

**EVALUATION OF SELECTED NAMIBIAN
ETHNO-MEDICINAL PLANTS FOR ANTI-HIV
PROPERTIES**

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ABSTRACT

Namibian ethno-medicinal plants have not yet been evaluated for their efficacy in inhibiting the activities of Human Immunodeficiency Virus (HIV) reverse transcriptase (RT) and their toxicological effects to mammalian cells have not yet been reported. Hence the aims of this study were: (1) to evaluate selected Namibian plants for their biological activities against RT; (2) to evaluate their toxicity to mammalian cells, and (3) to isolate and characterize the active compounds from selected plant extracts. Colorimetric assays were used to evaluate RT inhibition and cell viability. Absorbance for RT inhibition assay was read at 405 nm and that of cytotoxicity at 492 nm with a plate reader using standard procedures. Non-linear regression sigmoidal curves were used to determine the inhibitory concentration required to reduce the activity of RT by 50% (IC₅₀) and cytotoxicity concentration required to reduce the viability of cell by 50% (CC₅₀) for each sample. Purification of extracts was done with thin layer chromatography (TLC) and structural elucidation was done with Nuclear Magnetic Resonance (NMR). The study found 40 crude extracts (from 15 plants) to have concentration-dependent inhibitory activities against RT. Most of the samples had very low toxicity to Vero cells (with 31 out of 40 extracts having viability above 50% at the highest tested concentration). Variable inhibitory activity on RT were observed with fractions extracted with different solvents. Ethyl Acetate fractions had the best RT inhibitory activities when compared to other solvents. Six compounds were isolated and three of the structures were fully elucidated. D-Pinitol (a carbohydrate) was isolated from *Diospyros mespliformis* leaves. This is the first reported isolation of D-Pinitol from this plant species. A novel compound, 2 α ,3 β ,19 α ,23-Tetrahydroxyolean-1-en-28 β -D-glycopyranoside (a triterpenoid glycoside), was isolated from *Terminalia prunioides* roots, and was obtained in acetylated and non-acetylated forms. This is the first study to isolate and/or elucidate the structures of compounds active against HIV RT from Namibian ethno-medicinal plants.

Keywords: Active compounds; Cytotoxicity; Ethno-medicinal plants; HIV/AIDS; Inhibitory concentration; NMR, Thin Layer Chromatography; Reverse transcriptase

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LIST OF ABBREVIATIONS

3TC:	Lamivudine
ABC:	Abacavir
AIDS:	Acquired Immune Deficiency Syndrome
ALT:	Alanine Aminotransferase
ANC:	Ante Natal Care
ART:	Anti-Retroviral Therapy
ARV(s):	Anti-Retroviral(s)
AZT:	Zidovudine
CC₅₀:	Cytotoxic Concentration
CD4:	Cluster of Differentiation 4
COSY:	Correlated spectroscopy
CSIR:	Council for Scientific and Industrial Research
d4T:	Stavudine
ddC:	Zalcitabine
ddI:	Didanosine
DEPT:	Distortionless enhancement polarization transfer
DMSO:	Dimethylsulfoxide
DNA:	Deoxyribonucleic acid
ELISA:	Enzyme-Linked Immuno-Sorbent Assay
FTC:	Emitricitabine
GRN:	Government of the Republic of Namibia
HAART:	Highly Active Antiretroviral Therapy
HBsAg:	Hepatitis B surface Antigen
HIV:	Human Immunodeficiency Virus
HMQC:	Heteronuclear multiple quantum coherence
HPLC:	High Performance Liquid Chromatography

HRP:	Horseradish Peroxidase
HSQC:	Heteronuclear single quantum coherence
IC₅₀:	Inhibitory Concentration
LPV/r:	Lopinavir + ritonavir
MAWF:	Ministry of Agriculture, Water and Forestry
MET:	Ministry of Environment and Tourism
MoHSS:	Ministry of Health and Social Services
MTA:	Material Transfer Agreement
MTS:	[3-(4,5-dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium
NBRI:	Namibia Botanical Research Institute
NMR:	Nuclear magnetic resonance spectroscopy
NNRTI:	Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI:	Nucleoside Reverse Transcriptase Inhibitor
NVP:	Nevirapine
RNA:	Ribonucleic Acid
RT:	Reverse Transcriptase
THPs:	Traditional Health Practitioners
TLC:	Thin Layer Chromatography
ULN:	Upper limit of normal
VLC:	Vacuum Liquid Chromatography
WHO:	World Health Organization

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DECLARATIONS

I, Marius Hedimbi, hereby declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institute of higher education.

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Date: 20 April 2015

Marius Hedimbi

CHAPTER 1: INTRODUCTION

1.1. Biology and Phylogenetic of HIV

HIV is the cause of the spectrum of disease known as Human Immune Virus/Acquired Immune Deficiency Syndrome (HIV/AIDS). HIV is a retrovirus that primarily infects components of the human immune system such as CD4⁺ T cells, macrophages and dendritic cells. It directly and indirectly destroys CD4⁺ T cells (Alimont, Ball & Fowke, 2003). HIV is a member of the genus Lentivirus, (ICTV, 2002a) part of the family of Retroviridae (ICTV, 2002b). Lentiviruses share many morphological and biological characteristics. Many species of mammals are infected by lentiviruses, which are characteristically responsible for long-duration illnesses with a long incubation period (Lévy, 1993).

Lentiviruses are transmitted as single-stranded, positive-sense, enveloped RNA (ribonucleic acid) viruses. Upon entry into the target cell, the viral RNA genome is converted (reverse transcribed) into double-stranded DNA (deoxyribonucleic acid) by a virally encoded reverse transcriptase that is transported along with the viral genome in the virus particle. The resulting viral DNA is then imported into the cell nucleus and integrated into the cellular DNA by a virally encoded integrase and host co-factors (Smith & Daniel, 2006). Once integrated, the virus may become latent, allowing the virus and its host cell to avoid detection by the immune system (Martínez, 2010). Alternatively, the virus may be transcribed, producing new RNA genomes and viral proteins that are packaged and released from the cell as new virus

particles that begin the replication cycle anew (Martínez, 2010). The general structure of the HIV is shown in **figure 1**.

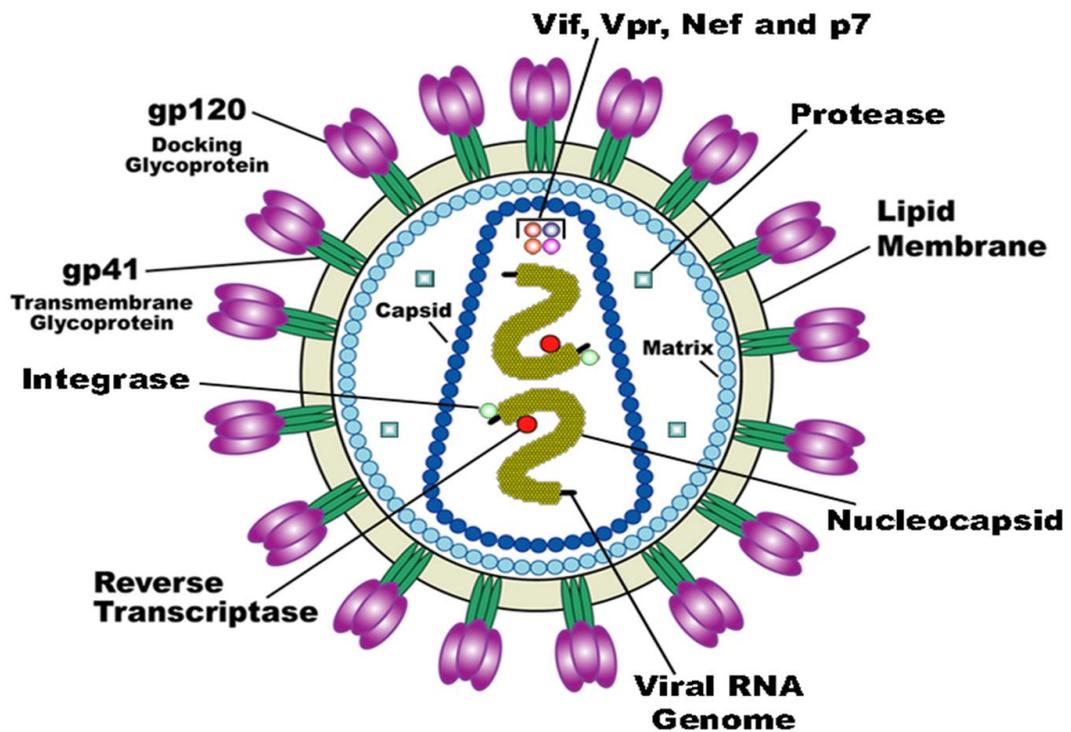


Figure 1: General structure of the Human Immune Virus (HIV) (US National Institute of Health, 2005).

Two types of HIV have been characterized: HIV-1 and HIV-2. The phylogeny of the two types of HIV is shown in **figure 2**. HIV-1 is the virus that was originally discovered. It is more virulent, more infective and is the cause of over 99% of HIV infections globally (Gilbert et al. 2003; Cos, Vanden, Bruyne & Vlietinck 2004). The lower infectivity of HIV-2 as compared with HIV-1 implies that fewer people exposed to HIV-2 will be infected per exposure. Because of its relatively poor

capacity for transmission, HIV-2 is largely confined to West Africa (Reeves & Doms, 2002).

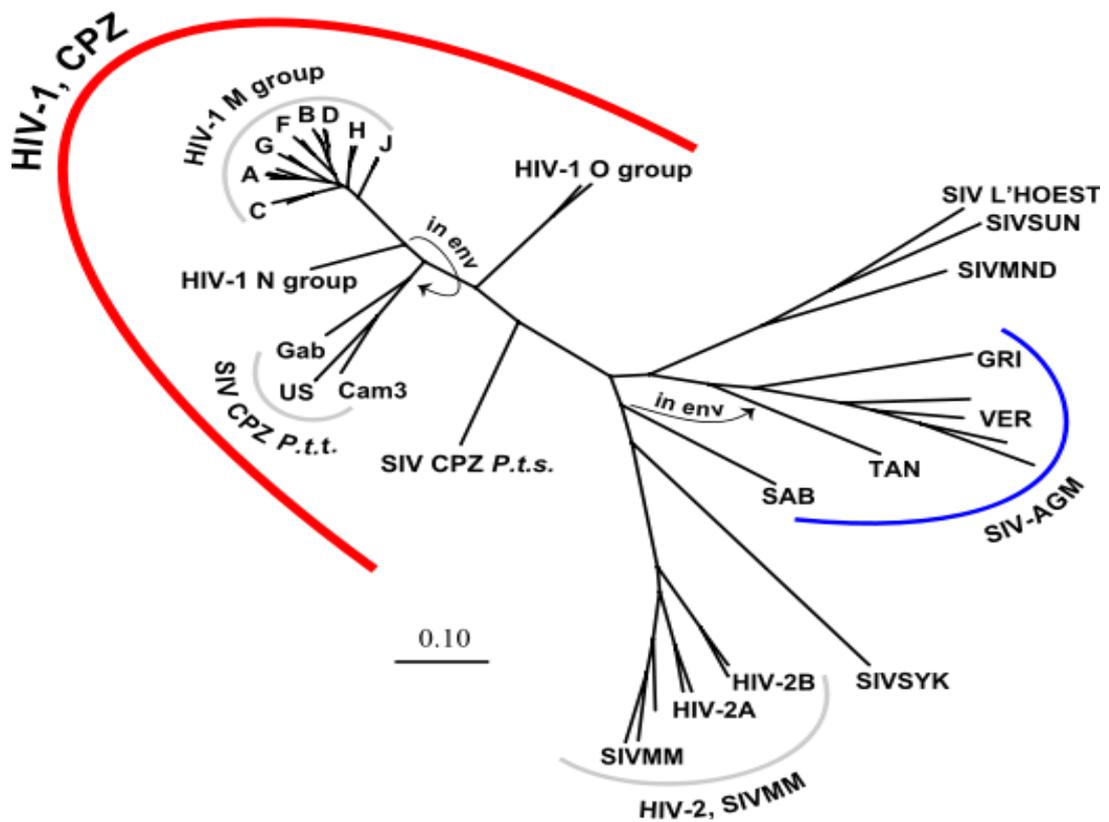


Figure 2: Phylogenetic tree of the Simian immune virus (SIV) and HIV viruses (Los Alamos National Laboratory, 1998).

1.2. Epidemiology of HIV/AIDS

Globally, an estimated 35.3 million people were living with HIV in 2012 (Biadgilign & Aklilu, 2013; UNAIDS, 2013). This is an increase from previous years as more people are receiving the life-saving antiretroviral therapy (ART). There were 2.3 million new HIV infections globally, showing a 33% decline in the number of new

infections from 3.4 million in 2001. At the same time the number of AIDS deaths is also declining with 1.6 million AIDS deaths in 2012, down from 2.3 million in 2005 (UNAIDS, 2013).

Sub-Saharan Africa is the hardest region hit by the HIV pandemic (**Fig. 3**). Furthermore, sub-Saharan Africa accounted for about 70% of all new HIV infections globally in 2012 (**Fig. 4**) (Biadgilign & Aklilu, 2013; UNAIDS, 2013). However, since 2001, the annual number of new HIV infections among adults in sub-Saharan Africa has declined by 34%. This decline is remarkable, considering that New HIV infections have been on the rise in recent years in other parts of the world especially in Eastern Europe and Central Asia. Furthermore, new HIV infections continue to rise in the Middle East and North Africa (UNAIDS, 2013). The annual number of new HIV infections among adults and adolescents decreased by 50% or more in 26 countries between 2001 and 2012. However, other countries are not on track to halve sexual HIV transmission, which underscores the importance of intensifying prevention efforts (UNAIDS, 2013).

Although trends in sexual behavior in high prevalence countries have generally been favorable over the last decade, recent surveys in several countries in sub-Saharan Africa have detected decreases in condom use and/or an increase in the number of sexual partners. Efforts to reduce transmission related to sex work and men who have sex with men remain insufficient, as evidenced by recent trends in prevalence among these groups (UNAIDS, 2013). However, prospects for strengthening prevention

efforts have never been more promising, as a series of highly effective biomedical prevention tools have emerged in recent years to buttress the prevention benefits of behavioral and structural approaches. Specifically momentum accelerated in 2012 towards the scale-up of one such biomedical intervention, voluntary medical male circumcision, which is reported to reduce the rate of transmission (to male) by as much as 60% (UNAIDS, 2013).

1.3. Reduction of HIV infection among women and children

As a result of sustained progress in Anti-retroviral therapy (ART) provision, the world has the potential to reach at least 90% of pregnant women living with HIV with antiretroviral interventions by 2015 (UNAIDS, 2013). Antiretroviral coverage among pregnant women living with HIV reached 62% in 2012, and the number of children newly infected with HIV in 2012 was 35% lower than in 2009. However, achieving the global goal of reducing the number of children newly infected by 2015 will require similar scale-up of other prevention strategies, including primary HIV prevention for women and access to contraception and other family planning services (UNAIDS, 2013). However, substantially greater efforts are needed to link pregnant women and children to HIV treatment and care. Pregnant women living with HIV are less likely than treatment-eligible adults overall to receive antiretroviral therapy, and treatment coverage among children living with HIV in 2012 was less than half the coverage for adults (UNAIDS, 2013).

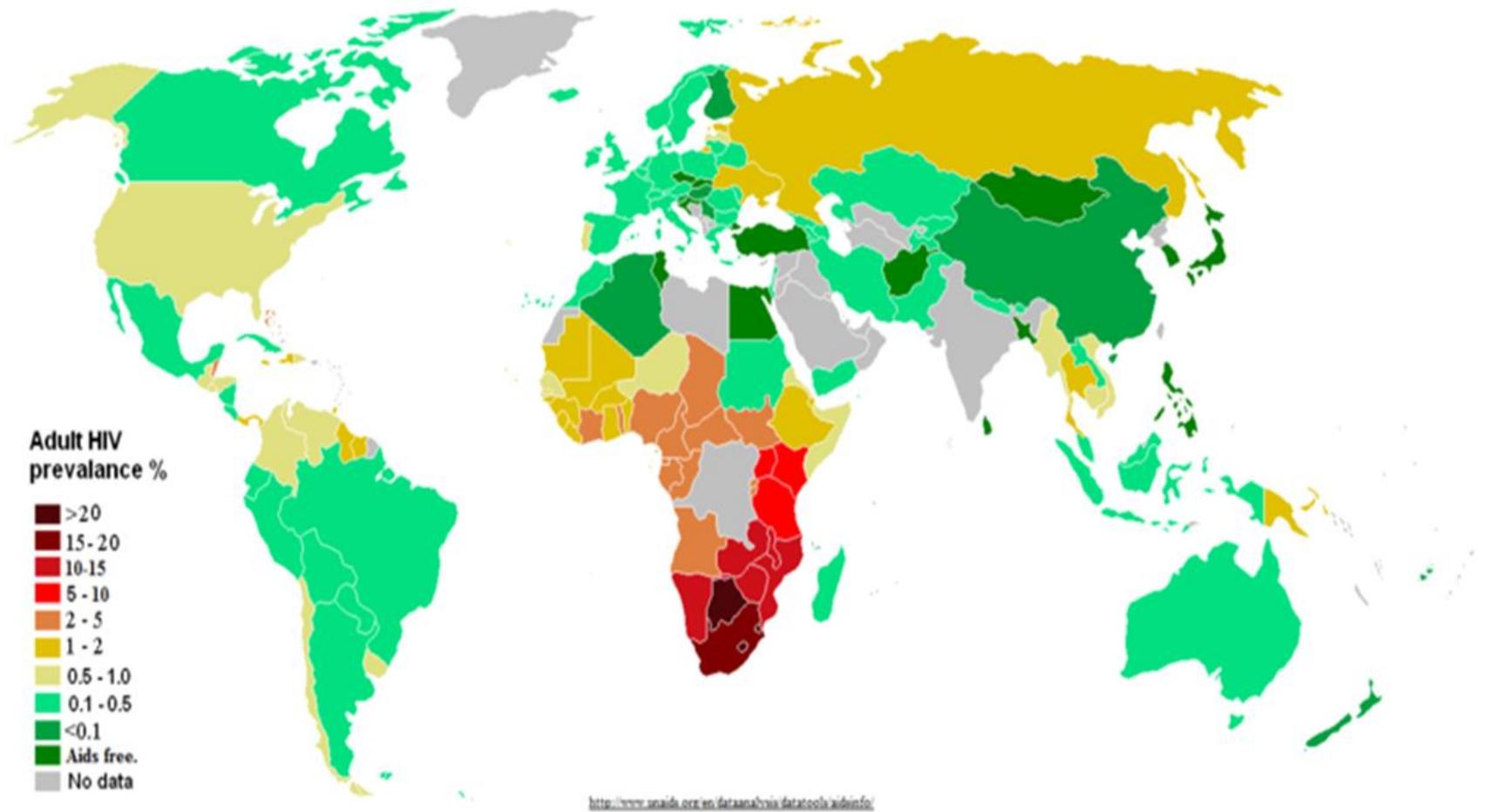


Figure 3: Estimated global prevalence of HIV/AIDS among young adults (15–49) per country (UNAIDS, 2013).

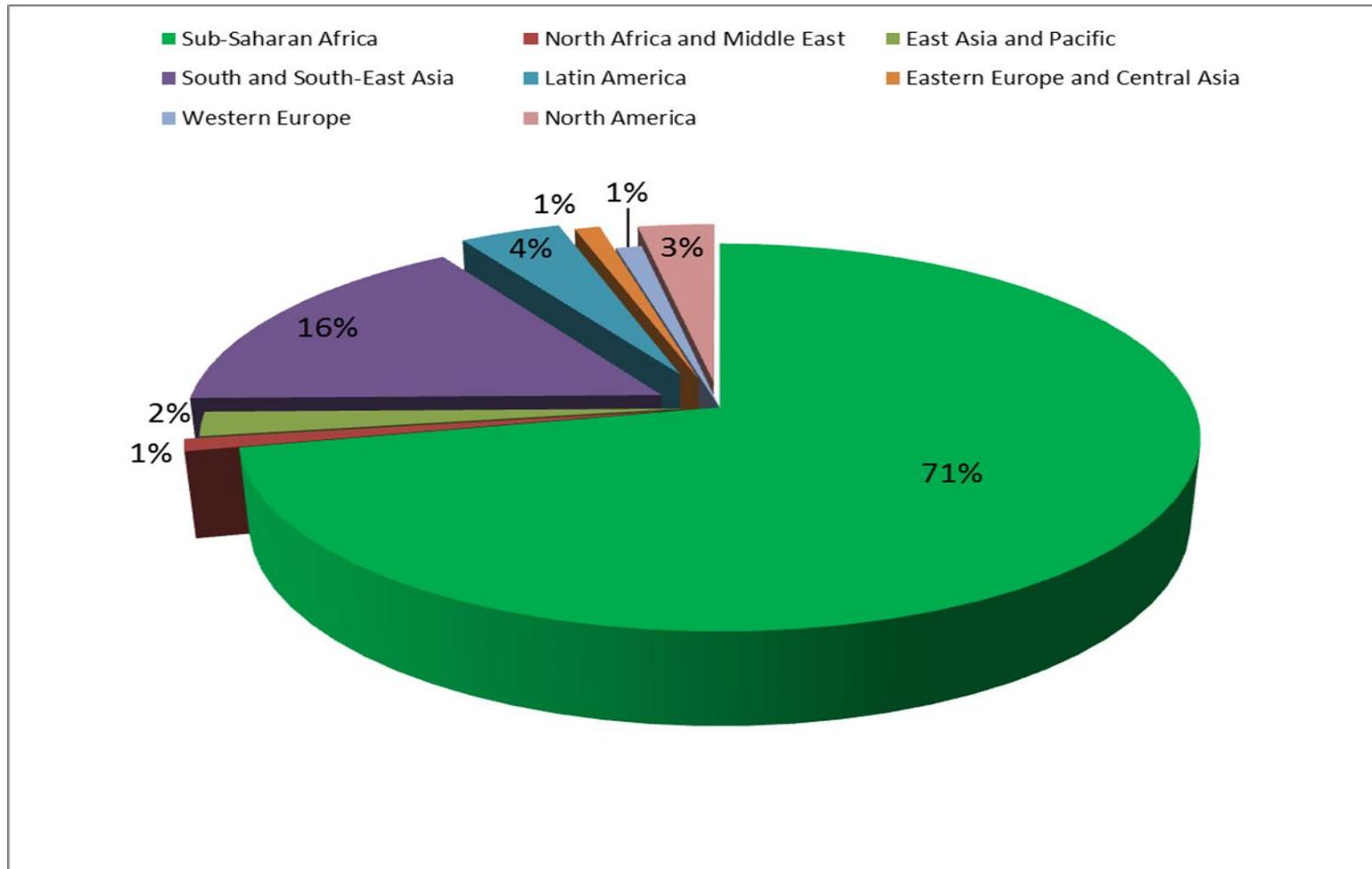


Figure 4: Global percentage of new HIV/AIDS infections by region (UNAIDS, 2013).

1.4. HIV/AIDS prevalence in Namibia

Namibia has an estimated population approaching 2.2 million people and a population growth rate projected at 1.87%. In 2010, nearly 60% of the population was under the age of 24, two-thirds of which was estimated to be under the age of 18 (PEPFAR, 2012). Namibia is classified as an upper-middle income country, with an estimated gross national income per capita of US\$ 6,206 in 2010. However, substantial income inequalities exist. Namibia's Gini coefficient, an international measure of income and wealth distribution, ranks among the highest in the world (UNDP, 2011).

Despite the strength of Namibia's formal economy (upper middle income), the country faces several similar health and development challenges that confront lower-income neighbors in sub-Saharan Africa. Namibia ranks 105 out of 169 countries on the United Nations' Human Development Index, a measure of countries' progress on indicators in Health, Education and Income (UNDP, 2011). HIV/AIDS remains a significant source of morbidity and mortality, and a major drain on national and international resources for health (PEPFAR, 2012). In 2008/09, the national HIV/AIDS response consumed 27.5% of total national expenditures on health (GRN, 2012).

In 2009, the ministry of Health and Social Services in Namibia published a report on the behavioral and social drivers of the epidemic, which vary across the country's

geographic regions (MoHSS, 2010). The study highlighted the following social, cultural and economic risk factors: Multiple and concurrent partnerships, inconsistent condom use, low HIV risk perceptions, varying levels of male circumcision, alcohol misuse, intergenerational and transactional sex, mobility of populations, and emerging pockets of “most-at-risk populations” (MARPS). It was also observed that the HIV/AIDS epidemic is mature, generalized, and driven by heterosexual and mother-to-child transmission (GRN, 2012).

A study by Chinsembu, Kasanda, Shimwooshili-Shaimemanya & Zealand (2011) reported coital debut ranging from 12-14.5 years among high school students in Namibia. The same study also revealed (amongst others) that, thinking one cannot get HIV if they were faithful to one sexual partner (low risk perception), having a boyfriend or girlfriend, alcohol consumption, and cigarette smoking were positively associated with sexual intercourse. All these factors, coupled with low level of condom-use, (could) contribute to high HIV/AIDS prevalence in the country.

1.4.1. Overall prevalence

The first case of AIDS in Namibia was identified in 1986 (GRN, 2008). Since then, HIV infection has spread rapidly throughout the country. Since 1992, Namibia has been monitoring the prevalence of HIV through anonymous unlinked HIV sentinel surveillance of pregnant women attending antenatal care (ANC) at public facilities across the country. From the first sentinel surveillance survey in 1992 among

pregnant women attending ANC when the HIV prevalence was 4.2%, the epidemic rose to 15.4% in 1996 and peaked in 2002 at 22.0% (GRN, 2008), before declining to 19.7% in 2004 (GRN, 2012) and 16.9 in 2014 (GRN, 2014) (**Fig. 5**).

Currently, the estimated number of new HIV infections among adults is decreasing, which could demonstrate the impact of prevention programmes. Approximately 10,685 people were newly infected with HIV during 2013/14 and projected as 9,784 in 2014/15. The estimated number of new infections coupled with high uptake of ART, has resulted in an estimated 250,942 adults and children living with HIV/AIDS in Namibia in 2014 (GRN, 2014). Approximately 250,000 children aged 18 years old or younger were orphans or vulnerable children (OVC) in Namibia (PEPFAR, 2012); around 28% of these OVC (69,000) had been orphaned by HIV/AIDS (MoHSS, 2010; PEPFAR, 2012). In a sentinel survey carried out in 2014, the overall national HIV prevalence among pregnant women stood at 16.9% (GRN, 2014) (**Fig. 5**). The overall HIV prevalence of 16.9% in 2014 (GRN, 2014) represents a slight reduction from 18.8% in 2010 and 18.2% in 2012 (**Fig. 5**).

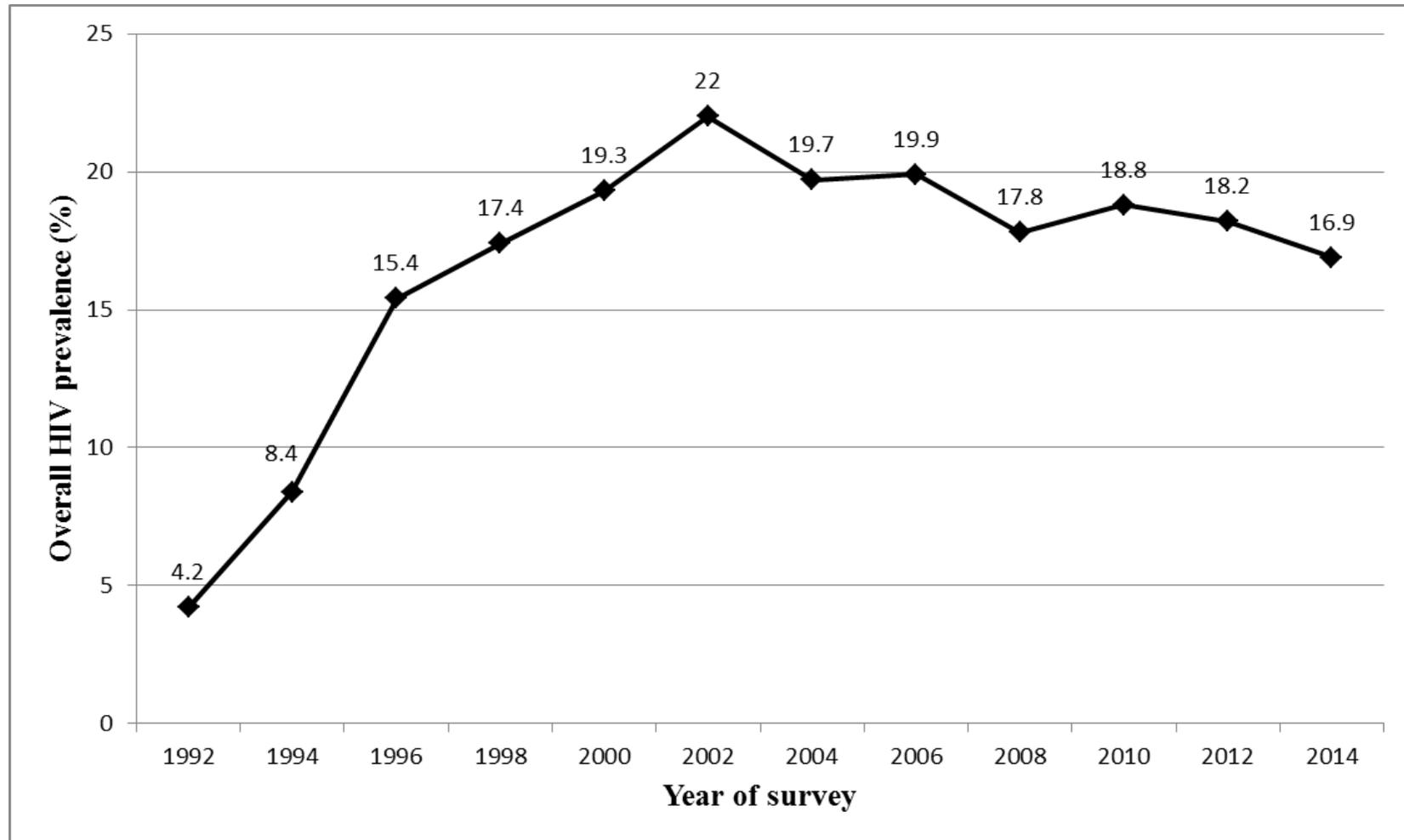


Figure 5: HIV prevalence in Namibia among pregnant women attending ante natal care (GRN, 2014).

1.4.2. Site level prevalence

Site level HIV prevalence varied considerably among sites in 2014. The sites with the highest HIV prevalence among pregnant women attending ANC were Katima Mulio (36.0%) followed by Rundu (24.1%), Engela (22.8%), Onandjokwe (22.4%) and Usakos (21.9%). The sites with the lowest HIV prevalence were Opuwo (3.9%), Windhoek Central Hospital (4.1%), Okakarara (9.0), Rehoboth (9.1%), and Swakopmund (10.5%). The Median HIV prevalence among sentinel sites was 14.1% (GRN, 2014). Site HIV prevalence level in different health districts in Namibia from 2006 to 2014 is given in **figure 6** (GRN, 2014). From 2012 to 2014, a decrease in the HIV prevalence was observed at 23 sites (out of 35 sites = 66%). An increase in the HIV prevalence between 2012 and 2014 was also observed at some sites of which the greatest increases observed at Usakos (12.2% to 21.9%), Katutura state hospital (14.4% to 19.6%), Okahao (16.3% to 20.6%) and Gobabis (9.9% to 12.7%) (GRN, 2014). This shows that, while there is a variable HIV prevalence in the country, prevalence remains high and various means of interventions (such as use of ethno-medicines) continues to be used in alleviating the burden of HIV/AIDS.

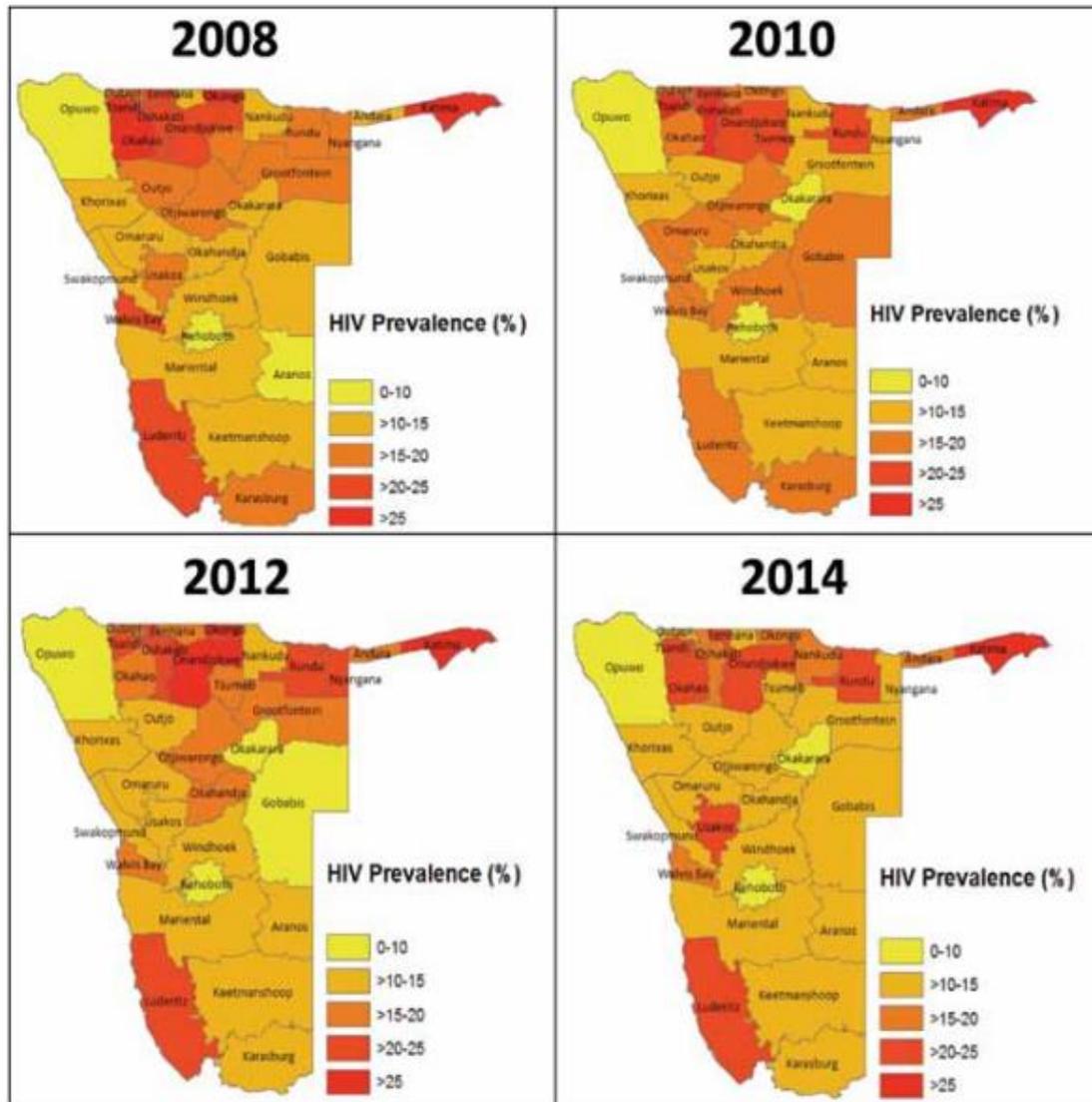


Figure 6: Site HIV prevalence level in different health districts in Namibia from 2006 to 2012 (GRN, 2014).

1.4.3. Prevalence by age group

By age group, HIV prevalence was observed to be highest among women aged 40-44 years (30.6%) and women aged 35-39 years (30.3%). HIV prevalence was lowest among women aged 15-19 years (5.8%) and women aged 20-24 years (9.8%) (**Table 1**). In 2014, the lowest HIV prevalence among women aged 15-24 years was observed in Opuwo (0.0%) followed by Omaruru (1.3%) while the highest HIV prevalence among women aged 15-24 years was observed in Katima Mulilo (24.3%) and Engela (14.3%). In 13 (37%) out of 35 sites, more than one quarter of the women within the older age group (25-49 years) were HIV positive (GRN, 2014). Since 2006, the overall HIV prevalence among the older age group (25-49 years) appears to be stabilizing while the overall HIV prevalence in the younger age group (15-24 years) appears to be decreasing. The overall HIV prevalence for the youth (15-24 years) was 8.3% in 2014 (**Table 1**) which shows a decline from 15.2% in 2004 to 14.2% in 2006, 10.6% in 2008 and 10.3% in 2010 and 8.9% in 2012 (GRN, 2012).

Table 1: HIV prevalence (%) in Namibia by age group in 2014 (GRN, 2014).

Age group	Total tested	Positive	Prevalence (%)
Young versus old			
15-24	3,560	297	8.3
25-49	4,167	1006	24.1
All age groups			
15-19	1,335	78	5.8
20-24	2,225	219	9.8
25-29	1,733	299	17.3
30-34	1,277	357	28.0
35-39	823	249	30.3
40-44	307	94	30.6
45-49	27	7	25.9
Overall	7,727	1,303	16.9

1.4.4. Prevalence in rural and urban areas

Overall, the HIV prevalence among women residing in rural areas (16.3%) is similar to HIV prevalence among women residing in urban areas (17.4%). The HIV prevalence among women residing in urban or rural areas is almost similar within each age group, except for women age 40-44 years, among whom there is a higher HIV prevalence in rural areas (33.2%) compared to urban areas (26.7%) while among women age 45-49 years is higher in urban areas (33.3%) compared to rural areas (20.0%) (GRN, 2014).

The 2014 National HIV sentinel survey collected data on the antiretroviral therapy (ART) status of women who participated in the surveillance survey. Overall, 49.1% of all women who tested HIV positive during the survey were already on ART. The percentage of HIV positive women who were already on ART was highest in the older age groups (73.3% among HIV positive women age 40-49 years and 66.7% among HIV positive women age 35-39 years) and lowest in the youngest age group (16.7% among women age 15-19 years) (GRN, 2014).

1.5. Problem statement

The shortcomings of conventional ART continue to open new interests in the use of ethno-medicinal plants and other natural products for the management of HIV/AIDS. Thus, more effective and less toxic anti-HIV agents are still needed. Alternative approaches, including herbal therapies, particularly anti-infective or immune-modulating medicinal herbs and the structural modification of lead compound, and

phytochemical compounds from plants may provide an alternative remedy. Despite various reports on the use of Namibian ethno-medicinal plants to treat ailments associated with HIV/AIDS (Chinsembu, 2009; Chinsembu and Hedimbi, 2010; Chinsembu, Hedimbi and Mukaru, 2011; Hedimbi and Chinsembu, 2012), there is no report in the literature to evaluate their biological efficacy on HIV.

1.6. Aims of the study

This study hence aimed to evaluate several ethno-medicinal plants from Namibia for their inhibitory activities on HIV-1 reverse transcriptase (RT) as well as their toxicity to mammalian (Vero) cells. The study also aimed to isolate and characterize the active compounds from selected active plant extracts.

1.7. Specific objectives

1. To evaluate *in vitro* medicinal properties of Namibian plants extracts against HIV-1 reverse transcriptase.
2. To determine the cytotoxicity of these plant extracts to mammalian cells using Vero cell lines.
3. To elucidate structures of isolated compounds and test their *in vitro* inhibitory activity against RT.

CHAPTER 2: LITERATURE REVIEW

2.1. General background

African traditional medicine and traditional healing has been in existence since well before the advent of Western medicine. This includes plant, mineral and animal remedies, and spiritual therapies including participation in ritual ceremonies (Mandizadza & Chavunduka, 2013). Globally, there is an observed increase in popularity and use of traditional medicine and ‘alternative’ therapies, such that collaboration between traditional and conventional medicine is inevitable and vital.

The estimates by WHO (2010) that 80 per cent of people in developing countries and the African region utilize traditional medicine for their primary health care, has provided a rallying point and rationale for recognition of Traditional Health Practitioners (THPs) and traditional medicine in national health policy (Mandizadza & Chavunduka, 2013). The omnipresence of THPs in most developing countries, the exorbitant health fees which are beyond the reach of many, and the shortage of health personnel coupled with the cultural acceptability of traditional medicine make a strong case for acceptance of traditional medicine by many people in response against HIV/AIDS (Mandizadza & Chavunduka, 2013). Hence, there is a strong need to scientifically evaluate the efficacy of various plants used in HIV/AIDS therapies by various communities.

2.2. Management of HIV/AIDS infections

It was estimated that 2 million deaths due to AIDS-related illnesses occurred worldwide in 2008; this was about 10% lower than in 2004 (UNAIDS, 2013). The decline in new infections and AIDS-deaths may be attributed to the scale-up of ART program, especially in the developing world. As of December 2008, approximately 4 million people in low- and middle-income countries were on ART, representing a 10-fold increase over five years (UNAIDS, 2013). In eastern and southern Africa, ART coverage rose from 7% in 2003 to 48% in 2008 (UNAIDS, 2013).

Irrespective of these encouraging trends, a number of challenges/problems have been encountered following the introduction of ART and in relation to its expansion (Biadgilign & Aklilu, 2013). The need to maintain/achieve equitable access, failure to reach those who haven't known their sero-status, and difficulty of convincing people living with HIV and AIDS, who are reluctant to receive the therapy until they see AIDS-related illness, are some of the challenges observed along the expansion of ART (Biadgilign & Aklilu, 2013). Poor adherence (often leading to drug resistance), complete discontinuation of the treatment, some long-term and short-term side-effects resulting from ART management, are other key problems occurring following ARV (antiretroviral) therapy.

2.3. HIV life cycle as a target for therapeutic interventions

The replicative cycle of HIV is comprised of ten steps that may be adequate targets for chemotherapeutical intervention. Most of the substances identified as anti-HIV agents interfere with one of these steps of HIV replicative cycle (Kostova, Raleva, Genova & Argirova, 2006). These steps are shown in **figure 7**: (1) viral adsorption to the cell membrane, (2) fusion between the viral envelope and the cell membrane, (3) uncoating of the viral nucleocapsid, (4) reverse transcription of the viral RNA to proviral DNA, (5) integration of the proviral DNA into the cellular genome, (6) DNA replication, (7) transcription of the proviral DNA to RNA, (8) translation of the viral precursor mRNA to mature mRNA, (9) maturation of the viral precursor proteins by proteolysis, myristoylation, and glycosylation, and (10) budding, virion assembly, and release (Kostova et al. 2006; Chen, Hoy & Lewin, 2007).

Step 4 is a key step in the replicative cycle of retroviruses, which makes it distinct from the replicative cycle of other viruses, is the reverse transcription catalyzed by reverse transcriptase (Kostova et al. 2006).. Another target for therapeutic intervention is step 9, particularly the proteolysis of precursor proteins by HIV protease. The majority of chemotherapeutic strategies have, therefore, focused on the development of retroviral enzyme inhibitors (Chen, Hoy & Lewin, 2007).

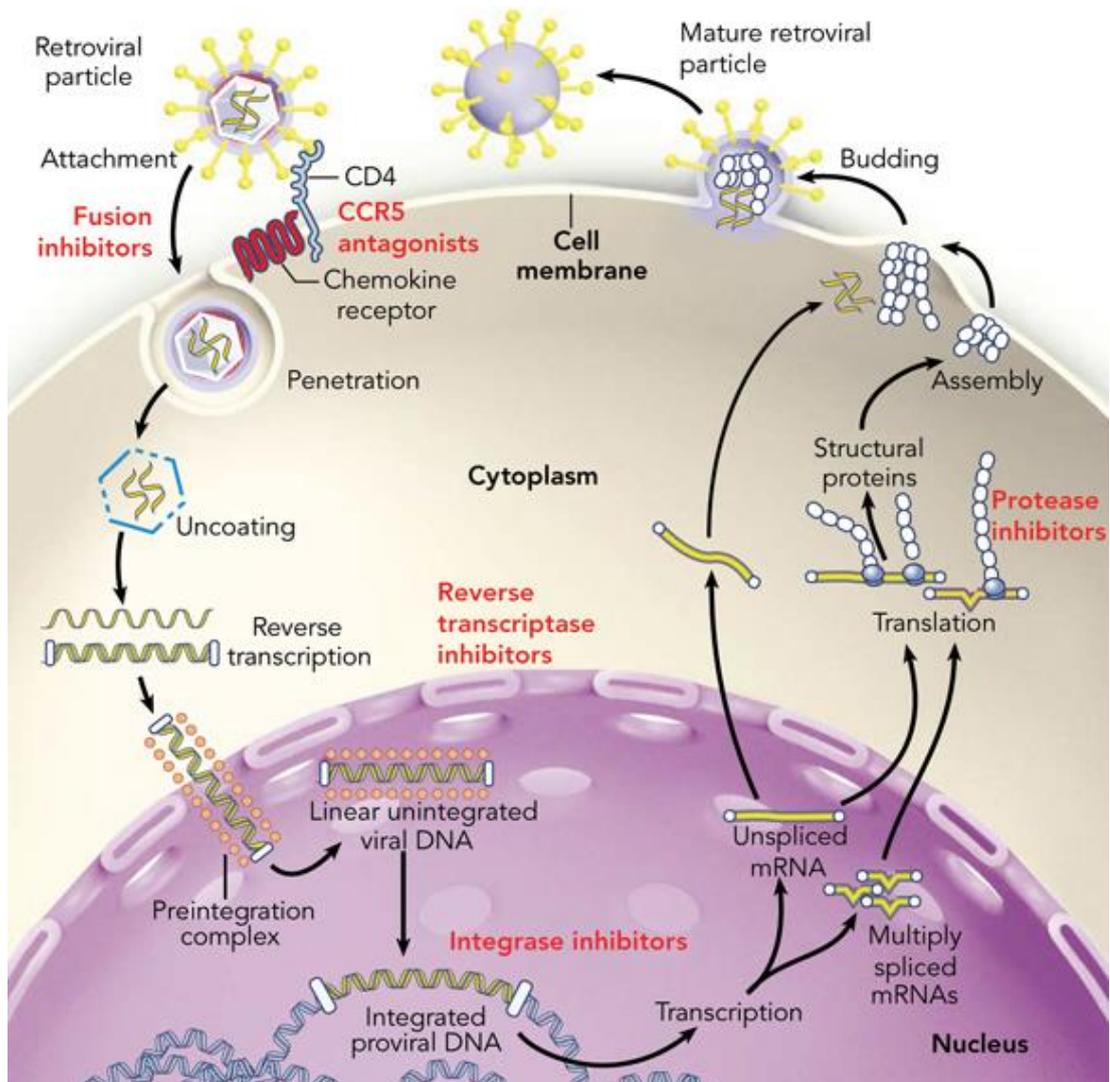


Figure 7: General life cycle of HIV (Chen et al. 2007).

2.4. Role of reverse transcriptase in HIV replication

RT has two enzymatic activities; a DNA polymerase that can copy either a DNA or an RNA template, and an RNase H that cleaves RNA. The two enzymatic functions of RT, polymerase and RNase H, convert the RNA into a double-stranded linear DNA (Sarafianos, Marchand, Himmel, Parniak, Hughes & Arnold, 2009). This conversion takes place in the cytoplasm of the infected cell. After DNA synthesis has been completed, the resulting linear double-stranded viral DNA is translocated to the nucleus where the viral DNA is inserted into the host genome by integrase. This inserted DNA copy, called a provirus, is the source of both viral genomic and viral messenger RNAs, which are generated by the host DNA-dependent RNA polymerase (Sarafianos et al. 2009).

Although other viral proteins help RT carry out the reactions that convert the viral RNA into DNA, RT contains all the necessary enzymatic activities for the conversion. Like many other DNA polymerases, RT requires both a primer and a template. The end product of the reverse transcription process is the substrate for integrase (Sarafianos et al. 2009).

2.5. Classes of medications used in management of HIV/AIDS

Several medications that target one or more steps in the development of HIV are in use. It is important to note that all these medications were obtained by chemical synthesis, none originated from natural sources.

- a) **Nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs)** include medications such as AZT (3'-Azidothymidine): Zidovudine and Retrovir; DDI (2',3'-dideoxyinosine): Didanosine and Videx; DDC (2',3'-didexoycytidine): Zalcitabine and Hivid; D4T (Didehydrothymidine): Stavudine and Zerit; 3TC (2'-deoxy-3'-thiacytidine): Lamivudine and Epivir; ABC (Abacavir succinate): Ziagen; and Tenofovir (Tenofovir disoproxil fumarate): Viread (De Clercq, 2000; Cihlar & Ray, 2010).
- b) **Non-nucleoside reverse transcriptase inhibitors (NNRTIs)** are commonly used in combination with NRTIs to help keep the virus from multiplying (De Clercq, 2000; Cihlar & Ray, 2010). Examples of NNRTIs are Efavirenz (Sustiva), NVP (Nevirapine): Viramune; DLV (Delavirdine): Rescriptor; and Etravirine (Intelence) (De Clercq, 2000; Cihlar & Ray, 2010). Rilpivirine (Edurant), the newest member of this class of drugs, was approved 2011 (Cohen et al. 2011; Molina et al. 2011; O'Neal, 2011).
- c) Two complete HIV treatment regimens that is a **combination of two NRTIs and one NNRTI** in one pill taken once a day are available Atripla (Cihlar & Ray, 2010) and Complera (O'Neal, 2011). Atripla is a combination of Efavirenz, emtricitabine, and Tenofovir. Atripla was approved for use in 2006 (Cihlar & Ray, 2010). Complera is a combination of Rilpivirine, Emtricitabine, and Tenofovir. This combination pill was approved in 2011 as another first-line treatment for HIV infection in patients who need to start therapy (O'Neal, 2011).
- d) **Protease inhibitors (PIs)** are medications that interrupt virus replication at a later step in its life cycle, preventing cells from producing new viruses

(Mehellou & De Clercq, 2010). These include Ritonavir (Norvir), a Lopinavir and Ritonavir combination (Kaletra), SQC (Saquinavir mesylate): Invirase; Indinavir sulphate (Crixivan), Amprenavir (Agenerase), Fosamprenavir (Lexiva), Darunavir (Prezista), Atazanavir (Reyataz), Tipranavir (Aptivus), and NFV (Nelfinavir mesylate): Viracept (De Clercq, 2000; Mehellou & De Clercq, 2010). Using PIs with NRTIs reduces the chances that the virus will become resistant to medications (De Clercq, 2000; Mehellou & De Clercq, 2010).

- e) **Fusion and entry inhibitors** are newer agents that keep HIV from entering human cells. Fusion inhibitors block the virus from being able to merge with the host cell (i.e. CD4 cell) after binding. EFV (Enfuvirtide): Fuzeon) is the only drug available in this group. It is given in injectable form like insulin. Another drug called Maraviroc (Selzentry), a CCR5 entry inhibitor, binds to a protein on the surface of the human cell and can be given by mouth. Both drugs are used in combination with other anti-HIV drugs (Mehellou & De Clercq, 2010).
- f) **Integrase inhibitors** stop HIV genes from becoming incorporated into the human cell's DNA. This is a newer class of drugs recently approved to help treat those who have developed resistance to the other medications. Raltegravir (Isentress) was the first drug in this class approved by the FDA in 2007 (Mehellou & De Clercq, 2010).

ARV drugs stop viral replication and delay the development of AIDS. However, they also have side effects that can be severe (Sharma, 2011). With long-term application, these regimens lead to the onset of marked side effects by developing adverse reactions, resulting in many metabolic disorders in the bodies of such patients (Stenzel & Carpenter, 2000). This phenomenon, in turn, exerts a negative impact on adherence (Andrews & Friedland, 2000), which has been considered one of the major causes of the emergence of drug-resistant variants of the virions causing AIDS (Sharma, 2011). It is reported that HIV-1 selects some drug-resistant mutants due to anti-HIV-1 drug pressure (Kartsonis & D'Aquila, 2000; Richman, 2001), and discontinuation of the ARV treatment due to the side effects of anti-HIV-1 chemotherapeutics further aggravates the situation (Sharma, 2011).

2.6. Interventions targeting HIV reverse transcriptase

Because of its central role in the life-cycle of HIV, RT has been a key target for the development of antiviral therapies. Nearly half of the anti-AIDS drugs target the polymerase activity of RT (Sarafianos et al. 2009). The approved anti-RT drugs belong to one of two broad classes: nucleoside RT inhibitors (NRTIs) and non-nucleoside RT inhibitors (NNRTIs). Most standard three-drug regimens involve two NRTIs combined with either a PR inhibitor or an NNRTI; other combination therapies, some of which involve the entry/fusion inhibitors, are used to treat some of the patients who have failed the standard therapies (Sarafianos et al. 2009).

For NRTIs to be effective against HIV, they must be taken up by the host cell and then phosphorylated by cellular enzymes to convert them to their active form, the NRTI triphosphates. The efficiency of this conversion to the active metabolite and the stability of NRTIs (and their triphosphates) in the presence of catabolic enzymes are important considerations in antiviral therapies, because these factors help determine the concentration of the inhibitor in the blood stream that is required for the NRTI to be effective (Sarafianos et al. 2009). NNRTIs are non-competitive inhibitors and do not interfere directly with the binding of either the dNTP or the nucleic acid substrates of RT. The structural data suggest that NNRTI-binding distorts the position of the primer grip, thus affecting the alignment of the primer terminus with the polymerase active site, which could affect the chemical step of viral DNA synthesis (Sarafianos et al. 2009).

2.7. Recommended HAART regimens in Namibia

Namibia has one of the highest levels of treatment coverage in sub-Saharan Africa. In 2009, when the eligibility threshold for ART enrolment was at CD4 count of less than 200 cells/ μ l, an estimated 88% of Namibians in need of treatment were receiving it (MoHSS, 2010; PEPFAR, 2012). In 2011, Namibia changed the CD4 ART enrolment eligibility threshold to less than 350 cells/ μ l. Because of this sudden increase in the denominator of patients eligible for ART, treatment coverage was estimated to have declined from its peak near 90% to approximately 76% (MoHSS, 2010; PEPFAR, 2012).

Since 2003, the Ministry of Health and Social Services (MoHSS) has been implementing an antiretroviral (ARV) programme in Namibia's public health facilities. Treatment with Highly Active Antiretroviral Therapy (HAART) started with 6 pilot hospitals in 2003. This was rapidly rolled out to involve all 34 state hospitals in Namibia. Since then, ART services have been rolled out to the entire country, making these services accessible to those in need (MoHSS, 2010). As of 2010, more than 80% of patients in need of ART are receiving ART, 64% of patients on ART are women and 16% of patients on ART are children (MoHSS, 2010). Furthermore, as of 2012, over 105 000 HIV-positive persons were receiving ARVs from public health facilities (GRN, 2012).

As of 2014, Namibia rolled out the new ART guidelines that resulted in changing the CD4 threshold for ART eligibility for adults from 350 to 500 cells/ μ l (GRN, 2014). In addition, all pregnant women, all children under 15 years old, all hepatitis B and TB/HIV co-infected patients, and HIV-positive persons whose partners are HIV-negative are eligible for ART irrespective of CD4 count. Furthermore, the Ministry of Health and Social Services (MoHSS) has adopted task shifting by allowing trained nurses to initiate ART. In addition, the MoHSS has recruited and trained Health Extension Workers (HEW) who serve as the link between health facilities and communities. They also started absorbing health professionals that were previously paid for by development partners/aid agencies (GRN, 2014). All of these factors are expected to contribute to an increased proportion of HIV positive people receiving ART.

Recommended HAART regimens in Namibia consist of a combination of 2 NRTIs plus an NNRTI or PI. Considerations in the selection of ARV regimens include potency, side-effect profile, the potential for maintenance of future treatment options, convenience of the regimen (pill burden, frequency of intake, absorption), coexistent conditions (e.g., TB and hepatitis B), pregnancy or the risk thereof, the use of other medications and potential medication interactions, costs, and required conditions for storage (MoHSS, 2010).

The first line regimen for HAART in adults in Namibia is tenofovir (TDF) 300 mg + (lamivudine) 3TC 300 mg daily plus Nevirapine (NVP) 200 mg twice daily. Due to metabolism issues and increased risks of hypersensitivity reactions, NVP treatment is always initiated as once daily therapy for the first 14 days, and then it is increased to twice daily. NVP is not to be given to women with a CD4 count of >350 or men with a CD4 count of >400 cells/mm³. The recommended first line regimen in Namibia has changed from zidovudine/lamivudine/nevirapine (AZT/3TC/NVP), which has become an alternate first line regimen. This change was made in keeping with the 2009 WHO Guidelines which were based on the growing body of evidence that AZT based regimens are associated with anemia and limitation of future treatment options compared with TDF based regimens. Stable patients on AZT/3TC/NVP, and who have no adverse effects, are continued on this regimen. Patients who do not tolerate TDF or AZT, are started on Stavudine (D4T) (MoHSS, 2010). **Table 2** gives information on recommended HAART regimens in Namibia.

Table 2: Recommended HAART regimens in Namibia (MoHSS, 2010).

First Line ART regimens		
Target population	2010 ART guidelines	Remarks
HIV+ARV-naïve adults	TDF/3TC/NVP (preferred first line)	AZT/3TC/NVP (Alternative to preferred first line). Use efavirenz (EFV) if CD4 >350.
HIV+ARV-naïve pregnant women	TDF/3TC/NVP	EFV included as a NNRTI option where CD4 >350 after first trimester. Benefits of NVP outweigh risks where CD4 count is 250 to 350 cells.
HIV/TB co-infection	TDF/3TC/EFV	ART should be initiated as soon as possible in all HIV/TB co-infected patients with active TB (as soon as TB Rx is tolerated and within 8 weeks of commencement).
HIV/HBV co-infection	TDF/3TC/NVP if alanine aminotransferase (ALT) <5 TDF/3TC/EFV if ALT >5	HBsAg positive clients with CD4>350 whose ALT is >2x ULN (upper limit of normal) ULN or ALT<2x ULN but with HBeAg+ are eligible for Rx regardless of WHO clinical stage. NNRTI regimens that contain both TDF/3TC are required.

Second -Line ART regimens

HIV+ adults	AZT/TDF/3TC/LPV/r (lopinavir + ritonavir)	Where standard first line regimens were used
HIV+ pregnant women	As above (adults)	
HIV/TB co-infection	As above (adults)	Adjust dose of RTV: i.e., LPV/r 400mg/400mg
HIV/HBV co-infection	AZT/TDF/3TC/LPV/r	

2.8. Toxicities due to antiretrovirals

After prolonged application, the different anti-HIV drugs have been reported to induce varying forms of toxicity, such as peripheral neuropathy, myopathy, and pancreatitis. These toxic symptoms have been reported to emerge due to treatment of the patients with some of the nucleoside analogs such as zidovudine (AZT) (Wilde & Langtry, 1993), didanosine (ddI) (Faulds & Brogden, 1992), zalcitabine (ddC) (Whittington & Brogden, 1992; De Clercq, 2009), and stavudine (d4T) (Cihlar & Ray, 2010). However, the intensity of symptoms and severity of toxicity vary with each nucleoside analog, e.g. AZT, ddI, and ddC, exert cytotoxic effects on human muscle cells, and trigger functional alterations in mitochondria (Feng, Johnson, Johnsoni & Anderson, 2001). In addition, AZT has also been reported to cause myopathy in HIV-1-infected individuals (Benbrick, Chariot & Bonavaud, 1997).

Antiretroviral toxicities can occur in a wide range from mild and self-limiting (AZT-associated headaches) to long-term and disabling (D4T-associated peripheral neuropathy) and even to potentially fatal (NRTI-associated lactic acidosis). Some toxicities are class related; others are related to one particular ARV. The frequency and severity of class-related toxicities also vary among the medicines within the same class (MoHSS, 2010). Some foods can enhance or inhibit the absorption, metabolism, distribution, and excretion of medication and, therefore, affect the medication's efficacy and the overall health of the individual. For example, some foods decrease the absorption of ddI. Medications such as PIs are absorbed better with fatty meals (MoHSS, 2010).

ABC and ddI can be considered as backup options in case of AZT or TDF toxicity or contraindication. However this regimen is poorly tolerated and some patients may have to complete TB treatment before restarting ARV treatment due to unacceptable gastrointestinal side effects (MoHSS, 2010). Both nevirapine and efavirenz (EFV) are included in first line regimen options for HAART and they have similar potency. The most common toxicities experienced with nevirapine are rash and liver toxicity. The most common toxicities experienced with efavirenz are central nervous system/psychiatric effects and rash. These toxicities usually occur during the first few weeks of treatment (MoHSS, 2010). Nevirapine hepato-toxicity is more common in women who initiate nevirapine with CD4 counts > 350 cells/ml and in men with CD4 counts > 400 cells/ml. Consequently, nevirapine should not be used for initial therapy in individuals with CD4 counts above 350 cells/ml. It can however be safely continued in patients whose CD4 counts have risen above these levels during the course of treatment (MoHSS, 2010).

ARV-associated toxicities are summarized in **Table 3**. The limitations of ARV programmes prompt the need to investigate some new, safer anti-HIV-1 molecules that are cost effective (Keiser, Nassar & Kvanli, 2001) and with few or no toxic effects in patients (Sharma, 2011). If toxicity is observed in a patient due to first line ARVs, they have to change to another drug and these are summarized in **Table 4**.

Table 3: Toxicities associated with ARV (Pham & Flexner, 2005).

Potentially	Fatal Associated drugs
Pancreatitis	Didanosine (ddl), Stavudine (D4T), Lamivudine (3TC)
Hypersensitivity reactions	Abacavir (ABC), Nevirapine (NVP)
Toxic epidermal necrolysis (TEN) or Steven's Johnsons Syndrome	Nevirapine (NVP)
Lactic Acidosis	All NRTIs
Hepatotoxicity	NNRTIs, Lopinavir (LPV), Ritonavir (RTV)
Haematological toxicity	Anaemia, Zidovudine (AZT)
leucocytopenia	
Disabling	Fatal Associated drugs
Peripheral neuropathy	Didasone (ddl), Stavudine (D4T), Lamivudine (3TC)
Osteonecrosis/osteoporosis	Origin uncertain (PIs?)
Long Term	Fatal Associated drugs
Lipoatrophy	NRTIs
Fat accumulation	PIs
Hyperlipidaemia	Efavirenz (EFV), PIs
Lactic Acidosis	All NRTIs
Insulin Resistance	NRTIs, PIs

Table 4: Changing first line ARV in case of toxicity (WHO, 2006).

Medicine	Toxicity	ARV Substitution
TDF	TDF related Renal insufficiency	Switch from TDF to AZT
AZT	AZT related severe haematological toxicity	Switch from AZT to TDF
NVP	NVP related severe hepatotoxicity and NVP related moderate rash (but not life threatening) NVP related life threatening rash (Stevens Johnsons Syndrome)	Switch from NVP to EFV Switch from NVP to PI
D4T	D4T related neuropathy or pancreatitis or D4T related lipoatrophy/lipohypertrophy	Switch from D4T to TDF

2.9. Role of RT mutations in development of resistance

Drug resistance remains a central challenge in HIV therapies. HIV-1 replication is error prone (Coffin, Hughes & Varmus, 1997) and the errors that arise during the viral life-cycle, together with the rapid replication of the virus in patients, allow the virus to escape the host immune system and develop resistance to all of the available drugs (Coffin et al. 1997). The virus evolves sufficiently rapidly that, unless the therapy is well-designed, resistance will develop in all treated patients (Sarafianos et al. 2009). Two basic types of NRTI-resistance mechanisms are known for HIV-1 RT: (1) one resistance mechanism (exclusion) involves enhanced discrimination at the time the NRTI-TP is incorporated, and (2) The second mechanism involves the selective removal of the NRTI from the end of the viral DNA after it has been incorporated by RT (Boyer, Sarafianos, Arnold & Hughes, 2001; Sarafianos et al. 2009).

Most of the NNRTI resistance mutations are found in and around the NNIBP, K103N and Y181C which are the most frequently observed resistance mutations in patients treated with the approved NNRTIs (Sarafianos et al. 2009). NNRTI resistance mutations can occur singly, or in combinations. It has been reported that a number of mutations in the connection, or RNase H subdomains of RT, can enhance resistance to both NRTI and NNRTI inhibitors of RT (Delvis-Frankenberry, Nikolenko, Barr & Pathak, 2007; Hachiya et al. 2008).

2.10. Potential of plants in the management of HIV/AIDS

Herbal medicines provide rational means for the treatment of many diseases that are obstinate and incurable in western systems of medicine (Vermani & Garg, 2002). Phytomedicines are regaining patient acceptance because they have fewer side effects, are relatively less expensive, are easy to use (Short, 2006), and have a long history of use (Vermani & Garg, 2002). Medicinal effects of plants tend to normalize physiological function and correct the underlying cause of the disorder (Murray & Pizzorno, 1999). Furthermore, medicinal plants are renewable in nature unlike the synthetic drugs that are obtained from non-renewable sources of basic raw materials such as fossil sources (Samanta, Mukherjee, Prasad & Suresh, 2000). Cultivation, gathering, and selling of medicinal plants can also be a source of income for poor families (Reihling, 2008).

Africa is also awash with fake AIDS cures (Amon, 2008). Despite the rich African repertoire from which to select medicinal plants, traditional herbal medicines are still not well-researched (Mills, Cooper, Seely & Kanfer, 2005), and African knowledge of herbal remedies used to manage HIV/AIDS is scanty, impressionistic and not well documented (Kayombo, Uiso, Mbwambo, Mahunnah, Moshi & Monda, 2007). HIV/AIDS is a relatively new human disease, with few ethnobotanical treatments, but logical associations of treatments for other likely viral infections (such as hepatitis B) and closely linked disease states or symptoms (wasting, diarrhoea, lymphadenopathy, skin lesions, cough, and genital ulcers) can increase the prospect of finding new plant leads as potential anti-HIV agents (WHO, 1989a,b; Cardellina & Boyd, 1995; Lewis & Elvin-Lewis, 1995). Natural products can be selected for

biological screening based on ethnomedical use, random collection and a chemotaxonomic approach i.e. screening of species of the same botanical family for similar compounds. However, the follow-up and selection of plants based on literature leads would seem to be the most cost-effective way of identifying plants with anti-HIV activity (WHO, 1989a).

Sub-Saharan Africa has rich plant biodiversity and a long tradition of medicinal use of plants with over 3,000 species of plants used as medicines (Van Wyk & Gericke, 2000; Scott, Springfield & Coldrey, 2004). Several of these plants may contain novel anti-HIV compounds. Thus, it is important to search for novel antiretroviral agents which can be added to or replace the current arsenal of drugs against HIV (Klos, Van de Venter, Milner, Traore, Meyer & Oosthuizen, 2009). In the past decade, there has been a sustained bio-prospective effort to isolate the active leads from plants and other natural products for preventing transmission of HIV and management of AIDS (Asres, Bucar, Kartnig, Witvrouw, Pannecouque & De Clercg, 2001; Vermani & Garg, 2002). Screening of plants based on ethno-pharmacological data increases the potential of finding novel anti-HIV compounds (Farnsworth, 1994; Fabricant & Farnsworth, 2001).

2.11. Techniques used in isolation and identification of compounds

The common way of preparing crude extracts from plant material is by solvent extraction of macerated plant material (Kapewangolo, 2014). Different solvents yield different compounds from plant material, depending on their polarity. Thin layer chromatography (TLC) is one of the preliminary basic techniques used to screen

crude natural compounds (Kapewangolo, 2014). TLC is a prescribed standard protocol 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, Bazytko & Staszewska., 2010; Kapewangolo, 2014). A 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). The detection and confirmation of separated components on a TLC plate can be observed by visualizing colored substances. The TLC plates can be inspected under ultraviolet (UV) light for fluorescent components or by using selective detection (spray) reagents (Kamatou, Van Zyl, Davids, Van Heerden, Lourens & Viljoen, 2008). TLC data provides an idea of the number of compounds/substances present in a crude extract (Martelanc, Vovk & Simonovska, 2009; Kapewangolo, 2014).

For complete separation and structural identification of compounds from a plant material, TLC will need to be combined with other analytical techniques (Kapewangolo, 2014) such as nuclear magnetic resonance (NMR). NMR is an excellent tool that gives detailed structural information of organic molecules (Wu, Wang & Simon, 2004). Proton (^1H) NMR and carbon-13 NMR are the most commonly used NMR techniques. ^1H NMR identifies hydrogen atoms/protons in an organic molecule while ^{13}C NMR allows the identification of carbon atoms in organic molecules (Kapewangolo, 2014).

CHAPTER 3: MATERIALS AND METHODS

3.1. Collection of plant samples

3.1.1. Background

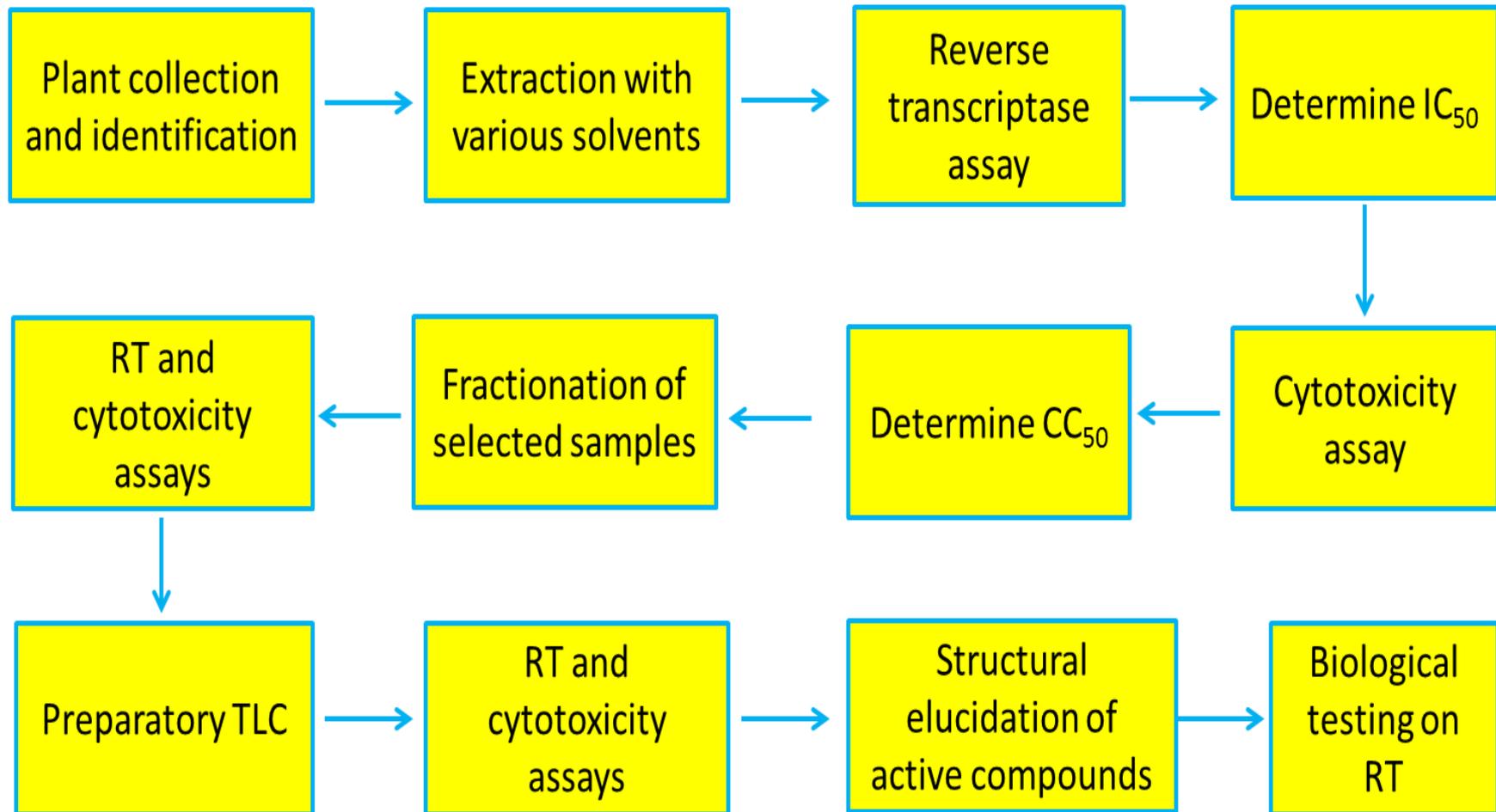
The overview of the whole study is outlined in **scheme 1**. This study was a follow up on previous studies on ethnomedicinal plants used by traditional health practitioners (THPs) in Namibia as reported by Chinsembu and Hedimbi (2010 a,b) and Chinsembu et al. (2011). In those studies, several THPs were asked “which plant(s) they use to treat someone who is suffering from HIV/AIDS”. This study then followed up on some of the reported plants and attempted to scientifically evaluate their potential in inhibiting the life cycle of HIV, based on reverse transcriptase assay.

3.1.2. Study sites

The plants were collected in Ohangwena region (Endola village), Kavango-west region (Mpungu village), and Oshikoto region (Omupupa village) in Namibia and are presented in **Table 5**. The locations where the plants were collected are shown in **Scheme 2**.

3.1.3. Collection and identification of plant samples

Once a plant was identified for collection, all plant parts (where possible) were collected, irrespective of which plant part is used by THP(s). The collection was based on the availability of the plant at the time of the collection. The first plant collection was done in March and April 2012, and another collection was done in September 2013, and in January and September 2014. Two duplicate plant samples were preserved and identified at Namibia Botanical Research Institute (NBRI). Plant materials were air-dried in the dark and crushed into powder. All biological assays were conducted at Council for Scientific and Industrial Research (CSIR), Biosciences laboratories. Several documents were obtained from several government institutions to ensure that the study conforms to the country's rules and regulations regarding the research in biomaterials: a research permit was obtained from Ministry of Environment and Tourism (MET) (**Appendix C**), a phytosanitary certificate was obtained from Ministry of Agriculture, Water and Forestry (MAWF) (**Appendix D**), a material transfer agreement (MTA) was obtained from Namibia Botanical Research Institute (NBRI) (**Appendix E**), an export permit was obtained from MET (**Appendix F**), memorandum of understandings (MoU) were signed between UNAM and two traditional healers which addressed issues of access and benefit sharing (ABS) from plants they identified and finally a MoU was signed between UNAM and CSIR.



Scheme 1: Outline of major steps that were followed in the study.

Table 5: Plants used in the study

	Collecti on ID	Oshiwambo name	Place of collection	Scientific name	Family	Part collected
1.	MTH23	Okatanga kamufifi	Omupupa Village, Oshikoto region	<i>Acanthoscyocis naudinianus</i>	Cucurbitaceae	LV, TB
2.	MTH13	Omupopo	Omupupa Village, Oshikoto region	<i>Albizia anthelmintica</i> Brongn.	Fabaceae (Mimosoideae)	DB, LB, LV, RT, ST
3.	MTH17	Omuve	Endola village, Ohangwena region	<i>Berchemia discolor</i> Neck. Ex DC	Rhamnaceae	DB, LV, RT, ST
4.	MTH15	Omunghudi	Omupupa Village, Oshikoto region	<i>Boscia albitrunca</i> (Burch) Gilg and C. Benedict	Capparaceae	DB, LB
5.	MTH03	Omusati	Endola village, Ohangwena region	<i>Colophospermum mopane</i> (Kirk ex Benth.) Kirk ex J.Léon.	Fabaceae (Caesalpiniodeae)	DB, LB, LV, RT, ST
6.	MTH08	Omukuku	Endola village, Ohangwena region	<i>Combretum imberbe</i> Wawra	Combretaceae	DB, LB, LV, RT, ST

7.	MTH07	Omboo	Endola village, Ohangwena region	<i>Commiphora glandulosa</i>	Burseraceae	LB, LV, RT, ST
8.	MTH11	Ombango	Omupupa Village, Oshikoto region	<i>Croton gratissimus</i> Burch.	Euphorbiaceae	LB, LV, RT, ST
9.	MTH10	Ongete	Omupupa Village, Oshikoto region	<i>Dichrostachys cinerea</i> (L.) Wight and Arn	Fabaceae (Mimosoideae)	DB, LB, LV, RT, ST
10.	MTH01	Omwandi	Endola village, Ohangwena region	<i>Diospyros mespiliformis</i> Hochst. Ex. A. DC.	Ebenaceae	DB, LB, LV, RT, ST
11.	MTH04	Omukwiyu	Endola village, Ohangwena region	<i>Ficus sycomorus</i>	Moraceae	DB, RT, ST
12.	MTH18	Iidimba	Endola village, Ohangwena region	<i>Pechuel-Loeschea</i> <i>leubnitiziae</i>	Asteraceae	LB, RT, ST
13.	MTH12	Omupanda	Omupupa Village, Oshikoto region	<i>Philenoptera nelsii</i>	Fabaceae (Papilionoideae)	LB, LV, RT, ST
14.	MTH16	Eenghushe	Elyambala village, Oshana region	<i>Sansevieria aethiopica</i> Thunb.	Asparagaceae	ST, RT

15.	MTH19	Omunghete	Mpungu constituency, Kavango west region	<i>Schinziophyton rautanenii</i> Hutch. Ex Radcl.-Sm	Euphorbiaceae	DB, LB, LV
16.	MTH04	Omwoongo	Endola village, Ohangwena region	<i>Sclerocarya birrea</i> (A. Rich.) Hochst.	Anacardiaceae	DB, LB, RT
17.	MTH20	Omuhongo	Omakango village, Ohangwena region	<i>Spirostachys africana</i>	Euphorbiaceae	DB, LB, LV, RT, ST
18.	MTH09	Omunghama	Omupupa Village, Oshikoto region	<i>Terminalia pruniodes</i>	Combretaceae	DB, LB, LV, RT, ST
19.	MTH14	Omwoolo	Omupupa Village, Oshikoto region	<i>Terminalia sericea</i>	Combretaceae	DB, LB, LV, RT, ST
20.	RH01	Unknown	Provided by THPs	Unknown	Unknown	LV, RT
21.	RH02	Unknown	Provided by THPs	Unknown	Unknown	WP
22.	MTH06	Oshipeke	Endola village, Ohangwena region	<i>Ximenia american</i>	Olacaceae	LB, LV, RT, ST
23.	MTH02	Omukekete	Endola village, Ohangwena region	<i>Ziziphus mucronata</i>	Rhamnaceae	DB, LB, LV, RT, ST

Key: LV=Leaves; TB=Tuber; DB=Dead bark; LB=Live bark; RT=Roots; ST=Stems; WP=Whole plant.

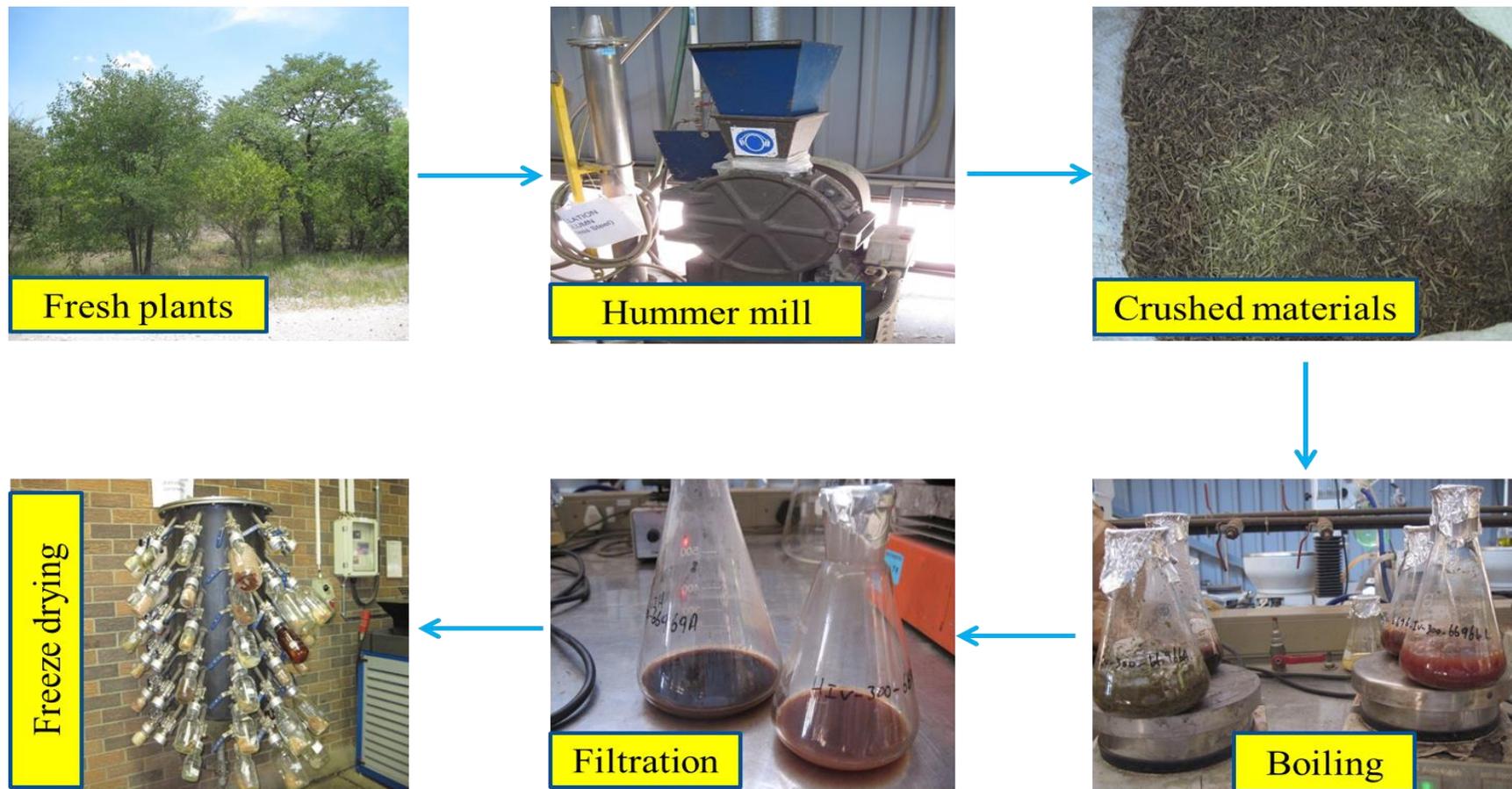
3.2. Extraction of plant samples

3.2.1. Crude extracts

Dry plant materials were grounded to powder using a Hippo™ hummer mill. The materials were then extracted with boiling water (**Scheme 3**) and organic extraction was carried out with Dichloromethane (DCM: CH₂Cl₂; 99.5%, Merck) and Methanol (MeOH: CH₃OH; 99.5%, Merck) (1:1;v/v) (**Scheme 4**). Organic extraction with DCM:MeOH was carried out by soaking samples in the solvent for 12 hours. The solid particles were then filtered off and rewashed with the solvent 3 times (by re-suspending for 12 hours) and filtered. Samples were then concentrated using a rotary evaporator at 60 °C and then completely dried in a fume hood (**Scheme 4**). Aqueous extraction was carried out by boiling the samples in double distilled water and filtering. The solid particles were then filtered off and rewashed with the solvent 3 times (by boiling). Water was removed by using a freeze dryer (**Scheme 3**).

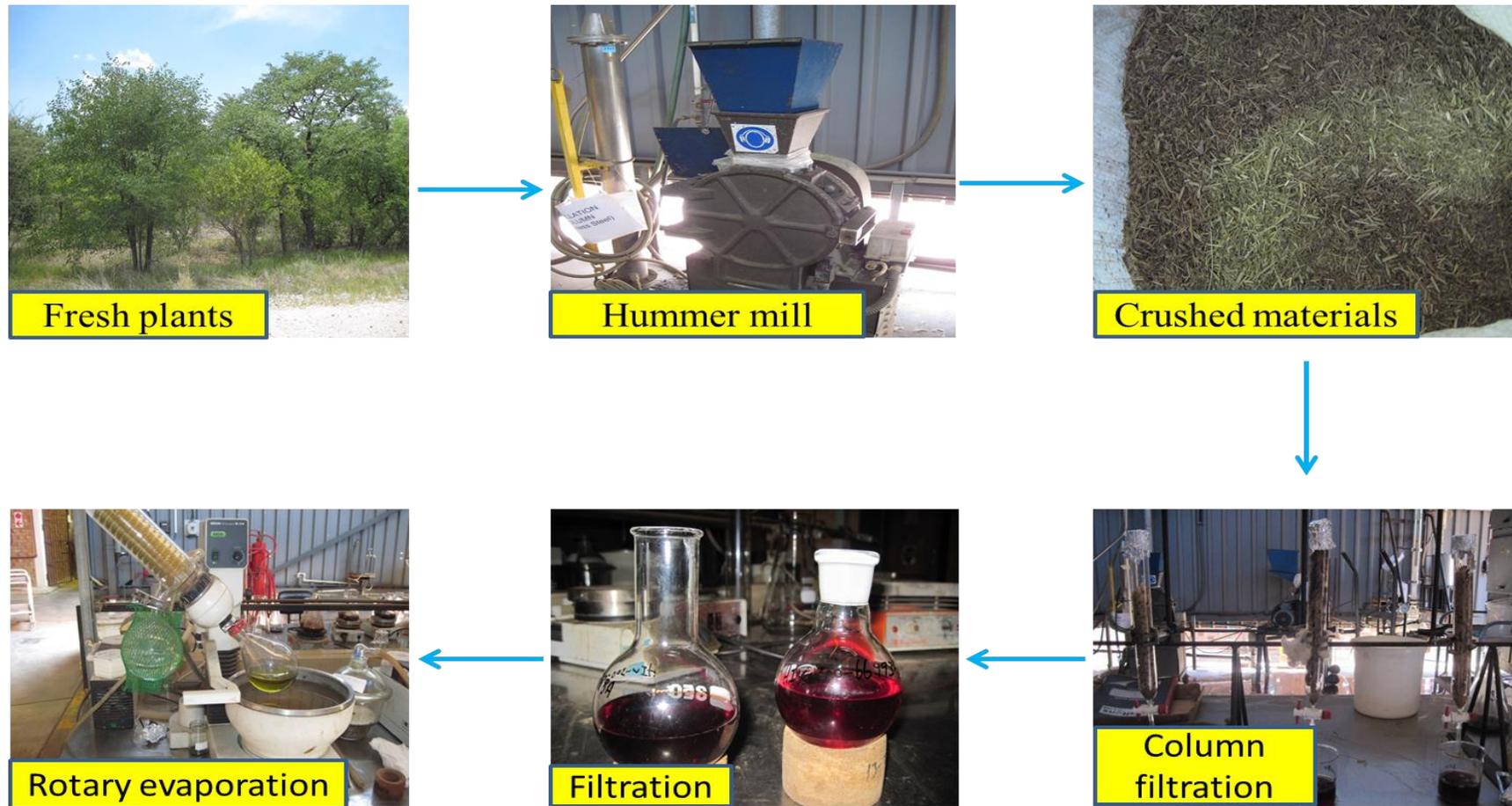
3.2.2. Fractionation of samples

Samples selected for fractionation were fractionated using vacuum liquid chromatography (VLC) to obtain different fractions from each extract. The following solvent systems, with increasing polarity were used successively: a) 10% Acetone (CH₃COCH₃; 99.5%, Merck) (in DCM); b) 50% Acetone (in DCM); c) 20% MeOH (in DCM; d) 100% Acetone; e) 100% Ethyl Acetate (C₄H₈O₂; 99.5%, Merck); and f) 100% MeOH. Samples were then concentrated using a rotary evaporator at 60 °C and then completely dried in a fume hood.



Scheme 3: Flowchart of aqueous (water) extraction of plant samples.

(CSIR Standard operating procedures (SOP) on natural products and structural elucidation, 2012)



Scheme 4: Flowchart of organic extraction of plant samples.

(CSIR Standard operating procedures (SOP) on natural products and structural elucidation, 2012)

3.2.3. TLC profiling and purification of compounds

Thin layer chromatography (TLC, silica gel coated 60 F₂₅₄, 20x20 cm, Merck) profiles were used to determine the right solvent mixture (mobile phase) for separation of different compounds present in selected bioactive fractions. The TLC plates were visualized under UV light (short wavelength at 254 nm, and long wavelength at 365nm). All compounds were visualized using the vanillin-sulfuric acid reagent (prepared by dissolving 1g of vanillin powder in 99 ml concentrated Sulphuric acid, H₂SO₄) as reported by Taketa, Gondorf, Breitmaier & Schenke, (2002). The compounds were then purified using preparative TLC plates (Sigma-Aldrich; 2000 microns).

3.2.4. Structural elucidation of isolated compounds

Structural elucidation of isolated compounds was done with nuclear magnetic resonance (NMR) 600 MHz. Confirmation of the structure was done using various NMR studies: ¹H NMR (number of protons) and ¹³C NMR (number of carbons) and distortionless enhancement polarization transfer (DEPT) (number of Carbon skeletons); and 2 dimensional (2D) studies for the structures done with: correlated spectroscopy (COSY) (short range proton-proton correlation), heteronuclear single quantum coherence (HSQC) (short range proton-carbon correlation) and heteronuclear multiple quantum coherence (HMQC) (long range proton-carbon correlation).

3.2.5. Acetylation procedure

Compounds that were too polar to completely dissolve in any available solvent (in the lab) were acetylated in order to make them less polar and hence soluble in organic solvents. This process made it easy to elucidate the structure with NMR studies by using the data obtained from the acetylated sample. Acetylation of samples was performed by the addition of pyridine-acetic anhydride (1:1) to the sample in the round-bottomed flask. The reaction mixture was then stirred for 48 h at room temperature. Ethanol (3 ml) was added to react with the excess acetic anhydride. The resultant mixture was poured into ice-chloroform mixture and the acetylated product was extracted two to three times by liquid-liquid partitioning in a separating funnel. The organic phases were combined and the solvent removed by rotovapor evaporation (Peter, 2007).

3.3. Reverse transcriptase inhibition assay

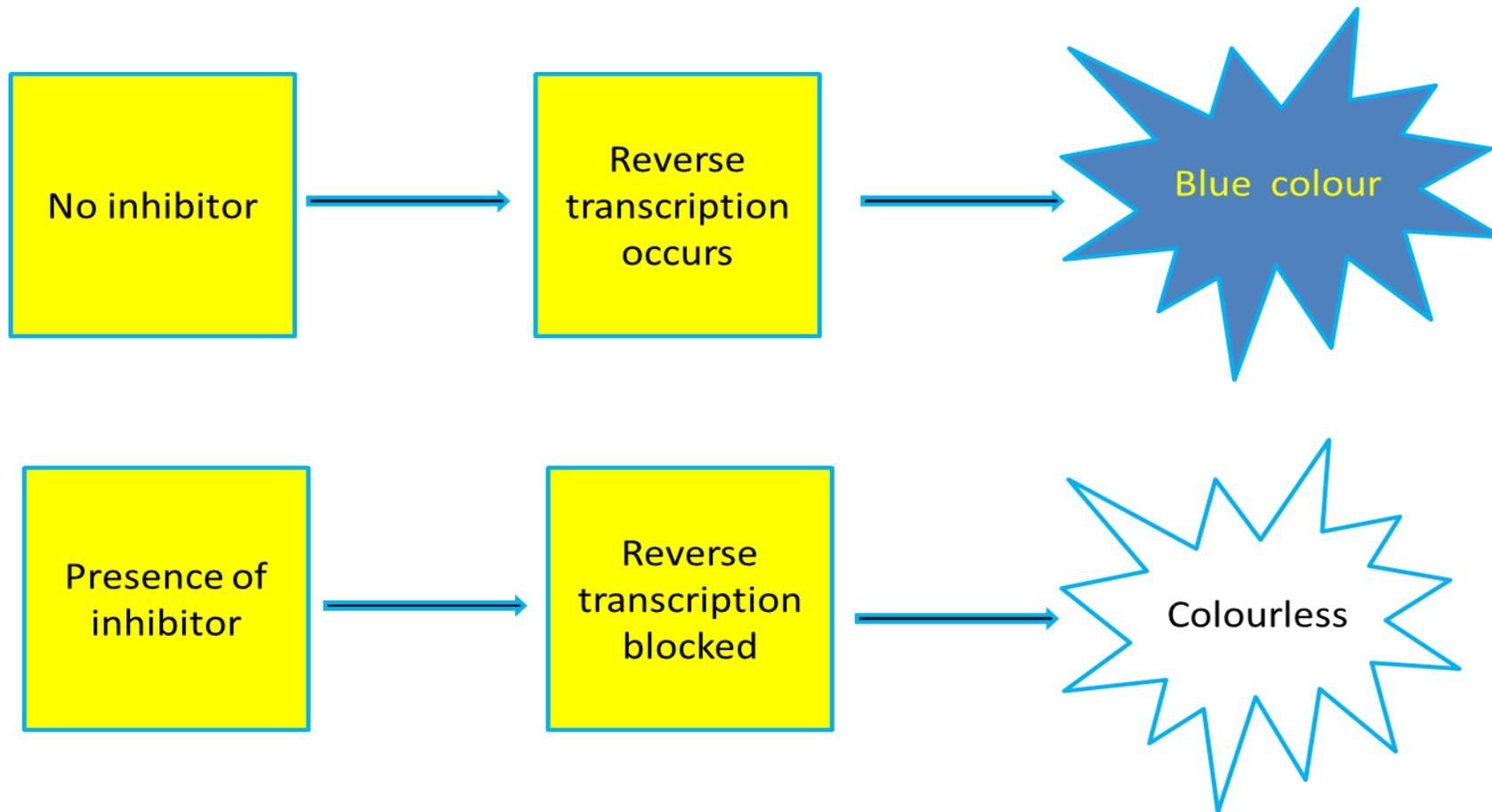
3.3.1. Background

This assay is based on a sandwich-ELISA (enzyme-linked immunosorbent assay) protocol employing the Roche™ colorimetric reverse transcriptase (RT) enzyme kit (cat# 11 468 120 910) (Roche reverse transcriptase assay, 2010). This immunoassay is used for quantitative determination of retroviral RT activity by incorporation of digoxigenin (DIG) and biotin-labeled dUTP DNA (deoxyuridine-triphosphatase Deoxyribonucleic acid). Biotin and DIG-labeled nucleotides are incorporated into cDNA strands polymerized on an RNA template by the action of HIV-1 RT. The

cDNA products are bound to streptavidin-coated wells of 96-well plate inserts (micro plates, MP modules), and their associated DIG-moieties detected by incubation with anti-DIG antibodies conjugated to horseradish peroxidase (HRP). The amount of bound antibody is quantitated by incubation with a colorimetric HRP substrate, followed by absorbance reading at 405 nm (Roche reverse transcriptase assay, 2010). The overview of RT assay is shown in **scheme 5**.

3.3.2. Preparation and storage of kit components

All solutions were kept on ice during the experiment. RT enzyme was reconstituted by adding 250µl of sterile double distilled water (sddH₂O) to the RT lyophilizate (vial 1) and vortexed. The reconstituted enzyme was aliquoted into PCR microcentrifuge tubes to avoid repeated freezing and thawing. The reconstituted enzyme was then stored at -70°C until use. The reaction mixture was prepared by adding 430µl sddH₂O to the template lyophilizate (vial 4) and vortexed. One millilitre of incubation buffer (bottle 2) was then added to each vial of nucleotides (4 x vial 3) and vortexed. Then 100 µl of reconstituted template (vial 4) was added to each vial of nucleotides and vortexed. The reaction mixture was then stored at -20°C until use. Anti-DIG-POD (anti-digoxigenin-peroxidase) was prepared by adding 500 µl of sddH₂O to the Anti-DIG-POD lyophilizate (bottle 6) and then vortexed. The solution was stored at 4°C until use. The washing buffer was prepared by adding 225ml sddH₂O to each bottle of washing buffer (3 x bottle 7) and then vortexed. The solution was stored at 4°C until use (Roche reverse transcriptase assay, 2010).



Scheme 5: Overview of RT assay in the presence and absence of an inhibitor.

(CSIR, SOP, Biological assays guide, 2012).

3.3.3. Screening of test compounds

The major steps involved in carrying out RT assay are outlined in **scheme 6**. High through-put screening using six (10-fold) serial dilutions (100mg/ml-0.001mg/ml) for each sample in dimethyl Sulfoxide (DMSO) was carried out for the crude extracts. Fractionated extracts and both semi and pure compounds were tested at a starting concentration of 1mg/ml. The RT enzyme was diluted in lysis buffer to achieve 20 µl diluted RT/well (=2ng RT/well). Twenty microlitres of the following were added to wells in a 96-well round bottom plate: lysis buffer (bottle 5) (positive control wells); pre-diluted RT (all wells, except blank control); test compounds (test compound wells); and a reaction mixture (vial 3) was added to all wells with samples. The plates were sealed with parafilm and incubated at 37 °C for 1 hour (Roche reverse transcriptase assay, 2010).

Ready to use microplate (MP) modules were placed in a strip frame 5 minutes before the end of incubation period. Samples (60µl) were transferred from the 96-well plates to the MP modules in the strip frame. The wells were then sealed using a self-adhesive cover foil (parafilm) and incubated at 37°C for 1 hour. A concentration 200µl/well Anti-DIG-POD working dilution was prepared, 10 minutes before the end of incubation period, by diluting an appropriate volume of Anti-DIG-POD solution (bottle 6) in conjugate dilution buffer (bottle 8) (Roche reverse transcriptase assay, 2010).

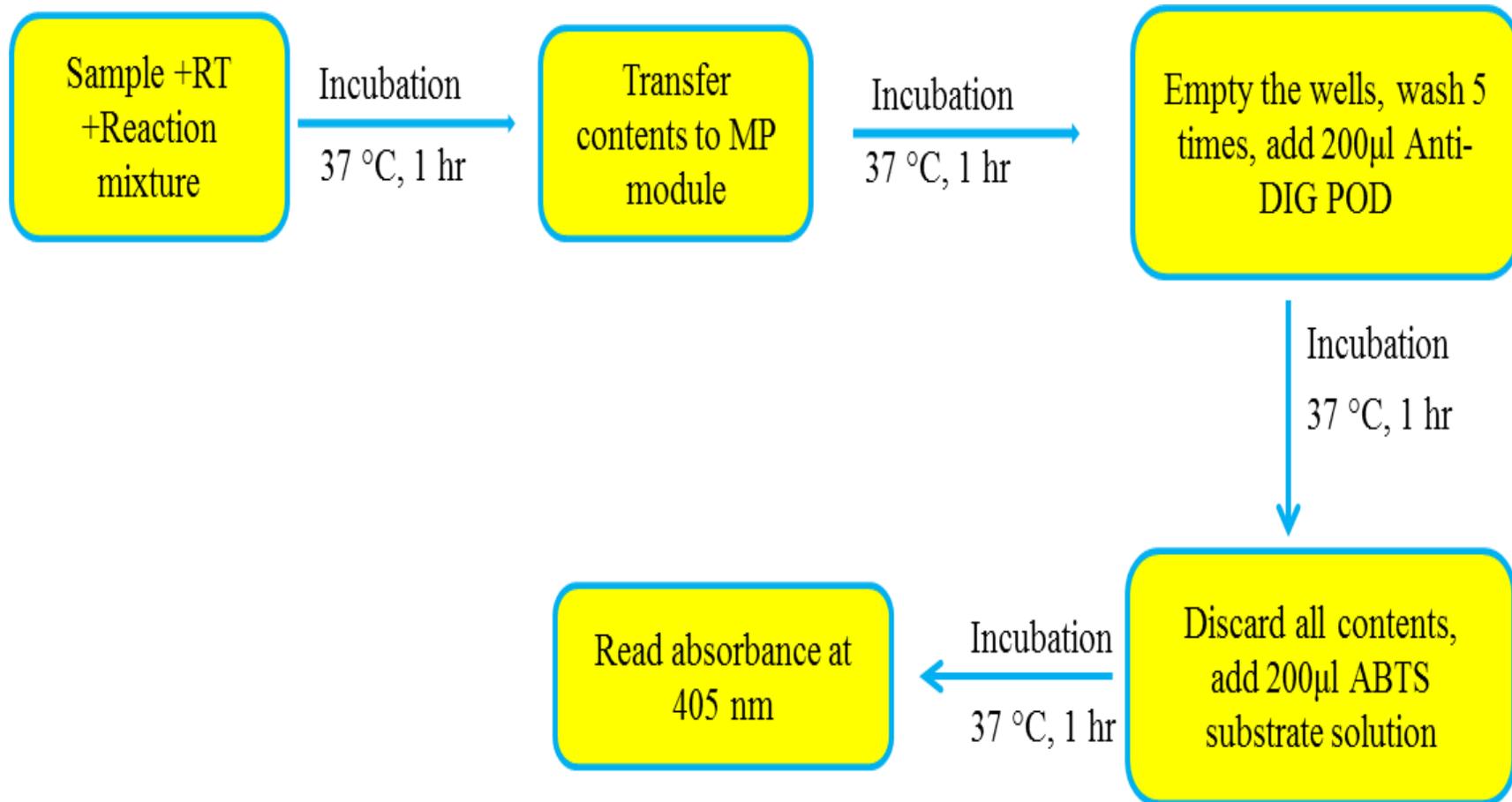
After incubation, the self-adhesive cover was removed and the wells were emptied by quickly inverting the plate over the sink. No pipette is used at this stage because it will damage the DNA bound to the surface of the plate. The wells were washed 5 times with 200µl washing buffer (bottle 7) per well for about 30 seconds each using a multichannel pipette. The last washing buffer was removed from the wells and 200µl of Anti-DIG-POD working dilution was immediately added to each well using a multichannel pipette. The plates were sealed with parafilm and incubated at 37°C for 1 hour (Roche reverse transcriptase assay, 2010).

ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) substrate solution (200µl/well) was prepared 30 minutes before end of incubation period, by dissolving ABTS tablet(s) in substrate buffer (1 ABTS tablet is dissolved in 5ml of substrate buffer). The solution is then covered with aluminum foil and kept in the dark at room temperature until use. Following the incubation period, the wells were emptied and 200µl of ABTS substrate solution was added to each well using a multichannel pipette. The plates were then incubated in the dark at room temperature for about 2-5 minutes until color development occurs. Absorbance was read at wavelength of 405 nm using a Tecan Infinite F500 plate reader. The enzyme (RT) activity was calculated using the following equation (Roche reverse transcriptase assay, 2010):

$$\text{Enzyme activity (\%)} = \frac{[\text{Absorbance}_{\text{compound}} - \text{Absorbance}_{\text{Blank}}]}{(\text{Absorbance}_{\text{positive}} - \text{Absorbance}_{\text{Blank}})} \times 100.$$

Blank wells do not contain RT (lysis buffer was added instead of RT), RT activity in these wells is therefore 0%. Positive control wells contains RT, but no inhibitor was added (lysis buffer was added instead of the compound), RT activity in these wells is therefore 100%. Nevirapine, a commercially available drug that is currently used as non-nucleoside RT inhibitor, was used as a standard. RT in 10% DMSO (dimethyl Sulphoxide) was used as sample control (this indicates the level of RT inhibition due to the presence of the solvent used to dissolve the compounds).

Dose-response curves with non-linear regression sigmoidal curves were used to determine the 50% inhibitory concentration (IC_{50}) of plant extracts using GraphPad Prism, version 6 software (GraphPad Prism User Guide, 1995-2014) (**Appendix A**). This was interpreted as the concentration of the extract that reduced RT activity by 50% when compared to controls.



Scheme 6: Outline of major steps in carrying out RT assay.

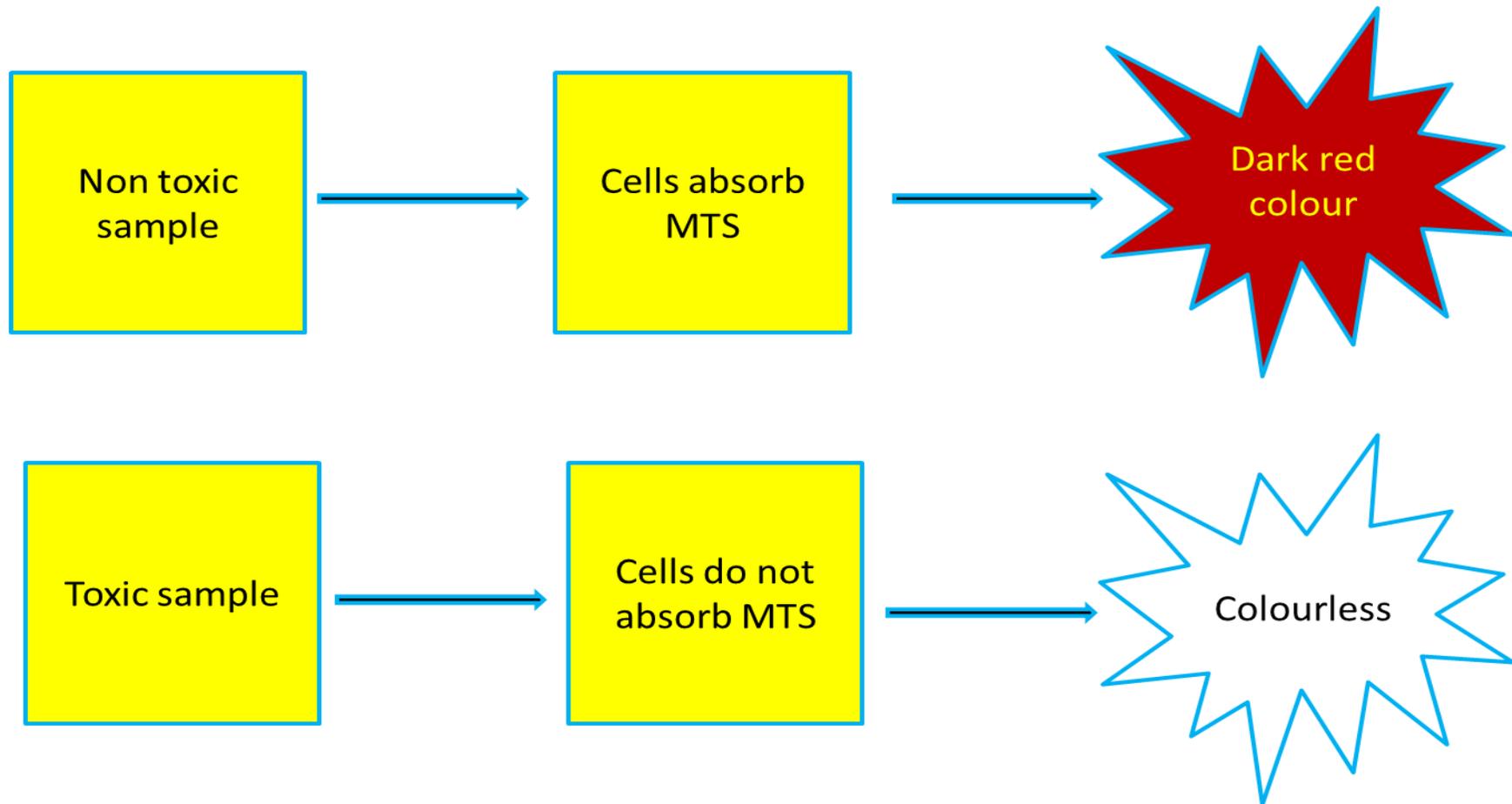
(CSIR, SOP, Biological assays guide, 2012).

3.4. Cytotoxicity assay

3.4.1. Background

The assay used in this study is based on the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (from Promega[™]) (Promega CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay, 2012) which is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The CellTiter 96[®] AQueous One Solution Reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl) -5- (3-carboxymethoxyphenyl)-2- (4-sulfophenyl) -2H-tetrazolium, inner salt; MTS] and an electron coupling reagent phenazine ethosulfate (PES). PES has enhanced chemical stability which allows it to be combined with MTS to form a stable solution. The MTS-tetrazolium compound (Owen's reagent) is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by either NADPH (nicotinamide adenine dinucleotide phosphate hydrogen) or NADH (nicotinamide adenine dinucleotide hydrogen) produced by dehydrogenase enzymes in metabolically active cells (Berridge & Tan, 1993).

Assays were performed by adding 20 μ l of the CellTiter 96[®] AQueous One Solution Reagent directly to culture wells, incubating for 1–4 hours and then recording the absorbance at 490 nm with a 96-well plate reader. The quantity of formazan product measured by the absorbance at 490 nm is directly proportional to the number of living cells in culture. The background of this assay is presented in **scheme 7**.



Scheme 7: Overview of cytotoxicity assay in the presence/ absence of a toxic/non-toxic sample.

(CSIR Standard operating procedures (SOP) of biological assays, 2012).

3.4.2. Cell culturing

3.4.2.1. Maintenance of cells

All cell culturing procedures were performed in a lamina flow hood and aseptic techniques were applied. Vero cells were grown and maintained in tissue culture flasks (25 cm³) at 37° C, 5% CO₂ and 90% humidity conditions. Vero cells are derived from the kidney epithelial cells of an African green monkey (*Chlorocebus* sp.; formerly called *Cercopithecus aethiops*). They were maintained in sterile 199 media supplemented with 10% (v/v) heat inactivated Fetal Bovine Serum (FBS) and 0.05 % gentamycin. The culture medium was changed every second day. (CSIR, SOP, Biological assays guide, 2012).

3.4.2.2. Propagation of cells

Once the cells were 80-90% confluent, the cell monolayers were washed three times with phosphate buffered saline (PBS) and the solution was discarded. To detach the cell monolayer from the surface of the flask, 2 ml of trypsin/EDTA (0.25% (v/v) / 0.53 mM EDTA) solution was spread on the monolayer cell surface and the flask was incubated at 37 °C for less than 3 min to assist detaching. After the cells were detached, 5 ml of the culture medium (199 Media) (BioWhitaker[®], with Earle's Balanced Salt Solution and with L-Glutamine and HEPES) was used to neutralise the trypsin action and the cell suspension was split in a ratio of 1:5 (CSIR, SOP, Biological assays guide, 2012).

3.4.2.3. Counting of cells

To count the cells after the trypsinisation step, the cells were pelleted by centrifugation at 3000 revolutions per minute (rpm) at 4°C for 5 min. The supernatant was discarded carefully to make sure the pellet was not disturbed. The same amount of PBS was added to wash the cells and subjected to centrifugation again. The supernatant was discarded and a small amount of cells in the pellet was transferred into a sterile 15 ml centrifuge tube. An adequate amount of 199 Media was added and mixed well in order to disperse the cells to form a suspension consisting of single cells (i.e. not agglomerates of cells) (CSIR, SOP, Biological assays guide, 2012).

A volume of 0.1 ml of the cell suspension was aliquoted into a 1 ml sample tube and 0.1 ml of 0.4% trypan blue stain solution was added and mixed thoroughly and allowed to stand for 2 min. With a cover-slip in place, a small amount of trypan blue-cell suspension was transferred to a chamber of a haemocytometer (Naubauer). This was done by carefully touching the edge of the cover-slip with the pipette tip and allowing the chamber to fill by capillary action. Cells were enumerated microscopically using light microscope (Carl Zeiss, North America, USA). Live cells do not take up the trypan blue dye while dead cells do, which allowed calculation of the percentage viable cells. Dilutions of cell suspensions were done according to the cell counts to achieve the specific density required (20,000 cells/well). The plates containing 20,000 cells/well (in 96-well plates) were incubated at 37°C for 48–72

hours in a humidified, 5% CO₂ atmosphere (CSIR, SOP, Biological assays guide, 2012).

3.4.2.4. Cryopreservation of cells

The pellet from the centrifuged cells were cryopreserved in culture medium (199 Media) containing 50% FBS and 10% sterile glycerol and were slowly frozen to -20°C overnight and transferred to liquid nitrogen at -196°C until further use. Cells were frozen at density between 5-10 x 10⁶ cells/ml (CSIR, SOP, Biological assays guide, 2012).

3.4.2.5. Resuscitation of cells

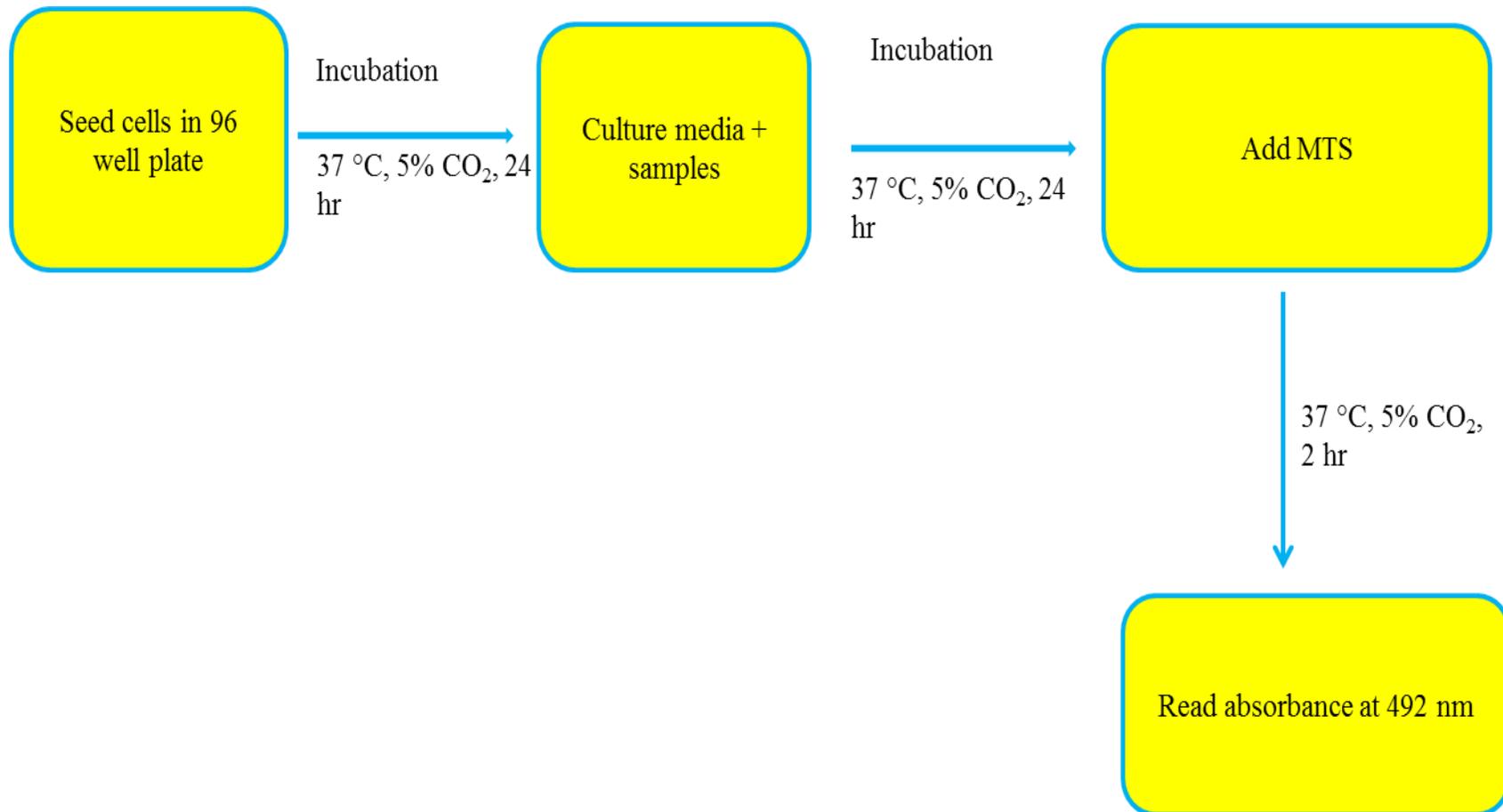
The cryopreserved cells were harvested by quickly immersing a cryovial in a 37 °C water bath for 2 min, which was then centrifuged at 2000 rpm for 5 min. Cells were resuscitated in culture medium at 37 °C and grown as described above (CSIR, SOP, Biological assays guide, 2012).

3.4.3. Testing cytotoxicity of compounds

The outline of major steps involved in the cytotoxicity assay is presented in **scheme 8**. Cells were seeded at a concentration of 20,000 cells/well. CellTiter 96® AQueous One Solution Reagent was thawed for 10 minutes in a water bath at 37°C. Before adding the test compounds, the media (in which cells were incubated) was removed and cells were washed with PBS. 100µl of culture media was added to each well and 10µl of test compounds was added to wells. Six (10-fold) serial dilutions (1-0.00001mg/ml) for each sample were carried out. Fractionated extracts and both semi

and pure compounds were tested at a starting concentration of 1mg/ml. Emetine® was used as a standard (CSIR, SOP, Biological assays guide, 2012).

Cells were incubated with respective extracts at 37°C and 5% CO₂ for 24 hours. 20µl of MTS was added to each well and incubated at 37°C and 5% CO₂ for 2 hours. Absorbance was read at 492 nm. Background absorbance was first read and then subtracted from the reading containing the cells and the extracts. Cell viability was calculated using the following equation: **Cell Viability = (absorbance of extract/absorbance of control) x 100** (Promega CellTiter 96® Aqueous One Solution Cell Proliferation Assay, 2012). No compound was added to the control wells of (only the growth media was added, viability was therefore 100%). Emetine was used as a standard. Dose-response curves with non-linear regression sigmoidal curves were used to determine the 50% cytotoxic concentration (CC₅₀) of plant extracts using GraphPad Prism, version 6 software (GraphPad Prism User Guide, 1995-2014) (**Appendix B**). This was interpreted as the concentration of the extract that reduced cell viability by 50% when compared to controls (CSIR, SOP, Biological assays guide, 2012).



Scheme 8: Outline of major steps in cytotoxicity assay.

(CSIR, SOP, Biological assays guide, 2012).

CHAPTER 4: RESULTS

4.1. Inhibitory activities of crude extracts on HIV-1 RT enzyme

A total of 40 extracts (from 15 plants) were found to have concentration-dependent inhibitory activities against RT (**Table 6**). Of the 40 extracts, 28 (from 13 plants) were extracted with DCM:MeOH, while the remaining 12 extracts (from 9 plants) were extracted with water. At concentration of 0.1 mg/ml, RT activity below 10% was observed in *C. imberbe* roots, *F. sycomorus* leaves, *T. sericea* live bark, roots, and leaves; while RT activity below 25% was recorded with extracts from *S. african* dead bark, *T. sericea* stem and dead bark. At concentration of 0.01 mg/ml, RT activity of below 50% was observed with extracts from *T. sericea* stem, dead and live bark, roots and leaves. RT activity of below 85% at concentration of 0.01 mg/ml was observed in 9 extracts (*B. albitrunca* leaves, *C. gratissimus* stem, *C. mopane* roots, *F. sycomorus* dead bark and stem, *T. sericea* stem, dead bark, live bark, roots and leaves) (**Table 6**). The general reactions of RT inhibition assay is shown in **Picture 1**.

Table 6: Database of RT activity of selected plant extracts.

Scientific name	Sample ID	Solvent	Part	RT activity (%)*					
				100 (mg/ml)	10 (mg/ml)	1 (mg/ml)	0.1 (mg/ml)	0.01 (mg/ml)	0.001 (mg/ml)
<i>A. anthelmintica</i>	3-976	Water	ST	-1.7	0.5	16.6	80.0	100.3	113.4
<i>A. anthelmintica</i>	3-978	Water	LB	-1.1	3.9	45.6	69.7	85.2	91.0
<i>A. naudinianus</i>	3-897	DCM: MeOH	TB	-0.7	12.1	66.8	85.4	89.0	97.1
<i>B. albitrunca</i>	3-955	Water	LV	1.5	27.8	69.7	89.5	93.5	98.3
<i>B. albitrunca</i>	3-956	Water	LB	5.0	13.8	81.7	99.9	107.4	80.3
<i>C. glandulosa</i>	2-605	DCM: MeOH	LB	-7.7	-7.3	31.6	77.4	109.6	115.9
<i>C. gratissimus</i>	2-615	DCM: MeOH	LV	-6.8	-6.9	65.6	101.8	111.5	104.0

<i>C. gratissimus</i>	2-616	DCM: MeOH	ST	-6.6	13.4	92.1	98.8	106.7	81.2
<i>C. gratissimus</i>	3-972	Water	LV	-0.9	10.7	49.1	88.0	87.1	125.6
<i>C. imberbe</i>	3-905	Water	LV	-46.8	0.6	7.8	71.9	89.9	108.3
<i>C. imberbe</i>	3-896	DCM: MeOH	RT	3.1	-0.3	3.2	3.1	100.3	108.4
<i>C. mopane</i>	3-987	DCM: MeOH	LB	-0.4	-0.4	16.7	86.9	89.3	101.9
<i>C. mopane</i>	3-989	DCM: MeOH	RT	-1.3	2.2	61.5	72.7	73.4	79.0
<i>C. mopane</i>	3-990	DCM: MeOH	LV	-0.5	-0.2	47.0	102.0	104.0	113.0
<i>D. mespiliformis</i>	3-936	DCM: MeOH	LV	-0.4	-0.6	17.0	78.7	89.8	88.4
<i>D. mespiliformis</i>	3-937	DCM: MeOH	LV	-0.7	5.7	86.2	88.1	89.3	99.3
<i>D. mespiliformis</i>	3-981	DCM: MeOH	ST	-1.0	2.2	63.7	63.1	78.9	101.9

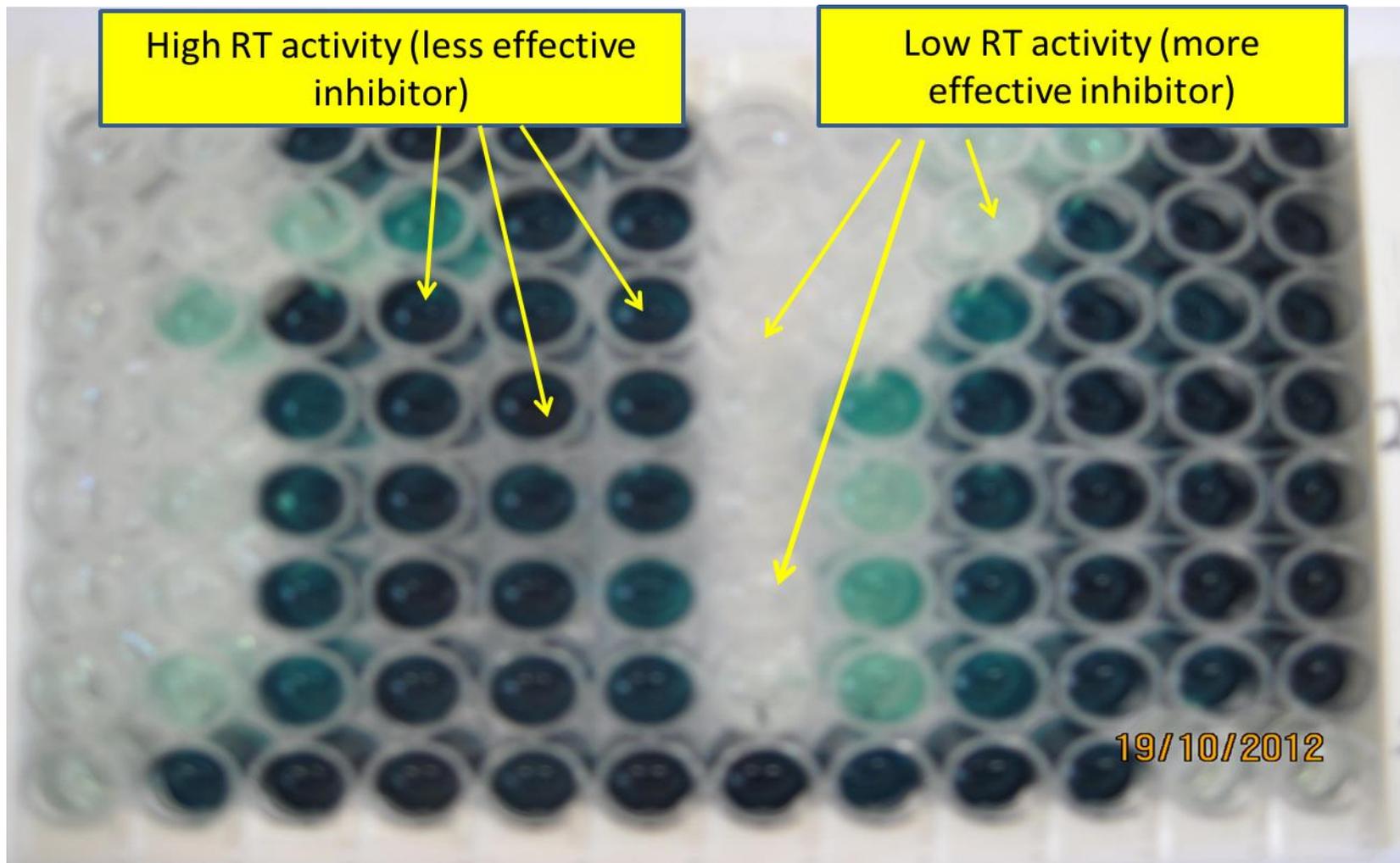
<i>F. sycomorus</i>	3-953	Water	LV	-0.7	-0.1	-0.4	6.5	81.2	102.2
<i>F. sycomorus</i>	3-954	Water	RT	-1.3	-1.3	-0.1	103.8	104.7	105.4
<i>F. sycomorus</i>	3-994	DCM: MeOH	RT	1.2	38.6	97.6	103.2	102.4	102.4
<i>F. sycomorus</i>	3-995	DCM: MeOH	DB	-2.1	17.6	84.4	84.9	88.6	75.4
<i>F. sycomorus</i>	3-996	DCM: MeOH	ST	-2.0	45.4	51.0	64.0	78.4	57.0
<i>S. africana</i>	3-961	Water	DB	-0.6	-0.2	4.9	24.1	101.2	110.8
<i>S. africana</i>	2-645	DCM: MeOH	LB	-23.7	48.6	48.1	96.9	103.0	106.6
<i>S. rautanenii</i>	2-640	DCM: MeOH	LV	-24.3	-23.7	-22.7	69.6	110.7	104.3
<i>S. rautanenii</i>	2-641	DCM: MeOH	DB	-23.6	-23.3	25.4	71.7	80.4	120.8
<i>S. rautanenii</i>	3-928	Water	LV	0.1	5.7	11.4	86.4	86.0	88.0

<i>S. rautanenii</i>	2-642	DCM: MeOH	LB	-23.6	-18.6	42.2	108.4	88.3	94.5
<i>T. pruniodes</i>	3-935	DCM: MeOH	RT	-1.8	-1.4	33.6	43.6	88.2	104.4
<i>T. sericea</i>	2-625	DCM: MeOH	ST	-76.7	-75.4	7.3	13.4	27.8	50.0
<i>T. sericea</i>	2-626	DCM: MeOH	DB	-73.7	-74.3	-28.5	14.3	31.8	64.8
<i>T. sericea</i>	2-627	DCM: MeOH	LB	-77.2	-74.7	-73.8	-62.5	28.2	89.5
<i>T. sericea</i>	2-628	DCM: MeOH	RT	-75.5	-74.8	-74.1	-71.0	-35.9	73.8
<i>T. sericea</i>	2-629	DCM: MeOH	LV	-75.3	-74.5	-73.7	-46.4	43.9	84.3
<i>T. sericea</i>	3-918	Water	LV	0.05	32.9	60.5	96.1	97.1	113.9
<i>T. sericea</i>	3-992	DCM: MeOH	LB	0.03	-1.0	62.1	100.9	103.9	100.7
Unknown	3-938	DCM: MeOH	WP	-0.4	35.8	94.1	96.3	97.6	93.7

<i>Z. mucronata</i>	3-950	Water	ST	-0.4	0.2	85.2	95.6	96.8	106.3
<i>Z. mucronata</i>	3-982	DCM: MeOH	ST	-1.3	1.7	82.4	86.4	86.7	91.4
<i>Z. mucronata</i>	3-983	DCM: MeOH	LV	-0.5	0.1	66.2	87.6	99.1	102.4

Key: LV=Leaves; TB=Tuber; DB=Dead bark; LB=Live bark; RT=Roots; ST=Stems; WP=Whole plant.

*The study reports the amount of RT that remained active after being exposed to different samples (extracts). This means that, the lower the RT activity, the more effective is the sample (extract) in inhibiting RT activity.



Picture 1: Colorimetric reaction of RT inhibition assay.

4.2. Cytotoxicity of crude plant extracts

Most of the samples had very low toxicity to Vero cells (with 31 out of 40 extracts having viability of above 50% at the highest concentration tested; 1 mg/ml) (**Table 7**). Thirty six samples had viability of 90% or more at the lowest tested concentration of 0.00001 mg/ml, except for 4 samples that had viability of above 80% and these were: *C. imberbe* roots (82.2%), *C. glandulosa* live bark (85.4%), *F. sycomorus* dead bark (85.7%) and *S. rautanenii* leaves (89.6%) (**Table 7**). The general results of cell proliferation assay is shown in **Picture 2**.

Table 7: Cytotoxicity of plant extracts based on MTS cell proliferation assay.

Scientific name	Sample ID	Solvent	Part	Cell viability (%)*					
				1 (mg/ml)	0.1 (mg/ml)	0.01 (mg/ml)	0.001 (mg/ml)	0.0001 (mg/ml)	0.00001 (mg/ml)
-	Emetine (µM/ml)	-	-	53.2	52.9	56.6	53.6	56.1	56.1
<i>A. anthelmintica</i>	3-976	Water	ST	40.3	87.5	93.4	91.9	92.5	93.0
<i>A. anthelmintica</i>	3-978	Water	LB	24.2	72.9	92.0	98.6	99.3	100.8
<i>A. naudinianus</i>	3-897	DCM: MeOH	TB	55.6	82.6	83.8	84.8	92.9	94.8
<i>B. albitrunca</i>	3-955	Water	LV	80.9	88.0	88.2	91.1	94.8	99.8
<i>B. albitrunca</i>	3-956	Water	LB	96.2	91.9	92.0	92.3	92.4	102.6
<i>C. glandulosa</i>	2-605	DCM: MeOH	LB	17.0	23.3	52.1	70.7	88.5	85.4

<i>C. gratissimus</i>	2-615	DCM: MeOH	LV	27.0	85.6	82.2	84.0	88.6	94.1
<i>C. gratissimus</i>	2-616	DCM: MeOH	ST	26.7	83.4	86.4	89.8	91.9	92.0
<i>C. gratissimus</i>	3-972	Water	LV	98.9	99.9	98.9	95.2	106.1	116.6
<i>C. imberbe</i>	3-905	Water	LB	104.1	107.0	101.2	104.0	102.6	104.6
<i>C. imberbe</i>	3-896	DCM: MeOH	RT	81.0	84.3	81.1	81.3	83.7	82.2
<i>C. mopane</i>	3-987	DCM: MeOH	LB	62.6	88.5	89.5	90.3	93.7	93.0
<i>C. mopane</i>	3-989	DCM: MeOH	RT	87.1	93.8	92.3	94.8	110.5	174.3
<i>C. mopane</i>	3-990	DCM: MeOH	LV	76.4	77.4	79.0	88.5	88.8	92.8
<i>D. mespiliformis</i>	3-936	DCM: MeOH	LV	77.9	85.9	82.1	89.9	98.4	95.2
<i>D. mespiliformis</i>	3-937	DCM: MeOH	LB	70.8	82.4	80.2	79.3	88.9	92.8

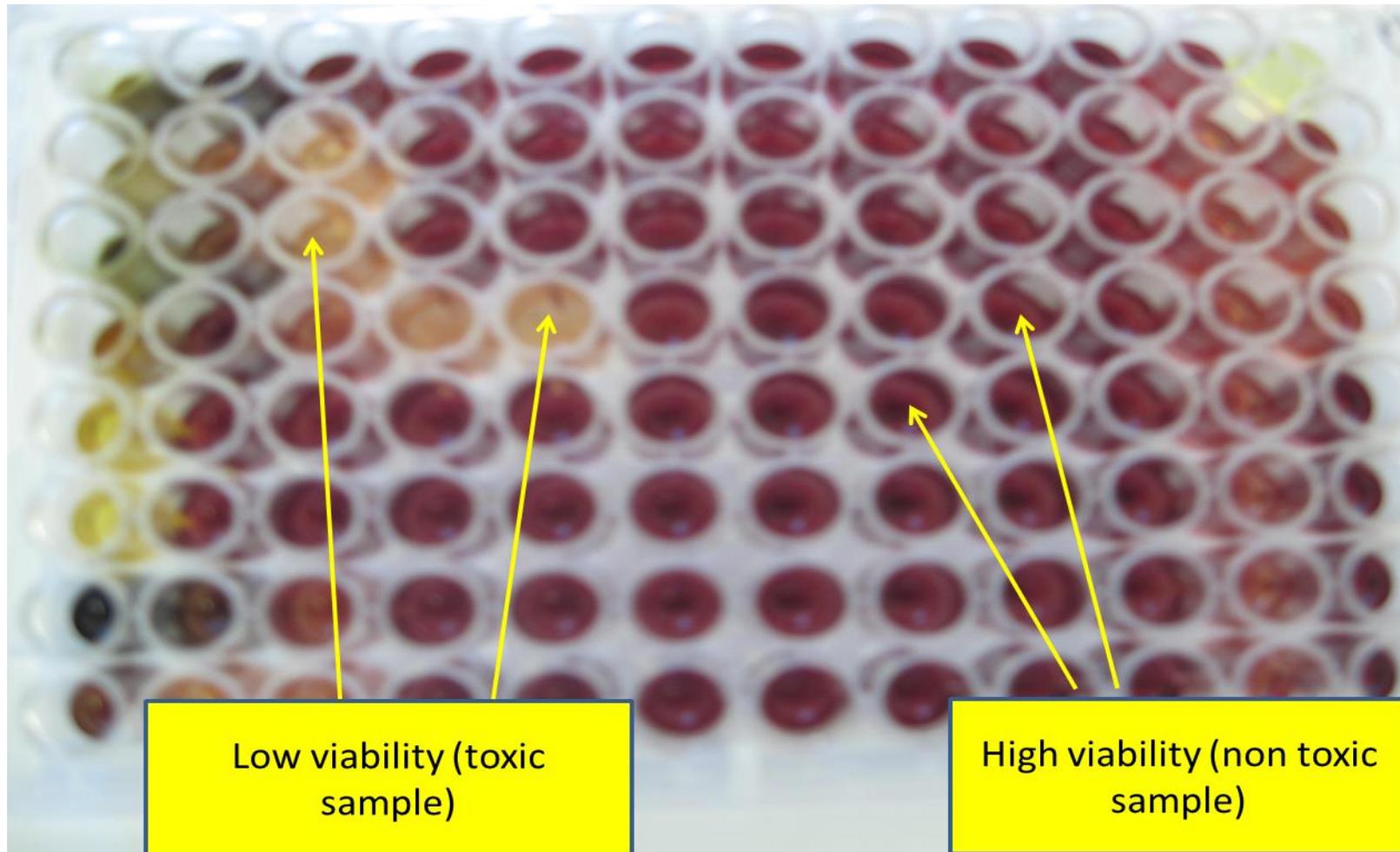
<i>D. mespiliformis</i>	3-981	DCM: MeOH	ST	71.8	85.7	90.1	89.4	97.8	103.8
<i>F. sycomorus</i>	3-953	Water	LV	93.1	95.8	96.4	98.9	100.6	99.3
<i>F. sycomorus</i>	3-954	Water	RT	82.1	93.2	92.5	93.1	94.4	99.1
<i>F. sycomorus</i>	3-994	DCM: MeOH	RT	36.7	81.4	86.7	83.8	89.3	98.9
<i>F. sycomorus</i>	3-995	DCM: MeOH	DB	76.2	84.2	83.2	88.6	84.4	85.7
<i>F. sycomorus</i>	3-996	DCM: MeOH	ST	41.8	74.6	80.8	80.3	86.1	95.0
<i>S. africana</i>	3-961	Water	DB	97.1	99.8	101.6	103.9	102.9	102.9
<i>S. africana</i>	2-645	DCM: MeOH	LB	62.5	77.6	78.7	91.6	81.1	97.8
<i>S. rautanenii</i>	2-640	DCM: MeOH	LV	54.4	77.3	81.6	79.5	88.9	89.6
<i>S. rautanenii</i>	2-641	DCM: MeOH	DB	43.2	71.1	79.2	85.0	93.8	93.0

<i>S. rautanenii</i>	3-928	Water	LV	65.3	92.0	91.5	89.2	89.8	101.5
<i>S. rautanenii</i>	2-642	DCM: MeOH	LB	39.7	73.5	74.3	77.3	81.8	99.9
<i>T. pruniodes</i>	3-935	DCM: MeOH	RT	82.8	95.7	96.7	90.9	91.8	108.2
<i>T. sericea</i>	2-625	DCM: MeOH	ST	72.8	87.8	84.5	83.0	90.2	126.0
<i>T. sericea</i>	2-626	DCM: MeOH	DB	56.3	79.5	81.8	86.2	86.5	95.7
<i>T. sericea</i>	2-627	DCM: MeOH	LB	63.8	75.7	81.4	79.5	87.5	94.4
<i>T. sericea</i>	2-628	DCM: MeOH	RT	49.8	82.3	83.3	87.5	92.0	111.1
<i>T. sericea</i>	2-629	DCM: MeOH	LV	88.7	85.2	86.4	92.4	89.0	92.9
<i>T. sericea</i>	3-918	Water	LV	98.9	98.7	98.3	98.1	101.2	107.5
<i>T. sericea</i>	3-992	DCM: MeOH	LB	84.8	93.6	91.7	92.4	95.2	102.8

Unknown	3-938	DCM: MeOH	WP	85.9	100.0	101.2	109.2	100.3	114.8
<i>Z. mucronata</i>	3-950	Water	ST	78.3	101.5	90.1	95.4	97.4	105.1
<i>Z. mucronata</i>	3-982	DCM: MeOH	Stem	80.0	89.5	83.6	86.8	85.1	90.8
<i>Z. mucronata</i>	3-983	DCM: MeOH	LV	57.8	92.5	94.4	105.1	105.7	111.0

Key: LV=Leaves; TB=Tuber; DB=Dead bark; LB=Live bark; RT=Roots; ST=Stems; WP=Whole plant.

*The study reports the amount of viable cells that remained after being exposed to different samples (extracts). This means that, the lower the viability, the more toxic the sample (extract) is to cells.



Picture 2: Colorimetric reaction of cell proliferation assay of Vero cells with MTS.

4.3. IC₅₀ and CC₅₀ of crude extracts

The inhibitory activities of extracts were ranked based on their IC₅₀ values according to the criteria given in (Table 8). The extracts with the most effective inhibitory activity against RT are those with low IC₅₀ values. The toxicities of extracts were ranked based on their CC₅₀ values according to the criteria given in (Table 9). The less toxic plants are those with high CC₅₀ values.

Table 8: Criteria used in ranking RT inhibitory activities of samples based on their IC₅₀ values.

IC ₅₀ values (mg/ml)	Classification of RT inhibition
IC ₅₀ < 0.5	Potent
0.5 ≤ IC ₅₀ < 1	Moderate
1 ≤ IC ₅₀ < 5	Weak
IC ₅₀ ≥ 5	Not potent

Table 9: Criteria used in ranking toxicities of samples based on their CC₅₀ values.

CC ₅₀ values (mg/ml)	Classification of toxicity
CC ₅₀ > 10	Not toxic
10 ≥ CC ₅₀ > 5	Low toxicity
5 ≥ CC ₅₀ > 1	Toxic
1 ≥ CC ₅₀	Very toxic

Nine extracts had IC_{50} values of 0.05 mg/ml or lower: *C. imberbe* roots, *F. sycomorus* leaves, *S. africana* dead bark, *T. pruniodes* roots and *T. sericea* stem, dead and live bark, roots and leaves (**Table 10**). Another 12 extracts had IC_{50} values of less than 1 mg/ml: *A. anthelmintica* stem and live bark, *C. mopane* live bark and leaves, *C. imberbe* live bark, *C. glandulosa* live bark, *C. gratissimus* leaves, *F. sycomorus* roots and *S. rautanenii* leaves, dead and live bark, and leaves (**Table 10**). The plants with the highest IC_{50} values were *S. african* live bark (8.72 mg/ml); *F. sycomorus* roots (7.18 mg/ml) and sample 3-938 (unidentified) with IC_{50} value of 6.19 mg/ml (**Table 10**).

Samples with high CC_{50} values of 10 mg/ml or were: *C. gratissimus* leaves (88.77 mg/ml), *T. sericea* leaves (79.74 mg/ml), *F. sycomorus* roots (63.73 mg/ml) and leaves (63.11 mg/ml), *S. africana* (dead bark), *D. mespiliformis* leaves (29.14 mg/ml), *B. albitrunca* live bark (20.16 mg/ml), and *C. mopane* roots (12.02 mg/ml) (**Table 10**). A total of 10 samples had CC_{50} of less than 1 mg/ml: *A. anthelmintica* stem and live bark, *C. glandulosa* live bark, *C. gratissimus* leaves and stem, *F. sycomorus* roots and stem, *S. rautanenii* dead and live bark, and *T. sericea* roots (**Table 10**). The extracts with low CC_{50} has potential of being toxic if used at high concentrations. Selective index (SI) which is the ratio between IC_{50} and CC_{50} , were calculated. The high the SI value, the more significant is the difference between IC_{50} and CC_{50} .

Table 10: Summary of IC₅₀, CC₅₀ and selective indices of plant extracts based on their potency and toxicity.

Scientific name	Sample ID	Solvent	Plant part	IC ₅₀ * (mg/ml)	Classification of RT activity	CC ₅₀ ** (mg/ml)	Classification of toxicity	SI value ***
-	Emetine	-	-	-	-	0.20	Very toxic	-
<i>F. sycomorus</i>	3-953	Water	LV	0.02	Potent	63.11	Not toxic	3 155.0
<i>S. africana</i>	3-961	Water	DB	0.05	Potent	33.86	Not toxic	677.2
<i>C. imberbe</i>	3-905	Water	LB	0.19	Potent	18.78	Not toxic	98.84
<i>F. sycomorus</i>	3-954	Water	RT	0.34	Potent	63.73	Not toxic	187.4
<i>T. sericea</i>	2-625	DCM: MeOH	ST	0.001	Potent	7.68	Low toxicity	7 680.0
<i>T. sericea</i>	2-629	DCM: MeOH	LV	0.005	Potent	7.14	Low toxicity	1 434.0
<i>T. pruniodes</i>	3-935	DCM: MeOH	RT	0.05	Potent	9.47	Low toxicity	189.4

<i>T. sericea</i>	2-626	DCM: MeOH	DB	0.003	Potent	1.17	Toxic	390.0
<i>T. sericea</i>	2-627	DCM: MeOH	LB	0.004	Potent	1.42	Toxic	355.0
<i>C. imberbe</i>	3-896	DCM: MeOH	RT	0.03	Potent	3.90	Toxic	130.0
<i>S. rautanenii</i>	2-640	DCM: MeOH	LV	0.14	Potent	1.02	Toxic	7.286
<i>S. rautanenii</i>	3-928	Water	LV	0.29	Potent	1.82	Toxic	6.276
<i>C. mopane</i>	3-987	DCM: MeOH	LB	0.33	Potent	1.64	Toxic	4.970
<i>T. sericea</i>	2-628	DCM: MeOH	RT	0.001	Potent	0.86	Very toxic	860.0
<i>S. rautanenii</i>	2-641	DCM: MeOH	DB	0.24	Potent	0.51	Very toxic	2.125
<i>A. anthelmintica</i>	3-976	Water	ST	0.30	Potent	0.72	Very toxic	2.400
<i>C. gratissimus</i>	3-972	Water	LV	0.94	Moderate	88.77	Not toxic	94.44

<i>C. mopane</i>	3-990	DCM: MeOH	LV	0.94	Moderate	2.68	Toxic	2.851
A.	3-978	Water	LB	0.57	Moderate	0.30	Very toxic	0.526
<i>anthelmintica</i>								
<i>S. rautanenii</i>	2-642	DCM: MeOH	LB	0.75	Moderate	0.46	Very toxic	0.613
<i>C. mopane</i>	3-989	DCM: MeOH	RT	1.08	Weak	12.02	Not toxic	11.13
<i>T. sericea</i>	3-918	Water	LV	2.38	Weak	79.74	Not toxic	33.50
D.	3-936	DCM: MeOH	LV	2.72	Weak	29.14	Not toxic	10.71
<i>mespiliformis</i>								
<i>B. albitrunca</i>	3-956	Water	LB	2.92	Weak	20.16	Not toxic	6.904
<i>T. sericea</i>	3-992	DCM: MeOH	LB	1.40	Weak	9.95	Low toxicity	7.107
<i>Z. mucronata</i>	3-982	DCM: MeOH	ST	2.20	Weak	5.12	Low toxicity	2.327

<i>B. albitrunca</i>	3-955	Water	LV	2.78	Weak	6.45	Low toxicity	2.320
<i>D. mespiliformis</i>	3-981	DCM: MeOH	ST	1.06	Weak	2.38	Toxic	2.245
<i>Z. mucronata</i>	3-983	DCM: MeOH	LV	1.44	Weak	1.53	Toxic	1.063
<i>A. naudinianus</i>	3-897	DCM: MeOH	TB	1.70	Weak	1.09	Toxic	0.641
<i>Z. mucronata</i>	3-950	Water	ST	2.39	Weak	3.71	Toxic	1.552
<i>D. mespiliformis</i>	3-937	DCM: MeOH	LB	2.59	Weak	2.10	Toxic	0.811
<i>F. sycomorus</i>	3-995	DCM: MeOH	DB	3.09	Weak	4.62	Toxic	1.495
<i>C. glandulosa</i>	2-605	DCM: MeOH	LB	0.41	Weak	0.002	Very toxic	0.005
<i>F. sycomorus</i>	3-996	DCM: MeOH	ST	1.28	Weak	0.60	Very toxic	0.469

<i>C. gratissimus</i>	2-615	DCM: MeOH	LV	1.43	Weak	0.41	Very toxic	0.287
<i>C. gratissimus</i>	2-616	DCM: MeOH	ST	3.43	Weak	0.47	Very toxic	0.137
Unknown	3-938	DCM: MeOH	WP	6.19	Not potent	6.31	Low toxicity	1.019
<i>S. africana</i>	2-645	DCM: MeOH	LB	8.72	Not potent	3.45	Toxic	0.396
<i>F. sycomorus</i>	3-994	DCM: MeOH	RT	7.18	Not potent	0.55	Very toxic	0.077

Key: LV=Leaves; TB=Tuber; DB=Dead bark; LB=Live bark; RT=Roots; ST=Stems; WP=Whole plant.

*IC₅₀ is interpreted as the concentration of the sample (extract) that reduced RT activity by 50% when compared to controls. This means that, the lower the IC₅₀ value, the more effective is the sample (extract) in inhibiting RT activity.

**CC₅₀ is interpreted as the concentration of the sample (extract) that reduced cell viability by 50% when compared to controls. This means that, the higher the CC₅₀ value, the less toxic is the (extract) to cells.

4.4. RT activity of fractionated plant extracts

Four plant samples were shortlisted for further analysis based on their IC₅₀, CC₅₀ and their selective index. Availability of plant materials at the the time of collection was also taken into account. The selected plants are shown in **Table 11**. The samples were fractionated using different solvent systems and their yields recorded (**Table 12**). For *T. sericea* stem, RT activity of 15.8%, 3.9% and 17% was obtained with 20% MeOH in DCM, 100% Ethyl Acetate and 100% MeOH, respectively compared to 7.3% activity with crude extracts (**Fig. 8**). RT activity of *T. pruniodes* roots were 3.5% (100% acetone), 1.2% (ethyl acetate), 12.9% (MeOH) and 33.6% for crude extracts (**Fig. 9**). From *D. mespiliformis* leaves, RT activity of 24.1% was obtained with 100% ethyl acetate (solvent) while the activity of crude extracts was 17% (**Fig. 10**). Furthermore, RT activity of *D. mespiliformis* stem were 44.5% (100% acetone) and 47% (100% MeOH), compared to 63.7% for crude extracts (**Fig. 11**).

Table 11: Plants shortlisted for fractionation.

Scientific name	Sample ID	Solvent	Plant part	IC ₅₀	CC ₅₀	SI value
<i>D. mespiliformis</i>	3-981	DCM: MeOH	Stem	Weak	Toxic	2.245
<i>D. mespiliformis</i>	3-936	DCM: MeOH	Leaves	Weak	Not toxic	10.71
<i>T. pruniodes</i>	3-935	DCM: MeOH	Roots	Potent	Low toxicity	189.4
<i>T. sericea</i>	2-625	DCM: MeOH	Stem	Potent	Low toxicity	7 680.0

Table 12: Yields of different fractionated plant samples

Scientific name	Part	Crude (g)	10%Acetone /DCM (mg)	50%Acetone /DCM (mg)	20%MeOH /DCM (mg)	Acetone (mg)	Ethyl Acetate (mg)	MeOH (mg)
<i>D. mespiliformis</i>	LV	120.9	532.8	330.4	24.8	597.8	554.6	436.5
<i>D. mespiliformis</i>	ST	114.4	836.7	216.3	147.9	414.9	467.2	380.6
<i>T. pruniodes</i>	RT	111.4	707.0	159.1	238.5	2357.0	2662.4	861.4
<i>T. sericea</i>	ST	112.2	836.7	216.3	147.9	414.9	467.2	380.6

Key: LV=Leaves; TB=Tuber; DB=Dead bark; LB=Live bark; RT=Roots; ST=Stems; WP=Whole plant

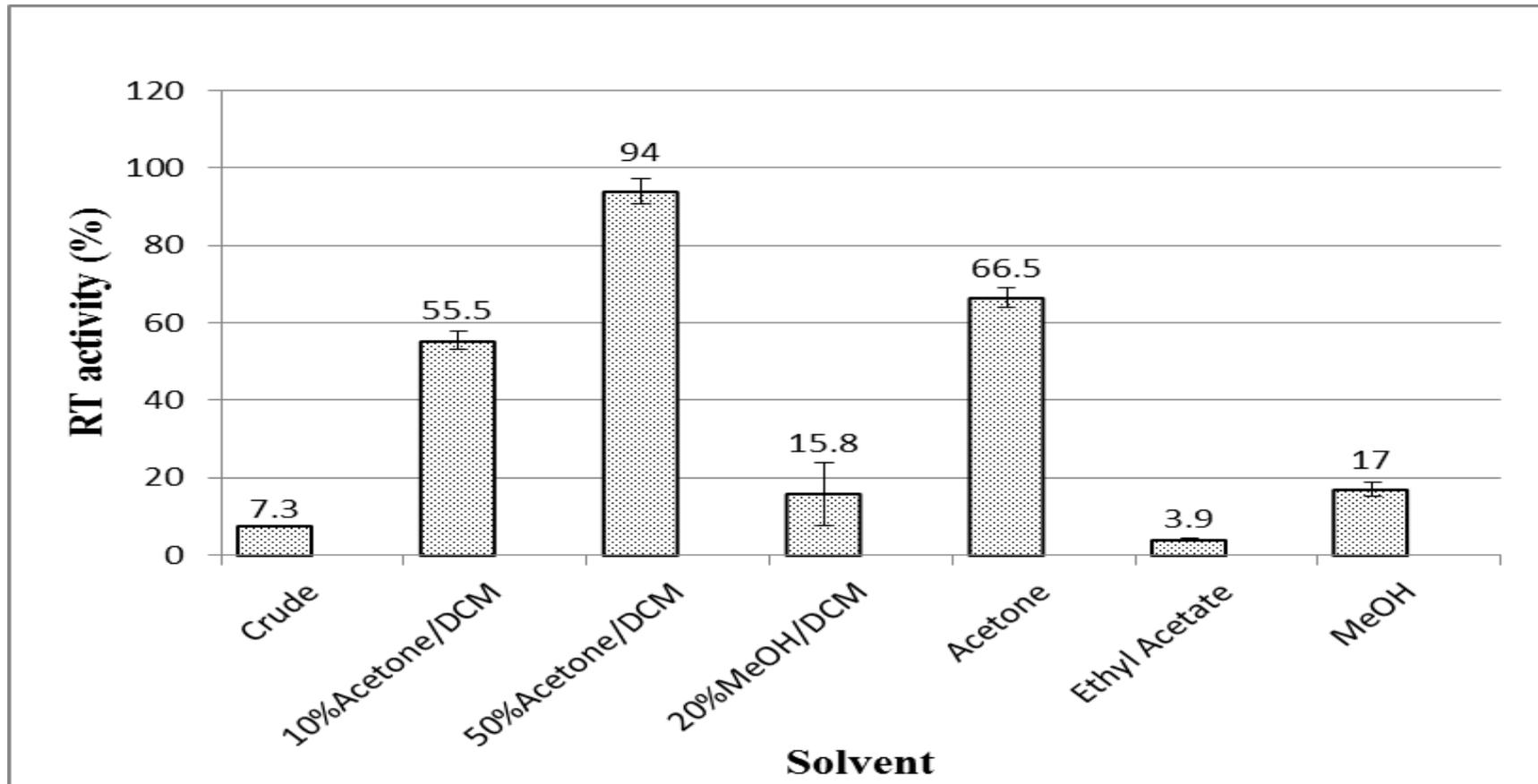


Figure 8: RT activity of *T. sericea* stem fractions at concentration of 1mg/ml. Means \pm SD of two replicates are presented.

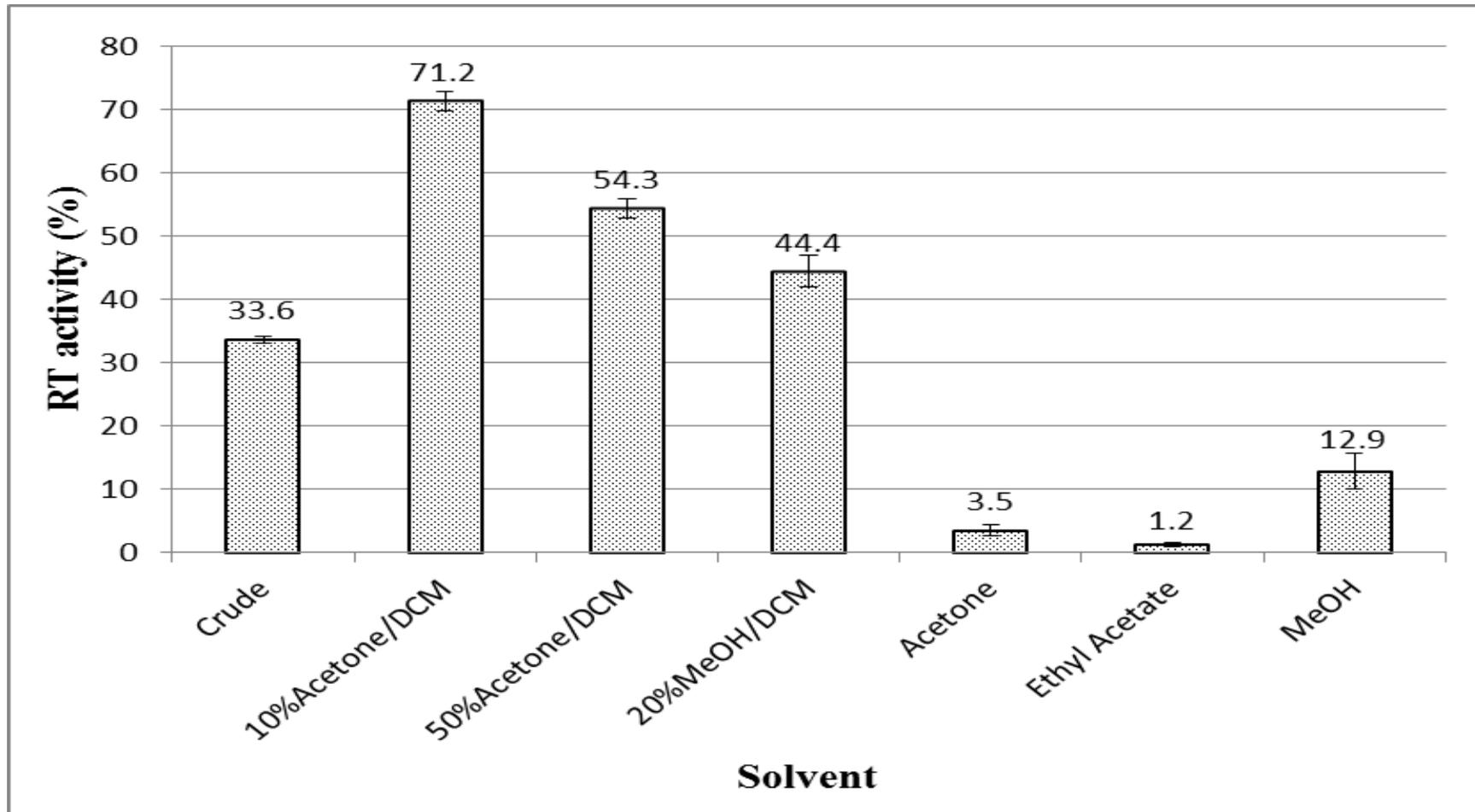


Figure 9: RT activity of *T. pruniodes* roots fractions at concentration of 1mg/ml. Means \pm SD of two replicates are presented.

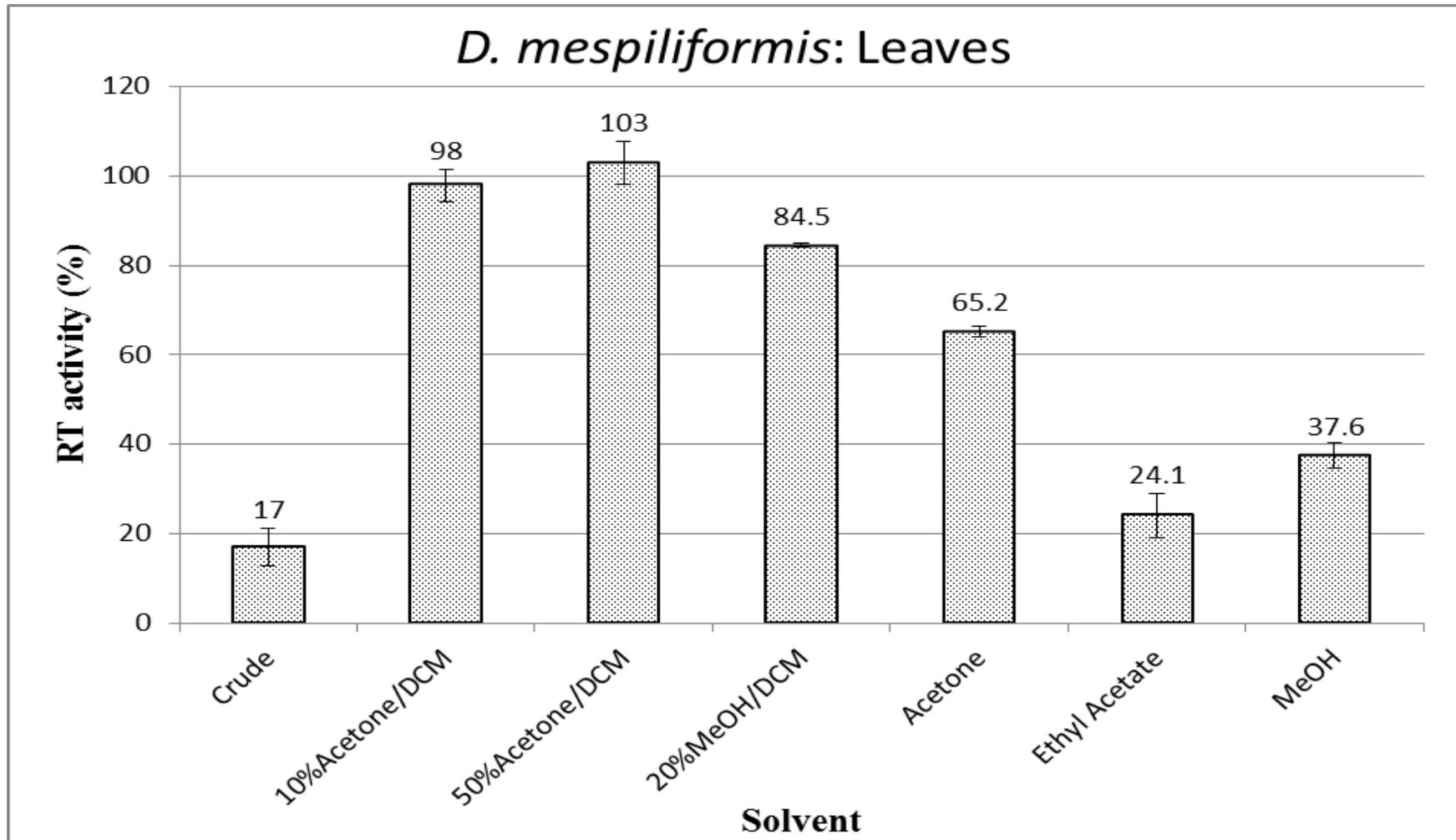


Figure 10: RT activity of *D. mespiliformis* leaves fractions at concentration of 1mg/ml. Means \pm SD of two replicates are presented.

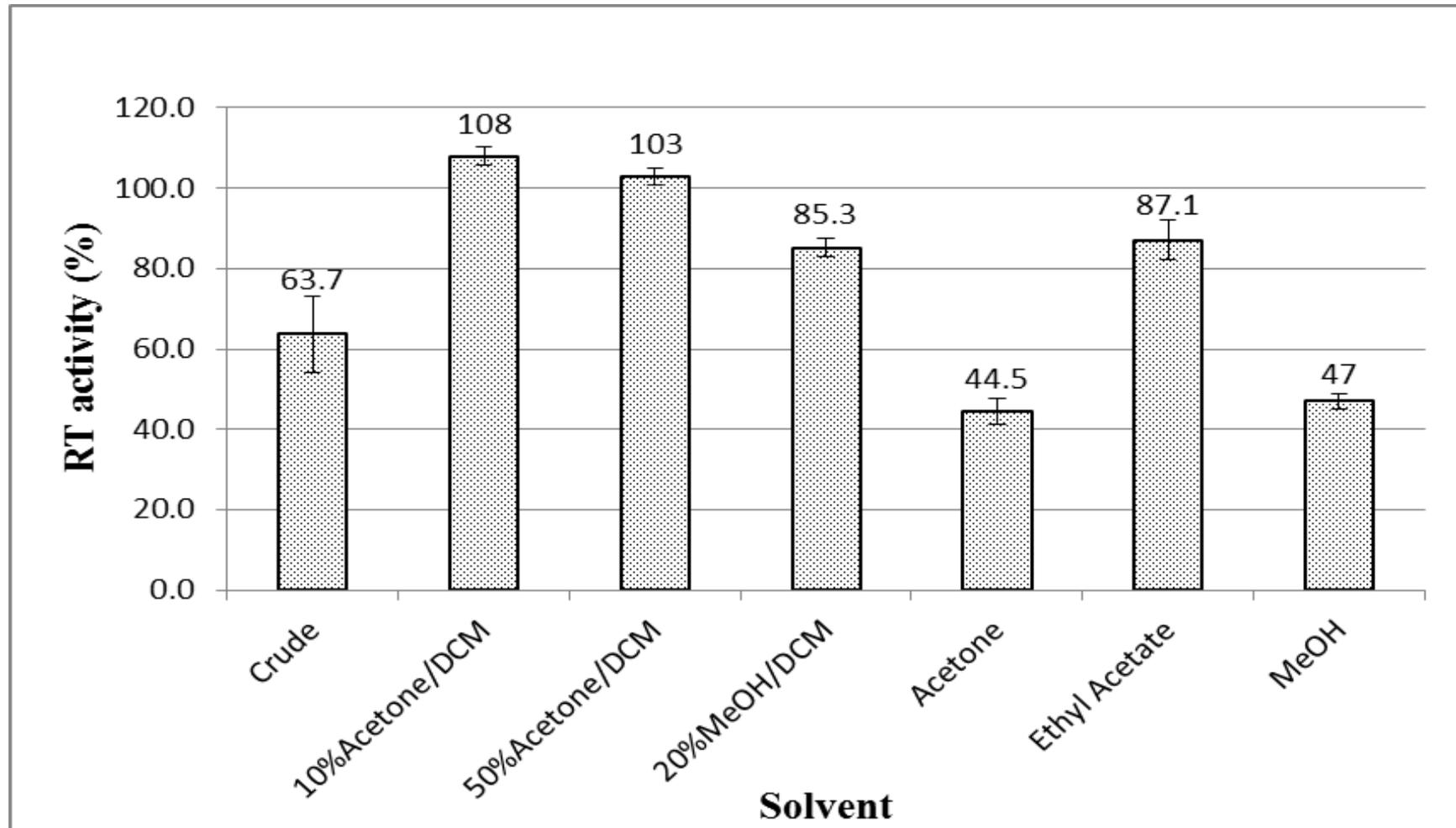


Figure 11: RT activity of *D. mesipiliformis* stem fraction at concentration of 1mg/ml. Means \pm SD of two replicates are presented.

4.5. Cytotoxicity properties of fractionated plant extracts

High viability of 95% or more was obtained with all crude extracts of all plant samples. In *T. sericea* stem, the only viability below 50% was with 50% acetone in DCM (**Fig. 12**). Low viability was obtained with 10% acetone and 50% acetone in DCM with fractions of *T. pruniodes* root (**Fig. 13**), and *D. mespiliformis* leaves (**Fig. 14**). Furthermore, high viability was observed with 20% MeOH in DCM in samples from *D. mepiliformis* leaves (**Fig. 14**) and stem (**Fig. 15**).

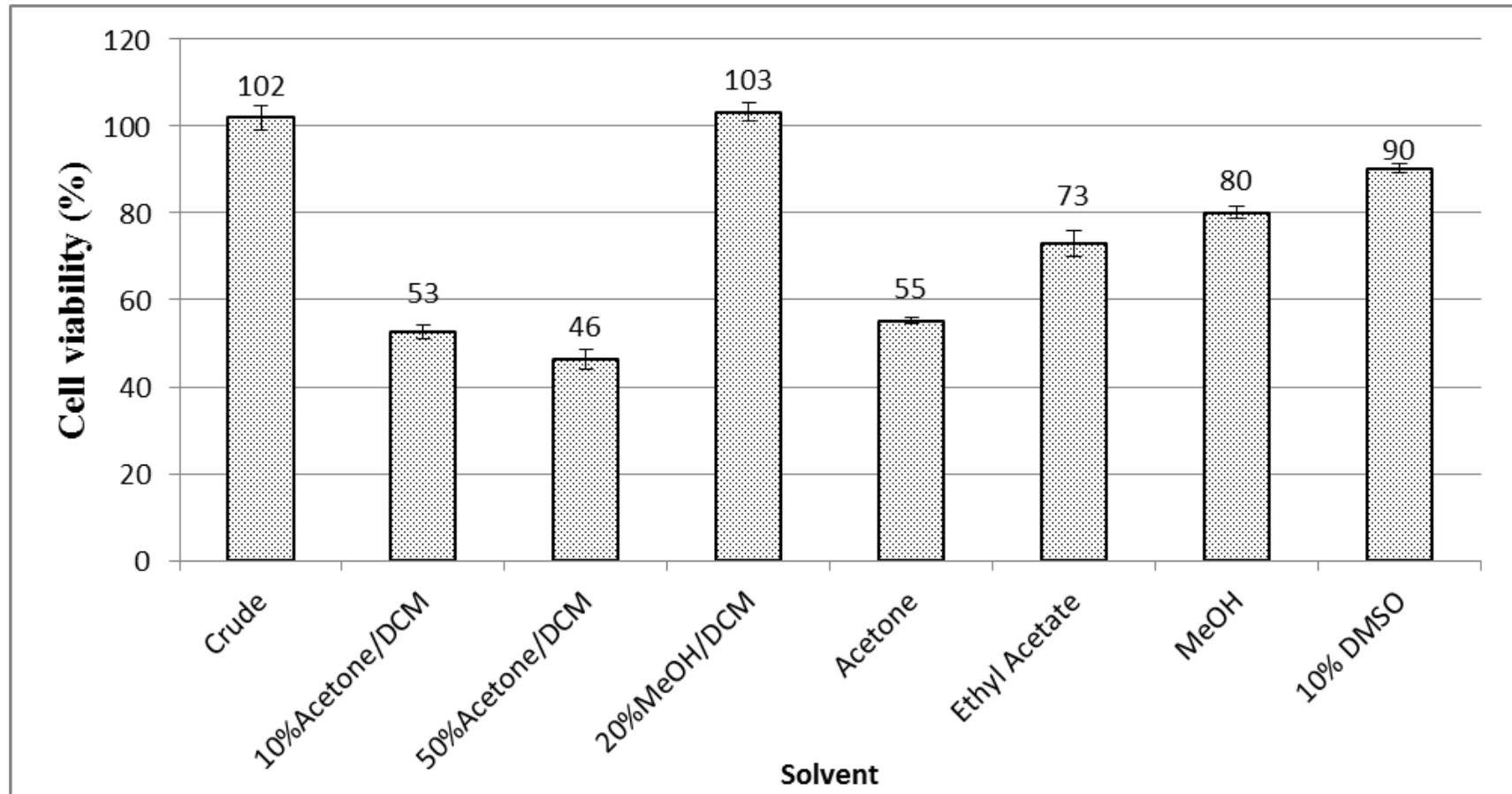


Figure 12: Cytotoxicity of *T. sericea* stem fractions on vero cells at concentration of 1mg/ml. Means \pm SD of two replicates are presented.

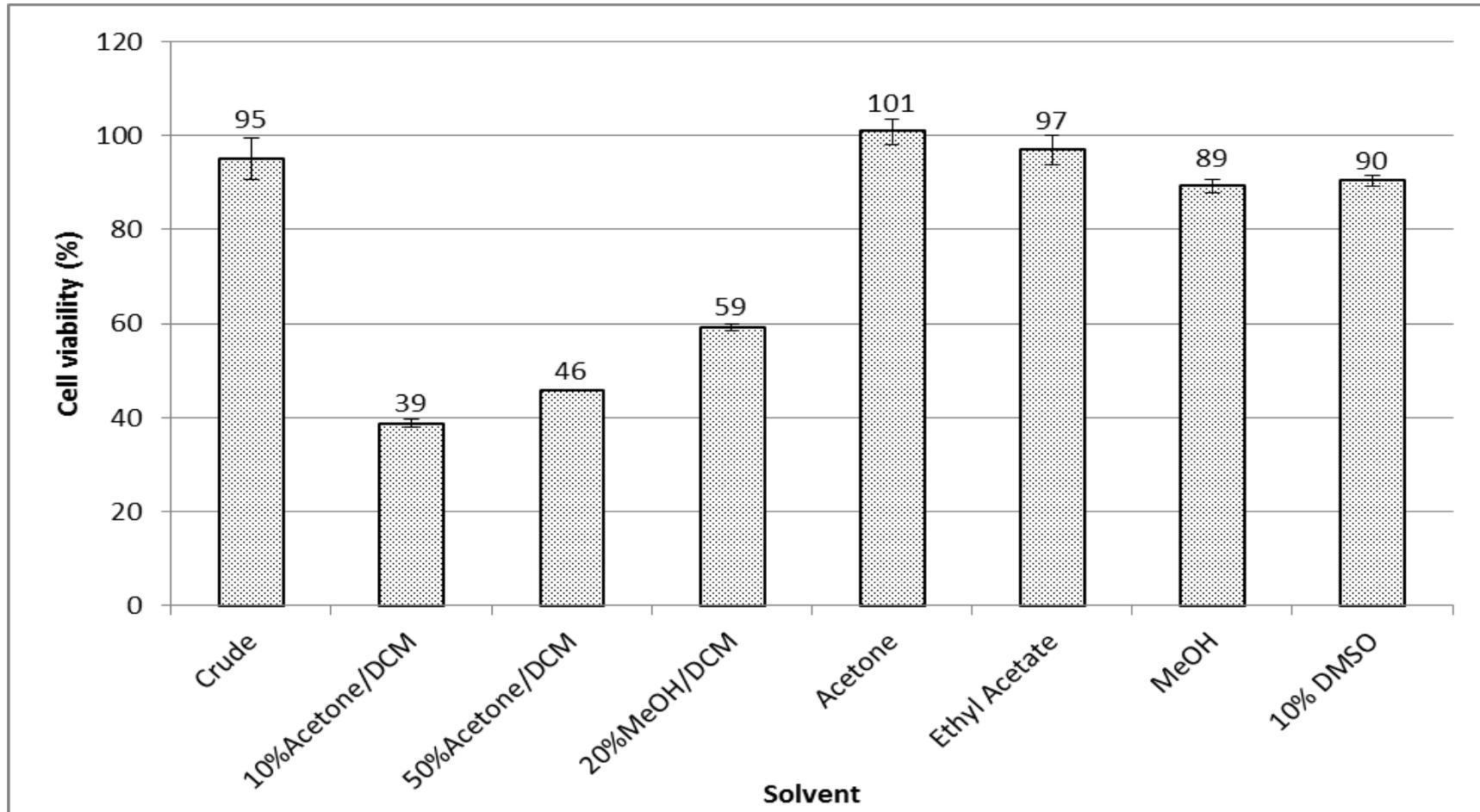


Figure 13: Cytotoxicity of *T. prunioides* root fractions on vero cells at concentration of 1mg/ml. Means \pm SD of two replicates are presented.

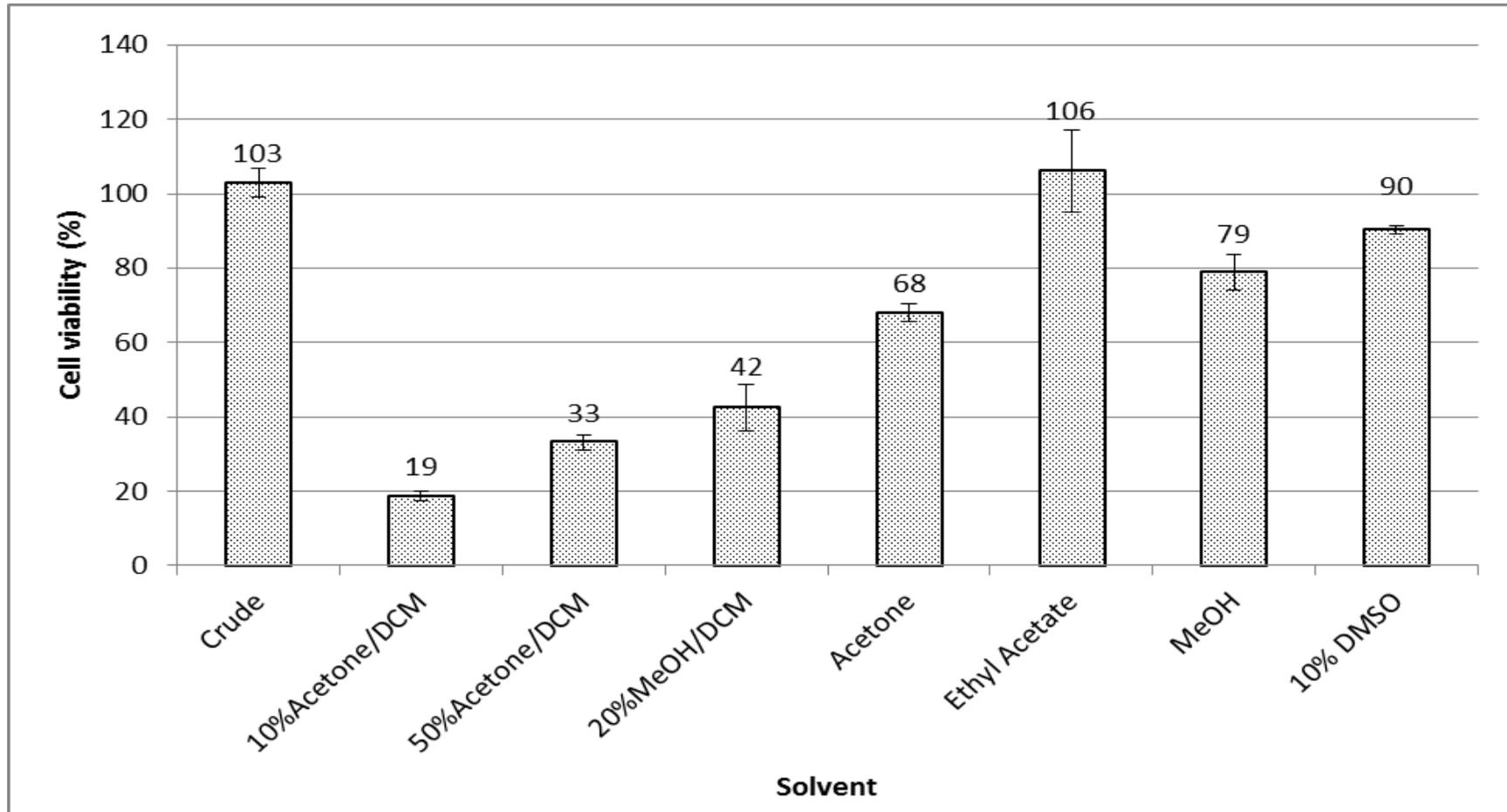


Figure 14: Cytotoxicity of *D. mespiliformis* leaves fractions on vero cells at concentration of 1mg/ml. Means \pm SD of two replicates are presented.

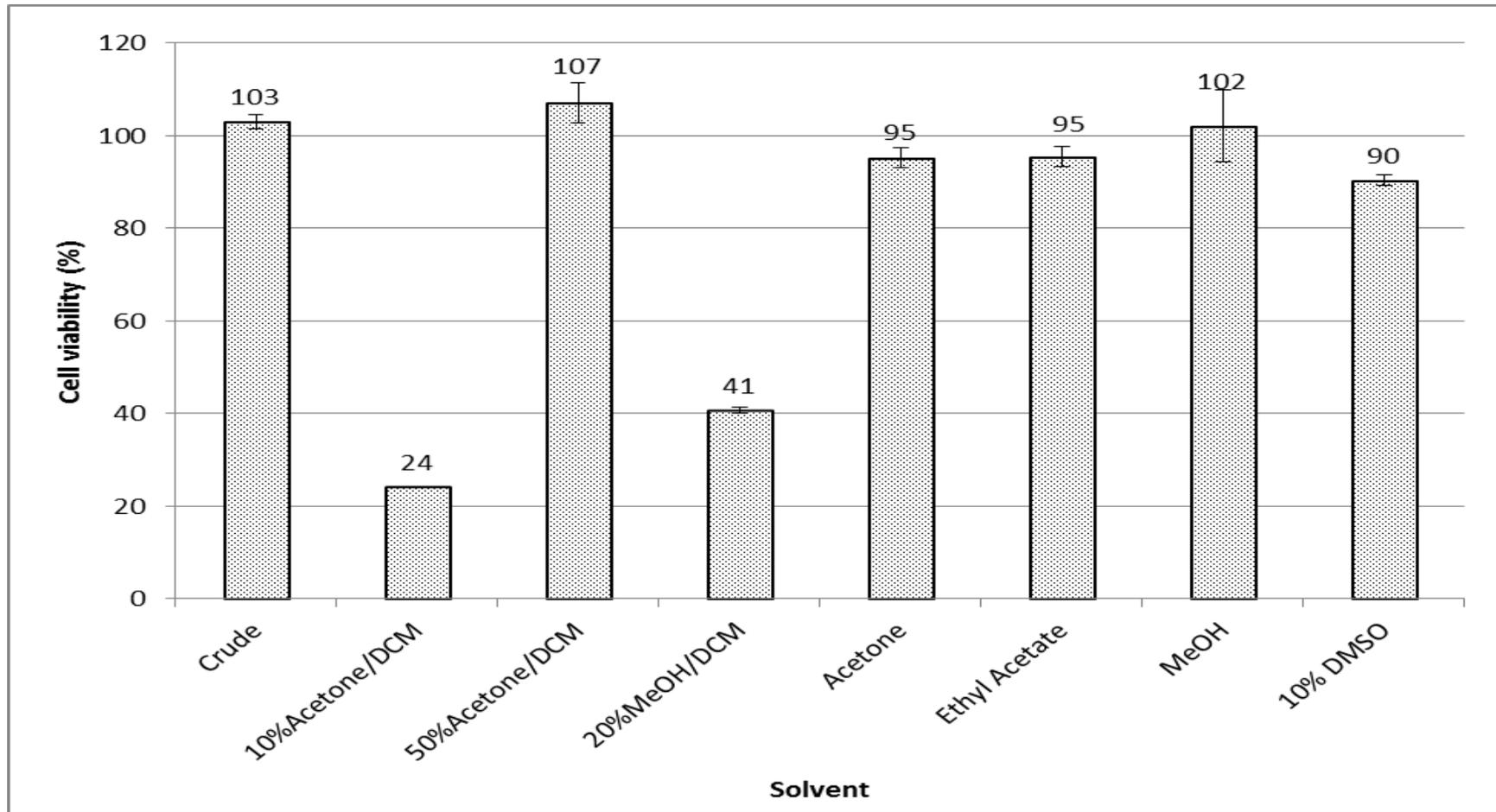


Figure 15: Cytotoxicity of *D. mespiliformis* stem fractions on vero cells at concentration of 1mg/ml. Means \pm SD of two replicates are presented.

4.6. RT activities of pure and semi-purified compounds

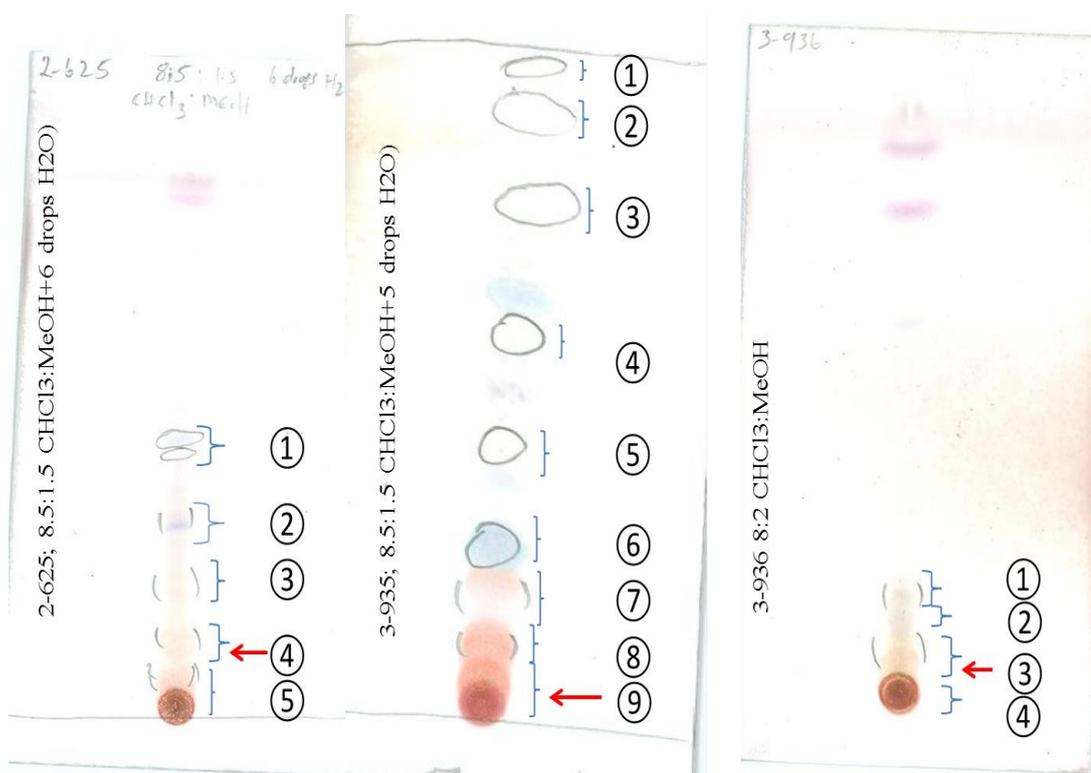
Four fractions were chosen for purification into pure compounds with reversed phase (glass coated) preparatory TLC plates (Silica gel 60, F₂₅₄, 20x20 cm). They were chosen based on their low RT activity as compared to the RT activity of their crude extracts. The active fractions were ethyl acetate for *T. sericea* (stem) (3.9%), *T. pruniodes* (roots) (1.2%) and *D. mespiliformis* leaves (24.1%) while acetone was the active fraction for *D. mespiliformis* stem (44.5%). All the fractions had viability ranging between 95-107%, except for *T. sericea* fractions with viability of 73%. The RT activities and viability of selected fractions are presented in **Figure 16**.

4.6.1. RT activities of semi-pure compounds

Several impure (semi-pure) compounds were obtained from each fraction and their inhibitory activities on RT as compared to the rest of compounds from respective fractions are shown in **figures 17-20**. Compound no. 4 was the active one for *T. sericea* with RT activity of 40%, whereas compound no. 9 was the active one for *T. pruniodes* with RT activity of 1.2%. For *D. mespiliformis* leaves and stems, compounds no. 3 were the active ones with RT activities of 1.3% and 34.1%, respectively. The TLC profiles of fractionated (semi-pure) compounds are shown in **Picture 3**.

4.6.2. IC₅₀ of the most active semi purified compounds on RT

Serial dilutions of the active compounds were done in order to determine their IC₅₀. The respective IC₅₀'s were: 1.788mg/ml (*T. sericea*); 0.000014mg/ml (*T. pruniodes*); 0.1376mg/ml and 1.1484mg/ml for *D. mespiliformis* leaves and stems, respectively (Figure 21).



Picture 3: TLC profiles of fractions from 3 plants.

The arrow points to the region with inhibitory activity against RT.

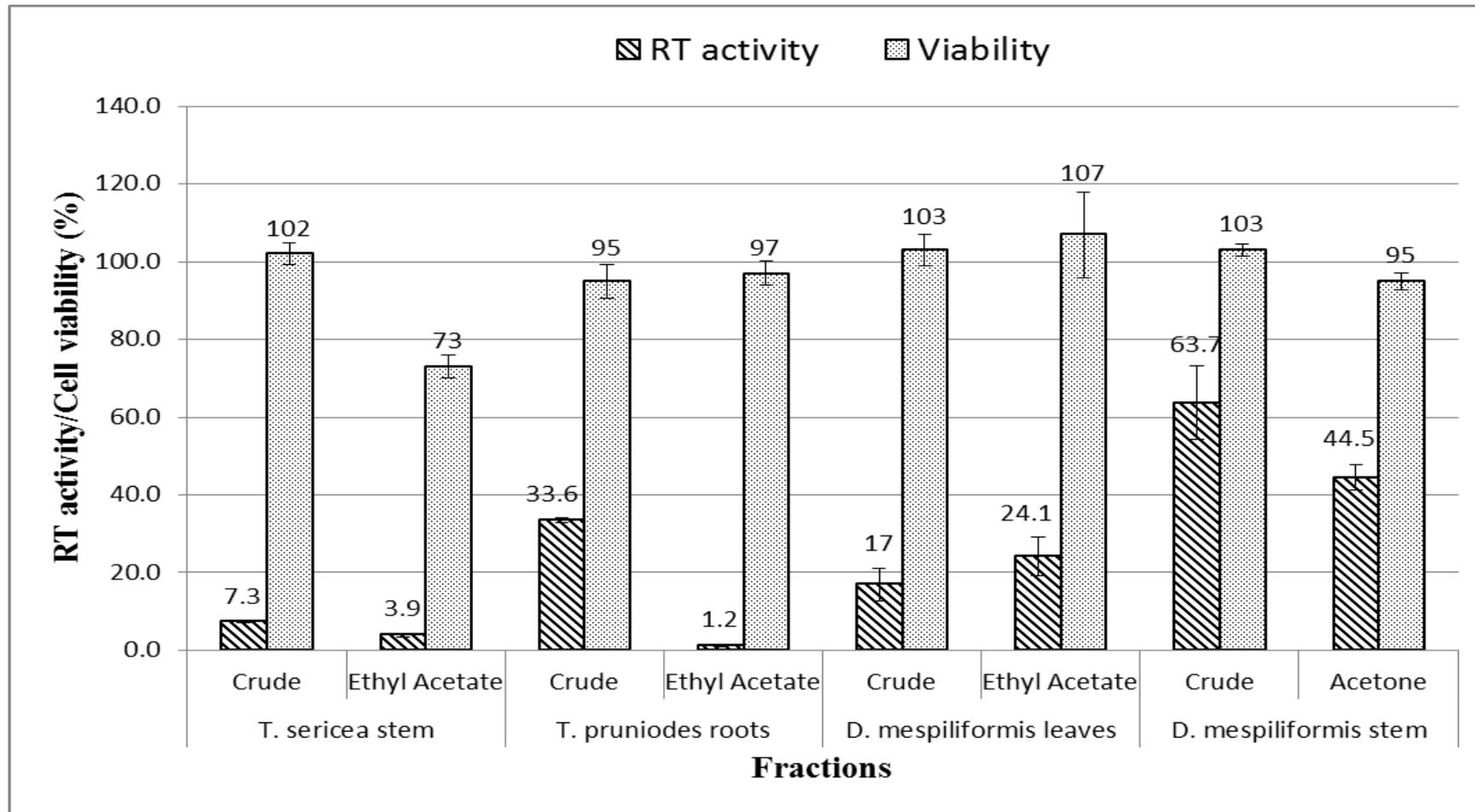


Figure 16: RT activity and cell viability of fractions selected for isolation of compounds. Means \pm SD of two replicates are presented.

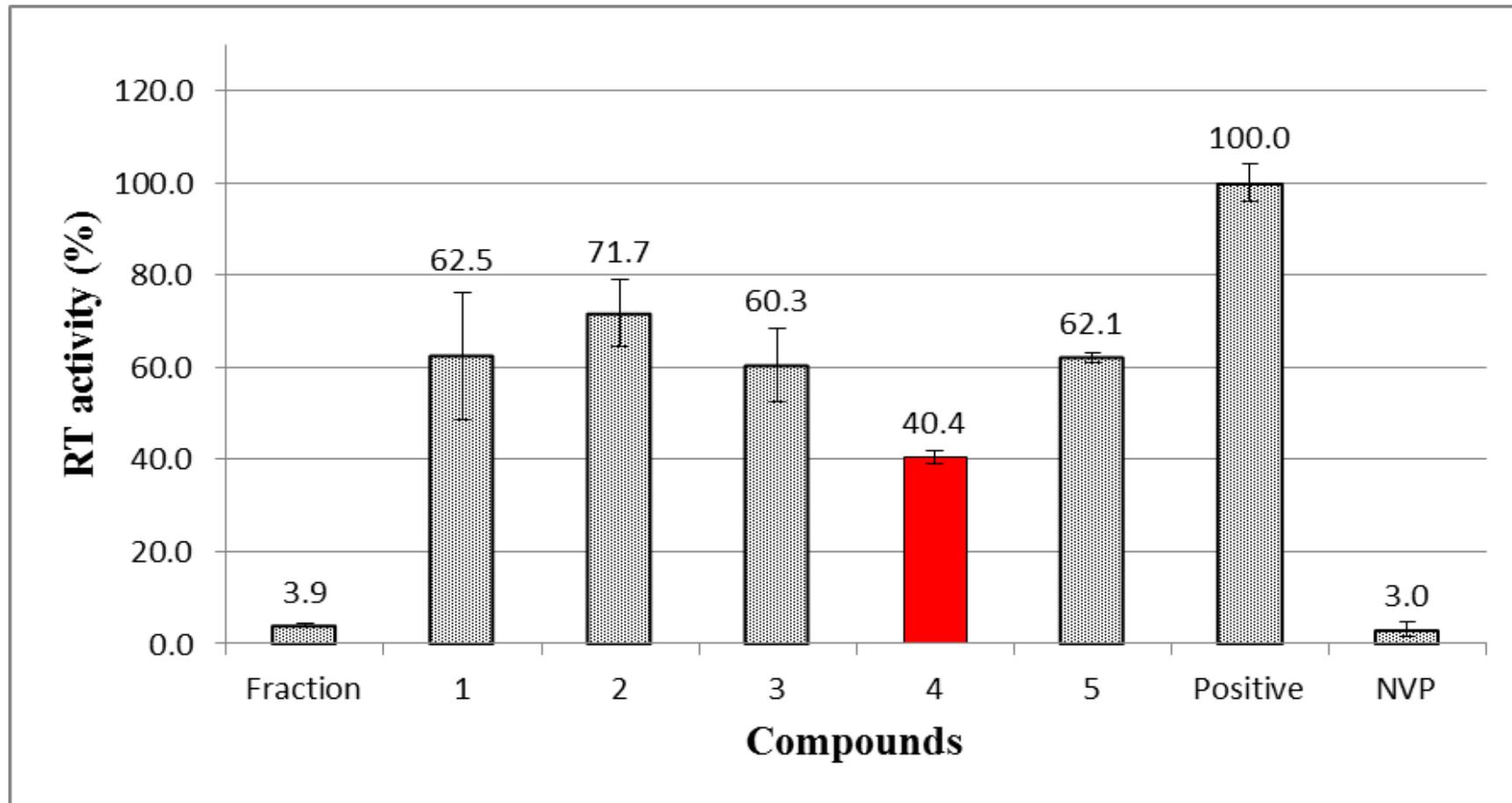


Figure 17: RT activity of semi-purified compounds from *T. sericea* stem. Means \pm SD of two replicates are presented.

The compound highlighted in red is the one which was selected for further purification.

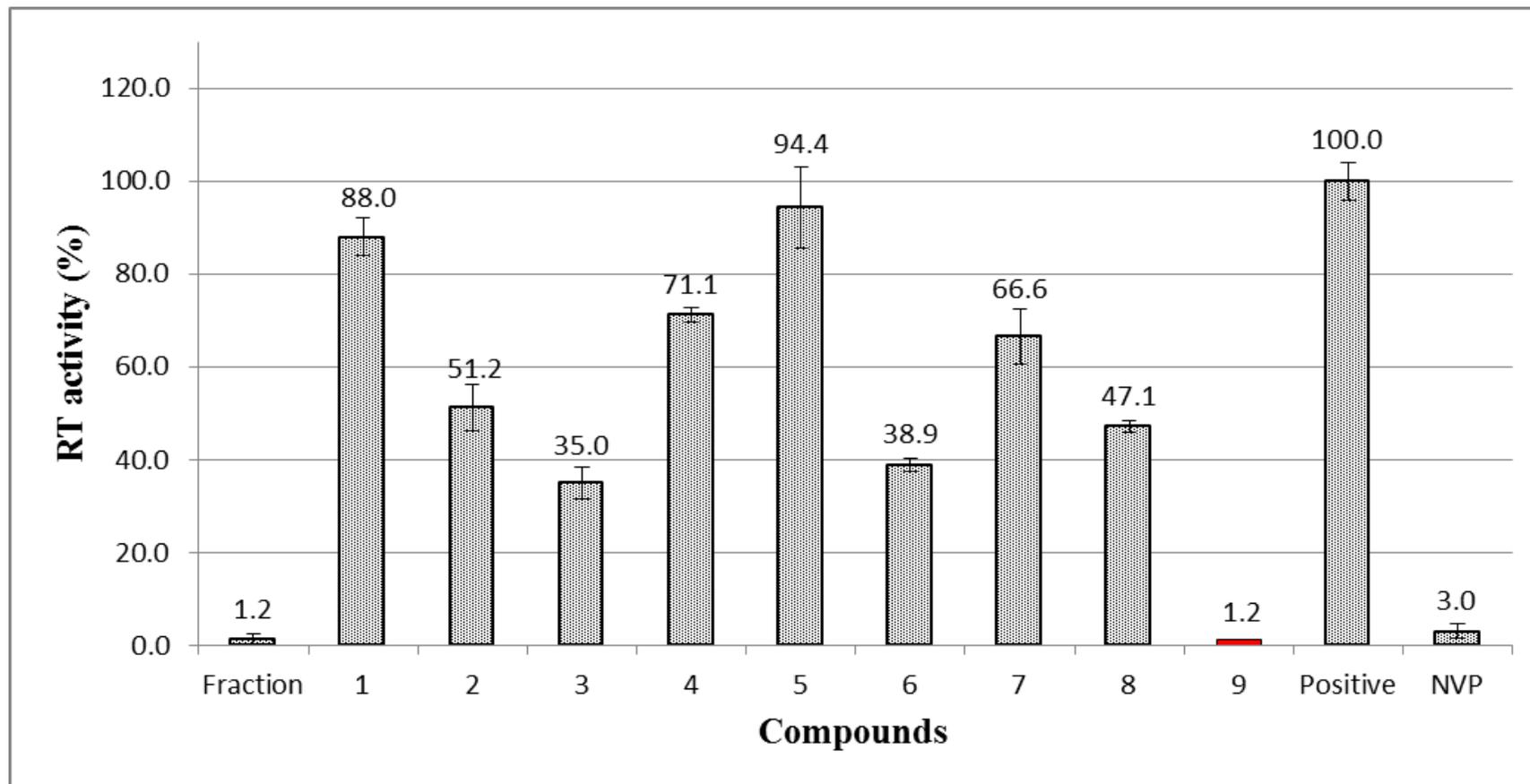


Figure 18: RT activity of semi-purified compounds from *T. pruniodes* roots. Means \pm SD of two replicates are presented.

The compound highlighted in red is the one which was selected for further purification.

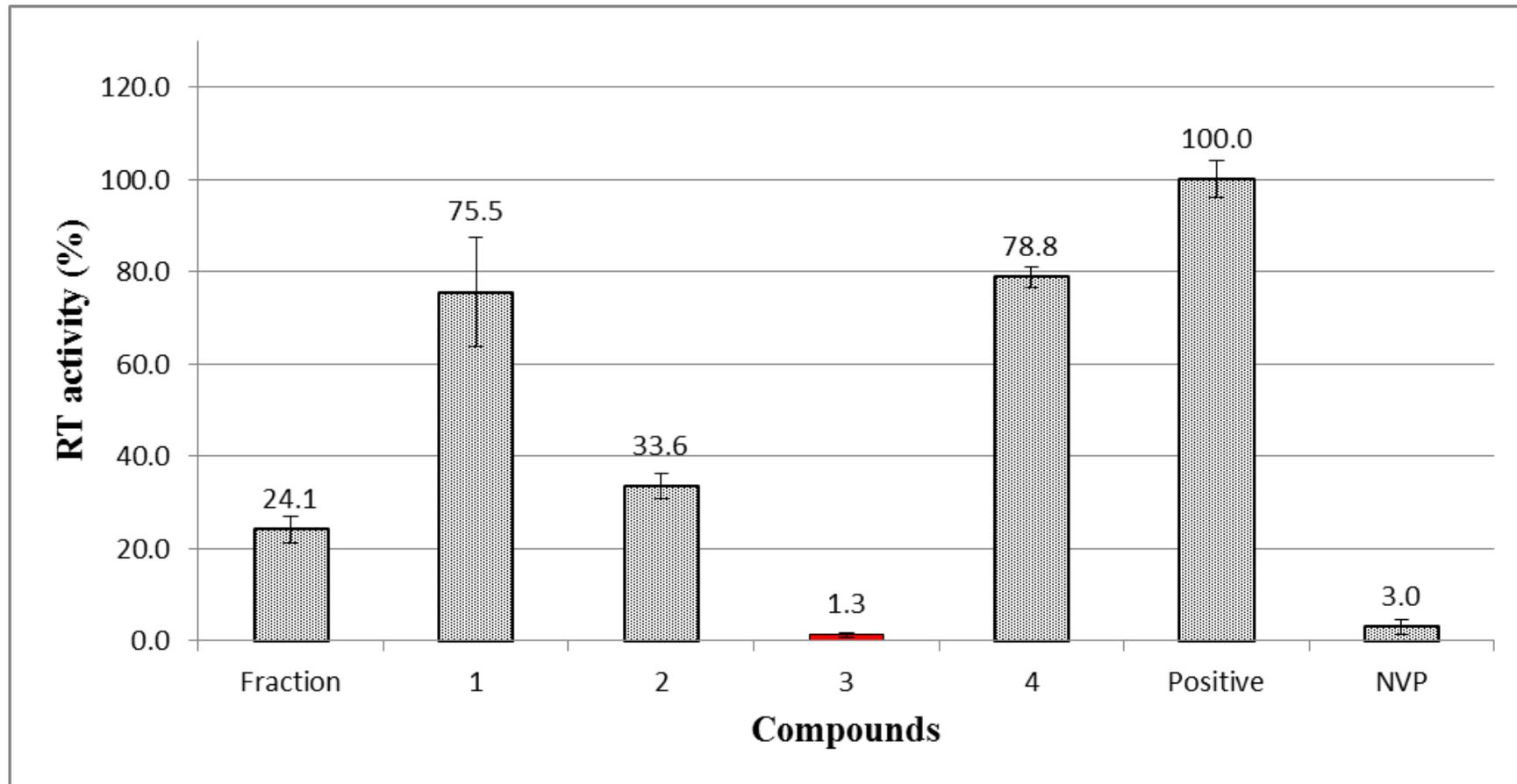


Figure 19: RT activity of semi-purified compounds from *D. mespiliformis* leaves. Means \pm SD of two replicates are presented.

The compound highlighted in red is the one which was selected for further purification.

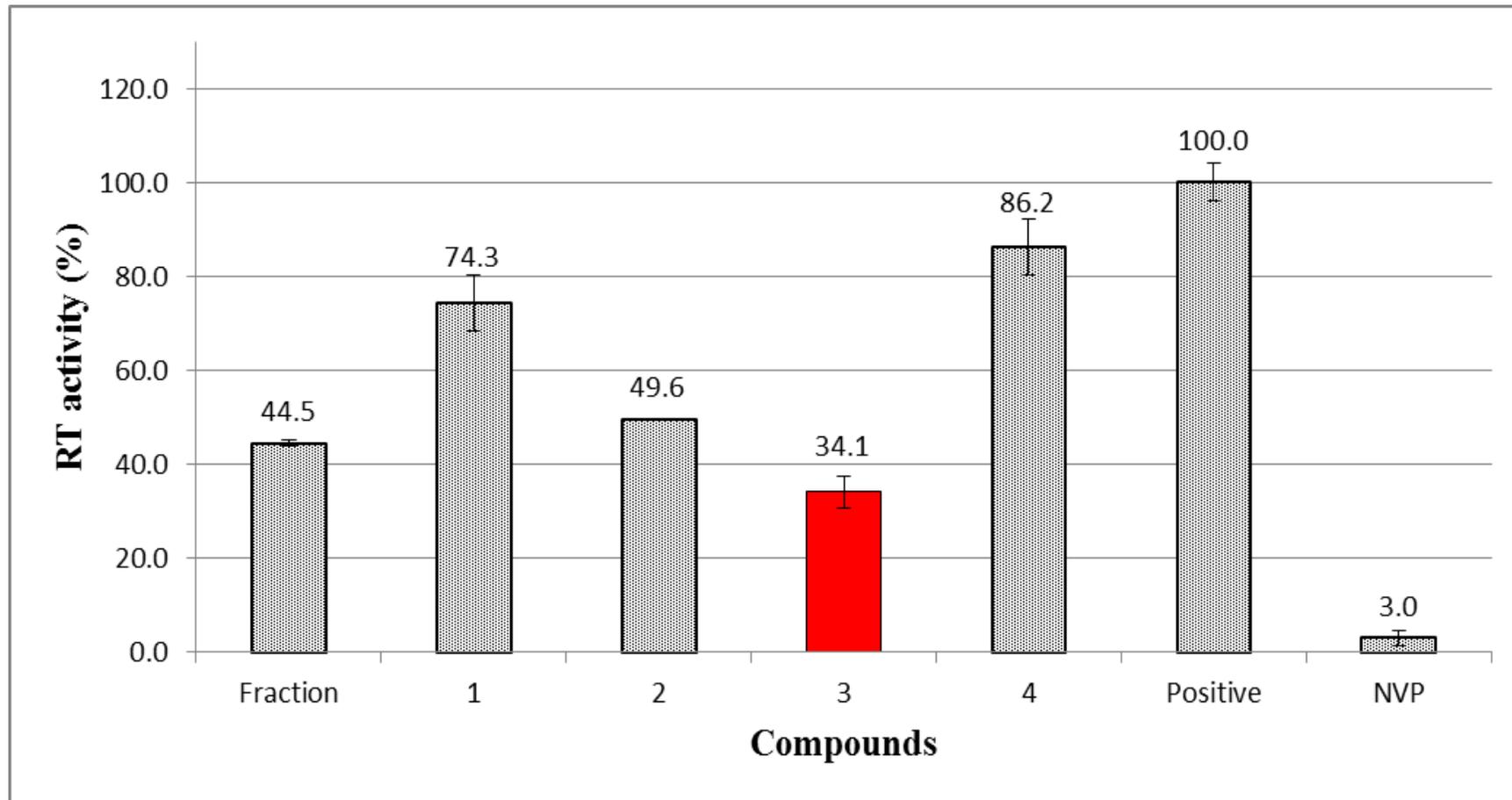


Figure 20: RT activity of semi-purified compounds from *D. mespiliformis* stem. Means \pm SD of two replicates are presented.

The compound highlighted in red is the one which was selected for further purification.

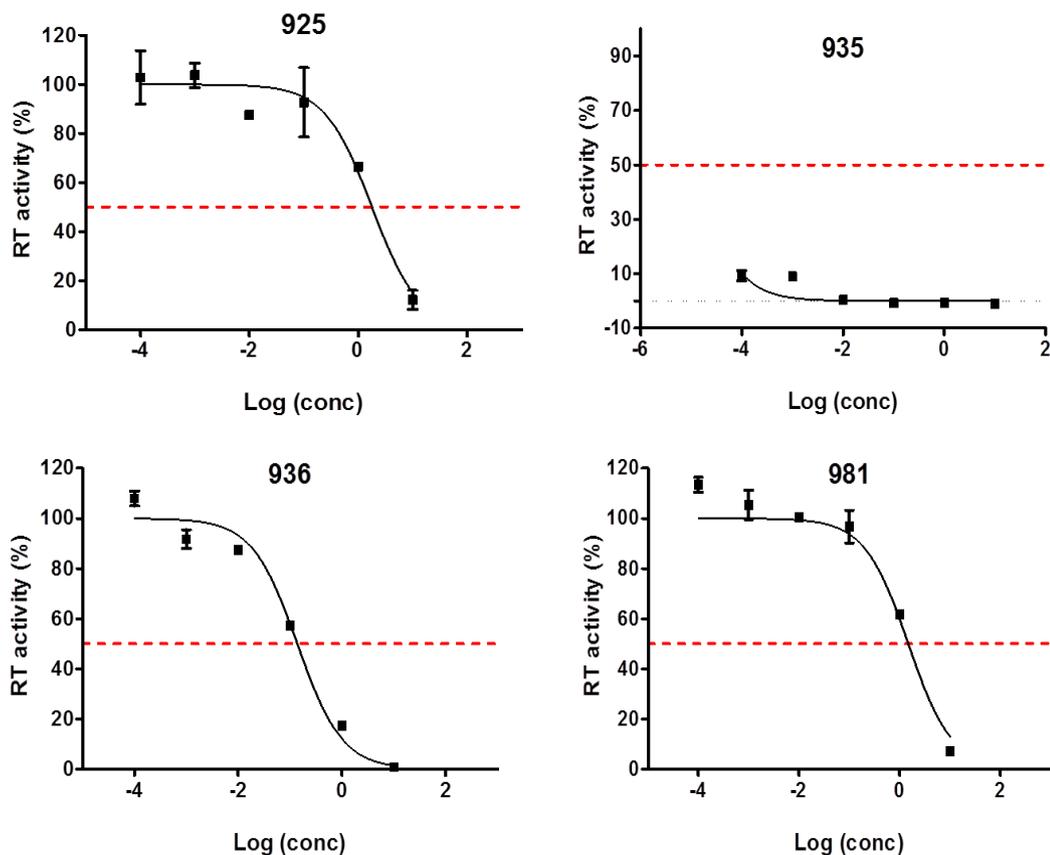


Figure 21: IC₅₀ of semi-purified compounds.

2-625=*T. sericea* stems; 3-935=*T. pruniodes* roots; 3-936=*D. mespiliformis* leaves, and 3-981=*D. mespiliformis* stems.

4.6.3. RT activities of purified compounds

The semi-pure compounds were further purified and 3 pure compounds were isolated and their inhibitory activities were tested on RT. The RT activities of the purified compounds were 1.6% (*T. sericea*); 0.8% (*T. pruniodes*); and 7.2% (*D. mesipliformis*), while that of Nevirapine was 2.8%. The inhibitory activities of purified compounds as compared to unpurified compounds are shown in **Figures 22-24**. The TLC profile purified compounds are shown in **Picture 4**.

4.6.4. IC₅₀ of the pure compounds on RT

The IC₅₀'s of the purified compounds were determined: 0.06 mg/ml (*T. sericea*) (Epigallocatechin); 0.006 mg/ml (*T. prunioides*) (triterpenoid glycoside); and 0.06 mg/ml (*D. mespliformis*); (Naphthoglycoside/Anthraquinoline glycoside) while that of Nevirapine was 4.966μM (**Figure 25**). The IC₅₀'s of identified structures (of compounds) were: 5.75 mg/ml (A2: non acetylated) and 10.13 mg/ml (Ac-C: acetylated) ($2\alpha,3\beta,19\alpha,23$ -Tetrahydroxyolean-1-en-28β-D-glycopyranoside) both isolated from *T. prunioides*; and 0.63 mg/ml for D-Pinitol isolated from *D. mespliformis* (**Figure 26**).



Picture 4: TLC profiles of purified compounds.

The red arrows points to the compound with the most inhibitory activity against RT, while the blue arrow points to the compounds whose structures were elucidated.

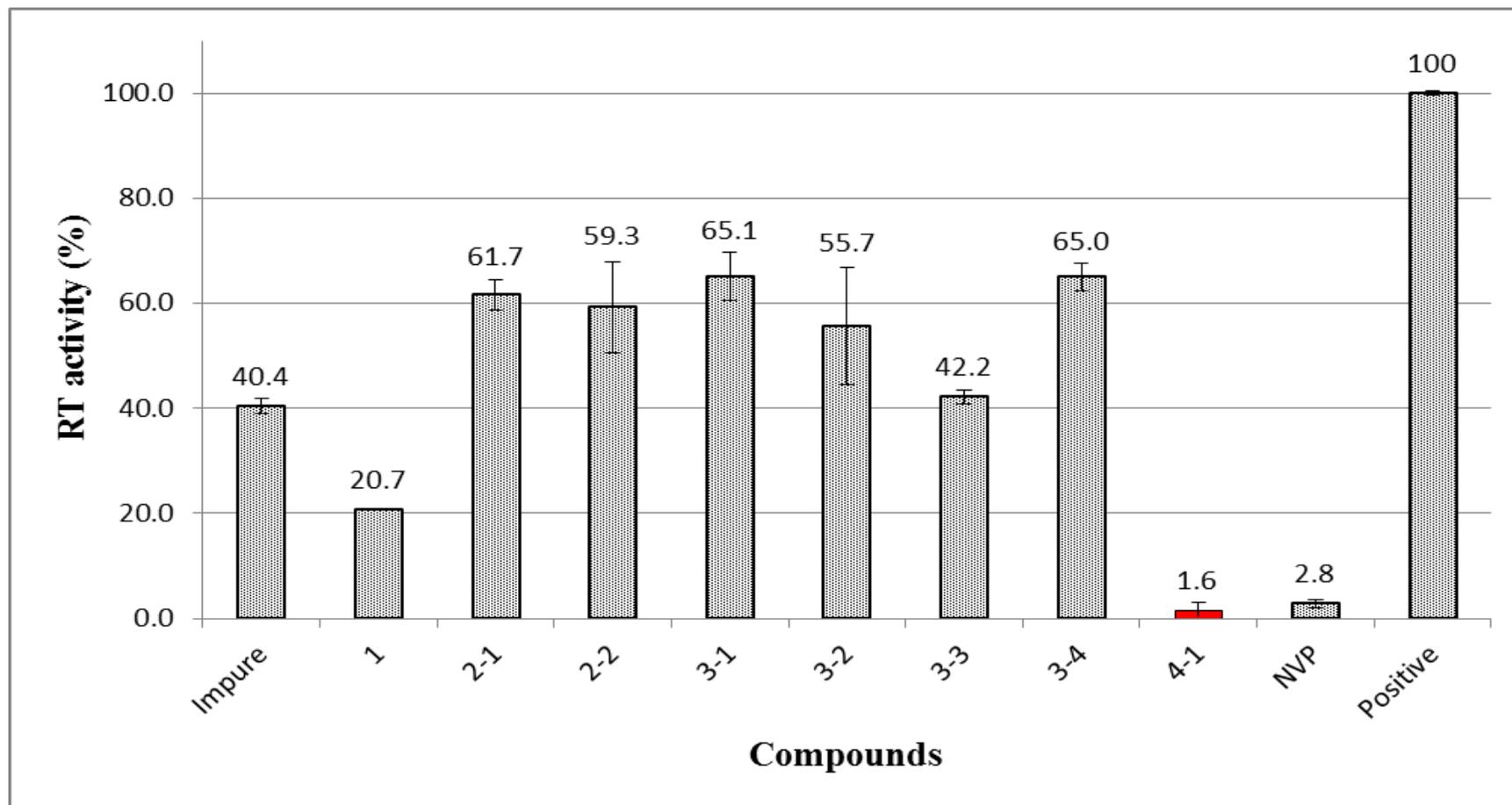


Figure 22: RT activity of purified compounds from *T. sericea* stem. Means \pm SD of two replicates are presented.

Compound 4-1 is **Epigallocatechin**. The compound with lowest RT activity is highlighted in red.

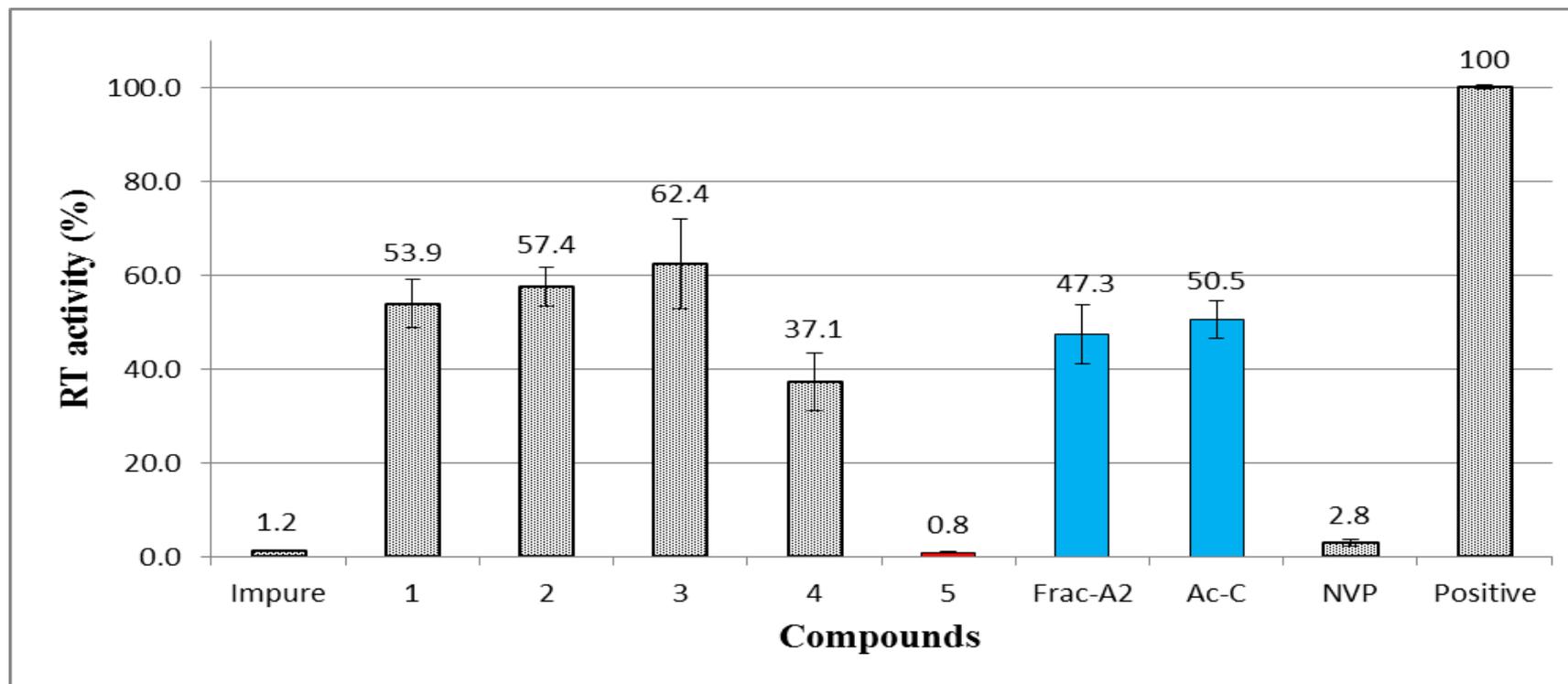


Figure 23: RT activity of purified compounds from *T. pruniodes* roots. Means \pm SD of two replicates are presented.

The compound with lowest RT activity is highlighted in red. Compounds highlighted in blue are those whose structures were elucidated. Frac A2 and Ac-C is non-acetylated and acetylated forms of the same compound and were identified as $2\alpha,3\beta,19\alpha,23$ -Tetrahydroxyolean-1-en-28 β -D-glycopyranoside (Masilinic acid group). Compound 5 is a Triterpenoid glycoside (preliminary). The compound with lowest RT activity is highlighted in red.

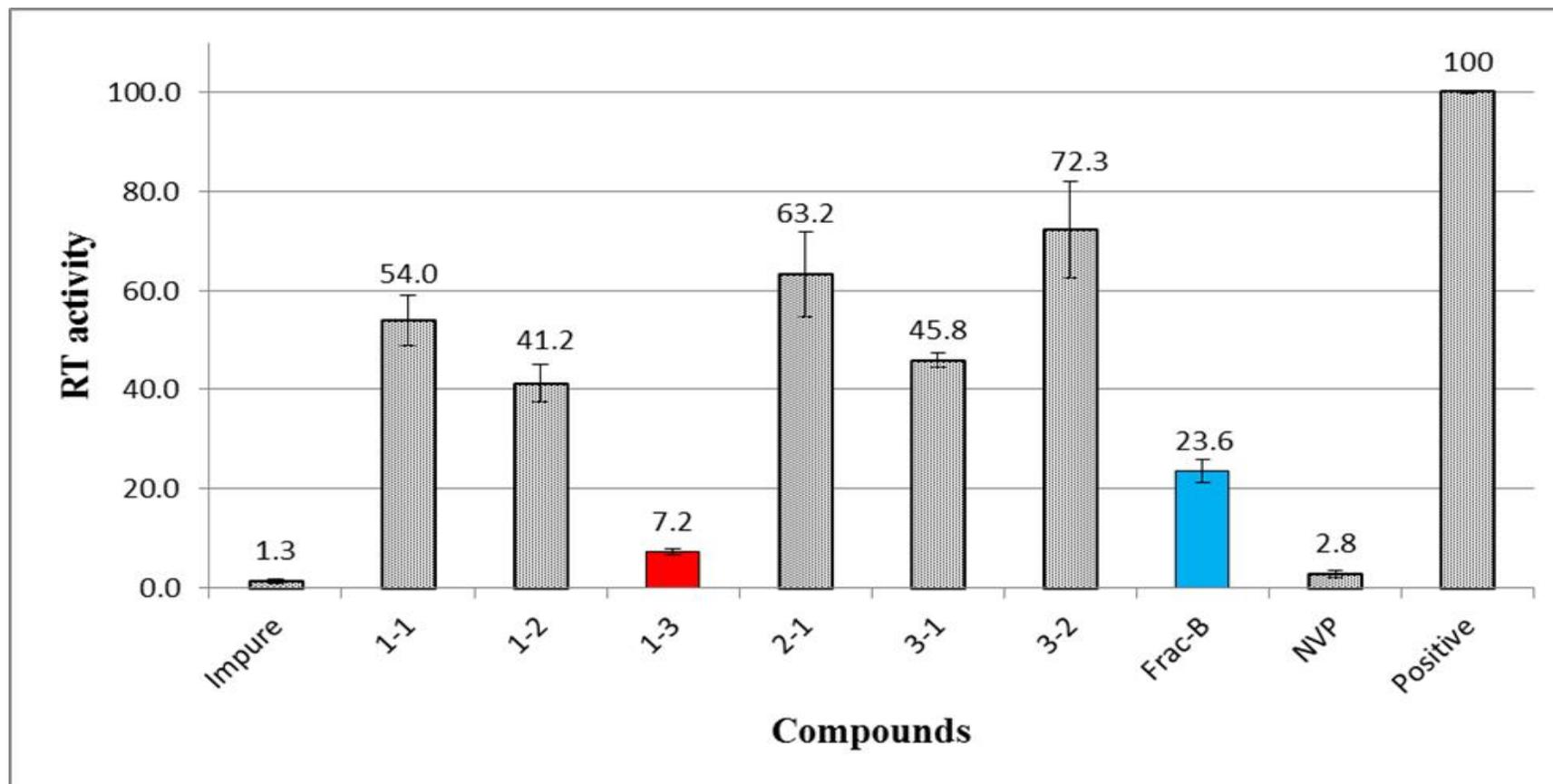


Figure 24: RT activity of purified compounds from *D. mespiliformis* leaves. Means \pm SD of two replicates are presented.

The compound with lowest RT activity is highlighted in red. Compound highlighted in blue is the one whose structure was elucidated. Frac B was identified as D-Pinitol (a sugar). Compound 1-3 is a Naphthoglycoside/Anthraquinoline glycoside.

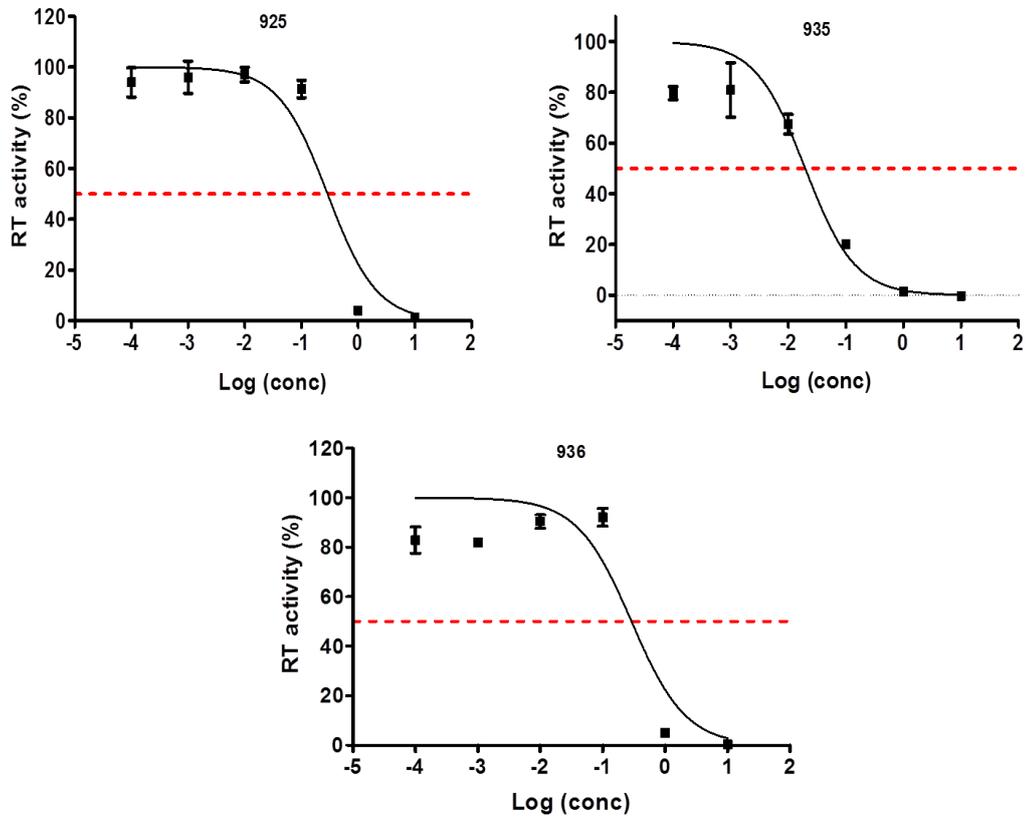


Figure 25: IC₅₀ of purified compounds whose structures were not fully elucidated.

2-625= *T. sericea* stems; 3-935= *T. pruniodes* roots; and 3-936= *D. mesipiliformis*

leaves.

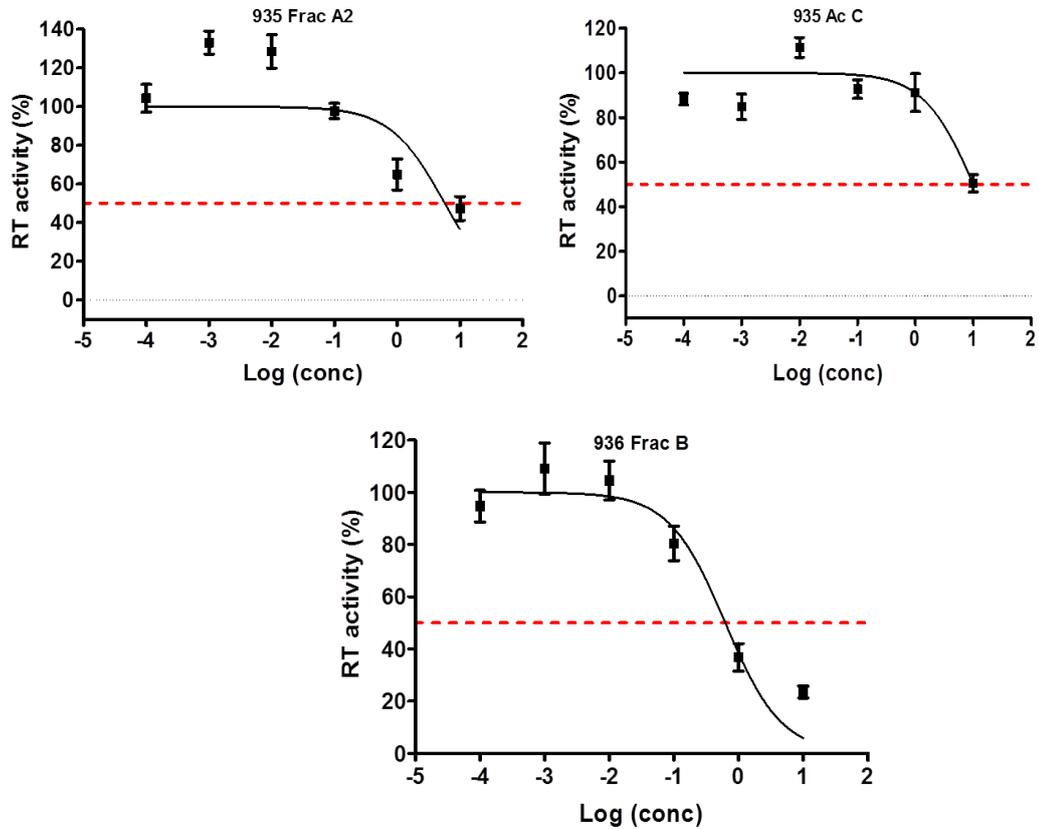


Figure 26: IC₅₀ of purified compounds whose structures were fully elucidated.

3935 Frac A2 (non-acetylated) and 3-935 Ac-C (acetylated) both isolated from *T. prunioides* and identified as **2 α ,3 β ,19 α ,23-Tetrahydroylean-1-en-28 β -D-glycopyranoside** and 3-936 Frac B isolated from *D. mespliformis* was identified as **D-Pinitol**.

4.6.5. Toxicity of purified compounds

Toxicities of purified compounds were determined by calculating their CC_{50} values. The compounds had higher CC_{50} values which are indicative of their low toxicities to Vero cell lines used in the study. The following CC_{50} were recorded: 10.39mg/ml (*T. sericea*); 10.92mg/ml (*T. pruniodes*); and 11.63mg/ml (*D. mespiliformis*) (**Figure 27**).

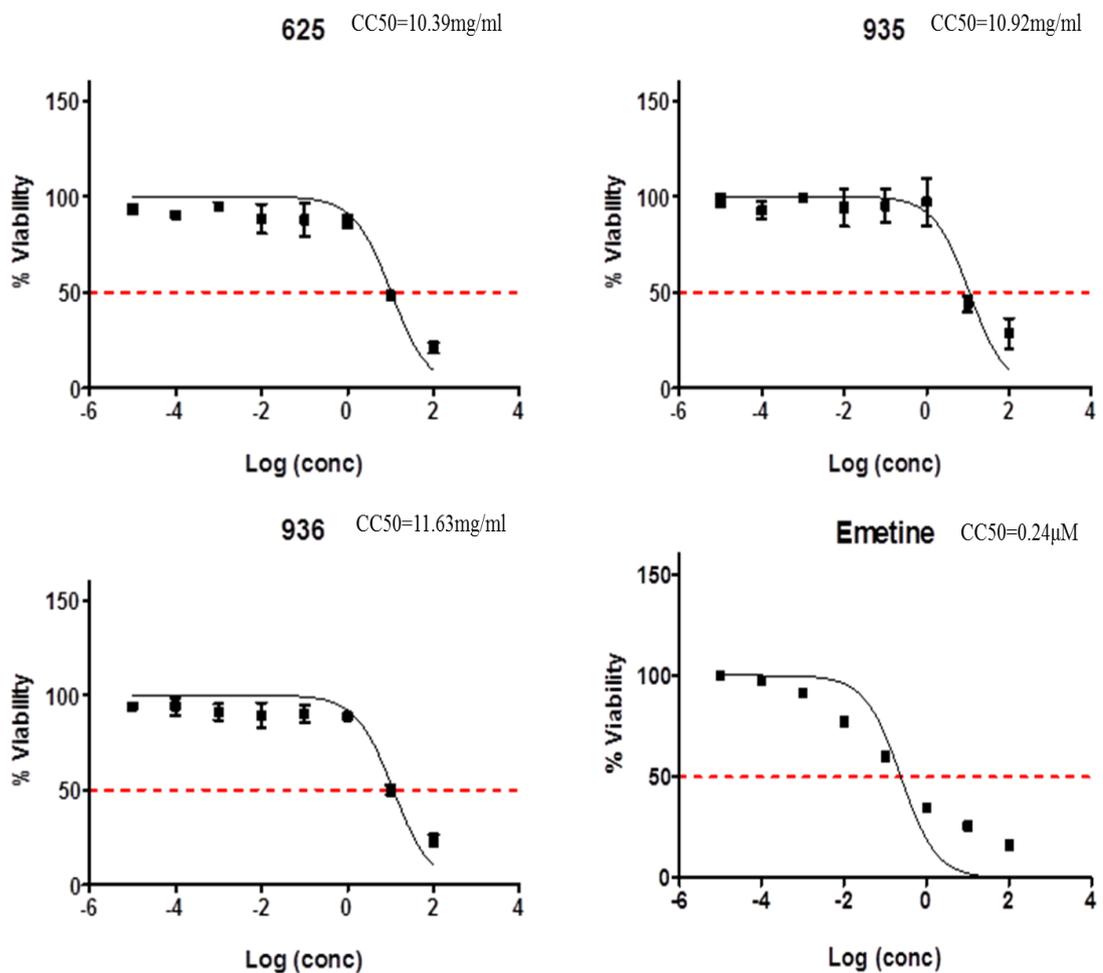


Figure 27: CC_{50} of purified compounds (Unidentified), tested on Vero cells.

Means \pm SD of two replicates are presented: 625= *T. sericea* (stem); 935= *T. pruniodes* (roots) and 936= *D. mespiliformis* (leaves).

4.7. Structural identification of the isolated compounds

a) D-Pinitol

The compound from *D. mesipiliformis* was identified as D-Pinitol. The compound was obtained as a light yellow powder. The chemical formula is $C_7H_{14}O_6$ (**Figure 28**), and molecular weight of 194.18. ^{13}C NMR (methanol (CD_3OD), 600MHz) revealed the presence of methoxy group (OCH_3) at 60.86, 84.55 (C-5), 73.91 (C-5), 73.47 (C-2), 73.17 (C-1), 72.33 (C-6), 71.66 (C-4). In its 1H NMR (CD_3OD , 600MHz), H-1 at 3.33, H-2 at 3.66, H-3 at 3.77, H-6 at 3.82, and H-4 and H-5 at 3.97 (**Figure 29**).

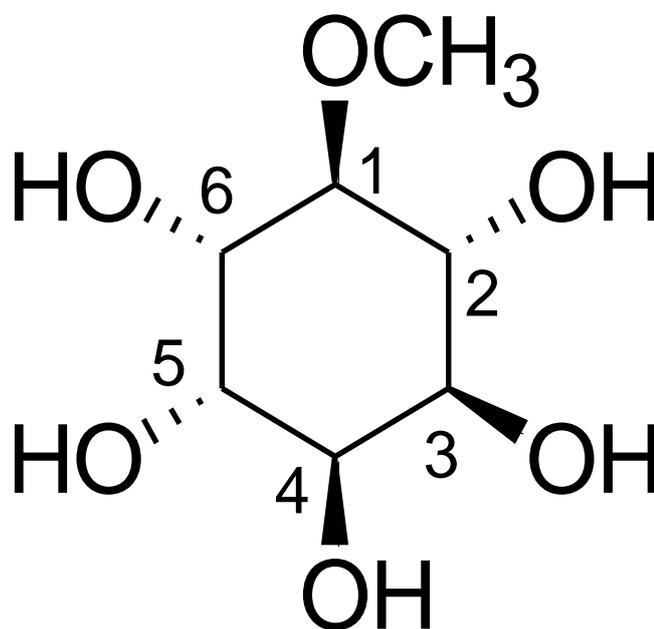


Figure 28: Structure of D-Pinitol isolated from *D. mesipiliformis* leaves.

b) 2 α ,3 β ,19 α ,23-Tetrahydroxyolean-1-en-28 β -D-glycopyranoside

Compound A-2 (non-acetylated) was obtained as a light yellow powder (with a slight tint of green) while compound Ac-C (acetylated) was obtained as white powder. The 2D NMR data (COSY, HSQ and HMBC) of acetylated compound was used to confirm the structure of the non-acetylated compound (A2). The structural transformation of non-acetylated and acetylated form of the structure is illustrated in **Figure 30**. The chemical formula of A2 is C₃₆H₅₈O₁₁ and has a molecular weight of 666.84. The chemical formula of Ac-C is C₄₈H₇₀O₁₇ and has a molecular weight of 918.46. The final structures of the non-acetylated (A2) and acetylated (Ac-C) compounds from *T. prunioides* are given in **Figures 31** and **32**, respectively. Both structures for the acetylated and non-acetylated compounds were novel (not reported before). Both class of compounds are triterpenoid glycosides.

For NMR studies; A2 was dissolved in DMSO, while Ac-C was dissolved in Chloroform (CDCl₃). ¹H NMR spectrum of both A2 and Ac-C revealed a triterpene moiety upfield, between 2.5 and 0.9 (**Figure 33**). ¹³C NMR revealed a sugar moiety between 85-60 and a triterpene moiety at 55-15 (**Figure 34**). DEPT have shown that in both structures there are 6 CH₃ carbons, 10 CH₂ carbons, 12 CH carbons and 8 quaternary carbons (30 carbons on the skeleton and 6 on the sugar; total 36 carbons) (**Figure 35**).

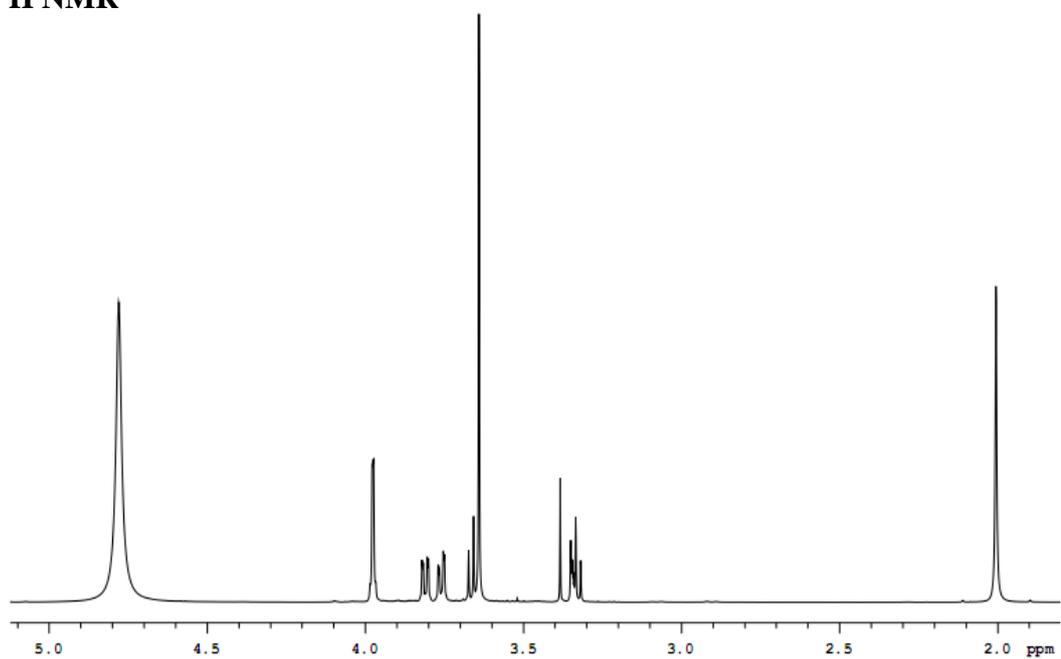
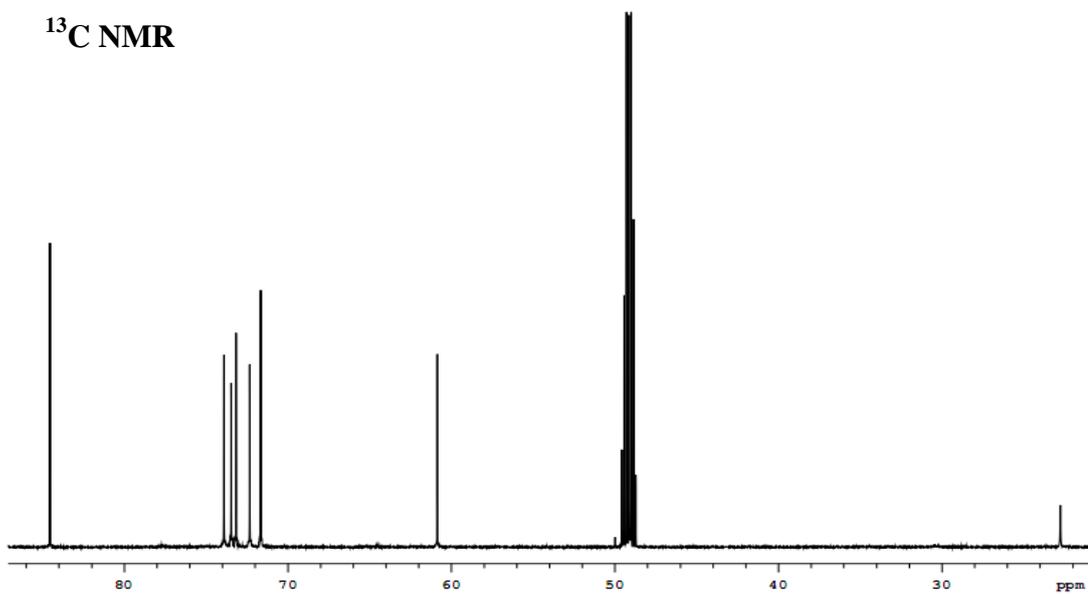
^1H NMR ^{13}C NMR

Figure 29: ^1H NMR and ^{13}C NMR spectra of D-Pinitol.

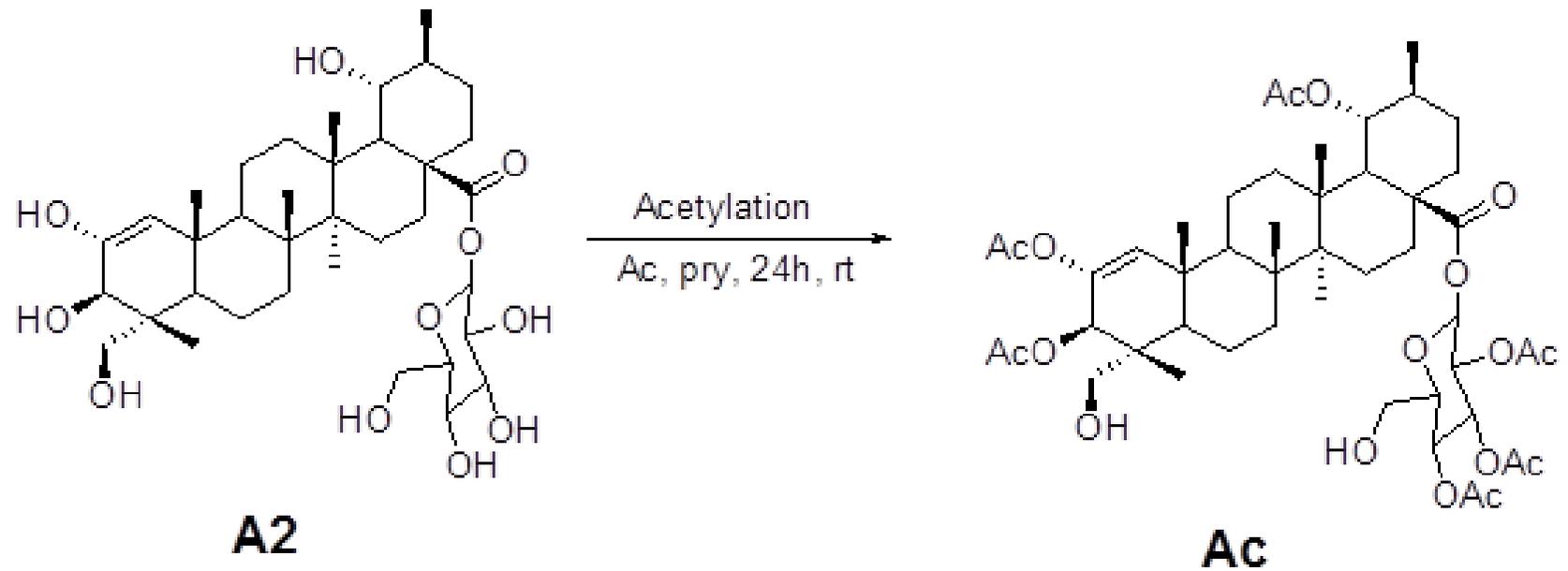


Figure 30: Structural transformation of 2 α ,3 β ,19 α ,23-Tetrahydroxyolean-1-en-28 β -D-glycopyranoside.

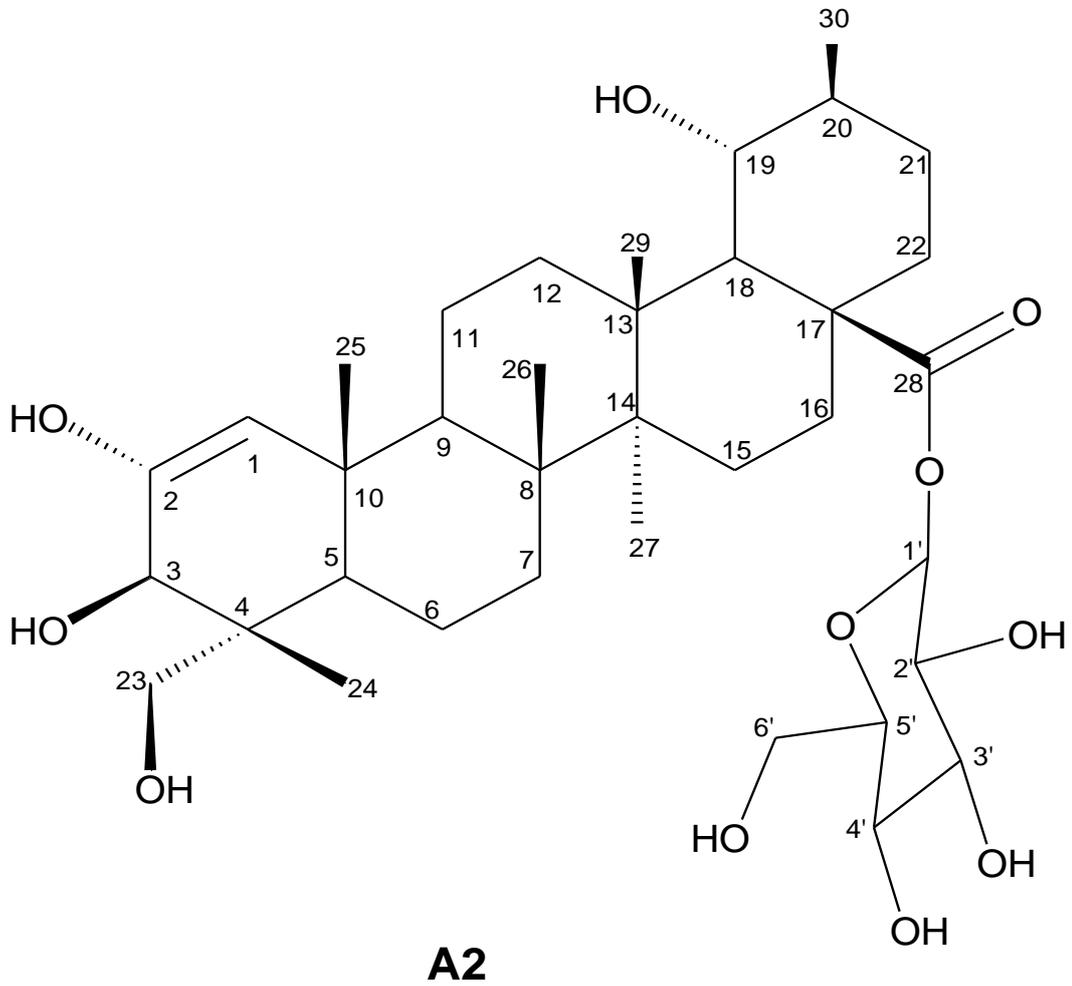


Figure 31: Non acetylated structure of 2 α ,3 β ,19 α ,23-Tetrahydroxyolean-1-en-28 β -D-glycopyranoside.

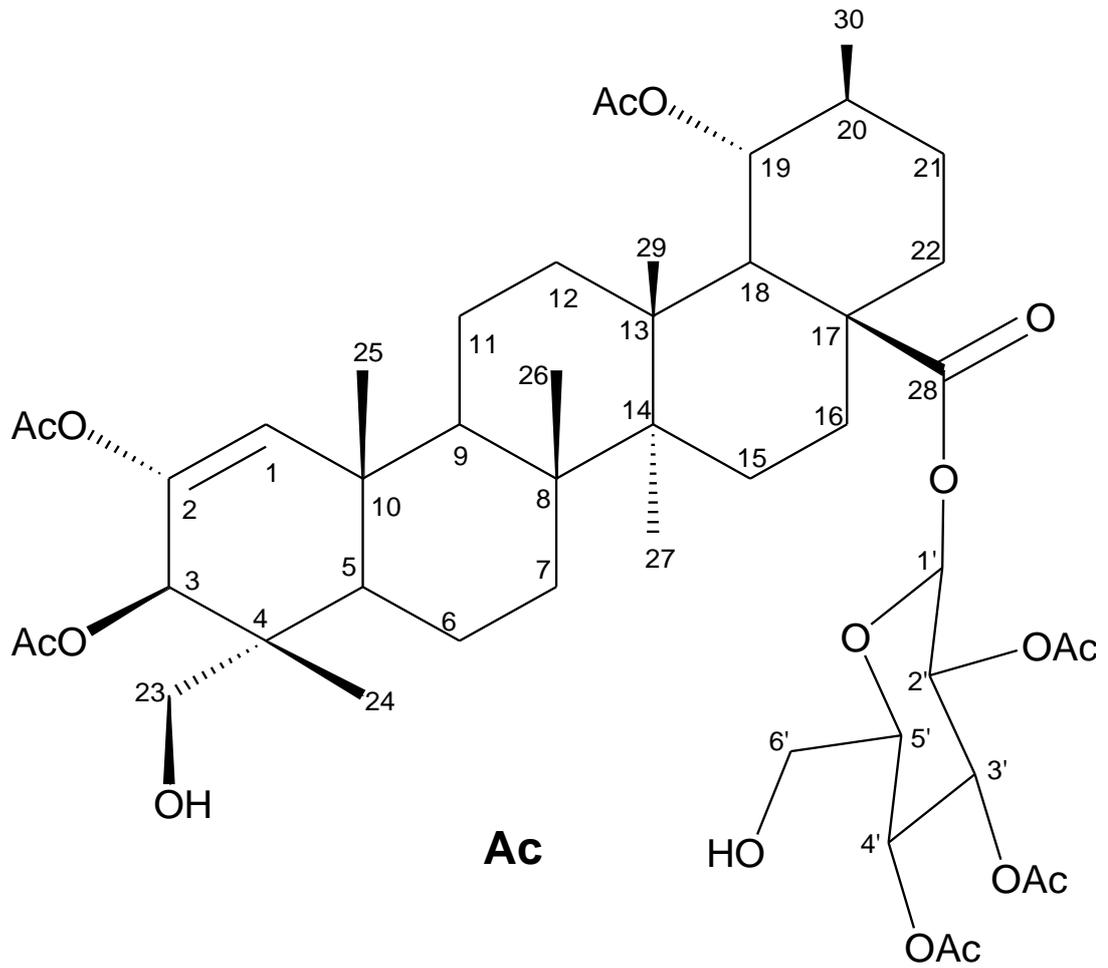


Figure 32: Acetylated structure of 2 α ,3 β ,19 α ,23-Tetrahydroxyolean-1-en-28 β -D-glycopyranoside.

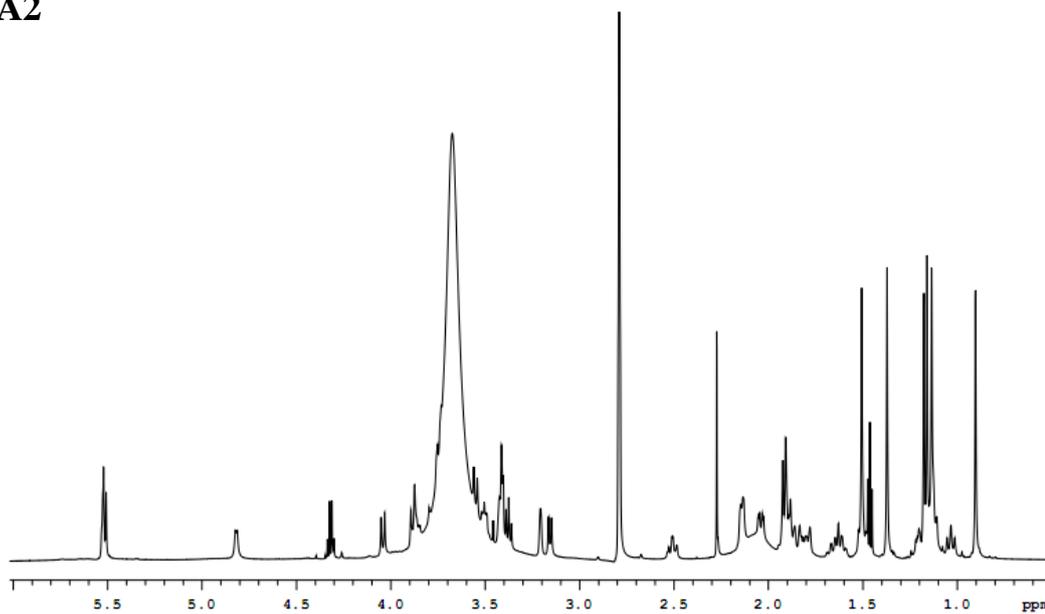
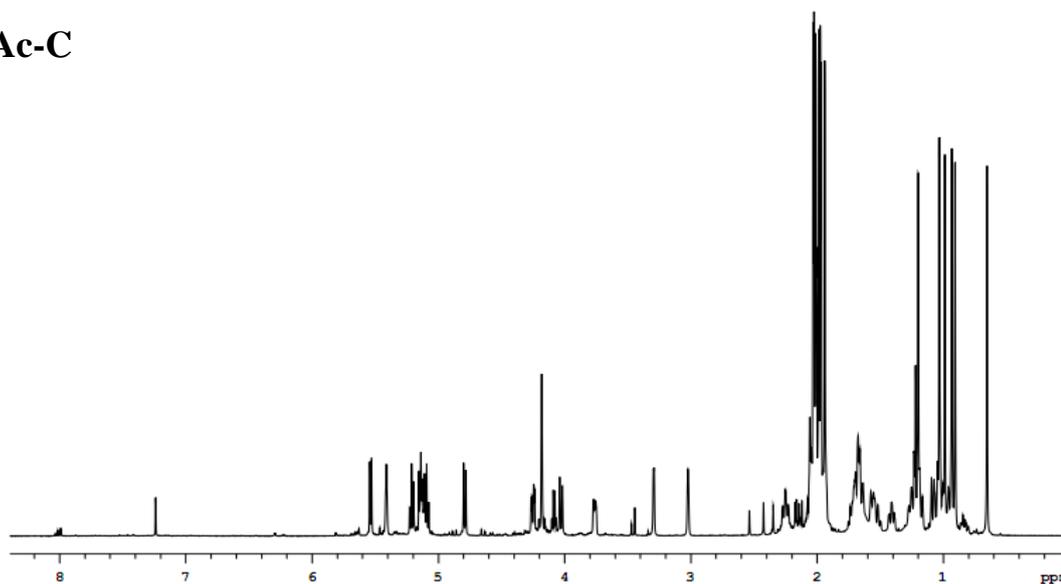
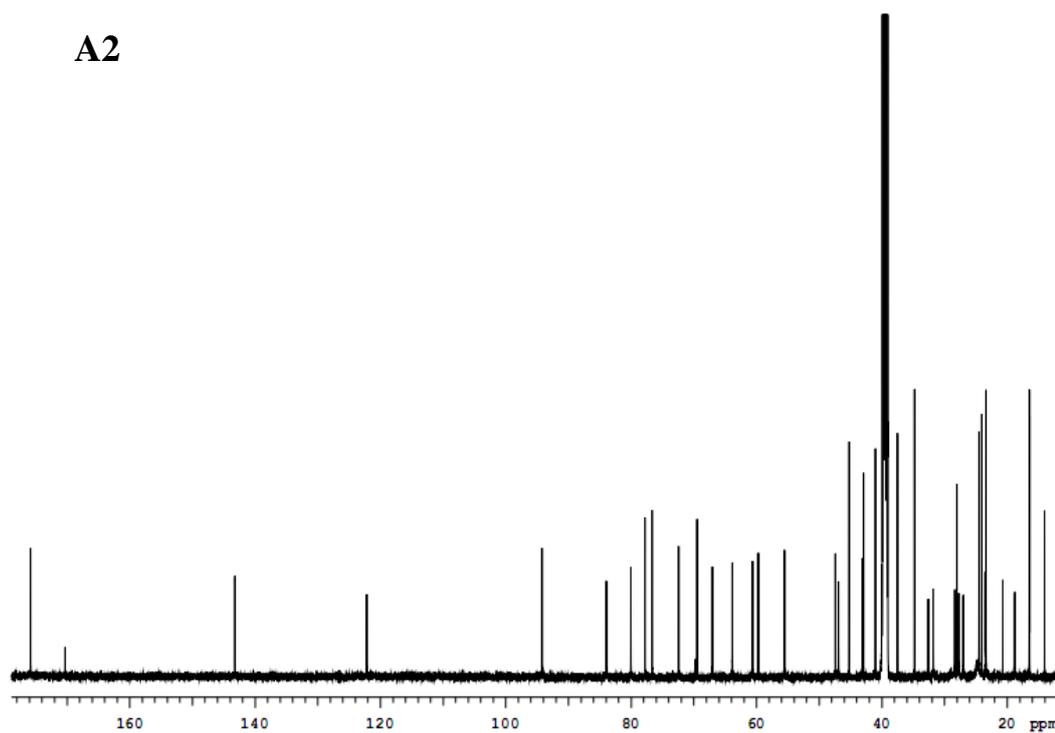
A2**Ac-C**

Figure 33: ^1H spectra of acetylated (Ac-C) and non acetylated (A2) compounds.

A2



Ac-C

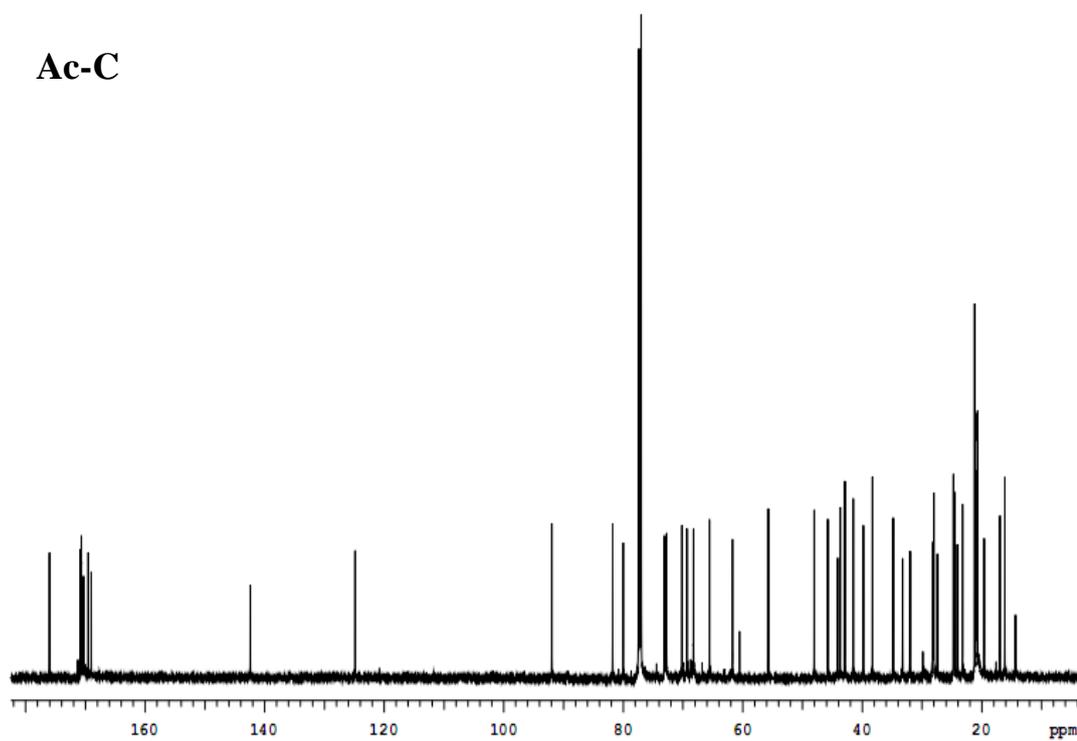


Figure 34: ^{13}C spectra of acetylated (Ac-C) and non acetylated (A2) compounds.

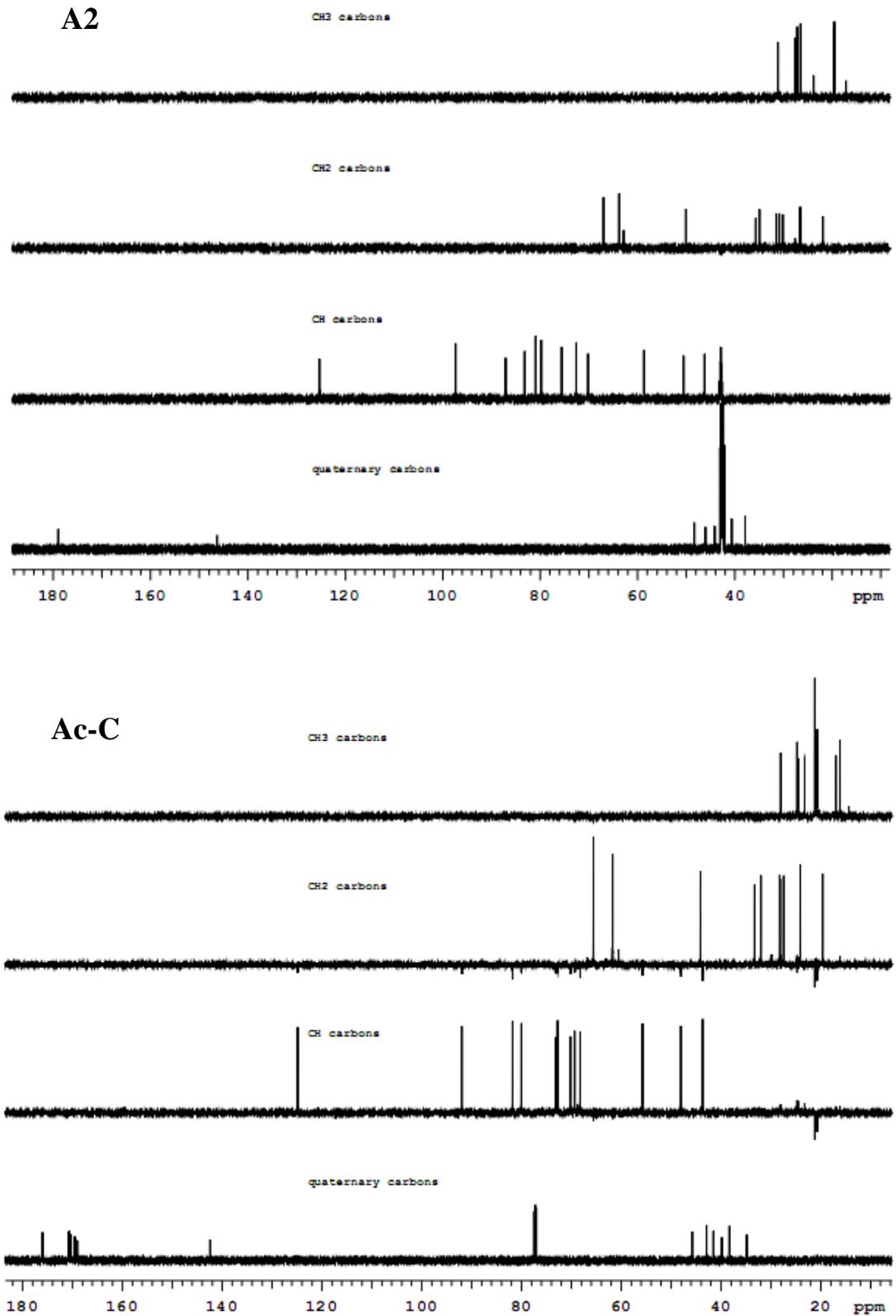


Figure 35: DEPT spectra of acetylated (Ac-C) and non acetylated (A2) compounds.

CHAPTER 5: DISCUSSION

5.1. Potential activity against HIV reverse transcriptase

Despite many HIV-infected persons have access to antiretroviral drugs, some still use ethno-medicinal plants and other natural products to treat opportunistic infections and offset side-effects from antiretroviral medication (Hardon, Desclaux, Egrot, Simon, Micollier & Kyakuwa, 2008). Medicinal plants and other natural products including mushrooms are used as primary treatment for HIV-related problems such as skin disorders, nausea, depression, insomnia, and body weakness (Babb, Pemba, Seatlanyane, Charalambous, Churchyard & Grant, 2004). In the case of rural communities, formal biomedical services are also hardly accessible. Thus, whilst the majority of HIV/AIDS patients rely on ART, some still have faith in the use of traditional medicines. Understandably, HIV/AIDS patients are vulnerable in their choice of treatments, such that some of them do vacillate from conventional ART program to traditional medicines and vice versa; they want to have the best of both worlds (Hardon et al. 2008; Chinsebu, 2009).

Several plants in this study were found to have inhibitory activities against HIV RT which implies some efficacy in the use of ethno-medicinal plants by traditional healers in Namibia. A total of 15 extracts were very potent and 4 extracts were moderate against HIV-1 reverse transcriptase. Therefore, the findings of this study may explain why traditional healers in Namibia continue using these plants against HIV infections and associated opportunistic infections.

There has been a good level of support for the use of medicinal plants in the management of HIV/AIDS. As early as 1989, the World Health Organization (WHO) had already voiced the need to evaluate ethnomedicines and other natural products for the management of HIV/AIDS (WHO, 1989a). Thus, African governments expressed the need for a concerted, systematic and sustained effort at both local and regional levels to support and biochemically validate African traditional medicines (UNAIDS, 1998). To popularize this commitment, the Organization of African Unity (African Union) Heads of State and Government declared the period 2000-2010 as the Decade of African Traditional Medicine. In addition, the Director General of WHO, declared 31st August of every year as the African Traditional Medicine Day (Homsy, King, Tenywa, Kyeyune, Opio & Balaba 2004). All these initiatives demonstrate the need to mainstream and institutionalize traditional medicine into the formal health care system.

The importance of investing in the high growth sectors of biotechnology and phytomedicine was also articulated in the founding document of the New Partnership for Africa's Development (NEPAD), and adopted by the African Biosciences Initiative (NEPAD, 2001; African Biosciences Initiative, 2005). Therefore, results of this study confirmed the potential that medicinal plants have on general wellbeing of the population in the management of HIV/AIDS. Moreover, it renders credibility to such initiatives and provides hope that such initiatives can yield useful results in combating HIV/AIDS.

5.2. Cytotoxicity against mammalian cell lines

A total of 16 extracts were found to be not toxic or have low toxicity. Another 24 were toxic or very toxic to cells especially when used at higher concentrations. This observation is very important as it is indicative that, some medicinal plants can be dangerous to humans if consumed in high doses. Most of medicinal plants are given in a form of herbal supplements and this practice can also pose problems when it comes to toxicities of such herbs.

In a national guidelines by Ministry of Health and Social Services on Traditional therapies and supplements (MoHSS, 2010), it is recommended that traditional therapies and supplements for people living with HIV (PLHIV) should be used with caution and guidance from health workers. Some traditional herbs can help enhance the flavour of foods, but when taken in large quantities and with less balanced meals, can potentially interact poorly with medications leading to toxicities. Hence, herbal supplements that claim to boost the immune system or cure diseases should be discouraged as these are often very expensive and tend to replace nutritious foods. The guidelines further recommends traditional herbs and remedies are acceptable to use, but should always be used with caution when taking multiple medications (MoHSS, 2010).

Of the 24 extracts (in this study) that were toxic, 10 were very toxic and 3 extracts (out of ten) were potent against RT. This means that while the extracts appears to be

active (potent) against RT, it is not clear if the activity observed were due to their toxicities or active compounds contained in these plants. In practice, this could mean that it is not safe to prescribe plant extracts to a patient based solely on its potency against RT. Such an extract could lead to toxicities in the host if consumed in large quantities or in combination with other medications (both pharmaceutical and herbal). Hence, while RT assay shows potency for some of the extracts, care should be taken in administering them to avoid complications due to possible toxicities in the host (**Table 10**).

5.3. Isolated compounds

Six compounds were isolated in this study. The structures of three of the compounds were not fully elucidated; however the available NMR data suggest that they could be: Epigallocatechin isolated from *T. sericea* stems; Triterpenoid glycoside isolated from *T. prunioides* roots; Naphthoglycoside/Anthraquinoline glycoside isolated from *D. mespliformis* leaves. Another three structures were fully elucidated: D-Pinitol isolated from *D. mespliformis* leaves and a novel compound (which is being reported for the first time) 2 α ,3 β ,19 α ,23-Tetrahydroxyolean-1-en-28 β -D-glycopyranoside isolated from *T. prunioides* roots, which was obtained in acetylated and non-acetylated forms. This is the first study to isolate and/or elucidate the structures of compounds active against HIV RT from Namibian ethno-medicinal plants. This is a significant accomplishment considering that primary data of known active compounds and mechanisms of action are available from studies mostly done outside Namibia.

In this study, two triterpenoid glycosides ($2\alpha,3\beta,19\alpha,23$ -Tetrahydroxyolean-1-en- 28β -D-glycopyranoside: obtained in acetylated and non-acetylated forms) were isolated from *Terminalia prunioides* roots. A similar compound, $2\alpha,3\beta,19\alpha,24$ -Tetrahydroxyolean-12-en-30-oic acid, has been isolated from *Terminalia glaucescens* (Atta-ur-Rahman, Choudhary, Akhtar, Shujaat & Ngounou, 2005). In another study, $2\alpha,3\beta,19\beta,23,24$ -pentahydroxy-11-oxo-olean-12-en-28-oic acid 28-O- β -D-gluco-pyranosyl ester has been isolated from *Pteleopsis suberosa* (Combretaceae) (De Leo, Tommasi, Sanogo, D'Angelo, Germano, Bisignano & Braca, 2006; Wang, Ali, Li, Shen & Khan, 2010). Another 2 compounds, $2\alpha,3\beta,19\beta$ -trihydroxy-11-oxo-olean-12-en-28-oic acid 28-O- β -D-glucopyranosyl ester and $2\alpha,3\beta,23$ -trihydroxy-11-oxo-olean-12-en-28-oic acid 28-O- β -D-glucopyranosyl ester were isolated from *Terminalia arjuna* and *Terminalia chebula*, respectively (Wang et al. 2010). However, all these reported compounds have their double bonds at carbon 12 and carbon 13, instead of between carbon 1 and 2 as in the compounds isolated in this study from *T. prunioides*.

One active compound, D-Pinitol, was isolated from *Diospyros mespiliformis* in the present study. This is the first report on the isolation of D-pinitol from this plant. Previous research on the phytochemistry of the stem barks or wood of *Diospyros mespiliformis* has revealed the presence of triterpenes: α -amyrin-baurenol, trihydroxy-triterpenoid acid, α -amyrin, β -sitosterol, lupeol, betulin and Betulinic acid, Naphthoquinones (e. g. diospyrin), Isodiospyrin, Diosquinone and Plumbagin (Lajubutu, Pinney, Robert, Odelola & Oso, 1995; Mohamed, El Nur, Choudhary &

Khan, 2009). Ethanol and water extracts of *Diospyros decandra* (Ebenaceae) were tested for its inhibitory activities on HIV integrase with $IC_{50}=69.9\mu\text{g/ml}$ and $IC_{50}=27.8\mu\text{g/ml}$, respectively (Tewtraku, 2009).

Other medicinal properties have also been reported for other species from Ebenaceae family. Antimicrobial activity revealed that both the extracts of *D. mespiliformis* were active against *Shigella* sp, *Escherichia coli*, and *Streptococcus* sp (Shagal, Kubmarawa & Alim, 2012). This supports the claims of efficacy reported in folk use of the plant in the treatment of disease caused by some pathogens and if further purified can be used to source novel antibiotics. *Diospyros ehretioides* (Ebenaceae) has anti-Leishmanicidal activity (Takahashi, Fuchino, Satake, Agatsuma & Sekita, 2004).

The fact that we managed to isolate active compounds in this study supports data from other researchers who managed to isolate compounds that are active against several stages of HIV from medicinal plants. Several researchers have reported different chemical compounds which are known to be active against HIV reverse transcriptase. Some HIV reverse transcriptase inhibitors included: michellamines (Boyd et al. 1994); triterpene (Li, Sun NJ, Kashiwada & Sun L, 1993); coumarins and isoflavone derivatives (Yao, Wainberg & Parniak, 1992); caffeic acid tetramers (Kashiwada, 1995); (+)-calanolide A (Xu, Flavin & Jenta, 2000); hypericin and 3-hydroxy lauric acid (Birt et al. 2009); gallotannin (Bessong, Obi, Andreola, Rojas,

Pouysegou, Igumbor et al. 2005); flavonone-xanthone glucoside (Wang, Hou, Liu, Lin, Gil & Cordell, 1994); linearol (Bruno, Rosselli, Pibiri, Kilgore & Lee, 2002); catechins 1-5 (Moore & Pizza, 1992); cepharanthine (Ma et al. 2002); galloylquinic acids (Moore, Westall, Ravenscroft, Farrant, Lindsey & Brandt, 2005; Moore, Lindsey, Farrant & Brandt, 2007); velutin (Wang & Ng, 2001); nitidine (Tan, Pezzuto, Kinghorn & Hughes, 1991); oleanolic acid (Sakurai, 2004); nigranoic acid (Sun, 1996); sulfated polysaccharides (Nakashima et al. 1987); triterpene lactone (Chen et al. 1999); and harmine (Ishida, Wang, Oyama, Cosentino, Hu & Lee, 2001).

Generally, the IC_{50} s of extracts (in this study) increased with further purifications. This could be due to the fact that most compounds are removed during each stage of purification, thereby increasing the concentration of the active compound(s). Interestingly, the compounds whose structures were elucidated did not show good activities against RT. This could mean that the actual active compound(s) were missed during the isolation stages. It is also possible that several compounds are needed to interact in order to increase the biological activities before final purification. Hence, it is possible that once the compound is fully purified, it will lose some activities due to absence of other compound(s) that might have been responsible for pharmacological effects observed before isolation into pure compounds. Another possibility is that the compound(s) might degrade fast when present in pure form, hence further resulting in reduction in its biological activity.

Although Namibia has a wealth of medicinal plants, information on the active compounds against HIV are either lacking or insufficient (Chinsembu, 2009; Chinsembu & Hedimbi, 2010). Hence this study is one of the first steps to ensure that initial screening and isolation of pure compounds from Namibian medicinal plants is carried out and documented. Partnerships with research centers in the region that have strong capacity in drug discovery from natural products should be encouraged in order to fast track discovery and isolation of biologically active compounds from more Namibia medicinal plants.

5.4. Plants with activities against HIV and HIV-related infections

5.4.1. Active compounds from Combretaceae family

Several plants from Combretaceae family have been reported to possess medicinal properties. A piperidine-flavone-related alkaloid isolated from *Buchenavia capitata* (Combretaceae), showed activity in both anti-HIV and anti-cancer cell-based screens (Beutler, Cardellina, McMahon, Boyd, Kashman & Cragg, 1992; Singh, Bharate & Bhutani, 2005). The ethanolic extract of the fruit of *Terminalia bellerica* (Combretaceae), one of the commonly used plants in the Indian traditional systems of medicine, yielded lignans which possessed anti-HIV activity in vitro (Valsaraj et al. 1997; Singh et al. 2005). Gallic acid and galloyl glucoses isolated from *Terminalia chebula* (Combretaceae) exhibited HIV integrase inhibitory (Hurinanthan, 2014). The acetone fraction of *Combretum paniculatum* showed selective inhibition of HIV-1 replication with selectivity index of 6.4 and afforded cell protection of viral-induced cytopathic effect of 100% (Hurinanthan, 2014).

In another study, antibacterial, anti-HIV-1 protease and cytotoxic activities of aqueous ethanolic extracts from *Combretum adenogonium* (Combretaceae) were evaluated with promising results (Mushi, Mbwambo, Innocent & Tewtrakul, 2012). The methanol extract of the leaves of *Terminalia sericea* (Combretaceae) was found to strongly inhibit the polymerase ($IC_{50} = 7.2 \mu\text{g/ml}$) and the ribonuclease H ($IC_{50} = 8.1 \mu\text{g/ml}$) activities of HIV-1 (Bessong et al. 2005). Other health benefits have also been attributed to *T. prunioides* and *T. Sericea*. Acetone and methanol fractions of these two plants were reported to have strong anti-oxidant and oxygen scavenging activities (Masoko & Eloff, 2008).

5.4.2. Active compounds from other plants

In other similar studies, several chemical compounds have been reported to interfere with HIV entry into cells while others were active against HIV reverse transcriptase, integrase, protease, and general replication, and others are reported to inhibit syncytia formation. Most of the entry inhibitors were lectins such as: agglutinins from *Galanthus nivalis* and *Hippeastrum* that hybrid stopped the spread of HIV among cells (Saidi et al. 2007); BanLec, a jacalin-related lectin that binds to glycosylated viral envelopes blocked HIV-1 entry into cells (Swanson, Winter, Goldstein & Markovitz, 2010); cyanovirin, an 11 KDa protein isolated from *Nostoc ellipsosporum*, targeted gp120 proteins and blocked fusion of HIV-1 to lymphocyte membranes (Gustafson et al. 1997; Balzarini et al. 2004); glycoprotein complexes from *Ganoderma* mushrooms inhibited HIV-1 gp120 binding to CD4 cells (Lindequist, Niedermeyer & Julich, 2005); a code-named compound, PJ-S21, from

Punica granatum inhibited the binding of gp120 to cells expressing CXCR4 receptors (Neurath, Strick, Li & Debnath, 2004); and *Phytolacca americana* pokeweed antiviral protein (PAP), a 29 KDa ribosome-inactivating protein that removes adenine from rRNA of prokaryotic and eukaryotic ribosomes was found to be a potent microbicide (Tumer, Hwang & Bonness, 1997; Uckun et al. 1998; D'cruz, Waurzyniak & Uckun, 2004). Other active constituents included: diterpene lactones (Calabrese et al. 2000) and a coumarin named wedelolactone (Yao et al. 1992) inhibited cell-to-cell transmission of HIV-1; prostratin, a 12-deoxyphorbol, inhibited HIV-1 entry into lymphocytes (Park et al. 2009); and rosmarinic acid isolated from *Melissa officinalis* inhibited fusion of HIV-1 to cells (Geuenich et al. 2008).

In other studies, many active compounds from plants have been reported to inhibit general HIV replication. Diterpene lactones from *Andrographis paniculata* (Otake et al. 1995) and *Tripterygium wilfordii* (Duan, Takaishi, Bando, Kido, Imakura & Lee, 1999); triterpene lactone and lancilactone from *Kadsura lancilimba* (Chen et al. 1999); biflavonoids from *Rhus succedanea* (Lin, Anderson, Flavin & Pai, 1997); lanostane-type triterpenes from *Polyalthia suberosa* (Li et al. 1993); suksdorfins from *Lomatium suksdorfii* (Yu, Morris-Natschke & Lee, 2007); wedelolactone (a coumarin) and orobol (an isoflavone derivative) from *Arctium lappa* (Yao et al. 1992); caffeic acid tetramers from *Arnebia euchroma* (Kashiwada, 1995); celastrol from *Celestrus hindsii* (Kuo YH & Kuo LMY, 1997); cordatolides from *Callophyllum cordato-oblongum* (Dharmaratne, Tan, Marasinghe & Pezzuto, 2002); hypericin and 3-hydroxy lauric acid from *Hypericum perforatum* (Birt et al. 2009);

sulfonated polysaccharides from *Mentha piperita* and *Prunella vulgaris* (Hauber I, Hohenberga, Holstermanna, Hunsteinb & Hauber J, 2009); linearol from *Sideritis akmanii* (Bruno, et al. 2002); cepharanthine from *Stephania cepharantha* (Ma et al. 2002); cyanovirin from *Nostoc ellipsosporum* (Gustafson et al. 1997); oleanolic acid from *Xanthoceras sorbifolia* (Sakurai, 2004); and harmine from *Symplocos setchuensis* (Ishida et al. 2001).

Furthermore, several active compounds isolated from plants were observed to be HIV protease inhibitors as reported by other researchers: ganoderiol, ganodermanontriol, and ganoderic acid B (a triterpene) from *Gonoderma* mushrooms (Lindequist et al. 2005); lignins from *Gonoderma* and *Inonotus obliquus*, commonly known as chaga mushroom belonging to the Hymenochaetaceae family (Ichimura, Watanabe & Maruyama, 1998); uvaol and ursolic acid from *Crataegus pinatifida* (Min, Jung & Lee 1999), and maslinic acid (a triterpene acid) from the plant *Geum japonicum* (Xu, Zeng, Wan & Sim, 1996), both from the Rosaceae family; limonoids (Manners, 2007), including limonin and nomilin, secondary metabolites from citrus fruit species belonging to the Rutaceae family (Battinelli et al. 2003); and curcumin from *Curcuma* species was also shown to be active against HIV-1 integrase (Itokawa, Shi, Akiyama, Morris-Natschke & Lee, 2008).

Other studies also reported that some active compounds of plant origin were found to inhibit syncytia formation, a property of HIV that makes infected and healthy CD4 cells to fuse and form one giant cell with as many as 500 nuclei. Syncytia-inhibiting compounds included: diterpene lactones (Calabrese et al. 2000); michellamines A and B (Boyd et al. 1994; Manfredi et al. 1991); and limonoids (Sunthitikawinsakul et

al. 2003). Eight natural compounds prevented HIV-induced cytopathic effect: sulfated lentinan (Suzuki H, Okubo, Yamazaki, Suzuki K, Mitsuya & Toda, 1989); sulfonated polysaccharides (Witvrouw et al. 1994); xanthohumol (Wang, Ding, Liu & Zheng, 2004); cordatolides (Dharmaratne et al. 2002); laxofloranone (Bokesch, Pannel & Cochran, 2001); ganoderiol, ganodermanontriol, and ganoderic acid (Lindequist et al. 2005); sulfated (1-3)- β -D-glucan with (1-6)- β -D-glucosyl branches (Lindequist et al. 2005); and palicourein (Bokesch et al. 2001).

In another study, 50 different compounds (belonging to tannins, terpenoids, flavonoids, flavones, alkaloids, coumarins, lignans, lignin-polysaccharide complexes, and lectins), found in 40 different plant species, were reported to inhibit adsorption, viral fusion, HIV reverse transcriptase, integrase, protease, syncytium formation and interference with cellular factors (Cowan, 1999). Other compounds isolated from medicinal plants such as castanospermine, hypericin, pseudohypericin, interferons, glycyrrhizin, avarol and avarone inhibited replication of HIV-1 and other retroviruses (Lin et al. 1997). Gossypol, a polyphenolic bissesterpene isolated as a racemic mixture from cottonseed has selective activity against HIV-1 but the exact mechanism was unknown (Lin et al. 1997). Flavonoid derivatives inhibited HIV-1 reverse transcriptase (Moore & Pizza, 1992). Suramin inhibits HIV-1 reverse transcriptase in a non-specific way (Moore & Pizza, 1992). Sulphated polysaccharides were potent *in vitro* inhibitors of HIV-1 and HIV-2 adsorption, fusion or penetration, induced cytopathogenicity, and antigen expression. They also inhibited reverse transcriptase and RNAase H, essential enzymes for retrovirus replication (Talyshinsky et al. 2002).

5.5. Other anti-HIV benefits from plants

In this study, 17 extracts were considered as having weak activity and another 4 extracts as very weak against RT. This observation does not mean that traditional healers were wrong or lied about their purported activities. Traditional healers do not report on or are unaware of the mode of action of many of their remedies. Furthermore, HIV has other enzymes which could be susceptible to the tested extracts. Hence, while a large number of extracts was not active against RT, there is still a big potential and possibility that they could be active against other stages of the HIV. In addition to the anti-RT activities of plants reported in this study, plants possess other benefits which were not reported in this study, which are either directly or indirectly active against HIV/AIDS and other related diseases. For example, Coumarins and naturally occurring benzopyrene derivatives from several plant species were reported to possess antioxidant, anti-inflammatory, antithrombotic, antiviral, anticarcinogenic, antiallergic, hepatoprotective, and anti-HIV properties (Kostova et al. 2006).

Some plant-derived compounds have been reported to possess activities against HIV-related symptoms. L-canavanine from *Sutherlandia frutescens* had anti-viral activity against HIV but interacted with the efflux of nevirapine (Brown, Heyneke, Van Wyk & Hamman, 2008). D-pinitol, also from *Sutherlandia frutescens*, had been suggested as a treatment for wasting in cancer and AIDS patients though evidence was scanty (Brown et al. 2008). (+)-Catechin (flavonoid), bergenin (a C-galloyl-glycoside) and betulinic acid from *Peltophorum africanum* were used to treat diarrhoea, dysentery,

sore throat wounds, HIV/AIDS, and other sexually transmitted infections (Theo et al. 2009; Kashiwada et al. 2000). These reports of plants having activities against a wide range of infections supports the logic of traditional healers in concluding that a particular plant can be used against HIV. In essence, traditional healers never specifically said that their plants particularly target HIV RT, hence there is still potential that they are active against other sorts of HIV-related infections.

CHAPTER 6: CONCLUSIONS

Currently, most of studies of medicinal plants with potential to manage HIV/AIDS in Namibia are mainly based on ethnobotanical data, with little or no scientific data to back up the claims of their purported benefits. To the best of our knowledge, this is the first study to scientifically evaluate and report on the biological activities of Namibian medicinal plants against HIV reverse transcriptase. The findings of this study will contribute to establishing a database of Namibian medicinal plants with potential anti-HIV activities.

Specific outcomes of the study:

1. The study found 40 crude extracts (from 15 plants) to have concentration dependent inhibitory activities against RT. Variable inhibitory activity on RT were observed with fractions extracted with different solvents. Ethyl Acetate fractions had the best RT inhibitory activities when compared to other solvents.
2. Most of the samples had very low toxicity to Vero cells (with 31 out of 40 extracts having viability above 50% at the highest tested concentration.
3. Six compounds were isolated and three of the structures were fully elucidated.
 - a) D-Pinitol (a carbohydrate) was isolated from *Diospyros mespliformis* leaves. This is the first reported isolation of D-Pinitol from this plant species.

- b) A novel compound, $2\alpha,3\beta,19\alpha,23$ -Tetrahydroxyolean-1-en-28 β -D-glycopyranoside (a triterpenoid glycoside), was isolated from *Terminalia prunioides* roots, and was obtained in acetylated and non-acetylated forms.

CHAPTER 7: RECOMMENDATIONS

Several activities listed below as recommendations were not possible to carry out in this study either because they were out of scope of the study or there were no adequate funding available to carry them out:

- Biological assays to test the efficacy of these plants on other enzymes of HIV life cycle (such as protease and integrase) should be carried out.
- Due to high toxicities of a large number of plants tested in this study, awareness campaigns to educate traditional healers on the dangers posed by some of the plants they use in treatment of HIV/AIDS patients is advisable.
- Isolation of possible active compounds from all plants classified as potent or very potent should be prioritized and carried out.
- Testing of several Namibian medicinal plants (as many as possible) should be carried out in order to establish a national database of possible lead plants.
- Synthesis of active drug analogues, similar to active compounds obtained in this study, should be carried out to reduce the possibility of overexploitation of promising plants.

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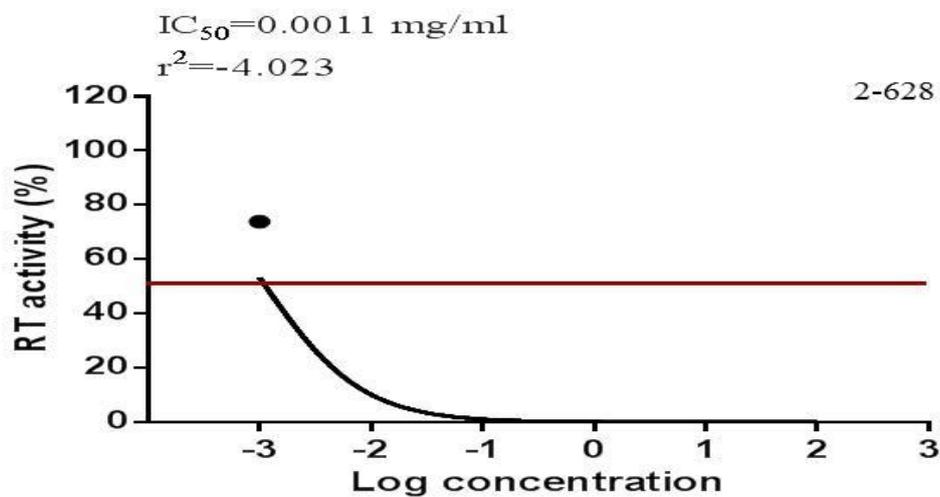
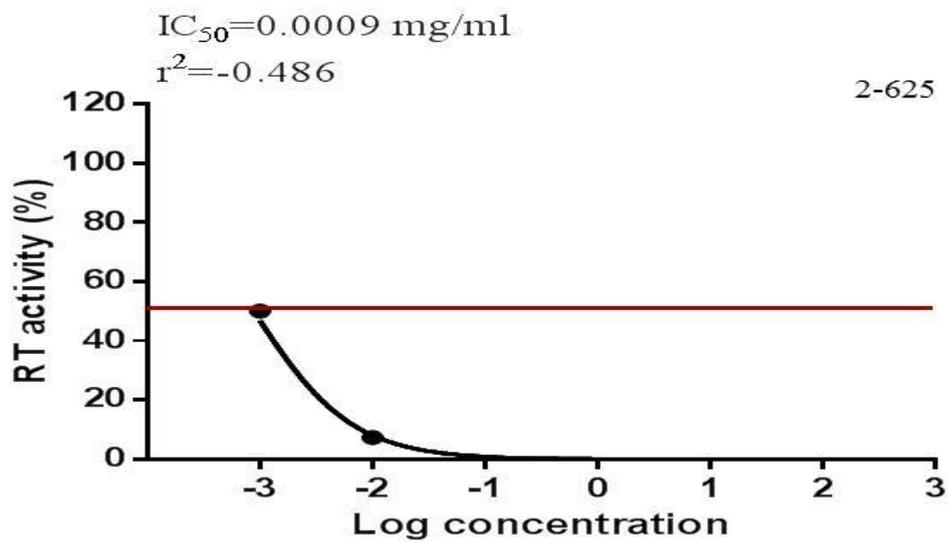
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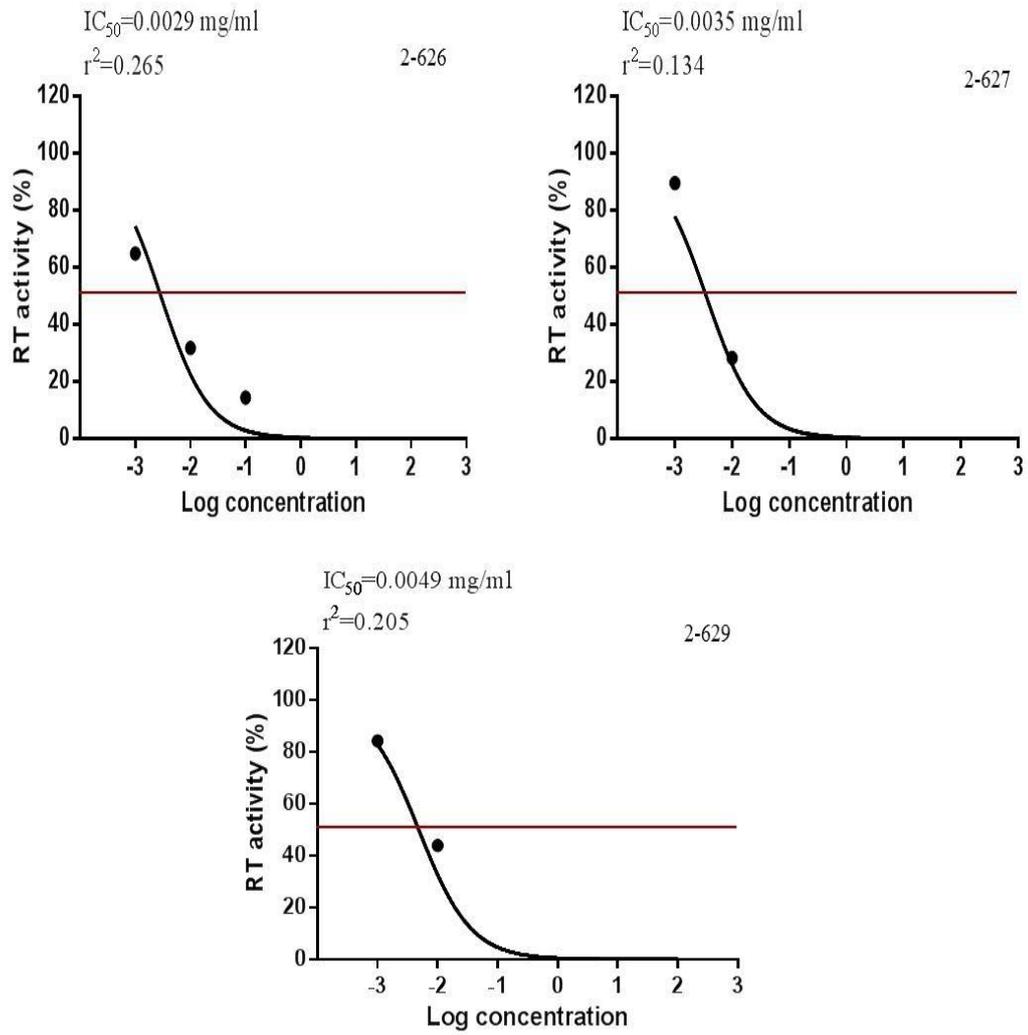
APPENDICES

APPENDIX A

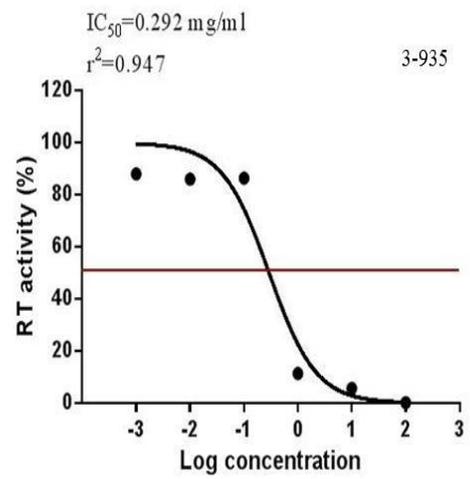
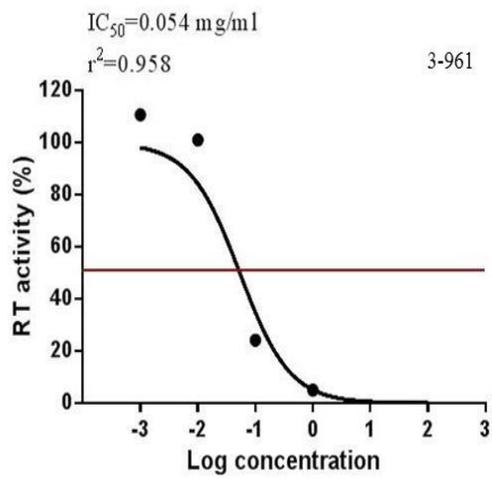
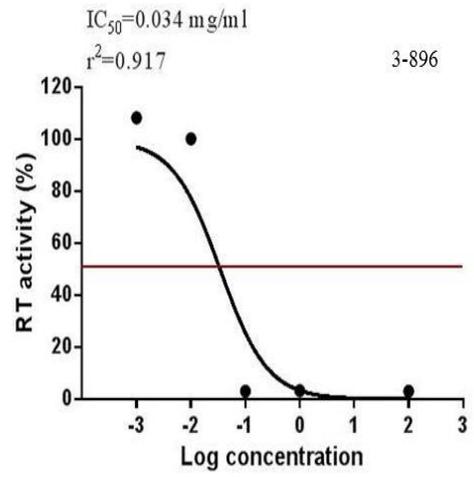
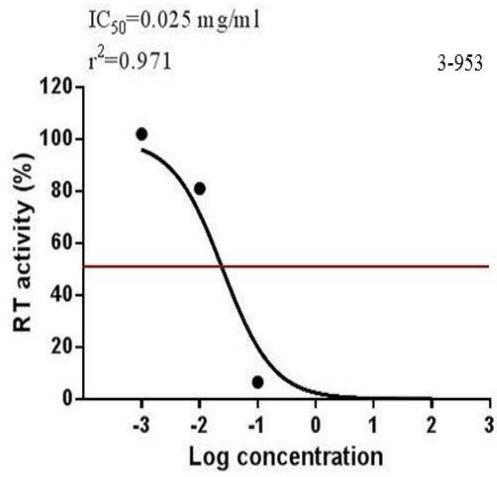
Dose-response curves with non-linear sigmoidal curves to determine IC_{50} of plant extracts. This was based on colorimetric assay to determine inhibitory activity of RT enzyme by the plant extracts.



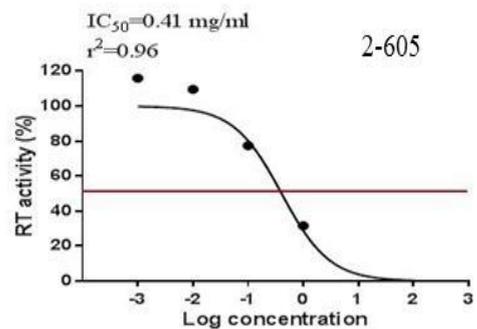
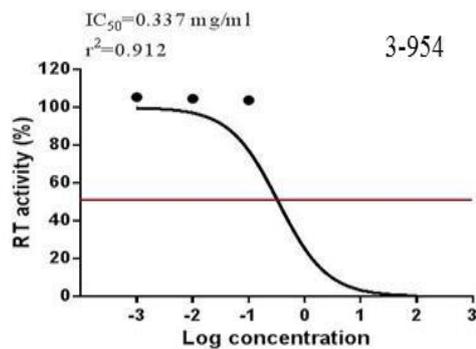
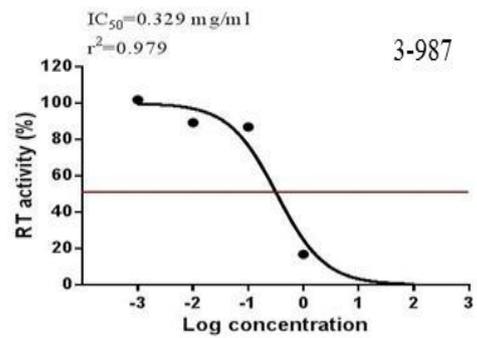
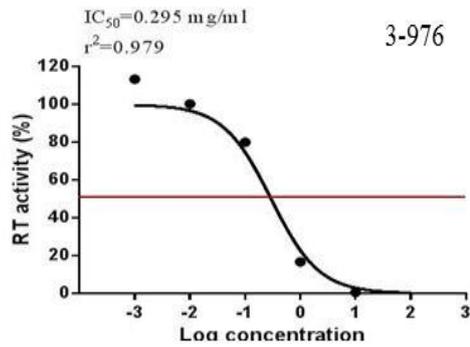
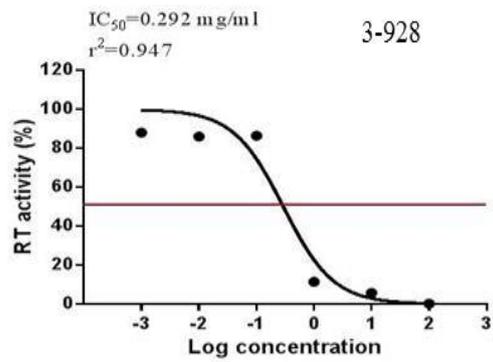
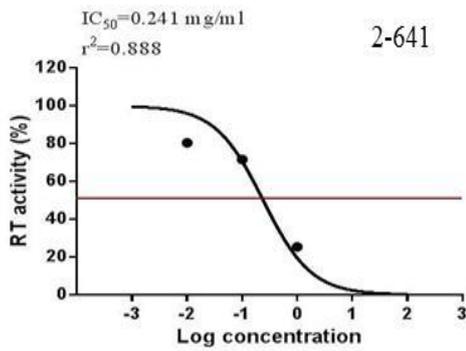
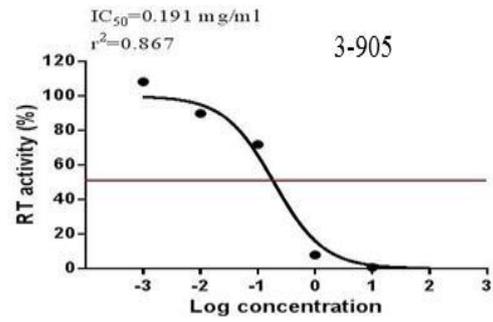
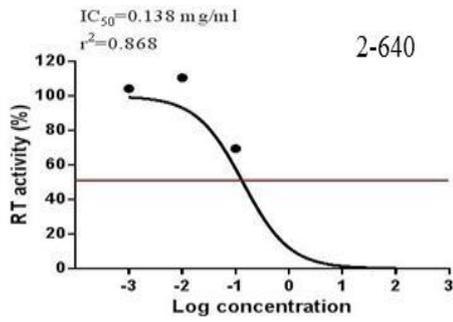
IC_{50} of very potent plant extracts



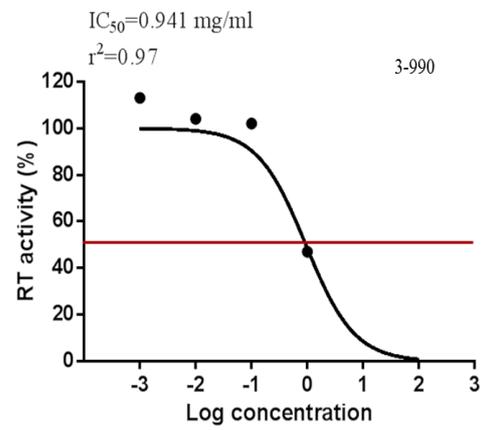
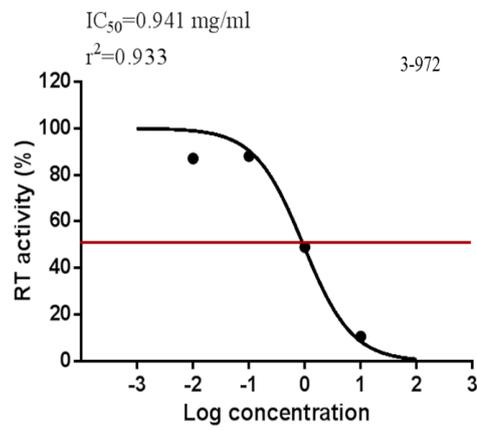
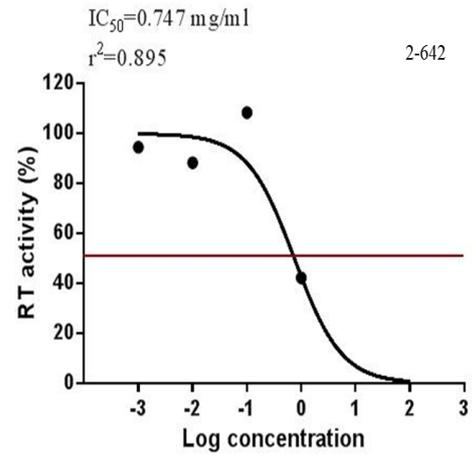
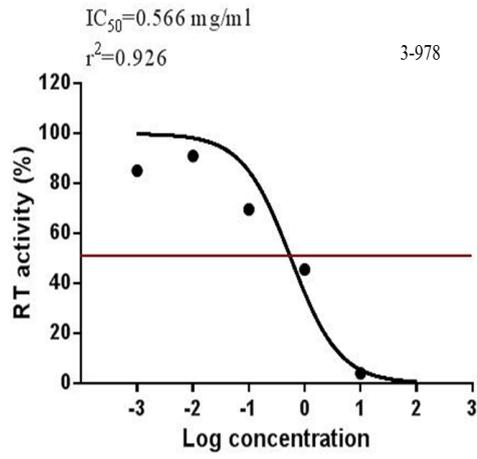
IC₅₀ of very potent plant extracts



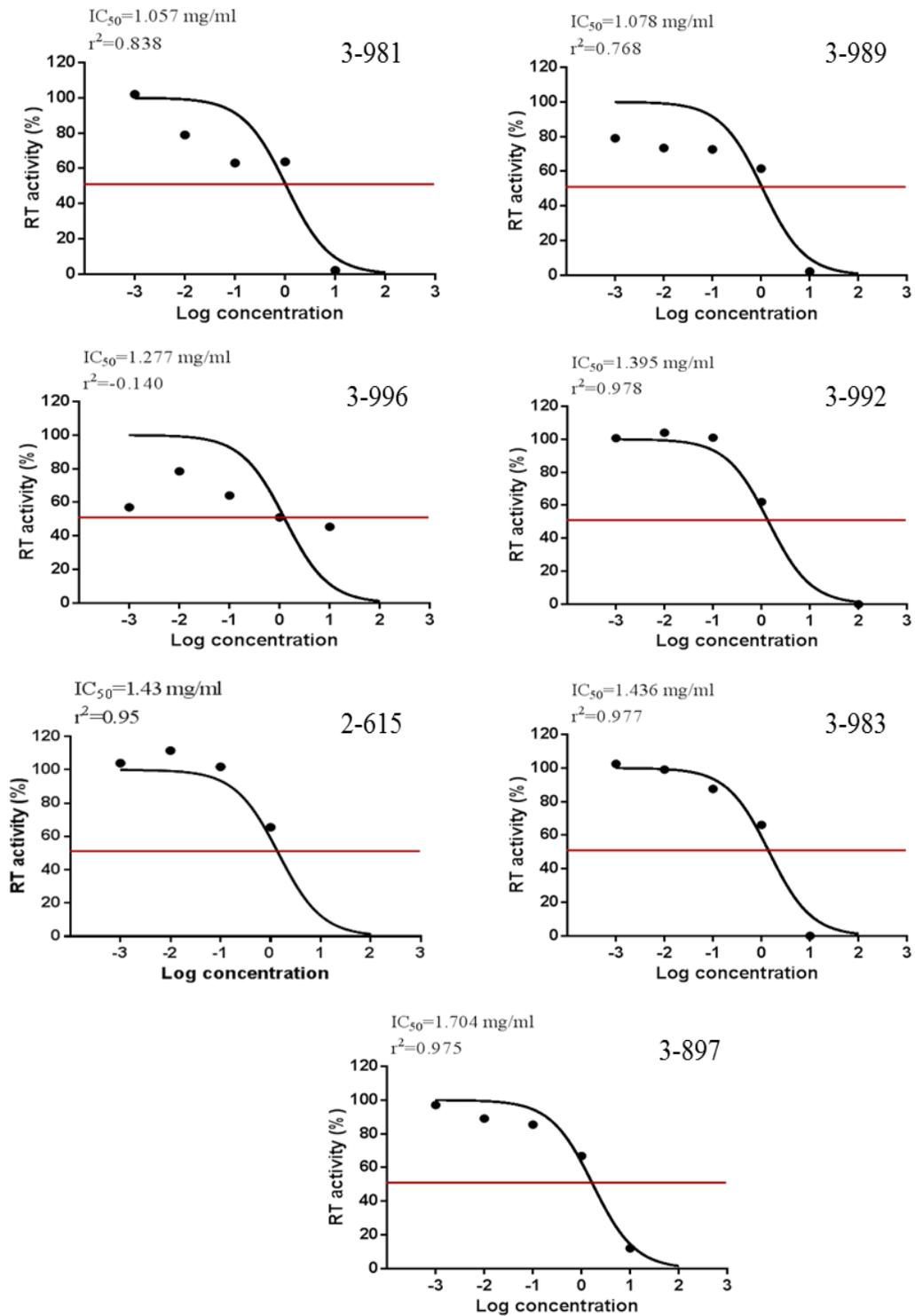
IC₅₀ of very potent plant extracts



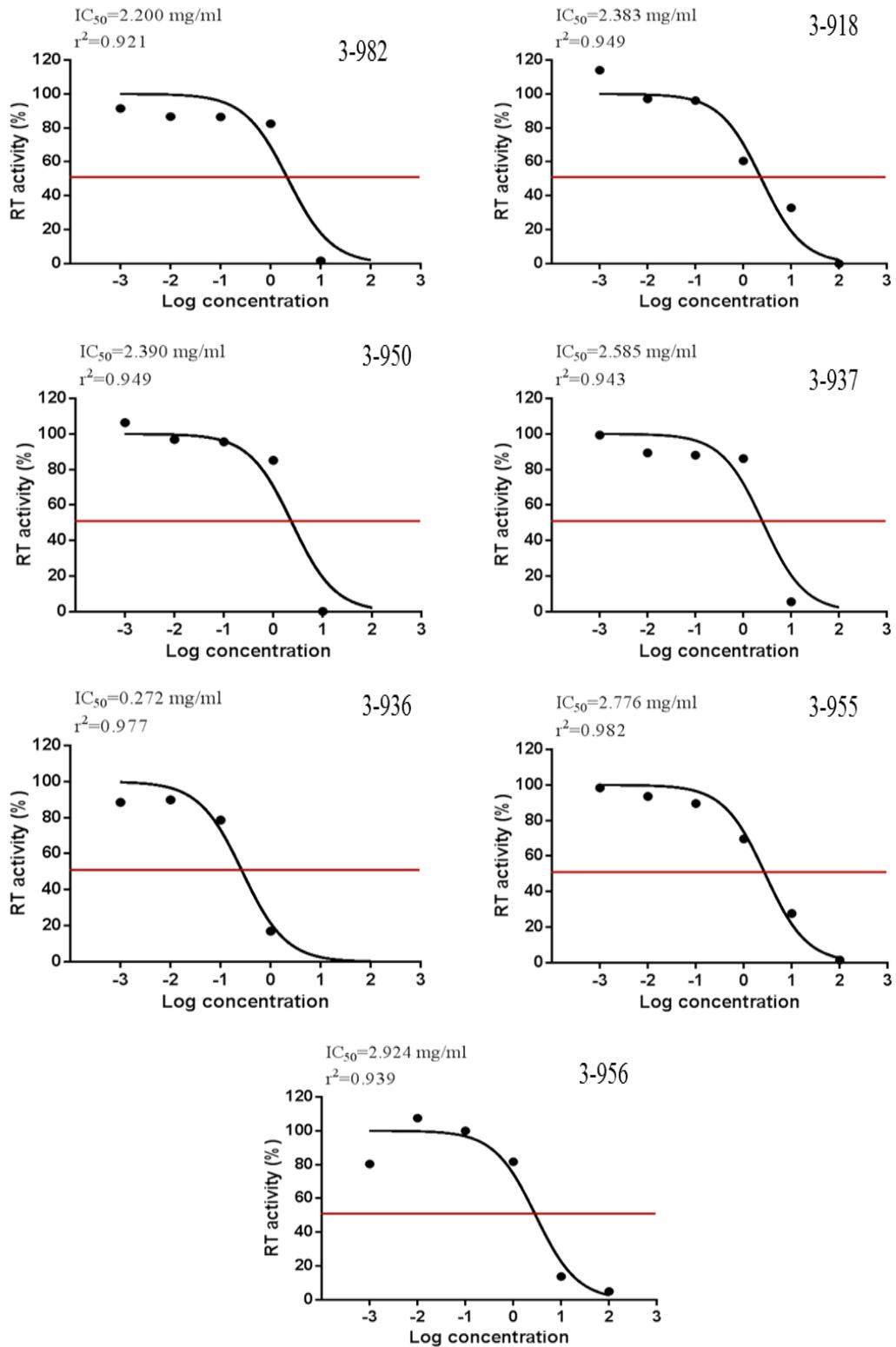
IC₅₀ of very potent plant extracts



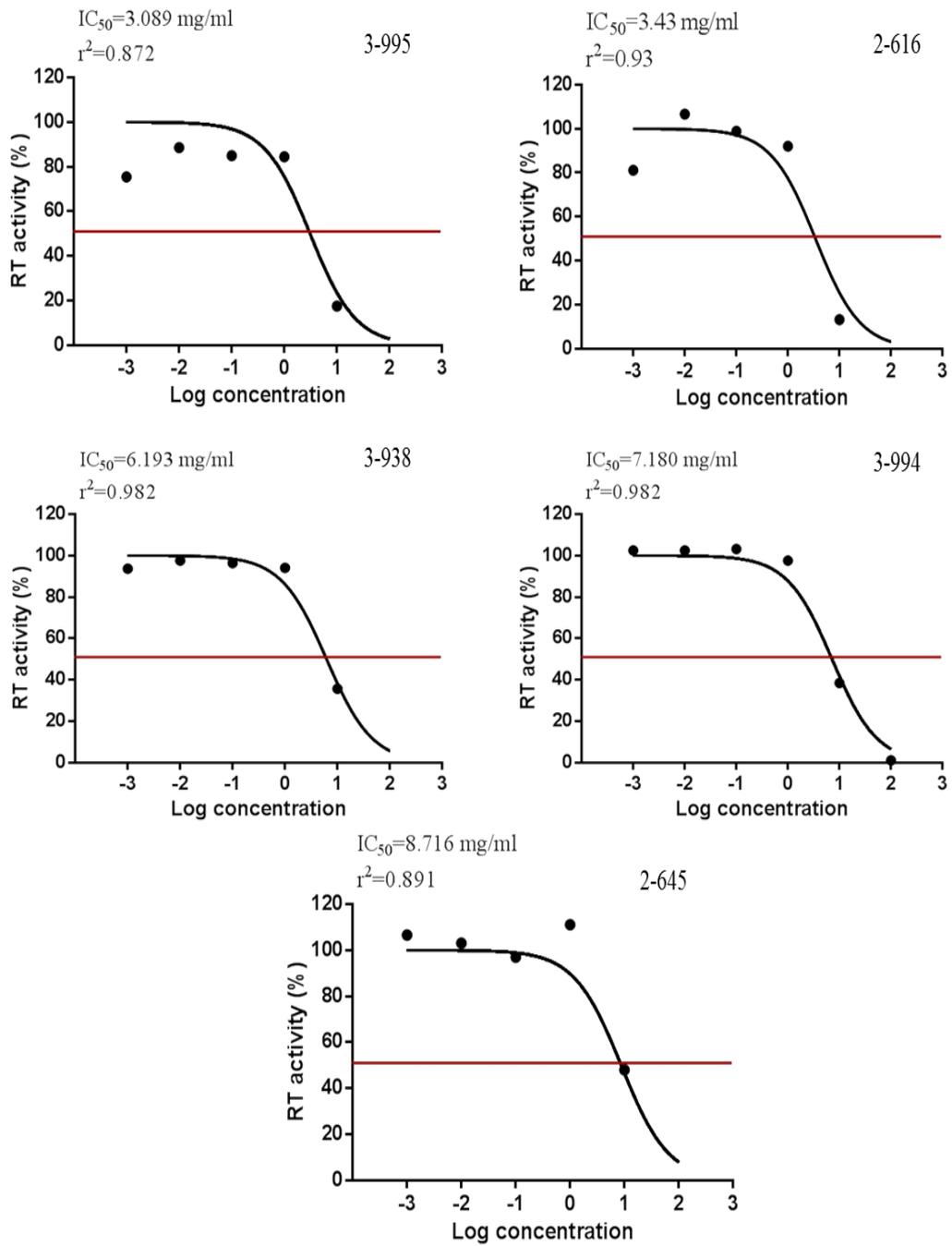
IC_{50} of potent plant extracts



IC₅₀ of plant extracts with low potency



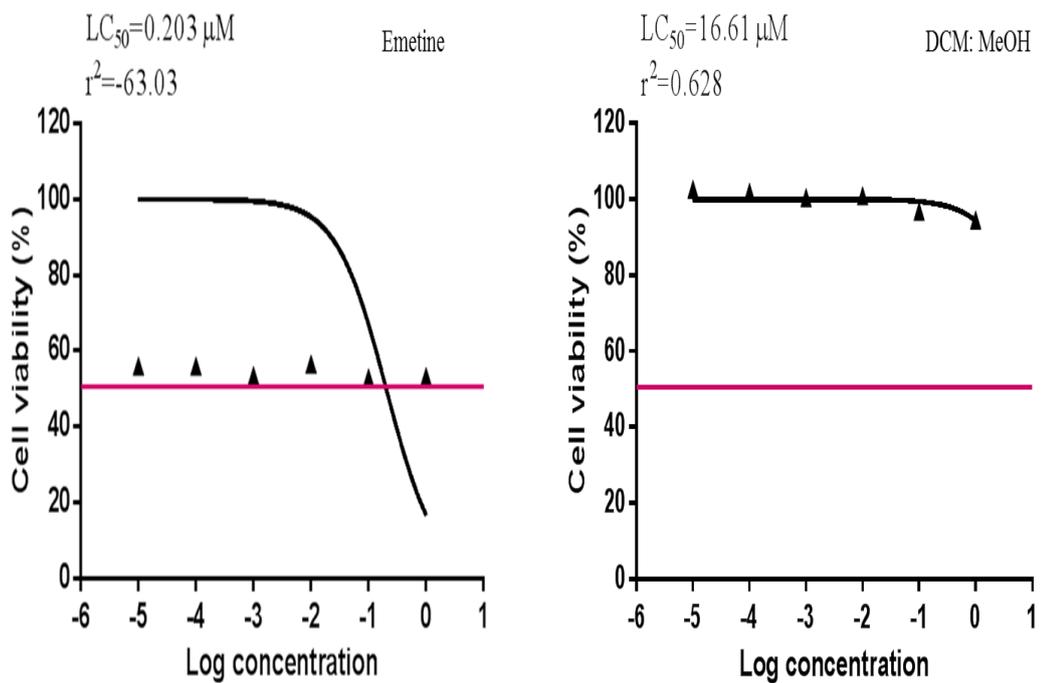
IC_{50} of plant extracts with low potency



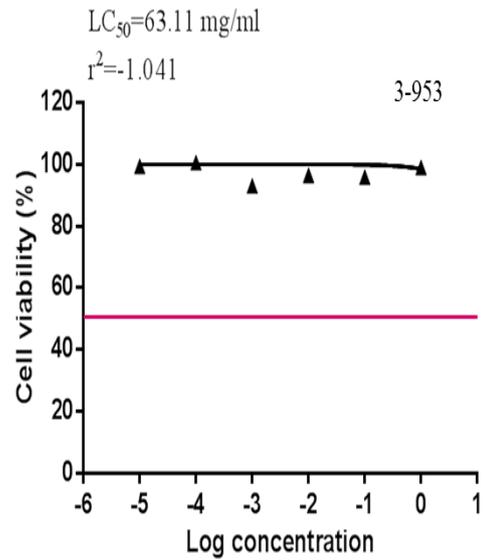
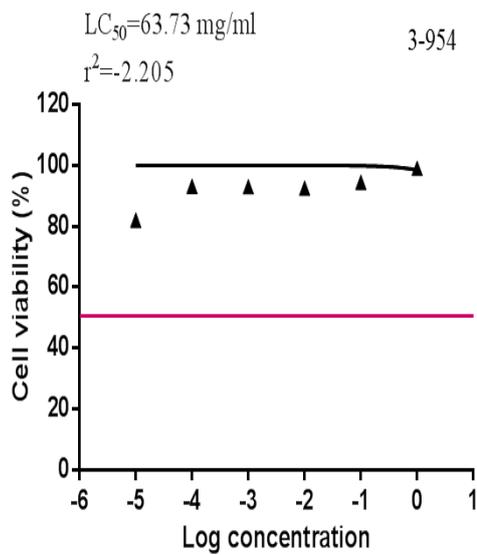
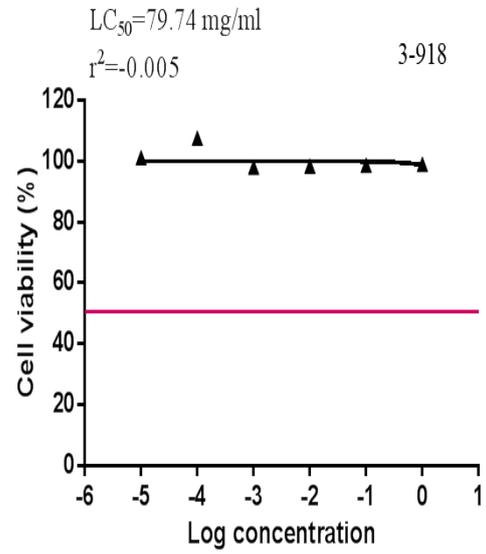
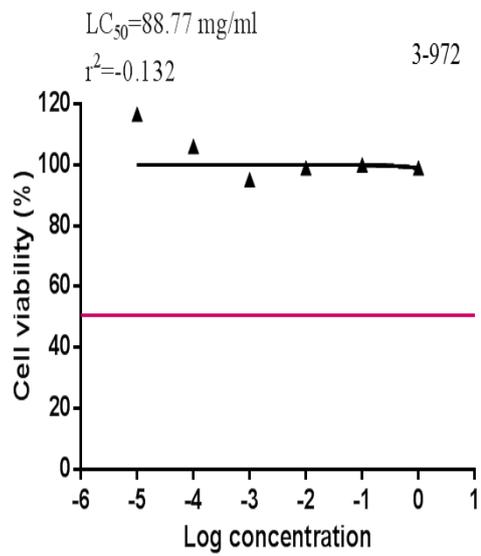
IC₅₀ of plant extracts that are not potent

APPENDIX B

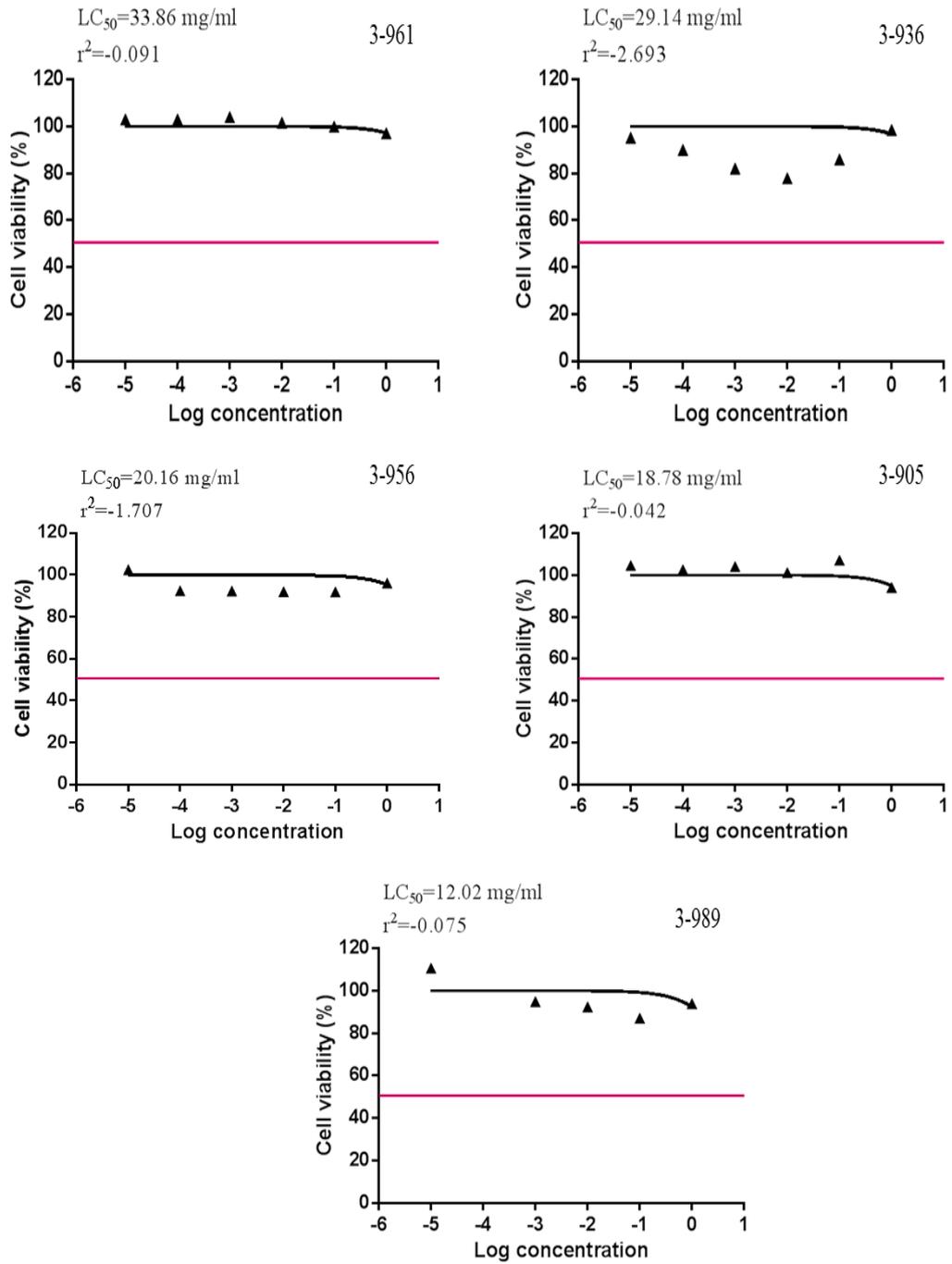
Dose-response curves with non-linear sigmoidal curves to determine CC_{50} of plant extracts. This was based on colorimetric cell proliferation assay for determining viability of vero cells based on reduction by MTS.



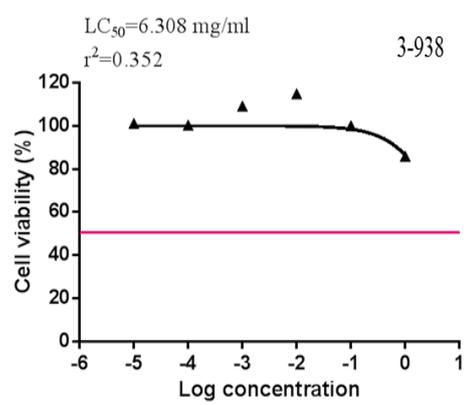
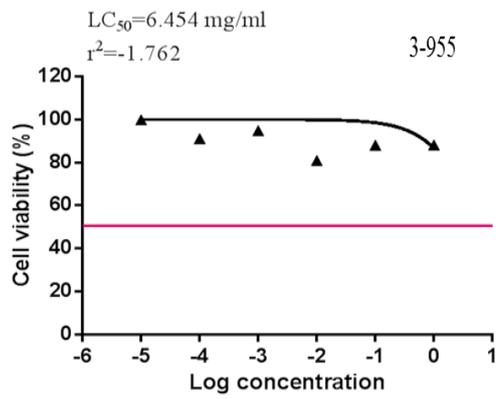
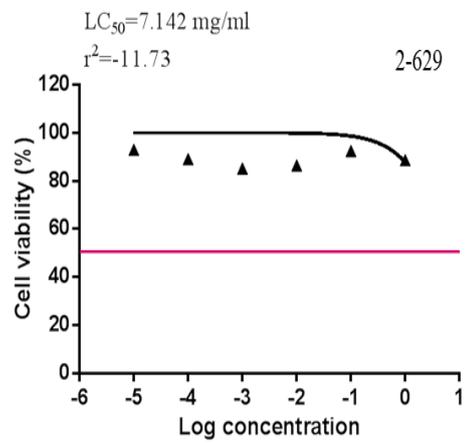
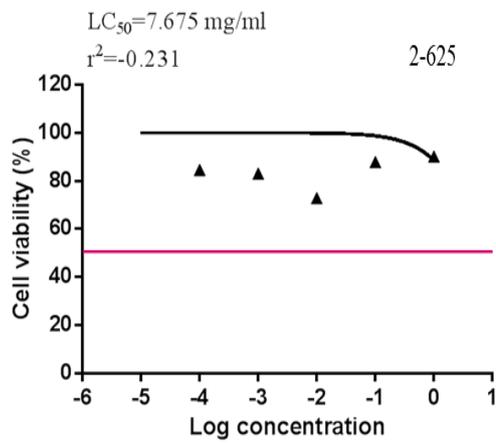
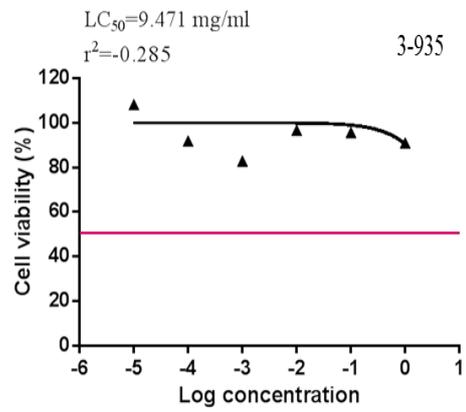
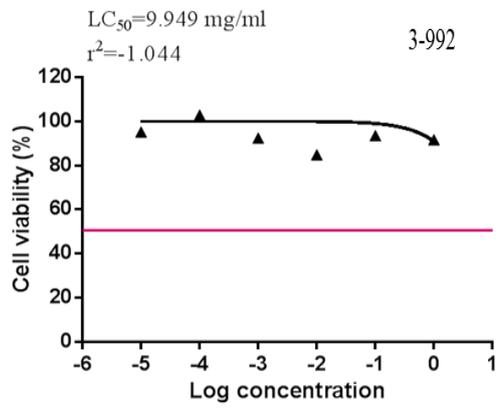
CC_{50} of Emetine (standard) and DCM/MeOH (the solvent)



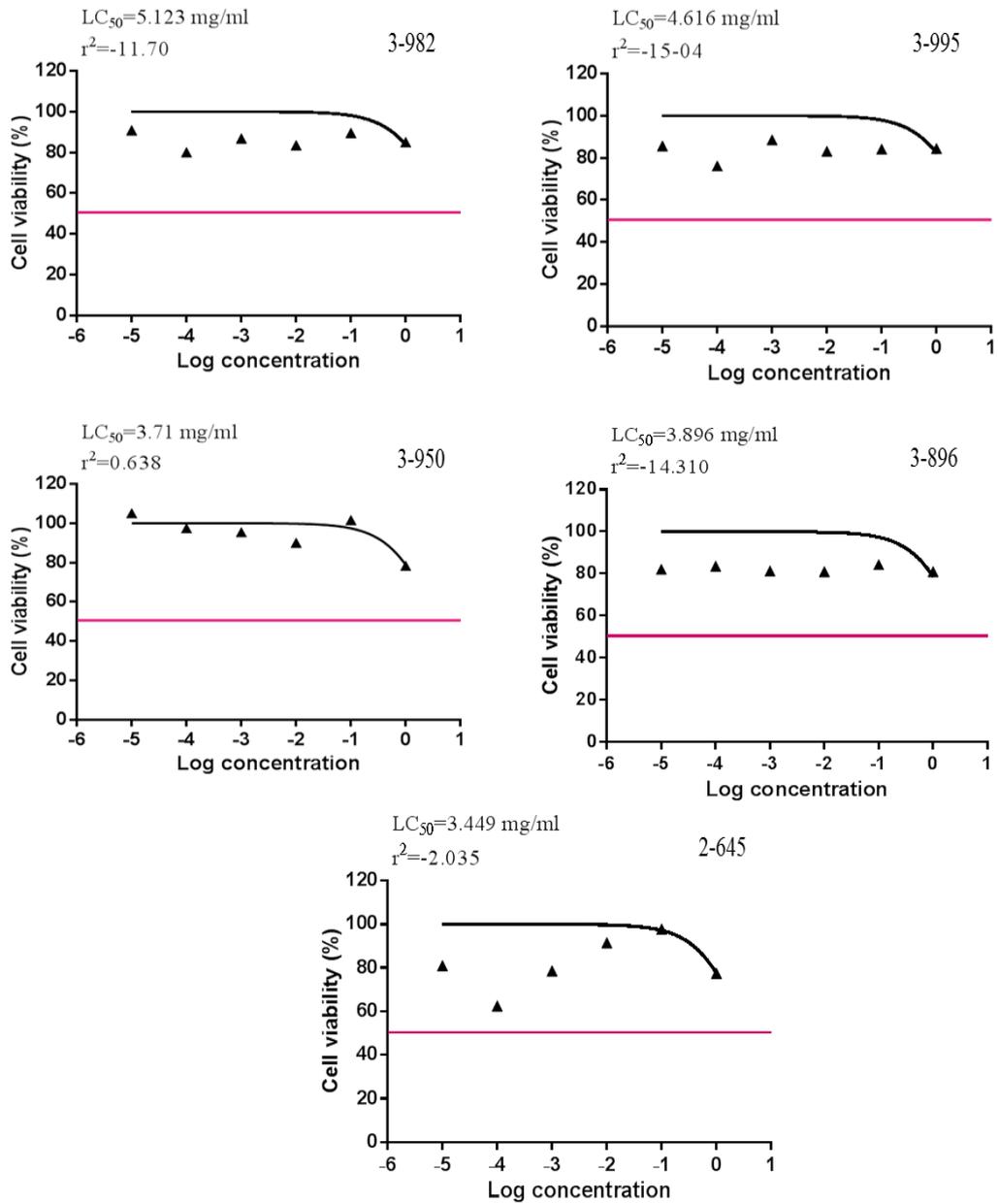
CC₅₀ of plant extracts that are not toxic



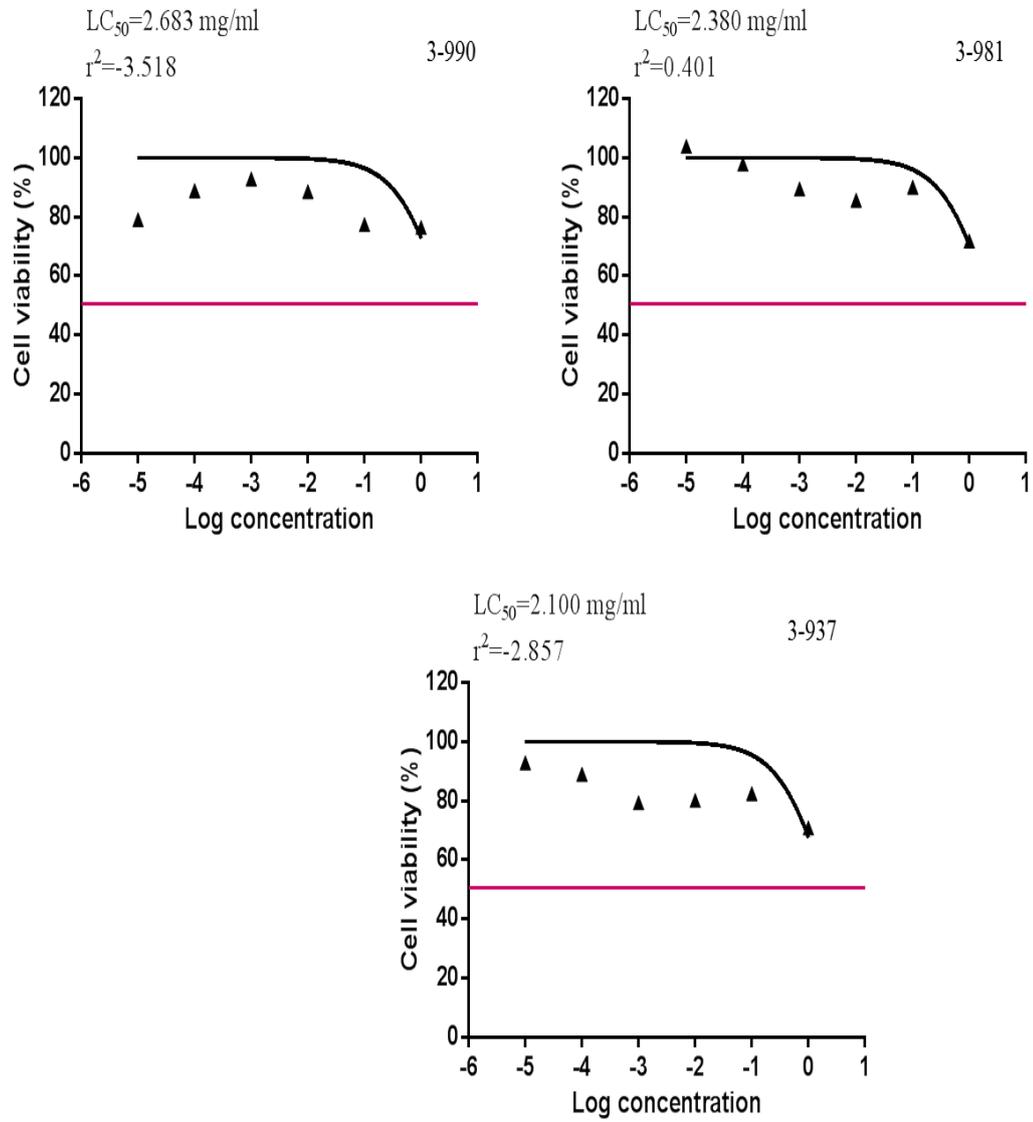
CC₅₀ of plant extracts that are not toxic



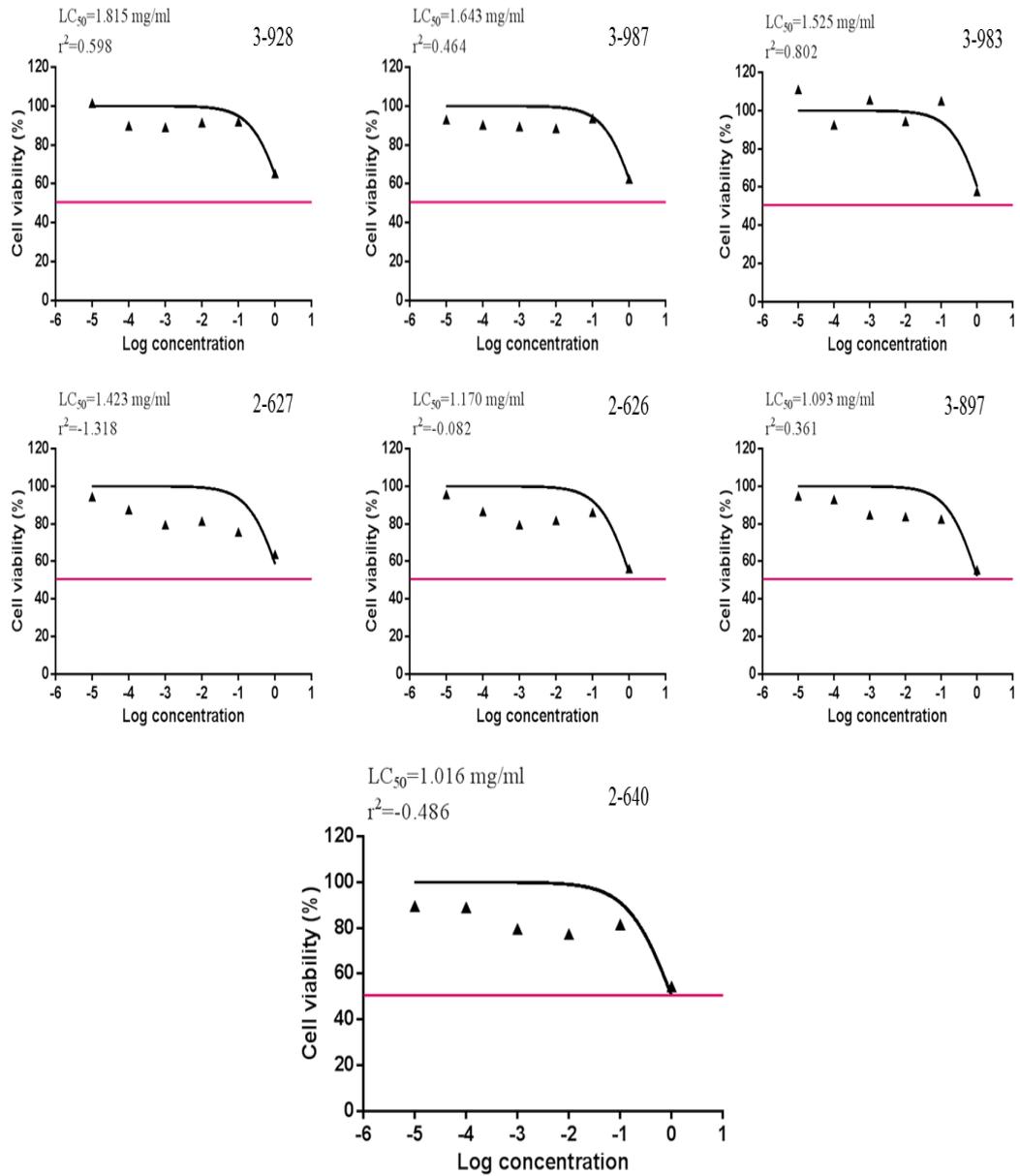
CC₅₀ of plant extracts with low toxicity



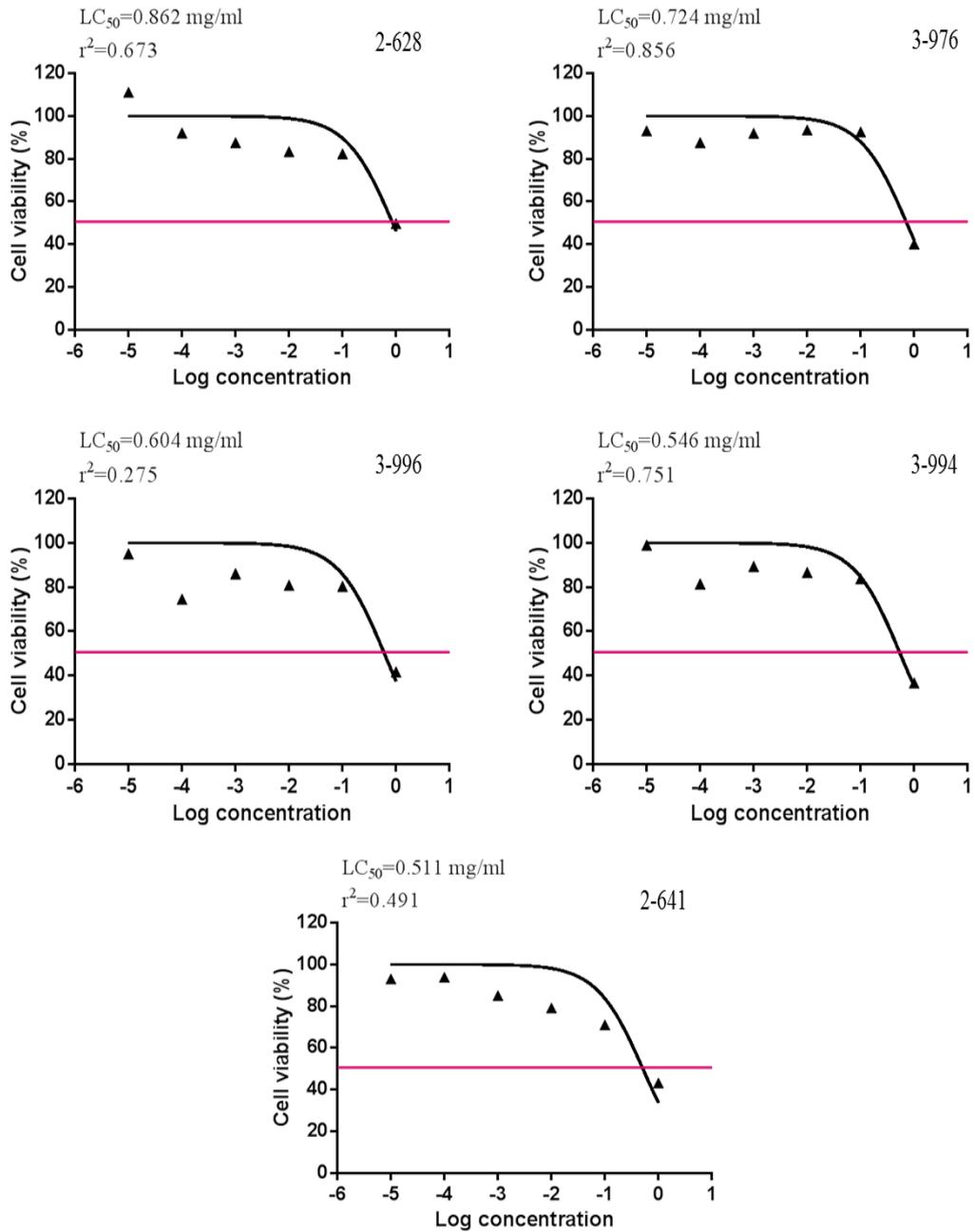
CC₅₀ of plant extracts that are toxic



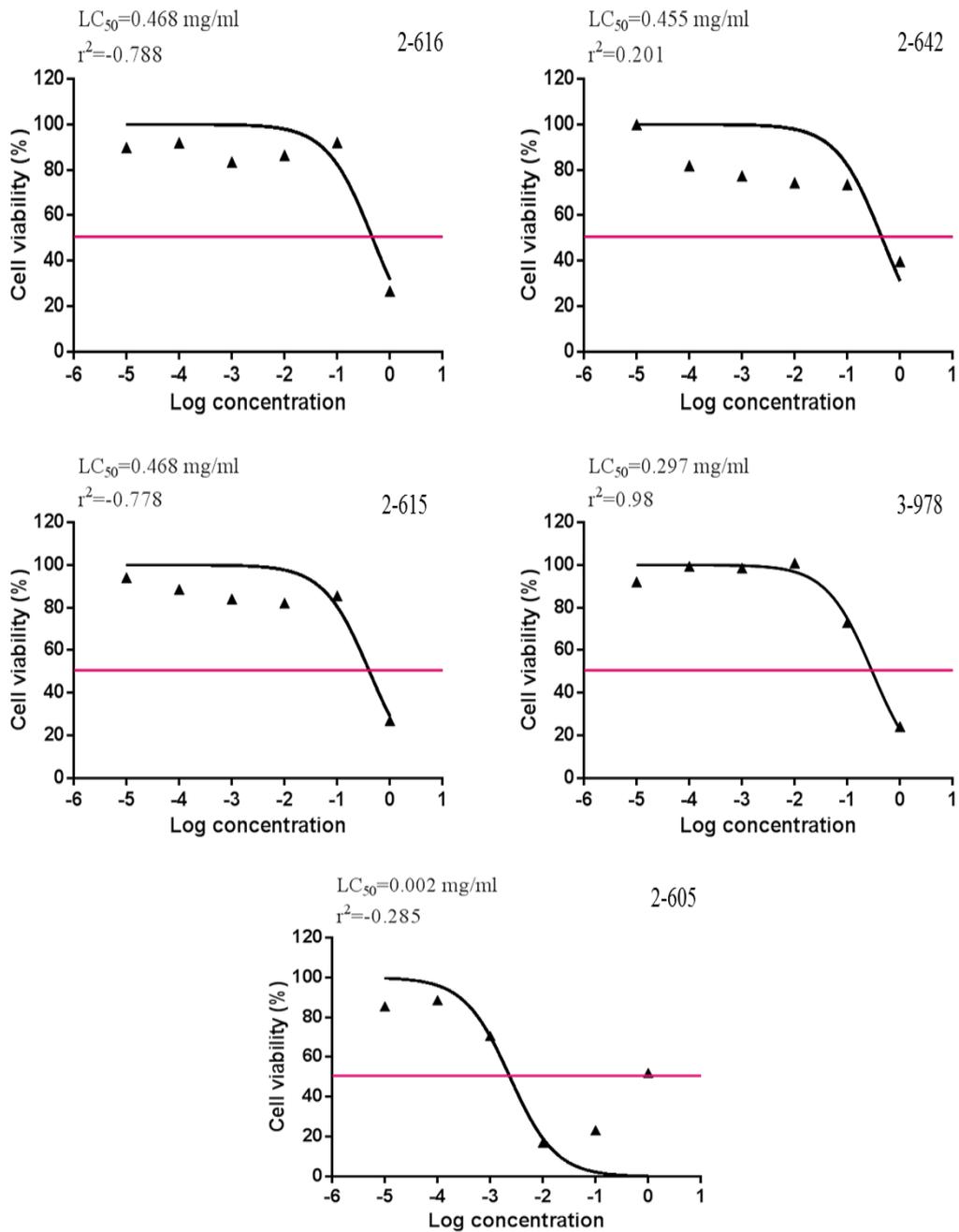
CC_{50} of plant extracts that are toxic



CC_{50} of plant extracts that are toxic



CC₅₀ of plant extracts that are very toxic



CC₅₀ of plant extracts that are very toxic

APPENDIX C

Plant collection permit obtained from the Ministry of Environment and Tourism (MET).

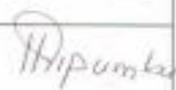
 MINISTRY OF ENVIRONMENT AND TOURISM				
<h3>RESEARCH/COLLECTING PERMIT</h3>				
Permit Number 1682/2012 Valid from 16 March 2012 to 28 February 2013				
Permission is hereby granted in terms of the Nature Conservation Ordinance 1975 (Ord. 4 of 1975) to:				
Name:	Mr. M. Hedimbi			
Address:	University of Namibia 340 Mandume Ndemufayo Avenue P/Bag 13301 Windhoek Namibia			
Coworkers:	Dr. K. Chinsembu, Dr. D. Mumbengegwi, Dr. R. Hans and Ms. H. Amputu			
To conduct a study on evaluation of selected Namibian plants for anti-HIV properties through Namibia excluding protected areas, subject to attached conditions.				
IMPORTANT: This permit is not valid if altered in any way.				
 Authorising Officer	<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="text-align: center;"> MINISTRY OF ENVIRONMENT AND TOURISM REPUBLIC OF NAMIBIA </td> </tr> <tr> <td style="text-align: center;"> 26 MAR 2012 </td> </tr> <tr> <td style="text-align: center;"> WINDHOEK Private Bag 13306, Windhoek Tel: 2842111 • Fax: 255561 </td> </tr> </table>	MINISTRY OF ENVIRONMENT AND TOURISM REPUBLIC OF NAMIBIA	26 MAR 2012	WINDHOEK Private Bag 13306, Windhoek Tel: 2842111 • Fax: 255561
MINISTRY OF ENVIRONMENT AND TOURISM REPUBLIC OF NAMIBIA				
26 MAR 2012				
WINDHOEK Private Bag 13306, Windhoek Tel: 2842111 • Fax: 255561				
IMPORTANT This permit is subject to the provisions of the Nature Conservation Ordinance, 1975 (Ordinance 4 of 1975) and the regulations promulgated thereunder, and the holder is subject to all such conditions and regulations.				
<small>Enquiries: Conservation Scientist, email matheus@met.na Private Bag 13306, Windhoek, Namibia</small>				

RESEARCH/COLLECTING PERMIT CONDITIONS

1. All collection must be conducted in non-conservation areas.
2. The Regional Nature Conservation official must be notified prior to arrival in fieldwork area, and must be shown your permit.
3. A material transfer agreement (MTA) must be signed with the NBRI for any plant material and/or extracts or derivatives thereof to be exported prior to the export thereof.
4. Duplicate voucher specimens of all plants collected are to be lodged with the National Herbarium at the NBRI.
5. A copy of the final report be submitted to the NBRI reference library
6. The permission of the land owner is required to work/collect on private lands.
7. The permission of the concession holder is required to work/collect in concession areas.
8. The permission of the communal authority is required to work/collect in communal areas.
9. No commercial filming will be permitted without prior approval by the Ministry of Environment and Tourism.
10. No palaeontological and or archaeological samples may be taken without a permit from the National Monuments Council.
11. The specimens and their derivatives may be used for the purposes of this study only and may not be patented, commercialised, donated or sold to a third party without the written consent of the Ministry of Environment and Tourism.
12. All results (raw materials) or technology derived directly or indirectly from this research must be made available free of charge without reservations to the Ministry of Environment and Tourism.
13. Please submit a report on the work conducted under this permit to this office not later than one month after the expiry of this permit.
14. Applications for renewal of this permit must reach this office at least two months prior to the expiry of this permit.
15. Habitat destructive collecting methods must not to be used.
16. Local Veterinary export regulations will apply.
17. Foreign (destination) wildlife import, and veterinary import permits may be required. Please file copies with our Permit Office.
18. All field teams must be in possession of the permit (or copy) and permits must accompany the transport of specimens.
19. You are subject to all conditions listed on the entry permit to any of the protected areas, unless specifically exempted.
20. It is your responsibility to make the necessary contacts and arrangements as specified above.

APPENDIX D

Phytosanitary certificate obtained from the ministry of Agriculture, Water and Forestry (MAWF).

 REPUBLIC OF NAMIBIA	GOVERNMENT REPUBLIC OF NAMIBIA PHYTOSANITARY CERTIFICATE		ORIGINAL						
			Serial Number Certificate Number 14967						
To: The Plant Protection Organisation of South Africa		Place of Issue Windhoek, Namibia							
DESCRIPTION OF CONSIGNMENT									
1. Name and Address of Exporter Marius Redimbi Unam, P/Bag 13301 Windhoek, Namibia		2. Declared name and address of consignee Dr. Phiyani CSIR Pretoria, South Africa							
3. Number and description of packages Crush Plant Material		4. Distinguishing marks							
5. Place of origin Namibia		5. Declared means of conveyance Road//Air							
7. Declared point of entry Noordoewer/Ariamsvlei/HKIA									
8. Name of produce and quantity declared <table style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 30%;">Name of produce</th> <th style="width: 30%;">Botanical name</th> <th style="width: 40%;">Quantity</th> </tr> </thead> <tbody> <tr> <td>50 Grams Containers with blue Caps</td> <td></td> <td></td> </tr> </tbody> </table>				Name of produce	Botanical name	Quantity	50 Grams Containers with blue Caps		
Name of produce	Botanical name	Quantity							
50 Grams Containers with blue Caps									
<small>This is to certify that the plants, plant products or other regulated articles described herein have been inspected and for tested according to appropriate official procedures and are considered to be free from the quarantine pests specified by the importing contracting party and to conform with the current phytosanitary requirements of the importing contracting party, including those for regulated non-quarantine pests</small>									
DISINFESTATION AND / OR DISINFECTION TREATMENT									
9. Date		10. Treatment							
11. Chemical (Active ingredient)		12. Duration and Temperature							
13. Concentration The plant materials are going for further Research Purpose.		14. Additional information							
15. Additional declaration									
 Stamp of Organisation 12/06/2012		17. Signature of Authorized Officer 							
16. Date of Issue		18. Name of Authorized Officer H. Lipumbu							
<small>No financial liability with respect to this Certificate shall be attached to the Government Republic of Namibia or to any of its officers or representatives.</small>									

APPENDIX E

Plant export permit obtained from Ministry of Environment and Tourism (MET).

MINISTRY OF ENVIRONMENT AND TOURISM Directorate Scientific Services Private Bag 13300 Windhoek, Namibia Enquiries: Chief Warden, Permit Office	PERMIT No.:	88189
	VALID FROM:	08 June 2012
	VALID TO:	08 Dec. 2012
	RECEIPT No.:	MET 17644
GENERAL PERMIT		
Name: <u>Marius Hedimbi</u>		
Residential Address: _____		
Postal Address: <u>Private Bag 13301 Windhoek</u>		
Permission is hereby granted in terms of the Nature Conservation Ordinance 1975 (Ord. 4 of 1975)		
<p>To export the following plant material from Windhoek in Rep. of Namibia to University of Toronto in Canada. (See attached list) or CSIR, Pretoria in R.S.A.</p>		
Remarks - Conditions		
<p>- Subject to MTA signed with NBR I and Import regulations</p>		
IMPORTANT: This permit is not valid if altered in any way		
 Authorizing Officer		
IMPORTANT		
This permit is subject to the provisions of the Nature Conservation Ordinance, 1975 (Ordinance 4 of 1975) and the regulations promulgated thereunder, and the holder is subject to all prescribed conditions and regulations.		

APPENDIX F

Material transfer agreement (MTA) obtained from Namibia Botanical Research Institute (NBRI).



NATIONAL BOTANICAL RESEARCH INSTITUTE AGREEMENT FOR THE SUPPLY OF BIOLOGICAL MATERIAL FROM THE REPUBLIC OF NAMIBIA FOR SCIENTIFIC RESEARCH PURPOSES

The National Botanical Research Institute (NBRI) is a subdivision within the Directorate of Agricultural Research and Training (DART) of the Ministry of Agriculture, Water and Forestry (MAWF) and its mission is "to promote the understanding, conservation and sustainable use of Namibian plants for the benefit of all".

In its work, the NBRI intends to honour the letter and spirit of the 1992 Convention on Biological Diversity, the 1973 Convention on International Trade in Endangered Species of Wild Fauna and Flora, the 2004 International Treaty on Plant Genetic Resources for Food and Agriculture, all other relevant international instruments to which Namibia is a signatory, and all national laws concerning biodiversity.

On receipt of the Agreement signed by the Recipient below and because the Recipient has agreed to comply with the terms and conditions set forth in this Agreement, the NBRI will supply to the Recipient such of the biological material (the Material)¹ requested by the Recipient as is, in the NBRI's judgement, reasonable and appropriate.

Such biological Material as is supplied to the Recipient shall be accompanied by a copy of this Agreement and an itemised list ("the Schedule") of the biological material being supplied, clearly stating the identity and quantity of Material supplied, the Purpose for which it is supplied, and any additional conditions that might apply to the supply of the Materials.

The Recipient acknowledges that he/she is acting as a duly authorised representative of the institution he/she represents, and that the terms of this Agreement shall be binding on all present and future employees of his/her organisation, for as long as this agreement remains in force.

The supply of any and all Biological Material by the NBRI to the Recipient, including any Material to be supplied under this agreement, will be subject to the following conditions:

1. Subject to clauses 2 and 4 below, the Recipient may use the Material and progeny or derivatives thereof (such as modified or unmodified extracts) for non-commercial purposes only.
2. The Recipient will provide NBRI with a fair and equitable share of any benefits obtained by the Recipient arising out of any utilisation by the Recipient of the Material or its progeny or derivatives, including benefits such as research results, copies of papers, acknowledgement of the NBRI as the Source of the Material.
3. Under this Agreement, the Recipient may not commercialise² the Material or any progeny or derivatives thereof.

¹ "Biological Material" shall include plants, plant parts or propagation material (such as seeds, cuttings, roots, bulbs, corms or leaves) fungi or other fungal material and any other material of plant, microbial, fungal or other origin and the genetic resources contained therein.

"Genetic Resources" shall mean any biological material of actual or of potential value containing functional units of heredity including DNA, transferred under this Agreement and its progeny and derivatives, including modified or unmodified extracts and purified compounds.

² "Commercialise" and "Commercialization" shall include but not be limited to any of the following: sale, filing a patent application, obtaining or transferring intellectual property rights or other tangible or intangible rights by the sale or licence or in any other manner, commencement of product development, conducting market research and seeking pre-market approval.

M.H

S

4. If, at any point, in the future the recipient wishes to commercialise the Material or its progeny or derivatives, the Recipient must first obtain the written permission of the NBRI. Any commercialisation to which NBRI agrees will be subject to a separate agreement between the Recipient and NBRI consistent with NBRI's policy.
5. **The Recipient may not transfer** the Material or any progeny or derivatives thereof to any third party² other than the Recipient or NBRI without the prior informed consent, in writing, of NBRI and then only under legally binding written agreement containing terms no less restrictive than those contained in this Agreement unless otherwise agreed in writing by NBRI. The Recipient agrees to take every reasonable precaution to prevent the material coming into the possession of any unauthorised third party.
6. NBRI makes no representation or warranty of any kind whether express or implied:
 - (a) as to the identity, safety, saleability or fitness for any particular purpose of the Material or its progeny or derivatives or
 - (b) that the Material provided to the Recipient under this Agreement is or will remain free from any further obligation to obtain prior informed consent from, to share benefits with or to comply with restrictions on use imposed by the national or local authorities. The Recipient will indemnify NBRI from any and all liability arising out of the Material or its progeny or derivatives and their use.
7. The biological Material is provided at no cost, or with an optional transmittal fee solely to reimburse the NBRI for its collection, preparation and distribution costs. If a handling fee is requested the amount will be indicated here:

² "Third Party" shall mean any person other than the NBRI and the Recipient.

M.H

S

I understand that any Material supplied to me by NBRI pursuant to this Agreement will be subject to, and I agree to comply with, the conditions above, and the additional conditions in the Schedule attached.

SIGNED BY: MAH

for and on behalf of

Name of the Recipient: Marius Hedrick

Title: MI

Date: 17 April

Address of the Recipient:
University of Manitoba
340 Main Street West
Winnipeg, Manitoba
R3S 0V6

SIGNED BY: [Signature]
for and on behalf of the NBRI

Name: S. J. CARR

Title: SENIOR AGRICULTURAL RESEARCHER

Date: 17 APRIL 2012



