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SCREENING OF A HERBAL PREPARATION (PHEKO) FOR ANTI HIV-1 REPLICATION PROPERTIES

A DISSERTATION SUBMITTED IN FULFIMENT OF THE REQUIREMENTS FOR A MASTER OF SCIENCE (MICROBIOLOGY) DEGREE

BY

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University of Venda

SOUTH AFRICA

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MAY 2014

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DECLARATION

I, **NONTOKOZO DAPHNEY MATUME**, hereby declare that the dissertation for the award of a Master of Science degree in Microbiology at the University of Venda, hereby submitted by me, has not been submitted for a degree at this or any other University, and that it is my work in design and execution and that all the reference materials contained therein have been duly acknowledged.

Signature..... Date..05 May 2014

Supervisor..... Date..5/5/14

Co-Supervisor..... Date..05 May 2014

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ACKNOWLEDGEMENTS

Glory be to God Almighty for all He has done. It is by his grace, endless love and divine purpose this work came to completion. Thank you Lord.

Courage, inspiration and guidance are essential for a production of quality and well trained students. To Prof P.O. Bessong, thank you so much for being all of these, I'm so grateful that you had such great faith in me and gave me an opportunity to learn and acquire skills in the field of research. I would not have asked for a better supervisor, you are simply the best.

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I would also like to thank the National Research foundation for funding this study.

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DEDICATION

I dedicate this dissertation to the following people;

My mother Priscilla Mkhabela,

My brothers and sister Xolani, Mzwake and Lindokuhle

My late Grandmother and Uncle, Anna Shiyiwe Nsingwane and Barnny Makhehlani Mkhabela

My aunts Thuli Mkhabela, Sokolile Mkhabela and Gugu Bulunga

My uncle Mandla Mkhabela.

This is for their undying love, support and encouragements throughout my study.

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ABSTRACT

Infection with Human Immunodeficiency Virus (HIV) is of global public health concern. Highly active antiretroviral therapy (HAART) significantly reduces morbidity and mortality due to AIDS, the outcome of HIV infection. However, due to the limitations of HAART, alternative forms of treatment, such as herbal therapies, are common particularly in developing communities. The current study investigated the anti-HIV effects of a herbal preparation (Pheko) for inhibitory effects against potential therapeutic targets of HIV.

Several extract types of Pheko were assayed for inhibitory effects against HIV-1 reverse transcriptase (RT) and integrase (IN) in a cell-free method. Additionally, the methanol extract was studied for anti-replicative properties against pseudoviruses of HIV-1 subtypes B and C of wild and resistant types. Viruses used included wild type and resistant mutants of NL4-3 (HIV-1 subtype B), wild type IndieC (HIV-1 subtype C).

The cytotoxicity of extracts against the human cell lines (U87.CD4.CCR5 and Vero T35) was also evaluated with the Lactate dehydrogenase (LDH) for cell death and MTT (3-[4, 5-Dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide) for cell viability. Methanol extract of Pheko showed the highest cell death with a CC_{50} of 0.5mg/ml using the LDH assay, and water extract showed the least cell death with the CC_{50} of 1mg/ml. Evaluation of cell viability using MTT assay showed that at the maximum concentration (2mg/ml), dichloromethane extract inhibited cell viability more than other extracts with 81% inhibition, and methanol inhibited 57% of cell viability.

The water extract of Pheko inhibited integrase with an IC_{50} of 0.125mg/ml. Dichloromethane extract was most inhibitory to RT with an IC_{50} of 0.003 mg/ml, and the water extract was the least inhibitory with an IC_{50} of 12.5 mg/ml. The methanol extract also showed excellent HIV-1 RT inhibition (IC_{50} of 0.02mg/m).

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Transformation of competent cells were successfully generated and successfully used to prepare plasmids for HIV-1 subtype C-based pseudoviral packaging. In the pseudovirus assays, the methanol extract inhibited RT activity of the HIV-1 subtype C virus (IndieC) with an IC_{50} of 0.025 µg/µl. Of interest was the strong inhibition of the NRTI and NNRTI resistant strains of HIV-1 subtype B (NL4-3) by the methanol extract with IC_{50} values of 0.035 µg/µl and 0.021 µg/µl, respectively. Low selective indices were observed when U87.CD4.CCR5 cell line where used as target for the pseudoviruses (range 0.01-0.023).

In the current study only the methanol extract was investigated in the pseudovirus assay for anti-HIV replicative property. Future work will particularly look at the water extract. In conclusion, this preliminary data shows that the methanol extract of Pheko can potentially inhibit HIV-1 Subtype C and viruses resistant to nucleoside RTI and non-nucleoside RTI.

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LIST OF ABBREVIATIONS

ABC	Abacarvir
AIDS	Acquired immunodeficiency syndrome
ARV	Antiretroviral
amp	Ampicillin
AZT	Zidovudine
BMI	Body mass index
CC_{50}	50% Cytotoxic concentration
CCR5	Cysteine-cysteine chemokine receptor 5
CD4	Cluster of differentiation 4
CDC	Centre for disease control
cDNA	Complementary deoxyribonucleic acid
CRFs	Circulating recombinant forms
CPE	Cytopathic effects
DNA	Deoxiribonucleic Acid
DPBS	Dulbecco's phosphate buffered saline
DMEM	Dulbecco's modified eagle medium
EFV	Efavirenz

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NRTI	Nucleoside reverse transcription inhibitors
NTD	N-terminal domain
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NVP	Nevirapine
p24	Capsid protein
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PCMV	Plasmid cytomegalovirus
PEN/STREP	Penicillin/Streptomycin
PIC	Pre integration complex
PR	Protease
RNA	Ribonucleic acid
OD600	Optical density 600
SIVcpz	Chimpanzee simian immunodeficiency virus
SOC	Super optimal broth
SQV	Saquinavir
TAE	Tris-acetate-EDTA
UNAIDS	Joint United Nations Programme on HIV/AIDS
USA	United States of America

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UV	Ultraviolet
µl	Microlitre
µM	Micromolar
V	Voltage
WHO	World HealthOrganization

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CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Human Immunodeficiency Virus (HIV) is a lentivirus which belongs to the Retroviridae family and causes Acquired Immunodeficiency Syndrome (AIDS), a condition in humans which results in the progressive failure of the immune system, allowing life threatening opportunistic infections and cancers to thrive. This virus can be transmitted through sexual contacts, contaminated blood products and from mother to child transmission.

HIV was discovered in 1983 after the discovery of AIDS between 1980 and early 1981(CDC, 1982). When patients showed symptoms of *Pneumocystis carinii* pneumonia (Gottlieb *et.al.*, 1981), which is a rare opportunistic infection that was known to present itself in people with much compromised immune systems. Soon thereafter, additional patients developed a previously rare skin cancer called Kaposi's sarcoma (Friedman-kien *et.al.*, 1981). Since then the virus has been driving the epidemic and has killed lots of people. At the end of 2012 an estimated 35.3 million people were living with HIV worldwide (UNAIDS 2013)The human immunodeficiency virus is known to have originated from non-human primates in sub-Saharan Africa and in the late 19th or early 20th century was transferred to humans (Korber *et.al.,* 2000). HIV-1 appears to have originated in southern Cameroon through the evolution of SIV (cpz) (Gao *et.al.*, 1999), a Simian Immunodeficiency Virus that infects wild chimpanzees. The closest relative of HIV-2 is SIV from sooty mangabey (Keele *et.al.*, 2006). HIV differs from many viruses in that it has very high genetic variability.

There is currently no cure for HIV infection. The main treatment for HIV is antiretroviral treatment which aims at keeping the amount of HIV in the body at low levels. Current

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treatment is with highly active antiretroviral therapy (HAART) composed of multiple anti-HIV drugs, which include a nucleoside reverse transcriptase inhibitor, a protease inhibitor and a non-nucleoside reverse transcriptase inhibitor. This has been highly beneficial to many patients since its introduction in 1996, when the protease inhibitor based HAART initially became available (Pallella *et.al.*, 1998). One major problem in HIV treatment is that the virus has a prolific and highly error prone replication process. The drugs used in the treatment have many disadvantages and limitations. The shortcomings of these drugs continue to open new vistas in the use of ethnomedicinal and other natural products for the management of HIV/AIDS. South Africa is one of the countries with rich plant diversity and a long tradition of medicinal use of plants with approximately 3000 species of plants used as medicine (van Wyk *et.al.*, 1997).

1.2 LITERATURE REVIEW

1.2.1 EPIDEMIOLOGY OF HIV

At the end of 2012, an estimated 35.3 million people were living with HIV worldwide, and32.1 million of the 35.3 million are adults, 17.7 million are women and 3.3 million are children under the age of 15 (http://www.nursingceu.com/courses/446/index-nceu.html). In 2012, an estimated 2.3 million people were newly infected with HIV, 260 million were children under the age of 15 and 2 million were adults. The total number of deaths in 2012 is 1.6 million, of which 1.4 million are adults and 210 000 were children. About 6 300 new HIV infections a day in 2012 and about 95% are in low and middle-income countries and 700 are in children under the age of 15, 5 500 are in adults aged 15 years and older of whom almost 47% are among women and 39% are among young people aged 15-24 (UNAIDS 2013).

Sub- Saharan Africa remains the region most heavily affected by HIV. In 2011, about 69% of all people living with HIV resided in sub- Saharan Africa, a region with only 12% of the global population. In 2012 adults and children living with HIV are estimated at 25 million, newly infected with HIV is 1.6 million. An estimated 1.2 million adults and children died of AIDS. In

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the Middle East and North Africa an estimated [illegible] people were living with HIV in 2012 and AIDS claimed an estimated 17000 lives in that region (UNAIDS 2013).

In Central and North America there were estimated 48000 new HIV/AIDS infected Americans in 2012. This region has currently 1.4 million people living with HIV/AIDS. As for North Africa and the Middle East, approximately 260000 people are living with HIV and an estimated 32000 people became newly infected in 2012, while an estimated 17000 adults and children died of AIDS (UNAIDS 2013).

In Eastern Europe and Central Asia an estimated 1.3 million people living with HIV and 130 000 were newly infected in 2012, and HIV/AIDS has claimed 91000 lives in 2012. As for Western and Central Europe, there were 29000 new cases of HIV in 2011 bringing the number of people living with HIV/AIDS to 860000, and an estimated 760 000 people in this region died of AIDS (UNAIDS 2013).



Figure 1.1: Global prevalence of HIV/AIDS (UNAIDS 2013).

The epidemic continues to be severe in South Africa where there are more people living with HIV, with an estimated 6.4 million than any other country in the world (HSRC, 2013).

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KwaZulu-Natal has recorded a notable decrease in HIV prevalence which is promising, whereas Mpumalanga has recorded an increase in the past four years. There has been notable drop in the 2011 HIV prevalence in KwaZulu-Natal with an estimate of 37.4%, indicating a decline by 2.1% from 39.0%. Mpumalanga province has shown an increase in estimated HIV prevalence of 2.0% from 34.7% in 2009 to 36.7% in 20011. There was also an increase in HIV prevalence in Free State from 30.6% in 2010 to 32.5% in 2011, and the North-West from 29.6% in 2010 to 30.2% in 2011. Limpopo is showing a steady increase from 21.4% in 2009 to 22.1% in 2011, see figure 1.2 (National Antenatal Sentinel, 2012).

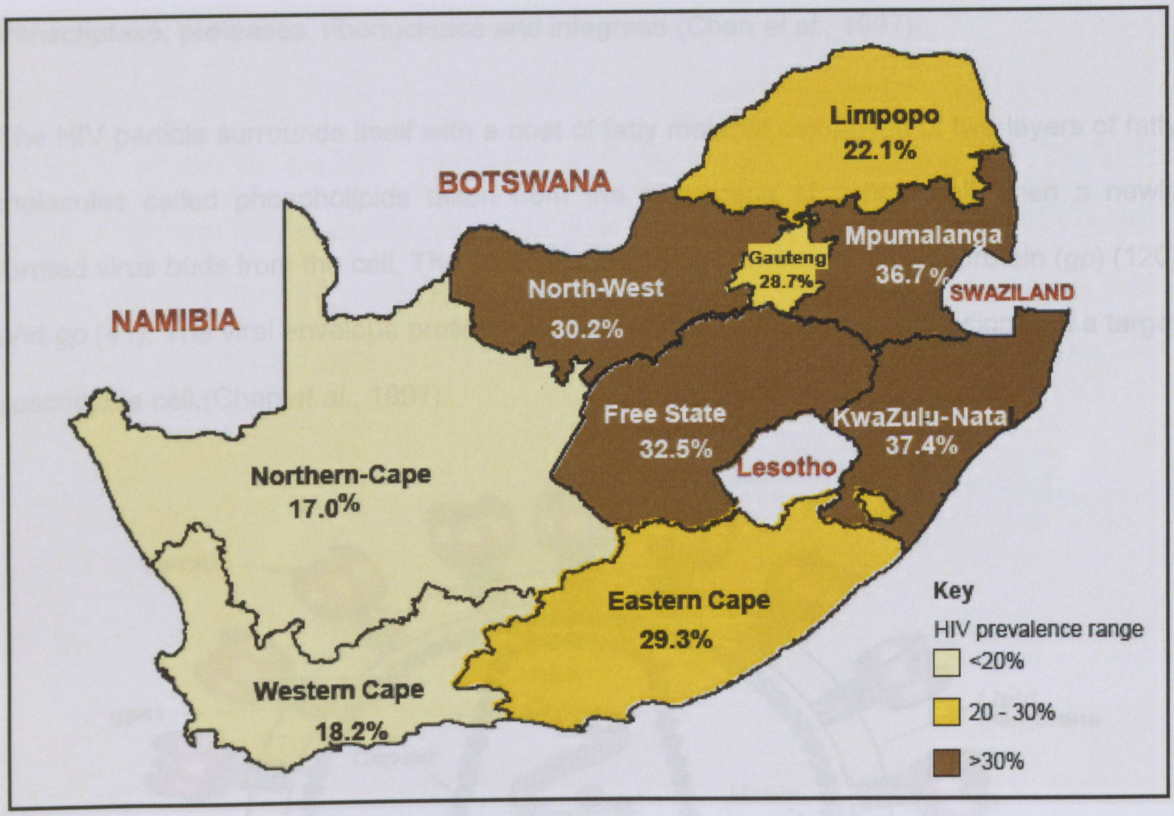


Figure1.2: National prevalence of HIV/AIDS (National Antenatal Sentinel, 2012).

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1.2.2 STRUCTURE AND GENOMIC ORGANISATION OF HIV

Human Immunodeficiency Virus has a different structural composition compared to other retroviruses. Outside of a human cell, a mature HIV virion exists as a roughly spherical particle with a diameter between 100-150 nanometers (figure 1.3). The central core consists of two copies of positive single-stranded RNA that codes for the virus nine genes enclosed by a conical capsid composed of 2,000 copies of the viral protein p24, a matrix protein p17, as well as p9 and p7. Proteins which function in binding tightly the single-stranded RNA, and also enzymes needed for the development and replication of the virion such as reverse transcriptase, proteases, ribonuclease and integrase (Chan *et.al.*, 1997).

The HIV particle surrounds itself with a coat of fatty material composed of two layers of fatty molecules called phospholipids taken from the membrane of a host cell when a newly formed virus buds from the cell. The envelope protein is composed of glycoprotein (*gp*) (120) and *gp* (41). The viral envelope proteins are essential for attachment and fusion with a target susceptible cell.(Chan *et.al.*, 1997).

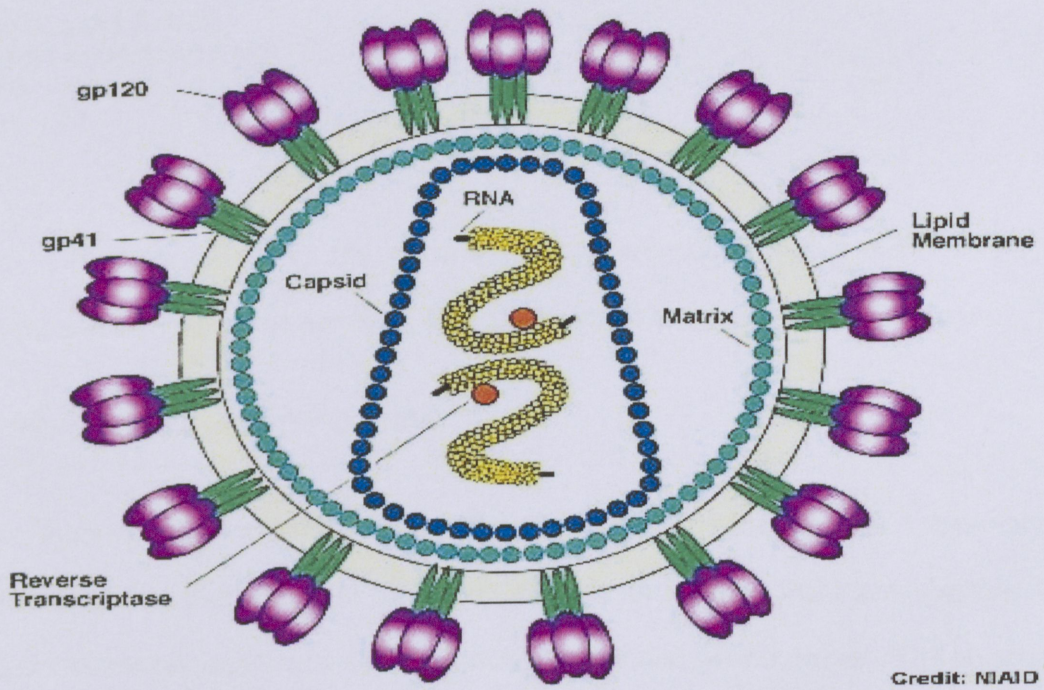


Figure 1.3: The structure of HIV (http://www.niaid.nih/gov/factsheets/graphics/howhiv.jpg)

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The virus nine genes encoded by the two copies of the positive single-stranded RNA are *gag*, *pol*, and *env*, *tat*, *rev*, *nef*, *vif*, *vpr*, *vpu*, and sometimes a tenth *tev*, which is a fusion of tat, *env and rev* (figure 1.4). Two of these genes which are the *gag* and *env* are classified as structural proteins. The six genes *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* (or *vpx* in the case of HIV-2), are regulatory genes for proteins that control the ability of HIV to replicate and cause disease (Chan *et.al.*, 1997). The *pol* gene codes for viral essential enzymes namely; reverse transcriptase, protease and integrase.

All these regulatory genes have specific functions that control the ability of the virus to infect cells and produce new viral copies of new viruses. For example, the *Vpr* protein (p14) arrests cell division at G2/M and *Nef* protein (p27), down-regulates CD4 (the major viral receptor), as well as the MHC class I and class II molecules (Stumptner *et.al.*,2001). The *Vpu* protein (p16) influences the release of new virus particles from infected cells. The ends of each strand of HIV RNA contain an RNA sequence called the long terminal repeat (LTR). Regions in the LTR act as switches to control production of new viruses (Schwartz *et.al.*, 1996).

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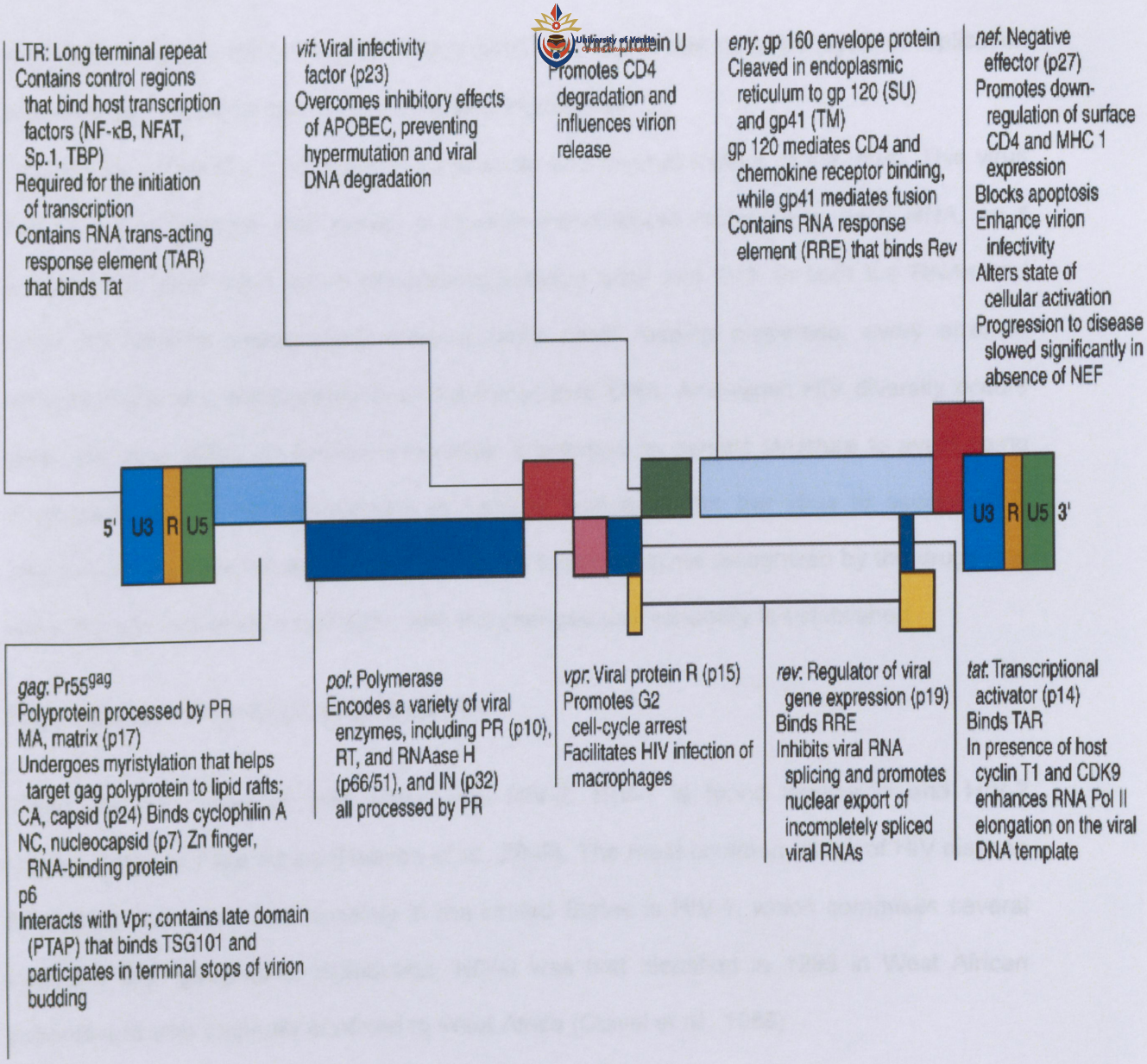


Figure 1.4: Organization of the genome of HIV provirus, together with the summary description of its nine genes encoding 15 proteins (http://www.accessmedicine.com).

1.2.3 GENETIC VARIABITITY OF HIV

Human immunodeficiency virus (HIV) differs from many viruses in that it has very high genetic variability, that amongst the same subtype of viruses, there are changes in the genome that makes each virus distinct though related and yet not clones. The diversity is a result of the fast replication cycle, with the generation of about 10^{10} virion every day, coupled

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with high mutation rates of approximately 3x10^{-5} mutations per nucleoside base per cycle of replication and recombinorgenic properties of reverse transcriptase.

High genetic diversity of HIV is due to external and internal factors of the virus. This virus has a diploid genome that carries a reverse transcriptase molecule in each RNA, so it creates high error rates when transcribing jumping back and forth in both the RNAs and since the reverse transcriptase enzyme lacks proof reading properties, every error or mistake made are incorporated in to the transcribed DNA. And again HIV diversity occurs when the virus elicits an immune response; it changes its genetic structure to avoid being recognized by the immune system as foreign. And again for the virus to escape drug pressure, it changes the amino acid that codes for the enzyme recognized by the drugs and when the cell replicates it replicates with the changes and variability is established.

1.2.4 GENETIC NOMENCLATURE OF HIV

There are two types of HIV: HIV-1 and HIV-2. HIV-1 is found worldwide and HIV-2 predominates in West Africa (Reeves *et al.*, 2002). The most common cause of HIV disease throughout the world and certainly in the United States is HIV-1, which comprises several subtypes with geographic distribution. HIV-2 was first identified in 1986 in West African patients and was originally confined to West Africa (Clavel *et.al.*, 1986)

The strains of HIV-1 can be classified into four groups: the "major" group M, the "outlier" group O and two new groups, N and P. These four groups may represent four separate introductions of simian immunodeficiency virus into humans.

Group O appears to be restricted to west-central Africa and group N a strain discovered in 1998 in Cameroon - is extremely rare. In 2009 a new strain closely relating to gorilla simian immunodeficiency virus was discovered in a Cameroonian woman. It was classified into HIV-1 group P (Plantier, 2009). More than 90% of HIV-1 infections belong to HIV-1 group M.

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Within group M, there are known to be at least nine genetically distinct subtypes or clades. These are subtypes A, B, C, D, F, G, H, J and K, see figure 1.5 (WHO, 2011).

Recombinant strains that infect more than one person are known as circulating recombinant forms or CRFs (Osmanov *et.al.,* 2002). For example, the CRF A/B is a mixture of subtypes A and B.

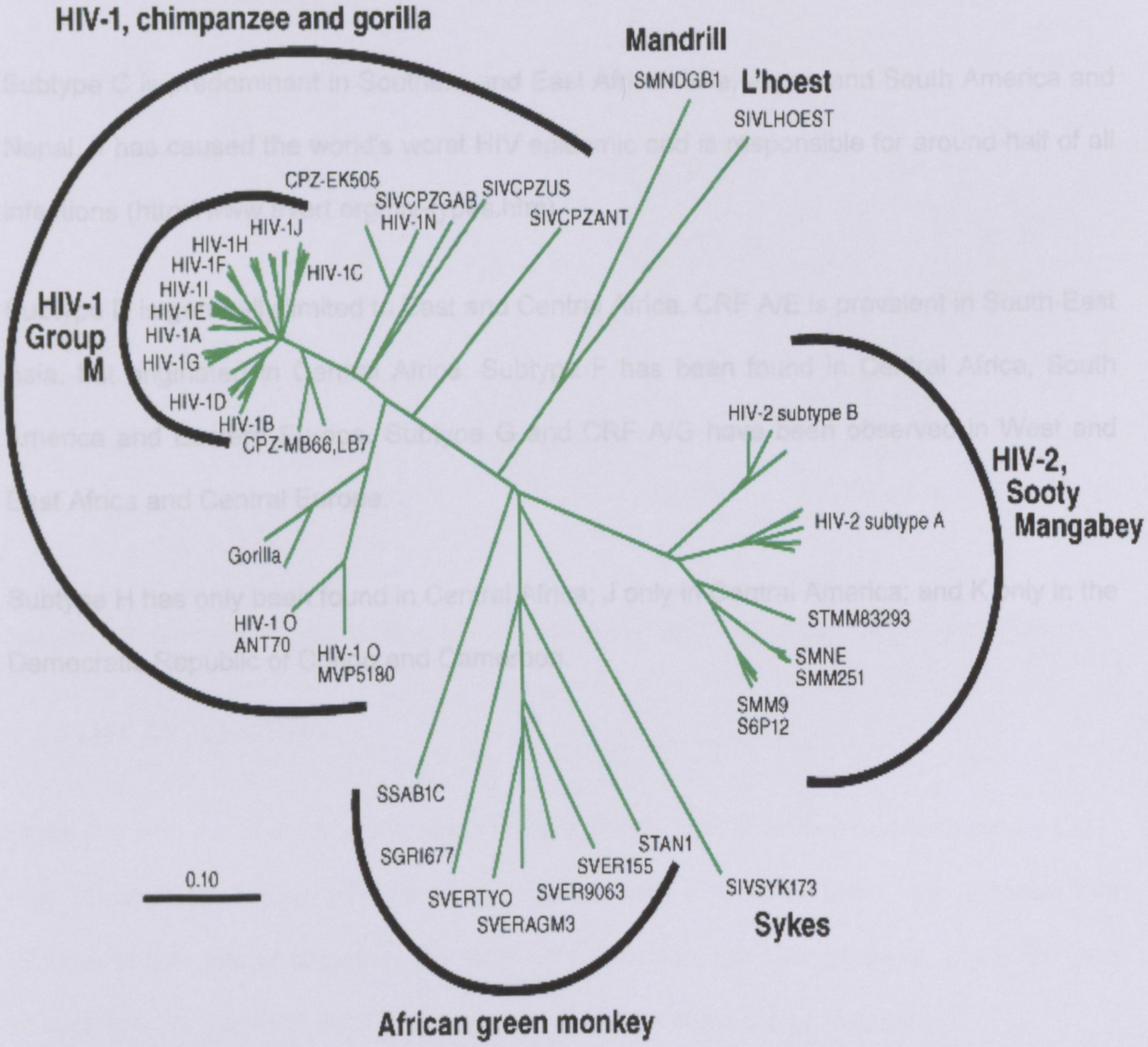


Figure 1.5: Phylogenic relationship of HIV and SIV. This phylogenetic tree is based on the complete genome of primate immunodeficiency viruses (http://www.accessmedicine.com).

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Subtype A and CRF A/G predominate in West and Central Africa, with subtype A possibly also causing much of the Russian epidemic (http://www.avert.org/hiv-types.htm).

Historically, subtype B has been the most common subtype in Europe, the Americas, Japan and Australia and is the predominant sub-type found in Europe (Le Vu *et.al.*, 2010). Although this remains the case, other subtypes are becoming more frequent and now account for at least 25% of new HIV infections in Europe (European CDC 2012).

Subtype C is predominant in Southern and East Africa, Asia, Japan and South America and Nepal. It has caused the world's worst HIV epidemic and is responsible for around half of all infections (http://www.avert.org/hiv-types.htm).

Subtype D is generally limited to East and Central Africa. CRF A/E is prevalent in South-East Asia, but originated in Central Africa. Subtype F has been found in Central Africa, South America and Eastern Europe. Subtype G and CRF A/G have been observed in West and East Africa and Central Europe.

Subtype H has only been found in Central Africa; J only in Central America; and K only in the Democratic Republic of Congo and Cameroon.

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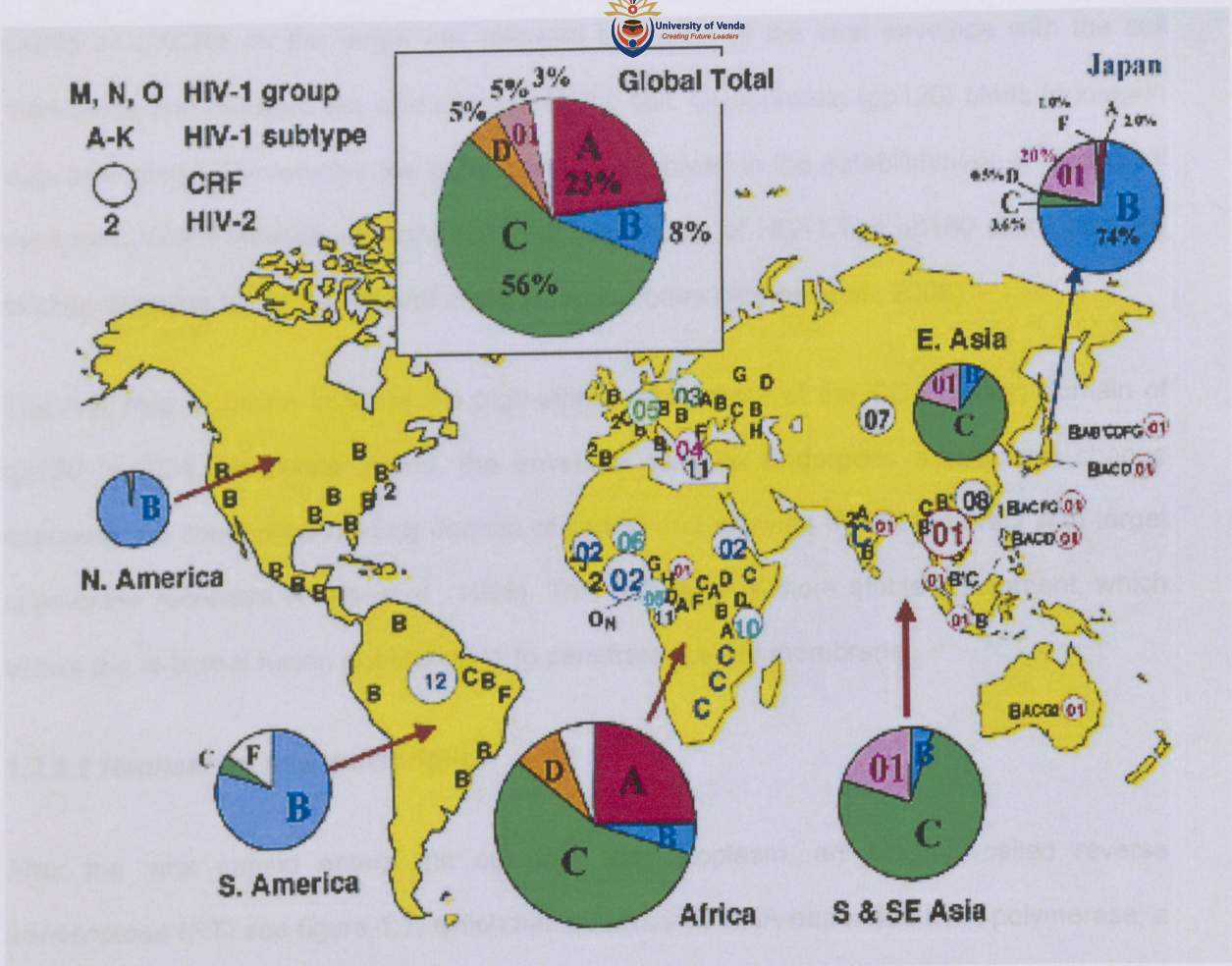


Figure 1.6: Geographic distribution of HIV subtypes (http://www.mapsofworld.com/)

1.2.5 LIFE CYCLE OF HIV

Once HIV is in the body, it targets and infects a certain type of white blood cell called a $CD4^+$ cell. (www.niaid.nih.gov) HIV takes over these cells and turns them into factories that produce thousands of copies of the virus. HIV goes through several steps, which are also drug targets, to establish itself in the human body. The steps are as follows:

1.2.5.1Binding

Human immunodeficiency virus infects or enters macrophages and $CD4^+$ T cells by adsorption of the glycoprotein (gp160) on its surface to chemokine receptors generally either

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CCR5 or CXCR4 on the target cell followed by fusion of the viral envelope with the cell membrane and releases the viral capsid into the cell. Glycoprotein (gp120) binds to integrin $\alpha_4\beta_7$ activating LFA-1whichis the central integrin involved in the establishment of virological synapses, which facilitate efficient cell-to-cell spreading of HIV-1.The gp160 spike contains binding domains for both CD4 and chemokine receptors (Arthos *et al.*, 2008)

The first step in fusion involves the high-affinity attachment of the CD4 binding domain of gp120 to CD4, and once bound, the envelope complex undergoes a structural change exposing the chemokine binding domain of gp120 and allowing them to interact with target chemokine receptors (Chan *et.al.*, 1998). This allows for a more stable attachment, which allows the N-termal fusion peptide gp41 to penetrate the cell membrane.

1.2.5.2 Replication and transcription

After the viral capsid enters the cell into the cytoplasm, an enzyme called reverse transcriptase (RT) see figure 1.7, which has activities as RNA-depended DNA polymerase, a DNA polymerase enzyme that transcribes single-stranded RNA into single-stranded DNA, and also a DNA-depended DNA polymerase which synthesizes a second strand of DNA complementary to the reverse transcribed single stranded cDNA after degrading the original mRNA with its RNaseH activity. Together the cDNA and its complementary, form a double stranded viral DNA which is then transported into the nucleus (Zheng *et.al.*, 2005).

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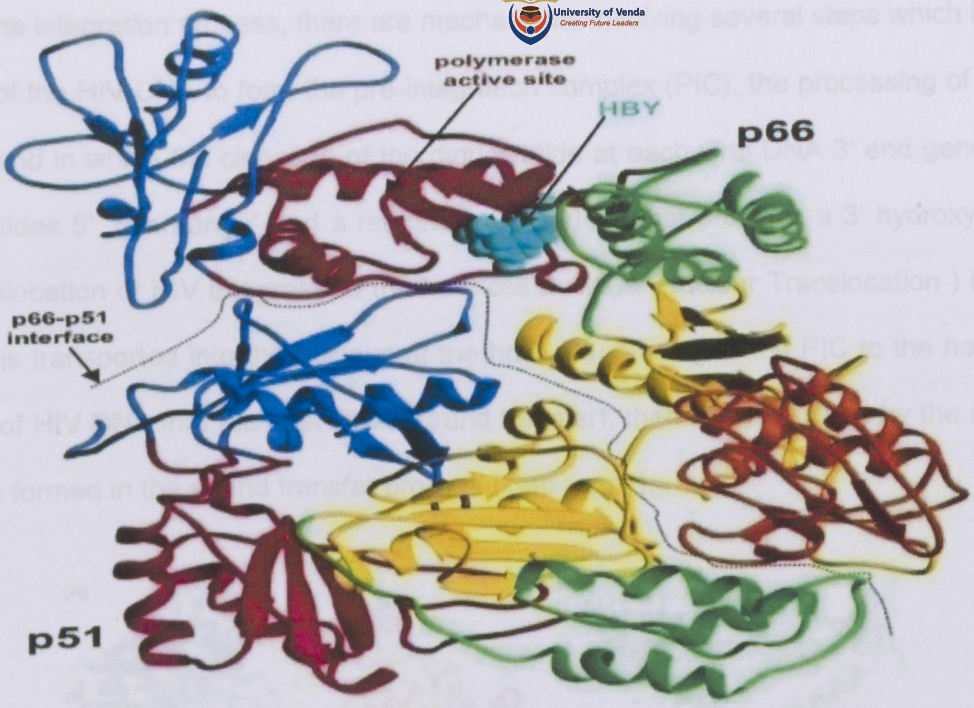


Figure 1.7: The structure of HIV-1 RT with a bound NNRTI. The p66 subunit is on the upper right; the p51subunit is on the lower left. The interface between the subunits is shown by a dotted line. The individual sub domains of the two subunits are color coded: blue, fingers; red, palms; green, thumbs; yellow, connection domains. The RNaseH domain is gold and the NNRTI (HBY) is cyan. The approximate position of the polymerase active site is indicated by an arrow. The figure was prepared from the coordinates of (Hsiou *et.al.*, 1998).

When the viral DNA has been reverse transcribed, it is then incorporated into the host DNA. The integration of the HIV DNA into the host DNA is a crucial step in the HIV life cycle and is catalyzed by the enzyme integrase (IN). This protein which is 288 amino acid in length contains three domains which are Amino (N)-termal domain, the central catalytic domain and the carboxy (C)-termal domain.The HIV integrase can exist in a form of a monomer, dimmer, tetramer and possible even octomer. Each particle has estimated 40-100 copies of the integrase enzyme (Farnet *et.al.*, 1990)

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During the integration process, there are mechanisms involving several steps which includes binding of the HIV DNA to form the pre-integration complex (PIC), the processing of the HIV DNA 3' end in which the cleavage of the dinucleotide at each viral DNA 3' end generates a dinucleotides 5' "overhangs" and a reactive intermediate that contains a 3' hydroxyl group, the translocation of HIV integrase to the host cell nucleus (Nuclear Translocation) in which the PIC is transported into the nucleus of the host cell, binding of the PIC to the host DNA, transfer of HIV DNA into the host DNA (strand transfer), then lastly followed by the repair of the gaps formed in the strand transfer process (Gap repair).

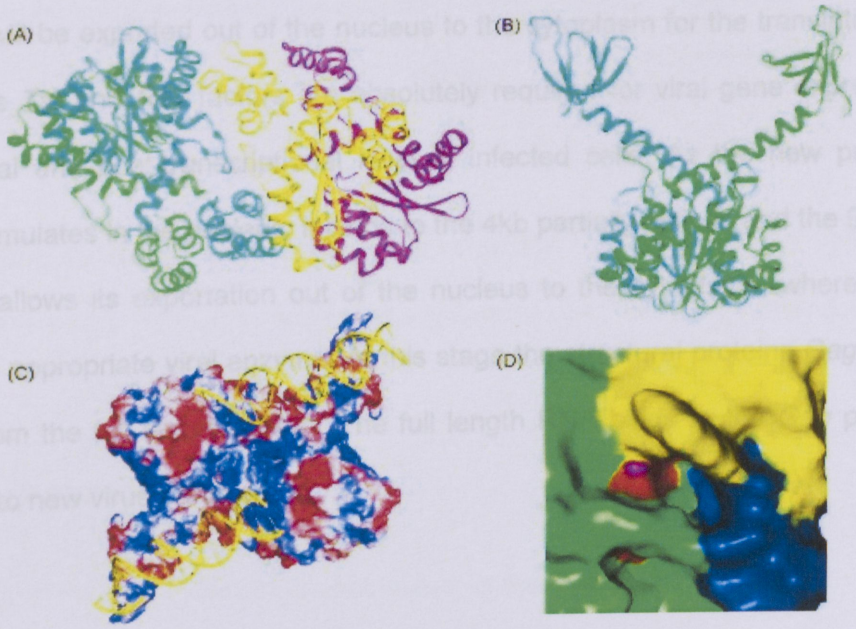


Figure 1.8: Crystal structure of HIV integrase (A and B) and the DNA binding domain.(A) Crystal structure of the NTD and CCD domains of integrase showing a model of an IN tetramer composed of a dimer of integrase dimers. Each individual integrase monomer (composed of a NTD and CCD) is shown in a distinct color. (B) Crystal structure of an integrase dimer composed of the CCD and CTD domains. (C) Hypothetical space filling model of an integrase tetramer in which each monomer contains all three domains of integrase (courtesy of X. Chen). Electrostatic surface potential is also shown (positive in blue; negative in red). A ribbon model of the viral DNA bound to the tetrameric integrase complex (in Trans binding mode) is also shown. The viral DNA ends are coordinated

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together in close proximity. Host chromosomal DNA (not shown) most likely lies in the central groove (D) Close up view a model of an integrase active site showing how an induced hydrophobic pocket is formed upon viral DNA binding (Chen *et.al.*, 2008).

Once the virus is integrated into the host chromosome, the cell will start making new HIV proteins. The proteins are the building blocks for new HIV viruses. The viral proteins are produced as polyproteins. The integrated DNA provirus is transcribed into different types mRNA, which is then spliced into smaller pieces and these smaller pieces are exported from the nucleus into the cytoplasm where they are translated into regular proteins the spliced 2kb mRNA will be exported out of the nucleus to the cytoplasm for the translation of *nef*, *tat* and *rev*. The *Tat* and *Rev* factors are absolutely required for viral gene expression at the transcriptional and post-transcriptional level in infected cells. As the new produced *Rev* protein accumulates in the nucleus, it binds to the 4kb partially spliced and the 9kb unspliced mRNA and allows its exportation out of the nucleus to the cytoplasm, where they will be translated to appropriate viral enzyme. At this stage the structural proteins *Gag* and *Env* are produced from the full length mRNA. The full length RNA binds to the *Gag* protein and is packaged into new virus particles.

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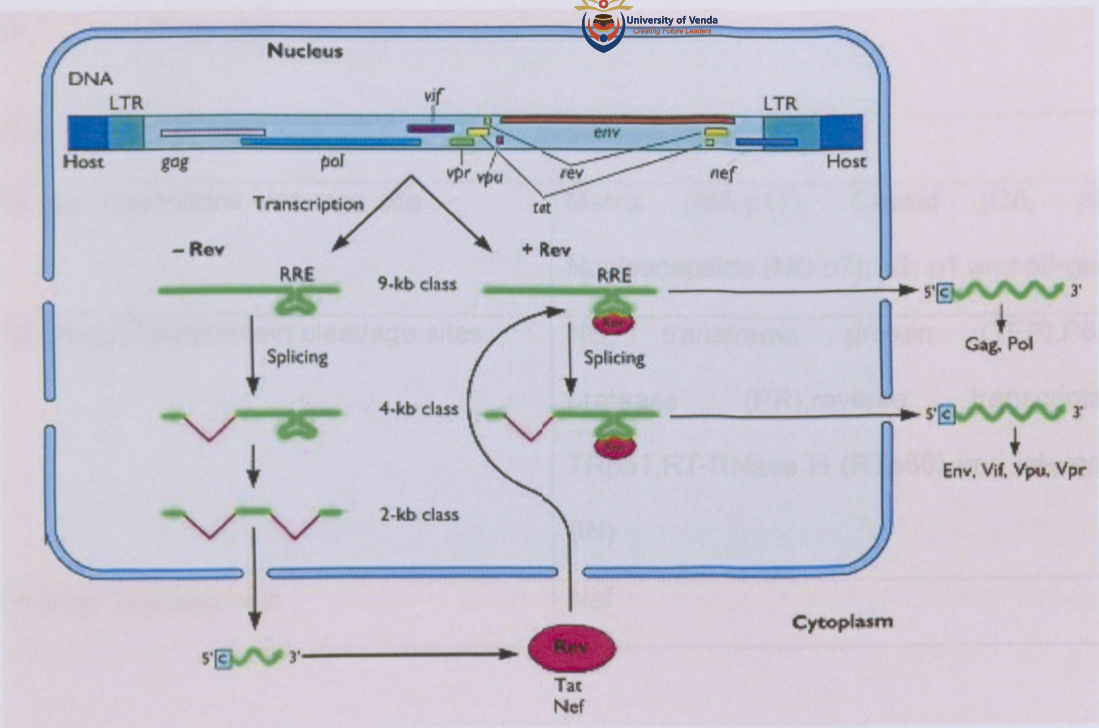


Figure 1.9: The illustration of the exportation of mRNA transcript out of the nucleus to the cytoplasm. (Wenny's viriology webnotes, powered by Wenliang Zhang, last update 14/09/2007).

1.2.5.3 Assembly and release

The final step of the viral cycle is the assembling of the new HIV-1 virons which begins at the plasma membrane of the host cell. The *Env* polyprotein *gp* 160 goes through the endoplasmic reticulum and is transported to the Golgi complex where it is cleaved by the host's protease called furin and processed into two HIV envelope glycoprotein (*gp* 41) and (*gp* 120), then transported into the plasma membrane of the host cell, *gp* 41 anchors the *gp* 120 to the membrane of the infected cell. Maturation occurs either in the forming bud or in the immature virion after it buds from the host cell. During maturation, HIV protease cleaves the polyproteins into individual factional HIV proteins and enzymes. The various structural components then assemble to produce a mature HIV virion (Geldorblom, 1997). The mature virus is then able to infect another cell.

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Table 1.1: Protease cleavage sites and their products

Protease cleavage sites	Viral proteins
Pr55-gag polyproteins cleavage site	Matrix (MA,p17), Capsid (CA, p24), Nucleocapside (NC,p7), p2, p1 and p6-gag
Pr160 gag-pol polyprotein cleavage sites	NC, transframe protein (TFP),P6pol, protease (PR),reverse transcriptase, TRp51,RT-RNase H (RTp66) and intergrase (IN)
Nef protein cleavage site	Nef

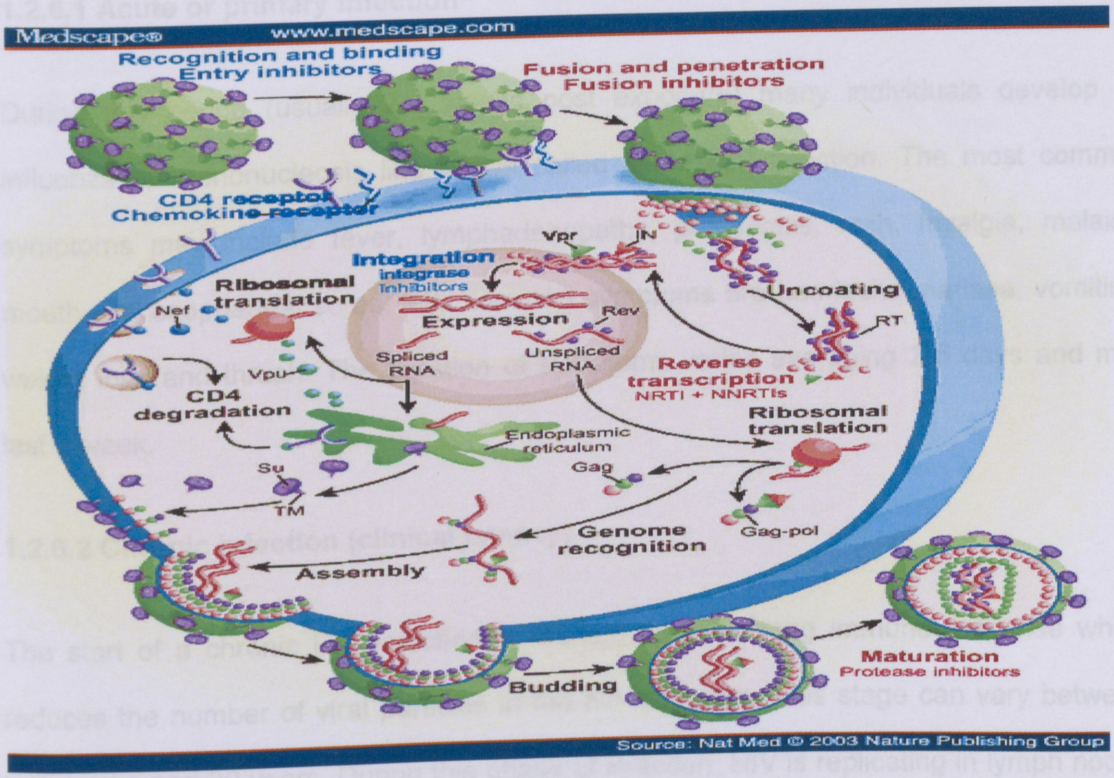


Figure 1.10: Illustration of the life cycle of HIV and the inhibition sites of ARVs (Nat Med © 2003 Nature Publishing Group)

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1.2.6 PATHOGENESIS OF HIV

Upon transmission to a new host, HIV infects vital cells of the human immune system, cells such as the helper T cells $CCR5^{+}CD4^{+}$ effector memory T cells, macrophages and dendritic cells. Depletion of such cells of the immune system leads to low levels of $CD4^{+}$ T cells through mechanisms such as direct viral killing of infected cells, increased rates of apoptosis in infected cells and killing of infected $CD4^{+}$ cells by $CD8^{+}$ cytotoxic lymphocytes that recognize infected cells. When the $CD4^{+}$ T cell number declines below a critical level, cell-mediated immunity is lost, and the body becomes progressively more susceptible to opportunistic infections and ultimately resulting in AIDS (Grossman *et.al.*, 2006).

1.2.6.1 Acute or primary infection

During this period (usually 2-4 weeks post exposure) many individuals develop an influenza or mononucleosis like illness called acute HIV infection. The most common symptoms may include fever, lymphadenopathy, pharyngitis, rash, myalgia, malaise, mouth and esophageal sores, less common symptoms are headache, nausea, vomiting, weight loss and thrush. The duration of symptoms varies averaging 2-5 days and may last a week.

1.2.6.2 Chronic infection (clinical latency)

The start of a chronic HIV infection is marked by the strong immune response which reduces the number of viral particles in the blood stream. This stage can vary between two weeks and 20 years. During this phase of infection, HIV is replicating in lymph nodes which becomes swollen in response to large amount of the virus that becomes trapped in the follicular dendritic cells (FDC) network (Burton *et.al.*, 2002). Tissues that are rich in CD4 T cells may also become infected, and viral particle accumulates both in infected cells and as free virus. Individuals in this phase are still infectious.

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1.2.6.3 Late stage of HIV (AIDS)

When the immune system is weakened and the CD4 T cell number declines below critical levels of 200 cells per µl, cell-mediated immunity is lost and the immune system becomes susceptible to a variety of opportunistic microbes. The symptoms often seen in this phase of infection include drastic weight loss, respiratory tract infection, prostatitis, skin rashes, and oral ulceration. *Pneumocystis jirovecii* is common and often fatal. In the final stages of AIDS, infections with cytomegalovirus or *Mycobacterium avium complex*are more prominent and these infections often lead to death.

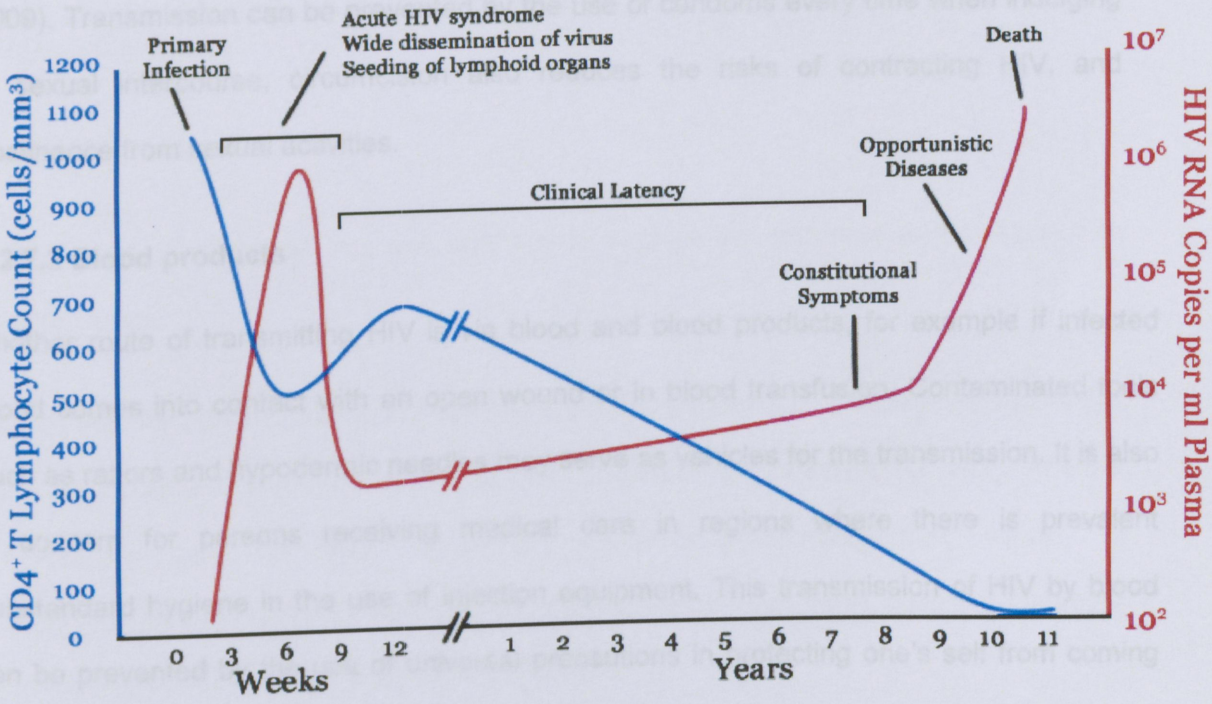


Figure 1.11A general graph representing a relationship between HIV copies (viral load) and CD4 counts over the average course of untreated HIV infection (http://www.emedicine.medscape.com/article/2061054-overview)

CD4+ T cell count (cells per µl) HIV RNA copies per ml of plasma

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Three main transmission routes for HIV have been identified.

1.2.7.1 Sexual transmission

Sexual transmission is the major route of transmitting HIV, and the majority of infections are acquired via unprotected sexual intercourse. This occurs when one infected partner's sexual secretion comes in to contact with the genital, oral, or rectal mucous membrane of the other partner. It is found that in high-income countries, the risk of female-to-male transmission is 0.04% per act and male-to-female transmission is 0.08% per act. For various reasons, these rates are 4 to 10 times higher in low-income countries (Boily *et.al.*, 2009). Transmission can be prevented by the use of condoms every time when indulging in sexual intercourse, circumcision also reduces the risks of contracting HIV, and abstinence from sexual activities.

1.2.7.2 Blood products

Another route of transmitting HIV is via blood and blood products, for example if infected blood comes into contact with an open wound or in blood transfusion. Contaminated tools such as razors and hypodermic needles may serve as vehicles for the transmission. It is also of concern for persons receiving medical care in regions where there is prevalent substandard hygiene in the use of injection equipment. This transmission of HIV by blood can be prevented by the use of universal precautions in protecting one's self from coming into contact with blood (Franceschi *et.al.*, 1995).

1.2.7.3 Mother-to-child transmission

The transmission of the virus from a mother to a child can occur at three different levels: it can occur during pregnancy, at childbirth, and via breast feeding. Transmission risks can be lowered when a combination of antiretroviral drug treatment and caesarian section are

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available. Mother to child transmission via breast feeding can be prevented by complete avoidance of breast feeding. However breast feeding together with the provision of extended antiretroviral prophylaxis to the infant can lower the risks of mother-to-child transmission (Coovadia, 2004).

1.2.8 LABORATORY DIAGNOSIS OF HIV

1.2.8.1 Enzyme linked immunosorbent assay (ELISA) is commonly used in the screening of infectious diseases including HIV. Screening for antibodies against the virus has become increasingly more effective and specific since HIV testing began in the early 1980'S.Its small disadvantage is that it can produce false positives and that is why an HIV diagnostic laboratory must confirm repeated ELISA screen positive results by a confirmatory assay.

1.2.8.2 P24 antigen

They are also EIA-based that use antibody to capture the disrupted p24 antigen from patient serum.

1.2.8.3 Western blot is commonly used as confirmatory test. It is immuno-blot that allows for the visualization of antibodies to the structural polypeptides of HIV. Another confirmatory test for HIV diagnosis is radioimmunoprecipitation assay. In immunoprecipitation assays, radiolabelled viral proteins react with patient's serum to produce radioactive antigen-antibody complexes.

1.2.8.4 Polymerase Chain Reaction (PCR) is a method used in cases where antibody testing may be insufficient to determine whether a patient is infected. It is necessary to perform PCR, a nucleic acid amplification method that allows detection of viral antigens in suspected infected individuals. Polymerase chain reaction is used to measure viral load, an important parameter in treatment monitoring.

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1.2.9 TREATMENT OF HIV

There is currently no cure for HIV/AIDS and the main treatment for HIV is antiretroviral treatment which aims at keeping the amount of HIV in the body at a low level. This stops any weakening of the immune system and allows it to recover from any damage that the virus might have caused.

Classical treatment methods have targeted primarily the reverse transcription step (reverse transcriptase inhibitors), the protease cleavage step (protease inhibitors), and the viral DNA integration step (integrase inhibitors), viral attachment (attachment inhibitors), the co-receptor binding step (co-receptor inhibition), and the fusion step (infusion inhibitors). Current treatment consists of a combination therapy also referred to as highly active antiretroviral therapy (HAART), and HAART has been highly beneficial in reducing the mortality and morbidity due to HIV.

Current HAART options are a combination consisting of at least three drugs belonging to at least two types or classes of antiretroviral agents. The most common drug combination in treatment initiation consists of two NRTIs combined with either an NNRTI or a boosted protease inhibitor. Ritonavir in small doses is the most commonly used booster. An example of the common antiretroviral combination is the two Nucleoside Reverse Transcriptase Inhibitors (NRTIs) zidovudine and lamivudine, combined with the Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) efavirenz.

The decline in new infections and AIDS deaths may be attributed to the scale-up of antiretroviral therapy programmes, especially in the developing countries. The remarkable in access to life-saving ART continues in 2012, fully 1.6 million more people were ART in low and middle-income countries at the end of 2012 compared with the year earlier the largest annual increase ever with the greatest contribution coming from African region

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increased to 9.7 million from 2002-2012. Nevertheless substantial additional efforts are needed to enable 15 million people to access ART in 2015 (UNAIDS 2013).

1.2.10 SOUTH AFRICAN HIV TREATMENT GUIDELINE 2013

In South Africa, standardized eligible criteria for starting ARV treatment for adults and adolescents is when the $CD4^+$ cell count is $\leq 350/mm^3$irrespective of WHO clinical stage, or in patients co-infected with TB/HIV and pregnant women or breast feeding, or patients showing clinical symptoms of AIDS irrespective of the $CD4^+$ count as well as patients with MDR or XDR TB (multidrug resistance or extensively drug resistance tuberculosis), and those in WHO stage 3/4irrespective of CD4 counts.

Patients requiring fast track, in which ART initiation will be within 2 weeks of being eligible are pregnant woman or breast feeding eligible for lifelong ART, patients with very low $CD4^+$ cell count <200, patients at stage 4 with irrespective of CD4 count and patients with MDR/XDR TB with CD4 count <50.

The first line regimen for all new patients needing treatment, including pregnant woman is tenofovir (TDF), lamivudine (3TC) or emtricitabine (FTC) with efavirenz (EFV) or nevirapin (NVP). For TB co-infection patients EFV is preferred, and for woman of child bearing age, not on reliable contraception NVP is preferred.

For patients who are on d4T based regimen with no side effects, they take d4T, 3TC with EFV/NVP. Patients remain on d4T if well tolerated, and an early switch will be required if there is toxicity. High BMI, low Hb, older females, who have a contraindication to TDF and renal disease, they take zidovudine (AZT), lamivudine (3TC) and EFV/NVP.

Second line regimen of patients failing on a d4T or AZT based 1st line regimen is TDF, 3TC/FCT with LPV/r. Those who fail on a TDF based 1st line regimen, take AZT, 3TC and LPV/r.

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Eligibility criteria for starting ART regimen for infants and children, reflecting to all children less than 1 year of age, and also children 1-5 years with clinical stage 3 or 4 or CD4 count is ≤25% or CD4 count is <750 cells/mm^3. Children ≥ 5 years to 15 years with clinical stage 3 or 4 or CD4$^+$ cell count ≤ 350 cells/µl, children less than 1 year of age, in stage 4 and with MDR/XDR TB are required to start ART after 2 weeks of being eligible.

The first line regimen for all infants and children under 3 years is abacavir (ABC), lamavudine (3TC) and liponavir/ritonavir (LPV/r), and children 3 years of age or over are given abacavir (ABC), lamivudine (3TC), efavirenz (EFV). Those currently on d4T based regimen with no side effects continue and substituted when lipodystrophy suspected.

Second line regimen for children above 3 years who have failed ABC, 3TC and EFV based regimen are given AZT, ddI, LPV/r and those who failed first and second line based regimen are referred to specialists (UNAIDS, 2013).

1.2.11 HISTORY OF THE USE OF PLANTS AS MEDICINES

The medicinal use of natural products/compounds derived from plants precedes recorded human history, probably by thousands of years. The knowledge of the medicinal plants before the writing of books was transmitted orally. It is known that the first written text on the use of medicinal plants is about 4000 years old and appears in a clay small board in the Sumerian culture. Palaeoantropological studies at the cave site of Shanidar, located in the Zagros mountains of Kurdistan in Iraq, have suggested that more than 60000 years ago, Neanderlands might have been aware of the medicinal properties of various plants, as evidenced by pollen deposits on one of the graves at the site (Solecki.,1975). Over the ensuring millennia, humankind discovered and made use of an enormous range of natural compounds.

In 1500 B.C, ancient Egyptians wrote the Ebers papyrus, which contains information on over 850 plant medicines, includinggarlic, lumpercannabis, castro bean, aloe, and mandrake

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(Sumner 2000). In India, Ayurveda medicine has used many herbs such as turmeric, possibly as early as 1900 B.C (Sanskrit writing from around 1500 B.C, such as the Rig veda, are some of the earliest available documents detailing knowledge that formed the basis of the Ayuveda system (Sumner, 2000). The Chinese emperor Shen Nung is said to have written the first Chinese herbal, the Pen Tsao. The Pen Trao lists 365 medical plants and their uses including elphedra that introduced drugs that are known and used in modern generation times.

The earliest known Greek herbals were those of Diocles of carystus. Greek and Roman medicinal practices, as preserved in the writings of Hippocrates eg De herbis et Curis and western medicines (Loudon *et.al.*, 2002). Sometime between 50 and 68 A D, a Greek physician known as Padanius Dioscorides wrote *De materia medica*, a compendium of more than 600 plants, 35 animal products and ninety minerals. *De materia medica* remained the authorative reference of herbalism into the 17th century (Collins 2000). Theophrastus wrote historia plantarum in the fourth century BC, which was the first systematization of the botanical world (Grene, 2004).During the early middle ages, Bonedictine Monasteria were the primary source of medicinal knowledge in Europe and England.

Through our evolution, the importance of natural products for medicine and health has been acknowledged by our earliest ancestors used plants to heal and treat diseases as well as injuries, like chewing certain herbs to relieve pain, or wrapped leaves around wounds to improve healing. In the past decade, natural plants took a secondary role in drug discovery, and drugs development. After the advent of molecular biology and combinatorial chemistry, was made possible the rational design of chemical compounds to target specific molecules.

Modern chemistry has ushered in a new era for the study and use of natural products. Analytical and structural chemistry have provided the tools to purify various compounds and to determine their structures which in turn, has given insight into their action in the human body. In 1805, the German pharmacist Friedrich Wihelm Sertumer between the years 1783

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to 1841, isolated morphine from opium, and became both the first pure naturally derived medicine and the first to be commercialized in 1826, and from there purified plants, compounds were preferred in the manufacturing of drugs than crude extracts.

Subsequently a large number of well-known natural compounds were identified, analyzed and synthesized; salicin from *Salix alba* (white willow) see figure 1.12, emetine from *Cephaelis ipecacuanha* (ipecacuanha), strychine and brucine from *Strychnos nux –vomica* (strychnos) quinine from *Cinchona legeriana* (conchna back), colchicines from *Colchicum autmale* (colchicum), caffeine from *Coffea erabica*, nocotina from *Nocotian tabacum* ,atropine from *Atropa belladonna* and cocaine from *Erythroxylum coca*. Many of these compounds are still widely used as drugs.

The structural analysis of natural compounds and the ability to synthesize them allowed chemists to modify them in order to suppress or enhance certain characteristics such as solubility, efficiency or stability in the human body (Newman, 2008) estimates that 60% of the drugs that are now available are either directly or indirectly derived from natural products. Natural products have also been an invaluable source of inspiration for organic chemists to synthesize novel drug candidates (Beghyn *et.al.*,2008,; Koehn & Cater;2005). Below are some of the plants that have a long history of use and of which some drugs used today were derived from.



Figure 1.12: Bark of a willow tree.

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It contains large amounts of salicylic acid, which is the active metabolite of aspirin. Willow bark has been used for millennia as an effective pain reliever and fever reducer (Lichterman *et.al.*,2004)



Figure 1.13: Dandelion (*Taraxacum officinale*).

This plant contains a large number of pharmacologically active compounds, and has been used for centuries as an effective laxative and diuretic, and as a treatment for bile or liver problems (Schutz *et.al.*,2006)



Figure 1.14: Bark of the Cinchona tree.

This bark contains quinine, which is now a widely prescribed treatment for malaria (http://www.rain-tree.com/quinine.ttm).

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Several studies have been conducted and some are ongoing and have demonstrated the inhibitory properties of a variety of crude plant extracts as well as characterized phytomolecules against different stages of the virus life cycle. Some of these studies focused their quest on ethnobotanical survey of plants used to treat various forms of diseases. There are a few plant derived compounds such as Papavarine, Glycorrhizin and Trichosanthin which have been found to have promise and have been evaluated in AIDS patients. These developments have shown that useful anti-HIV agents could be obtained from plant sources (Vlietinck *et.al.*, 1998, De clereq 2000, Kong *et.al.*, 2003).

Studies have also indicated the potential of local medicinal plants for anti-HIV activity. Such studies include the one done by Asres and Colleagues (Asres *et.al* 2001). They investigated the polar and non-polar extracts from 21 Ethiopian medicinal plants comprising of 14 plant families on HIV-1 and HIV-2 replication. They found that the acetone fraction of indice 6.4 of the leaves of *Combretum Paniculatum* and the methanol fraction of indice 4.9 of the leaves of *Dodonaea Anqustifolia* strongly inhibited the HIV-1 replication. These extracts also prevented the virus induced cytopathic effect by almost 100% using MT-4 cells as targets. And the activity against HIV-2 was greatly achieved by the acetone extract of *C.paniculatum* (EC_{50}= 3µg/ml) and a selective index of 32 was noted for this extract.

Another study done was on Sudanese medicinal plants, in which forty-eight methanol and aqueous extracts showed inhibitory activity on the virus replication, and 19 extracts showed inhibitory effects on HIV-induced cytopathic effects (CPE) on MT-4 cells. In this study plants were screened against protease (PR) by an HPLC assay. The methanol extracts of the bark and pods of *Acacia nolitica*, the leaves of *Euphorbia granulaia* and the stem bark of *Maytenus senegalensis*, together with the aqueous extract of the pods of *A.nilotica* and the stem-bark of *M. Senegalansis* showed considerable inhibitory effects against HIV-1 PR. Extracts from the leaves of *Combretum hartmannianum* used in Sudan in the treatment of

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malaria and other tropical diseases proved to inhibit HIV-1 RT at the concentration of 66µg/ml (Ali *et.al.*,2002).

In central Africa, particularly Cameroon representing the region with the largest diversity of HIV variants, a plant compound known to inhibit HIV has been isolated in the south west of Cameroon. The plant compound includes atropisomeric naphthylisoquinoline alkaloids dimmers, michellamines A, B and C. Michallamine B has been found to inhibit HIV induced cell killing and viral replication in a variety of human cell lines and in human peripheral blood leukocytes as well as monocytes. Michallamine B was also inhibitory on clinical strains of HIV-1 as well as the strains of HIV-2 (Cos *et.al.*, 2002).

In an in vitro investigation of selected Rwandan plants used in the traditional medicines for the treatment of infections even in rheumatoid disease, it has been reported that an aqueous fraction from the leaves of *Tithonia diversifolia* has a strong anti-replicative properties with a high selective index of 461. el-Meklcawy and colleagues in 1995 studied Egyptian plants for the activity against HIV-1, and methanol extracts of the fruits of *Phylanthus emblica* isolated Putranjivain A with a potent inhibitory effects of HIV-1 RT (IC_{50}= 3.9 µM). The mode of action of Putranjivain was seen to be non- competitive with respect to the substrate, but competitive with respect to the template primer.

Laboratory based investigation of South African medicinal plants against HIV have focused on seeking inhibitory properties of water and organic extracts, and extracted isolated compounds targeting the essential enzymes responsible for the virus replication, reverse transcriptase (RT), integrase (IN), and protease (PR). The selection of anti-HIV evaluation has been mainly done by the ethnobotanical survey with the help of the knowledge traditional healers have of plants they use in the treatment of disease and viral related disease.

One of the South African in vitro studies to directly assess the inhibitory effects of medicinal plants on HIV using a radioactive cell free assay was conducted and a strong inhibition of

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(IC_{50}= 7.5 µg/ml) by the methanol extract of the leaves of *Terminalia sericea* (Combretaceae) against the ability of the RT to form a complementary DNA strand was observed.This extract also inhibited the RNase H activity by similar magnitude (IC_{50}= 8.1µg/ml). Another study describes the anti-HIV on the RT by water extract of the leaves of *Lebostomon trigonus* (Boraginaceae) with a 50% inhibitory concentration IC_{50} of 49.0µg/ml. Minimal inhibition was also noted for S*utherlandia frutescens* L.Br (Fabaceae), stem bark and roots of *Peltophorum africanum sond* (Fabaceae) strong inhibited RT functions, and the 3' end processing of the IN (Bessong *et.al.*, 2005).



Figure 1.15: S*utherlandia frutescens*

Current study by Andrea and co-workers investigated the inhibitory properties of a tea infusion prepared from *Artemisia annua*, see figure 1.16, which has been used in the treatment of malaria for the past 10 to 20 years. The results obtained showed that this tea infusion have anti-HIV activity with IC_{50} values as low as 2.0µl/ml (Lubbe *et.al.,* 2012), and now people have started using *Artemisia annua* to treat HIV/AIDS (Willcox *et.al.*, 2011).

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Figure 1.16: *Artemisia annua*

A South Africa deciduous tree *Peltophorum africanum* has been long used by traditional healers in the treatment of diarrhoea, dysentery, sore throat, wounds, venereal disease, infertility and also HIV/AIDS. From this tree, lead compounds butanol and ethyl acetate have been isolated from the stem bark and screened for the inhibitory activities against HIV-1. This was carried out using MAGI CCR5^{+} cells, which were derived from Hela cervical cancer cells and express CD4, a chemokine receptor CCR5 and HIV-LTR β-galactosidase. The plant extracts of the stem bark of *Peltophorum africacum* that were extracted using butanol and ethyl acetate and butanol showed inhibitory activity against HIV-1 CXCR4 (X4) and CCR5 (R5) tropic viruses (Theo *et.al.*,2009).

Anti-HIV properties of selected plants that are used traditionally in the treatment of sexually transmitted diseases were also investigated. The extracts of the plants were evaluated for the inhibition against α-glucohydrolase, reverse transcriptase (RT) and viral protein (NF-$_{KB}$and Tat). *Terminalia sericea* extracts IC_{50}= 92mg/ml inhibited considerable α-glucosidace inhibition which was better than acarbose (IC_{50}= 131mg/ml) under the assay condition and in the RT assay, *T.sericea* also showed good inhibitory activity (IC_{50}= 43mg/ml) which was higher than that of the reference drug Adriamycin (IC_{50}=100mg/ml). The ethyl acetate extracts of *Elaeodendron transvaalense* exhibited the most potent inhibitory activity in both the NF-$_{KB}$ and Tat assays with inhibitory activity of 76% and 75% respectively, at a

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concentration of 15mg/ml. The acetone and chloroform extracts of *E.transvealense* and *Zanthoxylum davyi* again showed good activity in the NF-$_{KB}$ and Tat assays (Tshitalange *et.al.*,2008).

The Phyllanthus species have been in the medicinal folk in many countries as antimicrobials and or antioxidants. Extracts of *Sesen phyllanthus Sp* has been screened for the activity of anti-HIV reverse transcriptase (Eldeen *et.al.*, 2010). It was observed that a strong inhibition of HIV-RT was obtained by the*Phallunthus pulcher* (IC_{50}= 5,9µg/ml) followed by *P. urinaria* and *P. pyrtifolius* (IC_{50} of 10.4 and 12.7µg/ml respectively). These results supported the reported traditional uses of Phyllantius Sp and this work contributes positively to the efforts towards scientific evaluation of the use of Phyllanthus species in traditional medicines. Sutherlandia extracts were found to contain inhibitory compounds active against HIV target enzymes, while aqueous Lobostemon leaf extract contain a potent HIV-1 RT inhibitors .

Anti-HIV activity of five selected South African medicinal plants extracts, *Bulbine alooides* (L.) Wild.(Asphodelaceae),*Crinum macowani* Baker (Amaryllidacaee).*Hypoxis sobolfera* (Jacq) Nel (Hypoxidaceae), *Leonitis leonurus* (L.) R.Br. (*Lamiaceae* and *Tulbaghia violacea verv* (Liliaceae) used for the treatment of various ailments were also investigated. The aqueous and ethanol extracts of these plants were tested for inhibition in HIV infected CEM.NK^{R}-CCR5 cells, and against HIV-1 RT and HIV PR, and the results showed that in CEM.NK^{R}-CCR5 cells, ethanol extracts of *Leonotis leorurus* inhibited HIV-1 significantly (33% reduction in HIV-1 P24 $p< 0.05$). HIV-1 RT inhibition (≤ 50% was observed for extracts of *Bulbine alooides* (aqueous and ethanol extracts), *Hypoocis sobolifera* (aqueous and ethanol and *Leonotis leanurus* (aqueous). HIV-1 PR inhibition was observed for aqueous extracts of *Hypoxis sobolifera*, *Bulbine alooides* aqueous and ethanol and *Leonotis leaonurus* ethanol. Only ethanolic extracts of *Builbine alooides* and *Leonotis leanurus* retained HIV-1 PR inhibition in IC_{50} of 94µg/ml and 120µg/ml, respectively (Klos *et.al.*, 2009).

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Study surveys on medicinal plants used by traditional healers in the treatment and management of HIV/AIDS are of great importance for collaboration with biomedical scientists in the identification of candidate plants that may contain novel anti-HIV agents. A study done in Namibia presented a five-step contextual model of initiating collaboration with Namibian traditional healers for anti-HIV agents to be indentified (Chinsembu 2009). It is imperative that traditional medicines used to treat HIV/AIDS opportunistic infection are subjected to scientific validation.

In a literature survey by Chinsembu and Hedimbi, 55 plant families containing 95 plant species and other natural products were found to contain anti-HIV active compounds that included diterpenes, triterpenes, biflavonoids, coumarins, caffeic acid tetramers, hypericin, gallotannins, galloylquinic acids, curcumins, michellamines, and limonoids. These active compounds inhibited various steps in the HIV life cycle. However, further studies are needed to determine their interactions with current regimes of antiretroviral drugs (Chinsembu *et.al.*, 2010)

2.1 STUDY RATIONALE

Current available therapeutics against HIV/AIDS markedly reduce the mortality and morbidity rates. Antiretroviral drugs target steps of the virus life cycle, such as the action of the reverse transcriptase, protease, integrase, viral attachment and fusion.

Thus, while acknowledging that current antiretroviral drugs are vitally important in improving the quality and prolonging the lives of HIV/AIDS patients, these drugs still have many disadvantages. These include drug resistance, toxicity, limited availability, high cost and lack of a curative effect.

The shortcoming of conventional ART continues to open new avenues in the use of ethnomedicinal plants and other natural products for the management of HIV/AIDS. Plants and other natural products present a large repertoire for the potential isolation and identification of novel anti-HIV active compounds. Several studies to demonstrate the effects

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of plant and natural products against HIV/AIDS have been conducted and some are still ongoing.

The current generation of pseudovirus-based assays has several advantages over traditional replicating virus systems in peripheral blood mononuclear cell (PBMCs) culture. Firstly; pseudoviruses are replication-incompetent and can be safely handled outside an expensive biosafety level 3 laboratory. Secondly; the generation of a new pseudovirus stock simply requires transfection of a suitable mammalian cell line such as the HEK 293T cell. Third, neutralization or entry inhibitor assay can be performed in a continuous cell line, thus alleviating the requirement of primary donor cells. These elements together ensure that pseudovirus-based assays are precise, reproducible and easy to standardize.

This study aimed at screening a herbal preparation known as Pheko used in the treatment of HIV/AIDS in South Africa. Data obtained in this study will provide fundamental clues to potential benefits in the use of the preparation. The combined use of enzymatic and cell based assays will provide robust data to this effect.

2.2 GENERAL OBJECTIVE

The general objective of the study wasto evaluate the anti HIV-1 replication properties of Pheko, a herbal preparation, using enzymatic and cell based assays.

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2.3THE SPECIFIC OBJECTIVESWERE:

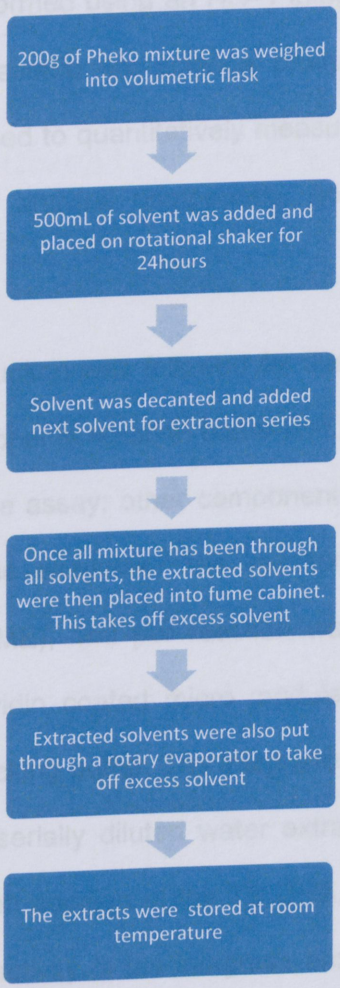
1) To screen the water and methanol extracts of Pheko for anti -reverse transcriptase and integrase activity using recombinant enzymes.
2) To screen water and methanol extracts of Pheko for anti -protease and anti - reverse transcriptase activities in an HIV-1 psueodovirus assay.

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2.1 AVAILABILITY OF HERBAL PREPARATION (PHEKO)

Pheko is a herbal preparation of a mixture of five different plants. The mixture was obtained from the indigenous knowledge system laboratory MRC in Cape Town. It was extracted using the following solvents; Dichloromethane, Ethyl acetate, Methanol, Hexane as well as the water.

2.1.2 Extraction method for Pheko mixture



Solvent series:

1. Hexane
2. DCM
3. Methanol
4. Ethyl Acetate
5. Water

Figure 2.1: Extraction method of Pheko mixture.

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2.2 SCREENING OF PHEKO IN CELL FREE ASSAYS

2.2.1 Reconstitution of Pheko extracts for testing

Aqueous and non-polar Pheko extracts were prepared by weighing 0.1g and completely dissolved in autoclaved distilled water and filtered in syringe filter macro membrane, 87 psi max1/tray 50/case non-pyrogenic sterile with 26mm sterile filters with 12ml Norm Ject syringe. Stock concentration 0.1mg/ml was prepared and serial dilutions in reaction buffer ranging from 1mg/ml to 0.06mg/ml were prepared for the assay.

2.2.2 Integrase assay

The assay was performed using an HIV-1 integrase assay kit, (Version 3.0 cat number EZ-1700) from XpressBio life science products, MD, USA. This assay is a non-radioactive assay used to quantitatively measure the integrase activity, the effect of the interacting proteins, antiviral compounds, and test substances on HIV-1 integrase activity.

Manufacturer's instruction was followed for this assay and performed in qua-duplicate. Briefly, the kit components reaction buffer and blocking buffer were prewarmed at 37ºC for 10 min before the assay; other components were prewarmed at room temperature except the integrase enzymes. The reaction buffer was activated with 14.5M β-mercaptoethanol (BME), 1ml per reaction was activated with 0.4µl BME pro to the assay. The streptavidin coated micro modules were coated with the DS DNA then blocked with the blocking buffer. The integrase was loaded on the DS DNA followed by the addition of the serially diluted water extract of Pheko from the range of 1mg/ml to0.06mg/ml and then addition of the TS DNA. The enzymatic assay was then carried out to evaluate the activity of the integrase enzyme and plates were read at 490nM in microtiter plate reader. A graph of the activity of the enzyme against concentration was plotted. Azide was used as a pocontrol in this assay.

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2.2.3 HIV-1 Reverse transcriptase assay

Plant extracts for the RT assay were prepared as follows;

A stock solution of 0.1g/ml of water extract of Pheko prepared in 2.2.1 was used for this assay and serially diluted from 25mg/ml to 0.003mg/ml. And for the non- polar solvents Pheko extracts; ethyl acetate, methanol and dichloromethane, were prepared by weighed 0.1g of each extract and dissolved in 1ml 1 DMSO and filtered using syringe filter macro membrane, 87 psi max1/tray 50/case non-pyrogenic sterile with 26mm sterile filters with 12ml Norm Ject syringe to prepare a stock concentration of 0.1g/ml each extract. And from the stock 1mg/ml was prepared and serially diluted to a concentration of 0.003mg/ml in lysis buffer.

The assay was performed using a Reverse transcriptase-assay colorimetric kit (Version 13.0, Cat.No. 11468120910) from Roche Diagnostics GmbH (Mannheim, Germany). The manufacturer's instruction was followed in performing the assay and was done in duplicates. Briefly, the entire kit component were removed from the refrigerator and thawed in ice. A 96 well plate was prepared and added to each well 20µl of 4ng recombinant HIV-1 RT diluted in lysis buffer and 20µl of the reaction mixture containing a template-primer hybrid and 10M dUTP/dTTP as well as 20µl of Pheko extracts from a concentration of 25mg/ml to 0.003mg/ml in duplicate were added to the wells. AZT from a concentration of 1µM to 0.0625µM was used as a control and incubated for an hour 37ºC

After an hour incubation appropriate MP modules were prepared for the enzymatic activity evaluation, 60µl of the reaction mixture was then transferred into the MP modules coated with streptavidin, covered with foil and incubated for an hour at 37ºC. The incubation was followed by the addition of 200µl anti-digoxigenin-peroxide solution then incubated again for another hour. 200µl ABTS substrate was added and incubated at room temperature for 20 minutes until an intense green color developed. The absorbance of each well was recorded

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at 405 nm with the reference wavelength at 4[illegible]. A graph illustrating the activity of the RT against the range concentration of different extract was plotted.

2.3 SCREENING OF PHEKO IN CELL BASED ASSAY.

2.3.1 Cytotoxicity of Pheko

Cytotoxicity of Pheko was evaluated in U.87.CD4/CCR5 cells with HIV receptor CCR5. Another cell line used was Vero T35 cell line from monkey kidney. The cytotoxicity was performed using two different assays, which are; LDH cytotoxicity assay kit (Promega, Madison, WI, USA) and MTT assay (Sigma). In preparation for the assays the cell lines were prepared as follows:

2.3.1.1 Propagation of the U87.CD4.CCR5/ Vero T35 cells

To create a cell line suitable for the evaluation of the anti-HIV activity of potential CCR5 antagonist U87.CD4 cell line were used as a starting point, and was transfected with chemokine receptors to produce the U87.CD4.CCR5 cell line which is highly used in HIV research.

The Vero cell line was initiated from kidney of a normal adult African green monkey, this cell line is mostly used in research for evaluation of cytotoxicity of medicinal plants. The cell lines were obtained from the National Institute of Health (NIH, AIDS Reagent Program).

They were propagated in complete 500ml DMEM medium, supplemented with 50ml of FBS, 56µl pyromycin, 3.36ml of G418 and 5ml of PEN/STREP. Cells were thawed in a water bath at 37^{0}C until 90% thawed. In a drop wise fashion media was added to vial then removed from vial and placed in a 15ml tube and brought up to 10ml with complete medium and spun at 1200rpm for 10min. The supernatant was removed and added 10ml media to the tube and mixed so no clumps are formed and then added to 10cm

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plates. The plates were incubated at 37ºC humidified environment with 5% CO_2 and monitored every day until 80% confluence.

2.3.1.2 Seeding of cells for the assays

When cells were about 80% confluent, plates were removed from the incubator washed two times with PBS (-Mg, -Ca) then trypsinised with 0.05% trypsin to detach the cells on the bottom of the plate. Cells were resuspended with 3ml media and mixed, 50µl of the cell suspension was mixed with 50µl of tryptan blue stain and cells were counted using a hemacytometer. And 20 000 cells/ml in 100µl growth media were seeded in two separate 96 well plates for both the assays, and incubated at 37ºC 5% CO_2 for 24 hours.

2.3.1.3 Preparation of extracts for testing

From the 0.1g/ml stock concentrations of each extract prepared, serial dilutions into DMEM media were prepared from 1mg/ml to 0.006mg/ml for the LDH cytotoxicity and for the MMT cytotoxicity assay 2mg/ml to 0.006mg/ml dilutions were prepared. When cells have attached on the bottom of the wells after 24 hours of incubation, the media was then aspirated from the plates and different concentrations of the test substance were then added in duplicate. Wells for controls, one without extracts and the other with percentage DMSO >1 were also included in both the plates with different cell type and the plates were incubated for 48 hours.

2.3.1.4 LDH assay

Lactate dehydrogenase is a stable cytosolic enzyme present in many different types of cells which is an indicator for cellular toxicity. This is a colorimetric assay which measures the number of dead cells by the release of the LDH in the media when cell wall is damaged. It is a biomarker for cellular cytotoxicity and cytolysis. The assay is ideal for high-through output

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screening and provides a safe alternative to radioactive cytotoxicity assay. It is convenient, robust, flexible and non-radioactive. The released LDH can be quantified by a coupled enzymatic reaction. Firstly, LDH catalysis the conversion of lactate to pyruvate via reduction of NAD+ to NADH. Secondly, diaphoresis uses NADH to reduce a tetrazolium salt INT to a red formazan product. Therefore the level of formazan formation is directly proportional to the amount of LDH released in the medium.

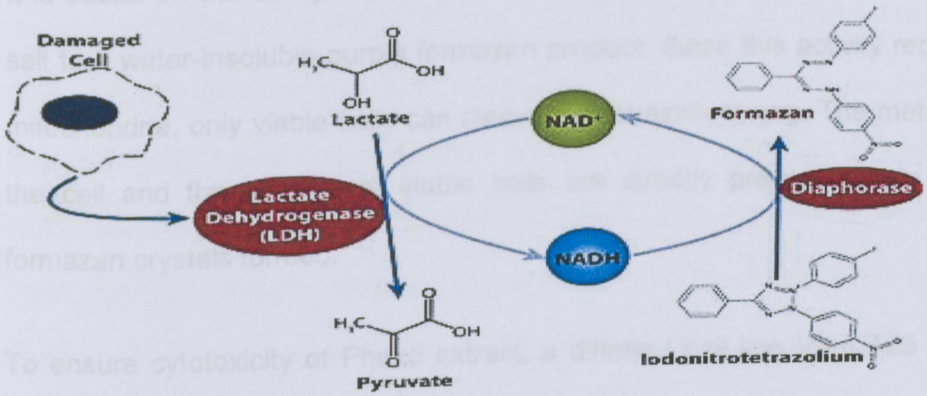


Figure 2.2: Illustration of the LDH assay principle (https://caymanchem.com/pdfs/1008883.pdf)

The plate with U.87.CD4.CCR5 cells were removed from the incubator after the 48 hour incubation and evaluated for any contamination. The plate layout was designed as follows; first row had only the growth media as negative control, followed by untreated wells for standard and lysed wells for positive control, test substances in duplicate and wells with DMSO as assay standard. Then 15µl of lysis buffer was added on the wells designated lysed and the plate was incubated for an hour at 37°C. After the incubation 50µl of the supernatant was transferred to a new assay plate and 50µl of substrate was added in all the wells and incubated at room temperature protected from light for 30 minutes. After the incubation period, the reaction was stopped by the addition of 50µl stop solution and read in a microtiter plate reader at wavelength 450nm.

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2.3.1.5 MTT assay

The MTT (3-[4,5-Dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide) assay is a non-radioactive quantitative colorimetric assay used for in vitro measurement of the cytotoxicity and metabolic activity of cell culture subjected to different culture conditions. It is based on the ability of viable cells to reduce the yellow water soluble tetrazolium salt to a water-insoluble purple formazan product. Since this activity requires functional mitochondria, only viable cells can cleave the tetrazolium ring. The metabolic activity in the cell and the number of viable cells are directly proportional to the amount of formazan crystals formed.

To ensure cytotoxicity of Pheko extract, a different cell line Vero T35 which does not express the HIV receptor was used with the MTT assay. The plate with the Vero T35 cells was removed from the incubator and cell viability was evaluated as follows; 200µl of sterile MTT (0.5mg/ml) was added in all the wells and incubated for 4 hours at 37ºC, and after the incubation, supernatant was aspirated from the wells and 200µl of DMSO was added to dissolve the formazan crystals. The plate was read on a microtiter plate reader at 450nM.

2.3 CHEMICAL PRODUCTION OF COMPETENT DH5α *E. COLI* CELLS.

DH5α is the most frequently used *E. coli* strain for routine cloning application. This strain is non-pathogenic and was developed by D. Hanahan as a cloning strain with multiply mutations that enable high-efficiency transformations. They were produced for the transformation of plasmids for pseudoviral production, and were produced as described in the following procedure;

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2.3.2.1 Starter culture preparation and treatment with calcium chloride

For the production of competent DH5α *E.coli* cells, one colony of commercial DH5α *E. coli* from LB Agar plate without antibiotics was inoculated into 2ml LB medium and incubated overnight at 37^{0}C while shaking at 222rpm to prepare starter culture. After overnight incubation,100ml of LB without antibiotics was inoculated with 1ml saturated overnight culture and incubated at 37^{0}C while shaking vigorously at 250 rpm until OD_{600}= 0.3 (2-3 hours). The culture was chilled on ice for 15 min, as well as 0.1M $Cacl_2$ and 0.1M $CaCl_2$ plus 15% glycerol also on ice. The cells were then transferred into pre-chilled sterile 2ml microcentrifuge tubes and centrifuged at 3000 rpm for 10 min at 4^{0}C to pallet the cells. The supernatant was discarded and cells were placed on ice and re-suspended with 800µl of 0.1M cold $CaCl_2$ solution and kept on ice for 30 min. They were again centrifuged for 10 min at 3000rpm at 4^{0}C to pallet cells. The supernatant was discarded and cells resuspended in 120µl 0.1M cold $CaCl_2$ solution plus 15% glycerol, at that point, cells were placed at 4^{0}C overnight, for this increased competency of cells. Finally cells were stored at -80^{0}C.

2.3.2.2 Evaluation of competency of the produced DH5α *E.coli* cells by transformation of plasmids

The pCMVIndeC*gag-pol*RREIndieC (4199), Env plasmid (HXB 3865) and Luciferase reporter gene plasmid (3683) were transformed to test for the competency of the produced cells and for subsequent experiments.

Description of plasmids.

- **PlasmidCMVgag/polRREIndieC** is a 9.1 kb in size and ampicillin resistant. Its host bacterium is DH5α and archived number is 4199. This plasmid was constructed by Wen at the University of Virginia USA. It is a subtype C plasmid with gag/pol and RRE genes inserted and essential for replication of HIV-1, the CMV acts as a promoter for transcription.

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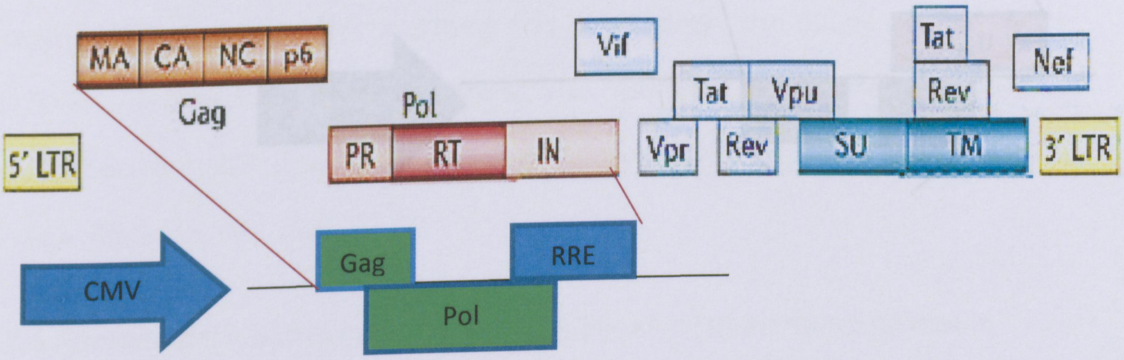


Figure 2.3: A schematic presentation of the genomic organization of HIV-1 showing the location of the plasmid genes.

- **Luciferase plasmid**: Archive number 3683 is a 10kb in size and ampicilin resistant. The host bacterium for this plasmid is BL21, it grows very slowly when transformed in DH5α, and it is also constructed by Wen in the US.

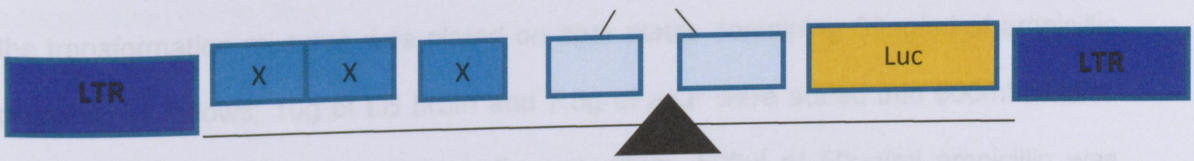


Figure 2.4: A schematic presentation of the luciferase plasmid, with a deletion and insertion of genes. The gag/pol genes were deleted represented by X and the genes inserted are the tat and luciferase genes. The long terminal repeat (LTR) and the gene tat are responsible for gene regulation and expression. The luciferase gene acts as a reporter gene.

- **Envelope plasmid**: It is 4kb in size with an archive number HXB2 3865. This plasmid is also ampicillin resistance and constructed by Wen.

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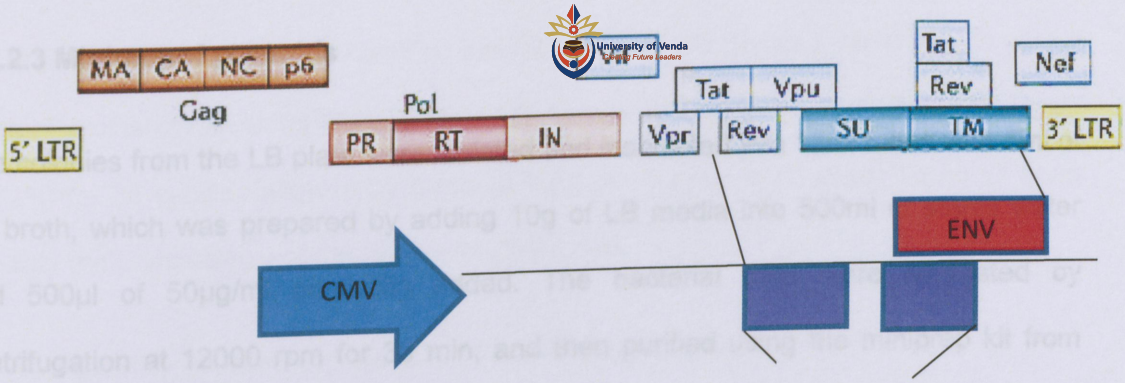


Figure 2.5: A schematic presentation of the genomic organization of HIV-1 showing where the envelop gene is located. The boxes in purple represent the Rev gene which is essential for the exportation of mRNA during transcription.

These plasmids were transformed as follows; 1µl of each plasmid was mixed with 50µl of produced DH5α competent cells in separate 13ml tubes. The mixture was heat shocked at 45^{0}C and 900µl of pre-warmed SOC was added. The mixture was incubated in an orbital shaker at 37^{0}C, shaking at 225 rpm for 45 min. Three hundred µl each of the transformation mixtures was plated on agar plates containing 50µg/ml of ampicillin prepared as follows; 10g of LB broth and 7.5g of agar were added into 500ml distilled water, autoclaved and when the media was warm, 500µl of 50µg/ml ampicillin was added and poured plates. The plates were incubated at 37C overnight.

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2.3.2.3 Miniprepof plasmids

The colonies from the LB plate were isolated and inoculated into 13ml tubes with 5ml of LB broth, which was prepared by adding 10g of LB media into 500ml of sterile water and 500µl of 50µg/ml ampicillin added. The bacterial cells were harvested by centrifugation at 12000 rpm for 30 min, and then purified using the miniprep kit from Promega, Madison, WI, USA.

2.3.2.4 Agarose gel electrophoresis and quantification of miniprep products

The gel was prepared by mixing 0.5g of agarose powder with 50ml of TAE buffer, and dissolved completely in a microwave oven. Stained with ethidium bromide, and allowed to solidify in a tray. Miniprep products and DNA molecular marker were loaded into the wells and electrophoresis performed at a constant voltage of 100V for 40 minutes. The gel was visualized for correct band size under UV transillumination using a gel documentation instrument (Syngene, Frederick, USA).

The miniprep products were quantified using a nanophotometer. One 1µl of eluent (EB buffer) was used as reference, and then 1µl of sample was quantified to get the concentration of the DNA.

2.3.2.5 Insert verification by PCR.

The CMVgag/polRREIndieC plasmid transformed was used as template DNA for the amplification of partial gag/pol using gagP1 and pol2 forward and reverse primers. This was done to draw conclusions on the competency of the cells as well as the effectiveness of DNA transformed by these cells. The reaction was performed in 50µl, with 10X PCR buffer, 0.2µM final concentration of forward and reverse primers, 0.1mM final concentration of dNTps (dCTP, dTTP, dGTP, dATP), 5mM final concentration of $MgCl_2$, 0.04 U/µl final concentration of Taq polymerase and 38.1µl of PCR water. All

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these reagents were added in a PCR tube with 1µl of the plasmid DNA. The following thermal profile: 2 min at 94^{0}C for 1 cycle, 15 sec at 94^{0}C and 1 min at 55^{0}C and 2 min at 72^{0}C for 35 cycles, followed by 5 min at 72^{0}C for 1 cycle were used. This was done to verify the competency of the produced DH5α cells.

2.3.2.6 Agarose gel electrophoresis of amplified products

The gel was prepared by mixing 0.5g of agarose powder with 50ml of TAE buffer, and dissolved completely in a microwave oven. Stained with ethidium bromide, and allowed to solidify in a tray. PCR products and DNA molecular marker was loaded into the wells and electrophoresis performed at a constant voltage of 100V for 40 minutes. The gel was visualized for correct band size under UV transillumination using a gel documentation instrument (Syngene, Frederick, USA).

2.3.3 SUBTYPE C PSEUOVIRUS PRODUCTION

2.3.3.1 Propagation of the 293T cell lines

The Human Embryonic kidney 293T cell (HEK 293T) obtained from the National Institute of Health (NIH) was used in this experiment because they readily take up DNA using various gene transfer vehicles such as the calcium phosphate.

They were propagated into a complete medium supplemented with, 50ml of calf serum or fetal bovine serum (FBS) was added and also 550µl of 50mg/ml gentamycin. Cells are usually frozen in 1ml aliquots, so the ampoule was removed from the nitrogen freezer and quickly thawed by shaking in a 37^{0}C water bath; the outside was wiped with an alcohol soaked tissue. The cells were then aspirated with a pipette and quickly added to 10cm plates that have 9ml of complete medium and left for a while until the cells got established. The 10ml suspension was mixed up and down to gentle mix to avoid clamps. The plates were incubated at 37^{0}C (5% CO2). The cells had attached in

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about 4-8 hours, but were instead incubated overnight. When most cells had attached, plates with cells were removed from the 37ºC (5% CO2) incubator and the media was aspirated off the plates and replaced with fresh medium until the plates were 50-80% confluent for splitting.

2.3.3.2 Preparation of 293T cells for transfection

Plates were removed from the incubator and examined for defects, and these plates with adherent cells were washed with 10ml of DPBS (Dulbecco's phosphate buffered saline). To detach the cells, 1ml of trypsin was added and the plates were incubated at 37^0C (5% CO_2) for 2-5 min. To resuspend cells 6ml of IMDM (Iscove's Modified Dulbecco's Medium) containing 50ml of FBS and 550µl of 50mg/µl was added in the plates, and 9ml of the IMDM was added into 3 clean plates and 1ml of the resuspended cells were added to each plate to a volume of 10ml. The plates were incubated at 37^0C (5% CO_2) overnight.

2.3.3.3 Splitting cells prior to transfection

After overnight incubation, plates were removed from the 37^0C (5% CO_2) incubator and the medium was aspirated from the plates, and the plates were washed twice with 10ml of DPBS (Dulbecco's phosphate buffered saline). To detach the cells, 1ml of trypsin was added and the plates were incubated at 37^0C (5% CO2) for 2-5 minutes. Cells were resuspended in 6ml of IMDM (Iscove's modified Dulbecco's medium) containing 50ml of FBS and 550µl of 50mg/µl. To count the cells, 50µl of the cell suspension and 50µl of the trypan blue was mixed and placed on a hemacytometer and observed under the microscope to count the cells. After the cell count, cell density of $3\times10^6$was seeded in 10cm plated and a density of 1.5×10^6 cells into 6cm plates and incubated until 60% confluent.

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2.3.3.4 Transfection

Transfection was done using the Calcium phosphate transfection kit (Promega, Madison, WI, USA) to transfect DNA into 293T cells and it was done in duplicate in both the 10cm and 6cm plates. Three hour prior to transfection, plates were removed from the incubator and changed the media and plates were taken back to the incubator. Transfection mixture was prepared as shown in table 2.1

Table 2.1: Transfection mixture

For a 10cm dish containing 10ml of media	**For a 6cm dish containing 5ml of media**
To a tube labeled Awas added; 50µl of 2M $CaCl_2$ 222µl of 20µg luciferase DNA 18.7µl of 4199 ((pCMVgag/polRREIndieC) DNA 21.7µg of HXB2 envelope DNA and 237.6µl of sterile water to a volume of 500µl **To a tube labeled B added was**; 500µL of 2X Hepes Buffered Saline (HBS)	**To a tube labeled A was added**; 25µl of 2M $CaCl_2$ 111µl of 20µg luciferase DNA 7µl of 15µg 4199 (pCMVgag/polRREIndieC) DNA 10.9µl of 5µg HXB2 envelope DNA and 118.8µl of sterile water to a volume of 250µl **To a tube labeled B was added**; 250µL of 2X Hepes Buffered Saline (HBS)

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Using 1ml serological pipette, solution A was slowly added dropwise into solution B while bubbling air through solution B with another pipette until solution A was depleted. Fine precipitate was formed and the solution was incubated at room temperature for 30 minutes. The plates were then removed from the incubator and precipitates were added drop wise in either a 10cm or 6cm the plates with media and cells and incubated in a humidified CO_2 incubator.

2.3.3.4.1 Post transfection

After 6 hours of incubation media was aspirated from the 10cm plates and replaced with 10ml fresh media and replaced with 6ml media in the 6cm plates. The plates were incubated for 72 hours at 37°C (5% CO_2) incubator.

2.2.3.4.2 Viral stock harvest

After 72 hour incubation, cells were harvested as follows;

The plates were removed from the incubator and cells were transferred into 15ml tubes and harvested at 1500 rpm for 5 minutes. Supernatant was transferred into new 15ml tubes and transferred on ice to prepare aliquots in 2mlcentrifuged tubes and kept in -80 freezers.

2.2.4 PSEUDOVIRAL SCREENING ASSAY

2.2.4.1 Preparation of U87.CD4.CCR5 cell lines for infection

The cells U87.CD4.CCR5 used to evaluate cytotoxicity were cells used for infectivity assay. They were prepared as follows;

The cells were removed from the incubator and washed with 4ml of PBS per 10cm dish. Cells detached from the dish by adding 2ml of PBS/ 5mM EDTA with 2ml Mg_2SO4 and

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incubated at 37°C for 3-5 minutes, 10ml of RPMI media was added and mixed up and down. The cells were counted and diluted to 10x104 cells in 100µl complete media per well.

2.2.4.2 Anti Reverse transcriptase activity

For the RT assay 96 well plates seeded with the same density of cells in 100µl as in 2.2.4.2 were prepared. Each plate layout was design for testing methanol extract of Pheko in parallel with AZT and NVP as drug controls with a subtype C constructed psueovirus. Subtype B psuedovisuses; 4018 (pNL43, a NRTI's resistant virus), 4012 (pNL43 wild type) and 4073 (NNRTI's resistant virus) Peudoviruses were used as controls for tor the virus. In each well 75µl Dilutions of methanol extracts of Pheko from 0.001µg/µl to 1000µg/µl were added in triplicates as well as dilutions of AZT and NVP from 0.001µM/µl to 1000µM/µl. In each well 25µl of diluted viral stock was added and plates were incubated for 48 hours.

2.2.4.3 Analysis with luciferase assay

After the 48 hours of incubation plates were removed from the incubator and analysed using the luciferase assay (Promega) performed as follows;

The media was completely aspirated from the wells and30µl of 1X passive lysis was added in each well and incubated at room temperature protected from light for 30 minutes. After incubation 100µl of luciferase buffer was added to each well just before plate reading. The plates were read on the luminometer with the Promega luciferase protocol, Injection volume of luciferine was 50µl each well.

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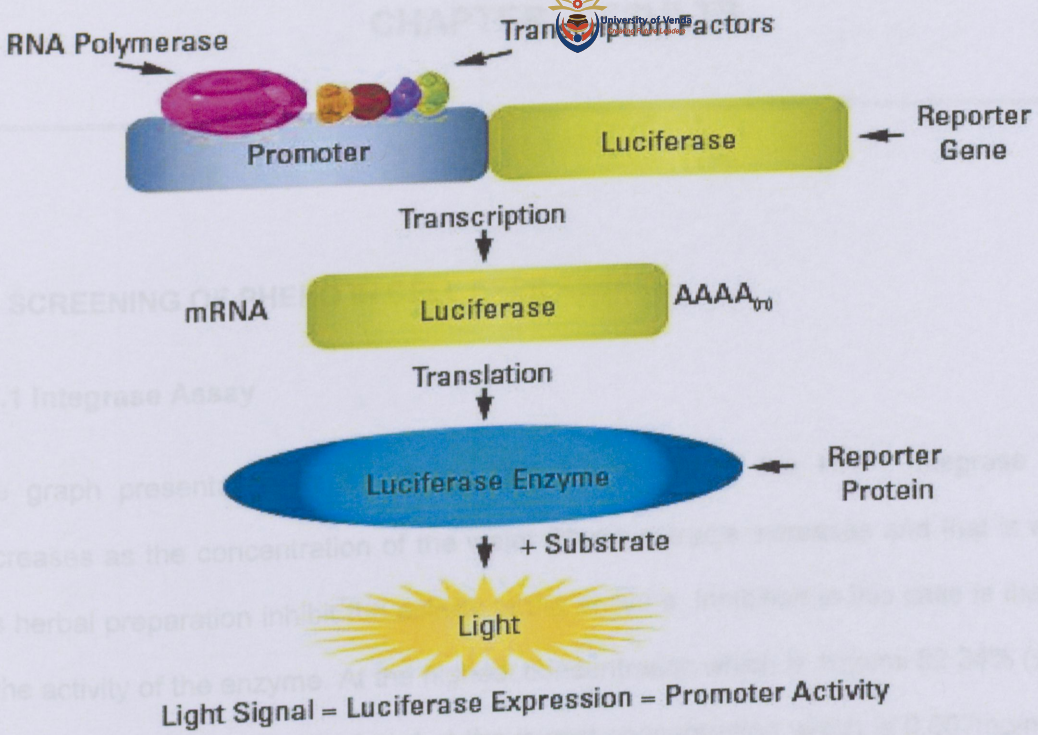


Figure 2.3: Illustration of the luciferase enzyme as a reporter

(http://www.piercenet.com/method/luciferase-perorter)

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3.1 SCREENING OF PHEKO IN CELL FREE ASSAYS

3.1.1 Integrase Assay

The graph presentation in 3.1 shows that the activity of the HIV-1 Integrase enzyme decreases as the concentration of the water Pheko extracts increases and that is evidence this herbal preparation inhibit the activity of the enzyme. Inhibition in this case is the inverse of the activity of the enzyme. At the highest concentration which is 1mg/ml 82.34% (±0.47) of the enzyme activity is inhibited and at the lowest concentration which is 0.007mg/ml 6.82% (±0.09) of the enzyme activity is inhibited, see figure 3.1 .Water extract shows an IC_{50}= 0.125mg/ml. In this assay, Azide was used as positive control and 0.15% concentration of Azide inhibited 54% of integrase as manufacture's protocol indicates that IC_{50} of enzyme is by 0.15% (HIV-1 integrase assay kit, version 3.0, and catalog number EZ-1700).

Table 3.1: Illustration of the IC_{50} and standard deviation of Pheko water extract and Azide.

Pheko extract and control	IC_{50}	SD
Water extract	0.125mg/ml	0.21
Azide	0.15%	0.01

The illustration of the decrease of enzyme activity as the extract concentration increases is seen in the graph below.

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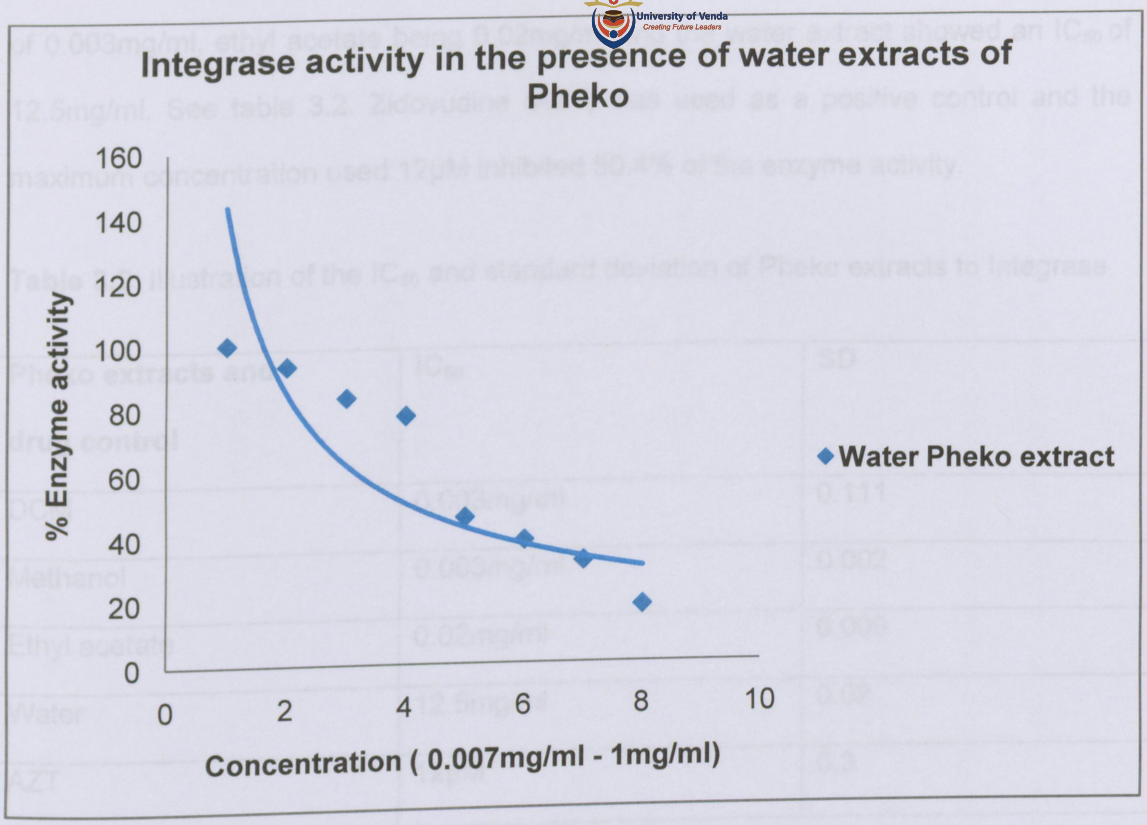


Figure 3.1: Nonlinear fit normalized curve (Macrosoft Excel 2010)) of reverse transcriptase activity in the presence of Pheko water extract. Enzyme activity decreased with increase in substance concentration (dose dependent activity).

3.1.2 Reverse transcriptase assay

To evaluate the activity of RT enzyme in the presence of Pheko as an inhibitor, four types of extracts were used which are; dichloromethane, methanol, water and ethyl acetate. The graph in figure 3.2 demonstrates that the percentage activity of the enzyme decreases as the concentration of extract increase. From the lowest concentration 0.003mg/ml ethyl acetate, methanol and dichloromethane shows inhibition in enzyme activity and at 0.01mg/ml this extract continues to inhibit the activity of the enzyme. Water extracts show no inhibition of enzyme activity from 0.003mg to 6.25mg/ml. From 6.25mg/ml concentration a decrease of enzyme activity was observed. Dichloromethane extract showed IC_{50} of 0.003mg/ml, methanol showed IC_{50}

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of 0.003mg/ml, ethyl acetate being 0.02mg/ml and the water extract showed an IC_{50} of 12.5mg/ml. See table 3.2. Zidovudine (AZT) was used as a positive control and the maximum concentration used 12µM inhibited 50.4% of the enzyme activity.

Table 3.2: Illustration of the IC_{50} and standard deviation of Pheko extracts to Integrase

Pheko extracts and drug control	**IC_{50}**	**SD**
DCM	0.003mg/ml	0.111
Methanol	0.003mg/ml	0.002
Ethyl acetate	0.02mg/ml	0.009
Water	12.5mg/ml	0.02
AZT	12µM	0.3

Water extract of Pheko required higher concentration to inhibit 50% of integrase than the other extracts, with an IC_{50} of 12.5mg/ml. Ethyl acetate showed the least IC_{50} of 0.02mg/ml.

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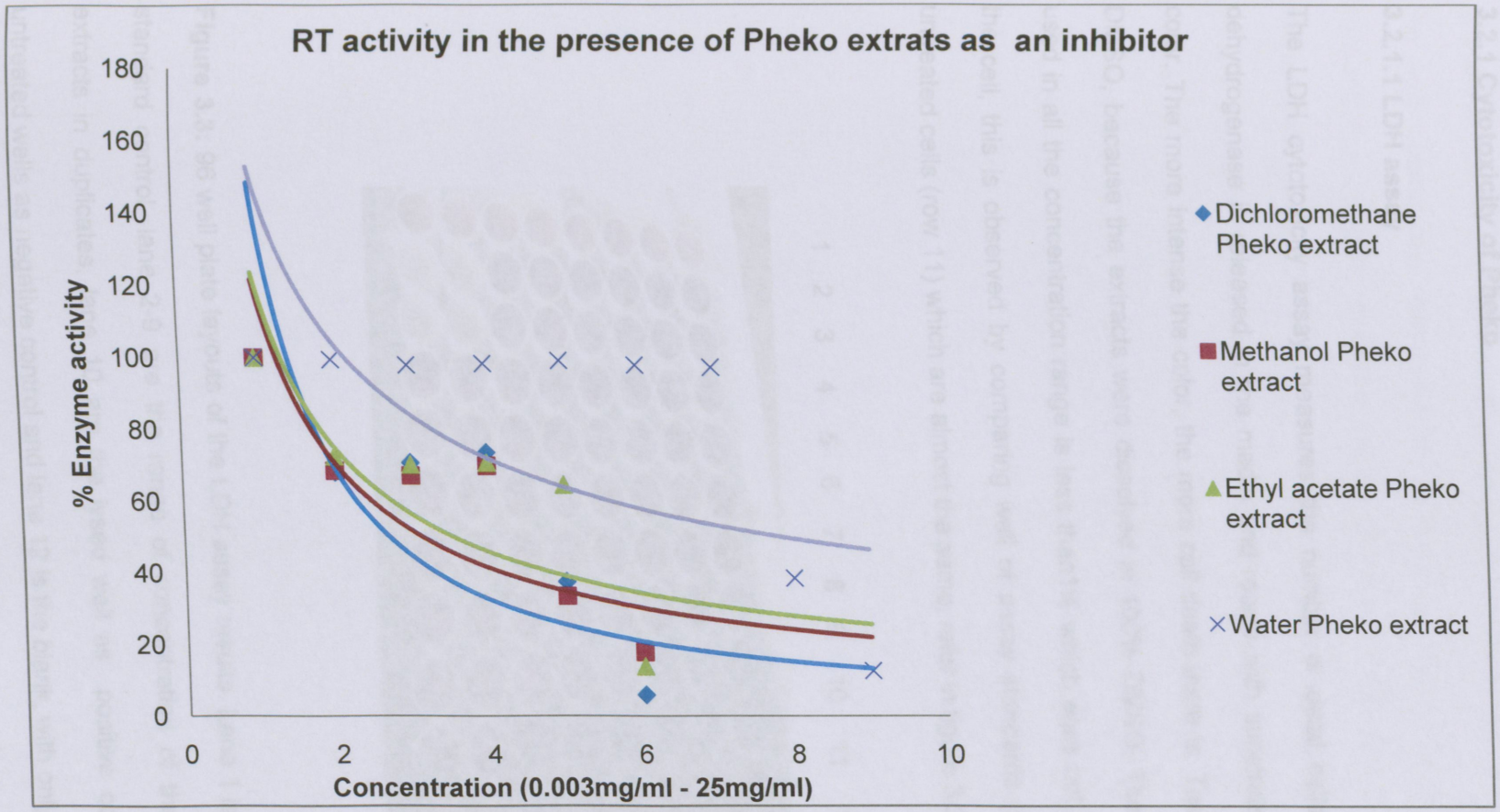


Figure 3.2: Nonlinear fit normalized curve (Microsoft Excel 2010)) of reverse transcriptase activity in the presence of different types of Pheko extracts. Enzyme activity decreased with increase in substance concentration (dose dependent activity). Dichloromethane and methanol extracts were most inhibitory.

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3.2 SCREENING OF PHEKO IN CELL BASED ASSAY

3.2.1 Cytotoxicity of Pheko

3.2.1.1 LDH assay

The LDH cytotoxicity assay measures the number of dead cells when the lactate dehydrogenase is released in the media and reacts with substrate to produce a red color. The more intense the color, the more cell death there is. The assay standard is DMSO, because the extracts were dissolved in 100% DMSO. The amount of DMSO used in all the concentration range is less than1% which does not have any effect on the cell, this is observed by comparing well of assay standards (row 1) and well of untreated cells (row 11) which are almost the same, refer in figure 3.3

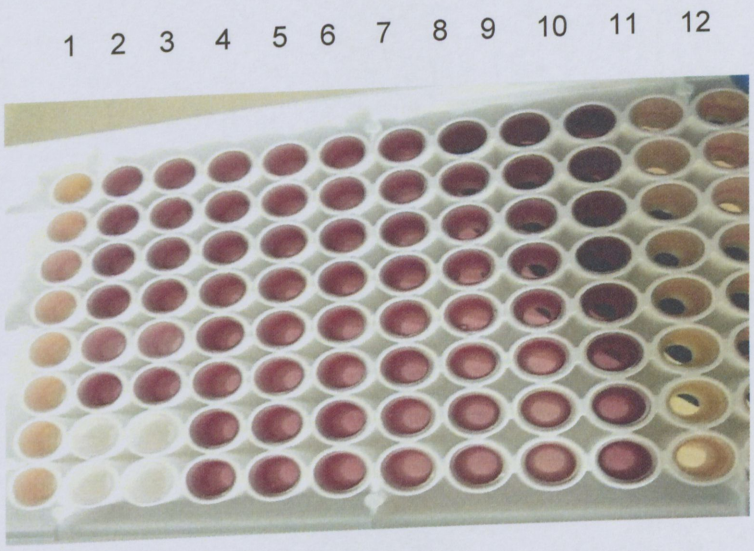


Figure 3.3: 96 well plate layouts of the LDH assay results. Lane 1 is wells of DMSO as standard control, lane 2-9 are the range of concentration of the non-polar Pheko extracts in duplicates, lane 10 are the lysed well as positive control, lane 11 are untreated wells as negative control and lane 12 is the blank, with only growth media.

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The assay was performed in duplicate and the percentage cytotoxicity was calculated as follows; OD (optical density) of sample subtract the OD of untreated divide by the OD of positive control subtract the OD of untreated multiply by 100. As the concentration of the test substances decreased low cytotoxicity was observed. At the highest concentration (1mg/ml) methanol shows 89.3% (± 0.018) cell death than the other extracts with a CC_{50} of 0.5mg/ml, followed by the ethyl acetate with 69.3% (±0.035) and CC_{50} of 0.5mg/ml, dichloromethane with 53.1% (±0.047), CC_{50} of 1mg/ml and hexane with 47.5% (±0.004) and CC_{50} of 1mg/ml. The water extract showed the least cell inhibition with CC_{50} of 1mg/ml. At the lowest extract concentration tested (0.06mg/ml) there was about 20% of cell death for all extracts (Table 3.3).

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Table 3.3: *Cytotoxicity profiles of extracts of Pheko against U87.CD4.CCR5 cell line as determined by the LDH assay*

	EXTRACT TYPE AND CONCENTRATION														
	Ethyl acetate					**Hexane**					**Methanol**				
OD	1mg/ml	0.5mg/ml	0.2mg/ml	0.125mg/ml	0.06mg/ml	1mg/ml	0.5mg/ml	0.2mg/ml	0.125mg/ml	0.06mg/ml	1mg/ml	0.5mg/ml	0.2mg/ml	0.125mg/ml	0.06mg/ml
Mean	0.65	0.552	0.38	0.35	0.33	0.52	0.48	0.4	0.351	0.35	0.771	0.61	0.467	0.365	0.319
SD	0.035	0.069	0.03	0.01	0.004	0.004	0.019	0.007	0.005	0.012	0.018	0.007	0.014	0.041	0.002
% Cell death	**69.3**	**53.15**	**45.1**	**22.5**	**18.11**	**47.46**	**41.52**	**28.07**	**19.95**	**19.82**	**89.29**	**62.64**	**38.79**	**22.29**	**14.7**

	Dichloromethane					**Water**				
OD	1mg/ml	0.5mg/ml	0.2mg/ml	0.125mg/ml	0.06mg/ml	1mg/ml	0.5mg/ml	0.2mg/ml	0.125mg/ml	0.06mg/ml
Mean	0.55	0.45	0.37	0.345	0.325	0.034	0.0195	0.018	0.018	0.015
SD	0.047	0.025	0.025	0.02	0.013	0.0014	0.0007	0.0014	0.001	0.001
% Cell death	**53**	**33.8**	**21.1**	**16.6**	**14.1**	**47.2**	**24.4**	**22.04**	**20.4**	**17.3**

$$\% \text{ of Cell death} = \frac{\text{OD Sample} - \text{OD Untreated}}{\text{OD Control - OD Untreated}} \times 100$$

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Table 3.4: Illustration of the 50% Cytotoxic or lethal dose of U87.CD4.CCR5 cell by Pheko extracts in an LDH assay.

Pheko extracts	**CC_{50}/LD_{50}**
Ethyl acetate	0.5mg/ml
Hexane	>1mg/ml
Methanol	0.2-0.5mg/ml
Dichloromethane	1mg/ml
Water	>1mg/ml

The narrative nonlinear dose response curve plot results showing the percentage of dead cells against a range of concentration of different extracts is shown in figure 3.4.

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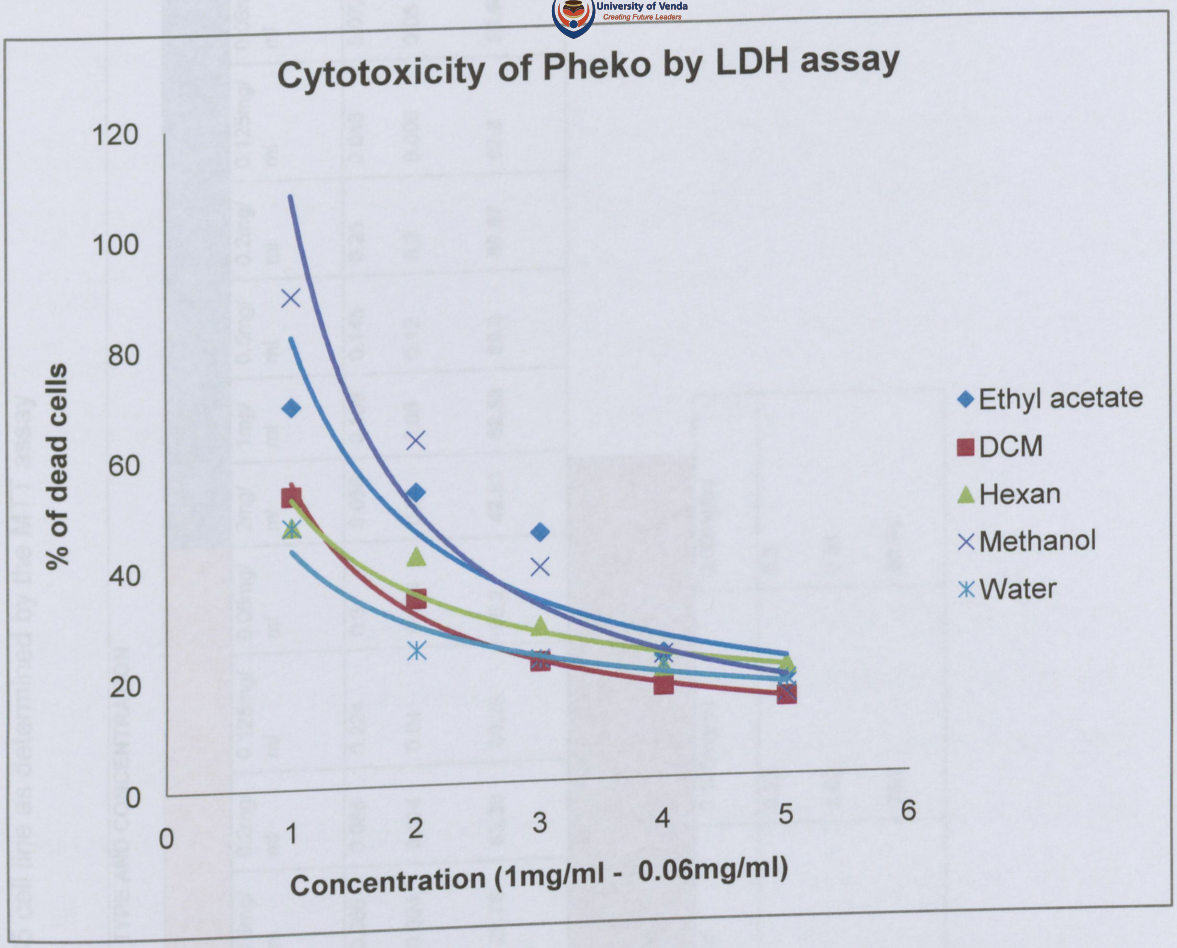


Figure 3.4.: Nonlinear fit normalized curve (Microsoft Excel 2010) of the percentage of dead cells against different extract types of Pheko. The methanol extract was shown to be the most toxic substance to U87.CD4.CCR5 cells with an IC_{50} of 0.5mg/ml.

3.4.1.2 MTT assay

Cytotoxicity of Pheko extracts was ensured by using a different cell line Vero T35 with the MTT assay being the best known method among enzyme based assays for determining mitochondrial dehydrogenase activity in living cells. The percentage of viable cells increases as the concentration decrease. Methanol shows a CC_{50} of 2mg/ml, Ethyl acetate CC_{50} of 0.5mg/ml, Hexane CC_{50} of 0.2mg/ml and dichloromethane CC_{50} of 0.2mg/ml. See table 3.5

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Table 3.5: Cytotoxicity profiles of extracts of Pheko against Vero T35 cell line as determined by the MTT assay

	EXTRACT TYPE AND CONCENTRATION																	
	Ethyl acetate						Hexane						Methanol					
Optical Density	2mg/ml	1mg/ml	0.5mg/ml	0.2mg/ml	0.125mg/ml	0.06mg/ml	2mg/ml	1mg/ml	0.5mg/ml	0.2mg/ml	0.125mg/ml	0.06mg/ml	2mg/ml	1mg/ml	0.5mg/ml	0.2mg/ml	0.125mg/ml	0.06mg/ml
Mean	0.23	0.07	0.13	0.20	0.25	0.028	0.016	0.05	0.066	0.088	0.224	0.3	0.056	0.108	0.145	0.25	0.043	0.072
SD	0.07	0.05	0.05	0.04	0.03	0.04	0.004	0.001	0.004	0.014	0.04	0.004	0.01	0.08	0.12	0.2	0.006	0.05
% Viable cells	**25.44**	**34.5**	**41.4**	**66.02**	**85.2**	**91.2**	**21.44**	**21.66**	**28.76**	**63.38**	**80.25**	**95.2**	**42.83**	**52.53**	**69.8**	**89.87**	**97.8**	**98.96**

	Dichloromethane					
Optical Density	2mg/ml	1mg/ml	0.5mg/ml	0.2mg/ml	0.125mg/ml	0.06mg/ml
Mean	0.015	0.061	0.07	0.09	0.23	0.3
SD	0.004	0.003	0.001	0.01	0.02	0.01
% Viable cells	**18.26**	**20.1**	**24.3**	**32.4**	**76.7**	**98.76**

% Cell Viability = OD sample/ OD control × 100

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Table 3.6: Illustration of the 50% Cytotoxic or lethal dose of U87.CD4.CCR5 cell by Pheko extracts in an MTT assay.

Pheko extracts	**CC_{50}/LD_{50}**
Ethyl acetate	0.5mg/ml
Hexane	>0.2mg/ml
Methanol	2mg/ml
Dichloromethane	>0.2mg/ml

Methanol extract of Pheko was highly toxic to the U87.CD4.CCR5 cells. To show the relationship between the percentage cell viability and concentration,,a graph was plotted using the nonlinear dose response curve plots (Microsoft Excel 2010). The graph represents the cytotoxicity percentage as an inverse of cell viability against the range of extract concentration. The number of viable cells decreased as the concentration increased. At the maximum concentration of 2mg/ml, dichloromethane (DCM) extract inhibits cell viability more than other extracts with 81.7% (±0.004) inhibition, and methanol at the maximum concentration inhibits 57.2% (±0.01).

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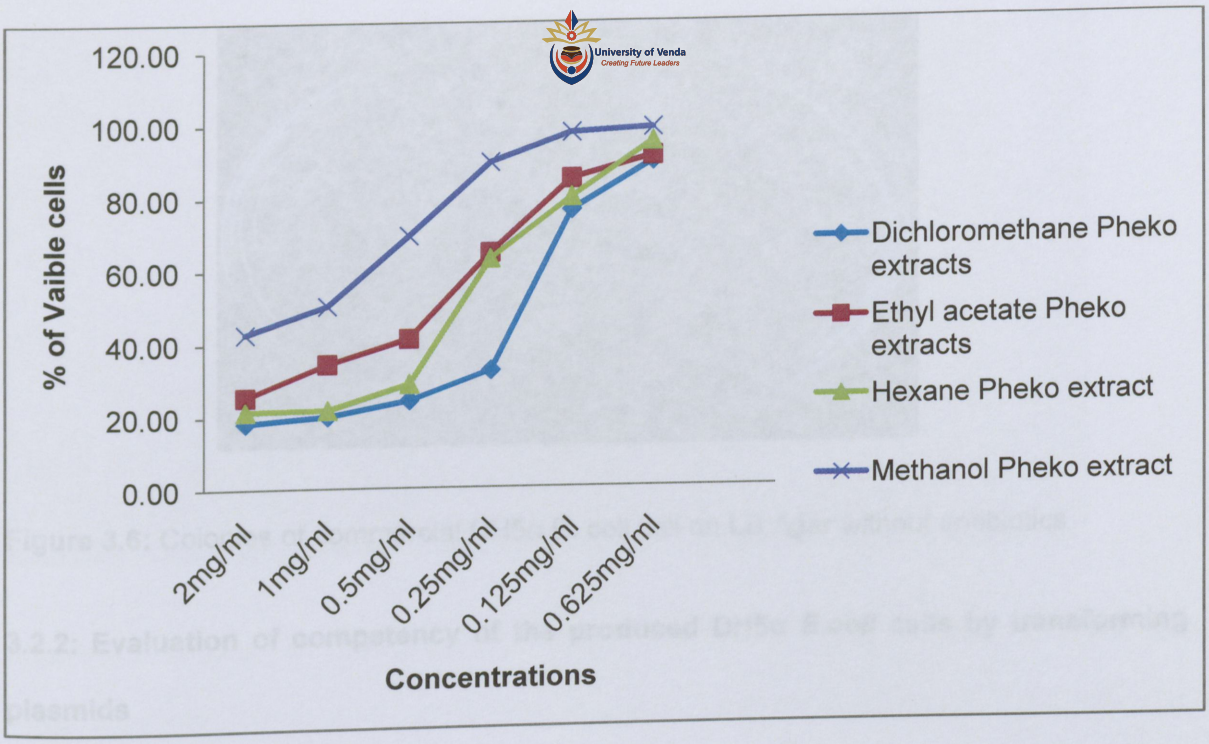


Figure 3.5: Line graph (Microsoft Excel 2010) for percentage of cell viability against different Pheko extracts. The graph presents the cytotoxicity of Pheko on Vero T35 cell line. Cell viability decreases as the concentration increases.

3.2 CHEMICAL PRODUCTION OF DH5α *E.coli* CELLS

3.2.1 Starter culture preparation and treatment with calcium chloride.

When DH5α*E.coli*cells grown on LB Agar without antibiotics grows into distinct colonies see figure3.6. One colony was inoculated into LB broth media to prepare starter culture that was again used to prepare large amount of culture and harvested the cells for treatment with calcium chloride at a lag phase OD_{600} was 0.3.

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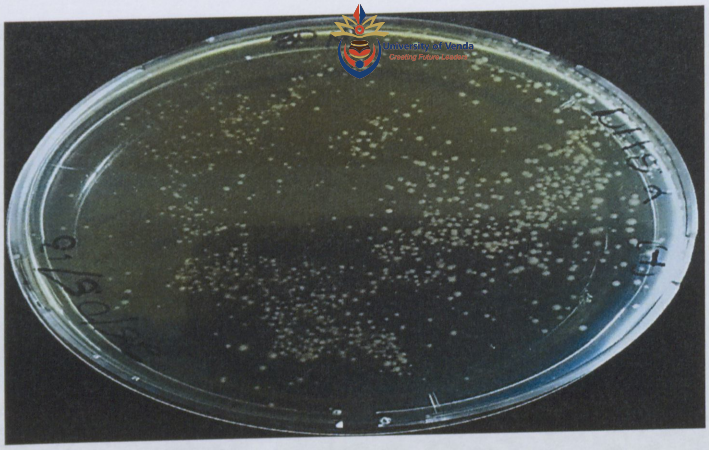


Figure 3.6: Colonies of commercial DH5α E. coli cell on LB Agar without antibiotics

3.2.2: Evaluation of competency of the produced DH5α *E.coli* cells by transforming plasmids

The produced cells were competent because they were able to transform the plasmids CMVgag/polIndieCRRE, Luciferase and envelope and grow into distinct colonies see figure 3.7 below. The transformation efficiency in CFU/µg DNA of these cells was calculated as follows;

$$\frac{\text{Number of colonies} \times (\text{total volume of transformation mixture}) / (\text{Total volume spread})}{\mu\text{g of plasmid used for transformation}}$$

- ✓ **The transformation efficiency calculated was 3.4×10^5 µg/DNA.**

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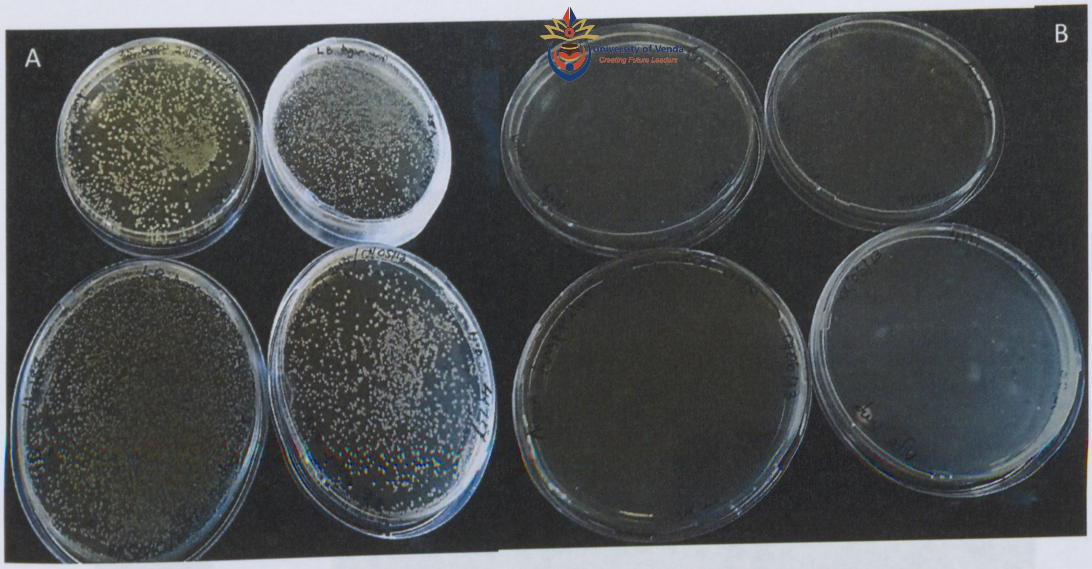


Figure 3.7: Colonies of CMVgag/polRREIndieC (4199), Luc (3863), HXB2 Env (3865) and Vsvg plasmids transformed using the produced competent cells. A represents the transformants and B represents the negative control (untreated cells).

3.2.3 Mniprep of CMVgag/polRRE (4199), Luc (3863) and Env (3865) plasmids

High quality of DNA was obtained from miniprep and quantified using a nanophotometer. Expected sizes of plasmids were assessed in 0.8% agarose gel electrophoresis, see figures below.

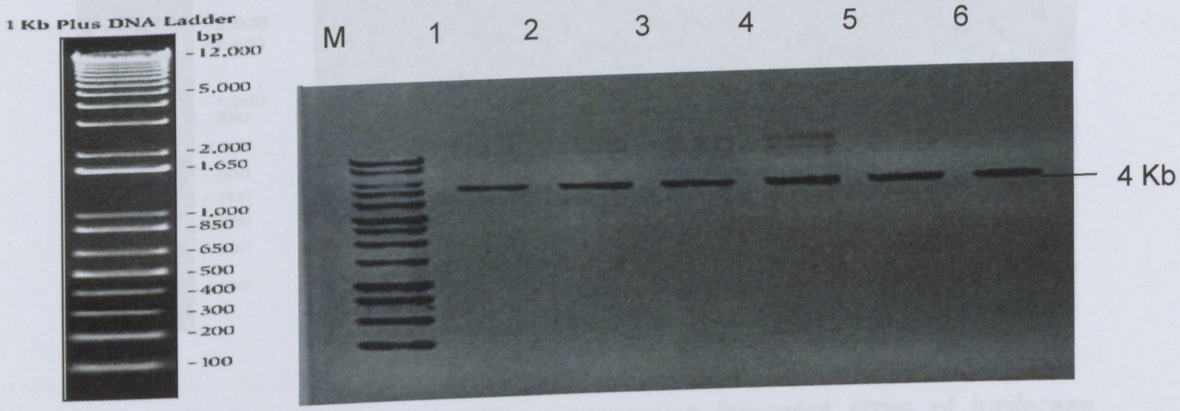


Figure 3.8: Gel electrophoresis profile, representing fragment size of envelope plasmid after miniprep. Lane M= molecular weight marker (1kb plus), lane 1-6 is the expected 4kb envelope size.

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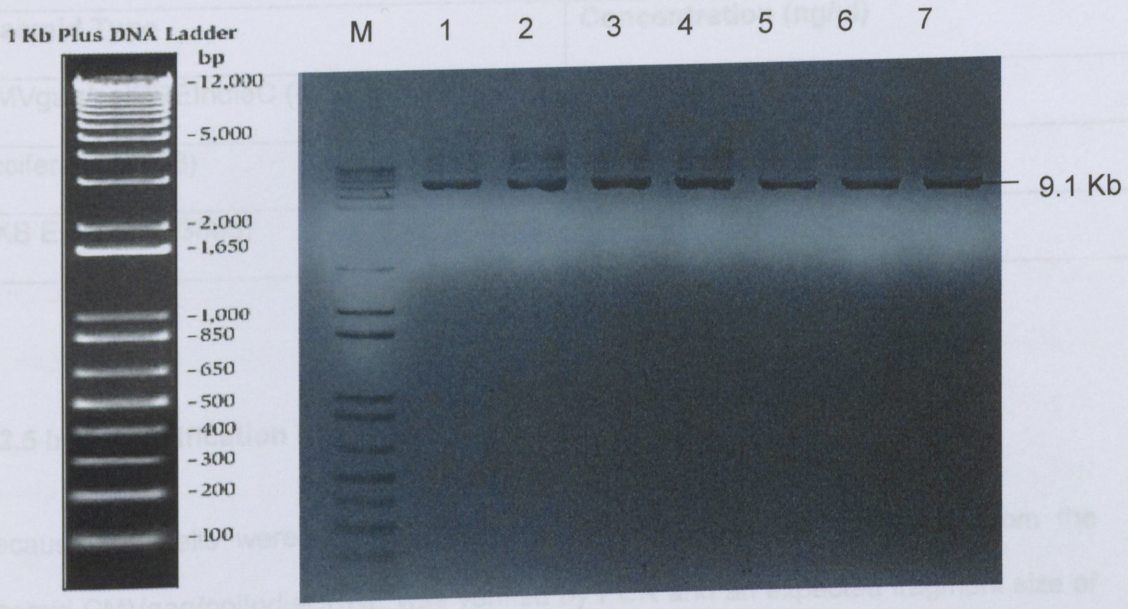


Figure 3.9: Gel electrophoresis profile, representing fragment sizes of plasmid CMVgag/polRREIndieC (4199) after miniprep. Lane M= molecular weight marker (1kb plus), lane 1-6 is the expected 9.1 kb size of the plasmid.

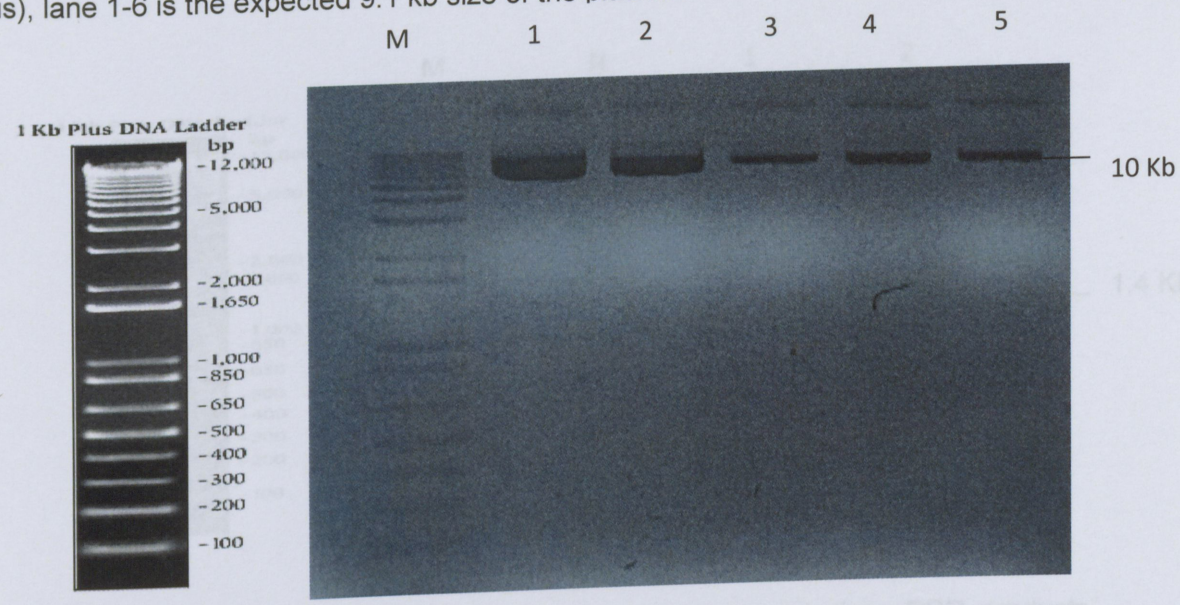


Figure 3.10: Gel electrophoresis profile, representing fragment sizes of luciferase plasmid (3863) after miniprep. Lane M= molecular weight marker (1kb plus), lane 1-5 is the expected 10 kb size of the plasmid.

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Table 3.7: Concentration of plasmids quantified by nanaphotometer

Plasmid Type	Concentration (ng/µl)
CMVgag/polRREIndieC (4199)	0.09
Luciferase (3683)	0.802
HXB Envelope (3685)	0.231

4.2.5 Insert verification by PCR

Because the cells were competent and transformed plasmids, the insert from the plasmid CMVgag/polIndieCRRE was verified by PCR and an expected fragment size of 1.4 Kb partial gag/polwas assessed in 0.8% agarose gel electrophoresis, see figure 4.6 below.

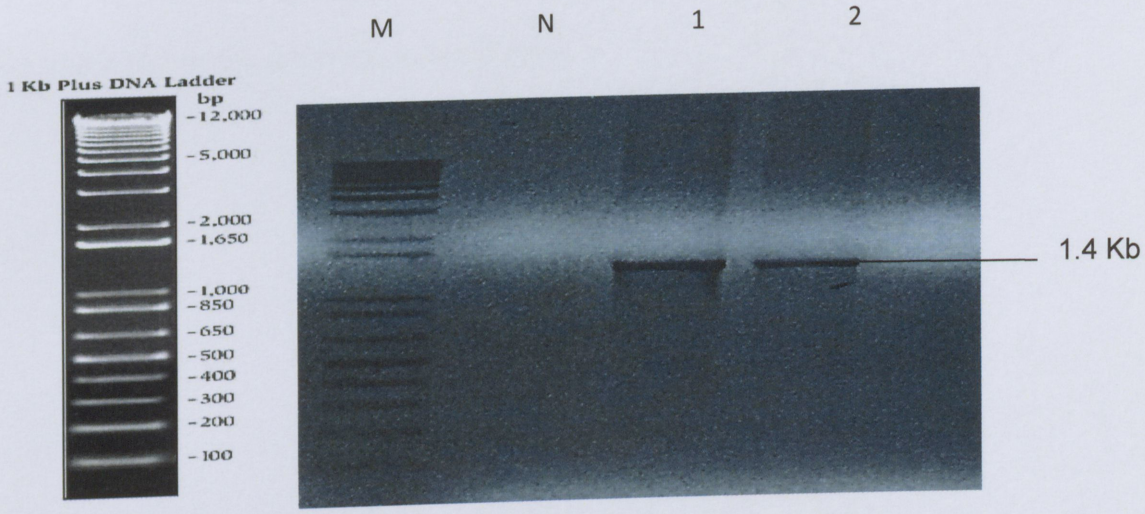


Figure 3.11: Gel electrophoresis profile, representing fragment sizes PCR products after amplification. Lane M= molecular weight marker (1kb plus), N is the negative control lane 1is positive control and lane 2 is 4199 used as the template.

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3.2 ANTI REVERSE TRANSRIPTASE ACTIVITY OF METHANOL PHEKO EXTRACT IN CELL BASED ASSAY

The methanol Pheko extract was evaluated for anti-reverse transcriptase activity in a replication defective single round HIV-Luc reporter system with subtype C and B peudoviruses. The reverse transcriptase inhibitors, zidovudine and nevirapine were used as control substances. All 4 pseudoviruses were inhibited by the methanol extract of Pheko in a dose dependent manner. Fifty percent inhibitory concentrations against the 4 pseudoviruses were as follows: 0.025µg/µl (IndieC, wild type), 0.035µg/µl (NL4-3, resistant to NRTI), 0.022µg/µl (NL4-3 wild type), and 0.021µg/µl (NNRTI resistant virus) (Figure 3.6)

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Table 3.8: Illustration of the IC_{50} of the four types of pseudovirus used in cell based assay.

	50% INHIBITORY CONCENTRATION (IC_{50})			
	IndieC	**NL4-3 NRTIs-resistant virus**	**NL4-3 wild type**	**NL4-3 NNRTIs-resistant virus**
Methanol	0.025µg/µl	0.0035µg/µl	0.022µg/µl	0.21µg/µl
AZT	0.025µg/µl	0.035µg/µl	0.022µg/µl	0.021µg/µl
NVP	0.041µg/µl	0.029 µg/µl	0.038µg/µl	1.813µg/µl

When comparing methanol and AZT, they both showed the same IC_{50} for IndieC, NL4-3 NRTIs- resistant virus and NL4-3 wild type, and showed the highest IC_{50} than AZT for the NL4-3 NNRTIs-resistant virus. NVP showed a higher IC_{50} for IndieC and much higher IC_{50} for NL4-3 NNRTIs-resistant virus, because NVP is a NNRTI and resistant virus required a higher concentration to show effect.

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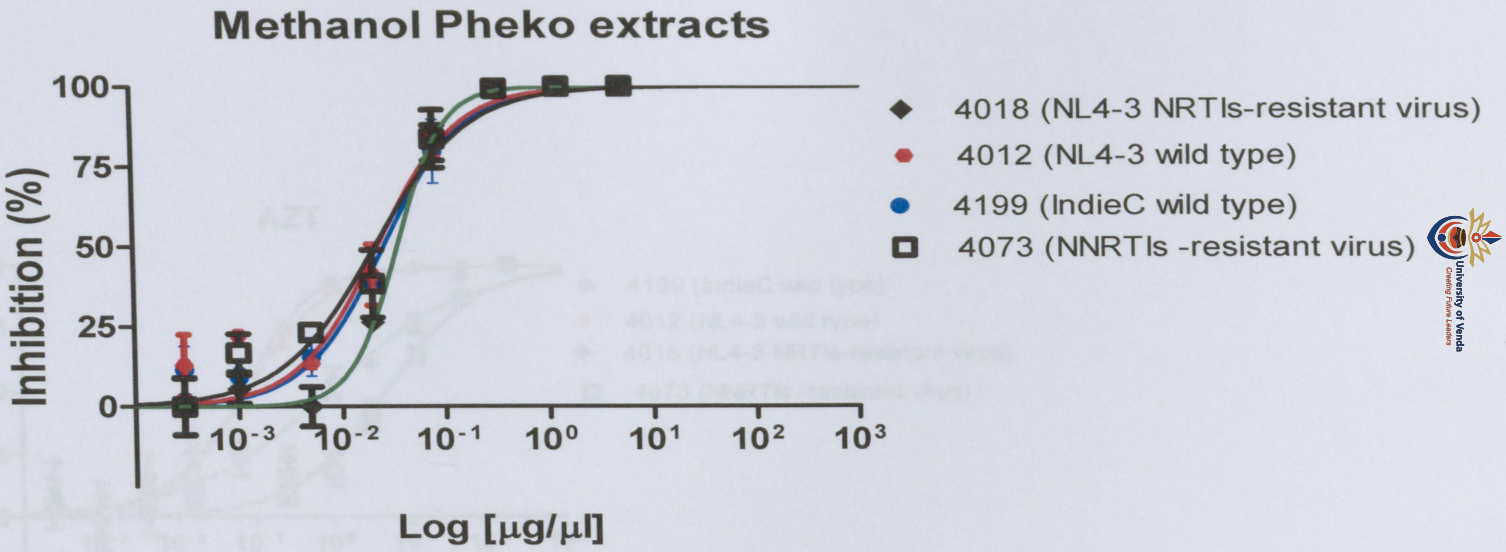


Figure 3.12: Dose-dependent inhibition profile of 4 pseudoviruses in the presence of methanol extract of Pheko. A nonlinear fit transform normalize curve is shown. The extract inhibited all the viruses including those resistant to non- nucleoside reverse transcriptase and nucleoside reverse transcriptase inhibitors.

Inhibition of the viruses by AZT and NVP are shown in figures 3.13 and 3.14 respectively. It was observed that inhibition of the resistant viruses occurred at much higher concentrations of AZT and NVP. The control AZT tested in all the pseudoviruses showed inhibition as the concentration increased. $IC_{50}$for all 4 Pseuoviruses were as follows: 0.025µg/µl (IndieC, wild type), 0.035µg/µl (NL4-3, resistant to NRTI), 0.022µg/µl (NL4-3 wild type), and 0.021µg/µl (NNRTI resistant virus)

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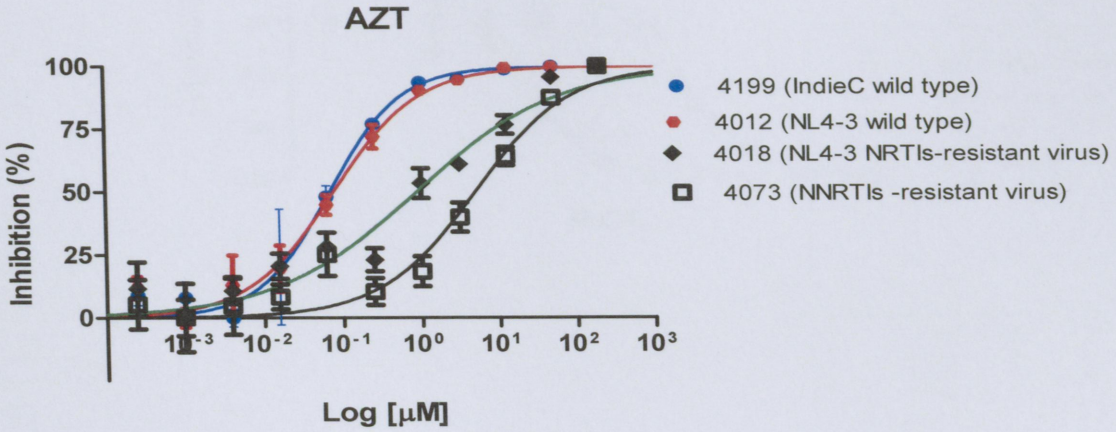


Figure 3.13: Dose-dependent inhibition profile of 4 pseudoviruses in the presence of AZT.A nonlinear fit transform normalize curve is shown. The drug inhibited all the viruses with the NNRTs resistant viruses showing resistance to the drug.

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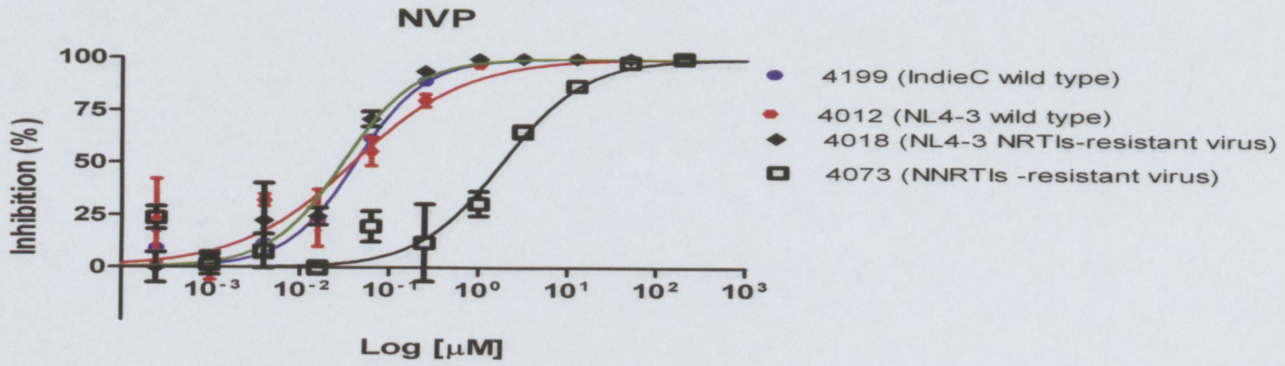


Figure 3.14: Dose-dependent inhibition profile of 4 pseudoviruses in the presence of NVP.A nonlinear fit transform normalize curve is shown. The drug inhibited all the viruses with the NRTs resistant virus showing resistance to the drug

To determine the effectiveness of Pheko extract, a selectivity index (SI) was calculated. Highest SI demonstrates the greatest overall antiviral activity of the extract.

Table 3.9: Selective index for HIV-1 RT cell based inhibition by methanol extract

Extract Type	CC_{50}	IC_{50}	SI
Methanol	0.5mg/ml	4199=0.025µg 4012=0.025 4918=0.035 4073=0.021	0.02 0.02 0.01 0.023

Selection Index = CC_{50}/IC_{50}

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The disadvantages and limitations of conventional ART continues to open new vistas in the use of ethnomedicinal plants and natural products for the management of HIV/AIDS (Chinsembu *et.al.*, 2009).The use of natural products/compounds derived from plants precedes recorded human history, probably by thousands of year. Plant products may have negative effects when used and its constituents are not known. Therefore cytotoxicity evaluation of plants extracts and their active principle is required for its effective therapeutic use. The herbal preparation Pheko (a mixture of 5 plants) is in the management HIV/AIDS patients. This study investigated the cytotoxicity of several extract types of Pheko and their in-vitro inhibitory properties against HIV therapeutic targets.

In this study, the methanol extract of Pheko was the most toxic to U87.CD4.CCR5 cell line in a LDH assay, while the water extract showed the least toxicity. U87.CD4.CCR5 cells carries the receptor CD4 and co-receptor CCR5, they were established by transfecting U87.CD4 with the chemokine receptor CCR5 (Katrien *et.al.*,2004). These cells are widely used in HIV research studies. On the other hand, dichloromethane was the most toxic to Vero cells in an MTT assay. Vero cells are derived from the kidney of an African green monkey (*Cercopithecus aethiops*) in the 1960s, Vero cells are one of the most common mammalian continuous cell lines used in research. This anchorage-dependent cell line has been used extensively in virology studies (Nicole *et.al.*, 2009). Pheko is consumed as a water extract and it would be expected to less toxic to humans. However, in vivo toxicity experiments would be needed to confirm this.

HIV-1 polymerase gene consists of essential replication enzymes which are reverse transcriptase, integrase and protease. Extracts of Pheko were investigated for their inhibition

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against HIV-1 recombinant reverse transcriptase and integrase. These are essential enzymes in the replication HIV. Methanol, dichloromethane, ethyl acetate and water extracts were evaluated. At the maximum concentration 1mg/ml the water extract inhibited 82.4% of the integrase activity and at the minimum concentration of 0.007mg/ml it inhibited 6.9% in a cell-free assay. These results suggest that Pheko potentially inhibits HIV-1 integrase. In the RT assay, the water extracts showed less inhibitory activity. No inhibition was observed at a concentration range of 0.003mg/ml to 6.25mg/ml. The methanol, ethyl acetate and dichloromethane showed enzyme inhibition from the minimum concentration and as the concentration increase, enzyme activity was gradually decreasing such that at the concentration of 0.05mg/ml enzyme activity was extinguished.

DH5-alpha strain is the most frequently used *E. coli* for routine cloning application. This train was developed by D. Hanahan with mutation supporting blue/white screening. Other mutations increase insert stability and improve the quality of plasmid DNA prepared from miniprep (http://www.melab.com/DH5-Alpha-competent-e.-coli-html). These cells are used in many applications with high transformation efficiency. In this study, an in-house protocol for the production of these cells was established.

The HIV-1 infection begins with HIV-1 envelope Gp120 binging to the CD4 and chemokine co-receptor CCR5 for M-Tropic strains or CXCR4 for T-tropic strains. And for it to establish itself inside the cell, its genome has to be transcribed to DNA by the action of reverse transcriptase and integrated inside the host cell through the action of integrase. Protease cleaves the gag-pol polyprotein to generate vital viral components.

The methanol extracts of Pheko was investigated for activity against RT in a proliferating cell replication defective single round HIV-Luc reporter system. The replication defective HIV-Luc reporter has the HIV-1 env genes inactivated and the firefly luciferase gene in place of the HIV-1 nef. Such a design allowed in-trans complementation of any viral envelope

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proteinincluding HIV-1 env and prepared subtype C pseudotyped viruses with M-tropic HIV-1 HXB2 envelope. Group M viruses are driving the pandemic, with subtype C being the circulating strain in South Africa; therefore it was convenient to screen Pheko using C-viruses. Drug controls and viral control used demonstrated robust data on the effectiveness of methanol Pheko extract. Pheko extract inhibited all the viruses, including the viruses resistant to AZT and NVP with a selective index of about 0.02 for all viruses tested.

The controls AZT, a nucleoside reverse transcriptase inhibitor and NVP, a non-nucleoside reverse transcriptase showed less inhibition of the drug resistant viruses, but with the methanol Pheko extract inhibition of all viruses was seen. At low concentrations of the extracts inhibition in viruses was seen.

The limitations for this study are, not all Pheko extracts were evaluated in all the assays performed. Hence Pheko is a herbal preparation of a mixture of 5 different plants, individual constituents of Pheko were not assayed. However this is the first report of Pheko extract on HIV inhibition work and future work will look at these limitations.

In conclusion, this preliminary data show that extracts of Pheko can inhibit subtype C and drug resistance viruses with acceptable selective indices

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