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In vitro anti-HIV-1 properties of ethnobotanically selected South African plants used in the treatment of sexually transmitted diseases

by

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I declare that the thesis, which I hereby submit for the degree of PhD Medicinal Plant Science (option) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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LIST OF ABBREVIATIONS

Abbreviation	Explanation
AIDS:	acquired immunodeficiency syndrome
^{13}C-NMR:	carbon-nuclear magnetic resonance
COSY:	correlated spectroscopy
DNA:	deoxyribonucleic acid
DMSO:	dimethylsulfoxide
DPPH:	1,2 –diphenyl-2-picrylhydrazyl
HIV:	human immunodeficiency virus
HMBC:	heteronuclear multiple bond correlation
HMQC:	heteronuclear multiple quantum correlation
^{1}H-NMR:	proton-nuclear magnetic resonance
LTR:	long terminal repeat
MRNA:	messenger ribonucleic acid
NF-κB:	nuclear factor kappa B
NMR:	nuclear magnetic resonance
NOESY:	nuclear overhauser effect spectroscopy
PBS:	phosphate buffer saline
RT:	reverse transcriptase
STD:	sexually transmitted disease
Tat:	transactivating regulatory protein
TB:	tuberculosis
TLC:	thin layer chromatography
UV:	ultra violet

WHO:	World Health Organization
XTT:	2,3-bis- [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide

Summary

In vitro anti-HIV-1 properties of ethnobotanically selected South African plants used in the treatment of sexually transmitted diseases

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Department of Plant Science

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Extracts of ten ethnobotanically selected medicinal plants used in the treatment of STD's were investigated for their anti-HIV properties against enzymes and proteins that play a role in the HIV life cycle. The antiviral activity was studied through the luciferase-based assay targeting the HIV promoter activation induced by either the HIV-1 Tat protein or the cellular transcription factor NF-κB, both required for efficient HIV-1 replication. Of the ten plant extracts investigated *Zanthoxylum davyi* and *Elaeodendron transvaalense* showed the most promising results. These extracts also showed specific luciferase inhibitory activity in the Hela-Tet-ON assay and did not show significant toxicity on MT2 cell line. The plant extracts were also tested against some enzymes (glycohydrolase and reverse transcriptase) that play a significant role in the HIV life cycle. *Senna petersiana* and *Terminalia sericea* showed to be potential inhibitors of both glycohydrolase and reverse

transcriptase enzymes.Futher phytochemical studies of *E. transvaalense* have led to the isolation of four known triterpenes [lup-20(30)-ene-3,29-diol, (3α)-(9Cl)] (**1**), [lup-20(29)-ene-30-hydroxy-(9Cl)] (**2**), Ψ – taraxastanonol (**3**), β-sitosterol (**4**) a catechin 4' –*O*- methylepigallocatechin (**5**), the rarely found phenolic derivative, atraric acid (**6**) and the depside, atranorin (**7**). The activities of Compound **6** and **7** were not analyzed further because of the low amount isolated. To evaluate the antiviral activity of the other five isolated compounds, NF-κB, anti-Tat and viral replication assays were performed. Only lup-20(29)-ene-30-hydroxy-(9Cl) (**2)** inhibited NF-κB activity at a low concentration of 10 µg/ml. Lup-20(30)-ene-3,29-diol, (3α)-(9Cl) (**1)** and Ψ – taraxastanonol (**3)** showed anti-NF-κB inhibition at a higher concentration of 50 µg/ml. The activities of the isolated compounds were not significant in other anti-HIV assays. All five isolated compounds were further analyzed for cytotoxicity using the XTT assay on Vero and MCF-7 breast cancer cell lines. Compound **2** demonstrated greater than 50 % growth inhibition at 25 µg/ml. The crude extract and other isolated compounds showed very little or no toxicity at the same concentration. The isolated compounds were also tested in the HIV-reverse transcriptase assay and none of these compounds displayed any RT activity. These results support the ethnomedicinal uses of these plants to some extent.

Keywords: Cytotoxicity; Terpenoid; HIV**;** NF-κB; *Elaeodendron transvaalense*

CHAPTER 1: Introduction

1.1 Background

Plants have not only provided mankind with food, clothing, flavours and fragrances, but have also served humanity to treat different ailments. According to the World Health Organization (WHO), about three-quarters of the world population rely on plants for the treatment of many illnesses. 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 (Van Wyk & Gericke, 2000). Therefore it is logical to expect biological 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 resurgence 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 (Houghton, 1996). Many useful drugs have been developed from medicinal plants used in traditional medicine in the treatment of a variety of illnesses. According to Gilani & Atta-ur-Rahman (2005), “The use of plants, plant extracts or plant-derived pure chemicals to treat diseases is a therapeutic modality, which has stood the test of time”. Most of the clinical drugs that are currently in use were derived from plants and developed because of their use in traditional medicine. Asprin (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 (Gilani & Atta-ur-Rahman, 2005).

1.2. HIV/AIDS

1.2.1 HIV life cycle

Acquired immunodeficiency syndrome (AIDS), is a global health problem affecting more than 42 million people worldwide (Figure 1.1 & 1.2). Human immunodeficiency virus (HIV) is the pathogen that causes AIDS, a complex array of disorders resulting from the deterioration of the immune system (Silva *et al.*, 2004). The infected individuals become susceptible to opportunistic pathogens such as common microbes and often suffer from tuberculosis (TB), pneumonia and rare forms of cancer. HIV uses the macrophages and T cells as sites for reproduction and production of multiple copies of viral genetic material ready to infect new viral hosts. During each round of infection more cells of the immune system are damaged or killed. The host cells produce antibodies and helper T-cells in order to fight the virus, but eventually the virus prevails and opportunistic diseases associated with AIDS appear (Gurib-Fakim, 2006).

HIV-1 infection (Figure 1.3) begins with a virus binding to a susceptible cell (step 1). Following binding, membrane fusion facilitated in part by the CKR-5 protein, results in the introduction of the HIV-1 (step 2). Reverse transcription of the viral RNA (step 3) and the integration of resulting DNA copy into the host-cell chromosomes ensues (step 4). Once integrated in the chromosome,

the transcriptal activity of the HIV-1 provirus is regulated in part by the virally encoded tat protein (step 5). Following synthesis of a full- length viral RNA, viral mRNAs can be produced. The HIV-1 rev protein controls this process and makes mRNA transcripts available in the cytoplasm for the translation of viral

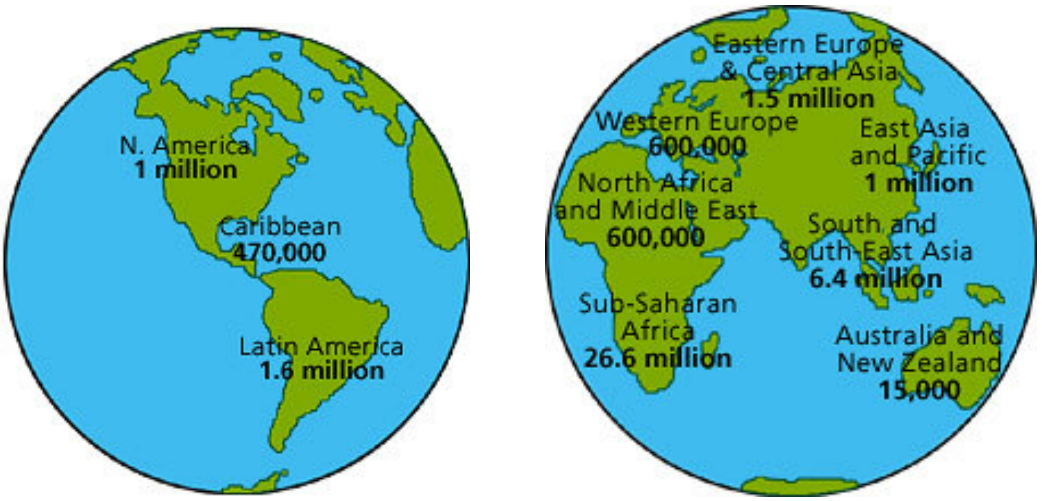

Figure 1.1 Worldwide HIV infections in 2005 (www.scripps.edu)

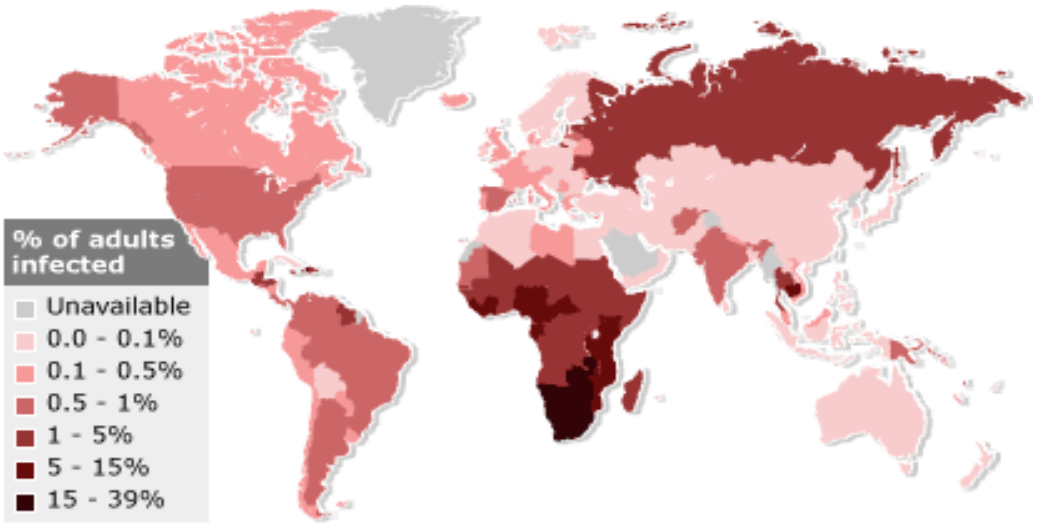

Figure 1.2 Worldwide HIV prevalence rates in 2005 (www.unaids.com)

proteins (step 6). Synthesis of viral structure proteins proceeds (step 7), HIV-1 particles assemble (step 8), and acquire viral envelope proteins as they bud through the host-cell membrane. The viral polyproteins are cleaved by viral protease during or shortly after budding, generating mature infectious virions (step 9) (Perry, 1997).

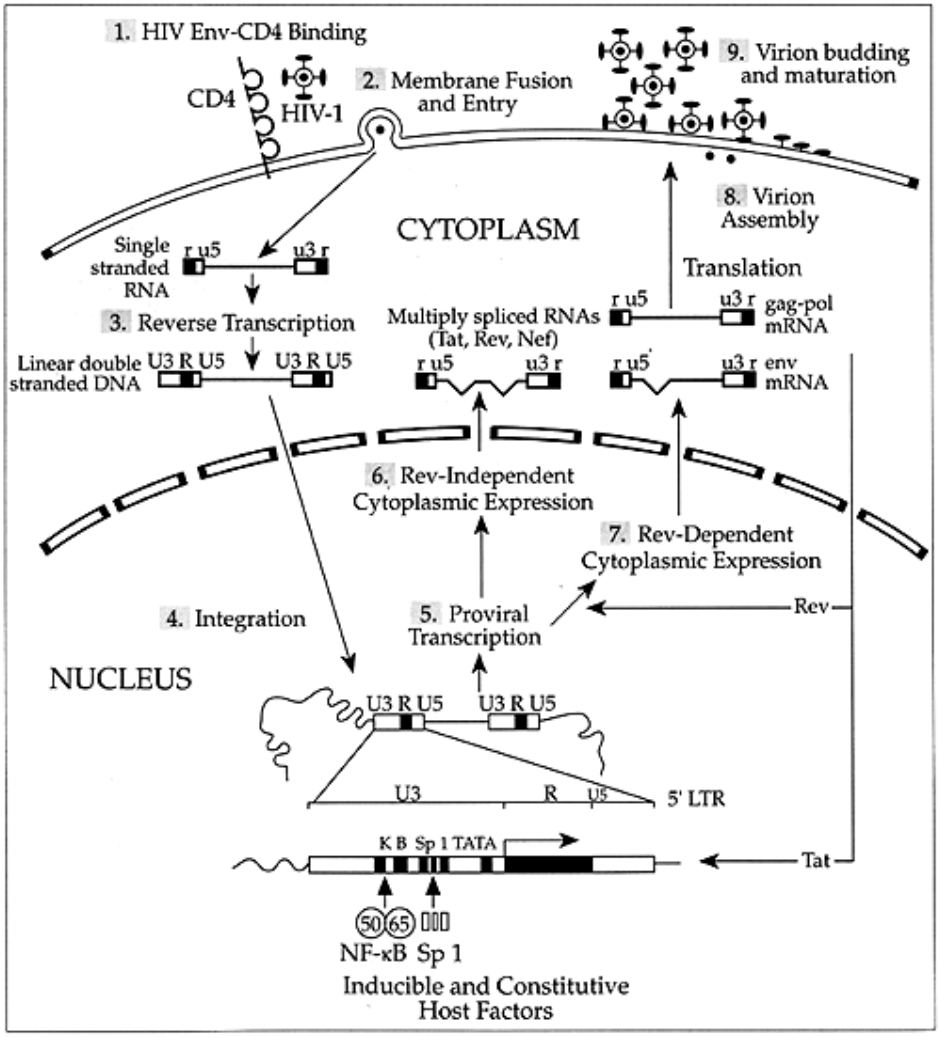

Figure 1.3 The replication cycle of HIV-1 (Perry, 1997).

1.2.2 HIV treatment and research on natural products

The antiretroviral agents which are currently available for the treatment of HIV infection targets enzymes essential to the life cycle of the virus. HIV reverse transcriptase (RT) is crucial for viral replication, HIV protease facilitates maturation and infectivity of virion particles and HIV intergrase mediates HIV intergration into the host genetic material (Ng *et al*., 1997). More than twenty drugs have already been licensed for HIV treatment, including formulations of both individual and combined antiretroviral agents. Specific inhibitors of several stages of the viral cycle, including viral attachment and entry are subject to preclinical investigation or have already entered clinical trials (Notka *et al*., 2003). Efforts to find other anti-HIV agents have been mainly focused on the development of drugs that targets viral proteins, which are essential for viral replication. The current antiviral therapy presents important limitations such as side effects and appearance of mutant viruses that are resistant and makes drugs which are currently in use to be insufficient to maintain a safe theraupeutic arsenal against HIV (Bedoya *et al*., 2006; Ma *et al*., 2002).

There is a global need or demand for broader, safer and cheaper drugs for the treatment of HIV infection. One of the approaches is to find anti-HIV agents from medicinal plants. New anti-HIV compounds from natural sources are often reported, some are essentially unproven and others with distinct promise based on *in vitro* research. Natural products have been a consistently successful source in drug discovery and may offer more opportunities to find anti-HIV drugs or lead compounds (Wang *et al*., 2006).

A number of medicinal plants have been screened and resulted in the isolation of some active compounds with inhibitory activity on HIV. One of the most promising anti-HIV compounds, calanolide was isolated from a Malaysian tree belonging to the Garcinia family. Calanolide, a coumarin, is now being tested in human trials (Gurib-Fakim, 2006). Other coumarins with anti-HIV activity have been reported by Bedoya *et al*. (2005) and Uchiumi *et al*. (2003). Active compounds like benzo[c]phenanthridine decarine and others isolated from *Zanthoxylum ailanthoides* (Figure 1.4) also showed anti-HIV activity in acutely infected H9 cells as reported by Cheng *et al*. (2005).

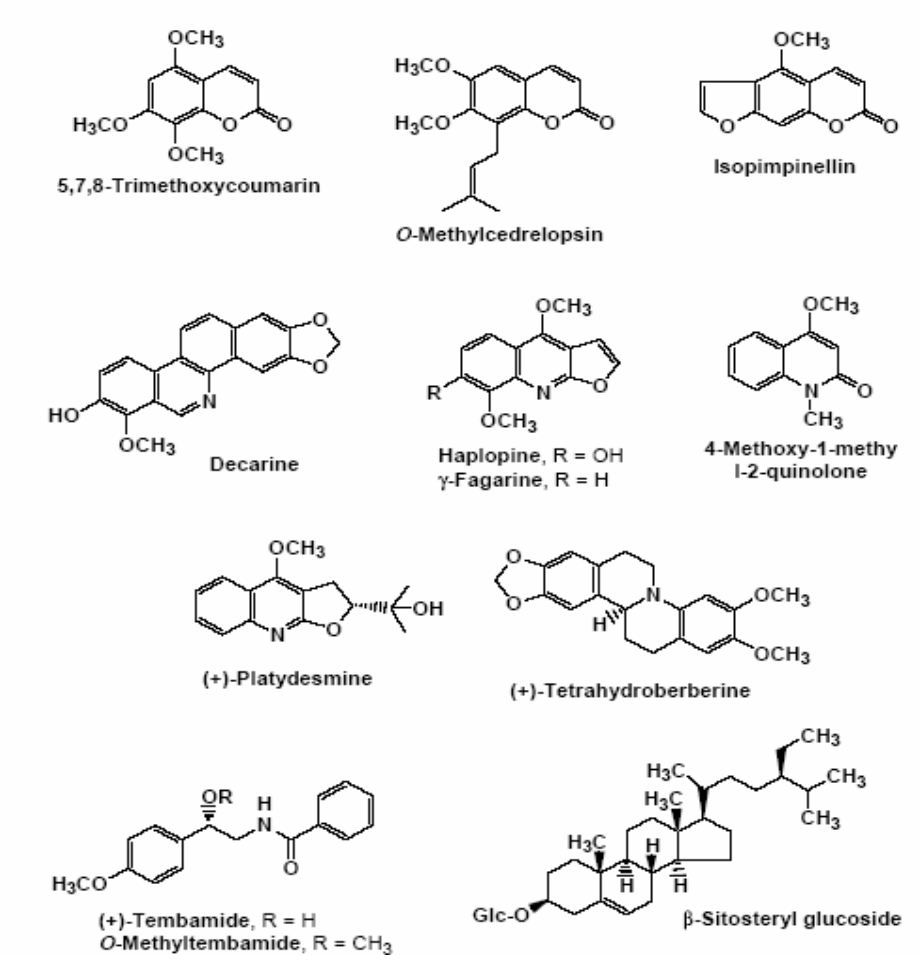

Figure 1.4 Anti-HIV constituents obtained from root bark of *Zanthoxylum ailanthoides* (Cheng *et al*., 2005).

A new kind of potential AIDS treatment by using long terminal repeat (LTR) inhibitors has now emerged since there are various factors that increase HIV activity by activating the LTR. Viral activation can be caused by the enhanced expression of the TAT gene that produces the protein, NF-κB which is normally present in the body in low concentrations. Each of these proteins binds to the LTR and activates HIV (Figure 1.5). The search for LTR inhibitors also led to the investigation of medicinal plants. Reports have shown that anti-NF-κB and anti-Tat agents from medicinal plants could be possible therapeutic agents against HIV infection. These agents seem to have the capacity of inhibiting HIV activation from latency, mainly through interference of NF-κB and Tat functions (Bedoya *et al.*, 2005 and Akesson *et al.*, 2003).

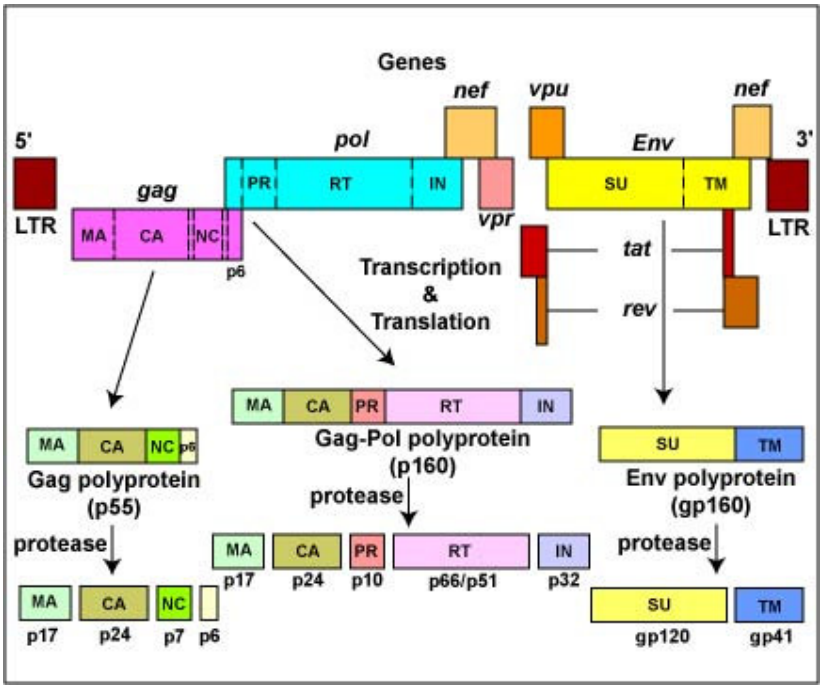

Figure 1.5 The RNA genome of HIV-1 (www.aids.harvard.edu)

1.2.3 Nuclear factor kappa B (NF-κB) and viral Tat transactivator

NF-κB constitutes a family of transcription factors that is important for cellular functions such as cell cycle progression and proliferation (Akesson *et al.*, 2003). It plays an important role in the regulation of a multitude of genes (Figure 1.6) involved in cell survival and it has also been implicated in inflammatory diseases such as rheumatoid arthritis, multiple sclerosis, asthma and atherosclerosis.

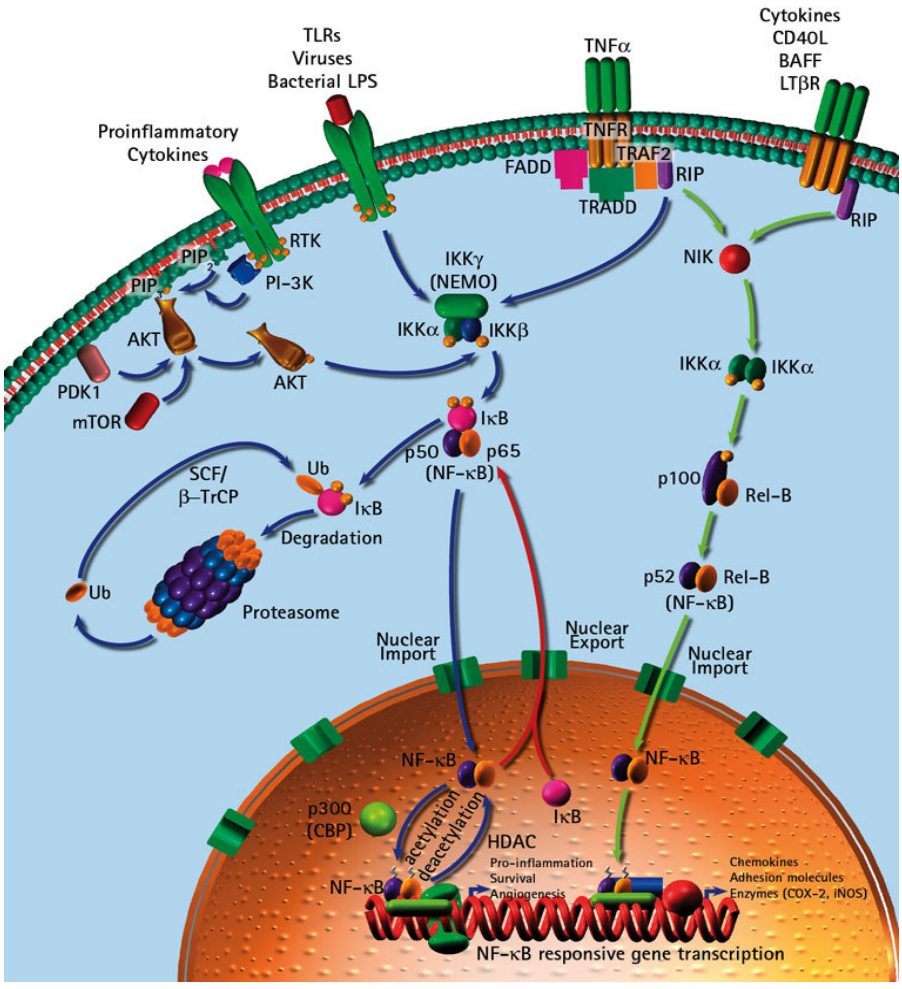

Figure 1.6 Nuclear factor kappa B (NF-κB) pathway (www.emdbioscience.com)

When cells are not stimulated, NF-κB is retained in the cytoplasm in a complex with the inhibitor of κB (IκB) proteins. NF-κB can be activated by stimuli including pro-inflammatory cytokines TNF-∝ which promotes phosphorylation of the IκB proteins followed by degradation of IκB and subsequent translocation of NF-κB into the nucleus (Lindgren *et al*., 2001). The Tat protein of HIV-1 is a small polypeptide of 101 amino acid essential for the transcription of viral genes and for viral replication. Tat activates HIV-1 transcription by promoting the assembly of transcriptionally active complexes at the LTR through interaction with TAR (Figure 1.7).

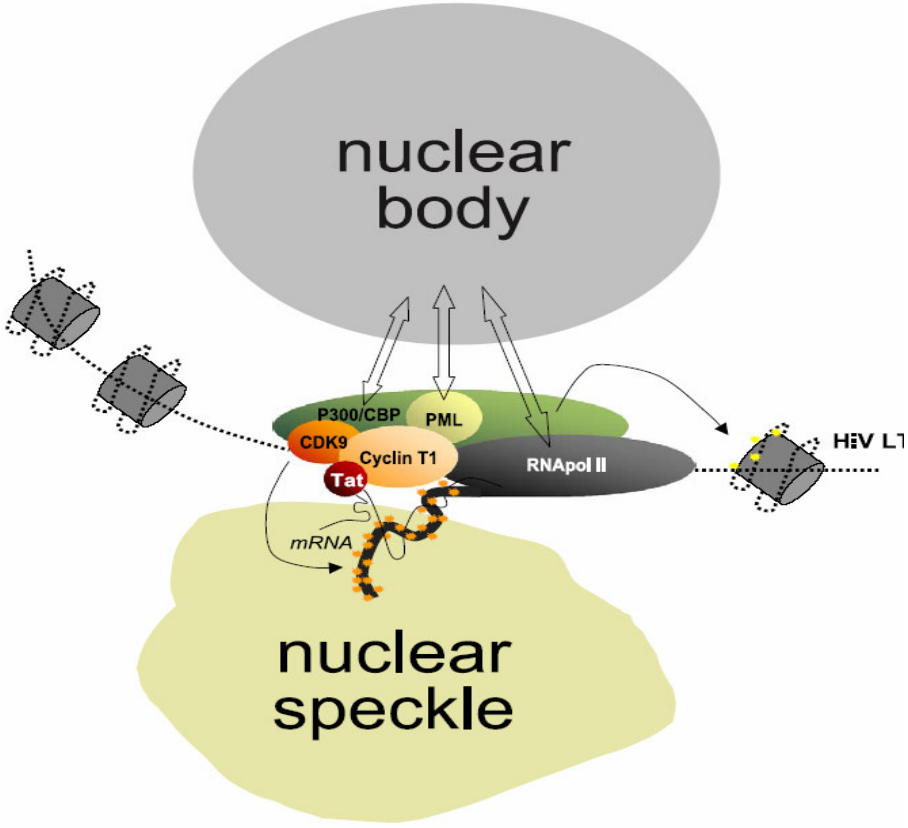

Figure 1.7 A model for regulation of Tat mediated transcriptional activation of the chromatinized HIV LTR promoter (www.clarku.edu/faculty/shuo/homepage/research).

Upon transcriptional activation, multiple spliced, short transcripts arise, encoding for viral accessory proteins including Tat and Rev. These accessory proteins enhance viral transcription and promote the expression and export of the late, unspliced RNAs from the nucleus (Marcello *et al*., 2004).

1.3 Aims and objectives of the study

HIV continues to pose an unprecedented 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. Several studies have demonstrated the inhibitory properties of variety of crude plant extracts, as well as isolated compounds against different stages of HIV life cycle. Compounds such as papavarine, glycyrrhizin and trichosanthin were seen to have promise and have been evaluated in AIDS patients (Bessong & Obi, 2006).

South African plant extracts such as *Lobostomon trigonus* (Boraginaceae), and the gallotannin isolated from *Peltophorum africanum* (Fabaceae) strongly inhibited HIV reverse transcriptase functions (Bessong *et al*., 2005). People often claim that their medicinal plant remedies could improve an AIDS patient's quality of life. However, the efficacy of these remedies or plants has mostly not been proved (Woradulayapinij *et al*., 2005).

The main objectives of the study were to determine the:

- anti-HIV activity of plant extracts and isolated compounds against glycohydrolase, reverse transcriptase, NF-κB and Tat proteins.
- cytotoxicity of isolated compounds.

1.4 Plant selection

The plant selection for this study was based on interviews with three traditional healers and a literature review of traditional medicinal plant usage in South Africa. Four of the selected plants (*Clerodendrum glabrum*, *Polianthes tuberosa*, *Rotheca myricoides* and *Senna occidentalis*) were collected after the interview response given by the late Mr Anthony, who was a traditional healer in Soshanguve and often received HIV/AIDS patients. He worked closely with medical doctors and his patients showed an increase in CD4 counts after treatment with the abovementioned plants.

Anredera cordifolia, *Elaeodendron transvaalense*, *Rauvolfia caffra* and *Zanthoxylum davyi* are used by Vhavenda people (Limpopo Province) in the treatment of sexually transmitted diseases (STDs) and two traditional healers (Mr T. Ramudingane and Mrs V. Nemutandani) interviewed claimed that these plants can also help AIDS patients. *Senna pertesiana* and *Terminalia sericea* also used traditionally by Vhavenda people to treat STDs and recent

reports have shown these plants to possess some compounds with anti-HIV activity (Eldeen, 2006; Mabogo, 1990).

It was not possible to select plants used historically for treatment of AIDS, since it does not seem to have been known as a disease until the early 1980s. However, this study investigated *in vitro* anti-HIV properties of some ethnobotanically selected plants used in the treatment of sexually transmitted diseases. It is commonly recognized that bioactive compounds are more likely to be discovered from screening guided by traditional medicine than from random screening (Lee & Houghton, 2005).

1.5 Scope of the thesis

The activity of crude extracts against glycohydrolase and reverse transcriptase enzymes is described in Chapter 2. NF-κB, Hela-Tat and cytotoxicity assays on plant extracts are described in Chapter 3. Chapter 4 deals with the isolation and identification of pure compounds from *E. transvaalense* extract. Chapter 5 describes the anti-HIV activity of isolated compounds and Chapter 6 describes their cytotoxicity. Chapter 7 comprises of the general discussion and conclusions.

1.6 Hypothesis

The extracts and/or isolated compounds from medicinal plants used to treat STD's will be active against HIV.

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CHAPTER 2:

Activity of crude plant extracts against glycohydrolases and reverse transcriptase

2.1 Introduction

Plant products have attracted attention as possible anti-HIV drugs targeted on the specific steps of the viral life cycle, such as viral attachment and entry as well as on essential enzymes and proteins that play a role during viral genome transcription (Matsuse *et al*., 1999). The approved medications that are currently in use are mainly restricted to target the two viral enzymes, protease and reverse transcriptase (RT). These inhibitors are very expensive and this has led to a global demand for broader, safer and also cheaper medications (Notka *et al*., 2004). A variety of plant extracts have been found to inhibit the reverse transcription process and glycohydrolase enzymes (Collins *et al*., 1997).

Glycohydrolase enzymes are found in the Golgi apparatus of eukaryotic cells. These are responsible for glycosylation of proteins. Inhibition of the

glycohydrolase enzymes has been found to decrease the infection caused by HIV virion, as the HIV glycoproteins are highly glycosylated (Collins *et al*., 1997).

Reverse transcriptase is an enzyme that reads the sequence of HI viral RNA nucleic acids that have entered the host cell and transcribes the sequence into complementary DNA. Without reverse transcriptase, the viral genome cannot be incorporated into the host cell; as a result a virus won't reproduce. Reverse transcriptase is therefore the principal target enzyme of antiretroviral drugs such as Nevarapine and Delavirpine that are used to treat HIV infected patients (De Clercq, 2007; Woradulayapinij *et al*., 2005).

This part of the study focuses on the screening of 10 South African medicinal plants (Table 2.1) associated with the treatment of STD's. Activity of the crude extracts against glycohydrolases and reverse transcriptase will be determined.

2.2 Materials and Methods

2.2.1 Plant Material

All the plant material (roots and stem bark) were collected based on their traditional uses against sexually transmitted diseases (Syphilis, gonorrhea, herpes as well as HIV) and some literature reports on their biological activity as antimicrobial agents. More information on the use of these medicinal plants was gathered through interviews with traditional healers as explained in chapter 1.

The plants investigated (Table 2.1) were collected from Venda in the Limpopo Province (South Africa). Voucher specimens were prepared and identified at the H.G.W.J. Schweikerdt Herbarium, University of Pretoria.

2.2.2 Preparation of extracts

Thirty gram of powdered roots or stem bark were extracted twice for 2 hours with 300 ml of chloroform, acetone, ethyl acetate or water and filtered. The extracts were then concentrated to dryness under reduced pressure and the residue was freshly dissolved in appropriate buffer on each day of experiment for the assays. Depending on the assay, extract that could not dissolve in appropriate buffer were first dissolved in DMSO and later diluted to different concentrations needed for a particular assay. .

Table 2.1: Medicinal plants investigated in this study for anti-HIV activity.

Species	Family	Part used	Voucher no	Other ethnobotanical information
Anredera cordifolia (Ten.) Steenis	Basellaceae	Stem tubers	Smit 085981	Pain, inflammation (Tornos *et al*., 1999)
Clerodendrum glabrum E. Mey var. glabrum	Lamiaceae	Roots	Van Wyk 51839	Malaria (Clarkson *et al*., 2004)
Elaeodendron transvaalense (Burtt Davy) R.H. Archer	Celastraceae	Stem bark	Tshikalange 092524	Stomach ache, fevers, diarrhoea (Van Wyk *et al*., 1997)
Polianthes tuberosa L	Agavaceae	Roots	E.T 29	Ornamental (Huang *et al*., 2001)
Rauvolfia caffra Sond.	Apocynaceae	Stem bark	Hemm 39291	Diarhoea, abdominal complaints (Palgrave, 1977)
Rotheca myricoides (Hochst.) Vatke	Lamiaceae	Roots	Van Wyk 45727	Malaria (Muregi *et al*., 2007)
Senna occidentalis (L.)	Fabaceae	Roots	Lubbe 075884	Malaria (Tona *et al*., 2004)
Senna petersiana (Bolle) Lock	Fabaceae	Roots	Van Wyk 070978	Fevers, skin infections (Coetzee *et al*., 2000)
Terminalia sericea Burch. ex DC.	Combretaceace	Roots	Van Rensburg 38564	Diabetes, diarrhea (Moshi & Mbwambo, 2005)
Zanthoxylum davyi (I.Verd.) P.G. Waterman	Rutaceae	Roots	Lubbe 078130	Chest pains, wounds, toothache, coughs (Tarus *et al*., 2006)

2.2.3 Glycohydrolase enzymes

To measure the inhibition of the glycohydrolase enzymes, α - glucosidase (Sigma, MO, USA) and β- glucuronidase (Roche Diagnostics, Germany) were used with their corresponding substrates ρ-nitrophenyl-α-D-glucopyranose and ρ-nitrophenyl-β-D-glucuronide in a 96-well microtitre plate in a colorimetric enzyme based assay. The assay was perfomed according to Collins *et al*., (1997). The substrates and enzymes were dissolved in 50 mM morpholinol thanesulfonic acid-NaOH, pH 6.50. The enzyme was calibrated relative to an enzyme concentration of 0.25 µg/ml.

To test enzyme inhibition, each well of the microtitre plate had a reaction volume of 200 µl containing 2mM substrate, 40 mM buffer, enzyme and the plant extracts at 200 µg/ml concentrations. The extracts were allowed to interact with the enzyme for five minutes before the substrate was added. This reaction was allowed to proceed for 15 minutes and terminated with 60 µl of 2 M glycine NaOH, pH 10. The assay was carried out in triplicate. Control 1 only contained the buffer (40 µl) and substrate (20 µl) (no enzyme and extracts were added). For the second control the enzyme, buffer and substrate were added for the reaction to take place. The absorbance was read on a microtitre plate reader at 412 nm. Results were analysed using the formula:

Percent Inhibition = Sample absorbance/Control 1 absorbance X 100

2.2.4 HIV reverse transcriptase (RT) assay

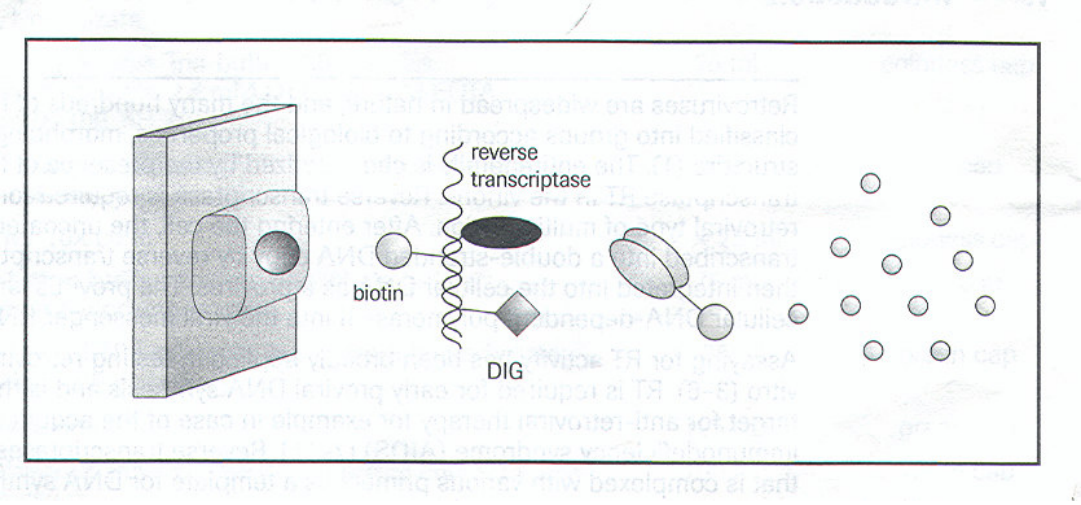

Figure 2.1: Reverse transcriptase colorimetric assay principle (Roche, 2005)

From the 10 plant extracts tested for glycohydrolase enzymes, only the four best plants were selected for RT activity testing. The effects of plant extracts on RT activity *in vitro* were evaluated with recombinant HIV – 1 enzyme, using a non-radioactive HIV–RT colorimetric (Figure 2.1) ELISA kit from Roche, Germany (Ayisi, 2003; Harnett *et al.*, 2005). The concentration of the extracts was 200 ug/ml. Extracts which reduced activity by at least 50 % were considered active (Woradulayapinij *et al.*, 2005). Doxirubicin was used as a positive control at 100 µg/ml. The assay was carried out in triplicate. Control 1 only contained the buffer and reaction mixture (no enzyme and extracts were added). For the second control the enzyme and reaction mixture were added for the reaction to take place. The absorbance was read on a microtitre plate reader at 412 nm and a reference wavelength of 490 nm. Results were analysed using the formula:

Percent Inhibition = Sample absorbance/Control 1 absorbance X 100

2.3 Results and discussion

The results shown in Table 2.1 demostrate the inhibition percentage of plant extracts against glycohydrolase enzymes, *α- glucosidase* and *β- glucuronidase*. The extracts of *Ceodendrum glabrum*, *Polianthes tuberosa* and *Senna occidentalis* showed no inhibition against *α- glucosidase*. The other seven extracts showed inhibition percentage ranging from 4.00 ± 0.01 to 90.00 ± 0.01, with the extracts of *Senna petersiana* (80.00 % inhibition) and *Terminalia sericea* (90.00 % inhibition) being the most active. *Elaeodendron transvaalense* and *Zanthoxylum davyi* showed weaker inhibition (not significant) of 23.00 and 22.00 respectively.

The inhibition percentage of *β- glucuronidase* by the extracts ranged from 1.40 ± 0.01 to 90.00 ± 0.01, with the extract of *P. tuberose* showing no inhibition. The most active extracts against *β- glucuronidase* were *S. petersiana* and *T. sericea* which exhibited 80.00 ± 0.01 and 90.00 ± 0.01 % inhibition respectively. All the extracts were tested at 200 µg/ml to determine their inhibitory activity against each enzyme and 50.00 % inhibition or higher were taken as significant. In this study the most promising inhibition against glycohydrolase enzymes tested was obtained with extracts of *S. petersiana* and *T. sericea*.

Table 2.2: Inhibition of glycohydrolases (percent) in the presence of medicinal plant extracts at 200 µg/ml concentration

Plant	Extract	*α- glucosidase*	*β- glucuronidase*
		Inhibition % (S.D)	Inhibition % (S.D)
Anredera cordifolia	70 % Acetone	11.00 ± 0.00	33.00 ± 0.04
Clerodendrum glabrum	Water	ni	4.00 ± 0.01
Elaeodendron transvaalense	Chloroform	23.00 ± 0.02	10.00 ± 0.00
Polianthes tuberosa	70 % Acetone	ni	ni
Rauvolfia caffra	70 % Acetone	10.00 ± 0.00	6.00 ± 0.01
Rotheca myricoides	Water	4.00 ± 0.01	1.40 ± 0.01
Senna occidentalis	70 % Acetone	ni[a]	6.00 ± 0.00
Senna petersiana	70 % Acetone	80.00 ± 0.03	80.00 ± 0.00
Terminalia sericea	Ethyl Acetate	90.00 ± 0.01	90.00 ± 0.01
Zanthoxylum davyi	70 % Acetone	22.00 ± 0.01	14.00 ± 0.10

ni[a] : no inhibition

The *in vitro* inhibitory activities of the extracts against the reverse transcriptase (RT) enzyme are shown in Table 2.3. Only *T. sericea* exhibited the most notable activity of 94 % against RT. *E. transvaalense* (8 % inhibition) and *Zanthoxylum davyi* (20 % inhibition) showed weaker inhibition which was not significant.

Table: 2.3: Effect of plant extracts (200 µg/ml) on the activity of recombinant HIV –1 reverse transcriptase.

Extracts	Inhibition %	Standard deviation
Control 1	100	± 0.02
Control 2	0	± 0.10
E. transvaalense	8.00	± 0.00
S. petersiana	ni*	± 0.00
T. sericea	94.00	± 0.00
Z. davyi	20.00	± 0.02
Doxorubicin (100 µg/ml)	71.00	± 0.17

*ni, no inhibition

The previous reported biological activities (not on HIV) of *T. sericea* were mainly attributed to triterpenoids, saponins and tannins (Steenkamp *et al*., 2004, Bombardelli *et al*., 1974, Fyhrquist *et al*., 2006). Eldeen *et al.* (2006) reported the isolation of the lignan, Anolignan B from *T. sericea* root extract. This compound was first isolated from *Anogeissus acuminata* and was reported as a constituent

acting with Anolignan A to inhibit the enzyme HIV-RT (Eldeen *et al*., 2006).The ethyl acetate extract of *T. sericea* in this study showed the most notable inhibition of reverse transcriptase enzyme and this could possibly be attributed to the abovementioned chemical constituents reported previously. Further phytochemical studies need to be conducted in order to determine the activity of individual compounds against reverse transcriptase enzyme.

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CHAPTER 3:

NF-κB, Hela-Tat and cytotoxicity assays on plant extracts

3.1 Introduction

The human immunodeficiency virus type 1 (HIV-1) infects CD4+ T-lymphocytes and macrophages and its genetic material is integrated into the infected cell's genome. Upon integration the virus remains transcriptionally silent and this allows the infected cell to escape currently used antiretroviral drugs. In latently infected cells, viral transcription can be reactivated with various stimuli, including, phorbol esters and cytokines (Marcello *et al*., 2004). When cells are activated, transition from latency to HIV gene expression occurs and requires the converted action of cellular transcription factors and regulatory HIV proteins (Bedoya *et al*., 2005). Among these proteins the HIV cellular transcription factor NF-κB and Tat are required for efficient HIV-1 replication. These proteins regulate the post-integration phase of the viral cycle, which preferentially occurs in activated cells on the long terminal repeat promotor (LTR). The viral regulatory proteins and cellular factors represent potential targets that should be considered in the search for anti-HIV-1

agents, because they determine the extent of HIV-1 gene transcription and the level of viral replication in the infected cells (Sancho *et al*., 2004).

Because of a persistent and urgent need for a preventive HIV vaccine, interest in the anti-HIV activity of traditional medicinal plants has now gained momentum. Because of their low cost, plants have been increasingly explored for production of biomedicine and vaccines (Karasev *et al*., 2005). Several plant-derived substances including phenylcoumarins and plant proteins have showed good anti-HIV activity that can be related to inhibition of NF-κB and Tat proteins (Akesson *et al*., 2003; Reddy *et al*., 2004; Marquez *et al*., 2005). This part of the study is aimed at evaluating anti-NF-κB and anti-Tat activity and cytotoxicity of medicinal plant extracts that are used traditionally in the treatment of STD's.

3.2 Materials and methods

3.2.1 Plant material

Plant material were collected and extracted in different solvents as described in Chapter 2.

3.2.2 Cell lines

MT2 cells were cultured in RPMI 1640 medium (Gibco BRL), containing 10 % fetal bovine serum, 2 mM glutamine, penicillin (50 IU/ml) and streptomycin

(50 µg/ml). MT-2 cells were cultured at 37 ºC in a 5 % CO_2 humidified atmosphere and splinted twice a week. The 5.1 cell line (obtained from Dr. N. Israel, Institut Pasteur, Paris, France) was maintained as MT2 cell line but the media was supplemented with 100 µg/ml G418 (Gibco BRL). Both Hela-Tat-luc and HeLa-Tet-ON cell lines were maintained in DMEM (Gibco BRL) in the presence of 100 µg/ml of hygromycin (Invitrogen) and 100 µg/ml of G418 (Gibco BRL). These cell lines were maintained at 37 ºC in a 5 % CO_2 humidified atmosphere and splinted when confluent.

3.2.3 NF-κB assay

To determine the anti-NF-κB activity of the selected extracts, an NF-κB-dependent luciferase assay was used. The 5.1 cell line was a Jurkat-derived clone stably transfected with a plasmid containing the firefly luciferase gene driven by the HIV-LTR promoter. This promoter is highly dependent on NF-κB activation induced by TNFα. Therefore high expression of luciferase activity reflects NF-κB activation through the canonical pathway.

The assay was done as described by Marquez *et al*., (2005). Briefly, 5.1 cells were pre-incubated with increasing concentrations of the extracts for 30 min and then stimulated with TNFα (2 ng/ml) for 6 h. Then, the cells were lysed in 25 mM Tris phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1 % Triton X-100, and 7 % glycerol. Luciferase activity was measured using an Autolumat LB 953 following the instructions of the luciferase assay kit (Promega) and protein concentration was measured by the Bradford method (Sancho *et al*.,

2004). The background obtained with the lysis buffer was subtracted in each experimental value and the specific transactivation was calculated as RLU/µg protein (relative light units) and the results were expressed as the percent of inhibition where 100 % activity was assigned to transcriptional activity induced by TNFα alone (Campagnuolo *et al.*, 2005). All the experiments were repeated at least four times.

3.2.4 Hela-Tat-Luc assay

To identify potential anti-Tat extracts, another luciferase-based cell system (HeLa-Tat-Luc cells) was used. The HeLa-Tat-Luc cells were stably transfected with the plasmid $pcDNA_3$- TAT together with reporter plasmid LTR-Luc. Therefore the HIV-1 LTR is highly activated in this cell line as a result of high levels of intracellular Tat protein. Cells (10^5 cells/mL) seeded the day before the assay, were treated either with the CDK9 inhibitor DRB, as a positive control, or with the plant extracts at the highest concentration of 50 µg/ml. After 12 h, the cells were washed twice with PBS and the luciferase activity measured as indicated previously for 5.1 cells. All the experiments were repeated at least four times.

3.2.5 Hela-Tet-ON-Luc assay

Compounds considered to be active in both NF-κB (>50 % inhibition) and Tat (>30 % inhibition) assays, were subsequently submitted to evaluation with

a Hela-Tet-ON assay to discard nonspecific luciferase inhibitory activity (Sancho *et al*., 2004).

The cells (10^5 cells/mL) were seeded the day before the assay, and then stimulated with doxycycline (1µg/mL) in the presence or absence of the extracts for 6 h. Then, the cells were washed twice in PBS, lysed and the luciferase activity measured as described (Sancho *et al*., 2004). All the experiments were repeated at least four times.

3.2.6 Cytotoxicity assay

MT2 cells (10^5/ml) were seeded in 96-well plates in complete medium and treated with increasing doses of the extracts for 36 h. Samples were then diluted with 300 µl of PBS and incubated for 1 min at room temperature in the presence of propidium iodide (10 µg/ml). After incubation, cells were immediately analyzed by flow cytometry. All the experiments were repeated at least four times and the results were calculated as a percentage of cell death by GraphPad software. Only extracts of *E. transvaalense* and *Z. davyi* were analysed as they had the best activity in NF-κB and Hela-Tat assays.

3.3 Results and discussion

The extracts were examined for the ability to inhibit NF-κB and Tat proteins which play a role in HIV replication. The toxicity of the active extracts to the cells was also investigated. From the results (Tables 3.1 and 3.2 and Figures 3.1 and 3.2) obtained in this study, the extracts of *Elaeodendron transvaalense, Zanthoxylum davyi* and *Polianthes tuberosa* showed activity in NF-κB and Hela-Tat assays at the highest concentration tested (50 μg/ml). The chloroform extract of *E. transvaalense* showed potent inhibitory activity (> 60 %) at the lowest concentration (1 μg/ml) tested in the NF-κB assay. Both acetone and ethylacetate extracts of *E. transvaalense* also showed a high inhibition, 67 % and 77 % respectively at a 25 μg/ml concentration, whereas *Zanthoxylum davyi* extract demonstrated moderate activity (Figures 3.1 and 3.2).

All the plant extracts were also analysed for their anti-Tat activity in the HeLa-Tat-luc assay. Chloroform and ethyl acetate extract of *E. transvaalense* showed a high Tat inhibitory activity of greater than 70 % at 15 μg/ml. The acetone extract of *E. transvaalense* demonstrated lower activity (>50 %), while *Z. davyi* exhibited moderate activity at 50 μg/ml (Figure 3.2). Those extracts showing anti-NF-κB and anti-Tat activities were also found to be specific in the HeLa-Tet-On assay.

The active extracts were also analysed for cytotoxicity to determine whether the activity was due toxicity. The results (Table 3.3) showed that,

these extracts did not cause significant cell death in the MT2 cell line. The acetone (22.7 %), ethyl acetate (27.6 %) and chloroform (17.1 %) extracts of *E. transvaalense* showed lower cell death percentages after 36 hours at the concentration tested (15 µg/ml). The acetone extract of *Z. davyi* showed little toxicity of 2.4 % cell death. These results indicate that, at the concentrations tested, anti- NF-κB and anti-Tat activity was not due to cellular toxicity.

The use of plant extracts or plant derived synthetic compounds targeting cellular proteins required for efficient HIV-1 replication and transcription has opened new avenues for scientific research in the management of AIDS. According to Marquez *et al*. (2005), plant-derived antiviral compounds interfering with the HIV-1 LTR promoter regulatory proteins are unlikely to generate drug-resistant HIV strains if proven useful for patients.

The results shown here are reported for the first time in these species and suggest that some of the extracts studied might contain chemical compounds that can have an effect on HIV inhibition.

Table 3.1 Results of anti-HIV evaluations for all plant extracts tested at 50 µg/ml

Plant name	Extracts	5.1 NF-κB (Inhi %)[a]	± SD	Hela-TAT-luc (Inhi %)[a]	± SD	Hela-TET-ON-luc (Inhi %)[b]
Anredera cordifolia	70 % acetone	ni		8.70	± 0.00	s
Clerodendrum glabrum	Water	ni		ni		s
Elaeodendron transvaalense	Chloroform	87.80	± 0.17	85.00	± 0.20	s
	Ethyl acetate	50.70	± 0.25	61.10	± 0.13	s
	70 % acetone	34.80	± 0.00	28.70	± 0.24	s
Polianthes tuberosa	70 % acetone	67.40	± 0.30	51.20	± 0.11	s
Rauvolfia caffra	70 % acetone	ni		ni		s
Rotheca myricoides	Water	ni		9.50	± 0.01	s
Senna occidentalis	70 % acetone	5.800	± 0.13	ni		s
Senna occidentalis	Water	ni		ni		s
Senna petersiana	70 % acetone	22.10	± 0.13	ni		s
Terminalia sericea	Chloroform	ni		ni		s
	Ethyl acetate	ni		ni		s
	Water	ni		nt		nt
Zanthoxylum davyi	70 % acetone	50.10	± 0.01	77.00	± 0.2	s

[a] Data are represented as % of inhibition over positive control (i.e., TNF treated cells in 5.1; and untreated in HeLa-Tat- Luc).

[b] S: specific (inhibition < 15%)

nt: not tested.

ni: no inhibition

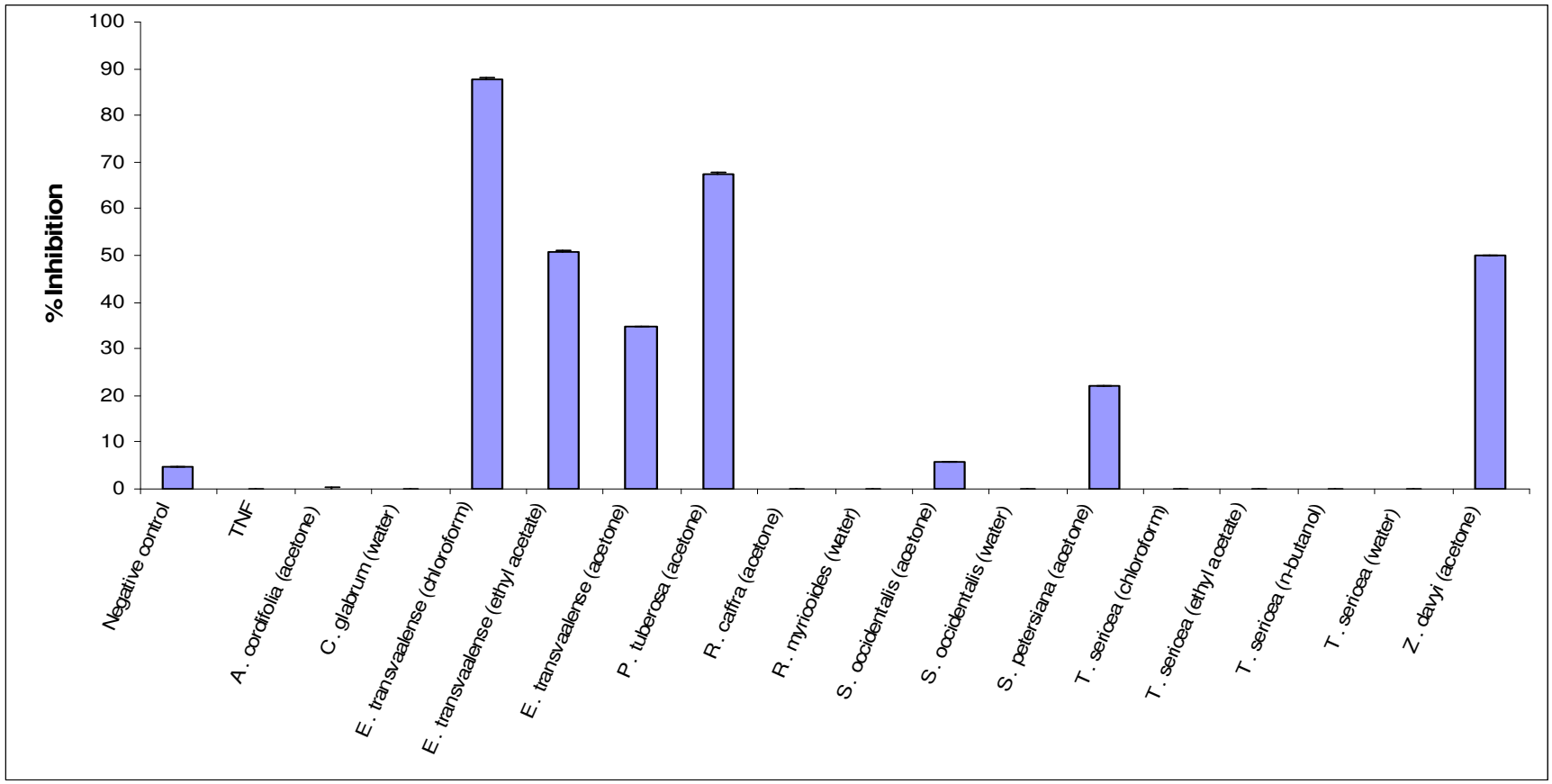

Figure 3.1 Graph showing the anti-NF-κB activity of plant extracts at a 50 µg/ml concentration.

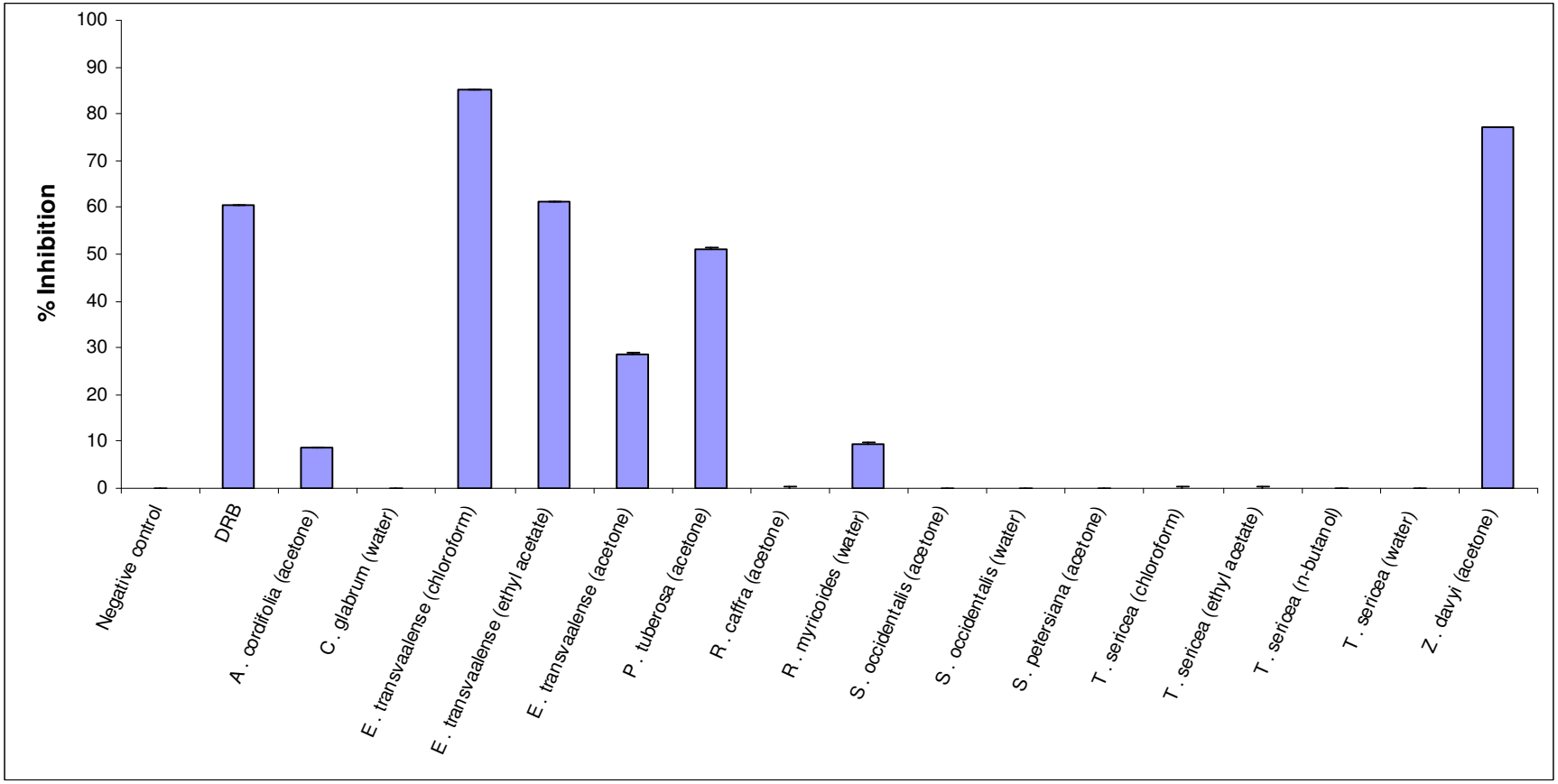

Figure 3.2 **Graph showing the anti-Tat activity of plant extracts at a 50 µg/ml concentration.**

Table 3.2 Results of anti-HIV evaluations of plant extracts that showed activity

Plant	Extracts	5.1 NF-κB (Inhi %)[a]				HeLa- Tat-Luc (Inhi %)[a]			HeLa- Tet-ON (Inhi %)[a]
		Concentration (µg/ml)							
		1	5	15	25	1	5	15	50
E. transvaalense	70% Acetone	0.0 ± 0.2	45.0 ± 0.3	54.0 ± 0.2	67.0 ± 0.4	0.0 ± 0.0	22.0 ± 0.0	43.0 ± 0.1	S
E. transvaalense	Chloroform	64.0 ± 0.6	64.0 ± 0.4	64.0 ± 0.1	73.0 ± 0.2	28.0 ± 0.1	66.0 ± 0.0	76.0 ± 0.2	S
E. transvaalense	Ethyl acetate	79.0 ± 0.3	72.0 ± 0.4	75.0 ± 0.0	77.0 ± 0.2	63.0 ± 0.0	66.0 ± 0.2	75.0 ± 0.0	S
Z. davyi	70% Acetone	34.0 ± 0.1	48.0 ± 0.3	54.0 ± 0.1	57.0 ± 0.1	1.4 ± 0.1	25.0 ± 0.0	50.0 ± 0.2	S

[a] Data are represented as % of inhibition over positive control (i.e., TNF treated cells in 5.1; and untreated in HeLa-Tat- Luc).

[b] S: specific (inhibition < 15%).

Table 3.3 Cell death (necrosis) percentage at 6, 24 and 32 hours intervals.

Plant	Extracts	Concentration (µg/ml)	6 hrs	24 hrs	32 hrs
Control			1.5 ± 0.0	2.1 ± 0.0	2.2 ± 0.5
E. transvaalense	acetone	1.0	2.1 ± 0.0	2.4 ± 0.5	2.7 ± 0.3
		5.0	2.7 ± 0.1	7.6 ± 0.2	10.9 ± 0.0
		15.0	4.2 ± 0.7	18.1 ± 0.0	22.7 ± 0.2
	ethyl acetate	1.0	2.0 ± 0.1	2.8 ± 0.1	4.8 ± 0.2
		5.0	3.4 ± 0.2	8.6 ± 0.6	14.5 ± 0.2
		15.0	2.6 ± 0.1	13.2 ± 0.2	27.6 ± 0.1
	chloroform	1.0	2.2 ± 0.0	9.5 ± 0.0	3.9 ± 0.5
		5.0	2.7 ± 0.3	7.6 ± 0.1	12.1 ± 0.3
		15.0	5.5 ± 0.1	14.7 ± 0.1	17.1 ± 0.0
Z. davyi	acetone	1.0	1.5 ± 0.4	1.9 ± 0.3	2.1 ± 0.2
		5.0	1.4 ± 0.2	1.6 ± 0.4	1.9 ± 0.1
		15.0	1.4 ± 0.2	1.8 ± 0.2	2.4 ± 0.1

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CHAPTER 4:

Isolation of compounds from *Elaeodendron transvaalense* extract

4.1 Introduction

Extracts from *Elaeodendron transvaalense* (Burtt Davy) (Celastraceae) have been used in traditional medicine by the Vhavenda people of South Africa (Limpopo province) to treat coughs, diarrhoea, stomach ailments, herpes and sexually associated diseases. Stem bark is mostly used to prepare infusions and decoctions (Mabogo, 1990). Other medicinal uses of *E. transvaalense* are shown in Table 4.1. Traditional healers prescribe it presently to people who are suffering from HIV/AIDS (Bessong *et al*., 2005). Dimethyl–1,3,8,10–tetrahydroxy–9- methoxypeltogynan and three pentacyclic triterpenes have been isolated from its bark which is also reported to contain 13.4 % catechol tannin (Hutchings,1996).

Other species belonging to the same family (Celastraceae) have been used in the Amazonian region against cancer, rheumatism and inflammation (Nakagawa *et al*., 2004). Previous reports have shown that species from the Celastraceae family contain biologically active metabolites with antimicrobial and cytotoxic activities (Sansores-Peraza *et al*., 2000). The aim of this part of study was to isolate compounds from *E. transvaalense* .

4.1.1 Plant description

E. transvaalense is a shrub or small tree (about 5 m high) occurring in forests and quite often on rocky outcrops in mountainous regions. The bark is generally smooth and has a grey colour. Leaves are often clustered on reduced lateral shoots, oblong in shape, about 50 mm long and 20 mm wide. The leaf margin is sometimes toothed. The flowers are greenish in colour and produce oblong, yellow to dark orange, berry-like fruits, which are edible (Figure. 4.1).

a **b**

Figure 4.1 (**a**) Bark and (**b**) branches of *E. transvaalense* (Van Wyk *et al.*, 1997).

The species is widely distributed in the north-eastern parts of South Africa. It also occurs along the coastal parts of KwaZulu-Natal and in Mpumalanga, Gauteng and the Limpopo province (Van Wyk, 2000).

4.1.2 Medicinal uses

E. transvaalense is used as remedy for several diseases (Table 4.11). The bark is extensively used for cleaning of the stomach and used as an enema for stomach ache, diarrhoea and fever (Mabberley, 1981).

Table 4.1: Other medicinal uses of *E. transvaalense*

Disease	Place	Preparation	References
Menorrhagia	Zimbabwe	Infusion	Gelfand, 1985
Haemorrhoids	South Africa	Unspecified	Hutchings , 1996
			Van Wyk & Gericke, 2000
Stomach cramps	South Africa	Decoction	Pujol, 1988
Diarrhoea	South Africa	Decoction	Mabberley, 1981
			Van Wyk, 1997
Herpes simplex	South Africa	Decoction	Felhabert, 1997
			Mabogo, 1990
Herpes zoster	South Africa	Decoction	Felhabert, 1997
Stroke	South Africa	Decoction	Felhabert, 1997
Gout	South Africa	Decoction	Felhabert, 1997
Rash	Southern Africa	Infusion	Van Wyk, 2000
HIV/AIDS	South Africa	Infusion	Bessong *et al.*, 2005

4.1.3 Chemistry

Elaeodendron species are rich in gallotannins, proanthocyanidins and a few other phenolic compounds like elaeocyanidin (Figure 4.2) have been isolated from this species (Van Wyk *et al.*, 1997).

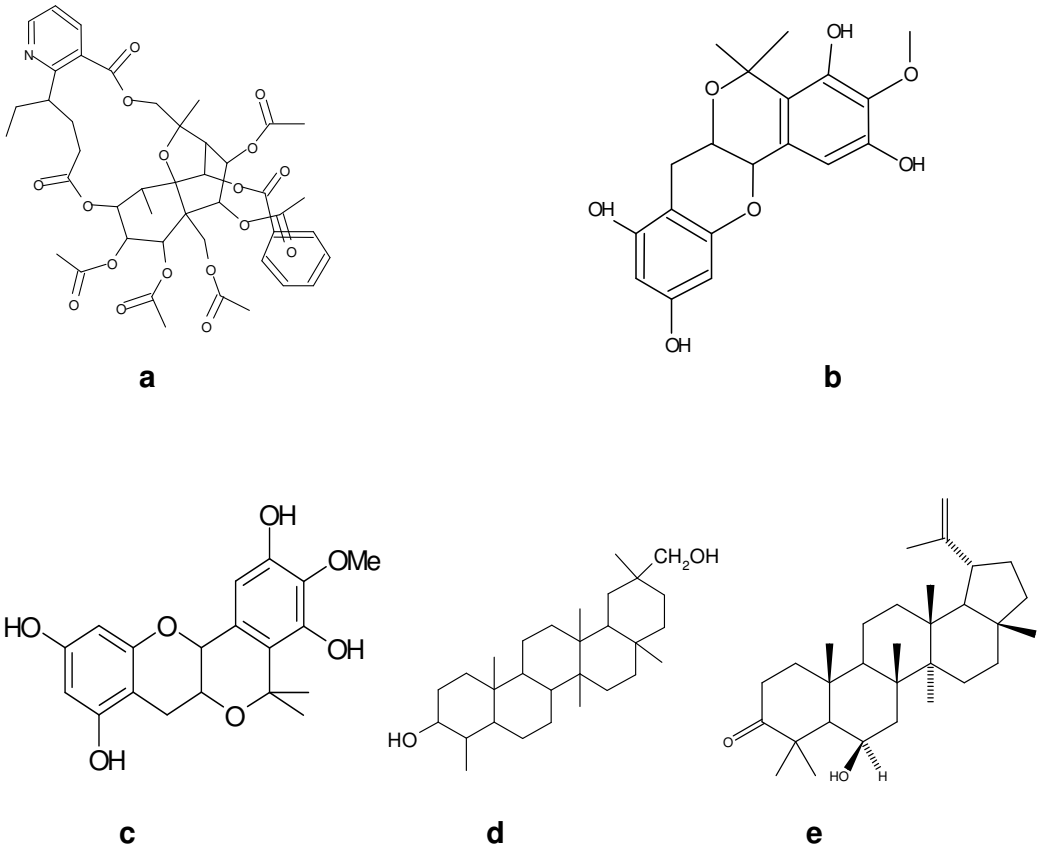


Figure 4.2 Compounds isolated from *E. transvaalense*: (**a**) cassinine, (**b**) elaeocyanidin, (**c**) 6R, 13R-11, 11-dimethyl-1, 3, 10-tetra-hydroxy-9-methoxypeltogynan, (**d**) canophyllol and (**e**) 6-β-hydroxy-lup-20(30)-en-3-one (Drewes & Mashimbye, 1993, Drewes *et al.*, 1991).

4.2 Materials and methods

4.2.1 Plant material

Stem bark of *E. transvaalense* was collected in Venda (Northern Limpopo). A voucher specimen is preserved in the HGWJ Schweickerdt herbarium at the University of Pretoria (Tshikalange 092524).

4.2.2 Preparation of extracts

In unreported results, different fresh extracts (acetone, chloroform, ethyl acetate and ethanol) of *E. transvaalense* were tested for their ability to inhibit NF-κB and Tat proteins. The ethanol extract exhibited the best activity and led us to isolate compounds from this extract.

Stem bark of *E. transvaalense* was collected and left to dry at room temperature for two weeks. The dried powder stem bark was placed in a container and soaked in ethanol. The container was closed and left in a dark cupboard for three days at room temperature before the extract was filtered and concentrated to dryness under reduced pressure (40 ^{0}C). The residue was soaked again in ethanol and filtered. The filtrates were dried with a rotary evaporator to give a total mass of 150 g (extract).

4.2.3 Isolation and identification of compounds

A 10 cm diameter glass column (Figures 4.3 and 4.4) was filled with 1.5 kg silica gel. The extract (120 g) was dissolved in a minimal amount of solvent

and mixed with 200 g silical gel. The column was eluted with a solvent gradient of hexane: ethyl acetate in 100:0 to 0:100 ratios. The column was then washed with ethyl acetate:methanol (9:1) and 100 % methanol. 45 fractions of 50 ml each were collected; fractions containing the same compounds as determined by TLC plates were combined and concentrated to dryness under reduced pressure. TLC plates of 11 pooled fractions (A-K) were developed with hexane: ethyl acetate 9:1, 7:3 and 3:7. Fraction I yielded a pure compound (**1**). Other fractions were crystallized and yielded 5 pure compounds (**2**, **3**, **5, 6** & **7**). TLC plates were examined under UV light (254 and 366 nm) after development and also dipped in vanillin (15 g vanillin, 500 ml ethanol and 10 ml concentrated 98 % sulphuric acid) and heated to detect compounds not absorbing UV. Fractions G and H were combined and subjected to a second column eluted with a solvent gradient of hexane: ethyl acetate in 100:0 to 0:100 ratios which resulted in isolation of compound **4.**

4.3 Results and discussion

The column chromatography (Figure 4.3) yielded 45 fractions (Figure 4.5) which were pooled together according to their TLC profile and resulted in 11 pooled fractions (Figure 4.6). From these pooled fractions seven compounds were isolated (Figure 4.7).

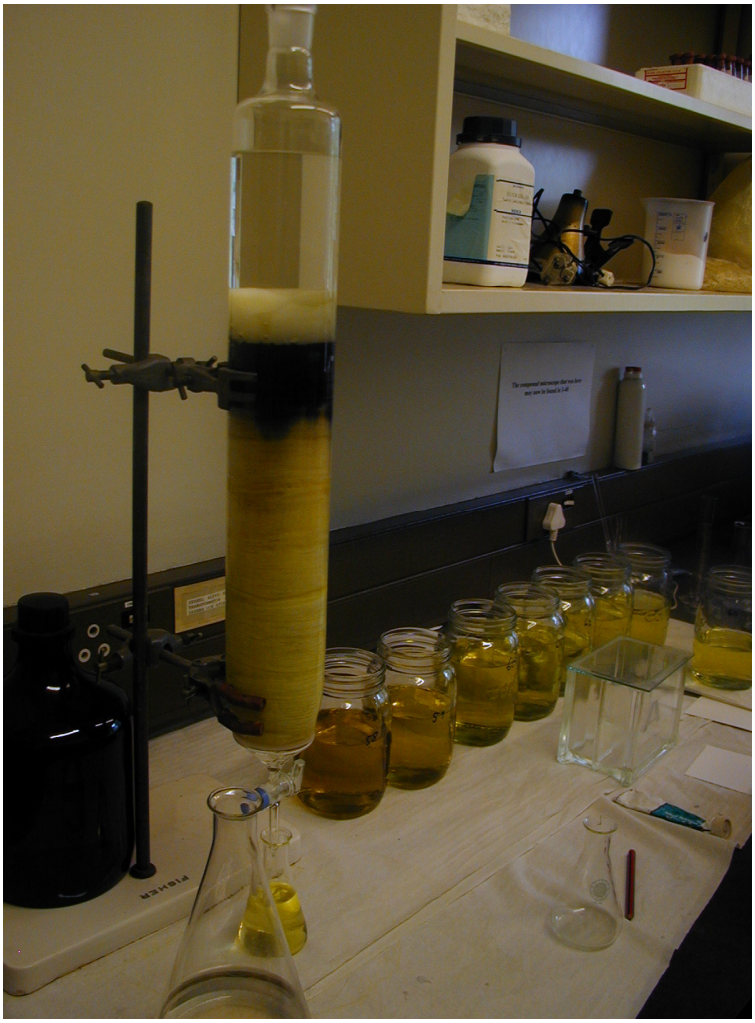


Figure 4.3 Column chromatography

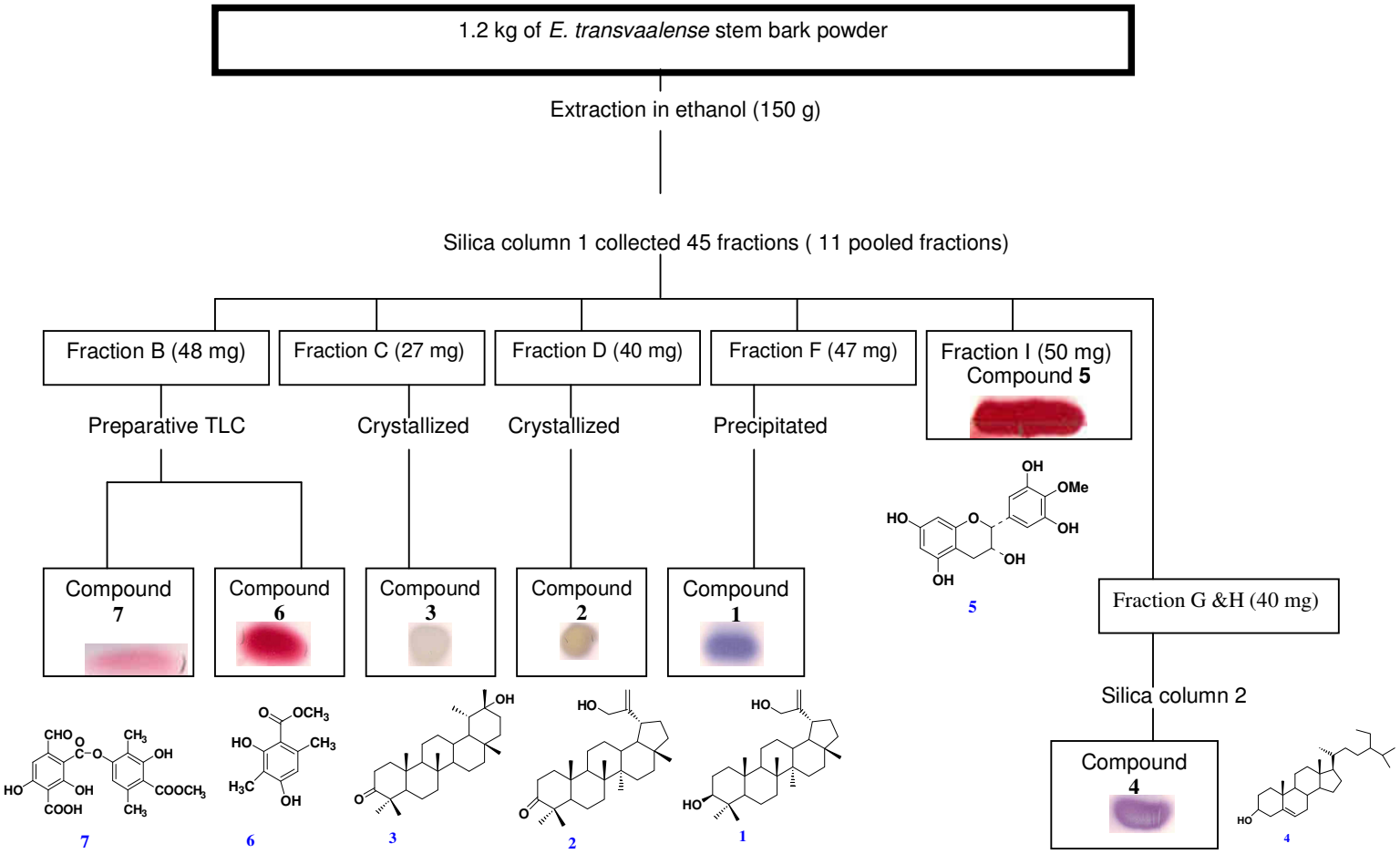


Figure 4.4: Schematic presentation of isolation steps followed.

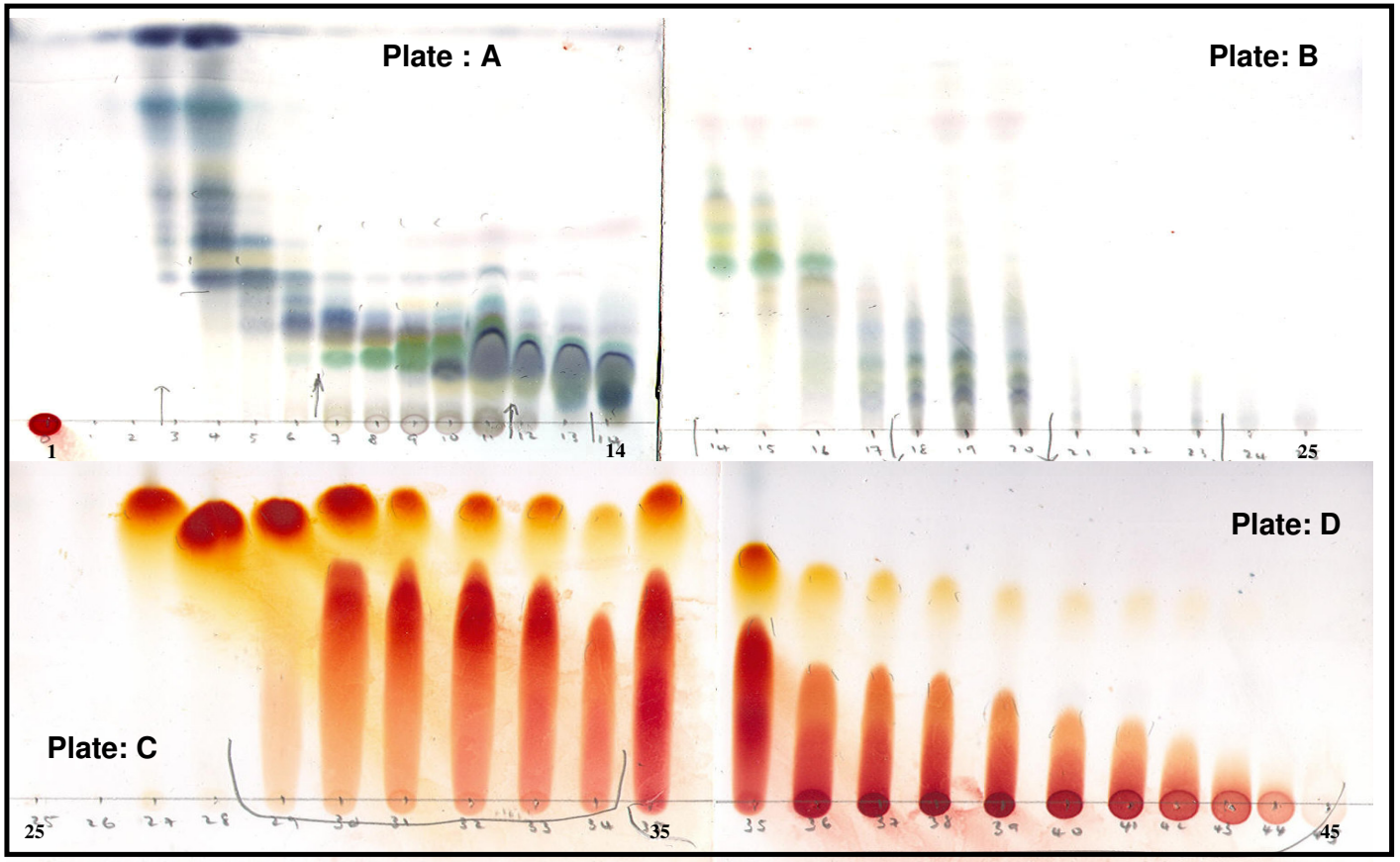


Figure 4.5 Fractions from the first silica column on TLC plates sprayed with Vanillin reagent. Plate A and B developed with hexane: ethyl acetate (9:1 and 7:3), Plate C and D fractions developed with hexane: ethyl acetate (1:9).

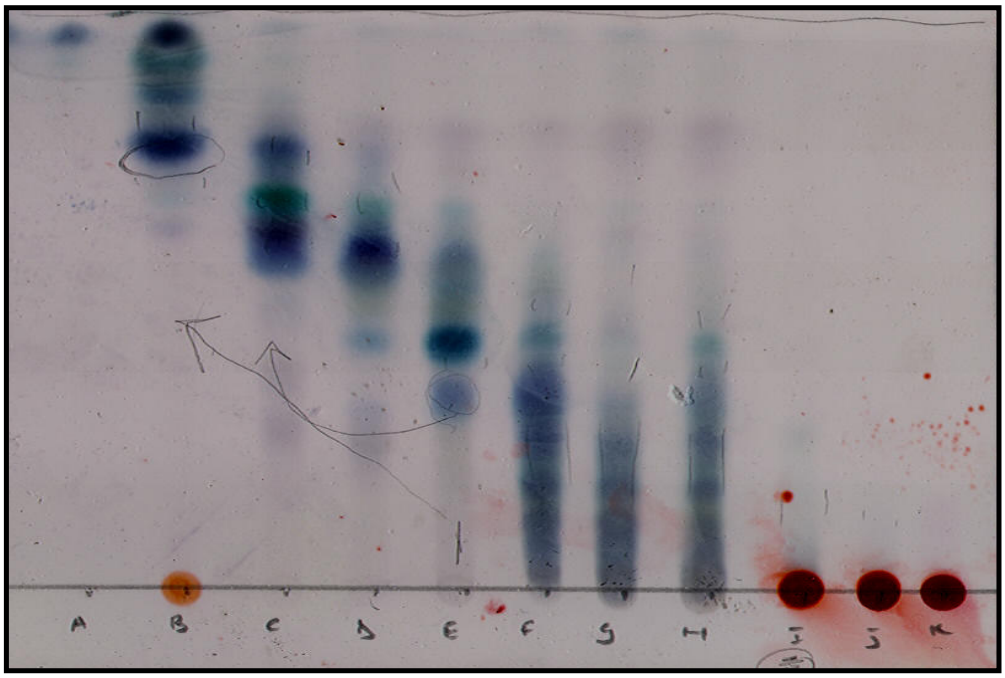


Figure 4.6 The 11 pooled fractions (silica column 1) TLC plates sprayed with Vanillin reagent.

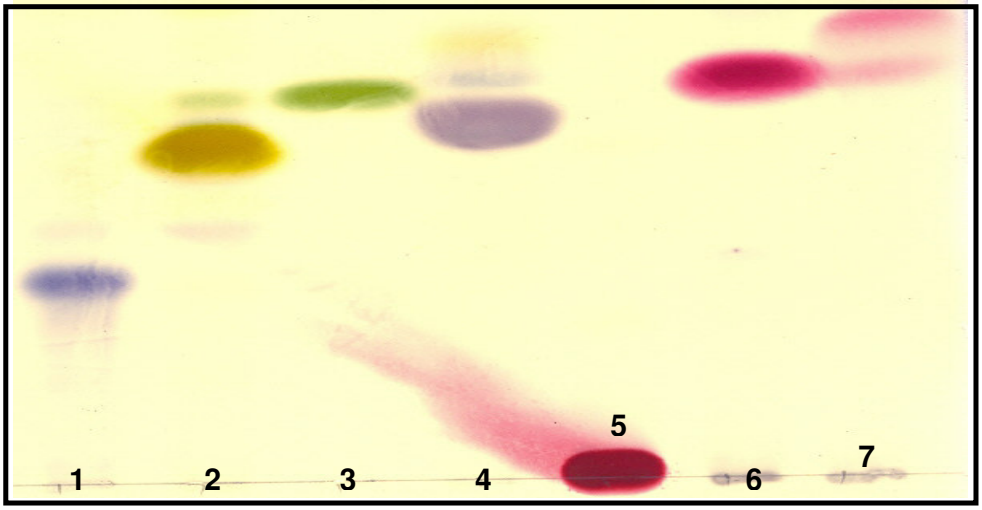
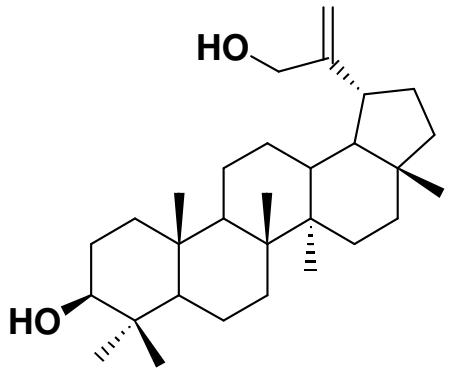


Figure 4.7 Isolated compounds as seen on TLC plates sprayed with Vanillin reagent.

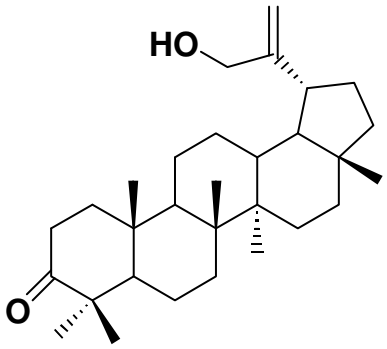
4.3.1 Triterpenoids isolated

Fraction F contained a white precipitate that was washed with ethyl acetate to give one pure compound **1** (Figure 4.7) as determined by TLC and other spectroscopic methods (Table. 4.2).



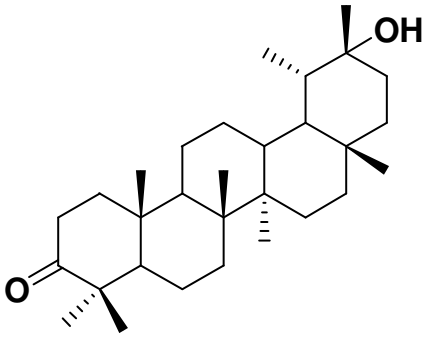
1

lup-20(30)-ene-3,29-diol, (3α)-(9CI)



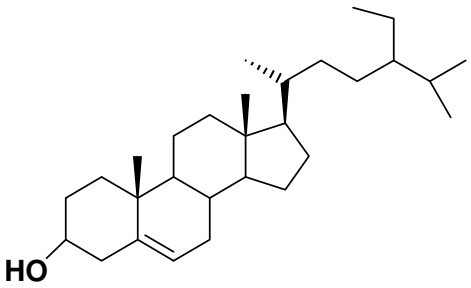
2

lup-20(29)-ene-30-hydroxy-(9CI)



3

Ψ – taraxastanonol



4

β-sitosterol

Figure 4.8 Structures of isolated triterpenes**.**

Compound **1** showed in its ^{1}H-NMR (Figure 4.8) spectrum a triterpenoid pattern with six methyl singlets at δ_H 0.76 , 0.78 , 0.83 , 0.95, 0.97 and 1.03, two olefinic protons at δ_H 4.95 (d 1.4 H), 4.88 (s) attached to carbon at δ_C 106.9, both protons correlated in HMBC (Figure 4.10) with the carbon at δ_C 65.1, another two protons attached to a oxygen bearing carbon at $^{\delta}$H 4.11 (d, J=14.8 Hz), **406** (d, J=14.8 Hz), and a proton δ_H 3.16 (dd, J=10.4, 5.7 Hz) on carbon at δ_C 79.0. The ^{13}C – NMR spectrum of compound 1 is shown in Figure 4.9. The data obtained with 2D NMR experiments HMBC, HMQC, COSY and NOESY (Figures 4.10 & 4.11) supported the structure for compound **1** (Abdel-Mogib, 1999). This compound has been previously isolated from the whole plant extract of *Daphne oleoides*, which is used as a purgative and the infusion of the leaves is used to treat gonorrhoea and applied to abscesses (Ullah *et al*., 1999).

Fractions C and D were crystallized to give compounds **2** and **3** as shown previously in Figure 4.3. Both compounds were obtained as white crystals, but showed different colours on TLC plates. Compound **2** showed similar signals in ^{1}H-NMR (Figure 4.12) with the previous compound except for the disappearance of the C-3 proton and the appearance of a carbonyl carbon in ^{13} C-NMR (Figure 4.13) at δ_C 218.2, which indicate the enzymatic oxidation of the hydroxyl group into the corresponding ketone group in compound **2**. This was supported by the HMBC correlation between C-3 and the H-23 and H-24 methyls and previously reported data (Fang et al., 1984). Compound **3**'s structure was supported by data published by Anjaneyulu *et al*. (1999) and Hinge *et al*. (1966). This compound was also isolated from resin of *Protium*

heptaphyllum and has shown analgesic effects (Susunaga *et al*., 2001 & Rudiger *et al*., 2007).

Fractions G and H were combined and subjected to a second silica gel column 2 and one pure compound (**4**) was obtained. This compound was identified as β - sitosterol when spectra were compared to published data (Prozesky, 2004). ^{13}C – NMR data (Table 4.2) of all the triterpenes isolated form *E. transvaalense* was also supported by other published data (Scleich *et al*., 2006, Sasunaga *et al*., 2001, Mahato & Kundu, 1994, Ullah *et al*., 1999 & Burns *et al*., 2000).

Table 4.2: ^{13}C – NMR data of tritepernoids isolated compounds (1-3).

C	**1** δC	**2** δC	**3** δC
1	38.7 t	39.6	39.6
2	27.4 t	34.1	33.8
3	79.0 d	218.3	218.0
4	38.9 s	47.3	47.4
5	55.3 d	54.9	54.8
6	18.3 t	19.7	19.8
7	34.3 t	33.6	34.1
8	40.9 s	40.8	41.4
9	50.4 d	49.7	49.1
10	37.1 s	36.9	36.7
11	21.0 t	21.6	22.2
12	26.7 t	26.7	26.7
13	38.0 d	31.8	39.0
14	43.0 s	42.9	43.2
15	27.4	27.4	26.8
16	35.5 t	35.4	38.1
17	43.0 s	43.0	35.6
18	49.0 d	48.8	47.3
19	43.8 d	43.8	38.7
20	154.8 s	154.7	73.5
21	31.8 t	31.8	35.4
22	39.9 t	39.8	37.7
23	28.0 q	26.7	26.8
24	15.4 q	21.0	21.1
25	16.1 q	16.0	16.2
26	16.0 q	15.8	16.1
27	14.6 q	14.5	14.8
28	17.7 q	17.7	17.8
29	106.9 t	106.8	17.8
30	65.1 t	65.0	30.3

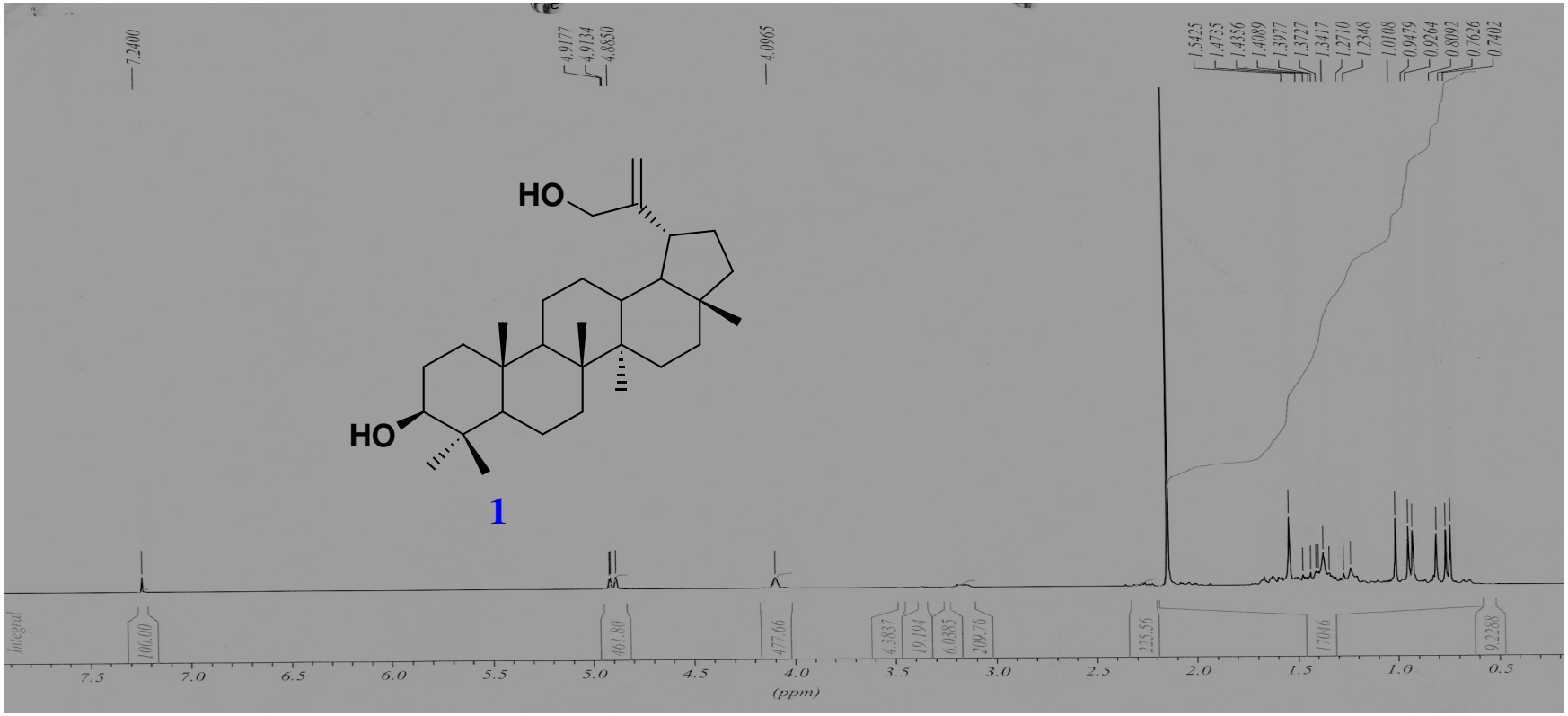


Figure 4.9 ^{1}H – NMR spectrum of Compound **1**

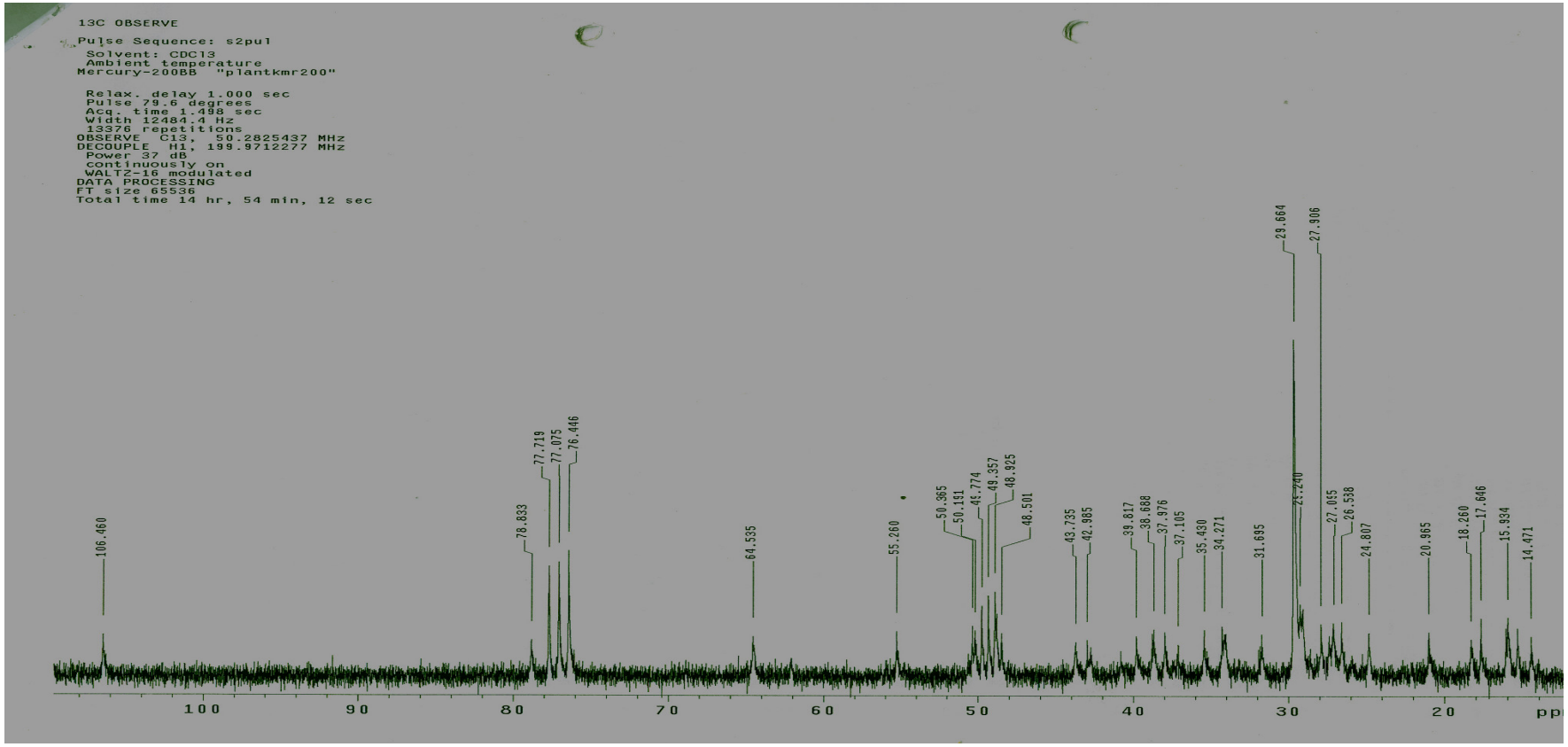


Figure 4.10 ^{13}C – NMR spectrum of Compound **1**

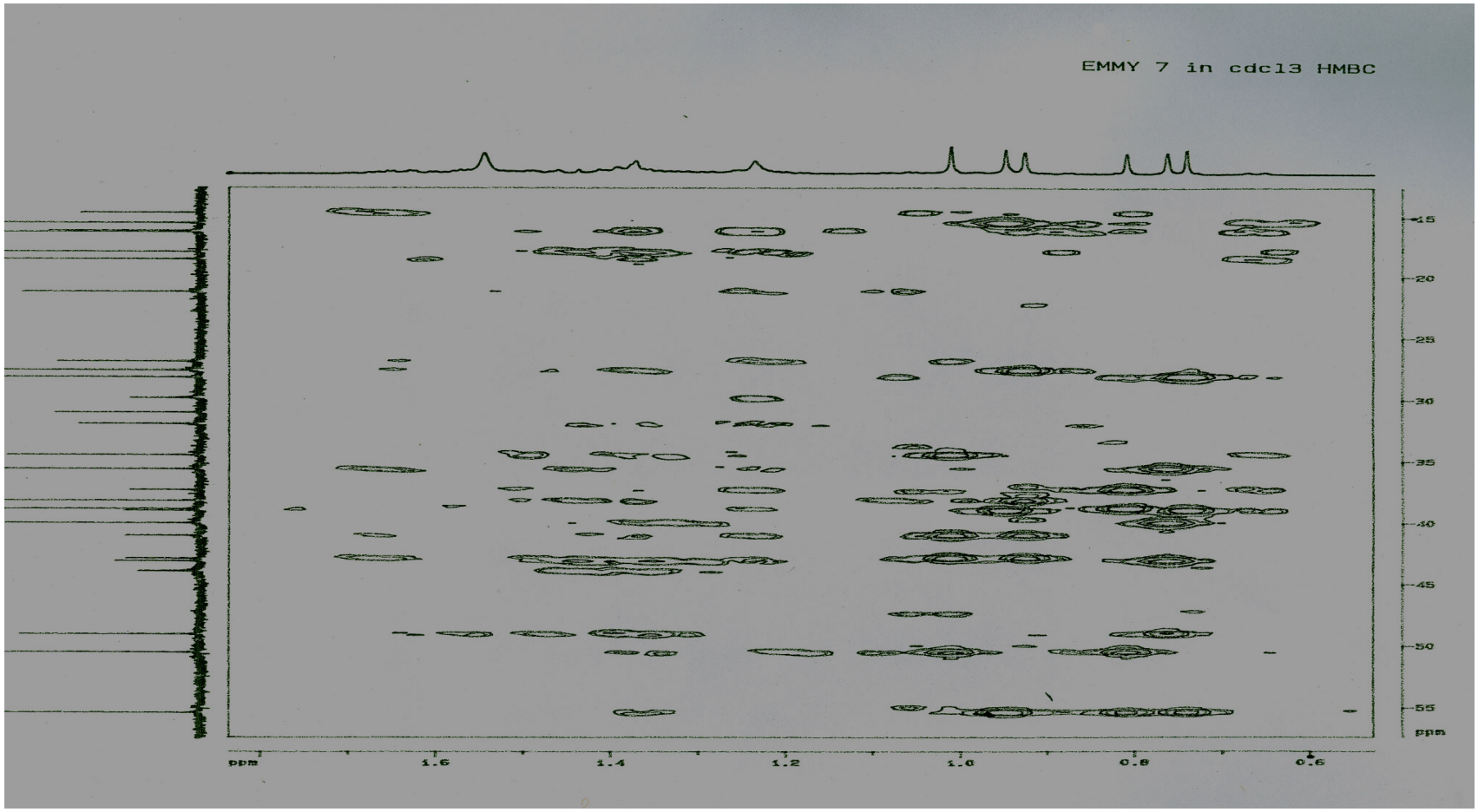


Figure 4.11 HMBC spectrum of Compound **1**

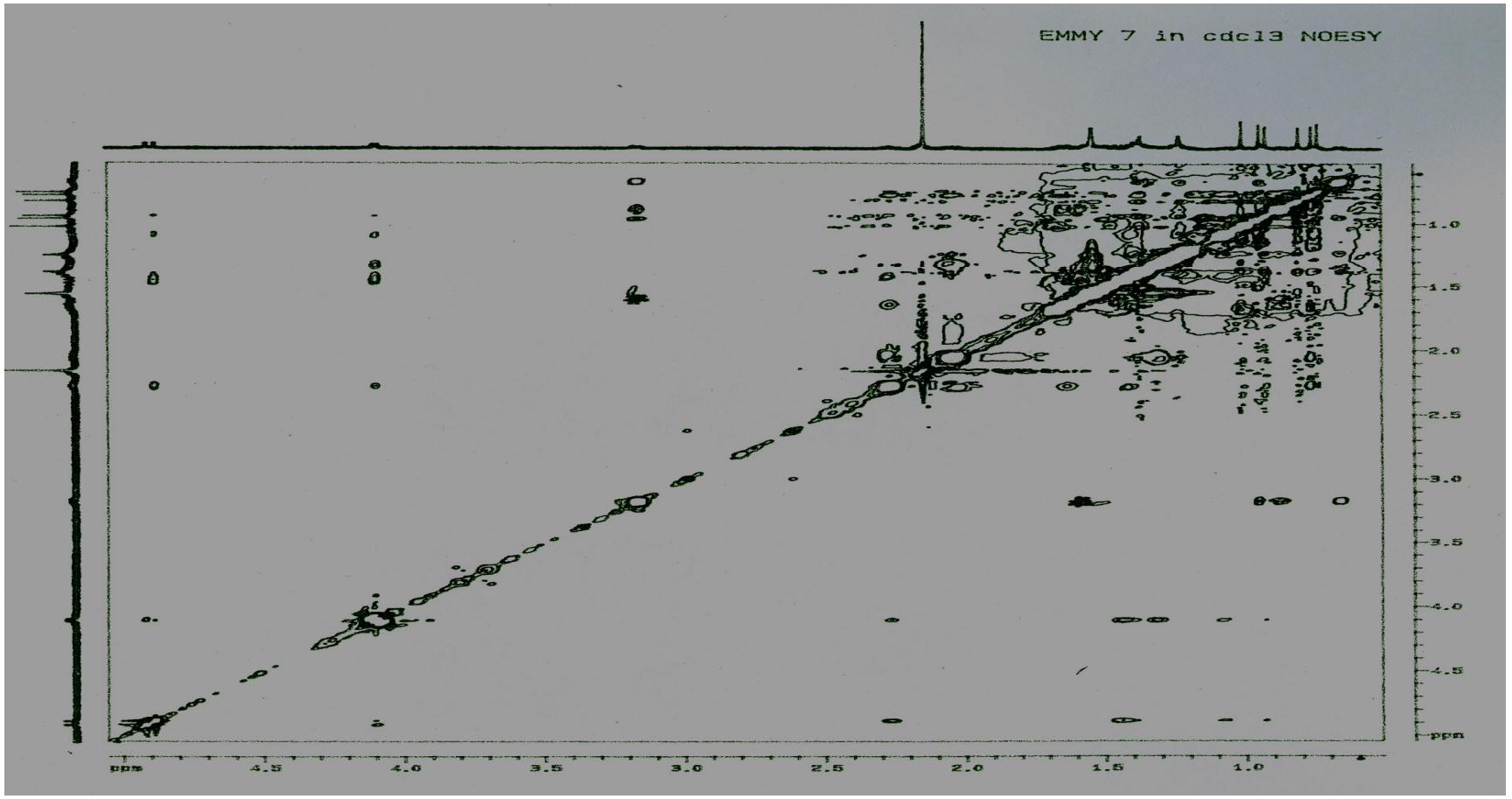


Figure 4.12 NOESY spectrum of Compound **1**

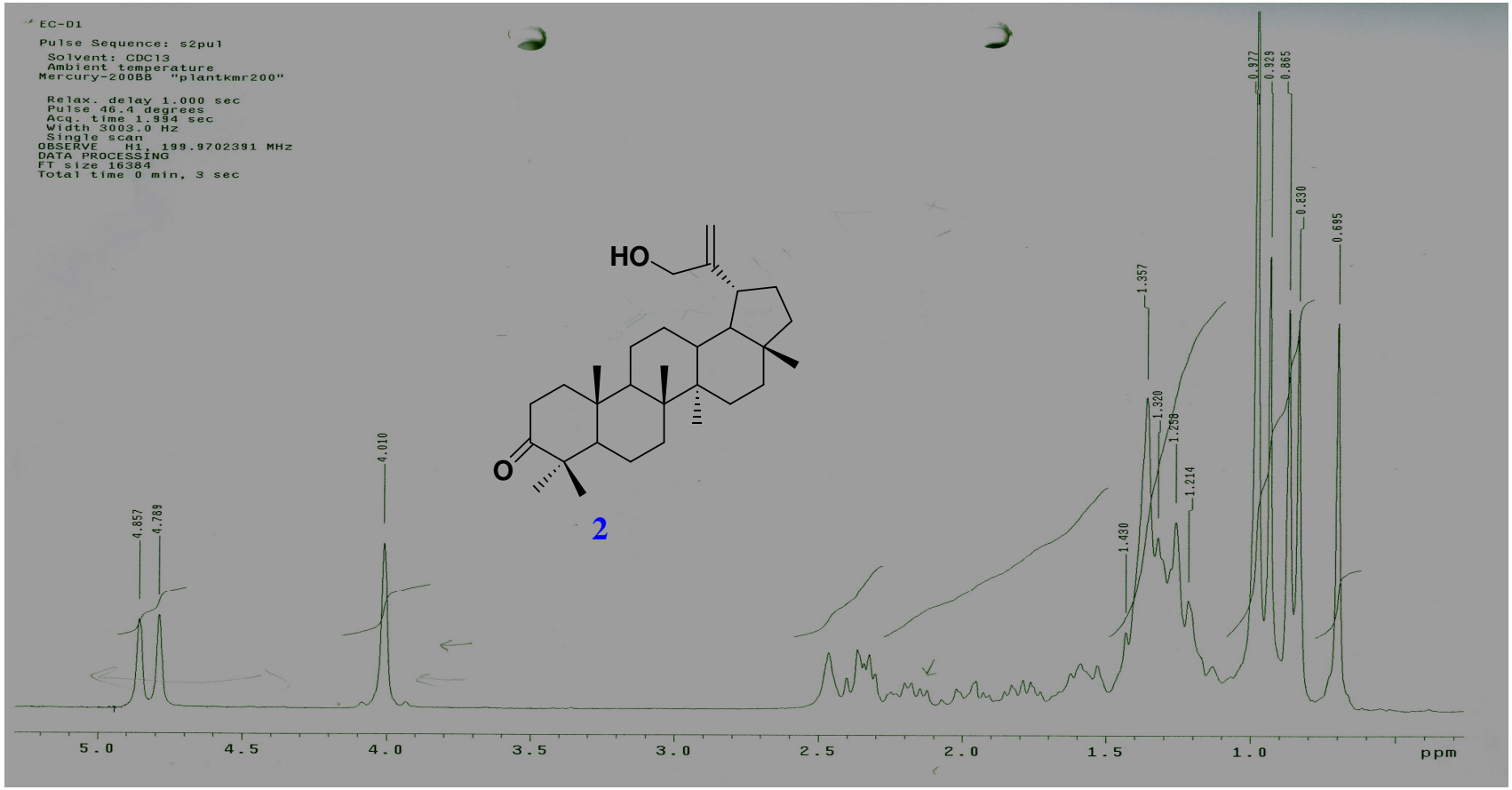


Figure 4.13 ^{1}H – NMR spectrum of Compound **2**

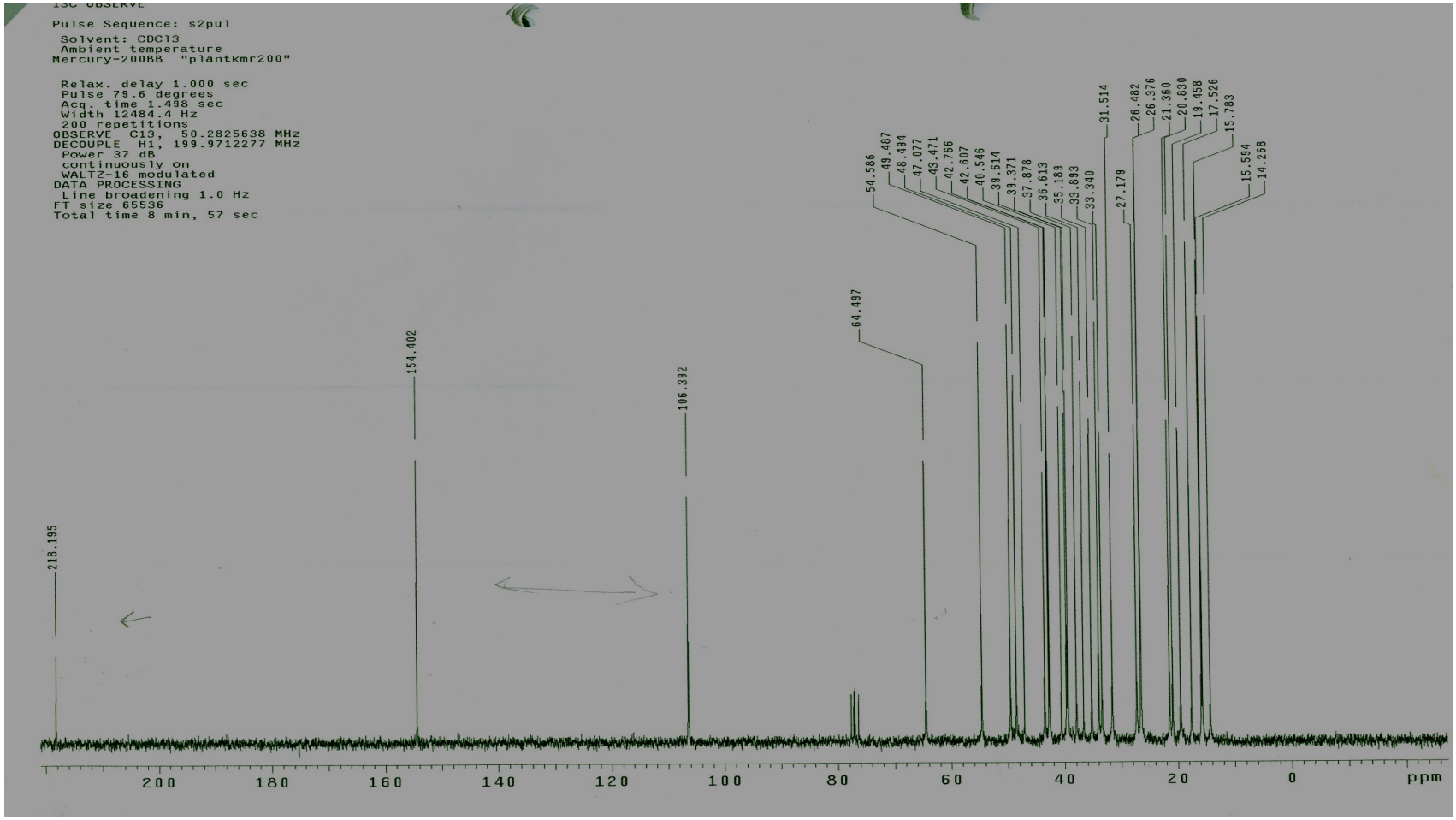


Figure 4.14 ^{13}C – NMR spectrum of Compound **2**

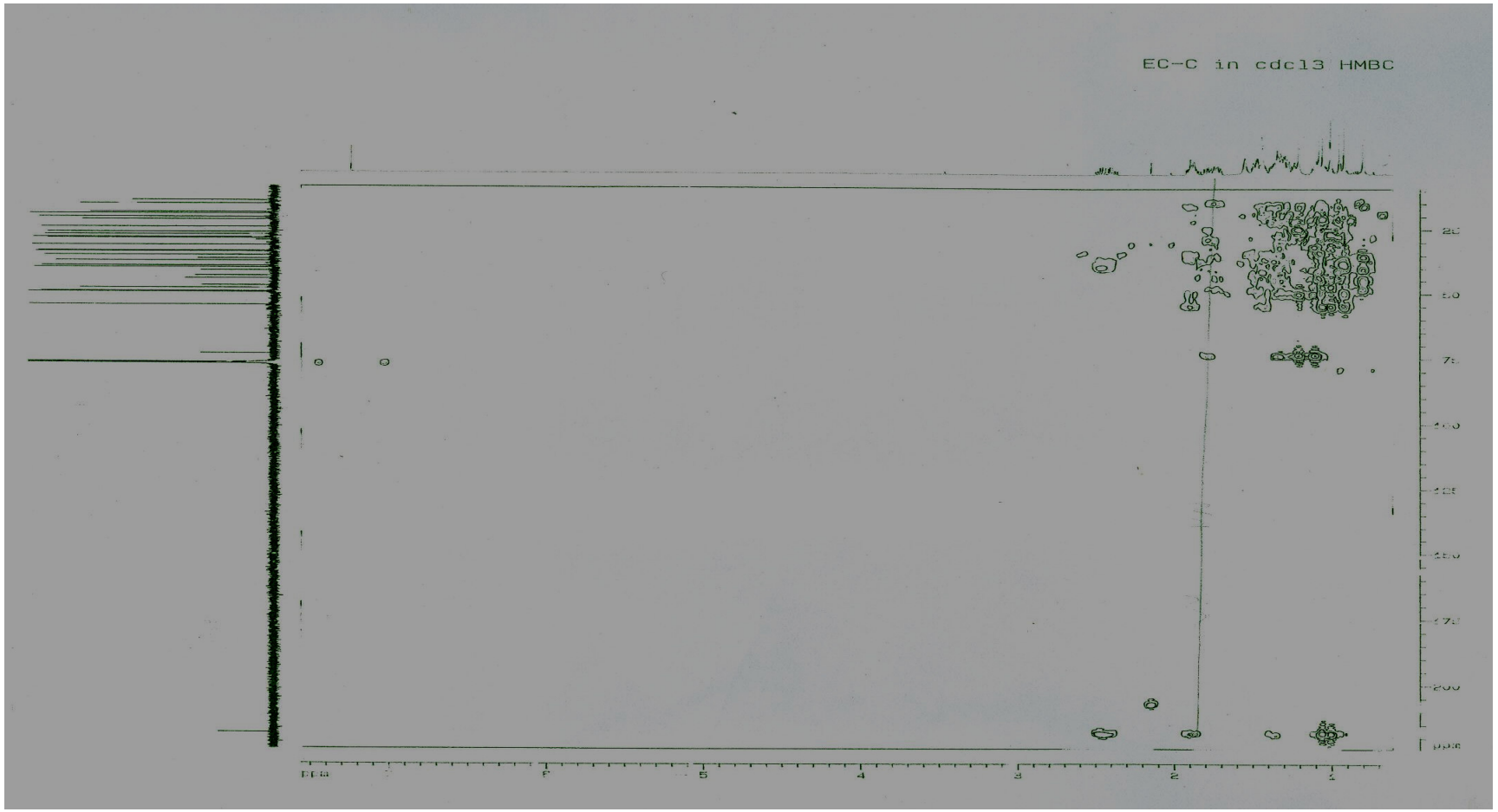


Figure 4.15 HMBC spectrum of Compound **3**

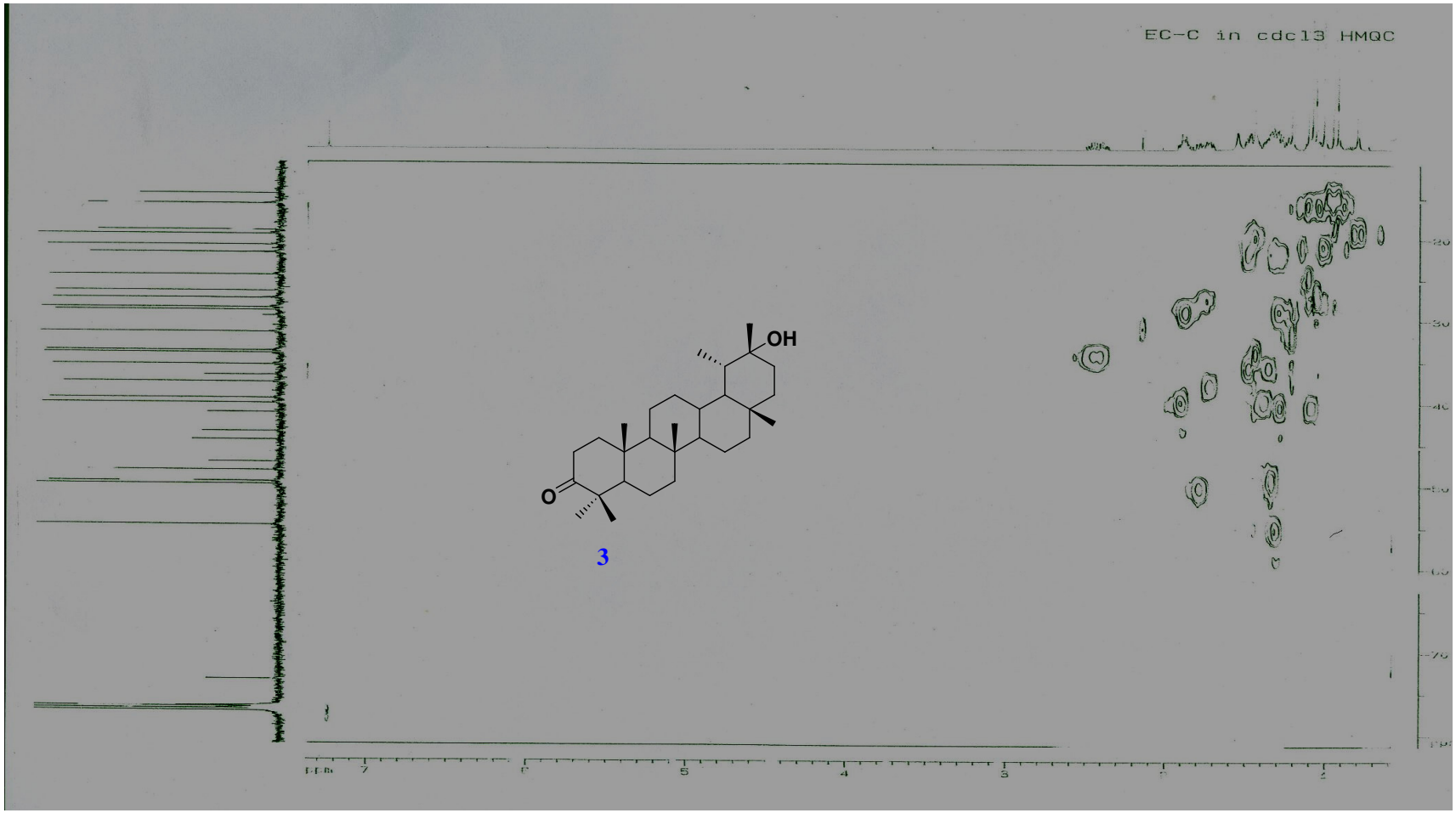
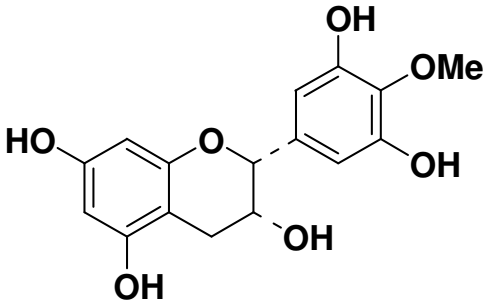


Figure 4.16 HMQC spectrum of compound **3**

4.3.2 Methylepigallocatechin

Fraction I contained one pure compound **5,** (Figure 4.16) as determined by TLC and other spectroscopic methods. It was obtained as a brown powder (50 mg). The ^{1}H-NMR (Table 4.3 and Figure 4.17) spectrum showed four aromatic protons at δ_H 5.71 (d, J=2.3), 5.89 (d, J=2.3), 6.40 (2H, d, J= 0.6), two methane protons at δ_H 4.68 (d, J=0.8) and 3.45 (s br), methylene protons at δH 2.45 (J=4.4) and 2.70 (J=3.2), and one methoxyl group at δ_H 3.65 (s). The ^{13}C-NMR spectra (Figure 4.18) indicated the presence of two methine carbons attached to an oxygen function (δ_C 78.6, 65.6), a methylene carbon (δ_C 28.8 t), 12 aromatic carbons δ_C 156.3 (s), 95.8 (d), 157.2 (s), 94.8 (d), 156.9 (s), 99.2 (s), 135.6 (s), 106.8 (x2C, d each), 150.7 (x2c, s each), 135.2 (s), and a methoxyl carbon (δ_C 60.3 q). The coupling constant between protons at δ_H 4.68 and 3.45 is 2.3=Hz which indicated ββ relative configuration. The above spectroscopic data indicated that compound **5** is (-)4'-*O*-methylepigallocatechin which have been isolated from the same genus previously (Drewes & Mashimbywe, 1993). Hussein *et al.* (1999) reported anti-HIV -1-protease activity for this compound. .



(-)4'-*O*-methylepigallocatechin (**5**)

Figure 4.17 Structure of compound **5**

Table 4.3 ^{1}H – NMR and ^{13}C – NMR data of compound **5.**

Position	$^{\delta}H$	$^{\delta}C$
2	4.68 d (J= 0.8)	78.6 d
3	3.45 (J= 3.9)	65.6 d
4 a 4 b	2.73 dd (J=3.2) 2.87 dd (J= 4.4	28.8 t
5		156.3 s
6	5.71 d (J=2.3)	95.8 d
7		157.2 s
8	5.89 d (J= 2.3)	94.8 d
9		156.9 s
10		99.2 s
1´		135.6 s
2', 6'	6.40 d (J= 0.6)	106.8 d
3',5'		150.7 s
4'	3.65	135.2 s
OMe		60.3 q

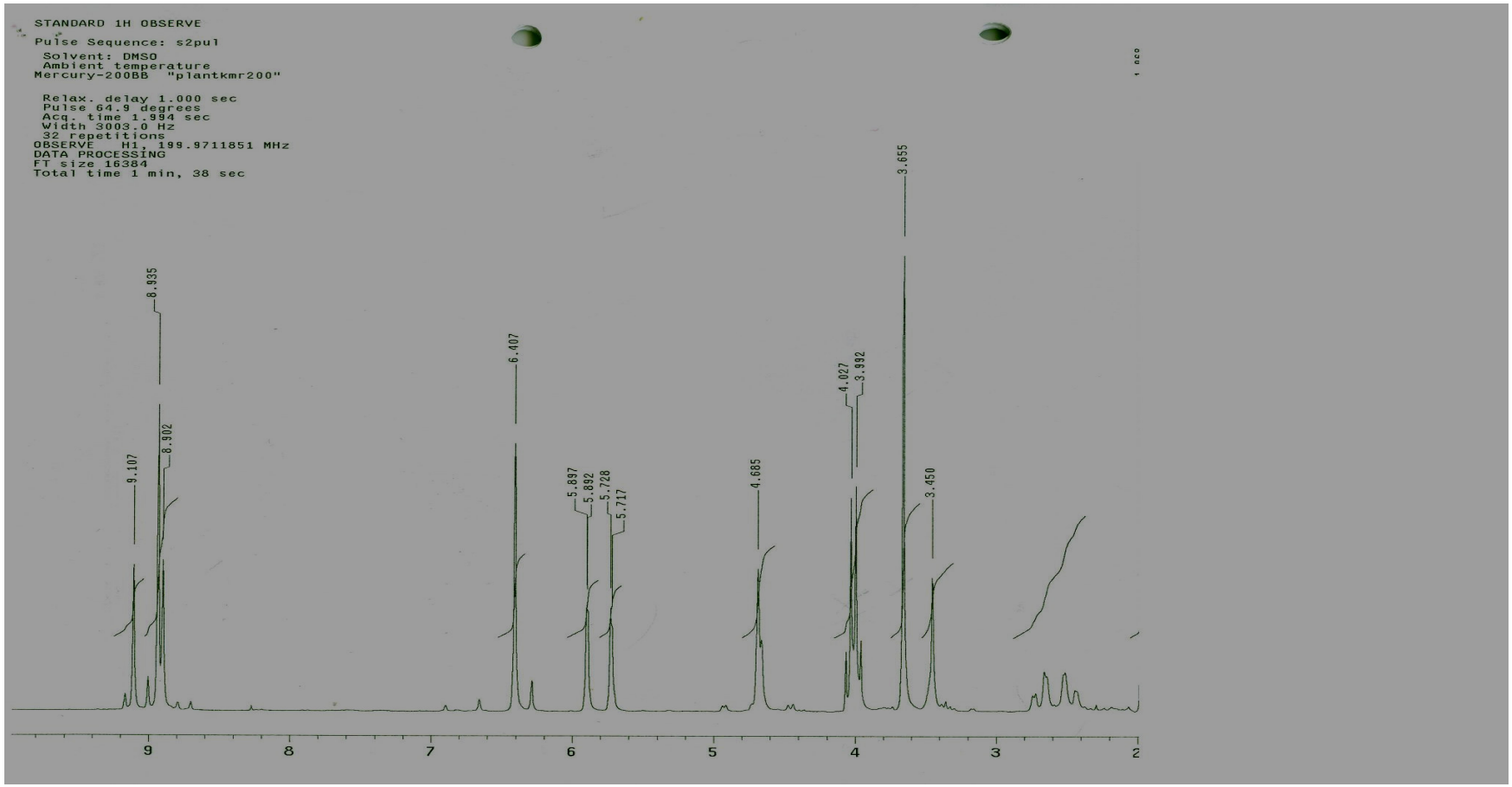


Figure 4.18 ^{1}H – NMR spectrum of Compound **5**

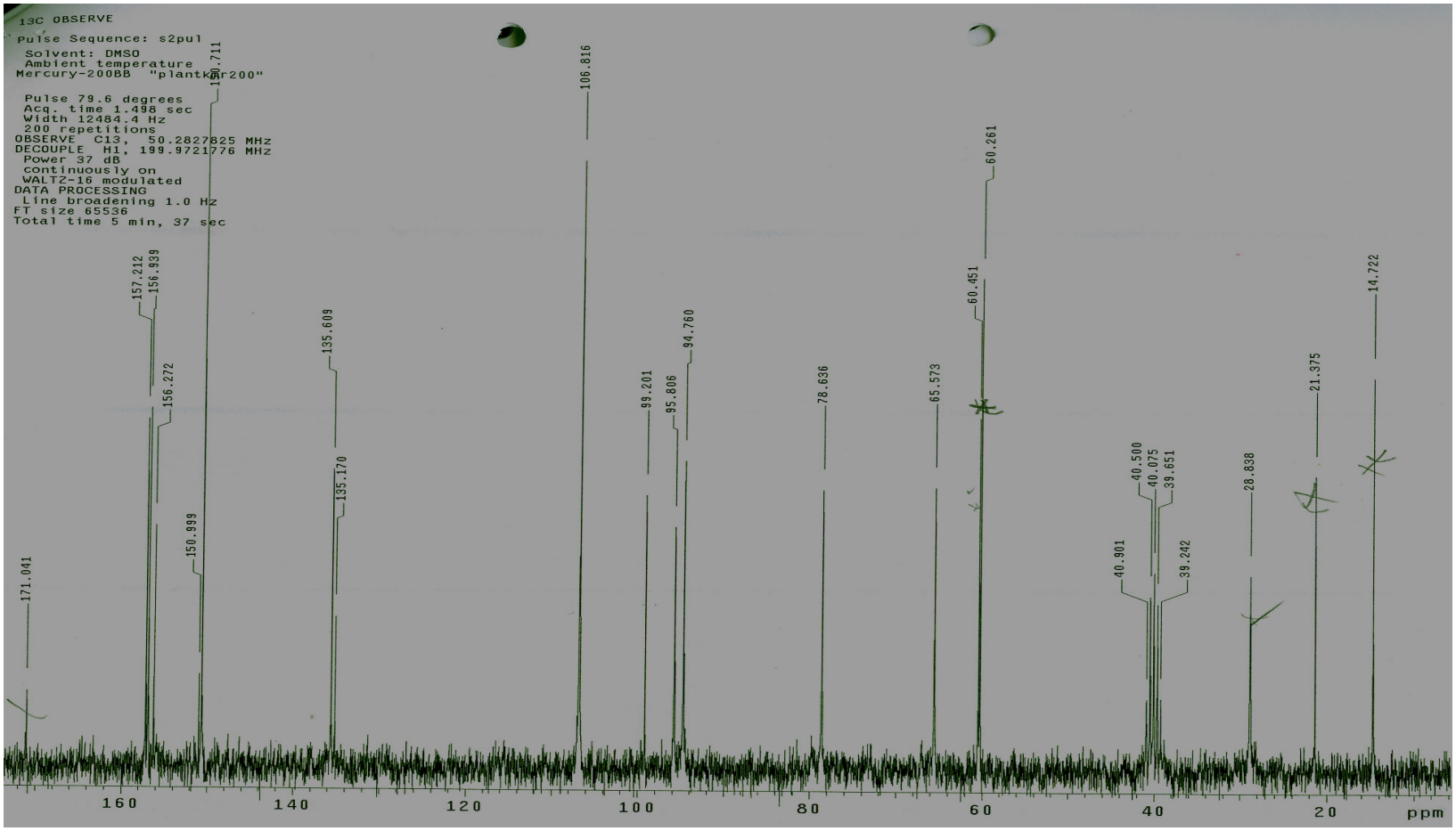
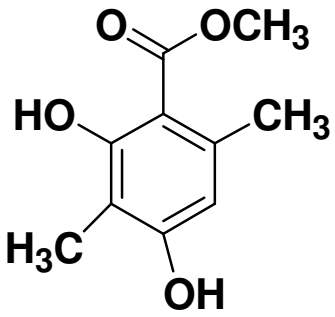


Figure 4.19 ^{13}C – NMR spectrum of Compound **5**

4.3.3 Phenolic derivative and depside

Compounds **6** and **7** (Figures 4.19 and 4.20) were obtained through preparative TLC of fraction B which was developed in hexane:chloroform:water (10:4:1). The plate was half developed, dried and redeveloped fully.



Atraric acid **(6)**

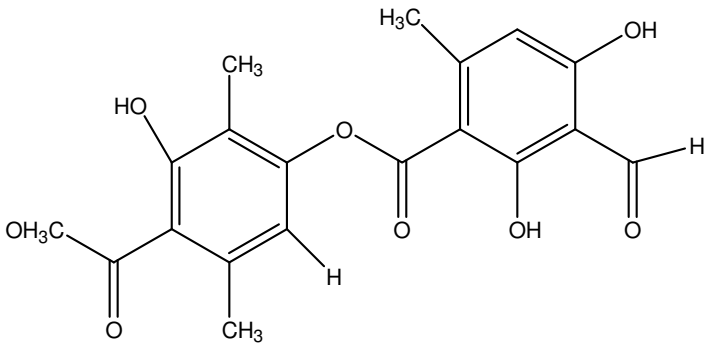
Figure 4.20 Structure of compound **6**

Compound **6** was formed as crystals. The ^{13}C-NMR (Figure 4.22) revealed the presence of 10 carbons, including six of which five where substituted on a benzene ring, including two hydroxyls (δ_C 163.1, 158.2) and one methylated (δ_C 51.8) carboxyl group (δ_C 172.6), in addition to two aromatic methyl groups (δ_C 24.1, 7.6) ^{1}H-NMR (Figure 4.21) spectral data showed two aromatic methyls δ_H 2.43, 2.07 singlets and an aromatic proton at δ_H 6.19.

The foregoing data indicated that the isolated compound is methyl 2,4-dihydroxy-3,6-dimethylbenzoate a phenolic derivative (atraric acid). This was confirmed by 2D-NMR spectra e.g. HMBC, HMQC, COSY. The NMR spectral data are in agreement with those previously reported (Gormann *et al.*, 2003; Lee *et al.*, 2001).The compound was isolated for the first time from lichens (Cooke & Down,1971), however it was reported to be a constituent in higher

plants such as *Alseodaphne andersonni*, *Acer nikoense*, *Dianella revolute*, *Frullania brasiliensis*, *Pygeum africanum* and *Xylosma velutina* (Bardon *et al*., 2002 and Schleich *et al*., 2006). It is difficult to say whether this compound is a secondary metabolite of *E. transvaalense* or is the result of lichens that colonized the stem bark, producing polyketides through the acetate-polymalonate pathway.

The LR MS for compound **7** (Figure 4.20) showed a peak at J 75 (MH^+) corresponding to $C_{19}H_{18}O_8$, this was supported by ^{13}C and DEPT NMR analysis of the compound.



Atranorin (**7**)

Figure 4.21 Structure of compound **7**

^{1}H-NMR data showed four singlets signals at δ 3.97, 2,67,2.53 and 2.07 corresponding to four methyl groups (Figure 4.23), one of them (3.97) esterified to the carboxyl group at C-4 and the others attached to the positions C-6, 2, 5. The spectra also showed three singlet signals, each intergrated for one proton, at δ 6.39, 6.50, 10.30 corresponding to two aromatic protons at C-5 and 6, the third (10.30) is belonging to the aldehydic group at C-8 (δ_c 193.8) (Figure 4.24).

This data indicated the compound to be the depside, atranorin as depicted in Figure 4.20, and is in agreement with those data reported for the same compound. Also this was supported by 2D NMR data, COSY, HSPC, HMBC and NOESY (Figures 4. 23 and 4.24). Compound **7** was also isolated originally from lichens (Santos *et al*., 2004) but recent literature reported it's presence in higher plants as well (Athukoralage *et al*., 2001).

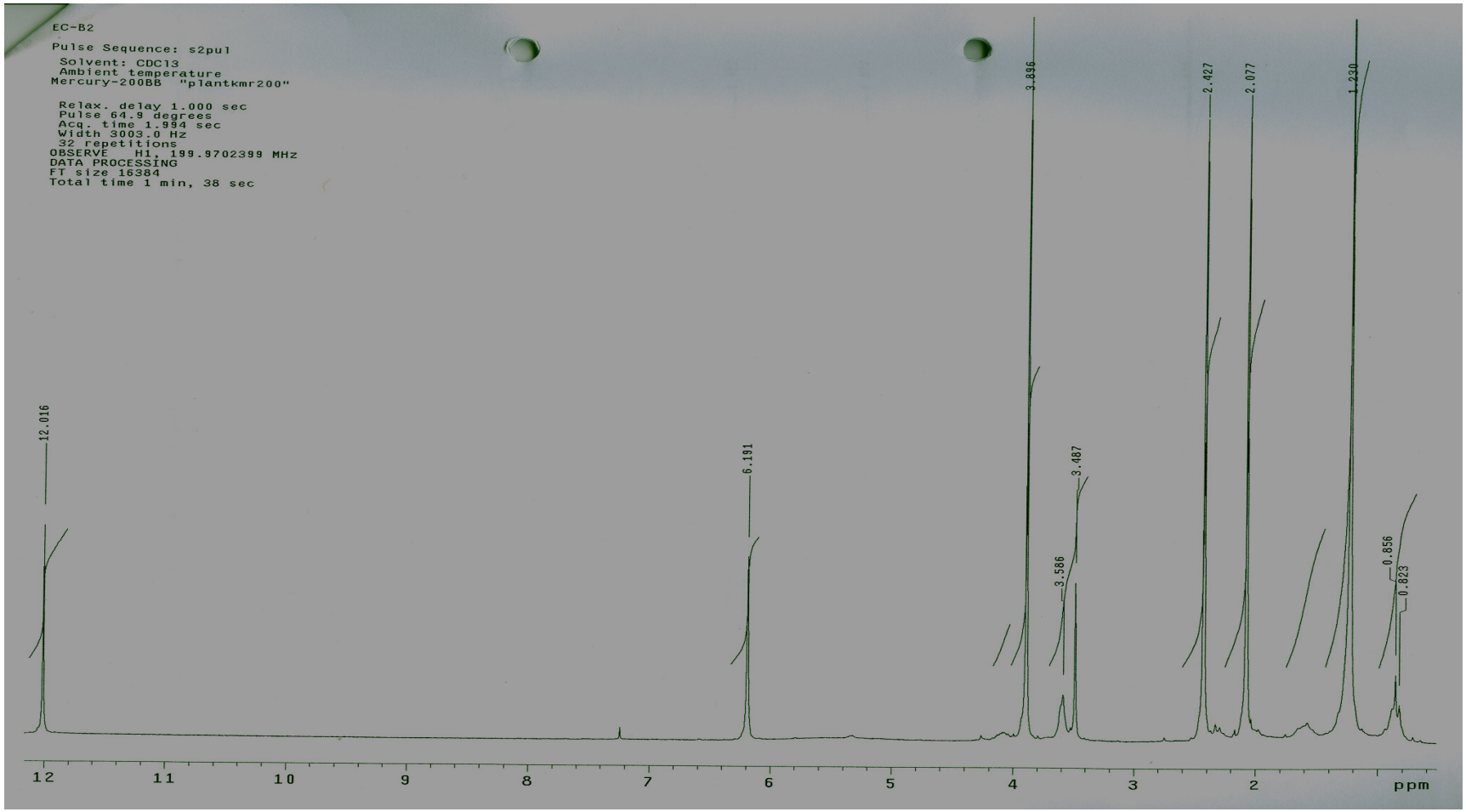


Figure 4.22 ^{1}H – NMR spectrum of Compound **6**

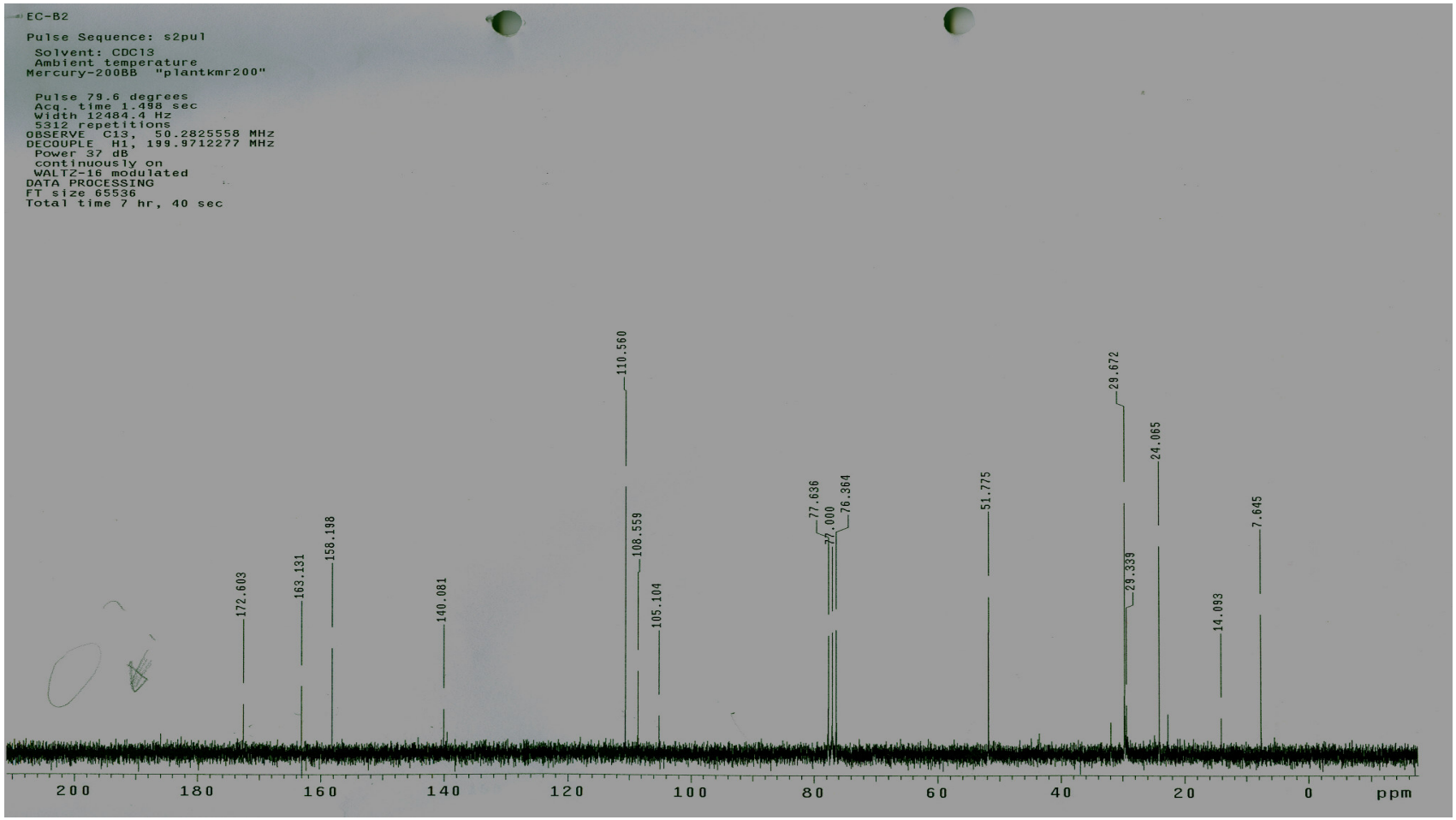


Figure 4.23 ^{13}C – NMR spectrum of Compound **6**

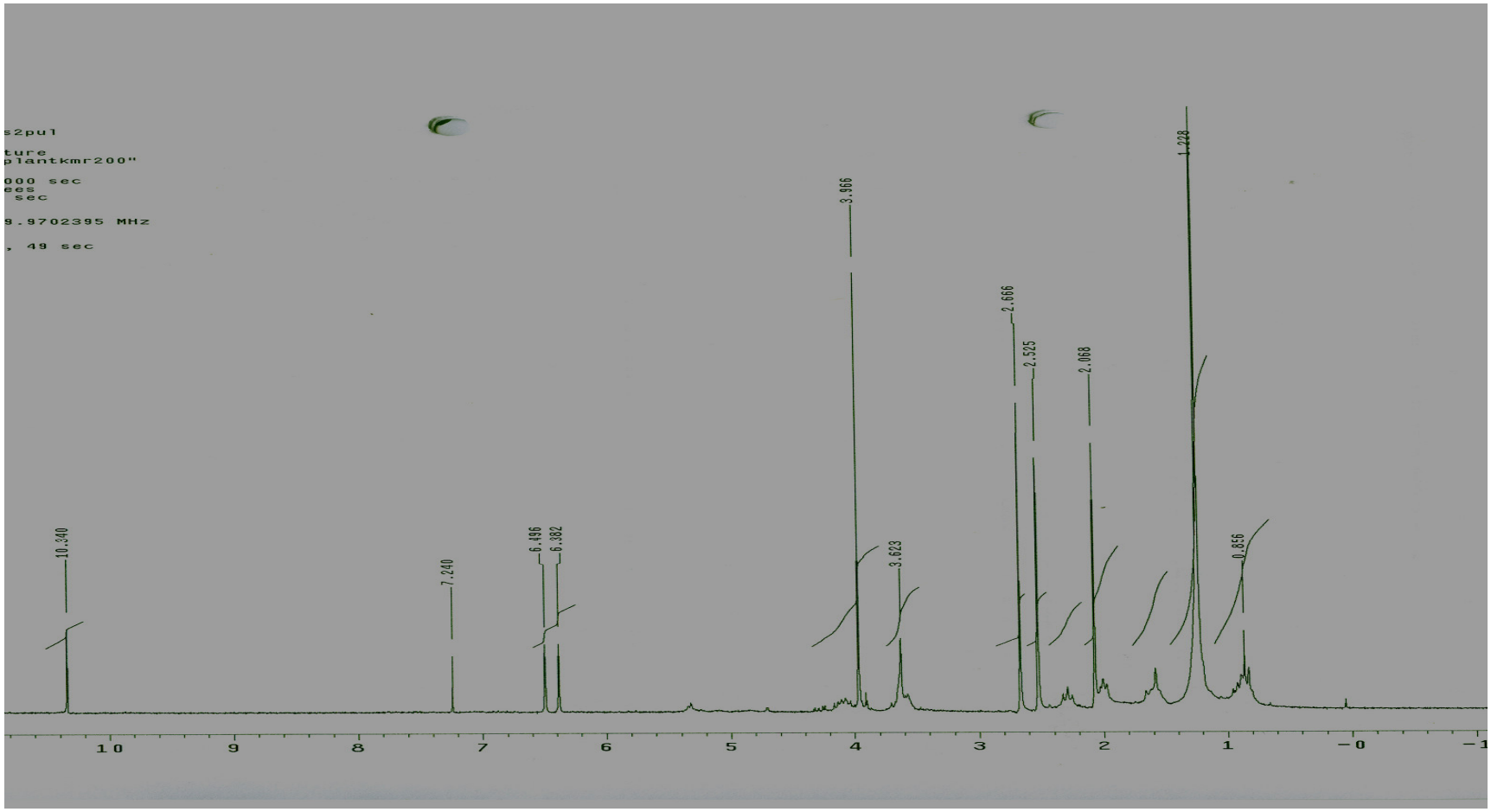


Figure 4.24 ^{1}H – NMR spectrum of Compound **7**

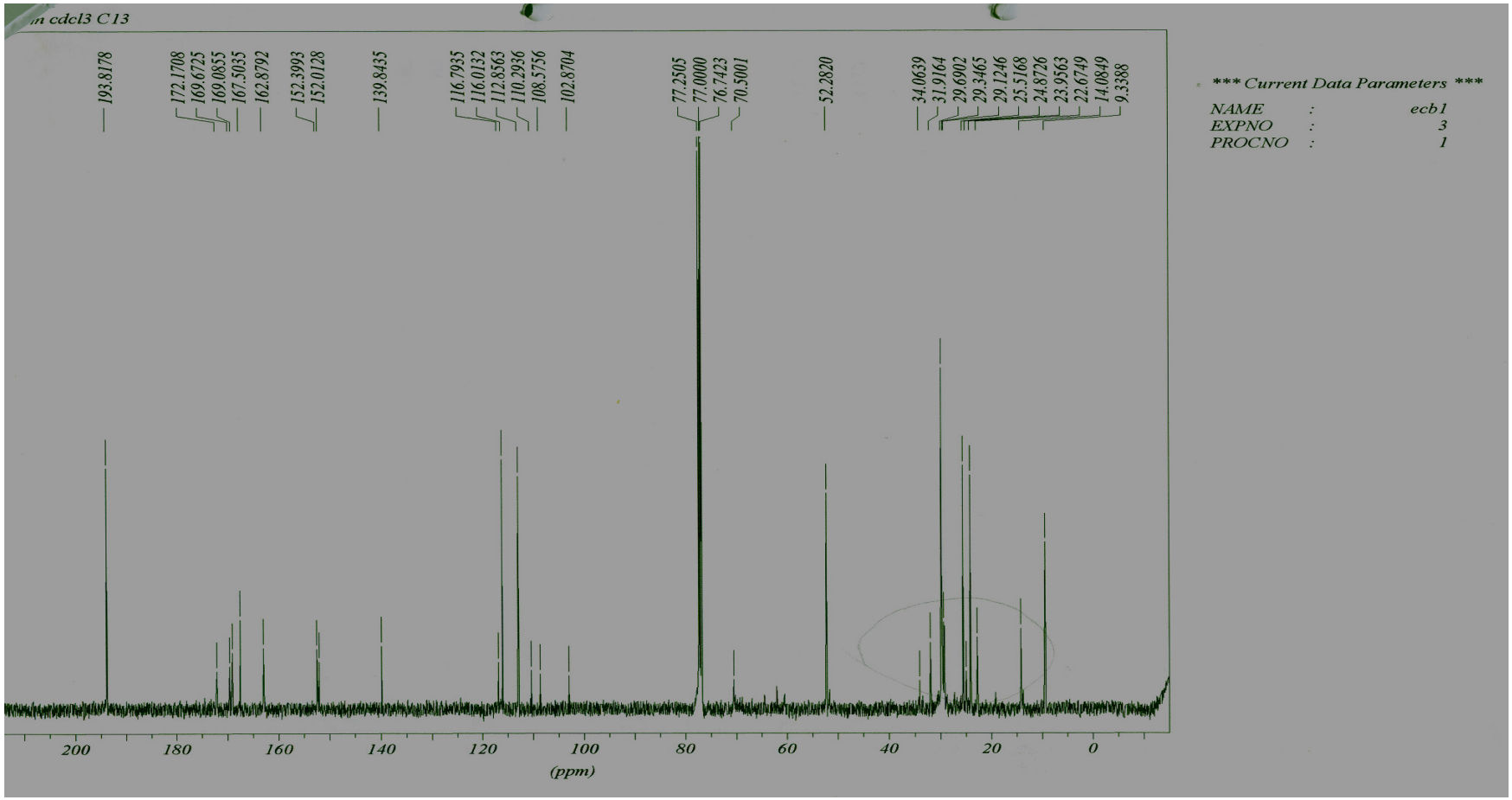


Figure 4.25 ^{13}C – NMR spectrum of Compound **7**

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CHAPTER 5:

Anti-HIV activity of compounds isolated from *Elaeodendron transvaalense*

5.1 Introduction

The use of natural or synthetic compounds targeting cellular proteins involved in the HIV replication has paved a way for new research in the management of AIDS (Marquez *et al*., 2005). Many compounds isolated from medicinal plants have been investigated *in vitro* for inhibitory effects on HIV-1 replication (Campagnuolo *et al*., 2005; Sandur *et al*., 2006; Cheng *et al*., 2005).

The transcription factor NF-κB is one of the key regulators of genes involved in the immune/inflammatory response as well as in HIV-1 gene regulation. The post-integration phase of the viral cycle preferentially occurs in the activated cells and is regulated by collaboration action of the viral regulatory protein Tat (Trans-activation of transcription) and cellular factors interacting with the long terminal repeat promoter (LTR), which determines the extent of HIV-1 gene transcription and the level of viral replication in the infected cells (Marquez *et al*., 2005; Peireira *et al*., 2000). In this chapter we

report the anti-HIV activity of five compounds isolated from *E. transvaalense* since compounds **6** and **7** were obtained in very small amounts.

5.2 Materials and methods

5.2.1 C-Med 100®

C-Med 100® was used as a positive control, it is a patented extract from *Uncaria tomentosa*, Cat's claw (U.S. patent 6,039,949) supplied by CampaMed (New York, NY, USA*). U. tomentosa* is a vine indigenous to South America and has been used for generations as an "immuno modulator" (Akesson *et al*., 2003). The extract is water-soluble and ultra-filtered to remove high molecular weight (MW) conjugates (>10,000 Da). The extract contains carboxy-alkyl-esters (CAE) as active ingredients (8–10%) and is almost free of oxindole alkaloids. The active (CAE) components have indeed been identified as benzoic acid analogs such as quinic acid (U.S. patent application EL781388471US, filed March 7 2002 and Åkesson *et al*., unpublished data). The extract contains no detectable gram negative bacteria and endotoxin. It was dissolved in RPMI medium 30 min before use (Akesson *et al*., 2003).

5.2.2 Transient transfection and luciferase activity analysis

The reporter construct containing the NF-κB binding sequences and the luciferase reporter gene were previously described (Parra *et al*., 1997). Jurkat T cells were transiently transfected with the construct using the lipofectin

method as described by the manufacturer (Life Technologies). Briefly, 2 g reporter plasmid was mixed with 10 L lipofectin and added to $3x10^6$ Jurkat cells. The cells were rested for 22 hours, pooled and pre-cultured for 2 hours in the presence or absence of C-Med 100®

After 6 hours of stimulation, the cells were harvested, washed twice in phosphate-buffered saline (PBS) and treated with 100 µl reporter lysis buffer according to the manufacturer's recommendations (Promega, Madison, WI). Twenty microliters of each lysate were assayed for luminescence with luciferase assay substrate (Promega) in a MicroLumat LB 96 P luminometer (EG&G Berthold, Wallac Sverige AB, Upplands Väsby, Sweden).

5.2.3 Hela-Tat-Luc assay

To identify potential anti-Tat extracts, another luciferase-based cell system (Hela-Tat-Luc cells) was used. The Hela-Tat-Luc cells were stably transfected with the plasmid $pcDNA_3$- TAT together with reporter plasmid LTR-Luc. Therefore the HIV-1 LTR was highly activated in this cell line as a consequence of high levels of intracellular Tat protein. Cells (10^5 cells/mL), seeded the day before the assay, were treated either with the CDK9 inhibitor DRB, as a positive control, or with the plant extracts. After 12 h, the cells were washed twice with PBS and the luciferase activity measured as indicated previously for 5.1 cells.

5.2.4 Anti-HIV-1 replication

Isolated compounds with either specific anti-NF-κB or anti-Tat activities (or both) were subjected to anti-HIV screening using a luciferase-based infectious recombinant virus. This recombinant virus assay is a reliable and sensitive test to detect anti-HIV activity because of two main reasons, firstly it evaluates direct viral replication and secondly the luciferase measurement provides a sensitive assay of HIV replication.

5.2.4.1 Production of VSV-pseudotyped recombinant viruses

High titre VSV-pseudotyped recombinant virus stocks were produced in 293T cells by co-transfection of pNL4-3.Luc.R^- E^- (AIDS Research and Reference Reagent program, NIAID, National Institutes of Health) together with the pcDNA$_3$-VSV plasmid encoding the vesicular stomatitis virus G-protein, using the calcium phosphate transfection system. Supernatants, containing virus stocks, were harvested 48 h post-transfection and were centrifuged 5 min at 500 x *g* to remove cell debris, and stored at -80ºC until use.

5.2.4.2 VSV-pseudotyped HIV-1 infection assay

This recombinant virus integrates into cell chromosomes and expresses the firefly luciferase gene and consequently luciferase activity in infected cells correlates with the rate of viral replication. Therefore, Jurkat T cells (10^6/ml)

were plated on a 24-well plate and were pre-treated with the extracts for 30 minutes. After pre-treatment, cells were inoculated with virus stocks and, 24 h later cells were washed twice in PBS and the luciferase activity measured as described above. The results are represented as the percentage of activation (considering the infected and untreated cells 100% activation).

5.2.4.3 HIV reverse transcriptase (RT) assay

Reverse transcriptase assay was done as described in chapter 2.

5.3 Results and discussion

The results of anti-HIV activity of compounds isolated from *Elaeodendron transvaalense* are shown in Table 5.1. This study reports that, from the five isolated compounds tested, only lup-20(29)-ene-30-hydroxy-(9Cl) (**2)** inhibits NF-κB activity at a low concentration of 10 μg/ml. Lup-20(30)-ene-3,29-diol, (3α)-(9Cl) (**1)** and Ψ – taraxastanonol (**3)** showed anti-NF-κB inhibition at a higher concentration of 50 μg/ml.

Regarding the anti-Tat of the isolated compounds, Lup-20(30)-ene-3,29-diol, (3α)-(9Cl) (**1)** displayed 22 % inhibition (not significant) at 25 μg/ml. lup-20(29)-ene-30-hydroxy-(9Cl) (**2)**, Ψ – taraxastanonol (**3),** β-sitosterol (**4)** and 4' –*O*- methylepigallocatechin (**5)** showed no inhibitory activity in the Hela – Tat-Luc assay. All the compounds isolated were not active in the RV assay.

Table 5.1 Results of anti-HIV activity assays of compounds isolated from *Elaeodendron transvaalense*

Compound	Antiviral activity[a]						
	5.1 + TNF[a]					HeLa- Tat-Luc[a]	RVA[a]
	1 µg/ml	10 µg/ml	25 µg/ml	50 µg/ml	1 mg/ml	25 µg/ml	25 µg/ml
lup-20(30)-ene-3,29-diol, (3α)-(9Cl) **1**	ni	20.0	40.0	64.0	na	22.0	ni
lup-20(29)-ene-30-hydroxy-(9Cl) **2**	11.0	51.0	72.0	73.0	na	ni	ni
Ψ – taraxastanonol **3**	ni	33.0	57.0	61.0	na	ni	ni
β-sitosterol **4**	ni	ni	11.0	23.0	na	ni	ni
4' –*O*- methylepigallocatechin **5**	ni	ni	18.0	47.0	na	ni	ni
Cmed (positive control)	na	ni	na	na	100	na	na

[a] Data are represented as % of inhibition (i.e., TNF treated cells in 5.1; and untreated in HeLa-Tat- Luc).

ni, no inhibition; na, not assayed.

The isolated compounds were also tested in the HIV-reverse transcriptase assay and none of these compounds displayed any RT activity. Interestingly, the mini-review by Ng *et al*., 1997 reported HIV-RT inhibition (IC_{50} = 7.2nM) by (-)-epigallocatechin isolated from *Calophyllum inophyllum*. It is therefore possible that the 4' –*O*-methylepigallocatechin (**5)** isolated in this study did not inhibit the reverse transcriptase enzyme because of the interference by the presence of methyl group.

The use of plant extracts or plant derived synthetic compounds targeting cellular proteins required for efficient HIV-1 replication and transcription has opened new avenues for scientific research in the management of AIDS. It has been reported that natural coumarins and derivatives isolated from *Marila pluricostata* can display anti-HIV activity through different mechanisms. These compounds may bind to cellular proteins that regulate HIV gene expression, inhibition of reverse transcriptase and interference with viral integration (Bedoya *et al*., 2005; Uchiumi *et al.,* 2003).

According to Marquez *et al*. (2005), plant-derived antiviral compounds interfering with HIV-1 LTR promoter regulatory proteins are unlikely to generate drug-resistant HIV strains if proven useful for patients. Anti-HIV drugs that are presently on the market are known to be reasonably effective, but there are some problems associated with their use, namely the emergence of resistant strains and serious side effects (Uchiumi *et al*., 2003).

NF-κB and Tat proteins play an important role on HIV-1-LTR transcription (Bedoya *et al*., 2006). However all the compounds tested showed no anti-Tat activity and in the RV assay none of the compounds could inhibit HIV replication at the highest concentration tested. Inhibition of transcriptional activity of HIV-1 LTR promoter by plant extracts or compounds through a signaling pathway that involves NF-κB transcription factor, can have a potential therapeutic role in the management of AIDS most probably in combination with other anti-HIV drugs (Marquez *et al*., 2005).

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CHAPTER 6:

Cytotoxic activity of *Elaeodendron transvaalense* extracts and isolated compounds

6.1. Introduction

Plants have been an indispensable source of natural products for relief from illness for many years (Graham *et al.,* 2000). Secondary metabolites of plants possess many biological activities since they serve either as protective agents against various pathogens (e.g. insects, fungi or bacteria) or growth regulatory molecules (e.g hormone-like substances that stimulate or inhibit cell division and morphogenesis (Cragg and Newman, 2005; Sturdíková *et al*., 1986). These physiological effects make some of them potentially anti-cancerous, due to either their direct cytotoxicity on cancer cells or modulation of tumor development, and eventually tumor inhibition. Plants have a long history of use in the treatment of cancer and more than 3000 plant species have been reported by Cragg and Newman, (2005). Many plant extracts and isolated compounds have been tested *in vitro* for cytotoxicity by using different human cell lines (prostate, stomach, liver colon etc.) as well as animal cells such as monkey kidney cells (Don *et al*., 2006; Lamidi *et al*., 2005, Al-Fatimi

et al., 2005, Jo *et al* 2005). Cell culture toxicity testing is a valuable and inexpensive approach for short term testing. A test should be able to provide information on the dose-effect relationship including the dose range for potential exposure and risks to humans. Cytotoxicity of plant extracts and isolated compounds should be evaluated before their impact in drug discovery is taken into consideration (Lall & Meyer, 2000).

People with AIDS are at high risk of developing certain cancers (e.g Kaposi's sarcoma and cervical cancer) and other opportunistic infections. In the present study, the cytotoxicity activity of *Elaeodendron transvaalense* and isolated compounds has been investigated against Vero and breast cancer cells.

6.2. Materials and methods

6.2.1. Plant material

Stem bark of *E. transvaalense* was collected in Venda (Northern Limpopo). A voucher specimen is preserved in HGWJ Schweickerdt herbarium at the University of Pretoria (Tshikalange 092524).

6.2.2 Preparation of extracts and isolation of compounds

The ethanol extract was prepared as described in 4.2.2 and compounds were isolated as described in 4.2.3.

6.2.3. Cell culture

The cytotoxicity of the *E. transvaalense* extract and isolated compounds was tested against Vero and breast cancer cell lines. Cells were cultured in Eagle's minimal essential (MEM) supplemented with 1.5 g/L sodium bicarbonate, 2 mM L-glutamine , 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10 µg/ml penicillium, 10 µg/ml streptomycin, 0.25 µg/ml fungizone, and 10 % fetal bovine serum at 37 ^{0} C in a humidified atmosphere with 5 % CO_2. Cells were subcultured in a 1:6 ratio every second to third day after trypsinization of confluent cultures.

6.2.4. Toxicity screening (XTT viability assay)

A colorimetric XTT assay system was utilized to determine the cytotoxicity of the plant extract and isolated compounds (Abid-Essefi *et al.*, 2004). On the first day of the experiment, a 100 µl dilution of the crude extract/pure compound was dispensed into cell-containing wells of sample plates in triplicate (Figures 6.1-6.2). The final concentrations of crude extract in the wells were 0.39, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00, 200.00 µg/ml. The final concentrations of pure compounds in the wells were 0.19, 0.39, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00,100.00 µg/ml. Control wells received a final concentration of 1 % (for crude extract) or 0.5 % (for pure compounds) DMSO in complete medium. Doxorubicin and zelaralenone were used as positive controls.

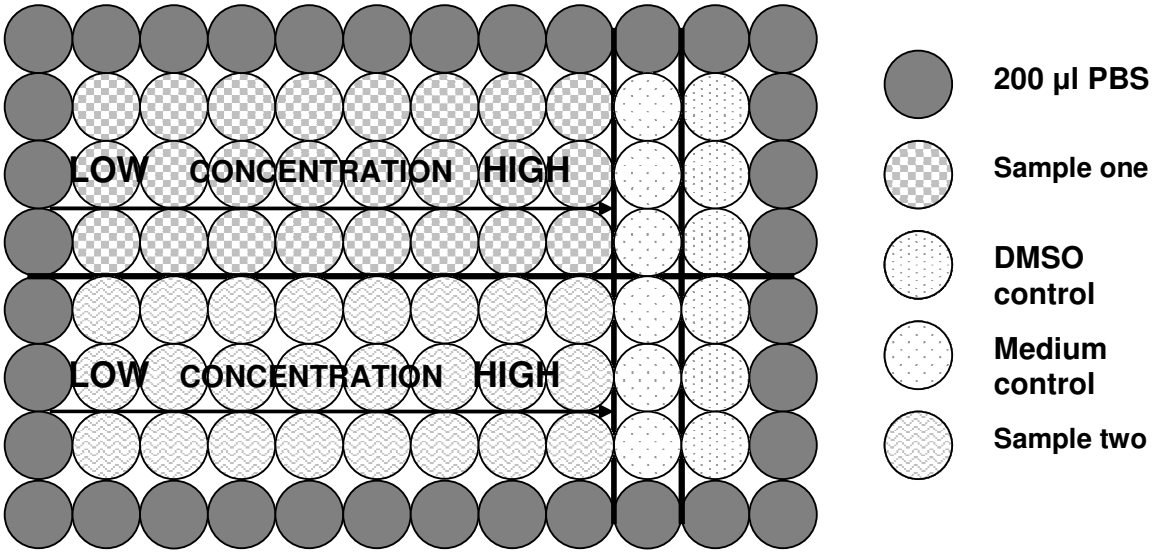

Figure 6.1 Plate design for cytotoxicity assay

Reference plates (without cells) were also prepared that contained 100 µl of medium and 100 µl of diluted extract/compound, in duplicate. Plates were then returned to 37 ^{0} C in a humidified atmosphere with 5 % CO_2 for another 3 days. On the 4th day, 50 µl of sodium-2,3-bis- [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) reagent was added to the wells and incubation commenced for 1-4 hours. The optical densities of the wells were measured at 450 nm (690 nm reference wavelength). The 690 nm reference wavelength values were subtracted from their corresponding 450 nm wavelength values. Reference plate values were then subtracted from their corresponding sample plate values. Cell viabilities were assessed by comparing sample values to the control values.

6.3. Results and discussion

The crude extract of *E. transvaalense* and isolated compounds were evaluated *in vitro* for their inhibitory ability against the growth of both Vero and MCF-7 cell lines. These cell lines were inhibited by all the compounds at the highest concentration tested (200 µg/ml), except Ψ – taraxastanonol (**3)**. The results (Table 6.1 and Figure 6.2) obtained from the calculation made from the spectrophotometer readings, indicated that the crude extract, Ψ – taraxastanonol (**3)** and 4' –*O*- methylepigallocatechin (**5)** have little or no toxicity on Vero cells by exhibiting IC_{50} values of greater than 100 µg/ml. Similarly the crude extract and taraxastanonol (**3)** exhibited IC_{50} values of greater than 100 µg/ml in MCF-7 cell line. lup-20(30)-ene-3,29-diol, (3α)-(9Cl) (**1)** and β-sitosterol (**4)** showed weaker activity with IC_{50} values ranging from 78 to 96 µg/ml in both Vero and (breast) cancerous cells.

From the isolated compounds only lup-20(29)-ene-30-hydroxy-(9Cl) (**2)** showed to be potent inhibitor with IC_{50} values of 25 µg/ml in Vero cells and 19 µg/ml in MCF-7 (breast) cancerous cells. This findings is consistent with observation by Fang *et al*. (1984) which showed no significant inhibition of KB carcinoma cell growth at the concentration lower than 20 µg/ml by similar compounds.

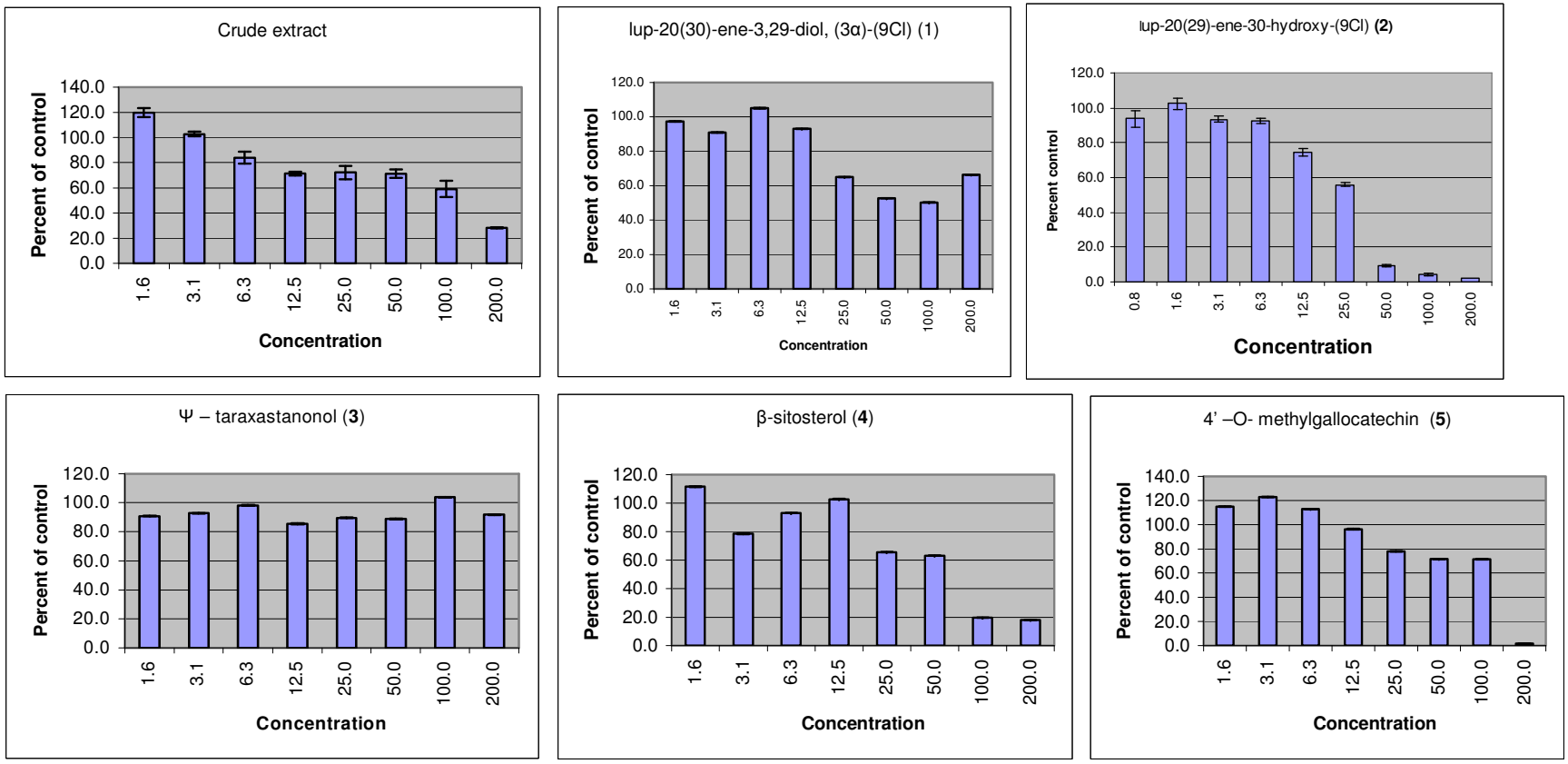

Figure 6.2 Effect of *E. transvaalense* crude extract and isolated compounds (µg/ml) on the growth of the normal Vero cell line.

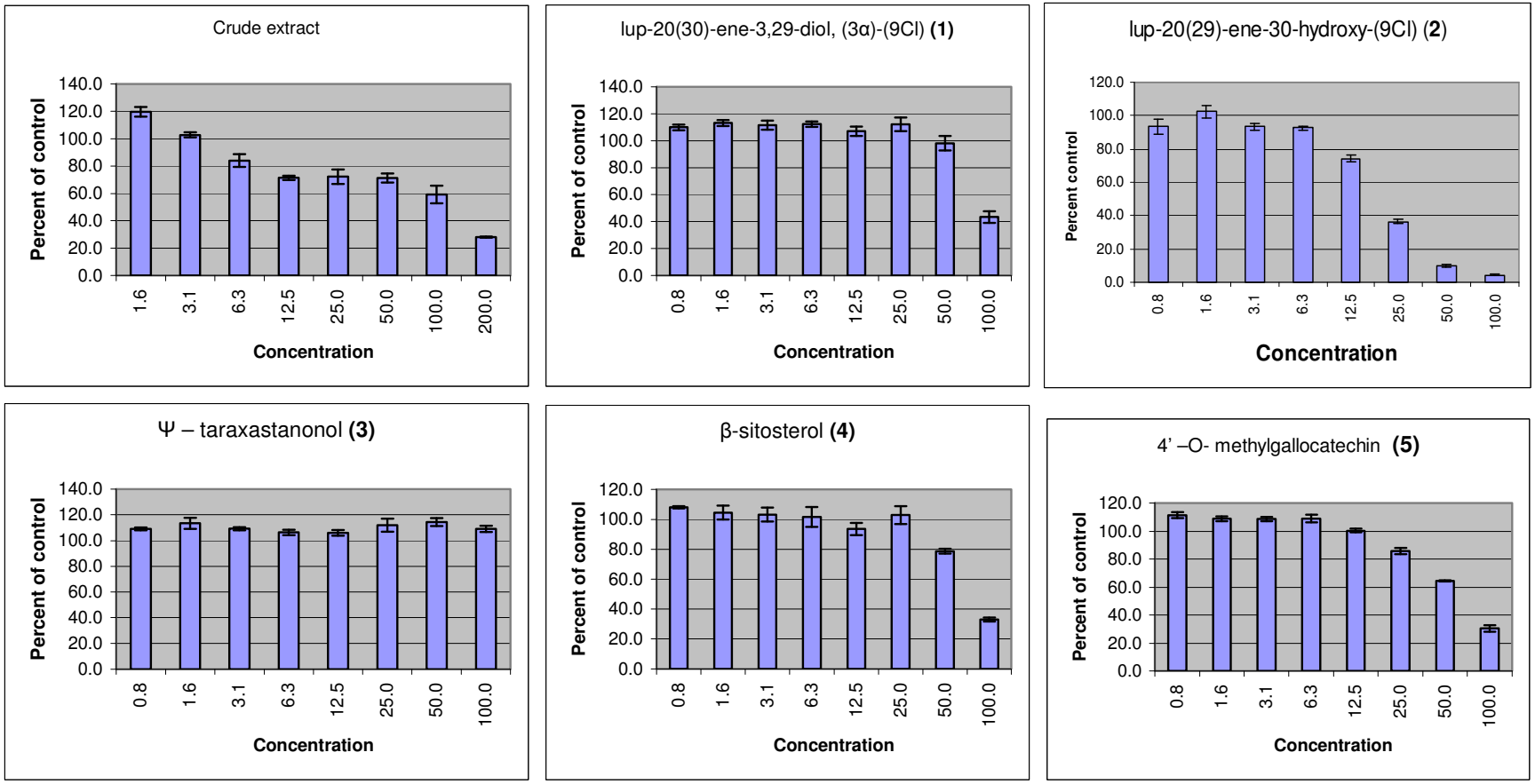

Figure 6.3 Effect of *E. transvaalense* crude extract and isolated compounds (µg/ml) on the growth of the cell line MCF-7.

Table 6.1 IC_{50} of the crude extract and isolated compounds from *E. transvaalense* after 4 days on Vero and breast cancer (MCF-7) cells.

Plant extract / compound	Vero	MCF-7
	IC_{50} (µg/ml) ± SD	
Extract	> 100.0 ± 3.6	> 100 ± 0.271
lup-20(30)-ene-3,29-diol, (3α)-(9Cl) **1**	93.0 ± 3.9	96.01 ± 2.883
lup-20(29)-ene-30-hydroxy-(9Cl) **2**	25.1 ± 3.3	19.40 ± 2.204
Ψ – taraxastanonol **3**	>100.0 ± 0.0	> 100 ± 0.115
β-sitosterol **4**	82.0 ± 2.8	78.94 ± 5.454
4' –*O*- methylepigallocatechin **5**	> 100.0 ± 3.3	66.61± 3.236
Doxorubicin (Positive control)	Na	0.009 ± 0.107
Zelaralenone (Positive control)	2.6 ± 0.3	2.4 ± 0.488

SD,Standard deviation.

Na, not assayed

In vitro cytotoxicity is necessary to define basal cytotoxicity such as the intrinsic ability of a compound to cause cell death as a result of damage to several cellular functions. This assay is also necessary to define the concentration range for more detailed *in vitro* testing to provide information on parameters such as genotoxicity or programmed cell death (Bouaziz *et al.,* 2006). It is difficult to conclude that *E. transvaalense* or compound **2** are not active against cancer cell lines, because some plants are reported to have a cytotoxic effect on cancer cells, whereas other plants activate several parameters of the immune system as a strategy to destroy cancer (Steenkamp and Gouws, 2006). Low toxicity of *E. transvaalense* extract confirms the findings of cytotoxicity studies of the same species reported by Bessong *et al*., (2005). Cao *et al*. (2006), reported the isolation of triterpene saponins which showed significant cytotoxicity activity against various cell lines. According to Kaviarasan *et al*. (2007), epigallocatchin-3-gallate can protect chang liver cells against ethanol-induced cytotoxicity and apoptosis.

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CHAPTER 7:

General discussion and conclusion

7.1 Introduction

About 39 million people are living with HIV globally and it has left no part of the world untouched. Sub-Saharan Africa is the region with the largest (> 60 %) burden of the AIDS epidemic; data also indicate that the HIV incidence rate has peaked in most countries. The progress of HIV/AIDS in the developing countries has multidimensional impact. The mortality of people who are suffering from HIV remains an immense problem. Although antiretroviral drugs are available, they are too expensive, not readily available to everyone and present important limitations such as side effects and appearance of mutant viruses that are resistant. There is a global need for broader, safer and cheaper drugs for the treatment of HIV infection. One of the approaches is to find anti-HIV agents from rich wealth of medicinal plants that many developing countries are endowed with. If the use of these plants is backed up with more scientific evidence, many people from developing countries will benefit by resorting to plant remedies. Many scientific reports have showed that many plants, most of which are used traditionally for the treatment of different ailaments have proven effective in suppressing HIV replication. New anti-HIV compounds from natural sources are often reported,

some are essentially unproven and others with distinct promise based on *in vitro* research. In this study ten ethnobotanically selected plants were investigated for their anti-HIV properties.

7.2 Activity of crude extracts against glycohydrolases and reverse transcriptase enzymes

Activity of crude extracts against glycohydrolase and reverse transcriptase enzymes was investigated using a colorimetric based assay and non-radioactive HIV –RT ELISA kit from Roche. The results indicated that the extracts of *Senna petersiana* and *Terminalia sericea* are inhibitors of glycohydrolase enzymes. *T. sericea* contains compounds such as triterpenoids, saponins and tannins and the inhibitory activity of this extract against reverse transcriptase enzyme could possibly be attributed to that.

7.3 NF-κB, Hela-Tat and cytotoxicity assays on plant extracts

The antiviral activity of the crude extracts was studied using a luciferase-based assay targeting the HIV-1 promoter activation induced by the cellular transcription factor (NF-κB) and Tat protein. Acetone, chloroform and ethylacetate extracts of *Elaeodendron transvaalense* and Zanthoxylum *davyi* showed to be potent anti- NF-κB inhibitors. The active extracts were found to be specific in the HeLa-Tet-On assay and being less toxic in the necrosis MT2 assay and therefore contain chemical compounds that can perhaps act

together synergistically to inhibit the HI virus. These results support the studies done by Cheng *et al*. (2005) who reported anti-HIV activity in acutely infected H9 cells of compounds isolated from *Zanthoxylum ailanthoides.*

7.4 Isolation of compounds from *Elaeodendron transvaalense* extract

The silica column chromatography of *E. transvaalense* extract yielded 11 pooled fractions and their phytochemical studies led to the isolation of four known triterpenes [lup-20(30)-ene-3,29-diol, (3α)- (9Cl)] **(1)** , [lup-20(29)-ene- , 30-hydroxy- (9Cl)] **(2)** , (ψ - taraxastanonol) (**3)**, (β-sitosterol) **(4)**, catechin (4'-O-Methylgallocatechin) **(5)**, a phenolic derivative (atraric acid) (**6)** and a depside (atranorin) (**7)**.

7.5 Anti-HIV activity of pure compounds isolated from *Elaeodendron transvaalense*

To evaluate the antiviral activity of the isolated compounds, NF-κB, anti-Tat and viral replication assays were performed. Only lup-20(29)-ene-30-hydroxy-(9Cl) (**2)** inhibited NF-κB activity at a low concentration of 10 μg/ml. Lup-20(30)-ene-3,29-diol, (3α)-(9Cl) (**1)** and Ψ – taraxastanonol (**3)** showed anti-NF-κB inhibition at a higher concentration of 50 μg/ml. The activity of the isolated compounds were not significant in other anti-HIV assays.

7.6 Cytotoxic activity of *Elaeodendron transvaalense* extracts and isolated compounds

The crude extract of *E. transvaalense* and isolated compounds showed little or no toxicity on both the Vero and MCF-7 (breast) cell lines in the XTT assay. Compounds **6** and **7** were not investigated for cytotoxiciy because of the low amount isolated. Compound **2** was found to be toxic against Vero and breast cancer cells with IC_{50} values of 25.1 µg/ml and 7.4 µg/ml respectively.

7.7 Conclusion

Traditional medicine is used to meet the primary health care needs in many countries, including the treatment of AIDS. Several medicinal plant products or traditional medicines have been prescribed to treat AIDS patients. Traditional knowledge and practice will probably have an important role in bioprospecting in the future and might provide a safer and more cost effective platform for drug discovery (Wang *et al*., 2006). The crude extract of *E. transvaalense* has showed good *in vitro* anti-HIV properties, but the compounds isolated only showed anti-NF-kb activity, with no anti-Tat activity and no HIV replication inhibition. The use of the extract cannot be overlooked, since the compounds might act synergistically to produce better activity. In future people may accept informal medicine if activity can be demonstrated

and quality control standards are introduced to guarantee efficacy and safety (Houghton, 1996).

This *in vitro* study supports and reinforces the value of ethno-pharmacology in the search for bioactive substances.