Chapter 9

In vitro cytotoxicity tests of the developed extracts

9.1. Introduction

Cytotoxicity testing using cell cultures is a rapid, standardized, sensitive, and inexpensive means to determine whether a material contains significant quantities of biologically harmful extractables. The high sensitivity of the tests is due to the isolation of the test cells in cultures and the absence of the protective mechanisms that assist cells within the body. A mammalian cell culture medium is the preferred extractant because it is a physiological solution capable of extracting a wide range of chemical structures, not just those soluble in water.

Toxicity is usually divided into two types, acute or chronic, based on the number of exposures to a poison and the time it takes for toxic symptoms to develop. Acute toxicity is due to short-term exposure usually a high dose, whereas chronic exposure is due to repeated or long-term exposure usually involving low doses.

Acute toxicity tests are short-term tests designed to measure the effects of a product on species during a short portion of their life span. The tests, which typically run for between 48 and 96 hours, usually measure the effects of products on the survival of a species. The results of these tests are often reported as an 'EC50', which is the effective concentration of a test sample that causes a specific effect to 50% of the cells. Chronic tests are used for low-level exposure for long periods, and are designed to measure effects on development, growth and reproductive success or failure.

Standard acute toxicity tests with aquatic macro-invertebrates have long played a major role in aquatic hazard and risk assessments, especially at a "screening" level of evaluation. A number of alternative tests have been proposed for rapid screening and are freshwater rotifer (*Branchionus calyciflorus*), brine shrimp (*Artemia salina*), lettuce (*Luctuca saliva*), mysid shrimp (*Mysidopsis hahia*), fathead minnow (*Pimephales promelas*) (McLaughlin, 1991). These tests are useful in situations where their rapidity and relative low cost make it practical to screen large numbers of samples for preliminary indications of toxicity. In this project the brine shrimp assay was used.

There are number of *in vitro* toxicity tests where different cell lines are used. Each test use different indicators. Some of this tests are Neutral red uptake cytotoxicity assay, one disadvantage of the Neutral Red assay is the possibility that deceptively low cell viability or cell number readings will result in those cases where a chemical has a relatively selective effect upon the lysosomes/endosomes of the cell. Hyaluronan gel cell toxicity test, a cell toxicity assay was devised to test for the effect of leachable chemicals and break-down products of the HA construct on CF-31 adhesion and proliferation (Borenfreund and Puerner, 1985). In this project the MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used based.

9.1.1. The brine shrimp assay

The brine shrimp lethality assay is considered a useful tool for preliminary assessment of toxicity. Brine shrimp is popularly known as sea monkeys and are crustaceans that live in saline environments. Their eggs (actually cysts), which can be inexpensively purchased from pet stores, hatch quickly and the larvae, termed a nauplius (plural, nauplii) are sensitive to small doses of biologically active chemicals. One indicator of the toxicity of a substance is LD_{50}, which refers to the amount (*i.e.,* lethal dose or concentration) of a substance that kills 50% of the test organisms. Activities are considered significant if the LD_{50} is less than 30 µg/ml (=0.03 mg/ml) (Geran *et al*., 1972). In this bioassay, the mortality of brine shrimp that are incubated in the test solution is recorded. Although the brine shrimp assay provides no information on the mechanism of action, it is a very useful preliminary tool in assessing the toxicity of extracts.

Brine shrimp assay has number of advantages like, experimental simplicity, sensitivity, reproducibility, ease of handing, lack of continuous culturing, short exposure time and lower costs. The main disadvantage is that one cannot extrapolate the results to toxicity to mammals (McGaw and Eloff, 2005).

9.1.2. The MTT cytotoxicity assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, (Mosmann, 1983), is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals which are largely impermeable to cell membranes, thus resulting in their accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals that are solubilized. The number of surviving cells is directly proportional to the level of

the formazan product created. The colour can then be quantified using a spectrophotometer (Mosmann. 1983).

The MTT cytotoxicity assay is considered a major advance in toxicity testing and it is the most widely used *in vitro* cytotoxicity assay. It is rapid, sensitive, versatile, quantitative and highly reproducible. It is also adaptable to a large-scale screening relevant for most cells. MTT reduction correlates to indices of cellular protein and viable cell number (Timmins, 2002).

However there are cell lines that do not metabolise MTT well or do not show an acceptable colorimetric profile for control cells. Production of the MTT product is dependent on the MTT concentration in the culture medium. The kinetics and degree of saturation are dependent on cell type. The assay is less effective in the absence of cell proliferation. The presence of glutathione-*S*-transferase (a normal enzyme that protects cells) can reduce the MTT independent of toxicity. These cells give high background and potentially false positives. MTT cannot distinguish between cytostatic and cytocidal effect. Individual cell numbers are not quantitated and results are expressed as a percentage of control absorbance. The test is less effective if cells have been cultured in the same media that has supported growth for a few days, which leads to underestimation of control and untreated samples. Certain types of drugs (i.e. interferon) can induce formazan production (MTT) and/or mitochondrial activity. Increased production of formazan will potentially give false positives with these drugs (Timmins, 2002).

9.2. Materials and Methods

9.2.1. Extracts tested

Acetone extracts of *C. imberbe*, *C. nelsonii*, *C. albopuntactum* and *T. sericea* were tested based on previous chapters results.

9.2.2. The brine shrimp assay

Brine shrimp (*Artemia salina*) eggs were hatched in a beaker filled with 3.8% aqueous sodium chloride in the dark. After 48 hours, the phototrophic nauplii were collected using a Pasteur pipette. Newly hatched nauplii were concentrated just above the unhatched eggs on the bottom. Since the nauplii are positively phototropic (attracted to light), shining a light in the middle of the container and shading the container at the bottom helps direct them to an area where they can be easily harvested. The nauplii were counted macroscopically in the stem of the pipette against

a lighted background. Approximately 10 – 15 shrimps were transferred to each well of 96 – well microplates containing the samples. The concentrations in which each extract was tested ranged from 0.1 – 2 mg/ml. The plates were kept in the dark. Survivors were counted after 24 hours of incubation and the percentage of deaths at each concentration and controls (salt water alone) were determined under the microscope. Podophyllotoxin (Sigma) was used as a positive control. The toxicity of the extracts to brine shrimps was determined in triplicate and the average percentage of live shrimps calculated.

9.2.3. The MTT cytotoxicity assay

Viable cell growth after incubation with test compound is determined using the tetrazolium-based colorimetric assay (MTT assay) (Mosmann, 1983). Vero kidney cells (monkey) of a subconfluent culture were harvested and centrifuged at 200 x *g* for 5 min, and resuspended in growth medium to 2.4 x 10^3 cells/ml. The growth medium used was Minimal Essential Medium (MEM, Highveld Biologicals) supplemented with 0.1% gentamicin and 5% foetal calf serum (Highveld Biologicals). A total of 200 µl of the cell suspension was pipetted into each well of columns 2 to 11 of a sterile 96-well microtitre plate. Growth medium (200 µl) was added to wells of columns 1 and 12 to minimize the “edge effect” and maintain humidity. The plates were incubated for 24 h at 37ºC in a 5% CO_2 incubator, until the cells were in the exponential growth phase. The Minimal Essential Medium was aspirated from the cells using a fine tube attached to a hypodermic needle, and replaced with 200 µl of test compound at differing concentrations (0.001 to 1 mg/ml) serial dilution prepared in growth medium. The cells were disturbed as little as possible during the aspiration of medium and addition of test compound. Each dilution was tested in quadruplicate. Untreated cells and positive control (berberine chloride, Sigma) were included. The microtitre plates were incubated at 37ºC in a 5% CO_2 incubator for 5 days.

After incubation, 30 µl MTT (Sigma, stock solution of 5 mg/ml in PBS) was added to each well and the plates were incubated for a further 4 h at 37ºC. After incubation with MTT the plates were centrifuged for 10 min at 1500 rpm. The medium in each well was carefully removed, without disturbing the MTT crystals in the wells, followed by adding 150 µl fresh phosphate buffer saline (PBS) to each well. The microtitre plates were again centrifuged for 10 min at 1500 rpm and the PBS removed from the wells. After washing with PBS, the MTT formazan crystals were dissolved by adding 50 µl DMSO to each well. The plates were shaken gently until the MTT solution was dissolved. The amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader (Titertek Multiscan MCC/340) at a test wavelength of 540 nm

and a reference wavelength of 690 nm. The wells in column 1, containing medium and MTT but no cells, were used to blank the plate reader. The LC_{50} values were calculated as the concentration of test compound resulting in a 50% reduction of absorbance compared to untreated cells.

9.2.4. Statistics

The line regression tool was used from Microsoft Excel.

9.3. Results

9.3.1. The brine shrimp assay

Brine shrimp assay results were analysed by plotting percentage mortality of brine shrimps against the different concentrations of the *C. nelsonii, C. imberbe, C. albopuntactum* and *T. sericea* extracts tested. The curves plotted for each extract had a percentage fit of 98, 73, 96 and 83% respectively. The equations of the curves are clearly indicated on the following figures (**Figure 9.1 – 9.4**). These four extracts were chosen because of their high antifungal activity based on MIC and bioautography assays.

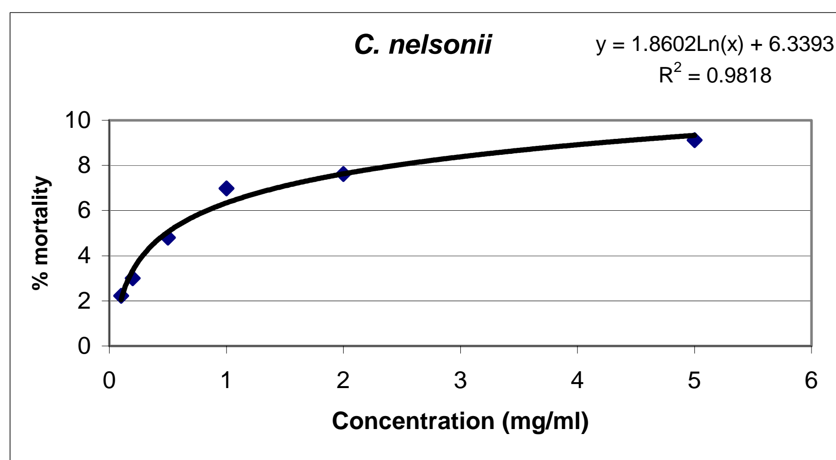

Figure 9.1. Brine shrimp assay mortality after exposure to *C. nelsonii* extract

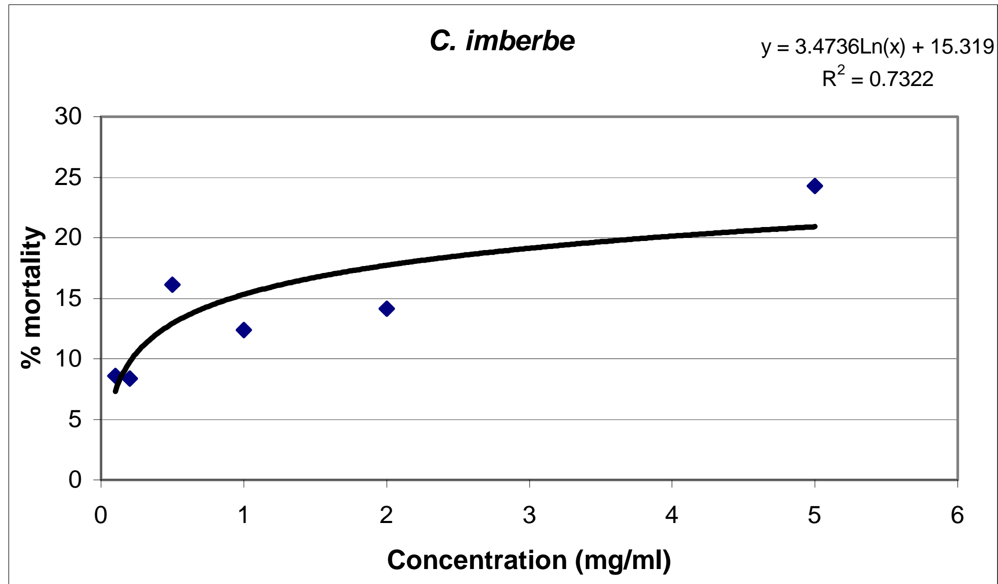

Figure 9.2. Brine shrimp assay mortality after exposure to *C. imberbe* extract

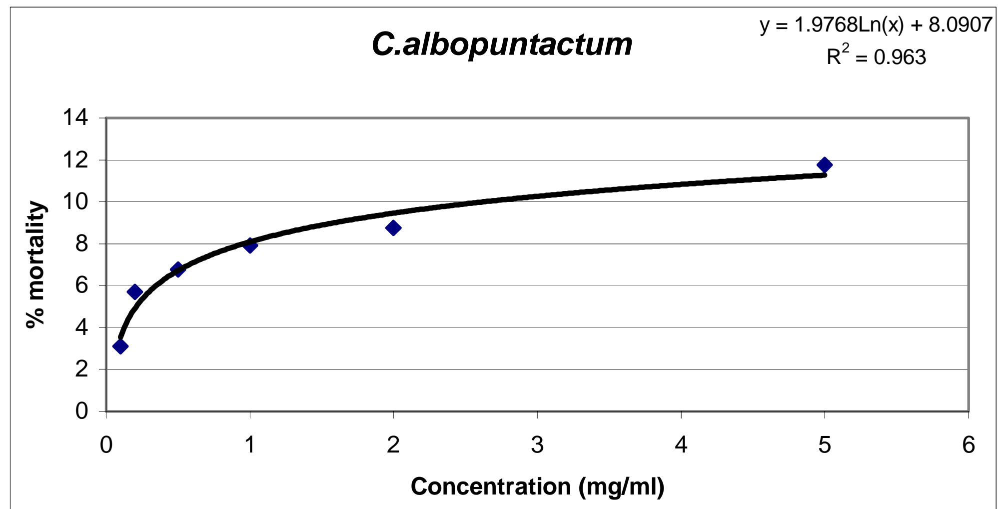

Figure 9.3. Brine shrimp assay mortality after exposure to *C. albopunctactum* extract

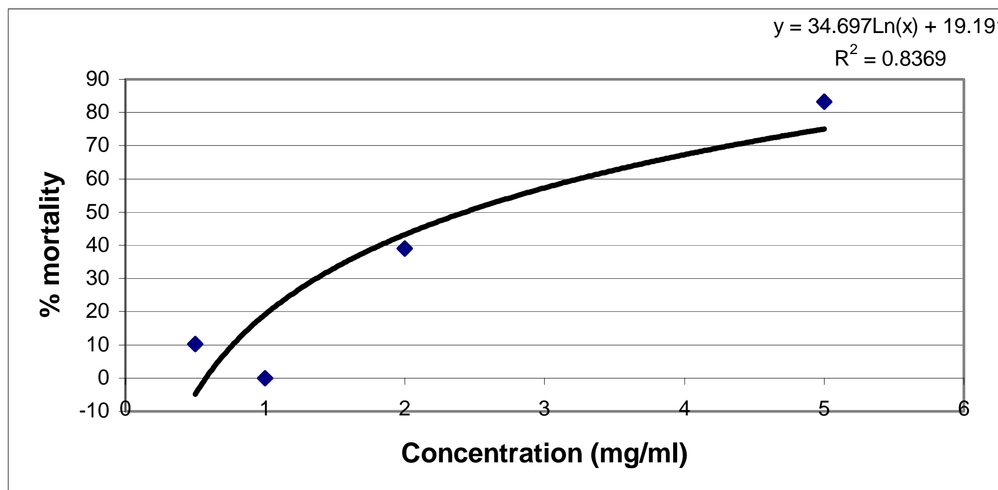

Figure 9.4. Brine shrimp assay mortality after exposure to *T. sericea* extract

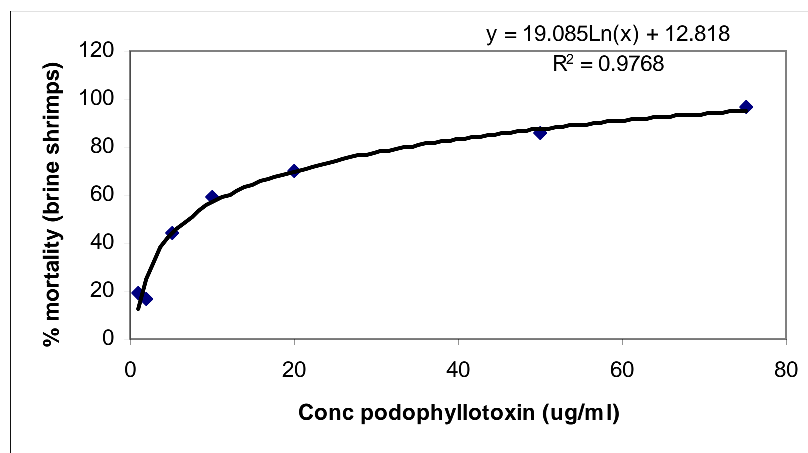

Figure 9.5. Brine shrimp assay curve of Podophyllotoxin (Positive control)

The LC_{50} value was calculated by substituting 50% for y into the curve equations. *In vitro* studies using the brine shrimp assay showed that *C. nelsonii, C. imberbe, C. albopuntactum* and *T. sericea* extracts to be relatively non toxic with LC_{50} values of 3.16, 2.30, 3.05 and 2.43 mg/ml respectively compared to 7 µg /ml for the podophyllotoxin standard.

9.3.2. The MTT cytotoxicity assay

The curve for the berberine chloride standard had a 91.64% fit and gave an equation of y = -0.4386x + 1.0009. The LC_{50} was calculated by substituting for y half the value of absorbance at 540 nm for the control (0.721).

The LC $_{50}$ value for berberine chloride was therefore 4.347 µg/ml (published results give an LC_{50} value of 10 µg/ml) (**Figure 9.6**).

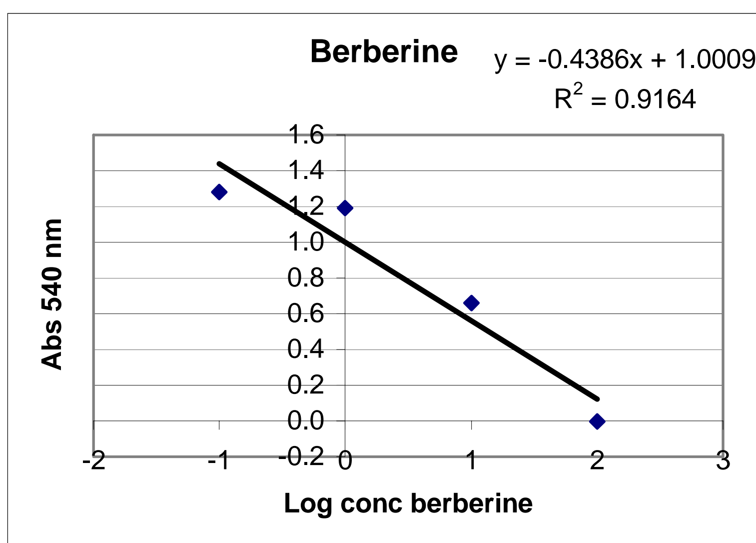

Figure 9.6. MTT cytotoxicity assay curve for Berberine chloride.

Cytotoxicity of the tested extracts was analysed at 540 nm for 1 mg/ml, 0.1, 0.01 and 0.001 mg/ml concentrations.

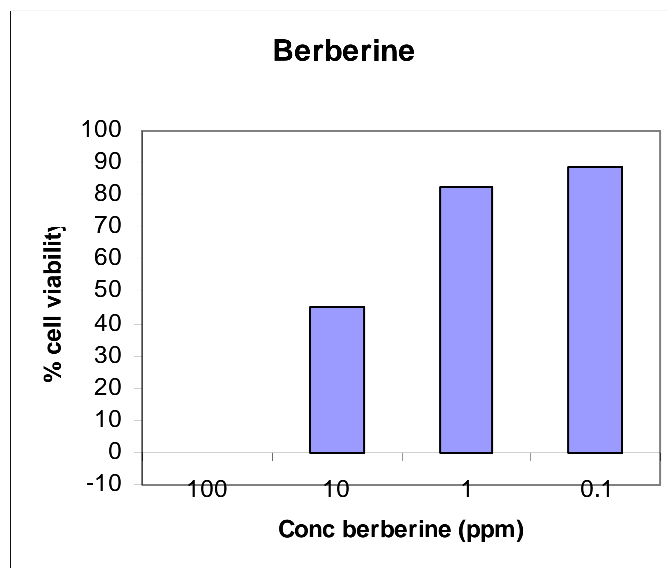

Figure 9.7. Percentage cell viability of berberine different concentration

Table 9.1. Results showing absorbance values at 540 nm for the various optimal extract concentrations.

Extracts	Conc.(mg/ml)	Log conc.	Ave abs 540	SD
C. imberbe	1	0.000	0.019	0.008
	0.1	-1.000	1.182	0.039
	0.01	-2.000	1.214	0.076
	0.001	-3.000	1.296	0.106
C. nelsonii	1	0.000	0.025	0.008
	0.1	-1.000	0.756	0.367
	0.01	-2.000	1.191	0.132
	0.001	-3.000	1.214	0.116
T. sericea	1	0.000	0.123	0.081
	0.1	-1.000	0.841	0.053
	0.01	-2.000	1.165	0.111
	0.001	-3.000	1.168	0.103
C. albopunctactum	1	0.000	0.003	0.005
	0.1	-1.000	1.086	0.085
	0.01	-2.000	1.146	0.055
	0.001	-3.000	1.225	0.063

The results were analysed by plotting the logarithm of different concentrations of the extract versus absorbance values at 540nm. The *C. imberbe*, *C. nelsonii*, *T. sericea* and *C. albopunctactum* curves had a percentage fit of 67.29, 86.39, 82.38 and 69.37 % respectively and the equation of the curves were y = -0.3861(x) + 0.3485; y = -0.4001(x) + 0.1961; y = -0.346(x) + 0.3054 and y = -0.3724(x) + 3063 respectively.

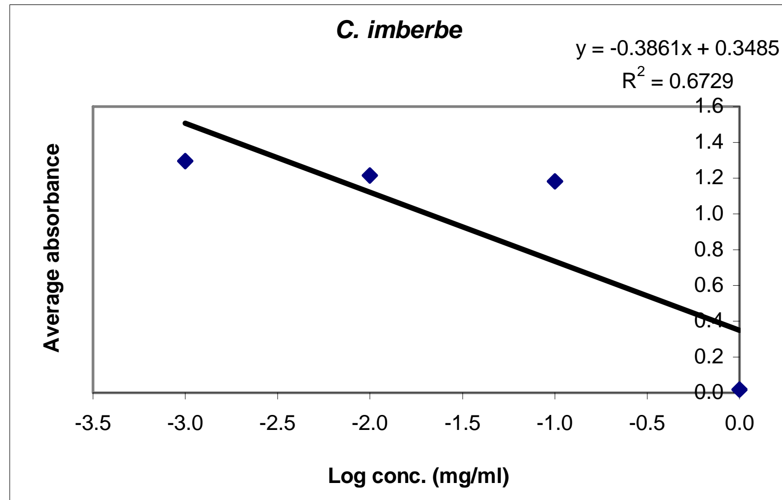

Figure 9.8. MTT cytotoxicity activity of *C. imberbe* extract against Vero cells.

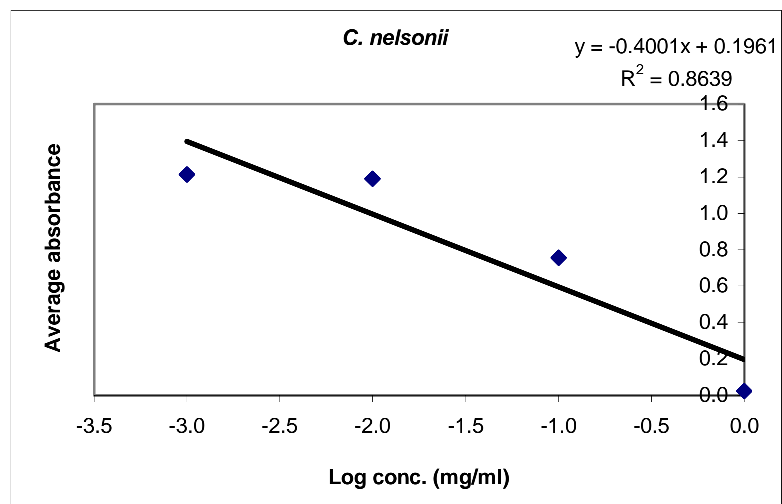

Figure 9.9. MTT cytotoxicity activity of *C. nelsonii* extract against Vero cells.

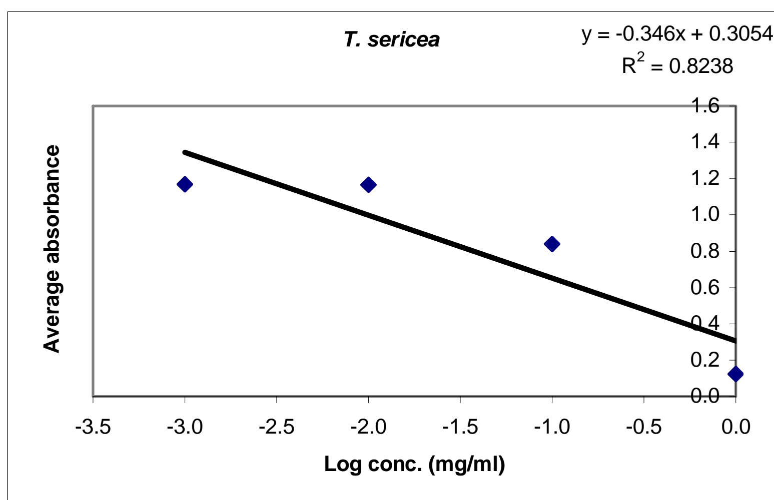

Figure 9.10. MTT cytotoxicity activity of *T. sericea* extract against Vero cells.

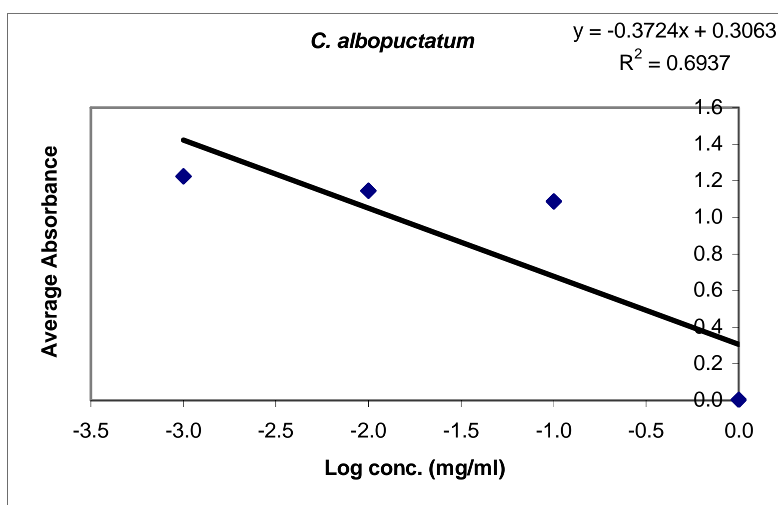

Figure 9.11. MTT cytotoxicity activity of *C. albopunctactum* extract against Vero cells.

LC_{50} untreated was calculated by substituting for y by half the value of absorbance at 540 nm for the control (0.645). The LC_{50} was a relatively non-toxic value of 168.6, 75.7, 102.9 and 121.7 µg/ml for *C. imberbe*, *C. nelsonii*, *T. sericea* and *C. albopunctactum* respectively compared to 4.347 µg/ml of the berberine chloride standard.

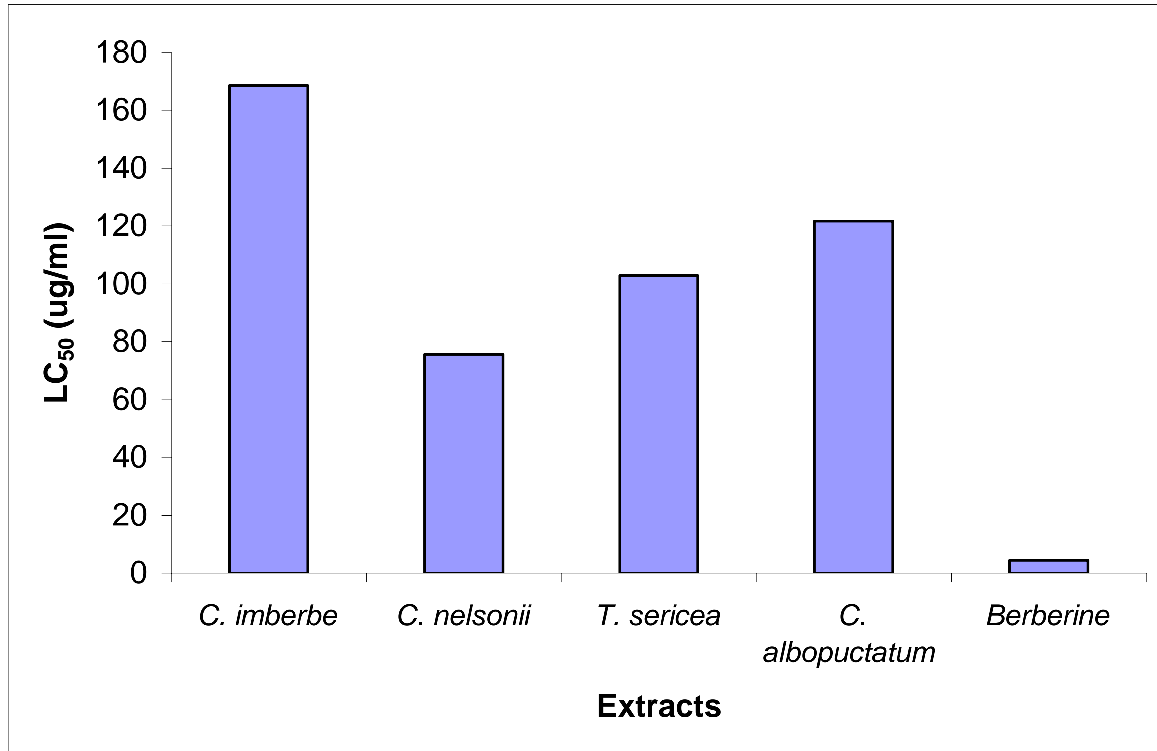

Figure 9.12. LC_{50} of the tested extracts

The relative safety margin indicates the number of times the effective concentration is lower than the LC_{50} concentration of the optimal extract and is calculated using the LC_{50} and MIC values.

Table 9.2. Relative safety margin (using LC $_{50}$ value from the brine shrimp assay and the MTT cytotoxicity assay) of the optimal extract.

Microorganisms	**MIC (mg/ml)**				**LC_{50}/MIC**							
					Brine Shrimp assay				**MTT assay**			
	C. imberbe	*C. nelsonii*	*T. sericea*	*C. albopuntactum*	*C. imberbe*	*C. nelsonii*	*T. sericea*	*C. albopuntactum*	*C. imberbe*	*C. nelsonii*	*T. sericea*	*C. albopuntactum*
C. albicans	2.5	0.04	0.64	0.64	1.26	57.50	4.77	3.80	0.09	1.89	0.16	0.19
C. neoformans	0.16	0.04	0.08	0.08	19.75	57.50	38.13	38.38	1.05	1.89	1.29	1.52
A. fumigatus	2.5	0.16	0.16	0.08	1.26	14.38	19.06	30.38	0.07	0.47	0.64	1.52
S. schenckii	2.5	0.02	0.02	0.08	1.26	115.0	152.5	30.38	0.07	3.79	5.15	1.52
M. canis	0.04	0.02	0.02	0.02	79.00	115.0	152.5	121.5	4.22	3.79	5.15	6.09
Average	**1.54**	**0.06**	**0.18**	**0.18**	**20.5**	**71.9**	**73.4**	**44.9**	**1.1**	**2.37**	**2.48**	**2.17**

9.4. Discussion

The *in vitro* cytotoxicity of three *Combretum* and one *Terminalia* species extract were investigated. These four extracts were chosen because of their good *in vitro* antifungal activity and we considered of using them in *in vivo* studies in animal models. The toxicity of the extracts to *Artemia salina* nauplii and monkey Vero cells were evaluated, because herbal medicines are perceived as safe, yet there is little knowledge on the potential toxicity of these indigenous plants. Responses varied for the different extracts and between the two assays, but brine shrimps responded less sensitively than the monkey Vero cells. Only acetone extracts were used, because it was found not to be toxic to fungi. In this study it was used as control and it was found not to have effect on *A. salina* nauplii and Vero cells at the concentrations used.

The results on brine shrimps indicated that the four leaf extracts have LC_{50} values above 20-30 µg/ml, the recommended cut-off point for detecting cytotoxic activity (Geran *et al*., 1972). Podophyllotoxin toxin standard had LC_{50} of 7 µg /ml, which is well within the cut-off value.

A crucial point in discussing the relevance of LC_{50} values obtained in the brine shrimp assay is the question of whether the mortality data can be tied to a more specific activity. A general correlation of brine shrimp toxicity with special types of bioactivity seems invalid. However, in various cases it has been shown to be possible. Fang *et al.* (1991) investigated the usefulness of the brine shrimp assay as an antitumour pre-screen for plant extracts and was able to determine a positive correlation between brine shrimp lethality and cytotoxicity towards 9KB cells (cell line derived from the human carcinoma of the nasopharynx used as an assay for antineoplastic agents), while Solis *et al.*, (1993) found the brine shrimp assay was not predictive for compounds requiring metabolic activation, since the brine shrimp lack the necessary cytochrome P-450 enzyme. No published work on whether brine shrimp can be used to detect specific activity of antimicrobial agents was found.

The MTT assay was done using one cell line (monkey Vero cells). It is known that different cell lines might exhibit different sensitives towards a cytotoxic compound or extracts. The use of more than one cell line is therefore considered necessary in the detection of cytotoxic compounds or extracts. Kamuhabwa *et al* (2000), used three human cells (HeLa, HT29 and A431) of different histological origin in their study and slight differences were observed.

However, in certain cell types the situation seems to be more complex than that. In a study with a cell line derived from rat brain tumours it was reported that the mitochondria are not the exclusive site of MTT reduction (Liu *et al.*, 1997). It was observed that various sub-cellular fractions could reduce MTT when supplied with NADH or NADPH and the intracellular MTT formazan granules did not accumulate in mitochondria, endoplasmic reticulum, or Golgi apparatus, but partially co-localize with endosomes or lysosomes. Furthermore, based on inhibition experiments it was concluded that the investigated cellular MTT-reductase is an N-ethylmaleimide sensitive flavin oxidase. Although these studies made the exclusive role of mitochondria in MTT reduction questionable, they did not question the validity and usefulness of the MTT assay because even if the MTT assay measures endocytosis, it would be based on a fundamental feature of living cells (Liu *et al.*, 1997).

The LC_{50} value for berberine chloride was 4.347 µg/ml, which is toxic. The normal LC_{50} of berberine is 0.141-0.148 µg/ml (Vennestrom and Klayman, 1988). *C. imberbe* had the highest LC_{50} of 168.6 µg/ml and *C. nelsonii* had the lowest which was 75.7 µg/ml. *C. albopunctactum*, *T. sericea* and *C. nelsonii* were 1.38, 1.64, and 2.23 less active than *C. imberbe*. The choice of cell number initially plated into the 96 well plate was determined, such that the control cells undergo

8-9 divisions during the incubation period before reaching 80 –90 % confluency. The number of cells in the well was ruled out as one of the causes of high values based on the above reason. Again number of cells were sufficient to enable detection of cell death and growth inhibition effects. If larger cell numbers and shorter assay times are used the cultures rapidly become confluent and cells destined to die as a result of the toxic effect of the test extract may still be metabolically active at the point where cell number is estimated. These can result in overestimation of survival and an underestimation of the toxic potential of the extracts.

The MTT assay is a well-established method used to assess mitochondrial competence (Freshney, 2000). Using this assay we found that the four extracts did not suppress mitochondrial respiration in monkey kidney cells. Only *C. imberbe* was closer to the cut-off value (200 µg/ml), which was used by other authors. However according to the criteria of the American National Cancer Institute, the LC_{50} limit to consider a crude extract promising for further purification to isolate biological active (toxic) compounds is lower than 30 µg/ml (Suffness and Pezzuto, 1990).

MTT assay formation of the formazan product correlated well with the number of surviving cells, although not always in a strictly linear fashion. The assessment of results was carefully interpreted. If surviving fraction is calculated directly from the ratio of absorbances, an estimation and not an absolute value, of the cell numbers will occur. Calibration curves of each extract were constructed, but at some point some of the values were far from the curves, maybe that is one of the reason the values were high. The MTT assay has several drawbacks: it is not readily adaptable for use with static cell populations or those of low mitochondrial activity. Certain compounds may selectively affect the mitochondria of the cells resulting in a greatly overestimated/ underestimated level of toxicity. Different cell lines are likely to give different absorbance levels when at similar degrees of confluence. Lastly MTT is mutagenic and, therefore must be handled with care.

The relative safety margin was calculated because these extracts were to be used in *in vivo* in rats. The relative safety margin indicates the number of times the effective concentration is lower than the LC_{50} concentration of the optimal extract and is calculated using the LC_{50} and MIC values. The extracts were relatively non-toxic, which means that the relative safety margin (LC_{50}/MIC) of the optimal extract was large (Table 9.2). This allows for large quantities of the optimal extract to be incorporated in treatment without causing toxic reactions. This will be discussed in detail in the following chapters.

The relative safety margins (RSM) in the MTT assay were high compared to those in the brine shrimp assay. RSM of *C. imberbe*, *T. sericea* and *C. albopunctactum* in *C. albicans* were 0.09, 0.16 and 0.19 respectively. *C. nelsonii* and *T. sericea* had high RSM values against *S. schenckii* and *M. canis*, therefore high amount of the material must be used in treatment. The *C. imberbe* acetone extract had the lowest RSM values, which means a lower amount can be used in the treatment of the test pathogen. The results of the brine shrimp assay correspond with those of the MTT assay with very low RSM values. But this was expected because the same MIC values were used.

Are these “toxic” concentrations relevant in traditional use? When most of these plants are used in traditional medicine, an infusion of about 50 g (estimated) leaves are soaked in 1 L of water for 24 h and taken orally, three times a day which is way below the detected toxicity. Even in the absence of information on the pharmacokinetics of the extracts, it is evident that the concentrations at which we observed either inhibition of mitochondrial respiration or loss of the cell membrane integrity are never relevant in traditional use. It is, however, important to know them as more concentrated forms may well be formulated as medicines even herbal medicines

9.5. Conclusion

We found that extracts of four plants used in traditional medicine are not toxic at therapeutic levels. Responses varied for the different extracts and bioassays but the brine shrimp assay generally responded less sensitively to the impact of the cyanotoxins than the monkey Vero cells. Therefore, further investigations are now needed to establish the exact mechanism of action and identify the bio-ingredients of each extract in order to explain the therapeutic efficacy, and this will be covered in the next chapters. Further extensive biological evaluations will also be carried out.