Screening and phytochemical characterization of a South African herbal concoction for anti-HIV-1 activity

Vincent Nkosinathi Hlatshwayo

A dissertation submitted to the Faculty of Science under the School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master in Science.

Johannesburg, June 2017

DECLARATION

I, **Vincent Nkosinathi Hlatshwayo** (**551762**), am a student registered for the degree of MSc in the academic year 2015.

I hereby declare the following:

I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.

I confirm that the work submitted for assessment for the above degree is my own unaided work except where explicitly indicated otherwise and acknowledged.

I have not submitted this work before for any other degree or examination at this or any other University.

The information used in the Thesis/Dissertation/Research Report **HAS NOT** been obtained by me while employed by, or working under the aegis of, any person or organisation other than the University.

I have followed the required conventions in referencing the thoughts and ideas of others.

I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.

Signature _____ - day of <u>01/06/17</u> **YEAR**

ABSTRACT

In South Africa, the anti-HIV-1 activity of various indigenous plants has not been studied extensively. Most of the phytochemical screening work has focused on anti-cancer activity with less attention given to infectious diseases. A large proportion of South Africans (70-80%) still rely on traditional medicines for treatment of various ailments. And, therefore, there is a need to evaluate and validate the effectiveness of the traditional medicines. The aim of this study was to identify, screen, phytochemically characterize and isolate bioactive compounds from a South African herbal extract that exhibit the best anti-HIV-1 activity. Three extracts were prepared: an ethanol extract, a dereplicated ethanol extract and an aqueous extract from a herbal concoction comprised of a mixture of six plants. These herbal concoctions were investigated for anti-HIV-1 subtype C activity.

Phytochemical profiling of the ethanol- and dereplicated ethanol- extracts from the herbal concoctions showed the presence of intermediate polar compounds (flavonoids, alkaloids, sugars and terpenes) for both extracts, while the aqueous extract contained predominantly highly polar compounds.

Anti-HIV-1 screening of the three extracts showed that the ethanol and dereplicated ethanol herbal- extracts had the best anti-reverse transcriptase activity. The ethanol extract had mean IC_{50} values of 56.53, 53.96 and 55.39 µg/ml against MJ4, Du179 and CM9 HIV-1 subtypes C isolates, respectively. The dereplicated ethanol extract had mean IC_{50} values of 51.87, 47.56 and 52.81 µg/ml against MJ4, Du179 and CM9 HIV-1 isolates, respectively. The aqueous extract was inactive against HIV-1 activity.

Moreover, both the ethanol- and dereplicated ethanol- extracts showed activity against HIV neutralization. The ethanol- and dereplicated ethanol- extracts had mean IC_{50} values of 36.33 and 32.06 µg/ml, respectively. Furthermore, they also potently neutralized Vesicular stomatitis virus (VSV) yielding mean IC_{50} values of 24.91 and 20.82 µg/ml for ethanol- and dereplicated ethanol- extracts, respectively. All extracts were inactive against Murine leukemia virus (MLV).

The isolation and phytochemical characterization of the bioactive compound(s) was done by utilizing various chromatographic and spectroscopic methods. Four homoisoflavanoids were isolated and tested for anti-HIV-1 subtype C activity. Three compounds (**1**, **3a** and **3b**) were inactive while compound **2** was found to be bioactive against HIV-1 reverse transcriptase (RT) and yielded mean IC_{50} values of 7.23 ± 1.88, 12.83 ± 0.41 & 12.81 ± 0.10 µg/ml for MJ4, CM9 and Du179 HIV-1 subtype C isolates, respectively. Compound **2** had a mean CC_{50} value of 23.08 ± 0.1981 µg/ml against HEK293T cells.

Overall, the data suggested that ethanol- and dereplicated ethanol- herbal extracts possess direct and indirect anti-HIV-1 activity. They possess a cocktail of phytochemicals that can inhibit HIV-1 RT, HIV-1 entry. Furthermore, these extracts possess phytochemicals that can lower the activation of inflammatory responses during an infection and, hence, reduction in the number new cells infected during the course of HIV-1 infection. Moreover, they possess phytochemicals that have antioxidant activity which, in relation to HIV infection, results in a boosted immune system response in order to ward off the virus.

DEDICATION

I dedicate this dissertation with love to my mom, Cordelia Hlatshwayo and dad, Themba Mgabhi. For supporting, encouraging and teaching me to dream big always.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
APOBEC3G/ A3G	apolipoprotein B mRNA–editing enzyme, catalytic polypeptide like 3G
ART	Antiretroviral therapy
AZT	Zidovudine
BSA	Bovine serum albumin
CCR5	C-C chemokine receptor type 5
CDC	Centre for Disease Control
cDNA	complimentary Deoxyribonucleic acid
CXCR4	C-X-C chemokine receptor type 4
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FRET	Forster resonance energy transfer
gp120	Envelope glycoprotein 120 kDa
GPS	Global positioning system
HAART	Highly Active Antiretroviral Therapy
HIV	Human immunodeficiency virus
HIV-I & II	Human immunodeficiency virus type 1 and 2
HIV-I Δ*vif*	Human immunodeficiency virus type 1 deficient in *vif* gene

HPLC/MS	High pressure liquid chromatography/mass spectrometry
HR-QTOF-MS	High-resolution quadrupole time-of-flight mass spectrometry
IN	Integrase
LPV	Lopinavir
LRT	Long terminal repeat
mRNA	messenger Ribonucleic Acid
MS	Mass spectrometer
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
Nef	Negative regulatory factor
NMR	Nuclear magnetic resonance
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NPs	Natural products
NRTIs	Nucleoside reverse transcriptase inhibitors
NVP	Nevirapine
PIs	Protease inhibitors
PR	Protease
Rev	Regulator of expression of virion proteins
RNA	Ribonucleic Acid
RT	Reverse transcriptase
SA	South Africa
SIV	Simian immunodeficiency virus

SIV_{gor}	Simian immunodeficiency virus infecting Gorillas
Tat	Trans-activator of transcription
UNAIDS	United Nations Program on HIV and AIDS
UPLC-HDMS	Ultraperformance liquid chromatography high-definition mass spectrometry
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein unique

1   CHAPTER 1

Literature review

1.1   HIV/AIDS background

Thirty years after the identification of Human Immunodeficiency Virus (HIV), a cure for the HIV infection still has to be found. HIV is believed to have been a result of the genetic mutation of the virus that has been endemic in the wild life of some areas in Central Africa (Tortora, Funke and Case, 2010). Genetic analysis of the HI virus led to the conclusion that the virus evolved from the monkey infecting virus known as: Simian immunodeficiency virus (Maartens et al., 2014). Human immunodeficiency virus/ acquired immunodeficiency syndrome (HIV/AIDS), is a type of zoonotic disease which is caused by two viral strains, namely, HIV-1 and HIV-2. HIV-1 is the primary virus strain infecting the human race worldwide and is related to SIV infecting chimpanzees in Central Africa (Maartens, et al., 2014; Tortora et al., 2010). HIV-2, on the other hand, is a less virulent strain mainly found in the West African region. HIV-2 has arisen from the SIV infecting sooty mangabey monkeys in West Africa (Maartens, et al., 2014; Tortora et al., 2010). HIV-2 is limited to West Africa and causes a similar illness as HIV-1, but progression to immunodeficiency is slower and is less infectious than HIV-1 (Sharp and Hahn, 2011)

The HIV epidemic emerged following infections with simian immunodeficiency viruses that naturally infect African primates; it is believed that bush meat hunters were probably the first group to be infected with HIV (Sharp and Hahn, 2011). Cross-species transmission from other primates into humans probably occurred by blood contamination through hunting and handling of 'bushmeat' (Lever, 2009).

The HIV infects vital immune system cells such as CD4+ T cells, monocytes, macrophages, and dendritic cells (Tortora et al., 2010; Cos et al., 2004). These cells harbour infectious HIV for years. After infection, the person is in an asymptomatic period which may last for several months to years, prior to the development of Acquired Immunodeficiency Syndrome (AIDS). HIV causes profound immunosuppression (Cos et al., 2002) leading to these observed clinical symptoms: weight loss, persistent fever, night sweats, diarrhoea, headache and opportunistic infections such as thrush, lymphadenitis, skin rashes, shingles, chicken pox, Kaposi sarcoma,

pneumonia, oesophageal candidiasis, mycobacterial infection, cryptococcosis and toxoplasma encephalitis (Vermani and Garg, 2002). It is important to note that not everyone experiences all these symptoms when they are diagnosed with AIDS.

Acquired immunodeficiency syndrome (AIDS) is defined as a multifactorial disease portrayed by gradual obliteration of a person's immune system whereby the white blood cells (especially T-lymphocytes) become deficient (Cos et al., 2004) leading to observable clinical symptoms that are associated with immune deficiency and autoimmune inflammation (Barbaro and Klatt, 2002; Cos et al., 2002; Vermani and Garg, 2002). The decline in CD4+ T-cell count due to HIV infection occurs by means of a series of processes, including apoptosis of uninfected bystander cells, direct viral killing of infected cells, and killing of infected CD4+ T cells by CD8+ cytotoxic white blood cells (Dambuza et al., 2005; Cos et al., 2002; Tortora et al., 2010). Research indicates that lower levels of CD4+ cells render patients susceptible to opportunistic diseases which lead to the development of AIDS.

Memory mature T-helper cells expressing the surface CD4 protein receptor (CD4+ T-cells) that are infected by HIV are regarded as the most important cellular reservoirs for this virus. The existence of this memory cell reservoir is due to the biological ability of these cells to survive for long periods of time, ranging from months to years, thereby equipping the host with the ability to respond rapidly to previously encountered infections (Deeks, 2003; Saksena and Potter, 2003). Together with viral load, CD4+ T-cell count is important in tracking the progress of HIV infection towards the AIDS state. The CD4 count is also used to select patients for antiretroviral therapy (ART) and assessment of the therapeutic effect following treatment (Dambuza et al., 2005). According to the Centre for Disease Control (CDC) and World Health Organization (WHO), a patient is diagnosed with AIDS if the CD4+ T cells count is equal to or less than $200/mm^3$ as shown in **Figure 1.1**.

Category A: Asymptomatic or chronic lymphadenopathy

Category B: Symptomatic. Early indications of immune failure

Category C: AIDS indicator conditions

1. 1–2 months following initial infection, the population of HIV in blood peaks at about 10,000,000/ml.
2. 1–2 months following initial infection, population of CD4 T cells plunges.
3. Seroconversion. Antibodies against HIV appear. HIV population in blood crashes.
4. HIV in blood stabilizes at steady state of 1000 to 10,000/ml.
5. CD4 T cell population declines steadily.
6. Huge but indefinite numbers of HIV in lymphoid tissue, in latent or proviral form (see Figure 19.13). At least 100 billion HIV generated each day for years, mostly by infected T cells. (Darker color below line indicates viral load in lymphatic tissue.)
7. Clinical AIDS: CD4 T cell population 200/mm³.
8. Rise of HIV in blood as immune system breaks down.

HIV population in blood

CD4 T cell blood concentration (cells/mm³) — Blood plasma HIV/RNA (millions of copies/ml)

1–2 months — Years

Figure 1.1: Viral load and CD4 count with the progression of HIV-1 infection. Red line represents CD4+ T cell population and grey represents HIV population in blood. (Taken from Tortora et al., 2010)

1.2   HIV/AIDS epidemiology and statistics

HIV-1 is classified into distinctive groups named M (main), N (non-M or O) and O (outlier) (Sharp and Hahn, 2011; Tortora et al., 2010). These groups represent three separate transmission events from chimpanzees (Sharp and Hahn, 2011). Recent studies have found another group called P which is closely related to the simian immunodeficiency virus infecting gorillas (SIVgor) and it is believed that it most likely arose from an independent gorilla to human transmission (Sauter et al., 2011; Sharp and Hahn, 2011).

Analogous to HIV-2, the HIV-1 groups: N, O, and P are limited to West African countries. The HIV group M, which is the causative agent of the global HIV/AIDS pandemic, emerged about a century ago and is made up of nine clades: A–D, F–H, J, and K. HIV-1 subtype C is predominantly found in Africa and India, and was responsible for 48% of cases of HIV-1

infections in 2007 worldwide (Hemelaar et al., 2011). Subtype B is predominantly found in the Americas, Western Europe and Australia. Currently, recombinant subtype strains of HIV are becoming more common (Hemelaar et al., 2011).

The apparent genetic diversity of HIV-1 is due to the lack of proofreading ability of reverse transcriptase which makes it error prone resulting in higher rates of mutation.

The epidemiology of HIV/AIDS especially in developing countries is further influenced (among other factors) by men who sleep with men (Birrell et al., 2013), sharing of needles in the case of intravenous drug users and addicts (Degenhardt et al., 2010). The epidemic in southern Africa is spreading mainly through heterosexual exposure, high rates of labour migration, simultaneous sexual partnerships, gender inequalities, and the limited distribution of condoms (Beyrer, 2007).

Since the recognition of HIV as a causative of AIDS in 1983 until 2013, 78 million people have been infected and 39 million people have died due to HIV/AIDS (Barre-Sinoussi et al., 1983; UNAIDS, 2016). UNAIDS (2016) estimated that in 2015, 36.7 million people were living with HIV and WHO estimated the worldwide mortality rate of HIV/AIDS to be 1.1 million people in 2015 (UNAIDS, 2016). Sub-Saharan Africa is the most affected region, accounting for approximately 52% of people living with HIV/AIDS globally (UNAIDS, 2016).

In the sub-Saharan African region, South Africa (SA) has the highest HIV burden. WHO estimated that the number of people living with HIV in SA by the end of 2015 was 7 million people which increased from 5.3 million people during 2005 (UNAIDS, 2016). SA has 12.2% HIV prevalence which is the fourth highest prevalence globally (UNAIDS, 2015). In comparison with high-income countries, low in-come countries have a higher mortality rate due to HIV/AIDS (Antiretroviral Therapy Cohort Collaboration, 2010).

Tuberculosis is a major cause of illness and death in low-income and middle-income countries, especially in Africa (Glaziou et al., 2013). Findings of a study by Sonnenberg et al., (2005) conducted in SA in the pre-antiretroviral therapy era, revealed that TB infections doubled within a year following HIV infection, and the incidence increased due to increased number of people that are HIV positive. HIV-related TB mortality is decreasing globally

(Glaziou et al., 2013), but the majority of HIV-positive people in Africa die of undiagnosed TB (Cohen et al., 2010).

In 2013, the WHO reported that in 2012, 11.7 million people in low-income and middle-income countries had started antiretroviral therapy. The expanding access to antiretroviral therapy, as seen from the WHO projections, markedly changed the global epidemiology of HIV infection. The global prevalence of HIV has increased from 31 million in 2002, to 35.3 million in 2012, due to increased access to antiretroviral therapy, thus enabling HIV-positive people to live longer while harbouring the virus (Zaidi et al., 2013).

The number of new HIV infections in children decreased globally by 38% between 2009 and 2012 (UNAIDS, 2013), due to increased access to antiretroviral therapy to prevent mother-to-child HIV transmission. Despite this progress, there is still a gap in addressing the distribution and access to antiretroviral therapy between children and adults. The access is much lower in children than adults (UNAIDS, 2013).

Antiretroviral Therapy Cohort Collaboration studies (2010) indicated that about 50% of all deaths in people on antiretroviral therapy in high-income countries are not due to AIDS, but were caused by non-AIDS related diseases such as cancers (23.5%), cardiovascular disease (15.7%), and liver disease (14.1%). Liver disease is common, mainly because of co-infection with hepatitis B and C, which share similar routes of transmission with HIV (Joshi et al., 2011).

1.3 HIV structure, life-cycle, infectivity and pathogenicity

HIV belongs to the Retroviridae family and the genus, Lentivirus. Lentiviruses are characterized by a long incubation period and their ability to infect non-dividing cells (Sierra et al., 2005; Tortora et al., 2010). HIV is a retrovirus, which has a positive-sense genomic RNA strand and uses virally encoded enzymes such as reverse transcriptase (RT) to synthesize a double-stranded DNA molecule which is the persisting viral genome in infected cells. There are two species under this genus of HIV as previously stated, HIV-1 and HIV-2.

Infectious, mature HIV virions have a spherical shape of 100-120 nm in diameter and have a host derived lipid bilayer (with viral-encoded projecting gp120 proteins) surrounding the core of the virus formed by p17 protein molecules (matrix proteins) (Male et al., 2006; Roitt et al., 2001; Sierra et al., 2005; Tortora et al., 2010). HIV is composed of two single-stranded, positive-sense RNA molecules that make up its genetic material. The RNA molecules are bound to p7 proteins forming a nucleocapsid. The genome together with three enzymes called reverse transcriptase (RT), integrase (IN) and protease (PR) and the 6 accessory proteins: Vif, Tat, Vpu, Vpr, Nef, and Rev are enveloped by capsid proteins p24 to form a conical capsid (Roitt et al., 2001). The mature HIV virion structure is depicted in (**Figure 1.2**).

The HIV genome is made up of two identical 9.2 kb positive-sense, single-stranded RNA molecules (Sierra et al., 2005). The RNA molecules are composed of nine genes (gag, pol, env, tat, rev, nef, vif, vpr and vpu) which encode proteins essential in the life-cycle, pathogenesis and structure of HIV (Chinen and Shearer, 2002; Sierra et al., 2005) as seen in (**Figure 1.3**). The first three genes encode the structural proteins and the remaining six genes encode regulatory proteins to promote viral transcription, mature virion release and/or viral pathogenesis (Chinen and Shearer, 2002; Sierra et al., 2005).
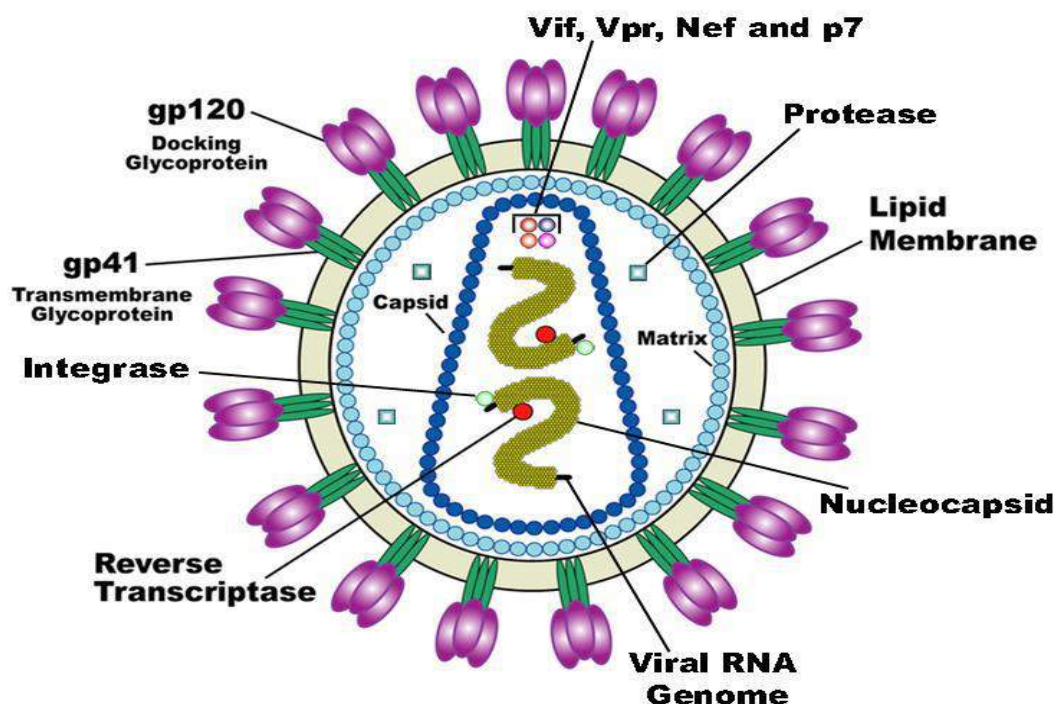
Figure 1.2: Mature HIV-1 virion structure with some of the regulatory proteins. (Sierra et al., 2005)
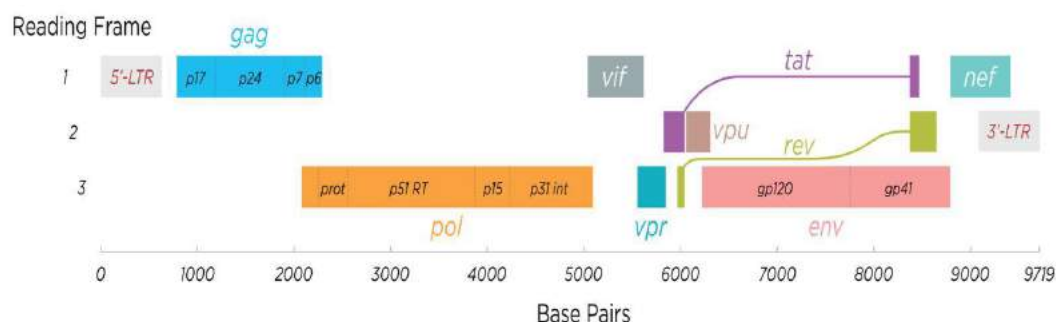
Figure 1.3: Diagrammatical representation of the HIV-1 RNA genome. (Sierra et al., 2005)

During an infection of the CD4+ T-cells (**Figure 1.4**), the virus attaches to the host cell via gp120/CD4 receptor interaction in the presence of a chemokine co-receptor (CCR5 or CXCR4). This interaction results in the fusion of the virus and the host cell. Following viral fusion, the virus-encoded enzyme, RT, copies the RNA genome into a double-stranded complimentary DNA (cDNA) which is integrated into the host genome by IN and cellular co-factors (Male et al., 2006; Roitt et al., 2001). Once the HIV virus genome is integrated into the host chromosomes, there are two events that are possible: either the virus becomes inactive (thereby forming what is known as a provirus) and the diseased cell continues to

function ordinarily; or the virus becomes active and replicates to yield $\approx 10^8$ virions/day which will infect other cells (Roitt et al., 2001).
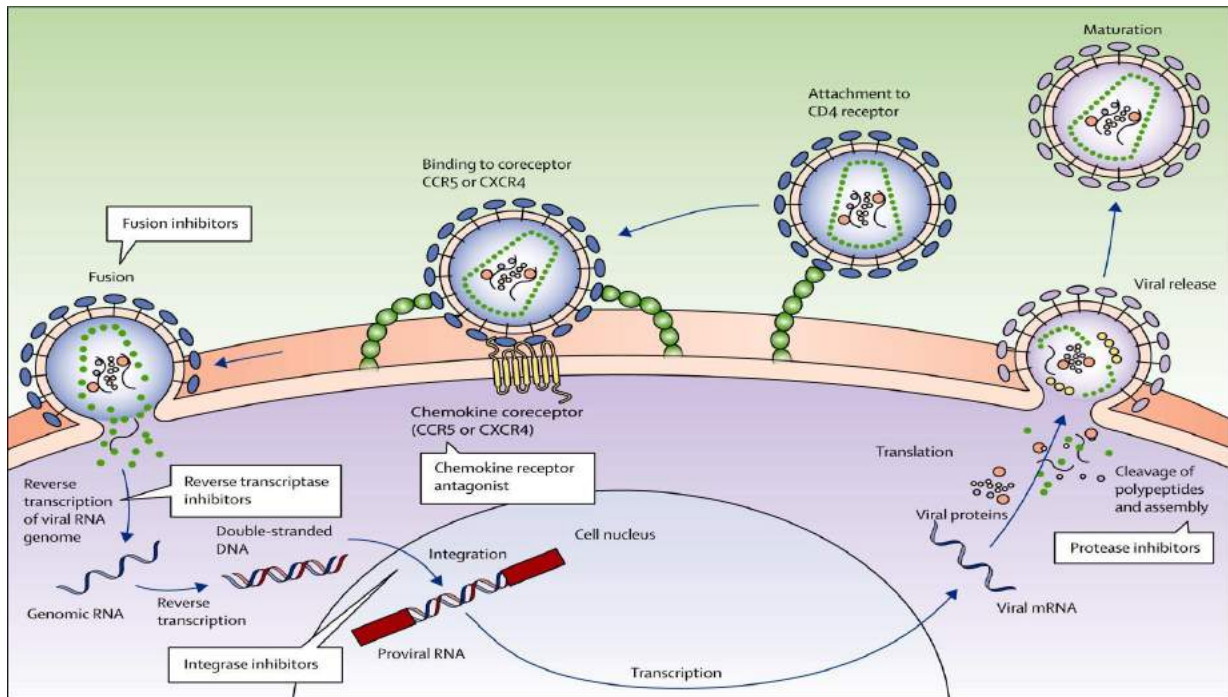
Figure 1.4: HIV-1 life cycle, pathogenesis and sites of action of current ARV drugs. (taken from Walker and colleagues, 2014)

The various enzyme-dependent HIV lifecycle steps needed for the production of infectious virions, as seen from **Figure 1.4**, renders HIV vulnerable to potent inhibitors for each step of the cycle. This is the basis for the development of ARVs against HIV.

1.4   Highly Active Antiretroviral Therapy (HAART)

The past three decades have seen a huge drive in the search for effective chemotherapeutic agents to treat HIV infection. To develop effective anti-HIV agents, one needs to fully understand the structure, genetics and life-cycle of the virus. Research so far has focused on developing agents that target specific steps in the HIV life-cycle. This is necessary because these steps are important in determining the infectivity and pathogenicity of the virus.

The most significant advance in the medical management of HIV-1 infection has been the treatment of patients with antiviral drugs which can suppress HIV-1 replication to undetectable levels. The discovery of HIV-1 as the causative agent of AIDS together with an ever-increasing understanding of the virus replication cycle have been instrumental in pioneering effective antiretroviral drugs against HIV. To date, there are several Food and Drug Administration (FDA) approved drugs available for treatment of HIV-1 infections (**Figure 1.5**). These drugs are distributed into six distinct classes based on molecular mechanism and resistance profiles: nucleoside-analogue reverse transcriptase inhibitors (NRTIs), non–nucleoside reverse transcriptase inhibitors (NNRTIs), integrase inhibitors, protease inhibitors (PIs), fusion inhibitors, and co-receptor antagonists (Clavel and Hance, 2004). So far, no inhibitors pioneered against the viral accessory proteins have been approved by the FDA. Inhibitors against Tat are still in clinical trials (Arts and Hazuda, 2012).

To treat HIV, the above inhibitor drugs are used concurrently. This method of treatment is known as highly active antiretroviral therapy (HAART) which is defined as the use of a combination of the antiretroviral drugs for the treatment of HIV infection (Clavel and Hance, 2004). Current HAART regimen is comprised of three antiretroviral drugs, in most cases having two nucleoside analogues and a protease inhibitor or a non-nucleoside reverse transcriptase inhibitor (Clavel and Hance, 2004). Alternative regimens include a boosted PI-based regimen or an INSTI-based regimen (integrase strand transfer inhibitor). The combination of drugs from different classes in the treatment of HIV is effective in reducing drug resistance development.
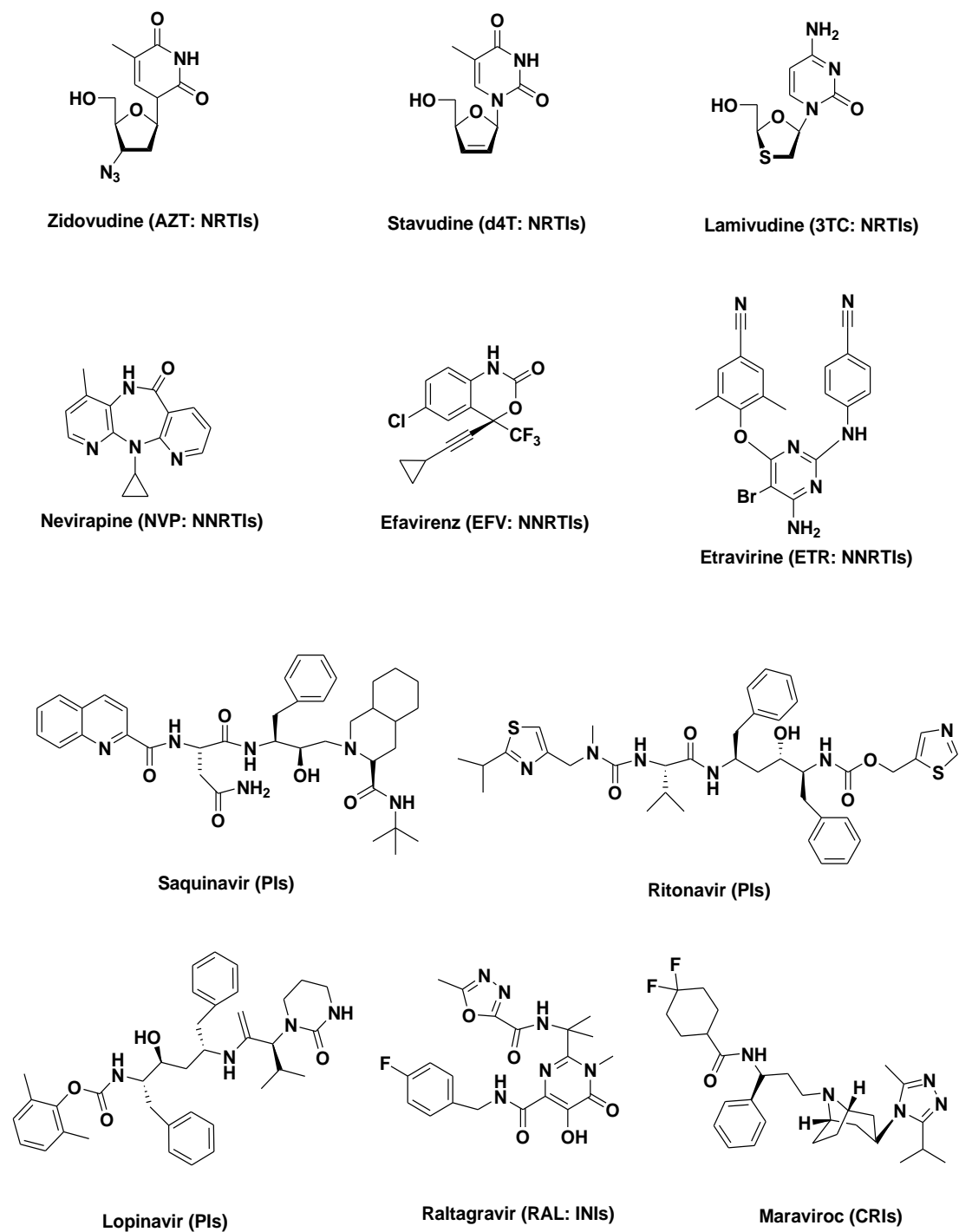
Figure 1.5: Some of the FDA-approved ARVs for the treatment of HIV/AIDS.

1.4.1 Mode of action of ARVs and mechanism of drug resistance by HIV-1

Nucleoside-analogue reverse transcriptase inhibitors (NRTIs), are pro-drug molecules that require phosphorylation by the cellular kinases in order to enact their antiviral function (Arts and Hazuda, 2012). The pro-drug molecule lacks a 3'-OH group in their sugar moiety, thus preventing the formation of the 3'-5' phosphodiester bond between the nucleoside triphosphates utilised in HIV proviral DNA synthesis, which in turn results in DNA synthesis termination by the reverse transcriptase enzyme (Arts and Hazuda, 2012). Resistance to NRTIs is usually regulated by two mechanisms: ATP-dependent pyrophosphorolysis, which is the removal of NRTIs from the 3'-end of the nascent chain and reversal of chain termination, and/or increased discrimination between the native deoxyribonucleotide substrate and the inhibitor (Arts and Hazuda, 2012).

NNRTIs are non-competitive inhibitors that inhibit HIV-1 RT by binding and inducing the formation of a hydrophobic pocket proximal to the active site, thereby resulting in spatial conformational change of the substrate binding site and reduction in polymerase activity (Arts and Hazuda, 2012). NNRTIs resistance usually arises from amino acid substitution with the K103N and Y181C being the most common mutations (Arts and Hazuda, 2012). These mutations confer resistance to first-line ART and hence patients have to be enrolled to the second-line ART which is costly.

Integrase inhibitors target the strand transfer reaction which is one of the two functions of integrase. The other function of integrase is to process the 3'-end of the HIV DNA for successful incorporation into the host genome. The mechanism of action of the inhibitors is through the binding of the integrase/viral DNA specific complex and then sequestration of the magnesium ion co-factors essential in the integrase active site (Arts and Hazuda, 2012). Resistance to these inhibitors is through mutation in the active site of the integrase near the amino acid residues that coordinate the magnesium co-factors (Arts and Hazuda, 2012).

Protease inhibitors fall within the class of competitive inhibitors. They bind strongly to the active site and prevent the cleaving of gag and gag-pol polyproteins to yield mature virions. Resistance to these inhibitors is through the amino acid changes near the enzyme's active site which results in poor binding affinity for the inhibitor (Arts and Hazuda, 2012).

Entry inhibitors disrupt entry events used by HIV. HIV uses several host proteins for attachment, fusion and release of the virus core into the cytoplasm. CCR5 antagonists are allosteric inhibitors that work by binding to the hydrophobic pocket of the trans-membrane helices of CCR5, thereby stabilising the receptor conformation and thus the interaction with the V3 loop of gp120 is inhibited (Arts and Hazuda, 2012). Drug resistance to entry inhibitors, more especially the CCR5 antagonist, is mediated by four factors: tropism switching (utilisation of CXCR4 instead of CCR5), fast rate of entry of the viral core, utilisations of the inhibitor-bound receptor and increased affinity for the receptor (Arts and Hazuda, 2012).

HIV drug resistance is influenced by high virus production and turnover ($\approx 10^7$-10^8 virions produced per day). Furthermore, the error prone RT, introduces mutations in every virion produced and, hence, creating a heterogeneous virus population of quasispecies (Arts and Hazuda, 2012; Clavel and Hance, 2004). Although other mutations have also been found, including insertions, duplications and recombination; studies have shown that mutations introduced in every viral genome transcribed by RT are predominantly base substitutions (Arts and Hazuda, 2012; Clavel and Hance, 2004).

Despite the success in the development of drugs that have revolutionized the lives of HIV positive patients enabling them to live with HIV for decades, a search for a cure is still on going. The development of new and effective antiretroviral drugs to treat HIV is crucial because of the fact that the virus is constantly evolving as proven by the observed mutations leading to drug resistance to the currently used antiretroviral chemotherapy regimens. New strategies under development include: gene therapy (e.g. Berlin patient) (Younai, 2013), targeting innate immune responses with targets including APOBEC3G, tetherin (that blocks viral release) and TRIMα (that destabilizes the HIV capsid and interferes with reverse transcription) (Ali et al., 2012; Ellegard et al., 2011; Monajemi et al., 2012; Shankar et al., 2012; Shi et al., 2011); 'LEDGF/p75', an integrase enzyme cofactor, whose antagonists are being studied for their ability to suppress HIV replication (Hu et al., 2012). Other possible ARV mechanisms involve the endogenously secreted antimicrobial peptides such as defensins, lactoferrins, secretory leukocytes protease inhibitor and Trappin-2/Elafin. These peptides have shown to possess strong anti-HIV properties (Ellegard et al., 2011; Shankar et al., 2012).

Other avenues include looking at natural products for a cure. This approach is inspired by the fact that current HAART treatment is associated with high cost, adverse effects, emergence of drug resistance, and the narrow spectrum of activity which has limited the therapeutic usefulness (Klos et al., 2009). Conversely, natural products may have better efficacy, safety and affordability. Several natural products, mostly of plant origin, have been shown to possess promising activities that could assist in the prevention of the disease. Many of these anti-HIV agents have other medicinal uses as well (Asres, 2005), which afford them further potential as novel leads for the development of new drugs that can deal with both the virus and the various disorders that characterize HIV/AIDS.

2   CHAPTER 2

2.1   PLANTS AND THEIR USE AS TRADITIONAL MEDICINES

Ever since the evolution of mankind, humans have relied on nature for survival. Plants in particular, have played a pivotal role in providing mankind with all the necessities to survive in an ever-changing environment. Plants (e.g. trees and herbs) have provided our forefathers, and us, with food-stuffs, shelters, clothing, means of transportation (e.g. boats), fertilizers, flavours and fragrances, and, importantly, medicines (Gurib-Fakim, 2007).

Plants provide people with the primary metabolites such as carbohydrates, proteins, fats and vitamins. Furthermore, plants produce secondary metabolites (alkaloids, phenols, terpenes etc.) which are essential for both humans and the plant because they form a chemical defence mechanism in response to infection by microorganisms, predation, insects, herbivores and they also play a crucial role in aiding the plant to survive and adapt to environmental changes (Pengelly, 2004). Secondary metabolites were considered to be useless by-products of the synthesis of primary metabolites. However, through the science of Pharmacognosy (''the study of the physical, chemical, biochemical and biological properties of drugs, drug substances, or potential drugs or drug substances of natural origin as well as the search for new drugs from natural sources'') and Ethnobotany ("the study of the relationship between humans and plants in all its complexity"), it was observed that these products are the basis of the healing properties of medicinal plants. Neuwinger (1994) found that constituents with special properties often occur in a high concentration and sometimes with greater purity in a particular medicinal plant.

The link between humans and healing plants dates back to ancient times, sometime around 5000 years-back according to the Sumarien records (Swerdlow, 2000). However, archaeological records suggest even earlier use of medicinal plants (Raskin et al., 2002). Plants have formed the foundation of advanced traditional medicine systems, namely, Ayurvedic, Unani, Chinese, African & Australian and Central & South American. These traditional systems have provided a wealth of knowledge regarding the discovery of pharmaceutical products which are used nowadays (Gurib-Fakim, 2006).

African traditional medicine, itself, is the oldest and perhaps most diverse of all traditional medicine systems. African traditional medicine in its varied forms is a holistic system involving both body and mind. The healer typically diagnoses and treats the psychological basis of an illness before prescribing medicines to treat the symptoms. The Khoi-San people of southern Africa, nowadays considered to be the most ancient of all cultures, have an extraordinarily diverse materia medica (medicinal plants and other materials) which typically includes general tonics, fever remedies, sedatives, diuretics, laxatives and numerous wound healing plants (van Wyk and Wink, 2004). This is not exceptional, however, when considering that of the estimated 300 000 species of plants which exist throughout the world (Cotton, 1996), southern Africa possesses approximately 24 000 taxa of 368 families, including more than 10% of the world's vascular plant flora on less than 2.5% of the Earth's land surface (Germisthuizen and Meyer, 2003).

Evidence also shows that the vast majority of people on this planet still rely on their traditional materia medica for their everyday health care needs (WHO, 2013). It is also a fact that one quarter of all medical prescriptions are formulations based on plant-derived substances or plant-derived synthetic analogues (pharmacophores). According to WHO, 80% of the world's population, primarily those of developing countries, rely on plant-derived medicines for their healthcare (WHO, 2011).

Phytomedicines have been shown to be affordable, effective and safe. The latter being mainly because when plant medicines have been used by these traditional cultures for centuries, they seldom show evidence of serious side effects (Heinrich et al., 2004). It is only within the last few decades that research results generated have given us a much better understanding of the scientific rationale behind many natural remedies.

Phytomedicines often contain a mixture of substances that have additive or even synergistic effects. Phytomedicines may have direct or indirect effects on several different biochemical pathways and receptors in the body-mind continuum that may all contribute directly and indirectly to restore equilibrium (van Wyk and Wink, 2004).

2.2   The use of traditional medicine in the South African context

In South Africa, a majority of the black population consult with traditional healers. Traditional healers have knowledge of the healing properties of several indigenous herbs and

use the leaves, roots and stems of individual herbal plants or a cocktail of different herbal plants to treat different diseases.

As previously stated, SA has approximately 24 000 taxa of 368 families, including more than 10% of the world's vascular plant flora (about 30 000 plant species) and of these, only 20% are used in traditional medicine (Cherry, 2005). Furthermore, approximately 4000 species of this 20% are used in traditional medicine. Only, about 350 species are the most commonly used and traded medicinal plants (van Wyk and Wink, 2004). This just indicates that, so far, we have not utilized this precious knowledge from our forefathers. Some of these plants have been shown to have antifungal, antibacterial, antiviral and also anti-amoebic properties (McGaw et al., 2000).

The problem is that the usefulness of these traditional remedies has not been systematically investigated, and the country (SA) is, therefore, not in a position to benefit from this indigenous knowledge base. Thus, a rational scientific evaluation is needed!

2.3   The significance of medicinal plants in drug discovery

One of the reasons why it is imperative to do research on traditional medicines is mainly because, a large portion of the world's population relies on plant-derived medicines for their primary healthcare according to WHO records. Furthermore, as stated above, a majority of South Africans consult with traditional healers. Lastly, it is mainly due to the fact that many of the drugs that are used nowadays are either plant-derived or modelled from plant-derived molecules as will be shown below.

To conduct research on medicinal plants and their use by humans, it is important to look at the two most important scientific fields in this regard; namely, ethnobotany and pharmacognosy. These are interdisciplinary fields of research that look specifically at the empirical knowledge of indigenous people concerning medicinal substances, their potential health benefits and the potential toxicity risks associated with such remedies (Heinrich et al., 2004). Through these two fields, it has been shown that many drugs that are commonly used today, came into use through bioscientific investigation of plants used by people throughout the world.

A review article by Balunans and Kinghorn (2005) presents evidence that despite the recent interest in molecular modelling, combinatorial chemistry, and other synthetic chemistry techniques by pharmaceutical companies and funding organizations; natural products, and particularly medicinal plants, remain an important source of new drugs, new drug leads, and new chemical entities (NCEs).

Newman and colleagues (2003), showed that during the time period between 1981 and 2002, natural product research accounted for approximately 48% of NCEs which medicinal chemists (in the pharmaceutical industry) use to generate new drugs. Furthermore, they showed that in the specified time period, approximately 28% of NCEs were natural products or natural product-derived. Again, they showed that of all the synthetic small molecule compounds made during this time, 20% of them were modelled based on natural product pharmacophores.

Butler (2004) showed that in 2001 and 2002, approximately a quarter of the best-selling drugs worldwide were natural product-derived. An analysis of all drugs approved worldwide

between 1981 and 2006 showed that 34% of all small-molecule drugs are natural products or their direct semisynthetic derivatives (Zhang and Wilkinson, 2007). Furthermore, evidence was presented that four new drugs (Arteether, Galantamine, Nitisonone and Tiotropium) derived from medicinal plants were approved by the FDA and they were in the USA market. These drugs are used to treat malaria, Alzheimer's disease, tyrosinaemia and chronic obstructive pulmonary disease (COPD) respectively.

Cherry (2005) declared that about 50% of the models of the Western developed drugs are derived from plant materials. Farnsworth et al., (1985) also showed that 25% of the prescribed drugs in the United States and Canada were modelled on plant-based products and plant metabolites are used worldwide as drugs.

Studies by Newman and colleagues (2007), indicate that of all the approved small-molecule anticancer drugs (total 155), 47% are either natural products or direct semisynthetic derivatives of them. Similarly, the same trend of dominance by natural products is observed among the anti-infective small-molecules, and astonishingly more than 75% of approved antibacterial drugs are natural products or their semisynthetic derivatives (74 out of 98). Furthermore, during the period 2000–2006, approximately 50% of the approved small-molecule drugs were still those related to natural products. This just indicates how different the health system we now know today would be without natural products!

The benefits of using natural products in drug discovery may be related to the fact that their chemical structures have been biologically prevalidated by evolutionary selection which defines structural prerequisites for binding to proteins targets. For example, an analysis of over 154 000 natural products showed that the majority of them have molecular volumes ranging from 100 to 500 $Å^3$ (Koch et al., 2005). The authors further showed that the volumes found in over 18 000 binding cavities of protein targets range from 300 to 800 $Å^3$. Therefore, the average volumes of natural products correlate with the average dimensions of protein cavities. Of note is that protein ligands often do not fill the entire volume of a given protein cavity. Although many natural products from plants and microorganisms are not meant to bind to human proteins, many human proteins consist of the same building blocks and contain similar structural domains to the targets with which natural products have coevolved (Koch et al., 2005). This goes to show why it is important to keep looking for NCEs from plants rather than solely relying on synthetic chemistry to generate NCEs.

Studies also show that natural products have distinct structural characteristics from synthetic molecules. For example, when Grabowski and colleagues (2007) analysed NPs by either a size-independent 'chemistry space filter' or 'support-vector-machine' approach, NPs exhibited better scores of 'drug-likeness' than synthetic compounds. Furthermore, they showed that NPs contain on average twice as many oxygen atoms and three times fewer nitrogen atoms than synthetic drug molecules. They also contain a slightly higher number of hydrogen-bond donors than do synthetic drugs. NPs contain approximately four times more chiral centres and far fewer aromatic rings, which gives NPs better selectivity when binding to stereo-defined sites such as those found in many protein targets.

Some of the plant-derived drugs used today to treat various ailments are shown below in **Figure 2.1**
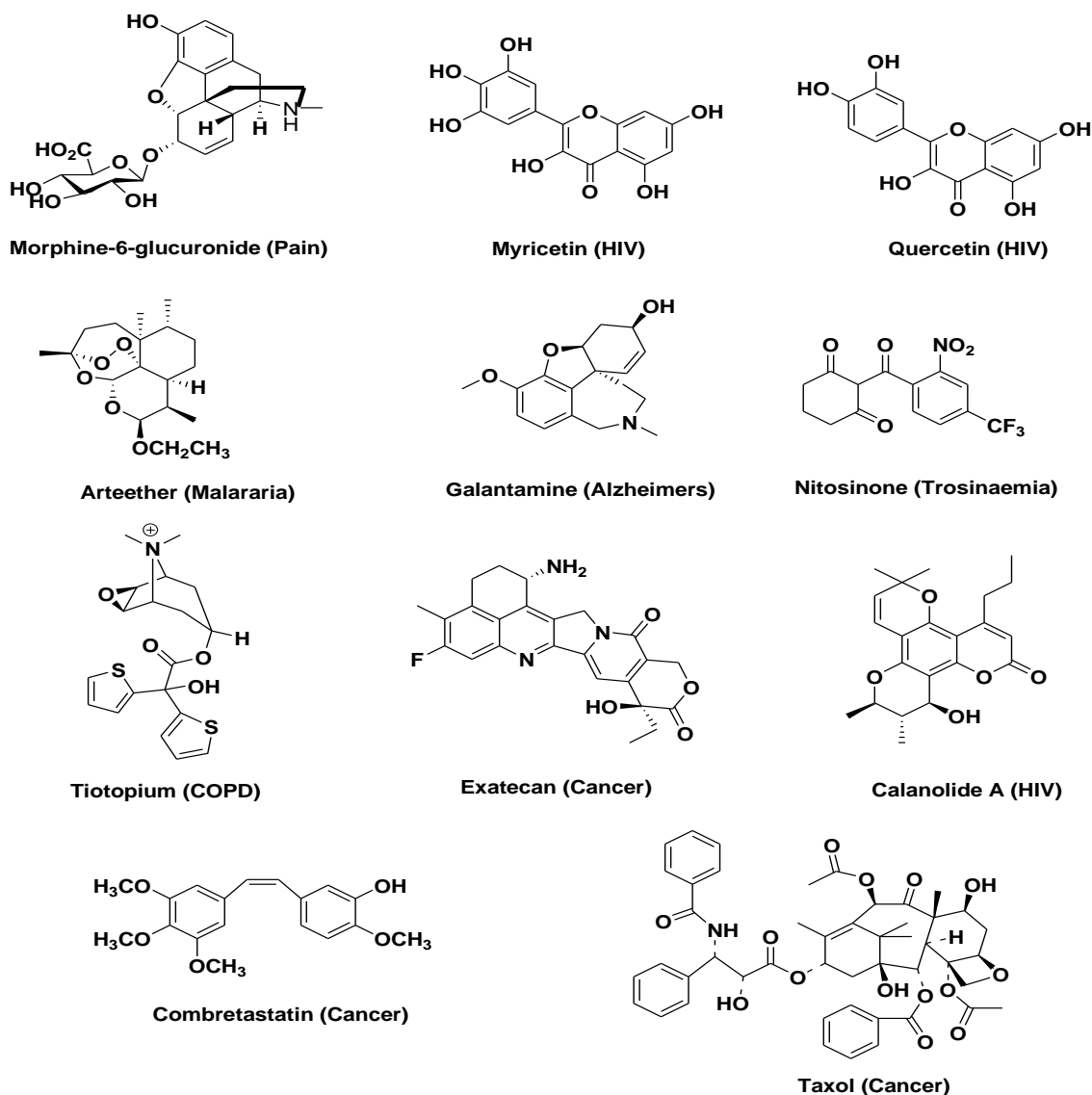
Figure 2.1: Chemical structures of plant-derived drugs used to treat various ailments

The evidence presented above clearly depicts why it is imperative to continue to conduct research in the field of natural products for drug discovery. NPs account for more drugs produced worldwide than chemically synthesized small molecules. Plants have yielded pharmaceutically active drugs due to the fact that plant-derived small-molecules have chemical structures that have been biologically prevalidated by evolutionary pressures to selectively and effectively interact with their protein targets to either inhibit or activate them.

2.4 Medicinal plants and HIV/AIDS

The urgent need for new anti-HIV/AIDS drugs is of a global concern. In addition to the obvious economic and commercial hurdles, HIV/AIDS patients are faced with diverse difficulties associated with the currently approved anti-HIV drugs. Adverse effects, the emergence of drug resistance and the narrow spectrum of activity have limited the therapeutic usefulness of the various reverse transcriptase and protease inhibitors that are currently available on the market. However, it should be noted that ARVs have extended the life span of HIV-positive patients and made AIDS a manageable disease. The above-mentioned disadvantages associated with the use of ARVs has driven many scientists to look for new anti-retrovirals with better efficacy, safety and affordability. As has always been the case in the search for cures, natural sources offer great promise. Several natural products, mostly of plant origin, have been shown to possess promising activities that could assist in the prevention and/or betterment of the disease.

Through scientific screening of plants, a number of medicinal plants have been reported to possess anti-HIV properties (Chinsembu and Hedimbi, 2010; Cos et al., 2004; Singh et al., 2011; Yang et al., 2001). The anti-HIV properties of plant compounds are through the interaction or inhibition of the viral enzymes RT, PR or IN (Cos et al., 2004; Klos et al., 2009; Singh et al., 2011; Vermani and Gard, 2002; Yang et al., 2001). For example, Klos and colleagues (2009) showed that ethanolic extracts of *Bulbine alooides* and *Leonotis leonurus* possess HIV-1 PR inhibition. **Table 1** below depicts the plants used to treat HIV/AIDS.

Table 1: Plant-derived compounds that possess anti-HIV-1 activity and their targets

Plant	Compound	Class	Target	Reference
Quercus rubra	Faicalein, quercetin, myricetin, baicalin	Flavonoid	Reverse transcriptase	Cowan, 1999
Cephaelis ipecacuanha	Psycotrines	Alkaloid		Tan et al., 1991
Coriandrum sativum	Coriandrin	Coumarin		Vlietinck et al., 1998
Hyssop officinalis	Caffeic acid	Tannin		Houghton et al.,
Tryptarygium wilfordii	Salaspermic acid	Flavonoid		1994
Momordica charantia,	MAP30, GAP31, DAP32, DAP30	Proteins	Integrase	Wang et al., 1999
Gelonium				Lee-Huang et al.,
multiflorum	Quercetin	Flavonoid		1991
	Thalassiolin A, B & C			Cowan 1999
Quercus rubra				Rowley et al., 2002
Thalassia testudinum				
Rosmarinus officinalis	Carnosolic acid	Terpenoids	Protease	Vlietinck et al.,
	Ursolic acid	Terpenoids		1998
Geum japonicum	Ganoderic acid-a	Terpenes		Cowan 1999
Ganoderma lucidum	Ganoderiol F			Yadav et al., 2009
	Ganodermontriol			
	Ganoderic acid B			
Coriandrum sativum	Glycocoumarin	Coumarine	Adsorption	Vlietinck et al.,
	Licopyranocoumarin			1998
Schumanniophyton magnificum	Schumannificine 1	Alkaloid		Houghton et al., 1994
Prunella	Prunellin	*PS		Cowan 1999

*PS= Polysaccharides

No single herbal plant has been used to treat HIV patients so far; but instead herbal concoctions have been used. Herbalists usually use herbs that seem to have logical association with the AIDS disease. The herbs used are those usually used for the treatment of viral infections such as hepatitis B and those used for the treatment of the clinical symptoms of AIDS viz. wasting, diarrhoea, lymphadenopathy, skin lesions, cough, haemoptysis and genital ulcers (Klos et al., 2009).

Despite all the evidence presented above, SA is still at its infancy with regards to utilizing the rich plant biodiversity in order to harness its benefit for human health. Thus, we have only scratched the surface of this wonderful resource of natural chemicals with its vast potential for the development of new drugs for medicinal use. This is why it is imperative to continue with natural product research.

2.5 Medicinal plants used in this study

For the purpose of this study, six medicinal plants were investigated. The plants were collected from Kwa Zulu Natal (KZN). Different parts of the plants were used for extraction, viz. leaves, stems, bark and rhizomes. These plants are used by the traditional health practitioner (THP) to treat HIV positive patients. A literature review on these plants was also conducted so as to ascertain whether studies were conducted with regard to HIV research and also to evaluate if they possess antiviral and antimicrobial activity. Table 2 depicts the family, species name, traditional name and plant parts used in this study. Detailed information on each plant is given below.

Table 2: List of plants to be evaluated for anti-HIV-1 activity in this study

Family	Plant species	Traditional name	Plant part used
Amaryllidaceae	*Clivia miniata*	Mayime	Leaves and rhizomes
Canellaceae	*Warbugia salutaris*	Isisbhaha	Bark
Hyacinthaceae	*Eucomis autumnalis*	Umathunga	Bulbs
Hyacinthaceae	*Scilla natalensis,*	Inguduza	Bulbs
Malvaceae	*Hibiscus surattensis L.*	Ucathucathu	Stems
Solanaceae	*Solanum aculeastrum*	Intuma	Fruits

2.5.1 *Scilla natalensis*

Background

This is a bulbous perennial plant with tall plumes of blue flowers. The wild squill belongs to the family Hyacinthaceae (hyacinth family). It has a large bulb covered with firm, hardened, papery brown or purplish tunics (bulb scales) (van Wyk et al., 1997). The plant is selectively toxic to mammals. It is said to be poisonous to livestock, mainly when the young leaves appear in spring. Apparently, it is toxic to humans when raw. It is reported that even the sap burns the skin. It is said that for any preparations to be taken internally, the plant must first be heated (Hutchings et al., 1996; van Wyk et al., 1997).

Common names

Scilla natalensis is commonly known as the Wild squill (English), Blue squill (English), Blue hyacinth (English), Blouberglelie (Afrikaans), Blouslangkop (Afrikaans), Inguduza (Zulu) (South African Department of Agriculture, Forestry and Fisheries, 2013; van Wyk et al., 1997).

Figure 2.2: *Scilla natalensis* **flowers and bulb** (Courtesy: Van Wyk et al., 1997)

Location

It is deciduous, growing during summer and dormant in the winter. *S. natalensis* is found in Eastern Cape, Kwa Zulu Natal, Free State, Mpumalanga provinces and also in Swaziland and Lesotho. *S. natalensis* is found in various habitats which range from sunny slopes, rocky hills,

cliffs, damp cliff faces, near waterfalls, moist depressions, streams and wetlands edges. It is usually found in groups or as solitary specimens (South African Department of Agriculture, 2013; van Wyk et al., 1997).

Medicinal uses

In South Africa, the bulb is used medicinally and is one of the most popularly traded traditional herb items in KwaZulu-Natal. Warmed fresh bulb scales, slightly burned bulb scales and decoctions of the bulb are used externally as ointments for wound-healing, to treat sprains, fractures, boils and sores and to draw abscesses. The ash from a burnt plant, and the bulb in powdered form, is rubbed into cuts and scratches, and over sprains and fractures. Decoctions are taken as enemas for female infertility and to enhance male potency and libido. It is also known to be used as a purgative, a laxative, for internal tumors, and is used in conjunction with other ingredients in infusions taken during pregnancy to facilitate delivery and in treatments for chest pain and kidney troubles (Hutchings et al., 1996; South African Department of Agriculture, 2013; van Wyk et al., 1997).

The plant has significant analgesic and antimicrobial activity, and has been reported to possess phytochemicals that have anti-inflammatory and anti-mutagenic properties (Hutchings et al., 1996; van Wyk et al., 1997).

Chemical compounds

Compounds isolated from this plant species include the following: Scillaren A, Proscillaridin A and Scillarenin. The latter compound has been shown to have antiviral activity (Sato et al., 1974).

2.5.2   *Solanum aculeastrum*

Background

This is an evergreen perennial shrub with lobed discolorous leaves with sharp prickles found on the branches and it has a poisonous apple-like yellow fruit. Apple of Sodom belongs to the family Solanaceae.

Common names

Solanum aculeastrum is commonly called the Apple of Sodom, goat apple, poison apple (English), bitterappel, doodenappel, gifappel (Afrikaans) and intuma (Zulu) (Hutchings et al., 1996; van Wyk et al., 1997).

Figure 2.3: *Solanum aculeastrum* fruit (Courtesy: Van Wyk et al., 1997)

Location

This plant is found in various regions in Africa. In southern Africa, the plant is commonly found in Limpopo, Mpumalanga, Kwa Zulu Natal, Western Cape and Eastern Cape provinces and Swaziland (van Wyk et al., 1997). This plant appears not to be discriminative in terms of the area of the land it grows. The plant grows naturally in grassland, woodland and in forest margins. It grows in both gentle and steep slopes and also in all soil types ranging from sandy, clay loam and reddish-brown clay loam soil (van Wyk et al., 1997). In South Africa, the plant prefers regions with higher rainfall of more than 700 mm per year and is found from 275- 1780 m altitudes.

Medicinal uses

Medicinally it is used in the Zulu tradition to treat various ailments. The boiled fruit pulp with carefully removed seeds is used as a purgative agent. Ash from the burnt fruit is rubbed into scarifications over painful parts for the relief of rheumatism (Huchings et al., 1996). The bitter fruit is used to treat ringworms in cattle and horses and also anthrax. The Transkei people use the fruits decoctions to cleanse the nave of new born babies. The vhaVendas use the plant as an antiemetic agent (Huchings et al., 1996; van Wyk et al., 1997).

Chemical compounds

The plant is reported to possess Solanine which is a glycoalkaloid poison possessing fungicidal and pesticidal properties. The plant also possesses Tomatidine, Solasodine, Tolamargine, Tomatidenol and Tomatine (Hutchings et al., 1996).

2.5.3 *Eucomis autumnalis*

Background

This is a summer-growing deciduous bulb plant. Pineapple Flower grows in grassland, forest, swamps and river banks. The plant does not grow in the drier areas. It belongs to the Hyacinthaceae family of plants which was previously known as the Liliaceae (lily family). The bulbs are large (8-10 cm diameter), ovoid in shape, and give rise to a rosette of large, broad, soft-textured leaves (van Wyk et al., 1997).

Common names

This plant is commonly known as the Pineapple Flower (English), Pineapple Lily (English), Wildepynappel, Krulkoppie (Afrikaans) and Umathunga (Zulu).

Figure 2.4: *Eucomis autumnalis* **flowers and bulb** (Courtesy: Van Wyk et al., 1997)

Location

Eucomis autumnalis is found in Africa, Eurasia and North America but most richly represented in southern Africa (van Wyk et al., 1997). In South Africa Pineapple Flower is found in Limpopo, Mpumalanga, Gauteng, Free State and Eastern Cape provinces (van Wyk et al., 1997).

Medicinal uses

Although the bulb is toxic, *Eucomis autumnalis* is used medicinally in South Africa. Decoctions of the bulb in water or milk are usually administered as enemas for the treatment of lower backache, to assist in post-operative recovery, and to aid in healing fractures. The aqueous concoctions are also used for a variety of ailments, including urinary diseases, stomach ache, fevers, colic, flatulence, hangovers and syphilis, and to facilitate childbirth.

E. clavata which is a subspecies of *E. autumnalis*, is used for the treatment of coughs and respiratory ailments, biliousness, lumbago, blood disorders, venereal diseases and to prevent premature childbirth (Hutchings et al., 1996).

Chemical compounds

Previous studies by Finckh and Tamm, (1970); Sidwell and Tamm, (1970) showed that the homoisoflavanoids are some of the chemical constituents of this plant species. These metabolites have recently been shown by Famuyiwa and colleagues (2013) to possess the anticancer, bactericidal, anti-Alpha glucosidase and antioxidant activity. Of note from these activities is the anti-α-glucosidase activity which has been implicated in preventing HIV fusion. Several compounds have been isolated from this plant species and they fall into different phytochemical classes. The isolated compounds include: Eucomnalin, 3,9-dihydroeucomnalin, Autumnariol, Autumnariniol and Eucosterol.

2.5.4   *Clivia miniata*

Background

This is a perennial evergreen herbal plant with pseudobulbs and/or thick rootstocks belonging to the family Amaryllidaceae. It has dark green strap-shaped leaves that emerge from the underground stem (Hutchings et al., 1996). The plant produces brilliant orange flowers that are trumpet shaped. Flowering occurs mainly during spring around August/November periods and sometimes flowering also occurs at other times of the year (van Wyk et al., 1997).

Common names

Commonly the plant is known as Bush lily (English); Boslelie (Afrikaans); umayime (Zulu).

Figure 2.5: *Clivia miniata* **flowers and rhizomes** (Courtesy: Van Wyk et al., 1997)

Location

Bush lily is endemic to SA. The bush lily plants seen around the world have been obtained from SA and breeders around the world, especially Japan, produced variants of this plant species. It is found naturally along the eastern region of South Africa, typically, in Kwa Zulu Natal, Eastern Cape, Mpumalanga provinces and also Swaziland. There is no specific habitat for this plant and, as such, it is found in subtropical coastal forest to valleys in high altitude forest. Bush lily grows in soil that is well drained and humus rich and in dappled shade area, often in large colonies.

Medicinal uses

Medicinally, the plant is used to treat various conditions. The Zulu tribe uses the roots infusions to treat snake bites and to apply the infusions on the wound. It is are also taken to induce or facilitate childbirth, to treat fevers. The Xhosa tribe use the bulb decoctions to treat infertility and urinary complaints (Hutchings et al., 1996). Crude root and leaf extracts have been shown to possess antiviral activity against poliomyelitis virus and measles virus (Hutchings et al., 1996).

Chemical compounds

Compounds isolated from this plant include: Lycorine, Clivacetine, Clivoine, Cliviasine and Clividine.

2.5.5   *Hibiscus surattensis L.*

Background

This is an annual prostrate or climbing herbaceous plant which is characterized by having prickly stems with recurved prickles and the ovoid or globular shaped fruit (as seen in **Figure 2.6**). The plant belongs to the family Malvaceae.

Common names

Commonly the plant is known as wild sour; bush sorrel (English), Liane oseille (French), Isigezo, Ucathucathu, Uvemvane (Zulu) (Hutchings et al., 1996).

Figure 2.6: *Hibiscus surattensis* **L. stems, leaves and flowers** (Courtesy: van Wyk et al., 1997)

This plant is distributed in KwaZulu-Natal, Limpopo and Mpumalanga provinces in South Africa. The habitats that provide ideal growth parameters for this plant species include grasslands and forest edges found in lowland and medium altitudes up to 1700 m. It grows well in regions with an average rainfall of 1000-1600 mm annually. *Hibiscus surattensis* L is also found in mashes, neglected fields and farms and in coastal habitats such as sand dunes. *H. surattensis* L is found on a wide variety of soil types (Hutchings et al., 1996; van Wyk et al., 1997).

Medicinal uses

The pounded leaf and stalk lotions mixed with *Warbugia salutaris* and any kind of fat are used by the Zulus to treat penile irritations and sores. Milky liquid from the strained leaves and stalks are mixed with water and injected into vagina or penis for the treatment of discharge or sores from venereal diseases. The leaves are rich in mucilage and hence in some parts of Africa like, Nigeria, the plant is cooked and consumed as a soup. In Senegal, the plant is used as a moisturizer (Hutchings et al., 1996; van Wyk et al., 2000).

2.5.6   *Warbugia salutaris*

Background

This is an evergreen, slender tropical forest tree that grows up to 20 m tall. The plant has characteristic dark green, glossy leaves and white to greenish flowers. *W. salutaris* belong to the plant family called Canellaceae.

Common names

It is commonly known as pepper-bark tree (Eng.); peperbasboom (Afr.); Isibhaha (Zulu); manaka (Venda); shibaha (Tsonga) (Jager et al., 1996)

Figure 2.7: *Warbugia salutaris* tree and bark (Courtesy: Van Wyk et al., 1997)

Location

This plant grows naturally along the east region of South Africa as far as KwaZulu-Natal, Eastern Cape and also it occurs in eastern and northern Gauteng province and across Swaziland. It also occurs in Malawi. The ideal growth habitat that allows propagation of this plant is forests and kloofs (van Wyk, 2008).

Medicinal uses

Medicinally, pepper-bark tree is used to treat several ailments by various tribes in South Africa and some parts of Africa. Zulus use powered bark together with a spoonful of cold water and *Cannabis sativa* leaves to treat dry coughs. Tree barks are also used as emetics and

purgatives for fever complaints and rheumatism. Sometimes the bark is used to treat chest pains such as intercostal neuralgia. The powered bark mixed with fat is used to treat penile irritation, venereal diseases. Lotions prepared by mixing *Hibiscus surratensis* with the pounded leaves with stalks are applied to the penis in cases of inflammation of the urethra, sores and other irritations. In West Africa, the bark and the roots are used to treat influenza, fevers and gastro-intestinal disorders (Hutchings et al., 1996).

Chemical compounds

The bark is reported to have tannins, drimane sesquitepenoids and some alkaloids with antimicrobial activity (Hutchings et al., 1996). Warburganal, polyoidal, salutarisolide, muzigadial, ugandensidial, isopolygodial, mukaadial and mannitol are some of the compounds isolated from this plant (Van Wyk et al., 2000).

Rationale

In South Africa, the anti-HIV-1 activity of various indigenous plants has not been studied extensively. Most of the work has focused on anti-cancer activity with less attention given to infectious diseases. South Africa, to date, is faced with a high HIV prevalence and incidence rates that presents issues with ensuring that all individuals in need of HAART treatment receive it. Furthermore, even people who have access to HAART are still faced with toxic side effects from drugs used for treatment and viral resistance to this treatment which renders it less efficacious.

These challenges justify the need for a continued search for new drug candidates with better activities such as low toxicity and affordability. It has been documented that most of the African and Asian countries still rely on traditional medicines to treat various ailments including infectious diseases like, HIV. There are fewer side effects that have been documented with regards to using traditional medicines. Furthermore, it has been shown that plant-derived small molecules exhibit better scores of 'drug-likeness' than synthetic compounds. Plant-derived NCEs have provided scaffolds for a majority of FDA approved drugs for treating various conditions including HIV and cancer. Also, they have chemical structures that have been biologically prevalidated by evolutionary selection for binding with protein targets. Plant-derived compounds have more chiral centers and far fewer aromatic rings, which gives NPs better selectivity when binding to stereo-defined protein sites compare to synthetic drugs. The above-mentioned drug properties are crucial in drug discovery because they ensure drug flexibility, specificity and reduce the drug resistance profile of a pathogen targeted.

Hence, the aim of this study is to identify, screen, phytochemically characterize and isolate bioactive compound(s) from a concoction presented by traditional herbalists suggesting that "a cocktail of these six plants: *Clivia miniata, Warbugia salutaris, Eucomis autumnalis, Scilla natalensis, Hibiscus surattensis* L. induces anti-HIV proliferation in HIV-infected individuals''. However, this has never been tested or validated scientifically, thus the interest of this particular study in investigating the claimed hypothesis.

Aim

The study is aimed at determining the anti-HIV-1 activity of a South African herbal concoction and characterization of the potential bioactive compound(s) therein.

Objectives

- To determine the toxicity of the crude extracts and compounds

- Determine HIV viral inhibition by the plant extracts and isolated compounds using pseudovirion infection TZM-b1 assay

- Evaluate whether the extracts can inhibit HIV-1 reverse transcriptase and protease activities

- Phytochemical profiling of the herbal mixture extracts

- Isolation of major phytochemical compounds from the herbal mixture

- Structure elucidation and characterization of potential bioactive compound(s)

3   CHAPTER 3

OVERVIEW OF THE METHODS AND MATERIALS

Phytochemistry studies on medicinal plants are important because they afford a phytochemical profile of the plant extract. Through phytochemical profiling of the plant extracts, therapeutic compounds can be identified which can justify the use of that plant by traditional health practitioners (THPs) to treat various ailments. Phytochemical studies also play an important role in modern drug discovery as the characterized isolated compounds can be used as drug leads. Plant-derived compounds can be chemically modified to enhance bioactivity, bioavailability and reduce toxicity during the development of novel drugs.

In this study, the extraction procedures that were followed are based on those used by the traditional health practitioners and the scientifically accepted extraction procedures (i.e. using organic solvents) for standardization and comparison of the actives present in the different preparations.

Therefore, this chapter focuses on the materials and methods used in this study, such as the following:

- preparation of dried plant material and preparation of extracts
- chemical profiling of extracts using Ultra-Pressure Liquid Chromatography-Mass Spectrometry (UPLC-MS)
- isolation, purification and identification of compound(s)
- screening of extracts and isolated compounds for toxicity
- screening the extracts and isolated compounds against HIV enzymes: RT, PR, and for inhibition of HIV entry into cells

The following studies were conducted in the Biosciences Unit of the Council for Scientific and Industrial Research (CSIR) based in Pretoria.

3.1 PLANT COLLECTION

The ethnobotanical method was used for the collection of plant material used in this study. This method relies on the knowledge and use of the medicinal plants by local, indigenous people and, in this case, traditional health practitioners. Plants were collected from Kwa Zulu Natal (KZN) province in South Africa (**Table 3**).

Table 3. The plant species used in this study and their location within South Africa.

Plant species	Location	GPS coordinates
Clivia miniata	Ngwavuma (KZN)	$27^0$07'55"S; $31^0$59'39.9"E
Eucomis autumnalis	Bergville (KZN)	28^o44'0" S; 29^o22'0" E
Hibiscus surratensis L.	Mondlo (KZN)	27^o58'58.76" S; 30^o43'3.68" E
Scilla natalensis	Bergville (KZN)	28^o44'0" S; 29^o22'0" E
Solanum aculeastrum	Bergville (KZN)	28^o44'0" S; 29^o22'0" E
Warbugia salutaris	Ngwavuma (KZN)	$27^0$07'55"S; $31^0$59'39.9"E

Harvested plants were washed with distilled water until no foreign object was observed and damaged leaves were removed so as to reduce contamination and false positive results in biological screening studies. The plant material was oven dried at 30–60 °C. The drying conditions such as temperature and time (days) varied from plant to plant due to the different plant parts used. Dried material was pulverized and stored at room temperature (25 °C) prior to extraction.

3.2   PLANT EXTRACTION

3.2.1   Maceration

The principle of the extraction method used in this study

This is basically the extraction of phytochemicals through soaking the crushed plant material in an organic solvent such as methanol, ethanol, chloroform, dichloromethane etc. or non-organic solvent such as water at a particular temperature for a defined period of time. Sometimes a mixture of the organic solvents is used for extraction of phytochemicals (e.g. dichloromethane: methanol). Maceration according to Raman & Houghton (1998) "is the extraction of phytochemicals or other chemicals involving bringing the solid material to be extracted (that has been thoroughly dried) into contact with the extraction solvent for a period of time, followed by separation of the solution from the solid debris". This technique exploits the polarity principle of chemicals (i.e. "Like dissolves like"). Depending on the type (polarity) of chemical targeted for a particular study, different organic solvents with different polarity can be used to extract compounds that are either polar or non-polar. One of the advantages of this method is that compounds which degrade at elevated temperatures are also extracted and not degraded. This method was used in this study to prepare organic extracts for all six plants and also for the herbal mixture.

For extraction with water, heat is often required so as to maximize extraction. This method is often used by traditional healers to prepare decoctions which are given to their patients. In this extraction method, dried plant material is placed in pure distilled water and then boiled at a defined temperature and duration (as specified by the traditional healer). To ensure even and controlled temperature conditions, a heating mantle, water bath or oil bath can be used. The preparation of aqueous extracts for all six individual plants and the herbal decoction used in this study, was done through the use of an oil bath whereby the soaked solution was boiled using an oil bath. This method was chosen over the water bath method because the dried plant material had to boil at 98-100 $^{\circ}C$ similar to that used by the traditional health practitioner. A water bath would, therefore, not be appropriate since water boils at 100 $^{\circ}C$ and would, therefore, evaporate off over time.

Preparation of aqueous extracts

Dry powdered plant material (100 g) of each plant was used in the preparation of the herbal decoction. The dried, ground plant material of six plants were combined and then added to 6 L of high-purity dH_2O and boiled at a temperature of 98 ^{o}C for 1 hour while stirring using a 3-neck round-bottom flask half submerged in an oil bath. The temperature of the boiling solution was monitored with a thermometer. After cooling, the mixture was filtered using a sterile white cloth and the resulting filtrate was then further filtered by using Whatman number 1 filter paper with a pore size of 11 μm (Sigma-Aldrich, USA).

Water extracts of the six individual plants were also prepared following the aforementioned procedure using 30 g of plant material and 350 mL high-purity dH_2O. Following filtration, the filtrate was dried and concentrated overnight using a Genevac speed vacuum concentrator (United Scientific, USA) The following day the extracts were further dried under reduced pressure using a vacuum pump. The extracts were stored at -20 ^{0}C until it was required.

Preparation of organic extracts

The organic solvent used for extraction in this study was absolute ethanol (Sigma-Aldrich, USA). The choice of solvent was motivated by the fact that it has low toxicity, evaporates off easily, has antimicrobial activity, does not cause complexation or dissociation of the extract, it extracts higher amounts of polyphenols, bioactive flavonoids as well as aromatic and organic compounds (Lapornik et al., 2005; Bimakr et al., 2010; Wang et al., 2010 & Cowan et al., 1999).

The ethanolic herbal extract was prepared by mixing 100 g of the individual dried plant material resulting in 600 g dried herbal plant material which was then macerated with 6 L of absolute ethanol overnight at 28 ^{o}C in a shaker at 180 rpm. Following maceration, the extract was filtered with 11 μm Whatman filter paper (Sigma-Aldrich, USA). The resulting filtrate was concentrated by evaporating off the organic solvent using the Buchi Rotavapor R-II (Buchi Labortechnik AG, Switzerland) at a temperature of 64 ^{o}C. Furthermore, each of the extracts was further dried under reduced pressure using compressed air at room temperature for 24 hours.

The extracts of the six individual plants were prepared by following the aforementioned procedure but using 100 g plant material in a 1 L absolute ethanol instead. Extracts to be screened for biological activity were stored at -20 ^{o}C until required.

3.3   LIQUID-LIQUID PARTITIONING

The principle of liquid-liquid (LL) partitioning

This method entails using two solvents with different polarity (polar: nonpolar). The main principle of this method is that the solubility of uncharged substances in any phase at a fixed temperature is dependent on its similarity in polarity to the liquid phase (i.e. "like dissolves like"). In this method, a dry extract is solubilized and then mixed with another solvent (that has a different polarity index to the solvent used for solubilizing the extract) in a separating funnel and shaken vigorously. This mixing of different solvents results in the formation of two layers (usually called phases) that have different chemical composition. Polar compounds in the extract will be distributed to the polar phase and nonpolar compounds to the nonpolar phase. This method is suitable for concentrating and "cleaning-up" the extract, i.e. removal of nonessential phytochemicals such as chlorophyll and nonpolar compounds when targeting polar compounds for a particular study.

Preparation of the "clean" extract used in isolation of potential bioactive compounds

Liquid-liquid partitioning was employed in this study to separate the extracts based on polarities and to obtain a higher concentration of polar compounds. The ethanolic organic extract was first dissolved in methanol/water (9:1) and then partitioned with hexane three times using a separating funnel. The methanol/water polar layer which has more polar phytochemicals present in it was collected and concentrated under reduced pressure by using a Buchi Rotavapor R-II (Buchi Labortechnik AG, Switzerland) at 60 ^{o}C. The resulting concentrated extract was further dried under stream of air using compressed air in a fume hood overnight at room temperature. This extract was used for the isolation of potential bioactive compounds.

3.4   CHROMATOGRAPHY

Chromatography is used in the separation of individual constituents within a sample on the basis of their physical properties, e.g. molecular size, shape, charge, volatility, solubility and/or adsorptivity. Different chromatographic systems are characterized by the form of chromatographic bed, nature of the mobile and stationary phases and the method of separation employed (Reed et al., 2007).

3.4.1   Thin layer chromatography (TLC)

The principle of TLC

Chromatography is a technique whereby a sample mixture is separated on the basis of its physiochemical differences e.g. molecular size, shape, charge, volatility, solubility and adsorptivity. A complete chromatographic system is comprised of a stationary phase, mobile phase, delivery system and detection system. The types of stationary phase adsorbents most commonly used in chromatography are silica gel (SiO_2 x H_2O) and alumina (Al_2O_3 x H_2O). Both of these adsorbents are polar, but, alumina is more so. A mobile phase can either be liquid or gas and these carry the sample through the stationary phase and by eluting the sample from the chromatographic bed. Sample separation is through the interaction of the sample with the stationary phase, the stronger the interactions, the more time the sample spends on the stationary phase and, hence, it will be eluted later or have a smaller relative frontal mobility value (R_f) in a TLC separation. The inverse applies when the sample has weak interactions with the stationary phase.

TLC is a sensitive, fast, simple, and inexpensive analytical technique. TLC involves spotting the sample to be analyzed near one end of a sheet of glass or aluminum that is coated with a thin layer of an adsorbent. The sheet, which can be the cut into various sizes, is placed on end in a glass tank containing a shallow layer of solvent. As the solvent rises up the stationary phase by capillary action, differential partitioning occurs between the components of the sample mixture in the stationary adsorbent phase (Fried and Sherma, 1996; Houghton and Raman, 1998).

Some common uses of TLC:

1. To determine the number of components in a mixture.

2. To determine the identity of two substances.

3. To monitor the progress of a reaction.

4. To determine the effectiveness of a purification.

5. To determine the appropriate conditions for a column chromatographic separation.

6. To monitor column chromatography (Fried and Sherma, 1996)

A large number of spray reagents have been produced which, when sprayed onto separated bands/spots on the TLC sheet, results in the formation of different colors from colorless substances. Some of the spray reagents are very specific, but many others will react with various compounds. Therefore, these spray reagents can be used for the detection and identification of particular compound classes present within the sample mixture. (Houghton and Raman, 1998).

Application of TLC in this study

We first used thin layer chromatography (TLC) to obtain an appropriate solvent system for the isolation of compounds using vacuum liquid chromatography and column chromatography. This was done to select the type/class of compounds that was going to be isolated and also to determine the chemical profile of the extracts (i.e. detect the major compound types in the individual extracts). Natural products have both fluorescent and quenching properties. These properties are exploited in the isolation of phytochemicals by using TLC and viewing the separated phytochemicals under UV light.

Aluminum backed TLC plates that pre-coated with silica gel (Merck, SIL G-25 UV $_{254}$, 20 cm x 20 cm) were used to obtain the profiles of the individual plant extracts following separation. These plates have a fluorescent agent incorporated into the silica stationary phase so as to give a uniform, brightly colored background when exposed to UV light. Visualization of the separated phytochemicals was done under UV light (254 and 366 nm,) short and long wavelengths respectively and spraying with vanillin-H_2SO_4 reagent, followed by baking the TLC plate at $100^{\circ}C$ for 1 minute. The use of vanillin-H_2SO_4 reagent was due to the fact that it is not specific for a particular class of compounds like other chromogenic sprays. Sulfuric

acid (H_2SO_4) oxidizes many compounds and hence the vanillin-H_2SO_4 is the ideal reagent to use for getting a profile of the extracts prior to extraction.

In the case of isolating alkaloids, Dragendorff chromogenic reagent was used which is specific for alkaloids.

3.4.2 Vacuum liquid column chromatography (VLC)

Principle of VLC

This is a form of column chromatography that is useful for obtaining fractions rapidly and saves time. It is similar to column chromatography with the exception of using a Büchner funnel (which is relatively short as a column), a Büchner flask for collecting the eluents and reduced pressure to facilitate the mobile phase elution from the column. The principle is based on separating a sample mixture applied onto a tube (column) that is tightly packed with porous particles and allowed to permeate the column under reduced pressure in the presence of a suitable mobile phase solvent. Some variations of VLC include a separating funnel in place of the Büchner flask so as to prevent the disassembling of the apparatus during the collection of eluent aliquots.

Fractionation of crude extracts

The process of isolating compounds from the crude extract began by fractionating the crude extract. Fractionation of the crude ethanolic herbal concoction extract was achieved by using VLC.

A column was dry packed with silica gel 60, (35-75 µm diameter size, Merk, Germany). The packing was done under vacuum to achieve maximum packing density. The solvent was poured and sucked dry under vacuum (reduced pressure) so as to saturate the column prior to the actual fractionation procedure. The sample was loaded in the form of a dry slurry and the vacuum reapplied to settle the sample. Cotton wool was then placed on top of the dry slurry to prevent disruption of the slurry during addition of the eluting solvent. The column was then eluted with an appropriate solvent mixture (or solvent of increasing polarity). In each step, the column was allowed to dry completely and be solvent free. Fractions of 200 ml were collected and were dried at ambient temperature overnight.

3.4.3   Dereplication of ethanol organic extract

Dereplication refers to the removal of unwanted compounds within extracts which, when present, might interfere with the bioassay system and give false positives. The material to be removed from the extracts fall in either of two categories:

1. those present in large quantities which may render isolation difficult and may also interfere with the test assay system. Chlorophyll and carotene pigments fall into this category especially if the extract was prepared from leaves since these have higher quantities of this material.

2. Substances that interact non-specifically with a wide range of enzyme systems. The most notorious group in this class are the polyphenolic substances known as tannins. These compounds form crosslinks with many protein targets, and thus, inhibit biological systems. These compounds should always be removed in drug discovery research that is focused on discovering novel drug leads.

Studies by Cardellina et al., (1993) at the NIH indicated that tannins present in plant extracts are responsible for false positive results especially in HIV studies on medicinal plants. Thus, in the present study, tannins were removed from the herbal concoction to avoid getting false positive results. Tannins were removed by using a protocol outlined by Raman and Houghton (1998) which was slightly modified. Polyvinylpolypyrrolidone (PVPP) (Sigma-Aldrich, USA) was utilized instead of polyamide resins. A VLC column was packed with PVPP instead of silica gel, saturated with dH_2O for 30 mins and then allowed to elute prior to loading the herbal concoction sample mixture to be dereplicated. The sample mixture was dissolved in a minimum volume of methanol and applied to the column. The column was eluted with absolute HPLC-grade methanol until the eluent was clear (Raman and Houghton, 1998).

3.4.4   Column chromatography (CC)

Principle of CC

This is the oldest form of chromatography that is useful for obtaining extract fractions and purifying sample mixtures. Here, a column is packed with a solid stationary phase (such as silica gel), a   sample mixture is then applied to the column and a mobile phase is allowed to move down the column under gravity. The contents in the sample mixture will move down

the column with the mobile phase but at different rates due to their physicochemical interactions with the stationary phase. The separated sample mixture constituents are collected as aliquots. The separation of the sample mixture can be achieved by using either a gradient or an isocratic mobile phase whereby, in the latter case, there is no altering of the mobile phase mixture composition. The stepwise gradient elution on the other hand, is characterized by altering the composition of the mobile phase by either increasing or decreasing the polarity of the solvent system. Major drawbacks of this method are that it is time-consuming and results in poorly resolved 'bands' which require repeated purification steps to achieve good resolution.

Isolation of major compounds from VLC fractionated extracts

This method was employed in order to purify the fractions obtained from VLC and to isolate phytochemicals. Various column sizes were used depending on the amount of sample available and the purification stage. The separation of fractions was generally carried out on a column using silica Gel-60 (35-70 μm diameter) under gravity. Equal volumes of 100 ml were collected and dried overnight at ambient temperature. The fractions were spotted onto a TLC plate to determine whether they could be combined; thus, concentrating the major compounds in those fractions and also to determine whether another round of purification by CC was required or not.

3.4.5 Preparative thin layer chromatography (prep-TLC)

The principle of prep-TLC

This technique is similar to the normal TLC technique. The only difference is the amount of sample applied, the way it is applied and the visualization/detection method. A thin horizontal band of sample not greater than 40 mg is applied at the base of the plate in prep-TLC as compared to applying a spot on a normal TLC plate. A non-destructive method should be used (i.e. avoid chromogenic spray reagents) for visualization of the isolated compounds. If there is no suitable non-destructive method then one can use the spray reagent but only spraying the edge of the plate while approximately 90% of the plate is covered and not exposed to the reagent. The plate is developed much like the way a TLC plate is developed. This technique is only applied at the end of purification, prior to structure elucidation and/or biological testing.

Application of prep-TLC for the isolation of compounds

Isolation and purification of compounds which were visible under UV light and also reactive to the vanillin-H_2SO_4 reagent was achieved by using this technique. This method involves applying a semi-pure sample in the form of a band approximately 1.5- 2 cm from the bottom of the plate. The plate is then developed in a solvent system which has been shown to separate the components present in the semi-pure sample.

Glass plates pre-coated with silica gel (Merck, SIL G-25 UV $_{254}$, 20 cm x 20 cm) were used for isolation of compounds. The samples were loaded onto the plates by using a Pasteur pipette. The plates were then developed in a chromatography tank with the appropriate solvent system and the compounds of interest were detected under UV light and by vanillin-H_2SO_4 or Dragendorff reagent. The bands were then scraped off the plate and dissolved in a solvent system used for the separation and then filtered to remove the silica gel from the filtrate containing compounds of interest. Furthermore, the silica gel was washed with methanol to maximize the recovery of the isolated compounds. The solvent used for recovering the isolated compounds was evaporated overnight at ambient temperature.
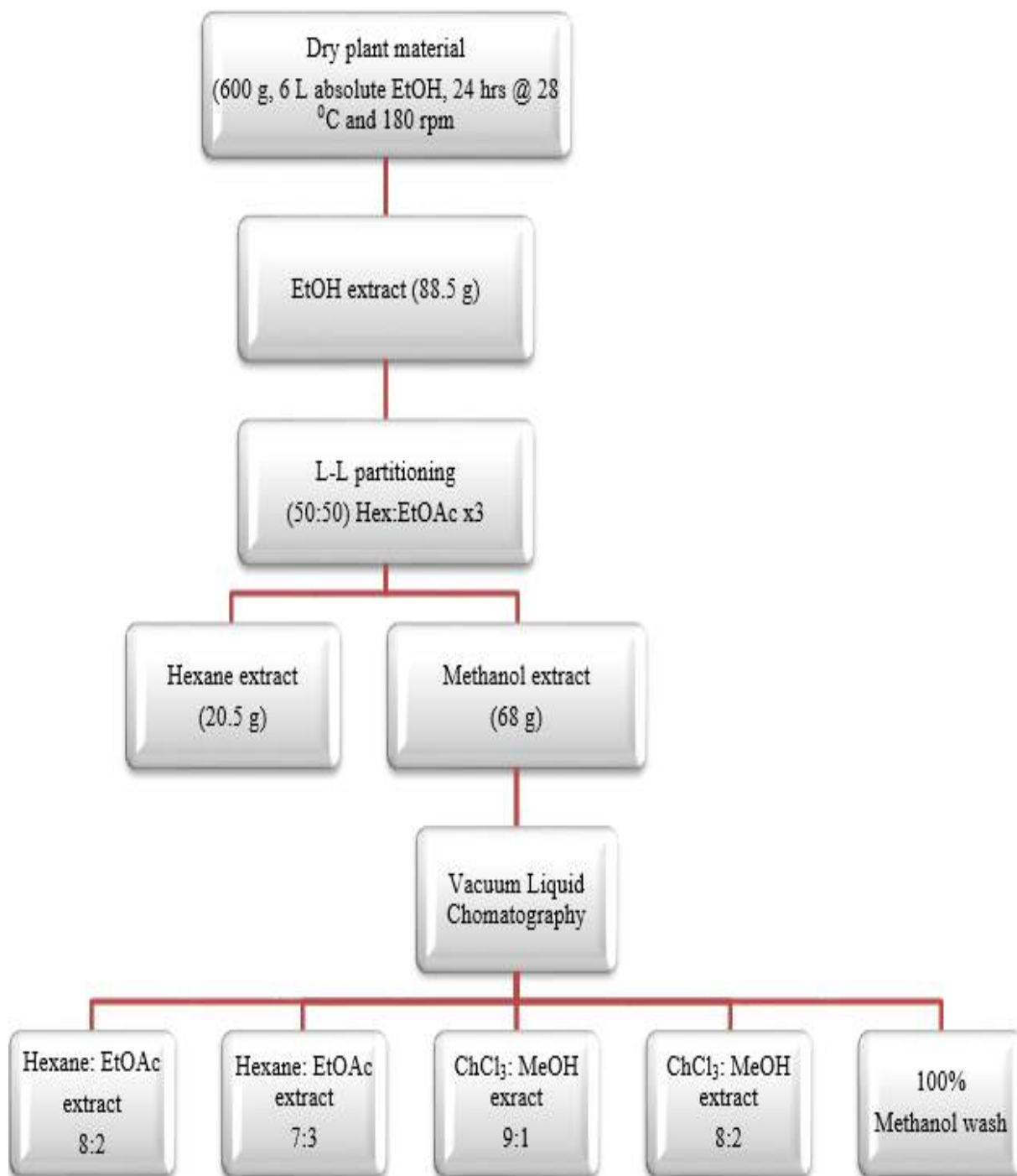
Figure 3.1: Schematic representation of the fractionation of the ethanol herbal concoction.

The flow chart shows the step-by-step fractionation, the conditions, the solvent systems for the different chromatographic methods used to get the various fractions. chloroform ($CHCl_3$), hexane (Hex), methanol (MeOH), ethyl acetate (EtOAc) were used in different ratios (v/v) in the solvent systems.

3.4.6   Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS)

The principle of UPLC-MS

UPLC is a highly developed powerful analytical technique with a wide range of variations mainly used as preparative tools and to obtain chemical fingerprints of plant material (Holme and Peck, 1998). UPLC is a development of column chromatography and allows for improved resolution compared to its counterparts, such as, HPLC. UPLC columns are packed with small sized particles with a narrow size distribution. Flow rate and column dimensions can be adjusted to minimize band broadening. The required pressures are supplied by pumps that can withstand the types of chemicals present.

In addition to the normal phase columns, there are also reverse phase (RP) columns. The latter normally involves the use of a polar solvent (water, methanol, acetonitrile etc.) and a nonpolar surface. RP-UPLC is the method of choice for larger non-volatile molecules.

The commonly used detector (a UV detector) in HPLC and UPLC systems not only places constraints on the solvents that can be used but is also limited to absorbing compounds. Refractive index detectors, although considered "universal", cannot easily be used with solvent gradients. Recently, the evaporative light-scattering detector has emerged as a universal detector. UPLC has been coupled to mass spectrometry to enhance its performance.

Coupling of a mass spectrometer (MS) and Quadrupole Time-of-Flight (Q-TOF) analyzer to UPLC results in a faster analysis time, accurate determination of elemental composition of the molecule, accurate molecular weight determination, and identification of the separated molecules. This technique has been useful in the drug development process. MS alone has been used for lead compound discovery, structural analysis, synthetic development, combinatorial chemistry, pharmacokinetics and drug metabolism (Pavia *et al.,* 2009).

Application of UPLC-QTOF-MS for plant extract profiling and accurate mass determination of isolated compounds

UPLC-QTOF-MS was used for plant metabolite profiling, and accurate mass determination of the isolated compounds. To obtain the phytochemical profile of the plant extracts and the herbal concoctions, the chromatographic separation was done by utilizing a Waters BEH C8

column with the dimensions (150 mm × 2.1 mm, 1.7 μm) at a constant column temperature of 60 °C. A binary mobile phase solvent system was used which was a mixture of acetonitrile (eluent B), water (eluent A), and 0.1% formic acid (Romil Chemistry, UK). The initial conditions were 80 :20 (A:B) at a flow rate of 0.4 ml min⁻¹ and kept constant for 1 min. A stepwise gradient separation method was used to change the chromatographic separation conditions. The overall runtime was 20 min and the injection volume was 5 μl. The PDA detector was scanned between 200 and 500 nm (1.2 nm resolution) and set for collecting 20 spectra s⁻¹. Following the chromatographic separation and detection by UPLC-PDA detection, Q-TOF was conducted so as to get the masses of the separated molecules within the extracts. For detailed conditions for MS-QTOF refer to section **3.6. Table 4** below depicts a stepwise gradient elution timetable used for the chromatographic separation of the molecules within the extracts.

Table 4: Stepwise gradient elution timetable profile

Time (minutes)	Flow rate (ml min⁻¹)	% A (H₂O):0.1% FA*	% B (Acetonitrile):0.1% FA*
Initial	0.4	80	20
1.00	0.4	80	20
15.00	0.4	20	80
15.10	0.4	5	95
17.00	0.4	5	95
18.00	0.4	80	20
20.00	0.4	80	20

*FA= formic acid

3.5   Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear Magnetic Resonance (NMR) spectroscopy is the study of the interaction of electromagnetic radiation (EMR) with matter. NMR spectroscopy is specifically concerned with the interaction of radio frequency (RF) of EMR with unpaired nuclear spins of atoms in the presence of a strong external applied magnetic field. It is fundamental in organic, inorganic and analytical chemistry since it is used for the investigation of molecular structures and dynamics. NMR allows chemists to study 3D structures of bio-molecules at high resolution. It allows the detection of bio-molecule's atomic nuclei and deduction of the

chemical environment the nuclei are in, within the molecule, thus, providing information regarding the structural dynamics. It is routinely used for chemical structure elucidation of simple molecules using simple one-dimensional technique (1D-NMR) and complex two-dimensional NMR (2D-NMR). Two-dimensional techniques (2D-NMR) are used to determine the structure of more complicated molecules (Pretsch et al., 1977; Kumar et al., 2013) such as natural products compounds.

3.5.1 1D-Proton NMR (^{1}H-NMR)

The principle

Proton NMR is both a qualitative and quantitative technique used for studying different protons in a compound under study. A ^{1}H-NMR experiment involves exposing hydrogen nuclei to an applied magnetic field to generate two spin states of unequal energy. The low energy spin state nuclei absorb RF energy to facilitate changing the orientation of the spin state with respect to the applied magnetic field. As this happens, signals arising from absorption of RF as a function of intensity against frequency (chemical shift) are plotted to offer a proton spectrum (Kumar et al 2013).

The ^{1}H-NMR plot gives information regarding the area under the peak which is crucial in determining the number of protons present in the molecule. It gives the position of the signals (the chemical shift, δ) which reveals information regarding the chemical and electronic environment of the protons. And lastly, it provides the splitting pattern of the protons which is used to determine the number of neighboring (vicinal or geminal) protons (Derome, 1987; Ernst, 1987; Abraham et al., 1988; Silverstein et al., 2005; Kumar et al., 2013).

Structure determination of the isolated compounds

^{1}H-NMR was used to obtain the proton spectrum which was crucial in evaluating the purity of the isolated compounds, data regarding the number of protons, splitting pattern and the type of protons present in the isolated compounds. All ^{1}H-NMR experiments were performed on either the Varian 600 MHz or 400 MHz spectrophotometer at the CSIR. All NMR spectra were recorded at 30 °C and the chemical shifts were recorded in parts per million (ppm) frequency referenced to the solvent shift. Coupling constants (J-values) are calculated as observed in the ^{1}H-NMR spectrum. Deuterated methanol (CD_3OD) was used for polar

compounds and deuterated chloroform (CDCl$_3$) was used for semi-polar and/or non-polar compounds.

3.5.2   1D-Carbon NMR (^{13}C-NMR)

The principle

Similar to proton NMR, carbon NMR involves exposing carbon nuclei (^{13}C) to the applied magnetic field to generate two spin states of unequal energy. The low energy spin state nuclei absorb RF energy to facilitate changing the orientation spin state with respect to the applied magnetic field. As this happens, signals arising from the absorption of RF as a function of intensity against frequency (chemical shift) are plotted to obtain a carbon spectrum. The signals in ^{13}C-NMR experiments normally appear as singlets because of the decoupling of the attached protons. Different techniques of recording of the 1D carbon NMR experiments have been developed so that it is possible to differentiate between the various types of carbons; such as, primary, secondary, tertiary and quaternary from the 1D ^{13}C-NMR plot. The range of the chemical shift values differs between the ^{1}H (normally 0-10 ppm) and ^{13}C NMR (normally 0-200 ppm) because of the two nuclei having different numbers of electrons around their corresponding nuclei as well as different electronic configurations (Derome, 1987; Ernst, 1987; Abraham et al., 1988; Silverstein et al., 2005).

Determination of the carbon backbone of the isolated compounds

^{13}C-NMR experiments were conducted to verify the purity of the isolated compounds, (as seen in the proton spectrum), to identify the number of carbons and also the type of carbons present in the isolated compounds. All ^{13}C-NMR experiments of the isolated compounds were done on either the Varian 600 MHz or 400 MHz spectrophotometer. All NMR spectra were recorded at 30 °C and the chemical shifts were recorded in ppm frequency referenced to the solvent shift. Deuterated methanol (CD$_3$OD) was used for polar compounds and deuterated chloroform (CDCl$_3$) was used for semi-polar and/or non-polar compounds.

3.6 High-Resolution Time-Of-Flight Mass Spectrometry (HR-TOF-MS)

Principle of mass spectrometry

Mass spectrometry is an analytical technique that involves generating charged particles (ions) from molecules of the analyte. The generated ions are analyzed to provide information about the molecular weight of the compound, the elemental composition and its chemical structure.

There are many types of mass spectrometers and different sample introduction techniques which allow a wide range of samples to be analyzed. All mass spectrometers consist of three distinct regions that can be described as the ionizer, the ion analyzer, and the detector. The variations of MS are mainly due to the differences in the type of ionizer and ion analyzer they are coupled to. These analyzers have different properties and hence the choice of a particular ionizer and ion analyzer is governed by the type of chemical analysis that is required (i.e. evaluation of non-volatile or volatile compounds and/or exact mass or a rough molecular mass estimate of the compound). Some of the widely-used ionizers include Electron Impact (EI), Chemical Ionization (CI) and Electrospray Ionization (ESI). The ion analyzers include Quadrupole Time-of-Flight (Q-TOF) and Ion Cyclotron Resonance (ICR). Q-TOF allows for a more accurate mass analysis (Snyder et al., 1979).

Application of HRTOF-MS

This method was used to determine the molecular mass of the isolated compounds. HR-QTOF-MS provides accurate mass of the compounds to four decimal places which results in a decreased probability of misidentifying a compound. The conditions for MS-QTOF were as follows: SYNAPT G1 High Definition Mass Spectrometer operating in electrospray ionization (ESI) mode to detect the compounds of interest. Leucine enkephalin (50 pg ml^{-1}) was used as reference calibrant to obtain typical mass accuracies between (556.2771 / 554.2615 Da). The MS was operated in both positive and negative ionization modes (ESI+ and ESI-) with a capillary voltage of 2.5 kV, sample cone voltage of 30 V, extraction cone voltage of 4 V. The scan time was 0.1 s covering the m/z range of 100 to 1,000 Da. The source temperature was 120 °C and the desolvation temperature was set at 450 °C. Nitrogen gas was used as the nebulization gas at a flow rate of 800 L h^{-1}. The software used to control the hyphenated system and perform all data analysis was MassLynx 4.1 (SCN 704).

3.7   BIOLOGICAL ASSAYS

The following studies were conducted at the National Institute of Communicable Diseases (NICD), a division of the National Health Laboratories Services (NHLS) in Sandringham.

3.7.1   Cell lines

HEK293T

Human HEK293T cell line (AIDS Reagent Program NIH, USA) which was originally known as 293tsA1609neo, is a highly transfectable derivative of human embryonic kidney 293 cells (HEK293), which contains the SV40 T-antigen (DuBridge et al., 1987). This cell line is competent to replicate vectors carrying the SV40 region of replication. It gives high titres when used to produce retroviruses. It has been widely used for retroviral production, gene expression and protein production (NIH AIDS Reagent Program).

TZM-bl cell line

The TZM-bl cell line is a derivative of the HeLa cell line (AIDS Reagent Program NIH, USA). TZM-bl is a CXCR4 positive cell line which is genetically engineered to express both the CCR5 chemokine co-receptor and CD4 receptor. The cells are also engineered to express the two reporter genes, Luciferase and β-galactosidase, which are both under the control of an HIV-1 promoter. The TZM-b1 cells are highly susceptible to infection with various HIV-1 isolates and SIV and SHIV strains (Montefiori, 2005).

3.7.2   Tissue Culture

Cells were grown and maintained in 75 cm^2 tissue culture flasks (T-75) (Greiner, Germany) with Dulbecco's modified Eagle's medium (DMEM) growth medium (Gibco, USA) supplemented with 10% FBS (fetal bovine serum) (HyClone, USA). The media was further supplemented with final concentrations of 0.05 mg/ml antibiotic gentamycin (Sigma Aldrich, USA) and 0.025 HEPES buffer (Gibco, USA). Cells were routinely maintained at 37 °C in a 5% CO_2 in a humidified incubator. Cells were maintained by replacing old growth medium (DMEM) with equal amount of fresh growth medium after rinsing once with 5 ml PBS when not confluent. The culture flasks were routinely examined for changes using an inverted microscope and this determined the frequency of media changes. The cells were sub-cultured

when at near-confluency, at least once per week, and excess sub-cultures were preserved at ≥ -70 °C in DMEM growth medium supplemented with 20% FBS and 10% DMSO (dimethyl sulfoxide) (Sigma-Aldrich, USA).

Tissue culture flasks with an estimated confluency of 70-80% were used for all the experiments. To harvest the cells to be used for toxicity studies, old media was discarded and cells were washed once with phosphate buffered saline (PBS) (Gibco, USA). Following washing, cells were trypsinized for 3 minutes in a humidified atmosphere incubator at 37 ^{0}C and 5% CO_2 with 2 ml of 50% trypsin solution for HEK293T and 100% trypsin solution for TZM-bl cells. Trypsinization was stopped by resuspending the trypsinized cells in 8 ml DMEM.

Cell counting was performed by using the trypan blue exclusion technique with a ratio of (90:10 v/v) of trypan blue: cell suspension, respectively (Miyoshi et al., 1982). Hemocytometer slides were used for cells counting. Only the translucent cells under the inverted light microscope were counted as viable cells and dark blue stained cells were considered non-viable. The number of viable cells per ml was calculated using the following formula:

$$cells/_{ml} = \frac{sum\ of\ cells\ in\ four\ squares}{4} \times dilution\ factor \times haemocytometer\ factor (\mathbf{10000})$$

Cell counting, using the above formula, is useful in calculating the number of cells to seed per well during an experiment.

All cell culture procedures were carried out in a Class III biological safety cabinet. The unit was swabbed /sterilized with 70% ethanol before each use.

3.7.3   Viral strains

MJ4

The following reagent was obtained through the NIH AIDS Reagent Program, (Division of AIDS, NIAID, NIH) HIV-1 MJ4 Infectious Molecular Clone (pMJ4) from Drs. Thumbi Ndung'u, Boris Renjifo and Max Essex.

pMJ4 is an infectious HIV-1 subtype C molecular clone from a southern African (Botswana) isolate. For a detailed construction of this plasmid refer to Ndung'u et al., (2001).

CM9

The following reagent was obtained from the NHLS (NICD). It was isolated from a 30 year old female in 1999 in Johannesburg, South Africa. The viral isolates were obtained by using PBMC co-cultured with phytohemagglutinin (PHA)-activated donor PBMC. CM9 is a subtype C HIV-1 that uses both CCR5 and CXCR4 chemokine receptors.

For detailed full-length genome analysis of this HIV-1 isolate, refer to Papathanasopoulos et al., (2002).

Du179

The following reagent was obtained from the NHLS (NICD). It was obtained from an individual that was infected in KZN in 1998. Du179 is a subtype C HIV-1 that uses both CCR5 and CXCR4 chemokine receptors.

For a detailed characterization of this isolate, refer to van Harmelen et al., (2001)

Vesicular stomatitis virus (VSV)

The following reagent was obtained through the NIH AIDS Reagent Program, (Division of AIDS, NIAID, NIH) pHEF-VSVG from Dr. Lung-Ji Chang.

This construct is a VSV-G envelope eukaryotic expression vector. Transfection of this construct in mammalian cells produces a high concentration of VSV-G which can be used for pseudotyping retroviral- and lentiviral-based vectors.

For a detailed construction of this plasmid refer to Chang et al., (1999).

HIV-1 93MW965 (MW) molecular construct

The following reagent was obtained through the NIH AIDS Reagent Program, (Division of AIDS, NIAID, NIH) from Dr. Paolo Miotti and the UNAIDS Network for HIV.

The virus was isolated from seropositive individuals from the following countries: Malawi (MW), India (IN), Kenya (KE), Brazil (BR), South Africa (ZA), Tanzania (TZ), or China

(CN). Subtyping determined by sequencing a short sequence of the gag and/or env genes shows that this is a subtype C HIV-1 isolate that uses CCR5 chemokine receptor.

Murine leukemia virus (MLV) molecular construct

The following reagent was obtained through the NIH AIDS Reagent Program, (Division of AIDS, NIAID, NIH) SV-A-MLV-env from Dr. Nathaniel Landau and Dr. Dan Littman.

This construct contains the amphotropic murine leukemia virus env gene linked to the MLV LTR, and an SV40 origin.

For a detailed construction of this plasmid refer to Landau et al., (1991).

We used more than one viral isolate in this study because we wanted to confirm the anti-HIV-1 activity by the ethanol-, dereplicated ethanol- extracts and the isolated compound **2**. Anti-HIV-1 RT activity was tested against the following HIV isolates: MJ4, CM9 and Du179.

Secondly, we wanted to evaluate if the ethanol- and dereplicated ethanol- extracts have a specific or non-specific neutralization activity (i.e. can the extracts specifically prevent HIV-1 entry or can it also inhibit other viruses from infecting their host cells?). Specific neutralization activity was tested against the following viral strains: HIV-1 (MW), VSV and MLV.

3.8   Production of HIV-1 subtype C pseudoviruses

Production of HIV-1 subtype C pseudoviruses

Principle of the HEK293T assay

The assay uses human host cells to produce virus-like particles (pseudoviruses) with foreign viral envelope proteins that are capable of single-cycle infection. Env-pseudoviruses are only capable of a single round of infection and contain a firefly luciferase reporter gene (*luc*); hence, precise quantitation of viral infection is possible 48–72 h post-infection.

For transfection, 1.24×10^7 HEK293T cells were seeded in 10 ml supplemented DMEM in a culture dish 5 hours prior to addition of a transfection mixture comprising plasmid DNA, DMEM and polyethylenimine (PEI) (Polysciences, Inc., Warrington, PA, USA) that

facilitated DNA transport into the cells. The HIV-1 (HIV-1 subtype C) gag-pol construct, p8.9MJ4 (Naldini et al., 1996), VSV-g envelope construct, pMD.G (Naldini et al., 1996), and firefly luciferase reporter construct pCSFLW (Demaison et al., 2002) at a ratio of 1:1:1.5 respectively were mixed gently with 150 µl plain DMEM and 10.5 µl of 1 mg/ml of the transfection reagent (PEI). The mixture was incubated at room temperature for 15 minutes prior to adding it dropwise to the plated HEK293T cells. Following transfection, the cells were incubated at 37 ^{0}C, 5% CO_2 for 48 hours. Virus-containing supernatants were harvested at 48 hours post transfection. Viral titers were determined by infecting HEK293T cells with serial dilutions of virus-containing supernatants. After 48 hours, the expression of firefly luciferase was determined with the Bright-Glo luciferase assay substrate (Promega, Fitchburg, WI, USA) on a Victor3 multilabel reader (PerkinElmer, Waltham, MA, USA). Virus inocula were standardized to produce a luminescence of 1×10^4 to 1×10^5 relative light units (RLU) after 48 hours of incubation with HEK293T cells in drug-free medium. **Figure 3.2** below shows a diagrammatical representation for the production of pseudoviruses that have a luciferase reporter RNA.
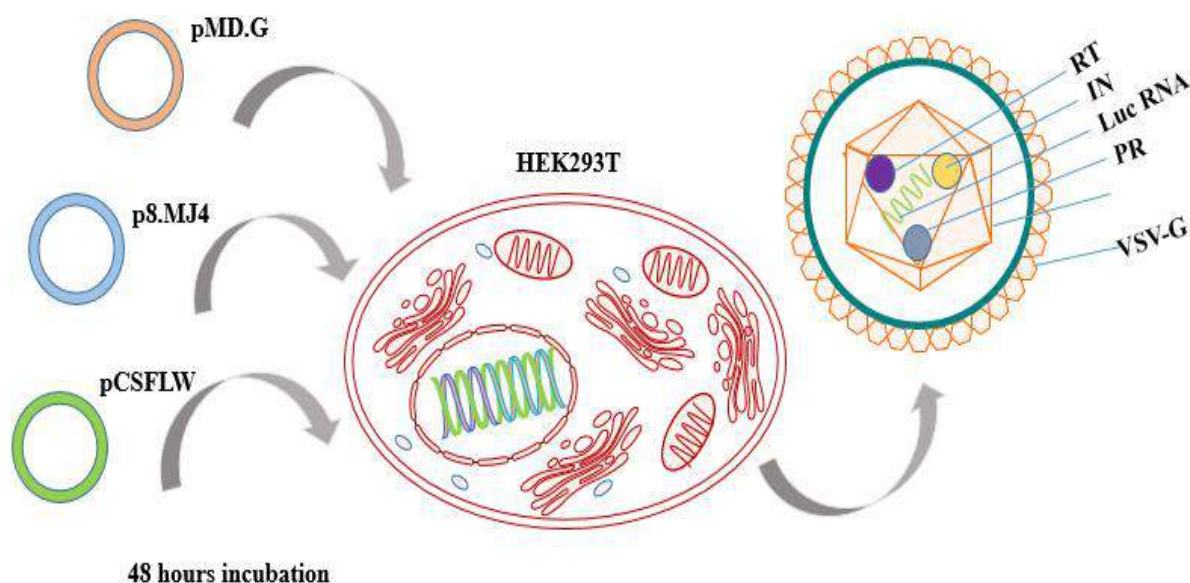
Figure 3.2: Diagrammatic representation of the production of HIV-1 subtype C pseudoviruses with luciferase reporter RNA

Three plasmids were used to generate MJ4 HIV-1 subtype C pseudoviruses; namely, pMD.G, p8.MJ4 and pSCFLW. These plasmids encode genes responsible for Vesicular stomatitis virus glycoprotein envelope (VSV-G), HIV enzymes together with gag-polyprotein, and firefly luciferase RNA, respectively. Co-transfection of HEK293T cells with the three

plasmids was facilitated by polyethylenimine (PEI). Cells were then incubated for 48-72 hours at 37 ^{0}C, 5% CO_2 in a humidified incubator. The resultant pseudoviruses, after co-transfection, had all the required components for successful single-cycle infection: protease (PR), reverse transcriptase (RT), integrase (INT), capsid, luciferase RNA (Luc RNA) and Vesicular stomatitis virus glycoprotein envelope (VSV-G). The generation of the other HIV isolates (CM9 and Du179) used the same method and the only exception being the different HIV plasmids.

The production of pseudovirions for the neutralization assay used the same protocol with the only exception being the molecular constructs (i.e. *HIV-1 93MW965* (MW); VSV & MLV)

3.9   Cytotoxicity assay

Test sample preparation

The samples to be tested for toxicity and anti-HIV activity were dissolved in 100% DMSO and further diluted with plain media so as to prepare working concentrations. The DMSO stocks for all test samples were prepared to a final concentration of 10 mg/ml and stored at -70^{0}C. Working concentrations were prepared by making a 200 µg/ml solution from a 10 mg/ml DMSO stock solution. The 200 µg/ml was further subjected to a 3-fold serial dilution with complete media. The highest test sample concentration used to test for toxicity was 100 µg/ml. The minimum concentration was 0.00169 µg/ml.

3.9.1   CellTiter 96 Aqueous One Solution Cell Proliferation assay

Principle of the assay

The CellTiter 96 AQueous One Solution, (Promega, USA) was used to evaluate toxicity of the extracts and isolated compounds. This kit contains the tetrazolium salt, MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-caboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] and an electron coupling reagent, phenazine ethosulfate. This is a colorimetric assay and is used to measure cellular proliferation or viability and cytotoxicity. The pale-yellow substrate, MTS, is converted to formazan by dehydrogenase enzymes (which cleave the tetrazolium ring to produce soluble formazan crystals in the presence of an electron coupling reagent) found in the mitochondria of viable cells. The solubilized MTS formazan can then be measured using a spectrophotometer at a wavelength of 490nm to quantify cellular

viability. Since MTS can only be reduced in metabolically active cells, it can thus be used to determine the number of viable cells versus non-viable cells.

Application

An MTS assay was conducted to measure the cytotoxicity effects of the ethanol-, dereplicated ethanol-, aqueous herbal concoction- extracts and isolated compounds on the cell lines outlined above (HEK293T and TZM-bl). Fifty microliters of cell suspension with 2×10^4 HEK293T cells were seeded per well into 96-well microtiter plates. Cells were treated with 50 μl of varying concentrations of the herbal extracts (100, 33.33, 11.11, 3.70, 1.23, 0.412, 0.137, 0.0457, 0.0152, 0.00508 and 0.00169 μg/ ml, w/v) and the isolated compounds (100, 33.33, 11.11, 3.70, 1.23, 0.412, 0.137, 0.0457, 0.0152, 0.00508 and 0.00169 μg/ ml, w/v), respectively, for 48 hours at 37 ^{0}C, 5% CO_2. The total volume per well was 100 μl. The MTS solution was added to each well at 20:100 μl, v/v and incubated at 37 ^{0}C, 5% CO_2 for 1 hour and the absorbance read at 490 nm on a Versa max tunable microplate reader (Separations, Molecular devices, USA). Each plate contained duplicates of test samples (treatment), drug blank, vehicle control (DMSO) and untreated control. At least two independent experiments were conducted on all the samples.

Toxicity determination for TZM-bl cells was slightly different to that of HEK293T cells. The total number of cells (1×10^5) in 150 μl of cell suspension, were seeded per well. Also, DEAE-dextran was added to the media to enhance infection (Monteferoli et al., 2009). Forty microliters of 5 mg/ml DEAE-dextran was added to 10 ml cell suspension prior to seeding the cells. Cells were treated with 100 μl of varying concentrations of the herbal extracts (100, 33.33, 11.11, 3.70, 1.23, 0.412, 0.137, 0.0457, 0.0152, 0.00508 and 0.00169 μg/ ml, w/v) and incubated for 48 hours at 37 ^{0}C and 5% CO_2 atmosphere. The same treatments were conducted for isolated compounds (100, 33.33, 11.11, 3.70, 1.23, 0.412, 0.137, 0.0457, 0.0152, 0.00508 and 0.00169 μg/ ml, w/v) and incubated for 48 hours at 37 ^{0}C and 5% CO_2 atmosphere. The total volume per well was 250 μl. Prior to adding the MTS solution, 150 μl of supernatant was removed from each well. MTS was then added to each well at 20:100 μl, v/v and incubated at 37 ^{0}C, 5% CO_2 for 1 hour and the absorbance read at 490 nm on a Versa max tunable microplate reader (Separations, Molecular devices, USA). Each plate contained duplicates of the test samples (treatment), drug blank, vehicle control (DMSO) and cell

control (untreated). Two independent experiments were conducted for all the samples. The percentage cell viability was calculated by using the following formula:

$$\% \: cell \: viability = \frac{Abs_{490} \: of \: treatment - Abs_{490} \: drug \: blank}{Abs_{490} \: cell \: control}$$

3.10 *In Vitro* Single-Cycle Non-Replicating Phenotypic Assay

Anti-HIV activity of potential compounds is evaluated by either using enzymatic assays or whole-cell assays. The former assay is important in that it helps with determining the IC_{50} of the compounds tested. However, the drawback of this assay is that it lacks the ability to determine the toxicity of the compound(s) toward the host cells. And, conversely, whole-cell assays can determine both the CC_{50} (cytotoxic concentrations) and IC_{50} (inhibitory concentrations). Whole-cell assays are especially useful in initial high throughput screening of potential effective compounds. This is because they can provide a researcher with information regarding the toxicity of an active compound, thus early chemical modifications to reduce toxicity and enhance target specificity of the compound can be conducted to avoid unnecessary animal tests, and to reduce the number of late-stage failures in drug discovery & development.

To determine the anti-HIV activity of the test samples, several concentrations were tested. The concentrations used were slightly cytotoxic because at the highest concentrations tested (100 µg/ ml), the organic extracts and compounds had IC_{80} values of 20-15%, as determined from the cytotoxicity studies. These slightly-cytotoxic concentrations were serially diluted using a 3-fold dilution factor. **Figure 3.3** below depicts a diagrammatical representation of a typical *in vitro* single-cycle, non-replicating phenotypic assay. Derivatives of this assay include RT- and PR- phenotypic assays, as shall be shown in **Figures 3.4** & **3.5** below.
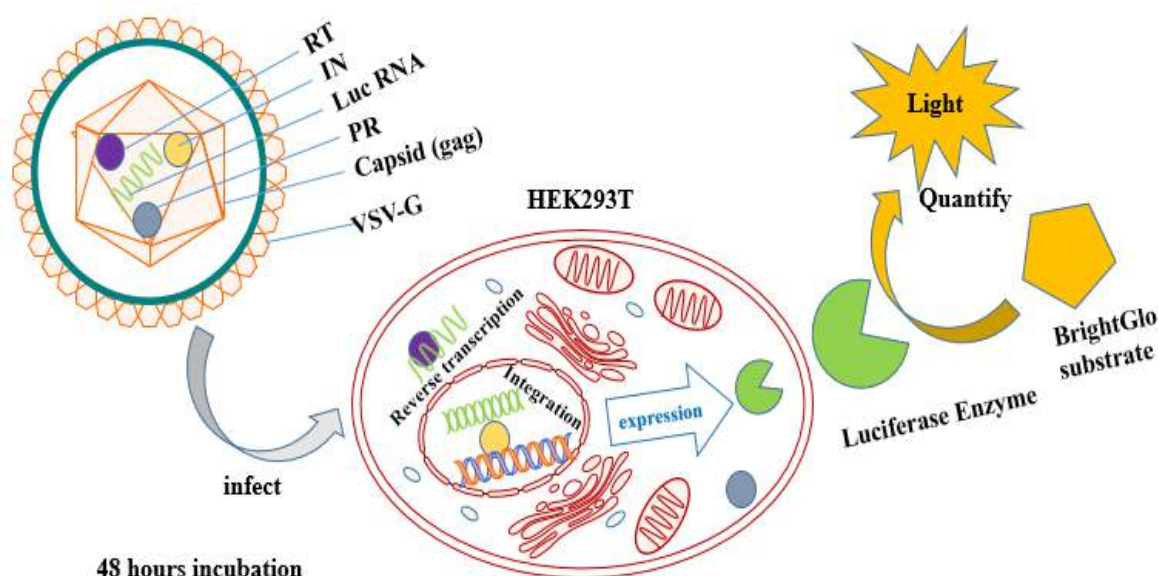
Figure 3.3: Diagrammatical representation of the HIV single-cycle non-replicative phenotypic assay

Principle of the phenotypic assay

A novel phenotypic assay to test for drug susceptibility of HIV isolate was developed by Gupta et al., (2010); Naldini et al., (1996) and Naldini et al., (2006). The assay is a whole-cell based assay that uses HEK293T cells to measure infectivity activity of HIV pseudoviral isolates following treatment with potential inhibitory drug candidates. The assay is a single-round infection luciferase reporter assay that uses HIV pseudoviruses with luciferase RNA instead of HIV RNA. During an assay, the pseudoviruses are allowed to infect HEK239T, and as a result the luciferase RNA is reverse transcribed by HIV RT and integrated into the host genome by the HIV INT. The integrated luciferase RNA is then translated to a luciferase enzyme protein by host factors during the RNA translation process. The expression of luciferase enzyme is directly proportional to the activity of infection. During an infection, that is to say, when there are drug inhibitors of HIV, a maximum or higher luciferase expression is obtained and, hence, higher luminescence (see **Figure 3.3**).

While, on the other hand, if the treatment is inhibiting any of the three HIV enzymes, the infectivity of those treatments will be lowered (see **Figures 3.4-3.5** below). Therefore, to quantify the infectivity activity, a luciferase substrate is added and the conversion of this substrate to a colorimetric by-product is measured as a function of relative light units (RLU) by a luminometer.

3.10.1 Principle of the phenotypic reverse transcriptase (RT) assay

This assay is directed at evaluating RT inhibitors. The principle of the assay is that inhibition of RT will result in no reverse transcription of the single-stranded Luc RNA and, hence, it will not be integrated into the host genome. This implies that no RNA translation of Luc RNA to protein will occur and, therefore, no luciferase enzyme to convert the substrate to a colorimetric by-product that can be quantified (see **Figure 3.4** below).
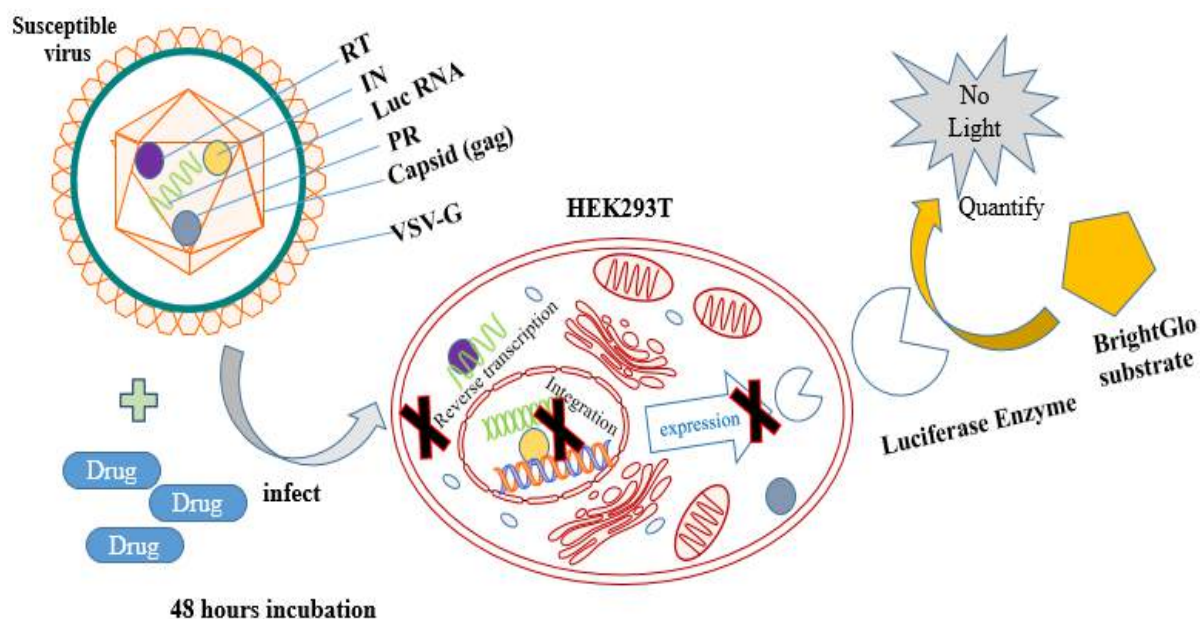
Figure 3.4: Diagrammatical representation of the phenotypic HIV-RT single-cycle, non-replicative assay

Application

Three plasmids were utilized for the production of virus-like particles: pMD.G to facilitate viral entry, pCSFLW for the detection of viral integration by bioluminescence, and p8.MJ4 for the expression of HIV-1 subtype C PR-RT. Plasmids were co-transfected into HEK293T cells in which cotransfection was facilitated with polyethylenimine as discussed above.

For drug susceptibility assays, duplicate serial dilutions of all test samples (3 herbal extracts and the isolated compounds) and positive controls nevirapine (NVP) and zidovudine (AZT) (NIH AIDS Reagent Program) were prepared in 96-wells cell culture plates to which standardized virus inocula and 2×10^4 HEK293T cells per well were added. After incubation for 48 hours 37 ^{0}C and 5% CO_2 atmosphere, the expression of luciferase was determined

using a Bright-Glo luciferase substrate solution. The substrate solution was added to each well in the ratio 100:100µl, v/v and incubated for 2 minutes at 25 ^{0}C and the contents, thereafter, transferred to a black 96-well plate and the luminescence (Relative Light Units: RLU) measured on a Victor3 multilabel reader (PerkinElmer, Waltham, MA, USA).

A total of three HIV isolates (MJ4, CM9 and Du179) were used in this study. The initial screening used the MJ4 isolate and the test samples that showed anti-RT activity were further screened against the additional isolates (CM9 and Du179). Each virus was screened in at least two independent assays for each test sample, and the 50% inhibitory concentration (IC$_{50}$) values were calculated.

3.10.2 Principle of the phenotypic protease (PR) assay

This assay is directed at evaluating PR inhibitors. In this assay, pseudoviruses are produced in the presence of PR inhibitors (PI) or test compounds and the produced pseudoviruses (supernatant) used to infect freshly plated cells that can express luminescence which can be quantified. The logic behind this approach is that if there's a PI during pseudovirion production, then the end result will be fewer/no pseudoviruses produced due the fact that no cleavage of gag and gag-polyprotein and viral maturation processes will occur and, hence, no budding of new pseudoviruses will happen. This implies that if the supernatant (supposed to be rich with pseudoviruses) was to be used in a new experiment to test for anti-PR activity by quantifying expression of luciferase (as in the RT assay), the RLU readings on the luminometer will be very low. Since the RLU reading is directly proportional to protease activity in this assay, low RLU is indicative of low virus infectivity activity. The following diagram (**Figure 3.5**) depicts the effects of inhibiting PR in this assay.
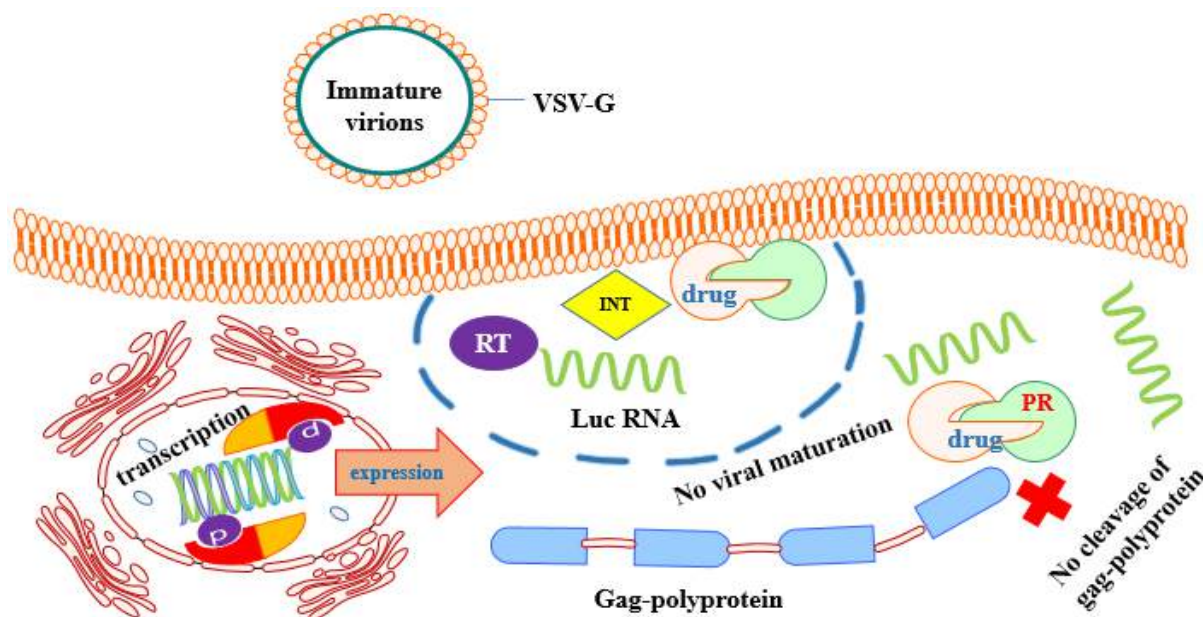
Figure 3.5: Diagrammatical representation of the phenotypic HIV-PR single-cycle, non-replicative assay

The diagram shows a series of events that occur when pseudoviruses are produced in the presence of an HIV protease inhibitor (PI). The gag and gag-pol polyproteins are expressed from the p8.MJ4 plasmid DNA (not the integrated luciferase or viral DNA). Also, the pseudoviruses are formed, but they are immature. Infection will occur because the pseudovirions will contain the VSV-g proteins on the surface. The signal RLU output will be lower because of the uncleaved gag, RT and INT. The PI will inhibit PR from cleaving gag and gag-pol polyproteins to yield immature virions. This will result in a supernatant that either has fewer functional pseudoviruses or none depending on the concentration of the drug used. The supernatant obtained from this reaction is used in the PR single-cycle, non-replicative phenotypic assay.

Application

This assay has three steps which makes it slightly different to the RT assay. The first step is to produce pseudoviruses in the presence of the test compounds/drugs. The second step is to use the pseudovirus containing supernatant for treating fresh cells. The last step is to evaluate anti-PR activity.

Plasmids were co-transfected into HEK293T cells, in which cotransfection was facilitated with polyethylenimine and incubated for 24 hours at 37^0 C and 5% CO_2 atmosphere.

Following transfection, the supernatant was removed and the transfected cells washed with PBS, trypsinized and harvested as discussed above and plated in a 96-well plate. 100 µl of cells were plated into wells containing duplicate serial dilutions of all test samples (3 herbal extracts and the isolated compounds), vehicle control, cell control (untreated) and the positive control Lopinavir (LPV) (NIH AIDS Reagent Program) and incubated for 24 hours at 37 ^{0}C and 5% CO_2 atmosphere.

For the drug susceptibility PR assay, 25 µl of supernatant was transferred into a new 96-well plate in which 75 µl of fresh 2.025×10^4 HEK293T cells were seeded and incubated for 48 hours at 37 ^{0}C and 5% CO_2 atmosphere. The expression of luciferase was determined using a Bright-Glo luciferase substrate solution. The substrate solution was added to each well at the ratio 100:100 µl, v/v and incubated for 2 minutes at room temperature and the contents, thereafter, transferred to a black 96-well plate and the luminescence (Relative Light Units: RLU) measured on a Victor3 multilabel reader (PerkinElmer, Waltham, MA, USA).
The MJ4 HIV isolate was used for this assay and was screened in at least two independent assays for each test sample.

3.10.3 Neutralization assay (viral entry TZM-b1 assay)

Principle of the assay

This is a luciferase (Luc) reporter gene assay that measures HIV neutralization as a function of Tat-regulated Luc reporter gene expression reduction after a single round of HIV infection in TZM-b1 cells (Montefiori, 2005). The assay is designed for single infection because of the use of Env-pseudotyped viruses generated by co-transfection of HEK293T cells with HIV-*env* expressing plasmid plus an HIV backbone plasmid containing all the other HIV genes except the *env* gene.

For the purpose of this study, this assay was used to evaluate HIV-1 viral entry inhibition following treatment of cells (TZM-b1) with test samples in the presence of the pseudoviruses expressing HIV-1 gp160 glycoprotein. The assay was also used to determine the CC_{50} and IC_{50} of the test samples. CC_{50} is defined as the concentration of the drug that causes 50% cytotoxic effect. HIV-1 93MW965 isolate was used as the pseudovirion for this assay.

Studies on VSV and MLV were also conducted in order to determine if the samples have a specific or non-specific entry inhibition mechanism.

HIV entry inhibitory activity was tested by initially diluting the MW HIV pseudoviruses 3-fold as dictated by the titration experiments to standardize virus inocula. Fifty microliters of standardized virus per well was incubated with the various serially diluted test sample concentrations for an hour prior to adding TZM-bl cells. This one hour incubation step is done in order to allow the test samples to interact with the virus (gp160 to be specific). Following the one hour incubation, 100 µl of 1×10^4 DEAE-dextran treated TZM-bl cells were seeded per well in a 96-well plate. The plates were incubated for 48 hours at 37 ^{0}C and 5% CO_2 in a humidified atmosphere.

The total volume per well was 250 µl (i.e. 100 µl drug, 50 µl virus, 100 µl cells). After the 48-hours incubation, prior to adding the Bright-Glo luciferase assay substrate solution, 150 µl of supernatant was carefully removed from each well. A volume of hundred microliters of Bright-Glo luciferase assay substrate solution was then added to each well at the ratio (100:100 µl, v/v) and incubated for 2 minutes at room temperature in the dark. Thereafter, the contents were transferred to a black 96-well plate and the luminescence (Relative Light Units: RLU) measured on a Victor3 multilabel reader (PerkinElmer, Waltham, MA, USA). Each plate contained duplicates of test samples (treatment), virus control (cell infected with virus), vehicle control (DMSO), positive control (virus treated with broadly neutralizing antibodies such as VRC01 (a CD4 antagonist)) and cell control (untreated & not infected cells). At least two independent experiments were conducted for all the samples. Percent activity was calculated by using the following formula:

$$\% \ entry \ inhibition = \frac{RLU \ of \ treated \ sample}{RLU \ of \ virus \ control \ sample} \times 100$$

The 50% inhibitory concentration (IC$_{50}$) and 50% cytotoxic concentration (CC$_{50}$) values were calculated and estimated by using GraphPad Prism 5$^{®}$ (GraphPad Software, Inc. La Jolla, CA, USA) and Microsoft Excel (using linear forecasting algorithm to estimate a value that will have 50% activity).

3.11 Statistical analysis

The results of each series of experiments (performed in duplicate) are expressed as the mean values ± standard deviation (SD) of the mean. Levels of statistical significance were calculated using the paired student t-test when comparing two groups or by one-way and factorial analysis of variance (ANOVA) for comparison of more than two groups. ANOVA Post Hoc analysis: Bonferroni was also conducted. P-values of $\leq$ 0.05 were considered significant. The calculations were conducted by using the GraphPad Prism 5® (GraphPad Software, Inc. La Jolla, CA, USA) software.

4   CHAPTER 4

Results and Discussion

Brief overview of the results and discussion

Three herbal extracts were prepared by macerating with absolute ethanol and water. A portion of the ethanol extract was dereplicated to make the second extract. And lastly, an aqueous extract was prepared according to the method used by traditional health practitioners. The ethanol-, dereplicated ethanol- and aqueous- extracts were prepared from a herbal concoction comprised of a mixture of six plants. This herbal concoction was investigated for anti-HIV-1 subtype C activity.

The extracts were phytochemically characterized by thin layer chromatography (TLC) and/or ultra-performance liquid chromatography so as to chemically profile what type of compounds/phytochemicals are present in the different extracts. The ethanol- and dereplicated ethanol- extracts of the herbal concoction showed the presence of intermediate polar compounds (flavonoids, alkaloids, sugars and terpenes) while the aqueous extract had predominantly highly polar compounds.

Among the three extracts, the ethanol extract and the dereplicated ethanol extract of the herbal concoction showed moderate anti-reverse transcriptase activity. (The ethanol extract had a mean fifty percent inhibitory concentration (IC_{50}) of 56.53, 53.96 and 55.39 µg/ml against the MJ4, Du179 and CM9 HIV-1 isolates, respectively. The dereplicated ethanol extract had a mean IC_{50} of 51.87, 47.56 and 52.81 µg/ml against MJ4, Du179 and CM9 HIV 1 isolates, respectively.

Moreover, both extracts showed activity against HIV entry. These extracts were able to neutralize HIV-1 and, hence, reduce HIV replication and infection. The ethanol and dereplicated ethanol extracts had mean IC_{50} activities of 36.33 and 32.06 µg/ml, respectively. Furthermore, they also potently neutralized Vesicular stomatitis virus (VSV) yielding mean IC_{50} values of 24.91 and 20.82 µg/ml for ethanol- and dereplicated ethanol- extracts, respectively.

The isolation and phytochemical characterization of bioactive compounds using various chromatographic and spectroscopic methods yielded four homoisoflavones. Furthermore, the data also shows that the isolated homoisoflavones belong to either of the three basic structural types: 3-benzyl-4-chromanone (Compound **1)**, 3-benzylidene-4-chromanone (compound **2)** and 3-benzyl-chrom-2-en-4-one & 3-benzylidene-4chromanone types (Compound **3**, an isomer composed of the two types). Three of these compounds (**1**, **3a** and **3b**) were inactive while compound **2** was found to be bioactive against HIV-RT and yielded the mean IC_{50} values of 7.228 ± 1.88, 12.83 ± 0.412 & 12.81 ± 0.1020 µg/ml against MJ4, CM9 and Du179 HIV-1 subtype C isolates, respectively. Compound **2** induced cytotoxicity in a concentration dependent manner with a calculated mean CC_{50} value of 23.08 ± 0.1980 µg/ml against HEK293T cells.

The positive controls used for anti-RT, anti-PR and HIV-1 neutralization were NVP, LPV and VRC01, respectively. NVP had mean IC_{50} values of 0.19, 0.13 and 0.12 µg/ml against MJ4, CM9 and Du179, respectively. LPV had an IC_{50} value of 5.78 µg/ml. Lastly, VRC01 control had mean IC_{50} values of 0.08, 0.15 and 0.04 µg/ml against MW, VSV and MLV, respectively.

These results suggest that the South African herbal extract used by herbalists possesses dual activity against HIV-1: the interaction of the homoisoflavones with the HIV-1 RT and/or blocking HIV entry into host cells during an infection. Additionally, the extract has broad spectrum activity, as seen from its ability to inhibit VSV and possessing homoisoflavonoid phytochemicals that can deal with other disorders that are linked to HIV infection such as oxidative stress and acute/chronic inflammatory reaction.

4.1 Chemical profiling of the herbal concoction and plant extracts

Aim: To determine the metabolic profile of the herbal extracts

Several scientific studies have found that determining the chemical profile of plant extracts helped in identifying some of the phytochemicals present and also provided guidance for further research on pharmacological activity in support of traditional uses.

Metabolomic profiling of plant extracts using either qualitative and/or quantitative analyses of cellular metabolites afford a comprehensive view of the biochemical status of an organism (Fiehn et al., 2000; Summer et al., 2003 and Fukusaki et al., 2005). Secondary metabolites are generally diverse in their respective physicochemical properties (molecular weight, polarity, solubility, volatility). Multiple analyses are required to elucidate all of the metabolome of an organism. There are different metabolomic approaches that have been developed for the varied secondary metabolites. These include metabolite target analysis (qualitative and quantitative analysis of specific metabolites related to a specific metabolic reaction); metabolite fingerprinting (sample classification by rapid, global analysis); metabonomics (evaluation of tissues and biological fluids for changes in endogenous metabolite levels); and metabolite profiling (identification and quantification of a selected number of pre-defined metabolites, generally related to a specific metabolic pathway) (Duun et al., 2005; Fukusaki et al., 2005 and Allwood et al., 2008).

In the present study, we have attempted to phytochemically/chemically profile the herbal extracts utilized for the anti-HIV-1 screening and characterization. To achieve this goal, we have used thin layer chromatography (TLC) and Ultra-performance liquid chromatography-high definition mass spectrometry (UPLC-HDMS).

4.2 Thin-layer chromatography (TLC)

Secondary metabolite chemical profiling using TLC analysis of the ethanol extracts of the six individual plants together with the herbal concoction allowed partial phytochemical characterization of the extracts. The TLC plate image (**Figure 4.1**) below shows the separation of the extracts. TLC was used for obtaining estimate phytochemical profiles of the extracts and provided an indicative separation of the constituents of the samples. Terpenes (pink stain), flavonoids (yellow-orange stain), sugars and alkaloids (blackish-brown stain)

were found to be present in the ethanol extracts of *W. salutaris, S. natalensis, E. autumnalis, S. aculeastrum,* and *C. miniata,* respectively.
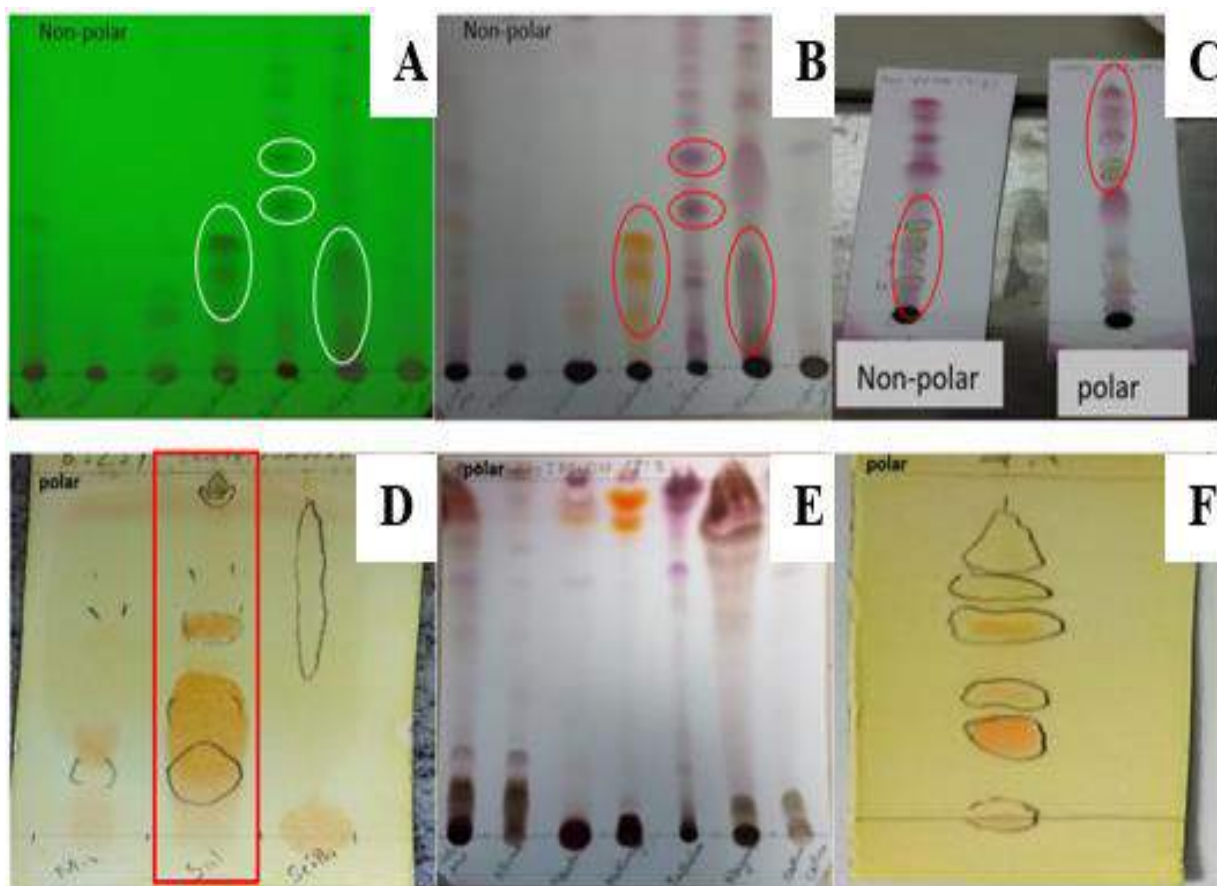
Figure 4.1: UV and vanillin sprayed chemical profiles of the individual plants and the ethanol herbal concoction.

The seven lanes represented above in (**a**), (**b**) and (**e**) (from left to right) correspond to the ethanol herbal-, *S. aculeastrum-, S. natalensis-, E. autumnalis-, W. salutaris-, C. miniata-* and *H. surratensis-* extract, respectively.

Figure 4.1 (**a**) shows the UV chromatogram profile of the different extracts. The separation was achieved by using non-polar hexane:ethyl acetate (7:3) solvent system. **Figure 4.1** (**b**) depicts the chromogenic chromatogram profile of Figure 4.1 (**a**) visualized by using vanillin-H_2SO_4 reagent. **Figure 4.1** (**c**) depicts the chromogenic chromatogram profiles of the ethanol herbal concoction separated by non-polar hexane:ethyl acetate (7:3) and polar chloroform:methanol (8:2)) solvent systems. **Figure 4.1** (**d**) depicts the chromogenic chromatogram profile of the ethanol herbal concoction, *S. aculeastrum* and *S. natalensis*

visualized by using Dragendorff reagent. Figure 4.1 (**e**) depicts the chromogenic chromatogram profile of **Figures 4.1** (**a**) and (**b**) but separated by using chloroform:methanol (8:2) polar solvent system and visualized by using vanillin-H_2SO_4 reagent. **Figure 4.1** (**f**) depicts the chromogenic chromatogram profile of *C. miniata* separated by using ethyl acetate: methanol (5:1) and visualized by using Dragendorff reagent. Visualization was done under UV at 254 and 366 nm wavelengths and spraying with vanillin-H_2SO_4 or Dragendorff reagent followed by baking the TLC plate at 100 °C for 1 minute.

Choosing a suitable solvent system to isolate compounds from plant extracts entails trying out several combinations of organic solvents that have different polarities. A good solvent system should be able to properly separate the different phytochemicals within the extract. We have conducted these separation studies, and found that the use of a polar system comprised of chloroform and methanol (8:2 v/v) can fractionate the herbal concoction **Figure 4.1 (c)**.

However, the use of this solvent system for isolating compounds using column chromatography resulted in fractions that were not poorly resolved. The desired intermediate polar phytochemicals co-migrated with the solvent front and hence eluted early. Therefore, a different solvent system that will retard the elution of polar compounds and, hence, yield properly resolved fractions was investigated. We found that the use of a non-polar system composed of hexane: ethyl acetate (7:3 v/v) as seen in **Figure 4.1** (**c**) would result in retarding the elution of polar phytochemicals.

Figure 4.1 (**c**) also shows the complex phytochemical composition of the herbal concoction when compared with individual plant extracts as seen in **Figure 4.1** (**a, b** and **e**). In general, **Figure 4.1 c** depicts a phytochemical profile of the herbal extract which shows the presence of different phytochemicals. It showed that terpenes (stain pink), flavonoids (yellow-orange), sugars and alkaloids (blackish-brown) were present in *W. salutaris, S. natalensis, E. autumnalis, S. aculeastrum,* and *C. miniata*, respectively.

Ethanol extract of *S. aculeastrum* in **Figure 4.1** (**a** and **b**) lane 2 shows that it contains alkaloids and sugars as major compounds but the separation was poor with this solvent system; hence, only a black intense spot at the baseline is observed. Conversely, **Figure 4.1** (**e**) lane 2 shows partial separation of the alkaloids that were retarded at the baseline in

Figure 4.1 (**b**) which is indicative that this extract contains polar compounds. The presence and abundance of alkaloids in this plant extract was also confirmed by the alkaloid specific Dragendorff spray reagent as seen from the highlighted box in **Figure 4.1** (**d**).

As expected, our results correlate with literature studies by Harbone et al., 1993 and Usubillaga et al., 1997 who have shown that the solanum species is rich in glycosidic steroidal alkaloids. Several pharmacological activity studies (Hostettmann et al., 1982; Marston et al., 1985; Young et al., 1996; Wanyoyi et al., 2002 and Laban et al., 2015) of the genus Solanum have shown that these types of alkaloids are responsible for most of the observed biological activity such as anti-molluscidal, antimicrobial activity and anti-gonorrheal activity (Kokwaro et al., 2009).

Ethanol extract of *S. natalensis* as seen in lane 3 of **Figure 4.1** (**a**, **b** and **e**) also did not separate well in both polar and non-polar solvent systems. Both **Figures 4.1** (**a** and **b**) show a similar phytochemical pattern. Furthermore, in **Figure 4.1**(**a**), faint dark spots can be seen which indicates that the plant has UV-active compounds and thus some level of double bond conjugation (indicative that metabolites such as aromatic compounds are present in this extract). **Figure 4.1** (**b**), on the other hand, shows the chromogenic chromatogram of **Figure 4.1** (**a**), and from it, faint bands of the same compounds are seen in **Figure 4.1** (**a**) staining yellow-orange. This color reaction is indicative of flavonoids, which are known to possess some aromatic rings. **Figure 4.1** (**b**) corroborates what is seen in **Figure 4.1** (**a**). A literature review of the phytochemicals present in *S. natalensis* confirms the presence of flavonoids; specifically, homoisoflavones and the fact that they are one of the major constituents of this plant species (Crouch et al., 1999; Moodley et al., 2004 and Mulholland et al., 2013).

The ethanol extract of *E. autumnalis* as seen in lane 4 for both **Figure 4.1** (**a**) and (**b**) was poorly resolved. Both **Figure 4.1** (**a**) and (**b**) show a similar phytochemical pattern. The chromatogram of the extract of the bulbs was characterized by three main bands, which occurred as dark spots under UV and turned a striking orange when treated with vanillin-H_2SO_4 reagent. The observed orange color is indicative of the presence of flavonoids **Figure 4.1** (**b**). These flavonoids are able to quench UV light and thus we observe pronounced dark spots in **Figure 4.1** (**a**). This UV light quenching ability is attributed to the fact that they possess double bond conjugation properties (i.e. aromatics). A literature search of the phytochemicals present in *E. autumnalis* confirms the presence of flavonoids; specifically,

homoisoflavanoids and the fact that they are the major constituents of this plant species (Sidwell et al., 1970; Farkas et al., 1971; Tamm et al., 1972 and Mulholland et al., 2013).

The ethanol extract of *W. salutaris* **Figure 4.1** (**a**), (**b**) and (**e**) lane 5 depicts distinctively different phytochemical patterns under UV at 254 and 366 nm wavelengths and after spraying with vanillin-H_2SO_4 reagent. **Figure 4.1** (**a**) shows that *W. salutaris* contains fewer UV-active compounds whereas **Figure 4.1** (**b**) and (**e**) show an array of different compounds present in this extract. *W. salutaris* separated well with the non-polar system **Figure 4.1** (**b**) compared to the polar system **Figure 4.1** (**e**). The presence of terpenes was observed which typically stain pink/purple when treated with vanillin-H_2SO_4 reagent. **Figure 4.1** (**b**) again shows that this extract possesses quite a few non-polar compounds which have migrated up the TLC plate. Furthermore, the presence of the major constituents of *W. salutaris* are also observed. The two circled spots in **Figure 4.1** (**a**) and (**b**) lane 5, belong to the two major *W. salutaris* phytochemicals known as Warburganal and Polygodial which are both UV-active and chromogenic with R_f values of approximately 0.39 and 0.47, respectively.

Our results are in agreement with the results of the studies by Drewes et al., 2001 which have shown that the bark of this plant contains more of Warburganal and Polygodial which have approximately similar R_f values, UV-activity and chromogenic activity. A literature search of the phytochemicals present in *W. salutaris* confirms the presence of terpenes; specifically, drimane sesquiterpenoid aldehydes, which are the major constituents of this plant species (Zschocke et al., 2000; Drewes et al., 2001).

The ethanol extract of *C. miniata*, **Figure 4.1** (**a**, **b** and **e**) lane 6 depicts distinctively different phytochemical patterns under UV at 254 and 366 nm wavelengths and after spraying with vanillin-H_2SO_4 reagent. **Figure 4.1** (**a**) shows that *C. miniata* contains fewer UV-active compounds whereas **Figure 4.1** (**b** and **e**) show an array of different compounds present in this extract. This also shows the importance of using various visualization techniques when conducting phytochemical profiling studies using TLC. Both **Figure 4.1** (**a**) and (**b**) lane 6, also show that the alkaloids retarded at the baseline are both UV-active and chromogenic. **Figure 4.1** (**f**) shows the properly separated alkaloids that were retained at the baseline in **Figure 4.1** (**b**) and visualized by Dragendorff reagent. The solvent system used in **Figure 4.1** (**f**) was EtOAc: MeOH (5:1) and, therefore, by comparing **Figure 4.1** (**a**), (**b**), (**e**) and (**f**) it was concluded that this was the solvent system that would be used to isolate the alkaloids

present in this extract. A literature search for the phytochemicals present in *C. miniata* confirmed the presence of alkaloids; specifically, the isoquinoline type that are the major constituents of this plant species These compounds are implicated in the antiviral activity induced by this plant species (Ieven et al., 1982; Ieven et al., 1983; Gabrielsen et al., 1992; Szlávik et al., 2004 and Li et al., 2005).

The ethanol extract of *H. surratensis* (lane 7) in **Figure 4.1** (**a**), (**b**) and (**c**) show that this extract contains very few phytochemicals. A literature search for the phytochemicals present in *H. surratensis* yielded no results. As it stands, it seems that there are no phytochemical studies conducted on this plant species. The observation that the ethanol extract of this plant contains fewer phytochemicals can be attributed to the fact that different plant parts contain differential amounts of phytochemicals. This differential phytochemical composition is dictated largely by the function these plant parts play (i.e. storage, transport and manufacturing of plant nutrients). As one would expect, the stems of most plants would contain fewer phytochemicals because they function to transport these phytochemicals back-and-forth to-and-from the roots and leaves. Therefore, this might, in part, explain why this extract contains fewer phytochemicals (see **Table 2** for plant parts used in extract preparation).

4.3   Chemical profiling of herbal extracts by UPLC-HDMS

Particular base peak intensities (BPI) chromatograms have been included here and show the chemical profiles of some of the extracts obtained from the different solvents.

For the purpose of comparison of the chemical profiles from the various extracts, attention will be placed on the ESI- and ESI+ BPI chromatograms (depending on which show better ionization of the compounds). The ESI- chromatograms of the ethanolic herbal extract, dereplicated ethanolic herbal extract and aqueous herbal extract are discussed below.

To corroborate the above observations, and phytochemically profile these extracts, Ultra performance liquid chromatography coupled to high definition mass spectrometry (UPLC-HDMS) was used for the analyses. This is because UPLC-HDMS can yield properly resolved base peak intensities (BPI) peaks corresponding to individual compounds present within the extract. UPLC-HDMS allows qualitative analysis of the BPI peaks and, thus, it is possible to

determine more accurately the elemental composition of the isolated molecules, accurate molecular weight, identification and structure elucidation of the separated compounds.

Figures 4.2 a, b and **c** shows visual examination of the BPI chromatograms at the negative electro spray ionization (ESI⁻) mode of the different herbal extracts. The chromatograms indicate clearly that the ethanol- and dereplicated ethanol- extracts have different phytochemicals when compared to the aqueous extract. Therefore, as expected, we can see that the different solvents used for extraction induced differential secondary metabolite changes. This is demonstrated by the increase and decreases of molecular ion peak intensities, the appearance of new molecular ion peaks and suppression of some molecular ion peaks which are present in other BPI chromatograms of the extracts.
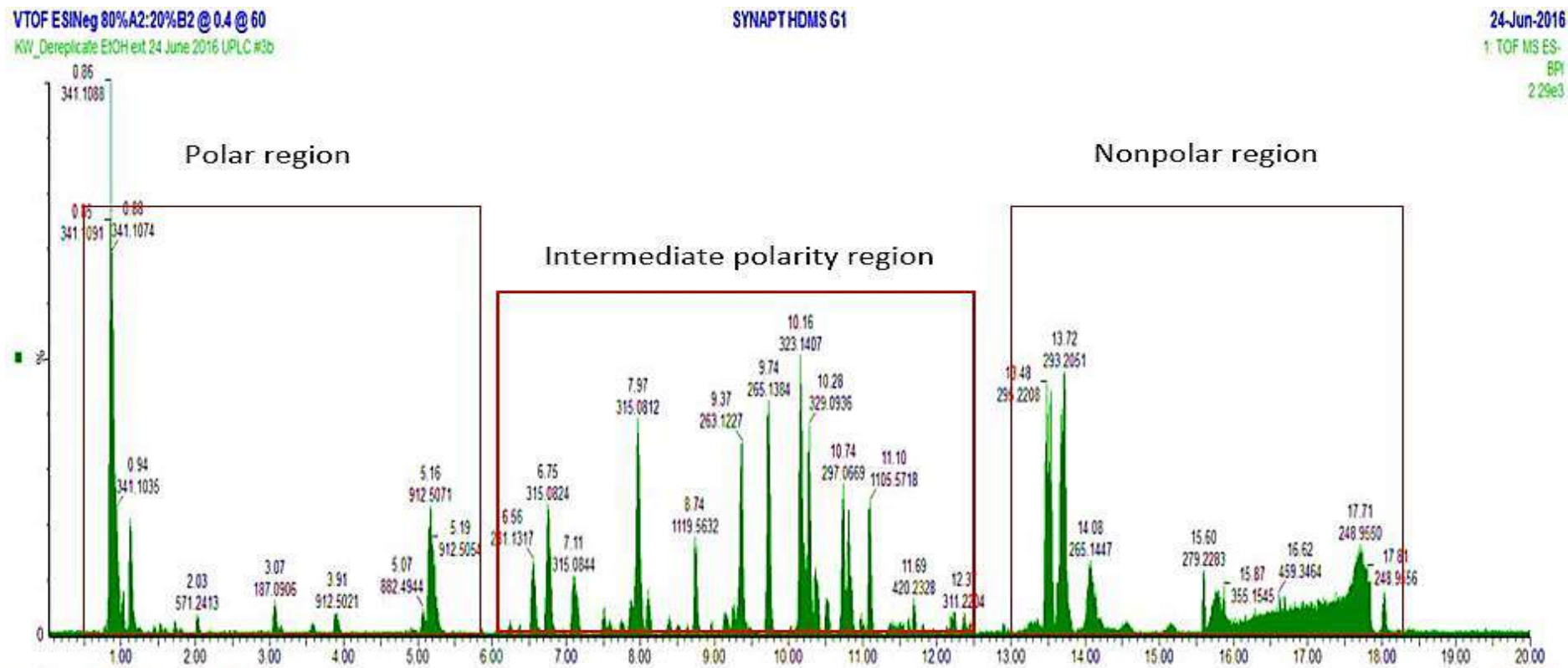
Figure 4.2: The BPI chemical profile chromatogram of the dereplicated ethanol extract of the herbal concoction in ESI- mode.

The profile shows the retention times and molecular masses of the various compounds present in the extract. The polarity range of the column is depicted by the red boxes (i.e. from left to right; polar region, intermediate polar region and nonpolar region). The y-axis shows the percentage intensity and the x-axis shows the time gradient used in running the UPLC separation.
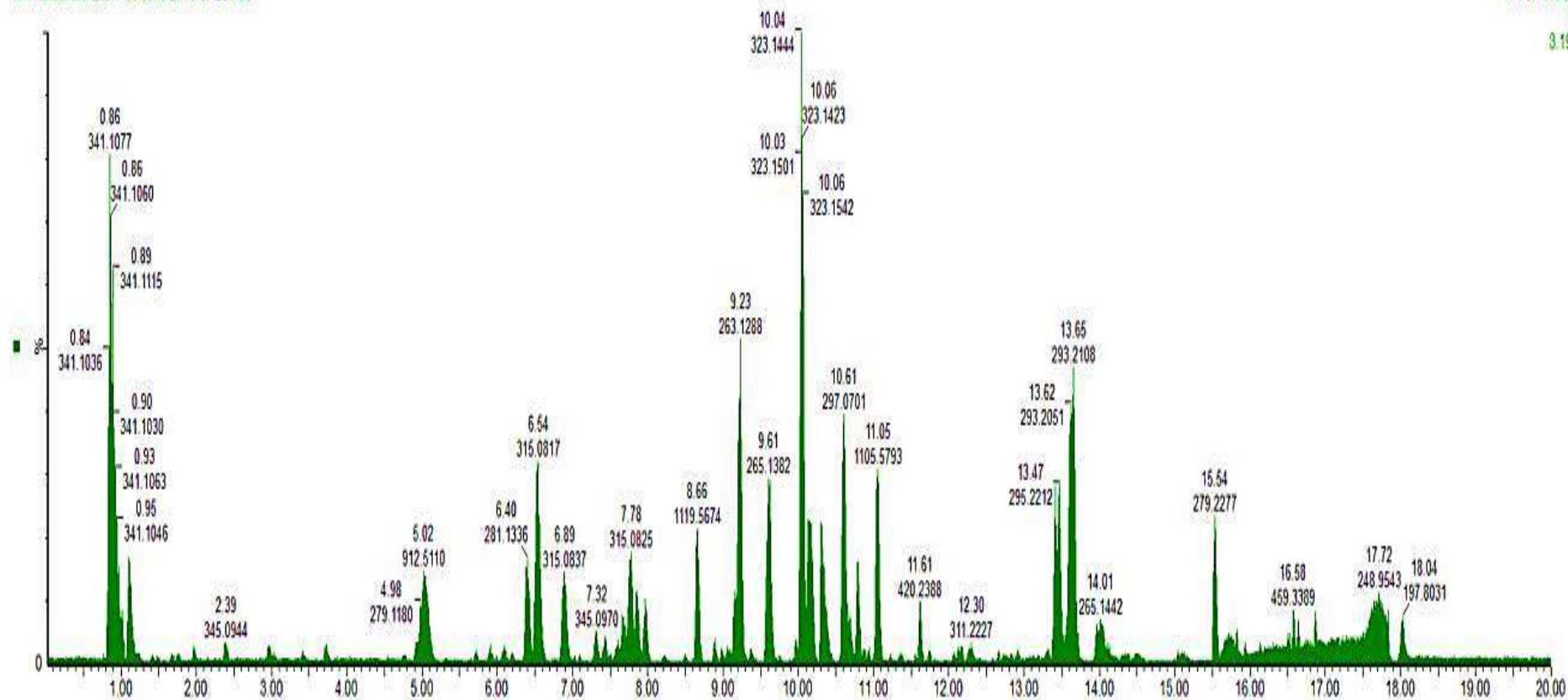
Figure 4.3: The BPI chemical profile chromatogram of the ethanol extract of the herbal concoction in ESI- mode.
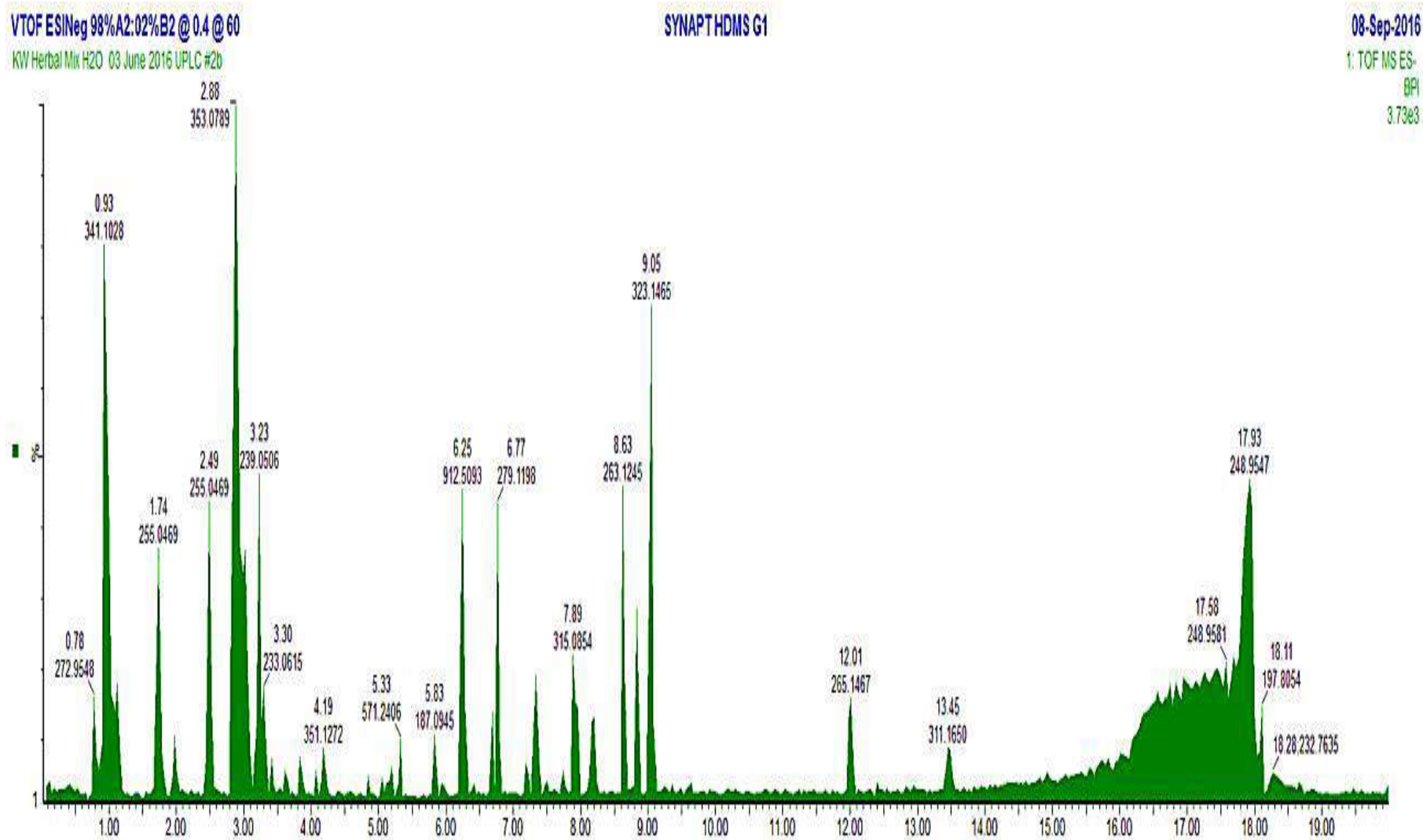
Figure 4.4: The BPI chemical profile chromatogram of the aqueous extract of the herbal concoction in ESI- mode.

Upon visual inspection of the BPI chromatograms of the three herbal extracts, it is evident that the organic solvent (ethanol) extracted secondary metabolites ranging from non-polar to mid-polar whereas those extracted by water (aqueous extract) are polar. The dereplicated ethanol extract profile (**Figure 4.2**) shows that there is not much difference observed when compared to the BPI chemical profile chromatogram of the ethanol extract profile (**Figure 4.3**). And, with regard to the polarity classes, both extracts (**Figures 4.2** and **4.3**) have similar compounds. Visual examination of these two BPI chemical profiles shows that the only difference is the reduced molecular ion peak intensity of the intermediate polar compounds for the dereplicated ethanol extract. This suggests that the dereplication of the ethanol extract has resulted in removal of some of the intermediate polyphenols.

The ethanol extract BPI chemical profile (**Figure 4.3**) shows that it is composed of various phytochemical classes, including fewer polar- metabolites (eluting at 1-6 mins), high intensity intermediately polar metabolites (6-12 mins) and few nonpolar metabolites (13-20 mins). The ethanol extract is, therefore, composed mostly, of intermediate polar metabolites when compared to dereplicated ethanol extract as shown by pronounced peak intensities (**Figure 4.3**) versus (**Figure 4.2**).

The aqueous extract profile (**Figure 4.4**) as expected, revealed that the extract contains mostly polar metabolites when compared to ethanol extracts (**Figures 4.2** and **4.3**) which showed the dominance of intermediate region.

Furthermore, examining both the TLC and UPLC-HDMS data, the ethanol extract would be an ideal extract to use for the isolation of bioactive compounds as it contains appreciable variation of metabolites.

Possible implications of these results in relation to the bioactivity of these extracts

Based on the results obtained for the chemical profiling of the herbal extracts, there are several possible outcomes to expect in the screening of these extracts for anti-HIV activity. The following hypotheses are proposed:

1.   The ethanol extract will induce moderate anti-HIV activity.

This is substantiated by the fact that this extract has various phytochemicals present and, thus, an increased probability of having phytochemicals that have antagonistic activity to the bioactive compounds present in this extract.

 2.   The dereplicated ethanol extract will induce increased activity when compared to the ethanol extract.
 3.   The dereplicated ethanol extract will induce slightly increased cytotoxic activity when compared to the ethanol extract.

The removal of antagonistic polyphenolic phytochemicals will result in enhanced cytotoxic activity. The fact that this extract contains fewer polar phytochemicals means that the more abundant intermediate polar compounds will not have bioavailibity issues (i.e. they can cross the cellular membrane and, thus, be able to reach intracellular targets such as RT and PR). With regard to toxicity, the extract will cause slightly higher cytotoxic activity because "beneficial" polyphenols that were antagonizing to the cytotoxic compounds may have been non-selectively dereplicated together with the tannins.

4   The aqueous extract will be inactive.
5   The aqueous extract will be non-cytotoxic.

This extract will be inactive because there are fewer intermediate polar compounds and more polar compounds extracted. This presents challenges with the bioavailability of the compounds. Polar compounds have challenges with passing though the lipid bilayer and, therefore, they will not reach intracellular targets such RT and PR in a whole-cell assay system such as those used in this study. The extract will be non-cytotoxic because polar

compounds will not be able to interact with intracellular proteins such as Cytochrome P450 (CYT P450 that are implicated in triggering toxicity effects.

4.4   Bioactivity of the herbal extracts

Aim: This study section is aimed at evaluating the anti-HIV-1 activity of the herbal extracts

The objectives followed to realise the aim:

➢   Determine the cytotoxicity of the extracts

➢   Evaluate if the extracts have anti-reverse transcriptase activity

➢   Evaluate if the extracts have anti-protease activity

➢   Evaluate if the extracts can neutralize HIV and, therefore, inhibit HIV entry

4.5   Cytotoxicity studies

The antiviral activity of plant extracts against HIV infection is occasionally a result of their toxic effects and subsequently might result in inaccurate conclusions. Therefore, to exclude the non-specific antiviral effect, the toxicity of plant extracts on HEK293T and TZM-bl cells was assessed using the TMS assay as outlined in the methods and materials (**section 3.9.1**). The CC_{50} values of the herbal extracts were obtained and are summarized in **Table 5** below. Briefly, the ethanol-, dereplicated ethanol- and aqueous- herbal concoction extracts yielded approximately 42, 4.0 and 51% cell viability respectively at 100 µg/ml when compared with the untreated cell control of HEK293T cells. The ethanol- and dereplicated ethanol- herbal extracts were cytotoxic to HEK293T and TZM-bl cells in a dose-dependent manner. Both of the positive controls (AZT and NVP) were not cytotoxic at the maximum and minimum concentrations tested 100-0.0017 µg/ml, respectively (data not shown). The vehicle control (DMSO) did not affect the cell viability at all concentrations including those evaluated for anti-reverse transcriptase activity (see **Figures** below). In this study CC_{50} values for plant herbal extracts ≤ 40 µg/ml were considered toxic and CC_{50} ≥ 100 µg/ml were considered non-toxic.

4.6 Anti-reverse transcriptase activity studies

Given that HIV-1 is a retrovirus, reverse transcriptase (RT) is one of the important virally encoded enzymes that catalyzes the conversion of viral RNA to proviral DNA, thus, presenting an important target for discovering antiretroviral therapeutics. Consequently, the RT activity was evaluated in the presence and absence of extracts using a novel phenotypic drug susceptibility assay as outlined in the methods and materials (**section 3.10.1**). The IC50 values of the herbal extracts and the positive controls that were obtained are summarized in **Tables 5** and **6**. Briefly, the ethanol-, dereplicated ethanol- and aqueous- herbal extracts at 100 µg/ml yielded approximately 18, 1.0 and 72% of RT activity, respectively when compared with the untreated cell control.

Graphical representation of the cytotoxicity results by all the herbal extracts evaluated in this study, DMSO and anti-reverse transcriptase by the extracts and the positive controls for all the HIV isolates are shown in **Figures 4.5** to **Figure 4.7**.

Table 5: *In vitro* **cytotoxicity and anti-HIV activity of the herbal extracts using HEK293T and TZM-bl cells**

Plant extracts	Viral strain	*CC_{50} (µg/ml)	*IC_{50} (µg/ml)	*SI
HEK293T Cells				
Ethanol extract	MJ4	92.95	56.53	1.644
	Du179		53.96	1.723
	CM9		55.39	1.671
Dereplicated ethanol extract	MJ4	66.17	51.87	1.276
	Du179		47.56	1.391
	CM9		52.81	1.253
Aqueous extract	MJ4	100.9	Inactive	-
	Du179		Inactive	-
	CM9		Inactive	-
TZM-bl cells				
Ethanol extract	MW	72.51	36.33	1.996
	VSV		24.91	2.912
	MLV		52.87	1.372
Dereplicated ethanol extract	MW	61.98	32.06	1.933
	VSV		20.82	2.978
	MLV		43.12	1.437
Aqueous extract	MW	193.0	Inactive	-
	VSV		Inactive	-
	MLV		Inactive	-

*CC_{50} (the concentration required to kill 50% of the cells) was estimated by using Graph Pad Prism 5® and Microsoft Excel (using linear forecasting algorithm to estimate a value that will have 50% activity).

*IC_{50} (the concentration required to inhibit 50% of the reverse transcriptase activity) was estimated by using Graph Pad Prism 5® and Microsoft Excel (using a linear forecasting algorithm to estimate a value that will have 50% activity).

*SI is the ratio of cytotoxicity to inhibitory activity = CC_{50}/IC_{50}

Table 6: Cytotoxicity and inhibitory activity of positive controls

Positive controls	Viral strain	*CC_{50} (µg/ml)	*IC_{50} (µg/ml)
HEK293T Cells			
NVP	MJ4	**>100**	**0.189**
	Du179		**0.130**
	CM9		**0.119**
AZT	MJ4	**>100**	**0.0826**
	Du179		**0.0535**
	CM9		**0.0182**
LPV	MJ4	**>100**	**5.775**
TZM-bl cells			
VRC01	MW	**n/a**	**0.0815**
	VSV	**n/a**	**0.153**
	MLV	**n/a**	**0.0390**

*CC_{50} (the concentration required to kill 50% of the cells) was estimated by using Graph Pad Prism 5[®] and Microsoft Excel (using linear forecasting algorithm to estimate a value that will have 50% activity).

*IC_{50} (the concentration required to inhibit 50% of the reverse transcriptase activity) was estimated by using Graph Pad Prism 5[®] and Microsoft Excel (using a linear forecasting algorithm to estimate a value that will have 50% activity).

n/a: not available (VRC01 is a potent CD4 binding site broadly neutralizing antibody, therefore it is not toxic).

4.7 Cytotoxicity and anti-reverse transcriptase (RT) activity of the ethanol extract

In this study, general extraction procedures and plant extract preparations were followed, some of which mimic the traditional preparations (see **section 3.2**) and others more scientifically accepted extraction procedures (i.e. using organic solvents for extraction). These were done for standardization purpose and comparison of the activities present in the different preparations (see **section 3.2** and **3.4**).

The ability of the ethanol herbal extract to inhibit HIV-1 RT was investigated using a novel RT phenotypic drug susceptibility assay. Cells were infected with either of the three HIV-1 isolates, namely, MJ4, Du179 and CM9. Following infection, cells were then treated with the ethanol extract at various concentrations followed by a 48 hour incubation. Culture supernatant was collected after 48 hours for Luciferase expression estimation by luminescence. AZT and NVP were included as positive controls and the results expressed as percent virus activity relative to control (100%). Cytotoxicity due to vehicle control (DMSO) was also evaluated and results expressed as percentage viability relative to control. Data points are representative of mean percent activity $\pm$ SD of two independent experiments: n=4; $p < 0.05$ was considered significant. **Figures 4.5** to **4.7** below show the graphical representation of the findings from these studies.
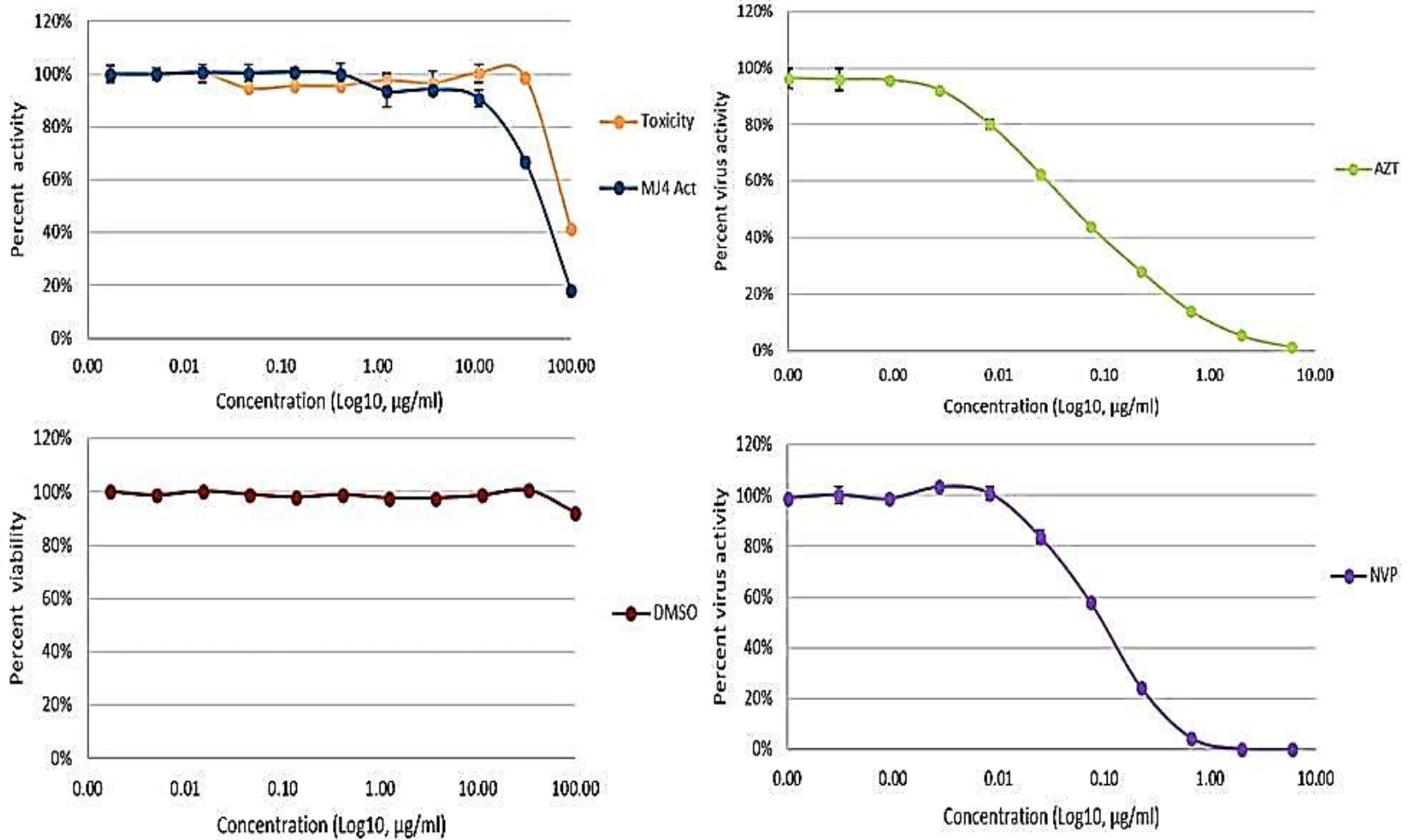
Figure 4.5: Reverse transcriptase phenotypic drug susceptibility profile of MJ4 HIV-1 subtype C isolate to the ethanol extract
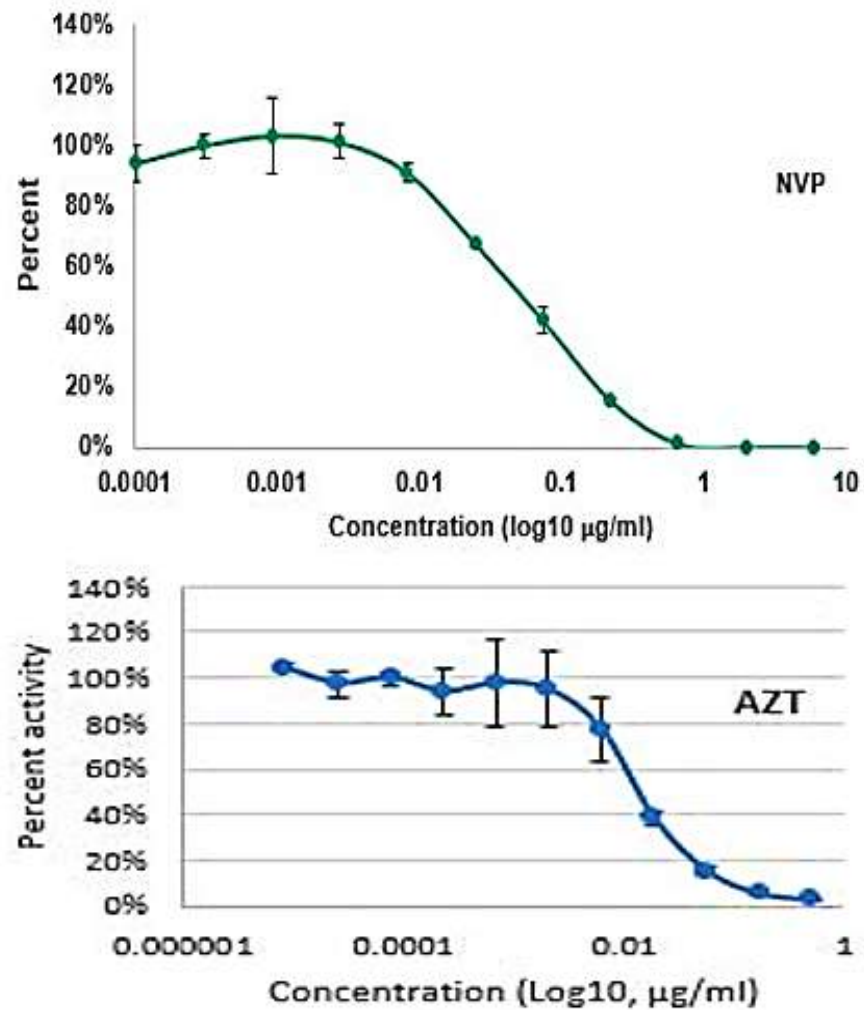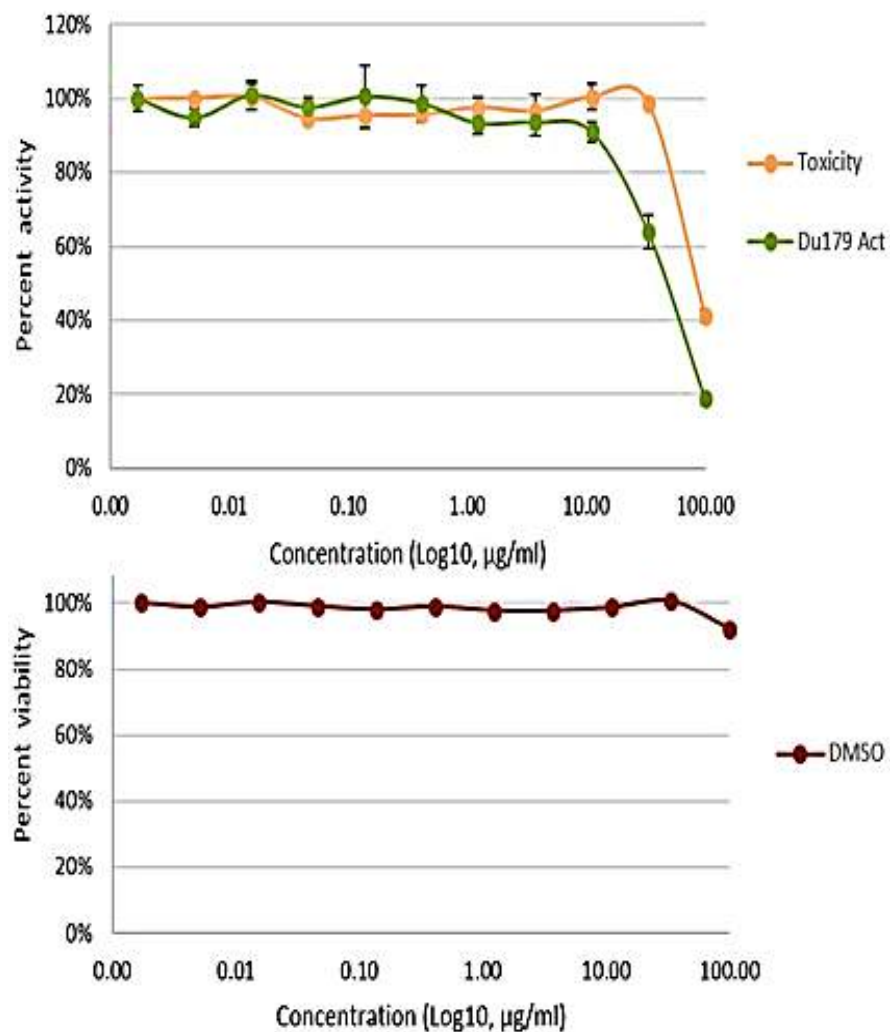
Figure 4.6: Reverse transcriptase phenotypic drug susceptibility profile of Du179 HIV-1 subtype C isolate to the ethanol extract.
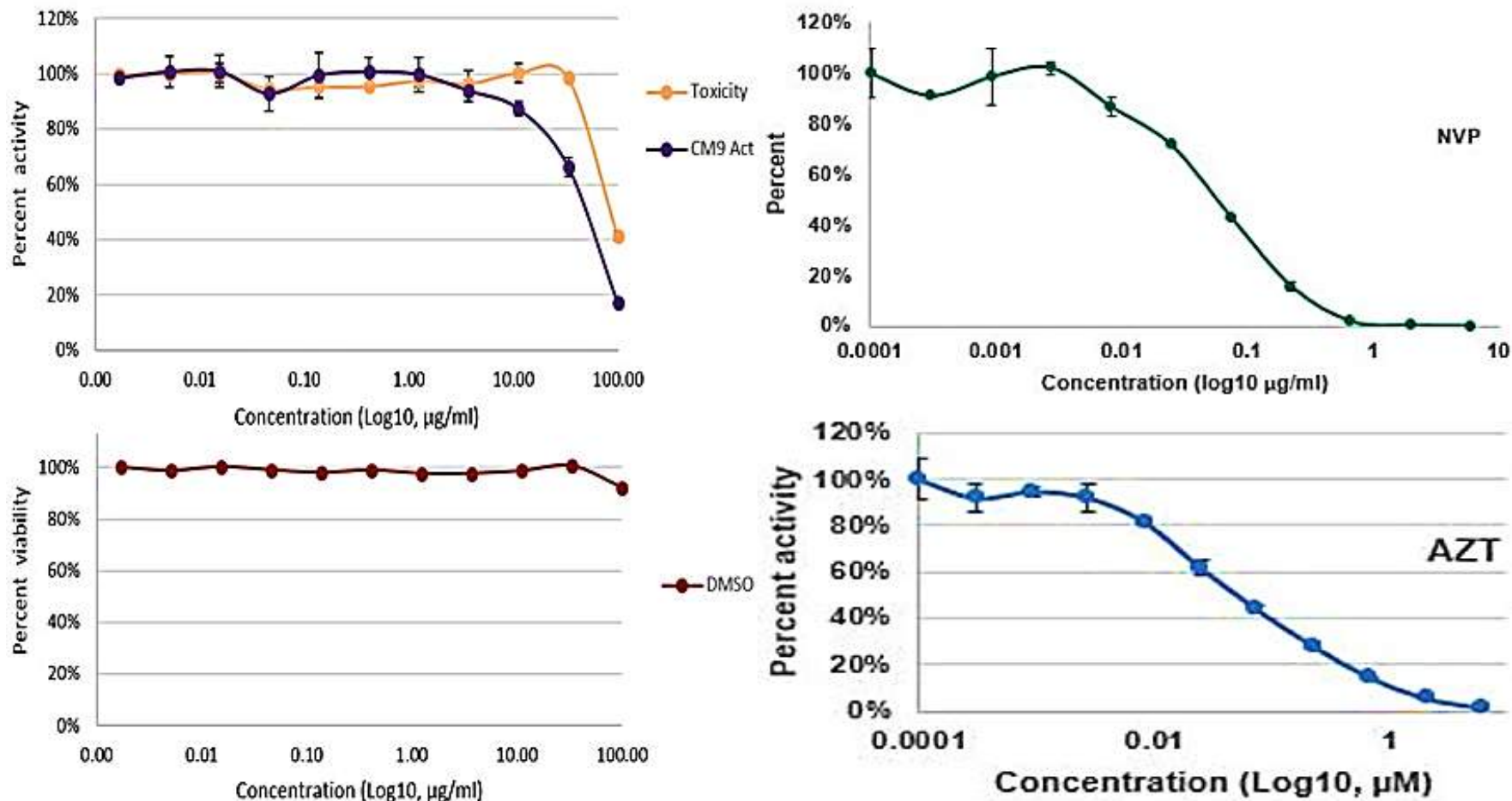
Figure 4.7: Reverse transcriptase phenotypic drug susceptibility profile of CM9 HIV-1 subtype C isolate to the ethanol extract.

4.8 Cytotoxicity and anti-reverse transcriptase (RT) activity of the dereplicated ethanol extract

As indicated earlier (**section 3.4.3**), studies by Cardellina et al., (1993) indicated that tannins present in plant extracts are responsible for the false positive results observed especially in studies of HIV and medicinal plants. Therefore, in the present study, we attempted to remove tannins from the ethanol herbal extract with the aim of determining whether they were responsible for the observed anti-HIV-1 activity or whether other compounds within the extract were responsible for the observed activity. Tannins were removed as outlined in the Methods and Materials section and this extract ("dereplicated ethanol extract") subjected to the same anti-HIV investigations as the ethanol herbal extract. Below (**Figures 4.8** to **4.10**) are the findings of both cytotoxicity and anti-RT activity by this extract.

Phenotypic drug susceptibility of the MJ4 HIV-1 subtype C isolate to the dereplicated ethanol extract was assessed using a novel RT phenotypic drug susceptibility assay. Cells were infected with either three of the HIV-1 isolates, namely, MJ4, Du179 and CM9. Infected cells were then treated with the dereplicated extract at various concentrations followed by a 48 hour incubation. Culture supernatant was collected after 48 hours for Luciferase expression estimation by luminescence. Results are expressed as percentage of activity relative to control (100%). The toxicity to HEK293T cells was also investigated. AZT and NVP were included as positive controls and their results expressed as percent virus activity relative to control (100%). Cytotoxicity due to vehicle control (DMSO) was also evaluated and results expressed as percentage viability relative to control. Data points are representative of mean percent activity $\pm$ SD of two independent experiments: n=4; p< 0.05 was considered significant.
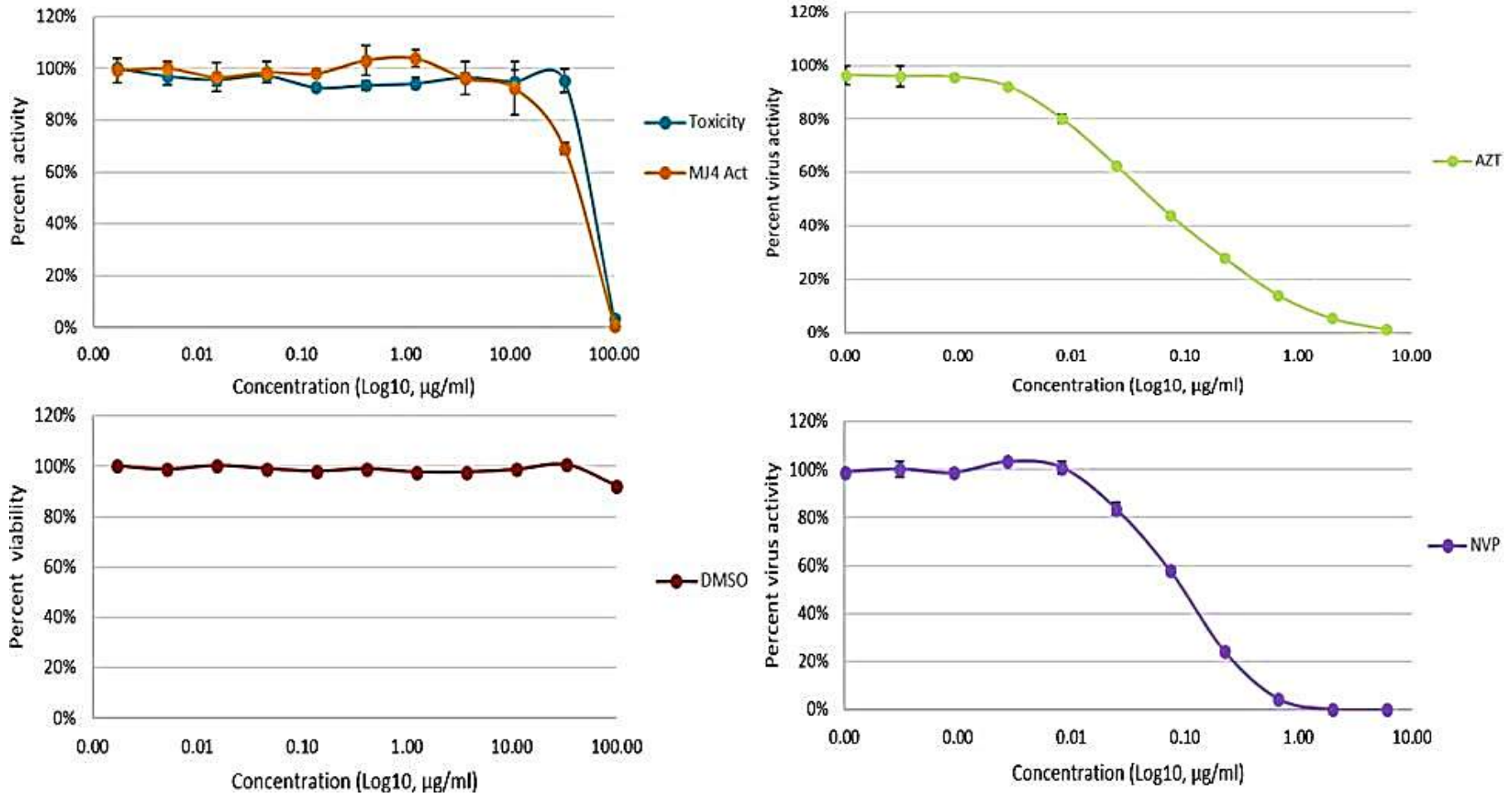
Figure 4.8: Reverse transcriptase phenotypic drug susceptibility profile of a MJ4 HIV-1 subtype C isolate to dereplicated ethanol extract.
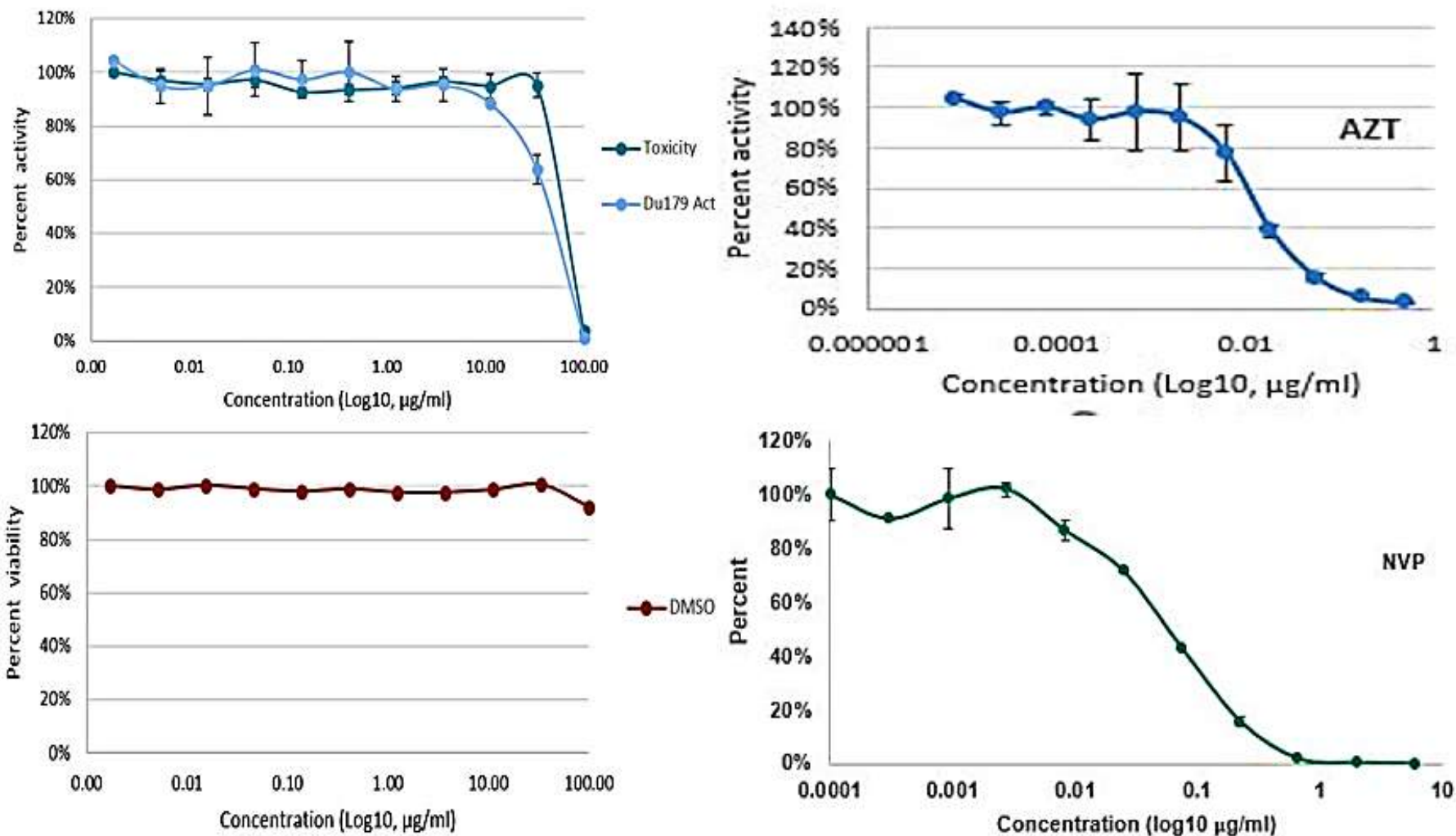
Figure 4.9: Reverse transcriptase phenotypic drug susceptibility profile of a Du179 HIV-1 subtype C isolate to dereplicated ethanol extract.
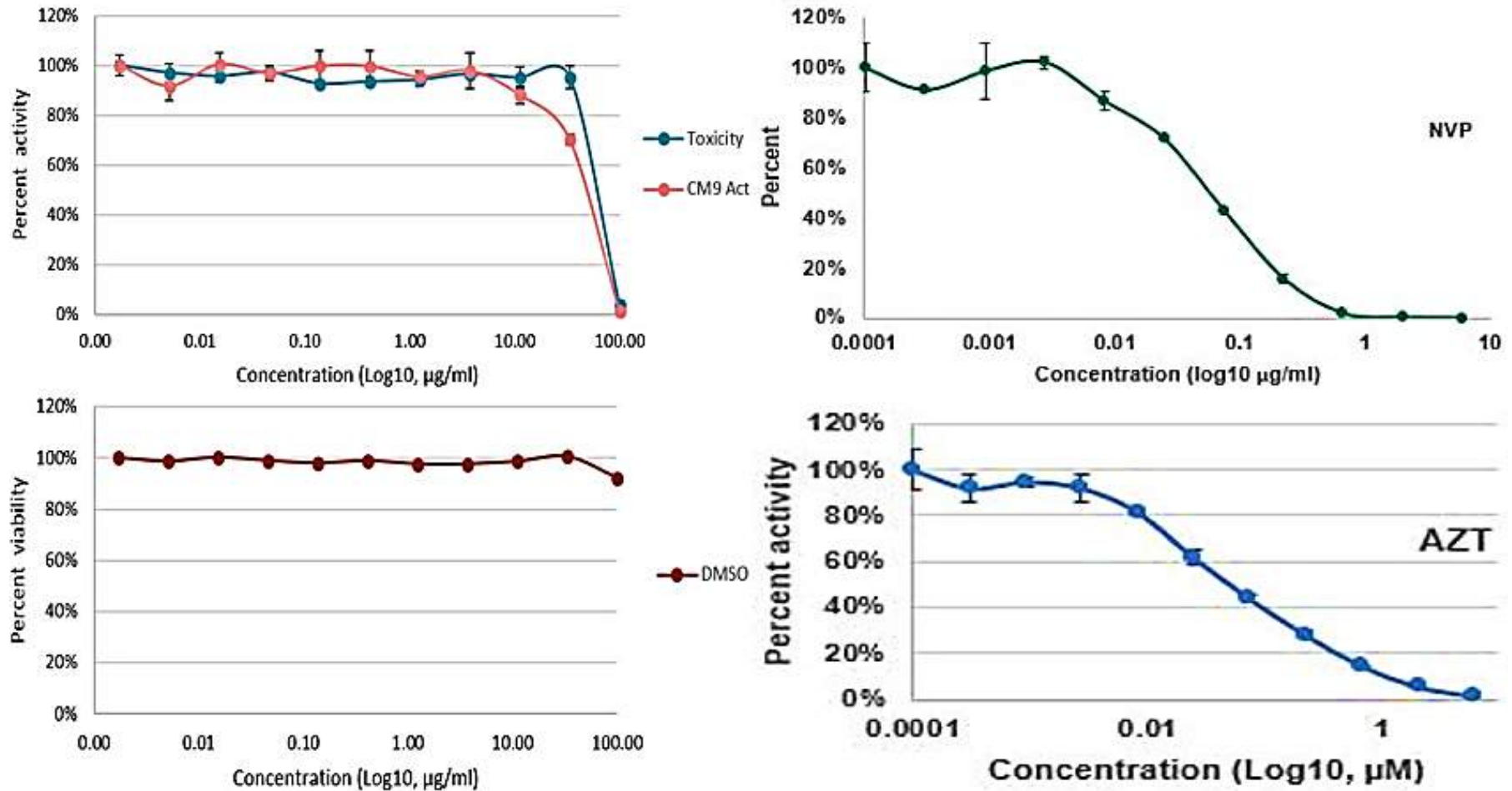
Figure 4.10: Reverse transcriptase phenotypic drug susceptibility profile of a CM9 HIV-1 subtype C isolate to dereplicated ethanol extract.

4.9 Cytotoxicity and *in vitro* phenotypic reverse transcriptase (RT) activity of the aqueous herbal concoction

In this section, the traditional healer's extraction technique was mimicked as outlined in the Methods and Materials section. This yielded an aqueous extract which was used to evaluate its ability to inhibit HIV infection. **Figure 4.11** below depicts the findings of this extract treatment with regard to cytotoxicity and anti-RT activity.

Phenotypic drug susceptibility of the MJ4 HIV-1 subtype C isolate to aqueous herbal extract was assessed using a novel RT phenotypic drug susceptibility assay. Cells were infected with an HIV-1 MJ4 isolate and treated with extracts at various concentrations followed by a 48 hour incubation. Culture supernatant was collected after 48 hours for Luciferase expression estimation by luminescence. Results are expressed as percentage of activity relative to control (100%). The toxicity to HEK293T cells was also investigated. AZT and NVP were included as positive controls and their results expressed as percent virus activity relative to control (100%). Cytotoxicity due to vehicle control (DMSO) was also evaluated and results expressed as percentage viability relative to control. Data points are representative of mean percent activity $\pm$ SD of two independent experiments: n=4; $p < 0.05$ was considered significant.

The extract was not active and, thus, we only screened for anti-RT activity against the MJ4 HIV-1 subtype C isolate and not the other isolates (i.e. Du179 and CM9). This was also done for all inactive extracts not only for anti-RT but for other assays as well.
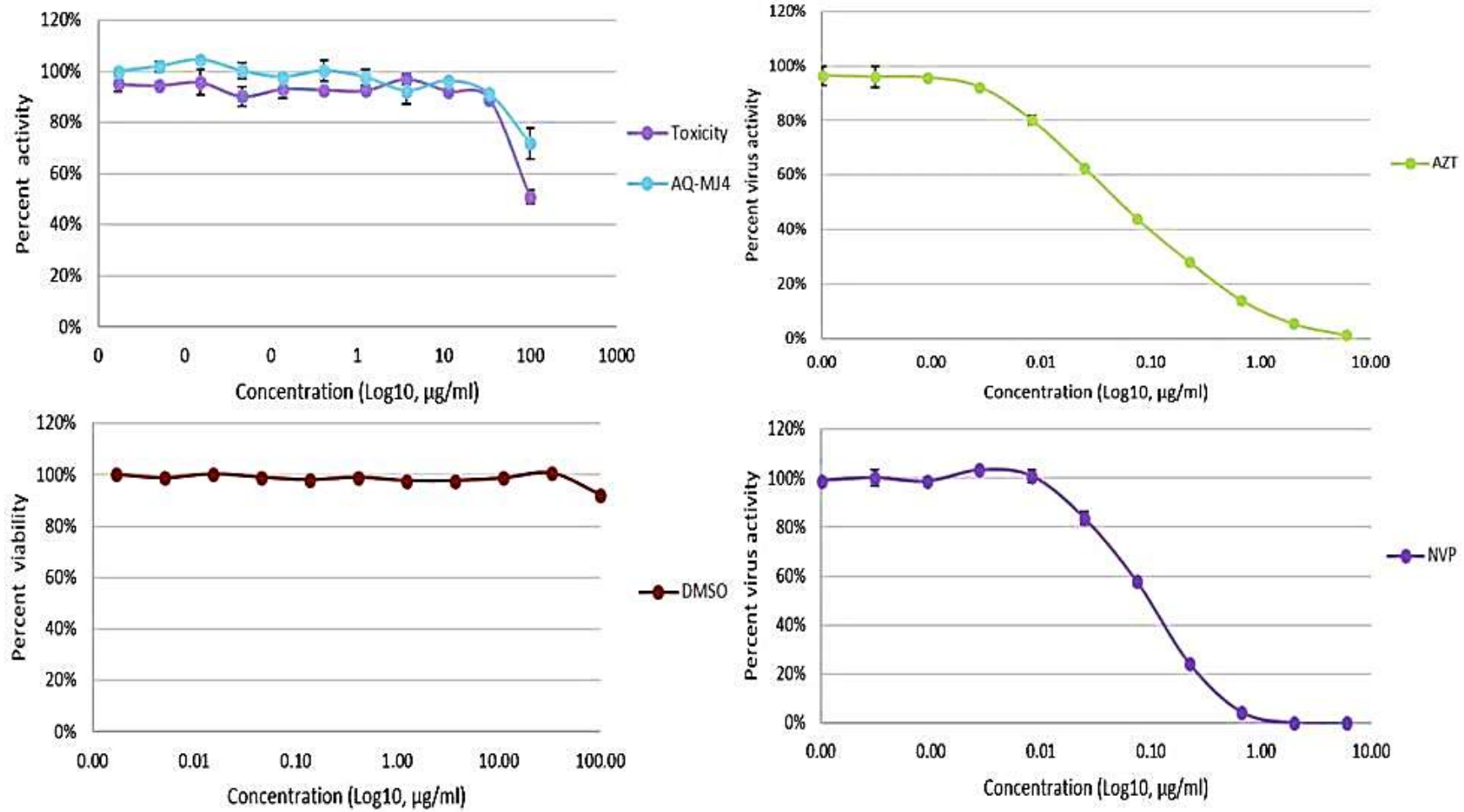
Figure 4.11: Reverse transcriptase phenotypic drug susceptibility profile of a MJ4 HIV-1 subtype C isolate to aqueous extract.

4.10 Description and discussion of the anti-RT activity by the herbal extracts

Figures. **4.5-4.7** show the toxicity of the ethanol herbal extract to HEK293T cells ("toxicity" curve). The extract yielded a mean CC_{50} value of 92.95 ± 23.32 µg/ml. Furthermore, the dereplicated ethanol herbal extract yielded a mean CC_{50} value of 66.17 ± 2.300 µg/ml and both extracts induced cytotoxicity to HEK293T cells in a concentration dependent manner and found to be non-toxic at concentrations below 100 µg/ml.

Comparison of the ethanol extract and the dereplicated ethanol extract showed that the removal of tannins enhanced the cytotoxicity of the dereplicated extract. This data suggests that during the dereplication process, phytochemicals that had a protective effect were also removed, thus, enhancing cytotoxicity. Also, the dereplication process improved the inhibitory activity of the dereplicated extract slightly as seen from the slight change of the IC_{50} values, before and after tannin removal. This is also indicative of the removal of phytochemicals that had antagonistic activity to the active phytochemicals. The aqueous extract on the one hand yielded a mean CC_{50} value of 100.9 ± 8.969 µg/ml and was found to be non-toxic to HEK293T cells at all concentration ranges.

Vehicle control (DMSO) did not affect the cell growth and, therefore, the observed cytotoxicity and anti-RT activity is a result of treatment with the ethanol-, dereplicated ethanol- and aqueous- extracts. The positive controls (AZT & NVP) behaved as expected for the anti-RT assay and, thus, it was concluded that the assay was valid and proceeded with data analysis.

The shifting of anti-RT activity curves ("MJ4-, Du179- and CM9- Act") to the left and downwards of the "toxicity" curve is indicative of inhibitory activity, while a shift to the right and upwards indicates no inhibitory activity. From the plots above (**Figures 4.5-4.10**), it is evident that all three viruses behave the same with regard to drug susceptibility following treatment with ethanol- and dereplicated ethanol- extracts at various concentrations.

Table 5 has a summary of the mean IC_{50} values of all viral isolates (MJ4, CM9 and Du179) for all the treatments investigated. From this table, the IC_{50} values lie within the 50-55 µg/ml range, which indicative of a moderate inhibitory activity exerted by the two extracts (ethanol- and dereplicated ethanol- extracts). Potent inhibitory activity by extracts lies below the 50-55 µg/ml range ($IC_{50} \leq 50$ µg/ml) while inactivity is expected to be $IC_{50} \geq 100$ µg/ml. Therefore,

in this study, it was shown that the aqueous herbal extract has an IC_{50} value above 100 µg/ml and, hence, this extract was considered to be inactive against HIV-1 RT.

Figures 4.5-**4.10** furthermore, show that from concentration 33.33 µg/ml, the activity of HIV-1 RT starts to decline and eventually it is completely inhibited at 100 µg/ml by the dereplicated ethanol extract and partially inhibited by the ethanol extract. It should be noted that the observed, complete and partial inhibitory activity at 100 µg/ml is attributed to cytotoxicity rather than true HIV-1 RT inhibition. At 33.33 µg/ml, toxicity is calculated as 1.000% and anti-HIV-1 RT activity as 33.00, 33.00 and 36.00% against MJ4, CM9 and Du179 respectively, for the ethanol extract. While, on the other hand, the dereplicated ethanol extract at the same concentration showed anti-HIV-1 RT activity to be 31.00, 30.00 and 36.00% for MJ4, CM9 and Du179, respectively.

Overall, these results suggest that both the ethanol- and dereplicated ethanol- herbal extracts have moderate activity against HIV-1 RT at 33.33 µg/ml. Tan et al (1991) have found that crude plant extracts that yield IC_{50} values in the range of 10-50 µg/ml usually have a single or fewer RT inhibitors in low concentration in the extract. We, therefore, assume that these extracts have fewer phytochemicals that can inhibit HIV-RT.

Statistical analysis, using factorial ANOVA followed by Bonferroni post hoc analysis, indicated that the viral isolate (MJ4, Du179 and CM9) and the relationship between dosage and virus subtype do not have a significant effect ($p > 0.05$) to the observed anti-HIV-1 RT activity. However, both Two-way and factorial ANOVA followed by Bonferroni post hoc analysis indicated that dosage alone (i.e. concentration treatment of 33.33 µg/ml) is accountable for the observed significant difference ($p < 0.001$) between toxicity and anti-RT activity induced by these two ethanol extracts.

A possible explanation for the observed anti-HIV-1 RT activity of the ethanol extracts is that these extracts encompass a plethora of phytochemicals such as terpenes, flavonoids, alkaloids and polysaccharides as depicted and discussed in **section 4.1**, **Figure 4.1** (**c**). Moreover, chemical analysis of these ethanol extracts as depicted in **section 4.3**, **Figures 4.2** and **4.3,** implies that intermediate polar compounds are responsible for the observed anti-RT activity.

Other studies by Bedoya et al., (2001); Rukunga et al., (2002); Bessong et al., (2004); Bessong et al., (2005); and Bessong et al., (2006), have also reported that organic extracts

from medicinal plants are more inhibitory than the aqueous extracts in screening for anti-HIV activity. This is because organic solvents such as ethanol, methanol, chloroform etc. extract a range of phytochemicals that are relatively less polar when compared to the water-extracted phytochemicals. Additionally, studies by Hughes et al., (2008) showed that there is a link between toxicity, activity and the physiochemical properties of drugs/phytochemicals. The authors found that more polar drugs in whole-cell systems have a decreased activity and toxicity when compared with more non-polar drugs. Furthermore, they showed that drugs with intermediate polarity have moderate activity with occasional toxicity.

This trend was indeed observed in this study. As previously stated, our findings from **section 4.3** (**Figures 4.2 and 4.3**) showed that the ethanol extracts are mostly composed of intermediate polar phytochemicals. Biological screening (**Figures 4.5-4.10**) of these extracts showed that they possess moderate activity and therefore they may contain single/fewer RT inhibitors, and also, they are non-cytotoxic at various concentrations excluding 100μg/ml.

Contrary to the above findings, the aqueous extract was found to be inactive and non-cytotoxic at all concentrations. There are several possible explanations for the observed findings of the treatment with the aqueous extract. Firstly, this might be due to the fact that the preparation of aqueous extract mimicking the traditional healer resulted in degrading thermoliable phytochemicals that may be potentially bioactive.

Secondly, the aqueous extract is mostly comprised of polar phytochemicals rather than intermediate polar compounds (**Figure 4.4**). This presents issues with the bioavailability of the compounds within the extract. This, is because polar compounds have been shown to have higher log P values which is related to poor absorption of these compounds through biological membranes and, hence, their distribution in tissues and intracellular targets such HIV-1 RT and PR in a whole-cell assay system is decreased (Edward et al., 2003).

The fact that this extract is non-cytotoxic is ascribed to it having more polar phytochemicals and, thus, their inability to interact with intracellular proteins such as the 10 nuclear receptors (androgen, estrogen a, estrogen b, glucocorticoid, liver X, mineralocorticoid, peroxisome proliferator-activated receptor g, progesterone, thyroid a, thyroid b), four members of the cytochrome P450 enzyme family (1A2, 2C9, 2D6, 3A4), a cytosolic transcription factor (aryl

hydrocarbon receptor) and a potassium ion channel (hERG) which are implicated in triggering toxicity effects (Vedani et al., 2015).

Statistical analysis revealed that there is no significant difference ($p > 0.05$) between cytotoxicity and anti-RT activity for the aqueous extract which indicates that this extract is inactive at all the concentrations tested for anti-RT activity.

To predict the relative safety profiles of these extracts for consumption by patients, we calculated the selective index (SI). This is the ratio between the toxic and the therapeutic dose and is used as a measure of its relative safety. "Safe to consume" drugs/therapeutic products by patients have minimum SI values > 5 to start of with. The SI values of the ethanol- and dereplicated ethanol- extracts are in the range of 1-2 which is below the minimum SI values for "Safe to consume" drugs/therapeutic products. Therefore, an SI value < 5 indicates that the extract/drug should be taken with care and in lower quantities to avoid severe side effects and drug poisoning.

4.11 Cytotoxicity and *in vitro* phenotypic protease (PR) activity of the ethanol extract of the herbal concoction

HIV-1 protease is one of the most important enzymes in the HIV life cycle. It functions at post-entry level. Protease is responsible for facilitating the assembly and association of the viral components at the host cell membrane which then buds off as immature virions. Protease activity continues after budding from the host cell to ensure maturation into a fully infectious virion. Because of this, it is also an ideal target as an antiretroviral therapeutic. We have also screened our extracts against HIV-1 subtype C protease using a novel protease phenotypic drug susceptibility whole-cell assay system and the results are shown in **Figure 4.12** to **Figure 4.14**.

The extracts (ethanol-, dereplicated ethanol- and aqueous- herbal extracts) were evaluated for anti-HIV-1 PR activity utilizing a novel protease phenotypic drug susceptibility assay. Cells were infected with an HIV-1 MJ4 isolate and treated with extracts at various concentrations followed by a 48 hours incubation. Culture supernatant was collected after 48 hours for Luciferase expression estimation by luminescence. Results are expressed as percentage of activity relative to control (100%). The toxicity to HEK293T cells was also investigated. Lopinavir (LPV) was included as a positive control and DMSO as a vehicle control with results expressed as percentage activity and viability relative to control. Data points are representative of mean percent activity $\pm$ SD of two independent experiments: n=4; p< 0.05 was considered significant.

Upon inspection and analysis of the data obtained for the anti-PR activity by all three extracts, it is evident that all three extracts do not possess anti-PR activity. No left and downward shift was observed for all extracts. Extract treatment resulted in no difference between activity and toxicity. The activity plots (**Figures 4.12-4.14**) overlap with toxicity plots.

In all experiments, the vehicle control (DMSO) did not affect the cell growth and, hence, the observed cytotoxicity and anti-PR activity is a result of the ethanol, dereplicated ethanol and aqueous extract treatments. The positive control Lopinavir (LPV) behaved as expected for anti-PR assay and, thus, it was concluded that the assay was valid and proceeded with data analysis.

Statistical analysis using a student t-test also confirmed these findings. There was no significant difference between activity (treated samples) and untreated samples. Calculated p-values greater than 0.05 ($p > 0.05$) at all the concentrations screened were obtained.
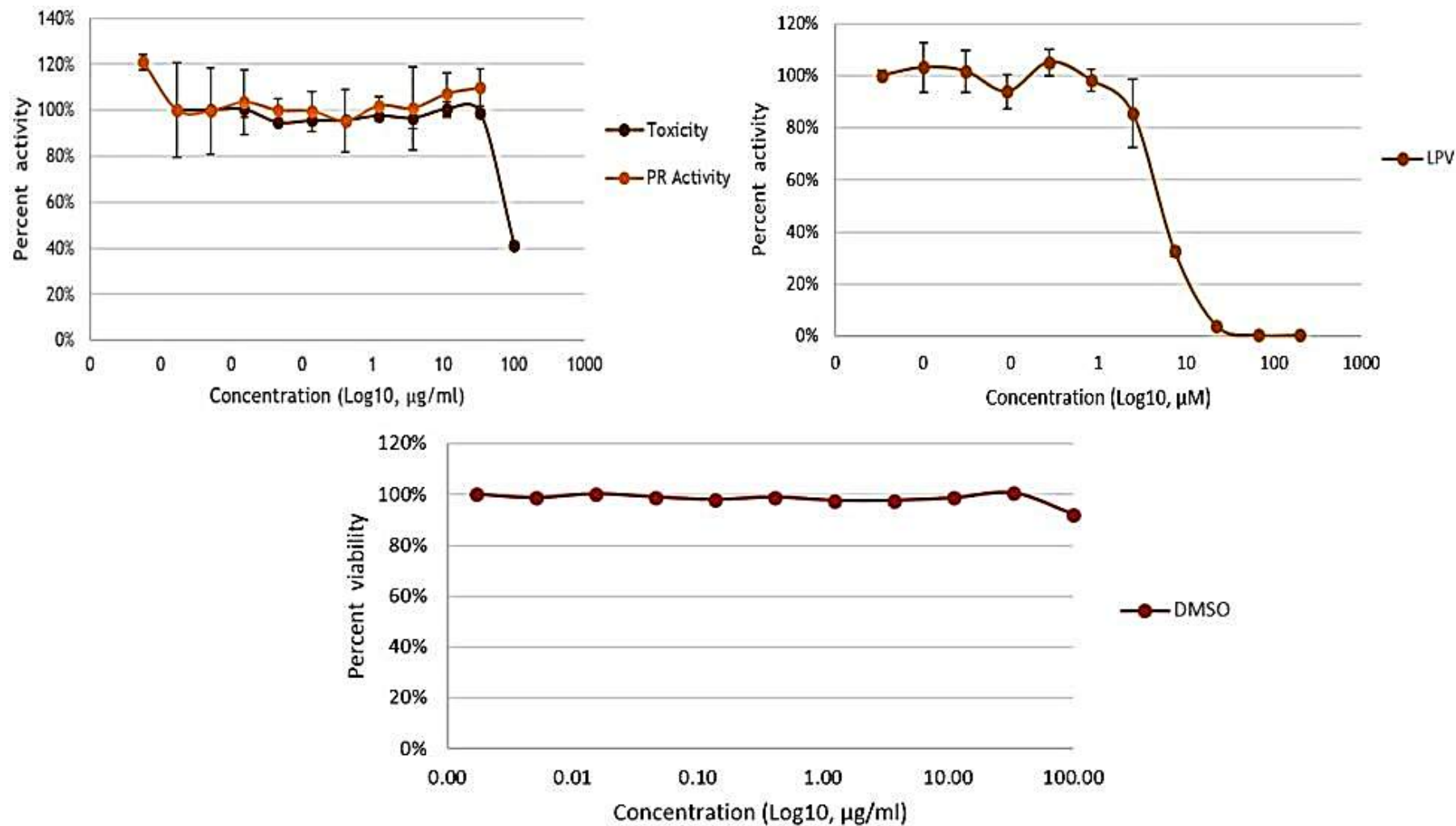
Figure 4.12: Protease phenotypic drug susceptibility profile of MJ4 HIV-1 subtype C isolate to the ethanol extract.
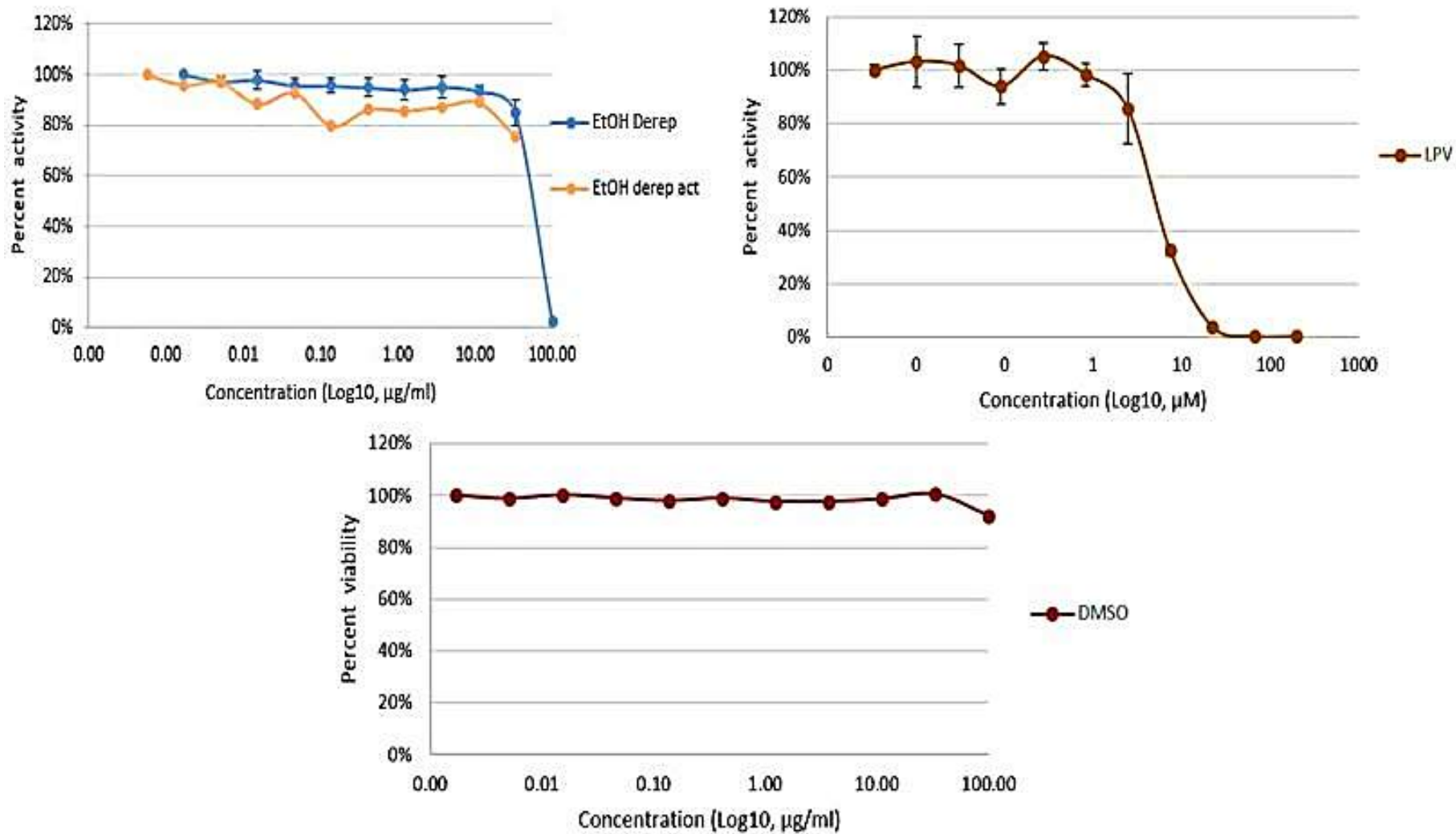
Figure 4.13: Protease phenotypic drug susceptibility profile of a MJ4 HIV-1 subtype C isolate to the dereplicated ethanol extract.
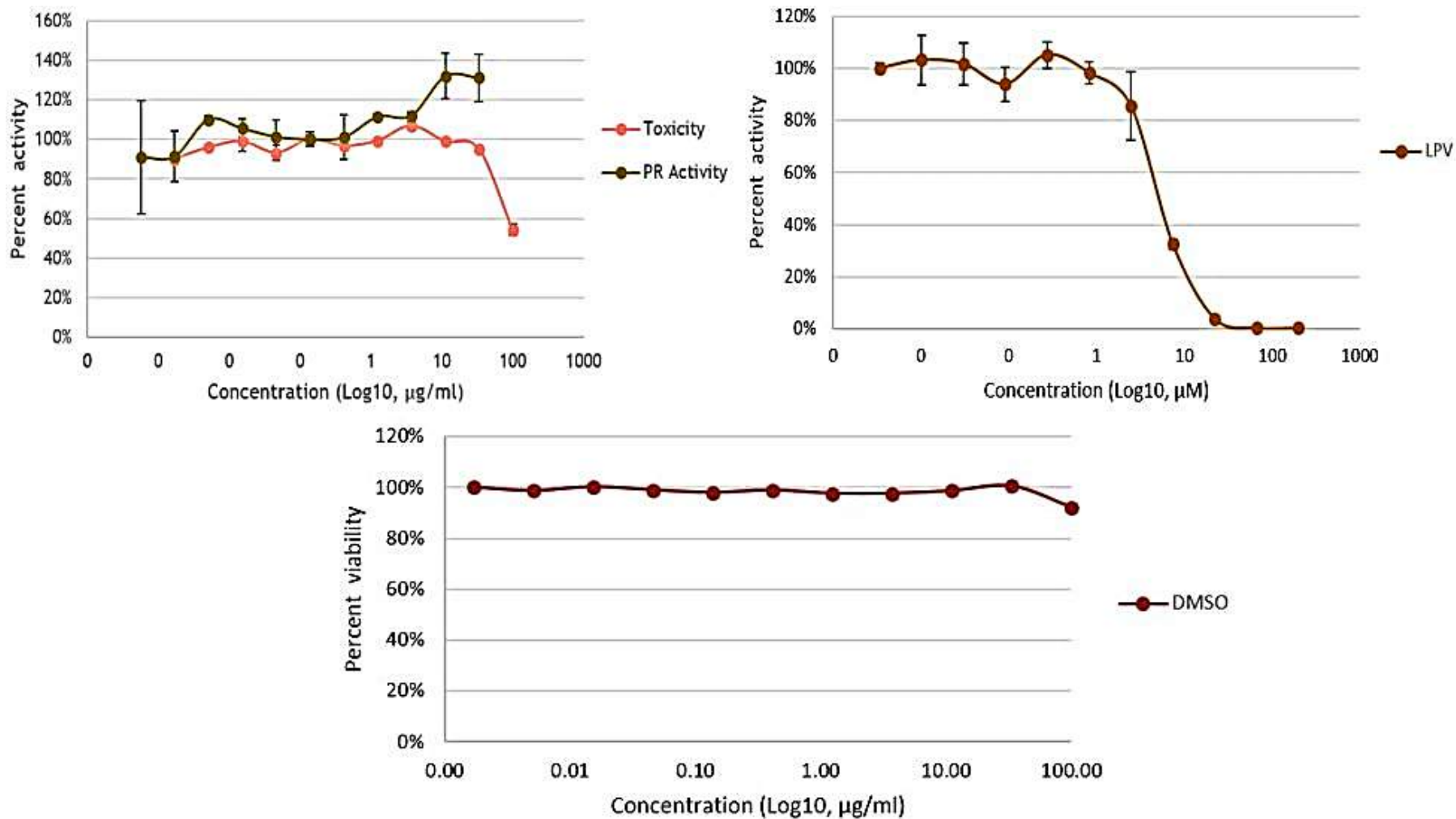
Figure 4.14: Protease phenotypic drug susceptibility profile of a MJ4 HIV-1 subtype C isolate to the aqueous extract.

4.12 Neutralization activity (viral entry TZM-b1 assay)

Given that the ethanolic extracts showed anti-HIV activities against different HIV strains, their anti-HIV mechanisms were explored further. The HIV entry process, is made up of three stages: gp120-CD4 electrostatic interaction, gp120-CCR5/CXCR4 chemokine interaction and membrane fusion. These are regarded as attractive targets for chemotherapeutic intervention, because blocking HIV entry into its target cell leads to suppression of viral infectivity, replication and the production of progeny virions.

The possibility of the ethanolic extracts to inhibit HIV-1 subtype C entry into target host cells was also investigated in this study. The entry inhibition specificity of the extracts (i.e. whether the extracts are only HIV-1 specific in their inhibition or not) was evaluated and, hence, two other non-HIV viruses namely, Vesicular stomatitis virus (VSV) and Murine leukemia virus (MLV) were used. To accomplish this, the neutralization TZM-bl assay was used.

The results obtained for anti-HIV infection suggest that the ethanol extract and the dereplicated ethanol extract have activity whereas the aqueous herbal extract does not. Both empirical and visual evaluation and analysis of the data and the plots, confirm that these two extracts have anti-HIV entry activity as seen by the downward and left shifting of the curves in **Figure 4.15** and **Figure 4.18**.

However, these extracts were not HIV specific in their entry inhibitory activity since there was activity also obtained against VSV (**Figure 4.16** and **Figure 4.19**). The inhibitory activity against VSV was more potent when compared to that of HIV at 33.33 µg/ml. Furthermore, cytotoxicity against VSV was lower and slightly higher for MW HIV-1 isolate when treated with ethanol- and dereplicated ethanol- extracts respectively. The extracts were cytotoxic against MLV (**Figures 4.17** and **4.20**) and this was confirmed by statistical analysis which showed that there is no significant difference between toxicity and activity at all concentrations. The aqueous extract was also found to be inactive against the MW HIV-1 isolate following empirical and statistical analysis at all concentrations tested. **Figures 4.15-4.21** below depicts graphically the above stated observations. VRC01 (a CD4 antagonist) and DMSO were included as positive and vehicle controls respectively. Data points are

representative of mean percent activity $\pm$ SD of two independent experiments: n=4 and p< 0.05was   considered   significant.
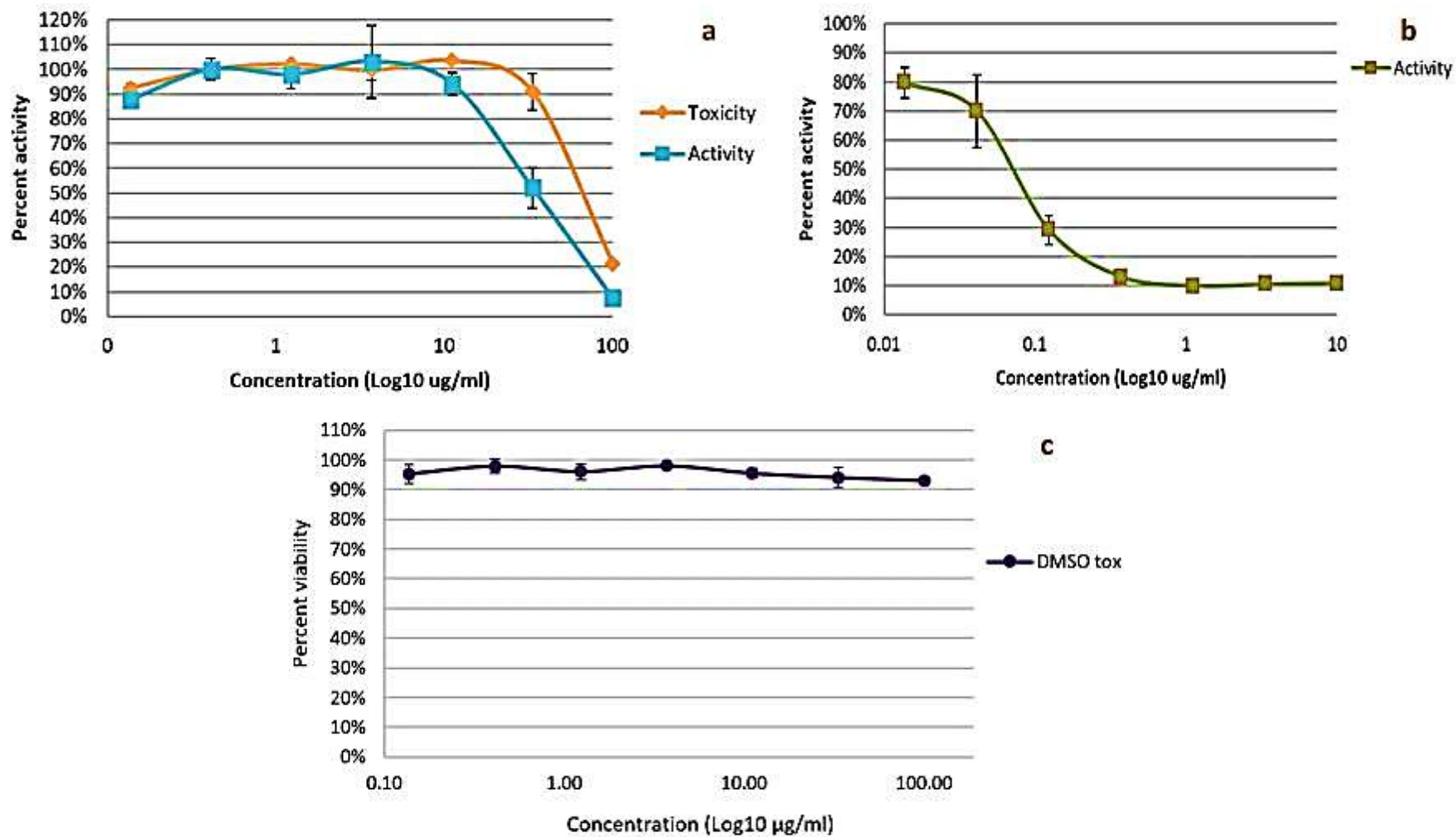
Figure 4.15: HIV-1 (MW isolate) neutralization profile by the ethanol extract.
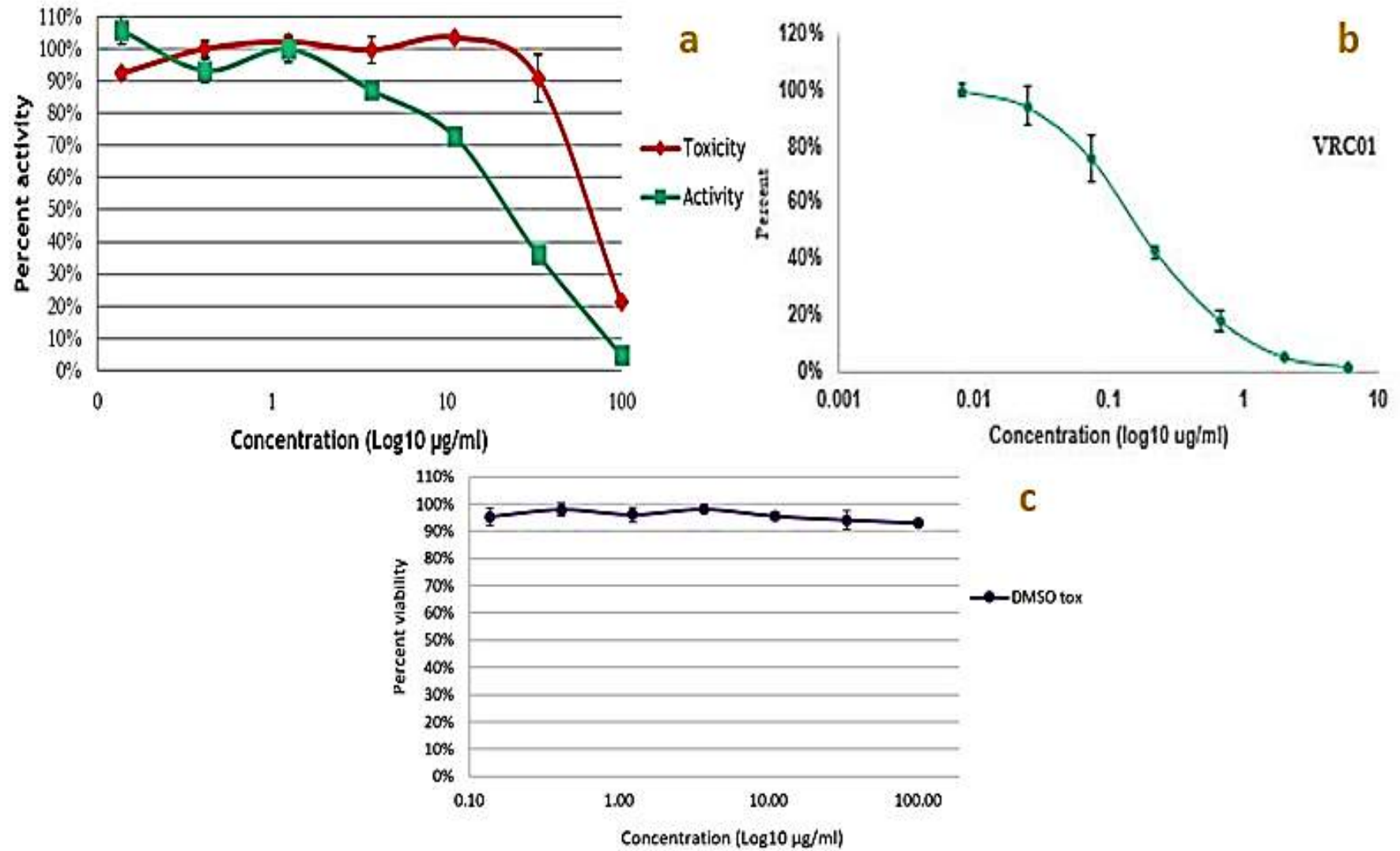
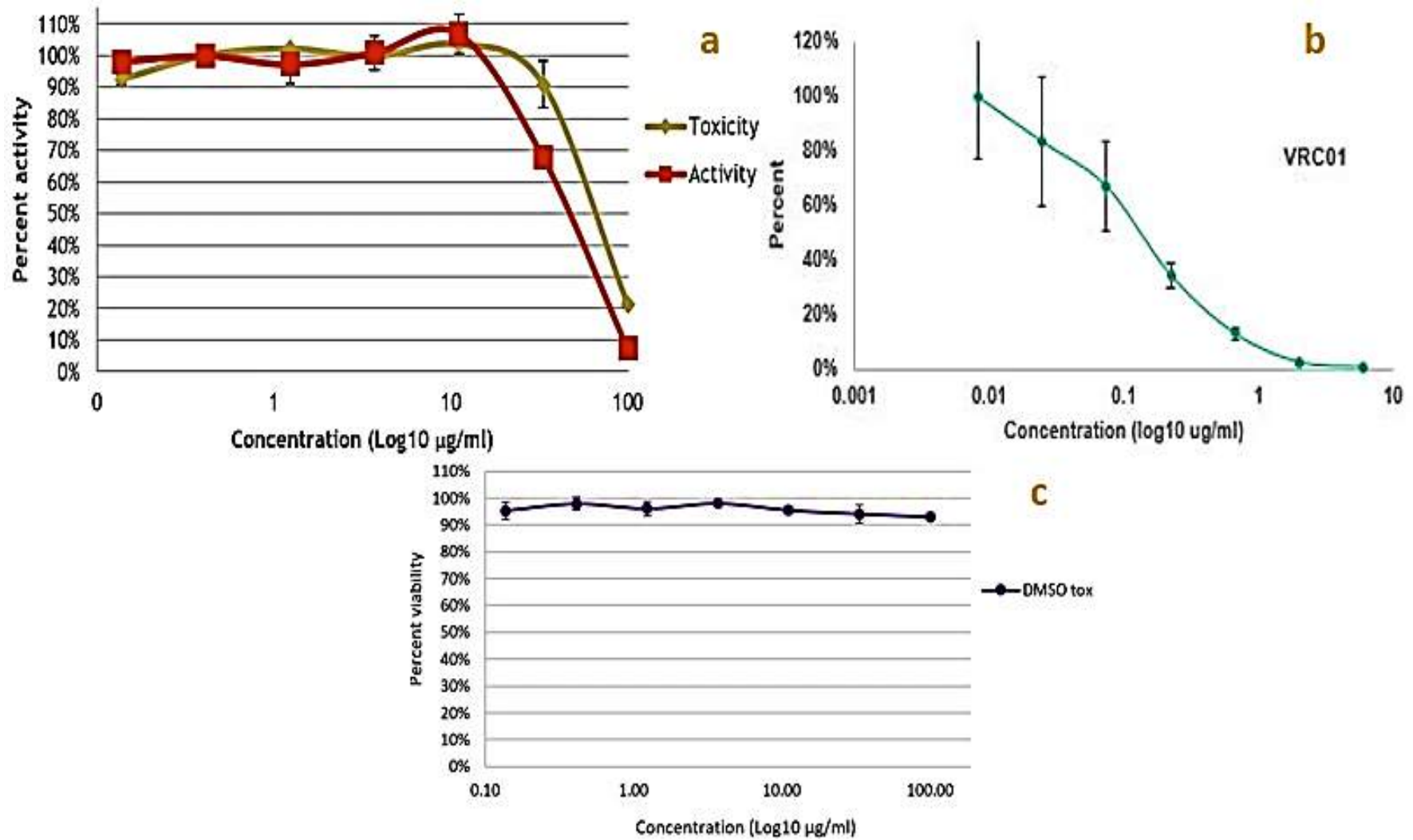**Figure 4.16: VSV neutralization profile by the ethanol extract.**

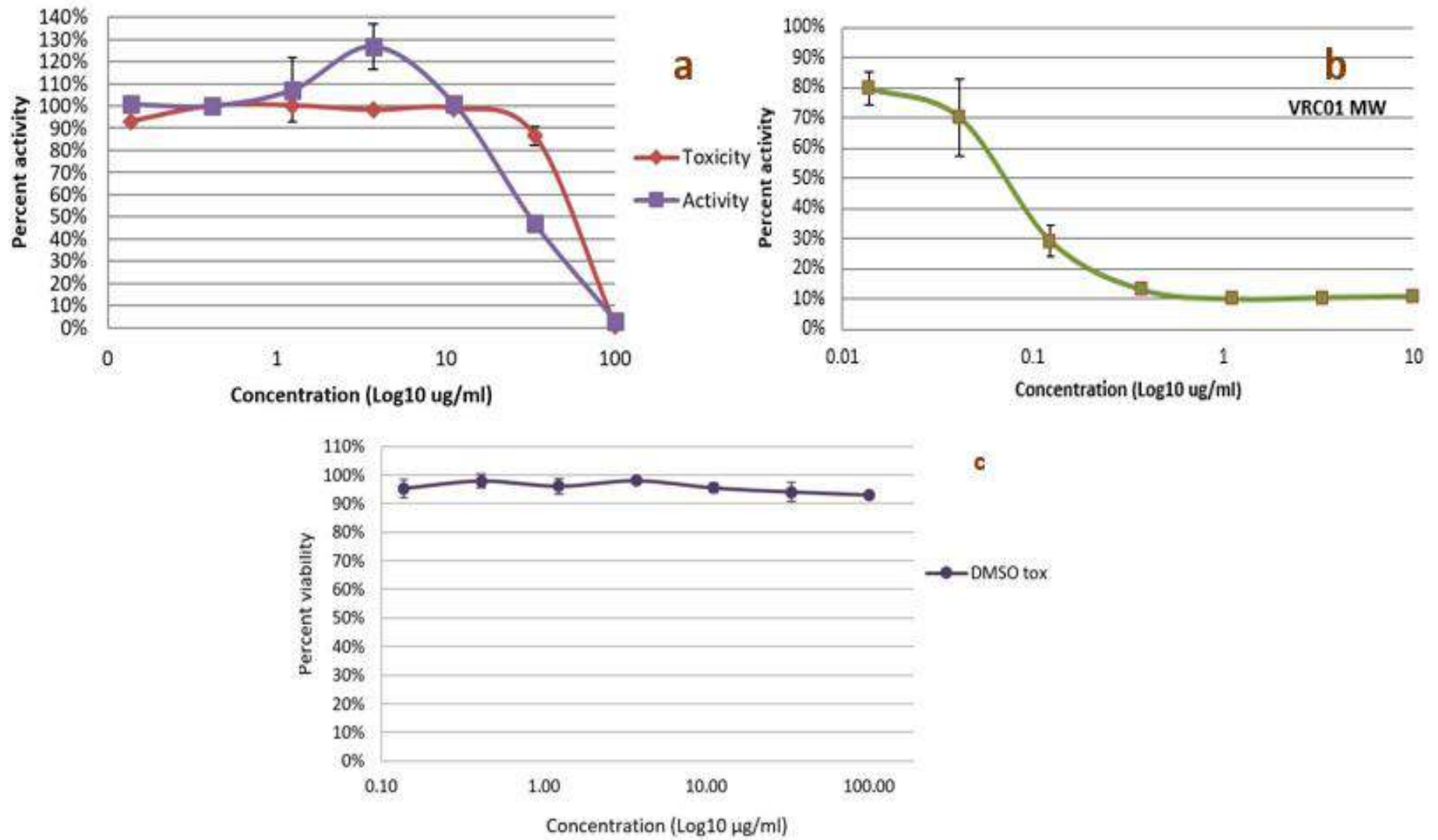**Figure 4.17: MLV neutralization profile by the ethanol extract.**

Figure 4.18: HIV-1 (MW isolate) neutralization profile by the dereplicated ethanol extract.
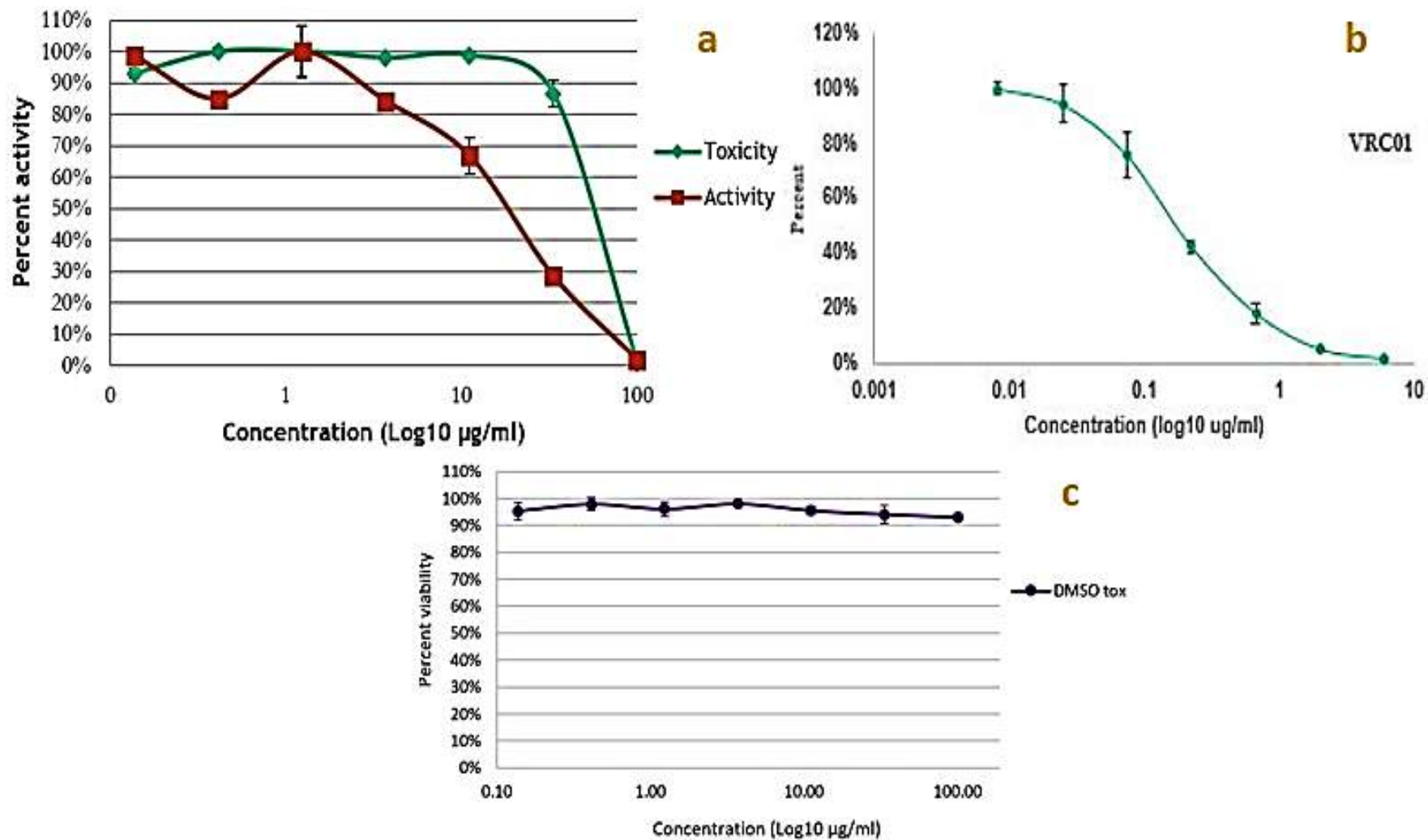
Figure 4.19: VSV neutralization profile by the dereplicated ethanol extract.
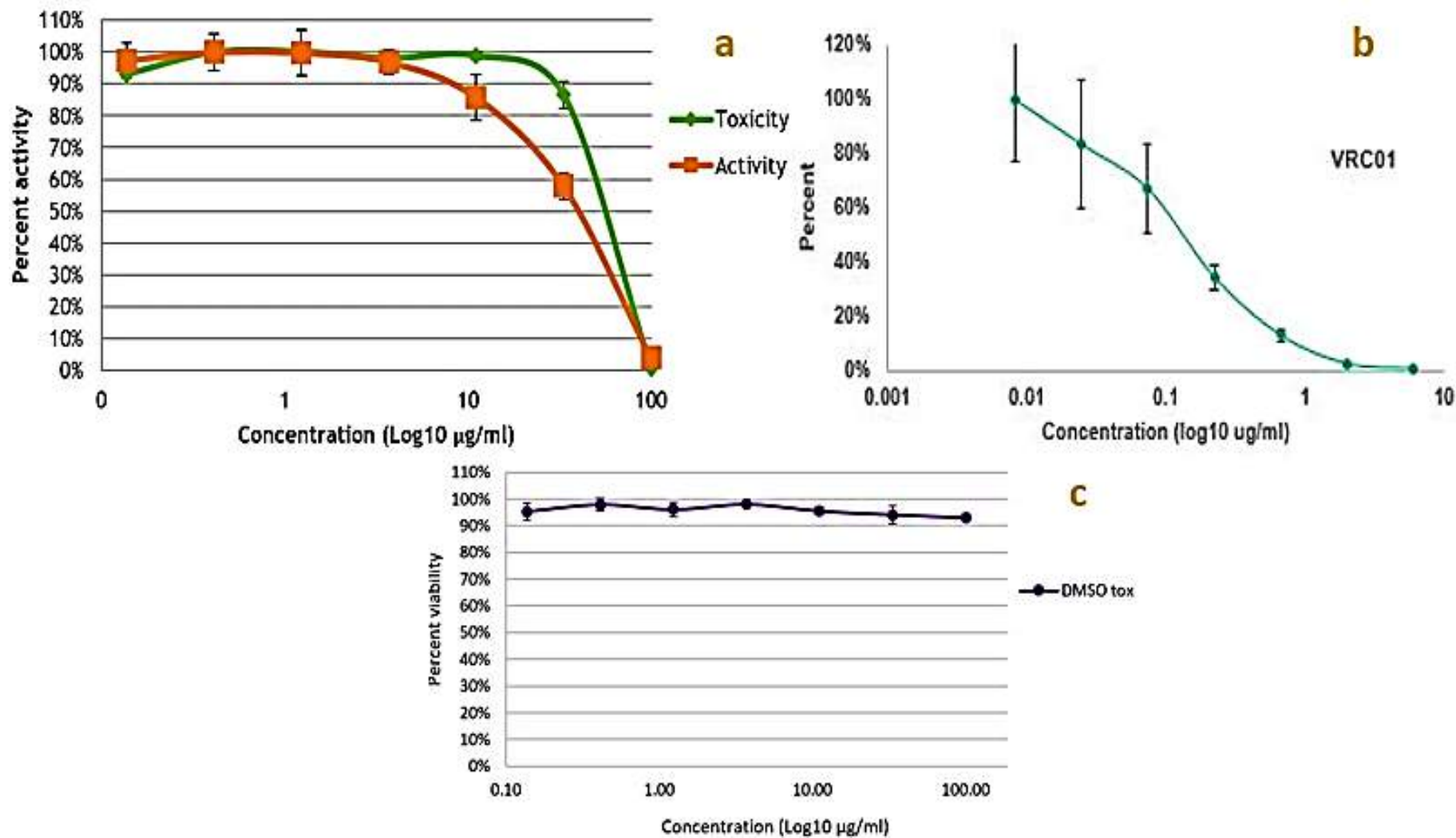
Figure 4.20: MLV neutralization profile by the dereplicated ethanol herbal extract.
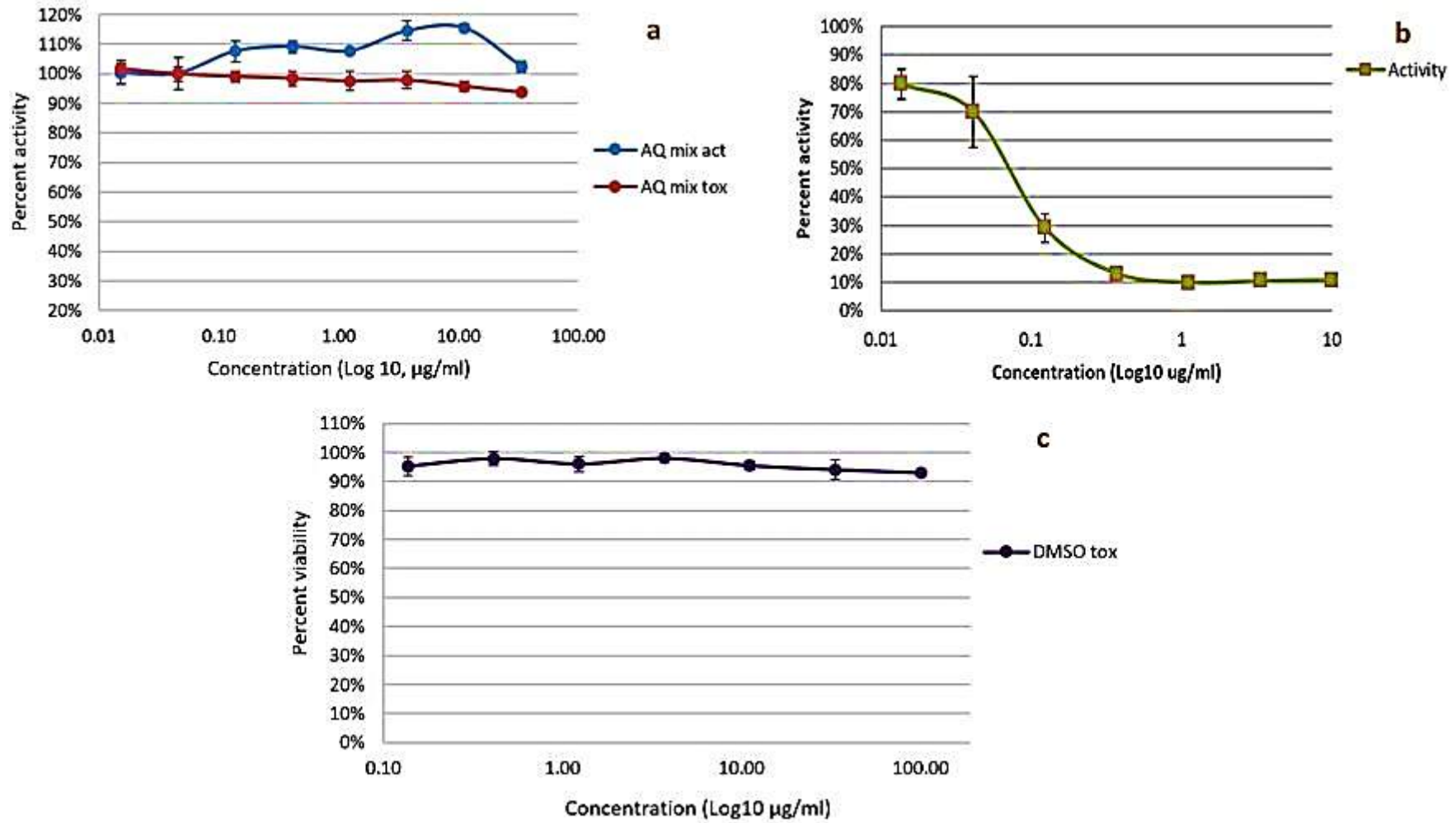
Figure 4.21: HIV-1 (MW isolate) neutralization profile by the aqueous herbal extract.

4.13 Description and discussion of the anti-HIV-1 infection (HIV-1 entry inhibition)

The ability of the extract to neutralize an HIV-1 MW isolate was evaluated utilizing the TZM-bl assay. The ethanol extract yielded a mean IC_{50} of 36.33 ± 1.101 µg/ml and inhibited HIV-1 entry at 33.33 µg/ml (blue curve, **Figure 4.15 a**). Furthermore, the neutralization of VSV by the extract was also evaluated and yielded a mean IC_{50} of 24.91 ± 1.350 µg/ml and significantly inhibited VSV entry at 11.11 and 33.33 µg/ml (green curve, **Figure 4.16 a**), $p > 0.01$. The neutralization of MLV by the ethanol extract yielded a mean IC_{50} of 52.87 ± 0.3121 µg/ml (red curve, **Figure 4.17 a**). The ethanol extract was non-cytotoxic ($CC_{50} = 72.51 \pm 23.32$ µg/ml).

The ability of the dereplicated ethanol extract to neutralize HIV-1 MW isolate was evaluated and yielded a mean IC_{50} of 32.06 ± 1.342 µg/ml. The extract significantly inhibited HIV-1 MW entry at 33.33 µg/ml (**Figure 4.18 a**), $p < 0.05$. Moreover, the extract was able to neutralize VSV and it showed a mean IC_{50} of 20.82 ± 2.251 µg/ml. The extract significantly inhibited HIV-1 entry at 11.11 and 33.33 µg/ml (brown curve, **Figure 4.19 a**), $p < 0.01$. The extract further showed some activity against MLV with a mean IC_{50} value of 43.12 ± 2.201 µg/ml (brown curve, **Figure 4.20 a**). The extract yielded a CC_{50} value of 61.963 ± 0.000 µg/ml against TZM-b1 cells.

The aqueous extract was inactive against MW HIV-1 isolate (brown curve, **Figure 4.21 a**) and non-cytotoxic (CC_{50}: 192.9 ± 2.202 µg/ml) to TZM-b1 cells. VRC01 (a CD4 antagonist, **Figure 4.21 b**) was included as a positive control and DMSO as a vehicle control (**Figure 4.21 c**) in all experiments, with results expressed as percentage activity and percentage viability relative to control. Data points in all experiments are presentative of mean percent activity $\pm$ SD of two independent experiments: n=4, and $p < 0.05$ was considered significant.

Statistical analysis of the data for the ethanol extract at 33.33 µg/ml using students-t test and Two-way ANOVA followed by Bonferroni post hoc correction has shown that there is a significant difference ($p < 0.05$) between anti-HIV entry and toxicity. Also, for the dereplicated ethanol extract there was a significant difference ($p < 0.01$). At this particular concentration (33.33 µg/ml), the ethanol- and dereplicated ethanol extracts inhibited viral entry by 48.00 and 53.12% while cytotoxicity was computed to be 9.101 and 13.42%, respectively.

However, these extracts were not HIV specific in their entry inhibitory activity since there was activity also obtained against VSV (**Figure 4.16** and **Figure 4.19**). The inhibitory activity against VSV was more potent (71.6%) when compared to that of HIV (63.9%) at 33.33 µg/ml. Furthermore, cytotoxicity against VSV was 9.1 and against HIV 13.4% for ethanol and the dereplicated ethanol extracts, respectively. The extracts also had some activity against MLV but that was actually due to cytotoxicity and this was further confirmed by statistical analysis which showed that there is no significant difference between the toxicity and activity at all concentrations.

These results suggest that the ethanolic extracts have phytochemicals that possess anti-HIV entry activity and also that these compounds have potent activity against VSV. The exact mode of action by these extracts is not yet known since specific tests for either of the three possibilities i.e. gp120-CD4, gp120-CCR5/CXCR4 interactions or fusion between the virus and the target host cell were not conducted.

However, HIV neutralization by the extracts were tested. Other studies have also shown that medicinal plants possess anti-HIV entry activity (Rege et al., 2010; Modi et al., 2013). These studies have attributed the anti-HIV entry activity by most medicinal plants to be due to the presence of flavonoids, alkaloids and glycosides. This is indeed what has been shown above and this explains the observed activity by these herbal extracts which were shown to possess mostly flavonoids, alkaloids and glycoside "sugars" (see section 4.1 and 4.2, **Figure 4.1**). These extracts also contain antiviral alkaloids from *Clivia miniata* (Ieven et al., 1982) and hence these might be the key active principles.

Generally, based on the findings obtained in this study, we conclude that the South African herbal concoction does indeed possess anti-HIV 1 activity. This anti-HIV activity is mediated by inhibition of the enzyme reverse transcriptase and also neutralization of HIV. However, the exact bioactive compounds responsible for the observed activity have not yet been identified and isolated.

Therefore, in the following section (Chapter 5), the isolation and characterization of the potentially bioactive phytochemicals was done.

5   CHAPTER 5

5.1   Isolation of the major compounds from the ethanol extract of the herbal concoction

Plants provide people with primary metabolites such as carbohydrates, proteins, fats and vitamins. Furthermore, it has been proposed that plants produce secondary metabolites (alkaloids, phenols, terpenes etc.) which are essential for both humans and plants because they form a chemical defense mechanism in response to infection by microorganisms, predation, insects and herbivores. Furthermore, they also play a crucial role in aiding the plant to survive and adapt to environmental changes (Pengelly, 2004). Secondary metabolites were considered to be useless by-products of the synthesis of primary metabolites. However, through the science of Pharmacognosy (''the study of the physical, chemical, biochemical and biological properties of drugs, drug substances, or potential drugs or drug substances of natural origin as well as the search for new drugs from natural sources'') and Ethnobotany ("the study of the relationship between humans and plants in all its complexity") it was observed that these products are the basis of the healing properties of medicinal plants. Neuwinger, (1994) found that constituents with special properties often occur in a high concentration and sometimes with great purity in a particular medicinal plant.

In this chapter, we explore further the observed anti-HIV activity of the ethanol extract by conducting studies to identify the active constituents. These studies included compound isolation, structure elucidation, characterization and biological testing. These studies were done in order to investigate the molecular mechanism of action of the inactive and active phytochemicals.

This study is, to the best of our knowledge, the first reported phytochemical and biological study of the ethanol extract of the herbal concoction. In an effort to identify potential anti-HIV compounds from the ethanol herbal extract, several chromatographic methods (see section 3.4) were used to fractionate the extracts and major compounds were isolated. The isolated compounds were further purified and subjected to NMR spectroscopic experiments. The phytochemical investigation of this extract led to the isolation of several compounds.

However, this section will only report on four compounds (i.e. four homoisoflavones). These were chosen for further studies based on their physiochemical properties (i.e. the "minor" differences within the same phytochemical class). Furthermore, these different compounds were chosen in order to show how the stereochemistry and molecular substitutions influence the bioactivity of phytochemicals belonging to the same class. And, again, we also wanted to identify which type of phytochemical category would yield a better bioactive pharmacophore scaffold to be further investigated in drug discovery and drug design research. The structures of these compounds were partially elucidated based on their 1D-NMR: ^{1}H, ^{13}C and MS spectral data.

The flow diagram, **Figure 5.1** below, shows the steps followed in the isolation of the homoisoflavone phytochemicals.
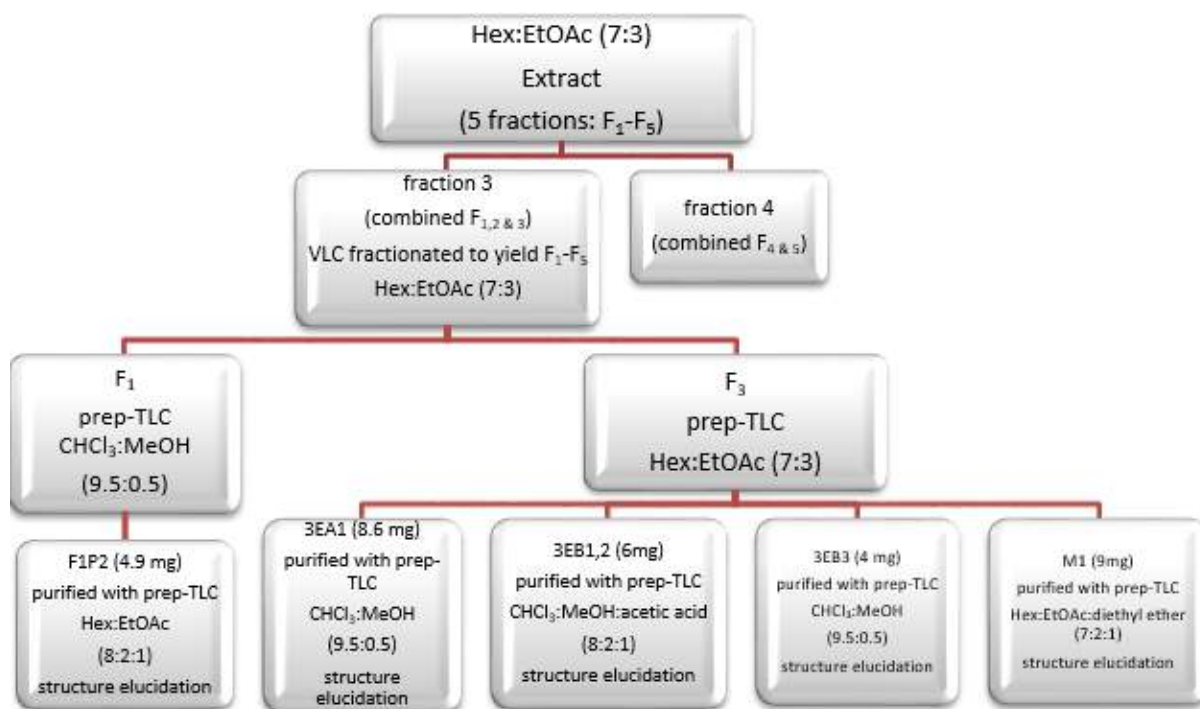

Figure 5.1: **Schematic representation of the isolation process of flavonoid compounds from the ethanol extract of the herbal concoction.**

Compounds F1P2, 3EA1, 3EB1.2, 3EB3 and M1 were isolated from the hexane: ethyl acetate fraction of the ethanol extract of the herbal concoction. Compounds 3EB1.2 and M1, upon analysis of their proton and carbon NMR spectra, were found to be exactly the same as compounds 3EA1 and F1P2, respectively.

5.2   Compound characterization and structure elucidation

5.2.1 Characterization of compound 1 (3EA1)

The ^{1}H-NMR spectrum (**Figure 5.3**) revealed the presence of two singlets resonating at δ_H 3.709 and 3.766 ppm integrating for three protons each, indicative of two methoxy groups. Five aromatic protons are observed at the region between δ_H 5.8-7.5 ppm, a sharp singlet resonated at δ_H 5.921 ppm and the other 4 peaks resonated at the δ_H 6.87 and 7.204 ppm region. Deuterated acetone solvent peaks are seen at 2.033 and 2.048 ppm.

The ^{13}C-NMR spectrum (**Figure 5.4**) showed compound **1** as having 18 carbons in its structure. A carbonyl carbon signal with a chemical shift δ_C 196.34 ppm was also observed. The ^{13}C spectrum also revealed the presence of methoxy carbons at δ_C 54.564 and 60.037 ppm. The aromatic ring signature signals are also observed between δ_C 113.879-158.514 ppm. The deuterated acetone solvent peaks are observed at 28.510 and 205, 265 ppm. Both spectra are in complete agreement with each other, the ^{13}C-NMR spectrum confirms what is seen in the ^{1}H-NMR spectrum of this compound. These chemical shift signals are indicative of flavonoids specifically the 3-benzyl-4-chromanone Homoisoflavone subclass (Mulholland et al., 2013).

The UPLC-MS chromatogram of the pure isolated compound **1** (**Figure 5.5**) showed a signal peak in the intermediate polarity region with a calculated mass of *m/z*: 329.1006 in the ESI-mode and a mass of *m/z*: 331.1262 in the ESI+ mode which then correlates with an actual mass of *m/z*: 330.0967 for this compound. This molecular mass was further confirmed by High Resolution Quadrupole Time Of-Flight-Mass Spectrometry (HR-QTOF-MS) (**Figure 5.6**) which also yielded the same value.

HR-QTOF-MS analysis also predicted the molecular formula of the compound to be $C_{18}H_{17}O_6$ with a double bond equivalent (DBE) of 10 which suggests that the compound could have two aromatic rings and two double bonds or one double bond plus a ring. Considering all of the above data, we propose the backbone chemical structure for compound **1** to be that of a 3-benzyl-4-chromanone homoisoflavone derivative as shown in **Figure 5.2** below.
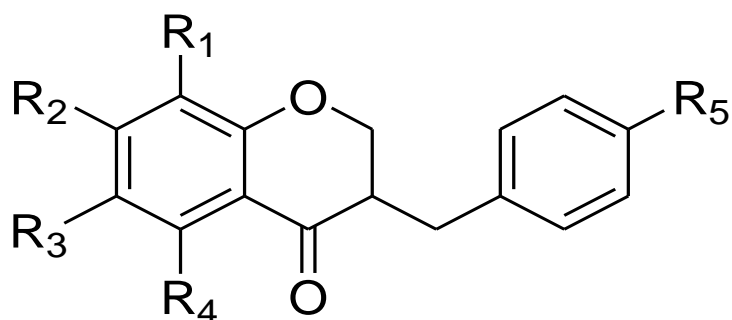
Figure 5.2: The chemical backbone structure of compound 1, a 3-benzyl-4-chromanone homoisoflavone derivative.

The "R" indicates the presence of a substituent. In order to get to the *m/z*: 330.0967 molecular mass (18 carbons, 17 hydrogens and 6 oxygens), we need the following substituents as seen from the proton-, carbon NMR and QTOF-MS prediction: H; 2 x OH; 2 x OCH$_3$. The proposed structure above complies with the predicted DBE of 10.
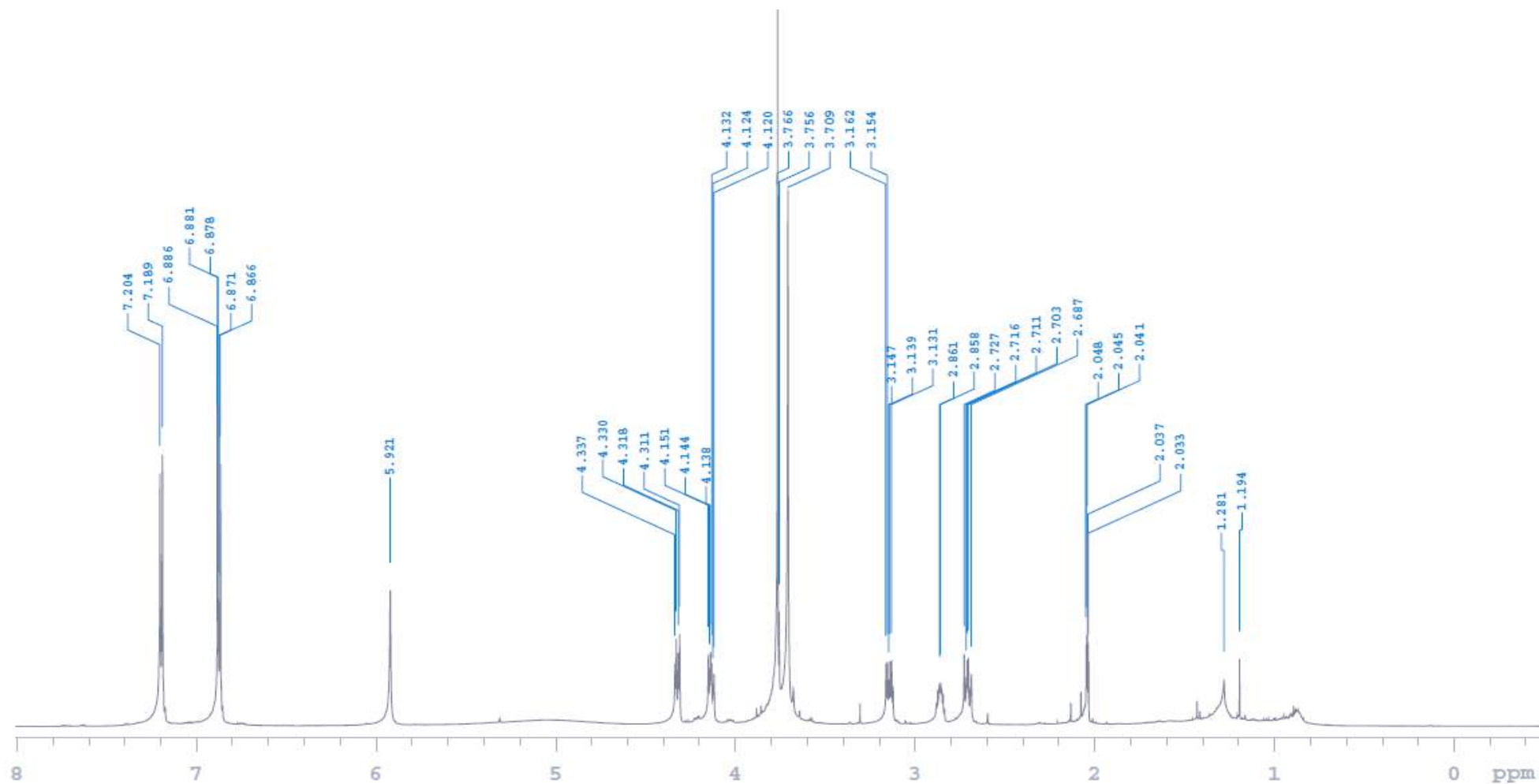
Figure 5.3: ¹H-NMR spectrum of compound 1 (3EA1) isolated from the ethanol extract of the herbal concoction.
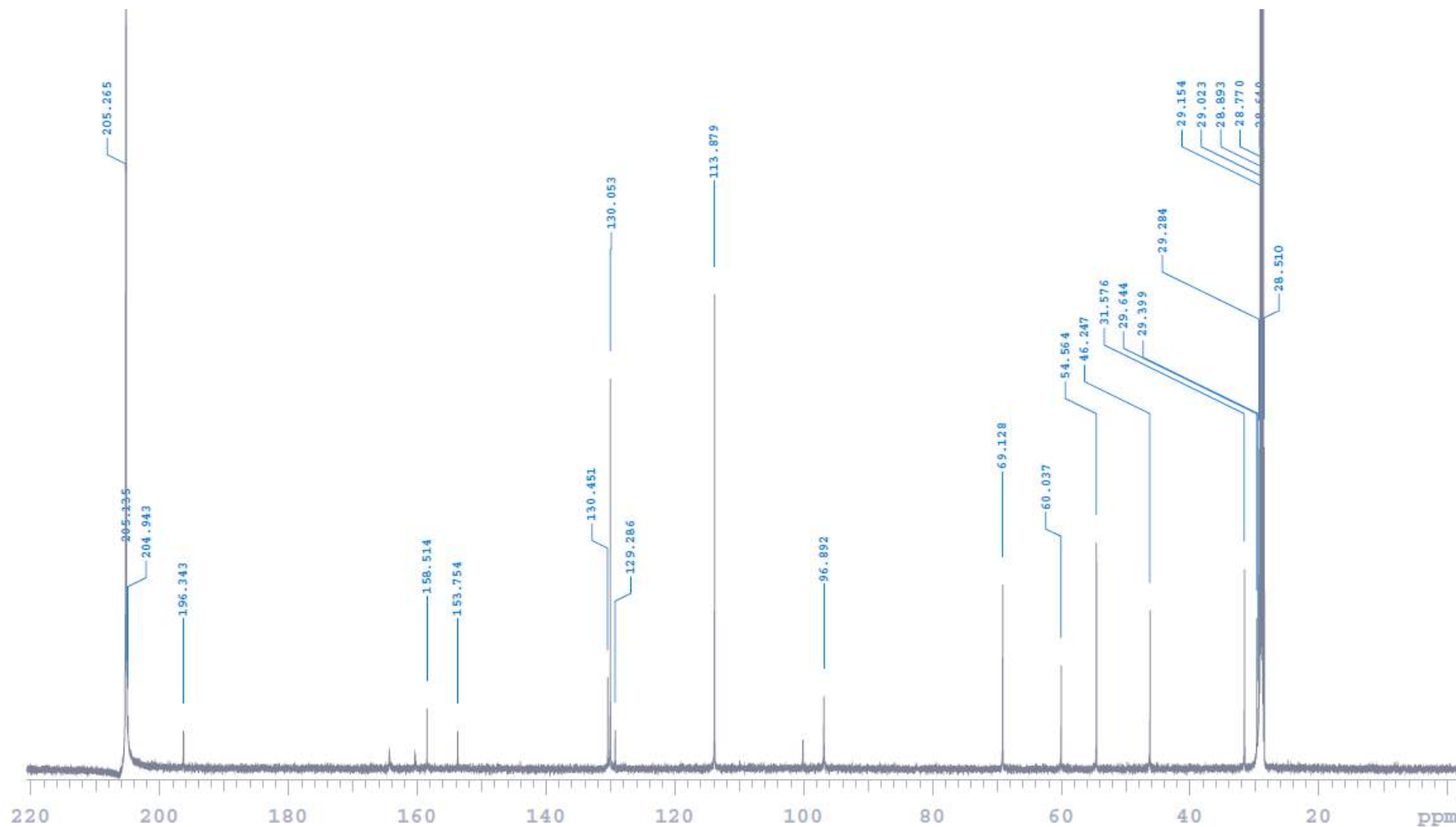
Figure 5.4: ^{13}C-NMR spectrum of compound 1 (3EA1) isolated from the ethanol extract of the herbal concoction.
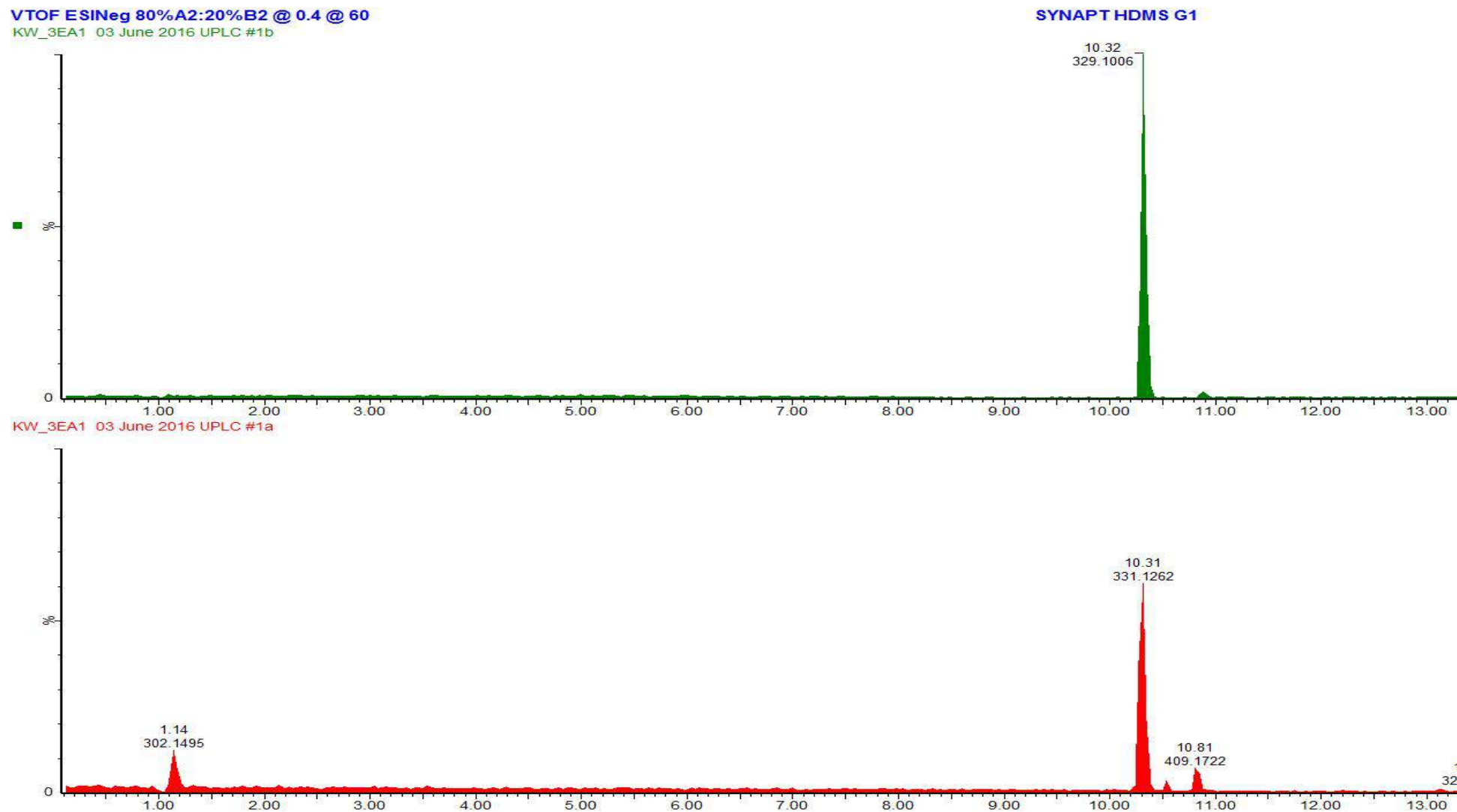
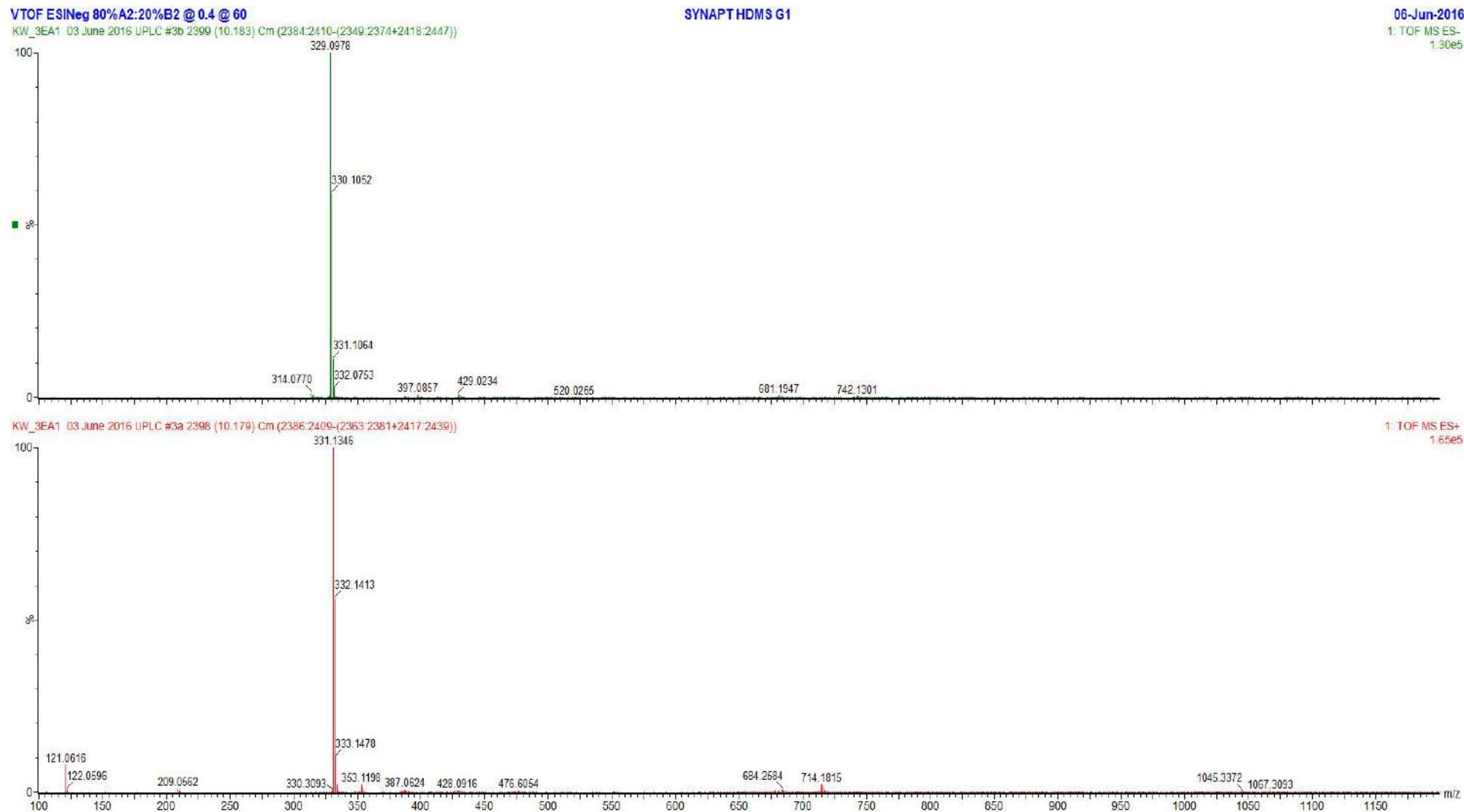**Figure 5.5: UPLC-MS (ES- and ES+) chromatograms for compound 1.**

Figure 5.6: HR-QTOF-MS (ES- and ES+) for compound 1.

5.2.1.1 Bioactivity of 3EA1 (compound 1)

Our results suggest that compound 1 is non-cytotoxic at concentrations below 11.11 µg/ml against HEK293T cells. It exhibited a mean CC_{50} value of 14.31 ± 7.16 µg/ml against the HEK293T cells (**Figure 5.7**).

This compound was found to be inactive against HIV-1 subtype C RT (Figure 5.7) when compared to the cytotoxicity plot, (no downward and left shift of the "3EA1 act" plot). Compound 1 was also inactive against PR compared with the cytotoxicity plot (no downward and left shift of the "3EA1 act" plot as seen in **Figure 5.8**.

Furthermore, compound 1 was also non-toxic to TZM-bl cells with a mean CC_{50} value of 88.39 ± 13.17 µg/ml and was inactive at neutralizing the HIV-1 MW isolate.

Statistical analysis of this data also confirmed that this compound is indeed inactive against HIV-1. No significant difference between toxicity and activity (RT, PR and Neutralization) was observed following a student t-test, a Two-way ANOVA and a Bonferroni post hoc analysis. The p-value was found to be greater than 0.05 at all concentrations tested.

5.2.1.2   Cytotoxicity and RT activity
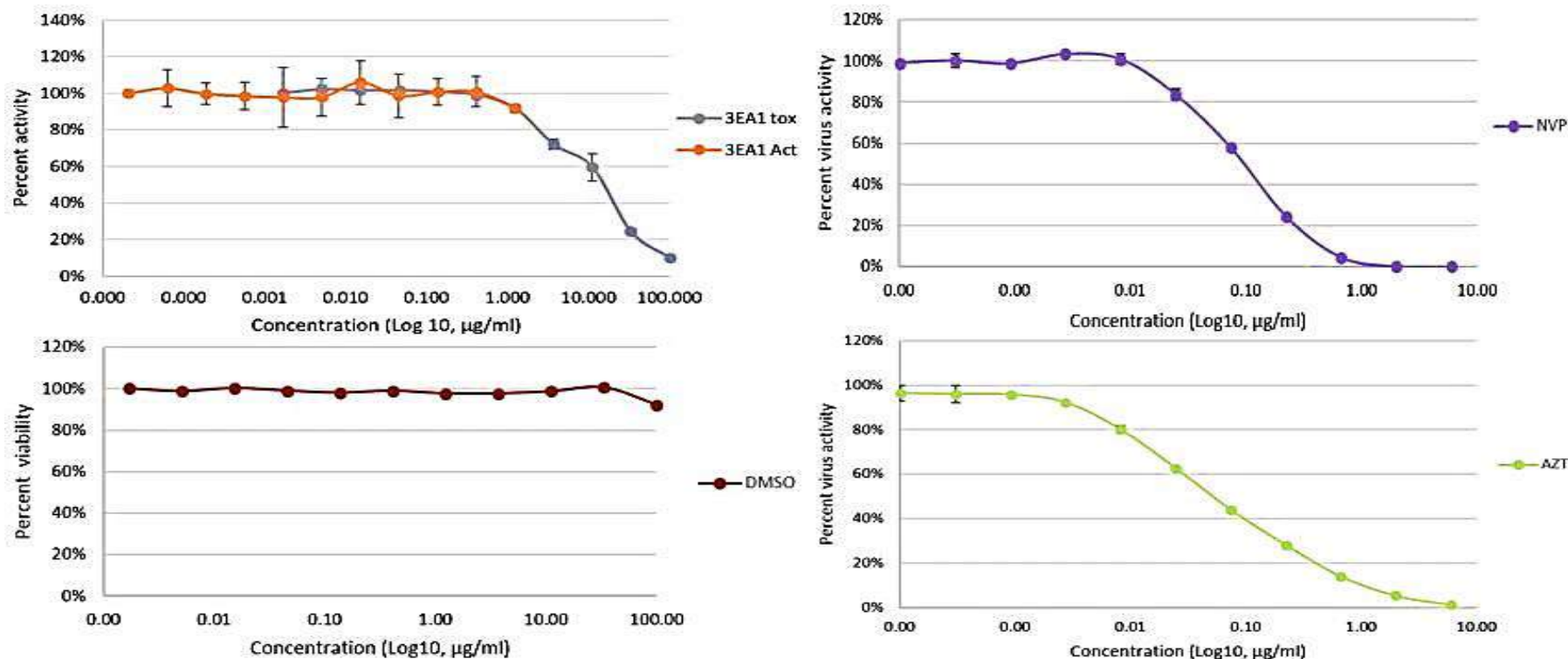
Figure 5.7: Reverse transcriptase phenotypic drug susceptibility profile of MJ4 HIV-1 subtype C isolate to compound 1.

The ability of compound 1 to inhibit HIV RT was investigated using a novel RT phenotypic drug susceptibility assay. Cells were infected with a HIV-1 MJ4 isolate and treated with compound 1 at various concentrations for 48 hours. Data points are representative of mean percent activity $\pm$ SD of two independent experiments: n=4; $p < 0.05$ was considered significant. Plots of Zidovudine ("AZT") and Nevirapine ("NVP") represent positive controls and plot of "DMSO" represents the vehicle control.

5.2.1.3 Cytotoxicity and PR activity
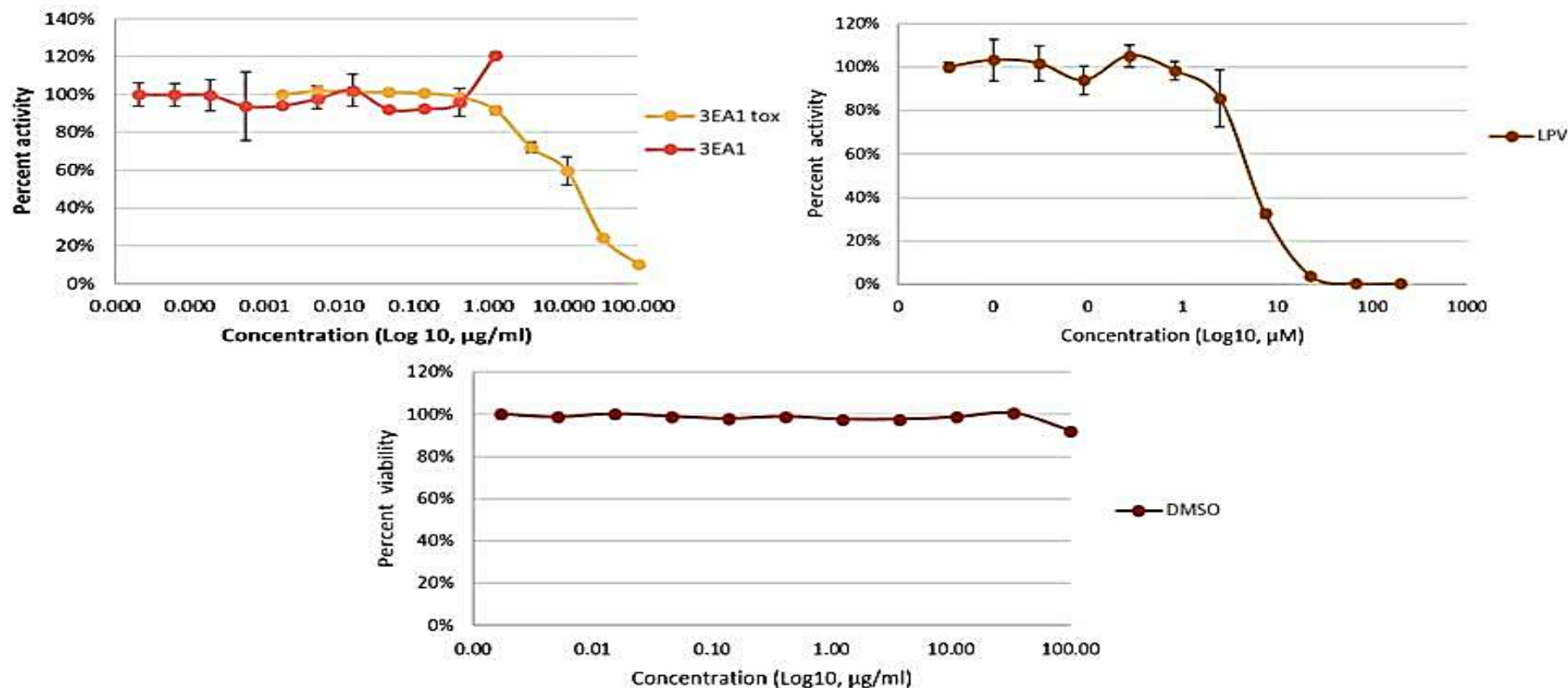
Figure 5.8: Protease phenotypic drug susceptibility profile of MJ4 HIV-1 subtype C isolate to compound 1.

Compound **1** was evaluated for anti-HIV protease activity utilizing a novel protease phenotypic drug susceptibility assay. Cells were infected with HIV-1 MJ4 isolate and treated with compound **1** at various concentrations for 48 hours. Data points are presentative of mean percent activity $\pm$ SD of two independent experiments: n=4; $p< 0.05$ was considered significant. Plots of "LPV" represent positive control and plot of "DMSO" represents the vehicle control.

5.2.1.4 Cytotoxicity and Neutralization activity
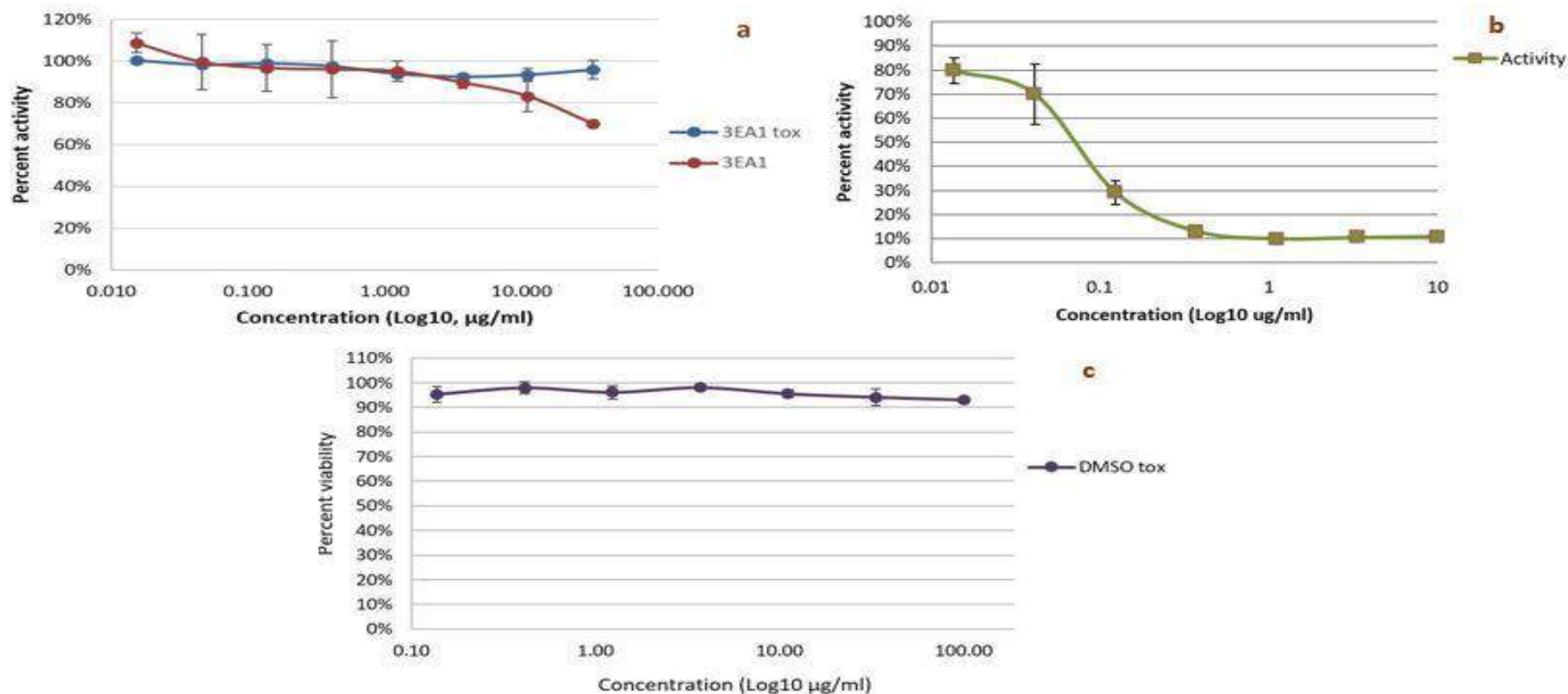
Figure 5.9: HIV-1 (MW isolate) neutralization profile for compound 1.

The ability of this compound to neutralize a HIV-1 MW isolate was evaluated utilizing the TZM-bl assay. The extract yielded a mean CC_{50} of 88.39 ± 13.17 µg/ml. Data points are representative of mean percent activity $\pm$ SD of two independent experiments: n=4; $p < 0.05$ was considered significant. Plots of "activity" represent positive control and plot of "DMSO" represent the vehicle control.

5.2.2   CHARACTERIZATION OF COMPOUND 2 (3EB3)

Upon viewing the ^{1}H-NMR spectrum (**Figure 5.11**) of this compound, some interesting signals were observed. The spectrum revealed the presence of one singlet resonance integrating to three protons at δ_H 3.86 ppm which correlates to a methoxy group. Two sharp singlet aromatic proton signals are seen at the δ_H 5.813 and 5.866 ppm. The spectrum also showed the presence of two doublet signals in the 7.047-7.396 ppm region which suggest that compound **2** is para substituted. There appears to be an ethylene signal at δ_H 7.679 ppm. The deuterated acetone signal is seen at 2.033-2.077 ppm

The ^{13}Carbon-NMR spectrum (**Figure 5.12**) showed compound **2** having 17 carbons in its structure. A carbonyl carbon signal at δ_C 183.679 ppm was also observed. The ^{13}C spectrum also revealed the presence of a methoxy carbon at δ_C 54.863 ppm. The aromatic ring signature signals are also observed at δ_C 114.231 and 133.203 ppm. An ethylene carbon was also observed at δ_C 135.089 ppm. Both spectra are in complete agreement with each other. ^{13}C confirms what is seen in the ^{1}H-NMR spectrum of this compound. These chemical shift signals are indicative of flavonoids, especially the homoisoflavones subclass, and specifically belonging to the 3-bezylidene-4-chromanone category of homoisoflavones (Mulholland et al., 2013).

The UPLC-MS chromatogram of the pure isolated compound **2** (**Figure 5.13**) showed a signal peak in the intermediate polarity region and a retention time of 10.87 minutes with a calculated mass of *m/z*: 297.0710 in the ESI- mode and a mass of *m/z*: 299.0911 in the ESI+ mode. These mass values correlate with an actual mass of *m/z*: 298.0732 for this compound. This molecular mass was further confirmed by HR-QTOF-MS (**Figure 5.14**) which also yielded the same value. Q-TOF/MS analysis also predicted the molecular formula of the

compound to be $C_{17}H_{14}O_5$ with a double bond equivalent (DBE) of 11 which suggests that the compound could have two aromatic rings and three double bonds or two double bonds plus a ring. Taking all of the above data into consideration, we propose the backbone chemical structure for compound **2** to be that of an (E)-3-benzylidene-4-chromanone homoisoflavone as shown below.
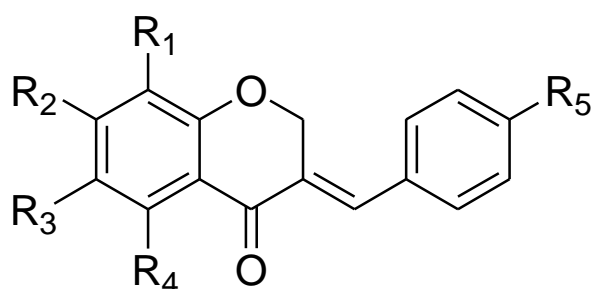
Figure 5.10: Chemical backbone structure of compound 3a: a (E)-3-benzylidene-4-chromanone homoisoflavone derivative.

In order to get to the *m/z*: 298.0732 molecular mass, 17 carbons, 14 hydrogens and 5 oxygens, we need the following substituents as seen from the proton-, carbon NMR and Q-TOF-MS prediction: OH; OH; OCH_3; H. The proposed structure above complies with the predicted DBE of 11.
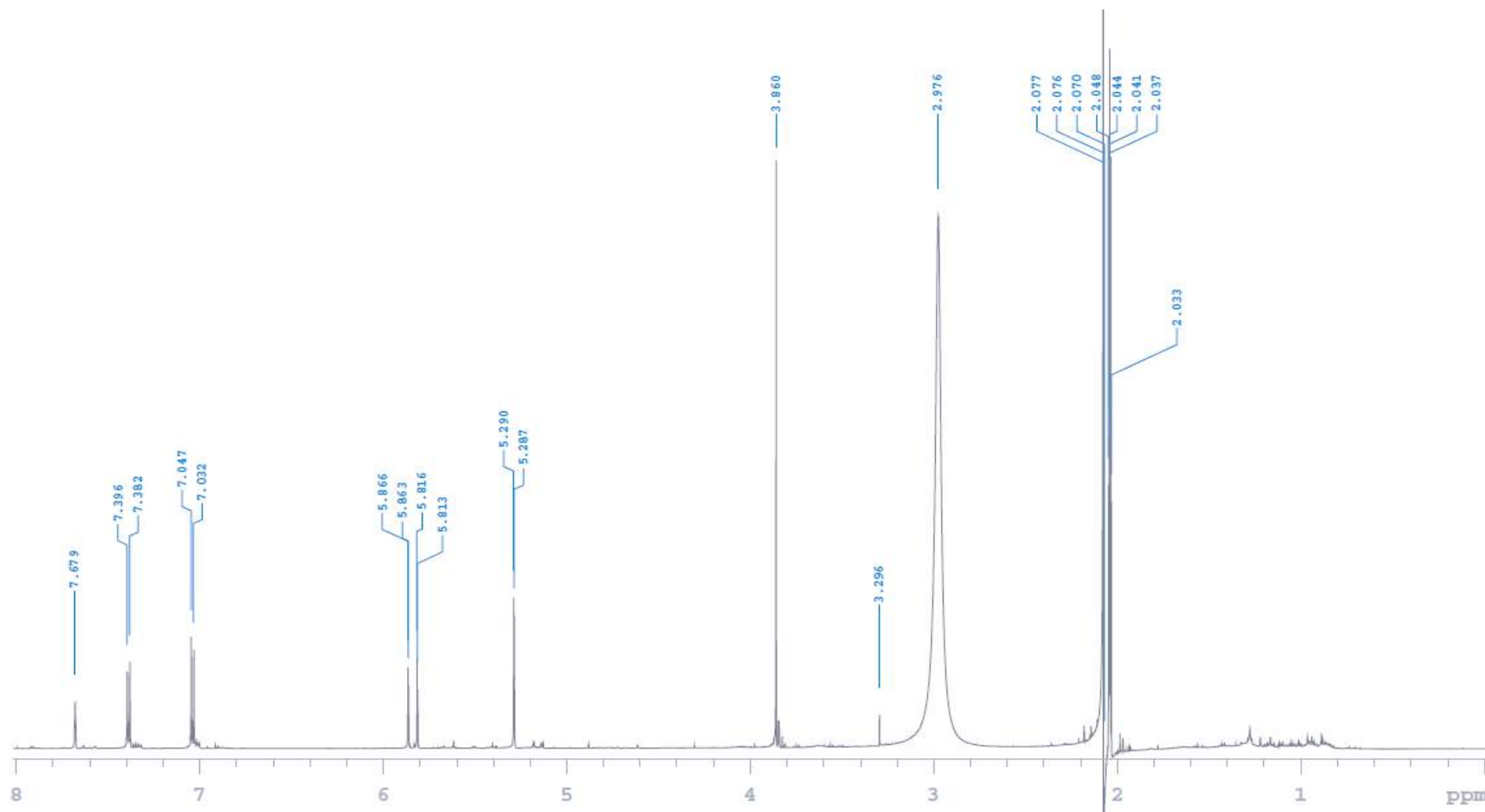
Figure 5.11: ^{1}**H-NMR spectrum of compound 2 (3EB3) isolated from the ethanol extract of the herbal concoction.**
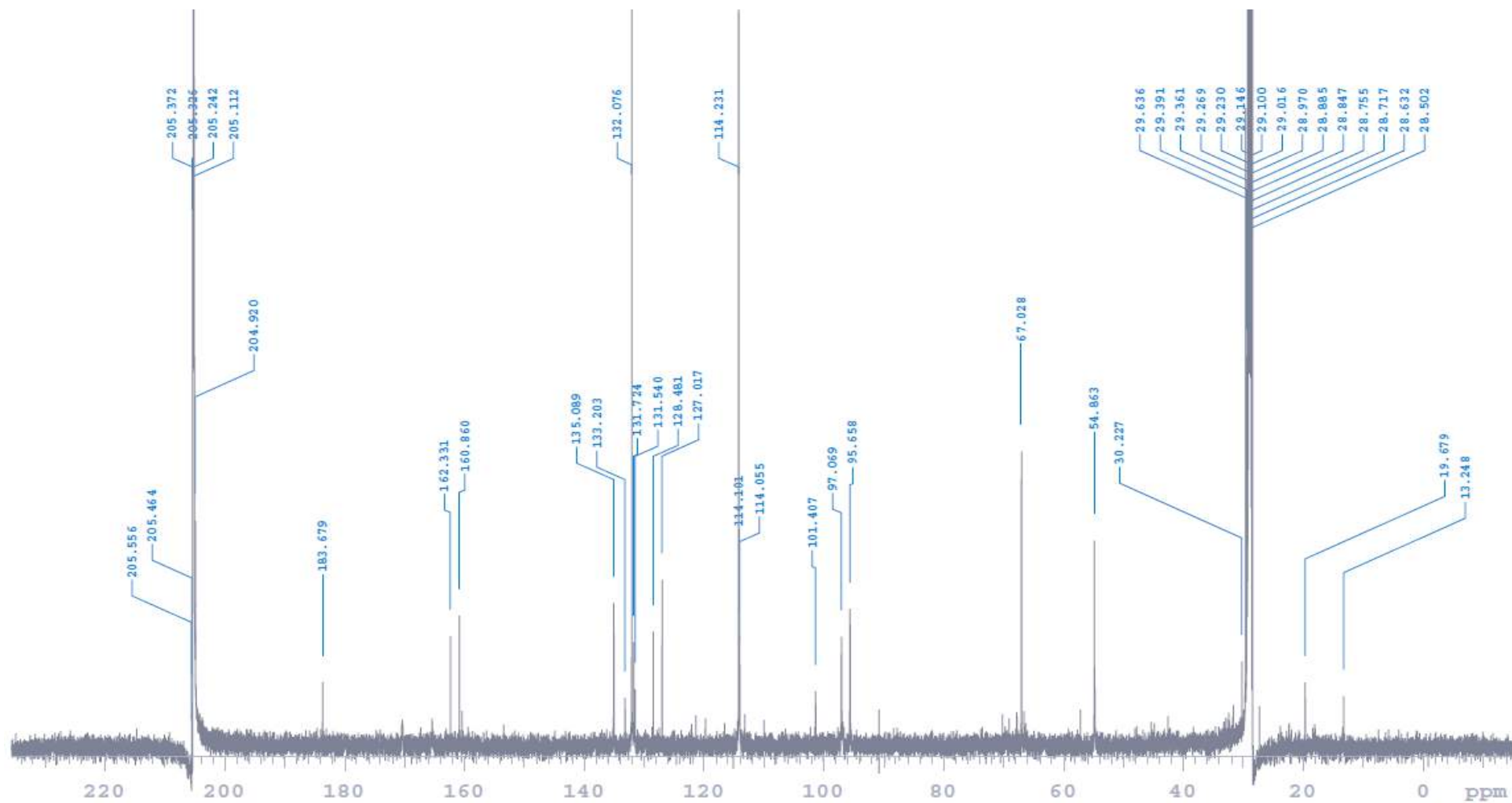
Figure 5.12: ¹³C-NMR spectrum of compound 2 (3EB3) isolated from the ethanol extract of the herbal concoction.

Figure 5.13: UPLC-MS (ES- and ES+) chromatograms for compound 2.
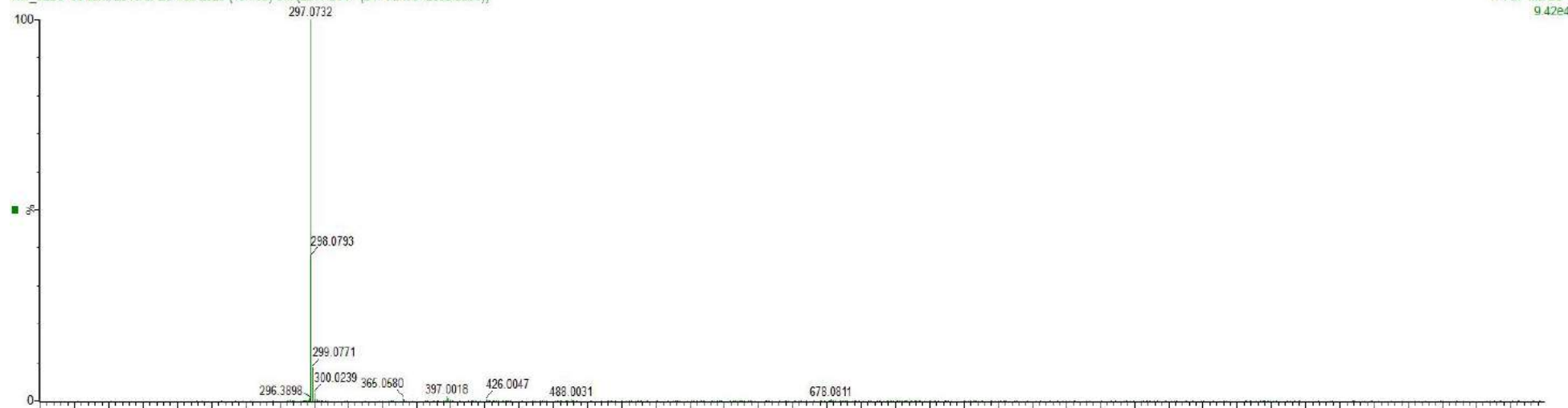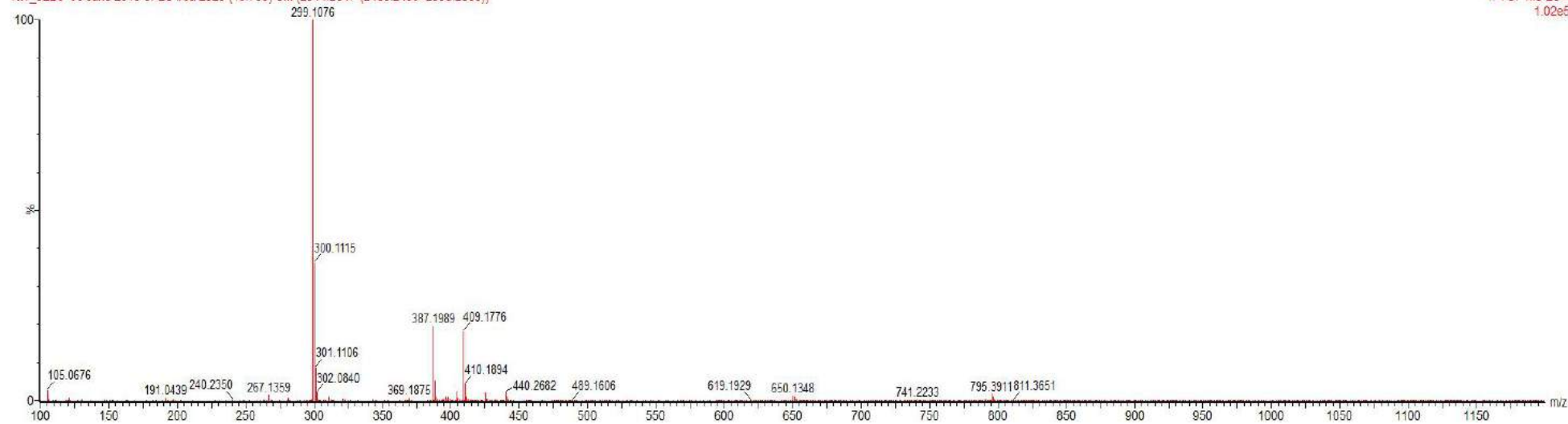
Figure 5.14: HR-QTOF-MS (ES- and ES+) for compound 2.

5.2.2.1   Bioactivity of 3EB3 (compound 2)

Figure 5.15 presents evidence that compound **2** was active against HIV-RT and **Table 7** depicts the mean IC_{50} and CC_{50} values of this compound. Based on the obtained IC_{50}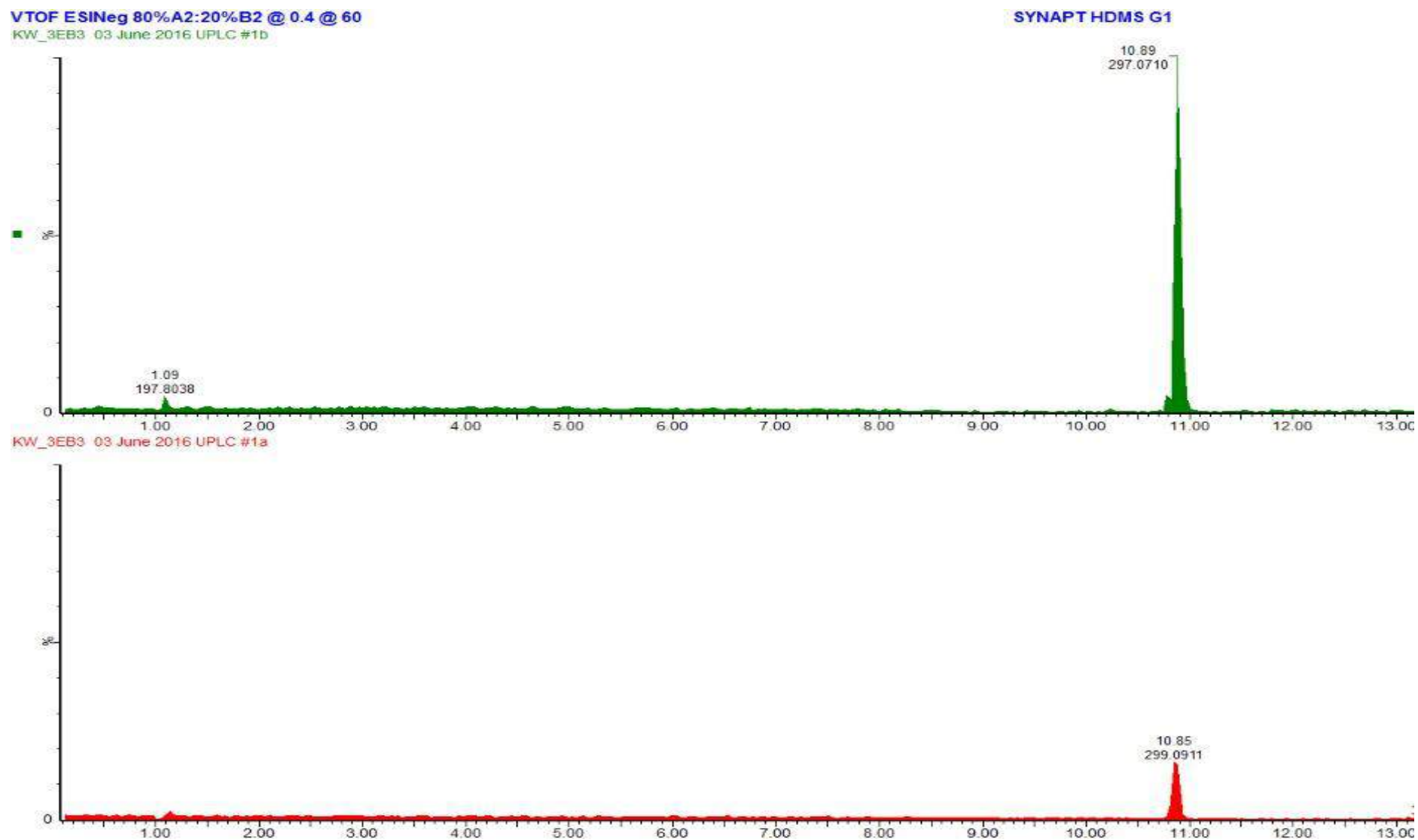 and CC_{50} values, the selectivity index (SI) was calculated and found to be 3.19, 1.78 and 1.80 for MJ4, CM9 and Du179, respectively. These SI values are an indication that compound **2** would have to be administered with caution if it was to be used as a therapeutic agent.

To evaluate if indeed there is a significant difference between the means of anti-HIV-1 RT activity and cytotoxicity, statistical analysis of this data was conducted. Statistical tests included a two-way ANOVA and a Bonferroni post hoc test. The findings of these analyses confirmed that there is a statistically significant difference between cytotoxicity and anti-HIV RT activity at particular treatments.

There was no significant difference between the mean toxicity, the anti-PR activity and the neutralization activity after treatment of the MJ4 isolate with compound **2** (see **Figures 5.16** and **5.17**). The student t-test, Two-way ANOVA and the Bonferroni post hoc analysis have also confirmed these observations. The p-value was found to be greater than 0.05 at all concentrations tested. **Figures 5.15** to **5.17** show the graphical representation of these findings.

5.2.2.2 Cytotoxicity and RT activity
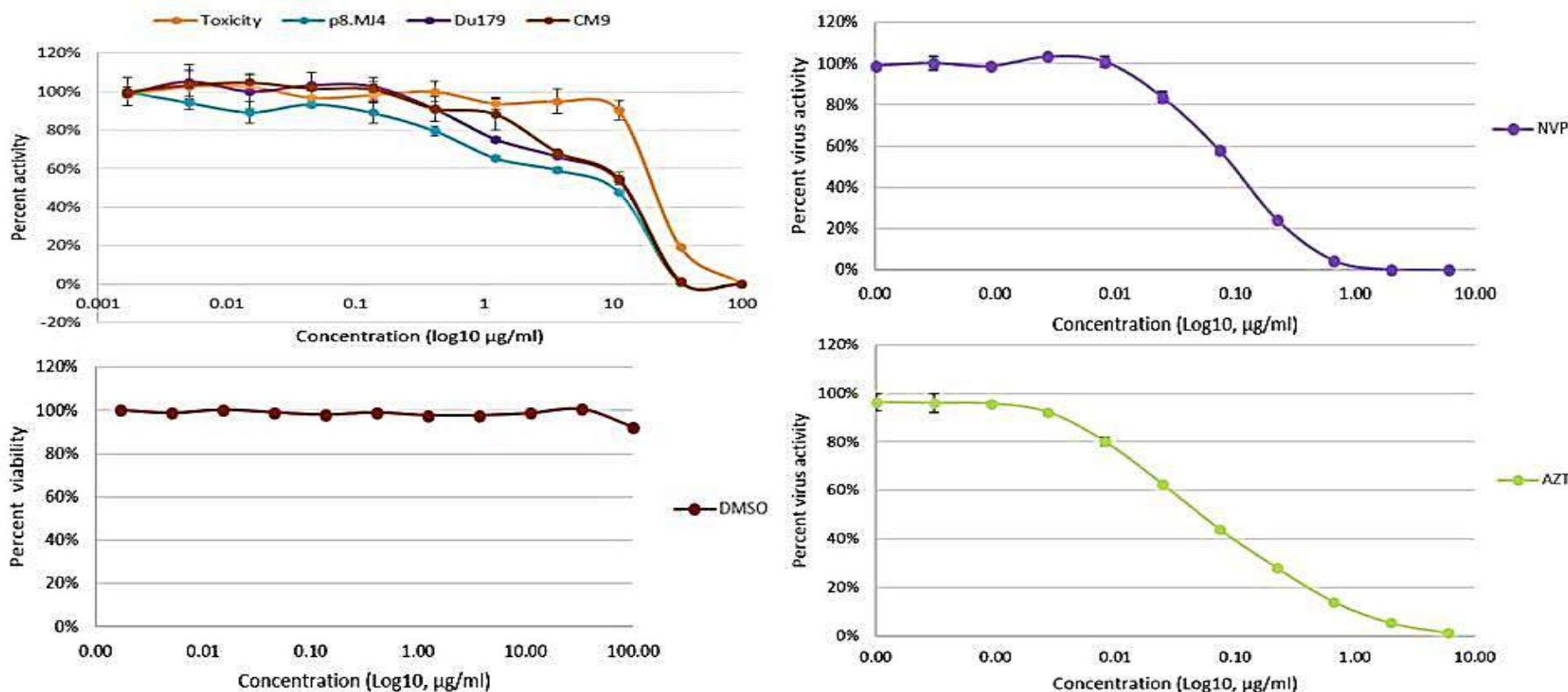
Figure 5.15: Reverse transcriptase phenotypic drug susceptibility profile of all three HIV-1 subtype C isolates to compound 2.

The ability of compound **2** to inhibit HIV-1 RT was investigated by a novel drug susceptibility phenotypic assay. Zidovudine (AZT) and Nevirapine (NVP) were used as positive controls and their results expressed as percent virus activity relative to control. Cytotoxicity due to vehicle control (DMSO) was also evaluated and the results expressed as percentage viability relative to control. Data points are presentative of mean percent activity $\pm$ SD of two independent experiments: n=4; $p< 0.05$ was considered significant.

5.2.2.3   Cytotoxicity and PR activity
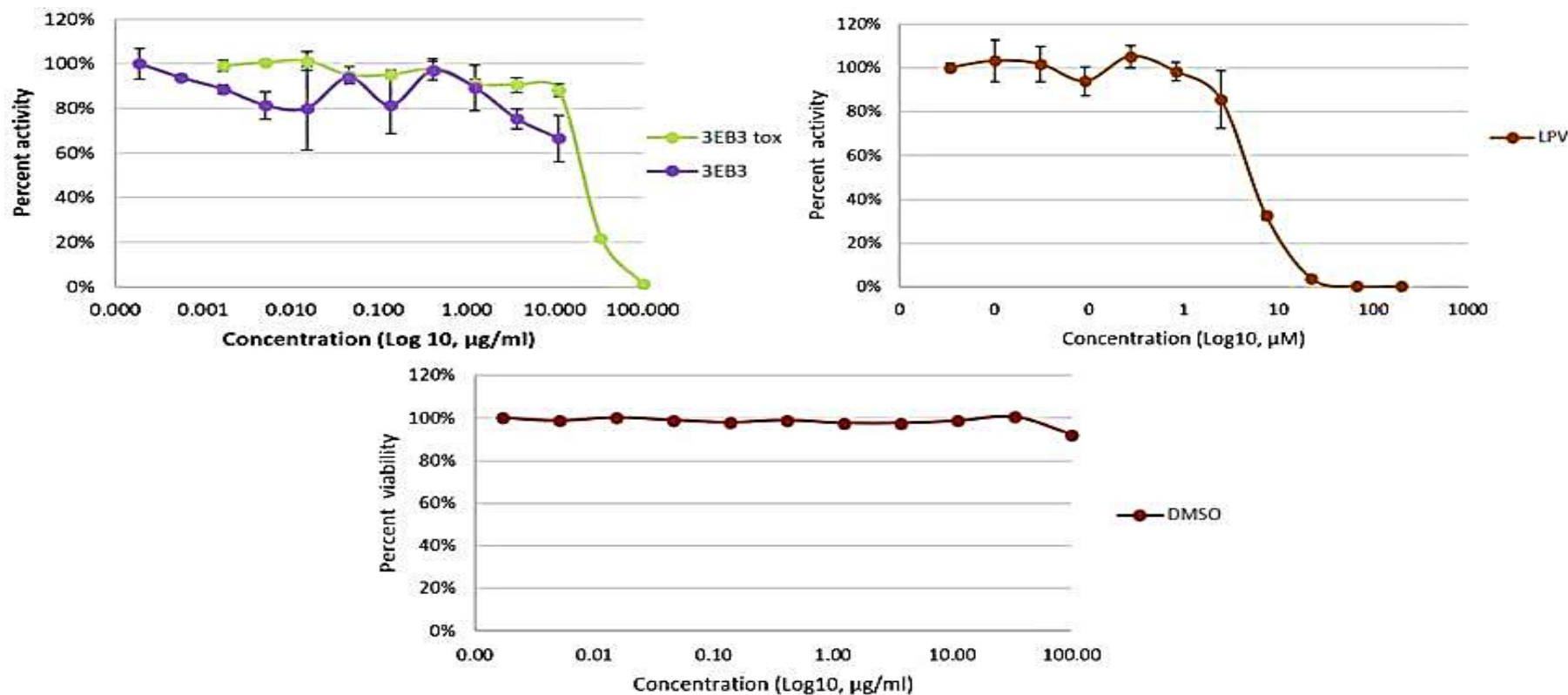
Figure 5.16:Protease phenotypic drug susceptibility profile of MJ4 HIV-1 subtype C isolate to compound 2.

To evaluate if compound **2** has anti-HIV protease activity, a protease phenotypic drug susceptibility assay was conducted. Both compounds were inactive against HIV-1 protease. Lopinavir (LPV) was included as a positive control and DMSO as a vehicle control with results expressed as percentage activity and viability relative to control. Data points are presentative of mean percent activity $\pm$ SD of two independent experiments: n=4; $p < 0.05$ was considered significant.

5.2.2.4 Cytotoxicity and Neutralization activity
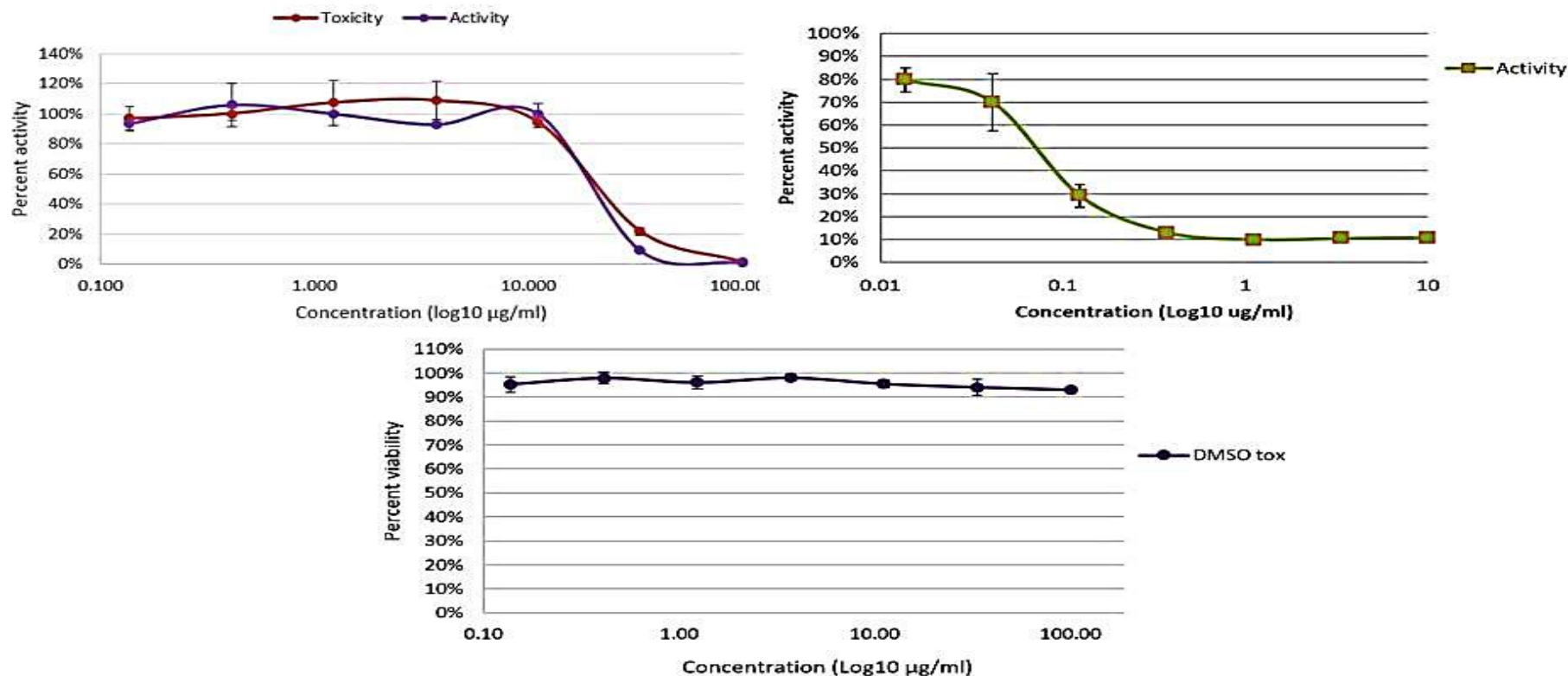
Figure 5.17: HIV-1 neutralization profile for compound 2.

The TZM-bl assay was used to evaluate the ability of the isolated compounds to neutralize HIV-1 MW isolate. Compound **2** was inactive for preventing HIV entry. VRC01 (CD4 antagonist) was included as a positive control and cytotoxicity due to "DMSO tox" vehicle control was also evaluated with results expressed as percentage activity and percentage viability relative to control. Data points are presentative of mean percent activity $\pm$ SD of two independent experiments: n=4; $p < 0.05$ was considered significant.

Table 7: Cytotoxicity and anti-RT activity of compound 2 against three HIV-1 subtype C isolates (MJ4, CM9 and Du179)

Cpd	IC_{50} (µg/ml)			CC_{50} (µg/ml)	
	Viral isolates			Cell-lines	
	MJ4	CM9	Du179	HEK293T	TZM-bl
2	7.23 ± 1.88	12.83 ± 0.41	12.81 ± 0.1021	23.08 ± 0.1981	23.072 ± 1.825

Cpd= compound

Bonferroni post hoc test revealed that the significant difference between anti-RT of the MJ4 HIV isolate and toxicity was observed between the concentration ranges of 1.23 ($p<0.001$), 3.70 ($p<0.001$) and 11.11 µg/ml ($p<0.001$). The difference for the CM9 HIV isolate was observed at 3.70 ($p<0.001$) and 11.11 µg/ml ($p<0.001$). Lastly, for the Du179 HIV isolate, the difference was seen from 1.23 ($p<0.01$), 3.70 ($p<0.001$) and 11.11 µg/ml ($p<0.001$). **Table 8** depicts the evaluation of the mean toxicity and anti-RT activities at the above-mentioned concentrations.

Table 8: The mean toxicity and anti-RT activity values of the concentrations that are statistically significant for all three HIV viral isolates when treated with compound 2

Dosage (µg/ml)	Viral isolates					
	MJ4		CM9		Du179	
	*Act (%)	§Tox (%)	Act (%)	Tox (%)	Act (%)	Tox (%)
11.11	51.3 ± 1.2	12 ± 3.8	45.9 ± 3.5	12 ± 3.8	46.0 ± 1.9	12 ± 3.8
3.70	39.75 ± 1.56	9.6 ± 6.3	32.6 ± 1.39	9.6 ± 6.3	33.8 ± 0.78	9.6 ± 6.3
1.23	27.6 ± 9.40	9.1 ± 3.9	Ns	Ns	24.4 ± 1.47	9.1 ± 3.9

*Act = percent activity; §= percent toxicity; ns= not significant

Furthermore, Bonferroni post hoc test has revealed that there is a significant difference between the mean activity within the viral isolates. The difference is seen between MJ4 and CM9 at 1.23 µg/ml ($p<0.05$) and also between MJ4 and Du179 at 0.412 µg/ml ($p<0.05$). There was no difference was observed between CM9 and Du179 HIV isolates ($p>0.05$).

The implications of the above statistical analysis are that, compound **2** is active against HIV-1 RT and it seems to be more selective, on average, for the MJ4 HIV-1 RT isolate when compared to the CM9 and Du179 isolates (see **Figure 5.15**).

5.2.3 CHARACTERIZATION OF COMPOUND 3a AND 3b (F1P2)

The ^{1}H NMR spectrum of compound **3** showed that this compound was in fact composed of two compounds with a slight difference in the chemical structure i.e. regioisomers (see **Figure 5.20**). From this point forth, these isomers shall be referred to as compounds **3a** and **3b**. The ^{1}H NMR spectrum shows a few interesting proton chemical shift signals (δ_H). The presence of two singlets with an integration of three protons at δ_H 3.87 and 3.83 ppm, indicate that compounds **3a** and **3b** have a methoxy functional group. And, the fact that two peaks for this functional group are observed, further correlate with the assumption that this compound was isolated as an isomer. Methylene signals (CH$_2$) were also observed at δ_H 3.708 and 5.36 ppm corresponding to H-9 and H-2 of compound **3b** and **3a**, respectively. A methyl signal was also observed at δ_H 2.07 ppm which suggests that these compounds have a methyl group as one of their functional groups. Furthermore, the spectrum showed a singlet at δ_H 5.89 ppm which is indicative of a substitution at the "A" ring of homoisoflavones. The spectrum also showed the presence of two sets of doublet signals in the δ_H 6.9-7.9 ppm region which suggest that compounds **3a** and **3b** are para substituted. Two sets of singlet ethylene signals at δ_H 6.99 and 7.71 ppm were observed which belong to H-9 and H-2 of compound **3b** and **3a**. The presence of these pairs of ethylene signals and other pairs of signals further confirm that compound **3** was indeed isolated as a regioisomer-type of a compound.

The ^{13}Carbon-NMR spectrum (**Figure 5.21**) showed compound **3 to** have 18 carbons in its structure. However, visual inspection of **Figure 5.21** showed more than 18 carbon signals, which is due to varied chemical shifts of the regioisomers. The ^{13}C-NMR spectrum also revealed the presence of two methoxy carbons at δ_C 60.04 and 60.099 ppm. The aromatic ring signature signals are also observed in the δ_C 97.39 to 134.77 ppm range. Two ethylene carbons were also observed at δ_C 139.13 and 160.15 ppm corresponding to C-9 and C-2 of compounds **3a** and **3b**. Carbonyl carbon signals at δ_C 184.71 and 186.71 ppm were also observed. Both spectra are in complete agreement with each other (i.e. the ^{13}C-NMR confirms what is seen in the ^{1}H-NMR spectrum of this compound). These chemical shift signals are indicative of flavonoids; especially, the homoisoflavones subclass and specifically belonging to the **3-bezylidene-4-chromanone** (**3a**) and **3-benzyl-chrom-2-en-4-one** (**3b**) categories of homoisoflavones (Mulholland et al., 2013)

The UPLC-MS chromatogram of the pure isolated compound **3** (**Figure 5.22**) showed two signal peaks with retention times of 10.54, 10.78, 10.53 and 10.74 minutes in the intermediate polarity region and both peaks have equal mass in both ESI- and ESI+ modes. This further confirms the assumption that this compound is a regioisomer. The peaks have a calculated mass of *m/z*: 327.0815 in the ESI- mode and a calculated mass of *m/z*: 329.1046 in the ESI+ mode which then correlates with an actual mass of *m/z*: 328.0931 g/mol for this compound.

This molecular mass was further confirmed by Q-TOF-MS (**Figure 5.23**) which also yielded the same value. Q-TOF-MS analysis also predicted the molecular formula of the compound to be $C_{18}H_{16}O_6$ with a double bond equivalent (DBE) of 11 which suggests that the compound could have two aromatic rings and three double bonds or two double bonds and a ring. Based on the data provided above, we propose the backbone chemical structure for compound **3a** to be that of the **(E)-3-benzylidene-4-chromanone** type and for compound **3b** to be that of the **3-benzyl-chrom-2-en-4-one** homoisoflavone type as shown below.
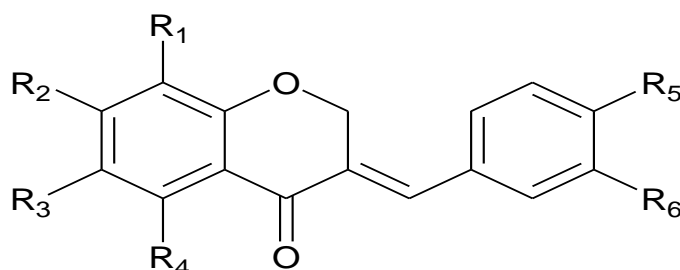
Figure 5.18: Chemical backbone structure of compound 3a, a (E)-3-benzylidene-4-chromanone homoisoflavone derivative.
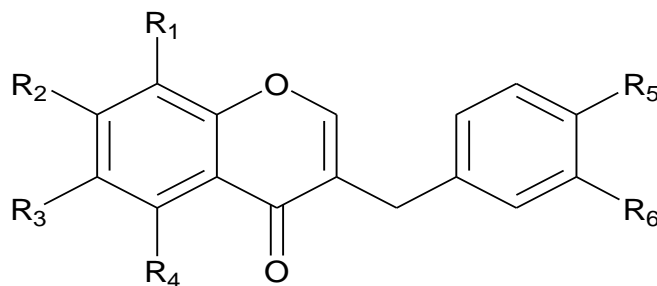
Figure 5.19:Chemical backbone structure of compound 3b, a 3-benzyl-chrom-2-en-4-one homoisoflavone derivative.

The proposed structures are in agreement with the predictions. However, the exact spatial positioning of the functional groups could not be extrapolated and, therefore, other techniques such as 2D NMR spectroscopy which exploit the short- and long-range correlations of the protons to carbons within a molecule need to be further conducted. the latter techniques will aid in determining the exact positions of these functional groups. Determining the exact position of these substituents is imperative because it will allow us to understand the structure-activity relationship between the ligand (compound) and its protein target (enzyme, receptor etc.). Therefore, this will assist further investigations into understanding the molecular mechanism of action of these compounds (i.e. answering questions such as: what is the mode of binding of the compound at the active site? Which functional groups are needed to elicit a biological response? and at which carbon position should they be substituted in order to elicit biological activity?).
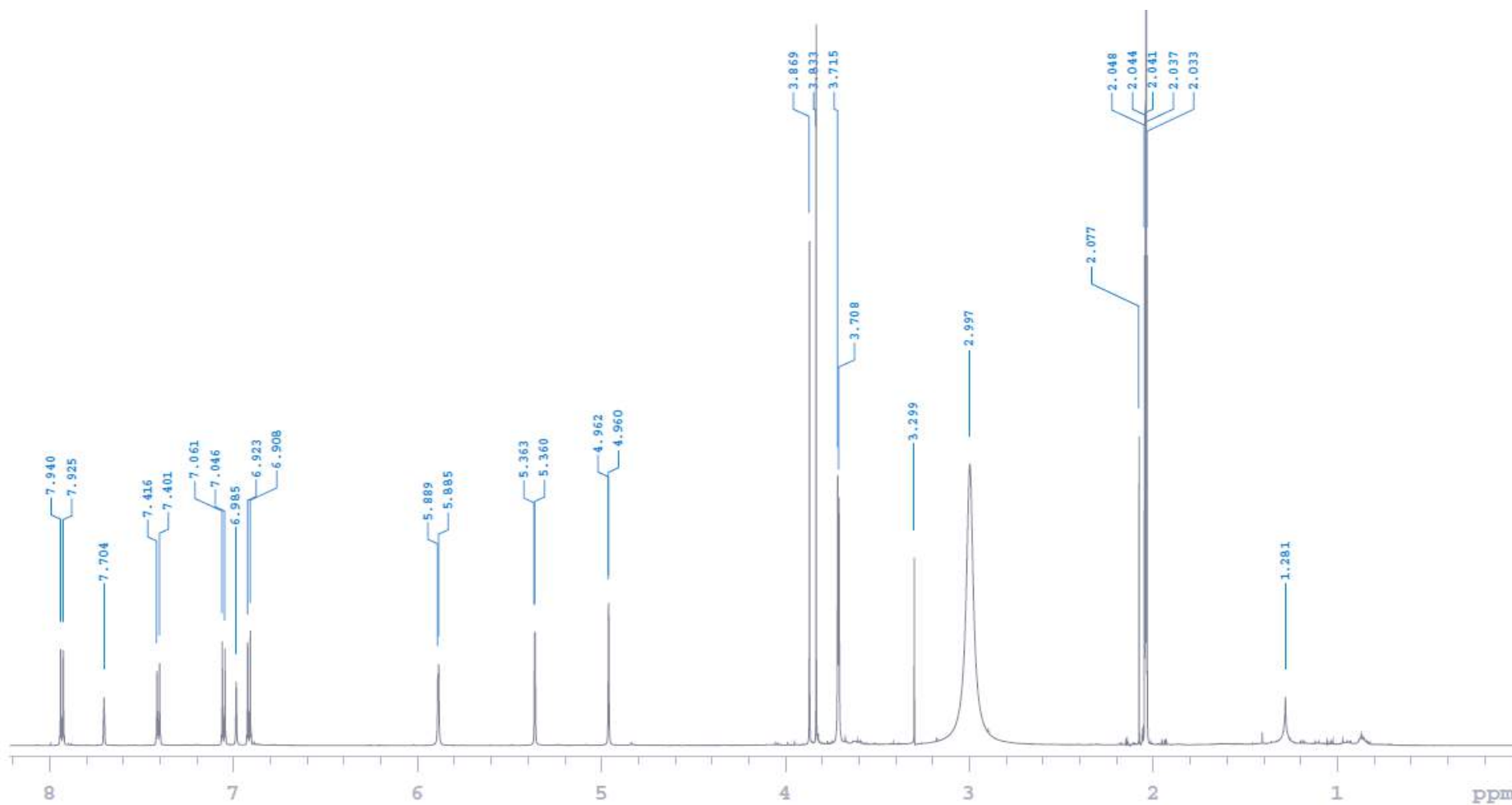
Figure 5.20: ¹H-NMR spectrum of compound 3 (F1P2) isolated from the ethanol extract of the herbal concoction.
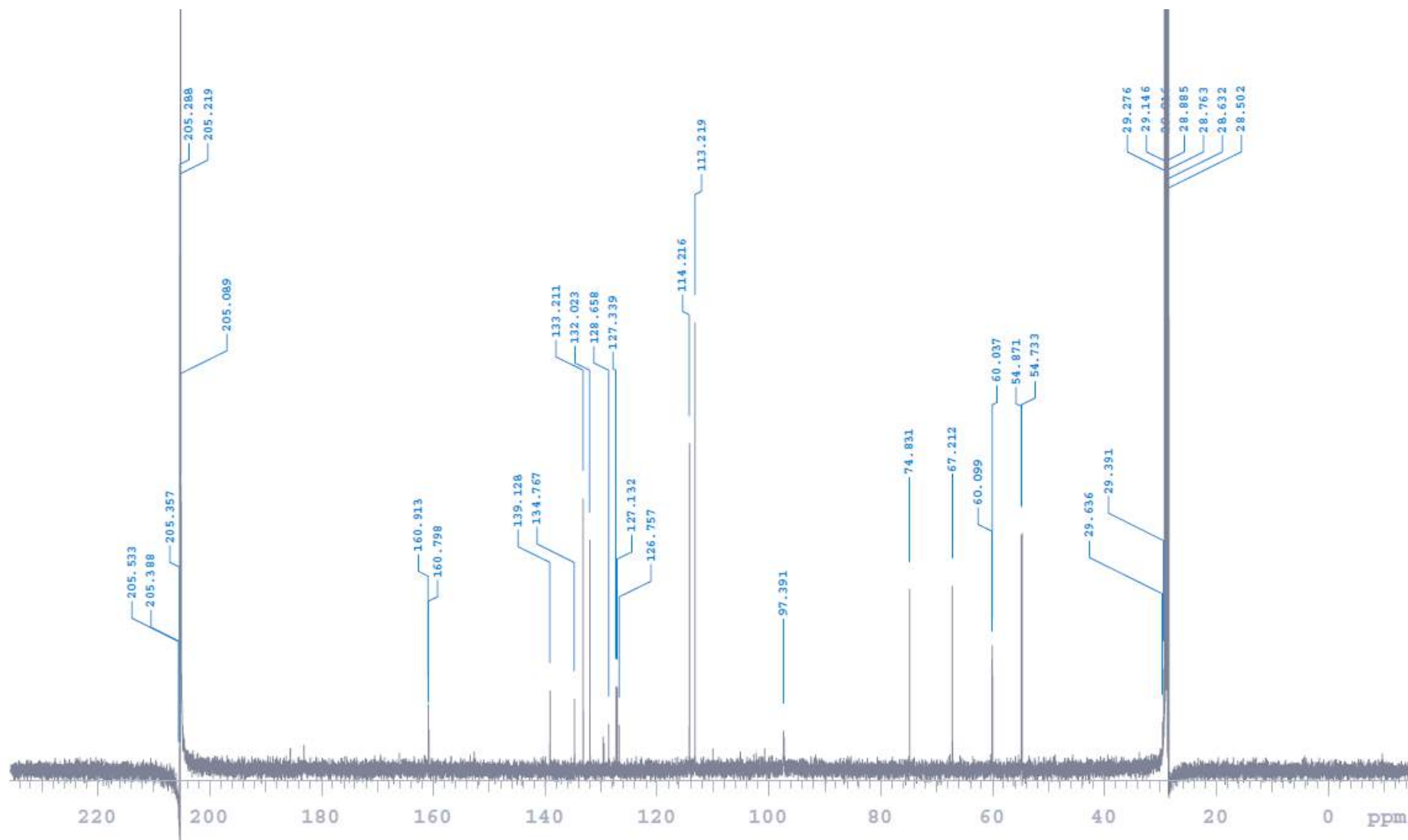
Figure 5.21: ^{13}C-NMR spectrum of compound 3 (F1P2) isolated from the ethanol extract of the herbal concoction.
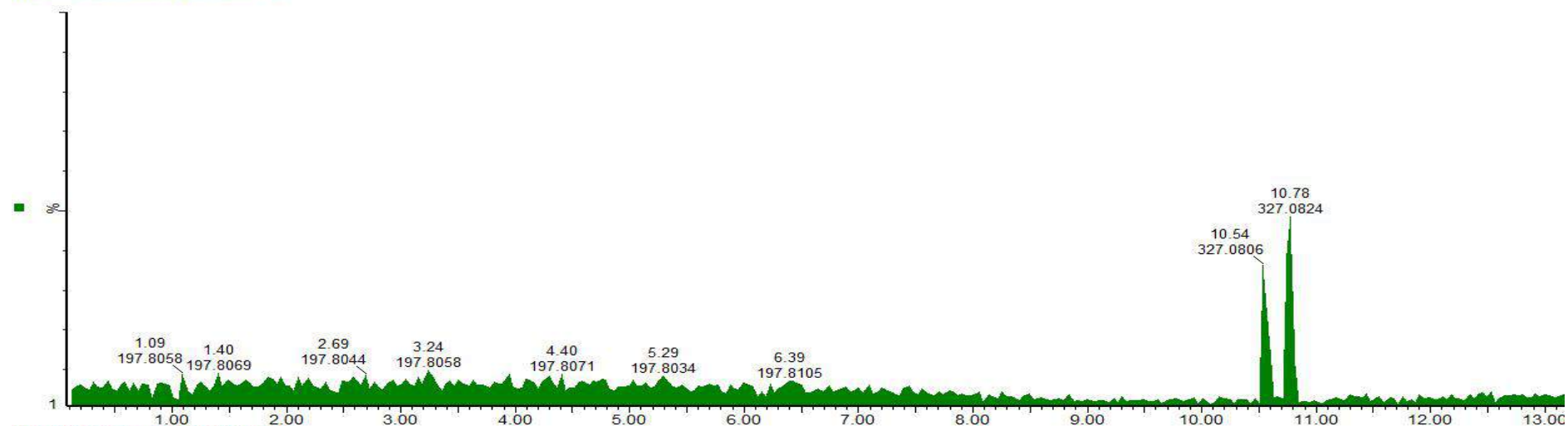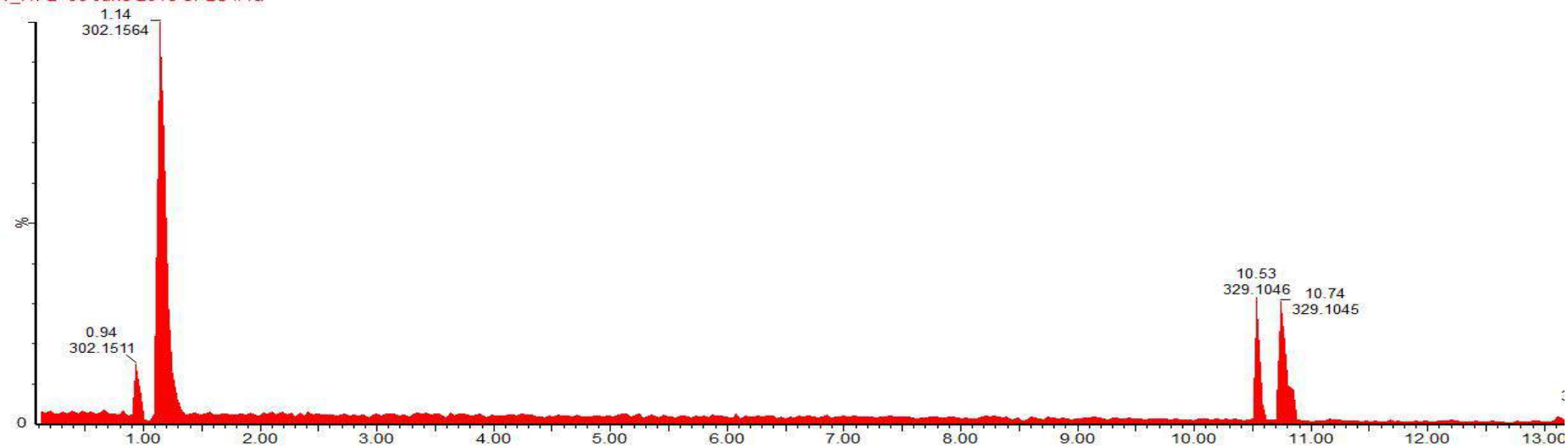
Figure 5.22: UPLC-MS (ES- and ES+) chromatograms for compounds 3a and 3b.
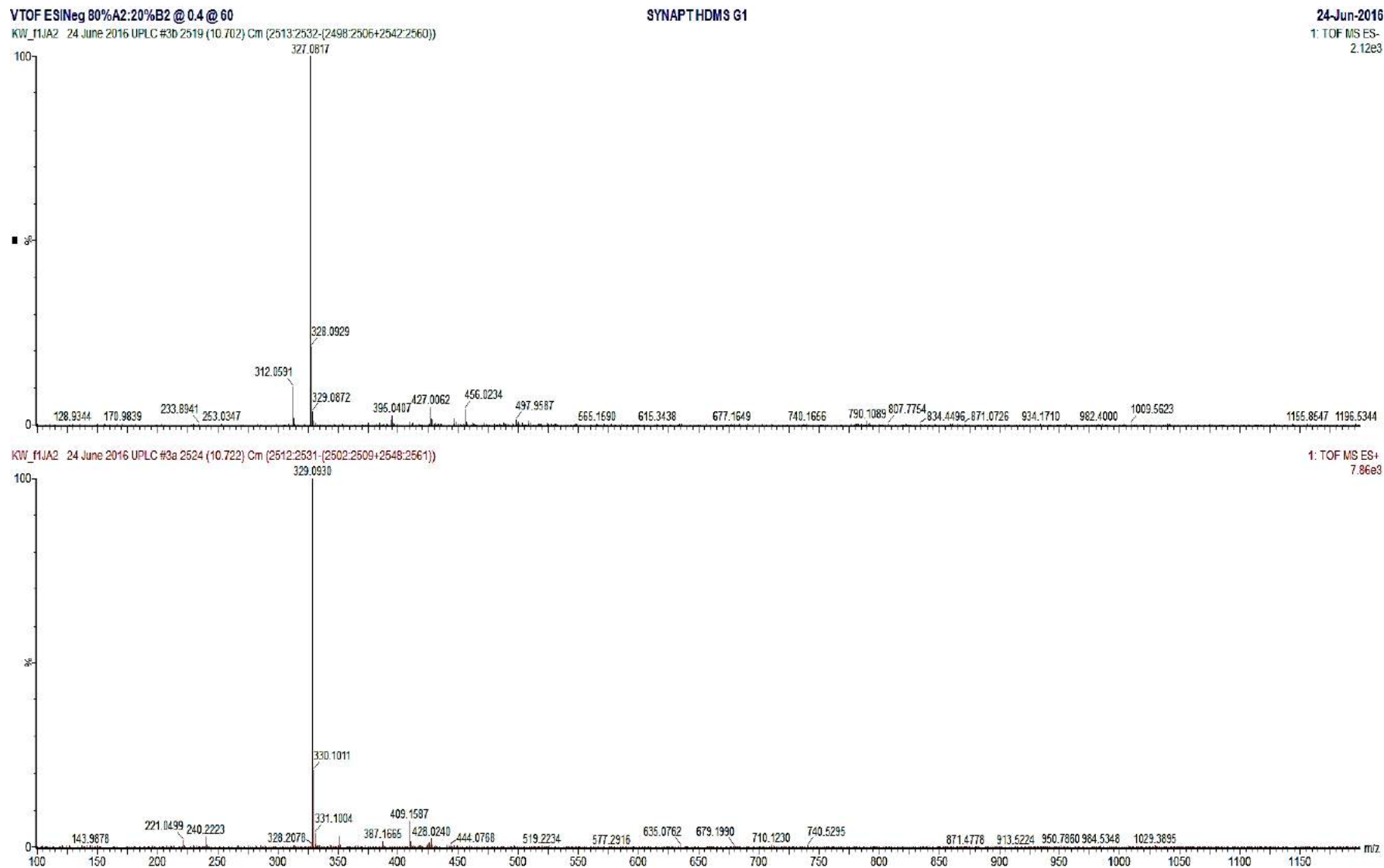
Figure 5.23: HR-QTOF-MS (ES- and ES+) for compounds 3a and 3b.

5.2.3.1 Bioactivity of compound 3 (F1P2)

As shown in **Figures 5.24-5.26**, the data suggests that compound **3** exerted its cytotoxic effect in a concentration dependent manner against HEK293T cells. Compound **3** exhibited a mean CC_{50} value of 19.32 ± 1.004 µg/ml against HEK293T cells.

Compound **3** was found to be inactive against HIV-1 subtype C RT and PR. Furthermore, it was also non-toxic to TZM-bl cells with a mean CC_{50} value of 20.933 ± 2.375 µg/ml. Compound **3** was inactive in neutralizing HIV-1 subtype C.

Statistical analysis of this data using the student-t test and Two-way ANOVA followed by the Bonferroni post hoc test, also confirms that there is no significant difference between anti-HIV-1 RT, PR, neutralization activity and cellular toxicity following treatment with this compound at all various concentrations tested starting from 100 to 0.00169 µg/ml. Therefore, this implies that compound **3** was inactive against HIV-1. The p-value was found to be greater than 0.05 at all concentrations tested.

5.2.3.2 Cytotoxicity and RT activity
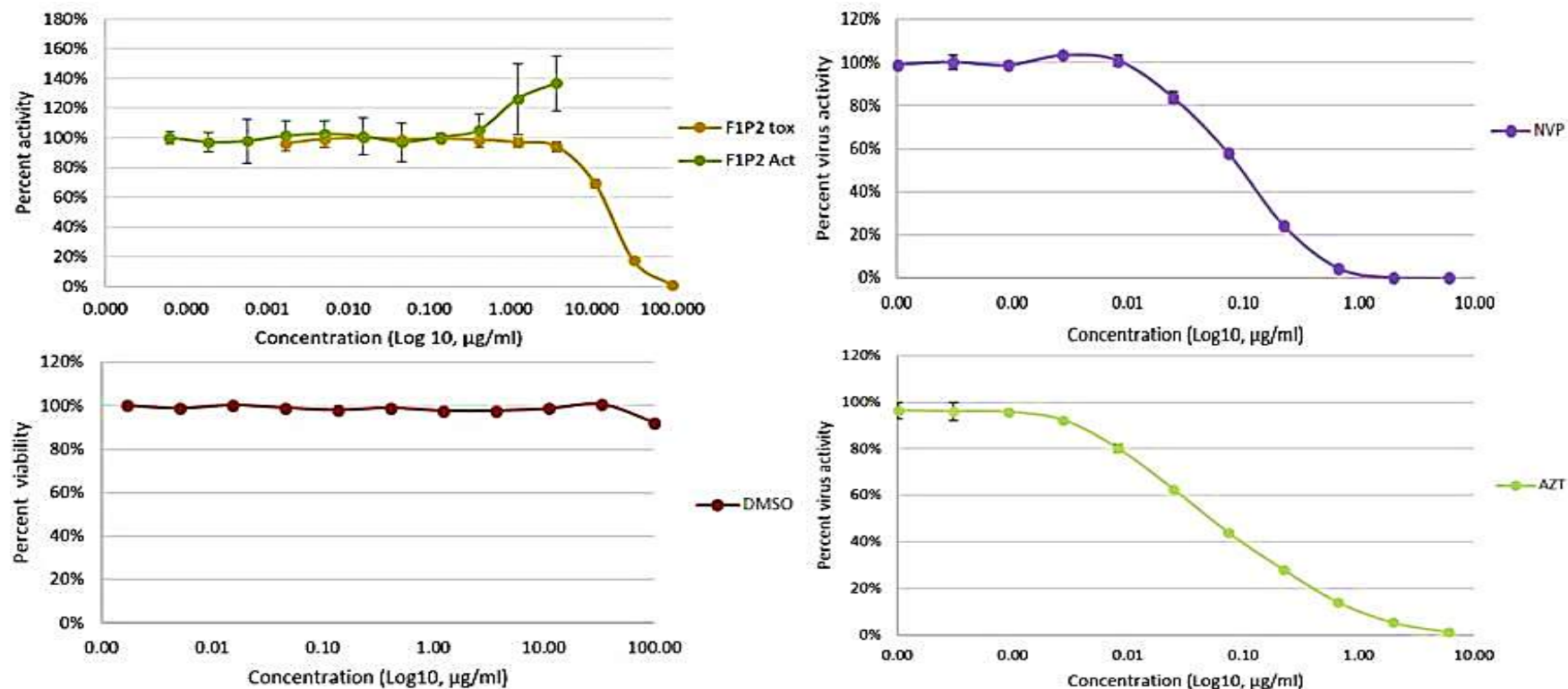
Figure 5.24:Reverse transcriptase phenotypic drug susceptibility profile of MJ4 HIV-1 subtype C isolate to compound 3

The ability of compound **3** to inhibit HIV-1 RT was investigated using a novel RT phenotypic drug susceptibility assay. Cells were infected with HIV-1 MJ4 isolate and treated with compound **3** at various concentrations for 48 hours. Data points are presentative of mean percent activity $\pm$ SD of two independent experiments Plots Zidovudine ("AZT") and Nevirapine ("NVP") represent positive controls and a plot of "DMSO" represents the vehicle control. n=4; $p < 0.05$ was considered significant.

5.2.3.3   Cytotoxicity and PR activity
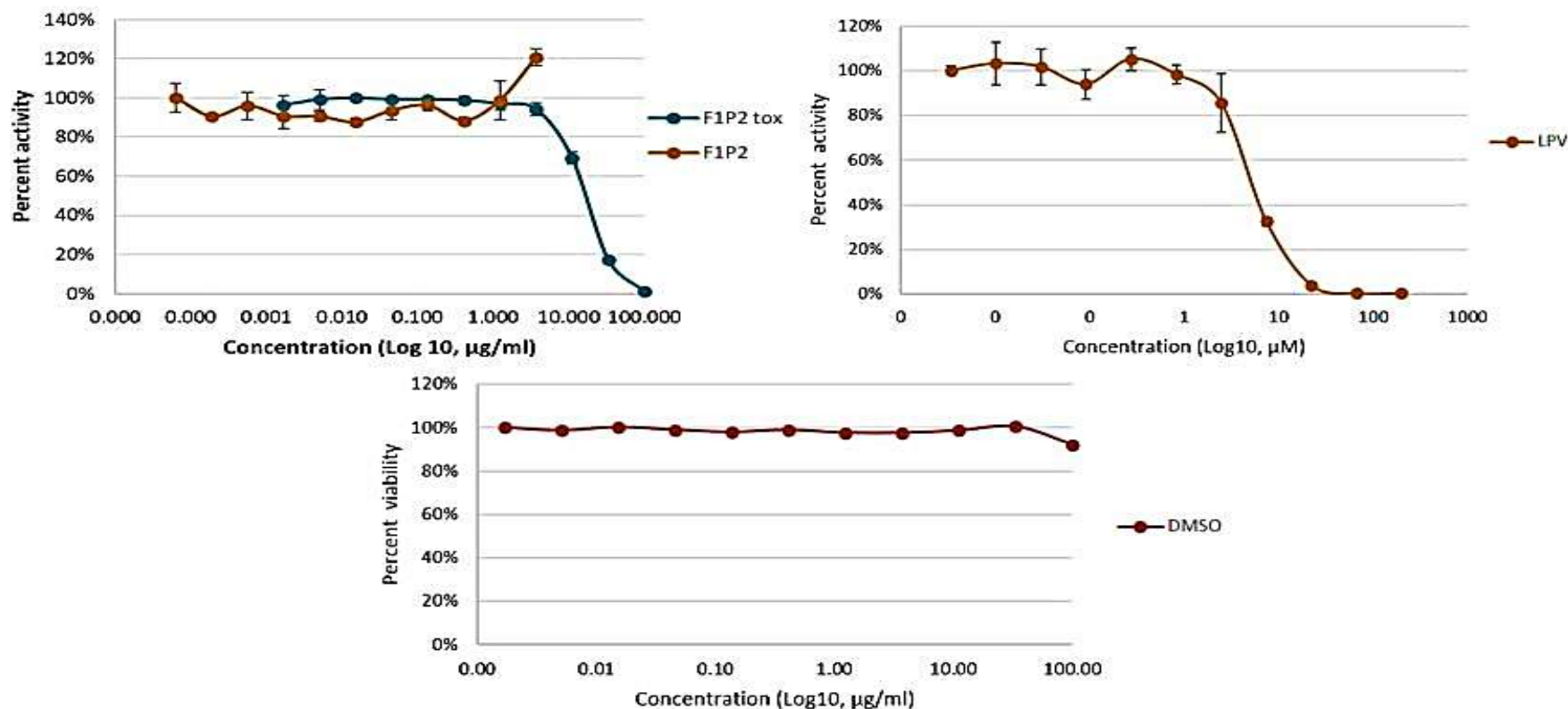
Figure 5.25: Protease phenotypic drug susceptibility profile of MJ4 HIV-1 subtype C isolate to compound 3

Compound **3** was evaluated for anti-HIV protease activity utilizing a novel protease phenotypic drug susceptibility assay. Cells were infected with HIV-1 MJ4 isolate and treated with compound **3** at various concentrations for 48 hours. Data points are representative of mean percent activity ± SD of two independent experiments. Plots of "LPV" represent positive control and plot of "DMSO" represents the vehicle control. n=4; $p < 0.05$ was considered significant.

5.2.3.4 Cytotoxicity and Neutralization activity
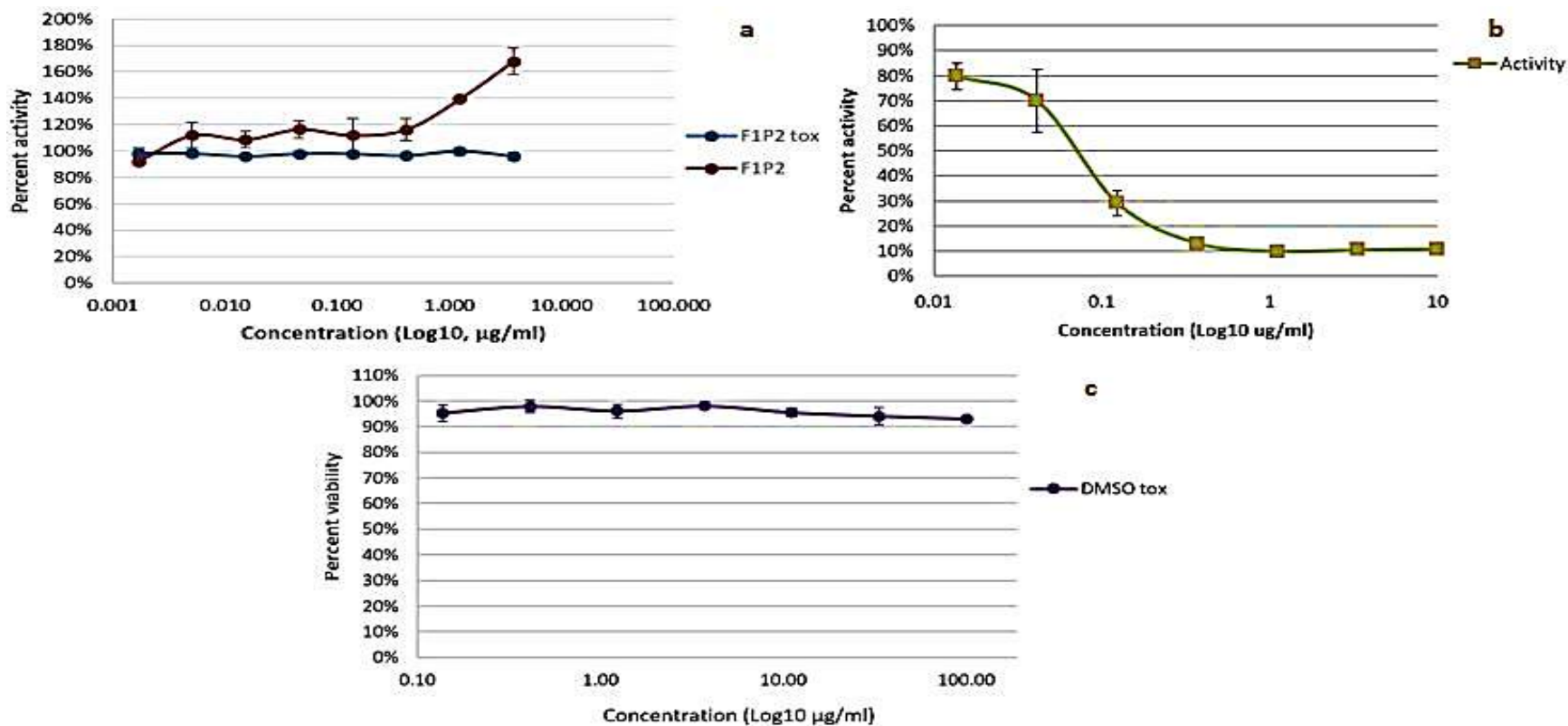
Figure 5.26: HIV-1 neutralization profile of compound 3

TZM-bl assay was used to evaluate the ability of the isolated compounds to neutralize HIV-1 MW isolate. Compound **3** was inactive for preventing HIV entry. VRC01 (CD4 antagonist) was included as a positive control and cytotoxicity due to DMSO vehicle control was also evaluated with results expressed as percentage activity and percentage viability relative to control. Data points are representative of mean percent activity ± SD of two independent experiments: n=4; p< 0.05 was considered significant.

6   DISCUSSION

The results presented here show that we have managed to isolate flavonoids specifically belonging to the homoisoflavones class. Homoisoflavonoids belong to a small group of naturally occurring oxygen heterocycles (Bohler et al., 1976). Structurally, these types of compounds are classified as derivatives of the 4-chromanone pharmacophore (Farkas et al., 1971 and Perjesi at al., 2008). Homoisoflavanones are characterized by a sixteen-carbon skeleton which has either a chromanone, chromone or chromane moiety attached to a benzyl or benzylidene group at the C-3 position (Namikoshni et al., 1987). There are three basic structural types of homoisoflavanones; namely, 3-benzyl-4-chromanone, 3-benzyl-3-hydroxy-4-chromanone and 3-benzylidene-4-chromanone. The latter structural type can undergo isomerization of the exocyclic double bond to form an endocyclic double bond between C2 and C3 thus forming a chromone derivative (Namikoshni et al., 1987).

The first homoisoflavanones to be isolated were eucomin and eucomol (Dewick et al., 1975). Subsequently, several compounds have been isolated from different genera within the *Hyacinthaceae* family including *Eucomis, Merwilla, Ledebouria, Veltheimia* and *Drimiopsis* (Mulholland et al., 2013). This particular 4-chromanone scaffold (Homoisoflavonoid) is one of the most important pharmacophores in medicinal chemistry and biologically too, it has been shown to possess several pharmacological activities including anti-cancer (Wall et al., 1989; Miadokova et al., 2002 and Perjesi et al., 2008), antifungal (Nakib et al., 1990), anti-monoamine oxidase (Desideri et al., 2011), antioxidant (Siddaiah et al., 2006 and Siddaiah et al., 2007), antiviral (Desideri et al., 1997; Quglia et al., 1999 and Tait et al., 2006), anti-inflammatory (Du Toit et al., 2010 and Hung et al., 2010), antihistaminic and antiallergic (Kirkiacharian et al., 1989), antitubercular (Yempala et al., 2012 and Roy et al., 2013) and anti-protein kinase C activity (Lin et al., 2008).

Two of the most important pharmacological activities that these compounds possess especially in relation to HIV/AIDS, are anti-inflammatory and antioxidant activities. Both of these pharmacological activities have been implicated in decelerating the progress of HIV infection to AIDS in HIV-positive patients (Cos et al., 2004 and Singh et al., 2005). Oxidative stress, which is a result of an imbalance between natural antioxidants and reactive oxygen species production, causes a broad range of activities including altering viral

replication, immune functions and apoptosis (Singh et al., 2005). Singh and colleagues have shown that in an HIV positive patient, the natural antioxidant response (i.e. glutathione, ascorbic acid and other natural antioxidants) is lowered, thus, resulting in an accumulation of ROS and oxidative DNA damage which, in turn, results in accelerated progression to the AIDS state in these patients.

With regard to anti-inflammatory activity, it is well documented that during an infection, the early events of an immune response include inflammation. This is a process whereby the white blood cells are activated to express interleukins which, in a feedback loop mechanism, will also activate white blood cell expression and production of other chemical factors that will activate and recruit cells like macrophages, thus ensuring that the pathogenic microbes do not spread to the rest of the body. However, the HIV virus relies on this mechanism (since it infects white blood cells) to ensure its replication (Boinon et al., 2005; Paiardini and Muller-Trutwin, 2013).

Therefore, a treatment that not only focuses on treating HIV alone but also considers these two factors is ideal. This would ensure the down-modulation of pro-inflammatory cytokines and, therefore, a reduced inflammation process. Furthermore, fewer white blood cells will be infected. Moreover, the reduction of ROS accumulation will ensure that oxidative DNA damage is prevented and fewer immune cells will undergo apoptosis, therefore, ensuring decelerated progression to the AIDS state.

In this study, a series of compounds including alkaloids and flavonoids have been isolated. However, we report on only four flavonoids which were partially characterized. As seen in **section 5.2.1-5.2.3**, the data shows that these flavonoids specifically belong to the homoisoflavones class and are of three basic structural types. Compound **1** belongs to the 3-benzyl-4-chromanone type, while compound **2** belongs to the 3-benzylidene-4-chromanone type. Compound **3** is an isomer composed of the 3-benzylidene-4-chromanone type and the 3-benzylidene-4-chromanone type that has undergone isomerization of the exocyclic double bond to an endocyclic double bond forming a chromanone type of homoisoflavonone. These compounds have been tested for anti-HIV-1 subtype C activity.

These different structural types have been shown to possess different bioactivities. Studies by Tait et al., (2006) investigating the antiviral activities of substituted homoisoflavonoids on

enteroviruses have shown that, generally, the 3-benzylidene-4-chromanone type has a pronounced potent activity and they are slightly toxic when compared to the 3-benzyl-4-chromanone type. The latter compound class has been shown to be inactive and non-toxic. They further showed that the chomanone type "3-benzyl-chrom-2-en-4-one" has moderate activity and toxicity. A structure-activity relationship evaluation of the substituted homoisoflavonoids showed that the presence of a methoxy group at C-5 and C-7 resulted in loss of activity and greater cytotoxicity against HeLa and BGM cells by the 3-benzyl-4-chromanone types. Whereas the presence of a methoxy at C-4' of the 3-benzylidene-4-chromanone type resulted in pronounced antiviral activity.

Moreover, Roy et al. (2013) found that of all the homoisoflavonoid analogues they tested for antitubercular activity, only the 3-benzylidene-4-chromanone type was potent at inhibiting the efflux pump of *mycobacterium smegmatis* which makes it resistant to most of antitubercular medicines. The authors further showed that the positioning of the substituents on the homoisoflavonoid structural backbone and the type of substituents can alter the mycobacterial activity drastically.

A study by Desideri and colleagues (2011), which looked at a different angle from antimicrobial to anti-neurodegeneration activity of homoisoflavonoids, showed that all three homoisoflavonoid structural types were active at inhibiting the two monoamine oxidase isoforms (MAO-A and MAO-B) but they showed selective inhibitory activity against the MAO-B isoform. Additionally, the 3-benzylidene-4-chromanone type was more potent even at nanomolar concentrations while the 3-benzyl-4-chromanone type was active only at micromolar concentrations. They also showed that the presence of an endocyclic double bond, as seen in the 3-benzyl-chrom-2-en-4-one types, resulted in a significant reduction of inhibitory activity and loss of activity of some analogues depending on the substitution pattern and the type of functional groups present in those analogues.

Corresponding to what the above studies have found, we also observed the same trend in our investigation of the anti-HIV activity of homoisoflavonoids. Looking at **Figures 5.7-5.9** for compound **1,** we see that the 3-benzyl-4-chromanone types are less toxic against HEK293T and TZM-bI cells and inactive against HIV-1 subtype C. Moreover, it was observed that compound **2**, the 3-benzylidene-4-chromanone type, is active against the RT of three HIV-1 isolates (MJ4, CM9 and Du179), while inactive against HIV PR, HIV neutralization and

moderately cytotoxic to HEK293T and TZM-bI cells (see **Figures 5.15-5.17**). Lastly, compound **3**, a mixture of the chomanone "3-benzyl-chrom-2-en-4-one" and the 3-benzylidene-4-chromanone type, was found to be inactive against HIV RT, PR and neutralization and moderately cytotoxic to HEK293T cells (see **Figures 5.24-5.26**). Figure 6 below shows the proposed chemical structures of the isolated compounds.

compound 1

compound 2

compound 3a
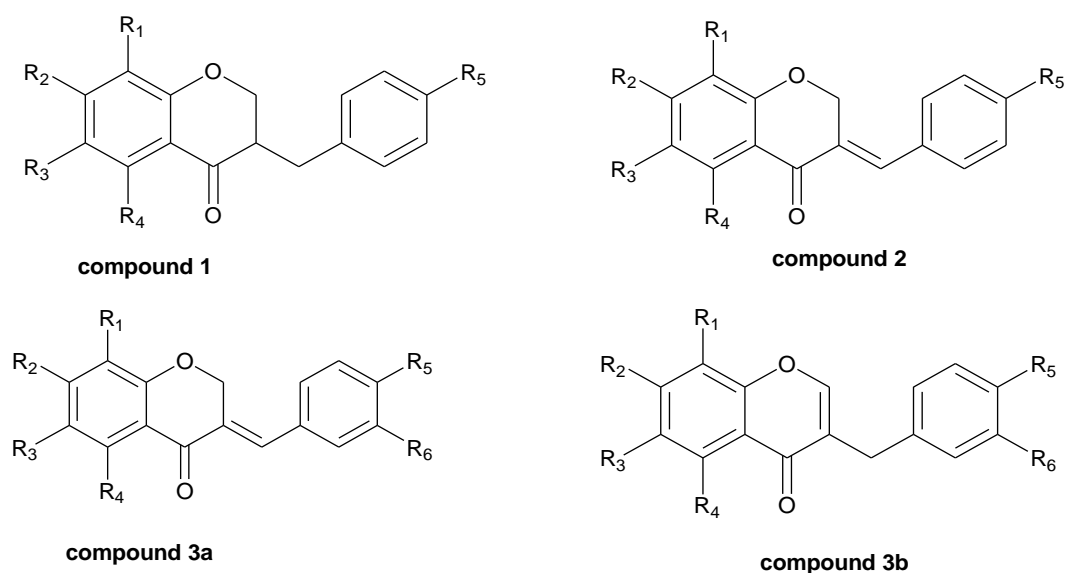
compound 3b

Figure 6.1: Backbone structures of the proposed compounds 1, 2, 3a and 3b

When comparing compound **1** and compound **2** structurally, we propose that the loss of activity for compound **1** could be a result of the physiochemical properties of this compound such as the flexibility of compound **1** which is due to the lack of an exocyclic double bond. It could also be the type and substitution pattern of the functional groups.

Structural comparison of compound **2** and compound **3**, specifically looking at the 3-benzylidene-4-chromanone isomer **3a**, we see that compound **3** is bulkier than compound **2** because of the extra functional group at C-3' "R_6" which could present issues with regard to binding of this compound in the active site. Additionally, because compound **3** was isolated as two isomers, it is possible that these two compounds could have an antagonistic effect on each other and, hence, we could not observe any biological activity. This, in relation to pharmacological activity, could be the reasons why **3a** was inactive while **2** was active.

In summary, compound **2** could be active because it has a molecular weight less than 500 g/mol. Therefore, *C*log P would be less than 5 (Lipinski et al., 1997) and it also appears to be

less bulky, reduced steric hindrance which will allow for easier access to the active site of RT. Compound **1** is not active probably because it lacks a double bond between C-3 and C-9 which would make this compound flexible and prevent it from forming stable bond interactions in the active site. Furthermore, it has 2 methoxy functional groups and a hydroxyl group which could be attached at either C-5, C-7 or C-8 of the chromane moiety and C-4' which results hampering the bioactivity as seen from the studies of Desideri et al., (2011); Roy et at., (2013) and Tait et at., (2006).

We, therefore, further assume that the reasons why compounds **1** and **3** were inactive while **2** was active is because of their backbone structures and more so, the type of functional groups attached and their spatial positioning on the backbone structure of these compounds. However, these assumptions can only be verified by X-ray crystallography and computational studies utilizing fully elucidated chemical structures.

To further discuss and account for the lack of direct anti-HIV-1 activity by compounds **1** and **3**, we propose that these could have anti-inflammatory and antioxidant activities which have been implicated in accelerating the progress of HIV infection. As stated above, studies have shown that homoisoflavones possess inhibitory activity against the enzymes Cox-1 and Cox-2 which are crucial in initiating the inflammatory action. (Du Toit et al., 2010). The authors found that the 3-benzyl-4-chromanone types have potent activity against prostaglandin synthesis and Cox 1 & 2 enzyme activity when compared to the 3-benzylidene-4-chromanone type such as compound **2**.

The chomanone "3-benzyl-chrom-2-en-4-one" compounds have also been shown to possess antioxidant properties. They were more potent when compared to 3-benzyl-4-chromanone types. Furthermore, the type of functional group also played a role in enhancing their antioxidant activity i.e. a hydroxyl group favored potent activity while a methoxy group favored moderate activity (Rao et al., 2008).

Overall, our data suggest that the ethanol- and dereplicated ethanol- herbal extract has direct and indirect anti-HIV 1 activity. These extracts possess a cocktail of phytochemicals that can inhibit HIV-1 RT and HIV-1 entry while, on the other hand, ensuring that the inflammatory responses activated during the infection are reduced thus reducing the number new cells infected during the course of the HIV 1 infection. Moreover, it possesses phytochemicals that

have antioxidant activity which, in relation to HIV infection, results in a boosted immune system to ward off the virus. This, then, translates to a decelerated progression of the AIDS state of patients who are HIV positive.

FUTURE WORK

The main objective will be to elucidate the chemical structures of these compounds and to compute their molecular masses utilizing the capabilities of both 1D & 2D-NMR spectroscopy and HR-Q-TOF-MS. These techniques will provide data pertaining to the type and/or class of compound, chemical substituents and spatial location of the substituents within the molecules. This will, therefore, enable the construction of 2D chemical structures of these molecules to be used to obtain the accurate molecular mass and molecular fragmentation patterns.

Furthermore, the compounds whose chemical structures and molecular masses have been solved could be used in the molecular modeling and molecular dynamic simulation studies. These studies are done in order to understand how these molecules interact chemically with their protein targets at the molecular level. These techniques afford us the ability to evaluate and predict the stable chemical configurations and chemical bonding interactions of these compounds.

The findings of these studies may have important implications/applications in the fields of chemistry which include extensive knowledge application of analytical techniques in the industry. Also, the knowledge and understanding gained from these studies could lead to the discovery of new molecules which possess special characteristics important in pharmaceutical industries and may also have broader applications in the field of science and engineering.

7 REFERENCES

Abraham, R. J., Fisher, J., Loftus, P. (1988). Introduction to NMR Spectroscopy, John Wiley and Sons, Chichester. London.

Al Nakib, T., Bezjak, V., Meegan, M. J., Chandy, R. (1990). Synthesis and antifungal activity of some 3-benzylidenechroman-4-ones, 3-benzylidenethiochroman- 4-ones and 2-benzylidene-1-tetralones. Eur. J. Med. Chem, 25, 455–462.

Ali, A., Wang, J., Nathans, R. S., Cao, H., Sharova, N., Stevenson, M., and Rana, T. M. (2012). Synthesis and structure-activity relationship studies of HIV-1 virion infectivity factor (Vif) inhibitors that block viral replication. ChemMedChem 7, 1217-1229.

Allwood, J. W., Ellis, D. I., Goodacre, R. (2008). Metabolomic technologies and their application to the study of plants and plant-host interactions. Physiol. Plant, 132, 117–135.

Arts, E. J., and Hazuda, D. J. (2012). HIV-1 antiretroviral drug therapy. Cold Spring Harbor perspectives in medicine. 2, 1-24.

Asres, K., Seyoum, A., Veeresham, C., Bucar, F and Gibbons, S. (2005). Naturally derived anti-HIV agents. Phytotherapy Research, 19, 557–581.

Barbaro, G., and Klatt, E. C. (2002). HIV infection and the cardiovascular system. AIDS Reviews 4, 93-103.

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

Beignon, A. S, McKenna, K., Skoberne, M., Manches, O., DaSilva, I., Kavanagh, D. G., Larsson, M., Gorelick, R. J., Lifson, J. D., Bhardwaj, N. (2005). Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor-viral RNA interactions. J Clin Invest.,115,3265–3275.

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

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

Beyrer, C. (2007). HIV epidemiology update and transmission factors: risks and risk contexts--16th International AIDS Conference epidemiology plenary. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America 44, 981-987.

Bimakr, M. (2010). Comparison of different extraction methods for the extraction of bioactive flavonoid compounds from spearmint (Mentha spicata L.). Food Bioproduction Process, 1-6

Birrell, P. J., Gill, O. N., Delpech, V. C., Brown, A. E., Desai, S., Chadborn, T. R., Rice, B. D., and De Angelis, D. (2013). HIV incidence in men who have sex with men in England and Wales 2001-10: a nationwide population study. The Lancet Infectious diseases 13. 313-318.

Bohler, P., Tamm, C. (1967). The Homo-isoflavones, a new class of natural product. Isolation and structure of eucomin and eucomol. Tetrahedron Letters. 36, 3479-3483

Borman, A. M., Quillent, C., Charneau, P., Dauguet, C., and Clavel, F. (1995). Human immunodeficiency virus type 1 Vif- mutant particles from restrictive cells: role of Vif in correct particle assembly and infectivity. Journal of Virology. 69, 2058-2067.

Cardellina, J. H., Munro, M. H. G., Fuller, R. W., Manfredi, K. P., McKee, T. C., Tischler, M., Bokesch, H. R., Gustafson, K. R., Beutler, J. A., Boyd, M. R., (1993). A chemical screening strategy for the dereplication and prioritization of HIV-inhibitory aqueous natural products extracts. Journal of Natural Products. 56 (7), 1123-1129.

Chang, L.J., Urlacher, V., Iwakuma, T., Cui, Y., Zucali, J. (1999). Efficacy and safety analyses of a recombinant human immunodeficiency virus type-1 derived vector system. Gene Ther 6,715-728.

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

Clavel, F., and Hance, A. J. (2004). HIV drug resistance. The New England journal of Medicine 350, 1023-1035.

Cohen, T., Murray, M., Wallengren, K., Alvarez, G. G., Samuel, E. Y., and Wilson, D. (2010). The prevalence and drug sensitivity of tuberculosis among patients dying in hospital in KwaZulu-Natal, South Africa: a postmortem study. PLoS medicine 7, 1-8.

Cos, P., Hermans, N., De, B. T., Apers, S., Sindambiwe, J. B., Witvrouw, M., De, C. E., Vanden, B. D., Pieters, L., and Vlietinck, A. J. (2002). Antiviral activity of Rwandan medicinal plants against human immunodeficiency virus type-1 (HIV-1). Phytomedicine: *International Journal of Phytotherapy and Phytopharmacology*. 9, 62-68.

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

Cowan, M. M. (1999). Plant products as antimicrobial agents. Clinical Microbiology Reviews, 12(4). 564-582.

Crouch, N. R, Bangani, V., Mulholland, D. A., (1999). Homoisoflavanones from three South African Scilla species. Phytochemistry. 51, 943-946.

Deeks, S. G. (2003). Treatment of antiretroviral-drug-resistant HIV-1 infection. Lancet. 362, 2002-2011.

Degenhardt, L., Mathers, B., Vickerman, P., Rhodes, T., Latkin, C., and Hickman, M. (2010). Prevention of HIV infection for people who inject drugs: why individual, structural, and combination approaches are needed. Lancet. 376, 285-301.

Demaison, C., Parsley, K., Brouns, G., Scherr, M., Battmer, K., Kinnon, C., Grez, M. and Thrasher, A. J. (2002). High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. Human Gene Therapy, 13, 803–813.

Derome, A. E. (1987). Modern NMR techniques for Chemistry Research, Pergamon Press, Oxford.

Desideri N., Bolasco A., Fioravanti R., Proietti Monaco L., Orallo F., Yáñez M., Ortuso F., Alcaro S. (2011). Homoisoflavonoids: Natural Scaffolds with Potent and Selective Monoamine Oxidase-B Inhibition Properties. J. Med. Chem. 54, 2155–2164.

Desideri, N., Olivieri, S., Stein, M. L., Sgro, R., Orsi, N., Conti, C. (1997). Synthesis and anti-picornavirus activity of homo-isoflavonoids. Antiviral Chem. Chemother, 8, 545–555.

Du Toit, K., Drewes, S. E., Bodenstein, J. (2010). The chemical structures, plant origins, ethnobotany and biological activities of homoisoflavanones. Nat. Prod. Res. 24, 457–490

Du Toit, K., Elgorashi, E. E., Malan, S. F., Drewes, S. E., van Staden, J., Crouch, N. R., Mulholland, D. A. (2005). Anti-inflammatory activity and QSAR studies of compounds isolated from Hyacinthaceae species and Tachiadenus longiflorus Griseb. (Gentianaceae). Bioorg. Med. Chem, 13, 2561–2568.

DuBridge, R. B., Tang, P., Hsia, H. C., Leong, P. M., Miller, J. H. and Calos, M. P. (1987). Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Molecular Cell Biology, 7, 379–387.

Dunn, W. B., Ellis, D. I. (2005). Metabolomics: Current analytical platforms and methodologies. Trends Anal. Chem., 24, 285–293.

Ellegard, R., Shankar, E. M., and Larsson, M. (2011). Targeting HIV-1 innate immune responses therapeutically. Current opinion in HIV and AIDS 6, 435-443.

Ernst, R. R., Bodenhausen, G. and Wokaun, A. (1987). Principles of nuclear magnetic resonance in one and two dimensions. Claredon Press, Oxford.

Fan, L., and Peden, K. (1992). Cell-free transmission of Vif mutants of HIV-1. Virology. 190, 19-29.

Fiehn, O., Kopka, J., Dormann, P., Altmann, T., Trethewey, R. N., Willmitzer, L. (2000). Metabolite profiling for plant functional genomics. Nat. Biotechnol. 18, 1157–1161.

Fried, B., Sherma, J. (1996). Practical thin-layer chromatography: a multidisciplinary approach. CRC Press, Inc. United States of America.

Fukusaki, E., Kobayashi, A. (2005). Plant metabolomics: Potential for practical operation. J. Biosci. Bioeng. 100, 347–354.

Fuloria, N. K. and Fuloria, S. (2013). Structural elucidation of small organic molecules by 1D, 2D and Multi-dimensional-solution NMR spectroscopy. Analytical & Bioanalytical techniques,11, 1-8.

Gabrielsen, B., Monath, T. P., Huggins, J. W., Kefauver, D. F., Pettit, G. R., Groszek, G., Hollingshead, M., Kirsi, J. J., Shannon, W. M., Schubert, E. M., Dare, J., Ugarkar, B., Ussery, M. A., Phelan, M. J. (1992), J. Nat. Prod. 55, 1569.

Glaziou, P., Falzon, D., Floyd, K., and Raviglione, M. (2013). Global epidemiology of tuberculosis. Seminars in respiratory and critical care medicine 34, 3-16.

Grabowski, K. and Schneider G. (2007) Properties and architecture of drugs and natural products revisited. Curr Chem Biol 2007, 1:115-127.

Gurib-Fakim, A. (2006). Medicinal plants: traditions of yesterday and drugs of tomorrow. Molecular Aspects of Medicine. 27, 1–93.

Harbone, J. B and Dey, P. M. (1993). Alkaloids and Sulphur Compounds, Vol. 8, Waterman, P.G. (Ed.), London, pp 475-496.

Heinrich, M., Barnes, J., Gibbons, S., Williamson, E. M. (2004) Fundamentals of Pharmacognosy and Phytotherapy. Elsevier Science Limited. Spain.

Hemelaar, J., Gouws, E., Ghys, P.D., and Osmanov, S. (2011). Global trends in molecular epidemiology of HIV-1 during 2000-2007. AIDS 25, 679-689.

Holme, D. J. and Peck, H. (1998). Analytical chemistry: third edition. England: Prentice Hall.

Hosseini I, Mac Gabhann F. (2013). APOBEC3G-Augmented Stem Cell Therapy to Modulate HIV Replication: A Computational Study. PLoS ONE, 8(5), 1-16

Hostettmann, K., Kizu, H., Tomimori, T. (1982). Molluscicidal properties of some saponins. Planta Medica, 44, 34–35.

Houghton, P.J., Raman, A. (1998) Laboratory Handbook for the Fractionation of Natural Extracts. Chapman & Hall. London.

Hu, G., Li, X., Zhang, X., Li, Y., Ma, L., Yang, L.M., Liu, G., Li, W., Huang, J., Shen, X., et al. (2012). Discovery of inhibitors to block interactions of HIV-1 integrase with human LEDGF/p75 via structure-based virtual screening and bioassays. Journal of Medicinal Chemistry 55, 10108-10117.

Hughes, J. D., J. Blagg, D. A. Price, S. Bailey, G. A. Decrescenzo, R. V. Devraj, E. Ellsworth, Y. M. Fobian, M. E. Gibbs, R. W. Gilles, N. Greene, E. Huang, T. Krieger-Burke, J. Loesel, T. Wager, L. Whiteley, and Y. Zhang. (2008). Physiochemical drug properties associated with in vivo toxicological outcomes. Bioorg. Med. Chem. Lett. 18:4872-4875.

Hung, T.M., Thu, C. V., Dat, N. T., Ryoo, S.W., Lee, J. H.; Kim, J. C., Na, M., Jung, H. J., Bae, K., Min, B. S. (2010). Homoisoflavonoid derivatives from the roots of Ophiopogon japonicus and their *in vitro* anti-inflammation activity. Bioorg. Med. Chem. Lett, 20, 2412–2416.

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

Ieven, M., Van den Berghe, D. A., Vlietinck, A. J. (1983). Plant antiviral agents. Influence of lycorine on the growth pattern of three animal viruses. Planta Medica. 49, 109–114.

Ieven, M., Vlietinck, A. J., Van den Berghe, D. A., Totte, J. (1982). Plant antiviral agents. Isolation of alkaloids from *Clivia miniata* Regel (Amaryllidaceae). Journal of Natural Products. 45, 564–573.

Joshi, D., O'Grady, J., Dieterich, D., Gazzard, B., and Agarwal, K. (2011). Increasing burden of liver disease in patients with HIV infection. Lancet 377, 1198-1209.

Kessler, H., Gehrke, M., and Griesinger, C. (1988). Two-dimensional NMR spectroscopy, principles and survey of the experiments. Ang. Chem. 100(4), 507-54.

Kirkiacharian, S., Tongo, H. G., Bastide, J., Bastide, P., Grenie, M. M. (1989). Synthese et activites angioprotectrice, anti-allergique et antihistaminique de benzyl-3 chromones (homo-isoflavones). Eur. J. Med. Chem, 24, 541–546.

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

Koch, M. A., Schuffenhauer, A., Scheck, M., Wetzel, S., Casaulta, M., Odermatt, A., Ertl, P. and Waldmann, H. (2005). Charting biologically relevant chemical space: a structural classification of natural products (SCONP). Proc Natl Acad Sci U S A, 102:17272-17277.

Laban, L. T., Anjili, C. O., Mutiso, J. M., Ingonga, J., Kiige, S. G., Ngedzo, M. M and Gicheru, M. M. (2015). Experimental therapeutic studies of *Solanum aculeastrum* Dunal. on Leishmania major infection in BALB/c mice. Iran J Basic Med Sci, 18, 64-71.

Landau N. R, Page K. A, Littman D. R. (1991). Pseudotyping with human T-cell leukemia virus type-I broadens the human immunodeficiency virus host range. J Virol 65,162-169.

Lapornik B., Prosek M and Wondra, A.G. (2005). Comparison of extracts prepared from plant by-products using different solvents and extraction time. Journal of Food Engineering. 71: 214-222.

Lapornik, B., Prosek, M. and Wodra, A.G. (2005). Comparison of extracts prepared from plant by-products using different solvents and extraction time. Food Engineering, 71, 214-222.

Lever, M., L. (2009). HIV: the virus. Medicine, 37(7), 313-316.

Li, M. F., Gao, J. R. Mascola, L. Stamatatos, V. R. Polonis, M. Koutsoukos, G. Voss, P. Goepfert, P. Gilbert, K. M. Greene, M. Bilska, D. L. Kothe, J. F. Salazar-Gonzalez, X. Wei, J. M. Decker, B. H. Hahn, and D. C. Montefiori. (2005) Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J. Virol, 79, 10108-10125.

Li, S. Y., Chen, C., Zhang, H. Q., Guo, H. Y., Wang, H., Wang, L., Zhang, X., Hua, S. N., Yu, J., Xiao, P. G., Li, R. S., Tan, X. (2005). Antiviral Res. 67, 18.

Lin, L. G., Xie, H., Li, H. L., Tong, L. J., Tang, C. P., Ke, C. Q., Liu, Q. F., Lin, L. P., Geng, M. Y., Jiang, H., Zhao, W. M.; Ding, J., Ye, Y. (2008). Naturally occurring

homoisoflavonoids function as potent protein tyrosine kinase inhibitors by c-Src-based high-throughput screening. J. Med. Chem, 51, 4419–4429.

Maartens, G., Celum, C., and Lewin, S. R. (2014). HIV infection: epidemiology, pathogenesis, treatment, and prevention. Lancet 384, 258-271.

Madani, N., and Kabat, D. (2000). Cellular and viral specificities of human immunodeficiency virus type 1 vif protein. Journal of virology 74, 5982-5987.

Marston, A., Hostettmann, K. (1985). Plant molluscicides. Phytochemistry, 24, 639–652.

Miadokova, E., Masterova, I., Vlckova, V., Duhova, V., Toth, J. (2002). Antimutagenic potential of homoisoflavonoids from Muscari racemosum. J. Ethnopharmacol., 81, 381–386.

Modi, M., Vinu, N., Pancholi, B., Kulshrestha, S., Rawat, A, K. (2013). Anti-HIV-1 activity, protease inhibition and safety profile of extracts prepared from *Rhus parviflora*. BMC Complement Altern Med. 13, 1-9.

Monajemi, M., Woodworth, C. F., Benkaroun, J., Grant, M., and Larijani, M. (2012). Emerging complexities of APOBEC3G action on immunity and viral fitness during HIV infection and treatment. Retrovirology 9, 35.

Moodley, N., 2004. PhD Thesis. The chemical investigation of the amaryllidaceae and hyacinthaceae.University of Natal, South Africa.

Mulholland, D. A., Schwikkard, S. L., Crouch, N. R. (2013). The chemistry and biological activity of the Hyacinthaceae. Nat. Prod. Rep, 30, 1165–1210.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M, and Trono, D. (1996). *In vivo* gene delivery and stable transduction of non-dividing cells by a lentiviral vector. Science, 272, 263–267.

Naldini, L., U. Bloemer, P. Gallay, D. Ory, R. Mulligan, F. H. Gage, I. M. Verma, and D. Trono. 1996. *In vivo* gene delivery and stable transduction of non-dividing cells by a lentiviral vector. Science 272:263–267.

Ndung'u, T., Renjifo, B. and Essex, M. (2001). Construction and analysis of an infectious human immunodeficiency virus type 1 subtype C molecular clone. J Virol 75(11), 4964-4972.

Neuwinger, H. D. (1994) African Ethnobotany: Poisons and Drugs Chemistry Pharmacology Toxicology. Chapman & Hall. Federal Republic of Germany.

Newman, D. J, and Cragg, G. M. (2007). Natural products as sources of new drugs over the last 25 years. Journal of Natural Products, 70:461-477.

Papathanasopoulos, M. A., Cilliers, T., Morris, L., Mokili, J. L., Dowling, W., Birx, D. L and McCutchan, F. (2002). Full-Length Genome Analysis of HIV-1 Subtype C Utilizing CXCR4 and Intersubtype Recombinants Isolated in South Africa. AIDS Research and Human Retroviruses. 18, 879-886.

Pavia, D. L., Lampman, G. M., Kriz, G. S. and Vyvyan, J. R. (2009). Introduction to spectroscopy. Fourth edition. Brooks/Cole. USA.

Perjesi, P., Das, U., De Clercq, E., Balzarini, J., Kawase, M., Sakagami, H., Stables, J. P., Lorand, T., Rozmer, Z., Dimmock, J. R. (2008). Design, synthesis and antiproliferative activity of some 3-benzylidene- 2,3-dihydro-1-benzopyran-4-ones which display selective toxicity for malignant cells. Eur. J. Med. Chem., 43, 839–845.

Pretsch, E. and Clerc, J. T. (1997). Spectra Interpretation of Organic Compounds, Weinheim, VCH. London.

Quaglia, M. G., Desideri, N., Bossu, E., Sgro, R., Conti, C., (1999). Enatioseparation and anti-rhinovirus activity of 3-benzylchroman-4-ones. Chirality 11, 495–500.

Raskin, I., Ribnicky, D. M., Komarnytsky, S., Ilic, N., Poulev, A., Borisjuk, N., Brinker, A., Moreno, D. A., Ripoll, C., Yakoby, N., O' Neal, J. M., Cornwell, T., Pastor, I., Fridlender, B. (2002) Plants and human health in the twenty-first century. Trends in Biotechnology 20:522-531.

Rege, A. A., Ambaye, R. Y. and Deshmukh, R. A. (2010). In-vitro testing anti-HIV activity of some medicinal plants. Ind. J. Natl. Prod. Res. 1(2), 193–199.

Roy, S. K., Kumari, N., Gupta, S., Pahwa, S., Nandanwar, H., Jachak, S. M. (2013). 7-Hydroxy-(E)-3-phenylmethylene-chroman-4-one analogues as efflux pump inhibitors against Mycobacterium smegmatis mc2155, Eur. J. Med. Chem. 66, 499-507.

Saksena, N. K., and Potter, S. J. (2003). Reservoirs of HIV-1 *in vivo*: implications for antiretroviral therapy. AIDS reviews 5, 3-18.

Sanders, J. K.M, and Hunter, B. K. (1993). Modern NMR Spectroscopy, 2nd. Edition, Oxford University Press, Oxford.

Sauter, D., Hue, S., Petit, S. J., Plantier, J. C., Towers, G. J., Kirchhoff, F., and Gupta, R. K. (2011). HIV-1 Group P is unable to antagonize human tetherin by Vpu, Env or Nef. Retrovirology 8, 103.

Shankar, E. M., Velu, V., Vignesh, R., Vijayaraghavalu, S., Rukumani, D.V., and Sabet, N. S. (2012). Recent advances targeting innate immunity-mediated therapies against HIV-1 infection. Microbiology and Immunology 56, 497-505.

Sharp, P. M., and Hahn, B. H. (2011). Origins of HIV and the AIDS pandemic. Cold Spring Harbor perspectives in medicine.1, 1-23.

Shi, J., Zhou, J., Shah, V. B., Aiken, C., and Whitby, K. (2011). Small-molecule inhibition of human immunodeficiency virus type 1 infection by virus capsid destabilization. Journal of Virology 85, 542-549.

Siddaiah, V., Maheswara, M., Rao, C. V., Venkateswarlu, S., Subbaraju, G. V. (2007). Synthesis, structural revision, and antioxidant activities of antimutagenic homoisoflavonoids from Hoffmanosseggia intricata. Bioorg. Med. Chem. Lett, 17, 1288–1290.

Siddaiah, V., Rao, C. V., Venkateswarlu, S., Krishnaraju, A. V., Subbaraju, G. V. (2006). Synthesis, stereochemical assignments, and biological activities of homoisoflavonoids. Bioorg. Med. Chem, 14, 2545–2551.

Sidwell, W. T., Tamm C., (1970). The homo-isoflavones 111). Isolation and structure of 4'O-methyl-punctatin, autumnalin and 3,9-dihydro-autumnalin. Tetrahedron Letters 7, 475-478.

Silverstein, R. M., Webster, F. X. and Kiemle, D. J. (2005). Spectrometric identification of organic compounds. 7th edition, John Wiley and sons Inc, USA.

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

Snyder, L. R. and Kirkland, J. J. (1979). Introduction to modern liquid chromatography. Wiley, New York.

Sonnenberg, P., Glynn, J. R., Fielding, K., Murray, J., Godfrey-Faussett, P., and Shearer, S. (2005). How soon after infection with HIV does the risk of tuberculosis start to increase? A retrospective cohort study in South African gold miners. The Journal of Infectious Diseases 191, 150-158.

Sumner, L. W., Mendes, P., Dixon, R. A. (2003). Plant metabolomics: Large-scale phytochemistry in the functional genomics era. Phytochemistry, 62, 817–836.

Swerdlow, J. (2000). Nature's Medicine. Plants That Heal. National Geographic Society. USA.

Szlávik, L., Gyuris, A., Minárovits, J., Forgo, P., Molnár, J., Hohmann, J. (2004). Alkaloids from Leucojum vernum and Antiretroviral Activity of Amaryllidaceae Alkaloids. Planta Med. 70(9), 871-873.

Tait, S., Salvati, A. L., Desideri, N., Fiore, L. (2006). Antiviral activity of substituted homoisoflavonoids on enteroviruses. Antiviral Res, 72 (3), 252–255.

Usubillaga, A., Aziz, I., Tettmanzi, M. C., Waibel, R and Achenbach, H. (1997). Steroidal alkaloids from Solanum sycophanta. Phytochemistry, 44, 537-543.

van Harmelen, J., Williamson, C., Kim, B., Morris, L., Carr, J., Karim, S.S and McCutchan, F. (2001). Characterization of full-length HIV type 1 subtype C sequences from South Africa. AIDS Research and Human retroviruses,17,1527-1531.

Van Wyk, B., Wink, M. (2004) Medicinal Plants of the World. 1st edition. Briza Publications. Pretoria.

Van Wyk, B. E., Van Oudtshoorn, B., Gericke, N. (1997). Medicinal plants of South Africa. Briza Publications. Pretoria, pp 7-22, 24, 130-131, 150-151.

Vermani, K., and Garg, S. (2002). Herbal medicines for sexually transmitted diseases and AIDS. Journal of ethnopharmacology 80, 49-66.

Walker, B. R., Colledge, N. R., Ralston, S.H., Penman, I. (2014). Davidson's Principles and Practice of Medicine, 22nd edn. London, UK: Churchill Livingstone.

Wall, M. E., Wani,M. C., Manikumar, G., Taylor, H., McGivney, R. (1989). Plant antimutagens, 6. intricatin and intricatinol, new antimutagenic homoisoflavonoids from hoffmanosseggia intricata. J. Nat. Prod, 52, 774–778.

Wang, G. X. (2010). *In vivo* anthelmintic activity of five alkaloids from Macleaya microcarpa against Dacylogyrus intermedius in Carassius auratus. Veterinary Parasitology, 171, 305-313.

Wanyonyi, A. W., Chhabra, S. C., Mkoji, G., Udo, E and Njue, W. N. (2002). Bioactive steroidal alkaloid glycosides from *Solanum aculeastrum*. Phytochemistry, 59, 79-84.

WHO (World Health Organization). (2011). The World Traditional Medicines Situation, in Traditional medicines: Global Situation, Issues and Challenges. Geneva 3,1–14.

Yang, S. S., Cragg, G. M., Newman, D. J., Bader, J. P. (2001). Natural product-based anti-HIV drug discovery and development facilitated by the NCI developmental therapeutics program. Journal of Natural Products 64, 265-277.

Young, C. K., Kingstone, D. G., Quing-Ming, C., Leslie, G. (1996). Bioactive steroidal alkaloids from Solanum umbelliferum. Journal of Natural Products, 59, 283–285.

Zaidi, J., Grapsa, E., Tanser, F., Newell, M. L., and Barnighausen, T. (2013). Dramatic increase in HIV prevalence after scale-up of antiretroviral treatment. AIDS 27, 2301-2305.

Zhang and Wilkinson. (2007). Drug discovery beyond the 'rule-of-five' Current Opinion in Biotechnology, 18:478–488.