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IN-VITRO BIOACTIVITY OF FRACTIONS FROM A LOCAL MEDICINAL PLANT ON HIV-1 REPLICATION, AND SELECTED FUNGAL AND BACTERIAL PATHOGENS

By

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Declaration

I, Awelani Mirinda Mutshembele, hereby declare that the dissertation for the award of a Masters degree in Microbiology at the University of Venda, hereby submitted by me, has not previously been submitted for a degree at this or any other university, and that it is my own work in design and execution and that all the reference materials contained therein have been duly acknowledged.

Signature:..... Date: 13 MARCH 2009

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Finally to the Almighty God, for His amazing grace in my life. Let all the praise be to him!

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Dedication

I would like to dedicate this dissertation to my very special PARENTS:

The late Mr Itani Daniel Mutshembele & Mrs Tshilidzi Mercy Mutshembele

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SUMMARY

In an earlier pilot study, a crude methanol extract from the stem-bark of *Peltophorum africanum* was evaluated for anti-HIV activity. The extract showed a strong inhibition against HIV-1 RT DNA polymerase and RNase H activities. An oxidized gallotannin from the extract also inhibited HIV-1 reverse transcriptase and integrase in an enzyme cell- free system (Bessong *et al.*, 2005). To broaden, the scope of the investigation on *P. africanum* it was important to evaluate different fractions from the plant obtained with different solvent system, in an attempt of identify other possible inhibitory compounds. In addition, it was important to evaluate extracts of the plant for anti-HIV replicative properties in infectious cultures.

Methanol crude extract, fractions of the extract, and isolated compounds were evaluated for bioactivity against HIV-1 *reverse transcriptase* (RT). Fractions and compounds with high inhibition were also tested against viral replication in infectious cultures. Fractions were obtained by silica gel column chromatography and further purified by Sephadex-LH 20 column chromatography. Fractionation led to the isolation of a white powdered compound which was identified as bergenin by spectroscopic methods. Inhibition of HIV-1 RT was performed using a RT colorimetric assay. A total of 28 fractions were obtained. Fraction C2 inhibited RT activity by 92% at 250 µg/ml. Other fractions such as B4, B6, D3 and D6 gave 87%, 84%, 78% and 50% inhibition respectively. C2 had significant inhibition at 100 and 250 µg/ml ($P< 0.05$). In infectious cultures, evaluating intracellular mode of action, bergenin did not inhibit HIV-1 replication, while the strongest inhibition was observed for fraction C2 with a mean IC_{50} of 9.5 µg/ml based on p24 antigen production. C2 shows the best activity in the cell-free anti HIV assay and in infectious cultures. In addition C2 did not exhibit any cytotoxicity at concentrations less than 250 µg/ml in a CellTiter Cell Proliferation Assay. C2 is worthy of further chemical and biological analysis.

Bioactivity of fractions of the methanol crude extract of the stem bark of *Peltophorum africanum* against the fungi *Candida albicans*, and *Cryptococcus neoformans*; and the bacteria *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* was also investigated. To evaluate the inhibition of the plant extract and fractions on these fungi

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and bacteria, the minimum inhibitory concentration (MIC), and bioautographic profiles were assessed. Twenty eight fractions were initially screened by bioautography and MIC. Pooling was done for similar fractions based on bioautographic activity. After two rounds of pooling five fractions gave the best active profiles against one or several of the organisms. These fractions were B4, D1, D7, H1, and H3. Based on the bioautography and MIC, B4 was the most active against *P. aeruginosa, S. aureus, E. coli*, and *E. faecalis*. The question to answer is whether B4 has a broad spectrum or non-specific mode of inhibition. Further studies will elucidate the chemical constituent or constituents of B4 and its mode of action.

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PUBLICATION EMANATING FROM THIS WORK

Mutshembele AM Obi CL, Eloff JN and Bessong PO. Inhibitory properties of extracts fractions and principles from *Peltophorum africanum* (Sond) on clinical isolates of *Candida albicans* and *Cryptococcus neoformans*. In preparation for Phytotherapy Research.

Bessong PO, **Mutshembele AM,** Obi CL and Eloff JN. Inhibition of HIV-1 subtype C replication by fractions and principles from a South African medicinal plant. In preparation for Pharmaceutical Biology.

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5.2. ^{13}C NMR of C1 (bergenin) 105

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%	Percentage
°C	Celsius degree
µg	microgram
µl	Micro litre
^{13}C NMR	Carbon 13 nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
ABTS	2,2-Azino-bis (3-Ethylbenzthiazoline-6-sulfonic acid)
AIDS	Acquired immune deficiency syndrome
AMV	Avian Myeloblastosis Virus
Anti-DIG-POD	Anti-digoxigenin-peroxidase
ARV	Antiretroviral drug
AZT	Azidovudine thymide
BAW	Butanol/acetic acid/water
BEA	Benzene/ethanol/ammonia
CAEV	Caprine arthritis encephalitis virus
$CD4^{+}$ T Cells	Cluster of differentiation Positive T Cells
CCR3	CC- chemokine receptor 3
CCR5	CC-chemokine receptor 5
CDC	Centres for Disease Control and Prevention
CEF	Chloroform/ethyl acetate/formic acid
cm	centimetre
CPE	Cytopathic effect
CRF	circulating recombinant forms
CXCR4	C-X-C-chemokine receptor 4
DCM	Dichloromethane
dH_2O	Distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dT	Deoxythymidine
EIAV	Equine infectious anemia virus

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ELISA	Enzyme linked immunosorbent assay
EMW	Ethyl acetate/methanol/water
env	envelope gene
FAO	Food and Agricultural organization
FCS	Fetal calf serum
FI	Fusion inhibitor
g	gram
gag	group associated antigens
GC	Gas Chromatography
GI	Gastrointestinal tract
gp	Glycoprotein
HAART	Highly active antiretroviral therapy
HBV	Hepatitis B virus
HIV	Human immunodefiency virus
HIV-1 RT	HIV type 1 reverse transcriptase
HIV-2	HIV type 2
HPLC	High performance liquid chromatography
HSV-2	Herpes simplex virus type 2
HTLV	Human T-cell lymphotrophic virus
IC_{50}	50 percent inhibitory concentration
INT	p-iodonitortetrazolium
LTR	long terminal repeat
MALDI-Tof	MALDI test of flight
MeOH	Methanol
mg	milligram
MHB	Mueller Hinton Broth
MIC	Minimum inhibitory concentration
ml	millilitre
M-MuLV	Moloney-Murine Leukaemia Virus
MP	Microplate
MS	Mass Spectrometry

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nef	negative factor
Nm	Nanometer
NMR	Nuclear Magnetic Resonance
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitors
NtRTI	Nucleotide reverse transcriptase inhibitor
OI	Opportunistic infection
PBMCS	Peripheral blood mononuclear cells
PBS	Primer binding site
PCR	Polymerase chain reaction
PI	Protease inhibitor
PIC	Preintegration complex
pol	polymerase gene
Poly A-dT	Polyadenylic acid-deoxythymidine
ppm	part per million
PR	Protease
RDDP	RNA dependent DNA polymerase
rev	regulator of virion
Rf	Retardation factor
RNA	Ribonucleic acid
RNase H	ribonuclease H
RT	Reverse transcriptase
SARS	Severe acute respiratory syndrome
SDA	Sabouraud dextrose agar
SIV	simian immunodeficiency virus
tat	trans-activator of transcription
TLC	Thin layer chromatography
tRNA	Transfer RNA
TTP	Thymidine triphosphate
UNAIDS	United Nations Joint Programme on HIV/AIDS
vif	virus infectivity factor

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vpr	viral protein r
vpu	viral protein u
vpx	viral protein x
WHO	World Health Organization

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CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. Background of HIV/AIDS

Infection with the human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS) remains one of the greatest public health problem worldwide. The first cases of HIV infections and AIDS were diagnosed in the USA in 1981 amongst homosexual drug abusers (Centers for Disease Control, 1981; Barré - Sinoussi *et al.*, 1983). Essentially, the infection spread so dramatically that by 1987 cases had been diagnosed in virtually all countries of the world. The pandemic had touched virtually all aspects of the social fabrics of most nations (UNAIDS, 2007).

Despite the advances made in the containment of the pandemic, several challenges remain to be overcome. For example current treatment options are not curative, there is no vaccine to prevent infections, and in some countries access to drugs is still limited.

1.2. Global epidemiology of HIV/AIDS

The Joint United Nations Programme on AIDS (UNAIDS) estimated that AIDS has killed more than 25 million people since it was first recognized in 1981, making it one of the most destructive epidemics in recorded history. Globally, between 30.6 and 36.1 million people currently live with HIV. In 2007, between 1.8 and 4.1 million people were newly infected and between 1.9 and 2.4 million people with AIDS died of which 330 000 were children under the age of 15 years. Figures 1.1 and 1.2 show the geographical distribution of adults and children estimated to be infected with HIV, and the distribution of death from AIDS during 2007 respectively (UNAIDS, 2007).

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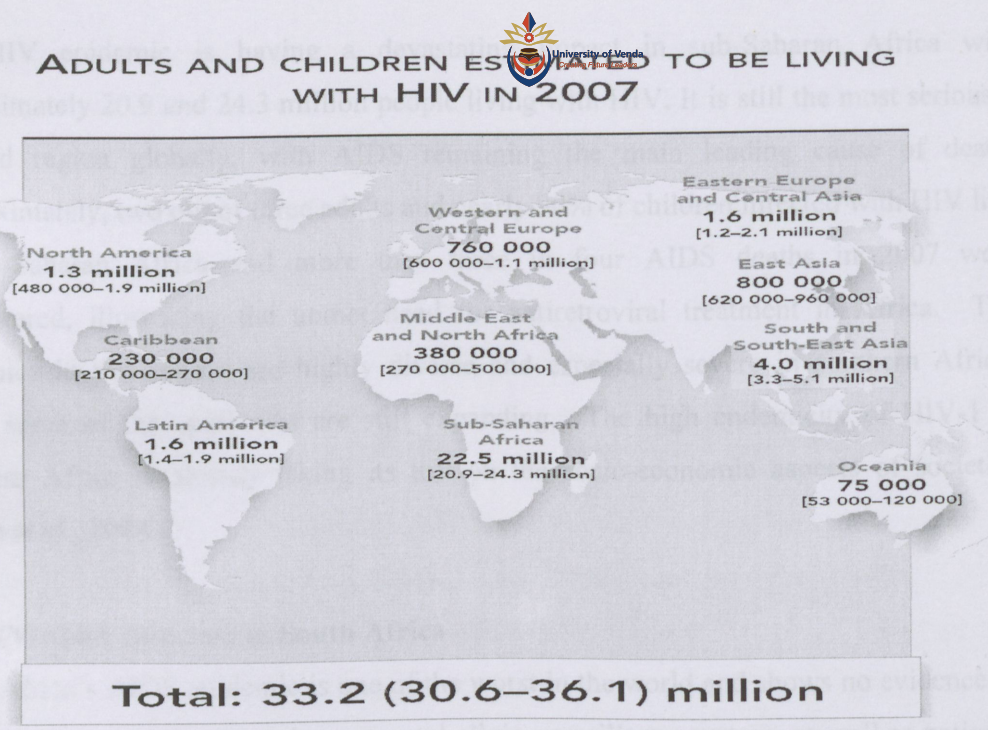


Figure 1.1: Global estimates for adults and children living with HIV in 2007 (UNAIDS, 2007).

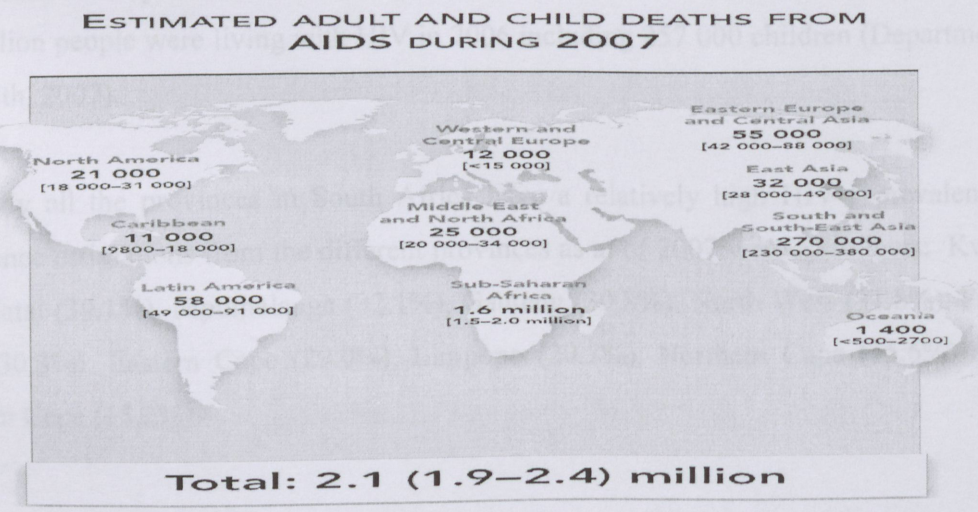


Figure 1.2: Global estimates for adult and children deaths from AIDS during 2007 (UNAIDS, 2007).

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The HIV epidemic is having a devastating impact in sub-Saharan Africa with approximately 20.9 and 24.3 million people living with HIV. It is still the most seriously affected region globally, with AIDS remaining the main leading cause of death. Approximately, two out of three adults and nearly 90% of children infected with HIV live in sub-Saharan Africa and more than three in four AIDS deaths in 2007 were encountered, illustrating the unmet need for antiretroviral treatment in Africa. The epidemics in this region are highly diverse and especially severe in southern Africa, where some of the epidemics are still expanding. The high endemicity of HIV-1 in Southern Africa is already taking its toll on the socio-economic aspects of societies (Rosen *et al*., 2004).

1.3. HIV/AIDS epidemic in South Africa

South Africa's AIDS epidemic is one of the worst in the world and shows no evidence of a decline. Based on its extensive antenatal clinic surveillance system, as well as national surveys with HIV testing and mortality data from its civil registration system an estimated 29% of pregnant women were living with HIV in 2006. An estimated 18.3% of individuals 15-49 years old were living with HIV in the same period. Generally, about 5.4 million people were living with HIV in 2006 including 257 000 children (Department of Health, 2007).

Generally all the provinces in South Africa have a relatively high HIV-1 prevalence. Prevalence estimations from the different provinces as at of 2007 were as follows: Kwa-Zulu Natal (39.1%), Mpumalanga (32.1%), Gauteng (30.8%), North West (31.8%), Free State (30.3%), Eastern Cape (29.0%), Limpopo (20.7%), Northern Cape (15.6%) and Western Cape (15.2%).

Before 2004 antiretrovirals were available only through private health institutions in South Africa; and due to scarcity and high cost the majority of patients could not afford them. In early 2004 a nationwide antiretroviral delivery programme started to provide antiretrovirals to adults who have a CD4 count of less than 200/µl or a WHO stage 4 diseases and to children with at least recurrent hospitalization due to HIV related disease.

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The South African drug regimen guideline makes use of stavudine, lamivudine and efavirenz as the first-line therapy for adults without prior history of antiretroviral use, with nevirapine replacing efavirenz for women of child bearing age. The second-line therapy comprises zidovudine, didanosine and lopinavir/ritonavir (Department of Health, 2006).

1.4. Taxonomy, morphology and structure of HIV

HIV is a retrovirus because in the course of its replication the genetic material which is RNA is reverse transcribed to DNA. It is also a lentivirus (lentiviridae) due to the long incubation period between infection and disease manifestation. Other examples of retroviruses include oncoretroviruses, with human T-cell lymphotrophic virus (HTLV) as the human prototype, spumaviruses with human foamy virus as the human prototype and lentiviruses, with HIV as the human prototype. Other lentiviruses include equine infectious anemia virus (EIAV), visna virus, caprine arthiritis encephalitis virus (CAEV), bovine immunodeficiency virus, feline immunodeficiency virus, simian immunodeficiency and human immunodeficiency virus (HIV) (Mayo & Pringle, 1998; Hirsch *et al*., 1999; Hahn *et al*., 2000).

There are two types of HIV: HIV-1 and HIV-2. These two viruses have been identified for almost 20 years as the major cause of AIDS (Barré -Sinoussi *et al*., 1983, Gallo *et al*., 1984 & Clavel, 1986). Both HIV-1 and HIV-2 are of primate origin. The probable origin of HIV-1 is the Central African chimpanzee (*Pan troglodytes troglodytes*), while it has been shown that HIV-2 may have originated from the Sooty Mangabey (*Cercocebus atys*), an Old World monkey of Guinea Bissau, Gabon, and Cameroon. HIV-1 is the variant driving the epidemic worldwide. Most of the literature review presented here-in will focus on HIV-1, and allusion to differences for HIV-2 will be made appropriately.

The HIV-1 virion has a diploid genome comprising two positive single stranded RNA molecules. It is roughly spherical in shape and about 120 nm in diameter (McGovern *et al*., 2002). The single stranded RNA is tightly bound to nucleocapsid proteins, p7 and enzymes such as reverse transcriptase, protease, and integrase necessary for viral

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replication (Jetz *et al.*, 2000). A matrix composed of the viral protein p17 (matrix) surrounds the capsid (p24) ensuring the integrity of the virion particle. This is in turn surrounded by the viral envelope which is composed of a phospholipid bilayer originating from the membrane of the infected cell when a newly formed virus particle buds from it (Robey *et al.*, 1985; Lama & Trono, 1998; Hahn *et al.*, 1999).

Embedded in the viral envelope are proteins from the host cell and about 70 copies of a complex HIV protein that protrudes through the surface of the virus particle. The viral envelope proteins consists of a surface glycoprotein (gp 120), and the transmembrane glycoprotein (gp 41) (Veronese *et al.*, 1985; Hammache *et al.*, 1998; Poignard *et al.*, 2000). The envelope glycoproteins are essential for attachment and fusion with target cells to initiate the infectious cycle (Figure 1.3) (Chan *et al.*, 1997).

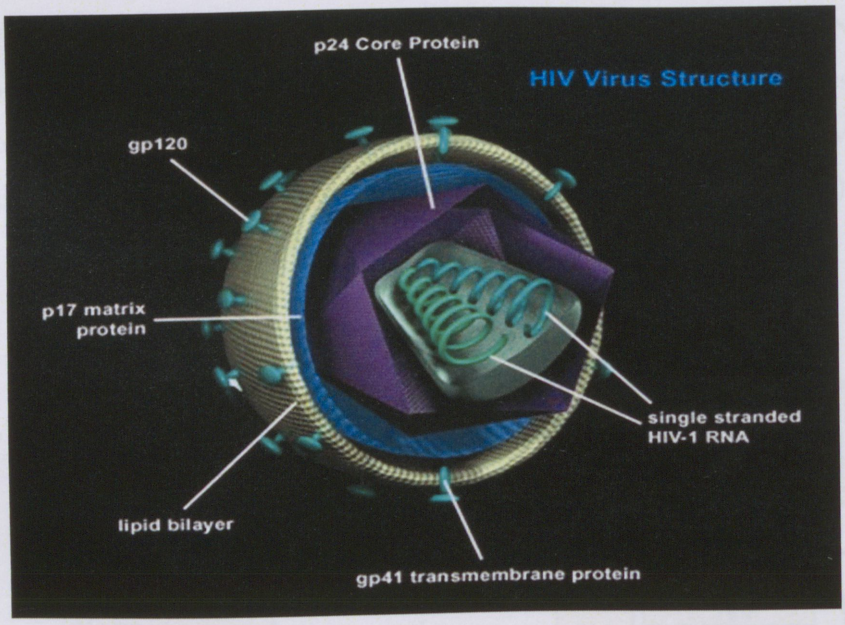


Figure 1.3: Organization of the HIV-1 virion showing the major internal and external viral components required for the viral replication, fusion, entry and replication infected cell (*www. niaid.nih.gov*).

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1.5. HIV-1 genes and gene products

HIV contains nine open reading frames that are encoded in the RNA genome. The major genes are the group associated antigen (*gag),* polymerase (*pol)* and envelope (*env)* genes. *Env* gene codes for a precursor glycoprotein 160 (gp160), that is cleaved by cellular enzymes to form gp120 and gp 41. *Gag* codes for viral capsid, matrix and nucleopcapsid proteins which are structural proteins. The *pol* gene codes for reverse transcriptase, protease and integrase (Robey *et al.*, 1985). In addition there are six regulatory or accessory genes for proteins that control the ability of HIV to infect cells, produce new copies of virus and influence disease progression (Figure 1.4). Table 1.1 shows the function of regulatory or accessory genes (Beer *et al.*, 1999; Yamada & Iwamoto, 2000, Fackler *et al.*, 20001; Schaeffer *et al.*, 2001).

The protein encoded by *nef* appears necessary for the virus to replicate efficiently, and the *vpu* encoded protein influences the release of new virus particles from infected cells Table 1.1 (Sherman *et al.*, 2002). The ends of each strand of HIV contain RNA sequence called long terminal repeat (LTR). Regions in the LTR act as switches to control production of new viruses and can be triggered by proteins from either HIV or the host cell (Strair & Mellors, 1994; Sherman & Greene, 2002; Sherman *et al.*, 2002).

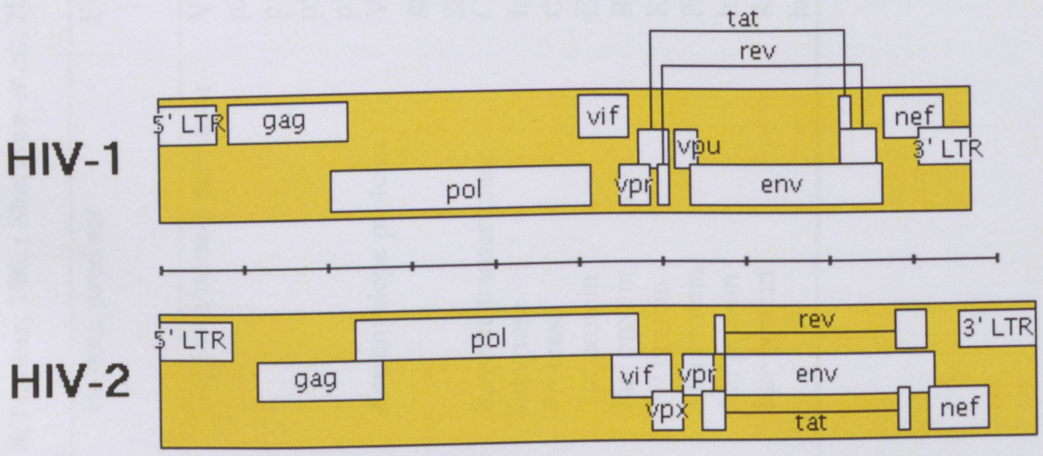


Figure 1.4: A diagrammatic representation of HIV-1 and HIV-2 genes (hiv-web.lanl.gov/content/immunology/pdf/2000/intro/GenomeMaps.pdf).

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Table 1.1: HIV-1 genes, protein products and the functions of regulatory and accessory genes (Strair & Mellors, 1994; Sherman & Greene, 2002; Sherman *et al.*, 2002).

HIV gene	Protein product	Function in life cycle of HIV
Gag	*Gag*/core protein precursor	Viral core proteins: p24: major capsid protein. p17: matrix protein, nuclear import of preintegration complex. p9: binds to viral RNA. p7: binds to viral RNA.
Env	*Env*/envelope proteins	Viral envelope proteins: gp 120: major envelope protein mediates virion binding to cell receptor (CD4). gp 41: mediates fusion of viral envelope and cell membrane.
Pol	Reverse transcriptase	Converts single-stranded viral RNA to viral DNA duplex.
	Integrase	Integrates viral DNA duplex into host cell genome.
	Protease	Cleaves core precursor viral polyprotein into structural and functional core proteins
Tat	*Tat* protein	Essential regulatory protein, trans-activates expression of all viral genes.
Rev	*Rev* protein	Regulatory protein activates expression of HIV structural and enzymatic genes.
Vif	*Vif* protein	Role in viral budding and infectivity of free virions.
Vpu	*Vpu* protein	Promotes release of budding virions from host cell.
Nef	*Nef* protein	Increases viral infectivity; down regulation of CD4.
Vpr	*Vpr* protein	Role in the nuclear import of the preintegration complex. Activates HIV replication in latently infected cells.

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1.6. Genetic nomenclature and variability of HIV

Phylogenetic analysis of HIV-1 isolates from diverse geographical origins, have revealed that they can be subdivided into groups, subtypes, sub-subtypes and circulating recombinant forms (CRFs) (Robertson *et al.,* 1999). HIV-1 has been classified into three groups, namely HIV-1 group M (Major), HIV-1 group O (Outlier) (Gurtler *et al*., 1994), and HIV-1 group N (non-M/non-O) (Simon *et al*., 1998). These three groups may represent three separate introductions of simian immunodeficiency virus (SIV) into humans. Group O appears to be restricted to West-central Africa and group N discovered in 1998 in Cameroon is extremely rare. More than 90% of HIV-1 infections belong to HIV-1 group M. Within group M there are known to be nine genetic subtypes (clades). These are subtypes A, B, C, D, F, G, H, J, and K. Within some subtypes, further phylogenetic structure has been identified, leading to a classification into sub-subtypes. Subtype A and F are subdivided into two sub-subtypes, A1, A2 and F1, F2 respectively. Subtypes within group O and N are not yet defined (Figure 1.5) (Myers *et al*., 1992; Louwagie *et al.,* 1993; Janssens *et al*., 1994; Kostrikis *et al.,* 1995; Leitner *et al*., 1995; Louwagie *et al*., 1995; Roques *et al*., 1999; Triques *et al*., 2000).

HIV-2 is very distinct from HIV-1. Subtypes within HIV-2 are known as groups, because sequences from these viral clades are nearly as distant from one another as are from the M, N and O groups of the HIV-1 virus and both the sequence and the epidemiology of HIV-2 viruses suggest that each clade of virus was the result of a separate Sooty mangabey-to-human transmission event. HIV-2 is classified into five subtypes namely A, B, C, F, or G (HIV sequence database, 2007). Figure 1.5 shows a schematic representation of HIV genetic classification.

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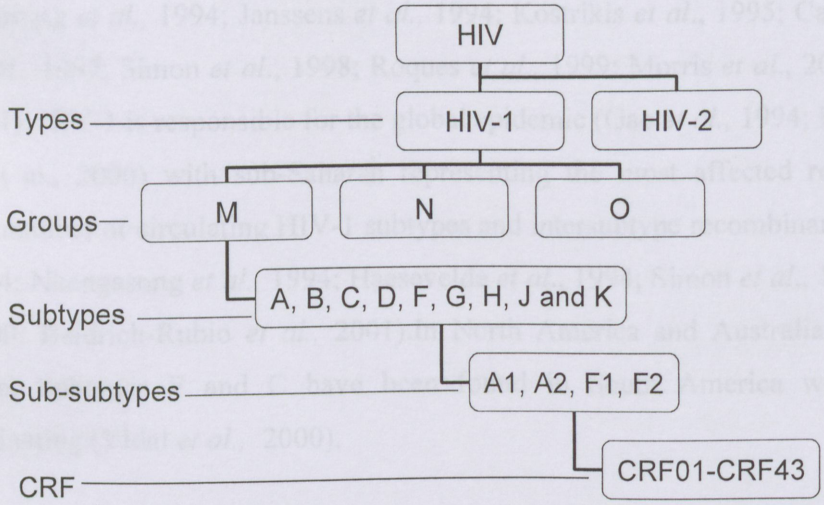


Figure 1.5: Schematic representation of HIV-1 and HIV-2 phylogenetic analysis showing groups, subtypes, sub-subtypes and CRFs. The majority of strains found belong to Group M. HIV-2 comprising subtypes A-G not presented in this figure. Sub-subtypes have been described for subtypes A and F. (HIV sequence compendium, 2007).

Certain isolates of group M were realized to be clustered with different subtypes in different regions of their genomes when phylogenetic analyses were performed (Robertson *et al.,* 1995). These mosaic HIV-1 genome have been identified in several, apparently unlinked, individuals and play a major role in the global AIDS epidemic and they were designated as circulating recombinant form (CRFs) (Robertson *et al.,* 1999). Currently described CRFs found in the literature are shown in Table 1.2. A variant formerly designated as subtype E based on the *env* gene sequence was latter shown to be a recombinant between clades A and E (Gao *et al.,* 1996), though a non-recombinant clade E has not been found. Interclade A/E possesses *gag* and *pol* genes, which are homologous to clade A viruses, but the *env* gene comes from an as yet undetected clade E. A similar discovery was made for hitherto described subtype I. subtypes E and I are now referred to as CRF01_AE and CRF04_cpx, respectively.

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All known HIV-1 subtypes circulate in Africa with subtypes C and A predominating (Nkengasong *et al.,* 1994; Janssens *et al.,* 1994; Kostrikis *et al.*, 1995; Carr *et al.*, 1996; Obi *et al.*, 1997; Simon *et al.*, 1998; Roques *et al.*, 1999; Morris *et al.*, 2000; Ayouba *et al.*, 2001). HIV-1 is responsible for the global epidemic (Gao *et al.,* 1994; Hu *et al.,* 1996; Hahn et al., 2000) with sub-Saharan representing the most affected region with the highest number of circulating HIV-1 subtypes and intersubtype recombinants (Janssens *et al.*, 1994; Nkengasong *et al.,* 1994; Haesevelde *et al.*, 1994; Simon *et al.*, 1998; Corbet *et al.*, 2000; Baldrich-Rubio *et al.,* 2001).In North America and Australia, subtype B is common. Subtypes F and C have been found in South America with subtype B predominating (Vidal *et al.,* 2000).

Table 1.2: Identified circulating recombinant forms and their subtypes components. U= unclassified sequence (HIV sequence compendium, 2007).

Name	Reference strain	Recombination Subtypes
CRF01_AE	CM240	A, E
CRF02_AG	IbNG	A, G
CRF03_AB	Kal153	A, B
CRF04_cpx	94CY032	A, G, H, K, U
CRF05_DF	VI1310	D, F
CRF06_cpx	BFP90	A, G, J, K
CRF07_BC	CN54	B', C
CRF08_BC	GX-6F	B', C
CRF09_cpx	96GH2911	CRF02, A, U
CRF10_CD	TZBF061	C, D
CRF11_cpx	GR17	A, CRF01, G, J
CRF12_BF	ARMA159	B, F
CRF13_cpx	96CM-1849	A, CRF01, G, J, U
CRF14_BG	X397	B, G
CRF15_01B	99TH.MU2079	CRF01, B
CRF16_A2D	KISII5009	A2, D
CRF17_BF	ARMA038	B, F
CRF18_cpx	CU76	A1, F, G, H, K, U
CRF19_cpx	CU7	A1, D, G
CRF20_BG	CB228	B, G
CRF21_A2D	99KE_KER2003	A2, D
CRF22_01A1	CM53122	CRF01, A1
CRF23_BG	CB118	B, G
CRF24_BG	CB378	B, G
CRF25_cpx	02CM_1918LE	A, G, U
CRF26_AU		A, U
CRF27		Not yet published

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Name	Reference strain	Recombination Subtypes
CRF28_BF	BREPM12609	B, F
CRF29_BF	BREPM16704	B, F
CRF30_0206	00NE36	CRF02, CRF06
CRF31_BC	04BR142	B, C
CRF32_06A1	EE0369	CRF06, A1
CRF33_01B	05MYKL007	CRF01, B
CRF34_01B	OUR2275P	CRF01, B
CRF35_AD	AF095	A, D
CRF36_cpx	NYU830	A, G, CRF01, CRF02
CRF37_cpx	NYU926	A, G, CRF01, CRF02, U
CRF38		Not yet published
CRF39		Not yet published
CRF40		Not yet published
CRF41		Not yet published
CRF42_BF		B, F
CRF43		Not yet published

One of the most dramatic changes in the global AIDS pandemic has been the emergence and rapid spread of HIV-1 subtype C viruses, which currently accounts for approximately 50% of all global infection (Esparza & Bhamarapravti, 2000; Kantor & Katzenstein, 2004; Hamelaar *et al.,* 2006). HIV-1 subtype C is becoming increasingly predominant relative to other subtypes being detected with increasing frequency in sub-saharan Africa, East Africa, China, India, and South America in countries like Argentina, Paraguay, Uruguay and Brazil (Renjifo *et al.*, 1998; Koch *et al.*, 2001; Hoelscher *et al.,* 2002; Gerhardt *et al.,* 2005; Siddappa *et al.,* 2004; Sanchez-Merino *et al.*, 2003; Carrion *et al.,* 2004).

Genetic diversity is the hallmark of HIV-1 biology and this may impact on diagnostic, drug and vaccine design and development (Kellam & Larder, 1995; Hu *et al.,* 1996). Genetic variability is attributed to the lack of a proof reading function of reverse transcriptase, introducing substitution at a rate of approximately $3x10^{-5}$ nucleotide changes per site per replication. Secondly, there is high virus replication up to 10^{10} per day which sustains HIV infection *in vivo.* Thirdly, there is rapid emergence of drug resistance which is caused by *in vivo* selection pressure. These mechanisms generate viral variants at extraordinary rates and represent a major force driving HIV-1 evolution in infected populations worldwide (Gao *et al.*, 1996; Jetz *et al.*, 2000).

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More diverse viruses are produced from recombination than mutations from patients that are being infected with multiple HIV-1 strains (Zhu *et al.*, 1995; Kuwata *et al.*, 1997), and recombination have been noticed as they may occur quite frequently indicating this process also contributes importantly to HIV-1 diversification (Sabino *et al.*, 1994; Diaz *et al.*, 1995; Gao *et al.*, 1996; Burke, 1997; Salminen *et al.*, 1997; Takehisa *et al.*, 1999).

1.7. Life cycle of HIV

HIV enters macrophages and CD4 T cells by the adsorption of viral envelope glycoproteins on surface of target cells followed by fusion of the viral gp 41 with the cell membrane and the release of the HIV capsid into the cell (Chan *et al.*, 1998; Wyatt & Sodroski, 1998). The first step in fusion involves the high-affinity attachment of the CD4 binding domains of gp 120 to CD4, whereby there is conformational change, exposing binding domains of gp 120 and allowing them to interact with the target chemokine receptor (Chan *et al.*, 1998).

Two majors types of chemokine receptors are used by HIV. These are CCR5 (a β-chemokine receptor) or CXCR4 (an α-chemokine receptor) (Deng *et al.*, 1996; Dragic *et al.*, 1996; Alkhatib *et al.*, 1997). Some viruses make use of other coreceptors such as CXCR6, CCR3 and BOB in addition to CCR5 and CXR4 (Cilliers *et al.*, 2003). Early in HIV infection, the virus is a non-syncytial-inducing variant with tropism towards CCR5 receptors. Non-syncytial-inducing viruses do not induce fusion of T-cells (syncytium formation) in vitro. They grow relatively slowly but grow well in monocytes and lymphocytes. Sometimes at later stage of infection, the virus switches to a syncytial-inducing variant with tropism towards CXCR4 receptors (Philpott, 2003). Syncytium-inducing viruses grow more rapidly *in vitro* than non-syncytial –inducing viruses and have tropism to T cells only. Their emergence indicates the near total depletion of $CD4^+$ T cells and rapid progress of the disease to AIDS. In an individual AIDS patient there may be swarms of viruses with different tropism towards the two different chemokine receptors (Miedema *et al.*, 1994).

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After attachment of gp 120 to CD4 and either of the chemokine receptors, a conformational changes occurs on gp 120 exposing gp 41. Once this happens, gp 41 pierces the cell membrane of the target cell through a thrust-like action, in so doing emptying the viral contents into the host cell. Viral RNA and reverse transcriptase, integrase and ribonuclease enzymes are injected into the host cell at fusion (Chan *et al.*, 1998). Reverse transcriptase liberates the single stranded RNA from the attached viral proteins and copies it into a complementary DNA of 9 kb size. The reverse transcriptase then converts a complementary DNA strand to a double-stranded viral DNA. The reverse transcription of RNA to DNA is achieved through the following stages: a retrovirus-specific cellular transfer RNA (tRNA) hybridizes with a complimentary region called primer binding site (PBS). A DNA segment is extended from tRNA based on the sequence of the retroviral genomic RNA. The viral R and U5 sequences are removed by RNase H. The extended DNA segmented binds and hybridizes with the remaining R sequence at the 3' end. A DNA strand is extended from the 3' end followed by the removal of most of the viral RNA. Both tRNA and the remaining viral RNA are removed by RNase H. The PBS region of the second strand binds and hybridizes with the PBS region of the first strand this lead to the extension of DNA strands. The viral DNA is then transported to the cell nucleus of the cell as the preintegration complex (PIC) (Dvorin *et al.*, 2002; Sherman & Greene, 2002; Sherman *et al.*, 2002).

The integration of the viral DNA into the host cell's genome is then carried out by the viral enzyme integrase (Bouyac-Bertoia *et al.*, 2001). Through series of steps including 3' end processing reaction, strand transfer and integration, the viral DNA is inserted into the host cell DNA (Brown *et al.*, 1989; Vink *et al.*, 1991; Katz & Skalka, 1994). Integrated viral DNA (the provirus) may lie dormant, or be expressed when the infected cell is activated (Zheng *et al.*, 2005).

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The proviral gene is transmitted to the daughter cells when the host cell divides. As a result, HIV can persist in cells of an infected individual both as the RNA virus particles and as part host cellular genes. When the provirus becomes transcriptionally active, host cellular enzymes, RNA polymerases, carry out transcription of the integrated provirus DNA template and cellular mechanisms also complete RNA processing and transport to ribosomes in the cell cytoplasm. Viral protein synthesis, further processing into viral glycoproteins and transport to the cell membrane, where maturation of viral particles occurs through a budding process, is carried out by the cell's machinery. The viral protease is involved in the cleavage of the precursor *gag* and *gag-pol* polyproteins into the capsid, nucleocapsid, matrix and enzymatic proteins. This is part of the maturation process and it occurs during the formation of the viral particle (Wong-Stall, 1991. The life cycle of HIV-1 is summarised in Figure 1. 6.

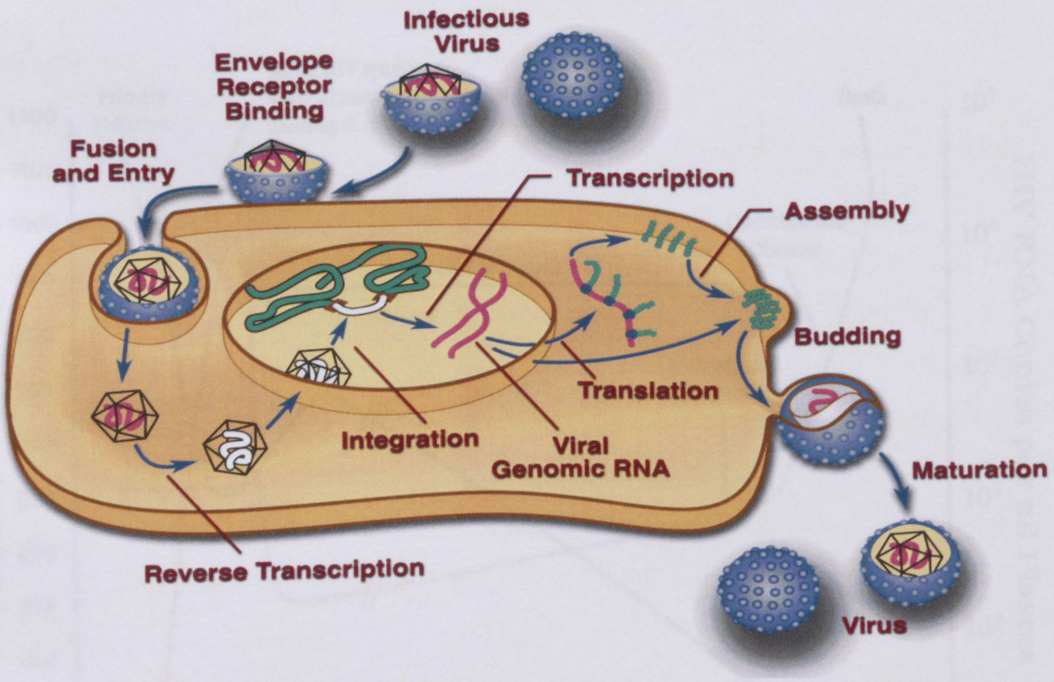


Figure 1.6: A schematic representation of the events occurring in the viral replication cycle of HIV-1 (*home.ncifcrf.gov/hivdrp/RCAS/replication.html*).

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1.8. Pathogenesis of HIV

There are generally three phases that characterize an individual who is infected with HIV (Pantaleo *et al.,* 1993; Fauci, 1993). During the early phase which occurs within a few weeks after infection, there is extensive viraemia accompanied by a large number of circulating $CD4^+$ T lymphocytes that are infected (Ho *et al.*, 1985). This stage comprises seroconversion and the symptoms experienced are similar to infectious mononucleosis which include fever, headache, body pains, diarrhea, and cough. The development of active humoral and cell-mediated immune responses decreases the circulating virus load and leads to the latent phase. Latent phase is characterized by low levels of virus and infected cells in circulation, with mild or unapparent disease symptoms and a gradual decline of $CD4^+$ T cells (Figure 1.7) (Goedert *et al.*, 1984; Walker *et al.*, 1987).

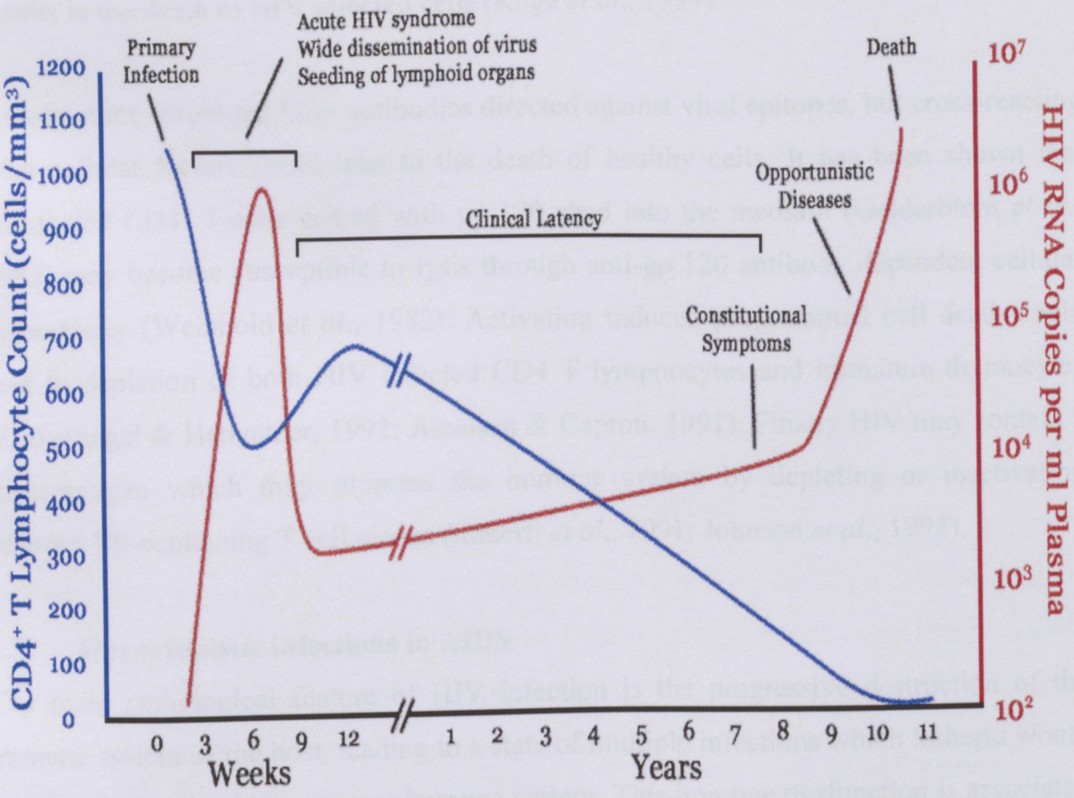


Figure 1.7: Graph showing HIV copies and CD4 counts over course of a typical HIV infection. As the virus replicates itself, as indicated by the red line, the number of T-helper cells in a person's blood decreases, as indicated by the blue line. (www.journaids.org/hivaidsoverview.php).

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HIV viral load increases and turnover of infected cells occurs during latent phase (Coffin *et al.,* 1995). Finally after several years of clinical latency there is viraemia during a 1-2 years period parallel with further deterioration of the immune and central nervous systems leading to opportunistic infections, disease symptoms and eventually death. HIV pathogenesis begins with immunosuppression due to the selective depletion of $CD4^+$ T helper/inducer lymphocytes (McCune, 1991).

$CD4^+$ T- cell death has been attributed to syncytium formation involving the formation of multinucleated cells that arise from the fusion between the HIV infected and uninfected $CD4^+$ T-cells (Sodroski *et al.*, 1986; Kjerns *et al.*, 1991). However, only occasional syncytia are observed in vivo (Rice, 1995). The fusion between the gp 120 of the budding virus and the surface CD4 of the cell, results in the death of infected cells without involving other cells. Massive virus budding may destabilize the plasma membrane and lead to cell death (Leonard *et al.,* 1988). Gp 160 mediated down regulation of CD4 also results in the death of HIV infected cells (Koga *et al.*, 1994).

Autoimmune responses from antibodies directed against viral epitopes, but cross-reacting with cellular factors could lead to the death of healthy cells. It has been shown that uninfected $CD4^+$ T-cells coated with gp 120 shed into the medium (Gelderblom *et al.,* 1985) may become susceptible to lysis through anti-gp 120 antibody dependent cellular cytotoxicity (Weinhold *et al.*, 1982). Activation induced programmed cell death could lead to depletion of both HIV infected CD4 T lymphocytes and immature thymocytes (Zinkernagel & Hengatner, 1992; Ameisen & Capron, 1991). Finally HIV may contain a super-antigen which may suppress the immune system by depleting or inactivating selected Vβ-containing T cell clones (Imberti *et al.*, 1991; Johnson *et al.*, 1992).

1.9. Opportunistic infections in AIDS

The main pathological feature of HIV infection is the progressive destruction of the immune system of the host, leading to a state of multiple infections which hitherto would have been contained by an intact immune system. This immune dysfunction is associated with the progressive depletion of the $CD4^+$ T-lymphocyte cells which are the main target

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cells of HIV infection. The correlation between CD4 cell count and immunodeficiency has become a valuable tool to assess progression of HIV/AIDS infection. At a CD4 count below 200 cell/µl most of the opportunistic infections (OI) begin to manifest. The spectrum of OI associated with HIV infection is quite wide and spans through microbial infections to some cancers.

In general the progression of HIV infection correlates most strongly with the loss of CD4 lymphocytes, the amount of HIV found in the plasma and the proportion of peripheral blood mononuclear cells (PBMCS) carrying provirus. More importantly, it has been documented that HIV associated deaths are not really due to the HIV itself but indeed to the associated OI. For most people living with HIV/AIDS who cannot afford antiretroviral effective management of OI as they manifest has been shown to have significant clinical benefits. Prompt diagnosis and effective management of these OI is also very important in patients on anti-retroviral. The profile and spectrum of OI vary from country to country and even among communities in the same country.

The HIV/AIDS associated infections are essentially of bacterial, viral, fungal and protozoan aetiologies. The fungal pathogens that have been globally reported as OI in HIV/AIDS include *Candida albicans, Cryptococcus neoformans, Pneomocystis carinii (jirovesi), Histoplasma capsulatum, Aspergillus species, Blastomycosis dermatitidis* and *Coccidiodiodes immitis* (Minamoto & Rosenberg, 1997; Muller *et al*., 1999).

C. neoformans is by far the most common life-threatening fungal pathogen in AIDS patients. Early in the epidemic, approximately 5 to 8% of these patients develop cryptococcal infection. The incidence of cryptococcosis has decreased where effective antiretroviral treatment is available (Holtzer *et al*., 1998; Michaels *et al*., 1999; Murphy *et al*., 2001). Meningoencephalitis is the most frequent manifestation of cryptococcosis in HIV-infected individuals. The clinical presentation of cryptococcal infections, particularly meningitis may be subtle, with few localizing signs or symptoms (Casadevall, *et al*., 1993; Barchesi *et al*., 1995; Patterson, 1997).

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C. albicans is well known as the most prevalent cause of candidiasis (Nucci *et al.*, 1998; Alviano *et al.*, 2005). There are many species of *Candida* but *C. albicans, C. tropicalis,* and *C. parapsilosis* are considered as the most important in HIV/AIDS. The frequent occurrence of mucous membrane candidiasis in HIV infected patients underscores the importance of T-cell mediated immunity in protection against superficial candidal infections, since immunosuppresion, rather than a novel or hypervirulent strain of *Candida* causes the high frequency of candidal infections. *Candida* causes two major forms of infection: mucocutaneous and systemic. Chronic mucocutaneous candidiasis is a syndrome characterized by T-cell dysfunction and persistent and recurrent candidal infection of the skin, nails and mucocutaneous membranes (Zhang *et al.*, 2002).

The major sites of candidal infections are the oral cavity, the gastrointestinal tract (GI) and the vagina (Odds, 1988; Fichtenbaum *et al.*, 2000). Candidiasis has become a major public health problem as an OI in HIV/AIDS. Studies have shown that oral candidiasis (thrush), is the most frequent AIDS-associated OI, with up to 90% of HIV-infected individuals suffering at least one episode during the course of their disease (Vasquez, 1999). The high incidence of oral candidiasis in HIV/AIDS patients has made candidiasis a leading fungal infection in this immune-suppressed population (Vasquez, 2000; Jankowaska, *et al.*, 2001).

Thrush, in HIV infected patients was described early in the epidemic and is independently predictive of progression to AIDS. The likelihood of thrush, which generally develops in patients with CD4 counts less than 500 cells/µl, increases as the CD4 counts decline. The standard treatment for thrush is clotrimazole, ketoconazole and fluconazole. Candidiasis of the GI frequently involves the oesophagus. In patients with advanced HIV disease with oesophageal candidias, oral thrush is almost always manifested. The diagnosis of candidal oesophagitis in an HIV-infected person is an indication of advanced HIV disease and is a sufficient criterion for the CDC AIDS defining diagnosis (Rex *et al.*, 1995).

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Treatment of candidiasis is complicated by the presence of strains of *Candida* that are resistant to the currently used antifungal agents (Perea *et al.*, 2001; Khan *et al.*, 2003). Currently used antifungal agents are not only limited in number, but many are in addition toxic and very costly (Mehta *et al.*, 2002). Relapse of *Candida* infection is very common (Debruyne, 1997) and this increases the burden of managing this OI. These factors prompt the need for the development of new antifungal agents in order to widen the spectrum of activities against *Candida* and combat strains expressing resistance to the available antifungals.

Moreover, the number of immunocompromised patients is on the increase due to great progress in the field of intensive care, organ transplantation and AIDS. HIV-positive patients have developed resistance to treatment with fluconazole, the most currently used antifungal. In some cases, resistance to fluconazole triggers cross-resistance to other ozoles or pathogen shift from *C. albicans* to less sensitive species such as *C. glabarta* and *C. krusei* (Bastert, 2001). Despite the existence of potent antifungal agents, the appearance of resistance or multiresistant strains imposes the need for a permanent search and development of new drugs (Silver & Bostian, 1993).

Bacterial infections common in HIV/AIDS include: *Mycobacterium tuberculosis, Mycobacterium avium* complex, *Streptococcus pneumonia, Haemophilus influenzae, Pseudomonas aeruginosa, Klebsiella pneumoniae, Norcadia asteroids Staphylococcus aureus,* and *Enterococcus faecalis* (Goozé, 1998).

S. aureus is one of the most common bacterial infections in AIDS patients. It causes cutaneous such as impetigo and systematic infections such as septicaemia (Augenbraun, 2000). *P. aeruginosa* is an important nosocomial pathogen causing significant morbidity and mortality. It is clear that *P. aeruginosa* as well as other *Pseudomonas* species are emerging as important opportunistic pathogens in AIDS . *P. aeruginosa* is a highly relevant opportunistic pathogen of immunocompromised individuals; it typically infects the pulmonary tract, urinary tract, burns, and wounds, and also causes other blood infections (Rolston *et al.*, 1987; Witt *et al.*, 1987; Fitchtenbaum *et al.*, 1995).

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E. faecalis can cause life threatening infections in humans, especially in the nosocomial environment. It causes endocarditics as well as bladder, prostate and epididymal infections (Pelletier, 1996; Ryan & Ray, 2004). Virulent strain of *E. coli* can cause gastroenteritis, urinary tract infections and neonatal meningitis (Abongo'o and Momba, 2008).

1.10. Transmission of HIV

HIV is transmitted through three main ways: sexual transmission, blood transmission, mother to child transmission. The majority of HIV infections are acquired through sexual intercourse with a partner who is infected with HIV. Globally between 75% and 80% of all HIV infections are transmitted through the sexual route. More than 70% of those engaged in sexual intercourse, is by heterosexual intercourse with approximately 5% to 10% of homosexual intercourse. Certain risk factors such as promiscuity, anal intercourse, prostitution appear to be responsible for an increased risk of heterosexual transmission (Quinn, 1994).

The use of blood or blood products accounted for a devastating epidemic among haemophiliacs in the 1980s. Infection through artificial insemination, skin grafts and organ transplants sharing of contaminated hypodermic needles or equipment (Des Jarlais *et al.*, 1992). The spread of HIV through blood transfusion has almost been brought to nil, since the introduction of HIV antibody testing of prospective blood donors. Through efficient screening of blood donors, observance of safety measures by laboratory and health care personnel and appropriate methods of preventing infection through sexual intercourse, HIV can be controlled (Chamberland & Khabbaz, 1998; Valdisseri *et al.*, 2003).

Mother to child transmission, occurs during pregnancy, at birth, and through breastfeeding. However, where drug treatment and Caesarean section is available, this can be reduced to 1%. Factors that influence the efficiency of mother to child transmission appear to include viral load, CD4 cell count and the level of neutralizing

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antibodies of the mother (Coll *et al.*, 1997; Sabatelli & Scarlatti, 2001). The use of antiretroviral such as zidovudine and nevirapine has helped in reducing the chances of transmitting HIV from mother to child during pregnancy at birth and during breast-feeding (Jackson *et al.*, 2003; Valdiserri *et al.*, 2003).

1.11. Laboratory diagnosis of HIV infection

There are two categories of tests for diagnosis of HIV infection. The first is the detection of antibodies in a patient's sample, and the second is the detection of the viral antigen (Rakundo *et al.*, 1997; Nkengasong *et al.*, 1998; Ayouba *et al.*, 2001). Commonly used specimens are serum, plasma, whole blood, and oral mucosal transudate.

Generally, HIV testing consists of screening for antibodies by using rapid tests, agglutination assays and enzyme linked immunosorbent assays (ELISA) (Gutler, 1996; Branson, 2002). The HIV rapid test can be very reliable if a good algorithm is used. They are generally cheap and easy to perform and very useful for surveillance purposes. HIV enzyme –linked immunosorbent assay (ELISA) requires more equipment, time consuming and technical skills. Western Blot is normally used to confirm results of rapid test (Gutler *et al.*, 1998; Nkengasong *et al.*, 1992). When a western blot test is used to detect HIV-1 infection, p24 antigen is one of the three major proteins tested for, along side gp 120/ gp 160 and gp 41 (Centers for Disease Control, 2001).

Rapid tests and ELISA are subject to false negative and positive results, while Western Blot is expensive and subject to indeterminate results. False results could also be due to the delay in the production of antibodies for several months after infection, as well as the evolution of highly divergent HIV strains with the propensity of escaping routine diagnostic tests . Specimens with nonreactive result from the initial ELISA are considered negative to HIV antibodies and a re-test with a new sample is recommended after three months. Samples reactive in initial testing are retested in duplicate to confirm initial observation (Mims *et al.*, 1998; Gutler *et al.*, 1998).

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The viral presence can be detected by the polymerase chain reaction to detect specific viral antigen or in the estimation of viral RNA load. P24 antigen test which is not routinely used is very specific but a negative result does not rule out infection. Lastly there is viral culture which is more time consuming, expensive and can take up to 4 weeks for results to be available (Luft *et al.*, 2004).

1.12. Treatment of HIV/AIDS

A turning point in the global control of the pandemic was the development of antiretroviral drugs (ARVs) (Hu *et al.*, 1996; Corbet *et al.*, 2000; UNAIDS, 2000). The main goal of ARV therapy is to decrease viral load and increase CD4 cell count. This will eventually lead to reduction of morbidity and mortality, enhance quality of life, and reduce transmission of HIV to infants and sexual partners (Palella *et al.*, 1998; Jaggy *et al.*, 2003. The first therapy became available in the late 1980s with the approval of zidovudine (AZT; 3′-azido- 3′-deoxythymidine) (Larder *et al.*, 1989).

Since the discovery of zidovudine as an antiretroviral agent HIV, drug therapy has been used widely in the treatment of AIDS. The loss of the therapeutic effect of zidovudine as a result of the acquisition of resistance was first recognized by Larder *et al* (1989) who demonstrated that HIV isolates from patients with advanced HIV-1 disease became less sensitive to the drug during the course of treatment (Larder *et al.*, 1989). Following this observation, many drugs that target different steps of the viral life cycle have been developed. As of mid-June 2002, ten inhibitors of HIV-1 reverse transcriptase (RT) and six inhibitors of HIV-1 protease (PR) have been licensed for clinical use. Classical monotherapy with zidovudine has been superseded by more powerful triple and quadruple combinations of drugs that include RT and PR inhibitors (Gulick *et al.*, 1997; Hammer *et al.*, 1997; Perelson *et al.*, 1997).

Since HIV develops resistance easily to monotherapy, treatment of HIV infection now consists essentially of highly active antiretroviral therapy (HAART). HAART has made an enormous contribution to the reduction of morbidity and mortality (Palella *et al.*, 1998). HAART is defined as a treatment with three or more ARV, typically two

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nucleoside or nucleotide reverse transcriptase (NRTIs) plus a non-nucleoside reverse transcriptase (NNRTIs) or a protease inhibitor (PIs). The main goal of HAART is to reduce the viral load to undetectable levels, allowing the immune system to rebound. (Finzi *et al.,* 1999; Marodon *et al.,* 1999; Appay *et al.,* 2002).

By the combination of several potent antiretroviral agents, viral replication is suppressed to such low levels that emergence of drug resistant HIV-1 variants, if not prevented, is at least delayed. In addition, CD4+ T-lymphocyte numbers increase, leading to a degree of immune reconstitution that is sufficient to reverse clinically apparent immunodeficiency. Widespread introduction of HAART in industrialised countries resulted in a striking decrease in morbidity and mortality, putting forward the hope that HIV-1 infection can be transformed into a treatable chronic disease (Hogg *et al*., 1998; Mocroft *et al*., 1998; Palella *et al*., 1998).

1.13. Drug classes and mechanisms of action

Different groups of ARVs based on the site and their mechanism of action are in use for the treatment of HIV/AIDS infection. The first group of ARVs is the non- nucleoside reverse transcriptase inhibitors (NNRTIs), it stops HIV production by binding directly onto reverse transcriptase enzyme thus preventing the transcription of viral RNA into viral DNA (Saag *et al*., 1993). The second group is the nucleoside reverse transcriptase inhibitors (NRTIs). These are prodrugs which are phosphorylated when taken up by cells, and cause chain termination when incorporated during elongation in the synthesis of the viral DNA molecule (Volberding *et al.,* 1990; McLeod & Hammer, 1992).

The third group is the nucleotide reverse transcriptase inhibitor (NtRTIs), it acts at the same stage of the viral life cycle as the NRTIs, but does not require to be phosphorylated. The fourth group comprises the protease inhibitors (PI). PI act by binding to and blocking the HIV protease enzyme that is essential to cleaving the *gag-pol* polyproteins (Miller *et al*., 1989; Wlodawer *et al.*, 1993; Raguin *et al.,* 2004; Johnson *et al.,* 2005). The fifth group is the fusion inhibitors (FI). They bind to the transmembrane protein and prevent fusion between the host cell and virus (Pomerantz & Horn, 2003). The sixth group is the

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integrase inhibitors. Integrase inhibitors interfere with the ability of the HIV DNA to insert itself into the host DNA through the 3'-end processing activity of integrase and thereby preventing the expression of viral proteins.

1.14. Limitations of currently used ARVs

There are several concerns about ARVs treatment. The main limitation is that currently used ARVs are not curative. There is also the issue of serious side effects, coupled with resistance development (Saitoh *et al.*, 2005). In addition, in some cases HIV patients have to take the drugs at various times during the day, which may lead to default and an increased chance of resistance development (Dybul *et al.*, 2002). Furthermore, ARVs are expensive and resource intensive and the majority of the world's HIV infected individuals cannot access treatment services.

Due to the high number of HIV infected people and the rapid emergence of drug-resistant strains, the demand for new antiviral therapeutics against HIV is great. Therefore there is a need to search for treatment alternatives. The alternative choice has to be less expensive, available to all who need it, curable and with manageable side effects. One of the possible approaches is the screening of traditional medicinal plants based on their ethnomedicinal data (Locher *et al.*, 1996; Vlietinck *et al.*, 1998).

1.15. The use of natural products in treatment of HIV infection

It was reported previously by World Health Organization (WHO) that about 80% of Africans use traditional medicines to treat various diseases (Farnsworth & Soerjarto, 1991). It has also been shown that many traditional medicinal plants have anti-microbial properties (Meyer *et al.*, 1996; Eloff, 1999; Lall & Meyer, 1999; 2000; Eloff *et al.*, 2001; Morris, 2002). The WHO estimates that 3.5 million people in developing countries rely on plant-based medicine for their primary health care. In Africa, which is home to 28.1 million of the world's 40 million AIDS patient, traditional healers are a crucial component of the health care system, and may be the only source of care and treatment for the poor and disenfranchised. Herbal therapies are growing rapidly in popularity both in the developing and industrial countries. The Food and Agriculture Organization (FAO)

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estimates that between 4000 and 6000 species of medicinal plants are traded internationally with China accounting for about 30% of exports. The benefits to be gained by screening plants for potential medical efficacy are substantial both economically and socially (Duke & Ayensu, 1985).

In African countries, traditionally used medicinal plants are sold in market places or prescribed by traditional healers in their homes (Fyhrquist *et al.*, 2002). Herbal medicine still plays a vital role in health care delivery systems especially in remote places where clinics and hospitals are sparsely located. In these communities, traditional herbalists operate closer to the people, taking advantage of biodiversity of plant species in such areas to cure various diseases and ailments (Gelfand *et al.*, 1985; Ndubani & Hojer, 1999). Although herbal medicine is well established in many cultures and traditions of Africans, and is still a way of life of almost 80% of the people in Africa (Cotton, 1996; Jager *et al.*, 1996), more information still has to be documented in scientific literature. Information on herbal medicine in Africa has been dominated by oral tradition (Van Wyk *et al.*, 1997). Because of the strong dependence on plants as medicines it is important to study their safety and efficacy (Farnsworth, 1994). Additional efforts to discover new medicinal compounds involve working with local and indigenous peoples. This would be of great value to global health, provided that the development and benefits gained from new molecular entities derived from biodiversity are equally distributed both to people who need the medication and the countries and regions from which they have been discovered (King, 1996).

A large proportion of the South African population use traditional medicine to serve the health needs of humans and animals. With the increasing acceptance of herbal medicine as an alternative form of health care, the screening of medicinal plants for bioactive compounds is important (Masoko *et al.*, 2005). Medicinal plants have become the focus of intense study recently in terms of conservation, and as to whether their traditional uses are supported by actual pharmacological effects or merely on folklore (Locher *et al.*, 1995). Many medicinal plants produce a variety of compounds of known therapeutic properties. Substances from plants that can either inhibit the growth of pathogens or kill

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them and have little or no toxicity to host cells are considered good candidates for developing new antimicrobial drugs (Masoko *et al.*, 2005). In recent years, antimicrobial properties of medicinal plants have been increasingly reported from different parts of the world and higher plants are still regarded as potential sources of new medicinal compounds (Cowan, 1999).

Antibiotic resistance has become a global concern (Westh *et al.*, 2004). There has been an increasing incidence of multiple resistances in human pathogenic microorganisms in recent years, largely due to indiscriminate use of antimicrobial drugs commonly employed in the treatment of infectious diseases. This has necessitated the search for new antimicrobial substance from various sources such as medicinal plants. Many plants belonging from different families have been screened for the search for new antibacterial agents and several herbs have been identified as potential sources of drugs (Shultes, 1992; Iwu, 2002). The screening of plant extracts and plant products for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotic prototypes (Plotkin, 1988; Meyer *et al.*, 1997; Afolayan, 2003; Fennel *et al.*, 2004; Tshikalange *et al.*, 2005).

Several plants have also been used to treat viral infections in the past. However, the first recognized interest in their evaluation as antiviral agent is the efforts of the Boots drug company (Nottingham, England) which screen 288 plants for anti- influenza activity (Chantrill *et al.*, 1952). Later studies have reported the inhibitory effects of medicinal plants extracts on the replication of several viruses, such as herpes simplex virus type 2 (HSV-2) (Debiaggi *et al.*, 1988), HIV (Vermani & Garg, 2002; Asres & Bucar, 2005), hepatitis B virus (HBV) (Huang *et al.*, 2006) and emerging viral infections associated with poxvirus and severe acute respiratory syndrome (SARS) virus (Kotwa *et al.*, 2005).

The molecular mechanisms associated with the antiviral effects of plant extracts may vary among different viruses. However, the potentials of plant extract to boost inherent antiviral defence of human body which involves an intricate immune system might utilize

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common pathways. In recent past, a number of studies have explored immunostimulatory properties of plant extracts having antiviral properties (Webster *et al.*, 2006).

1.16. Antimicrobial activities of South African plants

The rich diversity of medicinal plants coincides with the overwhelming HIV/AIDS burden in South Africa. Evidently, a good proportion of AIDS patients seek treatment from traditional healers who administer different preparations from a variety of plants. A South African plant deemed to be useful to HIV/AIDS patients is the use of infusions made from leaves and twigs of *Sutherlandia frutescens* subspecies microphylla (Fabaceae) also referred to as cancer bush. It is widely taken for their immuno-stimulatory property and for their reported ability to increase CD4 counts, decrease viral load, improve weight gain and generally re-establish vitality and a feeling of well being in AIDS patients (*http: //www.sutherlandia.org*) (Bessong, 2008).

The other popular plant is *Hypoxis* also referred to as the African potato or wonder herb. A decoction made from *H. colchicifolia* is normally used as an immuno-stimulatory in the treatment of arthritis, tumours, common colds and flu. Other plants that have been investigated for their ability to inhibit HIV include *Elaeodendron transvaalensis* (Calastraceae), *Bridelia micrantha* (Euphorbiaceae) and *Combretum molle* (Combretaceae) (Bessong *et al.*, 2005).

Several evaluations of South African plants for activity against bacteria have been documented. The use of plants in traditional medicine was validated by screening crude plant extracts against potential pathogens, and in some cases molecules with specific bioactivity were identified (Meyer *et al.*, 1997; Fennel *et al.*, 2004; Tshikalange *et al.*, 2005). Of interest are those organisms which are common opportunistic infection in AIDS related complex and AIDS. *Euclea natalensis* (Ebenaceae) afforded the known compound diospyrin and its amino acetate derivative found to be active against sensitive and resistant strains of *Mycobacterium tuberculosis,* the causative agent of tuberculosis and a case defining illness in AIDS (Lall & Meyer, 2001; Lall *et al.*, 2003). Obi *et al* (2002, 2003) reported the antibacterial activity of aqueous and methanol extracts of

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medicinal plants, used in the Venda Region of South Africa, against enteropathogens such as *Campylobacter* responsible for *Campylobacter*-associated diarrhoea in HIV/AIDS patients with substantial morbidity and mortality (Quinn, 1997; Coker *et al.*, 2002). Martini and Eloff (1998) isolated unidentified active compounds from *Combretum erythrophyllum* that inhibited the growth of *Staphlyococcus aureus*. A preliminary screening showed that *Peltophorum africanum* possesses antioxidant and antibacterial compounds (Bizimenyera *et al.*, 2005). Methanol and aqueous extracts of *Dombeya rotondifolia* (Hochst), *Terminalia sericea* and *Gunnera perpensa* are bactericidal against *S. aureus*, implicated in skin infections (Miller *et al.*, 2003).

Several investigations have also been done on antifungal activity of South African plants. Motsie and colleagues (2003) reported the antifungal effect of extracts from *Polygala myrtifolia* (Polygalaceae) and *Glycyrrhiza glabra* (Fabaceae) against *Candida albicans,* responsible in the most part for oral and oesophageal thrush common in HIV/AIDS patients (Lattif *et al.*, 2004). Masoko *et al* (2006) reported that *Terminalia* and *Combretum* species possess substantially antifungal properties. Three unidentified compounds isolated and characterized from *C. nelsonii* showed excellent antifungal activities (Masoko, 2006). Antifungal activity from the leaves of *Curtisia dentate* has also been reported (McGaw *et al.*, 2007). The observations from screening assays, and the isolation and identification of antimicrobial compounds tend to explain the rational for the use of plants in traditional medicine against diseases of bacterial, viral or fungal etiology.

1.17. STUDY RATIONALE

In an earlier pilot study, a crude methanol extract from the stem-bark of *Peltophorum africanum* was evaluated for anti-HIV activity. The extract showed a strong inhibition against HIV-1 RT DNA polymerase and RNase H activities. An oxidized gallotannin from the extract also inhibited HIV-1 reverse transcriptase and integrase in an enzyme cell- free system (Bessong *et al.*, 2005). To broaden, the scope of the investigation on *P. africanum* it is important to evaluate different fractions from the plant obtained with different solvent system, in an attempt of identify other possible inhibitory compounds. In

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addition, it is important to evaluate extracts of the plant for anti-HIV replicative properties in infectious cultures.

On the other hand, AIDS is characterized by fungal and bacterial infections, and it is therefore also essential to screen extracts of *P. africanum* against important bacteria and fungi pathogens incriminated in AIDS. The evaluation of extracts of *P. africanum* against different pathogens will provide a collection of data that will be useful in deciding further action regarding the most important inhibitory property inherence in the extracts of the plant. The general aim of the present study was to further characterized fractions and compounds from *P. africanum* on HIV and on clinical fungal and bacterial isolates.

1.18. INVESTIGATIONS PRESENTED IN THIS DISSERTATION

This dissertation is presented in the following format. Chapter one presents a review of literature on the biology of HIV, and opportunistic infections in AIDS and on the use of herbal preparations in the treatment of infectious diseases in general and particularly against HIV and OI common in AIDS. It ends with a synopsis of the use of the medicinal plants in South Africa on HIV, bacterial and fungal infections. Chapter two describes investigations of several fractions derived from *P. africanum* on the activity of HIV-1 *reverse transcriptase* in a cell free enzymatic assay and in infectious cultures. Chapter three deals with studies of fractions from *P. africanum* on selected bacterial and fungal pathogens. Each investigative chapter comprises an abstract, introduction, study objectives, materials and methods, results, discussion and conclusion, and references. The dissertation concludes with recommendations for further investigations based on the results described herein.

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CHAPTER TWO

INHIBITORY PROPERTIES OF *PELARGONIUM AFRICANUM* (SOND) EXTRACTS ON HIV-1 REPLICATION

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2.1. ABSTRACT

Methanol crude extract, fractions of the extract, and isolated compounds were evaluated for bioactivity against HIV-1 *reverse transcriptase* (RT). Fractions and compounds with high inhibition were also tested against viral replication in infectious cultures. Fractions were obtained by silica gel column chromatography and further purified by Sephadex LH-20 column chromatography. Fractionation led to the isolation of a white powdered compound which was identified as bergenin by spectroscopic methods. Inhibition of HIV-1 RT was performed using a RT colorimetric assay. A total of 28 fractions were obtained. Fraction C2 inhibited RT activity by 92% at 250 mg/ml. Other fractions such as B4, B6, D3 and D6 gave 87%, 84%, 78% and 50% inhibition respectively. C2 had significant inhibition at 100 and 250 mg/ml ($P< 0.05$). In infectious cultures evaluation for intracellular mode of action, Bergenin did not inhibit HIV-1 replication, while the strongest inhibition was observed for fraction C2 with a mean IC_{50} of 9.5 µg/ml based on p24 production. C2 shows the best activity in the cell-free anti HIV assay and in infectious cultures. In addition C2 did not exhibit any cytotoxicity at concentrations less than 250 µg/ml in a CellTiter Cell Proliferation Assay. C2 is worthy of further chemical and biological analysis.

2.2. INTRODUCTION

Human immunodeficiency virus (HIV) is responsible for substantial degree of morbidity and mortality worldwide. Globally other medicinal plants that have also been tested against RT. This includes *Andrographia paniculata* Nees. (Acanthaceae) indigenous to Indonesia and India (Otake *et al.*, 1995); *Anogeissus acuminate* Roxb. Ex DC. Guill. and Perr (Combretaceae), indigenous to Bangladesh, India, Burma, Thailand and Vietnam (Rimando *et al.*, 1994); *Lepidobotrys staudtii* Engl. (Lepidobotryaceae) indigenous to Cameroon (Bokesh *et al.*, 1996); *Maprounea africana* Muell-Arg. (Euphorbiaceae) indigenous to Central African Republic and Tanzania (Beutler *et al.*, 1995; Pengsuparp *et al.*, 1994

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The shortcomings of antiretrovirals (ARVs) have led to the rampant use of medicinal plants for treatment of HIV/AIDS in endemic regions such as South Africa. Examples of plants frequently used to treat AIDS in South Africa include *Sutherlandia frutescens, Hypoxis colchicifolia,* and *H. hemerocallidea* (Bessong, 2008).

Improved access to ARVs in developing countries is accompanied by the simultaneous use of herbs. In terms of efficacy is very important to know the interaction between the uses of ARVs simultaneously with herbal preparations. It was reported previously that the *H. hemerocallidea* (African potato) has the potential to interact with HIV-drug metabolizing enzymes by its near total inhibition of the action of the cytochrome P-450 system CYP3A4 normal isoform activity, moderation of the activity of drug transporter p-glycoprotein and stimulation of the activity of pregnane X receptor (Mills *et al.*, 2005). The pharmacology of *Hypoxis* reveals hypoxoside as an important constituent, alongside β-sistosterol, stigmasterol, β-sistosterol glycoside and stigmastanol, which have biological activity. *S. frutescens* showed a near complete inhibition of CYP3A4, moderated the activity of p-glycoprotein and activated pregnane X function by more than two-fold in a dose-dependent manner. Drug resistant, drug toxicity and treatment failure could have result from interaction when ARVs and herbs were taken together (Mills *et al.*, 2005).

P. africanum is a woody plant commonly found in Southern Africa (Palmer & Pitman, 1972). Its heartwood and leaves have been reported to be rich source of tannins (El Sherbeiny *et al.*, 1977; Bam *et al.*, 1988; Bam *et al.*, 1990), the bark in local traditional medicine is claimed to be a remedy for abdominal disorders, diarrheas, dysenteries and infertility, wounds, toothache, sore throat, tuberculosis (Wild & Gelfand, 1959; Watt & Breyer-Brandwijk, 1962; Arnold & Gulumian, 1984; Van der Merwe, 2000; Van Wyk & Gericke, 2000; Obi *et al.*, 2003). Cytotoxicity experiments performed previously using a human epitheloid cervical carcinoma cell line to indicate that *P. africanum* could be safe (Bessong *et al*, 2005).

Phytochemical investigation of the stem bark of *P. africanum* previously led to the isolation of a coumaric acid ester of bergenin and nonbergenin (Tschitschibabin *et al.*, 1929; Kalidhor *et al.*, 1981; Taneyama *et al.*, 1983; Bessong *et al.*, 2005). Phytochemists have also found several condensed flavonoids, a novel cyanomaclurin analogue (Bam *et al.*, 1988), profisetinidic-type-4-aryflavan-3-ols and related δ-lactones in the heartwood of *P. africanum*

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flavonoids are also hydroxylated phenolic substances but occur as a C6- C3 linked to an aromatic ring. Their activity is probably due to their ability to complex with extra cellular and soluble proteins and to complex with bacterial cell wall (Bam *et al.*, 1990).

2.3. OBJECTIVES OF THE STUDY

2.3.1. General objectives:

The general objective of this aim of the study was to investigate the antiviral activities of methanol crude extract of the stem-bark of *P. africanum*, and several fractions from the crude extract to inhibit the RNA-dependent-DNA polymerization function of recombinant HIV-1 RT in a cell-free assay, and to evaluate fractions of the methanol extract and isolated compounds on HIV-1 replication using infectious cultures. This objective was addressed through the following specific objectives:

2.3.2. Specific objectives of the study

The specific objectives were:

1. To prepare several fractions from the stem bark of *P. africanum* using different solvents systems.
2. To screen obtained fractions for biological activity against HIV-1 RT.
3. To identify fractions and compounds with activity against HIV-1 RT.
4. To determine inhibition of HIV-1 replication by fractions using infectious cultures.

2.4. MATERIALS AND METHODS

2.4.1. Identification and collection of plant material

Stem bark of *P. africanum* was collected in April 2006 from a mature tree growing naturally within Vhembe district, Limpopo, South Africa. The plant was identified by Mr. P. Tshisikhawe of the Department of Biological Sciences, Botany Unit, University of Venda, South Africa. A voucher specimen BP01 has been deposited in the herbarium of the Botany unit at the University of Venda.

2.4.2. Preparation of crude methanol extract

Plant material was rinsed thrice with distilled water, dried in the oven (37°C) for two weeks. Dried plant material was chopped into small pieces and ground into fine powder using a grinder (Reicht, Labotec, S.A.). Two hundred grams of powdered material was stirred in 1litre of methanol (analytical grade, Merck®) and left overnight on a platform shaker

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qualitative Whatman filter paper No.3 (W&R, England, UK). Residues were reconstituted twice with one liter of methanol for further extraction. Filtrates were evaporated to dryness using a rotary evaporator (RE200, Bibby Sterillin, Ltd, UK) at 40°C. Crude extract was stored in the dark at 4°C for subsequent fractionation experiments (Bessong *et al.*, 2005).

2.4. 3. Solvent-solvent fractionation of crude methanol extract

The extract was fractionated using solvents of different polarities. Three solvents were selected: hexane which is non-polar, dichloromethane (DCM) which is intermediate and n-butanol (Merck®) which is polar. The use of solvent of different polarity was an attempt to extract a diversity of compounds from the crude methanol extract. In other words non-polar solvent will dissolve non-polar compounds, intermediate solvent will dissolve intermediate compounds and polar solvent will dissolve polar compounds.

One hundred and forty gram of methanol crude extract was dissolved in 10% distilled water (dH_2O) and methanol (MeOH) mixture and thoroughly mixed. The mixture was suspended in 500 ml of 10% dH2O: MeOH mixture and added into 2000 ml separating funnel and successively extracted 3 times with 500 ml hexane (500 ml x 3), (500 ml DCM x 3), (500 ml n-butanol x 3). The content of the separating funnel was thoroughly shaken and the immiscible part transferred into new flask and concentrate using Rotavapor (Büchi Rotavapor R-114) at 50°C. An empty bottle was weighed out and labeled and then add the concentrated extract and leave air dry. This is done for quantitative determination of extract. N-butanol extract of 46 g, DCM extract of 36.3 g and water extract of 35 g was obtained.

2.4.4. Thin layer chromatography (TLC)

Thin layer chromatography employs glass or aluminum plates pre-coated with the sorbent (e.g. silica gel 60 F_{254}) to varying thickness depending on the amount to be loaded. The compound mixture is loaded both in the preparative or analytical plates at around 2cm from the bottom and lowered in a tank containing the solvent. The solvent migrates up the plates and separates the compound mixture according to the polarity of the components. Several reagents are used for visualization of the separated materials. TLC has the advantage of being a highly cost-effective qualitative technique in as much as a large number of samples can be analysed or separated simultaneously. The few drawbacks include poor detection of

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2.4.6. Chromatographic and TLC on the n-butanol fraction

Forty gram of the dried butanol fraction obtained from the crude methanol extract was subjected to Sephadex column chromatography, eluted with ethanol then with 30% acetone/water. Thin layer chromatography plates were prepared using butanol/acetic acid/water (BAW) (4:1:5) as a solvent system. One hundred and fifty eight fractions were collected and monitored by TLC. Fractions with similar profiles were combined and further purified by Sephadex column chromatography eluted with ethyl acetate/methanol/water (EMW) (7:3) solvent system. TLC plates were run using EMW (7:3), visualized using UV light and then sprayed with fresh vanillin. Thereafter, the plates were heated at 110°C for a few minutes for optimal colour development.

Subsequently, the crude methanol extract was further fractionated using dichloromethane, n-butanol and hexane and eluted with different proportions of dichloromethane/methanol (Figure 2.1). This was done to increase the chances of isolating different compounds. Figure 2.2 is a flow diagram that describes this second fractionation process. Fractions B1-B8, D1-D7 and H1-H6 were obtained.

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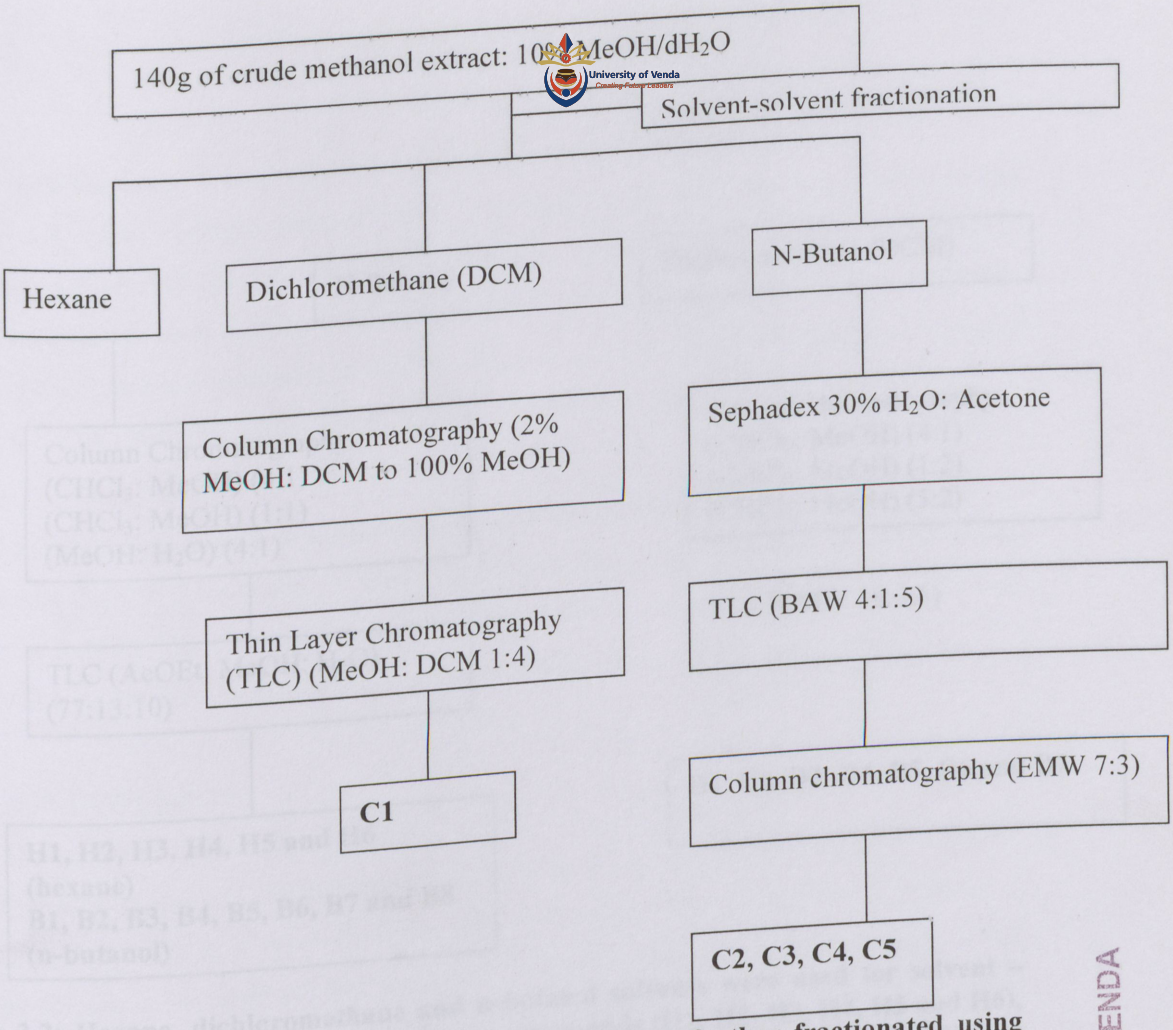


Figure 2.1: Dichloromethane and n-butanol solvents were further fractionated using different solvents in order to obtain different compounds (C1, C2, C3, C4 and C5). Column chromatography using (Silica gel 60F_{254} Merck®), eluted with MeOH: DCM ranging from 2% to 100% MeOH was performed with DCM extracts. To collect fractions of similar profiles, TLC was performed eluted with MeOH: DCM (1:4). N-Butanol fractions were separated by Sephadex LH-20 using 30% H_2O: Acetone as an eluent. Fractions of similar profiles were obtained when TLC was performed using n-butanol/acetic acid/water (BAW) (4:1:5). Similar profiles obtained were combined and further separated by column chromatography and eluted with ethylacetate/methanol/water (EMW) (7:3).

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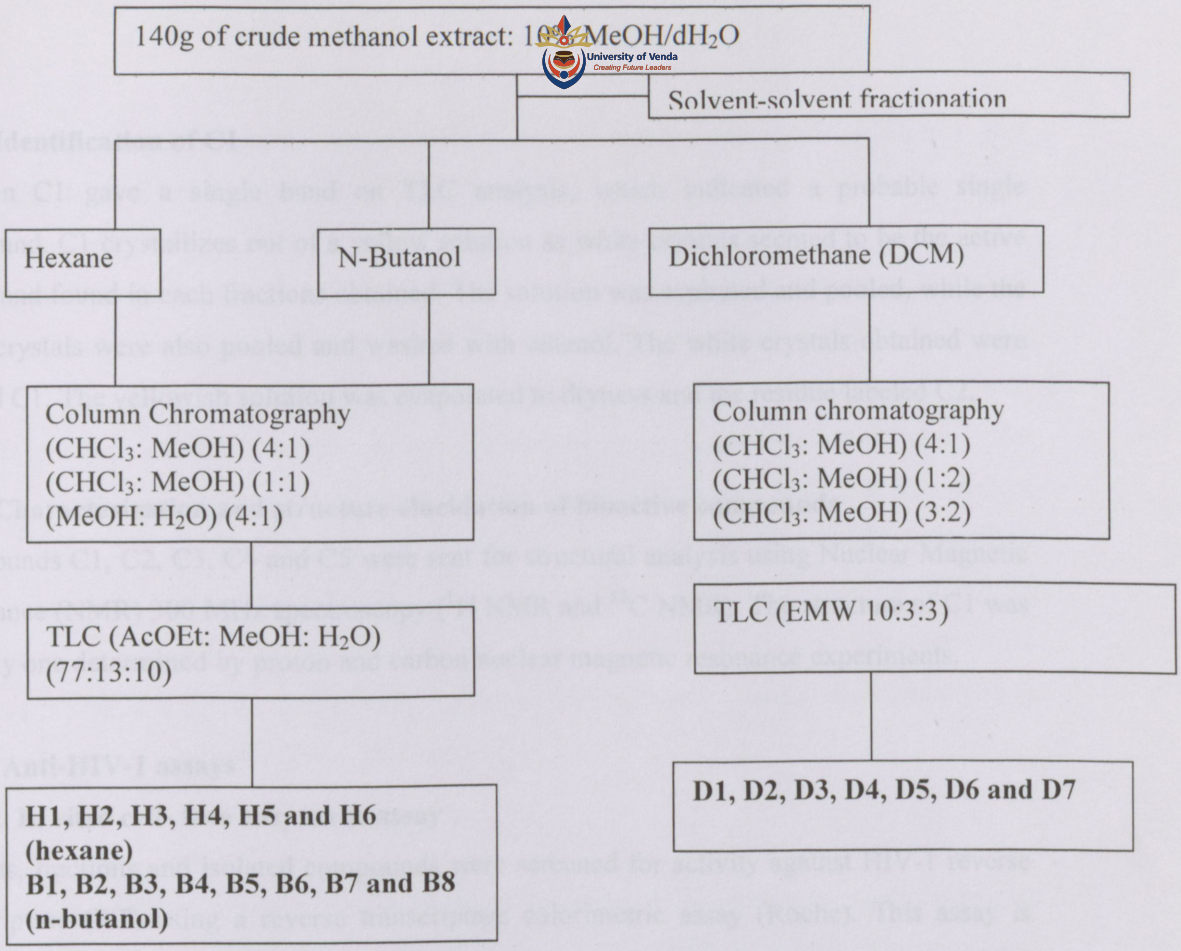


Figure 2.2: Hexane, dichloromethane and n-butanol solvents were used for solvent – solvent fractionation to obtain extra different compounds (H1, H2, H3, H4, H5 and H6), (D1, D2, D3, D4, D5, D6 and D7) and (B1, B2, B3, B4, B5, B6, B7 and B8) according to their polarities. Column chromatography using (Silica gel 60F_{254} Merck®), eluted with $CHCl_3$: MeOH ranging from 4:1, 1:1 to 100% MeOH was performed. To collect fractions of similar profiles TLC was performed eluted with MeOH: DCM (1:4). Fractions obtained were further purified using Sephadex LH -20 eluted with $CHCl_3$: MeOH (2: 1).

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2.4.7. Identification of C1

Fraction C1 gave a single band on TLC analysis, which indicated a probable single compound. C1 crystallizes out of a yellow solution as white crystals seemed to be the active compound found in each fractions obtained. The solution was aspirated and pooled, while the white crystals were also pooled and washed with ethanol. The white crystals obtained were labeled C1. The yellowish solution was evaporated to dryness and the residue labeled C2.

2.4.8. Characterization and structure elucidation of bioactive compounds

Compounds C1, C2, C3, C4 and C5 were sent for structural analysis using Nuclear Magnetic Resonance (NMR) 300 MHz spectroscopy (^{1}H NMR and ^{13}C NMR). The structure of C1 was the only one determined by proton and carbon nuclear magnetic resonance experiments.

2.4.9. Anti-HIV-1 assays

2.4.9.1. In vitro cell- free enzymatic assay

Extracts, fractions and isolated compounds were screened for activity against HIV-1 reverse transcriptase (RT) using a reverse transcriptase colorimetric assay (Roche). This assay is designed for use in research studies, as a method for the quantitative determination of RT activity in cell cultures and other biological samples. The assay has been shown to be useful for the determination of RT activity derived from a variety of retroviruses, including HIV-1, HIV-2, simian immunodeficiency virus (SIV-1), Avian Myeloblastosis Virus (AMV) and Moloney-Murine Leukaemia Virus (M-MuLV) (Ukkonen *et al.*, 1988). This assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly (A)-oligo (dT) (Garry *et al.*, 1990). It avoids the use of [^{3}H] – or [^{32}P] - labeled nucleotides employed for the setup of classical RT assay. In place of radiolabeled nucleotides, digoxigenin – and biotin-labeled nucleotides in an optimized ratio are incorporated into one and the same DNA molecule, which will be freshly synthesized by the RT. The detection and quantification of synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol: Biotin-labeled DNA binds to the surface of microplate (MP) modules that will be precoated with streptavidin. An antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD), will bind to the digoxigen-labeled DNA. The peroxidase enzyme catalyzes the cleavage of the substrate 2,2-Azino-bis (3-Ethylbenzthiazoline-6-sulfonic acid)(ABTS) producing a coloured reaction product. The

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absorbance measured as optical densities is determined using a microplate (ELISA) reader and is directly correlated to the level of RT activity in the sample.

A stock concentration of each compound (1 mg/ml) was made by first dissolving in DMSO (2.5%). Three hundred µg/ml of samples were prepared to a final volume of 40 µl in lysis buffer. To this, 40 µl of reaction mixture and 40 µl of enzyme was added making final concentration of samples to be 100 µg/ml. Controls included: negative control (lysis buffer (80 µl + reaction mixture 40 µl), positive control (40 µl lysis buffer, 40 µl reaction mixture and 40 µl enzyme), known inhibitor (40 µl inhibitor, 40 µl reaction mixture, 40 µl enzyme). Final reaction volume was 120 µl. The reaction was then continued according to manufacturer's protocol. The crude extract and a total of 28 fractions were screened. These included B1-B8, C1-C5, D1-D7, H1-H6. Compound C1 earlier isolated and characterized as bergenin (a known compound) was also tested.

2.4.9.2. Infectious culture assay

Five fractions (B4, B6, C1, C2, D3) were tested for intracellular mode of action targeting an HIV-1 subtype C virus. Inhibition of virus from infecting PBMC in the presence of test compounds was estimated by p24 antigen production evaluation.

Each compound was re-suspended in DMSO. Compounds were initially dissolved in 1.5 ml DMSO, except compound C2 which was dissolved in 1.2 ml DMSO. Stock concentrations of 20 mg/ml were prepared as outlined in Table 2.1, using RPMI (10% fetal calf serum (FCS) + IL-2, with the exception of C2 (at 20 mg/ml after the addition of 1.2ml DMSO). Solutions formed either immediately or after vortexing (turbulent suspensions), and the appearance of the stock solutions were dark brown in colour, excluding compound C1 which was clear.

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Table 2.1: Preparations of 20 mg/ml stock solution of test compounds.

Compound	Mass (mg)	Volume of DMSO added (ml)	DMSO stock Concentration (mg/ml)	20mg/ml Stock	
				Vol. DMSO stock added (µl)	Vol. RPMI + IL-2 (µl)
B4	73.0	1.5	48.67	411	589
B6	134.0	1.5	89.33	229	771
C1	123.0	1.5	82.00	244	756
C2	24.0	1.2	20.00	500	0
D3	87.0	1.5	58.00	345	655

2.4.9.3. Test drug dilution preparation

A starting concentration (2x) at 1000 ug/ml was prepared in RPMI (10% FCS) + IL-2 for each compound. Addition of each compound resulted in a colorimetric pH change at the highest concentrations. The starting dilution was used to sequentially prepare 2 fold dilutions with RPMI (10% FCS) + IL-2, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml and 31.25 µg/ml. The PBMC inhibition assay requires an initial 2x concentrated stock of each compound dilution in order to obtain the desired final concentration of 1X. Thus compounds were tested at a final concentration of 500 µg/ml to 15.625 µg/ml. Each dilution was used immediately in the PBMC inhibition assay, and the remainder stored at -20°C for 4 days until required.

2.4.9.4. Characteristics of viruses used

All five compounds were tested for potential inhibitory activity against the primary HIV 1 subtype C viral isolate, 05ZAFV5. Isolate 05ZAFV5 is a CCR5 tropic virus and exclusively uses the CCR5 co-receptor for cell entry.

2.4.9.5. PBMC inhibition assay procedure

The PBMC inhibition assay determines the susceptibility or resistance of HIV-1 primary isolates to various drugs (range of drug concentrations). Briefly, CD8-depleted PBMCs from HIV-1 seronegative donors were infected with 1000 $TCID_{50}$ of primary virus in the presence

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or absence of increasing drug concentrations on a 96 well plate. The assay was performed in triplicate using two different PBMC donors. On day 4, 125 µl of cell suspension from each well was discarded and replaced with 150 µl of each of 1X drug concentrations (stored at -20°C on day of assay set up). Cultures were monitored for 7 days post infection, and then supernatant fluid was harvested. HIV p24 antigen was quantified (Murex p24 ELISA kit; Abbott) and the concentrations were used to establish the percentage inhibition and an IC_{50} for each test drug. The percentage inhibition data was generated in excel and transferred into GraphPad Prism 5. An XY graph was created and analysed by nonlinear regression. Data was presented as a plot of the log (inhibitor) against response (variable slope). GraphPad Prism 5 determines the IC_{50} values (Figure 2.5).

Prior to harvesting supernatant on days 4 and 7, the cells were evaluated microscopically for obvious cytopathic effects (CPEs) due to the presence of drug. AZT (a nucleoside reverse transcriptase inhibitor), was used as a positive control at final concentrations of 10, 2, 0.4, 0.08, 0.016, and 0.0032 µM. The CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) was used to monitor for potential cell death, and subsequently reduction in viral replication, as a result of cytotoxicity from each concentration of AZT and the 5 test compounds, according to the manufacturers instructions.

2.5. RESULTS AND DISCUSSION

2.5.1. Yield of methanol crude extract

From 3700 g of pulverized stem bark of the plant under investigation, 414.13 g of crude methanol extract was obtained; this gives a weight by weight yield of 11.2%. Percentage yield is influenced by the degree of pulverization of material, the chemical nature of the solvent and the duration of contact of solvent and solute. Methanol is also seen as a solvent capable of dissolving many phytochemicals (Cowan, 1999). The output realized, using methanol as the extractant, is comparable to that of other investigators. Shaphiullah *et al.* (2003) reported a 1.0% yield from the whole plant of *Ludwigia hyssopifolia*; and Pramanik *et al.* (2005) reported a 9.38% methanol extract yield from the bark of *Albizzia lebbeck*.

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2.5.2. Identification of pure compounds

Fractions with similar profiles on preparative TLC analysis were combined to give fractions C1 and C2. Fraction C1 crystallized with ethanol to give a white crystalline compound, which displayed a single band on further TLC analysis indicating that it was probably a single compound. Fraction C2 gave an orange coloured compound. Chromatography on the n-butanol residue provided several fractions that were subjected to TLC analyses. Analysis of the TLC profiles led to the isolation of four fractions and they were labelled C2, C3, C4 and C5. It is worthwhile to note that the C2 obtained from the 1-butanol fraction had a similar profile as the C2 that was isolated from the dichloromethane fraction, hence both were designated C2. The other fractions required further purification for spectroscopic analyses. The hydrogen and 13-Carbon spectra of C1 in addition to consultation with structure data base showed that the molecule is bergenin (Figure 2.3), (See Appendix 5.1 and 5.2 for spectra), a known C-galloylglycoside (Taneyama *et al.*, 1983), and previously isolated from the roots of *Astilbe thunbergii* (Han *et al.*, 1998); and from the aerial parts of *Fluggea virosa* (Pu *et al*, 2002). Bergenin has been reported to possess a moderate activity against HIV-1 gp120/CD4 interaction (Paciente *et al.*, 1996); as well as antiarrhythmic (Pu *et al.*, 2002) and hepatoprotective (Lim *et al.*, 2000) properties. The identification of C2, C3, C4 and C5 were inconclusive.

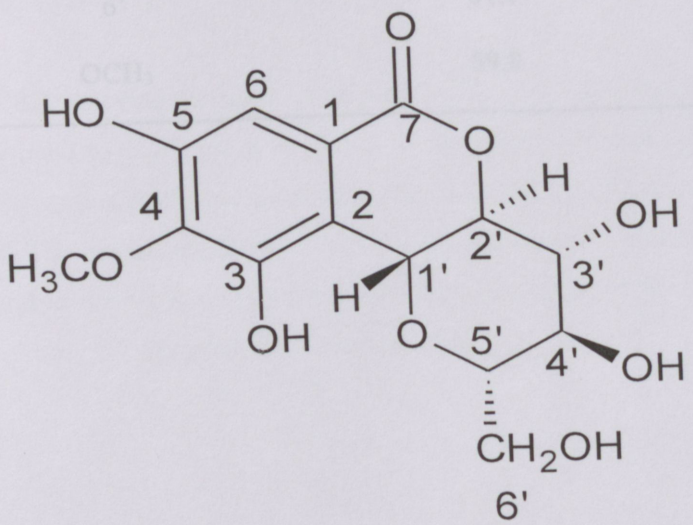


Figure 2.3: Structure of bergenin (C1) obtained from dichloromethane fractionation and n-butanol fractions of the crude methanol extract of *P. africanum* Sond.

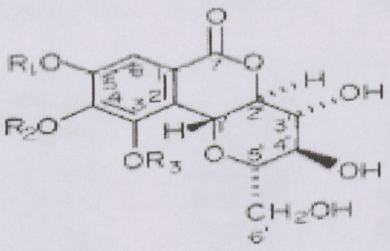
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Table 2.2: ^{13}C NMR chemical shifts of bergenin in DMSO-d_6

Carbon No.	Chemical shift in ppm
1	118.0
2	115.9
3	148.0
4	140.6
5	150.9
6	109.4
7	163.3
1'	72.1
2'	73.7
3'	79.8
4'	70.7
5'	81.7
6'	61.1
OCH_3	59.8

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1 $R_1 = R_2 = R_3 = H$
Norbergenin

2 $R_1 = R_3 = H$, $R_2 = Me$
Bergenin

3 $R_1 = R_2 = R_3 = Me$
Tri-*O*-methylnorbergenin

*Part 3 in the series "Studies on *C*-Glycosides in Higher Plants". For Part 2 see Taneyama, M. and Yoshida, S. (1979) *Bot. Mag.* **92**, 69.

Figure 2.4: Structure of norbergenin, bergenin and trio-O-methylnorbergenin isolated from *Saxifraga stolonifera*.

Taneyama *et al* (1982) reported the same compound in *Saxifraga stolonifera* which was the same compound isolated by Kalidhar *et al* (1981) in *Woodfordia fruticosa* (Luthraceae) and called it norbergenin. In this study, we now report the confirmation of its structure as isolated from *P. africanum*. The compound isolated and its derivatives were identical in every way with norbergenin and its derivatives as describes by Taneyama *et al* (1982) and Kalidhar *et al* (1981) (Figure 2.4; Table 2.2; Appendix 5.1 and Appendix 5.2).

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Table 1. ^{13}C NMR chemical shift of norbergenin (1), bergenin (2) and tri-*O*-methylnorbergenin (3)

Carbon No.	1		2	3
	CD_3OD–D_2O (2:1)	DMSO-d_6	DMSO-d_6	DMSO-d_6
1	117.49 *s*	116.08 *s*	118.03 *s*	126.45 *s*
2	114.08 *s*	112.76 *s*	115.93 *s*	119.05 *s*
3	143.31 *s*	142.43 *s*	148.04 *s*	150.80 *s*
4	140.93 *s*	139.61 *s*	140.59 *s*	147.70 *s*
5	146.58 *s*	145.94 *s*	150.92 *s*	152.82 *s*
6	111.25 *d*	109.30 *d*	109.50 *d*	109.00 *d*
7	166.65 *s*	163.73 *s*	163.34 *s*	163.54 *s*
1′	73.88 *d*	72.27 *d*	72.17 *d*	71.00 *d*
2′	75.14 *d*	73.78 *d*	73.73 *d*	73.97 *d*
3′	80.99 *d*	79.82 *d*	79.82 *d*	80.16 *d*
4′	71.44 *d*	70.90 *d*	70.71 *d*	70.41 *d*
5′	82.40 *d*	81.57 *d*	81.77 *d*	81.60 *d*
6′	62.33 *t*	61.25 *t*	61.16 *t*	61.11 *t*
OMe	—	—	—	61.10 *q*
	—	—	59.84 *q*	60.60 *q*
	—	—	—	56.00 *q*

21.15 MHz. TMS as int. standard.

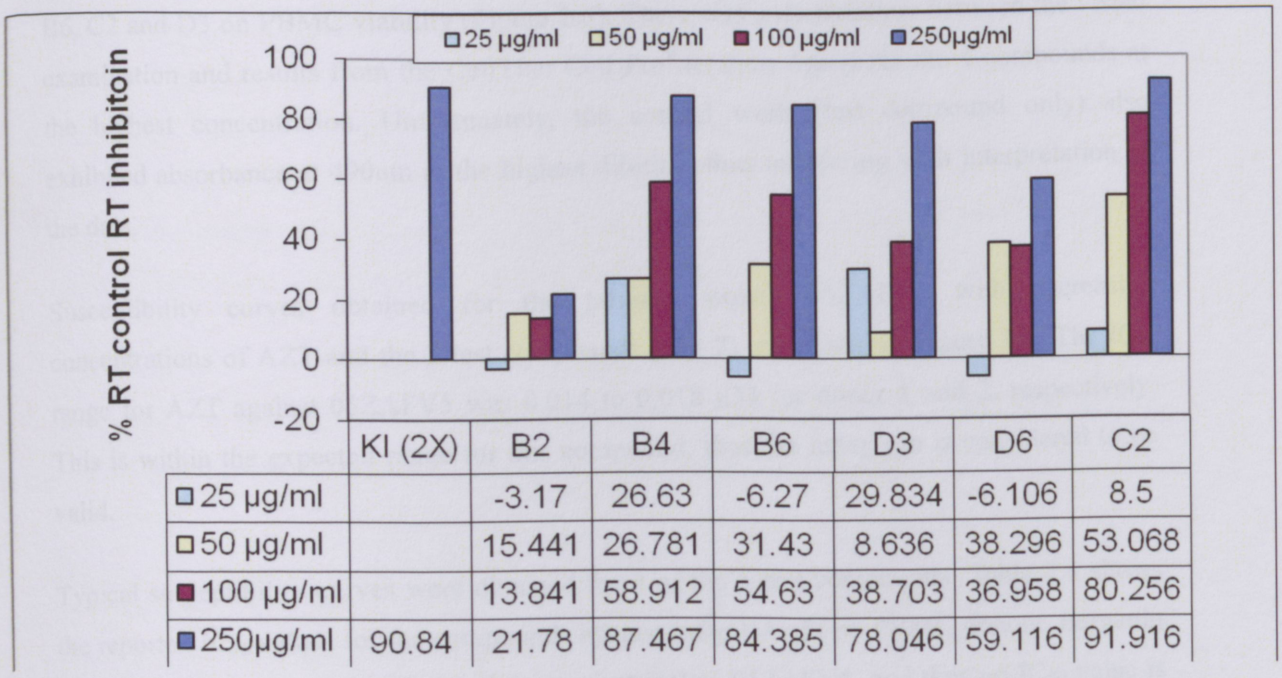
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2.5.3. Bioactivity of fractions and compounds

2.5.3.1 Anti-HIV-RT assays

A total of 28 fractions from the methanol crude extract were screened in a cell-free, ELISA-based assay to determine inhibition of HIV-1 RT. Six fractions (B2, B4, B6, C2, D3, and D6) showed inhibition activity at a screening at 100 µg/ml. Based on this, the fractions were tested at 4 different concentrations (25, 50, 100 and 250 µg/ml). A dose dependent inhibition was observed (Figure 2.5). B4 and C2 exhibited inhibitions above 80% at 250 µg/ml concentration. C2 maintained significant inhibition at 100 and 250 µg/ml ($p<0.05$) in repeat experiments. No inhibition of HIV-1 RT was observed for C1, which was initially identified as bergenin, a known compound.



	KI (2X)	B2	B4	B6	D3	D6	C2
25 µg/ml		-3.17	26.63	-6.27	29.834	-6.106	8.5
50 µg/ml		15.441	26.781	31.43	8.636	38.296	53.068
100 µg/ml		13.841	58.912	54.63	38.703	36.958	80.256
250µg/ml	90.84	21.78	87.467	84.385	78.046	59.116	91.916

Figure 2.5: Inhibition of HIV-1 RT by selected plant extract fractions. The assay is a colorimetric ELISA-based evaluation employing a cell-free enzymatic protocol. KI is a known inhibitor of RT. Percentage inhibition is expressed relative to an untreated control sample.

It is interesting to note that the crude methanol extract from which all fractions were derived using different solvent systems did not show activity against HIV-1 RT in the assay used. As reported previously Bessong *et al* (2005), the crude methanol extract inhibited HIV-1 RT by 98.2% with an IC50 of 8 µg/ml. The biological activity was measured for the ability of the

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extract to abolish the RNA-dependent-DNA polymerase (RDDP) activity of HIV-1 RT. The inhibition was estimated by evaluating the incorporation of methyl-3H thymidine triphosphate ([3H]TTP) by the enzyme using polyadenylic acid-oligodeoxythymidilic acid (polyA-dT) as the template-primer in the presence and absence of plant extract. It is difficult to explain this apparent discrepancy since the two assays are based on a similar principle.

2.5.3.2. Anti-HIV replication properties of fractions and bergenin

2.5.3.2.1. *Cytotoxicity and fifty percent inhibitory concentration (IC_{50}).*

Microscopic examination of the 5 test compound plates revealed cytotoxicity at the highest dilution (500 µg/ml), and to a lesser extent at the second highest (250 µg/ml) dilution. As expected, there was no evidence of cytotoxicity or morphological changes due to AZT. Results from the CellTiter Cell Proliferation Assay confirmed the effect of compounds B4, B6, C2 and D3 on PBMC viability (Figure 2.6). There was a discrepancy between the visual examination and results from the CellTiter Cell Proliferation Assay for the 4 compounds at the highest concentration. Unfortunately, the control wells (test compound only) also exhibited absorbance at 490nm at the highest dilution, thus interfering with interpretation of the data.

Susceptibility curves obtained for the primary isolate 05ZAFV5 with decreasing concentrations of AZT and the 5 test compounds (day 7) are shown in Figure 2.6. The IC_{50} range for AZT against 05ZAFV5 was 0.014 to 0.018 µM for donor 1 and 2, respectively. This is within the expected range for this compound, thus the assay run is considered to be valid.

Typical susceptibility curves were obtained for 4 of the 5 test compounds. Table 2.3 shows the reported IC_{50} values for the compounds against infection of two PBMC donors. Bergenin did not exhibit any antiretroviral activity against isolate 05ZAFV5, and thus no IC_{50} value is reported.

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Table 2.4: IC_{50} values of compounds against virus 05ZAFV5 on two PBMC donors

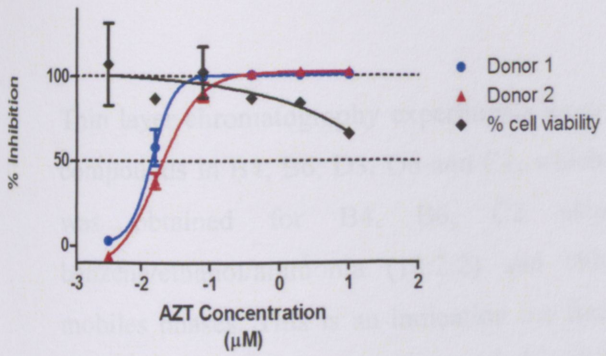
Compound	Fifty percent inhibitory concentration (IC_{50})		Mean	Standard deviation
	PBMC 1	PBMC 2		
B4	26.9 µg/ml	33.3 µg/ml	30.1	4.53
B6	16 µg/ml	20 µg/ml	18	2.83
C2	4.2 µg/ml	14.8 µg/ml	9.5	7.50
D3	24.3µg/ml	34.5 µg/ml	29.4	7.21

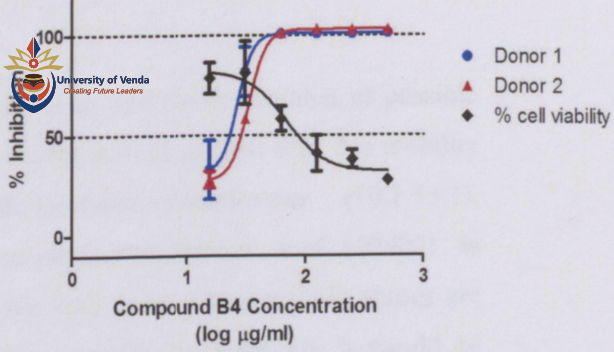
However, these data must be interpreted with caution because the percentage inhibition noted in the two highest concentrations of compounds tested (500 and 250 µg/ml) correlate with the detected cell cytotoxicity. Because cell viability is affected by the presence of test compounds, virus replication is prevented due to the lack of viable targets and is incorrectly interpreted as inhibition. However, since the inhibitory activity is maintained throughout the range of compound concentrations tested, it is likely these compounds exert anti-HIV activity but within the lower concentration spectrum since higher concentrations are cytotoxic.

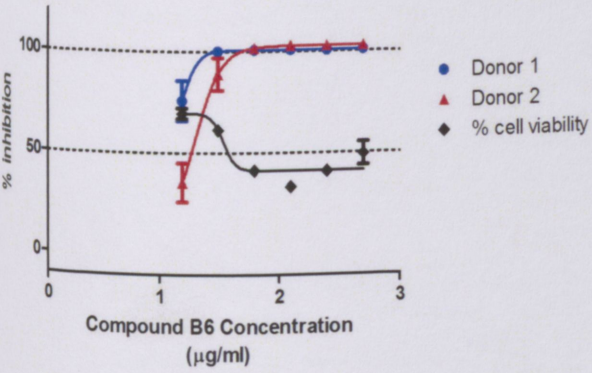
The effect of the test compounds on PBMC viability is either a direct effect of the compounds, or alternatively due to the growth medium pH change seen during the test drug dilution preparation. A different cytotoxicity assay may be needed to adjust for possible cross interference.

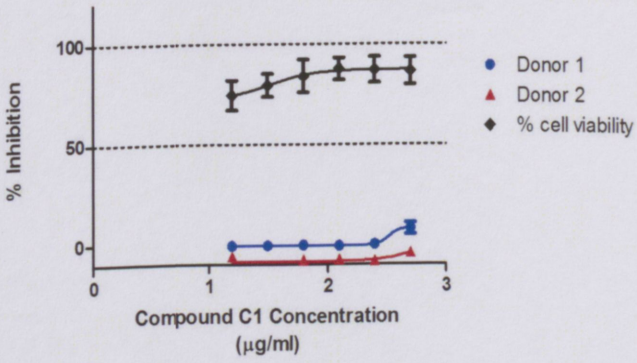
Susceptibility curves and cytotoxicity data were generated for each of the 5 test compounds provided and AZT, using isolate 05ZAFV5. Four of the 5 compounds exhibit antiretroviral activity at low concentrations, but were cytotoxic at the higher concentrations. However, the best inhibition was observed for compound C2 (Figure 2.6).

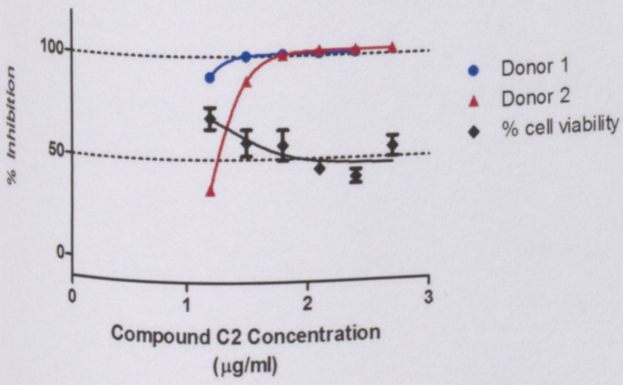
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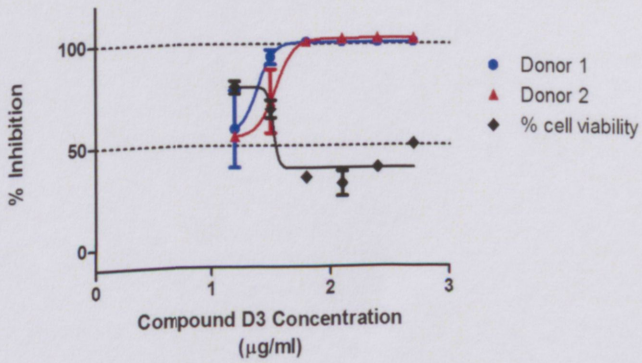


Figure 2.6: The effect of the test compounds on PBMC viability. Data is presented as a plot of the log (inhibitor) against response (variable slope). GraphPad Prism 5 was used to determine the IC_{50} values.

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Thin layer chromatography experiments were carried out to find out the number of possible compounds in B4, B6, D3, D6 and C2, which have shown activity against RT. No mobility was obtained for B4, B6, C2 using ethylacetate/methanol/water (10:1.35:1), benzene/ethanol/ammonia (18:2:2) and chloroform/ethylacetate/formic acid (10:8:2) as mobiles phases. This is an indication the fractions are very polar. Other mobile phases are being assessed to provide the needed mobility. Once mobility is achieved, it would be possible to have an idea of the complexity of these fractions.

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CHAPTER THREE:

BIOACTIVITY OF CRUDE EXTRACTS AND FRACTIONS OF *PELTOPHORUM AFRICANUM* ON SELECTED FUNGAL AND BACTERIAL PATHOGENS

3.1. ABSTRACT

Bioactivity of fractions of the methanol crude extract of the stem bark of *Peltophorum africanum* against the fungi *Candida albicans*, and *Cryptococcus neoformans*; and the bacteria *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* was investigated. To evaluate the inhibition of the plant extract and fractions on these fungi and bacteria, the minimum inhibitory concentration (MIC), and bioautographic profiles were assessed. Twenty eight fractions were initially screened by bioautography and MIC. Pooling was done for similar fractions based on bioautography activity. After two rounds of pooling five fractions gave the best active profiles against one or several of the organisms. These fractions were B4, D1, D7, H1, and H3. Based on the bioautography and MIC, B4 was the most active against *P. aeruginosa, S. aureus, E. coli*, and *E. faecalis*. The question to answer is whether B4 has a broad spectrum or non-specific mode of inhibition. Further studies will elucidate the chemical constituent or constituents of B4 and the mode of action.

3.2. INTRODUCTION

AIDS is a syndrome characterized by a dysfunctional immune system and compounded by opportunistic infections especially of fungal and bacterial etiologies. The positive effect from the use of a particular plant extract in AIDS could be as a result of several factors: antiviral, antibacterial, or antifungal properties, or a boost to the immune system. Consequently, it is acceptable to reason that since other pathogens contribute to the AIDS pathology, it would be worthwhile to evaluate inhibitory properties of the plant extracts against a selection of fungi and bacteria commonly incriminated in AIDS. In this regard, bioactivity against the fungi *Candida albicans*, and *Cryptococcus neoformans*; and the bacteria *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*, which are common among the

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opportunistic pathogens in HIV/AIDS patients were investigated for their sensitivity to a local medicinal plants (Minamoto & Rosenberg 1997; Muller *et al* 1999).

Cryptococcus neoformans is by far the most common life-threatening fungal pathogen in AIDS. Where it accounts for approximately 5 to 8% of all opportunistic infections. It has been reported that meningitis is by far the most common form of cryptococcal infection in patients with advanced HIV disease (Casadevall, *et al*., 1993; Barchesi *et al*., 1995; Patterson, 1997).

There are many species of *Candida* but *C. albicans, C. tropicalis,* and *C. parapsilosis* are considered as one of the most important in HIV/AIDS. The frequent occurrence of mucous membrane candidiasis in HIV infected patients underscores the importance of T-cell mediated immunity in protection against superficial candidal infections. Thus immunosuppresion, rather than a novel or hypervirulent strain of *Candida* causes the high frequency of candidal infections either mucocutaneous or systematic in HIV infected patients (Zhang *et al.*, 2002).

The major sites of Candidal infection are the oral cavity, the gastrointestinal tract (GI) and the vagina. Candidiasis has become a major public health problem as an OI of HIV/AIDS. Studies have shown that oral candidiasis, mostly commonly characterized by development of oral thrush, is the most frequent AIDS-associated OI, with up to 90% of HIV-infected individuals sufferings at least one episode during the course of their disease (Vasquez, 2000).

P. aeruginosa is a highly relevant opportunistic human pathogen most commonly affecting immunocompromised patients, such as those with cystic fibrosis or AIDS (Shanson, 1990; Elkin & Geddes, 2003). *E. faecalis* and pathogenic *E. coli* have been implicated in the pathology of diarrhoea. *S. aureus* is well known as a cause of skin infections. Opportunistic diseases cause substantial morbidity, necessitate toxic and expensive therapies, resulting in hospitalization and shorten the survival of people living with HIV infection (Moore & Chaisson, 1996).

Due to the increasing resistance of bacterial isolates for antibiotics, more efforts are being made to find alternatives to available antibacterial and antifungal components (Berkowitz, 1995). The use of medicinal plants as traditional medicines is well known in rural areas of many developing

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countries (Sandhu & Heinrich, 2005; Gupta *et al.*, 2005), were communities use folk medicines for the treatment of common infections (Rojas *et al.*, 2006). Of particular note are the previous in vitro studies that have revealed the activity of several South African medicinal plants against a good number of bacteria and viruses pathogenic to humans (Meyer *et al.*, 1996; Martini & Eloff, 1998; Eloff, 1999; Lall & Meyer, 2000; Eloff *et al.*, 2001; McGaw *et al.*, 2000, 2001; Lin *et al.*, 2002; Obi *et al.*, 2002, 2003; Manana, 2003; Bessong *et al.*, 2005, 2006, 2008; Samie *et al.*, 2005).

Active compounds of interest that can be used to inhibit fungi or bacteria can be isolated either by using solvents that have selectivity based on polarity, H-donor/acceptor or dipole moment (Snyder & Kirkland, 1979), or alternatively solvents that would extract a wide range compounds (Eloff, 1998a). Different methods for determining minimum inhibitory concentration (Masoko *et al.*, 2005) and bioautography (Masoko & Eloff, 2006) was developed for evaluating antibacterial and antifungal activity of extracts and isolated compounds. On the selected fungi toxicity of different solvents (dimethyl sulphoxide, acetone, methanol and ethanol) was investigated using microdilution method and *p*-iodonitrotetrazolium violet (INT) as indicator (Eloff, 1998b).

3.3. OBJECTIVES OF THE STUDY

3.3.1. General objectives of the study

The general objective of this arm of the study was to investigate the antimicrobial activities of methanol crude extract of the stem-bark of *Peltophorum africanum*, and several fractions from the crude extract on selected bacterial and fungal opportunistic pathogens in AIDS.

3.3.2. Specific objectives of the study

The specific objectives were:

1. To determine the inhibitory properties of the methanol crude extract of *P. africanum* and fractions thereof on bacteria namely *S. aureus*, *P. aeruginosa*, pathogenic *E. coli*, and *E. faecalis*. To evaluate the inhibition of the plant extract and fractions on these bacteria, the minimum inhibitory concentration, and bioautographic profiles was assessed.

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2. To determine inhibitory properties of the methanol crude extract of *P. africanum* and fractions thereof on fungi namely *C. albicans* and *C. neoformans*. This was investigated by similar methods as for the antibacterial evaluations.

3.4. MATERIALS AND METHODS

3.4.1. Test organisms

Two fungi namely, *C. albicans* and *C. neoformans* were used in this study. They were isolated and cultured from clinical cases that were not treated prior to sampling. Cultures were obtained from the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. These fungi are the most common and important disease-causing fungi of animals and human beings (Masoko *et al.*, 2005). These organisms were maintained on 6.5% (w/v) Sabouraud dextrose agar (SDA) (Oxoid, Basingstoke, UK).

Two Gram-positive and two Gram-negative bacteria were also used in this study: *S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. coli* pathogenic and opportunistic bacteria to people infected with HIV/AIDS. They were isolated and cultured from clinical cases that were not treated prior to sampling in the Department of Veterinary, Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. An inoculum of each bacterial strain was suspended in 5 ml of Mueller Hinton broth (Difco, Detroit, MI) and incubated overnight at 37°C.

3.4.2. Antibacterial assays

3.4.2.1. Determination of minimum inhibitory concentrations (MIC)

The minimum inhibitory concentration (MIC) was determined using the microplate serial dilution method. Residues C1-C5, H1-H6, B1-B7 and D1-D8 were dissolved in acetone to a concentration of 10 mg/ml. Distilled water (100 µl) was placed in each well and 100 µl of samples were added in each of the first well of the relevant series. Bacterial cultures were transferred into fresh Mueller Hinton broth (MHB), and 100 µl of this was added to each well. This reduces the concentrations in the wells to 50%. After all extracts has been diluted in the first well 100 µl was transferred from it into the next well. The concentration in this well would then be 25% of the original the next would be 12, 5% and so on. The process was repeated till the last lane of the plate. The first 100 µl from the last row was discarded to ensure that the wells contain

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100 µl of the extract. The first column will therefore be having a series of two dilutions of the first extract. After overnight incubation, 40µl of 0.2 mg/ml of *p*-iodonitrotetrazonium (INT) (Sigma) was added to each raw. The microplates were examined for colour change after 24 hours of incubation. The lowest concentration where growth is inhibited was noted and recorded and the minimum inhibitory concentration (MIC) values of the extract were calculated from the original concentration of the extracts. The positive control comprised gentamycin; and the negative control comprised acetone, this was to ensure that the extraction solvent had no inhibitory effect of its own (Eloff, 1998).

3.4.2.2. Thin layer chromatography (TLC) and antibacterial bioautography

Thin layer chromatography (TLC) plates were prepared in duplicates for each organism. One hundred µg of compound to be tested was spotted on the TLC plates and developed in the different mobile systems: ethyl acetate/methanol/water (40:5.4:4:5) [EMW] (polar/neutral); chloroform/ethyl acetate/formic acid [CEF] (intermediate polarity/acidic); benzene/ethanol/ammonium hydroxide [BEA] (non-polar/basic) (Kotze & Eloff, 2002). The plates were dried overnight under a stream of air to remove excess solvent. TLC were sprayed with excess concentrated broth culture suspensions of actively growing cells of *E. coli*, *E. faecalis*, *P. aeruginosa* and *S. aureus,* followed by incubation overnight at 37°C in a clean chamber at 100% relative humidity. After overnight incubation, the plates were sprayed with 2 mg/ml solution of INT. Clear zones on chromatogram indicated inhibition of growth after incubation for about an hour (Begue & Kline, 1972).

3.4.3. Antifungal assays

3.4.3.1.Determination of minimum inhibitory concentration (MIC)

3.4.3.2.Thin layer chromatography (TLC) and antifungal bioautography

The same procedures as described in Sections 3.4.2.1 and 3.4.2.2 were followed for the investigation of antifungal activity. Except that cultures were grown on Sabouraud agar for 3-5 days. Sabouraud broth was prepared in 250 ml bottles. Cultures were transferred into broth from agar with a sterile swab.

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3.5. RESULTS AND DISCUSSION

3.5.1. Minimum inhibitory concentrations (MIC)

Minimum inhibitory concentration (MIC) is the lowest concentration of test sample dilution expressed in mg/ml, which inhibited all visible bacterial growth on the TLC plate. Twenty eight fractions were initially screened by bioautography and MIC against selected fungi and bacteria. Pooling was done for similar fractions based on bioautography activity and this gave 12 fractions (H1, H2, H3, H4, C2, C5, B4, B6, D1, D3, D4 and D7). The twelve fractions were again subjected to bioautography. Five of the 12 fractions gave the best active profiles against one or several of the organisms. These fractions were B4, D1, D7, H1, and H3. Total activity and percentage activity was calculated for these fractions and details are presented in Table 3.1.

MIC values of the fractions that show inhibition of bacteria on bioautography TLC plates were determined against six test organisms *Candida albicans*, *Cryptococcus neoformans*, *E. coli, S. aureus, P. aeruginosa* and *E. faecalis*. Based on MIC values fraction H1 was the most active against *C. neoformans* (MIC = 0.08), B4 was the most active against *C. albicans* (MIC = 0.16), *S. aureus*, *E. coli E. faecalis* and *P. aeruginosa* (MIC = 0.31). A strong activity was also observed for H3 against *P. aeruginosa* (MIC = 0.31). Gentamycin used a positive control for the antibacterial assay and gave an MIC of 0.02 mg/ml.

To find out which fractions contained the largest total quantity of antimicrobial components a relative measure was develop by calculating the total activity. The total activity was calculated from MIC values obtained per 1 gram extract (i.e. quantity in mg extracted from 1 gram of sample divide by MIC in mg/ml). This is an accurate measure of the total value of a plant because it takes into account the MIC and total quantity present in 1 gram. Total activity indicates the volume to which the bioactive compounds present in 1 gram sample can be diluted and still inhibit growth target organism. Based on the bioautography and MIC, B4 is the fraction of interest since it shows the highest total activity in which 1 gram of B4 can be diluted and still inhibit growth of fungi/bacteria (*C. neoformans* =28000, *C. albicans* =54250, *S. aureus* = 28000, *E. coli* = 28000, *E. faecalis* = 28000 and *P. aeruginosa* = 28000). Percentage total activities of B4 were (*C. neoformans* =73, 72%, *C. albicans* = 98.17%, *S. aureus* = 92.62, *E. coli* = 96.53, *E. faecalis* = 92.18 and *P. auruginosa* = 87.72%). B4 showed strong biological activity against six

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tested organisms. The question to answer is whether B4 has a broad spectrum or non-specific mode of inhibition. Further studies will elucidate the chemical constituent or constituents of B4 and their mode of action.

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Table 3.1: Antifungal and antibacterial characterization of selected plant fractions

	Minimum inhibitory concentrations (mg/ml)					
Fractions	*Cryptococcus neoformans*	*Candida albicans*	*Staphylococcus aureus*	*Escherichia coli*	*Enterococcus faecalis*	*Pseudomonas aerug*
B4 (8630mg)	0.31	0.16	0.31	0.31	0.31	0.31
D1 (470mg)	0.63	2.5	2.5	2.5	2.5	2.5
D7 (360mg)	0.63	2.5	2.5	2.5	2.5	2.5
H1 (660mg)	0.08	2.5	2.5	2.5	2.5	2.5
H3 (1030mg)	2.5	2.5	0.63	2.5	0.63	0.31

	Total Activity/ml					
Fractions	*Cryptococcus neoformans*	*Candida albicans*	*Staphylococcus aureus*	*Escherichia coli*	*Enterococcus faecalis*	*Pseudomonas aerug*
B4	28000	54250	28000	28000	28000	28000
D1	746	188	188	188	188	188
D7	571	144	144	144	288	144
H1	8250	264	264	264	264	264
H3	412	412	1634	412	1634	3322

	Percentage total activity					
Fractions	*Cryptococcus neoformans*	*Candida albicans*	*Staphylococcus aureus*	*Escherichia coli*	*Enterococcus faecalis*	*Pseudomonas aerug*
B4	73.72	98.17	92.62	96.53	92.18	87.72
D1	1.96	0.34	0.62	0.64	0.62	0.59
D7	1.5	0.26	0.48	0.49	0.95	0.25
H1	21.72	0.28	0.87	0.97	0.87	0.83
H3	1.08	0.78	5.4	1.42	5.38	10.4

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3.5.2. Bioautography of compounds isolated from hexane, dichloromethane and n-butanol fractions

Initially, all extracts obtained from hexane, dichloromethane and n-butanol fractions were tested, by bioautography in order to obtain the ones that have activity against selected fungi. Even though some of the extracts showed similarity on the TLC profiles using different mobile phases according to the polarity, they were tested in order to see if probably they contain the same active ingredients. The following TLC chromatograms show the profile of the various fractions obtained from the stem bark of *P. africanum* and evaluated for antimicrobial activity.

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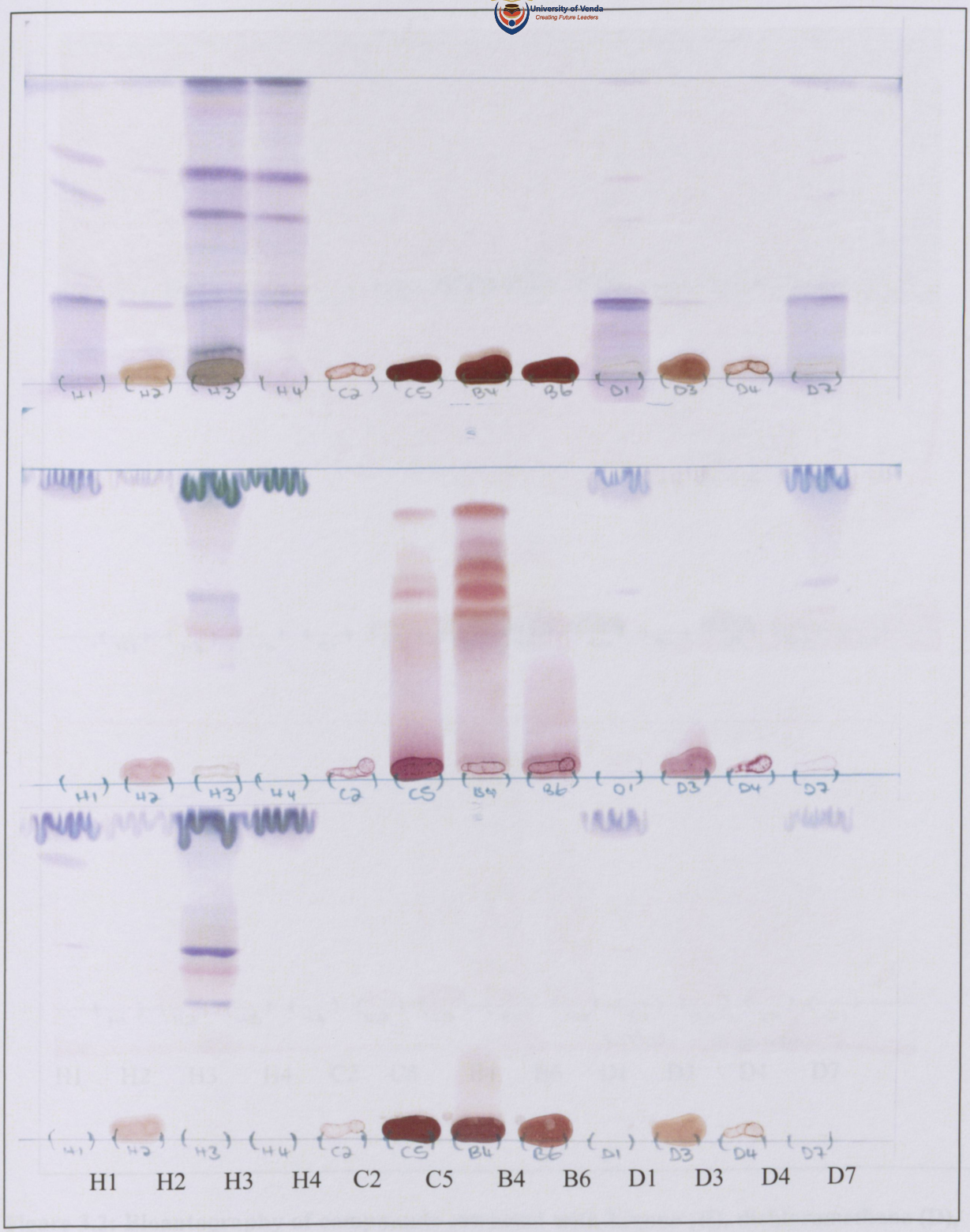


Figure 3.1: Chromatograms of compounds extracted with hexane (H), dichloromethane (C& D), n-butanol (B) fractions developed in BEA (top), EMW (centre) and CEF (bottom) solvent systems and sprayed with vanillin-sulphuric acid.

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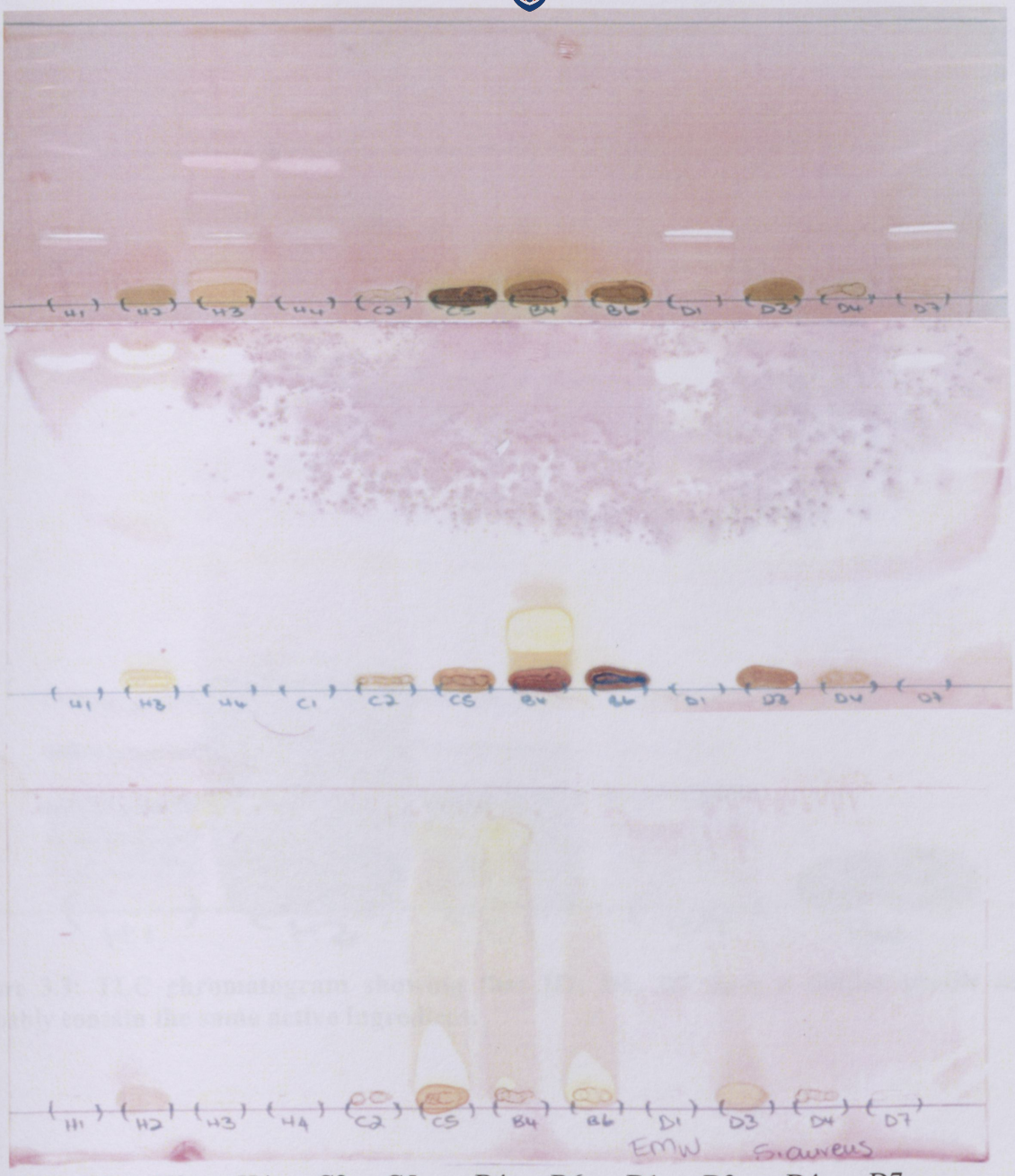


Figure 3.2: Bioautography of compounds extracted with hexane (H), dichloromethane (D), n-butanol (B) fractions developed in BEA (top), EMW (centre) and CEF (bottom) solvent systems sprayed with *C. albicans* (top) *C. neoformans* (centre) and *S. aureus* (bottom). White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of tested organisms.

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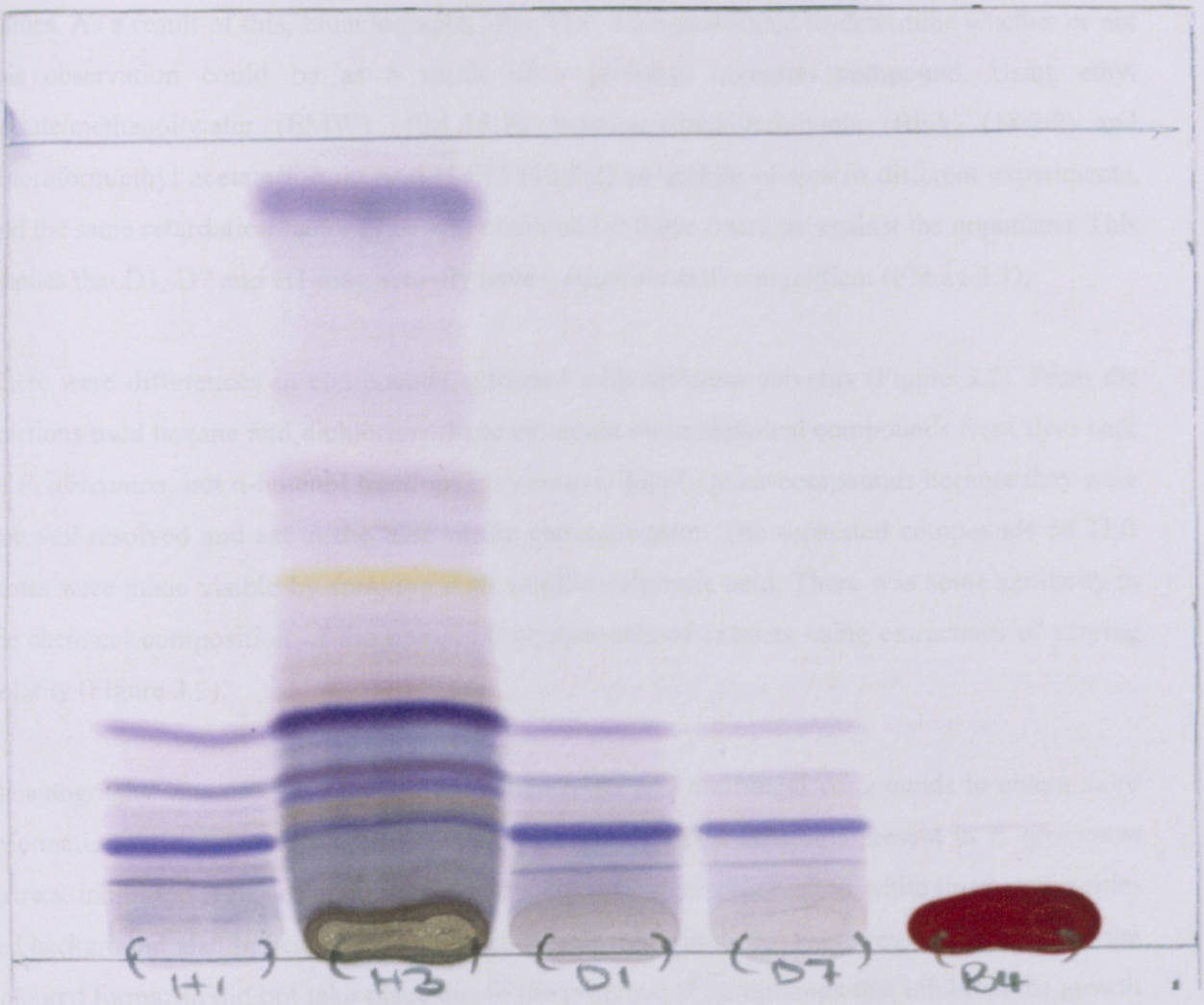


Figure 3.3: TLC chromatogram showing that H1, D1, D7 have a similar profile and probably contain the same active ingredient.

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Generally, fractions D1, D7 and H1 showed similar activity profiles judging from the MIC values. As a result of this, bioautographs after TLC were performed to determine whether or not this observation could be as a result of a probable common compound. Using ethyl acetate/methanol/water (EMW) (10:1.35:1), benzene/ethanol/ammonia (BEA) (18:2:2) and chloroform/ethyl acetate/formic acid (CEF) (10:8:2) as mobile phases in different experiments, and the same retardation factor (7.5) was obtained for these fractions against the organisms. This implies that D1, D7 and H1 may actually have a common active ingredient (Figure 3.1).

There were differences in compounds extracted with different solvents (Figure 3.2). From the fractions used hexane and dichloromethane extracted more chemical compounds from stem bark of *P. africanum*, but n-butanol fractions may contain highly polar compounds because they were not well resolved and sat at the base of the chromatogram. The separated compounds on TLC plates were made visible by spraying with vanillin-sulphuric acid. There was some similarity in the chemical composition of the non-polar components of extracts using extractants of varying polarity (Figure 3.2).

Bioautography was used to separate the antibacterial and antifungal compounds to obtain more information on the diversity of antibacterial and antifungal compounds present in *P. africanum* extract. Inhibition zones of antibacterial and antifungal were observed as white lines on a purple-red background also indicated by a star sign. White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of tested fungi (Figure 3.3).

A representative bioautogram of some of the fractions against the different bacteria and fungi is shown (Figure 3.3). To make it easier to evaluate the results of the biochromatograms in the data has been tabulated (Table 3.2 to 3.7). Success in isolating compounds from plant material is largely dependent on type of the solvent used in the extraction procedure. Compounds obtained using different solvents (hexane (H), dichloromethane (D) and n-butanol (B) are shown in Table 3.2 *C. neoformans* about 20 compounds, Table 3.3 *C. albicans* about 13 compounds, Table 3.4. *E. coli* about 8, Table 3.5 *S. aureus* about 8 compounds, Table 3.6 *P. auruginosa* about 2 compounds and Table 3.7 *E. faecalis* about 3 compounds. Due to time constrain this compounds

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were not evaluated in order to be able to contain the exact number of compound isolated. Some of the compounds obtained they might be one compounds basically because of the solvent system used. Hexane and dichloromethane were the best extractants, extracting active compounds of interest. The bioautograms of pates run in the CEF and EMW systems showed that the compounds were not well separated and the active compounds sat at the bottom of the chromatograms. Some of the compounds obtained were joined together making it difficult to calculate the exact retardation factor value (Figure 3.3).

BEA was the best solvent system separated the highest number of active compounds (Table 3.2 to 3.7), followed by CEF and EMW. This implies that polar systems separated les active compounds compared to non-polar system. The wide diversity in polarity of the antimicrobial components may provide clinically useful leads. In other cases there were growth, but no inhibition was observed even though MIC values indicated antibacterial and antifungal activity. The non-activity of these extracts in bioautography may possibly be explained by evaporation of active compounds during removal of the TLC eluents or by the disruption of synergism between active constituents caused by TLC separation (Masoko & Eloff, 2006) in this study is indicated in Figure 3.3 using CEF (bottom) as a mobile phase.

3.5.3. Retardation (Retention) factor (R_f)

After obtaining the bioautography results the retardation (retention) factors were measured, in order to know where the compounds that are active can be found on the respective TLC plates when compared with the retardation factor obtained from the normal TLC chromatogram that was not sprayed with an organism. Retardation factor (R_f) is calculated as the distance covered by the solvent system front divided by the distance covered by the extract. The values indicate where the targeted could be obtained from the original TLC plate performed using different mobile phases. The retardation factors of the fractions investigated under different mobile systems are shown in Table 3.2 to 3.7.

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Table 3.2: R_f values of bioautography of compounds obtained from hexane dichloromethane and n-butanol fractions, sprayed with *C. neoformans* using BEA, EMW and CEF as a mobile phases.

Fractions	R_f		
	BEA	EMW	CEF
Hexane			
H1	0.1	No activity	0.88
H2	No activity	No activity	No activity
H3	No activity	No activity	No activity
H4	0.2	No activity	0.86
H5	0.1	No activity	0.88
H6	No activity	No activity	No activity
n-Butanol			
B1	No activity	No activity	No activity
B2	No activity	No activity	No activity
B3	No activity	No activity	No activity
B4	0.16	0.84;0.42;0.17	0.48;0.34;0.18
B5	0.05	No activity	0.03
B6	No activity	0.14;0.42	No activity
B7	0.05	No activity	No activity
Dichloromethane			
D1	0.45; 0.48	No activity	0.96
D2	No activity	No activity	No activity
D3	0.45	No activity	0.93
D4	No activity	No activity	No activity
D5	No activity	No activity	No activity
D6	No activity	No activity	No activity
D7	0.36;0.41;0.45; 0.48	No activity	0.96
D8	No activity	No activity	No activity

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Table 3.3: R_f values of bioautography of compounds obtained from fractions of hexane dichloromethane and butanol, sprayed with *C. albicans* using BEA, EMW and CEF as a mobile phases.

Fractions	R_f		
	BEA	EMW	CEF
Hexane			
H1	0.43;0.47	No activity	No activity
H2	0.47	No activity	No activity
H3	0.47	No activity	No activity
H4	0.47	No activity	No activity
H5	0.47;0.43	No activity	No activity
H6	No activity	No activity	No activity
n-Butanol			
B1	No activity	No activity	No activity
B2	No activity	No activity	No activity
B3	No activity	No activity	No activity
B4	No activity	0.84;0.42;0.17	No activity
B5	No activity	No activity	No activity
B6	No activity	No activity	No activity
B7	No activity	No activity	No activity
Dichloromethane			
D1	0.15	No activity	0.86
D2	No activity	No activity	No activity
D3	0.15	0.44;0.30;0.07	0.86
D4	No activity	No activity	No activity
D5	No activity	No activity	No activity
D6	No activity	no activity	No activity
D7	0.13	No activity	0.83
D8	No activity	No activity	No activity

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Based from bioautography results of *C. neoformans, C. albicans* and MIC results of *C. neoformans, C. albicans, E. coli, P. aureginosa, S. aureus* and *E. faecalis* only those fractions that inhibited fungi and bacteria were selected for further bioautography. The results shows that the same compounds that were active against fungi were also active against bacteria. This is being indicated in Table 3.4 to 3.7. As from Table 3.4 to table 3.7 only those fractions that were showing inhibition from the results of fungi tested they were tested for their activity against bacteria. Instead of testing all the fractions since most of them were showing same profile based on their activity.

Table 3.4: R_f values of bioautography of compounds obtained from fractions of hexane, dichloromethane and n-butanol, sprayed with *E. coli* using BEA, EMW and CEF as a mobile phases.

Fractions	R_f		
	BEA	**EMW**	**CEF**
H1	0.51;0.26;0.24	0.6	No activity
H2	0.26	No activity	No activity
H3	0.54; 0.26; 0.24	0.6	0.3
H4	0.54; 0.26;0.24	No activity	No activity
C2	No activity	No activity	No activity
C5	No activity	0.2	0.3
B4	No activity	No activity	No activity
B6	No activity	0.2	No activity
D1	0.26; 0.24	0.6; 0.5	No activity
D3	No activity	No activity	No activity
D4	No activity	No activity	No activity
D7	0.26; 0.24	0.6; 0.5	No activity

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Table 3.5: R_f values of bioautography of compounds obtained from fractions of hexane dichloromethane and n-butanol, sprayed with *S. aureus* using BEA, EMW and CEF as a mobile phases.

Fractions	R_f		
	BEA	EMW	CEF
H1	0.3; 0.2	No activity	No activity
H2	No activity	No activity	No activity
H3	0.6;0.3;0.2	0.5	0.6;0.4
H4	0.6;0.3;0.2	No activity	No activity
C2	No activity	No activity	No activity
C5	No activity	0.3	0.3
B4	No activity	No activity	0.3
B6	No activity	0.2	No activity
D1	0.3;0.2	0.6	No activity
D3	No activity	No activity	No activity
D4	No activity	No activity	No activity
D7	0.3;0.2	0.6	No activity

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Since there was no activity on H2 fraction it was replaced with C1 fraction which was characterized as bergenin, only for *P. aeruginosa* and *E. faecalis*.

Table 3.6: R_f values of bioautography of compounds obtained from fractions of hexane dichloromethane and n-butanol, sprayed with *P. aeruginosa* using BEA, EMW and CEF as a mobile phases.

Fractions	R_f		
	BEA	EMW	CEF
H1	0.26	No activity	No activity
H3	0.26	No activity	No activity
H4	0.26	No activity	No activity
C1	No activity	No activity	No activity
C2	No activity	No activity	No activity
C5	No activity	No activity	No activity
B4	No activity	No activity	0.3
B6	No activity	No activity	No activity
D1	0.26	No activity	No activity
D3	No activity	No activity	No activity
D4	No activity	No activity	No activity
D7	0.26	No activity	No activity

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Table 3.7: R_f values of bioautography of compounds obtained from fractions of hexane dichloromethane and n-butanol, sprayed with *E. faecalis* using BEA, EMW and CEF as a mobile phases.

Fractions	R_f		
	BEA	EMW	CEF
H1	0.24	No activity	0.9
H3	No activity	No activity	1
H4	No activity	No activity	No activity
C1	No activity	No activity	No activity
C2	No activity	No activity	No activity
C5	No activity	No activity	No activity
B4	No activity	No activity	No activity
B6	No activity	No activity	No activity
D1	0.24	No activity	0.9
D3	No activity	No activity	No activity
D4	No activity	No activity	No activity
D7	0.24	No activity	0.9

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Fractions obtained from hexane and dichloromethane solvent-solvent fractionation was found to be the best antifungal and antibacterial inhibitors. Most compounds were isolated from the non-polar solvent (hexane). Therefore antifungal and antibacterial activities were not mainly due to polar compounds. A number of fractions showed promising biological activity especially B4 which showed the highest total activity. Further phytochemical studies on B4 would reveal its chemical nature.

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CHAPTER FOUR:
GENERAL CONCLUSION AND RECOMMENDATION FROM THIS STUDY

This study describes the phytochemical analysis and the biological activity of fractions and isolated compounds of the stem bark of *Peltophorum africanum* on HIV-1 replication and selected pathogenic fungi namely *Cryptococcus neoformans*, *Candida albicans* and bacteria namely *Escherichia coli, Staphylococcus aureus, Pseudomonas aureginosa* and *Enterecoccus faecalis*. A fraction C2 was found to have significant inhibitions on HIV-1 *reverse transcriptase* in a cell-free system. C2 also inhibited HIV replication when its intracellular mode of activity was evaluated in HIV-1 subtype C cultures.

The chemical constituent of C2 is still undetermined. It is recommended that the structure of C2 be fully elucidated in order that future structure could be performed to identify the functional group or groups and seek possible synthetic route for the molecule. In addition, although this showed that C2 was not toxic below 250 µg/ml to human cells, it is important to evaluate the toxicity of C2 in an animal model. This will also add information on the possibility of C2 being classified as a molecule with a pharmaceutical lead potential.

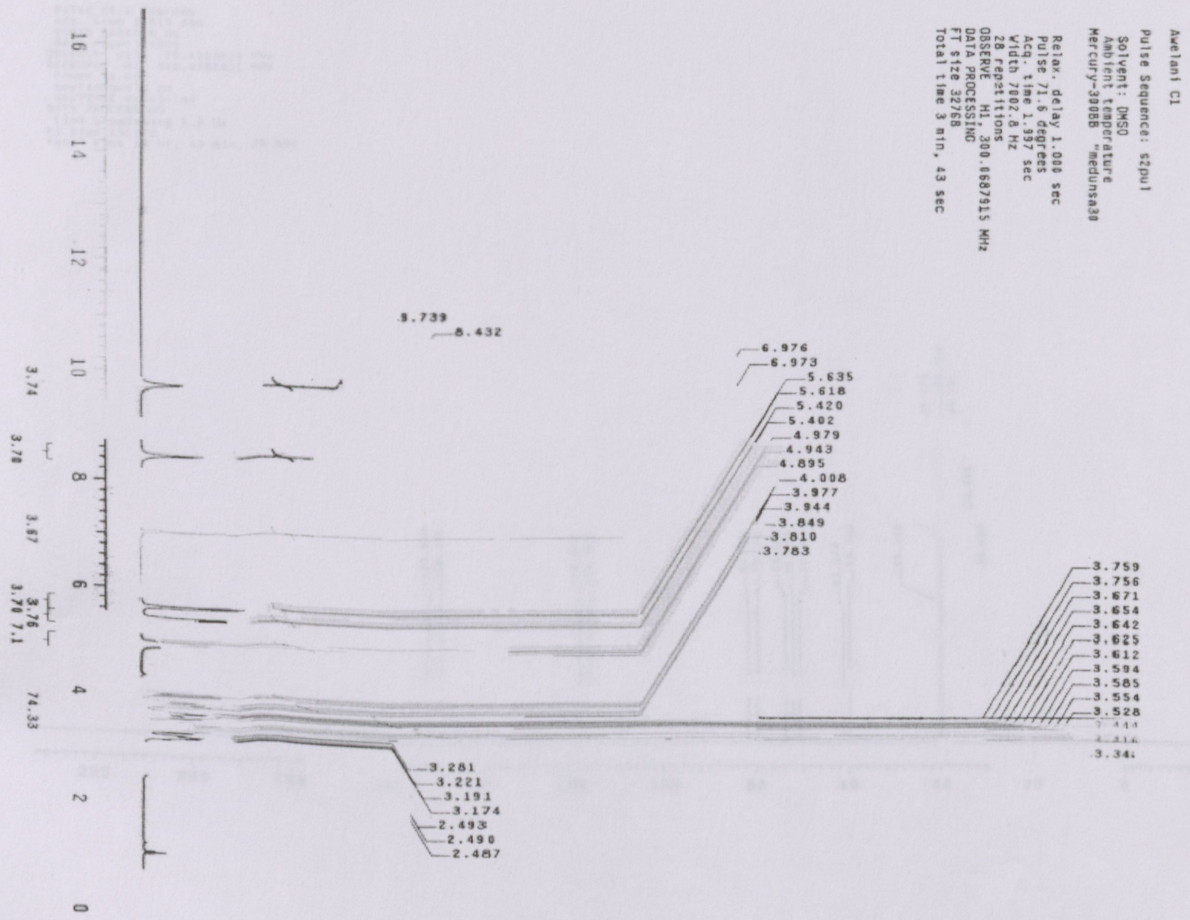
Another fraction B4, obtained from the n-butanol pre-fraction was shown to have potent activity against *C. neoformans* and *C. albicans* important opportunistic pathogens in AIDS. It is recommendation that further studies be done to fully understand the constituents of B4 and isolate and characterize the active ingredient(s).

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APPENDIX 5

5.1. ^{1}H NMR of C1 (bergenin)



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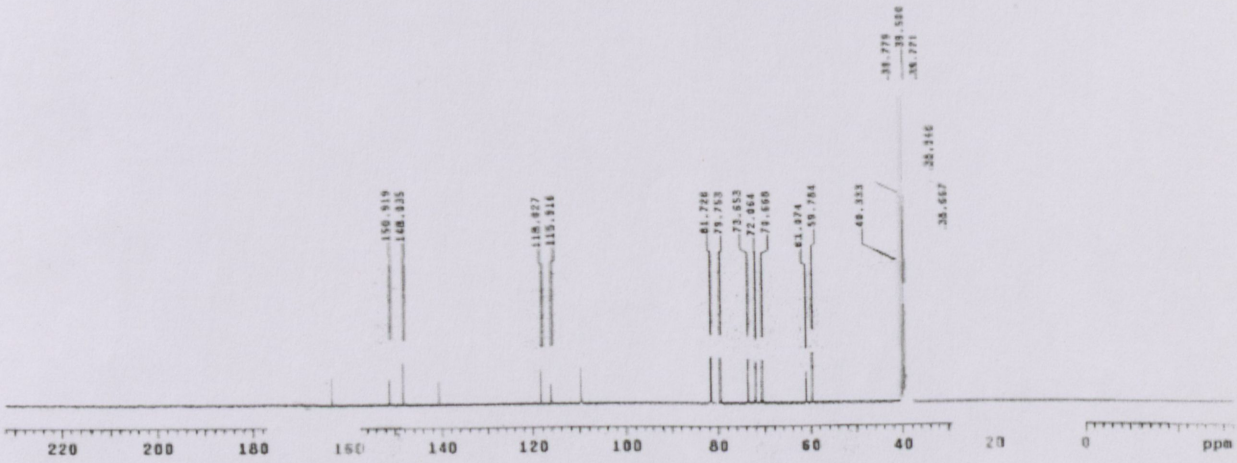
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5.2. ^{13}C NMR of C1 (bergenin)

Avelani C1

Pulse Sequence: s2pul
Solvent: DMSO
Ambient temperature
Mercury-300BB "wedunsa33

Pulse 82.1 degrees
Acq. time 1.815 sec
Width 22080.0 Hz
8648 repetitions
OBSERVE C13, 75.4523920 MHz
DECOUPLE H1, 300.0792831 MHz
Power 41 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.8 Hz
FT size 131072
Total time 16 hr, 43 min, 28 sec



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