Anti-HIV activity of selected South African medicinal plants

VASHKA HURINANTHAN

Submitted in complete fulfilment for the Degree of Doctorate of Technology (Biotechnology) in the Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa.

FINAL COPY APPROVED FOR EXAMINERS

_____	_____
SUPERVISOR **Professor B. Odhav**	**DATE**
_____	_____
CO-SUPERVISOR **Dr R. Parboosing**	**DATE**

REFERENCE DECLARATION

I, **Ms Vashka Hurinanthan** - (Student number: 20515779), **Prof Bharti Odhav** and **Dr R. Parboosing** do hereby declare that in respect of the following dissertation:

Title: Anti-HIV activity of selected South African medicinal plants

(i) As far as we have ascertained : no other similar dissertation exists; the only similar dissertation(s) that exist(s) is/are referenced in this dissertation as follows:

_____

_____

(ii) All references as detailed in the dissertation are complete in terms of all personal communications engaged in and published works consulted.

_____ _____

Signature of student Date

_____ _____

Signature of supervisor Date

_____ _____

Signature of co-supervisor Date

AUTHOR'S DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof Bharti Odhav** and **Dr Raveen Parboosing**

_____

Student's signature (Vashka Hurinanthan)

Date: _____

DEDICATION

This work is dedicated to my late Mother/ Best Friend

Parusmanie Hurinanthan

ACKNOWLEDGEMENTS

I would like to extend my sincere appreciation and gratitude to:

- My father, without whom I would not be the person I am today, thank you for loving and guiding me each day to be a better person .
- GOD, without belief, one has no hope.
- My supervisor and mentor, Prof. B. Odhav, Department of Biotechnology and Food Technology, Durban University of Technology, for her guidance, support, love and friendship throughout this project .
- My co-supervisor, Dr Raveen Parboosing, NHLS labs, Inkosi Albert Luthuli Central Hospital, thank you for your expert advice and use of the laboratory facilities.
- Anneta Naidoo, NHLS labs, Inkosi Albert Luthuli Central Hospital, for helping me and guiding me with virology techniques.
- The Staff of NHLS laboratory, Inkosi Albert Luthuli Central Hospital, for all the time and effort in teaching me the virology techniques.
- The Staff of School of Chemistry, University of KwaZulu Natal, Pietermaritzburg Campus for the use of their machines.
- My friend, Alveera Singh, thank you for being my rock in this difficult journey of post-gradraute studies.
- All my friends and colleagues at the Department of Biotechnology and Food Technology, Durban University of Technology for their support and assistance in the laboratory throughout this project.
- The National Research Foundation (NRF, South Africa), for funding this project.
- My family for their love, support and prayers, during the good and bad times.
- Nevas Heeralall, my partner, for his encouragement, love and support.
- And finally to Zorro, Elizabeth and Pablo my angels, who brighten up my darkest days.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AIDS	**:**	Acquired immunodeficiency syndrome
ART	**:**	Antiretroviral therapy
ARV	**:**	Antiretroviral
ATV	**:**	Atazanavir
AZT	**:**	Zidovudine
CC_{50}	**:**	50 % cellular cytotoxicity concentration
CCM	**:**	Complete culture medium
DMSO	**:**	Dimethyl sulfoxide
DNA	**:**	Deoxyribonuclease acid
DPPH	**:**	1,1-diphenyl-2-picrylhydrazyl radical
EC_{50}	**:**	50 % effective inhibitory concentration
HAART	**:**	Highly active antiretroviral therapy
HIV	**:**	Human immunodeficiency virus
HIV-1	**:**	Human immunodeficiency virus type 1
HIV-2	**:**	Human immunodeficiency virus type 2
HPLC	**:**	High performance liquid chromatography
PBS	**:**	Phosphate buffered saline
RNA	**:**	Ribonucleic acid
RT	**:**	Reverse transcriptase
SI	**:**	Selectivity index
SIV	**:**	Simian immunodeficiency virus
$TCID_{50}$	**:**	Tissue culture infectivity dose at 50 %
TLC	**:**	Thin layer chromatograph
UPLC-MS		Ultra performance liquid chromatography coupled to MS
UV-Vis	**:**	Ultraviolet-visible spectrophotometry
LPV	**:**	Lopinavir
NMR	**:**	Nuclear Magnetic Resonance
XTT	**:**	2,3-bis[2-Methoxy- 4-nitro-5-sulfophenyl]- 2H-tetrazolium-5- derivative carboxanilide

ABSTRACT

South Africa has the largest number of people infected with HIV/AIDS. It also has more than 30 000 species of plants and many of these have a long tradition of medicinal use. It is highly likely that the treatment for HIV will come from this traditional knowledge. The need for effective preventative and therapeutic agents for HIV remains an urgent global priority. The aim of this study was to screen selected South African medicinal plants for anti-HIV activity and to identify and characterise an active compound from a plant that can be used for HIV treatment. The aqueous and methanolic extracts of the roots, leaves, flowers and stems of thirty eight plant species (108 extracts) were screened for anti-HIV activity. The plants which had anti-HIV activity were further screened for anti-reverse transcriptase activity. Thirty-two extracts exhibited varying degrees of anti-HIV activity. *Cleome monophylla*, *Dichrostachys cinerea* and *Leonotis leonurus* aqueous leaf extracts had anti-HIV-1 reverse transcriptase activity.

The aqueous extracts of *D. cinerea* showed the best anti-HIV activity with a Selectivity Index of 43.5 and significant anti-HIV-1 reverse transcriptase activity. Crude phytochemical screening of *D. cinerea* showed that it had tannins, saponins, flavonoids and alkaloids but did not contain any phlobatannins, terpenoids, steroids or phenols. *D. cinerea* displayed a high degree of free radical scavenging activity with an IC_{50} of 25 µg/ml, therefore the anti-HIV activity could be attributed to the flavonoids present in the plant.

Bio-guided fractionation was used to isolate and purify the active compound from the *D. cinerea* extract. Compounds were isolated by thin layer chromatography and were tested for anti-HIV-1 and anti-reverse transcriptase activity. From these results the active compound was identified, and purified using preparative TLC. The active compound was characterised by High Performance Liquid Chromatography, Ultraviolet-visible spectrophotometry, and Ultra Performance liquid chromatography coupled to MS/MS. Structural elucidation was performed using Nuclear Magnetic Resonance. From these results, it was deduced that the compound isolated from *D. cinerea* was a catechin.

In this study we show that the catechins present in *D. cinerea* are responsible for the anti-HIV-I activity and inhibits the reverse transcriptase activity which is a key factor in the progression of HIV. Potentially, these results can be used to develop a new drug for the treatment of HIV or as a cost effective therapeutic agent in treating HIV-infected individuals with oxidative stress.

CHAPTER ONE: LITERATURE REVIEW

1.1 INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is a clinical syndrome that is the result of infection with human immunodeficiency virus (HIV) which causes profound immunosuppression (Cos *et al.*, 2002). It has been a serious, life threatening health problem since the first case was identified in the early 1980's, and is the most rapidly spreading disease of the century (Cos *et al.,* 2004, Yadav *et al.,* 2009). Worldwide AIDS is the fourth biggest killer (Chinsembu and Hedimbi., 2009). United Nations Programme on HIV/AIDS., (2013) has estimated that there were 23.5 million people living with HIV in sub-Saharan Africa in 2011, which includes 3.1 million children (United Nations Programme on HIV/AIDS., 2013). An estimated 1.7 million people were newly infected with HIV in 2011 alone (United Nations Programme on HIV/AIDS., 2013). For 2011, Statistics South Africa estimated the total number of people living with HIV at approximately 5. 38 million (Stats SA., 2011). Clearly the need for new anti-HIV drugs is not only an African concern but a global one.

Antiretroviral therapy (ART) is treatment for people living with HIV. The standard treatment consists of a combination of at least three drugs (often called "highly active antiretroviral therapy" or HAART) that seeks to suppress the HIV replication cycle, and reduce the likelihood of the virus developing resistance. HIV variants isolated from patients following prolonged AZT therapy show reduced susceptibly to AZT alone (Clercq., 1995). The potential of HIV to become resistant to ARV has become an increasing concern since it was first reported (Clercq., 1995). Despite the different treatment regimes this disease is still not controlled and the epidemic continues. Chinsembu and Hedimbi., (2010), reported that the dynamics of poverty played a vital role in the acquisition and spread of AIDS. Furthermore, the Antiretroviral therapy (ART) programs that are currently rolled out are not sustainable due to their heavy reliance on donor funding to maintain hospitals and clinics infra-stuctures (Chinsembu and Hedimbi., 2010). Admission to ART has many shortcomings, such high demand of bed space, cost of transport to hospitals, shortages of qualified members of medicinal fraternity, and serious side effects of ART (Chinsembu and Hedimbi., 2010). HIV-infected patients have also been associateded with lipodystrophy (Chinsembu and Hedimbi., 2010). Antiretroviral therapy is significant in improving the life of people living with HIV,

however the drugs have many disadvantages, including resistance, toxicity, limited availability, high cost, and lack of curative effect (Chinsembu and Hedimbi., 2010). These shortcomings continue to open new avenues for the use of ethno-medicinal plants and other natural products in the management of HIV/AIDS.

Southern Africa has a rich plant biodiversity and a long tradition of medicinal use of plants with over 30 000 species of plants used as medicines (Van Wyk *et al.*, 2002). With South Africa's remarkable biodiversity and cultural diversity, there are more than 3 000 species of plants that are used as traditional medicines (Van Wyk *et al.*, 2002). Several of these plants may contain novel anti-HIV compounds. Due to the rapid emergence of drug resistant virus strains, the development of effective therapies for human immunodeficiency virus type 1 (HIV-1) infection is dependent upon the identification of novel therapeutic agents with low toxicity (Locher *et al.*, 1996). There has been much attention directed to Southern Africa for the identification of novel therapeutic agents with low toxicity for the treatment of HIV-1. This is due to the fact that medicinal plants are an important aspect of the daily lives of most South Africans. The colossal repository of plants, with many still unexplored provides a foundation for the discovery of novel therapeutic agents.

1.2 HIV AND AIDS

HIV is a lentivirus (a member of the retrovirus family) that leads to AIDS, a condition that causes the immune system to fail, hence leading to life threatening opportunistic infections (Hemelaar., 2012). Lentiviruses are characteristic of a single stranded, positive-sense, enveloped RNA (Roitt *et al.*, 2001). When the virus enters the target cell, the viral RNA genome is then converted to a double stranded DNA (cDNA) by a virally encoded reverse transcriptase that is present in the virus (Male *et al.*, 2006). This DNA is then incorporated into the host genome with the aid of intergrase, other cellular co-factors; the genome can be transcribed in the host DNA (Roitt *et al.*, 2001). Once the HIV virus genome is intergrated into the host chromosomes, there are two possibilities either it becomes inactive and the diseased cell continues to function ordinarily; or the virus becomes active and duplicates large quantities of virus particles which are released to infect other cells (Roitt *et al.*, 2001).

Two major strains of HIV have been identified so far, HIV-1 and HIV-2. Worldwide it is refered to simply as HIV (Chinsembu and Hedimbi., 2010). HIV-1 is responsible for the majority of HIV infections globally, is most pathogenic and causes over 99% of infections, therefore most research is done on HIV-1 (Cos *et al.*, 2004; Chinsembu and Hedimbi., 2010; and Hemelaar., 2012).

HIV primarily infects vital cells in the human immune system such as T-helper cells (specifically CD4+ T-cells), macrophages and dendritic cells (Roitt *et al.*, 2001). HIV-1 entry to macrophages and CD4+ T-cells is mediated through interaction of the virion envelope glycoprotein (gp 120) with the CD4+ T-cells receptors on the target cells, and also with chemokine co-receptors (Roitt *et al.*, 2001). HIV infection leads to a decrese in the number of CD4+ T-cells through three key factors firstly by the virus destroying diseased cells through the lysis of cells; secondly the rate of apoptotic cells increase in diseased HIV infected cells; and thirdly by CD 8 cytotoxic lymphocytes that distinguish and kill diseased HIV CD4+ T-cells (Prescott *et al.*, 1993).

Opportunistic infections are common when CD4+ T-cells numbers drop below a certain level this is attributed to decrease in cell mediated immunity (Roitt *et al.*, 2001). Once infected with HIV it takes an average of ten years for AIDS to develop. AIDS is defined by, either the presence of an AIDS opportunistic infection or a CD4+ T-cells count of less than 200 cells/µl. However it has been noted that in some persons, progress to AIDS is faster, sometime in less than 2 years, whereas there is the other variation in which infected untreated persons have not developed AIDS in over 25 years of documented infection (Male *et al.*, 2006). The rate of disease progression in HIV infection is associated with both viral load and CD4+ T-cells counts as shown in Figure 1, (Adapted from Lewthwaite and Wilkins., 2009)

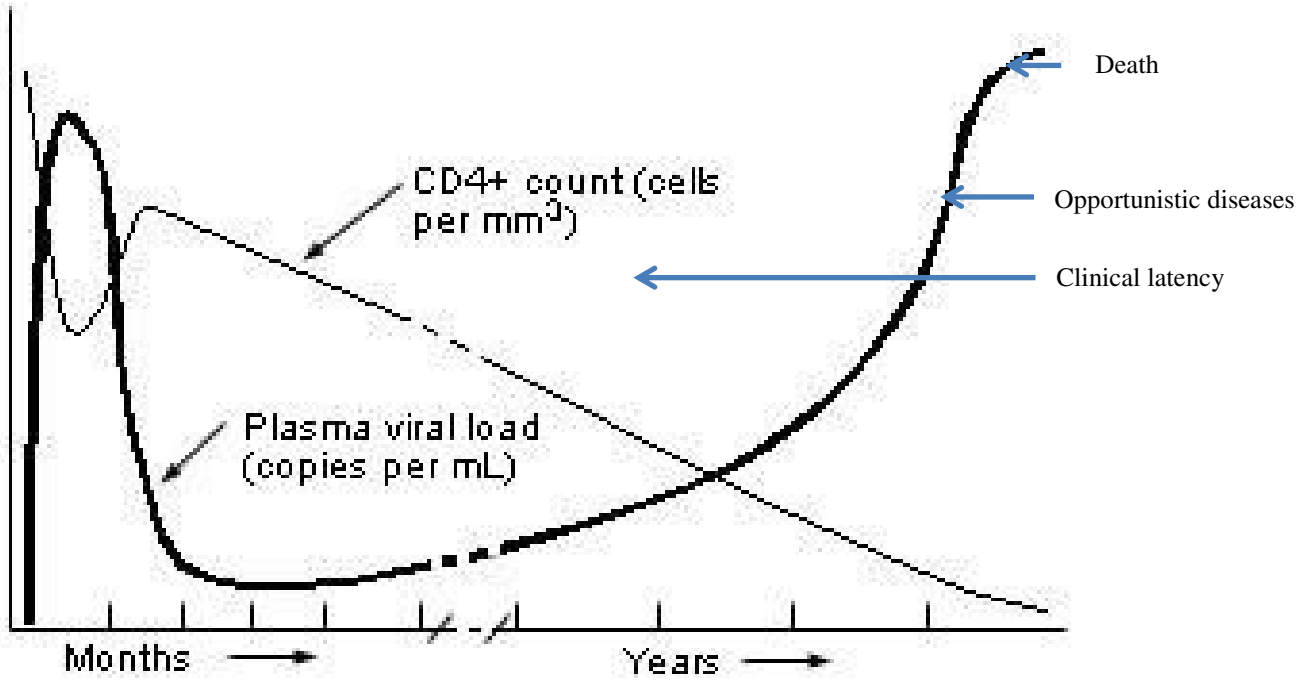


Figure 1: Viral load/ CD4+ T-cells changes over time following HIV infection

(Adapted from Lewthwaite and Wilkins., 2009)

1.2.1. The history of HIV and AIDS

HIV is thought to have originated in non-human primates in Sub-Saharan Africa and transferred to humans in the early 20th century, from multiple zoonotic transmission of simian immunodeficiency virus (SIV) (Hemelaar., 2012). Several HIV lineages have been produced through independent zoonotic transmission events from non-human primates to humans (Lewthwaite and Wilkins., 2009). HIV-1 is the most pathogenic and causes over 99% of HIV infections (Cos *et al.,* 2004). HIV-1 groups M and N originated directly, but independently, from SIV*cpz* found in chimpanzee *Pan troglodytes troglodytes*. In West Central Africa. HIV-1; Group O and P are derived through independent transmission of SIV*gor* from Western Lowland gorillas (*Gorilla gorilla* ssp. *Gorilla*). In Cameroon, the primate reservoir of HIV-2 has been clearly identified as the sooty mangabey (*Cercocebus atys*) infected with SIV*smm* (Hemelaar., 2012). The first documented proof of humans being infected with HIV was identified from two samples in 1959 and 1960, a serum sample and lymph node biopsy sample respectively which is lodged at Kinshasa in the Democractic Republic of Congo (Hemelaar., 2012).

1.2.2. HIV and AIDS in Africa

UNAID's has estimated 23.5 million people living with HIV in sub-Saharan Africa in 2011, which includes 3.1 million children; an estimated 1.7 million people were newly infected with HIV in 2011 alone (United Nations Programme on HIV/AIDS., 2013). The majority of adult HIV infections in sub-Saharan Africa resulted from unprotected sexual intercourse (United Nations Programme on HIV/AIDS., 2013).

In 2003 6.1 million people were receiving antiretroviral therapy in sub-Saharan Africa, and in 2011, 8 years the number increased by 100 000, to 6.2 million people (United Nations Programme on HIV/AIDS., 2013). United Nations Programme on HIV/AIDS reported that in 2011 from all the sub-Saharan countries the most noteable progress was seen in South Africa, Zimbabwe and Kenya, with 300 000; 100 000; 150 000 people newly enrolled for treatment; respectively (United Nations Programme on HIV/AIDS., 2013). Sub- Saharan countries such as Botswana, Namibia, Rwanda and Swaziland have accomplished great levels of coverage with regard to treatment plans in 2011 (United Nations Programme on HIV/AIDS., 2013).

In sub-Saharan Africa, the number of people dying with AIDS has decreased from 1.8 million in 2005 to 1.2 million in 2011 this could be attributed to the increased access to HIV treatment (United Nations Programme on HIV/AIDS., 2013). More than 80% of antiretroviral drugs dispensed in sub-Saharan Africa are imported (United Nations Programme on HIV/AIDS., 2013). Statistics South Africa reported in 2011, that the total estimated population was 50.59 million people of which approximately 5.38 million people were HIV positive or living with HIV (Stats SA., 2011).

The rate of disease progression in Africa is shown in Figure 2. Antiretroviral therapy distribution has improved dramatically in Sub-Saharan Africa. Antiretrovirals are costly drugs and are not always an option for most African countries. As some African countries lack the health system infrastructure needed to adequately administer complex drug regimens, therefore these therapeutic drugs are unlikely to be available to everyone. Hence efforts should focus on alternative means of preventing HIV infection and treatment of AIDS by short course treatment regimes or a single drug treatment.

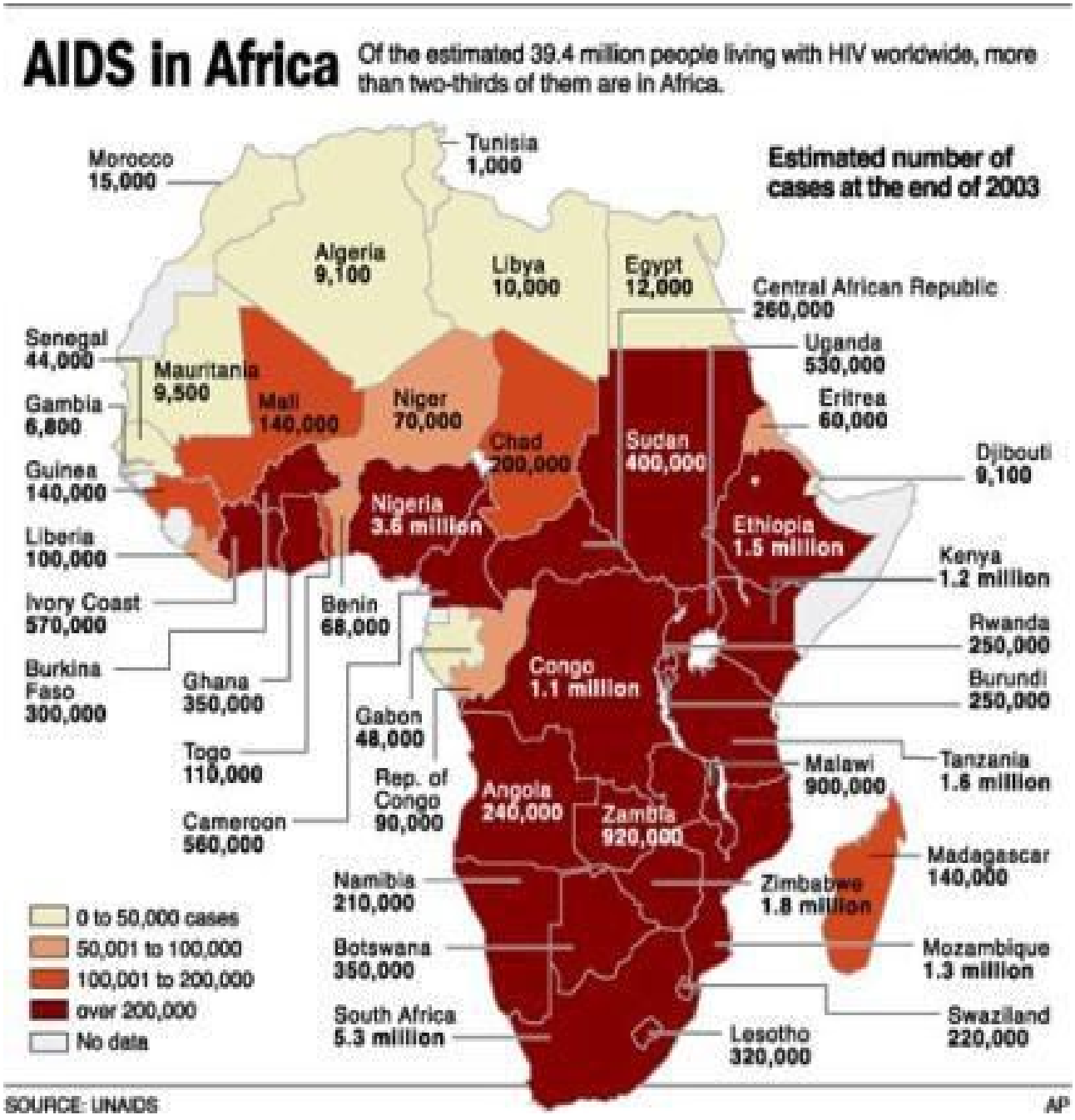


Figure 2: **AIDS in Africa demographic (United Nations Programme on HIV/AIDS., 2013)**

1.3 HUMAN IMMUNODEFICIENCY VIRUS

1.3.1 The classification

Order: Virales

Family: Retroviridae

Subfamily: Orthoretrovirinae

Genus: Lentivirus

Species: Human Immunodeficiency Virus type 1 (HIV-1)

Human Immunodeficiency Virus type 2 (HIV-2)

The HIV virus belongs to the Family of Retroviridae, Genus Lentivirus. There are two species in this genus, HIV-1 and HIV-2. The virus HIV-2 is structurally similar to HIV-1 but the distinguishing feature is that the virus responsible for the main AIDS epidemic in central Africa is named HIV-1 and the related viral strain found mainly in West Africa is named HIV-2 (Clavel *et al.,* 1986). HIV-2 infection differs from HIV-1 in being inherently resistant to non-nucleoside reverse transcriptase inhibitors (NNRTI's) and patients have lower viral loads, slower CD4+ T-cell decline and a 12 fold slower progression to AIDS (Tristem *et al.*, 1988; Cos *et al.*, 2004; Chinsembu and Hedimbi., 2010; and Hemelaar., 2012).

HIV-1 can be further subdivided into different groups: M, O, N and P and HIV-2 groups: A-H (Hemelaar., 2012). The magnitude of the epidemic caused by each group differs significantly (Hemelaar., 2012). HIV-1 group P has the least number of infected individuals with only two people, followed by Group N with handful of detected cases, Group O, causes a few thousand infections in West – Central Africa, and HIV-1 group M is responsible for the global HIV pandemic, (Hemelaar., 2012).

HIV-1 Group M is further subdivided into nine subtypes (A-K) with numerous sub-subtypes (e.g. A1-A4) and circulating recombinant (e.g. CRF01_AE) forms (Lewthwaite and Wilkins., 2009). HIV-1 Group O sequence diversity is high, which has led to a classification of sequences into clades I-V. Each clade is genetically distinct from another. HIV-1; Group M, Group N and Group P are closely related (Hemelaar., 2012).

1.3.2 Structure of HIV-1

HIV is an enveloped retrovirus and 100-120 nm in diameter as shown in Figure 4. It is composed of single stranded diploid RNA, that codes for the virus's nine genes enclosed by a conical capsid composed of 2000 copies of the viral protein p24 (Roitt *et al.*, 2001). Each enveloped virus expresses 72 glycoprotein projections composed of gp120 and gp 41, the glycoprotein gp120 of HIV binds avidly to cell surface of CD4 + T-cells molecules and initiates fusion with the cell, involving gp 41 (Roitt *et al.*, 2001). The single stranded RNA is firmly bound to the nucleocapsid proteins, p 7. The enzymes needed for the development of the virion such as reverse transcriptase, proteases, ribonuclease and intergrase, ensures the integrity of the virion particle (Roitt *et al.*, 2001). The viral envelope is derived from the host cell and contains some host cell membrane proteins such as HLA class 1 and 11 molecules (Male *et al.*, 2006).

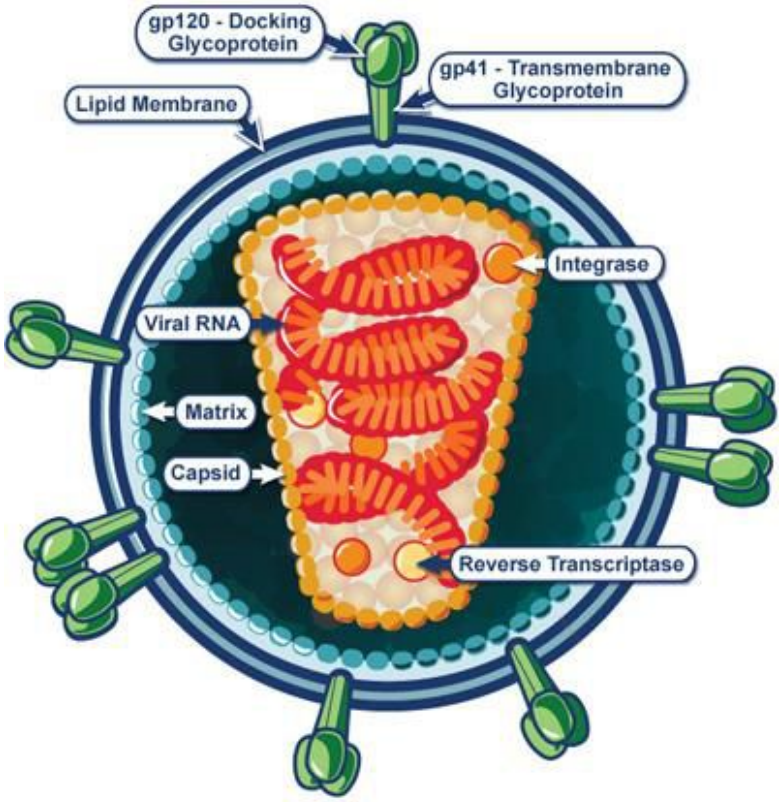


Figure 3: Human Immunodeficiency Virus Structure (United Nations Programme on HIV/AIDS., 2013)

1.3.3 Infection life-cycle of HIV-1

The life-cycle of HIV starts with the attachment of the virus to the CD4+ T-cell receptor on the host cell surface, and ends with the production of new viral particles that bud off from the new host cell (Male *et al.*, 2006). There are three HIV enzymes which are crucial to the life cycle and development of a mature virion particle (Male *et al.*, 2006). Firstly the HIV intergrase (IN) enzyme facilitates HIV incorporation into the host chromosome (cDNA), secondly HIV reverse transcriptase (RT) enzyme which is vital for the viruses' replication and finally the HIV proteases (PR) enzyme essential for the production of viral polyproteins into functional enzymes and structural proteins, thereby facilitating maturation and infectivity of the virion particles (Ng *et al.*, 1997).

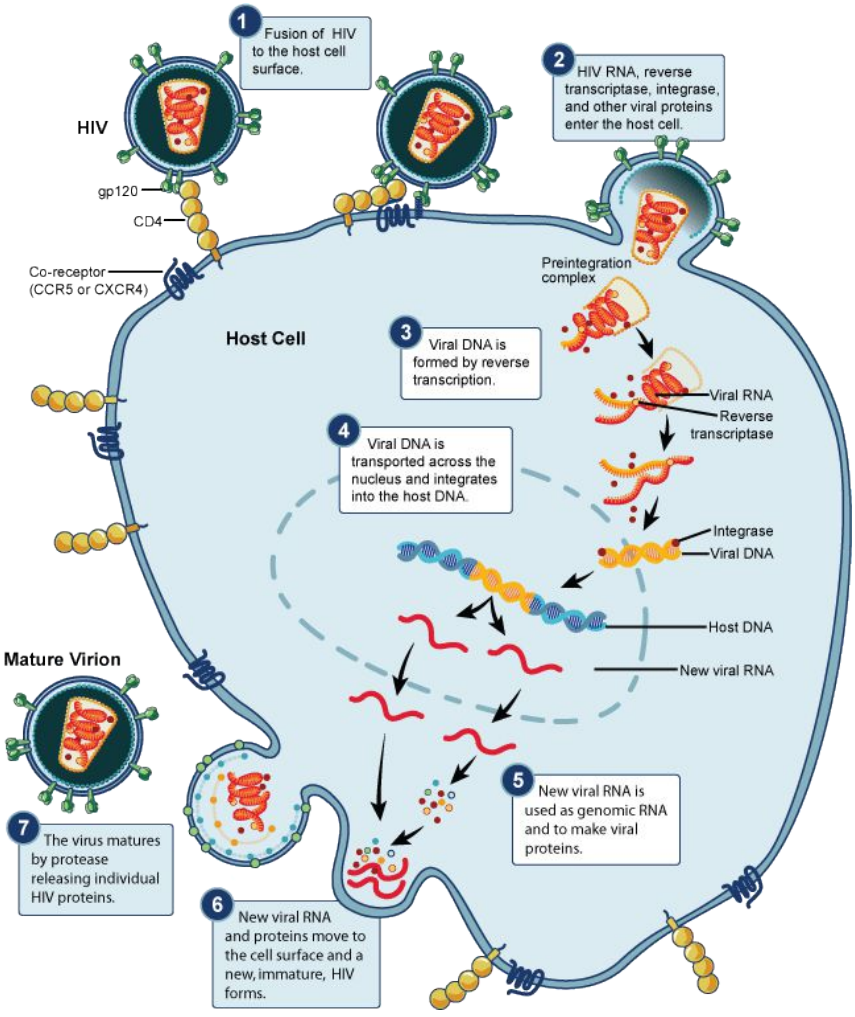


Figure 4: Life cycle of HIV (United Nations Programme on HIV/AIDS., 2013)

The basic steps of the HIV-1 life cycle are outlined in Figure 4. CD 4 + antigens are the main receptor for viral entry. It is present on CD 4+ T lymphocytes, monocytes, dendritic cells and brain microglia (Male *et al.*, 2006). The gp120 protein of the viral surface binds to a CD4 + receptor on the host cell surface by a mechanism that involves conformational changes that expose binding sites for the chemokine receptors (CCR5 or CXCR4), which then serve as co-receptors for viral entry (Prescott *et al.*, 1993). The co-receptor protein is a part of the host cell. The co-receptor is important in the successful binding of HIV-1 to the host cells (Ng *et al.*, 1997). HIV-1 infectivity is sometimes compromised in individuals who have mutant (non-functioning) CCR5 proteins and those agents that bind to CCR5 and CXCR4 (thus blocking HIV-1 from binding to these receptors) hence reducing viral infectivity (Male *et al.,* 2006). When the virus successfully attachs to the host cell, the virus is then able to pass through the host cell membrane, by fusing the viral envelope to the plasma membrane of the host cell (Ng *et al.*, 1997).

Upon entry of the virus into the host cell reverse transcription begins, where the viral single-stranded RNA genome is converted into double-stranded DNA (Roitt *et al*, 2001). Reverse transcription is a crucial step in the HIV-1 life cycle since it prepares the viral genome for the successful integration into the host chromosome. (Prescott *et al.,* 1993). Due to HIV reverse transcriptase being very error-prone, which results in nucleotide mismatches causing a high mutation rate and combined with the lack of proof-reading by reverse transcriptase, this causes great genetic diversity in the HIV viral genome (Roitt *et al*, 2001). Once reverse transcription is accomplished, the viral genome which is now a double-stranded DNA molecule and the proteins associated with reverse transcription, can then enter the nucleus of the host cell, and the viral genome which is incorporated within the host chromosome is termed a provirus (Prescott *et al.,* 1993).

Integration is a process by which, the HIV-1 genome becomes integrated into the host cell's chromosome, this is essentially done in three-steps, firstly the host chromosome and the viral DNA are arranged and prepared for their intergration, secondly the host chromosome and the viral DNA are then intergrated together and finally the host chromosome and the viral DNA are chemically bonded together which is termed post-integration repair. (Roitt *et al*, 2001).

The provirus DNA can either start manufacturing infectious viral particles or become a latent infection, this is dependent on critical host factors of whether the host cell is in an activated or inactivated state (Roitt *et al*, 2001).

In an activated cellular arrangement, the provirus DNA is copied by the host cell's RNA polymerase II to create viral RNA, and these transcripts are spliced into messenger RNAs for the translation to form HIV regulatory proteins (Tat, Rev, others) or precursor proteins encoded by the gag, pol, and env genes however the core protein precursor is only cleaved by the HIV protease (Male *et al.*, 2006). The infectious virion particle is assembled to have a core which contains RNA (2 copies), proteins (p24, p17, p9, p7) and pol enzymes (protease, reverse transcriptase and intergrase enzymes) which is pushed to the cell membrane to acquire envelope proteins (gp 120 and gp 41) and this then buds off using the plasma membrane to infect healthy cells (Roitt *et al*, 2001).

1.3.4 Antiretroviral (ARV) therapy

Antiretroviral therapy (ART) is treatment for people infected with HIV using ARV (Meintjes *et al.*, 2012). Highly active antiretroviral therapy (HAART) is the standard treatment plan, which consists of a combination of at least three drugs that causes suppression of HIV replication cycle. These three drugs are used in order to reduce the likelihood of the virus developing resistance. The oldest combination is AZT/3TC, but a number of other 2-3 fixed dose combinations such as LPV/r, ATV/r and DRV/r, are now available in Southern Africa (Meintjes *et al.*, 2012). The potential of HIV to become resistant to ARV has become an increasing concern since it was first reported that HIV variants isolated from patients following prolonged AZT therapy showed reduced susceptibly to AZT alone (Clercq., 1995).

With new drugs being developed and researched, and the use of drug combinations, antiviral therapy for HIV infection seems to be at a more advanced stage right now compared to several years ago. Table 1, lists the approved drugs used for treatment in South Africa. These changes are brought about by the better understanding of the disease (Lipsky., 1996). The first drug to be approved for use in HIV infection, Zidovudine, is a dideoxynucleoside, a structure that lacks a hydroxyl group on the 3' position in the ribose ring (Lipsky., 1996). The life cycle of HIV infection shows several susceptible pathways that can be inhibited by pharmaceutical agents/ARV. These classes of ARV agents are described in Table 1. Although

these drugs dramatically improved the quality of life and survival of HIV-infected individuals, antiretrovirals are still not able to completely eradicate HIV from the body. Antiretrovirals sometimes induce long term toxicity, and eventually lead to the development of drug resistant HIV strains (Pereira *et al.*, 2004). Combinations of drugs that target different viral steps reduce the risk of developing, or delay, the development of resistance to individual drugs (Meintjes *et al.*, 2012).

One of the characteristics of HIV infection is the rapid development of a genetically complex (quasispecies) population from a primarily limited number of virus particles, this is the major obstacles to eradication of HIV (Smyth *et al*., 2012). This quasispecies responed rapidly to selective pressures imposed by either the immune system or ART, and one of the main reasons for the failure of vaccines development (Smyth *et al*., 2012).

According to Department of Health of South Africa there are standardised national ART regimens for adults and adolescents (Department of Health., 2013)

All infected indivuals needing treatment, including pregnant women are treated with Tenofovir and Emtricitabine (or Lamivudine) and Efavirenz (Department of Health. 2013). Department of Health., 2013 states that adolescents usually treated with Abacavir and Lamivudine and Efavirenz, there are alternative ARV combinations if there is any contra-indications to any of the drugs regimens listed above such as:

- Contra-indications to EFV is replaced with either Tenofovir and (Emtricitabine or Lamivudine) and Nevirapine
- Contra-indication to TDF is replaced with either Zidovudine and Lamivudine and Efavirenz or (Nevirapine)
- Contraindication to TDF and AZT either Stavudine and Lamivudine is used

Table 1: Classes of ARV agents adapted from Lipsky., 1996 and Meintjes *et al.*, 2012

CLASS	ABBREVIATION	MECHANISM OF ACTION / PATHWAY INHIBITED	EXAMPLES OF ARV	COMMON ADVERSE DRUG REACTIONS OF ARV
Chemokine receptor antagonist	CRA	Entry can be blocked by the chemokine receptor antagonist	maraviroc (MVC)	• MVC: low fever, itching, nausea, gastro-intestinal upset, loss of appetite, dark urine, clay-colored stools; jaundice
Nucleoside and nucleotide reverse transcriptase inhibitors	NRTIs/ NtRTIs	Reverse transcription is blocked by the nucleoside and nucleotide reverse transcriptase inhibitors	zidovudine (AZT), didanosine (DDI), lamivudine (3TC), stavudine (D4T), abacavir (ABC), fumarate (TDF) emtricitabine (FTC), nevirapine (NVP), tenofovir disoproxil efavirenz (EFV), etravirine (ETR), and rilpivirine (RPV).	• AZT: Bone marrow suppression, gastro-intestinal upset, headache, myopathy, hyperlactataemia / steatohepatitis and lipo-atrophy. • DDI: Peripheral neuropathy, pancreatitis, nausea, diarrhoea and hyperlactataemia/ steatohepatitis. • 3TC: Anaemia and hyperlactataemia / steatohepatitis • D4T: Peripheral neuropathy, lipo-atrophy, hyperlactataemia / steatohepatitis, pancreatitis and HIV associated neuromuscular weakness syndrome (HANWS). • ABC: Hypersensitivity reaction and hyperlactataemia/ steatohepatitis. • TDF: Renal failure, tubular wasting syndrome, reduced bone mineral density and hyperlactataemia/ steatohepatitis. • FTC: Palmar hyperpigmentation and hyperlactataemia/ steatohepatitis • NVP: Rash and hepatitis • EFV: Central nervous system symptoms, rash and hepatitis
Intergrase strand transfer inhibitors	InSTIs	Integration is blocked	raltegravir (RAL) and elvitegravir (ELV)	• RAL: Rash, headache and gastro-intestinal upset
Protease inhibitors	Pis	Virus maturation is blocked	amprenavir (APV), atazanavir (ATV), darunavir (DRV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), saquinavir (SQV), tipranavir (TPV) and ritonavir ®	• IDV: Kidney stones, unconjugated hyperbilrubinanaemia, gastro-intestinal upset, hair loss, hyperglycaemia, headaches and dyslipidaemia. • ATV: unconjugated hyperbilrubinanaemia, dyslipidaemia, renal stones and hepatitis. • LPV/r: gastro-intestinal upset, dyslipidaemia and hepatitis. • DRV: gastro-intestinal upset, dyslipidaemia, hepatitis, and rash • SQV: gastro-intestinal upset, dyslipidaemia, hepatitis, and hyperglycaemia

1.4 ANTI- HIV ACTIVITY OF PLANTS

Plants have structural diversity and a vast range of biological activities, therefore plant secondary metabolites represent a huge repository for novel anti-HIV drugs that may be functional against resistant HIV strains. A number of medicinal plants have been reported to have anti-HIV properties and these are reviewed by Yang *et al.*, (2001); Cos *et al.*, (2004); Singh *et al.*, (2005); McRae *et al.*, (2007); and Chinsembu and Hedimbi., (2010). Bioactivity-guided fractionation of these crude extracts has provided a platform for the discovery of novel anti-HIV compounds. These new compounds must have two crucial qualities:

(i) They must be specific for HIV-1;
(ii) Interfere minimally with the cellular processes of the host.

With the emergence of drug resistant HIV variants in patients who are being treated with ARV, the search for other effective inhibitors of HIV has accelerated. There are millions of different compounds that can be found within the thousands of plant species worldwide. They are usually classified into distinct classes of compounds based on similar characteristics. A variety of secondary metabolites obtained from plants have shown moderate to good anti-HIV activity. The main classes of compounds from plants include: flavonoids, terpenes, alkaloids, saponins, phenolics and coumarins (McRae *et al.*, 2007), as summarised in Table 2.

Alkaloids are the most commonly investigated group of plant compounds due to their highly active nature and being easily extracted from plant material (McRae *et al.*, 2007). A variety of alkaloids such as Castanospermine, Papaverine, Michellamine B, FK-3000 (morphine related) have been found to possess HIV-inhibitory activity (Singh *et al.*, 2005). Castanospermine was isolated from the plant *Castanospermum austral* (Family Fabaceae) and has shown inhibition of HIV replication and syncytium formation (Singh *et al.*, 2005). Papaverine an alkaloid obtained from the plant *Papaver sominiferum* (Family: Papaveraceae) has been shown to inhibit HIV replication and reduce production of HIV proteins (Yadav *et al.*, 2009). Michellamine B which was isolated from the leaves of *Ancistrocladus korupensis* (Family: Ancistrocladus) demonstrated inhibition of HIV replication and syncytium formation (Singh *et al.*, 2005).

Flavonoids have been reported to possess a whole array of biological activity (Singh *et al.*, 2005; McRae *et al.*, 2007; and Yadav *et al.*, 2009) and this is due to their antioxidant properties. In a healthy individual there is a balance within the body with the antioxidant

defence system. Oxidative stress results from an imbalance and has been implicated in a variety of disorders such as cancer, Parkinson's disease and AIDS (Singh *et al.*, 2005). From the leaves of the plant *Monotes africanus* (Family: Dipterocarpaceae), 6, 8-diprenylaromadendrin and 6,8-diprenylkaempferol were isolated and exhibited HIV-inhibitory activity in the XTT-based, whole-cell screen (Yadav *et al.*, 2009). Quercetin 3-*O*-(2-galloyl) *a*-L-arbinopyranose, isolated from the ethanolic extract of *Acer okamotoanum* (Family: Aceraceae), possessed anti-HIV-1 integrase activity (Yadav *et al.*, 2009).

Comarins are most abundant in grasses and have a broad spectrum of activity ranging from antimicrobial, antiviral, antithrombotic and anti-inflammatory activity (McRae *et al.*, 2007). Calanolides (Calanolide A, Calanolide B) are isolated from various species of *Callophyllum spp* (Family: Clusiaceae) and have non-nucleoside specific reverse transcriptase inhibitors and protects cells from cytopathic effect (Yadav *et al.*, 2009). Suksdorfin is isolated from the fruits of *Lomatium suksdorfii* (Family: Apiaceae) and inhibits HIV replication in the T cell line (Singh *et al*., 2005).

Phenolic compounds have shown great potential as virucidal compounds in several viral systems, because polyphenols act by associating with the proteins of viral particles and/or host cell surfaces, resulting in reduction or prevention of viral absorption. Repandusinic acid isolated from the aqueous extract of *Phyllanthus niruri (*Family Euphorbiaceae) inhibits HIV-1 reverse transcriptase (Yadav *et al.*, 2009). Catechin isolated from *Detarium microcarpum* (Family: Caesalpiniaceae) has demonstrated anti-HIV activity by blocking the binding of gp-120 to CD 4 cells and inhibits reverse transcriptase activity (Yadav *et al.*, 2009).

In the past decade much progress has been made in the discovery of potent anti-HIV agents from plants. Plants have been used as leads for the discovery of novel compounds, because of their specific activity and low toxicity (Singh *et al.*, 2005). Although no plant derived drug is currently in clinical use to treat AIDS, there is much going on research in this area. However, calanolide A (a class of coumarin derivatives from the tropical rainforest tree, Calophyllum *spp*) is in phase II clinical trials for assessment of its long term anti-HIV activity (Singh *et al.*, 2005). Calanolide A is also being used in combination with other approved anti-HIV agents to determine its anti-HIVactivity (Singh *et al.*, 2005).

Table 2: Main classes of compounds found in plants with anti-HIV activity adapted from a review article by Yadav *et al.*, (2009).

Main Classes	Example	Mode of Action	Family/ Species
Alkaloids	Castanospermine	Inhibits the enzyme α-glucosidase and synergistic effect with Zidovudine against HIV-1 and HIV-2 without any toxicity	*Castanospermum austral* Family: Fabacae
	Papaverine	Inhibits HIV replication *in vitro* and reduces HIV protein production	*Papaver sominiferum* (Family: Papaveraceae)
	Michellamine B	Inhibits reverse transcriptase and cellular fusion and syncytium formation	*Ancistrocladus korupensis* (Family: Ancistrocladaceae)
	FK-3000 (morphine related)	Inhibits the cytopathic effects of HIV-1 on MT-4 cells	*Stephania cepharantha* (Family: Menispermaceae)
Coumarins	Calanolides, Calanolide A, Calanolide B	Non-nucleoside specific reverse transcriptase inhibitors Protects cells from cytopathic effect	*Callophyllum sp,* (Family: Clusiaceae)
	Suksdorfin	Inhibits HIV replication	*Lomatium suksdorfii* (Family: Apiaceae)
	Imperatorin	Anti-HIV and antiviral activity	*Ferula sumbul* (Family : Umbelleferae)
Flavonoids	6,8-diprenylaromadendrin 6,8-diprenylkaempferol	Exhibited HIV-inhibitory activity in the XTT-based, whole-cell screen	*Monotes africanus* (Family: Dipterocarpaceae)
	Quercetin 3-*O*-(2-galloyl) *a*-L-arbinopyranose	Possessed anti-HIV-1 integrase activity	*Acer okamotoanum* (Family: Aceraceae)
	Robustaflavone Hinokiflavone	Showed strong inhibition of the polymerase of HIV-1 Rtase in *in vitro* assay	*Rhus succedanea* (Family: Anacardiaceae)
	Wikstrol B	Showed good activity against HIV-1 in *in vitro* studies	*Wikstroemia indica* (Family: Thymelaeaceae)
Saponins	Escins	Moderate anti-HIV-1 protease activity	*Aesculus chinensis* (Family: Hippocastanaceae)

Main Classes	Example	Mode of Action	Family/ Species
Phenolics	Repandusinic acid	Inhibited HIV-1 Rtase	*Phyllanthus niruri* (Family Euphorbiaceae)
	Catechin	Blocks the binding of gp-120 to CD 4 cells and Inhibits reverse transcriptase activity	*Detarium microcarpum* (Family: Caesalpiniaceae)
	Guttiferone A	Cytoprotection of CEM-SS cells from HIV-1 infection	*Symphonia globulifera* (Family: Guttiferae)
	Gallic acid	Exhibits HIV intergrase and Reverse transcriptase activity	*Terminalia chebula* (Family: Combretaceae)
Quinones	Conocurvone	Showed potent anti-HIV activity	*Conospermum incurvum* (Family: Proteaceae)
	Dianthroquinone Hypericin	Non-human retroviruses as well as human retroviruses in lymphocytes and it has also inhibited HIV-1 Rtase	*Hypericum perforatum* (Family: Hypericaceae)
Lignans	Phyllamyricin B, Retrojusticidin B	Exhibits strong inhibition of HIV-Rtase	*Phyllanthus myrtifolius/ P. urinaria* (Family Euphorbiaceae)
	Demethoxyepiexcelsin	Good anti-Hiv activity	*Listea verticillata* (Family: Lauraceae)
Terpenes	Betulinic acid, Platanic acid Oleanolic acid	Exhibited anti-HIV activity in H9 lymphocyte cell	*Syzigium claviflorum* (Family. Myrtaceae)
	Uvaol Ursolic acid Oleanolic acid	Showed potent inhibitory activity against HIV-1 protease. Inhibited HIV-1 replication in acutely infected H9 cells	*Crataegus pinatifida* (Family: Rosaceae) *Xanthoceras sorbifolia* (Family: Sapindaceae)
	Moronic acid	Showed significant anti-HIV activity	*Myrceugenia euosma* (Family: Myrtaceae)
	1*b*-hydroxymaprounic 3-*p*-hydroxybenzoate 2*a*-hydroxymaprounic acid 2,3-*bis*-*p*-hydroxybenzoate	Active against HIV-1 Rtase	*Maprounea Africana* (Family: Euphorbiaceae)
	ganoderic acid-*a* ganoderiol F ganodermontriol ganoderic acid B ganoderiol B ganoderic acid C1	Inhibits HIV-1 induced cytopathic effects in MT-4 cells also possessed HIV-1 protease inhibitory activity	*Ganoderma lucidum* (Family: Polyporaceae)
	Phorbol ester prostratin	Showed potent HIV inhibitory property	*Homalanthus nutans* (Family: Euphorbiaceae)

1.5 OVERVIEW OF PROJECT

The need for effective preventative and therapeutic agents for HIV/AIDS remains an urgent global priority and this research is a small step in this direction. The aim of this study was to screen medicinal plants of South Africa and identify and characterise bioactive compound/s from plants that might be used for HIV treatment. The study was designed in three phases:

- **Phase one**: Literature based desktop survey on medicinal plants of South Africa traditionally used for any microbial diseases. A comprehensive list of potential active plants was documented;
- **Phase two**: Screening of the plants by testing the extracts for inhibiting viral proliferation. The plants that demonstrated activity were further evaluated for reverse transcriptase activity. The active plant was identified;
- **Phase three**: From the active plant, the active compound was identified using thin layer chromatography, high performance liquid chromatography, ultra performance liquid chromatography and finally nuclear magnetic resonance. The identified compound was evaluated for inhibiting viral proliferation and for reverse transcriptase activity.

AIM: To investigate the potential anti-HIV activity of medicinal plants and to isolate and characterize the active compounds responsible.

OBJECTIVES:

1. Survey of the literature, of plants which are used traditionally in South Africa
2. Screening of selected plants for cellular cytoxicity and anti-HIV activity.
3. Isolate and characterize the active compound from plant showing anti-HIV activity.

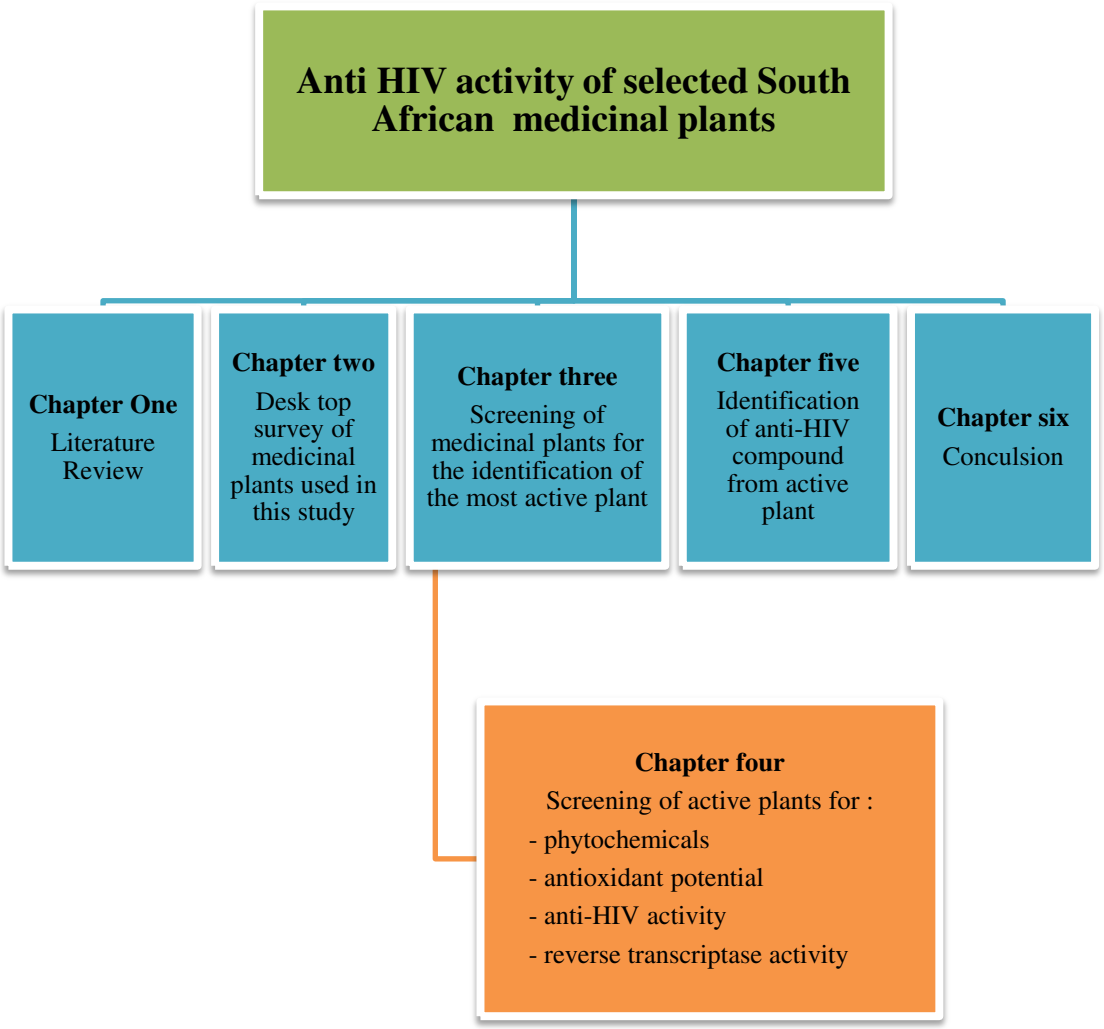


Figure 5: Overview of project

CHAPTER TWO: SURVEY OF MEDICINAL PLANTS

2.1 INTRODUCTION

Medicinal plants are widely used in traditional cultures all over the world (Van Wyk and Wink., 2004). There is a perception that African people sometimes visit traditional healers even before western medicine is considered. Natural products and their derivatives represent more than 50% of all the drugs in clinical use in the world (Gurib-Fakim., 2006). Historically plants have been the basis for the treatment of a number of human ailments. During the last 40 years, at least a dozen potent drugs have been derived from plants such as (Gurib-Fakim., 2006):

- Diosgenin was derived from *Dioscorea spp* on which all contraceptive agents have been based;
- Vinblastine and vincristine, are powerful anti-cancer agents from the Citrus family which have been isolated from *Catharanthus roseus*; and
- Digoxin is a cardiotonic agent which is used to treat heart failure isolated from *Digitalis spp*

The present study finds support in the views of Fabricant and Farnsworth., (2001) who stated that the goals of using plants as sources of therapeutic agents are :

i. To identify novel compounds as useful pharmaceuticals e.g., digoxin, morphine, taxol, vinblastine and vincristine;
ii. To yield compounds either novel or known as pharmaceuticals with either lower toxicity or increased acitivity that could be patented e.g., metformin, nabilone, oxycodon, taxotere, teniposide, verapamil, and amiodarone;
iii. To use entire plant or portion thereof as a homeopathic treatment, e.g., cranberry and echinacea

Recently, the isolation and identification of novel compounds from plants has increased. Given the diversity of plant species present in South Africa, this represents a huge repository for novel compounds, which have the potential to become drugs. Analysing traditionally used plants has the added benefit of scientifically determining whether its use in traditional medicine is justified. Therefore this literature survey provides an evidence-based contribution to our understanding of plants that can be used in the identification of new compounds for the treatment of HIV/AIDS

2.2 METHODOLOGY

A literature search was performed using the key words “medicinal plants of South Africa” in the database; of Science direct via the university link:
(http://www.sciencedirect.com.dutlib.dut.ac.za:2048/)
This was the primary information used. Within the processs of the literature search, which lasted 3 months, only peer reviewed journal articles, written in the English language were used. Pay-to view articles were not included. However secondary information was obtained from library books which are referenced herein. Taxonomic families and species of plants, active compounds and their modes of action and traditional uses were documented from both primary and secondary forms of literature sources. The search was not restricted to any time frame. The inclusion criteria were:

a. Plants should have been known by their scientific names
b. Plants of South Africa traditionally used for the treatment of any microbial disease.
c. Active compounds were isolated or known

2.3 RESULTS

Of the 120 journal articles found during the search only ± 40 of them met the predetermined inclusion criteria. Out of the total number of articles reviewed only about a third were included in this study.

The literature survey documented about 33 plant families containing 38 plant species that are used traditionally in South Africa to treat various ailments and the active compounds of these plants were isolated or known. Several compounds suchs as flavonoids, terpenes, alkaloids, saponins, tannins, phenolics and coumarins have been reported to possess an array of biological activity. Details of the family name, scientific name, common name, part of plants used, traditional uses, active compounds and literature sources are listed in Table 3

Table 3: List of South African plants to be investigated for their Anti-HIV activity

FAMILY NAME	SCIENTIFIC NAME	COMMON NAME	PART USED OF PLANT	TRADITIONAL USES	ACTIVE COMPOUNDS
1. Amaranthaceae	*Alternanthera sessile* (L.) R. Br.	Sessile joyweed	Leaves	The plant enhances the secretion of milk in new mothers (Naples., 2005) Used as a remedy against intestinal cramps, diarrhoea, dysentery intestinal disorder, hepatitis, tight chest, bronchitis, asthma, lung troubles, to stop bleeding and as a hair tonic (Singh., 2009).	It contains abundant carotene, therefore it is used for curing night blindness (Naples., 2005).
	Achyranthea aspera (L.)	Prickly Chaff-flower	Leaves	Fever, especially malarial fever, dysentery, asthma, hypertension and diabetes (Chakraborty *et al.*, 2002).	Roots contains ecdysterone and oleanolic acid. Leaves and stems contains ecdysterone, alkaloids (betaine type or betalaine), aliphatic dihydroxyketone : 36,47-dihydroxy-henpentacontan - 4 - one (Chakraborty *et al.*, 2002).
	Amaranthus dubius (Mart.)	Wild spinach	Leaves, Inflorescence and seeds	Used for haemorrhage, anaemia, constipation, stomach pains and kidney complaints (Grubben and Denton., 2004).	The leaves contain considerable amounts of β-carotene, niacin, thiamin, riboflavin and ascorbic acid High content of hydrocyanic acid and oxalic acid (Grubben and Denton., 2004).
	Amaranthus hybridus (L.)	Rough pigweed	Leaves and Flowers + fruit	Used to treat anaemia, chronic fatigue, heavy menstrual bleeding intestinal bleeding, diarrhoea and coughs (Beekrum., 2003). Relieve constipation. Externally, leaves can be used as a poultice for bleeding wounds and infusions can be splashed onto the skin to alleviate burning and itchy skin (Grubben and Denton., 2004).	Various leaf extracts of *A. hybridus* (methanolic, hexane, ethyl acetate, dichloromethane) were found to contain varied types of pharmacologically active compounds such as flavonoids, steroids, terpenoids and cardiac glucosides (Maiyo *et al.,* 2010).
	Amaranthus spinosus (L.)	Spiny amaranth	Leaves	Leaves and roots relieve burns, abscesses, inflammatory swellings and bruises (Kirtikar and Basu., 2001).	Amaranthoside, amaricin, stigmasterol glycoside, spinoside, quercetin and betaine (Kirtikar and Basu., 2001; Zeashan *et al.*, 2009)**.**

FAMILY NAME	SCIENTIFIC NAME	COMMON NAME	PART USED OF PLANT	TRADITIONAL USES	ACTIVE COMPOUNDS
2. Acanthaceae	*Asystasia gangetica* (L.) T. Anderson	Hunter's spinach	Leaves	Anti-helmintic agent and is also used for relieving childbirth pains, stiff neck, nose bleeding, stomach aches and fever (Beekrum., 2003). Analgesic and treats epilepsy and urethral discharge (Grubben and Denton., 2004).	Salidroside, apigenin, ajugol, megastigmaneglucoside and benzyl β-D-glucopyranoside (Akah *et al.*, 2003).
3. Asteraceae	*Bidens pilosa* (L.)	Beggar's ticks	Leaves and Flower	Anti-inflammatory, anti-diabetic, diuretic, anti-rheumatic and antibiotic agents (Changa *et al.*, 2007).	Chalcones, flavonoids, polyacetylenes, glucosides and diterpenes (Chiang *et al.*, 2004).
4. Buddlejaceae	*Buddleia saligna* (L.)	False Olive	Leaves	Lowers blood pressures, colic, coughs, colds, sore eyes, urinary problems and as purgatives (Houghton *et al.*, 2003).	Flavonoids (luteolin, 6-hydroxyluteolin) and phenylethanoids (verbascoside, echinacoside), (Houghton *et al.*, 2003).
5. Capparidaceae	*Capparis tomentosa* (L.)	Woolly caper-bush	Leaves	Rheumatism, insanity, jaundice, malaria, headache, coughs and pneumonia (Van Wyk *et al.*, 2002).	Stachydrine, and 3-hydroxy-4-methoxy-3-methly-oxindole (Raven *et al.*, 1999).
6. Mesembryanthemaceae	*Carpobrotus dimidiatus* (L.) L. Bolus	Natal sour fig	Leaves	Dysentery, laryngitis, soothing cure for blue bottles stings. Ringworm, eczema, dermatitis, herpes, nappy rash, thrush, cold sores, cracked lips, chafing skin allergic reactions, ear ache and eye infections (Van Wyk *et al.*, 2002).	Tannins, malic acid and citric acid (Van Wyk *et al.,* 2002).
7. Apiaceae	*Centella asiatica* (L.) Urban	Gotu kola	Leaves	Depression, insomnia, stress and memory loss (Ganachari *et al.*, 2004). Treat fevers, diarrhoea, leprosy, stress, tubercolosis and cancer (Jayathirtha and Mishra., 2004).	Asiaticoside, madecassic, several monoterpenoids and some Sesquiterpenoids (Van Wyk *et al.*, 2002).
8. Pedaliaceae	*Ceratotheca triloba* (Bernh.) E. Mey ex Hook	Wild foxglove	Leaves	Painful menstruation, stomach cramps, nausea, fever and diarrhoea (Tregold *et al.,* 1986).	Three anthraquinone derivatives and one steroid, 9, 10 anthracenedione, 1-hydroxy-4-methylanthraquinone, 5, 8-dimethoxy-2, 3, 10, 10a-tetrahydro-1H-phenanthrene-4, 9-dione and androst-5-ene-3, 17, 19-triol (Mohanlall., 2011).

FAMILY NAME	SCIENTIFIC NAME	COMMON NAME	PART USED OF PLANT	TRADITIONAL USES	ACTIVE COMPOUNDS
9. Chenopodiaceae	*Chenopodium album* (L.)	Fat-hen	Leaves, Fruit +flowers	Infusion for urethral itch or poulticed onto burns (Duke., 1992).	Phenolic compounds (Akula and Odhav., 2008).
10. Capparaceae	*Cleome monophylla* (L.)	Spindle- pod	Leaves, Fruit + flowers and Roots	Rubbed onto the skin to relieve pneumonia; as an infusion it is used as eyewash (Tregold *et al.,* 1986).	No compounds have been isolated from this plant.
11. Fabaceae	*Dichrostachys cinerea* (Wight et Arn)	Sickle-bush	Leaves	Diarrhoea, toothaches and earache abdomininal pain, coughs and pneumonia (Van Wyk and Gericke., 2003)	Polyphenols especially flavonoids and tannins (Raven *et al.*, 1999).
12. Asteraceae	*Elytropappus rhinocerotis* (L.) Less.	Renosterbos	Leaves	Indigestion, dyspepsia, ulcers, stomach cancer and influenza (Van Wyk *et al.*, 2002).	Rhinocerotinoic acid (Van Wyk *et al.*, 2002).
13. Polygonaceae	*Emex australis* (Steinh.)	Devil's thorn	Leaves , Crowns and Roots	Unspecified parts of the plant are used to treat stomach and intestinal complaints (Dold and Cocks., 2000).	*E. australis* also displayed antioxidant activity owing to the presence of phenolic compounds in the plant. (Akula and Odhav., 2008).
14. Meliaceae	*Ekebergia capensis* (Sparrm.)	Cape ash, Dogplum	Leaves	Venereal diseases, chronic cough, emetic, backache, headache, dysentery and skin diseases (Mulaudzi *et al.*, 2011).	Phenolics: Flavonoids Gallotannin Condensed tannins (Mulaudzi *et al.*, 2011).
15. Moraceae	*Ficus sur* (Forssk.)	broom cluster fig	Leaves	Pulmonary tuberculosis, treatment of burns, alleviate thirst, sore throat, peptic ulcers (Kunle *et al.*, 1999).	Two pentacyclic triterpenoids of oleanane and ursene structures (Kunle *et al.*, 1999).
16. Asteraceae	*Galinsoga parviflora* (Cav.)	Gallant soldier	Flower heads and Roots	Treat colds and sores (Matu and Van Staden., 2003). Wound healing (Schmidta *et al.*, 2009).	Flavonoids: apigenin: 7-_-d-glucoside, luteolin7-d-glucoside (Schmidta *et al.*, 2009).
17. Amaranthaceae	*Guilleminea densa* (Moq.)	Small Matweed	Leaves	Sores, fever, vomiting and diarrhoea (Singh., 2009).	Vitamins A, B1, B2, B3 and iron (Singh., 2009).
18. Gunneraceae	*Gunnera perpensa* (L.)	River pumpkin	Leaves	Induce labour and child birth, rheumatic fever, swellings, menstrual pain and stomach bleeding (Van Wyk *et al.*, 2002).	Celastrin (Van Wyk *et al.*, 2002).

FAMILY NAME	SCIENTIFIC NAME	COMMON NAME	PART USED OF PLANT	TRADITIONAL USES	ACTIVE COMPOUNDS
19. Heteropyxidaceae	*Heteropyxis natalensis* (Harv.)	Lavender tree	Leaves	Colds, nose bleeding, bleeding gum and menorrhagia (Van Wyk *et al.*, 2002).	Monoterpenoids mainly β-ocimene. 1,8-cineole, limonene, linalool and myrcene (Van Wyk *et al.,* 2002).
20. Malvaceae	*Hibiscus sabdariffa* (L.)	Hibiscus	Calyx+flowers	Herbal tea, appetite loss, colds, catarrh of the respiratory tract, circulatory ailments, expectorant, laxative, diuretic, allergic eczema and various skin conditions (Van Wyk and Wink., 2004).	Organic acids (Hibiscus, ascorbic, citric, malic and tartaric acid) and polysaccharides: Arabinan, arabinogalactan, galacturonic acid, rhamnose, galactose and arabinose, and anthocyanins (Van Wyk and Wink., 2004).
21. Acanthaceae	*Justicia flava* (Vahl.)	Yellow justicia	Leaves and Flower+seeds	Fever, yaws and diarrhoea in children (Grubben and Denton., 2004).	Sterols and salicyclic acid were isolated from the leaves, stems and roots of *J. flava*. In addition, the leaves contain the three lignins, helioxanthin, Iso-lariciresinol and justicinol as well as docosanoic acid (Grubben and Denton., 2004).
22. Lamiaceae	*Leonotis leonurus* (L.) R. Br.	Wild Dagga	Leaves and Flowers	Coughs, colds, influenza, bronchitis, hypertension (Van Wyk *et al.*, 2002). jaundice, cardiac problems, asthma, hemorrhoids, headaches, chest ailments, bronchitis, epilepsy and earaches. (Malan *et al.*, 2006).	Rosmarinic acid and other derivatives of caffeic acid. (Raven *et al.,* 1999). Diterpenoids such as marrubiin (labdane type lactones) (Van Wyk *et al.*, 2002).
23. Cucurbitaceae	*Momordica balsamina* (L.)	Balasam apple	Leaves	Anti-helmintic, treat fevers, uterine bleeding, syphilis, rheumatism and skin disorders (Grubben and Denton., 2004). Hypoglycaemic and anti-malarial activity (Ramalhete *et al.,* 2009).	Ramalhete *et al*., 2009 reported on the isolation and structural elucidation of seven compounds Balsamina; balsaminapentaol, balsaminol A, balsaminol B, cucurbalsaminol B, cucurbita-5,23(*E*)-diene-3-,7-,25-triol and karavilagenin E.
24. Polygonaceae	*Oxygonum sinuatum* (Meissn.)	Stars talk	Leaves	Treat boils, tonsillitis, gastric ulcers, malaria, hepatitis and coughs (Grubben and Denton., 2004).	No compounds have been isolated from this plant

FAMILY NAME	SCIENTIFIC NAME	COMMON NAME	PART USED OF PLANT	TRADITIONAL USES	ACTIVE COMPOUNDS
25. Geraniaceae	*Pelargonium. sp* (L.)	Rabassam	Leaves	Diarrhoea and bronchitis (Van Wyk *et al.*, 2002).	Tannins,phenolic compounds, umckalin and monoterpenoids such as (+)-isomenthone (Van Wyk *et al.*, 2002).
26. Solanaceae	*Physalis viscosa* (L.)	Sticky gooseberry	Leaves	Tonic, sedative, laxative, diuretic, urinary disorders and inflammatory diseases (Beekrum., 2003).	Contain withanolides such as 4β-hydroxywithanolides and its 5,6-desoxi analogue, withaphysanolides and withanolide related pregnanes such as 4-hydroxy-5β, 6β-epoxypregn-2-ene-1,20-dione (Silva *et al.*, 1993).
27. Portulacaceae	*Portulaca oleracea* (L.)	Purslane	Leaves and Roots	Infusions for ear-ache, treatment for worms and poultice for bruises and burns. It is also used for pain and stomach-ache, boils, bug bites, colic, dermatitis, indigestion and snake bite (Duke., 1992).	No compounds have been isolated from this plant.
28. Myrtaceae	*Syzygium cordatum* (Hochst.)	Waterbessie	Leaves	Respiratory ailments, tuberculosis, stomach complaints, diarrhoea and emetic (Van Wyk *et al.*, 2002).	Phenolic compounds such as triterpenoids (Van Wyk *et al.*, 2002).
29. Fabaceae	*Senna occidentalis* (L.)	Coffee senna	Leaves	Leaf extracts are used as an analgesic, anti-inflammatory, antibacterial, antifungal, antiviral, febrifuge, purgative and immunostimulant and used as a purgative and laxative (Demel and Teketay., 1996).	Anthraquinone compounds are common in this species.
30. Solanaceae	*Solanum nodiflorum* (Jacq.)	Black nightshade	Leaves , Roots and Fruits + flowers	Anti-phlogistic, anti- phoretic, diuretic, emollient, narcotic, purgative, sedative and cancerous sores (Beekrum., 2003).	Green berries contain solanine (Tredgold *et al.*, 1986).

FAMILY NAME	SCIENTIFIC NAME	COMMON NAME	PART USED OF PLANT	TRADITIONAL USES	ACTIVE COMPOUNDS
31. Asteraceae	*Sonchus oleraceus* (L.)	Sow thistle	Leaves and Roots	Teatment of diarrhoea, used to treat warts, applied as a poultice to inflammatory swellings (Auld and Meld., 1992).	15-O- *β* -glucopyranosyl-11 and *β*,13-dihydrourospermal (Elkhayat., 2009).
	Taraxcum officinale (F. H. Vigg)	Common Dandelion	Leaves and Roots	Diuretic, stomach, liver, gall bladder, rheumatic complaints and eczema (Van Wyk and Wink., 2004).	Sesquiterpene lactones (tetrahydroridentin B, taraxacolide β-D-glucoside), phenolic acids (taraxacoside), triterpenoids and taraxasterol (Van Wyk and Wink., 2004).
32. Lamiaceae	*Tetradenia riparia* (Hochst.)	Misty Plume Bush	Leaves	Respiratory ailments (coughs, colds, sore throat), mouth ulcers, stomach ache, diarrhoea, influenza, fever, malaria, swollen legs and headache (Van Wyk *et al.*, 2002).	Ibozol, a diterpenediol, and various similar diterpenoids such as 8(140,15-isopimaradiene-7,18-diol and α-pyrones such as umuravumbolide (Van Wyk *et al.*, 2002).
33. Alliaceae	*Tulbaghia violacae* (Harv.)	Wild garlic	Leaves	Fever, colds, asthma, tuberculosis, enemas for stomach problems, cancer of the oesophagus (Van Wyk *et al*., 2002).	Two sulphur componds similar to alliin (Van Wyk *et al*., 2002)

2.4 DISCUSSION

Various traditional plants are used in South Africa for the treatment of an assortment of illnesses and also as part of everyday diet. A detailed description of each plant and its medicinal uses as well as habitats and distribution are provided below. For easy of reference an overview of all plants used in this study is presented in Table 3.

2.4.1 *Alternanthera sessile*

Figure 6: ***Alternanthera sessile***

A. sessile is an aquatic plant which belongs to the family Amaranthaceae, and is commonly called sessile joyweed. It is a perennial herb with prostrate stems, rarely ascending, often rooting at the nodes. Leaves are thin to broadly elliptic in shape. Flowers are situated over the stem, are round in shape and are shiny white in colour. Leaves along with the flowers and tender stems are used as a vegetable. The juice of this plant, deemed beneficial to the eyes, is an ingredient in the making of medicinal hair oils and kajal (Naples., 2005). The leaves show high concentration of Vitamin A (β-carotene) and can used for the treatment of its deficiency in conditions related to poor vision, skin aliments (Naples., 2005). The plant leaf extract has been used as a galactogogue (Naples., 2005). The phytochemical anaylsis of the leaves yielded positive results for the presence of sitosterol, stigmasterol, campesterol, spinasterol, oleanolic acid rhamnoside, 24-methylene cycloartenol, cycloeucalenol, lupeol, 5-cc-stigmasta-7-enol and palmitate (Naples., 2005).

2.4.2 *Achyranthea aspera*

Figure 7: ***Achyranthea aspera***

A. aspera belongs to the family Amaranthaceous and is commonly called Prickly Chaff-flower. It is an erect annual herb with a woody base. It has stems that are ribbed, simple or branched from the base, often with tinged purple colour (Srivastav *et al.*, 2011). The leaves are dark green, thick, ovate and elliptic in shape. Fine hair are present on both sides of the leaves. The flowers are greenish white, numerous in axillary.

The main chemical constituent present is achyranthine. Chemical investigations of the seeds reported the isolation and identification of saponins A and B (D-Glucuronic Acid, β-galactopyranosylester of D-Glucuronic Acid). Other constituents isolated were oleanolic acid, amino acids and hentriacontane. (Srivastav *et al.*, 2011). Chakraborty *et al*., (2002), reported that the roots have ecdysterone and oleanolic acid while the leaves and stems have ecdysterone, alkaloids (betaine type or betalaine), aliphatic dihydroxyketone (36,47-dihydroxyhenpentacontan-4-one). It has been used as folk medicine. According to Ayurvedic medicine, it is useful in the treatment of vomiting, bronchitis, heart disease, piles, itching abdominal pains, ascites, dyspepsia, dysentery, blood diseases and skin aliments (Srivastav *et al.*, 2011). Also used to treat fever, especially malarial fever, dysentery, asthma, hypertension and diabetes (Chakraborty *et al.*, 2002).

2.4.3 *Amaranthus dubius*

Figure 8: ***Amaranthus dubius***

A. dubius belongs to the family Amaranthaceous. It is a flowering plant, also known as wild spinach or spiny amaranth (Odhav *et al.*, 2007) and it is widespread throughout the humid lowland tropics. The plant grows up to 150 cm tall, has slender to stout stems and simple, spiral leaves without stipules. It is a protected weed cooked as a pot herb in many African countries. The leaves are generally recommended as a healthy food options cause of the high antioxidant properties and iron content (Van Wyk *et al.*, 2002 and Odhav *et al.*, 2007). The leaves are used in the treatment of various aliments such as internal bleeding, anaemia, constipation, gastrointestinal pains and kidney complaints (Grubben and Denton., 2004). The phytochemical analysis of the leaves demonstrate a chemical profile of β-carotene, niacin, thiamin, riboflavin and ascorbic acid (Van Wyk *et al.*, 2002). The leaves are not suitable for fresh consumption by humans due to the elevated levels of hydrocyanic acid and oxalic acid present in the leaves (Grubben and Denton., 2004).

2.4.4 *Amaranthus hybridus*

Figure 9: ***Amaranthus hybridus***

A. hybridus belongs to the family Amaranthaceae. It is a popular nutritious leafy vegetable that is cultivated for the leaves which are eaten as spinach. The plant is rich in minerals, proteins and vitamins and is thus consumed widely in Southern Africa (Mellem., 2008). It is a 2.5 m high, erect, branched, perennial herb that reaches maturity in six weeks. It is found on wastelands or near old kraals. The leaves are edible and can be taken as an infusion to treat anaemia, chronic fatigue, heavy menstrual bleeding, intestinal bleeding, diarrhoea and coughs (Beekrum., 2003). The juice of the whole plant is used to relieve constipation. Externally, the crushed leaves can be used as a poultice for bleeding wounds and infusions can be splashed onto the skin to alleviate burning and itchy skin. Leaf infusions also have cosmetic uses as a cleansing rinse for oily skin since it tightens pores and moistens the skin (Grubben and Denton., 2004). Various leaf extracts of *A. hybridus* (methanolic, hexane, ethyl acetate, dichloromethane) were found to contain varied types of pharmacologically active compounds such as flavonoids, steroids, terpenoids and cardiac glucosides (Maiyo *et al.,* 2010).

2.4.5 *Amaranthus spinosus*

Figure 10: ***Amaranthus spinosus***

A. spinosus is another member of the Amaranthaceae family and is commonly referred to as spiny pigweed, thorny amaranth or prickly amaranth. The plant grows annually as an erect, branched monoecious herb and it is found mostly on roadsides and wastelands. Rural communities in Africa consume this plant as a non-conventional leafy vegetable. A number of traditional uses have been documented for this plant. It improves appetite, biliousness, blood diseases, burning sensations, bronchitis, piles, leucorrhoea and also acts as a laxative, diuretic and antipyretic. The leaves and roots are used to relieve burns, abscesses, inflammatory swellings and bruises (Kirtikar and Basu., 2001). The chemical constituents of this plant include; amaranthoside, amaricin, stigmasterol glycoside, spinoside, quercetin and betaine (Kirtikar and Basu., 2001; and Zeashan *et al.*, 2009)**.**

2.4.6 *Asystasia gangetica*

Figure 11: ***Asystasia gangetica***

A. gangetica (Family Acanthaceae), also known as Hunter's spinach and creeping foxglove is an erect, clambering herb that can grow up to 1.25 m. It has green, oval shaped, smooth leaves and is generally found along roadsides and waterbanks, in semi-water logged areas (Akah *et al.,* 2003). The leaves are good sources of proteins, amino acids, sugars, lipids, fibres and minerals, accounting for its potential as a food source. The plant is used as an antihelmintic agent and is also used for relieving childbirth pains, stiff neck, nose bleeding, stomach aches and fever (Beekrum., 2003). The sap is applied to sores, wounds and piles. A leaf decoction is used as an analgesic and treats epilepsy and urethral discharge (Grubben and Denton., 2004). Known compounds of *A. gangetica* include; salidroside, apigenin, ajugol, megastigmane glucoside and benzyl b-D-glucopyranoside (Akah *et al.,* 2003). The traditional medicinal uses of this plant are based on its antihelmintic and anti-parasitic properties. Research also shows that the leaf extracts are effective in the local treatment of asthma (Akah *et al.*, 2003).

2.4.7 *Bidens pilosa*

Figure 12: ***Bidens pilosa***

B. pilosa, commonly known as black jack belongs to the Asteraceae family, the largest flowering plant family in the world. This plant inhabits mainly plantations, roadsides and wastelands thriving in moist, nutrient-rich soil. Its characteristic features include the petiolate leaves, erect, ramified stems and the flowers which have white ray florets and yellow disk florets. The plant is used traditionally in several forms of treatment, namely as anti-inflammatory, anti-diabetic, diuretic, anti-rheumatic and antibiotic agents (Chiang *et al.*, 2004). The juices of the leaves are used to dress wounds and as a drink for ulcers (Beekrum., 2003). Previous studies reported on the bioactivities of *Bidens pilosa* plant extracts such as, anti-hyperglycemic (Ubillas *et al.*, 2000), anti-hypertensive (Dimo *et al.,* 2001), anti-leukemic (Chiang *et al*, 2004) and anti-microbial (Khan *et al,* 2001) activities. Phytochemical studies have reported on the isolation of several photochemical constituents from the plant namely, chalcones, flavonoids, polyacetylenes, glucosides and diterpenes (Chiang *et al.*, 2004).

2.4.8 *Buddleia saligna*

Figure 13: ***Buddleja saligna***

B. saligna belongs to the family Buddlejaceae and is commonly called false olive. It grows to an evergreen shrub (small tree), growing up to 15 m in height. The bark becomes longitudinally furrowed with age. The branchlets are quadrangular in section, and are winged. The leaves are dark green, smooth and hairless, while the underside is clothed in pale stellate hairs. The honey - scented flowers are cream or white.

It is traditionally used to treat many ailments. The leaves are used to treat coughs, tuberculosis and colds and the roots as a laxative, for diabetes, as well as for thrush and sores (Houghton *et al.*, 2003). This tree is popular with bee farmers because it produces a large amount of pollen and nectar. It is sometimes grown as a bonsai. The wood has a very fine grained texture and is sometimes used to make furniture or wood ornaments. Also makes excellent fire wood as it burns with intense heat. There is little published literature dealing with the secondary chemistry of *Buddleja saligna* but studies by Houghton *et al*., (2003) demonstrated that this genus is known to contain iridoids, acetylated iridoids, saponins, sesquiterpenoids and flavonoids. Flavonoids such as luteolin, 6-hydroxyluteolin and phenylethanoids (verbascoside, echinacoside) have been isolated from the aerial parts of the plant (Houghton *et al.*, 2003).

2.4.9 *Capparis tomentosa*

Figure 14: ***Capparis tomentosa***

C. tomentosa belongs to the family Capparaceae commonly referred to as 'Woody Caper Bush' in English or in Afrikaans as the Wollerige Kapperbos (Windadri., 2001).

It is a scrambling shrub, sometimes developing into a tree that can grow as high as 10 m tall. The twigs and leaves are yellow-green in colour and are covered in soft, velvety hairs. The oblong leaves are approximately 50 × 20 mm, with a pair of sharp, hook-like thorns at the junction of the stem and leaf base. The white and pink flowers have multiple stamens as shown in Figure 14. The fruit is pink to orange in colour, round and stalked. The seeds are surrounded by fleshy, grey fruit pulp (Windadri., 2001).

It is used as a popular medicine for rheumatism, insanity, snakebite, chest pain, jaundice, malaria, headache, coughs, pneumonia, constipation, infertility and to prevent abortions. It is also used to treat leprosy, tuberculosis and gonorrhea (Van Wyk and Gericke., 2003).

The phytochemicals present are alkaloids, L-stachydrine, saponin glycosides, tannins, sterol, polyphenols, flavonoids and anthranoids, (Sama and Ajaiyeoba., 2006; and Oluwole *et al.*, 2007). Two alkaloids, Stachydrine, and 3-hydroxy-4-methoxy-3-methly-oxindole have been isolated from *C. tomentosa*. The oxindole compound has been found to possess weak anti-spasmodic activity, substantiating the traditional use of *Capparis species* for their anti-inflammatory and anti-convulsive properties. Stachydrine has been widely researched in the treatment of rheumatism (Van Wyk *et al*., 2002).

2.4.10 *Carpobrotus dimidiatus*

Figure 15: ***Carpobrotus dimidiatus***

C. dimidiatus belongs to the family Mesembryanthemaceae and is commonly referred to as Natal sour figs, whilst the Khoi name is ghaukum (Van Wyk *et al.*, 2002). It is a robust, flat-growing, trailing perennial, rooting at nodes and forming dense mats. The succulent horizontal stems curve upwards at the growing point (Malan and Notten., 2006). The smooth fleshy leaves are erect, triangular in cross section and often reddish-green in colour. The rose-purple flowers are large and fleshy and develop into fragrant fleshy fruit with a jelly-like, somewhat slimy, sour sweet fruit pulp, which contains a multitude of small brown seeds.

The leaf juice is astringent and mildly antiseptic, it is mixed with water and swallowed to treat gastric aliments such as dysentery and spasms and it is used as a gargle to relieve oesophagal disorders such as laryngitis and throat infections (Van Wyk and Gericke., 2002). It is also used as a soothing lotion for skin aliments suchs as burns, bruises, scrapes, cuts, grazes and sunburn, ringworm, eczema, dermatitis, herpes, nappy rash, cold sores, cracked lips and chafing skin (Van Wyk *et al.*, 2002). The leaf is also famous for the cure of blue bottle stings (Van Wyk *et al.*, 2002). The leaf extracts contain malic acid, citric acid and other calcium salts which may be attributed to soothing blue bottle stings (Watt and Breyer-Brandwijk., 1962). The tannins present as active compounds in the leaf juice, make it mildly antiseptic and highly astringent. *Carpobrotus species* display phytochemical profile of flavonoids, tannins, alkaloids, phytosterols and aromatic acids. The flavonoids present in *Carpobrotus species* are rutin, neohesperidin, hyperoside, catechin and ferulic acid which are shown to have biological properties such as antimicrobial, antioxidant and anti-inflammatory activity (Van der Watt and Pretorius., 2001; and Springfield *et al.*, 2003).

2.4.11 *Centella asiatica*

Figure 16: ***Centella asiatica***

C. asiatica is a member of the Apiaceae family and is also referred to as Marsh pennywort (Odhav *et al.*, 2007). It is a perennial creeper with long stems and heart shaped bright green leaves. It grows abundantly in moist areas such as riverbanks and is distributed widely in tropical and subtropical areas. *C. asiatica* has many traditional medicinal uses in various parts of the world. In the Ayurvedic system of medicine, it is used to treat central nervous system disorders such as depression, insomnia, stress and memory loss (Ganachari *et al.*, 2004). In China, it is used in the treatment of leprosy, scar formation and for wound healing purposes (Yen *et al.*, 2001). Other medicinal actions of this plant include its ability to treat fevers, diarrhoea, leprosy, stress, tubercolosis and cancer (Jayathirtha and Mishra., 2004). The crushed leaves can be mixed with aqueous cream to form a paste that can be applied to skin ailments such as eczema, wounds and sores. The plant also has anti-bacterial, anti-fungal, anti-inflammatory and anti-tumor properties (Grubben and Denton., 2004). This could be attributed to the asiatic acid or madecassic acid and their respective glycosides, asiaticiside and madecassoside which are the bioactive terpene acids found in *C. asiatica* (Inamdar *et al.*, 1996). Park *et al.,* (2005) found that asiatic acid decreased the viability and induced apoptosis in human melanoma SK-MEL-2 cells. A study by Shukla *et al.*, (1999) shows that, asiaticoside, has wound healing activity, promotes fibroblast proliferation and increases the level of enzymatic and non-enzymatic anti-oxidants.

2. 4.12 *Ceratotheca triloba*

Figure 17: ***Ceratotheca triloba***

C. triloba belongs to the family Pedaliaceae, commonly called Wild foxglove. *Ceratotheca* means horned capsules and the species name *triloba* alludes to the leaves. The wild foxglove grows in the summer rainfall areas of South Africa, especially the grasslands and grows up to 1.5-2 metres in height. The leaves are soft, green, long and divided into 3 lobes with a bluntly serrated margin. The white or mauve foxglove-like flowers are carried in pairs up the stems in tall, sparsely flowered spikes. The bottom flowers open first and form fruits while new buds are still developing at the tip of the stem. *C. triloba* in traditional medicine is used to treat painful menstruation, stomach cramps, nausea, fever and diarrhoea (Tredgold., 1986). In terms of traditional leafy vegetables, *C. triloba* serves as a good source of energy and magnesium (Odhav *et al.*, 2007). Three anthraquinones have been isolated from *C. triloba* 9, 10 anthracenedione, 1-hydroxy-4-methylanthraquinone, 5, 8-dimethoxy-2, 3, 10, 10a tetrahydro-1H-phenanthrene-4,9-dione and androst-5-ene-3,17,19-triol (Mohanlal *et al.*, 2011).

2.4.13 *Chenopodium album*

Figure 18: ***Chenopodium album***

C. album belongs to the Chenopodiaceae family. It is commonly referred to as fat hen and in *isi*Zulu as Isiwisa (Odhav *et al.,* 2007). Is a fast-growing weedy annual plant that's sometimes regarded as a weed. It tends to grow upright at first, reaching heights of 10 –150 cm but falls over after flowering (due to the weight of the foliage and seeds) unless supported by other plants. The leaves are toothed, roughly diamond-shaped and broad, they are waxy-coated, with a whitish/ greyish coat on the underside. The small flowers grow in small cymes to form dense bunches and are creamish white in colour. It is consumed regularly as cooked spinach, with mielie meal (Odhav *et al.,* 2007). Infusion of the leaves is used to treat urethral itch or poulticed onto burns (Duke., 1992).

2.4.14 *Cleome monophylla*

Figure 19: ***Cleome monophylla***

C. monophylla belongs to the family Capparaceae and is commonly referred to as spindle-pod. *C. monophylla* is an erect annual herb, occurring in a wide variety of habitats. The stems and leaves have fine glandular hairs. The leaves are simple, narrowly ovate in shape. Flowers are mauve with purple markings on the petals. Flowers form a fruit that is a narrowly linear capsule. Rubbed onto the skin to relieve pneumonia; as an infusion it is used as eyewash (Tregold *et al.,* 1986). Leaves are applied to sores, roots chewed for coughs and the whole plant is used externally for swellings and inflammation.

2.4.15 *Dichrostachys cinerea*

Figure 20*: Dichrostachys cinerea*

D. cinerea belongs to the family Fabaceae. It is commonly referred to as sickle bush or Sekelbos, Marabou-Thorn, Kalahari Christmas tree and Chinese lantern tree. In *isi*Zulu it is called as Ugagane, Umthezane and Umzilazembe (Pacific Island Ecosystem at Risk (Pier)., 2005). It is widely distributed throughout South Africa, and has now encroached upon many overgrown bushveld regions. Despite this undesirable ecological impact, it is a valuable medicinal plant (Van Wyk and Gericke., 2003). *D. cinerea* is a small tree or strongly growing shrub which grows up to 8 m in height and has an untidy growth pattern with thorny branches. It forms impenetrable thickets in overgrazed bushveld (Van Wyk and Gericke., 2003). The feather like leaves show similarities with the leaves of the *Acacia species*. The characteristic flowers hang in clusters from branches, bright pink fertile flowers in the upper portions of the cluster, whereas the sterile, bright yellow flowers are at the bottom of the cluster. The curved and coiled seedpods are borne in clusters on long stalks (Pier., 2005).

The Haikum Bushmen of Namibia chew the fresh leaves to treat diarrhoea, toothaches and earache (Van Wyk and Gericke., 2003). It is also applied directly to snakebites (Van Wyk *et al.*, 2002). Extracts of the leaves and bark, as well as powdered bark are used for wound healing (Van Wyk *et al.,* 2002). Infusions of the roots are used to treat abdominal pain, coughs and pneumonia (Van Wyk and Gericke., 2003). Powdered roots are sniffed to curb nose bleeds, whilst the leaves and roots are smoked to relieve head colds, and to treat

tuberculosis and for treatment of epilepsy (Van Wyk and Gericke., 2003). In Sri Lanka, it is commonly used for traditional medicinal purposes as an aphrodisiac and for eye diseases (Wijesundara., 2003). In Sudan, it is used for the treatment of wounds (Eisa *et al.,* 1999) and in Zimbabwe it is frequently used in the treatment of sexually transmitted diseases (Kambizi and Afolayan., 2001).

The phytochemicals present in the leaves are saponins, tannins, flavonoids, sterols and triterpenes (Eisa *et al.*, 1999). The large family Fabaceae is characterized by impressive phytochemical diversity. These include polyphenols, flavonoids, tannins and alkaloids. In some genera e.g. Genista, Cytisus and Laburnum, the phytochemicals such as catechin, quinolizidine alkaloids, including cystisine and sparteine are common (Raven *et al.*, 1999). Another group of important natural products present are isoflavonoids that are known for their oestrogenic activity, and coumarin which is used as an anticoagulant (Raven *et al.*, 1999).

Furthermore other important chemicals with biological properties have been described in yet other genera of this family. *Cystisus scoparius* yields sparteine which is used for cardiac arrhythmias (Raven *et al.*, 1999). *Melilotus officinalis* has dicoumarol from which the anticoagulant drug warfarin was developed (Raven *et al.*, 1999). *Physostigma venenosum* is used on the tips of West African traditional poison arrows. It also has a cholinesterase inhibitor, physostigmine which is used as a myotic in glaucoma, in post-operative paralysis of the intestine and to counteract atropine poisoning (Manosroi *et al.*, 2004). Recent studies by Bessong *et al.*, (2005) shows that *Peltophorum africanum* has RNA dependent, DNA polymerase activity of reverse transcriptase, which could have activity against human immunodeficiency virus type 1.

2.4.16 *Electropappus rhinocerotis*

Figure 21: ***Electropappus rhinocerotis***

E. rhinocerotis is a much-branched grey to grey-green aromatic shrub which is 0,6 - 2,5m in height. It comes from the family Asteraceae. The leaves are minute, numerous, and pressed to the stem, usually woolly on both surfaces. The flowers are inconspicuous, yellow, tubular, born in capitula of mostly, 3 florets and well developed. The fruit has prominent longitudinal ribs (Dekker *et al.*, 1988). It is common on dry clay flats and slopes throughout the Western and Eastern Cape Provinces, up to Namaqualand (Dekker *et al.*, 1988).

E. rhinocerotis has traditionally been used in the treatment a number of aliments; in young children it's used to treat stomach aliments such as gripe, diarrhoea and flatulence; and for adults it is used for gastric disorders and as a bitter tonic to stimulate appetite (Dekker *et al.,* 1988). Phytochemical analysis of the plant indicated the occurrence of cardiac glycosides, saponins, tannins and reducing sugars (Dekker *et al.*, 1988). From the leaves and stem a labdane diterpene, rhinocerotinoic acid, has been isolated (Dekker *et al.*, 1988). Not much is known about this plant as it was not studied for its biological activities and phytochemical properties.

2.4.17 *Emex australis*

Figure 22: ***Emex australis***

E. australis, also known as Devil's thorn is an herbaceous plant of the Polygonaceae family. It is an annual herb spreading from a dense rosette with a thick taproot system. The leaves are pear-shaped, broadly rounded and dull green in colour (Factsheet., 2005). The plant inhabits suptropical and temperate regions, mainly with sandy and loamy soils. When consumed in large quantities, the leaves have a laxative effect and also contain oxalates (Factsheet., 2005). The leaves have also been used as a treatment in Southern Africa to relieve biliousness and to stimulate appetite. Decoctions of the roots are used for stomach cramps and are also given to infants suffering from restlessness and constipation. Unspecified parts of the plant are used to treat stomach and intestinal complaints (Dold and Cocks., 2000). *E. australis* also displayed antioxidant activity owing to the presence of phenolic compounds in the plant (Akula and Odhav., 2008).

2.4.18 *Ekebergia capensis*

Figure 23: ***Ekerbegia capensis***

E. capensis belongs to the family Meliaceae commonly known as Cape ash or in *isi*Zulu as umnyamathi. It grows to a 10-12 m tree; bark is grey in colour with a rough texture. It has compound leaves with 7 or 9 oblong leaflets. The flower is pink to white which forms round reddish berries in the summer. The bark is mainly used in traditional medicine to treat dysentery and heartburn (Watt and Breyer-Brandwijk., 1962). The leaves are sometimes used to treat venereal diseases, chronic cough, emetic, backache, headache, dysentery and skin diseases (Mulaudzi *et al.*, 2011). The roots are used to treat chronic cough, headaches, scabies (Hutchings., 1996). The chemical compounds of *Ekebergia* species are poorly known (Van Wyk *et al.*, 2002). Mulaudzi *et al.*, (2011) reports that pharmacological activity may be due to phenolics such as flavonoids; gallotannin or condensed tannins. Seeds contain a limonoid ekebergin as the major constituents, however none was found in the bark or timber (Van Wyk *et al.*, 2002). Limonoids have shown activity against insect's as a anti-feedants and have been used to treat intestinal parasites (Van Wyk *et al.*, 2002).

2.4.19 *Ficus sur*

Figure 24: ***Ficus sur***

F. sur belongs to the family Moraceae, it is commonly called broom cluster. Is a large, fast-growing, evergreen tree, reaching up to 35 m in height, with large, oval, green leaves borne on a massive, spreading crown. Attractive purplish figs are carried in large clusters on the lower parts of the trunk. New growth of figs have a striking pinkish colour. Attracts a wide variety of fruit-eating birds, some of them rare visitors that are only seen when the figs are ripe. A white milky fluid or latex exudes from any part of the plant which is damaged. Depending on the locality which this plant is found, it is used to treat different conditions, for example the Zulus drink a traditionally preparation of the root and bark for pulmonary tuberculosis and an infusion of the leaf and bark to improve milk production in cows (Kunle *et al.*, 1999).

Throught out Africa this plant is used traditionally, in Tanganyika a extract of the bark is used to promote milk production in women and cows; while in West Africa afflictions of the eye are treated with the leaves of this plant (Kunle *et al.*, 1999). The latex is used in Zaire for the treatment of burns, while in the northern part of Nigeria, the fresh young aerial root along with the inner bark is chewed to treat sore throat, and the leaves chewed and swallowed three times per day for approx. 6 weeks as a remedy for peptic ulcers (Kunle *et al.*, 1999). The active compounds are thought to be two pentacyclic triterpenoids of oleanane and ursene structures (Kunle *et al.*, 1999).

.

2.4.20 *Galinsoga parviflora*

Figure 25: ***Galinsoga parviflora***

G. parviflora belongs to the family Asteraceae. It has several common names including, Galinsoga and gallant soldier. Is an herbaceous plant that has leaves that are, ovate acuminate, light green,with margins finely dentate. Flower heads small, numerous, button-like with yellow tube-like disk florets and 5 white lingular margin florets. It is eaten as a potherb, raw or cooked. Sometimes dried and ground into a powder then used as a flavouring in soups. When rubbed onto the body, the plant is useful in treating nettle stings (Beekrum., 2003). The juice of the plant is applied to wounds; as it helps to coagulate the blood of fresh cuts and wounds (Beekrum., 2003). The leaves are boiled to treat colds and sores (Matu and Van Staden., 2003). *G. parviflora* has flavonoids: apigenin: 7-d-glucoside, luteolin-7-d-glucoside which have been shown to promote wound healing (Schmidta *et al.*, 2009). In much of the world it is considered a weed.

2.4.21 *Gulleminea densa*

Figure 26: ***Gulleminea densa***

G. densa belongs to the family Amaranthaceae commonly called small matweed. It is a perennial herb that has a woolly, prostrate mat-forming texture. The plant forms flowers that are yellowish cream to off-white in colour. The leaves are narrowly elliptic in shape and the upper surface is smooth, with long matted white hairs on the lower surface. It is found on roadsides, in grassland and open woodland, usually in well-trodden disturbed places (Hyde and Wursten., 2002). Singh., (2009) reported that *G.densa* has:

- Appropriate amounts of ash, fibre and fat.
- Large amounts of Vitamin B_1, could be used for treatment of beriberi.
- High amounts of Vitamin B_2, hence used for prevention of ariboflavinosis.
- Vitamin B_3 found in this plant can help with the treatment of pellagra.
- Appropriate amounts of Vitamin A, to help prevent night blindness, xerophthalmia and keratomalacia.
- The plant has high levels of iron can be used to preclude anaemia.
- Have antibacterial effects against a range of bacteria so it can prevent/treat diarrhoea, vomiting and bronchitis that are caused by bacterial infections.
- Repellent properties against the mosquito which could be used for personal protection especially by rural communities where chemical protection is very expensive.

2.4.22 *Gunnera perpensa*

Figure 27: ***Gunnera perpensa***

G. perpensa belongs to the family Gunneraceae. Is commonly called river pumpkin or in *isi*Zulu as ugobho. It is a perennial, herb that grows up to 1 m tall; and is found in marshy areas especially river banks. It has large round leaves that arise from a central tuft near the top of the apex, just above the soil level. The margin of the leaves is irregularly toothed. They produce tiny reddish brown flowers in dense groups on a long thick stem, the male flowers occur on the upper part of the cluster while the female occurs at the bottom. Main medicinal use is to induce labour and as an ante-natal medication to tone the uterus (Hutchings., 1996). It is also used in the expulsion of the placenta (Watt and Breyer-Brandwijk., 1962). It is also used to treat stomach trouble, rheumatic fever, swelling, menstrual pain and bleeding or applied externally for the dressing of wounds and for psoriasis (Van Wyk *et al.*, 2002). There is very little information regarding the chemistry of *G.perpensa*. In 1962 Watt and Breyer-Brandwijk reported the presence of a bitter principle celastrin.

2.4.23 *Heteropyxis natalensis*

Figure 28: ***Heteropyxis natalensis***

H. natalensis belongs to the family Myrtaceae. Commonly reffered to as lavender tree and in *isi*Zulu as inkunzi or uhuzu. It grows to a height of 10 m with a branched trunk, which has dense leafy branches; and a strong aromatic smell. They have inconspicuous yellowish flowers which form dry capsules. The leaves and roots are used in traditional medicine. Leaf infusions are used to treat colds, nose bleeds and bleeding gums (Watt and Breyer-Brandwijk., 1962). The roots are used to treat menorrhagia and fresh leaves for weaning babies (Hutching., 1996). The essential oils contain a wide range of monoterpenoids, mainly β-ocimene, 1.8-cineole, limonene, linalool and myrcene. (Van Wyk *et al*., 2002)

2.4.24 *Hibiscus sabdariffa*

Figure 29: ***Hibiscus sabdariffa***

H. sabdariffa belongs to the family Malvaceae and is commonly called Hibiscus. It is an annual growing shrub that can grow up to 4 m tall, it has lobed leaves and bright red flowers with a single stamen. The hibiscus flowers are most commonly used in traditional medicine. It is used as an herbal tea, which has a sweet sour taste, sometime as a nutritive colourful additive.

Flower extracts are used to treat appetite loss, colds, catarrh of the respiratory tract, circulatory ailments, expectorant, laxative, diuretic, allergic eczema and various skin conditions (Van Wyk and Wink., 2004). The active ingredients are organic acids (Hibiscus, ascorbic, citric, malic and tartaric acid) and polysaccharides (arabinan, arabinogalactan, galacturonic acid, rhamnose, galactose and arabinose, and anthocyanins (3- sambubiosides of delphinidin and cyaniding) (Van Wyk and Wink., 2004). The deep red colour of the flowers is due the anthocyains present (Van Wyk and Wink., 2004).

2.4.25 *Justicia flava*

Figure 30: ***Justicia flava***

J. flava belongs to the family Acanthaceae and is commonly called yellow justicea. Yellow justicea is a perennial herb or shrublet that grows up to 450 mm high. The leaves are lanceolate or broadly ovate, with the leaf stalk growing up to 25 mm long. *J. flava* is widespread in parts of tropical and Southern Africa and is found in different veld types, more commonly in disturbed habitats, growing in sunny or semi-shady areas. It is able to tolerate dry conditions. In a study conducted to determine the potential antihypertensive properties of plants, *J. flava* demonstrated ACE (angiotensin-converting enzyme) inhibition activity (Science in Africa., 2007).

This plant has various medicinal uses in many parts of Africa. In Tanzania, the leaves have emetic properties while in the Ivory Coast and Ghana, the leaves of the plant are used either as a rub or consumed to treat convulsions and feverish pains in babies, as well as yaws and diarrhoea in children (Grubben and Denton., 2004). Sterol, salicyclic acid and three lignins, helioxanthin, isolariciresinol and justicinol as well as docosanoic acid were isolated from *J. flava* (Grubben and Denton., 2004).

2.4.26 *Leonotis leonurus*

Figure 31: ***Leonotis leonurus***

L. leonurus belongs to the family Lamiaceae and is commonly referred to as Wild dagga (Van Wyk and Gericke., 2003). The *isi*Zulu name is Umunyane, the Sotho name is Lebake, the Xhosa name is Umfincafincane and the Shona name is Umhlahlampetu (Van Wyk *et al.*, 2002). *L. leonourus* is distributed over a large part of South Africa (Van Wyk *et al.*, 2002) and it is a shrub that grows between 2 to 5 m in height and has a substantial, woody foundation, with branches that are pale brown. The entire plant has a strong odour. The characteristically hairy leaves, located opposite each other on the stem, are elongated, narrow and toothed along the upper half. The flowers are bright orange in colour, hairy and tubular in shape. They are borne in rounded groups neatly arranged at the tips of the branches. The name leonurus (meaning lion's ears) was given to the plant due to the resemblance of the flowers to lion's ears.

There are a number of traditional uses recorded. The stems, leaves and flowers are the main parts used (Van Wyk *et al.*, 2002; Van Wyk and Gericke., 2003). The KhoiKhoi first used it as a tobacco and later introduced it to settlers as a medicine to help alleviate ailments of the chest (Malan and Notten., 2006). The Zulu, Xhosa and Khoikhoi people make a tea of the

flowers for a soothing cough and cold remedy, also sometimes used effectively in the treatment of jaundice, cardiac problems, asthma, haemorrhoids, headaches, chest aliments, bronchitis and epilepsy (Van Wyk *et al.*, 2002; Van Wyk and Gericke., 2003). It is known that a tea of the leaves, used to be drunk daily by the older generation for water retention, obesity and digestive tract problems, intestinal worms and constipation (Malan and Notten., 2006). The plant has been smoked in order to relieve epilepsy (Van Wyk and Gericke., 2003). The leaves and roots have been used as a remedy for snakebites, insect bites, and stings. Decoctions have been applied as local dressings for boils, eczema, and ailments such as itching and muscle cramps. Herbal decoction can be used internally to treat coughs, colds, influenza, bronchitis, hypertension and headaches. Leaf infusions are used to treat hepatitis and asthma (Van Wyk *et al.*, 2002).

L. leonurus contains volatile oils as well as several unusual diterpenoids (Labdane type lactones). One such isolated diterpenoid is Marrubin which is the main diterpenoid lactone found in the European herb, Marrubium vulgare (White Horehound) that is traditionally used to treat acute bronchial coughs. The exact pharmacological effect, however, is yet unknown (Van Wyk *et al.*, 2002). Some plants from the Lamiaceae family has shown to accumulate monoterpenoid glycosides, rosmarinic acid and other derivatives of caffeic acid (Van Wyk *et al.*, 2002; Van Wyk and Gericke., 2003). Rosmarinic acid has shown to be medicinally significant, because it demonstartes non-specific complement activation by the inhibition of biosynthesis of leukotrienes this causes an anti-inflammatory effect and antiviral activity (Raven *et al.*, 1999).

Plants from the family Lamiaceae contain various phenolic compounds such as flavonoids, phenolic diterpenes, monoterpenoid glycosides (iridoids), rosmarinic acid and other derivatives of caffeic acid (Raven *et al.*, 1999; and Erdemoglu *et al.*, 2006). Essential oils from the leaf and flower of *L. leonurus* illustrate diverse phytochemicals such as limonene, (Z)-β-ocimene, terpinene, caryophyllene, humulene and germacrene (Raven *et al.*, 1999). Other members from this family that are important include *Lavandula angustifoli* and *Mentha arvensis* which have a mild sedative, carminative and spasmolytic activity (Erdemoglu *et al.*, 2006).

2.4.27 *Momordica balsamina*

Figure 32: ***Momordica balsamina***

M. balsamina belongs to the family Curcurbitaceae and is commonly called African cucumber. *M. balsamina* is widespread throughout Africa and is found in all the provinces of South Africa except the Western Cape. The balsam apple, as it is known, occurs in sandy and calcareous soil, clay and loam. It thrives in areas such as grasslands, savannah and river bank vegetations and forest margins. The monoecious, perennial herb has a tuberous rootstock, mostly annual stems and broadly ovate to orbicular waxy leaves (Hutchings *et al.*, 1996). The leaves and young fruits of this plant are cooked and eaten as a vegetable in many parts of Africa. Its medicinal uses are wide and diverse. It is used as an anti-helminthic (fruits, seeds and leaves) and also used to treat fevers and uterine bleeding (leaves) and syphilis, rheumatism and skin disorders (Grubben and Denton., 2004). The bitter taste of *M. balsamina* is due to the presence of saponins. Other compounds which have been isolated from the plant include a ribosome inactivating protein called momordin-11 and the caffeic acid ester, rosmarinic acid which has shown anti-inflammatory, antiviral and antioxidant activities (Grubben and Denton., 2004). Ramalhete *et al.,* (2009) reported on the isolation and structural elucidation of seven compounds Balsamina, balsaminapentaol, balsaminol A, balsaminol B, cucurbalsaminol B, cucurbita-5,23(*E*)-diene-3-7-25-triol and karavilagenin E.

2.4.28 *Oxygonum sinuatum*

Figure 33: ***Oxygonum sinuatum***

O. sinuatum belongs to the family Polygonaceae and is commonly called Stars Talk. *O. sinuatum* is an erect, annual herb that has green to red-brown pubescent stems, white or pink flowers and grows up to 1 m tall. It is found mainly as a weed on fields or on waste grounds and prefers well-drained loamy soils. The plant is distributed in parts of eastern and southern Africa, where the leaves are eaten raw or boiled as a vegetable. The raw leaves of *O. sinuatum* have an acidic taste while in powdered form, the taste is mild. Medicinally, the leaves are used to treat boils and the stems are chewed to treat tonsillitis. The whole plant is used for treating gastric ulcers, malaria and hepatitis while the leaf sap is used for coughs (Grubben and Denton., 2004). The juice of the leaves is used for fungal and eye infections (Maundu *et al.*, 1999).

2.4.29 *Pelargonium sp*

Figure 34: ***Pelargonium. sp***

Pelargonium. sp belongs to the family Geraniaceae and is commonly called Geranium or in *isi*Zulu as ishaqa. It's a perennial herbaceous plant that has a tuberous root from which a rosette of leaves bud. The leaves are round to heart shaped. The flowers are usually pink occasionally white and they bloom on a tall slender stalk. The fleshy roots are used fresh or dried in traditional medicine. Van Wyk and Wink., (2004) report that the root extracts are used to treat acute and chronic infections of the nose, ears and chest, and as a supportive treatment for tuberculosis and chronic bronchitis. In South Africa it is commonly grown around pathways to homes to deter snakes. The main active ingredients are coumarins, gallic acid derivatives, oligomeric proanthocyanidins, and flavonoids (Van Wyk and Wink., 2004).

2.4.30 *Physalis viscosa*

Figure 35: ***Physalis viscosa***

P. viscosa, also known as sticky gooseberry belongs to the Solanaceae family. It is a perennial plant that grows up to 1.8 m high and inhabits coastal areas and mountains. It is an erect, sprawling plant with bell-shaped greenish-yellow flowers and yellow berries. The plant is used as a tonic, sedative, laxative, and diuretic. The juices of the berries are used in urinary disorders and inflammatory diseases (Beekrum., 2003). The aerial parts of *P. viscosa* were found to contain withanolides such as 4-beta-hydroxywithanolides and its 5,6-desoxi analogue, withaphysanolides and withanolide related pregnanes such as 4-beta-hydroxy-5-beta and 6-beta-epoxypregn-2-ene-1,20-dione (Silva *et al.*, 1993). Withanolides are a group of steroidal lactones that are known for their broad spectrum of biological activity (Silva *et al.*, 1993). No toxicity data for this plant is reported.

2.4.31 *Portulaca oleracea*

Figure 36: ***Portulaca oleracea***

P. oleracea is commonly called Purslane and belongs to the Portulacaceae family. It is consumed both raw or cooked. Whole plant is used in treatment of bacterial bacillary dysentery, diarrhoea, haemorrhoids and enterorrhagia (Beekrum., 2003). Infusions of the leaves are used for ear-ache, treatment for worms and as a poultice for bruises and burns. It is also used for pain and stomach-ache, boils, bug bites, colic, dermatitis, indigestion and snake bite (Duke.,1992). Little is known about this plant and there is no literature on its biological activities and phytochemical properties.

2.4.32 *Syzgium cordatum*

Figure 37: ***Syzygium cordatum***

S. cordatum belongs to the family Myrtaceae. It is commonly known as water berry in English and in *isi*Zulu it is called umdoni. It is a medium sized tree, which grows up to 15 m in height. It has a rough bark. The leaves are broad, somewhat circular in shape and bluish-green in colour. It has cream to pinkish flowers which have numerous stamens and are produced in clusters on the tips of the branches. The flowers form an oval shaped fruit which are red to purple in colour. These berries are edible though not very tasty. The bark is reddish pink in colour when fresh. The bark is most commonly used in traditional medicine; however the leaves and roots are used as well sometimes. The plant is used to treat respiratory ailments, tuberculosis, stomach complaints and diarrhoea (Hutchings., 1996). The wood and bark contain proanthocyanidins, pentacyclic triterpenoids such as arjunolic acid, friedelin and epifriedelin, steroidal triterpenoids such as β-sitosterol, gallic acid, ellagic acid and various gallic acid derivatives (Van Wyk *et al.*, 2002). The exact pharamacological action of this plant is not known.

2.4.33 *Senna occidentalis*

Figure 38: ***Senna occidentalis***

S. occidentalis belongs to the Fabaceae family and is also known as coffee senna or cassia senna. The species varies from a semi-woody annual herb in warm areas to a short-lived perennial shrub in frosty areas. The crushed foliage of the plant has an unpleasant odour. The compound, alternate leaves have four to six pairs of glabrous leaflets, which are ovate to ovate-lanceolate and pointed at the tip (Demel and Teketay., 1996). Many phytoactive chemicals present in the tissues of the plant supports numerous applications in folk medicine. Leaf extracts are used as an analgesic, anti-inflammatory, antibacterial, antifungal, antiviral, febrifuge, purgative and immunostimulant. The foliage of the plant is poisonous and generally avoided by livestock (Timm and Riet-Correa., 1997). Anthraquinone compounds are common in this species and contribute to its widespread use as a purgative and laxative (Timm and Riet-Correa., 1997).

2.4.34 *Solanum nodiflorum*

Figure 39: ***Solanum nodiflorum***

S. nodiflorum belongs to the family Solanaceae and is commonly called white nightshade. It is consumed regularly by the local community as potherbs and the fruit is eaten green (Beekrum., 2003). It is used as an anti-phlogistic, anti-phoretic, diuretic, emollient, narcotic, purgative, sedative and to treat cancerous sores (Beekrum., 2003) Its toxicity is not clear but the green berries contain solanine (Tredgold *et al.*, 1986). Not much literature exists on this plant. There are no reports of its biological activities and phytochemical properties.

2.4.35 *Sonchus oleraceous*

Figure 40: ***Sonchus oleraceous***

S. oleraceous belongs to the family Asteraceae also known as sow thistle. It is an erect annual herb that grows from 30-110 cm in height and inhabits fields, roadsides, pastures and waste areas. The leaves are thin, soft and dark green in colour while the flowers blossom yellow and are 5-6 mm in diameter. The distinguishing feature of the plant are its hollow stems which exude latex (a whitish milk) if damaged (Auld and Meld., 1992). The plant is used in the treatment of diarrhoea. The latex in the sap is used to treat warts while the leaves are applied as a poultice to inflammatory swellings. An infusion of the leaves and the roots are used as a febrifuge and tonic (Auld and Meld., 1992). A phytochemical study of the roots of S. oleraceous revealed two compounds; 15-O-β-glucopyranosyl-11 and β,13-dihydrourospermal that displayed cytotoxic activity against mouse lymphoma and rat brain cancer cells as well as antibacterial activity against *Staphylococcus aureus and Escherichia coli* (Elkhayat., 2009). *S. oleraceous* was also reported to have free radical scavenging activity, likely due to the phenolic and flavonoid compounds present in the plant (Yin *et al.*, 2007). Yin *et al.,* 2007 also showed that the ethanolic extracts showed considerable activity against the proliferation of stomach cancer cells. Thus, they deduced that the plant might be a good source of food and natural antioxidants.

2.4.36. *Taraxicum officinale*

Figure 41: ***Taraxicum officinale***

T.officinale belongs to the family Asteraceae. It is commonly known as dandelion. They are a perennial herb, with a characteristic rosette of markedly toothed leaves. It has a solitary yellow flower on a hollowed unbranched stalk. All parts of the plant ooze a bitter milky juice when it is damaged. The fresh or dried leaves, roots or both are used in traditional medicine. The young leaves are eaten raw as salad and roots which are rich in inulin is dried and roasted as a coffee substitute (Van Wyk and Wink., 2004). Due to the high inulin content it is good for patients with diabetes. It is used mainly as a diuretic but also as an appetite stimulating tonic. It may also be used for stomach, liver, gall bladder, rheumatic complaints and eczema (Van Wyk, and Wink., 2004). The main constituents are Sesquiterpene lactones (tetrahydroridentin B, taraxacolide β-D- glucoside), phenolic acids (taraxacoside) and triterpenoids (taraxasterol) (Van Wyk and Wink., 2004).

2.4.37 *Tetradenia riparia*

Figure 42: ***Tetradenia riparia***

T. riparia belongs to the family Lamiacceae. It is commonly called ginger bush, and in *isi*Zulu iboza. It's a shrub like plant that is multi-branched, and usually grows one to 3 m in height. Stems and leaves have glandular hairs and are somewhat succulent. The large rounded leaves are very characteristics because the margins have rounded teeth. Male and female flowers are found on separate plants. The male flowers are 80 mm long and are less dense compared to the female flowers which are 20 mm in length. Leaves are used in traditional medicines (Van Wyk *et al.*, 2002). Plant extracts are used to treat respiratory ailments such as coughs, colds, sore throat and mouth ulcers (Hutchings., 1996). Watt and Breyer-Brandwijk (1962) have reported the leaves are used to treat stomach ache, diarrhoea, influenza, fever and malaria. The active compound is ibozol, a diterpene diol, and various similar diterpenoids (Van Wyk *et al.*, 2002). This plant has been shown to contain large concentrations of α-pyrones (Van Wyk *et al.*, 2002).

2.4.38 *Tulbaghia violacae*

Figure 43: ***Tulbaghia violacae***

T. violacae belongs to the family Alliaceae. It is commonly called wild garlic, and in *isi*Zulu isihaqa. It is a bulbous plant with thin long narrow leaves. On thin slender stalks flowers occur in groups of ten or more, which are generally mauve or light purple. This species is grown through South Africa in domestic gardens. The leaves are sometimes eaten as vegetables or raw in a salad (Van Wyk *et al.*, 2002). The bulbs and leaves are generally used as traditional medicine. The plant smells like garlic. The plant is traditionally used to treat fever, colds, asthma and tuberculosis (Watt and Breyer-Brandwijk., 1962). Decoctions are used to treat stomach ailments and for enema's (Hutchings., 1996). The active compounds found in the plant are two sulphur compounds which are similar to alliin which is found in true garlic (Van Wyk *et al.*, 2002)**.**

CHAPTER THREE: SCREENING OF MEDICINAL PLANTS FOR ANTI – HIV ACTIVITY

3.1 INTRODUCTION

There is a need to intensify the screening of plant material, based on ethno-pharmacological data and indigenous knowledge. This will provide the backbone for the isolation and identification of novel compounds that could have anti-HIV activity (Chinsembu and Hedimbi *et al.,* 2009).

The World Health Organization in 1989, recommended that traditional healers be included in the national responses to HIV/AIDS, with the aim of evaluating ethnomedicines for the management of HIV/AIDS (WHO, 1989). The World Health Organisation, memorandum "*In vitro* screening of traditional medicines for anti-HIV activity"; from the WHO meeting 1989; 87: 613-618 stated that:

(i) There is a need to evaluate elements of traditional medicine, particularly medicinal plants that might yield effective and therapeutic agents; and

(ii) It has been estimated that 25 % of approved drugs prescribed in the USA have been developed from plant material and the information leading to the discovery of the active drugs has often been from traditional uses. In many cases a single plant product was the intial lead that resulted in the development.

A variety of plant species are being used by AIDS patients without any experimental evidence of anti-HIV activity (Bessong and Obi., 2006). In South Africa, many patients with a hospital diagnosis of AIDS sought alternative treatment from traditional healers; because standard anti-viral therapies were seen as too expensive (Cos *et al.*, 2002), however that is no longer the case as treatment is now free. Despite this many people are culturally inclined to visit the traditional healer who uses medicinal plants. Plant derived anti-HIV compounds are widely distributed in nature (Singh *et al.,* 2011). Therefore screening medicinal plants provides an opportunity for the discovery of HIV-1 inbitors with lower or no toxicity and side effects (Narayan *et al*., 2013). Medicinal active substances harvested from plants, come from any part of the plant but the most commonly used parts are the leaves (Narayan *et al*., 2013). Various plants have already been investigated for anti-HIV activity, some of them are listed in Table 4.

Table 4: Plants with anti-HIV activity

Species	Part of plant used	SI value	Reference
Adansonia digitata	Flowers	5	Locher *et al*., (1996)
Aspilia pluriseta	Leaves	12	Cos *et al.*, (2002)
Aleurites moluccana	Stems	10	Locher *et al*., (1996)
Calotropis gigantea	Flowers	24	Locher *et al*., (1996)
Cuscuta sandwichiana	Stems	9	Locher *et al.*, (1996)
Eugenia malaccensis	Leaves	109	Locher *et al*., (1996)
Justicia reptans	Leaves	5.7	Bedoya *et al.,* (2008)
Neurolaena lobata	Leaves	3.8	Bedoya *et al.,* (2008)
Pipturus albidus	Bark	68	Locher *et al*., (1996)
Pluchea indica	Leaf	94	Locher *et al.,* (1996)
Pouteria viridis	Leaves	6.3	Bedoya *et al.,* 2008
Psychotria hawaiiensis	Bark	23	Locher *et al*., (1996)
Rumex bequaertii	Leaves	11	Cos *et al.*, (2002)
Scaevola sericea	Leaves	31	Locher *et al*., (1996)

Plants can be selected for anti-HIV activity based on: ethno-medical use, random screening or a chemotaxonomic approach (screening of species of the same botanical family for similar compounds). Bessong and Obi., (2006), stated that when selecting plants , the selection of plants based on literature leads seems to be the most cost effective way of identifying plants with anti-HIV activity. Screening is an essential tool for new drug discovery (Weislow *et al.*, 1989). Plant compounds either as a pure compound or as standardized plant extracts provide unlimited opportunities for new drug leads because of the chemical diversity which plants possess (Cos *et al.*, 2006). It must be remembered that the crude plant extracts are highly impure compared to the pure anti-HIV compounds such as lopinavir. It may be that the amount of active compound extracted is a very small percentage of the extract.

3.2 METHODOLOGY

3.2.1 Collection and preparation of plant material

The leaves roots, stems and flowers (whole plant) of selected plants (± 3 kg fresh weights) were collected from the greater Durban area in KwaZulu-Natal, South Africa. These plants were collected and identified using taxonomic keys. Herbarium specimens were prepared and are lodged at the Ward Herbarium as voucher specimens. The plants scientific name, family name, common name, and parts that were used are listed in Table 5. After collection, the plants were de-leafed, washed repeatedly with distilled water until no foreign material remained (damaged leaves were removed) and dried in an oven (Memmert B. Owen Jones limited, South Africa) at 25°C for 48 h. The dried plant material was milled in an industrial grinder (Retsch Gmbh, West Germany) and stored in labelled Schott bottles in a cool dark place.

3.2.2 Sample Preparation

Methanolic and aqueous extracts of the dried plant material were prepared according to the procedure outlined by Jeremy and Whiteman., (2003) with minor modifications.

Methanolic extract: Fifty grams of the powdered plant material was weighed out, stirred in 200 ml of 80% methanol (v/v) and agitated for 24 hours. This was filtrated with Whatman No. 1 filter paper. The resultant filtrate was removed by evaporation using a roto-evaporator (Buchi RE) which was connected to a water bath set at a temperature of 50°C. The remaining slurry was freeze dried in a Freeze Dryer (Virtis Benchtop) and used for all subsequent experiments.

Aqueous extract: Fifty grams of the powdered plant material was stirred in 200 ml of distilled water and agitated for 24 hours, before centrifuging at 8000 rpm for 10 min. The supernatant was filtered using Whatman No. 1 filter paper and freeze dried in a Freeze Dryer (Virtis Benchtop) to form a powder. This was used for all subsequent experiments.

The yield from 100 g of dried powdered plant material was calculated as follows:

$$\text{Product yield} = \frac{\text{Amount of product}}{\text{Amount of plant material used}} \times 100$$

Table 5: The biodata and parts of plants used in this study

Scientific name	Family name	Common name	Source	Part
1. *Achyranthes aspera*	Amaranthaceae	Devils Horsewhip	Reservoir hills	Leaves
2. *Alternanthera sessile*	Amaranthaceae	Sessile joyweed	Reservoir hills	Leaves
3. *Amaranthus dubius*	Amaranthaceae	Wild Spinach	Reservoir hills	Leaves, inflorescence & seeds
4. *Amaranthus hybridus*	Amaranthaceae	Rough pigweed	Reservoir hills	Leaves, flowers &fruit
5. *Amaranthus spinosus*	Amaranthaceae	Spiny amaranth	Reservoir hills	Leaves
6. *Asystasia gangetica*	Acanthaceae	Creeping foxglove	Reservoir hills	Leaves
7. *Buddleja saligna*	Buddlejaceae	False olive	Reservoir hills	Leaves
8. *Bidens pilosa*	Asteraceae	Blackjack	Reservoir hills	Leaves
9. *Capparis tomentosa*	Capparaceae	Woody capper bush	Reservoir hills	Leaves
10. *Carpobrotus dimidiatus*	Mesembryanthemaceae	Natal sour figs	Park rynie	Leaves
11. *Centella asiatica*	Apiaceae	Marsh pepperwort	Pinetown	Leaves
12. *Ceratotheca triloba*	Pedaliaceae	Wild foxglove	Reservoir hills	Leaves
13. *Chenopodium album*	Chenopodiaceae	Fat hen	Reservoir hills	Leaves , Fruit and flowers
14. *Cleome monophylla*	Capparaceae	Spindle-pod	Reservoir hills	Leaves , Fruit &flowers, Roots
15. *Dichrostachys cinerea*	Fabaceae	Sickle bush	Reservoir hills	Leaves
16. *Elytropappus rhinocerotis*	Asteraceae	Rhinoceros bush	Eastern Cape	Leaves
17. *Emex australis*	Polygonaceae	Devils thorn	Reservoir hills	Leaves , Crowns, Roots
18. *Ekerbegia capensis*	Meliaceae	Cape ash	Reservoir hills	Leaves
19. *Ficus sur*	Moraceae	Broom cluster figs	Reservoir hills	Leaves
20. *Galinsoga parviflora*	Asteraceae	Gallant solider	Reservoir hills	Flower heads , Roots

Scientific name	Family name	Common name	Source	Part
21. Guilleminea densa	Amaranthaceae	Small matweed	Reservoir hills	Leaves
22. Gunnera perpensa	Gunneraceae	River pumpkin	Reservoir hills	Leaves
23. Heteropyxis natalensis	Heteropyxidaceae	Lavender tree	Reservoir hills	Leaves
24. Hibiscus sabdariffa	Malvaceae	Roselle	Reservoir hills	Calyx-flowers
25. Justicia flava	Acanthaceae	Yellow justicia	Reservoir hills	Leaves, Flower & seeds
26. Leonotis leonurus	Lamiaceae	Wild dagga	Leaves : Shongweni Flowers : Randles Nursery	Leaves , Flowers
27. Momordica balsamina	Cucurbitaceae	African cucumber	National Botanical Institute	Leaves
28. Oxygonum sinuatum	Polygonaceae	Stars talk	Reservoir hills	Leaves
29. Pelargonium. sp	Geraniaceae	Geranium	Reservoir hills	Leaves
30. Physalis viscosa	Solanaceae	Sticky goose berry	Park rynie	Leaves
31. Portulaca oleracea	Portulacaceae	Purslane	Verulam	Leaves , Roots
32. Syzygium cordatum	Myrtaceae	Water berry	Reservoir hills	Leaves
33. Senna occidentalis	Leguminosae	Cassia senna	Reservoir hills	Leaves
34. Solanum nodiflorum	Solanaceae	White nightshade	Reservoir hills	Leaves , Roots , Fruits & flowers
35. Sonchus oleraceus	Asteraceae	Sow thistle	Reservoir hills	Leaves , Roots
36. Taraxcum officinale	Asteraceae	Dandelion	Reservoir hills	Leaves , Roots
37. Tetradenia riparia	Lamiaceae	Ginger bush	Reservoir hills	Leaves
38. Tulbaghia violacae	Alliaceae	Wild garlic	Reservoir hills	Leaves

3.2.3 Screening of plant extract for anti- HIV activity

This study was conducted at the National Health Laboratories Services, Inkhosi Albert Luthuli Central Hospital**.**

3.2.3.1 Cell Line

The MT-4 cell line, was used in this study, it is a transformed human T-cell leukemia virus type 1 (HTLV-1). It was derived from a 50 year old Japanese male with generalised lymphadenopathy and heptosplenomegaly (adult T-cell leukaemia) by co-culture of his peripheral leukocytes with male umbilical cord lymphocytes. The cells carry HTLV-1 and support the growth of HIV. For this study the cells were obtained from The National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital. The cells were grown in 75 cm^2 tissue culture flasks (T 75) (Greiner, Germany) with RPMI 1640 medium (Sigma, South Africa) supplemented with 10% heat-inactivated fetal bovine serum, FBS (Sigma, South Africa). The cells were sub-cultured in fresh media at a concentration of 6×10^5 cells/ml every 3-4 days. The cell count and viability were measured by a Trypan blue dye exclusion technique (Miyoshi *et al.*, 1982).

3.2.3.2 Cell maintenance

After the MT-4 cells were incubated over a 3 day period, they were then sub-cultured in RPMI, and stock cultures were stored at -70ºC in a biofreezer until required. Cell maintenance was performed according to protocols obtained from Miyoshi *et al.,* (1982). All cell culture procedures were carried out in a Class III biological safety cabinet. The unit was swabbed /sterilized with 70% ethanol before each use. The cells were grown aseptically in 75 cm^2 tissue culture flasks (T 75) (Greiner, Germany) in Complete Culture Medium (CCM), (RPMI-1640, containing 10% FBS and supplemented with antibiotics (penicillin [10 000 U/ml, streptomycin sulphate [10 000 U/ml] and sodium pyruvate [1mM]). CCM was then filter sterilized with a 0.22 µm millpore filter (Sigma, South Africa). The cultures were incubated at 37°C in a humidified incubator (Snjiders Hepa, United Scientific Group, Cape Town, South Africa) with an atmospheric condition of 5% CO_2. The culture flasks were routinely examined for changes using an inverted microscope and this determined the frequency of media changes.

3.2.3.3 The percentage cell viability

After the MT-4 cells were incubated over a 3 day period, the percentage cell viability was determined using a haemocytometer. The number of viable cells was determined by using a cell suspension, which was mixed with 0.2% Trypan Blue [Biowhittaker, Wakersville (USA)] (v/v 1:1). This mixture was drawn across the grid by capillary action. Only the viable (translucent) cells that lay within, or that touched, the left or top boundary, where counted. The number of viable cells per ml in the original sample was calculated as follows:

Cells/ml = Average number of cells per primary square X 10^4 dilution factor

3.2.3.4 Storage of cells

Storage of cells was performed according to protocols obtained from Miyoshi *et al*., (1982). The cells were pelleted and washed twice with pre-warmed Phosphate Buffered Saline (PBS), pH 7.2, then re-suspended in 0.5 ml FCS and cooled on ice. A 20% dimethylsulphoxide (DMSO) in RPMI-1640 solution was prepared as the cryopreserving agent and placed on ice. Equal aliquots (0.5 ml) of the cell suspension and the cryoprotective agent were added to a cryotube (Corning, South Africa). The tubes were transferred to the thermos flask and kept overnight at -80°C. The cells were subsequently transferred to liquid nitrogen and stored until required.

3.2.3.5 Regeneration of cells

Regeneration of cells was performed as described in protocols obtained from Freshney., (1987). Cells were removed from the liquid nitrogen, swabbed with 70% ethanol and rapidly thawed. The cells was then transferred to 20 ml of pre-warmed 10% CCM in 75 cm^2 tissue culture flasks and incubated at 37°C in a humidified incubator with a 5% CO^2.

3.2.3.6 Inoculation of MT-4 cells with HIV-1 ($HTLV_{111B}$)

Virus was prepared according to the procedure outlined by Pauwels *et al*., (1988). HIV was prepared from the supernatant of the MT-4 cells infected with HIV-1 $HTLV_{IIIB}$. A continuous supply of HIV-1 ($HTLV_{111B}$) at high titres was required for antiviral testing. To ensure that HIV was readily available the virus was cultured with HIV susceptible cells MT-4. These cells produced very high titres of virus which was observed by cytopathic effect. To the active cells which was grown aseptically in 75 cm^2 tissue culture flasks (T 75) (Greiner,

Germany), a 1000 µl of virus HIV-1 supernatant was added. This virus/cell mixture culture was incubated for 3 days, after which they were centrifuged at 1500 rpm for 5 min. A 1000 µl of virus supernatant was pipetted into cryo-vials and stored in a biofreezer at -70˚C.

3.2.3.7 $TCID_{50}$ of HIV-1 $HTLV_{IIIB}$ in MT4 cells

Tissue Culture Infectivity Dose at 50% ($TCID_{50}$) is defined as that dilution of virus required to infect 50% of inoculated cell cultures (Cos *et al*., 2006). $TCID_{50}$ of $HTLV_{IIIB}$ in MT-4 cells was determined using the method of Pauwels *et al*., (1988). To the exponentially growing MT-4 cells, a cell count using trypan blue was performed. Colourless RPMI was used to re-suspend the cells at a concentration of 6 X 10^5 cells/ml. Fifty microlitres of cells were add to each well, rows B-G Columns 2-11. Followed by 150 ul of CCM was added to rows B-G, columns 2-11 (Shown in Figure 44). 50 µl of virus was added to column 2 and rows B-G. With a multichannel pipette a serial dilution (1in 4) was performed from column 2 to column 10, (rows B-G). Column 11, (rows B-G), was the cell control (no virus added). The plate was incubated at 37°C in a humidified incubator with a 5 % CO_2 for 5 days. PBS was added to rows A and H, columns 1 and 12 (Red in diagram). This prevents evaporation of media. The plates were evaluated for cytopathic effect (degenerative changes in cells, which is associated with the multiplication of HIV-1), from day 1 to day 5. And the cytopathic effect was recorded and expressed as a percentage value. The Spearman-Karber method was used to determine the $TCID_{50}$ (Pauwels *et al*., 1988).

Column → Rows ↓	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Figure 44: Design plan for $TCID_{50}$

$$\text{Log } TCID_{50} = L\text{-}d\ (s\text{-}0.5)$$

L = log of lowest dilution of $HTLV_{IIIB}$ (constant of 0.6)
d = difference between dilution steps (constant of - 0.6)
s = sum of positive wells

3.2.3.8 Effect of plant extracts on MT-4 cells infected with HIV virus ($HTLV_{IIIB}$)

The XTT assay is based on the bioreduction of the XTT salt (2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt) yielding an orange formazan. The effect of the extracts was assayed using XTT assay according to the method described by Weislow *et al.,* (1989). The bioreduction is enhanced by the presence of an electron coupling agent such as phenazine methosulfate (PMS). Formazan production is indicative of the number of viable cells, therefore an increase or decrease in cellular viability results in a change in the amount of formazan formed, which indicates the degree of anti-HIV (EC_{50}) or cellular cytotoxicity (CC_{50}) caused by the plant extract.

The assay was carried out in 96 well, flat bottomed microtitre plates (Cellstar, Greiner, Germany). Fifty micro-litres of the plant extracts (1000 µg/ml) were serially diluted in a 2 fold series. The highest concentration being tested was 500 µg/ml and the lowest concentration 4 µg/ml. Fifty micro litres of MT-4 cells ($\pm$ 6 $\times 10^5$) cells were added to each well. In the control wells either 50 µl Dimethyl sulfoxide (DMSO) or 50 µl media was added (no plant extracts), and to all wells 50 µl of virus was added. The plate was incubated in a 37°C in a humidified incubator with a 5% CO_2 for 5 days. Then XTT salt with PMS was made up in colourless RPMI and 20µl of this solution was added to each test well and control well. The plate was further incubated at 37°C in a CO_2 incubator for 4 h, the absorbance was read at 450 nm with a reference wavelength of 620 nm using an ELISA plate reader. The results were analysed using Magellan software (Tecan Trading, Switzerland). The 50 % cellular cytotoxicity concentration (CC_{50}) was evaluated. The above protocol was used, however 50 µl of complete CCM was added instead of virus.

The phenol red indicator contained in regular RPMI may compromise the optical density measurement of the XTT assay. Therefore colourless RPMI was used in all experiments.
To negate possible false results due to the plant extracts containing colour pigment, the plates where read on the ELISA plate reader at 450 nm with a reference wavelength of 620 nm first before the addition of the XXT reagent. These results were subtracted from the results obtained after assay. The percentage viability was determined using the formula below:

$$\% \text{ Cell Viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

All data were calculated from the average optical density of the readings (triplicate). The 50% cellular cytotoxicity concentration (CC_{50}) was defined as the concentration of test compound that reduces the absorbance of the mock-infected (un-infected) control by 50%. The effective concentration at 50% (EC_{50}) is defined as the concentration of the test compound that achieves 50% protection in infected cultures according to the following formula:

$$\frac{(OD_T)_{HIV} - (OD_C)_{HIV}}{(OD_C)_{MOCK} - (OD_C)_{HIV}}$$

where:

$(OD_T)_{HIV}$: is the optical density measured at a given concentration of the test compound in HIV-infected cells.

$(OD_C)_{HIV}$: is the optical density measured for the control untreated HIV-infected cells.

$(OD_C)_{MOCK}$: is the optical density measured for the control untreated (with no virus) cells.

The selectivity index (SI) of the test compound is calculated as the ratio of CC_{50}/EC_{50}.

3.3 RESULTS

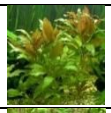
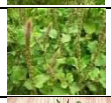
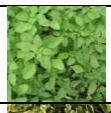
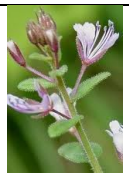
A total of 110 plant extracts from 35 plant species were investigated for their antiviral activity against HIV-1 and cellular cyto-toxicity to MT-4 cells. The anti-HIV and cellular cyto-toxicity activity was measured using a tetrazolium-based colorimetric assay. And the EC_{50} and IC_{50} values was determined. The results of the screening are shown in Table 6. This screening led to the discovery of thirty two extracts from 19 species displayed varing degrees of anti-HIV activity expressed as a SI value. The infection of MT-4 cells with virus to obtain a 200 X $TCID_{50}$ (1:5 v/v of virus/media) was considered adequate for this study

Amaranthus dubius methanolic leaf extract (SI 1.62); *Amaranthus hybridus* aqueous and methanolic leaf and flower extracts respectively (SI 1.72 and 1.52); *Asystasia gangetica* aqueous and methanolic leaf extracts (SI 5.54 and 2.66); *Bidens pilosa* methanolic leaf extract (SI 6.36); *Capparis tomentosa* aqueous and methanolic leaf extracts (SI 0,646 and 4.079); *Carpobrotus dimidiatus* aqueous leaf extract (SI 2.07); *Centella asiatica* methanolic leaf extract (SI 0.79); *Ceratotheca triloba* aqueous and methanolic leaf extracts (SI 5.6 and 2.97); *Cleome monophylla* methanolic leaf extract (SI 10.87); *Dichrostachys cinerea* aqueous leaf extract (SI 43.5); *Emex australis* aqueous and methanolic crown extract (SI 0.76 and 0.4) and the methanolic leaf extract (SI 1.6); *Gunnerea perpensa* aqueous leaf extract (SI 3.9); *Justicia flava* methanolic leaf extract (SI 1.499); *Leonotis leonurus* aqueous and methanolic leaf extracts (SI 24.37 and 3.26) and the aqueous flower extract (SI 4.7); *Oxygonum sinuatum* methanolic leaf extract (SI 1.73); *Pelargonium. sp* aqueous and methanolic leaf extracts (SI 3.76 and 1.25); *Portulaca oleracea* methanolic leaf extract (SI 2.49) and the aqueous and methanolic root extracts (SI 0.85 and 2.75); *Syzygium cordatum* aqueous and methanolic leaf extracts (SI 2.06 and 1.93); *Taraxcum officinale* methanolic leaf extract (SI 2.96).

Plant extracts that displayed an SI value of > 5 was consider significant, and these are *A. gangetica* aqueous extract (SI 5.54); *B. pilosa* methanolic leaf extract (SI 6.36); *C. triloba* aqueous leaf extract (SI 5.6); *C. monophylla* methanolic leaf extract (SI 10.87); *D. cinerea* aqueous leaf extract (SI 43.5); *L. leonurus* aqueous and methanolic leaf extracts (SI 24.37).

Plants demonstrating CC_{50} of less than 40 µg/ml were considered extremely toxic and were not further evaluated. The methanolic extracts of *Physalis viscosa* (leaves) and *Solanum nodiflorum* (roots) were toxic in a dose dependent manner, with a CC_{50} of 34 µg/ml and 39 µg/ml respectively.

Table 6: Plants and their product yield, EC_{50}, CC_{50} and SI values were determined

Plant		Part	Extraction	Product yield (%)	[a] EC_{50} (ug/ml)*	[b] CC_{50} (ug/ml)*	[c] SI
Alternanthera sessile		Leaves	Aqueous	10	n.a	96	-
			Methanolic	23	n.a	79	-
Achyranthes aspera		Leaves	Aqueous	11	n.a	92	-
			Methanolic	33	n.a	77	-
Amaranthus dubius		Leaves	Aqueous	13	n.a	70	-
			Methanolic	1	212	345	1.62
		Inflorescence & seeds	Aqueous	8	n.a	346	-
			Methanolic	23	n.a	143	-
Amaranthus hybridus		Leaves	Aqueous	11	225	389	1.72
			Methanolic	19	n.a	151	-
		Flowers&fruit	Aqueous	28	n.a	397	-
			Methanolic	3	179	272	1.52
Amaranthus spinosus		Leaves	Aqueous	9	n.a	300	-
			Methanolic	10	n.a	222	-
Asystasia gangetica		Leaves	Aqueous	26	110	611	5.54
			Methanolic	28	145	389	2.66
Bidens pilosa		Leaves	Aqueous	37	n.a	100	-
			Methanolic	22	76	484	6.36
		Fruit flower	Aqueous	12	n.a	318	-
			Methanolic	7	n.a	154	-
Buddleia saligna		Leaves	Aqueous	13	n.a	104	-
			Methanolic	11	n.a	111	-
Capparis tomentosa		leaves	Aqueous	23	171	110	0.646
			Methanolic	19	91	373	4.079
Carpobrotus dimidiatus		Leaves	Aqueous	15	178	370	2.07
			Methanolic	22	n.a	113	-
Centella asiatica		Leaves	Aqueous	16	n.a	350	-
			Methanolic	19	162	128	0.79
Ceratotheca triloba		Leaves	Aqueous	36	51	289	5.6
			Methanolic	40	182	540	2.97
Chenopodium album		Leaves	Aqueous	16	n.a	242	-
			Methanolic	6	n.a	240	-
		Fruit and flowers	Aqueous	8	n.a	167	-
			Methanolic	6	n.a	362	-
Cleome monophylla		Leaves	Aqueous	13	n.a	333	-
			Methanolic	18	26	278	10.87
		Fruit and flowers	Aqueous	11	n.a	62	-
			Methanolic	12	n.a	323	-
		Roots	Aqueous	12	n.a	109	-
			Methanolic	12	n.a	500	-

Plant		Part	Extraction	Product yield (%)	[a] EC_{50} (ug/ml)*	[b] CC_{50} (ug/ml)*	[c] SI
Dichrostachys cinerea		Leaves	Aqueous	18	6.9	302	43.5
			Methanolic	22	n.a	106	-
Elytropappus rhinocerotis		Leaves	Aqueous	21	n.a	244	-
			Methanolic	15	n.a	76	-
Emex australis		Leaves	Aqueous	5	n.a	91	-
			Methanolic	14	58	94	1.6
		Crowns	Aqueous	4	115	88	0.76
			Methanolic	15	148	58	0.4
		Roots	Aqueous	4	n.a	269	-
			Methanolic	12	n.a	54	-
Ekcebergia capensis		Leaves	Aqueous	10	n.a	100	-
			Methanolic	7	n.a	107	-
Ficus sur		Leaves	Aqueous	9	n.a	71	-
			Methanolic	16	n.a	63	-
Galinsoga parviflora		Flower heads	Aqueous	18	n.a	337	-
			Methanolic	11	n.a	200	-
		Roots	Aqueous	8	n.a	108	-
			Methanolic	8	n.a	78	-
Guilleminea densa		Leaves	Aqueous	11	n.a	94	-
			Methanolic	4	n.a	86	-
Gunnera perpensa		Leaves	Aqueous	9	127	489	3.9
			Methanolic	12	n.a	443	-
Heteropyxis natalensis		Leaves	Aqueous	6	n.a	114	-
			Methanolic	16	n.a	-	-
Hibiscus sabdariffa		Calyx-flowers	Aqueous	42	n.a	113	-
			Methanolic	41	n.a	97	-
Justicia flava		Leaves	Aqueous	22	n.a	102	-
			Methanolic	21	146	219	1.499
		Flower&fruit	Aqueous	9	n.a	90	-
			Methanolic	6	172	328	1.91
Leonotis leonurus		Leaves	Aqueous	32	16	391	24.37
			Methanolic	15	99	322	3.26
		Flowers	Aqueous	17	15	101	4.7
			Methanolic	9	n.a	89	-
Momordica balsamina		Leaves	Aqueous	23	n.a	154	-
			Methanolic	12	n.a	90	-
Oxygonum sinuatum		Leaves	Aqueous	17	n.a	239	-
			Methanolic	3	188	325	1.73
Pelargonium. sp		Leaves	Aqueous	13	87	77	3.76
			Methanolic	25	240	299	1.25

Plant		Part	Extraction	Product yield (%)	[a] EC_{50} (ug/ml)*	[b] CC_{50} (ug/ml)*	[c] SI
Physalis viscosa		Leaves	Aqueous	19	n.a	331	-
			Methanolic	22	n.a	34	-
Portulaca oleracea		Leaves	Aqueous	9	n.a	110	-
			Methanolic	21	119	299	2.49
		Roots	Aqueous	13	183	155	0.85
			Methanolic	9	255	426	2.75
Syzygium cordatum		Leaves	Aqueous	13	121	251	2.06
			Methanolic	3	174	335	1.93
Senna occidentalis		Leaves	Aqueous	24	n.a	103	-
			Methanolic	16	n.a	107	-
Solanum nodiflorum		Leaves	Aqueous	13	n.a	86	-
			Methanolic	17	n.a	70	-
		Roots	Aqueous	8	n.a	101	-
			Methanolic	9	n.a	39	-
		Fruits &stem	Aqueous	11	n.a	86	-
			Methanolic	20	n.a	147	-
Sonchus oleraceus		Leaves	Aqueous	12	n.a	107	-
			Methanolic	22	n.a	296	-
		Roots	Aqueous	4	n.a	109	-
			Methanolic	4	n.a	55	-
Taraxcum officinale		Leaves	Aqueous	19	n.a	58	-
			Methanolic	34	160	475	2.96
		Roots	Aqueous	8	n.a	187	-
			Methanolic	5	n.a	125	-
Tetradenia riparia		Leaves	Aqueous	8	n.a	133	-
			Methanolic	13	n.a	116	-
Tulbaghia violacae		Leaves	Aqueous	1	n.a	91	-
			Methanolic	2	n.a	168	-
Lopinavir		-	-	-	0.00102	32.75	32239.64

[a] **EC_{50}** = 50% effective inhibitory concentration
[b] **CC_{50}** = 50% cellular cytotoxicity concentration
[c] **SI** = selectivity Index (CC_{50}/ EC_{50})
n.a = no anti- HIV activity
- = unable to calculate SI value
***n** = 3

The plants that demonstrated significant anti-HIV, and cellular cytotoxicity are shown in Figures (45-52). These graphs show the relationship between the anti-HIV activity and the cellular cytotoxicity activity of the plant extract.

The percentage cell viability of MT-4 cells which were treated with aqueous leaf extract of *A. gangetica* is shown in Figure 45. The uninfected and infected cells were significant at concentration of 110 and 511 µg/ml respectively (Table 6). The ratio between the uninfected (IC_{50}) and infected cells (EC_{50}) determines the selective index which was found to be 5.54.

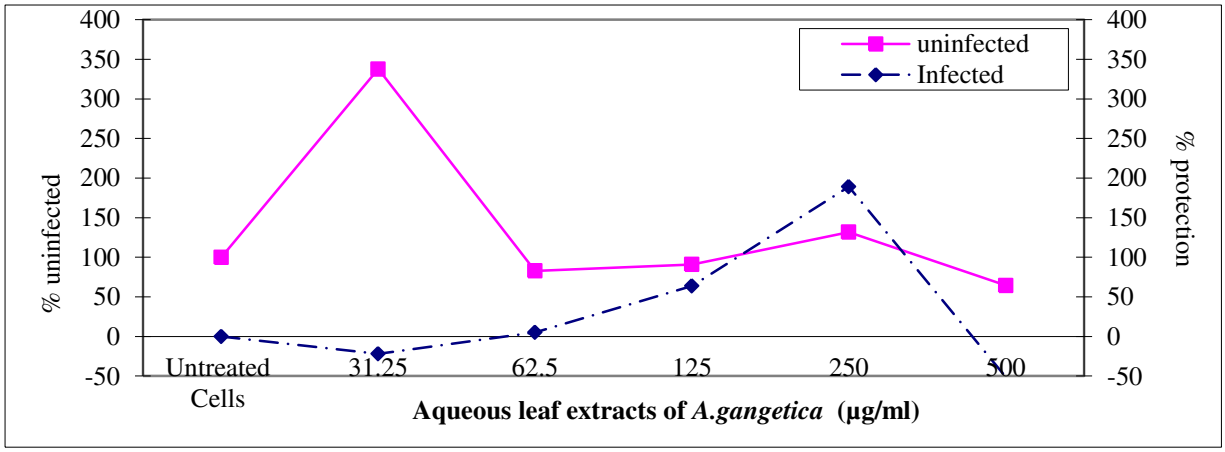


Figure 45: Anti- HIV (infected) and cellular cytotoxicity (unifected) activity of *A.gangetica*

The percentage cell viability of MT-4 cells which were treated with methanolic leaf extract of *B.pilosa* is shown in Figure 46. The uninfected and infected cells were significant at concentration of 76 and 484 µg/ml respectively (Table 6). The ratio between the uninfected (IC_{50}) and infected cells (EC_{50}) determines the selective index which was found to be 6.36.

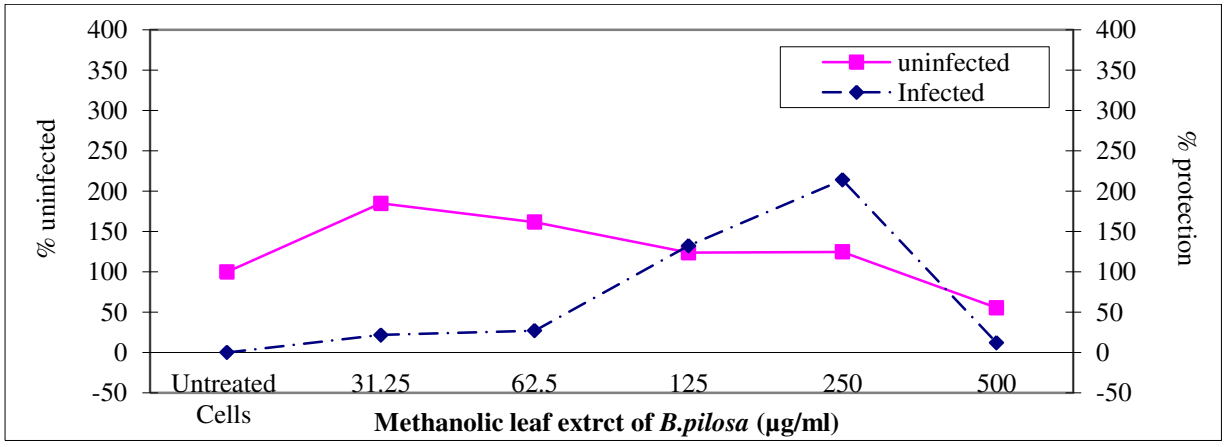


Figure 46: Anti-HIV (infected) and cellular cytotoxicity (unifected) activity of *B.pilosa*

The percentage cell viability of MT-4 cells which were treated with aqueous leaf extract of *C. triloba* is shown in Figure 47. The uninfected and infected cells were significant at concentration of 51 and 289 µg/ml respectively (Table 6). The ratio between the uninfected (IC $_{50}$) and infected cells (EC_{50}) determines the selective index which was found to be 5.60.

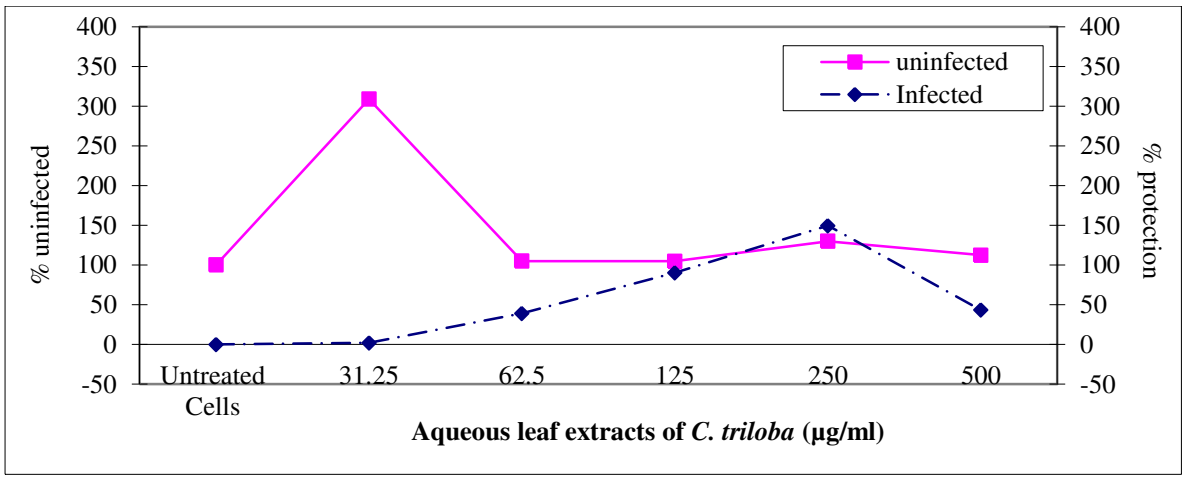


Figure 47: Anti-HIV (infected) and cellular cytotoxicity (unifected) activity of *C. triloba*

The percentage cell viability of MT-4 cells which were treated with methanolic leaf extract of *C.monophylla* is shown in Figure 48. The uninfected and infected cells were significant at concentration of 26 and 278 µg/ml respectively (Table 6). The ratio between the uninfected (IC $_{50}$) and infected cells (EC_{50}) determines the selective index which was found to be 10.87.

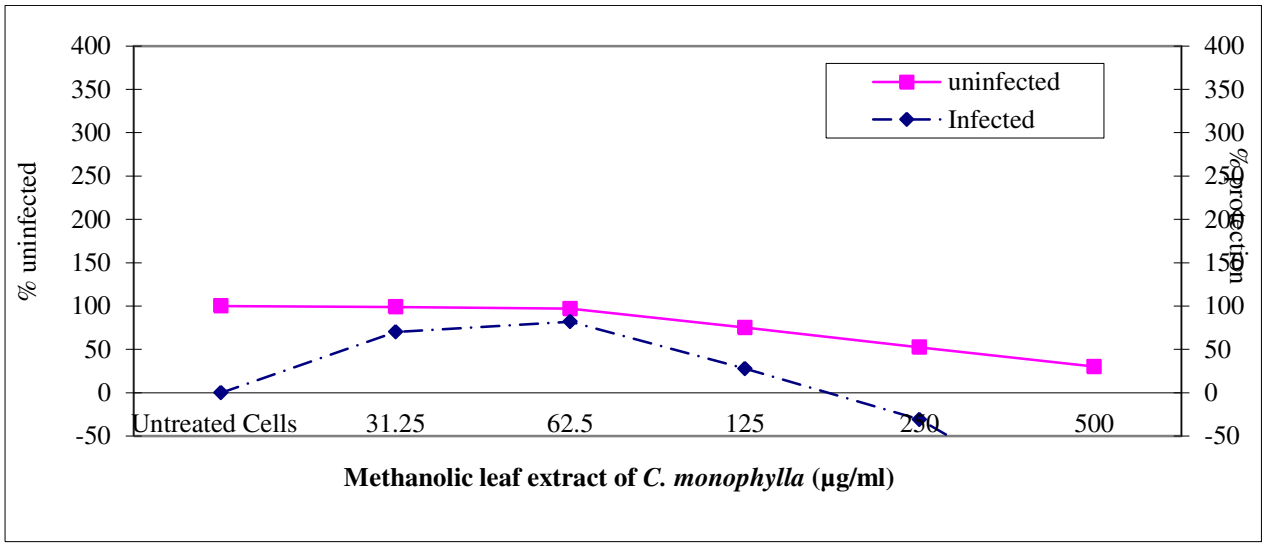


Figure 48: Anti-HIV (infected) and cellular cytotoxicity (unifected) activity of *C.monophylla*

The percentage cell viability of MT-4 cells which were treated with aqueous leaf extract of *L. leonurus* is shown in Figure 49. The uninfected and infected cells were significant at concentration of 16 and 391 µg/ml respectively (Table 6). The ratio between the uninfected (IC_{50}) and infected cells (EC_{50}) determines the selective index which was found to be 24.37.

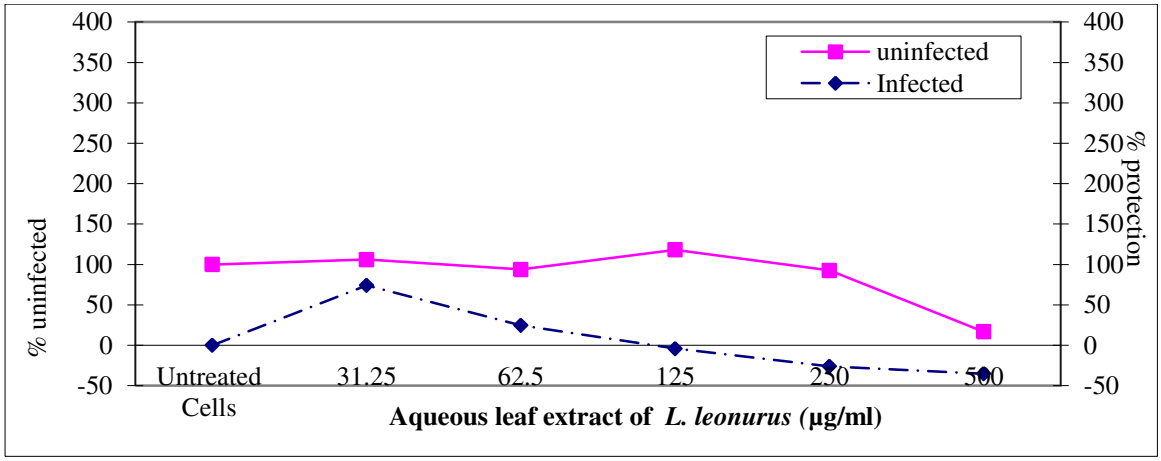


Figure 49: Anti-HIV (infected) and cellular cytotoxicity (unifected) activity of *L.leonurus*

The percentage cell viability of MT-4 cells which were treated with methanolic leaf extract of *D. cinerea* is shown in Figure 50. The uninfected and infected cells were significant at concentration of 6.9 and 302 µg/ml respectively (Table 6). The ratio between the uninfected (IC_{50}) and infected cells (EC_{50}) determines the selective index which was found to be 43.5.

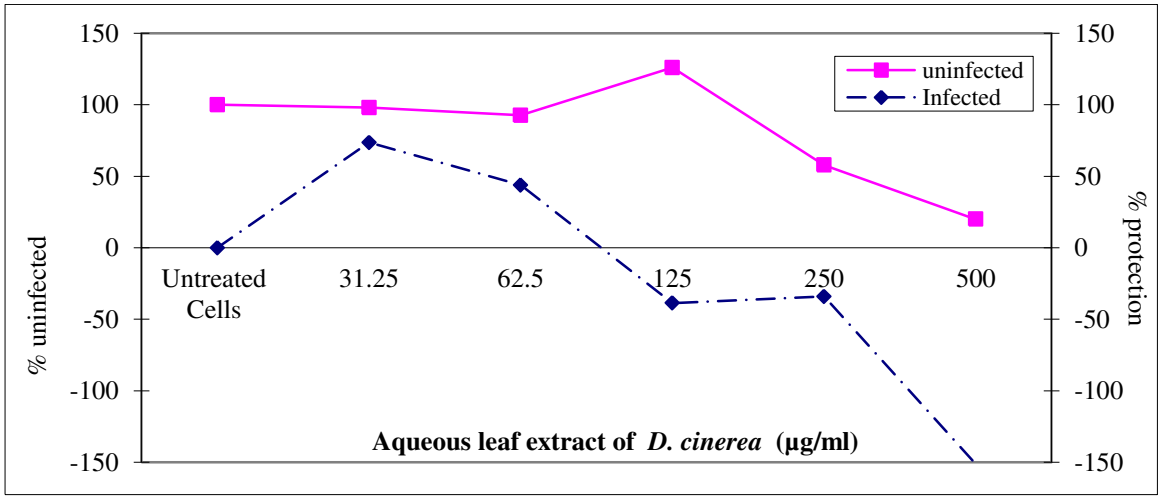


Figure 50: Anti-HIV (infected) and cellular cytotoxicity (unifected) activity of *D.cinerea*

P. viscosa methanolic leaf extract demonstrated a sharp decline in % cell viability on the MT-4 cellls; this is due to the cytotoxic effect of the plant extract from a concentration higher than 31.5 µg/ml. The extract does not demonstrate a protective effect/ anti-HIV, i.e. the % protection does not reach 50%. This is shown in Figure 51

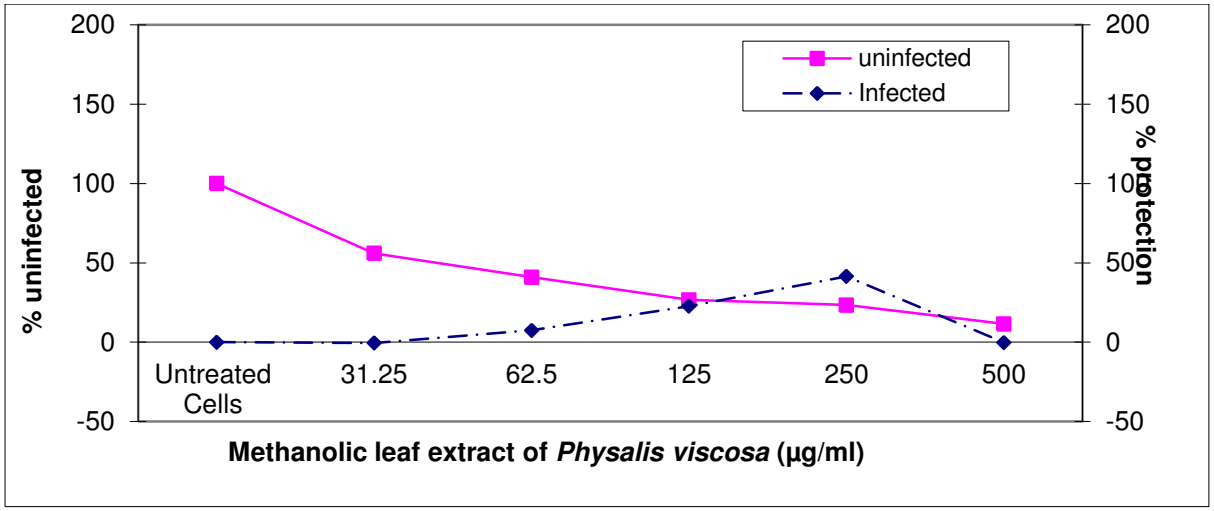


Figure 51: Anti-HIV (infected) and cellular cytotoxicity (unifected) activity of *P. viscosa*

S. nodiflorum methanolic root extract demonstrated a sharp decline in percentage cell viability on the MT-4 cellls; this is due to the cytotoxic effect of the plant extract at concentration higher than 31.5 µg/ml. The extract does not demonstrate a protective effect/ anti-HIV, i.e. the % protection does not reach 50%. This is shown in Figure 52.

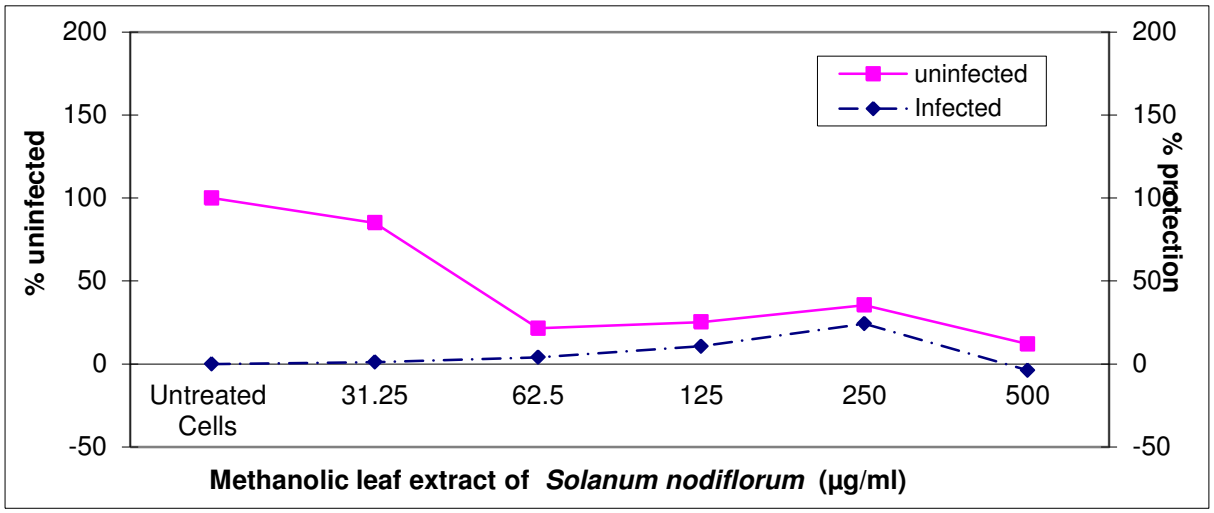


Figure 52:Anti-HIV (infected) and cellular cytotoxicity (unifected) activity of *S. nodiflorum*

3.4 DISCUSSION

The yield from 100 g of fresh plant material is shown in Table 6. Although fresh or dried plant material can be used as a source for secondary plant components, in these experiments dried material was used since:

(i) Traditional healers frequently use dried plant material

(ii) The time delay between collecting plant material and processing makes it difficult to work with fresh material; and

(iii) There are fewer problems associated with the large scale extraction of dried plant material.

The cellular cytotoxicity of extracts was screened in cells representative of the immune system especially those known to be host cells for HIV infection. Hence the MT-4 cells were selected for both anti-HIV activity (EC_{50}) using whole virus and cellular cytotoxicity screening (CC_{50}). The effects of plant extracts on infected cells was routinely analysed over a 5 day incubation period (Pauwels *et al.*,1988). Since toxicity studies were performed over a 5 day period, it was also assessed whether this incubation time could also be used for the anti-HIV studies, thus keeping the experimental conditions consistent throughout. Most of the extracts tested were stimulatory to human lymphocytic MT- 4 cells. In this experiment plants demonstrating CC_{50} of less than 40 µg/ml were considered extremely toxic and were not further evaluated. These were the methanolic extracts of *Physalis viscosa* (leaves) and *Solanum nodiflorum* (roots) showed cellular cytotoxicity in a dose dependent manner, with a CC_{50} of 34 µg/ml and 39 µg/ml respectively. Both shown in Figure 51 and Figure 2 respectively. Both *P. viscosa* and *S. nodiflorum* belonging to the family Solanaceae were not screened further. In this family the major active compound present is withanolide-type structures. Withanolides are classically defined as a group of C-28 ergostane-type steroids with a C-22, 26 δ-lactone group, first isolated from the genus *Withania* (Kindscher *et al.*, 2012). Withanolides are a group of steroidal lactones that are known for their broad spectrum biological activity (Silva *et al.,* 1993). Khalighi-Sigaroodi *et al.,* 2012, reported on the cytotoxicity of some plants species of Solanaceae family. This could be attributed to the many alkaloids present in this family such as solanine (Tredgold *et al.*, 1986). Both the methanolic and aqueous leaf extracts of *D. cinerea* were not toxic, and displayed an CC_{50} of 106 µg/ml and 302 µg/ml respectively. Neondo *et al*., (2012) reported that the aqueous and methanolic extraction of *D. cinerea* showed no toxicity even at a concentration of 2000 µg/ml, which is 4 fold higher than tested in this study.

A total of 108 plant extracts from 38 plant species were investigated for their antiviral activity against HIV-1 (Table 6). The anti-HIV activity was measured using a tetazolium based colorimetric assay in infected MT-4 cells. Infection with virus (1:5 v/v of virus/media) to obtain a 200 X $TCID_{50}$ was considered adequate for this study since other studies have indicated successful infection over a range of $TCID_{50}$, from as low as 1× $TCID_{50}$ to 1000 × $TCID_{50}$ (St Clair *et al.*, 1995). The medicinal plants which yielded the most promising anti-viral activity in this broad screening for anti-HIV activity are among some of the common medicinal plants used in traditional medicines. From the 108 extracts tested 32 extracts (Highlighted in blue in Table 6), exhibited varying degrees of anti-HIV activity. *A.gangetica* (aqueous), *B. pilosa* (methanolic), *C. monophylla* (methanolic), *C. triloba* (aqueous), *D. cinerea* (aqueous) and *L. leonurus* (aqueous) extracts gave the highest SI values in the screening of crude extracts for anti-viral activity with SI values > 5. Parametric statistics were considered unnecessary since these samples were merely screened to determine anti-HIV activity.

Many scientists such as Tommasi *et al.*, (1998); Bedoya *et al.*, (2001); Rukunga *et al.*, (2002); Bessong *et al.*, (2004); Bessong *et al.*, (2005); and Bessong *et al.*, (2006), have shown that methanolic extracts from medicinal plants are more inhibitory than the aqueous extracts in screening for anti-HIV activity. However in this study the aqueous extracts of *D. cinerea* and *L. leonurus* showed the highest anti-HIV activity, indicating that the active compound/s are hydrophilic. This is in keeping with studies of Locher *et al.*, (1996); and Bot *et al.*, (2007) who reported that phytochemicals extracted with water are more commonly effective inhibitors of HIV virus. The results suggest that the separation of polar and non-polar compounds can increase the chance of finding highly active anti-viral compounds with low cytotoxicity (Cos *et al.*, 2004). *D. cinerea* and *L. leonurus* aqueous extracts showed strong anti-HIV activity with SI of 43.5 and 23.5, respectively. *D. cinerea* is not known to be used traditionally as a treatment of viral diseases and there is no literature of its anti HIV-activity. *L. leonurus* is used traditionally and is reported in literature to demonstrate anti-HIV activity. According to Klos *et al.*, (2009) it was found that the ethanolic leaf extract of *L. leonurus* showed anti-HIV properties that were associated with HIV-1 PR inhibition. *A. gangetica, B. pilosa, C. monophylla, C. triloba, D. cinerea* and *L. leonurus* (aqueous) was screened further for potential anti-HIV activity.

CHAPTER FOUR: CHARACTERIZATION OF THE SIX ACTIVE PLANTS WITH ANTI-HIV ACTIVITY

4.1 INTRODUCTION

Medicinal plants are integral to the health and wellbeing of individuals and communities. The medicinal value of these plants is attributable to a single chemical compound that produces a definite physiological action on the human body (Edeoga *et al*., 2005). Hence the identification of these compounds starts with simple phytochemical analysis.

The qualitative and quantitative distribution of phytochemicals differs from plant to plant, organ to organ, age, various ecological and climatic factors (Khan *et al.*, 2011). Plants have been shown to possess phytochemical compounds, with varying pharmacological activities, one such activity is antioxidant activity. Flavonoids are widely distributed in plants giving them the ability to scavenge free radicals by single electron transfer (Choi *et al.*, 2002). Polyphenols and flavonoids which are isolated from plants are generally antioxidants, which has show remarkable biological activity such as antibacterial, anti-carcinogenic, anti-inflammatory, antiviral, anti-allergic, estrogenic and immune stimulating effects (Edeoga *et al*., 2005). The antioxidant activity is mainly due to their redox properties which allow them to act as reducing agents (Akula *et al.*, 2008).

Plants have provided man with many useful compounds. Most of the natural products are secondary metabolites and about 12 000 such products have been isolated thus far (Khan *et al.*, 2011). There are many approaches to the identification of these compounds. Chemicals present universally in all plants can be classified as primary and secondary metabolites (Khan *et al.*, 2011). Primary metabolites are essential for the growth of plants such as proteins, amino acids, sugars and chlorophyll etc. Secondary metabolites are compounds that do not participate in normal metabolism or growth of the plants, but are involved in protecting the plant from, disease (from fungus, bacteria, viruses, and pests), and to overcome extreme conditions of stress (Khan *et al.*, 2011). Some of the secondary metabolites are alkaloids, steroids, terpenoids, essential oils, flavours, fragrance, colours and pigments. The qualitative and quantitative distribution of these metabolites differs from plant to plant (Khan *et al.*, 2011).

Depending on the structure, and the biological activity of plant secondary metabolites, plants represent a huge repository for novel anti-HIV drugs. Furthermore, there is an urgent need for safer and cost effective treatment in developing countries that rely heavily on traditional medicines. Thus, compounds from plants can provide a complementary strategy against HIV. Indeed more than 50 compounds with antiviral activity have already been isolated from plants (Bedoya *et al.*, 2001; Cos *et al.*, 2002; Bedoya *et al.*, 2002; Bessong *et al.*, 2004; Bessong *et al.*, 2005; and Bessong *et al.*, 2006).

Bioactivity guided fractionation of crude extract has provided many compounds for treating diseases. Most of them have their origins in secondary metabolites from plants. Anti-HIV plant compounds described are inhibitors of HIV-1 and/or RT and include aliphatic ketones and aldehydes, terpenoids, alkaloids, coumarin derivatives, flavanoids, xanthones, tannins, polysaccarides, proteins and phenols (Singh *et al.*, 2005). Potential anti -HIV targets from plant derived substance groups are: betulinic acid, (inhibit virus entry); betulinic acid derivatives calanolides/inophyllums (inhibit reverse transcriptase); flavonoids (inhibit entry, reverse transcriptase), integrase and transcription; mannose-specific plant lectins (inhibit viral entry) and sulphated polysaccharides (inhibit entry and transcription). Several more exist and are reviewed by Cos *et al*., (2004).

Alkaloids are found in low concentrations relative to the phenolic compound which is offset by their high biological potency in vegetative tissues (Khan *et al.*, 2011). Alkaloids are found in higher concentration in storage tissues (roots, fruits, seed) as compared to the green leaves (Walton and Brown., 1998). Alkaloids and glycosides are complex substances and are distributed in large varieties of plants (Khan *et al.*, 2011). Alkaloids and glycosides are sometimes poisonous but some have still shown potential to possess medicinal properties (Khan *et al.*, 2011).

Plants synthesize numerous different aromatic substances, which are usually phenolic compounds. Flavonoids are polyphenolic compounds that produce the flavour as well as pigment (such as red and blue) in many plants. (McRae *et al.,* 2007). Flavonoids have been reported to possess a number of biological activities, attributed to their antioxidant properties. In a healthy (non-infected) person, the production of reactive oxygen species (ROS) is balanced with the bodies' natural antioxidant defense system (Singh *et al.*, 2005). The role of oxidative stress in HIV disease appears to be quite broad and may involve alterations in viral

replication, immune function, and apoptosis (Cos *et al.*, 2004). An increasing number of studies that support the theory that oxidative stress are involved in the progression of HIV to AIDS (Cos *et al.*, 2004; and Singh *et al.*, 2005). In a HIV-postive person tissue levels of antioxidants such as glutathione, ascorbic acid, α-tocopherol and selenium have been shown to be low whilst there is an increase in the levels of products from lipid peroxidation and oxidative DNA damage, free radicals. (Singh *et al.*, 2005). There is compelling proof on the presence of oxidative stress in a HIV positive individual, which may accelerate the rate at which HIV progresses to AIDS. A dietary intervention with antioxidants could be an inexpensive method with existing HIV strategies such as HAART to treat HIV positive individuals. Therefore there is a clear need for studies on the effect of plant derived antioxidants on the HIV virus.

The search for new anti-HIV drugs is mostly targeted at specific steps of the viral life cycle. In the life-cycle of HIV, specific mechanisms are targeted, such as viral attachment, entry, viral genome transcriptation (reverse transcriptase activity), processing of viral proteins by protease (protease activity) or integration of viral DNA into host DNA (integration activity). All these steps are crucial in the infective ability of the virus (Matsuse *et al.*, 1999). The reverse transcriptase enzyme is very important, and if it is inhibited effectively, it could either stop the infection process or it could lower the rate of infection. The most effective treatment of HIV infected patients in recent years is the use of RT inhibitors with a combination of either protease or intergrase inhibitors (Matsuse *et al.,* 1999). Thus it is likely that new therapeutic compounds for anti-HIV activity will probably be based on reverse transcriptase activity. This necessitates the search for new RT inhibitors from a plant, due to the toxicity of current drugs and emergence of drug resistant viruses (Matsuse *et al.,* 1999).

The current therapy for HIV is focused on the use of reverse transcriptase inhibitors because the first four anti-HIV drugs that have been approved for the treatment of HIV infection (AZT, DDI, DDC, and D4T) belong to Nucleoside and nucleotide reverse transcriptase inhibitors (Clercq., 1995). Therefore the first target for most scientists, when looking for plants that have anti-HIV activity is to screen for anti-RT activity. The HIV-RT controls three consecutive functions; (RNA transcriptase to DNA, degradation of RNA template and duplication of the remaining strand); and since RNA directed DNA synthesis does not occur in healthy cells, RT activity is considered as one of the most important targets in the search for a anti-HIV substance (Cos *et al.*, 2004). Once the virus is inside the host cell, the viral

single-stranded RNA genome is converted into double-stranded DNA (Prescott *et al.,* 1993). During this reverse transcription process, a DNA-copy of viral RNA has to be formed; and if the DNA-copy is not formed, the viral RNA genome becomes susceptible to destruction by cellular enzymes hence halting viral infection. This is the target for reverse transcriptase inhibitors. There are many plants that have displayed reverse transcriptase activity and some of these plants are listed in the Table 7 below.

Table 7: Plants with Reverse Transcriptase Activity

Plant	Family	Compound	Reference
Acalypha macrostrachya	Euphorbiaceae	Diterpenoid esters	Matsuse *et al.,* (1999)
Callophyllum sp	Clusiaceae	Calanolides, Calanolide A and Calanolide B	Yadav *et al.*, (2009)
Combretum Molle	Combretaceae	Iridoid glucosides	Bessong *et al.,* (2005)
Hyptis lantanifolia	Labiatae	Gallic acid	Matsuse *et al.,* (1999)
Terminalia chebula	Combretaceae	Gallic acid	Yadav *et al.*, (2009)
Terminalia sericea	Combretaceae	Resveratrol-3-*O*-d-rutinoside	Bessong *et al.,* (2005)
Tuberaria lignosa	Cistaceae	Ellagitannins	Bedoya *et al.,* (2001)
Momordica charantia	Cucurbitaceae	momordicin I, momordicin II cucurbitacin B	Singh *et al.,* (2005); and Bot *et al.*, (2007)
Phyllanthus myrtifolius	Euphorbiaceae	Phyllamyricin B, Retrojusticidin B	Yadav *et al.*, (2009)

In this study, *A.gangetica* (aqueous)*, B.pilosa* (methanolic), *C.monophylla* (methanolic)*, C.triloba* (aqueous), *D. cinerea* (aqueous) and *L. leonurus* (aqueous) extract gave the highest SI value in the intial screening of crude extracts for anti-viral activity (Table 6). These plants were selected for phytochemical screening for tannins, phlobatannins, saponins, flavanoids, steroids, terpenoids, alkaloids and antioxidant activity and were rescreened for anti-HIV-1 activity and anti-reverse transcriptase activity.

4.2 METHODOLOGY

4.2.1 Phytochemical analysis of active plant extracts

The analysis was carried out on the powdered samples using standard procedures described by Harborne., (1973); Trease and Evans., (1989); and Sofowara., (1993),

4.2.1.1 Test for tannins

Half gram (0.5 g) of the dried powdered samples of *A. gangetica, B. pilosa*, C. *monophylla, C. triloba*, *D. cinerea* and *L. leonurus* leaves was each boiled in 20 ml of distilled water respectively. A few drops of 0.1% ferric chloride solution was added to half the volume of filtrate. The appearance of an intense green or brownish green or a blue-black colouration is indicative of the presence of tannins in the test sample. This was confirmed by adding a few drops of iodine in a second portion of the filtrate which yielded a faint bluish colouration.

4.2.1.2 Test for phlobatannins

One gram (1 g) of the dried powdered samples of *A. gangetica, B. pilosa*, *C. monophylla, C. triloba*, *D. cinerea* and *L. leonurus* leaves was mixed in 20 ml of distilled water respectively and filtered. The mixture was boiled with 1% aqueous hydrochloric acid. The presence of phlobatannins was observed by the presence of a red precipitate at the base of the test tubes.

4.2.1.3 Test for saponin

Two grams (2 g) of *A. gangetica, B. pilosa*, *C. monophylla, C. triloba*, *D. cinerea* and *L. leonurus* leaves was boiled in 20 ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion. Presence of saponins was indicated by the formation of a heavy emulsion.

4.2.1.4 Test for flavonoids

Two grams (2 g) of *A. gangetica, B. pilosa*, *C. monophylla, C. triloba*, *D. cinerea* and *L. leonurus* leaves was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The

mixture was filtered and 4 ml of the filtrate was shaken and added to 1 ml of dilute ammonia solution. A yellow colouration indicated a positive test for flavonoids.

4.2.1.5 Test for steroids

Five hundred milligrams (500 mg) of *A. gangetica, B. pilosa*, *C. monophylla, C. triloba*, *D. cinerea* and *L. leonurus* powdered leaf sample was mixed with 2 ml of acetic anhydride respectively. This was followed by the addition of 2 ml sulphuric acid. The colour change from violet to blue or green was indicative of the presence of steroids.

4.2.1.6 Test for terpenoids (Salkowski test)

Five millilitre (5 ml) of each extract of *A. gangetica, B. pilosa*, *C. monophylla, C. triloba*, *D. cinerea* and *L. leonurus* leaves was mixed in 2 ml of chloroform respectively, and concentrated H_2S0_4 (3 ml) was carefully added to form a layer. A reddish brown colouration at the interface was indicative of the presence of terpenoids. The presence of steroids was determined by blue-green interfaces between layers.

4.2.1.7 Test for alkaloids

Five Hundred milligrams (500 mg) of *A. gangetica, B. pilosa*, *C. monophylla, C. triloba*, *D. cinerea* and *L. leonurus* leaves was mixed in 8 ml of 1% hydrochloric acid, warmed, and filtered respectively. 2 ml of the filtrate was treated with a few drops of freshly prepared Dragendorffs reagent (Sofowara., 1993). A positive result is indicated by the presence of precipitate.

4.2.1.8 Test for phenols

The ferric chloride test was conducted using *A. gangetica, B. pilosa*, *C. monophylla, C. triloba*, *D. cinerea* and *L. leonurus* powdered leaf samples. The extract was prepared by mixing 5 mg of *A. gangetica, B. pilosa*, *C. monophylla, C. triloba*, *D. cinerea* and *L. leonurus* leaves in 1 ml of distilled water respectively. To this, a few drops of neutral 5% ferric chloride solution was added. A dark green colour indicated the presence of phenols.

4.2.2 Antioxidant Activity of active plant extracts

The plant material was prepared according to Jeremy and Whiteman., (2003), and was used for the 1.1-diphenyl-2-picrylhydrazyl radical (DPPH) photometric assay (Choi *et al*., 2002).

4.2.2.1 The 1.1-diphenyl-2-picrylhydrazyl radical (DPPH) photometric assay

The anti-oxidative properties of *A. gangetica, B. pilosa*, C. *monophylla, C. triloba*, *D. cinerea* and *L. leonurus* leaves were tested using the DPPH photometric assay described by Choi *et al*., (2002). The freeze dried aqueous plant material (1000 µg/ml) was diluted to final concentrations of 1000 µg/ml, 500 µg/ml, 250 µg/ml, 100 µg/ml, 50 µg/ml, and 10 µg/ml in methanol. Rutin (1000 µg/ml) found in the plant *Fagopyrum esculentum*, was used as a comparative standard (control).

One millilitre of a 0.3 mM DPPH in methanol was added to 2.5 ml of *A. gangetica, B. pilosa*, C. *monophylla, C. triloba*, *D. cinerea* and *L. leonurus* solution. The test was performed on different concentrations and was allowed to react at room temperature for 30 minutes. One ml methanol plus plant extract solution (2.5 ml) was used as a blank and DPPH solution and 2.5 ml ethanol was used as a negative control. The positive control was DPPH solution (1 ml) plus 2.5 ml 1 mM Rutin. Each test was carried out in triplicate and results are expressed as the mean ± standard deviation. The absorbance values were measured in a Varian Cary 1E UV-visible spectrophotometer at 518 nm and the average absorbance values was converted into the percentage antioxidant activity, using the following equation:

$$\text{Scavenging capacity \%} = 100 - \left(\frac{(\text{Abs of sample} - \text{Abs of blank})}{\text{Abs of negative control}} \times 100 \right)$$

4.2.3 Re-screening of active plants for anti-HIV virus.

A. gangetica, B. pilosa, C. *monophylla, C. triloba*, *D. cinerea* and *L. leonurus* leaf extracts were re-screened using, XTT assay according to a method described by Weislow *et al.,* (1989) and was performed as decribed in Chapter 3 (Section 3.2.3).

4.2.4 Reverse transcriptase activity of the active plant extracts

This assay was performed using the Colorimetric Reverse Transcriptase Assay (Roche). For the quantification of the inhibitory effect of reverse transcriptase inhibitors, all steps of the Reverse Transcriptase Assay, including the RT reaction, were performed directly in the 96 well plates supplied with the kit. Inhibitory activity of reverse transcriptase inhibitors is usually calculated as percentage inhibition as compared to a sample that does not contain an inhibitor. Therefore, an HIV-1-RT calibration curve was not required.

4.2.4.1 Assay procedure

HIV-1 reverse transcriptase was reconstituted from the lyophillizate (500 ng) in 250 µl double distilled autoclaved water to yield a final concentration of 2 ng/ µl. The plant extract were prepared in DMSO, after which a 50/50 dilution with lysis buffer (Tris-buffer : 50 mM Tris , 80 mM potassium chloride, 2.5 mM DTT, 0.75 mM EDTA and 5% Triton X-100, pH 7.8) was prepared to yield a concentration of 500 µg/ml.

To each test well 20 µl of lysis buffer, reaction mixture (polyadenylic acid x pentadecathymidylic acid) and plant extract was added. For the positive controls 20 µl of diluted recombinant HIV-1 reverse transcriptase (at a concentration of 2 ng/µl) and AZT (at concentration of 1 mM solution) was added separately. Lysis buffer was used as a negative control. This was then covered and incubated for 2 h at 37°C. The reaction mixture was completely removed and then washed 5 times with 250 µl of washing buffer per well for 30 s each. The entire washing buffer was carefully removed.

Then 200 µl of anti-DIG-POD (Anti-digoxigenin-peroxidase, polyclonal anti body), working dilution was added to each well and covered with a cover foil and incubated for a further 1 h at 37°C. The solution was then completely removed and washed 5 times with 250 µl of washing buffer per well for 30 s each and the washing buffer was completely removed. Then

200 µl of absorbance substrate enchancer solution was added to each well and incubated for ± 20 mins at a temperature of 25°C until colour development (green color) was sufficient for photometric detection. The samples were then analysed using a microplate (ELISA) reader, at an absorbance of 405 nm (reference wavelength: 490 nm).

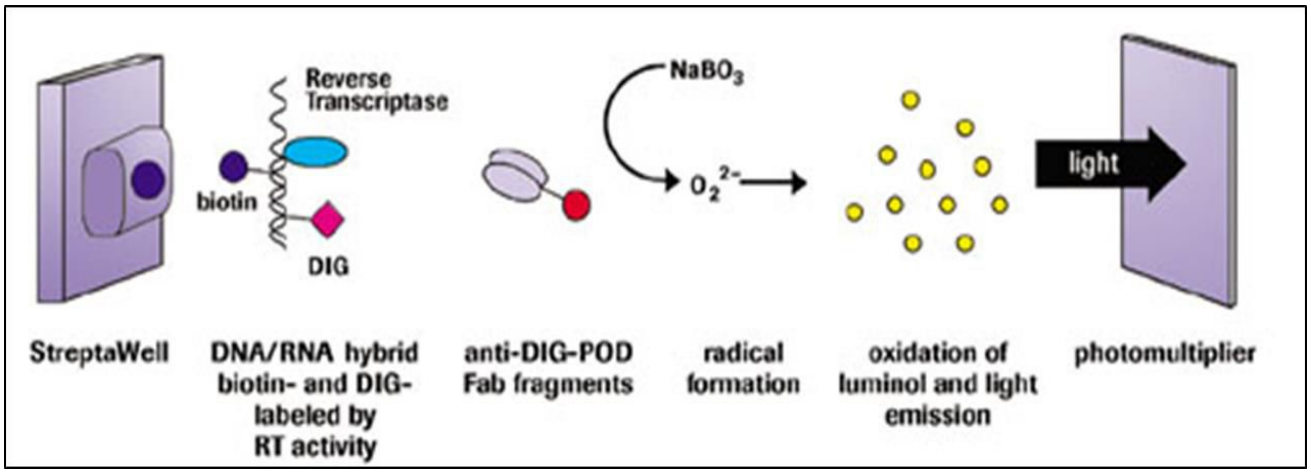


Figure 53: Test principle

The resulting signal intensity is directly proportional to the actual RT activity. The samples were analysed by comparing the inhibitory activity of RT inhibitors (plant extract), and calculating the percentage inhibition as compared to a sample that did not contain an inhibitor, using the following equation:

$$\text{Percentage RT inhibition} = 100 - \left(\frac{\text{Extract sample}^{(A405\text{ nm} - A\ 490\text{ nm})}}{\text{Control}^{(A405\text{ nm} - A\ 490\text{ nm})}} \times 100 \right)$$

4.3 RESULTS

4.3.1 Phytochemical analysis of the leaves of active plant extracts

The phytochemical analysis of the leaves of A. *gangetica, B. pilosa*, C. *monophylla, C. triloba*, *D. cinerea* and *L. leonurus* are summarized in Table 8. Flavonoids and saponins were present in all plants tested and all plants lacked the presence of phlobatannin.

A. gangetica showed the presence of tannins, saponin, flavonoids, steroids and phenol and did not contain phlobatannins, alkaloids or terpenoids. *C. triloba* displayed the presence of tannins, saponin, flavonoids, steroids and alkaloids and did not contain any terpenoids or phenol. *B. pilosa* showed the presence of tannins, saponin, flavonoids, steroids, alkaloids and terpenoids but it did not have any phenols. *C. monophylla* displayed the presence of saponin, flavonoids, steroids alkaloids and phenol and did not contain any terpenoids or tannins. *D. cinerea* displayed the presence of tannins, saponin, flavonoids and alkaloids and did not contain any phlobatannins, terpenoids, steroids or phenol. *L. leonurus* exhibited the presence of tannins, saponin, flavonoids and steroids and did not have any phlobatannins, alkaloids, terpenoids or phenol.

Table 8: Phytochemicals analysis of active plant extracts

Plants	Phytochemical test*							
	Tannins	Phlobatannins	Saponin	Flavonoids	Steroids	Alkaloids	Terpenoids	Phenol
A.gangetica	+	-	+	+	+	-	-	+
C.triloba	+	-	+	+	+	+	-	-
B.pilosa	+	-	+	+	+	+	+	-
C.monophylla	-	-	+	+	+	+	-	+
D. cinerea	+	-	+	+	-	+	-	-
L. leonurus	+	-	+	+	+	-	-	-

***(n=3)**

4.3.2 Antioxidant activity of active plant extracts

The highest degree of free radical scavenging activity was shown by *D.cinerea* with an IC_{50} of 25 µg/ml followed by *L. leonurus* IC_{50} of 100 µg/ml, *C. triloba* IC_{50} of 300 µg/ml, *B. pilosa* IC_{50} of 380 µg/ml, *C. monophylla* IC_{50} of 420 µg/ml, and *A. gangetica* IC_{50} of 430 µg/ml. Reduced IC_{50} values suggest a greater DPPH radical scavenging capacity therefore representing a greater antioxidant activity. Rutin displayed an IC_{50} value of 30 µg/ml.

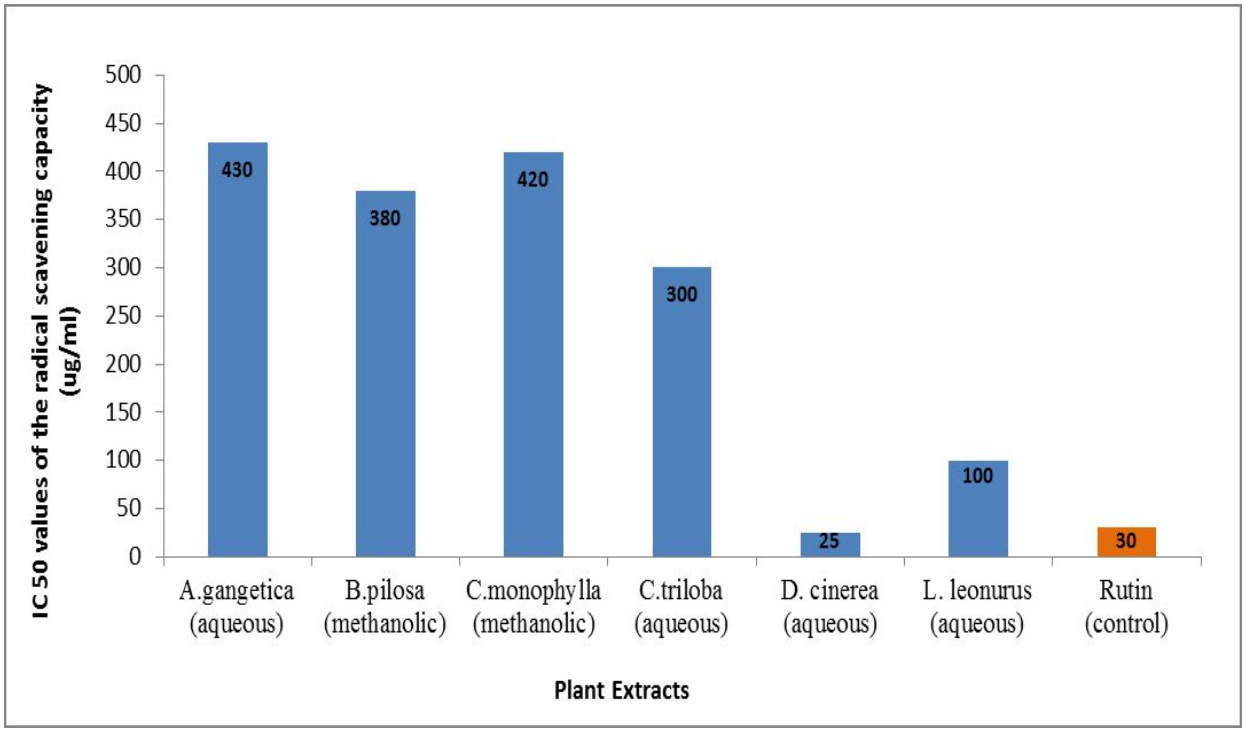


Figure 54 : The IC_{50} values of the free radical scavenging capacity of active plant extracts

(*n=3)

4.3.3 Re-screening of active plants for anti-HIV virus.

The plant extracts *A. gangetica* (aqueous), *C. triloba* (aqueous), *B. pilosa* (methanolic), *C. monophylla* (methanolic), *D.cinerea* (aqueous) *L. leonurus* (aqueous) were re-screened for confirmation of anti-HIV activity and cellular cytotoxicity. From the 6 plants screened *D.cinerea* gave the highest SI value of 44.20, followed by *L. leonurus* with an SI value of 26.07, *C. monophylla* with an SI value of 10.92, *B. pilosa* with an SI value of 6.33, *C. triloba* with an SI value of 5.70, and *A. gangetica* with an SI value of 5.58. The EC_{50} and CC_{50} and SI values of each plant are shown in Table 9. The results obtained in this re-screening confirmed the initial anti-HIV activity shown in Table 6.

Table 9: SI value of the active plants

Plant Extracts	[1] **EC $_{50}$ (ug/ml)***	[2] **CC $_{50}$ (ug/ml)***	[3]**SI**
A. gangetica (aqueous)	112	625	5.58
C. triloba(aqueous)	50	285	5.70
B. pilosa (methanolic)	75	475	6.33
C. monophylla (methanolic)	25	273	10.92
D.cinerea (aqueous)	7	310	44.20
L. leonurus (aqueous)	15	391	26.07
Control: Lopinavir	**0.00102**	**32.75**	**32239.64**

[1] **EC_{50}** = 50% effective inhibitory concentration

[2] **CC_{50}** = 50% cellular cytotoxicity concentration

[3] **SI** = selectivity Index (CC 50/ EC 50)

***n** = 3

4.3.4 Reverse transcriptase activity of active plant extracts

The results for the average percentage HIV-1 RT inhibition can be seen in Figure 55. The percentage inhibition of control and extracts were calculated relative to uninhibited HIV-1 RT. All 6 plant extracts showed some degree of HIV-1 RT inhibition, but those which displayed ≥ 50% HIV-1 RT inhibition was considered significant (Klos *et al*, 2009). Extracts displaying siginifcant HIV-1 RT activity were plant extracts *C. monophylla*, *D.cinerea* (aqueous), *L. leo*nu*rus* (aqueous) with a HIV-1 RT activity 58%, 82%, 75% respectively.

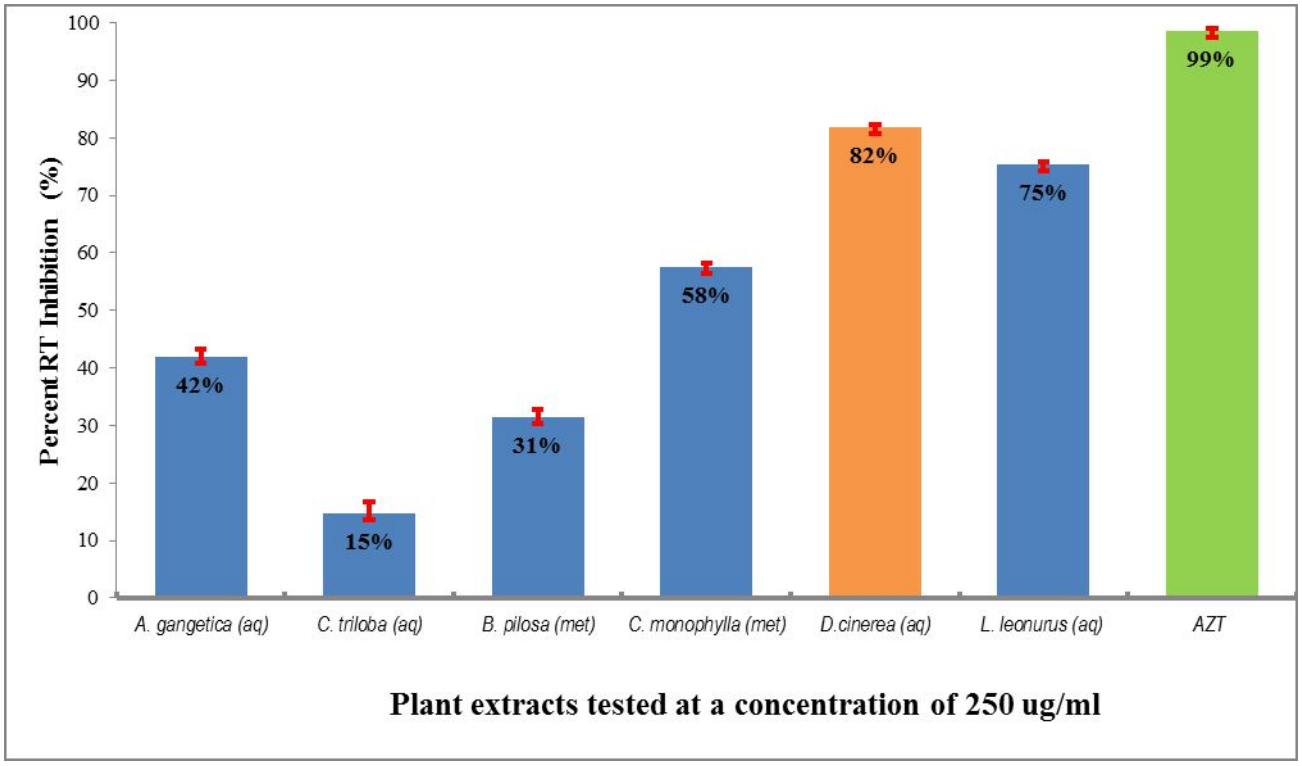


Figure 55: HIV-1 RT inhibition assay of selected plants

- The control was AZT, tested at 1mM solution.
- (n = 3).

4.4 DISCUSSION

A. gangetica, B. pilosa, C. *monophylla, C. triloba*, *D. cinerea* and *L. leonurus,* were selected because they displayed anti-HIV activity (Chapter 3).

The phytochemical analysis of the leaves of A. *gangetica, B. pilosa*, C. *monophylla, C. triloba*, *D. cinerea* and *L. leonurus* is shown in Table 8. The phytochemical analysis show that flavonoids and saponins were present in all plants tested and all plants lacked the presence of phlobatannin. *A. gangetica* showed the presence of tannins, saponin, flavonoids, steroids and phenol and did not contain phlobatannins, alkaloids and terpenoids. Kensa., (2011) found similar results when they screened *A. gangetica* for phytochemicals, and found saponins, tannins, flavonoids and steroids. *C. triloba* showed the presence of tannins, saponins, flavonoids, steroids and alkaloids and did not contain any terpenoids and phenol. However, Mohanlall *et al.,* (2011) reported the presence of phlobatanins, saponins, and steroids in *C. triloba*. *B. pilosa* showed the presence of tannins, saponin, flavonoids, steroids, alkaloids and terpenoids but it did not have any phenols. Khan *et al.,* (2001) reported that *B. pilosa* contained tannins, saponins, flavonoids, steroids, alkaloids and triterpenoids which are similar to this study. There are no reports on the phytochemical profile of *C. monophylla,* but this study found the presence of saponin, flavonoids, steroids alkaloids and phenol and did not contain any terpenoids or tannins. *D. cinerea* showed the presence of tannins, saponins, flavonoids and alkaloids and did not contain any phlobatannins, terpenoids, steroids and phenol. Eisa *et al*, (1999) and Neondo *et al.*, (2012)reported that the aqueous extraction of *D. cinerea* revealed the presence of tannins, flavonoids and saponins and this is in accordance with the results. *L. leonurus* showed the presence of tannins, saponin, flavonoids and steroids and did not have any phlobatannins, alkaloids, terpenoids and phenol in this study. Oyedemi *et al.*, (2011) reported the presence of flavonoids, tannins, and saponins in *L. leonurus*.

The presence or absence of phytochemicals in different plant samples can be attributed to geographical origin, enviromental factors and the storage of the plant material after harvesting (Van Wyk *et al* ., 2002; and Van Wyk and Gericke., 2003). From the plants studied, most of the leaves were rich in alkaloids flavonoids, tannins, saponins and steroids. They are known to demonstrate medicinal activity (Edeoga *et al.*, 2005) however the flavonoids and saponins concentration will differ among the selected plant species due to them belonging to different families hence giving them varying ability for biological activity

such as their anti-HIV activity. Kaul *et al.*, 1985 has demonstrated that the tannins and polyphenols such as flavonoids do inhibit viral reverse transcriptase.

Antioxidant activity of the leaves of *A. gangetica, B. pilosa*, C. *monophylla, C. triloba*, *D. cinerea* and *L. leonurus* is shown in Figure 54. Odhav, *et al* (2007) and Akula and Odhav., (2008), reported that the *A. gangetica, B. pilosa*, *C. monophylla, C. triloba* methanolic extracts displayed better radical scavenging activity than the aqueous extracts; the methanolc extracts displayed scavenging activity of greater than 80% whereas the aqueous was less than 40 %. In contrast to this study the aqueous extracts of *C. triloba*, *D. cinerea* and *L. leonurus* showed IC_{50} values of 25, 100, 300 µg/ml respectively compared to the methanolic extracts of *A. gangetica, B. pilosa*, and C. *monophylla* which showed IC_{50} values of 430, 380, 420 µg/ml respectively. Frum and Viljoen., (2006) noted that the methanolic extract of *L. leonurus* was far more effective antioxidant than the aqueous extract due to the fact that flavonoids and tannins are more readily soluble in methanol than in water. However the aqueous extracts of *L. leonurus* demostrated better anti-HIV activity. The antioxidant activity of plants from the family Lamiaceae was reported to be attributed mainly to the presence of phenolic compounds such as rosmaric acid, flavonoids and diterpenes, because of their ability to scavenge free radicals and active oxygen species such as singlet oxygen, superoxide anion radical and hydroxyl radicals (Erdemoglu *et al.*, 2006 and Nurgun *et al.*, 2006). Elevated levels of reactive oxygen species (ROS) have been established to be present at the onset of the HIV infection (Kashou and Agarwal., 2011). Therefore plant derived antioxidants may show a promising and cost-effective therapeutic approach in treating HIV-infected individuals (Cos *et al*., 2004).

From the 6 plants screened *D.cinerea* gave the highest SI value of 44.20, followed by *L. leonurus* with an SI value of 26.07, *C. monophylla* with an SI value of 10.92, *B. pilosa* with an SI value of 6.33, *C. triloba* with an SI value of 5.70, and *A. gangetica* with an SI value of 5.58. The anti-reverse transcriptase activities of extracts displaying significant activity are *C. monophylla*, *D. cinerea* and *L. leonurus* when compared to controls AZT.

B. pilosa belongs to the family Asteraceae. Bessong *et al.,* 2006 reported that *Venonia stipulacea* from the Asteraceae showed anti-HIV-RT activity in the acetyl acetate and n-butanol fraction. *C. monophylla* and *D. cinerea* are not known to be used traditionally and there is no literature of their anti HIV-activity. *D. cinerea* belongs to the family Fabaceae, Bessong *et al.*, 2005 reported that *Mucana coriacea* and *Surtherlandia fructescens* both from

the Fabaceae family showed anti-HIV RT activity. Klos *et al*., (2009) established that the ethanolic extract of *L. leonurus* possesses anti-HIV properties associated with anti- HIV-1 protease inhibition. In this study the aqueous leaf extract of *L. leonurus* displayed anti-HIV-1 RT activity. *L. leonurus* could be the key to a novel anti-HIV drug which inhibits both RT and PR inhibition. More research is required to confirm this activity.

Table 10 : Summary of results obtained

Plants	**Phytochemicals**	**Antioxidant activity (IC_{50} : µg/ml)**	**Selective Index**	**Anti- Reverse transcriptase activity**
A. gangetica	tannins, saponin, flavonoids, steroids and phenol	430	5.58	42%
B. pilosa	tannins, saponin, flavonoids, steroids, alkaloids and terpenoids	380	6.33	15%
C. monophylla	saponin, flavonoids, steroids alkaloids and phenol	420	10.92	31%
C. triloba	tannins, saponin, flavonoids, steroids and alkaloids	300	5.70	58%
D. cinerea	**tannins, saponin, flavonoids and alkaloids**	**25**	**44.20**	**82%**
L. leonurus	tannins, saponin, flavonoids and steroids	100	26.07	75%

A summary of the results obtained in this chapter is shown in Table 10. Based on this information, the plant with the best results from all activity screened was *D.cinerea.* It was found that *D.cinerea* had tannins, saponin, flavonoids and alkaloids present. *D.cinerea* had the highest; antioxidant activity with an IC_{50} of 25 µg/ml; anti-HIV-1 activity with a selective index of 44.20 and anti-reverse transcriptase activity of 82%. A survey of the literature showed that *D.cinerea* belongs to the family Fabaceae (Van Wyk and Gericke., 2003) and the predominant phytochemicals present in this family are flavonoids (Eisa *et al*., 1999). The family Fabaceae is characterized by impressive phytochemical diversity. One of the predominant flavonoids present is catechin (Raven *et al.*, 1999). Furthermore catechins have been shown to have anti-HIV activity by inhibiting reverse transcriptase (Nance *et al.,* 2008; Chinsembu and Hedimbi., 2009; Chinsembu and Hedimbi., 2010; Zhao et al., 2012; and Narayan *et al.*, 2013). Therefore, *D.cinerea* was investigated further for the identification of the active compound.

CHAPTER FIVE: IDENTIFICATION OF ANTI– HIV COMPOUND FROM *Dichrostachys cinerea*

5.1 INTRODUCTION

Numerous studies have been done on the isolataion of plant compounds which may be used in the treatment of HIV infection (Ng *et al.,* 1997; Cos *et al.,* 2004; Singh *et al.*, 2005; Chinsembu and Hedimbi., 2009; Singh *et al.,* 2011; and Narayan *et al.*, 2013). Recently Narayan *et al.*, (2013) reviewed compounds isolated from plants as potential anti-HIV drugs and this is shown in Table 11.

These isolated compounds are phytochemicals, usually secondary metabolites. Some of the most promising phytochemical compounds isolated are alkaloids, flavonoids, or terpenes. HIV inhibitory activities have been found in various types of alkaloids, example, Michellamines A and B isolated from the plant *Ancistrocladus korupensis,* which inhibit reverse transcriptase, late stage cellular fusion and syncytium formation (Yadav *et al.,* 2009). Some flavonoids have been shown to have HIV inhibitory potential and also in reducing oxidative stress (Cos *et al.*, 2004). Bioflavonoids, robustaflavone and hinokiflavone have been isolated from *Rhus succedanea* and displays inhibition of the polymerase of HIV reverse transcriptase (Narayan *et al.*, 2013). Celasdin B, a terpene has been isolated from *Celastrus hindsii* and demonstrated anti-HIV activity.

One of the most promising results shown by a compound isolated from a plant is 4-propyldipyranocoumarins (commonly know as Calanolides) from a tree from the tropical rainforest called *Calophyllum lanigerum* Miq. var. *austrocoriaceum* (Cos *et al.,* 2004; Singh *et al.*, 2005; Chinsembu and Hedimbi., 2009; and Narayan *et al.*, 2013). Calanolides compounds show potent activity against HIV-1 RT and display a unique sensitivity profile to nucleoside and nucleotide reverse transcriptase-resistant viruses and its synergistic effect with other anti-HIV drugs such as lamivudine, nelfinavir and AZT (Cos *et al.*, 2004). Currently Calanolides is the only plant derived compound to be evaluated in a clinical trial to determine its safety and pharmacokinetics in both healthy volunteers and HIV positive patients.

Table 11: Compounds isolated from plants as potential anti-HIV drugs adapted from a review article by Narayan *et al.*, 2013

Compound Isolated	Plant	Mode of action
Michellamines A and B	*Ancistrocladus korupensis*	Inhibits reverse transcriptase, late stage cellular fusion and syncytium formation
Bioflavonoids, robustaflavone and hinokiflavone	*Rhus succedanea*	Inhibition of the polymerase of HIV reverse transcriptase
Lectin	*Galanthus nivalis*	Stops the spread of HIV among lymphocytes by targeting gp 120 glycoprotein
Flavonoid gallate ester	*Acer okamotoanum*	Inhibits intergrase enzyme
Triptonine A and B	*Tripterygium hypoglaucum*	*In vitro* anti-HIV activity
Celasdin B		*In vitro* anti-HIV activity
Diterpene lactones	*Tripterygium wilfordii*	*In vitro* anti-HIV activity
Xanthohumol	*Humulus lupulus*	Inhibitory activity, induces cytopathic effect and reverse transcriptase activity
Dicaffeoyl-quinic acids	*Achyrocline satureioides*	Irreversible inhibition against intergrase
Protostanes, garcisaterpenes A and C	*Garcinia speciosa*	Inhibitory effect against reverse transcriptase
Ligins	*Inonotus obliquus*	Inhibits protease activity
Calanolide A and B	*Calophyllum sp*	Inhibitory activity, induces cytopathic effect and reverse transcriptase activity
Flavones – xanthone glycoside	*Swertia franchetiana*	Inhibitory effect against reverse transcriptase
Saponins and alkaloids	*Acacia auriculiformis*	RNA dependendant- DNA polymerase activity of HIV reverse transcriptase
Gallotannin	*Peltophorum africanum*	Inhibition of ribonuclease-H activity of reverse transcriptase
Gallic acid and galloyl glucose	*Terminalia chebula*	Inhibition of ribonuclease-H activity of reverse transcriptase and intergrase activity.
Guttiferone A	*Symphonia globulifera*	*In vitro* anti-HIV activity

Fabaceae is the second largest family of medicinal plants, and contains over 490 medicinal plant species, most of which have been used in traditional medicines. *D.cinerea* belongs to the family Fabaceae and there are 8 species that display varying degrees of anti-HIV activity, (Table 12). *Glycyrrhiza uralensis*, *Glycyrrhiza lepidota* and *Glycyrrhiza glabra*, which are all generally used in traditional medicines, have inhibitory effects on HIV replication *in vitro*.

Table 12 : Plants from the Fabaceae family that have displayed anti-HIV activity.

Species	Compound isolated	Mechanism of action	Reference
1. *Acacia auriculiformis*	Saponins and alkaloids	RNA dependendant-DNA polymerase activity of HIV reverse transcriptase	Narayan *et al.*, (2013)
2. *Castanospermum australe*	Castanospermine	Inhibition of HIV replication and syncytium formation	Singh *et al.,* (2005); Yadav *et al.*, (2009) and Chinsembu and Hedimbi ., (2010)
3. *Eriosema montanum*	-	*In-vitro* anti-HIV bioassay	Cos *et al.*, (2006)
4. *Glycyrrhiza glabra*	Glycyrrhizin	Inhibits HIV induced plaque formation in MT-4 cells	Singh *et al.,* 2011
5. *Glycyrrhiza lepidota*	Diprenylated bibenzyl-1	*In-vitro* anti-HIV bioassay	Singh *et al.,* (2005); and Yadav *et al.*, (2009)
6. *Glycyrrhiza uralensis*	Glycyrrhizin	Inhibitory effects on HIV replication in vitro	Singh *et al.,* (2005)
7. *Indigofera arrecta*	-	*In-vitro* anti-HIV bioassay	Cos *et al.*, (2002)
8. *Peltophorum africanum*	Flavonoids: Gallotannin, catechin and betulinic acid	Inhibition of ribonuclease HIV activity of reverse transcriptase	Chinsembu and Hedimbi ., (2009); Chinsembu and Hedimbi., (2010); and Narayan *et al.*, (2013)
9. *Sutherlandia frutescens*	Triterpenoids: L-canavanine and D-pinitol	HIV reverse transcriptase and In-vitro HIV bioassay	Van-Wyk and Albrecht., (2008); and Chinsembu and Hedimbi., (2010)

Acacia auriculiformis, *Peltophorum africanum* and *Sutherlandia frutescens* show reverse transcriptase activity. The compounds associated with this anti-HIV activity are saponins, alkaloids, flavonoids (gallotannin, catechin and betulinic acid), and triterpenoids (L-canavanine and D-pinitol).

Bio-guided fractionation is a procedure whereby plant extracts are chromatographically fractionated until a pure biologically active compound is isolated. Each fraction produced during the fractionation of the plant extract is tested in a bioassay system. The active compound is then identified and characterised. Bio-guided fractionation method is commonly employed in drug discovery research. It is efficient because it is able to link the identified compound with particular biological activity

Bio-guided fractionation was used to isolate and identify the active compound from *D. cinerea*. To isolate the single compound from the extract, two chromatographic techniques were employed; column chromatography resulted in co-eluted compounds therefore thin layer chromatography (TLC) was used. Compounds were tested for anti-viral and reverse transcriptase activity. From these results the active compound was identified, and bulked by preparative TLC and collected. The active compound was characterised by High Performance Liquid Chromatography (HPLC), Ultraviolet-visible spectrophotometry (UV-vis), Ultra Performance liquid chromatography coupled to MS/MS (UPLC-MS). Structure elucidation was performed using Nuclear Magnetic Resonance (NMR).

5.2 METHODOLOGY

The aqueous leaf extracts of *D. cinerea* has shown anti-HIV activity (Figure 55) and antioxidant activity (Figure 54), therefore only the aqeous extraction was used to isolate and characterize the active compound from *D. cinerea.*

5.2.1 Optimization of TLC for maximum fractions /compounds

Before performing Preparative Thin Layer Chromatography, various solvent systems were evaluated for best separation of the compounds present in the aqueous leaf extracts from *D.cinerea*. The optimization of TLC for maximum fractions were performed according to a methods described by Wagner *et al.*, 1984. Ten to fifteen micro-litres (10-15 µl) of a 100 mg /ml solution of *D. cinerea* was loaded on Merck TLC F_{254} / Silica gel 60 plates. The solution was spotted onto the baseline of a 10 cm X 10 cm TLC plate and allowed to dry. Before the plate was developed the TLC chamber was allowed to equilibriate for 1 h with the various solvent systems such as**:**

- Hexane- ethyl acetate (1: 9)
- Hexane- ethyl acetate (9: 1)
- Ethyl acetate-formic acid-glacial acetic acid -water (100:11:11:27)
- Toluene- ethyl acetate (93:7)
- Ethyl actetate- methanol- water(100:13.5:10)

After the plate had been developed, it was air dried and the bands were examined, firstly under ultraviolet light 254 and 360 nm (Camag, Universal UV lamp TL-600) and then sprayed with Vanillin sulphuric spray reagent (Amarowicz *et al.,* 2005). The TLC plate was sprayed with spray reagent after which it was heated at 110 °C in an oven for 5- 10 min to allow for development of colour changes (Carr and Rogers., 1987). Migration rates were calculated using the following equation:

$$RF = \frac{\text{Distance of sample from baseline}}{\text{Distance of solvent front from baseline}}$$

5.2.2 Preparative Thin Layer chromotography for *D. cinerea*

The crude extract of *D.cinerea* was separated on prepared TLC plates which were prepared on glass sheets (20cm × 20cm). To prepare a single plate, 6g of 60F silica gel G (Unilab, Saruchem, South Africa) was added to 15 ml of double distilled water to form a slurry which was mixed to ensure that all air bubbles were removed. It was then carefully poured onto a clean glass sheet. This was followed by air drying for 1 h till it formed a firm layer. This was then placed in an oven and allowed to be activated by baking each plate for ± 2 h at 110 °C.

The ethyl acetate-methanol- water (100:13.5:10) solvent system was the best solvent system for screening of plant phytochemicals from *D.cinerea*. About 60-85 µl of a 100 mg /ml solution of *D. cinerea* was spotted on the prepared TLC silica gel plates (20 cm X 20 cm). TLC was performed at ambient temperature after 1 h chamber saturation period. The run times of each plate was approximately 1 h. The developed plate was allowed to air dry and thereafter followed by the appropriate detection for development.

A small section on the side of the plate was sprayed with vanillin spray reagent and heated with a heating gun, whilst protecting the rest of the plate with foil. Components were easily visualized and marked. After spraying the plate with water to facilitate easier removal of components, the bands were scraped off the glass plate. This was repeated twenty times to obtain sufficient volume of concentrated compounds for further analysis. The components were collected into separate beakers and crushed to a fine powder using a glass rod. The isolated bands were desorbed with ethyl acetate-methanol (v/v).

The suspension was then sonicated for 30 sec and left to stand for 12 h. This was repeated twice for effective de-absorption of the active compound from the silica. This was followed by centrifugation at 4000 rpm for 20 min for the separation of the silica and the supernatant. The supernatant was then extracted using a pipette and filtered into vials using a 0.45 µm filter. These extracts were concentrated by allowing the solvents to be evaporated through air drying in a cool dark room under a laminar flow. The weight of isolated compounds was noted. These purified compounds were tested for anti-HIV activity.

5.2.3 Anti-HIV activity of isolated compounds

The isolated compounds were screened using, XTT assay (Weislow *et al.,* 1989), according to the method outlined in Chapter 3 (Section 3.2.3)

5.2.4 Reverse transcriptase activity of isolated compounds

The isolated compounds were screened using, Colorimetric Reverse Transcriptase Assay (Roche), according to the protocol outlined in Chapter 4 (Section 4.2.4)

5.2.5 Characterization of active compound by UV Vis Spectroscopy

Determination of the characteristic absorbances of the active isolated compound from the leaves of *D. cinerea* was achieved through spectrophotometric analysis according to Bark *et al.*, (2011). Commercial standards of catechin and epicatechin (Sigma-Aldrich, Inc) which were dissolved in methanol/ ethyl acetate (v/v) at a concentration of 10 µg/ml were used. The isolated compound was diluted in methanol/ ethyl acetate (v/v) at a concentration of 20 µg/ml. The isolated compound and standard were placed in a glass cuvette and analysed using a Varian-Carey 100 UV-Vis spectrophotometer. The samples were subjected to a UV scan in the 200-800 nm range. Relative absorbance's and shifts in spectrum for the isolated compound was recorded using Cary Win-UV programme. A glass cuvette containing distilled methanol/ ethyl acetate (v/v) was utilized as the reference (blank) during spectrophotometric analysis.

5.2.6 Characterization of active compound by HPLC

HPLC analysis was carried out according to the method of Benavides *et al.*, (2005) using commercial standards of catechin and epicatechin (Sigma-Aldrich, Inc) which were dissolved in acetonitrile (100 µg/ml). The active compound (100 µg/ml) was also dissolved in acetonitrile. Analyses were performed on a Merck- Hitachi LaChrom system (Darmstadt, Germany) consisting of a D7000 system controller, four pumps (D7400), a Merck- Hitachi LaChrom (L-7200) auto injector and an Merck- Hitachi LaChrom (L-7200) UV-VIS detector (λ = 280 nm). Separation of the analytes was performed at 25 ˚C on a Phenomenex Luna C18 column, 5 µm particle size, 250×4.6 (Merck, Darmstadt, Germany). The analytes were eluted isocratically at a flow rate of 1 ml/ min using an acetonitrile; (0.05%) triflouroacetic acid (pH 3.0) solution (83:17 v/v). The injection volume was 10 µl.

5.2.7 Characterization of active compound by UPLC-MS.

The UPLC separation was performed according to Facassetti *et al.*, (2011) with a Waters Acquity UPLC equipped with a binary solvent pump, an auto sampler and a photo array detector. The column was a BEH-C18 column (1.7 µm, 100 × 1.7 mm, Waters). The mobile phases consisted of A : water/ acetic acid (0.05% v/v) and methanol. The elution gradient is reported in Table 13.

Table 13: Solvent concentration in the eluting gradients

TIME (min)	A (%)	B (%)
0.0	90	10
5.0	83	17
7.5	70	30
8.5	65	35
8.8	0	100
9.3	90	10
11	90	10

Detection was carried out using a photo array detector at wavelength of 280 and 320 nm for the active isolated compound from the leaves of *D.cinerea.* The injection volume was 2 µl and the column was maintained at 25˚C

5.2.8 Characterization of active compound by 13C NMR

After UP-LC it was noted that the compound needed further purification. The sample was re-run on the Preparative Thin Layer chromotgraph. The samples were weighed and dissolved in maximum 2 ml deuterated solvents used for NMR [Merck]. In the studies, Deuterated methanol, CD_3OD was used as the solvent of choice, although other solvents were also attempted, because of its good ability to dissolve a wide range of compounds. The samples were then pipetted into NMR tubes with the aid of a Pasteur pipette and sent to of the Chemistry Department, University of Kwazulu Natal (Pietermaritzburg Campus). ^{1}H NMR was run at 400 MHz and ^{13}C at 75 MHz using the solvent signal tetramethylsilane (TMS), $(CH_3)_4Si$ as reference on a BRUKER Avance III NMR system. The spectra were interpreted.

5.3 RESULTS

5.3.1 Optimization of the separation technique of compounds from *D. cinerea*

Before performing Preparative Thin Layer Chromatography, various solvent systems were evaluated for best separation of the compounds present in the aqueous leaf extracts from *D.cinerea*. And the best mobile phase was of Ethyl actetate- methanol- water (100:13.5:10) (adapted from Wagner *et al*, 1984) sprayed with Vanillin sulphuric spray reagent. At the optimized conditions the TLC results showed the aqueous extracts of *D. cinerea* gave 7 distinct bands (Figure 56 D). In Figure 56 A; B; C the plant extracts did not move from the baseline.

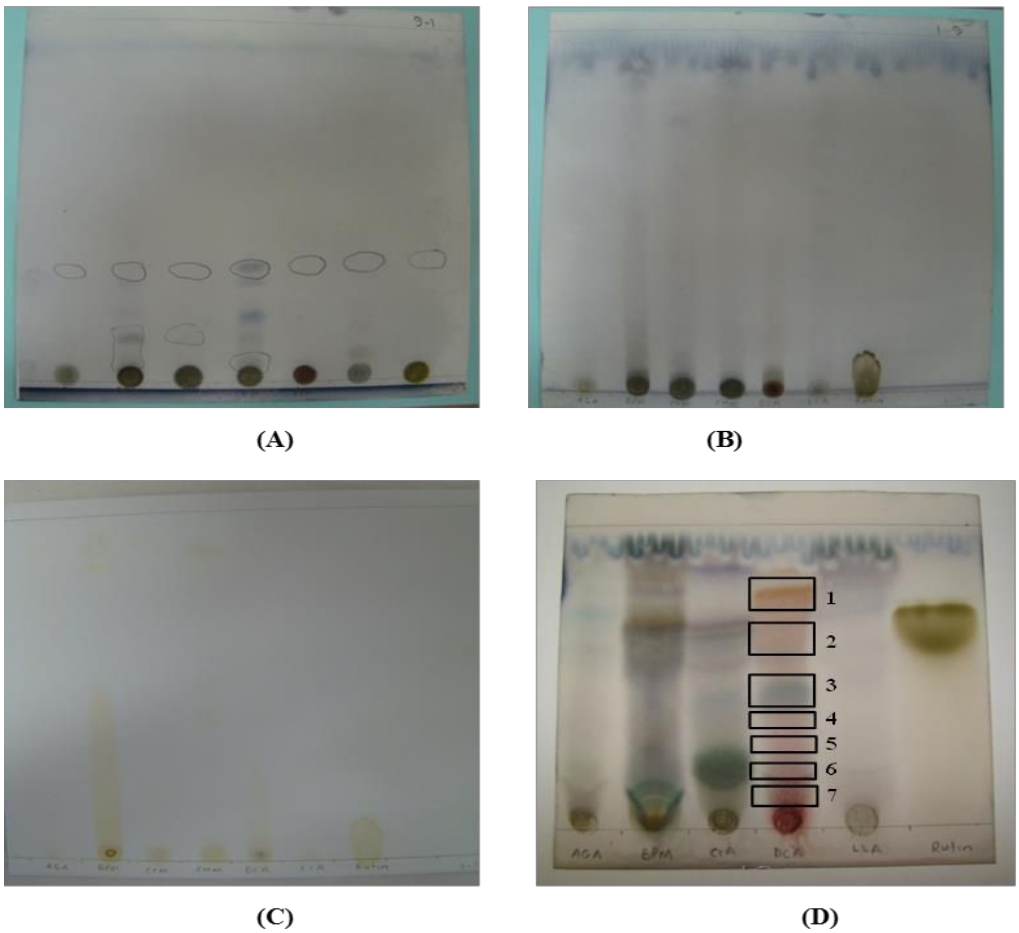


Figure 56: TLC of *A. gangetica, B. pilosa, C. monophylla, C. triloba, D. cinerea and L. leonurus* sprayed with Vanillin sulphuric spray reagent.

(A): Hexane- ethyl acetate (1: 9)
(B): Hexane- ethyl acetate (9: 1)
(C): Toluene- ethyl acetate (93:7)
(D): Ethyl acetate – methanol- water (100: 25: 15)

5.3.2 Isolation of compounds with Preparative Thin Layer chromatography

TLC of fractions (1-7) collected during preparative TLC of the aqueous leaf extract of *D. cinerea* and controls catechin and epicatechin are shown in Figure 57. The R_f values and description of the compounds isolated are listed in Table 14. The standard catechin displayed a darker pink/orange colour with the R_f value of 0.82 on the TLC plate after spraying with vanillin sulphuric spray reagent and this corresponds with the spot of the aqueous extracts for *D. cinerea.* These purified compounds were further analysed for anti-HIV activity and Reverse transcriptase activity

Table 14: R_f values of the bands isolated from *D. cinerea*

Band	Colour	R_f value
1	Pink/orange	0.82
2	Pinkish blue	0.73
3	Purple	0.61
4	Blue/brown	0.50
5	Grey/brown	0.46
6	Pink	0.43
7	Pink/red	0.25

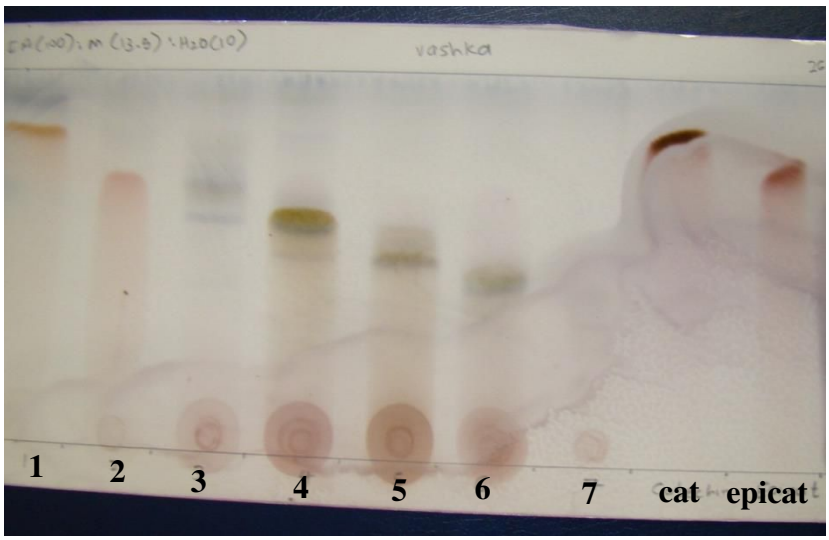


Figure 57: TLC of fractions (1-7) collected during preparative TLC of of *D. cinerea* and controls catechin and epicatechin.

5.3.3 Anti-HIV activity of isolated compounds

The 7 compounds isolated from the aqueous extract of *D.cinerea* were screened for anti-HIV activity and cellular cytotoxicity**.** Compound 1 demonstrated even stronger anti-HIV activity *in vitro* with the SI value of 90.44 compared to the crude plant extract which demonstated an SI of 44.20 (Table 9). Hence the isolated compound activity increased two-fold. Compound 2 did demonstrate weak anti-hiv activity but this could be attributed to poor isolatation of bands in the TLC plates, and could be residue of compound 1, as compound 1 is directly above compound 2. The 5 other compounds isolated did not demonstrate any anti-hiv activity.

Table 15: SI values of isolated compounds.

Compound/ Plant	[1] **EC $_{50}$ (ug/ml)**	[2] **CC $_{50}$ (ug/ml)**	[3]**SI**
Compound 1	4.5	407	90.44
Compound 2	115	375	3.26
Compound 3	n.a	146	-
Compound 4	n.a	441	-
Compound 5	n.a	449	-
Compound 6	n.a	264	-
Compound 7	n.a	257	-
Lopinavir	0.00102	32.75	32239.64

[a] **EC_{50}** = 50% effective inhibitory concentration

[b] **CC_{50}** = 50% cellular cytotoxicity concentration

[c] **SI** = selectivity Index (CC 50/ EC 50)

n.a = no anti- HIV activity

- = unable to calculate SI value

***n** = 3

5.3.4 Reverse transcriptase activity of the active compounds

The results for the average percentage HIV-1 RT inhibition for the isolated compounds can be seen in Figure 58. The percentage inhibition of control and extracts were calculated relative to uninhibited HIV-1 RT diluted in lysis buffer. All 7 plant extracts showed some degree of HIV-1 RT inhibition, but those which displayed ≥ 50 % HIV-1 RT inhibition were considered significant (Klos *et al.*, 2009). Compound 1 and 2 displayed signifcant HIV-1 RT activity, with a HIV-1 RT activity of 89% and 41% respectively. Compound 2 was not characterised further as it was assumed that it was possibly was mixed with Compound 1.

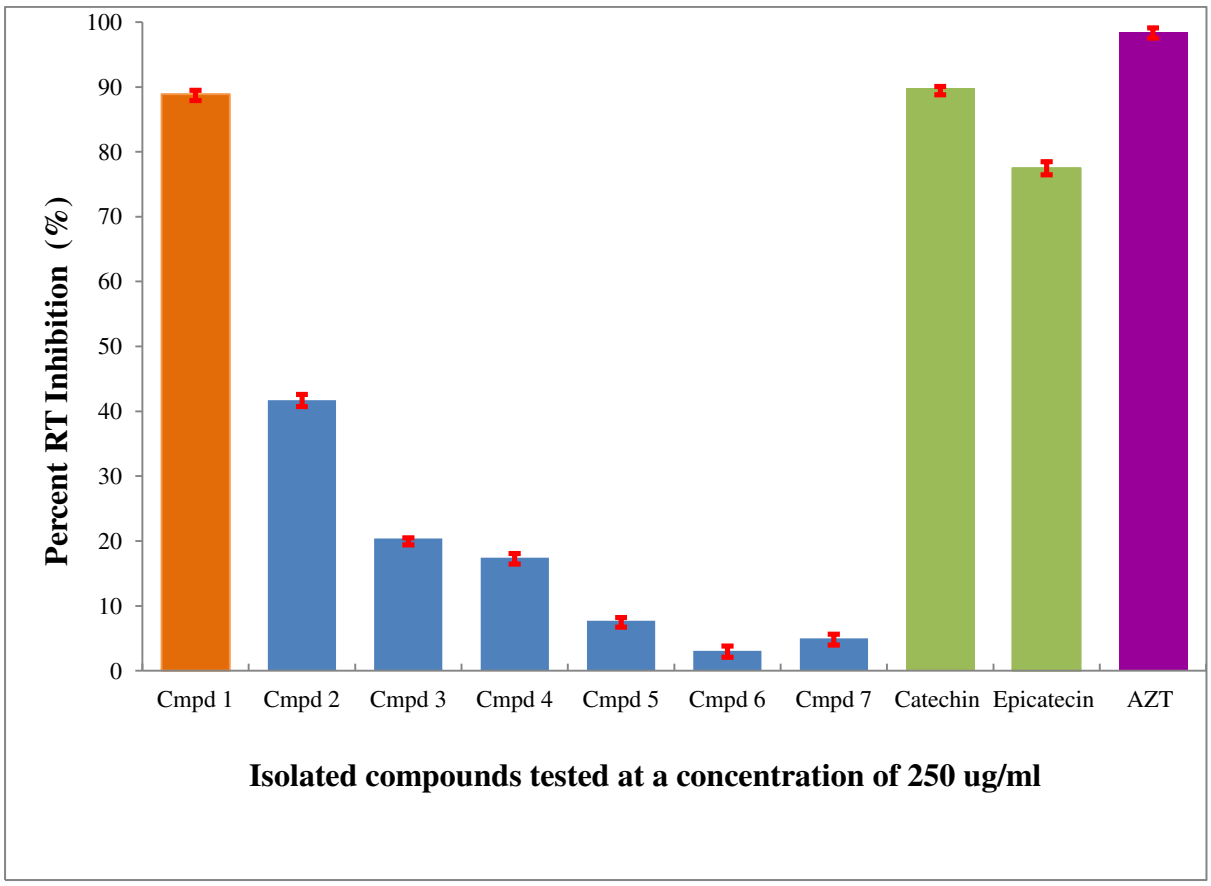


Figure 58: HIV-1 RT inhibition assay of the isolated compounds from *D.cinerea*.
The control was AZT, tested at 1mM solution.
(n = 3)

5.3.5 Characterization of active compound by UV-Vis Spectroscopy

The UV-vis spectroscopy was used to further confirm the presence of catechin in the isolated active compound. The UV-Vis absorption spectrum of isolated active compound overlaps with that of catechin which confirms the presence of catechin. The UV-vis absorption spectrum of isolated active compound displays a strong absorption at 288 nm (Figure 59) which corresponds with the peak of catechin also at 288 nm. The UV-vis absorption spectrum of epicatechin displays a strong absorption at 285 nm.

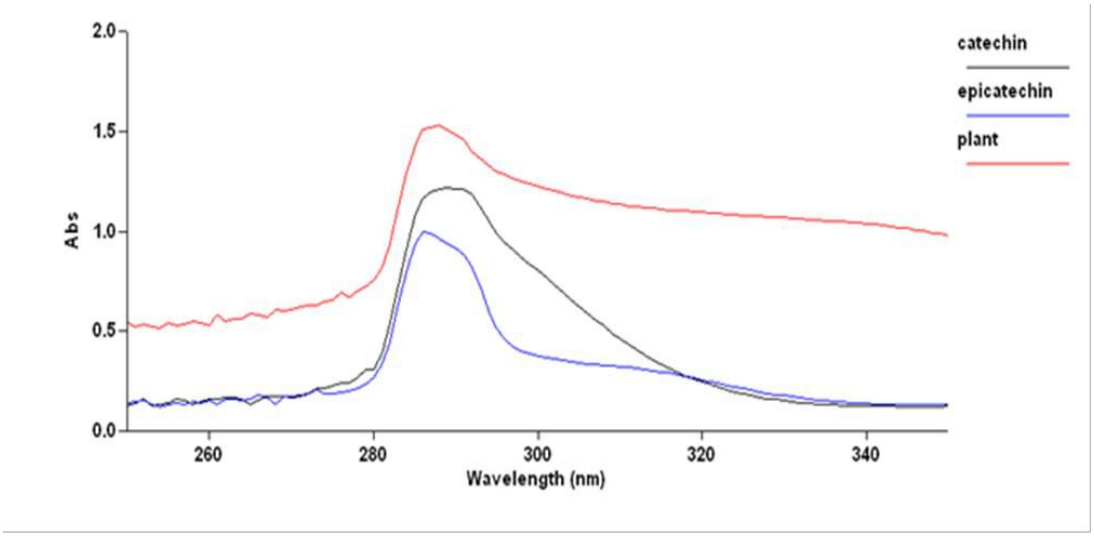


Figure 59: UV spectra of the active compound from *D.cinerea*

5.3.6 Characterization of active isolated compound by HPLC

In the HPLC analysis of active isolated compound from the leaves of *D.cinerea* attempts to distinguish between catechin and epicatechin (the closest in structure, that catechin could be confused with) were made. From the chromatograph it was noted that solvent of Phenomenex Luna C18 column and acetonitrile; 0.05% triflouroacetic acid (pH 3.0) solution (83:17, v/v) demonstrated good baseline resolution of the interested peaks. The retention time for catechin standard (Figure 61) was observed at t_R = 2.50 min and epicatechin standard (Figure 60) was observed at t_R = 2.44 min. The peak for catechin in the active isolated compound from the leaves of *D.cinerea* (Figure 62) was observed at retention time of t_R = 2.51 min using the same mobile phase gradient and instrumental parameters as for standards. In this way HPLC was able to corroborate the conclusion drawn from TLC and UV-vis anaylsis that the flavonoid presents in the active isolated compound from the leaves of *D.cinerea* is indeed catechin and not epicatechin.

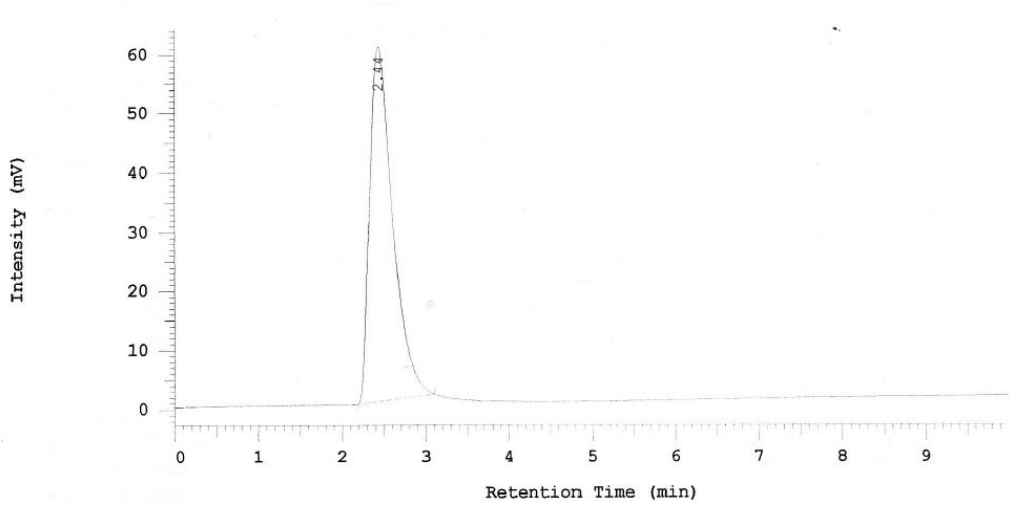


Figure 60: HPLC chromatograms showing epicatechin

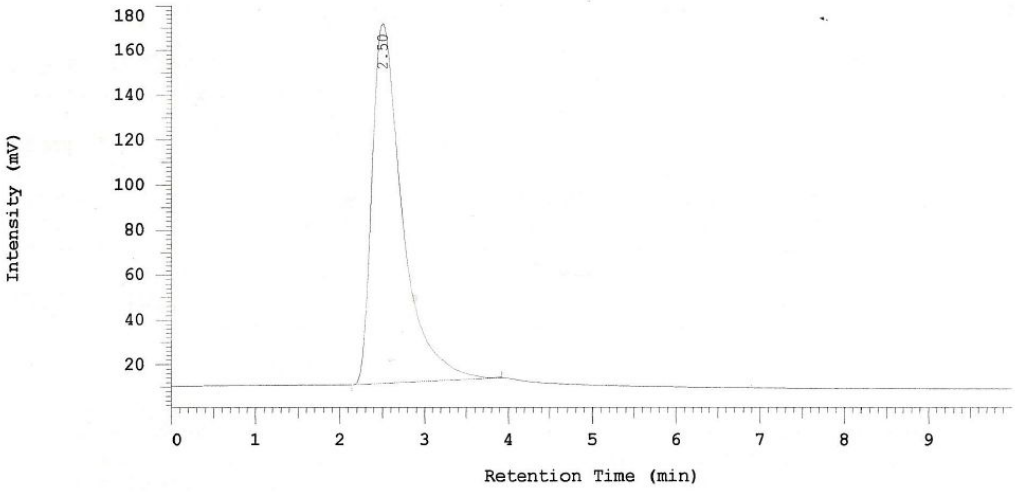


Figure 61: HPLC chromatograms showing catechin.

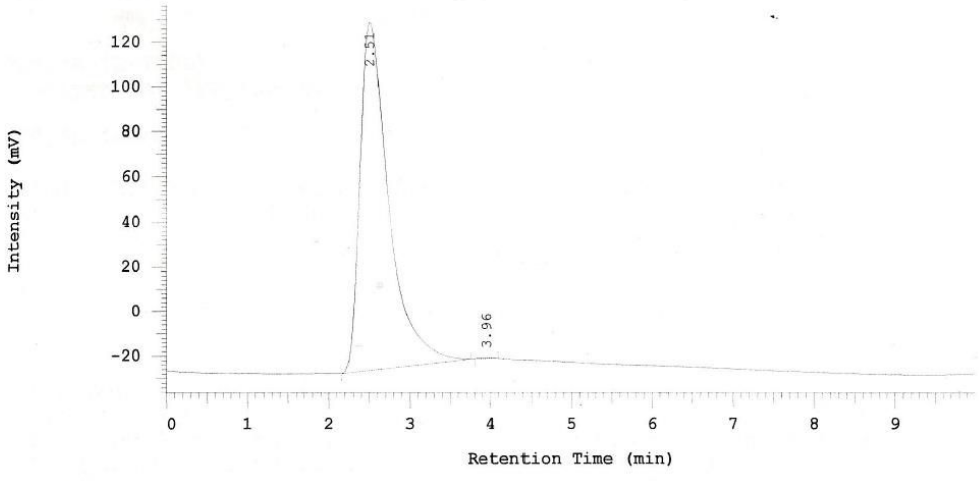


Figure 62: HPLC chromatograms of active isolated compound.

5.3.7 Characterization of active compound by UPLC-MS.

UPLC-MS data spectrum showed that the main peak that was eluted was catechin, since it had a molecular ion of *m/z* of 291 (ringed in blue). This was detected in the positive ion mode of the EPI scan. The fragmented ions of catechin are 273 (*m/z*) and 139 (*m/z*) respectively and are shown in green in Figure 63.

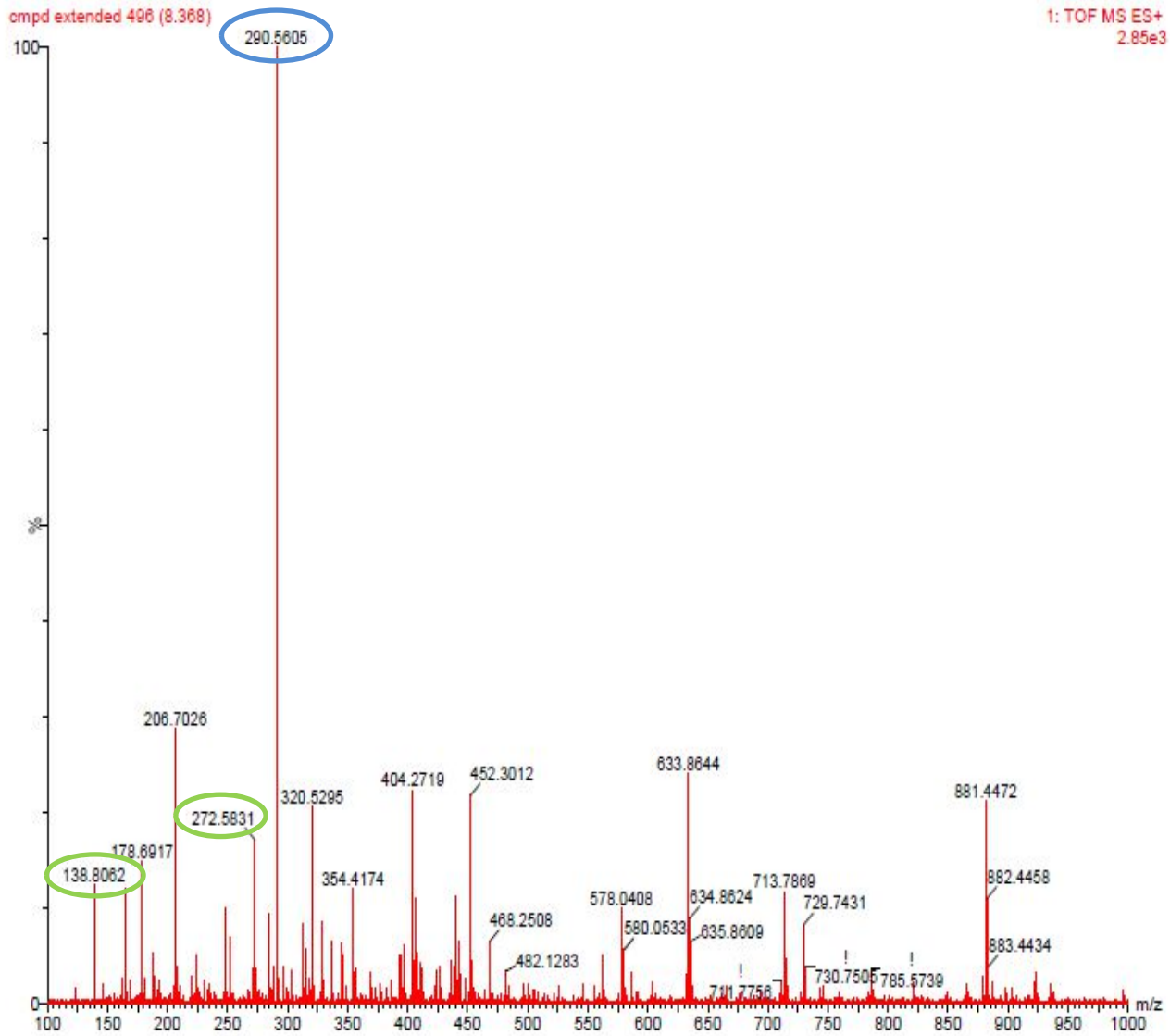


Figure 63: Mass spectrum of isolated compound from the leaves of *D.cinerea*

5.3.8 Characterization of active compound by 13C NMR spectroscopy

From the NMR results it was noted that the sample had a residue of ethyl acetate which showed up as peaks at (173.04), (61.56), (20.87), (14.47) and (30.74). From the resulting peaks which are (146.42), (129.78), (122.06) and (110.39), it was concluded that the compound is a pyrogallol as shown in Figure 64.

.

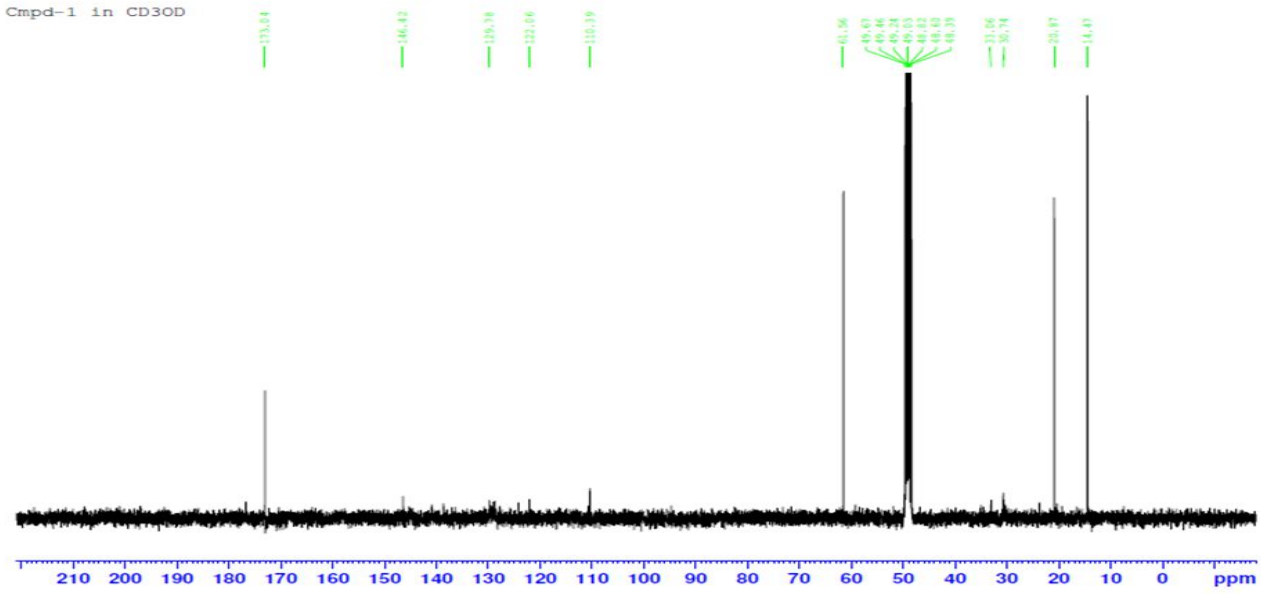


Figure 64: NMR Carbon 13 spectrum of active isolated compound from the leaves of *D.cinerea*

Catechin and pyrogallol are related. When catechin isolated from a plant material goes through dry distillation (processes such as heating above its decomposition point), pyrogallol is formed (Prabhu *et al*., 2012). The sample was expanded in the UPLC-MS data spectrum, and the peak of pyrogallol identified, since it had a molecular ion of *m/z* of 126 (ringed in orange).

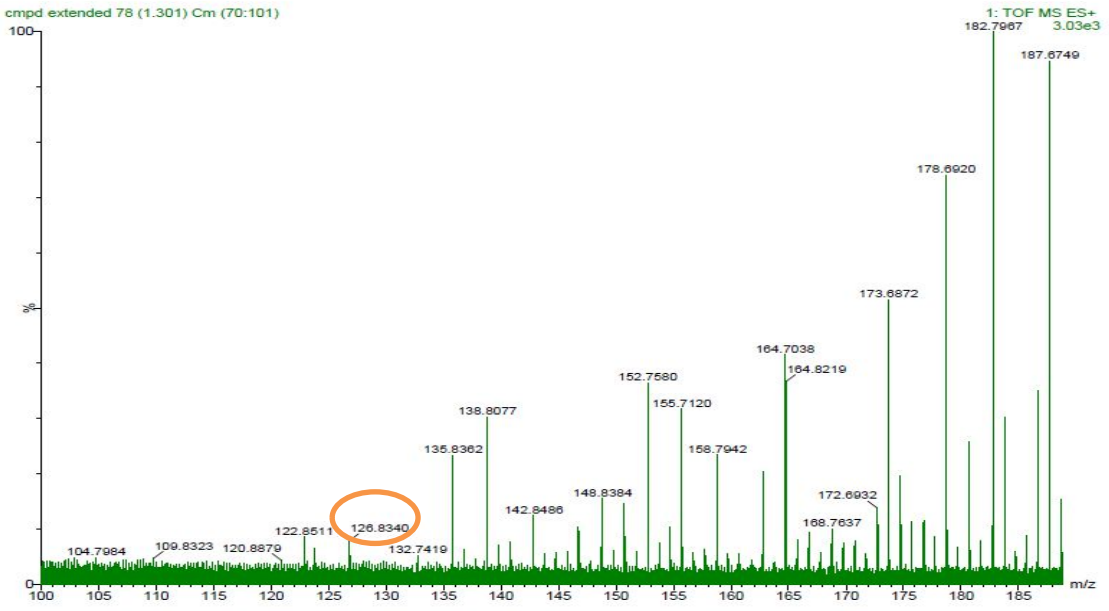


Figure 65: Mass spectrum of active isolated compound from the leaves of *D.cinerea* expanded

5.4 DISCUSSION

Bio-guided fractionation was used to isolate and identify the active compound from aqueous extracts of the leaves of *D. cinerea*. TLC plates, showed that the aqueous extracts of *D. cinerea* gave 7. Band 1 was most pronounced with an intense pink/orange colour spot when sprayed with vanillin sulphuric spray reagent. The standard of catechin displayed a darker pink/orange colour with the R_f value of 0.82 on the TLC plate after spraying with vanillin sulphuric spray reagent. This corresponds with Band 1, R_f value of 0.82, from the aqueous extracts of *D. cinerea* (Figure 57). The colour and R_f of catechin was also reported by Maoela *et al.*, (2009) where catechin was isolated from *Carpobrotus quadrifidus*. The purified compounds Band 1-7 were further analysed for anti-HIV activity and reverse transcriptase activity and this led to the identification of the active compound being Band 1.

Active compound was further analysed by UV-vis, HPLC, UP-MS and NMR. The UV-vis spectroscopy was used to further confirm the presence of catechin in the active compound. The Uv-Vis absorption spectra of isolated active compound overlapped with that of catechin which confirmed the presence of catechin. The isolated active compound displays a strong absorption at 288 nm (Figure 59) and this corresponds with the peak absorption of catechin standard also at 288 nm. The UV-vis absorption spectrum of epicatechin displayed a strong absorption at 285 nm. Amarowicz *et al*., (2003) and Amarowicz *et al.,* (2005) confirmed the presence of catechin from green tea with TLC analysis using vanillin sulphuric spray reagent and UV spectral data in which, catechin gave UV absorption at 288 nm and epicatechin 278 nm. Maoela *et al*, (2009) also reported that catechin isolated from *Carpobrotus quadrifidus* when dissolved in ethylacetate shows a strong absorption band at 288 nm also. Many other researchers Amarowicz *et al*., (2003); Amarowicz *et al.,* (2005); Hye *et al.*, (2009); and Maoela *et al.*, (2009), have all shown that catechin and related compounds display a UV around the 285 nm region in the UV-vis spectrum. From the UV-vis spectrum and literature it can be deduced that the active compound that was isolated is most likely a catechin.

HPLC analysis of active compound (Figure 62) attempts to distinguish between catechin (Figure 61) and epicatechin (Figure 60); the closest in structure, that catechin could be confused with, were made. The retention time for catechin standard was observed at t_R = 2.50 min and epicatechin standard was observed at t_R = 2.44 min. The peak for catechin in the active isolated compound was observed at retention time of t_R = 2.51 min using the same

mobile phase gradient and instrumental parameters as for standards. In this way HPLC was able to corroborate that the conclusion drawn from TLC and UV-vis anaylsis that the flavonoid presents in the active isolated compound from the leaves of *D. cinerea* is indeed catechin.

The UPLC-MS results showed the peak eluting was identified as catechin, since it had a molecular ion of *m/z* of 291, and this was detected in the positive ion mode of the EPI scan (Hye *et al.*, 2009; Srivastava *et al*., 2010; Chau *et al.*, 2011; and Francisco *et al*., 2011). Hye *et al.*, (2009) and Chau *et al.*, (2011) and reported that the protonated and fragmented ions of catechin are 291 (*m/z*), 273 (*m/z*) and 139 (*m/z*). These fragmentation ions at 273 (*m/z*) and 139 (*m/z*) result from a respective loss of 18 Da (291-273) and 152 Da (291-139) respectively. Benavides *et al*., (2005) and Chau *et al.*, (2011), both have reported that catechin produces *m/z* of 139 as a characteristic ion in the positive ion mode with the loss of a 152 Da in a *Retro Diels Reaction*. This is summarized in Table 16. Zeeb *et al.*, (2000), reported that the presence of the *m/z* 139 established that the compound is catechin and that the A-ring was unmodified.

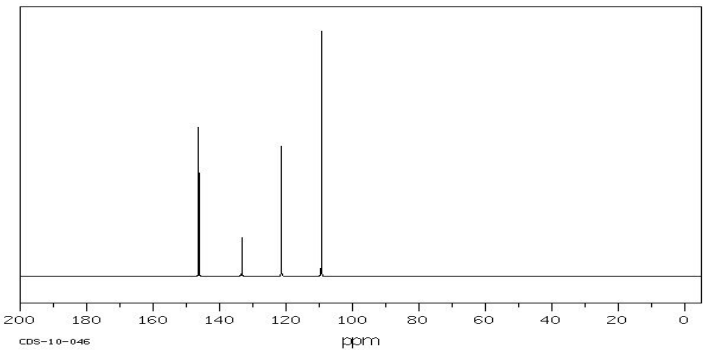
Table 16: Molecular and fragment ions of catechin in *D.cinerea* after UPLC-MS

Positive ionization [a,b]				
Compound	**Molecular weight**	**MS1 $[M+H]^+$ (*m/z*)**	**MS 2 Fragment ions (*m/z*)**	
Catechin	290	291	273	139

*([a] Chang *et al* 2011 and [b] Chua et al, 2011)

It was noted that the sample needed to be further purified before NMR. Samples were purified through re-running on TLC and isolating the active band. This was then screened with NMR. The NMR results showed that the sample had a residue of ethyl acetate which showed up as peaks at (173.04), (61.56), (20.87), (14.47) and (30.74). From the resulting peaks which are (146.42), (129.78), (122.06) and (110.39), it was concluded that the compound is a pyrogallol. This was done by reference to a spectral database: Spectral Database for Organic Compounds SDBS, 2013 (www.riodb01.ibase.aist.go.jp). The sample was then re-run on LC-MS looking for the molecular weight of pyrogallol which is 126.1 (*m/z*), shown in Figure 65.

SPECTRUM : 13C NMR of C6 H6 (pyrogallol)



ppm	Int.	Assign.
146.26	605	1
133.29	155	2
121.41	530	3
109.34	1000	4

Figure 66 : SPECTRUM of C6 H6: pyrogallol (www.riodb01.ibase.aist.go.jp)

Pyrogallol is a colorless compound that occurs naturally in trace amounts (Harborne., 1973). It was first discovered by destructive distillation of the plant extracted catechin, by heating catechin above its decomposition point, "pyrogallol" is formed (Prabhu *et al*., 2012). Therefore it was deduced the compound isolated from *D.cinerea* was indeed a catechin.

The initial screening of aqueous extracts of *D.cinerea* displayed very promising results. The aqueous leaf extracts were not toxic, and displayed a CC_{50} of 302 µg/ml and showed good anti-viral activity EC_{50} of 6.9 µg/ml and SI of 43.5. These results prompted an investigation of the active constituents of the aqueous extract of *D. cinerea*. This led to the isolation of 7 main fractions/ compounds which were tested for *in vitro* anti-HIV activity and screened for mechanism of action by evaluating the anti-reverse transcriptase activity. The active compound 1 was identified as catechin. When the isolated compound was compared to the commercially purchased catechin the anti-RT activity was similar with 89 % and 90 % respectively. The positive control for the anti-RT activity was AZT which showed inhibition of 99 %.

From the results it can be deduced that the isolated compound, catechin displayed stronger anti-HIV activity when compared to the crude plant extract of *D.cinerea.* In the *in-vitro* screening the EC_{50} decreased from 7 µg/ml in the case of the crude extract to 4.5 µg/ml in the isolated compound. In the reverse transcriptase activity there was a ± 8 % increase from the crude plant extracts in comparsion to the isolated compound, catechin, which was 82% and 89% respectively.

The inhibitory nature of catechin against HIV-1 has been well studied, however the selectivity and mechanistic action have not been investigated when isolated from *D. cinerea.* Chang *et al.*, (1992); Tommasi *et al.*, (1998); Nance *et al.*, (2008); and Zhao *et al.*, (2012) have shown that catechin and catechin derivatives are important natural antioxidants and are capable of inhibit HIV-1. Chang *et al.*, (1992) reported that catechin and catechin derivatives isolated from green tea were found to inhibit the activities of cloned human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT). Nance *et al.*, (2008); and Zhao *et al.*, (2012) stated that catechin has been shown to inhibit not only reverse transciptase activity of HIV-1 but also:

i. Blocks the binding of gp 120 protein to CD_4;
ii. inhibits p24 antigen;
iii. inhibits intergrase activity;
iv. inhibits protease activity; and
v. reduce oxidative stress caused by HIV-1 infection

Catechins anti-HIV activity is well documented. The presence of catechin has never been reported in the aqueous leaf extract *D. cinerea.*

CHAPTER SIX: CONCLUSION

South Africa has a wide array of medicinal plants, and in literature plant isolated compounds have been proven to be used for the treatment of a wide spectrum of diseases. Due to the emergence of drug resistance and side effects of current available drugs for the treatment of HIV-1, greater effort is being put into the search for novel and effective anti-HIV treatment. Therefore the identification and screening of medicinal plants with potential as anti-HIV compounds is justified.

A total of 108 plant extracts from 38 plant species were investigated for cellular cytotoxicity and antiviral activity against HIV-1 (Table 6). Most of the extracts did not exhibit cellular cytotoxicity to the MT- 4 cells, even at the maximum concentration tested (500 µg/ml). The methanolic extracts of *Physalis viscosa* (leaves) and *Solanum nodiflorum* (roots) were toxic at dose dependent manner ie as concentrations increase so did cellular cytotoxicity. From the 108 extracts tested 30 extracts exhibited varying degrees of anti-HIV activity (SI value > 5). However aqueous extracts of *D. cinerea* and *L. leonurus* demonstrated the best anti-HIV activity, indicating that the active compounds are hydrophilic in nature. *D. cinerea* and *L. leonurus* aqueous extracts showed strong anti-HIV activity with SI of 43.5 and 23.5, respectively. *D. cinerea* is not known to be used traditionally as a treatment of viral diseases and there is no literature of its anti HIV-activity. *L. leonurus* is used traditionally and is reported in literature to demonstrate anti-HIV activity. In this study it was used a positive control to validate the methodology, and *D.cinerea* was found to be deserving of further investigation.

Analysis of phytochemicals and antioxidant activity of the leaves of *A. gangetica, B. pilosa, C. monophylla, C. triloba, D. cinerea and L. leonurus* was investigated. The phytochemical analysis of the leaves of *A. gangetica, B. pilosa, C. monophylla, C. triloba, D. cinerea* and *L. leonurus* (Table 8), showed that the leaves were rich in alkaloids flavonoids, tannins, saponins and steroids. Flavonoids and saponins were present in all plants tested, and are known to demonstrate medicinal activity. However the flavonoids and saponins concentrations will differ among the selected plants due to them belonging to different families therefore giving them varying ability for biological activity such as their anti-HIV activity. *D. cinerea* displayed the presence of tannins, saponin, flavonoids and alkaloids and did not contain any phlobatannins, terpenoids, steroids or phenol. The antioxidant activity of

the leaves of *A. gangetica, B. pilosa, C. monophylla, C. triloba, D. cinerea* and *L. leonurus* is displayed in Figure 54. The highest degree of free radical scavenging activity was displayed by *D.cinerea* with an IC_{50} of 25 µg/ml. *D. cinerea* displayed potential as an antioxidant, and may show promise as a cost effective therapeutic agent in treating HIV-infected individuals with oxidative stress which has been linked to the progression of HIV to AIDS.

Bio-guided fractionation was used to isolate and identify the active compound from the extract. To isolate and identify the single active compound from the extract, thin layer chromatography was used. The results showed that the aqueous extracts of *D. cinerea* gave 7 distinct bands which where visible when sprayed with Vanillin sulphuric spray reagent. The 7 bands were simultaneously screened for anti-HIV activity and identification. Band 1 was found to be the most active compound.

After the isolation of the compound, analysis of the compound was performed using Ultraviolet-visible spectrophotometry, the Uv-vis absorption spectra of isolated active compound overlapped with that of catechin which confirmed the presence of catechin. The isolated active compound displayed a strong absorption at 288 nm (Figure 59) and this corresponded with the peak absorption peak of catechin standard also at 288 nm.

The HPLC analysis of active isolated compound, attempts to distinguish between catechin and epicatechin were made. The retention time for catechin standard was observed at t_R = 2.50 min (Figure 60) and epicatechin standard was observed at t_R = 2.44 min (Figure 61). The peak for catechin in the active isolated compound was observed at retention time of t_R = 2.51 min (Figure 62) the same instrumental parameters was used. In this way HPLC was able to corroborate the conclusion drawn from TLC and UV-vis analysis that the flavonoid presents in the active isolated compound from the leaves of *D.cinerea* is a catechin and not a epicatechin. From the UPLC-MS results, catechin was identified , as it had a molecular ion of *m/z* of 291 This was detected in the positive ion mode of the EPI scan, the protonated and fragmented ions of catechin 291 (*m/z*) are 273 (*m/z*) and 139 (*m/z*) as shown in Figure 63
After UPLC-MS the sample was further purified, before structure elucidation was performed using Nuclear Magnetic Resonance. The resulting peaks which are (146.42), (129.78), (122.06) and (110.39), suggests that the compound is a pyrogallol. Catechin and pyrogallol are related. When catechin isolated from a plant material goes through destructive distillation

and processes such as heating above its decomposition point, pyrogallol is formed. Therefore it was deduced the compound isolated from *D. cinerea* was indeed a catechin.

D. cinerea showed HIV-1 reverse transcriptase inhibition, of 82%. The isolated compound catechin showed 7% increase, to 89 %. Both the extract and isolated compound displayed promising results *in vitro* to be developed into new drugs for the treatment of HIV-1, but more research on the effects *in vivo* is essential.

This is the first report of the isolation of catechin from the aqueous leaves extract of *D. cinerea*. Catechin isolated from *D. cinerea* show anti-HIV activity because of its :

- Ability to inhibit viral proliferatation *in-vitro* by disrupting HIV lifecycle through inhibition of reverse transcriptase.
- Ability to reduce oxidatative stress therefore decreasing the rate at which HIV progresses to AIDS.

Potentially, these results can be used to develop a new drug for the treatment of HIV. There is scope for future work to evaluate the diferences between catechin isolated from *D. cinerea* and from tea.

REFERENCES

Akah P.A., Ezike A.C., Nwafor S.V., Okoli C.O., and Enwerem N.M. 2003. Evaluation of the anti-asthmatic property of *Asystasia gangetica* leaf extracts. Journal of Ethnopharmacology. Vol 89, pp 25-36.

Akula U., and Odhav B., 2008. In vitro 5- Lipoxygenase inhibition of polyphenolic antioxidants from undomesticated plants of South Africa. Journal of Medicinal Plants Research. Vol 2(9), pp. 207-212.

Amarowicz R., Shahidi F., and Wiczkowski W. 2003. Separation of individual catechins from green tea using silica gel column chromatography and HPLC. Journal of Food Lipids Vol 10, pp 47-56.

Amarowicz R., Maryniak A., and Shahidi F. 2005. TLC Separation of methylated (-)-Epigallocatechin -3-Gallate. Czech. Journal of Food Science. Vol 23(1), pp 36-39.

Auld B.A., and Meld R.W. 1992. 'Weeds: An illustrated botanical guide to the weeds of Australia.' . Inkata Press: Melbourne.

Bark K.M., Yeong J.E., Yang J.I., Yang I.K., Park C.H., and Park H.R. 2011. Spectroscopic studies on the oxidation of Catechin in Aqueous Solution. Korean Chemical Society. Vol 32 (9), pp 3343-3347.

Bedoya L.M., Palomino S.S., Abad M.J., Bermejo P., and Alcami J. 2001. Anti-HIV activity of medicinal plant extracts. Journal of Ethanopharmacology. Vol 7(1), pp 113-116.

Bedoya L.M., Sanchez-Palomino S., Abad M.J., Bermejo P., and Alcami J., 2002. Screening of selected plant extracts on human immunodeficiency virus. Phytotherapy Research. Vol 16 (2), pp 550-554.

Bedoya L.M., Alvarez A.,Bermejo M., Gonzalez N., Beltran M., Palomino S.S., Cruz S.M., Gaitan I., Olmo E.D., Escarcena R., Garcia P.A., Caceres A.S.F., and Alcami J. 2008 Guatemalan plant extracts as virucides against HIV-1 infection. Phytomedicine. Vol 15, pp 520-524.

Beekrum S. 2003. Nutritional value of indigenous plants for human consumption. Master's degree in Technology: Food Technology dissertation, Department of Food Technology, Durban Institute of technology , Durban, South Africa.

Benavides B., Montoro P., Bassarello C., Piacente S., and Pizza C. 2005. Catechin derivatives in *Jatropha macrantha* stems: characterization and LC/ESI/MS/MS quali-quantitative analysis. Journal of Pharmaceuticals and Biomedical Analysis. Vol 40, pp 639-647.

Bessong P.O., Obi C.L., Igumbor E., Andreola M.L., Litvak S. 2004. *In vitro* activity of three selected South African medicinal plants against human immunodeficiency virus type 1 reverse transcriptase. African Journal of Biotechnology. Vol 3(10), pp 555-559.

Bessong P.O., Obi C.L., Andréola M.L., Rojas L.B., Pouységu L., Igumbor E., Meyer J.J.M., Quideau S., and Litvak S. 2005 Evaluation of selected South African medicinal plants for inhibitory properties against human immunodeficiency virus type one reverse transcriptase and integrase, Journal of Ethnopharmacology. Vol 99(1), pp 83-91.

Bessong P.O., Rojas L.B., Obi L.C., Tshisikawe P.M., and Igunbor E.O. 2006. Further screening of Venda medicinal plants for activity against HIV type 1 reverse transcriptase and integrase, African Journal of Biotechnology. Vol 5(6), pp 526-528.

Bessong P.O., and Obi C.L. 2006. Review: Ethno-pharmacology of human immundeficiency virus in South Africa. African Journal of Biotechnology. Vol 5 (19), pp 1693-1699.

Bot Y.S., Mgbojkwe L.O, Nwosu C., Abimiku A., Dadik J., and Damshak D. 2007. Screening of the fruit pulp extract of Momordica balsamina for anti-HIV property. African Journal of Biotechnology. Vol 6(1), pp 47-52.

Carr J.D., and Rogers C.B. 1987. Chemosystematic studies of the genus *Combretum* [Combretaceae], I. A convenient method of identifying species of this genus by a comparison of the polar constituents extracted from leaf material. South African Journal of Botany. Vol 53(1), pp 173–176.

Chakraborty A., Brantner T., Mukainaka Y., Nobukuni M., Kuchide T., Konoshima H., and Nishino H. 2002. Cancer chemopreventive activity of *Achyranthes aspera* leaves on Epstein-Barr virus activation and two-stage mouse skin carcinogenesis. Cancer Letters. Vol 177 (1), pp 1-5.

Chang C. W, Hsu F.L., and Lin J.Y.1992. Inhibitory Effects of Polyphenolic Catechins from Chinese Green Tea on HIV Reverse Transcriptase Activity. Journal of Biomedical Science, Vol 1 (3), pp 163-166.

Chang C. L., Wu R.T., and Aziz R.A. 2011. Quantification of (+)-catechin and (-)epicatechin in coconut water LC-MS. Journal of Food chemistry. Vol 126 (1), pp 710-717.

Changa, S.B., Chianga Y.M., Changa C.L.T., Yeha H.H., Shyura L.F., Kuoc Y.H., Wub T.K., and Yanga W.G. 2007. Flavonoids, centaurein and centaureidin, from Bidens pilosa, stimulate IFN-γ expression. Journal of Ethno-pharmacology. Vol 112 (2), pp 232–236.

Chinsembu K.C., and Hedimbi M., 2009. Model and experiences of initiating collaboration with traditional healers in validation of ethnomedicines for HIV/AIDS in Namibia. Journal of Ethnobiology and Ethnomedicine. Vol 5 (30), pp 567- 576.

Chinsembu K.C., and Hedimbi M., 2010. Full Length Research Paper: Ethno-medicinal plants and other natural products with anti-HIV active compounds and their putative modes of action. International Journal for Biotechnology and Molecular Biology Research. Vol 1 (6), pp 74–91.

Choi C.W., Kim S.C., Hwang S.S., Choi B.K., Ahn H.J., Lee M.Y., Park S.H., and Kim S.K. 2002. Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay guided comparison. Plant Science. Vol 163(1), pp 1161-1168.

Chiang Y.M., Chuang D.Y., Wang, S.Y., Kuo Y.H., Tsai P.W., and Shyur L.F. 2004. Metabolite profiling and chemopreventive bioactivity of plant extracts from *Bidens pilosa.* Journal of Ethnopharmacology. Vol 9, pp 409-419.

Chua L.S., Latiff N.A., Lee S.Y., Lee C.T., Sarmidi M.R., and Aziz R.A. 2011. Flavonoids and phenolic acids from *Labisia pumila* (Kacip Fatimah). Journal of Food Chemistry. Vol 127 (10), pp 1186-1192.

Clavel F., Guétard D., Brun-Vézinet F., Chamaret S., Rey M.A., Santos-Ferreira M.O., Laurent A.G., Dauguet C., Katlama C., Rouzioux C. 1986. Isolation of a new human retrovirus from West African patients with AIDS. Science. Vol 18(233)-4761, pp 343-346.

Clercq De E. 1995. Reviews: Antiviral therapy for human immunodeficiency virus infections. Clinical microbiology Reviews. Vol 8 (2), pp 200-239.

Cos P., Hermansa N.,De Bruynea T ., Apersa S., Sindambiwea J.B., Witvrouwb M., De Clercqb E., Vanden Berghea D., Pietersa L., and Vlietincka A.J. 2002. Antiviral activity of Rwandan medicinal plants against human immunodeficiency virus type-1 (HIV-1). Phytomedicine. Vol 9 (1), pp 62–68.

Cos P., Maes L., Berghe D.V., Hermans N., Pieters L., and Vlietinck A. 2004. Plant substances as anti-HIV agents selected according to their putative mechanism of action. Journal of Natural Products. Vol 67 (2), pp 284-293.

Cos P., Vlietinck A.J., Vanden Berghe D., and Maes L., 2006. Review: Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'. Journal of Ethnopharmacology. Vol 106 (3), pp 290-302.

Dekker T.G., Fourie T.G., Elmare M., Snykers F.O., and Van der Scgyf C.J. 1988. Studies of South African plants Part 7: Rhinocerotinoic acida labdane diterpene with anti-inflammatory properties from *Elytropappus rhinocerotis*. South African Journal of Chemistry. Vol 41, pp 33-35.

Demel T, and Teketay D. 1996. The effect of different pre-sowing seed treatments, temperature, and light on the germination of five *Senna* species from Ethiopia. New Forests. Vol 11 (2), pp 155-171.

Department of Health. 2013. The South African antiretroviral treatment guidelines: Date of Implementation 1 April 2013. Last accessed: August 2013. http://www.doh.gov.za/docs/policy/2013/ART_Treatment_Guidelines_Final March2013.pdf

Dimo T., Azay J., Tan P.V., Pellecuer J., Cros G., Bopelet M., and Serrano J.J.2001. Effects of the aqueous and methylene chloride extracts of *Bidens pilosa* leaf on fructose-hypertensive rats. Journal of Ethnopharmacology. Vol 76 (3), pp 215-21.

Dold A.P., and Cocks M.L. 2000. The trade in medicinal plants in the Eastern Cape Province, South Africa. South African Journal of Science. Vol 98 (1), pp 234 -252.

Duke J.A. 1992. Handbook of Edible Weeds. CRC Press, London.

Edeoga H.O., Okwu D.E., and Mbaebie M. 2005. Phytochemical constituents of some Nigerian medicinal plants. African Journal of Biotechnology. Vol 4 (7), pp 685-688.

Eisa M.M., Almagboul A.Z., Omer M.E.A., and Elegami A.A. 1999. Antibacterial activity of Dichrostachys cinerea. Fitoterapia. Vol 71, pp 314-327.

Elkhayat E.S. 2009. Cytotoxic and Antibacterial Constituents from the Roots of *Sonchus oleraceus* L. Growing in Egypt. Pharmacognosy Magazine. Vol 5 (1), pp 324-328.

Erdemoglu, N., Turan, N.N., Cakõcõ, I., Sener, B. and Aydõn, A. 2006. Antioxidant activities of some Lamiaceae plant extracts, Phytotherapy Research. Vol 20 (1), pp 9-13.

Fabricant D. S., and Farnsworth N.R. 2001. The Value of Plants Used in Traditional Medicine for Drug Discovery. Environmental Health Perspectives. Vol 109 (1), pp 70-75.

Factsheet, 2005. Plants of Australia : *Emex australis*. Last accessed: April 2013. (http://www.iewf.org/weedid/Emex_australis.html).

Fracassetti D., Lawrence, N., Tredoux A.G.J., Tirelli, A., Nieuwoudt H.H., and Du Toit, W.J. 2011. Quantification of glutathione, catechin and caffeic acid in grape juice and wine by a novel-performance liquid chromatography method. Journal of Food Chemistry. Vol 128, pp 1136-1142.

Francisco P.P., Costas-Mora I., Romero V., Lavilla I., and Bendicho C. 2011. Advances in miniaturized UV-Vis spectrometric systems. Trends in Analytical Chemistry. Vol 30 (10), pp 1637- 1648.

Fransworth N.R., Akerele O., Bingel S.A., Soejarto D.D., and Guo Z. 1985. Medicinal plants in therapy. World Health Organization. Vol 63, pp 965-981.

Freshney R. I. 1987. Culture of animal cells: A manual of basic technique. A.R. Liss (New York), ISBN 0845142410.

Frum, Y. and Viljoen, A.M. 2006. In vitro 5-Lipoxygenase and Anti-Oxidant Activities of South African Medicinal Plants Commonly Used Topically for Skin Diseases, Journal of Pharmacological and Biophysiological research. Vol 19 (6), pp 1-7.

Ganachari M.S., Babu V., and S.S. Katare, 2004. Neuropharmacology of an extract derived from *Centella asiatica*. Pharmaceutical Biology. Vol 42, pp 246-252.

Grubben, G. J. H., and Denton, O. A. 2004. Plant resources of Tropical Africa- 2. Nordic Journal of Botany. Vol 23 (3) , pp 298- 324.

Gurib-Fakim, A. 2006. Medicinal plants; Traditions of yesterday and drugs of tomorrow. Molecular Aspect of Medicine. Vol 27 (1), pp 1-93.

Harborne J.B., 1973. Photochemical Methods: A Guide to Modern Techniques of Plant Analysis. Chapman A.& Hall. London, pp: 279.

Hemelaar J., 2012. Review: The origin and diversity of the HIV-1 pandemic. Trends in Molecular Medicine. Vol 18 (3), pp 182-92.

Houghton P.J. 2003. Wound-healing properties of *Buddleja globosa* (Buddlejaceae). Paper presented at Congress: 2003 Years of Natural Products Research: past, present and future, Amsterdam 2003.

Hutchings A. 1996. Zulu medicinal plants. Natal University Press, Pietermaritzburg.

Hyde M., and Wursten B. 2002. Flora of Zimbabwe: Species information: *Guilleminea densa.*(Willd. ex Roem & Schult) Moq. Flora of Zimbabwe. Last accessed : June 2012. (www.zimbabweflora.co.zw/speciesdata/species.php).

Hye M.A., Taher M.A., Ali M.Y., Ali M.U., and Zaman S. 2009. Isolation of (+) - catechin from Acacia Catechu (Cutch Tree) by a Convenient Method. Journal of Scientific Research. Vol 1 (2), pp 300-305.

Inamdar E.K., Yeole R.D., Ghogare A.B., and De Souza N.J. 1996. Determination of biologically active constituents in *Centella asiatica*. Journal of Chromatography A. Vol 74 (2), pp 127-130.

Jayathirtha M.G., and Mishra S.H. 2004.Preliminary immunomodulatory activities of methanol extracts of *Eclipta alba and Centella asiatica.* Phytomedicine. Vol 11(4), pp 361-365.

Jeremy C.K.L., and Whiteman, M. 2003. Antioxidant activities of some Chinese herbs. Free Radical Biology and Medicine. Vol 26, pp 1231-1237.

Kambizi L,. and Afolayan A.J. 2001. An ethanobotanical study of plants used for the treatment of sexually transmitted disease (Njovhera) in Guruve District, Zimbabwe. Journal of Ethnopharmacology. Vol 77, pp 5-9.

Kashou A.H ., and Agarwal. A., 2011. Oxidants and Antioxidants in the Pathogenesis of HIV/AIDS. The Open Reproductive Science Journal. Vol 3 (1), pp 154-162.

Kaul T.N., Middlerton Jr E., and Ogra P.L. 1985. Antiviral effect of flavonoids on human viruses. Journal of medicial Virology. Vol 15, pp 71-79.

Kensa V.M. 2011. Studies on phytochemical profile and antimicrobial Activity on *Asystasia gangetica* (l.) T.Anderson. Plant Sciences Feed. Vol 1 (7), pp 112-117.

Khalighi-Sigaroodi F., Ahvazi M., Yazdani D., and Kashefi M. 2012. Cytotoxicity and Antioxidant Activity of Five Plant Species of Solanaceae Family from Iran. Journal of Medicinal Plants. Vol 11 (43), pp 1-13.

Khan M.R., Kihara M., and Omoloso A.D. 2001. Anti-microbial activity of *Bidens pilosa, Bischofia javanica, Elmerillia papuana* and *Sigesbekia orientalis*. Fitoterapia. Vol 72, pp 662-665.

Khan A.M., Qureshi R.A., Ullah F., Gilani S.A., Nosheen A., Sahreen S., Laghari M.K., Laghari M.Y., Rehman S.U., Hussain I., and Murad W. 2011. Phytochemical analysis of selected analysis medicinal plants of Margalla Hills and surroundings. Journal of Medicinal Plants Research. Vol 5 (25), pp 6017-6023.

Kindscher K., Long Q., Corbett S., Bosnak K., Loring H., Cohen M., and Timmermann B.N. 2012. A Review : The Ethnobotany and Ethnopharmacology of Wild Tomatillos, *Physalis longifolia* Nutt., and Related *Physalis* Species. Botany. Vol 38 (2), pp. 1–13.

Kirtikar K.R.,and Basu B.D. 2001. Indian Medicinal Plants, Vol 1. Lalit Mohan Basu, Allahabad, India, pp. 35-45.

Klos M., Van de Venter M., Milne P.J., Traore H.N., Meyer D., and Oosthuizen., V. 2009. In vitro anti-HIV activity of five selected South African medicinal plant extracts. Journal of Ethnopharmacology. Vol 124 (2), pp 182-188.

Kunle O.O., Shittua U. A., Nasipuria R.N., Kunlea O.F., Wambebea C., and Akahb P.A., 1999. Gastrointestinal activity of *Ficus sur*. Fitoterapia. Vol 70 (1), pp 542-547.

Lewthwaite P., and Wilkins E., 2009. Natural history of HIV/AIDS. Medicine. Vol 37 (7), pp 333–337.

Lipsky J.J. 1996. Antiretroviral drugs for AIDS. Lancet. Vol 348 (6), pp 800-803.

Locher C.P., Witvrouw M., De Bethune M.P., Burch M.T., Mower H.F., Davis H., Lasure A., Pauwels R., Clercq E.D., and Vlietinck A.J. 1996. Antiviral activity of Hawaiian medicinal plants against human immunodeficiency Virus Type -1 (HIV-1). Journal of Phytomedicine. Vol 2(3), pp 259-264.

Maiyo Z.C., Ngure1 R.M., Matasyoh J.C., and Chepkorir R. 2010. Full Length Research Paper: Phytochemical constituents and antimicrobial activity of leaf extracts of three *Amaranthus* plant species. African Journal of Biotechnology. Vol 9 (21), pp 3178-3182

Malan C., and Notten A. 2006. Kirstenbosch National Botanical Garden, South African National Biodiversity Institute's plant information Accessed: March 2012. (Website: www.plantzafrica.com).

Maoela, M.S., Arotiba., O.A., Baker, P.G.L., Mabusela, W.T., Jahed, W., Songa, E.A., and Iwuoha, E.I. 2009. Electroanalytical determination of catechin flavonoid in ethyl acetate extracts of medicinal plants. International Journal of Electrochemical science. Vol 4 (2), pp 1497-1510.

Male D., Brostoff J., Roth D.B., and Roitt I. 2006. Immunology (7 th edition). Harcourt Publishers Ltd., Canada.

Manosroi J., Dhumtanom P. and Manosroi A. 2004. Anti-proliferative activity of essential oil extracted from Thai medicinal plants on KB and P388 cell lines, Cancer Letters. Vol 235, pp 114–120.

Matsuse T.l., Lim Y.A., Hattori M., Correa M., and Gupta M.P .1999. A search for anti-viral properties in Panamanian Medicinal Plants - The effect on HIV and essential enzymes. Journal of Ethnopharmacology. Vol 64(1), pp 15-22.

Matu E.N, and Van Staden J. 2003. Antibacterial and anti-inflammatory activities of some plants used for medicinal purposes in Kenya. Journal of Ethnopharmacology, Vol 87 (1), pp 35–41.

Maundu P. M., NgugI G. W., and Kabuye C. H. S. 1999. Traditional food plants of Kenya. Kenya Resource Centre for Indigenous Knowledge, National Museums of Kenya, 270 pages

McRae J., Yang R., Crawford E., and Palombo E. 2007. Environmentalist. Vol 27 (2), pp 165-174.

Meintjes G., Maartens G., Boulle A., Conradie F., Goemaere E., Hefer E., Johnson D., Mathe M., Moosa Y., Osih R., Rossouw T., Van Cutsem G., Variava E., Venter F., and Spencer D. 2012. Guidelines for antiretroviral therapy in adults', South African Journal of HIV Medicine. Vol 13, pp 114–130.

Mellem J.J., 2008. Phyto-remediation of heavy metals using *Amaranthus dubius.* Master's degree in Technology: Food Technology dissertation, Department of Food Technology, Durban Institute of Technology , Durban, South Africa.

Miyoshi I., Yoshimoto S., Fujishita M., Taguchi H., Kubonishi I., Niiya K., and Minezawa M. 1982. Natural adult T-cell leukemia virus infection in Japarese monkeys. Lancet. Vol 658, pp 143-156.

Mohanlall V., Steenkamp P., and Odhav B. 2011. Isolation and characterization of anthraquinone derivatives from *Ceratotheca triloba* (Bernh.) Hook.f. Journal of Medicinal Plants Research. Vol. 5 (14), pp 3132-3141.

Moore P.S., Pizza C. 1992. Research communication: Observations on the inhibition of HIV-1 reverse transcriptase by catechins. Journal of Biochemistry. Vol 288, pp 717-719.

Mulaudzi R.B., Ndhala A.R., Kulkarni M.G., Finnie J.F., and Van Staden J. 2011. Antimicrobial properties and phenolic contents of medicinal plants used by the Venda people for conditions related to venereal diseases. Journal of Ethnopharmacology. Vol 135, pp 330-337.

Nance C.L., Siwak E.B., and Shearer W.T. 2008. Preclinical development of the green tea catechin, epigallocatechin gallate, as an HIV-1 therapy. Journal of Allergy and Clinical Immunology. Vol 12 (24), pp 459-465.

Naples M.L. (2005). Weeds of rain fed lowland rice fields of Laos & Cambodia. Unpublished M.Sc Thesis, University of Leiden, The Netherlands.

Narayan L.C., Rai V.R., and Tewtrakul S. 2013. Review Article : Emerging need to use phytopharmaceuticals in the treatment of HIV. Journal of Pharmacy Research. Vol 6, pp 218-223.

National Institute of Allergy and Infectious Diseases (NIAID). 2013 – NIH : HIV/AIDS. Accessed: March 2013. (http://www.niaid.nih.gov).

Neondo J.O., Mbithe C.M., Njenga P.K., and Muthuri C.W. 2012. Phytochemical characterization, antibacterial screening and toxicity evaluation of Dichrostachys cinerea. International Journal of Medicinal plant Research. Vol 1 (4), pp 032-037.

Ng T.B., Huang B., Fong W.P., and Yeug H.W. 1997. Mini-review: Anti-Human immunodeficiency virus (anti-HIV) natural products with special emphasis on HIV reverse transcriptase inhibitors. Life Sciences. Vol 61 (10), pp 933-949.

Nurgun, E., Küpeli, E., Erdem, A. and Ihsan, Y. 2006. Antioxidant activities of some Lamiaceae plant extracts. Journal of Ethnopharmacology. Vol 119(1), pp172-178.

Odhav B., Beekrum S., Akula U.S., and Baijnath H. 2007. Preliminary assessment of nutrional value of traditionally leafy vegetables in Kwa Zulu Natal, South Africa. Journal of Food Composition and Analysis. Vol 20, pp 430-435.

Oluwole O.G.A., Sukati N.A., Dlamini P.S. and Sibandze F.G. 2007. Some Swazi plants phytomedicines and their constituents. African Journal of Biotechnology. Vol 6 (3), pp 267-272.

Oyedemi S.O., Yakubu M.T., and Afolayan A.J. 2011. Anti-diabetic activities of aqueous leaves extract of *Leonotis leonurus* in streptozotocin induced diabetic rats. Journal of Medicinal Plants Research. Vol 5 (1), pp 119-125.

Pacific Island Ecosystems at Risk (Pier). 2005. Dichrostachys cinerea, Australian/New Zealand Weed Risk Assessment adapted for Hawaii, Research directed by C. Daehler (UH Botany) with funding from the Kaulunani Urban Forestry Program and US Forest Service, Last updated 3 March 2005.

Park B.C., Bosire K.O., Lee E.S., Lee Y.S., and Kim J.A., 2005. Asiatic acid induces apoptosis in SK-MEL-2 Human melanoma cell. Cancer Letters. Vol 218 (2), pp 81–90.

Pauwels R., Balzarini J., Baba M., Snoeck R., Schols D., Herdewijn P., Desmyter J., and De Clercq E. (1988) Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. Journal of Virological Methods. Vol 20, pp 309-321.

Pereira C.F., Judith T.M., and Paridaen L. 2004. An overview: Anti-Hiv Drug Development. Current Pharmaceuticals Design. Vol 10, pp 4005-4037.

Prabhu K.H., and Bhute A.S. 2012. Review: Plant based natural dyes and mordnats. Journal of Natural Product Plant Resource. Vol 2 (6), pp 649-664.

Prescott L.M., Harley J. P. and Klein D. A. 1993. Microbiology. 2nd edition. Brown publishers, USA.

Ramalhete C., Mansoor T.A., Mulhovo S., Molna J., and Ferreira M.U. 2009. Cucurbitane-Type Triterpenoids from the African Plant *Momordica balsamina*. Journal of Natural Products. Vol 72 (11), pp 209 – 215.

Raven P.H., Evert R.F. and Eichhorn S.E. 1999. Families yielding important phytopharmaceuticals, 6th edition. W.H. Freeman, New York..

Roitt I., Brostoff J., and Male D. 2001. Immunology (6 th Edition). Mobsy International Ltd. London.

Rukunga G.M., Kofi-Tsepko M.W., Kurokawa M., Kageyama S., Mungai GM., and Muli J.M. 2002. Evaluation of the HIV-1 transcriptase inhibitory properties of extracts from some medicinal plants in Kenya. African Journal of Health Sciences. Vol 9 (2), pp 81-90.

Sama W. and Ajaiyeoba E.O. 2006. Phytochemical and antimicrobial studies of *Capparis thonningii* and *Capparis tomentosa*. Pharmacognosy Magazine.Vol 2 (6), pp119- 122.

Schmidta C, Fronzaa M., Goettertb M., Gellerb F., Luikb S., Floresc E. M. M., Bittencourtd C.F., Zanettie G.D., Heinzmanne B.M., Lauferb S., and Merforta I. 2009. Biological studies on Brazilian plants used in wound healing. Journal of Ethnopharmacology. Vol 122 (3), pp 523–532.

Science in Africa. 2007., Potential antihypertensive properties of *Justicia Flava*. Accessed Last: June 2009. (http://www.unesco.org/science/science_africa.pdf).

Scudiero D.A., Shoemaker R.H., Paull K.D., Monks A., Tierney S, Nofziger T.H., Currens M.J., Seniff D., and Boyd M.R.1988. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Research. Vol 48(17), pp 4827-4833.

Shukla A., Rasik A.M., and Dhawan D.N. 1999. Asiaticoside induced elevation of antioxidant levels in healing wounds. Phytotheraphy Research. Vol 13 (2), pp 50-54.

Silva G.L., Pacciaroni A., Oberti J.C., Veleiroa A.S., and Burtona G. 1993. A pregnane structurally related to withanolides from Physalis viscosa. The International Journal of Plant Biochemistry. Vol 34 (3), pp 871–873.

Singh A., 2009. Bioactivity of Famine food plants from the family: Amaranthaceae. Master's degree in Technology: Food Technology dissertation, Department of Food Technology, Durban Institute of technology, Durban, South Africa.

Singh D., Singh B., and Goel R.K. 2011. Review: Traditional uses, phytochemistry and pharmacology of *Ficus religiosa*. Journal of Ethnopharmacology. Vol 134 (3), pp 565-58.

Singh I.P., Bharate S.B., and Bhutani K.K. 2005. Review Article: Anti-HIV natural products. Current Science. Vol 89 (2), pp 269-289.

Smyth R.P., Davenport M.P., Mak J. 2012. The origin of genetic diversity in HIV-1. Virus Research.Vol 169 (2), pp 415-429.

Sofowora A. 1993. Medicinal Plants and Traditional Medicines in Africa. Chichester John Wiley & Sons. New York, pp: 97-145.

Spectral Database for Organic Compounds: SDBS Spectral Database for Organic Compounds SDBS (www.riodb01.ibase.aist.go.jp) Last accessed: March 2013.

Springfield E.P., Amabeoku G., Weitz F., Mabusela W. and Jhonson Q. 2003. An assessement of two *Carpobrotus species* extracts as potential antimicrobial agents. Phytomedicine. Vol 10 (5), pp 434-439.

Srivastava B., Sharma B.K., Singh U., Yashwant B., and Sethi N. 2010. Review Article: Ultra performance liquid chromatography (UPLC)-a chromatography technique. International Journal of Pharmaceutical Quality Assurance. Vol 2 (1), pp 19-25.

Srivastav S., Singh P., Mishra1 G., Jha1., K.K., Khosa R.L., 2011, *Achyranthes aspera* - An important medicinal plant: A review, Journal of Natural Product and Plant Resources. Vol 1 (1), pp 1-14.

Statistics South Africa. 2011. Census 2011: HIV/AIDS. Accessed July 2012. (http://www.statssa.gov.za/census2011/default.asp).

St. Clair M., Martin J.L., Tudor-Williams G., Bach M.C., Vavro C.L., King D.M., Kellam P., Kemp S.D, and Larder B.D. 1995. Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. Science. Vol 253, pp 1557-1559.

Timm C.D., and Riet-Correa F. 1997. Plants toxic to pigs. Journal Ciência Rural. Vol 27(3), pp 521-528.

Tommasi N.D., Piacente S., Rastrelli L., Mahmood N., and Pizza. 1998. Anti-HIV activity directed fractionation of the extracts of *Margyricarpus setosus.* Pharmaceutical Biology. Vol 36 (1), pp 29–32.

Trease G.E., and Evans W.C. 1989. Pharmacology 11th Edn., Bailliere Tindall Ltd., London, pp: 60-75.

Tregold N.H., Biegel H.M., Mavi S., and Ashton H. 1986. Food of Zimbabwe. Mambo press, Bulawayo.

Tristem M., Mansinho K., Champalimaud J.L., Ayres L., and Karpas A. 1988. Six New Isolates of Human Immunodeficiency Virus Type 2 (HIV-2) and the Molecular Characterization of One (HIV-2_{CAM2}). Journal of General Virology. Vol 70 (2) , pp 479-484.

Ubillas R.P., Mendez C.D., Jolad S.D., Luo J., King S.R., Carlson T.J., and Fort D.M. 2000. Anti-hyperglycaemic acetylenic glucosides from *Bidens pilosa*. Planta Medica. Vol 66, pp 82-83.

United Nations Programme on HIV/AIDS (UNAIDS), 2013. Regional fact sheet : SUB-SAHARAN AFRICA. Accessed: March 2013 (communications@unaids.org).

Van der Watt, E. and Pretorius, J.C. 2001. Short communication: Purification and identification of active antibacterial components in *Carpobrotus edulis*. Journal of Ethanopharmacology. Vol 76, 87-91.

Van Wyk, B., Van Oudshoorn, B. and Gericke, N. 2002. Medicinal Plants of South Africa. Pretoria: Briza Publications. ISBN-1-875093095.

Van Wyk, B. and Gericke, N. 2003. Peoples Plants –a guide to useful plants of South Africa. Briza Publications: Pretoria. ISBN-1-875093192.

Van Wyk B., and Wink M. 2004. Medicinal Plants of the world. Briza Publications: Pretoria. ISBN-1-875093443.

Van Wyk B.E., and Albrecht C., 2008. Review: A review of the taxonomy, ethnobotany, chemistry and pharmacology of Sutherlandia frutescens (Fabaceae). Journal of Ethnopharmacology. Vol 119(1), pp 620–629

Wagner H., Bladt S., and Zgainski E. M. 1984. Plant Drug Analysis, Springer-Verlag, Berlin ISBN 0-387-13195-7

Walton J.N., and Brown D.E., 1998, Chemical from plants perspectives on plant secondary products. Imperial College Press, London UK, pp. 2-5.

Watt J.M. and Breyer-Brandwijk M. G. 1962. The medicinal and poisonous plants of Southern and Eastern Africa, (2nd edition). E. & S. Livingstone, Edinburgh. pp 53–54.

Weislow O.S., Kiser R., Fine D.L., Bader J., Shoemaker R.H., and Boyd R. 1989. New Soluble-Formazan Assay for HIV-1 Cytopathic Effects: Application to High-Flux Screening of Synthetic and Natural Products for AIDS-Antiviral Activity. Journal of the National Cancer Institue. Vol 81, pp 577-586.

Wijesundara D.S.A. 2003. Status of medicinal plants research in Sri-lanka and plans for inventory and documentation of medicinal plants in Sri Lanka. Paper presented at the Asia Pacfic Medicinal plant research meeting Kuala Lumpur, Malaysia.

Windadri F.I. 2001. Plant Resources of South-East Asia No. 12(2): Medicinal and poisonous plants 2. Backhuys Publisher, Leiden, Netherlands, pp. 138- 141.

World Health Organisation.1989. *Invitro* screening of traditional medicines for anti-HIV activity: Memorandum from WHO meeting. Bulletin of the World Health Organisation. Vol 87, pp 613-618.

World Health Organisation. 2011. Global Health Observatory (GHO):Situation and trends. Accessed: July 2012. (http://www.who.int/gho/hiv/epidemic_status/cases_all/en/).

Yadav I.K., Jaiswal D., Singh H.P., Mishra A., and Jain D.A. 2009. Anti-HIV drugs from natural sources. Pharmaceutical Research. Vol 1(2), pp 93-109.

Yadav N., Vasudeva,N.,Singh,S. and Sharma K.S., 2007, Medicinal properties of genus Chenopodium Linn, Natural Product Radiance. Vol 6 (2), pp 131-134.

Yang S.S., Cragg G.M., Newmann D.J., and Bader J.P. 2001. Natural Products –Based Anti-HIV Drug Discovery and Development Facilitated by the NCI. Journal of Natural Products. Vol 64 (5), pp 265 – 277.

Yen G.C., Chen H.Y., and Peng H.H. 2001. Evaluation of the cytotoxicity, mutagenicity and antimutagenicity of emerging edible plants. Food Chem. Toxicol. Vol 39, pp 1045-1053.

Yin J., Si C.L., and Wang M.H. 2007. Antioxidant Activity of Flavonoids and Their Glucosides from *Sonchus oleraceus* L. J. Appl. Biol. Chem. Vol 51 (2), pp 57-60.

Zhao Y., Jiang F., Liu P., Chen W., and Yi K. 2012. Catechins containing a galloyl moiety as potential anti-HIV-1 compounds. Journal of drug discovery today. Vol 17(11/12), pp 630-635.

Zeashan H., Amresh G., and Khan G.H. 2009. Anti-nociceptive activity of *Amaranthus spinosus* in experimental animals. Journal of Ethnopharmacology. Vol 122 (3), pp 189-222.

Zeeb D.J, Nelson B.C., Albert K., and Dalluge J.J. 2000. Separation and identification of twelve catechins in tea using liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry. Journal of Analytical Chemistry. Vol 72 (1), pp 5020–5026.