Cape Peninsula
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Bioactive compounds in a Manayi traditional medicinal product from East London

By

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DECLARATION

I, Mongikazi Mbandezelo, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed **Date**

ABSTRACT

In Africa, herbal medicines are often used as primary treatments for a variety of ailments and diseases including HIV/AIDS and for HIV-related problems. In general, traditional medicines are not well researched scientifically in controlled studies, and are poorly regulated. Since the pharmacological effectiveness of natural products is affected by several native and foreign factors, studies on the variations of chemical composition and biological activity of these medicines are necessary.

The processes of investigating plants to identify chemical substances are of great interest to natural product researchers because there is a need to discover new drugs for treating old and new diseases. These facts underscore an urgent need to develop new anti HIV and AIDS drugs with fewer or no side effects. Research into drug discovery and development using natural products is increasingly becoming better established. Marine organisms as a source of natural products delivered numerous novel compounds with multiple pharmacological properties. Natural products give endless opportunities for discovering novel compounds that can be used as drugs or backbones of drug leads. Manayi is a natural product that has been used to treat and manage people with HIV, but no scientific studies have been done to prove its efficacy on the HIV under controlled conditions

For the purposes of this study, cooked and uncooked Manayi product was evaluated for its efficacy on HIV *in vitro*. Manayi samples were collected in East London, Eastern Cape and sequentially extracted with hexane, chloroform, dichloromethane, butanol, methanol, and water as a series of increasingly polar solvents for its bioactive chemical constituents. The extracts were tested against HIV-1 enzyme *in vitro* and their potential *in vitro* cytotoxicity. The effect of Manayi extracts were evaluated in *vitro* with recombinant HIV-enzyme, using a non-radioactive HIV-RT colorimetric ELISA from Roche and its effects on Integrase enzyme. Cytotoxicity (The ability to kill cancer cells) of the extracts was determined using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazodium bromide) assay.

Phytochemical analyses of Manayi were also done to determine the possible chemical groups present in the product. Phytochemical analyses of the crude product would assist in the chemical purification of the drug and physicochemical properties were also determined to standardize the crude drug for safety and effective system of traditional medicine. Two extracts with the highest percentage yield and highest HIV activity against integrase enzyme were selected for further investigation. Using chromatographic fractionation process, 14 fractions were isolated and those fractions were evaluated using Thin Layer Chromatograph (TLC). Fraction 8 named MC1 (Manayi Compound 1) (0%water: 100 methanol) of uncooked water extract exhibited only one compound and was the purest. The compound of this fraction was then chemically characterized using FTIR, NMR and LCMS.

Manayi crude extract showed no activity against HIV-1 Reverse Transcriptase, but inhibitory activity was shown for the HIV integrase enzyme assay. The Hexane extract showed the highest most potent inhibitory effect of 99.4% at 50 µg/ml, and was followed by Chloroform extract with 93.6% inhibitory effect also at 50 µg/ml. Cytotoxicity results of the cooked and uncooked Manayi extracts in Vero cells reveal that the product is not toxic in all the tested extracts. Qualitative phytochemical analysis of the product showed the presence of proteins, glycosides, phenolic, tannins, saponins and gums. The results of physicochemical properties indicate that the Manayi product has mainly organic compounds as reflected in their different content of total ash, acid soluble ash, water soluble ash, sulphated ash, alcohol soluble extractives and water soluble extractives. Physiochemical tests showed that the values of total ash, acid insoluble ash, water soluble ash, sulphated ash, alcohol extractive and water extractive for uncooked Manayi were 62.5, 60, 60, 40, 3 and 17 %. For cooked Manayi the total ash, acid insoluble ash, water soluble ash, sulphated ash, alcohol extractive and water extractive were 62.5, 20, 60, 40, 3 and 7 % respectively.

Bioassay guided fraction using different chromatographic and spectroscopic techniques in the analysis of the cooked and uncooked Manayi led to the isolation of one compound. Further purification is required since the compound appeared to be not pure when analysed by NMR. LCMS analysis of the same compound revealed that it may be a mixture of palmitic and oleic acids.

Further purification and chemical characterizations are needed to conclusively identify the chemical nature and structure of the bioactive compounds in Manayi, by comparing their molecular ions (m/z) to those validated.

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DEDICATION

This thesis is dedicated to the sake of God, my creator and my Master, my uncle, the teacher and messenger, who taught me the purpose of life and the importance of education. My dearest mother, who led me through the valley of darkness with light of hope and support (May your beautiful soul rest in peace). My beloved family, particularly my dearest aunt, Nowayilesi Stofile, who was there for me when things were rough, my friends who encouraged and supported me, and all the people in my life who touch my heart I dedicate this research

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GLOSSARY

AIDS- Acquired Immunodefiency Syndrome

ARV- Antiretroviral drugs

ART- Antiretroviral Therapy

AZT- Azidothymine is an anti HIV drug that reduces the amount of virus in the body.

CCR5- Chemokine receptor that is a protein on the surface of white blood cells that is involved in the immune system as it acts as a receptor for chemokines.

CD4- Is the type of white blood cells that play a major role in protecting your body from

CXCR4- Is a protein coding gene. Diseases associated with CXCR4 include whim syndrome and usual interstitial pneumonia.

DMSO- Dimethyl Sulfoxide

DNA- Deoxyribonucleic acid, is the hereditary in humans and almost all other organism

FBS- Foetal Bovine serum HIV-Human Immunodefiency Virus

FTIR-Fourier Transform Spectroscopy

HAART- Highly active anti-retroviral therapies

HIV-Human Immunodefiency Virus

HRP- Horseradish peroxide

LCMS- Liquid Chromatography Mass Spectroscopy

MMT- is a colorimetric assay for assessing cell metabolic activity.

NMR-Nuclear Magnetic Resonance

NNRTIS- is an Non-Nucleoside, Reverse Transcriptase Inhibitor that bind to block HIV reverse transcriptase (an HIV enzyme)

P24- Antigen is a viral protein that makes up most of the viral core.

P450- are a major source of variability in drug pharmacokinetics and response

RNA-Stands for ribonucleic acid. syndrome

RPMI 1640- is a general purpose media with a broad range of applications for mammalian cells, especially hematopoietic cells.

RT- Reverse Transcriptase

SADC- Southern African Development Community

SPE- Solid Phase Extraction

TLC-Thin Layer Chromatograph

UNAIDS-Joint United Organisation programme on HIV and AIDS

SPE- Solid Phase Extraction

WHO- World Health Organisation

CHAPTER ONE: INTRODUCTION

1 Introduction

1.1 What is HIV

Human immunodeficiency virus (HIV) is the causative agent in Acquired Immunodefiency Syndrome (AIDS), an infectious medical conditions that has been spreading at an alarming rate worldwide, Africa being the hardest hit continent with more than 70 % people infected with HIV (Essex, 1999). There are two related but distinct types of HIV: HIV-1 and HIV-2 (Fletcher et al, 2002). HIV-1 is the most pathogenic and causes over 99 % of HIV infections (Cos et al, 2004). HIV-2 is also known to cause AIDS but is much less prevalent, being present in fewer and isolated geographic locations such as West Africa. Therefore, most research is done on HIV-1 (Klos et al, 2009). AIDS infections remain one of the leading causes of death globally (NIAID, 2001). There is an increase in the number of people who are dying with HIV and AIDS as there is no cure as yet. According to the Joint United Organization Programme on HIV/AIDS (UNAIDS), the number of people living with HIV/AIDS worldwide was estimated at 33.4 million in 2008, which is 20 % higher than the number in 2000 (UNAIDS, 2009). It was estimated that 2 million deaths due to AIDS-related illnesses occurred worldwide in 2008, and this was 10 % lower than in 2004 (UNAIDS, 2009).

Current antiretroviral (ARV) drugs are vitally important to improve the quality of life and prolong life of HIV/AIDS patients. However, these drugs have many disadvantages, which include but not limited to resistance, toxicity, negative side effects, limited availability, high cost and lack of any curative effect. Thus, it is important to search for improved antiretroviral agents, which can be added to or replace the current drugs in the anti-HIV armamentarium (Klos et al, 2009). There is a need for new ARV treatment methods that are safe, effective and well tolerated. Resistance of the virus to ARV drugs poses challenges to the eradication of the disease. Side effects of ARVs, and the knowledge of non-curative effects of ARVs, drive patients to seek alternative treatment, which includes the use of traditional medicine. Natural products used as traditional medicine are purported to have health benefits for people with HIV but there is no scientific proof to support such serious claims.

1.2 Structure and the life cycle of HIV

HIV belongs to the class of viruses called retroviruses, which carry genetic information in the form of RNA. HIV uses the macrophages and T cells as sites for reproduction and production of several copies of viral genetic material ready to transmit new viral hosts. Throughout each round of infection more cells of the immune system are damaged or killed. The host cells produce antibodies and helper T-cells in order to fight the virus, but eventually the virus prevails, and opportunistic diseases associated with AIDS appear (Gurib-Fakim, 2006). HIV

protease, the third virally encoded enzyme, is required in this step to cleave a viral polyprotein precursor into individual mature proteins. The viral RNA and viral proteins assemble at the cell surface into new virions, which then bud from the cell and are released to infect another cell. The extensive cell damage from the destruction of the host's genetic system to the budding and release of virions leads to the death of the infected cells (Brik and Wong, 2003).

The HIV-1 life cycle as well as the inhibiting strategies targeted against the numerous stages in the viral life cycle is summarized in figure 1.1.

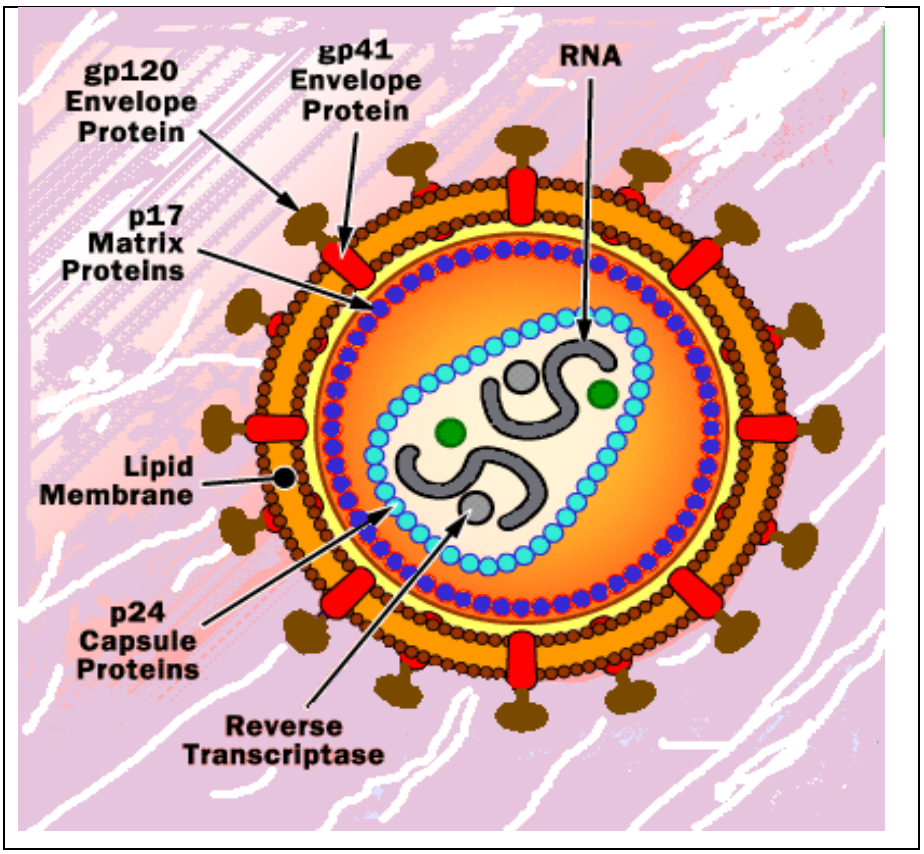


Figure 1.1: structure of HIV extracted from http;//static,howstuffworks.com/gif/aids-hiv-anatomy.gif-accessed 30 June 2009

HIV infections are initiated by binding the virion gp120 surface subunit (SU protein) to the CD4 receptor. The SU protein is attached to the virus by a non-covalent binding to the gp41 transmembrane subunit (TM protein (Nielsen et al, 2005)). The yellow b-strand forms an antiparallel b-sheet with residues in CD4. The domain is probably oriented with the viral membrane near the N terminus and the cellular membrane near the bridging sheet (from Kwong et al. (1998) with permission).

1.3 The use of natural products on HIV and AIDS

Natural products such as medicinal plants and marine organisms provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversities they possess (Cos et al, 2004). According to the World Health Organization (WHO), more than 80 % of the world's population rely on traditional medicine for their primary healthcare needs. The use of herbal medicines, for instance in Asia, represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases (Duraipandiyan et al, 2006). The use of natural products with therapeutic properties goes back to the origin of human civilization, and plants were amongst the main sources of medicine (De Pasqual, 1984).

Southern Africa has a long history in plant usage for medicinal purposes by indigenous people known as traditional healers and/or herbalists. Their recognition was revealed when Boots Drug Company (Nottingham, England) took efforts to identify compounds as antiviral agents. Therefore, medicinal plants represent great potential sources for the development of potential anti-HIV drugs with less side effects (Singh et.al, 2005; Schaeffer and Krylov, 2000).

The product being investigated in this research belongs to Mr Xhegolakhe “Manayi” Ntsonto, a traditional herbalist from Ntsholomnqa, near East London, in the Eastern Cape Province. Mr Ntsonto has been treating HIV and AIDS patients exclusively since 2003 with anecdotal claims of success, and has kept very neat records for each of his patients. He treats only HIV patients who present proof of being HIV positive. He has collected and documented data on pathology, viral load and CD4 cell counts. The Medical Research Council-Indigenous Knowledge System (MRC-IKS) formulated 350-mg capsules made of Manayi and the prescription is taken six times a day.

1.4 The purpose of the Investigation on the research

For the purposes of this project, biological efficacy, safety testing, drug purification and chemical characterization of bioactive molecules were researched on the Manayi product. Manayi product is a traditional medicinal marine natural product that is used in treating and managing HIV infections. Research on the product is conducted by the MRC-IKS unit. The purpose of the research is to validate its use in the treatment and management of HIV infections. This product is has been used for a long-time by the traditional healer but no scientific work has been conducted to prove if it has anti HIV properties or can boost the immune system and other health benefits to people living with HIV. The purpose of this study is to scientifically validate the traditional use of the Manayi product and find possible active chemical compounds in it.

1.5 Problem statement:

- There is an increase in the number of people, who are dying with HIV and AIDS as there is no cure as yet, and there can be no doubt that HIV and AIDS is no longer only a public health challenge and is having devastating impact on the continent.
- Poverty, lack of adequate medical facilities, inadequate education, cultural/social barriers and political inertia are a few of the complex factors that facilitate the spread of the disease.
- Resistance of the virus to anti-Retroviral treatment possesses challenge to the eradication of the disease.
- There are claims by traditional healers that their natural products have health benefits for people with HIV but there is no scientific proof to support such serious claims.
- Side effects of ARVs make patients seek alternative treatment.
- Against this backdrop, there is a need to develop treatments that are cheap, safer, effective, and well tolerated.

1.6 The main aims of the study:

- To investigate the HIV-1 inhibitory properties, cytotoxicity, and phytochemical and physiochemical properties in order to determine whether the traditional claims are supported by actual pharmacological effects. The idea was to link the HIV/AIDS ethnomedicinal use of the Manayi product to *in vitro* studies in order to validate the anecdotal claims in favour of Manayi product.

1.7 Objectives of the study:

- To extract and isolate bioactive compounds from the Manayi product.
- To chemically identify active Anti-HIV and/or immune boosting compounds from the Manayí product.
- To run phytochemical screening and physiochemical analysis
- Isolate, purify and chemically characterize compounds from active extracts and fractions using MRC-IKS method
- To fingerprint these compounds and also to use other chromatographic techniques, such as Thin Layer Chromatography (TLC), Liquid Chromatography-Mass Spectrometry (LC-MS) and Fourier Transform Infrared Spectroscopy (FTIR).

1.8 Research questions/hypothesis and Investigative questions: Null Hypothesis:

- Manayi products do not contain bioactive compounds that are active or that can boost the human immune system.

1.9 Delimitations of the study

The necessity for active preventive and beneficial agents for HIV/AIDS remains a vital global priority in the World and this research is a small step towards this direction. The main aim of this study was to screen cooked and uncooked Manayi product against HIV/AIDS enzymes. The study was designed in four phases:

- **Phase one**: literature review was based on medicinal plants that are traditionally used for the treatment of HIV and AIDS. A comprehensive list of potential active plants was discussed.
- **Phase two**: screening of the products was performed by testing the extracts for inhibitory viral activities such as reverse transcriptase and integrase enzyme. The cooked and uncooked Manayi products were further analysed for their cytotoxicity.
- **Phase three**: The phytochemical and physiochemical analysis of the each cooked and uncooked Manayi was determined.
- **Phase four**: From the active extracts, fractionation was done using solid phase extraction and the fractions were pooled in TLC to determine which ones are pure. A fraction that showed one spot was further analysed using FTIR, LC-MS and NMR.

CHAPTER 2: LITERATURE REVIEW

2.1 The use of natural product/medicinal plants in HIV/AIDS

Anti-retroviral therapy (ART) has brought renewed hope for many people living with HIV. However, they do not offer a cure, and they produce many side effects. For these and other reasons, many HIV positive people turn to alternative medicines for help. Some people use alternative medicine because of desperation for cure or because of their strong cultural beliefs in traditional medicine instead of standard Western Medicine. Traditional herbal use has been reported to be common among individuals with chronic and advanced HIV disease. In Africa, traditional herbal medicines are often used as primary treatment for HIV/AIDS and for HIV-related problems including dermatological disorders, nausea, depression, insomnia and weakness. The use of traditional herbal medicines by AIDS patients after HIV diagnosis was noted in a study in Uganda (Langlois-Klassen, et al, 2007). Despite a lack of evidence on effectiveness and the possibility of serious side effects, some African ministries of health currently promote traditional medicines for the treatment of HIV and associated symptoms.

Two principal African herbal remedies used for HIV/AIDS treatment in sub-Saharan Africa include *Hypoxis hemerocallidea* (African potato - an immune system stimulant) and *Sutherlandia*. These two herbal remedies are currently recommended by the South African Ministry of Health for HIV management (SADC, 2002). Natural sources, particularly plants, are an excellent source of anti-HIV agents. The screening and growth of natural products and chemically synthesized compounds have been developed as medication for HIV infections. South Africa has a variety of plants with just 3000 of these species that have been identified to have been used in traditional medicine across the country (Van Wyk and Gericke, 2000; Scott et al, 2004).

2.2 Most studied and commonly used anti HIV natural products:

Mills et al. (2005a and 2005b) have reported that the herbal remedy has been suggested for HIV management, which was shown to cause an improvement in CD4 count together with a decrease of viral load in AIDS patients. It is hoped that this treatment management will delay the development of HIV into AIDS. They additionally reported that *Sutherlandia* contains inhibitory compounds active against HIV target enzymes. Canavanine, which was found to be present in the extracts, has also been reported to have antiviral activity against influenza and retroviruses. *Sutherlandia* extracts have also been reported to have effects on cytochrome (P450 3A4) metabolism, together with activation of the pregnane X-receptor, which are involved in anti-retroviral metabolism.

Mills, Foster et al. (2005), have further indicated that factors which need to be taken into consideration with HIV treatment are risk of treatment failure, induced viral resistance or subsequent drug toxicity. They also considered that uncontrolled human consumption of *Sutherlandia* extracts could affect anti-retroviral drug metabolism, leading to bi-directional drug

interactions and loss of therapeutic efficacy. *Sutherlandia* has also recently been shown to interact with the permeability glycoprotein (P-gp) receptor, to allow for increased absorption of anti-retroviral drugs (such as Amprenavir) into the cell system, which could lead to drug intoxication, but had no significant interaction with the drug itself (Katerere and Rewerts, 2011).

In 2008, Artan made an investigation on the screening of anti HIV activities of compounds isolated from a medicinal plant called *Rhus chiness*. *Rhus chiness* was found to inhibit HIV reverse transcriptase. Four extracts, viz, petroleum ether, ethyl acetate, butanol and water were tested but only petroleum ether inhibited the synctium fortation and HIV-1 antigen at non-cytotoxic concentrations. However, the same extract was unable to inhibit HIV-1 entry into host cells (Wang et al, 2006).

The use of medicinal plants for AIDS-related conditions is common in South Africa. A South African medicinal plant called *Peltrophorum africanum* was found to inhibit HIV-1 activity and it contains three active compounds, namely methanol, ethyl acetate and butanol extracts (Andros et al, 2009). Methanol extracts showed anti HIV-1 activity with R5 virus with a selective index (SI) of 3. The ethyl acetate extracts showed anti HIV-1 activity with an X4 virus with a selective index of 189 and the butanol extracts showed anti HIV-1 activity with R5 virus with a selective index of 37 and X4 virus with a selective index of 11. Selectivity index is the relative effectiveness of investigational product in inhibiting viral replication compared to inducing cell growth (Badisa et al., 2009). The selectivity index was calculated as the ratio of cellular cytotoxicity (CC_{50}) and concentration of an inhibiter when the response is reduced by half (IC_{50}): (SI)=CC_{50}/IC_{50}. The results indicate that *Peltrophorum africanum* in ethyl acetate extracts possesses a high selectivity index.

Several compounds were found to interfere with HIV entry into cells while others were active against HIV reverse transcriptase, intergrase, protease, and general replication. Some phytochemicals were also potent to activators of HIV replication and expression in latently - infected T-cells, and others were known to inhibit syncytia formation (Kazhila et al, 2010). Table 2.1 lists other plants that are confirmed to have anti HIV activity and as well as their modes of action against HIV.

Table 2.1: Plants and other natural products with known active compounds and modes of action against HIV

Family species	Active constituents	Mechanism of Action	References
Acacia catechu (Mimosa family)	Catechins (chemical family such as flavonoids; (+)-catechin, & (-1) epitacatechin (cis)	Anti-HIV-1 activity	Nutal et al, 2013
Agaricaceae Lentinus edodes (Berk.) Singer	Sulfated lentinan	Prevent HIV-induced cytopathic effect	Suzuki et al, 1989
Conospermum incurvum	Conocurvone	Showed potent anti-HIV activity by a novel mechanism	Anuya et al, 2015
Combreatacea Combretum molle R.Br. ex G.Don	Gallotannin	Inhibits HIV-1 reverse transcriptase	Bessong, et al,2006

Table 2.1 (continued): Plants and other natural products with known active compounds and modes of action against HIV

Family species	Active constituents	Mechanism of Action	References
Geum japonicum	Maslinic acid	Inhibits HIV-1 protease	Xu et al 1996
Menispermaceae *Stephania cepharantha*	Cepharanthine	Inhibits HIV replication	Ma et al, 2002
Rosaceae *Crataegus pinatifida*	Uvaol and ursolic acid	Inhibits HIV-1 protease	Min, et al 2009
Sanguisorba Magnolili	Aqueous extract	Inhibits HIV activity in an in vitro MTT assay	Bedoya et al, 2001
Sazygium Claviflorum	Bevirimit (PA-457)	Inhibits the final step of HIV Gas protein processing	Heider et al, 2010
Tuberaria Lignosa	Water-soluble polar substance (aqueous extract)	Exhibited anti-HIV activity in an in vitro MTT assay	Bedoya et al, 2001

2.3 Identification of HIV drugs and/or immune boosters

It is important to understand HIV replication life cycle when doing studies on HIV-1 drug discovery. HIV-1 Reverse Transcriptase (HIV-1 RT) has been the target of numerous approved anti-AIDS drugs that are key components of Highly Active Anti-Retroviral Therapies (HAART). It remains the target of extensive structural studies that continue unabated for almost twenty years. The crystal structures of wild-type or drug-resistant mutant HIV RTs in the free form or in complex with substrates and/or drugs have offered a valuable prospect into the enzyme's folding and its interactions with DNA and dNTP substrates, as well as with nucleotide reverse transcriptase inhibitor (NRTI) and non-nucleoside reverse transcriptase inhibitor (NNRTIs) drugs. These studies have been used to interpret a large body of biochemical results and have paved the way for innovative biochemical experiments designed to elucidate the mechanisms of catalysis and drug inhibition of polymerase and RNase H functions of RT. In turn, the combined use of structural biology and biochemical approaches has led to the discovery of novel mechanisms of drug resistance and has contributed to the design of new drugs with improved potency and ability to suppress multi-drug resistant strains (Kamalendra et al, 2010).

2.4 HIV enzymes

There are three HIV enzymes, namely, HIV-1 reverse transcriptase, HIV-1 protease and HIV-1 integrase. Reverse transcriptase enzyme was the first to be targeted in HIV-1 drug development. Azidothymidine (AZT) in table 2.2, which is a nucleoside analogue RT inhibitor, was the first drug to be approved for treatment of HIV (Mitsuya, 1985). United State Food and Drug Administration (US FDA) has approved eight nucleosides and three nucleotide reverse transcriptase inhibitors (NRTIs and NtRTIs) so far. They inhibit HIV RT by chain termination (Jochmans, 2008) because of the following structural properties such as dideoxynucleoside analogues which lack both 2'- and 3'-OH groups on their sugar moiety, modifications that includes replacing the 3'-OH group with other chemical functions such as H, N_3 etc or an elimination of 3'-position by changing the ribose ring. In addition, most NRTIs have a broad-spectrum antiretroviral activity as a result of the antimetabolites of natural nucleosides. Currently approved NRTI drugs in table 2.2, are AZT (1), Lamivudine (2), Didanosine (3), Zalcitabine (4), Stavudine (5), Abacavir (6), and Emtricitabine (7). Only one NtRT drug, Tenofovir disoproxil fumarate (8), has been approved by US FDA.

Table 2.2: Currently approved NRTIs drugs (Haung et al., 1998)

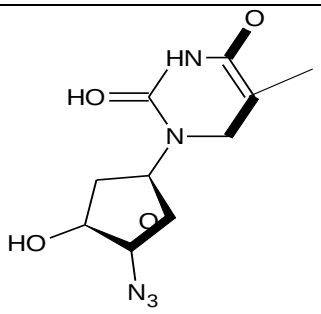
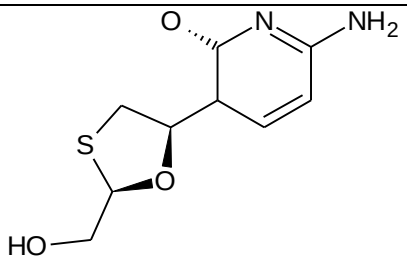
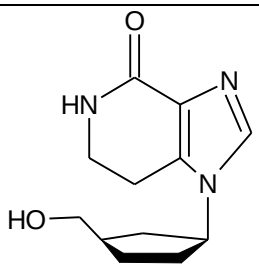
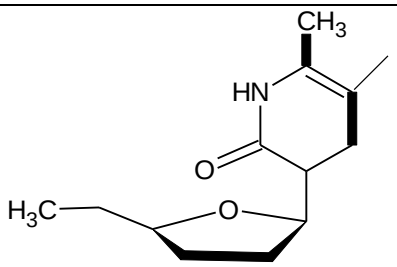
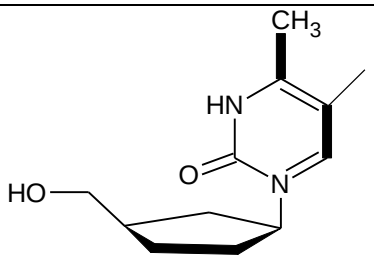
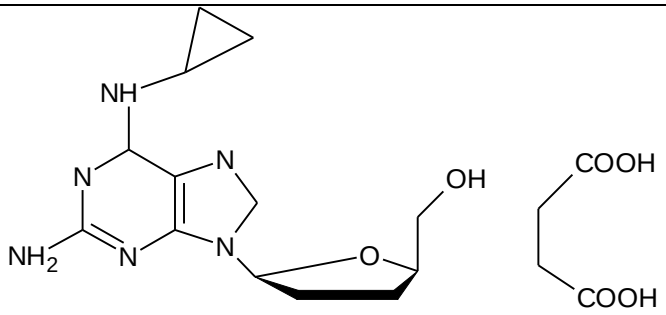
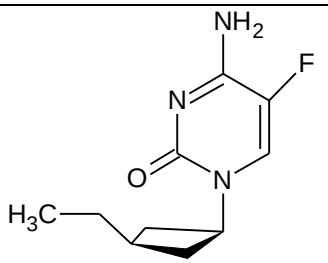
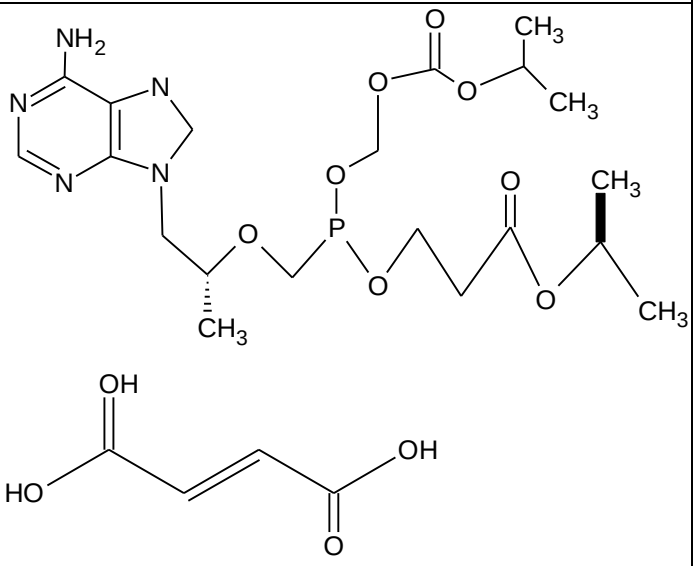
Drug name	Structure
1. Azidothymidine	
2. Lamivudine	
3. Didanosine	
4. Zalcitabine	
5. Stavudine	

Table 2.2 (Continued): currently approved NRTIs drugs (Haung et al., 1998)

Drug name	Structure
6 Abacavir	
7 Emtricitibate	
8 Tenofovir Disoproxil Fumarate	

Jochmans (2008) reviewed the second important group of HIV-1 RT inhibitors called non-nucleoside RT inhibitors (NNRTI). One drug that was reported to have easy administration and shows potent activity on HIV-1 is Efavirenz, and it is the most popular NNRTI prescribed to patients infected with HIV. There are also two other NNRTIs approved so far, and these are Nevirapine and Delaviridine mesylate. The protease inhibitors were second to be developed in HIV-1 research. They were designed to bind the viral protease with high affinity and could occupy more space than the natural substrates (Wensing et al, 2010).

Ten protease inhibitors in table 2.3 are currently approved for clinical uses: Saquinavir (1), Ritonavir (2), Indinavir (3), Nelfinavir (4), Amprenair (5), Lopinavir (6), Atazanavir (7), Tipranavir (8), Fosamprenavir (9) and Darunanir (10).

Table 2.3: Ten Protease inhibitors that are currently approved (Lv et al., 2015)

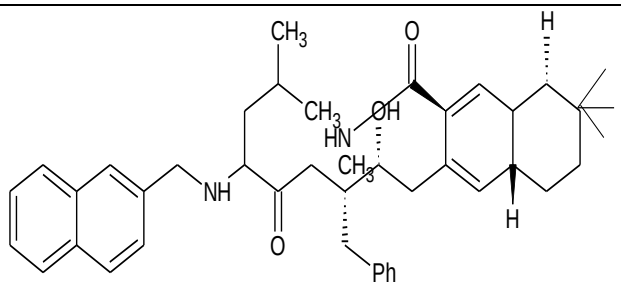
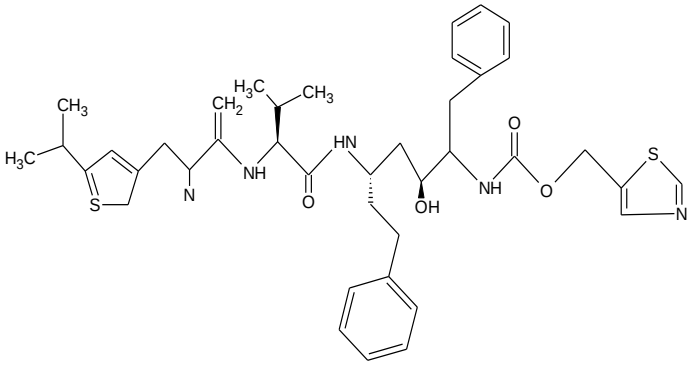
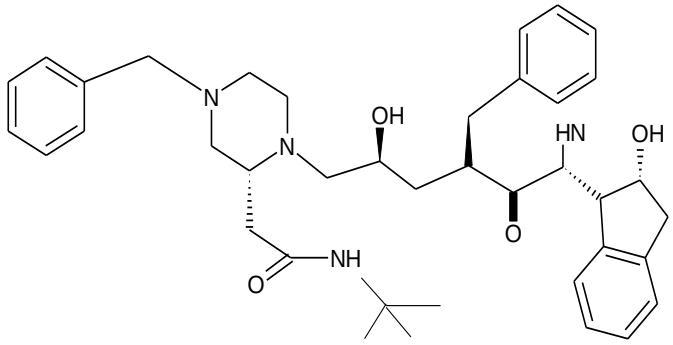
Compound	Structure
1. Saquinavir	
2. Ritonavir	
3. Indinavir	

Table 2.3 (Continued): Ten Protease inhibitors that are currently approved (Lv et al., 2015)

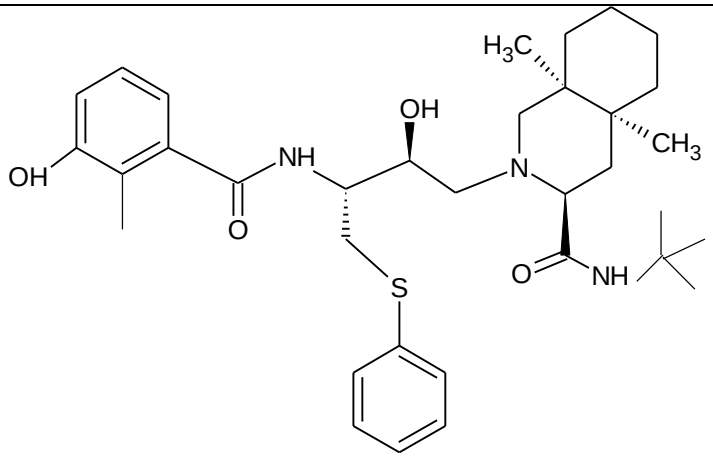
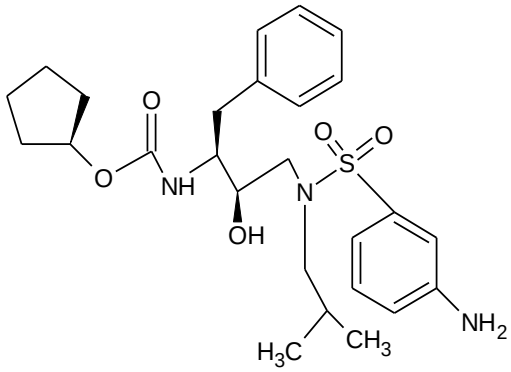
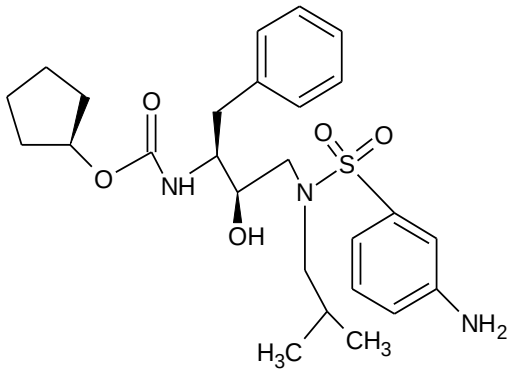
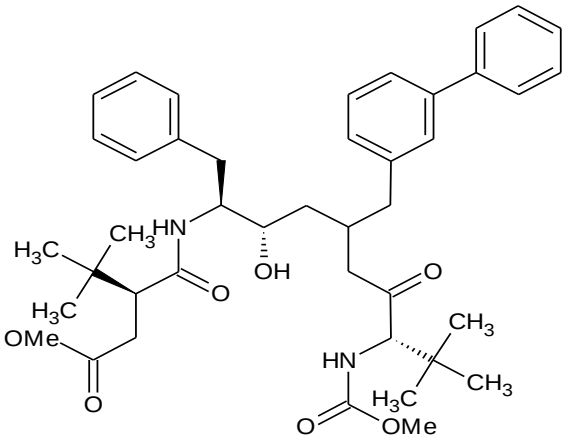
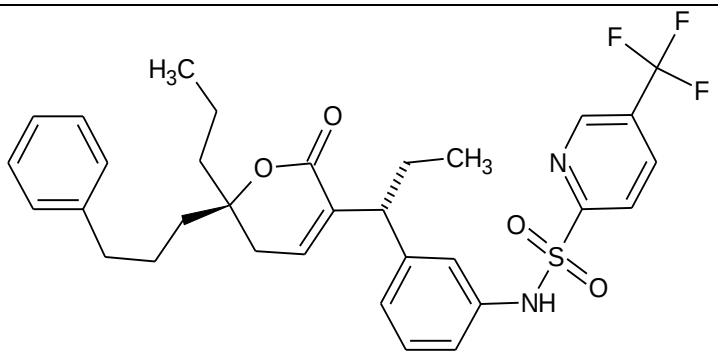
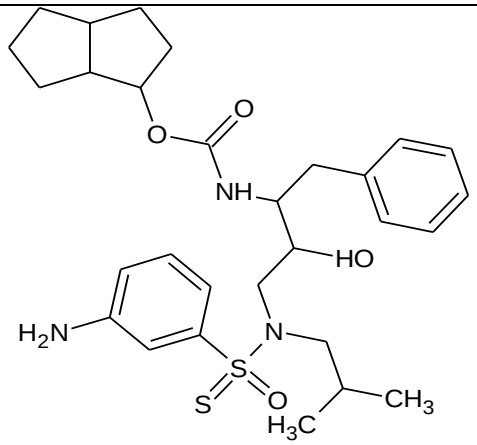
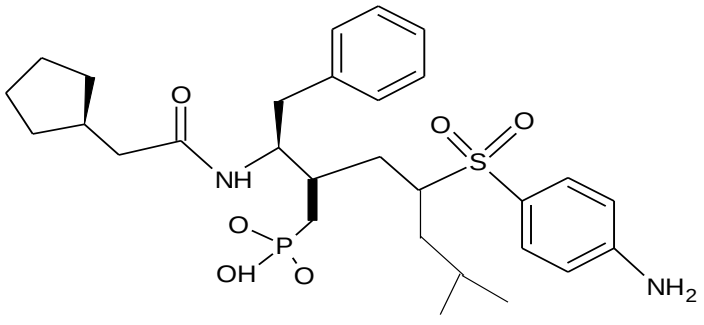
Compound	Structure
4. Nelfinavir	
5. Amprenavir	
6. Lopinavir	

Table 2.3 (Continued): Ten Protease inhibitors that are currently approved (Lv et al., 2015)

Compound	Structure
7. Atazanavir	
8. Tripranavir	
9. Darunavir	
10. Fosamprenavir	

Integrase inhibitors were the last to be developed as part of the therapy for HIV-1. Kinetic studies of DNA-integrase complexes led to the proposal of a model explaining the formation of an active complex (Faure et al., 2005). It was found that the activity in integrase monomers and multimers was due to cross-linked tetrameric form of Intergrase (IN) and is the minimal oligomer that performs full-site integration of the substrate carrying LTRs. There are seven compounds that are reported to inhibit integrase, but the first drug to be clinically approved by US FDA is Raltegravir. The six integrase inhibitor compounds are Quercetin (1), Caffeic acid (2), Phenethyl ester (3), Curcumin (4), L-chicoric (5), a Tyrphostin (6) and its Derivative 6, 7-dihydroxynaphtho-2-yl (Artico et. al, 1998). These integrase are illustrated in table 2.4.

Table 2.4: Six Integrase inhibitor compounds (Pommier et al., 2005)

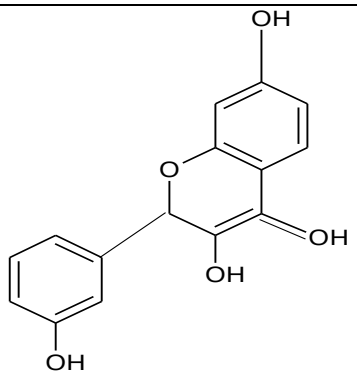
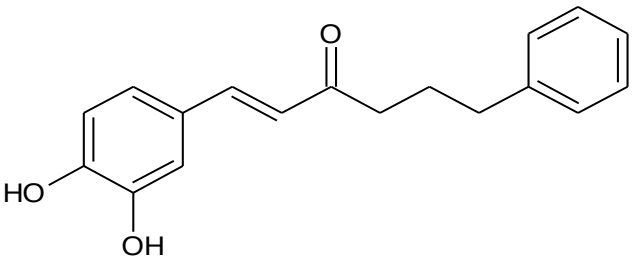
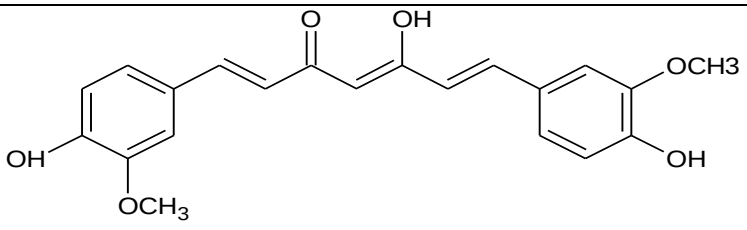
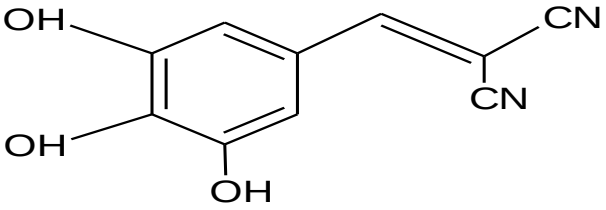
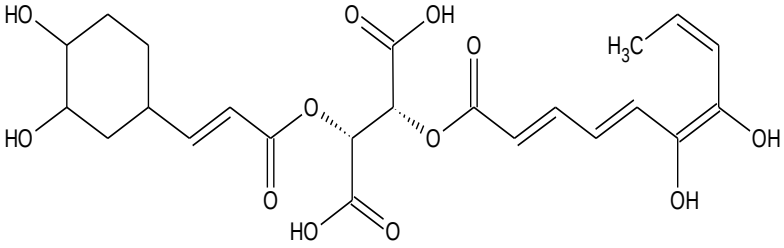
Compound	**Structure**
1. Caffeic acid	
2. Phenethyl ester	
3. Curcumin	

Table 2.4 continued: six integrase inhibitor compounds (Pommier et al., 2005)

compound	Structure
4. Tyrphostin	
5. 6,7dihydroxynaphtho-2-yl	

2.5 Phytochemical studies

The importance of plants is well known to us. The plant kingdom is a treasure house of potential drugs, and in recent years there has been an increasing awareness about the importance of medicinal plants. Drugs from plants are easily available, less expensive, safe, efficient, and rarely have side effects. Plants which have been selected for medicinal use over thousands of years constitute the most obvious choice of examining the current search for therapeutically effective new drugs such as anticancer drugs [1], antimicrobial drugs [2], and antihepatotoxic compounds [3]. According to World Health Organization (WHO), medicinal plants would be the best source to obtain drug varieties. About 80% of the population from developed countries use traditional medicines, which have compounds derived from medicinal plants. However, such plants should be investigated to better understand their properties, safety, and efficacy (Yadav et al, 2011).

Despite great advances in drug design, failure to treat HIV patients completely is still a major challenge. However, medicinal plants are still known to have the richest source of leading bioactive compounds, together with their derivatives. Therefore, natural products, especially plants, are a great source for the development of new generation of anti-HIV drugs, which would be more effective with less side effects. Analysing the phytochemicals in medicinal plants provides scientists with an insight into how medicinally effective plants are, and understanding their effectiveness can lead to the development of new curative drugs. A lot of medicinal plants with many different compounds have been screened as HIV inhibitors and were found to inhibit nearly all stages of HIV life cycle (Wang, 2004).

Phytochemical compounds, including alkaloids, found in medicinal plants are illustrated in table 2.5 bellow. They are Schumannificine (1), Pentachumannificine (2), Pentagalloyglucose (3), Baicalin (4), Repandusinic acid (5), Inophyllum (6), Gossypol, Platanic acid (7), 6, 6' Bieckel (8), and Arctigenin acid (9).

Table 2.5: Phytochemical compounds that are found in natural medicine (Dias et al., 2012)

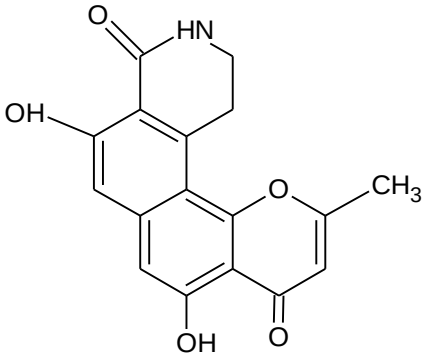
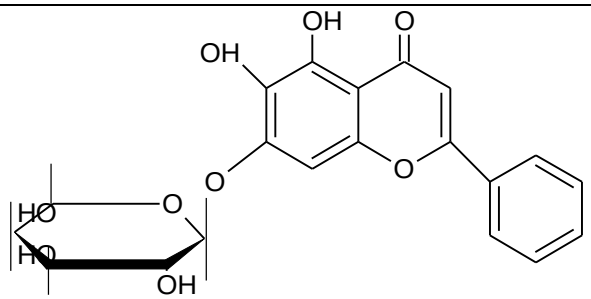
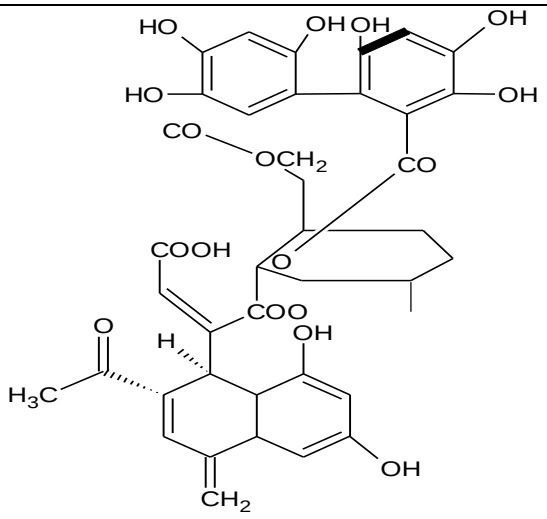
Phytochemical compound	structure
1. Pentagalloylglucose	
2. Baicalin	
3. Repandusinic acid	

Table 2.5 (continued): Phytochemical compounds that are found in natural medicine (Dias et al., 2012)

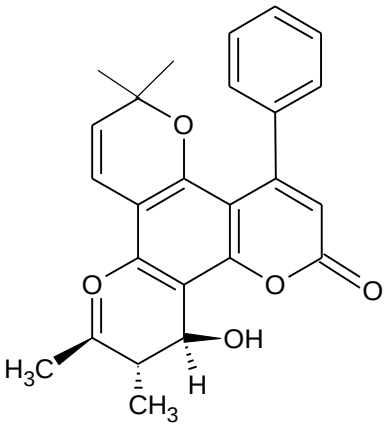
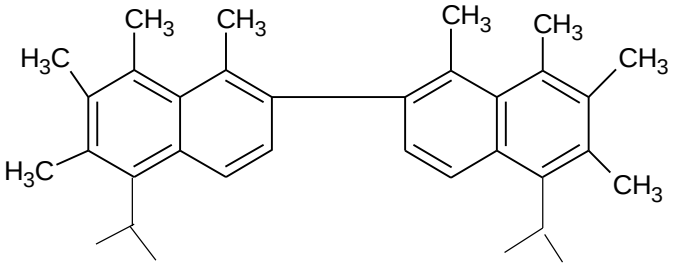
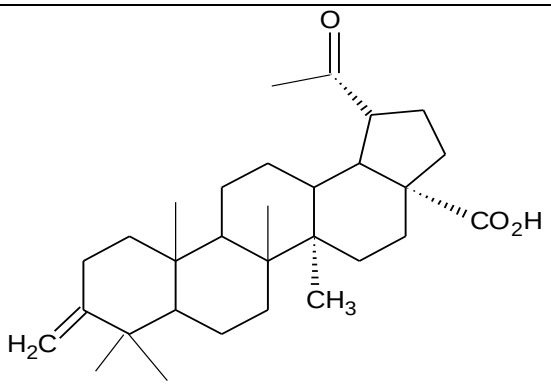
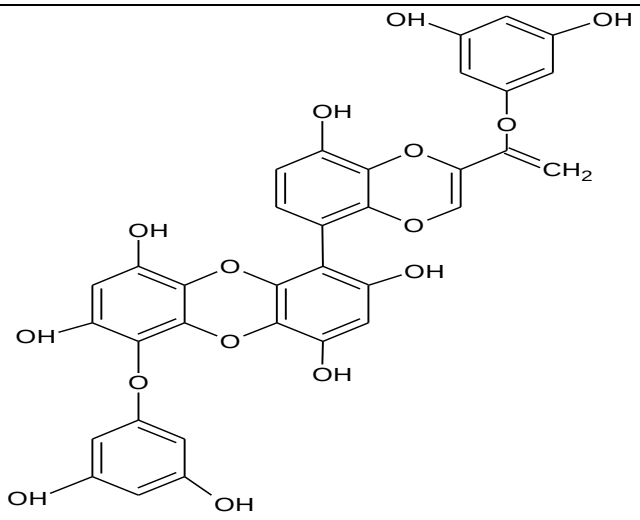
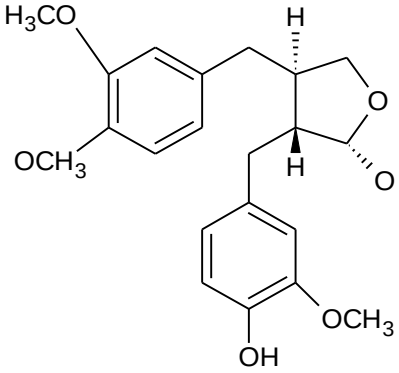
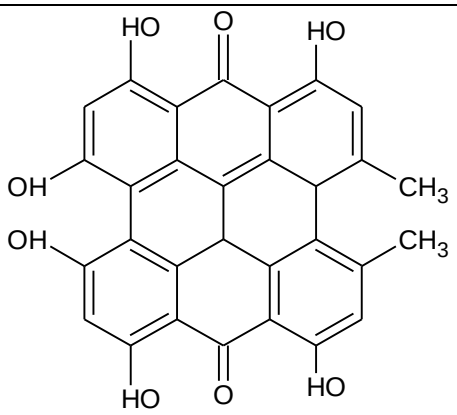
Phytochemical compound	Structure
4. Inophyllum	
5. Gossypol	
6. Platanic acid	
7. 6, 6' Bieckol	

Table 2.5 (continued): Phytochemical compounds that are found in natural medicine (Dias et al., 2012)

Phytochemical compound	Structure
8. Arctigenin	
9. . Hypericin	

These compounds are synthesized by primary or secondary metabolism of living organisms. Secondary metabolites are chemically and taxonomically extremely diverse compounds with obscure functions. They are widely used in human and veterinary therapy, agriculture, scientific research and countless other areas. A large number of phytochemicals belonging to several chemical classes have been shown to have in vitro inhibitory effects on all types of microorganisms. Plant products have been part of phytomedicines since time immemorial. This can be derived from barks, leaves, flowers, roots, fruits, and seeds. Knowledge of the chemical constituents of plants is desirable because such information will be of value for the synthesis of complex chemical substances (Yadav, 2011).

2.6 Techniques used in this research

This section will discuss the techniques that were employed in this study. These techniques focused on the assessment of the effects of plant extracts on cells (toxicity), and the bioassay of HIV antiviral replication activities. 3-[4, 5-dimethylthaizol-2yl]-2, 5-diphenyltertazolium bromide (MTT) Assay is a colorimetric assay for assessing cell metabolic activity. The MTT assay is used to determine the toxic effects of the crude plant extracts on cells, as well as to indirectly assess

their anti-viral qualities in conjunction with the viability assay (ELISA) technique. Several techniques were applied in the isolation of active compounds from Manayi crude extracts. These techniques are Thin Layer Chromatography (TLC), Solid Phase Extraction (SPE), Liquid Chromatography Mass Spectrometry (LCMS), Nuclear Magnetic Resonance (NMR) Spectroscopy and Fourier Transform Infrared (FTIR) Spectroscopy.

2.6.1 Thin Layer Chromatography

TLC is a simple, quick, and inexpensive procedure that gives a researcher a quick answer as to how many components are in a mixture (Sasidharan et al., 2011). TLC can be used to identify active ingredients in traditional medicines; however, there is uncertainty if this technique could differentiate between closely related species. Hahn-Deinstrop (2000) and many researchers have made use of TLC and declared this technique to be a good tool for the analysis of drug substitution and adulteration. Research conducted so far has also shown TLC to be a good technique for analysis of botanical products in the investigation of additional substitutions (Ntloedibe, 2001). TLC is also used to confirm the identity of a compound in a mixture when the retention factor (Rf) of a compound is compared with the Rf of a known compound (Sasidharan et al., 2011). The pure compounds are then used for the determination of structure and biological activity. TLC also includes the additional tests, which involve the spraying of phytochemical screening reagents, which cause colour changes according to the phytochemicals existing in a plant extract, or by viewing the plate under the UV light. This has also been used for confirmation of purity and identity of isolated compounds. Bio-autography is a useful technique to determine bioactive compounds with antimicrobial activity from a plant extract (Sasidharan et al, 2011).

Traditionally, a bioautographic technique has used the growth inhibition of microorganisms to detect anti-microbial components of extracts chromatographed on a TLC layer. This method has been considered as the most efficacious assay for the detection of anti-microbial compounds (Shahverdi, 2007). Bio-autography localizes antimicrobial activity on a chromatogram using three approaches: (I) direct bio-autography, where the micro-organism grows directly on the thin-layer chromatographic (TLC) plate, (ii) contact bio-autography, where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact and (iii) agar overlay bio-autography, where a seeded agar medium is applied directly onto the TLC plate (Hamburger and Cordell, 1987; Rahalison et al, 1991). The inhibition zones produced on TLC plates by one of the above bioautographic technique will be used to visualize the position of the bioactive compound with antimicrobial activity in the TLC fingerprint with reference to Rf values (Homans and Fuchs, 1970).

Characteristic features of TLC include analysis of many samples and comparison of their phytochemical profiles on the same plate. Results can be stored and communicated as images (picture, videos or scanned) and the choice of mobile and stationary phases is flexible (Cimpoiu, 2006). Identification of compounds can be done using three different mobile phases or three different phases with one mobile phase to develop the fingerprint of the same extracts and standards. If the difference in Rf values is less than 0.03 then the compounds are identified without further isolation (Nyiredy and Glowniak, 2001).

Visualization of separated compounds is achieved by colour in daylight or by fluorescent quenching on 254 nm and 366 nm UV light. Visualization of chromatography under UV light at 366 nm shows orange yellow bands for flavonoids and blue fluorescent bands for phenolic acids (Males et al, 2001). There are chromogenic spray reagents for specific classes of compounds. Examples are vanillin/sulphuric acid solution, anisaldehyde and ferric chloride-potassium ferricynide bands for phenolic compounds (Wettasinghe et al., 2001).

2.6.2 Solid phase extraction

Solid Phase Extraction (SPE) is the crucial first step of cleaning impurities in the analysis of medicinal plants, because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization. The basic operation includes steps such as pre-washing, drying of plant materials or freeze drying, grinding to obtain a homogenous sample and often improving the kinetics of analytic extraction and also increasing the contact of sample surface with the solvent system. Proper actions must be taken to assure that potential active constituents are not lost, distorted or destroyed during the preparation of the extract from plant samples. If the plant was selected on the basis of traditional uses (Fabricant and Farnsworth, 2001), then it is essential to prepare the extract as described by the traditional healer in order to mimic as closely as possible the traditional ‘herbal’ drug.

The selection of a solvent system largely depends on the specific nature of the bioactive compound being targeted. Different solvent systems are available to extract bioactive compounds from natural products. The extraction of hydrophilic compounds uses polar solvents such as methanol, ethanol or ethyl-acetate. For extraction of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol in ratio of 1:1 are used. In some instances, extraction with hexane is used to remove chlorophyll (Cos et al., 2006). To be able to separate and identify definite families of phenolic or individual compounds from complex food matrices, the approach of SPE is very often the solution to this problem (Bremner et al., 1999).

2.6.3 Fourier-transform infrared spectroscopy (FTIR*)*

FTIR has proven to be a valuable tool for the characterization and identification of compounds or functional groups (chemical bonds) present in an unknown mixture of plant extracts (Eberhardt et al., 2007; Hazra et al., 2007**)**. In addition, FTIR spectra of pure compounds are usually so unique that they are like a molecular "fingerprint". For most common plant compounds, the spectrum of an unknown compound can be identified by comparison to a library of known compounds. Samples for FTIR can be prepared in a number of ways. For liquid samples, the easiest is to place one drop of the sample between two plates of sodium chloride. The drop forms a thin film between the plates. Solid samples can be milled with potassium bromide (KBr) and then compressed into a thin pellet, which can be analyzed or can be placed directly to ATR. Another method involves dissolving solid samples in a solvent such as methylene chloride, and placing the solution onto a single salt plate. The solvent is then evaporated off, leaving a thin film of the original material on the plate (Sasidharan et.al, 2011).

Thangarajan and many other researchers (2012), while analyzing the ethanolic extracts of *Ichnocarpus frutescens* by FTIR, identified functional groups such as amino acids, amides, amines, carboxylic acid, carbonyl compounds, organic hydrocarbons and halogens. Ramamoorthi and Kannan (2007) screened the bioactive group of chemicals in the dry leaf of powder of *Calotropis gigantean* by FTIR analysis and the strong absorption reveals the presence of amino acids. In addition, FTIR spectroscopy is proved to be a reliable and sensitive method for detection of molecular composition (Patil and Patil, 2006).

2.6.4 Liquid Chromatography-Mass spectrometer (LCMS)

Liquid Chromatography Mass Spectroscopy (LC-MS) is an important hyphenated technique for quantitative analysis of drugs in biological fluids. Because of high sensitivity and selectivity, LC-MS has been used for pharmacokinetic studies, and metabolite. This manuscript gives a comprehensive analytical review, focusing on chromatographic separation approaches column packing materials, column length and mobile phases as well as different acquisition modes for quantitative analysis of natural products.

LC-MS/MS techniques provide specific, selective quantitative results with reduced sample preparation. It is the choice of interest because it is highly sophisticated and is considerably powerful for detection of low and high molecular weight compounds. New methods have enabled the determination of drugs that were formerly difficult to detect by conventional methods of analysis as well as time consuming procedures have been replaced by faster more comprehensive

and robust assays. Good sensitivity and high throughput are key factors for the LC-MS/MS approaches used in drug analysis (Haneef, 2013).

In order to study possible applications of extracts or compounds derived from extracts, methods to screen for biological activity, and separation methods with bioassays and spectroscopic techniques such as NMR and MS are very attractive tools. It was reported that LC-MS was considered as the method of choice because of its sensitivity and selectivity in comparison to wavelength specific UV detection. The method of LC-MS is usually used in finding new compounds, analysing complex components from nature, and drug analysis (Kraus et al., 2002). Lu and many other researchers (2013) reported on LC-MS analysis of the extracts, isolation of bioactive compounds via bioassay-guided separation techniques including LC- bioassays, and structure determination by MS of the bioactive functional groups obtained by carefully analysing total ion chromatograms. This could supply structurally informative ion patterns. Thus, LC-MS was mainly used in the study. The results of the investigation also demonstrated that LC-MS is a highly sensitive detection method that can assist in the identification of new sources of target families of natural products.

2.6.5 Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance (NMR) imaging has recently become an accepted technique in the medical practitioner's armory. NMR spectroscopy is a subtly different application of the same physical principles underlying NMR imaging, but the clinical potential for this modality is currently still under evaluation. The most important application of clinical NMR spectroscopy is for the noninvasive monitoring of changes in metabolite levels and intracellular pH of intact tissues during physiological stress or in response to pharmacological agents or disease (Cady et.al.1985). NMR is one of the most powerful spectroscopic methods, because it is able to use atomic connectivity to provide not only a full structural assignment but also information about the spatial geometry, conformation, and stereochemistry (Lorena et al, 2013).

Proton nuclear magnetic resonance (1H-NMR, or simply NMR) is among the techniques that have been used to look for differences between remedies and control samples. The term "NMR" encompasses both solvent mobility studies and analytical studies. In analytical studies, also called spectroscopy, the results are displayed as a graph or spectrum plotting of concentration against a variable called chemical shift. There are also more complex applications of NMR such as imaging and two-dimensional NMR that are not relevant to the study of discrete remedy samples. The chemical shift of a proton in a molecule in a sample reflects the (time-averaged) amount of magnetic shielding provided by the electrons making up the covalent or hydrogen bond(s) in which

the proton participates, with greater electron density generally correlating with lower chemical shift numbers. Chemical shifts are measured in units of parts per million (ppm) deviations**.** Recently the NMR technique is applied in quantitative analysis in order to establish the contamination of a drug (Mistry et al, 1999). It is also used in the characterization of the configuration of drug products and in quantitation of drugs in pharmaceutical formulations and biological fluids (Salem et al, 2006; Reinscheid, 2006). Numerous evaluations on the application of NMR in pharmaceuticals have been published (Holzgrabe et al, 2005; Malet- Martino and Holzgrabe, 2011).

CHAPTER THREE: METHODOLOGY

3.1 PHARMACOLOGY

3.1.1 Introduction

HIV has affected many people in many ways. The infected people also carelessly or accidentally infect others. Thus, the different stages in the HIV replication cycle are a clear indication that the present targets of the virus need new interventions. In this study, the focus is on the discovery of possible bioactive molecules on the Manayi product. The initial screening of the crude extract of the natural product for the inhibitory effect against reverse transcriptase and integrase was carried out using a Colorimetric RT Elisa for RT incisory, and Xpresso Bio for Integrase. The results for the initial screening have led to the prioritisation of the Manayi product for further investigation. This section elaborates on pharmacological studies in this research.

3.1.2 Materials and Methods

The reagents used include ammonia, methanol, hexane, dichloromethane, acetone, butanol, formic acid, ethyl acetate, sulphuric acid, sodium hydroxide, copper sulphate, Nihydrin, pyridine, sodium nitroprusside, sodium picrate, phloroglicinol, ethanol and dimethyl sulfoxide. All of them were purchased from Sigma. These reagents were used on each day of experiment in different analyses discussed in this chapter.

3.1.3 Preparation of crude extract

Samples of Manayi were collected in a rural area of Eastern Cape called Ntsholomnqa, South Africa. They were separated into product A (uncooked Manayi which is a raw material) and product B (cooked Manayi). Product B was prepared by boiling product A in water, followed by filtering and cooling. The filtrate was the product used as medicine dispensed for patients. The residue is powdered and used as such. In preparation for the scientific research, the extraction process for each product A and B was done sequentially. 100 g of each cooked and uncooked were accurately weighed to two decimal places using top pan balance. Each weighed product was extracted three times with 500 ml hexane at room temperature for 12 hours. During the extraction period the mixture was shaken using a mechanical shaker. The extraction mixtures were then filtered using 90mm Whatman filter paper and the filtrate was evaporated using a Rotovapor. After extracting the products with hexane, the filtrate was dried in order to remove any residual hexane. The dried residues were repeated each time using chloroform, dichloromethane, butanol, methanol and water, sequentially. The extraction processes resulted in six extracts for each of the Manayi product A and B. All the organic extracts were concentrated to dryness using a Rotovapor and left in a fume hood to dry.

3.1.4 Detection of anti-HIV Reverse Transcriptase activity using Reverse Transcriptase Assay Colorimetric version 13.0

The Reverse Transcriptase Assay (see figure 3.1) is designed for the quantitative determination of RT activity in cell culture and other biological samples. The assay is used to determine the propagation of retroviruses in retrovirus-infected mammalian cells in a culture. The assay is also used for *in vitro* screening for RT inhibitors. Without reverse transcriptase, the viral genome cannot be incorporated into the host cell, and as a result the virus will not replicate (De Clercq, 2007; Woradulayapinij et al, 2005). Therefore, Reverse Transcriptase is one of the principal drug target enzymes for anti-retroviral drugs such as Nevarapine and Delavirpine.

The reverse transcriptase assay technique takes advantage of the ability of reverse transcriptase to synthesis Deoxyribonucliec acid (DNA), starting from the template/primer hybrid poly (A) x Oligo $(dT)_{15}$. Alternatively, the flexibility of the assay allows for the use of a template/primer hybrid of individual choice. The detection and quantification of synthesis DNA as a parameter for RT activity follows a sandwich Enzyme-linked immunosorbent assay (ELISA) protocol: Biotin-labeled DNA binds the surface of microplate (MP) modules that have been precoated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD), binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate ABTS is added. The peroxidase enzyme catalyses the cleavage of the substrate by producing a coloured reaction product. The absorbance of the samples is determined using the microplate (ELISA) reader and is directly correlated to the level of RT activity in the sample.

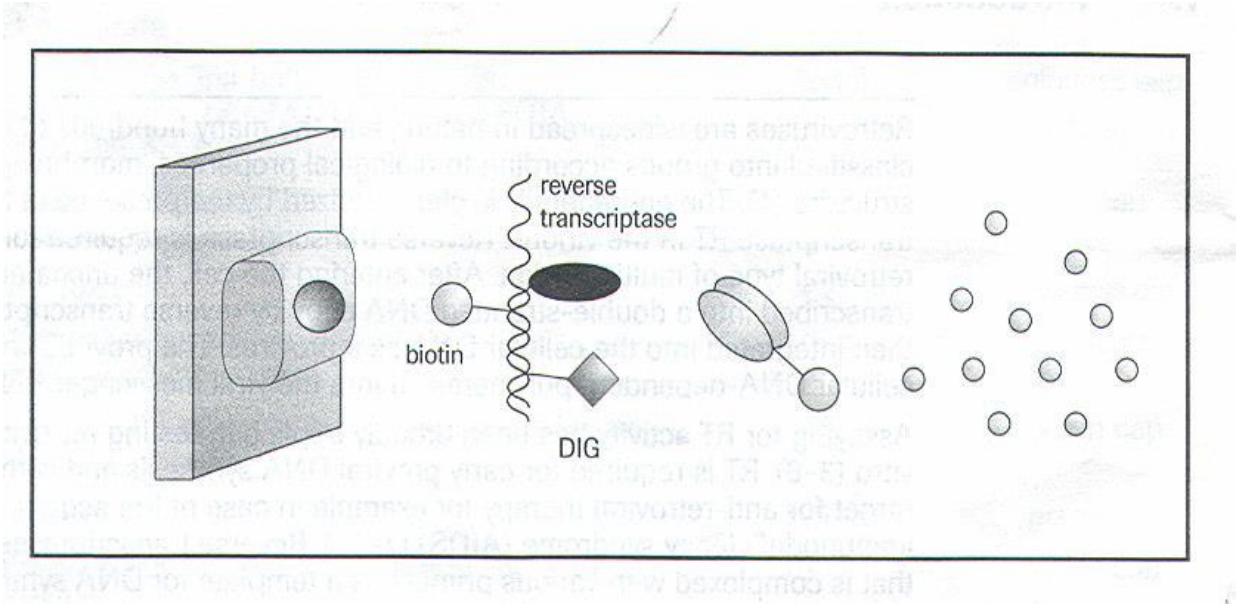


Figure 3.1: Reverse transcriptase colorimetric assay principle (Roche, 2005)

Both cooked and uncooked Manayi extracts were tested for their inhibition of RT to determine the presence of reverse transcriptase activities. The effects of product extracts on RT activity in vitro were evaluated with recombinant HIV-enzyme using a non-radioactive HIV-RT colorimetric (see figure 3.1) ELISA kit from Roche, Germany. The recombinant HIV-1 Reverse Transcriptase was diluted to 200-300 ng/ml in the lysis buffer supplied with the kit. Odimune was used as the positive control at a concentration range of 6.25 and 12.5 µg/ml. Odimune contains three HIV medications such as Efavirenz, FTC and Tenofovir. Positive control and extract concentration was done in duplicate. The extracts were first solubilized in 20 µl dimethyl sulfoxide (DMSO) to a final volume of 1 ml to make a stock solution of 2 mg/ml. The plate layout was as follows: Each test well contained 20 µl of diluted recombinant HIV-1 Reverse Transcriptase (0.2-0.3 mg) and 20 µl of the reaction mixture. Negative control wells contained 20 µl of lysis buffer and 20 µl of reaction mixture. Positive control wells contained 20 µl of diluted recombinant HIV-1 Reverse Transcriptase (0.2-0.3 mg), 20 µl of diluted buffer containing 20 µl of DMSO, and 20 µl of the reaction mixture. The final concentration in each well was 150 µg/ml. The wells of the microtiter plate were then covered and sealed with plastic covers that came with the kit and incubated for an hour. After incubation at 37 ^{0}C for an hour, the wells were washed five times with 250 µl of wash buffer per well for 32 seconds each. The washing buffer (250 µl wash buffer) was then carefully removed, and 200 µl of anti-peroxidase (DIG-POD) working dilution was dispensed into each well. The wells were covered and incubated at 37 ^{0}C for an hour. The wells were then washed in the same manner as before. The wash buffer was carefully removed from the wells, and 200 µl of ABTS substrate was dispensed into the wells. The plates were incubated for 10-30 minutes at room temperature (15-25 ^{0}C). The absorbance of the samples was measured using PherASTAR, high-throughput screening microplate reader at 405 nm reference wavelength and 492 nm. The percentage RT enzyme inhibition activities of the extract were calculated as follows:

$$3.1.4.1\ \text{Percentage RT inhabitory} = 100 - \frac{\text{extract sample}^{\,\text{A405nm}-\text{A492nm}}}{\text{positive control}^{\text{A405nm}-\text{A492nm}}}\ \text{x}\ 100$$

3.1.5 Detection of Anti-HIV-1 Integrase Activity version 3.0

The Manayi extract was screened using anti-HIV-1 activity in order to determine its inhibitory effect on HIV-1 Integrase. Enzyme activity was measured according to the supplier's instructions using a non-radioactive integrase colorimetric kit supplied by XpressBio. In the assay, 100 µl of donor substrate (DS) DNA was added to each well and incubated for 30 minutes at 37 ^{0}C. The liquid (100 µl donor substrate) was removed from the plates and the plate was washed 5 times with 300 µl of 1x buffer which was prepared by mixing 20X wash buffer concentrate with 950 ml sterile distilled water. 200 µl of blocking buffer was added to each wells and subsequently washed 3

times with 200 µl of the reaction buffer. The integrase enzyme was diluted 1:300 (2 µl HIV-1 integrase and 958 µl reaction buffer). The liquid from the plate wells was aspirated and washed three times with 200 µl reaction buffer. The azide was used as a positive control. 100 µl of the integrase enzyme (positive control) was added to each well and incubated for 30 minutes at 37 ^{0}C. The medium from the plate wells was aspirated and washed three times with the reaction buffer. The extracts that were initially dissolved in 50 µl DMSO and 50 µl of 0.30 % sodium azide (positive control) were added to the respective wells and incubated for 5 minutes at room temperature. The concentration range used for all extracts was 50 and 100 µg/ml. After 5 minutes of incubation at room temperature, 50 µl of the target substrate (TS) DNA was added to each well and gently mixed. The plate was incubated for 30 minutes at 37 ^{0}C. The liquid was then removed and the wells were washed 5 times with 300 µl of washing buffer. 100 µl TMB antibody solution was added into each well and incubated for 30 minutes at 37 ^{0}C. 100 µl TMB stock solution was directly added to the wells containing TMB substrate. The absorbance of the wells was measured at 450 nm using a PheraStar microplate. The plates were measured within 10 min of adding TMB stop solution. The HIV integrase inhibition by the plant extracts was measured as percentage of the inhibition that occurred with HIV-1 integrase in the absence of an inhibitor in the same solvent (DMSO) as the extracts. The experiment was done two times to confirm the results. The experimental data to determine the percentage inhibition was analyzed as follows:

- The mean absorbance blank (reaction buffer + negative control) was determined.
- The mean standard deviation (SD) and coefficient of variation (CV) [(SD/mean) X100] for background corrected absorbance of integrase alone (positive control) and test solution replicate wells were calculated.
- The data was then converted to percentage control activity by dividing the mean absorbance of test extracts by that of integrase control and multiplied by 100%.
- Therefore, the mean absorbance of the test solutions divided by the mean integrase control activity multiplied by associated CV provides the percentage adjusted standard deviation. The CV = % SD. The experimental data is shown in appendix D.

3.1.6 Cytotoxicity of Manayi Extracts

3.1.6.1 Introduction

The cell growth is determined by counting viable cells using trypan blue exclusion dye. The method is however, not selective and cannot be used to distinguish between dead and dying cells. The MTT assay is the most preferred assay to determine the cell viability in culture. The basis of the MTT assay is that the Yellow MTT (3-(4, 5-Dimethylthaiazol-2-yl)-2, 5-diphenyltetrazolium

bromide, a tetrazole is reduced to purple formazan in the mitochondria of living cells. The absorbance of this coloured compound can be determined after solubilization of the crystalline dye with DMSO and measuring the absorbance at a wavelength of between 500 and 600 nm. This enzymatic reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of living cells. For best results, cell numbers should be determined during log growth stage. The section bellow discusses how cytotoxicity of the Manayi product was determined.

3.1.6.2 Method of cytotoxicity

Cytotoxicity of cooked and uncooked Manayi was determined using MTT assay. The crude extract samples tested for cytotoxicity were those from chloroform (U1), dichloromethane (U2), butanol (U3) and water (U4) solvents. These extracts were analyzed using four concentrations. Cytotoxicity on the hexane extract for both cooked and cooked Manayi could not be done because the extract did not dissolve in both DMSO and methanol. Cells in an exponential growth phase were trypsinized, counted using a Neabauer haemocytometer and diluted to a density of 30 000 cells/ml. The cells were seeded into a 96-well plate at 6000 cells/well in 200 µl aliquots in RPMI 1640:10 % FBS. Plates were incubated at 37 ^{0}C for 24 hours to allow cells to attach. The Manayi extracts were screened for cytotoxicity at concentrations ranging from 6.25 to 50 µg/ml. Cisplatin served as a posting drug control in concentrations ranging from 10 to 100 µM. Following the initial 24 hour incubation period, the growth medium was removed and 200 µl aliquots of the Manayi extracts, and a positive control (cisplatin) were added and the plates were incubated for a further 48 hours. After the 48-hour incubation period, the medium was replaced with 200 µl MTT (sigma, 0.5 mg/ml in RPMI 1640:10 % FBS). The 200 µl TTM was removed after 4 hours incubation at 37^{0}C, and the resulting purple product was dissolved in 100 µl DMSO in each well. The plates were agitated for 60 seconds and the absorbance was measured at 540 nm on a PherStar microplate reader from BMG LABTECH. The experiment was done in triplicate to confirm the results. All the incubation steps were carried in a 37 ^{0}C humidified incubator with 5 % CO_2. The percentage inhibition of cytotoxicity was analysed as follows;

$$\text{3.1.6.2.1 Percentage cell viability} = \frac{\text{mean absorbance in test well}}{\text{mean absorbance in control test well}} \text{ x } 100$$

3.2 CHEMISTRY

3.2.1 Introduction

This section looks at the chemistry of the bioactive compounds in a Manayi product. Phytochemicals are biologically active, naturally occurring compounds found in plants, which provide health benefits to humans in addition to those attributed to macronutrients and micronutrients (Hater et al, 1999). Once a new compound has been isolated from a plant material, the determination of its molecular structure and a study of its properties are distinct problems of pure chemistry. Phytochemistry is concerned primarily with two problems, (1) a study of the chemical composition of plants/natural product and (2) explanation of the various plant processes in which chemical phenomena are concerned. The first problem includes: (a) qualitative detection of plant components; (b) the actual isolation of plants components; and (c) quantitative estimation of the plant components with actual isolation. Qualitative and quantitative phytochemical analysis was done.

Qualitative analysis was carried out for the determination of the presence or absence of steroids, flavonoids, proteins, amino acids, reducing sugars, glycosides, phenolic compounds, tannins, saponins, anthraquinones and gums through the use of the modifications of the methods by Trease et al., (1989) and Harbone et.al. (1984), as detailed below.

3.2.2 Preparation of test solutions for phytochemical tests

Approximately 30 g of each cooked and uncooked Manayi was dissolved in 200 ml water and 200 ml methanol, resulting in four test solutions.

3.2.3 Detection of phytosterols

Detection of phytosterols was carried out using two different approaches listed below:

3.2.3.1 Liberman-Burchard test

Approximately 2 ml each of the prepared solutions was dissolved in 1 ml chloroform. To each mixture, 2 ml of concentrated sulphuric acid and 1 ml acetic anhydride were also added. The solutions were shaken. A reddish violent colour indicates the presence of steroids.

3.2.3.2 Salkowiski test

Approximately 2 ml of each of the prepared test solutions from 3.2.2 were extracted in 1 ml of chloroform in a separating funnel. The aqueous layer was decanted. 1 ml of concentrated sulphuric acid was added to the chloroform layers and the solutions were shaken. The presence

of a reddish-blue colour in the chloroform layer and green fluorescence in the acid layer indicates the presence of steroids.

3.2.4 Detection of flavonoids

Approximately 5 ml of each of the four prepared samples of water and methanol filtrates were hydrolyzed with 10 % v/v sulphuric acid and allowed to cool. The solutions were extracted with diethyl ether and divided into 3 portions. Each of the portions was diluted with 1 ml ammonia, 1 ml sodium bicarbonate and 1 ml of 0.1N sodium hydroxide. The development of a yellow colour indicates the presence of flavonoids.

3.2.5 Detection of proteins and Amino acids

Proteins and amino acids were determined using Biuret test, Nihydrin test and Xanthoprotein test as described below.

3.2.5.1 Biuret test

Approximately 1 ml of 40 % sodium hydroxide (NaOH) (v/m) and two drops of 1 % copper sulphate were mixed with 5 ml of each test solutions in 3.2.2. A violent colour indicates the presence of proteins.

3.2.5.2 Ninhydrin test

Two drops of a freshly prepared 0.2 % Ninhydrin reagent was added to 1 ml of each four test solutions in 3.2.2 and heated. A blue colour indicates the presence of amino acids or peptides

3.2.5.3 Xanthoprotein test

Approximately 10 ml of each four solutions were treated with 1 ml of concentrated nitric acid. Formation of yellow colour indicates the presence of proteins.

3.2.6 Detection of Glycosides

The test for glycosides was carried out using three different tests classified in the following methods:

3.2.6.1 The legal test

Approximately 2 ml of each of the solutions in 3.2.2 was dissolved in pyridine and sodium nitroprusside solutions. The test solutions were made alkaline using a 0.5 molar sodium hydroxide. A pink red colour indicates the presence of glycosides.

3.2.6.2 Baljet test

Sodium picrate solution was added to 1 ml of each four test solutions described in 3.2.2 and the development of a yellow to orange colour indicates the presence of glycosides.

3.2.6.3 Borntrager's test

Two millimeters of diluted sulphuric acid was added to each 2 ml test solutions prepared in 3.2.2. The solutions were boiled and the samples were filtered. The filtrates were extracted with chloroform. The organic layers were separated. To the organic layers, a few drops of ammonia were added. The presence of a pink to red colour in the organic layer indicates a positive test for glycosides.

3.2.7 Determination of phenolic compounds and tannins

Phenolic compounds and tannins were carried using 5 % ferric chloride and 10 % potassium dichromate as described in 3.2.7.1 and 3.2.7.2:

3.2.7.1 5 % ferric chloride

Approximately 5 ml of each water and methanol filtrates for both cooked and uncooked Manayi were allowed to react with 1 ml of 5 % ferric chloride solution. Greenish black coloration indicates the presence of tannins. Approximately 2 ml of each test solutions described in 3.2.2 were mixed in alcohol and one drop of neutral ferric chloride (5 %) solution was added. Formation of an intense blue colour indicates the presence of phenols.

3.2.7.2 10 % potassium dichromate

Approximately 5 ml of each methanol and water filtrate for both cooked and uncooked Manayi was treated with 1 ml of 10 % aqueous potassium dichromate solution. Formation of a yellowish brown precipitate indicates the presence of tannins. 5 ml of the four test solutions described in 3.2.2 was treated with 1 ml of 10 % lead acetate solution in water. A yellow coloured precipitate indicates the presence of tannins.

3.2.8 Detection of saponins

Approximately 10 ml of each solution in 3.2.2 was mixed with 5 ml of distilled water and shaken vigorously. Stable persistent foam indicates the presence of saponins.

3.2.9 Detection of pentose

Two millimeters of the solutions were dissolved in concentrated hydrochloric acid phloroglicinol (1:1) and heated in a test tube for 5 minutes. The development of a red colour confirms the presence of pentose sugars.

3.2.10 Detection of anthraquinones

Approximately 3 ml of each test solution prepared in 3.2.2 was transferred into a test tube. 1 ml of 10 % sodium hydroxide solution was added to each tube. A red colour indicates the presence of anthraquinones.

3.2.11 Detection of Gums

To 25 ml of ethanol, 10 ml of each of the four test solutions was added slowly with constant stirring. A white or cloudy precipitate indicates the presence of gums.

3.3 Quantitative analysis (determination of ash and extractive values)

Natural products play a significant role in health care programs, mainly in developing countries. Nowadays, numerous medicinal plants and their products are still in use as home remedies. Documentation of research work carried out on traditional medicine is required (Mulla et al, 2010). Total ash and acid-insoluble ash content are significant to demonstrate the quality as well as the purity of herbal medicine (Rao et al, 2009). As a result, it is vital to make an effort towards standardization of the plant material to be used as medicine.

3.3.1 Total Ash.

Approximately 4 g of each cooked and uncooked Manayi was weighed into a silica crucible that was preheated at 600 ^{0}C, cooled and weighed. The crude drug powder was spread evenly as a fine layer at the bottom of the crucible. The crucible was incinerated by gradually increasing the temperature to make the crude drug a dull red hot until it was free of carbon. The crucible was cooled and weighed. The percentage of total ash was calculated as follows;

$$3.3.1.1\ \%\ \text{total ash} = \frac{\text{weight of total ash}}{4\ \text{g of Manayi product}} \times 100\ \%$$

3.3.2 Acid-insoluble ash

It is determined from the total ash obtained in 3.3.1. The pre-weight total ash was boiled with 25 ml of diluted hydrochloric acid for 10 minutes. The insoluble ash was collected using an ashless filter paper and washed with hot water. The insoluble ash was transferred into a silica crucible and the crucible was ignited, cooled and weighed. The residue was ignited in the furnace to get a constant weight. The percentage of the insoluble ash based on the weight of the air dried crucible drug was calculated as follows:

$$3.3.2.1\ \%\ \text{acid insoluble ash} = \frac{\text{weight of total acid insoluble ash}}{4\ \text{g of Manayi product}} \times 100\ \%$$

3.3.3 Water soluble ash

The total ash was obtained as indicated in procedure in 3.3.1. The sample, containing 25 ml of water, was heated up to 600 ^{0}C for 10 minutes. The insoluble ash was collected on an ashless filter paper and washed with hot water. The insoluble ash was transferred into a silica crucible and ignited in the furnace to get a constant mass. The crucible was cooled and weighed. The weight of the insoluble ash was subtracted from the weight of the total ash. The difference in weight was due to the water soluble ash. The percentage soluble ash was calculated as follows:

$$3.3.3.1\ \%\ \text{water soluble ash} = \frac{\text{weight of water soluble ash}}{4\ \text{g of Manayi product}} \times 100\ \%$$

3.3.4 Sulphated Ash

The silica crucible was heated to redness for 10 minutes, cooled in a desiccator and weighed. Approximately 4 grams of each cooked and uncooked Manayi was ignited in an electric furnace until the products were charred. The crucibles were cooled and the residues were moistened with 1 ml H_2SO_4, heated gently until the white fumes were no longer evolved, and ignited at 800^{0}C until all black particles disappeared. The crucibles were allowed to cool. Few drops of H_2SO_4 were added and heating was continued. The product was cooled and weighed. This was repeated until a constant mass was obtained. The percentage sulphated ash was calculated as follows;

$$3.3.4.1\ \%\ \text{sulphated ash} = \frac{\text{weight of sulphated ash}}{4\ \text{g of Manayi product}} \times 100\ \%$$

3.4 Determination of extractive values

The determination of extractive values is a useful means of evaluating crude drugs which cannot be readily estimated by other means. Extractive values can be estimated using water and alcohol soluble extractives. Extractive values also determine the nature of the constituents present in a crude drug.

3.4.1 Alcohol soluble extractive value

Approximately 5 g of each cooked and uncooked Manayi material was weighed and macerated with 100 ml of ethanol in a closed container for 20 hours. The samples were shaken for six hours and allowed to stand, soaked for 18 hours and filtered using a Whatman filter paper. After the samples were filtered, 25 ml of each filtrate was evaporated in a flat bottom shallow dish, dried at

105 ^{0}C, cooled and weighed. The percentage ethanol soluble extractive value was calculated with reference to the air-dried product.

3.4.2 Water soluble extracted value

Approximately 5 g of each cooked and uncooked Manayi material was weighed and macerated with 50 ml of chloroform and water at 80 ^{0}C separately for 24 hours. The samples were shaken for six hours and allowed to stand, soaked for 24 hours and filtered. After filtration, 25 ml of the filtrates was evaporated in a flat bottom shallow dish, dried at 105 ^{0}C, cooled and weighed. The percentage water-soluble extractive material was calculated with reference to the air dried product.

3.5 Analysis of extract profiles using TLC

Six crude solvent extracts of each of cooked and uncooked Manayi product were analysed using TLC to determine chemical constituents present in Manayi product. 10 µl of dissolving solvent extract was spotted at a concentration of 10 mg/ml. The plates were developed using mobile phases with various combinations of methanol (M), chloroform (C), ethyl acetate (E), ammonia (Am), water (W) and formic acid (F) at different ratios to create an eluting solvent of varied polarities. The combinations of eluting solvent mixtures that were used are:

1. Eluting solvent :C:M (9:1)
2. Eluting solvent: E: M: Am (90:20:15) and
3. Eluting solvent: E: M: W: F (50:6.5:5:2).

The TLC plates were developed in eluting solvent mixtures 1, 2, and 3. The TLC plates were viewed under UV light at 270nm and 360nm. They were then sprayed with vanillin/H_2SO_4 solution and heated at 100 ^{0}C to allow colour development (FAO/IAEA, 2000).

3.6 Isolation and identifications of compounds

Two crude extracts from uncooked Manayi were chosen for further investigation. These were chloroform and water extracts. The extracts were chosen because they contain the highest activity in HIV-1 integrase enzyme and have the highest percentage yield. Hexane extract was found to have more HIV-1 activity enzyme compared to all other solvent extracts, but further investigations were not done because of low percentage yield (See appendix A). The water extract was chosen because it is the solvent used by the traditional healer to prepare the product and also due to the fact that it had a high percentage yield.

Fractionation started by conditioning the sorbent with 100% methanol and equilibrated with 100 % water. 1.0235 g of the crude chloroform and 1.0999 g of the crude water solvent extract were weighed, dissolved in 20 ml of water and loaded on the Carbon 18 (C18) sorbent SPE columns. The natural extracts were eluted with different percentages of methanol (v/v): 0 %, 20 %, 40 %,

50 %, 60 %, 80 % and finally 100 % methanol, so that fourteen separate fractions were obtained at the end of the process. All fractions were evaporated to dryness under vacuum using a rotary evaporator. The fourteen fractions were collected and quantified using TLC, and later analyzed using FTIR, NMR and LC/MS to identify possible compounds.

3.7 Finger printing fractions on Manayi product using Fourier Transform Infrared Spectroscopy (FTIR)

Chloroform and water crude solvent extracts that showed HIV-Integrase enzyme activity were fractionated. The procedure to fractionate the extracts is described in 3.6. After fractionation, the fractions were quantified using TLC and fractions with same Rf value were pooled together. After the fractions were quantified using TLC, 100 % methanol fraction of a mobile phase of E: M: Am (90:20:1.5) from uncooked water extract showed one spot and produced the highest percentage yield. This fraction was selected for further investigation and labeled Manayi compound 1 (MC1), and was further subjected to spectroscopic analysis for identification of the functional groups in the bioactive compounds. FTIR analysis was performed using a Perkin Elmer IR Spectroscopy instrument, which was used to detect the characteristic peaks and their functional groups. A small amount of the sample was loaded on ATR (Attenuated Total Reflectance), and the fraction was scanned in the range of 4000-650 cm^{-1}. Each analysis was repeated twice for spectrum confirmation. The FTIR spectrum was used to identify functional groups of active components based on peak value in the region of infrared radiation.

3.8 Analysis of compounds using NMR and LC/MS

The MC1 fraction which showed only one spot on TLC and high percentage yield was analyzed with NMR for structural elucidation. Proton (^{1}H) and Carbon (^{13}C) spectra were recorded with a Bruker Advance 600 MHz NMR spectrometer. A freeze dried MC1 was dissolved in methanol. The compound MC1 was also analyzed using micromass LCT premier TOF-MS. The column used was a Symmetry (Waters) C18 column (250 × 4.6 mm). A 25-µl sample was injected using the system's auto sampler. Solvent A contained 5 % formic acid in water, and solvent B was a HPLC-grade methanol. The UV response during LC/MS was monitored at 360 nm, which is the highest absorbance wavelength for each set of components as determined from prior HPLC studies. The LC/MS was operated in the positive-ion mode using the electrospray ionization (ESI) source and the manufacturer's recommended operating conditions. An infusion was dissolved in methanol and analyzed in low resonance mass spectroscopy and high resonance mass spectroscopy.

CHAPTER FOUR: RESULTS AND DISCUSSION

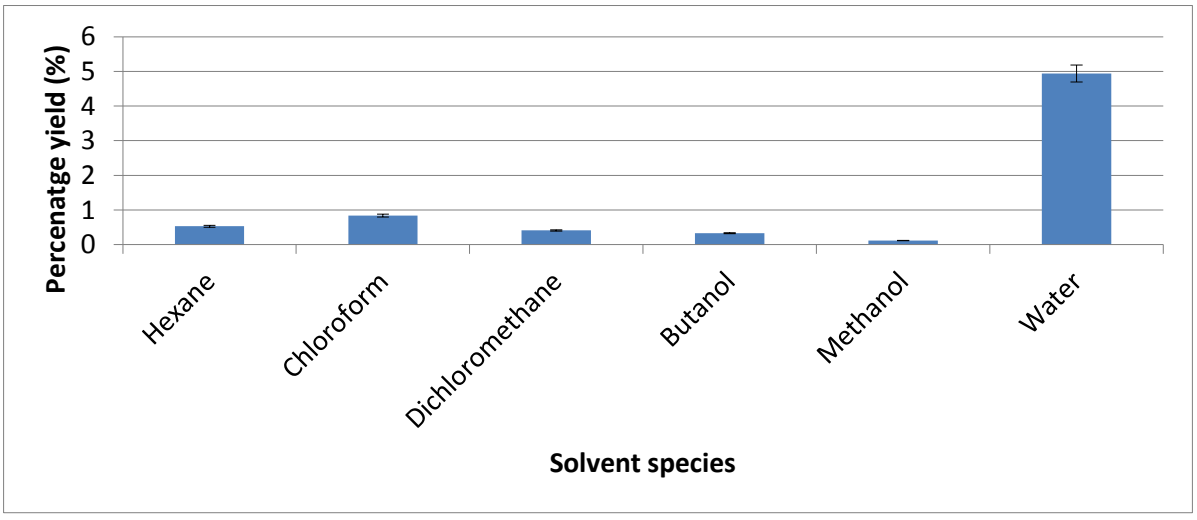
4.1 Pharmacological studies

Resistance is the result of mutations that emerge in the viral proteins targeted by antiretroviral agents. Antiretroviral drugs have many disadvantages which include development of resistance to drugs, many side effects, limited availability, high cost and lack of curative effects (Klos et al, 2009). An initial screen of the crude extracts for the presence of RT inhibitory and Integrase activity using a Colorimetric RT Elisa for RT inhibition, and Xpresso Bio for Integrase has led to the prioritization of a product with chemically pure fractions and extracts for further investigation.

4.1.1 Percentage yield of the extracts

Manayi products were selected for screening of HIV activities. Even though in traditional medicine water is used as an extractant, not all compounds are extracted in water, for example, if the bioactive compounds are non-polar, they are not extracted (Ellof, 1988). To target polar and non-polar constituents for bioactivity testing in this study, powders of each cooked and uncooked Manayi were extracted using hexane, chlrorform, dichloromethane, butanol, methanol and water sequentially. The yield of the extracts varied with the type of solvent used, see figure 4.1. The crude water extract of uncooked Manayi resulted in the largest yield of 11 % when compared to other solvent extract yields. This was followed by chloroform with 8 % of uncooked Manayi, while water and chloroform for cooked Manayi resulted in low percentage yield of (1 %) and (4 %) respectively.

a) Cooked Manayi



b) Uncooked Manayi

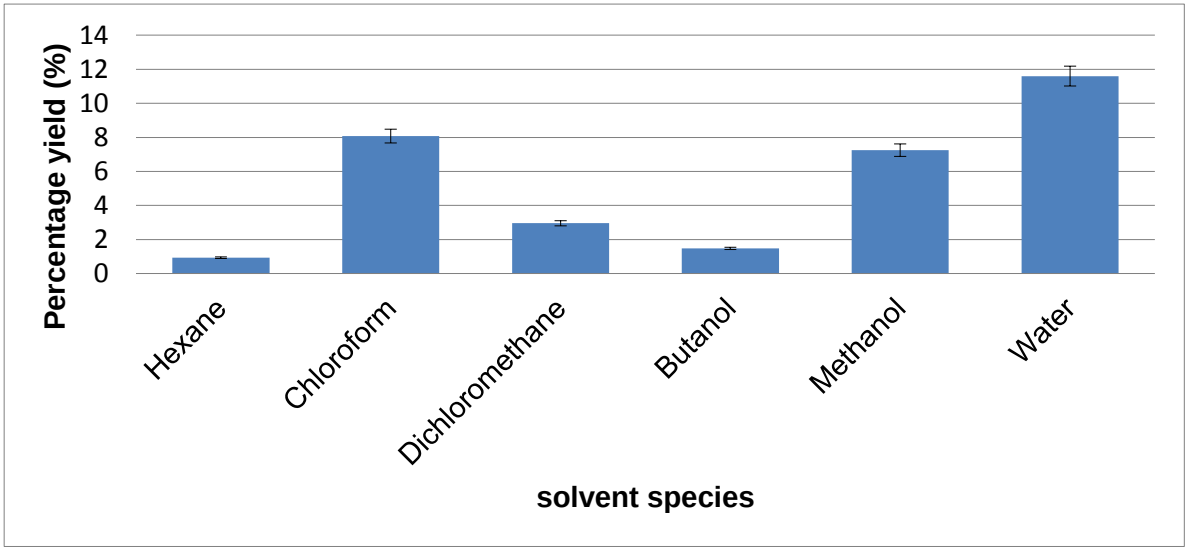


Figure 4.1: Percentage yield of cooked (a) and uncooked Manayi (b) crude extracts using hexane, chloroform, dichloromethane, butanol, methanol and water.

Water extract for each cooked and uncooked Manayi showed the best percentage yield and the hexane extract for cooked and uncooked Manayi has the low percentage yield. The high percentage yield obtained with water maybe related to the presence of large quantities of more polar compounds in the product. The results of the percentage yield of each cooked and uncooked Manayi are shown in Appendix A.

4.1.2 Analysis of cooked and uncooked Manayi using reverse transcriptase activity and Integrase activity enzyme.

Extracts of one selected natural product called Manayi were used in the treatment of HIV and AIDS and were investigated for their anti-HIV properties against enzymes that play significant role in the cycle. The antiviral first activity inhibition was studied through the Reverse Transcriptase HIV-1 Assay from Roche. Inhibition of recombinant HIV RT by the crude Manayi product extracts was investigated. Extracts from the product were pooled and tested at 150 µg/ml final concentration. The percentage inhibitory activity was calculated with reference to the positive control and then subtracted from 100 to give the percentage inhibition. All the crude extracts of the cooked and uncooked Manayi (see appendix A) were tested for their ability to inhibit recombinant HIV RT in a Colorimetric reverse transcriptase Elisa from Roche. Odimune was used as the positive control.

The results in figure 4.2 show that no inhibition was obtained with all the crude extracts of the cooked and uncooked Manayi product tested, and this shows that the product does not possess an RT inhibitor. Extracts RT inhibitor experimental data is shown in Appendix C.

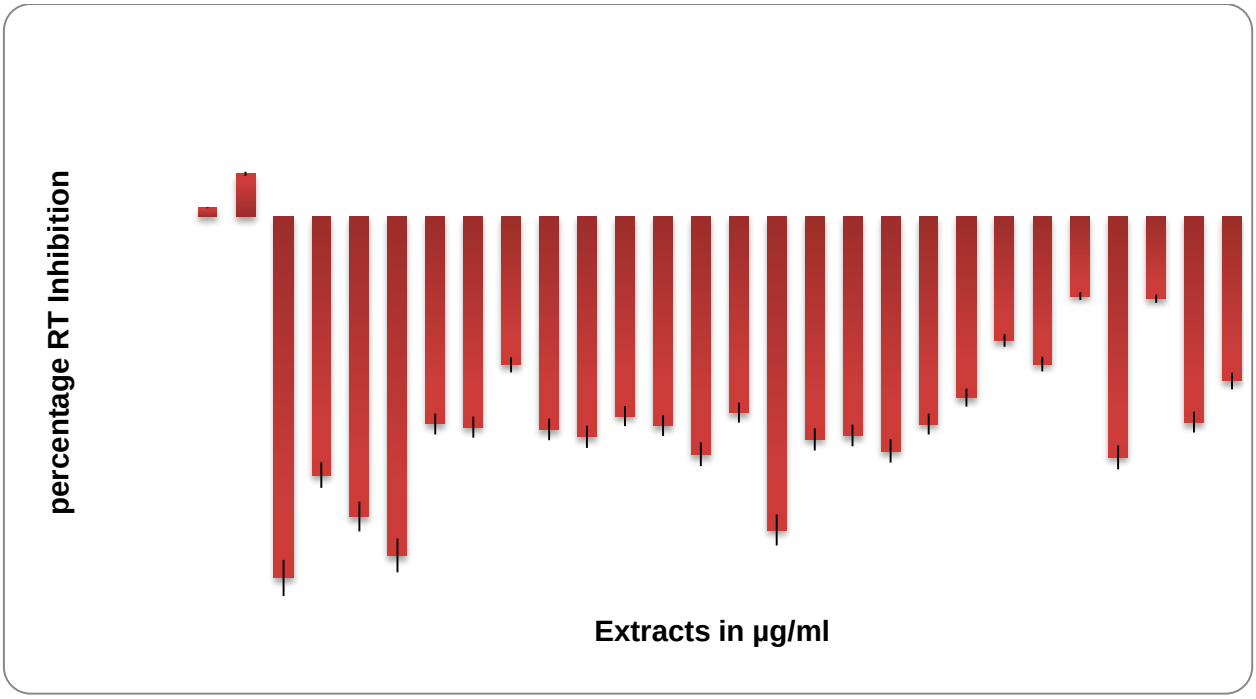


Figure 4.2: Percentage inhibition of HIV-1 RT by cooked and uncooked Manayi product used by the Eastern Cape Herbalist to treat people with HIV. Extr (1) uncooked 90:10 (MeOH:H_2O), extr (2) uncooked hexane, extr (3) cooked hexane, extr (4) uncooked dichloromethane, extr (5) cooked dichloromethane, extr (6) uncooked butanol, extr (7) cooked butanol extr (8) uncooked water, extr (9) cooked water, extr (10) uncooked methanol (11) cooked methanol, extr (12) uncooked 80:20 (MeOH:H_2O) and extr (13) uncooked 60:40 (MeOH:H_2O). Percentage inhibition by positive control (Odimune) were 13.502 % at 6.25 µg/ml and 64.437 % at 12.5 µg/ml.

The second activity has been tested against HIV-1 Integrase enzyme from XpressBio. The XpressBio activity kit is a non-radioactive assay used to quantitatively measure integrase activity and the effects of the test compounds on HIV-1 integrase activity (XpressBio, life science products). Sodium azide is included in the kit as a positive control that inhibits HIV-1 catalytic activity. The experimental data for HIV-1 integrase enzyme activity is described in 3.1.5. Typical assay results for buffer/no integrase, integrase alone/no azide, and integrase plus azide are tabulated in Appendix D for inhibition of the HIV-1 integrase when treated with azide. Hexane crude extract from uncooked Manayi leaves exhibited the most potent inhibitory activity against HIV-1 Integrase enzyme with a percentage value of 99.4 % at 50 µg/ml and 104.8 % at 100 µg/ml and an IC_{50} of 7.7, followed by chloroform extract with a percentage value of 93.6 % at 50 µg/ml and 60.2 % at 100 µg/ml and an IC_{50} of 2.2. Cooked Manayi also has the highest potent inhibition in hexane extract with a percentage value of 81.8 % at 50 µg/ml and 33.0 % at 100 µg/ml, and an IC_{50} of 2.2 followed by water with a value of 66.2 % at 50 µg/ml and 42.9 % at 100 µ/ml, and an IC_{50} of 1.8. Results of the percentage inhibition are shown in figure 4.3. The values of IC_{50} were

calculated by drawing scatter graphs (see figure 4.4) where the Y axis is the percentage inhibition. The slope equation for the graph (Y=mx+c or Y=mx-c) was then determined. M and C values are presented in the equation itself. IC_{50} represents the concentration of the tested agent that is required for 50 % inhibition of the cell viability. IC_{50} was calculated for an extract with percentage inhibition above 50 %. The IC_{50} was calculated according to cell biology protocols.

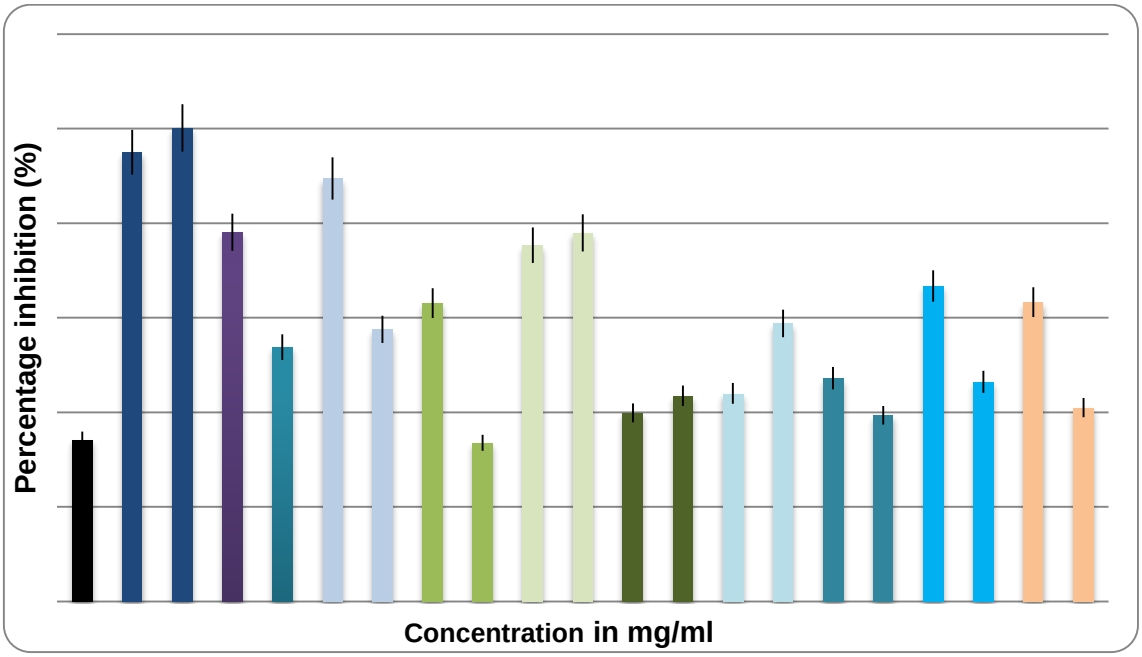
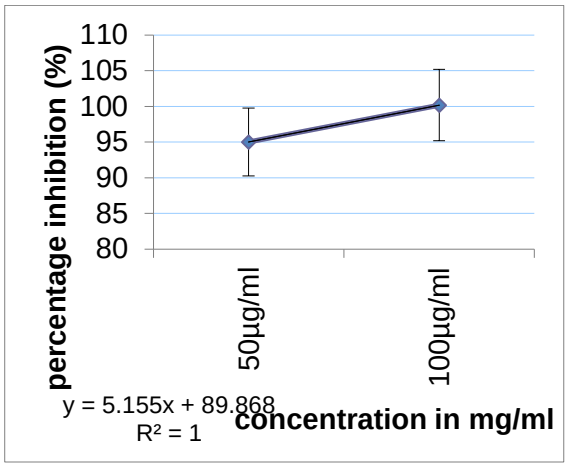
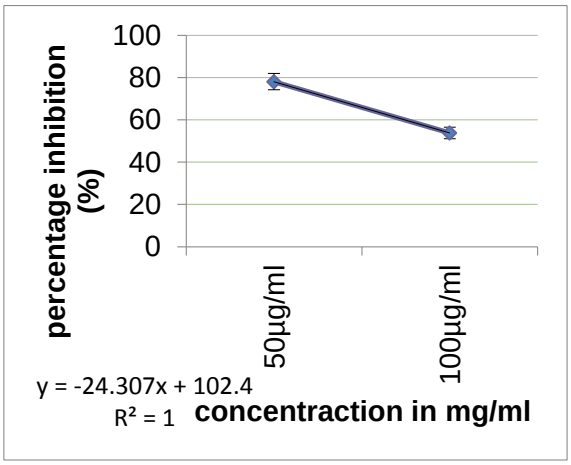


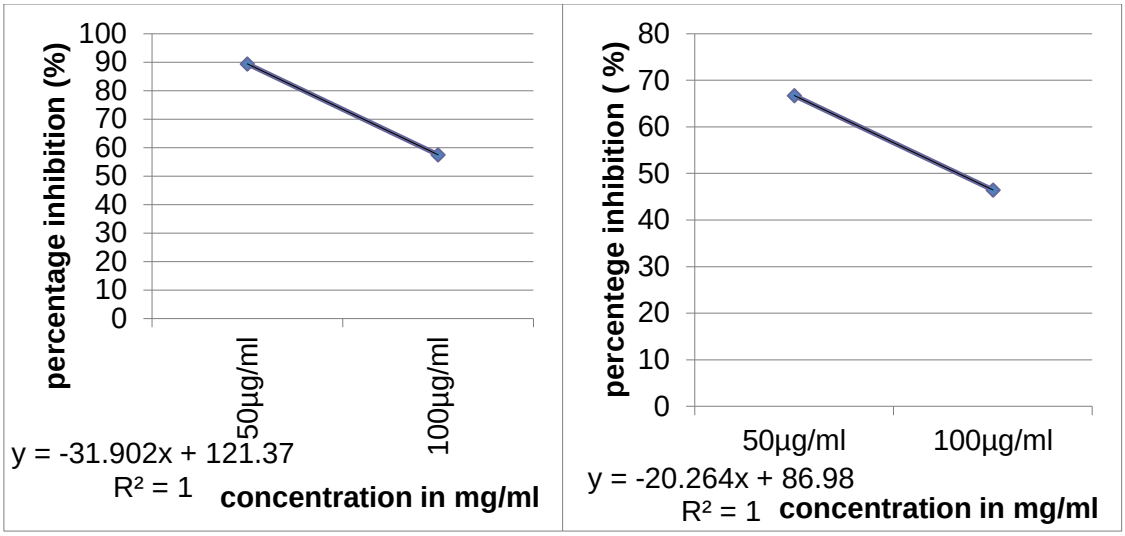
Figure 4.3: Percentage inhibition of HIV-1 integrase enzyme. Extract (1) uncooked Hexane, extract (2) cooked hexane, extract (3) uncooked chloroform, extract (4) cooked chloroform, extract (5) uncooked dichloromethane, extract (6) cooked dichloromethane, extract (7) uncooked butanol, extract (8) cooked butanol, extract (9) uncooked water, extract (10) cooked water. The percentage inhibition of positive control (Azide) was 35.761 at 12.5 µg/ml.



(a) Uncooked hexane



(b) cooked hexane



(c) Uncooked chloroform **(d) uncooked water**

Figure 4.4: % inhibition of the most effective extracts graphs for the determination of IC_{50}

The above results in figure 4.3 indicate that cooked and uncooked Manayi extracts have the potential to be used as enzyme inhibitors against sexually transmitted infections. Figure 4.4 shows IC_{50} values for inhibitions at 50% and more. The high IC_{50} in hexane crude extracts shows that hexane extract is more effective than chloroform and water extracts.

4.1.3 Results on the cytotoxicity of Manayi product

The potential cytotoxicity of twelve solvent extracts, six of each cooked and uncooked Manayi was evaluated in the screening against Vero cells using MTT viability assay. The crude extracts were screened using four concentrations (6.25, 12.5, 25 and 50 µg/ml). Both cooked and uncooked Manayi extracts at the concentrations tested were found not cytotoxic on Vero cells in vitro. Their IC_{50} could not be calculated because no percentage inhibition is above 50 %. Fig 4.5 and 4.6 are the graphs for cooked and uncooked Manayi % inhibition cytotoxicity on Vero cells

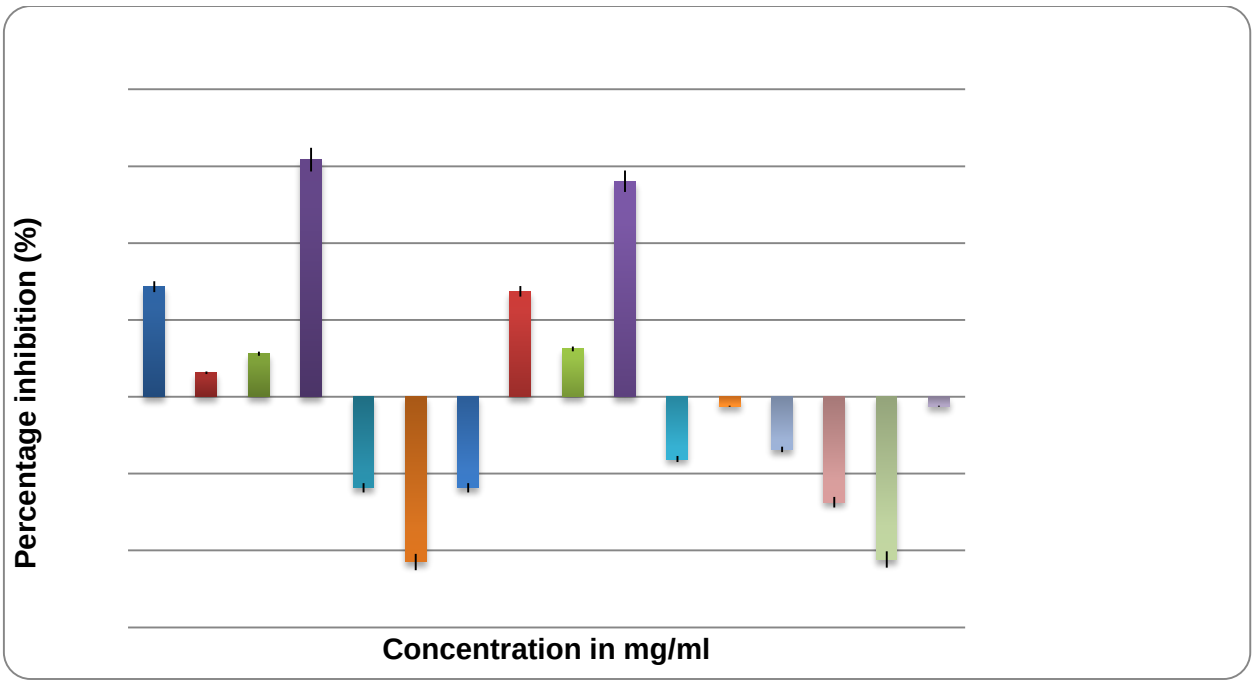


Figure 4.5: Percentage inhibition of cooked Manayi against Vero cells

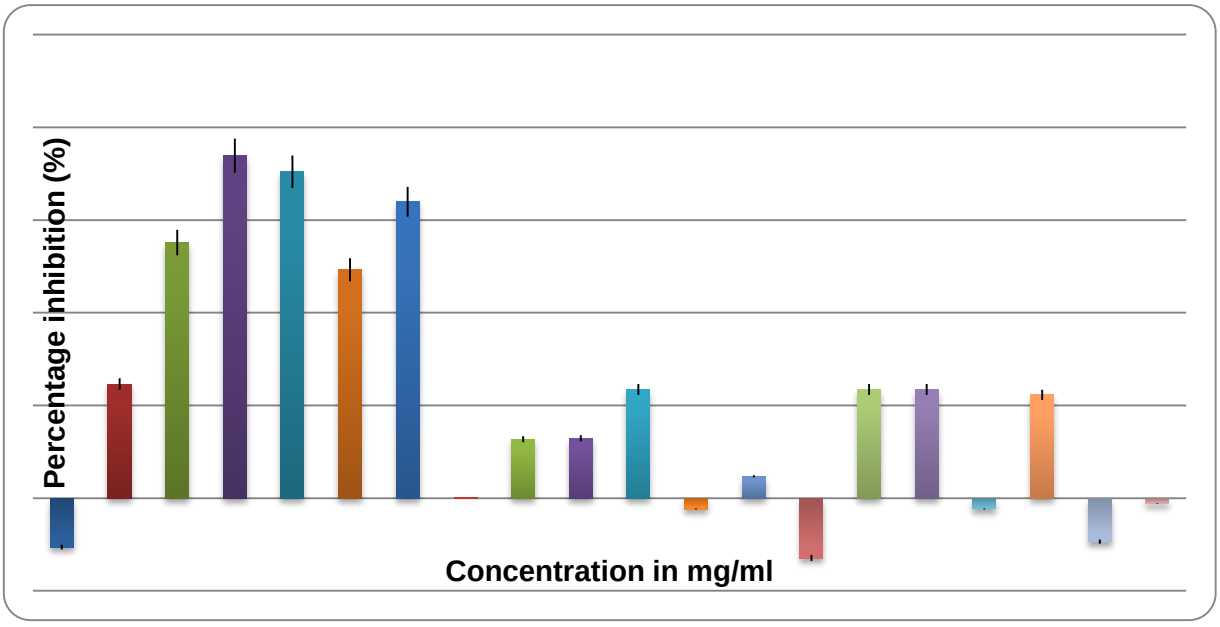


Figure 4.6: Percentage inhibition of uncooked Manayi against Vero cells

Different assay are required to distinguish between effects on specific or general cytotoxicity.

4.2 Chemistry studies results:

The investigation of natural products used in traditional medicine requires the successful integration of evidence on chemical composition of extracts, pharmacological activities of isolated compounds as well as indigenous knowledge of traditional therapy. The procedure of separation and identification of principal compounds from a complex of mixtures needs a number of particular resources as well as comprehensive knowledge, specialised equipment and skills. Therefore, a move towards natural product investigation, which is further driven by outstanding advances in plant extract technology, biotechnology and analytical chemistry, is essential.

4.2.1 Qualitative phytochemical analysis of cooked and uncooked Manayi product

Preliminary analysis of phytochemicals is a valuable in the detection of bioactive compounds present in medicinal plants/natural products and may subsequently lead to drug discovery and development. In the present chapter, qualitative and quantitative analysis of cooked and uncooked Manayi was applied in order to relate their presence with bioactivities of the products. Screening of cooked and uncooked Manayi using chloroform and methanol solvents for extraction was performed to confirm or disprove the presence of steroids, flavonoids, proteins, and amino acids, reducing sugars, glycosides, phenolic compounds, tannins, saponins, anthraquinones and gums. The description of cooked and cooked Manayi is discussed in section 3.1.3.

Results in table 4.1 show the screening of methanol and water extracts of cooked and uncooked Manayi product based on phytochemical and physiochemical tests. These tests reveal the presence of various bioactive secondary metabolites which might be responsible for their medicinal attributes. The observations and interpretations made in phytochemical tests are presented as follows:

4.2.1.1 Steroids

- Lieberman-Burch test: The absence of reddish violent colour was an indication of the absence of steroids

- Salk-wish test: The absence of reddish-blue colour in the chloroform layer and green florescence in the acid layer meant that there are no steroids.

4.2.1.2 Flavonoids

A yellow coloration was observed in all the extracts indicating the presence of flavonoids except for the cooked methanol extract.

4.2.1.3 Proteins and amino acid

- Biret tests: Violent colour on cooked water and uncooked water extract was an indication of the presence of proteins.
- Nihydrin test: Absence of the blue colour in all extracts indicates the absence of amino acids
- Xantho-protein: the formation of a yellow colour in cooked and uncooked water extracts indicates the presence of proteins

4.2.1.4 Reducing sugars

- Fehling's solution test: Absence of reducing sugars was confirmed by the absence of a brick red precipitate.
- Benedict's test: The absence of reddish brown precipitate indicated the absence of carbohydrates.

4.2.1.5 Glycosides

A pink red colours solution on legal test confirmed the presence of glycosides in all extracts.

4.2.1.6 Phenolic compounds and tannins

- 5 % ferric chloride: Blue green colour confirmed the presence of phenols and tannins.
- Potassium dichromate test: Brown precipitate was formed which confirms the presence of phenols.

4.2.1.7 Saponins

Formation of a soluble emulsion on cooked water extract confirmed the presence of saponins.

4.2.1.8 Anthraquinones

The absence of a pink, violet or red coloration in the ammonia layer indicated the absence of free anthraquinones in all extracts.

4.2.1.9 Gums

The presence of the persistent foam on the cooked water extract was an indicator of the presence of gums.

The preliminary phytochemical analysis indicates the nature of phytoconstituents present in different solvent extract. The results are presented in table 4.1. The preliminary phytochemical analysis results of cooked and uncooked Manayi product using methanol and water reveals the

presence of various bioactive secondary metabolites (**+++**) such as proteins, glycosides, phenolic, tannins, and saponins and the absence of various secondary metabolites (**---)** such as steroids, reducing sugars, anthraquinones and gums. The presence of these secondary metabolites is well known to have curative activity against several human pathogens, and therefore could suggest their traditional usefulness for the treatment of various diseases (Hassan et al., 2004). In table 4.1 it was also revealed that most of the biologically active phytochemicals were present in uncooked and cooked water extract. In other words, the results confirmed the presence of therapeutically potent compounds in cooked and uncooked Manayi. It revealed that glycosides were predominantly found in all the four extracts, followed by flavonoids, which were found in four extracts except cooked methanol extracts of portion 1. Steroids, reducing sugars, saponins, anthraquinones, and gums were not found in all four extracts.

Table 4.1: Results of phytochemical analysis of cooked and uncooked Manayi extracts

	Qualitative and quantitative phytochemical analysis			
Tests	**Uncooked Methanol extract**	**Uncooked water extract**	**Cooked Methanol extract**	**Cooked water extract**
Steroids (Liebermann)	**---**	**---**	**---**	**---**
Steroids (Salkowski)	**---**	**---**	**---**	**---**
Flavonoids				
Portion 1	**+++**	**+++**	**---**	**+++**
Portion 2	**+++**	**+++**	**+++**	**+++**
Portion 3	**+++**	**+++**	**+++**	**+++**

(+++) = Presence, (---) = Absent

Table 4.1 (continued): Results of phytochemical analysis of cooked and uncooked Manayi extracts continued.

	Qualitative and quantitative phytochemical analysis			
tests	**Uncooked Methanol extract**	**Uncooked water extract**	**Cooked Methanol extract**	**Cooked water extract**
Proteins				
Biurets's	**---**	**+++**	**---**	**+++**
Nihydrin	**---**	**---**	**---**	**---**
Xanthoportein	**---**	**+++**	**---**	**+++**
Reducing sugars				
Fehling's solution	**---**	**---**	**---**	**---**
Benedict's solution	**---**	**---**	**---**	**---**
Glycosides				
Legal test	**+++**	**+++**	**+++**	**+++**
saponins	**---**	**+++**	**---**	**+++**
Anthraquinones	**---**	**---**	**---**	**---**
Gums	**---**	**---**	**---**	**---**

(+++) = Presence, (---) = Absent

4.3 Physiochemical analysis of cooked and uncooked Manayi

The determination of ash content is frequently helpful for the discovery of low-grade products, exhausted drugs and excess of sandy or earthy matter. It is more especially applicable to powdered drugs (Prajapati and Patel. 2012). The purpose of charring plant material is to eliminate all traces of organic matter, which might otherwise interfere in its analysis. Charring at too high temperatures may also result in the development of complex silicates, which are not soluble in hydrochloric acid and an apparent loss of some constituents may result from it. The acid insoluble ash, for example, the ash insoluble in dilute hydrochloric acid, is often of much value than the total ash. The water soluble ash is used to detect the presence of material soluble in water. The water soluble ash shows greater reduction than the total ash and is therefore used as an important indicator for the presence of exhausted material substituted for the genuine articles (Verma, 1988). Analysis of ash provides the list of various elements present in the plant. The total ash is particularly important in the evaluation of drug purity, for example, the presence or absence of foreign inorganic matter such as metallic salts and/or silica.

Extractive values are useful to evaluate the chemical constituents present in the crude drug and also help in the estimation of specific constituents soluble in a particular solvent. The variation in extractable matter in various solvents is suggestive of the fact that the formation of the bioactive principles of medicinal plants is influenced by a number of intrinsic and extrinsic factors. Bioactive principles, also referred to as plant secondary metabolites, are derived from the products of plant primary metabolites, which are associated with the process of photosynthesis viz., carbohydrates, amino acids, and simple lipids. High extractive values for alcohol- and water-soluble extracts reveal the presence of polar substances like phenols, tannins and glycosides, as reported by Baravalia and others, 2011.

The medicinal importance of cooked and uncooked Manayi product was thoroughly investigated for their physicochemical characters including their solvent extractive values (Table 4.2), total ash values, safety and standardisation for their safe use. The physicochemical evaluation was conducted on these crude drugs because they are used by the traditional healer to treat HIV patients. Physicochemical properties that include total ash, acid insoluble ash, water soluble ash, sulphated ash and extractive values were analysed using the United State Pharmacopoeia-National formulary (2003). The extractive values were analysed using ethanol, chloroform and water as described in 3.4.1 and 3.4.2. This study revealed that the highest water soluble ash was from both cooked and uncooked Manayi (60 %), and the highest water extractive value was for uncooked Manayi (17 %). Ethanolic extractive values for cooked and uncooked Manayi were both 3 %. The total ash content indicated that both cooked and uncooked Manayi yielded 62.5 %, both of which are much higher than the standard ash values given in the World Health Organisation

(2005) monographs for medicinal plant or products. This indicates that these samples were highly adulterated and substandard.

Table 4.2: results of physiochemical analysis of cooked and uncooked Manayi

	Uncooked Manayi extract	cooked Manayi extract
Ash values in %		
Total ash	62.5	62.5
Acid soluble ash	60	20
Water soluble ash	60	60
Sulphated ash	40	40
Extractive values in %		
Alcohol extractive	3	3
Water extractive	17	8

Determination of extractive values, ash values and active components (flavonoids, proteins, glycosides, phenolic, saponins and gums) plays a significant role for standardisation of indigenous crude drugs.

4.4 Qualitative analysis of cooked and uncooked Manayi by TLC

Thin Layer Chromatography (TLC) was used to identify compounds present in the product extracts. The extracts were done using hexane, chloroform, dichloromethane, butanol, methanol and water sequentially. We had to determine the optimal mobile phase such as, (9:1) (C: M), (50:65:5:2) (E: M: F: Am), and (90:20:1.5).

The compounds profiles of the crude extract of Manayi product investigated are presented in the following figure 4.7 (a) to (f).

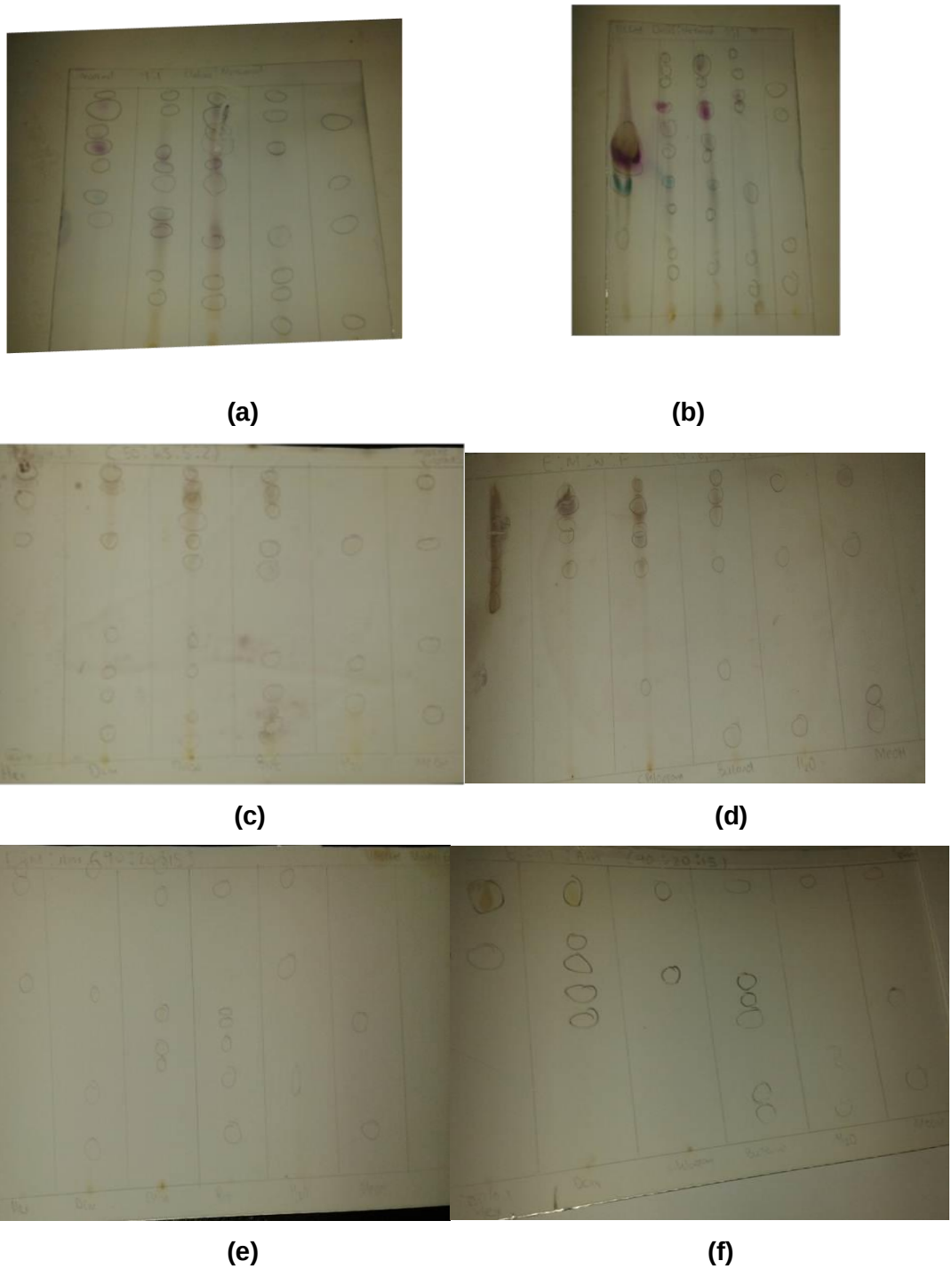


Figure 4.7: TLC profiles of (a) and (b) (9:1) (C:M); (c) and (d) (50:65:5:2) (E:M:W:F); and (e) and (f) (90:20:1.5) (E:M:Am) uncooked and cooked Manayi product respectively.

The development of the chromatograms was done in a closed TLC tank in which the atmosphere was saturated with the eluent. The TLC profiles of the cooked and uncooked Manayi product

shown in figure 4.7 (a) to (f) are crude solvent extracts of hexane, chloroform, dichloromethane, butanol, methanol and water, respectively, with three mobile phases of different polarities. Each of the six extracts and fractions discussed on the isolation and identification on section 3.6 were first checked by the TLC technique on analytical plates over silica gel-G of 0.2 mm thickness. These plates were developed in the following solvent ratios: chloroform: methanol (9:1), ethyl acetate: methanol: ammonia s (90:20:15), and ethyl acetate: methanol: water: formic acid (50:6.5:5:2).

Both Uncooked and cooked Manayi crude extracts from chloroform and dichloromethane yielded the highest number of compounds/spots. Mobile phases (9:1) (C: M) and (50:65:5:2) (E: M: W: F) eluted the most spots and had the best separation. Spots were also visualized by exposure of the plates to a vanillin/H_2SO_4 reagent. The chromatograms revealed a complex mixture of compounds which exhibited different coloured reactions with vanillin/H_2SO_4 spray reagent. The classes of compounds in the extracts include terpenoids (purple or bluish purple) (Taganna et al., 2011) and phenolics such as flavonoids (yellow, pinkish or orange colour) with vanillin/H_2SO_4 reagent. The presence of the phenolic components both in cooked and uncooked Manayi extracts was confirmed by blue and purple colour spots while that of flavonoids by a yellow spot (Rijke et al., 2006) when vanillin/H_2SO_4 was used as a developing reagent in a (C: M) (9:1) mobile phase. At the optimised conditions, the TLC results show the dichloromethane of uncooked Manayi extract gave 10 bands figure (4.7(c)), therefore dichloromethane extracts have more compounds than other extracts.

Two extracts that contains more yield and have the highest anti-integrase HIV activity were chosen for further investigations. These extracts are Chloroform and water, chloroform with more yield and more inhibition against HIV-Integrase activity, water has been chosen as it has the highest percentage yield and also being the solvent used by the traditional healer to prepare the product.

4.5 Preliminary isolation study

Bioassays and phytochemical screening of the product described above showed the presence of different primary and secondary bioactive molecules like proteins, glycosides, phenolic compounds, tannins, and saponins. The results for bioassays are discussed in 4.1.2 and observations of phytochemical analysis are summarised in table 4.1

4.5.1 Percentage yield of fractions

The general procedure of fractionation of the selected crude extract for further investigation is described in section 3.6. The results of percentage yield of the fractions are shown in appendix B. Figure 4.8 shows that fractions 1 to 8 are crude chloroform extract and fractions 9 to 14 are crude water extract all from uncooked Manay. A fraction is a quantity collected from a sample or batch of a substance in a fractionating separation process. In such a process, a mixture is separated into fractions, which have the same compositions that vary according to a gradient. The results described in figure 4.8 revealed that the crude water extract, fraction 14 (0 % H_2O) resulted in the percentage yield of (29 %), followed by crude chloroform extract, fraction 7 (0 % H_2O) with 20 %. Fraction 1 from crude chloroform extract (100 % H_2O) has the lowest yield of 0.90 %. Fractionation was not done on cooked Manayi due to low percentage inhibition in integrase enzyme activity and low percentage yield on the extraction of the product.

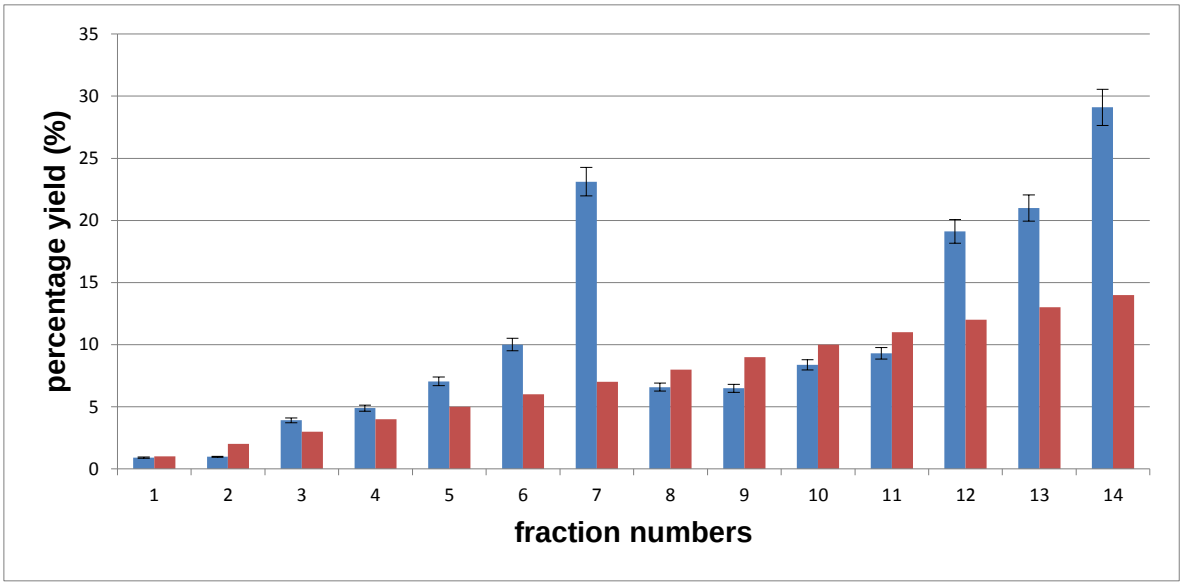


Figure 4.8: percentage yield of fractions of two uncooked Manayi, with solvents of different polarities such as crude chloroform extract faction (1-7) and crude water extract, fraction (8-14)

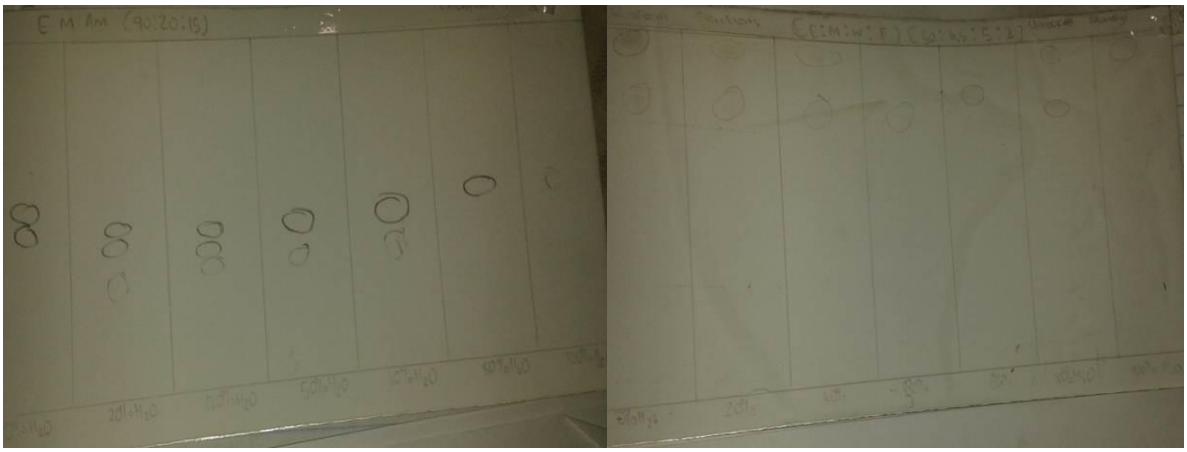
4.5.2 TLC analysis profiles of fractions

Thin layer chromatography also confirmed the different bioactive compounds. Therefore, more investigation was required to identify these compounds. The identifications were done by selecting the best active extracts for bioassay processes on anti HIV. The next step was to carry out preliminary separation procedures (fractionation) in order to simplify the complex crude extract and then verify the compound fractions before large scale isolation procedures were performed (as discussed in 3.8). In this section, identification of bioactive compounds is described as both these extracts are confirmed to be a source of bioactive compounds due to the results of phytochemical and TLC analysis analysed in this research. The methods to prepare these fractions are discussed in chapter 3. Solid phase extraction (SPE) was used to clean-up the crude

extracts, whereby it was separated into seven elution fractions according to polarity crude extract. The following figure 4.9 is a TLC profile of fractions of chloroform and water crude extracts.

Chloroform fractions

a). E: M: Am (90:20:1.5) b) E: M: W: F (50:65:5:2)



Water

c) E: M: Am (90:20:1.5) d) E: M: W: F (50:65:5:2)

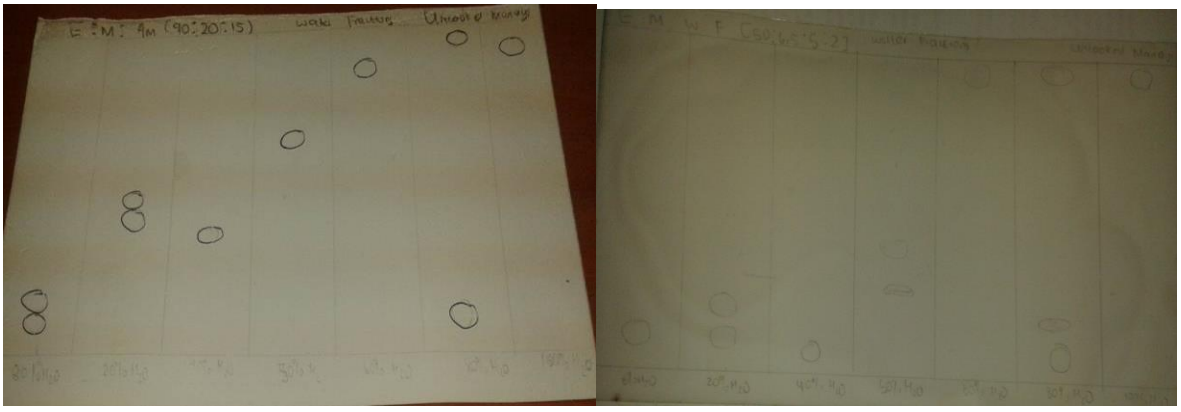


Figure 4.9: TLC profiles of (a) E: M: Am (90:20:1.5)and (b) E: M: W: F (50:65:5:2)); (c) and (d) E: M: Am (90:20:1.5) and E: M: W: F (50:65:5:2) uncooked Manayi chloroform and water fractions respectively.

Solid phase extraction yielded 14 fractions. Thin layer chromatography analysis was carried out on chloroform and water fractions where E: M: W: F (50:65:5:2) and E: M: Am (90:20:1.5) mobile phases were used. Chloroform and water fractions, using E: M: Am (90:20; 1.5) mobile phase, revealed homogeneous spots which is an indication that the mobile phase used might show a good resolution if adopted for fractionation. In figure 4.9, TLC plate (a), 100 % water and 80 % water fractions were pooled together because they both contain one spot and the same Rf value of 0.53. Water fraction (100 % methanol) in figure 4.9 (c) with one spot showed good resolution under UV and yielded a high percentage yield and was chosen for further identification

of possible bioactive compounds using FTIR, NMR and LC-MS. The Rf values of fractions are ranged from 0.08 to 0.99 and are shown in tables 4.3 and 4.4.

Table 4.3: R_f values of uncooked chloroform fractions

Fractions	Solvents	E:M:W:F(50:6.5:5:2)	E:M:Am(9:20:15)
1	100 %H_2O	0.95	0.53
2	80 % H_2O	0.81 0.95	0.53
3	60 % H_2O	0.86	0.38 0.53
4	50 % H_2O	0.84	0.38 0.53
5	40 % H_2O	0.84 0.99	0.35 0.38 0.53
6	20 % H_2O	0.84 0.99	0.34 0.43 0.48
7	0 % H_2O	0.84 0.99	0.50 0.53

Table 4.4: R_f values of uncooked water (H_2O: MeOH)

Number of fractions	**Solvents**	**E:M:W:F(50:6.5:522)**	**E:M:Am(9:20:15)**
8	100 % H_2O	0.98	0.92
9	80 % H_2O	0.10 0.18 0.98	0.12 0.96
10	60 % H_2O	0.97	0.86
11	50 % H_2O	0.27 0.41	 0.63
12	40 % H_2O	0.08	 0.36
13	20 % H_2O	0.13 0.23	 0.39 0.45
14	0 % H_2O	0.13	0.10 0.18

4.6 FTIR analysis of compound MC1

100 % methanol fraction from water extracts showed to have one spot with Rf 0.13, therefore further investigation was conducted and the compound was named MC1. FTIR, NMR and LCMS techniques have been applied in trying to elucidate its molecular structure and identify the compound. The FTIR spectroscopic technique was used for easy and rapid characterisation and identification of various functional groups responsible for the Manayi's medicinal properties. The FTIR technique involves relating the absorption bands to organic functional groups. Since different bands have different vibrational frequencies, the presence of bands can be detected by identifying the characteristic vibrational frequencies as an absorption band in the IR spectrum. Data analysis in table 4.5 shows frequencies, bonds and functional groups identified during analysis.

An unknown compound that is brown when observed under UV was analysed using FTIR technique. The FTIR spectrum in figure 4.10 displayed hydrogen bonded alcohol bands of 3328.1 cm^{-1} and alkenes (1634.87 cm^{-1}), which appeared to be a C=C bend as shown in table 4.5.

Table 4.5: analysis table of FTIR

Frequency in cm^{-1}	**Bond**	**Functional groups**
3328.1	OH	Hydrogen bonded alcohols
1634.87	C=C	Alkenes

4.7 Analysis of compound MC1 using NMR and LCMS

Compound MC1, a brown crystalline substance, was analysed for carbon and proton on NMR and was found to be impure. Further purification could not be done due to a small amount of the available sample. Therefore, further extraction and purification is required in order to be able to elucidate structure for this compound. Carbon-13 and proton NMR spectra for this compound are shown in figure 4.11 and 4.12. Electro-spray Time-of-Flight Mass Spectroscopy (TOF-MS) analysis was done, and it yielded a clear fragmentation pattern. The analysis was done with low and high resonance liquid chromatography mass spectroscopy (HR-LC-MS). The fragmentation spectra of the molecular ion of MC1 in low resonance spectroscopy with fragment numbers and structures are shown in figure 4.13 and that of high resonance spectroscopy are shown in figure 4.14.

Accurate masses and elemental compositions of fragments from MS/MS analysis are shown in table 4.6. The molecular ion peaks are highlighted in bold. For low and high resonance spectra m/z are 281.2459 and 281.2481, respectively. Molecular formulae $C_{18}H_{34}O_2$ with m/z 281.2459 and $C_{16}H_{29}O_2$ with m/z 253.2147 were found to correspond to oleic acid and palmitic acid, respectively (royal society of chemistry, 2016). Oleic acid and palmitic acid are long

hydrocarbon chain carboxylic acids, known as fatty acids. They are usually produced by hydrolysis of common animal and vegetable fats and oils. Oleic acid and palmitic acid are saturated fatty acids of 12-, 14-, 16-, and 18-carbon lengths. Oleic acid is an 18-carbon cis-mono unsaturated fatty acid (Liebert, 1987).

Table 4.6: Fragment numbers, accurate masses, and elemental compositions fragments of MC1 for low resonance mass spectrometer

Fragment	**m/z**	**Formulae**	**Corresponding name of compound**
Molecular ion	281.2459	$C_{18}H_{34}O_2$	Oleic acid
F3	253.2147	$C_{16}H_{29}O_2$	Palmitic acid

Table 4.7: Fragment numbers, accurate masses, and elemental compositions fragments of MC1 for high resonance spectrometer

Fragment	**m/z**	**Formulae**	**Corresponding name of compound**
F7 (Molecular ion)	281.2468 281.2454 281.2454	 $C_{18}H_{33}O_2$ $C_{14}H_{29}N_6$	Oleic acid

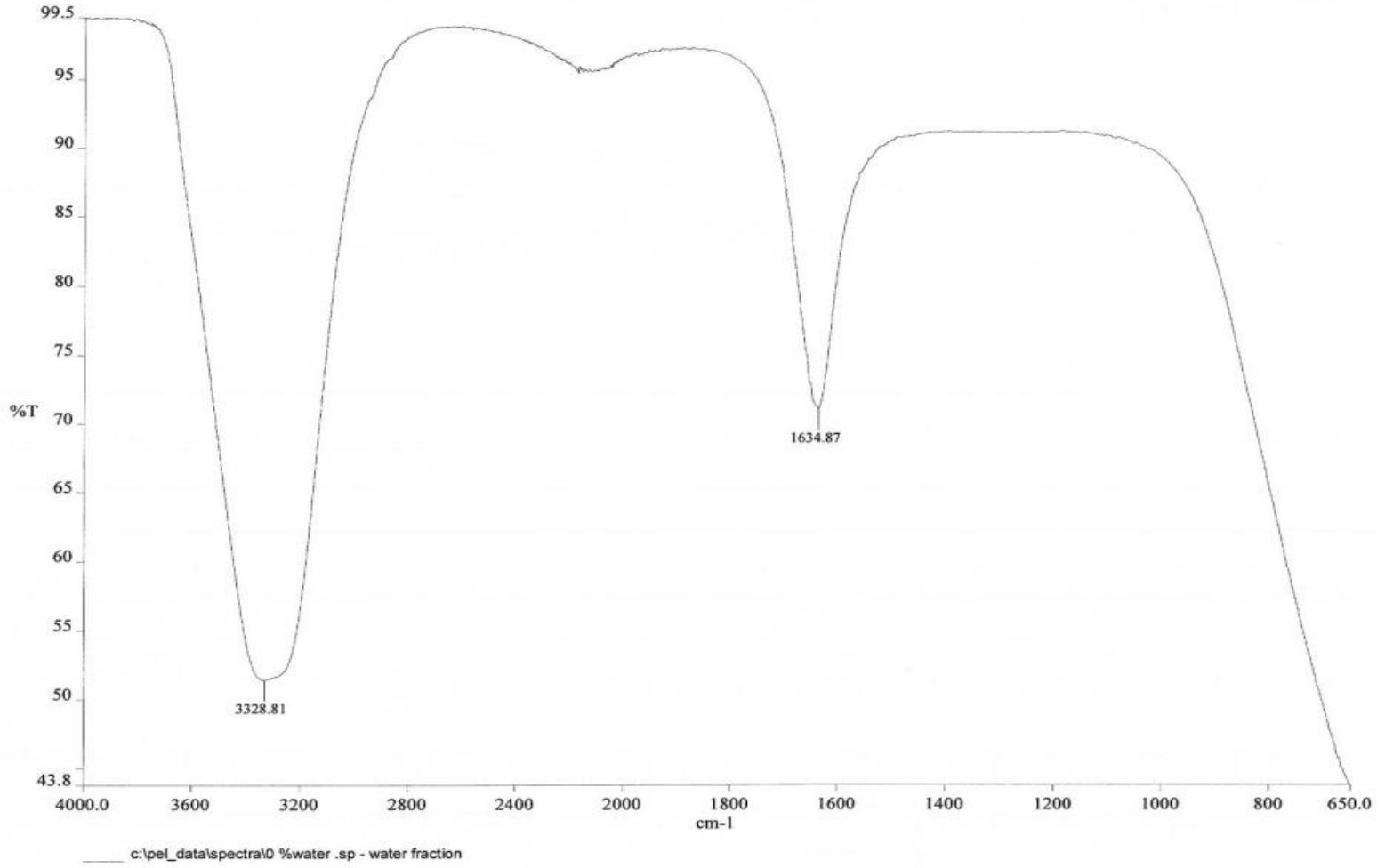


Figure 4.10: FTIR spectra of MC1

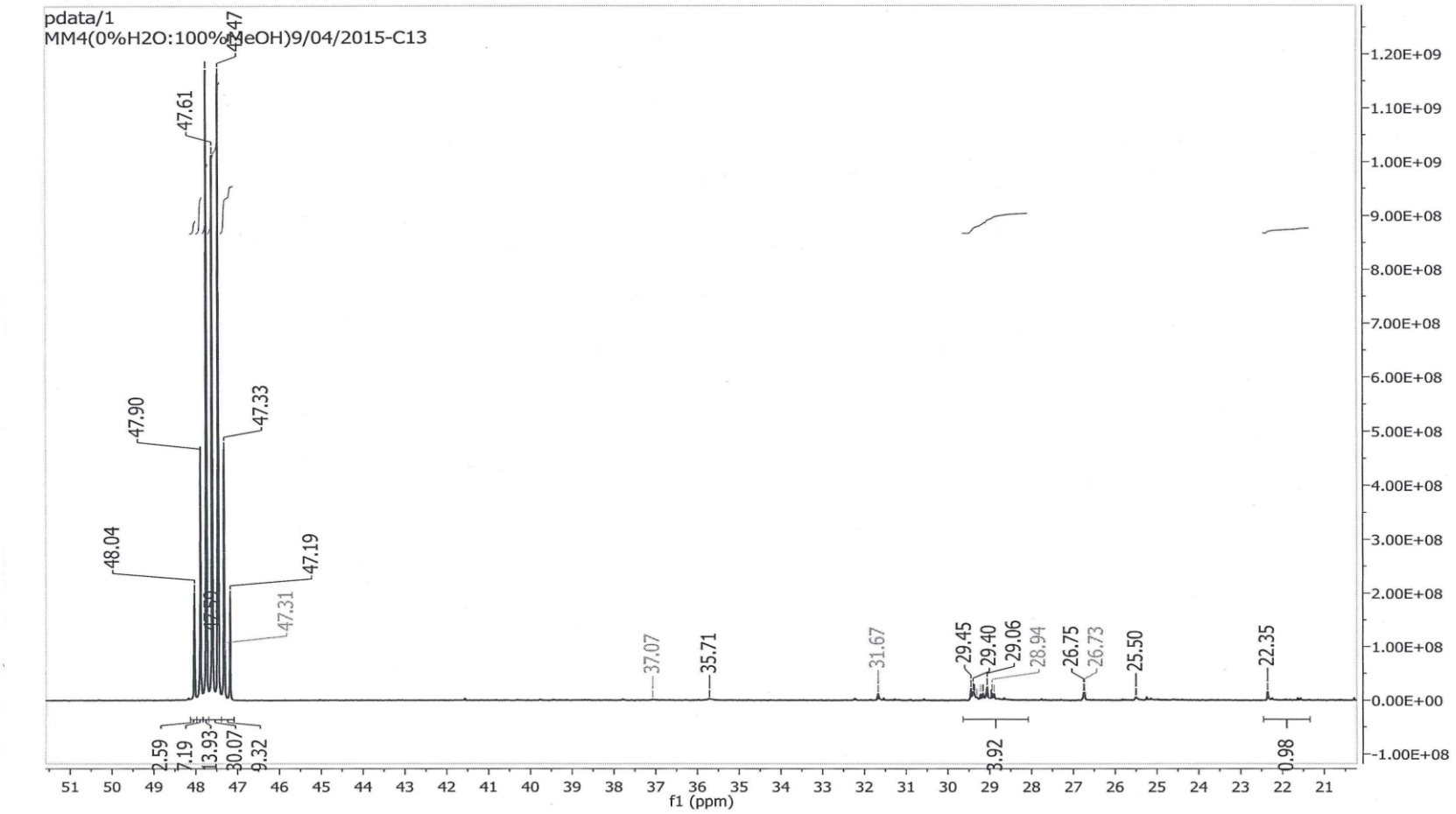


Figure 4.11: proton spectrum of MC1

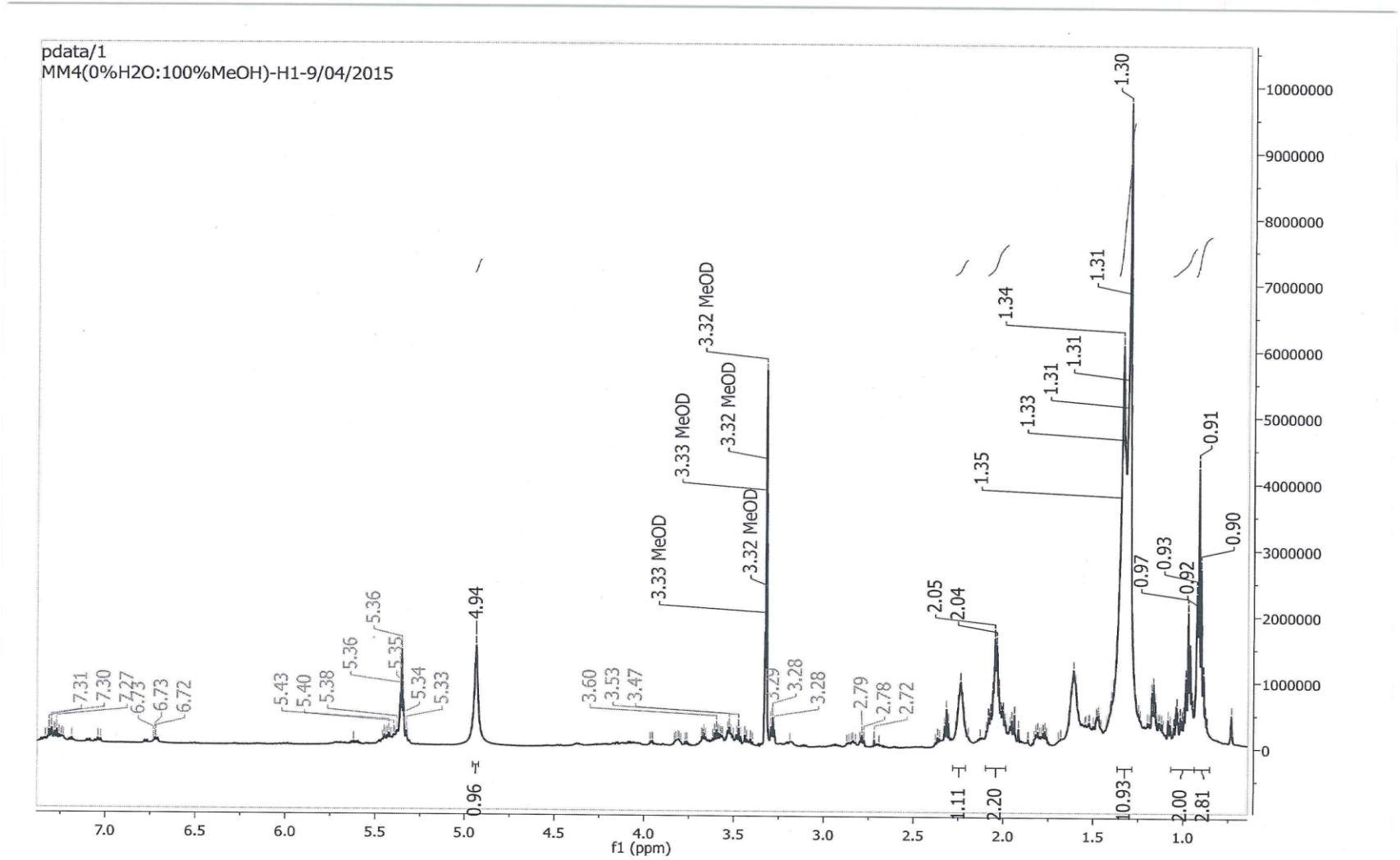


Figure 4.12: Carbon spectrum of MC1

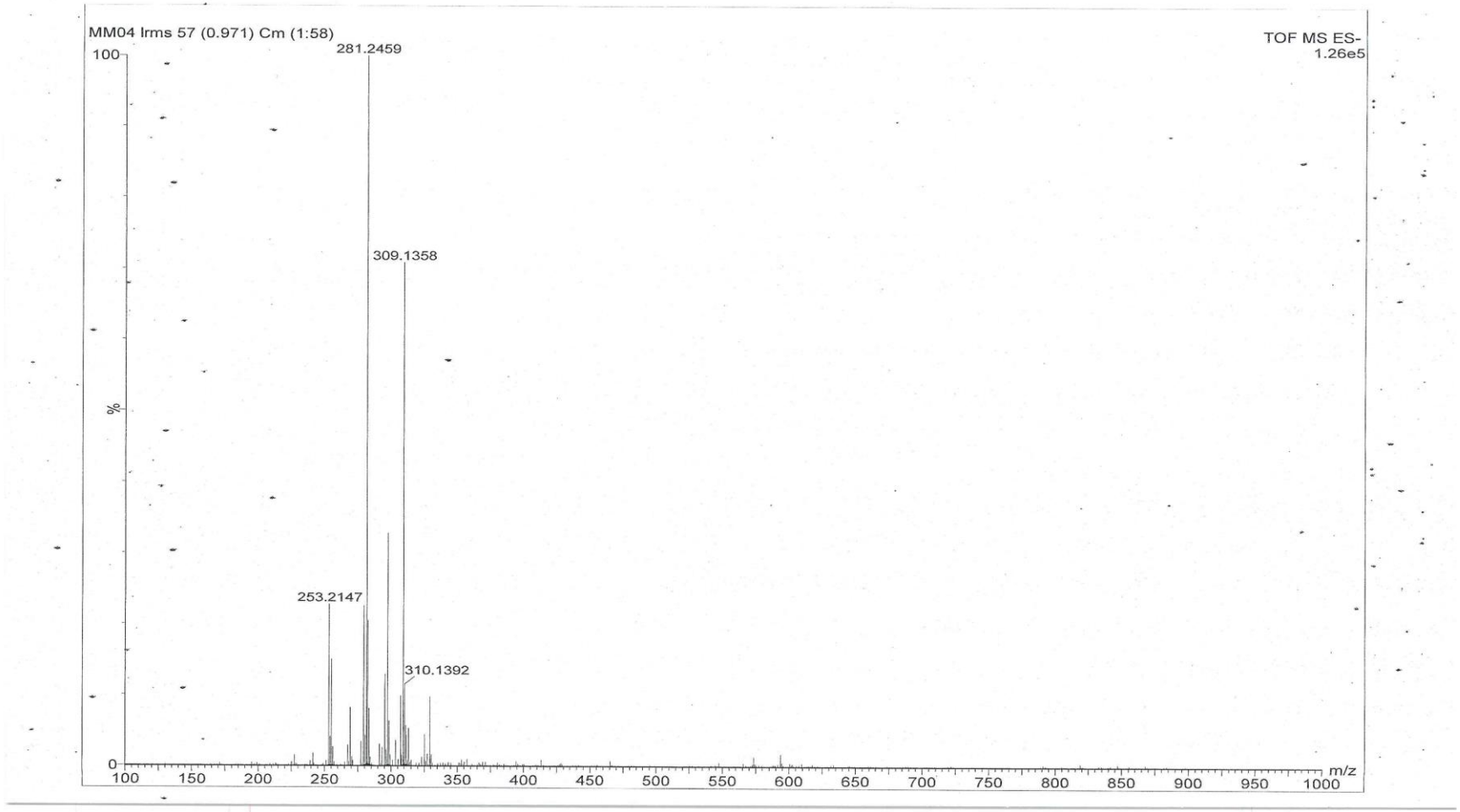


Figure 4.13: spectra of compound MC1 in low resonance mass spectroscopy

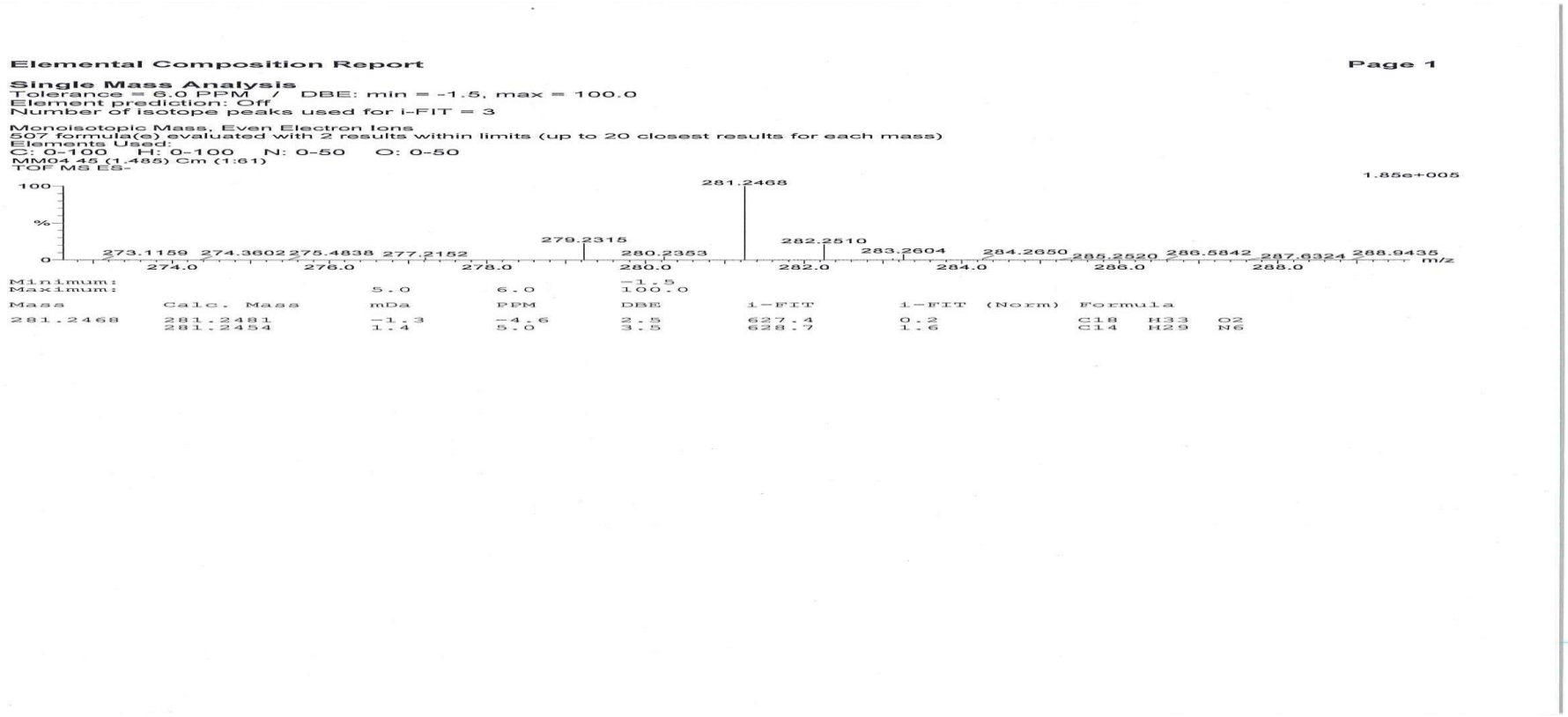


Figure 4.14: spectra of compound MC1 in high resonance mass spectroscopy

CHAPTER FIVE
GENERAL CONCLUSION RECOMMENDATIONS

5.1 Conclusion

The aim of this study was to investigate bioactive compounds in a Manayi mixture used to treat HIV patients. Various solvents were used to extract dried, cooked and uncooked Manayi. The highest extraction yield was obtained from using water in both Manayi species, while extraction with hexane gave the lowest yield. The high yield of water and low yield of hexane extracts might be influenced by the polarities of solvents (Luque de Castro, 1994 & Romdhane, 2002).

This study has shown the presence of certain phytochemical compounds and bioactive compounds against HIV activities from the Manayi extracts. Flavonoids, proteins, glycosides, phenolic and saponins are highly potent bioactive compounds and could perhaps be responsible for most activities shown by the Manayi products. Manayi product is highly active in integrase HIV activity for all the tested dissolving solvent extracts. The cytotoxicity of the tested extracts cell lines in vitro was examined using the MTT assay. Cytotoxicity results reveal that the product is not toxic with all the tested concentrations, therefore it is safe to consume. *In vitro* assays can be used to some extent to predict human toxicity and for the general screening of chemicals for potential toxic effects (Scheers et al, 2001).

NMR results on compound MC1 showed the product to have impurities, therefore more purification is required. LC/MS using low resonance and high resonance spectroscopy reveals that the product contains a mixture of oleic and palmitic acids. It was reported that oleic acid has been associated with the reduction in blood pressure and lower incidence of hypertension (Tera et al., 2008). Palmitic acid displays mild antioxidant and anti-atherosclerotic properties in animal studies when taken in moderation according to a Korean study published in a 2010 edition of the "Journal of Medicinal Food", but it is not as effective as oleic acid.

The results of the present study have a potential in the treatment and management of HIV and may enhance the natural product uses, showing the potential of these plants in the treatment of HIV. The study forms a basis for further phytochemical and pharmacological studies to isolate and characterize the bioactive components necessary for the development of the new drugs that are safe and effective. Further studies are needed to carry out bioassays on fractionated essential oils (palmitic and oleic acids) since the product is a protein, in order to isolate active compounds that can be useful in the management of HIV and AIDS.
Cooked and uncooked Manayi products, with their good integrase activity and relatively low toxicity, have the potential for development as anti-HIV agents.

5.2 Recommendations

From the outcomes of the study, the following recommendations for future work can be made:

- The crude extracts have shown a good integrase activity, relatively high percentage inhibition to all the tested extracts and possess a potential in the development of standardised phytomedicines. Further studies on in-vitro assay and toxicity on fractions are necessary to further confirm the efficacy and safety of the plants.
- More *in vitro* confirmatory tests using other assays and/or *in vivo* tests are still required.
- Further research on purification is also recommended to isolate the plant's active compounds in order to understand their modes of action.

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Appendinces

Appendix A: Data analysis of % yield for cooked and uncooked Manayi extracts

Uncooked Manayi extracts	**Mass of extract**	**% yield**
Hexane	0.9341	0.93
Chloroform	8.0858	8.08
Dichloromethane	2.9280	2.96
Butanol	1.4770	1.48
Methanol	7.2541	7.25
Water	11.6069	11.60
Cooked Manayi extracts		
Hexane	0.0261	0.53
Chloroform	0.3350	0.84
Dichloromethane	0.0122	0.41
Butanol	0.1319	0.33
Methanol	0.0152	0.12
Water	4.9430	4.94

Appendix B: data for percentage yield of the fractions of chloroform extract and water extract

Number of fraction	**Mass of fraction**	**% yield**	**Number of fraction**	**Mass of Fraction**	**% yield of fraction**
Chloroform fraction			Water fraction		
1	0.0092	0.90	8	0.0723	6.58
2	0.0092	0.97	9	0.0713	6.49
3	0.0400	3.91	10	0.0920	8.37
4	0.0500	4.89	11	0.1022	9.30
5	0.0513	5.02	12	0.2101	19.11
6	0.0618	6.04	13	0.2215	20.14
7	0.2073	20.26	14	0.3200	29.10

Appendix C: Data analysis of cooked Manayi and uncooked Manayi against HIV-1 reverse transcriptase

Extracts	**Mass (mg)**	**absorbance**	**Ave abs**	**std**	**Corrective values**					
Blank			0.092	0.098	0.102	0.097				
-control			0.132	0.129	0.139	0.133		0.036		
-control			0.597	0.4		0.499		0.401		
Odimune	2.5 mg	6.25 µg/ml	0.338	0.571	0.424	0.444	0.118	0.347	86.498	**13.502**
Odimune		12.5 µg/m	0.259	0.246	0.215	0.240	0.023	0.143	35.563	**64.437**
Extr 1 uncooked 90:10MeOH:H_2O	2.8 mg	100 µg/ml	2.419	3.449	2.173	2.680	0.677	2.583	643.874	**-543.872**
		50 µg/ml	1.938	2.127	2.113	2.059	0.105	1.962	489.074	**-389.074**
Extr 2 uncooked hexane	3.3 mg	100 µg/ml	1.811	1.698	1.742	1.750	0.057	1.653	412.048	**-312.048**
		50 µg/ml	1.535	2.013	1.758	1.769	0.239	1.671	416.618	**-316.618**
Extr 3 cooked hexane	1.7 mg	100 µg/ml	1.629	2.691	1.534	1.951	0.642	1.854	462.152	**-362.152**
		50 µg/ml	1.097	1.169	0.719	0.995	0.242	0.898	223.764	**-123.764**
Extr 4 uncooked hexane	2.5 mg	100 µg/ml	2.087	2.842	2	2.310	0.463	2.121	551.475	**-451.475**
		50 µg/ml	2.51	2.218	2.904	2.544	0.344	2.447	609.888	**-509.888**
Extr 5 cooked hexane	2.2 mg	100 µg/ml	1.749	1.369	2.137	1.752	0.384	1.654	412.381	**-312.846**
		50 µg/ml	1.547	1.868	1.36	1.592	0.257	1.494	372.497	**-272.497**

Appendix C (continued): Data table analysis of cooked Manayi and uncooked Manayi against HIV1 reverse transcriptase

Extracts	**Mass (mg)**	**absorbance**	**Ave abs**	**std**	**Corrective values**					
Extr 6 uncooked dichloromethane	2.8 mg	100 µg/ml	1.642	1.049	1.489	1.393	0.308	1.296	323.058	**-223.058**
		50 µg/ml	2.552	1.339	1.462	1.784	0.668	1.687	420.523	**-320.523**
Extr 7 cooked dichloromethane-	0.7 mg	100 µg/ml	1.389	1.494	1.285	1.389	0.105	1.292	322.061	**-222.061**
Extr 8 uncooked Butanol	2.7 mg	100 µg/ml	2.172	1.637	1.238	1.682	0.469	1.585	395.098	**-295.098**
		50 µg/ml	2.552	1.339	1.462	1.784	0.668	1.687	420.523	**-320.523**
Extr 9 cooked butanol	1.9 mg	100 µg/ml	2.488	2.714	1.97	2.391	0.381	2.293	571.666	**-471.666**
Extr 10 Uncooked H_2O	2.5 mg	100 µg/ml	1.456	2.023		1.740	0.401	1.642	409.348	**-309.348**
		50 µg/ml	1.551	1.431		1.491	0.085	1.394	347.403	**-247.403**
		50 µg/ml	2.229	1.579	1.722	1.843	0.342	1.746	435.231	**-335.231**
Extr 11 Cooked H_2O	2.5 mg	100 µg/ml	1.857	1.921	1.505	1.761	0.224	1.664	414.707	**-314.707**
		50 µg/ml	2.306	1.937	1.558	1.934	0.374	1.836	457.748	**-357.748**

Appendix C (continued): Data table analysis of cooked Manayi and uncooked Manayi against HIV1 reverse transcriptase

Extracts	**Mass (mg)**	**absorbance**	**Ave abs**	**std**	**Corrective values**					
Extr 12 uncooked 80:20 MeOH:H_2O	2.5 mg	100 µg/ml	1.929	2.216	1.342	1.829	0.445	1.732	431.658	**-331.658**
		50 µg/ml	2.018	1.418	1.672	1.703	0.301	1.605	400.166	**-300.166**
Extr 13 uncooked 60:40MeOH: H_2O	2.5 mg	100 µg/ml	1.768	1.73	1.96	1.819	0.123	1.722	429.248	**-329.248**
		50 µg/ml	1.441	2.078	2.223	1.914	0.416	1.817	452.846	**-352.846**

Appendix D: Data analysis of cooked and uncooked Manayi extracts against HIV-1 intergrase activity

									CV	% Activity	% adjusted SD	Ratio of extr inh/ratio of contr ihn
	Blank		0.121	0.127	0.116	0.121						
	Enzyme		1.335	1.158	1.098	1.0197	1.076	0.123	10.294	100.000		1.000
	Azide		0.456	0.556		0.506	0.385	0.071	13.974	35.761	4.997	0.358
Uncooked Hex	Extract 1	50 µg/ml	0.872	1.509		1.191	1.069	0.450	37.835	99.396	105.161	0.994
		100 µg/ml	1.424	1.073		1.249	1.127	0.248	19.879	104.788	20.958	1.048
Cooked Hex	Extract 2	50 µg/ml	1.033	0.967		1.000	0.879	0.047	4.667	81.686	3.638	0.817
		100 µg/ml	0.201	0.752		0.477	0.355	0.390	81.766	33.018	33.051	0.330
UncookedChlro	Extract 3	50 µg/ml	1.194	1.062		1.128	1.007	0.093	8.275	93.585	23.453	0.936
		100 µg/ml	0.767	0.771		0.769	0.648	0.003	0.368	60.211	0.237	0.602
Cookedchloro	Extract 4	50 µg/ml	0.801	0.862		0.832	0.710	0.043	5.187	66.021	5.688	0.660
		100 µg/ml	0.593	0.405		0.499	0.378	0.133	26.640	35.110	14.167	0.351
UncookedDCM	Extract 5	50 µg/ml	0.866	1.072		0.969	0.848	0.146	15.032	78.804	33.740	0.788
		100 µg/ml	0.994	1.003		0.999	0.877	0.006	0.637	81.546	0.660	0.815
UncookedBUT	Extract 7	50µg/ml	0.59	0.643		0.617	0.495	0.037	6.079	46.033	6.147	0.460

Appendix D (continued): Data analysis of cooked and uncooked Manayi extracts against HIV-1 integrase activity.

				cv	**% activity**	**% adjusted SD**	**Ratio of extract inh/ratio of control inh**
UncookedBut		100 µg/ml	0.775	0.791		0.783	0.662
CookedButanol	Extract 8	50 µg/ml	0.599	0.706		0.653	0.531
		100 µg/ml	0.653	0.475		0.564	0.443
UncookedWater	Extract 9	50 µg/ml	0.971	0.773		0.872	0.751
		100 µg/ml	0.624	0.664		0.644	0.523
CookedWater	Extract 10	50 µg/ml	0.748	0.919		0.834	0.712
		100 µg/ml	0.654	0.511		0.583	0.461

Appendix E: Data analysis of cytotoxicity in uncooked Manayi against Vero cells

	Control	Uncooked HEXU1 6.25	U1 12.5	U1 25	U1 50	UncooedChloroU3 6.25	U3 12.5	U3 25	U3 50	UncookedDCM U5 6.25	U5 12.5
	0.112	0.105	0.101	0.093	0.087	0.095	0.104	0.093	0.104	0.111	
	0.115	0.118	0.126	0.102	0.093	0.093	0.1	0.098	0.116	0.117	0.109
	0.114	0.127	0.093	0.099	0.098	0.093	0.095		0.092	0.1	0.111
Average	0.114	0.117	0.107	0.098	0.093	0.094	0.100	0.096	0.104	0.109	0.110
%Variable		102.639	93.842	86.217	81.525	82.405	87.683	84.018	91.496	96.188	96.774
% inhibi		-2.639	6.158	13.783	18.475	17.595	12.317	15.982	8.504	3.812	3.226
STDEV		0.011	0.017	0.005	0.006	0.001	0.005	0.004	0.01	0.009	0.001

Appendix E (continued): Data analysis of cytotoxicity in uncooked Manayi against Vero cells

Uncooked Manayi	Control	5 25	U5 50	UncookedButanolU7 6.25	U7 12.5	U7 25	U7 50	UncookedH2OU9 6.25	U9 12.5	U9 25	U9 50
	0.112	0.112	0.12	0.1	0.117	0.106	0.101	0.128	0.102	0.113	0.116
	0.115	0.102	0.115	0.102	0.115	0.114	0.116	0.106	0.111	0.125	0.116
	0.114	0.107	0.108	0.135	0.12	0.101	0.104	0.109	0.109	0.111	0.11
Average	0.114	0.107	0.114	0.112	0.117	0.107	0.107	0.114	0.107	0.116	0.114
%Variable		94.135	100.587	98.827	103.226	94.135	94.135	100.587	94.428	102.346	100.293
%Inhibition		5.865	-0.578	1.173	-3.226	5.865	5.865	-0.587	5.572	-2.346	-0.293
STDEV		0.005	0.006	0.020	0.003	0.007	0.008	0.012	0.005	0.008	0.003

Appendix F: Data analysis of cytotoxicity cooked Manayi against Vero cells

	Control	CookedHexC2 6.25	C2 12.5	C2 25	C2 50l	CookedChlroC4 6.25	C4 12.5	C41 25	C4 50	CookedDCMC6.25	C6 12.5	C6 25
	0.108	0.101	0.1			0.118		0.1	0.098	0.108	0.083	0.108
	0.108	0.098	0.109	0.107	0.094	0.111	0.115	0.114	0.092	0.096	0.098	0.124
	0.107	0.099	0.107	0.101	0.087	0.111	0.122	0.126	0.109	0.107	0.095	0.102
Average	0.108	0.099	0.105	0.104	0.091	0.113	0.119	0.113	0.100	0.104	0.092	0.111
%Variable		92.835	98.442	97.196	84.579	105.919	110.748	105.919	93.146	96.885	85.981	104.050
%inhibitio		7.165	1.558	2.804	15.421	-5.919	-10.748	-5.919	6.854	3.115	14.019	-4.050
STDEV		0.002	0.005	0.004	0.005	0.004	0.005	0.013	0.009	0.007	0.008	0.011

Appendix F (continued): Data analysis of cytotoxicity in cooked Manayi against Vero cells

	C6 50	CookedButanolC8 6.25	C8 12.5	C8 25	C8 50	CookedH2OC10 6.25µg/ml	C10 12.5 µg/ml	C10 25µg/ml	C10 50µg/ml
	0.1	0.099	0.117	0.121	0.111	0.094	0.085		0.059
	0.112	0.133	0.111	0.112	0.095	0.108	0.084	0.08	0.078
	0.111	0.1	0.115	0.122	0.117	0.089	0.096	0.072	0.072
average	0.108	0.111	0.114	0.118	0.108	0.097	0.088	0.076	0.070
%variable	100.623	103.427	106.854	110.592	100.623	90.654	82.555	71.028	65.109
%inhibition	-0.623	-3.427	-6.854	-10.592	-0.623	9.346	17.445	28.972	34.891
STDEV	0.007	0.019	0.003	0.006	0.011				