Isolation of an anti-HIV compound from *Elaeodendron croceum* (Thunb.) DC.

by

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Submitted in partial fulfillment of the requirements for the degree

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in the Faculty of Natural and Agricultural Sciences

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Promoter: Prof. J. J. M. Meyer

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Summary

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HIV/AIDS threaten more than 40 million people worldwide and more than 5 million in South Africa alone. There is no cure for the disease yet, and novel drugs need to be discovered to make any progress in combating the disease.

Twelve extracts from indigenous South African plants were analysed, of which one, *Elaeodendron croceum,* showed exceptionally good inhibition of transcription factors and a recombinant HIV strain in the HeLa-TAT-Luc and MT-2 VSV-pseudotyped recombinant virus assays. The pure compound isolated from this extract seemed to be

the most toxic of all the samples, with toxicity of only 25% at a concentration of 100 µg/ml. When the concentration is increased, the toxicity increased slowly from 15% at a concentration of 0.195 µg/ml until it reached 25% toxicity at a concentration of 100 µg/ml. The active concentration of the compound against HIV is much lower at 100 ng/ml with an inhibition of approximately 90% of the recombinant virus. The therapeutic index of 250 makes it a promising possibility to be studied further for the compound to be used as a drug.

The semi-purified extract and the pure compound were tested for its toxicity on VERO cells. The semi-purified extract had no toxicity up to a concentration of 50 µg/ml and the pure compound had toxicity of 20 % up to a concentration of 25µg/ml. The active concentration of 100 ng/ml for the VSV-Pseudotype assay is much lower than the start of toxicity at 25 µg/ml, and leaves a margin of activity before the toxicity level is reached.

Both the extract and pure compound shows promising results *in vitro* to be developed into a medicine to be used against HIV, but need more research on the effects *in vivo.* Using an extract is easier, cheaper and faster than isolating a pure compound from the extract. It might also be possible that the extract could be prepared as a tea and its use could be very accessible.

Chapter 1

Introduction & Background

1.1 General introduction and background

South Africa is a country rich in resources, however many of these are not yet used to its full capacity. In South Africa there are about 25 000 plant species, a diversity that is found in very few countries. This accounts for 10 % of the world's plant species. About 70% of these species are also endemic to South Africa (Torssel, 1997).

Poverty and the lack of knowledge support the spread and mortality rates of several diseases in our country. Most of these diseases are curable, but the treatment method and cost account for the increasing infection and mortality rates. Many people in South Africa still rely on traditional medicine, because it is their only access to health care. 80% of people living in developing countries are almost completely dependent on traditional medicine for their primary health care needs, and higher plants are known to be the main source of drug therapy in traditional medicine (Torssel, 1997). The development of affordable medicines providing solutions to the diseases in our country is therefore critical.

The need for development of new medicines are being realised all over the world, as pathogens become drug resistant. Medicines effective against these drug-resistant organisms are frequently in demand all over the world. This study is one of only a few that investigates South African plant treasures. One of our indigenous plants might prove to be effective in inhibiting the growth of the most feared virus ever – the Human Immunodeficiency Virus (HIV) that eventually leads to Acquired Immune Deficiency Syndrome (AIDS). The path of drug discovery to find useful and effective

treatments for patients is still a long and steep one, which might prevent many of these plant extracts to be commercialised as a treatment against HIV.

More than a decade has passed since the emergence of HIV and yet the impact of HIV/AIDS still remains a major threat and continues to have a devastating effect on the population. Nowhere has the impact been more devastating than in sub-Saharan Africa where more than 11 million people have died of it. The World Health Organisation (WHO) and the joint United Nations Programme on HIV/AIDS (UNAIDS, 2005) estimated that 30 million people have been living with HIV/AIDS in Africa by 2000 (UNAIDS, 2005) and there was no decrease in this number by 2003. The Population Reference Bureau estimated that by 2002 nearly 5 million of the population in South Africa was already infected with HIV. This is the highest number of HIV infections in any country in Africa (Population Reference Bureau, 2002). The Department of Health estimates that, on average, 1500 people are infected daily with the virus (HIV/AIDS case studies in South Africa, 2002). HIV and three HIV-related diseases namely tuberculosis, influenza and pneumonia are the major causes of death in South Africa. More than a million South Africans are dying each year of HIV/AIDS. According to UNAIDS (2004) there have been 2.3 million deaths by the end of 2004 due to HIV/AIDS (Statistics South Africa, 2002). Statistics South Africa reported that 9% of all deaths can be contributed to HIV, but the Medical Research Council (MRC) believes that the deaths due to HIV are closer to 25% (UNAIDS 2005). The percentage of deaths for each of the HIV related diseases is given in Figure 1.1.

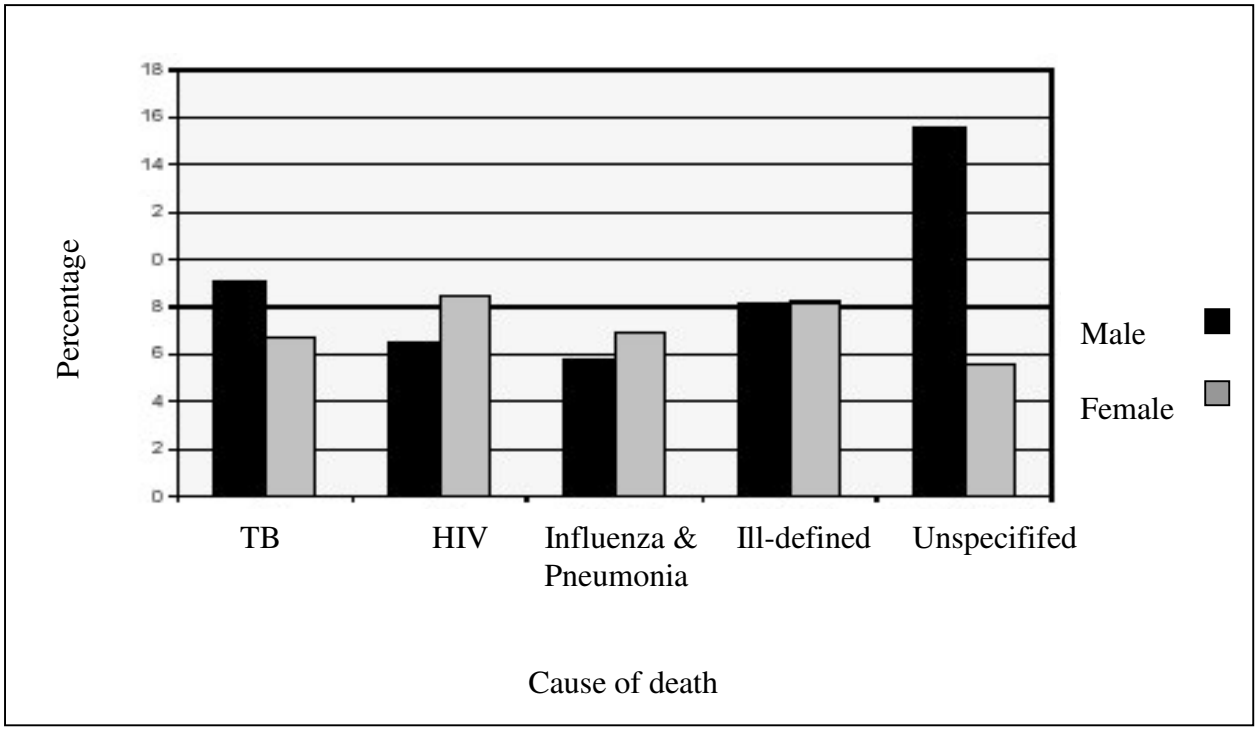


Figure1.1 Causes of death in South Africa, by sex from 1997 to 2001 (Statistics South Africa, 2002).

1.2 Background on traditional medicine

"Nature distributed medicine everywhere". – Pliny the Elder, circa A.D. 77

While the invention of synthetic chemistry in the 1930s reduced our reliance on the natural world as our sole source of medicines, an electrifying renaissance is well under way as we search the far corners of our planet for healing compounds. Within the course of the past decade, this quest has gone from being a marginal exercise to a mainstream concern all over the world. Mother Nature has been devising extraordinary compounds for more than 3.5 billion years, and new technologies increasingly facilitate our ability to discover them. New technologies therefore

enhance, rather than diminish nature's value as a source of healing compounds (Plotkin, 2000).

Plants have fed the world and cured its ills since time immemorial. A vast knowledge of medicinal and poisonous plants have therefore accumulated. Most of this knowledge still only exists as verbal tradition and only a fraction is yet available to science. Less than 10% of plants have been subjected to investigations of secondary metabolites and their effects. In order to evaluate the prospects of medicinal plant research we must know something about the results already obtained. History is important since it gives us the key to the present and should help in planning the future (Hedberg, 1987).

According to The World Health Organisation, up to 80% of Africans – or more than a half billion people visit traditional healers for some or all of their medical care. In Africa and in many developing countries, medical services are limited or unobtainable for the majority of the population (Iwu & Wootten, 2002). In South Africa today the same phenomenon is found. Many people still uses a wide variety of plants in their daily lives as food, water, shelter, fuel, medicine and for other important necessities in life. Modern health care and educational changes shifted populations from rural to urban areas, and these changes all contribute to the erosion of the indigenous knowledge of our country (Van Wyk & Gericke, 2000).

It is difficult to determine when scientific research started on plants used in traditional medicine. It would probably be safe to date it back to the late 18th century when investigations were carried out on the effect of *Digitalis* (Whithering, 1785).

Up to early 1800 drugs were used raw or as simple extracts. A new epoch was initiated when morphine was isolated for the first time. From there the interest seemed to have gradually diverged from research on medicinal plants to research on plant medicines. The research became increasingly orientated towards the chemical aspects and manufacturing of pure compounds like strychnine, caffeine and quinine (Hedberg, 1987).

The improving possibilities to synthesise desirable products meant that searching in the plant kingdom for remedies diminished. The discovery of the oncolytic properties in the alkaloids of *Catharanthus roseus* probably turned the wheel to research of plant products again. This discovery has in 30 years resulted in hundreds of scientific papers and stimulated the search for other anti-tumour agents of plant origin (Hedberg, 1987). The case of *Catharanthus roseus* is by no means unique, as many of our modern medicines are based or modelled on compounds occurring in plants which have been used for hundreds or thousands of years in traditional medicine (Hedberg, 1987).

More Americans are visiting "traditional healers" than physicians. The ineffectivity of Western medicines in treating certain disorders leads people to investigate alternatives. The future of Western healing is probably not in alternative medicines, but complementary medicines, which brings together the best of different healing traditions (Plotkin, 2000).

To ensure that these medicines will be available for research and further discovery, more controlling measures need to be implemented. Environmental destruction and degradation remain the major threats to the use of traditional medicines as complementary medicine to Western medicines. Overpopulation, deforestation, pollution and wildlife trade threaten endangered species, and even our own species as well (Plotkin, 2000).

1.3 Objectives and hypothesis

Due to the alarming statistics of HIV/AIDS over the world and the rich plant species diversity of South Africa, a project was launched between the Department of Botany at the University of Pretoria and the University of Cordoba in Spain to investigate the anti-HIV properties of some indigenous plants in South Africa.

It was important to isolate and identify the active components of the most promising extract, which is however a very lengthy and costly method. It was therefore also important to determine the effectiveness and toxicity of the crude and semi-purified extracts which have a much lower cost of preparation.

The hypothesis of the thesis was that plant extracts from selected indigenous South African plants would be effective against HIV. These plants were selected on their antibacterial and antiviral activity determined in previous studies. If the extracts inhibited HIV, it would be necessary to isolate and identify the pure compounds from the plant extracts for further studies, and to determine the possibility of developing these into commercial products.

The thesis comprises of ten chapters. Chapter one provides a general background on the study. It also includes a literature review on the genus *Elaeodendron* and the species *Elaeodendron croceum.* Chapter two discusses the HIV/AIDS disease, the effects it has on humankind and its pathogenesis. Chapter three describes the preparation of the plant extracts, the steps followed to isolate the active ingredient, and also the structure elucidation. In Chapters four and five the different assays performed to determine the efficacy and toxicity of the crude extract, the semi-purified extract and the pure compound is discussed. The importance of the use of different assays and toxicity tests is highlighted in these chapters. Chapter six gives an overview of the isolated compound digitoxigenin-glucoside and the cardiac glycosides. It describes the uses and characteristics of this group of compounds. Chapter seven is a general discussion and conclusion of all the work covered in the thesis and Chapter eight contains the summary of the study. The individuals, groups and companies that contributed to the study are acknowledged in chapter nine, and the last chapter, Chapter ten has all the references used during the course of the study.

1.4 Plant selection

All the selected plants had previously been shown to have good antibacterial and antiviral properties in other studies at the Department of Botany at the University of Pretoria. It is however not a 100% effective method, as viruses act very differently from bacteria and fungi, and the results from the anti-HIV assays could give negative results. Even the mode of action between viruses differs completely, and it is therefore important to prepare as many solvent extracts as possible to increase the efficacy of plant screening. Four different anti-HIV assays were used to further

increase the possibility of finding compounds that inhibited the virus using different mechanisms.

Twelve species were extracted according to a specific extraction procedure that will be discussed in Chapter 3. The only extract from the 12 species that showed inhibition of the virus, was the crude ethanolic extract of *Elaeodendron croceum.* As promising results were obtained from *E. croceum* the rest of the study focussed on the isolation and identification of the active compounds from this plant extract.

1.5 The genus *Elaeodendron* Jacq.

Elaeodendron is one of the largest genera in the Celastraceae family, consisting mostly of trees and shrubs. The African species are more frequently found in southern Africa and the eastern lowland parts of the continent (Archer & Van Wyk, 1998).

This genus comprises of evergreen or rarely deciduous shrubs to trees. The bark occasionally contains layers of yellow pigment, with the lenticels usually prominent. The leaves are opposite to subopposite, or occasionally alternate. The petals are cream to greenish with erect stamens. The flowers also contain a disc with the ovary immersed and adnate to the disc. The fruits are drupaceous, spheroid, white to yellow with a smooth surface. The seeds are brownish and flattened with fleshy cotyledons (Archer & Van Wyk, 1998).

1.6 *Elaeodendron croceum* (Thunb.) DC.

This is a medium to tall evergreen tree with a greyish bark containing layers of powdery yellow pigment. The lenticels are very prominent on the bark, and the leaves are opposite, elliptic, dark green above and a paler green below. The inflorescences are inconspicuous containing flowers with whitish green petals, a disc and an inconspicuous style and stigma. The fruits are drupaceous, ellipsoid and cream in colour or pale brown with fleshy cotyledons (Archer & Van Wyk, 1998). The typical characteristics are shown in an artist's representation in Figure 1.2 and photographs in Figures 1.3 and 1.4.

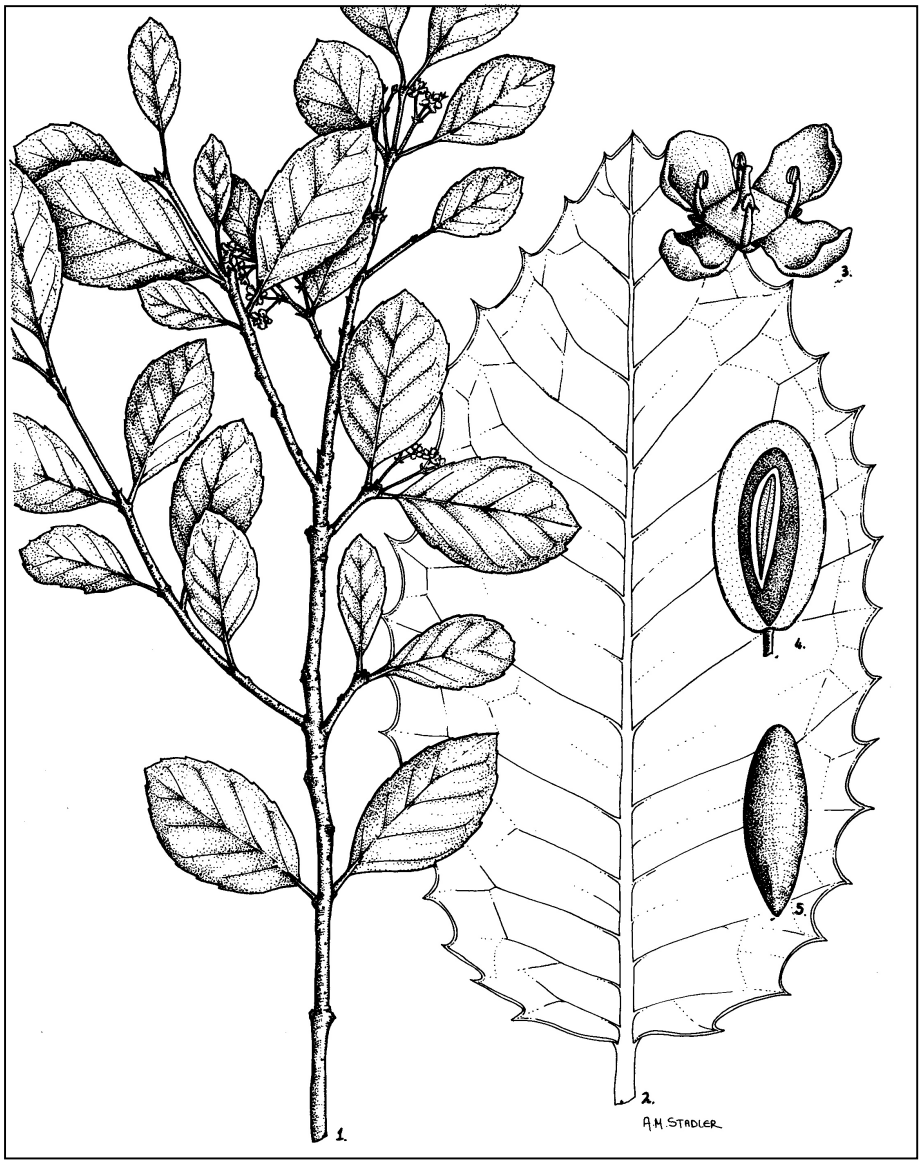


Figure 1.2 An artist's representation of the aerial parts of *E. croceum*. 1 = part of branch, 2 = leaf, 3 = flower, 4 = fruit and 5 = seed (Archer & Van Wyk, 1998).

Figure 1.3 Photograph showing the shiny leathery leaves of *E. croceum.*

Figure 1.4 Photograph showing the olive-shaped fruit.

This tree occurs on the margins of coastal and montane forests from near Ladismith in the Western Cape to Northern KwaZulu-Natal in the east. It is also found in isolated spots along the Mpumalanga and Eastern Zimbabwean escarpment (Figure 1.5). It is most abundant in the southern Cape forests where it was once popular for its bright yellow, durable wood.

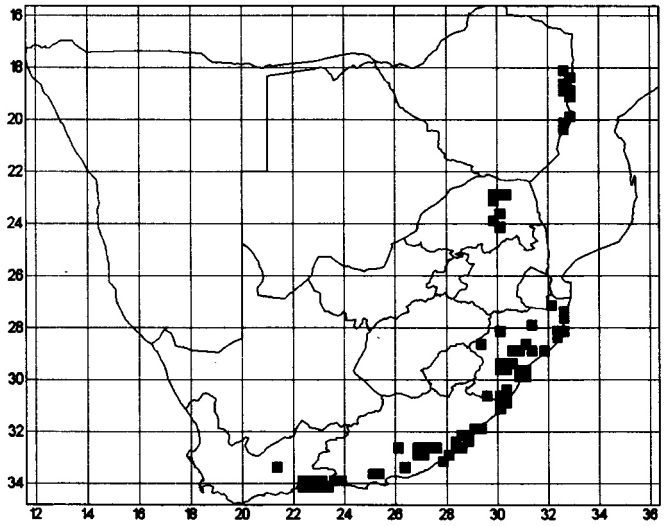


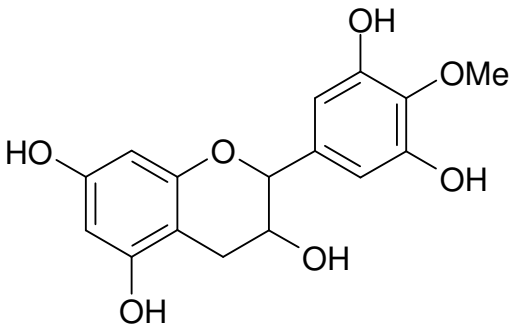
Figure 1.5 Distribution of *E. croceum* (Archer & Van Wyk, 1998).

Thunberg already described the uses of the fine and durable wood in 1794. The wood was used for making all kinds of furniture, building material, wagons as well as buttercasks. The wood is also excellent for firewood and produces long-lasting coals (Van Wyk *et al.,* 2000). Most parts of the plants are poisonous and valued for medicinal and magical properties (Watt & Breyer-Brandwijk, 1962).

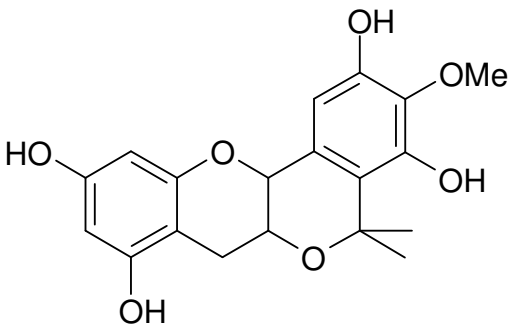
Several vernacular names, including 'Saffron', 'Common Saffron', 'Saffron wood' and 'geelhout' (yellow wood) were first recorded by Thunberg (1794). Other names include umbomvane (Xhosa/Zulu), ikhukhuzi (Xhosa/Zulu), ummakhankatha (Xhosa) and izinama (Zulu) (Von Breytenbach *et al.,* 2002). The names commonly refer to the yellow pigment found in the bark, which lead to confusion with *Podocarpus* spp. (Archer & Van Wyk, 1998).

1.7 Compounds previously isolated from *Elaeodendron croceum*

The only compounds that have been isolated from *E. croceum* were identified and described by Drewes and Mashimbye (1993). The compounds isolated are mostly terpenoid and flavonoid structures which are shown in Figures 1.6-1.8.

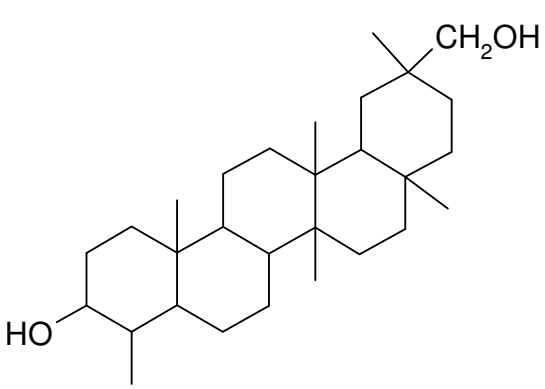


A

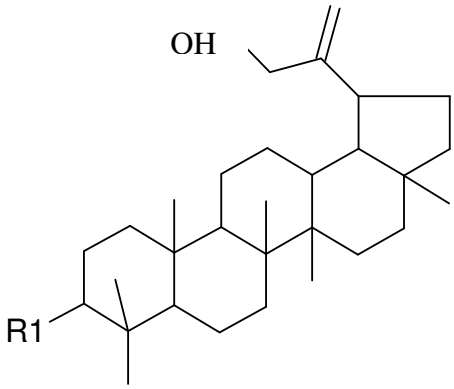


B

Figure 1.6 A=(-)-4'-O-methyl epigallocatechin & B=(+) 6R, 13R-11, 11-dimethyl-1, 3, 8, 10-tetra-hydroxy-9-methoxy-peltogynan.



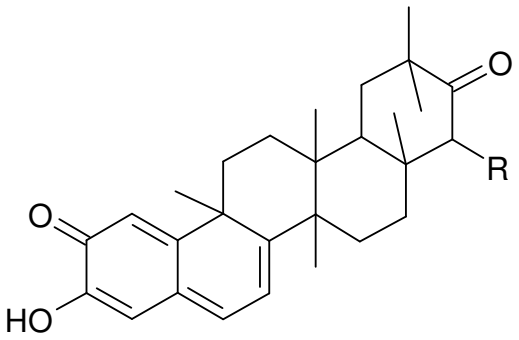
A



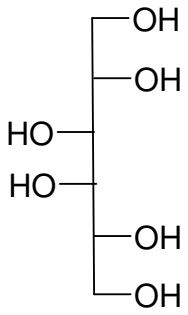
B

R1= O	30-hydroxylup-20(29)-en-3-one
R1= β-OH	30-hydroxylupeol

Figure 1.7 A= Canophyllol & B=30-hydroxylup-20(29)-en-3-one and 30-hydroxylupeol.



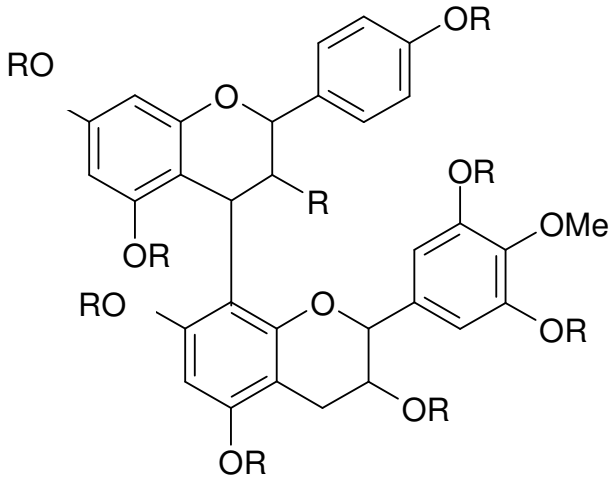
A



B

R= OH Tingenin B

R= H Tingenone



C

R= OH Ouratea proanthocyanidin

R= Ac Ouratea proanthocyanidin-nona-O-acetate

Figure 1.8 A=Tingenin B and Tingenone, B=Galacticol & C=Ouratea proanthocyanidin and Ouratea proanthocyanidin-nona-O-acetate.

Anti-tumour activity has been discovered for only one compound namely tingenone. The other compounds are not very well-known compounds, and more research needs to be conducted to identify the potential of them (Drewes & Mashimbye, 1993).

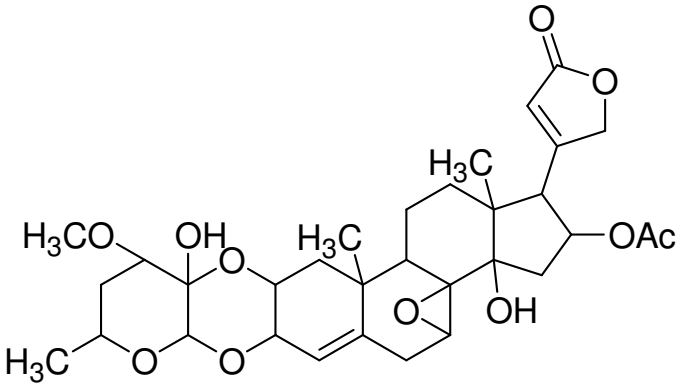
1.8 Compounds previously isolated from other *Elaeodendron* spp.

Several authors have isolated and identified compounds from this genus, and the compounds isolated cover a broad spectrum of different structures. A number of these compounds exhibit medicinal value, and is already used as traditional medicine to treat several diseases. Previous phytochemical investigations of some *Elaeodendron* species have revealed the presence of cardiac steroids from the bark of *E. glaucum* (Anjaneyulu & Naranyana, 1980), tetranortriterpenoid cardiac glycosides (Shimada *et al.,* 1985), triterpenoid quinine methides (Fernando & Gunatilaka, 1989 & Fernando *et al.,*1988) and flavonoids from the root bark of *E. balae* (Weeratunga & Kumar, 1985 & Weeratunga *et al.,* 1984). Other uses include antifeedant chemicals from *E. buchananii* (Tsujino *et al.,* 1995 and Tsanuo *et al.,* 1993) and a steroidal compound with moderate cytotoxicity against leukaemic cells from the root bark of *E. buchananii* (Kubo & Fukuhara, 1990).

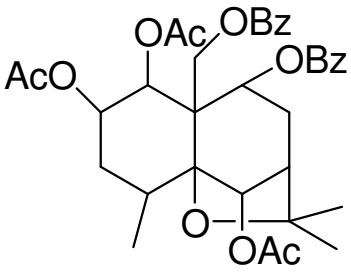
Some of the uses and structures of the compounds that have been isolated from *E. buchananii, E. glaucum, E. transvaalensis* and *E. balae* are described and given below.

1.8.1 *Elaeodendron buchananii*

This tropical tree grows in east Africa and is poisonous to animal stock and human beings. Ingestion of its leaves, fruits and bark is said to cause sudden death. The compounds related to these deaths are shown in Figure 1.9. Chewing of the plant has been said to cure diarrhoea and dried powdered roots can be used in treatment of wounds and the primary symptoms of syphilis (Tsujino *et al.,* 1995). The compounds isolated from this plant often exhibit anti-feedant activity against the nut grass worm (Tsujino *et al.,* 1995) and a lepidopteran (Tsanuo *et al.,* 1993).



A

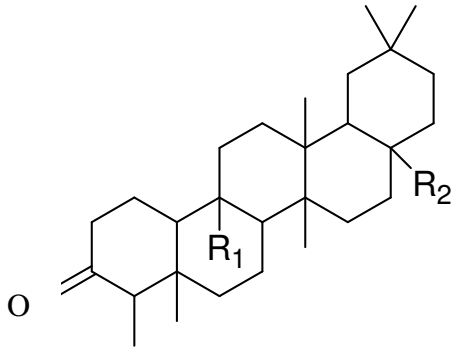


B

Figure 1.9 A=A glycoside of 2α,3β-14-trihydroxy-16α-acetoxy-14β-carda-4,20(22)-dienolide-7β,8β-epoxide (buchaninoside) & B=Mutangin (OBz=Benzoyl, Ac=Acetate).

1.8.2 *Elaeodendron glaucum*

A number of compounds and several derivatives have been isolated from this species. The isolated compounds which include several cardiac glycosides (Shimada *et al.*, 1985) form part of the compounds isolated from *E. glaucum.* The compounds are shown in Figures 1.10 & 1.11.



A

$R_1 = R_2 = Me$

$R_1 =$ Me; $R_2 = CHO$

$R_1 =$ Me; $R_2 = CH_2OH$

$R_1 =$ Me; $R_2 = CH_2OAc$

$R_1 =$ Me; $R_2 = OAc$

$R_1 = CHO$; $R_2 = Me$

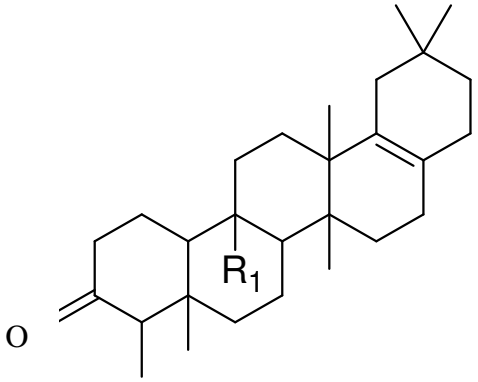
$R_1 = CH_2OH$; $R_2 = Me$

$R_1 = CH_2OAc$; $R_2 = Me$

$R_1 = Me$; $R_2 = OH$

$R_1 = Me$; $R_2 = OAc$

$R_1 = CH_2OH$; $R_2 = OH$

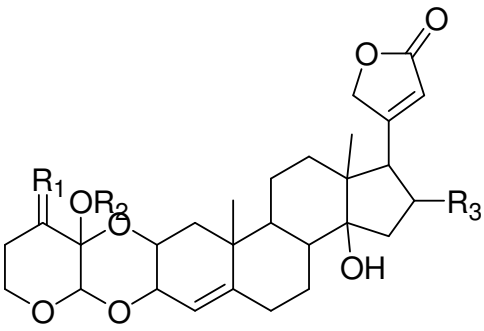


B

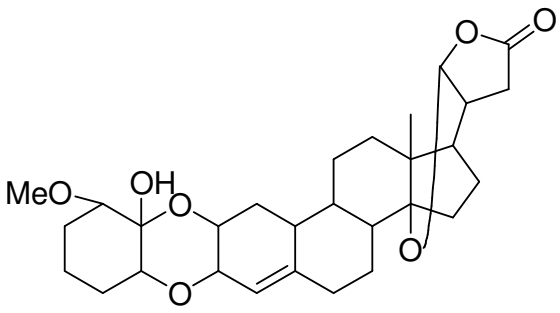
R_1=Me

R_1=CH_2OH

Figure 1.10 A & B=Compounds isolated from *E. glaucum* (Anyaneyulu & Narayana, 1980).



A



B

R_1 = H, OMe; R_2 = H; R_3 = H

R_1 = H, OMe; R_2 = Ac; R_3 = H

R_1 = OMe,H; R_2 = H; R_3 = H

R_1 = OMe, H; R_2 = Ac; R_3 = H

R_1 = H, OMe; R_2 = H; R_3 = OAc

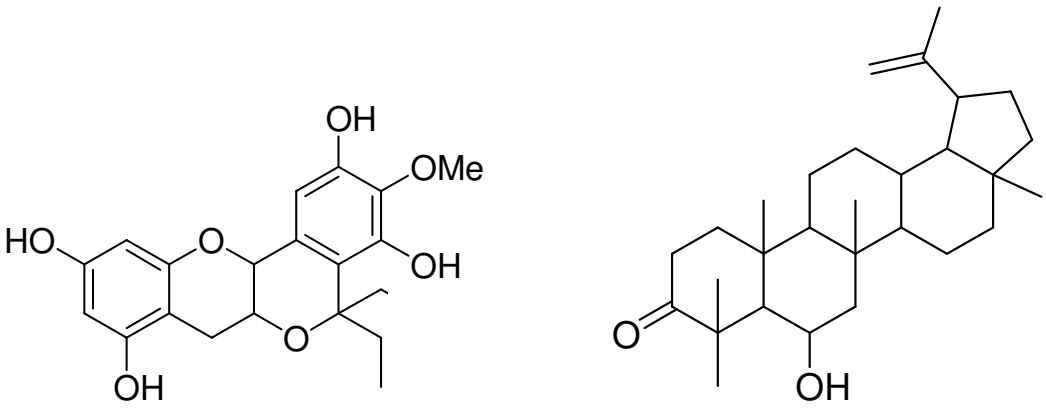
R_1 = OMe, H; R_2 = H; R_3 = OAc

R_1 = OMe, H; R_2 = H; R_3 = OH

Figure 1.11 A=Structures of elaeodendroside and related compounds & B=Isocardenolide (Shimada *et al.,* 1985).

1.8.3 *Elaeodendron transvaalensis*

E. transvaalensis is a well-known plant and used to treat several conditions. Venda and Zulu people drink large quantities of the bark infusion as a general stomach conditioner. It is also used to prepare an enema to relief stomachaches and fevers. Four compounds have been isolated from the root bark of this species and the two basic structures are given in Figure 1.12 (Drewes *et al.,* 1991).

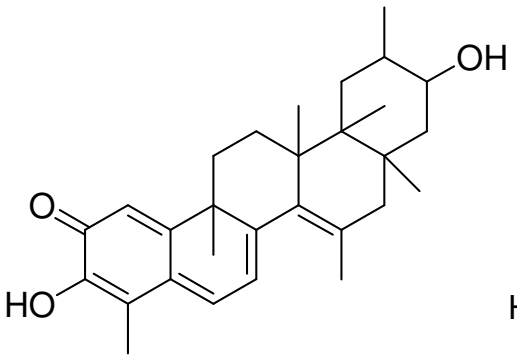


A B

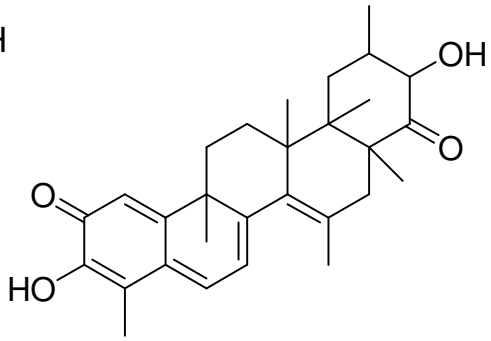
Figure 1.12 A=(+)-11,11dimethyl-1,3,8,10-tetrahydroxy-9-methoxypeltogynan & B= 6-β-hydroxy-lup-20(30)-en-3-one.

1.8.4 *Elaeodendron balae*

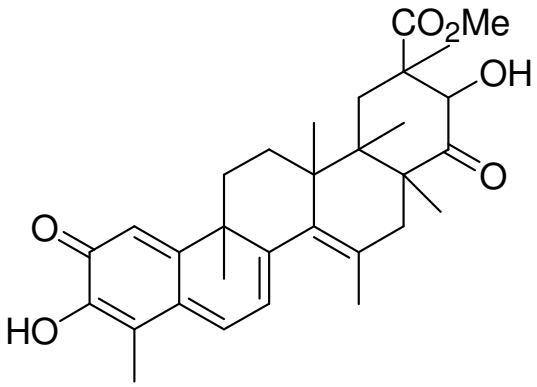
Several authors isolated compounds and derivatives from *E. balae*, which are shown in Figure 1.13. Very little medicinal data is available on any of these compounds (Tezuka *et al.,* 1993, Fernando & Gunatilaka, 1989, Fernando *et al.,* 1988 and Weeratunga & Kumar, 1985).



A



B



C

Figure 1.13 A=Balaenol , B=Balaenonol & C=Netzahualcoyone.

1.9 Discussion

HIV/AIDS is a threat to people all over the world. The fact that the disease is still spreading daily, underlines the fact that Africa, and especially sub-Saharan Africa will still feel the effect on several levels such as the economy and social impact for many years to come. The urgent need for a safe and cheap treatment against this virus is critical and needs urgent attention (Smith *et al.,* 2001).

Plants are used to treat many people in developing countries, and since 80% of the world's population resides in developing countries, about 64% of the total population utilizes plants as drugs (Torssel *et al.,* 1997). Our country contains such a wealth of plant species (10% of the world's species), and we are therefore in a good position to discover and develop new medicines.

The *Elaeodendron* plant family have been the focal point of many research investigations determining their ethnobotanical uses and several are being used as treatments already. None of these species have been indicated to treat any viral infections, and therefore also not HIV. The extract of *E. croceum* is not used as a treatment, because of its toxicity.

Chapter 2

Literature review on Human Immunodeficiency Virus (HIV) and Acquired Immune Deficiency Syndrome (AIDS)

2.1 General

HIV is a disease that is feared all over the world. It was already discovered in 1981, and 24 years later this disease has reached devastating effects with millions affected all over the world. The disease was first recognised when small clusters of young homosexual men in American cities were reported to suffer rare opportunistic infections like *Pneumocystis carnii* and Kaposi's sarcoma (Hochauser & Rothenberger, 1992). Initially it was not sure if the disease was a "gay disease" and if it was spread by other means as well. By early 1982 reports of Acquired Immune Deficiency Syndrome (AIDS) in recipients of blood transfusions and pooled clotting factors, as well as among injecting drug users indicated that an infectious agent was to blame. The appearance in Africa and in Haiti suggested that the unknown pathogen was already widespread in countries all over the world (Mims *et al.*, 1999).

In 1983 Francoise Barré-Sinoussi and colleagues isolated their first virus from a patient at the Institut Pasteur in France. The patient had persistent lymphadenopathy and the virus was named the lymphadenopathy-associated virus. By April 1984 the French group had already isolated two more, one from an AIDS patient. A month later Gallo's group at the US National Institutes of Health (NIH) reported retroviruses that they named human T-lymphotropic virus type III or HTLV-III (Gallo *et al.*, 1984). Levy *et al.*, 1984 also independently isolated AIDS-related retroviruses. The term Human Immunodeficiency Virus (HIV) was adopted in 1986 (Smith *et al.*, 2001).

By 1986 the drug zidovudine (AZT) had become available, though its effectiveness was still to be measured. As the decade of the 1980's advanced, it became clear that the effects of HIV infection were variable and not necessarily confined to the life-threatening conditions identified in the official definitions of AIDS (Anderson & Wilkie, 1992).

HIV positive refers to a condition where the person is infected with the virus. This does not necessarily mean that the patient will show symptoms, or will feel any different than a HIV negative person. It is only until the disease progresses to AIDS when the person starts to show symptoms and it is often recognised by the development of HIV-related diseases such as pneumonia and tuberculosis.

Several stages in the development of an HIV infection to the condition of AIDS have been identified (Figure 2.1). After infection with the virus, the person will enter the window period, with no signs or symptoms indicating infection. The virus will infect mostly CD4 cells. CD4 receptor sites on helper T-cells serves as a marker to distinguish them from other T-cells. That is the reason for these cells to be named CD4 cells or T4 cells. After infection the body's immune system will produce antibodies against the foreign virus particles called antigens. It takes six to eight weeks for these antibodies to be produced. The virus can not be detected during the window period, because conventional HIV tests test for the antibodies produced against the virus, which is not present in high enough quantities during the first six to eight weeks. People are therefore advised to repeat the test after eight weeks to eliminate the window period, and detect the antibodies that would have been formed after eight weeks or longer.

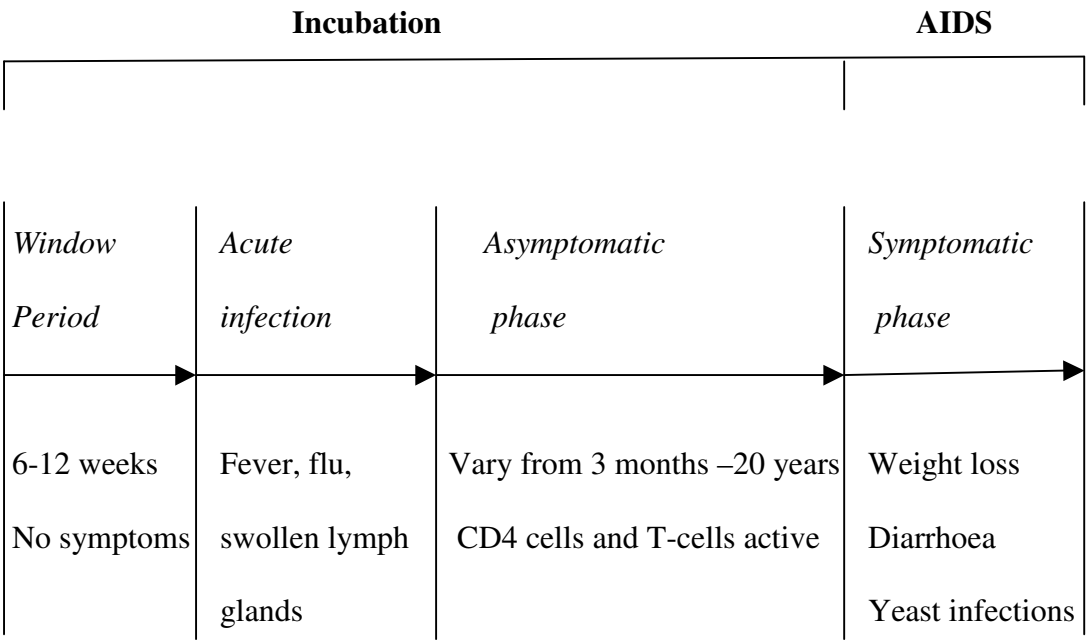


Figure 2.1: Timeline of HIV infection, with associated symptoms and duration.

After the window period a short acute period follows with very mild symptoms like flu, fever and swollen lymph glands, which could last for a day or two. The symptoms are so mild and common that few people would recognise these symptoms as warnings signs of HIV infection. People with HIV may remain healthy and show no symptoms for many years during the asymptomatic phase. This phase varies between individuals, depending on the strength of the body and the immune system, and might only last for a few months or could continue for many years. This phase has been monitored in individuals for twenty years or even more. Later in the course of infection, harmful changes to the immune system may be observed, and the development of HIV-related problems might occur. These people can also develop opportunistic infections (OI) and cancers that can be life threatening. OI will only

surface during the symptomatic phase which also indicates the onset of the infection turning into AIDS (Hochshauser & Rothenberger, 1992).

The clinical manifestations of AIDS include OI that thrive in the immuno-suppressed host. Some of these are common microbes that are seldom pathogenic in immuno-competent individuals. *Pneumocystis carinii*, *Candida albicans* and *Aspergillus* are fungal infections that do not cause more than a mild infection in healthy individuals. Various latent herpes virus infections frequently become reactivated to cause severe illness and AIDS. Some of these OI are therefore correlated with the stage of degeneration caused by HIV (Figure 2.2). Herpes simplex virus 1 (HSV-1), Herpes simplex virus 2 (HSV-2) and varicella-zoster viruses can develop life-threatening OI's in AIDS. The Epstein-Barr virus and Kaposi's sarcoma herpes virus allow tumours to occur at higher frequency during AIDS than in healthy persons (Mims *et al.*, 1999).

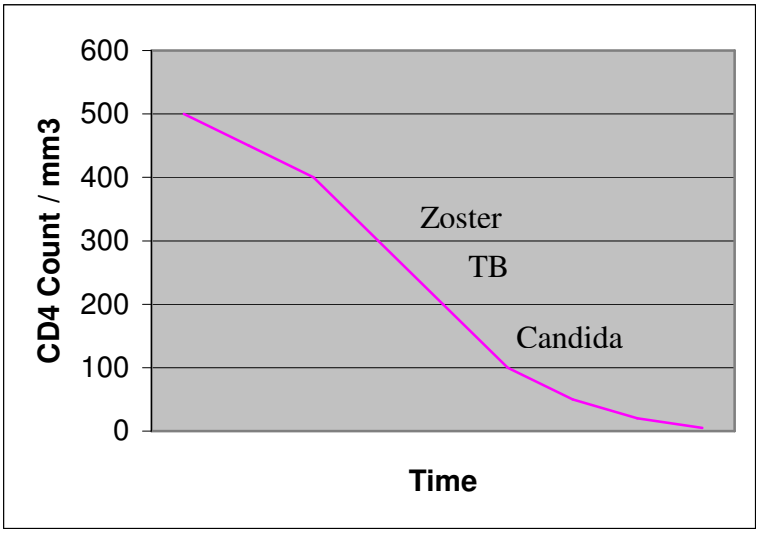


Figure 2.2 Correlation between the number of CD4 cells, and different OI associated with decreased CD4 levels (Smith *et al.,* 2001).

HIV comprise two distinct viruses, HIV-1 and HIV-2, which differ in origin and gene sequence. In 1986, a similar but not identical virus, HIV-2 was identified among people with AIDS of West African origin. So far, there have been comparatively few instances of infection with HIV–2 in Europe and the USA (Anderson & Wilkie, 1992). Both viruses cause AIDS with similar symptoms, although central nervous system (CNS) diseases may be more frequent in HIV-2. It appears that HIV-2 is less virulent than HIV-1 as HIV-2 takes longer to progress to AIDS. In some cases however it has been found that HIV-2 progressed at a similar pace as HIV-1 (Smith *et al.*, 2001).

Since 1986 numerous research projects have been conducted on HIV, and today much more is known about the virus, its infection, pathogenesis and the effects on the body. The genomes of the two types of viruses (HIV-1 and HIV-2) compare very well and only small differences can be detected. There are a variety of types that form the HIV group of viruses, and the fact that viruses mutate continuously make treatment and drug development a difficult task. This virus illustrates Darwinian selection perfectly. It is this selectivity that is responsible for the resistance developed against every new drug that is introduced to stop virus infection (Smith *et al.*, 2001). There are several complementary reasons for this great diversity. The process of reverse transcription does not include an editing device to correct mutations. The RNA genomes of retrovirus particles are also diploid and genetic recombination occurs during reverse transcription. Each infected individual therefore possesses an immense pool of HIV variants, allowing substantial genetic and antigenic drift to occur within each infected individual. It is the high rate of replication that provides the conditions for numerous immune escape and drug-resistant mutants to be regenerated. During the long

asymptomatic incubation period before AIDS develops, the virus is not latent, but is actively replicating producing millions of virions per day (Anderson & Wilkie, 1992).

Humans harbour three major groups of HIV-1, named M, N and O, with group M representing all the subtypes or clades A-H that have spread to cause the worldwide pandemic. HIV-1 groups N and O, in contrast are largely confined to Gabon, Cameroon and their neighbouring countries. The gene sequences of M, N and O are however very different from each other. HIV-2 is endemic to West Africa, but has spread to Europe and India. HIV-2, like HIV-1 are also subdivided into a number of major groups.

2.2 Structure of a virus

Viruses are much smaller than other disease causing organisms. The basic structure of a virus consists of the envelope, capsid and the core of genetic material in the form of either RNA or DNA (Figure 2.3). The envelope is additional to the capsid in some viruses protecting the virus. The basic structure of the virus particles are similar but there is some genetic variability. The capsid surrounds the genetic material, the viral RNA and reverse transcriptase, and it consists of two coats of core proteins namely p18 and p24. These protein coats, in particular p24, are of importance in testing for the presence of the virus (Anderson & Wilkie, 1992). The lipid membrane that makes up the outer envelope of the virus and the gp 120 protein together with another protein gp 41, to which it is anchored, protect the inner parts of the virus containing the RNA and essential enzymes (Hochhauser & Rothenberger, 1992).

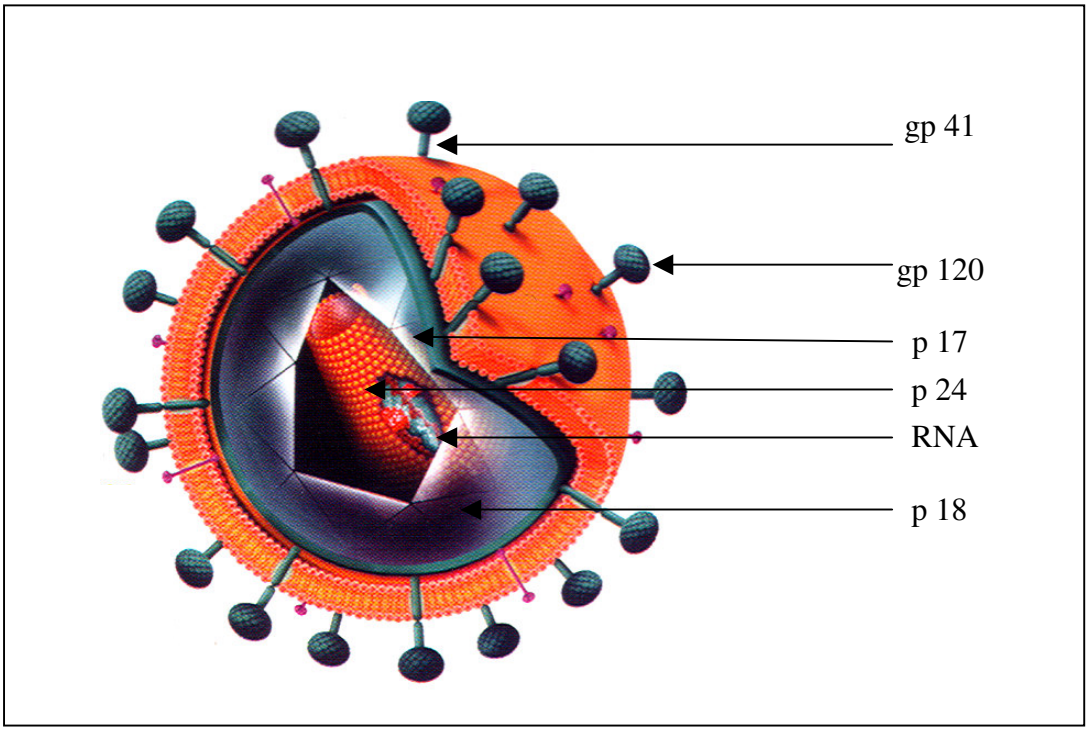


Figure 2.3 The basic structure of HIV (Mims *et al.,* 1999).

HIV comprises of a very small genome, with only nine genes (Figure 2.4). In common with all retroviruses, the gag gene encodes the structural proteins of the core (p24, p7, p6), and matrix proteins of the virus particle (p17), and the env genes encodes for the glycoproteins (gp120, gp41) that comprise the viral envelope antigens. These antigens will interact with the cell surface receptors (Smith *et al.*, 2001). The pol gene encodes the enzymes crucial for viral replication namely reverse transcriptase (RT) to convert RNA into DNA, integrase (IN) to incorporate the viral DNA into the host genome and protease (PR) to cleave the precursor gag and pol genes into their component parts. RT and PR inhibitors represent the current generation of anti-retroviral drugs given in combination to lower the viral load. The

tat gene encodes a protein that promotes transcription or production of HIV RNA from the DNA provirus while rev ensures that the correctly processed mRNA and genomic RNA is exported from the nucleus to the cytoplasm. The function of the other accessory HIV genes is not well understood (Smith *et al.*, 2001).

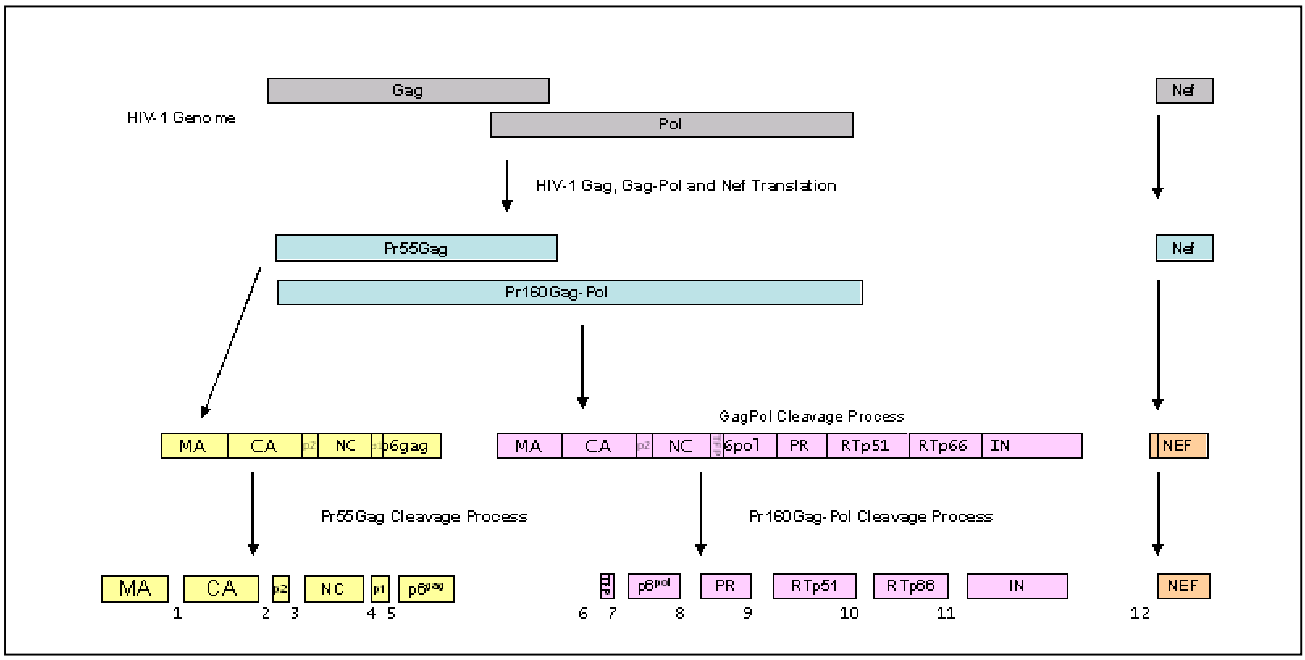


Figure 2.4 The genome of HIV (Mims *et al.,* 1999).

2.3 Pathogenesis

HIV is part of a group of viruses called retroviruses. These viruses contain RNA as genetic material and not DNA. The information then needs to be changed into the form of DNA before it can be incorporated into the genetic material of the host. The DNA is then built into the genetic blueprint of the host cell (Figure 2.5).

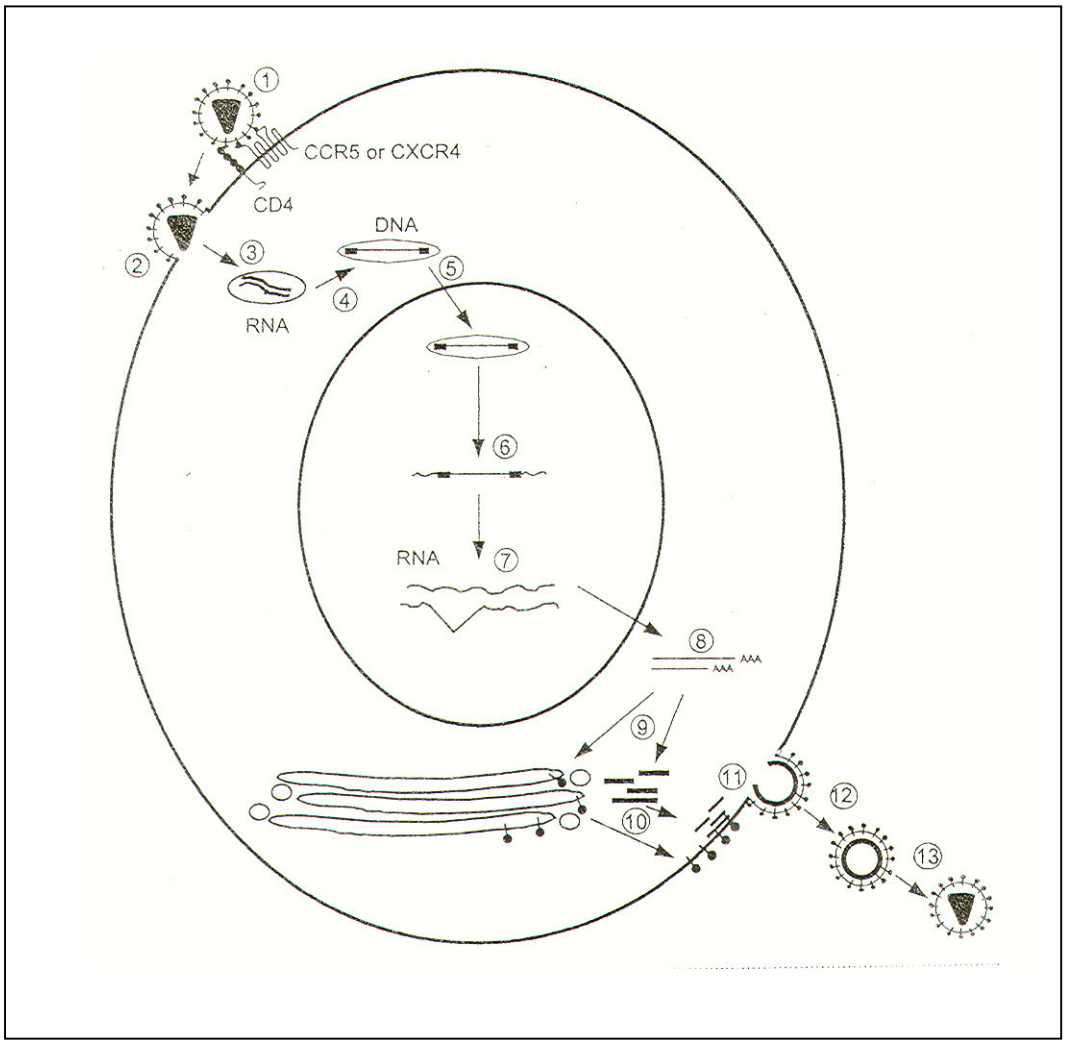


Figure 2.5 Stages in the replication cycle of HIV. (1) Attachment, (2) fusion, (3) entry, (4) RT, (5) nuclear transport, (6) chromosomal integration of DNA, (7) transcription of RNA, (8) nuclear transport of RNA, (9) translation and processing, (10) membrane transport, (11) assembly, (12) budding and (13) assembly (Smith *et al.,* 2001).

The genetic information of all cellular organisms, which allow them to copy themselves, is contained in their DNA or RNA. The DNA is then replicated to produce multiple copies of the virus, with the DNA encoding all the information needed to build an exact same copy. DNA is used to produce RNA that is in turn responsible for protein production. These proteins will form a new cell and in the case of viruses a copy of the virus (Anderson & Wilkie, 1992).

The first step of viral entry into a cell occurs when a protein on the viral envelope, known as gp 120 binds to a molecule on the surface of the CD4 cell. The CD4 receptor sites are present in considerable quantities in certain cells of the immune system namely the T-helper lymphocytes. These cells are also the targets of HIV. There are some CD4 receptor sites on the surface of other cells of the body, such as monocytes, macrophages and in micoglial cells within the brain. The virus can gain entry into all these cells by binding to their CD4 receptors (Hochhauser & Rothenberger, 1992). It became apparent that CD4 receptors are necessary for attachment, but not sufficient for entry into the host cell. Some HIV-2 strains do not depend on CD4 receptors at all. Two chemokine receptors, CCR5 and CXCR4 were identified as co-receptors to CD4 that permit virus entry. These co-receptors have a secondary binding function, assisting binding of the virus to the receptor, and it also opens up the cell wall for the virus to enter. In HIV-1 the CCR5 co-receptor proved to be the most important factor, but later during the course of infection CXCR4 utilising viruses emerged. These strains are more virulent than the initial strains and hasten the depletion of CD4 cells (Smith *et al.*, 2001).

After a virus particle has gained entry into a host cell, the genetic material in the core of the virus becomes integrated into the DNA of the cell. The virus now has 'access' to the cell's own machinery for reproduction, and it will persist like that during the lifetime of the cell. If the virus is integrated into the genetic material, the genetic material may remain latent in the cell, although the cell remains viable (Anderson & Wilkie, 1992).

The enzyme responsible for the translation of RNA to DNA is called reverse transcriptase (RT). Once the RNA is converted to DNA the genetic material can be incorporated into the host genome. When the virus DNA is incorporated into the genetic material of the host, it uses the host's processes to replicate it's own DNA, and to reproduce itself (Anderson & Wilkie, 1992).

RT and integrase (IN) which also integrate DNA into the host genome are the markers for retroviruses. The integrated DNA can remain latent, and be passed to daughter cells during chromosomal replication and cell division. Full replication in T-lymphocytes usually results in cell death, whereas in macrophages lower levels of virus replication permit the host cell to survive for longer periods. Macrophages represent a substantial virus reservoir in the infected host (Smith *et al.*, 2001).

A great deal is known about the dynamics of HIV replication *in vivo*, but there is still little understanding of what eventually tips the balance of infection away from host immunity towards the development of AIDS. It also inhibits the production of an effective vaccine against the infection (Mims *et al.*, 1999).

Figure 2.6 shows the typical course of HIV infection. Primary infection causes a transient fever in the symptomatic phase. The viral load increases sharply within the first three to six weeks that are called the window period. The viral load decreases then concomitantly with the appearance of cytotoxic T-lymphocytes. Following primary infection, HIV is never eliminated and it becomes latent. It will however stay active in the asymptomatic phase, but at a much lower level than in primary infection. The vast majority of untreated people infected with HIV eventually succumb to an AIDS-related death. The asymptomatic period varies greatly among HIV-infected individuals. Progression to AIDS may be within 9-10 years and sometimes even longer, with slowly declining levels of CD4 lymphocytes. These individuals called long-term non-progressors maintain healthy levels of CD4 cells and low levels of viral load for longer periods, whereas others may progress to AIDS within three to five years. CD4 cell replacement is probably playing a major role in determination of the progression to AIDS (Smith *et al.*, 2001).

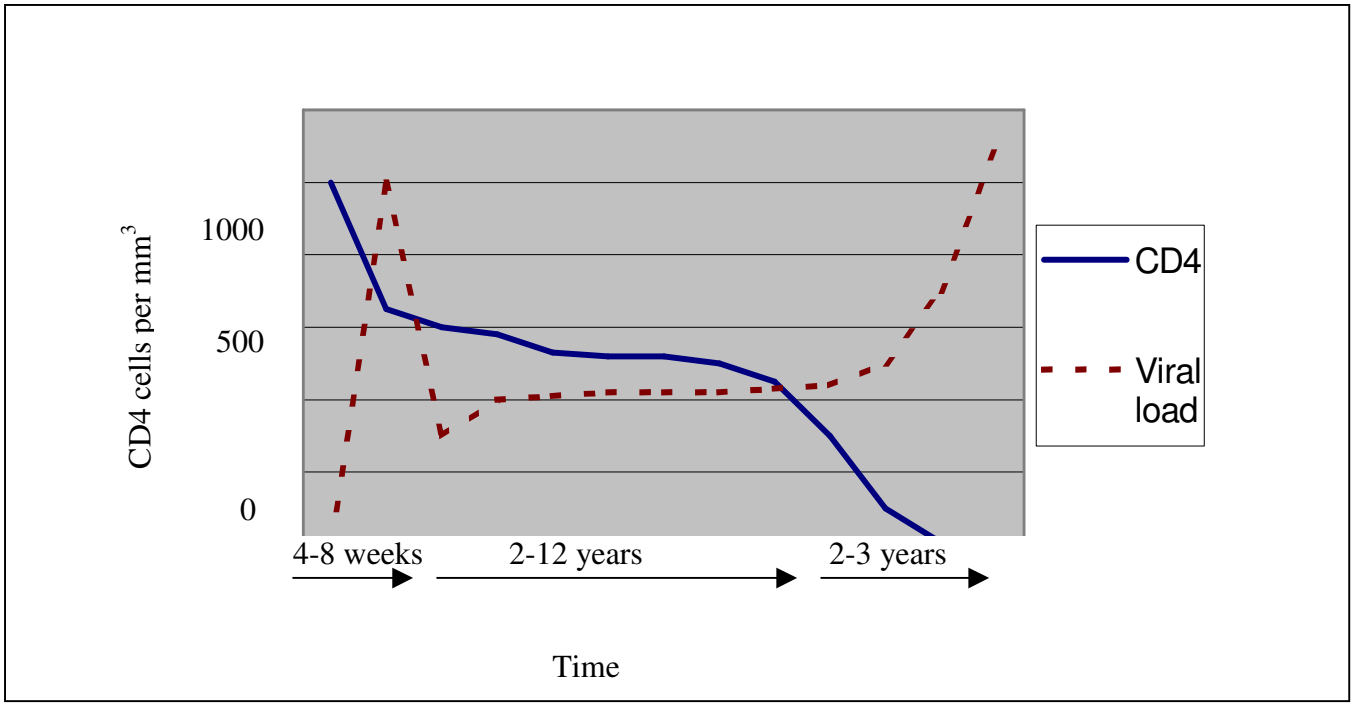


Figure 2.6 Relationship between the CD4 count and the corresponding viral load. 4-8 weeks represents the window period, 2-12 years represents the asymptomatic phase and 2-3 years represents AIDS (Smith *et al.*, 2001).

The overall HIV replication and T-lymphocyte turnover has been estimated to be extremely high, with approximately 10^9 new virions and 3.5 x 10^7 new cell infections per day (Smith *et al.*, 2001). Rising HIV viral load levels and falling CD4 cell counts lead to the onset of AIDS. The phenotype may also change during the course of the infection. These variants tend to have a selective advantage when transmitted from a late stage person to a newly infected one. These variants lead to a fast progression towards AIDS once the host immune system is sufficiently damaged (Mims *et al.*, 1999).

It is macrophage infection that leads to wasting syndrome and CNS disease in AIDS. Micoglia is a type of macrophage in the brain. Their infection leads to signalling of cytokines and chemokines, leading to a loss of neurons and dementia that sometimes occurs in AIDS. Dendritic cells are also infected by HIV. These cells include Langerhans cells of the mucous membranes and these may be a target during sexual transmission. These cells carry HIV to the lymph nodes, where CD4-positive lymphocytes become infected.

2.4 Current anti-retroviral drugs and their mode of action

Anti-retroviral drugs are used to combat the action of HIV. Treatment with these drugs is complex and the field changes rapidly. The drugs currently available inhibit the action of two enzymes vital for the replication of HIV, reverse transcriptase and protease (AIDS Bulletin, 2005).

Drugs that inhibit reverse transcriptase (RT) are called RT inhibitors and are found in two forms: nucleoside and non-nucleoside. The nucleoside RT inhibitors suppress the RT enzyme because they are analogues to the enzyme, and will therefore prevent the enzymes from binding to the active site. AZT is an example of a nucleoside RT drug. The non-nucleoside inhibitors are also RT inhibitors, but they bind to the RT enzymes, and therefore eliminate RT enzymes from producing DNA from the RNA injected into the cell by the virus. Delaviridine is an example of a non-nucleoside RT inhibitor drug currently in use (AIDS Bulletin, 2005).

Drugs that inhibit protease are called protease inhibitors. Protease inhibitors inhibit protease action, which is responsible for cleaving the viral proteins in their active components. These medicines such as Indinavir and Ritonavir bind to the protease active site, and prevent the binding of protease enzymes to cleave the proteins.

Anti-retroviral drugs do not destroy HIV infection, but effectively suppress viral replication. The usual combinations are two nucleoside reverse transcriptase inhibitors together with a protease inhibitor or a non-nucleoside reverse transcriptase inhibitor. This is called highly active anti-retroviral therapy (HAART). HAART treatment starts when antigen effects are decreased in the body, and the immune system is therefore not as effective as before. A combination of three drugs commonly used is for example: nevirapine, stavudine and lamivudine.

All these drugs have side-effects. The most common side effects of the nucleoside reverse transcriptase inhibitors are nausea, headache, muscle pain, insomnia and sometimes anaemia and other blood abnormalities. The non-nucleoside reverse transcriptase inhibitors can cause rashes, fever, nausea, headache and liver problems. All the protease inhibitors cause abnormal fat distribution, high cholesterol and triglycerides, and resistance to the hormone insulin that is involved in the metabolism of glucose to fat (AIDS bulletin, 2005).

2.5 The immune system

The immune system of the body that provides protection from disease is a very complex system. It identifies and deals with the very large number of potentially

harmful microorganisms that exist in our environment (Hochhauser & Rothenberger, 1992). Aside from the physical barriers to infection such as the skin and mucous membranes, the body possesses a wide array of chemical agents and specific types of cells that can detect and destroy microorganisms. There is a broad distinction between non-specific defences and specific defences against disease in the human body. The immune system evolved as a defence against infectious disease. Specific immunity is only called into play when microorganisms bypass the non-specific mechanism (Underwood, 2000). Many non-specific mechanisms prevent invasion of microorganisms and are given below:

- Mechanical barriers are highly effective and their failure results in infection.
- Secretory factors present effective chemical barriers to many organisms.
- Cellular factors include leukocytes and macrophages that phagocytose and kill microorganisms.
- Complements are a complex series of interacting plasma proteins (Underwood, 2000).

The non-specific mechanism consists of phagocytes, macrophages, neutrophills and killer cells. A large number of cells are known as phagocytes. Individual types such as macrophages and neutrophills, can act against a wide range of microorganisms. They act by detecting the foreign particle, binding to it and engulfing it. HIV however can gain entry into macrophages. Natural killer cells are also important, and are capable of directly attacking and killing virus-infected, or cancerous body cells.

Interferons and complements form a significant part of the body's general (non-specific) chemical defence. Interferons are a class of small proteins which are

released by virus-infected cells. The interferons assist in protecting uninfected cells from viral entry as well as mobilising the immune system. The term complement refers to a group of different plasma proteins that act to kill bacteria and several other cells. They also enable macrophages and neutrophils to adhere to and engulf microorganisms more rapidly, and intensify the body's inflammatory response to infection (Hochhauser & Rothenberger, 1992).

Specific defences produce a defence that is precisely targeted against specific microorganisms. These microorganisms produce antigens in the infected host, which the immune system will recognise and destroy or neutralise (Anderson & Wilkie, 1992). The immune system has four essential features:

- Specificity
- Diversity
- Memory
- Recruitment of other defence mechanisms

A specific immune response consists of two parts: a specific response to an antigen and a non-specific augmentation of the effect of that response. There is always a quicker and larger response the second time a particular antigen is encountered. An immune response has two phases: the recognition, involving antigen-presenting cells and T-lymphocytes, in which the antigen is being recognised as being foreign. The effector phase follows in which antibodies and effector T-lymphocytes eliminate the antigen, often by recruiting non-specific mechanisms such as complement or macrophage activation (Underwood, 2000).

2.5.1 Antigens

Antigens are substances able to provoke an immune response and react with the immune products. They react with both the T-cell recognition receptor and with the antibody. Antigens are conventially divided into thymus dependent and thymus-independent antigens. Thymus dependent antigens require T-cell participation and provoke the production of antibodies where the thymus-independent antigens require no T-cell co-operation for antibody reproduction (Underwood, 2000).

B-cells within the immune system are involved in the production of antibodies. Antibodies are made up of different types of immunoglobulin (Ig). They are able to recognise foreign proteins or sugars on the surface of antigens, and they will bind to that antigen. Memory cells are produced which is capable of being activated on subsequent encounters with an infectious agent. Antibodies circulate within the blood or lymph where they can bind to bacteria, free viruses or bacteria produced toxins (Anderson & Wilkie, 1992).

2.5.2 Antibodies

Humoral immunity is dependent on the production of antibodies and their actions. All antibodies belong to the immunoglobulin class of proteins and are produced by plasma cells, derived from B-lymphocytes (Underwood, 2000). Antibodies act against infectious agents in various ways. Phagocytes, complement or activated T-cells mark, destroy and neutralise toxic chemicals produced by bacteria, by binding to specific sites on viruses that prevent the viruses from binding to receptor sites on tissue cells. Antibodies play therefore an important role in destruction of micro-organisms, although they cannot penetrate the cells, and therefore have a limited

function in preventing the replication of viruses within the cells (Anderson & Wilkie, 1992).

2.5.3 T-cell receptors

There are mainly four types of T-cells:

- Cytotoxic T-cells (killer T-cells)
- Delayed hypersensitivity T-cells (memory cells)
- Helper T-cells
- Suppressor T-cells

Like B-cells, T-cells are committed to a given antigen. T-cells can recognise antigens that are attached to or displayed on the surfaces of cells. The cytotoxic T-cells or killer T-cells, can be activated to recognise cells which are displaying these antigens. The killer T-cells will then destroy the cells containing the antigens (Anderson & Wilkie, 1992). CD4 T-cells differentiate either into inflammatory or helper cells (Underwood, 2000). CD8 T-cells produce cytotoxins with which they eliminate tumour cells and target cells infected with viruses and other microorganisms (Haslett *et al*., 1999).

T-cells play an important role in regulating the overall activity of the immune system. The helper T-cells activate and co-ordinate the immune response. Once they are activated, they stimulate the production of other T-cells including killer T-cells and B-cells that initiate the process of producing antibodies. Suppressor T-cells slow or stop the activity of T-cells and B-cells once the infection is suppressed. When the memory

cells recognise the same antigen again, they release chemicals which enhance the defence system against the antigen (Hochhauser & Rothenberger, 1992).

2.5.4 Cytokines

Cytokines are soluble mediators secreted by lymphocytes or by macrophages. They act as stimulatory or inhibitory signals between cells. Cytokines which act between cells of the immune system are called interleukins, while those which induce chemotaxis of leukocytes are called chemokines. All the chemokines share the same common features:

- Short half lives
- Rapid degradation
- Local action within the environment of cells
- May act on cytokine receptors on the surface of the cell of production to promote further activation and differentiation
- May affect multiple organs in the body
- Exhibit overlapping functions

The immune system consists therefore of antibodies that are produced to eliminate and destroy antigens which are foreign bodies to the immune system. T-cells have different functions in strengthening and activating the immune response while cytokines stimulate and transport signals between the cells. All of these components work together during HIV infection to oppose the virus.

2.5.5 The effect of HIV on the immune system

One of the most important targets of HIV is the T-cells. The principle way in which HIV compromises the immune system is by damaging the helper T-cells by binding to the CD4 receptor sites which are present on helper T-cells. Later the number of T-cells decline markedly, and the normal ratio of helper to suppressor T-cells are disturbed. The helper T-cells cannot recognise the antigens and the activation of the immune system is suppressed. This leads to considerable problems in the normal functioning of the immune system in the body. It appears as if HIV has a less damaging effect on the other cells of the defence system. The decline in the body to defend itself is sometimes described as immuno-compromised, and these people may be subject to a range of OI (Anderson & Wilkie, 1992).

CD4 receptors sites on helper T-cells serves as a marker to distinguish them from other T-cells. That is the reason for these cells to be named CD4 cells or T4 cells. Killer T-cells and suppressor cells which can be detected by a CD8 marker on the surface are often referred to as CD8 cells or T8 cells (Anderson & Wilkie, 1992).

2.5.6 Antibody tests

As HIV infects the body and the immune system, the foreign antigens will be recognised by the host immune system. This will lead to the production of antibodies against the viral antigens. The detection of the virus is normally achieved by taking a blood sample and detecting antibodies to HIV in the serum. The most common methods used to test for the presence of antibodies to HIV are the ELISA (enzyme-linked immuno-absorbent assay) and the Western Blot test.

If the antibodies are present in the serum the person is HIV positive. If a negative result was obtained, it means that the antibodies were not detected but it might give incorrect results if a person was infected in the recent past. It takes three to eight weeks for antibodies to be produced. This period is also known as the window period of HIV infection. People that have been exposed to a risk of infection in the recent past are advised to repeat the test after three months to eliminate the window period, as the test only detects antibodies in the serum (Hochhauser & Rothenberger, 1992).

2.5.7 HIV antigen tests

This type of test directly detects the antigen of the viral material itself. The tests in clinical settings identify the p14 protein found in the core of the virus. This test can play a part in detecting HIV shortly after infection. Circulating HIV material including p14 can be detected soon after infection but prior to the development of antibodies. In most individuals the level of p14 antigens declines to undetectable quantities in the body as the body begins to produce specific antibodies to HIV. If the level of antibodies falls later during infection, the p24 antigen generally reappears in the serum. This also indicates a decline in the functioning of the immune system (Anderson & Wilkie, 1992).

2.5.8 Monitoring the effects of HIV

An important test for monitoring the functioning of the immune system is the CD4 (T4) lymphocyte count. The normal range of the CD4 count is 500 to 1500 per mm^3 blood. If the CD4 count drops below 200 per mm^3, a more rapid development of symptoms will be experienced (Anderson & Wilkie, 1992). This will be the start of developing OI, because the immune system does not function properly. People

reaching this stage, normally start the use of anti-retroviral treatment (ART) in South Africa, that takes them back to the asymptomatic phase where the CD4 count increases and stabilises for another undetermined period of time.

2.6 Statistics on HIV/AIDS

2.6.1 Sub-Saharan Africa

Sub-Saharan Africa has just over 10% of the world's population, but is home to more than 60% of all people living with HIV (De Oliveira, 2005). Of the 5.6 million new infections in 1999, two thirds occurred in sub-Saharan Africa, and almost a quarter in south and southeast Asia. Africa has been, and continues to be hardest hit. Subtype C, mainly found in sub-Saharan Africa, now accounts for almost 50% of all new infections (Figure 2.7). This subtype may prove to be a virulent strain.

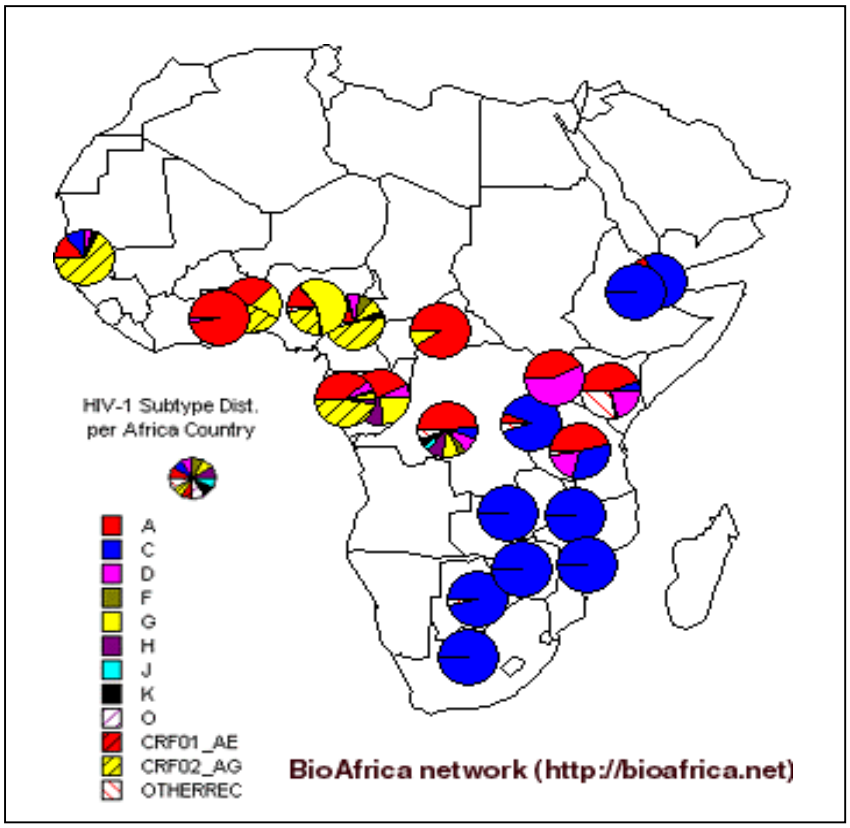


Figure 2.7 Diagram indicating the different subtype distribution in Africa (De Oliveira, 2005).

It is not only the type of virus that determines the prevalence, but also co-infections that favours the transmission of HIV. It is now clear that sexual transmission is enhanced by the presence of other sexually transmitted diseases (STDs), and helps to explain the rapid spread of HIV in countries with high occurrence of STDs. Herpes simplex virus 2 (HSV-2) is highly prevalent in many developing countries, and could increase the risk of HIV transmission by causing genital ulcers that provide a portal of entry for the virus (Smith *et al.*, 2001).

The AIDS estimates for sub-Saharan Africa, at the end of 2004 are given in Table 2.1 below.

Table 2.1: Statistics of HIV/AIDS infection in sub-Saharan Africa (UNAIDS, 2004).

Category	**Percentage or number**
Adult (15-49) HIV prevalence rate	7.4%
Adults and children living with HIV (0-49)	25 400 000
Women (15-49) living with HIV	13 300 000
Adults and children newly infected with HIV in 2004	3 100 000
Adults and child deaths due to AIDS in 2004	2 300 000

HIV infection is becoming endemic in sub-Saharan Africa. The havoc wrought will shape the lives of several generations of Africans. Southern Africa offers only faint hints of impending declines in HIV prevalence. With the exception of Angola each country in this region is experiencing national prevalence of at least 10%. This means that an estimated 11.4 million people are living with HIV in the nine sub-Saharan African countries. This is almost 30% of the global number of people living with HIV in an area where only 2% of the total world population resides (UNAIDS, 2004).

Across the region, women are disproportionately affected with HIV. On average there are 13 women living with HIV for every 10 infected men, and the gap continues

to grow. In most countries women are also infected at an earlier age than men. Recent studies suggest that there are on average 36 young women living with HIV for every 10 young men in sub-Saharan Africa (UNAIDS, 2004).

2.6.2 South Africa

The latest results released at the end of 2003, estimated that 5.3 million South Africans were infected with HIV, the largest number of individuals living with the virus in a single country. Unfortunately, there is no sign yet of a decline in the epidemic. Latest data suggest prevalence levels are still increasing in all age groups, except for pregnant women older than 40 years of age (UNAIDS, 2004).

The HIV and AIDS estimates for South Africa, at the end of 2003 are given in Table 2.2 below.

Table 2.2: Statistics of HIV/AIDS infection in South Africa.

Category	**Percentage or number**
Adult (15-49) HIV prevalence rate	21.5%
Adults living with HIV (15-49)	5 100 000
Women (15-49) living with HIV	2 900 000
Adults and children living with HIV (0-49)	5 300 000
AIDS deaths (adults and children) in 2003	370 000

The national HIV infection rate among pregnant women attending antenatal services in 2003 was 27.9%. Commitment to tackling the epidemic in South Africa is backed by increased domestic financial resources. In 2003, the government approved a Comprehensive National Plan on HIV and AIDS Care Management and Treatment, which provides access to antiretroviral treatment to more than 1.4 million South Africans by 2008 (UNAIDS, 2004).

These figures coincide with the release of a report on the National Indicators of the Demographic Impact of HIV/AIDS in South Africa, 2004 by the Centre for Actuarial Research (CARe) at UCT, The Burden of Disease Research Unit of the MRC and the AIDS Committee of the Actuarial Society of South Africa (ASSA). The report also shows that 5 million out of a total of 46 million South Africans (11%) are infected with HIV. It is also speculated that the population growth of 0.8% is set to fall to half of that level by 2010. In the absence of ART AIDS deaths would be expected to rise to nearly 500 000 by 2010. With ART the number is expected to fall to 380 000. The life expectancy at birth in South Africa is currently 50 years (AfroAIDS info, 2004).

Chapter 3

Extract preparation and isolation and identification of active compounds from *Elaeodendron croceum*

3.1 Introduction

From the twelve extracts tested against HIV, the extract of *Elaeodendron croceum* was identified as most active. The extract was prepared according to a specific preparation procedure proposed by the University of Cordoba, Spain.

After determining the activity of the extract, bio-guided fractionation was used to isolate and identify the active compound from the extract. To isolate the single compound from the extract, several chromatographic techniques such as thin layer chromatography (TLC), column chromatography and liquid-liquid fractionation were used. Several columns were needed to obtain a pure compound. Precipitation of the compound from the semi-purified fractions made it easier to isolate the compound.

After isolation, analysis such as one-dimensional and two-dimensional nuclear magnetic resonance (NMR) was used to determine the structure of the compound. Two-dimensional NMR analyses proved to be most useful, as several of these analyses were needed to confirm the structure. It was determined that the active compound is digitoxigenin-glucoside, a compound that belongs to the cardiac glycosides.

3.2 Materials and Methods

3.2.1 Selection of plants

Several South African plants were tested against HIV. The selection was done on previous antibacterial, antifungal and antiviral activity found in these plants. For each

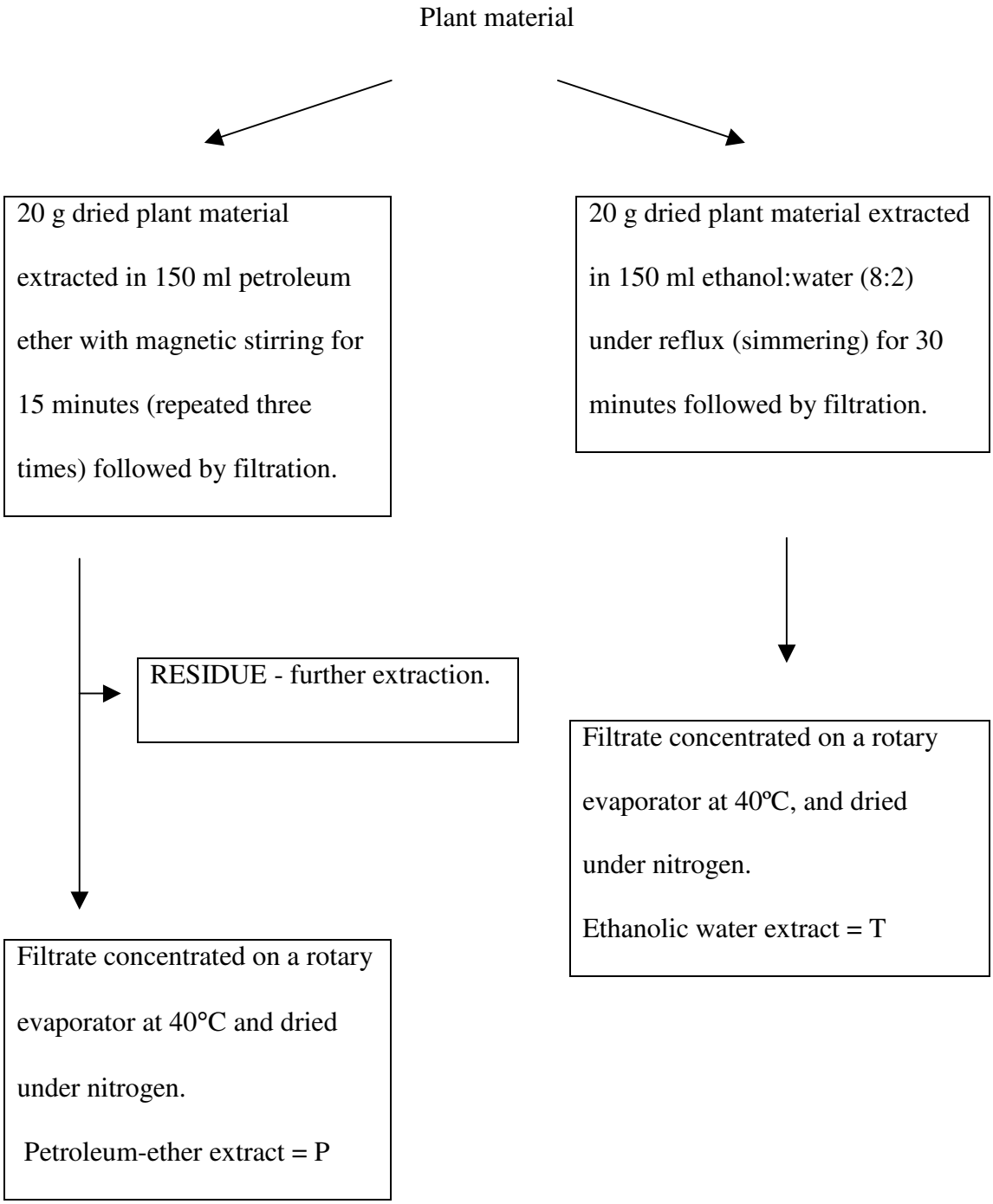
plant a herbarium specimen had been deposited in the H.G.W.J. Schweickerdt Herbarium (PRU) at the University of Pretoria. A total of four extracts were prepared for each plant, to maximise the extraction of different polarity compounds from the plant. Extracts were prepared by using petroleum ether, ethanolic water (8:2), ethyl acetate and methanol. The twelve plants selected are given in Table 3.1. For each plant the herbarium specimen number and the type of extracts prepared are given. For some of the extracts the residue was only a small amount and were therefore not used further in the bioassays.

Table 3.1 The twelve species analysed for anti-HIV activity, their herbarium specimen numbers and the type of extracts prepared (P = Petroleum ether, T = Ethanolic water, E = Ethyl acetate and M = Methanol).

Plant	**Herbarium specimen number**	**Extracts prepared**
Croton pseudopulchellus	(E A Prozesky 91)	P, T
Helichrysum melanacme	(N.L. 16)	P, T
Nidorella anomala	(N.L. 28)	P, T
Nidorella auriculata	(N.L. 29)	P, T
Ekebergia capensis	(G. Prinsloo 10)	P, T
Rapanea melanophloes	(G. Prinsloo 12)	P, T, E
Euclea natalensis	(G. Prinsloo 13)	P, T, E, M
Polygala myrtifolia	(G. Prinsloo 5)	P, T, E
Elaeodendron croceum	(G. Prinsloo 11)	P, T
Bidens pilosa	(G. Prinsloo 4)	P, T
Cryptocarya latifolia	(G. Prinsloo 14)	P, T
Protasparagus africanus	(G. Prinsloo 15)	P, T

3.2.2 Extract preparation

The plant extracts were prepared according to the procedure in Figure 3.1.



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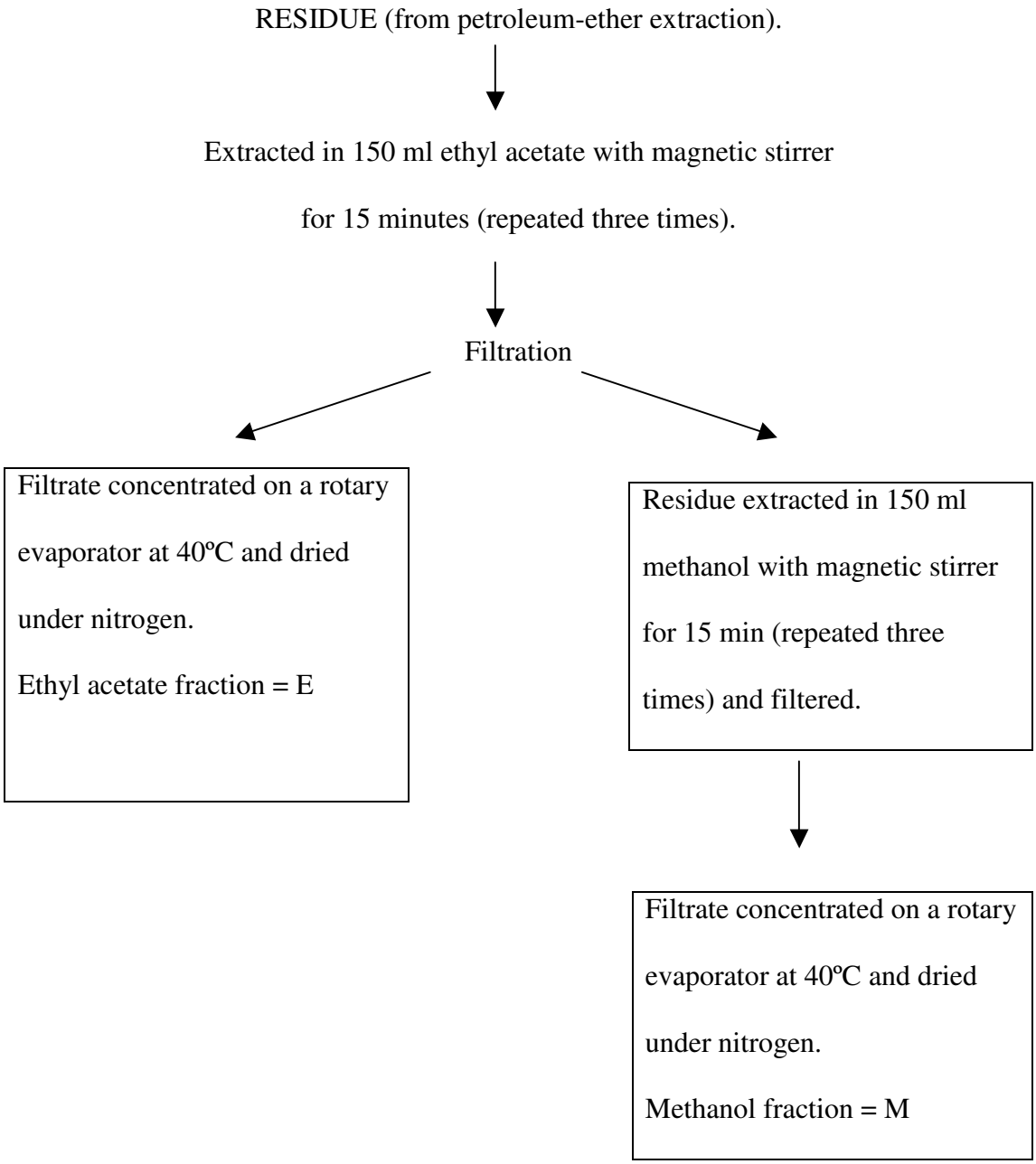


Figure 3.1 Extraction procedure.

After preparation of these extracts, it was analysed at the University of Cordoba in Spain for activity against HIV. The *E. croceum* ethanolic extract was selected for the isolation of active compounds, because it had the best anti-HIV activity. The HeLa-Tat-Luc and MT-2 VSV pseudotype assays and results will be discussed in Chapter 4.

3.2.3 Isolation of active compounds

Liquid-liquid fractionation was used to remove a large quantity unwanted compounds from the fraction. The extract volume was measured and an equal amount of chloroform was added for fractionation, and the process was repeated three times. The green chloroform layer that was below the red water layer was removed. The green chloroform extract was combined and concentrated on a rotary evaporator. This extract was then separated on a silica gel column.

The *E. croceum* extract was separated on a dry silica column (silica gel 60). The concentrated extract was mixed with a small amount of dry silica gel powder. The extract and silica gel powder mixture was allowed to dry overnight, to allow complete evaporation of the solvent. The extract and silica gel mixture was then added to the prepared dry silica gel column, and covered with a layer of clean dry silica gel powder. A variety of solvents from apolar to polar were used, starting with the apolar solvents. The following solvents were used during the separation process: hexane, ethyl acetate, acetone, ethanol and methanol. The concentrations were changed from 100% solvent to 70:30, 50:50, 30:70 ratios with the next solvent. Hexane removed most of the non-polar compounds from the column, and was used until all the yellow and orange compounds eluted from the column. As the coloured bands eluted from the column, the concentration of hexane was decreased and the concentration of ethyl acetate slowly increased. As the ethyl acetate was increased, a yellow and later a dark green band eluted form the column. Most of the green colour eluted from the column as the ethyl acetate reached a 100% concentration in the column. After all the colour bands had eluted from the ethyl acetate solvent, the acetone concentration was

increased very slowly. After acetone, ethanol and methanol were used as solvents. The concentration of ethanol and methanol was increased very slowly as to allow all the compounds to elute from the column before the polarity changed too much. After the ethanol concentration was increased it seemed as if most of the compounds were already eluted from the column. Methanol was used to clear compounds that were still trapped in the column. Very few compounds eluted from the column at this stage.

The fractions were concentrated and evaluated by means of thin layer chromatography (TLC) on silica gel 60 aluminium plates. The best separation of the compounds on the TLC plate was obtained by using a mobile phase of chloroform: methanol (95:5).

The TLC plates were evaluated and similar fractions were combined and the combined fractions were tested again for anti-HIV activity. After selection of the active fractions, these fractions were separated on a silica column again. A dry silica column had been prepared exactly as described with the separation of the crude extract. A smaller column had to be used, as the fraction quantity was much less than the original extract. The same solvents were used as with the previous separation method, although smaller quantities were used as most of the unwanted compounds had been removed from the fractions with the previous column.

The smaller fractions contained a maximum of five compounds, and some of the fractions were already purified to contain only a single pure compound. All these fractions were tested against HIV to determine which fractions or compounds inhibited the virus. The fraction containing the active compound was dried by rotary

evaporation, until only a small volume of the solvent was left. The concentrated fraction was then exposed to air to allow evaporation of the last solvents from the fraction. As the fraction was allowed to dry, a white precipitate formed. The precipitate was then removed and purified further by firstly adding a small amount of ethyl acetate to it to dissolve the impurities. The precipitate did not dissolve in ethyl acetate but did in methanol. The purified compound was identified as the active compound and analysed by spectroscopic methods to identify it.

The figure below (Figure 3.2) is a summary of the isolation process of the active compound.

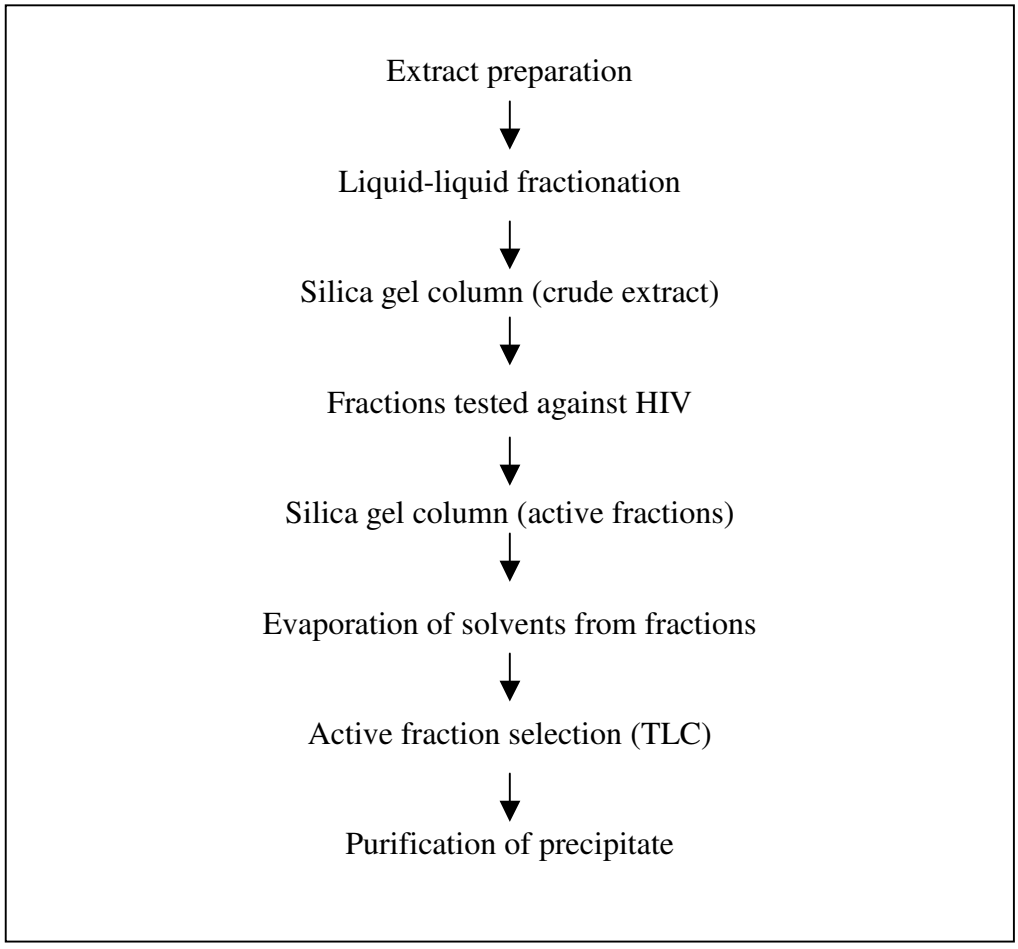


Figure 3.2 Diagrammatic representation of the isolation process.

3.2.4 Identification of the active compound

To identify and determine the structure of a compound can be a lengthy and difficult process, as several methods are used to determine the structure. The purified compound was identified by using ^{1}H-NMR, ^{13}C-NMR, HMQC, HMBC, COSY and NOESY analyses.

One-dimensional proton and carbon nuclear magnetic resonance (NMR) spectra were obtained first to get an idea of the type of compound. These analyses were performed in deuterated methanol on a 300 MHz Varian NMR apparatus. The two-dimensional NMR analyses were obtained on a 500 MHz Brücker apparatus. The results were compared with published data to determine the structure, and to obtain information on the specific compound that had been isolated.

3.3 Results

The anti-HIV activity results of the plant extracts and isolated compound will be discussed in Chapter 4.

Figure 3.3 shows a TLC plate with the fractions obtained from the first silica gel column using a solvent system of chloroform: methanol (95:5).

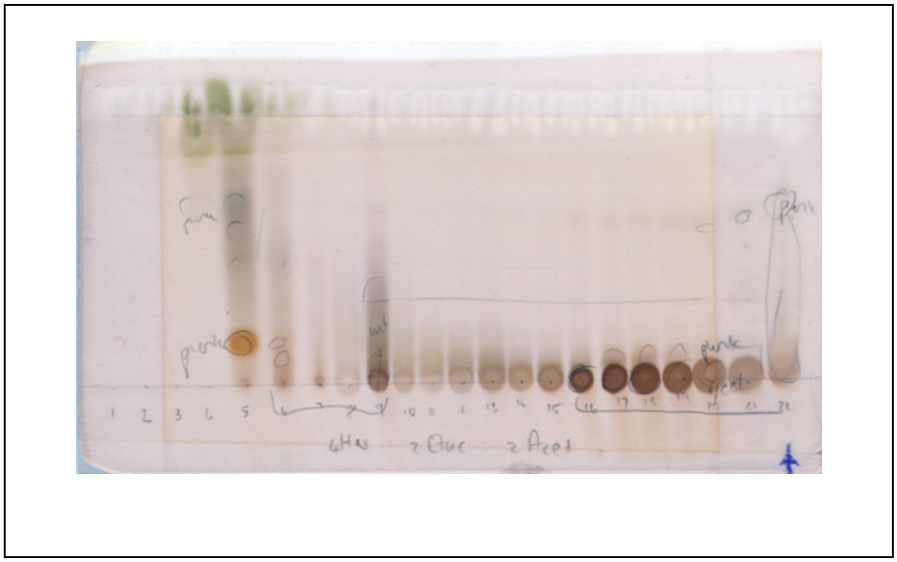


Figure 3.3 TLC (silica gel 60) plate showing column separation of the ethanolic water extract. The TLC was developed with a solvent system of chloroform: methanol (95:5).

The presence of the compound was confirmed by using TLC plates. After spraying the plates with vanillin colour regent, the characteristic blue colour could be used to identify the compound. Figure 3.4 shows the purified compound on a TLC plate. The compound proved to be a large molecular mass compound with an aglycone and a sugar moiety which is responsible for the striped effect on the TLC plate. It was eventually identified as digitoxigenin-glucoside (Figure 3.5). The structure consists of a terpenoid with an attached glucose molecule and a lactone ring. The compound forms part of the well-known group of cardiac glycosides.

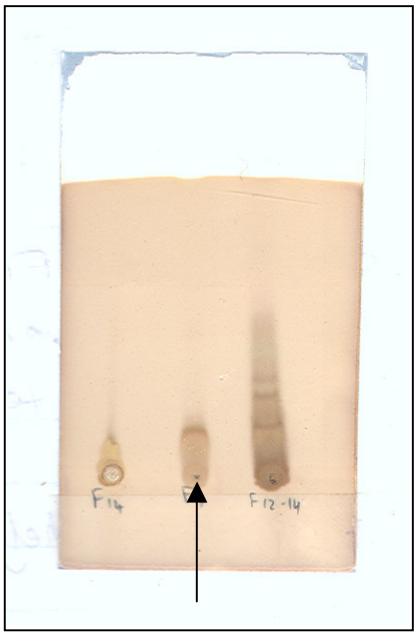


Figure 3.4 TLC plate showing the purified compound digitoxigenin-glucoside, isolated from the active fraction.

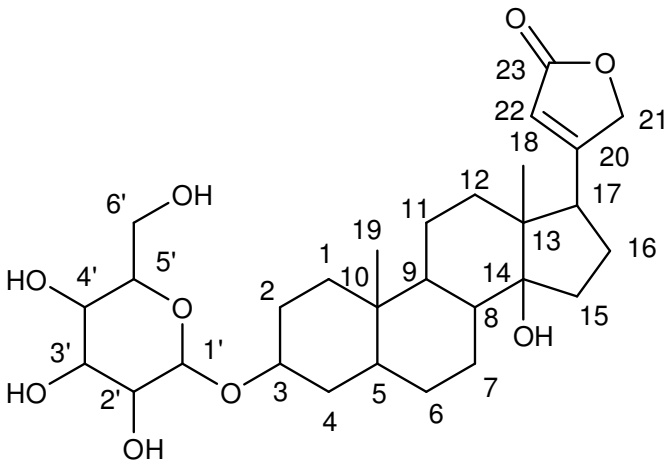


Figure 3.5 Structure of digitoxigenin – 3-O-glucoside.

Figure 3.6 shows the one-dimensional ^{1}H-NMR of the compound, with the chemical shift values given in Table 3.2 together with the ^{13}C-NMR results.

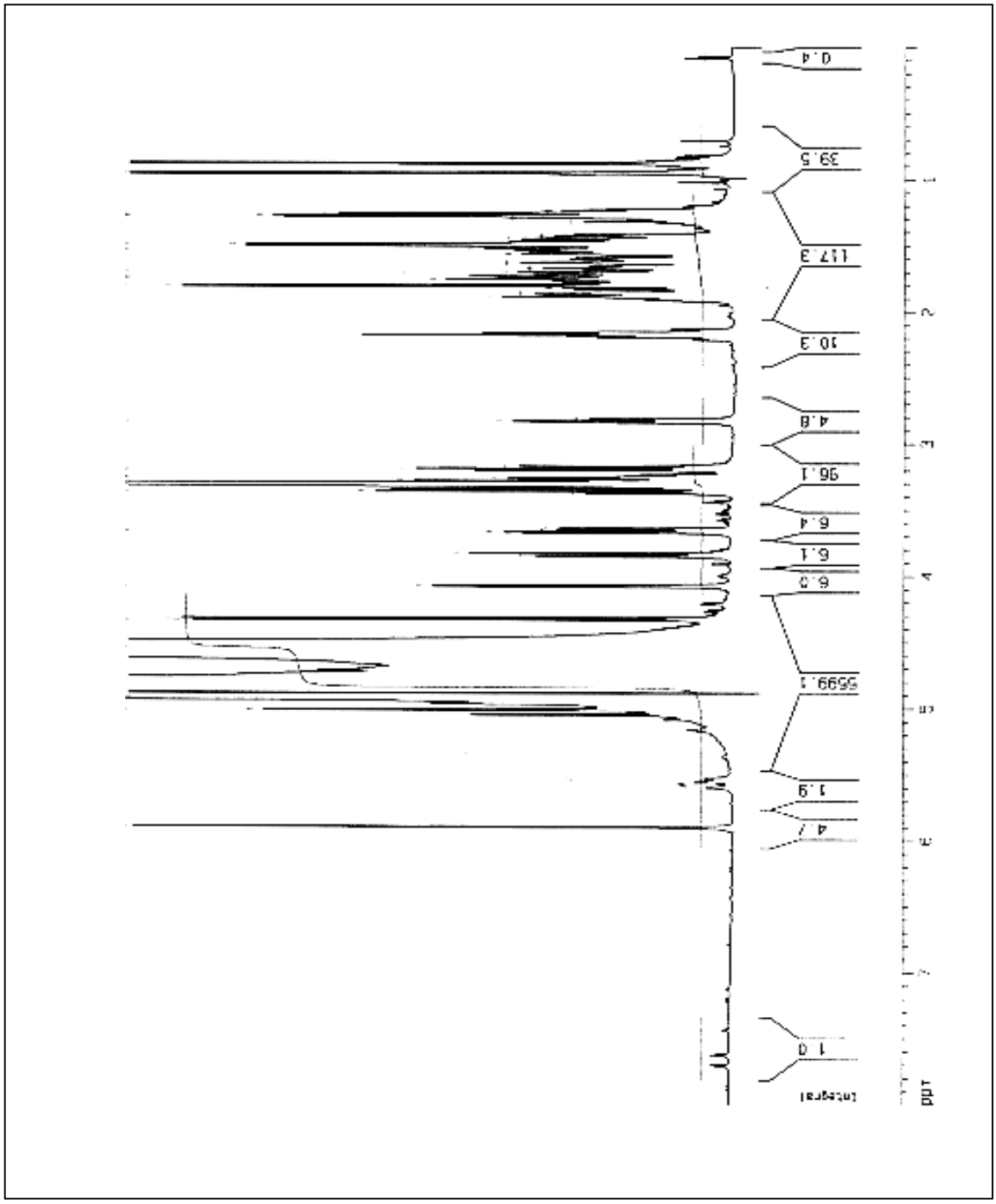


Figure 3.6 ^{1} H-NMR of purified digitoxigenin-glucoside in deuterated methanol.

Figure 3.7 shows the ^{13}C-NMR spectrum of digitoxigenin-glucoside ranging from 0-200ppm. The ^{13}C-NMR of the isolated compound correlates well with values published by Rathore *et al.* (1985) and by Cheung *et al.* (1981). The ^{13}C-NMR, HPLC and MS values have been published by Kawaguchi *et al.* (1989).

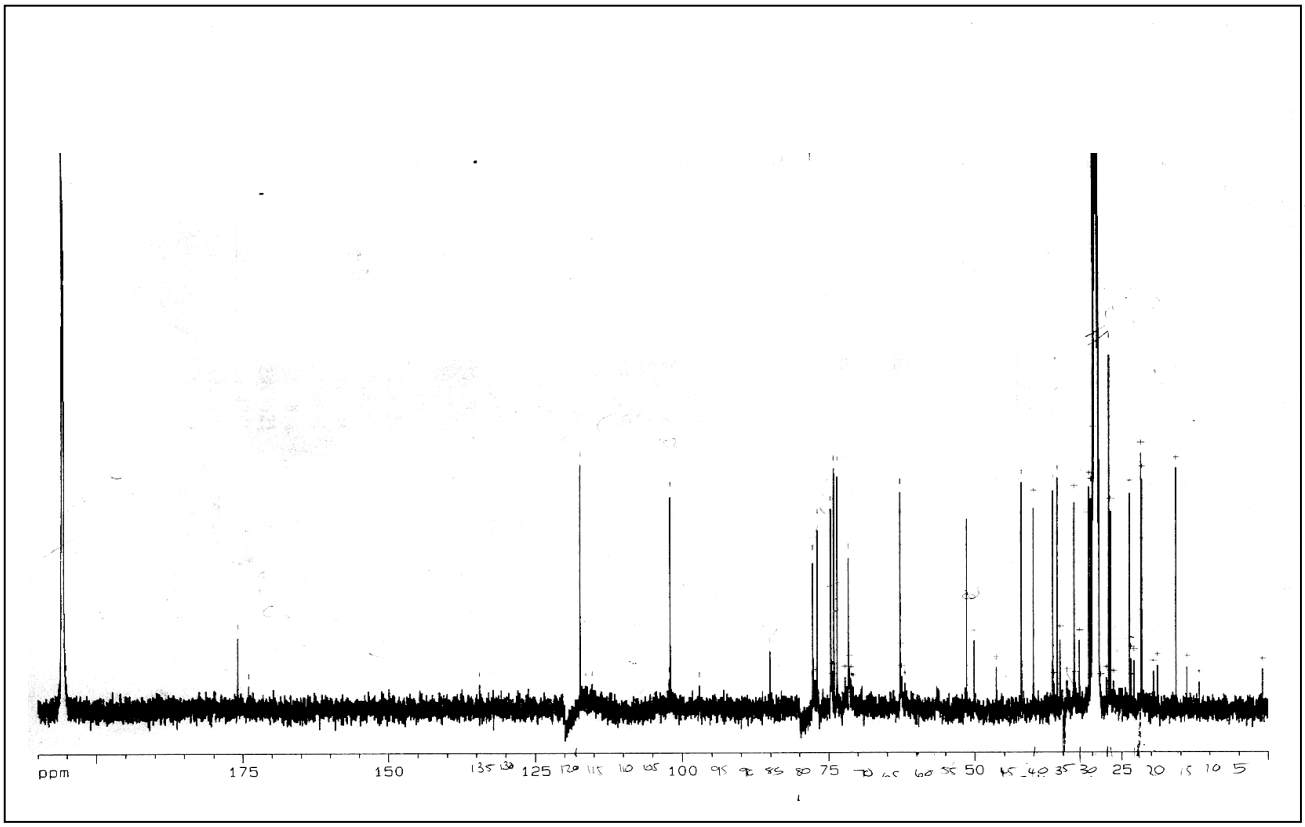


Figure 3.7 ^{13}C-NMR of digitoxigenin-glucoside in deuterated methanol.

Table 3.2 ^{1}H-NMR and ^{13}C-NMR values for digitoxigenin-glucoside in deuterated methanol.

C-atom	^{13}C-chemical shift	^{13}C-chemical shift Rathore *et al.*,1985	^{1}H-chemical shift
1	30.9	30.06	2.83, 2.6t
2	28.0	29.61	2.23, 1.84t
3	72.0	70.54	3.3d
4	34.0	35.20	2.23, 1.78t
5	36.8	36.64	1.7d
6	27.7	26.56	1.9, 1.27t
7	22.4	21.21	1.45, 1.28t
8	42.4	41.79	1.67d
9	36.9	35.74	1.8d
10	37.5	35.20	
11	22.5	21.28	1.81, 1.28t
12	41.0	40.03	1.5, 1.5t
13	52.0	49.63	
14	86.0	85.34	
15	31.3	33.08	1.45, 1.58t
16	27.8	26.92	1.9, 2.23t
17	50.0	51.03	2.85d
18	16.4	15.74	0.75q
19	24.1	23.62	0.95q
20	172.0	174.78	
21	75.8	73.44	4.9, 5.0t
22	117.7	117.50	
23	172.1	174.78	5.9d
1'	102.6	95.01	4.3d
2'	75.7	90.49	4.08d
3'	77	81.96	3.32d
4'	75.5	75.57	3.15d
5'	77	78.18	3.22d
6'	63.1	68.95	3.58, 3.79d

COSY is a two-dimensional NMR analysis showing the hydrogen atoms that are attached to specific carbon atoms. Hydrogen atoms in close proximity will be indicated for each carbon atom. The COSY spectrum of the isolated compound is given in Figure 3.8.

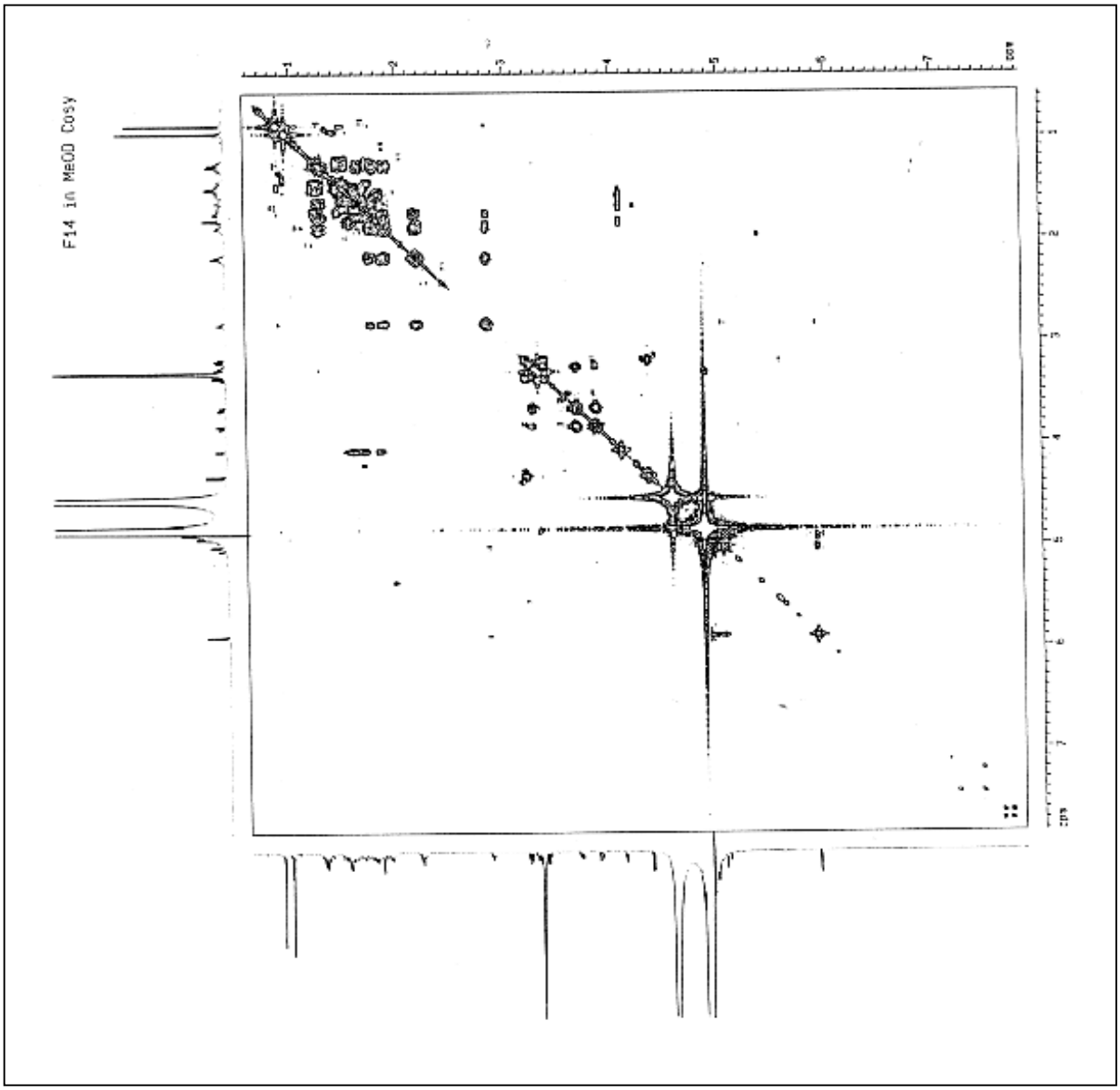


Figure 3.8 COSY analysis for digitoxigenin-glucoside in deuterated methanol.

HMBC spectra show the linked hydrogen and carbon atoms in the structure over short distances of one or two C-H bonds (Figure 3.9).

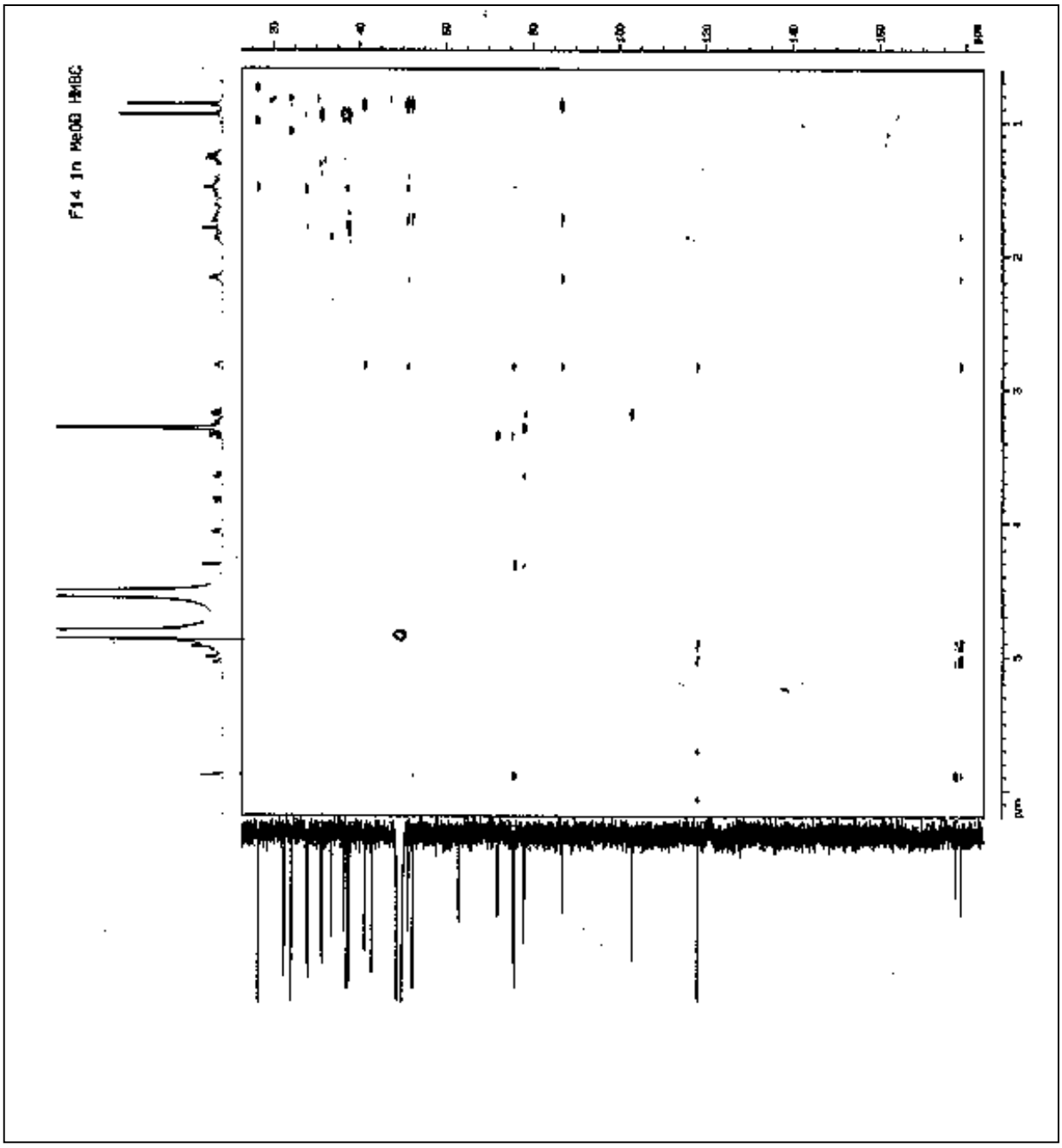


Figure 3.9 HMBC for digitoxigenin-glucoside in deuterated methanol.

The HMBC spectrum showed cross-peak connectivity between Me-18/C-17, C-13, C-12, C-14; Me-19/C-1, C-5, C-9, C-10; H-17/C-13, C-21, C-14, C-20, C-23; H-1`/C-3; CH_2-17/C-22, C-20, C-23; H-23/C-21, C-20, C-23. The cross-peak connectivity for digitoxigenin-glucoside is shown in Figure 3.10.

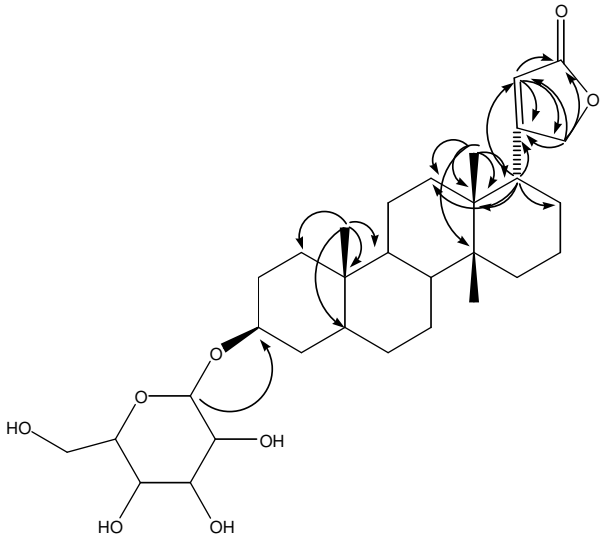


Figure 3.10HMBC cross-peak connectivity for digitoxigenin-glucoside.

The HMQC spectrum (Figure 3.11) indicates the linked hydrogen and carbon atoms in the structure over longer distances with more than two hydrogen atoms attached to a single carbon atom.

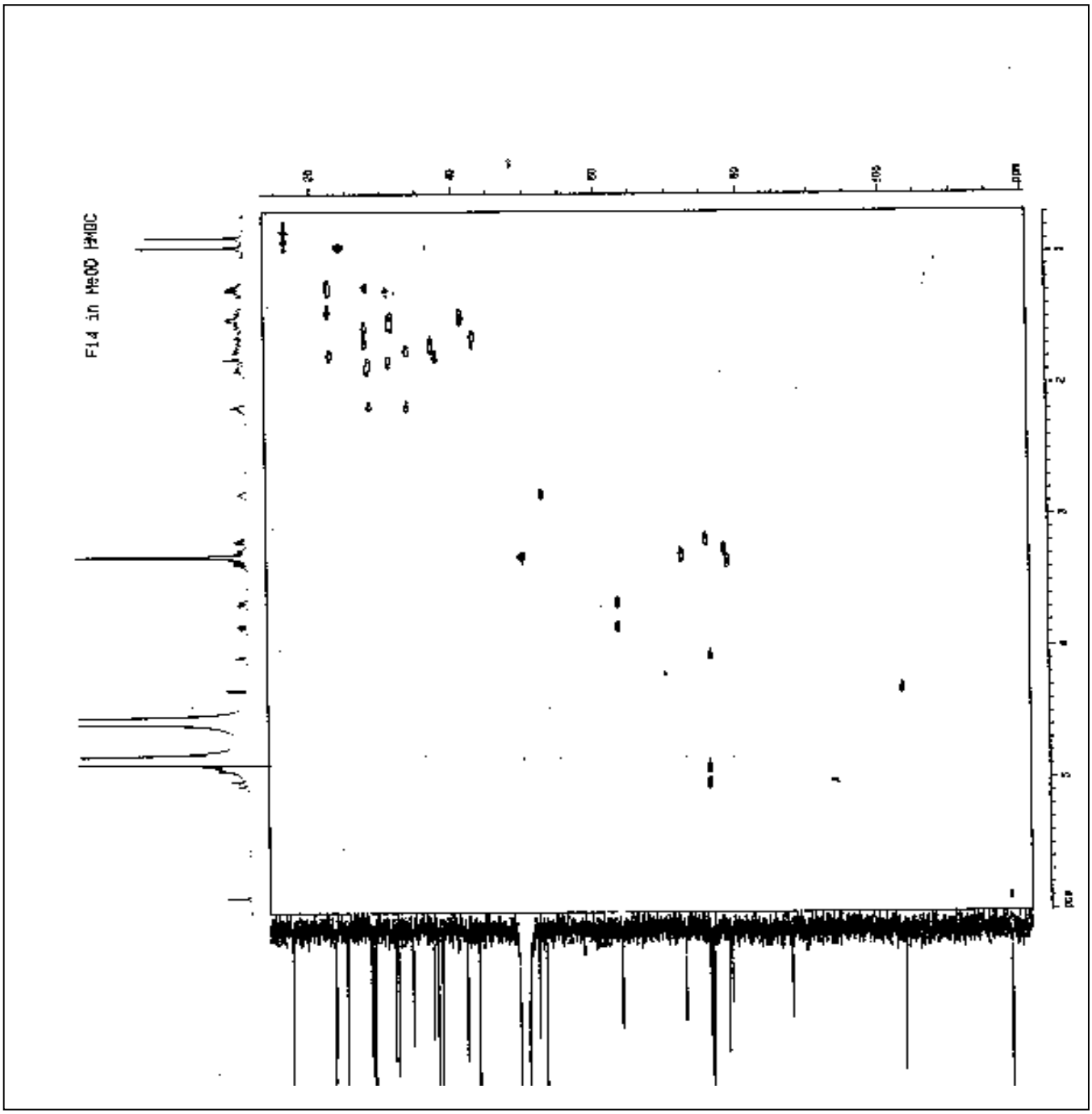


Figure 3.11 HMQC for digitoxigenin-glucoside.

3.4 Discussion

Bio-guided fractionation was used to identify and eventually isolate the active compound from the crude extract. From the twelve plant extracts that were prepared, only the extract from *E. croceum* showed promising results against the anti-HIV assays that have been used in the study.

The elucidation of the chemical structure of digitoxigenin – 3-O-glucoside was carried out using one- and two-dimensional NMR. The ^{1}H-NMR of digitoxigenin – 3-O-glucoside showed the presence of two singlet methyl groups at δ_H 0.89 and 0.92, and signals at δ 5.86 (t, J = 1.8 Hz), 9.83 (dd, J = 18.1, 1.8 Hz), 7.99 (dd, J = 18.1, 1.8 Hz) indicating the presence of an α, β – unsaturated lactone a doublet proton at 4.34 (d, J = 6.6) of β –orientation and an anomeric proton of the attached glucose. ^{13}C-NMR spectrum of digitoxigenin – 3-O-glucoside showed 29 carbons (2 methyls, 11 methylene, 11 methine and 5 quaternary carbons). Signals at δ_C 77.3, 77.0, 75.7, 75.8, 62.1 and 102.6 indicated the presence of a glucose molecule. According to the NMR data (Table 3.2, Figures 3.6-3.11), the compound was identified as digitoxigenin 3-O-glucoside (Figure 3.5), a known compound, which was isolated from *Digitalis lanata* (Singh & Rastogi, 1970) before, but with no medicinal uses related to its antiviral activity.

The compound forms part of the cardiac glycosides, which is known for its potent cardiac activity. These molecules are also known for their toxicity, and might explain the activity of the compound against HIV. It was therefore important to determine the toxicity of the compound and the extract. The toxicity results are discussed in Chapter five.

Chapter 4

Anti-HIV activity of *Elaeodendron croceum* and isolated compound

4.1 Introduction

Several anti-HIV tests have been used in this study to test for the anti-HIV activity of the extract and isolated compound obtained from *Elaeodendron croceum*. It is important to use a variety of tests as the active compounds may have different modes of action. A combination of different tests and a combination of different extracts increases the possibility to find an active compound for a certain microorganism. The assays used in this study tested the crude extracts, purified fractions and also the pure isolated compound digitoxigenin-glucoside.

Not all the assays showed equally good results, and the assays that showed the most promising results were the MT-2 VSV-pseudotype and the HeLa-Tat-Luc assays. The MT-2 VSV-pseudotype assay uses a recombinant virus, and does not test the enzymes or promoters of the virus. HeLa-Tat-Luc is an assay that tests the inhibition of transcription factors and it measures the transcription of the viral DNA. The other assays performed were the NF-kB and reverse transcriptase assays that showed no inhibition with the extracts or digitoxigenin-glucoside.

Reverse Transcriptase (RT) is an assay performed on one of the important enzymes HIV uses to produce DNA from the RNA that is found in the core of the virus. This enzyme reverses the normal process of transcription of DNA to make RNA that will be used to produce the useful protein products. This reverse process is very important in retroviruses as RNA needs to be incorporated into the host genome, and to achieve this DNA is needed as the genetic material in the nucleus, as the nucleus only consists of DNA. Once the viral DNA is produced in the cytoplasm, the DNA is transported

into the nucleus where another enzyme integrase (IN) will incorporate the viral DNA randomly into the host genome. Once this is achieved the viral DNA takes over all the functions of the nucleus, and the nucleus is now used as a "factory" to produce viral RNA to be transported to the cytoplasm. These RNA fractions will now be transcribed into proteins to form new viral particles (Mims *et al.,* 1999).

The RT enzyme is therefore a very important enzyme in the duplication process of viruses and the whole infection process of the host nucleus. If this enzyme is inhibited effectively, it could either stop the infection process or it could at least lower the infection process of the viruses into the host cells. Most anti-retroviral treatment (ART) medicines focus on these important processes to lower the reproduction process of the virus, and therefore decrease the viral load in the blood stream of the host.

NF-κB is an ubiquitous mammalian transcription factor. NF-κB plays an important role in the transcription of several genes, especially genes that encode for pro-inflammatory cytokines. Concurrent with NF-κB's role in inflammation is its influence in some cancer causing events such as transcribing of anti-apoptotic genes that promote cell survival activity. Inhibition of NF-κB has been postulated and tested as a means to target apoptotic events in cancerous tissue (Bremner *et al*., 2004). The Rel/NF-κB family of transcription factors are involved in different processes such as embryonic development, apoptosis control, regulation of inflammation and activation of the immune system. In addition NF-κB is the major inducible regulatory element involved in long terminal repeat (LTR) transactivation and HIV replication in CD4 lymphocytes. NF-κB is therefore an attractive target in diseases affecting the

immune system, since it is an important link in the pro-inflammatory response in mammals (Sancho *et al.,* 2004).

The HIV Tat protein is a strong transactivator of the viral LTR promoter by at least two mechanisms. The first one is mediated by Tat interaction with TAR, resulting in an overall 100-fold increase of the transcription rate, thus promoting RNA synthesis, protein expression and subsequent virion spread. TAR is a cis-activating stem-loop RNA structure called a transactivating response element present in the HIV-1 LTR promoter. Through interaction with TAR, Tat recruits a host cell protein kinase complex p-TEFb (CDK9 and CycT) that binds to the stem loop region of TAR. As a consequence of the p-TEFb recruitment to the HIV-1 promoter complex, the C-terminal domain (CTD) of the RNA polymerase II is phosphorylated, increasing the efficiency of transcription elongation. The second mechanism is based on the interaction of Tat with cellular transcription factors bound to the enhancer region of HIV-1 LTR. Accordingly, a functional interaction between Tat and NF-κB has been described (Sancho *et al.,* 2004).

The VSV pseudotype assay uses a recombinant virus, that makes it a very directed method to test HIV. Pseudotyped vectors can be used to introduce genes into cells or to study the entry process of the virus from which the outer shell of the recombinant virus is derived (Sanders, 2002).

Pseudotyping in its original sense means that one or more of the structural proteins of a virus particle are not encoded by the nucleic acid carried by the virus. Using this broad definition, pseudotyped viruses include any recombinant viral gene transduction

system that is important during genome packaging expressed by helper proteins from defective genomes in the viral producer cell. The common current usage in the gene transfer field dictates that a pseudotyped virus is one in which the outer shell originates from a virus that differs from the source of the genome and replication apparatus (Sanders, 2002).

Pseudotyped vectors have several experimental and clinical applications. The outer shell through its interaction with cellular receptor molecules plays a major role in determining the movement of the virus. These vectors may have an altered stability or interaction with the host immune system that increases its efficacy (Sanders, 2002).

4.2 Materials and Methods

4.2.1 Materials

The extracts were prepared and the compound isolated as described in Chapter 3.

4.2.2 Reverse Transcriptase

The reverse transcriptase colorimetric assay, takes advantage of the ability of reverse transcriptase to synthesise DNA, starting from the template/primer hybrid. Digoxigenin- and biotin- labled nucleotides in an optimised ratio are incorporated into the same DNA molecule, which is synthesised by RT. The detection and quantification of synthesised DNA as a parameter for RT activity follows a sandwich ELISA protocol: Biotin–labelled DNA binds to the surface of the microtiter plate (MTP) modules that have been precoated with streptavidin. An antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD) will bind to the digoxigenin-

labled DNA. After the peroxidase substrate ABTS is added, the peroxidase enzyme catalyzes the cleavage of the substrate, producing a coloured reaction product. The absorbance of the samples can be determined using a microtiter plate (ELISA) reader and is directly correlated to the level of RT activity in the sample (Roche Applied Science manual, 2004). The method is illustrated in Figure 4.1.

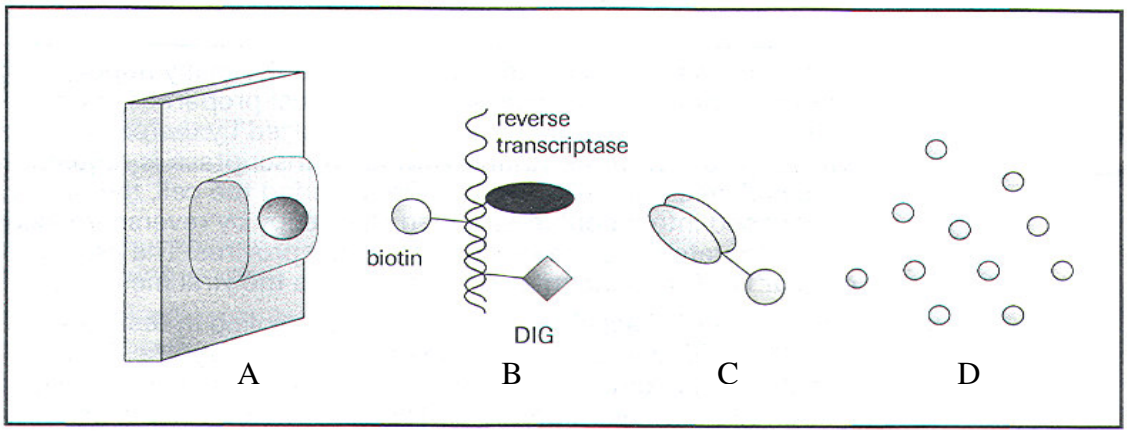


Figure 4.1 A=Streptavidin-coated MTP, B=DNA/RNA hybrid, biotin- and DIG-labeled by RT activity, C=Anti-DIG-POD Fab-fragment and D=POD substrate (ABTS).

The assay is nuclease sensitive, and the water is sterilised or autoclaved and redistilled. Heat-sterilised laboratory ware was used where possible. The HIV-1 RT was prepared by reconstituting the lyophilizate in 250 µl autoclaved redistilled water at a final concentration of 2 ng/µl. The reaction mixture was prepared by reconstituting the template in 430 µl of autoclaved redistilled water. 1ml of the incubation buffer was added per vial nucleotides and 100 µl of the reconstituted template was also added to each vial containing the diluted nucleotide solution. The lysis buffer is a ready to use mixture and the Anti-DIG-POD was prepared by reconstituting the lyophilizate in 0.5 ml autoclaved redistilled water. To prepare the

anti-DIG-POD working concentration, the reconstituted anti-DIG-POD was diluted with the ready to use conjugate dilution buffer to a final concentration of 40 ng/µl. The working dilution should be prepared immediately before use and should not be stored. A washing buffer was prepared by adding 225 ml autoclaved redistilled water per bottle washing buffer. The reconstituted solution is stable and can be stored. The ABTS substrate solution is prepared by dissolving the ABTS powder mixture in the bottle of substrate buffer and mixed by stirring (Roche Applied Science manual, 2004).

The assay was performed with an assay kit bought from Roche Applied Science. The instructions given in the assay were followed precisely, and three repetitions of each concentration and extract (6mg/ml) were used to ensure that the results were accurate.

4.2.3 NF-κB (Nuclear factor kappa B)

The NF-κB assay was performed in Spain at the University of Cordoba, according to method of Bremner *et al.,* (2004) explained below.

Cell maintenance

HeLa cells were maintained in Dulbecco's Minimum Essential Medium (DMEM-Invitrogen) supplemented with 10% foetal bovine serum and antibiotics (complete media). The cells were incubated at 37°C in a 5% CO_2 humidified atmosphere and split when confluent. The stable transfected HeLa cells were cultured in complete medium containing 100 µg/ml hygromycin B. The carrying solvent of 1% dimethylsulfoxide (DMSO) compared with the cells of the unstimulated controls had no effect.

Interleukin-6/luciferase (IL-6/Luc) assay.

HeLa cells were stably transfected with a luciferase reporter gene controlled by the IL-6 promoter. IL-6 is one of the target genes for activated NF-κB, therefore the luciferase produced can be measured as an IL-6 dependent measurement of the activation with a high light incidence or inhibition with a low light incidence of NF-κB.

Cells were washed with phosphate-buffered saline (PBS) and cleaned using trypsin/EDTA. The cells were then seeded in 12-well plates each well containing 1 ml of media incubated for 18-20 hours. Following this incubation, test samples were added to the cell media. Each sample was dissolved in DMSO to give a 10 µg/ml concentration. 10 ng of each sample was added to the wells to give a final concentration of 100 ng/ml. Cells were exposed to the compounds or plant extracts for 1hour, at which point the stimulant Phorbol 12-myristate 13-acetate (PMA) was added (PMA, 50 ng/ml, final concentration). The cells were incubated at 37°C for a further seven hours before cell harvesting.

100 µl of the luciferase lysis reagent was added to each well and left for 15 minutes to complete lysing of the cells. 15 µl from each well was then added to a 96-well plate in preparation for the automated reading of the luciferase reaction. An Anthos Lucy 1 luminometer/photometer was used to record the resulting luminometric readings. A luciferase assay system that consisted of the luciferase substrate and a luciferase buffer were used to dissolve the substrate. In each well, 50µl of the substrate was automatically added by Lucy 1 and the resulting luminometric reading recorded following a reaction time of 10s. Stimulated cells without a sample and resting cells

without stimulation were included as positive and negative controls to monitor assay consistency. Active samples were identified as being those with a reading that was 80% lower than the positive control value.

Luciferase assays in 5.1 cells

The cell line contains a plasmid in which the reporter luciferase gene is driven by a HIV LTR promoter and its responsiveness to the NF-κB activator cytokine TNF-α. To determine NF-κB dependent transcription of the HIV-1 LTR-luc, 5.1 cells were preincubated for 30 minutes with the compounds, followed by stimulation with TNF-α (2 ng/ml) for 6 hours. The cells were lysed in 25 mM Tris–phosphate at a pH of 7.8, 8 nM $MgCl_2$, 1 mM 1,4-dithio-DL-threitol (DTT), 1% Triton X-100, and 7% glycerol. The luciferase activity was measured using an Autolumat LB 953 (EG & G Berthold, USA). The readings were compared to non-stimulated cells and this represented the index of transactivation as the maximum levels of TFN-α-induced NF-κB activation (100%) (Sancho *et al.,*2004).

4.2.4 HeLa-Tat-Luc

HeLa-Tat-Luc contains the same reporter plasmid as 5.1 cells and the Tat gene is regulated by the Cytomegalo Virus (CMV) promoter. Therefore the HIV-1 LTR is 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 either treated with the CDK9 inhibitor DRB, as a positive control, or with three doses of the compounds tested. After 12 hours, the cells were washed twice with PBS and the luciferase activity measured as indicated for 5.1 cells (Sancho *et al.,* 2004).

4.2.5 VSV Pseudotype assay

The most common pseudotype protein for the HIV-1 vector is the vesicular stomatitis virus glycoprotein (VSV-G). The VSV-G pseudotype helps to stabilise the vector particles and broadens the tropisms of the vector since the receptor for VSV-G is a phospholipid (Heuntelman, 2003).

Recombinant virus assay

The recombinant virus assay (RVA) is a reliable and sensitive test to detect anti-HIV activity because of two main reasons:

- it evaluates direct viral replication in contrast to indirect protection of a cytopathic effect and
- the luciferase measurement provides a sensitive assay of HIV replication, because the test is performed in a single cycle of virus replication in 48 hours.

The latter is particularly important, because if only partial inhibition of HIV replication is achieved or the compound has a short half-life, the viral replication could occur easily in the classical MTT test (multiple cycles of viral replication produced in 7 days of culture).

The anti-HIV assay used was prepared by using a recombinant virus. These viruses were obtained by transfection of 293-T with the NL 4.3 Luc plasmid using the calcium phosphate method. 1 X 10^5 MT-2 cells were seeded in 96-well microtiter culture plates and were infected with the recombinant virus, previously titrated (100 000 RLUs/well). These cells were prepared in the presence and absence of the samples to be analysed at different concentrations, in a final volume of 200µl of

RPMI medium. The infected cells were incubated at 37°C in 5% CO_2. At 48 hours post-infection, the antiviral activity was assayed measuring luciferase activity of the infected cells with the Luciferase Assay System Kit with Reporter Lysis Buffer (Promega). Since the luciferase activity obtained is proportional to the infection rate, decreased activity shows the antiviral effect of the sample (Sancho *et al.,* 2004).

4.3 Results

The results of the four different tests that were performed namely the reverse transcriptase, NF-κB, HeLa-Tat-Luc and the VSV-pseudotype assays are given below. The active extract of *E. croceum* and the purified compound digitoxigenin-glucoside showed no inhibition in the reverse transcriptase or the Nf-κB assays. The HeLa-Tat-Luc and the MT-2 VSV-pseudotyped assays proved to be very successful with an inhibition of more than 82% in the HeLa-Tat-Luc assay and more than 85% inhibition in the VSV pseudotype assay. The other eleven extracts that were prepared had no inhibition on any of the four tests that were performed on them.

The following graphs show the results obtained of three semi-purified fractions and two pure compounds from *E. croceum* after separation on the silica gel column for the Hela-Tat-Luc, and the MT-2 VSV-pseudotype assays.

Figure 4.2 shows the results obtained from the HeLa-Tat-Luc assay. The expression of the DNA is given as a percentage. A control was included with 100% transcription. F1, F2 and F3 were fractions obtained from the column, F4 is pure

digitoxigenin-glucoside and F5 is an unidentified pure compound. The best results of approximately 85% inhibition was obtained with digitoxigenin-glucoside.

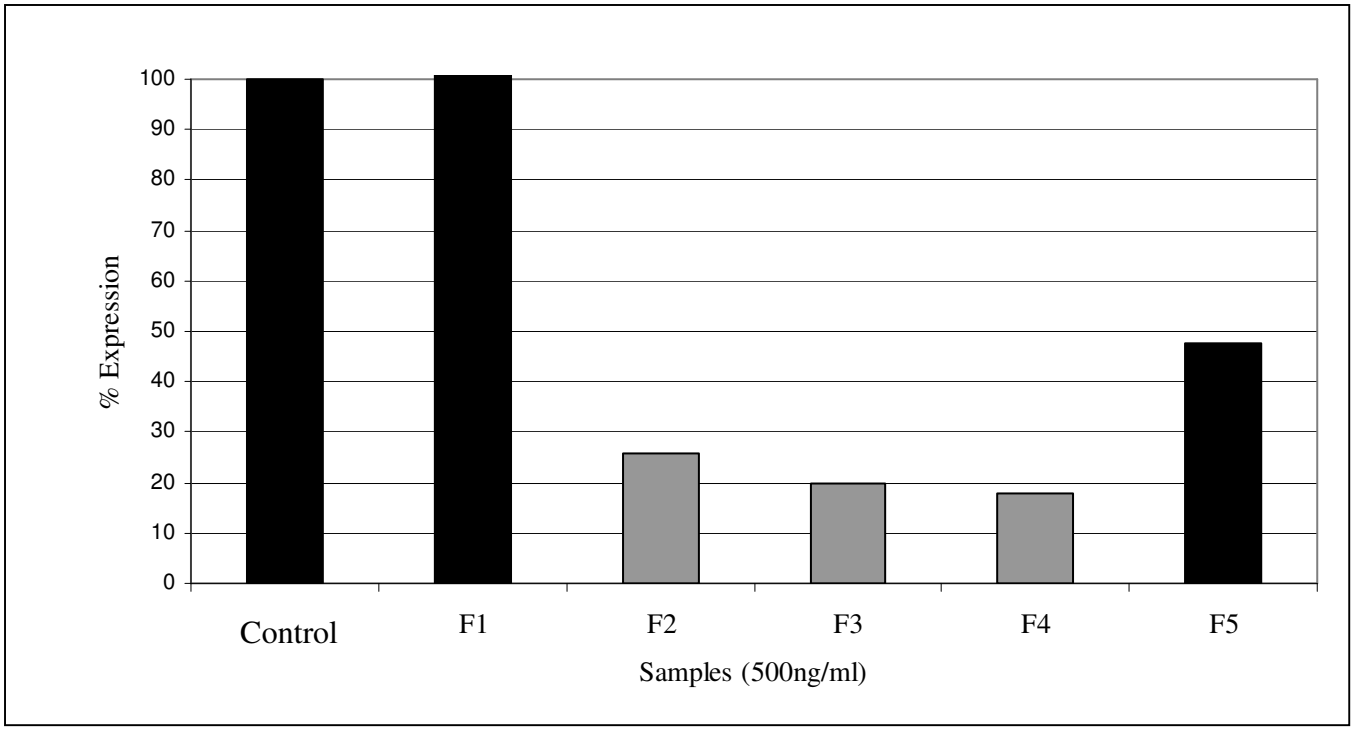


Figure 4.2 Graph showing the HeLa-Tat-Luc assay results. The first bar shows the control and F1-F3 indicates the silica gel column fractions and pure compounds F4 (digitoxigenin-glucoside) and F5 (pure unidentified compound) of the extract analysed.

Figure 4.3 shows the results obtained from the MT-2 VSV-pseudotype assay. The results are expressed as inhibition of the viral growth. The control indicates 100% growth of the recombinant virus. F2 and F3 are fractions obtained from the column, and F4 and F5 were pure compounds. F4 was the pure compound digitoxigenin-glucoside and F5 a pure unidentified compound. Inhibition of approximately 90% was obtained from digitoxigenin-glucoside at a very low concentration of 100 ng/ml.

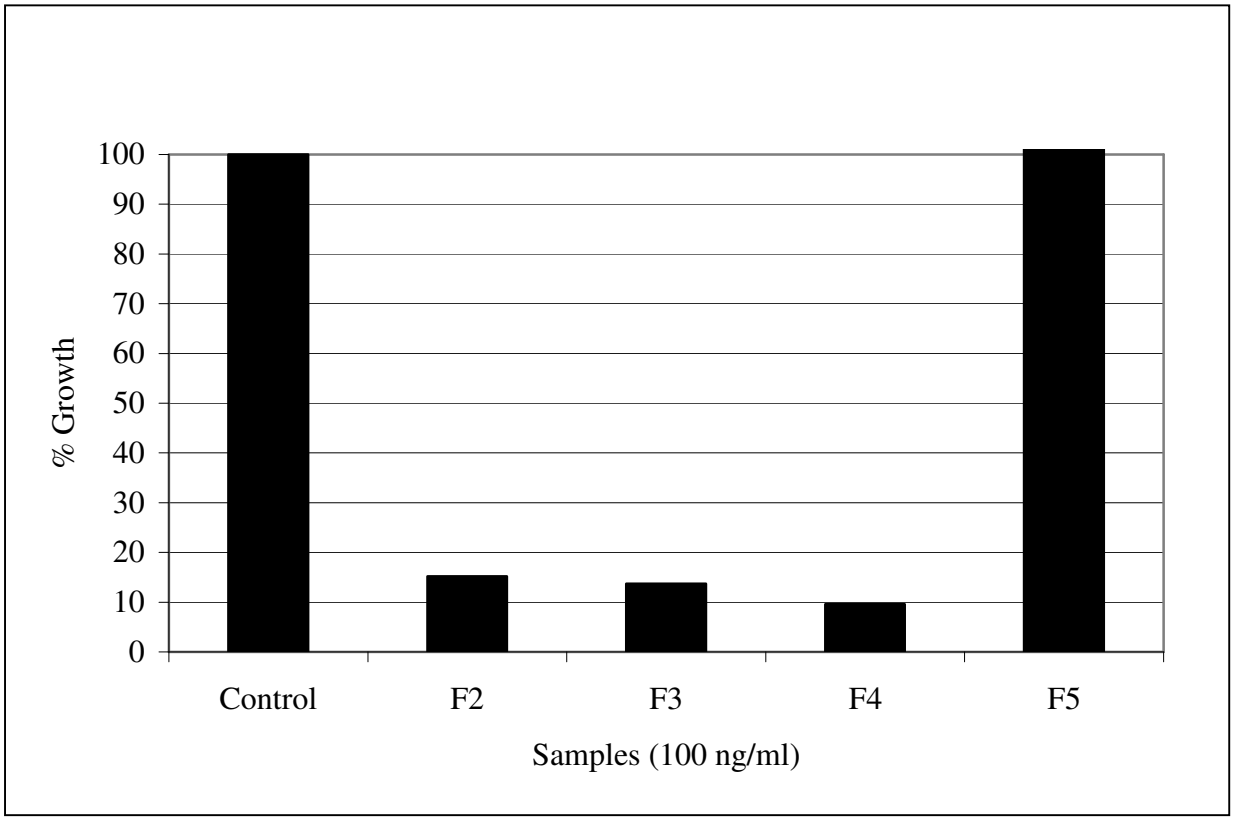


Figure 4.3 Graph showing the results from the MT-2 VSV-pseudotype assay. The first bar shows the control. F2 and F3 indicate the fractions and F4 (digitoxigenin-glucoside) and F5 (a pure unidentified compound) are compounds of the extract added to the assay.

4.4 Discussion

Four different assays against HIV were used do determine if the extract inhibited HIV. The differences in the results from the various tests emphasise the importance of performing different tests on a single sample to determine if all the possible mechanisms were tested.

The best results were obtained with the HeLa-Tat-Luc and the MT-2 VSV-pseudotype assays. The HeLA-Tat-Luc assay is an assay targeting transcription factors used for transcription during the infection process of the virus.

More than 85% inhibition was obtained with the pure compound digitoxigenin-glucoside at a concentration of 500 ng/ml. The other fractions showed inhibition as well, but at much lower levels than were shown for digitoxigenin-glucoside.

The MT-2 VSV-pseudotype assay is an assay using a recombinant virus, and not only certain enzymes or enzyme products. This is a more direct method to test compounds and extracts against a virus. The best results were obtained with this assay, with more than 90% inhibition on the growth of the virus with the use of digitoxigenin-glucoside at a concentration of 100 ng/ml. The semi-purified extract gave the same results against the virus, which also makes it a promising possibility to develop a treatment from the extract. Using an extract is easier, cheaper and faster than isolating a pure compound from the extract. It might be possible that the extract could be prepared as a tea and its use could be very accessible.

The activity of digitoxigenin-glucoside was very high in both the HeLa-Tat-Luc assay and the MT-2 VSV-pseudotype assay. It is however a cardiac glycoside of which many are toxic compounds. The active compound could therefore also be a toxic compound that effectively kills the virus, but also the living cells?

Chapter 5

Toxicity of *Elaeodendron croceum* extract and isolated compound

5.1 Introduction

It is very important to determine the toxicity of an extract or compound if it shows promising *in vitro* results against a pathogenic microorganism. The isolated compound and extract from *Elaeodendron croceum* showed good anti-HIV activity, but it is possible that its activity is due to its toxicity. The cardiac glycosides are very toxic compounds in general, and its use needs to be controlled very strictly (Altman *et al.,* 1988). Examples of the cardiac glycosides include uzarin, scillarin A, digoxin, digitoxin and uzarigenin. The toxicity tests were performed on VERO cells, used to indicate the general toxicity of a compound or extract. Digitoxigenin-glucoside and the semi-purified extract were tested at several concentrations.

5.2 Materials and methods

5.2.1 Preparation of the Minimal Essential medium (MEM)

The toxicity screen was performed on Vero cells that were cultured in minimal essential medium (MEM). This medium was prepared by adding 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 penicillin, 10 µg/ml streptomycin, 0.25 µg/ml fungizone and 10% fetal bovine serum to 800 ml distilled water. After all the components were mixed, the pH was adjusted to 7.4 with HCl. The medium was then filter-sterilised through a 2 µm filter by vacuum-filtration, divided into 250 ml aliquots, and stored at 4°C. This medium was then used to culture the cells at 37ºC in a humidified atmosphere with 5% CO_2. Cells were subcultured in a 1:6 ratio every second to third day after trypsinisation of confluent cultures (American Tissue Culture Collection).

5.2.2 Preparation of cells for toxicity screen

On day 0, confluent cultures were trypsinised and diluted in complete MEM to a concentration of 1×10^5 cells/ml. In the outer wells of a 96-well plate(s), 200µl of medium was dispensed. All inner wells received 100µl (1×10^4 cells) of the cell suspension. The plate was incubated overnight at 37ºC in a humidified atmosphere with 5% CO_2 (Sigma-Aldrich cell culture manual, 2005-2006).

5.2.3 Preparation of crude extract and pure compound

On day 1, stock solutions of the crude extract and pure compound were prepared in DMSO at 20 mg/ml. For the crude extract, 40 µl of the DMSO stock were added to 1960 µl medium to obtain a final volume of 2 ml. 1 ml of this mixture was added to the next tube with 1 ml medium and it was mixed properly, and the procedure is repeated. The final concentrations of the crude extract dilutions were: 0.195, 0.391, 0.781, 1.563, 3.125, 6.25, 12.5, 25, 50, 100, 200 and 400 µg/ml. For the pure compound, 20 µl of the DMSO stock were added to 1980 µl medium to obtain a final volume of 2 ml. 1 ml of this mixture was added to the next tube with 1 ml medium and it was mixed properly, and the procedure is repeated. The final concentrations of the pure compound dilutions were: 0.098, 0.195, 0.391, 0.781, 1.563, 3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/ml. A DMSO control was prepared by adding 20 µl DMSO in 2 ml complete medium. The plate with cells was transferred from the incubator to the laminar flow hood. 100 µl of each extract or compound dilution were added in triplicate to 100 µl of cells in the inner wells. DMSO and a growth medium control were also included. The plates were incubated for 3 days in the incubator.

On day 4, enough XTT reagent for all the plates were prepared. For each 1 ml XTT, 20 µl PMS and 50 µl of XTT reagent were added to each well. The plates were placed back in the incubator and left for 1 hour. If the colour development was not intense after 1 hour, it was incubated for longer. The contents of the wells were briefly mixed with a multi-channel pipette. The plates were read with the KC Junior program by selecting the "XTT assay" protocol. The outlay of the microtitre plate is given in Figure 5.1.

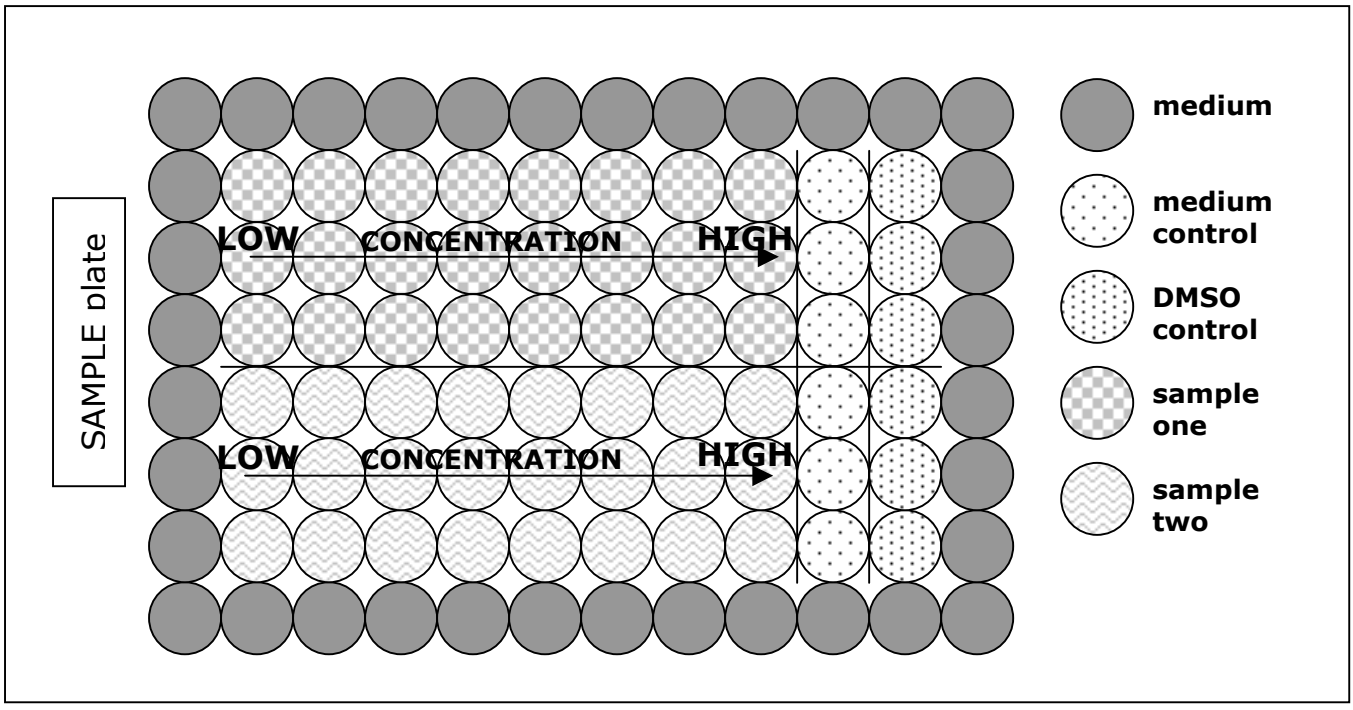


Figure 5.1 Outlay of the microtitre plate for toxicity analysis on VERO cells.

5.3 Results

Although the plant species and the cardiac glycosides are well known for their toxicity (Altman *et al.,* 1988), the toxicity of the extract and isolated compound were well below the anti-HIV active concentration. It seems as if the specific structure of the isolated compound is responsible for the decrease in its toxicity. The isolated compound contains a single glucose moiety where most of the other cardiac glycosides contain several sugar moieties. The type of sugar moiety also play an important role in the activity and toxicity of the compound.

Toxicity for the crude extract, semi-purified extract and the pure compound is shown in Figures 5.2-5.4.

The crude extract showed very low toxicity to the Vero cells. The toxicity of all the concentrations showed toxicity less than 20% of the control and well below the active concentration of 100 ng/ml.

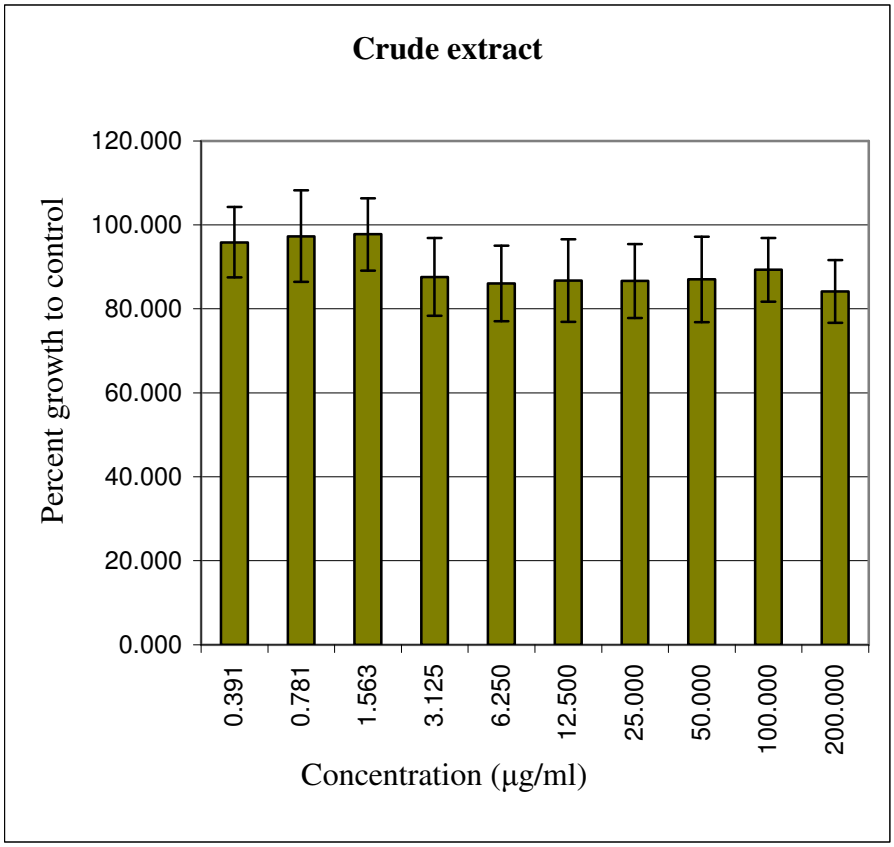


Figure 5.2 Toxicity of the crude ethanolic water (8:2) extract.

The semi-purified extract also showed very good results in the toxicity tests. Concentrations of 0.391 µg/ml to 50 µg/ml showed no toxicity on the VERO cells. At a concentration of 100 µg/ml there was a drop to 80 % growth compared to the control and at 200 µg/ml a further decrease to 50%. The active concentration of the semi-purified extract is 100 ng/ml and the toxicity to the cells is 0% at this concentration. The therapeutic index is therefore 500 at a concentration 50 µg/ml where there were no toxicity. It would seem as if the most toxic compounds had been removed from the crude extract by the process of liquid-liquid fractionation with chloroform. The results for the semi-purified extract are given in Figure 5.3.

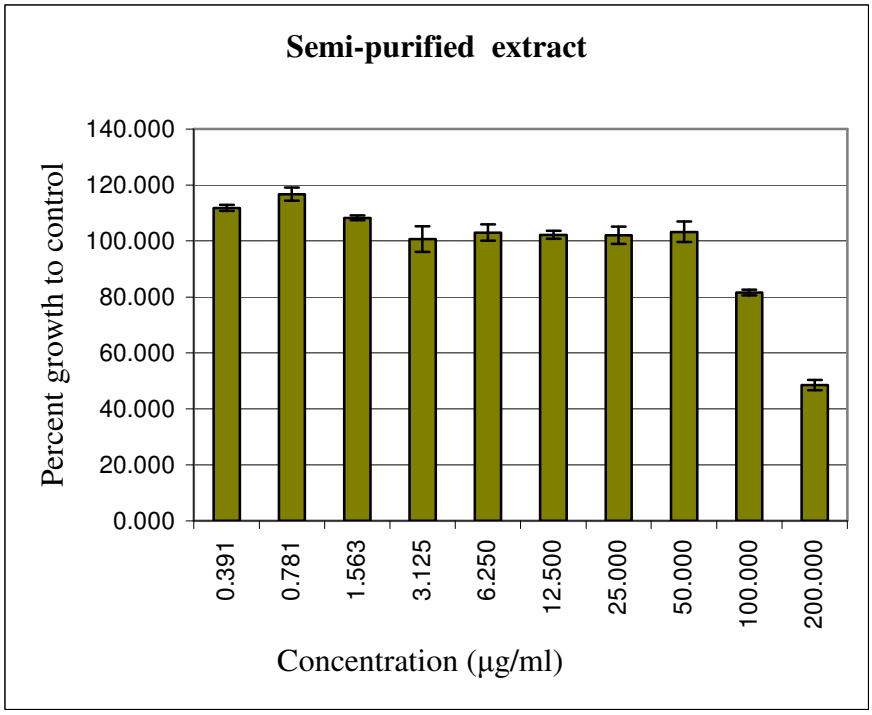


Figure 5.3 Toxicity of the semi-purified extract.

The pure compound was more toxic than the crude extract in toxicity as was expected. For concentrations of 0.195 µg/ml to 25 µg/ml the toxicity compared to the control is still very good with toxicity between 10% and 20%. At concentrations of 50 µg/ml and 100 µg/ml the toxicity slightly increased to approximately 25 % (Figure 5.4). The therapeutic index at a concentration of 25 µg/ml is therefore 250, before the toxicity control drop below 20%.

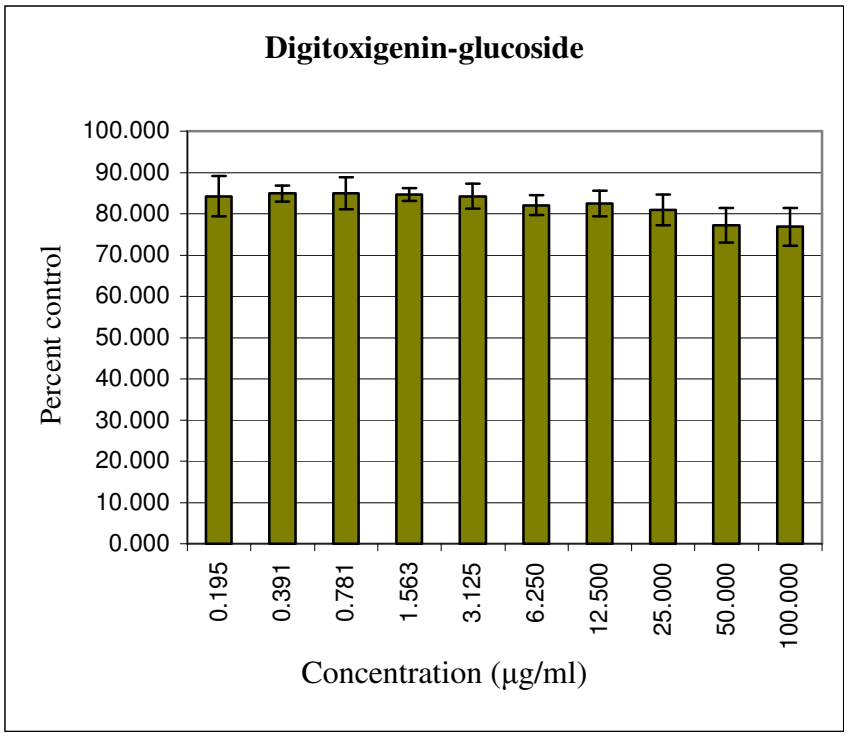


Figure 5.4 Toxicity of digitoxigenin-glucoside.

5.4 Discussion

The toxicity results of all the samples tested were satisfactory for most of the concentrations having toxicity of less than 20%. The crude extract showed very good toxicity results with all the concentrations showing toxicity of less than 20%. The extract therefore has the potential to be developed a medicine, as it seems to have a low toxicity on the VERO cells.

The semi-purified extract showed the lowest toxicity values for concentrations below 100 µg/ml having 0% toxicity. It would seem as if the most toxic compounds were removed from the extract by liquid-liquid fractionation with chloroform. The semi-purified extract would seem to have potential as a medicine against HIV when

considering the *in vitro* toxicity results against VERO cells. The active concentration of 100 ng/ml is much lower than the toxic concentrations of 100 µg/ml. There is a therapeutic index factor of 1000 between the concentrations which would make the extract an attractive target to be developed as a medicine.

The pure compound seemed to be the most toxic of all the samples, with toxicity of 25% at 100 µg/ml. When the concentration is increased, the toxicity increased slowly from 15 % at a concentration of 0.195 µg/ml until it reached 25% toxicity at a concentration of 100 µg/ml. The active concentration of the compound against HIV is much lower at 100 ng/ml with an inhibition of approximately 90% of the recombinant virus. The therapeutic index factor difference of 250 makes it a promising possibility to be studied further for the compound to be used as a drug.

The compound could potentially be produced synthetically in the laboratory by the Königs-Knorr condensation reaction, by partial hydrolysis of digitoxigenin, rather than isolating the compound from the plant material (Kawaguchi *et al.,* 1989). The *in vitro* toxicity level proves to be quite low at the active concentration of the compound, and the concentration can even be increased to have a higher inhibition than 90% against the recombinant virus.

The use of the semi-purified extract as a medicine, could be considered as well because the active compound will be used with the extract, and it potentially decrease the risk of side effects. The extract is not toxic at any of the concentrations up to 50 µg/ml.

Preparation of the extract could be easily done with minimal apparatus, and the active concentration of the extract is much lower than the toxicity concentration of the extract. Even if the concentration of the extract is increased a thousand times, the extract would still not be toxic in terms of toxicity against VERO cells.

It can therefore be concluded that the semi-purified extract and digitoxigenin-glucoside could be possibly used safely against HIV. These results were however al obtained *in vitro*, and still need to be explored *in vivo*. There is a large margin between the active concentration and the concentrations where the extract and the compound become toxic to the cells. The next step would be to test and determine the side effects and secondary effects of the extract or the compound on cells, to secure the safety of the use as a medicine or drug.

Chapter 6

Review on digitoxigenin-glucoside and related cardiac glycosides

6.1 Introduction

The virtues of foxglove (*Digitalis purpurea),* against a disease called dropsy, were already documented by Withering in 1785. Withering investigated this plant, and only after a decade of investigations published the results of his findings (Withering, 1785) showing that foxglove could be used to treat dropsy. Dropsy was known as an ailment characterised by swelling of the limbs and torso, which we know today is due to inadequate pumping action of the heart (Balick & Cox, 1996). The retention of fluid that swelled the dropsy patient's body was clearly alleviated by administration of foxglove, but the connection between dropsy and inadequate pumping action of the heart was not properly understood in Withering's day. Withering observed that foxglove "has the power over the motion of the heart, to a degree yet unobserved in any other medicine". Withering foresaw that "this power may be converted to salutary ends". He began prescribing foxglove for cases of dropsy, but gave it in doses much too large. He also discovered that standardising of the dosage was important. He found that the dose vary considerably in the plant during different seasons of the year, and found that the dose could be controlled by gathering and drying the leaves at late flowering. He soon began prescribing leaf infusions and later ground powdered leaves. His administration was astonishingly successful in the treatment of dropsy (Balick & Cox, 1996).

Powdered foxglove leaf is still prescribed in tablet or capsule form to treat congestive heart failure. *Digitalis* has been affixed to this crude drug as well as to the cardiac glycosides isolated from foxglove in the early twentieth century. These compounds are named after their powerful action on the heart. They increase the force of heart

contractions and allow the heart more time to rest between contractions. More than 30 cardiac glycosides have been isolated from dried foxglove leaves, including digitoxin and digoxin (Balick & Cox, 1996).

The compound isolated during this study from *E. croceum,* digitoxigenin-glucoside, was first isolated from *Digitalis lanata* (Humber *et al.,* 1983) and falls in the class of the cardiac glycosides with a structure that is very similar to digoxin and digitoxin. The compound shows however less toxicity than what is generally found with the cardiac glycosides. The medicinal uses of this compound are restricted to its cardiotonic activity (Humber *et al*., 1983). The *in vitro* inhibitory activity of this compound against HIV as described in this study could be a new application for the cardiac glycosides. This chapter highlights the important facts of the isolated compound, but also give some interesting information on the toxicity and medicinal uses of the group of cardiac glycosides.

The structural similar compounds that form the cardiac glycosides owe their name to their biological activity which is mainly the increase in the contractibility force of the heart by inhibiting the enzyme Na^+, K^+-ATPase. The enzyme is the only receptor for the cardiac glycosides and is responsible for the active extrusion of intercellular Na^+ in exchange for extracellular K^+. Digoxin and digitoxin are the two most widely used digitalis inotropes with an estimated two million patients receiving these cardiac glycosides in the USA. The acute toxicity of cardiac glycosides is due to their arrhythmogenic action causing cardiac arrest (Steyn & Van Heerden, 1998).

These cardiac glycosides have been mainly isolated from *Digitalis* spp. which is commonly named the foxgloves or purple foxgloves from the family Scrophulariaceae. The compounds from this species exhibit a therapeutic dose which is close to the toxic dose which causes anorexia, nausea, salivation, vomiting, diarrhoea, headache, drowsiness, disorientation, delirium, hallucinations and death may result. Due to their exceedingly narrow therapeutic index, digoxin and cardiac glycosides in general, are among the most hazardous drugs in routine use (Budavari *et al.,* 1989).

6.2 Digitoxigenin – 3-O-glucoside

The isolated compound (Figure 6.1) is a derivative of digitoxigenin and was isolated previously from *Digitalis lanata* (Humber *et al.,* 1983). The compound has a similar structure to the well-known compounds such as digoxin and digitoxin. Several derivatives and variants of these compounds are found, with varying biological uses and toxicity.

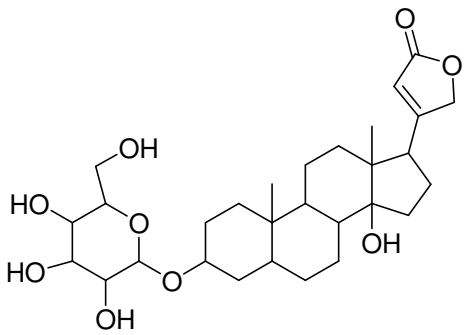


Figure 6.1 Digitoxigenin–3-O-glucoside.

Digitoxigenin-glucoside can be prepared from digitoxigenin with the Königs-Knorr condensation reaction, by partial hydrolysis of digitoxigenin. Digitoxigenin can be bought from Aldrich: D10320-9, Fluka: 37020 & Sigma: D5753. It has also been synthesised by cell cultures from *Digitalis purpurea* by feeding the cultures digitoxigenin (Kawaguchi *et al.,* 1989).

Another characteristic of the cardiac glycosides is that their cardiac activity and toxicity increase with an increase in the number of monosaccharides. The conformational distribution of the glycosidic moiety was postulated to be the major determinant of the biological activity of these cardenolides. The steroid aglycone provides the major part of the binding energy to the receptor, whereas the glycoside portion plays a secondary role in stabilising the cardenolide receptor complex (Steyn & Van Heerden, 1998).

Digitoxigenin-glucoside only contains one glucose molecule in contrast to three monosaccharides in digitoxin and digoxin. It is therefore expected to be less potent but lower in toxicity. It has also been found that the toxicity and lipophilicity are related in activity. When the hydrophilicity increases with the increasing number of monosaccharides, the toxicity will increase as well (Biagi *et al.,* 1991).

Digitalis and its preparations are known to have positive inotropic effects on the heart muscles. Digitoxigenin-glucoside were effective at $2x10^{-7}$M drug concentration (Takiura *et al.,*1974) and it was found that digoxin (0.112 µmol/kg) and digitoxigenin-glucosides (0.056 µmol/kg) produced similar increases in myocardial contractibility. Digitoxigenin-glucoside was also faster in onset of action, but had a shorter duration

of action. The shorter onset and duration of action may be due to more rapid drug-receptor association followed by rapid equilibrium of the drug with other tissues and fluid compartments. The results suggested that the rapid onset and short duration of the effect are a function of the glucose moiety. It was reported that the rapid onset and what appears to be a reduced tendency to accumulate may confer clinical potential for these analogues such as digitoxigenin-glucoside (Altman *et al.,* 1988).

Low acute toxicity (LD_{50}) in mice and high inotropic potency in guinea-pigs of digitoxigenin-glucoside prompted more studies on its cardiovascular effects. After a transient fall in contractibility during the infusion of digitoxigenin-glucoside, the contractibility increased relatively rapid to peak at 45 minutes, and declined to pre-treatment levels at 180 minutes. The hypertensive response was significantly greater than digoxin and was followed by a hypotensive response which reached a peak at 60 minutes. The heart rate was slightly reduced at time of infusion (Altman *et al*., 1988).

Radioactivity is used to determine the metabolism of the compounds after infusion. For digitoxigenin-glucoside the total plasma radioactivity was complete at 30 minutes and 60 minutes for digoxin. Digitoxigenin-glucoside appear to undergo fairly rapid metabolism when compared to digoxin (Altman *et al,*. 1988).

6.3 Related cardiac glycosides

6.3.1 3,14-Dihydroxycard-20(22)-enolide (digitoxigenin)

3,14-dihydroxycard-20(22)-enolide is the basic structure of a variety of compounds related to the cardiac glycosides. All the variants resemble the same structure as shown in Figure 6.2. Several variants of this compound are found with digitoxigenin one of these variants.

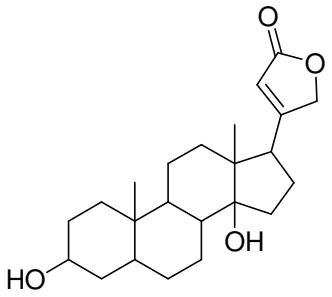


Figure 6.2 3,14-dihydroxycard-20(22)-enolide.

Digitoxigenin has been isolated from several plant families including Scrophulariaceae, Asclepiadaceae, Apocynaceae and Periplocaceae. The compound has been used as a cardiotonic agent with a LD_{50} (mus, oral) of 26.2 mg/kg. Other variants of this compound include urizeginen, 3-epiuzarigenin, 3-epidigitoxigenin, allouzarigenin and uzarigenin that is also a cardiotonic agent (Dictionary of Natural Products, 2005).

The LD_{50} of the compound has been determined for digitoxigenin at 36 nmole/10 g weight. The LD_{50} values are at least several times as high as those of the digitose series (Takiura *et al.,*1974).

Guinea pig atrial studies showed that the configuration of the A/B ring junction did not influence the cardiotonic activity of digitoxigenin significantly. The substitute groups showed a significant influence on the cardiotonic activity. Glucosidation decreased the potency of uzarigenin a variant of digitoxigenin by 63%. Conjunction with rhamnose increased the potency of both (Brown & Thomas 1984).

It was found that the sugar residue which is found in digitoxigenin-glucoside has a profound influence on inotropic potency on isolated left atrium of the guinea pig. Digitoxigenin-glucoside was 2.8 times more potent than digitoxigenin. Digoxin with three sugar residues was 2.1 times as effective as digitoxigenin. Digitoxin was however 8.8 times as effective as digitoxigenin (Brown *et al.*, 1981).

The derivatives of digitoxigenin are given in Table 6.1 with the toxicity and medicinal uses indicated for those that have been studied. The isolated digitoxigenin-glucoside is one of these derivatives and is also listed in the table.

Table 6.1 Derivatives of digitoxigenin, their uses and toxicity. Gpg = guinea pig, orl = orally and ivn = intravenous (Dictionary of Natural Products, 2005).

Name	Toxicity	Uses
Beaumontoside	It is very toxic intravenous (no values provided)	Cardiotonic agent
Cerberin	LD_{50} (cat, ivn) of 0.147 mg/kg	Cardiotonic agent
Digitoxigenin digitaloside	LD_{50} (cat, ivn) of 0.2 mg/kg	-
Digitoxigenin-glucoside	LD_{50} > 310 nm/10g body weight	Cardiotonic agent
Echujin	Highly toxic: LD_{50} (cat) of 0.3 mg/kg	It has been used as arrow poison
Evomoside	LD_{50} (cat, ivn) of 0.278mg/kg	-
Glucodigifucoside	It is very toxic intravenous	-
Lanatoside	Toxicity of LD_{50}(gpg, orl) 100 mg/kg and LD_{50} (rat, ivn) of 16 mg/kg	Cadriotonic agent
Neriifolin	LD_{50} (cat, ivn) of 0.2 mg/kg	-
Purpureaglycoside	Toxicity of LD_{50} (cat, ivn) 0.33 mg/kg	Cardiotonic agent
Ramnodigin	-	Cardiotonic agent
Solanoside	It is very toxic intravenous	-
Somalin	-	Antineoplastic agent
Vallaroside	Very toxic by intravenous route	Cardiotonic agent
Wallichoside	-	Cardiotonic agent

6.3.2 Digoxin

The compound digoxin (Figure 6.3) was extracted from *Digitalis lanata*, and the biological uses include inotropic activity and it is also a cardiotonic agent. This compound is well known and currently used as a drug to increase adequate pumping of the heart. Some of the commercial names used for this drug include Cordioxil, Davoxin, Digacin, Dilanacin, Dixina, Dokim, Dynamos, Lanacordin, Lanicor, Lanoxin, LenoxiCaps, Lenoxin, Longdigox, Neo-Dioxanin, Rougoxin, Stillacor and Vanoxin (Budavari *et al.,* 1989).

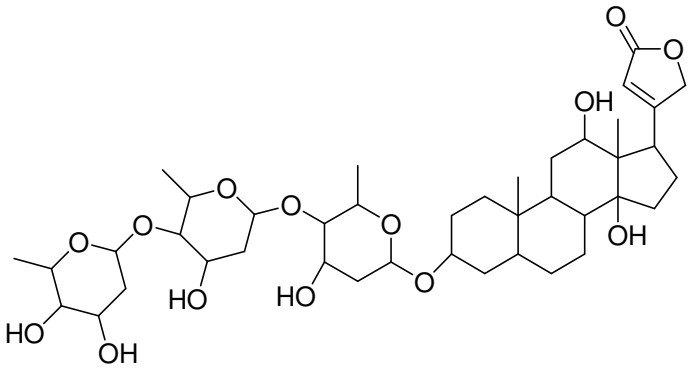


Figure 6.3 Digoxin.

6.3.3 Digitoxin

Digitoxin (Figure 6.4) is a structural derivative of digoxin and the compound was extracted from several plants including *Digitalis purpurea, D. lanata* and other *Digitalis* species (Humber *et al.,* 1983). The biological uses for the plant include inotropic activity and it is also a cardiotonic agent. The compound is currently used as a drug to improve the pumping action of the heart and some of the commercial names include Digitalin, Asthenthilo, Cardigin, Carditoxin, Coramedan, Digicor, Digilong, Digimed, Digipural, Ditaven, Digisidin, Lanatoxin, Myodigin, Purodigin, Tardigal and Unidigin (Budavari *et al.,* 1989).

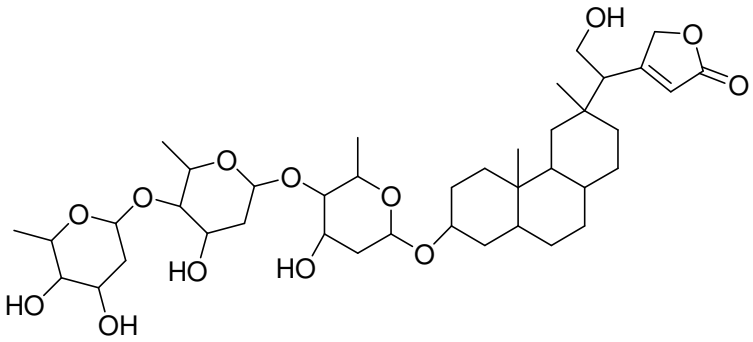


Figure 6.4 Digitoxin.

6.3.4 Actodigin

Actodigin (Figure 6.5) is a synthetic isomer of digitoxigenin-glucoside with a modified lactone ring (Brown *et al.*, 1981). It seems as if the biological properties of this molecule arise because the isomeric lactone alters the drug-receptor interaction in such a way that the sugar portion is directed away from the sugar-binding site on the receptor (Brown *et al.*, 1981).

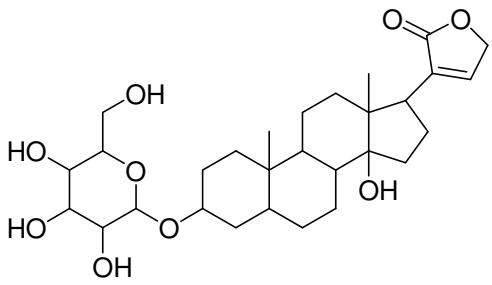


Figure 6.5 Actodigin.

Studies performed on actodigin's genin shows that the genin's ability to inhibit Na^+/K^+-ATPase can be largely explained by its lactone carbonyl oxygen position and molecular conformation (Cheung *et al.,* 1981). The D ring of actodigin was found to be a half chair; unlike the natural digitalis D rings like digitoxigenin-glucoside which exist in an envelope. The glucose make however an unexpectedly large contribution to the activity. Table 6.2 shows the I_{50} values of the Na^+/K^+-ATPase activity of actogdigin, its genin, digitoxigenin and its glycoside.

Table 6.2 I_{50} of selected cardiac glycosides on Na^+/K^+-ATPase activity.

Steroid	**I_{50} without preincubation (M)**	**I_{50} with 10 minutes preincubation (M)**
Actodigin	1.0×10^{-6}	1.0×10^{-6}
Actodigin genin	7.0×10^{-5}	7.0×10^{-5}
Digitoxigenin	3.5×10^{-7}	3.5×10^{-7}
Digitoxigenin-glucoside	3.1×10^{-7}	1.3×10^{-7}

The data show the remarkable effect of glucose on actodigin's Na^+/K^+-ATPase inhibiting potency. Actodigin is 70 times more active than its genin. In contrast, digitoxigenin-glucoside is only slightly more active than its genin digitoxigenin. The glucose of actodigin is having a much greater effect on its Na^+/ K^+-ATPase inhibitory activity than the glucose of digitoxigenin-glucoside. This confirms that binding of a glycoside to the Na^+/K^+-ATPase proceeds in two steps: first binding of the genin to the enzyme, and second binding of the sugar to the enzyme (Cheung *et al.,* 1981).

6.3.5 Glycyrrhizic acid

The compound glycyrrhizic acid (Figure 6.6) is not part of the group of cardiac glycosides, but is included in the review because of its anti-HIV activity, and its general structure shows correlation with the cardiac glycosides. The compound has been tested as a long-term treatment and as a combination therapy with AZT or DDI on HIV-1 carriers (Ikegami *et al.,* 1996). The efficacy of this compound as an oral monotherapy (150-225 mg/day) for 5 to 10 years and as a combination therapy with AZT or DDI on HIV-1 carriers was evaluated on the viral RNA levels in plasma samples. Patients who started the monotherapy at an early stage had low or undetectable levels.

Other biological uses include its anti-inflammatory activity and its antihaemorrhagic activity. It has adverse effects of hypermineralocorticoidism that result from intake. The toxicity for the compound is LD_{50} (rat, orl) 3000 mg/kg and LD_{50} (rat, ipr) 2000 mg/kg.

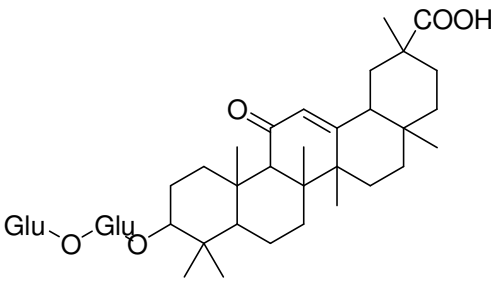


Figure 6.6 Glycyrrhizic acid (Glycyrrhizin).

6.4 Discussion

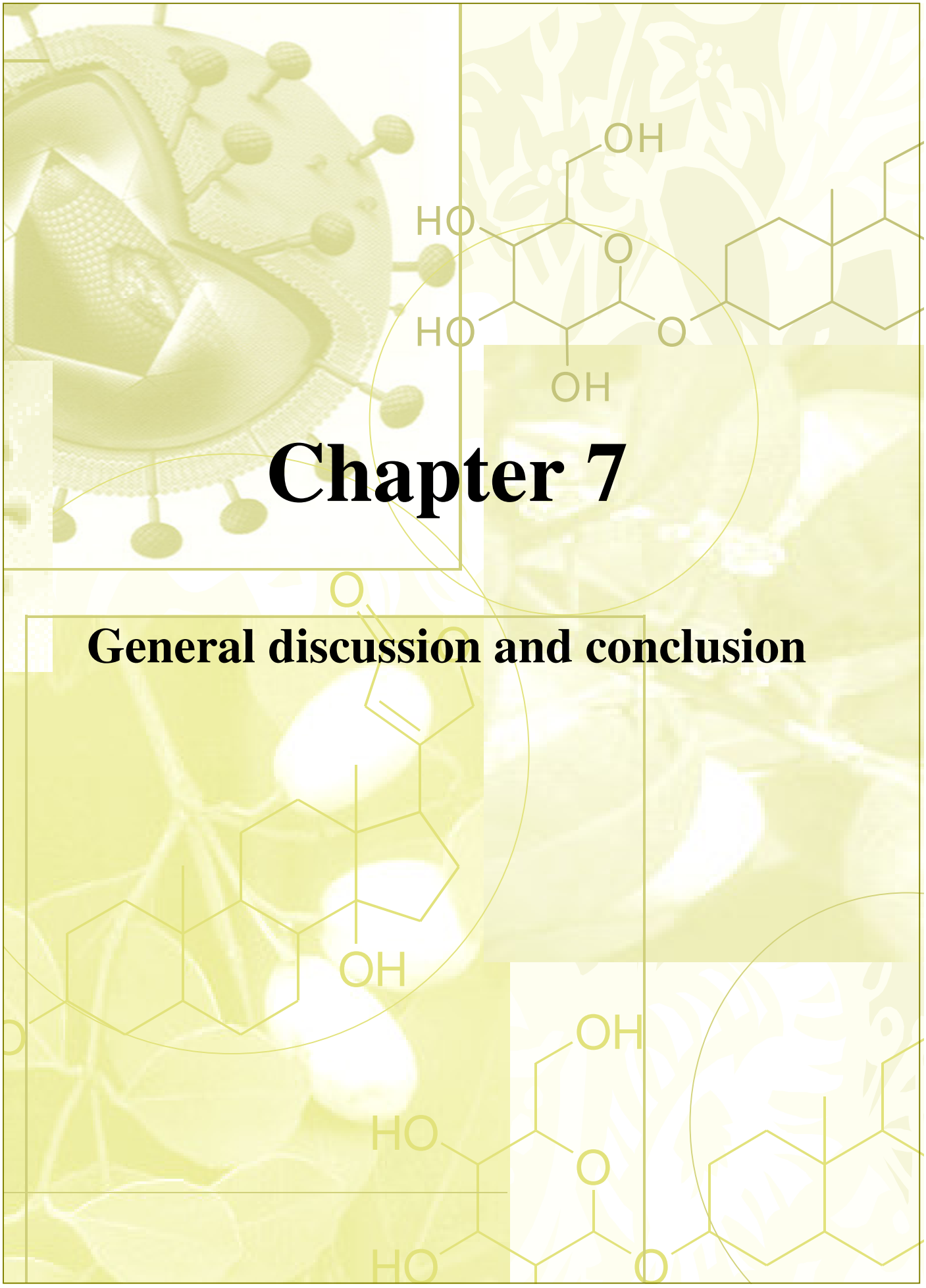
Cardiac glycosides are known for their cardiac activity with more than 2 million users in USA. Digitoxigenin-glucoside is part of that group of compounds and has also been tested for its cardiac activity. If the structure of digitoxigenin-glucoside is compared to the cardiac glycosides, it is strikingly similar in major parts of the compound. The major difference between these compounds is the number and type of sugar moieties attached to the terpenoid structure.

Its low toxicity could be ascribed to its one glucoside compared to other cardiac glycosides such as digoxin. It was also postulated that the potency depends on the number and type of sugar moieties. Digitoxigenin-glucoside has other effects such as hypertension, and it is also known as a fast onset compound that does not have a lasting effect on the heart. This in itself is not always negative, as certain applications need a fast reaction without long-term effects.

The compound was synthesised by several methods, and makes it an easy option to prepare the compound synthetically instead of isolating the compound from the plant

material. Digitoxigenin is easily obtainable from chemical suppliers, and digitoxigenin-glucoside can therefore be easily synthesised in the laboratory.

In 1974 Takiura *et al.,* stated that derivatives such as digitoxigenin-glucoside (LD_{50} > 310 nm/10 g body weight) are much safer compounds to be used than digitoxigenin (LD_{50} = 36 nm/10 g body weight) because of its lower toxicity and decrease in potent effects.



Chapter 7

General discussion and conclusion

Since the discovery of HIV in 1981, 25 years ago, there has not been any solution to the ever-growing disease. Many vaccine trials have not resulted in any usable information, and still we have no method to kill the virus, and to help the people already suffering from the disease. Current medicines are only selectively effective, and the prevalence- and death rate of the disease increase every year in South Africa. Very little progress has been made in any of the related fields of HIV. Currently there is very little hope that the disease will be contained and overcome before it is too late.

South Africans are still ignorant about the infection and prevalence rate of HIV in the country. South Africa has the highest rate of infection in the world, and more than 5 million of the population are already infected with the virus. The mid-2005 population of South Africa is estimated at 46.9 million. The latest results available for South Africa at the end of 2003 indicated that 5.3 million had already been infected with the virus. The overall estimate of HIV-prevalence rate is approximately 10%, while the prevalence rate for adults aged 15-49 is estimated at 17%. When this is compared to the prevalence rate of Sub-Saharan Africa of 7.4%, South Africa compare badly to other sub-Saharan countries (Statistics South Africa, 2002).

A cure for HIV is definitely not within reach for a few years to come. Research contributed very little to understanding the mechanism and progress of the virus during infection. The need for an effective drug to contain the virus becomes more evident as more people are realising that they are dying from AIDS. This does not only have a social implication for families, but an enormous economic impact on every level of the country as well.

In this study twelve extracts were prepared and tested against HIV. Only one of the extracts showed inhibition of the virus. All the extracts were however potent anti-microbial extracts, and shows that anti-microbial activity is therefore not an accurate indicator of HIV activity. The extract prepared from the leaves of the indigenous tree *Elaeodendron croceum*, showed promising results. The extract was purified and from the extract a very active pure compound had been isolated. The crude extract was tested first for antiviral activity against HIV, with an inhibiting activity of the VSV-pseudotype assay of more than 80%. Silica gel columns and liquid-liquid fractionation were used to obtain a semi-purified extract. The semi-purified extract had comparable activity to the crude extract with inhibition of approximately 85%. The chloroform extract is easily prepared from the crude extract, and the stable compounds at room temperature make the preparation of the extract easy and usable in any type of environment.

The chloroform extract was purified further by column chromatography using silica gel columns. To obtain the pure compound, fractions containing the compound were identified by TLC, and after evaporation of the last solvents, the pure compound precipitated and formed white crystals.

To identify the compound it was necessary to use several analytical methods. NMR was the most useful method to determine the structure and position of the substituents on the structure. For confirmation of the positions of the substituents, it was necessary to use two-dimensional NMR. The purified active compound had been identified by these methods as digitoxigenin-glucoside. The compound yielded even

better results on the anti-HIV assays than was found with the crude and chloroform extracts. The compound inhibited the virus in the HeLa-Tat-Luc assay with 85% and the recombinant virus with approximately 90%.

Toxicity tests were performed to determine if the compounds could be used on living cells without having high cell toxicity. *Elaeodendron* spp. are known for their toxic compounds, and it was therefore expected that this plant extract would be toxic as well. Traditional healers often use toxic compounds as medicine. It is important to ensure the safety of these compounds taken, by controlling the dosages of the intake.

The extract and the pure compound of *E. croceum* were not toxic as expected. The pure compound was more toxic than the crude- and the semi-purified extracts, but still not very toxic. The toxicity did increase rapidly when the concentration of the semi-purified extract was increased. The active concentration of 100 ng/ml for digitoxigenin-glucoside was much lower than the toxic concentration of 25 µg/ml. The chloroform extract was less toxic than the pure compound at 100 ng/ml, and it only became toxic at concentrations of 50 µg/ml. These in vitro results seem to indicate that the extract and pure compound from *E. croceum* are not as toxic as expected. These results are however on in vitro tests only and need to be confirmed by in vivo results as well.

The isolated compound belongs to the extended group of cardiac glycosides. The structure is derived from digitoxigenin which is similar in structure to digitoxin and digoxin. All these compounds are very toxic, but they are also used medicinally because of their cardiotonic activity. Extensive research was done on the group of

cardiac glycosides determining their effectivity and toxicity when used as a cardiotonic. Several derivatives and variants were produced to decrease the toxicity but still keep their cardiotonic potency (Rathore *et al.,* 1985), (Humber *et al.,* 1983) & (Takiura *et al.,* 1974).

Digitoxigenin was tested for its cardiotonic and Na^+/K^+-ATPase activity. It was found that the sugar attachment improves the binding ability of the compound and it increased the activity of this compound (Rathore *et al.,* 1985). Digitoxigenin-glucoside only contains one glucose moiety in comparison to the three sugar moieties of digoxin and digitoxin. The orientation of the glycoside also determines its activity. β-D-glycosides such as the compound isolated were more effective than the α-D-glycosides. Most of the naturally occurring glycosides exhibit the β-D-orientation, as found in digitoxigenin-glucoside.

The type and number of sugar molecules are also correlated with the toxicity of the cardiac glycosides. Less sugar molecules decrease the toxicity of the compound, but it has a negative influence on the potency of the compound. Digitoxigenin-glucoside is therefore less toxic than most of the cardiac glycosides that contain more than one sugar molecule.

In terms of cardiac activity, a less potent compound would not be useful, although it would be less toxic than other similar compounds. Other applications of the less toxic cardiac glycosides like the isolated compound are therefore worth considering.

No previous antiviral activity had been shown for digitoxigenin-glucoside or any of the related cardiac glycosides. This is therefore the first report of the cardiac glycosides being active against viruses, and specifically HIV. As the compound was very active against the virus in the VSV-pseudotype assay using a recombinant virus, it is possible that digitoxigenin-glucoside could inhibit the virus directly. This assay focuses on the viral structure and not on enzymes and enzyme products responsible for the survival of the virus. The decreased toxicity is a big advantage, as the concentration of the compound can still be increased significantly before the start of toxicity to the cells.

This compound is also different from anti-retroviral treatment (ART) used at present that blocks or inhibits some of the viral enzymes or enzyme products. These medicines cannot eliminate the virus from the body, but can only reduce the viral load in the blood. Medicines used today are only useful to keep a person in the asymptomatic phase, and prolongs the development into AIDS with the progress of other opportunistic infections (OI). Digitoxigenin-glucoside is unique in its activity targeting the virus itself, and the activity is probably not because of its toxicity, but because of a specific interaction with the virus.

As the glucose moiety is responsible for attachment in the heart, the glucose might also play a role in attachment to the virus. The active concentration of 100 ng/ml is more than 250 times less than the toxic concentration of 25 µg/ml determined on the VERO cells. It can therefore potentially be a very useful drug in combination with current ART to lower the viral load, but also eliminating the virus from the blood stream. More *in vivo* experiments are however necessary to determine its activity.

There would always be the problem with the viral DNA that is incorporated into the host DNA. The DNA would stay in the cells, and it would be ideal to eliminate the virus before it enters the nucleus to be integrated into the genome.

Considering the prospects of this compound to be used as a medicine, there are still some obstacles to overcome. Once the virus enters the nucleus, there is no stopping the replication process of the virus, as it will always be part of the genome. Prevention is still however, better than a cure, as the drug will only lower the viral load by eliminating the virus from the blood system. This will in itself reduce the risk of transmitting the virus to another person. It would still be better to prevent the virus from infecting the cells, than to lower the viral load and to eliminate the virus from the blood stream.

A medicine to eliminate the virus from the blood stream would be a great advantage to the drugs that are currently in use. It would increase and strengthen the efficacy of ART. As the compound could be synthesised in a laboratory, harvesting of the plant would not be necessary, and the species would not be endangered.

Further research and funding could pave the way for digitoxigen-glucoside or related cardiac glycosides to reach the markets as ART drugs. Although new medicines could reach the market, prevention will still play the biggest role in combating the virus from depleting the work force of our country.

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