In vitro anti-HIV activities of *Sutherlandia frutescens* and *Lobostemon trigonum* extracts

By

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Contents

Abbreviations

a	Alpha
Å	Angstrom
A	Adenosine
ABTS	[2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid]
Abu	Gamma aminobutyryl
ACK	ammonium chloride potassium
AIDS	Acquired Immunodeficiency Syndrome
Ala	Alanine
Asn	Asparagine
Asp	Aspartate
AZT	Zidovudine
ß	Beta
bp	Base pair(s)
BSA	Bovine serum albumin
° C	Degrees Celsius
C	Cytosine
CA	Capsid
CAM	Complementary/alternative medicine
CCR5	Chemokine co-receptor number 5
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
CPT	cell preparation tube
CXCR4	Fusin (lester) co-receptor
Cys	Cysteine
DEPC	Diethyl pyrocarbonate

DIG	Digoxigenin
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
ds	Double-stranded
DTP	Developmental Therapeutics Program
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbant assay
FasL	Fas ligand
FCS	Foetal calf serum
?	gamma
g	gram
g	Gravitational acceleration
G	Guanine
GABA	?-Aminobutyric acid
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
Gp	glycoprotein
HAART	Highly active antiretroviral therapy
HCl	Hydrogen chloride
His	Histidine
HIV	Human Immunodeficiency Virus
IC_{50}	Concentration at which there is 50% inhibition
IgG	Immunoglobulin G
Il-2	Interleukin 2
Ile	Isoleucine
IN	Integrase

kDa	Kilodalton
l	Litres
LB	Luria-Bertani
LiCl	Lithium chloride
LTR	Long terminal repeat
M	Molar
mA	Milliamps
MA	Matrix protein
MCS	Multiple cloning site
Mes	2-Morpholinoethanesulphonic acid monohydrate
mg	Milligrams
$MgCl_2$	Magnesium Chloride
MHC	Major histocompatibility complex
ml	Millilitres
mM	Millimolar
$MnCl_2$	Manganese Chloride
mRNA	messenger RNA
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,2-diphenyltetrazolium bromide
NaCl	Sodium chloride
NC	Nucleocapsid
NCI	National Cancer Institute
Nef	Negativity factor
NF-aB	Nuclear factor alpha B
NFAT	Nuclear factor of activated T-cells
ng	nanograms
NGO	Non-governmental organization
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institute of Health
NK	Natural killer

nm	Nanometres
NMR	Nuclear magnetic resonance
no.	Number
OD	Optical density
OH	Hydroxyl
p24	core protein
PBMCs	peripheral blood mononuclear leukocytes
PHA	Phytohaemagglutinin
PIC	Pre-integration complex
POD	Peroxidase
Pol	Polymerase
PR	Protease
Pro	Proline
RAU	Rand Afrikaans University
RCT	Random clinical trials
Rev	Regulator of viral gene expression
RNA	Ribonucleic acid
RNase	Ribonuclease
RRE	RNA response element
RT	Reverse transcriptase
SA	South Africa
SA-HRPO	streptavidin conjugated to horseradish peroxidase
sCD4	soluble CD4
Ser	Serine
TAE	Tris-acetate-EDTA
TAR	Transactivation response element
Tat	Transcriptional activator

TBP	TATA box binding protein
TE	Tris-EDTA
T_H cells	T-lymphocyte helper cells
Thr	Threonine
TMB	Tetramethylbenzendine
TNF	Tumour necrosis factor
tRNA	Transfer ribonucleic acid
Tyr	Tyrosine
U	Unit
µg	Micrograms
µl	Microlitres
µM	Micromolar
UPE	University of Port Elizabeth
US	United Sates
USA	United States of America
UV	ultraviolet
V	Variable
V	Volts
v/v	volume per volume
Val	Valine
Vif	Viral infectivity factor
Vis	Visible
Vpr	Viral protein R
vs.	versus
WHO	World Health Organisation
w/v	weight per volume
XTT	2, 3-bis [2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide
$ZnCl_2$	Zinc chloride

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Abstract

In vitro screening for anti-HIV activity in extracts from *Sutherlandia frutescens* and *Lobostemon trigonum*

Currently, the approved anti-HIV drugs on the market only target the three HIV enzymes: reverse transcriptase, protease and more recently, integrase. Due to the limited nature of the current therapy, it is possible that a multi-drug resistant virus can emerge. The main concerns in developing countries however, are the expense and availability of the drugs and because of this, it is essential to investigate all alternatives. Traditional medicine offers many advantages as compared to allopathic treatment in so far as being relatively cheaper, accessible and it is broadly accepted in the population groups of the developing countries. Little is known though, of the exact efficacy and toxicity of these remedies so it is vital that these possible leads be investigated thoroughly. For the purpose of this study, two plants, *Sutherlandia frutescens* and *Lobostemon trigonum* were studied to ascertain their potential anti-HIV activity. *Sutherlandia* has received international attention as a possible cheap herbal remedy to improve the health of AIDS sufferers. Anecdotal evidence from health workers claim that HIV-infected patients on *Sutherlandia* treatment have shown improved CD4 counts, decreased viral loads and a general improvement in well-being.

Extracts were prepared from dried leaves and flowers in methanol, ethanol, acetone, methylene dichloride or distilled water. Sulphated polysaccharides have been described extensively in literature with regards to their anti-HIV activity, so as a form of dereplication; an ethanol precipitation was performed on the aqueous extracts to remove sulphated polysaccharides.

A toxicity study was performed on all crude extracts using uninfected peripheral mononuclear blood cells (PBMCs) isolated from whole blood. To measure anti-HIV activity, HIV-infected PBMCs were cultured with each of the crude extracts and cell viability measured using the tetrazolium salt, XTT. HIV-infected CEM-NK^{R}-CCR5 cells were also used and supernatant from the viral studies was tested for the HIV antigen p24.

Results varied greatly between assays but with the inclusion of a point-scale system to evaluate the extracts it was clear that overall the organic extracts of the *Sutherlandia* flowers, especially the acetone extract (SFA), showed great anti-HIV potential. SFA in every case decreased p24 levels and in the toxicity study did not decrease cell proliferation. With the HIV-infected PBMCs SFA actually helped improve cell proliferation despite the infection.

To determine the specific anti-HIV activity, all crude extracts were tested for inhibition of HIV-I reverse transcriptase, the glycohydrolase enzymes: a-glucosidase, ß-glucosidase, ß-glucuronidase, HIV-I integrase and HIV-II protease. No significant inhibition was seen with these experiments except for the HIV-I RT assay. The aqueous extract of the *Lobostemon* leaves produced an inhibitor of HIV-RT with a very low IC_{50} value of 0.049mg/ml. Some inhibitory effect was lost with the removal of the sulphated polysaccharides and the addition of BSA to the assay, but still 64% inhibition of the HIV-RT remained, which confirmed that the inhibitor could be something novel, and not of the polysaccharide or tannin compounds.

Keywords: ***Sutherlandia, Lobostemon,*** **anti-HIV activity, reverse transcriptase, protease, glycohydrolase enzymes, integrase, plant extracts, traditional medicine.**

1 Introduction

Human Immunodeficiency Virus (HIV) is the etiological agent of Acquired Immunodeficiency Syndrome (AIDS) and since its discovery more than 20 years ago, 60 million people have been infected worldwide (http://www.unaids.org). AIDS is defined as the presence of clinical signs associated with immune deficiency but excludes other known causes of immunosuppression, such as cancer or malnutrition (Klatt, 2002). Symptoms of HIV-infection include night sweats, weight loss, persistent fever, diarrhoea, thrush, headache, lymphademopathy, skin rashes, recurrence of varicella zoster virus infection, Kaposi sarcoma, pneumonia, Candida esophagitis, disseminated atypical mycobacterial infection, cryptococcal meningitis and toxoplasma encephalitis (Vermani and Garg, 2002). There are two types of HIV, namely types I and II but for the duration of this review, HIV will be used to refer to HIV-I, which is the retrovirus that is responsible for the AIDS pandemic (http://www.unaids.org; Klatt, 2002). Throughout the world HIV is the fourth leading cause of death but in Sub-Saharan Africa, it ranks first (http://www.unaids.org).

In 2002, 2.4 million people died in Africa from AIDS-related diseases and 29.4 million now live with it in Africa (http://www.unaids.org). This amounts to greater than 45 million cumulative HIV infections in Africa as of the end of 2001 (Weidle *et al.*, 2002). In South Africa (SA) alone, it is estimated that one in nine people are infected with HIV (Eckert and Kim, 2001; http://www.unaids.org). Figure 1.1 shows the global estimates of the HIV/AIDS epidemic as of the end of 2003 while figure 1.2 gives the estimated deaths that occurred in 2003 from HIV/AIDS.

At first, doctors treated HIV-infected patients with bone marrow transplants, lymphocyte transfusions and thymic transplants, which due to their invasive nature and failure, were stopped (Klatt, 2002). Now patients are treated with a cocktail therapy of three or more drugs and this treatment is known as Highly Active Antiretroviral Therapy (HAART) (Klatt, 2002). Current therapy has allowed for people infected with HIV to live longer and healthier lives by lowering the viral load and slowing the rate at which the patient progresses to the symptomatic stage of AIDS, but it does not cure the infection (Eckert and Kim, 2001). HIV is able to establish latent reservoirs early in infection that current therapy

cannot target (Levy, 2002). As a consequence, an infected patient must remain on therapy, which is not viable as several situations may occur:

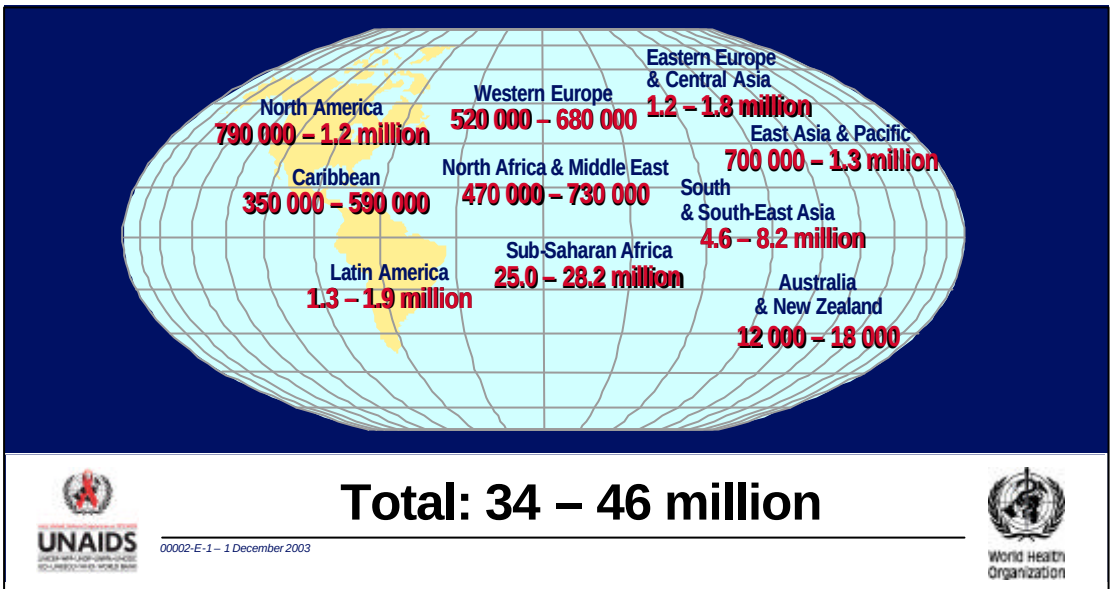

Figure 1.1: Estimated numbers of adults and children living with HIV/AIDS as of the end of December 2003 (http://www.unaids.org)

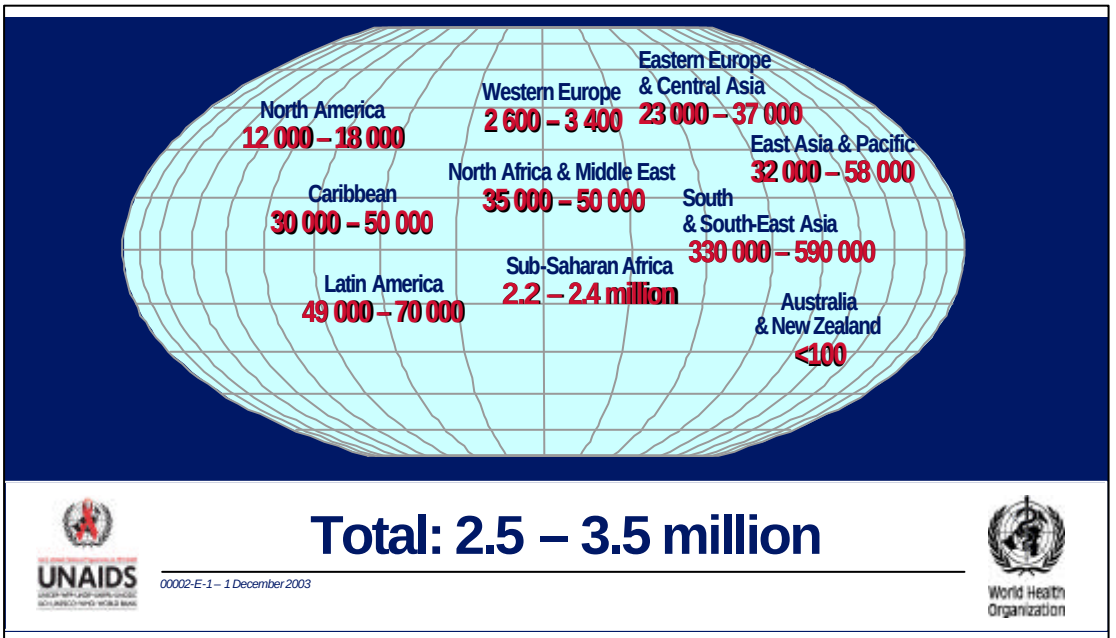

Figure 1.2: Estimated adult and children deaths from HIV/AIDS during 2003 (**http://www.unaids.org**)

- HAART is very toxic and about 20% of patients can not tolerate the treatment or become intolerant over the course of therapy and suffer many side-effects (Eckert and Kim, 2001; Moore and Stevenson, 2000).
- Because of the high virus turnover and error-prone replication of HIV, resistance arises to the small molecular drugs (Eckert and Kim, 2001). Drug-resistant HIV is rapidly becoming a problem and in one study, they showed that 16% of HIV-infected people were infected with variants that had resistance to known antiretroviral agents (Klatt, 2002; Moore and Stevenson, 2000).

As of the end of July 2003, there are now 19 approved anti-HIV drugs on the United States market including the combination medications (Table 1.1) (www.thebody.com). However these drugs only target two HIV enzymes; reverse transcriptase (RT) and protease (PR) while one drug blocks fusion but this is difficult to use and very expensive (Eckert and Kim, 2001; Klatt, 2002; Moore and Stevenson, 2000, www.thebody.com). Since only three steps have been targeted, the possible emergence of a virus immune to all treatment is quite likely and so it is essential that the other steps in the HIV lifecycle be exploited (Klatt, 2002). At present, there is a clinical trial in progress that is investigating a drug that targets HIV integrase (IN) (Greene and Peterlin, 2002; Hazuda and Felock, 2000).

Table 1.1:Pharmacological agents for antiretroviral therapy (wwww.thebody.com)

Nucleoside Reverse Transcriptase Inhibitors	Zidovudine	Zalcitabine
	Didanosine	Stavudine
	Emtricitabine	Abacavir
	Lamivudine	
Non-nucleoside Reverse Transcriptase Inhibitors	Nevirapine	Delavirdine
	Efavirenz	
Nucleotide Reverse Transcriptase Inhibitors	Tenofovir	
Protease Inhibitors	Saquinavir	Indinavir
	Ritonavir	Nelfinavir
	Amprenavir	Lopinavir
	Atazanavir	
Fusion Inhibitors	Enfuvirtide	

But the story in developing countries with regards to anti-HIV therapy is of course, an entirely different matter, where the main problems are the expense and access to treatment.

In 2000, the cost per patient per year for HAART was approximately US$10 000 but since then, prices have come down for generic forms of treatment to US$300 (Moore and Stevenson, 2000; www.unaids.org). This is still beyond many people's means in developing countries. Because of this, an alternative must be found.

1.1 Traditional Medicine

There has been a worldwide move towards the use of traditional medicines due to the concerns over the more invasive, expensive and potentially toxic mainstream practices (Cowan, 1999; WHO 1, 2002). Its popularity is due in large course to the desire for more personalised health care and greater public access to health information (Greene and Peterlin., 2002). Not only is this form of treatment popular in developing countries but it is also gaining favour in developed countries as can be seen in figure 1.3. Traditional medicine includes medicines from plant, animal or mineral sources, spiritual therapies, manual techniques and exercise but this review will be focusing on herbal medicines (WHO 1, 2002). The term traditional medicine is used when the therapy is part of the country's culture or history and if it is not, e.g. acupuncture practices in Europe, then it is referred to as complementary/alternative medicine (CAM) (WHO 1, 2002).

It is not only the general populace that has taken this form of treatment more seriously but also researchers; pharmaceutical companies and health institutes are focusing more attention on the search for new phytochemicals. Many herbal remedies have proven to have a real effect as compared to placebos (figure 1.4) (WHO 1, 2002). Twenty-five to fifty percent of all current pharmaceuticals are derived from plants (Cowan, 1999). Up till now, we have relied on bacterial and fungal sources for antimicrobial activity but plants are rich in secondary metabolites that have been shown to have *in vitro* antimicrobial activity (Cowan, 1999). It has been foreseen that current antibiotics have a limited life span and lack the ability to fight viral infection (Cowan, 1999). With the trend of extinction in animal and plant species, it is vital to fast track the discovery of novel phytochemicals but pharmaceutical companies are holding back due to the uncertainty about whether proprietary claims can be made (Cowan, 1999).

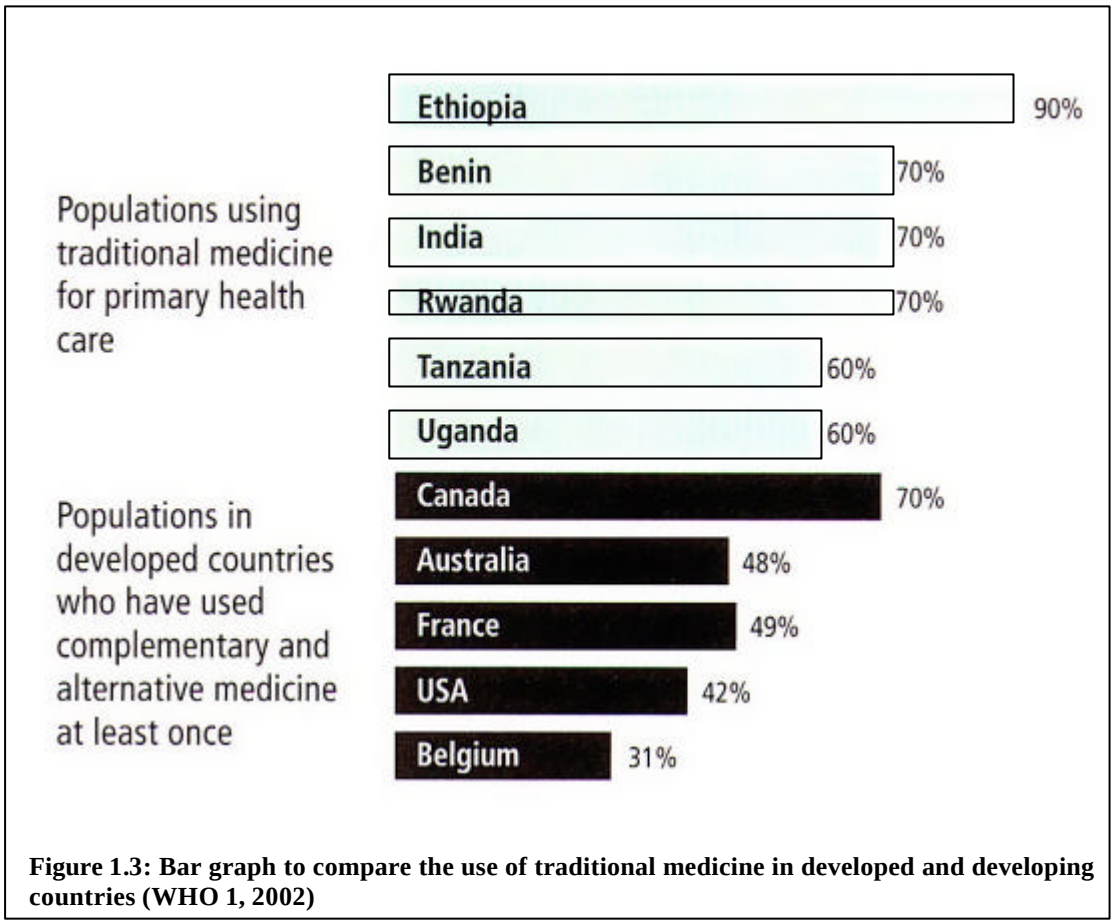

Figure 1.3: Bar graph to compare the use of traditional medicine in developed and developing countries (WHO 1, 2002)

In Africa, it is not so much the case that traditional medicines offer an alternative but rather that it is in fact the only source of treatment for many people (WHO 2, 2002; Morris, 2001). In places like Africa, Asia and Latin America, traditional medicine is used to meet the primary health care needs and it has been argued that traditional medicine is carrying the burden of clinical institutes for the AIDS epidemic in Africa (WHO 1, 2002; WHO 2, 2002). The World Health Organisation (WHO) has estimated that one-third of the global population lacks access to essential drugs and in poorer regions of Africa and Asia, this number is greater than 50% (WHO 2, 2002).

There are several advantages to using traditional medicines:

1. Diversity and flexibility (WHO 2, 2002).
2. Accessibility (WHO 2, 2002). Where allopathic practitioners are mainly in cities and urban areas, traditional healers reach the rural population and in South Africa they outnumber practitioners 10:1 (Morris, 2001). In Uganda there is one traditional healer for every 200-300 people but the ratio is 1:20 000 for trained medical personnel (Bodeker *et al.*, 2000).

3. Affordable (WHO 2, 2002).
4. Broad acceptance among many population groups in developing countries where traditional medicine often forms part of a wider belief system (WHO 2, 2002). In Africa traditional medicine is used by 80% of the population and globally this figure is 60% (WHO, 1989; WHO 1, 2002).
5. Low manufacturing costs (WHO 2, 2002).
6. No need for a high level of technical input (WHO 2, 2002).
7. It is of growing economic importance (WHO 2, 2002). The world markets for herbal medicines is now at US$ 60 billion and from May 1996 to May 1998, the USA herbal sales have increased by 101% (WHO 2, 2002).

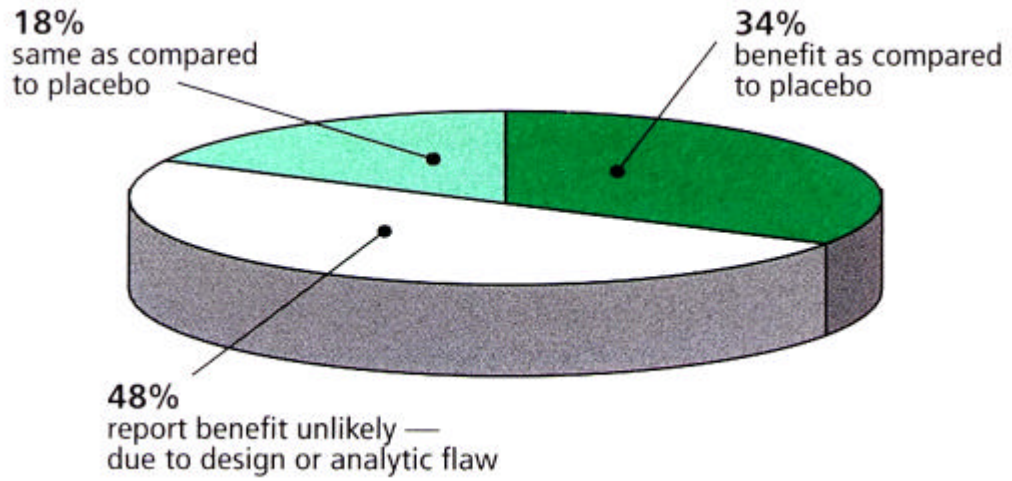

Figure 1.4: Pie chart showing evidence of efficacy for some herbal remedies. Percentage of random clinical trials (RCT) showing benefit of herbal medicines based on 50 RCT with 10 herbal medicines for 18 therapeutic indications (WHO 1, 2002)

There are of course disadvantages too, but these are problems that can be rectified. Traditional medicine is recognised with varying degrees by governments (WHO 1, 2002). By the end of 2000, 25 countries have reported to having a traditional medicine national policy for defining its role in the national health care system and WHO has established committees and policies regarding traditional medicine (WHO, 1989; WHO 2, 2002).

Long-term use of traditional medicine demonstrates its safety but proper investigation needs to be done to disprove systemic toxicity, carcinogenicity, teratogenicity and show efficacy (WHO, 1989, WHO 2, 2002). One must also check that it does not affect other

treatments, for example St John's Wort reduces the HIV-PR inhibitor, Indinavir, to ineffectual levels in the blood (WHO 2, 2002). WHO guidelines for traditional medicine states that if the medicine is in customary use, with no side-effects, fast-track toxicology studies can be done and the treatment can go straight to phase III clinical trials (Morris, 2001). Many centres have been set up in Africa in collaboration with WHO, focussing on traditional medicine research (Figure 1.5.) (WHO 2, 2002). South Africa is not one of these countries (WHO 2, 2002).

Figure 1.5: African countries that have institutes that carry out traditional medicine research (WHO 2, 2002)

Another problem surrounding the extensive use of traditional medicine is the protection of indigenous knowledge and protection of plant species. The vast majority of plant genetic resources and other forms of biodiversity seem to originate from developing countries, countries that lack the capacity to protect them. This has already happened with *Hypoxis hemerocallidea* (or *H. rooperi*) better known as the wild African potato. Since receiving a great deal of publicity about its use in HIV/AIDS therapy, this plant species has now become threatened (WHO 2, 2002). It is, therefore, important that if one was to commercialise a traditional medicine, that the plant could be cultivated easily (Houghton, 1996).

All these problems can be overcome with proper policies and sound scientific research. This way, countries like South Africa can make use of all the advantages offered by traditional medicine, especially in the fight against AIDS.

1.2 Secondary metabolites

In herbal remedies, the medicinal properties can largely be attributed to secondary metabolites that are abundant in plants. Plants have the ability to produce an almost endless range of secondary metabolites, especially aromatic substances. About 12 000 have been described so far but it is believed this only covers about 10% of the secondary metabolites available (Cowan, 1999).

These compounds are used in plant defence against microbes, herbivores and insects and the fact that plants have developed metabolites for defence against plant viruses leads one to believe that it is only logical that these could possibly be used in antiviral studies elsewhere. Secondary metabolites are also produced by plants and used for odours (e.g. terpenoids), pigmentation (e.g. quinones and tannins) and flavour (e.g. terpenoid capsaicin). Table 1.2 gives a list of the major classes of compounds that have shown multiple examples of antimicrobial and antiviral activity (Cowan, 1999).

An advantage using antiviral compounds from plants is the fact that plants can synthesise complex molecules with specific stereochemistry, often something that a chemist would not have thought of, and if active could possibly have a novel mode of action (Houghton, 1996). It is because of the immense potential of natural products that the Developmental Therapeutics Program (DTP) of the National Cancer Institute (NCI) has been providing support for internal and external anti-HIV drug discovery since 1987 (Yang *et al.*, 2001). Products from plants have been discovered to inhibit HIV at nearly all stages of the viral lifecycle, including sulphated polysaccharides to inhibit HIV viral attachment and calanolide A from the tropical rainforest tree *Calophyllum lanigerum* that inhibits HIV RT (Yang *et al.*, 2001). Many more anti-HIV products have been discovered but as yet the mode of action is unknown (Yang *et al.*, 2001).

Table 1.2: Major classes of antimicrobial and antiviral compounds from plants (Cowan, 1999)

Class	Subclass	Example	Mechanism
Phenolics	Simple phenols	Catechol	Substrate deprivation
		Epicatechin	Membrane disruption
	Phenolic acids	Cinnamic acid	
	Quinones	Hypericin	Bind to adhesins, inactivates enzymes
	Flavonoids	Chrysin	Bind to adhesins, binds to cell wall
	Flavones	Abyssinone	Inactivates enzymes, inhibits HIV reverse transcriptase
	Flavonols	Totarol	?
	Tannins	Ellagitannin	Binds to proteins, inactivates enzymes, Substrate deprivation, membrane disruption etc.
	Coumarins	Warfarin	Interaction with eukaryotic DNA (anti-viral)
Terpenoids and essential oils		Capsaicin	Membrane disruption
Alkaloids		Berberine	Intercalate into cell wall/DNA
		Piperine	
Lectins and polypeptides		Mannose-specific agglutinin	Block viral fusion
		Fabatin	Form disulfide bridges
Polyacetylenes		8S-Heptadeca-2(Z),9(Z)-diene-4,6-diyne-1,8-diol	?

1.3 Description of HIV

There has been a great deal of focus worldwide, especially in Asia, on the use of traditional herbal medicines against AIDS and many Chinese medicines have been shown to have an

inhibitory effect on HIV (Au *et al.*, 2001). To understand how new drugs can target HIV, one must understand the structure and lifecycle of HIV.

HIV belongs to the family of RNA viruses, lentivirus and was first known as human T-lymphocytotrophic virus type III or lymphadenopathy associated virus (Au *et al.*, 2001). The virus consists of a bar shaped core that contains two short RNA strands (~ 9200 nucleotides) that make up the genome, RT, PR, ribonuclease H (RNase H), and IN which are all encased in an outer lipid membrane (figure 1.6). Projecting from the envelope are 72 "spikes" that are the glycoproteins (Klatt, 2002).

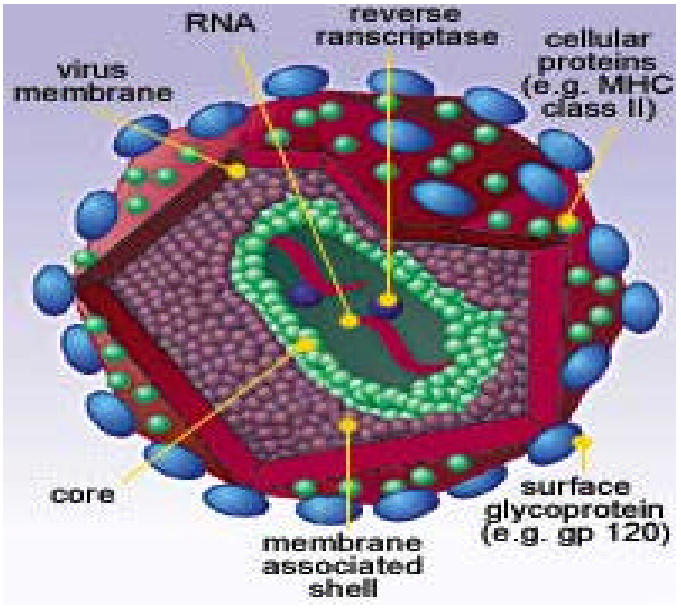

Figure 1.6: Structure of HIV virion (http://www.schoolscience.co.uk)

The genome has three major genes, *env*, *gag*, and *pol* that encode the major structural components. The *env* gene encodes for the glycoprotein gp160, which is the precursor for gp120 and gp41. The *gag* gene produces the core nucleocapsid proteins, p55, p24, p40, p17 (matrix) and p7 (nucleocapsid). *Pol* encodes the p66/p51 (RT), p11 (PR) and p32 (IN) (Klatt, 2002).

Accessory genes, *tat*, *rev*, *nef*, *vif*, *vpr* and *vpu*, encode for proteins that play a vital role in viral replication and maturation and their functions can be seen in figure 1.7 (Greene and Peterlin, 2002).

The probability of finding a single inhibitor of HIV is very slim, so the more targets exploited the better. An overview of the main steps in the HIV lifecycle is shown below and are illustrated in figure 1.8 (Greene and Peterlin, 2002; Klatt, 2002):

1. HIV, via the glycoprotein gp120, attaches to the CD4 receptor of the host cell.
2. This leads to conformational changes in gp120 and it binds to a co-receptor.
3. Gp 41 is released and is inserted in the target cell's lipid membrane and fusion occurs.
4. The virion uncoats and RT converts the (RNA) genome to complementary double-stranded DNA (ds cDNA).
5. The viral DNA is transported to the nucleus and is integrated into the host genome.
6. Viral DNA is transcribed and translated to produce viral proteins.
7. Viral proteins are cleaved/processed by HIV PR to give functional proteins.
8. RNA and proteins are transported to the cell membrane and assembled.
9. A new virus buds off, forming a new lipid envelope from the host membrane.

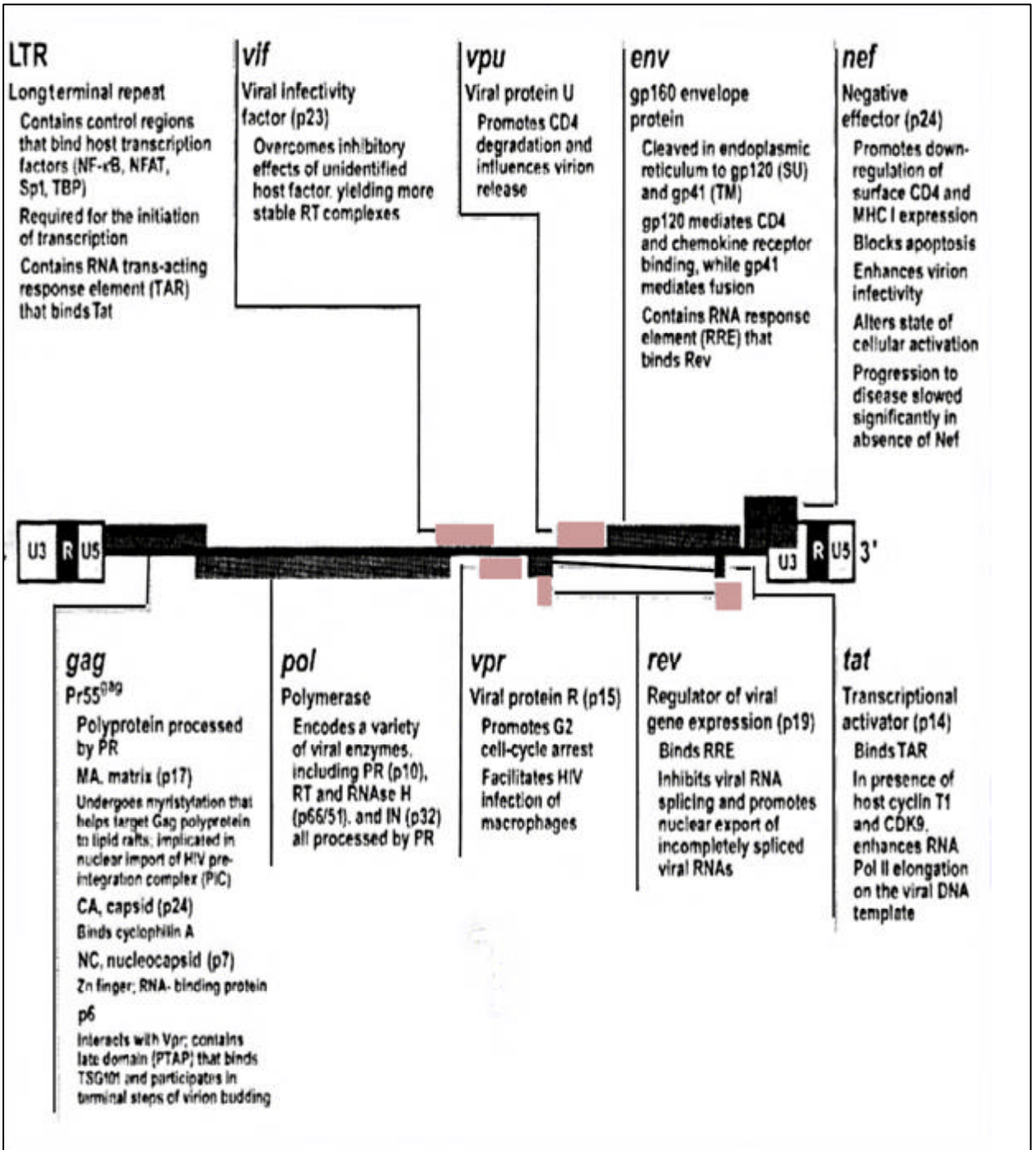

Figure 1.7: An overview of the organisation of the ~9 kilobase genome of the HIV provirus. Included is a summary of the functions of its nine genes encoding 15 proteins (Greene and Peterlin, 2002)

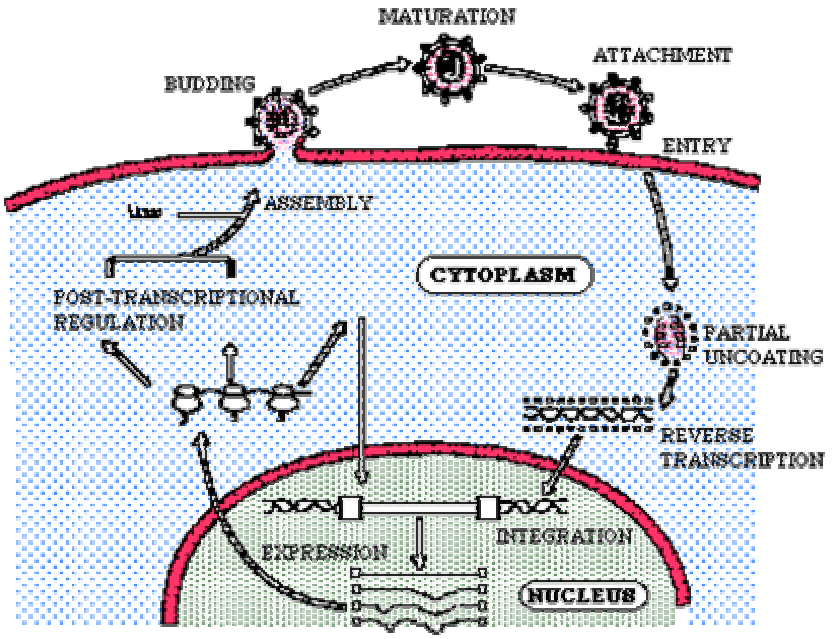

Figure 1.8: Overview of the HIV life cycle (www.tulane.edu)

1.4 HIV Targets for therapy

As can be seen, there are several stages of the HIV lifecycle that are potentially vulnerable to specific inhibitors. These steps can be divided into pre-entry steps and post-entry steps and can be defined as to whether it is a viral target or a host cell target. Essentially, it is ideal to target viral factors rather than disturb the normal functioning of a host cell factor that could possibly produce toxic effects. However, by targeting host factors, one can sometimes avoid the problem of viral resistance (Moore and Stevenson, 2000).

Although studies have focused on targeting other steps in the viral lifecycle besides the PR and RT, progress is often hampered due to the properties of the inhibitor. *In vitro* activity does not necessarily translate into *in vivo* activity and sometimes the inhibiting compound may be toxic to the host, expensive to manufacture in bulk, not bioavailable or may have a limited life span. Some compounds may simply be impractical to implement like proteins and peptides that need to be injected daily (Moore and Stevenson, 2000).

The following paragraphs go into detail about the targets that have been investigated to see if inhibition of these could affect viral replication.

1.4.1 Pre-entry steps

1.4.1.1 Virus-cell attachment

It is best to target entry steps, to stop target cells from being infected and prevent the possible risk of forming reservoirs of infection. Figure 1.9 illustrates the steps involved in the entry of HIV. Anti-HIV molecules that stop the virus before infection, could be used as a prophylactic and provide leads for an effective vaccine (Eckert and Kim, 2001).

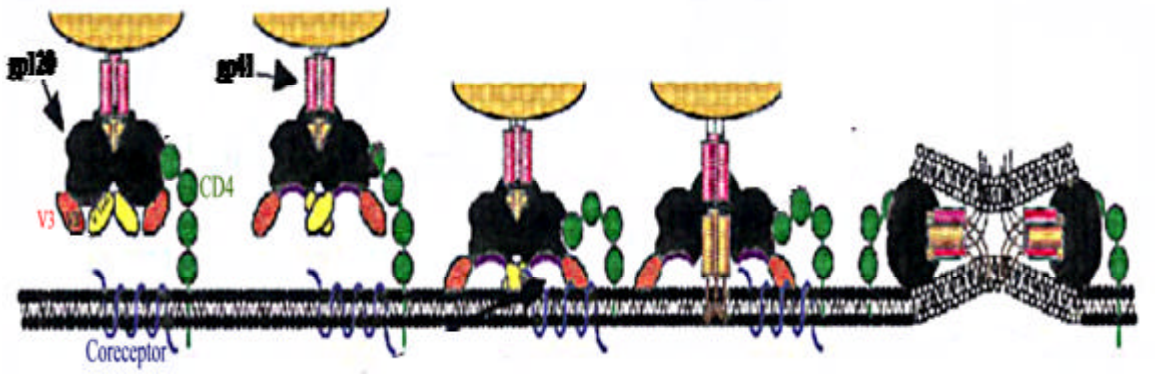

Figure 1.9: Entry steps in the HIV lifecycle. Gp120 recognises the host CD4 and upon attachment there is a conformation change of the gp120 revealing a chemokine receptor-binding site on the V3 loop. Gp120 binds to the chemokine receptor and with the gp41 fusion occurs between the two cells (LaBranche *et al*., 2001)

The mechanism behind the initial attachment of the virus to the target cell is not well understood but it is believed to involve positively charged regions on the virus and negative heparan sulphate proteoglycans on the target cell. This procedure could be strain-specific. Strong cationic peptides can inhibit this stage by interacting with the cell but lack any real clinical value (Moore and Stevenson, 2000).

1.4.1.2 Gp120-CD4 binding

The viral protein required for fusion is first synthesised as a gp160 product that is proteolytically cleaved as a surface subunit, gp120 (figure 1.10), and a transmembrane unit, gp41. The whole protein consists of (Eckert and Kim, 2001):

1. Cleavable amino-terminal signal sequence to direct it to the endoplasmic reticulum of the host cell, where the protein is highly glycosylated.
2. Ectodomain. Gp120 is the subunit responsible for recognising and binding to CD4.
3. Stop transfer region that forms a transmembrane helix in order to anchor the protein into the viral membrane.
4. Cytoplasmic tail.

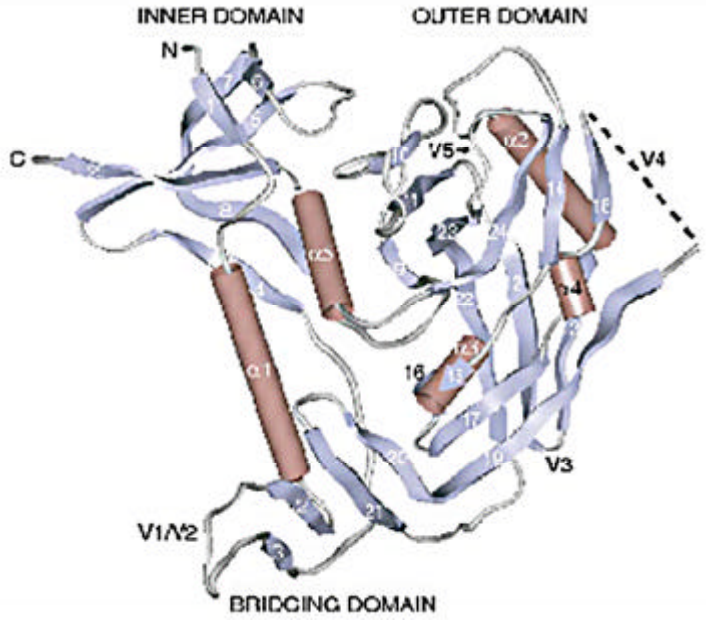

Figure 1.10: High-resolution x-ray structure of the core gp120 (Eckert and Kim, 2001).

CD4, found on the target cell, is a glycoprotein of 55 kDa that plays a role in the host immune system by mediating efficient association of T-lymphocyte helper cells (T_H) with antigen-bearing cells (Deen *et al.*, 1988). This is the essential receptor used by HIV to attach to the target cell (Dalgleish *et al.*, 1984). Cells with the CD4 receptor include the cells of the mononuclear phagocyte system, mainly blood monocytes and tissue macrophages, T and B lymphocytes, natural killer cells, dendritic cells, haematopoietic

stem cells, endothelial cells, microglial cells in the brain and gastrointestinal epithelial cells (Klatt, 2002).

HIV-II has the ability to bind to $CD4^-$ cells and some strains of HIV-I have been shown, *in vitro*, to use heparans and galactocerebroside to aid attachment (Clapham *et al.*, 1999; Fantini, 1999).

Gp120 has five variable regions, V1-V5 (figure 1.10), interspersed with conserved regions (Eckert and Kim, 2001). The binding surface is large and present on all three gp120 domains and consists of variable and conserved regions (Eckert and Kim, 2001). Once gp120 binds to CD4, conformation changes occur that exposes a chemokine receptor site on the V3 loop (Eckert and Kim, 2001; Fantini, 1999; Moore and Stevenson, 2000).

Potential exists to design an inhibitor to gp120. Soluble CD4 (sCD4) has been used to "mop up" gp120 and act competitively with target cell CD4 (Deen *et al.*, 1988). At first, this showed great efficacy with lab strains of HIV but later it was seen to be ineffective with primary isolates (Eckert and Kim, 2001). In fact, in some cases, sCD4 served to enhance infectivity by inducing the conformation changes in gp120 and cause its release to give a fusion active virion that could even infect $CD4^-$ cells (Eckert and Kim, 2001; Hart *et al.*, 1991).

The crystal structure of gp120 offered a more feasible option by revealing a small pocket that can accommodate an inhibitor in the binding site and prevent binding of the gp120 to the target cell (Moore and Stevenson, 2000). Inhibitors can also be designed to target the V3 loop to change the conformation of the gp120 to a format unfavourable for binding (Moore and Stevenson, 2000).

Antibodies have been designed to target CD4, but there are obvious concerns about immune suppression by depleting CD4 cells. Recent developments have produced antibodies that do not deplete CD4 cells *in vivo* (Moore and Stevenson, 2000).

1.4.1.3 Gp120 and co-receptor interactions

Once gp120 has bound to CD4, it is in the correct conformation for binding to a co-receptor (Moore and Stevenson, 2000).

The HIV co-receptors belong to the seven-transmembrane spanning, guanine-nucleotide-binding (G)-protein-coupled receptor superfamily and there has been shown to be 11 possible co-receptors for HIV-I and HIV-II (Clapham *et al.*, 1999; Eckert and Kim, 2001; Greene and Peterlin, 2002; Moore and Stevenson, 2000). However, *in vivo* studies indicate that the main receptors are CCR5 and CXCR4 (Moore and Stevenson, 2000). The normal function of these co-receptors is to bind chemokines that signal the cells to migrate along a chemokine gradient in an immune response (Moore and Stevenson, 2000). Chemokine receptors are found in lipid rafts (cholesterol and sphingolipid-enriched microdomains) in the plasma membrane and these rafts mirror the lipid bilayer of the virus to provide a better environment for fusion (Greene and Peterlin, 2002). Removing cholesterol from either the virus or target cell, results in a greatly reduced viral infectivity (Greene and Peterlin, 2002).

CCR5 receptors are found mainly on memory T-lymphocytes, macrophages and dendritic cells and the strain of HIV that shows this tropism is referred to as a R5 virus (Clapham *et al.*, 1999; Eckert and Kim, 2001). The majority of initial infections are with R5 strains and as disease progresses, viruses that use the co-receptor CXCR4 (X4 viruses), emerge (Clapham *et al.*, 1999; Eckert and Kim, 2001; Greene and Peterlin, 2002). It is unclear whether strains switch from R5 to X4 or have the capacity for multiple receptor recognition (Clapham *et al.*, 1999). CXCR4 is widely expressed and can be found on macrophages but it is unclear whether X4 viruses actually infect macrophages (Clapham *et al.*, 1999; Moore and Stevenson, 2000). The candidate cell types for first-line infection are at and below the mucosal membrane and these include dendritic langerhans cells and macrophages, which probably explains the initial infection of R5 strains (Clapham *et al.*, 1999).

Blocking the co-receptors is a promising target for anti-HIV therapy, as these are static, not prone to mutations and each has a specific ligand that blocks HIV infection (Eckert and Kim, 2001).

One to two percent of Caucasians have a homozygous 32 base pair (bp) deletion of the gene encoding CCR5. This makes it possible for these individuals to be resistant to R5 strains and they seem to live a normal life despite the absence of CCR5 (Eckert and Kim,

2001; Greene and Peterlin, 2002; Moore and Stevenson, 2000). Therefore, it seems possible to block CCR5 with little toxic side effects. A heterozygous deletion of CCR5 or a mutation in the CCR5 promoter region slows progression to AIDS (Eckert and Kim, 2001).

CXCR4 may be more problematic as homozygous deletions in mice result in fatal embryonic phenotypes, but there is still hope, as inhibitors of CXCR4 do not seem to be toxic in mice (Moore and Stevenson, 2000).

Using co-receptor ligands to block HIV could cause undesirable immune responses or interfere with normal signalling in the inflammatory pathway. For this reason, modified chemokines, antibodies and peptides have been investigated in the hope of finding an inhibitor that does not induce chemotaxis (Clapham *et al.*, 1999; Eckert and Kim, 2001; Moore and Stevenson, 2000).

There are three different ways in which inhibitors of chemokine receptors could act (Moore and Stevenson, 2000):

1. Competitive binding to block gp120
2. Downregulation of the co-receptor
3. Alter the state of the cell as to produce an ineffectual host environment for the virus.

1.4.1.4 Gp41-mediated fusion

Once again, after binding of the gp120 to the co-receptor, the glycoprotein undergoes conformation change so that the trimeric gp41 protein's hydrophobic amino-terminal is exposed. This is immediately inserted into the target cell's lipid membrane (Moore and Stevenson, 2000). Gp41 then forms a hairpin loop, bringing both viral and target cell membranes in close proximity for fusion (Figure 1.11) (Eckert and Kim, 2001).

Possible targets include a highly conserved hydrophobic groove in the amino-terminal region, since some residues lining this are critical for membrane fusion (Eckert and Kim,

2001, Moore and Stevenson, 2000). Small cyclic peptides designed to target this are in clinical trials and they are referred to as D-peptides (Root *et al.*, 2001).

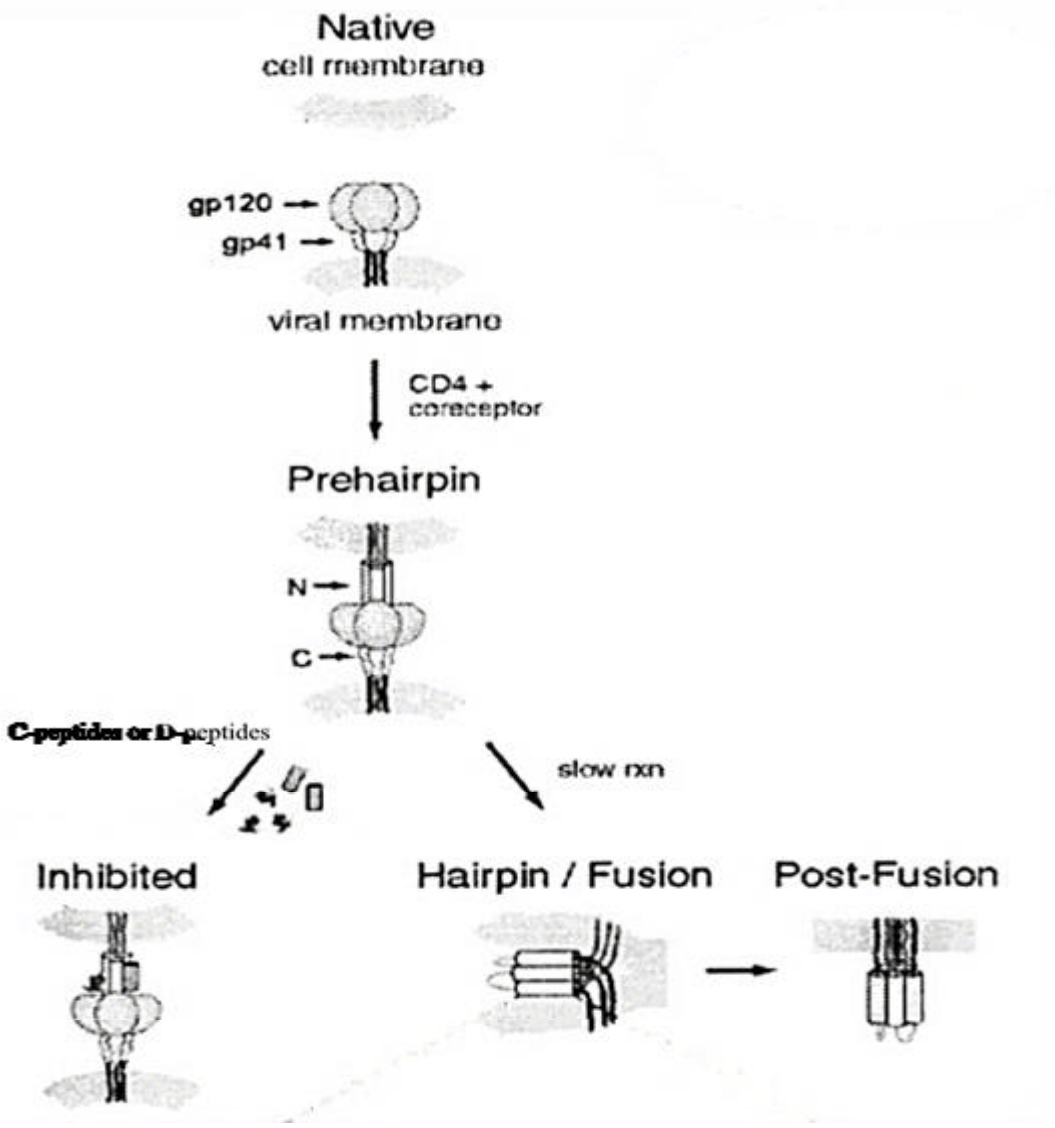

Figure 1.11: Schematic representation of the current working model for viral membrane fusion (Eckert and Kim, 2001).

Synthetic peptides have also been designed from the carboxyl-terminal region (C-peptides), which target the amino-terminal region and disrupt the formation of the trimer of hairpin structures (Eckert and Kim, 2001; Root *et al.*, 2001). The same has been done with the carboxyl-terminal region with the design of N-peptides (Eckert and Kim, 2001; Root *et al.*, 2001).

1.4.1.5 Escape Mutants

Using an entry inhibitor could potentially lead to a selection of viruses with altered tropisms or "escape" mutants (Eckert and Kim, 2001; Moore and Stevenson, 2000). Amino acid changes can occur to reduce the affinity for the inhibitor in the case with gp41 and gp120 or viruses could become CD4 independent (Moore and Stevenson, 2000). With resistance to co-receptor inhibitors, viruses could bind to the inhibitor in an insensitive manner, switch from CCR5 to CXCR4 or vice versa or use a different co-receptor altogether as is shown in figure 1.12 (Moore and Stevenson, 2000).

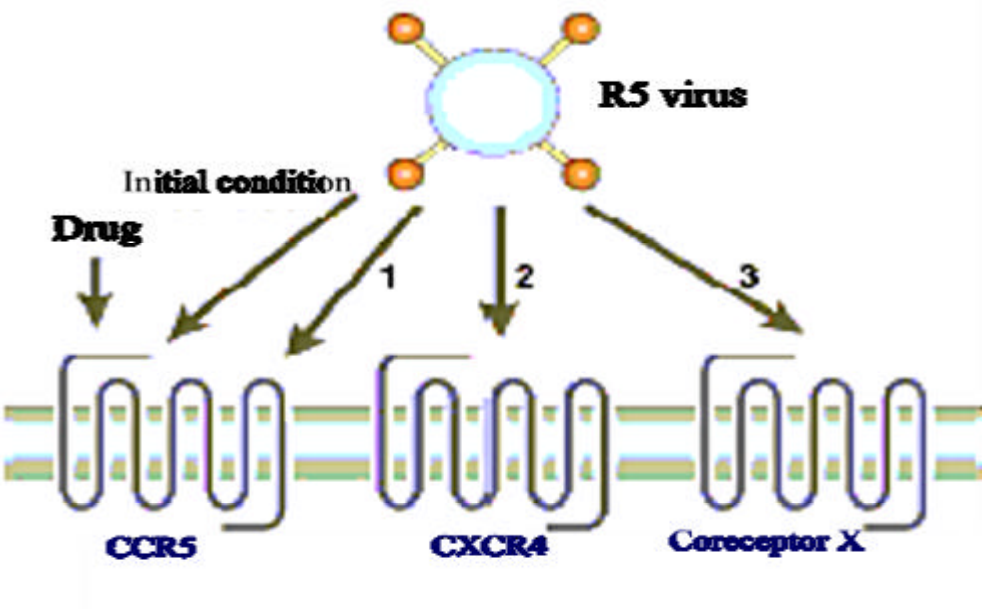

Figure 1.12: Escape pathways for a CCR5-targeted inhibitor of the HIV-1 entry.
1) Use receptor in a drug-insensitive manner. 2) Switch to use of CXCR4.
3) Use a new alternative receptor (Moore and Stevenson, 2000).

1.4.2 Post-entry Targets

1.4.2.1 Uncoating

Fusion of the virion leads to the release of an RT complex consisting of diploid RNA, transfer RNA (tRNA) primer, RT, IN, matrix protein (MA), nucleocapsid protein (NC), viral protein R (Vpr) and host proteins into the target cell by uncoating them from the capsid core (figure 1.13) (Greene and Peterlin, 2002).

The process is thought to involve phosphorylation and actions by the viral proteins negativity factor (Nef) and viral infectivity factor (Vif) (Greene and Peterlin, 2002). Nef is associated with a universal pump and probably works by changing the pH to mediate uncoating (Greene and Peterlin, 2002). Vif stabilises the RT complex and it is thought to block defensive cell factors that could prevent formation of infectious virions (Moore and Stevenson, 2000). The host protein, cyclophilin A helps in uncoating and binds to the HIV capsid protein (Braaten *et al.*, 1996; Moore and Stevenson, 2000). Budding virion particles package cyclophilin A into the new viral particle (Moore and Stevenson, 2000).

Cyclosporin A inhibits cyclophilin A and leads to the release of non-infectious virions but this has not been expanded on, as it is highly immunosuppressive and there is the possibility of HIV becoming cyclophilin A-independent (Moore and Stevenson, 2000).

1.4.2.2 Reverse Transcriptase

Since the HIV genome consists of RNA, it must first be converted from RNA to DNA to complete its lifecycle and produce new virions (figure 1.13). This is done in three stages (Davis *et al.*, 1991):

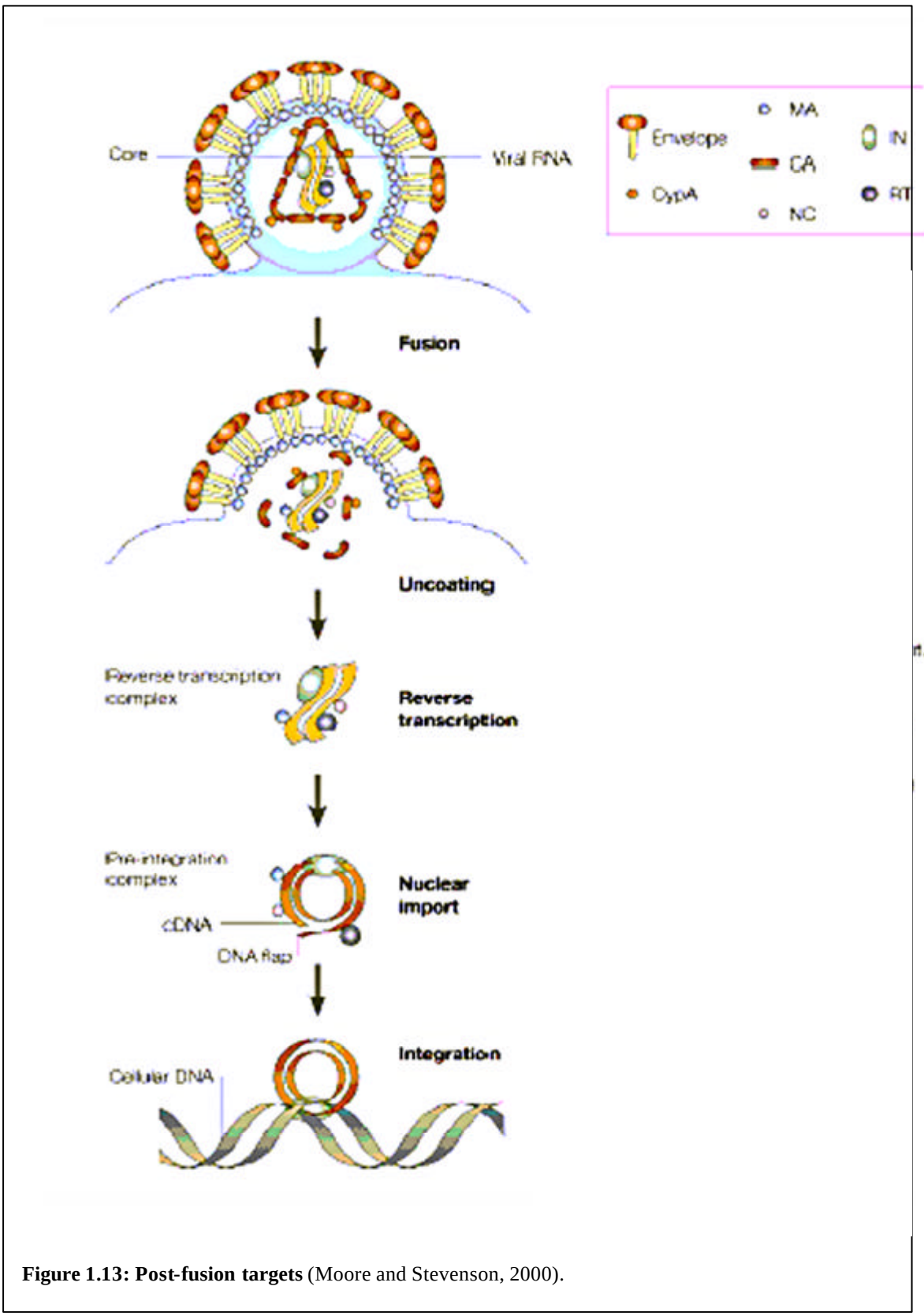

Figure 1.13: Post-fusion targets (Moore and Stevenson, 2000).

1. RNA-dependent DNA polymerase activity, involving RT makes a cDNA copy of the RNA template to form a RNA: DNA hybrid.
2. RNase H removes the tRNA primer via endonucleolytic action and selectively cleaves the phosphodiester bonds of the RNA during the synthesis of the positive strand of DNA.
3. The host enzyme, DNA-dependent DNA polymerase, makes a copy of the cDNA to give a double-stranded DNA complex.

RT is quite unique to retroviruses and consequently makes a good target (Palca, 1991). RT consists of two subunits, one of 66 kDa and the other 55 kDa (Davis *et al.*, 1991). The amino-terminal region of both units is identical and it is speculated that the 55 kDa unit is derived from the 66 kDa unit by proteolytic cleavage (Davis *et al.*, 1991; Palca, 1991,). Sequence homology studies show the polymerase activity to be at the amino-terminal region (Davis *et al*, 1991; Palca, 1991).

The carboxyl-terminal region of the 66 kDa unit contains the RNase H domain and the active site for both the RT and RNase are physically and functionally separate (Davis *et al.*, 1991). RNase H isolated on its own does not seem to function and probably requires the polymerase domain for correct binding (Davis *et al.*, 1991).

Besides designing inhibitors to the catalytic sites, RNase H has two metal binding sites and a third for uranium and blocking these sites has potential for inhibiting RNase activity (Davis *et al.*, 1991; Palca, 1991). There are many inhibitors of RNase H but none have made it to the clinical setting (Moore and Stevenson, 2000).

Inhibitors designed for RT can be divided into those that inhibit elongation in transcription by incorporating themselves into the growing cDNA chain or those that inhibit RT directly.

The first lines of drug therapy ever used against HIV were nucleoside RT inhibitors (nucleoside analogues) (Klatt, 2002). RT shows a higher affinity for the nucleoside analogues over the natural nucleotide substrate and these serve as chain terminators in the growing DNA chain as they lack a 3' hydroxyl (OH) group (Balzarini and De Clercq, 1996). These need to be phosphorylated before they become active, which is a rate-limiting step (Balzarini and De Clercq, 1996; Klatt, 2002). Because of the rate-limiting

phosphorylation step, nucleotide cyclic analogues were designed and these act in the same manner as the nucleoside analogues but they do not need to be phosphorylated (Klatt, 2002).

Non-nucleoside RT inhibitors are relatively non-toxic and are specific for the HIV RT. These do not need to be metabolised but resistant strains emerge more rapidly against these inhibitors as compared to nucleoside analogues. The non-nucleoside inhibitors interact with a hydrophobic pocket close to the catalytic site (non-substrate site) (Balzarini and De Clercq, 1996).

1.4.2.3 Nuclear Import

Completion of the reverse transcription, gives rise to a pre-integration complex (PIC) that consists of the double-stranded viral cDNA, IN, MA, Vpr, RT and host proteins and this needs to be transported to the nucleus (figure 1.13) (Craige, 2001; Greene and Peterlin, 2002). Inhibition of this step would confine the PIC to the cytoplasm and prevent any new infectious virions from being produced.

Positive-strand synthesis occurs in a discontinuous manner and because of this a triple helical DNA domain of 99bp (DNA flap) is generated and it is believed to be essential in nuclear targeting (Greene and Peterlin, 2002; Moore and Stevenson, 2000). Mutations in the DNA flap impair nuclear import and inhibit viral replication significantly (Moore and Stevenson, 2000).

Three other karyophiles implicated include IN and Vpr, which have nuclear localisation signals, and MA which has a nuclear localisation signal recognised by importin a and ß (Greene and Peterlin, 2002; Moore and Stevenson, 2000).

1.4.2.4 Integrase

The process of integration of the viral cDNA requires four steps and is an essential step in the HIV lifecycle (figure 1.14) (Craige, 2001):

1. Two nucleotides are removed from the 3'end of a conserved CA dinucleotide to create a 5' overhang and exposed hydroxyl groups for joining to the host DNA. This process is known as 3'end processing (Au *et al.*, 2001; Craige, 2001).
2. Host DNA is cleaved in a jagged manner, 5 bp apart (Au *et al.*, 2001; Thomas and Brady, 1997).
3. The pair of processed viral DNA ends are inserted into the target DNA and the 3'ends of viral DNA are covalently joined to the 5'ends of the target DNA. This process is known as strand transfer (Craige, 2001).
4. The viral 5' overhang is removed and host polymerase repairs the gaps and ensures complete ligation between the viral and host DNA (Craige, 2001).

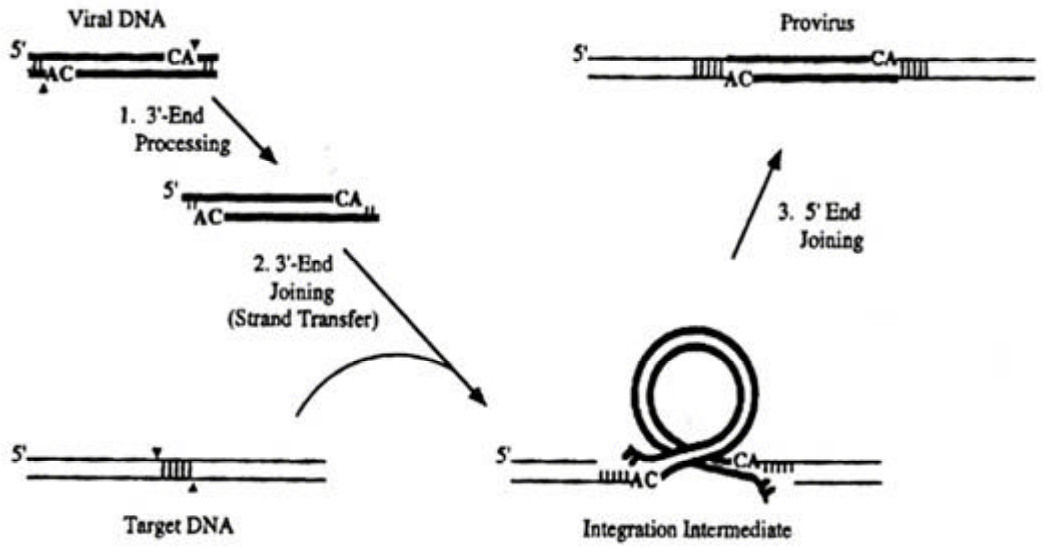

Figure 1.14: Pathway of retroviral DNA integration. Thick lines represent viral DNA, and thin lines represent target DNA. The conserved CA dinucleotide is indicated at the viral DNA end. Filled triangles represent sites of cleavage or 3'-end joining (Chow, 1997).

There is no known analogue in humans of IN, so it is an ideal target. The reason why there are no drugs on the market to date inhibiting IN, is because the study of IN has been problematic experimentally. IN solubilises poorly and is difficult to crystallize (Craige, 2001; Thomas and Brady, 1997). It is known that IN consists of three domains and acts as a multimer, but the structure of the active complex and the relationship between the three domains remains unclear (figure 1.15) (Craige, 2001). *In vitro*, IN can function as a dimer but stereochemical analysis suggests that for *in vivo* activity, a tetramer is necessary (Craige, 2001).

The catalytic domain, consisting of residues 5-212 (core), contains the acidic residues Asp^{64}, Asp^{116} and Glu^{152}. Mutational and crystallography studies reveal that all three are in close proximity, co-ordinate a divalent metal ion and define the active site. The whole structure is very flexible; therefore binding of DNA must dictate the correct conformation (Craige, 2001).

The amino-terminal domain, consisting of residues 1-49, has conserved His and Cys residues with a bound zinc which makes it very similar to the zinc co-ordinating residue of a zinc finger but its structure is very different (Craige, 2001; Thomas and Brady, 1997). The function of this domain remains unclear but it could play a role in multimerisation (Thomas and Brady, 1997).

The carboxyl-terminal domain (residues 213-288) has the ability to bind to DNA non-specifically and because the provirus is inserted non-specifically into the host genome, it was thought that this was the function of this domain (Craige, 2001). However, the DNA binding function has been found to be located in the catalytic domain (Craige, 2001). The carboxyl-terminal domain also has a putative nuclear localisation signal (Thomas and Brady, 1997).

Inhibition of IN could be achieved by disrupting the multimer formation but finding a specific agent to do this is problematic (Thomas and Brady, 1997). Inhibitors of the integrase reaction could act by changing the surface of DNA or interfere with the

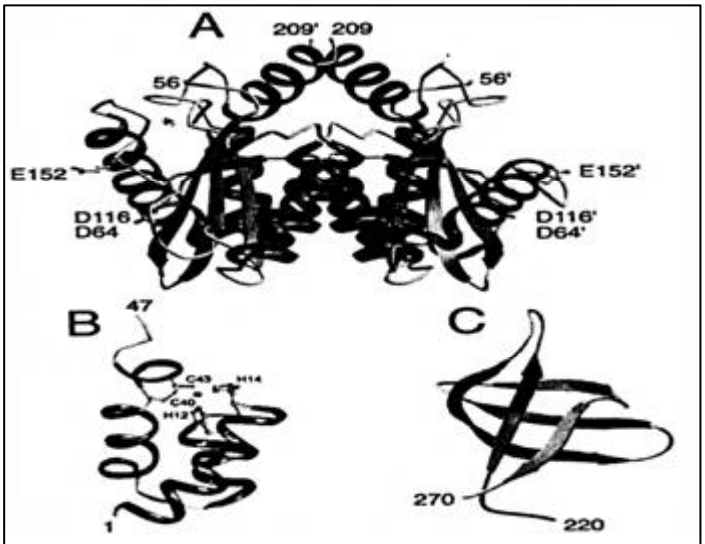

Figure 1.15: Structures of three domains of HIV-I integrase. A) Core domain. B) Amino-terminal domain. C) Carboxyl-terminal domain (Craige, 2001)

physical binding of DNA but again there is the problem of specificity (Thomas and Brady, 1997). Long terminal repeats (LTR) at the ends of HIV DNA are highly conserved in all strains and integrase interacts with these so it could be used as a target for DNA binding compounds that subsequently inhibit IN (Moore and Stevenson, 2000; Thomas and Brady, 1997).

It is better to target the catalytic activities of 3'end processing and strand transfer to ensure less interference with normal host functioning by inhibiting the IN active site (Moore and Stevenson, 2000; Thomas and Brady, 1997). Inhibitors that block the strand transfer activity all seem to have diketo acid moieties that are very toxic but this structure may not be necessary for activity as not all compounds with this moiety are inhibitory (Hazuda and Felock, 2000; Moore and Stevenson, 2000). Work is being done to find useful compounds without this structure to bring to clinical trials (Hazuda and Felock, 2000; Moore and Stevenson, 2000).

1.4.2.5 Protease

Another step in the HIV lifecycle that has been successfully targeted is the proteolysis stage. Inhibition of this or a mutation in the HIV protein responsible, PR, leads to

immature virions that are non-infectious (Meek *et al.*, 1990; Wlodawer and Vondrasek, 1998).

The HIV-PR is responsible for processing HIV proteins to give the correct sized and functional proteins and to do this requires specific amino acid or conformation recognition (Ringe, 1994). PR prefers aromatic residues, Pro and hydrophobic residues, which indicates it tolerates variety (Ringe, 1994). Examples of proteins that are cleaved by PR are the p55 from *gag*, which is processed to produce functional p17, p24, p7, p6, and p160, the precursor glycoprotein, which is cleaved to form functional gp120 and gp41 (Meek *et al.*, 1990).

Analysis of the amino acid sequence of HIV-PR, has revealed the signature sequence, Asp-Thr-Gly and it has been shown that HIV-PR is related to eukaryotic aspartic proteases (pepsin-like proteases) and is inhibited by the eukaryotic protease inhibitor, pepstatin (Wlodawer and Vondrasek, 1998). This places HIV-PR in the class of aspartic proteases. The active conformation of HIV-PR is a homodimer and the active site is similar to pepsin-like proteases (Wlodawer and Vondrasek, 1998). The active site (Asp^{25}-Thr^{26}-Gly^{27}) can accommodate six to eight amino acids, is situated between the subunits and is stabilised by a network of hydrogen bonds (Meek *et al*, 1990; Ringe, 1994; Wlodawer and Vondrasek, 1998). The Asp residues are donated from each subunit and tightly bind a water molecule and it is thought that this plays a role in general acid-base catalysis (Ringe, 1994). Displacing this could lead to inactivation (Ringe, 1994).

The fact that HIV-PR is similar to cellular aspartic proteases makes it a difficult protein to target but there are some differences. Even though central features of the retroviral and cellular aspartic protease catalytic sites are similar; the residue following the signature sequence is Ala in retroviruses and Ser/Thr in pepsin-like proteases (Wlodawer and Vondrasek, 1998). Another difference between the two types of proteases is the “flap “ which refers to a ß-hairpin that covers the active site and participates in binding (Wlodawer and Vondrasek, 1998). This is singular in pepsin-like proteases and in retroviral proteases there are a pair of two-fold related flaps (Wlodawer and Vondrasek, 1998). The molecular weight of HIV-PR is also less than two-thirds that of pepsin (Wlodawer and Vondrasek, 1998). Despite the differences, HIV-PR inhibitors are known to inhibit cellular/host aspartic acid proteases (Strickler and Goldberg, 1998). It has been shown that HIV-PR

inhibitors inhibit the enzyme group called cathepsins, which are responsible for the degradation of glucagon, insulin and insulin-like growth factor (Strickler and Goldberg, 1998).

The design of inhibitors has been based on two main strategies. Inhibitors designed to mimic good substrates and inhibitors from structure-based studies. (Wlodawer and Vondrasek, 1998). Many analogues have been found but it is difficult to find inhibitors that don't interfere with normal cellular proteases (Klatt, 2002). Information from nuclear magnetic resonance (NMR) spectroscopy, crystallography and computational studies, have focused on finding the "best-fit" inhibitor of the HIV-PR active site (Wlodawer and Vondrasek, 1998). Work done at the Cambridge Crystallography Data Base using shape complementary algorithms and *in vitro* assays prove that the haloperidol structure (similar to a soccer ball) is the best fit (figure 1.16) (Ringe, 1994).

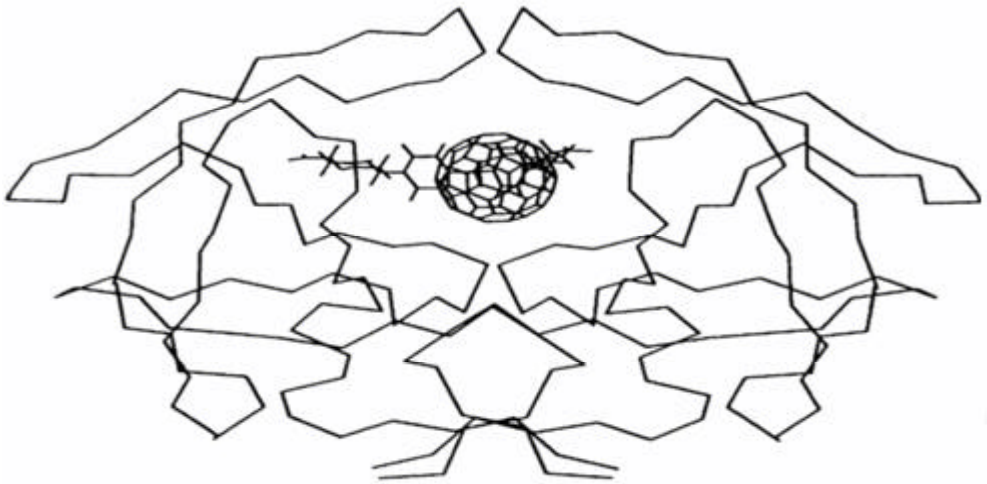

Figure 1.16: Model of soluble C_{60} fullerene ('bucky ball") in the active site of the HIV-I protease (Ringe, 1994).

1.4.3 Other possibilities

Most work has focused on the major steps in the HIV lifecycle and the main structural/functional proteins but attention is starting to focus on the accessory proteins as a means to inhibit HIV/AIDS.

Transcriptional activator (Tat) is critical for gene expression and regulation and binds to the HIV-LTR transactivation response element (TAR) to increase viral transcription initiation and elongation plus it superactivates the HIV promoter (Marble and Key, 1997). Without Tat, initiation of transcription can still occur but the RNA polymerase II will separate from the template more readily so that incomplete transcription of the HIV genome occurs (Marble and Key, 1997). Tat also has the ability, along with gp120, to trigger the release of cytokines that promote a T_{H2} response when a T_{H1} response is needed to direct CD8 cells against virus infected cells (Klatt, 2002). An increase in the T_{H2} response also leads to more CD4 target cells being produced and activated (Klatt, 2002).

Antibodies that sequester Tat in the cytoplasm and block its transport to the nucleus are potent inhibitors (Marble and Key, 1997). Small chemicals exist already for Tat inhibition and it is just a matter of them being viable in an *in vivo* system and clinical setting (Greene and Peterlin, 2002).

HIV Vpr is found packaged with virions and studies making recombinant Vpr showed that progeny viruses were less infectious. Expression, assembly and release were not affected (Key *et al.*, 1998). This is another accessory protein that can possibly be targeted.

Another target is Nef, which promotes the production and release of infectious virions. The absence of Nef has been shown to slow the progression to AIDS. Nef plays a role in uncoating but has many other functions as well. This protein can activate Fas ligand (FasL), which induces apoptosis in bystanding cells that express the FasL receptor e.g. CD8 cells and it blocks apoptosis in the host cell by binding to signal regulating kinases. Nef has also been found to decrease the major histocompatibility complex I (MHC I) receptor on the host cell to reduce the risk of the host cell being recognised by CD8 cells (Greene and Peterlin, 2002).

The regulator of viral gene expression (Rev) plays an important function in ensuring that the viral pre-mRNA is transported to the cytoplasm. Absence of this protein has resulted in the viral mRNA being sequestered in the nucleus and subsequently being degraded, which suggests that Rev is another potential target (Malim and Cullen, 1993).

Viral nucleocapsid proteins promote viral genomic RNA assembly before the new virion is formed and all have zinc finger motifs where the His and Cys residues are co-ordinated by a zinc cation. Small molecules that displace the zinc ion impair RNA packaging and impair the progeny virion's infectivity but this lacks specificity (Moore and Stevenson, 2000).

1.5 Objectives of this study

South Africa is a country with a rich source of untapped phytochemicals and it has an established culture of traditional medicine that could provide hope for many AIDS sufferers. These medicines need to be studied to confirm and verify if there is indeed any anti-HIV activity. Information from the study can then be used to verify the usage of the plant and develop further market products.

The purpose of this study was to investigate various extracts from *Sutherlandia frutescens* and *Lobostemon trigonum*, which are commonly used in traditional medicine, and to perform *in vitro* assays to evaluate the potential anti-HIV activity of these medicinal herbs.

In 2001 an association was formed with traditional healers from Nyangazezizwe Traditional Healers Association, the Port Elizabeth Traditional Healers Association and the Department of Biochemistry and Microbiology at UPE to look for scientific evidence of herbal remedies efficacy. This project forms part of a research group in the laboratory that is investigating the medicinal properties of herbal remedies and plants used by the healers. Other projects include investigating anti-cancer, anti-diabetic, anti-coagulant and anti-microbial effects of some herbal remedies and plants.

2 Plant extracts

2.1 Introduction

In the selection process of choosing medicinal plants, there are several ways in which one can go about deciding which plants to look at. One way is to look at plants with compounds of unusual structure or to do a random screening as is done at the NCI. The selection process that has proven the most successful though, is to focus on those plants with reported historical use. This, however, is not always easy with HIV studies, as AIDS has only been known since the 1980's (Houghton, 1996).

2.1.1 Sutherlandia

For the purpose of this study, two plant species commonly used in South Africa in traditional medicines, *Sutherlandia frutescens* and *Lobostemon trigonum* were studied.

Sutherlandia frutescens has received international attention as a cheap herbal medicine that can improve the health of AIDS patients (New Straits Times-Management Times, 12 November 2001). Anecdotal evidence from doctors and health care workers have reported HIV-infected people using *Sutherlandia* treatments; gaining weight, having improved CD4 counts, decreased viral loads, improved appetites and general mood improvements (Chaffy and Stokes, 2002; comm. from Phyto Nova). Charles Wambe of the National Institute for Pharmacological Research and Development in Nigeria, along with WHO collaboration, has started a pilot trial with *Sutherlandia* and HIV-infected patients based on these reports (Chaffy and Stokes, 2002; Morris, 2001). Phyto Nova, a small company, has for the last two years been distributing *Sutherlandia* tablets as a supplement to AIDS sufferers with no reported adverse effects, while studies done in mice given 1500mg/kg orally, showed no adverse effects (comm. from Phyto Nova). Studies done at the Medical Research Council in South Africa have also shown that feeding *Sutherlandia* to vervet monkeys at nine times the recommended dose produced little or no side effect (Müller, 2002). Although much

work has been done on *Sutherlandia* there is nothing published that identifies how *Sutherlandia* may be helping AIDS patients.

There are six species of *Sutherlandia* all of which are endemic to South Africa but there is much inter-population variation and the classification of the subspecies is unclear (Moshe, 1998). The genus has also been renamed recently to *Lessertia* but is still referred to by its more favoured name of *Sutherlandia* (Müller, 2002).

Figure 2.1: **Picture of *Sutherlandia frutescens*** (vanWyk *et al*., 1997).

Family: *Sutherlandia*

Tribe: *Galegeae*

Genera: *Sutherlandia*

Species: *frutescens*

Sutherlandia frutescens is a one metre high bush characterised by hairy leaves, large red flowers and bladder-like pods (Figure 2.1) (van Wyk et al., 1997). In preparations of traditional medicines, the leaves are mainly used but sometimes everything above ground level is used (van Wyk et al., 1997). A bitter tonic is made that is used to treat colds, flu, cancer, chicken pox, diabetes, varicose veins, piles, inflammation, liver problems, backache, rheumatism, fever, poor appetite, indigestion, gastritis, oesophagitis, peptic ulcers, dysentery, heart failure, kidney problems, stress and urinary infections (van Wyk and Gericke, 2000; van Wyk *et al.*, 1997). Because of its multiple medicinal uses, it has been referred to as Cancerbush/"kankerbos" and "insisa", Zulu for, "dispels the darkness" (van Wyk *et al.*, 1997)

The components of *Sutherlandia* have been investigated and it has been found to contain a high yield of amino acids, which could explain its medicinal properties, substantial triterpenoids, detectable flavonoids and no alkaloids or monoterpenoids (Moshe, 1998). Four compounds found in *Sutherlandia* that have been described are L-canavanine, ?-aminobutyric acid (GABA), pinitol and a triterpenoid designated SU1 (comm. from Phyto Nova; Moshe, 1998).

Sutherlandia seeds are rich in L-canavanine, [2 amino-4 (guanidinooxy) butyric acid], a structural analogue of arginine (figure 2.2) (Moshe, 1998; van Wyk *et al.*, 1997). This compound has already been patented and it has been found to have anti-tumourigenic properties and antimicrobial and antiviral activity (Crooks, 1994). Canavanine, a non-protein amino acid, also inhibits nitric oxide synthase, which is a benefit in heart failure (Li *et al.*, 2001; van Wyk and Gericke, 2000).

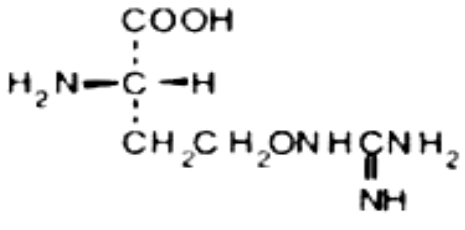

Figure 2.2: Structure of L-canavanine (Moshe,1998)

Pinitol, another patented compound, is found in significant levels in *Sutherlandia*. This compound is an anti-diabetic agent with hypoglycaemic activity and it is thought to treat wasting seen in cancer and AIDS patients (figure 2.3) (Ostlund, 1996; van Wyk and Gericke, 2000). Studies performed in rats have shown that pinitol also has a significant anti-inflammatory effect (Singh *et al.*, 2001).

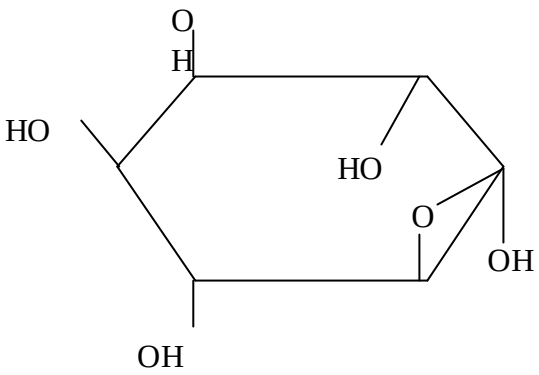

Figure 2.3: Pinitol structure (Cowan, 1999).

GABA is an inhibitory neurotransmitter, which may account for the use of *Sutherlandia* in treating stress (Morris, 2001; van Wyk and Gericke, 2000). It has also been found useful to treat inflammatory diseases (Schrier *et al.*, 2001).

The triterpenoid, SU1, has shown to have immune-stimulating activity (comm. from Phyto Nova). *In vitro* and *in vivo* studies have shown triterpenoids to act against various diseases, contain anti-viral activity and have anti-cancer activity by preventing the proliferation of cancer cells (Moshe, 1998).

French scientists in the Paris-based non-governmental organisation (NGO) Afrique Initiative, are busy working with *Sutherlandia* and have discovered that *Sutherlandia* contains tumour necrosis factor (TNF) inhibitors. Excessive TNF drives wasting and this could explain the weight gain seen in cancer and AIDS patients. (comm. from Phyto Nova).

2.1.2 *Lobostemon*

Little is known about the chemistry of *Lobostemon* and the only work done on *Lobostemon* has been botanical to help characterise and classify the species and sub-species.

One species, *Lobostemon fruticosis,* has been looked at more closely with regards to its medicinal properties. It is also known as "agdaegeneesbos" (Afrikaans; meaning eight day healing bush) due to its apparent ability to heal a condition in eight days. Another name used is "douwurmbos", as it is believed to treat ringworm. Decoctions are used to treat wounds, skin disease, ringworm and ulcers while infusions are used for general internal problems and to purify the blood (van Wyk and Gericke, 2000; van Wyk *et al.*, 1997).

L. fruticosus is closely related to comfrey (*Symphytum officinale*) and borage (*Borago officinales*). For this reason it is thought that *L. fruticosus* may have similar compounds. Comfrey has allantoin, which is well known for its wound-healing properties. Comfrey also has pyrrolizidine alkaloids like symphytine but this is a cumulative toxin (van Wyk and Gericke, 2000; van Wyk *et al.*, 1997). Traditional healers also believe this plant to have anti-HIV properties.
Unfortunately however, this plant is very difficult to find and almost impossible to cultivate so it was decided to use another *Lobostemon* species, *Lobostemon trigonum* with the hopes that there would be an overlap in medicinal properties. A preliminary study done previously in this department by S. Boudler (2001) with *L.trigonum* also showed promising results in *in vitro* RT assays and deserves further investigation.

2.2 Materials and methods

Plant material was collected in one batch from the Eastern Cape region in October 2002 and classified by the Botany department at UPE. The plant material was separated into flowers and leaves and oven-dried at 80°C until dry and brittle. For *Sutherlandia frutescens*, the leaves and flowers were used, where as only leaves were used for *Lobostemon trigonum* (*L. trigonum* was not flowering at time of collection). The dried plant parts were ground to a fine texture with a homogeniser.

Researchers have used several different techniques of extraction and a variety of solvents when examining plant extracts. A more common method is to soak crushed dry plant material in cold or warm water, homogenize and then filter (Au *et al.*, 2001; Collins *et al.*, 1997; Cowan, 1999). Although using cold water ensures that protein components are not destroyed, it is possible to lose active compounds in the filtering process.

It was important to mimic traditional medicine preparation as much as possible, and in the case of *Sutherlandia* a decoction or alcoholic tincture is prepared (Houghton and Raman, 1998; van Wyk *et al.*, 1997). For *Lobostemon*, infusions are made (van Wyk *et al.*, 1997).

For the purpose of this study, a range of solvents were used to ensure that most of the potential active components were extracted. Distilled water, high-grade ethanol, methanol, acetone and methylene dichloride were used for both *Sutherlandia* and *Lobostemon* so that comparative studies could be done. Ethanol and methanol are commonly used and can extract aromatic and saturated organic compounds that have been identified to be anti-microbial (Cowan, 1999). Acetone has been neglected in the study of plant material and in terms of its potential to solubilize antimicrobials from plants, its ease of removal from the fraction and the biohazard risk, it is thought to be one of the more promising solvents (Cowan, 1999). Table 2.1 shows the compounds that can potentially be removed from the plant material with each solvent.

For each solvent, 20g to 25g of the crushed plant material was used depending on availability and in the case of the *Sutherlandia* flowers only 4g was used. Aqueous extracts were prepared by leaching the crushed plant material in 100 to 200ml double distilled water in a 1l flask in the dark at 4°C. This was left to soak for four days and each day the extract was filtered using Whatman no 1 paper, and fresh water was added. Supernatant was then lyophilised. Extracts from the organic solvents were prepared in much the same way but the acetone, methanol and ethanol extracts were left at room temperature to soak. No methylene dichloride extract was prepared from the *Sutherlandia* flowers as there was not enough plant material. Organic solvent extracts were dried by rotary evaporation at a temperature not greater than 60°C. Most of the organic extracts were extremely oily, so to further dry the extracts, they were re- dissolved in the minimum amount of relevant solvent and double distilled water was added so the final volume of solvent was 10% v/v. This was then lyophilised.

Table 2.1: **Solvents used for active compound extraction** (Cowan, 1999)

Water	Ethanol	Methanol	Chloroform	Dichloromethanol	Ether	Acetone
Anthocyanins	Tannins	Anthocyanins	Terpenoids	Terpenoids	Alkaloids	Flavonols
Starches	Polyphenols	Terpenoids	Flavonoids		Terpenoids	
Tannins	Polyacetylenes	Saponins			Coumarins	
Saponins	Flavonols	Tannins			Fatty acids	
Terpenoids	Terpenoids	Xanthoxylines				
Polypeptides	Sterols	Totarol				
Lectins	Alkaloids	Quassinoids				
	Propolls	Lactones				
		Flavones				
		Phenolles				
		Polyphenols				

In order to test the extracts in biological assays, the dried material was reconstituted in dimethylsulphoxide (DMSO). DMSO serves to sterilize the extract and once diluted (3%) should have no effect on the biological assays (Houghton and Raman, 1998). Extracts were stored at 4°C in the dark at 15mg/ml. For cell-based work, extracts were prepared in a laminar flow hood using aseptic techniques and sterile DMSO to give a final concentration of extract at 6mg/ml. To ensure that the aqueous extracts were solubilized, sterile double distilled water was added first (10% v/v) and then DMSO (90% v/v).

2.2.1 Removal of sulphated polysaccharides

At the NCI, an automated system has been developed to screen large volumes of terrestrial plants, lichens, cultured cyanobacteria, marine invertebrates and algae and it was found that 15% of the aqueous extracts showed anti-HIV activity (Cardellina *et al.*, 1993). This high percentage is more than likely due to recurring compound classes. Because of this, a system known as dereplication technology was designed to overcome this problem. Sulphated polysaccharides have consistently showed activity in anti-HIV assays and it is believed to function by destabilizing the glycoprotein complex and/or inhibiting reverse transcriptase (Greene and Peterlin, 2002; Wagner and Farnsworth, 1991). Many

compounds from this class have already been described extensively in the literature as having anti-HIV activity (Wagner and Farnsworth, 1991). To determine if any potential anti-HIV activity seen in the aqueous extracts was due to sulphated polysaccharides, a portion of the extract for each of the *Sutherlandia* flowers and leaves and *Lobostemon* leaves was subjected to a 50% ethanol precipitation.

Half of the dried extract material was dissolved in the minimum amount of cold double distilled water to which an equal volume of 100% ethanol was slowly added, with stirring. This was left to stir at 4°C for 30 minutes then filtered using Whatman no. 1 paper. The supernatant was then lyophilised (Houghton and Raman, 1998)

2.3 Results and discussion

Table 2.2 represents the extract yields obtained, along with descriptions of their appearance

As can be seen in Table 2.2, a higher percentage yield was produced from the aqueous extracts for all three batches of plant material as compared to those of the organic extracts. In general, the organic extracts produced a very oily substance that if redissolved in solvent and water, then lyophilised, gave a powder that was easier to work with. This, however, could not be achieved with the acetone and methylene dichloride extracts as the oil was too thick. Extractions performed on the *Lobostemon* leaves yielded the least material as compared to *Sutherlandia* extracts.

For the removal of the sulphated polysaccharides, all the aqueous extracts left behind a gel-like residue on the filter paper, with *Lobostemon* giving the most.

Table 2.2: Plant extract yields and descriptions

Solvents		*Lobostemon*	*Sutherlandia*	
		Leaves	**Leaves**	**Flowers**
Aqueous	*Yield*	18.8%	27.1%	22.9%
	Description	Dark brown, shiny flakes	Fluffy, dark green flakes	Light yellow fluffy powder
	Code	LLW	SLW	SFW
Ethanol	*Yield*	2.9%	4.3%	7.9%
	Description	Dark green, sticky powder	Dark green powder. Highly hydroscopic	Light green, sticky powder
	Code	LLE	SLE	SFE
Acetone	*Yield*	4.7%	4.7%	7.6%
	Description	Dark green oil	Dark green, viscous oil	Green, viscous oil
	Code	LLA	SLA	SFA
Methanol	*Yield*	5.2%	11.5%	12%
	Description	Dark green dry material but looks oily	Fluffy, light brown. Highly hydroscopic	Dark yellow powder. Highly hydroscopic
	Code	LLM	SLM	SFM
Methylene dichloride	*Yield*	2.3%	2.5%	n/a
	Description	Viscous, dark green oil	Dark green, viscous oil	n/a
	Code	LLD	SLD	n/a

Yield was determined as the dry weight of the extract (E) divided by the weight of the plant material used for the extract (P) multiplied by 100 (E/P x 100).

n/a: not available/applicable

3 Cell-based Assays

3.1 Introduction

Guidelines laid out by WHO, with respect to the screening of traditional medicine for anti-HIV activity, suggest that a minimum of a single T-cell culture assay be performed (WHO, 1989). Other cell-free based systems to test anti-HIV activity are very specific and not only do they not give an indication of the cytotoxicity, but they may not pick up any anti-viral activity as determined by the nature of the assay. It may also be necessary for the compound to be metabolised before becoming active against the virus, as is the case with nucleoside inhibitors like Zidovudine (AZT) (Balzarini and De Clercq, 1996; Klatt 2002).

To study the effects of plant extracts on HIV, it is necessary to use a mammalian cell line, preferably primate in origin. In literature there is no consensus on which cell line to use or how to determine cytotoxicity and anti-viral effect.

In the study of cell cultures, there is often an overlap in the definition of viability and proliferation. "Viability" is the term used to describe healthy cells while "proliferation" describes dividing cells. Since only healthy cells can divide there is a tendency to interchange the two terms. To measure cell viability/proliferation, many different dyes can be employed. A direct measurement of cell viability uses trypan blue stain. Using a light microscope, cell counts are made to determine the number of viable cells (unstained) in culture as compared to dead cells (Traore and Meyer, 2002). Only the dead cells can take up the dye through damaged membranes. Although a direct measurement is considered a better tool, this assay is not suited for working with a large number of samples as is often done in traditional medicine/plant extract screening. Dilutions and counts would need to be made of each extract, at each concentration, in triplicate and this would be extremely labour intensive

An indirect method of determining cell proliferation/viability is to use a metabolic assay with a tetrazolium salt such as 3-(4, 5-dimethylthiazol-2-yl)-2,2-diphenyltetrazolium

bromide (MTT) or 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT). These dyes can only be metabolised by healthy viable cells to form a coloured product, e.g. XTT is reduced by the mitochondrial dehydrogenases (Ayala-Fierro *et al.*, 2000). This method has been adopted by the NCI to use in their screening of samples for anti-HIV or anti-cancer activity (Cardellina *et al.*, 1993; Weislow *et al.*, 1989). The MTT/XTT assay offers a quantitative determination of HIV-I induced cytopathic effects, as well as that of the extracts, it is non-radioactive and has been found to be sensitive and give accurate data. The XTT assay can also be automated and is better suited for screening of a large number of samples (Weislow *et al.*, 1989).

MTT, after cellular reduction, forms a coloured insoluble formazan product so an additional step must be included to solubilize the product before absorbance can be read. The additional step can possibly introduce more room for human error so it is preferential to use XTT as this produces a soluble formazan product as seen in figure 3.1. (Weislow *et al.*, 1989).

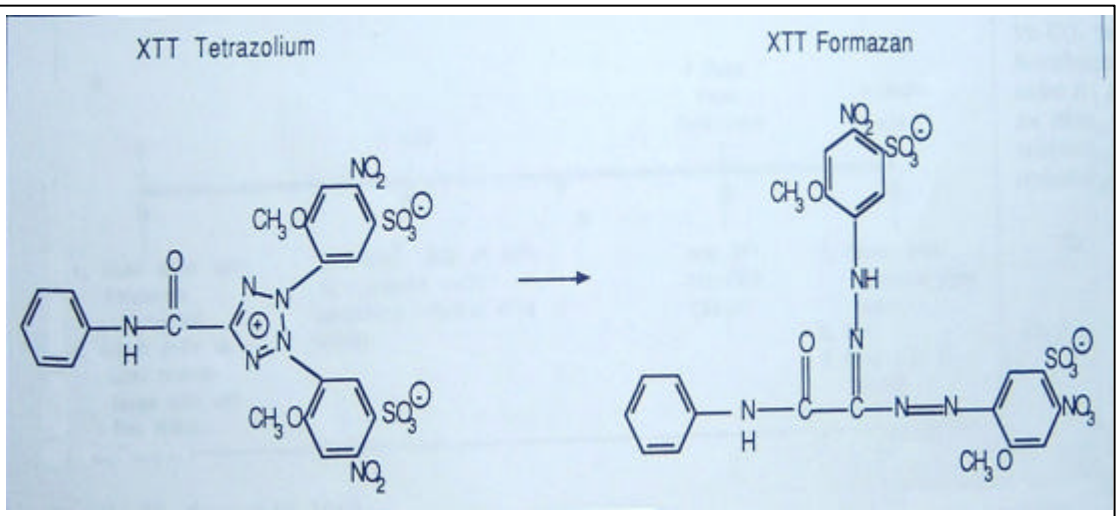

Figure 3.1: Chemical structures of XTT (colourless) and XTT formazan (orange) (Weislow *et al.*, 1989).

However, problems do occur with the XTT assay. It has been found that inaccuracies can occur due to human pipetting errors and the cell-free reduction of the tetrazolium salts by some plant extracts, therefore giving false positives (Bruggisser *et al.*, 2002; Weislow *et al.*, 1989). It is therefore essential to correlate the results with another assay such as core protein (p24) as this measures the viral infectivity directly.

3.2 Materials and methods

3.2.1 Reagents

3.2.1.1 Complete medium

Cells were maintained and cultured in complete medium consisting of RPMI 1640 containing 2mM L-glutamine (Sigma, MO USA). This was supplemented with 10 % (w/v) heat-inactivated (56°C, 30 minutes) foetal calf serum (FCS) and a mixture of antibiotics that consisted of 0.01% (v/v) antimytotic solution [penicillin G (10mg/ml), streptomycin sulfate (10mg/ml) and fungizone (25µg/ml)] and 0.01% (v/v) of 1% gentamycin sulphate.

3.2.1.2 Phytohaemagglutinin (PHA)

PHA is a lectin that has been isolated from red kidney beans, that is non-immune in origin and has the ability to agglutinate cells and precipitate complex carbohydrates (Roitt *et al.*, 2001). This is used to cause mitogenic stimulation of the uninfected peripheral blood mononuclear leukocytes (PBMCs) and therefore stimulate proliferation of both the B- and T-cells despite antigen specificity (Goldsby *et al.*, 2000).

3.2.1.3 Interleukin 2 (IL-2)

Interleukin-2 is added to infected PBMCs to stimulate cell proliferation of T-lymphocytes, natural killer cells and B-lymphocytes (Ortaldo *et al.*, 1984). It has been found in a recent study, that the lack of *in vitro* proliferation of HIV-specific $CD4^+$ T cells in viremia is probably due to diminished IL-2 but these cells still occur at a relatively high frequency in peripheral blood. With the addition of exogenous IL-2, proliferation *in vitro* is greatly improved (Iyasere *et al.*, 2003).

3.2.2 Cells

Two cell types were used in the viability assays. The first was PBMCs, which were isolated from blood using cell preparation tubes. Blood was drawn from healthy uninfected donors for the toxicity study, and from HIV-infected patients, attending the IC2 clinic at Helen Joseph Government Hospital in Auckland Park, for the viral study. PBMCs belong to the leukocyte group and have the advantage that the cells should contain all the necessary receptors (i.e. CCR5 and CXCR4) for HIV-I infection (Bleul *et al.*, 1997).

The second cell type, CEM-NK^R-CCR5, is a human T-lymphoblastic cell line obtained through the AIDS Research and Reference program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), National Institute of Health (NIH) (MD USA) from Dr Alexandra Trkola. These cells were transformed with a retroviral vector to express human CCR5 and show resistance to natural killer cell-mediated lysis and do not secrete infectious virus (Howell *et al.*, 1985; Trkola *et al.*, 1999). The HIV-I subtype C virus preferentially uses the CCR5 co-receptor for infection and it is this subtype that is prevalent in South Africa (http://www.unaids.org). These cells were maintained in complete medium. All incubations were done at 37°C, 5% CO_2 and 95% humidity for all the cells.

3.2.3 Cell counts

To determine the cell number and percent viability, aliquots of cells were diluted 10-fold with trypan blue solution (0.4% in 0.85% saline solution) and from this 10µl of the solution was transferred to a haemocytometer. Cell counting was done using a light microscope and stained (dead cells) and unstained cells (viable cells) were counted. The percentage of viable cells was calculated as the number of live cells / the total number of cells, x 100.

3.2.4 Virus

Virus stocks were prepared at the Rand Afrikaans University (RAU) in the department of Biochemistry and Chemistry from HIV and PBMCs obtained from the SA blood transfusion services and prepared according to the protocols described by E. Hill (2003).

3.2.5 Blood collection and cell preparation

Before drawing blood, informed consent was obtained from both healthy donors and patients while ensuring complete anonymity and confidentiality. Information acquired gave the sex, age, race and whether the patient was on HIV therapy or not.

To obtain PBMCs, 7 ml of blood was drawn into sodium citrate containing Vacutainer blood tubes, CPT^{TM} cell preparation tubes (Becton Dickinson Vacutainer systems, France) and PBMCs were isolated within 8 hours according to the protocol given by Becton Dickinson with the CPT^{TM} tubes. CPT^{TM} tubes contain gel and density gradient medium and after centrifugation, red blood cells are collected at the bottom of the gel plug and PBMCs and plasma remain at the top of the plug.

Plasma and PBMCs were then carefully added to a sterile centrifuge tube and washed once with complete medium. The PBMC pellet was then washed again with complete medium containing 30% (v/v) ammonium chloride potassium (ACK), so to lyse any red blood cell contamination.

PBMCs from uninfected donors were placed in complete medium with 2 µg/ml PHA (Sigma, MO USA) at 2 x 10^5 cells/ml and incubated for 3 days before being used in the viability assays. However, PBMCs isolated from infected patients, were used in the viability assays immediately once the cells were diluted to 2 x 10^5 cells/ml with complete medium and 0.001% (w/v) IL-2 (Sigma, MO USA) added.

3.2.6 Viability Assays

3.2.6.1 Toxicity study

For the toxicity study only uninfected PBMCs were used and the microtitre plate was set up according to figure 3.2.

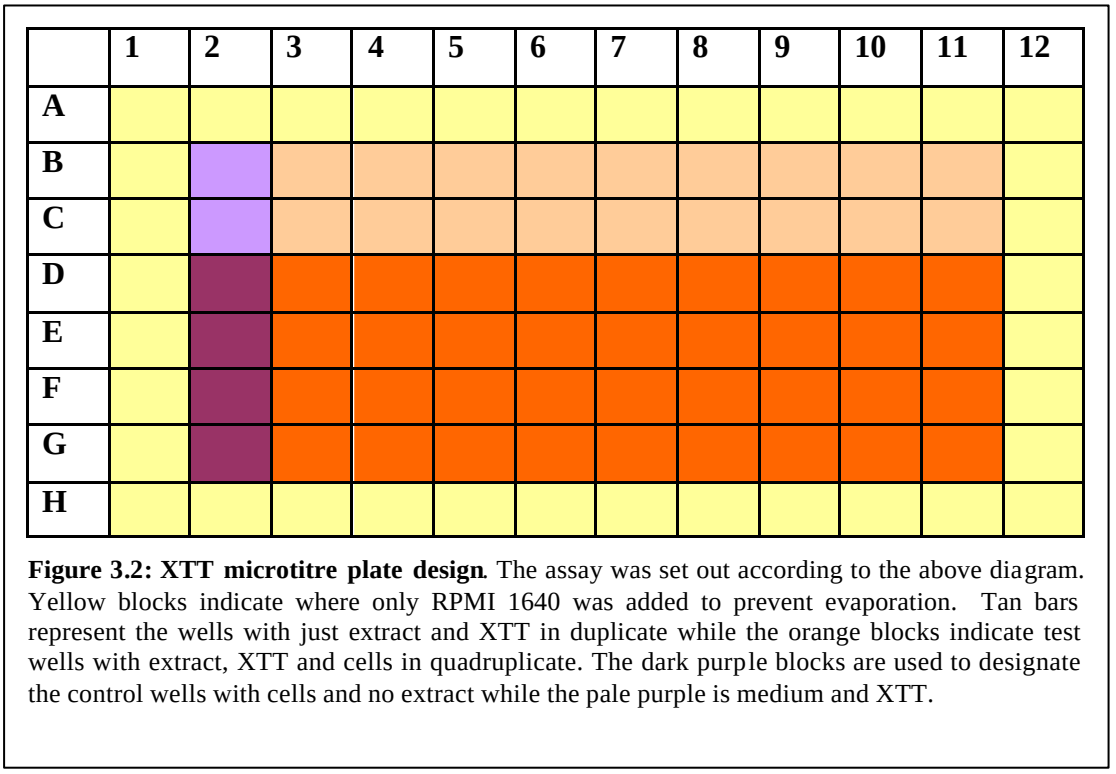

Figure 3.2: XTT microtitre plate design. The assay was set out according to the above diagram. Yellow blocks indicate where only RPMI 1640 was added to prevent evaporation. Tan bars represent the wells with just extract and XTT in duplicate while the orange blocks indicate test wells with extract, XTT and cells in quadruplicate. The dark purple blocks are used to designate the control wells with cells and no extract while the pale purple is medium and XTT.

Exponentially growing PBMCs were centrifuged for 10 minutes at 180g and supernatant discarded. The cells were then resuspended in complete medium, cell counts made (viability =95%) and cells diluted to 2 x 10^5 cells/ml. To the microtitre plate, 100µl of cells was added to the appropriate wells.

All the outside wells contained 50µl RPMI to prevent evaporation of the inner test wells and the second column contained only cells in medium to act as the untreated control. Plant extracts were tested in quadruplicate at a final concentration of 0.2mg/ml, 0.02mg/ml and 0.002mg/ml. The second and third rows of each plate (rows B and C) contained only the plant extract and medium so as to determine the background absorbance given by the extracts at each of the concentrations. The final volume in each well was 200µl.

The plates were incubated for 5 days after which viability of growing cells was assessed using the tetrazolium salt XTT from the Cell Proliferation kit (Roche Diagnostics, Mannheim Germany). XTT was prepared by adding 5ml XTT labelling reagent to 0.1ml electron coupling reagent and 50µl of this solution was added to all the inner wells as they were and the covered plate incubated at 37°C, 5% CO_2 and 95% humidity.

After four hours incubation, optical density was read at 450nm with the reference wavelength at 690nm. Cell viability was calculated using the formula [(A450nm-A690nm) of the test wells – (A450nm-A690nm) control wells]/(A450nm-A690nm) control wells multiplied by 100 to give the percent difference in cell viability as compared to the control.

3.2.6.2 Viral assay

For this study both infected PBMCs and CEM-NK^R-CCR5 cells were used. These experiments were performed in the laboratories of Dr Debra Meyer (Department of Chemistry and Biochemistry, RAU) due to the presence of the live HIV in the experiments. In the case of the infected PBMCs, the XTT assay was set up as in section 3.2.6.1 with the exception that 10µl of XTT was used to detect viable cells as opposed to 50µl in the toxicity study.

In the case of the CEM-NK^R-CCR5 cells, exponentially growing cells were counted using trypan blue and diluted to 2 x 10^5 cells/ml. This cell suspension was then centrifuged at 180g for 10 minutes and the pellet resuspended in 1.6ml HIV supernatant (section 3.2.4) to which polybrene (Sigma, MO USA) was added to a final concentration of 5 µg/ml. Polybrene facilitates cell and viral particle contact in cell culture (Gallo *et al.*, 1989).

The HIV-infected CEM-NK^R-CCR5 cells were then incubated for 2 hours with gentle shaking after which the XTT assay was set up as in section 3.2.6.1. Only the plant extracts SLW, SLW-PS, SLA, SLM, SFW, SFW-PS, SFA, SFM, LLW, LLW-PS, LLA and LLM were tested at a final concentration of 0.2mg/ml with the CEM-NK^R-CCR5 cells.

3.2.7 p24 assay

The HIV-I p24 Antigen Assay is an enzyme-linked immunosorbant assay (ELISA) that is used to detect and quantify HIV-I p24 core protein. This is a highly sensitive assay and p24 levels can be used to correlate viral infectivity and disease progression.
Culture supernatant from the viral assays (100µl) was transferred to the murine monoclonal-coated 96-well plate for the p24 assay. The protocol was followed exactly as described by the manufacturer (Beckman Coulter, Miami Florida USA), and for each plate, positive (HIV-I p24 Antigen reagent) and negative (human- [negative for HIV-I antigen(s), anti-HIV-I and II, HbsAa, and anti-HCV]) controls were set up, along with a standard curve.

To all the wells, 20µl lysis buffer was added and the plate incubated for one hour at 37°C. After the incubation step, the plate was washed with the provided wash buffer and 200µl biotinylated human anti-HIV-I Immunoglobulin G (IgG) was added and the plate incubated for another hour at 37°C. Again the plate was washed and streptavidin conjugated to horseradish peroxidase (SA-HRPO) was added to the wells with a further 30-minute incubation at room temperature. Tetramethylbenzendine (TMB) was added after another wash step and incubated at room temperature for 30 minutes. To stop the reaction, 50µl COULTER stopping reagent was added and absorbance measured at 450nm.

Using the standard curve (Appendix A) the level of p24 could be calculated for each test well and was compared as a percentage of that of the untreated control as described in section 3.2.6.1

3.3 Results and discussion

3.3.1 Toxicity study

Figure 3.3 shows the results obtained from the toxicity studies done in three separate XTT experiments. The results are represented on the same y-axes scales for all the experiments for ease of comparing the individual experiments. The scale has been limited from -1000% to 1000% but some bars exceed these points. As can be seen, there is very little correlation between the three experiments. It has been found in cell culture assays that there are many

factors that can influence the outcome and these are difficult to correct for. The assays are very sensitive to the health status of the donor at the time of bloodletting and even the conditions under which the experiment is performed as has been already experienced at RAU. To eliminate some variation, blood was drawn from a single donor but this was done at different time points. In figure 3.3A the blood was drawn one month prior as compared to the last two experiments and these latter experiments were also performed at a different laboratory (Department of Chemistry and Biochemistry, RAU).

Bars above the x-axis (figure 3.3) show results where the extract has had a positive influence on the proliferation of the cells as compared to the untreated control. Bars below the x-axis represent those extracts that had a negative effect on cell proliferation as compared to the untreated control. In the first experiment many extracts showed toxicity but in the next two assays done later, this pattern changed which again could have been influenced by the nature of the cells in those particular experiments.

The only pattern seen in all three assays, is that SLW, SFW, SFW-PS and SFE showed no inhibition of proliferation at even the highest concentration of 0.2mg/ml and that the extracts LLW-PS and SLD showed inhibition of proliferation in all three assays at 0.2mg/ml. At the lower concentrations of 0.02mg/ml and 0.002mg/ml, PBMCs with all the extracts showed an increase in cell proliferation in most of the assays, except for the first assay where even at lower concentrations SLM, SLD, LLW-PS, LLA and LLM were problematic. In particular the last two experiments gave very high absorbance readings in comparison to the untreated control (cells and XTT). The differences seen between the first and last two experiments could also be influenced by the change in lab conditions (University of Port Elizabeth (UPE) vs. RAU) for example incubator temperature, humidity and CO_2.

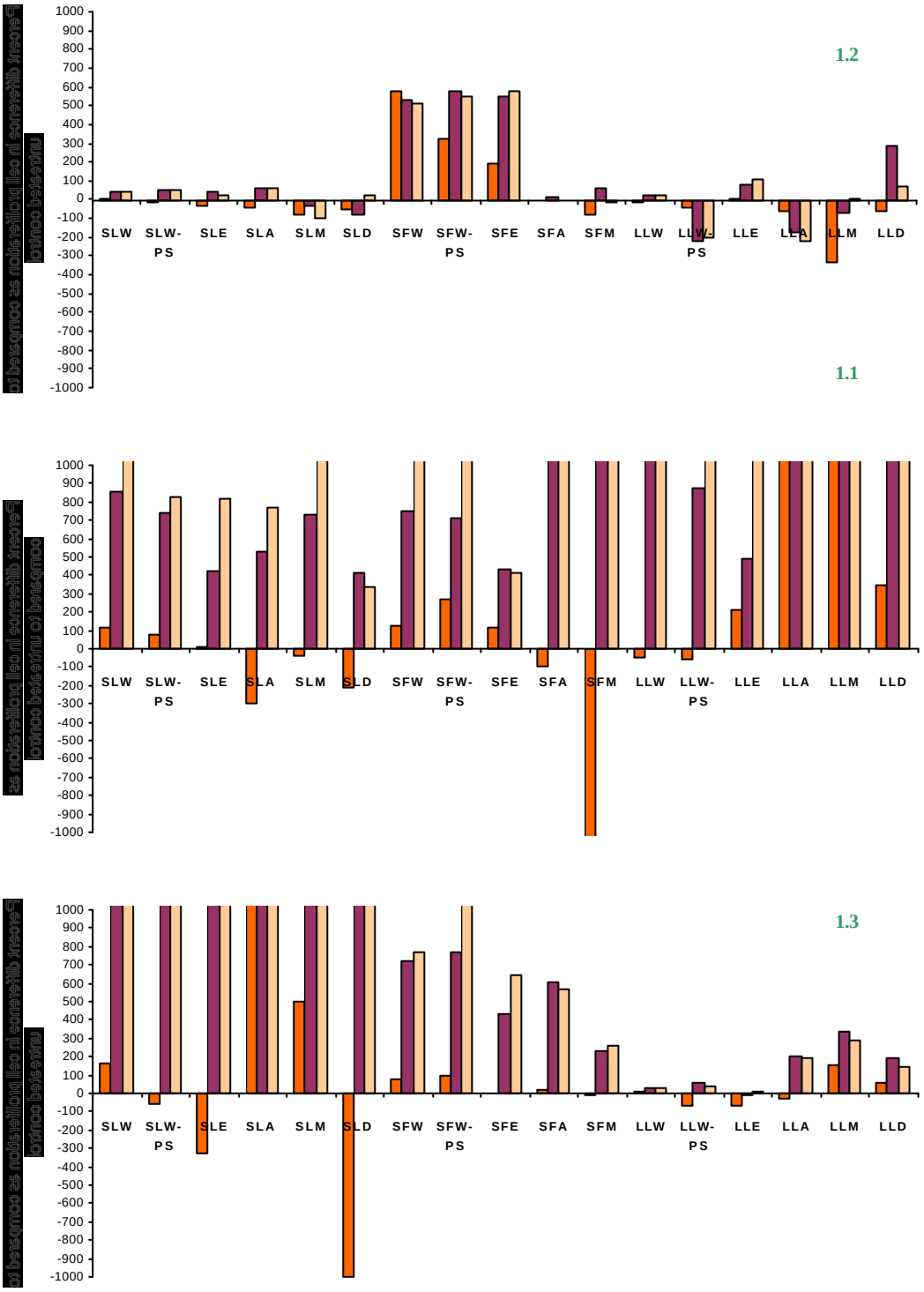

Figure 3.3: Toxicity of plant extracts towards uninfected PBMCs. Extracts were diluted in RPMI 1640 and tested at 0.2mg/ml, 0.02mg/ml and 0.002mg/ml. Results are the mean of quadruplicate test wells (corrected for the background readings of the extracts) compared to the mean of the untreated control (difference) and this is then represented as the percent difference as compared to the control. Standard deviation was within 10% of the mean (n=4). A, B and C represent individual experiments performed on PBMCs from a single donor.

In some cases the percent difference was less than –100%, which is a result of the test wells giving a negative result. A negative result was obtained because the extract controls (background controls) gave a higher reading than those of the test wells that contained the extract plus the cells. This could have come about due to the extracts being able to reduce the XTT as previously described and even though there is an equal amount of extract in the test wells as in the control wells, it is possible that the cells could have influenced the extract (i.e. metabolised) so that the plant extract, in the test wells, lost some reductive activity.

3.3.2 Viral assays

3.3.2.1 PBMCs

In the case of the viral assay, where the effect of the plant extracts on an established HIV infection was tested, three separate patients donated blood. In figure 3.4 and figure 3.5, patients were on anti-HIV medication while figure 3.6 represents blood from a patient that was not on any treatment for the HIV infection.

Looking at the results individually first, one can see discrepancies between the XTT assay data and those from the p24 assay, again highlighting the necessity to do an assay to confirm the XTT viral results. As explained earlier false results could be obtained through interactions of the plant extract and XTT.

In figure 3.4A it seems all the extracts helped improve cell proliferation significantly (=50%) at concentrations of 0.02mg/ml and lower. Extracts, SFW, SFW-PS and SFE could not be tested at the lowest concentration of 0.002mg/ml due to the limited amount of PBMCs available but already at 0.02mg/ml there is an improvement in cell proliferation. Higher concentrations of SLW, SLM and SFA (0.2mg/ml) did not significantly improve cell proliferation while at this concentration SLE, SLA and LLD had a negative influence on cell proliferation.

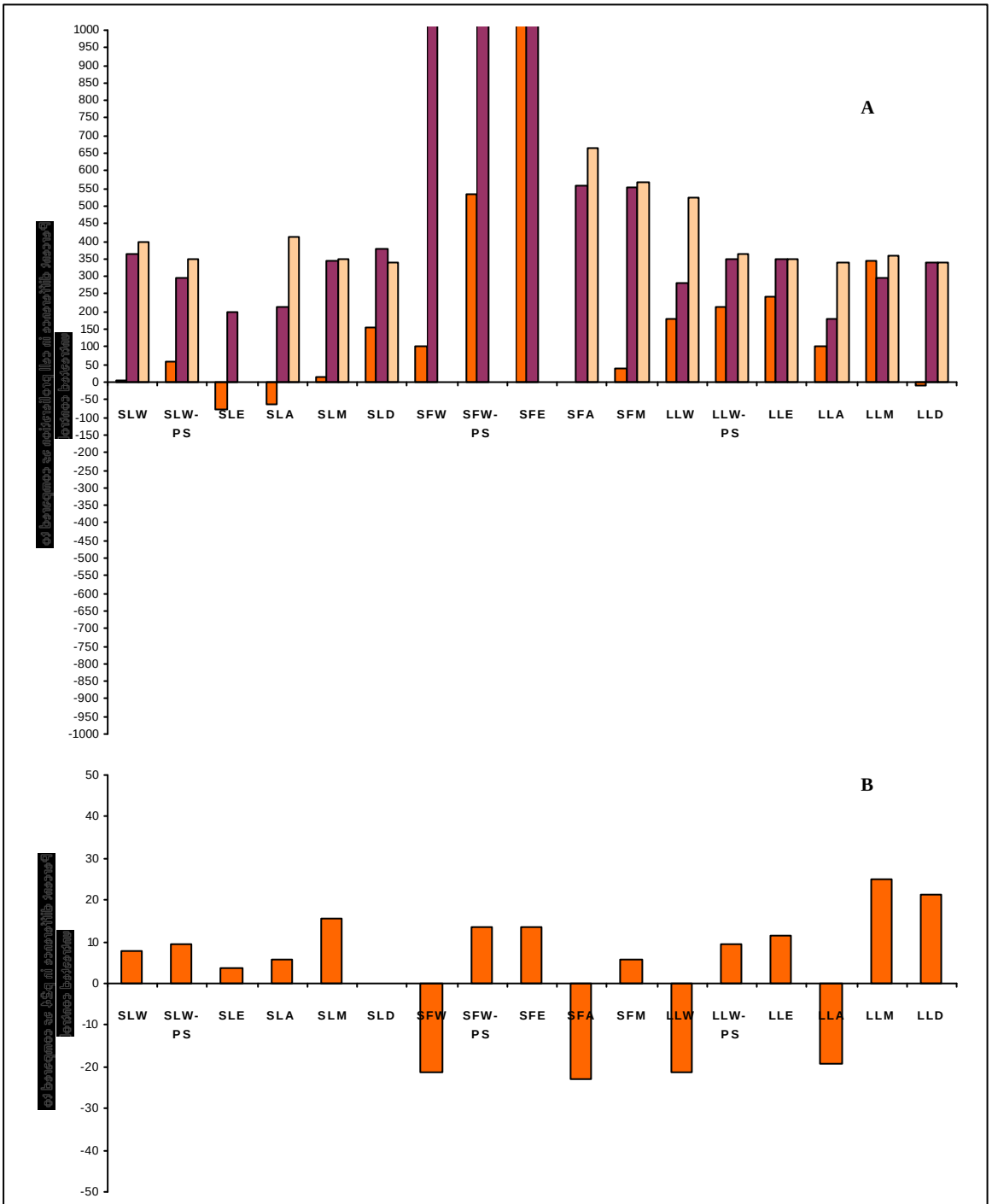

Figure 3.4: Viral assays with infected PBMCs from patient one in the presence of plant extracts. A, XTT assay with plant extracts at 0.2mg/ml, 0.02mg/ml and 0.002mg/ml. Results are the mean of quadruplicate test wells (corrected for the background readings of the extract) compared to the mean of the quadruplicate control readings (difference) and this is then represented as a percent difference as compared to the control. Standard deviation was within 20% of the mean (n=4). B, p24 assay using supernatant from the XTT assay containing plant extracts at 0.02mg/ml. Results were obtained from a single determination from pooled quadruplicate test wells and the difference calculated as compared to the untreated control supernatant. This was then represented as a percentage difference as compared to the control.

For all the p24 assays done on PBMCs the supernatant was removed from the wells containing extract at 0.02mg/ml. Due to the number of extracts tested and number of patients looked at, only a single reading was done of the pooled supernatant of quadruplicate test wells. Results from the p24 assay (figure 3.4B) from the first patient show a different pattern from that of the XTT assay results. Only extracts SFW, SFA, LLW and LLA confirm the XTT data with reduction in p24 levels. The rest of the extracts actually seem to help improve viral infectivity but only by = 25% increase in p24 levels. These extracts could be helping to make the cells more susceptible to the virus. SLD had no effect.

There seems to be less improvement in cell proliferation in the presence of the virus in patient two (figure 3.5A). Even at lower concentrations, extracts SFW, SFW-PS and SFE inhibited cell proliferation while at only the higher concentration of 0.2mg/ml were the extracts SLW, SLA, SLM and SLD inhibitory of cell proliferation. In comparison the p24 data (figure 3.5B) suggests that all the extracts decrease p24 levels except for SLW. In the XTT assay, extracts SLM, SFW, SFW-PS and SFE at 0.02 mg/ml decreased cell proliferation but in the p24 assay they reduced p24 levels. This could mean that the reduction in p24 is not due to inhibition of the virus but simply the indiscriminate killing of healthy/infected cells. Correlation of the XTT assay with the p24 results showed that extracts SLW-PS, SLE, SLA, SLD, SFA, SFM, LLW, LLW-PS and LLE all showed a 50% or more improvement in cell proliferation and a reduction in p24 levels. Extracts LLA, LLM and LLD were not tested with this patient as there was not enough PBMCs.

With regards to the results obtained from patient three who was not on any treatment, very few extracts showed an improvement in cell proliferation in the HIV-infected PBMCs (figure 3.6A). Only the highest concentration (0.2mg/ml) of SLW-PS, LLW and LLA and the lower concentrations of SFE showed a significant improvement in cell proliferation. At all concentrations, the extracts SFA, SFM, LLW-PS, LLM and

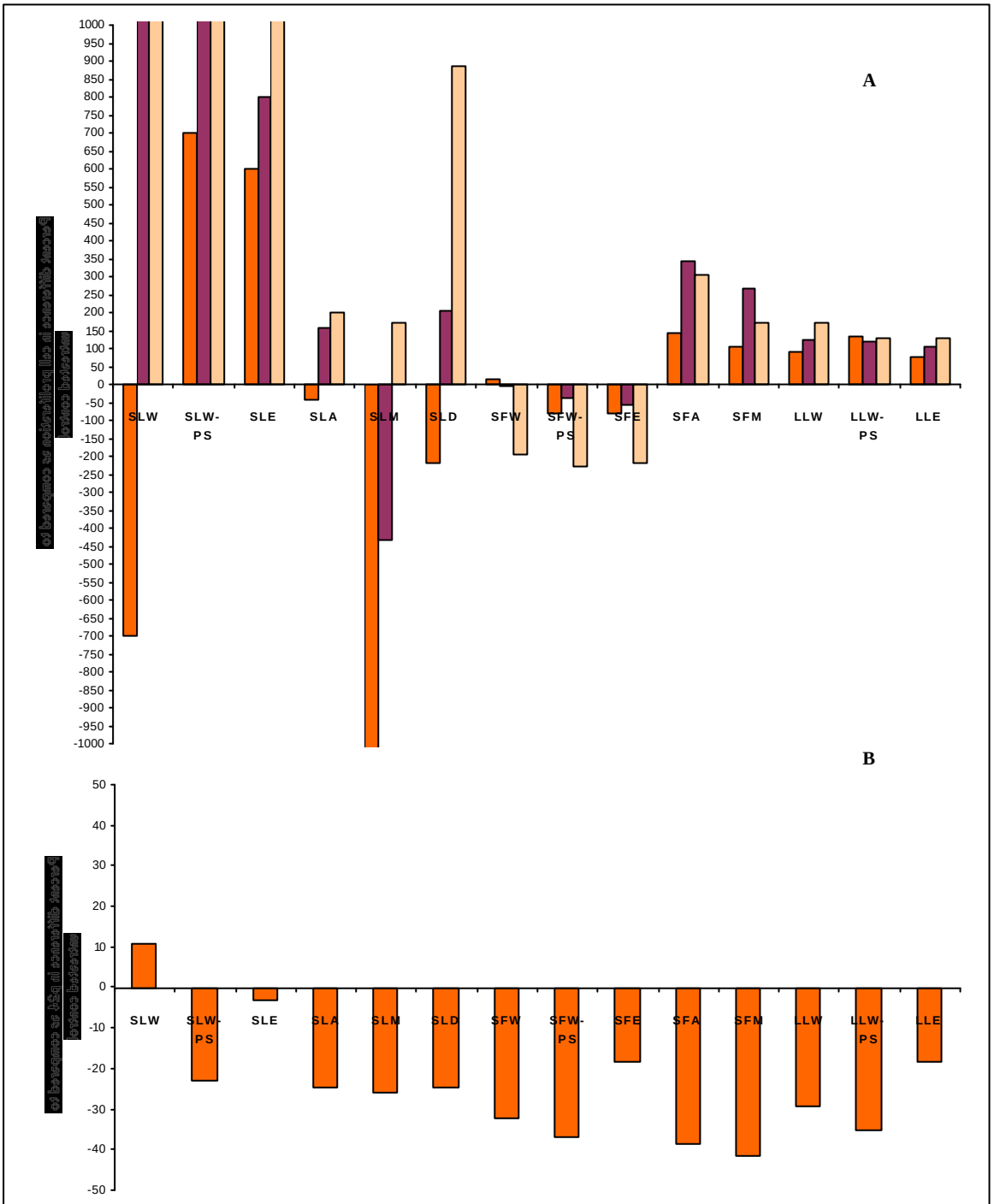

Figure 3.5: Viral assays with infected PBMCs from patient two in the presence of plant extracts. A, XTT assay with plant extracts at 0.2mg/ml, 0.02mg/ml and 0.002mg/ml. Results are the mean of quadruplicate test wells (corrected for the background readings of the extract) compared to the mean of the quadruplicate control readings (difference) and this is then represented as a percentage difference as compared to the control. Standard deviation was within 20% of the mean (n=4). B, p24 assay with supernatant from the XTT assay containing plant extracts at 0.02mg/ml. Results were obtained from a single determination from pooled quadruplicate test wells and the difference calculated as compared to the untreated control supernatant. This was then represented as a percentage difference as compared to the control.

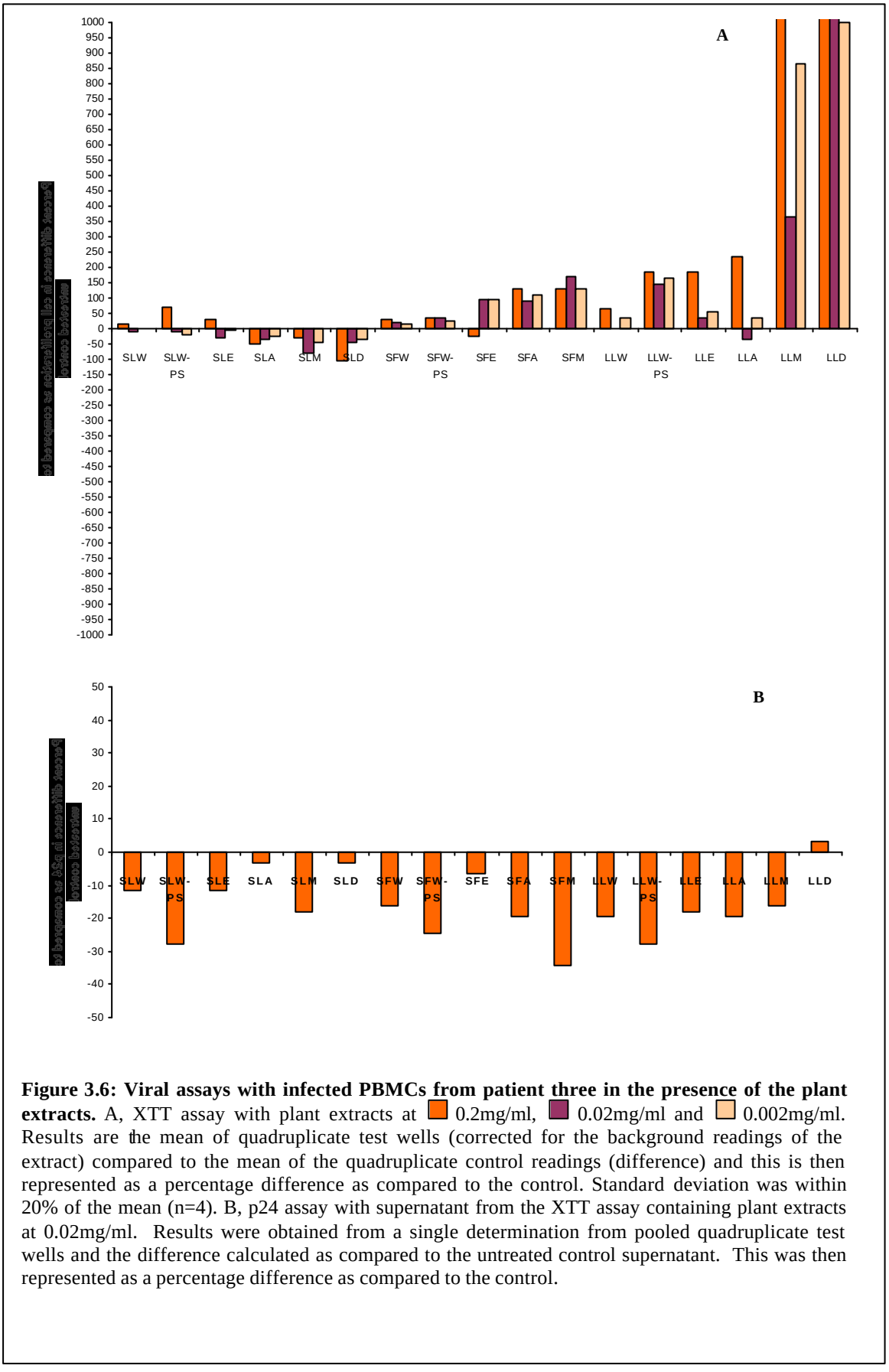

Figure 3.6: Viral assays with infected PBMCs from patient three in the presence of the plant extracts. A, XTT assay with plant extracts at 0.2mg/ml, 0.02mg/ml and 0.002mg/ml. Results are the mean of quadruplicate test wells (corrected for the background readings of the extract) compared to the mean of the quadruplicate control readings (difference) and this is then represented as a percentage difference as compared to the control. Standard deviation was within 20% of the mean (n=4). B, p24 assay with supernatant from the XTT assay containing plant extracts at 0.02mg/ml. Results were obtained from a single determination from pooled quadruplicate test wells and the difference calculated as compared to the untreated control supernatant. This was then represented as a percentage difference as compared to the control.

LLD improved cell proliferation significantly while for LLE the middle concentration of 0.02mg/ml improved cell proliferation by less than 50%. Again in the p24 assay (figure 3.6B), there is reduction in p24 levels in the presence of all the extracts at 0.02mg/ml except for LLD. There was correlation with the XTT assay as all the extracts that showed a 50% or more improvement in cell proliferation, showed a reduction in p24 levels except for LLD for which p24 levels were slightly increased. Extracts that showed a decrease in cell proliferation but also reduced levels of p24 were seen in all the *Sutherlandia* leaf extracts and LLA. Again this may be attributed to the indiscriminate killing of healthy/infected cells.

Comparing results between patients shows that there is no clear pattern being formed. The fact that two patients were on treatment greatly influences the results. Information about what treatment the patient was on and for how long could not be obtained and it has been shown that there can be interactions between plant medication and the HIV treatment (WHO 1, 2002). In addition, it could not be established for how long the patient had been infected with the virus. There is also the problem of not knowing the strain of virus that the patient has or the level of fitness of the virus and therefore the susceptibility of the virus to inhibitors. The p24 values for the patients from the cultured, untreated PBMCs were 7.6 pg/ml for patient one, 9.6 pg/ml for patient two and patient three gave 9.9 pg/ml which are very low values in themselves.

Overall, none of the extracts reduced p24 levels by more than 50%. The most reduction was seen in the second patient where SFM was able to reduce p24 levels by 42%.

3.3.2.2 CEM-NK^{R}-CCR5

To determine the effect of the extracts in an early phase infection, a viral study was done using HIV-infected CEM-NK^{R}-CCR5 cells. What was found during this experiment is that by including 3% (v/v) DMSO in the control wells, there was about a 70% reduction in cell proliferation (results not shown). In another study done in the lab it was shown that 3% (v/v) DMSO had little effect on the proliferation of uninfected PBMCs. The difference could be attributed to different cell type used.

When comparing the test wells with the control wells containing DMSO the results are highly distorted and the percentage difference becomes very large. For figure 3.7A, test wells were compared to untreated controls containing no DMSO. In this assay extracts were tested at 0.2 mg/ml but the ethanol and methylene dichloride extracts were not available at the time of testing so they were not included. Overall none of the extracts showed a significant improvement in cell proliferation in the presence of HIV. It can be seen that there is a difference in activity in all the aqueous extracts with and without sulphated polysaccharides. For the leaf extracts, once sulphated polysaccharides were removed there was an increase in toxicity and in the aqueous *Sutherlandia* flower extracts there is a slight improvement in proliferation once the sulphated polysaccharides are removed. This difference is mirrored in the p24 results (figure 3.7B) but here it seems that with the removal of the sulphated polysaccharides p24 levels are now reduced by up to as much as 44.8% as seen with SFW-PS. Extracts SFA, LLA and LLM also showed a slight decrease in p24 levels but only with LLM was there also an increase in cell proliferation.

3.3.3 Comparison of cell results

It is very difficult to come to any conclusions with respect to the nature and activity of the extracts with the cell assays as results vary from blood sample to blood sample. To get a better idea, all results from the extracts at 0.02 mg/ml for the PBMC assays and the data from the CEM-NK^R-CCR5 experiments were evaluated by a point-scale system to obtain a percentile index of activity. Ultimately the allocation of points is subjective so for this reason three different versions of these point systems were done.

The first system, table 3.1, shows the point-scale in a very simplified manner where no negative points are allocated while a point is allocated for any positive activity. For the toxicity study one point is allocated if there is an increase in cell proliferation and nothing if there is no increase or a decrease in proliferation as compared to the

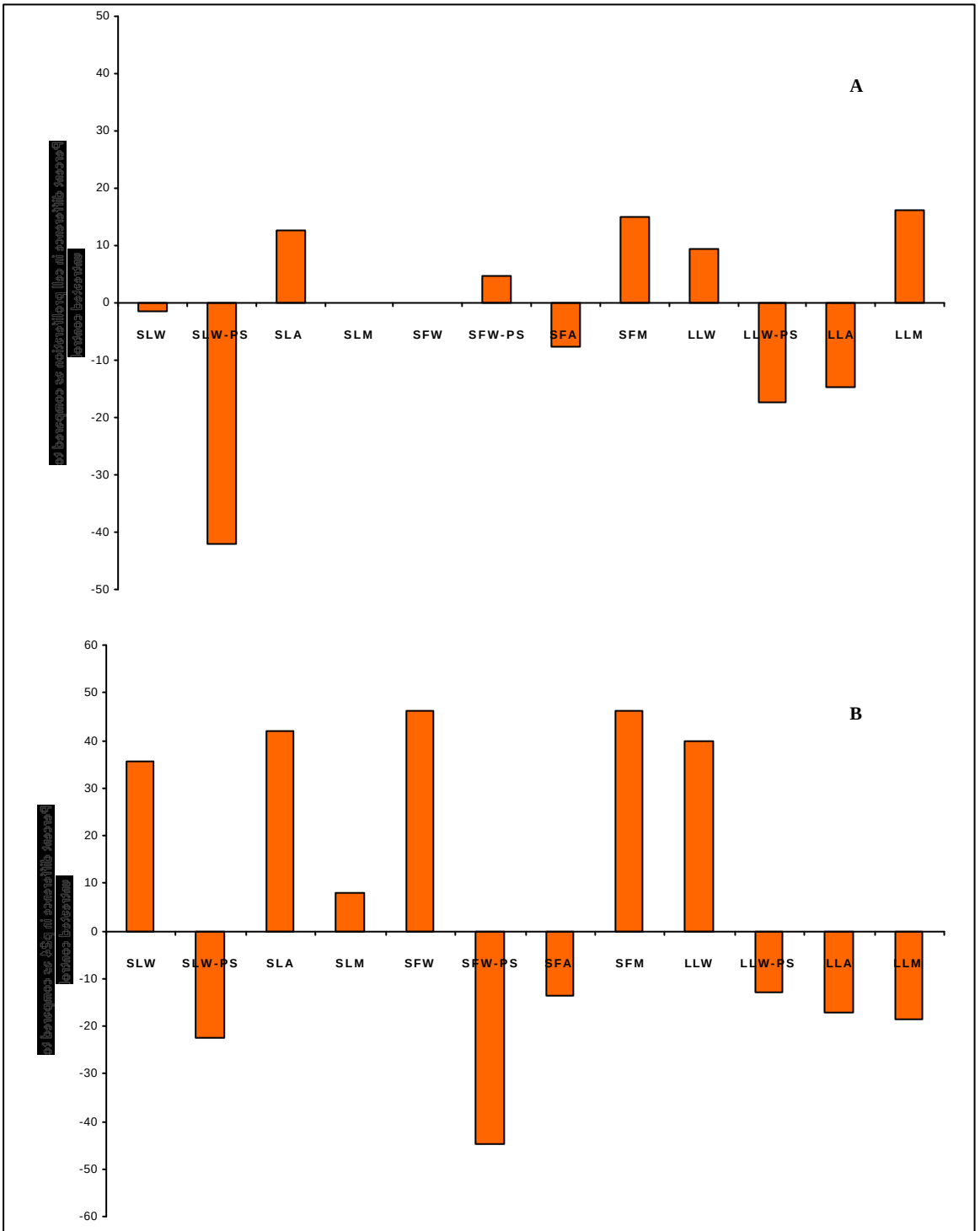

Figure 3.7: Viral assays with infected CEM-NK^{R}-CCR5 cells and plant extracts. A, XTT assay with plant extracts at 0.2 mg/ml. Results are the mean of quadruplicate test wells (corrected for the background readings of the extract) compared to the mean of the quadruplicate control readings (difference) and this is then represented as a percentage difference as compared to the control. Standard deviation was within 10% of the mean (n=4). B, p24 assay with supernatant from the XTT assay. Results are the average of duplicate test wells and the difference is calculated as compared to the untreated control supernatant. This was then represented as a percentage difference as compared to the control.

control. Results from the infected PBMC XTT experiments were slightly more weighted where two points were given for a 50% or more increase in cell proliferation, one for any proliferation between 0% and 50% and nought for no increase or a decrease in proliferation. The reason for this difference is that I believe an increase in proliferation in the presence of HIV to be more significant. For this project it is important to first determine if the extract is not toxic and then if it can improve cell proliferation in the presence of the HIV. It is not important whether the extract can increase proliferation of healthy PBMCs. However for the CEM-NK^R-CCR5 viral study the percent difference never exceeded 50%, which is probably because the results were compared to the untreated control without DMSO (see section 3.3.2.2). The maximum points that could be allocated then is only one point for an increase in proliferation and nothing for no increase or a decrease in proliferation. All p24 results were treated the same with one point for a decrease in p24 levels and nothing for an increase in p24 levels.

From table 3.1 it is seen that the acetone extract of the *Sutherlandia* flowers (SFA) gave the best results overall showing an increase in proliferation in all three toxicity studies, a significant increase in cell proliferation (≥50%) in all three PBMC viral assays and a decrease in p24 levels in all the assays. Only in the CEM-NK^R-CCR5 cells was there a slight decrease in proliferation of 7.6%, but considering the standard deviation was within 10% of the mean this may not necessarily be significant. SFM and LLW also proved to be promising extracts with increases in cell proliferation in all assays and in most cases decreasing the levels of p24. Extracts, which were not tested in all the assays, scored high points in most cases except for SLD. However this could be influenced by the missing data and upon the inclusion of these results from further experiments it could confirm the extracts as potential anti-HIV leads or it may not.

SLM scored the lowest points with only 50%. In most cases the extract was able to improve cell proliferation in uninfected PBMCs and infected CEM-NK^R-CCR5 cells but with an established infection of the PBMCs this was not achieved. Also in only 50% of the cases was SLM able to reduce p24 levels. The aqueous extract from the *Sutherlandia* leaves scored only 57%. Although in the PBMC assays the extract generally increased cell proliferation, it failed in most cases to reduce p24 levels.

Table 3.1: Summary of cell results presented using a simple point-scale evaluation

Extracts	Toxicity study			Viral study (PBMCs)						Viral study (CEM-NK^{R}-CCR5)		Total	Percent[f] (%)
	XTT[a]			*XTT*[b]			*p24*[c]			*XTT*[d]	*p24*[e]		
Experiment	1	2	3	1	2	3	1	2	3				
SLW	1	1	1	2	2	0	0	0	1	0	0	8	**57**
SLW-PS	1	1	1	2	2	0	0	1	1	0	1	10	**71**
SLE	1	1	1	2	2	0	0	1	1	n/a	n/a	9	**75**
SLA	1	1	1	2	2	0	0	1	1	1	0	10	**71**
SLM	0	1	1	2	0	0	0	1	1	1	0	7	**50**
SLD	0	1	1	2	2	0	0	1	1	n/a	n/a	8	**67**
SFW	1	1	1	2	1	1	1	1	1	1	0	11	**77**
SFW-PS	1	1	1	2	0	1	0	1	1	1	1	10	**71**
SFE	1	1	1	2	0	2	0	1	1	n/a	n/a	9	**75**
SFA	1	1	1	2	2	2	1	1	1	0	1	13	**93**
SFM	1	1	1	2	2	2	0	1	1	1	0	12	**86**
LLW	1	1	1	2	2	1	1	1	1	1	0	12	**86**
LLW-PS	0	1	1	2	2	2	0	1	1	0	1	11	**77**
LLE	1	1	0	2	2	1	0	1	1	n/a	n/a	9	**75**
LLA	0	1	1	2	n/a	0	1	n/a	1	0	1	7	**64**
LLM	0	1	1	2	n/a	2	0	n/a	1	1	1	9	**82**
LLD	1	1	1	2	n/a	2	0	n/a	0	n/a	n/a	7	**78**

[a] One point was given for an increase in cell proliferation and 0 for no increase or a decrease in proliferation at 0.02mg/ml.
[b] Two points were given for a 50% or more increase in cell proliferation, 1 for less than 50% increase and 0 for no increase or a decrease in proliferation at 0.02mg/ml.
[c] One point was given for a decrease in p24 levels and 0 for no decrease or an increase in p24 levels.
[d] One point was given for an increase in cell proliferation and 0 for no increase or a decrease in proliferation.
[e] One point was given for a decrease in p24 levels and 0 for no decrease or an increase in p24 levels.
[f] Points were summed and presented as a percentage of the total points that could be obtained per section taking into account that some assays were not performed on all extracts (n/a).

In general, the aqueous extracts of *Lobostemon* leaves and *Sutherlandia* flowers showed little difference between extracts with and without sulphated polysaccharides. With SLW once the sulphated polysaccharides were removed there was a marked increase in points (57% to 71%) contributed mainly by the increased ability of the extract to reduce p24 levels.

There is, however, some problems with this point-scale system. Weighting the viral studies could influence how the extracts are looked at overall considering the "outside" influences on the results as discussed in section 3.3.2.1. Another problem is that the p24 data is given the same importance as the toxicity data even though results from the infected PBMCs came from a single reading. Quantitative aspects are also ignored in most cases giving the same weight to 10% proliferation increase in the toxicity study as to that of 100% increase. For a comparison, a second evaluation was made of the data taking these points into consideration.

In table 3.2, a more quantitative approach has been taken. For all the proliferation studies one point was allocated to increased proliferation at 0% to 100%, two points for proliferation 101% to 200% and three points for proliferation increases greater than 200%. No points were given if proliferation decreased. Results from the p24 assays were evaluated in the same manner as in table 3.1 with one point for a decrease in p24 levels and nothing if there was an increase or no change in p24 levels. Overall the percentage values obtained for all the extracts were decreased except for the LLD extract where the value rose from 78% to 82%. SFA still gave a high percentage value in this new evaluation of 72 % and SLM still scored the lowest points at 48% while LLE and LLA were not far behind. Of the other two extracts that showed promise in table 3.1 only SFM still retained a high score while LLW dropped to 56%. The score slightly improved with SFE so that out of all the extracts evaluated in this table it came second. Again as in the first evaluation there was little difference between extracts with or without sulphated polysaccharides with the aqueous extracts of *Lobostemon* leaves and *Sutherlandia* flowers.

Table 3.2: Summary of cell results presented using a weighted proliferation point-scale evaluation

Extracts	Toxicity study			Viral study (PBMCs)						Viral study (CEM-NK^R-CCR5)		Total	Percent[f] (%)
	XTT[a]			*XTT*[b]			*p24*[c]			*XTT*[d]	*p24*[e]		
Experiment	1	2	3	1	2	3	1	2	3				
SLW	1	3	3	3	3	0	0	0	1	0	0	14	**56**
SLW-PS	1	3	3	3	3	0	0	1	1	0	1	16	**64**
SLE	1	3	3	2	3	0	0	1	1	n/a	n/a	14	**67**
SLA	1	3	3	3	2	0	0	1	1	1	0	15	**60**
SLM	0	3	3	3	0	0	0	1	1	1	0	12	**48**
SLD	0	3	3	3	2	0	0	1	1	n/a	n/a	13	**62**
SFW	3	3	3	3	0	1	1	1	1	0	0	16	**64**
SFW-PS	3	3	3	3	0	1	0	1	1	1	1	17	**68**
SFE	3	3	3	3	0	2	0	1	1	n/a	n/a	16	**76**
SFA	1	3	3	3	3	1	1	1	1	0	1	18	**72**
SFM	1	3	3	3	3	2	0	1	1	1	0	18	**72**
LLW	1	3	1	3	2	0	1	1	1	1	0	14	**56**
LLW-PS	0	3	1	3	2	2	0	1	1	0	1	14	**56**
LLE	1	3	0	3	1	1	0	1	1	n/a	n/a	11	**52**
LLA	0	3	3	2	n/a	0	1	n/a	1	0	1	11	**52**
LLM	0	3	3	3	n/a	3	0	n/a	1	1	1	15	**71**
LLD	3	3	2	3	n/a	3	0	n/a	0	n/a	n/a	14	**82**

[a, b, d] Three points given for = 200% proliferation, two points given for cell proliferation between 200% and 101%, 1 for less than 100% increase and 0 if no increase or a decrease in proliferation at 0.02mg/ml.

[c e] One point was given for a decrease in p24 levels and 0 for no decrease or an increase in p24 levels.

[f] Points were summed and presented as a percentage of the total points that could be obtained per section taking into account that some assays were not performed on all extracts (n/a).

Combining qualities from both tables 3.1 and 3.2, the third point-scale system (table 3.3) keeps the more quantitative analysis of the viral studies for both the PBMCs and the CEM-NK^R-CCR5 cells, as in table 3.2 but the toxicity studies are scored in the same manner as those in table 3.1. Again the p24 data is treated in the same manner as in both tables.

SFA and SFM score the highest points in this system after LLD and these extracts have consistently scored high in all the evaluations. Again LLM and LLD score high percentages but considering the number of assays excluded (in the case of LLD four assays were not done) the relevance of the high points is unclear especially since in all the p24 assays done, LLD did nothing to decrease the p24 levels. In the second table SFE scored the highest percentage but in this evaluation it drops slightly but overall still has a high relative percentage compared to the rest of the extracts. The lowest points scored is still by SLM with LLA not far behind. As with the other tables there is little difference between extracts with or without sulphated polysaccharides with the aqueous extracts of *Lobostemon* leaves and *Sutherlandia* flowers but in the case of the *Sutherlandia* leaves the aqueous extract continues to have improved score upon the removal of the sulphated polysaccharides.

Table 3.3: Summary of cell results presented using a weighted viral point-scale evaluation

Extracts	Toxicity study			Viral study (PBMCs)						Viral study (CEM-NK^{R}-CCR5)		Total	Percent[f] (%)
	XTT[a]			*XTT*[b]			*p24*[c]			*XTT*[d]	*p24*[e]		
	1	2	3	1	2	3	1	2	3				
SLW	1	1	1	3	3	0	0	0	1	0	0	10	**53**
SLW-PS	1	1	1	3	3	0	0	1	1	0	1	12	**63**
SLE	1	1	1	2	3	0	0	1	1	n/a	n/a	10	**67**
SLA	1	1	1	3	2	0	0	1	1	1	0	11	**58**
SLM	0	1	1	3	0	0	0	1	1	1	0	8	**42**
SLD	0	1	1	3	2	0	0	1	1	n/a	n/a	9	**60**
SFW	1	1	1	3	0	1	1	1	1	0	0	10	**53**
SFW-PS	1	1	1	3	0	1	0	1	1	1	1	11	**58**
SFE	1	1	1	3	0	2	0	1	1	n/a	n/a	10	**67**
SFA	1	1	1	3	3	1	1	1	1	0	1	14	**74**
SFM	1	1	1	3	3	2	0	1	1	1	0	14	**74**
LLW	1	1	1	3	2	0	1	1	1	1	0	12	**63**
LLW-PS	0	1	1	3	2	2	0	1	1	0	1	12	**63**
LLE	1	1	0	3	1	1	0	1	1	n/a	n/a	9	**60**
LLA	0	1	1	2	n/a	0	1	n/a	1	0	1	7	**47**
LLM	0	1	1	3	n/a	3	0	n/a	1	1	1	11	**73**
LLD	1	1	1	3	n/a	3	0	n/a	0	n/a	n/a	9	**82**

[a] One point given for an increase in cell proliferation and 0 if no increase or a decrease in proliferation at 0.02mg/ml.

[b, d] Three points given for = 200% proliferation, two points given for cell proliferation between 200% and 101%, 1 for less than 100% increase and 0 if no increase or a decrease in proliferation at 0.02mg/ml.

[c, e] One point was given if there was a decrease in p24 levels and 0 if no decrease or an increase in p24 levels.

[f] Points were summed and presented as a percentage of the total points that could be obtained per section taking into account that some assays were not performed on all extracts (n/a).

4 *In vitro* screening

4.1 Introduction

Results from the cell-based assay help identify those extracts with potential anti-HIV activity but from these results one cannot conclude the actual mechanism. For this, more specific *in vitro* assays must be performed that determine the inhibitory effect by studying each HIV target separately. Unfortunately each laboratory follows their own procedure and there is a severe lack of standardization in *in vitro* screening (Cowan, 1999, Vlietinck and van den Berghe, 1991). This makes inter-laboratory comparisons difficult.

As can be seen from Chapter one, there are a great deal of potential targets that can be inhibited but there is not an established, standardised assay for each of these targets to determine inhibitory effect. No single assay is ideal in determining potential anti-HIV activity and each assay is limited. It is therefore essential to use multiple systems and cell-based assays so a broad perspective of the potential inhibitory effect of the plant extracts can be obtained.

An outline of the assays performed for the *in vitro* screening can be seen in figure 4.1.

4.2 Materials and methods

4.2.1 Reverse transcriptase

For the purpose of this study the effect of the crude extracts on reverse transcription was tested using a non-radioactive HIV-RT colourimetric ELISA kit from Roche Diagnostics, Germany. The protocol outlined in the kit was followed after the reverse transcriptase enzyme was calibrated (Appendix A).

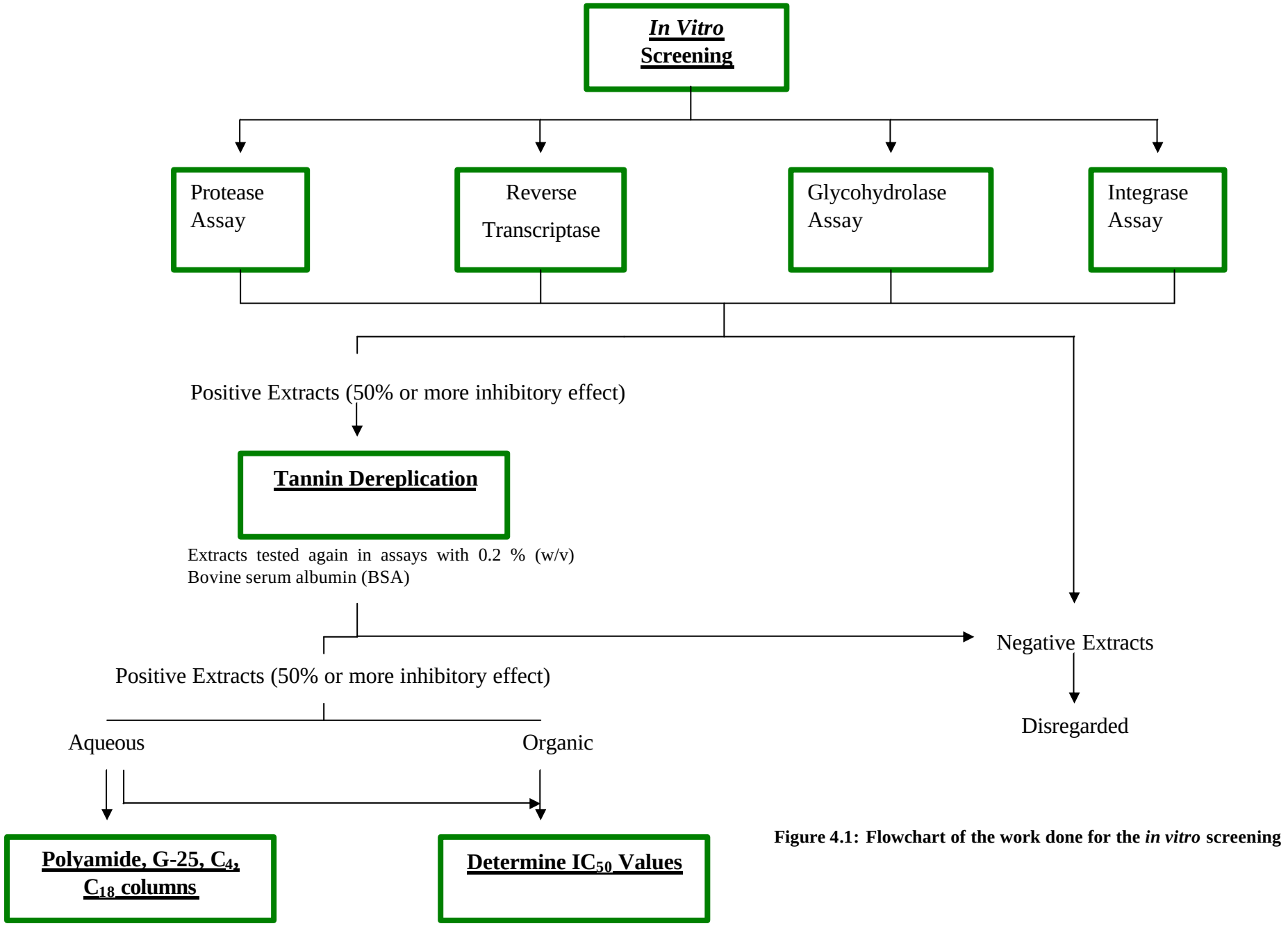

Figure 4.1: Flowchart of the work done for the *in vitro* screening

In brief, the kit instructions were that HIV-RT be added to the wells of the streptavidin-coated microtiter plate at 2ng per well along with the plant extract diluted in lysis buffer to a final concentration of 0.2mg/ml. A 20µl aliquot of reaction mixture containing the template/primer hybrid that is biotin-labelled and nucleotides that are digoxigenin-labelled (DIG), were then added and the reaction allowed to incubate for two and a half hours at 37 °C. The plates were then washed five times with washing buffer. An antibody to DIG, conjugated to peroxidase (POD) was added to the wells for one hour at 37 °C to detect bound nucleotides containing the DIG-label. In the final step, the POD substrate, [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)] (ABTS) was added to detect POD, which converted the substrate to a coloured product that was measured at 412 nm (reference wavelength 492 nm) using the Labsystems Multiskan MS microtiter plate reader. An increase in inhibition of the reverse transcription reaction will result in a decrease in the coloured product. Extracts were tested in duplicate initially and later fractions tested in triplicate. Controls included RT with no extract as the untreated control and a blank with just ABTS.

The extracts were assayed under nuclease-free conditions using sterile, diethyl pyrocarbonate (DEPC)-treated (Sigma, MO USA) double distilled water and autoclaved pipette tips and reaction vials. Gloves were worn at all times and the workbench was treated with RNase Away™ (Molecular Bio-products, inc. CA USA).

4.2.1.1 Tannin-depleted assay for HIV RT

Tannins are widely found in plants and most HIV-inhibitory effects attributed to plant material are due to tannins (Cardellina *et al*., 1993). When this step was included in the NCI's experimental procedures, they found that of the extracts that had anti-HIV activity, 90% lost this ability after tannin removal (Cardellina *et al.*, 1993). Tannins have been shown to inhibit HIV-RT but are non-specific and inhibit a large range of enzymatic pathways (Cowan, 1999; Houghton and Raman, 1998; Nakashima *et al.*, 1992). Although tannins show antiviral effects, they also are known to cause possible liver damage, have carcinogenic potential and have anti-nutritional activity (Notka *et al.*, 2003). At the NCI, 0.1% (w/v) BSA is included in their *in vitro* studies to adsorb

the non-specific cross-linking abilities of the tannins and to determine if the inhibitory effects seen are due to tannins (Natural Product-based Drug Discovery Workshop, Rhodes University, Grahamstown, South Africa, 6 April 2003).

Crude extracts that showed a 50% or more inhibitory effect in the RT assay were tested again as described in section 4.2.1 and BSA fraction V, (Miles Laboratories Research Products, SA) was included to a final concentration in the well of 0.2% (w/v). Extracts that retained an inhibitory effect in the presence of 0.2% (w/v) BSA were examined further to determine the IC_{50} values in the presence of BSA (0.2%, w/v).

4.2.1.2 Characterization of positive fractions

Aqueous extracts that remain inhibitory in the tannin-depleted assays, were applied to four separate solid phase extraction cartridges. A cartridge containing polyamide resin was used to remove tannins from the extracts as it has been shown to effectively bind all polyphenolic compounds (Cardellina *et al.*, 1993). One gram of polyamide resin (Discovery DPA-6S, Supelco, PA USA), pre-swollen in water, was packed into 12ml syringes fitted with frits (Supelco, PA USA). An aliquot of the dry extract, 5mg, was dissolved in 500µl double distilled water, applied to the column and eluted in four fractions: 2ml water, 2ml water-methanol (1:1, v/v), 5ml methanol, and again 5 ml methanol. These samples were evaporated, weighed, dissolved in DMSO-water (1:1, v/v) and tested in the RT assay with 0.2% (w/v) BSA as described in section 4.2.1.

Sephadex G-25 cartridges provide information about the approximate molecular size and weight of the compound. Bonded-phase cartridges, C_4 (300 Å) and C_{18} (60 Å) help determine the relative polarity of the active compound. The bonded-phase cartridges C_4 (Supelclean^{TM} LC-4 SPE tubes, Supelco, PA USA) and C_{18} (Supelclean^{TM} ENVI-18 SPE tubes, Supelco PA USA) were preconditioned with three column volumes of methanol, methanol-water (2:1 and 1:2, v/v) and water respectively. For Sephadex G-25 cartridges, 1g of medium grade gel (Pharmacia Fine Chemicals, Sweden) was pre-swollen in 6ml of double distilled water in a 12ml

syringe and rinsed with several column volumes of water. For each of the three columns, 5mg of the dry crude extract was dissolved in 500µl of double distilled water and applied to the column. In the case of the bonded-phase columns, the extract was eluted in three column volumes of water, water-methanol (2:1 and 1:2, v/v) and methanol. The Sephadex G-25 cartridges were developed with double distilled water, collecting fractions of 2ml, two of 750µl and one of 5ml. Samples were treated in the same manner as those from the polyamide resin (Cardellina *et al.*, 1993).

Absorbance readings were taken of the fractions obtained from the Sephadex G-25 column at 280nm using a Perkin Elmer Lambda 25 UV/Vis spectrophotometer to help characterize the fractions.

4.2.2 Glycohydrolase enzymes

This assay is often included in studies of anti-HIV activity. Glycohydrolase enzymes are found in the host cell's Golgi apparatus of eukaryotic cells and are responsible for glycosylation of proteins. Inhibition of the glycohydrolase proteins has been found to decrease the infectivity of the HIV virion, as the HIV envelope proteins are highly glycosylated. a-Glucosidase has been found to be partly responsible for the glycosylation of HIV gp120 (Collins *et al.*, 1997).

To measure the inhibition of the glycohydrolase enzymes; a-glucosidase (Sigma, MO USA), ß-glucosidase (Sigma, MO USA) and ß-glucuronidase (Roche Diagnostics, Germany) were used with their corresponding substrates ?-nitrophenyl-a-D-glucopyranose (Sigma, MO USA), ?-nitrophenyl-ß-D-glucopyranose (Sigma, MO USA) and ?-nitrophenyl-ß-D-glucuronide (Sigma, MO USA) in a colourimetric 96-well microtiter plate based assay, determining the amount of ?-nitrophenol released. The method described by Collins *et al.*, (1997) was followed with modifications.

Substrates and enzymes were dissolved in their appropriate 50mM buffers [2-Morpholinoethanesulphonic acid monohydrate (Mes)-NaOH (Sigma, MO USA) pH 6.5 for a-glucosidase and ß-glucuronidase and sodium acetate pH 5.6 for ß-

glucosidase] and a calibration curve for each enzyme was established to determine the correct concentration of enzyme to use in inhibitory assays (Appendix A).

It was determined that the optimal enzyme concentration per well for the glycohydrolase enzymes was 0.17µg for a-glucosidase and 0.25µg for both ß-glucosidase and ß-glucuronidase. To test for enzyme inhibition with the crude extracts, the assay was performed in quadruplicate at 25°C with duplicate wells for a background control of each extract that included the substrate. A reaction volume of 200µl contained 2mM substrate, enzyme and the crude extract at 0.2mg/ml. Firstly, the test sample was allowed to interact with the enzyme for five minutes before adding the respective substrate. This reaction was allowed to proceed for 15 minutes before terminating with 60µl of 2M glycine-NaOH, pH 10. Absorbance was read at 412nm using a Labsystems Multiskan MS microtiter plate reader.

4.2.2.1 Tannin-depleted assay for glycohydrolases

Extracts that showed a 50% or more inhibitory effect in the glycohydrolase assays were tested again as described in section 4.2.2 but including 0.2% (w/v) BSA in the 200µl reaction volume to negate any possible effect of tannins. If extracts retained their inhibitory effect they were assayed further with 0.2% (w/v) BSA to determine IC_{50} values.

4.2.3 Integrase

For this assay, recombinant HIV-I IN is not commercially available but HIV-I_{NL4-3} IN protein (F185H/C280S) was kindly supplied through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (MD USA) from Dr. Robert Craige in a limited amount of 50µg in 1M NaCl, 20mM Hepes pH 7.5, 100µM $ZnCl_2$, 2mM dithiothreitol (DTT), 10% (v/v) glycerol buffer. As with all reagents from this programme, only one aliquot is supplied per laboratory and so the following assay was restricted to one experiment with no calibration of the enzyme.

A non-radioactive ELISA based HIV-IN assay was used as described in Chang *et al.*, (1996) with modifications by Au *et al.*, (2000). DNA is immobilised to the wells of a microtitre plate to which viral DNA and HIV-I IN is added, the reaction allowed to occur and then the plate washed. If there is integration, the biotin-labelled viral DNA will be integrated into the immobilised DNA and the biotin can be detected using streptavidin conjugated to alkaline phosphatase. The alkaline phosphatase is measured using its substrate ?-nitrophenyl phosphate which when cleaved results in a coloured product. An increase in yellow-coloured product is an indication that integration has occurred

4.2.3.1 Preparation of target DNA

4.2.3.1.1 Large-scale DNA extraction

Plasmid DNA was extracted from cells using the method adapted from Ish-Horowics and Burke (1981) and Sambrook *et al.*, (1989).

Cultures were prepared by adding 500µl of 50mg/ml ampicillin (Roche Diagnostics, Germany), to 500ml of Luria-Bertani (LB) medium (Appendix B), and inoculating with a single bacterial colony of *E.coli* DH5a transformed with pPCR-Script^{TM} plasmid (Novagen, CA USA; Appendix C). The culture was incubated at 37°C overnight and harvested by centrifugation at 5000g for 15 minutes at 4°C. The supernatant fluid was discarded and the pellet was resuspended in 18ml of Solution I (Appendix B) for 5 minutes at room temperature to which 0.5g of lysozyme was added. Once the pellet was resuspended, 40 ml of Solution II (Appendix B) was mixed into the suspension and this was kept at room temperature for 10 minutes. Ice-cold Solution III (Appendix B) was added (20ml), and the mixture was kept on ice for 10 minutes. The mixture was centrifuged for 15 minutes at 15 000g at 4°C. Supernatant was placed in aliquots in vials and 0.6 volume of isopropanol was added to the supernatant, mixed and incubated at room temperature for 10 minutes. Nucleic acids were recovered by centrifugation at 5000 rpm for 15 minutes using a benchtop microfuge, Häger Designs HM2. The resulting pellet was washed in 70% ethanol for 10 minutes at 14 000 rpm in the Häger Designs HM2 microfuge. The pellet was left

to dry and resuspened in the required volume of Tris-EDTA (TE), pH 8 buffer (Appendix B). The plasmid DNA solution was stored at 4°C.

4.2.3.1.2 DNA purification

To extract and purify DNA from a solution the method described in Sambrook *et al.* (1989) was used.

If the solution was less than 200µl, it was made up to this volume with sterile double distilled water. An equal volume of phenol:chloroform was mixed with the solution and then centrifuged at 14 000 rpm for 2 minutes in a Häger Designs HM2 microfuge. After centrifugation, the top aqueous layer was removed to a fresh tube and an equal volume phenol-chloroform-isoamylalcohol (25:24:1) was added and mixed. The solution was then centrifuged in a Häger Designs HM2 microfuge for two minutes at 14 000 rpm and the top aqueous layer was transferred to a fresh tube. The phenol-chloroform-isoamylalcohol purification step was repeated and once the top aqueous layer had been transferred to a fresh tube, a 1/10 volume of 4M LiCl (Appendix B) and 3 volumes of 100% ethanol were added to precipitate the DNA. This was kept at -20°C for 30 minutes after which the DNA was pelleted by centrifugation for 15 minutes at 14 000 rpm in a Häger Designs HM2 microfuge. The resulting pellet was washed with 70% ethanol with centrifugation in a Häger Designs HM2 microfuge for 10 minutes at 14 000 rpm. The pellet was resuspended in the required volume of TE buffer pH 8 and stored at 4°C.

The concentration of plasmid DNA from each preparation was determined by measuring absorbance of a diluted sample (1:100, v/v) in an ultra violet (UV) spectrophotometer (Perkin Elmer Lambda 25 UV/Vis spectrometer) at a wavelength of 260nm. The concentration was calculated with the knowledge that 1 OD unit is equal to 50 µg/ml of DNA (Sambrook *et al.*, 1989).

4.2.3.1.3 Linearization of plasmid DNA

Sma I (**CCC⁻GGG**) will cleave pPCR-Script within the multiple cloning site (Appendix C), and this linearizes the plasmid with blunt ends. Digestion of 60µg pPCR-Script was carried out using 10µl of *Sma* I (Roche Diagnostics, Germany) (1 U/µl) with 4µl of 10X buffer A (Roche Diagnostics, Germany) in a total volume of 40µl (adjusted with double distilled water) at 37°C overnight. After digestion, the linearized DNA was checked by agarose gel electrophoresis, using a submerged horizontal slab gel system of 1% (w/v) agarose dissolved in Tris-acetate-EDTA (TAE) buffer pH 8 (Appendix B), to verify that all the DNA had digested. DNA was visualised using ethidium bromide (Appendix B) at a concentration of 200ng/ml and an UV light source at 254nm on an UV transilluminator. Gel tracking dye was first added to DNA samples before loading them into the wells. The bromophenol blue in the dye also made it possible to monitor the migration of the DNA in the gel. Once the DNA samples were loaded into the wells, electrophoresis was performed at 60V and 100mA, constant voltage.

A 250bp ladder molecular weight marker, XVI (Roche Diagnostics, Germany; Appendix B), was included in all agarose gel electrophoresis alongside DNA samples, so that DNA fragment sizes could be determined.

4.2.3.1.4 Purification of plasmid from agarose gel

Upon determining which band corresponded with the digested pPCR-Script plasmid, the band was excised from the gel, cut into small pieces and placed inside a vial. Using a gel extraction kit (Qiagen, Germany) and following the instructions, linearized plasmid was isolated from the gel and again checked with agarose gel electrophoresis to ensure only linearized DNA was present. The DNA concentration was determined as described in section 4.2.3.1.2.

4.2.3.2 Preparation of microtitre assay plates

A hundred microlitres per well containing 1µg target DNA, *Sma I*-linearized pPCR-Script, was coated onto the well of a Nunc-immuno Maxisorb^{TM} (Nunc, Denmark) plate in the presence of 2M NaCl. This was incubated for two hours at 37°C then washed three times with distilled water. Blocking unreacted areas of the plate was done with 250µl of 1% (w/v) BSA in phosphate-buffered saline for one hour at 37°C. Plates were stored at 4°C for no longer than a week and kept in blocking buffer.

4.2.3.3 Preparation of Donor DNA

A pre-cleaved donor DNA substrate with blunt ends was prepared by annealing VU5BR (5'biotin-GTG TGG AAA ATC TCT AGC AGT-3') and VU5 (5'-ACT GCT AGA GAT TTT CCA CAC-3') (Integrated DNA Technologies, South Africa) corresponding to the HIV-1 U5 sequence, in 10mM Tris-HCl, pH 8.0, 1mM EDTA and 0.1M NaCl at 80°C for one minute, followed by 30 minutes at room temperature.

4.2.3.4 Integration assay

The prepared plate was used within one week from its preparation and was first washed in double distilled water three times before using directly in the integration assay. Each well of the DNA coated plate received 50µl of solution consisting of 10mM Tris-HCl, pH 7.4, 10mM $MnCl_2$, 100 µg/ml BSA, 10mM DTT, 16mM NaCl, 1% glycerol, 2.5pmol biotin-labelled donor DNA, 40pmol HIV-IN and 0.2 mg/ml diluted plant extract.

After a one-hour incubation at room temperature, the wells were washed three times with double distilled water. Biotinylated-DNA, immobilised in the wells due to integration, was detected with 100µl of streptavidin-conjugated alkaline phosphatase at 1 µg/ml in blocking buffer and this was incubated for another hour at room temperature after which the wells were washed with distilled water. This was followed by colourimetric detection using 50µl of 1mg/ml ρ-nitrophenyl phosphate in

10% (v/v) diethanolamine buffer (pH 9.8) containing 0.5mM $MgCl_2$. The colour generated by the alkaline phosphatase reaction was measured at 412nm. Inhibition would result in a decrease in the coloured product.

4.2.4 Protease Assay

4.2.4.1 HIV Protease

The HIV-II PR was kindly supplied by the NIH AIDS Research and Reference Reagent Program, NIAID, NIH, MD USA from Bret Shirley and Mr. Michael Cappola, Boehringer Ingelheim Pharmaceuticals, Inc. in the form of 100µg in 100mM sodium phosphate (pH 8), 50mM NaCl buffer (Rittenhouse *et al.*, 1990). Unfortunately the protease from HIV-I was unavailable but both these enzymes share 50 % sequence homology. There are 5 amino acid differences in the residues that form the S_1 and S_2 substrate binding pockets of the HIV-I and HIV-II PR's that causes only a small difference in the shape of the binding pockets, although it is known that some inhibitors of HIV-I PR lack activity with HIV-II PR (Tong *et al*, 1993). Inhibitors of HIV-II bind in the same manner as those of HIV-I (figure 4.2), in an extended manner and there are only three amino acid subsitutions between the PR's that are in direct contact with ligands (Mulichak *et al*., 1993). These substitutions are the amino acids 32 (Val to Ile) and 47 (Ile to Val) which form part of the P2/P2' binding subsite and 82 (Val-Ile) (Mulichak *et al*., 1993). The first two substitutions mentioned are opposite complimentary to each other, so there is no change in the size of the binding site just a subtle change in shape (Mulichak *et al*., 1993).

4.2.4.2 Fluorometric HIV-PR Assay

The procedure for the fluorometric detection of HIV-PR activity was carried out as described by Au *et al.*, (2000) after the HIV-II protease enzyme was calibrated (Appendix A).

The fluorogenic substrate, DABCYL-?-Abu-**Ser-Gln-Asn-Tyr-Pro-Ile-Val**-Gln-EDANS (Bachem, Switzerland) was added to a 200µl reaction sample at a concentration of 10µM (bold lettering indicates the sequence corresponding to the HIV-I *gag* polyprotein sequence from residue 128 to 135 and the amino acids underlined indicate the site of cleavage) (Tomasselli *et al*., 1990). Included in the reaction volume was 100nM HIV-II protease, reaction buffer (0.1M sodium acetate, 1M NaCl, 1mM EDTA, 1mM DTT, 10% DMSO, 1 mg/ml BSA, pH 4.7) and the diluted plant extract at 0.2 mg/ml. This was incubated at 37°C for two hours. The fluorescence intensity is indicative of protease activity and was measured in a Fluoroskan Ascent FL plate reader (Thermolabsystems) at an excitation wavelength of 355 nm and emission wavelength of 460 nm.

Since the reaction buffer already contains a high percentage of BSA, it was decided that extracts that showed a strong inhibitory effect need not be tested again with 0.2% (w/v) BSA to determine if the results seen were due to the non-specific nature of the tannins. IC_{50} values were determined for extracts showing 50% or more inhibition in the fluorometric HIV-II protease assay.

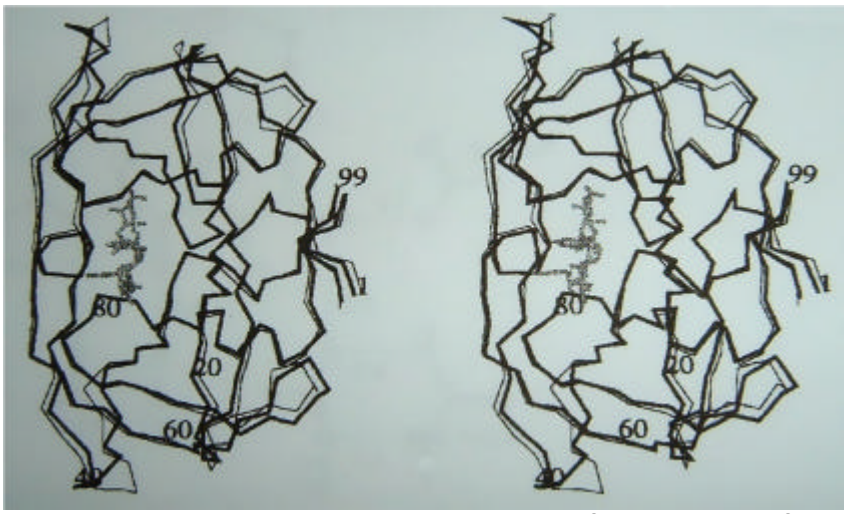

Figure 4.2: Stereo diagram showing the superposition of the structures of both HIV-I (thin line) and HIV-II (thick lines) protease dimers. The inhibitor of both, BI-LA-398, is indicated by the stippled line. (Tong *et al*., 1993).

4.3 Results and discussion

4.3.1 Reverse transcriptase

In the RT assay (figure 4.3), the aqueous extracts of the *Sutherlandia* and *Lobostemon* leaves showed significant inhibition as well as the methanol and methylene dichloride extract of the *Lobostemon* leaves. All the extracts showed some inhibitory effect except the ethanol and methanol extracts of *Sutherlandia* leaves and acetone extracts of *Sutherlandia* flowers and *Lobostemon* leaves. Overall it was mainly in the leaf extracts that RT inhibition was seen. In the aqueous extracts, no significant differences were seen once the sulphated polysaccharides were removed.

Extracts SLW, SLW-PS, LLW, LLW-PS, LLM and LLD were tested again in the RT assay in the presence of 0.2% (w/v) BSA to remove any possible effects seen with tannins (figure 4.4). With each extract there was a loss of the RT inhibition. Only the aqueous *Lobostemon* leaf extracts, with or without sulphated polysaccharides, were still significantly inhibitory of RT (96.45 % and 63.53 %, respectively) in the presence of BSA (0.2%, w/v).

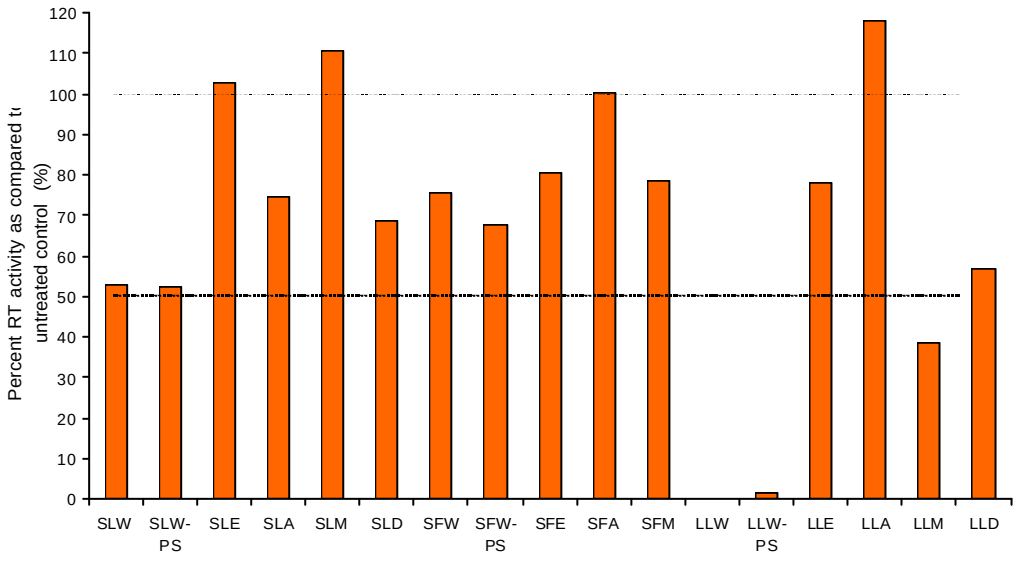

Figure 4.3: Percent RT activity with plant extracts at 0.2 mg/ml as compared to the untreated control. The DMSO aliquots of plant extract were diluted in lysis buffer to a final concentration in the well of 0.2mg/ml. Refer to table 2.2 for an explanation of the codes. Results are expressed as the mean of the duplicate test wells as a percentage of that of the mean of the duplicate control readings. Dashed lines mark the RT activity at 50% and 100% (---).

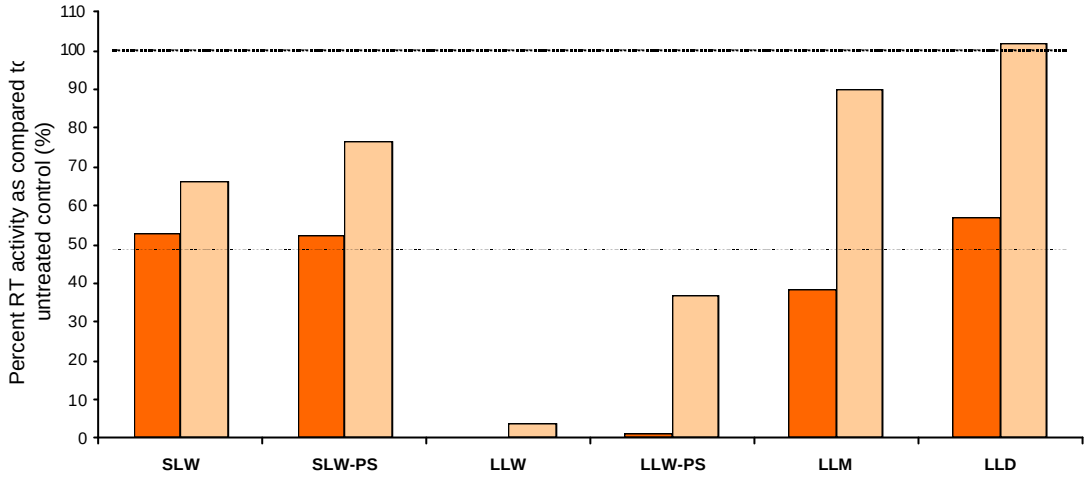

Figure 4.4: Percent RT activity with plant extracts at 0.2 mg/ml with or without 0.2% (w/v) BSA as compared to the untreated control. The DMSO aliquots of plant extract were diluted in lysis buffer to a final concentration in the well of 0.2mg/ml and BSA was added to a final concentration of 0.2% (w/v). Orange bars represent results obtained without BSA and the tan bars represent results with BSA. Results are expressed as the mean of the duplicate test wells as a percentage of that of the mean of the duplicate control readings. Dashed lines mark the RT activity at 50% and 100% (---).

The aqueous *Lobostemon* leaf extract without sulphated polysaccharides (LLW-PS) was tested again to determine the IC_{50} value in the RT assay (figure 4.5). Results from this experiment gave a low IC_{50} value of 0.049 mg/ml, indicative of a strong inhibitory effect on HIV-I RT by this plant extract.

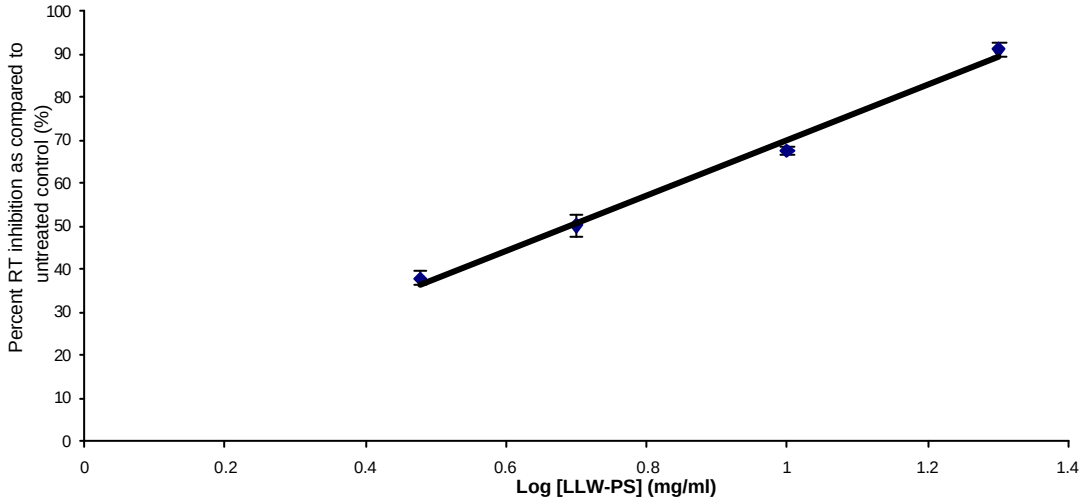

Figure 4.5: Determination of RT IC_{50} by extract LLW-PS. Concentration values for LLW-PS were multiplied by 100 and then represented as a log value along the x-axis. The control was taken as 100% and results were subtracted from this to give percent inhibition. Results were calculated as the mean for each LLW-PS concentration as a percentage of the mean of the untreated control. Error bars represent percent standard deviation (n=4). The correlation coefficient is 0.9928 and the equation is y = 64.145x +5.8873.

To partially characterise the inhibitory compound in the LLW-PS extract, the extract was applied to four columns as described in section 4.2.1.2. The fractions collected were tested in the RT assay in the presence of 0.2% (w/v) BSA (figures 4.6 and 4.7). Fractions from the polyamide gel showed significant inhibition after the removal of tannins and in the presence of BSA confirming that the inhibitory compound is not a tannin compound. Significant inhibition of RT was seen in the first fractions of the C_4 and C_{18} columns, which indicates the compound might be highly polar (figure 4.6). The fact that the same pattern was seen for these two columns may also suggest that they were simply overloaded. Since the IC_{50} value is very low, the suggested 5 mg of dry plant extract to load per column may have been too high and the pattern seen in the assay may not be a true reflection of the properties of the compound (Cardellina *et al.*, 1993).

The UV-spectral properties of the four fractions from the G-25 column and the inhibitory pattern in the RT assay (figure 4.7) suggests that the inhibitory compound is possibly 1000-5000 Daltons in size (as the inhibitory activity elutes in the first three fractions) and could be a peptide as it is very large and is one of the compounds isolated only in water extracts which explains why no activity was seen when the other solvents were used (Cowan, 1999).

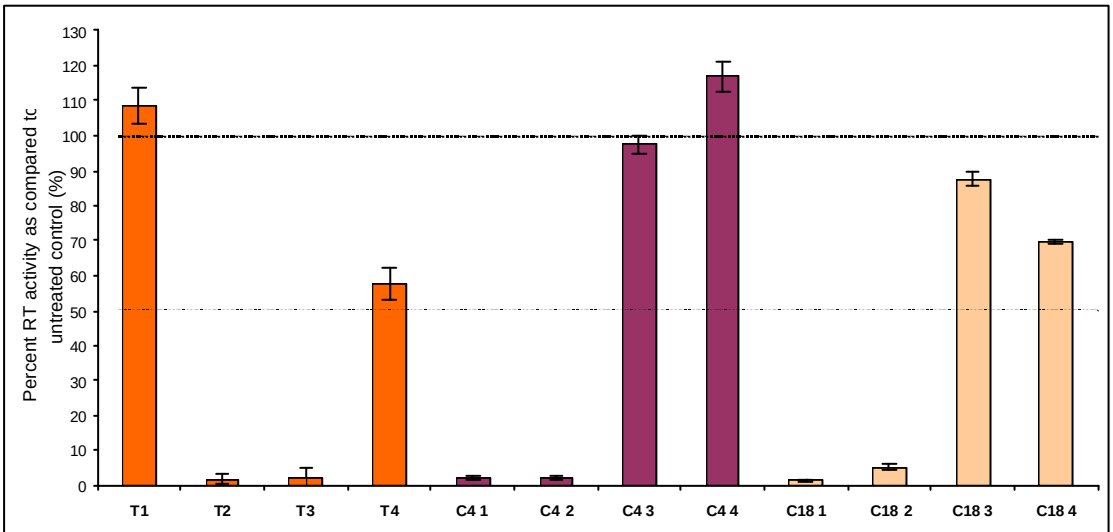

Figure 4.6: Percent RT activity with LLW-PS fractions with 0.2 %(w/v) BSA as compared to the untreated control. The LLW-PS fractions collected from the columns were dried, dissolved in 50% (v/v) DMSO and diluted in lysis buffer. Orange bars represent fractions collected from the polyamide column (T 1-4), purple bars represent the fractions from the C_4 column and the tan bars represent the four fractions collected from the C_{18} column. Results are expressed as the mean of the triplicate test wells as a percentage of that of the mean of the duplicate control readings. Dashed lines mark the RT activity at 50% and 100% (---). Error bars indicate standard deviation.

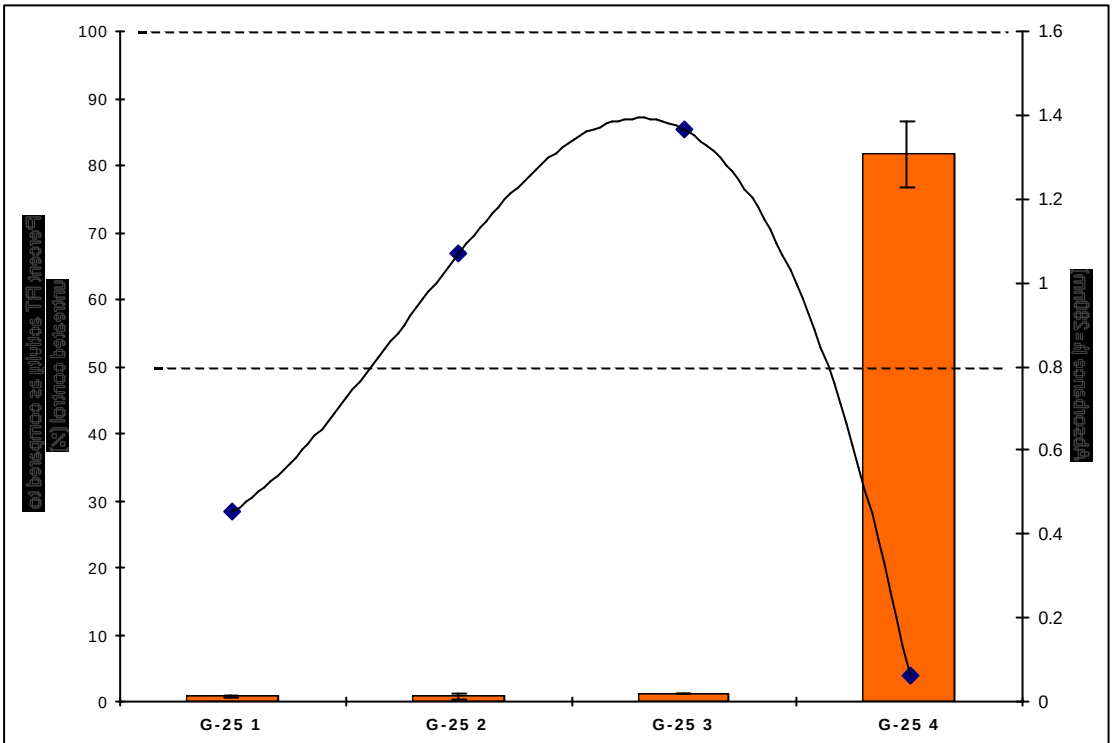

Figure 4.7: Percent RT activity with LLW-PS G-25 fractions and the absorbance readings of these fractions at 280nm. The LLW-PS fractions collected from the G-25 column were dried, dissolved in 50% (v/v) DMSO and diluted in lysis buffer. Results are expressed as the mean of the triplicate test wells as a percentage of that of the mean of the duplicate control readings. Blue points indicate the absorbance readings at 280nm of the four fractions. Dashed lines mark the RT activity at 50% and 100% (---). Error bars indicate standard deviation (n=3).

4.3.2 Glycohydrolase enzymes

In the glycohydrolase enzyme assays (figure 4.8), it is seen that there appears to be some concentration dependence in ß-glucosidase and ß-glucuronidase activity when the concentration of plant extract is varied. This, however, is not as evident in the a-glucosidase assays but may occur in another concentration range. Significant inhibition of the a- and ß-glucosidase enzymes was seen with all extracts made from the *Lobostemon* leaves, though not as strong in the ß-glucosidase assay. The methylene dichloride extract of the *Sutherlandia* leaves showed strong inhibition in both glucosidase enzyme assays as well, and the methanol extract showed some inhibition in the ß-glucosidase assay.

All the extracts inhibited the ß-glucuronidase enzyme although inhibition was slightly weaker for the aqueous *Sutherlandia* flower and leaf extracts and the methylene dichloride *Lobostemon* leaf extract (figure 4.8C).

In each enzymatic assay, no differences were observed in the aqueous extracts of *Sutherlandia* and *Lobostemon*, with or without sulphated polysaccharides, suggesting that any inhibition observed in the glycohydrolase enzyme assays is independent of such compounds.

To confirm that inhibition seen in the glycohydrolase assays was independent of tannins, enzymatic assays were repeated with 0.2mg/ml of extract in the presence of 0.2% (w/v) BSA (figure 4.9). In the glucosidase assays, all the *Lobostemon* fractions were retested as well as the methylene dichloride extract of the *Sutherlandia* leaves. The methanol extract from the *Sutherlandia* leaves was also included in the ß-glucosidase assay. In the case of the ß-glucuronidase, all the extracts were retested except for LLD, SFW and SFW-PS.

In most cases, the original inhibitory effect of each extract seen in the assays, was decreased upon the addition of BSA. In a- and ß-glucosidase assays no significant inhibition was retained with the plant extracts in the presence of BSA (0.2%, w/v). Although inhibition was decreased after the addition of BSA in the ß-glucuronidase assay, all extracts remained significantly inhibitory of the enzyme except for the aqueous extracts of the *Lobostemon* leaves. Interestingly, the aqueous extracts of the *Sutherlandia* leaves showed a significant improvement in inhibition of the ß-glucuronidase once BSA was included. The p-value for SLW with or without BSA was 0.00327 and for SLW-PS, the p value was 0.0002 using the two-tailed student T-test. A possibility exists that the BSA could adsorb/block an inhibitor of the ß-glucuronidase inhibitory compound, thus allowing the ß-glucuronidase inhibitor to interact with the enzyme. ß-glucuronidase has a broad substrate specificity that reflects a non-discriminatory active site and this could be the reason why most of the extracts were able to inhibit the enzyme (Collins *et al.*, 1997).

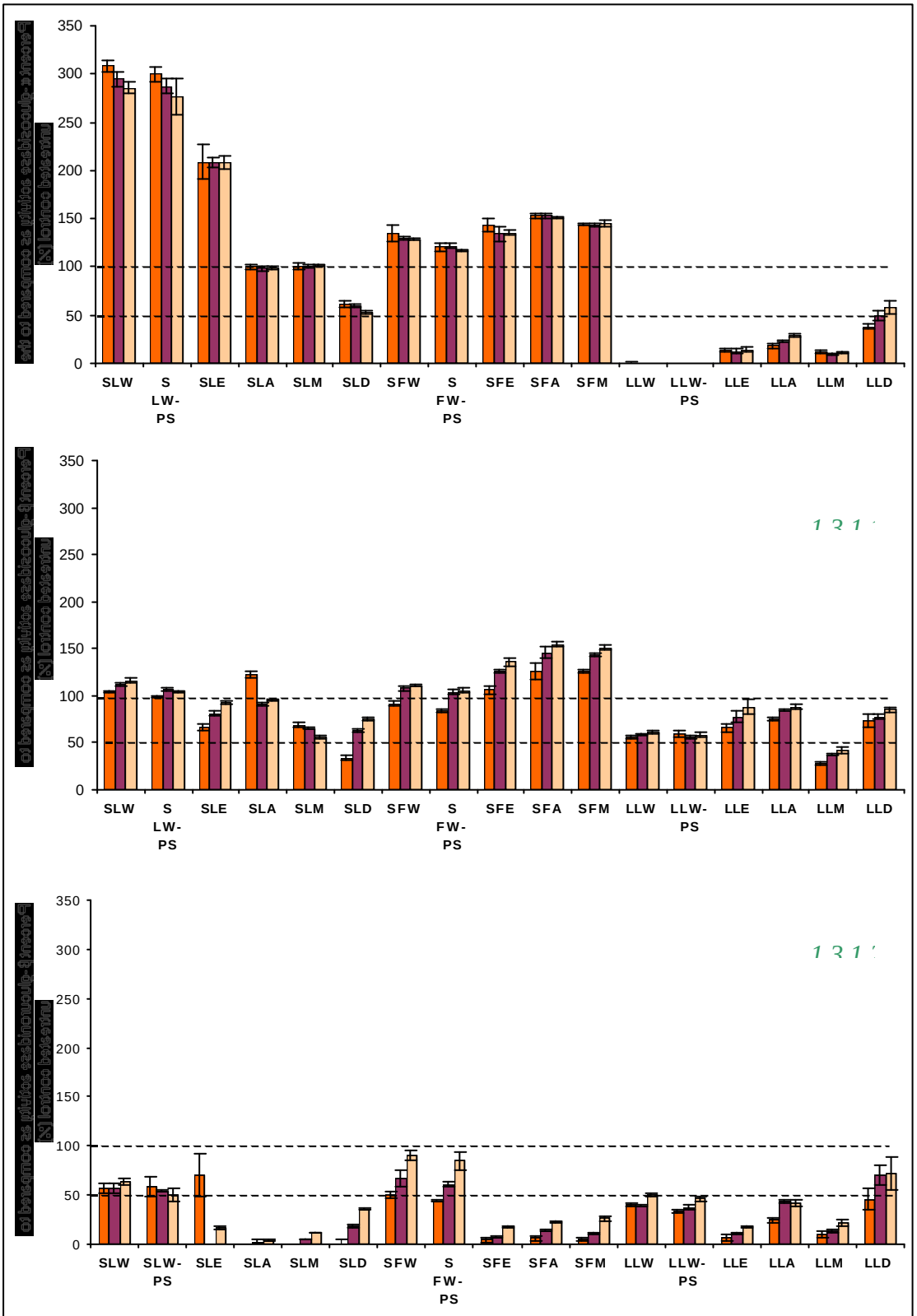

Figure 4.8: Percent glycohydrolase activity with plant extracts as compared to the untreated control. The DMSO aliquots of plant extract were diluted in relevant enzyme buffer to a final concentration in the well of ■ 0.5 mg/ml, ■ 0.2mg/ml and ■ 0.1 mg/ml. (A) a-glucosidase (B) ß-glucosidase (C) ß-glucuronidase. Results are expressed as the mean of the quadruplicate test wells (minus the mean of the background readings of the extract) as a percentage of that of the mean of the triplicate control readings. Dashed lines mark the glycohydrolase activity at 50% and 100% (---). Error bars indicate standard deviation (n=4).

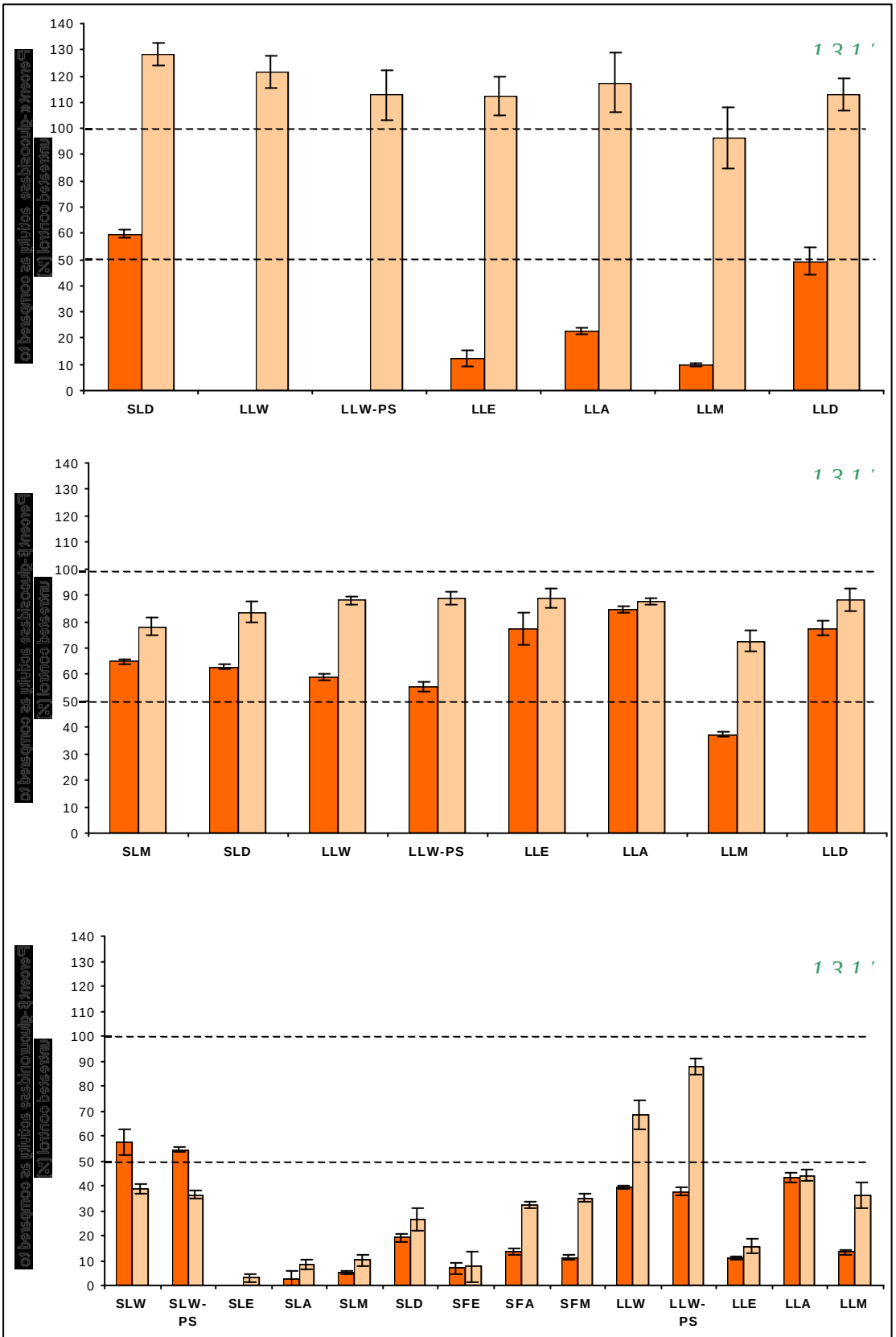

Figure 4.9: Percent glycohydrolase activity with plant extracts at 0.2 mg/ml in the presence of 0.2% (w/v) BSA as compared to the untreated control. The DMSO aliquots of plant extract were diluted in relevant enzyme buffer to a final concentration in the well of 0.2 mg/ml. Orange bars represent results obtained without BSA and tan bars with BSA. (A) a-glucosidase (B) ß-glucosidase (C) ß-glucuronidase. Results are expressed as the mean of the triplicate test wells (minus the mean of the background readings of the extract) as a percentage of that of the mean of the triplicate control readings. Dashed lines mark the glycohydrolase activity at 50% and 100% (---). Error bars indicate standard deviation (n=4).

4.3.3 Integrase

Unfortunately the IN assay did not work and no results were obtained for even the positive control. The problem may have been with the enzyme itself. As can be seen in figure 4.10, there was complete digestion of the pPCR-Script^{TM} plasmid (Novagen, CA USA) and that this preparation yielded enough DNA to coat each well with 1 µg of digested plasmid. The process of coating the plate with DNA is uncomplicated and so it is unlikely that a problem occurred at this step.

In similar HIV-I IN assays where the IN protein was used from the NIH AIDS Research and Reference Program (MD USA), the amount of IN was quadrupled in the assay as compared to the established methods laid out by Chang *et al.*, (1996) and Chow (1997) as was the case with Abd-Elazem *et al.*, (2002). This suggests that the recombinant HIV-I_{NL4-3} IN may not be very active and that for this experiment to work, the amount of HIV-1 IN should have in turn been quadrupled in the ELISA-based assay. However this could not have been done in practice due to the limited amount of enzyme provided, which at the higher concentration would allow one to test only two extracts in triplicate. For this assay all the extracts needed testing in at least duplicate, so to achieve this 40pmol of IN was used as suggested by Chow (1997).

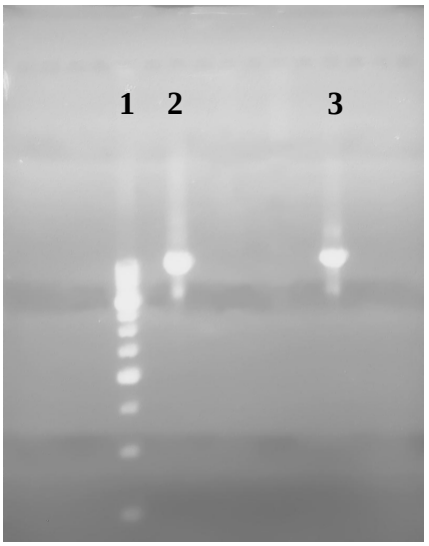

Figure 4.10: A 1% agarose gel showing the digested pPCR-Script plasmid. Lane 1 is the 250bp ladder, molecular marker, XVI. Lane 2 and 3 show the *Sma* I digested pPCR-Script plasmid at 3000bp. Digestion was carried out as described in section 4.2.3.1.3

4.3.4 Protease assay

From figure 4.11, it is clear that none of the extracts showed any significant inhibition of the HIV-II PR (=50 %), though this does not rule out the possibility that the extracts may show some activity against HIV-I PR. Only the extracts LLW and LLM showed some slight inhibition of the HIV-II PR at 29.4% and 30%, respectively.

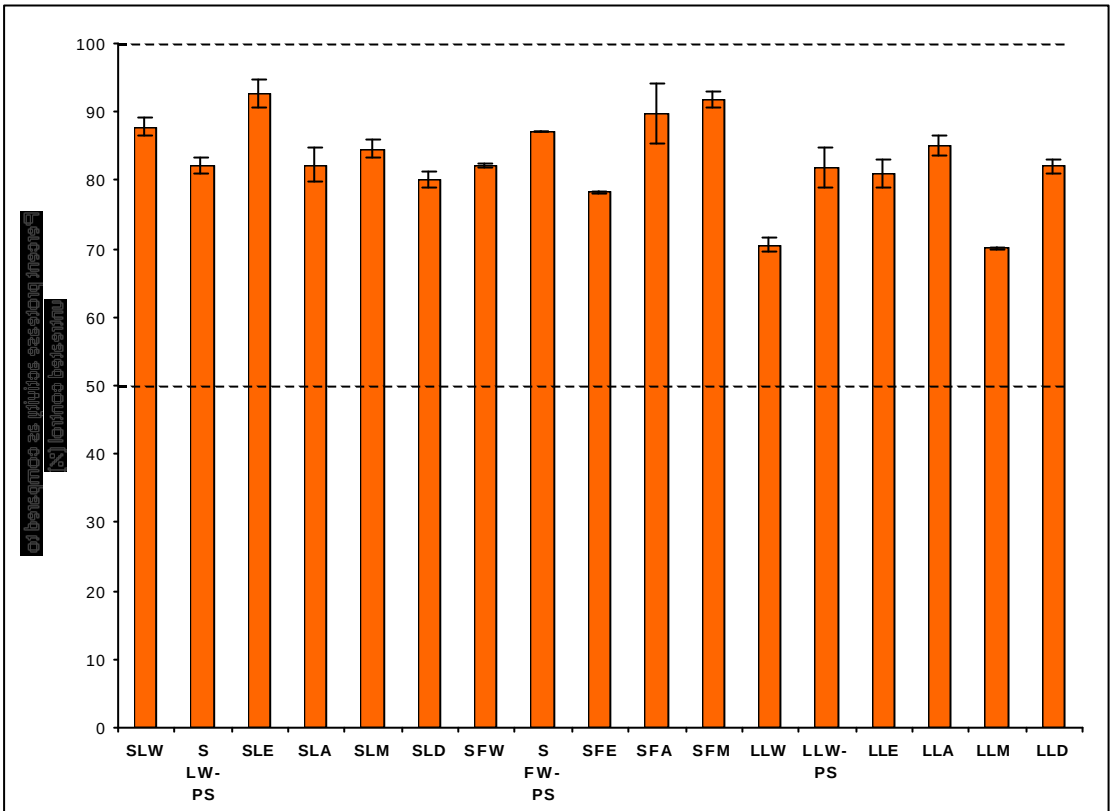

Figure 4.11: Percent protease activity with 0.2 mg/ml plant extract as compared to the untreated control. The DMSO aliquots of plant extract were diluted in protease buffer to a final concentration in the well of 0.2mg/ml. Results are expressed as the mean of the triplicate test wells (minus the mean of the background readings of the extract) as a percentage of that of the mean of the duplicate control readings. Dashed lines mark the protease activity at 50% and 100% (---). Error bars indicate standard deviation (n=3).

No further tests were performed with the extracts in the protease assay because of the low inhibition shown.

5 Conclusion

5.1 Cell results

Setting up a point-scale system greatly helps to clarify the cell results and gives a better overall impression of how the extracts fared. When screening many extracts to determine which have potential anti-HIV activity, this serves as a useful tool to determine which of the extracts show promise and which can be carried forth to the more specific enzymatic assays. Considering the cost of some of the enzymatic assays (e.g. the RT kit used in this project) it could help to bring down expenses in the screening process especially when one includes the dereplication steps of removing sulphated polysaccharides and negating the effects of tannins.

Of the three systems used to evaluate the results (table 3.1, 3.2 and 3.3), the last system described in table 3.3 is probably the best as it weighs the viral assays higher than those results from the toxicity assay. For this study I believe the viral assay results hold more importance than those from the toxicity study as it is only important that the extract does not inhibit normal proliferation in healthy cells, not increase proliferation. If more significant results were obtained from the p24 assays (=50% decrease in p24 levels) this would have been weighted as well but since this was not the case, the p24 data was treated the same for each evaluation. Although the CEM-NK^R-CCR5 cells do not give as wide a range of results as those found with the PBMCs, it should be evaluated in the same manner as was done with table 3.3.

Even with the different ways of evaluating the extracts there are some consistencies. The organic extracts from the *Sutherlandia* flowers scored very high percentages with SFA scoring the highest out of the three organic extracts in two of the three tables. With all three systems it is seen that SLM scored the lowest and so can probably be disregarded in further analysis along with SLW, SFW and SFW-PS as seen in table 3.3. Although LLA scored low points as well, its exclusion from some of the

experiments done may have influenced the score, as is the case with the high score for LLD.

Looking closer at the results, the XTT assays gave very high absorbance readings for the test wells as compared to the untreated controls and this could have influenced results greatly. There may have been several contributing factors. Firstly extracts could have been highly mitogenic and therefore stimulated the proliferation of immune cells. Secondly the extracts themselves could have been contributing to the absorbance values by reducing the XTT (Bruggisser *et al.*, 2002; Weislow *et al.*, 1989). To overcome this, a pre-screening assay should be performed to compare the results of extracts with XTT to controls of XTT and controls of just extract. A third problem is that of the DMSO toxicity. As was the case in the CEM-NK^R-CCR5 viral study, DMSO may have damaged the control cells that contain virus so that the test wells seemed to have very high cell proliferation in comparison. Although DMSO at 3% (v/v) did not affect the uninfected PBMCs, in a compromised state of the HIV-infected PBMCs, this could have been a problem too. To account for this, a second control should be included in all assays with just cells and medium with no DMSO.

In literature, the XTT assay has been shown to be fairly accurate and give reproducible data but in most cases the systems used have been semi or fully automated (Traore and Meyer, 2002; Weislow *et al.*, 1989). To do this experiment manually requires many manipulations, therefore allowing room for human error. Despite this, it seems the best assay available when choosing to screen a large number of extracts for toxicity. Ultimately, to confirm results, a trypan blue assay could be incorporated, but for the amount of extracts tested here this was not feasible as there were too many extracts to test in this manner in a single day and in the time available at RAU.

In the viral PBMC study, inter-patient variation was also a problem but due to time constraints and number of extracts, little could be done to match patients. The viral studies were performed by the author at RAU and this was made possible by a mobility grant from NRF but it only allowed for a two-week stay in Johannesburg and HIV-infected patients visited the hospital on Thursdays only. Potential leads from this screening process should be tested again in the viral assays but this time it should

be expanded further with more patients that are matched according to age, viral load, CD4 counts and they should all be either not on any medication or on the same type of treatment. Knowing the stage of infection is especially important when doing the p24 assay, as false negative results can occur if the patient is in the early stages of infection and the free antigen could be below detection level. As was the case in this project the p24 levels were very low so to overcome this, infected PBMCs should be co-cultured with uninfected PBMCs (instructions supplied with p24 kit, Beckman Coulter Miami Florida USA).

Between the XTT and p24 assays there are many differences but since a single p24 reading was taken for the PBMCs and a duplicate for the HIV-infected CEM-NK^R-CCR5 cells, one cannot conclude decisively but only pinpoint potential leads to follow up. The XTT assay measures the health and proliferation of cells while the p24 assay measures the level of p24 core protein (viral load). Although one expects an overlap of results, these assays can be mutually exclusive at times. An increase in cell proliferation does not necessarily mean a decrease in p24 levels. Extracts could be immune-enhancing and therefore stimulate cell proliferation while having no impact on the virus directly. There is some controversy about the role of immune-enhancers in HIV infections as it is the immune cells themselves that are the target for HIV infection and an increase in immune cells may only be providing more host cells for infection; hence an increase in p24 levels could be seen (Fidler and Weber, 2000). In figure 3.4, 3.5 and 3.6 many extracts show a marked increase in proliferation but p24 levels were raised as well, for example in figure 3.4, LLM increased p24 levels by 25% even though proliferation of cells was improved by more than 50%.

Conversely, a reduction in p24 levels may not correlate with improved cell proliferation, as extracts could be quite toxic and kill infected cells as well as healthy cells. This is seen with assays performed in this project; in figure 3.5 SLM reduces cell proliferation by more than 50% despite the reduction of p24 levels by 26%. In the toxicity study (figure 3.3) though SLM proved to increase cell proliferation two out of the three assays. As explained earlier the problems with the XTT assays could account for this discrepancy but it may also be possible that in the uninfected PBMCs, the extract was metabolised by the healthy cells to form a less toxic product.

5.2 Enzyme targets

Only in the RT assay did any of the extracts show significant inhibition (=50%) being retained in the presence of BSA. The aqueous extract of the *Lobostemon* leaves produced an inhibitor of HIV-RT with a very low IC_{50} value of 0.049mg/ml (figure 4.5). Some inhibitory effect was lost with the removal of the sulphated polysaccharides and the addition of BSA to the assay, but still 64% inhibition of the HIV-RT remained, which confirmed that the inhibitor could be something novel, and not of polysaccharide or tannin compounds. Although it is not clear the exact nature of the compound, one can conclude that it is polar in nature and around 1000-5000 daltons in size, plus the compound is very stable. Due to the nature of the extraction process and conditions in the laboratory, the extract went through several manipulations but still retained the strong inhibitory effect against the HIV-RT. Extracts SLW, SLW-PS, LLM and LLD also inhibited HIV-RT but when BSA was included, this inhibitory effect was decreased (figure 4.4) indicting that the effect seen in these extracts was probably due to tannins.

Looking back at the cell results, the LLW and LLW-PS extracts scored high points in table 3.1 and average results with table 3.3 with both extracts being able to reduce p24 levels in three out of the four p24 assays done. One cannot conclude however that the results from the cell work are related to RT inhibition. In many cases when mutation studies were done it was found that an alternative inhibitory effect such as an entry step inhibition was responsible for the results seen in the cell-based assays but was just not picked up in the *in vitro* screening (De Clercq, 2000).

In the traditional medicine setting, the *Lobostemon* extract offers some hope as it is a strong inhibitor of HIV-RT and in the preliminary results, seems to be non-toxic. Preparation of this extract would be cheap and simple and would only require that crushed leaves be soaked in water. However, a concern is the availability of the plant, as it seems difficult to cultivate.

In the rest of the assays no significant inhibition was seen of the glycohydrolase enzymes and HIV-II PR (figures 4.9 and 4.11, respectively). In literature where

glycohydrolase enzymes are included in the study of plant extracts for anti-HIV activity, BSA is never included (Collins *et al*., 1997). What was seen in this project was that nearly all the inhibitory effect seen with the extracts (figure 4.8) was probably due to tannins, suggesting that positive results of inhibition seen elsewhere may be only due to tannins. It is therefore a good idea to include BSA in all the glycohydrolase enzyme assays to confirm results. Concern over the effect of BSA on the enzymes themselves was tested and it was shown that there was no effect (results not shown).

None of the extracts showed any significant inhibition of the HIV-II PR (=50%) but this does not necessarily mean that the extracts could not show inhibition of HIV-I PR. With HIV protease inhibitors there is some overlap in inhibition of both HIV-I and HIV-II e.g. BI-LA-398 (Phe-Val-Phe-f(CH_2NH)-Leu-Glu-Ile-amide) but many inhibitors are also specific for one protease (Mulichak *et al*., 1993; Salto *et al*., 1994; Tong *et al*., 1993).

5.3 Future prospects

Work should definitely be continued with the LLW-PS extract with regards to its toxicity and anti-viral activity in cell-based assays. The extract should first be run through a polyamide gel to remove the tannins and only the tannin-free fraction examined further. Trypan blue and/or flow cytometry should be included to confirm XTT and p24 results. To confirm that the inhibitory effect of the extract is related to the inhibition of RT, a mutational study could be included as well.

The extract should also be fractionated and active fractions further characterized by mass spectrometry and NMR. Activity could be lost, however, upon fractionation as compounds could be working synergistically.

From the cell results, those extracts that scored high points in table 3.3, especially SFA, and SFM should be tested further in more extensive cell-based assays. The effect of the extract at different stages of infection should be done as well as this could establish where in the HIV lifecycle the extract helps the infected cells. The

extracts could be added in the cell-based assays before infection, soon after infection and later when the infection has been established in the cells. These extracts should also be tested again in the *in vitro* screening but with the use of HIV-I PR. The HIV-IN assay should be repeated but with either more of the HIV-1_{NL4-3} IN or a more active enzyme. The UPE laboratory has two different strains of bacteria transformed for each of the HIV-I IN and HIV- I PR but no pure enzyme has been isolated yet. It would be ideal to also look at the inhibition of viral attachment. This was originally going to be performed with the use of a kit but this is no longer commercially available and this assay can only be performed otherwise in cell-based work using transformed cells.

During this study, cell-based assays were performed at RAU on two short visits and this made it difficult to do everything due to the length of incubations (7-8 days per experiment). So, in order to complete the above suggestions, it is essential that the facilities to perform the cell-based work with HIV-infected cells, be readily available and at hand.

Many positive aspects have come from this project. A useful system has been established to evaluate results from cell-based assays in determining which extracts may show potential anti-HIV activity and the aqueous extract of *Lobostemon* leaves has been established as a potent inhibitor of HIV-I RT. Also, procedures have been somewhat standardised and established in the laboratory with problems having been identified so that work can continue on this project with satisfying results.

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7 Appendix A

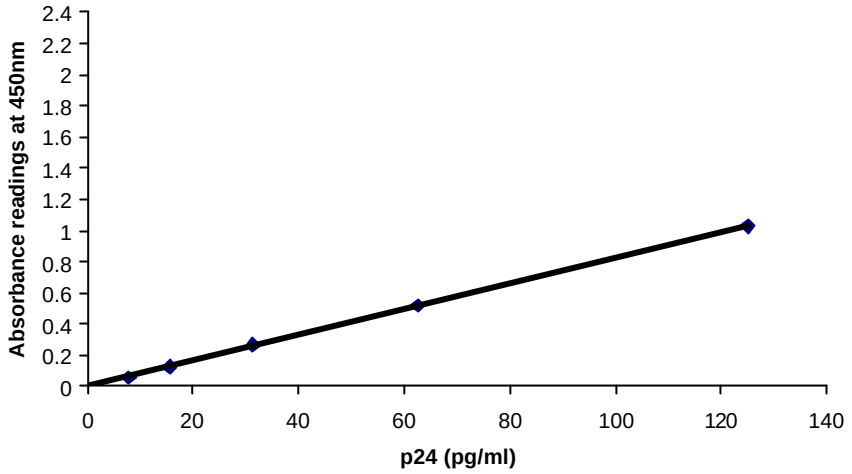

Figure 7.1: Standard curve for p24 measured at 450nm in the infected PBMC assay. Results were calculated as the mean of duplicate absorbance readings for each p24 concentration. The correlation coefficient is 0.9998 and the equation is y = 0.0082x**.**

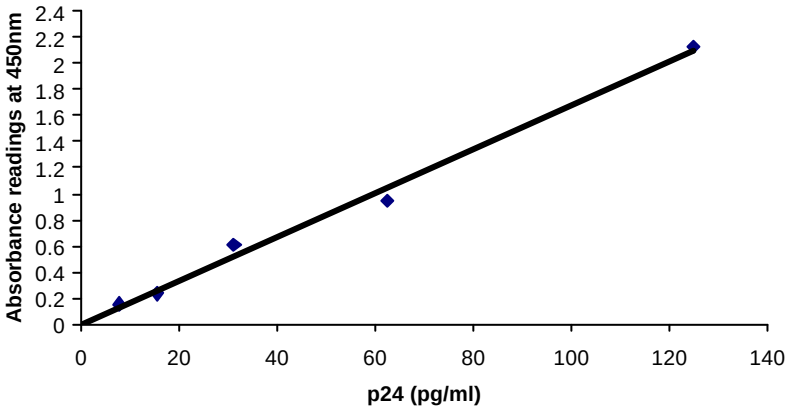

Figure 7.2: Standard curve for p24 measured at 450nm in the infected CEM-NK^{R}-CCR5 cells**. Results were calculated as the mean of duplicate absorbance readings for each p24 concentration. The correlation coefficient is 0.9935 and the equation is y = 0.0167x.**

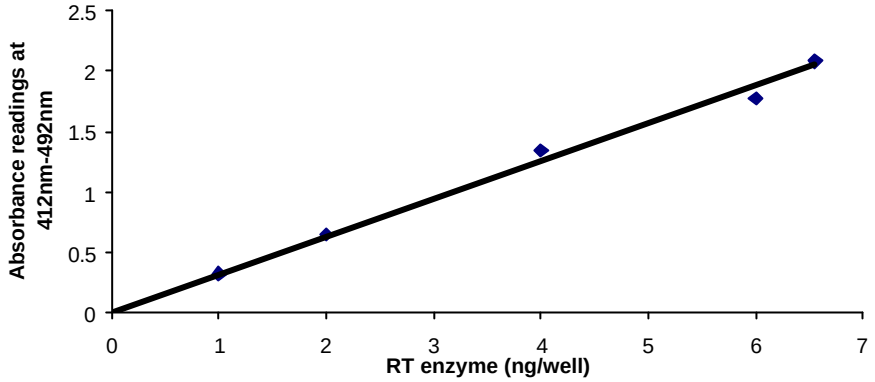

Figure 7.3: Standard curve for HIV-I RT measured at 412nm with reference wavelength at 492nm. Results were calculated as the mean of duplicate absorbance readings for each RT concentration. The correlation coefficient is 0.9942 and the equation is y = 0.3134x.

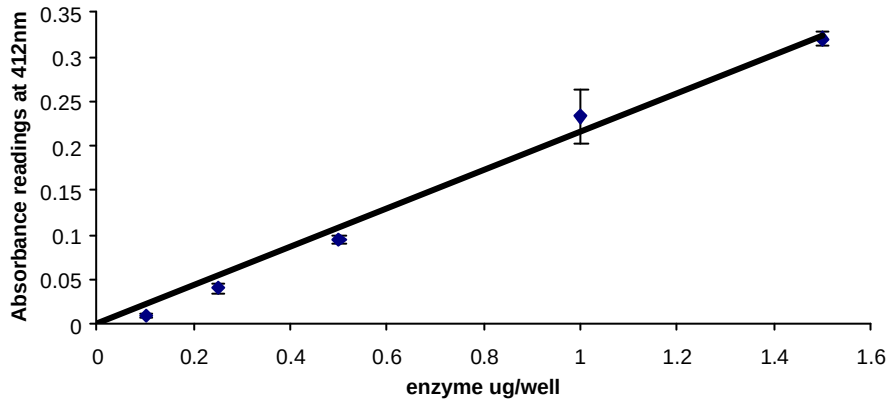

Figure 7.4: Standard curve for a-glucosidase measured at 412nm. Results were calculated as the mean absorbance reading for each enzyme concentration. Error bars represent standard deviation (n=4). The correlation coefficient is 0.9883 and the equation is y = 0.216x.

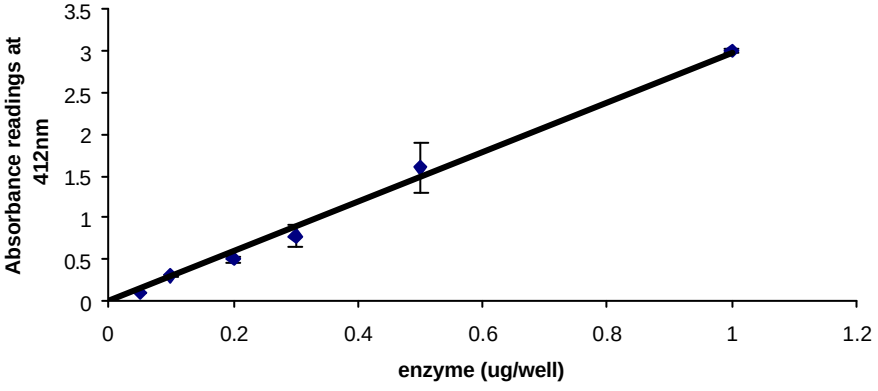

Figure 7.5: Standard curve for ß-glucosidase measured at 412nm. Results were calculated as the mean absorbance reading for each enzyme concentration. Error bars represent standard deviation (n=4). The correlation coefficient is 0.9936 and the equation is y = 2.9935x.

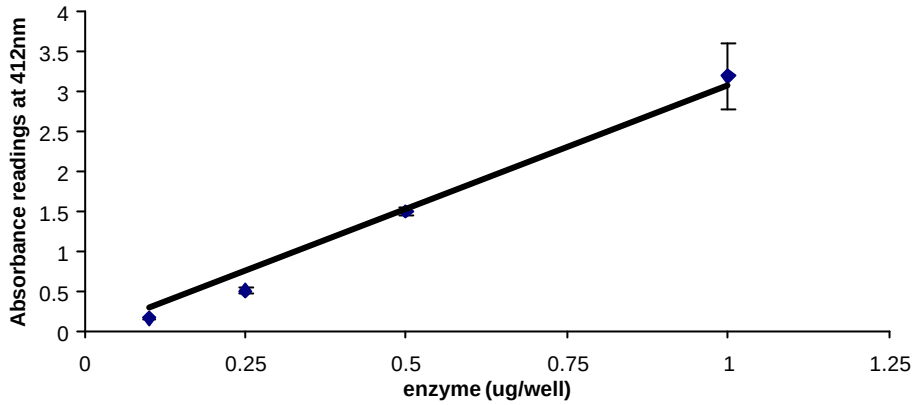

Figure 7.6: Standard curve for ßglucuronidase measured at 412nm. Results were calculated as the mean absorbance reading for each enzyme concentration. Error bars represent standard deviation (n=4). The correlation coefficient is 0.9818 and the equation is y = 3.0959x.

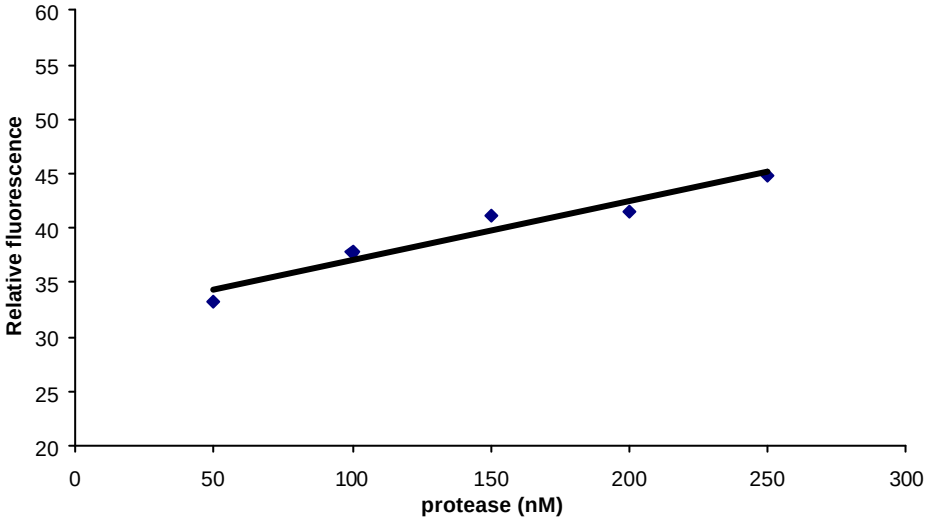

Figure 7.7: Standard curve for HIV-II PR measured at an excitation wavelength of 355nm and emission wavelength of 460nm. Results were calculated as the mean of duplicate absorbance readings for each enzyme concentration. The correlation coefficient is 0.9422 and the equation is y = 0.0538x + 31.644.

8 Appendix B

Note: volumes were adjusted with distilled water unless otherwise indicated

LB Medium (1l)

10g Tryptone

5g Yeast extract

10g NaCl

Mix and autoclave

4M LiCl (100ml)

16.96g LiCl

Autoclave

Solution I (100ml)

2.5ml 1M Tris-HCl (pH 8)

10ml 0.5M EDTA (pH 8)

5ml 20% (w/v) glucose

Solution II (100ml)

2ml 10M NaOH

10ml 10% (w/v) SDS

Solution III (100ml)

60ml 5M Potassium acetate

11.5ml glacial acetic acid

Autoclave and store at 4℃

50X Tris-acetate-EDTA/TAE buffer

242g Tris base

57.1ml glacial acetic acid

100ml 0.5M EDTA (pH 8)

Autoclave

Tris-EDTA/TE buffer (1l)

10mM Tris-HCl (pH 8)

1mM EDTA (pH 8)

Autoclave

9 Appendix C

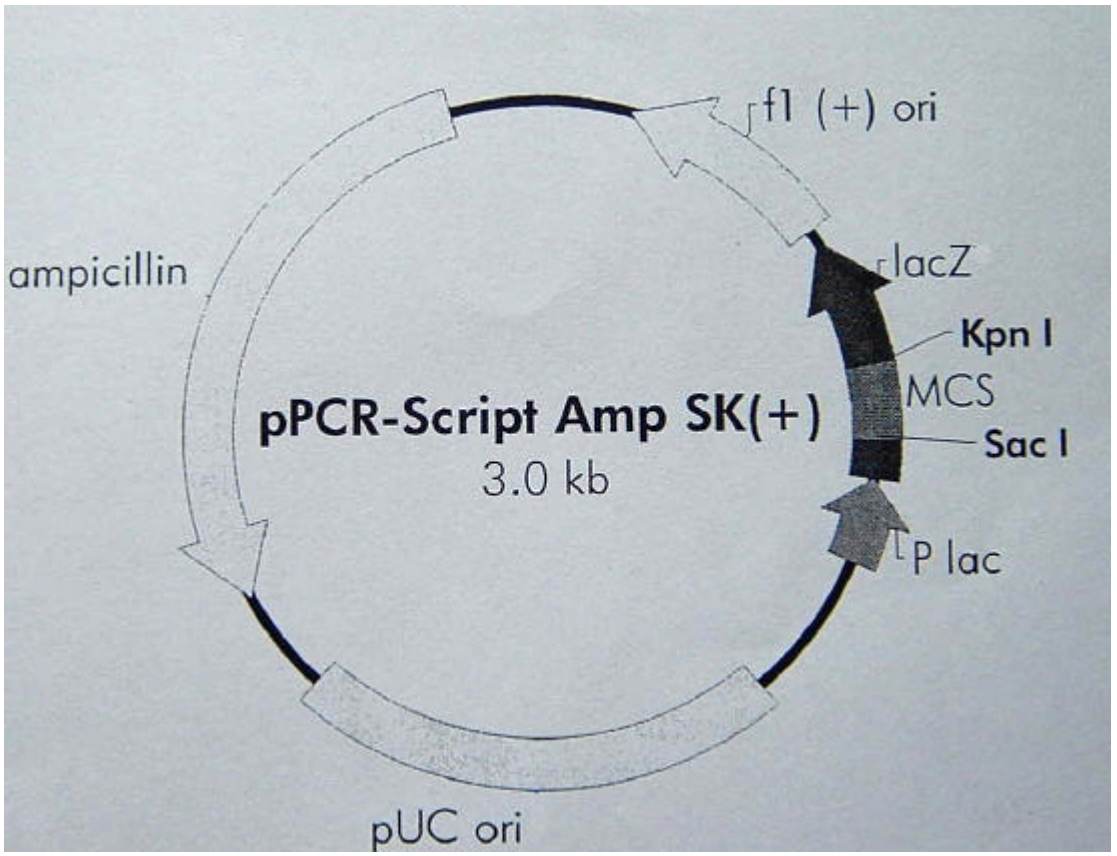

Figure 9.1: pPCR-Script Amp SK (+) vector map showing the multiple cloning site (MCS).