Lamiaceae plant extracts and isolated compounds demonstrate activity against HIV/AIDS

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s11124416

Submitted in partial fulfillment of the degree

***Philosophiae Doctor* Biochemistry**

In the Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria

13th December 2013

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DEDICATION

To my parents, Reverends' Josef and Aino Kapewangolo, and my siblings (Ndapanda, Aino, Homateni and Nangula) for being my rock throughout the years and for cheering me on to succeed. This journey could have not been possible without your unconditional love and support. This is for us.

Family portrait (1988)

ACKNOWLEDGEMENTS

My deepest gratitude goes out to God almighty for all His blessings. My sincere appreciation and gratitude goes out to the following people and institutions for their contribution in the course of the study:

- Prof. Debra Meyer, my main supervisor, thank you for all your guidance and mentorship throughout this project.
- Dr. Martha Kandawa-Schulz, my co-supervisor, for all the encouragement, guidance and for always believing in me.
- My colleagues and friends at the department of Biochemistry at the University of Pretoria: thank you for your advice, assistance, support, friendship and for creating for me a home away from home.
- The HIV research team at the University of Pretoria. Words cannot express my gratitude and love for each one of you.
- Prof. Ahmed A. Hussein for his guidance during initial extraction and purification work.
- Special thanks to Prof. Charles de Koning at the School of Chemistry, University of the Witwatersrand for providing assistance with NMR analysis.
- Dr. Justin Omolo at the department of Traditional Medicine, National Institute for Medical Research, Tanzania for assistance and guidance with NMR spectra interpretation.
- The staff at the University of Botswana (UB), Chemistry department for the use of their facilities for some of my purification work. Special thanks to Dr. Kenmogne Marguerite and Mr Chi Fru from the University of Yaounde, Cameroon (who were visiting UB at that time) for their guidance with column chromatography.
- Special thanks to Dr. Maya M. Makatini at MINTEK, South Africa: for not only assisting with purification and data interpretation but also providing great advice.
- To my family, thank you for all the love, motivation and endless support.
- Southern African Biochemistry & Informatics for Natural Products (SABINA), my primary funders.
- Ministry of Education, Namibia; for extra research funding.
- The Margaret McNamara Memorial Fund (MMMF) for the 2012 fellowship.

PREFACE

A section of the research presented in this thesis has been published in a peer-reviewed journal. Additional manuscripts have been submitted for publication and are listed at the end of this section. Portions of this research have also been presented at national and international conferences.

Publication

- Kapewangolo, P., Hussein, A.A., Meyer, D., 2013. Inhibition of HIV-1 enzymes, antioxidant and anti-inflammatory activities of *Plectranthus barbatus*. *Journal of Ethnopharmacology*. **149**: 184-190.

Awards

The following awards were received by Kapewangolo P. in the course of this study:

- Southern African Biochemistry and Informatics for Natural Products (SABINA); PhD Scholarship for study at the University of Pretoria (14 September 2009).

- In September 2010, additional funding for running and subsistence from the Namibia Government and Training Program (NGSTP).

- Recipient of Margaret McNamara Memorial Fund (MMMF) was awarded on 9 February 2012.

Conferences

- Kapewangolo P. and Meyer D. 2012. Poster presentation entitled: Anti-HIV-1 and inflammatory properties of selected Lamiaceae plants. First Pan-African Summer School in Nanomedicine (4-10th November 2012, Pretoria, South Africa).

- Kapewangolo P. and Meyer D. 2012. Poster presentation entitled: HIV-1 inhibitory and anti-inflammatory properties of South African Lamiaceae species. 8th Conference of the Federation of African Immunological Societies (2-5th December 2012, Durban, South Africa).

- Kapewangolo P. and Meyer D. 2013. Poster presentation: HIV-1 inhibitory and anti-inflammatory properties of selected Lamiaceae plants. 7th Regional Plant Biotech Forum (9th April 2013, Pretoria, South Africa).

- Kapewangolo P., Omolo J.J. and Meyer D. 2013. Poster presentation: Novel triterpenes activate latent human immunodeficiency virus expression. What Will it Take to Achieve an AIDS-free World? (3-5th November 2013, San Francisco, California, USA).

Manuscripts submitted for publication:

- Kapewangolo P., Kandawa-Schulz M., Meyer D. (2013). Anti-HIV activity of *Ocimum labiatum* extract and isolated pheophytin-a. Submitted to *Phytomedicine*.

- Kapewangolo P., Omolo J.J., Kandawa-Schulz M., Meyer, D. (2013). Triterpenoids from *Ocimum labiatum* activates latent HIV-1 expression: potential for use in adjuvant therapy. Submitted to *Antiviral Research*.

- Kapewangolo P., Omolo J.J., Meyer D. (2013). *Ocimum labiatum* demonstrates anti-inflammatory and antioxidant activities. Submitted to *BMC Journal of Inflammation*.

SUMMARY

Lamiaceae plant extracts and isolated compounds demonstrate activity against HIV/AIDS

by

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Co-supervisor: **Dr. Martha Kandawa-Schulz (University of Namibia)**

Department: **Biochemistry**

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Background: HIV/AIDS remains a major health concern worldwide and the number of people infected in Sub-Saharan Africa continues to increase. This despite increased awareness and availability of HIV drugs in most countries. The success of current HIV-1 drugs is overshadowed by the emergence of drug resistant viral strains and the adverse side-effects they may cause. It is these limitations and many more that drives the continuous search for better HIV treatments. Research into drug discovery and development using natural products is becoming better established. With natural products, there are endless opportunities for discovering novel compounds which either ends up as final drugs or as backbones of drug leads.

Methods: In this thesis, sixteen Lamiaceae (mint) plants were investigated for inhibitory properties against HIV-1 as well as for beneficial immune enhancing effects. This family of plants is commonly used in traditional medicine preparations for the treatment of various ailments including those that are virus induced.

Cytotoxicity of the plant material was determined using tetrazolium dyes and the results subsequently confirmed with flow cytometry and real-time cell analysis. Direct enzyme assays were used to determine the inhibitory properties of the extracts and isolated compounds against HIV-1 protease (PR), reverse transcriptase (RT) and integrase (IN). The effect of the plant materials was also evaluated in an *in vitro* model of chronic and latent infection by measuring HIV-1 p24 protein secretion of an infected cell line (U1) following treatment. Most HIV-infected individuals only seek treatment during the chronic stages of disease and latent reservoirs of the virus perpetuate treatment. The

immune modulating properties were determined by quantitating the effects of plant extracts/compounds on Th1/2/17 cytokine production in human mononuclear cells using the cytometric bead array technology. Finally, anti-oxidant and anti-inflammatory properties were also assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide colorimetric assays respectively.

Results and discussion: The 50% cytotoxic concentration (CC_{50}) of the extracts was between 4.2 and 100 µg/ml. Of the sixteen, extracts from six plants (*Ocimum labiatum*, *Ocimum serratum*, *Plectranthus barbatus*, *Plectranthus neochilus*, *Salvia apiana* and *Stachys byzantina*) were active against HIV-1. Four plants (*P. neochilus*, *O. serratum*, *S. apiana* and *S. byzantina*) demonstrated moderate inhibitory properties against HIV-1 PR, RT and IN (40-49%) and three of these plants (*O. serratum, S. apiana and S. byzantina*) significantly ($p<0.05$) suppressed HIV-1 replication in U1 cells. The most exciting data was obtained from extracts of *P. barbatus* and *O. labiatum* which demonstrated inhibition classified as good (>50%) against HIV-1 PR (IC_{50}s 62 ±0.2 and 49.8 ±0.4 µg/ml), reduced the production of pro-inflammatory cytokines at non-cytotoxic concentrations and demonstrated strong antioxidant properties (IC_{50} values 13 ±0.8 and 15.8 ±0.3 µg/ml). *O. labiatum* extract also suppressed HIV-1 expression in U1 cells, significantly ($p<0.05$). In addition, one of the extracts (*P. ciliatus*) had anti-cancer potential with CC_{50} values <10 µg/ml.

O. labiatum extract was purified to yield a chlorophyll derivative, pheophytin-a (phy-a); triterpene isomers (3-hydroxy-4,6a,6b,11,12,14b-hexamethyl-1,2,3,4,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydropicene-4,8a-dicarboxylic acid), amyrin and a labdane diterpenoid (labda-8(17),12*E*,14-triene-2R,18-diol). Phy-a inhibited HIV-1 PR with an IC_{50} value of 44.4 ±1.5 µg/ml. The triterpenes activated latent HIV-1 (a serious obstacle in the eradication of the virus) while the diterpenoid reduced the production of pro-inflammatory cytokines. These activities were observed at non-toxic concentrations of these compounds. There is an ongoing search for novel compounds that are able to activate latent HIV-1 to use in conjunction with HAART. If the triterpenes were to progress to clinical use, their use would be in activating latent virus for eradication by existing treatments.

Conclusion: The findings presented in this thesis provide some scientific explanation for the anecdotal success of some Lamiaceae plants used traditionally to manage

HIV/AIDS. The findings also conform to recommendations by the scientific community regarding the validation of the beneficial effects of plant products used traditionally.

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LIST OF IMPORTANT ABBREVIATIONS

AAA	Abdominal aortic aneurysms
AIDS	Acquired immune deficiency syndrome
ARV	Antiretroviral
AZT	2,2'-Azinobis (3-ethylbenzthiazoline sulfonic acid)
CC_{50}	50% cytotoxic concentration
CFSE	Carboxylfluorecein succinimidyl ester
CI	Cell Index
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
ELISA	Enzyme linked immunosorbent assay
FDA	Food and drug administration
HAART	Highly active antiretroviral therapy
HDAC	Histone deacetylases
HIV-1	Human immunodeficiency virus type 1
HPLC	High performance liquid chromatography
IC_{50}	50% inhibitory concentration
IL	Interleukin
IFN-γ	Interferon gamma
IN	Integrase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide
NF-κβ	Nuclear factor kappa beta
NMR	Nuclear magnetic resonance
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleos(t)ide reverse transcriptase inhibitors
PBMCs	Peripheral blood mononuclear cells
PKC	Proteinase kinase C
PMA	Phorbol 12-myristate 13-acetate
POD	Peroxidase
PR	Protease
RA	Rheumatoid arthritis

RT	Reverse transcriptase
RT-CES	Real time cell electronic sensing
TLC	Thin-layer chromatography
TNF	Tumor necrosis factor

CHAPTER 1

INTRODUCTION

The human immunodeficiency virus (HIV) is the causative agent of the acquired immunodeficiency syndrome (AIDS), a degenerative disease of the immune system that results in life-threatening opportunistic infections and malignancies (UNAIDS, 2013). The number of people living with HIV worldwide reached an estimated 35.3 million by 2012 with 23.5 million of infected people residing in Sub-Saharan Africa (UNAIDS, 2013). In 2012, the number of new HIV infections worldwide was 2.3 million and an estimated 70% of the new HIV infections were recorded in Sub-Saharan Africa. This made Africa the continent with the highest number of HIV infections worldwide (UNAIDS, 2013).

Existing antiretroviral (ARV) drugs when used in combinations (called HAART) control the progression and replication of HIV resulting in prolonged patient survival. These benefits are however compromised by the fact that ARVs do not completely eradicate the virus and this leads to the development of drug resistant viral strains as a consequence of the mutations that emerges in the viral proteins targeted by antiretroviral agents (Clavel and Hance, 2004; Palmer et al., 2011). HIV patients sometimes discontinue the use of ARV drugs due to liver toxicity (which is one of the side effects reported for some drugs e.g. nevirapine). The shortcomings of existing drugs provide the rationale for continued research into the discovery of new HIV drugs with less or no side effects (Colafigli et al., 2009).

Plants have a long history in the treatment of various disease or symptoms. Medicinal plants are readily available and plant-based products have been known to have minor or no side effects making them safer options as medicinal drugs (Itokawa et al., 2008).

Although the use of bioactive natural products as herbal drug preparations dates back hundreds, even thousands of years especially in African and Asian communities, the isolation of their bioactive compounds for modern drug discovery and development only started in the 19th century (Choudhary and Khan, 2011; Liang and Fang, 2006). In recent years the use of herbs for treatment has increased noticeably as a result of continued funding of this kind of research and more recognition of traditional medicines

in formal medicine. Plant based substances form part of many medicines and there are about 60% of natural products used as drugs worldwide (Newman and Cragg, 2012). It has been well documented that natural products played critical roles in modern drug development, especially for the formulation of antibacterial, anti-viral and antitumor agents. Naturally occurring pure compounds have become medicines, dietary supplements, and other useful commercial products (Itokawa et al., 2008; Liang and Fang, 2006; Vermaak et al., 2010).

Natural products with antiviral and immunomodulating effects are viewed as possible sources of new compounds to inhibit HIV and treat AIDS (Itokawa et al., 2008). Plant constituents have been reported to inhibit HIV at nanomolar concentrations (César et al., 2011; Geuenich et al., 2008; Prinsloo et al., 2010; Stoddart et al., 2007). In this study, selected plants from the Lamiaceae family were explored for anti-HIV and immune system supporting properties.

Lamiaceae is a plant family, commonly known as mints, with 980 species distributed in Southern Africa alone (Klopper et al., 2006). Plants belonging to the Lamiaceae family are used traditionally to treat viral and microbial ailments (Van Wyk et al., 2009) even in the absence of scientific data on efficacy. Surveys carried out in Uganda, Tanzania and Namibia on the use of medicinal plants for HIV/AIDS treatment revealed that Lamiaceae plants, especially those from the genera *Ocimum*, *Plectranthus* and *Leonotis*, are used traditionally in managing HIV/AIDS and opportunistic infections associated with the disease. Leaves from these plants are commonly administered orally by traditional medicine practitioners as decoctions or infusions for the treatment of HIV/AIDS, tuberculosis, oral candidiasis, herpes simplex etc. (Chinsembu and Hedimbi, 2010; Kisangau et al., 2007; Lamorde et al., 2010). Based on this information, the present study was aimed at screening sixteen plants from the Lamiaceae family for scientific evidence supporting traditional success. Previous *in vitro* studies have demonstrated extracts of some Lamiaceae plants, for example peppermint, sage and lemon balm as possessing HIV-1 inhibitory properties (Amzazi et al., 2003; Geuenich et al., 2008; Klos et al., 2009). However, the specific anti-HIV components from these plants have not been identified yet. The present study was designed to not only identify anti-HIV extracts but to also isolate and identify constituents from bioactive extracts.

The tendency of Lamiaceae plants to be successful as traditional medicines may be due to the ability of the extracts to enhance the immune system of infected individuals. HIV suppresses the immune system by regulating a number of factors including cytokines (Ayyavoo et al., 1997). Compounds that restore immune system function should therefore prove detrimental to HIV infection. Testing plant extracts and isolated compounds for immunomodulatory properties should provide insight into the ability of these plants to either enhance or suppress the immune system.

The next chapter (2) provides a literature review of topics relevant to this study. The hypothesis, purpose of the study, specific aims and screening strategy are provided at the end of chapter 2. The specific aims of the study are addressed in five experimental chapters. Chapters 3-5 present data on the effect of selected Lamiaceae extracts on HIV. An HIV inhibiting plant constituent was isolated from *O. labiatum* and is explained in chapter 5. In chapter 6 the investigation of the effect of isolated triterpenes on latent HIV-1 is presented. Inflammation and immunomodulating properties of an extract and isolated diterpenoid are explained in chapter 7. The experimental chapters are followed by an overall discussion of the findings, significance and limitations of the project as well as future recommendations (chapter 8). A comprehensive list of references is provided in chapter 9. Supplementary data and a published manuscript on this work are provided in the appendix (chapter 10).

CHAPTER 2

LITERATURE REVIEW

2.1 HIV/AIDS drug development

Since the discovery of the first ARV drug, azidothymidine (AZT), HIV infection has become a manageable chronic infection with improved life expectancy and quality (Esté and Cihlar, 2010). This can be said with confidence for developed countries but challenges remain in most developing countries due to limited resources amongst other obstacles (Esté and Cihlar, 2010). In some parts of the world, HIV trends are still cause for concern (Figure 2.1).

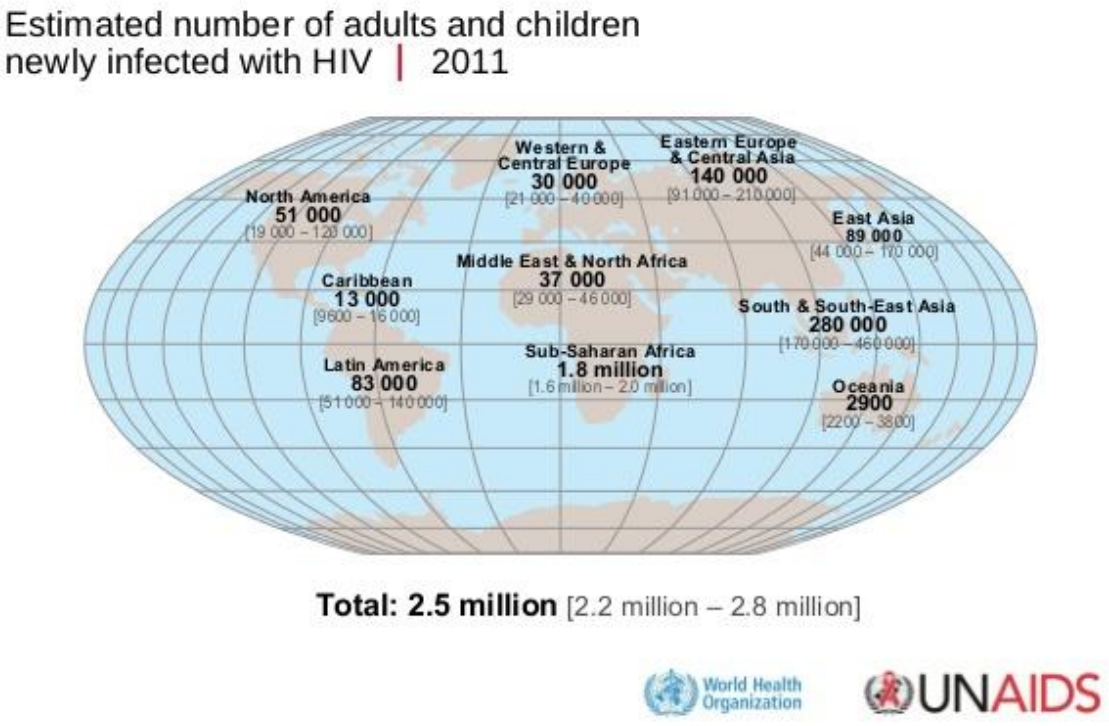

Figure 2.1: A global view of adults and children newly infected with HIV in 2011 (UNAIDS, 2012)**.**

Since 2001, the number of people newly infected in Africa has increased by 35% (UNAIDS, 2012). The incidence of HIV infection in Europe and Asia began increasing in the late 2000s after having remained relatively stable for several years (UNAIDS, 2012) Despite the introduction and successes of highly antiretroviral therapy (HAART), the number of new infections continues to rise worldwide (UNAIDS, 2012) and this is mainly

due to the emergence of multidrug resistant HIV strains and their transmission, which render existing drugs ineffective (Esté and Cihlar, 2010). Drug discovery for HIV targets different steps in the replication cycle of the virus (Figure 2.2). The current study will be looking for new RT and PR inhibitors which also have other activities (e.g. immune supportive and less toxic).

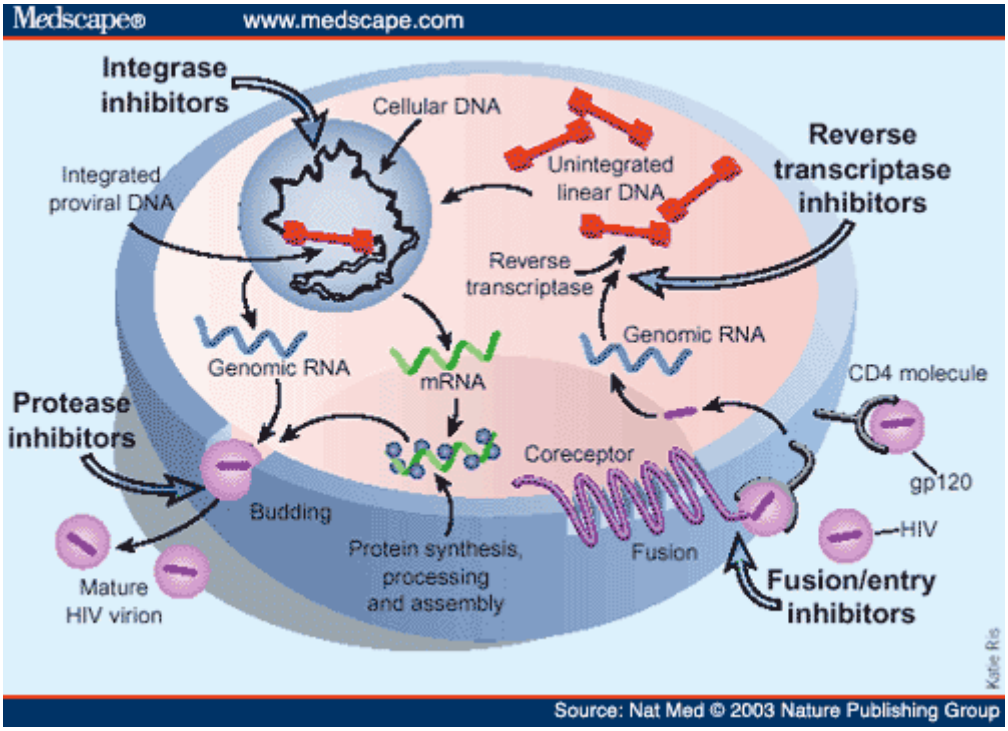

Figure 2.2: HIV life cycle and targets for antiretroviral therapy (Fauci, 2003)**.**

The mechanism of action of AZT, the first drug discovered to block HIV replication in cell culture, is through selective inhibition of HIV-1 reverse transcriptase (RT) by the enzyme's triphosphate metabolite (Cihlar and Ray, 2010). The demonstration of AZT's activity led to the establishment of the first class of ARV drugs known as nucleoside and nucleotide RT inhibitors (NRTIs) (Cihlar and Ray, 2010). In total, thirteen NRTIs are currently available for clinical application and more are being developed or being identified through drug discovery efforts (Cihlar and Ray, 2010). Long-term NRTI therapy has reported limitations with the most common being drug-drug interactions and emergence of drug resistance strains (Cihlar and Ray, 2010).

Non-nucleoside RT inhibitors (NNRTIs) is another class of ARV drugs that was identified after NRTIs. This class of drugs target HIV-1 RT using a different mechanism

which is through interaction with the enzyme and in the process induce conformational changes that prevents the catalytic activities of HIV-1 RT (de Béthune, 2010). NNRTIs are specific for HIV-1 which makes them selective drugs of the virus (de Béthune, 2010). Nevirapine and efavirenz were the first NNRTI generation and further research has led to the discovery and development of more NNRTIs (de Béthune, 2010). HIV-1 resistance with NNRTIs has been reported and this is mainly due to the emergence of drug resistant viruses which compromise the efficacy of the drugs (de Béthune, 2010).

After the production of the viral DNA copy from HIV-1 RNA by HIV-1 RT, the DNA copy is integrated into the host genome by HIV-1 integrase (IN) and this process forms a functional integrated proviral DNA (McColl and Chen, 2010). The discovery of IN inhibitors was not a straight path; raltegravir, the first inhibitor was only approved in late 2007 and was incorporated into the existing ARV treatment (McColl and Chen, 2010). The second IN drug, elvitegravir, was approved in August 2012 and is co-formulated with other drugs in a single tablet called Stribild, an initial treatment for HIV infection (Sax et al., 2012). Resistance to both raltegravir and elvitegravir was reported earlier on during *in vitro* studies and early clinical trials. Multiple IN mutations, resistant to both drugs, have been reported and are a major contributor to the inefficacy of IN drugs (McColl and Chen, 2010).

HIV-1 protease (PR) is another of the three essential enzymes (along with RT and IN) encoded by the viral gene and it is responsible for the production of mature infectious virus particles (Wensing et al., 2010). The development and design of specific HIV PR inhibitors was through understanding the structure of this enzyme (Wensing et al., 2010). It was the development of these inhibitors that resulted in the evolutionary triple combination therapy, HAART (Wensing et al., 2010). Drug resistance for all PR inhibitors has been reported and the limitations of these drugs were mostly due to low bioavailability and high pill burdens which resulted in reduced adherence (Wensing et al., 2010). When therapy was interrupted or failed, multiple PR resistance mutations occurred but advancements are being made with existing drugs by boosting with another drug called ritonavir in order to improve bioavailability (Wensing et al., 2010). Development of new inhibitors with better potency is also in the pipeline.

Setbacks of HIV-1 treatment that have been accumulating over the past years have forced the serious consideration of novel approaches of inhibiting HIV-1 replication (Adamson and Freed, 2010). These include looking into RNase H activity of the viral reverse transcriptase, uncoating of the viral core and integration of the viral DNA into host cell chromatin, virus assembly, maturation, viral budding and the various functions of the viral proteins (Adamson and Freed, 2010). Developing inhibitors targeting some of these highlighted steps of the HIV life cycle has shown some progress.

HIV entry into target cells is a complex process with multiple stages involving attachment to host cells, binding to receptors and membrane fusion (Tilton and Doms, 2010). Drugs that prevent these steps from happening are collectively called entry inhibitors even though they exhibit different mechanisms of action (Tilton and Doms, 2010). Maraviroc and enfuvirtide are two entry inhibitors approved for treatment in HIV-1 infection and a number of drugs are in development to add on to existing entry inhibitors (Tilton and Doms, 2010).

HIV/AIDS patients worldwide depend on the use of antiretroviral therapy to improve their life span and quality of life (Wang et al., 2008). However, the emergence of HIV strains resistant to the current drugs and the fact that ARVs do not eradicate HIV from patients completely, reduce the success rate of these drugs (Lipsky, 1996). Drug resistant HIV can either be caused during the normal viral reverse transcription phase by base substitution or during treatment if the drugs that are being used are unable to block viral replication which results in HIV variants with decreased drug susceptibility (Clavel and Hance, 2004). The high cost and toxicity of ARVs makes it difficult to maintain patients' loyalty to the drugs as well (Sliciano et al., 2003).

Alternative anti-HIV drugs with diverse mechanisms of inhibiting the virus is thus needed and should preferably involve new chemical entities.

A list of existing HIV treatments and the side-effects associated with each drug is provided in Table 2.1. A few of these drugs (hivid, agenerase and fortovase) have been discontinued due to fatal adverse reactions (http://www.fda.gov).

Table 2.1: FDA approved ARV drugs used in the treatment of HIV infection and their potential side-effects*.

Multi-class Combination products				
Brand Name	**Generic Name**	**Dosage and Administration‡**	**Adverse Reactions**	**Approval Date**
Atripla	efavirenz, emtricitabine and tenofovir disoproxil fumarate	One tablet once daily taken orally on an empty stomach.	Lactic acidosis/severe hepatomegaly with steatosis, post treatment exacerbation of hepatitis B, diarrhoea, nausea, fatigue, headache, dizziness, depression, insomnia, abnormal dreams, and rash	12-Jul-06
Complera	emtricitabine, rilpivirine, and tenofovir disoproxil fumarate	One tablet taken once daily with a meal.	Lactic acidosis/severe hepatomegaly with steatosis, post treatment acute exacerbation of hepatitis B, diarrhoea, nausea, fatigue, headache, dizziness, depression, insomnia, abnormal dreams, and rash	10-Aug-11
Stribild	elvitegravir, cobicistat, emtricitabine, tenofovir disoproxil fumarate	One tablet taken once daily with food.	Renal failure, lactic acidosis/severe hepatomegaly with steatosis, post treatment acute exacerbation of hepatitis B, nausea and diarrhoea	27-Aug-12
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)				
Brand Name	**Generic Name**	**Dosage and Administration‡**	**Adverse Reactions**	**Approval Date**
Combivir	lamivudine and zidovudine	One tablet twice daily	Hematologic toxicity, myopathy, lactic acidosis, exacerbations of hepatitis b, headache, nausea, malaise and fatigue, nasal signs and symptoms, diarrhoea, and cough	27-Sep-97
Emtriva[S*]	emtricitabine, FTC	One 200 mg capsule or 24 mL oral solution administered once daily	Lactic acidosis/severe hepatomegaly with steatosis, post treatment exacerbation of hepatitis B, headache, diarrhoea, nausea, fatigue, dizziness, depression, insomnia, abnormal, dreams, rash, abdominal pain, asthenia, increased cough, and rhinitis, skin hyperpigmentation	02-Jul-03

Epivir [S*]	lamivudine, 3TC	Dose of 300 mg/day oral solution administered with or without food	Lactic acidosis, post treatment exacerbations of hepatitis b in co-infected patients, headache, nausea, malaise, fatigue, nasal signs and symptoms, diarrhoea, fever and cough	17-Nov-95
Epzicom	abacavir and lamivudine	One tablet daily	Drug hypersensitivity, lactic acidosis, severe hepatomegaly, exacerbations of hepatitis, insomnia, depression/depressed mood, headache/migraine, fatigue/malaise, dizziness/vertigo, nausea, and diarrhoea.	02-Aug-04
Hivid [S*]	zalcitabine, dideoxycytidine, ddC (no longer marketed)	Tablet and solution discontinued	Clinical adverse reactions, severe peripheral neuropathy, pancreatitis, lactic acidosis, severe hepatomegaly with steatosis, hepatic failure and death.	19-Jun-92
Retrovir [S*]	zidovudine, azidothymidine, AZT, ZDV	Tablets, capsules and syrups 600 mg/day	Haematological toxicity, myopathy, lactic acidosis, headache, malaise, nausea, anorexia, vomiting, fever, cough, digestive disorders, anaemia and neutropenia.	19-Mar-87
Trizivir	abacavir, zidovudine, and lamivudine	One tablet twice daily	Hypersensitivity reactions, hematologic toxicity, myopathy, lactic acidosis and severe hepatomegaly, exacerbations of hepatitis B, nausea, headache, malaise and fatigue, and nausea and vomiting.	14-Nov-00
Truvada	tenofovir disoproxil fumarate and emtricitabine	One tablet once daily taken orally with or without food.	Lactic acidosis/severe hepatomegaly with steatosis, post-treatment acute exacerbation of hepatitis B, risk of drug resistance, diarrhoea, nausea, fatigue, headache, dizziness, depression, insomnia, abnormal dreams and rash.	02-Aug-04
Videx EC [S*]	enteric coated didanosine, ddI EC	Dosing based on body weight; 200-400 mg/day	Pancreatitis, lactic acidosis, hepatomegaly with steatosis, diarrhoea, peripheral neurologic symptoms/neuropathy, nausea, headache, rash, and vomiting.	31-Oct-00

Videx	didanosine, dideoxyinosine, ddI	Administered on an empty stomach at least 30 minutes before or 2 hours after eating. Dosing is based on body weight; 125-400 mg/day.	Pancreatitis, lactic acidosis, hepatomegaly with steatosis, diarrhoea, peripheral neurologic symptoms/neuropathy, abdominal pain, nausea, headache, rash, and vomiting.	09-Oct-91
Viread[S*]	tenofovir disoproxil fumarate, TDF	Dose of 300 mg/day taken orally without regard to food.	Lactic acidosis/severe hepatomegaly with steatosis, post treatment exacerbation of hepatitis, rash, diarrhoea, headache, pain, depression, asthenia, nausea, abdominal pain, insomnia, pruritus, vomiting, dizziness and pyrexia.	26-Oct-01
Zerit	stavudine, d4T	Dose of 30-40 mg taken every 12 h	Lactic acidosis, hepatomegaly with steatosis; pancreatitis, headache, diarrhoea, neuropathy, rash, nausea and vomiting.	24-Jun-94
Ziagen[S*]	abacavir sulfate, ABC	Dose of 600 mg/day	Hypersensitivity reactions, lactic acidosis, severe hepatomegaly, nausea, headache, malaise and fatigue, nausea and vomiting, dreams/sleep disorders, skin rashes, and ear/nose/throat infections.	17-Dec-98
Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs)				
Brand Name	**Generic Name**	**Dosage and Administration[ǂ]**	**Adverse Reactions**	**Approval Date**
Edurant	rilpivirine	One tablet taken once daily with a meal.	Fat redistribution or immune reconstitution syndrome, depression, insomnia, headache and rash.	20-May-11
Intelence[S*]	etravirine	Dose of 200 mg taken twice daily following a meal.	Stevens-Johnson syndrome, hypersensitivity reaction, toxic epidermal necrolysis, erythema multiforme, rash, diarrhoea and peripheral neuropathy.	18-Jan-08

Rescriptor	delavirdine, DLV	Dose of 400 mg taken three times daily.	Abdominal cramps, abdominal distention, abdominal pain (localized), abscess, allergic reaction, chills, edema (generalized or localized), epidermal cyst, fever, infection viral, lip edema, malaise, Mycobacterium tuberculosis infection, neck rigidity, sebaceous cyst, and fat redistribution.	04-Apr-97
Sustiva	efavirenz, EFV	One capsule/tablet daily on an empty stomach.	Serious psychiatric symptoms, nervous system symptoms, hepatotoxicity, rash, convulsions, immune reconstitution syndrome, fat redistribution, rash, dizziness, nausea, headache, fatigue, insomnia and vomiting.	17-Sep-98
Viramune	nevirapine, NVP	One tablet daily	Life-threatening (including fatal) hepatotoxicity and skin reactions.	21-Jun-96
Viramune XR	nevirapine, NVP	One tablet daily	Life-threatening (including fatal) hepatotoxicity and skin reactions.	25-Mar-11
Protease Inhibitors (PIs)				
Brand Name	**Generic Name**	**Dosage and Administration‡**	**Adverse Reactions**	**Approval Date**
Agenerase[S*]	amprenavir, APV	Capsule and oral solution discontinued	Mild to moderate gastrointestinal adverse events	15-Apr-99
Aptivus[S*]	tipranavir, TPV	Dose of 500 mg aptivus, co-administered with 200 mg ritonavir, twice daily	Hepatotoxicity, intracranial hemorrhage, diarrhoea, nausea, pyrexia, vomiting, fatigue, headache, rash and abdominal pain.	22-Jun-05
Crixivan[S*]	indinavir, IDV,	Two capsules taken every 8 h	Nephrolithiasis/urolithiasis, haemolytic anaemia, hepatitis, hyperglycaemia, rhabdomyolysis, hyperbilirubinemia, tubulointerstitial nephritis, fat redistribution.	13-Mar-96

Fortovase	saquinavir	Capsule and oral solution discontinued	Confusion, ataxia, weakness; acute myeloblastic leukemia; haemolytic anaemia; attempted suicide; Stevens-Johnson syndrome; seizures; severe cutaneous reaction; elevation of transaminases; thrombophlebitis; headache; thrombocytopenia; exacerbation of chronic liver disease, jaundice, ascites, quadrant abdominal pain; drug fever; bullous skin eruption and polyarthritis; pancreatitis leading to death; nephrolithiasis; thrombocytopenia and intracranial hemorrhage leading to death; peripheral vasoconstriction; portal hypertension; intestinal obstruction.	07-Nov-97
Invirase[S*]	saquinavir mesylate, SQV	Dose of 1000 mg/day taken in combination with ritonavir 100 mg twice daily.	New onset or exacerbations of diabetes mellitus, hyperglycaemia, elevated cholesterol and/or triglyceride concentrations, fat redistribution, immune reconstitution syndrome, worsening liver disease, haemophilia, nausea, vomiting, diarrhoea, fatigue, pneumonia and abdominal pain.	06-Dec-95
Kaletra[S*]	lopinavir and ritonavir, LPV/RTV	Three capsules taken twice daily with food.	Hypersensitivity, hepatotoxicity; new onset or exacerbations of diabetes mellitus, hyperglycaemia, immune reconstitution syndrome and fat redistribution; total cholesterol and triglycerides elevations, haemophilia, diarrhoea, nausea, abdominal pain, asthenia, vomiting, headache, and dyspepsia.	15-Sep-00

Lexiva[S*]	Fosamprenavir Calcium, FOS-APV	Dose of 1,400 mg taken twice daily/1,400 mg plus ritonavir 200 mg once daily/1,400 or 700 mg plus ritonavir 100 mg once daily	Hypersensitivity, transaminase elevations, new onset or exacerbations of diabetes mellitus, hyperglycaemia, immune reconstitution syndrome, fat redistribution and elevated triglyceride and cholesterol concentrations; acute haemolytic anaemia, haemophilia, nephrolithiasis, diarrhoea, rash, nausea, vomiting, headache and neutropenia.	20-Oct-03
Norvir[S*]	ritonavir, RTV	Dose of 600 mg taken twice daily with meals.	Sedative hypnotics, antiarrhythmic, hypersensitivity, hepatic reactions, pancreatitis, total cholesterol and triglycerides elevations, new onset or exacerbations of diabetes mellitus, hyperglycaemia; immune reconstitution syndrome, fat redistribution, haemophilia, abdominal pain, asthenia, headache, malaise, anorexia, diarrhoea, dyspepsia, nausea, vomiting, paraesthesia, circumoral paraesthesia, peripheral paraesthesia, dizziness and taste perversion.	01-Mar-96
Prezista	darunavir	Dose of 800 mg taken with ritonavir 100 mg once daily with food.	Drug-induced hepatitis, skin reactions, new onset diabetes mellitus or hyperglycaemia, fat redistribution, immune reconstitution syndrome, diarrhoea, nausea, rash, headache, abdominal pain and vomiting.	23-Jun-06
Reyataz[S*]	atazanavir sulfate, ATV	Dose of 300 mg taken with ritonavir 100 mg/day with food.	Cardiac conduction abnormalities, rash, hyperbilirubinemia, hepatotoxicity, nephrolithiasis, new onset or exacerbations of diabetes mellitus/hyperglycaemia, immune reconstitution syndrome and fat redistribution; haemophilia, nausea, jaundice/scleral icterus, abdominal pain, vomiting, insomnia, peripheral neurologic symptoms, dizziness, myalgia, diarrhoea, depression, and fever.	20-Jun-03

Viracept[S*]	nelfinavir mesylate, NFV	Dose of 1250 mg taken twice daily or 750 mg three times daily with a meal.	Diabetes mellitus/hyperglycaemia, haemophilia, fat redistribution, immune reconstitution syndrome, diarrhoea, leukopenia/neutropenia, nausea, rash, flatulence, anorexia and abdominal pain.	14-Mar-97
Fusion Inhibitors				
Brand Name	**Generic Name**	**Dosage and Administration‡**	**Adverse Reactions**	**Approval Date**
Fuzeon[ND]	enfuvirtide, T-20	Dose of 90 mg (1 mL) injected twice daily.	Injection site reaction, pneumonia, hypersensitivity, immune reconstitution syndrome, diarrhoea, nausea and fatigue.	13-Mar-03
Entry Inhibitors - CCR5 co-receptor antagonist				
Brand Name	**Generic Name**	**Dosage and Administration‡**	**Adverse Reactions**	**Approval Date**
Selzentry	maraviroc	Tablets: 150-600 mg taken twice daily	Hepatotoxicity, immune reconstitution syndrome, severe skin and hypersensitivity reactions, myocardial ischemia and/or infarction, postural hypotension, upper respiratory tract infections, cough, pyrexia, rash and dizziness.	06-Aug-07
HIV integrase strand transfer inhibitors				
Brand Name	**Generic Name**	**Dosage and Administration‡**	**Adverse Reactions**	**Approval Date**
Isentress	raltegravir	Dose: 400 mg film-coated tablet taken twice daily.	Severe, potentially life-threatening and fatal skin reactions; immune reconstitution syndrome, insomnia, headache, dizziness, nausea, fatigue, creatine kinase elevations, myopathy and rhabdomyolysis.	12-Oct-07

*This information is based on the U.S. Food and Drug Administration (FDA) http://www.fda.gov

‡Dosage specified is for adults and adolescents. Dosage for pediatric patients is calculated based on body weight (kg) and does not exceed adult dose. Dosage is also adjusted in patients showing adverse reactions to ARVs or demonstrating symptoms of other diseases/disorders.

[ND] Enfuvirtide (fusion inhibitor) was derived from a natural product and semisynthetic modifications were done (Newman and Cragg, 2012).

[S*] These drugs are made by total synthesis but the pharmacophore is from a natural product (Newman and Cragg, 2012).

2.2 Latent HIV-1: a major hurdle in HIV treatment

The previous section highlighted ARV therapy; how it inhibits HIV replication in patients and prevents progression to AIDS, and its potential side-effects. Drug resistant HIV strains remains a challenge to current treatment and the persistence of the virus during therapy in rare reservoirs remains a bigger challenge because there are no approved drugs to address latency.

Latent HIV is the existence of dormant virus particles usually residing in stable reservoirs (Figure 2.3) in infected individuals (Palmer et al., 2011). A viral reservoir, as defined by Dahl et al. (2010), can be a cell type or anatomical site where a replication competent form of the virus persists for a longer time as compared to the main pool of actively replicating virus.

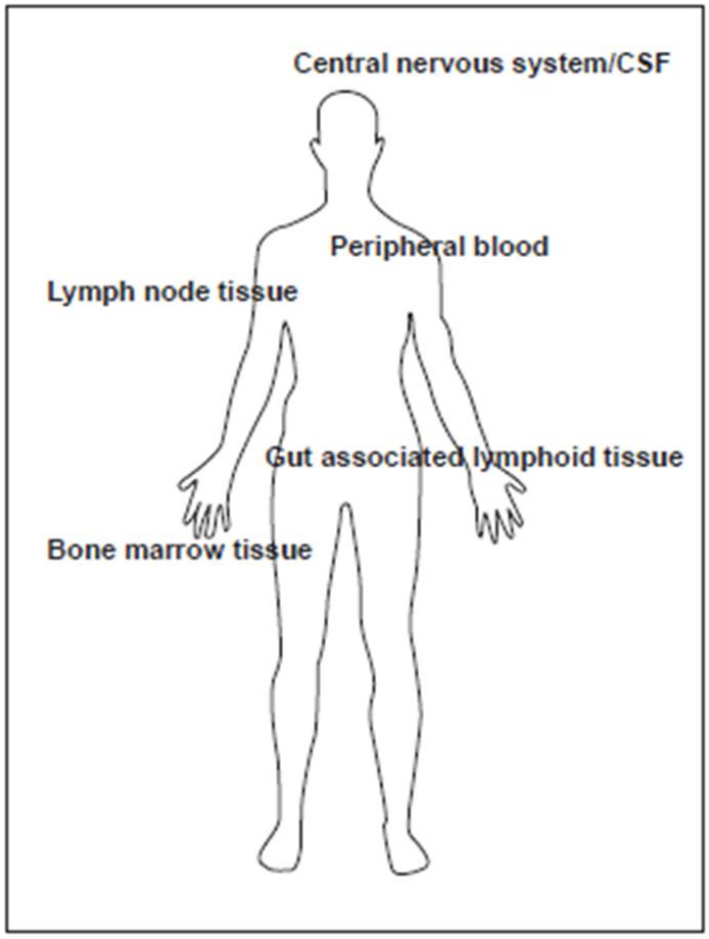

Figure 2.3: Possible HIV reservoirs. HIV can hide in the brain, lymph nodes, skin, peripheral blood, reticuloendothelial system, bone marrow and gastrointestinal cells (Palmer et al., 2011).

Studies have demonstrated that suppressive therapy drives HIV-1 RNA to less than 50 copies mL^{-1} in the plasma of infected patients (Palmer et al., 2011). However, ultrasensitive techniques have proven that most patients continue to host low levels of these viral particles (Palmer et al., 2011). There are various sources from which dormant virions arise including: HIV-infected cells that had lived for long and still have the ability to replicate and produce virus as well as ongoing replication in cells located in preserved sites (Figure 2.3) where drugs do not have full access to (Palmer et al., 2011). Figure 2.4 provides a simple illustration of how latent HIV is generated.

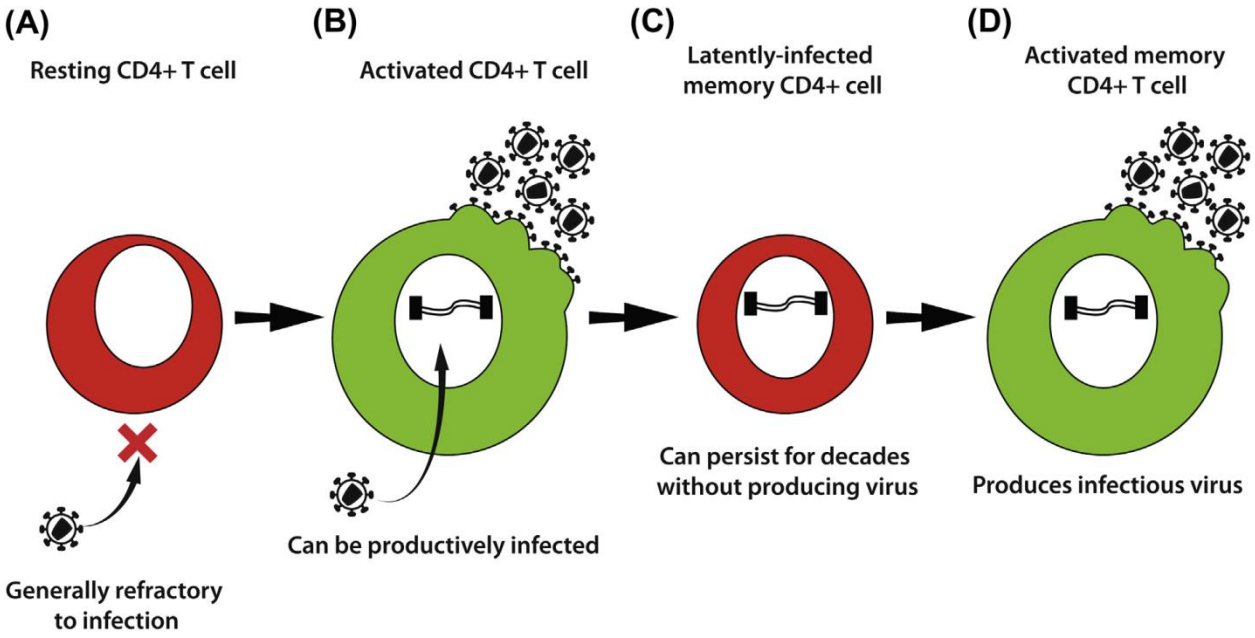

Figure 2.4: Generation of HIV latency. (A) Resting CD4+ T cells are generally refractory to infection. (B) However, upon stimulation by cytokines they become activated susceptible to HIV infection. Infection will generally result in death of the host cell, but a few of these cells survive and transition back to a resting state. (C) The transition results in a shut-down of HIV expression and production of a long-lived latently infected cell that harbors an HIV provirus that is not producing viral proteins. (D) Subsequent activation of this latently infected cell (possible many years later) results in re-initiation of virus expression and production of new infectious viral particles (Marsden and Zack, 2013).

Untreated HIV infection is generally characterized by billions of new virions and infected cells being produced and cleared everyday (Figure 2.5) (Marsden and Zack, 2013).

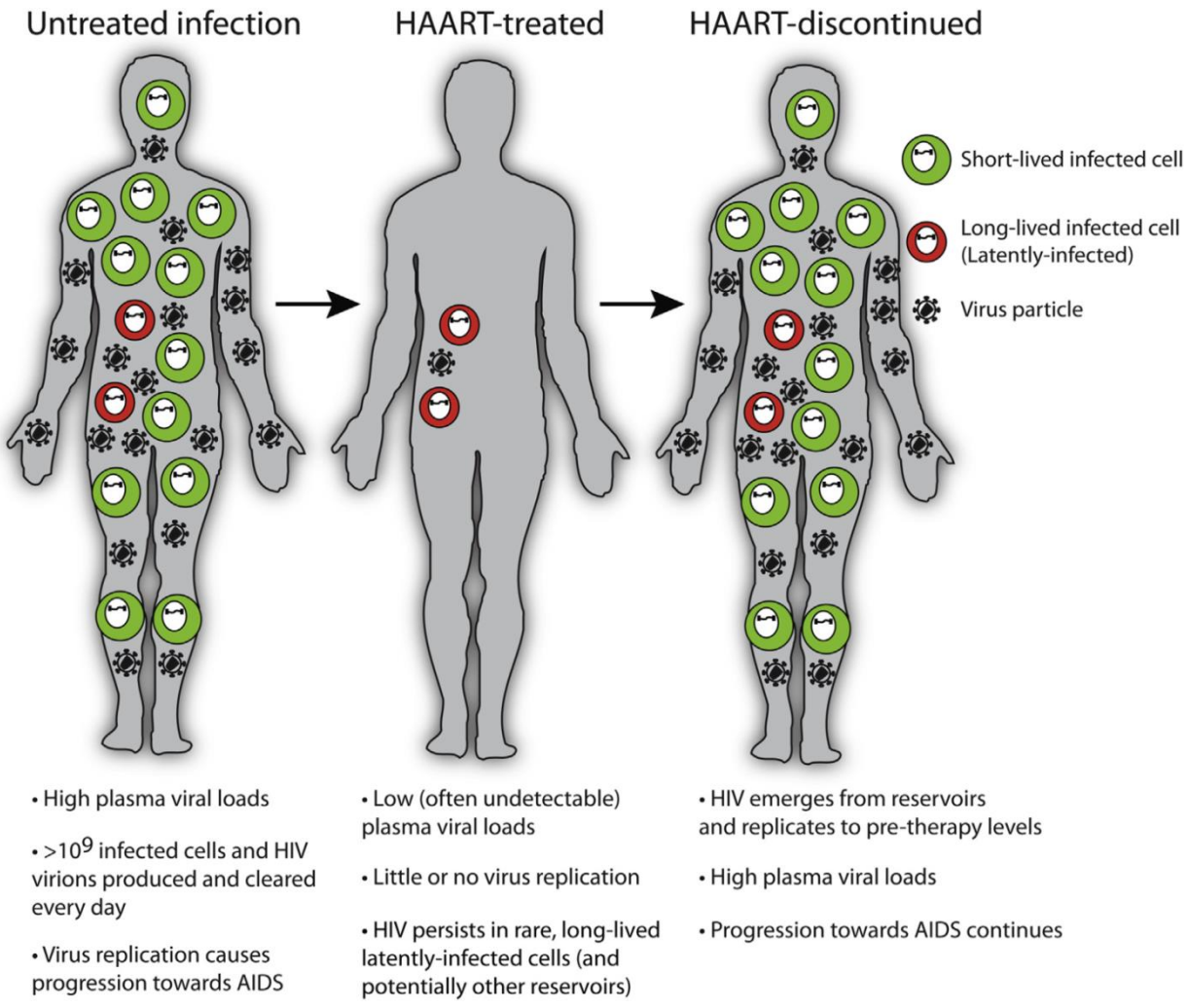

Figure 2.5: Effects of stopping HAART. HIV infection is associated with high levels of virus replication. HAART inhibits the vast majority of this replication, but HIV persists at low levels in cellular reservoirs in treated individuals. Discontinuing HAART for any reason, results in viral particles emerging from these reservoirs allowing replication to continue at pre-therapy levels (Marsden and Zack, 2013).

Studies have looked at molecules capable of activating latent HIV-1 expression as inductive adjuvant therapy for HAART (Imai et al., 2009; Kulkosky et al., 2001). Adjuvant therapy is generally defined as treatment that is provided in addition to the primary treatment. Traditionally, the term adjuvant is commonly used to describe cancer treatment known as adjuvant chemotherapy (Gray et al., 2007). Adjuvant chemotherapy is used to reduce the risk of cancer recurrence (Gray et al., 2007; Smith et al., 2007). Adjuvants are also used to boost immunization in what is referred to as vaccine adjuvant; a clinical term used to describe a new generation of vaccines with added

substances (diverse range of compounds and materials) to induce enhanced immune responses to vaccine antigens (Petrovsky and Aguilar, 2004). In the current context, adjuvant therapy refers to potential drug candidates that may supplement HAART by inducing expression of latent HIV, thereby allowing other drugs in the regimen to then eradicate newly activated viral particles (Kulkosky et al., 2001). The ultimate goal of this kind of therapy is to eliminate persistent viral reservoirs in infected individuals.

There are various strategies proposed for the purging of latently-infected cells and these approaches are detailed in Figure 2.6. Gene therapy is one possible approach; introducing genes that generate more effective immune responses against the virus (Marsden and Zack, 2013). The second major strategy is the 'activation/elimination' approach and this involves flushing out the latent virus by inducing it to express viral proteins (Palmer et al., 2011). Cytokines have been implicated to play a role in inducing HIV-1 expression in latently infected cells (Folks et al., 1987). Early clinical attempts to purge latent virus were done with interleukin (IL)-2 but viral rebound was still observed following termination of ARV (Chun et al., 1999). Inhibiting or activating certain enzymes has been reported to activate viral expression of dormant virions. Activating the proteinase kinase C (PKC) pathway is one of the strategy identified to activate latent HIV (McKernan et al., 2012). Another activation strategy involves inhibiting histone deacetylases (HDACs). HDACs are recruited to the HIV long terminal repeat (LTR) promoter creating a deacetylated LTR chromatin that seems to play a key contributory role in regulating HIV expression, and especially in maintaining proviral quiescence and latency (Archin et al., 2012; Marsden and Zack, 2013). Other mechanisms that may contribute to latency are the actions of histone methyltransferases, DNA methyltransferase, bromodomain proteins and the lack of positive transcription elongation factor b (pTEFb) in latently infected cells (Dahl et al., 2010; Marsden and Zack, 2013). Hence, PKC activators (McKernan et al., 2012) as well as inhibitors of HDACs (Archin et al., 2012) and other enzymes involved in maintaining HIV latency are under consideration for use in future clinical trials. These activators or inhibitors may be derived from natural or synthetic sources. Examples of natural products currently under investigation for activating latency are; prostratin and bryostratin (DeChristopher et al., 2012; Kulkosky et al., 2001; Rullas et al., 2004) and these are discussed in more detail in chapter 6.

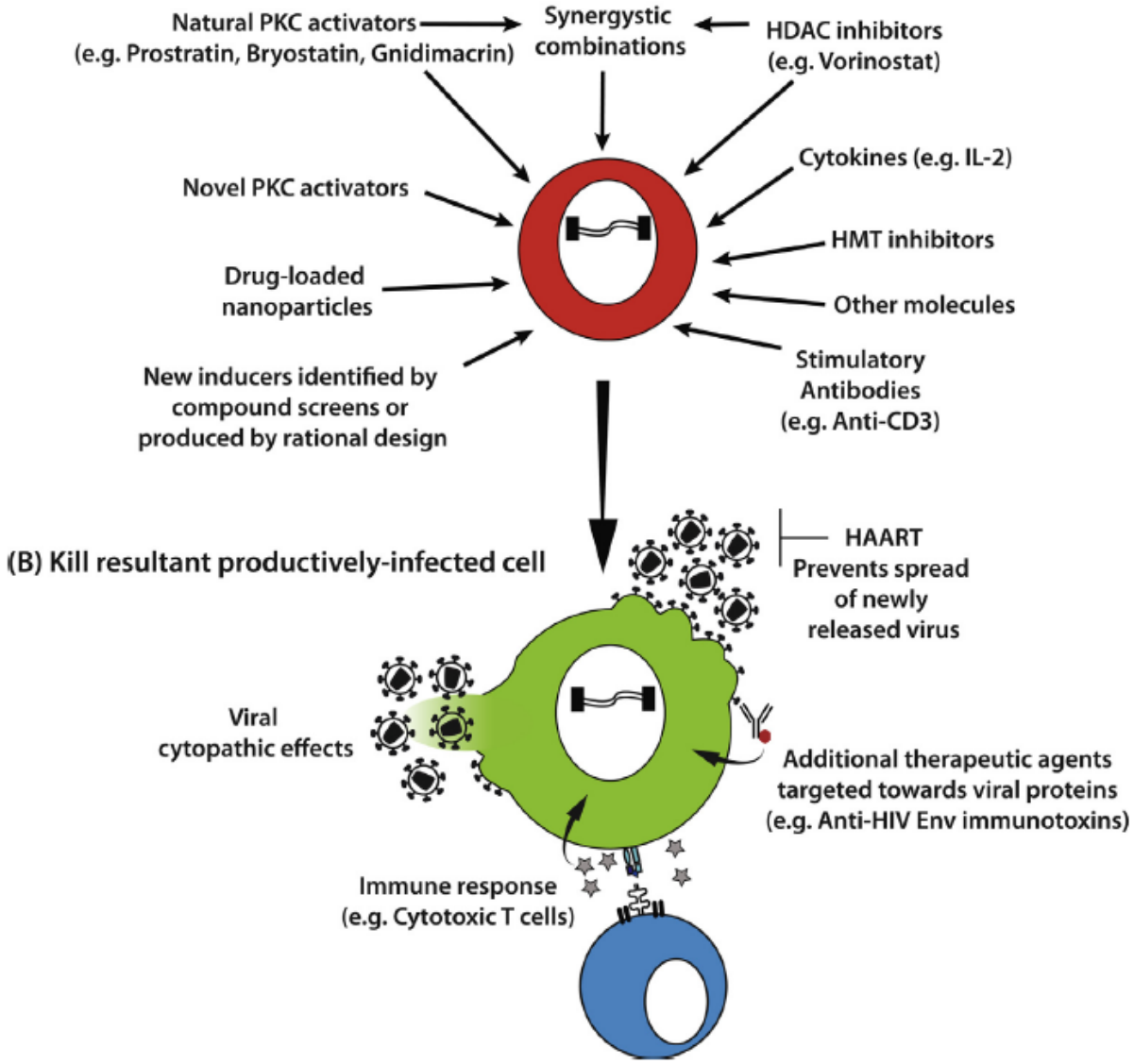

Figure 2.6: Activation/elimination approaches to eradicate latent HIV (Marsden and Zack, 2013)**.**

The first two sections of this chapter looked into the shortcomings of HIV/AIDS management which prompts continuous search for identification of novel treatment options that will either augment or replace existing drugs. The next section has provided background on Lamiaceae plants as sources of alternative anti-HIV agents. Lamiaceae is the plant family that was investigated in this thesis.

2.3 Lamiaceae plants as sources of alternative anti-HIV agents

Vast amounts of research has been carried out worldwide to explore the potential of plants as anti-HIV agents because plants have mechanisms to control plant virus attacks and these compounds may have activity against mammalian viruses as well. Plant products have been identified as possible anti-HIV drugs targeted at the different steps of the viral life cycle (Matsuse et al., 1999). These steps include preventing viral attachment and entry as well as inhibiting the enzymes that play a role in transcribing and integrating the viral RNA in the genome of the host cell (Harnett et al., 2005; Matsuse et al., 1999).

For centuries, plant extracts have been used to treat various disorders, not only because they tend to be without side-effects (Kaileh et al., 2007) but also because for many people in Africa, traditional medicines have been the only source of medical treatment (Harnett et al., 2005).

Medicinal plants have been a target in anti-HIV screening studies because of their relative low cost and availability (Bedoya et al., 2001; Lo et al., 2003; Tewtrakul et al., 2006). Mint plants, scientifically known as Lamiaceae have been used in the food and cosmetic industry for a long time and plants belonging to this family are currently being researched on as potential anti-HIV agents (Geuenich et al., 2008).

Various plants belonging to the Lamiaceae family have numerous traditional uses (Van Wyk et al., 2009). These plants are common in Southern Africa (Van Wyk and Gericke, 2000) and many of them have reported anti-viral properties (Brindley et al., 2009; Geuenich et al., 2008). The rest of this section will provide background information on the Lamiaceae species used in this study as well as (where possible/available) their potential link to HIV/AIDS treatment.

Leonotis leonurus, shown in Figure 2.7, is a shrub which grows up to 5 meters in height and its leaves, stems and sometimes roots are used traditionally to treat a wide range of ailments including colds, coughs, viral hepatitis, influenza, boils, bronchitis, headaches and high blood pressure. The plant is administered as leaf decoctions and infusions (Van Wyk and Gericke, 2000; Van Wyk et al., 2009). Decoction is an extraction method which involves boiling the plant material to obtain a liquid that can then be administered

to patients, on the other hand an infusion does not involve boiling but allows the plant material to be suspended over time (Lamorde et al., 2010). An ethanolic extract of the plant has been shown to have anti-HIV activity (Klos et al., 2009) and proximate analysis of *L. leonurus* showed the percentage protein, carbohydrate, crude fibre and fat of the leaves as 19.0%, 51.0%, 12.02% and 6.0%. The plant reportedly contains low levels of anti-nutrients as well as toxicants and demonstrated antioxidant and antibacterial activities making it a good choice as a potential high valued medicinal plant (Jimoh et al., 2010).

Figure 2.7: *Leonotis leonurus*

A study carried out by Klos et al. (2009) reported the ethanolic extract of *L. leonorus* to possibly contain novel compounds inhibiting HIV-1 PR. This was because the inhibition observed was not due to tannins. However, the specific compounds in *L. leonurus* responsible for HIV-1 PR inhibition have not been identified yet.

Mentha longifolia is an indigenous creeping herb that is found growing in wet places such as from Calvinia down to the Cape Peninsula through the Eastern Cape, Lesotho, Orange Free State, KwaZulu-Natal to Gauteng and Limpopo the Northern Province. The plant is widely spread in Southern Africa and parts of the plant are highly aromatic (Van Wyk and Gericke, 2000; Van Wyk et al., 2009). Plants from the genus *Mentha* are known for their essential oil production (Shaiq Ali et al., 2002). Research has shown that this essential oil has strong antimicrobial activities and this suggested its potential to be used as a natural preservatives in food to avoid food spoilage (Gulluce et al., 2007).

Traditionally, *M. longifolia* is used to treat coughs, colds, urinary tract infections and respiratory ailments. The leaves of the plant are the ones used mostly and are administered as an infusion or decoction that is orally ingested (Van Wyk and Gericke, 2000; Van Wyk et al., 2009).

A study carried out on Morrocan *M. longifolia* reported the methanolic and ethyl acetate extracts from the plant to inhibit HIV-1BaL infection by approximately 40 and 55%, respectively. Ethyl acetate extract was also reported to inhibit the HIV-1 RT by 50%. However, the bioactive components were not identified in both extracts (Amzazi et al., 2003).

Tetradenia riparia (Figure 2.8) is a multi-branched plant with succulent leaves and stems. This plant is found in South Africa, Namibia, Angola as well as east tropical Africa. The leaf infusion and decoction of this plant is traditionally used to treat fever, colds, cough, influenza, mouth ulcers, diarrhoea and malaria. The essential oil from the leaves has been found to have moderate anti-malarial activity against two strains of *Plasmodium falciparum* (Schwikkard and van Heerden, 2002; Van Wyk and Gericke, 2000; Van Wyk et al., 2009).

Figure 2.8: *Tetradenia riparia*

The genus *Salvia* like the plants previously described belongs to the Lamiaceae family. These plants are mostly added in the infusions of other Lamiaceae plants to treat ailments such as fevers, colds, influenza and abdominal cramps (Van Wyk and Gericke, 2000; Van Wyk et al., 2009). Solvent extracts of selected *Salvia* species were found to have antimalarial, antibacterial and anticancer activities (Kamatou et al., 2008, 2006). Traditional healers sometimes use a mixture of plant extracts to treat infections.
L. leonurus reportedly has synergistic effects against Gram-positive bacteria when combined with *Salvia chamelaeagnea* (Kamatou et al., 2006).

One of the significant, prolific and mostly used Lamiaceae genus in Southern Africa is *Plectranthus* (Rice et al., 2011). *Plectranthus* plays a huge role in traditional medicine with a wide variety of its species used to treat a wide range of conditions. Different parts of *Plectranthus* species (stems, leaves, roots and tubers) are used to treat various ailments (Rice et al., 2011). A literature search identified the following *Plectranthus* species, *P. barbartus*, *P. comosus* and *P. ciliatus* as some of the common medicinal plants used by persons with HIV/AIDS (Kisangau et al., 2007; Nagata et al., 2011; Semenya et al., 2013). These plants are used either on their own or in addition to ARVs.

Concurrent herb-ARV usage is becoming common in different areas of the world including Africa, North America and Europe (Nagata et al., 2011). There are various reasons for why herbal remedies are often used with HIV drugs and one of the reasons is to apparently counteract some of the negative side effects of ARV (Nagata et al., 2011). The inability of the current HIV regimen to completely eradicate the virus is another reason for concurrent herb-ARV use by patients (Nagata et al., 2011).

Ocimum is another Lamiaceae genus containing species used to alleviate various HIV/AIDS related conditions (Lamorde et al., 2010). *O. sauve*, *O. gratissimum*, *O. sanctum* and *O. basilicum* are used in managing HIV/AIDS and related opportunistic infections (Hegde et al., 2007; Lamorde et al., 2010; Prabhu et al., 2009; Rege et al., 2010). Pharmacological studies have proven extracts of some of these plants to inhibit HIV *in vitro* (Hegde et al., 2007; Rege et al., 2010).

Lamiaceae plants reportedly contain various terpenoid compounds. Terpenes are a large diverse class of naturally occurring organic-chemicals derived from five-carbon isoprene units. Most of the effective ingredients discovered from some medicinal extracts are terpenoid compounds (Salminen et al., 2008). Plant isolates containing terpenoids have been found to suppress nuclear factor-κB (NF-κB) signaling and this protein complex has been linked to the pathogenesis of inflammatory diseases, cancer, viral infection and autoimmune diseases (Salminen et al., 2008). *L. leonurus* reportedly contain diterpenoids, which are identified as the active ingredients of that plant.
M. longifolia and *T. riparia* plants contain numerous monoterpenoids which have also been identified as the active ingredients in those plants (Van Wyk et al., 2009).

Taken together the promising health benefits of the Lamiaceae plant family, it would be worthwhile to explore plants from this family for possible drug leads. The remainder of this chapter has provided a background on the successes and failures of developing plant products as possible HIV/AIDS drugs, contributory roles that plant products might play in alleviating HIV/AIDS through the antioxidation process and in modulating the immune system. This will be followed by a discussion on common drug discovery techniques used in screening and identifying promising compounds.

2.4 Status of plant products for treating HIV/AIDS

Information on existing HIV treatments was provided in section 2.1 and from what was supplied in table 2.1, it is evident that all drugs used in the current HIV regimen have adverse side-effects. This indicates that an immediate need for new anti-HIV drugs is a global concern and plant derived anti-HIV agents may present great promise (Singh et al., 2011).

Herbal or plant materials are traditionally used to manage HIV and infections that arise from this viral infection. The most common method of administration, as described in section 2.3, is by oral application of decoctions or infusions. Decoctions and infusions are commonly prepared using water or milk (Kisangau et al., 2007; Lamorde et al., 2010). For skin eruptions, powdered medicines are mixed with jelly and applied as ointments (Lamorde et al., 2010). Oral administration is done in variable doses and some traditional medicine practitioners advise their patients to take the medical preparation after meals while others do not give advice with regard to food intake (Chinsembu and Hedimbi, 2010; Lamorde et al., 2010; Omoruyi et al., 2012).

Traditional medicine is commonly administered in the absence of scientific validation. A number of plant derived compounds have been identified as promising anti-HIV agents and these compounds include alkaloids, flavonoids, coumarins, tannins, terpenes, phenolics, polysaccharides, chlorophyll derivatives etc (Singh et al., 2011; Wang et al., 2009; Zhang et al., 2003). The remainder of this section has provided examples of compounds from nature and their activities against HIV.

Alkaloids: Michellamines (A, B and C) are atropisomeric naphthylisoquinoline alkaloid dimers that were isolated from a Cameroonian plant, *Ancistrocladus korupensis* (Boyd et al., 1994). These alkaloids inhibited HIV-induced cell killing and viral replication in a variety of human cell lines (Boyd et al., 1994). Biochemical studies showed that michellamine B was active against a panel of biologically diverse laboratory and clinical strains of HIV-1, including the AZT resistant strain G910-6 and the pyridinone-resistant strain A17; the compound also inhibited several strains of HIV-2 (Boyd et al., 1994; Hallock et al., 1995). Michellamine D, a homolog of michellamines A-C underwent extensive preclinical evaluation as a potential HIV drug but was found to be highly toxic (Singh et al., 2011). Cepharanthine is a biscoclaurine alkaloid isolated from *Stephania*

cepharantha and was found to suppress HIV-1 LTR-driven gene expression through the inhibition of NF-kB activation (Okamoto et al., 1998). These are just a few of this class of compounds, a lot more alkaloids are reported in the literature with potential inhibitory activity against HIV-1 (Singh et al., 2011).

Flavonoids: This is one of the groups of compounds reported to inhibit all three HIV enzymes; RT, PR and IN (Singh et al., 2011). Flavonoids have also been shown to inhibit the attachment of viral gp120 to CD4+ cells as well as reducing viral replication *in vitro* (Singh et al., 2011). Flavonoids with potent anti-HIV activity include glucuronide, baicalin, quercetin, chrysin, epigallocatechin, thalassionlins A-C,taxifolin etc (Lee et al., 2003; Li et al., 2000a; Singh et al., 2011; Wu et al., 2003).

Coumarins: Calanolide is one of the most popular coumarins which inhibits HIV-1 RT. This compound was first isolated from a tropical tree (*Calophyllum lanigerum*) in Malaysia (César et al., 2011; Creagh et al., 2001). The safety and pharmacokinetics of this compound have already been evaluated and it is so far the only natural product undergoing HIV clinical trials (Creagh et al., 2001). Other types of coumarins showed anti-HIV activity and these include khellolactone, furanocoumarins, immperatorin, heraclenol etc (Singh et al., 2011).

Tannins: Tannins are classified as either hydrolysable or non-hydrolyzable (Chung et al., 1998) and for many years have been regarded as anti-nutritional by inhibiting the hydrolyses of proteins by trypsin, resulting in indigestible protein complexes (Horigome et al., 1988). This polyphenolic group is also reported to be carcinogenic and can cause toxicity of the liver. Tannins have antiviral properties and are good enzyme inhibitors; gallotannins were reported to inhibit reverse transcriptase inhibitor-resistant strains and block virus uptake but the tannin containing extracts were found to be very cytotoxic. The antiviral effect and cytotoxicity declined when gallotannins were removed from the extracts (Notka et al., 2003). Tannins are known to be non-specific inhibitors of microbial growth and a large tannin intake has a negative health impact (Chung et al., 1998).

Terpenes: This is a large group of medicinal compounds that mainly falls under three classes; diterpenes, triterpenes and sesquiterpenes (Singh et al., 2011). These cyclic

compounds have been reported to mostly inhibit HIV RT as well as prevent replication in cells. Betulinic acid (DSB), also known as PA-457 or bevirimat (BVM), potently inhibits HIV-1 replication by specifically blocking CA-SP1 cleavage resulting in antimaturation activity (Adamson and Freed, 2010; Stoddart et al., 2007). Even though clinical trials of berivimat indicated a significant and clinical reduction of the viral load in infected individuals, a high baseline drug resistance was also revealed (Dang et al., 2013). The high baseline drug resistance poses serious limitations to the clinical potential and further development of this terpene as a potential HIV drug (Dang et al., 2013). New berivimat derivatives are being developed to overcome the drug resistance of the parent compound (Dang et al., 2013). Other terpenes that have been shown to inhibit HIV include butenolide-3-epi-litsenolide D_2, lactone, lanicilacton C, limonoid etc (Singh et al., 2011).

Phenolics: Several phenolics have been highlighted to be of immense significance in managing chronic HIV/AIDS. Two of these phenolics, dicaffeoyl quinic acid and dicaffeoyl-tartaric acid, have been identified as potent and selective HIV-1 integrase inhibitors (Mcdougall et al., 1998). Other phenolics with anti-HIV effects are phloroglucinol alpha pyrone arzanol, which inhibit HIV-1 replication *in vitro* and the release of pro-inflammatory cytokines (Appendino et al., 2007); geraniin and corilagin are two other polyphenols that reportedly inhibit HIV-1 RT (Notka et al., 2003).

Polysaccharides: Sulphated polysaccharides, a class of compounds containing hemi-ester sulphated groups, are commonly found in marine algae (Shanmugam and Mody, 2000). This group of compounds reportedly show high antiviral activity against enveloped viruses including HIV (Damonte et al., 2004; Witvrouw and De Clercq, 1997). Calcium spirulan isolated from a blue-green alga, *Spirulina platensis*, was found to inhibit the replication of several enveloped viruses including herpes simplex, human cytomegalovirus, measles, influenza A, HIV-1 etc. by selectively inhibiting the penetration of virus into host cells (Hayashi and Hayashi, 1996). Nothing much is being done on developing calcium spirulan further as an anti-HIV agent.

Chlorophyll derivatives: Antiviral activity of chlorophylls have been documented by Bouslama et al. (2011) and Wang et al. (2009). Chlorophyll is a naturally occurring plant pigment and it is known to be highly unstable (Humphrey, 2004). The progressive

degradation of chlorophyll leads to the formation of similarly colored derivatives (Lanfer-Marquez et al., 2005). Commonly reported chlorophyll derivatives include chlorophylls a and b, pheophytins a and b, and pheophorbide a and b (Lanfer-Marquez et al., 2005). Pheophytin compounds have shown anti-hepatitis C as well as anti-herpes simplex properties (Sakdarat et al., 2009; Wang et al., 2009). Pheophorbide-a has been found to possess anti-HIV effect *in vitro* and also demonstrated activity against herpes simplex and influenza A viruses (Bouslama et al., 2011; Zhang et al., 2003).

Natural product leads against HIV exist and are in various stages of development even though nothing is in clinical use yet. Calanolide A (coumarin), the only natural compound undergoing clinical trials, continues to show promise in clinical tests. No synthetic modifications were done on the structure of calanolide A before clinical trials.

As indicated above, compounds isolated from natural sources hold potential as anti-HIV agents and further investigation needs to be done for some of these compounds to be developed as possible drug candidates. Natural products being good antioxidants might also play a role in reducing disease pathogenesis in infected individuals. Oxidative stress has been implicated in the increase of viral replication (amongst other aspects) and the next section explores natural products as potential modulators of oxidative stress.

2.5 Natural products and the alleviation of oxidative stress in HIV/AIDS

A number of studies reported an excessive production of reactive oxygen species (ROS) in HIV-infected individuals and this was regardless of the extent of their immunosuppression (Allard et al., 1998a, 1998b; Halliwel and Cross, 1991). Oxidative stress has been suggested as a contributing factor, during the course of HIV Infection, involved in many aspects of HIV pathogenesis such as viral replication, inflammatory responses, decreased immune cell proliferation, loss of immune function, cellular apoptosis, chronic weight loss, and increased sensitivity to drug toxicity (Gil del Valle et al., 2013). Oxidative metabolism pathways are reportedly involved in HIV infection and antiretroviral toxicity also has a potential impact on oxidative damage with the response contributing to co-morbidities (Gil del Valle et al., 2013).

Oxidative stress arises during an imbalance between oxidants and antioxidants resulting in the damage of both structure and function of tissues (Valko et al., 2006). Oxidants, reactive oxygen species (ROS), are naturally produced by various cells and cumulative ROS have been associated with a number of diseases, mainly neurodegenerative (Valko et al., 2006). ROS are also associated with chronic inflammatory diseases such as rheumatoid arthritis (RA) and the increased production of these ROS leads to tissue damage in RA patients (Gelderman et al., 2007; Mirshafiey and Mohsenzadegan, 2008). Other inflammatory diseases associated with cumulative ROS include for example, various types of periodontal diseases (Munksgaard, 1997).

Micronutrient supplementation, including antioxidants, is suggested to be beneficial as a cost-effective strategy for improving oxidative and nutritional status in HIV infection. The general strategy and combination of these interventions represent an important complementary approach for HIV infection treatment in the era of HAART (Gil del Valle et al., 2013).

Allard et al., (1998b) were able to demonstrate in their study that vitamin E and C supplementation significantly decreased oxidative stress in HIV-infected individuals. They were also able to prove that vitamin supplementation resulted in a viral load reduction and their data suggested clinical benefit that was worthy of clinical trials and which will especially be beneficial for the developing world due to the low cost of vitamins.

Plants are known to exhibit strong antioxidant activity (Gupta and Sharma, 2006) and natural product preparations may play a crucial role in reducing virus induced oxidative stress in HIV-1 infected individuals. Numerous plant extracts with anti-HIV properties have been reported to possess antioxidant activity (Aruoma et al., 1996; Chen et al., 2005; Katerere and Eloff, 2005; Nair et al., 2007) and some of these plants (*Hypoxis hemerocallidea* and *Sutherlandia frutescens*) are in use as African herbal medicines in the treatment of HIV.

2.6 Role of natural products in inflammation and immunomodulation

Plant extracts for immune enhancing purposes are already in clinical use e.g. Moducare, African potato (*Hypoxis hemerocallidea*), Astragalus (*Astragalus*

membranaceus), Pulsatilla (*Anemone patens*), Cancer Bush (*Sutherlandia frutescens*) etc. *Sutherlandia frutescens* is a multi-purpose medicinal plant found in Southern Africa and it is known for its antiviral and anticancer properties. The plant has been reported to possess superoxide and hydrogen peroxide scavenging activities which contributes to the plant's anti-inflammatory properties (Fernandes et al., 2004). Another medicinal plant (mentioned above) with immune supportive properties is *Hypoxis hemerocallidea* (African potato), this plant is popularly used in South Africa by HIV/AIDS patients to strengthen their immune system and the plant has a long history of use by traditional healers. The use of *H. hemerocallidea* and *S. frutescens* plants in managing HIV/AIDS is being supported by Ministries of Health and NGOs in all SADC countries (Mills et al., 2005).

Plant based products can act as immunostimulants or immunosuppressants. Natural products are potential anti-inflammatory drugs and are being investigated for potent immune modulation compounds. Inflammation is a way in which an organism responds to harmful stimuli and is one of the processes the immune system uses to modulate itself (Kim et al., 2010).

Inflammation has been reported to be important in the onset of various diseases such as cancer, diabetes and neurodegenerative diseases (García-Lafuente et al., 2009). In the presence of harmful substances, cells produce nitric oxide (NO) as part of an inflammatory response (Luiking et al., 2011). This is experimentally quantified by the Griess reagent which is a chemical that detects the presence of organic nitrites (Choi et al., 2001). Flavonoids have been reported to be potential anti-inflammatory agents and more research is still being carried out to determine their beneficial effects against cancer and cardiovascular diseases (García-Lafuente et al., 2009).

Cytokines, indicators of immune system modulation, are minute signaling protein molecules that are involved in intercellular communication. These proteins are largely secreted by the T-helper (Th) cells of the immune system as well as other cells in the nervous system including antigen presenting cells such as dendritic cells and macrophages (Elenkov et al., 2005). Two subsets of T-cells exist based on their distinct cytokine production pattern (Elenkov et al., 2005). Th1 is known to secrete interferon-γ (IFN- γ) and Interleukin (IL)-2 amongst other cytokines and Th2 secretes IL-4, IL-5, IL-

10, IL-13 etc (Elenkov et al., 2005). Some cytokines are known to be pro-inflammatory, for example IL-1 and TNF-α, and they need to be down-regulated/suppressed while dual function cytokines (pro- and anti) also exist (e.g. IL-6). Flow cytometry and ELISA are some of the common techniques used in cytokine detection (Bani et al., 2006).

Cytokines are associated with common human diseases especially those affecting the immune system and these include atopy/allergy, autoimmunity, chronic infections and sepsis (Elenkov et al., 2005). These diseases are characterized by a dysregulation of the pro- versus anti-inflammatory and Th1 versus Th2 cytokine balance. Specific cytokines are either up- or down-regulated during the pathogenesis of those diseases and one good example is in HIV/AIDS (Nixon and Landay, 2010). HIV infection results in the immune deficiency of affected individuals (Kedzierska and Crowe, 2001). The following cytokines: IL-2, 6, 10 and TNF have been linked to the pathogenesis of HIV (Kedzierska and Crowe, 2001; Williams et al., 2013). INF-ɣ is an interesting cytokine; it is generally produced during early stages of any viral infection but in the case of HIV infection this cytokine is apparently undetectable and it is only detected during the later stages when HIV progresses to AIDS (Francis et al., 1992).

The ability to regulate the production of certain cytokines can be of therapeutic importance. The previous section (2.4) highlighted a phenolic compound, phloroglucinol alpha pyrone arzanol, which has an ability to reduce HIV replication as well as possess anti-inflammatory properties by inhibiting the production of pro-inflammatory cytokines in primary monocytes qualifying it as a plant-derived anti-inflammatory and antiviral chemotype worth further investigation (Appendino et al., 2007). Flavonoids, another group of compounds, have been reported to be potential anti-inflammatory agents and more research is still being carried out to determine their beneficial effects against cancer and cardiovascular diseases (García-Lafuente et al., 2009).

Plant extracts with immune enhancing properties through the modulation of cytokine expression, *in vitro* and *in vivo*, have been documented (Spelman et al., 2006; Strzelecka et al., 2005; Talhouk et al., 2007; Yeşilada et al., 1997). Herbal medicines as immunomodulators may offer novel approaches in the treatment of a variety of diseases.

Plants investigated here were screened for immunostimulatory/modulatory activity, as well as antioxidant activities along with direct effects on HIV enzymes and other aspects of the viral life cycle. The ability of a single natural product to possess more than one health-related quality makes it possible for these products/compounds to offer many health benefits in a single dose when developed for clinical use.

2.7 Common drug discovery techniques

Drug discovery usually takes place in academic institutions and pharmaceutical companies. Figure 2.9 illustrates the processes involved in the discovery and development of drugs before they reach the market. The entire process usually takes about 10-15 years to just develop one new medicine. The next subsections have looked into techniques and bioassays commonly used in drug discovery from natural products, some of which were incorporated in the present study.

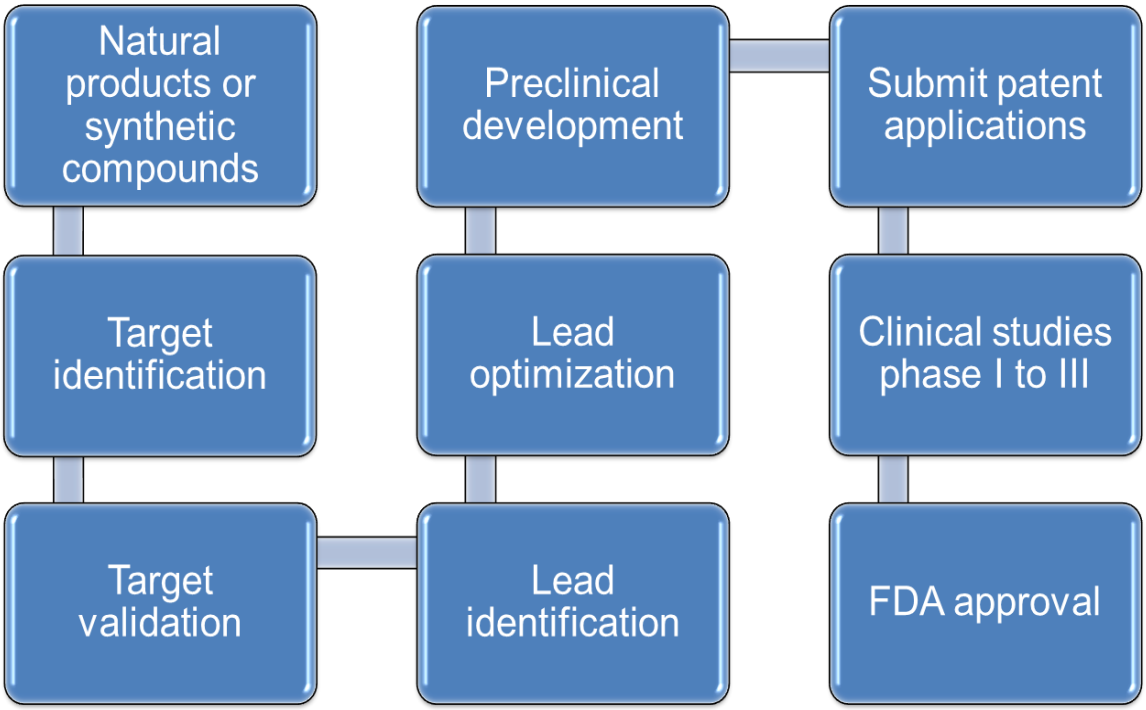

Figure 2.9: A typical drug discovery and development paradigm (Adapted from Choudhary and Khan, 2011; Erhardt, 2002).

2.7.1 Analytical techniques

The isolation and identification of a bioactive compound from a natural product requires a series of analytical techniques. Sample preparation is the first step in plant analyses. One of the popular ways of preparing crude extracts from plant material is by solvent extraction of macerated plant material (t'Kindt et al., 2009). Different solvents, depending on their polarity, yield different compounds from plant material. One of the preliminary basic techniques used to screen crude natural compounds is thin layer chromatography (TLC). It is an official method prescribed in some herbal monographs of many pharmacopoeias and it is mostly used to characterize the first fingerprints of a crude extract which is usually made up of many different components (Tomczyk et al., 2010). The solvent system used to separate crude extracts on a TLC plate depends on the polarity of the solvent that was initially used to extract the plant material (Poole and Dias, 2000). TLC is fast, easy and it does not require pretreatment of samples. Large amounts of samples can be analyzed at the same time using TLC (Martelanc et al., 2009).

The detection and confirmation of separated components on a TLC plate can be observed by visualizing in daylight for colored substances, inspection under an ultraviolet (UV) light source for fluorescent components or by using selective detection (spray) reagents. UV visualization is normally done at 254 or 366nm (Kamatou et al., 2008). TLC data provides an idea of the compounds/substances present in a crude extract, and can be done qualitatively or quantitatively (Martelanc et al., 2009).

For complete separation and structural identification of a bioactive component from a plant material, TLC will need to be combined with other analytical techniques. These other techniques are column chromatography (CC), high performance liquid chromatography (HPLC), gas chromatography (GC), mass spectroscopy (MS) and nuclear magnetic resonance (NMR) (Martelanc et al., 2009; Tringali, 2001). HPLC analysis is used in separation and identification of non-volatile constituents of plant extracts by comparing their retention times to standard substances/compounds (Bienvenu et al., 2002; Kamatou et al., 2006; Manojlović et al., 2010). HPLC is further used for bioassay-guided fractionation procedures of crude plant extracts which helps in targeting and isolation of active compounds (Su et al., 2005; Tringali, 2001).

In CC, the stationary phase, a solid adsorbent, is placed in a vertical glass (usually) column and the mobile phase, a liquid, is added to the top and flows down through the column (by either gravity or external pressure) (Bhalla et al., 1967). CC is generally used as a purification technique to isolate desired organic compounds from a mixture (Hussein et al., 2007; Macías et al., 2008; Wang et al., 2009).

GC is normally coupled with MS to analyze thermally stable and volatile samples such as essential oils from plant materials (Dagnac et al., 2005; Tringali, 2001). GC-MS coupling is known to produce increased resolution, sensitivity and selectivity, and is important in plant metabolite identification (Novotny et al., 2008). MS produces a fragment pattern containing mass-to-charge ratios of particles which helps in determining the molecular weight of sample constituents (Wu et al., 2004). The identification of the constituents is done by comparing their retention indices as well as spectral data to corresponding data in respective libraries (Kamatou et al., 2006).

NMR is an excellent tool that gives detailed structural information of organic molecules. It is able to provide a structural difference between compounds with the same molecular mass or formula (Wu et al., 2004). Proton (^{1}H) NMR and carbon-13 (^{13}C) NMR are the most commonly used NMR techniques. ^{1}H NMR identifies hydrogen atoms/protons in an organic molecule while ^{13}C NMR allows the identification of carbon atoms in organic molecules. There is a universal occurrence of protons in plant metabolites, this makes ^{1}H NMR a great starting tool in structural elucidation of unknown constituents of bioactive crude extracts (Banci et al., 2010; Fan and Lane, 2008).

2.7.2 Bioassays and viability/cytotoxicity profiling

Bioassays are used in the investigation of natural products as part of the screening methods in drug development. The main objective of bioassays is to determine whether samples under study have any desired (or detrimental) biological activity. Screening with bioassays helps in the selection of plant materials for secondary testing and they are also incorporated in guided fractionation of crude extracts in order to isolate the bioactive component (Tringali, 2001).

The tetrazolium dye assay 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide, commonly referred to as MTT, is one of the most popular assay used to measure

cytotoxicity and cell proliferation. This assay is dependent on the ability of the mitochondrial enzymes in live cells to reduce the water soluble tetrazolium salt into a water insoluble purple formazan product. The healthy cells absorb the purple formazan and the absorption of light is done at wavelengths ranging from 540-570nm (Abd-Elazem et al., 2002; Kamatou et al., 2008; Liu and Nair, 2010; Twentyman and Luscombe, 1987).

MTT reduction is associated with mitochondria, cytoplasm and with non-mitochondrial membranes including the endosome/lysosome compartment and the plasma membrane (Berridge et al., 2005). The net positive charge on tetrazolium salts like MTT appears to be the predominant factor involved in their cellular uptake via the plasma membrane potential (Berridge et al., 2005). However, a second generation of tetrazolium dyes (MTS, XTT and WST-1) forms water-soluble formazans and require an intermediate electron acceptor for reduction which is characterised by a net negative charge and is therefore largely cell-impermeable (Berridge et al., 2005). In other words, MTT reduction occurs inside the cells (cytoplasm) while that of second generation dyes occurs outside the cell via plasma membrane electron transport. The mechanism of the described dyes is illustrated in Figure 2.10.

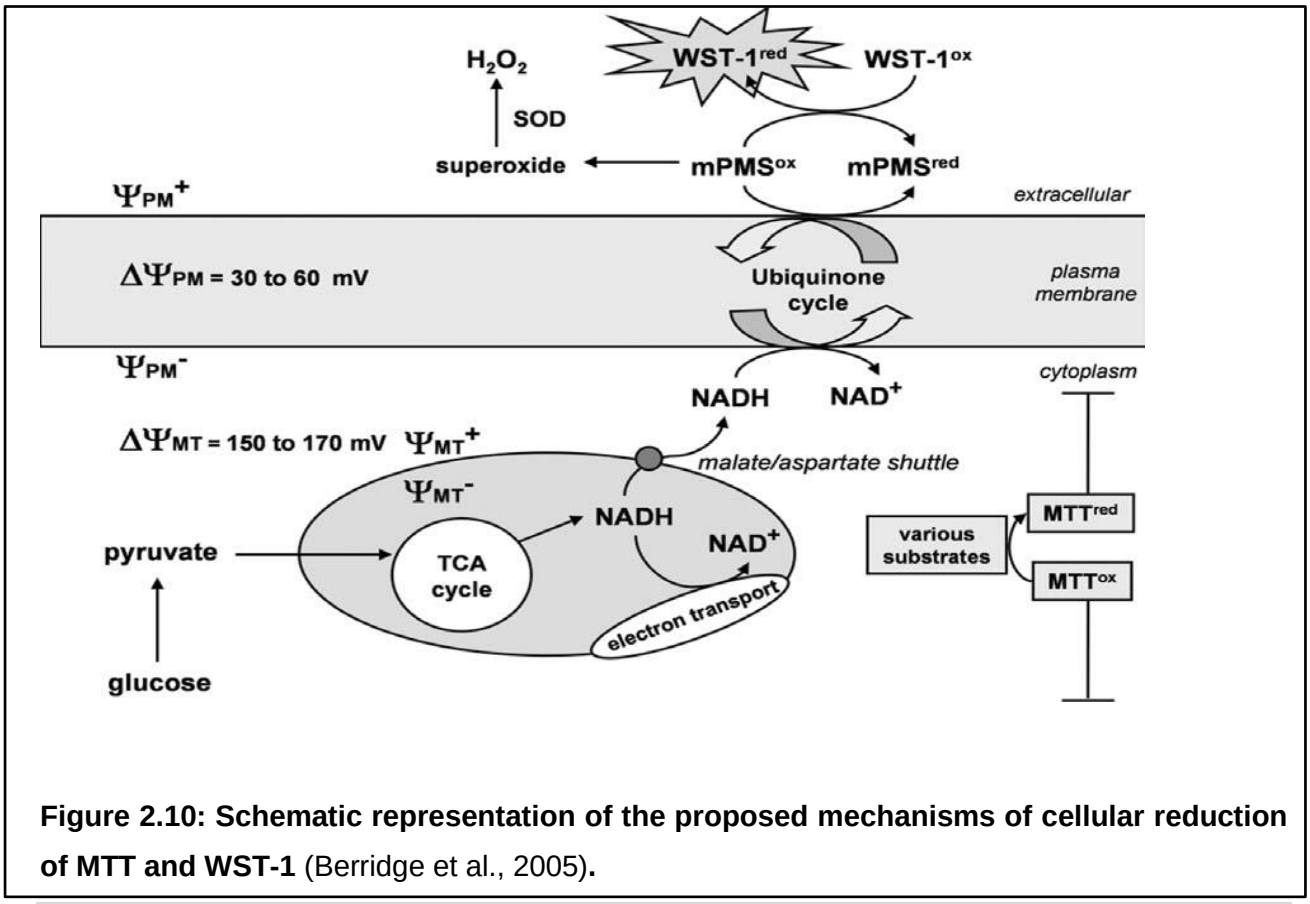

Figure 2.10: Schematic representation of the proposed mechanisms of cellular reduction of MTT and WST-1 (Berridge et al., 2005)**.**

Flow cytometry is a technique that measures and analyses cells in a suspension. Any particles with cell-like properties can be analysed with flow cytometry. It is used in many applications including measuring cell viability, cytotoxicity, apoptosis and intracellular antigens such as cytokines (Jaye et al., 2012). This technique works by measuring the fluorescence emitted from fluorescently labeled cells of interest, it measures single-particle emission and it is able to provide information on the configuration and internal complexity of cells (Jaye et al., 2012). A wide range of fluorescent labels/dyes called fluorochromes are used in this technique and each has a unique excitation and emission wavelength (Hadjoudja et al., 2009; Stanke et al., 2010).

In normal cell viability assays and techniques such as MTT and flow cytometry, the effect of a specific compound or extract is assessed at a single time point. This however does not explain the mechanism of action that takes place from the time point of sample addition (treatment) to the end-point for viability/toxicity analysis. These techniques overlook the possible multiple and kinetic effects that a certain extract/compound might possess (Abassi et al., 2009).

To overcome this limitation, a live cell morphological profiling process has been devised which is label/dye-free. It monitors the effects of substance addition to cells in real time. Measurement is done continuously, evaluating and displaying the data during the whole procedure (Abassi et al., 2009). The instrument is called real time cell analyzer (RTCA) or real time cell electronic sensor (RT-CES) and it has many cell based applications including cell proliferation, measuring cytotoxicity/apoptosis, cell adhesion and spreading, and cell migration and invasion (Figure 2.11). The major limitation of RTCA is that it only works with adherent cell lines, cells that can attach to the surface of a special tissue culture plate (Atienza et al., 2006).

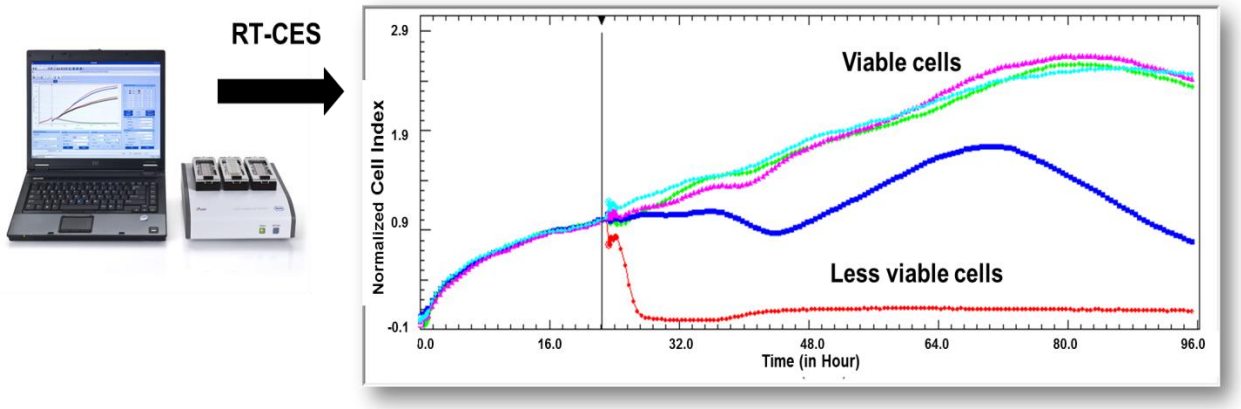

Figure 2.11: Real time cell electronic sensing device and representation of data.

RTCA uses microelectronic plates (E-plates) integrated with gold micro-electrode arrays on glass substrate in the bottom of the wells. Hence, the monitoring of the cells is microelectronic. The electrodes cover about 80% of the well bottom and a small electric field between electrodes is produced in the presence of media and a very low alternating current (AC) voltage of 10mV (Roche Diagnostics, Mannheim, Germany). This electric field is impeded by the presence of adherent cells and the degree of impedance is directly proportional to the number of cells attaching to the bottom of the well (Abassi et al., 2009; Atienza et al., 2006). Impedance values are converted to Cell Index (CI) values by the RTCA Software and these values appear on the y-axis on the generated results output chart (Figure 2.11). CI values reflect the number of cells, as well as morphological parameters such as size, shape and degree of cell attachment to the plates (Abassi et al., 2009; Atienza et al., 2006).

Assays to measure inflammation are becoming common practice in drug discovery screening and one of these techniques involves measuring effects on cytokine secretion. Because cytokines influence one another, there is a need to study a panel of these proteins simultaneously in what is referred to as multiplex cytokine analysis (Mitchell et al., 2009; Tateishi et al., 2010). Multiplex cytokine analysis technologies have become readily available in the past few years and there are two main formats that exist: multiplex sandwich ELISA and bead based assays (Lash et al., 2006). A comparison of the multiplex sandwich ELISA and bead based assay procedures was reported by Lash et al. (2006). According to the authors, each system had merits and there are several factors to consider when choosing a system for use. These factors

include range of analytes available, prospect of development of new analytes, dynamic range of the assay, sensitivity of the assay, the cost of equipment and consumables, ease of use and data analysis (Lash et al., 2006).

Cytometric Bead Array (CBA) is a flow cytometry application that was employed in this study (BD, Biosciences). This technique allows users to quantify multiple proteins simultaneously. The CBA system uses a broad dynamic range of fluorescence detection offered by flow cytometry and antibody-coated beads to efficiently capture analytes (BD, Biosciences). Each bead in the array has unique fluorescence intensity so that beads can be mixed and run simultaneously in a single tube allowing more cytokines to be measured in one assay. A bead array is generated, which is resolved in the red channel of a flow cytometer (Figure 2.12). This method significantly reduces sample requirements and time to results in comparison with traditional ELISA and Western blot techniques (BD Biosciences).

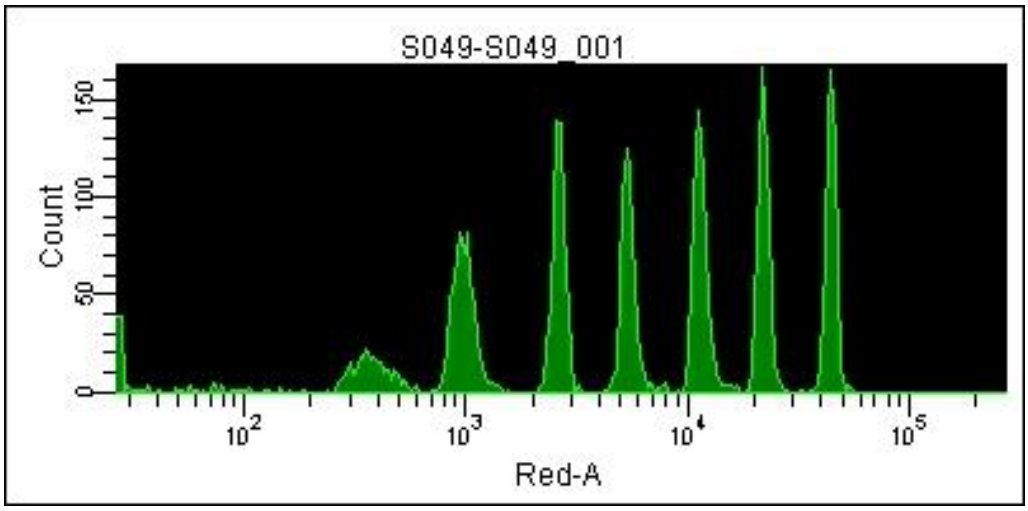

Figure 2.12: Seven bead populations with distinct fluorescence intensities.

Each peak represents a specific analyte.

There are other bioassays not detailed in this chapter used as screening strategies such as direct enzyme assays (HIV-1 PR, RT and IN), NO release and antioxidant assays. These assays are explained in detail in various methodology sections of this thesis.

2.8 Hypothesis

For this specific study, it was hypothesized that plants from the Lamiaceae family contain bioactive compounds able to inhibit HIV replication. Being nature-derived, these compounds should have other beneficial qualities as well (immunomodulation and anti-inflammatory/antioxidant activity). To investigate this hypothesis, the following objective was formulated and investigated through the described aims.

2.9 Purpose and objective of the study

The purpose of this study was to investigate extracts against HIV and where promising activity existed isolate active compounds from 16 Lamiaceae plants. The immune enhancing and antioxidant properties of the bioactive extracts and isolated compounds were to be investigated as well.

2.9.1 Aims

- The anti-HIV activity of fourteen Lamiaceae plants was investigated (chapter 3). Lamiaceae species have reported ethnobotanical uses including potential antiviral therapy.
- In chapter 4, research into one of the plants, *Plectranthus barbatus*, was undertaken. *P. barbatus* is documented in the literature as a plant traditionally used in HIV/AIDS treatment, however, the anecdotal evidence was not backed by empirical evidence. The immunomodulatory properties of this plant as well as cytotoxicity were also investigated.
- In chapter 5, bioassay-guided fractionation was undertaken to identify the constituents in *Ocimum labiatum* responsible for inhibiting HIV-1 PR. This plant has never been evaluated for anti-HIV-1 properties making this investigation novel.
- Chapter 6 was aimed at reporting on the evaluation of HIV-1 properties of triterpenoids isolated from *O. labiatum*.
- Research into the anti-inflammatory and antioxidant properties of *O. labiatum* was undertaken in chapter 7.

2.10 Screening strategy

The overall methodology for this study is summarized in the schematic illustration shown in Figure 2.13. In the figure, sixteen plants were collected from the herbarium of the University of Pretoria and all voucher specimens deposited in the herbarium. Organic extraction was carried out on fresh leaves of the plants to produce crude extracts and detailed explanations of the extraction procedure are provided in chapter 3 and appendix (Figure A3.1). Extracts were first screened for inhibitory properties against HIV-1 enzymes in direct enzyme assays. Only extracts from two plants, *P. barbatus* (chapter 4) and *O. labiatum* (chapter 5), inhibited HIV-1 protease (PR) by more than 50%. These two plants were further studied for other properties such as anti-inflammatory and antioxidant behavior. Of the two plants that inhibited HIV-1 PR well, *O. labiatum* demonstrated the strongest inhibition and was selected for bioassay-guided purification. Purification yielded compounds that inhibited HIV-1 PR (detail in chapter 5 and section 10.2 of the appendix), activated latent HIV-1 (detail in chapter 6 and section 10.3 of the appendix) and demonstrated anti-inflammatory properties (chapter 7). Information on the binding kinetics of pheophytin-a to HIV-1 PR is provided in the appendix (Figure A5.14). The remaining fourteen plants had moderate to weak inhibitory properties in the direct enzyme assays (PR and RT) but three of these plants demonstrated promising inhibition against HIV-1 expression (p24 antigen) in a chronically infected HIV cell line. Extracts were also tested for activity against HIV-1 integrase (IN) and two plant extracts showed moderate inhibition (detail in chapter 8 and section 10.1.2 of the appendix).

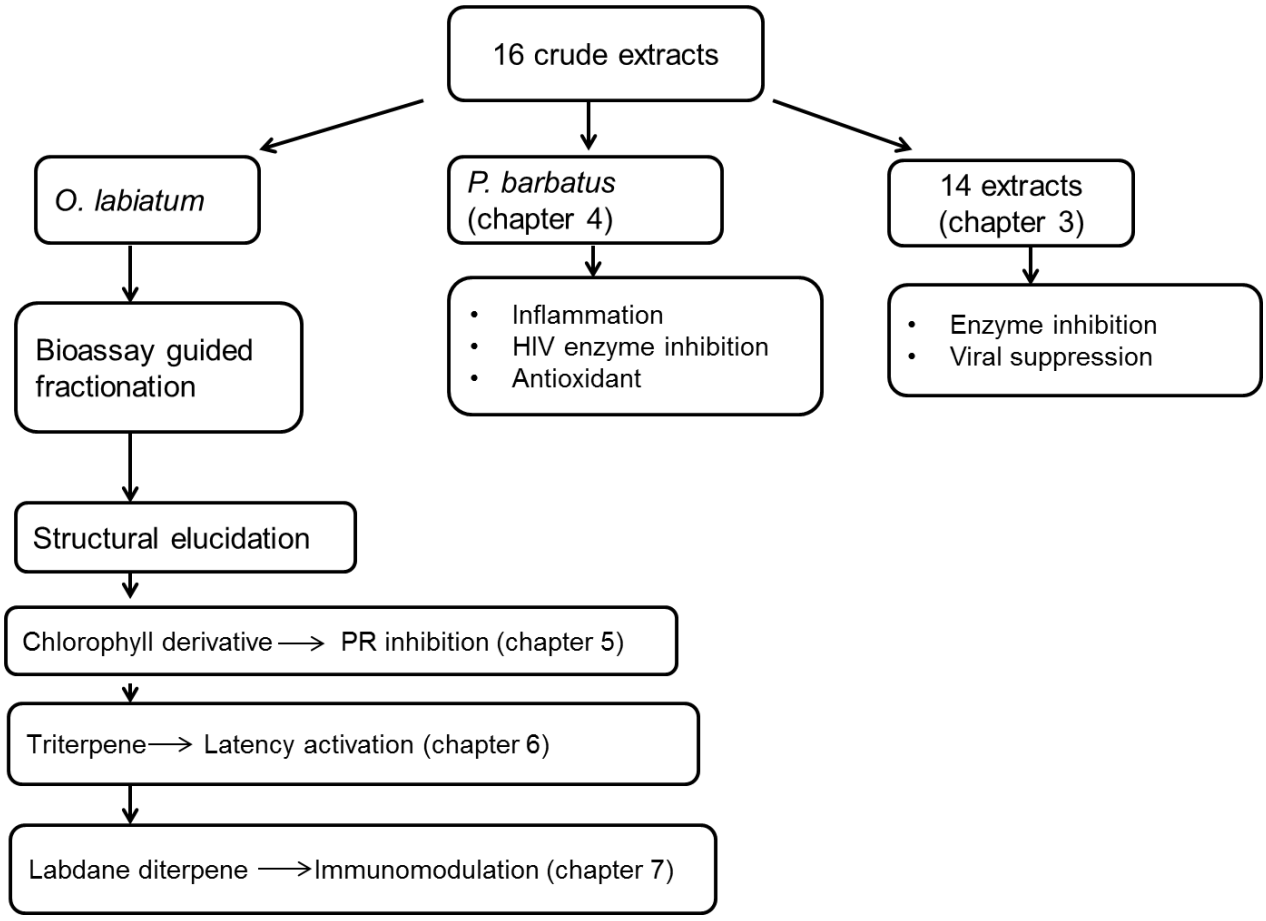

Figure 2.13: Work plan followed in this study.

2.11 Outputs

The research presented in this thesis was presented at various conferences and workshops including the 8th Conference of the Federation of African Immunological Societies, 2-5 December 2012, Durban, South Africa. A publication was generated from the work done on *P. barbatus* (Kapewangolo et al., 2013) and study chapters have been submitted to various journals for publication. These are indicated in the preface and on the front pages of the different data chapters.

CHAPTER 3

Lamiaceae plant extracts suppress HIV-1 expression in chronically infected monocytic cells

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Abstract

Background: Lamiaceae plants are traditionally used in African countries to treat viral and microbial ailments. Several Lamiaceae species are specifically used by traditional medicine practitioners to manage HIV/AIDS and associated opportunistic infections. The aim of this study was to evaluate the inhibitory properties of extracts of several Lamiaceae plants against HIV-1 protease (PR), reverse transcriptase (RT) and virus expression in a chronic infection model in order to contribute some scientific evidence for the apparently successful traditional use against HIV/AIDS.

Methods*:* Crude extracts were prepared from the leaves of fourteen Lamiaceae plants. These extracts were first tested for inhibitory activities against HIV-1 enzymes (PR and RT). Cytotoxicity was also investigated in TZM-bl cells and human peripheral blood mononuclear cells (PBMCs). Extracts that inhibited HIV-1 enzymes were screened for their ability to suppress HIV-1 replication in a chronically infected monocytic U1 cell line at non-cytotoxic concentrations because the enzyme inhibitory activity suggested a possible viral prevention mechanism.

Results: Phorbol 12-myristate 13-acetate (PMA)-induced HIV-1 expression, as determined by HIV-1 p24 antigen ELISA, was significantly ($p<0.05$) reduced in U1 cells by *Ocimum serratum*, *Salvia apiana* and *Stachys byzantina* extracts at non-cytotoxic

concentrations. The same extracts inhibited HIV-1 PR and RT. CC_{50} values of the three extracts ranged from 33.6 to >100 µg/ml which translates to low toxicity.

Conclusions: This study provides evidence that *O. serratum, S. apiana and S. byzantina extracts* inhibit HIV-1 replication, presumably by blocking HIV-1 enzymes PR and RT, and supports the traditional use of these plants in the treatment of HIV/AIDS.

Keywords: HIV-1, HIV-1 protease, HIV-1 reverse transcriptase, Lamiaceae.

3.1 Introduction

Plants contain many different compounds known to exhibit mechanisms for controlling plant virus attacks and these compounds may have activity against mammalian viruses as well (Kang et al., 2005; Matsuse et al., 1999). This is why a possible role for natural products in the fight against the transmission of the human immunodeficiency virus (HIV) or the associated acquired immune deficiency syndrome (AIDS) is routinely investigated.

Plant products have been identified as possible anti-HIV agents with many investigated as potential inhibitors of the different steps of the viral life cycle. These steps include those involving key enzymes that play a role in the replication of the viral RNA in the host cell (Harnett et al., 2005; Matsuse et al., 1999). HIV-1 reverse transcriptase (RT) is a multifunctional enzyme and its key function is to transcribe the viral genomic RNA to viral DNA. It is this viral DNA that is integrated in the host genome by HIV-1 integrase. HIV-1 protease (PR) is responsible for the processing of polyproteins which are catalytic viral proteins needed for virus maturation and efficient infection (Friedler et al., 1999; Todd et al., 1998). Most existing HIV treatment inhibits one of the mentioned enzymes.

Some of the crude plant extracts with reported anti-HIV-1 RT activity are those from *Hyptis lantanifolia* (Lamiaceae), *Sutherlandia frutescens* (Fabaceae) and *Lobostemon trigonus* (Boraginaceae) (Harnett et al., 2005; Matsuse et al., 1999). Calanolide is a potential HIV-1 RT inhibitor from the *Calophyllum* species, the only naturally derived potential HIV drug presently undergoing clinical trials (César et al., 2011). Other plants with potential HIV-1 inhibitory properties are *Hypoxis sobolifera* (Hypoxidaceae) and *Bulbine alooides* (Asphodelaceae) (Klos et al., 2009).

Lamiaceae (mint) family contain plants with a diversity of ethnobotanical uses (Van Wyk, 2011; Van Wyk et al., 2009). Southern Africa is one of the regions where these plants are found and communities in this region use Lamiaceae species to treat various ailments and traditional treatment usually occurs in the absence of scientific data on efficacy (Lukhoba et al., 2006; Van Wyk et al., 2009). Medicinal properties of Lamiaceae plants include antibacterial, antifungal and antiviral activity (Kamatou et al., 2006; Lukhoba et al., 2006; Prabhu et al., 2009). Some of the plants are also administered to patients with malaria (Asase et al., 2005). It is the apparent traditional success of Lamiaceae plants that prompts researchers to evaluate the plants' pharmacological activities by either finding evidence for the anecdotal success (Kapewangolo et al., 2013) or simply attempting to isolate potent novel compounds with promising antimicrobial properties (Alasbahi and Melzig, 2010a; Kamatou et al., 2008). The traditional use of Lamiaceae plants in managing HIV/AIDS is widely documented (Chinsembu and Hedimbi, 2010; Kisangau et al., 2007; Lamorde et al., 2010; Omoruyi et al., 2012; Semenya et al., 2013) and some traditional healers claim that their herbal prescriptions are side-effect free (Semenya et al., 2013). Lamiaceae species from *Leonotis*, *Ocimum*, *Plectranthus*, *Salvia*, *Stachys* and *Tetradenia* are commonly used to alleviate symptoms in HIV infected individuals (Kisangau et al., 2007; Lamorde et al., 2010; Semenya et al., 2013; Yamasaki et al., 1998). Mechanisms by which these species assist in improving the health of HIV affected individuals' or by which other antimicrobial (fungal or bacterial) effects are demonstrated needs to be assessed in order to identify potent drug candidates.

Searches for new therapies against HIV continue due to the growing problem of drug resistance and adverse side effects of existing drugs. In this study, fourteen Lamiaceae herbs were screened for inhibition of HIV-1 RT and PR as well as cytotoxicity. Some of the plants studied are used by traditional healers in treating HIV/AIDS (Omoruyi et al., 2012; Semenya et al., 2013). In addition; the extracts were studied for the ability to reduce HIV-1 expression in infected monocytic cells. Only extracts which demonstrated activity against RT and PR were investigated for the ability to inhibit viral expression because inhibition of these enzymes was suggestive of a mechanism of action for possible prevention of viral expression. It is however possible that extracts that did not inhibit RT and PR may still possess the ability to inhibit viral expression by other means (e.g maturation/assembly inhibition, inhibition of other viral enzymes etc.) Investigating plant products for various mechanisms of HIV-1 inhibition is becoming common practice

because some constituents demonstrate unique abilities, for example plant derived bevirimat is a potent HIV-1 maturation inhibitor (Stoddart et al., 2007).

3.2 Methods

3.2.1 Plant extracts preparation

The following Lamiaceae plants were collected during April (2011) from the Botanical garden of the University of Pretoria: *Lavandula lanata*, *Leonotis leonurus*, *Ocimum serratum*, *Plectranthus ciliatus*, *P. ecklonii*, *P. neochilus*, *Salvia africana-lutea*, *S. apiana*, *S. canariensis*, *S. disermas*, *S. greggii*, *S. leucantha*, *Stachys byzantina* and *Tetradenia riparia*. Plant identification was done in the H.G.W.J Schweikerdt herbarium of the University and voucher specimens are kept in the herbarium.

Fresh leaves were blended in ethanol (99.9%) and vacuum filtered. The filtrates were concentrated under reduced pressure at 50 °C using a rotary evaporator (Buchi, Flawil, Switzerland). The residue was re-dissolved in ethyl acetate to exclude highly polar tannin compounds; non-specific enzyme inhibitors. The ethyl acetate fractions were dried and stored in the dark at 4 °C until use. Extracts were reconstituted in dimethyl sulfoxide (DMSO; Sigma, St Louis, MO, USA), which provides a sterile environment, before each biological assay. Further dilutions to obtain desired extracts concentrations were done in either cell culture media for cytotoxicity or buffer for HIV-1 assays. The plants, together with their voucher numbers, laboratory codes and yields from extraction are summarised in Table 3.1.

Table 3.1: Lamiaceae extract information with yields (w/w) and extract codes.

Code	Plant identity	Voucher specimen number	Initial weight of fresh plant leaves (g)	Yield[a] (%, w/w)
1	*Lavandula lanata*	117692	66.8	34.1
2	*Leonotis leonurus*	117197	53.3	40.9
3	*Ocimum serratum*	117694	99.6	21.7
4	*Plectranthus ciliatus*	117199	86.7	17.3
5	*Plectranthus ecklonii*	117695	85.7	26.1
6	*Plecanthrus neochilus*	117696	282.9	7.4
7	*Salvia africana-lutea*	117706	67.0	58.2
8	*Salvia apiana*	117698	156.7	14.7
9	*Salvia canariensis*	117705	157.3	16.3
10	*Salvia disermas*	117699	89.4	41.6
11	*Salvia greggii*	117700	46.2	48.1
12	*Salvia leucantha*	117704	123.4	18.6
13	*Stachys byzantina*	117701	55.5	25.6
14	*Tetradenia riparia*	117702	120.5	17.5

[a]Yield represents the percentage recovery of dried extract per weight as compared to the original fresh plant material.

3.2.2 Cell viability and proliferation

The effect of the crude extracts on the viability of TZM-bl and U1 cells was determined by quantifying the amount of a formazan product metabolized by viable cells from MTT solution (Sigma, St Louis, MO, USA) (Mosmann, 1983). Cells were seeded in 96 well plates (Corning Incorporated, Corning, NY, USA) at 1 x 10^4 cells per well. Extracts were added to the cells at final concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 ug/ml. After a 72 h incubation, at 37 °C in humidified air with 5% CO_2, the plates were read at 550/690 nm using a microtiter plate reader (Multiskan Ascent; Thermo Labsystems; Waltham, MA, USA). Control wells included a negative control (cells and medium only), blank controls for each extract (extract and medium only), a toxicity control (Auranofin; Sigma, St Louis, MO, USA) (Mirabelli et al., 1985) and a DMSO control (percentage of DMSO similar to extracts in cells to ensure that this solvent did not cause cell death). The percentage viability was calculated relative to untreated control cells.

Ethical approval for obtaining blood samples from consenting donors was granted by the Faculties of Natural and Agricultural Sciences and Health Sciences Ethics Committees (EC080506-019; 163/2008, University of Pretoria, South Africa). Freshly isolated uninfected PBMCs were plated in 96 well plates (Corning Incorporated, Corning, NY, USA) at 1 x 10^5 cells per well and treated with crude extracts at final concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml. The number of viable cells was detected after 72 h using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution (Promega, Promega Corporation, Madison, WI USA). Control wells included a toxicity control (Auranofin); a known toxic compound with antitumor activity (Mirabelli et al., 1985), and the plates were read at 492/690 nm. The percentage viability was calculated relative to an untreated control of cells only.

3.2.3 HIV-1 RT assay

The effect of the crude extracts on reverse transcription was tested using an RT colorimetric assay kit from Roche Diagnostics (Mannheim, Germany) and a purified recombinant HIV-1 RT (Merck, Darmstadt, Germany). The assay was performed according to the method previously described (Fonteh et al., 2009). Extracts were tested at 100 and 50 µg/ml. The enzyme (0.2U) was incubated for 1 h with the extracts at 37 °C. Subsequent 1 h incubations involved addition of an antibody that is conjugated to peroxidase that binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate solution [2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)] is cleaved by the peroxidase enzyme, producing a colored reaction product. The absorbance of the samples was read at 405 nm with a reference wavelength of 492 nm using a microplate reader (Multiskan Ascent; Thermo Labsystems; Waltham, MA, USA) and was directly correlated to the level of RT activity in the sample. Doxorubicin (Sigma, St Louis, MO, USA), a known HIV-1 RT inhibitor, was used as a positive control (Kapewangolo et al., 2013).

3.2.4 HIV-1 PR fluorogenic assay

This assay was a modified version of the assay performed by Lam et al. (Lam et al., 2000). The fluorogenic HIV PR substrate 1 (Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(DABCYL)-Arg; Sigma, St Louis, MO, USA) was dissolved in DMSO to make a 1 mM stock. The stock fluorogenic substrate was diluted to 10 µM using assay

buffer (0.1 M sodium acetate, 1 M NaCl, 1 mM EDTA and 1 mM DTT, pH 4.7). An aliquot of the substrate (10 µM, 49 µl) and 1 µl of HIV-1 PR solution (1 µg/ml; Bachem, Bubendorf, Switzerland) were added to the reaction mixture in an assay buffer in the presence or absence (untreated control) of tested extracts (100 and 50 µg/ml) to make a final reaction volume of 100 µl. The mixture was incubated at 37 °C for 1 h in black 96 well assay plates. The fluorescence intensity was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a synergy microplate spectrofluorometer (Thermo Labsystems; Waltham, MA, USA). Acetyl pepstatin (AP; Sigma, St Louis, MO, USA) was used as a positive control for HIV-1 PR inhibition. The blank treatment consisted of assay buffer with only the substrate and an untreated control comprising enzyme and substrate was also included. The percentage inhibition was calculated based on the formula: 100 – [(Test reagent RFU – background RFU)/(untreated control RFU – blank) x 100] where RFU = relative fluorescence units.

3.2.5 Induction of HIV-1 expression

U1 cells are latently infected with HIV-1 and active HIV-1 replication can be induced by stimulation with phorbol 12-myristate 13-acetate (PMA; Sigma, St Louis, MO, USA). These cells are used as a model of latent and chronic HIV-1 infection (Folks et al., 1988; Kalebic et al., 1991). To study the effect of the extracts on PMA-mediated induction of HIV expression, U1 cells (1 x 10^5 cells/well) were pre-treated with non-cytotoxic concentrations of the extracts for 6 h at 37 °C in humidified air with 5% CO_2. PMA (2 ng/ml) was then added to the culture and incubated further. The supernatant was collected after 72 h and HIV activity was monitored by measuring HIV-1 p24 antigen in the culture supernatant.

3.2.6 Antiviral assay: measurement of HIV-1 p24 antigen

The p24 antigen level in the cell culture supernatant was measured using a RETRO-TEK HIV-1 p24 antigen ELISA (ZeptoMetrix Corporation, Buffalo, NY, USA). Special microplate wells coated with a monoclonal antibody specific for the p24 gag gene product of HIV-1 were used to capture the p24 antigen in the supernatant. The captured antigen was then reacted with a human anti-HIV-1 antibody conjugated to horseradish peroxidase (HRP) and the substrate was subsequently added resulting in color development as the HRP enzyme reacts. The optical density of each well was read at

450 nm using a microplate reader (Multiskan Ascent; Thermo Labsystems; Waltham, MA, USA).

3.3 Statistical analysis

The 50% cytotoxic concentration (CC_{50}) of each extract was obtained using Graphpad Prism (Graphpad Software Inc. CA, USA). This was computed as the concentration of the extract that reduced cell viability by 50% when compared to controls. Data for HIV-1 inhibition were analysed for statistical significance and expressed as mean values ±standard deviation (SD). The mean values were compared using Student's *t-test*. P values of <0.05 were considered statistically significant.

3.4 Results

The effects of the extracts on the viability of TZM-bl and PBMCs were tested at various concentrations in order to determine the CC_{50}s of the extracts. The CC_{50} values are displayed in Table 3.2. Auranofin, the positive control used for cytotoxicity, produced CC_{50} values less than 10 µM in both TZM-bl and PBMCs. *Plectranthus* extracts tested presented anti-cancer properties. These extracts were more toxic to the two cell types compared with extracts of the other Lamiaceae plants. Cytotoxicity of *P. ciliatus* stood out, CC_{50} values were <10 ug/ml in both TZM-bl and PBMCs.

Moderate HIV-1 enzyme inhibition was observed in five extracts. Extracts from *O. serratum* (3)*, P. ciliatus* (4), *P. neochilus* (6)*, S. apiana* (8) *and S. byzantina* (13) either inhibited HIV-1 PR or RT by more than 40% at 100 or 50 µg/ml (Table 3.3). The rest of the extracts demonstrated inhibition less than 40% at the tested concentrations. The recommended criteria for activity of crude extracts is generally at or below 100 µg/ml (Cos et al., 2006). Therefore, inhibitory activity at less than 100 µg/ml is considered good. Promising (better than moderate) inhibition worthy of further investigation is measured at ≥ 50%. As expected the controls exhibited positive enzyme inhibition. Doxorubicin inhibited HIV-1 RT activity with an IC_{50} less than 25 µg/ml and acetyl pepstatin inhibited HIV-1 PR with an IC_{50} of 0.3 µg/ml.

Table 3.2: Cytotoxic effects of Lamiaceae extracts.

		CC_{50} (µg/ml) ± SD[a]	
	Plant extract	TZM-bl	PBMCs
1	*Lavandula lanata*	45.8 ± 1.1	16.7 ± 0.1
2	*Leonotis leonurus*	>100	29.8 ± 0.2
3	*Ocimum serratum*	59.0 ± 0.9	81.7 ± 3.8
4	*Plectranthus ciliatus*[b]	9.7 ± 0.2	4.2 ± 0.0
5	*Plectranthus ecklonii*	33.7 ± 1.0	8.0 ± 0.1
6	*Plecanthrus neochilus*	33.0 ± 0.8	16.8 ± 0.2
7	*Salvia africana-lutea*	35.7 ± 0.7	16.1 ± 0.1
8	*Salvia apiana*	70.8 ± 14.9	34.0 ± 0.3
9	*Salvia canariensis*	33.9 ± 0.4	29.9 ± 0.1
10	*Salvia disermas*	60.8 ± 1.1	32.2 ± 0.2
11	*Salvia greggii*	>100	65.2 ± 0.9
12	*Salvia leucantha*	55.3 ± 2.3	26.1 ± 0.2
13	*Stachys byzantina*	>100	84.7 ± 0.1
14	*Tetradenia riparia*	35.5 ± 0.4	28.4 ± 0.1
	Auranofin[c]	<10 µM	<10 µM

[a]The values are 50% cytotoxic concentrations ± SD computed from repeats of six independent experiments and are indicating the effect of crude extracts on the viability of TZM-bl cells and PBMCs. [b]*P. ciliatus* extract is currently being studied for anticancer properties (Le Roux, 2013). [c]Positive control for toxicity.

Table 3.3: Inhibition of HIV-1 enzymes by Lamiaceae extracts at (A) 100 µg/ml (B) 50 µg/ml.

		Reverse transcriptase inhibition (%)		Protease inhibition (%)	
	Plant extract	A[a]	B[b]	A	B
1	*Lavandula lanata*	12.4 ±2.2	24.0 ±0.8	0.0	0.0
2	*Leonotis leonurus*	18.2 ±2.9	21.6 ±0.5	20.7 ±2.8	30.9 ±7.2
3	*Ocimum serratum*	30.4 ±3.4	41.4 ±2.5	30.3 ±1.3	36.9 ±0.2
4	*Plectranthus ciliatus*	19.1 ±0.5	13.8 ±0.6	44.2 ±2.3	42.6 ±4.4
5	*Plectranthus ecklonii*	21.8 ±2.8	21.9 ±0.8	39.5 ±3.9	0.0
6	*Plecanthrus neochilus*	20.5 ±4.2	10.1 ±2.1	46.3 ±3.3	35.5 ±7.5
7	*Salvia africana-lutea*	14.9 ±1.8	10.7 ±2.0	12.6 ±5.2	0.0
8	*Salvia apiana*	33.6 ±4.2	45.1 ±8.2	33.2 ±3.1	39.4 ±3.0
9	*Salvia canariensis*	14.5 ±3.1	24.5 ±5.2	0.0	7.8 ±0.2
10	*Salvia disermas*	25.4 ±5.0	23.0 ±2.2	31.2 ±3.3	31.9 ±7.5
11	*Salvia greggii*	4.5 ±2.4	21.0 ±0.5	37.2 ±3.8	31.6 ±2.9
12	*Salvia leucantha*	25.9 ±7.1	17.8 ±6.8	24.7 ±0.4	27.0 ±4.7
13	*Stachys byzantina*	28.3 ±0.6	22.9 ±2.2	47.0 ±5.2	40.9 ±3.6
14	*Tetradenia riparia*	26.5 ±4.9	17.9 ±4.9	36.6 ±3.9	0.0
	Doxorubicin[c]		95.9 ±0.8		
	Acetyl pepstatin[d]			98.6 ±3.5	

Each value represents the mean percentage inhibition ±SD from six independent experiments. Each plant extract was tested at [a](A) 100 and [b](B) 50 µg/ml. [c]A known inhibitor, doxorubicin (50 µg/ml), was used as a positive control for HIV-1 reverse transcriptase inhibition. *O. serratum* (3) and *S. apiana* (8) inhibited HIV-1 RT by more than 40%. [d]Acetyl pepstatin, (1 µg/ml) was used as a positive control for HIV-1 protease inhibition. *P. ciliatus* (4), *P. neochilus* (6) and *S. byzantina* (13) inhibited HIV-1 PR by more than 40% at the two tested concentrations.

Extracts that demonstrated moderate anti-HIV-1 activity were tested for their ability to suppress HIV-1 expression in the chronically HIV-1 infected U1 cell line. Due to the extreme cytotoxicity demonstrated by *P. ciliatus* extract in TZM-bl and PBMCs (Table 3.2), it was excluded from the HIV-1 expression study. Cytotoxicity in unstimulated U1 cells of HIV-1 enzyme inhibiting extracts was first determined and the CC_{50}s are shown in Table 3.4.

Table 3.4: Cytotoxicity of HIV-1 bioactive extracts on unstimulated U1 cells

	CC_{50} (µg/ml) ± SD
Plant extract	U1 cells
Ocimum serratum	76.6 ± 0.6
Plectranthus neochilus	24.6 ± 0.3
Salvia apiana	33.6 ± 0.4
Stachys byzantina	96.5 ± 2.0
Auranofin*	<10

*Positive control for toxicity

Extracts were then tested at concentrations lower than CC_{50} (viability >50%) for the ability to inhibit HIV-1 replication and the results are presented in Figure 3.1. *O. serratum*, *S. apiana* and *S. byzantina* significantly ($p<0.05$) reduced HIV-1 expression in chronically infected U1 cells with *S. byzantina* extract being the most significant in reducing viral replication (Figure 3.1A). *P. neochilus* also reduced HIV-1 replication in U1 cells, however, the difference was not significant ($p>0.05$) when compared to PMA stimulated control cells. U1 cells express minimal HIV-1 p24 protein under normal growth conditions. The levels of HIV-1 p24 core protein increases remarkably in the presence of PMA (stimulant) and reduction in p24 secretion is indicative of replication/activation inhibition (Kalebic et al., 1991). Viability of PMA-treated U1 cells in the presence/absence of extracts was again tested after the supernatant was collected and the data obtained confirmed that the ability of the extracts to reduce HIV-1 expression in U1 cells was not due to cytotoxicity because viability for all treatments was more than 60% (Figure 3.1B).

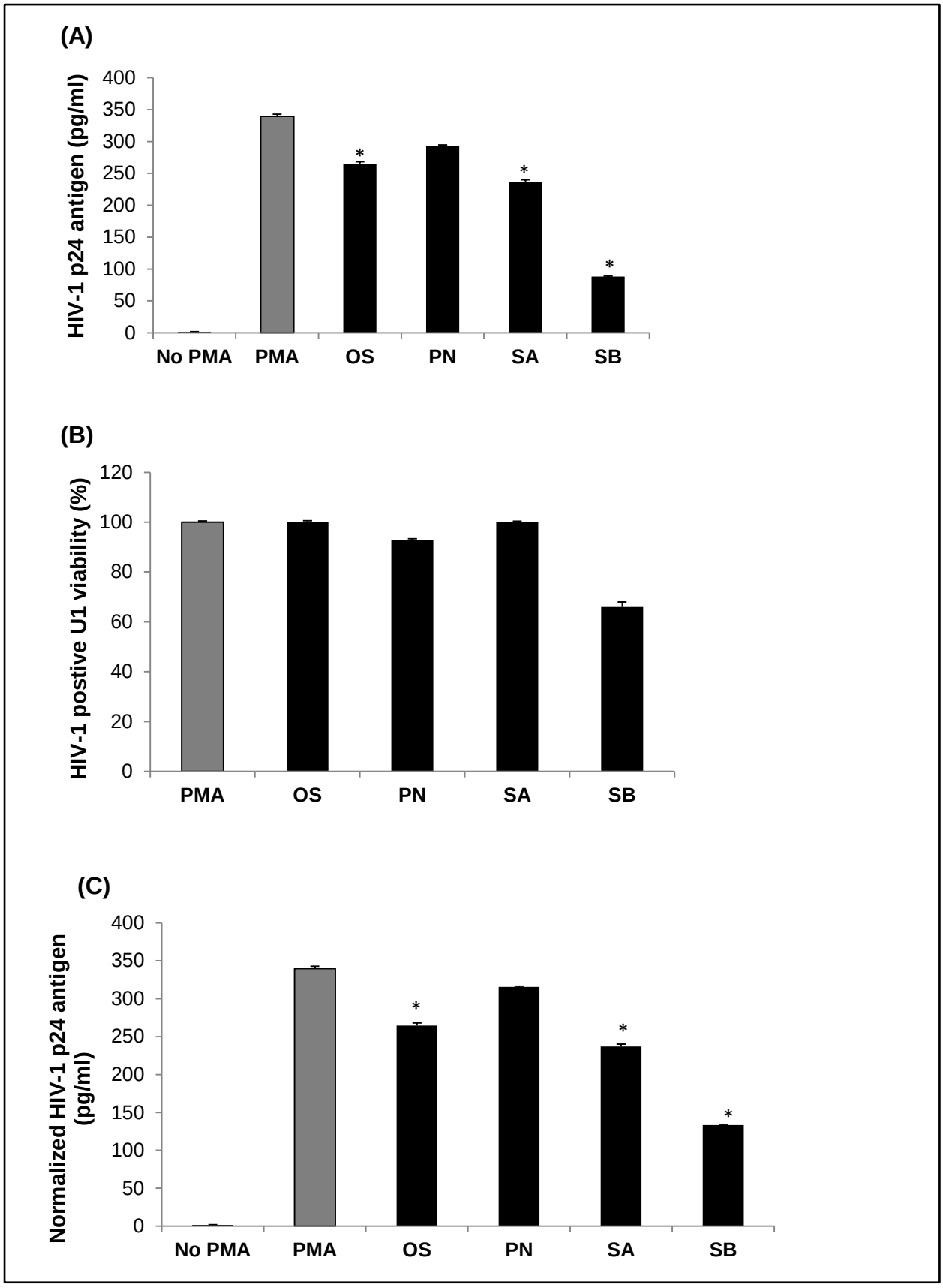

Figure 3.1: Inhibition of HIV-1 replication by selected Lamiaceae extracts in U1 cells. (**A**) Cells were incubated with non-cytotoxic concentrations of **OS**=*O. serratum* (50 µg/ml), **PN**=*P. neochilus* (12.5 µg/ml), **SA**=*S. apiana* (25 µg/ml) and **SB**=*S. byzantina* (50 µg/ml) for 72 h in the

presence of PMA (2 ng/ml). Controls included unstimulated (no PMA) and stimulated cells (PMA treated cells). HIV-1 expression was monitored by measuring HIV-1 p24 antigen in culture media. All the extracts tested reduced HIV-1 replication in U1 cells with OS, SA and SB (*) being significant ($p<0.05$). (**B**) Data demonstrating that HIV-1 inhibition in U1 cells by extracts was not affected by cytotoxicity. Viability of PMA-induced U1 cells in the presence of extracts was more than 50% after 72 h. **(C)** Normalized HIV-1 p24 antigen, taking cell number into account, still indicated significant ($p<0.05$) reduction of HIV-1 expression by OS, SA and SB (*).

3.5 Discussion

Many herbal extracts are reportedly used in managing HIV/AIDS, some with proven pharmacological activities (Harnett et al., 2005; Kapewangolo et al., 2013) and many others without (Chinsembu and Hedimbi, 2010; Lamorde et al., 2010; Semenya et al., 2013). The *in vitro* data of the present study suggests that some plants from the Lamiaceae family have anti-HIV activity.

All extracts, except for *O. serratum*, were more toxic to PBMCs than TZM-bl cells and this could be due to the different characteristics of the two cell types. TZM-bl is a genetically modified HeLa cell line that expresses large amounts of CD4 and CCR5 (Platt et al., 1998). TZM-bl cells contain integrated copies of the luciferase and ß-galactosidase genes under control of the HIV-1 promoter which makes the cell line highly sensitive to infection with diverse isolates of HIV-1 (Todd et al., 2012). TZM-bl cells are commonly used in HIV-1 infectivity studies as are PBMCs, containing important cells of the immune system (Fonteh et al., 2011; Todd et al., 2012). PBMCs are commonly incorporated in cytotoxicity studies, especially if the focus of the study is on a disease that affects the immune system (Oyaizu et al., 1993). *P. ciliatus*'s toxicity suggests possible anti-cancer properties and according to the National Cancer Institute (NCI), crude extracts with CC_{50} values <30 µg/ml are considered to potentially have anticancer activity (Talib and Mahasneh, 2010).

Most studies in the literature that looked at inhibitory properties of plant extracts on HIV enzymes report promising inhibitory activity (≥50%) at concentrations >100 µg/ml (Estari et al., 2012; Klos et al., 2009; Rege et al., 2010) which made the moderate inhibitory activity obtained at the concentrations used in this study worthy of further investigation. Studies have been done on the chemical constituents of *P. neochilus*, *S. apiana* and *S. byzantina* (Asnaashari et al., 2010; Caixeta et al., 2011; Khanavi et al., 2005; Taylor et

al., 2010). There are also reports on the antimicrobial properties of these plants (Adams and Garcia, 2005; Saeedi et al., 2008; York et al., 2011) but minimal information in the literature on *O. serratum*. None of the four Lamiaceae plants were previously investigated for anti-HIV activity making this study a first. The plants however belong to genera (*Ocimum*, *Plectranthus*, *Salvia* and *Stachys*) with reported antiviral properties (Kisangau et al., 2007; Mouhajir et al., 2001). Decoctions/infusions prepared from leaves of certain Lamiaceae plants are commonly administered orally to manage HIV/AIDS (Chinsembu and Hedimbi, 2010; Kisangau et al., 2007; Lamorde et al., 2010) and data obtained from this study partially explains the popular use of plants from this family. The results of the present study showed that *O. serratum*, *P. neochilus*, *S. apiana* and *S. byzantina* extracts have the ability to inhibit HIV-1 replication in chronically infected cells possibly by inhibiting HIV-1 RT and PR. GUT-70, a tricyclic coumarin isolated from the stem bark of *Calophyllum brasiliense* reportedly inhibits HIV-1 replication in PMA treated U1 cells through suppression of nuclear factor kappa B (NF- κβ); NF- κβ is an inducible cellular transcription factor known to play a major role in regulating HIV-1 gene expression (Kudo et al., 2013). Extracts in the present study might have reduced HIV-1 replication through suppression of NF- κβ but further analysis need to be carried out to confirm this likelihood. Lamiaceae plants contain various terpenoid compounds which are often the active ingredients discovered in some medicinal plant extracts (Salminen et al., 2008). The presence of terpenoids in these plants is linked to enhancing immunity and managing opportunistic infections in HIV/AIDS patients (Kisangau et al., 2007). Since it was crude extracts studied here, active compounds may be something other than terpenes and inhibitory activity may also be due to synergism of mixed compounds.

In South Africa, the use of traditional medicine among individuals with moderate and advanced HIV infection is common (Omoruyi et al., 2012; Peltzer et al., 2008). There is a continuous search for novel anti-HIV drugs because of the adverse side effects of existing drugs as well as the existence of HIV drug resistant strains (Brennan-Benson, 2009). Natural products remain an excellent source of novel compounds for drug discovery because toxicity of drugs from nature is rarely an issue (Park et al., 2009). The majority of drugs on the market are plant-derived (Newman and Cragg, 2012) and the area of infectious diseases is largely dependent on natural products and their structures for sources of better treatment. The anti-HIV-1 results from the present study lends support for further investigation of the bioactive extracts of the plant genera

mentioned here to validate the use of these plants in traditional medicine for managing HIV/AIDS and/or to identify potential drugs.

3.6 Conclusion

The work presented here continues the worldwide search for new inhibitors of HIV and is directly connected to the traditional use of Lamiaceae plants in HIV/AIDS treatment. Synergistic interactions between compounds in crude extracts have been reported to occur (Ma et al., 2009). Hence, the need to first test crude extracts before purification of bioactive constituents. This study shows that *O. serratum*, *P. neochilus*, *S. apiana* and *S. byzantina* contain inhibitory compounds active against HIV-1 replication possibly through inhibition of HIV-1 PR and RT. Isolating the active constituents will help establish exact mechanisms of inhibition as well as standardize the extracts for potential clinical translation.

3.7 Authors' contributions

DM designed the study; PK conducted the research and wrote the first draft of the manuscript. DM edited and revised the manuscript. Both authors approved and reviewed the data interpretation and wording in the final draft of the manuscript.

Acknowledgements

We would like to thank the following organisations for financial support: Southern African Biochemistry and Informatics for Natural Products (SABINA), Ministry of Education (Namibia), Technology Innovation Agency (TIA, RSA) and the University of Pretoria.

CHAPTER 4

Inhibition of HIV-1 enzymes, antioxidant and anti-inflammatory activities of *Plectranthus barbatus*

Published in *Journal of Ethnopharmacology*

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Abstract

Ethnopharmacological relevance

Plectranthus barbatus is widely used in African countries as an herbal remedy to manage HIV/AIDS and related conditions.

Aim of the study

To investigate the HIV-1 inhibitory, anti-inflammatory and antioxidant properties of *P. barbatus* and thereby provide empirical evidence for the apparent anecdotal success of the extracts.

Materials and methods

Ethanolic extract of *P. barbatus*'s leaves was screened against two HIV-1 enzymes; protease (PR) and reverse transcriptase (RT). Cytotoxicity of the extract was determined through measuring tetrazolium dye uptake of peripheral blood mononuclear cells (PBMCs) and the TZM-bl cell line. Confirmatory assays for cytotoxicity were performed using flow cytometry and real-time cell electronic sensing (RT-CES). The

free radical scavenging activity of the extract was investigated with 2,2-diphenyl-1-picrylhydrazyl while the anti-inflammatory properties of the plant extract were investigated using a Th1/Th2/Th17 cytometric bead array technique.

Results

P. barbatus extract inhibited HIV-1 PR and the 50% inhibitory concentration (IC_{50}) was 62.0 µg/ml. The extract demonstrated poor inhibition of HIV-1 RT. Cytotoxicity testing presented CC_{50} values of 83.7 and 50.4 µg/ml in PBMCs and TZM-bl respectively. In addition, the extract stimulated proliferation in HIV negative and positive PBMCs treated. RT-CES also registered substantial TZM-bl proliferation after extract treatment. The extract exhibited strong antioxidant activity with an IC_{50} of 16 µg/ml and reduced the production of pro-inflammatory cytokines indicating anti-inflammatory potential.

Conclusion

This is the first demonstration of the *in vitro* anti HIV-1 potential of *P. barbatus* including direct activity as well as through the stimulation of protective immune and inflammation responses. The low cytotoxicity of the extract is also in agreement with the vast anecdotal use of this plant in treating various ailments with no reported side-effects.

Keywords: *Plectranthus barbatus*, HIV-1 protease, HIV-1 reverse transcriptase, antioxidant, cytokines.

4.1 Introduction

Plectranthus is a genus that belongs to the Lamiaceae family. This genus is distributed worldwide and contains about 300 species with 45 of these being used ethnobotanically on the African continent (Lukhoba et al., 2006). *Plectranthus* species has numerous ethnomedicinal uses such as in antibacterial, antiviral, anti-malaria and antifungal phytotherapy (Lukhoba et al., 2006). *Plectranthus barabatus* Andr. is regarded as one of the most important medicinal species of the genus *Plectranthus*. *P. barbatus* originated from north-eastern Africa and is found in Sub-Saharan Africa as an invasive species (Rice et al., 2011). For centuries it has been used in folk medicine of Brazil, tropical Africa and China as well as in Hindu and Ayurvedic traditional medicine (Lukhoba et al., 2006; Maioli et al., 2010).

P. barbatus is used in antispasmodic therapy, where it is specifically used for the treatment of gastric disturbances associated with intestinal spasms (Almeida, 2003). In Kenya, the plant is reportedly used traditionally as antimalarial phytotherapy (Nguta et al., 2010a, 2010b). *P. barbatus* has also been reported to be a herbal remedy against candida which was proven in an *in vitro* pharmacological study (Runyoro et al., 2006).

A number of diterpenoids have been isolated from *P. barbatus.* These are the most common group of compounds found in *Plectranthus* plants (Abdel-Mogib et al., 2002). The medicinal properties of *P. barbatus* can probably be attributed to terpenoids.

Surveys carried out in Uganda, Tanzania and Namibia on the use of medicinal plants for HIV/AIDS treatment revealed *P. barbatus* as one of the commonly used herbal remedies in managing HIV/AIDS and the associated opportunistic infections. The aerial parts of this plant are administered orally by traditional medicine practitioners as a decoction or an infusion to treat HIV/AIDS, oral candidiasis, herpes simplex, hepes zoster and skin rashes (Chinsembu and Hedimbi, 2010; Kisangau et al., 2011, 2007; Lamorde et al., 2010). Exactly what the mechanism of action of the extract is, or why patients experience alleviation of symptoms (during or after its use) is unknown. Because HIV replicates in a highly oxidized environment (Gil del Valle et al., 2013), demonstrating substantial natural antioxidant activity in *in vitro* extracts of *P. barbatus* will contribute some mechanistic information. Also, direct anti-viral (as in the inhibition of viral enzymes) or anti-inflammatory activities, if exhibited by the extract, presents explanations for how the plant may slow disease progression. To be entirely useful, if

the preceding effects exist, these abilities must go hand in hand with a lack of, or very low cytotoxicity.

HIV infected individuals even when successfully treated with highly active anti-retroviral therapy (HAART), continue to deal with incidences of malignancy, cardiovascular disease (CVD), metabolic, bone, renal and liver disease, very much like aging populations (Nixon and Landay, 2010). There is also ample evidence supporting the notion that inflammation plays a role in these conditions in the general population, while also having become leading causes of morbidity and mortality in HIV-infected individuals in settings where HAART is routinely used. Compared with the general population, high-sensitivity C-reactive protein (hs-CRP), interleukin 6 (IL-6), d-dimer, and certain other inflammatory biomarkers are significantly elevated during HIV infection (Nixon and Landay, 2010). Any product/compound that decreases these types of inflammatory biomarkers therefore has a potential use in improving the quality of life of infected individuals. The balance between the pro-inflammatory cytokines IL-1, IL-6 and tumor necrosis factor-alpha, which up-regulate HIV expression, and IL-10, which can act both as an anti-inflammatory cytokine and a B-cell stimulatory factor plays an important role in the progression to AIDS (Breen, 2002). Because HIV/AIDS is also known to affect IFN and IL-17 (Williams et al., 2013) where their levels serve as indicators of disease progression, the anti-inflammatory behavior of the extract was investigated by assessing its effect on all the mentioned relevant pro-inflammatory cytokines simultaneously.

The aims of this study were therefore the investigation of the HIV-1 inhibitory properties, cytotoxicity, anti-inflammatory and antioxidant abilities of *P. barbatus* in order to determine whether the traditional claims are supported by actual pharmacological effects. The idea was to link the HIV/AIDS ethnomedicinal use of the plant to *in vitro* studies in order to validate the anecdotal claims in favour of *P. barbatus*. A lot of work has been done on *P. barbatus*; there is however nothing published on how or why extracts of this plant may be specifically assisting HIV/AIDS patients.

4.2 Material and methods

4.2.1 Plant material

Plectranthus barbatus Andr. leaves (74.8 g) were collected at the Botanical garden of the University of Pretoria during April 2011. Plant identification was done in the H.G.W.J Schweikerdt herbarium of the University and a voucher specimen (117198) is kept in the herbarium.

4.2.2 Extraction

Fresh leaves were blended in ethanol and filtered. The filtrate was concentrated under reduced pressure at 50 °C. The residue was re-dissolved in ethyl acetate to exclude highly polar tannin compounds, non-specific enzyme inhibitors. The ethyl acetate fraction (lipophilic fraction) was dried and stored in the dark at 4 °C until use. The ethyl acetate extract was reconstituted in dimethyl sulfoxide (DMSO), which provides a sterile environment, before each biological assay. Further dilutions to obtain desired extract concentrations were done in either cell culture media for cytotoxicity or buffer for HIV-1 enzyme assays.

4.2.3 Cytotoxicity assay using MTT and real time cell sensing

Tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used to assess the cytotoxicity of *P. barbatus* in TZM-bl cells (Mosmann, 1983). Briefly, cells were seeded in a 96-well tissue culture treated plate at 1 x 10^4 cells per well in the absence or presence of various gradient *P. barbatus* extract concentrations (100-3.125 µg/ml). Incubation was carried out for 72 h at 37 °C in humidified air with 5% CO_2. Auranofin was used as a positive control for cytotoxicity because it is a known toxic compound with antitumor activity (Mirabelli et al., 1985). Other control wells included a negative control (cells & medium only), background control for extract and a DMSO control. Plates were read on a Multiskan Ascent reader at 550/690 nm. The 50% cytotoxic concentration (CC_{50}) was calculated using Graphpad Prism (Graphpad Software Inc. California, USA).

In addition to using MTT for the detection of cytotoxicity a real time-cell electronic sensing (RT-CES) device, xCelligence (Roche Diagnostics, Mannheim, Germany) was used to monitor proliferation of the TZM-bl cells in the presence of *P. barbatus* extract. The system monitors cellular events in real time without the incorporation of labels by

measuring electrical impedance across interdigitated gold micro-electrodes integrated on the bottom of special tissue culture plates. Increasing attachment of cells to the electrodes increases electrode impedance which is displayed as Cell Index (CI) (Abassi et al., 2009; Atienzar et al., 2011). TZM-bl cells were seeded at 1 x 10^4 cells per well. Three concentrations of *P. barbatus* extract (100, 50.4 and 25 µg/ml) were tested alongside 10 µM auranofin, positive control for toxicity. An untreated TZM-bl cells control was also included. Cell treatment monitoring was carried out over a period of 72 h.

4.2.4 Effect of the extract on PBMC proliferation

Ethical approval for obtaining blood samples from consenting donors was granted by the Faculties of Natural and Agricultural Sciences and Health Sciences Ethics Committees (EC080506-019; 163/2008, University of Pretoria, South Africa). Freshly isolated uninfected and infected PBMCs were plated in 96 well plates (Costar, Corning Incorporated, USA) at 1 x 10^5 cells per well and treated with the extract at final concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml. The number of viable cells was detected after 72 h using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) solution (Promega, Promega Corporation, USA). Control wells included a toxicity control auranofin (Mirabelli et al., 1985) and the plates were read at 492/690 nm. The percentage viability was calculated relative to an untreated control of cells only and the CC_{50} values were determined using Graphpad Prism (Graphpad Software Inc. California, USA).

The flow cytometric analysis of lymphocyte proliferation was measured using carboxylfluorecein succinimidyl ester (CFSE). The technique monitors the effect of a treatment, in this case extract, on mitotic activity of the T cell population based on the visualisation of 8 to 10 discrete cycles of cell division (Fulcher and Wong, 1999; Lyons, 2000). HIV negative and positive PBMCs (1 x 10^6 cells/ml) were labelled with 5 mM CFSE and extract treatment of the labelled cells was done at two concentrations (100 and 50 µg/ml) for 72 h. Prior to flow cytometric analysis, propidium iodide was incorporated to exclude background staining from dead cells (Fonteh et al., 2011). Data (10 000 events) was acquired on a FACSAria (BD BioSciences, California, USA) and analysed using FlowJo Version 7.6.1 (TreeStar Inc., Oregon, USA).

4.2.5 HIV-1 protease fluorogenic assay

This assay was a modified version of the assay performed by Lam et al. (2000). The fluorogenic HIV protease (PR) substrate 1 (Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(DABCYL)-Arg) was dissolved in DMSO to make a 1 mM stock. The stock fluorogenic substrate was diluted to 10 µM using assay buffer (0.1 M sodium acetate, 1 M NaCl, 1 mM EDTA and 1 mM DTT, pH 4.7). An aliquot of the substrate (10 µM, 49 µl) and 1 µl of HIV-1 PR solution (1 µg/ml; Bachem, Switzerland) were added to the reaction mixture in an assay buffer in the presence or absence (untreated control) of the extract to make a final reaction volume of 100 µl. The mixture was incubated at 37 °C for 1 h in black 96 well assay plates. The fluorescence intensity was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a synergy microplate spectrofluorometer (BioTek, Analytical & Diagnostic products, South Africa). Acetyl pepstatin (AP) was used as a positive control for HIV-1 PR inhibition. The blank treatment consisted of assay buffer with only the substrate and an untreated control of enzyme and substrate was also included. The percentage inhibition was calculated based on the formula: 100 – [(Test reagent RFU – background RFU)/(untreated control RFU – blank) x 100] where RFU = relative fluorescence units.

4.2.6 HIV-1 reverse transcriptase colorimetric assay

The effect of the crude extract on reverse transcription was tested using a reverse transcriptase (RT) colorimetric assay kit from Roche Diagnostics (Mannheim, Germany) and purified recombinant HIV-1 RT (Merck, Darmstadt, Germany). The assay was performed according to a method previously described (Fonteh et al., 2009). Extract was tested at two concentrations 100 and 50 µg/ml. The enzyme (0.2U) was incubated for 1 h with the extract at 37 °C. Subsequent 1 h incubations involved addition of an antibody conjugated to peroxidase that binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate solution [2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)] is cleaved by the peroxidase enzyme, producing a colored reaction product. Two positive controls were used; doxorubicin, a known HIV-1 RT inhibitor (Kuete et al., 2010b; Mbaveng et al., 2011) and an in-house natural product inhibitor active against HIV-1 RT (Fonteh et al., 2009). The absorbance of the samples was read at 405 nm with a reference wavelength of 492 nm using a microtiter plate reader (Multiskan Ascent; Thermo Labsystems; USA) and was directly correlated to the level of RT activity in the sample.

4.2.7 Antioxidant activity: DPPH free radical scavenging assay

The free radical scavenging activity of the extract was evaluated using a modified method previously described by Kuete et al. (2010). The extract dissolved in ethanol was mixed with a 90 µM 2,2-diphenyl-1-picrylhydrazyl (DPPH) ethanol solution to give final extract concentrations of 6-100 µg/ml. Incubation of extract with DPPH was done in the dark at room temperature for 30 min. The absorbance values were measured at 550 nm (Multiskan Ascent; Thermo Labsystems; USA) and converted into percentage of antioxidant activity (Marxen et al., 2007). A known antioxidant, ascorbic acid, was used as a standard control. IC_{50} values were calculated using Graphpad Prism (Graphpad Software Inc. California, USA).

4.2.8 Anti-inflammatory activity: cytometric bead array

The effect of the extract on cytokine production was evaluated using the Human Th1/Th2/Th17 cytometric bead array (CBA) kit (BD Biosciences, San Jose, California) (Williams et al., 2013).

PBMCs were isolated from blood samples of healthy individuals (n=4). Cells were seeded at a concentration of 1 x 10^6 cells per well and treated with two non-cytotoxic concentrations of the extract, 25 and 50 µg/ml. The supernatant was collected after 24 h incubation and stored at -20°C until cytokine analysis. Cytokine levels in the supernatant was analysed following the protocol outlined in the kit. The supernatant (50 µl) was mixed with the cytokine capture beads and the detector reagent, phycoerythrin (PE)-conjugated detection antibodies, to form sandwich complexes. These complexes were measured using a FACSArray Bioanalyzer (BD Biosciences, San Jose, CA, USA) in order to identify the concentrations of specific cytokines. The limits of detection for each cytokine were as follow: 2.6 pg/ml for IL-2, 4.9 pg/ml for IL-4, 2.4 pg/ml for IL-6, 4.5 pg/ml for IL-10, 3.8 pg/ml for TNF, 3.7 pg/ml for IFN- γ and 18.9 pg/ml for IL-17A.

4.3 Results and discussion

4.3.1 Effect of P. barbatus extract on the viability of TZM-bl and PBMCs

The CC_{50} of *P. barbatus* extract in TZM-bl cells using MTT was 50.4 ± 2.7 µg/ml (Table 1) while RT-CES revealed a different cytotoxicity pattern. With RT-CES (Figure 4.1), 100 µg/ml (iii) of the extract resulted in <50% of TZM-bl cells being viable after 72 h compared to the control cells (i) and this was expected because of what was observed

with MTT. The cell index for 100 µg/ml (iii) was low compared to that of control cells (i) indicating the reduction of cell viability at that concentration. Other concentrations tested; 50.4 (iv) and 25 µg/ml (v) were not toxic towards TZM-bl cells. *P. barbatus* has been reported to generally have low toxicity (Figueiredo et al., 2010) and the data obtained in the current study suggests that *P. barbatus* extract concentrations ≥100 µg/ml may result in high toxicity. The CC_{50} for Auranofin, positive control for toxicity, was <10 µM in TZM-bl and PBMCs.

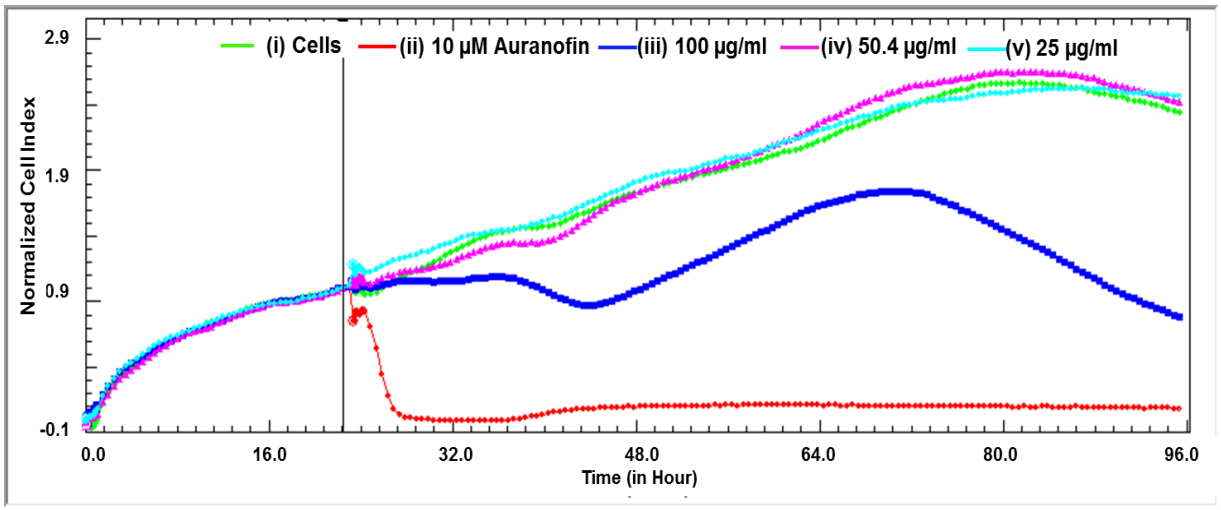

Figure 4.1: Effect of *P. barbatus* on TZM-bl cells as generated by real-time cell analysis. The cells were exposed to 100, 50.4 and 25 µg/ml of the extract and these are represented by the different colored curves. Auranofin (ii) was used as a positive control for toxicity. Each data point was normalized against the time point just before extract addition (24 h).

P. barbatus was less toxic to PBMCs with a CC_{50} of 84.7 ± 2.2 µg/ml (Table 4.1). These results were confirmed with flow cytometry whereby there was no significant difference ($p<0.05$) in lymphocytes proliferation of untreated HIV negative (-) PBMCs (42%) and lymphocytes treated with 100 µg/ml of the extract (40%). However, a marginally higher cell division of 47% was observed for lymphocytes treated with 50 µg/ml of the *P. barbatus* extract (Figure 4.2). Flow cytometry can be used on adherent or suspension cells while RT-CES is applicable to adherent cells, TZM-bl cells, explaining why the different techniques were applied for confirmation assays as they were.

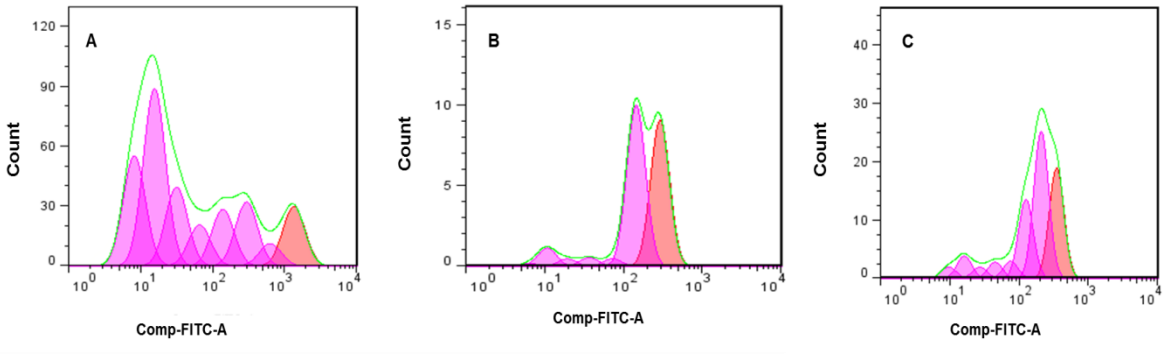

Figure 4.2: The 3-day response of CFSE-labelled HIV- PBMCs treated with the *P. barbatus* extract. The orange peak represents the parent cells and pink peaks are daughter populations as a result of cell division. (A) Cell division of 42% was observed in untreated PBMCs. (B) Parent cells of PBMCs treated with 100 µg/ml resulted in 40% division of parent to daughter cells. (C) PBMCs treated with 50 µg/ml of the extract had the highest cell division of 47%.

A different trend was observed in HIV positive (+) lymphocytes (Figure 4.3); in the absence of the extract the control cells resulted in 19% cell division (compared to the 42% in uninfected, untreated cells) and a 2.8 % cell division was observed with the parent cells of HIV+ lymphocytes treated with 100 µg/ml of the extract. A lymphocyte division of 54% was observed with cells treated with 50 µg/ml of the *P. barbatus* extract. The CFSE data suggests that the high extract concentration tested, 100 µg/ml (Figure 4.3B), was toxic to PBMCs while 50 µg/ml was less toxic (Figure 4.3C). CFSE analysis allowed for the tracking of daughter cells from the parent population of cells (Lyons, 2000) and the cell division into daughter cells was clearly observed. Lower concentrations of extract had a comparable proliferative effect on infected/uninfected cells.

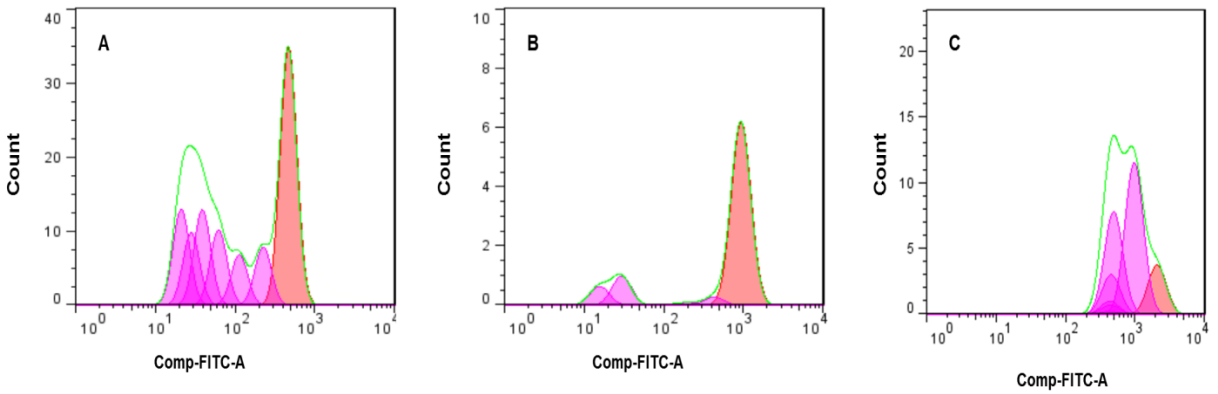

Figure 4.3: Effect of *P. barbatus* extract on the proliferation of CFSE-labelled HIV+ PBMCs. The orange peak represent the parent cells and pink peaks are daughter populations as a result of cell division. (A) %cell division in control PBMCs was 19% (B) Only 2.8% cell division was obtained with PBMCs treated with 100 µg/ml (C) A high cell division of 54% was observed in PBMCs treated with 50 µg/ml of the extract which was comparable to uninfected cells treated with a similar extract concentration.

Table 4.1: Cytotoxicity and HIV-1 inhibitory activities of *P. barbatus* ethanolic extract.

Sample	CC_{50} (µg/ml) ± SD[a]		IC_{50} (µg/ml) ± SD	
	TZM-bl	PBMCs	HIV-1 RT	HIV-1 PR
P. barbatus	50.4 ± 2.7	84.7 ± 2.2	>100	62.0 ± 0.2
Auranofin[b]	<10	<10	-	-
Acetyl pepstatin[c]	-	-	-	<0.3
Doxorubicin[d]	-		<25	
BBR[d]	-	-	0.3 ± 0.1	-

[a]The values represents CC_{50} and IC_{50} ± SD indicating the effect of *P. barbatus* extract on the viability of TZM-bl cells and PBMCs (detected with MTT and MTS) as well as the inhibitory effect on HIV-1 enzymes.
[b]Positive control for toxicity (concentration in µM)
[c]Known inhibitor (positive control) of HIV-1 protease.
[d]Positive control for HIV-1 reverse transcriptase (BBR concentration in mg/ml)

4.3.2 Anti-HIV-1 potential of P. barbatus

P. barbatus extract inhibited HIV-1 PR, 70% inhibition, at 100 µg/ml (IC_{50} = 62.0 ±0.2 µg/ml) (Table 4.1). The lowest concentration tested, 50 µg/ml, gave less than 50% HIV-1 PR inhibition. The positive control, acetyl pepstatin, inhibited HIV-1 PR by 97% at 10 µg/ml and its IC_{50} was <0.3 µg/ml. The extract had weak inhibitory activity against HIV-1

RT (<50%). Positive control for HIV-1 RT, doxorubicin, exhibited an IC_{50} <25 µg/ml. The IC_{50} of an in-house natural product inhibitor was 0.3 ± 0.1 mg/ml. These results suggests that the effectiveness of the traditional use of *P. barbatus* in managing HIV/AIDS (Chinsembu and Hedimbi, 2010; Kisangau et al., 2011; Lamorde et al., 2010) could be through the inhibition of HIV-1 PR. The use of herbal remedies for HIV/AIDS treatment is widely practised traditionally and it is of utmost importance to test the efficacy of plants used, in this case *P. barbatus*. There is no literature reporting the *in vitro* anti-HIV-1 activity of *P. barbatus*, making this study the first to link the *in vitro* inhibition of HIV-1 protease to the traditional use of this plant in HIV/AIDS treatment. Inhibition of HIV-1 PR could be attributed to diterpenoid compounds which are often described as the active ingredients identified in *P. barbatus* (Alasbahi and Melzig, 2010a, 2010b).

4.3.3 Antioxidant properties of P. barbatus

The results of the antioxidant activity of *P. barbatus* extract summarized in Figure 4.4 showed a dose-dependent response. The IC_{50} of the extract was 15.8 ± 0.3 µg/ml and vitamin C, the positive control, had an IC_{50} of 1.1 ± 0.02 µg/ml. The IC_{50} value of *P. barbatus* obtained in this study was twice lower than that previously reported by Maioli et al. (2010), where an IC_{50} of 35.8 ± 0.3 µg/ml was obtained in aqueous leaves extract of *P. barbatus* using a similar DPPH assay.

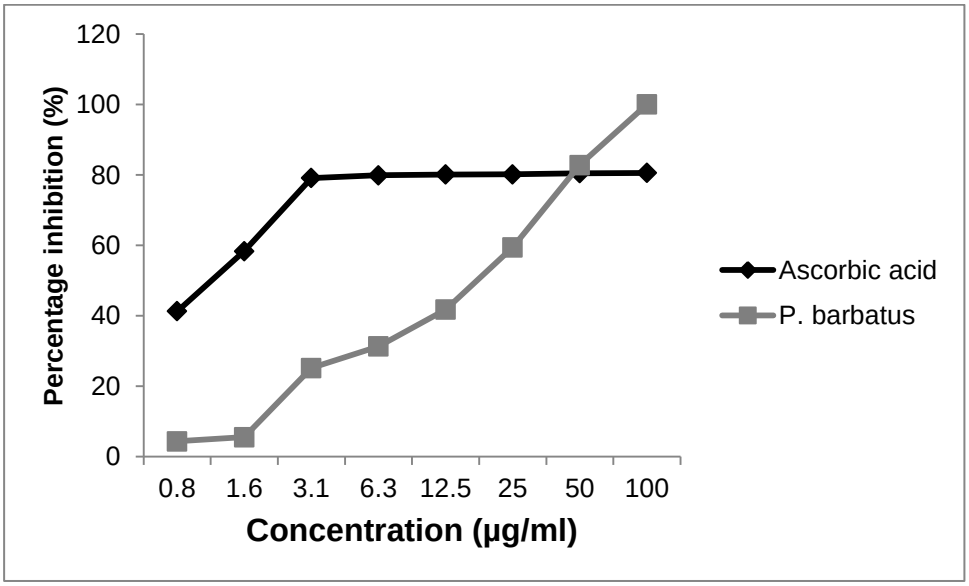

Figure 4.4: DPPH antioxidant potential of *P. barbatus* extract. Ascorbic acid was used as a positive control. IC_{50}s were 15.8 ± 0.3 and 1.1 ± 0.02 µg/ml for extract and ascorbic acid respectively.

The good antioxidant properties of *P. barbatus* possibly contribute to the reduction of the viral load in HIV-infected patients that traditionally uses this plant. Oxidative stress, which is caused by the accumulation of reactive oxygen species, has been linked to the activation of HIV-1 leading to the development of AIDS (Gil del Valle et al., 2013; Gil et al., 2003; Vaira et al., 1990). Antioxidants such as vitamin E and C have been shown to reduce oxidative stress in HIV patients and in the process contribute towards a reduction in viral load (Allard et al., 1998b).

4.3.4 P. barbatus inhibits pro-inflammatory cytokines

The two non-cytotoxic concentrations of *P. barbatus* tested, 50 and 25 µg/ml, resulted in the reduction of cytokine production for all cytokines tested including pro-inflammatory cytokines IL-2, IL-6, IL-10, TNF and IL-17A (Figure 4.5).

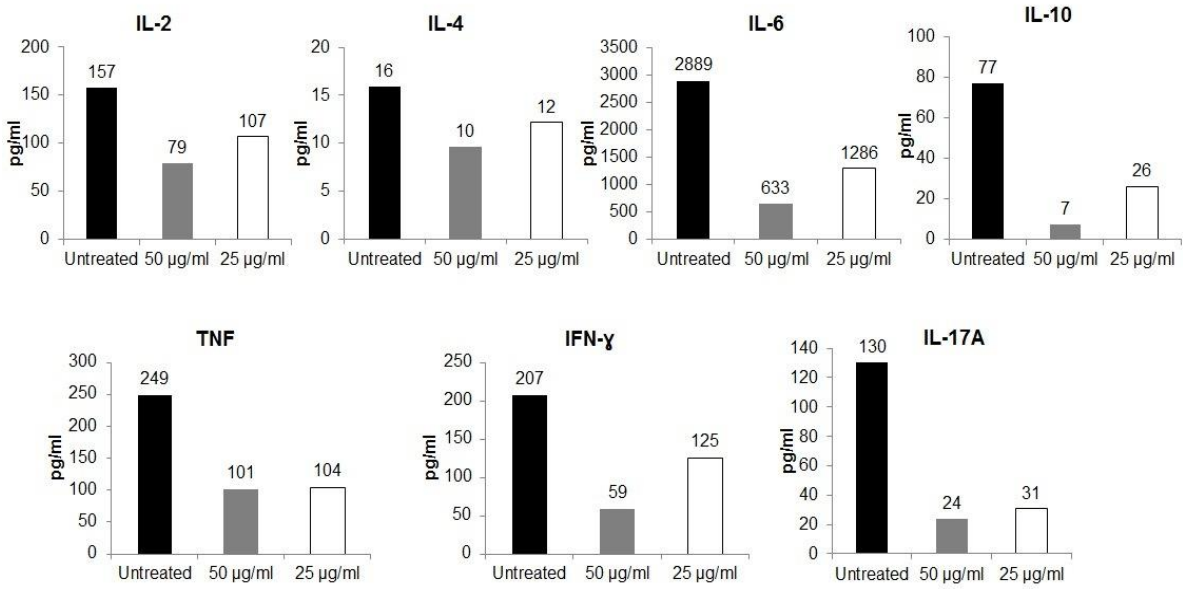

Figure 4.5: Effect of *P. barbatus* extract on cytokine production in PBMCs. The values are mean cytokine concentrations of PBMCs from 4 HIV negative individuals. Cells were exposed to medium only (untreated control) and two non-cytotoxic concentrations of the extract, 50 and 25 µg/ml. The extract significantly (p <0.05) reduced the production of IL-2, IL-6, IL-10, TNF, IFN- γ and IL-17A.

The ability of *P. barbatus* extract to inhibit inflammatory cytokines can be linked to its traditional use in treating various ailments (Lukhoba et al., 2006). Anti-inflammatory properties were previously detected by Matu and Staden (2003) in *P. barbatus* from Kenya when the methanolic extract of the leaves inhibited cyclooxygenase (COX-1), an enzyme involved in the cascade of events resulting in pain and inflammation. The

current study identified another anti-inflammatory mechanism of *P. barbatus* which is through the inhibition of pro-inflammatory cytokines. These results can also be linked to anti-HIV properties of this plant; low levels of IL-17A in HIV-1 infection is associated with non-progression (Yue et al., 2008). IL-17A along with other cytokines such as IL-2 and IL-6 are elevated during the different stages of HIV infection (Keating et al., 2011; Nixon and Landay, 2010; Worsley et al., 2010; Yue et al., 2008). Therefore lowering the production of these cytokines especially IL-17A could contribute to slow or non-progression of HIV infection. The IFN-γ paradox in HIV infection needs better understanding. There is a need to control antiretroviral effects of IFN-γ during HIV infection. The ability of the extract in this study to lower IFN-γ production can possibly be of clinical importance during late stages of HIV infection. High levels of IFN-γ have been detected during progression of HIV to AIDS with no reported anti-HIV effect (Abel et al., 2002; Biswas et al., 1992; Francis et al., 1992). From data presented here it seems possible that *P. barbatus* ingestion contributes to a slower disease progression during HIV infection by inhibiting cytokines involved in this pathogenesis.

The *in vitro* anti-HIV-1 data obtained in this study is the first for *P. barbatus*. The ability of *P. barbatus* to inhibit HIV-1 protease confirms the ethnobotanical claims of this plant and supports its continuous traditional use in managing HIV/AIDS and associated opportunistic infections. Synergistic interactions between compounds in crude extracts have been reported to occur (Ma et al., 2009). Hence, the need to first test crude extracts before purification of bioactive compounds. The study shows that *P. barbatus* extract contain inhibitory compounds active against HIV-1 PR. Since the chemistry of *P. barbatus* has been vastly reported in the past 30 years, it will be of interest for further investigation to be carried out through *in silico* analysis of the compounds isolated from *P. barbatus*. Further work will also involve purification of the active ingredient (s) in order to determine the exact mechanism of action that occurred with HIV-1 PR inhibition which seems to aid in the health of HIV/AIDS patients treated with that plant.

Acknowledgments

This research was supported by the Southern African Biochemistry and Informatics for Natural Products (SABINA), the Technology Innovation Agency (TIA) of South Africa), Margaret McNamara Memorial Fund (MMMF), the Namibian Ministry of Education and the University of Pretoria.

CHAPTER 5

Anti-HIV activity of *Ocimum labiatum* extract and isolated pheophytin-a

Submitted to *Phytomedicine*

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Abstract

Purpose: Plants from the *Ocimum* genus are traditionally used to treat HIV/AIDS and various other ailments. The aim of this study was to evaluate the anti-HIV activities of *O. labiatum* extract as well as identify the bioactive components through bioassay-guided screening.

Methods: Ethanolic extract of *O. labiatum* was tested for inhibitory activity against HIV-1 enzymes protease (PR) and reverse transcriptase (RT). The effect of the extract on HIV-1 replication in chronically infected U1 cells was also investigated, using an HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA). Bioassay-guided fractionation was performed to identify the bioactive components of the extract. The HIV-1 PR inhibiting fraction was purified through preparative high performance liquid chromatography (HPLC) yielding a chlorophyll derivative, which was identified by ^{1}H and ^{13}C nuclear magnetic resonance (NMR). Cytotoxicity of the extract and isolated compound was determined through tetrazolium dye uptake of viable TZM-bl cells, peripheral blood mononuclear cells (PBMCs) and U1 cells. Cytotoxicity data was confirmed by the use of a real-time cell electronic sensing (RT-CES) device which monitored proliferation/viability of adherent cells in real time.

Results: *O. labiatum* significantly ($p<0.05$) inhibited HIV-1 PR with an IC_{50} value of 49.8 ±0.4 µg/ml. The extract also significantly ($p<0.05$) reduced HIV-1 replication in U1 cells at a non-cytotoxic concentration (25 µg/ml) and presented weak inhibition (<50%) against HIV-1 RT at the highest concentration tested (100 µg/ml). CC_{50} values of the extract in TZM-bl, PBMCs and U1 cells were 62.6 ±0.6, 30.1 ±0.4 and 42.0 ±0.13 µg/ml respectively; demonstrating low toxicity. A chlorophyll derivative, pheophytin-a (phy-a), was identified as the active constituent from *O. labiatum* extract and it inhibited HIV-1 PR with an IC_{50} value of 44.4 ±1.5 µg/ml. The chlorophyll derivative demonstrated low toxicity in TZM-bl cells as determined by RT-CES and the CC_{50} value in U1 cells was 51.3 ±1.0 µg/ml.

Conclusion: This study provides the first evidence of the anti-HIV activity of *O. labiatum* and an isolated chlorophyll derivative, phy-a. These data underscores the medicinal importance of members of the *Ocimum* genus and provides evidence in support of further investigation of *O. labiatum* in particular for lead compounds against HIV-1.

Keywords: *Ocimum labiatum*; Pheophytin-a; HIV-1 protease; HIV-1 reverse transcriptase; HIV-1 replication; Real-time cell analyser.

5.1 Introduction

Natural products are being explored as possible contributors in the identification of potential inhibitors of the different steps of the HIV life cycle (Geuenich et al., 2008; Harnett et al., 2005; Newman and Cragg, 2012). The activities of compounds from plants against human viruses is attributed to the different mechanisms that plants have in fending off plant viral attacks (Kang et al., 2005). The search for better drugs against HIV/AIDS is ongoing, for many reasons including side-effects of existing HIV drugs which contribute to non-compliance and the development of drug resistant viral strains (Clavel and Hance, 2004).

A wide variety of phytochemicals with potential antiviral activity have been isolated from several hundred plant and herb species (Perera and Efferth, 2012). Types of compounds isolated include flavonoids, terpenoids, lignans, sulphides, polyphenolics, coumarins, saponins, proteins and chlorophyll derivatives (Perera and Efferth, 2012; Wang et al., 2009). The mechanisms of action of some of these phytochemicals include inhibiting the formation of viral DNA or RNA or inhibiting other viral reproduction steps (Perera and Efferth, 2012). Chlorophyll derivatives like pheophytin-a have been implicated in the inhibition of viral proteases like those in hepatitis C virus (Wang et al., 2009) while pheophorbide-a demonstrated activity against HIV (Zhang et al., 2003), herpes simplex and influenza (Bouslama et al., 2011).

The *Ocimum* genus is native to many countries worldwide and comprises 150 species. *Ocimum* species are widely used for the treatment of various ailments and most of these plants are incorporated in indigenous *ayurveda* medicine (Chandrasekaran et al., 2013; Prabhu et al., 2009). Plants from the *Ocimum* genus are traditionally used in managing HIV/AIDS and to treat opportunistic infections such as herpes simplex virus and chronic diarrhoea. One of the species from this genus, *O. gratissimum*, is documented as an herbal remedy administered in HIV/AIDS treatment in Uganda and Tanzania (Kisangau et al., 2007; Lamorde et al., 2010) and has been shown pharmacologically to inhibit an HIV-1 strain (Ayisi and Nyadedzor, 2003). Plant parts commonly used are leaves and a decoction of that is administered orally in variable doses over different time periods (Lamorde et al., 2010).

Leaf extracts when investigated for anti-viral qualities are tested on whole virus and viral proteins (enzyme inhibition) (Geuenich et al., 2008; Kapewangolo et al., 2013). It is also

important to assess toxicity of active compounds and extracts which is usually done with viability dyes as first assessment method which can then be confirmed by label-free methods involving real-time cell electronic sensing (RT-CES) (Kapewangolo et al., 2013). RT-CES is also useful when extracts or isolated compounds have intrinsic colours which may interfere with viability dyes.

Due to the various ethnobotanical uses of the *Ocimum* genus, most species of this genus have been extensively studied except for *O. labiatum* for which very little literature exists. Here, *O. labiatum* was investigated; for inhibitory properties against HIV-1 protease and reverse transcriptase, to evaluate its ability to suppress HIV-1 in a chronically infected cell line, to determine the plant's cytotoxicity as well as to isolate the HIV-1 bioactive components. The wide variety of diverse phytochemicals with multi-faceted modes of action that exist in a single plant makes it possible to use extracts and/or isolated compounds to explore various antiviral activities (Perera and Efferth, 2012).

O. labiatum is found in several Southern African countries and this plant has not yet been investigated for anti-HIV-1 activities. Hussein et al. (2007) isolated a number of compounds from *O. labiatum* of which 2α-Hydroxylabda-8(17),12*E*,14-trien-18-oic acid and Labda-8(17),12*E*,14-triene-2R,18-diol demonstrated activity against *Mycobacterium tuberculosis* and a human breast cancer cell line respectively. In the present study, the first time activity of *O. labiatum* against HIV-1 was evaluated.

5.2 Methods

5.2.1 General experimental procedures

^{1}H NMR spectra were recorded on a Bruker 400 MHz (MA, USA) spectrometer, using tetramethylsilane (TMS) as an internal standard and deuterated chloroform for dissolution of the sample. Thin layer chromatography (TLC) was carried out on Merck (Darmstadt, Germany) silica gel plates F_{254} (0.25 mm layer thickness) and visualized using UV lamp at 254 & 360 nm, and by spraying with vanillin-sulphuric acid. Column chromatography separations and purifications were performed on silica gel 60 (70-230 mesh) from Merck and on Sephadex LH-20 (Sigma, MO, USA). HPLC analysis was conducted to further purify the bioactive fraction. This was done with a Shimadzu preparative 6AD LC system equipped with a UV-visible (254 and 370 nm) detector, a manual injector 10AF, and a fraction collector FRC-10A (Shimadzu, KYT, Japan).

Aliquots (200 µl) were injected in a C18 Jupiter analytical column of 250 mm x 4.6 mm x 10 µm (particle size).

5.2.2 Plant material

Fresh aerial parts of *O. labiatum* were collected during February 2012 from the Botanical Garden of the University of Pretoria (S25° 45' 21" E28º 13' 51"). The specimen was authenticated by H.G.W.J Schweikerdt Herbarium of the University where the voucher specimen (117693) is deposited. The leaves were separated from the stems and extracted while fresh.

5.2.3 Extraction and isolation

The fresh leaves (894.6 g) were extracted with ethanol and the extract was concentrated *in vacuo*. The residue was dissolved in ethyl acetate (EtOAc) in order to exclude polar tannins which are non-specific enzyme inhibitors. The EtOAc portion was evaporated to dryness under reduced pressure and it yielded a residue of 20.7 g. The residue was subjected to column chromatography (silica gel 70-230 mesh) and eluted with a mixture of hexane:EtOAc of increasing polarity. The HIV-1 PR inhibiting fraction was eluted with 30-50% EtOAc. This fraction was further subjected to gel filtration chromatography (Sephadex LH-20) eluting with chloroform from which a sub-fraction (981.9 mg) was obtained. The sub-fraction was further purified by preparative HPLC, by eluting sample isocratically with acetonitrile/methanol (1:1, v/v) at a flow rate of 1.0 ml/min for 30 min, to afford a compound (10 mg) with spectral data in agreement with those of pheophytin-a (Smith et al., 1984).

5.2.4 HIV-1 protease and reverse transcriptase assays

The procedure for screening against HIV-1 protease (PR) was followed as previously described (Lam et al., 2000). This procedure uses a fluorogenic HIV Protease Substrate 1 Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(DABCYL)-Arg (Sigma, MO, USA), a synthetic peptide sequence that contains the cleavage site (Tyr-Pro) for HIV protease as well as two covalently modified amino acids for the detection of cleavage. The substrate was dissolved in dimethyl sulfoxide to make a 1 mM stock and this was further diluted in assay buffer to make a working solution of 10 µM. The substrate (49 µl) and 2 µl of HIV-1 PR (1 µg/ml; Bachem, Bubendorf, Switzerland) were incubated with samples for 1 h in black 96 well assay plates. Acetyl pepstatin (AP) was used as a positive control for HIV-1 PR inhibition. Other control wells included substrate in assay

buffer and an untreated enzyme control. The fluorescence intensity was measured in a synergy microplate spectrofluorometer (Thermo Labsystems, MA, USA) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

A reverse transcriptase (RT) colorimetric assay kit (Roche Diagnostics, MA, Germany) and a recombinant RT (Merck, Darmstadt, Germany) were used to test the effect of the crude extract on HIV-1 reverse transcription. The assay was performed according to the manufacturer's instructions. The enzyme was incubated for 1 h with three different concentrations of the extract (100, 50 and 25 µg/ml). Subsequent 1 h incubation steps included the binding of biotin-labelled DNA to the surface of microplate modules that have been pre-coated with streptavidin, and the addition of an antibody conjugated to peroxidase that binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate solution [2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)] is added and the peroxidase enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of the samples was read at 405 nm with a reference wavelength of 492 nm using a microtiter plate reader (Multiskan Ascent; Thermo Labsystems, MA, USA) and was directly correlated to the level of RT activity in the sample. Doxorubicin (Sigma, MO, USA), a known HIV-1 RT inhibitor, was used as a positive control (Kapewangolo et al., 2013).

The 50% inhibitory concentration was calculated using Graphpad Prism (Graphpad Software Inc. CA, USA).

5.2.5 HIV-1 p24 antigen ELISA

In order to study the effect of *O. labiatum* extract and the isolated compound, phy-a, on phorbol 12-myristate 13-acetate (PMA; Sigma, MO, USA)-mediated induction of HIV-1 expression. The assay was performed as previously described (Kalebic et al., 1991); U1 cells (latently infected monocytes) were seeded at a concentration of 1x 10^5 cells/well and pre-treated with non-cytotoxic concentrations of extract and compound. Previous studies have reported pre-treatment at various times depending on the kind of treatment desired (Kalebic et al., 1991; Kudo et al., 2013; Li et al., 2000b). Here, pre-treatment was done for 6 h (Kalebic et al., 1991) at 37˚C in 90% humidified air with 5% CO_2 and PMA (2ng/ml) was then added and further incubated for 66 h. Control cells included PMA-stimulated and unstimulated U1 cells. Supernatant samples were drawn after incubation, stored at -20°C, and analysed for HIV-1 p24 concentration using the

RETRO-TEK HIV-1 p24 antigen ELISA 2.0 (ZeptoMetrix Corporation, NY, USA). A standard curve was generated using a heat-inactivated HIV-1 p24 antigen standard provided by the manufacturer. The unknown concentration of HIV-1 p24 antigen in the supernatant of extract and compound treated cells was determined by linear regression analysis from the standard curve.

5.2.6 Cytotoxicity analysis of the extract and compound

Cytotoxicity of *O. labiatum* extract and isolated compound, phy-a, was assessed using TZM-bl, PBMCs and U1 cells. The cells (1 x 10^4 cells/well) in complete medium, containing antibiotics and fetal calf serum, were treated with various concentrations of the extract and compound (100-3.125 µg/ml) in 96-well tissue culture plates. Auranofin, a positive control for toxicity (Mirabelli et al., 1985) and untreated cells, were included as controls. Incubation was done for 72 h at 37 °C in a humidified incubator with 5% CO_2. Viability of TZM-bl and U1 cells was determined by quantifying formazan crystals that form as a result of reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, MO, USA) by dehydrogenases in viable cells. The plates were read at 550 nm (reference wavelength of 690 nm) using a microtiter plate reader (Multiskan Ascent; Thermo Labsystems, MA, USA). The percentage viability was computed relative to untreated control cells.

The effect of *O. labiatum* on the viability of peripheral blood mononuclear cells (PBMCs) was also evaluated. Ethics approval for obtaining blood samples from healthy consenting donors was granted by the Faculties of Natural and Agricultural Sciences and Health Sciences Ethics Committees (EC080506-019; 163/2008, University of Pretoria, South Africa). PBMCs were isolated by Histopaque (Sigma, MO, USA) density gradient centrifugation and were plated in 96 well plates at 1 x 10^5 cells/well. Cells were treated with the extract at final concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml. PBMCs better metabolized 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS; Sigma, MO, USA) and this was used to measure viability at 492/690 nm using a microtiter plate reader (Multiskan Ascent; Thermo Labsystems, MA, USA). Control wells included auranofin and cells in medium only. The 50% cytotoxic concentration was calculated using Graphpad Prism (Graphpad Software Inc. CA, USA).

In addition to using the tetrazolium dyes for viability, a real-time cell electronic sensing (RT-CES) device, xCELLigence (Roche Diagnostics, MA, Germany) was used to monitor viability in real time. The analyser only works with adherent cells due to its principle of cell impedance that is created as cells attach to the surface of interdigitated gold micro-electrodes integrated on the bottom of special tissue culture plates. The more cells attach to the electrodes, the larger the increases in electrode impedance and the values are displayed as Cell Index (CI) (Atienzar et al., 2011). A detailed procedure was followed as previously described (Kapewangolo et al., 2013); pre-titrated TZM-bl cells were plated at a concentration of 1 x 10^4 cells/well and treated with the extract and compound when the cell index was ±1 as per manufacturer's instructions. Two concentrations of the crude extract and compound (100 and 50 µg/ml) were tested for their effects on TZM-bl cells in real-time and this was monitored for 72 h. Control wells included 10 µM Auranofin and cells in media only.

5.3 Results

5.3.1 Elucidation of pheophytin-a chemical structure

The proton NMR spectrum of the isolated compound was similar to the spectrum of pheophytin-a (phy-a) (Figure 5.1). The chemical shifts (Table 5.1) were in agreement with those reported in the literature (Smith et al., 1984; Wang et al., 2009) and the propionic side chains in the spectrum (2.0-2.7 ppm) were identical to those illustrated by Smith et al. (1984). The proton resonances were properly assigned, except for protons of the phytyl ester (side-chain) and this is also in accordance with what Smith and his coworkers reported (1984) when they could not assign with confidence all protons of the phytyl ester. A thorough literature search indicated similar data; most studies reporting phy-a either did not take into account protons of the chain and reported molecular weights varied (Ina et al., 2007; Wang et al., 2009) and this explains the complexity of this compound, especially in its phytyl side-chain.

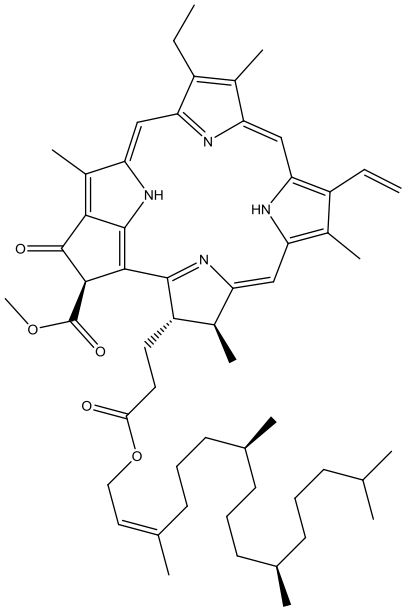

Figure 5.1: Chemical structure of a chlorophyll derivative pheophytin-a

Table 5.1: Proton NMR data of phy-a.

Position	Isolated ^{1}H δ ppm	Literature ^{1}H δ ppm*
α	9.38	9.38
β	9.52	9.52
δ	8.57	8.55
1a	3.4	3.4
2a	8	8
2b	6.31	6.28
2	6.17	6.18
3a	3.23	3.23
4a	3.69	3.68
4b	1.69	1.69
7	4.21	4.21
7a	2.63	2.63
7a'	2.33	2.34
7b	2.47	2.49
7b'	2.19	2.19
8	4.47	4.46
8a	1.81	1.8
10	6.26	6.26
10b	3.89	3.88
P-5/P-15	1.1, 1.3	1.0-1.3
P-7a, P-11a	0.86	0.85
NH	-1.6	-1.6

*(Smith et al., 1984)

5.3.2 HIV-1 PR inhibition

O. labiatum inhibited HIV-1 PR by more than 50% at both 100 and 50 µg/ml (Figure 5.2) and the IC_{50} value of the crude extract was 49.8 ±0.4 µg/ml. Weak inhibitory activities of the extract was obtained against HIV-1 RT (<50%) and this led to the HIV-1 PR fluorogenic assay being used as the guiding assay in the purification of the crude extract in order to obtain the specific component responsible for HIV-1 PR inhibition. The isolated compound, phy-a, inhibited HIV-1 PR (Figure 5.3) with an IC_{50} of 44.4 ±1.5 µg/ml.

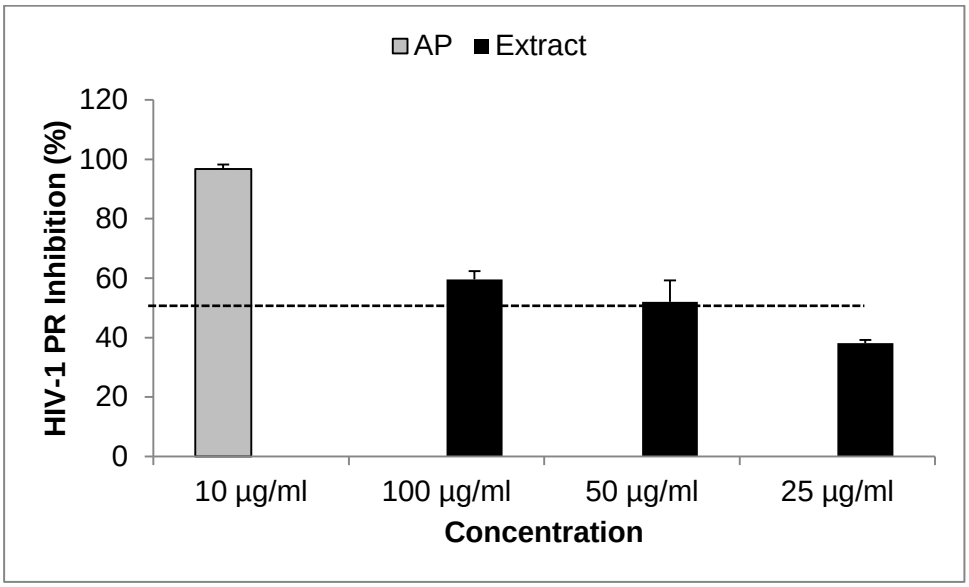

Figure 5.2: Effect of *O. labiatum* on HIV-1 PR. The extract, fluorogenic substrate and HIV-1 enzyme were incubated at 37°C for 1 h. The extract significantly ($p<0.05$) inhibited HIV-1 PR with >50% inhibition at 100 and 50 µg/ml. AP=Acetyl pepstatin a known protease inhibitor was used as control. The dotted line represents 50% inhibition cut-off.

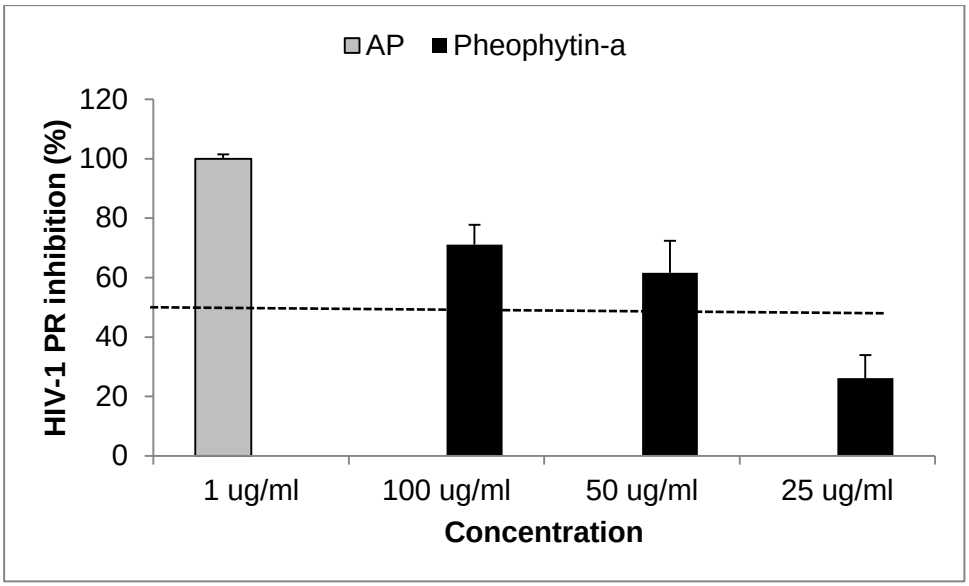

Figure 5.3: Effect of phy-a on HIV-1 PR. Phy-a, fluorogenic substrate and HIV-1 enzyme were incubated at 37 °C for 1 h. Phy-a significantly ($p<0.05$) inhibited HIV-1 PR with >50% inhibition at 100 and 50 µg/ml. The IC_{50} value was 44.4 ± 1.5 µg/ml. AP=Acetyl pepstatin a known protease inhibitor was used as control. The dotted line represents 50% inhibition cut-off.

5.3.3 Inhibition of PMA-induced HIV-1 expression by O. labiatum

U1 cells are latently infected with HIV-1 and treatment of these cells with PMA activates viral replication (Kalebic et al., 1991) which is measurable by assessing the viral core protein, p24, concentration in culture supernatant. The concentration of HIV-1 p24 antigen was significantly ($p<0.05$) reduced, in chronically infected U1 cells, in the presence of a non-cytotoxic concentration of the extract (Figure 5.4A). This possibly mean that *O. labiatum* contain compound/s with the ability to inhibit HIV-1 expression in U1 cells. Phy-a also reduced HIV-1 replication, however, the reduction was not significant ($p>0.05$) meaning that other compounds in *O. labiatum* contributed to the reduction of viral expression and not just phy-a. After culture supernatant was collected for HIV-1 p24 testing, the viability of the cells that remained after removal of supernatant was tested by addition of MTT dye. This was done to ensure that the ability of the extract and phy-a to inhibit HIV-1 replication was not due to cytotoxicity (Figure 5.4B).

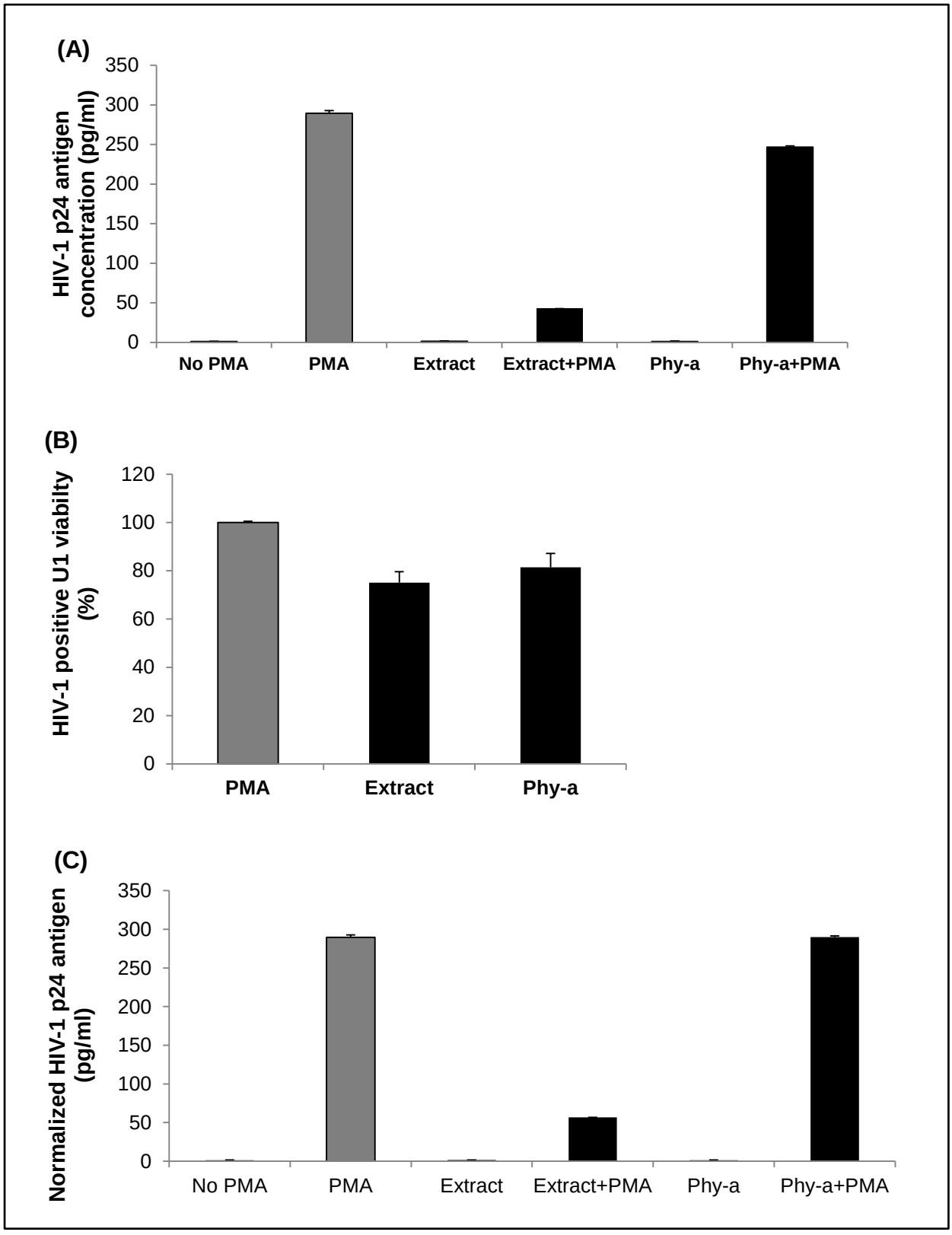

Figure 5.4: Effect of *O. labiatum* extract and phy-a on HIV-1 replication in U1 cells. (A) Cells were pre-treated with 25 µg/ml of extract and 12.5 µg/ml of phy-a for 6 h before viral stimulation with PMA. Controls included unstimulated U1 cells (No PMA), PMA stimulated cells (PMA) and unstimulated treated cells (Extract and phy-a only). **(B)** Viability of the tested concentrations in stimulated U1 cells was determined directly after removal of culture

supernatant to ensure that inhibition of viral expression was not due to cytotoxicity. **(C)** Normalized HIV-1 p24 antigen, taking into account cell viability, still indicated significant ($p<0.05$) reduction of HIV-1 expression by *O. labiatum* extract.

5.3.4 Effect of O. labiatum on TZM-bl and PBMCs

Having already demonstrated the limited toxicity of the extract (25 µg/ml) and purified phy-a (12.5 g/ml) in U1 cells, what remained was an assessment of higher concentrations of these materials in cell lines/types routinely used in HIV drug development studies. The cytotoxicity data obtained revealed that the highest concentration of the crude extract, active against HIV-1 PR, 100 µg/ml, was toxic to TZM-bl cells and PBMCs (Figure 5.5). The extract was more toxic to PBMCs compared to TZM-bl cells as depicted in Figure 5.5. Tetrazolium dyes MTT and MTS were used to determine the viability of TZM-bl and PBMCs respectively. The PBMC concentration (1 x 10^5 cells/well) used in this study did not adequately metabolize the viability dye MTT but properly metabolized MTS which is why the latter dye was used in the PBMC analyses and MTT for TZM-bl assessments. Tetrazolium dyes are metabolized differently by differently cell types. Considerable evidence indicates that the reduction of MTS occurs at the cell surface, or at the level of the plasma membrane via trans-plasma membrane electron transport and not inside a cell as is the case with MTT (Berridge et al., 2005). It is there for possible that MTT was unable to properly penetrate the membrane of PBMCs for proper intracellular metabolism and MTS which is metabolized on the cell-surface was easily metabolized by these mononuclear cells.

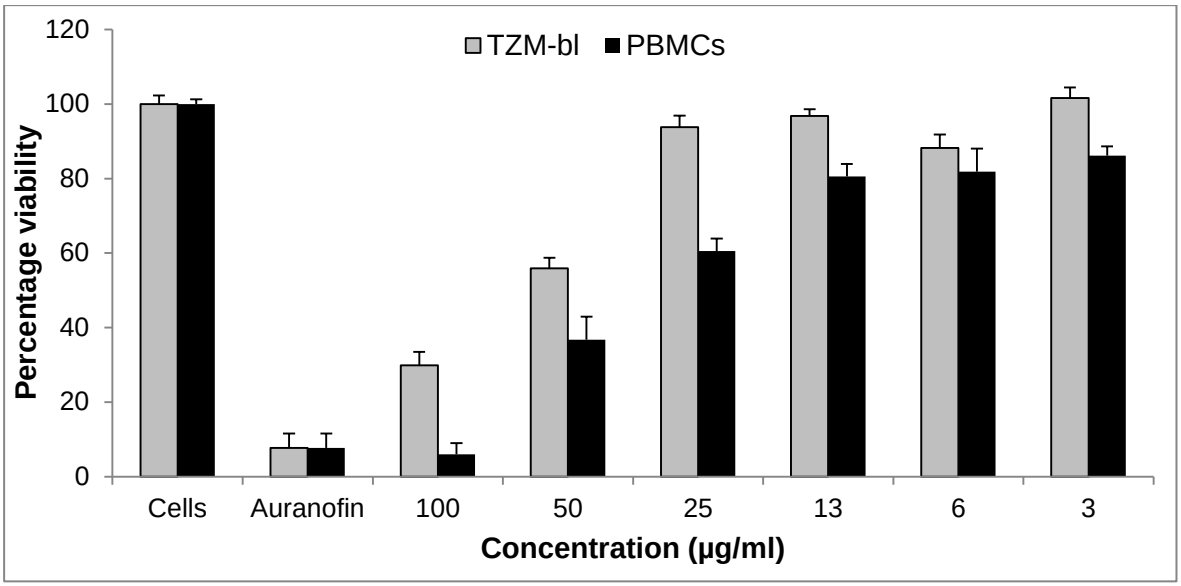

Figure 5.5: Effect of *O. labiatum* extract on the viability of TZM-bl and PBMCs. The cells were incubated with the extract at various concentrations for 72 h at 37 °C in a 5% CO_2 humidified environment. Controls included cells only and auranofin, a known toxic compound. Viability was assessed using tetrazolium dyes (MTS for PBMCs and MTT for TZM-bl cells).

The tetrazolium dye results were confirmed with a RT-CES device and the highest concentration tested against HIV-1 PR, 100 µg/ml, was found to reduce the viability of TZM-bl cells at 6h after treatment of cells with extract (Figure 5.6A, iii); this was observed when the cell index for that concentration was lower than that of control cells and was at the same level as auranofin (toxic control) at the end of incubation (96 h). The lowest HIV-1 PR inhibiting concentration tested, 50 µg/ml, resulted in more than 50% of the cells being viable at 96 h (Figure 5.6A, ii). A unique viability pattern was observed with TZM-bl treated with 50 µg/ml of the extract; cells had a high proliferation at 58-83 h of incubation and this could suggest high metabolism of the extract by the cells during that time period. As expected, the positive control, auranofin (iv) drastically reduced TZM-bl viability right after cell treatment (24 h). The isolated compound (Figure 5.6B) demonstrated low cytotoxicity at both concentrations tested (100 & 50 µg/ml). At 96 h, cell index for control cells (i) was 2.4 and that of phy-a was 1.8 (ii, 50 µg/ml) and 1.3 (iii,100 µg/ml) and this indicated that viability of both concentrations of the phy-a was >50% because the cell indexes were more than half the cell index of control cells (2.4÷2). The cell index (y-axis) is directly correlated to the number of cells in the wells and the higher the cell index the more viable the cells (Atienzar et al., 2011). The intrinsic colour of phy-a interfered with the absorbance of tetrazolium dyes at high concentrations (>50 µg/ml) that is why the dye-free technique, RT-CES, was directly

used to determine the viability of TZM-bl cells in the presence of phy-a. One of the limitations of the use of the MTT assay for screening particular compounds that are extracted from natural sources, for example polyphenols, is that these compounds can react with MTT and thus interfere with the screening results (Han et al., 2010). This explains the interference of phy-a (at high concentrations) with MTT absorbance in the present study.

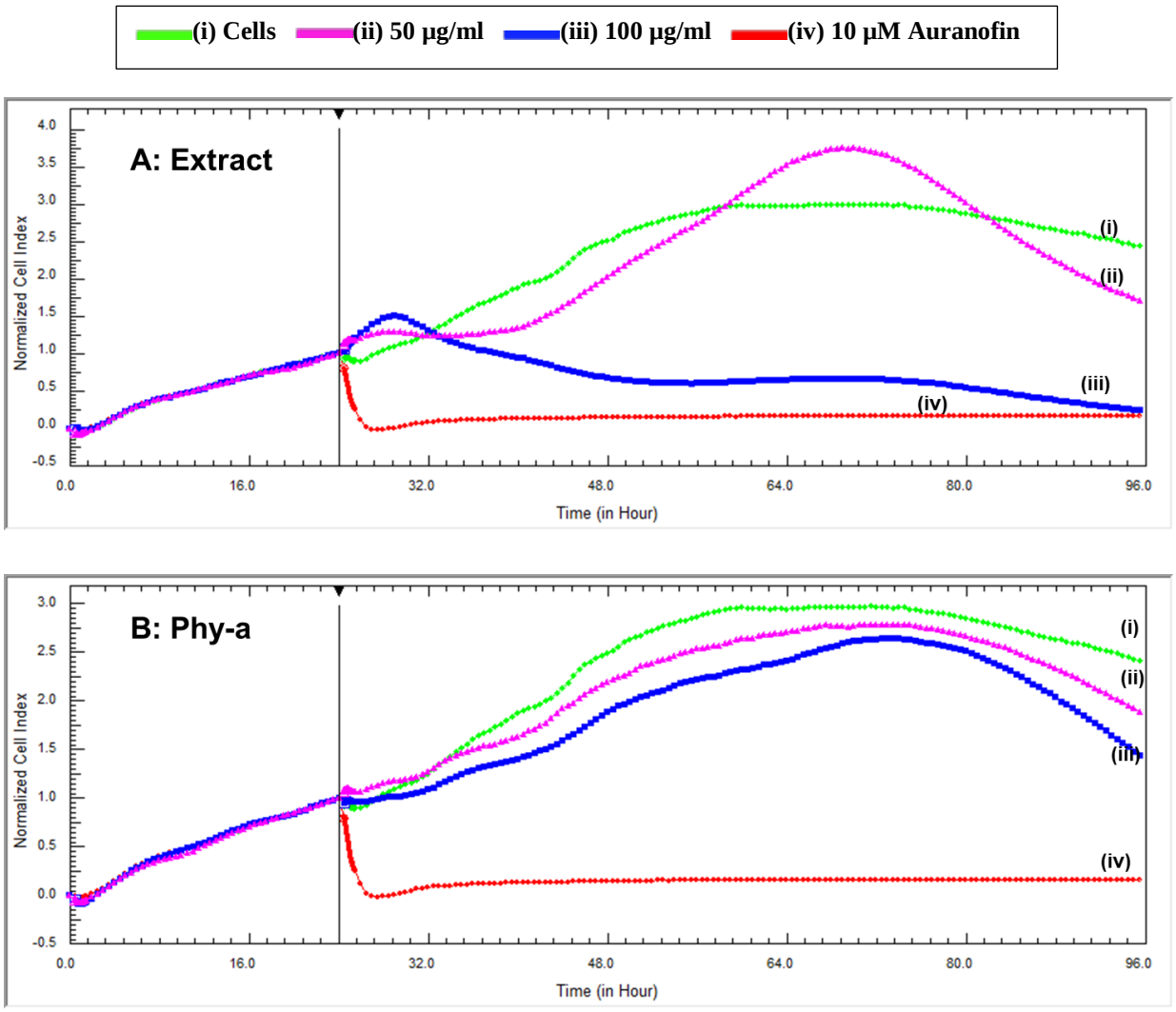

Figure 5.6: Real time cytotoxicity monitoring of *O. labiatum* extract (A) and phy-a (B) towards TZM-bl cells. The cells were seeded at 10,000 cells per well and were exposed to two concentrations of the extract and compound at 24 h when the cell index was ±1. Auranofin (iv) was used as a positive control for toxicity and cells only (i) were an untreated control. Each data point was calculated from duplicate values normalized against the time just before sample addition. The highest concentration of the extract inhibiting HIV-1 PR, 100 µg/ml (iii), was toxic to TZM-bl cells whilst 50 µg/ml (ii) which also inhibited the enzyme resulted in >50% cell viability. The isolated compound (B) demonstrated low cytotoxicity towards TZM-bl at both concentrations tested, 100 & 50 µg/ml.

5.4 Discussion

Even though *O. labiatum* has not been reported to be used traditionally in HIV/AIDS, similar plants *O. sauve* and *O. gratissimum* are administered traditionally to HIV/AIDS patients to manage the disease and related illnesses (Kisangau et al., 2007; Lamorde et al., 2010). The antiviral and antimicrobial properties reported on plants from the *Ocimum* genus could mean that these species generally contain antiviral properties as proven by the pharmacological data presented here.

A study by Geuenich et al. (2008) revealed extracts from three Lamiaceae plants to have potent activity against HIV-1 infection. These extracts were able to reduce activity of the viral particles by altering their density, rendering them unable to replicate (Geuenich et al., 2008). Presented here is the first report of the ability of *O. labiatum* extract to inhibit HIV-1 expression *in vitro* which supports emerging evidence of the ability of Lamiaceae plants to reduce HIV-1 replication either by hindering the whole virus or by directly inhibiting HIV-1 enzymes (Geuenich et al., 2008; Kapewangolo et al., 2013; Klos et al., 2009). The crude extract and purified compound were able to inhibit HIV-1 PR. In addition to enzyme inhibition, suppression of viral replication in a latent model was observed.

Cytotoxicity of the extract was noted at higher concentrations. *In vitro* cytotoxicity of compounds isolated from *O. labiatum* was previously reported (Hussein et al., 2007). A labdane diterpenoid compound isolated from this plant presented moderate cytotoxicity against breast cancer cells whereas another diterpenoid from the same plant did not present any cytotoxicity at the highest concentration tested in the same cells (Hussein et al., 2007). Oleanolic acids which is commonly isolated from *Ocimum* plants including *O. labiatum* presented strong toxicity against solid tumor cancer cell lines (Hussein et al., 2007; Prabhu et al., 2009). Cytotoxicity varies in compounds isolated from *O. labiatum* and cytotoxicity at high concentrations is usually attributed to oleanolic acid. The chlorophyll derivative, phy-a, isolated from *O. labiatum* in this study was generally non-toxic at high concentrations.

Chlorophyll is the most widely distributed natural pigment with strong antioxidant activity found in leaves and most other plant parts (Lanfer-Marquez et al., 2005). Chlorophyll is highly unstable and its breakdown leads to the formation of chlorophyll derivatives with

the most common derivatives being chlorophyll-a and b, phy-a and b as well as pheophorbide-a and b (Lanfer-Marquez et al., 2005).

Phy-a isolated from *Lonicera hypoglauca* (Caprifoliaceae), was previously reported by Wang et al. (2009) to be a potent anti-hepatitis C virus (HCV) compound by binding to the active site of HCV-NS3 protease. Makatini et al. (2012) worked with compounds structurally similar to phy-a and explained the mechanism of action of long chain inhibitors with carbonyl groups against HIV-1 protease. According to the authors' findings, HIV-1 protease converts ketones of the long chain inhibitors to hydroxyl groups, and then the newly formed hydroxyl group binds to the active pocket of the enzyme (Makatini et al., 2012). Phy-a contains regions with ketones which possibly inhibited HIV-1 PR in this study. In another study, three pheophytin compounds isolated from *Clinacanthus lutans* (Acanthaceae) were shown to have anti-herpes simplex (HSV) activity; the three pheophytins inactivated HSV before cell entry (Sakdarat et al., 2009). Another chlorophyll isolate pheophorbide-a, relatively abundant in green plants, purified from *Vatica cinerea* (Dipterocarpaceae) demonstrated anti-HIV activity by inhibiting HIV infectivity in HOG.R5 cells (Zhang et al., 2003). Pheophobide-a isolated from another natural source, *Opuntia ficus-indica* (Cactaceae), showed potent virucidal effects on HSV and influenza A virus (Bouslama et al., 2011). Based on the current results as well as those previously reported, it can be deduced that chlorophyll derivatives have virucidal effects against enveloped viruses HIV, HSV and HCV.

There is no existing literature indicating known HIV inhibitory properties of *O. labiatum* and phy-a, making this *in vitro* HIV study a first for this plant and the isolated chlorophyll derivative. The data obtained from this study demonstrated that *O. labiatum* has potent inhibitory activity against HIV-1. The identification of a bioactive compound, phy-a, adds to additional compounds to consider for further development against HIV/AIDS especially in the light of evidence that these types of chlorophyll derivatives inhibit HIV replication.

Acknowledgements

The authors want to thank the funders; Southern African Biochemistry and Informatics for Natural Products (SABINA), Ministry of Education (Namibia), the Technology Innovation Agency (TIA, RSA), Margaret McNamara Memorial Fund (MMMF) and the University of Pretoria. Special thanks to the University of Botswana (Chemistry

Department) and Mintek, South Africa for assistance with purification and NMR analyses.

CHAPTER 6

Triterpenoids from *Ocimum labiatum* activates latent HIV-1 expression

Submitted to Antiviral Research

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Abstract

Purpose: HIV reservoirs in infected individuals are a major contributor to the failure of current regimens in eradicating infection. These viral latent pools, as they are known, contribute to the re-emergence of drug resistant HIV strains. Antiretroviral strategies need to be modified to take into account latent HIV which is why compounds are being developed as possible adjuvants for highly active antiretroviral therapy (HAART). In the current study, compounds isolated from *Ocimum labiatum* were investigated for possible effects on the induction of HIV-1 expression in a latently infected cell line.

Methods: Crude extract was prepared from fresh leaves of *O. labiatum*. Compounds were purified from the crude extract by column chromatography and analysed using high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR). Cytotoxicity of the samples was measured in cells using tetrazolium dye and flow cytometry. The effect of the isolated constituents on HIV-1 expression was investigated in a latently infected monocytic U1 cell line and primary cells from blood of infected patients on HAART by measuring the HIV-1 p24 antigen released in culture

media. The effect of the compounds on cytokine production from human mononuclear cells was determined by cytometric bead array technology. A fluorometric assay was used to investigate the effect of compounds on histone deacetylase (HDAC) in a HeLa cell lysate.

Results: Isolated triterpene isomers (HHODC) significantly ($p<0.05$) induced HIV-1 expression in a dose-dependent manner in U1 cells at concentrations that were non-cytotoxic (4, 6 and 8 µg/ml). The results were comparable with those of a positive control, prostratin, currently being considered for human clinical trials to purge latent HIV. The isomers further induced viral expression from PBMCs of HIV-1 infected patients on HAART. HHODC up-regulated the production of IL-2, IL-4, IL-6, TNF and IFN-γ suggesting a possible latent activating mechanism. Inhibition of HDAC by HHODC was minimal at concentrations activating latent HIV-1.

Conclusion: These findings suggests that the isolated triterpene isomers show promise as candidates for supplementing current HIV drugs by inducing the expression of latent HIV-1 enabling dormant virus eradication from HIV-infected individuals.

Keywords: *Ocimum labiatum*, novel triterpenes, latent HIV-1, HIV-1 eradication.

6.1 Introduction

HIV-1 infection is not eradicated by highly active antiretroviral therapy (HAART). The drugs are unable to eradicate latent HIV-1 which has the ability to escape immune surveillance or clearance (Kulkosky and Bray, 2006). Current HIV-1 regimens have the ability to reduce the viral load to an undetectable level; but complete eradication of the virus cannot be achieved (Kulkosky and Bray, 2006; Marcello, 2006; McKernan et al., 2012).

Viral reservoirs that are established early on during HIV-1 infection are a major contributor to the development of drug resistant HIV-1 infection (Marcello, 2006). These reservoirs remain unaffected by anti-retroviral drugs and have the ability to replenish systemic infection once treatment is interrupted (Marcello, 2006). HIV-1 infected individuals on treatment tend to stop taking drugs in the long run due to adverse side-effects such as metabolic complications (Leary et al., 2012; Mills and Nabiryo, 2013). It

is this kind of interruption that leads to activation of HIV-1 cellular reservoirs leading to the development of drug resistant HIV-1 strains (Marcello, 2006). Therapeutic targeting of HIV-1 requires further investigation and current therapies need modification in order to take latent HIV-1, a major hurdle in HIV eradication, into account (Palmer et al., 2011; Tyagi and Bukrinsky, 2012). A number of clinical trials were conducted with the aim of targeting latent viral reservoirs and the drugs used in these trials were unable to completely purge the highly stable viral reservoirs (Gallastegui et al., 2012; Kulkosky and Bray, 2006). Panobinostat, an experimental anti-cancer drug, is currently being used in a phase II clinical trial that aims to assess the safety and ability of the drug to purge HIV-1 from latent reservoirs of HIV-infected patients on HAART (Rasmussen et al., 2013). Another promising HIV latent activator that is currently being considered for human clinical trials to augment HAART is prostratin, a phorbol ester isolated from a tropical plant *Homalanthus nutans* (Euphorbiaceae). Prostratin has the unique ability to block HIV-1 infection and yet induce latent proviral expression (Kulkosky et al., 2001; Rullas et al., 2004). Bryostratin, a marine macrolide, isolated from *Bugula neritina* (Linnaeus) can induce HIV from latency (Marsden and Zack, 2013; Pettit et al., 1982). Because the isolation of bryostratin from natural sources is expensive and problematic; total synthesis was recently achieved and the *in vivo* safety and efficacy of the new compound is under investigation (DeChristopher et al., 2012).

Nature remains a great source of novel compounds and most existing drugs are derived from natural sources (Itokawa et al., 2008; Newman and Cragg, 2012). Lamiaceae is a plant family with numerous ethnobotanical uses (Rice et al., 2011; Van Wyk et al., 2009). Plants from this family are rich in terpenoids, a class of compounds commonly associated with the medicinal activity of these plants (Hussein et al., 2007; Macías et al., 2008). Various terpenoids reportedly inhibit different stages of the HIV-1 life cycle *in vitro* including the inhibition of viral enzymes like protease (Wei et al., 2008) responsible for cleaving viral proteins, the required components of new virus particles (Todd et al., 1998). Betulinic acid is a terpene that was discovered to be a potent compound for inhibiting HIV-1 replication by blocking viral maturation (Li et al., 2003; Stoddart et al., 2007).

In the present study, triterpenes isolated from *Ocimum labiatum* (Lamiaceae) were investigated for possible effects on HIV-1 replication in a latently infected monocytic U1 cell line. The isolated terpenes demonstrated the ability to activate latent virus,

suggesting possible use as adjuvants in conjunction with HAART. Adjuvant therapy in this context means the use of a compound (in this case a natural product) in concert with HAART; so that the former can activate latent virus replication while the latter drugs can then inhibit this replication and potentially eradicate latent reservoirs. There is a continuous search for novel compounds to include in future clinical trials (Gallastegui et al., 2012) and the present report is a contribution towards that goal.

6.2 Methods

6.2.1 General

^{1}H and ^{13}C NMR were recorded on Bruker 400 MHz (Massachusetts, USA) NMR spectrometer using tetramethylsilane (TMS) as an internal standard. The solvents used for NMR spectra were deuterated chloroform and deuterated methanol. TLC was carried out on Merck (Darmstadt, Germany) silica gel plates F_{254} with layer thickness of 0.2 mm and visualized under UV light and by staining with vanillin-sulphuric acid, followed by heating. Column chromatography separation and purification were performed on silica gel 60: 70-230 mesh (Merck, Darmstadt, Germany) and Sephadex LH-20 (Sigma, MO, USA). HPLC was conducted with a Shimadzu preparative 6AD LC system equipped with a UV-visible (215 and 254 nm) detector, a manual injector 10AF, and a fraction collector FRC-10A (Shimadzu, KYT, Japan). Aliquots (200 µl) were injected in a C18 Jupiter analytical column of 250 mm x 4.6 mm x 10 µm (particle size).

6.2.2 Plant material

Leaves of *Ocimum labiatum* were collected in February 2012 from the Botanical Garden of the University of Pretoria. A voucher specimen is deposited in the H.G.W.J Schweikerdt Herbarium of the University (117693).

6.2.3 Isolation and identification of compounds

Fresh leaves (894.6 g) were extracted in ethanol. After filtration, the filtrate was concentrated under vacuum and the residue was re-dissolved in ethyl acetate to exclude highly polar tannin compounds. The ethyl acetate fraction was partitioned on silica gel with n-hexane-ethyl acetate (100:0 to 0:100) successively to afford fractions which were further purified with Sephadex LH-20, eluted with chloroform. Separation over a Sephadex column yielded compounds 1 (24.9 mg) and 2 (250 mg). The spectral data for compound 1 were in agreement with information in the literature for amyrin

(Kushiro et al., 1998; Martelanc et al., 2009). Compound 2 (Figure 6.1) was identified from the NMR spectra to be a new triterpenoid 3-hydroxy-4,6a,6b,11,12,14b-hexamethyl-1,2,3,4,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydropicene-4,8a-dicarboxylic acid (abbreviated to HHODC). The spectral assignments for the new compound are presented in Table 6.1. Compound 2 will be referred to as HHODC in the rest of this report. Compounds were reconstituted in dimethyl sulfoxide (DMSO), which provides a sterile environment, before each biological assay. Further dilutions to obtain desired compound concentrations were done in cell culture media.

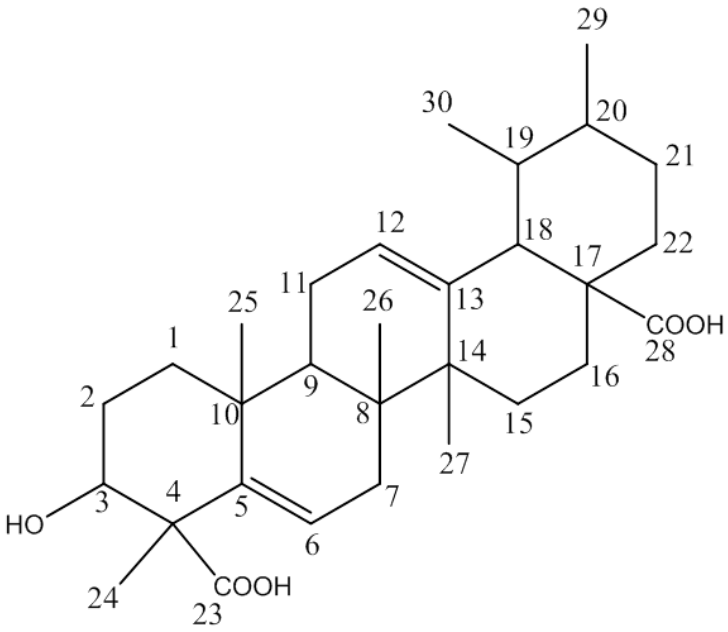


Figure 6.1: New triterpenoid isolated from *Ocimum labiatum*.

Table 6.1: ^{1}H- and ^{13}C-NMR data of compound 2 (400 MHz, in CD_3OD).

Position	^{13}C δ ppm	^{1}H δ mult
1	36.7 (*t*)	1.41, 2H, (*m*)
2	27.3 (*t*)	1.61, 2H, (*m*)
3	78.3 (*d*)	2.89, 1H (*m*)
4	55.3 (*s*)	
5	143.8 (*s*)	
6	122.2 (*d*)	5.26, 1H, (*m*)
7	33.9 (*t*)	2.02, 2H, (*s*)
8	39.0 (*s*)	
9	47.8 (*d*)	1.54, 1H, (*m*)
10	30.2 (*s*)	

11	26.4 (*t*)	2.15, 2H, (*m*)
12	125.4 (*d*)	5.25, 1H, (*m*)
13	138.2 (*s*)	
14	41.3 (*s*)	
15	27.8 (*t*)	1.35, 2H
16	25.0 (*t*)	1.64, 2H
17	48.1 (*s*)	
18	52.9 (*d*)	2.25, 1H
19	38.6 (*d*)	1.71, 1H
20	38.4 (*d*)	1.67, 1H
21	30.4 (*t*)	1.58, 2H
22	33.5 (*t*)	1.98, 2H
23	180.3 (*s*)	
24	18.1 (*q*)	1.18, 3H (*s*)
25	20.1 (*q*)	1.14, 3H (*s*)
26	14.9 (*q*)	0.93, 3H (*s*)
27	23.9 (*q*)	1.00, 3H (*s*)
28	180.5 (*s*)	
29	23.1 (*q*)	0.80, 3H
30	16.3 (*q*)	0.87, 3H

6.2.4 Cytotoxicity

Cytotoxicity of the compounds in TZM-bl and U1 cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, MO, USA) (Mosmann, 1983). Briefly, cells were seeded at $1x10^4$ cells/well in 96-well plates and incubated with various concentrations of the compounds for 72 h at 37 °C in a humidified incubator with 5% CO_2. After 72 h, MTT solution was added to the cells and incubated for an additional 2 h. The formazan crystals produced by viable cells were dissolved in 50 µl of 1 M hydrochloric acid in propanol, and the absorbance was measured at 550/690 nm using a Multiskan Ascent microplate reader (Thermo Labsystems; MA, USA).

6.2.5 Stimulation of latent HIV-1

U1 cells were seeded at $1x10^5$/well in a 24-well format and treated with desired concentrations of HHODC, prostratin or phorbol myristate acetate (PMA) (Sigma, MO, USA). Prostratin and PMA are known latent HIV-1 inducers (Kulkosky et al., 2001). A population of untreated U1 cells was included as a control. All stimulations were

performed in triplicate. Supernatants were drawn after 72 h, stored at -20°C, and analysed for HIV-1 p24 antigen concentration using a RETRO-TEK HIV-1 p24 antigen ELISA kit (ZeptoMetrix Corporation, NY, USA). To establish that the induced virus production was caused by activation of HIV-1 expression and not due to toxicity, MTT was added to U1 cells after harvesting supernatant. In a separate replica experiment, U1 cells were labelled with a fluorescent dye (carboxylfluorecein succinimidyl ester; CFSE) prior to treatment with samples for 72 h. Propidium iodide was incorporated to exclude background staining from dead cells (Kapewangolo et al., 2013). Data was acquired on a FACSAria (BD BioSciences, California, USA).

6.2.6 Activation of HIV-1 expression from PBMCs of infected individuals on HAART

Ethical approval for obtaining blood samples from consenting donors was granted by the Faculties of Natural and Agricultural Sciences and Health Sciences Ethics Committees (EC080506-019;163/2008,University of Pretoria, South Africa). Fresh whole blood from two infected patients receiving HAART was treated to density centrifugation on a Ficoll–Hypaque (Sigma, MO, USA) gradient to obtain peripheral blood mononuclear cells (PBMCs). Cells were seeded at $5x10^5$/well in a 24-well format and treated with HHODC (4, 6 & 8 µg/ml) and prostratin (0.05 & 0.1 µM). Supernatant was drawn after 72 h and analysed for p24 concentration using a RETRO-TEK HIV-1 p24 antigen ELISA kit (ZeptoMetrix Corporation, NY, USA).

6.2.7 Effect of HHODC on histone deacetylase activity

The effect of HHODC on histone deacetylase (HDAC) activity was investigated. HDAC inhibition is one of the mechanisms attributed to the activation of latent HIV-1 in viral reservoirs (Rasmussen et al., 2013). A fluorometric HDAC assay kit (Sigma, MO, USA) was used for this assay. The assay provides a simple enzymatic reaction for the detection of HDAC activity using a substituted peptide as a substrate. The peptide has an acetylated lysine residue and a bound fluorescent group. The kit provides HeLa cell lysate as a source for HDAC activity. The first step of the reaction is deacetylation of the acetylated lysine side chain by HDAC and the second step involves the cleavage of the deacetylated substrate by the developer solution, releasing a free highly fluorescent group. HHODC was incubated with HDAC at various concentrations. Trichostatin A (TSA) was used as a positive control for HDAC inhibition (Wegener et al., 2003). Other controls included substrate in assay buffer and an untreated enzyme control.

Fluorescence was measured with the spectrofluorometer (Thermo Labsystems, MA, USA) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

6.2.8 Statistical analysis

Data for all experiments is presented as the mean ± SD with n=3-6 unless stated otherwise. Significant difference and CC_{50} values were computed using Graphpad Prism 5 (Graphpad Software Inc. CA, USA) and Student's t test for unpaired observations. A $p<0.05$ was considered significant. Flow cytometry data was analysed using FlowJo Version 7.6.1 (TreeStarInc., Oregon, USA).

6.3 Results

Column chromatography of an ethyl acetate fraction of *O. labiatum* leaves on silica gel followed by Sephadex LH-20 led to the isolation of two triterpenes. A known triterpene that is common in nature, amyrin (Kushiro et al., 1998) was identified as compound 1 while compound 2, the second triterpene,3-hydroxy-4,6a,6b,11,12,14b-hexamethyl-1,2,3,4,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydropicene-4,8a-dicarboxylic acid isolated appeared to be a mixture of isomers. HPLC analyses of HHODC showed two closely adjacent peaks on the HPLC chromatogram (Figure 6.2) which is typical of isomers (Zhou et al., 2013). Peaks 1 and 2 had the same retention time.

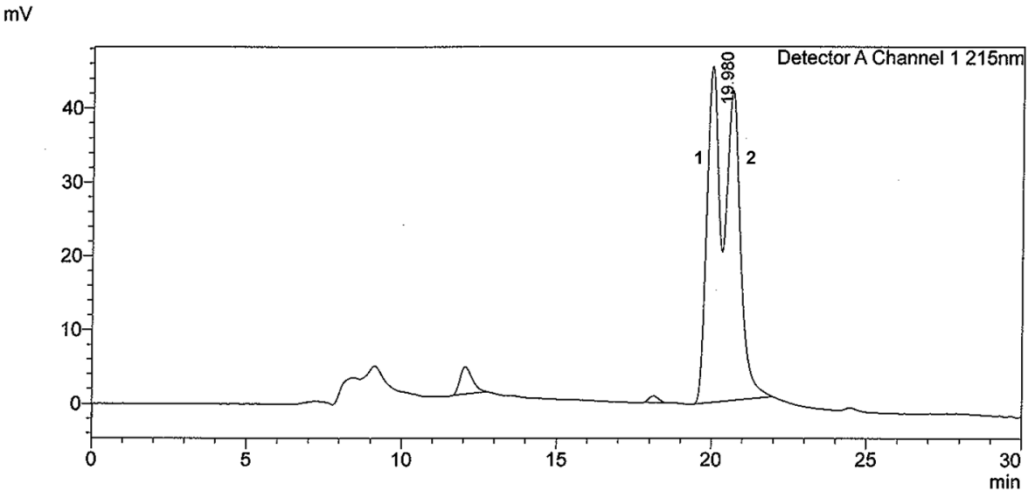
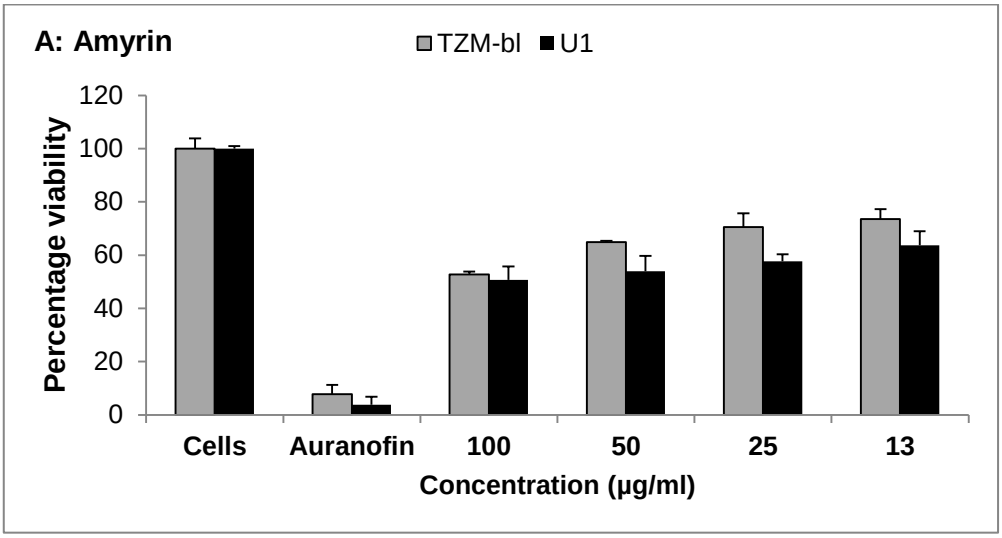


Figure 6.2: HPLC chromatogram of HHODC isomers.

Amyrin and HHODC demonstrated low toxicity in TZM-bl cells with 50% cytotoxic concentrations (CC_{50}s) of >100 µg/ml (Figure 6.3). Amyrin also demonstrated low

toxicity (Figure 6.3A) in U1 cells with a CC_{50} of >100 µg/ml. HHODC was more toxic in U1 cells (Figure 6.3B) exhibiting a CC_{50} of 8.2 ±0.1 µg/ml as computed using Graphpad prism 5 software. Auranofin was used as a toxicity control.



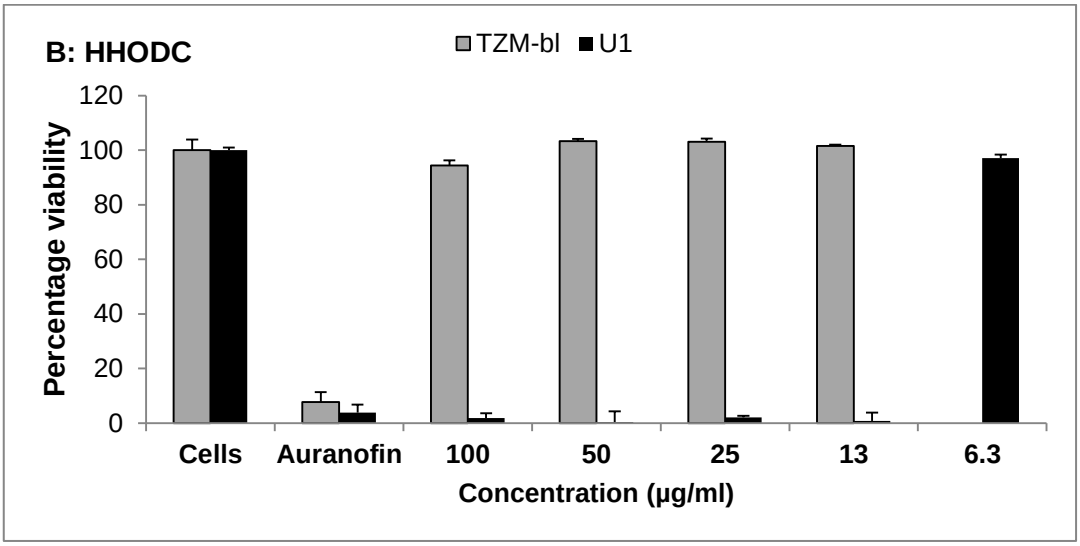


Figure 6.3: Cytotoxicity of isolated compounds on TZM-bl and U1 cells. Cells were incubated with the compounds for 72 h. **A**. In the presence of amyrin, viability of TZM-bl and U1 cells was >80% demonstrating low cytotoxicity. **B**. The isolated triterpene, HHODC, was more toxic to U1 cells than TZM-bl cells.

HHODC induced HIV-1 expression from U1 cells in a dose-dependent manner (Figure 6.4A). The viral up-regulation compared well to that induced by prostratin (Figure 6.4B). PMA actively induced viral production (Figure 6.4C) but it appeared to be cytotoxic at both concentrations tested (Figure 6.5). The second compound that was isolated in this

study, amyrin, did not induce HIV-1 expression in U1 cells at the tested concentration (3.1 µg/ml) (Appendix Figure A6.3). Unstimulated U1 cells were characterized by a state of relative latency and low detectable HIV-1 p24 antigen. The concentrations of HHODC and Prostration tested for HIV-1 expression were not cytotoxic as indicated in Figure 6.5. The idea was to test concentrations that resulted in cell viability of >90% in order to exclude cytotoxicity concerns. PMA (0.5, 1 & 2 µM) resulted in less than 20% of U1 cells being viable (Figure 6.5). Flow cytometry was used to confirm concentrations of HHODC that appeared non-cytotoxic when viability studies were performed using MTT (Figure 6.6).

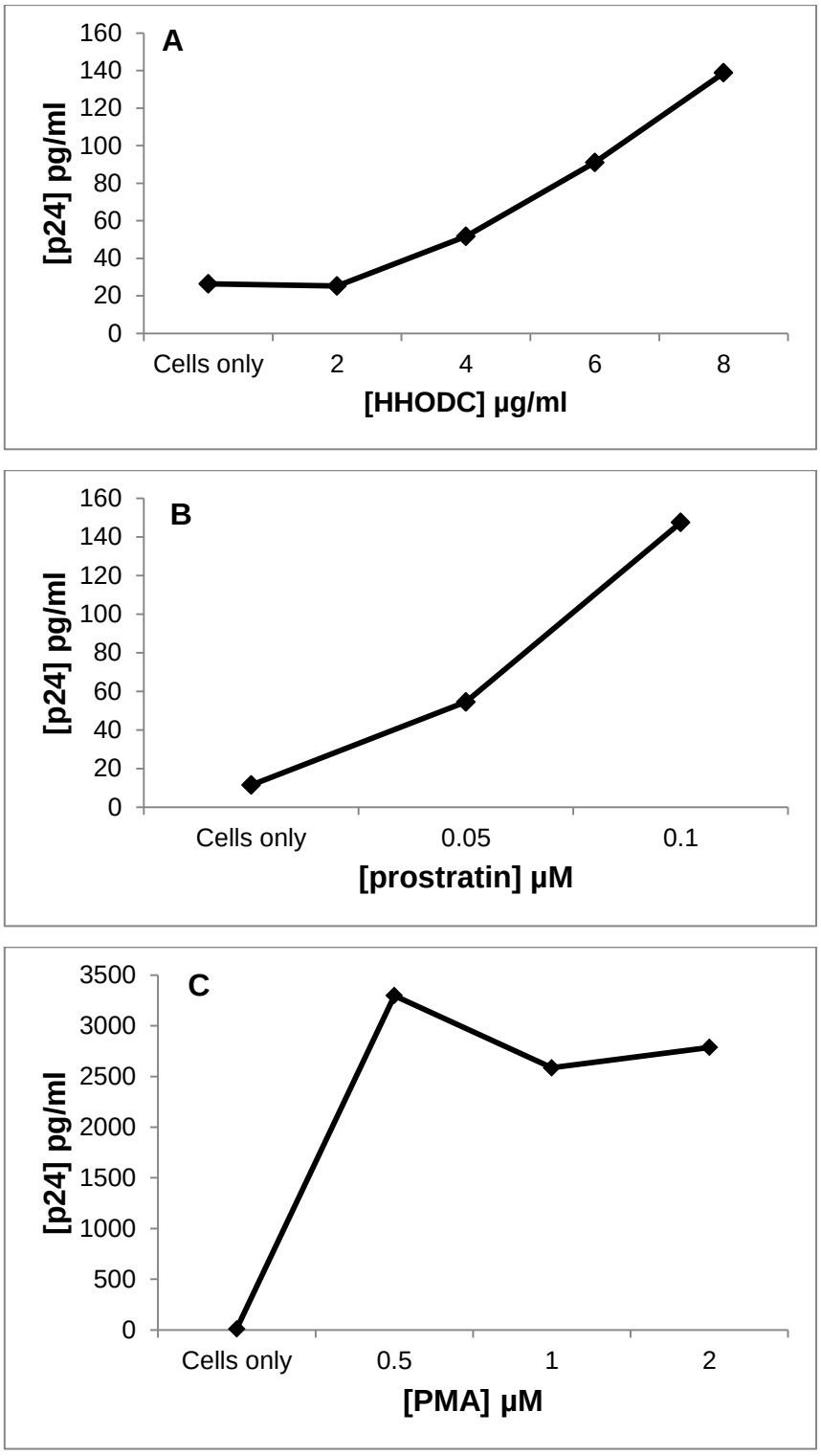


Figure 6.4: Effects of compounds on HIV-1 expression. U1 cells were treated with 2, 4, 6 and 8 µg/ml HHODC **(A)**. Supernatant was collected after 72 h incubation and quantitatively analysed for HIV-1 p24 antigen. HHODC activated latent HIV-1 expression in a dose-dependent manner. Prostratin **(B)** and PMA **(C)** were included as positive controls of latent HIV-1 activation in U1 cells.

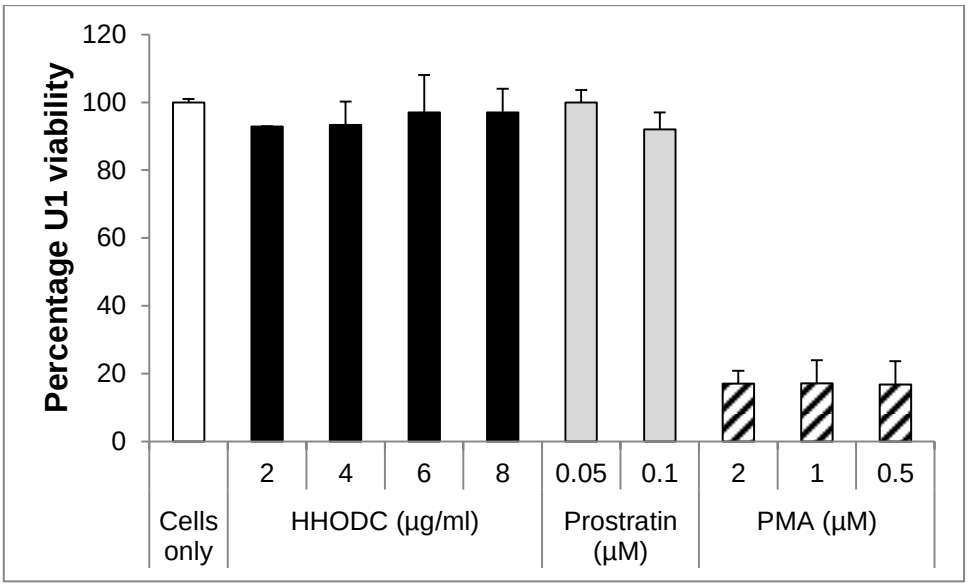


Figure 6.5: Effects of compounds on U1 viability upon viral stimulation.

Cytotoxicity was analysed by MTT and U1 viability was ≥90% at the concentrations of HHODC and prostratin tested for HIV-1 expression. PMA was cytotoxic to U1 cells.

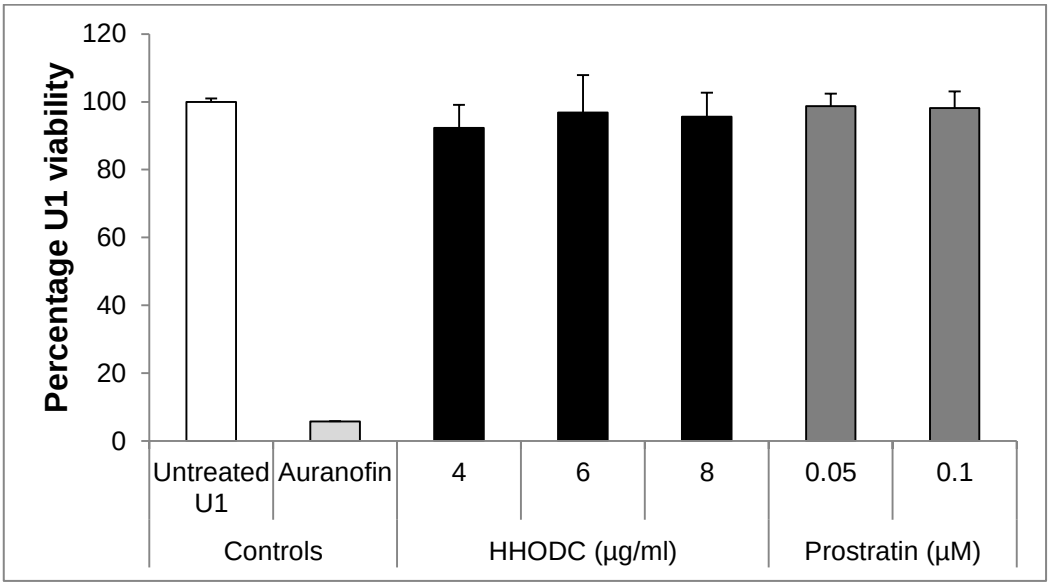


Figure 6.6: Effect of HHODC on U1 proliferation as determined by flow cytometry.

U1 cells labeled with CFSE were treated with active concentrations of HHODC and prostratin for 72 h. PI was included to exclude dead cells. Each bar reflects the mean of 2 trials ±SD.

The tumor promoting effect of PMA was evident right after addition to U1 cells (Figure 6.7). Images captured from a light microscope clearly showed grouping of cells treated with PMA. HHODC and prostratin did not have this cluster promoting effect on the cells.

PMA is widely reported to have clastogenic effect (Emerit and Cerutti, 1982; Kim et al., 2013; Yu et al., 2012). It damages chromosomes causing mutations and clustering of cells. The implication of this is that HHODC does not possess tumor promoting effect. Prostratin is known to lack this tumor promoting effect, explaining the similarity in cell morphology of prostratin treated and untreated U1 cells.

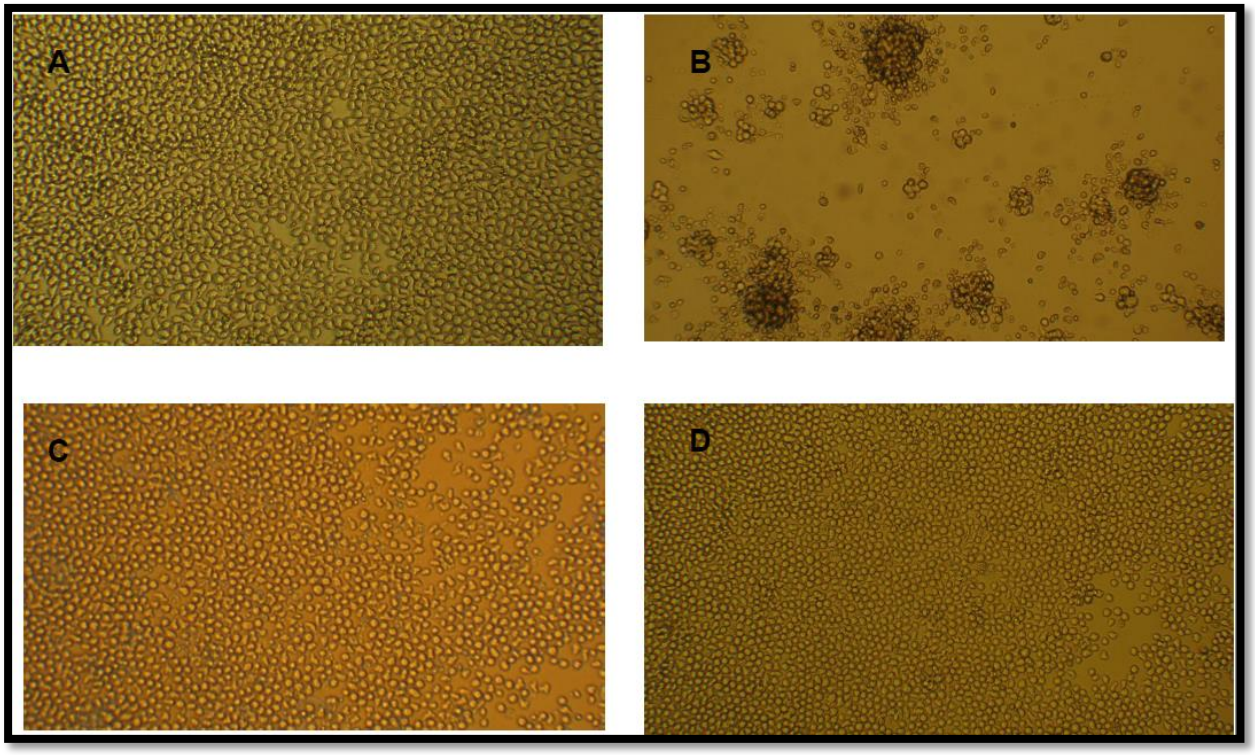


Figure 6.7: HHODC did not have clustering effect on U1 cells. Effect of samples on U1 cells was monitored with a light microscope. Untreated U1 cells **(A)** were used as a control. PMA **(B)** induced clustering effect upon addition to cells at all concentrations tested (0.5, 1 and 2 µM). HHODC at 8 µg/ml **(C)** and 0.1 µM prostratin **(D)** did not have the same effect. Images were captured before collecting supernatant for HIV-1 p24 analysis. These observations are representative of 3 different experiments.

HHODC had an effect on HIV-1 expression in cells isolated from 2 patients as illustrated in Figure 6.8. Cells from patient 1 elicited the most response for viral expression after treatment with 8µg/ml of HHODC. Cells from patient 2 also produced HIV-1 most notably at the two high concentrations tested for HHODC (6 and 8 µg/ml). Treatment with prostratin resulted in low up-regulation of p24 antigen production (Figure 6.8). Concentrations tested were not cytotoxic to PBMCs; viability was >90% (Appendix Figure A6.5).

HHODC was also investigated for its effect on an array of cytokines in HIV negative PBMCs and U1 cells using a Th1/Th2/Th17 cytometric bead array technique (Appendix Table A6.1). The compound significantly ($p<0.05$) increased the production of interleukin (IL)-6, tumour necrosis factor (TNF) and interferon (IFN)-γ in PBMCs and up-regulated the production of IL-2, IL-4, IL-6, TNF and IL-17A in U1 cells.

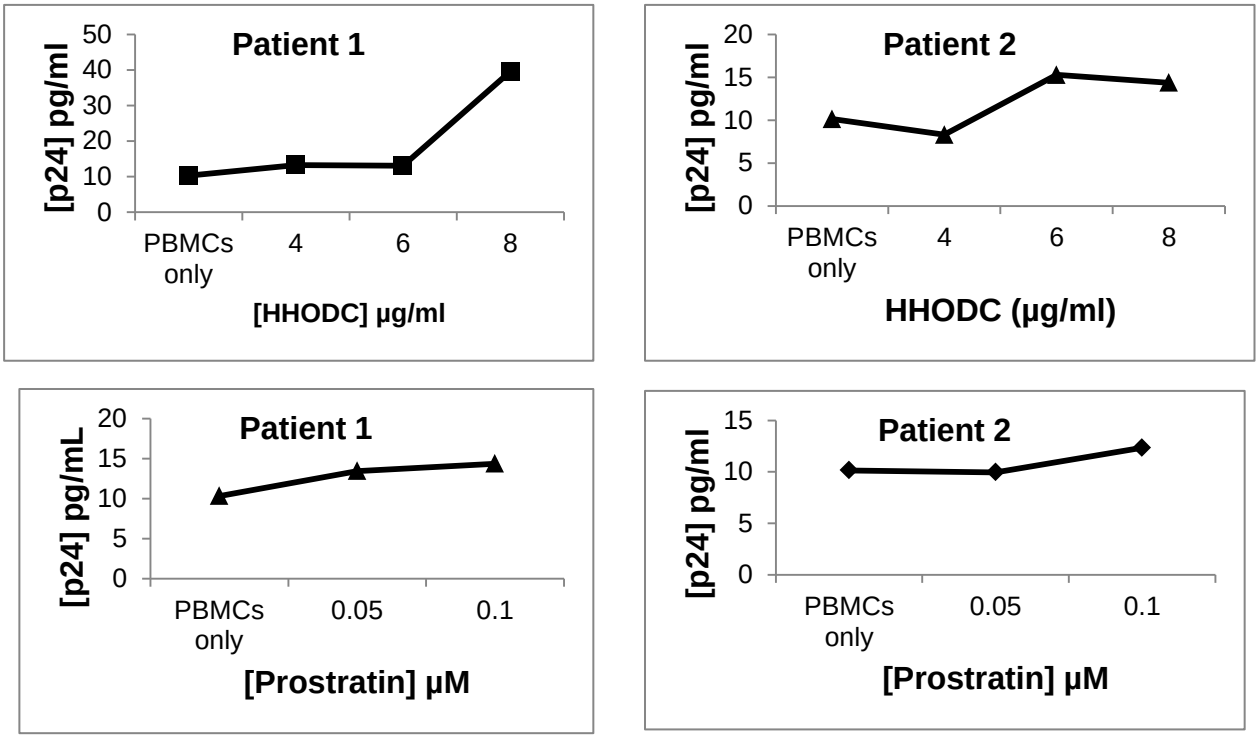


Figure 6.8: HHODC induction of HIV-1 expression from PBMCs of infected patients on HAART. PBMCs from HAART patients were treated with HHODC and prostratin as indicated. Supernatant was collected after 72 h to determine HIV-1 p24 antigen by ELISA. Each point represents the mean of 2 trials ±SD.

As illustrated in Figure 6.9, higher concentrations of HHODC (25 and 50 µg/ml) caused an increase in HDAC activity. Lower concentrations (6-12.5 µg/ml) demonstrated marginal HDAC inhibition.

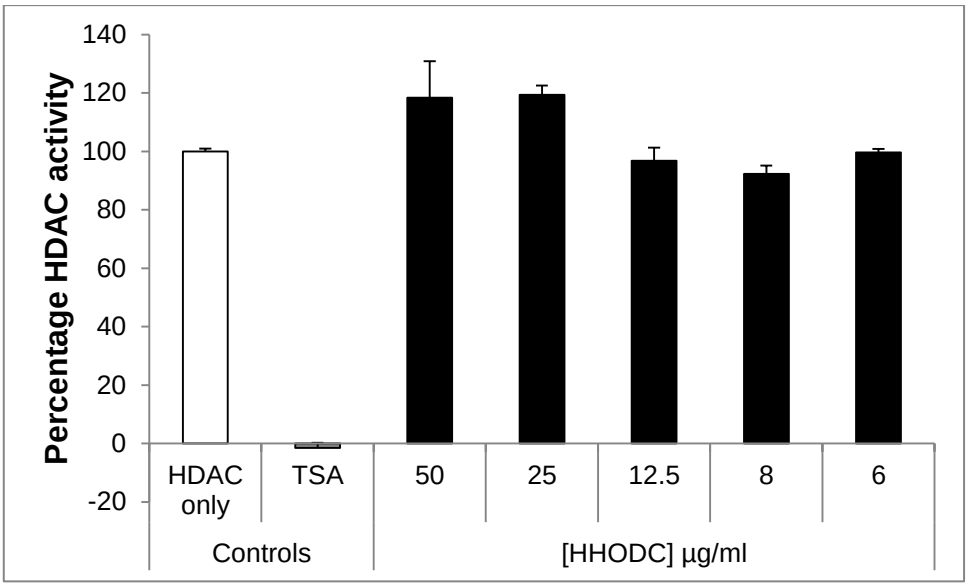


Figure 6.9: Effect of HHODC on HDAC activity. HDAC activity in HeLa cell lysate was measured by incubating substrate and samples in a reaction volume 100 µl. Marginal HDAC inhibition was observed with 8 and 12.5 µg/ml of HHODC, while higher HHODC concentrations increased enzyme activity.

6.4 Discussion

Current HIV drugs target different stages of the viral life cycle, however, none of the drugs in the regimens target latent HIV-1 particles in cellular viral reservoirs (Palmer et al., 2011). These reservoirs can replenish systemic infection (when treatment is interrupted) and also contribute to the development of drug resistant HIV-1 strains (Tyagi and Bukrinsky, 2012). Ideal antiretroviral therapy should also include drugs with the ability to activate latent HIV-1 reservoirs so that once activated other drugs in the regimen can then interrupt viral replication.

Here, a triterpene was isolated from *O. labiatum* (HHODC) with structure resembling that of a group of compounds that were identified virtually by Gallastegui et al. (2012) having the ability of activating latent HIV-1. In the study by Gallastegui et al. (2012) only one compound was found to be potent *in vitro* when combined with PMA and on its own did not activate the virus. In the present study, HHODC was able to activate latent HIV-1 in the absence of PMA and the viral expression was comparable to prostratin. The activation occurred at a non-cytotoxic concentration suggesting that HHODC has potential as an inductive adjuvant for HAART. HHODC also induced viral expression from primary cells isolated from HIV-1 infected patients on HAART, suggesting potential

clinical applicability. Amyrin, a common occurring triterpene in nature (Kushiro et al., 1998), was also isolated from *O. labiatum* leaves in the course of this investigation. It is the first time amyrin was isolated from this plant. Amyrin did not demonstrate HIV activation/inhibition potential when tested in the p24 ELISA assay. It is however a valuable compound because it has been reported to demonstrate hepatoprotective effects (Oliveira et al., 2005).

The viral activation approach is regarded as one of the major strategies for purging latently-infected cells. There are various mechanisms by which latent HIV can be activated. Prostratin and bryostratin, compounds from natural sources, induce HIV expression in latent cells by activating protein kinase C (PKC) (Marsden and Zack, 2013). PKC can be modulated by small molecular agents to induce the expression of latent HIV-1 from infected cellular reservoirs (McKernan et al., 2012). Inhibiting histone deacetylases (HDACs) is another mechanism by which latent HIV can be induced (Marsden and Zack, 2013). HDACs produce hypoacetylated nucleosomes at the HIV promoter region and this reduces access to transcription factors which contributes to the maintenance of HIV latency (Vigushin et al., 2001). HDAC inhibitors have been shown to activate HIV from latency (Rasmussen et al., 2013). Inhibiting HDAC results in the availability of transcription factors required for viral expression. A number of compounds have been identified as potential HDAC inhibitors with some examples being romidepsin, vorinostat and panobinostat (Archin et al., 2012; Marsden and Zack, 2013). The triterpenoid investigated in this study, HHODC, could be activating latent HIV expression through one of the described mechanisms or something completely novel because its structure differs from other latent activators. Alternatively, the minimal HDAC inhibition observed could be contributing to latent activation. In a different context, high HDAC activity has been linked to selectively kill human cancer cell lines in the presence of a masked cytotoxic agent (Ueki et al., 2013); in the present study, HHODC increased the activity of HDAC at higher concentrations. HHODC could be of importance in the field of cancer therapy due to its ability to increase HDAC enzymatic activity. In comparison to prostratin, it is possible that HHODC has the ability to activate the PKC pathway but this requires validation.

HHODC could also have induced viral expression by modulating an array of cytokines in U1 cells; cytokines implicated in the stimulation of latent HIV-1. Some of the cytokines affected by HHODC in this study, IL-2, IL-6, TNF and IFN-γ, are documented as being

among the many biomarkers affected by HIV-1 infection due to immune dysfunction (Nixon and Landay, 2010; Williams et al., 2013; Worsley et al., 2010). IL-6 is reportedly elevated during HIV infection and has been associated with mortality and opportunistic infections (Nixon and Landay, 2010; Williams et al., 2013). Worsley et al. (2010) reported an increase in IFN-γ levels in a group of HIV-infected individuals with immune reconstitution inflammatory syndrome. IL-2 was used in early clinical attempts to purge latent HIV-1 *in vivo* where the cytokine had a significant effect on latent cellular reservoirs, but viral rebound was still observed when antiretroviral therapy was discontinued (Marsden and Zack, 2013).

A study by Poli et al. (1994) reported the ability of IL-6 to synergistically induce the production of HIV-1 expression in U1 cells. IL-6 synergized with IL-1 in the upregulation of virus expression in the cells (Poli et al., 1994). In another study, IFN-γ was found to be a potent modulator of HIV-1 expression; direct stimulation of U1 cells with IFN-γ activated HIV-1 in U1 cells suggesting this cytokine to play an important role as an inducer of latent HIV-1 (Biswas et al., 1992). In the present study, the ability of HHODC to up-regulate IL-2, IL-4, IL-6, TNF and IFN-γ in monocytic cells could suggest that HHODC might have activated latent HIV-1 by up-regulating cytokines indicated to be inducers of HIV-1 expression in monocytes.

A number of studies reported the expression of IL-1, TNF-α and nuclear factor kappa B (NF-κβ) in PMA-treated cells (Folks et al., 1987; Kalebic et al., 1991; Poli et al., 1994). These studies confirm the ability of U1 cells to produce cytokines, supporting the data presented here. U1 cells were derived from a promonocytic cell line, U937, which has been reported to express various cytokines (Garrelds et al., 1999), further supporting possibility of cytokines production from these cells when stimulated with various agents. This partially explains the mechanism by which HHODC was able to activate latent HIV-1 in this study; the isomers possibly induced the production of IL-2, IL-6, TNF and IFN-γ which in turn activated latent HIV-1 from the cells.

6.5 Conclusion

In this study, a potential *in vitro* inducer of HIV-1 expression was identified from a natural source. The ability of this agent to activate latent HIV at a non-cytotoxic concentration may present an important contribution to the improvement of therapeutic

strategies to control HIV replication by targeting latent viral reservoirs. The data presented here provides promising evidence for the further investigation of HHODC's development as a potential HAART adjuvant.

Acknowledgements

This research was supported by the Southern African Biochemistry and Informatics for Natural Products (SABINA), the Technology Innovation Agency (TIA, South Africa), Margaret McNamara Memorial Fund (MMMF), the Namibian Ministry of Education and the University of Pretoria. Special thanks go to the University of Botswana (Chemistry Department), University of the Witwatersrand (School of Chemistry) and Mintek for assistance with purification and NMR analyses.

CHAPTER 7

Ocimum labiatum demonstrates anti-inflammatory and antioxidant activities

Submitted to *BMC Journal of Inflammation*

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Abstract

Background: Plants from the genus *Ocimum* are used as folk medicine for treating various diseases including inflammatory and immune-related diseases. Anti-inflammatory agents with fewer side-effects arising from natural sources should be cheaper to produce than current anti-inflammatory drugs. Here, *in vitro* evidence of *Ocimum labiatum*'s immune-enhancing and antioxidative properties is presented for the first time.

Methods: The anti-inflammatory effect of *O. labiatum* ethanolic extract was determined using a cytometric bead array (CBA) technique, which simultaneously measured seven cytokines. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging test was used for detecting antioxidant activity. The extract was examined for its effect on PHA-induced nitric oxide (NO) production in peripheral blood mononuclear cells (PBMCs). Purification of the plant extract yielded a labdane diterpenoid; labda-8(17),12*E*,14-triene-2R,18-diol. Anti-inflammatory behaviour of the isolated compound was also evaluated using CBA and NO analyses. Cytotoxicity was determined through the tetrazolium dye uptake by viable TZM-bl cells and PBMCs, and confirmed through a novel label-free real-time cell electronic sensing (RT-CES) device.

Results: *O. labiatum* extract significantly ($p<0.05$) inhibited the production of pro-inflammatory cytokines; IL-2, IL-4, IL-6 and IL-17A, at a non-cytotoxic concentration of 25 µg/ml. The isolated labdane diterpenoid compound significantly ($p<0.05$) decreased the production of all the pro-inflammatory cytokines except IL-6 at a non-cytotoxic concentration of 50 µM. The terpene up-regulated dually-active IL-6; which acts as pro- or anti-inflammatory protein depending on the challenge experienced during its production. The extract demonstrated strong antioxidant activity with an IC_{50} value of 13 ±0.8 µg/ml. The extract and isolated diterpenoid decreased the production of the inflammatory mediator NO, at non-cytotoxic concentrations. The CC_{50} of the extract in TZM-bl and PBMCs was 62.6 ±0.6 and 30.1 ±0.4 µg/ml respectively. The compound's CC_{50} of 112.6 ±0.2 µM in TZM-bl and 70 ±0.4 µM in PBMCs demonstrated very low toxicity. RT-CES confirmed tetrazolium dye assessed viability and also detected a unique growth pattern in the presence of plant materials that differed from untreated cells.

Conclusions: *O. labiatum* extract and the isolated diterpenoid demonstrated promising anti-inflammatory, antioxidant and practically non-toxicity properties. This expected behavior from a plant product is a promising first step towards the development of naturally derived anti-inflammatory drugs.

Keywords: *Ocimum labiatum*, Labdane diterpenoid, Inflammatory cytokines, Nitric oxide, Antioxidant, RT-CES

7.1 Background

The majority (60%) of approved drugs are either directly isolated or derived from natural products (Newman and Cragg, 2012). *Ocimum labiatum* belonging to the Lamiaceae family is widely distributed in Southern Africa and indigenously used for medicinal purposes (Van Wyk et al., 2009). Plants from the *Ocimum* genus are used traditionally to alleviate various disease symptoms such as pain, fever and inflammation, and the pharmacological activities of some extracts of these plants have been studied *in vitro* or *in vivo* without identifying the bioactive components (Giday et al., 2007; Masresha et al., 2012; Selvakkumar et al., 2007; Singh et al., 1996).

Lamiaceae plants are generally rich in terpenoids; a diverse class of naturally occurring organic-chemicals derived from five-carbon isoprene units. Plant isolates containing terpenoids have been found to suppress nuclear factor kappa B (NF-κβ) signalling (Salminen et al., 2008). NF-κβ is a protein complex linked to the pathogenesis of inflammatory diseases, cancer, viral infection and autoimmune diseases (Salminen et al., 2008). Some semisynthetic labdane diterpenoid derivatives have also been shown to have suppression effects against NF-κβ and inhibited the production of nitric oxide (NO) in macrophages (Girón et al., 2008). NO is an inflammatory molecule produced by inducible NO synthase in macrophages and it is involved in inflammation and immunoregulation (Kim et al., 2011; Lyu and Park, 2005). NO inhibitors may have therapeutic potential when related to inflammation which is a central feature of pathological conditions (Girón et al., 2008; Kim et al., 2011; Lyu and Park, 2005). Investigations of labdane diterpenoids suggest these compounds to have potential as alternative treatment for inflammatory diseases and further investigation is needed to identify the exact mechanism of action and pathways that are modulated by these

compounds. Two labdane diterpenoid compounds were isolated from *O. labiatum* by Hussein et al. (2007) one of which inhibited *Mycobacterium tuberculosis* and the other demonstrated moderate anti-cancer properties both *in vitro* (Hussein et al., 2007).

Plants from the Lamiaceae family are considered to be good sources of antioxidants due to the presence of high concentrations of phenolic compounds (Pedersen, 2000; Škrovánková et al., 2012). Antioxidants have the ability to dismutate reactive oxygen species (ROS) which are produced by the oxidation processes in various cells. Oxidative stress, caused by the accumulation of ROS in animal tissues, is a major cause of cell damage or death and is considered an instrumental process that leads to various cancers and other diseases (Valko et al., 2006). ROS in low concentrations act as significant cell signalling molecules and regulates the biological conditions of cytokines, hormones and growth factors. High levels of free radicals, however, overcome the normal cellular antioxidant defences and end up being cytotoxic to the biological system (Fang, 2004). These cumulative ROS are associated with a number of diseases including chronic inflammatory diseases (Mirshafiey and Mohsenzadegan, 2008). ROS have also been reported to be involved in the activation of NF-κβ by pro-inflammatory cytokines such as Tumor necrosis factor (TNF)-α (Fang, 2004). Given the importance of activated NF-κβ in inflammatory disease progression, suppression of this protein directly or through inhibition of ROS or pro-inflammatory cytokines preferably by antioxidants, remain therapeutically important because of the ability of the latter to combat pathogenic chain reactions initiated by free radicals.

The onset of common human diseases such as autoimmunity and chronic infections is characterized by a dysregulation of the T helper cell type 1 (Th1) and Th2 cytokine balance (Elenkov et al., 2005). Interleukin (IL)-6 has been reported to be responsible for Th17 cells induction to secrete IL-17, a pro-inflammatory cytokine (Infante-Duarte et al., 2000). Since cytokines are central mediators in major inflammatory diseases and impact one another's production and action (Elenkov et al., 2005), it is important to simultaneously measure more than one cytokine from all 3 subsets; Th1/Th2/Th17. This will allow for better assessment of immune and inflammation status with these proteins as potential indicators in prognostic and drug discovery studies (Elenkov et al., 2005; Williams et al., 2013).

Although many species of the *Ocimum* genus have been extensively investigated *in vitro*, this is not the case with *O. labiatum*. In this study, *O. labiatum* was investigated for

anti-inflammatory properties through inhibition or suppression of pro-inflammatory cytokines using the human Th1/Th2/Th17 cytometric bead array assay which allows for the quantification of multiple cytokines in a single sample. The effect of *O. labiatum* on NO production was also evaluated because there is ongoing research for potential anti-inflammatory agents from nature due to adverse side effects and high costs of existing anti-inflammatory drugs (Paul et al., 2006).

Plant extracts and their active compounds are expected to have antioxidant activities (Gupta and Sharma, 2006) which was therefore also investigated for *O. labiatum*. Investigating both antioxidant and anti-inflammatory potential for the same plant makes sense because the two properties are related in the sense that antioxidation prevents chains of reactions that eventually results in inflammatory diseases.

7.2 Methods

7.2.1 Plant extraction and compound isolation

Fresh leaves (894.6 g) of *O. labiatum* were collected during February (2012) from the Botanical garden of the University of Pretoria. Plant identification was done in the H.G.W.J Schweikerdt herbarium of the University and a voucher specimen (117693) is kept in the herbarium.

Fresh leaves were blended in ethanol and filtered. The filtrate was evaporated under reduced pressure at 50 °C using a rotary evaporator (Buchi, Flawil, Switzerland) and the residue was dissolved in ethyl acetate to obtain a lipophilic fraction. The ethyl acetate fraction was transferred to a pre-weighed vial and was evaporated to dryness at room temperature. The dried extract was stored in the dark at 4 °C until use. The extract was weighed out as needed and reconstituted in dimethyl sulfoxide (DMSO) before each biological assay. Reconstituting in DMSO surface sterilized the extract. Further dilutions to the desired extract concentrations were done in either cell culture media or buffer depending on the type of biological assay performed.

The ethyl acetate fraction (31.1 g) was subjected to column chromatography (Si gel 70-230 mesh) eluting with hexane (1.5 L) then with hexane-ethyl acetate (9:1, 4:1, 7:3, 3:2, 1:1, 3:7; 1.5 L each) and finally with ethyl acetate (1.5 L), collecting fractions of 500 mL each. The last fraction, with 100% ethyl acetate, yielded a pure compound in the form of white crystals (65 mg).

Labda-8(17),12*E*,14-triene-2R,18-diol (Figure 7.1): White crystals ($C_{20}H_{32}O_2$); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.32 (1H, dd, J = 10.5, 17.5 Hz, H-14), 5.40 (1H, t, J = 6.0, 12.5 Hz, H-12), 5.05 (1H, d, J = 17.5 Hz, H-15a), 4.88 (1H, d, J = 11.0 Hz, H-15b), 4.86 (1H, d, J = 1.5 Hz, H-17a), 4.49 (1H, d, J = 1.0 Hz, H-17b), 3.97 (1H, m, H-2), 3.42 (1H, d, J = 11.0 Hz, H-18a), 3.18 (1H, d, J = 11 Hz, H-18b), 2.38 (1H, m, H-11a), 2.37 (1H, m, H-11b), 2.03 (2H, m, H-7), 1.88 (1H, d, J = 10.5 Hz), 1.75 (3H, s, H-16), 1.63 (2H, m, H-6), 1.47 (1H, dd, J = 2.5, 12.5 Hz, H-5), 1.41 (2H, d, J = 11.5 Hz, H-3), 1.05 (2H, t, J = 11.5, 23.0 Hz, H-1), 0.81 (3H, s, H-20), 0.80 (3H, s, H-19); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 147.4 (C, C-8), 141.5 (CH, C-14), 133.6 (C, C-13), 133.4 (CH, C-12), 110.0 (CH_2, C-15), 108.5 (CH_2, C-17), 71.5 (CH_2OH, C-18), 65.5 (CHOH, C-2), 56.8 (CH, C-9), 47.9 (CH_2, C-1), 47.5 (CH, C-5), 44.6 (CH_2, C-3), 40.7 (C, C-10), 39.4 (C, C-4), 37.5 (CH_2, C-7), 23.5 (CH_2, C-6), 23.3 (CH_2, C-11), 18.5 (CH_3, C-19), 15.8 (CH_3, C-20), 11.8 (CH_3, C-16); MS *m/z* 304.2402 (calculated for $C_{20}H_{32}O_2$ [M^+] 304.2402).

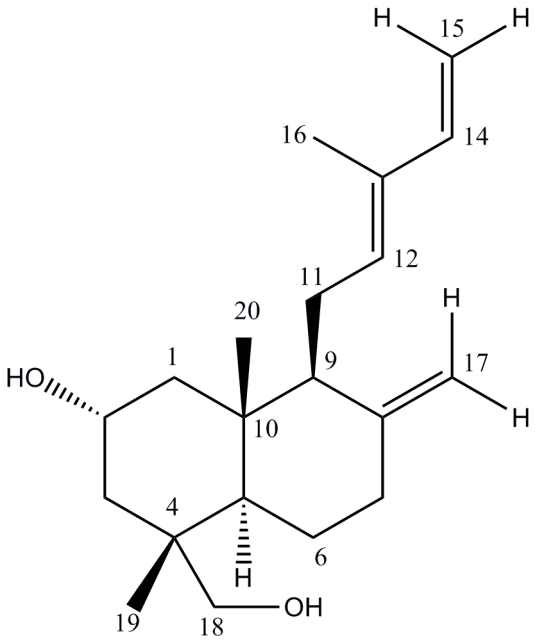
The ^{1}H and ^{13}C NMR spectra of compound **1** displayed resonances for an exocyclic methylene group H-17 at δ 4.86 (1H, d, 1.5) and 4.49 (1H, d, 4.49) both attached to carbon at δ 108.5. The spectra further showed the presence of C-9 side chain which was identical to those reported data by Hussein *et al*., 2007. The ^{1}H NMR spectra showed the presence of methine proton in a cyclohexane ring at δ 3.97 (1H, m), placed between two methylene substituents at δ 1.05 (2H, t, 11.5, 23.0) and 1.41 (2H, d, 11.5), suggesting C-2 was attached to a hydroxyl substituent, possibly as part of the labdane hydrocarbon skeleton. The spectroscopic data showed an AB system at δ 3.42 and 3.18 (1H, d, J = 11.0 each) of hydroxymethylene group attached to quaternary carbon C-4 (δ 71.5). The position of the hydroxyl group at the C-18 (δ 71.5) was further confirmed by the HMBC spectrum which showed correlation between the hydoxymethylene protons and the C-3 (δ 44.6), C-4 (δ 39.4), C-5 (δ 47.5) and C-19 (δ 18.5).

The ^{13}C NMR and DEPT spectra showed the presence of twenty carbon signals. These included, four quaternary carbons C-4, C-8, C-10 and C-13 at δ 39.4, 147.4, 40.7 and 133.6 respectively; one oxymethine carbon C-2 at δ 65.5; four methine carbons C-5, C-9, C-12 and C-14 at δ 47.5, 56.8, 133.4 and 141.5 respectively; one oxymethylene carbon C-18 at δ 71.5; seven methylene carbons C-1, C-3, C-6, C-7, C-11, C-15 and C-17 at δ 47.9, 44.6, 23.5, 37.5, 23.2,110.0 and 108.5 respectively; and three methyl carbons C-16, C-19 and C-20 at δ 11.8, 18.5 and 15.8 respectively as shown in Table 7.1.

Table 7.1: NMR spectral assignments for compound 1.

Carbon number	δ_C	δ_H, J (Hz)	HMBC	COSY
C-1 (CH_2)	47.9	1.05 (2H, t, 11.5, 23.0)		
C-2 (CHOH)	65.5	3.97 (1H, m)		
C-3 (CH_2)	44.6	1.41 (2H, d, 11.5)		
C-4 (C)	39.4			
C-5 (CH)	47.5	1.47 (1H, dd, 2.5, 12.5)		
C-6 (CH_2)	23.5	1.63 (2H, m)		
C-7 (CH_2)	37.5	2.03 (2H, m)		
C-8 (C)	147.4			
C-9 (CH)	56.8	1.88 (1H, d, 10.5)		
C-10 (C)	40.7			
C-11 (CH_2)	23.2	2.38 (1H, m)		H-11b
		2.37 (1H, m)		H-11a
C-12 (CH)	133.4	5.40 (1H, t, 6.0, 12.5)	C-9, C-11, C-14, C-16	
C-13 (C)	133.6			
C-14 (CH)	141.5	6.32 (1H, dd, 10.5, 17.5)	C-13, C-16	
C-15 (CH_2)	110.0	5.05 (1H, d, 17.5)	C-13, C-14	H-14
		4.88 (1H, d, 11.0)		H-14
C-16 (CH_3)	11.8	1.75 (3H, s)		
C-17 (CH_2)	108.5	4.86 (1H, d, 1.5)	C-7, C-9	H-17b
		4.49 (1H, d, 1.0)		H-17a
C-18 (CH_2OH)	71.5	3.42 (1H, d, 11.0)	C-3, C-4, C-5, C-19,	H-18b
		3.18 (1H, d, 11.0)		H-18a
C-19 (CH_3)	18.5	0.80 (3H, s)		
C-20 (CH_3)	15.8	0.81 (3H, s)		

The COSY spectrum depicted mostly correlations of the adjacent protons H-11a,b; H-14a,b; H-17a,b and H-18a,b. From the NMR spectroscopic data the molecular formula of compound **1** was determined to be $C_{20}H_{32}O_2$, corresponding to a theoretical exact mass of 304.24. The MS data showed a molecular ion M^+ peak at *m/z* 304.24 confirming the structure of compound **1**. The ^1H and ^{13}C NMR spectroscopic data for compound **1** compared well with the reported data by Hussein et al. (2007).



(1)

Figure 7.1: Chemical structure of the labdane diterpenoid Labda-8(17), 12E,14-triene-2R,18-diol.

7.2.2 Viability of TZM-bl cells using MTT

The effect of *O. labiatum* extract and isolated compound on the viability of TZM-bl cells was determined by quantifying the amount of a formazan product metabolized by viable cells from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma, MO, USA) as previously reported (Mosmann, 1983). Cells were plated in 96 well plates (Corning Incorporated, Corning, USA) at 1 x 10^4 cells per well and were treated with crude extract at final concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 ug/ml. In the case of the compound, the final concentrations were 3.125-100 µM. Viability was determined after a 72 h incubation at 37 °C in humidified air with 5% CO_2. Control wells included a negative control (cells and medium only), blank control for the extract (extract and medium only), a toxicity control auranofin; a known toxic compound with antitumour activity (Mirabelli et al., 1985), and a DMSO control (percentage of DMSO similar to extracts in cells to ensure that this solvent did not cause cell death). The plates were read at 550 nm using a microtiter plate reader (Multiskan Ascent; Thermo Labsystems; MA, USA), a reference wavelength of 690 nm was used and the percentage viability was calculated relative to untreated control cells. Fifty percent cytotoxic concentration (CC_{50}) of the extract and compound was obtained using Graphpad Prism (Graphpad Software Inc. CA, USA). This was computed as the

concentration of the extract/compound that reduced cell viability by 50% when compared to controls.

7.2.3 Viability of PBMCs using MTS

Ethical approval for obtaining blood samples from consenting donors was granted by the Faculties of Natural and Agricultural Sciences and Health Sciences Ethics Committees (EC080506-019; 163/2008, University of Pretoria, South Africa). Freshly isolated healthy peripheral blood mononuclear cells (PBMCs), stimulated with phytohemagglutinin-protein (PHA-P, 4 µg/ml), were plated in 96 well plates (Corning Incorporated, Corning, USA) at 1 x 10^5 cells per well and treated with the extract and compound at final concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml. The number of viable cells was detected after 72 h using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) solution (Promega Corporation, WI, USA). Control wells included a toxicity control auranofin and the plates were read at 492 nm (reference wavelength of 690 nm). The percentage viability was calculated relative to an untreated control of cells only and the CC_{50} values were determined using Graphpad Prism (Graphpad Software Inc. CA, USA).

7.2.4 Real Time Cell Analysis

Confirmatory cytotoxicity analysis for the crude extract and isolated compound was performed using a real time cell electronic sensing (RT-CES) device, xCELLigence (Roche Diagnostics, MA, Germany) to monitor proliferation of the TZM-bl cells in the presence of the extract and isolated compound. A detailed procedure was followed as previously described (Fonteh et al., 2011). The xCELLigence system monitors cellular events in real time without the incorporation of labels by measuring electrical impedance across interdigitated gold micro-electrodes integrated on the bottom of special tissue culture plates. Increasing attachment of cells to the electrodes increases electrode impedance which is displayed as cell index (CI) (Abassi et al., 2009; Atienzar et al., 2011). Cell titration was carried out as recommended by the manufacturer in order to determine an optimal cell number that reaches an index of ±1 after 24 h, before treatment with the samples. Each well was seeded with 10,000 cells, which was the ideal cell number obtained from the titration. The CC_{50} of *O. labiatum* extract obtained with an MTT assay was tested alongside 10 µM auranofin as a positive control for toxicity. Untreated cells were also included as controls. Cells were first allowed to

adhere for 24 h before treating them with extracts. The cellular effects of the extracts were monitored for 72 h and CI values were recorded. The compound was applied in the same manner. One concentration of the compound, 112.6 µM, CC_{50} determined with MTT was also monitored for its effect in real time for 72 h.

7.2.5 DPPH antioxidant assay

The potential antioxidant activity of *O. labiatum* extract and Labda-8(17),12E,14-triene-2R,18-diol was assessed on the basis of the scavenging ability of the test samples towards a stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. Test samples were prepared at a starting concentration of 100 µg/ml after mixing with 90 µM DPPH ethanol solution. A series of concentrations were tested for the samples in order to determine the 50% inhibitory concentration (IC_{50}). Samples were incubated with DPPH in the dark for 30min at room temperature. Absorbance values were measured at 550 nm (Multiskan Ascent; Thermo Labsystems; USA) and converted into percentage of antioxidant activity (Marxen et al., 2007). Ascorbic acid was used as a standard control and the background control was samples in ethanol only. IC_{50} was determined using Graphpad Prism (Graphpad Software Inc. California, USA). The percent inhibition was calculated by using the formula:

100 – [(Sample OD – Sample background OD)/(DPPH only OD) x 100]

7.2.6 Cytokine quantitation using cytometric bead array (CBA)

Ethical approval for obtaining blood samples from consenting donors was granted by the Faculties of Natural and Agricultural Sciences and Health Sciences Ethics Committees (EC080506-019; 163/2008, University of Pretoria, South Africa).Sample preparation: Blood samples were taken from healthy volunteers; 9 individuals for extract and 14 for compound. Freshly isolated PBMCs, stimulated with PHA-P (4 µg/ml), were seeded at 1 x 10^6 cells/well in order to get enough cytokine produced for quantitative detection. Incubation of the cells with non-cytotoxic concentrations of the extract (25 µg/ml) and compound (50 µM) was done for 24 h. The supernatant was collected and stored at -20°C until testing.

Quantitation: Cytokine levels were analysed in the tissue culture supernatant using a BD CBA human Th1/Th2/Th17 cytokine kit (BD Biosciences, CA, USA). The CBA kit simultaneously measured IL-2, IL-4, IL-6, IL-10, TNF, interferon gamma (INF-γ) and IL-

17A protein levels in a single sample using a FACSArray Bioanalyzer (BD Biosciences, CA, USA). The assay was performed according to the manufacturer's instructions. Briefly, the supernatant was thawed and 50 µl of each sample was mixed with the cytokine capture beads and the detector reagent, phycoerythrin (PE)-conjugated detection antibodies, to form sandwich complexes. The intensity of PE fluorescence of each sandwich complex reveals the concentration of that cytokine (Williams et al., 2013). The limits of detection for each cytokine was as follow: 2.6 pg/ml for IL-2, 4.9 pg/ml for IL-4, 2.4 pg/ml for IL-6, 4.5 pg/ml for IL-10, 3.8 pg/ml for TNF, 3.7 pg/ml for INF-γ and 18.9 pg/ml for IL-17A.

7.2.7 Nitrite and nitrate detection by colorimetric assay

The effect of extract and isolated compound on NO production was studied using an NO assay colorimetric kit (Calbiochem, CA, USA). In aqueous solution, NO is rapidly converted to nitrate and nitrite. Hence, for accurate determination of the total NO generated, both nitrate and nitrite levels must be monitored. Spectrophotometric quantitation of nitrite using only the Griess reagent does not measure nitrate. Therefore, the NADH-dependent enzyme nitrate reductase is used to convert the nitrate to nitrite prior to quantitation using the Griess reagent. NO was measured from PBMCs by plating cells in 96 well plates at 2.0 x 10^5 cells/well. Cells were pre-incubated for 1 h with non-cytotoxic concentrations of the crude extract (25 µg/ml) and labdane diterpenoid (50, 25 and 10 µM) before stimulating for NO with a non-cytotoxic concentration of PHA-P, 25 µg/ml and further incubating for 24 h. After the 24 h incubation, cell culture supernatant (50 µl) was collected and incubated with 1 U/µl of nitrate reductase in the presence of 0.2 mM NADH and 50 mM MOPS buffer, pH 7.0. After 20 min, Griess reagent was added and further incubated for 5 min at room temperature. The color was read at 550 nm (Multiskan Ascent; Thermo Labsystems; MA, USA). A standard curve was generated, using freshly prepared 0-100 µM potassium nitrate dissolved in assay buffer, to quantitate unknown nitrite in samples.

7.2.8 Statistical analysis

Data for all experiments is presented as the mean ± standard deviation (n=3-6). Since cytokine profiles vary from person to person, the cytokine concentrations obtained for each individual were log transformed in order to standardize the data and make it more comparable (Fung et al., 2013; Williams et al., 2013). Significant differences were

estimated using Graphpad Prism 5 (Graphpad Software Inc. CA, USA) and Student's t test for unpaired observations. A $p<0.05$ was considered significant.

7.3 Results

7.3.1 Cytotoxicity and antioxidant activity

The CC_{50} of crude *O. labiatum* extract was 62.6 ±0.6 µg/ml for TZM-bl cells and 30.1 ±0.4 µg/ml in PBMCs determined by viability dye MTT and MTS respectively. Cytotoxicity of the crude extract was confirmed with RT-CES. RT-CES monitored the effect of the extract on cell viability (Figure 7.2), in real-time and confirmed the CC_{50} value obtained with MTT. At 96 h, the cell index for untreated cells (i) was 2.5. Cells treated with 62.6 ±0.6 µg/ml crude extract (CC_{50}) resulted in sample uptake (dip in cell growth before 48 h) and metabolization (hump) as demonstrated by the pattern (ii) in Figure 2. When the extract was properly metabolized, a reduction in cell viability was observed. About 50% (half of cell index value of control cells) of treated cells remained alive at 96 hours of incubation.

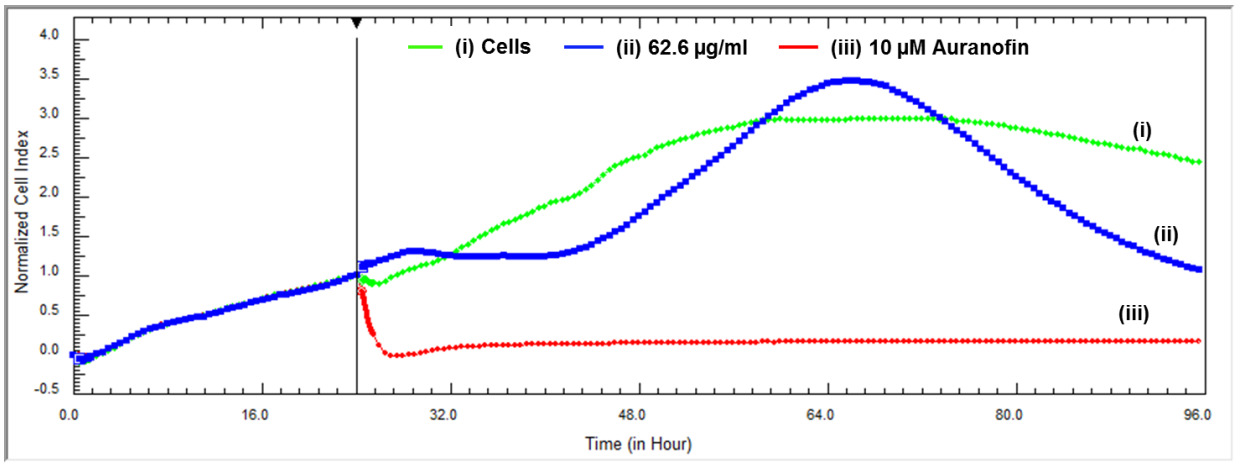


Figure 7.2: Real time monitoring of *O. labiatum* extract's effect on the viability of TZM-bl cells. Cells were exposed to 62.6 µg/ml (which was the CC_{50} value determined with a tetrazolium dye) of *O. labiatum*. Auranofin (iii) was used as a positive control for toxicity and caused cell death almost from the time of addition until end of incubation period (96 h). A unique growth pattern was observed in treated cells (ii) indicating extract uptake and metabolization which resulted in a cell index value equivalent to ½ cell index of control cells (i) at 96 h. The cell index curve was generated in real time. Each data point was normalized against the time just before extract addition.

The cytotoxicity of the labdane diterpenoid compound, Labda-8(17),12*E*,14-triene-2R,18-diol , was also studied using viability dyes MTT and MTS and it was found to cause CC_{50}s of 112.6 ±0.2 and 70 ±0.4 µM in TZM-bl and PBMCs respectively. The cytotoxicity results were again confirmed with RT-CES and these results are depicted in Figure 7.3. Extract uptake was observed before 48 h and metabolization of the sample resulted in a cell index value of 2.5 (ii) equivalent to that of untreated control cells (i) at 96 h. RT-CES results indicated that the CC_{50} of the compound obtained in TZM-bl cells was not cytotoxic. Since RT-CES does not involve the use of dye uptake or invasive methods to measure cell status, this suggests this technique to be more sensitive and accurate than MTT.

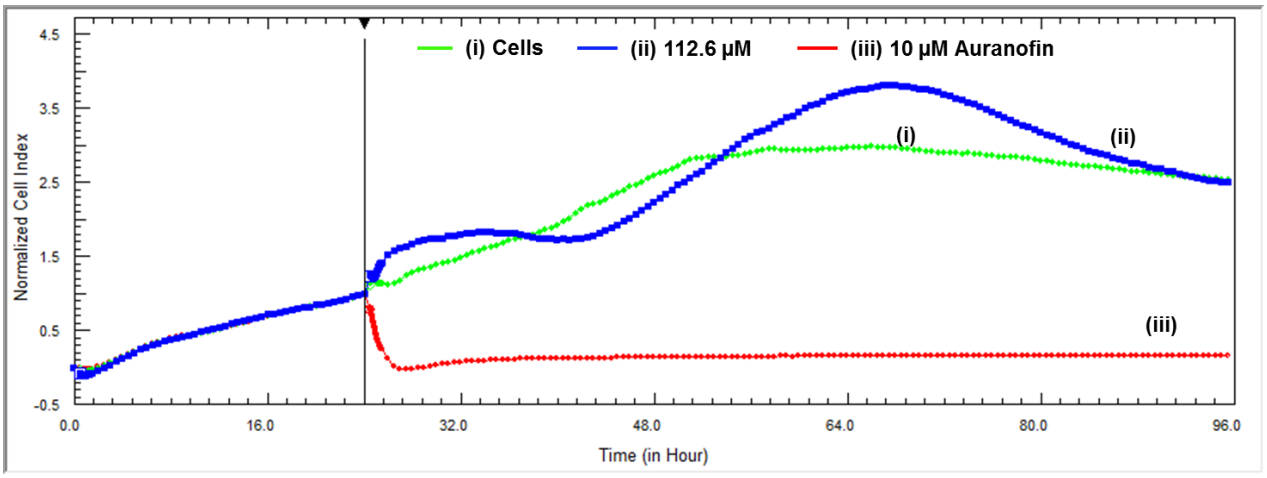


Figure 7.3: Cytotoxicity curve of TZM-bl cells exposed to 112.6 µM (CC50) of the labdane diterpenoid compound labda-8(17),12E,14-triene-2R,18-diol. Auranofin (iii) was used as a positive control for toxicity and caused cell death almost from the time of addition compared to almost constant cell viability in the presence of the compound. RT-CES demonstrated low cytotoxicity of the compound (ii), cell index at 96 h was similar to that of untreated cells (i). Each data point was normalized against the time just before compound addition.

The DPPH radical scavenging results for test samples and ascorbic acid as positive control are presented in Figure 7.4. The antioxidant activity of the extract was expressed as an IC_{50} value of 13 ±0.8 µg/ml. It is good to note that this concentration does not have any toxicity effect on the viability of TZM-bl and PBMCs. The labdane diterpenoid compound demonstrated less than 50% DPPH inhibition at all

concentrations tested in this study (0.8-100 µg/ml). The positive control, ascorbic acid, produced an IC_{50} value of 1.1 ±0.03 µg/ml.

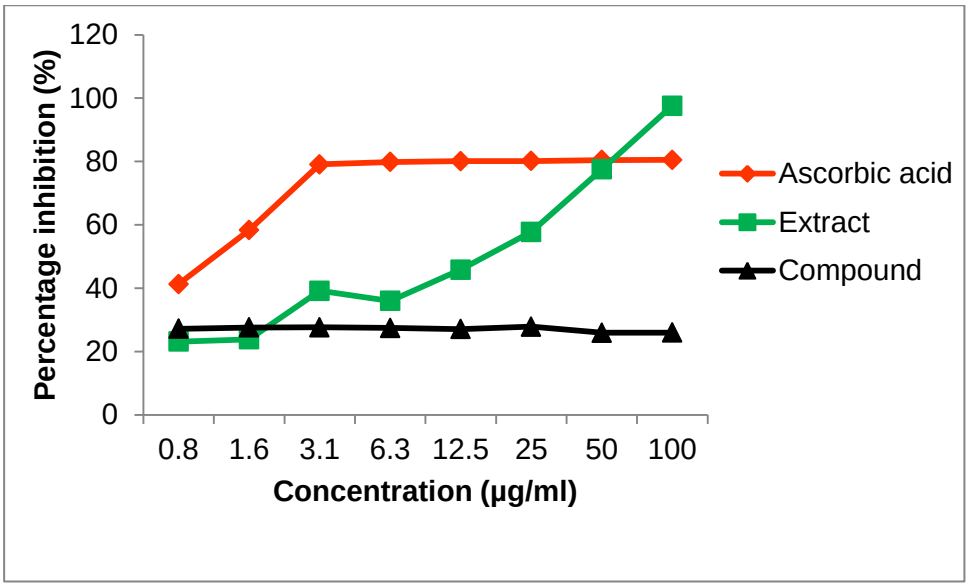


Figure 7.4: DPPH free radical scavenging activity of *O. labiatum* extract (squares) and the isolated compound (triangles). Ascorbic acid was used as positive control. Extract exhibited strong antioxidant activity while the diterpenoid compound did not.

7.3.2 Effect of O. labiatum on cytokine production

Using the Cytometric Bead Array (CBA) human Th1/Th2/Th17 cytokine kit (BD Biosciences, San Jose, CA, USA), 7 cytokines, IL-2, IL-4, IL-6, IL-10, TNF, INF-ɣ and IL-17A, were analysed and quantified (Fig. 7.5) following exposure of PBMCs to plant material. *O. labiatum* crude extract was tested for anti-inflammatory properties at 25 µg/ml, a non-cytotoxic concentration. Cytokine production in untreated PBMCs was used as a control for comparison to the production of cytokines in extract treated PBMCs. The extract significantly ($p<0.05$) decreased the production of pro-inflammatory cytokines IL-2, IL-4, IL-6 and IL-17A (Figure 7.5). For IL-10, INF-ɣ and TNF, there was no significant ($p>0.05$) difference detected in untreated and extract treated samples even though production of those cytokines was lowered by the extract.

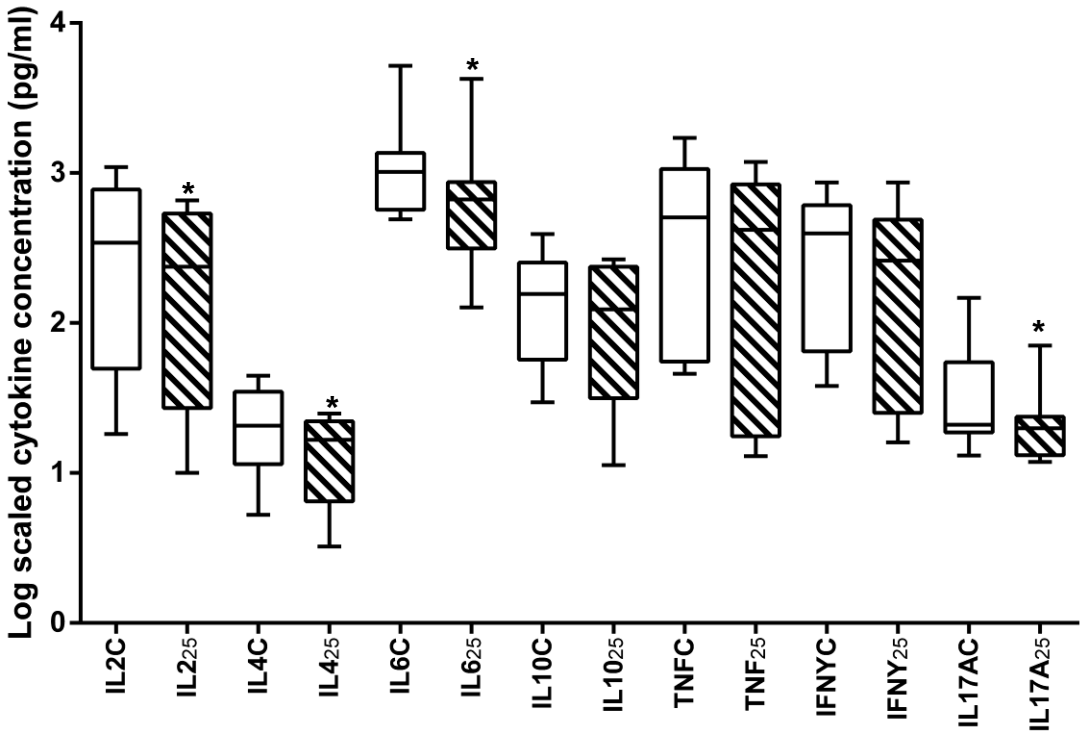


Figure 7.5: Effects of a non-toxic concentration of *O. labiatum* extract (25 µg/ml) on cytokine production in PBMCs. Cytokines were quantified using the CBA kit and flow cytometry. Cytokine concentrations were log transformed in order to make the data comparable. Empty bars represent untreated controls and black striped bars are extract treated samples. The crude extract significantly (*p<0.05) down-regulated levels of IL-2, IL-4, IL-6 and IL-17A.

7.3.3 Effect of labda-8(17),12E,14-triene-2R,18-diol on pro-inflammatory cytokines

The anti-inflammatory property of the purified labdane diterpenoid was also studied using the CBA human Th1/Th2/Th17 cytokine kit (BD Biosciences, San Jose, CA). The compound was tested at 50 µM, a non-toxic concentration. The results obtained were compared to untreated cells. The compound was found to significantly ($p<0.05$) inhibit the production of all cytokines tested except for IL-6 as illustrated in Figure 7.6. The production of IL-6, which acts as both an anti- and pro-inflammatory cytokine, was significantly ($p<0.05$) up-regulated.

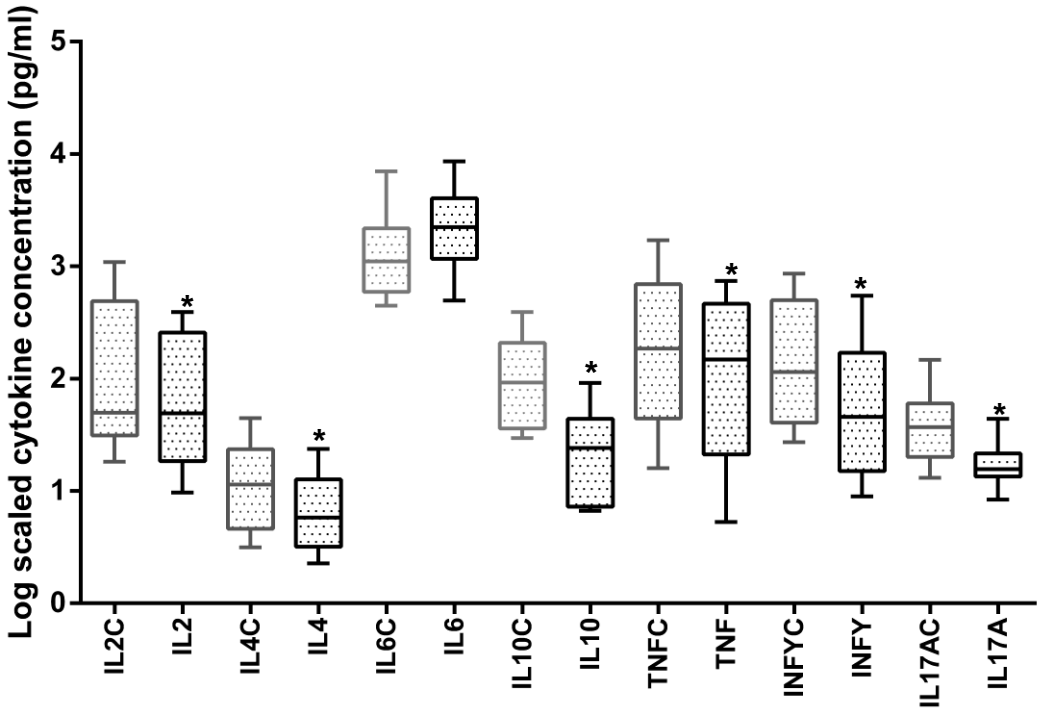


Figure 7.6: Influence of labda-8(17),12*E*,14-triene-2R,18-diol on cytokine production in PBMCs as quantitated by CBA analysis and flow cytometry. Cells were treated with a non-toxic concentration of 50 µM. Concentrations obtained of tested cytokines were log transformed in order to make the data comparable. Grey bars represent untreated controls and black bars are compound treated samples. IL-6 was the only cytokine that was up-regulated by the compound. The rest of the cytokines were significantly (*p<0.05) down-regulated.

7.3.4 NO levels in culture supernatant reduced by O. labiatum and labdane diterpenoid

The effects of the extract and compound on PHA-induced NO production in PBMCs were investigated by quantitating nitrite in culture medium using the Griess reaction. Unstimulated control cells, after 24 h of incubation, produced a negligible amount of NO (<10 µM). NO production in PHA stimulated cells was evident (43 µM), while no significant levels of NO were detected in the extract treated cells as illustrated in Figure 7.7. The 3 concentrations tested for the labdane diterpenoid compound significantly (p<0.05) decreased the level of NO in the cells with 25 µM of the compound being more significant by reducing NO production to an undetectable level (Figure 7.7).

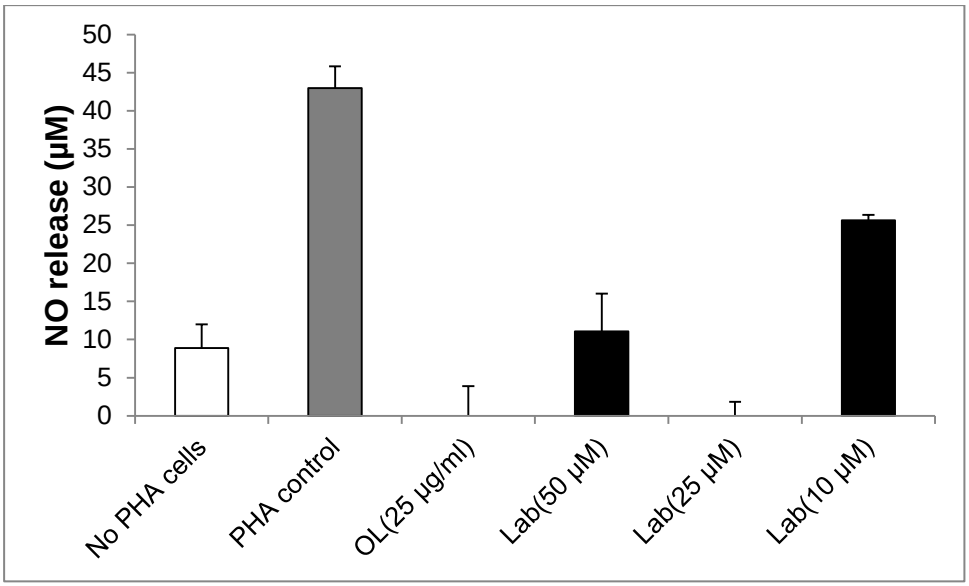


Figure 7.7: Inhibition of NO release by *O. labiatum* extract and labdane diterpenoid. PBMCs were pre-incubated for 1 h with the indicated concentrations of the extract (OL) and compound (Lab) and then stimulated with 25 µg/ml PHA for 24 h. Untreated cells were used as a control. Nitrite accumulation was measured with the Griess reagent. Extract and compound concentrations used were non-toxic. The results were expressed as mean ± SD.

7.4 Discussion

The data obtained in this study suggest that *O. labiatum* has immune supportive activities in addition to direct antioxidant abilities. RT-CES proved to be a more sensitive technique for detecting the effect of treatment on cell viability. The technique detected a unique cell growth pattern with extract and compound uptake and metabolism which would not have been observed with viability dyes. The use of conventional methods such as MTT or MTS has been reported to be insufficient if used alone since these methods only produce end-point data (Masarik et al., 2012). RT-CES, which is label-free, is able to capture important effects such as the onset and rate of cytotoxicity as well as other proliferation dynamics such as cytostasis (Fonteh et al., 2011). Sensitivity and accurate reflection using RT-CES has been attributed to the ability of the technique to reflect on the cell concentration and changes in cell morphology more than viability dyes (Masarik et al., 2012).

O. labiatum extract exhibited strong antioxidant activity while the isolated labdane diterpenoid, Labda-8(17),12*E*,14-triene-2R,18-diol, was not a good antioxidant

suggesting the antioxidant ability of the crude extract to be due to the presence of other compound(s). Non-terpenoid compounds present in the extract such as flavonoids are more likely to be responsible for antioxidant activity of plant extracts. Flavonoids, vitamins and polyphenols present in plants are known to be powerful antioxidants (Gupta and Sharma, 2006).

The bead array technology used in this study allowed for simultaneous detection of multiple cytokines in a single sample. Because cytokines influence one another, an imbalance in especially Th1 and Th2 cytokines can lead to the pathogenesis of acute and chronic inflammatory diseases (Elenkov et al., 2005). Therefore, in studies where cytokines are investigated as prognostic indicators (Williams et al., 2013), for therapeutic uses (Asadullah et al., 1998) or to assess their modulation by potential drugs (Watkins and Maier, 2003), it is important to study an array of these molecules representative of all 3 subsets (Th1/Th2/Th17) as was done here.

Lamiaceae terpenoids have been documented to have anti-inflammatory as well as antitumor effects (Banno et al., 2004). The isolation of the compound Labda-8(17),12*E*,14-triene-2R,18-diol from *O. labiatum* was first reported in 2007 (Hussein et al., 2007). The only investigations done on the labdane diterpenoid by these authors were anti-cancer testing against breast cancer cells and an anti-tuberculosis study in which the compound did not inhibit *M. tuberculosis* activity at the highest concentration tested but showed moderate cytotoxic effects against a breast cancer cell line (Hussein et al., 2007). According to the literature, some labdane diterpenoid compounds have been shown to possess immune enhancing properties (Girón et al., 2008) which encouraged the investigation of these specific compounds for anti-inflammatory and/or any immune supportive properties. The ability of the isolated compound to down-regulate pro-inflammatory cytokines, as was demonstrated here, can be of therapeutic importance. The crude extract suppressed IL-2, IL-4, IL-6 and IL-17A while the compound suppressed all cytokines tested except for IL-6 which was up-regulated. IL-6, which can act as a pro- or anti-inflammatory cytokine, is part of a network of cytokines that trigger or regulate immune responses and its increase by the compound in comparison to the inhibitory effect on pro-inflammatory cytokines contributes a beneficial balance to the host (Akira et al., 1990; Williams et al., 2013).

IL-2 and IL-17A have been reported to be responsible for activating pathogenic inflammation in a number of inflammatory skin diseases such as psoriasis (Krueger et

al., 2012; von Bonin et al., 2011). High levels of IL-17A are reportedly associated with several chronic inflammatory diseases including rheumatoid arthritis and multiple sclerosis (Hueber et al., 2010; Lock et al., 2002). Therefore, lowering these cytokines in such cases will be of clinical use to the affected individual.

The down-regulation of the following cytokines IL-4, IL-10 and TNF by the extract and compound can be of general therapeutic significance. Over expression of these cytokines has been linked to certain autoimmune diseases as well as activation of pathogenic inflammation (Krueger et al., 2012; von Bonin et al., 2011). Inhibiting IL-4 has been associated with alleviating allergies while the neutralization of IL-10 has been linked to the reduction of helminth infection by restoring the function of Th2 effector cells (Taylor et al., 2012).

The production of TNF was drastically inhibited by the labdane diterpenoid compound and this is a pro-inflammatory cytokine associated with cancers and autoimmune diseases (Grivennikov and Karin, 2011). TNF-α is regarded as a major pro-inflammatory cytokine and there is a continuous search for potential TNF-α inhibitors from natural products due to serious adverse side-effects of existing protein-based inhibitors (Paul et al., 2006). Overproduction of TNF-α in rheumatoid arthritis patients has been linked to increased ROS (Feldmann and Maini, 2001). Inhibiting high levels of this cytokine in such patients should also decrease ROS production and consequently inflammation and cellular damage. TNF-α has also been reported to induce NF-κβ production (Osborn et al., 1989) and this protein is inhibited by the presence of antioxidants (Beg et al., 1993). Here, *O. labiatum* showed antioxidant activity and the ability to lower the production of TNF-α meaning it could suppress NF-κβ production, further supporting this plant being potentially useful in diseases with pathogenesis enhanced by NF-κβ.

The reduction of INF-ɣ by the labdane diterpenoid in this study is suggestive of a therapeutic potential. INF-ɣ is reportedly involved in the inflammatory events underlying a vascular inflammatory condition called abdominal aortic aneurysms (AAA) (Szekanecz et al., 1994). A large quantity of INF-ɣ was reported (Szekanecz et al., 1994) in supernatants from AAA explant cultures that suggested this cytokine to be involved in AAA pathogenesis. Regulating the production of INF-ɣ in AAA patients by reducing its production could assist in alleviating that inflammatory condition. Another study (Oxenkrug, 2011) reported high production of INF-ɣ in the brain and periphery to be involved in activating certain metabolic pathways which leads to an inflammation

cascade that results in aging and aging-associated medical psychiatric disorders. Suppressing INF-ɣ in such cases could be beneficial in avoiding/minimising psychiatric disorders associated with aging.

The anti-inflammatory properties of *O. labiatum* by pro-inflammatory cytokines inhibition were supported by the ability of the extract and labdane diterpenoid to reduce the production of NO release. NO, which is regarded as a potent inflammatory mediator, was inhibited at concentrations that were not toxic indicating that the NO actions of the extract and compound are not attributable to cytotoxicity. The data presented here confirms the ability of diterpenoids to also possess anti-inflammatory abilities through NO inhibition as previously reported (Girón et al., 2008).

Antioxidation and anti-inflammation properties of plants are routinely investigated and are considered to be among the primary health benefits of natural products. In addition, antioxidative agents prevent the formation of a number of diseases including inflammatory diseases. The anti-inflammatory properties of the *O. labiatum* extract as well as that of the isolated compound strongly suggest the immune enhancing properties of this plant.

7.5 Conclusion

The *in vitro* data obtained from this study demonstrated for the first time that *O. labiatum* has potent anti-inflammatory and antioxidant activity. This work encourages further investigations of *O. labiatum*'s potential use as complimentary medicine in anti-inflammatory and antioxidant therapeutics and provides some empirical data for the already prominent anecdotal use in traditional medicines.

7.6 Authors' contributions

DM designed the study; PK conducted the research and wrote the first draft of the manuscript. JJO did the compound's structural elucidation and wrote that section of the manuscript. DM edited and revised the manuscript. All the authors approved and reviewed the data interpretation and wording in the final draft of the manuscript.

Acknowledgments

This research was supported by the Southern African Biochemistry and Informatics for Natural Products (SABINA), the Technology Innovation Agency (TIA, South Africa),

Margaret McNamara Memorial Fund (MMMF), the Namibian Ministry of Education and the University of Pretoria. The School of Chemistry, University of the Witwatersrand is thanked for running the NMR spectra.

CHAPTER 8

CONCLUDING DISCUSSION AND FUTURE WORK

8. OVERVIEW

Presented in this thesis is the bioactivity of selected Lamiaceae plant extracts and isolated compounds. This bioactivity includes HIV-1 enzyme inhibitory properties, the ability to significantly decrease whole virus replication (in a chronic-infection cell line model), activation of HIV-1 latency, anti-inflammatory and antioxidant properties. Before revisiting the data and providing concluding comments for each data chapter, a brief overview on the latest developments in HIV/AIDS is provided below.

The HIV/AIDS epidemic is now in its third decade and continues to be a global challenge since the discovery of the virus particle responsible in 1981. Research is ongoing and focuses on various aspects of the viral life cycle and its effect on the host to possibly identify a cure, vaccine or novel drugs to improve existing antiretroviral therapies (Stephenson and Barouch, 2013). The case of the Boston patients, whom were recently reported to be “virus-free” after receiving stem-cell transplants to treat lymphoma, is a promising development that is unlikely to be used generally because of the high risks and costs of transplants (http://www.nature.com/news/stem-cell-transplants-may-purge-hiv-1.13297, accessed 27/7/2013). Tests are ongoing to locate (traces of) the virus in the tissues of these patients. The possibility of finding viral traces in tissues does exist because viral reservoirs can include regions not affected by radiation/chemotherapy e.g. liver and brain. The “Berlin patient” was initially reported free of the virus after a bone marrow transplant and 5 years later traces of HIV were found in his tissues (http://www.npr.org/blogs/health/2012/06/13/154869103/traces-of-virus-in-man-cured-of-hiv-trigger-scientific-debate, accessed 27/7/2013).

The bottom line is that the development of affordable HIV treatment needs to be the focus of scientists especially here in Africa where the epidemic is more severe. A number of African scientists use what is freely available for research, and that is nature. Plants provide an endless source of compounds and has been a major contributor of novel medicinal compounds for centuries and drug discovery opportunities are endless with natural products (Newman and Cragg, 2012).

The present study screened sixteen Lamiaceae plants, a plant family with various ethnobotanical uses. This was done by investigating direct inhibition of HIV enzymes or viral replication as well as the possibility of activating latent HIV-1, a major hurdle in current HIV treatment. HIV/AIDS results in immune dysfunction which is characterized by a dysregulation of cytokine production. The effects of the HIV bioactive plant extracts/compounds on cytokine production in human blood mononuclear cells were also investigated, simultaneously measuring multiple cytokine proteins using multiplexing. Plant extracts are expected to contain antioxidants and perhaps also ant-inflammatory activities because of common medicinal plant constituents such as phenolic acids, flavonoids and terpenes to which those types of activities are attributed (Škrovánková et al., 2012). In addition, the effect of the plants on cytokine production and nitric oxide release (an inflammatory mediator) was assessed as well as experiments for determining antioxidant properties.

In the following sections, a summary of the major findings for each experimental chapter is provided as well as recommendations for future investigations. Novel contributions of the thesis chapters are also highlighted.

8.1 Chapter 3: Crude extracts inhibited HIV-1 replication

Of the sixteen studied, four plants; *Ocimum serratum*, *Salvia apiana*, *Stachys byzantina* and *Plectranthus neochilus* demonstrated moderate inhibitory properties against HIV-1 reverse transcriptase, protease and integrase (integrase data is provided in Figure A3.2 of the appendix). The following is a general classification of enzyme inhibition adapted from the literature: strong/potent inhibitor (>80%), good inhibitor (50-80%), moderate/appreciable inhibitor (35-49%) and a weak inhibitor (<35%) (César et al., 2011; Flockhart, 2007; Klos et al., 2009; Min et al., 2001) . These same plants were able to inhibit HIV-1 replication at non-cytotoxic concentrations with the inhibition by *S. byzantina* being the most significant ($p<0.05$). The findings of this study suggested the possible use of these plants as herbal remedies in managing HIV/AIDS. This is the first report of the anti-HIV-1 properties of the four plants. *Ocimum*, *Salvia* and *Plectranthus* are all genera consisting of species with numerous medicinal uses. Communities in developing countries still rely on herbal medications as administered by traditional practitioners for their primary care and this practise will probably never end (Zhang, 1998). Even though the Food and Drug administration has trouble approving herbal

remedies, during the past decades public interest in natural therapies has increased greatly even in industrialized countries; expanding the use of medicinal plants and herbal medicines. The World Health Organization (WHO)'s report on national policy on traditional medicine and regulation of herbal medicines looked at how different countries face major challenges in the development and implementation of the regulation of traditional, complementary/alternative and herbal medicines (WHO, 2005). WHO highlighted that traditional medicine will always maintain its popularity worldwide.

It is always of great interest to identify constituents that are responsible for bioactivity in crude extracts; future investigation should attempt to purify the crude extracts from the four plants in order to identify the active compounds. This was not done here because the study focused on two other plants with stronger activities than the four mentioned here.

8.2 Chapter 4: Inhibition of HIV-1 protease, antioxidant and anti-inflammatory activities of *Plectranthus barbatus* (published in Journal of Ethnopharmacology)

Plectranthus barbatus is one of the plants traditionally used to manage HIV/AIDS and the associated opportunistic infections. There were no reported *in vitro* studies to support the anecdotal traditional success of this plant. Chapter 4 was the first report on the inhibition of HIV-1 protease, stimulation of immune and inflammatory responses by an extract of this plant (Kapewangolo et al., 2013). The HIV-1 inhibitory properties were observed at concentrations that were not cytotoxic which explains, to an extent, why its decoctions are so widely used.

This plant is acclaimed in literature as a plant for the future because of its numerous ethnomedicinal uses (Rice et al., 2011). The chemistry of *P. barbatus* has been studied extensively. Future research should focus on purifying the extract for identification of the specific bioactive constituents. Other options may be to work with existing compounds of this plant and virtually identify the ones docking to HIV-1 enzyme models using docking software. Compounds identified virtually can then be verified *in vitro*. The main limitation with the second approach is that, novel compounds that have never been identified from this plant could be left out. Even though purification can be tedious, it is the only way novel compounds can be identified from natural sources and one can never know the potential of novel compounds until discovered.

If purified material do not demonstrate the activities observed with extracts, this would be an indication that more than one compound was working in synergy with others for the observed outcome.

8.3 Chapter 5: Potent HIV-1 inhibitor from *Ocimum labiatum*

In this chapter, bioactivity-guided screening identified pheophytin-a (phy-a) from *O. labiatum* as a good inhibitor (>50%) of HIV-1 protease. *O. labiatum* belongs to a genus, *Ocimum*, consisting of plants that are traditionally used to treat HIV/AIDS. This study is the first to report the *in vitro* activity of this plant against HIV-1. The data obtained here is in agreement with the successful use of *Ocimum* plants as herbal remedies in HIV-infected individuals. Not only did the extract inhibit HIV-1 protease, it also drastically reduced HIV-1 replication in a chronically infected cell line, U1. Phy-a was previously reported to have anti-hepatitis C virus (HCV) activity through the inhibition of HCV protease (Wang et al., 2009) and also anti-herpes simplex virus (HSV) activity by inactivating HSV before cell entry (Sakdarat et al., 2009). Activity against HIV-1 in this study was through inhibition of HIV-1 protease. Phy-a's structure consists of a lot of carbonyl groups. Makatini et al. (2012) was able to observe that HIV-1 protease had the ability of converting carbonyl groups to hydroxyl groups before allowing it to bind to its active site and this could possibly suggest the mechanism by which phy-a inhibited HIV-1 protease in this study. The present study is also the first to report HIV-1 inhibitory properties of phy-a. Phy-a is a complex molecule and its structure was elucidated by NMR spectra and comparison to literature. The mass of the compound could not be obtained with the Quadrupole time-of-flight (Qtof) mass spectrometer that was available as that technique could not ionize the molecule. Using Fast atom bombardment (FAB) mass spectrometry may assist in obtaining the molecular weight of phy-a and is recommended as a next step for this research. FAB makes use of a high energy beam of atoms that is bombarded on a molecule, ionizing it in the process to produce fragments that are analysed. It is mostly employed for high molecular weight compounds which previously were difficult or impossible to study by other ionisation methods (Barber et al., 1981).

8.4 Chapter 6: Latent HIV-1 activated by triterpenoids from *O. labiatum*

O. labiatum yielded a compound that was able to inhibit HIV-1 protease and also produced novel triterpene isomers (HHODC) that had the ability to activate latent HIV-1.

The crude extract was able to reduce viral replication as described in chapter 5 but did not activate latency. This could possibly mean that phy-a (and possibly other unidentified compounds) could be the dominant compound in the crude extract and masked the activity of HHODC, explaining why the crude extract only inhibited HIV-1 PR and replication, and did not induce latency.

The concentration at which HHODC activated HIV-1 in the latently infected cell line was not cytotoxic. Current drugs for HIV cannot eradicate reservoirs with latent viral particles in the cells of HIV-infected individuals. Latent HIV-1 produces new viruses that replenish the ones destroyed by treatment and it is for this reason that these reservoirs need to be targeted to possibly eradicate HIV during treatment. The idea is that compounds that target latent reservoirs could be used in conjunction with HAART. In this case, the latency activators are considered as functioning like the adjuvants and this study is the first of potential adjuvants from *O. labiatum*.

A number of cytokines have the ability to induce HIV-1 expression (Biswas et al., 1992; Poli et al., 1994). IL-6 and IFN-γ are some of those cytokines reported to be potent modulators of latent HIV-1 (Biswas et al., 1992; Poli et al., 1994). The present study looked at the effect of HHODC on cytokine expression and of all the cytokines tested HHODC only up-regulated the production of two cytokines, IL-6 and INF-γ. This finding suggests that the mechanism by which HHDOC activated latent HIV-1 could possibly be by inducing the production of IL-6 and INF-γ, and in turn these cytokines activated the dormant HIV-1 particles. IL-6 levels were generally high in control cells compared to other cytokines. According to the literature high levels of IL-6 in healthy individuals have been suggested to contribute to the age-related increase of prostaglandin E2 and nitric oxide production (Ferrucci et al., 2005; Navarro et al., 1989; Plowden et al., 2004). Another compound isolated in this study, amyrin, did not have any effect on cytokine production (appendix Figure A6.3) but has other medicinal qualities (hepatoprotective), reported by Oliveira et al. (2005).

Future work should look into the separation of the never before reported isomers (HHODC) and this should determine whether the bioactivity that was observed is obtainable with the individual compounds or whether the isomers work in synergy. HHODC should be further investigated as a potential adjuvant *in vitro* and *in vivo* by combining it with a specific HAART combo.

8.5 Chapter 7: Anti-inflammatory and antioxidant properties of *Ocimum labiatum*

A labdane diterpenoid compound was isolated from *O. labiatum*. This compound did not possess any HIV-1 inhibitory properties (appendix Figure A7.2) but was able to down-regulate a number of pro-inflammatory cytokines implicated in the pathogenesis of a number of inflammatory diseases. *O. labiatum* extract demonstrated antioxidant properties and antioxidation plays a role in the pathogenesis of inflammatory diseases (Mirshafiey and Mohsenzadegan, 2008), suggesting that *O. labiatum* could potentially slow down inflammatory disease progression. The extract and isolated compound were also able to reduce the production of an inflammatory mediator, nitric oxide. The anti-inflammation findings are a first for the plant and suggest further investigation of *O. labiatum* as a potential complimentary medicine for treatment of inflammation.

8.6 Revisiting hypothesis

In this study, it was hypothesized that “plants from the Lamiaceae family contain bioactive compounds that are able to inhibit HIV replication”. Being nature-derived, these compounds should have other beneficial qualities as well (immunomodulation and anti-inflammatory/antioxidant activity). In order to investigate this hypothesis several activities were undertaken.

8.6.1 Selecting plants and screening the crude extracts

Only plants from the Lamiaceae family were selected for this study and sixteen extracts were obtained from sixteen different plants. Plants were also selected based on the reported traditional uses of either the genus or species.

Extracts were prepared by first extracting with ethanol and the residue re-dissolved in ethyl acetate to exclude highly polar tannin compounds. Tannins are non-specific enzyme inhibitors (Klos et al., 2009) and are readily extracted by water as well as ethanol/methanol extraction. In order to search for specific enzyme inhibitors, tannins should be excluded from the extracts. Traditional healers use plant infusions or decoctions prepared with water and the possibility of those extracts inhibiting enzymes is very high due to the presence of tannins.

Of the sixteen plants, only *O. labiatum* and *P. barbatus* inhibited HIV-1 protease (PR) by more than 50% in the direct enzyme assays. The rest of the plants had inhibitions of

<50%. Perhaps it should be highlighted here that the concentrations selected for bioactivity screening of the crude extracts were 100 and 50 µg/ml. The selection of these concentrations was based on the recommended criteria for activity of crude extracts as ≤100 µg/ml (Cos et al., 2006). The two plants with good activity against HIV-1 PR were studied in more detail in order to properly evaluate other bioactive properties. Only *O. labiatum* was selected for bioassay-guided fractionation to identify the compounds of interest and the reason was that *O. labiatum* inhibited the HIV-1 enzyme at both concentrations tested (>50%) unlike *P. barbatus* that only had good inhibition at the highest concentration tested.

8.6.2 Further investigation of the two potent plants

P. barbatus was investigated for its ability to inhibit pro-inflammatory cytokines and that data together with the HIV-1 inhibitory properties was published (Kapewangolo et al., 2013) to support the traditional use of this plant in managing HIV/AIDS. Bioassay-guided purification of *O. labiatum* yielded a number of compounds with HIV-1 inhibitory properties as well as the ability to stimulate latent HIV-1. A different compound from the same plant possessed immune enhancing properties.

8.6.3 The other 14 extracts

After the identification of the two bioactive plants a decision had to be made about the other fourteen plants that gave less than 50% inhibition in direct enzyme assays.

One of these plants, *Plectranthus ciliatus*, demonstrated anticancer properties by exhibiting low CC_{50} values and was donated to a separate research project to be further evaluated for anti-cancer properties (Le Roux, 2013). From the remaining 13 plants, plants that moderately (40-49%) inhibited HIV-1 RT, IN or PR were tested for their influences on HIV-1 expression. Four plants were able to reduce HIV-1 expression in chronically infected U1 cells. This indicated that even though the inhibition of enzymes was moderate for these extracts, it was significant enough to reduce viral replication in infected cells.

8.7 Hypothesis accepted

Based on the findings of the overall study, the hypothesis could therefore be turned into the following fact, “plants from the Lamiaceae family contain bioactive compounds that

inhibit HIV replication (through inhibition of key enzymes in the viral life cycle) and have immunomodulatory and antioxidant effects"

8.8 Other questions

During the course of the study, other questions were raised and these are described in the next subsections.

8.8.2 Are the extracts capable of inhibiting HIV-1 integrase?

Integration of the viral DNA into the host genome is another important step in the HIV life cycle. Once the viral DNA is integrated, it becomes part of the host genome and that process cannot be reversed. Blocking the integration step in the life cycle of HIV will definitely block viral infection. In the present study, a non-radioactive HIV-1 integrase assay from XpressBio (Life Science products) was used to quantitatively measure integrase activity. None of the extracts from the 16 plants inhibited the enzyme by more than 50% (appendix Figure A3.2). One plant that stood out was *Ocimum serratum* with an inhibition of 47%, the same plant was selected for the HIV-1 expression study in chapter 3 and it was able to reduce HIV-1 replication in infected cells.

8.8.1 What are the effects of any isolated compounds on selected opportunistic infections?

The first compound isolated in this study was the labdane diterpenoid, labda-8(17),12E,14-triene-2R,18-diol. This compound did not possess any HIV-1 inhibitory properties nor the ability to activate latent HIV-1 (appendix Figure A7.2). Terpenoids isolated from natural sources have been previously reported to demonstrate antimycobacterial activity (Gordien et al., 2009). The compound was sent to the National Health Laboratory Service (NHLS), Center of Excellence in TB research for testing against *Mycobacterium tuberculosis* (strain no. H37R). A rapid radiometric method was used and isonicotinic acid hydrazide was included as positive control (Palomino et al., 2002). A minimum inhibitory concentration (MIC) of 320 µM was obtained which was an indication of very weak activity against *M. tuberculosis*. Hussein et al. (2007) analysed the same diterpenoid against *M. tuberculosis* (H37R) and did not find any inhibitory activity at the highest concentration tested (650 µM). Individuals infected with HIV are at a high risk of developing tuberculosis compared to those individuals not infected with the virus (UNAIDS, 2012). *M. tuberculosis*/HIV co-infection

has been a major treatment challenge as the immune system of co-infected individuals has to cope with two microorganisms.

Another opportunistic infection that was investigated was cervical cancer. Cervical cancer is regarded as an opportunistic infection because human papilloma virus, the causative agent of cervical cancer, is considered a risk factor for precancerous lesions among HIV-1 positive women (Patel et al., 2013). The labdane diterpenoid compound had a CC_{50} of 60 µM in cervical cancer cells which is also indicative of weak activity. Recommended stringent end-points for good activity of compounds is a CC_{50} of 25 µM (Cos et al., 2006). For future studies, it will be of interest to evaluate the anticancer properties of the triterpene isomer (HHODC) reported in chapter 6. HHODC had selective cytotoxic effects; at 100 µg/ml viability of TZM-bl was more than 50% in the presence of HHODC but the compound had a different cytotoxic effect in U1 cells where a low CC_{50} of 8.1 µg/ml was obtained. According to the National Cancer Institute (NCI), plant products with a CC_{50} <30 µg/ml are considered to potentially have anticancer activity (Talib and Mahasneh, 2010).

8.8.2 Strengths and limitations of in vitro assays

Drug discovery and validation of lead compounds varies among academic laboratories and pharmaceutical companies (Astashkina et al., 2012). The main focus is usually end-point assays that establish cell survival as was done in this study in the case of tetrazolium dyes and flow cytometry. Traditional toxicity assays measure general cell viability or cell proliferation end-points, and are not specific to toxicity mechanisms. Real-time monitoring, as was done in the present study, overcomes the end-point assay limitation by allowing the user to monitor in real-time toxicity mechanisms (Astashkina et al., 2012). Another limitation of traditional assays is the inability to distinguish between apoptosis and necrosis cell death mechanisms (Astashkina et al., 2012); flow cytometry fluorochromes such as annexin V and propidium iodide are able to measure apoptotic and necrotic cell deaths.

A key challenge in screening and developing drug candidates as therapeutic agents is accurate toxicity determination in humans. Nearly 3% of all drugs making it into the clinic from 1974 to 2007 were withdrawn from the market later due to adverse side effects, mainly toxicity (Kola, 2008). Despite rigorous testing *in vivo*, about 43% of compounds reportedly fail in phase III, and about 23% fail in registration due to

unforeseen side effects (Astashkina et al., 2012; Kola, 2008). This finding testifies to the need for innovation and development of advanced technologies and risk assessment techniques.

8.9 Conclusion

Nature as a source of novel bioactive agents continues to provide the best solution to the scientific community engaged in drug discovery (Newman and Cragg, 2012). The current study was able to prove that it is worth investigating the bioactivity of crude extracts as they serve as the first indicators of success. The findings of this study showed that bioactive compounds can be anything from terpenes to chlorophylls.

A total of 16 plants were investigated and two were singled out as promising candidates inhibiting HIV-1 PR (*P. barbatus and O. labiatum*). Compounds isolated from *O. labiatum* demonstrated anti-HIV and immune enhancing properties, with the latter being the ability to reduce the production of pro-inflammatory cytokines. Antioxidant activity as well as the suppression of NO an inflammatory mediator was also observed. Four plants *O. labiatum, O. serratum, S. apiana* and *S. byzantina*, showed promising suppression of HIV-1 replication at concentrations that were not toxic. In addition, *O. serratum* was a moderate inhibitor of HIV-1 integrase. Out of 16 members of Lamiaceae, 6 are promising (and traditionally used) anti-HIV candidates, while one plant has anti-cancer potential (*P. ciliatus*). All the bioactive plant extracts not yet investigated to the extent of *O. labiatum*, are worthy candidates for further investigation and in addition the promise of isolated compounds (phy-a, HHODC, amyrin, labdane diterpenoid) vary from direct inhibitors of HIV to supports of immune and inflammation systems.

Novel triterpenes with the ability to induce latent HIV-1 were isolated from *O. labiatum* and this study also provided a first time report on a chlorophyll derivative phy-a inhibiting HIV-1 PR. A labdane diterpenoid, labda-8(17),12*E*,14-triene-2R,18-diol, with immune enhancing properties was also reported in this study for the first time.

CHAPTER 9

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CHAPTER 10

APPENDIX

This section contains additional information and data which supports some of the main findings. Here, supplementary information is provided on crude extracts preparation, bioassay-guided purification, enzyme kinetics, NMR spectra and HPLC chromatograms of purified compounds as well as extra data on inflammation.

10.1 Chapter 3

10.1.1 Crude extract preparation

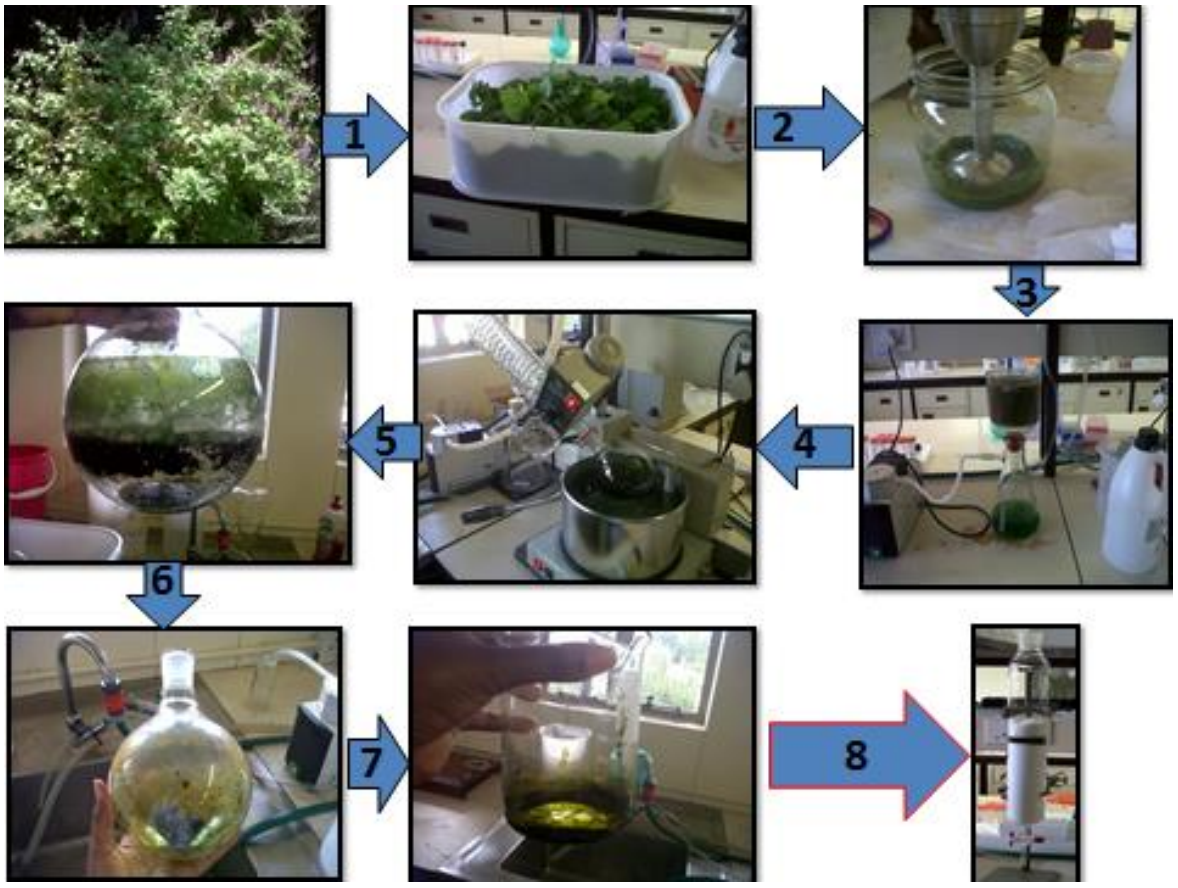

Figure A3.1: Schematic illustration of crude extracts preparation.

1- Plant collection

2- Maceration in ethanol

3- Filtration

4-Concentrating filtrate

5–6 Dissolving residue in ethyl acetate to exclude polar tannins

7 – Dried ethyl acetate fraction (ready for biological testing)

8 – Column fractionation of extract.

10.1.2 HIV-1 integrase assay

10.1.2.1 Principle of the assay

The XpressBio HIV-1 integrase assay kit is a non-radioactive assay used to quantitatively measure integrase activity and the effects of test compounds on HIV01 integrase activity (XpressBio, Life Science Products). Sodium azide is included in the kit as a positive control that inhibits HIV-1 integrase catalytic activity.

A

Sample	% Intergrase inhibition
Sodium Azide	103
1	29
2	23
3	24
4	23
5	26
6	**47 ±9**
7	21
8	43 ±9
9	36 ±4
10	15
11	8
12	6
13	10
14	12
15	-3
16	8

B

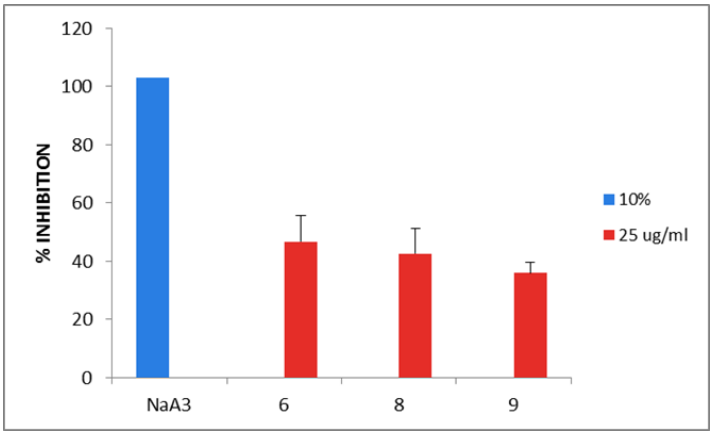

Figure A3.2: (A) Effect of all 16 crude extracts on the activity of HIV-1 integrase**. (B)** Crude extracts that showed potential inhibition against integrase but activity was <50%. Moderate enzyme inhibition was observed with extracts 6 and 8.

10.1.3 Principle of HIV-1 reverse transcriptase (RT) assay

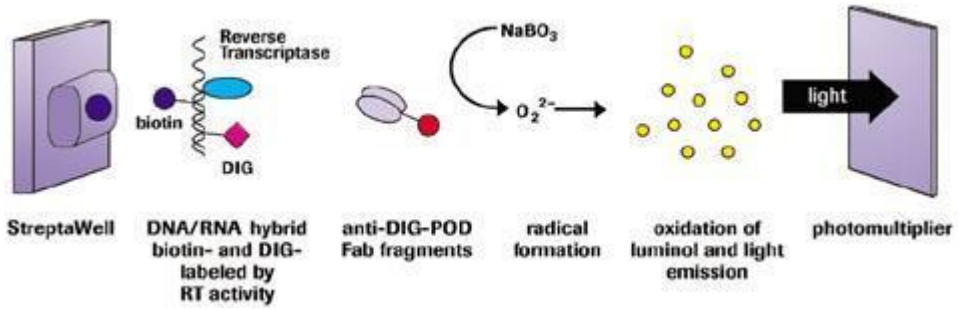

Figure A3.3: HIV RT assay.

The colorimetric principle takes advantage of the ability of RT to synthesize DNA, starting from the template/primer hybrid poly (A) x oligo $(dT)_{15}$. It incorporates digoxigenin (DIG)- and biotin-labeled nucleotides in an optimized ratio into one and the same DNA molecule, which is freshly synthesized by the RT. The detection and quantification of synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol: Biotin-labeled DNA binds to the surface of microplate (MP) modules that have been precoated with streptavidin. An antibody to DIG conjugated to peroxidase (POD) binds to DIG-labeled DNA. In the final step, the POD substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) is added and the POD enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of the reaction product is determined spectrophotometrically with a microplate reader and is directly correlated to the level of RT activity in the sample.

Most of the extracts tested demonstrated weak RT inhibition of <30% (Figure A3.5).

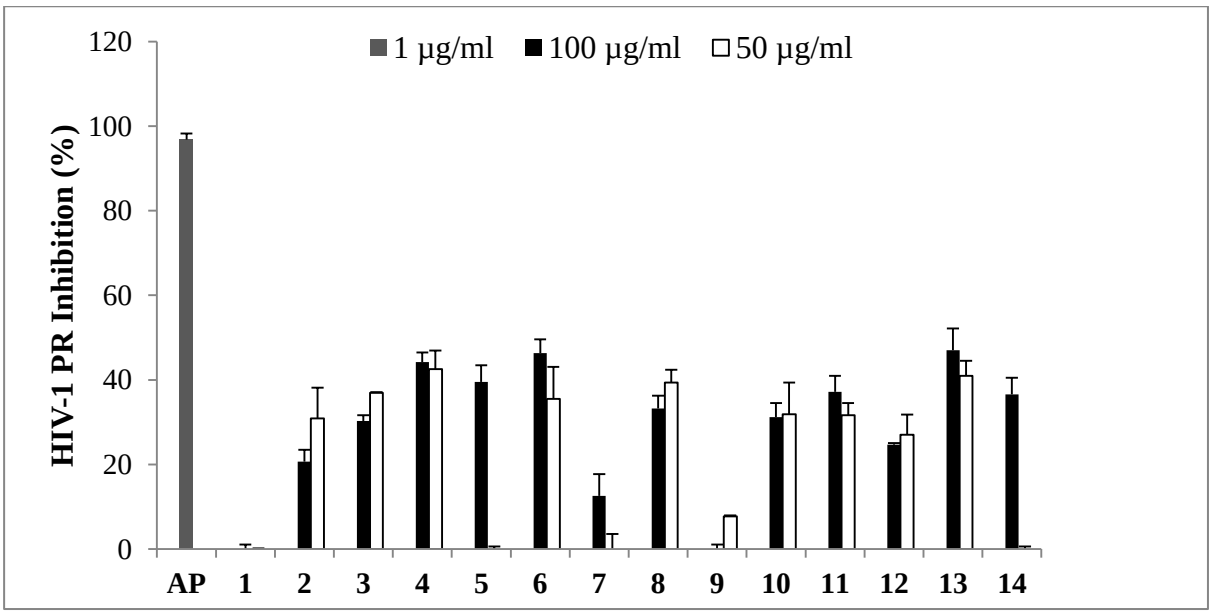

Figure A3.4: Effect of crude extracts on HIV-1 PR activity. The data represents the mean of multiple replicates of HIV-1 PR inhibition (relative to an untreated control with buffer only) of various Lamiaceae extracts, each tested at 100 and 50 µg/ml in the final reaction volume. AP = Acetyl pepstatin, was used as a positive control. *P. neochilus* (6) and *S. byzantina* (13) inhibited HIV-1 PR by more than 40% at the two tested concentrations.

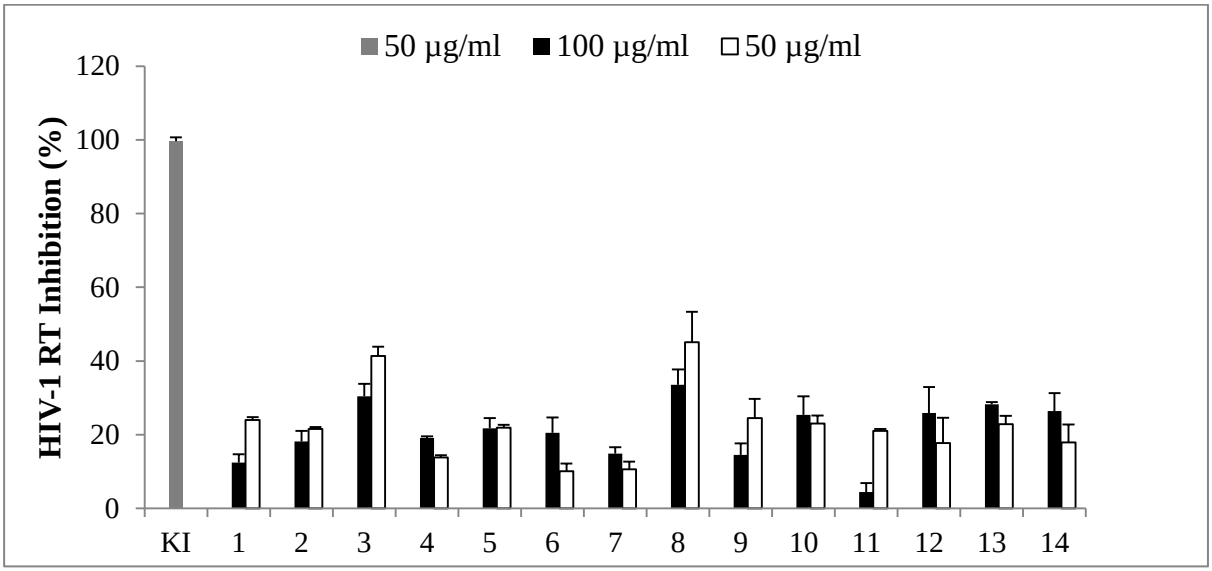

Figure A3.5: Effect of crude extracts on HIV-1 RT activity. The data represents the mean of multiple replicates of HIV-1 RT inhibition (relative to an untreated control) of various Lamiaceae extracts, each tested at a final reaction concentration of 100 and 50 µg/ml. A known inhibitor (KI), doxorubicin, was used as a positive control for HIV-1 RT inhibition. *O. serratum* (3) and *S. apiana* (8) inhibited HIV-1 RT by more than 40%.

10.2 Chapter 5: Bioassay-guided fractionation of *Ocimum labiatum*

10.2.1 Purification of crude extract

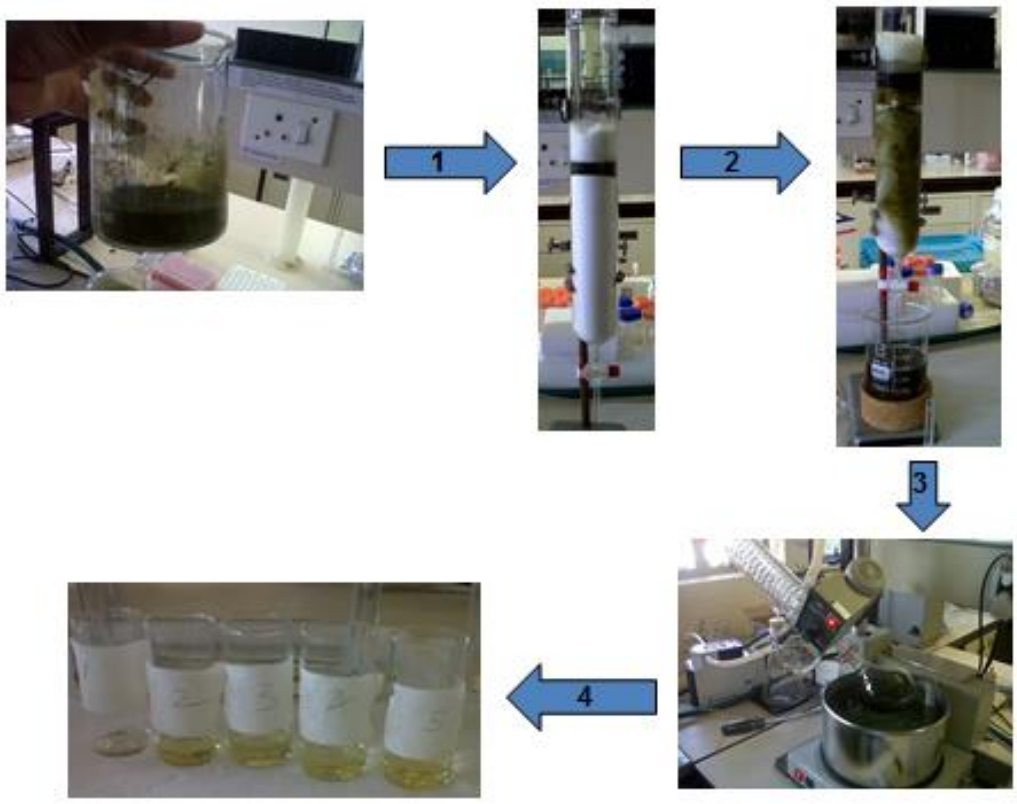

Figure A5.1: Schematic illustrations of crude extract purification using silica gel.

Extract was partitioned into polar and non-polar fractions (100:0 hexane – 0:100 ethyl acetate). The extract yielded 19 fractions which were screened for HIV-1 PR inhibiting properties.

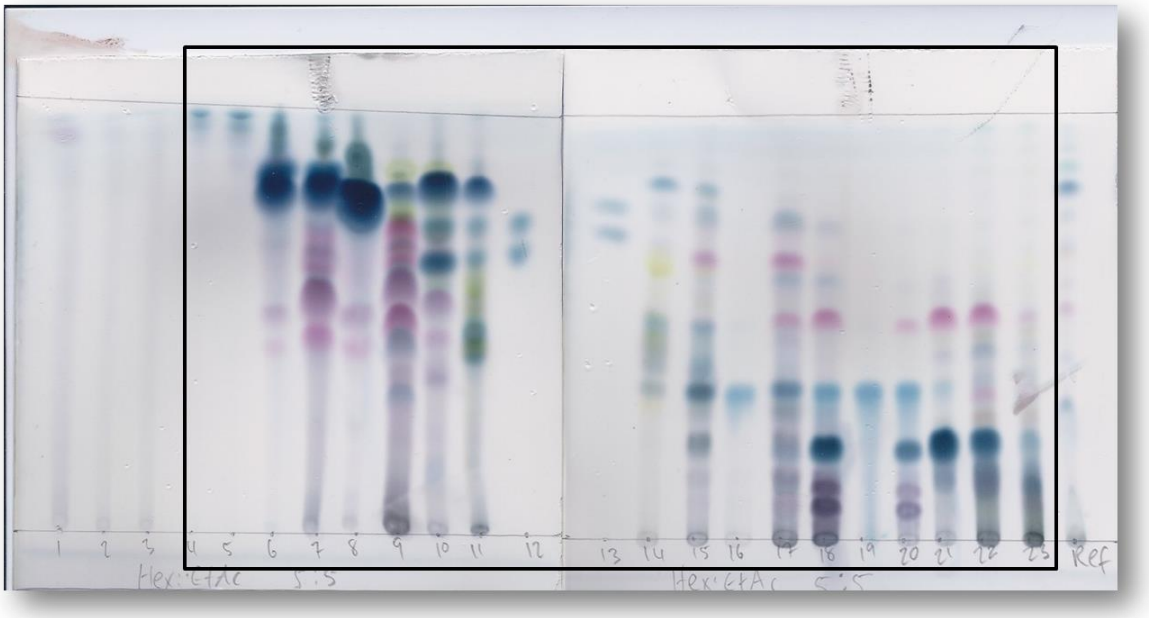

Figure A5.2: Fractions yielded from *O. labiatum* by column chromatography.

Fraction 16 came out as a pure compound; the labdane diterpenoid compound described in chapter 7.

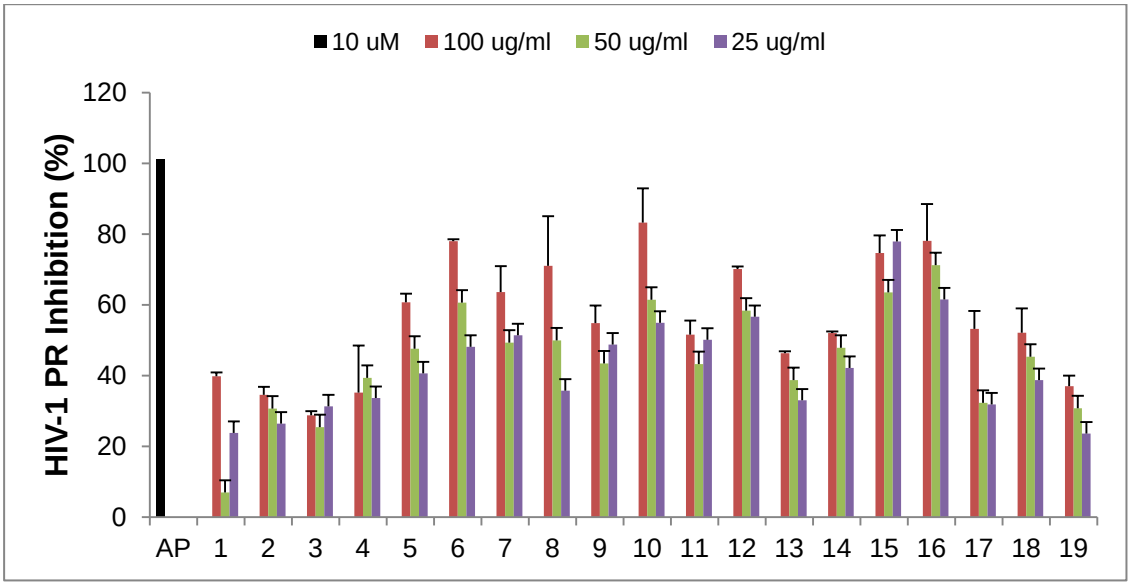

Figure A5.3: Inhibitory properties of *O. labiatum* fractions against HIV-1 PR.

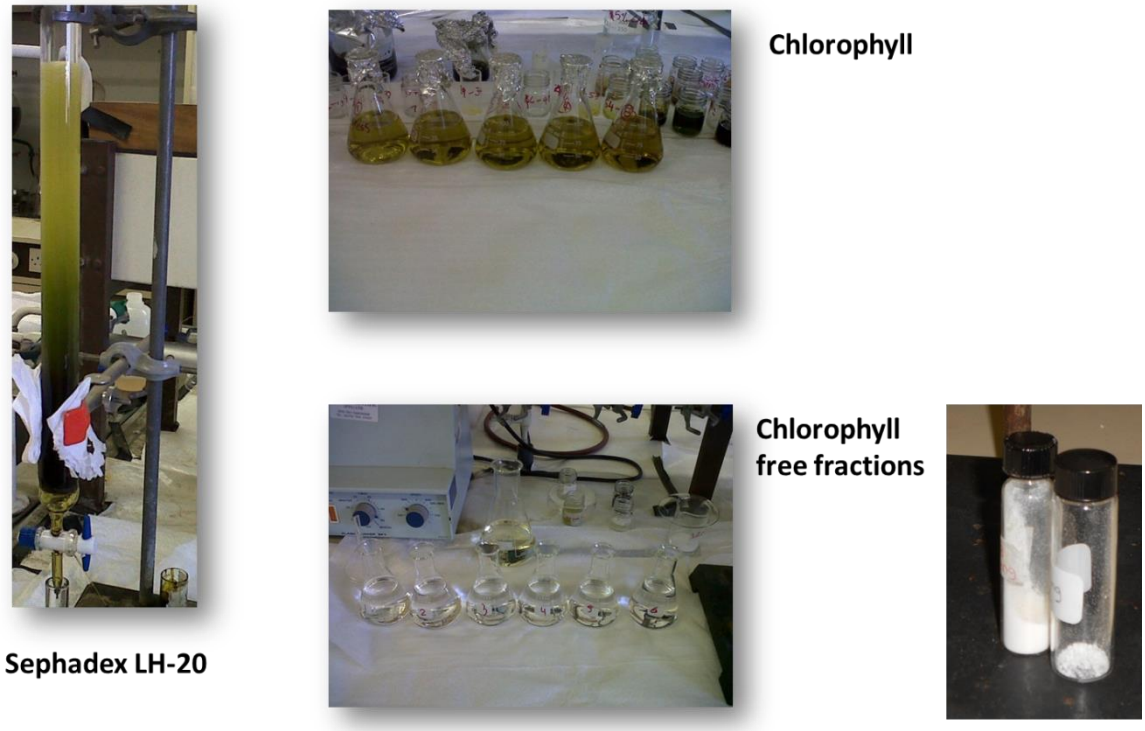

Figure A5.4: HIV-1 PR inhibiting fractions were further purified using sephadex.

Sephadex separates based on the size of the constituents. Chlorophyll was successfully removed from the fractions.

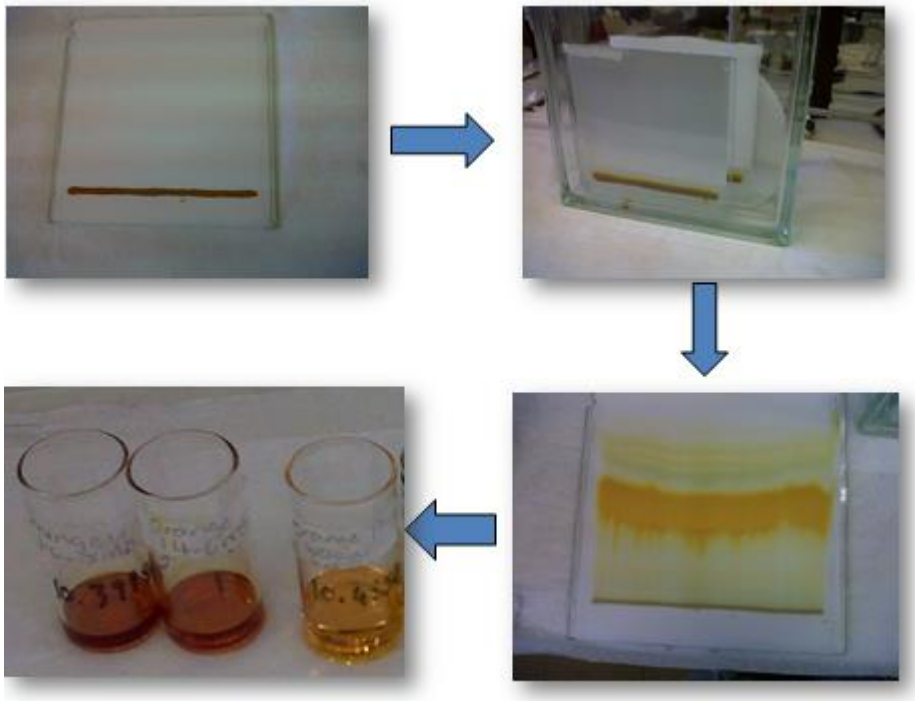

Figure A5.5: Preparative TLC was employed to separate a compound (PK04) that was closely attached to chlorophyll.

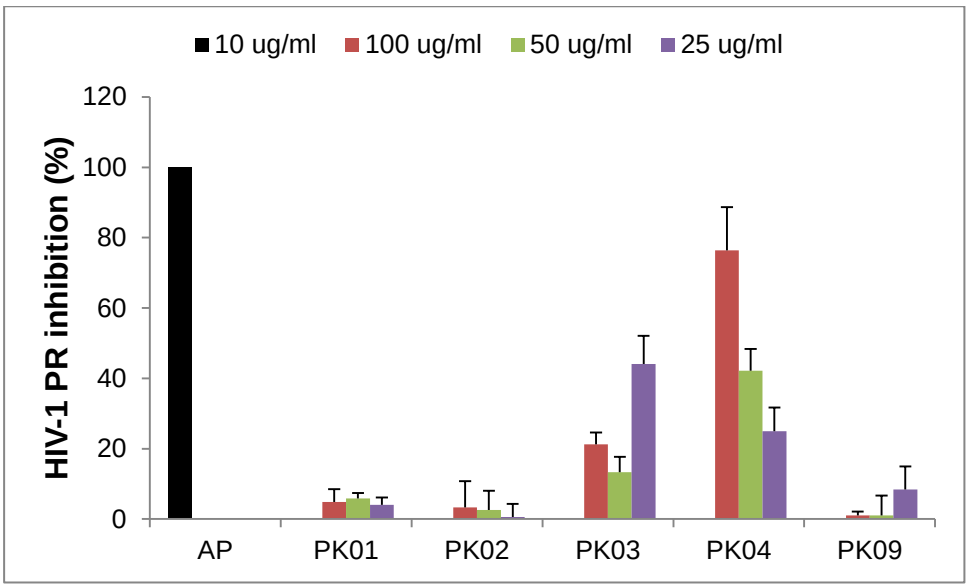

Figure A5.6: HIV-1 PR inhibitory properties of compounds purified from the chlorophyll free fractions. These included Amyrin, triterpenoid isomers and labdane diterpenoid compound. The compounds did not have activity against HIV-1 PR except for one compound, PK04. Only 4 mg of PK04 was obtained from purification and thus quantity was not sufficient for NMR analysis.

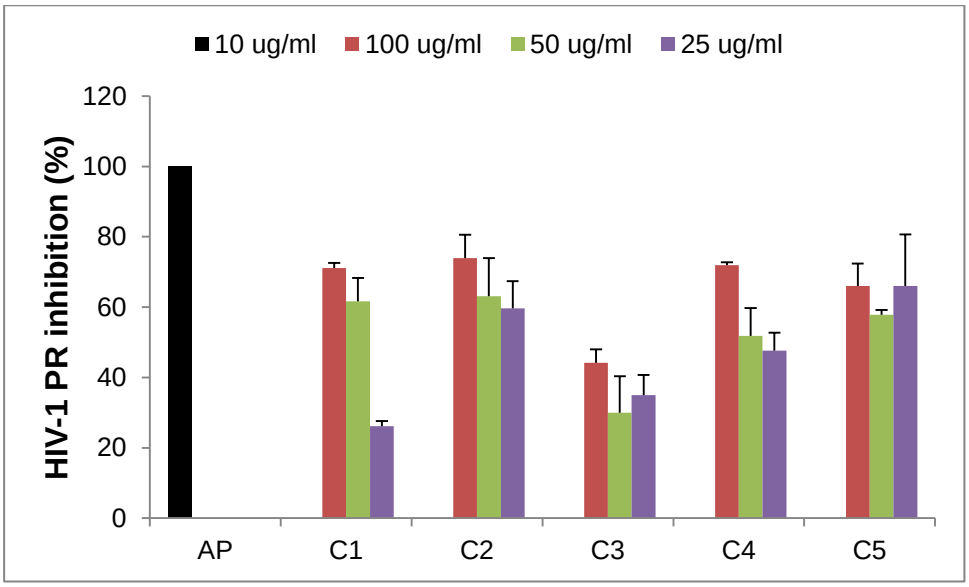

Figure A5.7: Bioactivity of chlorophyll fractions. HIV-1 PR inhibition retained by chlorophyll samples obtained with sephadex. Pheophytin-a (phy-a) was purified from fraction C1 using preparative HPLC and TLC. The quantity of the rest of the bioactive fractions (C2, 4 & 5) were not sufficient for further purification.

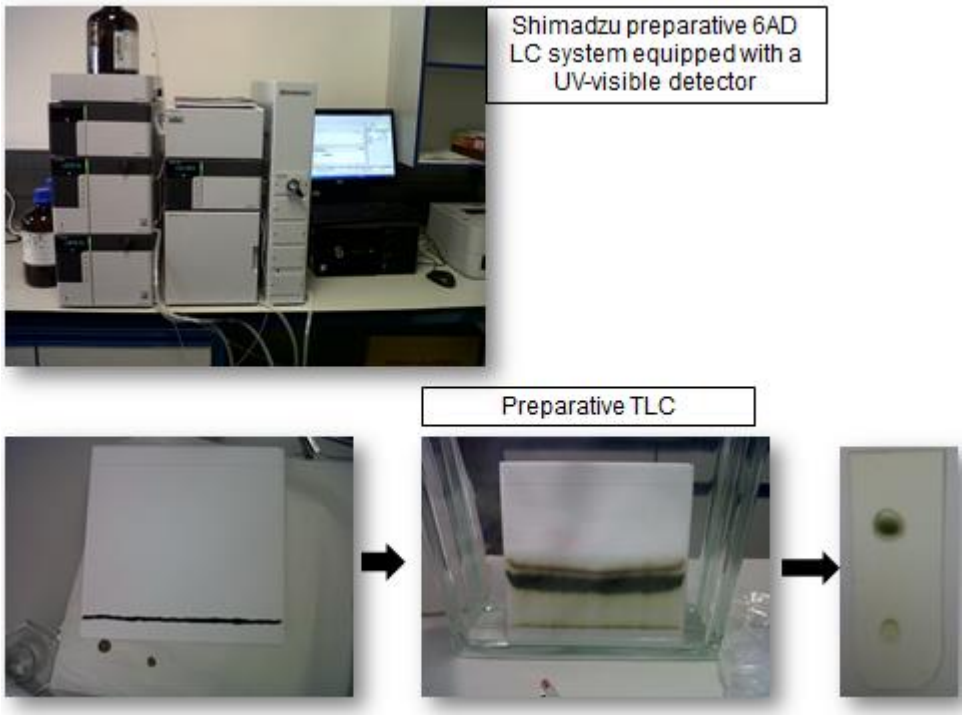

Figure A5.8: Purification of phy-a using prep- HPLC and TLC.

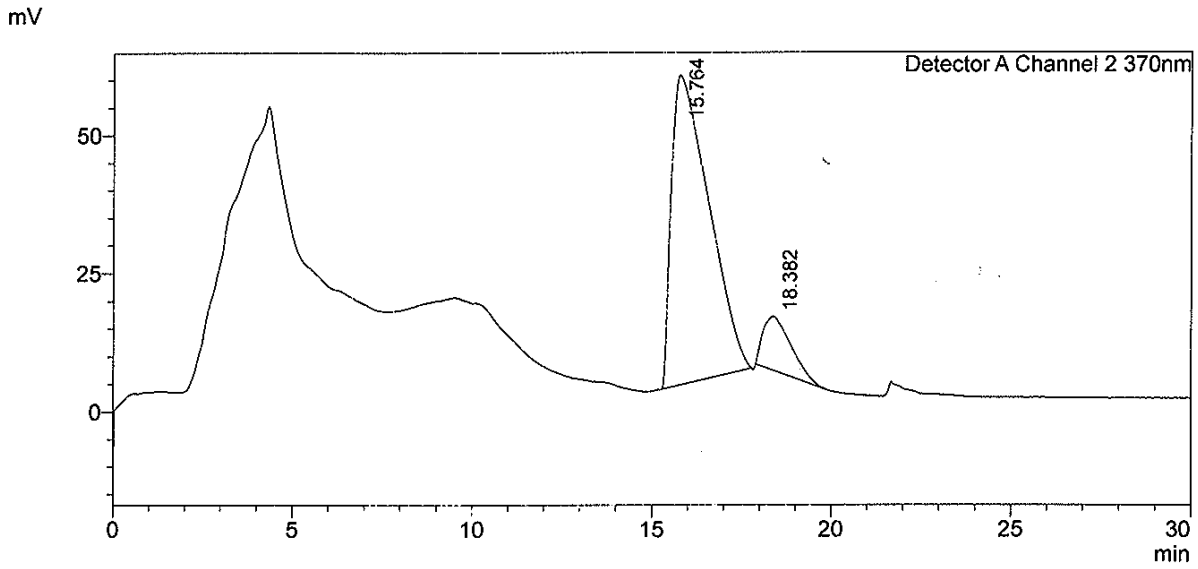

Figure A5.9: HPLC chromatogram of phy-a obtained by isocratic elution with acetonitrile/methanol (1:1) for 30 min. Retention time of 16 min compared well with the literature (Breemen et al., 1991).

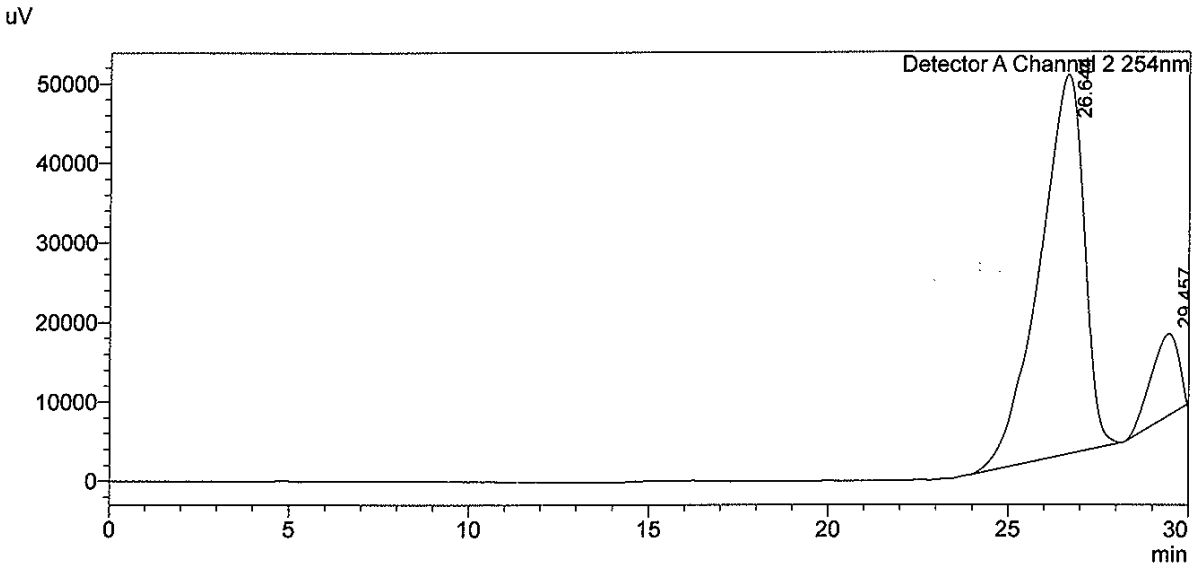

Figure A5.10: HPLC chromatogram of phy-a obtained by gradient elution for 30 min using methanol and water (5-95%) as mobile phase.

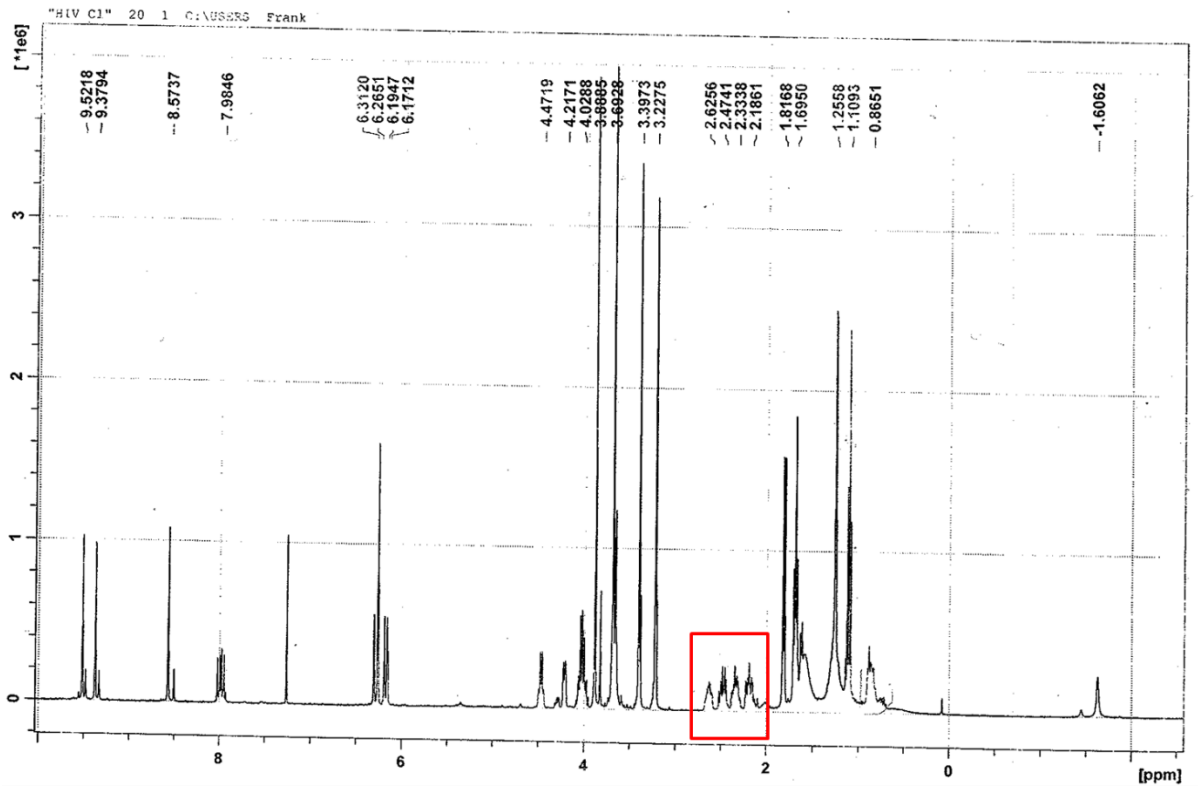

Figure A5.11: ^{1}H NMR of phy-a. Sample was analysed in deuterated chloroform.

Multiplets for the propionic side chains for phy-a in the spectrum, 2.0-2.7 ppm, are identical to those reported in the literature (Smith et al., 1984).

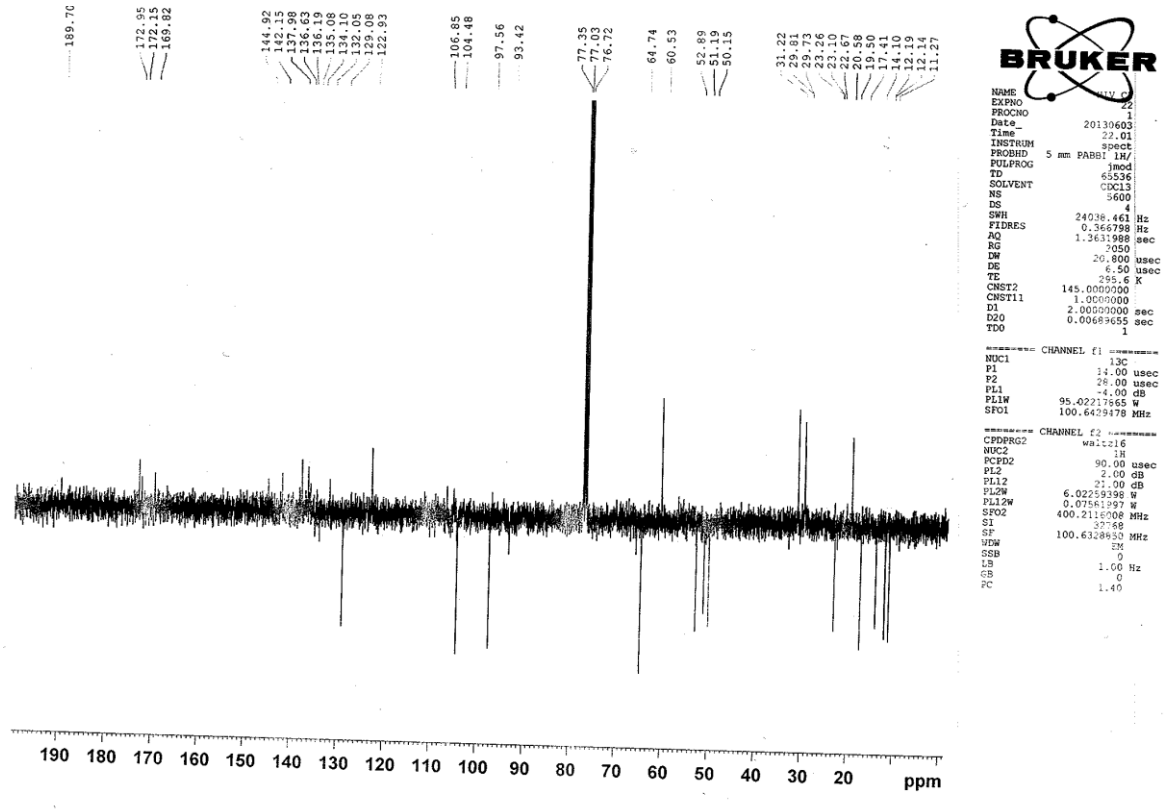

Figure A5.12: DEPT spectra of phy-a.

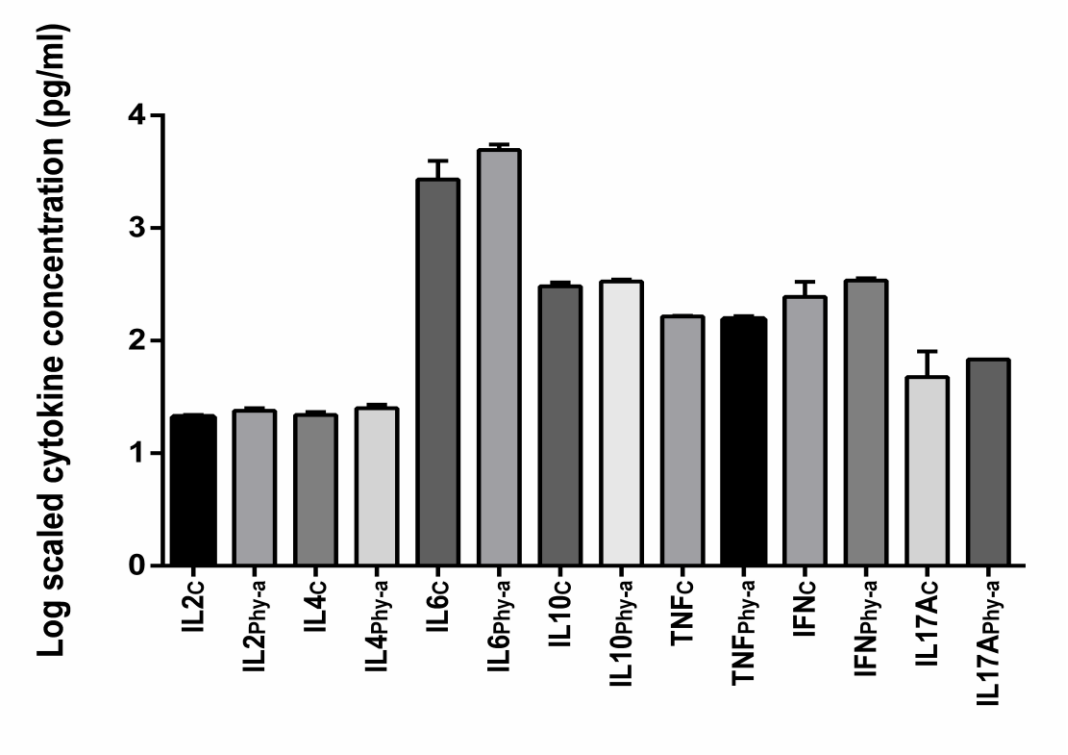

Figure A5.13: Effect of phy-a on cytokine production in PBMCs.

There was no significant ($p>0.05$) difference between samples treated with phy-a and control samples (e.g. IL2C vs IL2phy-a).

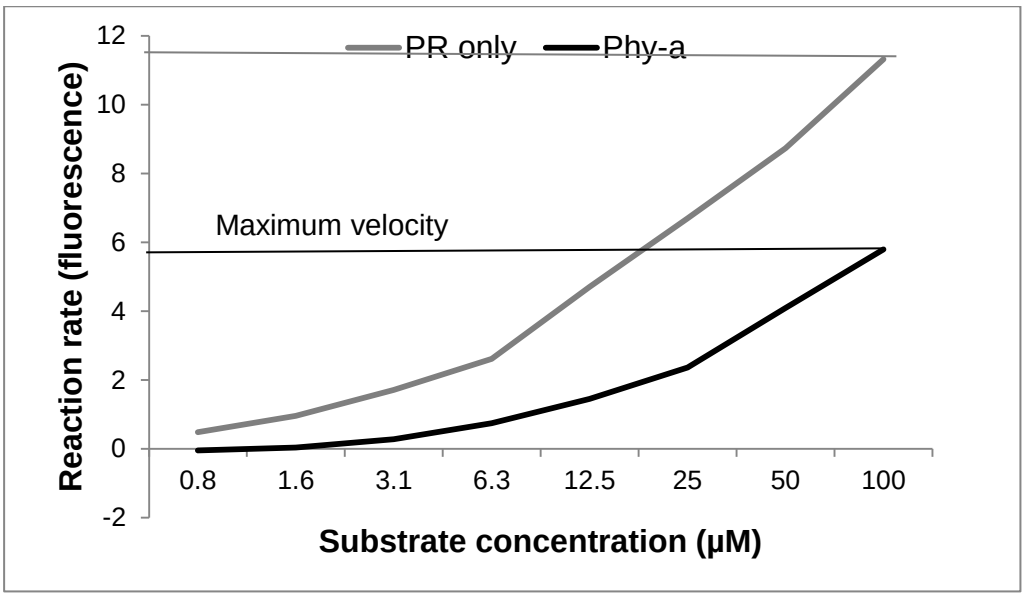

Figure A5.14: Effects of phy-a PR inhibition on enzyme kinetics.

From the kinetics study, phy-a was found to be a non-competitive inhibitor with a Km value = 13.0 µM (the value on the x-axis at ½ Vmax). The horizontal lines represent the maximum reaction rate for the sample and uninhibited enzyme. A non-competitive inhibitor has a Km value equivalent to that of the enzyme only. Enzyme activity in the presence and absence of sample was studied by varying the concentration of the substrate. Km value was obtained by determining the concentration of the substrate at half the maximum velocity (Vm) of the reaction.

10.3 Chapter 6: Latent HIV-1 study

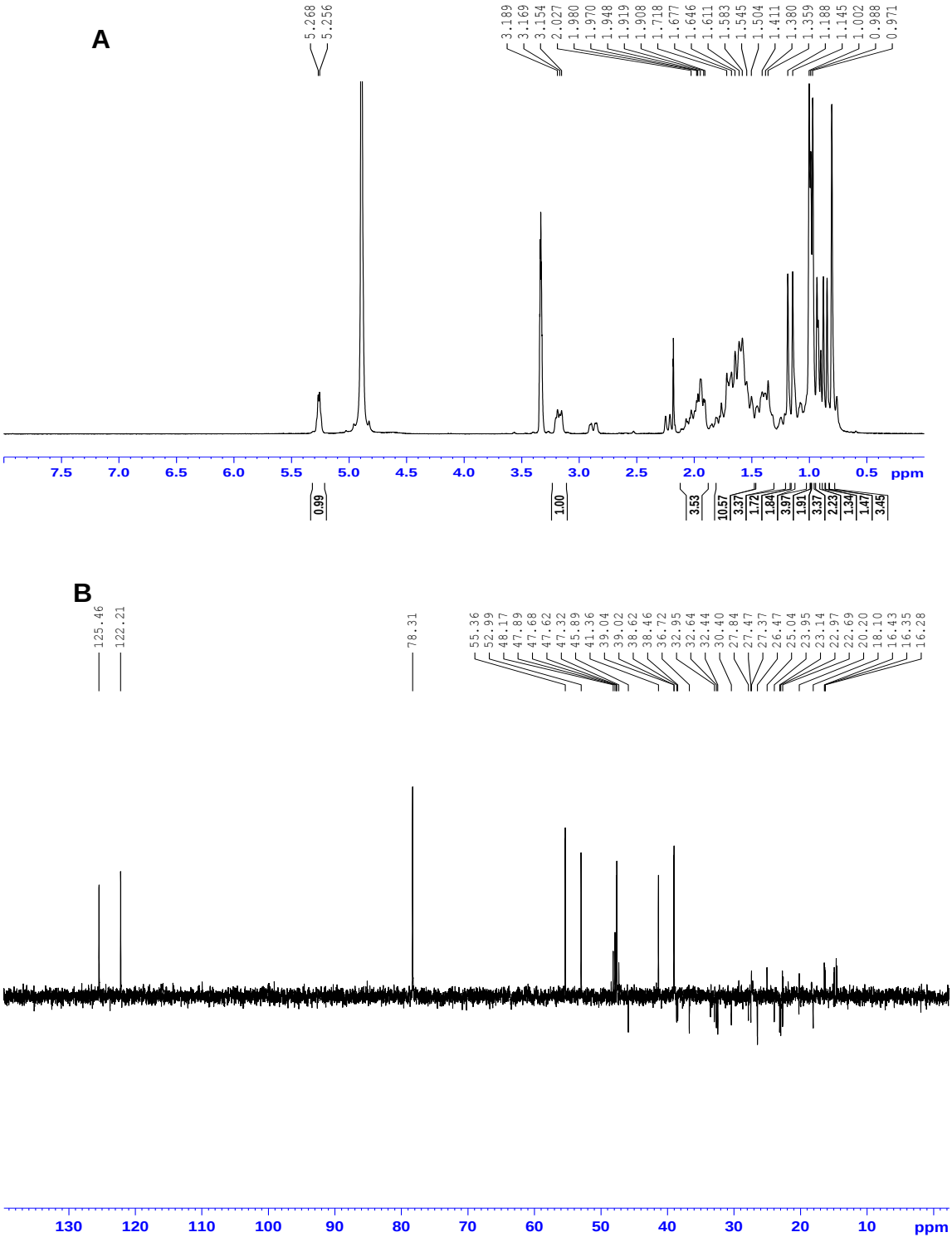

Figure A6.1: A. Proton NMR and **B.** DEPT spectra of PK in deuterated methanol.

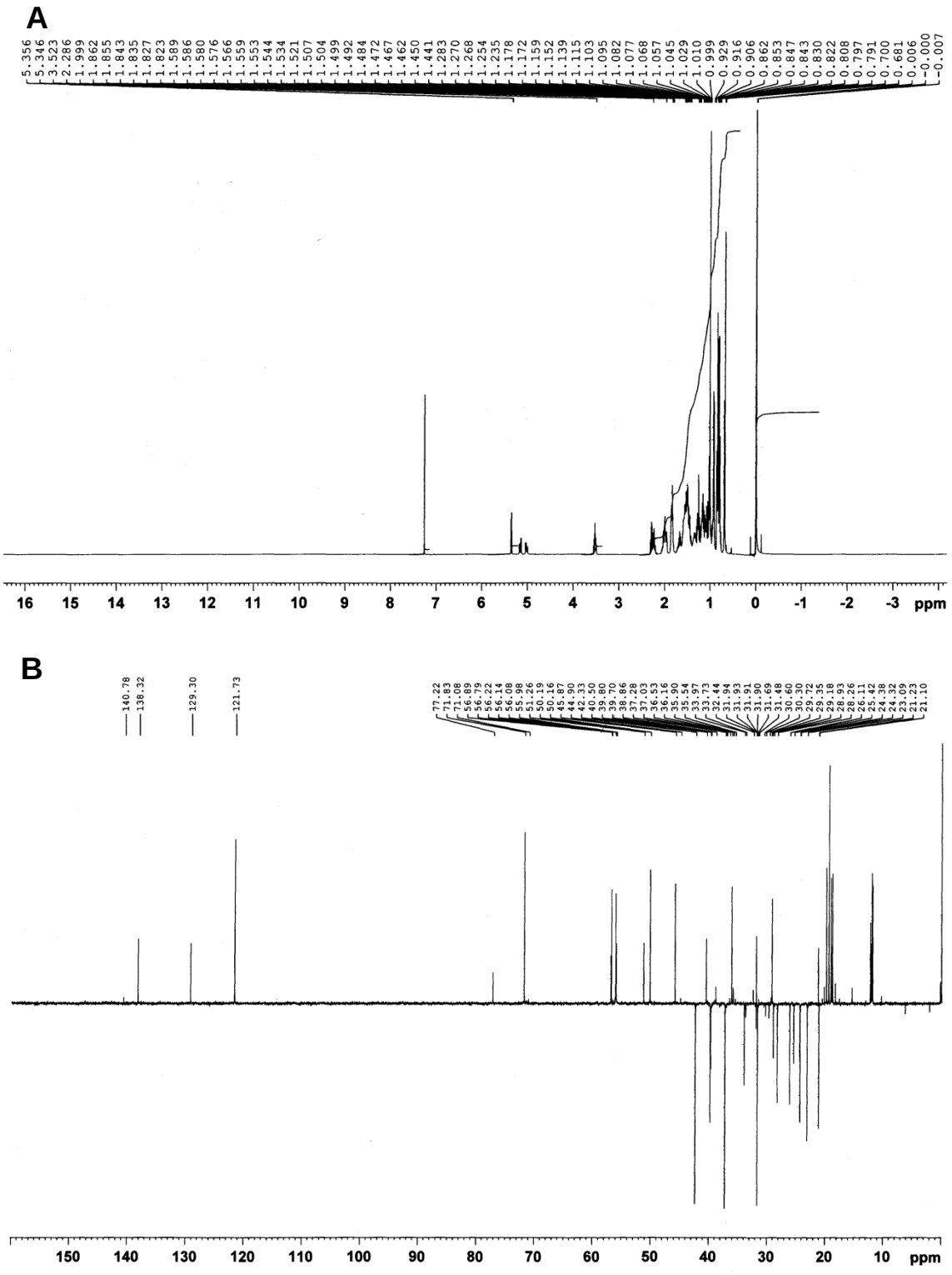

Figure A6.2: A. ^{1}H NMR and **B.** DEPT spectra of amyrin.

Table A6.1: Effect of HHODC on cytokine production in PBMCs and U1 cells

Cytokine concentrations in pg/ml						
	Uninfected PBMCs		U1 cells			
Analyte	Untreated	Treated with HHODC	No PMA	HHODC only	HHODC+PMA	PMA
IL-2	21.0 ±6.4	26.9 ±6.9	0	2.01 ±5.4	2.8 ±5.6	0
IL-4	22.0 ±7.2	24.1 ±7.5	0	1.9 ±5.0	1.8 ±5.0	1.5 ±6.1
IL-6	2801.7 ±21.9	14744.9 ±17.9	1.4 ±6.3	1.6 ±4.6	2.2 ±4.9	1.9 ±5.0
IL-10	304.4 ±22.6	191.7 ±24.8	0	0	3.2 ±5.5	10.0 ±6.3
TNF	164.1 ±26.5	528.7 ±27.7	1.7 ±4.4	2.2 ±4.9	6.7 ±5.11	2.0 ±4.5
IFN	251.0 ±22.9	842.6 ±21.0	2.5 ±4.6	1.8 ±4.7	0.0	0.0
IL-17A	50.9 ±5.6	31.6 ±5.6	0	9.0 ±4.4	9.0 ±4.2	0.0

The compound was tested at 6.3 µg/ml, the same concentration that activated latent HIV-1 in U1 cells. HHODC significantly ($p<0.05$) increased the production of IL-6 as compared to an untreated control. IFN-γ was also up-regulated significantly ($p<0.05$) by the compound. HHODC did not affect the production of IL-2, 4, 10, 17A and TNF.
In U1 cells, HHODC up-regulated the production of IL-2, 4, 6, TNF and IL-17A.

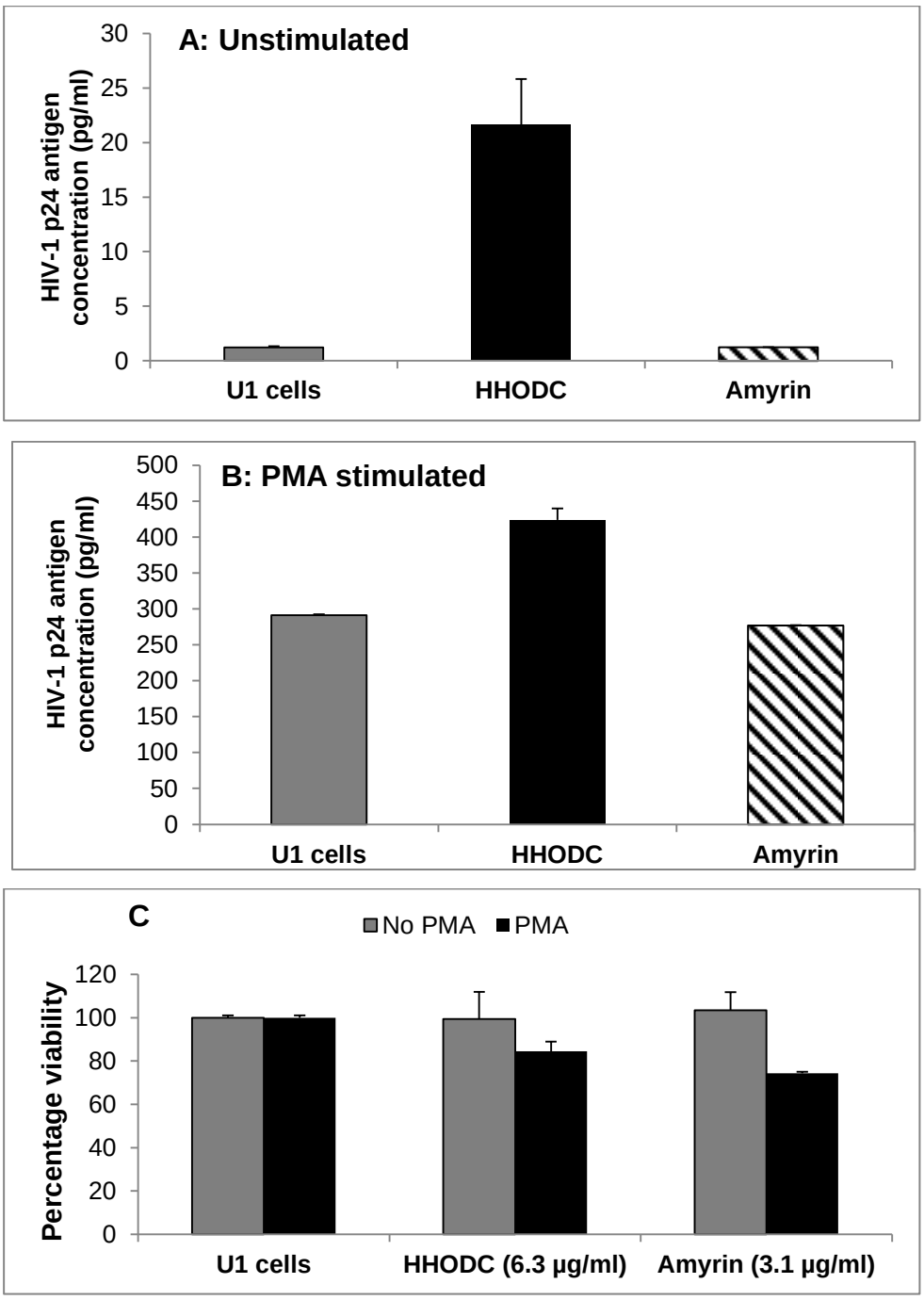

Figure A6.3: Effects of compounds on HIV-1 expression. A. Unstimulated cells were incubated with the samples and HHODC activated latent HIV-1 expression in the absence of a known stimulant. **B**. PMA was included as a known stimulant of latent HIV-1 activation in U1 cells and it synergized with HHODC by further increasing expression of HIV-1 p24 antigen concentration compared to control cells (U1 cells). There was no significant ($p<0.05$) difference in HIV-1 expression between the stimulated control cells and stimulated cells treated with amyrin. **C.** Cytotoxicity was analysed by MTT and U1 viability was ≥80% for the concentrations of compounds investigated for HIV-1 expression in both PMA stimulated and unstimulated cells.

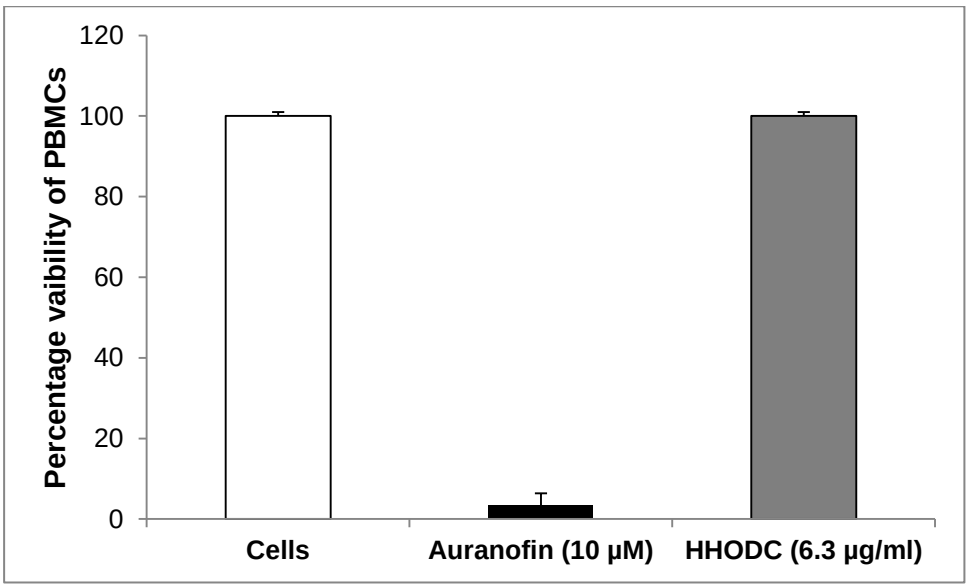

Figure A6.4: Effect of HHODC on the viability of PBMCs. The tested concentration, 6.3 µg/ml, was not toxic in PBMCs as illustrated. Auranofin was used as a positive control for toxicity.

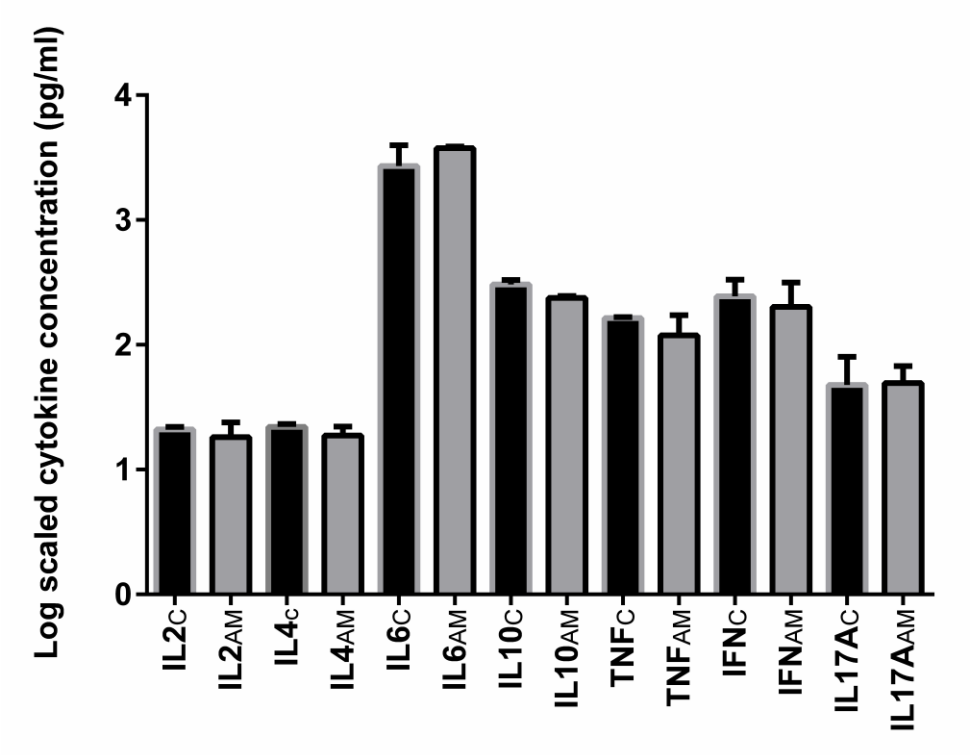

Figure A6.5: Effect of amyrin on cytokine production in PBMCs. There was no significant ($p>0.05$) difference between cells treated with amyrin and untreated cells.

10.4 Chapter 7: Anti-inflammatory and anti-oxidant properties of *Ocimum labiatum*

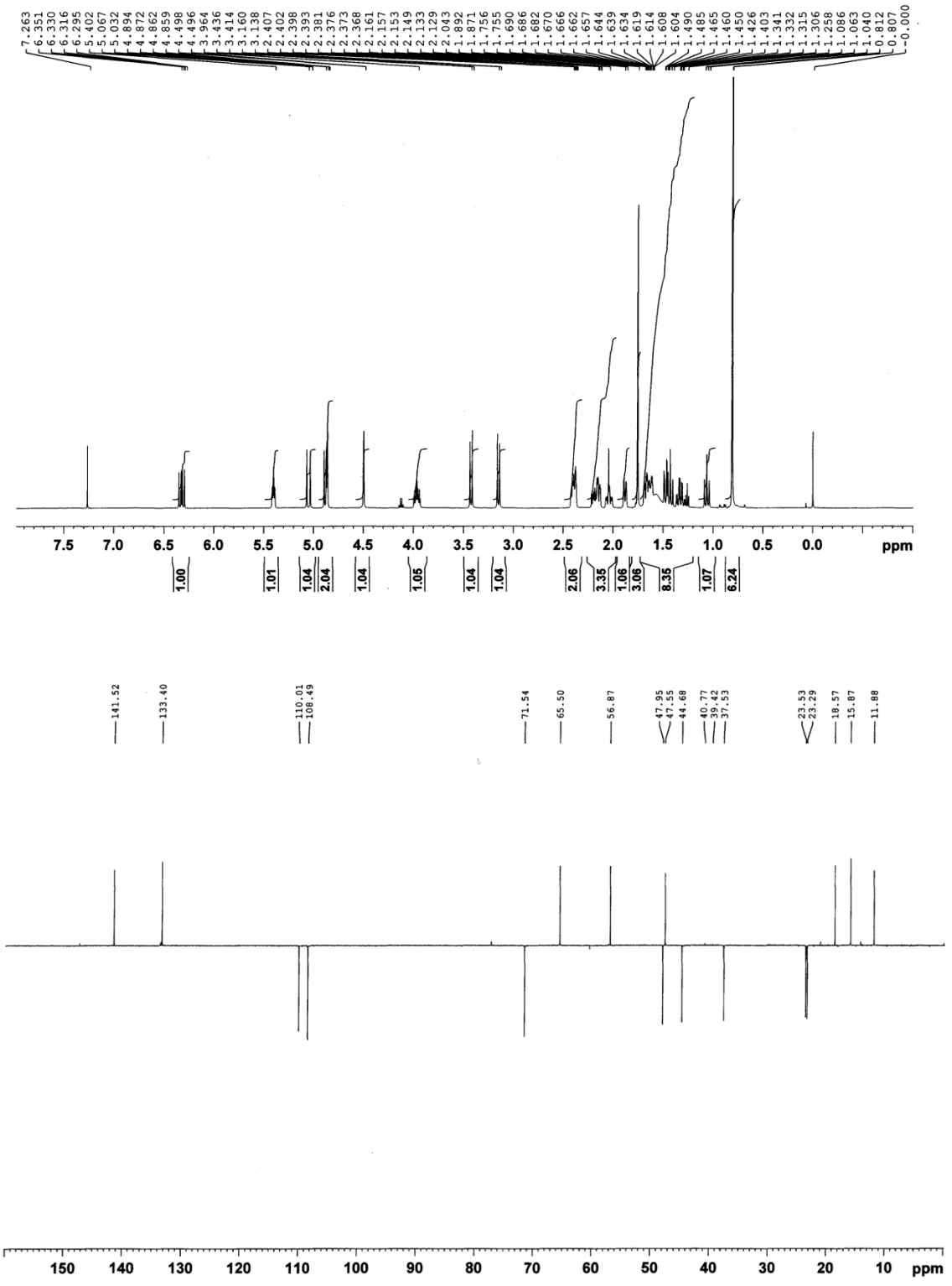

Figure A7.1: **A**. Proton NMR and **B**. DEPT spectra of the labdane diterpenoid, labda-8(17),12E,14-triene-2R,18-diol.

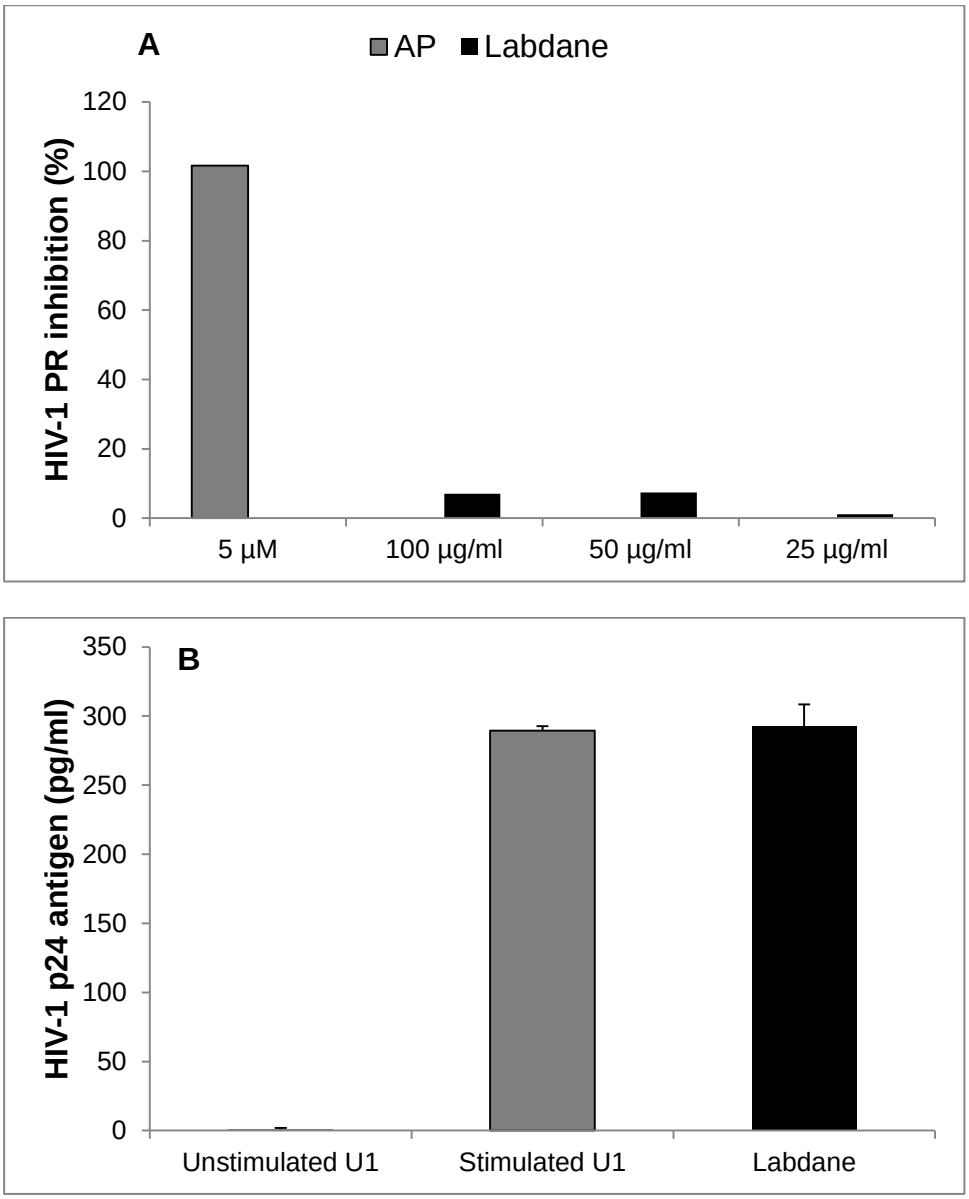

Figure A7.2: HIV-1 inhibitory properties of the labdane diterpenoid compound.

A. The compound had very weak inhibitory properties against HIV-1 PR (<10%). Acetyl pepstatin (AP) was used as a positive control for inhibition. **B**. There was no significant ($p>0.05$) difference in HIV-1 expression between stimulated cells treated with compound (labdane) and untreated stimulated U1 cells.

GLOSSARY

The following is a list of important terminologies used in this thesis, adapted from the following Basson, 2005 and Fonteh, 2011.

Acquired immunodeficiency syndrome (AIDS): AIDS is the advanced stage of HIV infection. It is marked by characteristic opportunistic infections and malignancies as well as a loss of helper T-lymphocytes.

Active site: Asymetric pocket on or near the surface of a macromolecule that promotes chemical catalysis when the appropriate sample or substrate binds.

Antibody: A class of large protein molecules that are produced in response to, and interact with, specific target molecules known as antigens.

Anti-inflammatory cytokine: Cytokine that prevents systemic inflammation.

Antioxidant: A molecule that inhibits the oxidation of other molecules.

Cell index: Unit for measuring cellular impedence or resistance on a real time cell electronic device, xCelligence. The value is directly related to the ability of adherent cells to attach to electrode plates.

Cell line: A population of cultured cells that have been subjected to chemical or viral transformation. Cell lines can be propagated indefinitely.

Chronic infection: An infection in which a virus or other pathogen is always present to some extent in the body. Chronic infections can be a persistent infection or latent infection.

Colorimetric: Is a method of determining the concentration of a chemical element or chemical compound in a solution with the aid of a colour reagent.

Cytokine: Small cell signalling proteins that influence the activity and behaviour of other cells. Some cytokines increase the intensity of an immune response, while others suppress it.

Crude extract: A concentrated form of an herb that is derived when the crude herb is mixed with water, alcohol, or another solvent and distilled or evaporated. Extracts may be either fluid or solid.

Enzyme-linked immunosorbent assay (ELISA): An assay for either quantitating an antibody or antigen by use of an enzyme-linked antibody and a substrate that forms a coloured reaction product. The product is commonly measured by spectrophotometric means.

Human immunodeficiency virus (HIV): A retrovirus that causes AIDS.

Immunomodulatory: An immunological change in which one or more immune system molecules are altered through suppression or stimulation.

Impedence: Resistance

In silico: Virtuall study performed using a computer and specially developed software.

In vitro: Refers to laboratory experiments performed outside the living organism.

Informed consent: The voluntary permission given by a person before participating in an experimental program after being informed of its purpose, procedures, risks and benefits.

Interferon: A group of diverse cytokines produced by infected cells in response to viruses, foreign nucleic acids or antigens. Interferons mainly have antiviral and cell-growth inhibitory activities.

Interleukins: A group cytokines produced by a wide variety of body cells. The function of the immune system depends in a large part on interleukins.

Latent HIV: The ability of HIV to lie dormant within a cell.

Monocyte: A type of white blood cell and are part of the innate immune system. Monocytes play multiple roles in immune function.

Peripheral blood mononuclear cells (PBMCs): The lymphocytes, monocytes and natural killer cells (cells that have a round nucleus) found in circulating blood, as opposed to those in lymphatic organs.

Pro-inflammatory cytokine: A cytokine that promotes systemic inflammation.

Protease: Any enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein.

Replication: The process by which a virus produces new virus particles or virions.

Reverse transcriptase: An enzyme found in retroviruses and few other viruses that enable them to make a DNA copy of their RNA genome.

TZM-bl: Is a genetically modified HeLa cell line that expresses CD4 and CCR5. The TZM-bl cell line is highly sensitive to infection with diverse isolates of HIV-1.

Virus particle: Complete, free-floating viral units as found in body fluids.**)**