CHAPTER 6:

Cytotoxic activity of *Elaeodendron transvaalense* extracts and isolated compounds

6.1. Introduction

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

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

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

6.2. Materials and methods

6.2.1. Plant material

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

6.2.2 Preparation of extracts and isolation of compounds

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

6.2.3. Cell culture

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

6.2.4. Toxicity screening (XTT viability assay)

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

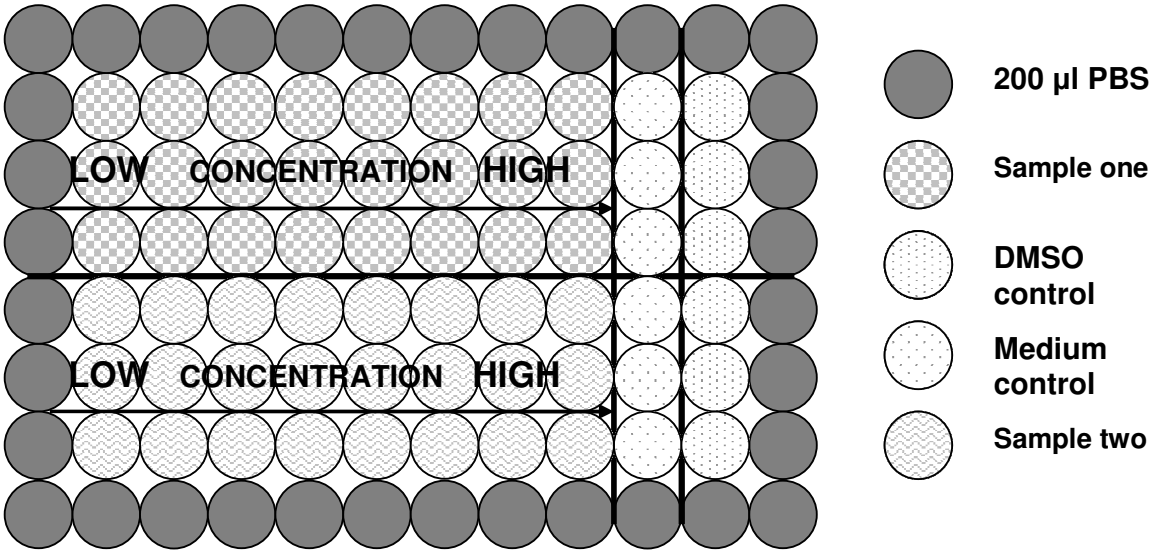

Figure 6.1 Plate design for cytotoxicity assay

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

6.3. Results and discussion

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

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

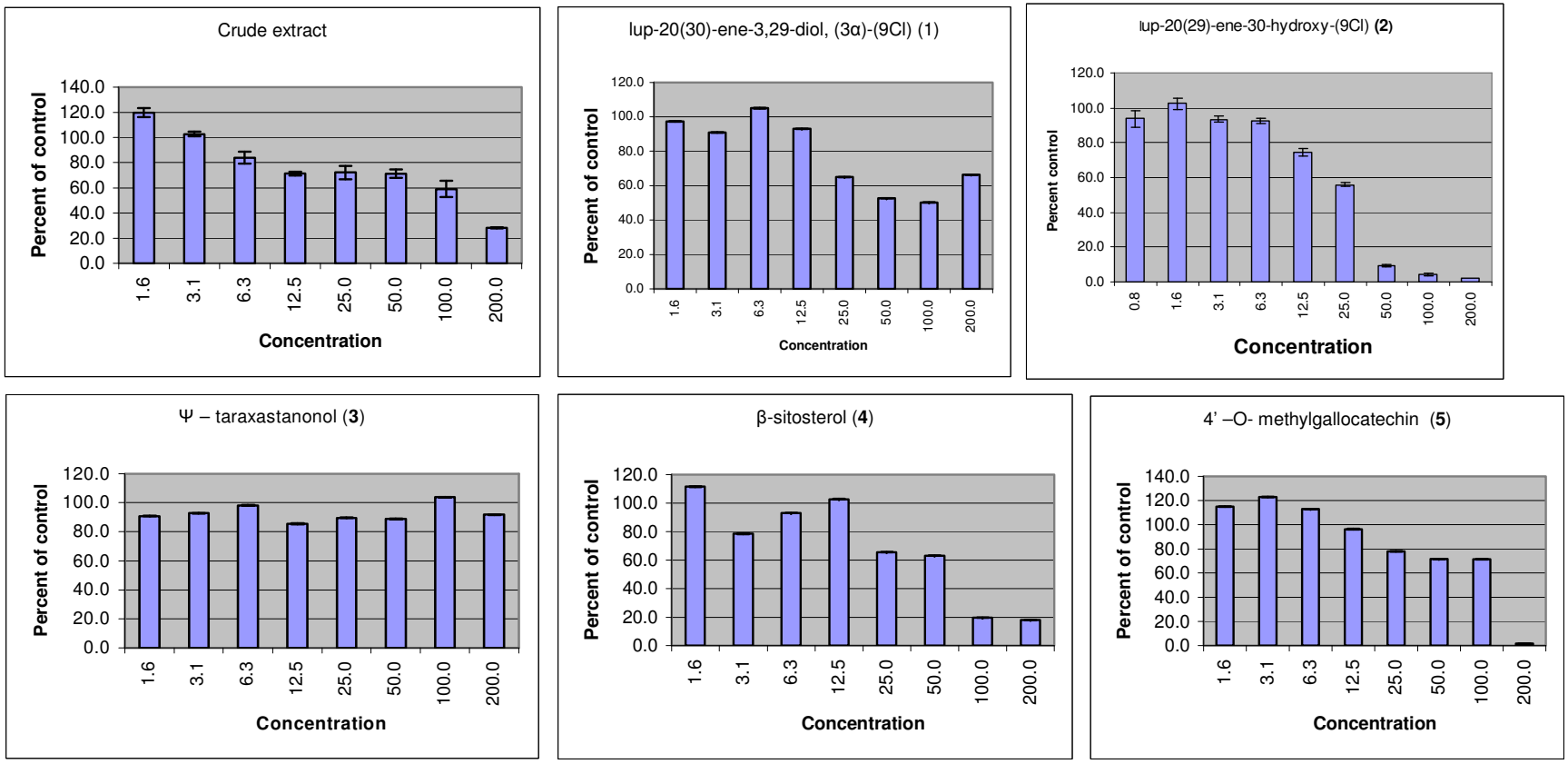

Figure 6.2 Effect of *E. transvaalense* crude extract and isolated compounds (µg/ml) on the growth of the normal Vero cell line.

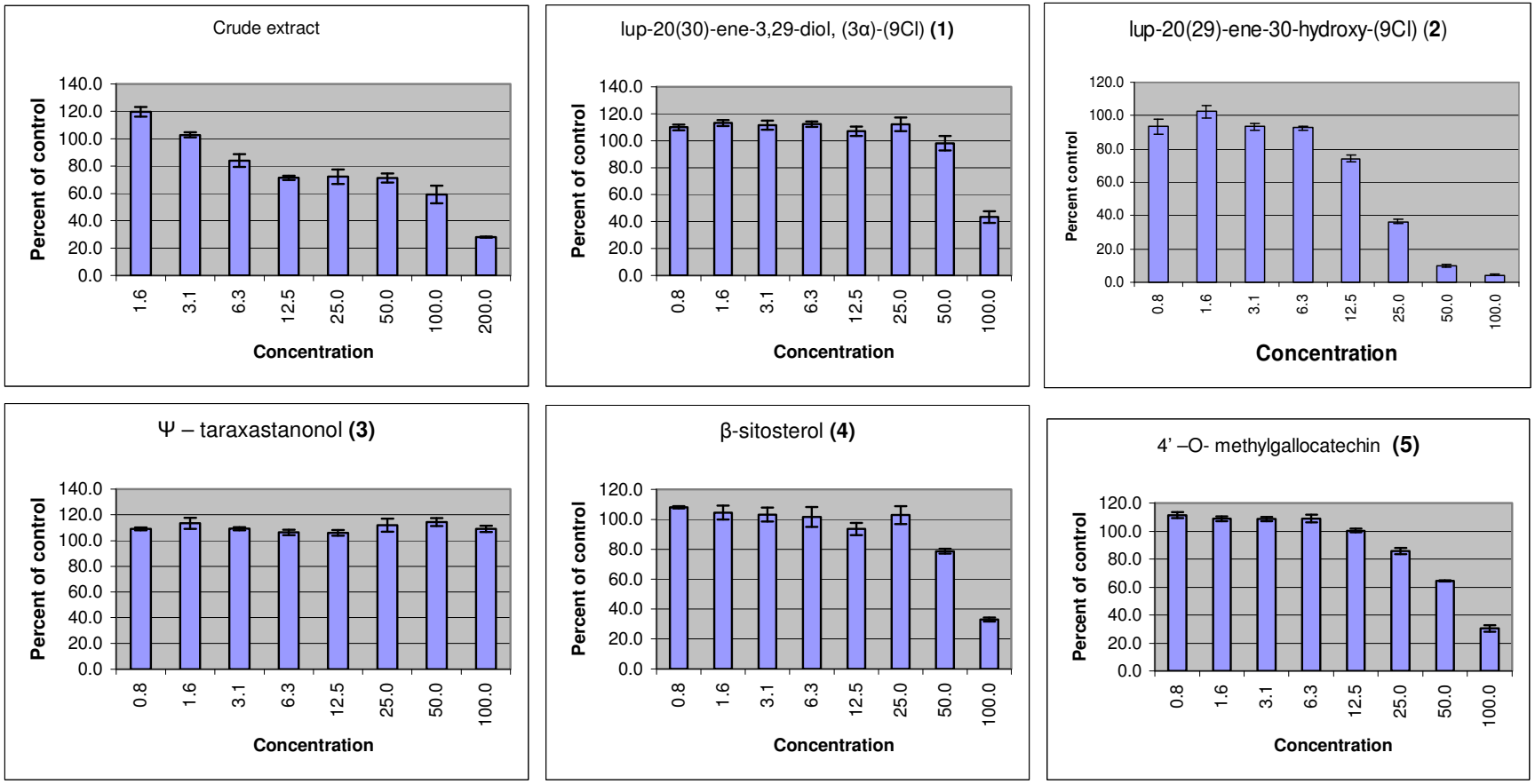

Figure 6.3 Effect of *E. transvaalense* crude extract and isolated compounds (µg/ml) on the growth of the cell line MCF-7.

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

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

SD,Standard deviation.

Na, not assayed

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

6.4 References

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CHAPTER 7:

General discussion and conclusion

7.1 Introduction

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

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

7.2 Activity of crude extracts against glycohydrolases and reverse transcriptase enzymes

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

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

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

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

7.4 Isolation of compounds from *Elaeodendron transvaalense* extract

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

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

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

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

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

7.7 Conclusion

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

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

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