ISOLATION AND CHARACTERISATION OF THE ACTIVE PHYTO-PHARMACEUTICAL INGREDIENT FROM *LOBOSTEMON TRIGONUS* FOR USE IN DEVELOPMENT OF A MICROBICIDE

By

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DECLARATION

I, Phindiwe Felicia Mbobela, hereby declare that Isolation and characterisation of the active phyto pharmaceutical ingredient from *Lobostemon trigonus* for the use in development of a microbicide is my own work and that all sources that I have used or quoted have been indicated and acknowledged by means of complete references.

…… Date…....

(Candidate)

Supervisor Date…….

Co-supervisor 1 Date…….

Co-supervisor 2 Date…….

DEDICATION

To my awesome Family

“My people are destroyed for lack of knowledge”

Hosea 4:6

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To my heavenly Father, thank you for your grace. I would not have gone this far if it was not for you.

ABSTRACT

The HIV-1 pandemic affects millions of people worldwide with approximately 70% of those affected residing in sub-Saharan Africa (SSA) relying on traditional medicines for treatment. The key aim of the study was to isolate and characterise an active phyto-pharmaceutical ingredient (API) from *L. trigonus* for use as a vaginal microbicide.

The aerial parts of *L. trigonus* were oven-dried at 80°C, ground and then extracted with boiling water for 30 minutes. Aqueous extracts were screened using an HIV-1 neutralization assay in TZM bl cells. Chromatographic and spectroscopic techniques were used to purify, isolate and identify the API.

The API (BP36-117-26464C) was identified as a polymeric macromolecule with IC_{50} = 0.04 µg/ml against HIV-1 HXB 2 subtype B. This activity is comparable to the ARV drug, enfuvirtide (IC_{50} = 0.02 µg/ml). The API consists of galacturonic acid polymer and a mixture of seven compounds. Its mode of action may involve inhibiting virus attachment.

The activity of this precipitate (BP36-117-26464C) tested against HIV-1 subtype C pseudovirions and shown to compare favorably with that of enfuvirtide (T20). The water-soluble nature of this API and its mode of action identified it as a potential microbicide. In the current form, the precipitate (API) would be difficult to develop as an oral treatment for HIV, as high-molecular weight agents often have poor bioavailability following oral administration. However, large molecules with potent anti-HIV activity are ideal for topical use and potent development as a microbicide.

KEY TERMS

Lobostemon trigonus, traditional uses*,* anti-HIV, vaginal microbicide, formulations, HIV-1 neutralisation assay, phytochemistry, nuclear magnetic resonance spectroscopy, bornesitol, rosmarinic acid.

ABBREVIATIONS

°C	Degrees Celsius
µg	Micrograms
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance Spectroscopy
^{13}C	Carbon 13
^{1}H NMR	Proton Nuclear Magnetic Resonance
^{1}H	Proton
2D	Two dimensional
Ac_2O	Acetic anhydride
AcOH	Acetic acid
AIDS	Acquired Immunodeficiency Syndrome
API	Active phyto-pharmaceutical ingredient
ART	Antiretroviral therapy
ARV	Antiretroviral
AZT	Zidovudine
BP36	Bio prospecting lead 36
BuOH	Butanol
CAPRISA	Centre for the AIDS Programme of Research in South Africa
CC	Column Chromatography
CC_{50}	50% Cytotoxicity Concentration

CCR_5	Chemokine co-receptor number 5
CD_4	Cluster domain 4
COSY	Correlation Spectroscopy)
CPE	Cyto Pathic Effect
CSIR	Council for Scientific and Industrial Research
CXCR4	Fusin (lester) co-receptor
d	doublet
dd	doublet of doublet
DEAE	Diethylaminoethanol
DEPT	Distortionless Enhancement by Polarisation Transfer
dH_2O	Distilled water
H_2O	Water
ELSD	Evaporative Light Scattering Detector
ESI TOF-MS	Electrospray ionization-time of flight-mass spectrometry
ESI-MS	Electrospray Ionization Mass Spectroscopy
EtOAc	Ethyl acetate
EtOH	Ethanol
GC- MS	Gas Chromatography-Mass spectrometer
GC	Gas Chromatography
GM	Ground aerial plant parts
HAART	Highly active antiretroviral therapy
Hex	Hexane

HIV	Human Immunodeficiency Virus
HIV-1 HXB 2	HIV-1 subtype B strain
HIV-1	Human Immunodeficiency- type 1
HIV-LTR	Human Immunodeficiency-Long terminal repeat
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HPV	Human papillomavirus
HSQC	Heteronuclear single-quantum correlation
HSV-1	Herpes Simplex Virus (HSV) – type 1
i.e.	That is
IC_{50}	Concentration at which there is 50% inhibition
IEC	Ion Exchange Chromatography
IPM 009	Dapivirine gel efficacy trial
L	Litre
L/L	Liquid-Liquid Chromatography
MeOH	Methanol
$MgSO_4$	Magnesium Sulphate
ML	Milli-litre
MS	Mass Spectrometer
MSM	Material Science and Manufacturing
MTS	Tetrazolium compound
MW	Molecular Weight

NMR	Nuclear Magnetic Resonance
NVP	Nevirapine
PBMC’s	Peripheral Blood Mononuclear Cells
PVPP	Polyvinylpolypyrrolidone
PY	Pyridine
RA	Rosmarinic acid
RT	Reverse Transcriptase
SA	South Africa
SEC	Size Exclusion Chromatography
STD’s	Sexual Transmitted Diseases
STI’s	Sexual Transmitted Infections
SSA	Sub-Saharan Africa
T20	Enfuvirtide
TB	Tuberculosis
TH	Traditional Healer
TLC	Thin Layer Chromatography
TMCS	Trimethylchlorosilane
TZM b1	HIV-1 reporter cell line developed from HeLa cells
UL	Unprocessed leaves
UPLC-MS	Ultra Performance Liquid Chromatography Mass Spectrometry
US	United States

US	Un-processed stem
UV	Ultra violet
VOICE	Vaginal and Oral Interventions to Control the Epidemic
WHO	World Health Organisation
Wits	University of Witwatersrand
δC	^{13}C chemical shift
δH	^{1}H chemical shift

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CHAPTER 1: INTRODUCTION

1.HIV/AIDS and anti-HIV agents from plants

1.1 HIV/AIDS: The Global Status

Human immunodeficiency virus (HIV) is the pathogen that causes Acquired Immune Deficiency Syndrome (AIDS), a complex array of disorders resulting from the weakening of the immune system. Infected individuals become vulnerable to opportunistic pathogens such as common microbes and often suffer from tuberculosis (TB), pneumonia and rare forms of cancer (Tshikalange 2008) . HIV uses macrophages and T-cells as target cells to produce multiple progeny virions ready to infect new viral hosts. During each round of infection more cells of the immune system are damaged or killed. The host produces antibodies and helper T-cells in order to fight the virus but eventually the virus prevails and opportunistic diseases associated with AIDS appear (Tshikalange 2008).

By 2011, 34 million people were infected with HIV. These included an estimated 0.8% of adults aged 15-49 years. The burden of the epidemic varies considerably between countries and regions and sub-Saharan Africa (SSA) remains most severely affected, with nearly 1 in every 20 adults (4.9%) living with HIV. This region accounts for 69% of the people living with HIV worldwide and shows an infection prevalence 25 times greater than in Asia (UNAIDS 2012).

1.1.1 Causes of high rate of HIV infection in South Africa

In South Africa, HIV is transmitted predominantly between heterosexual couples, with mother-to-child transmission being the other main infection route. Drivers of the epidemic in South Africa are intergenerational sex, multiple concurrent partners, low condom use, excessive use of alcohol and low rate of male circumcision. In relation to intergenerational sex, research has identified younger females having sex with older males as an important factor contributing to the spread of HIV. It has been

noted that such relationships are usually motivated by subsistence needs as well as being linked to materialism (Garcia-calleja & Barrerre 2010).

Shisana and colleagues found a higher HIV prevalence among teenage males and females who reported having sexual partners who are five or more years older than themselves. Owing to unequal power dynamics in such relationships, vulnerability may be exacerbated for young girls who do not have the skills and power to negotiate condom use (Global AIDS response progress report 2012).

1.1.2 The Need for Female Controlled Methods

Biologically, women are 2-4 times more likely than men to be infected with HIV resulting from unprotected sex. Likewise, from an economics viewpoint, women tend to show an economic need or dependency on men and are less able to assert their rights. This is reflected in social and cultural societal features reflecting gender norms about sexuality and gender-based violence. In essence, current methods of sexual and birth control (abstinence, fidelity, and condom use) often requires male consent, participation and cooperation (Wanyoike 2011).

The rapid increase of HIV infection among women in the world has been attributed primarily to sexual contact with HIV-infected men. While a range of contraceptives protect against unintended pregnancies, only condoms, for both males and females, provide dual protection by stopping HIV transmission and preventing unintended pregnancies. The lack of methods of HIV prevention that are controlled by women and girls, along with low levels of condom use, place women and girls at increased vulnerability to HIV infection (UNAIDS & WHO 2012).

Women need safe contraceptive and HIV prevention options that they can own and manage. New investments into research for female-controlled technologies that allow women to prevent both HIV and pregnancy are still essential.

1.2 HIV prevention Strategy: Microbicides as an alternative prevention method for women in the fight against HIV/AIDS

Although a variety of inhibitors that target different steps in HIV-1 entry have been developed, only maraviroc, a small-molecule CCR5 antagonist, and enfuvirtide, an oligo-peptide fusion inhibitor, are approved for clinical use. Several other drugs are currently active in the development pipeline, and entry inhibitors may play an important role in preventing acquisition of HIV-1 infection (Henrich & Kuritzkes 2013).

1.2.1 What are microbicides?

Microbicides are compounds that can be applied inside the vagina or rectum to protect against sexually transmitted infections (STIs), including HIV. They can be formulated as gels, creams, rings, or suppositories (see Fig 1). Microbicides may or may not have spermicidal activity.

To be truly female controlled, the ideal microbicide should be effective, safe, acceptable, affordable, colourless, odourless, stable, easy to store and use, available in formulations without any prescription. Microbicides offer many potential advantages for increasing a woman's control over her sexual life and for protecting women, men, and children from infection (Attawell 2001).

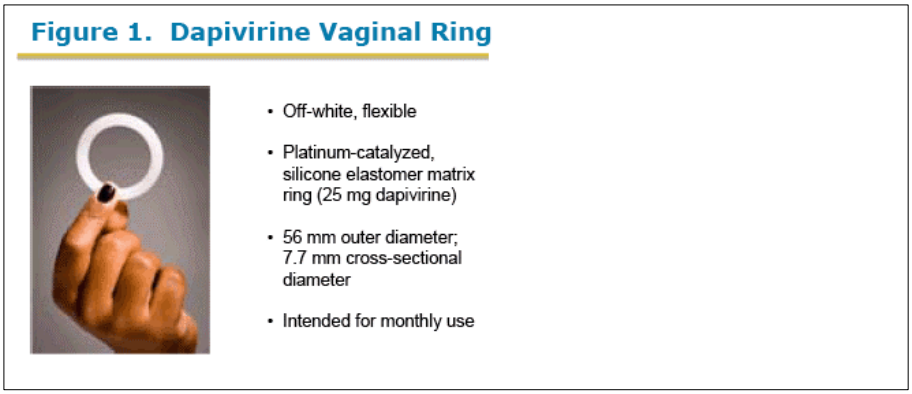


Figure 1: An example of a vaginal microbicide (http://www.natap.org/2012/CROI/croi_160.htm)

1.2.2 How does a microbicide work?

Various STD's including HIV pathogens can attack the body in multiple ways and an effective microbicide will help prevent infection by stopping this attack at one or more stages in the process (see Fig 2). The microbicides currently under development act in one or more of the following ways:

- Killing or inactivating pathogens. Some microbicides work by breaking down the surface or envelope of the virus or pathogen.
- Creating physical barriers. Microbicides could provide a physical barrier between pathogens and vulnerable cells in the epithelium (cell wall) of the vagina or rectum (Attawell 2001).
- Strengthening the body's normal defenses. The body has several naturally occurring defense mechanisms that a microbicide may be able to supplement or enhance. Lactobacilli, for example, are naturally occurring, "good" bacteria that helps protect the vagina by maintaining its acidic environment. This natural acidity helps foster an inhospitable environment for many pathogens, including HIV. Thus, the idea of developing a microbicide that supports the lactobacilli in performing this function is one potential mechanism of action being explored (Attawell 2001).
- Inhibiting viral entry. Some microbicides bind to viruses and bacteria to prevent them from binding to and infecting healthy cells.
- Inhibiting viral replication. Some candidate microbicides are being developed from the antiretroviral drugs that HIV-positive people use to lower the amount of virus in their bodies. Formulated as gels or creams, these drugs may be able to suppress replication of any HIV that enters the vagina or rectum during sex. If so, they could substantially lower the odds that the microbicide user will become infected or re-infected, if already HIV positive (Attawell 2001).

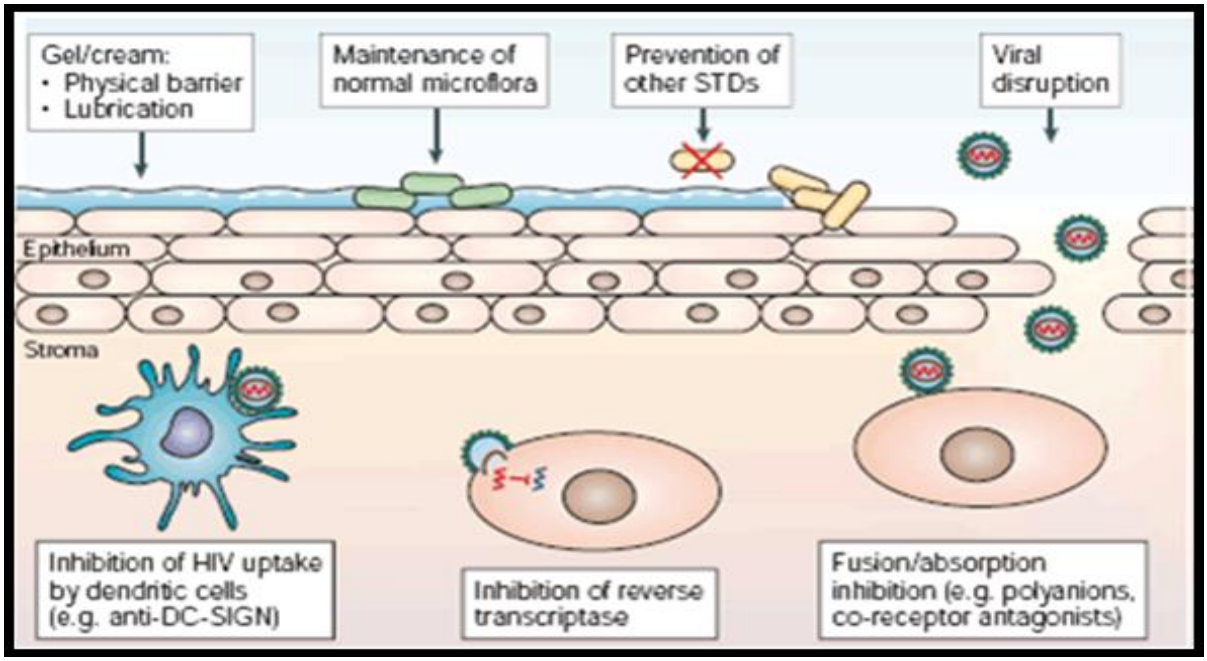


Figure 2: Various potential modes of microbicide action that may be effective against viral infection (Shattock & Rosenberg 2012)

1.2.3 Challenges in developing a microbicide

Ideally, a microbicide should not irritate tissue, it should quickly spread - this is especially important with tissue vulnerable to HIV infection - and it should remain *in situ* while walking and prior to sexual intercourse. A microbicide should be able to cross protective barriers such as membranes of the vagina or rectum and penetrate the surrounding tissue and nearby lymph nodes (Pebody 2011).

Ideally, it should not enter the blood stream where its effect can be diluted and possibly lead to side effects. As an example of this, if an ARV-based microbicide was used by a person with undiagnosed HIV infection, the possibility exists that systemically distributed microbicide could lead to the development of drug-resistant HIV and limit future treatment options. An ideal ARV-based microbicide, then, should consist of a drug which is not already used for HIV treatment and with limited absorption into the bloodstream.

This emphasises the advantage of a topical microbicide over oral pre-exposure prophylaxis. To increase compliant use, a feminine-oriented microbicide should be inexpensive, require no prescription, could be applied each day rather than when sex

is anticipated and could be dual-acting to simultaneously protect against pregnancy and HIV or single-acting to protect against HIV infection (Pebody 2011).

1.2.4 Vaginal Microbicide studies

In July 2010, the results of the CAPRISA 004 study were released. This blinded South African trial involved 889 sexually active women who were randomly selected to receive a microbicide gel containing 1% tenofovir (a nucleotide reverse transcriptase inhibitor) or a gel containing a placebo. The participants were at high risk of HIV infection, as indicated by the 9.1% annual incidence in HIV infection within the placebo group, while women in the test group showed a lower annual incidence of 5.6%. In other words, the study group showed a reduction of HIV incidence of 39% when compared to the placebo group.

Results were shown to be better in women who used the microbicide consistently. The study showed that women who used the microbicide on at least 80% of the days they had sex showed a risk of infection that was 54% lower than the placebo group. However, the fact that overall compliance was poor highlights the possibility that consistent microbicide use may be as challenging as consistent condom use (Karim *et al*; 2010).

Other South African studies include the, Vaginal and Oral Interventions to Control the Epidemic (VOICE) study which compared HIV infection rates in women using a tenofovir microbicide against a placebo and an efficacy trial, IPM 009 that will test the effectiveness of dapivirine (a non-nucleoside reverse transcriptase inhibitor), either as a gel or as a vaginal ring. In addition, other preliminary studies (for example, in animals or to test safety in humans) are underway. At least 13 different ARV-based microbicide studies involving primates demonstrated efficacy in blocking HIV infection. Some of these ARVs are also used for HIV treatment, while others such as dapivirine show anti-HIV activity but are not licensed for HIV treatment (Pebody 2011).

1.3 HIV treatment

Inhibiting the replication cycle stages has become an important part of treating and controlling HIV, although to date more inhibitors are still to be developed. Treating people with infected with HIV lowers the number of virions in their body and can dramatically reduce their risk of transmitting HIV to others, underscoring the importance of HIV testing and access to medical care and treatment (Centers for disease control and prevention (CDC) 2013). In fact, a landmark clinical trial in 2011 showed that people infected with HIV who began taking anti-HIV medications early, before their immune systems were significantly weakened, experienced a 96% reduction in their risk of transmitting HIV to their sexual partners (Centers for disease control and prevention (CDC) 2013).

Antiretroviral therapies have brought renewed hope for many people living with HIV. However, they do not offer a cure, and they can cause many side effects. HIV is treated with antiretrovirals (ARVs), which work against the HIV infection by slowing down the spread of the virus in the body (WHO *et al*; 2013). Patients tend to take three or more types of ARV medication. This is known as combination therapy or antiretroviral therapy (ART). Combining ARVs actually helps to reduce drug resistance as HIV can quickly adapt and become resistant to a single ARV. This means that the most common treatment for people just diagnosed with HIV involve taking just one or two pills a day. Different combinations of ARVs work for different people so the medicine they take is individual to them (WHO, UNICEF 2013).

Recent studies confirm that gains in life expectancy among people living with HIV receiving ART in low and middle-income countries are as remarkable as those previously documented in the high-income countries of Europe and North America**.** In South Africa, for example, data from six HIV treatment programmes in three provinces show that adults starting ART have a life expectancy of about 80% of normal if they start treatment before their CD_4 count drops below 200 cells/mm3ml (WHO, UNICEF 2013).

Researchers from Beth Israel Deaconess Medical Centre; 2013 have identified a potentially powerful new treatment for HIV infection that makes use of recently discovered antibodies to disable the virus, a finding that promises to energize research in both prevention and treatment. It was found that administering a round of

potent human antibodies to monkeys infected with a hybrid version of HIV caused the amount of virus in their bodies to reduce to low or undetectable levels that were sustained for weeks. The virus dropped to undetectable levels within three to seven days after an intravenous infusion of a single antibody in one of the studies (Beth Israel Deaconess Medical Center (BIDMC) 2013). However there is still a need for a search for a drug that will permanently eradicate this HIV virus.

1.4 HIV drug resistance problem - the need for prevention rather than a cure

Therapy designed to impact on HIV infection has come a long way. However, many challenges still remain such as drug resistance which is a widespread problem that limits the efficacy of antiretroviral treatment. As indicated above, for antiretroviral treatment to be effective in the long term, it has been found that combination therapy should be administered (Dambuza 2007).

In more developed countries, most HIV-1-infected patients are surviving longer with an improved quality of life where the progression to AIDS has been reduced as a result of this combined antiretroviral therapy. The term highly active antiretroviral therapy (HAART) is used to describe a combination of three or more anti-HIV drugs which include either a protease inhibitor or a non-nucleoside analogue reverse transcriptase inhibitor and two nucleoside analogue reverse transcriptase inhibitors. This combination can lead to prolonged virus suppression and immunological reconstitution (Dambuza 2007).

Despite the beneficial effects of HAART, there are serious side effects of social, economic and clinical importance. First of all, according to the research done by Dambuza (2007), most infected persons live in countries where treatment is not available or is unaffordable. Secondly, the efficacy of HAART has resulted in a relaxation of appropriate health measures that threaten an outbreak of epidemic infection, especially in western countries.

Lastly, antiretroviral drugs have several side effects, with different incidences and severities, including myopathy, migraine, dementia, ataxia, stroke-like episodes, hypertrophic cardiomyopathy, gastrointestinal complaints, increased bilirubin serum levels, nephrolitiasis, perioral paresthesias, diabetes mellitus, hypoparathyroidism,

hypogonadism, infertility, dysphagia, nausea, vomiting, intestinal pseudo obstruction, anemia, pancytopenia, pancreatitis, depression, amongst others (Dambuza 2007).

With speedy development into understanding the structure and function of HIV, it is important that the growing knowledge from laboratory research be quickly directed into not only developing more drugs which may stop the replication of HIV but also into microbicides which may prevent infection altogether.

1.5 Traditional and herbal medicine - a source of anti-HIV agents

Plants have not only provided mankind with food, clothing, flavours and fragrances, but have also served humanity to treat different ailments. Plants are always surrounded by an enormous number of potential enemies such as bacteria, viruses, fungi etc. By nature plants cannot avoid these enemies simply by moving away, they protect themselves through chemical defence systems (Lewu & Afolayan 2009).

Therefore, it is reasonable to expect biologically active compounds to be produced by plants as a chemical defence measure against their enemies. The search for biological active agents from plants is part of a wider recurrence of scientific interest to produce new chemotherapeutics. Plants synthesize very complex molecules with specific stereochemistry and can show biological activity with novel modes of action (Tshikalange 2008).

Many useful drugs have been developed from medicinal plants used in traditional medicine in the treatment of a variety of illnesses and their use as a therapeutic modality continues to be effective (Gilani & Atta-ur-Rahman 2005). Most of the clinical drugs that are currently in use were derived from plants and developed because of their use in traditional medicine. Asperin (antipyretic), atropine, digoxin, morphine (pain killer), quinine, respine (hypertension) and tubocurarine are a few examples of drugs, which were discovered through the study of ethnobotany (Magalhães *et al;* 2010*)*.

Traditionally, herbs and herbal products have been considered to be nontoxic and have been used by the general public and traditional medicinal practitioners worldwide to treat a range of illnesses (Barnes *et al*. 2007). However, there is

relatively little published scientific research about the safety and efficacy of these herbal treatments. It is, therefore, important that these medicines are properly researched so that the safe and effective ones can be identified and used, and the ones that can harm people are withdrawn (Barnes *et al*. 2007).

The World Health Organisation (WHO) estimated that around 80% of the population in Africa uses traditional medicines. In sub-Saharan Africa there is one traditional healer for every 500 people, whereas there is only one medical doctor for every 40 000 people. It has been estimated that 70% of the South African population consult traditional healers and that the entire industry is worth well over R250 million (Masupha *et al;* 2013)

Many people use traditional medicines to treat the symptoms of HIV and AIDS, and lots of claims have been made that some of these medicines can even cure HIV disease. Without scientific validation and proper clinical evaluation, these claims are considered as false, as no treatments, including ARVs, have been shown to cure HIV and AIDS (Vermani & Garg 2002).

Some herbal treatments may help by attacking the virus directly or by stimulating a person's immune system which then attacks the infected cells. Other studies have shown that in Europe herbal treatments are considered to be the most popular complementary medicine used by HIV-infected individuals. A substantial amount of research has been done and a lot more is in progress to isolate the active leads from plants for prevention of transmission of HIV and treatment of AIDS. These active principles may act by different mechanisms, targeting critical steps within the replication cycle of HIV (Vermani & Garg 2002).

Several plants have been screened for activity against STD's on the basis of ethnopharmacological data and some of these screening programs have yielded potential leads. Since then, medicinal plants have been used for the treatment of STDs and AIDS with little scientific evidence in traditional systems of medicine. In the last century enormous efforts were made to select the plants, isolate the active principles and screen the crude extract/fractions/compounds for activity against various sexually transmitted pathogens, and to elucidate their mechanism of action (Vermani & Garg 2002).

Studies have revealed that because of structural diversity of the natural products, they provide a large reservoir of compounds for screening of anti-HIV agents. Many compounds with anti-HIV-1 activity have been isolated from natural products and shown to inhibit HIV at nearly all stages of the viral life cycle (Moshi 2005). These compounds include alkaloids, sulphated polysaccharides, polyphenolics, flavonoids, coumarines, phenolics, tannins, triterpenes, lectins, phloroglucinols, lactones, iridoids, depsidones, O-caffeoyl derivatives, lignans, ribosome inactivating proteins, saponins, xanthones, naphthodianthrones, photosensitisers, phosholipids, quinines and peptides (Moshi 2005).

A local study also revealed that two South African plants, *Lobostemon trigonus* and S*utherlandia frutescens* have inhibitory activity against HIV-1 reverse transcriptase activity. Specifically the aqueous extract of *Lobostemon* leaves inhibited HIV-1 RT with an IC_{50} value of 49 µg/ml (Harnett *et al*; 2004).

1.6 Value of the study

People often claim that their traditional medicinal plant remedies could improve the quality of life of an HIV/AIDS patient. However, the efficacy of these remedies or plants has generally not been proved (Tshikalange 2008). South Africa is a country with a rich source of unexploited phytochemicals and it has an under-established culture of traditional medicine that could provide support for many HIV/AIDS victims. These drugs need to be studied to confirm and verify if they are effective as anti-HIV agents. In addition to contributing towards this body of knowledge, this study focuses on part of the development of a microbicide based on a South African indigenous plant used to treat HIV patients.

1.7 Aim of the current study

This study is part of a multidisciplinary consortium initiative comprising of several research groups within the Council for Scientific and Industrial Research (CSIR) as well as the Drug and Formulation group at the University of the Witwatersrand (Wits), South Africa. The CSIR Biosciences division has on-going collaborations with

Traditional Healers (TH) and indigenous knowledge holders on the use of medicinal plants in South Africa. This has led to the identification of a number of plants used to treat HIV.

One of these plants, *Lobostemon trigonus*, is being used by a private individual in the Eastern Cape to treat HIV-positive patients. The traditional use of the plant is as a tea prepared from the dried aerial parts of the plant. In support of these activities, previous studies by Harnett *et al*. (2004) and Dambuza (2007) showed that an aqueous extract of *L. trigonus* inhibits HIV-1 reverse transcriptase. These authors used a range of solvents to extract most of the potential active compounds present in this plant species.

The key aim of the current study is to isolate and characterise the most suitable API from *Lobostemon trigonus*, which will contribute to the overall development of a vaginal microbicide, ultimately leading to the empowerment of women to protect themselves from sexual transmission of HIV.

1.8 Objectives of the study

Infection with HIV continues to pose an extraordinary public health problem and current treatment options have not been satisfactory. The quest for effective curative or preventive therapies continues with plants increasingly seen as an alternative source for discovery of novel anti-HIV molecules. The objectives of the study are as follows:

- To perform plant extractions and optimise the aqueous extraction method
- To concentrate extracts using an ethanol precipitation method
- To optimise the ethanol precipitation method
- To biologically evaluate the different potential APIs
- To purify the API using chromatographic techniques such as column chromatography (CC), thin layer chromatography (TLC) and vacuum liquid chromatography (VLC) and semi preparative high performance liquid chromatography (HPLC) as guided by the tested biological efficacy of each extract

- To characterise the potential API using specialized analytical techniques including nuclear magnetic resonance (NMR) and ultra-performance liquid chromatography- mass spectrometer (UPLC-MS) and gas chromatography (GC)

CHAPTER 2: PRELIMINARY DATA COLLECTION

2. Primary identification stages of the potential API

2.1 *Lobostemon trigonus:* Literature review

Lobostemon belongs to the family of Boraginaceae and is mainly distributed in the Eastern and Western Cape regions of South Africa. A photograph of this plant is shown in Figure 3 below. Research on this plant has been limited to botanical studies to help characterise and classify the species and sub-species. According to the Angiosperm Phylogeny Group (APG III 2009) classification, Boraginaceae, the borage, or forget-me-not family, includes shrubs, trees, and herbs, totalling about 2750 species in about 150 genera worldwide.

The family is especially well represented in the Mediterranean region, but is also found in most temperate to subtropical areas. *Lobostemon Lehmann* (1830: 378), with 28 species, is the largest southern African genus in the Boraginaceae (Buys 2011). This genus is mainly confined to the winter-rainfall area of South Africa, occurring from Springbok to Mossel Bay and further eastward along the coast to around Grahamstown, where rain may occur throughout the year (Buys 2011).

Agtdaegeneesbos (eight day healing bush), Douwurmbos (dew worm bush), Luibos (lazy bush) and Geneesbos (healing bush) are the most common vernacular names for *Lobostemon* and these names are known to most rural folk in the Western Province of South Africa, and to those interested in South African medical plants in particular.

One *Lobostemon* species, *L. fruticosus,* has been studied with regards to its medicinal properties particularly as *L. trigonus*, the species investigated in the present study, is not easy to cultivate nor to find in the wild. Furthermore, several studies have shown significant anti-HIV activity, specifically *in vitro* reverse transcriptase inhibition (Harnett *et al*; 2004). However, there is still a need to further investigate this plant species.

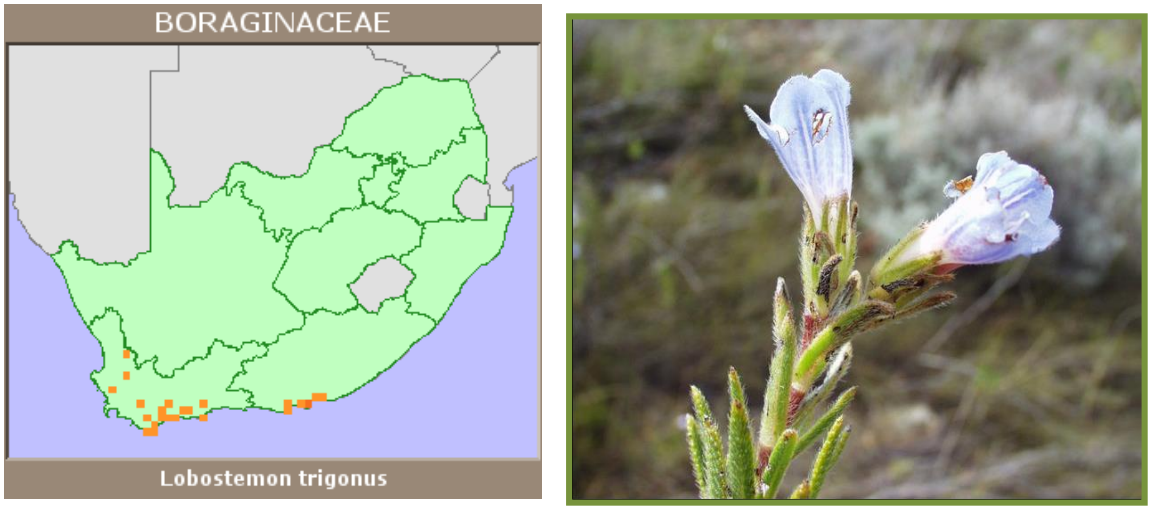


Figure 3: Left – regional distribution of *L. trigonus* (Foden & Potter 2005) Right **-** Picture of *Lobostemon trigonus* (Dambuza 2007)

2.1.1 Medicinal usage

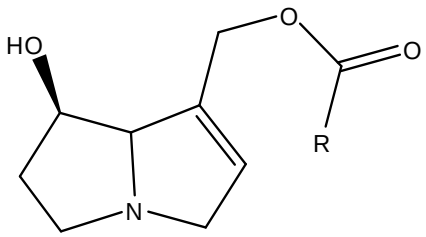
Lobostemon, according to its traditional name, has an apparent ability to heal a condition in eight days. Another name used is “douwurmbos”, as it is believed to treat ringworm. Decoctions are used to treat wounds, skin disease, ringworm and ulcers while infusions are used for general internal problems and to purify the blood. The remedies all require an infusion of the leaves or a paste is made by either pounding the leaves or chewing them, the slimy paste is then topically applied to the wound or affected area (Appidi *et al*; 2008)

2.1.2 Chemical constituents

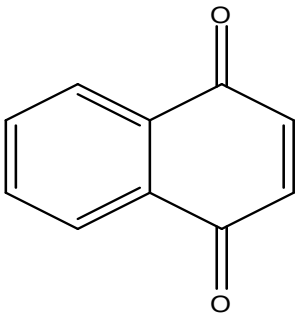
Little is known of the chemistry of this species, nor of others in this South African endemic genus. Members of the family are a common source of pyrrolizidine alkaloids and an example of this is shown as compound 1 in figure 4. These alkaloids accumulate in the liver and are toxic, thus, the use of food or products containing them should best be avoided (Martena 2010). As described in the study done by Sharma *et al*., (2009), various chemical constituents have been isolated and characterized from Boraginaceous plant species. These included naphthoquinone

derivative (compound 2, fig 4), bornesitol (compound 3, fig 4), phenolic acids, tannins and the ureide allantoin - the latter compound being particularly abundant in the root of comfrey, (*Symphytum officinale*).

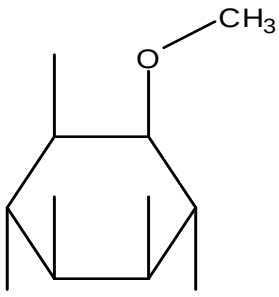
L. trigonus also contains sulphated polysaccharides, which proved to be one of the classes of compounds responsible for the anti-HIV activity. They appear to be present, as aqueous infusions are very viscous and set to a gel if in sufficient concentration (Harnett *et al*; 2004).



Compound 1: Pyrrolizidine alkaloids



Compound 2: Naphthoquinone derivative



Compound 3: A cylitol, bornesitol

Figure 4: Classes of the chemical constituents found in the *Lobostemon* genus

2.1.3 Biological properties

The Boraginaceae family has been shown to possess a high-molecular, water-soluble compound that was also isolated from the roots of *S. asperum* and to represent a new class of natural polyethers (Barbakadze, *et al*. 2000). Some plants species of the Boraginaceae family are a source of naphthaquinones (compound 2), red pigments which possess antibacterial, antifungal, anti-inflammatory and wound healing properties (Sharma *et al*; 2009).

2.2 Materials and Methods

Preliminary studies performed at CSIR Biosciences had revealed that the aqueous extract of the *Lobostemon trigonus* possessed anti-HIV properties. This preliminary research invented the current study, which focused on investigating the plant further for its anti-HIV properties. The overall intention was to isolate the API for use in the development of a microbicide.

The initial research stages were to produce an aqueous extract (spray dried (flow diagram 2) and freeze dried extract (flow diagram 1) and also to screen it against HIV-1 subtype B and/or C strains. The full details of the initial identification, purification and isolation stages of the API are described below. The detailed methodology is discussed in chapter 7.

2.2.1 Plant collection

Plant material was collected from the Port Elizabeth region in the Eastern Cape, South Africa. The aerial plant parts were harvested and transported to the CSIR in Pretoria, where it was dried, ground and stored for future use. The aerial plant parts were identified by the South African National Biodiversity Institute (SANBI Genspec number: 1492 44)

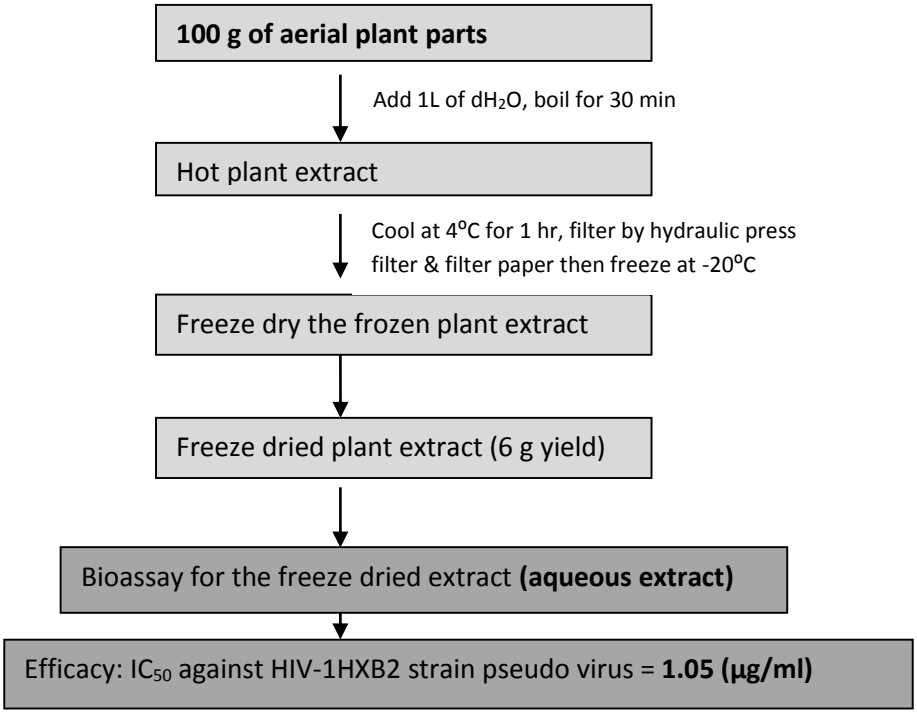
2.2.2 Determination of the anti-HIV activity

2.2.2.1 HIV Neutralization assay

This is the primary assay employed in the current study and is conducted as a routine assay within CSIR Biosciences. It uses molecularly cloned HIV-1 pseudovirions for greater reproducibility due to reagent stability. Envelope (env)-expressing plasmids are co-transfected with backbone plasmid into 293T cells to generate pseudo viral particles. These pseudovirions can infect cells but are unable to reproduce due to the lack of the complete genome resulting in single-round infection (Montefiori 2008).

These single-round infections are detected in genetically engineered cell lines that contain a Tat-responsive gene such as luciferase. TZM-bl cells (HeLa cell clones), engineered to express CD_4, CCR5 and endogenous CXCR4 contain integrated genes for firefly luciferase and *E.coli* ß-galactosidase under control of an HIV long terminal repeat sequence. Once the virus infects the cell the viral Tat proteins induces the expression of the luciferase reporter gene, the activity of this gene is detected and quantified by luminescence and is proportional to the infectious viral particles that have entered the cell (Montefiori D. C 2008). Detailed protocols of this assay are found in chapter 7.

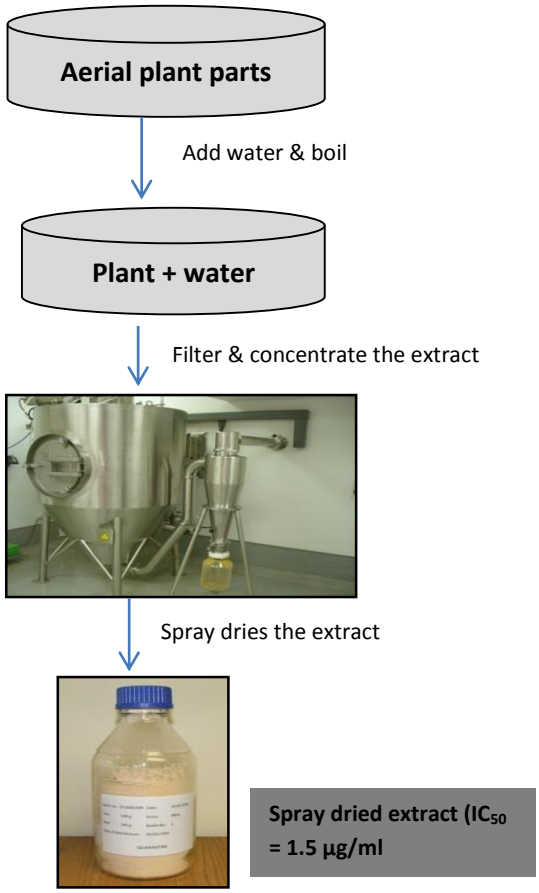
2.2.3 Preparation of the *L. trigonus* freeze-dried (aqueous) extract



Flow diagram 1: Preparation of the freeze-dried extract and its efficacy against HIV-1HXB2 pseudovirus.

2.2.4 Preparation of the spray dried (aqueous) extraction

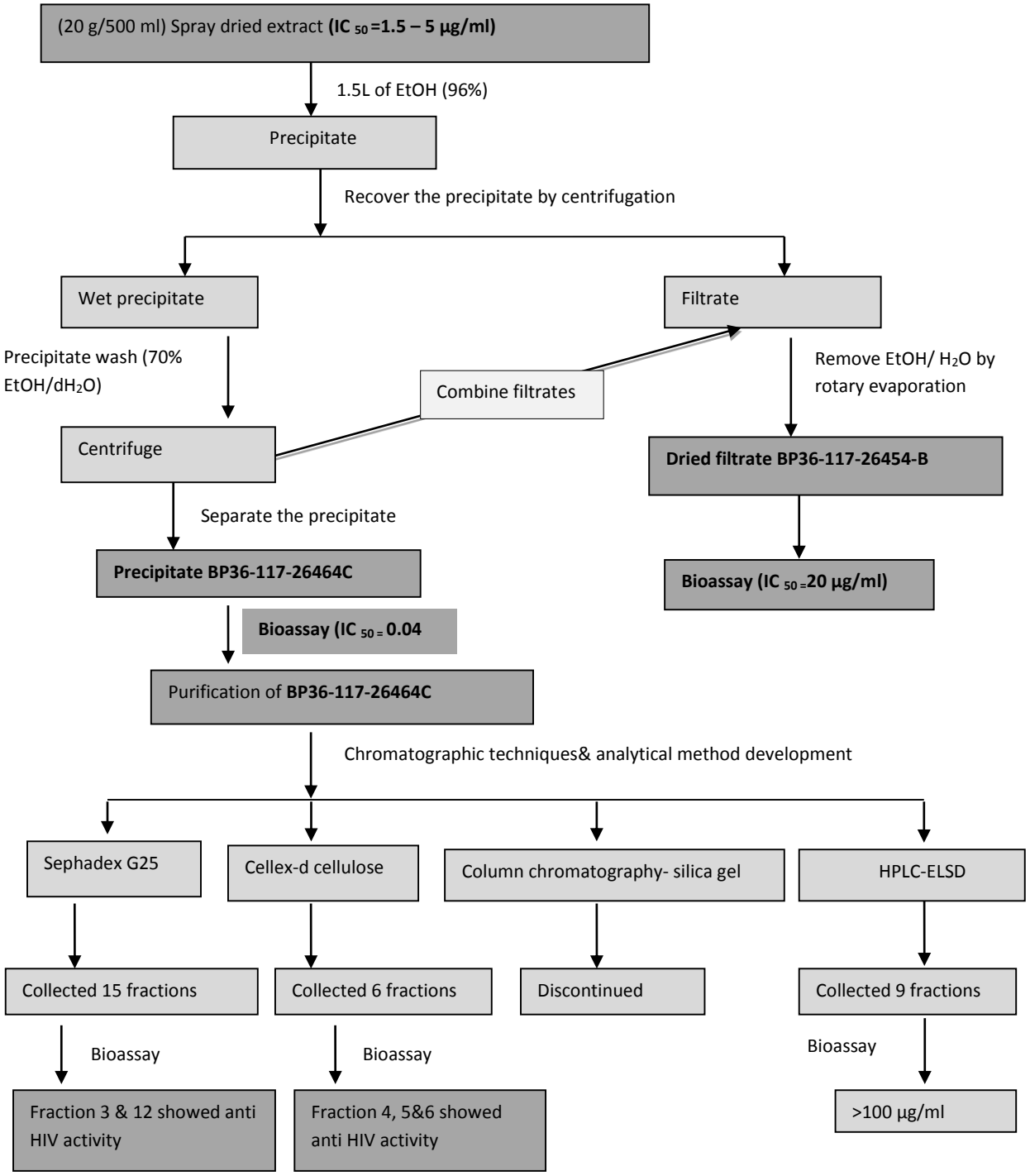
The extraction method that was developed led to a spray-dried extract of the plant which was also shown to have *in vitro* efficacy against laboratory-adapted HIV strain isolates (IC_{50} = 1.5 µg/ml). This was done to increase the scale of production and to examine the effect of extraction method on efficacy. The same procedure, ratio of plant material and water was followed as the above (flow diagram 1).



Flow diagram 2 : Production of spray-dried powder (aqueous) extract that provided sufficient material for bioassay-guided fractionation.

2.2.5 Bioassay-guided fractionation of the *L. trigonus* aqueous extract

Bioassay-guided fractionation based on *in vitro* activity against HIV-1HXB2 pseudovirions was conducted on the aqueous extract of *L. trigonus.* Flow diagram 3 below outlines the stepwise fractionation of the **spray-dried extract** (aqueous extract), later labelled as Lob-1-13A. All the extracts, fractions or compounds that showed activity are shaded in dark grey on the tables and on flow diagrams presented in this study.



Flow diagram 3: Flow diagram of the bioassay-guided fractionation of Lob-1-13A (spray dried extract)

As the aqueous extract showed moderate activity (IC_{50} = 1.5 – 5 µg/ml) against HIV-1 (HXB2 strain), it was then subjected to a liquid-liquid partitioning process, which was referred to as ethanol precipitation, in an attempt to purify and separate the chemical components that appeared to be responsible for the activity in this plant. The spray dried extract (20 g) was dissolved in distilled water (500 ml) to which was added 96% ethanol (1.5 L). The same method as described by Dambuza (2007) was used to remove the sulphated polysaccharides.

2.2.6 Details of a bioassay-guided fractionation

2.2.6.1 Silica gel column chromatography of the extract

Column chromatography is suitable for the physical separation of gram quantities of material. A solvent acts as the mobile phase while a finely divided solid surface acts as the stationary phase. The stationary phase will adsorb the components of the mixture to varying degrees. As the solution containing the mixture passes over the adsorbent, the components are distributed between the solvent and adsorbent surface. This process may be described by three-way equilibrium between the sample, the solvent and the adsorbent (Walker 2006).

Both the precipitate (**BP36-117-26464C)** and the aqueous extract (**Lob -1-13A)** were purified by silica gel column chromatography. Unfortunately, the separation was not successful as both samples were only soluble in water and it is not recommended to elute very polar components using water/MeoH on a column. In addition, it was not possible to purify the fractions on a silica gel column as it was time-consuming due to the extremely slow flow rate of the mobile phase. Chapter 7 has more details in this respect.

2.2.6.2 Size exclusion chromatography (SEC) using Sephadex G25

Size exclusion is a chromatographic technique in which molecules in solution are separated by their size, not by molecular weight. SEC is a widely used technique for

the purification and analysis of synthetic and biological polymers, such as proteins, polysaccharides and nucleic acids (Walker 2006).

Sephadex is available in different particle size grades. The different grades give chromatographic beads with different efficiencies and operating pressures. Sephadex G-25 type (20-50 µm bead size and 100-5000 molecular weight range) is recommended for separations of biomolecules such as peptides and other small biomolecules. As compounds migrate with the solvent through the gel, small molecules become included into the gel matrix, whereas larger ones are excluded and migrate at a greater rate (Walker 2006).

From the fractionation of the precipitate using Sephadex G 25, 15 fractions were generated and only fraction 3 (BP36-117-26486-III) and fraction 12 (BP36-117-26486-XII) showed significant activity. This is shown in Table 3. After it showed consistent anti-HIV activity, the precipitate **BP36-117-26464C** was then used as the reference sample extract in all the bioassays performed in the present study. All the fractions generated from the columns were then tested in comparison with this precipitate.

2.2.6.3 Ion exchange chromatography (IEC) using DEAE Cellulose (Cellex d)

Ion exchange column chromatography is a process that allows the separation of ions and polar molecules based on their charge (Walker 2006).This technique is limited to mixtures that contain components carrying a charge. In this form of chromatography, the sorbent is usually a polymeric resin like cellulose (cellex d), which contains charged groups and mobile counter ions that may exchange with ions of a component as the mobile phase migrates through the sorbent. Separation is achieved by differences in the affinity between ionic components and the stationary phase. DEAE cellulose (Cellex-d) is used as the resin. It allows fast flow rates especially after fines removal, suitable for negatively charged biopolymers

2.3 Results and Discussion

2.3.1 DEAE cellulose (Cellex-D) anion exchange column chromatography

This was employed to purify only the precipitate **BP36-117-26464C** as the precipitate was considered to be the component that was most active against HIV-1 HXB2 strain (0.04 µg /ml) and it was easy to dissolve compared to the spray dried extract.

Six fractions were collected from the DEAE Cellex-d cellulose column. As the purification and separation progressed, a synergy effect was observed, which means the components were observed to lose activity as they were separated from each other.

The fractions (F1, F2, and F3) did not show any significant activity, but fractions F4, F5, F6 showed some activity (see Table 1). When F1, F2 & F3 were combined, they showed no activity (IC_{50} value was >100 µg/ml) while a combination of fractions F4, F5 & F6 showed moderate activity. However, when F1, F2, F3, F4, F5 & F6 were combined, an increase in activity was noted (Table 2).

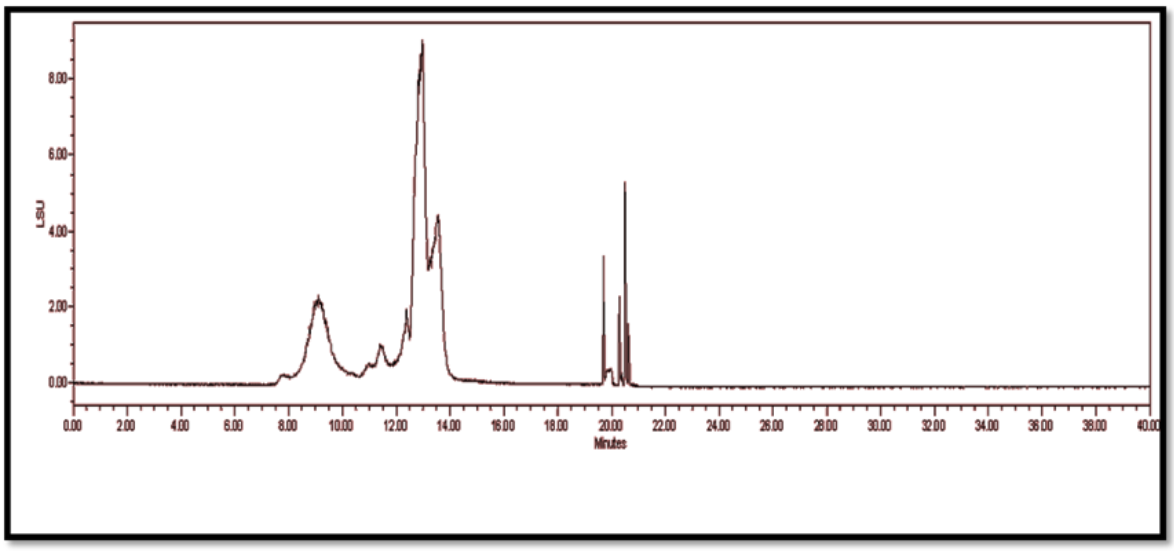
Another approach to further prove the synergy of the DEAE Cellex-d fractions was to combine the six fractions with pre-isolated compound from *L. trigonus* called bornesitol. Chapter 4, section 4.2.5 has the details as to how the compound was re-isolated. This compound is a *myo*-inositol methyl derivative found in several plant species. It was isolated as a pure compound (see fig. 14) from the aqueous extract of *L. trigonus.* When it was screened for activity, it showed no significant activity but when mixed with F1, F2, F3, F4, F5 and F6, the activity greatly improved (see Table 2). This proved that the activity of the individual fractions, including the compound bornesitol, act synergistically.

2.3.2 High Performance Liquid Chromatography - Evaporative Light Scattering Detection (HPLC-ELSD)

High Performance Liquid Chromatography (HPLC) is a form of column chromatography used to separate, identify, and quantify compounds. In this study, HPLC coupled with an ELS detector was identified as another possible technique for

further purification and for the fingerprinting of the precipitate (BP36-117-26464C) and spray-dried extract (Lob-1-13A) which were referred to as the potential Active Phyto-pharmaceutical Ingredients (API's). The problem with the method used was that the separation was done on a size exclusion column and the API size distribution appears to have spanned beyond the separation limit of the column.
Because of the nature of the chemical components in the sample, API, the successful separation and identification of these compounds in the API required a specific column and a detector suitable for these compounds. Therefore, it was difficult to develop a method that can separate these components and also difficult to identify how many compounds were in the mixture.

The ELSD did produce some results (see chromatogram 1) it is just that the API appears to have a very large molecular weight range. It was decided then to just collect a relatively few fractions (**fraction A, B, C, D, E, F, G, H & I).** These fractions were available based on the peak method which was employed to confirm their activity against HIV-1. All the peaks between 8 min – 14 min were collected as noted in chromatogram 1 below. However, when tested against HIV-1 HXB2, they showed an IC_{50} value of >100 µg/ml which was equated as no activity (see Table 1). This further proved that all the possible active compounds were possibly retained in the column which indicated that a better column was needed. Note that peaks around 20 min consisted of solvent peaks.



Chromatogram 1: HPLC-ELSD profile of the BP36-117-26464C – Precipitate (API)

Table 1: Efficacy of the aqueous extract, precipitate, two internal standards, sephadex G 25 fractions and DEAE cellex-d cellulose fractions: IC_{50} against HIV-1 HXB2 strain pseudovirus

Sample name	**Sample description**	**IC_{50} (μg/ml)**	**HIV-1 strain**	**Method**
Lob-1-13A	Aqueous extract	1.500	HXB2	Extraction
BP36-117-26464C	Precipitate	0.040	HXB2	EtOH precipitation
BP36-117-26454B	Filtrate	0.900	HXB2	Precipitate wash
BP36-117-26488-**F1**	Cellex-d fraction	>100	HXB2	Cellex-d column
BP36-117-26488-**F2**	Cellex-d fraction	>100	HXB2	Cellex-d column
BP36-117-26488-**F3**	Cellex-d fraction	>100	HXB2	Cellex-d column
BP36-117-26488-**F4**	Cellex-d fraction	6.000	HXB2	Cellex-d column
BP36-117-26488-**F5**	Cellex-d fraction	18.00	HXB2	Cellex-d column
BP36-117-26488-**F6**	Cellex-d fraction	21.00	HXB2	Cellex-d column
BP36-117-26486-**III**	Sephadex fraction3	14.03	HXB2	Sephadex G25 column
BP36-117-26486-**XII**	Sephadex fraction12	27.04	HXB2	Sephadex G25 column
BP36-117-26474-**A,B,C,D**	HPLC-ELSD 4 fractions	>100	HXB2	HPLC-ELSD
Enfuvirtide (T20)	Internal standard	0.020	HXB2	External sourced
Tenofovir (TFV)	Internal standard	0.400	HXB2	External sourced

Note that Tenofovir is the reverse transcriptase inhibitor and Enfuvirtide is the fusion inhibitor.

Table 2: Efficacy of the Cellex-d Cellulose fractions and their synergistic effect

Sample name	**Source**	**Combined fraction**	**HIV Strain**	**IC_{50} (μg/ml)**
BP36-117-26504-C (Bornesitol)	Aqueous extract	Pure compound	HXB2	>100
BP36-117-26504-C (Bornesitol)	Aqueous extract	**F1,F2,F3,F4,F5 & F6**	HXB2	**6.1**
BP36-117-26488-**F1-F3**	Cellex-d cellulose column	**F1, F2 & F3**	HXB2	>100
BP36-117-26488-**F4-F6**	Cellex-d cellulose column	**F4, F5,F6**	HXB2	**24.0**
BP36-117-26488-**F1-F6**	Cellex-d cellulose column	**F1,F2,F3,F4,F5 & F6**	HXB2	**12.0**
BP36-117-26464C	**Precipitate**	Reference sample	HXB2	**0.04**

2.3.3 Ethanol precipitation of the aqueous extract

Many compounds from this class (sulphated polysaccharides) have already been described extensively in the literature as having anti-HIV activity (Harnett *et al*, 2004; Dambuza 2007). This research also involved the removal of tannins and sulphated polysaccharides to further prove that potential anti-HIV activity seen in the aqueous extract of *L. trigonus* was not due to these class of compounds, indicating that there is another group or class of compounds responsible for the activity in this plant.

The filtrate named as **BP36-117-26454B** which in this present study is referred to as the precipitate wash**,** was dried and HIV-screened. Improved anti-HIV activity was observed for the precipitate (see flow diagram 3 above) – **BP36-117-26464C** (IC_{50} 0.04 μg/ml) compared to the aqueous extract, Lob -1-13A, which showed moderate activity (IC_{50}1.5 - 5 μg/ml). The activity of the precipitate against the HXB2 HIV-1 strain compared favourably with that of T20 and Tenofovir (Table 1).

2.3.4 Elementary Analysis of the precipitate (API)

Elemental analysis on carbon, hydrogen and nitrogen is the most essential and, in many cases, the only investigation performed to characterize and/or prove the elemental composition of an organic and/or aqueous sample. This aspect of analysis was very important, as it was going to give an indication of what the API was roughly made up of. The API (BP36-117-26464C) was sent for the elementary analysis using the Leco Truspec CHN Analyser. The percentage composition of elements in the API is summarised in table 3 below.

Table 3: Elementary composition of the API

Sample name	%C	%H	%N
BP36-117-26464C	41.8	4.605	1.1687

The results above (table 3) showed that the API is made up of 47.5 percent of carbon (C), hydrogen (H) and nitrogen (N). It was speculated that the other 52.5% could be oxygen (O) and/or other elements. Unfortunately, these speculations could not be verified. They are still to be investigated.

2.3 Conclusions

Both freeze dried and spray-dried aqueous extracts (Lob-1-13A) of the plant showed good anti-HIV activity (IC_{50} = 1-5 μg/ml) against laboratory-adapted pseudovirus in the neutralisation essay, but the precipitate (BP36-117-26464C) was the most potent with an IC_{50} of 0.04 μg/ml. Bioassay-guided fractionation of the aqueous spray-dried extract identified a polymeric molecule as the API showing anti-HIV activity in the same order of magnitude as Zidovudine (AZT), Enfuvirtide (T20), Tenofivir and Nevirapine (NVP).

The aqueous solubility of this API indicated that it was more amenable to formulation as a topical microbicide for the prevention of sexual transmission of HIV in women. A target-based, time of addition assay showed that the API acts as an HIV attachment inhibitor, thus, substantiating its use as a topical microbicide (Van Den Berg *et al*; 2010). As such, the API extracted from the aerial parts of *L. trigonus* has enormous potential to block HIV infection and, in addition, to further counteract the development of drug resistance by HIV. In future, drug resistance could be a major problem, and in this regard, multiple and/or non-specific methods of action could be a solution to such a problem.

In observing the physical properties of all the fractions obtained from Sephadex and Cellex-d cellulose columns, they were found to be brownish in colour, with a plastic and crystalline, sugar-like texture. In addition, they were all water soluble. All the physical properties of the fractions indicated that they were polymeric in nature or were molecules attached to sugars.

Finally, it didn't seem practical to continue purifying the individual fractions (from Sephadex G 25 and DEAE Cellex-d column) since they either lost or showed, at best, only moderate activity. Therefore, the partial characterization of the precipitate **BP36-117-26464C** (IC_{50} = 0.04 μg/ml) was prioritized since it showed superior activity and consistently positive results.

Elementary analysis for other suspected elements such as phosphorus (P), sulphur (S) or any other possible metals could not be determined. Only four elements were

detected, (carbon, hydrogen, O and nitrogen) and those were expected since the API was an organic sample, and the *lobostemon* genus was reported to be rich in nitrogen containing compounds such as pyrrolizidine alkaloids.

The main goal of characterization was to identify its structural components and perform fundamental analysis using nuclear magnetic resonance, gas chromatography and ultra- performance liquid chromatography mass spectrometry. This will be discussed further in chapter 4.

CHAPTER 3 : METHODS MODIFICATION

3. Process development

3.1 Process optimization

Most processes, be it physical, chemical or otherwise, can be improved. While designing experiments, they require a deep understanding of influences that achieve desirable performance. The need for an efficient and systematic decision-making approach drives the need for optimization strategies. This chapter provides an overall description of how the aqueous extractions of *L. trigonus* and ethanol precipitation methods were optimized in order to identify the best API for further development as a microbicide. Several variables were investigated, such as:

- Plant part (leaves, stems and combination of both)
- Pre-processing of plant material (fresh or dry, milled or un-milled)
- Extraction temperature (4°C and 95°C)
- Dissolution volume of an extract (between 200 ml – 400 ml)
- Precipitate wash composition (0%, 50% and 70% EtOH/H_2O)

At every stage of the process optimisation, the API yield and activity were assessed to identify the optimal variable. The HIV-1 pseudovirion neutralisation assay was used to evaluate activity. Note that all the extracts, fractions or compounds that showed activity are shaded in dark grey on the tables and flow diagrams.

Values such as IC_{50} indicate the activity of extracts, fractions, compounds and precipitate and IC_{50} is defined as the concentration of an inhibitor required to give 50% inhibition of an activity, where the smaller the value the greater the activity. Strain HXB2 is an HIV-1 strain used in the current study and compounds such as AZT (Reverse transcriptase inhibitor), Tenofivir (nucleotide reverse transcriptase inhibitor) and Enfuvirtide (fusion inhibitor) were the internal standards used. Optimisation experiments were conducted at laboratory scale.

3.1.1 Optimisation of the aqueous extraction method

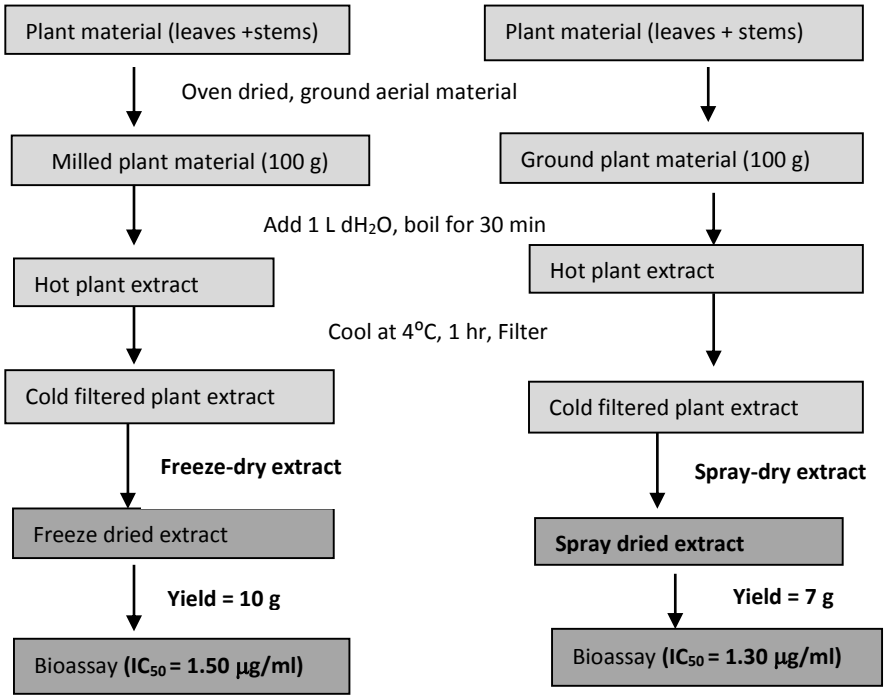
Optimization of the aqueous extraction method was carried out to investigate the effect on yield and activity by the following:

- Freeze-drying as an alternative to spray drying of aqueous extract
- Performing the aqueous extractions at 4°C and 95°C
- Extraction of leaves and stems separately instead of in combination

Several other potential variables could have been included, such as the microwave extraction, organic extraction instead of aqueous extraction, as they could have had an impact on the efficacy and yield, but because of the restricted time allocated for these experiments, it was impossible. So, only the primary variables were considered.

3.1.1.1 Freeze-drying versus spray-drying

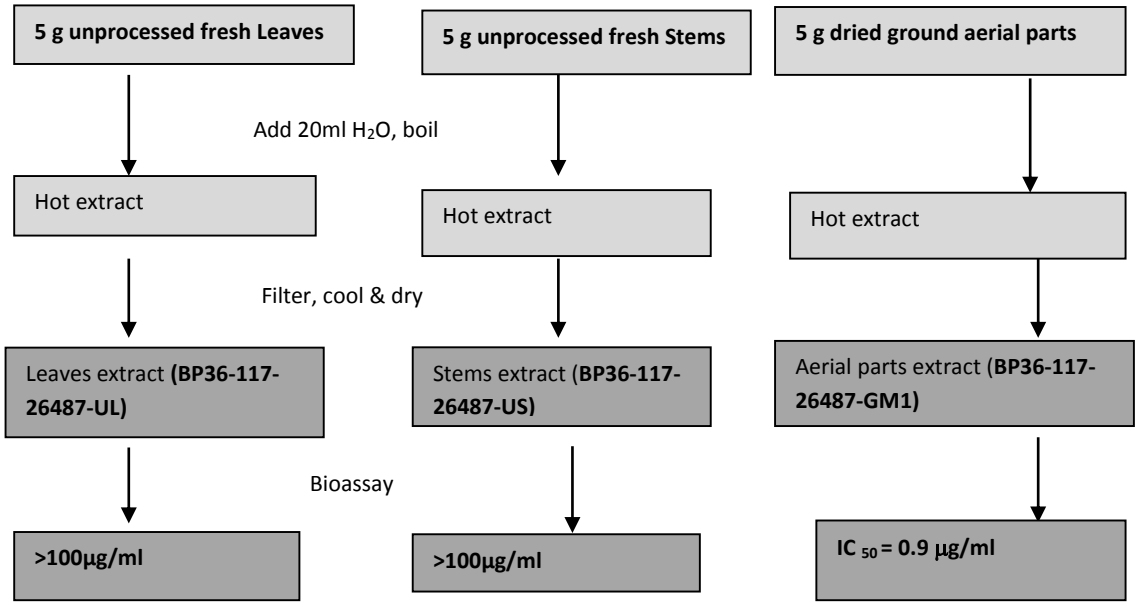
Preparation of the spray-dried extract was time-consuming and energy-intensive. (Flow diagram 2). This consisted of several processes and the spray dried extract was difficult to dissolve as it showed a tendency to gel. However, preparation of the freeze-dried extract was practically efficient and proved easier to re-dissolve. In terms of the efficacy, there was no difference as both extracts were active to the same order of magnitude (flow diagram 4 for the IC_{50} values). The best method to dry the extract was decided to involve freeze drying as it was fast, potent and produced material that was more readily soluble and in higher yield.



Flow diagram 4: Effect of eliminating the spray drying process and introducing freeze drying process after aqueous extraction leading to increased anti-HIV activity

3.1.1.2 Plant pre-processing and extraction temperature

Another variable investigated was whether the activity was restricted to either the leaves or stems. A portion of fresh leaves and stems of *L. trigonus* was collected and extracted separately with cold water (4^0C) and boiled water (95^0C) to also validate the effect of temperature on anti-HIV activity. The extraction and the efficacy were assessed on each plant part at the two temperatures in comparison with dried ground aerial parts (i.e. combination of leaves and stems) see flow diagram 5 below).

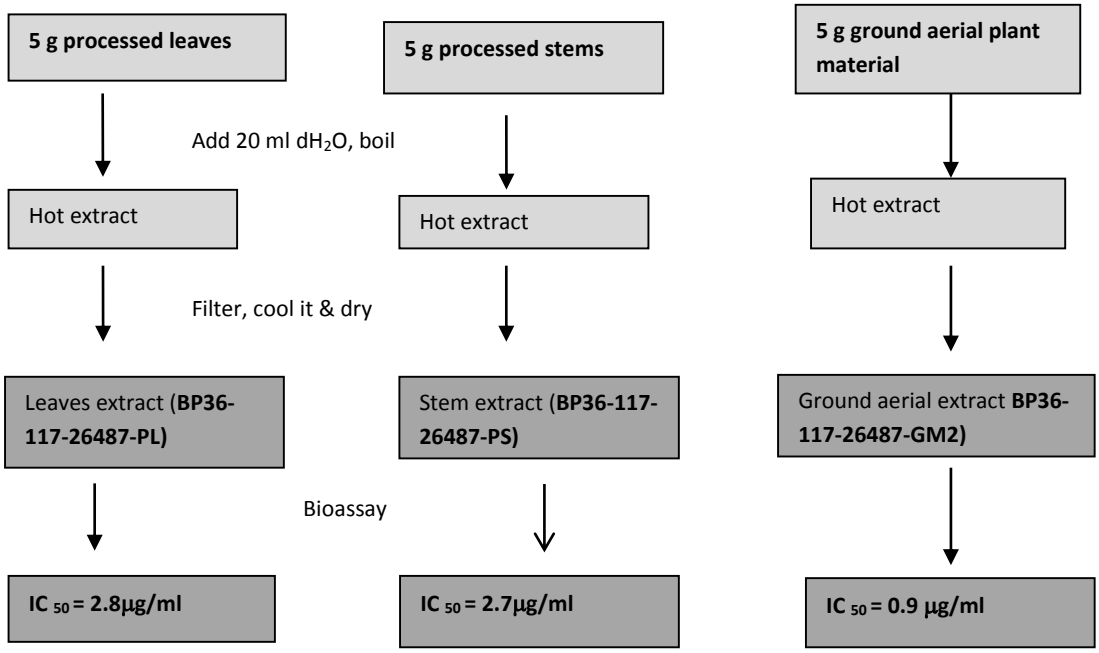


Flow diagram 5: Optimization of the production of the aqueous extraction: preparation of the aqueous extract at 95°C (unprocessed leaves vs. unprocessed stems vs. dried ground aerial parts). Abbreviations include UL (unprocessed leaves), US (unprocessed stems) and GM1 (Ground aerial parts).

The results demonstrated that the fresh leaves (**BP36-117-26487-UL**) and stems (**BP36-117-26487-US**) extracts at 95°C did not exhibit activity (see flow diagram 5 above) unless being processed by methods such as oven drying, crushing and milling. The same extraction process was repeated but with cold water at 4°C. No distinction was observed in terms of the efficacy on the cold water extracts of the leaves and stems as individual components. However, the dried ground aerial parts of the plant from which were extracted **BP36-117-26487-GM1**) (IC_{50} = 0.9 µg/ml) showed consistently good activity for both **4°C** and **95°C**.

3.1.1.3 Remarks on processed aerial plant parts

The leaves and the stems were processed as the individual components (oven dried at 80°C and partially milled). They were extracted with hot water (95°C) and found to show significantly increased activity (see flow diagram 6 below). Thus, it is possible that oven drying (temperature) and grinding (physical processing) aid in the formation and/or the extraction of the anti-HIV constituents. The same extraction experiment was repeated but at a different temperature (**4°C**) and the anti-HIV activity was the same.



Flow diagram 6: Optimization of the production of the aqueous extraction: preparation of the aqueous extract at **95°C** (processed leaves vs. processed stems vs. dried ground aerial parts.

It was then speculated that processing the plant material (i.e. addition of energy) aids in the release and extractability of the bioactive constituents, resulting in improved anti-HIV activity. This suggestion is supported by traditional healers who often use

their hands or a hammer to partially process the plants before they prepare the decoctions for their patients. However the scientific explanation is that, oven drying would break the cell membranes and that ground material consists of smaller particles that will be extracted much more efficiently than large particles. The temperature in terms of boiling water (95°C) or cold (4°C) water during extraction had no effect whatsoever and it appears that the most important variable in producing an API was the plant processing (oven drying and grinding/milling).

3.1.2 Optimization of the ethanol precipitation method

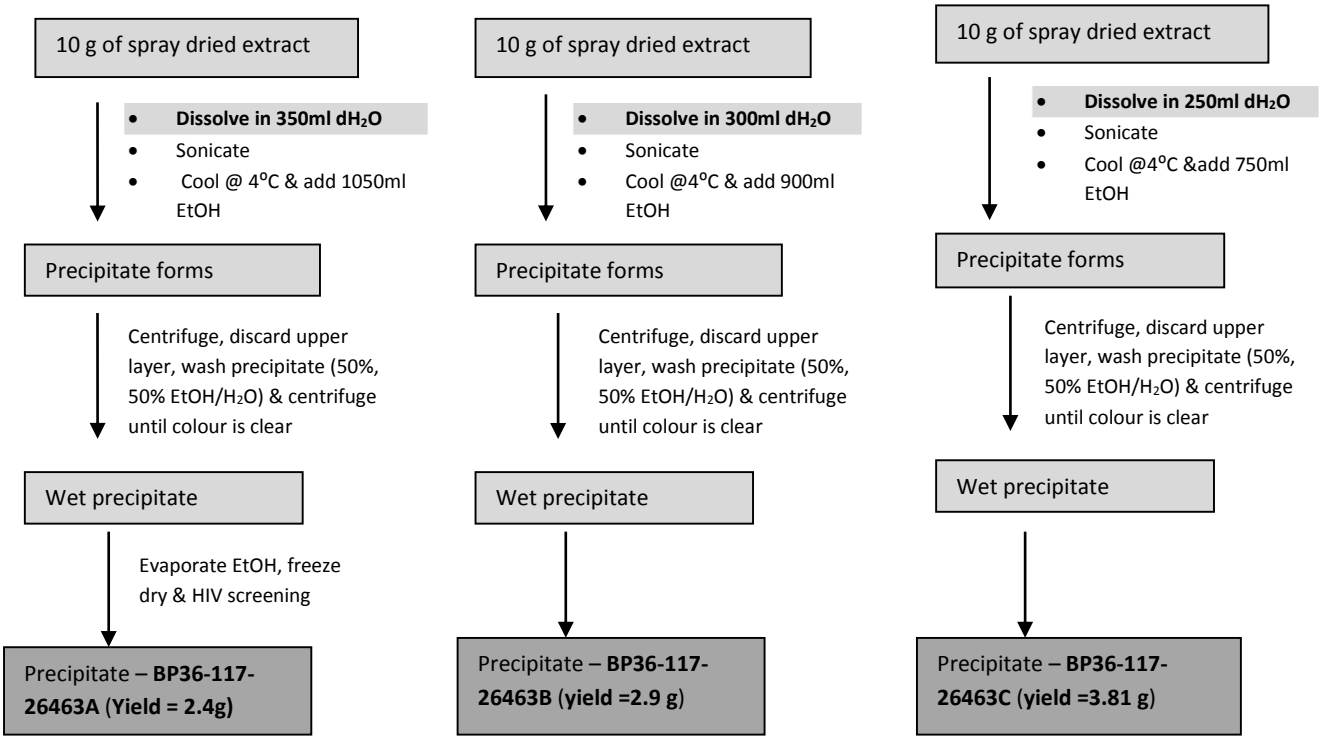
In order to optimise the ethanol precipitation method, two variables needed to be investigated:

- The volume of water (200-400 ml) required to dissolve the spray dried extract for ethanol precipitation process
- The solvent system (0%, 50% and 70% EtOH/H_2O) required to wash the precipitate during the ethanol precipitation process

Both variables were considered vital as they were observed to affect the efficacy and the yield of the API.

3.1.2.1 Details of the dissolution volume

The first variable to be optimised was the amount of water required to dissolve the spray dried extract. The main difficulty with the spray dried extract was that it was difficult to dissolve completely. In addition, this process was time consuming as this extract would gel and develop lumps. Determining the correct volume (200 ml - 400 ml) of water was vital to optimally dissolving 10 g of the spray dried extract for the ethanol precipitation and ultimately to produce the API with the greatest activity. The process is shown in the flow diagram 7 below.



Flow diagram 7: Optimization of ethanol precipitation – investigating the volume of water to dissolve 10 g of the extract

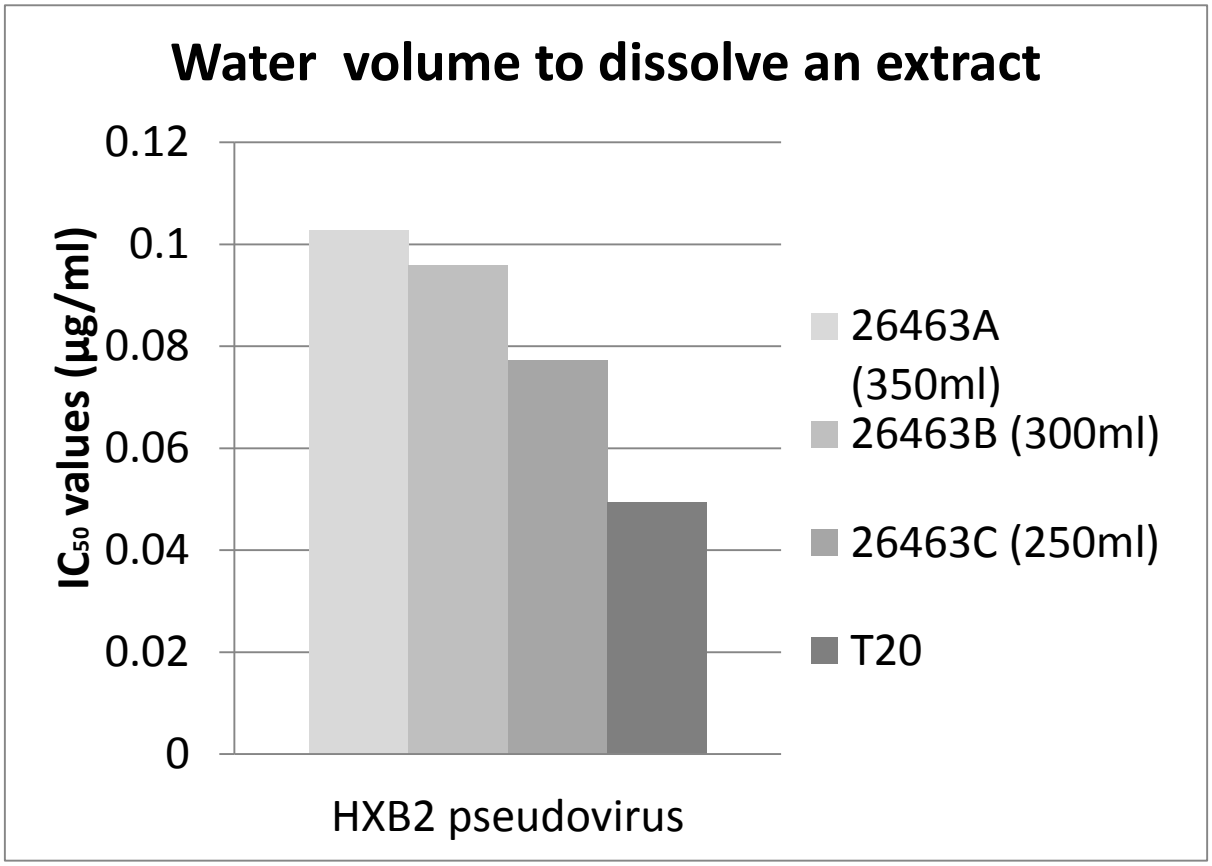


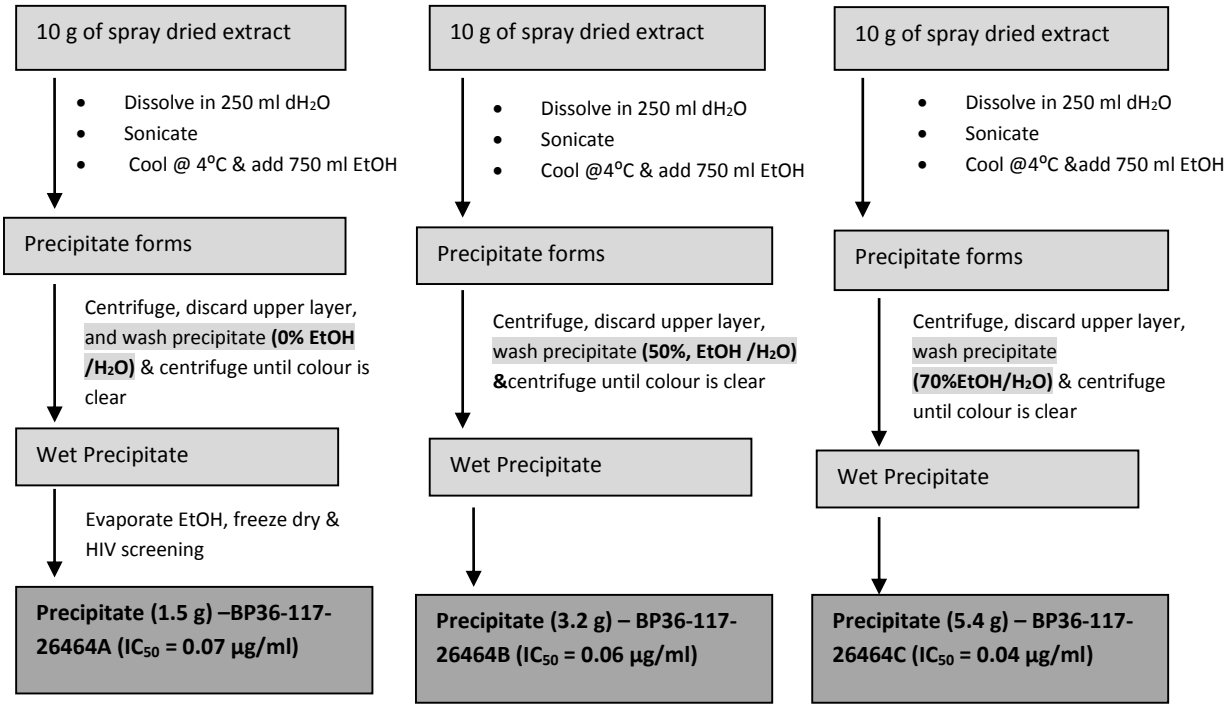
Figure 5: Use of the anti-HIV assay to select the optimal volume of water required to dissolve 10 g of the extract for precipitation.

Based on the efficacy and the yield, the amount of water selected to dissolve the spray dried extract was 250 ml (see flow diagram 7 and fig 5 above). This was expected as the lower the volume of water, the more concentrated would be the extract, the higher the yield leading to an increase in the anti-HIV activity.

Remarks: To dissolve 10 g of the extract in 350 ml of distilled water took about 7 hours, for 300 ml it took 8 hours and for 250 ml it took about 9 hours. As it was time consuming to perform these experiments, there was still then a need to investigate the best way or method of preparing a bigger batch of the precipitate (the API) or a bigger scale without having to waste time and resources.

3.1.2.2 Details of the Precipitate wash

To form a precipitate, the dissolved aqueous extract (250 ml) was mixed with 750 ml (1:3 ratios) of 96% ethanol. The precipitate was then recovered through centrifugation. To wash the precipitate, a mixture of ethanol & water was used until the solution (wash) was clear. It was observed that during the washing of the precipitate, some of the bioactive compounds were lost due to solubility in the wash solvent (see yields on flow diagram 8). To avoid this, an investigation of the ideal wash solvent (0%, 50% and 70% ethanol/water ratios) was conducted. All the other variables previously discussed were kept constant during the optimization process. The detailed method is shown below on the flow diagram 8.



Flow diagram 8: Optimization of ethanol precipitation involving between 0% - 70% EtOH/H_2O solvent ratios to wash the precipitate and its resulting efficacy. Internal standards (T_{20} - IC_{50} = 0.02 µg/ml and AZT – IC_{50} = 0.4 µg/ml)

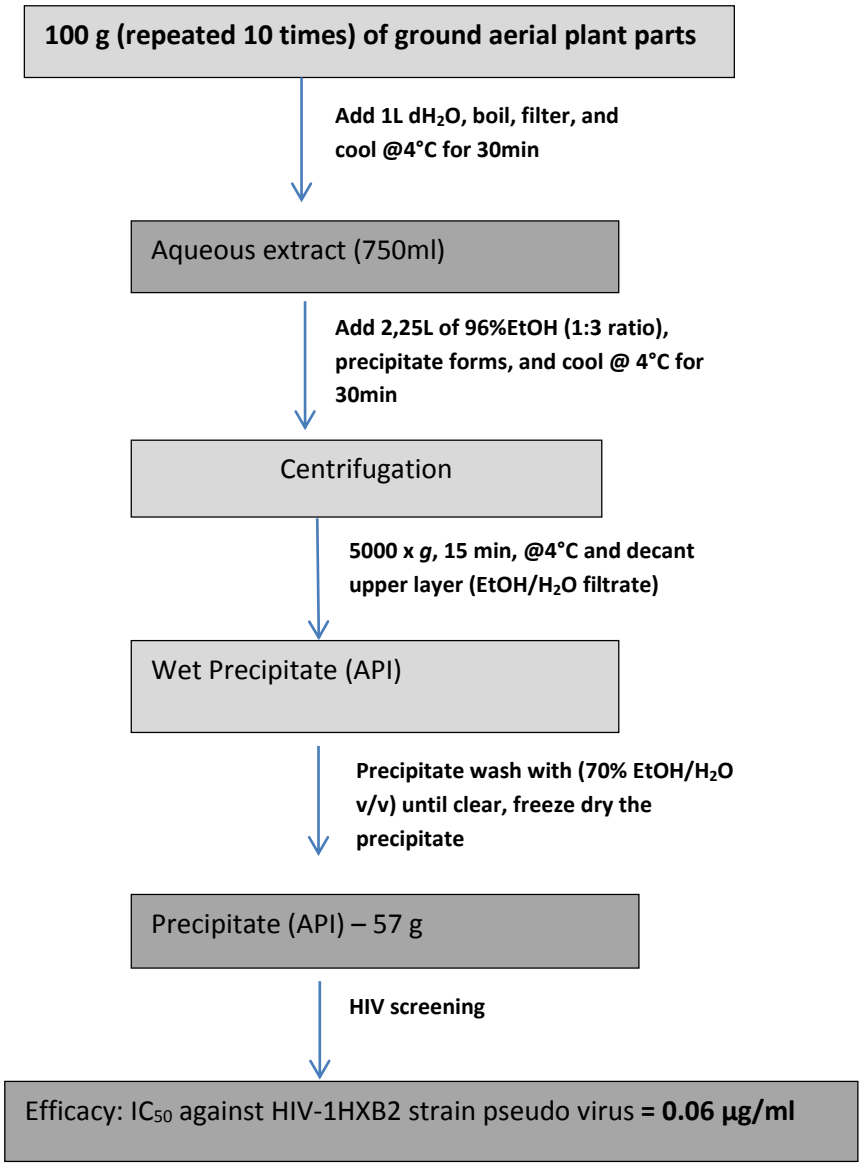
3.2 Conclusion

Optimization of the aqueous extraction method showed that hot water extraction was the best method. This was in line with the traditional use of the medicinal plant. Fresh leaves and stems did not exhibit any activity, possibly because they were harvested, washed and then extracted and the active components could have been diluted to reduce their extractability and activity. Although partially processed leaves and stems individually produced significant activity, a combination of leaves and stems, which are fully processed (dried and ground), gave the best activity. The same ratio of water to plant material was used for scale up productions of the API.

After the addition of 96% EtOH into the extract as showed in flow diagram 7, the precipitate formed immediately and the extract was then centrifuged to recover the precipitate that appeared as a jelly-like residue. The aqueous layer from the precipitation was decanted and was referred to as "filtrate" showed moderate activity (IC_{50} = 20.5 µg/ml), and the water soluble residual gel, which was referred to as the precipitate, was the most active (IC_{50} = 0.04 µg/ml). This activity compared favourably with virus fusion inhibitor Enfuvirtide (T20).

From the flow diagram 8 shown above, the most active API selected was that of **BP36-117-26464C** with an IC_{50}= 0.04 µg/ml. This was prepared from 10 g of extract in 250 ml, 70% EtOH/H_2O for precipitate wash. This selected API **(BP36-117-26464C)** was further purified and characterized as it was still a mixture of unknown active compounds. The purification and fractionation of the API is discussed in Chapter 4.

The same parameters and protocols as described above were up-scaled and used to produce a large scale batch of the API (57 g) – see flow diagram 9 below. In terms of plant parts, oven dried, ground aerial parts appeared to be best when it comes to preparation of the precipitate and for future experiments including formulations. **Please note below**: the illustration of how the large batch of the API was prepared for the formulation and other necessary *in vivo* and *in vitro* assays including the PBMC's assay.



Flow diagram 9: Illustration of the large-scale batch preparation of the API

This batch (flow diagram 9) was produced for the development of the API into a microbicide, an API that will be formulated into gels and caplets. These formulations are identified as the most suitable formulations for women to use to protect themselves from sexual transmission of the HIV and other STI's. More details concerning the formulations and development of the API as a microbicide is discussed in Chapter 5.

CHAPTER 4: PHYTOCHEMISTRY

4. Isolation and Characterization of the API

4.1 Isolation of the API

4.1.1 Introduction

Chapters 2 and 3 explained how the aqueous extractions and ethanol precipitation methods were modified and optimised, to ultimately produce the API **BP36-117-26464C**. This chapter contains a summary of how the API was purified and characterised using different high-end techniques.

At every stage of the purification process, the compounds and/or fractions were consistently assessed in terms of their bioactivity using the HIV-1 pseudovirion neutralisation assay. The IC_{50} values indicated the activity of the individual compounds and other activity-rich fractions and are shown in the flow diagrams and tables. In this assay, HXB2 is an HIV-1 strain used, AZT (reverse transcriptase inhibitor), Tenofivir (nucleotide reverse transcriptase inhibitor) and Enfuvirtide (fusion inhibitor) are the internal standards utilised. All the extracts, fractions or compounds that showed good activity (<20 µg/ml) are shaded in dark grey on the tables and flow diagrams, moderate activity (<50 µg/ml) is shown in light grey and lastly weak activity (>100 µg/ml) is represented by a very light, almost white grey colour.

4.1.2 Purification of the API

In this study, chromatographic techniques such as Liquid-liquid chromatography and thin layer chromatography (TLC) were used in order to purify the API. As described in chapter 2, the API could not be fractionated through a normal column or using High Pressure Liquid chromatography (HPLC - reverse phase) due to the polymeric nature of the API. The API was also discovered to consist of a high molecular weight compound/s that showed potent activity against HIV-1 pseudo virus (IC_{50} = 0.04 µg/ml).

For the API to be purified, it had to be re-extracted with different polar solvents (liquid-liquid partitioning) to release all the possible soluble compounds in the API. The API was also treated with a polymer called polyvinylpolypyrrolidone (PVPP) in order to adsorb all the polyphenols in the API. All these efforts were aimed at discovering different classes of compounds present like fatty acids, sugars, alkaloids and polyphenols. To fractionate the API so that compounds can be easily separated and isolated, a selection of chromatographic techniques were attempted.

4.1.2.1 The Use of the PVPP in the extraction of the polyphenols

Polyvinlypolypyrrolidone (**PVPP**) is an organic solvent and water insoluble polymer. It is a light and creamy white powder. It is also insoluble in strong acid minerals and in alkali with stable foam being produced. It is very expensive but can be reusable. It absorbs water and swells very rapidly generating a swelling force. This compound forms bonds similar to peptide bonds in protein and that is why it can precipitate tannins the same way as proteins do. It is a highly cross-linked modification of polyvinylpyrrolidone (PVP) (Magalhães *et al*; 2010).

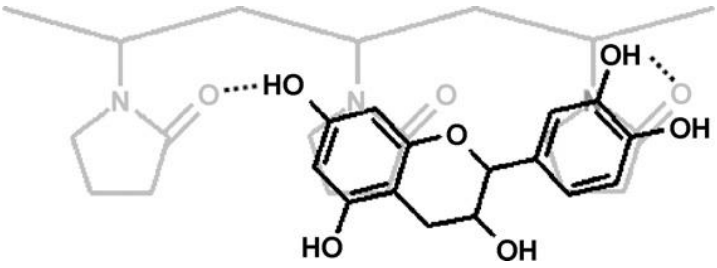


Figure 6: Illustration of hydrogen bonding between the proton donor from the polyphenol and the carbonyl group from PVPP.

As seen in Figure 6 above, it has been known for some time that phenolic compounds bind to polyvinylpolypyrrolidone (PVPP) (Magalhães *et al*; 2010). PVPP in the present study was used as a decolorizing agent (see chapter 5) and to adsorb the polyphenols which were thought to be partially responsible for the anti-HIV activity.

4.1.2.2 Detection of polyphenols such as flavonoids in the API

Flavonoids are water soluble polyphenolic molecules containing 15 carbon atoms. They can be visualized as two benzene rings (see fig 7) which are joined together with a short three carbon chain. They also consist of 6 major subgroups: chalcone, flavone, flavonol, flavanone, anthocyanins and isoflavonoids. Together with carotenes, flavanoids are also responsible for the colouring of fruits, vegetables and herbs (Magalhães *et al*; 2010).

With regards to detection, flavonoids normally produce yellow-brown spots on a white background when treated with iodine and ammonia vapour. Flavonoids may appear as dark spots on green background fluorescent when observed under UV light at 254 nm. Similarly under UV light at 366nm spot, colours depending on the structure of flavonoids can be yellow, green or blue fluorescent. It would be more clear and intense after being sprayed with the reagent.

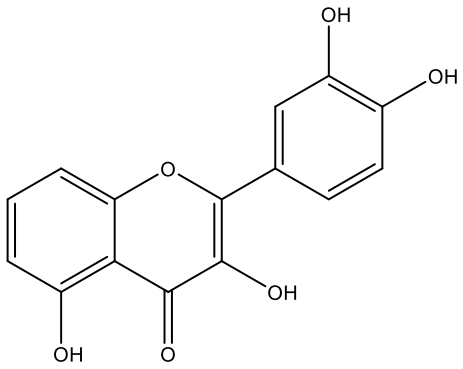
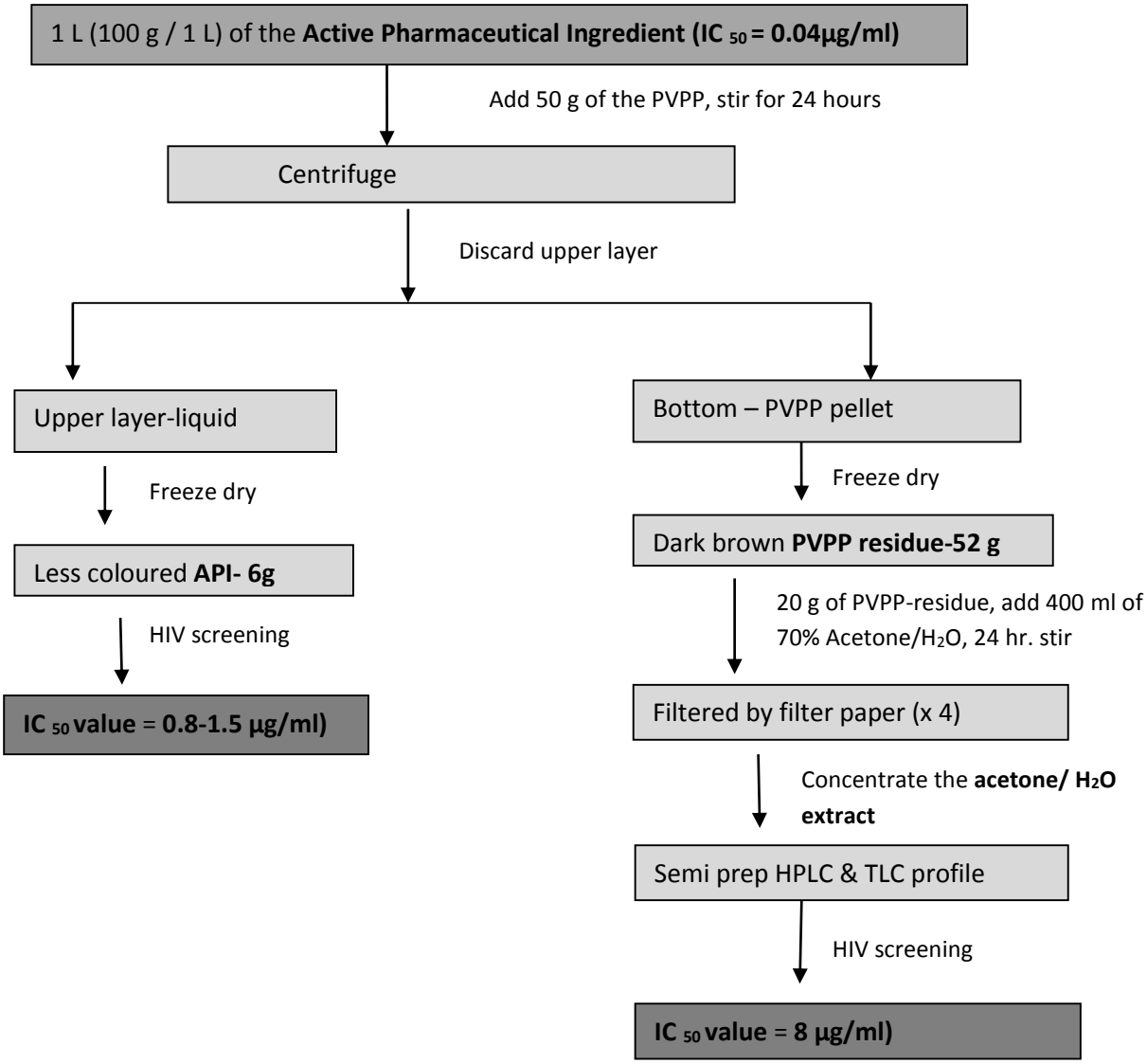


Figure 7: An example of a polyphenol compound

4.1.2.3 Separation of polyphenols in the API by PVPP

A liter (L) of the API was prepared as described in flow diagram 10 below, it was then mixed with 50 g of polymeric PVPP. The mixture was exhaustively mixed and stirred overnight for a complete adsorption of the polyphenols by the PVPP. The next day, the PVPP residue (pellet) and the filtrate (decolorized aqueous layer) were separated and recovered through centrifugation (5000 *x g*, 15min, at 4°C).

The PVPP residue and filtrate were freeze dried and weighed. The extraction/desorption of polyphenols was achieved by mixing 20 g of the PVPP residue with 400 ml of 70% Acetone/H_2O. This mixture was thoroughly stirred overnight for complete extraction. Finally, a light brown extract containing the polyphenols and PVPP was separated by centrifugation. After filtration through a filter paper to remove the very small particles of the PVPP still in the mixture, the acetone-water extract was vacuum dried so as to remove the water and acetone as shown in Flow Diagram 10 below. The extract was subjected to the biological assay for the anti-HIV activity. The same extract was also phytochemically screened for the presence of polyphenols.



Flow diagram 10: Flow diagram illustrating the extraction and biological efficacy of the Acetone/H_2O extract (containing the polyphenols)

The polyphenol rich extract (Acetone-water extract) exhibited anti-HIV activity with an IC_{50} of 8 µg /ml. Also the TLC profile of the extract confirmed the presence of polyphenols as expected but the intensity of the spots was very weak even though these compounds were UV active. For the separation and purification of these compounds, semi-prep HPLC was employed

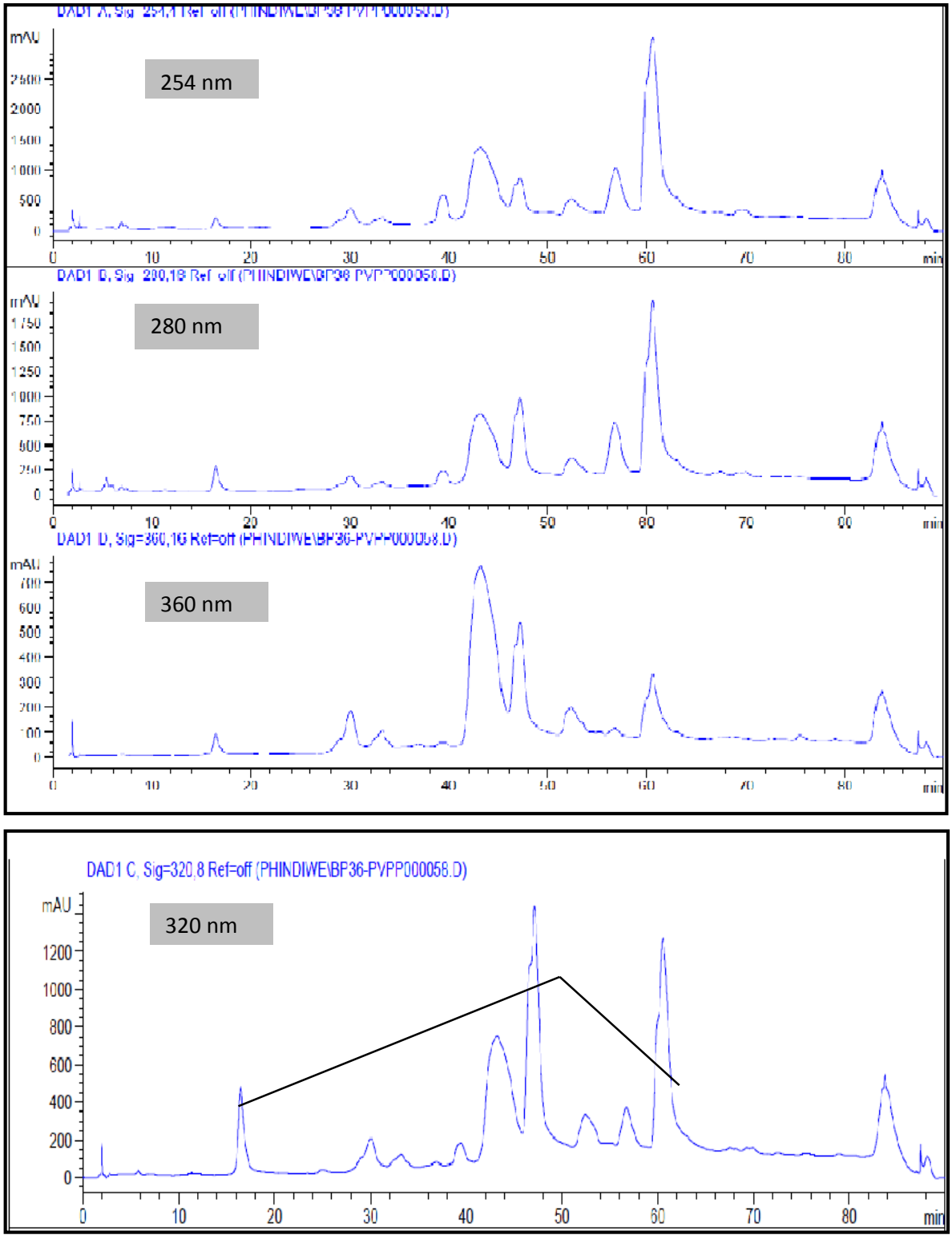
4.1.2.4 Semi prep HPLC-guided isolation of polyphenols in the API

Preparative high-performance liquid chromatography (prep-HPLC) is a method needed to satisfy the purity specifications required on a routine basis, and it is also an important industrial separation process for the isolation and purification of pharmaceuticals and other valuable products.

The precipitate (API) had initially been purified on a semi prep HPLC but no successfully separation was observed. Identifying the correct column was also a problem. Given this reason, it was then decided to determine the semi prep HPLC profile of the Acetone-H_2O extract from a PVPP extraction (explained above in flow diagram 10). It was the only extract subjected to the semi prep HPLC as it showed a well resolved profile, had a better anti HIV activity (IC_{50} = 8 µg/ml) than other extracts (table 4). The Acetone-H_2O extract showed four major peaks at 15-65 min retention time. Please see chromatogram 2 below.

Several runs were conducted so as to develop a reproducible method that can actually separate the compounds in the acetone-H_2O extract. Water and acetonitrile were the solvents used for the method development and optimization. A peak-based collection method was identified as the best collection method, and the best wavelength to detect the peaks was 320 nm. Please see chromatogram 2.

Several fractions were collected from the semi preparative HPLC. The fractions were subjected to phytochemical analysis and HIV screening. TLC analysis (iodine and ammonia spraying reagents), mass spectrometry and NMR analysis were also conducted. The fractions that showed the highest levels of purity were then shown to be flavonoids. The dark spots that were visualized under the 254 nm UV lamp and appeared blue under long (366 nm) wavelength, tested positive after being exposed to an iodine and ammonia vapour which further confirmed the presence of flavonoids.



Chromatogram 2: Semi preparative HPLC profile of the acetone-H_2O extract, at different wavelengths (254 nm, 280 nm, 360 nm and 320 nm).

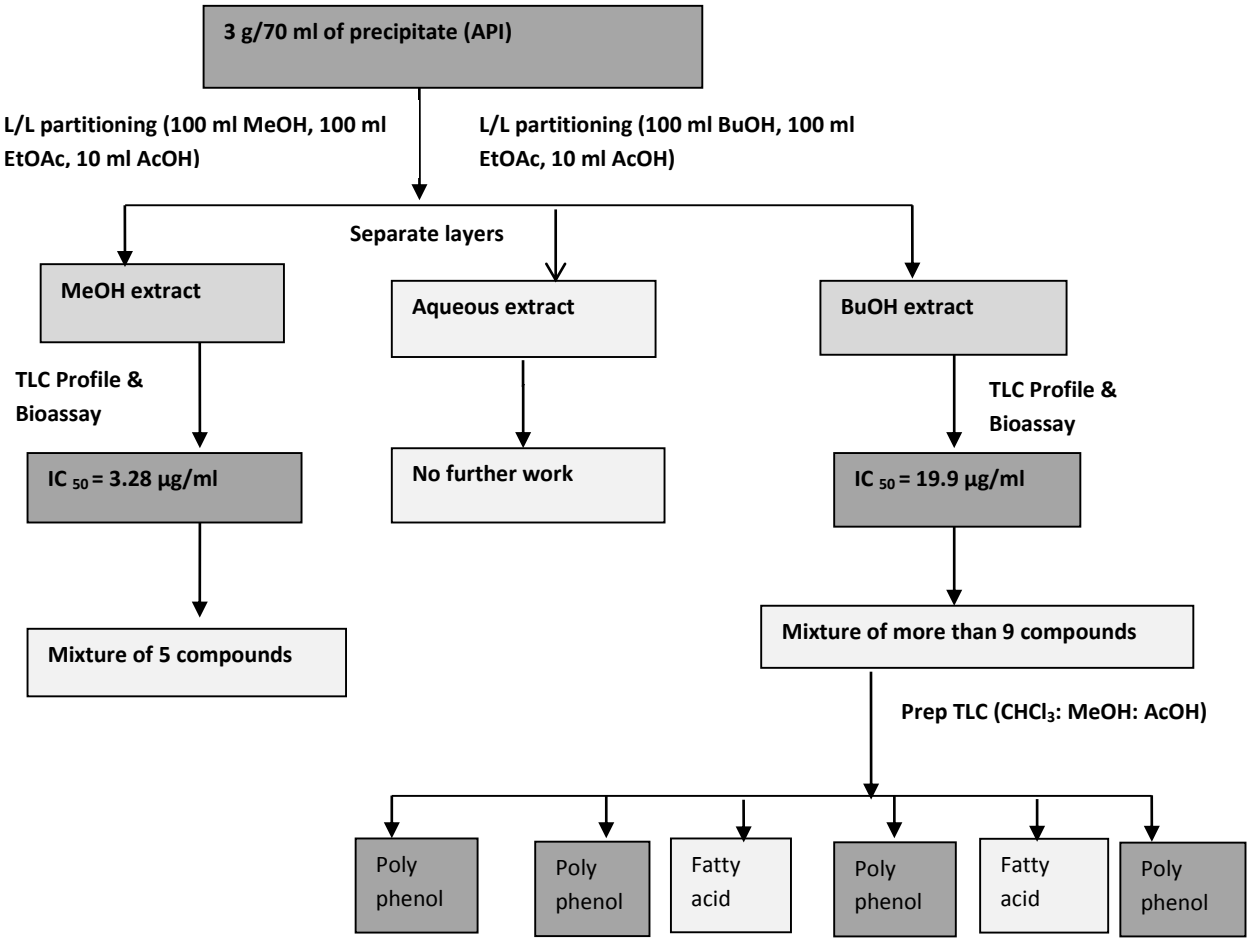
4.1.2.5 Phytochemical detection of Alkaloids in the API

Alkaloids are low molecular weight nitrogenous compounds and about 20% of plant species have been found to contain them. They are mainly involved in plant defense against herbivores and pathogens but can be utilized by humans (Roeder 1999). Plants containing these alkaloids have been used throughout history as poisons, but many of the alkaloids do have valuable pharmaceutical properties (Roeder 1999).

The plant investigated in the present study is from the Boraginaceae family which is known to possess pyrrolizidine alkaloids. These types of alkaloids are known to be effective plant toxins. This class of compound is primarily restricted to Boraginaceae, Asteraceae, Fabaceae, and Orchidaceae families (Roseman 2007).

4.1.2.6 Liquid- Liquid extraction of the API

Liquid–liquid extraction, also known as solvent extraction and partitioning, is a method to separate compounds based on their relative solubility in two different immiscible liquids, usually water and an organic solvent. It is an extraction of a substance from one liquid into another liquid phase (Berthod & Carda-Broch 2004). This technique was employed in an attempt to separate the compounds in the API (precipitate) and the flow diagram followed towards this end is shown below as Flow Diagram 11.



Flow diagram 11: Illustration of liquid-liquid partitioning of the API of *L trigonus,* the separation, isolation of compounds and anti-HIV activity of the organic layers (BuOH and MeOH).

4.1.2.7 TLC profiling of the extracts (BuOH and MeOH)

Anti-HIV screening revealed that the MeOH extract was a more active extract than the BuOH extract (see flow diagram 11 above). The TLC profile of BuOH revealed about nine (see fig 9 & 10) chemical components with relative high intensity whereas MeOH extract showed about five (see fig 8) with a low intensity. It was, thus, evident that the BuOH partitioning process extracted more compounds from the API than MeOH. That could be the reason why it was less active than MeOH, while another reason could be the compounds that were responsible for the anti-HIV were probably in low concentration in the BuOH extract.

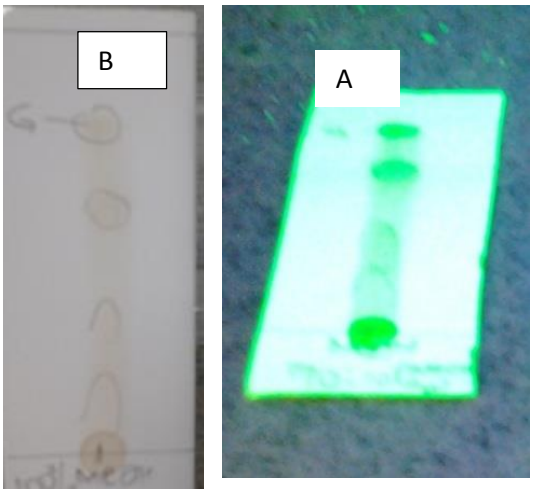


Figure 8: MeOH extract profile, TLC plate A, under UV lamp. TLC plate B, exposed to ammonia vapour for 15 min.

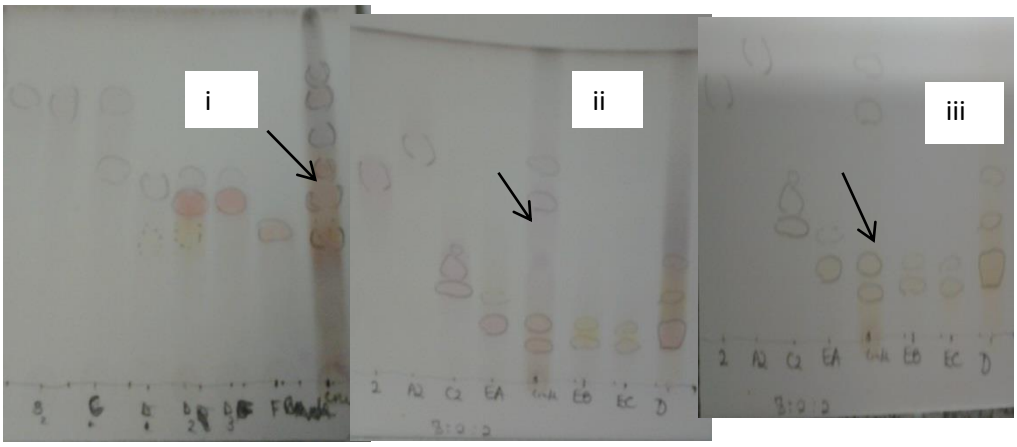


Figure 9: Separated compounds (labelled on the plates as 2,A2,C2,EA, EB,B,C,D1,D2,D3,F) from BuOH extract by prep TLC, plate (i) & (ii) were sprayed with vanillin and plate (iii), exposed to ammonia vapour (see chapter 7 for detailed methodology)

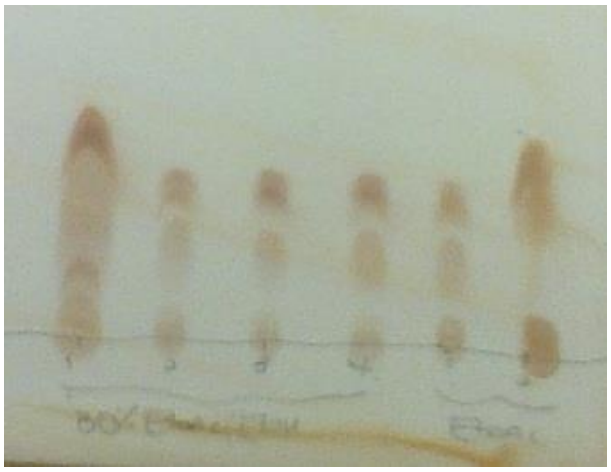


Figure 10: BuOH fractions from VLC column, TLC plate sprayed with ninhydrin (see chapter 7 for further details)

It was, therefore, concluded that BuOH extract should be purified further to isolate compounds. That inference was based on the fact that it had a better yield than MeOH. Compounds in the MeOH extract could not be separated even though it had a better activity. Prep TLC technique was employed to separate the chemical constituents showed on the plates above (fig 9 & 10). That brought about seven pure compounds being isolated but from the seven, only one compound was structurally elucidated (rosmarinic acid (BP36-117-26503-2), fig 12). BuOH extract was always used as the reference sample (labelled as “crude” on the TLC plate) in tracing the impurities on the pure compounds and/or fractions.

4.1.2.8 Nuclear magnetic resonance and its application

Nuclear Magnetic Resonance (NMR) spectroscopy is an analytical chemistry technique used in quality control and research for determining the content and purity of a sample as well as its molecular structure. For example, NMR can quantitatively analyze mixtures containing known compounds. For unknown compounds, NMR can either be used to match against spectral libraries. Once the basic structure is known, NMR can be used to determine molecular conformation in solution as well as

studying physical properties at the molecular level such as conformational exchange, phase changes, solubility, and diffusion.

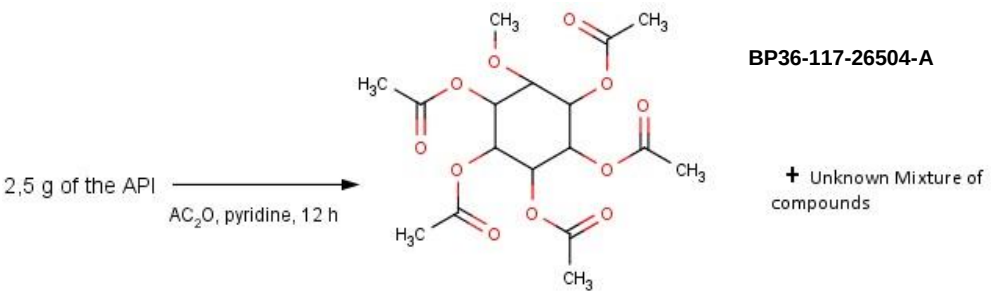
The in-house NMR Facility is currently home to four solution-state NMR spectrometers: Varian 400, 500, and 600 MHz spectrometers and a Bruker 800 MHz spectrometer. All spectrometers are equipped with multiple solution-state NMR probes for direct or indirect detection of $^{1}H/^{19}F$ and low frequency nuclei such as ^{13}C, ^{31}P, and ^{15}N. The 600 and 800 MHz spectrometers are also each equipped with a cryoprobe. This technique is used to identify and/or elucidate detailed structural information about chemical compounds. For example:

- Determining the purity of medicines before they leave the factory
- Identifying contaminants in food, cosmetics, or medications
- Challenging small molecule projects, such as studies of low-quantity of natural products

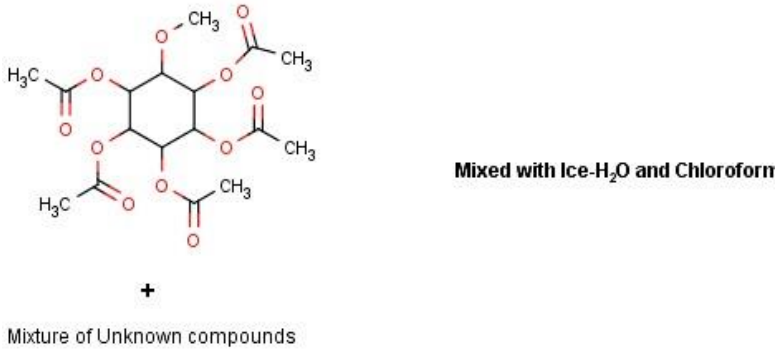
The principle behind NMR is that many nuclei have spin and all nuclei are electrically charged. If an external magnetic field is applied, an energy transfer is possible between the base energy to a higher energy level (generally a single energy gap). The energy transfer takes place at a wavelength that corresponds to radio frequencies and when the spin returns to its base level, energy is emitted at the same frequency. The signal that matches this transfer is measured in many ways and processed in order to yield an NMR spectrum for the nucleus concerned (http://www.chem.ucsb.edu/facilities#nmr).

4.1.2.9 Acetylation reaction of the API

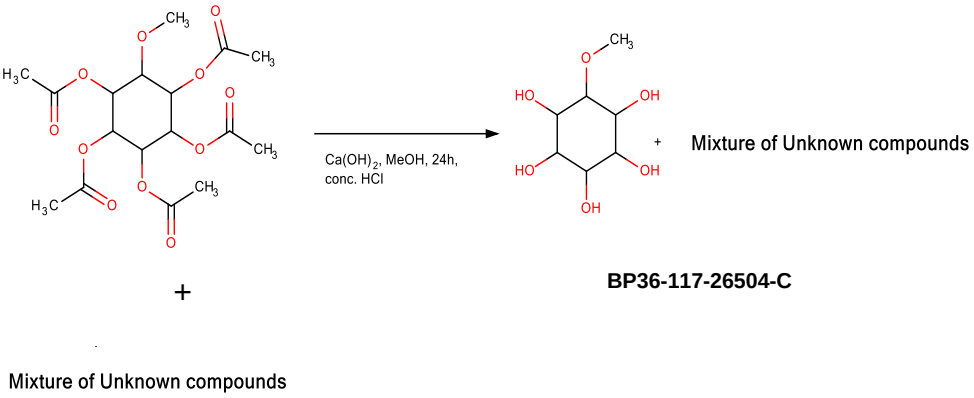
The last attempt to purify the API was to acetylate and hydrolyse the API. This was achieved by treating 2.5 g of the API with acetic anhydride and pyridine for 12 hrs. The reaction mixture was then poured over in a mixture of ice H_2O-chloroform and re-extracted. The layer of interest was the chloroform ($CHCl_3$) layer, which was then evaporated to yield 1.8 g of a crude chloroform fraction.



A) Acetylation step



B) Extraction step



C) Base hydrolysis reaction

Figure 11: Summary of the acetylation reaction process

4.2 Results and Discussion

From the above analyses, it was decided that the BuOH extract should be the one selected for further purification. All the general spraying reagents, ninhydrin, iodine, ammonia vapour and vanillin tested positive showing the presence of polyphenols, fatty acids and alkaloids in the BuOH extract (see fig 9 and 10 above).

To separate these compounds EtOAc: MeOH: DCM: FA and $CHCl_3$: MeOH: AcOH solvent systems were utilised during preparative thin layer chromatography (prep TLC) of the extract. The seven isolated compounds were identified as four polyphenols and three alkaloids. They were analysed using NMR and UPLC-MS However, only one compound was fully elucidated and characterised and it was identified as Rosmarinic Acid (RA) which is a known compound from the Boraginaceae family but to the best of our knowledge, it has never been reported from *L. trigonus*. The structure of this compound is shown in Figure 12 below.

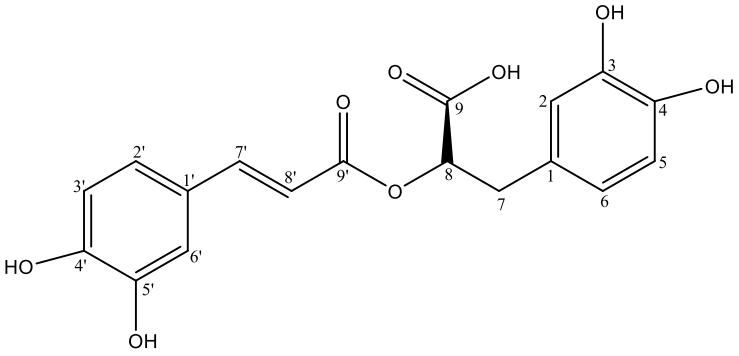


Figure 12: Compound **BP36-117-26503-2**

Rosmarinic acid is a well-known polyphenol which has been previously reported as an anti-oxidant and antiviral agent. Originally identified in rosemary (*Rosmarinus officinalis* L.), the structure was elucidated as an ester of caffeic acid and 3-(3, 4-dihydroxyphenyl) lactic acid (Bhatt *et al*. 2013).

This compound has also been reported to occur in several taxonomically non-related families of the plant kingdom. It has attracted much attention since it was identified to be the main compound responsible for the antiviral activity of lemon balm in treating

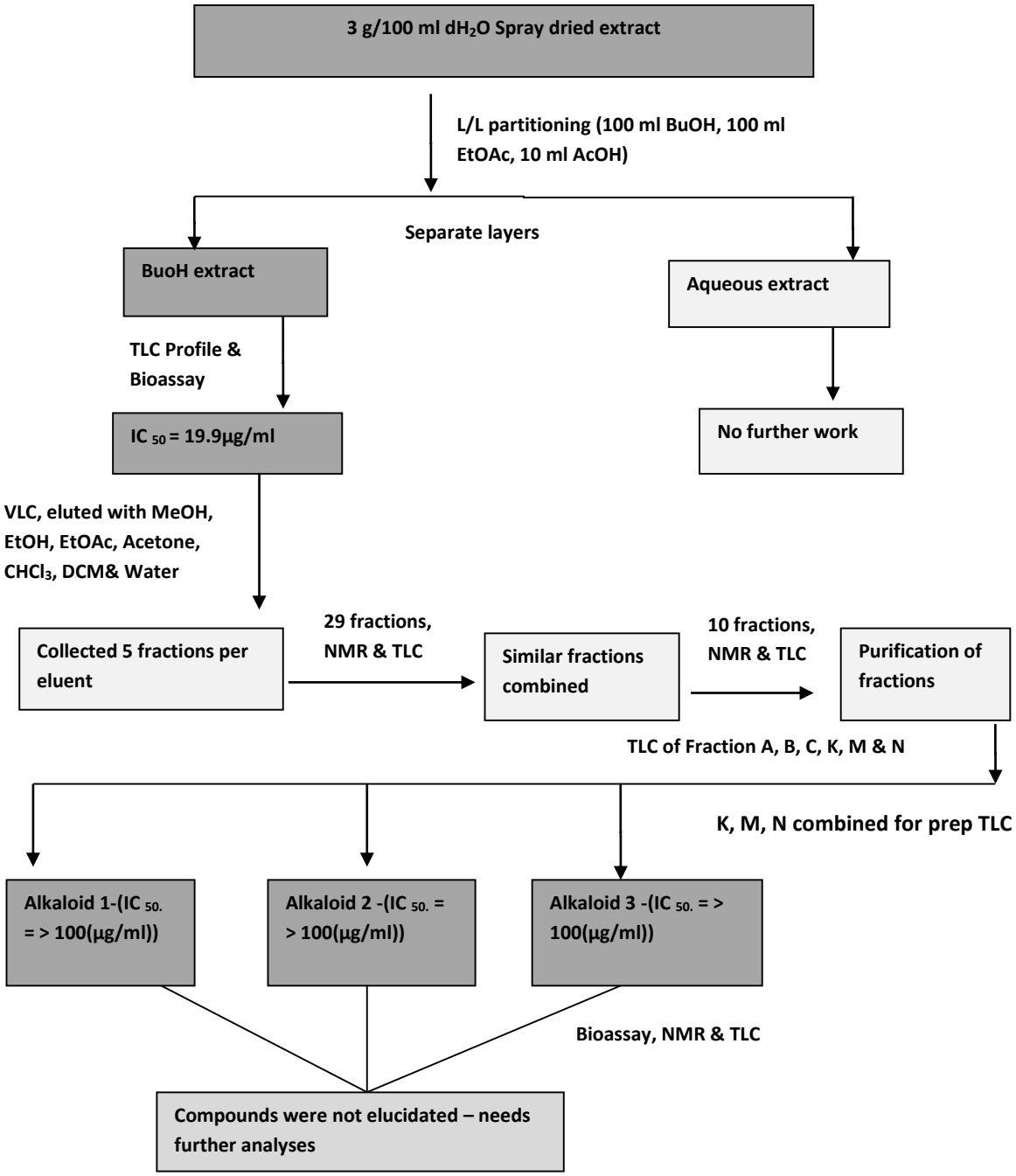
Herpes simplex (Toth *et al*; 2003). In the present study compound BP36-117-264503-2 (identified as Rosmarinic acid) showed weak anti-HIV activity with an IC_{50} value of 111 µg/ml.

Table 4: Summary of the anti-HIV results of the isolated compounds from the Liquid-liquid partitioning chromatography

SAMPLE NAME	PURITY	SOLVENT	IC_{50} (µg/ml)	METHOD
BP36-117-26464C (API)	mixture	Water	**0.04**	EtOH precipitation
BP36-117-26504-MeOH extract	mixture	Water	**3.26**	Liquid-liquid extraction
BP36-117-26504-Acetone/H_2O	mixture	DMSO	**8.83**	PVPP extraction
BP36-117-26504-Butanol extract	mixture	DMSO	**19.9**	Liquid-liquid extraction
BP36-117-26503-2	Polyphenol (pure)	DMSO	**111**	Prep TLC
BP36-117-264504-EB	Semi pure	DMSO	**357.31**	Prep TLC
BP36-117-264504-C2	Semi pure	DMSO	**242.88**	Prep TLC
BP36-117-264504-A2	pure	DMSO	**310.43**	Prep TLC
BP36-117-264504-EA	pure	DMSO	**496.89**	Prep TLC
BP36-117-264504-DE2	alkaloid (pure)	DMSO	**85.24**	Prep TLC
BP36-117-264504-F	Polyphenol (pure)	DMSO	**22.24**	Prep TLC
BP36-117-264504-(acetone 1)	Alkaloid (pure)	DMSO	**486.54**	Prep TLC
BP36-117-264504-(50% MeoH/Water)	Alkaloid (pure)	DMSO	**473.42**	Prep TLC

4.3 Isolation of alkaloids in the API

The alkaloids isolated in this present study could not be confirmed as pyrrolizidine alkaloids. Flow diagram 12 below illustrates clearly how the API was purified through Liquid-liquid partitioning and ultimately isolating three alkaloids shown on a TLC plate below - please see Figure 13.



Flow diagram 12: Indicating the step-by-step isolation of the alkaloids in the API

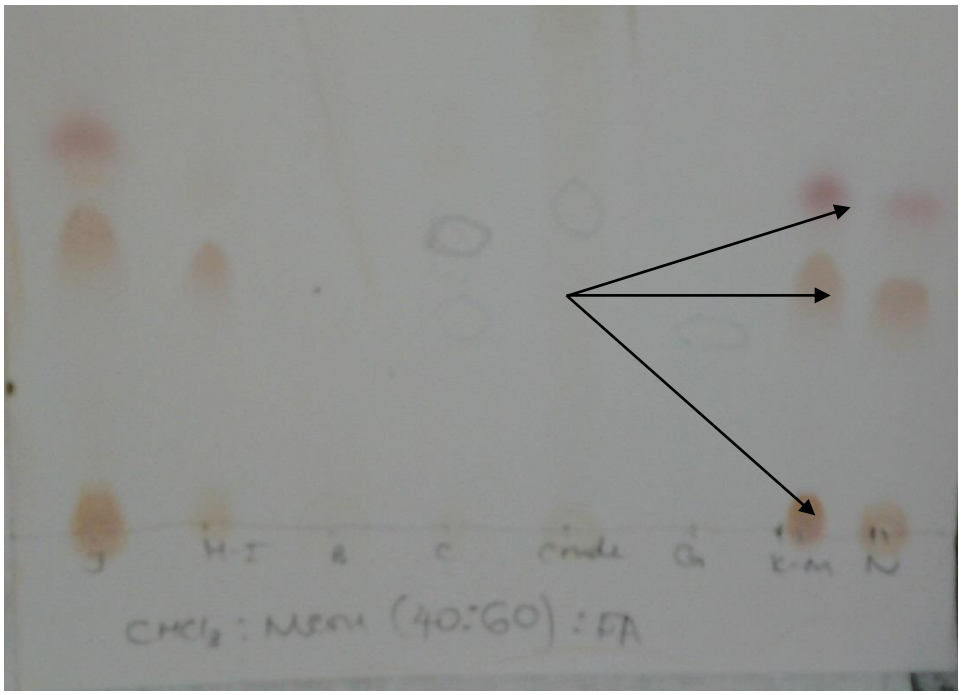


Figure 13: A TLC plate showing 3 alkaloids (three arrows pointing at three spots) that were determined using $CHCl_3$ (40): MeOH. (60)

The structural elucidation of the three alkaloids could not be determined due to the complexity of their structures.

4.4 Acetylation of the API

Section 4.2.5 describes this process broadly (see fig 11). The chloroform fraction was partitioned on silica gel and two major compounds were isolated using a Hexane: EtOAc (8:2) solvent mixture. The TLC plate was developed in Hex: EtOAc (6:4) solvent system. The two compounds were identified as methyl pentacetate (**BP36-117-26504-A)** and sucrose derivative/s **(BP36-117-26504-B**). The isolated compounds were then hydrolysed, to replace the acetates by OH groups resulting in two well-known compounds called Bornesitol or methyl inositol **(BP36-117-26504-C)** (fig 14) and sucrose in their pure form. They were then screened for anti-HIV activity.

The results showed no activity (an IC_{50} value of >500 µg/ml which was equated as no activity) from these compounds.

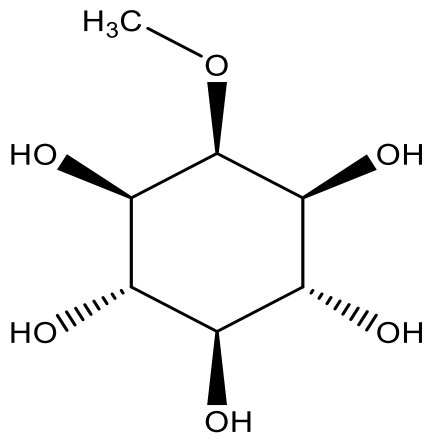


Figure 14: Compound **BP36-117-26504-C**

In trying to purify and separate all these compounds in the API, it appeared that these compounds may be inter-dependent or reliant on one another to produce significant anti-HIV activity, something that was experienced during the preliminary purification of the API (section 2.3.1). This could explain why it was not possible to purify the API and still retain the same magnitude of activity. There was no advantage in trying to separate the compounds in the API for formulation into a microbicide since formulations into a gel and caplets would be conducted using the API as a whole. Separation and isolation of the compounds in the API was attempted so that all the classes or individual compounds in the API can be identified.

4.5 Molecular characterization of the API

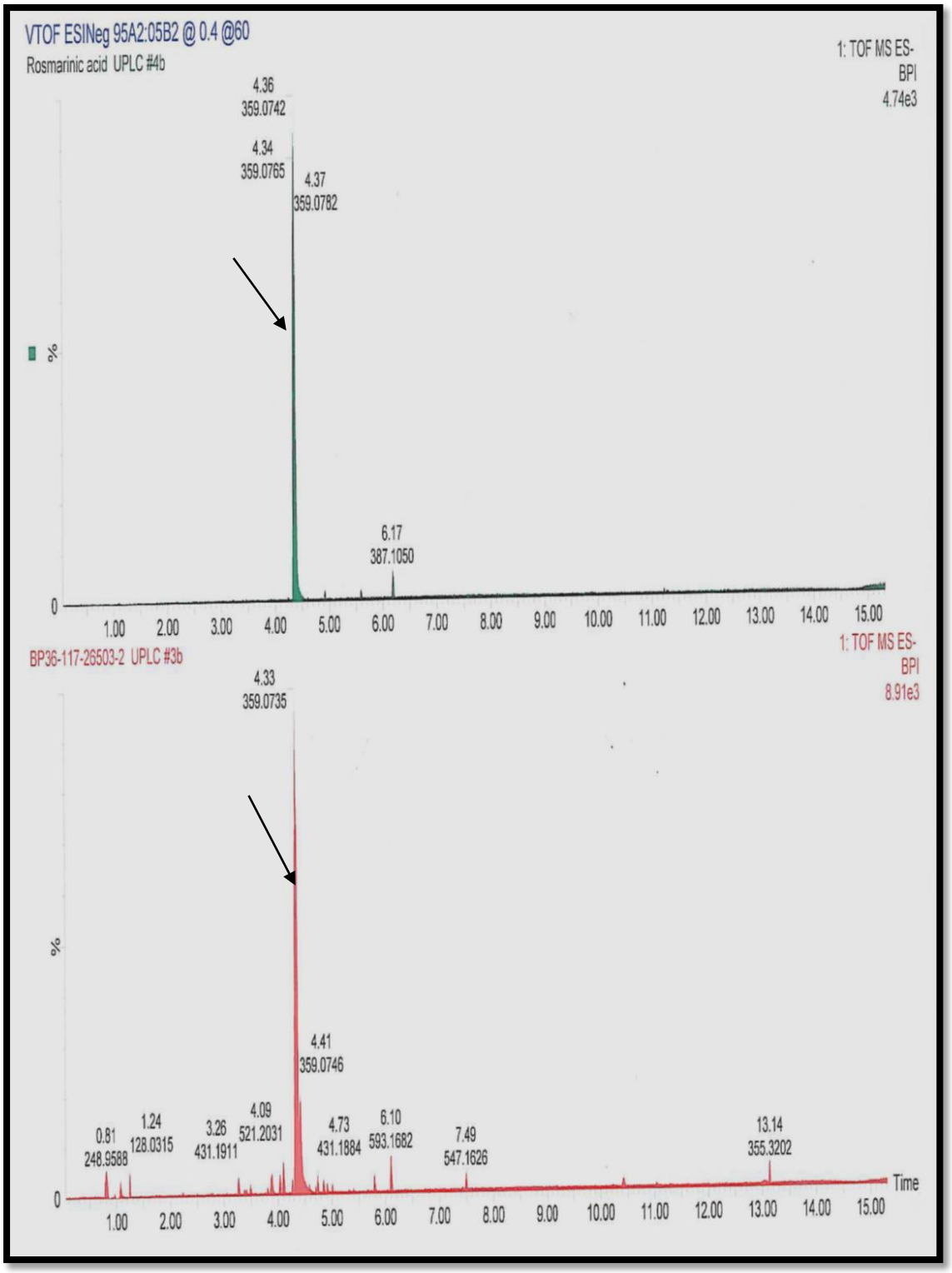
This section will provide details regarding the characterization of the API and the isolated compounds. Since it was discovered that the API was a big polymeric molecule, it was difficult to fully characterize it as it needed specialized techniques.

However, the isolated compounds and the API were partially characterized by NMR, GC and UPLC-MS / TOF MS ESI and HPLC-ESI.

4.5.1 Mass spectroscopy

Compound **BP36-117-264503-2** (Fig 12) was isolated as a yellow semi-solid powder. The TOF MS-ESI (chromatogram 3) showed a molecular ion $[M-H]^-$ peak at m/z 359 corresponding to a molecular formula $C_{18}H_{16}O_8$. This compound was identified as the known compound called rosmarinic acid. A standard of rosmarinic acid was purchased from Sigma-Aldrich for comparison. Mass spectrometer (MS), thin layer chromatography (TLC) (fig 15) and NMR (spectrum 1 and 3) of the standard provided a 100% match with the compound BP36-117-264503-2 (see chromatogram 3 below).

Figure 15: TLC plate of Rosmarinic acid (right spot) and **BP36-117-264503-2** (left spot)



Chromatogram 3: TOF MS-ESI profile of Rosmarinic acid and **BP36-117-264503-2**

BP36-117-26504-C was also analysed through High Resolution Mass spectrometry (MS). It showed a molecular ion peak of 194 g/mol corresponding to the molecular formula $C_7H_{14}O_6$. This compound was identified as the known compound bornesitol which to the best of our knowledge has never been isolated from this plant species before.

The API, on the other hand, was analysed using high resolution mass spectrometry and found to have a molecular weight in the region of 10 – 20 kDa. In the current form (active precipitate), it was considered difficult to develop the API as an oral treatment for HIV, as high-molecular weight agents often have poor bioavailability following oral administration.

However, large molecules with potent anti-HIV activity are ideal for topical use and potent development as a microbicide. Unlike the other carbohydrate microbicides which have poor anti-HIV properties (Balzarini 2007), *L. trigonus* produced an API with significant anti-HIV activity and was demonstrated to be ideal to be used topically as a microbicide (See chapter 5 for microbicide formulation studies).

4.5.2 HPLC-ESI

Polymerization is a process of reacting monomer molecules together in a chemical reaction to form polymer chains or three-dimensional networks. To confirm if there was any degree of polymerisation in the API, it was compared with two other polymers (poly-galacturonic and galacturonic acid), using HPLC-ESI.

4.5.2.1 Method

This was carried out by extracting the aerial plant parts in boiled and cold water. The aqueous extracts (potential API's) were collected at different times (T_0 –T_4) i.e. 5 samples were collected

Five grams of the aerial plant parts was extracted using 100ml of hot water at 90°C.

Five samples were collected at 2 hours different intervals. i.e. T_0, T_1…T_4.

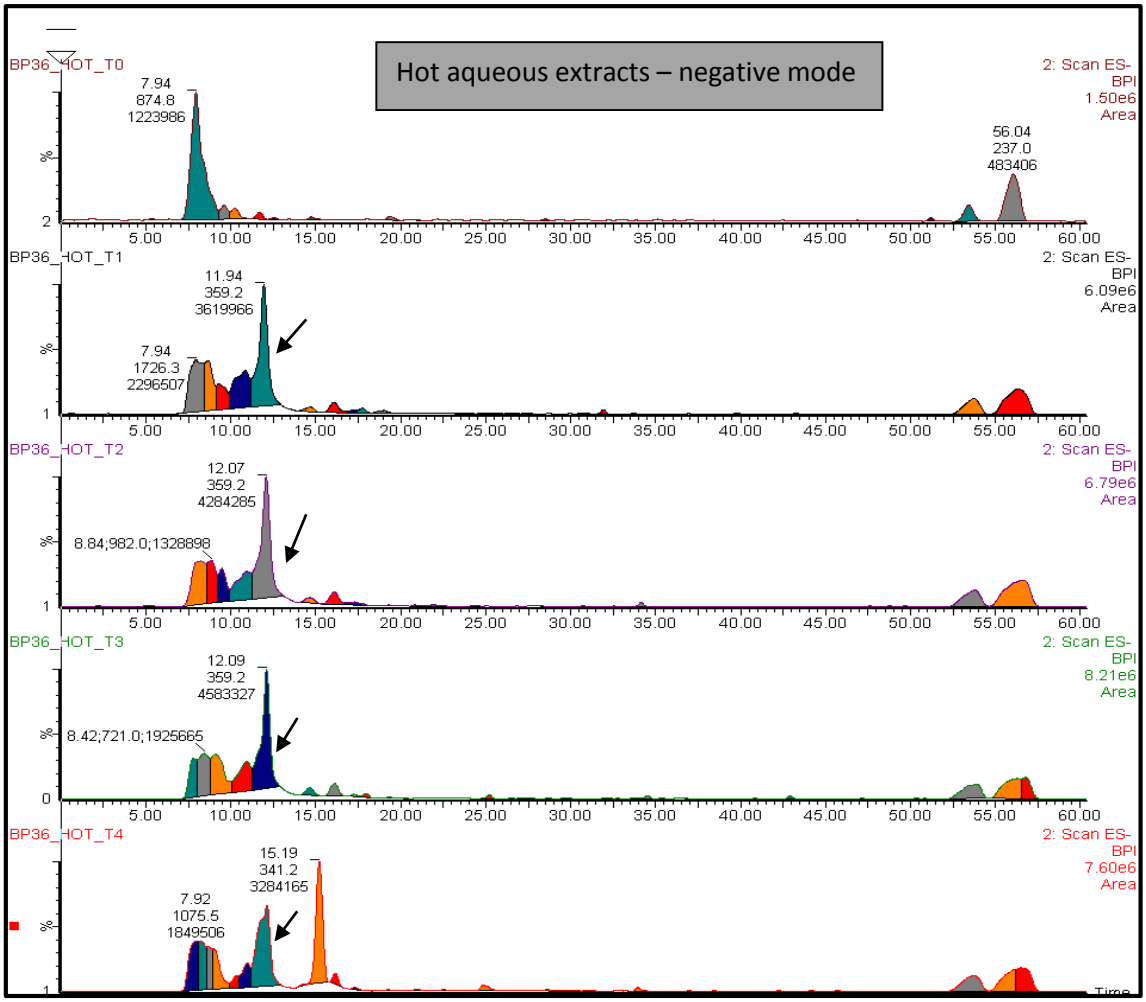
Two millilitres (2 ml) of each sample was analysed immediately using HPLC-ESI.

The same was done for the cold extracts, performed at 4°C with cold distilled water. Below are the chromatograms showing how the API behaves after a long extraction (cold and hot water).

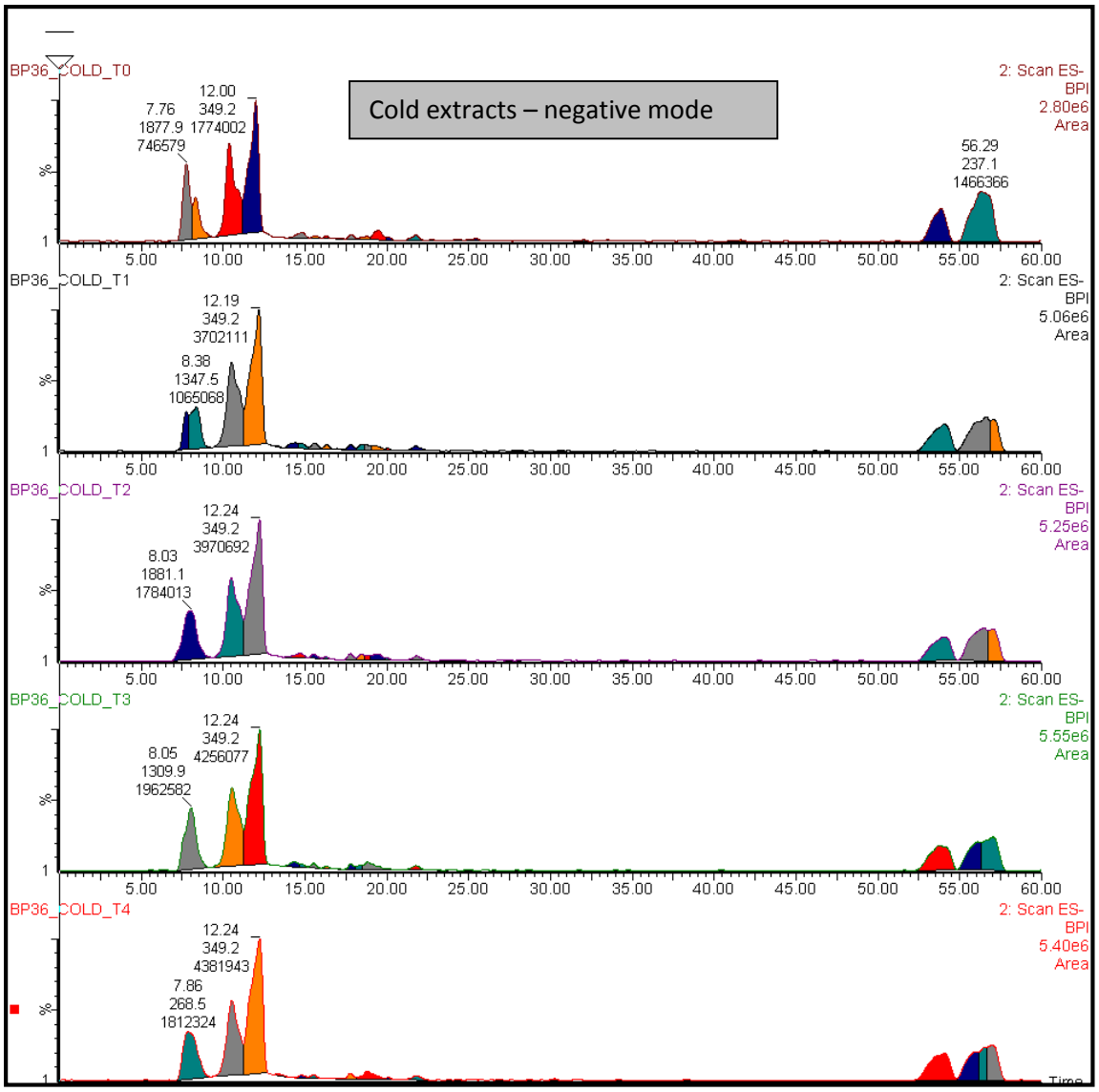
4.5.2.2 Results

The negative ion mode was more sensitive as it revealed more constituents and a better separation profile after a prolonged extraction. The more time the aerial parts of the plant were soaked in water (cold or hot), the more it polymerised (i.e. by the number of peaks appearing in the lower retention region of the chromatogram of T_1 – T_4). This also explained the differences in the activity between the extracts prepared from the processed (oven dried at 80°C and ground) and unprocessed aerial parts of the plant (fresh leaves and stems) please refer to chapter 3, section 3.1.1.2 for further details.

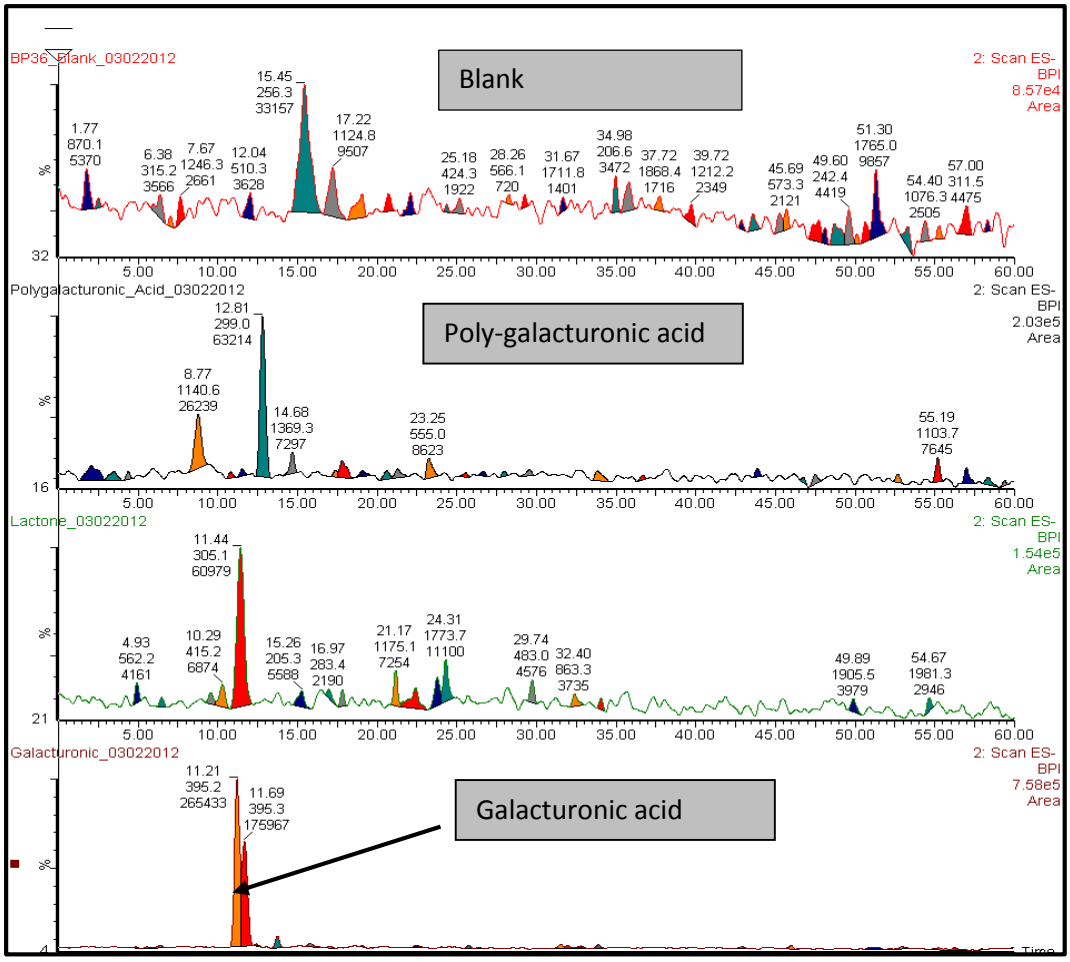
The HPLC chromatogram (see chromatogram 4 below) revealed that something was happening after at least 2 hours of extraction, as more peaks or components appeared. The API was compared with a galacturonic acid, which was assumed to be the main polymer it contained. This polymer was found to be present in massive amounts although it exhibited no anti-HIV activity. It had a retention time of between 10 – 15 minutes. See chromatograms below.



Chromatogram 4: ESI$^-$ negative ion of the hot extract



Chromatogram 5: ESI$^-$ negative ion of the cold extract



Chromatogram 6: ESI$^-$ negative ion of the three standards (polymers) and the blank

4.5.3 Gas chromatography of the API

Gas chromatography (GC) has been used as a powerful qualitative and quantitative tool for carbohydrate analysis. Prior to analysis by GC, compounds containing functional groups with active hydrogens such as COOH, OH, NH, and SH needed to be protected and converted into volatile derivatives, amenable to GC analysis. Compounds with these functional groups tend to form intermolecular hydrogen bonds, reducing volatility (Guan *et al*; 2010).

The current study describes a method for the qualitative and quantitative determination of free carbohydrates and polysaccharides in the API by GC. Their saccharide content was also compared. This was achieved through methanolysis

and silylation of five monosaccharides, namely, arabinose, xylose, mannose, glucose, galactose and mannitol as the internal standard and also two polymers namely, glucuronic acid and galacturonic acid.

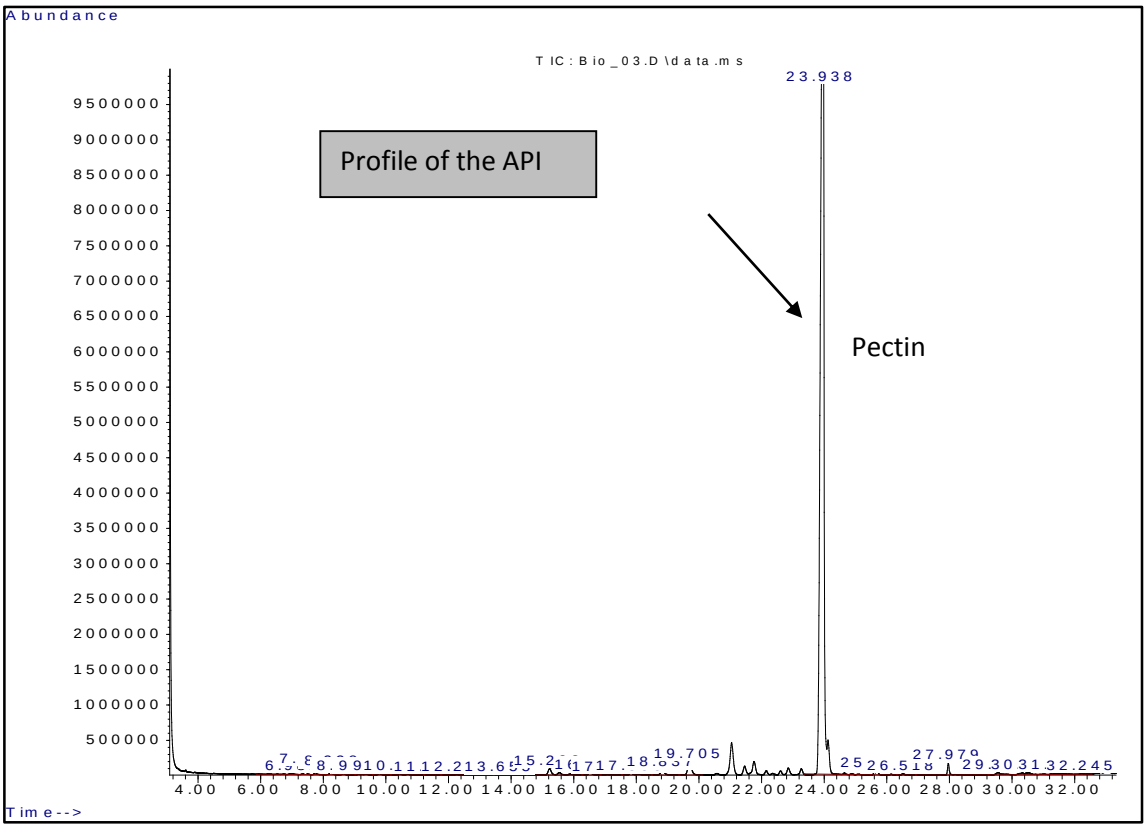
4.5.3.1 Methanolysis and Silylation process

During silylation which is the most widely used derivatisation scheme for GC, trimethylchlorosilane (TMCS), which is known to be the simplest reagent, was used in conjunction with HMDS and pyridine to improve the silylation of sugars and related compounds.

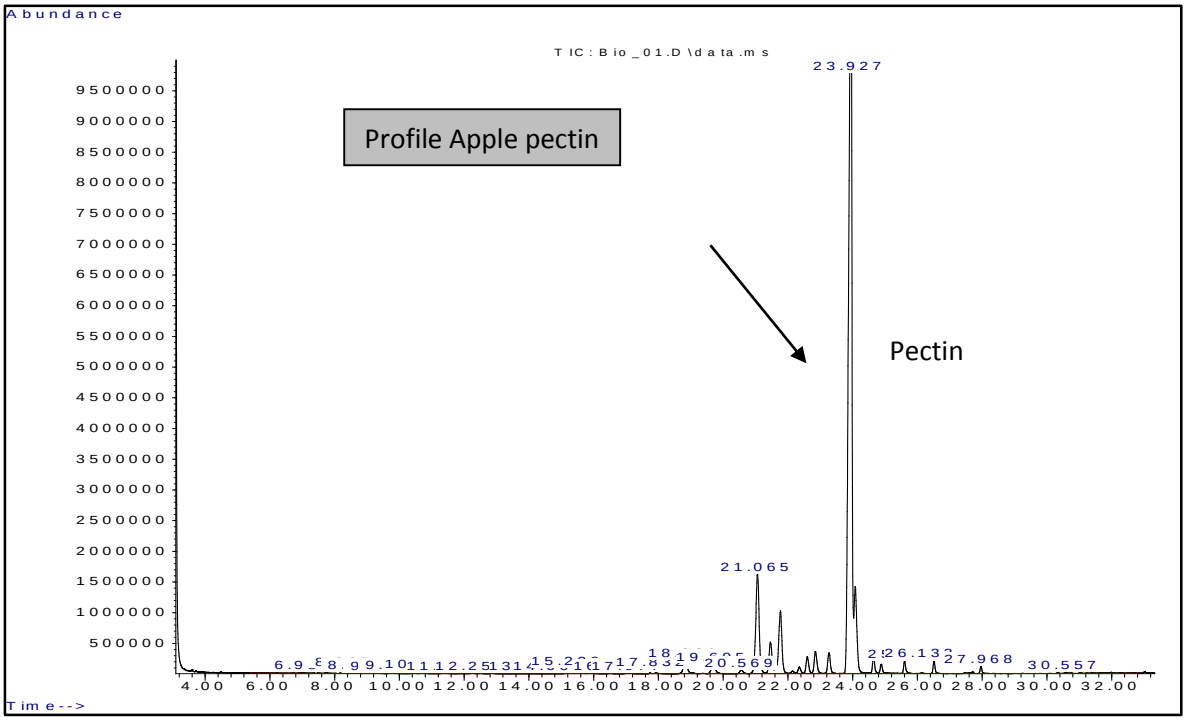
The results showed that the API (precipitate) contained more than 30% of carbohydrate content with galacturonic acid dominating at 36.3%. Please note the results in Table 5. Gas chromatography analysis also proved the API chemical composition to be the same as that of apple pectin. Please note chromatograms 7, 8 and 9. Galacturonic acid is the major component of pectin, which also explains why galacturonic acid is seen as the major compound in the API even though it does not have any anti-HIV properties. The order of how these sugars are held together in the API is still to be investigated.

Table 5: GC analysis of the API, illustrating the carbohydrates content**.**

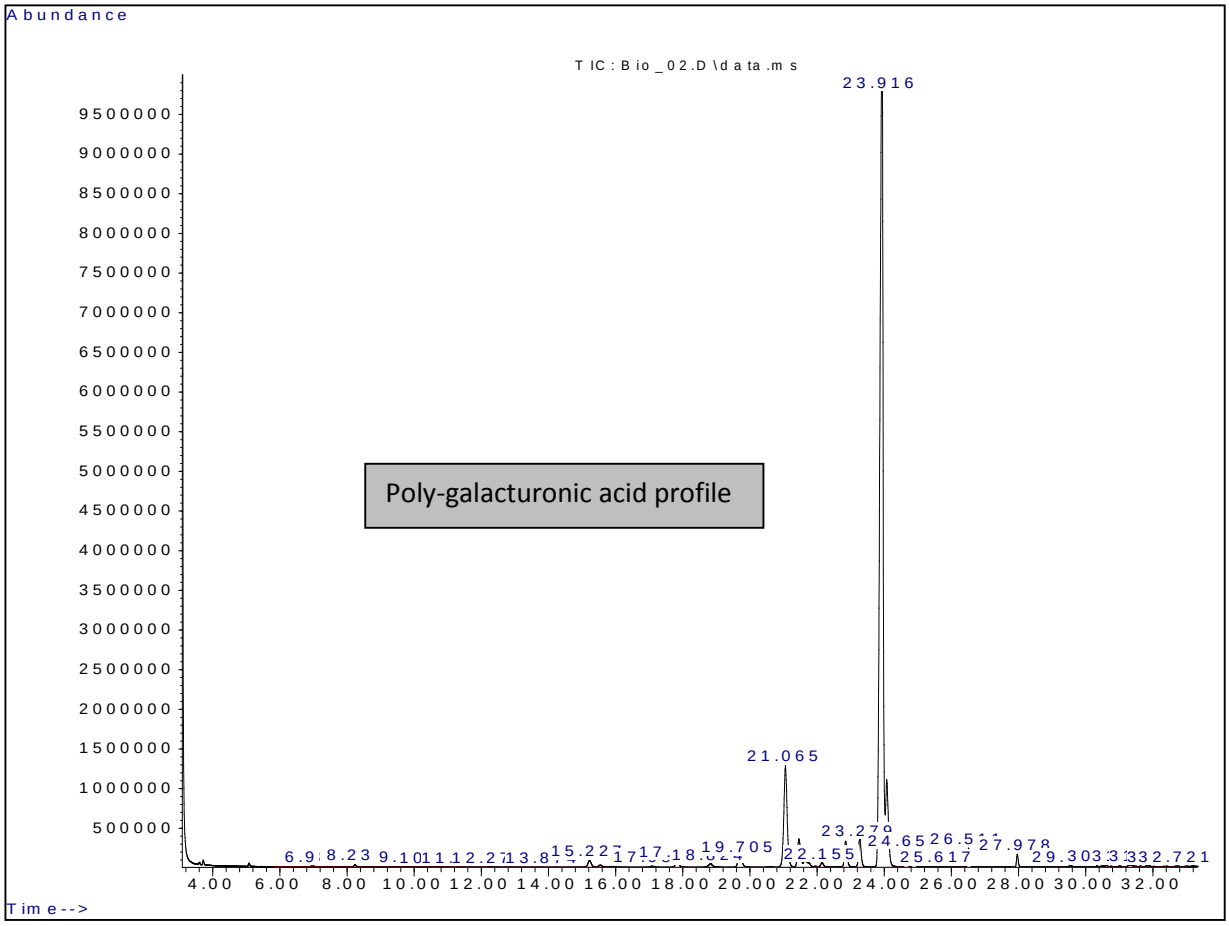
Name	% of total carbohydrate	Description
Arabinose	11.5	Monosaccharide
Rhamnose	5.5	Monosaccharide
Xylose	1.9	Monosaccharide
Galactose	10.5	Monosaccharide
Glucose	26	Monosaccharide
Mannose	4	Monosaccharide
Glucuronic acid	4.3	Polymer
Galacturonic acid	**36.3**	**Polymer**



Chromatogram 7: GC chemical profile of the API



Chromatogram 8: Apple pectin



Chromatogram 9: Poly-galacturonic acid

4.5.4 Structural elucidation of compound, BP36-117-26503-2

The NMR data of compound BP36-117-26503-2 is indicated in Figure 12, and is collated in Table 6. The ^{13}C NMR spectrum of this compound displayed (spectrum 4) a total of eighteen carbon signals. One CH_2 carbon, eight CH carbons, and seven non-protonated carbons were identified.

Two dimensional (2D's) NMR experiment, gHSQC, gCOSY, gHMBC allowed the correlation of all protonated carbon atoms with well-resolved protons and correlations between protons. From this information there was a construction of the structural fragment of the compound and later was confirmed to be rosmarinic acid, a known polyphenol. The proton (^1H) NMR spectrum of this compound (spectrum 3) displayed signals with chemical shifts and coupling constants comparable with literature data for rosmarinic acid.

Table 6: ^{13}C and ^{1}H NMR spectral data

Literature, CD_3OD Bruker Avance 500 MHz (Rosmarinic acid)		**Experimental, CD_3OD Varian 600MHz (BP36-117-26503-2)**	
^{13}C (δ)	**^{1}H (δ)**	**^{13}C (δ)**	**^{1}H (δ)**
174.3	7.52, 1H, d	178.6, C-9	7.51, 1H, d, H-7’
169.4,	7.04, 1H, d	170.3, C-9’	7.01, 1H, d, H-2’
149.5	6.91, 1H, dd	150.5, C-4’	6.90, 1H, dd, H-6’
145.7	6.75, 1H, d	147.9, C-7’	6.75, 1H, d, H-5’
145.0	6.70, 1H, d	147.8, C-3	6.65, 1H, d, H-2
144.9	6.69, 1H, d	147.1, C-4	6.61, 1H, d, H-5
143.2	6.57, 1H, dd	146.0, C-3’	6.60, 1H, dd, H-6
130.4	6.26, 1H, d	132.4, C-1	6.26, 1H, d, H-8’
128.2	5.19, 1H, dd	129.2, C-1’	5.08, 1H, dd, H-8
122.2	3.0, 1H, dd	124.1, C-6	3.1, 1H, dd, H-7a
121.4	3.00, 1H, dd	123.0, C-6’	2.98, 1H, dd, H-7b
117.6		118.7, C-2	
116.3		117.7, C-5	
116.1		117.4, C-5’	
115.8		116.9, C-8’	
115.3		116.4, C-2’	
77.8		78.9, C-8	
38.9		40.0, C-7	

d- doublet, dd – double of doublet

4.5.5 Structural elucidation of compound (BP36-117-26504-C)

The NMR data of compound BP36-117-26504-C (fig 14) is summarized in Table 7. The ^{13}C NMR spectrum of this compound (spectrum 7) displayed a total of seven carbon signals with 6 CHs and one OCH_3. The proton (^1H) NMR spectrum of this compound (spectrum 6) displayed signals with chemical shifts and coupling constants comparable with literature data for bornesitol.

Table 7: ^{13}C and ^1H NMR spectral data for BP36-11-26504-C (fig 14) and bornesitol (Ichimura, 1998)

Experimental in D_2O		**Literature in CD_3Cl**	
^{13}C (δ)	**^1H (δ)**	**^{13}C (δ)**	**^1H (δ)**
80.41	3.1, 1H, dd	80.52	3.19, dd
67.36	4.2, 1H,t	67.66	4.29, 1H, t
70.98	3.39, 1H, dd	71.09	3.48, 1H, dd
72.17	3.3, s	72.28	3.26, 1H, s
74.33	3.5, 1H, t	74.45	3.61, 1H, t
71.55	3.18, 1H, t	71.65	3.19, 1H, t
56.62		56.72	

4.6 Conclusion

After the API was partially characterized, about 57 g of it was produced for the development and formulation of the API into a microbicide. The active ingredient (BP36-117-26464C) in the spray dried extract (aqueous extract) was isolated and concentrated through an ethanol precipitation process. The plant drying process was

shown to contribute significantly to the formation of this API i.e. oven drying and grounding the aerial plant parts (chapter 3).
The API production method was optimised and refined, with the energy-intensive spray drying process being eliminated. The precipitate, which has been identified to consist of galacturonic acid and other small compounds, has shown potency against several HIV clinical isolates which was comparable and in certain cases better than T20 and Tenofivir, which are two commercial anti HIV drugs.

The biggest challenge in this present study has been the characterisation of the API as it is a complex carbohydrate with a high molecular weight. Although it is not critical to completely characterise the API, further preclinical work such as the release studies (chapter 5) are dependent on a means of detecting and quantifying the API. Due to its polymeric nature, the profiling of the API using standard analytical methods was not feasible. Several alternative techniques were investigated. Ultimately a method was developed that relies on the methanolysis of the API with the subsequent formation of silyl derivatives which were then analyzed by Gas Chromatography. Please see details of this in section 4.5.3.1.

Since the API also contains a large percentage of galacturonic acids, which are the building blocks of pectin, it was decided to compare the chemical profiles of polygalacturonic acid, apple pectin, citrus pectin, and galacturonic acid with that of the API. This polymeric material (API) was found to be very similar in chemical composition to apple pectin. Please see chromatograms 7, 8 and 9 indicating this.

Neither apple pectin nor polygalacturonic acid were found to have anti-HIV activity, so even if they are similar in chemical composition with the API, it doesn't mean these are responsible for anti-HIV activity in this plant. It is possibly the unique folding of this polymer or its metameric character that is responsible for the activity of the API.

Since the chemical composition of the API was similar to that of apple pectin and yet the pectin showed no ant-HIV activity, this required further investigation of the other components present in the API. For that purpose, chromatographic techniques were employed and four classes of compounds were detected phytochemically, that led to

isolation of eight compounds (1 methyl cyclitol, 3 alkaloids and 4 polyphenols). Due to complexity of the structures, only two compounds (Rosmarinic acid and Bornesitol) were structurally elucidated as noted in Figures 12 and 14.

The API was, therefore, shown to be a mixture of several small molecules and pectin as the major polymer. The presence of pectin in the API was first suspected when it showed a tendency of gelling (during the dissolution). Globally, pectin is known as the gelling agent. The suspicions were later proved during GC analysis by comparing their profiles. Their chemical composition was more or less the same. The eight other compounds that were isolated from the API all showed very weak activity as noted in Table 4. It was, therefore, concluded that all these compounds in the API contribute towards activity, which could explain why the more the API was purified the more it lost activity.

As the main aim of the present study was to identify the best active phyto-pharmaceutical ingredient (API) for further microbicide development into gels and caplets, the conclusion is that this would be the precipitate **BP36-117-26464C**.

CHAPTER 5: CONTRIBUTION OF THE STUDY

5. Development of the API (BP36-117-26464C) as a microbicide

5.1 Microbicides

The main aim of the present study was to isolate and characterize the API from *Lobostemon trigonus*, a plant used by a private traditional healer (TH) in the Eastern Cape region of South Africa, in order to facilitate its development as a microbicide.

Microbicides must meet certain standards in terms of colour, cost effectiveness of production and must be odourless, etc. This chapter will describe the methods used to remove the colour of the API and will provide a summary of the last stages of the development of the API as a microbicide.

The ultimate product (microbicide) will eventually help women to protect themselves from sexual transmission of HIV and other STI's. The best and most active API was selected based on the efficacy i.e. the precipitate named **BP36-117-26464C** with a determined IC_{50} value of 0.04 µg/ml as discussed in Chapter 3. A large-scale batch of the precipitate was prepared for this purpose and half of it was decolourised for gel formulation while the other half was stored for caplet formulations. These processes will be discussed in further detail below.

5.1.1 Microbicide formulations

As the number of HIV-infected women escalates worldwide, vaginal microbicides may help slow the spread of HIV/AIDS. In terms of HIV prevention, a microbicide is a product which is designed to be applied to the vagina or the rectum, in order to reduce the user's risk of HIV infection when HIV exposure occurs. While potential microbicides have most often been developed as gels or creams (similar to a lubricant), they could be developed in a number of different formulations (a coating for a condom, anal douches, tablets or pieces of film, vaginal rings or gels) (Obiero *et al*; 2012).

5.1.2 Pre-steps taken before development of the API into a microbicide

5.1.2.1 Decolourisation of the API

For the API to be developed and formulated into a gel**,** the dark brown colour of the API required removal. Different “decolourisation” techniques were used i.e. activated charcoal, $MgSO_4$, 7000, 10 000 & 12 000 dialysis membranes and a polymer, PVPP. These different techniques were all investigated and the best technique was selected based on the highest activity shown in the anti-HIV assay.

5.1.2.2 Gel formulation of the API

Topical gel preparation has remained one of the most popular and important pharmaceutical dosage forms. As a result, the therapeutics effects of the drugs are achieved effectively whereas the systemic side effects can be avoided or minimized. The formulation of an effective gel requires the use of an appropriate gelling agent, usually a polymer.

The preferred characteristics of such polymer include their inertness, safety, and biocompatibility with other ingredients, good adhesion to mucous membrane, and permission of drug permeation while not being absorbed into the body, irritation-free and preferably decomposable. When in the formulation, the polymer should exhibit good swelling, syneresis and rheological properties suitable for solidifying the system (Abrar *et al*; 2012).

Material Science and Manufacturing (MSM) have been involved in the formulation of the API into a classical microbicide gel. The dark colour of the API and the possible poor aesthetic appeal of this as a vaginal microbicide, led to a concerted effort to further purify/decolourise the API. MSM then focused their efforts on the formulation of the gel with the decolourised/purified form of the API and the characterisation thereof (rheological studies, shelf-life and stability, etc.), which was comparable with that of the coloured gel.

Besides the dark colour of the API posing a deterrent for further development, there was also the issue of compliance with a gel microbicide formulation. Hence, it was decided to prepare the gel for demonstration purposes only and not to pursue with the irritancy studies on this formulation and to rather focus on an alternative formulation (i.e. the caplet) which has a better chance of clinical success.

5.1.2.3 Caplet formulations of the API

As contraceptive technology has evolved, research on microbicide formulations and delivery now includes new formulation platforms for strategies able to provide protection against HIV infection even during unanticipated sex. These include slow-release intra-vaginal rings made of silicone or thermoplastic urethane, loaded with the API within the ring matrix or in a reservoir at its centre that could remain *in situ* for weeks or months (Buckheit *et al*; 2010).

The choice of delivery system depends on many factors, including the following:

• Active ingredient to be delivered

• Amount of active ingredient to be delivered

• Means or route that ingredient is to be delivered

• To which sites, at what rate, over what period of time, and for what purpose

The collaboration involving the University of Witwatersrand led to the development of a slow release intra-vaginal caplet technology incorporating the API and AZT. This allows the application of a dual-mechanism microbicide formulated into a caplet into the vagina, which can then last up to 72 days without application on a daily basis (Buckheit *et al*; 2010).

5.2 Methods and Materials

5.2.1 Decolourisation of API

Firstly, 50 g of activated charcoal was mixed with 10 g/400 ml of the API (IC_{50} = 0.04 µg/ml), in distilled water. The mixture was washed through a vacuum filter with 700 ml of lukewarm distilled water. The residue (charcoal) was decanted and the filtrate (colourless API) was freeze dried. The amount of the activated charcoal was reduced because; primarily the amount of charcoal used was too much and it was thought to have an impact on the activity but still the decolorized API was shown to have lost activity. This is shown in Table 8.

The second technique made use of $MgSO_4$ where four batches of 500 mg/100 ml of the API (precipitate) were treated with 1000 mg, 500 mg, 250 mg, and 25 mg of $MgSO_4$. The mixture was centrifuged and the salts formed were removed through dialysis. The colourless filtrate (API) was freeze dried (fig 16 (A)) and screened for anti-HIV activity.

A third decolourisation technique involved the use of dialysis where three separated 20ml aliquots of the API (precipitate) were dialysed through two membranes (7000, 10 000 & 12 000) membranes, using 1L of water as the medium. As for the PVPP, it was used as indicated in chapter 4, section 4.1.2.6.

5.2.2 Assaying of API against Herpes Simplex Virus

The HSV-1 assay is based on the ability of Herpes Simplex type 1 virus (HSV-1) to cause cytopathic effects (CPE) in Vero cells. The changes in cell morphology caused by infecting virus are called cytopathic effects (CPE). Compounds of interest (API) should be effective against formation of CPE on cells in the presence of virus. In this study, the cytopathic effect inhibitory method previously described by *(Schmidtke et al;* 2001) was used to evaluate antiviral activity of the API labelled as “BP36 precipitate and plant extracts (table 10).

5.3 Results and Discussion

5.3.1 Effects of decolourisation on anti-HIV Activity

All the decolorized API's were subjected to a bioassay and the results of these assays are shown on Table 8 below.

Table 8: Anti-HIV results of the decolourised API using different decolourisation techniques

Decolourisation technique	**Sample of interest**	**HIV strain**	**IC_{50} (µg/ml)**
Reference sample	**API (BP36-117-26464C)**	**HXB2**	**0.04**
Activated charcoal	decolourised API	HXB2	**>100**
7000 MW Dialysis membrane	decolourised API	HXB2	**0.8**
10 000 MW Dialysis membrane	decolourised API	HXB2	**0.08**
12500 MW dialysis membrane	decolourised API	HXB2	**0.2**
$MgSO_4$	decolourised API	HXB2	**10.82**
PVPP	decolourised API	HXB2	**0.3**

All the decolourised API's treated with $MgSO_4$ as described above also exhibited no activity except for the batch treated with 25 mg of $MgSO_4$ which showed significant activity (IC_{50} = 10.82 μg/ml). After the method was revised and the amount of $MgSO_4$ was reduced further, the decolorised API's showed an improved activity but the colour did not improve. It was then concluded that the more $MgSO_4$ added, the more the API loses its activity. The use of this technique was discontinued as it was going to be time consuming to decolourise little by little, since more than 30 g was the target for the formulation of the API. For that reason, $MgSO_4$ was eliminated as the decolourising agent, even though it was inexpensive.

The problem with the dialysis was that the API had a tendency to gel, because of the presence of pectin, and this blocked the membranes so that colour removal could not be achieved. There was not much of a diffusion reaction happening after blockage as anticipated. All the residue and filtrates were recovered and screened. As expected, the residue retained its dark colour and activity. Please note the results indicated in Figure 16 (D) and Table 8.

The last technique used Polyvinylpolypyrrolidone (PVPP)**,** a polymer which can also be used to extract polyphenols from the crude extract as discussed in Chapter 4. This technique worked well as PVPP removed the colour from the API and when the decolourised API was screened for anti-HIV activity it showed good activity (IC_{50} = 0.3μg/ml). This compares to the activity of the API before the colour was removed (IC_{50} = 0.04 μg/ml). This method was optimized and it showed consistent results in terms of efficacy but the removal of the colour was not complete.

As indicated, when PVPP was used for API decolourisation, most colour was removed from the API but there was an associated decrease in anti-HIV activity. This indicates that some other anti-HIV compounds were removed, possibly by adsorption to the PVPP. To test this, the PVPP residue was subjected to liquid-liquid (L/L) partitioning to extract all the possible compounds that were adsorbed by the PVPP. The PVPP residue was indeed shown to contain compounds that were ultimately separated by L/L partitioning and TLC, some of which showed marginal anti-HIV activity. Details of this are provided in Chapter 4.

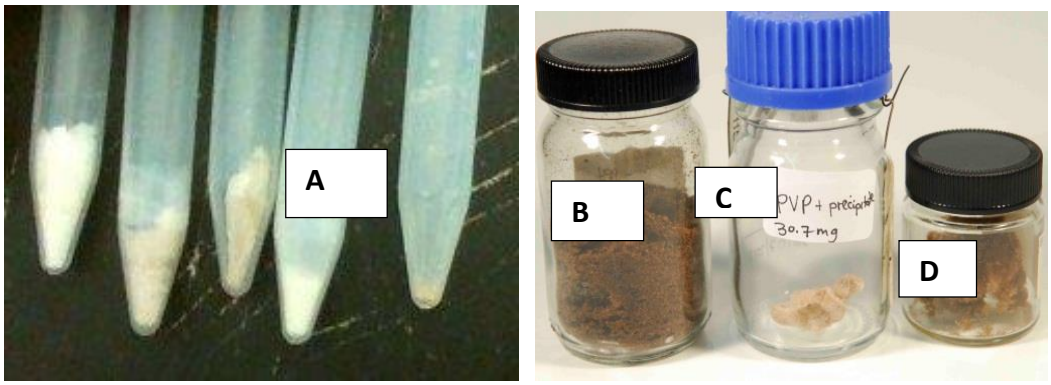


Figure 16: Decolourized potential API's. A is the API (white samples) treated with $MgSO_4$; B, API without any treatment; C, API with PVPP (light brownish) and D, dialyzed API (dark brown) using 10 000 and 7 000 MW membranes.

5.4 API as a potential microbicide candidate

For the development of an API into a gel or caplet formulation, grams of the API are required. In this study, more than 50 g of the API were prepared as it was important for the API to be repeatedly screened to test for reproducible anti-HIV activity. Several batches of the API were prepared and screened to show consistently high levels of activity (IC_{50} = 0.04 – 1.0µg/ml). The activity of the API **BP36-117-26464C** (0.04 µg/ml) against several HIV-1 subtype C pseudovirions compared favorably with that of T20 (0.02 µg/ml) and Tenofovir (0.40 µg/ml).

As part of another MSc study, the API was also screened against 10 subtype C clinical isolates and showed anti HIV activity against most of these isolates. A "time of addition" assay indicated that the API acts during the early stages of HIV infection, similar to the T20 and Maraviroc entry inhibitors, and may act as an attachment inhibitor but that it appears to lose its ability to inhibit infection after viral attachment. The data suggest that API is a potent antiviral and that it may act as an attachment inhibitor. This mode of action supported its potential use as a microbicide (Van Den Berg *et al*; 2010).

5.4.1 Evaluation of the API against other viruses

Herpes simplex viruses (HSVs) have a complex life cycle. Once the virus has infected the host, it persists for life as a latent infection that reactivates throughout the life span of the host. These viruses cause infections of the skin and mucous membranes of the mouth, eyes and genitals in humans.

There are two types of HSV, HSV type 1 has traditionally been associated primarily with oral-facial infections, whereas HSV-2 is generally associated with a genital infections transmitted through sexual activity (Emami *et al*; 2009). There was a need for this particular assay to be conducted since this virus can also be transmitted through sexual activities. Other sexually transmitted viruses (like gonorrhea and HSV-2) are still to be investigated. Table 10 below provides results of the anti-HSV assay.

Table 10. Results of the screening of the antiviral activity of the API against HSV-1

Sample ID	**Sample description**	**Extract type**	**Showed activity (concentration)**
BP36 **precipitate (API)**	**BP36-117-26464C**	H_2O	At 500 ug/ml
BP36 Acetone H2O	BP36-117-264504-Acetone/ H_2O	30% Acetone in H_2O	Weak activity (>500)
BP36 Methanol	BP36-117-264504-MeOH	Methanol	At 500 ug/ml
BP36 Butanol	BP36-117-264504-BuOH	Butanol	Weak activity (>500)
Acyclovir	Internal standard	purchased	5 ug/ml

BP36 precipitate (API) and methanol extracts (which was previously extracted through liquid-liquid extraction as described in Chapter 4 exhibited good antiviral activity at 500 ug/ml (% CPE inhibition >50%) against herpes simplex 1 virus. This is shown in Figure 17. The most active compounds are shown in sky blue (BP36 precipitate, API), purple (methanol extract) and lastly green (acyclovir, the internal standard). Both the precipitate and the methanol extract unfortunately could not be compared with the standard, as it showed very good activity at the lowest concentration, whereas the API and the methanol extract only showed a cytopathic effect at a high concentration (500ug/ml), which is not a good indication. Pure compounds need to be screened individually as they might show different results.

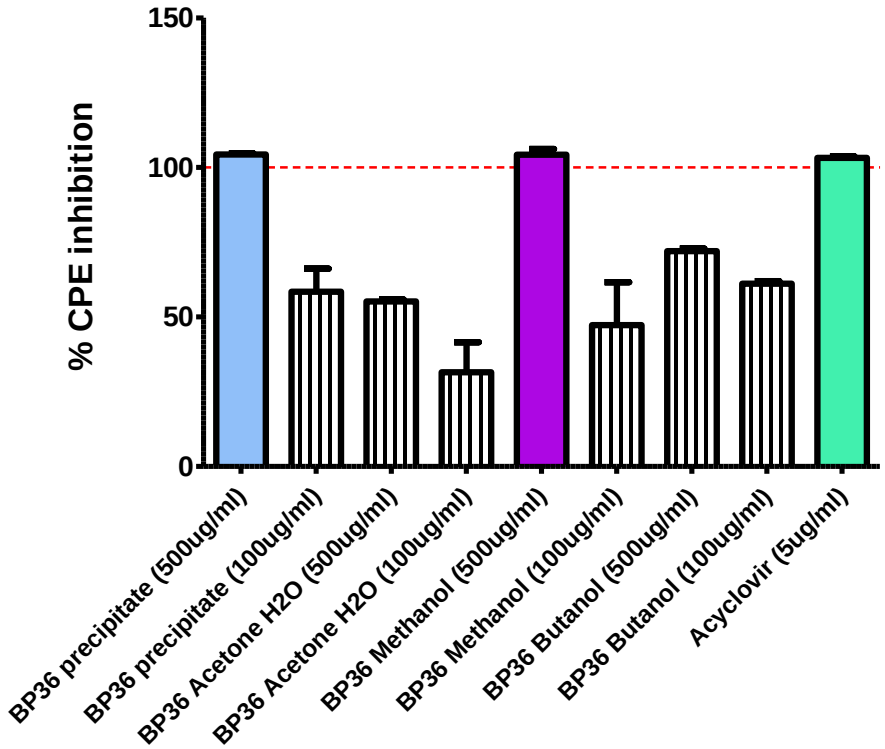


Figure 17: Antiviral activity of compounds at 500 ug/ml and 100 ug/ml and acyclovir (5 ug/ml) tested against Herpes simplex virus 2.

5.4.2 Formulations of the API

Formulation studies were conducted by two CSIR Biosciences research collaborators. The University of the Witwatersrand (Wits), Department of Pharmacy and Drug Delivery Platform, South Africa is responsible for the caplet formulation of the API. The second group, the Material Science and Manufacturing (MSM) Research Group is from within CSIR, and is responsible for the gel formulation.

To commence development of these formulations, both collaborators were provided with sufficient quantities of the API. For the gel formulations, a decolourised API was used, and for the caplet formulation the original isolated API (dark precipitate) was provided as there was no need to remove the colour of the API.

5.5 Conclusions

A suitable API has been identified, characterized accordingly and screened for anti-HIV activity against both a pseudovirus and peripheral blood mononuclear cells (PBMCs). It has been found to be a suitable microbicide candidate but there are still other studies required before it can be commercialized. The summary of other *in vivo* or *ex vivo* and *in vitro* studies are listed below:

- Evaluation against other STI's e.g. HSV-2, gonorrhoea
- Efficacy against other HIV strains in PBMC assay
- Preclinical toxicity studies i.e. irritancy
- pH stability
- *In vivo* pig vagina study

CHAPTER 6: GENERAL CONCLUSIONS

South Africa is a home to thousands of plant species, each an industry for unique and useful chemical compounds. Natural products and medicinal plants still remain an important starting point for drug discovery and the identification of novel anti- HIV agents. The historical success of natural products as anti- HIV agents and the large number of structurally diverse plant-derived compounds provide justification for the continued effort in this area.

The objective of this study was to isolate, identify and characterize the active phyto-pharmaceutical ingredients (API) from *L. trigonus*, a plant used traditionally to treat HIV. This active ingredient was ultimately going to contribute to the development of a microbicide, which will serve the purpose of empowering women to help protect themselves from sexual transmission of HIV.

Chapter 1 discussed the global status of HIV/AIDS and its potential prevention and treatment tools. Since women are so vulnerable to HIV and are the most affected in the world, an alternative prevention tool against this virus was investigated. This led to the identification of the API, being the precipitate **BP36-117-26464C**. It was identified to consist of a large polymeric molecule with significant anti-HIV activity. The API was also subjected to other biological assays like herpes simplex virus 1.

Chemical analysis of the API which included NMR analysis, HPLC profiling, UPLC-MS and also GC analysis revealed the nature of the API and the fact that it was still a mixture of unknown compounds which need separation for complete elucidation. Chromatographic techniques used in this study were employed to separate and isolate compounds in the API. Eight compounds were successfully isolated. During the phytochemical screening, four classes of compounds (flavonoids, alkaloids, fatty acids and sugars) were identified by the use of chemical spraying reagents (vanillin, iodine vapor, ammonia and nihydrin sprays). Only two compounds were successfully structurally elucidated and these were identified as rosmarinic acid and bornesitol. Rosmarinic acid showed weak anti-HIV activity (IC_{50} of 111 µg/ml) while bornesitol exhibited no activity.

Both compounds are well known, but have never been isolated from *L trigonus.* RA is known for its biological activities including anti-viral and anti-oxidant properties.

The other six (three alkaloids and three polyphenols) compounds also showed very weak to moderate anti-HIV activity and it appears that API activity is dependent on a combination of these components. This is reflected in Table 4.

The API has been identified as a galacturonic acid-rich polymer with other small molecules attached to it. The plant oven drying and grinding process was shown to contribute significantly to the formation of this polymer. The API production method was optimised and refined with the energy intensive spray drying process being eliminated.

As the main contribution of the present study was towards development of a microbicide to help protect and improve the lives of women against sexual transmission of HIV, it was, therefore, concluded that the precipitated mixture (BP36-117-26464C) of compounds was the best API, in terms of activity, for further development as the microbicide, particularly as it was water soluble.

Characterisation of the API, including its decolourisation, was important as the main problem of the API was the dark colour that needed to be removed while at the same time maintaining the anti-HIV activity of the compound. Different decolourisation techniques were employed to improve the chances of using the API in a microbicide gel. Polyvinylpolypyrrolidone (PVPP) was found to be the best decolourising agent to remove the colour of the API and also retain its activity. For the intra-vaginal caplet formulations, there was no need to decolourise the API.

The University of Witwatersrand, Johannesburg, South Africa and MSM, Pretoria, were two collaborators responsible for the formulation of the API as described in chapter 5. A large scale batch (57 g) was prepared for the two groups to undertake these studies. Before the final product (API) can reach the market, a series of biological assays are still outstanding. For instance, the vaginal studies are still underway where a pig model is being used to test for the irritancy and stability of the caplet.

Besides the dark colour of the API posing a deterrent for further development, there was also the issue of compliance with a gel microbicide formulation. Hence, it was decided to prepare the gel for demonstration purposes only and not to pursue with

the irritancy studies on this formulation and to rather focus on an alternative formulation i.e. the caplet which has better chance of clinical success.

In the current form, the precipitate (API) would be difficult to develop as an oral treatment for HIV, as high-molecular weight agents often have poor bioavailability following oral administration. However, large molecules with potent anti-HIV activity are ideal for topical use and potent development as a microbicide. Unlike the other carbohydrate microbicides which have poor anti-HIV properties, the API has been shown to have significant anti-HIV activity (Table 4).

The API was also screened against 10 sub type C clinical isolates and showed anti-HIV activity against most of these isolates. A target based time of addition assay (which shows the trend in which the active shows a reduced ability to block infection when added at different time points after completion of each stage of the viral life) showed that the API acts in the early stages of HIV as an entry (attachment) inhibitor, substantiating its use as a topical microbicide. The advantage of having an entry inhibitor is that it could help prevent infection of cells altogether whereas the classical interventions such as the Reverse Transcriptase inhibitors act only after infection.

Other ongoing studies involve determining a more detailed toxicity profile of the API, confirmatory screening of the API in a PBMC assay against clinically relevant strains of the virus and evaluation of the API for efficacy against other STIs.

6.1 Challenges during the study

Isolation of active phyto-pharmaceutical ingredients, which was one of the main objectives of the study, was challenging as the techniques employed were restricted to small scale. This proved a disadvantage because bioassay-guided fractionation requires a stepwise isolation of active compounds.

Having discovered a means of purification through re-extraction of the API using different solvents, the yields recovered were too low - hence the acetone-water, MeOH extracts could not be purified further even though the activity was significant. The concentration of the compounds of interest was relatively low.

After the API was identified, its characterization was extremely difficult, because it was a complex polymer with unknown small molecules attached to or embedded in it. It, therefore, needed specialized techniques more suited to polymeric molecules and time was spent in trying to find a suitable chromatographic technique that can purify and separate the active compounds. The only technique which proved successful could only separate the small molecules (polyphenols, alkaloids and sugars). The concentration of sugars and how they were arranged, structured and holding up together in the API could not be determined as well. Therefore, more efficient purification methods are required to isolate and characterize the large molecules in the API of *L. trigonus.*

As the study was of great importance in developing and identifying an API for use in a microbicide, the API had to be decolourised for the purpose of gel formulation. Time was invested in trying to find the right decolourising agent that would remove the colour of the API and yet retain the anti-HIV activity. PVPP, which was identified as the best polymer for decolourisation was very expensive, and could not be used for larger scale purifications.

Due to the complexity of the compounds, structural elucidation of the other six compounds could not be achieved and, hence, the structure of only two compounds is reported in the present study. Therefore, more investigation needs to be conducted on this plant in order to identify other small molecules as well as the polymeric molecule/s. The individual compounds will need to be tested for anti-HIV activity but all evidence to date indicates that the API is a mixture of molecules of various sizes which act together to produce anti-HIV activity.

CHAPTER 7: GENERAL MATERIALS AND METHODS

7. Experimental

7.1 Extract preparation

L. trigonus was initially brought to the CSIR by a traditional healer for HIV treatment. The plant was collected from Grahamstown, in the Eastern Cape region of South Africa. The aerial plant parts were identified by the South African National Biodiversity Institute (SANBI Genspec number: 1492 44).

The aerial parts of the plant were collected and dried in an oven at 30–60°C for 3 days. Dried aerial parts were ground to a coarse powder using a hammer mill and stored at 4°C prior to the extractions. The extraction procedure was carried out as per the traditional method of preparation. Distilled cold water was added to the ground extract, consistently boiled for 30 minutes and allowed to cool at 4°C for about 3 hours before filtering. For the scaling up production of the extract this procedure was repeated several times. The aqueous extract was concentrated by freeze drying for 3 days.

For the preparation of the precipitate (API), the aqueous freeze-dried extract was re-suspended in distilled water. The suspension was cooled at 4°C and mixed with 96% ethanol (EtOH). Immediately after the ethanol addition, a precipitate was observed. The precipitate was recovered through centrifugation (for 25 minutes at 5000 x *g* at 4°C). Both the filtrate (supernatant) and the precipitate (API) were freeze dried. The dried filtrate and the precipitate were stored at 4°C until use.

Three major extracts of *L. trigonus* were analysed and screened:

- **Lob-1-13A** – a spray dried extract prepared by water extraction via spray drying process. It showed significant anti-HIV activity.
- **BP36-117-26464C (API)** – a precipitate which was identified as the API was derived from the spray dried extract through the ethanol precipitation process described above. This was the most active API amongst others.

- **BP36-117-26454B** – a filtrate which was the supernatant during the ethanol precipitation. It also showed moderate activity against HIV-1

7.2 Chemical reagents

Analytical grade solvents for extraction and HPLC grade solvents for chromatography were purchased from Sigma-Aldrich and Merck. Formic acid (98.0–100.0%) was purchased also from Sigma Aldrich (South Africa). Reference materials rosmarinic acid (>98.0% purity), galacturonic acid, poly-galacturonic acid, pectin from citrus and pectin from an apple peel, xylose, Mannose, galactose, glucose and Arabinose (>98.0% HPLC purity) were purchased from Sigma Aldrich (South Africa). DEAE cellulose (Cellex-d), sephadex G 25 and silica gel 60 were purchased from Sigma Aldrich. Distilled water (H_2O) was used for all procedures. Finally, for the decolourisation of the API, activated charcoal, PVPP, $MgSO_4$ and dialysis membranes (10 000 and 12 500 MW) were all procured from Sigma.

7.3 Chromatography

7.3.1 Liquid-liquid partitioning

A standard liquid-liquid (L/L) partitioning process was performed on the spray dried aqueous extract (Lob-1-13A) and the precipitate- API **BP36-117-26464C** using various solvents. Both the extract and the precipitate (2 g) were dissolved into distilled water (50 ml) and re-extracted with the following solvents:

- Methanol / ethyl acetate / acetic acid (100:60:10)
- Butanol / ethyl acetate / acetic acid (100:60:10)
- Acetone / dH_2O (140: 60)

The organic layers were separated, evaporated individually under reduced pressure. These layers were purified, screened for HIV activity and ultimately chemically analyzed.

Extracts produced were:

- **BP36-117-264504** Methanol extract
- **BP36-117-264504** BuOH extract
- **BP36-117-264504** Acet/H_2O extract

7.3.2 Preparative thin layer chromatography

Preparative thin layer chromatography (prep TLC) and thin layer chromatography (TLC) were tools used to purify and separate compounds. Pool of fractions was purified by this technique. TLC Silica gel 60 F254 plates were purchased from Merck. Crude extracts and pure compounds were dissolved in methanol (AR grade), spotted and developed on a TLC plate with Chloroform (7): methanol (3): acetic acid (2) solvent systems. Before heating the plates in an oven at 80°C, the plates were first viewed under UV lamp, sprayed with four spraying reagents i.e. Ammonia and Iodine vapour for the detection of the polyphenolic compounds, vanillin: concentrated H_2SO_4 (1 g: 100 ml) for the general compounds like fatty acids and lastly ninhydrin (0.2g ninhydrin in 100 ml ethanol and heated to 110°C until spots appear) / Nitric acid: ethanol (50 drops conc. nitric acid in 100 ml ethanol. If necessary, heat to 120°C for the detection of the alkaloids and amines.

7.3.3 Gas chromatography (Silylation process)

The process of methanolysis and derivatisation was carried out as follows:-

Step 1: prepare a concentration of 20 mg/ml for every monosaccharide and the API

Step 2: take 5 µl from each (from the concentration in step 1) monosaccharide, add 80 µl of mannitol (standard) and combine them in one vial

Step 3: take 10 µl, 15 µl, 20 µl, 40 µl, 60 µl and 80 µl as described in step 2

Step 4: completely dry each in a Speedy Vac evaporator

Step 5 (methanolysis): add 400 µl of 4M MeOH/HCl in each, close the vials and heat the samples in the oven at 60°C for 24 hours

Step 6: add 500 µl of MeOH at least 4 times in each vial, let it evaporate under fume hood to a minimum volume of 20 µl

Step 7: removal of excess HCl by blowing N_2 gas into each vial until no smell

Step 8 (silylation): In anhydrous pyridine (2 ml), a mixture of HMDS (800 µl) and TMCS (400 µl) were combined and mixed well. 200 µl of the mixture was added to the hydrolysed and methanolysed 21 samples (sugars, polymers and the API). This reaction was held for 30 min at 60°C. The samples were centrifuged to remove the excess silica and then transferred to the GC glass vials with inserts. The derivatized monosaccharides were injected directly as a pyridine solution and analysed. This was done in triplicate so as to check for inconsistencies and to check if the reactions were complete as the silylation was very humid-sensitive.

7.3.4 Semi preparative HPLC

The active extract (**BP36-117-264501**-Acet/H_2O) generated from the PVPP crude extract was fractionated using the Agilent 1200 semi-preparative HPLC system consisting of an auto sampler, high pressure mixing pump, column oven and DAD detector. Sample concentration was 20 mg/ml in DMSO. Semi pure fractions which mainly contained flavonoids were evaporated on a GeneVac EZ-2 Plus evaporator at 40ºC using the pre-installed HPLC fractions vacuum settings. The fractions were then spotted on the TLC for the profile and NMR analysed.

7.3.4.1 Method parameters

Column:	Waters SunFire^{TM} Prep C_{18}
Column temperature:	45˚C
Flow rate:	4mL/min
Injection volume:	500 µL
Detector:	PDA (Photo diode array)
	Scanned wavelengths from 210 – 600 nm
Solvents:	A = 0.1 % Formic acid in ddH_2O
	B = Acetonitrile

Table 9: Solvent gradient

Time (min)	**% Solvent A 0.1 %FA in ddH_2O**	**% Solvent B Acetonitrile**	**Flow rate (mL/min)**
0	0	5	4
30	0	100	4
35	0	100	4
36	0	5	4
40	0	5	4

7.4 Nuclear magnetic resonance (NMR) spectroscopy

NMR data was acquired on several fractions and pure compounds including the API. The NMR used was a 600 MHz Varian, 5 mm probe. Structural characterization was carried out using a combination of 1D (^{1}H, ^{13}C) and various 2D Distortionless enhancement by polarisation transfer, Correlation spectroscopy, Heteronuclear single quantum coherence and Heteronuclear multiple bond correlation experiments. Structural elucidation of other compounds could not be determined due to their complexity. The concentration of the samples was 20 mg in 400 µl of the deuterated methanol (CD_3OD) water (D_2O) and chloroform (CD_3Cl).

7.5 HIV -1 Neutralisation assay protocol

7.5.1 Reagents

- Media: DMEM5
- Pseudovirus diluted according to titration experiments to give a concentration of 100 TCID50
- HIV-1 subtype A, B and C pseudovirions screened
- Cells: TZM-bl cells ($2x10^5$ cells/ml), 10 000 cells/well
- Compounds to be tested: all the pure compounds or active-rich fractions from *Lobostemon trigonus* including the API
- Bright Glo luciferase substrate solution (Promega)

7.5.2 Positive controls

- T20 (Entry/Fusion inhibitor)
- Tenofovir (Reverse transcriptase inhibitor)

7.5.3 Procedure

- Add 60 µl DMEM5 to all wells, add an extra 25 µl to cell control (CC) wells
- Add 15 µl compound (1 mg/ml) to bottom row wells in duplicate
- Prepare a serial 5-fold dilution in 15 µl of the compound by transferring 15 µl of the media to upper wells before discarding 15µl from the top row wells
- Add 25 µl virus (100 $TCID_{50}$) to all wells, except CC
- Add 50 µl TZM-bl cells ($2x10^5$ cells/ml) to all wells
- Incubate 48 hrs, 37°C, 5%CO_2
- Remove and discard 75 µl from all wells
- Add 50 µl Bright Glo luciferase substrate (Promega) to all wells
- Incubate 2 min
- Transfer 50 µl of media from all wells to a black, flat bottom NUNC plate
- Read luminescence with a luminometer

- Analyse results in Excel

7.6 Antiviral assay method

To test the potency of these compounds against herpes simplex virus 2 (HSV-2), Vero cells were seeded at $0.5x10^6$ cells/well in 24 well plate for 24 hours to obtain 80-90% monolayer confluence. Thereafter, the media was removed and the monolayer washed three times with PBS prior to virus adsorption. Virus at a multiplicity of infection (m.o.i) of 0.05 particle forming unit (p.f.u) was allowed to adsorb for 1 hour. At the end of adsorption, traces of virus were removed. Cell monolayer was then treated with 500 ug/ml or 100 ug/ml of compounds in DMSO.

A drug that is currently used to treat symptomatic herpes simplex virus, acyclovir, was used as a positive control at 5 ug/ml (Whitley 1993). The plate was then incubated for 5 days to allow virus-cytopathic effects formation. Cytopathic effect was measured by using a 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay kit (Promega Corporation (Madison, United State of America) according to the manufacturer's instructions.

The compound tetrazolium, is converted into a blue formazan dye by metabolically active mitochondria of viable cells (Mosmann 1983). At the end of the incubation period, cells were incubated with MTS solution for a minimum of 2 hrs, and the absorbance was measured at 492 nm using a spectrophotometric microtitre plate reader (Infinite, F500, Tecan group Ltd, Mannedorf, Switzerland).
The percentage of CPE inhibition was calculated by subtracting the mean value of virus-infected cell control (0%) from the measured absorbance, and resulting number was divided by the measured absorbance of uninfected cell control (100%) using equation (2) below:

CPE inhibition (%) = $100* [(OD_{cmp}- OD_{vc}/OD_{cmp})/ OD_{cc}]$(2)

Where, OD_{cmp} is the optical density of the test compound, OD_{vc} of the virus control and ODcc of cell control (untreated).

REFERENCES

Abrar, B., Anis, S., Tanu, B., Singh, S., 2012. Formuation and in-vitro evaluation of NSAID's gel. *Internatiomal journal of current pharmaceutical research*, 4(3), pp.3–5.

Appidi, J.R., Grierson, D.S., Afolayan, A.J., 2008. Ethnobotanical study of plants used for the treatment of diarrhoea in the Eastern Cape, South Africa. *Parkistan journal of biological sciences*, 15, pp.1961–1963.

Attawell, K., 2001. The case for microbicides: A global priority Second edi., United Kingodom: Population council & International family health.

Balzarini, J., 2007. Carbohydrate-binding agents: a potential future cornerstone for the chemotherapy of enveloped viruses? *Antiviral chemistry & chemotherapy*, 18(1), pp.1–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17354647.

Barbakadze, V.V. Kemertelidze, E.P. Shashkov, A.S. Usov, A.I., 2000. Structure of a new anticomplementary dihydroxycinnamate-derived polymer from Symphytum asperum (Boraginaceae). *Mendeleev Communications*, 10(4), pp.148–149. Available at: http://linkinghub.elsevier.com/retrieve/pii/S095994360070887X.

Barnes, J., Anderson, L.A. & Phillipson, J.D., 2007. Herbal medicines : Third edition. In *Pharmaceutical Press*.

Berthod, A. & Carda-Broch, S., 2004. Determination of liquid–liquid partition coefficients by separation methods. *Journal of chromatography A*, 1037(1-2), pp.3–14. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0021967304000445 [Accessed March 26, 2014].

Beth Israel Deaconess Medical Center (BIDMC), 2013. Monoclonal antibodies show promise as effective HIV therapy. *Nature*. Available at: http://www.sciencedaily.com/releases/2013/10/131030142412.htm.

Bhatt, R., Mishra, N. & Bansal, P.K., 2013. Phytochemical, pharmacological and pharmacokinetics effects of rosmarinic acid. *Journal of pharmaceutical and scientific innovation*, 2(2), pp.28–34. Available at: http://www.jpsionline.com/admin/php/uploads/202_pdf.pdf [Accessed April 9, 2014].

Buckheit, R.W., Watson, K.M., Morrow, K.M., 2010. Development of topical microbicides to prevent the sexual transmission of HIV. *National Institute of Health (NIH) public access*, 85(1), pp.1–38.

Buys, M.H., 2011. Revision of Lobostemon (Boraginaceae). *Phytotaxa 37*, 131, pp.1–3.

Centers for disease control and prevention (CDC), 2013. *Proven HIV prevention methods*,

Dambuza, N.S., 2007. *Isolation of bioactive metabolites with activity against HIV-1 target proteins from extracts of Sutherlandia frutescens and Lobostemon trigonus*. Nelson Mandela Metropolitan University (NMMU). Available at: http://dspace.nmmu.ac.za:8080/jspui/handle/10948/492.

Emami, S.A., Tayarani-najaran, Z., Ghannad, M.S., Karamadini, P.K., Karamadini, K.M., 2009. Antiviral activity of obtained extracts from parts of Cupressus sempervirens against Herpes Simplex Virus Type 1. *Iranian journal of basic medical sciences*, 12(3-4), pp.133–139.

Foden, W. & Potter, L. 2005. *Lobostemon trigonus* (Thunb.) H.Buek. National Assessment: Red List of South African Plants version 2014.1. Accessed on 2015/07/15

Garcia-calleja, J.M. Barrerre, B., 2010. Trends in HIV prevalence and sexual behaviour among young people aged 15-24 years in countries most affected by HIV. *Sexually transmitted infections (BMJ journals)*, 86 Suppl 2(22), pp.ii72–ii83. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3173837&tool=pmcentrez&rendertype=abstract [Accessed August 16, 2013].

Global AIDS response progress report, 2012. *Global AIDS response republic of South Africa*,

Guan, J. Yang, F. Li, S., 2010. Evaluation of carbohydrates in natural and cultured Cordyceps by pressurized liquid extraction and gas chromatography coupled with mass spectrometry. *Molecules (Basel, Switzerland)*, 15(6), pp.4227–4241. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20657437 [Accessed October 18, 2013].

Harnett, S.M. Oosthuizen, V. van de Venter, M., 2004. Anti-HIV activities of organic and aqueous extracts of Sutherlandia frutescens and Lobostemon trigonus. *Journal of ethnopharmacology*, 96(1-2), pp.113–119. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15588658 [Accessed August 19, 2013].

Henrich, J.T., Kuritzkes, D.R., 2013. HIV-1 entry inhibitors: Recent development and clinical use. *Current opinion in virology*, 3(1), pp.51–57. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23290628 [Accessed August 8, 2013].

Karim, A.Q., Frohlich, J.A., Gobler, C.A., Baxter, A., Sibeko, S., 2010. Effectiveness and safety of Tenofovir gel, an antiretroviral microbicide , for the prevention of HIV infection in women. *National Institute of Health (NIH) public access*, 329(5996), pp.1168–1174.

Lewu, F.B. & Afolayan, A.J., 2009. Ethnomedicine in South Africa : The role of weedy species. *African Journal of biotechnology*, 8(6), pp.929–934.

Magalhães, P.J., Vieira, J.S., Gonçalves, L.M., Pacheco, J.G., Guido, L.F., Barros, A.A., 2010. Isolation of phenolic compounds from hop extracts using polyvinylpolypyrrolidone: characterization by high-performance liquid chromatography-diode array detection-electrospray tandem mass spectrometry. *Journal of chromatography. A*, 1217(19), pp.3258–68. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19913228 [Accessed October 23, 2013].

Martena, M.J., 2010. *Safety of herbal preparations on the Dutch market*. Wageningen University.

Masupha P., Thamae L., P.M., 2013. *Analysis of Traditional Healers in Lesotho : Implications on intellectual property systems working paper series no . 68*,

Montefiori D. C, 2008. Protocol for measuring neutralizing antibodies against HIV-1 , SIV and SHIV using a luciferase reporter gene assay in TZM-B1 Cells. , (July), pp.1–11.

Moshi, M.J., 2005. Current and future prospects of integrating traditional and alternative medicine in the management of diseases in Tanzania. *Tanzania health research bulletin*, 7(3), pp.159–67. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16941942.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of immunological methods*, 65(1-2), pp.55–63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6606682.

N. Van Den Berg, P.F. Mbobela, P. Pillay, V. Maharaj, M.K., 2010. *Inhibition of HIV-1 by a natural compound*, Pretoria, South Africa.

Obiero, J., Mwethera, P.G., Hussey, G.D., Wiysonge, C.S., 2012. Vaginal microbicides for reducing the risk of sexual acquisition of HIV infection in women: systematic review and meta-analysis. *Biomedcentral infectious diseases*, 12(1), p.289.

Pebody, R., 2011. *Microbicides : Making it count briefing sheet 8*, England.

Roeder, E., 1999. Analysis of pyrrolizidine-alkaloids. *Current Organic chemistry*, 3, pp.557–576.

Roseman, G.M., 2007. *Analysis of pyrrolizidine alkaloids in Crotalaria species by HPLC-MS/MS in order to evaluate related food health risks*. University of Pretoria. Available at: etd-08032007-170633.

Schmidtke, M., Schnittler, U., Jahn, B., Dahse, H.M., Stelzner, A., 2001. A rapid assay for evaluation of antiviral activity against coxsackie virus B3, influenza

virus A, and herpes simplex virus type 1. *Journal of virological methods*, 95(1-2), pp.133–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11377720.

Sharma, R.A., Singh, B., Singh, D., Chandrawat, P., 2009. Ethnomedicinal, pharmacological properties and chemistry of some medicinal plants of Boraginaceae in India. *Journal of medicinal plants research*, 3(13), pp.1153–1175.

Shattock, R.J. & Rosenberg, Z., 2012. Microbicides: Topical prevention against HIV. *Cold Spring Harbor perspectives in medicine*, 2(2), p.a007385. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3281595&tool=pmcentrez&rendertype=abstract [Accessed August 19, 2013].

Toth .J, Mrlianova .M, Tekelova. D, K.M., 2003. Rosmarinic acid – an important phenolic active compound of Lemon Balm (Melissa officinalis L.). *Faculty of pharmacy*, pp.139–146.

Tshikalange, T.E., 2008. *In vitro anti-HIV properties of ethnobotanically selected South African plants in the treatment of sexually transmitted diseases*. University of Pretoria. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18809485.

UNAIDS, 2012. *National strategic plan on HIV, STI's and TB (2012 - 2016)*,

UNAIDS & WHO, 2012. HIV and hormonal contraceptIon.

Vermani, K. & Garg, S., 2002. Herbal medicines for sexually transmitted diseases and AIDS. *Journal of ethnopharmacology*, 80(1), pp.49–66. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11891087.

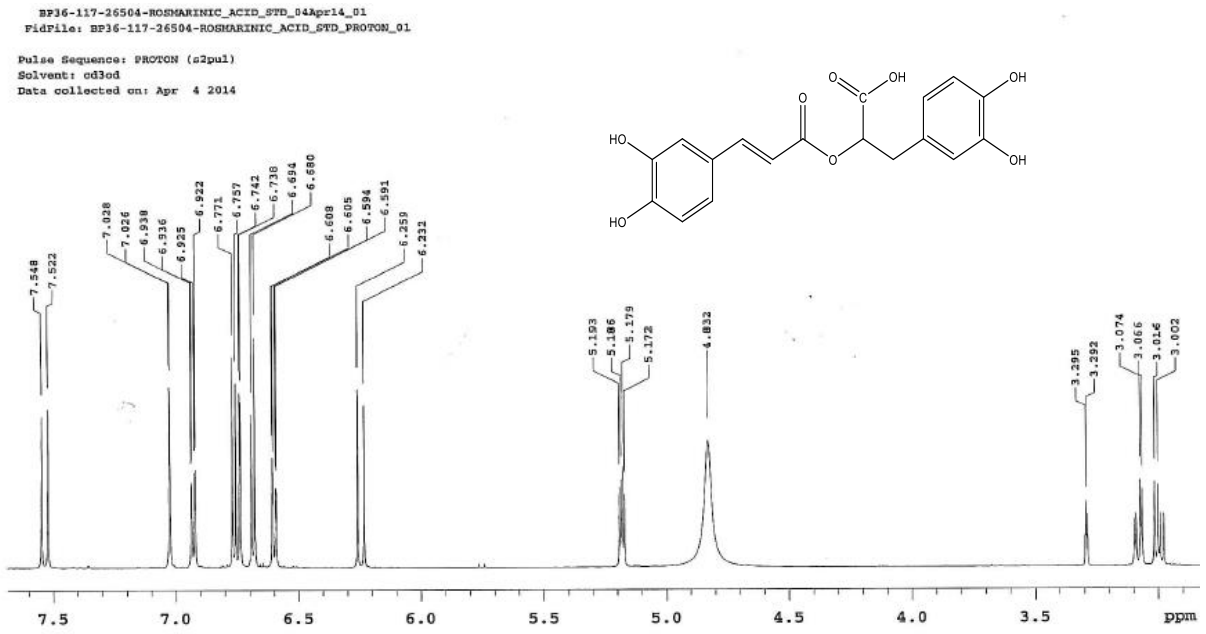
Walker, J.M., 2006. *Natural products isolation* Second edi. L. Gray, A.I. Satyajit, S. Zahid, ed., Totowa, New Jersey: Humana press : Methods in biotechnology.

Wanyoike, P.N., 2011. *The perceptions of rural Samburu women in Kenya with regard to HIV/AIDS: Towards developing a communication strategy*. University of South Africa.

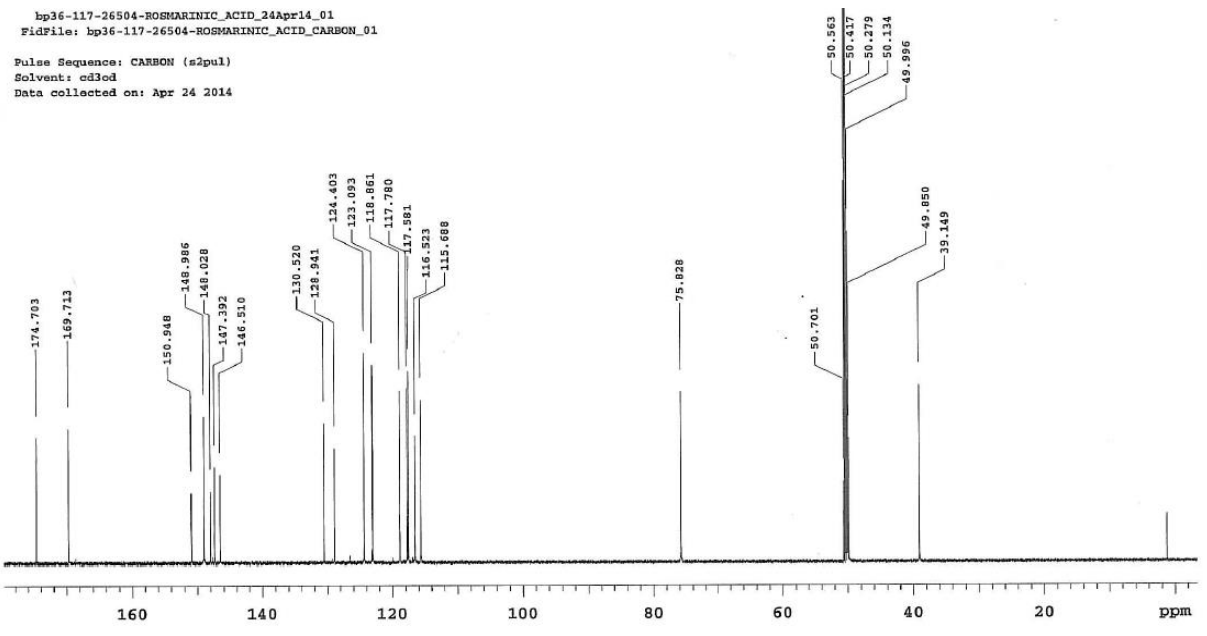
Whitley, R.J., 1993. Herpes simplex virus infections of women and their offspring: Implications for a developed society. In *Changes in human ecology and behaviour: Effects on infectious diseases*. Washington, DC: National academy of sciences, pp. 2441–2447.

WHO, UNICEF, UNAIDS., 2013. *Global update on HIV treatment 2013 : Results, impact and opportunities*, Malaysia. Available at: www.who.int.

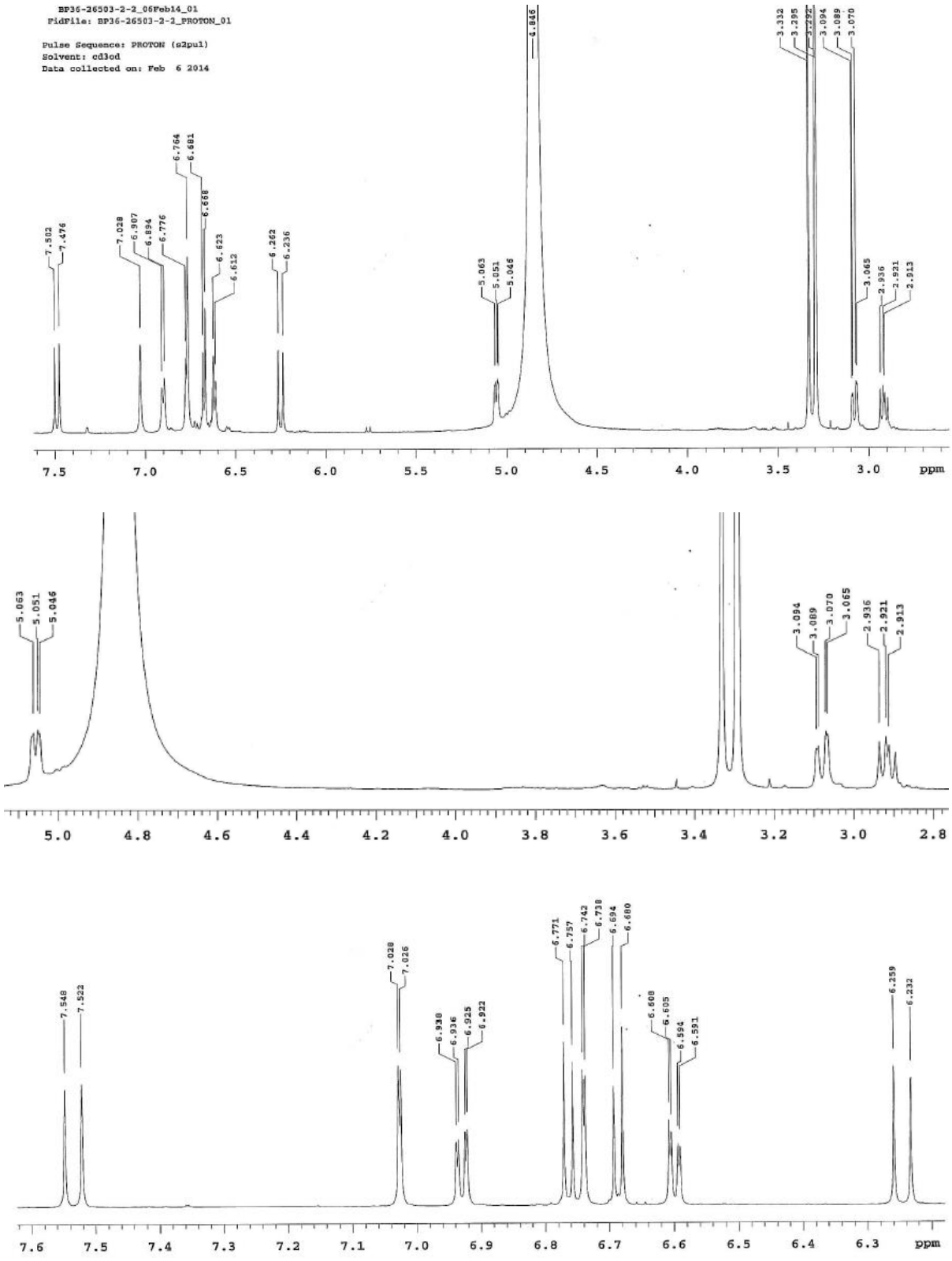
APPENDIX LIST OF SPECTRA



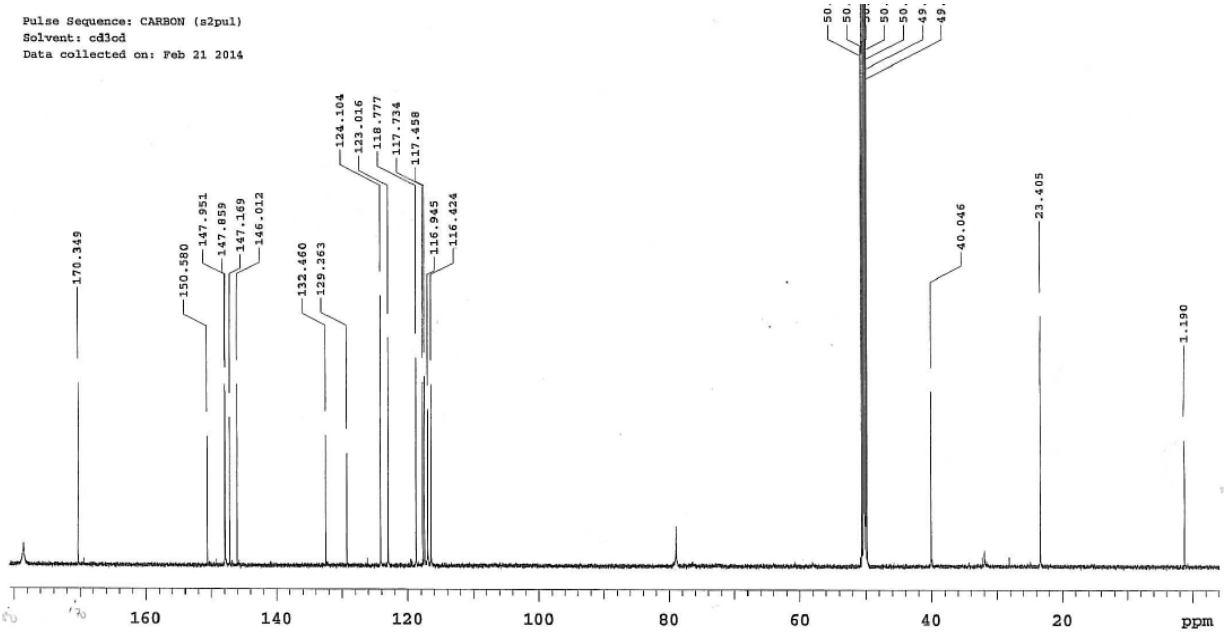
Spectrum 1: ^{1}H (Proton NMR) of Rosmarinic acid in CD_3OD (fig 12)



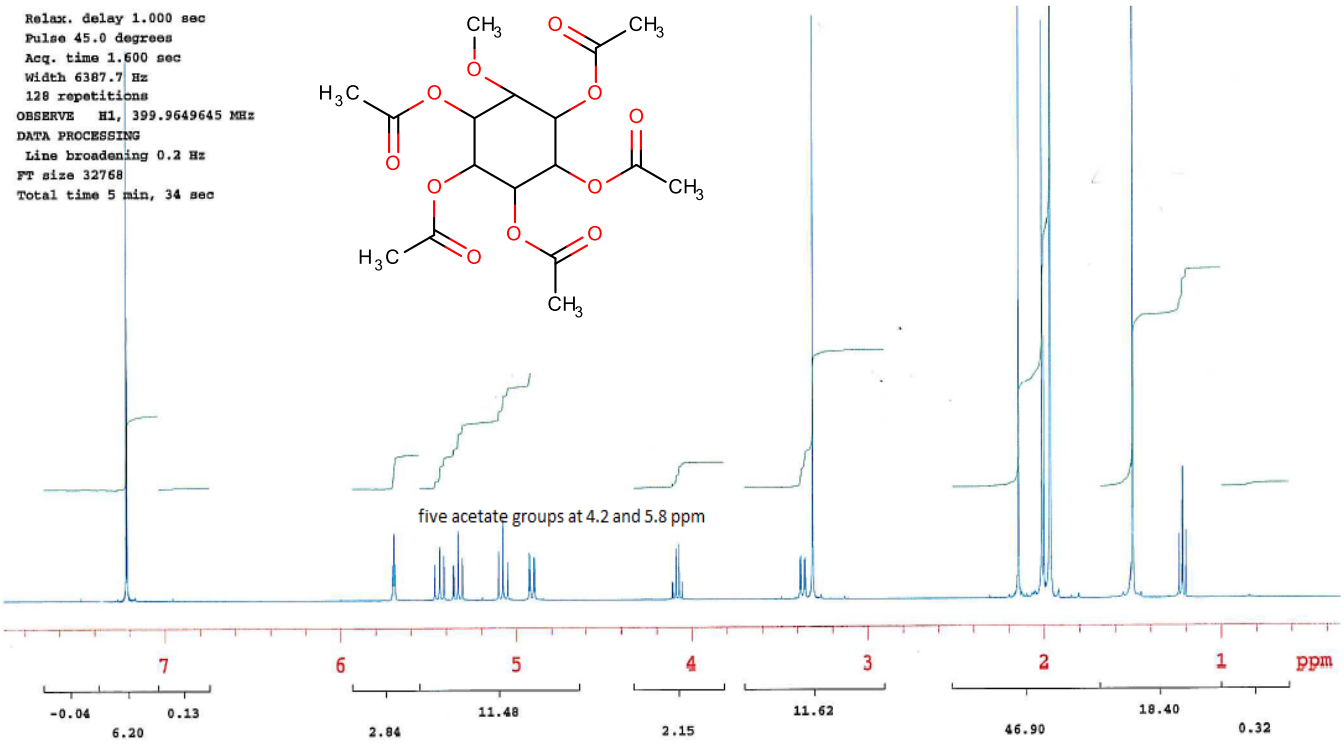
Spectrum 2: ^{13}C (Carbon NMR) of Rosmarinic acid (RA) in CD_3OD



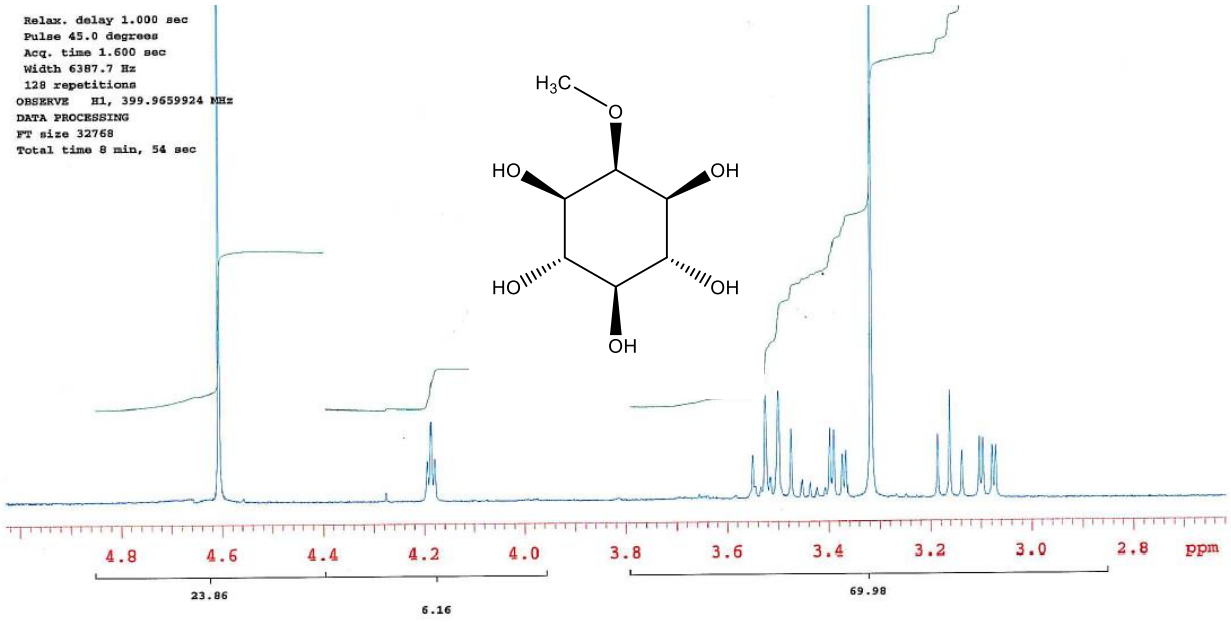
Spectrum 3: Proton NMR (^{1}H NMR) of BP36-117-264503-2 in CD_3OD (fig 12)



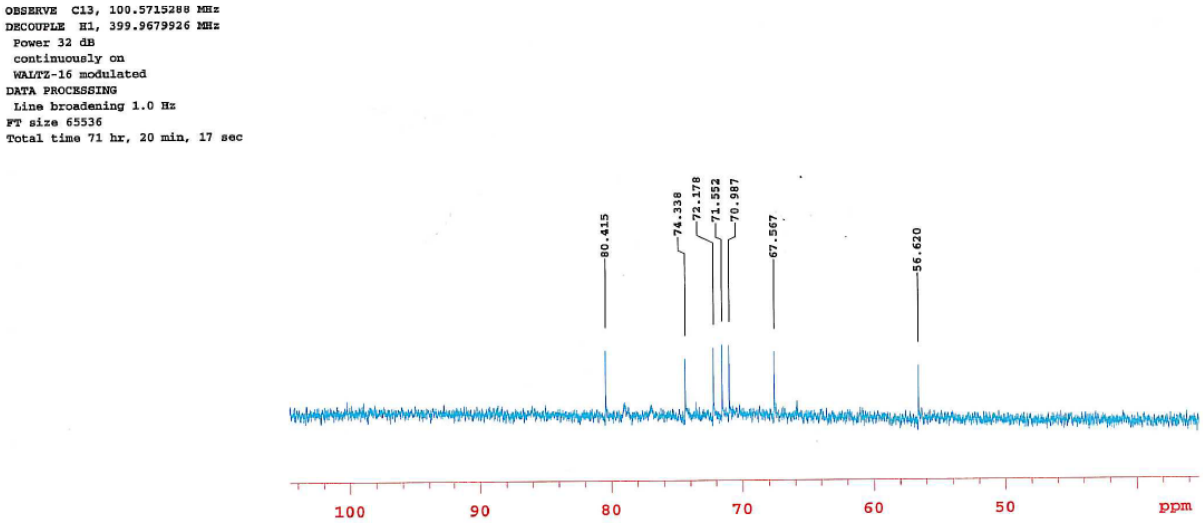
Spectrum 4: ^{13}C NMR of BP36-117-264503-2



Spectrum 5: ^{1}H NMR of BP36-117-26504-A (acetylated bornesitol – (fig 11) in CD_3Cl



Spectrum 6: ^{1}H NMR of BP36-117-26504-C (bornesitol – fig 14) in D_2O



Spectrum 7: ^{13}C NMR of BP36-117-26504-C (bornesitol– fig 14) in D_2O