Chapter 4

Comparative cytotoxicity studies of extracts of selected medicinal plants on different cell types

4.1. Introduction

Naturally derived plant products with medicinal value play an important role in health care systems, both in humans and animals. This is evident with the growing interest in their utilisation on a global perspective in the treatment of different ailments (Farnsworth and Morris, 1976; Farnsworth and Soejarto, 1985; Farnsworth, 1988; Balandrin, *et al.,* 1993). In the crude form, medicinal plants contain a diverse structural array of compounds with varying chemotherapeutic relevance that is harnessed traditionally through various modes of preparation. However, our current perspective of the cytotoxic effects of most medicinal plants utilised in traditional health systems is at a rudimentary stage including their long term effects on the majority of the population in resource poor settings who rely on these plants for solving their health problems.

The utilization of medicinal plants for the treatment of various ailments and the actualization of a non-toxic effect of these remedies depends on the contribution of various organs in the body. Even though the liver is the principal organ of metabolism-mediated clearance, the kidney possesses a distinctive physiology and metabolic pathways (Lohr *et al*., 1998) that help in the uptake, metabolism and elimination of various drugs and other chemicals from the body. A breakdown in the normal functioning of the kidney will result in renal selective toxicity, leading to the accumulation of these chemicals within the cells of the kidney.

The skin on the other hand is directly exposed to ultraviolet light, ozone and other environmental stress. These stress conditions can result in the generation of free radicals and reactive oxygen species (ROS) which are considered to be involved in inflammatory disorders and aging of the skin (Cross *et al.,* 1987). The skin is the first area of contact of any topically applied substance. Skin disorders such as burns, wounds, psoriasis, eczema, and fungal infections are some of the diseases for which traditional medicine has played a significant role and the relevance of the practice remains high (Alemayehu, 2001; Subbarayappa, 2001). Metabolism of substances in the

skin may play a role in the manifestation or amelioration of adverse effects through the topical route. It is of relevance under these circumstances to examine the effect of substances on the epidermis and dermis owing to the fact that different pathologies affect different layers, and the different layers of the skin have different functions.

A practical approach is needed for the safe evaluation of medicinal plants that can have potentially fatal adverse effects and have been presumed to contain acceptable toxicity profiles due to long term use. The proposition that drug toxicity should not only be defined solely by dose – response relationship, but also as a function of pharmacology, chemistry, metabolism, environmental and genetic risk factors (Li, 2004) warrants a thorough investigation of the toxic effects of medicinal plants. Since different constituents are present in crude plant extracts, drug–drug interactions based on pharmacological properties of inherent constituents can play a significant role in the safety or cytotoxic effects of extracts. As such, the understanding that drug toxicity invariably correlates with or the lack of metabolic conversion within the body (Koppal, 2004) may serve as a useful tool in the testing of the toxic effect of medicinal plants in vitro.

In work done in the Phytomedicine Programme we have focussed on selecting plants with high antimicrobial activities. Due to the difference in polarity of solvents and type of active constituents extracted from crude plant material (Eloff, 1998 Kotze and Eloff, 2002), the possibility is high that a similar phenomenon may influence the toxic effect of a plant extract depending on the type of solvent used for extraction. In previous projects, we have usually only determined the cellular toxicity at the end of the study. In many cases extracts with very promising activity were too toxic to use in further studies. The approach in this study was to determine toxicity at an early stage to select the best species for in depth further work entailing either isolating the bioactive compounds or by manipulating the extract to increase activity. If an extract contains a general metabolic toxin it would affect fungal as well as animal cells. It would be better to have an extract with a lower activity and higher safety because that indicates selective toxicity against the pathogen. The measurement of the viability of cells in culture has been evaluated by the metabolic reduction of soluble tetrazolium salt to insoluble formazan as a means of histochemical localization of enzyme activity in viable cells (Mosman, 1983; Alley *et al*., 1986). Hence, simultaneous cytotoxicity testing using different test systems is one way of testing the toxic effect of plant extracts which can provide information on the selective activity of the test substances on pathogens. This study was therefore aimed at evaluating the toxic effects of different extracts of seven South African medicinal plant species on Vero, Crandell feline kidney and bovine dermis cells in an *in vitro* toxicity study.

The antioxidant activity of extracts was also determined to investigate a possible correlation between good antioxidant activity and lower cytoxicity.

4.2. Materials and Methods

4.2.1. Plant collection and preparation

The plants used in this study were selected based on their traditional use in the treatment of various ailments and are represented in Table 4.1, together with their ethnomedicinal indication. The plants were collected and extracted using solvents of varying polarity as described in sections 3.1 and 3.2.

4.2.2. Determination of qualitative antioxidant activity of extracts

Thin layer chromatography plates (10 x 10 cm) were spotted with 100 µg (10 µℓ of 10 mg/mℓ) of the extracts as described in section 3.4 and sprayed with 0.2% 1-1-diphenyl-2-picryl-hydrazyl (DPPH) (Sigma®) in methanol as an indicator of antioxidant activity as described in section 3.5. Masoko, *et al*., (2005).

4.2.3. Determination of cytotoxicity of extracts

The cytotoxic effect of the different extracts of plants selected for the study was evaluated on Vero, CRFK and bovine dermis cells using the method of Mosmann (1983) as described in section 3.9.

Table 4.1. Plants used in the study and their ethnomedicinal indication

Plant name	Family	Voucher specimen number	Plant part	Indication	Reference
Acokanthera schimperi (A.DC) Benth. Var.rotundata Codd	Apocynaceae	NBG 584177	Leaves	For the treatment of headache, epilepsy, amnesia, eye disease syphilis, rheumatism	Abebe and Ayehu, 1993
Carissa edulis (Forssk.) Vahl.	Apocynaceae	PBG841631		Schistosomiasis	Ndamba *et al.,* 1994
Ekebergia capensis Sparrm	Meliaceae	NBG1322	Roots	Gastritis, hyperacidity, coughing	Pujol, 1990
Podocarpus henkelii Stapf ex Dallim. & jacks	Podocarpaceae	PBG818945	Bark and Sap	Canine distemper Chest pain, gall-sickness (animals)	Watt and Breyer-Brandwijk, 1962; Dold and Cocks, 2001
Plumbago zeylanica L.	Plumbaginaceae	NBG307004	Roots	Pneumonia	Van der Merwe *et al*., 2001
Schrebera alata (Hochst.) Welw.	Oleaceae	PBG584579	Leaves	-	-

4.3. Results and Discussion

4.3.1. Effect of extracting solvents on yield of extracts

Water is mostly used in folk remedies for extraction; however, this solvent does not extract a wide range of active constituents contained in plants (Eloff, 1998c). To target polar and non-polar constituents for bioactivity testing in this study, leaves of each selected plant were extracted using hexane, DCM, acetone and methanol separately. The yield of extracts varied with the type of solvents used (Figure 4.1). In most cases, methanol extracted the highest quantity followed by acetone, and hexane the least. Only with DCM extracts of *Annona senegalensis* and *Plumbago zeylanica,* and the hexane extract of *Carissa edulis,* was the yield higher than extracts prepared with acetone (Figure 4.1). The high extraction yield obtained with methanol may be related to the possible presence of a large quantity of more polar compounds in the selected plants. It may also correlate with the season of the year with leaves containing larger quantities of carbohydrates.

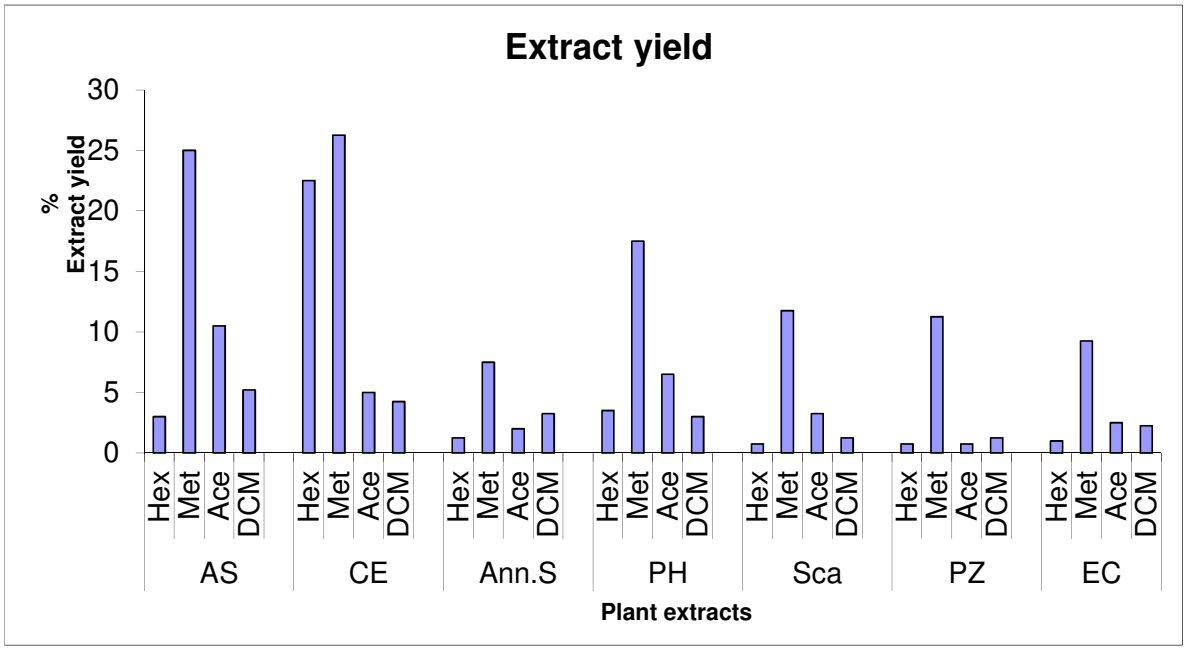

Figure 4.2. Percentage yield of plant material extracted using four different solvents for extraction. *As = Acocanthera schimperi, CE = Carissa edulis, Ann.s = Annona senegalensis, PH = Podocarpus henkelii, SCa = Schrebera alata, Pz = Plumbago zeylanica, EC = Ekebergia capensis, Hex = hexane, Met = methanol, Ace = acetone, DCM = Dichloromethane*

4.3.2. Cytotoxic effect of extracts on cells

4.3.2.1 Microscopic determination of cytotoxic effect of extracts of all the plants on different cell type

Apart from maintaining the stability of compounds in plant extracts during the extraction process (depending on whether they are thermo stable or labile), the choice of solvent used for extraction of plant material can also depend on what is intended with the extract (Eloff, 1998c). Different solvents, depending on their polarity, extract varying quantities of components in crude plant material that may be beneficial or harmful to biological systems. Hexane for instance extracts waxes, fats, and fixed oils while acetone extracts alkaloids, aglycones and glycosides. On the other hand, methanol extracts sugars, amino acids and glycosides while DCM will commonly extract alkaloids, aglycones and volatile oils (Houghton and Raman, 1998).

The cytotoxic effects of the hexane, DCM, acetone and methanol extracts of selected plants at concentrations ranging from 1 mg/ml to 0.001 mg/mℓ were tested on Vero, bovine dermis and CRFK cells by microscopic evaluation and using the MTT assay. Percent cell viability by microscopic evaluation was scored on a 5-point scale at different extract concentrations (5 = excellent and 1 = poor cell viability). According to the 5-point scale score (Table 4.2).

Table 4.2. Comparison of the cytotoxic effect of extractants at varying concentrations on different cell types based on a five point safety scale after microscopic evaluation (1–5)

Concentration (mg/ml)		1			0.1			0.01			0.001		
		Cells											
Plants		Vero	CRFK	B.D	Vero	CRFK	B.D	Vero	CRFK	B.D	Vero	CRFK	B.D
Plumbago zeylanica	Hexane	1	1	1	5	3	4	5	5	5	5	5	5
	DCM	1	1	1	1	2	1	5	5	5	5	5	5
	Acetone	1	1	1	1	1	2	5	3	5	5	4	5
	Methanol	3	1	2	3	1	4	5	5	5	5	5	5
Carissa edulis	Hexane	1	1	1	5	2	5	5	5	5	5	5	5
	DCM	1	1	1	1	2	1	1	5	3	3	5	5
	Acetone	1	1	1	5	1	5	5	3	5	5	3	5
	Methanol	1	1	1	1	5	5	4	5	5	4	5	5
Ekerbergia capensis	Hexane	1	1	1	3	5	1	5	5	5	5	5	5
	DCM	1	1	1	1	1	1	5	5	3	5	5	4
	Acetone	1	1	1	2	1	1	4	3	4	5	4	4
	Methanol	2	1	1	4	1	1	4	4	4	5	5	5
Annona senegalensis	Hexane	1	1	1	2	1	1	3	1	1	4	2	2
	DCM	1	1	1	1	1	1	4	2	1	5	2	1
	Acetone	1	1	1	2	1	1	3	1	1	4	1	1
	Methanol	1	1	1	1	1	1	1	1	1	1	2	2
Podocarpus henkelii	Hexane	1	1	1	5	1	3	5	4	4	5	4	5
	DCM	1	1	1	1	1	1	5	4	5	5	4	5
	Acetone	1	1	1	5	1	4	5	4	5	5	4	5
	Methanol	1	1	1	1	1	4	5	1	5	5	4	5
Schrebera alata	Hexane	1	1	1	5	3	3	5	5	5	5	5	5
	DCM	1	1	1	5	1	1	5	2	4	5	4	5
	Acetone	1	1	1	5	1	3	5	3	5	5	4	5
	Methanol	1	1	1	4	2	4	5	5	5	5	5	5
Acokanthera shimperi	Hexane	1	1	1	4	2	2	4	4	5	5	4	5
	DCM	1	1	1	1	1	1	1	1	1	3	1	1
	Acetone	1	1	1	1	3	1	1	4	1	1	4	1
	Methanol	1	1	1	1	3	2	1	5	5	1	5	5

To compare data for the different extractants, values for all the cell types and each plant specie at different concentrations from Table 4.2, were added (Table 4.3).

Table 4.3. Relative cytotoxicity of different extractants of plants on the different cell types and plant species at different concentrations

	Concentration (mg/mℓ)				
Extractant	1	0.1	0.01	0.001	Total
Hexane	21	65	91	96	273
DCM	21	27	72	83	203
Acetone	21	47	75	80	223
Methanol	25	50	81	89	245

In general, total values indicate the degree of toxic effect of solvent, plant or susceptible nature of cells to the different extracts. Low values represent high toxicity. In general the hexane extracts were the least toxic indicating that highly polar compounds were not toxic, possibly because they could not be absorbed through membranes. The intermediate polar extracts were generally the most toxic, possibly again because these compounds are better absorbed.

To determine which of plant had the lowest toxicity value for all the cell types at different concentrations, percent cell viability score by microscopic evaluation of the three cells of each plant specie from Table 4.2, were added (Table 4.4)

Table 4.4. Relative cytotoxicity of extracts of different extractants and cell types of different plant species at different concentrations

Plants	Concentration (mg/mℓ)				Total
	1	0.1	0.01	0.001	
Plumbago zeylanica	15	28	58	59	160
Carissa edulis	12	38	51	55	156
Ekebergia capensis	13	22	51	57	143
Annona senegalensis	12	14	20	27	73
Podocarpus henkelii	12	28	52	56	148
Schrebera alata	12	37	54	58	161
Acokanthera shimperi	12	22	33	36	103

Because low values are associated with toxicity, *Annona senegalensis* and *Acokanthera shimperi* extracts were the most toxic of plants of all the plants evaluated. (Table 4.4). These plants are toxic to animals and the cytoxicity is in line with the *in vivo* toxicity. P zeylanica and S. alata were the least toxic with C. edulis and P. henkelii having close to the same safety. C. edulis fruit are edible and the cytotoxicity data reflect this. Cells were more tolerant to the toxic effect of extracts at 0.01mg/mℓ and below in those plants that had moderate toxicity.

To determine which cells were the most sensitive all values for the different plant species and extractants from Table 4.2 were added (Table 4.5).

Table 4.5. Relative cytotoxicity of extracts of different extractants and different plant species on three cell types tested at different concentrations

Cell types	Concentration in (mg/mℓ)				Total
	1	0.1	0.01	0.001	
Vero	31	76	111	121	339
CRFK	28	49	100	111	288
B.D	29	64	108	116	317

Of the three cell types used CRFK was slightly sensitive followed by BD and Vero cells. This pattern was valid for all the concentration tested (Table 4.4). Vero and CRFK cells are both kidney derived cells, and may therefore be expected to show a similar response to the toxic effect of the extracts, but this was not the case in this study. At the highest concentration (1 mg/mℓ), all the extracts were very toxic to the cells with three exceptions where the methanol extracts were less toxic (Table 4.2). At the lowest concentration tested 0.001 mg/ml in most cases there was little cytotoxicity.

4.3.2.2. Determination of cytotoxicity by MTT assay

4.3.2.2.1. *Plumbago zeylanica*

Vero cell viability based on MTT assay following exposure to the different extracts of the same plant is presented in Figure 4.2. At the highest concentration (1 mg/mℓ), the hexane, DCM and acetone extracts of *Plumbago zeylanica* exhibited deleterious effects on the viability of Vero cells. However, with this species, the methanol extract at the

same concentration showed sustained cell viability of more than 40% (Fig. 4.2). At concentrations below 0.01 mg/mℓ, all the extracts had little effect on Vero cell viability. Unlike in the case of Vero cells, where the methanol extracts sustained cell viability, all the extracts showed deleterious effects on CRFK cells at 1 mg/mℓ (Fig. 4.3). The toxic effect at this concentration (1 mg/mℓ) was observed with the different extracts of all the tested plant extracts on CRFK cells. At concentrations where viability was sustained, a variation in the cytotoxic effect of extracts was observed between cells as could be seen with the methanol and hexane extracts on Vero, CRFK and bovine dermis cells at 0.1 mg/mℓ (Figs. 4.2, 4.3 and 4.4). At this concentration, the acetone and DCM extracts were toxic to all the cell types, with sustained cell viability only at lower concentrations (Table 4.2). Worthy of note is the cytotoxic effect of the methanol extract of *Plumbago zeylanica* on CRFK cells (Fig 4.3), which was not the case with Vero and bovine dermis cells at 0.1 mg/mℓ. A plausible reason for the observed effect may be related to the presence or lack of metabolizing enzymes that may influence the toxic effect of constituents present in extracts by CRFK cells, or a difference in bio-metabolism processes of substances in a variety of cell types. The hexane and acetone extracts of this plant at concentrations of 0.01 and 0.001 mg/mℓ showed increased cell viability of CRFK and Vero cells of more than 100% (Fig 4.2). This increase in cell viability at similar concentrations was also observed with extracts of the different plants and cell (Figs 4.2, 4.3 and 4.4). The proliferation in viable cells at these concentrations was very interesting to note. Some plant extracts may reduce MTT in the absence of cells (Shoemaker *et al*., 2004). Thus, the extracts were incubated in the absence of cells and the absorbance values subtracted from absorbance values after incubation in the presence of cells. Since medium-containing extracts were removed prior to the addition of MTT, it is unlikely that the extracts may have reduced MTT. This increase in viable cells at low concentrations may suggest a possible mitogenic effect or induction of expression of growth-stimulating substances evident by an increase in mitochondrial dehydrogenase activity as measured by MTT reduction.

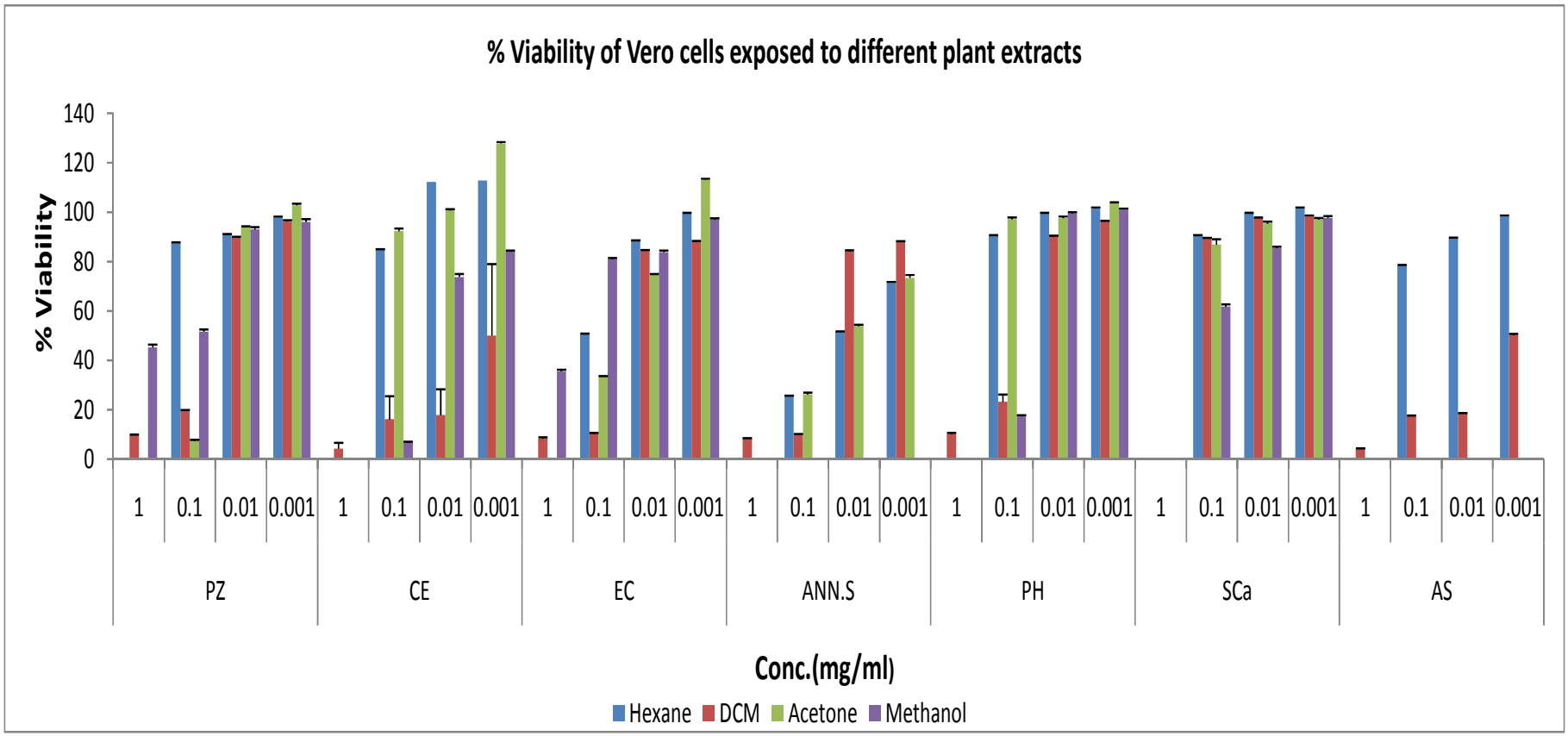

Figure.4.2: Viability of Vero cell exposed to extracts of different plant species extracted using solvents of varying polarity PZ = *Plumbago zeylanica*

CE = *Carissa edulis,* EC = *Ekebergia capensis,* ANN.S = *Annona senegalensis,* PH = *Podocarpus henkelii,* Sca = *Schrebera alata,*
AS = *Acokanthera schimperi*

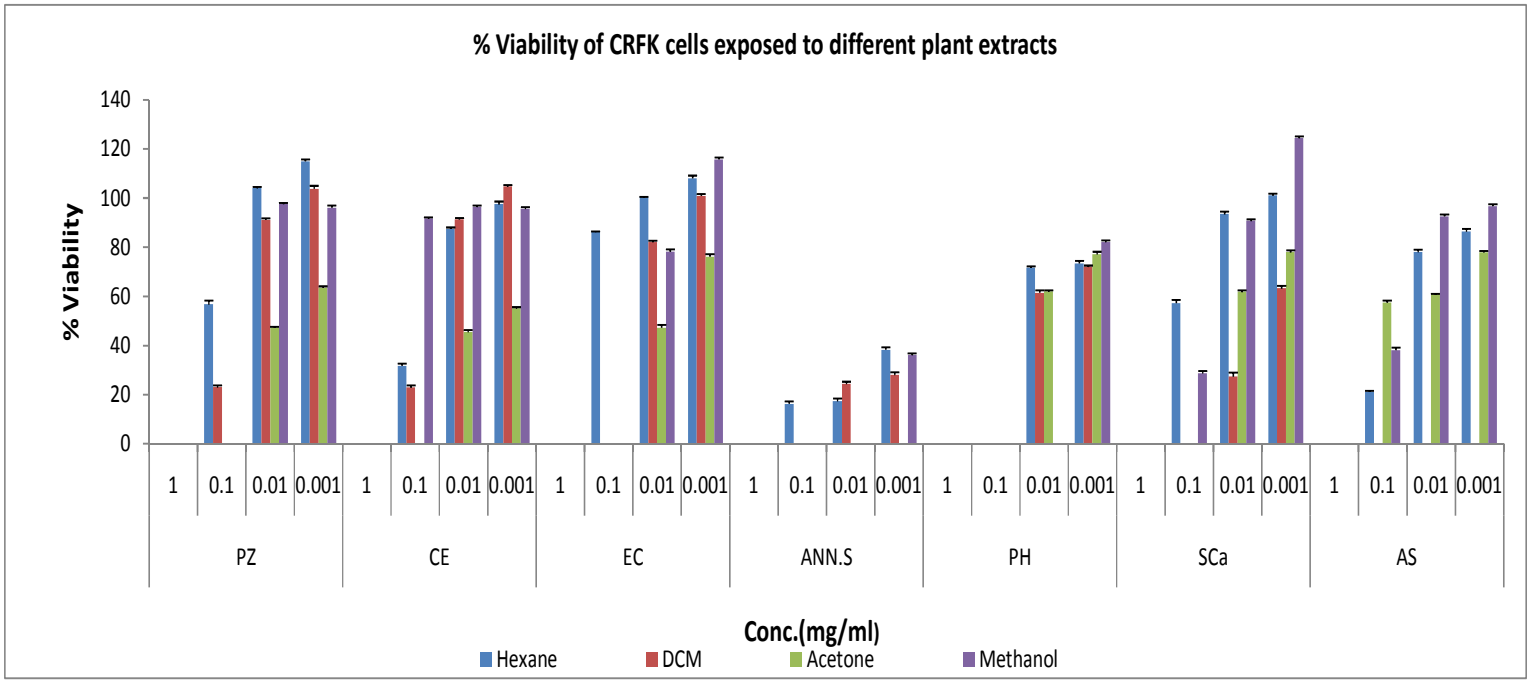

Figure 4.3: Viability of CRFK cell exposed to extracts of different plant species extracted using solvents of varying polarity, PZ = *Plumbago zeylanica,* CE = *Carissa edulis,* EC = *Ekebergia capensis,* ANN.S = *Annona senegalensis,*PH = *Podocarpus henkelii,* Sca = *Schrebera alata,* AS = *Acokanthera schimperi*

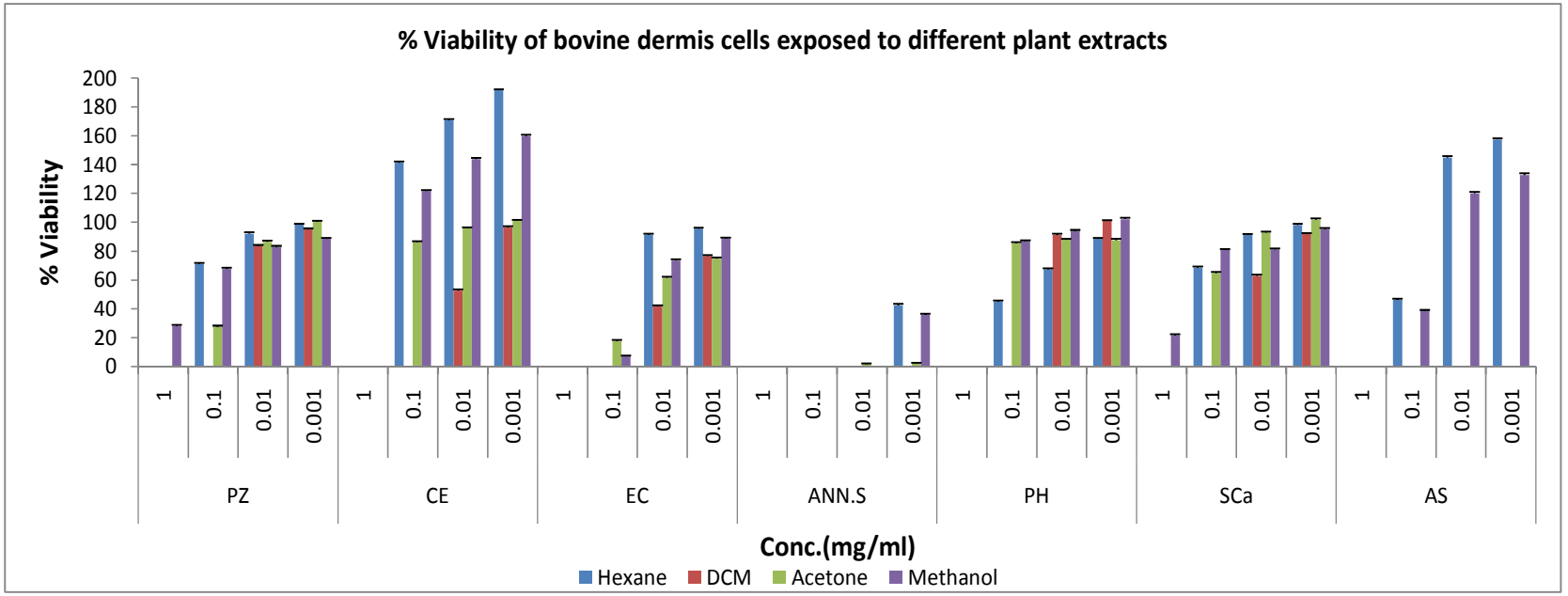

Figure.4.4: Viability of bovine dermis cell exposed to extracts of different plant species extracted using solvents of varying polarity PZ = *Plumbago zeylanica,* CE = *Carissa edulis,* EC = *Ekebergia capensis,* ANN.S = *Annona senegalensis,* PH = *Podocarpus henkelii,* Sca = *Schrebera alata,* AS = *Acokanthera schimperi*

The influence of serum on the MTT assay using cultured smooth muscle cells at 5% and 10% serum concentration has been reported (Zhang and Cox, 1996). In that study, the increase of 20% cell viability at 10% serum concentration in the MTT assay when counted using a haemocytometer led to no difference in total mitochondrial activity per cell. It is however, not clear whether the increase in cell viability observed at this concentration is due to unknown factors in serum necessary for the maintenance of the homeostatic mechanisms of the cells or a protective or inductive phenomenon resulting from the presence of one or more constituents present in these extracts. Factors that may appear to have some mitogenic activity may make the cells responsive to growth factors present in the serum-extract medium. Nonetheless, earlier studies to investigate the growth stimulating effect on cells show that growth promoting substances exhibit a high degree of specificity with varying cell types (Temin *et al.*, 1972).

4.3.2.2.2. *Ekebergia capensis*

The pattern of cell viability observed with the hexane and methanolic extracts of *Ekebergia capensis* on Vero and CRFK cells was similar to that of *Plumbago zeylanica* although percent viability and CC_{50} values varied in some extracts (Table 4.3). With this species, only the hexane extract showed viability at 0.1 mg/ml on CRFK cells while on bovine dermis cells (Fig. 4.4), none of the extracts was shown to sustain cell viability at 0.1 mg/mℓ. At lower concentrations however, all the extracts exhibited sustained cell viability on all three cell types.

4.3.2.2.3. *Annona senegalensis*

Extracts of *Annona senegalensis,* at 1 mg/mℓ and 0.1 mg/mℓ were cytotoxic to the cell types used in this study. However, at concentrations below 0.1 mg/mℓ, where the hexane, DCM and acetone extracts of this plant were toxic to CRFK and bovine dermis cells (Figs. 4.3 and 4.4), these extracts sustained the viability of Vero cells within the ranges of 40 - 80% (Fig. 4.2). The methanol extract on the other hand was toxic to all the cells at the concentrations tested (Table 4.3). It is interesting to note that despite the inhibition of cell viability by the methanol extract of this specie, the hexane, acetone and dichloromethane extracts were able to sustain moderate to excellent Vero cell viability at similar concentrations, whereas all the extracts were toxic to bovine dermis cells even at the lowest concentrations tested (Fig. 4.4).

The metabolic variation leading to susceptibility of cells to constituents present in the extracts is not very clear. It is possible that constituents cytotoxic to Vero cells in the methanol extract of *Annona senegaliensis* may not be extracted by the other solvents. Alternatively, the presence of constituents in the hexane, DCM and acetone extracts with possible protective effects on Vero cells and/or a toxic effect on bovine dermis cells cannot be ruled out. Some authors have reported the activity of many antioxidants to be higher in the epidermis than dermis of hairless mouse and human skins with the difference being greater in human skin (Shindo *et al.,* 1993). Furthermore, substances with antioxidant effects have been suggested to be helpful in the removal of reactive oxygen species (ROS) and can equally be readily oxidized in culture media with deleterious effects on cells *in vitro* (Rice-Evans, 2000; Long *et al*., 2000; Halliwell, 2003).

4.3.2.2.4. *Carissa edulis*

All the extracts of *Carissa edulis* at the highest concentration were toxic to the different cell types (Figs. 4.2, 4.3 and 4.4), while the hexane and acetone extracts at a lower concentration of 0.1 mg/ml were less toxic to Vero cells with cell viability greater than 70% (Fig. 4.2). At this concentration (0.1 mg/mℓ) except for the methanolic extract of this plant, all other extracts had deleterious effects on CRFK cells whereas on bovine dermis cells (Fig. 4.4), only the DCM extract had a cytotoxic effect. The extent of reduced cell viability of the DCM extract was even more evident on Vero cells at a much lower concentration of 0.01 mg/ml, which was not the case with the other extracts at this concentration (Table 4.2). The susceptible nature of Vero cells to DCM extracts, even at a lower concentration where the other cells showed sustained cell viability, may be indicative of the susceptible nature of Vero cells to substances present in the DCM extract of this specie. In the case of *Carissa edulis,* the presence of antioxidant compounds was only evident in the acetone extract. The acetone extracts of this plant showed excellent cell viability of Vero and bovine dermis cells at an even higher concentration of 0.1 mg/mℓ but this was not the case for CRFK cells (Table 4.2).

Both acetone and DCM can extract alkaloids and aglycones. Pascaline *et al.* (2011) in a general screening programme of medicinal plants identified the presence of alkaloids, saponins, terpenoids, glycosides and phenolics from the chloroform and methanolic extracts of *Carissa edulis.* Similarly, other authors have reported the presence of biologically active cytotoxic alkaloids from the genus *Carissa* (Ganapaty *et al.,* 2010). It is therefore likely that the

acetone extract of this plant contains substances with deleterious effects on CFRK cells that could not be ameliorated by the presence of antioxidant constituents in the extract. There could also have been a possible oxidation of the antioxidant constituents in culture media leading to deleterious effects as could be seen with CRFK cells (Table 4.2.).

4.3.2.2.5. *Podocarpus henkelii*

The variation in susceptibility of cells to different extracts was also observed with the extract of *Podocarpus henkelii.* The viability of Vero cells exposed to extracts of *P. henkelii* was similar to that observed with *Carissa edulis.* While none of the extracts of this plant at 1 mg/mℓ and 0.1 mg/mℓ showed viability of CRFK cells, only the DCM extract at similar concentrations showed deleterious effects on bovine dermis cells (Fig. 4.4). A similar trend in the susceptibility of cells to extracts of *Schrebera alata* and *Acokanthera schimperi*, was observed with all the cell types (Table 4.2).

The cytotoxic concentrations of all the plants were also calculated on the different cell types (Table 4.6). Of all the hexane extract of the different plants against all the cell types, *Carissa edulis* had the best CC_{50} value, followed by *Schrebera alata, Ekebergia capensis, Acokanthera schimperi, Podocarpus henkelii, Plumbago zeylanica* and *Annona senegalensis* in that order. DCM extracts of *Plumbago zeylanica* had the best CC_{50} value followed by *Podocarpus henkelii* and *Acokanthera schimperi* the least, while the acetone extracts of *Carissa edulis, Schrebera alata* and *Podocarpus henkelii* had the best CC_{50} value in that order. With methanol extracts *Plumbago zeylanica* and *Ekebergia capensis* had the best CC_{50} value and *Annona senegalensis* the least.

Table 4.6. CC_{50} values of different extracts of the same plant on different cell types

	Hexane			DCM			Acetone			Methanol		
	CC_{50} µg/mℓ			CC_{50} µg/mℓ			CC_{50} µg/mℓ			CC_{50} µg/mℓ		
	Vero	CRFK	Bov.Derm	Vero	CRFK	Bov.Derm	Vero	CRFK	Bov.Derm	Vero	CRFK	Bov.Derm
SCA	22	62	46	33	2	5	31	3	>1000	25	10	244
EC	43	43	14	27	14	2	30	5	12	678	4	13
PZ	36	5	21	43	44	7	32	3	14	>1000	11	243
PH	56	5	15	43	5	5	46	5	107	42	1	153
CE	76	89	>100	<0.001	6	4	>1000	>1000	71	10	28	112
ANN.S	16	1	1	27	<0.001	<0.001	8	<0.001	<0.001	<0.001	<0.001	<0.001
AS	30	30	37	<0.001	<0.001	<0.001	<0.001	50.1	<0.001	<0.001	16	52
BERB	10	9.8	3									

PZ = *Plumbago zeylanica,* CE = *Carissa edulis,* EC = *Ekebergia capensis,* ANN.S = *Annona senegalensis,* PH = *Podocarpus henkelii,* SCA = *Schrebera alata,* AS = *Acokanthera schimperi*, BERB = berberine

4.3.2.3 Antioxidant activity

To investigate the presence of substances with a protective effect acting via an antioxidant mechanism, the different extracts were analyzed for the presence of antioxidant constituents by spraying chromatograms with 0.2% 1-1-diphenyl-2-picryl-hydrazyl (DPPH) in methanol. Figure 4.5. represents those plant that had antioxidant constituents. The antioxidant constituents in some plants were highly polar and could not move from the bottom of the TLC plates following elution in different solvent systems.

Qualitative antioxidant activity studies revealed the presence of antioxidant compounds in the acetone, and methanol extracts of *Podocarpus henkelii* with more than 90% viability of Vero cells at 0.1 mg/mℓ and at 0.1 mg/mℓ, less than 20% cell viability respectively, representing a huge difference in cell viability despite the presence of antioxidant constituents in both extracts (Fig. 4.2). Because antioxidant compounds are usually relatively polar compounds it is not surprising that the more polar solvents extracted the most antioxidant compounds. Heo and Jeon (2009) illustrated the protective effect of antioxidants derived from marine algae against H_2O_2-induced Vero cell damage. Other authors have shown that structure–activity relationships of some compounds may be related to effective radical scavenging (Harborne and Williams, 2000; Op de Beck *et al.,* 2003). On the other hand, Aderogba *et al.,* (2007) demonstrated the toxic effect of a flavonol glycoside, myricetin-3-*O*-galactopyranoside isolated from *Bauhinia galpinii*, on Vero and bovine dermis cells. Although crude extracts were investigated in the present study, it is likely that the process of cell damage may not be associated with generation of free radicals, or the concentration of antioxidant was too low and could not protect the cells from the toxic constituents contained in the extract. This may explain the toxic effect of the DCM and acetone extracts of *Acokanthera shimperi* on bovine dermis and vero cells as well as the acetone and methanol extracts of *Annona senegalensis* on the viability of CRFK and bovine dermis cells.

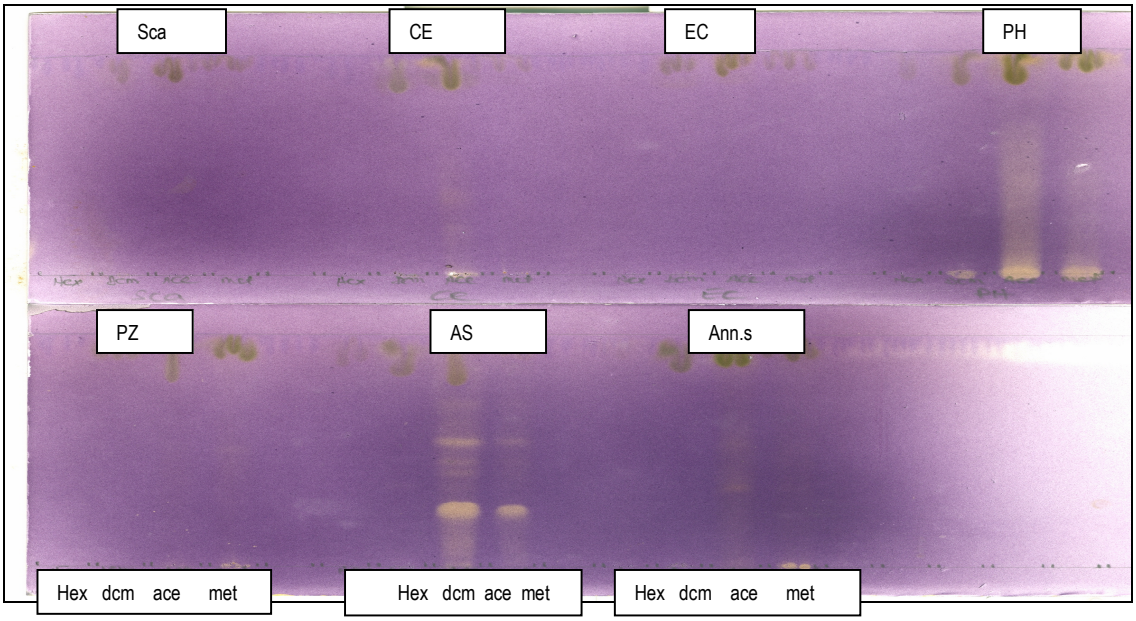

Figure 4.5. Thin layer chromatogram eluted in CEF indicating presence of antioxidant constituent in PZ = *Plumbago zeylanica,* CE = *Carissa edulis,* EC = *Ekebergia capensis,* ANN.S = *Annona senegalensis,* PH = *Podocarpus henkelii,* SCA = *Schrebera alata,* AS = *Acokanthera schimperi*

The presence of compounds with antioxidant activity in the different extracts of plants used in the study did not seem to protect the cells from the toxic effect of substances present in the extract. This may suggest that the toxicity is not related to the generation of free radicals. It may also be likely that the protective effect of constituents with antioxidant activity may be related to the type of cell in culture under study. Synergism and mechanism of action between natural products in combating antimicrobial infections is well documented (Hemaiswarya *et al*., 2008). However, this is not the case in ascertaining the principal component responsible for cytotoxic effects, especially with crude plant extracts.

4.5. Conclusion

It is frequently stated that a plant is toxic without specifying the extractant used to prepare the extract tested for toxicity. In traditional medicine, mainly aqueous extracts are used because other extractants or solvents are not available. Extracts of many plants are administered in traditional medicine without prior knowledge of their chemical composition, toxicity and efficacy. Extracts in their crude form may contain toxic principles that may not be of therapeutic relevance. This study evaluated the cytotoxic effects of different extracts of the same plant and the possible protective effect of antioxidant constituents present in the different extracts.

Between cells and plant species, hexane extracts of the different plant species was by far the least toxic on the different cell types, followed by methanol, dichloromethane and acetone the most toxic. *Annona senegalensis* and *Acokanthera shimperi* extracts were the most toxic of plants of all the plants evaluated. Hexane is a solvent of low polarity and commonly extracts waxes, fats and fixed oils (volatile oils). Although the classes of volatile oils present and the lipophilic nature of the extracts were not determined in this study, available reports suggest the non-cytotoxic effect of a majority of these classes of compounds on different cell types (Allahverdiyev *et al*., 2004; Zai-Chang *et al*., 2005; Orhan *et al*., 2009; Al-Kalaldeh *et al*., 2010). This may possibly explain why the hexane extracts had a less deleterious effect on the viability of the cells. Between extracts of the plants species and cells, *Annona senegalensis* and *Acokanthera shimperi* were the most toxic on the different cell types while *Plumbago zeylanica, Carissa edulis*, *Ekerbergia capensis, Podocarpus henkelii* and *Schrebera alata* had moderate toxicity. Chrandell feline kidney cells were the most susceptible to the toxic effect of the different plants and extracts while Vero cells were the most tolerant.

The response of cells under culture conditions, especially where the addition of substances is required for evaluation of cytotoxic effects, and the mechanisms by which these cells withstand the potential toxic effects of these substances is complex. This is further complicated by the scantiness of information available on changes in metabolic activity of mammalian cells at different cell densities. Findings in this study suggest that the protective effect of substances with antioxidant activity in culture may be related to metabolism of the type of cell in culture. The studies also show a difference in susceptibility of kidney-derived cells used in this study, which may have been in part due to the metabolic efficiency of the cells being influenced by enzymatic conversion or degradation of cytotoxic components present in extracts or due to species variation in the origin of cells. It also suggests the presence of substances in some plant extracts depending on the solvent used for extraction that may induce viable cell proliferation. It further illustrates that the choice of solvent used in extraction can have an influence on the cytotoxic potential of a given plant. This should therefore be considered in the selection of solvent used for extraction of plant materials for biological activity testing. It is interesting that many publications in the Phytomedicine Programme have shown that acetone extracts are generally by far the best extractant to detect antimicrobial compounds (Kotze and Eloff, 2002), this extractant also yielded the most toxic extracts. This may be related to the bioavailability of compounds of intermediate polarity to cells of microorganisms and animals. Due to the cytotoxic effects of *Annona senegalensis* and *Acokanthera shimperi,* they will not be considered as potential candidates as possible microbial activity that maybe observed with these extracts may be due to their toxic effect on pathogens. The next chapter will focus on the evaluation of the different extracts of the plants specie for antibacterial activity.