CHAPTER 6

CYTOTOXICITY OF THE ISOLATED COMPOUNDS

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6.1 Introduction

Drug development is a very expensive and time consuming process and only 1 in 5 000 drug candidates that enter pre-clinical testing makes it through the development process (Kraljevic *et al.,* 2004). One-third of these failures are due to unacceptable toxicity levels. An *in vitro* cytotoxicity assay is a rapid and cost effective tool to identify likely failures before a compound is entered into the costly development process and also helps to choose the optimal candidate.

Cytotoxicity can be measured by the following assays: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole); XTT (sodium 3-[1(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate); Trypan blue (TB); Sulforhodamine B (SRB); WST and the clonogenic assay (Mosmann, 1983; Wilson, 2000).

XTT and MTT assays operate on the same principle. They are standard colorimetric assays (that is assays which measure changes in colour) used for measuring cellular proliferation (cell growth). The principle of the MTT or XTT assays are based on the cleavage of the yellow tetrazolium salts MTT or XTT to purple or orange formazan (Figure 6.1), respectively in the mitochondria of living cells. A solubilization solution (usually either dimethyl sufoxide or a solution of the detergent sodium dodecyl sulphate in dilute hydrochloric acid) is added to dissolve the insoluble formazan product into a coloured solution. The absorbance of this coloured solution can be quantified by measuring at a wavelength of 400 nm using a spectrophotometer (ELISA reader) (Mosmann, 1983; Wilson, 2000).

This cleavage takes place only when mitochondria reductase enzymes are active, and therefore conversion is directly related to the number of viable (living) cells. When the amount of formazan produced by the cells treated with an agent is compared with the amount of formazan produced by the untreated control cells, the effectiveness of the agent in causing death of cells can be

deduced, by calculations using a dose-response curve (Mosmann, 1983; Roche, 2005). The cell proliferation kit II (XTT) was used for the cytotoxicity measurement in this study.

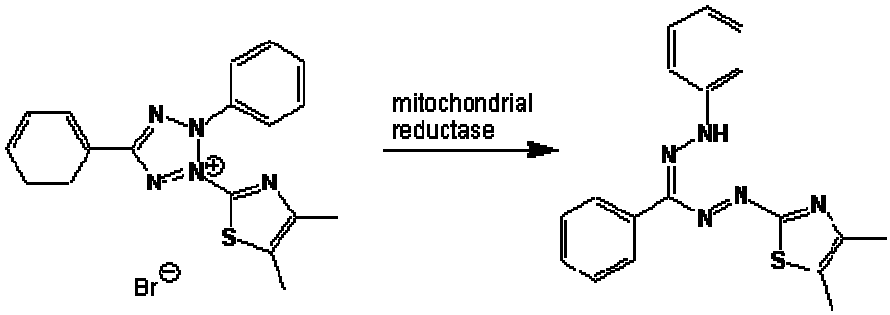

Figure 6.1: The reduction of yellow tetrazolium salt MTT to purple formazan (Roche, 2005).

6.2 Materials and Methods

6.2.1 Plant material

The leaves of *Croton steenkampianus* were collected at Thembe Elephant Park in northern KwaZulu-Natal as described in section 2.2.1.

6.2.2 Preparation of extract and isolation of compounds

The crude ethanol extract preparation was carried out as described in 2.2.2.1 and the isolation of the compounds as described in section 2.2.2.3.

6.2.3 Cell culture

The cytotoxicity of the crude extract and the isolated compounds were tested against Vero cell lines. Cells were cultured in Eagle's minimal essential media (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 bovin serum at 37 ºC in a humidified incubator set at 5 % CO_2.

6.2.4 Toxicity screening (XTT viability assay)

The XTT colourimetric assay was used to measure the cytotoxicity of the crude extract and the isolated compounds (Roche, 2005). On day one of the experiment, the outer wells of the 96- well plate (Figure 6.2) were filled with 200 µl of incomplete medium while the inner wells were filled with cell suspension. The plate was then incubated overnight at 37 ºC in a humidified incubator set at 5 % CO_2. The 100 µl of the crude extract/pure compound was dispensed into the cell-containing wells of the sample plate in triplicate. The final concentrations of the 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 the pure compounds in the wells were 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 % DMSO in complete medium. Doxorubicin and zelaralenone were used as positive controls. The plate was then incubated for 3 days.

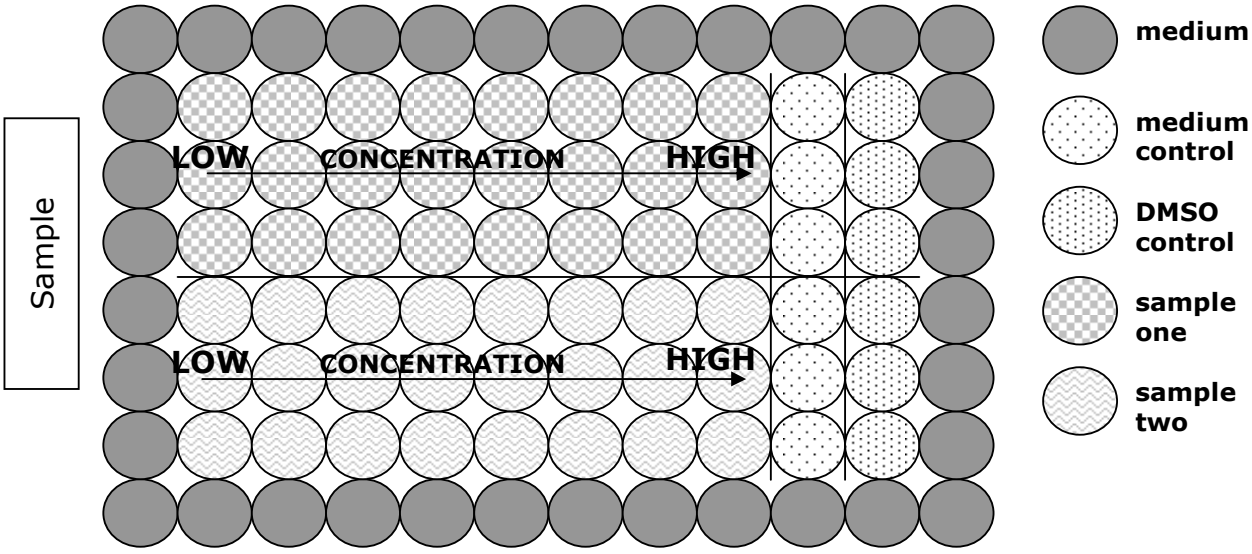

Figure 6.2: Sample plate design.

Reference plates (without cells), containing 100 µl of medium and 100 µl of diluted extract/compound, were also prepared in triplicate. These plates were also incubated at 37 ºC in a humidified incubator set at 5 % CO_2 for 3 days. On the 4th day, 50 µl of sodium 3-[1(phenylaminocarbonyl)-3,4-tetrazolium]-bis

(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) reagent was added to the wells and incubated for 1.4 hours. The colour changes were measured at 450 nm (690 nm reference wavelength). The program (KC Junior) used to read the plates automatically subtracted the values at 690 nm from the corresponding ones at 450 nm. Reference plate values were then subtracted from their corresponding sample values. Cell viabilities were assessed by comparing the sample values to the control values.

6.3 Results and Discussion

The results obtained for the cytotoxicity of the crude extract and pure compounds showed that they have little or no toxicity (Table 6.1 and Fig. 6.3).

Table 6.1: Cytotoxicity of the crude ethanol extract and compounds isolated from *C. steenkampianus* on Vero cells.

Plant extract/compound	**ID_{50} µg/ml**
Ethanol extract	45.0
Eriodictyol	Nt
Indane	248.2
Quercetin	33.6
Steenkrotin A	35.3
Steenkrotin B	305.9
Steenkrotin B acetate	Nt
Tamarixetin	53.8
Chloroquine	25.0

Nt = not done

All the isolated compounds as well as the extract had lower toxicity than chloroquine. The indane and steenkrotin B showed the lowest toxicity in this *in vitro* assay.

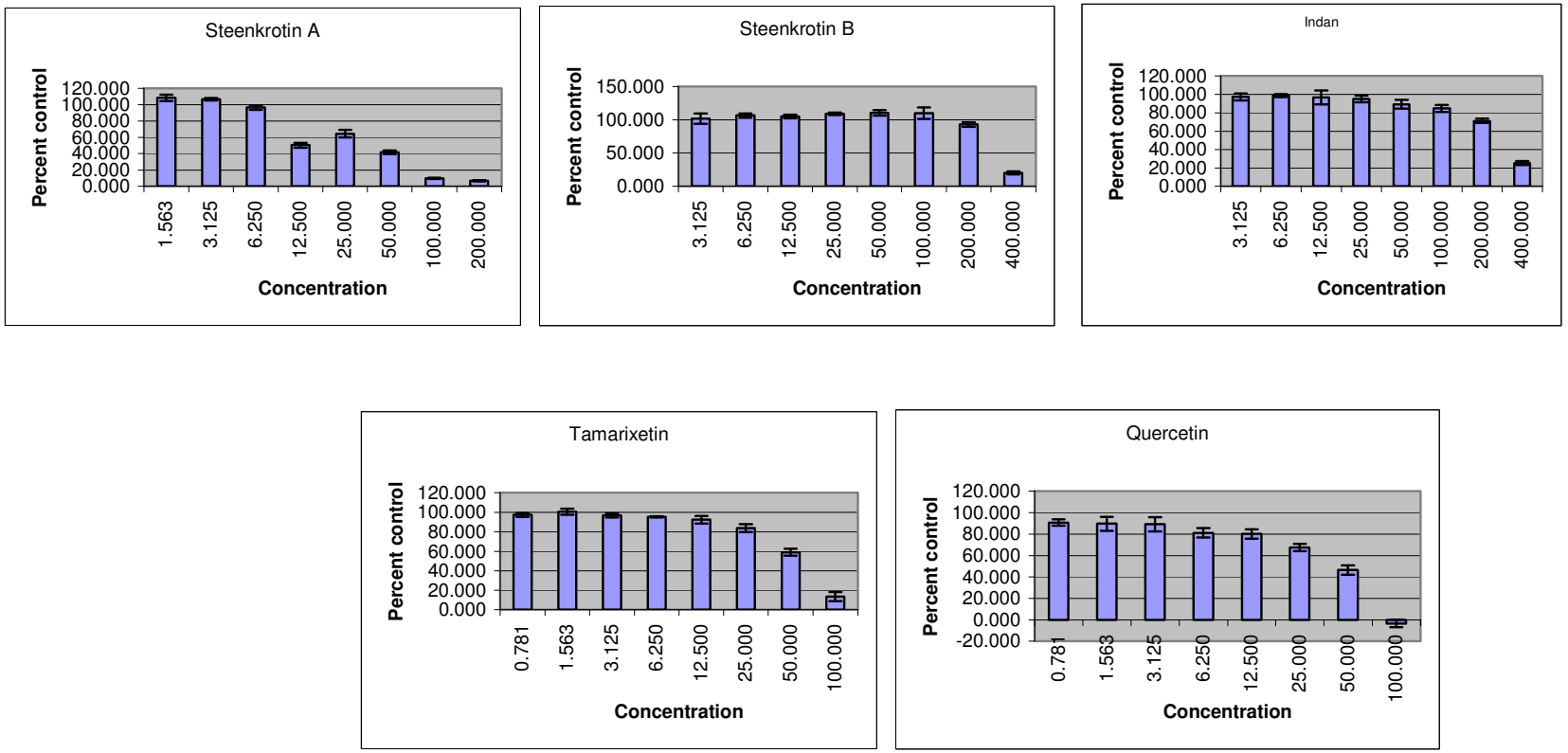

Figure 6.3: Activity of the isolated compounds on the growth of Vero cells in µg/ml.

Quercetin is the most toxic when its ID_{50} is compared to those of the crude extract and compounds isolated. However, It showed the highest antiplasmodial activity (0.34 and 0.73 µg/ml) at concentrations where its toxicity on Vero cells cause between 0% and 10% inhibition. Its therapeutic index for antimalarial activity is 46.0 to 98.8, which are not as high as the proposed index for a good remedy (Prozesky, 2004). However, it is being used as an important drug agent with very broad biological activities (Terao *et al.*, 1994; Hollman *et al.,* 1995).

Steenkrotin A showed no toxicity on the Vero cells at concentration of 1.5 to 6.3 µg/ml. This finding is very interesting and promising in that at this range it showed antiplasmodial activity (3.0 to 5.2 µg/ml) with corresponding therapeutic index of 7.0 to 11.8. Just as in the case of quercetin, its low therapeutic index based on this *in vitro* study may not mean that it will not be suitable for *in vivo* use. Nevertheless, Its suitability as a drug candidate requires further investigation *in vivo*.

Steenkrotin B did not show toxicity on Vero cells at concentration of 3.1 to 200 µg/ml. It is the least toxic of compounds isolated. However, its percentage inhibition increases from 0 to 80% at 400 µg/ml. Its antibacterial activities were at the concentrations where Vero cells' growth was not inhibited. Again, its therapeutic index (6.1) is very low compared to what is expected of a good drug. However, its very low toxicity properties might make it a promising therapeutic agent.

The indane did not inhibit the growth of Vero cells at concentrations between 3.1 and 25 µg/ml while between 50 and 200 µg/ml, the growth of cells dropped from 90 to 75%. The concentrations (50 to 100 µg/ml) where it has shown biological activities (anti-HIV and antibacterial) correspond to concentrations where Vero cells' growth was inhibited. Further evaluation needs to be performed to establish whether the observed biological activities are due to toxicity of the compound at these concentrations.

The crude extract showed lower toxicity (45.0 µg/ml) compared to the known drugs (chloroquine and quercetin). Its rich diverse component compounds which include flavonoids and terpenes known for their medicinal properties as well as its relatively low toxicity makes it a good candidate to be considered as medicine just like other species of the genus *Croton* being used traditionally for treatment of ailments (Pooley, 1993; Ngadjui *et al.,* 2002; Suarez *et al.,* 2006).

In conclusion, *in vivo* studies are needed to determine and establish the overall potential (safety, efficacy, suitability etc) of the crude extract and compounds as potential drug agents.

6.4 References

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