

Chapter 4

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

4.1 Introduction.....	82
4.2 Materials and methods.....	85
4.2.1 Materials.....	85
4.2.2 Reverse Transcriptase.....	85
4.2.3 NF- κ B and Tat.....	87
4.2.4 HeLa-Tat-Luc.....	89
4.2.5 VSV Pseudotype.....	90
4.3 Results.....	91
4.4 Discussion.....	93

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- κ B is an ubiquitous mammalian transcription factor. NF- κ B plays an important role in the transcription of several genes, especially genes that encode for pro-inflammatory cytokines. Concurrent with NF- κ B'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- κ B has been postulated and tested as a means to target apoptotic events in cancerous tissue (Bremner *et al.*, 2004). The Rel/NF- κ B 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- κ B is the major inducible regulatory element involved in long terminal repeat (LTR) transactivation and HIV replication in CD4 lymphocytes. NF- κ B 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- κ B 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- labeled 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-

labeled 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.

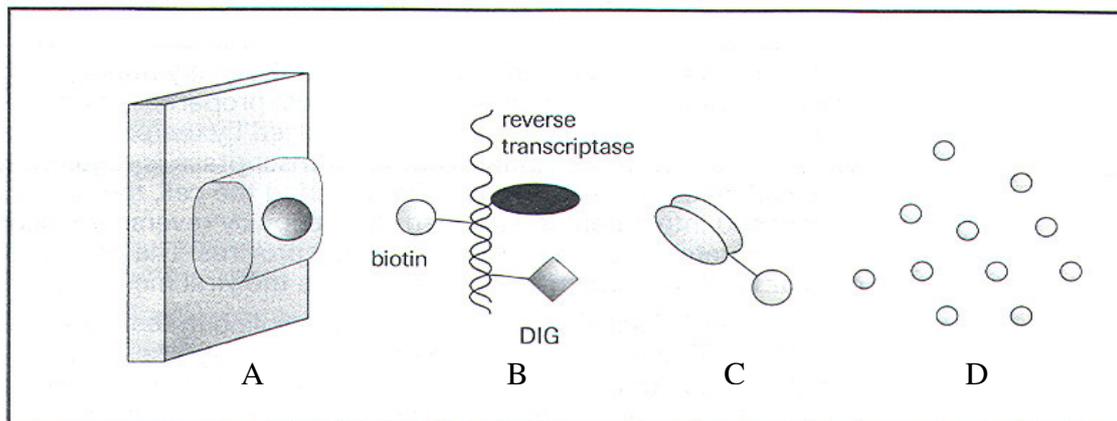


Figure 4.1 A=Streptavidin-coated MTP, B=DNA/RNA hybrid, biotin- and DIG-labeled by RT activity, C=Anti-DIG-POD Fab-fragment and D=POD substrate (ABTS).

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 $\text{ng}/\mu\text{l}$. 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/ μ l. 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- κ B (Nuclear factor kappa B)

The NF- κ B 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% CO₂ humidified atmosphere and split when confluent. The stable transfected HeLa cells were cultured in complete medium containing 100 μ g/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- κ B, 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- κ B.

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 μ g/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 1 hour, 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 μ l of the luciferase lysis reagent was added to each well and left for 15 minutes to complete lysing of the cells. 15 μ l 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, 50 μ l 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- κ B activator cytokine TNF- α . To determine NF- κ B 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 MgCl₂, 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 TNF- α -induced NF- κ B 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 (10⁵ 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. 1×10^5 MT-2 cells were seeded in 96-well microtiter culture plates and were infected with the recombinant virus, previously titrated (100 000 RLU/well). These cells were prepared in the presence and absence of the samples to be analysed at different concentrations, in a final volume of 200 μ l of

RPMI medium. The infected cells were incubated at 37°C in 5% CO₂. 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.

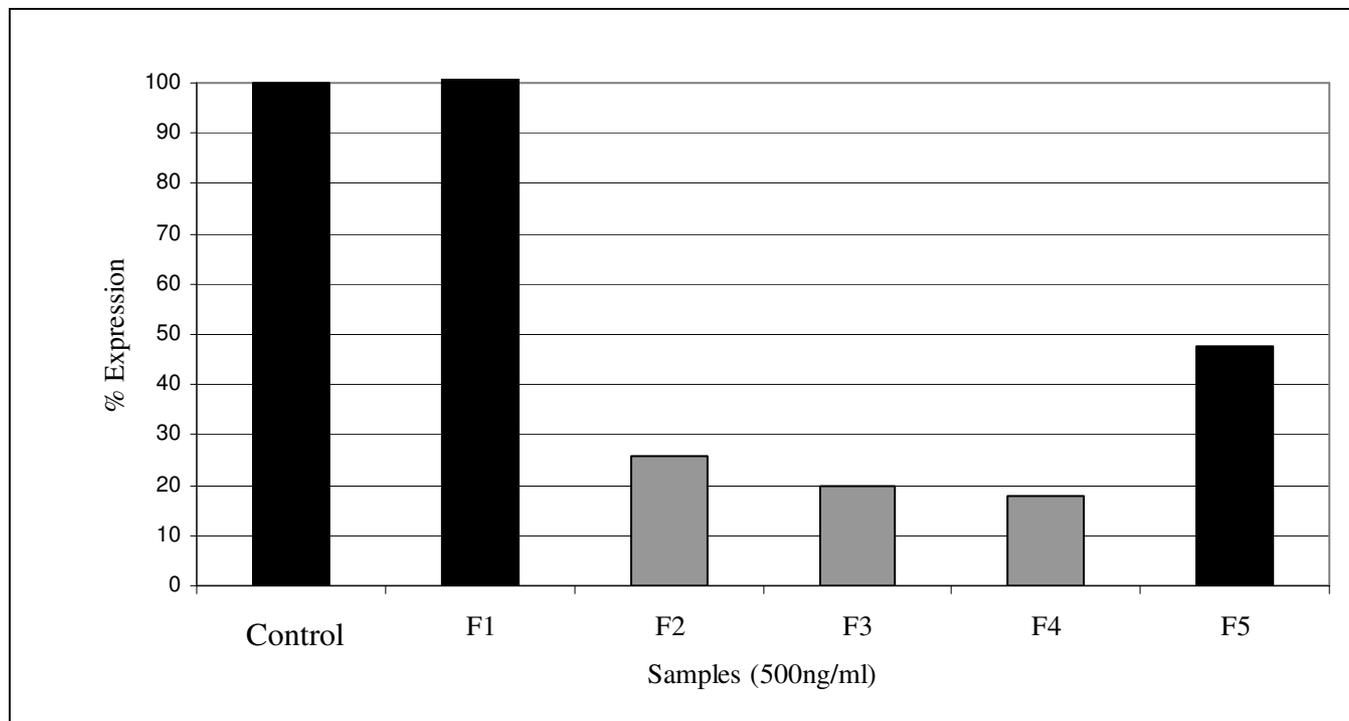


Figure 4.2 Graph showing the HeLa-Tat-Luc assay results. The first bar shows the control and F1-F3 indicates the silica gel column fractions and pure compounds F4 (digitoxigenin-glucoside) and F5 (pure unidentified compound) of the extract analysed.

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.

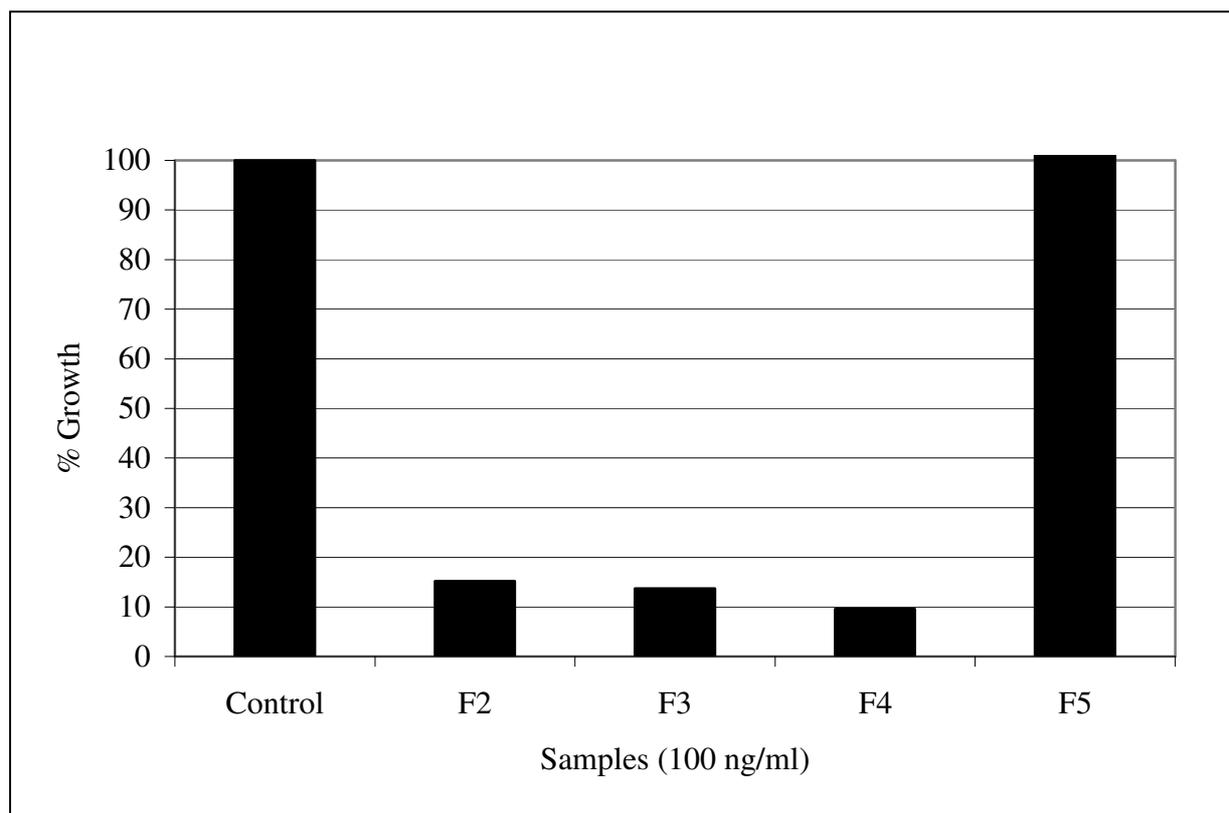


Figure 4.3 Graph showing the results from the MT-2 VSV-pseudotype assay. The first bar shows the control. F2 and F3 indicate the fractions and F4 (digitoxigenin-glucoside) and F5 (a pure unidentified compound) are compounds of the extract added to the assay.

4.4 Discussion

Four different assays against HIV were used to 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?