CHAPTER 5:

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

5.1 Introduction

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

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

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

5.2 Materials and methods

5.2.1 C-Med 100®

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acid analogs such as quinic acid (U.S. patent application EL781388471US, filed March 7 2002 and Åkesson *et al*., unpublished data).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

5.2.2 Transient transfection and luciferase activity analysis

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

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

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

5.2.3 Hela-Tat-Luc assay

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

5.2.4 Anti-HIV-1 replication

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

5.2.4.1 Production of VSV-pseudotyped recombinant viruses

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

5.2.4.2 VSV-pseudotyped HIV-1 infection assay

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

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

5.2.4.3 HIV reverse transcriptase (RT) assay

Reverse transcriptase assay was done as described in chapter 2.

5.3 Results and discussion

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

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

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

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

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

ni, no inhibition; na, not assayed.

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

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

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

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

5.4 References

AKESSON, C., LINDGREN, H., PERO., R.W., LEANDERSON, T. & IVARS, F. 2003. An extract of *Uncaria tomentosa* inhibiting cell division and NF-κB activity without inducing cell death. International Immunopharmacology 3: 1889-1900.

AKESSON, C., PERO, R.W. & IVARS, F. 2003. C-Med 100, a hot water extract of *Uncaria tomentosa*, prolongs lymphocyte survival *in vivo*. Phytomedicine 10: 23-33.

BEDOYA, L.M., BELTRAN M., SANCHO, R., OLMEDO, D.A., SANCHEZ-PALOMINO, S., DEL OLMO, E., LOPEZ-PEREZ, J.L., MUNOZ, E., SAN FELICIANO, A.S. & ALCAMI, J. 2005. 4-Phenylcoumarins as HIV transcription inhibitors. Bioorganic & Medicinal Chemistry Letters 15: 4447-4450.

BEDOYA, L.M., DEL OLMO, E., SANCHO, R., BARBOZA, B., BELTRAN, M., GARCIA-CADENAS, A.N., SANCHEZ-PALOMINO, S., LOPEZ-PEREZ, J.L., MUNOZ, E., FELICIANO, A.S. & JOSE ALCAMI. 2006. Anti-HIV activity of stilbene-related heterocyclic compounds. Bioorganic & Medicinal Chemistry Letters 16: 4075-4079

CAMPAGNUOLO, C., FATTORUSSO, E., PETRUCCI, F., TAGLIALATELA-SCAFATI, O., APPENDINO, G., MARQUEZ, N. & MUNOZ, E. 2005. A

prenylbisabolane with NF-kB inhibiting properties from Cascarilla (*Croton eluteria*). Bioorganic & medicinal chemistry letters 13: 4238-4242.

CHENG, M., LEE, K., TSAI, I. & CHEN, I. 2005. Two new sesquiterpenoids and anti-HIV principles from the root bark of *Zanthoxylum ailanthoides*. Bioorganic & Medicinal Chemistry Letters 13: 5915-5920.

MARQUEZ, N., SANCHO, R., BEDOYA, L.M., ALCAMI, J., LOPEZ-PEREZ, J.L., FELICIANO, A.S., FIEBICH, B.L. & MUNOZ, E. 2005. Mesual, a natural occurring 4-phenylcoumarin, inhibits HIV-1 replication by targeting the NFκ-B pathway. Antiviral Research 66: 137-145.

NG, T.B., HUANG, B., FONG, W.P. & YEUNG, H.W. 1997. Anti-Human Immunodeficiency Virus (Anti-HIV) natural products with special emphasis on HIV reverse transcriptase inhibitors. Life Sciences 61: 933-949.

SANDUR, S.K., ICHIKAWA, H., SETHI, G., AHN, K.S. & AGGARWAL, B.B. 2006. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) suppresses NF-kappa B-regulated gene products through modulation of p^{65} and Ikappa Balpha kinase activation, leading to potentiation of apoptosis induced by cytokine and chemotherapeutic agent. Journal of Biological Chemistry 281 (25): 17023-17033.

PARRA, E., VARGA, M., HEDLUND., G., KALLAND., T. & DOHLSTEN, M. 1997. Constimulation by B7-1 and LFA-3 targets distinct nuclear factors

that bind to the interleukin-2 promoter: B7-1 negatively regulates LFA-3-induced NF-AT DNA binding. Molecular and Cellular Biology 17: 98-108.

PEIREIRA, L.A., BENTLY, K., PEETERS, A., CHURCHILL, M.J. & DECON, N.J. 2000. A compilation of cellular transcription factor interactions with HIV-LTR promoter. Nucleic Acids Research 28: 663-668.

UCHIUMI, F., HATANO, T., ITO, H., YOSHIDA, T. & TANUMA, S. 2003. Transcriptional suppression of the HIV 1 promoter by natural compounds. Antiviral Research 58: 89-98.