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.

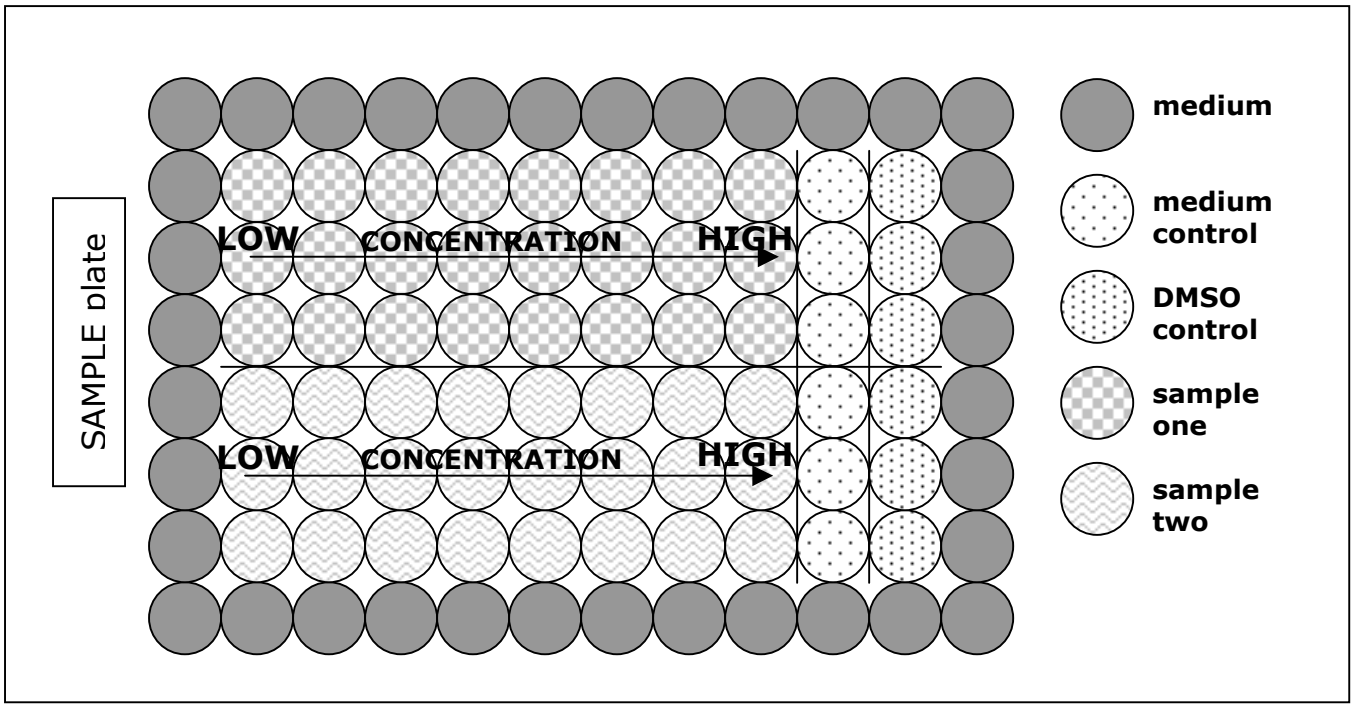

Figure 5.1 Outlay of the microtitre plate for toxicity analysis on VERO cells.

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.

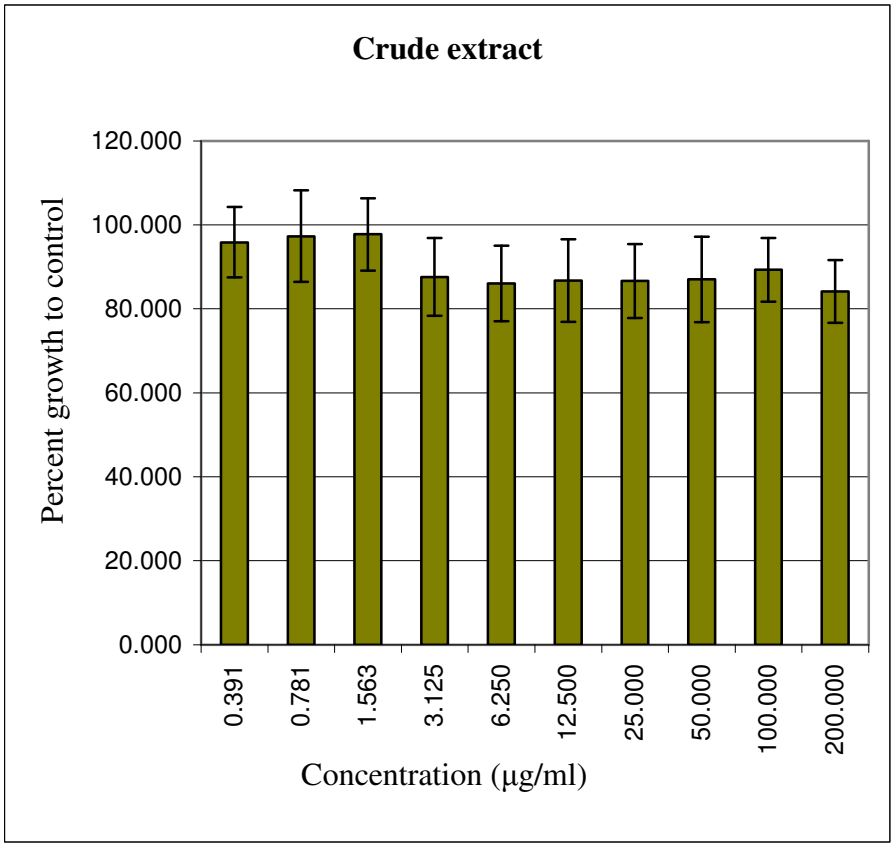

Figure 5.2 Toxicity of the crude ethanolic water (8:2) extract.

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.

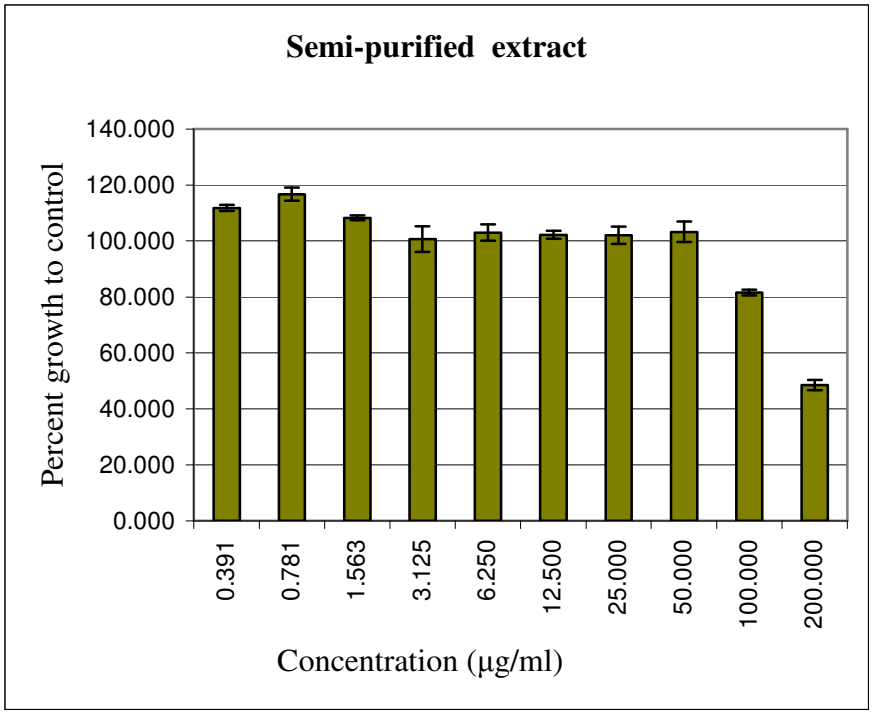

Figure 5.3 Toxicity of the semi-purified extract.

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%.

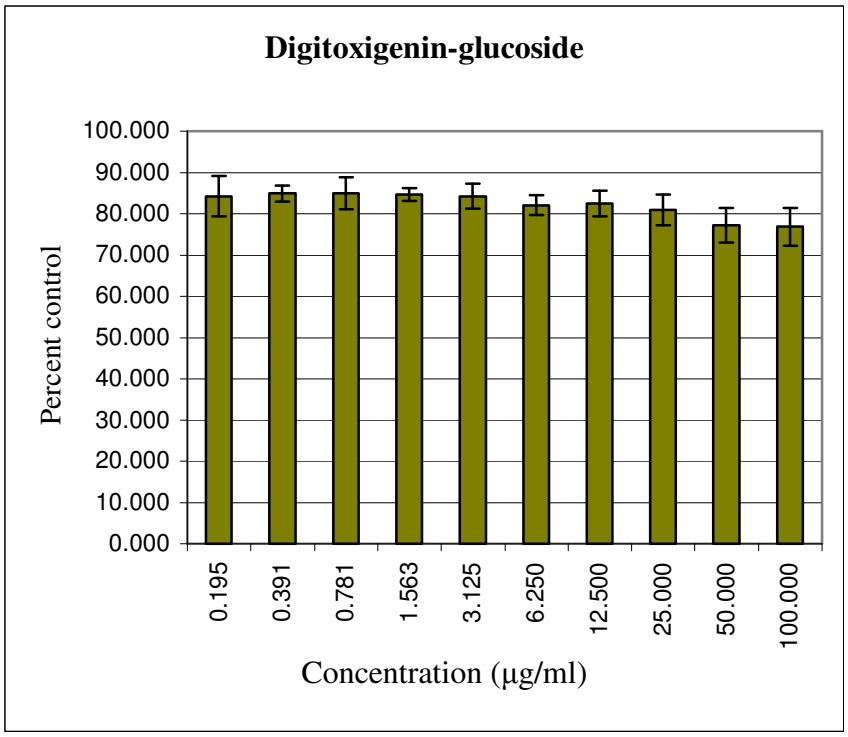

Figure 5.4 Toxicity of digitoxigenin-glucoside.

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.