude extract and fractions. The crude extract and fractions of *P. angolensis* were evaluated for their phenolic contents using Follin-Ciocalteu reagent. The antioxidant activities were evaluated using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and reducing power assays. Ultraviolet-Visible and Fourier transform infrared spectroscopy were used to assess the spectroscopic characteristics. We obtained 7 fractions from the crude extracts after column chromatography on silica gel 60. The results showed that fraction two (PaF2a) displayed the highest DPPH radical scavenging activity (18.7 µg/ml) but had the lowest phenolic content while fraction three (PaF2b) exhibited the highest reducing power activity (44.28 µg/ml) with high phenolic content. Spectroscopic details showed that PaF2a had maximum absorbance at 287.1 nm while PaF2b displayed maximum absorbance at 288.2 nm. The infra-red spectroscopy presented four main characteristic fingerprinting at 1606, 1518, 1444 and 1064 $cm^{-1}$ as fingerprints for *P. angolensis*. There was a difference in the wave number at C==C and C-O vibrations between PaF2a and PaF2b. In conclusion, this study has shown that PaF2a and PaF2b are the antioxidant rich fractions of *P. angolensis* stem bark and exhibited different spectroscopic characteristics.

**Keywords:** Antioxidant activity, *P. angolensis* DC., Fractions, Spectroscopic characteristics, Reducing power
**IPC Int. Cl.[8]**: C09K 15/00, B01, A62B 17/00, A01D 15/00

Oxidative stress occurs when there is an imbalance between the generation of reactive oxygen species (hydroxyl radicals, singlet oxygens, hydrogen peroxides, superoxide anions) and the antioxidant defense system in favor of the former during metabolic processes[1]. The accumulation of the oxidative species lead todamage of biological molecules resulting in aging and diseases such as Alzheimer, cancer, diabetes, cardiovascular disorders[2-4]. Plant offers a wide variety of natural antioxidants which can protect either biomolecules from degradation or the endogenous antioxidants from depletion through the quenching of reactive oxygen species[5]. Phenolics are important secondary metabolites that are wide spread in plant kingdom[6]. They are good antioxidants and are able to counteract oxidative stress by acting as reducing agents, hydrogen donators, singlet oxygen quenchers and metal chelators[7-9].

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*Pterocarpus angolensis* DC. belongs to the family of Fabaceae and is commonly knownas blood wood in English and *mutondo* in TshiVenda[10]. It is commonly used by traditional healers in the Venda region to treat malaria, venereal disease, pile, amenorrhoea, haematuria, bilharzia, headache, stomachaches, diarrhea, mouth sores and rashes[10-12]. Epicatechin and its derivatives isolated from ethanol crude extract of the bark have been reported to be active against *Staphylococcus aureus*[10]. Hydrodihydrochalcones, α-methyldeoxybenzoins and isoflavonoids have also been identified in the heart wood[13]. The aim of our study was to explore the antioxidant capacity and spectroscopic characteristics of *P. angolensis*.

## Methodology

### Materials

All materials and chemicals used for extraction, elution, chromatography and analyses were purchased from Merck (Darmstadt; Germany) or Sigma Aldrich (St Louis, MI; USA) or unless specified.

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# Anti-HIV, pro-inflammatory and cytotoxicity properties of selected Venda plants

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The use of medicinal plant species among rural communities has played a role in the development of various traditional medical systems. Such development has also accelerated the exploration of different plant species in order to correlate traditional practices to scientific findings. In the Vhembe district (Limpopo, South Africa), a vast majority of traditional medicine is indigenous based and knowledge of the pharmacological activities of plants is solely grounded on the understanding by traditional healers. The anecdotal claims by traditional healers to treat HIV using these plants and their safety are being investigated in this study. RAW 264.7, U937, MeWo and Vero cells were treated to various concentrations (50, 100, 125, 250 µg/mL) of *Elaeodendron transvaalense, Cassia abbreviata*, *Ornithogalum ornithogaloides*, *Ochna holstii*, *Lannea edulis*, *Elephantorrhiza elephantina*, *Coccinia rehmannii* and *Jatropha zeyheri* for anti-inflammation and cytotoxicity testing's. Reverse Transcriptase (RT) assay was used for the anti-HIV activity of the plants. There was no anti-inflammatory activity observed for the plants tested. However, in the absence of LPS stimulation there was an increase of NO production indicating that the extracts might have pro-inflammatory properties. The cytotoxicity observed in human tumor cancer cell lines U937 ($p < 0.0001$) and MeWo ($p < 0.0001$) was more pronounced with *O. orithogaloides* and *E. transvaalense*. However, cytotoxicity of the same extracts was not observed in both resting and activated macrophages (RAW cells). With the exception of *J. zeyheri*, all the extracts tested, induced reverse transcriptase inhibition with some of the extracts showing significant inhibitory activity ($p < 0.0001$). There was no cytotoxicity on mouse macrophage cell line. The study demonstrated the inhibitory potential of selected Venda plants and their pro-inflammatory properties however; further studies are needed in order to rule out other effects.

**Keywords:** Plant extract, Anti-inflammatory, Anti-HIV, Pro-inflammatory, Traditional medicine, Venda, South Africa

**IPC Int. Cl.[8]**: A61K 36/00, A61K 9/00, A61K 31/00, A61K 39/395, A61K 38/16, A61K 47/00, A61K 38/19

Traditional medicine is regarded as a very efficient alternative health care system especially in arears characterized by high rate of unemployment and lack of efficient services in government hospitals. The use of medicinal plants can also be attributed to their accessibility, affordability and cultural beliefs[1,2]. Medicinal plants are widely used in many third world countries for their benefits in treating a wide variety of ailments[3]. A number of natural products and other chemical substances constitute over 50 % of drugs with antioxidant, antiviral, antibacterial and antifungal activities[4,5]. The selection of plants based on ethnomedicinal usage increases the probability of finding therapeutic agents as opposed to random plant selection[6]. Traditional medicine is mainly used in making infusions and decoctions that can either be taken orally or applied topically. Some of the herbs are used to cleanse or drive away evil spirits[7]. The traditional knowledge is thought to be basic and can be practiced without any form of training although there is urgent need to document the indigenous knowledge in order to save them from over-harvestation, habitat degradation and also illegal collection[8,9].

The traditional knowledge of plants in relation to their use in the Vhembe district has not been well

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