CHAPTER THREE

Effects of HC and extracts of *E. hirta* on the fibroblast MRC-5 cell line

3.1. Introduction

Asthma is one of the most common respiratory complaints in the world as it affects an estimated 300 million people worldwide. The marked increase in the prevalence of asthma has made it a worldwide health concern and global figures are expected to rise to 400 million by the year 2025 (Masoli et al., 2004).

Over the years, corticosteroids have been very effective in the treatment of asthma but serious systemic adverse effects have been associated with prolonged use of these medications (Leonard and Sur, 2002). With increasing popularity of complementary and alternative medicine, research geared towards developing more effective alternative anti-asthma agents appears to be gaining much attention.

Herbal medicines are purported to show clinical efficacy with minimal side effects compared to mainstream treatments (Chevrier et al., 2005). A number of studies support the use of herbal medicines in asthma and as reported by Ming-Chun et al., (2005), herbal medicine intervention appears to be safe and effective alternative methods for treating asthma. In addition, many pharmaceutical products have been developed from traditionally used plant products (McRae et al., 2007). Continuous investigation of how herbal medicines work could lead to the development of additional effective medication for the treatment of asthma, allergies and many other health conditions (Bielory and Lupoli, 1999). The present study aims at investigating the possible toxic effects of *E. hirta* on the MRC-5 cell line.

The MRC-5 cell line is one of the most common cell lines used for cytotoxicity studies. This cell line is even more suitable for modelling *in vitro* conditions of a

respiratory tract disease like asthma because as it is derived from normal human diploid foetal lung fibroblasts. The advantages of using *in vitro* model systems are that cellular damage is easy to measure, the system is simple and easy to reproduce, only small amounts of the compound are required and several statistically significant experiments under controlled conditions can be undertaken.

In one previous study involving the MRC-5 cell line, the biocompatibility of the Erlanger silver intravenous catheter was investigated. The possible acute cytotoxicity of extracts from the catheter was studied using MRC-5 cells and lymphocytes. The extracts were not cytotoxic to MRC-5 human fibroblasts or to sensitized phytohemagglutinin (PHA)-stimulated human lymphocytes (Greil, 1999). In another study, the physiological concentrations of the dipeptide L-carnosine were found to prolong the lifespan of MRC-5 cells and strongly reduce the normal features of senescence (Holliday and McFarland, 2000).

MRC-5 cells have also been used in many other studies including screening of medicinal plants for cytotoxicity (Zirihi et al., 2005), antimicrobial and antioxidant activities (Mensah et al., 2006); in vaccine production (Wu et al., 2006); in virology studies (McCarter and Robinson, 1997; Maes et al., 2004); as well as in inflammation studies (Hase et al., (2003). Others include carcinogenicity studies (Merlin et al., 2002; Tomatsu et al., 2003); studies on the damaging effects of heavy metals (Yang et al., 1997), stem cell research (Rieske et al., 2005) as well as studies on radiation-induced damage effects (Jones et al., 2006).

In this study, different types of bioassay systems were used to measure cytotoxicity in the MRC-5 cell line. Cell number, cell viability and lysosomal membrane integrity were determined using the Crystal Violet (CV); 3-(4, 5-

dimethylthiazol-zyl) 2, 5-dimethyl tetrazolium bromide (MTT); and Neutral Red (NR) assays. HC was used as a pharmaceutical control for the plant extract because of its known anti-asthma effects. A study by McLendon et al., (2006) showed that different cortisol concentrations did not induce structural damage in MRC-5 cells throughout the experiment.

A pilot cell culture study was carried out to determine the appropriate concentration of HC to be used as control and later, the final cell culture tests were done using the aqueous and organic (hexane, dichloromethane, acetone) extracts of *E. hirta.* The individual effects of each solvent were also evaluated.

3.2. Hypothesis

This study was carried out to test the hypothesis that extracts of *E. hirta* have limited cytotoxic effects on cultured MRC-5 cells and such effects are not only dose-dependent but vary between the different solvent extracts used.

3.3. Aims of study

- To evaluate the possible cytotoxic effects of *E. hirta* extracts using *in vitro* assays (NR, MTT and CV) to analyze the viability and lysosomal membrane integrity of MRC-5 cells as well as MRC-5 cell numbers.

- To compare the effects of the aqueous and organic solvent (acetone, dichloromethane and hexane) extracts of *E. hirta* on the MRC-5 cell line.
- To compare the effects of HC with the effects of the different extracts of *E. hirta* extract on the MRC-5 cell line.

- To provide information on the concentrations of *E. hirta* and HC that are safe to use in the animal studies

3.4. Materials

3.4.1. MRC-5 cell line

The MRC-5 lung permanent cell line is a normal, finite cell line derived from human embryonic diploid lung fibroblasts (Jacobs et al., 1970; Freshney, 2000). MRC-5 cells are common and commercially available and were obtained from Highveld Biological Company, Johannesburg, South Africa.

3.4.2. HC and *E. hirta*

HC was used as a pharmacologic control for the extracts of *E. hirta* in this study. The drug was purchased from Elwierda Apteek/Pharmacy Wirdapark, Pretoria, South Africa. One hundred milligrams (100mg) of the powdered HC were dissolved in 2ml of injection water giving a concentration of 50mg/ml. This solution has a molarity of 0.138M and the MRC-5 cells were exposed to five times progressive dilutions of HC in the following molar concentrations: (0.00 µM, 22.8µM, 3.8µM, 0.63µM, 0.16µM and 0.0263µM). The pilot study showed that the significant cytotoxic effects of HC on MRC-5 cells mostly affected the CV and NR assays.

E. hirta was obtained from fields in the Gezina area of Pretoria, South Africa and was identified and verified by staff in the Department of Botany, University of Pretoria, South Africa by comparison with a voucher specimen of the plant available at the H.G.W.J. Schweikerdt Herbarium of the University. Aqueous and organic solvent (acetone, hexane and dichloromethane) extracts of the plant used for the various experiments were prepared in the Cell Biology Laboratory of the Department of Anatomy, University of Pretoria. The aboveground parts of the plant were dried at room temperature for one week and a fine powder was then

prepared and was subdivided into four 50 g samples. Each 50 g sample was extracted with distilled water and the three different organic compounds (acetone, dichloromethane and hexane), filtered and then dried on a rotary evaporator at 40°C.

A concentration of 0.1g/ml (each 50g sample dissolved in 500ml distilled water or 500ml solvent) was prepared for each solvent and the samples were stored frozen as the stock solutions. Later the stored samples were thawed and fifty microlitres (50µl) of the working solution added to the culture wells in five times progressive dilutions to determine the effects of the different extracts on the MRC-5 cell line.

3.4.3. Media, supplements, reagents and plastic ware

Eagles Minimum Essential Medium (EMEM) powder, Hanks Balanced salt solution (HBSS) and Foetal Calf Serum (FCS) were from Highveld Biological Company, Johannesburg, South Africa. Sartorius cellulose acetate membrane filters 0.22µm were from National Separations, Johannesburg, South Africa. Fixatives, acids and organic solvents, such as glutaraldehyde, hydrochloric acid (HCl), acetic acid, isopropanol, and formic acid were analytical grade and were purchased from Merck, Johannesburg, South Africa. Streptomycin sulphate, penicillin G (sodium salt), Amphotericim B and Trypsin were obtained from Life Technologies Laboratory supplied by Gibco BRL Products, Johannesburg, South Africa. Ethylene diamine tetra acetate (EDTA), Dimethyl sulphoxide (DMSO), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), sodium chloride (NaCl) and sodium hydrogen carbonate ($NaHCO_3$) were from Merck, Johannesburg, South Africa.

MTT [1- (4, 5-Dimethylthiazol-2-yl) -3, 5- diphenylformazan] and Crystal Violet

(CV) powder were obtained from Sigma-Aldrich, Atlasville, South Africa. Bovine serum albumin (BSA) was from Boehringer Mannheim, Randburg South Africa. Water was double distilled and deionised (ddH_2O) with a Continental Water System and sterilized by filtration through a Millex 0.2µm filter. Glassware was sterilized at –140^{0}C in a Prestige Medical Autoclave (Series 2100). Twenty four (24-well) and 96-well 25cm^2 and 75cm^2 cell culture flasks, 10ml and 5ml pipettes, 15ml and 50ml centrifuge tube, micro centrifuge tubes were obtained from NUNC^{TM} and supplied by AEC- Amersham, Johannesburg, South Africa.

3.5. Methods

3.5.1. Cultivation, maintenance and preservation of the MRC-5 fibroblast cell line

The MRC-5 fibroblast cell line was maintained in EMEM supplemented with 5% FCS and 1% antibiotic solution (EMEM/ 5% FCS). An antibiotic stock solution was prepared by mixing 10,000U/ml Penicillin G (sodium salt), 10,000µg/ml Streptomycin sulphate and 25µg/ml Amphotericim B in 0.85% saline. A volume of 10 ml of the working solutions was added to 1 litre of the prepared medium. The antibiotic solution was kept at -10 ^{o}C and thawed when needed. The media was sterilized by filtration through a 0.22µm membrane filters under aseptic conditions in a laminar flow cabinet. Aliquots of 100 ml were prepared and the medium was stored at 4^{o}C and warmed to 37^{o}C before use.

The MRC-5 cells were plated at a concentration of 5X10^4 cells per ml in 25cm^2 and 75cm^2 cell culture flasks and were maintained at 37°C and 5% CO_2 in a CO_2 - water-jacketed incubator from Forma Scientific. The cell culture medium was changed every three days or when the medium had become acidic. The cells were

later passaged once confluent with a 0.05% trypsin solution. A 10X Trypsin/EDTA stock solution of 5g/l Trypsin, 2g/l EDTA.4Na was prepared by mixing 0.25g Trypsin, 0.1g EDTA and 0.425g NaCl in 50ml Hanks buffer. Hanks buffer was prepared by dissolving 9.86g/ l Hanks salt and 0.35g/l $NaHCO_3$ in ddH_2O. A working solution of 1X was prepared by diluting the stock solution with DPBS that was stored at -10 ^oC and warmed up at 37^0C before use. The solutions were filtered through a 0.22µm membrane filter under aseptic conditions.

The cells were passaged by firstly removing the medium from the confluent monolayer. A volume of 0.08 ml/cm^2 trypsin working solution was added and the flask was placed at 37^0C for 5-10 minutes. The medium containing the detached cells was added to 10 ml medium. The cells were collected by centrifugation in a BTL Bench centrifuge from Baird and Tatlock at 6000xg (2000 rpm) for 5 minutes. The medium was removed and the cells were suspended in 10 ml fresh medium. The centrifugation step was repeated and the number of cells was determined by counting a 10µl aliquot of cells using a haemocytometer from Brand supplied by Merck, Johannesburg, South Africa.

The MRC-5 fibroblasts were either used for experiments described below or stored at –70°C. For storage, the cells were suspended in cell culture freezing medium at a concentration of $5x10^6$ cells per ml. The freezing medium was prepared by adding 10% DMSO and EMEM/5%FCS. One millilitre of the cell suspension was transferred to the 1.5ml freezing vials and stored by slow freezing (the vials were wrapped with tissue paper and placed into a large Styrofoam box) in a –70°C freezer.

The cells were stored for an indefinite period of time, with minimum loss of viability. The vials containing the MRC-5 fibroblasts were thawed rapidly by stirring the vial in warm water at 37^0C. The cells were suspended in EMEM containing 5% FCS to a volume of 15 ml. The cells were collected by centrifugation, the supernatant removed and the cells suspended in fresh medium. This step was repeated twice before the cells were suspended in a final volume of 3ml culture medium and plated in 25cm^2 cell culture flasks. For each experiment, cells were plated at a cell concentration of $2x10^4$ cells per ml in 24 well plates with the culture area of 1.9cm^2/well and were kept for 24 hours at 37°C and 5% CO_2 before conducting each experiment.

3.5.2. Exposure of cells to the treatment agents

Different experiments were done using MRC-5 cells. Some cells were exposed to HC and others were treated with a 0.1g/ml concentration of each of the stock solutions (aqueous, acetone, dichloromethane and hexane). In all experiments, cells in the first set of wells represented the controls and were left untreated. A five times dilution was done for the remaining wells by first adding 50µl of the treatment agents to the set of wells next to the control wells, mixing properly before transferring 100µl by pipette from these set of wells to the next, and again removing another 100µl to the next set of wells. The process was repeated in series for all the wells and the 100µl removed from the last set of wells was discarded.

After 24 hours of exposure cell number, cell viability and lysosomal membrane integrity were determined using the combined NR/MTT/CV assay. All experiments conformed to the ethical standards of the University of Pretoria.

3.5.3. The combined NR, MTT and CV (NR/MTT/CV) bioassay

In this study, cell viability, lysosomal membrane integrity and cell number were determined almost simultaneously using the combined NR/MTT/CV bioassay of Chiba and others (Chiba et al., 1998) with only a slight modification.

Briefly, 50µl of a 50µg/ml NR dye solution was first added to each well for uptake into lysosomes of viable cells. After an incubation period of 1-2 hours, 50µl of a 2.5mg/ml MTT solution was added. The yellow water-soluble MTT dye is converted by mitochondrial dehydrogenase in viable cells to insoluble purple/blue formazan (Mosmann, 1983).

After a further 1-2 hours, the culture medium was carefully removed, the plate carefully blotted dry and the cells rapidly fixed in 1% each of acetic acid and formalin in water for a few seconds. The fixative was decanted and the plate blotted dry before 100µl of the NR solvent (1% acetic acid in 50% ethanol) was added for extraction of NR. The extracted dye was then transferred to a 96-well plate. Likewise the formazan crystals were extracted by adding 100µl dimethyl sulphoxide (DMSO) and the extracted dye transferred to the 96-well plate.

The plates were later washed with water and dried before the attached cells were stained with 1% CV for 20 minutes. The CV dye was thereafter removed, the plates washed thoroughly with tap water, dried and the dye extracted with 100µl of a 10% acetic acid solution and transferred to the 96-well plate as well. Absorbency was measured at 570 nm, 450 nm and 590 nm using an EL900 plate reader for the NR, MTT and CV assays respectively (Borenfreund et al., 1990).

3.6. Data management and statistical analysis

The average of four independent experiments was determined and the data was expressed as percentage of the unexposed control wells. Graphs were plotted to show the effects of HC and the different plant extracts on lysosomal membrane integrity, cell viability and number. Tables were combined to obtain one final graph that is a summary of all experiments. All data was expressed as mean ± standard deviation (SD) and analysed for statistical significance using the Analysis of Variance (ANOVA) statistical package. A confidence level of 95% (i.e. $p < 0.05$) was considered statistically significant.

For the CV, MTT and NR assays, a one-way Analysis of Variance (ANOVA) was used to determine the presence or otherwise of statistically significant differences between unexposed control cells and the different dosage concentrations. For the solvent only experiments, a one-way ANOVA was used to compare between control and solvent extract dosage while a two-way ANOVA was used to compare each concentration of solvent with the solvent and plant extract.

In order to determine the differences in effects between the solvent only assays and the solvent and plant extract assays, comparisons were made between group-matched concentrations. The concentration range was designated ‘0-5’ where ‘0’ represents the control concentration, ‘1’ the lowest concentration and ‘5’ the highest concentration respectively. The 2-way ANOVA gives the results of statistically significant differences between corresponding concentrations in the solvent assays and the solvent with plant extract assays respectively.

3.6.1. Pilot study with HC

MRC-5 cells were exposed to a concentration range of 0-5.56 µM HC for 24 hours after which lysosomal membrane integrity, cell viability and number was determined using the NR/MTT/CV assay. From the data presented in *figure 3.3,* it is evident that HC had no effect on cell viability (MTT assay) but did cause a decrease in lysosomal membrane integrity and cell number. At concentrations of 1.11µM and 5.56µM, a significant decrease in NR ($p \leq 0.05$) was observed while at 5.56µM, a significant decrease in CV ($p \leq 0.05$) was observed.

Analysis of the mean results obtained showed that only the two highest concentrations of HC had statistically significant effects compared with the controls for the NR assay. There was no significant difference between the different dose concentrations for the MTT assay but a statistically significant difference was seen in the CV assay between control and the highest concentration.

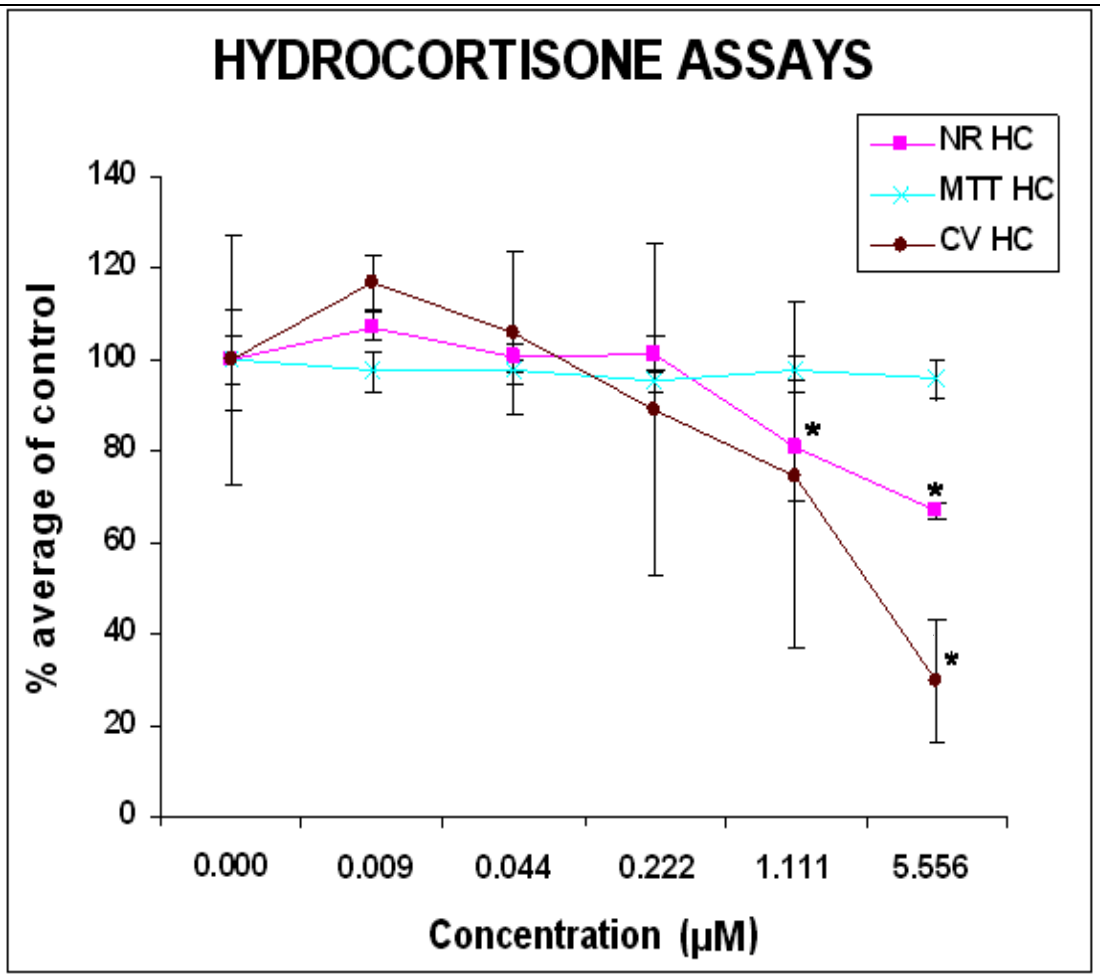


Figure 3.1: The effect of 0-5.56 µM HC on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=4) ± SD. *Differences are significant $p \leq 0.05$ compared to the least concentration.

In addition, there was no statistically significant difference between the three lowest concentrations but differences were observed between the highest NR concentration and the three lowest concentrations as well as between the second highest NR concentration and the three lowest concentrations respectively. In the case of the CV assay, statistically significant differences were seen only when the three lowest concentrations were compared with the highest concentration.

3.6.2. Treatment with the aqueous extract of *E. hirta.*

The aqueous extract of most plants generally contain lipophobic compounds such as polyphenolic compounds, triterpenes and flavonoids, and most of these compounds have potent anti-inflammatory and antioxidant properties (Lanhers et al., 1991; Sharma et al., 2007; Fecka and Turek, 2007). MRC-5 cells were exposed to 0-11.11 mg/ml of the aqueous extract of *E. hirta* for 24 hours after which lysosomal membrane integrity, cell viability and number were determined using the NR/MTT/CV assay.

The aqueous fraction of *E. hirta* had no significant effect on lysosomal membrane integrity. For the MTT assay that measures cell viability. There was a significant decrease in cell viability when the 0.02mg/ml and 0.09mg/ml concentrations were compared to the 2.22mg/ml and 11.11mg/ml ($p \leq 0.05$ and $p \leq 0.05$) respectively. However, from 0.44 - 11.11mg/ml concentrations, the aqueous extract of *E. hirta* caused an increase in value for the CV assay from 100% to 300% compared to the control ($p \leq 0.05$). This finding appeared to imply that the aqueous extract of *E. hirta* could cause a three fold increase in cell number. This is very unlikely an increase in cell number, since such an increase is usually associated with a corresponding increase in the number of mitochondria and lysosomes and therefore an increase in the values for the NR and MTT assays. It is possible that the plant proteins bind either the tissue culture surface or to the surface of the MRC-5 cells resulting in false high values.

However, the MTT assay mean showed statistically significant differences between the control and least concentration, as well as between the third highest and the highest concentration.

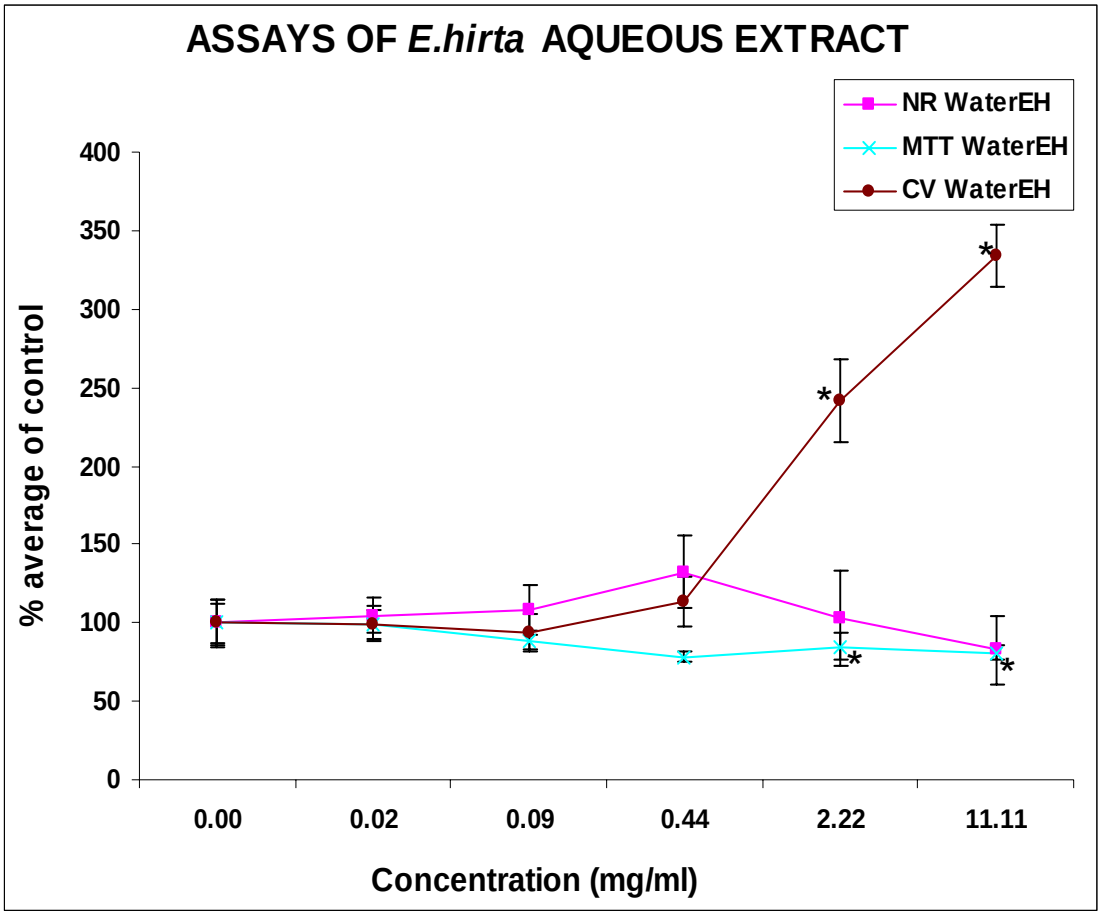


Figure 3.2: The effect of 0-0.11.11mg/ml aqueous extract of *E. hirta* on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=4) ± SD. * Differences are significant p ≤ 0.05 compared to lower concentrations and the control.

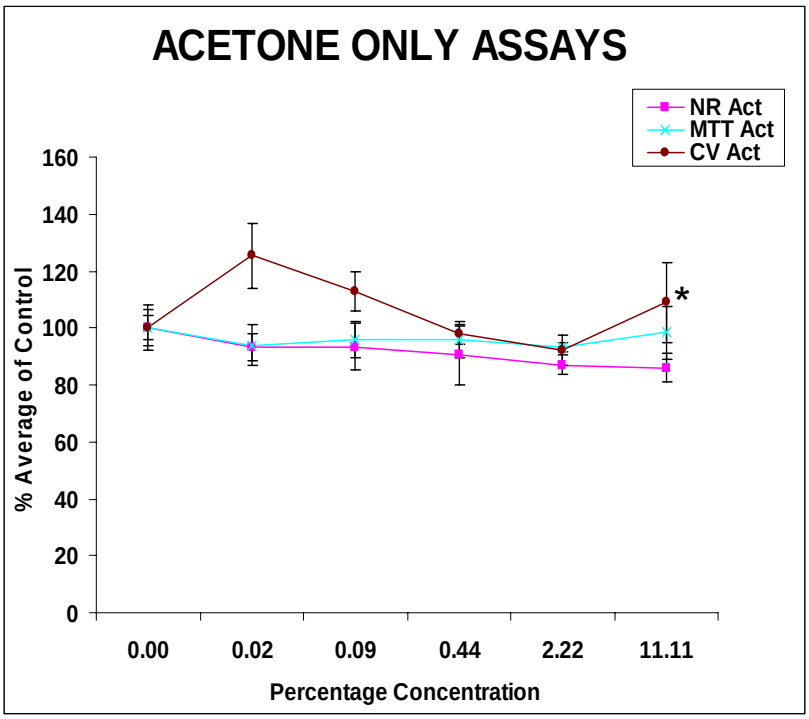
3.6.3. Effects of acetone and acetone extracts of *E hirta* on MRC-5 cells

Acetone extracts of plants generally contain glycerides, sterols, sterol esters, carotenoids, lipid soluble vitamins, monogalactoside diglycerides and sterol glycosides (Gouveia and Empis, 2003; Nyström et al, 2007). Acetone used as carrier itself can be cytotoxic to cells; therefore, the cytotoxicity of acetone in MRC-5 cells was determined. MRC cells were exposed to 0-11.11% acetone for 24 hours before lysosomal membrane integrity, cell viability and number was determined.

For acetone only assays (*Figure 3.3a*), following exposure to 11.11%, there was no significant difference in lysosomal membrane integrity and cell viability. However, the cell number appeared to rise initially to 125.4% before decreasing steadily until percentage concentration 2.22%; increasing again to 108.9% at the highest concentration. The differences found in cell number were significantly different from the controls as well as from 0.09% and 0.44% concentrations.

The MRC-5 cells in culture were also exposed to 0 - 11.11 mg/ml acetone extract of *E. hirta* (*Figure 3b*) and results show a steady progression between the different concentrations. Again, no statistical differences were observed between the control (without extract) and the different volumes/concentrations of the plant extract added to the cell culture medium of MRC-5 cells ($p \leq 0.05$ for both NR and MTT assays) (*Figure 3.3b*). Except at the 0.02 mg/ml concentration with 47% significant increase in cell number, all other differences in volumes/concentration were not significant ($p \leq 0.05$).

Two way ANOVA analysis showed significant differences in the NR and CV assay when corresponding concentrations of the acetone only and the acetone extract treatments were compared ($p \leq 0.05$ and $p \leq 0.05$)(*Figure 3.4b* and *c*). No statistical differences were observed for the MTT assay (*Figure 3.4c*).



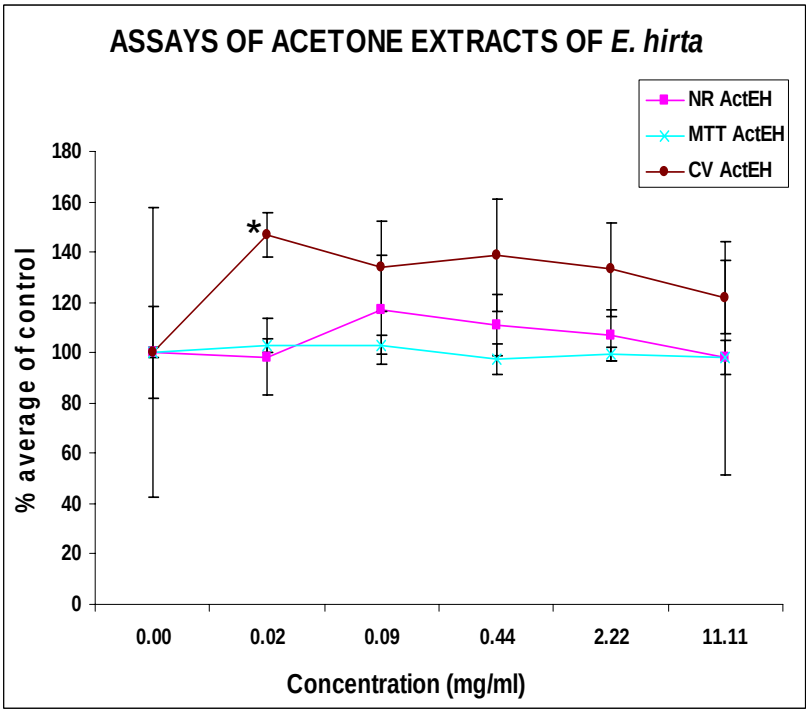


Figure 3.3: The effect of **(a)** 0-11.11% of acetone solvent, and **(b):** 0-11.11 mg/ml acetone extract of *E. hirta*, on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=2) ± SD. * Differences are significant at $p \leq 0.05$ compared to lower concentrations and the control.

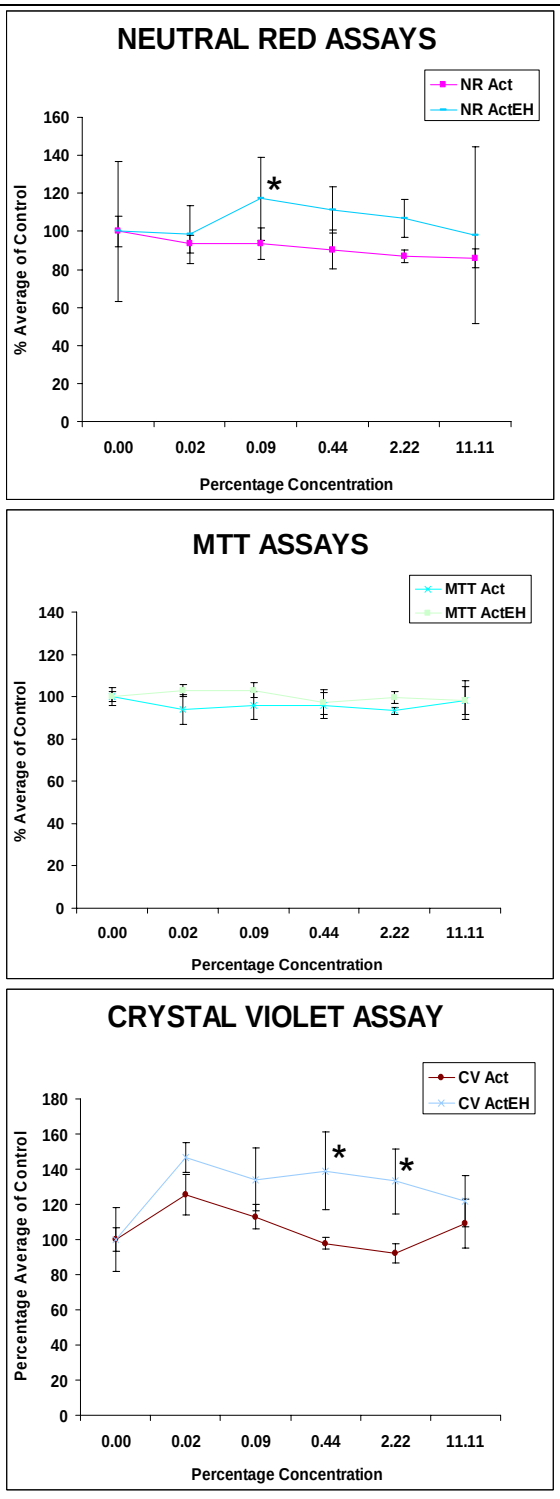


Figure 3.4: Comparison of the effects of 24-hour exposure to the carrier acetone and *E. hirta* acetone extracts on the MRC-5 cell line; **a)** NR, **b)** MTT and **c)** CV assay. *Differences are significant ($p \leq 0.05$) for NR and CV assays, each compared to ActEH.

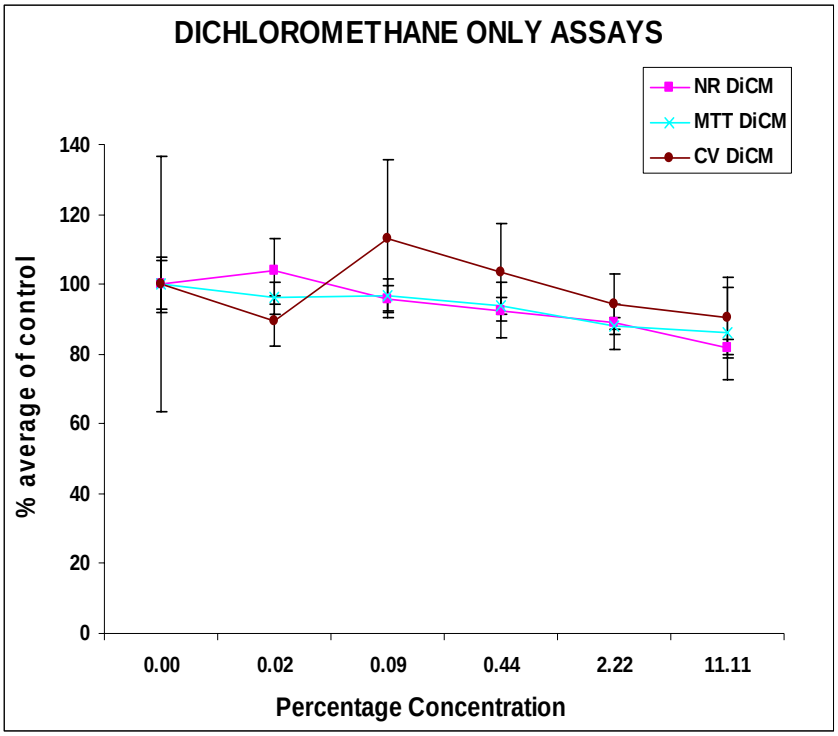
3.6.4. Effects of dichloromethane (DCM) and DCM extracts of *E hirta* on MRC-5 cells

Most DCM plant extracts usually contain sesquiterpene lactones lychnopholide, centratherin, goyazensolide and 15-desoxygoyazensolide (Grael et al., 2005), terpene and naphthalene derivatives (Jing et al., 2007) as well as β-Sitosterol, 5, 6-Trihydroxy-3′, 7-dimethoxyflavone, Daucosterin and Ursolic acid (Moghaddam et al., 2007). The MRC-5 cells were exposed to dichloromethane (DCM) alone as well as to DCM of *E. hirta.*

After 24 hours exposure to 0 - 11.11% DCM (*Figure 3.5a*) and DCM containing 0-11.11 mg/ml plant extract (*Figure 3.5b*), lysosomal membrane integrity, cell viability and number was determined by the NR, MTT and CV assays respectively.

For the DCM treatment (*Figure 3.5a*), a one-way ANOVA showed that there was no significant difference ($p \leq 0.05$) between the control (no DCM) and other concentrations for all three assays. Likewise, no significant differences were observed (*Figure 3.5b*) for all three assays of the DCM extracts of *E. hirta*.

When treatment with DCM was compared to the DCM extracts of *E. hirta* using two-way ANOVA analysis (*Figure 3.6*), no significant differences were seen in the NR and MTT assays. However, the two-way ANOVA showed that only the difference between the second highest concentrations (2.22%) of the CV assays in the two treatments was statistically significant. Comparisons between the two treatments at other concentrations were not statistically significant.



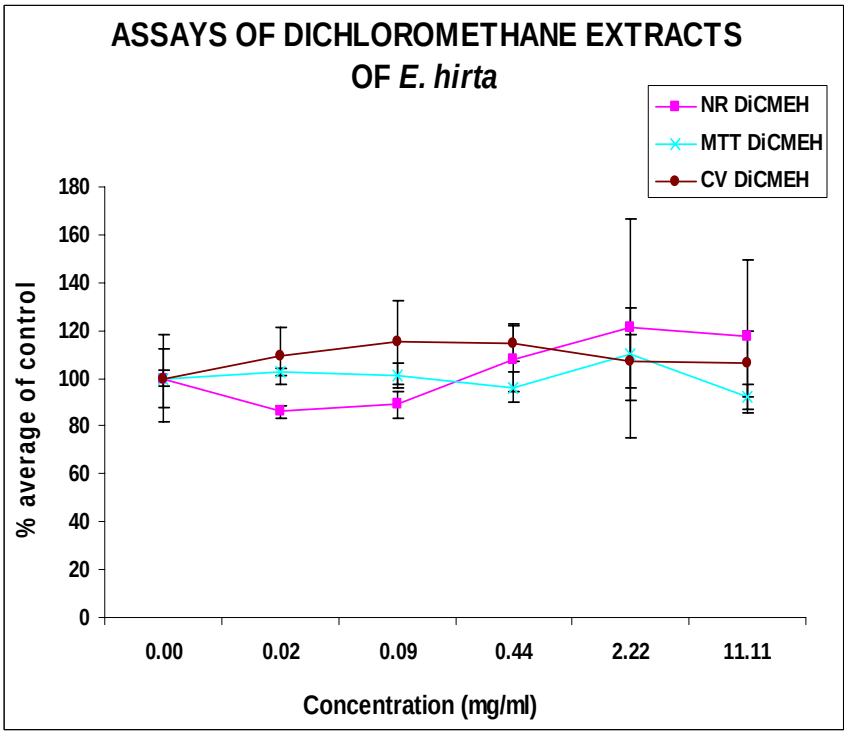
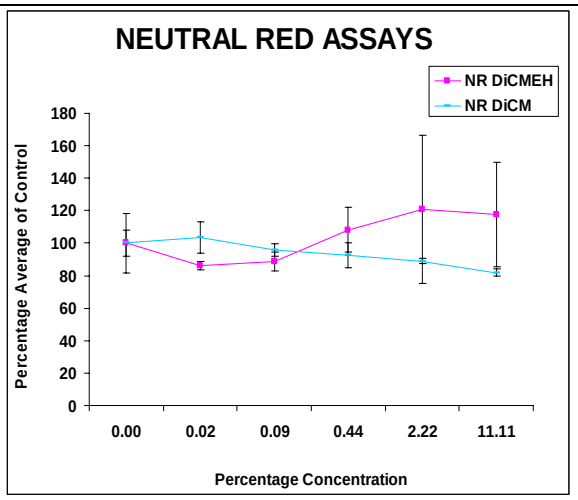
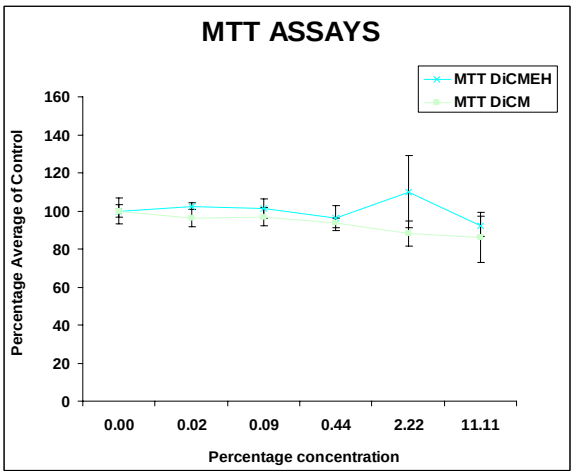


Figure 3.5: The effect of **(a)** 0-11.11% DCM and **(b)** 0-11.11 mg/ml DCM extract of *E. hirta*, on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=2) ± SD. *No significant differences at all concentrations.





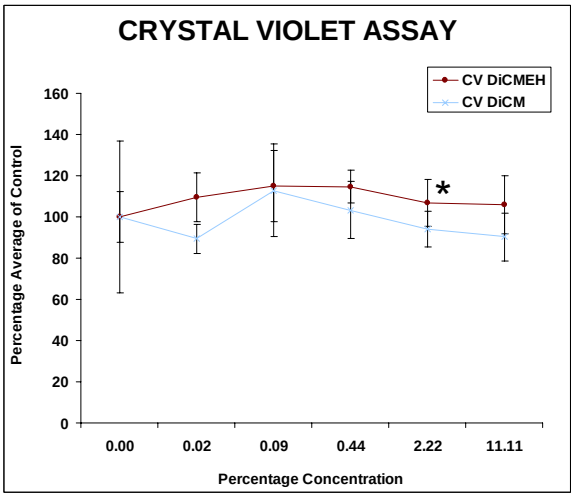


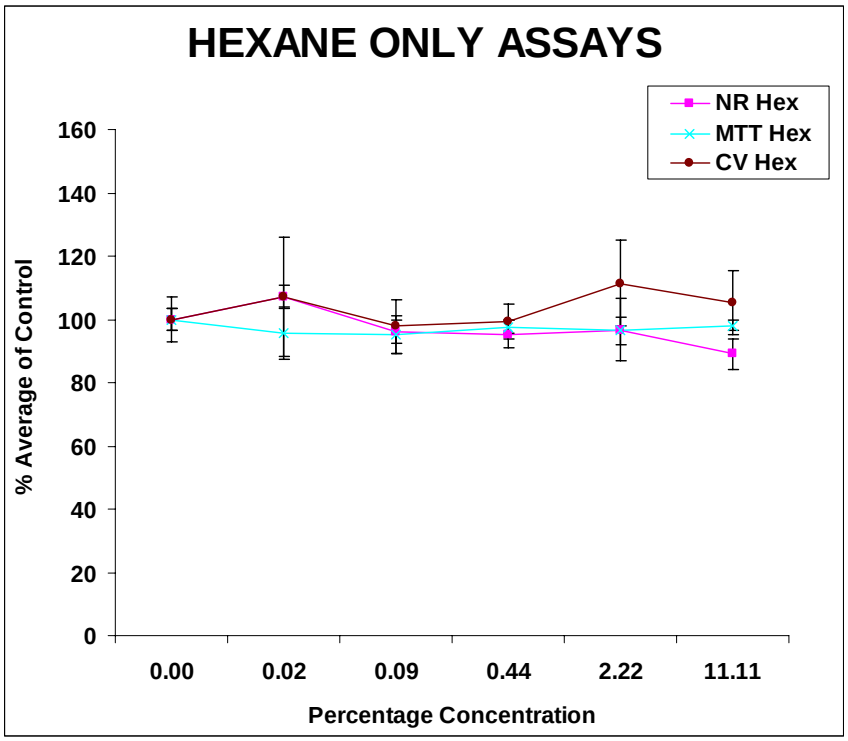
Figure 3.6: Comparison of the effects of 24-hour exposure to the carrier DCM and DCM extracts of *E. hirta* on the MRC-5 cell line; **a)** NR, **b)** MTT and **c)** CV assay. * Differences significant at $p \leq 0.05$ compared to the highest concentration.

3.6.5. Effects of hexane and hexane extracts of *E hirta* on MRC-5 cells

In most plant extracts, hexane extracts mainly contain terpenes, terpenic alcohols, terpenic aldehydes and ketones, xylene, dimethylformamide, and hexadecanoic acid (Quinn et al., 2007). The hot hexane extract of *E. hirta* was found to contain alpha and beta amyrin, sitosterol and taraxerone (Atallah and Nichola, 1972).

MRC-5 cells were exposed *in vitro* to 0-11.11% hexane alone as well as 0 - 11.11mg/ml hexane extracts of *E. hirta* for 24 hours. The effects of the hexane solvent alone on lysosomal membrane integrity, cell viability and number were determined using the combined NR/MTT/CV assays. Each assay in the two different experiments was tested for possible statistically significant differences between the control (no hexane or hexane extract of *E hirta*) and the different concentrations added. A two way ANOVA was used to determine differences between hexane alone and hexane *E. hirta* extract.

The one-way ANOVA test showed that treatment of MRC-5 cells with the hexane solvent alone did not show statistically significant differences between the different concentrations for the NR, MTT and CV assays (*Figure 3.7a*). Similarly, treatment with the hexane *E hirta* extract showed no significant differences at all concentrations for all three assays following (*Figure 3.7b*).



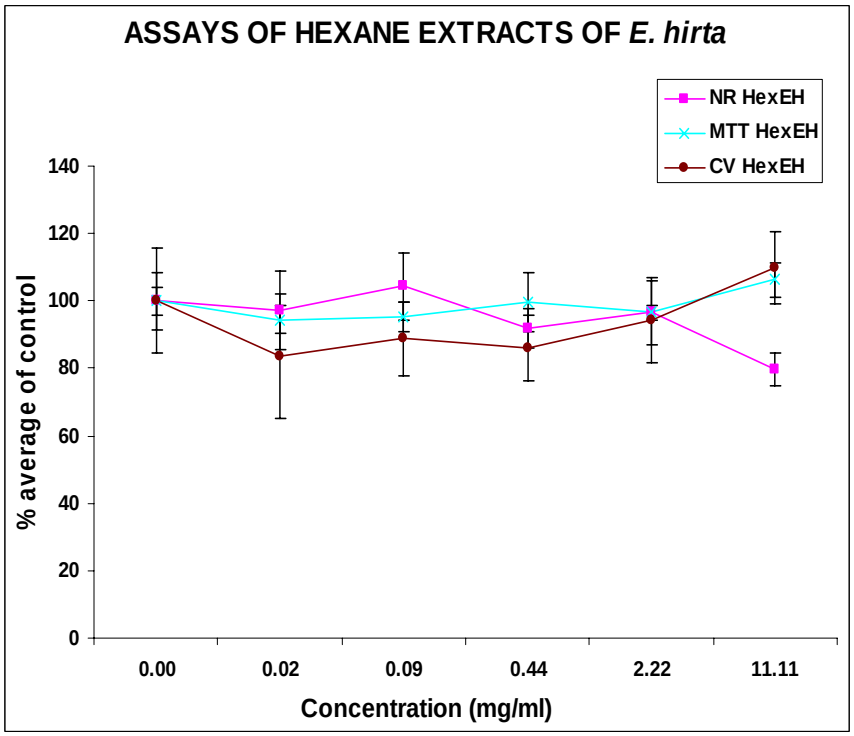
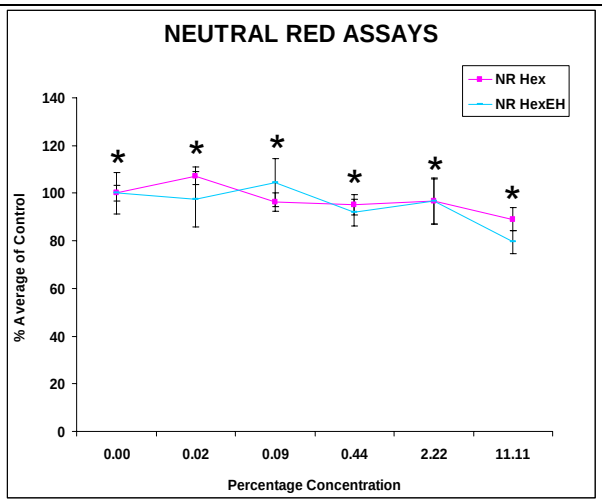
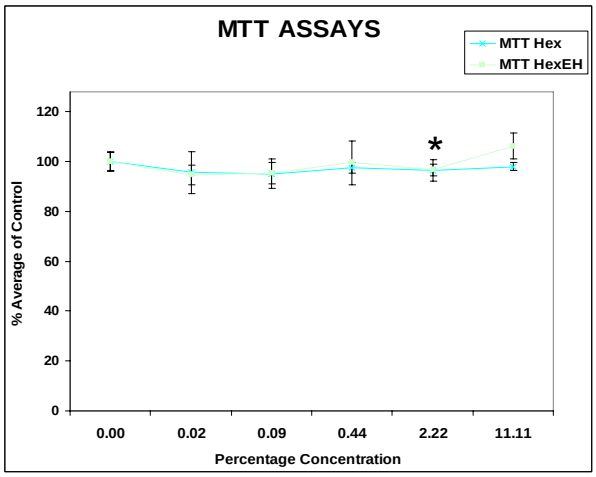


Figure 3.7: The effect of **(a)** 0-11.11% carrier hexane and **(b)** 0-11.11 mg/ml hexane extract of *E. hirta* on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=2) ± SD. No significant differences at all concentrations.

Comparison between the hexane-treated MRC-5 cells and those treated with the hexane extract of *E. hirta* was done for the different assay types (*Figure 3.8a-c*) using the 2-way ANOVA analysis. Corresponding NR values showed statistically significant differences ($p \leq 0.05$) at all the concentrations compared. On the other hand, no statistically significant differences were observed between the hexane only and the hexane extract treatments in the MTT assays except at 2.22% concentration. This appears to indicate that the effects of MTT were relatively similar in both treatments.

For the CV assays, all percentage average of control values in the hexane only treatment were higher than corresponding values of the hexane extract treatment at the same concentration range (except the highest concentration), as shown in *Figure 3.8c*. The differences between these values were also statistically significant ($p \leq 0.05$) except at the highest percentage concentration (11.11%). At this concentration, the value of the percentage average of control for the 'hexane only' treatment decreased while that for the hexane extracts treatment increased.





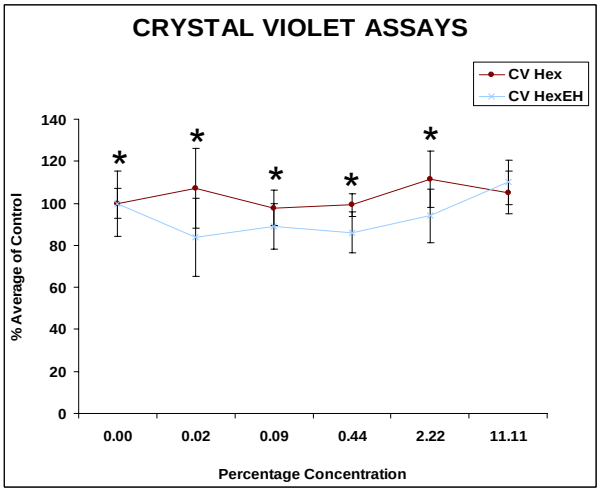


Figure 3.8: Comparison of the effects of 24-hour exposure to hexane and hexane extracts of *E. hirta* on the MRC-5 cell line; **a)** NR, **b)** MTT and **c)** CV assay.* Differences are significant ($p \leq 0.05$) as indicated for hexane only, compared to the hexane extract.

3.6.6. Analysis of the effects of the different solvents, compared with their *E. hirta* extracts and solvent extracts compared with water extract.

A summary of all the effects of treatment of MRC-5 cells with different solvents and extracts of *E hirta* as determined using the NR/MTT/CV assays is given in *table 3.1:*

Table 3.1: Effects of all solvent and *E hirta* extractas determined by NR/MTT/CV assays.

Solvent only				
Assay	**Water**	**Acetone**	**DCM**	**Hexane**
NR	NA	NSD	NSD	NSD
MTT	NA	NSD	NSD	NSD
CV	NA	SD (⇑ highest conc only)	NSD	NSD
***E hirta* Extract**				
Assay	**Water**	**Acetone**	**DCM**	**Hexane**
NR	NSD	NSD	NSD	NSD
MTT	SD (⇓ highest conc only)	NSD	NSD	NSD
CV	SD (⇑ highest two concs. only)	SD (⇑ lowest conc only)	NSD	NSD
Solvent versus solvent *E. hirta* extract				
Assay	**Water**	**Acetone**	**DCM**	**Hexane**
NR	NA	SD (⇑ in the 2nd lowest conc only, compared to ActEH)	NSD	SD in all (⇑ for hexane in all except 2nd lowest conc)
MTT	NA	NSD	NSD	SD (⇓ for hexane; 2nd highest conc. only)
CV	NA	SD (⇑ 2nd and 3rd highest concs only, compared to ActEH)	SD (⇑ in the 2nd highest conc only, compared to DCM)	All SD except highest conc (⇑ for hexane)

Key: **⇓ = decrease** **⇑ = increase**

3.6.6.1. Comparative effects in the NR assays

Only the differences between the effects of acetone and the acetone extract at the second highest concentration (2.22mg/ml) as well as all comparisons between hexane and hexane extracts were statistically significant ($p \leq 0.05$). Comparisons between hexane and hexane extracts all showed statistically significant differences.

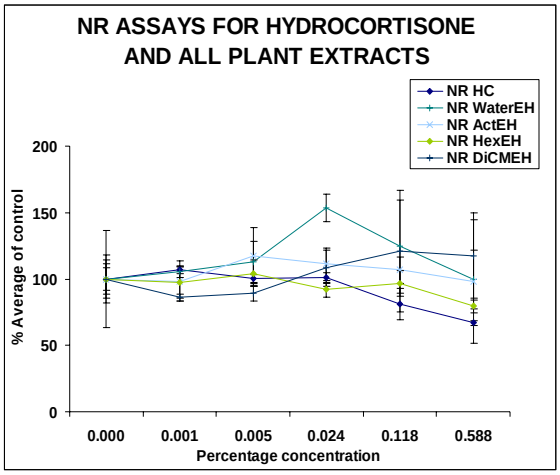
3.6.6.2. Comparative effects in the MTT assays

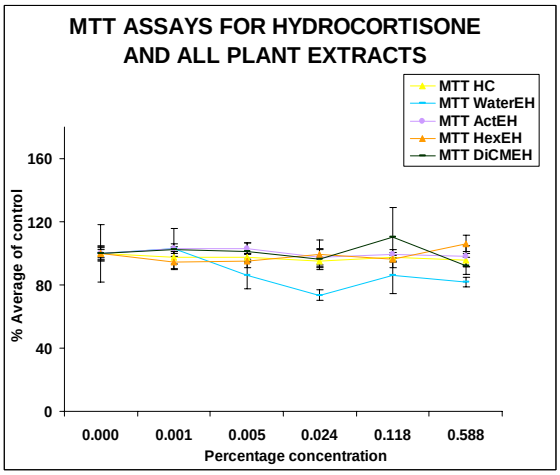
Only the highest two concentrations (0.118 mg/ml and 0.588 mg/ml) of the aqueous extracts of *E. hirta* were significantly different ($p \leq 0.05$) when compared to the control. In addition, the second highest concentrations (2.22 mg/ml) of the hexane and hexane extract of *E. hirta* showed statistically significant difference ($p \leq 0.05$).

3.6.6.3. Comparative effects in the CV assays

Most of the comparisons in the CV assays showed statistically significant differences. Statistically significant difference ($p \leq 0.05$) was observed in the acetone only treatment when the highest concentration was compared with the control as well as in the acetone extracts treatment when the lowest concentration was compared with the control. The highest two concentrations in the aqueous extract treatment also showed significant differences ($p \leq 0.05$) compared with the controls. In the hexane only treatment, all comparisons except at the highest concentration showed statistically significant differences ($p \leq 0.05$). In addition, only the second and third highest concentrations in the acetone versus acetone extract as well as the second highest concentration in the dichloromethane versus

dichloromethane extract comparisons showed statistically significant differences ($p \leq 0.05$).





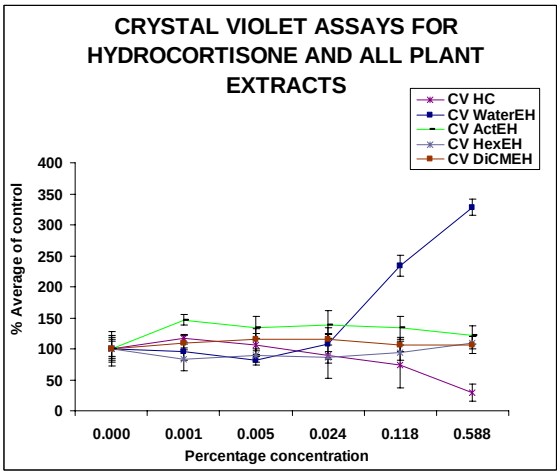


Figure 3.9a-c: Comparison of the effects of 24-hour exposure to HC, aqueous, acetone, DCM and hexane extracts of *E. hirta* on lysosomal membrane integrity, cell viability and number measured using the NR/MTT/CV assay. Data expressed as mean (n=2) ± SD. * Differences are significant at $p \leq 0.05$ compared to the hydrocortisone treatment.

3.6.7. Comparison between all organic solvent extracts and the aqueous extract of *E. hirta*.

At all concentrations and in all three assay systems used in this study, all the organic solvent (acetone, DCM and hexane) extracts used in this study generally showed statistically significant differences ($p \leq 0.05$) when compared with the aqueous *E. hirta* extract (*Figure 3.9a-c*). Some differences in the NR and CV assays in the acetone and water extract comparisons were however not significant at some concentrations (*table 3.2*).

NR values were relatively higher at most concentrations of all solvent extracts, indicating less toxic effects on lysosomal membrane integrity. However, most of the values for the MTT were much lower at all concentrations, indicating lower cell viability with the aqueous *E. hirta* extract treatment.

In the CV assays, there appeared to be a spike in cell numbers (that significantly differed from results obtained in the CV assays with organic solvent extracts) following addition of 2.22 mg/ml and 11.11 mg/ml aqueous *E. hirta* extract. The observed spike in cell numbers could not possibly be due to cell number increase and so the aqueous *E. hirta* extract could be said to have similar effects on cell number as other extracts.

3.6.8. Comparison between treatment with HC and treatments with all three organic solvent extracts of *E. hirta*.

Most herbal preparations of *E. hirta* used by traditional healers for the treatment of asthma are in aqueous solution, hence the need to investigate the effects of the aqueous extract. The organic solvent extracts were used in this study to determine their suitability for use. Different organic solvents may have effects that differ greatly as observed in previous studies (Jung et al., 2006), from the effects of the water extract or known anti-inflammatory medications like HC.

The effects of each of the different *E. hirta* extracts used in this study were compared with the effects of HC on the MRC-5 cells for every concentration of each assay type. The two-way ANOVA test was used to determine statistically significant differences (at $p \leq 0.05$) while graphs of the percentage averages of control plotted against concentrations (*figure 9a-c*) were used to compare the effects of the treatments.

3.6.8.1. Comparative effects in the NR assay

Figure 3.9a shows that for the NR assays, the hexane extract followed by the acetone extract, had effects that were similar in pattern to the effects of HC at all concentrations. The water extract showed a wide variation in pattern while the dichloromethane extract showed a sigmoid graphical pattern.

The observed graphical differences were tested statistically and results showed that only the differences between the HC and the aqueous extract treatments at all concentrations were statistically significant. The acetone extract showed significant differences only in the second lowest concentration; the hexane extract

showed significant differences between in the two lowest concentrations while the dichloromethane extract showed significant differences only in the two highest concentrations (*table 3.2*).

3.6.8.2. Comparative effects in the MTT assay

The graphs for the MTT assays (*figure 3.9b*) show similar graph patterns for both the acetone and hexane extracts as HC for all concentrations. The water extract showed a marked variation in pattern while the dichloromethane extract only showed variation in pattern at concentration 0.118mg/ml that later normalized at the highest concentration. In spite of the observed similarities in graphical patterns to the HC treatment, statistical analysis showed significant differences between the HC treatment and treatment with all the plant extracts at the different concentrations (*table 3.2*).

3.6.8.3. Comparative effects in the CV assay

The graphs of the acetone and the dichloromethane extract treatments at all concentrations show relative similarity in the patterns for the CV assays (*figure 3.9c*). In addition, only the graphs of these two treatments appear to resemble the HC graph. The water extract had a pattern that widely varied from the HC graph like in the other two assays while the hexane extracts had a graph pattern that differed from all other treatments.

Statistical analysis showed significant differences mostly in the two highest concentrations for all the three plant extract treatments relative to the HC treatment (*table 3.2*). In case of the aqueous extract, the lowest concentrations also showed significant differences when compared. Similarly, the differences

between HC and the hexane and DCM extracts treatment were also significant at the third lowest concentrations.

3.6.9. Comparative effects between the three organic solvent extracts

Comparisons between the different organic solvent extracts of *E. hirta* showed differences that were not statistically significant at $p \leq 0.05$ in most cases. Such differences were observed mostly in comparisons between the other two extract solvents and the DCM extract. Comparison between acetone and hexane showed only one significant difference at the lowest concentration. Other comparisons are given in *table 3.2*.

Table 3.2: Summary of the comparative effects of different *E hirta* solvent extracts as well as HC, as determined by the NR/MTT/CV assays.

Aqueous versus Solvent Extracts of *E hirta*				
Assay	**Water**	**Acetone vs. Water extract**	**DCM vs. Water extract**	**Hexane vs. Water extract**
NR	NA	SD (⇑ highest 3 concs. only)	SD (All)	SD (All)
MTT	NA	SD (All)	SD (All)	SD (All)
CV	NA	SD (⇑ lowest 2 concs. only)	SD (All)	SD (All)
***E hirta* Solvent Extracts compared**				
Assay	**Water**	**Acetone vs. DCM**	**Acetone vs. Hexane**	**Hexane vs. DCM**
NR	NA	SD (highest 3 concs only; ⇑ for DCM)	NSD	SD (highest 3 concs only;⇑ for DCM)
MTT	NA	SD (2nd highest conc only; ⇑ for DCM)	SD (lowest conc. only;⇑ for ACT)	SD (highest 2 concs; lowest conc. only;⇑ for DCM)
CV	NA	SD (highest conc. only;⇓ for DCM)	NSD	SD (lowest conc. only;⇑ for DCM)
Aqueous and Solvent *E. hirta* extracts versus HC				
Assay	**Water vs. HC**	**Acetone vs. HC**	**DCM vs. HC**	**Hexane vs. HC**
NR	SD (All)	SD (2nd lowest conc. only; ⇑ for ACT)	SD (highest 2 concs. only;;⇑ for DCM)	SD (lowest 2 concs. only;⇑ for HEX)
MTT	SD (All)	SD (All)	SD (All except 2nd lowest conc.;⇑ for DCM)	SD (All)
CV	SD (All except the 2nd and 3rd lowest concentrations;⇑ for water)	SD (highest 2 concs. only;⇑ for ACT)	SD (highest 3 concs. only;⇑ for DCM)	SD (highest 3 concs. only;⇑ for HEX)

Key: **⇓ = decrease** **⇑ = increase**

3.7. Discussion

Approximately 80% of the people in the world's developing countries rely on traditional medicines for their primary health care needs, and about 85% of traditional medicine involves the use of plant extracts (Vieira and Skorupa, 1993). Since most of these traditional plant remedies are used without expert prescriptions, there is need for scientific validation as well as *in vitro* and/or *in vivo* evaluation to understand their claimed effectiveness. Over the years, cell culture systems have provided the means for such scientific validation, commonly by evaluating medicinal plants for cytotoxicity (Zirihi et al., 2005).

The MTT assay is based on the reduction of the yellow coloured 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) by the enzyme mitochondrial dehydrogenase of metabolically active cells to a blue formazan which can be spectrophotometrically measured. Using the MTT colorimetric assay, the percentage of survived cells after treatment with the plant extract could be determined. Thus, the MTT assay measures cell viability.

The neutral red (NR) assay depends on the uptake of the neutral red dye solution into the lysosomes of viable cells and this uptake is proportional to the number of viable cells within the well and is usually expressed as percentage uptake by control cells. Thus, the NR assays measure lysosomal membrane integrity. The CV assay is a simple assay useful for obtaining quantitative information about the relative density of cells adhering to multi-well cluster dishes. The crystal violet dye stains DNA and upon solubilization, the amount of dye taken up by the monolayer can be quantitated in a spectrophotometer or plate reader. Thus, the CV assay measures protein levels and is an indirect measure of the cell number.

This study was carried out to test for the cytotoxic effects of the different extracts of *E. hirta* on the MRC-5 cell line using the NR, MTT and CV bioassay systems. The aqueous and organic solvent extracts (acetone, hexane and dichloromethane) were used. Separate experiments were carried out with the organic solvents alone to determine their individual effects and compare them with the effects of their corresponding plant extracts. In addition, the effects of the different extracts were compared with the effects of HC.

HC was chosen as a pharmaceutical control for the plant extract because of its known anti-asthma effects. The pilot study showed that HC like other treatment agents caused statistically significant differences between most of the concentrations in the NR assay indicating that its effect on lysosomal membrane integrity was concentration dependent.

The differences observed between the concentrations in the MTT assays of most of the treatments in this study were not statistically significant, implying that cell viability was not dependent on concentration difference. The crystal violet (CV) assays however showed statistically significant differences between concentrations mostly in the water, HC and acetone treatments, indicating that cell number differences could be concentration dependent.

The concentration dependence of the effects of treatment agents in bioassay systems has been previously studied. Findings from this particular study are similar to many reported findings from previous studies. In a study by Castro-Garza et al., (2007) the effects of Triton X-100 (known to dissolve the cell membrane leading to cell death) on a macrophage (THP-1 cell line) monolayer were investigated. The study found that the effects depended on the concentration

used, starting with a 0.05% concentration and reaching a maximum at 0.1%, measured with the CV assay. Another study by Greil et al., (1999) reported that a polyvinyl chloride (PVC) extract concentration of 15% killed MRC-5 cells completely but did not cause a decrease in the neutral-red uptake of the MRC-5 cells in the NR test. In addition, PVC-extract concentrations of 4.5% or less showed no cytotoxicity on MRC-5 cells in the MTT test.

The biological effects of the individual organic solvents used to prepare the plant extracts (acetone, dichloromethane and hexane) appear to differ from the effects of the plant extracts prepared with them. Results from this study show that acetone treatment differed significantly ($p \leq 0.05$) from treatment with the acetone extract of *E.hirta*. Similar results were observed for the hexane solvent versus hexane extract comparisons but the differences between the dichloromethane solvent and the DCM extract were not statistically significant.

A previous study showed that out of all the active solvent extracts, the DCM extracts of *Baccharis grisebachii* and *Pluchea sagittalis* had the greatest inhibitory activity (Pérez-García et al., 2001). The cells were stimulated using hydrogen peroxide, phorbol-12-myristate-13-acetate (PMA) or formyl-methionyl-leucyl-phenylalanine (FMLP) for reactive oxygen species (ROS) generation and sodium nitroprusside (SNP) or PMA for reactive nitrogen species (RNS) generation

In the present study, the effects of the different organic solvent extracts were also found to differ significantly ($p \leq 0.05$) from those of the aqueous extract. Observations of differences in effects of the different extracts of the same plant as seen in this study are consistent with previous studies. One such study by Šipailienė et al., (2005) showed that the methanol extract had the most effective

antimicrobial activity followed by the acetone extracts whereas the aqueous extracts either showed the lowest effectiveness or did not possess inhibitory effect in all eight bacterial test cultures studied.

3.8. Conclusion

At the concentrations used in this study, treatment of MRC-5 cells with HC in the pilot study showed toxic effects with increasing concentration. On the other hand, acetone, dichloromethane and hexane solvents showed very limited toxic effects on the MRC-5 cells *in vitro*, and less toxic effects were observed when the cells were exposed to the acetone, dichloromethane and hexane extracts of *E. hirta*. However, the aqueous extract showed the least toxic effects compared to all three solvent extracts.

The above results prove the hypothesis set for this cell culture study. Further studies done with different assay probes at different concentrations of the plant extracts or using the individual active compounds, as well as mechanism-of-action studies will be required.