AN INVESTIGATION INTO THE CHEMOPREVENTIVE PROPERTIES OF AN INDIGENOUS HERB, *AMARANTHUS LIVIDUS*, USING CANCEROUS CELL LINES

BY

DONELLA JOY WRIGHT

N. Dip.: Biotechnology (TN)

B.Med.Sci (Hons) (UND)

Submitted in partial fulfilment of the requirements for the degree of Master of Medical Science in the Department of Physiology, School of Medical Sciences, Nelson R. Mandela School of Medicine, University of KwaZulu–Natal, Durban

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ABSTRACT

Chemoprevention may be defined as the inhibition, delay or reversal of carcinogenesis by dietary compounds or their derivatives. "Imifino" is a collective name for many wild plants used predominantly by rural people as herbs in cooking. Many of these herbs possess medicinal properties. As the rural population is at higher risk of exposure to dietary carcinogens, such as mycotoxins, this pilot study was undertaken to determine whether the *Amaranthus lividus* plant held potential for use in chemopreventive strategies.

The plant leaves were extracted to obtain individual solvent fractions. Cytotoxic profiling of the fractions using the SNO oesophageal adenocarcinoma cell line and normal human lymphocytes was achieved using the methylthiazol tetrazolium salt bioreduction assay. The SNO cell line, the A549 lung adenocarcinoma cell line and normal human lymphocytes were utilised for the evaluation of the anti–mycotoxigenic potential of the plant fractions in combination with two important dietary carcinogens, aflatoxin B_1 and fumonisin B_1. A specific biomarker assay (the induction of reduced glutathione) was employed using the SNO cell line. Flow cytometry was also conducted to determine the apoptotic properties of the acetone fraction on normal human lymphocytes.

The results of the anti–mycotoxigenic study showed that certain fractions did have protective effects against both of the carcinogens tested. In addition, these effects were noted in the two cancerous cell lines, which were of different tissue origin. None of the fractions tested were toxic towards the normal human lymphocytes. The glutathione assay indicated that certain acetone fraction dilutions were inducive to reduced glutathione production.

This plant is a promising candidate for further investigation concerning chemoprevention and the rural community could be educated on the possible benefits of this herb.

AUTHOR'S DECLARATION

The experimental work presented in this manuscript represents the original work of the author and has not been submitted to any other university or tertiary institution. The contributions of others have been duly acknowledged in the text.

The research described in this study was carried out under the supervision of Professor AA Chuturgoon in the Department of Physiology, Nelson R. Mandela School of Medicine, University of KwaZulu–Natal, Durban, between July 2001 and September 2003.

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AF	acetone fraction
AFB_1	aflatoxin B_1
AFB_2	aflatoxin B_2
AFBO	aflatoxin B_1–8,9–epoxide
AFG_1	aflatoxin G_1
AFG_2	aflatoxin G_2
AFM_1	aflatoxin M_1
AFM_2	aflatoxin M_2
AFP_1	aflatoxin P_1
AFQ_1	aflatoxin Q_1
ANOVA	analysis of variance
B[*a*]P	benzyl–*a*–pyrene
^oC	degrees Celsius
Ca^{2+}	calcium cation
CANSA	Cancer Association of South Africa
cc	cubic centimetres
CCM	complete culture medium
CF	combination fraction
cm	centimetre
cm^3	cubic centimetres
CO_2	carbon dioxide
CV	cell viability
CYP	cytochrome P_{450}
DBP	dibenzo[*a*,*l*]pyrene

DCM	dichloromethane
ddH_2O	double distilled water
DLA	Dalton's lymphoma ascites
DMBA	9,10–dimethyl–1,2–benzanthracene
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EAC	Ehrlich ascites cells
EC	embryonal carcinoma
EDTA	ethylenediaminetetra–acetic acid
ELEM	equine leucoencephalomalacia
ELISA	enzyme linked immunosorbant assay
em	emission
EMEM	Eagles minimal essential medium
eq.	equivalent
EtOH	ethanol
ex	excitation
FA	fusaric acid
FB_1	fumonisin B_1
FB_2	fumonisin B_2
FB_3	fumonisin B_3
FB_4	fumonisin B_4
FCS	foetal calf serum
g	gravity
$g.mol^{-1}$	grams per mole
GSH	glutathione (reduced glutathione)
GSSG	glutathione disulphide (oxidised glutathione)

GST	glutathione–S–transferase
^3H	tritium
H_2O	water
HBSS	Hank's balanced salt solution
HEPA	high efficiency particulate air
HPLC	high performance liquid chromatography
I3C	indole–3–carbinol
IARC	International Agency for Research on Cancer
IL	interleukin
MeOH	methanol
mg/kg	milligrams/kilogram
mm	millimetre
mm^3	cubic millimetres
mM	millimolar
µg/ml	micrograms/millilitre
µl	microlitre
µm	micrometre
µM	micromolar
MTT	methylthiazol tetrazolium
N_2	nitrogen gas
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
nm	nanometers
NMBA	*N*–nitrosomethylbenzylamine
OC	oesophageal cancer
OPA	*ortho*–phthaldialdehyde

OSC	organosulphur compounds
PBS	phosphate buffered saline
PHS	prostaglandin H synthase
PI	propidium iodide
PS	phosphatidylserine
Rb	retinoblastoma
redox	reduction–oxidation
Res	*trans*–resveratrol
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
S	solvent control
S–1–P	sphingosine–1–phosphate
SA	South Africa
SCC	squamous cell carcinoma
T	transmission
TCA	trichloroacetic acid
TNF	tumour necrosis factor
UV	ultraviolet
WF	water fraction

CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

From antiquity to modern times, man has been obsessed with a quest for rejuvenation and an innate desire to extend his lifespan (Dhuley, 1997). The notion that the prevention of disease is far more beneficial than the curing of it is probably as old as the concept of restoring human health using medical intervention (Geschner *et al.*, 1998).

Cancer, currently the second leading cause of death in the western world, may outrank cardiovascular diseases in the United States and other developed countries in a few decades (Tamimi *et al.*, 2002) and is a problem of continuing magnitude. Combined with the failure of conventional chemotherapy of advanced and invasive disease to effect major reductions in the mortality rates for the common epithelial malignancies, including carcinoma of the lung, colon, breast, prostate and pancreas, there is clearly a need for new approaches to the control of cancer (Sporn and Suh, 2000).

Despite great advances regarding basic scientific knowledge relating to cancer, as well as in the clinical treatment and cure of malignancies such as some leukaemia's and lymphomas, the National Cancer Institute's stated goal of a 50% reduction in overall cancer mortality by the year 2000 was not met. On the contrary, death rates from some of the more common cancers continue to rise (Sporn and Suh, 2000).

For centuries, man has utilised plants as the primary therapeutic agent in medicines, initially as traditional preparations and then as pure active principles. Natural products and their derivatives represent more than half of all drugs in clinical use today, with 25% originating from higher plants. Thus, the estimated 500 000 higher plant species known form a vast pool for further investigation into plant products for the development of new drugs (Elgorashi *et al.*, 2002; Taylor and van Staden, 2002).

South Africa, particularly KwaZulu–Natal, has a rich history of indigenous knowledge on the use of traditional medicinal plants (Sewram *et al.*, 1998). Approximately three million people in this country use indigenous, traditional plant medicine for primary health care purposes (Louw *et al.*, 2002). Zulu traditional medicine is widely practised and Zulu medicinal plants are traded and used throughout Southern Africa. Some scientific basis has been shown for traditional treatment or the potential use of traditional Zulu medicinal plants, however many plants have not yet been screened and further research is required to validate traditional claims (Opoku *et al.*, 2000). Additionally, although plant extracts have been used to treat diseases according to knowledge accumulated over centuries, scientific research has discovered that some substances in these medicinal plants are potentially toxic and carcinogenic (Verschaeve *et al.*, 2004).

In most countries with frequent use of ethnomedical treatments, many traditional healers prepare herbal remedies or provide preparation instructions to local populations. The scientific evaluation of the efficacy of local ethnomedicinal preparations could provide indigenous populations with better access to effective drug treatment and improved health status for those who cannot afford the benefits of Western medicines. If organised and encouraged to only utilise safe, efficient herbal remedies, local traditional healers could be an enormous advantage in this regard, especially to rural populations (Kaido *et al.*, 1997).

1.2 HERBS WITH ANTICANCER ACTIVITY

Plants are well established as useful sources of clinically relevant antitumour compounds. Indeed, there have been worldwide efforts to discover new anticancer agents from plants. Numerous methods for the selection of plants that may contain new biologically active compounds exist, one being the ethnomedical data approach. In this instance, the selection of particular plants is based on prior information on the folk medicinal use of the plant (Kamuhabwa *et al.*, 2000).

Catharanthus roseus (Madagascar periwinkle) is traditionally used for the treatment of diabetes and rheumatism in South Africa. This plant is abundant in the KwaZulu–Natal region, where it grows as a garden weed. The leaves of this plant are more commonly utilised, although the roots are an official drug in France, being used for the supportive treatment of diabetes. In addition, alkaloid extracts of the aerial parts are used for the treatment of various forms of cancer, including breast and uterine cancers, as well as Hodgkin's and non–Hodgkin's lymphoma. With regards to cancer chemotherapy, two binary indole alkaloids, vincristine and vinblastine, are well known antitumour agents (van Wyk *et al.*, 1997). Intensive phytochemical investigations have yielded the isolation of approximately 90 alkaloids, including a group of around 20 dimeric alkaloids exhibiting antineoplastic activity (Hutchings *et al.*, 1996).

The cancer bush (*Sutherlandia frutescens*) is an old Cape remedy for stomach disturbances and internal cancers, with the hairy coastal variety of the species (*S. frutescens* var. *incana*) believed to be particularly effective against cancer. Mainly the leaves of this plant are used, although all above–ground parts are usually included in medicinal preparations. The plant is rich in amino acids, contains small amounts of saponins and no alkaloids (van Wyk *et al.*, 1997). Chemical studies on this plant have identified the component canavine, a non–protein α–amino acid, with antitumourigenic properties (Fernandes *et al.*, 2004).

Another widely distributed plant reported to possess antitumour properties is *Centella asiatica* (pennywort). This plant (usually the dried leaves) has been used to treat leprosy, wounds and cancer in South Africa and elsewhere. Additionally, *C. asiatica* is reported to possess antibacterial, antifungal, anti–inflammatory, anti–allergic and antipyretic activities. The triterpenoid saponin, asiaticoside, as well as several other triterpenoids may be responsible for the antitumour properties of this plant (van Wyk *et al.*, 1997). The leaves of this plant are reportedly cooked and eaten as a vegetable by Xhosa women (Hutchings *et al.*, 1996). A study by Babu *et al.* (1995) showed significant cytotoxic activity of both a crude methanol extract and a semi–purified acetone extract of *C. asiatica* against Ehrlich ascites tumour cells (EAC) and Dalton's lymphoma ascites tumour cells (DLA) in culture. These extracts also exhibited significant tumour reducing properties against both ascites and solid transplanted murine tumours in a prophylactic treatment model. The lack of toxicity towards the normal human lymphocyte control suggests that these extracts have selective toxicity towards tumour cells.

Members of the *Hypoxis* genus are reputed to possess antitumour activity (Hutchings *et al.*, 1996). They are tuberous perennials and the tuberous rootstock, known as a corm, is used in medicinal preparations. Some of the many traditional uses of *H. hemerocallidea* include treatment of testicular tumours, prostate hypertrophy and urinary infections (van Wyk *et al.*, 1997). Phytosterol glycosides extracted from the tubers of this species have shown activity against benign prostate hypertrophy (Hutchings *et al.*, 1996). The activity of this plant against adenoma of the prostate is ascribed to β–sitosterol (van Wyk *et al.*, 1996). In addition, hypoxoside A has been extracted from this plant. A registered patent referring to drugs containing this compound claims antitumour action in cancer patients and reports lack of teratogenicity and animal toxicity of extracts (Hutchings *et al.*, 1996). The aglycone of the hypoxoside, known as rooperol, is believed responsible for the anticancer, anti–HIV and anti–inflammatory action of this plant. Rooperol has several biological activities, being markedly antimutagenic and cytotoxic to cancer cells (van Wyk *et al.*, 1997).

1.3 IMIFINO

"Imifino" is a collective name for many wild plants used predominantly by rural people as herbs in cooking and includes the Black Jack and pumpkin plants. Many "imifino" have been found to have medicinal properties, for instance *Momordica* species have hypoglaecemic properties and have been used to treat diabetes (Hutchings, pers comm). The leaves of the wild garlic (*Tulbaghia violacea*) plant may be consumed as a vegetable and are used for the treatment of oesophageal cancer (van Wyk *et al.*, 1997). The importance of these under–exploited vegetables cannot be overestimated (Bhat and Rubuluza, 2002).

The particular plant of interest for this investigation has been identified as *Amaranthus lividus* (L.) subsp. Polygonoides (Moq.) Probost (Figure 1.1) and is commonly known as Imbuya among Zulu people. This plant is an indigenous summer annual and is widely distributed throughout southern Africa.

Figure 1.1: Photograph of *Amaranthus lividus*.

Usually only the leaves of these plants are used, although the stems of very young plants are sometimes also eaten. In most cases the leaves are picked, washed, cut and boiled. Spices and other flavourings such as garlic may also be added. This vegetable is then often served with meat and stiff pap, which is mainly consumed by the men. Alternatively, the mealie meal may be added directly to the boiled, seasoned leaves to make a stiff pap. Prepared in this manner, the "imifino" is mainly consumed by women (Gqaleni, pers comm).

People who consume regular quantities of the "imifino" leaves have been observed as being generally healthier than those who do not (Chuturgoon, pers comm). Therefore, it is thought that "imifino" may possess many properties that contribute to good health, one of these being chemoprevention.

1.4 OBJECTIVES

The primary objective of this investigation was to ascertain the possible chemopreventive properties of the indigenous edible herb *Amaranthus lividus*. This was achieved by determining the cytotoxicity of selected solvent extracts on two carcinoma cell lines of different tissue origins to establish whether the effects noted were cell line or tissue type specific. Effects on normal human lymphocytes were also assessed. Dose–response and time–dose–response studies were conducted. Specific biomarker analysis was performed to assess the chemopreventive potential of promising extracts. The effect of selected plant fractions on the apoptosis of normal human lymphocytes was also analysed.

CHAPTER TWO

LITERATURE REVIEW

2.1 NEOPLASIA

The term neoplasia literally means "new growth" (of cells or tissues) and the resultant new growth is referred to as a neoplasm (Medical Dictionary Online, 2005). However, there are many forms of new growth, such as repair, and the term neoplasm is reserved for abnormal new growth and means the same as tumour. Neoplasia involves the formation of benign (something that does not metastasise and treatment or removal is curative) and more particularly, malignant (tending to become progressively worse and to result in death) tumours or cancer (On–line Medical Dictionary, 2005). Although the term tumour was originally used in reference to swelling, it is now commonly used as a synonym for neoplasm (Loveday, 1993).

The new growth results from an inheritable change in a cell or group of cells, which allows them to escape from many of the normal homeostatic mechanisms that control cell proliferation. It thus seems likely that this change could occur in any cell capable of dividing. When it has occurred, the cell is said to be transformed (Evans, 1991).

It is important to differentiate between cell proliferation of normal tissue, even when as extensive as seen in wound healing, and cell proliferation associated with transformation. In normal tissue, proliferation is under some form of control and stops when a regulated end point is reached, such as healing. However, transformed cells usually continue to proliferate to the detriment of the host. Very occasionally tumours may regress, perhaps suggesting that in certain cases some tumours may not have fully lost, or may be able to regain, sensitivity to the normal proliferation control mechanisms (Evans, 1991).

2.2 THE CLASSIFICATION OF TUMOURS

There are two fundamental types of tumours, benign and malignant, which behave in different ways. Benign tumours remain localised and do not spread to other parts of the body. Malignant tumours, however, usually invade and destroy host tissue and may spread throughout the body. The term cancer is used to describe a malignant tumour (Ferguson, 1984; Evans, 1991; Sheldon, 1992).

Table 2.1: Primary differences between benign and malignant cells (Sheldon, 1992).

FEATURE*	BENIGN	MALIGNANT
MITOTIC FIGURES	Few and normal	Many and abnormal
NUCLEUS	Predominantly normal	Pleomorphic (variable and irregular)
TISSUE STRUCTURE	Usually normal	Dysplastic/anaplastic
FUNCTIONS	Usually normal	Lost or deranged
CAPSULE	Usually intact	Often lacking
METASTASIS	Never	Often
LOCAL INVASION	Rare	Common
FATALITIES	Rare	Often

* Note that while benign lesions never metastasise, exceptions exist for all other features listed.

A number of criteria are used in the classification of tumours including behaviour, appearance and origin.

2.2.1 BEHAVIOUR

Two major behavioural traits of tumours assist in their classification, namely their growth and their invasive and metastatic behaviour.

2.2.1.1 Growth

As mentioned, tumour growth differs from that of normal tissue in that it does not seem to be controlled by the normal regulatory mechanisms of the body. However, tumour growth is not autonomous, as it is still dependant on the host for nutrient supply and the removal of waste products. Although uncontrolled growth is taken as a hallmark of a tumour, they vary in their individual rate of growth, suggesting that this behavioural trait may not be particularly reliable for classification (Evans, 1991).

2.2.1.2 Invasion and Metastasis

Cells of benign tumours are often encased in a fibrous capsule and usually remain at their site of origin. On the other hand, malignant tumours rarely have a complete capsule and they frequently invade local tissues. In addition to their locally invasive character, malignant tumours often spread to remote sites after gaining access to the lymphatic or blood systems or to body cavities. The spread of malignant tumours culminating in the establishment of one or more secondary tumours at a remote site is known as metastasis. The ability to metastasise probably represents the most reliable criterion for distinguishing malignant from benign tumours (Ferguson, 1984; Evans, 1991).

2.2.2 APPEARANCES

Both the gross and microscopical appearances of tumour cells are used in their classification.

2.2.2.1 Differentiation

This refers to the degree of resemblance between the tumour and the normal tissue from which it was derived. Dysplastic tumours show partial resemblance to the parent tissue, whereas anaplastic tumours show almost none (Loveday, 1993). Generally the more highly differentiated a tumour is the better the prognosis. Pathologically speaking, tumour differentiation is usually assessed "naked eye" or by microscopical observation of histological sections. Whereas benign tumours usually resemble their tissues of origin in both naked eye and microscopical appearance, malignant tumours are often anaplastic and their origins may not be immediately obvious by either means. In addition, changes in the state of cell differentiation may be associated with changes in function. It is often found that whereas benign tumours may retain the functions of the parent tissues, these functions may occasionally be lost or abnormal in malignant tumours (Ferguson, 1984; Evans, 1991).

2.2.2.2 Cytology and Tumourigenicity

Recorded cytological changes occurring during the spontaneous transformation of a number of rodent cell lines showed that most cell lines that became transformed exhibited a progression of changes. These included an increase in cytoplasmic basophilia, increased size and number of nucleoli, increased nulcear: cytoplasmic ratio as well as retraction of the cytoplasm (Evans, 1991).

2.2.3 ORIGINS

Tumours originate from different tissues (histogenic differences), organs and cells and this is taken into account in their classification. The seven important types of malignant tumours are outlined below.

Table 2.2: Important malignant tumours and their tissues of origin (Ferguson, 1984; Evans, 1991).

TUMOUR TYPE	TISSUE OF ORIGIN
CARCINOMAS	These are malignant epithelial tumours and represent by far the most common type. Carcinoma *in situ* refers to an epithelium, particularly of the skin or cervix, which shows many malignant characteristics but does not invade the underlying tissue. Strictly speaking, carcinoma *in situ* is a premalignant change as invasion is lacking.
ADENOCARCINOMAS	Malignant tumours of glandular epithelial tissue.
MALIGNANT MELANOMA	A malignant tumour of pigmented epithelial cells (melanocytes).
SARCOMAS	Malignant tumours of connective tissue.
LEUKAEMIAS	Malignant tumours of precursor cells within the haemopoietic system.
LYMPHOMAS	Tumours of lymphoid cells.
TERATOCARCINOMAS	These malignant teratomas are tumours of multipotential embryonic tissue cells (embryonal carcinoma or EC cells).

2.3 CAUSES OF TUMOURS

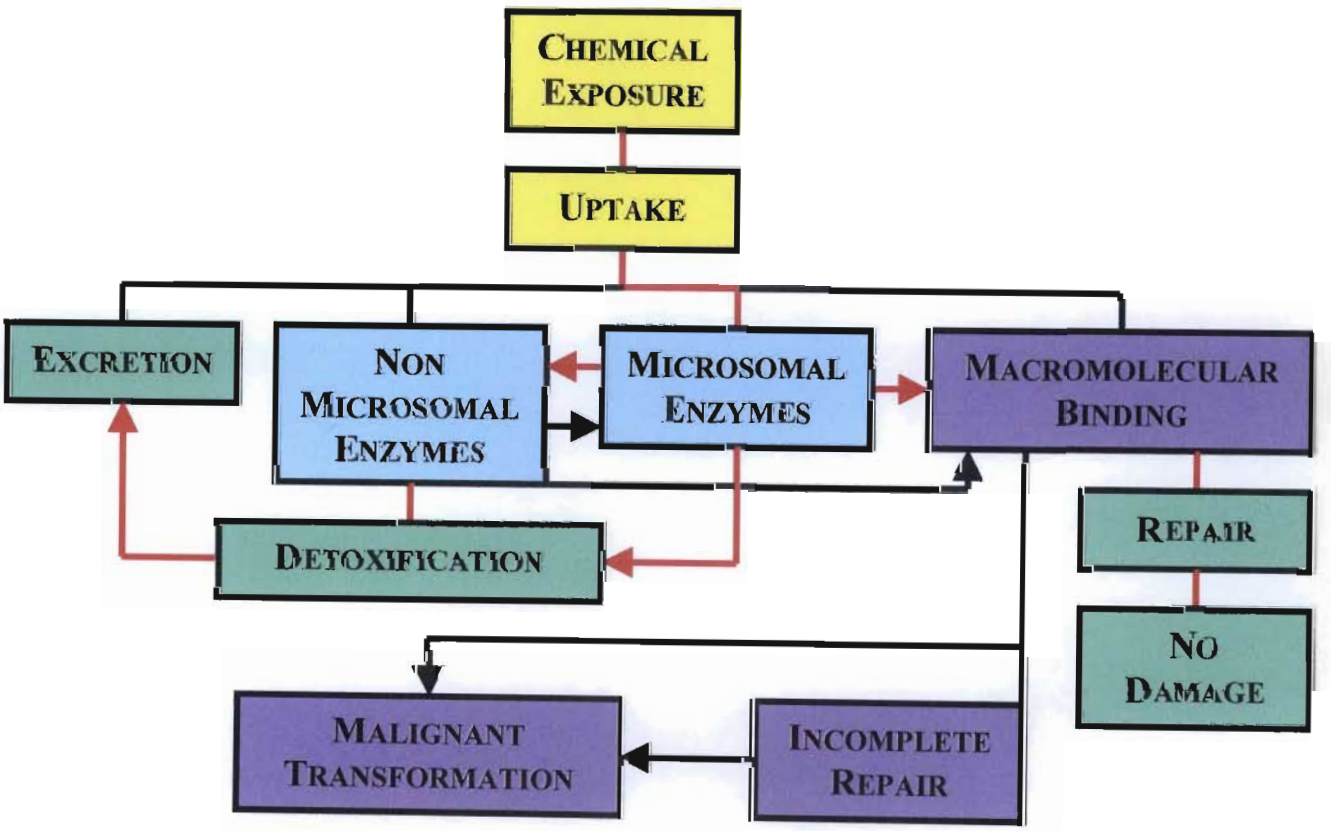
The ultimate mechanisms that cause cancer remain unknown. Important factors in carcinogenesis include intrinsic (internal) factors, such as genetic factors/heredity, as well as various extrinsic (external) factors, including chemical carcinogens, ionising radiation, transforming viruses and various environmental agents, for example mycotoxins. This does not imply that there may not be other causes or that only one factor is responsible in a given case (Ferguson, 1984; Evans, 1991).

In most, if not all cases, the basis of transformation is probably a mutation, which is a change in the primary structure of DNA. It is also likely to be influenced by epigenetic events, which are shifts in gene expression. However, the extent to which each of these forms of cellular change is involved in transformation is uncertain (Evans, 1991).

A role for mutational events in transformation is supported by the observations that most carcinogens are also mutagens and *vice versa* (although this is not always the case) and that many tumours show chromosomal abnormalities. In addition, many oncogenes (cancer causing genes) are found at or near sites of chromosomal abnormalities. Some involvement of epigenetic events is suggested by changes in the expression of surface antigens. Whatever its basis, transformation must involve a heritable change, as the daughter cells of the originally transformed cell retain the transformed nature, except under extraordinary circumstances (Evans, 1991).

2.3.1 BIOACTIVATION OF CARCINOGENS

Many environmental carcinogens are moderately chemically inert and relatively stable outside of the body and are referred to as procarcinogens. Once ingested and absorbed however, they are subjected to the xenobiotic detoxification processes. The normal biochemical defence against toxic chemicals is to attach functional groups to them to increase water solubility and aid in their excretion as harmless derivatives. However, chemical carcinogens may form highly reactive and chemically unstable intermediates during this detoxification process. Once formed, these intermediates can readily react with cellular macromolecules. Alkylation of genetic material by these substances is critically involved in malignant transformation and mutagenesis (Selkirk and MacLeod, 1982). The possible reactions occurring within a cell following exposure to xenobiotics are outlined in Figure 2.1 below.



The major pathways taken by cells to deactivate and remove toxic chemicals are shown in red. The purple blocks represent occurrences that may result in malignant transformation of the cell.

Figure 2.1: Metabolic processing of foreign chemicals (Adapted from: Selkirk and MacLeod, 1982).

The cytochrome P_{450} enzymes are largely responsible for primary metabolism resulting in the production of reactive metabolites and are referred to as Phase I enzymes. In contrast, secondary conjugating metabolism is principally detoxifying (Manson *et al.*, 1997), although this is not always the case. An example of this is the Phase II enzyme glutathione (GSH) transferase, which activates 1,2–dihaloethanes amongst other chemicals (Guengerich, 2000).

2.4 INITIATION AND PROMOTION

The application of a chemical carcinogen to a tissue is not followed by the presence of a tumour within a matter of days. The time between the exposure to a carcinogen and the appearance of a tumour is known as the latent period, which is variable, but may be as long as decades. It has been proposed that carcinogenesis is a multi–stage phenomenon. Most of the evidence to support this view stems from studies associated with chemical carcinogenesis. However, some viral oncogenes appear to cause tumours in a single step (Ferguson, 1984; Evans, 1991).

Two stages have been described in experimentally produced, chemically induced tumours. Firstly some form of initiation in which the conversion to neoplastic potential is created by exposure to a carcinogen, with subsequent promotion to reach the overt stage (Evans, 1991).

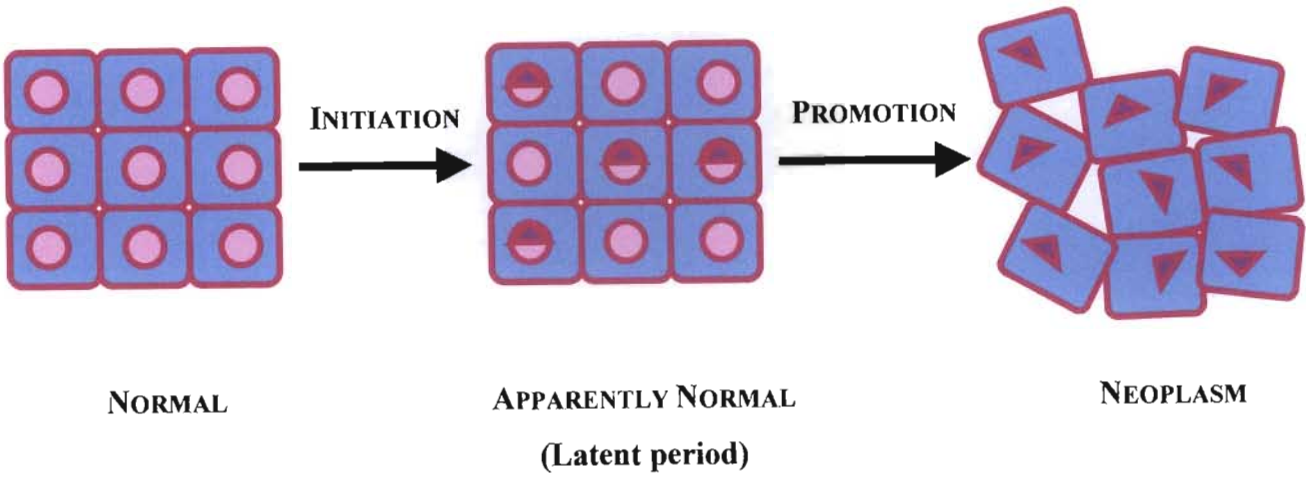


Figure 2.2: Schematic representation of the two steps in neoplasm production.

In Figure 2.2 the normal cells are shown on the left. Following initiation some of these cells may have changes in their nucleic acids, indicated by the triangles in the nuclei. After repeated exposure to agents (promotion), a tumour may appear.

Initiation is a relatively rapid and irreversible event, suggesting that this step involves mutational change. Thus, initiators are usually mutagens and as such they introduce sites of potential mistakes when DNA replication and transcription occur. Promotion usually occurs over a long period of time (the latency period) and may be reversible. It is thought that this step involves epigenetic changes. Promoters are usually not mutagens. They mostly enhance cell division, often due to a cascade of events during which various intracellular enzymes are phosphorylated by protein kinases. The eventual increase in cell proliferation mediated by promoters allows the initiation–induced mutations to be transmitted to daughter cells (Ferguson, 1984; Evans, 1991).

2.5 THE INVASIVE AND METASTATIC BEHAVIOUR OF MALIGNANT CELLS

As mentioned, the behaviour of tumours allows their categorisation into two general types, benign and malignant. Benign tumours do not invade surrounding tissues, although they do displace them as they increase in size. On the other hand, malignant tumours frequently invade and destroy local tissues and furthermore, they often spread or metastasise, giving rise to secondaries far from the original or primary tumour (Evans, 1991).

Local invasion is distinct from, but part of the process of metastasis. Some invasive tumours rarely metastasise, but all metastases involve an invasive step. In addition, not all cells within a tumour have the same potential to metastasise. Three cellular properties that are important in invasion have been identified, namely proliferation, motility and lytic action. There is no generalised scheme that adequately explains the invasive behaviour of all malignant tumours, however two general patterns have been proposed (Evans, 1991).

2.5.1 TYPE 1 INVASION

Often called invasive growth, it is characterised by malignant cells that invade normal surrounding tissue by *en masse* penetration. It is often shown by tumours of epithelial origin (carcinomas) and may be associated with a more or less general pattern of lytic activity in the immediate vicinity of the tumour. Proliferation rather than locomotion is the main agent of invasion where, following division, two cells have to adapt to a site previously occupied by only one. This growth pressure leads to invasion (Ferguson, 1984; Evans, 1991).

2.5.2 TYPE 2 INVASION

In contrast to Type 1, this type of invasion results from the migration of individual cells or cell islands. Lytic activity tends to be limited to the microenvironments of the invasive cells. Although locomotion contributes to this type of invasion, it is believed that proliferative activity is primarily responsible. Invading cells emerge from the tumour mass and migrate only a small distance before dividing again. This division contributes to cellular relocation. This type of invasion is predominantly shown in the sarcomas and by malignant melanomas (Ferguson, 1984; Evans, 1991).

2.6 THE PROBLEM OF METASTASIS

The spread of malignant tumours prevents successful treatment by simple excision of the primary growth. Thus, metastasis is the major problem facing the cancer clinician. Often metastases have already been seeded by the time the patient presents with a malignant tumour. In many cases, these metastases may not be immediately obvious, even by modern detection mechanisms, as they may consist of only one or very few cells. The nature of malignant cells provides the potential for widespread metastasis and as metastatic lesions may progress differently from their primary tumours, the effective treatment of metastatic disease presents a formidable problem (Evans, 1991).

2.6.1 THE NATURE OF METASTATIC CELLS

Two conflicting hypotheses have been proposed concerning the nature of the cell that gives rise to metastases. The Specialist Model suggests that metastasis is a non–random process requiring a specialised subset of cells that pre–exist in the tumour mass, as not all cells within a malignant tumour appear to have the same metastatic potential. In contrast, The Stochastic Model holds the view that the likelihood of a malignant cell forming a metastasis is entirely random and dependent on local events. In this model, there is no subset of specialist metastatic cells pre–existing before metastatic lesions develop. Instead, cells are thought to be recruited randomly into a transient metastatic pool. Regardless of whether metastatic variants pre–exist in a stable pool or move in and out of a transient pool, metastatic cells seem to represent only a small fraction of the total tumour cell population at any one time (Ferguson, 1984; Evans, 1991).

2.6.2 GENERAL PRINCIPLES OF THE METASTATIC PROCESS

A tumour may metastasise in the body by three main routes.

2.6.2.1 Haematogenic Spread

Tumour cells gain access to the blood usually via the venous circulation in which they may be transported to a distant site. Within the blood, tumour cells may interact with blood components and although some of these interactions may lead to tumour cell destruction, they may promote tumour cell lodgement in small blood vessels through clump formation. Once lodged within a vessel the tumour cell may extravasate into the tissue parenchyma where it can divide and establish a secondary growth (Sheldon, 1992).

2.6.2.2 Lymphatic Spread

Tumour cells probably enter the lymphatic system via lymph capillaries present near the edge of the tumour mass. Within lymph vessels, tumour cells may exist singly, as small clumps or may grow as a column within the vessel lumen before fragmenting. Tumour cells usually spread via the lymph vessels to lymph nodes where they may lodge. Tumour cells not destroyed within the lymph node may colonise it and use it as a base for the seeding of further metastases (Sheldon, 1992).

2.6.2.3 Surface Implantation

Cancer cells may seed onto cell surfaces, such as those lining the pleural and peritoneal cavities. The escape of malignant cells into the body cavities may give rise to ascites tumours in which the cells grow essentially in suspension, both singly and in clumps of various sizes. Ascites tumours usually invoke haemorrhagic and inflammatory reactions. They are associated with the accumulation of large volumes of fluid, which may often be fatal due to pressure effects on major organs (Sheldon, 1992).

The essential steps in the metastatic process can be summarised as follows. Firstly, there is local invasion of the surrounding tissues. This is followed by the detachment of one or a few cells from the primary tumour. These cells invade a blood vessel or lymph node, thereby gaining transport to a distant site where they may remain (primarily lymph nodes). Alternatively, the cells may leave the vessel to invade and colonise the distant tissues, giving rise to a metastasis (Ferguson, 1984; Evans, 1991).

2.7 MYCOTOXINS IN GENERAL

Mycotoxins are mutagens and carcinogens produced by many moulds, including *Aspergillus*, *Fusarium* and *Penicillium* species. These secondary metabolites have no effect on the growth of the producing organism and appear to be formed as a means of reducing the accumulation of precursor compounds produced during the primary growth phase (Jay, 1996). Some mycotoxins exhibit specific organ toxicity, as clearly demonstrated by animal experimentation as well as *in vitro* cytotoxicity testing.

Mycotoxin contamination of food products, particularly maize type products, is a major problem in rural areas especially in the Transkei region of South Africa, where the incidence of oesophageal cancer is extremely high. Poor storage facilities and lack of education regarding the dangers of consuming contaminated maize products exacerbate the problem. The absence of the producing organism upon culture does not necessarily indicate the absence of the mycotoxin in food or feed samples, as the fungus itself is not directly involved in the disease.

The diseases caused by exposure to mycotoxins, called mycotoxicoses, may result from ingestion, inhalation or direct skin contact with these compounds. Mycotoxicoses affect both humans and animals, including birds (Nelson *et al.*, 1993; Diaz and Boermans, 1994). In the 1960's, there was a severe outbreak of Turkey "X" disease in the United Kingdom in which over 100 000 turkey poults perished. It was found that Brazilian groundnuts used as feed were contaminated with aflatoxins, highly toxic metabolites of toxigenic *Aspergillus* species. Mycotoxins have since been of major concern as toxic contaminants of food and feeds (Diaz and Boermans, 1994).

Following the discovery of aflatoxins, intensive research on mycotoxins led to the identification of a variety of these compounds associated with adverse human and animal health effects (Diaz and Boermans, 1994). The ability of fungal spores to germinate at temperatures ranging from 15°C to 40°C facilitates the high incidence of mycotoxins in food materials (Calistru, 1995).

2.8 AFLATOXINS

The most well known and widely studied group of mycotoxins until recently is the aflatoxins, which are produced by various strains of *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. There are many closely related toxins in this group, however, only six have been identified as being highly toxic. These are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin M_1 (AFM_1), aflatoxin M_2 (AFM_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) (Jay, 1996).

Aflatoxin B_1 is the most carcinogenic of all naturally occurring aflatoxins and the producing organisms are capable of growing on a variety of foods and foodstuffs (Moon *et al.*, 1999). As aflatoxins cannot be eliminated from the human food supply completely, they represent a serious health concern for populations that are unable to store agricultural commodities adequately in order to limit mould growth (Roebuck, 2004).

Aflatoxicosis has been shown to increase the susceptibility of domesticated animals to infectious diseases and experimental evidence shows that AFB_1 induces hepatoma and other tumours in a wide range of animal species. Additionally, AFB_1 has demonstrated immunosuppressive activity in animal models (Moon *et al.*, 1999).

2.8.1 CHEMICAL PROPERTIES OF AFLATOXIN B_1

Aflatoxin B_1 (Figure 2.3) is a highly polar polyketide (C_{20}) formed from acetate units via the polyketide pathway. The methoxy–methyl group is supplied by methionine (Applebaum and Marth, 1981). Aflatoxin B_1 has a relative molecular mass of approximately 320.2 $g.mol^{-1}$.

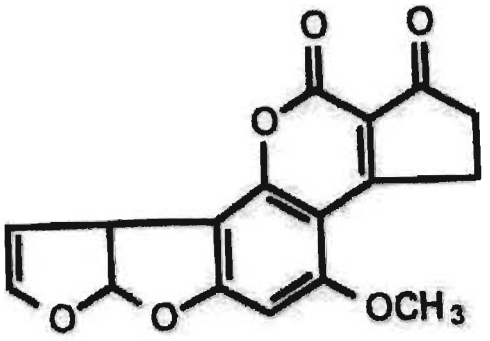


Figure 2.3: The chemical structure of aflatoxin B_1 (Massey *et al.*, 1995).

2.8.2 BIOTRANSFORMATION OF AFLATOXIN B_1

Aflatoxin B_1 is a procarcinogen and requires biotransformation in order to exert its carcinogenic potential. The principal enzyme system involved in the oxidative biotransformation of AFB_1 is the P_{450} system (Massey *et al.*, 1995). The cytochrome P_{450}s (CYP) constitute a superfamily of haemproteins that are important in the metabolism of xenobiotics. Three main CYP gene families are responsible for most hepatic drug metabolism, namely CYP1, CYP2 and CYP3. The CYP1 and CYP3 families are relatively simple, while the CYP2 gene family is complex, comprising numerous subfamilies (Lin and Lu, 1998). Bioactivation is not limited to one form of these enzymes. Studies have implicated CYP1A2, CYP2A3, CYP2B7 and both CYP3A3 and CYP3A4 as isoforms capable of AFB_1 activation (Massey *et al.*, 1995).

Oxidation of AFB_1 by the cytochrome P_{450}–dependant system is considered central in the toxicity exerted by this mycotoxin. The observed differences in species, strain or individual sensitivity to AFB_1 are largely related to variations in the expression of these enzymes (Guerre *et al.*, 1997).

The mutagenic and carcinogenic potential of AFB_1 results from its bioactivation to the highly reactive electrophile, AFB_1–8,9–epoxide (AFBO; Figure 2.4), the ultimate carcinogen (Yu *et al.*, 1994). The epoxide formed can be either an *endo*– or an *exo*–epoxide. Only the latter exerts DNA binding activity, leading to the formation of DNA and protein adducts (Massey *et al.*, 1995).

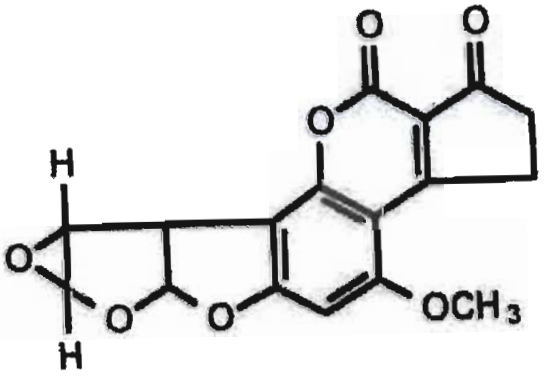


Figure 2.4: The chemical structure of the aflatoxin B_1 8,9 *exo*–epoxide (Massey *et al.*, 1995).

Microsomal prostaglandin H synthase (PHS) and cytosolic lipoxygenases can also catalyse the bioactivation of AFB_1. This occurs during the oxidation of arachidonic acid to lipid peroxy radicals, which are epoxidising agents for xenobiotics. This process is termed co–oxidation, as the epoxidation of AFB_1 occurs simultaneously with the oxidation of arachidonic acid. The action of these enzymes in AFB_1 transformation is especially important in extra–hepatic tissues, where P_{450} enzyme activity is reduced (Massey *et al.*, 1995).

2.8.3 DETOXIFICATION OF AFLATOXIN B_1

The electrophilic epoxide readily reacts with cellular macromolecules, however endogenous defence mechanisms exist which can protect the cell from this toxicity. These include conjugating reactions, active efflux from the cell and repair of AFB_1–DNA adducts. In many mammalian cell types, detoxification of the AFB_1–epoxide via phase II metabolism is of utmost importance (McLellan *et al.*, 1994).

The conjugation of activated AFB_1 with reduced glutathione (GSH) is thought to be the most important detoxification mechanism (McLellan *et al.*, 1994; Massey *et al.*, 1995). These reactions are catalysed by enzymes of the glutathione–S–transferases (GST's), a super family of proteins that catalyse the conjugation of GSH with a wide spectrum of xenobiotics possessing an electrophilic centre (Massey *et al.*, 1995).

Thus, AFB_1 species specificity is not only dependent on expression of the activating enzymes. Rather, its toxicity is based on a balance between the rate of primary activation and the rate of detoxification of the resulting metabolites or repair of cellular damage. These reactions are determined by the relative activity of the phase II detoxification enzymes (McLellan *et al.*, 1994).

The induction of phase II enzymes with subsequent enhancement of electrophile detoxification is recognised as a characteristic action of numerous chemopreventive agents. An example of such a compound is oltipraz, a dithioethione with potent phase II inducing capacity, which has been demonstrated to be an effective agent against AFB_1–induced hepatocarcinogenicity (Maxuitenko *et al.*, 1998).

2.8.4 TOXICOLOGICAL EFFECTS OF AFLATOXIN B_1

Since their discovery, aflatoxins have been shown to exert potential carcinogenic effects in a range of animal species and have been associated with mycotoxicoses in poultry and other domestic animals. In 1993 the International Agency for Research on Cancer (IARC) classified AFB_1 as a Group 1 chemical carcinogen (International Agency for Research on Cancer, 1993a).

Although sensitivity varies, AFB_1 is acutely toxic to most animal species, with the rat, duck and rainbow trout being among the most susceptible to toxic effects. In addition, AFB_1 is a potent hepatocarcinogen in these species and a powerful mutagen in both mammalian and bacterial cells (Croy *et al.*, 1978).

The AFB_1–epoxide reacts with guanine residue of DNA to form 2,3–dihydro–2–(N^7–guanyl)–3–hydroxy–AFB_1 (AFB_1–N^7–guanine). The formation of this adduct impairs DNA synthesis, as well as altering RNA metabolism (Wogan, 1968). In addition, AFB_1–epoxide binds covalently to albumin, with AFB_1–lysine adducts being the primary product (Sabbioni, *et al.*, 1987; Wild, *et al.*, 1990).

In 1993, Hsieh and Hsieh demonstrated the presence of AFB_1–DNA and –protein adducts, as well as AFB_1 and metabolites thereof, in cord blood specimens. This indicates that these toxic products may indeed cross the placental barrier and cause exposure to the foetus.

2.9 FUMONISINS

Fumonisins were first isolated from cultures of *Fusarium verticillioides* (formerly *F. moniliforme*) and chemically characterised by South African researchers in 1988, making them the most recently discovered group of mycotoxins (Marasas *et al.*, 1993). They are produced by various species of *Fusarium* fungi, although the primary producer is *F. verticillioides*, a common contaminant of maize, sorghum and other grains worldwide (Wang *et al.*, 1992; Riley *et al.*, 1993). The principal metabolite, fumonisin B_1 (FB_1), has subsequently been shown to cause equine leucoencephalomalacia (ELEM) in horses and ponies, porcine pulmonary oedema, as well as liver cancer in rats (Marasas *et al.*, 1993).

The effect of fumonisins on human health remains unclear. However, the consumption of contaminated maize has been associated with the high incidence of oesophageal cancer (OC) in the Transkei region of South Africa, as well as in some provinces of China, where there is high consumption of mouldy maize by the human population (Norred, 1993; Riley *et al.*, 1993; Diaz and Boermans, 1994). Fumonisins were classified in the Group 2B category as probably carcinogenic for humans by the IARC in 1993 (International Agency for Research on Cancer, 1993b).

2.9.1 CHEMICAL PROPERTIES OF FUMONISIN B_1

Fumonisins are long chain polyhydroxyl alkylamines with two propane tricarboxylic acid moieties esterified to hydroxyls on adjacent carbons. They are strongly polar, water–soluble compounds but are more soluble in acetonitrile–water and methanol (Scott, 1993). As they are moderately thermostable, fumonisins pose a potential risk to humans even following cooking (Alberts *et al.*, 1990).

At least seven fumonisins are known to be produced by *F. verticillioides*, namely FB_1, FB_2, FB_3, FB_4, FA_1, FA_2 and FA_3. Fumonisin B_1 (Figure 2.5) is the primary fumonisin produced in culture in addition to occurring naturally in maize and maize–based foods and feeds (Sydenham *et al.*, 1992). Fumonisin B_1 has a relative molecular mass of approximately 721 $g.mol^{-1}$.

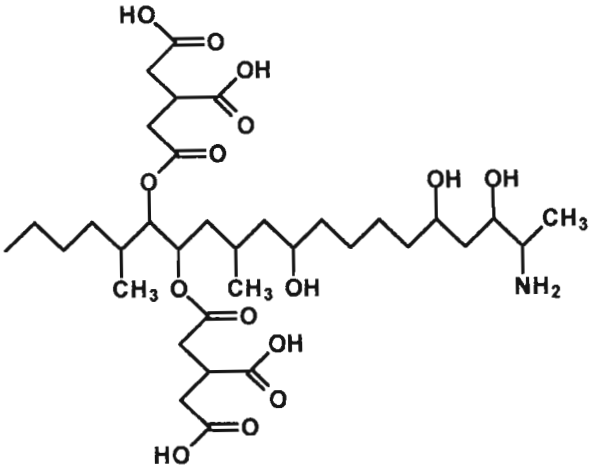


Figure 2.5: The chemical structure of fumonisin B_1 (Merrill *et al.*, 1995).

2.9.2 TOXICOLOGICAL EFFECTS OF FUMONISIN B_1

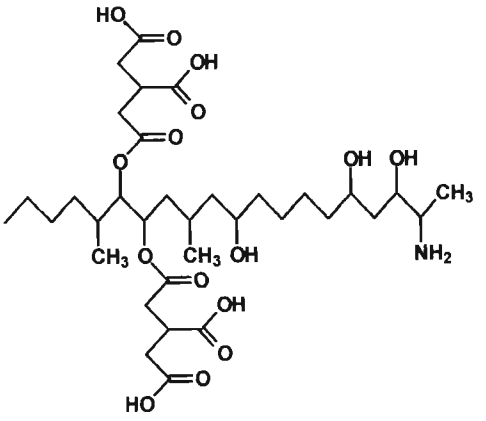
The toxicology of FB_1 is interesting as it appears to induce changes in the homeostasis of the cell, leading to either the proliferation or demise (apoptosis) of target cells. Due to the frequency and level of maize contamination, as well as toxicity to animals, serious concern exists regarding this dual role of fumonisins and the part they may play in human and animal diseases. Some of this concern emanates from emerging data linking the biological activity of FB_1 to apoptosis in animal cells (Caldas *et al.*, 1998).

Fumonisin B_1 is poorly absorbed and rapidly excreted in the faeces, much of it unmetabolised, leading to low plasma levels (Prelusky *et al.*, 1994). In rats, FB_1 mainly passes directly through the gastrointestinal tract and what little is absorbed is primarily excreted in the bile (Shephard *et al.*, 1994).

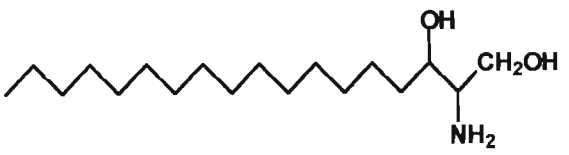
The uptake of FB_1 is very small in all animal models to date and there is no evidence suggesting that this is not the case in humans also. This toxin is either highly potent or its absorption is aided by other dietary factors, including alcohol and fat. It is improbable that FB_1 is modified into a more accessible form, by esterification for instance, or that there are transport systems to assist its passage (Dutton, 1996).

2.9.3 BIOCHEMICAL MODE OF ACTION OF FUMONISIN B_1

Fumonisins bear a remarkable structural similarity to sphinganine (Figure 2.6) and are inhibitors of *de novo* sphingolipid biosynthesis via activity on the key enzyme ceramide synthase. Sphingolipids are thought to be involved in the regulation of cell growth, differentiation and neoplastic transformation through participation in cell–cell communication, cell–substrate interactions, as well as possible interactions with cell receptors and signalling systems (Wang *et al.*, 1991).



FUMONISIN B_1



SPHINGANINE

SPHINGOSINE

Figure 2.6: The chemical structures of fumonisin B_1, sphinganine and sphingosine (Merrill *et al.*, 1995).

Sphingolipids are found in all eukaryotic as well as some prokaryotic organisms, where they are abundant in plasma membranes and related organelles, which are functionally associated with cellular responses to external stimuli, including growth factors, cytokines and extra–cellular proteins (Merrill *et al.*, 1995). They are the most structurally diverse class of membrane lipids, being composed of over 70 long chain bases, numerous amide–linked fatty acids and in excess of 300 headgroups. They are both structural and functional lipids, serving as ligands for extracellular matrix proteins and receptors on neighbouring cells, as well as for enteric bacteria and viruses. Sphingolipids are also important in cell regulation as modulators of growth factor receptors and as second messengers for an increasing number of agonists, including tumour necrosis factor–α (TNF–α), interleukin–1–β (IL–1–β) and nerve growth factor (Merrill *et al.*, 1997).

Since the fumonisins posses such striking structural similarity, it was hypothesised that the mechanism of action of these mycotoxins could be via the disruption of sphingolipid metabolism. As sphingolipids are highly bioreactive molecules involved in many important cellular processes, disruption of their metabolism could affect cell growth, differentiation and behaviour (Wang *et al.*, 1992; Shephard *et al.*, 1996).

In the sphingolipid biosynthetic pathway, ceramide synthase acylates the amino group of sphinganine with a fatty acid moiety to yield dihydroceramide. This is then converted to ceramide by the addition of the 4,5–trans–double bond and finally more complex sphingolipids by the addition of the appropriate headgroup. The turnover of these complex sphingolipids leads to the formation of sphingosine, which can undergo further catabolism or can be re–acylated by ceramide synthase to yield ceramide once again (Wang *et al.*, 1992; Shephard *et al.*, 1996). Ceramide has been shown to be a regulator of cell growth, differentiation and apoptosis (Wolf, 1994).

The cellular target of fumonisin has been demonstrated to be the enzyme N–acyltransferase, also referred to as ceramide synthase. This enzyme catalyses the addition of the fatty acid residue to sphinganine in the *de novo* biosynthesis of sphingolipids and the re–acylation of long chain bases arising from sphingolipid turnover (Figure 2.7). Disruption of sphingolipid metabolism appears to be the first step in the toxicity and carcinogenicity of fumonisins as they can both inhibit and stimulate growth (Wang *et al.*, 1991).

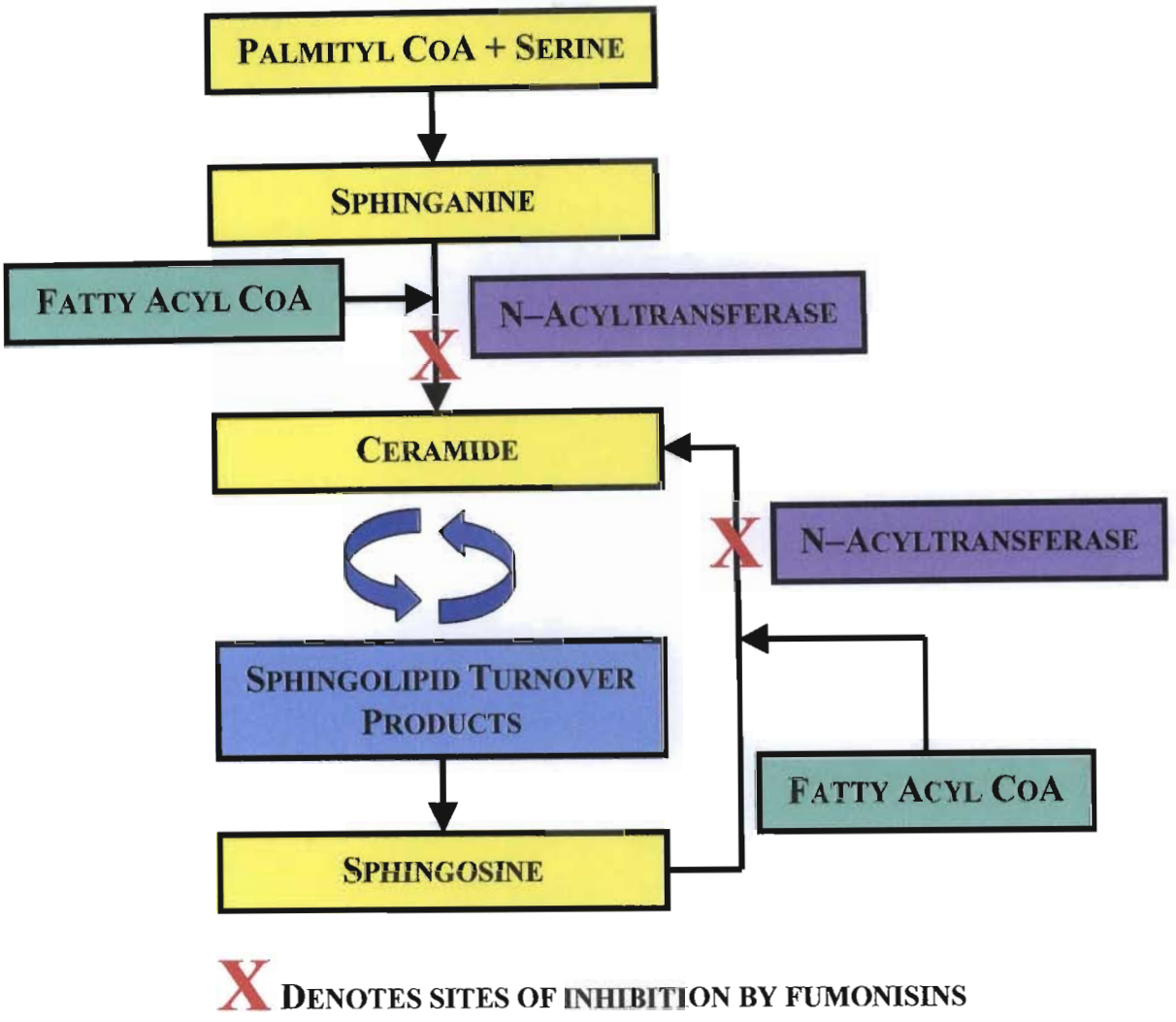


Figure 2.7: **Schematic representation of the sphingolipid turnover pathway with indication of the sites of fumonisin B_1 inhibition (Adapted from: Diaz and Boermans, 1994).**

The accumulation of sphinganine in cells exposed to fumonisins may lead to cell death, due to the cytotoxicity of long chain bases. However, it may also lead to cell proliferation as these compounds are mitogenic to certain cell types and affect diverse cell systems (Wang *et al.*, 1992).

The interruption of sphingolipid biosynthesis results in apoptosis in the liver and kidney of rodents as well as human cells. The increase in apoptosis in rodent livers and kidneys is accompanied by a compensatory increase in cell proliferation, a possible explanation for the tumour promoting properties of FB_1 (Howard *et al.*, 1998).

2.10 CHEMOPREVENTION

Recent progress in molecular biology and pharmacology has increased the likelihood that cancer prevention, either primary or secondary, will increasingly rely on interventions collectively termed chemoprevention (Tamimi *et al.*, 2002).

2.10.1 WHAT IS CHEMOPREVENTION?

Chemoprevention is a preventive strategy used to reduce the incidence of human cancer either by inhibiting the initiation and spread of carcinogenesis or by preventing exposure to high levels of carcinogens (Kim *et al.*, 1999). Thus, cancer chemoprevention can be simply defined as the treatment of carcinogenesis (Kelloff *et al.*, 2000), using specific diets, natural chemicals or synthetic analogues to reverse, suppress or prevent carcinogenic progression to invasive cancer (Sharma *et al.*, 1994; Geschner *et al.*, 1998; Reddy, 2000).

Many human cancers are preventable because their causes have been identified in the human environment (Geschner *et al.*, 1998). A large body of compelling evidence exists to either confirm or implicate various environmental factors in the development of a wide range of malignancies. Among the essential factors are chemical and physical carcinogens, infectious agents and lifestyle. Environmental factors are known to play important roles in the aetiology of human cancer. These include chemical carcinogens such as those found in cigarette smoke, dietary contaminants, for instance the mycotoxin AFB_1 and physical carcinogens, for example UV radiation (Minamoto *et al.*, 1999).

The finding that regular consumption of certain constituents of fruits and vegetables might provide some protection from this deadly disease has been the cause for great interest, amongst both the medical establishment and the general public (Geschner *et al.*, 1998).

2.10.2 CHEMOPREVENTIVE AGENTS

Chemopreventive agents have been classified into three broad categories with distinctly different functions. These are: agents that can prevent the formation of carcinogenic compounds from precursors; agents that can block the metabolic activation of carcinogens and agents that can suppress the expression of neoplasia in cells that have been previously exposed to an effective dose of carcinogen. Additionally, several agents have been shown to inhibit carcinogenesis via multiple mechanisms (Reddy, 2000).

2.10.3 PHYTOCHEMICALS AS CHEMOPREVENTIVE AGENTS

The human diet contains a large number of potential chemopreventive substances. The majority of dietary chemopreventive agents include various phytochemicals that occur in a variety of fruits and vegetables (Chung *et al.*, 1999; de Boer, 2001; Hudson *et al.*, 2003). There are many studies describing the chemopreventive properties of dietary constituents with respect to a range of carcinogens in a number of tissues and several species (Harttig and Bailey, 1998; Manson *et al.*, 1998). Studies in rodent–based systems have identified certain foods as being effective, with cruciferous vegetables, green tea and certain herbs and spices being of particular benefit (Manson *et al.*, 1997).

Flavonoids are polyphenolic compounds found in various foods of plant origin. It has been estimated that humans consuming high fruit and vegetable diets may ingest as much as one gram of these compounds daily (Ciolino *et al.*, 1999; Yang *et al.*, 2000). A large body of evidence exists to implicate bioflavonoids as having significant potential as chemopreventive agents against a number of different cancer types. A variety of effects in mammalian cells and tissues have been attributed to this class of phytochemicals (Van Dross *et al.*, 2003).

Silymarin is a polyphenolic flavonoid antioxidant isolated from the seeds of milk thistle. The primary active constituent of silymarin is silibinin and both compounds have been used clinically in Europe and Asia for the treatment of liver diseases. Animal model studies have revealed that pretreatment with silymarin or silibinin provides protection against hepatotoxicity by a variety of toxicants. Regarding chemoprevention, oral administration of silibinin to mice has been shown to significantly increase the activity of the phase II enzymes GST and quinone reductase in lung, liver, stomach, skin and small bowel tissues (Zhao and Agarwal, 1999). Another antihepatotoxic biflavonoid, kolaviron, obtained by extraction of the seeds of the edible *Garcinia kola*, has been shown to posses potential as a chemopreventive agent against AFB_1 genotoxicity and hepatocarcinogenicity *in vitro* (Nwankwo *et al.*, 2000).

Studies in experimental animal models have provided support for the protective effects of coffee against certain chemical carcinogens. The coffee–specific diterpenes cafestol and kahweol have been identified as two potentially chemopreventive agents in green and roasted coffee beans. The mechanisms by which coffee or its diterpene constituents exert their chemopreventive effects are not entirely known. However, it has been postulated that these diterpenes act as blocking agents by producing a co–ordinated enhancement of multiple enzymes responsible for the detoxification of carcinogens (Cavin *et al.*, 1998).

This hypothesis is supported by the finding that cafestol and kahweol markedly enhance GST activity in the liver and small bowel of experimental animals. In addition, these diterpenes may modulate the cytochrome P_{450} xenobiotic metabolising enzymes, thereby also altering carcinogen activation (Cavin *et al.*, 1998).

Joy *et al.* (2000) investigated the anti–tumour and anti–carcinogenic activity of *Picrorrhiza kurroa* extract in mice. *Picrorrhiza kurroa* is a major ingredient of many indigenous Himalayan medicinal preparations, particularly useful in the treatment of liver diseases, such as hepatitis and jaundice, as well as in the treatment of anaemia and asthma. Administration of the chemical carcinogen 20–methylcholanthrene resulted in 100% induction of sarcoma in control mice, whereas tumour incidence and tumour related deaths were significantly inhibited by the oral administration of *P. kurroa* extract. In addition, the extract was found to reduce the volume of transplanted solid tumours induced by DLA tumour cell lines and increase the lifespan of ascites tumour bearing mice.

Siamese cassia is a native Thai vegetable with some medicinal properties and has traditionally been used for the treatment of a number of ailments, including liver conditions and gastro–intestinal disturbances. It has been reported that ethanol extracts of this vegetable consist of phenolic compounds, which are the most interesting class of chemopreventive agents. Tepsuwan *et al.* (1999) demonstrated that dietary Siamese cassia leaves caused a significant reduction in the activities of some hepatic P_{450}–dependant monooxygenases, while increasing the activities of phase II detoxification enzymes. The authors also demonstrated the potential chemopreventive capacity of this plant. This was due to an inhibitory effect produced by leaves fed during the initiation phase of 9,10–dimethyl–1,2–benzanthracene (DMBA)–induced mammary gland carcinogenesis in female Sprague Dawley rats.

Indole–3–carbinol (I3C) is derived from cruciferous vegetables such as broccoli and Brussels sprouts. It has been shown to possess anti–carcinogenic activity in a number of models, both *in vivo* and *in vitro* and has attracted considerable attention as a potential anti–tumour agent for breast cancer. In addition, I3C has been shown to protect against carcinogen–induced tumours in a range of rodent models including liver, tongue, skin, mammary tissue and colon (Hudson *et al.*, 2003). Indole–3–carbinol has been demonstrated to be a bifunctional blocking agent, affecting both the initiation and promotion stages of carcinogenesis (Stresser *et al.*, 1994; Manson *et al.*, 1998).

Second only to water, tea is the most widely consumed beverage worldwide. Three types of tea are predominantly manufactured. These are black tea, in which most green leaf polyphenols are oxidised, green tea, where the oxidation of these polyphenols is prevented and oolong tea, where some of the polyphenols are oxidised (Jung and Ellis, 2001). Tea, especially green tea and its components, has been reported to inhibit experimentally–induced tumourigenesis in a variety of animal organ systems. These include skin, mammary gland, lung, liver, forestomach, small bowel, oesophagus, gall bladder, pancreas, uterus, nasopharynx and colon (Boone *et al.*, 2000; Steele *et al.*, 2000).

Curcumin is the major pigment in tumeric, a rhizome of the plant *Curcuma longa Lin.* It is used as a flavouring agent in curries and has been employed as a herbal medicine for inflammatory disease (Dinkova–Kostova and Talalay, 1999; Hong *et al.*, 1999). Curcumin (diferuloylmethane) is a phenolic compound possessing antioxidant, anti–inflammatory and free–radical scavenging properties (Limtrakul *et al.*, 2001). Experimental studies have shown that curcumin prevents tumourigenesis and mutagenesis, blocks DNA–adduct formation and inhibits angiogenesis (Dinkova–Kostova and Talalay, 1999).

Epidemiological studies have established an association between diets high in fruits, vegetables or fibre and a reduced risk of the development of colorectal (Kirlin *et al.*, 1999) and bladder cancer (Jacobs *et al.*, 2002). *trans*–Resveratrol (Res), a natural phytoalexin abundant in grapes and grape products, has been shown to possess chemopreventive activity by inhibiting cellular events associated with tumour initiation, promotion and progression (Holmes–McNary and Baldwin, 2000).

It is well known that both oil–soluble and water–soluble organosulphur compounds (OSC) are present in garlic and onions. Some of these, particularly the oil–soluble OSC, have shown chemopreventive potential in the initiation stage of carcinogenesis (Fukushima *et al.*, 2001). Various studies have demonstrated that garlic can slow the development of bladder, skin, stomach and colon cancers, although the inhibition of tumours by garlic seems to be most effective when the tumours are small (Craig, 1999).

Though many chemopreventive phytochemicals are only present in small quantities naturally, the chlorophylls can be abundant, especially in green leafy vegetables. For example, spinach leaves may contain between 2.6 and 5.7% of their dry weight as chlorophylls (Harttig and Bailey, 1998). It is thus important to explore the anticarcinogenic and antigenotoxic properties of these ubiquitous pigments. However, due to the insolubility of chlorophylls in aqueous solutions, chlorophyllin, a stable, water–soluble chlorophyll derivative has been used as an alternative in investigations (Chung *et al.*, 1999).

Harttig and Bailey (1998) investigated the chemopreventive effects of native chlorophylls as well as chlorophyllin on dibenzo [*a,l*] pyrene (DBP)–DNA adducts *in vivo* in rainbow trout. As a complete carcinogen, DBP is among the most potent environmental polycyclic aromatic hydrocarbons known and initiates tumours in the liver, stomach and swim bladder in the rainbow trout model. This study showed a highly significant suppression of DBP–DNA adduct formation in all treatment groups and represented the first demonstration of an anticarcinogenic property of chlorophylls in a vertebrate animal model. The finding suggested a potential role for dietary chlorophylls in the reduction of tumour initiation risk by chemicals present in the human diet, including certain polycyclic hydrocarbons, heterocyclic amines and mycotoxins.

CHAPTER THREE

EXTRACTION OF THE PLANT LEAVES

3.1 INTRODUCTION

The main component of liquid solutions is called the solvent. A smaller amount of another substance, termed the solute, is dissolved in this component. Liquid solutions form because of interactions between the solvent and solute molecules. These forces can be very weak or very strong. In either instance, in order for a solution to occur the solvent–solute interactions must be at least as strong as or stronger than those between solvent molecules and between solute molecules. Only when solvent–solute interactions prevail will solution be attained. This concept is often summarised in the simple expression "like dissolves like" (Odian and Blei, 1994). In order to extract many compounds from a sample, such as plant leaves, one must consider the fact that not all compounds of interest will be soluble in the same solvent.

3.1.1 SOXHLET EXTRACTION

Many different methods of solvent extraction exist, one of which utilises the Soxhlet apparatus. This method has been widely used by chemists in many fields for over a century. It is relatively simple to operate, utilises small sample volumes and does not require much specialised equipment beyond the glass sample chamber. The apparatus is designed such that the sample is not submitted to excessive heat, which may destroy some sample components. In addition, as the apparatus is sealed, volatile compounds are retained in the system for collection following cooling of the extract.

The Soxhlet apparatus comprises three separate components, namely the condenser, the sample chamber and solvent chamber, as demonstrated in Figure 3.1.

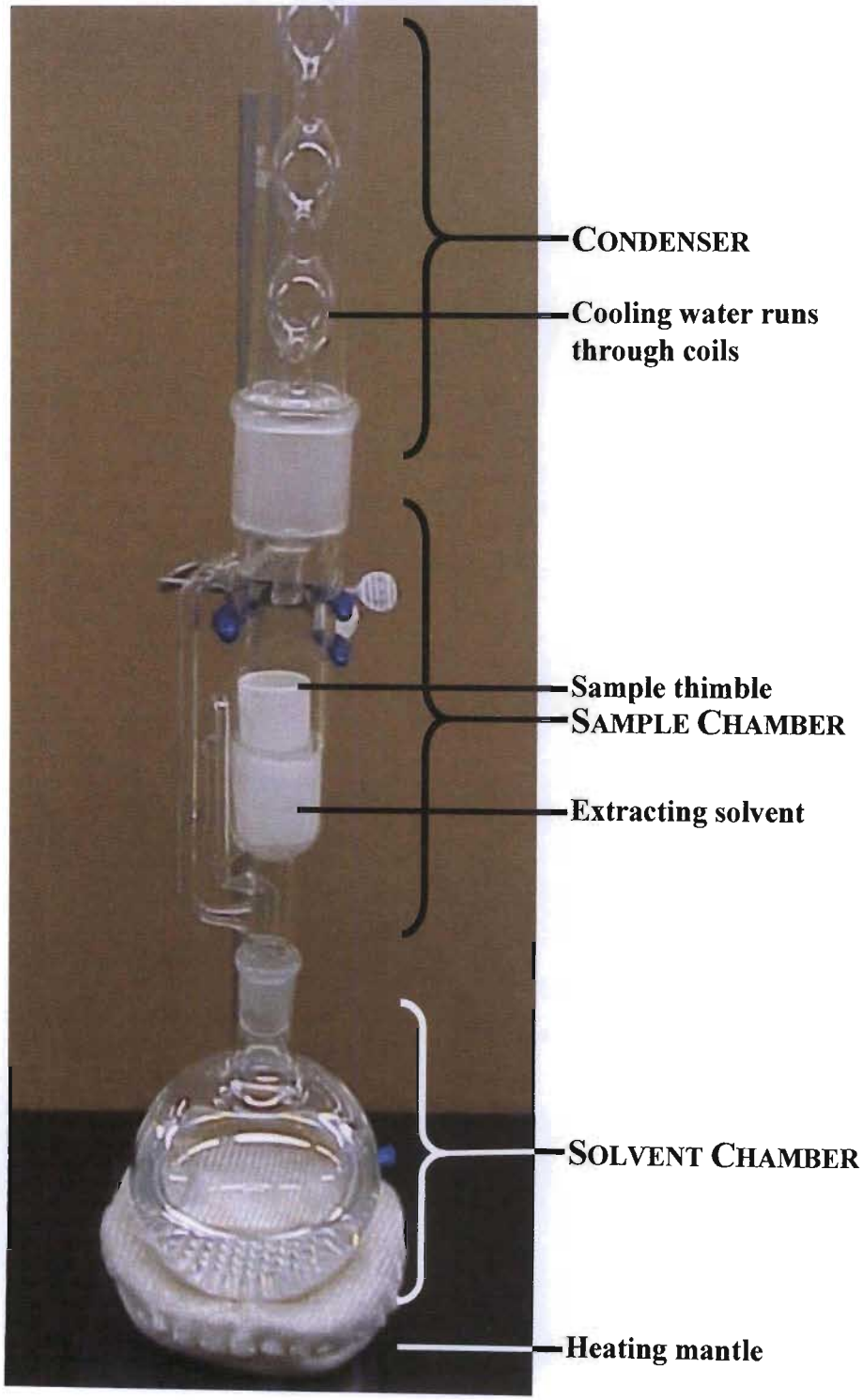


Figure 3.1: Photograph of the Soxhlet apparatus.

The condenser is the uppermost segment of the apparatus and is supplied with cool running water for the duration of the extraction procedure. The sample is contained within a special filter thimble placed into the upper chamber (sample chamber), which allows the solvent and extracted compounds to pass through without removing any particulate sample material. This facilitates the sequential extraction of samples with, among others, solvents of increasing polarity. The lack of particles in the final extract also aids in sample preparation, as no filtration of the solvent is required.

The solvent chamber, directly below the sample chamber, is a reservoir for the extraction solvent. This chamber, commonly a round-bottomed flask, sits in the heating device, such as a heating mantle. The two chambers are connected via two hollow glass tubes or "arms" that run along the outside of the sample chamber.

The "vapour arm" runs in a straight line from the top of the solvent chamber to the top of the sample chamber. The extraction solvent is boiled in the reservoir chamber and the vapours rise via this arm into the sample chamber, where they are exposed to the condenser. The condensed solvent drips onto the sample in the thimble and collects at the bottom of the sample chamber, now bearing a small concentration of the sample's extracted chemicals.

The "solvent return arm", also connecting the two chambers, takes an indirect route. Beginning at the bottom of the sample chamber, the arm runs upward to about half the chamber's height, then bends abruptly down until it enters the top of the solvent chamber. The liquid level rises simultaneously in the sample chamber and the connected solvent return arm. When the liquid in the return arm flows over the bend gravity takes over drawing it quickly down and a natural vacuum siphons most of the solvent from the sample chamber into the solvent reservoir.

The Soxhlet extractor carries out a series of cycles in this manner, the length of which are dependent on the temperature used for the particular solvent. The higher that the temperature used is above that of the boiling point of the solvent, the quicker the chamber will fill and empty. Thus, it is very important to operate the heating mantle at the optimum temperature for each solvent. Ideally, the solvent should boil slowly while the extractor runs through enough cycles to produce the desired concentration of extract. This not only ensures the safe operating of the apparatus, but also increases the contact time between the solvent in the sample chamber and the sample in the thimble, allowing for increased extraction of compounds of interest.

3.1.2 NUTRITIONAL REQUIREMENTS

The nutrients that must be ingested for survival may be divided into two groups. These are the macronutrients, which are required in large quantities, and micronutrients, which are required in much smaller amounts (Arms and Camp, 1995).

3.1.2.1 Macronutrients

The three classes of macronutrients are carbohydrates, proteins and lipids, which can all serve as energy sources since they can be catabolised into molecules that are respired to produce ATP. They also provide carbon atoms that can be utilised to produce organic molecules. The macronutrient that supplies most of the human population's energy requirements is starch. This carbohydrate is most commonly produced and stored by plants. All of the most important human food plants, including grains and potatoes, contain high concentrations of starch. Proteins are broken down into their amino acid constituents, most of which are utilised to produce the body's own proteins. Lipids are important components of all cell membranes. Steroid hormones are also lipids (Arms and Camp, 1995).

Macronutrients can be stored in the body until required for energy production. Carbohydrates are stored as glycogen in muscles and the liver and lipids are stored as fat. However, proteins cannot be stored. Excess protein is broken down into amino acids, which are deaminated and the remainder of the molecule is then processed as lipid or carbohydrate (Arms and Camp, 1995).

3.1.2.2 Micronutrients

Micronutrients are substances that are required in the diet in small quantities, as the body cannot produce them at all, or cannot produce them as quickly as they are needed. This group of nutrients can be divided into the vitamins, which are organic compounds and the minerals, which are inorganic. Various diseases result from deficiency of certain vitamins and minerals in the diet (Arms and Camp, 1995).

The vitamin requirements differ between species, as different animals can only produce some vitamins from precursor molecules. For example, vitamin C is required in the human diet, although many other animals can synthesise it for themselves. Vitamins required in the human diet are generally divided into two categories, either as water–soluble or lipid–soluble. Water–soluble vitamins are coenzymes required in metabolism and are readily excreted by the kidney. Lipid–soluble vitamins have diverse, poorly understood functions. As these vitamins are not water soluble, they must be processed by enzymatic action before they can be excreted by the kidney. Lipid–soluble vitamins that are not utilised immediately may be stored in the fatty tissue where they may accumulate to toxic levels if consumed in amounts larger than the body is able to process or use. Vitamin–rich foods include fresh fruit and vegetables as well as liver (Arms and Camp, 1995).

Some mineral nutrients are required in relatively large quantities. For instance, sodium and potassium are vital for the working of nerves and muscles in the body and large amounts of these minerals, particularly sodium, are excreted daily in the urine. Calcium is required for muscle activity and, together with phosphorous, is necessary in large quantities for bone development. Other minerals are known as trace minerals. Some of these are required in tiny amounts as cofactors of enzymes in various metabolic pathways, while the functions of others are poorly understood or unknown. Foods rich in minerals include meat, seafood, dairy products and spinach (Arms and Camp, 1995).

3.1.3 OBJECTIVES

The purpose of this study was to obtain crude solvent fractions from the leaves of the *Amaranthus lividus* plant for use in further experiments to determine the cytotoxic activity and possible chemopreventive potential of this plant.

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

All organic solvents were of ANALAR grade and were purchased from Merck SA. HPLC grade, double distilled water (ddH_2O) was used.

3.2.2 METHODS

3.2.2.1 Collection, Identification and Preparation of the Plant Material

Plants were collected from residential gardens where they grow naturally. An intact specimen was pressed, dried and sent to the Natal Herbarium for identification. The plant was identified as *Amaranthus lividus* (L.) subspecies Polygonoides (Moq.) Probost. A voucher specimen was deposited at the same herbarium (specimen identification number: Donella Wright No.1). The leaves were removed from the plants, rinsed in ddH_2O and finely chopped with a blade.

3.2.2.2 Preparation of the Acetone Fraction (Soxhlet Extraction)

For use in the Soxhlet apparatus, the fresh leaves were first lyophilised. A five gram portion of the lyophilised leaves was weighed out and extracted in the Soxhlet apparatus using solvents of increasing polarity, namely hexane, dichloromethane (DCM), acetone and methanol (MeOH). The volume of each solvent used in the extraction was 250ml.

Following an extraction period of 24 hours per solvent, the solvent fraction was removed from the flask. The flask was then rinsed with the following solvent before the addition of this solvent. All rinsings were discarded.

Heating of the solvents was achieved using a heating mantle with a temperature control attachment. The temperature differed for each solvent and was generally kept at 5°C below their respective boiling points, as shown in Table 3.1. As the system was subjected to vacuum throughout the extraction, the temperatures used were sufficient to provide a steady flow of condensed solvent vapours over the sample, ensuring adequate contact time between the solvent and the sample material.

Table 3.1: Solvents used in the Soxhlet extraction and their respective boiling points.

SOLVENT	BOILING POINT (°C)	T° OF HEATING MANTLE (°C)
HEXANE	69	65
DICHLOROMETHANE	40	35
ACETONE	56	51
METHANOL	65	60

For the purposes of this study, the effects of only one of the Soxhlet fractions, namely the acetone fraction, were investigated. This was based on results from previous studies, which demonstrated that this fraction had anti–microbial as well as cytotoxic activity towards the SNO oesophageal adenocarcinoma cell line (unpublished data).

This fraction was concentrated under vacuum on a rotary evaporator, reconstituted to a final volume of 100ml, aliquotted and dried in a heating block, under a stream of nitrogen gas. The nitrogen gas speeds the drying process by displacing solvent fumes from the vessel and helps prevent excessive heating of the sample, as the stream of gas is cool. In addition, the N_2 gas is chemically inert and will not react with any of the sample components.

These dried aliquots were stored at 4°C in the dark until required and for no longer than six months.

The yield of this fraction was determined by removing one millilitre of the reconstituted fraction (100ml) into a pre–weighed bottle. This aliquot was then dried completely and the bottle weighed to determine the amount of solids present in the fraction. This weight was then represented as amount solids per millilitre of fraction (either mg/ml or µg/ml as appropriate).

3.2.2.3 Preparation of the Water Fraction

In order to simulate the normal cooking process a water fraction was prepared. Fresh, chopped leaves were placed in a 250ml Schott bottle, brought to the boil in ddH_2O (1:10 w/v) and allowed to simmer for approximately 30 minutes. Constant mixing was achieved with the use of a magnetic stirrer bar. The mixture was allowed to cool and was then filtered through Whatman No. 5 filter paper using the Buchner apparatus. The water fraction was then concentrated using a rotary evaporator, aliquotted and dried on a heating block, under a stream of nitrogen gas. These dried aliquots were stored at 4°C in the dark until required and for no longer than six months. The yield of this fraction was determined as for the acetone fraction.

3.2.2.4 Preparation of Stock Solutions

Due to the highly toxic nature of acetone and its high volatility, leading to rapid evaporation from solution, the acetone fraction was reconstituted in absolute ethanol (EtOH). For all tests performed, an ethanol solvent control was performed simultaneously, ensuring that any effects noted were due only to fraction components and not to the ethanol solvent. A stock solution of the fraction was prepared in ethanol (10mg/ml) and sterilised using a 0.45µm millipore filter before use.

The water fraction was reconstituted with ddH_2O therefore, for all tests performed, a water solvent control was performed simultaneously, ensuring that any effects noted were due only to fraction components and not to possible dilution effects of the water solvent. A stock solution of the water fraction was prepared in ddH_2O (10mg/ml) and sterilised using a 0.45µm millipore filter before use. The water used in the preparation of the solvent control was similarly sterilised.

These stock solutions were prepared as required and stored at 4°C for no longer than two weeks.

3.3 RESULTS

3.3.1 The Acetone Fraction (AF)

The average yield for this fraction was approximately 200µg/ml. The fraction was very dark green in appearance indicating a high concentration of chlorophyll. In fact, upon reconstitution this fraction exhibited a darker green colour than the DCM fraction, which was more translucent.

The solution was clear (free from particulate matter) upon reconstitution with the ethanol solvent to a concentration of 10mg/ml. All of the compounds isolated in this fraction were soluble in the ethanol solvent at room temperature with minimal agitation required.

3.3.2 The Water Fraction (WF)

The yield obtained for this fraction was much higher than that of the AF, at approximately 7mg/ml. This was due to the different extraction methods and solvents employed.

As the AF was obtained from a sequential extraction procedure, with two preceding solvents, many compounds would have already been removed from the plant material. In addition, the Soxhlet extraction was conducted on lyophilised leaves, whereas the water extraction was carried out on fresh plant material. This would result in a completely different type of solution being obtained. The ratios of leaves to solvent were also different for the extractions.

This fraction was also green in colour however, the depth of the green hue was not as dark as for the AF. In addition, this fraction had a yellow–brown tinge when held up to the light and did contain some tiny particulates following reconstitution.

3.4 DISCUSSION

Due to the varying solubility of compounds, the two fractions obtained most likely contained different nutrients and other cofactors. The AF possibly contained lipid–soluble components, whilst the WF contained the water–soluble nutrients.

3.4.1 The Acetone Fraction

Carotenoids are lipid soluble compounds classified as xanthophylls, carotenes or lycopene. The most commonly available of the more than 600 naturally occurring carotenoids in the human diet is β–carotene. Carotenoids are abundant in yellow and green leafy vegetables and have antioxidant and other beneficial properties. Most importantly, these compounds are relatively non–toxic. Observational epidemiological data consistently indicates protective effects of carotene–rich vegetables against cancers of the lung and oesophagus, amongst others (Tamimi *et al.*, 2002).

As detailed in Chapter 2, dietary chlorophylls have been demonstrated to have a potential role in the reduction of tumour initiation by mycotoxins present in the human diet (Harttig and Bailey, 1998). As the AF was dark green in colour, it is reasonable to assume that this fraction contained a large proportion of chlorophylls. In support of this postulation, Harttig and Bailey (1998) used acetone to extract chlorophyll A from spinach powder. The yield of chlorophyll from the extract obtained following partial purification via ascorbate–silica gel chromatography was determined to be approximately 77%.

Lipid soluble vitamins have also been associated with decreased cancer risk. Long term tocopherol supplementation in the diet may reduce the risk of bladder cancer (Jacobs *et al.*, 2002). In addition, a strong inverse relationship between dietary intake of vitamin A and cancer development has been established. Retinoids have shown protective effects against lesions associated with cutaneous, oral and bronchial premalignancies in humans (Gescher *et al.*, 1998).

3.4.2 The Water Fraction

Many classes of nutrients are water soluble, such as polysaccharides, proteins, amino acids, minerals and the water–soluble vitamins.

The aqueous extracts of *Crocus sativus* L. (saffron) petals were found to contain flavonoids, tannins and anthocyanins, while the stigma aqueous extract contained alkaloids and saponins. This plant is used in traditional medicine for various purposes. Modern pharmacological studies have shown that saffron extracts possess radical scavenging, anti–inflammatory and anti–tumour activity (Hosseinzadeh and Younesi, 2002).

Many plant polysaccharides have been used in traditional medicine worldwide for the treatment of various ailments due to their numerous biological properties. These include anti–ulcer, anti–hepatitis, anti–inflammatory, wound healing and anti–neoplastic properties (Kim *et al.*, 1999).

3.5 CONCLUSION

Interest in a large number of traditional natural products has increased and it has been postulated that aqueous and ethanolic extracts from plants used in allopathic medicine are potential sources of antitumour agents. The selection of crude plant extracts for screening programs may currently be more beneficial in the initial stages than the screening of pure compounds isolated from natural products.

CHAPTER FOUR

PRE–SCREENING OF THE WATER AND ACETONE FRACTIONS FOR CYTOTOXIC EFFECTS USING CANCER AND NORMAL CELL LINES

4.1 INTRODUCTION

4.1.1 CELL CULTURE

Tissue culture is often a generic term referring to both organ and cell culture and the terms are often used interchangeably. Cell cultures are derived either from primary tissue explants or cell suspensions. Primary cell cultures typically have a finite life span in culture whereas continuous cell lines are, by definition, abnormal and often transformed cell lines (Freshney, 1987).

Cell culture is advantageous as the moral and ethical issues surrounding the use of animal models are avoided. In addition, *in vitro* studies allow for better uniformity and reproducibility of results and the use of human cell cultures allows for experimentation not possible *in vivo*. However, cell culture is a specialised technique requiring a certain level of skill and strict maintenance of aseptic conditions. The method is also very expensive, as costly equipment and reagents are required for the maintenance of the cells. Cell culture is, in fact, more expensive than working with live animals. In addition, exact reproduction of the *in vivo* situation is impossible using cell lines (Freshney, 1983).

4.1.2 EQUIPMENT AND REAGENTS

Specialised equipment is required for use in a cell culture facility. These include a laminar flow hood, a CO_2 humidified incubator and a phase contrast microscope. In addition, cells in culture have specific requirements with regards to the medium of growth.

4.1.2.1 Laminar Flow Hoods

There are two types of laminar flow hoods, namely vertical and horizontal. The vertical hood, also known as a biological safety cabinet, is best for working with hazardous organisms since the aerosols generated in the hood are filtered out before they are released into the surrounding environment. Horizontal hoods are designed such that the air flows directly at the operator hence they are not useful for working with hazardous organisms but provide the best protection for the cultures. Both types of hoods have continuous displacement of air that passes through a HEPA (high efficiency particulate air) filter, which removes particulate matter from the air. In a vertical hood, the filtered air blows down from the top of the cabinet whereas in a horizontal hood, the filtered air blows out at the operator in a horizontal fashion. These hoods are equipped with a short–wave UV light that can be switched on to sterilise the surfaces of the hood when it is not in use. However, only the exposed surfaces will be accessible to the UV light. The hood should be switched on 10–20 minutes prior to use to allow the air pressure to stabilise. To ensure sterility, all surfaces and reagent bottles must be wiped down with ethanol (70%) before and after each use. The hood must also be kept as free of clutter as possible because this will interfere with the laminar flow air pattern (Freshney, 1987).

4.1.2.2 Humidified CO_2 Incubators

The cells must be grown in an atmosphere of 5–10% CO_2 because the medium used is buffered with sodium bicarbonate/carbonic acid and the pH must be strictly maintained. Alternatively, HEPES buffer may be added to the medium to maintain this equilibrium. Culture flasks should have loosened caps to allow for sufficient gaseous exchange. The cells should be left out of the incubator for as little time as possible and the incubator doors should not be opened for very long. The humidity must also be maintained for cells growing in tissue culture dishes so a pan of water is kept filled at all times (Freshney, 1987).

4.1.2.3 Inverted Phase Contrast Microscopes

Inverted phase contrast microscopes are used for visualising the cell cultures *in situ*. These microscopes should be kept covered and the lights turned down when not in use. Cultures should be examined daily, observing cell morphology, the colour of the medium and the density of the cells (Freshney, 1987).

4.1.2.4 Culture Vessels

Anchorage dependent cells require a non–toxic, biologically inert and optically transparent surface that will allow cells to attach and movement for growth. The most convenient vessels are specially treated polystyrene plastic. These include petri dishes, multi–well plates, microtitre plates, roller bottles and screwcap flasks, which are disposable and supplied sterile. Suspension cells can be shaken, stirred or grown in vessels identical to those used for anchorage dependent cells (Freshney, 1987).

4.1.2.5 Growth Requirements for Cell Cultures

Mammalian cell cultures require a degree of simulation of the physiological conditions encountered in the body in order to grow successfully in the culture environment. Physiological parameters that must be maintained include temperature and pH and osmolality of the medium. Humidity is required, as previously discussed. In addition, visible light can have an adverse effect on cells, for instance, light induced production of toxic compounds can occur in some media. Therefore, cells should be cultured in the dark and exposed to room light as little as possible (Freshney, 1987).

In addition to the physiological requirements mentioned above, cell cultures have specific nutrient requirements, which are often empirical. Serum has many of growth promoting activities such as buffering of toxic nutrients by binding them. It has undefined effects on the interaction between the cells and the growth substrate and contains peptide hormones or hormone–like growth factors that promote healthy growth. It also neutralises trypsin and other proteases. Other medium requirements include sugars (usually glucose), amino acids (there are 13 essential amino acids) and vitamins (such as the B–group vitamins) as well as bulk ions and trace elements. These are either present in the medium as purchased, or must be added as supplements to the medium to ensure optimal growth of the cultures. Finally, although not required for cell growth, antibiotics are often used to control the growth of bacterial and fungal contaminants (Freshney, 1987).

4.1.2.6 Preservation of Cell Cultures

Tissue culture cells are preserved in liquid nitrogen. Freezing can be lethal to cells due to the damaging effects of ice crystal formation, alterations in the concentration of electrolytes, dehydration and changes in pH. To minimise these negative effects, several precautions are taken. A cryoprotective agent that lowers the freezing point, such as glycerol or dimethyl sulphoxide (DMSO), is added to the cells prior to freezing. The storage medium typically used consists of 90% serum and 10% cryoprotective agent. It is best to use healthy cells that are growing in the log phase and to replace the medium 24 hours before freezing. In addition, the cells are slowly cooled from room temperature to –70°C to allow the water to move out of the cells before it freezes. Cells are stored in liquid nitrogen as the growth of ice crystals is retarded at temperatures below –130°C. To maximise recovery of the cells when thawing, the cells are warmed very quickly by placing the tube directly from the liquid nitrogen container into a 37°C water bath with moderate shaking. As soon as the last ice crystal has melted, the cells are immediately diluted into warmed medium (Freshney, 1987).

4.1.2.7 Passaging of Cell Cultures

The cells are harvested when they have reached a population density that suppresses growth. Ideally, cells are harvested when they are semi–confluent and still in the log phase of growth. Cells that are not passaged and are allowed to grow to a confluent state can sometimes lag for a long time and some may never recover. Suspension cultures are passaged by dilution into fresh medium. Alternatively, the culture can be centrifuged and the cell pellet resuspended in fresh medium, which is then divided amongst the new culture vessels. When adherent cultures become semi–confluent, several methods are available to remove the cells from the growth surface, including mechanical and chemical methods (Freshney, 1987).

A rubber spatula can be used to physically remove the cells from the growth surface. This method is quick and easy but is also disruptive to the cells and may result in significant cell death. Proteolytic enzymes, such as trypsin or collagenase can be used, usually in combination with ethylenediaminetetra–acetic acid disodium salt (EDTA), and cause the cells to detach from the growth surface. This method is fast and reliable but can damage the cell surface by digesting exposed cell surface proteins. The proteolysis reaction can be quickly terminated by the addition of complete medium containing serum. Alternatively, EDTA can be used alone to detach cells and is not as harsh to the cells as trypsin (Freshney, 1987).

4.1.2.8 Measurement of Growth and Viability

The viability of cells can be observed visually using the inverted phase contrast microscope. Live cells are phase bright, with suspension cells typically being rounded and fairly symmetrical. Adherent cells form projections when they attach to the growth surface. Viability can also be assessed using the vital dye trypan blue, which is excluded by live cells but accumulates in dead cells. Cell numbers are determined using a haemocytometer (Freshney, 1987).

4.1.2 Enumeration of Viable Cells via the Trypan Blue Dye Exclusion Haemocytometer Method

Trypan blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. The method is based on the principle that live cells do not take up certain dyes, whereas dead cells do (Sigma, 2004). The cells are counted using a haemocytometer, which is a glass slide that is viewed under a light microscope. It consists of two identical counting chambers with a series of grids (Figure 4.1) and a fragile coverslip (Exercise in the use of the Hemocytometer, 2004).

A cell suspension is prepared and an aliquot of cells is thoroughly mixed with trypan blue stain. The sample is allowed to stand for 5–15 minutes at room temperature. With the cover slip in place, a small amount of trypan blue–cell suspension is transferred to both chambers of the haemocytometer. This is achieved by carefully touching the edge of the cover slip with the pipette tip so that each chamber fills by capillary action. Care must be taken to avoid over– or under–filling the chambers (Sigma, 2004).

Beginning with one chamber of the haemocytometer, all unstained cells in the 1mm centre square and four 1mm corner squares are counted. This is done for both chambers and the average viable cell number is calculated. Cells at the periphery of the chamber are not counted. Thus, according to Figure 4.1 below, cells on top and to the left touching middle line are included (green outline), while cells touching the middle line at bottom and right are not (red outline). If there are too many or too few cells to count, the procedure must be repeated either concentrating or diluting the original suspension as appropriate (Sigma, 2004).

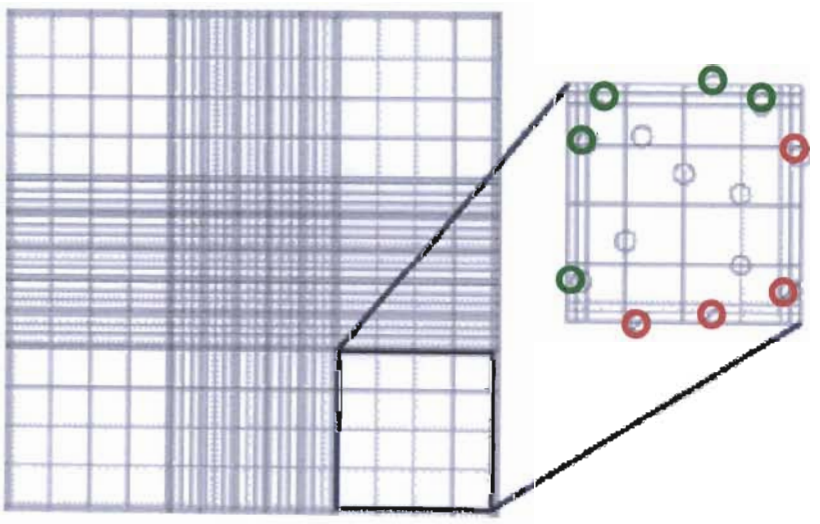


Figure 4.1: Counting chamber of the haemocytometer with detail depicting one grid and the cells to be included and excluded when counting (Adapted from: Exercise in the use of the Hemocytometer, 2004).

Each large square of the haemocytometer, with coverslip in place, represents a total volume of $0.1mm^3$ or $10^{-4}cm^3$. Since $1cm^3$ is equivalent to approximately 1ml, the total number of cells per ml can be determined from the following calculations (Sigma, 2004):

Cells/ml = Average of cells in 10 fields x dilution factor x 10^4

Total Cells = Cells/ml x original volume of culture suspension

4.1.3 TETRAZOLIUM SALT BIOREDUCTION ASSAYS

Tetrazolium salts are extensively used for a variety of research applications, including cell proliferation and cytotoxicity assays. In each instance, the tetrazolium salts are metabolically reduced to highly coloured precipitates called formazans, as demonstrated in Figure 4.2 (Berridge *et al.*, 1996). The prototype tetrazolium salt introduced by Mosmann (1983) for cellular bioassays was 3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyl tetrazolium bromide (MTT).

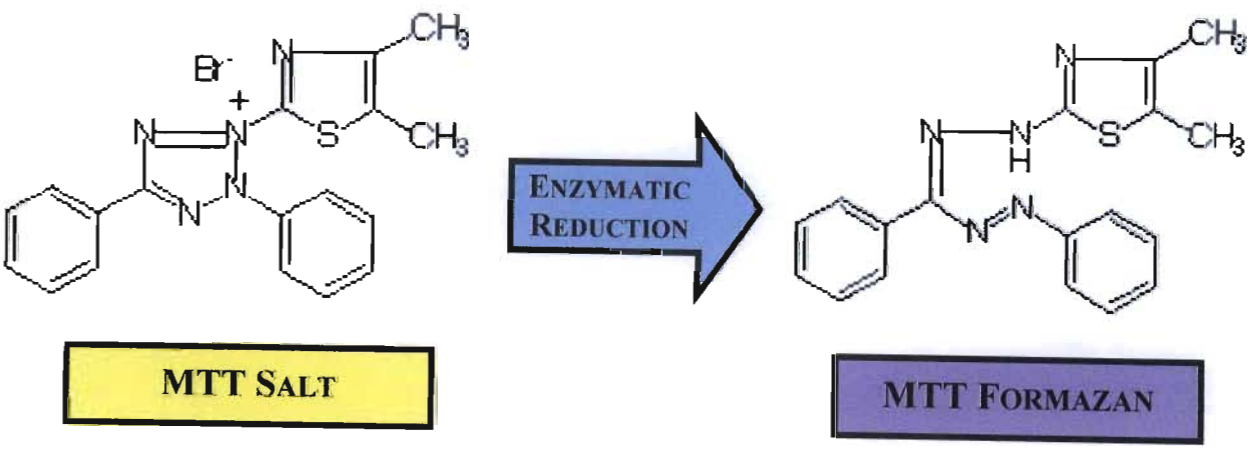


Figure 4.2: Chemical structures of the methylthiazol tetrazolium salt and its formazan product (Roche, 2004).

The reduction of tetrazolium salts, including MTT, is regarded as a convenient test for cell viability and an alternative to clonogenic and thymidine incorporation assays. However, despite extensive application of tetrazolium viability assays, the mechanism of cell–mediated reduction remains to be fully elucidated (Bernas and Dobrucki, 1999). Originally, it was believed that succinate tetrazolium reductase enzymes present in the respiratory chain of mitochondria in viable cells were responsible for the bulk of MTT reduction (Slater *et al.*, 1963; Altmann, 1976).

This theory has since been challenged and Berridge *et al.* (1996) demonstrated that the majority of cellular MTT reduction occurs extramitochondrially and was dependent on NADH and NADPH dependent enzymes of the endoplasmic reticulum. Although succinate can also act as an electron donor in MTT reduction, through mitochondrial succinate dehydrogenase in Complex II, the reaction is slow and contributes little to total cellular MTT reduction. This was demonstrated by the observation that cellular MTT reduction was unaffected by the addition of specific respiratory inhibitors. In addition, cellular reduction of MTT is sensitive to the concentration of glucose in the medium. This, together with the acute sensitivity of MTT responses to inhibitors of glucose transport into the cell, supports the view that reduction is primarily a measurement of the rate of glycolytic NADH production, rather than respiration.

The limited knowledge regarding the exact sites of MTT reduction results from a lack of suitable methodology for investigating the subcellular localisation and reduction of tetrazolium salts in intact, viable cells. To this end, Bernas and Dobrucki (2000) localised the formation of the formazan crystals, rather than the tetrazolium salt itself, utilising fluorescence and backscattered light confocal microscopy, in single cells *in vitro*. This study demonstrated that MTT reduction occurred intracellularly as well as in the region of the plasma membrane. The MTT salt readily traversed the plasma membrane and membrane integrity was maintained throughout treatment.

More recently, the same authors further elucidated the subcellular localisation of the sites of MTT reduction using the same methodology (Bernas and Dobrucki, 2002). They found that only a small portion of MTT–formazan (25–45%) was associated with the mitochondria, whereas most MTT is reduced in other cellular compartments, in the cytoplasm and in regions of the plasma membrane.

Major advantages of the MTT colorimetric assay are that many substances at various concentrations can be tested in duplicate and simultaneously (Carmichael *et al.*, 1985). Moreover, since the spectrophotometric analysis of an entire 96 well microtitre plate takes 30 seconds or less, a large volume of samples can be analysed in a day. Although MTT is a potential mutagen and certain precautions must be taken when using this chemical, the problems encountered when working with this substance are much less than those of handling and disposing of radioactive materials (Holt *et al.*, 1988).

4.1.4 CELL LINES AND TOXIN

The SNO oesophageal adenocarcinoma cell line was selected, as cancer of the oesophagus is the most common gastrointestinal malignancy amongst South African Blacks (Levy *et al.*, 1999). In addition, experiments were also performed using normal human lymphocytes freshly isolated from whole peripheral blood. These cells represent a primary cell culture of non–transformed, normal cells. The inclusion of these cells in this study allowed for the initial assessment of possible derogatory effects of the plant fractions on a normal cell population.

The SNO cell line was established by Bey *et al.* in Johannesburg, South Africa in 1976. A biopsy specimen from a 62–year–old, male Zulu patient (SN) was used to establish, maintain and propagate an epithelial oesophageal carcinoma (OC) cell line *in vitro*. The cells exhibit a squamous pattern of growth, which is maintained, even in heavily confluent cultures without piling up of the cells. Epidemiological evidence links geographic areas with a high prevalence of OC in humans to high levels of FB_1 occurrence (Myburg *et al.*, 2002; Theumer *et al.*, 2002), thus this toxin was selected for the SNO experiments.

Lymphocytes are the pivotal cells involved in the specific immunologic response in humans. They are small, nonphagocytic, mononuclear leucocytes that are either immunologically competent or precursors of such cells. Undifferentiated lymphocytes are derived from bone marrow stem cells. They migrate through the circulatory and lymphatic systems to secondary lymphoid tissues, producing lymphocyte colonies. Those that migrate to the thymus undergo processing to become T cells or T lymphocytes (**T**hymus), which comprise 70–80% of circulating lymphocytes. The remaining 20–30% of circulating lymphocytes is composed of the B class of lymphocytes, which differentiate in the bone marrow of adults, and the null cells, which lack T and B cell characteristics. In addition, T lymphocytes have the ability to recognise and destroy tumour cells (Prescott *et al.*, 1996).

In addition to the plant fractions, the effects of FB_1 were also assessed using the normal human lymphocyte cell population.

4.1.5 OBJECTIVE

The principal goal of this component of the study was to acquire cytotoxicity profiles for the crude WF and AF obtained from the Soxhlet extraction, as well as one of the mycotoxins to be used throughout this study. This would enable further experiments on appropriate concentrations of the fractions and FB_1. In addition, several incubation periods were evaluated to ascertain the most suitable time interval for use in further studies.

4.2 MATERIALS AND METHODS

4.2.1 MATERIALS

The SNO oesophageal adenocarcinoma cell line was purchased from Highveld Biologicals, Johannesburg. Normal human lymphocytes were isolated from fresh, whole blood via the Histopaque method as described later. All cell culture vessels, culture media and components were purchased from Adcock Ingram, SA. Histopaque®–1077, MTT salt, dimethyl sulphoxide (DMSO), trypan blue stain and FB_1 were purchased from Sigma Chemical Company, SA. Blood vials, sterile, single use, 10cc syringes, needles and millipore filters were purchased from Endomed, SA. All solvents were of HPLC grade and were purchased from Merck, SA, as were the sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate (anhydrous) and sodium chloride. HPLC grade, double distilled water was used.

Ethical approval for this study was obtained from the Research Ethics Committee on 7 May 2002 (Reference number: H146/01).

4.2.2 METHODS

4.2.2.1 Maintenance of the SNO Cell Line

The cells were grown as monolayer cultures in $25cm^3$ or $75cm^3$ culture flasks and maintained in complete culture medium (CCM; Appendix 1) at 37°C in a sterile cell culture incubator. Every second day the cells were washed with Hank's Balanced Salt Solution (HBSS) and the media was changed to maintain optimal growth and health of the cells. Once the cells occupied over 80% of the total growth area of the culture flask, confluence was attained and the cells were trypsinised to facilitate passaging of the cells into new culture vessels.

4.2.2.2 Isolation of Normal Human Lymphocytes

Whole human peripheral venous blood was collected in sterile, 5ml lithium heparin tubes. The number of tubes drawn each time was determined by the nature of the experiment. The source of the blood was a healthy 25 year old female donor.

Histopaque®–1077 was used to separate the whole blood. Histopaque®–1077 is a solution of polysucrose and sodium diazoate adjusted to a density of 1077g/ml (Sigma, 2004). The Histopaque® was stored at 4°C and warmed to 37°C in a sterile incubator prior to use.

The whole blood was carefully layered onto an equal volume of Histopaque® in sterile untreated blood vials. Care was taken not to disturb the tubes, as mixing would prevent the sedimentation of the different cell types. The tubes were then centrifuged at 400 x g in a bench top centrifuge (Eppendorf Model 5804 R, Merck, SA) for 30 minutes at room temperature, following which four distinct layers were observed (Figure 4.3).

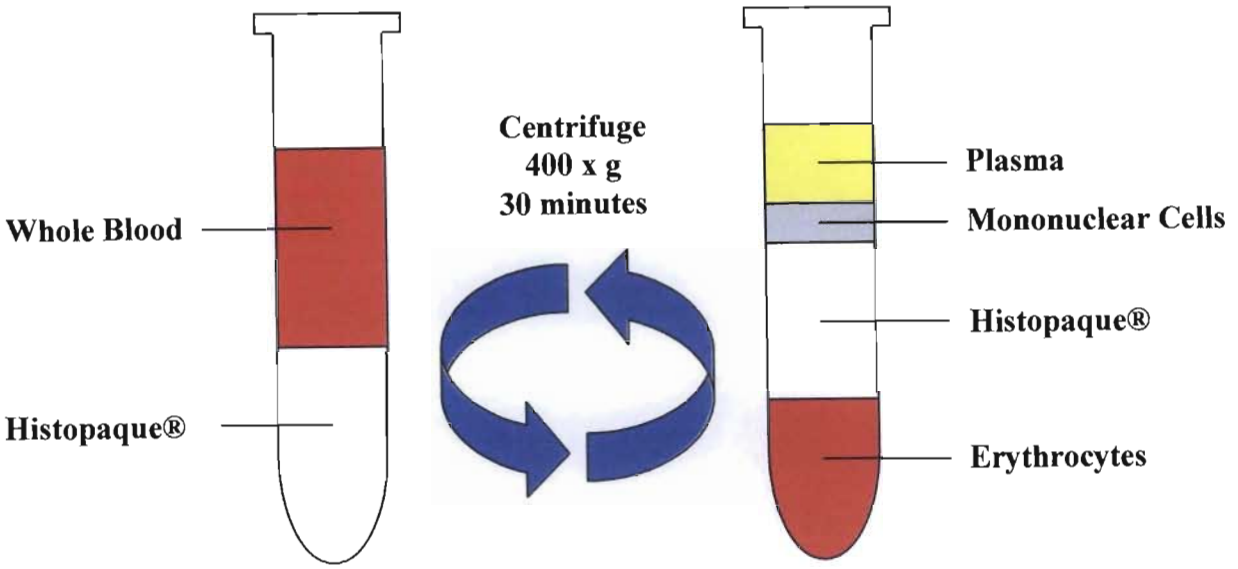


Figure 4.3: **The isolation of lymphocytes from whole human blood using Histopaque®–1077.**

The upper plasma layer was carefully aspirated to within 0.5cm of the second opaque layer using a sterile micropipette and discarded. The mononuclear cell layer, also referred to as the buffy coat, contained the lymphocytes, which were aspirated with a sterile micropipette and transferred to sterile vials.

The lymphocytes were then resuspended in 5ml sterile phosphate buffered saline (PBS; pH 7.4; Appendix 2) warmed to 37°C. The tubes were then centrifuged at 250 x g for 30 minutes at room temperature. This step was repeated twice and served as a rinse to remove extraneous debris. The remaining layers from the Histopaque® separation were discarded.

Following the final rinse, the supernatants were discarded and the pellets were resuspended in small aliquots of CCM (Appendix 1), pooled together and topped up to a final volume of 20–30ml with CCM warmed to 37°C.

4.2.3 PREPARATION OF DILUTIONS

4.2.3.1 Plant Fractions and Solvent Controls

The stock solutions (10mg/ml) prepared following extraction as described in Chapter 3 were used to prepare working stock solutions (1mg/ml) of both the WF and AF. Sterile water and ethanol were used to prepare 1mg/ml equivalent (eq.) working stock solutions of the respective solvent controls. These working stock solutions were prepared in CCM (appropriate for the cell line used) and were used in the preparation of all further dilutions.

For initial screening, a high (25µg/ml–100µg/ml) and a low (2.5µg/ml–10µg/ml) range of concentrations of the fractions and solvent controls were tested against the SNO cell line. Only the 5, 10, 50 and 100µg/ml concentrations were assessed on the normal human lymphocytes.

It is important to note that the stated concentrations are the final concentrations of the plant fractions or solvent controls in each well during the assay. Each sample well consisted of 100µl cell suspension and 100µl plant fraction or solvent control, giving a final volume of 200µl in each well. Thus, the initial concentrations of the dilutions prepared were doubled.

4.2.3.2 Mycotoxin Preparation

A 1mg/ml (1385µM) stock solution was prepared for FB_1. As FB_1 is water soluble, no solvent vehicle was required and the toxin was dissolved directly in CCM appropriate for the cell line used. This toxin stock solution was sterilised using 0.45µm millipore filters prior to use in the preparation of further dilutions.

As for the plant fractions, the initial screen was performed using a high (25μM–100μM) and a low (2.5μM–10μM) range of concentrations of FB_1 on the SNO cell line. Again, only the 5, 10, 50 and 100μM concentrations of FB_1 were tested on the lymphocytes.

These were the final concentrations of the toxin in the wells and as the composition of the sample wells were the same as for the plant fraction assays, dilutions were prepared at double the final concentrations desired.

4.2.4 TREATMENT PROTOCOL

The SNO cells were maintained in a sterile culture environment until confluence was attained, at which time the assay was conducted. The cells were trypsinised, counted at room temperature using the trypan blue haemocytometer method previously described and seeded in standard, sterile 96 well flat–bottomed microtitre plates at a density of 3×10^5 cells/well (100μl).

The normal human lymphocytes were used immediately upon isolation. These cells were also counted via the trypan blue haemocytometer method and seeded in standard, sterile 96 well microtitre plates at a density of 3×10^5 cells/well (100μl).

The SNO cells were exposed to the high and low concentration ranges of plant fractions, toxin or solvent controls for periods of 24, 48 and 72 hours and the lymphocytes for 12 hours in the culture environment. Following these incubation periods, the MTT cell viability bioassay was conducted.

Six well replicates per treatment were performed and the assay was conducted in triplicate. For both cell lines assayed, an untreated cell control was performed for all incubation periods.

4.2.5 The Methylthiazol Tetrazolium (MTT) Cell Viability Bioassay

4.2.5.1 SNO Cells

Following incubation, the supernatants from all wells were carefully aspirated with a sterile micropipette and discarded. All wells were treated with 5mg/ml MTT salt in HBSS (10µl; filter sterilised) and CCM (100µl) for four hours in the culture environment.

The salt was then removed and the cells incubated for 30 minutes with DMSO to allow for solubilisation of the formazan product. Spectrophotometric analysis was conducted using a Bio–Rad (Johannesburg, SA) multiwell plate reader at 595nm with a reference wavelength of 655nm.

4.2.5.2 Normal Human Lymphocytes

Following overnight incubation in the culture environment, the cells were pelleted via centrifugation of the microtitre plates for 30 minutes at room temperature in an Eppendorf centrifuge with a microplate rotor. The media was discarded and all wells were treated with 5mg/ml MTT in HBSS (10µl) and CCM (100µl) for four hours in the culture environment before the plates were centrifuged at room temperature for 30 minutes.

The MTT solution was discarded and all wells were treated with DMSO for 30 minutes to allow for the solubilisation of the formazan crystals. Spectrophotometric analysis was performed as for the SNO cells.

4.2.6 ANALYSIS OF DATA

4.2.6.1 Calculation of Cell Viability

The spectrophotometric analysis of microtitre plates yields readings of optical density measured as the absorbance of the light passing through the sample in the well. Despite efforts to ensure that all assays are uniform in preparation and cell numbers, there will be variations in the exact content of the sample wells between assays. This in turn will affect the absorbance readings obtained. Thus, in order to compare the results from different assays, the absorbance readings were converted to percentage cell viability (%CV) readings for each treatment using the following equation:

$$\%CV = \frac{\text{Absorbance (Test)}}{\text{Absorbance (Control)}} \times 100$$

Where: Absorbance (Test) = Absorbance reading obtained for the test well

Absorbance (Control) = Absorbance reading obtained for the appropriate control well

Cell viability for plant solvent controls and FB_1 were calculated against the untreated cell controls. The values for the plant fractions were calculated against their corresponding solvent controls. The untreated cell controls were taken to represent 100% CV.

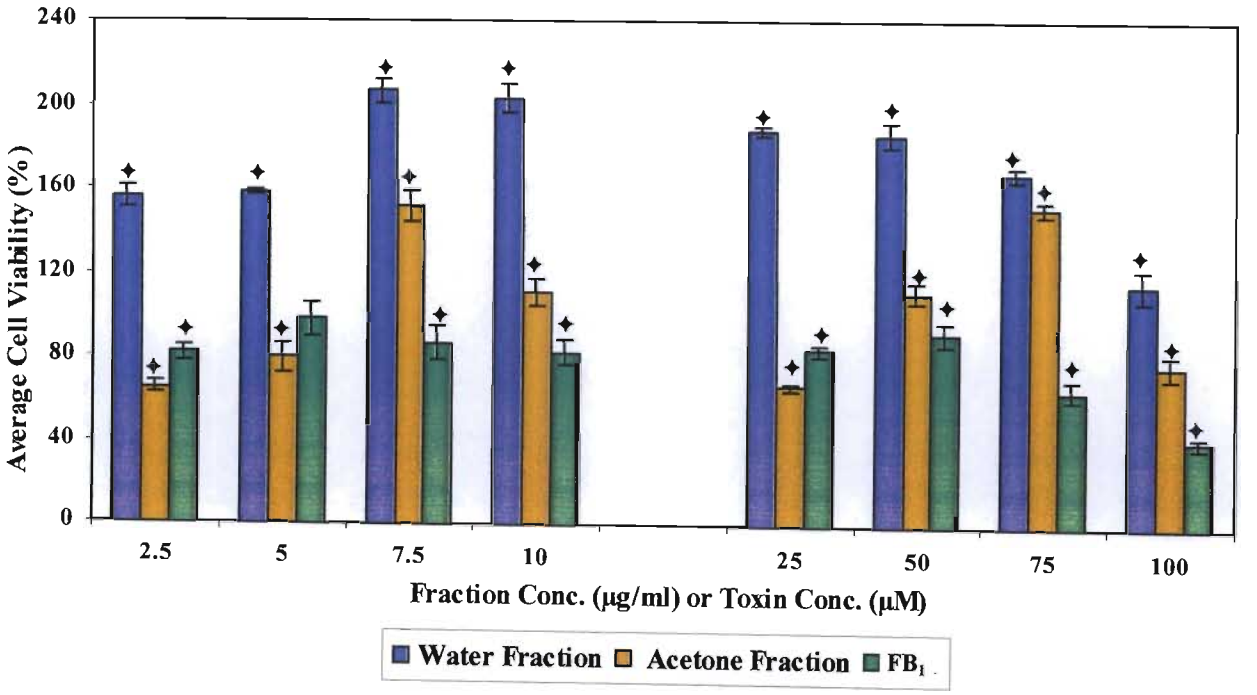
To allow for graphical representation of the results, means of the %CV values obtained were calculated for all assays. Standard deviation was also calculated for each treatment. These calculations were performed using Microsoft Excel for Windows 98. The graphs thus display the mean %CV ± the standard deviation for each treatment.

4.2.6.2 Statistical Analysis of Data

The Student's T–Test was performed on all data to determine significant differences between the fractions, toxin and their corresponding controls. The level of significance was set at $p < 0.05$ and these calculations were performed using Microsoft Excel for Windows 98. Statistical analysis for differences between treatments across the low and high concentration ranges was conducted using one–way analysis of variance (ANOVA), performed on SPSS for Windows version 11.

4.3 RESULTS

4.3.1 THE SNO CELL LINE



✦ Student's T-Test: $p < 0.05$ (Significance to control)

Figure 4.4: Cytotoxic profiles obtained for the water and acetone fractions, as well as fumonisin B_1, following 24 hours incubation with the SNO cell line.

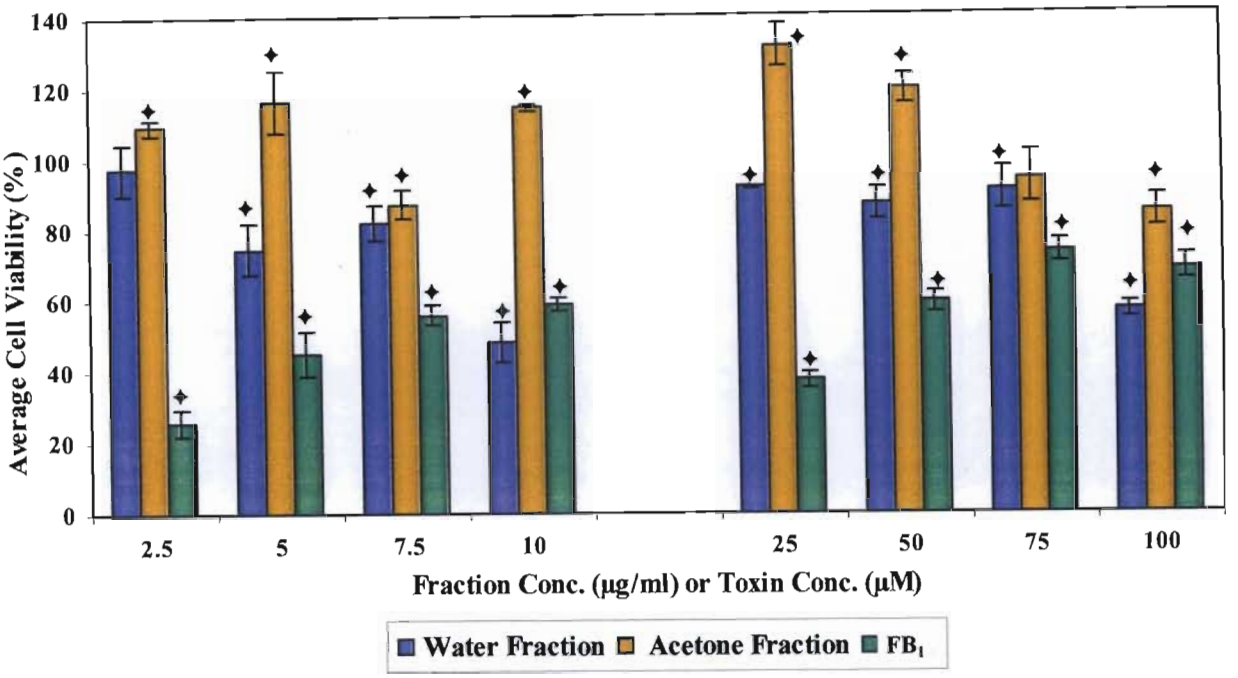
The WF stimulated proliferation of the SNO cells at low concentrations, resulting in a dose dependant increase in cell viability (Figure 4.4). The 7.5µg/ml and 10µg/ml concentrations resulted in cell viability significantly higher (206% and 203% respectively; $p < 0.001$) than that of the two lowest concentrations in this range (2.5µg/ml and 5µg/ml; 155% and 158% respectively).

The AF (low concentrations) also exhibited a dose dependant increase in cell viability. The 10µg/ml concentration did not conform to this trend, resulting in a significant decrease in cell viability compared to the 7.5µg/ml treatment ($p < 0.001$; Figure 4.4). However, the AF did not stimulate cell proliferation to the same extent as the WF and the lowest concentrations (2.5µg/ml and 5µg/ml) resulted in a significant reduction in cell viability (65% and 79% respectively; $p < 0.001$).

The high WF concentrations also exhibited proliferative activity however, a dose dependant decrease in cell viability was observed for this range (Figure 4.4). Although the highest WF concentration (100µg/ml) was seen to significantly reduce cell viability ($p < 0.001$) compared to all other concentrations in this range, the cell viability achieved was still 115%.

The dose dependant trend that was observed for the low concentrations of the AF was also evident in the high concentration range (Figure 4.4). Again, the 100µg/ml concentration was seen to deviate from this trend and result in lower cell viability (77%).

All concentrations in the low concentration range for FB_1 exhibited cell viability above 80%, with the 5µM concentration (97% CV) showing no significant difference from the untreated control (Figure 4.4). A dose dependant decrease in cell viability was observed from 25µM to 100µM FB_1, although only the two highest concentrations tested (75µM and 100µM) resulted in cell viability below 70% (65% and 41% respectively; Figure 4.4).



✦ Student's T-Test: $p < 0.05$ (Significance to control)

Figure 4.5: Cytotoxic profiles obtained for the water and acetone fractions, as well as fumonisin B_1, following 48 hours incubation with the SNO cell line.

Following 48 hours incubation with the SNO cells (Figure 4.5), the proliferative activity exerted by the WF was lost. A dose dependant decrease in cell viability was observed for the low concentration range, with cell viability ranging from 97% (2.5µg/ml) to 48% (10µg/ml). No statistical significance between the effects of the different concentrations was observed in the low concentration range for this incubation period.

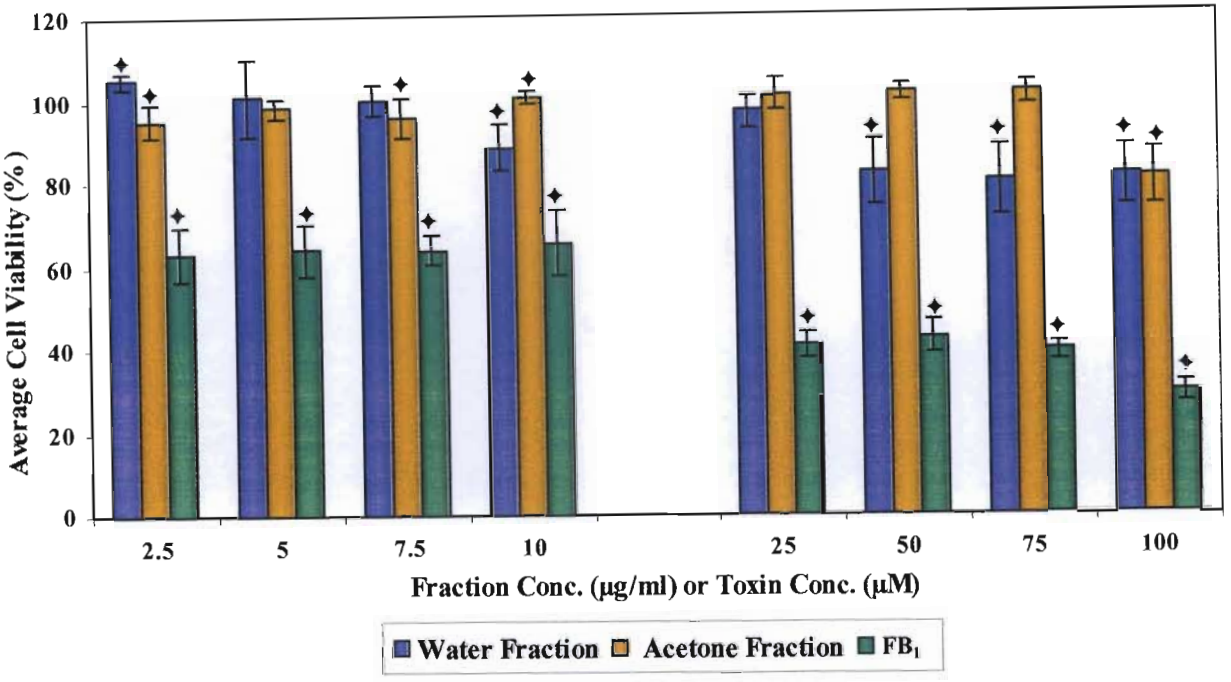
Contrary to the observations at 24 hours incubation (Figure 4.4), the AF (low concentration range) exhibited a proliferative effect on the SNO cells following 48 hours exposure. Only the 7.5µg/ml concentration exhibited cell viability significantly less than the other concentrations in this range (87%; $p < 0.001$).

The high concentration range for both fractions was seen to elicit a dose dependant decrease in cell viability (Figure 4.5). This trend was more pronounced for the AF, due to the high cell viability recorded for the lowest concentration in this range (25μg/ml; 131% CV), although the 100μg/ml concentration still produced cell viability of 84%.

The WF (100μg/ml) was observed to have the greatest effect on cell viability, resulting in 56% cell viability. As observed in the low concentration range, all cell viability for the WF was below 100% (91%–56% CV) in the high concentration range and no statistical significance was reported between the concentrations (Figure 4.5).

A dose dependant increase in cell viability was observed for the low concentration range of FB_1 at the 48 hour time interval. However, the maximum cell viability achieved was only 60% (10μM FB_1; Figure 4.5). In addition, the overall cell viability recorded was lower (25%–58% CV) than for the 24 hour treatment (81%–97% CV; Figure 4.4).

The high concentration range of FB_1 treatments also exhibited a dose dependant increase in cell viability for the 48 hour incubation period (Figure 4.5). Cell viability was again observed to be lower than at 24 hours (Figure 4.4), except for the 75μM and 100μM treatments, which demonstrated higher cell viability (24 hours: 65% and 41% respectively; 48 hours: 73% and 68% respectively).



✦ Student's T-Test: $p < 0.05$ (Significance to control)

Figure 4.6: Cytotoxic profiles obtained for the water and acetone fractions, as well as fumonisin B_1, following 72 hours incubation with the SNO cell line.

Following 72 hours treatment (Figure 4.6) with the WF at low concentrations, the dose dependant decrease in cell viability trend was maintained (105%–88% CV). However, the average cell viability observed was higher than at 48 hours incubation (97%–48% CV; Figure 4.5). Although still high (88% CV), the 10µg/ml concentration exhibited significantly lower cell viability than the other concentrations in this range ($p < 0.05$).

None of the AF concentrations in the low range inhibited cell viability (Figure 4.6). A slight dose dependent increase trend in cell viability was observed (95%–100%). Overall, the values were slightly lower than those obtained at 48 hours (87%–116% CV; Figure 4.5). No statistical significance was observed between the effects of treatments at the 72 hour interval for the low range of AF concentrations.

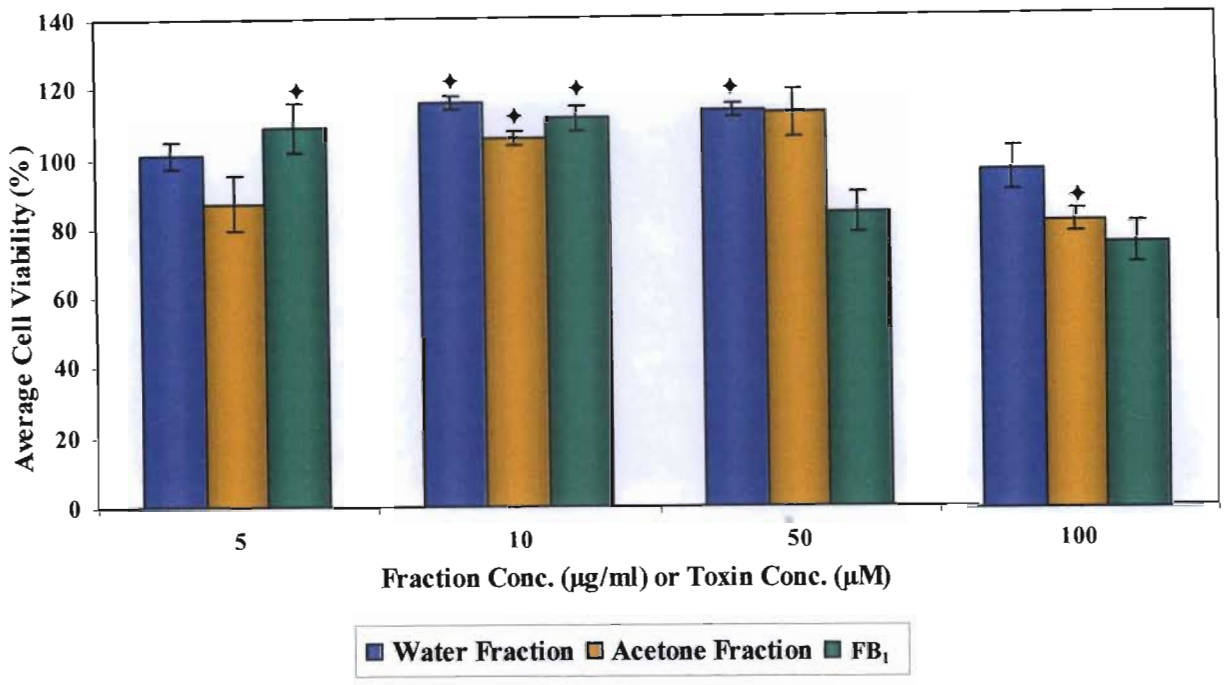
The dose dependant decrease in cell viability trend was maintained for the high concentrations of the WF at the 72 hour time interval (96%–79% CV, dose dependently; Figure 4.6). Again, these values exceeded the cell viability observed at 48 hours incubation (91%–56% CV; Figure 4.5). The cell viability recorded for the 25μg/ml treatment was significantly higher than for the other concentrations in this range ($p < 0.05$).

Treatment of the SNO cells with the high concentrations of the AF for 72 hours resulted in cell viability of 100% for all but the highest concentration (100μg/ml; Figure 4.6). This concentration resulted in a significant decrease in viability with regards to the other concentrations in this range, although the value achieved was still 80% CV ($p < 0.001$).

Treatment with the low concentrations of FB_1 exhibited a slight dose dependent increase in cell viability (62%–65%) with a concomitant lack of statistical significance between the results. The overall cell viability was increased from the 48 hour incubation period (25%–58% CV, dose dependently; Figure 4.5), thus the SNO cells appear to have recovered from the effects of these concentrations of FB_1 over this treatment period.

The stimulatory effect previously demonstrated (48 hours incubation; Figure 4.5) for the higher concentrations of FB_1 was lost, with a dose dependant decrease in cell viability being observed at the 72 hour interval (40%–29% CV; Figure 4.6). Similar cell viability readings were obtained for all but the highest concentration (100μM), which displayed a significant decrease in cell viability (29% CV; $p < 0.001$). This is a complete reversal to the dose dependent increase in cell viability trend observed for the 48 hour time interval (37%–73% CV; Figure 4.5).

4.3.2 NORMAL HUMAN LYMPHOCYTES



✦ Student's T-Test: $p < 0.05$ (Significance to control)

Figure 4.7: Cytotoxic profiles obtained for the water and acetone fractions, as well as fumonisin B_1, following overnight incubation with normal human lymphocytes.

The WF was not cytotoxic to the normal human lymphocytes (Figure 4.7) over the range of concentrations assayed. There was no excessive proliferation of these cells although the 10µg/ml and 50µg/ml concentrations caused a significant increase in cell viability with respect to the other two concentrations (115% and 113% CV respectively, $p < 0.05$).

Treatment with the AF (5µg/ml and 100µg/ml) resulted in lowered cell viability (87% and 81% respectively), although no pronounced cytotoxic or proliferative effect was noted at any concentration. As with the WF, the 10µg/ml and 50µg/ml concentrations caused a significant increase in cell viability (105% and 112% CV respectively; $p < 0.05$; Figure 4.7).

For both plant fractions, there was a dose dependant increase in cell viability from 5µg/ml–50µg/ml (Figure 4.7). A significant drop in cell viability at the 100µg/ml concentration was recorded for both fractions ($p < 0.005$) compared with the 10µg/ml and 50µg/ml concentrations.

Interestingly, the two low FB_1 treatments resulted in stimulation of lymphocyte proliferation (5µM: 108% CV; $p < 0.005$ and 10µM: 111% CV; $p < 0.003$; Figure 4.7). This represented a significant increase in cell viability compared to the two higher concentrations (50µM and 100µM), which showed decreased cell viability (84% and 75% CV respectively).

4.4 DISCUSSION

The MTT assay has been successfully applied for cytotoxicity screening of crude plant extracts. Betancur–Galvis *et al.* (1999) evaluated extracts from nine different medicinal plants traditionally used in Colombia for the treatment of a range of diseases. Using the MTT and Neutral Red colorimetric assays changes in cell viability in two different cell cultures with and without the plant extracts were determined. In addition, it was found that the MTT assay was suitable for evaluation of the effects of these extracts on the lytic activity of herpes simplex 2 virus.

4.4.1 THE WATER FRACTION

It has been postulated that cellular MTT reduction is dependent on the rate of glycolysis within the cell rather than the rate of respiration (Berridge *et al.*, 1996). If this is the case, then glucose–rich plant fractions could conceivably result in increased MTT reduction by highly metabolically active cells with increased NAD(P)H levels.

Additional NAD(P)H may also be generated in these cells via stimulation of the pentose phosphate pathway. The primary functions of this pathway are to produce NAD(P)H for reductive biosynthesis and to provide ribose–5–phosphate for nucleotide and nucleic acid biosynthesis. These functions are linked in that NAD(P)H is the ultimate electron donor in the reduction of ribonucleotides to deoxyribonucleotides for DNA synthesis. For these reasons, rapidly proliferating cells such as cancerous cells generally have a high activity of pentose phosphate pathway enzymes. High levels of glucose that cannot be sufficiently metabolised by glycolysis will be funnelled into the pentose phosphate pathway following conversion of glucose to glucose–6–phosphate (Matthews and van Holde, 1996). Thus, if the WF contains high levels of glucose, cell metabolism may be affected in such a way as to stimulate cell proliferation.

The WF could possibly contain many sugars, amino acids and water–soluble vitamins. This increased nutrient content of the media may account for the high rate of cell proliferation recorded for the WF at the 24 hour interval (150% < %CV; 2.5µg/ml–75µg/ml; Figure 4.4) for the SNO cell line. The 100µg/ml concentration may represent a point at which these constituents become too concentrated in the media and begin to cause osmotic stress to the cells.

Alternatively, as they are impermeable to the hydrophobic plasma membrane, water–soluble components require facilitated transport into the cell. Two general types of facilitated transport are known to occur, namely pore formation by transmembrane proteins and transport mediated by transmembrane carrier molecules. These systems are saturable, as any membrane contains a limited number of carrier proteins or pores and each of these can only accommodate one or a few molecules at a time (Matthews and van Holde, 1996). Higher concentrations of the WF (100µg/ml) may represent saturation of these transport mechanisms with slowing of the entry of nutrients into the cell and thus decreased metabolism of the active compounds.

The dose dependent decrease in cell viability observed for the WF following 48 hours incubation (Figure 4.5) may be attributed to degradation of the components of this fraction. Alternatively, it may also be indicative of complete utilisation of the active compounds by the cells, resulting in depletion of the nutrients and a slowing of metabolism. In addition, facilitated transport is slower than passive transport. It may therefore be postulated that due to the lower rate of entry into the cell, the cytotoxic effects of the water were delayed.

However, there was a loss of this cytotoxic action following 72 hours incubation, demonstrated in Figure 4.6, with an increase in cell viability from the 48 hour period (Figure 4.5). It is unclear why this would be the case. Possibly, progeny cells that have not been exposed to the fraction compounds for the entire 72 hour period would elicit similar effects (though not as pronounced) to the 24 hour incubation period (Figure 4.4).

4.4.2 THE ACETONE FRACTION

The AF may contain lipid–soluble vitamins and other nutritive factors, which are able to traverse the plasma membrane and gain entry into the cells via passive transport. This mechanism of transport is independent of carriers or pores and is driven by the concentration gradient of the compounds across the plasma membrane (Matthews and van Holde, 1996). This would explain the dose dependent increase in cell viability trend observed for the AF at 24 hours (Figure 4.4), due to increased nutrient concentration in the fraction dilutions.

As lipid–soluble components of the AF may not be restricted by receptor availability and may enter the cell by passive transport the effects of such components would become apparent quicker. This could account for the observation that the AF was more cytotoxic to the SNO cells at 24 hours than the WF (Figure 4.4).

In addition, the comparative loss of cytotoxic activity seen at 48 hours (Figure 4.5) for this fraction could be due to quicker utilisation and depletion of these components. Longer exposure of the SNO cells to the AF (72 hours; Figure 4.6) may have resulted in the accumulation of toxic metabolic products within the cells or surrounding medium. This would result in the decreased cell viability observed for many AF concentrations at this time interval compared to the 48 hour interval (Figure 4.5).

The loss of cytotoxic activity observed for the 48 and 72 hour incubation periods (Figures 4.5 and 4.6) also indicates that the cytotoxic activity of the AF is reversible. This is evidenced as the loss of cytotoxic action once the compounds have been utilised and removed from the medium with the cells being able to recover from the exposure and proliferate. This is an important observation to bear in mind when implementing chemopreventive strategies, as the compounds must be continuously administered in order to be effective.

4.4.3 FUMONISIN B_1

The lack of acute toxicity of FB_1 was apparent in both the 24 hour incubation for the SNO cells (Figure 4.4) and the overnight incubation with the normal human lymphocytes (Figure 4.7). As outlined in Chapter 2, the primary mechanism of toxicity of FB_1 is believed to involve the disruption of sphingolipid metabolism. The effects of this disruption may only reach toxic levels following 48 hours exposure of the SNO cells, especially to the low concentrations of FB_1 (Figure 4.5).

The *de novo* sphingolipid biosynthetic pathway begins with the condensation of serine with palmitoyl–CoA and rapidly proceeds to the biosynthesis of ceramide and more complex sphingolipids. The turnover of these more complex sphingolipids results in the production of ceramide, sphingosine and sphingosine–1–phosphate (S–1–P), which are either proven or suspected lipid–signalling molecules. Fumonisins potently inhibit the ceramide synthase enzyme responsible for the acylation of sphinganine and the reacylation of sphingosine. Ceramide synthase recognises both the amino group (sphingoid–binding domain) and the tricarballylic acid side chains (fatty acyl CoA domain) of FB_1. The binding of FB_1 to the catalytic site of ceramide synthase is the first event in the process of disruption of sphingolipid metabolism (Riley *et al*, 2001).

The consequences of this activity most likely to result in altered cell regulation include altered ceramide biosynthesis, increased intracellular concentrations of free sphingoid bases and their 1–phosphates as well as alterations in the cellular concentration of specific glycosphingolipids. The complete inhibition of ceramide synthase by FB_1 results in a rapid increase in the intracellular concentration of sphinganine (Wang *et al.*, 1991). However, the capacity of sphingosine kinase to degrade free sphinganine must be exceeded before this can occur. Partial inhibition of ceramide synthase may increase the rate of sphingoid base 1–phosphate biosynthesis in the absence of an apparent increase in the free sphinganine concentration. Free sphingosine concentration may also increase through fumonisin of the reacylation of sphingosine derived from sphingolipid turnover or dietary sources/growth medium (Riley *et al*, 2001).

Fumonisin exposure also results in imbalances in phosphoglycerolipid and fatty acid metabolism *in vitro*. The ability of cells to rapidly metabolise bioactive sphingoid bases into less bioactive products or into products such as S–1–P may protect the cell from toxicity associated with elevated free sphingoid bases and ceramide (Wang *et al.*, 1991).

The balance between endogenous concentrations of ceramide and S–1–P determines cell death or cell survival respectively and is maintained by the relative activities of various important enzymes in the *de novo* and sphingolipid turnover pathways (Riley *et al.*, 2001).

Numerous studies also propose that fumonisin–induced changes in essential enzymes involved in cell cycle regulation, differentiation and/or apoptosis are either secondary or primary sites of FB_1 action. Among these are alterations in the expression of cyclins, cyclin–dependant kinases and dephosphorylation of the retinoblastoma (Rb) protein (Riley *et al*, 2001). The Rb protein is known to be a negative regulator of cell growth as loss of Rb function results in uncontrolled cell growth and malignancies (Cooper and Shayman, 2001). Inactivation or deletion of Rb has been reported in numerous carcinomas including those of the breast, bladder and prostate, as well as in leukaemia (Wiman, 1993). Activation of the Rb protein by dephosphorylation leads to the sequestration of transcription factors believed to be essential for continued passage through the cell cycle. Thus, it is the dephosphorylated or hypophosphorylated form of Rb which prevents passage of the cell through the cell cycle (Cooper and Shayman, 2001).

If FB_1 does indeed dephosphorylate the Rb protein (either directly or indirectly), one would expect decreased cell viability readings compared to the untreated control cells, which would undergo unperturbed cell cycle progression and proliferation. This may account for the results obtained following 24 and 48 hours incubation with the SNO cell line shown in Figures 4.4 and 4.5 respectively.

Studies in our laboratories using the SNO cell line have indicated that FB_1 may be more cytotoxic at lower concentrations (Myburg *et al.*, 2002). However, the cell viability recorded here after 48 hours treatment of the SNO cell line (Figure 4.5) is much lower than in the report by Myburg and co–workers (2002). They reported cell viability over 80% for a concentration range of 2.16μM–34.6μM (doubling concentrations), which is more in line with the 24 hour results (Figure 4.4).

The high rate of cell death observed for the low concentrations of FB_1–treated SNO cells at 48 hours incubation (Figure 4.5) might have resulted in compensatory cell proliferation following 72 hours treatment with this range (Howard *et al.*, 1998; Figure 4.6). Conversely, the higher range of concentrations was seen to decrease cell viability from the 48 hour interval (Figure 4.6). It may be that the lower concentrations of FB_1 lost toxicity due to enzymatic modification of the entire dose of toxin, whereas this did not occur with the high concentration range. This would result in continued disruption of sphingolipid metabolism and further accumulation of toxic amounts of sphinganine.

Lymphocytes are the smallest white blood cells and are characterised by round, densely stained nuclei and a small amount of pale basophilic, non-granular cytoplasm. The amount of cytoplasm varies according to the state of activity of the lymphocyte (Wheater *et al.*, 1987). The relatively small proportion of the cell taken up by cytoplasm compared with the SNO cells may partially account for the similarity in cell viability between these two cell lines (Figures 4.7 and 4.4 respectively). The lymphocytes were expected to have decreased cell viability, as they represent a normal, untransformed cell line and should be more sensitive to the effects of toxic substances. However, as mentioned earlier in the discussion, the toxic effects exerted by FB_1 are dependent on balances between many enzyme systems (Riley *et al.*, 2001) and the effects appear to be chronic in nature. As these cells were only incubated overnight in combination with the toxin, it is not surprising that similar cell viability's were obtained.

4.5 CONCLUSION

High cell viability readings were obtained for the 24 hour incubation period for the SNO cell line (Figure 4.4), particularly for the WF. In addition, loss of FB_1 activity was observed for this cell line at the 72 hour incubation period (Figure 4.6). Thus, the 48 hour incubation period was selected for the time–dose response assays using the SNO cell line.

It was difficult to discern trends with regards to the optimal concentrations of plant fractions to use for further experiments. The concentrations selected could not be overtly cytotoxic, as this may result in complete cell death in further experiments, which would be undesirable.

As the 48 hour incubation period was selected, the 5µg/ml and 50µg/ml concentrations of plant fractions were chosen. These concentrations of the WF demonstrated cell viability representative of the average fraction effects for this incubation period (74% and 86% CV respectively). With regards to FB_1, the 5µM and 50µM also exhibited acceptable cell viability readings at 48 hours incubation period (45% and 73% CV respectively).

It is encouraging that the plant fractions were not cytotoxic to the normal human lymphocytes (Figure 4.7), as this may have precluded their potential use as protective agents.

CHAPTER FIVE

A TIME–DOSE ASSESSMENT OF THE CHEMOPREVENTIVE ABILITY OF THE PLANT FRACTIONS: ANTI–MYCOTOXIGENIC EFFECTS

5.1 INTRODUCTION

5.1.1 CHEMOPREVENTION AND OESOPHAGEAL CANCER

Oesophageal cancer (OC) occurs worldwide with a variable geographic distribution. It ranks eighth in order of cancer occurrence, combining both sexes (Stoner and Gupta, 2001) and is the fifth most common cause of cancer death (Kresty *et al.*, 2001). It is characterised by an advanced pathological stage at presentation, which is associated with poor prognosis. The overall 5 year survival rate of oesophageal cancer patients is still under 10% (Xu *et al.*, 1999).

This malignancy exists in two main forms, namely squamous cell carcinoma (SCC) and adenocarcinoma, each possessing distinct aetiological and pathological characteristics. More than 90% of OC's worldwide are squamous cell carcinomas. Squamous cell carcinoma of the human oesophagus has a multifactorial aetiology involving several environmental and/or genetic factors (Stoner and Gupta, 2001).

Epidemiological data supports the theory that consumption of fruits and vegetables, at levels that are behaviourally possible, decreases cancer risk at numerous sites, including the oesophagus. However, the cancer–inhibitory effects of food derived chemopreventive agents have traditionally been assessed individually, or as a few active constituents and at pharmacological doses (Kresty *et al.*, 2001). A "food–based" approach is emerging as an alternative to the use of single compounds in cancer chemoprevention.

In 2001, Stoner and Gupta outlined the results of a study using lyophilised strawberry pulp as an anti–initiation agent in *N*–nitrosomethylbenzylamine (NMBA)–induced rat oesophageal tumourigenesis. This product was chosen due to high ellagic acid content. The authors found that dietary administration of the berries reduced tumour multiplicity in the rat oesophagus, with a concomitant reduction in the formation of DNA adducts.

In the same year, Kresty *et al.* evaluated the inhibitory potential of lyophilised black raspberries in the F344 rat model during the initiation and post–initiation phases of oesophageal carcinogenesis. They concluded that dietary administration of lyophilised black raspberries inhibited events associated with both the initiation and promotion/progression phases of carcinogenesis. This was evidenced by decreased tumour incidence and multiplicity, adduct inhibition and inhibition of pre–neoplastic lesion formation.

5.1.2 CHEMOPREVENTION AND LUNG CANCER

Lung cancer is the most lethal cancer in the world, with the number of deaths from lung cancer exceeding the total combined number of deaths from breast, colon, prostate and cervical cancers (Mulshine and Smith, 2002). The mortality rate is high as no effective screening procedures exist and there is a high predilection for early spread leading to metastatic disease, which systemic therapies do not cure (Soriano *et al.*, 1999).

In addition to being the leading cause of cancer death, lung cancer is one of the few cancers to have a well–defined aetiology, namely the inhalation of tobacco smoke. However, not all cigarette smokers develop lung cancer and other factors must be considered. One area of investigation is into the enzymes responsible for carcinogen activation, degradation and DNA repair. These enzymes can exhibit gene deletion and polymorphisms, which can affect enzyme activity (Goodman, 2002).

It has been hypothesised that an individual's enzyme profile is associated with lung cancer risk. Genetic profiling could be used to counsel those at risk for lung cancer and select high–risk individuals for specific chemoprevention agents. The enzymes themselves and the pathways they regulate may also become potential targets for preventive agents and strategies (Goodman, 2002).

Epidemiological studies have indicated that lung cancer risk is inversely associated with the intake of carotenoid–containing fruits and vegetables. The mechanisms of carotenoid chemoprevention in animal models have not been elucidated, but may involve antioxidant activity, induction of detoxification enzymes and inhibition of cell proliferation (Holick *et al.*, 2002).

Holick *et al.* (2002) observed significant inverse associations between the dietary carotenoids lycopene, lutein, β–crytoxanthin and total carotenoids and lung cancer risk in a prospective study of male smokers. They also found that intakes of β–carotene, α–carotene and retinol were not associated with risk, as previously thought. Their results were consistent with the hypothesis that carotenoids other than β–carotene, or a combination of carotenoids, may have chemopreventive potential for lung cancer.

A vitamin A derivative that has been demonstrated to be an effective chemopreventive agent is 13–*cis*–retinoic acid. Soriano *et al.* (1999) found that this chemopreventive agent had synergistic growth–inhibitory effects with other agents with various inhibitory mechanisms of action.

5.1.3 CELL LINES AND TOXINS

The multistep nature of carcinogenesis provides numerous opportunities for interventions with chemopreventive agents targeted at specific mechanisms involved in the initiation, promotion and progression of cancers. Determination of the efficacy of such agents in the post–initiation stages, when the pre–malignant lesions are known to have developed, is vital with regards to the eventual clinical use of these agents in the secondary prevention of cancer (Rao *et al.*, 2001).

Two cancerous cell lines were used in this study. The SNO cell line was again utilised in conjunction with FB_1. In addition, the A549 (non small cell lung cancer) lung adenocarcinoma cell line was used to assess the effects of the plant fractions on various cell types and ascertain whether any effects noted were cell line specific. These cell lines are both transformed and could thus be utilised to assess any post–initiation effects exerted by the fractions. Normal human lymphocytes were again employed as a normal cell population to assess potential effects on the initiation steps of carcinogenesis by the fractions.

The A549 tumour cell line was initiated from the neoplastically transformed alveolar type II epithelial (AII) cell of a human pulmonary adenocarcinoma. These cells retained both the morphologic and biochemical characteristics of normal AII cells, including the ability to metabolise certain procarcinogens via oxidative pathways and form covalent carcinogen–DNA adducts in culture (Lieber *et al.*, 1976).

Palanee *et al.* (2000) demonstrated that AFB_1 was cytotoxic to the A549 lung adenocarcinoma cell line. The cytotoxic responses obtained for AFB_1 showed a similar trend to those of AFBO, possibly indicating the biotransformation of AFB_1 to AFBO by this cell line. Thus, AFB_1 was used to assess the effects of the plant fractions on the A549 cell line.

5.1.4 OBJECTIVES

This investigation determined possible inhibitory effects of the plant fractions the cytotoxicity of the mycotoxins under investigation. A secondary aim was to ascertain any relationship between the time of administration of these fractions and their potential effects.

5.2 MATERIALS AND METHODS

5.2.1 MATERIALS

The A549 lung adenocarcinoma cell line was purchased from Highveld Biologicals, Johannesburg, SA and the AFB_1 was purchased from Sigma Chemical Company, SA. All other reagents, chemicals and equipment were obtained from suppliers as specified in Chapter 4.

5.2.2 METHODS

5.2.2.1 Maintenance of Cell Lines

The A549 cell line was cultured and maintained as for the SNO cell line outlined in Chapter 4. Normal human lymphocytes were isolated as previously described and used immediately.

5.2.3 PREPARATION OF DILUTIONS

5.2.3.1 Plant Fractions and Solvent Controls

The working stock solutions (1mg/ml) of the WF and AF and their equivalent respective solvent controls prepared as described in Chapter 4 were used to prepare further dilutions.

Two concentrations of each fraction were selected for use on the SNO and A549 cell lines in these experiments, namely 5µg/ml and 50µg/ml. Only the 100µg/ml concentration of the AF and ethanol control was assessed on the normal human lymphocytes.

In addition to using the two fractions individually on the SNO and A549 cell lines, a combination fraction (CF) was prepared. This was done to elucidate any interactive effects between components in the two initial crude fractions. Equal volumes of the WF and AF were combined, such that the final concentration of each component was 5µg/ml or 50µg/ml. An equivalent solvent control was similarly prepared.

As in Chapter 4, the concentrations stated are the final concentrations of the plant fractions or solvent controls (individual or combination) in each well during the assay. Each sample well consisted of 100µl cell suspension, 50µl plant fraction and 50µl toxin, giving a final volume of 200µl in each well. The control wells were similarly prepared. Thus, the initial concentrations of the dilutions prepared were 20µg/ml (5µg/ml final concentration) and 200µg/ml (50µg/ml final concentration) for the individual fractions. The CF was prepared using equal volumes of 40µg/ml and 400µg/ml stocks of the WF and AF to prepare the 20µg/ml and 200µg/ml dilutions respectively. All solvent controls were similarly prepared.

5.2.3.2 Mycotoxins and Solvent Controls

As with the plant fractions, the stock solution of FB_1 prepared as described in Chapter 4 was used in the preparation of further dilutions.

For AFB_1, a 100μg/ml (320.2μM) stock solution was prepared. As this toxin is not water soluble, it required solubilisation prior to the addition of CCM. This was achieved using 140μl DMSO and 60μl ethanol per 10ml CCM required. The solvent control was similarly prepared. The AFB_1 toxin and solvent control stock solutions were sterilised using 0.45μm millipore filters prior to use in the preparation of further dilutions.

Again, two concentrations of toxin were chosen for use in these remaining experiments, namely 5μM and 50μM. The same concentrations were used for both toxins on all three cell lines under investigation. As with the plant fractions, these were the final concentrations of the toxins in the sample wells and 20μM and 200μM dilutions were prepared to yield the final concentrations desired.

5.2.4 TREATMENT PROTOCOLS

5.2.4.1 Anti–Mycotoxigenic Effects of the Plant Fractions

To assess the possible anti–mycotoxigenic effects of the individual WF and AF as well as the CF, the cells (100μl; 3 x 10^5 cells/well) were treated with the fractions (50μl) together with the toxin (50μl; appropriate for the cell line). Each fraction and solvent control concentration was tested against each toxin concentration and in the case of AFB_1, the toxin solvent control also.

Six well replicates were performed for each test combination and the assay was conducted in triplicate. Cells were incubated for 48 hours (SNO and A549 cells) or overnight (lymphocytes) in the culture environment, following which the MTT cell viability bioassay was performed as described in Chapter 4.

5.2.4.2 Controls

Due to the numerous variables in the tests, many controls were required to allow for interpretation of the results.

The test itself consisted of cells treated with fraction and toxin together. As the fractions all had solvent vehicles, one control consisted of plant solvent and toxin together. This was performed to assess any effects exerted by the plant solvent vehicle on toxin interaction with the cells.

In the case of AFB_1, the toxin also had a solvent vehicle. Two additional controls were required for this treatment, namely plant fraction with toxin solvent vehicle as well as plant solvent vehicle with toxin solvent vehicle. The latter served as the true control for the plant fraction with AFB_1 test and served to assess any combined effects of the two solvent vehicles.

In addition, standard controls were performed where cells were treated with each test component individually. In this case, the well volume of 200µl was maintained by the addition of 50µl CCM in place of the second component. Untreated cell controls were also performed for all experiments.

5.2.4.3 Treatment Schemes

Different treatment schemes were utilised to assess whether the order of exposure had an effect on the anti–mycotoxigenic capacity of the plant fractions. Three treatment schemes were employed for the two cancerous cell lines. The cells were either treated with the plant and toxin at the same time (Together) or the plant fractions were added 24 hours before the toxin (Plant First) or the plant fractions were added 24 hours after the toxin (Toxin First). The total incubation period for all schemes was 48 hours.

All of the aforementioned controls were performed for each scheme and treated accordingly. The normal human lymphocytes were assessed using the Together interval only with overnight incubation.

5.2.5 ANALYSIS OF DATA

5.2.5.1 Calculation of Cell Viability

Cell viability for all solvent only controls (plant and toxin) were calculated against the untreated cell control, as was the FB_1 toxin only control. All plant fraction only controls and the AFB_1 toxin only controls were calculated against their corresponding solvent only controls. The untreated cell controls were taken to represent 100% CV.

Treatments with plant fraction and FB_1 were calculated against the plant solvent only controls, due to the solubility of FB_1. For AFB_1 treatments, the plant fraction and AFB_1 test was calculated against the plant solvent and toxin solvent control. These calculations were performed to assess any combined solvent effects on the tests.

For the calculation of the effect of the plant fractions in the test containing plant fraction and toxin, the absorbance readings for this test were compared to those of the plant solvent and toxin control. Similarly, to assess toxin effects within this test, the absorbance readings were compared to those of the plant fraction only control (FB_1 assays) or to the plant fraction and toxin solvent control (AFB_1 assays).

To allow for graphical representation of the results, means of the %CV values obtained were calculated for all assays. Standard deviation was also calculated for each treatment. All calculations were performed using Microsoft Excel for Windows 98.

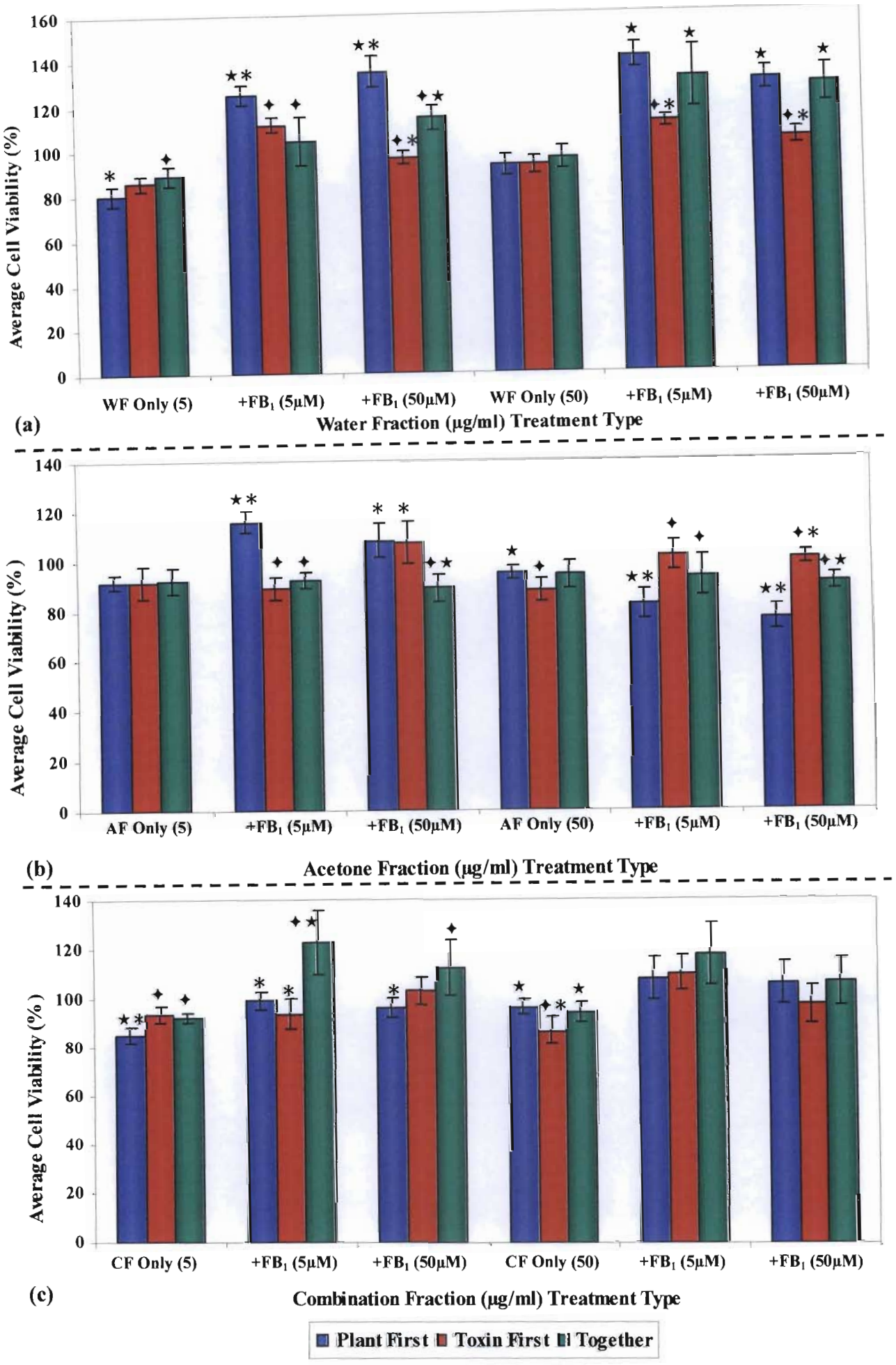
5.2.5.2 Statistical Analysis of Data

The Student's T–Test was performed on data where only two sets of results were to be compared. The level of significance was set at $p < 0.05$ and these calculations were performed using Microsoft Excel for Windows 98. In addition to ascertaining the level of significance against the corresponding controls, it was important to assess significance across the three treatment schemes. This was in order to determine the most effective treatment scheme. One–way ANOVA ($p < 0.05$), the test of homogeneity of variance and Bonferroni analysis were performed using SPSS for Windows version 11.

5.3 RESULTS

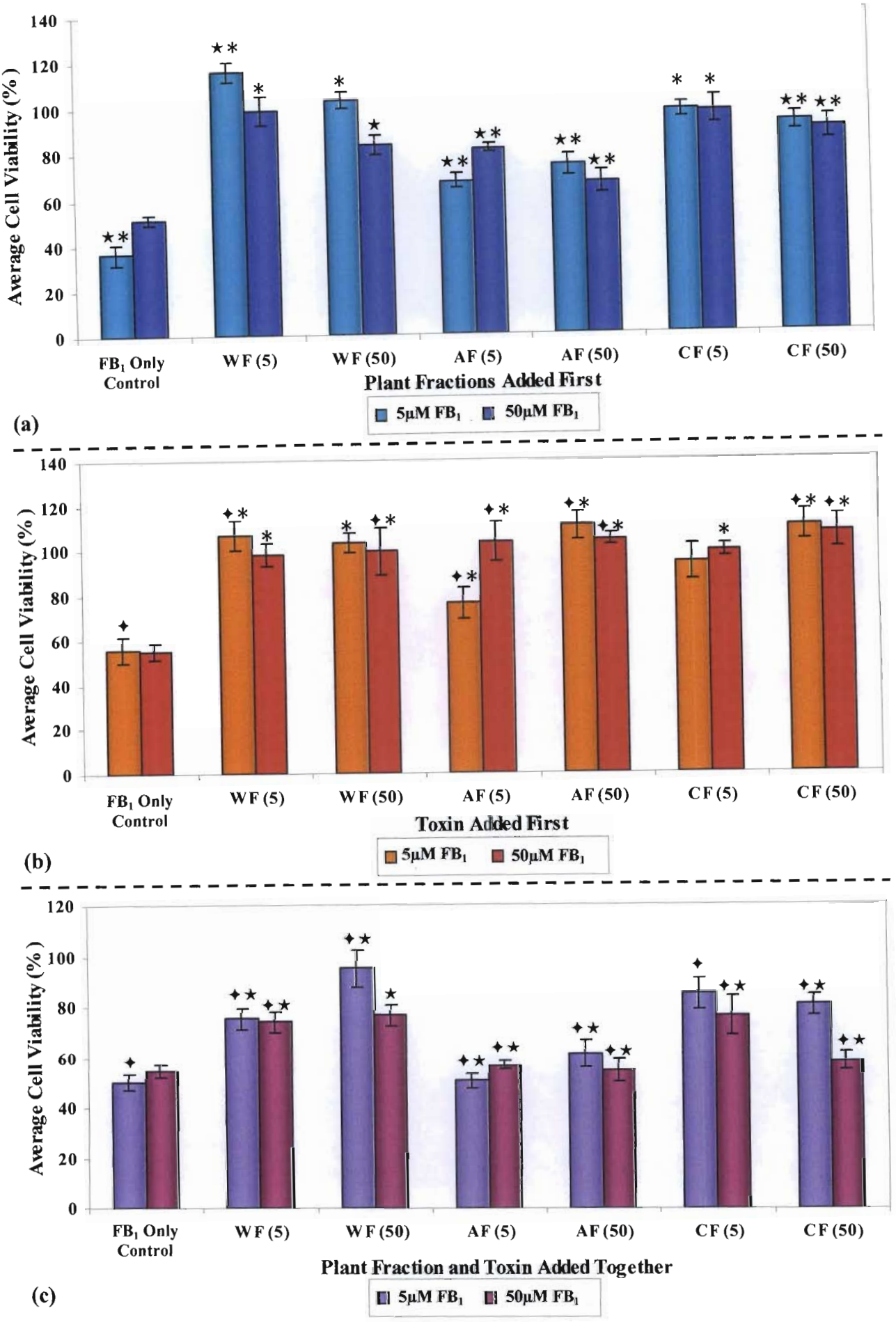
5.3.1 THE SNO CELL LINE

The test consisted of cells treated with both plant fraction and FB_1 according to the three treatment schemes. Within this test, the effects of the fraction in the presence of the toxin, as well as the effects of the toxin in the presence of the fractions were of interest. Therefore, two sets of cell viability were calculated, one to represent fraction effects and the other toxin effects.



One Way ANOVA: $p < 0.05$ (Significance to: ✦ Plant First; ★ Toxin First; ✻ Together Interval)

Figure 5.1: Cytotoxic evaluation of the fraction effects on SNO cells treated with a) water fraction, b) acetone fraction and c) combination fraction as well as fumonisin B_1, over the three treatment schemes.



One Way ANOVA: $p < 0.05$ (Significance to: ✦ Plant First; ★ Toxin First; ✻ Together Interval)

Figure 5.2: **Cytotoxic evaluation of the toxin effects on SNO cells treated with plant fractions and fumonisin B_1, according to the three treatment schemes: a) Plant First, b) Toxin First and c) Together.**

The fraction effects calculated for all three fractions demonstrated a trend of increased cell viability compared to the fraction only controls (Figure 5.1a–c) for both concentrations of FB_1. All of the WF and CF treatments exhibited higher cell viability than their respective fraction only controls. Only four of the AF treatments exhibited cell viability less than their fraction only controls (Figure 5.1b).

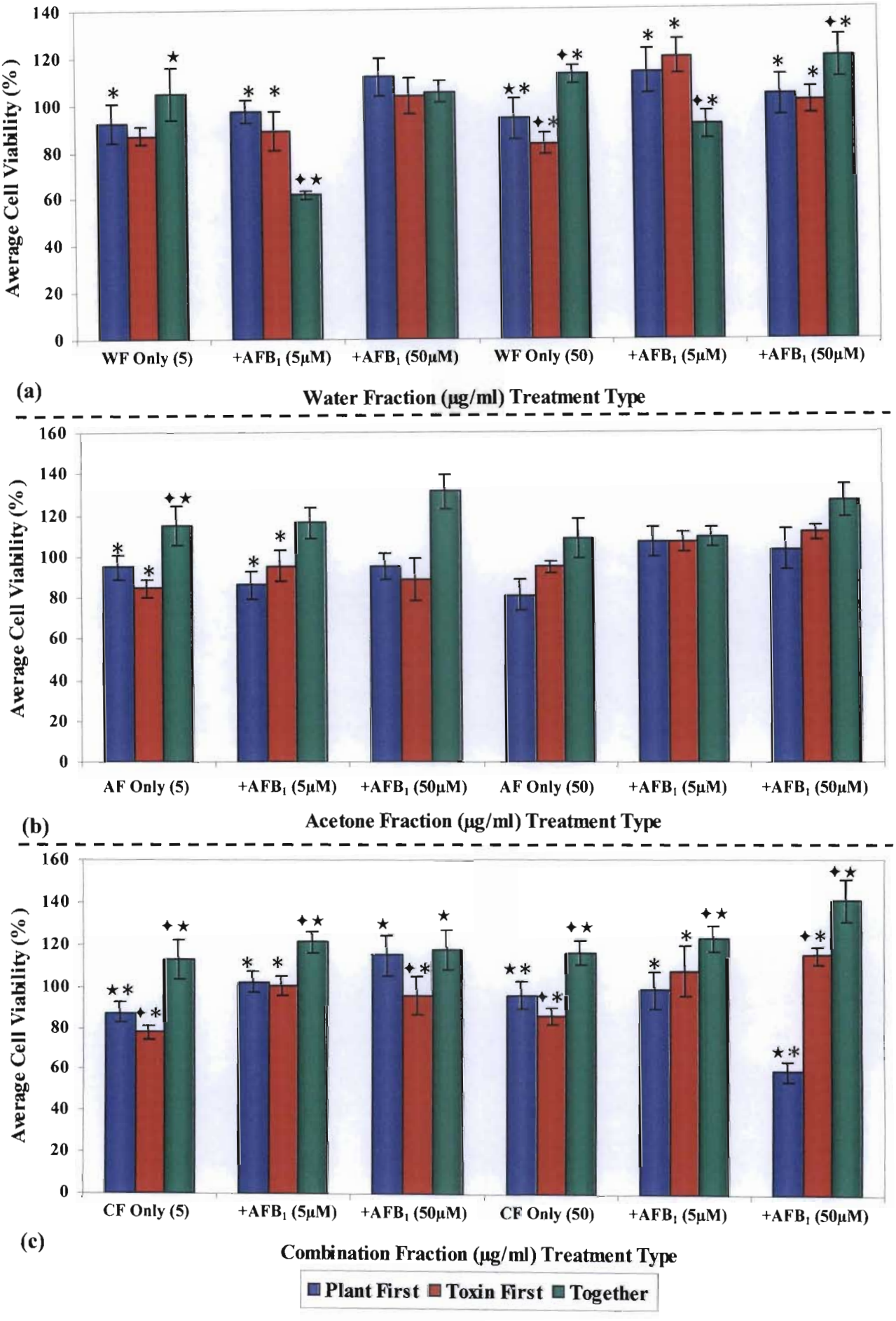
With regards to the toxin effects, all treatments with the WF resulted in decreased toxin effects, evidenced by an increase in cell viability from the toxin only controls. This trend was most pronounced in the FB_1 (5μM) treatments for both concentrations of the WF (Figure 5.2a–c).

Treatment with the AF also resulted in higher cell viability for the toxin effects compared to the toxin only controls. This effect was not as pronounced as for the WF and the Together treatment interval for both AF concentrations resulted in very small increases in cell viability from the controls for both FB_1 concentrations (Figure 5.2c).

The CF resulted in intermediate cell viability compared to the WF and AF. There was still an increase with respect to the toxin only controls and only one treatment for the Together interval showed cell viability close to the toxin only control value (Figure 5.2c).

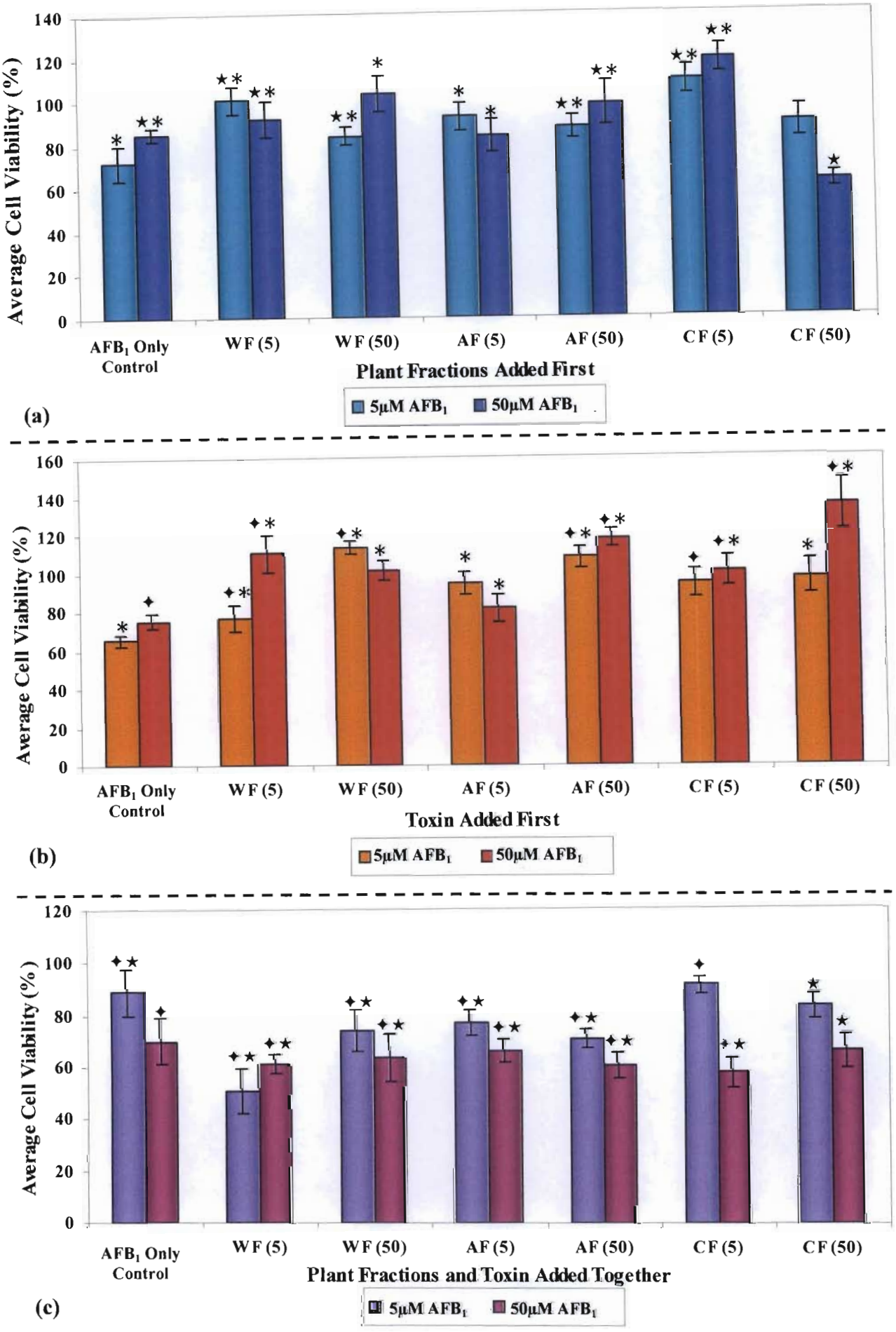
5.3.2 THE A549 CELL LINE

As with the SNO cell line, the test consisted of cells treated with both plant fraction and toxin (AFB_1) according to the three treatment schemes. Again, two sets of cell viability were calculated, one to represent fraction effects and the other toxin effects.



One Way ANOVA: $p < 0.05$ (Significance to: ✦ Plant First; ★ Toxin First; ✱ Together Interval)

Figure 5.3: **Cytotoxic evaluation of the fraction effects on A549 cells treated with a) water fraction, b) acetone fraction and c) combination fraction as well as aflatoxin B_1, over the three treatment schemes.**



One Way ANOVA: $p < 0.05$ (Significance to: ✦ Plant First; ★ Toxin First; ✻ Together Interval)

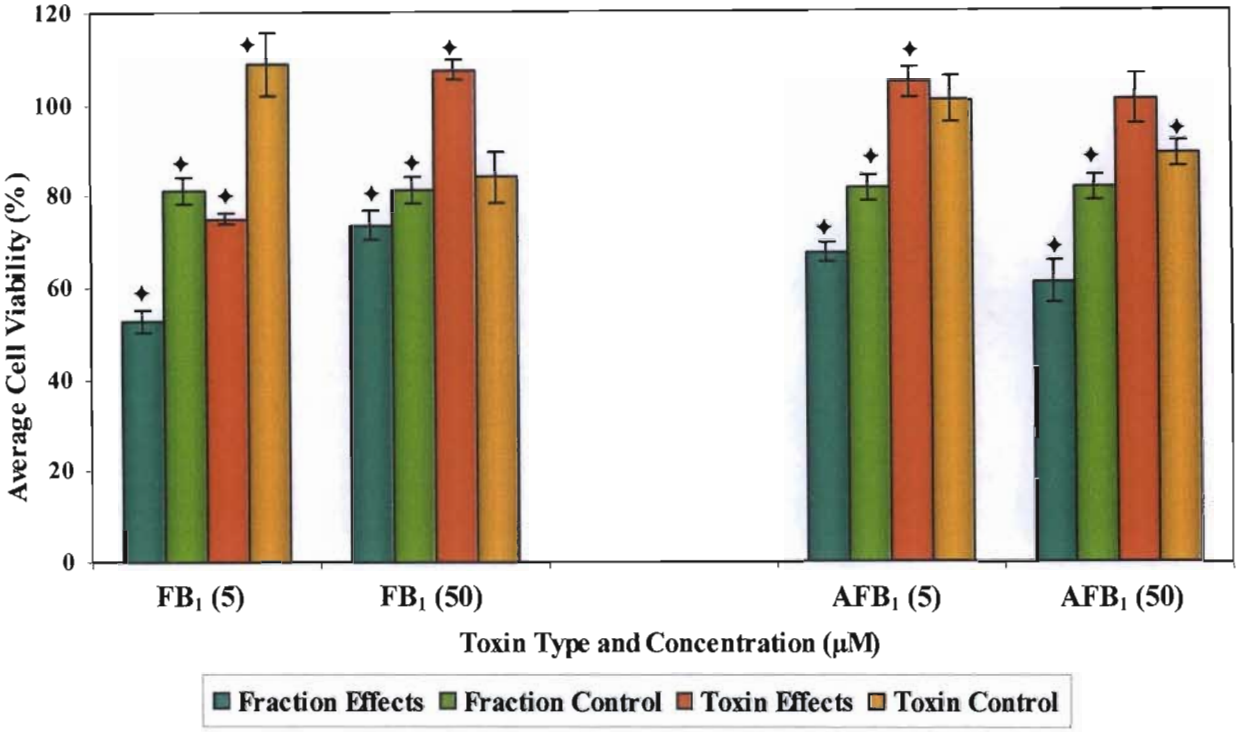
Figure 5.4: **Cytotoxic evaluation of the toxin effects on A549 cells treated with plant fractions and aflatoxin B_1, according to the three treatment schemes: a) Plant First, b) Toxin First and c) Together.**

The fraction effects (Figure 5.3a–c) calculated for the A549 cell line were again seen to display a trend of increased cell viability compared with the fraction only controls. For the WF, only the Together treatments with AFB_1 5μM for both concentrations of the fraction resulted in decreased cell viability for the fraction effects (Figure 5.3a). The Plant First interval resulted in decreased fraction effects for the AF (5μg/ml) for both concentrations of AFB_1 (Figure 5.3b). Only the CF (50μg/ml) with AFB_1 (50μM) for the Plant First interval resulted in decreased fraction effects compared with the fraction control (Figure 5.3c).

With the exception of CF (5μg/ml) with AFB_1 (5μM), all treatments at the Together interval resulted in decreased cell viability for the toxin effects at both concentrations of fractions and AFB_1 compared to the toxin only controls (Figure 5.4c). The only other treatment to have this effect was CF (50μg/ml) with AFB_1 (50μM) for the Plant First interval (Figure 5.4a). All other treatments resulted in cell viability above that of the toxin only controls, indicating decreased toxin effect.

5.3.3 NORMAL HUMAN LYMPHOCYTES

The normal human lymphocytes were only assessed using the Together treatment scheme with overnight incubation of cells with the AF (100μg/ml) and selected FB_1 and AFB_1 concentrations (5μM and 50μM). As for the SNO and A549 cell lines, toxin effects and fraction effects within this treatment scheme were calculated. These results, as well as the respective AF only (100μg/ml) and toxin only controls are represented in Figure 5.5.



✦ Student's T-Test: $p < 0.05$ (Significance to control)

Figure 5.5: Cytotoxic evaluation of the fraction effects and the toxin effects on normal human lymphocytes treated with acetone fraction, fumonisin B_1 and aflatoxin B_1, following overnight incubation.

This cell line was only assessed using the Together interval and one concentration of the AF (100µg/ml). The fraction effects for the AF were decreased for all treatments when compared with the fraction only control (Figure 5.5).

The FB_1 treatments demonstrated a dose dependant increase in cell viability with regards to fraction effects, with cell viability for the 5µM treatment significantly ($p < 0.05$) below that of the fraction only control. The FB_1 (5µM) treatment also resulted in significantly lower cell viability (52.6%) than the 50µM treatment (73.5%; $p < 0.001$). However, the cell viability for the 50µM treatment was not significantly different from the AF only control ($p = 0.23$).

The AFB_1 treatments demonstrated a different profile however, with a dose dependent decrease in cell viability being observed for the fraction effects as compared with the AF only control (Figure 5.5). The AFB_1 5µM treatment demonstrated significantly higher cell viability than the 50µM treatment ($p < 0.05$), although this value was still significantly below that of the AF control ($p < 0.005$).

The FB_1 5μM toxin effect was enhanced by the presence of the AF, as evidenced by a significant decrease in cell viability ($p < 0.02$) with respect to the FB_1 5μM only control (Figure 5.5). The FB_1 50μM treatment however, displayed decreased toxin effects, with a significant increase in cell viability observed ($p < 0.04$) compared to the toxin only control (Figure 5.5).

The toxin effects were decreased for both concentrations of AFB_1 tested (Figure 5.5). This effect was more pronounced in the 50μM AFB_1 treatment, resulting in significantly increased cell viability ($p < 0.02$) in comparison to the toxin only control.

5.4 DISCUSSION

Maize has frequently been found to be contaminated with FB_1 and AFB_1 (Palanee *et al.*, 2000; Ahsan *et al.*, 2001; Chelule, 2001; Turner, 2003) and comprises a significant portion of the staple diet of rural South African populations. In addition, co–contamination of cereal and grain based foods by toxigenic species of *Fusarium* and *Aspergillus* has been reported in several countries in recent years (Theumer *et al.*, 2003). It is thus likely that the rural community in South Africa is at risk of exposure to both of these toxins in their daily diets.

In a study conducted in our department, Chelule and colleagues (2001) examined the levels FB_1 in raw, stored maize as well as cooked maize–based foods in both rural and urban areas of KwaZulu Natal. This study found that the level of contamination of these foods was appreciably higher in the rural areas, with 32% of the maize samples containing FB_1 compared with only 6% in the urban areas. The average level of FB_1 was also significantly higher in the rural areas (2.2mg/kg) than in the urban areas (0.3mg/kg). Also of importance in this study was the finding that the cooking process did not appreciably degrade the FB_1 present in the raw maize, with 29% of the rural cooked maize samples containing detectable levels of FB_1.

Imifino is also an integral part of the diet in rural communities, where it is readily available for human consumption (Gqaleni, pers comm). In this study, the effects of two crude fractions obtained from the *Amaranthus lividus* imifino plant on the toxicity of the two important dietary mycotoxins, FB_1 and AFB_1, was assessed. A combination of the two plant fractions was also assayed for anti–mycotoxigenic activity. This was conducted on two cancerous cell lines of different tissue origin as well as a normal cell population in the form of human lymphocytes.

The results obtained indicate that these fractions possess anti–mycotoxigenic potential against both FB_1 and AFB_1. This was evidenced by the increase in the cell viability of cells treated with the plant fractions in conjunction with the mycotoxins compared to that of cells treated with the toxins only. This effect was observed in both of the carcinoma cell lines (Figures 5.2 and 5.4) as well as the normal human lymphocytes (Figure 5.5). The fractions could be exerting these inhibitory effects by various mechanisms.

As detailed in Chapters 2 and 4, it is widely accepted that FB_1 exerts its toxic action through interference with sphingolipid metabolism. This leads to accumulation of free sphingoid bases and their 1–phosphates, alterations in complex sphingolipids and decreased ceramide biosynthesis. Free sphingoid bases and ceramide can both induce cell death, therefore FB_1 inhibition of ceramide synthase can inhibit cell death induced by ceramide accumulation, but promotes free sphingoid base–induced cell death (Riley *et al.*, 2001).

Unexpectedly, the WF followed by the CF exhibited the greatest protective effect against FB_1 toxicity in the SNO cell line (Figure 5.2), with the AF showing the lowest activity. The AF was expected to have the highest activity in this model, as it contains the lipid–soluble components, which would be expected to affect lipid related mechanisms.

However, the presence of an amino group in the FB_1 molecule (Figure 2.5) facilitates its conjugation to protein molecules (Azcona–Olivera *et al.*, 1992). It is therefore possible that proteins present in the WF and CF interacted with the water soluble FB_1, effectively sequestering it from the ceramide synthase and providing protection to the cells.

In addition, FB_1 has been demonstrated to undergo modification in the presence of reducing sugars to form *N*–(carboxymethyl)fumonisin B_1. The reactions were temperature–dependant and occurred at physiologic pH (pH 7.5) in the presence of phosphate buffers. The optimum temperature for the reaction of FB_1 with glucose (20mM) was determined to be 78°C. At this temperature loss of FB_1 from the reaction mixture occurred at pH 7, with no significant increase in loss reported at pH 8.9. This loss in FB_1 was accompanied by an increase in the reaction product, *N*–(carboxymethyl)fumonisin B_1. Although the experiments regarding these reactions were conducted using pure solutions of reducing sugars and FB_1, the same derivative was isolated from raw corn samples. This indicates that such reactions also occurred in the corn under environmental growth conditions (Howard *et al.*, 1998).

No literature is available regarding the formation of this derivative *in vitro* or *in vivo*, however it may be postulated that the presence of the amino group would allow such reactions to occur under favourable conditions. Enzymatic activity or the concentration of reducing sugars in the WF and CF may result in the formation of this or similar derivatives within the cell, again rendering the FB_1 unavailable for interaction with ceramide synthase. Such modification of the FB_1 molecule may also facilitate its excretion from the cell into the surrounding medium.

As outlined in Chapter 2, aflatoxin B_1 requires biotransformation to the reactive exo–epoxide to exert its carcinogenic effect (Massey *et al.*, 1995). This reaction is catalysed by the CYP isoenzymes, the expression of which is species specific (Guerre *et al.*, 1997).

The CYP enzymes are also capable of metabolising AFB_1 to other products, including AFM_1, AFP_1 and AFQ_1, and production of these metabolites is considered to represent detoxification reactions. Another important detoxification pathway is the conjugation of AFBO to GSH via the activity of GST isoenzymes. Major determinants of susceptibility to AFB_1–induced hepatocarcinogenesis are thus AFBO production as well as GSH conjugating activity (Miyata *et al.*, 2004).

Native chlorophylls have been demonstrated to significantly suppress *in vivo* DNA adduct formation induced by DBP in the rainbow trout model, as described by Harttig and Bailey (1998). The implications with regards to AFB_1–DNA adduct formation remain to be elucidated however, these authors suggested a potential role for dietary chlorophylls in the reduction of this type of tumour initiation risk. The potentially high concentration of these ubiquitous plant pigments in the AF may account for the protective activity of this fraction against AFB_1 cytotoxicity in the A549 cell line (Figure 5.4b).

Also regarding the observed reduction in AFB_1 toxicity, Kim *et al.* (1999) investigated the role of polysaccharide fractions from various plant types in chemoprevention *in vitro*. An aloe (*Aloe barbadensis Miller*) polysaccharide at concentrations of 20–180µg/ml was found to significantly inhibit the formation of ^{3}H–B[*a*]P–DNA adducts by 20%–50% of the control in NCTC clone–1469 mouse normal liver cells. In the same study, a polysaccharide from *Ganoderma lucidum* was found to be a potent inducer of GST detoxification enzymes in the same cell line. A significant increase of 20%–30% in GST activity was observed. Carbohydrate analysis of the polysaccharides indicated that the aloe polysaccharide consisted mainly of mannose, while glucose was the principal monosaccharide in the *Ganoderma lucidum* polysaccharide (Kim *et al.*, 1999).

Similar polysaccharides may be present in the WF (Figure 5.4a), leading to decreased adduct formation or increased conjugation of AFBO to GSH with subsequent clearance from the cell. It has been suggested that high levels of expression of GST's, which inactivate the exo–epoxide, rather than low levels of the activating CYP enzymes, is pivotal in chemoprevention (Kelly *et al.*, 2000).

Contrary to this statement however, were the findings of Miyata and colleagues (2004). In a study on the effects of grapefruit juice consumption on AFB_1 toxicity in rats, they discovered that the administration of grapefruit juice significantly reduced AFB_1–induced hepatic DNA damage compared to control animals. This effect was attributed to a significant reduction in both the content and activity of the activating enzyme CYP3A in the livers of treated rats. The major GST subunit responsible for protection against AFB_1–induced hepatotoxicity in the rat is GSTA5. As there was no significant increase in GST activity, GSH content or GSTA5 expression, the protective effect of the grapefruit juice was not due to enhanced detoxification capacity. Also of importance was the finding that lipid–soluble components of the grapefruit juice, such as furanocoumarin derivatives, also significantly reduced DNA damage by AFB_1. This may be relevant with regards to the activity of the AF in this study.

It should be noted that the MTT assay has limitations regarding data interpretation, not the least of which is the continued ambiguity regarding the exact mechanism of cellular MTT reduction (Bernas and Dobrucki, 2002). Marionnet *et al.* (1997) compared the MTT assay with two other methods for measuring cell viability, namely cell counting and tritiated thymidine incorporation. It was found that the sensitivity of the MTT assay differed according to the drug treatment and the type of culture used. Often the growth inhibitory activity of the compounds tested was underestimated. Thus, the MTT assay should ideally be used in conjunction with other methods to assess drug effects on cell growth.

The most widely used methods to detect protective effects are *in vitro* tests with bacteria and stable mammalian cell lines. These models reflect the situation in laboratory rodents and humans only partly and misleading results have been obtained (Kassie *et al.*, 2002). In addition, the shortcomings of tissue culture models in general are many. The use of animal models for the evaluation of potential carcinogenic or indeed chemopreventive natural products is advantageous as the entire physiology and tissue specific metabolism of carcinogens is an important element of such assays. Most carcinogens undergo a cascade of activation and detoxification reactions in one or more tissues before exerting their carcinogenic potential. Such pathways cannot be truly replicated in cell based assays (de Boer, 2001).

5.5 CONCLUSION

The results of this investigation are significant in that the plant fractions displayed protective effects against the toxicity of both FB_1 and AFB_1 (Figures 5.2 and 5.4), in cells of different tissue origin. The lack of fraction toxicity against the normal human lymphocytes (Figure 5.5) is also encouraging from a chemopreventive standpoint.

This observation, together with the apparent protection from toxin cytotoxicity exerted by the AF (100µg/ml) on the normal cells, lead to the determination that this fraction in particular, warrants further investigations into potential chemoprotective effects.

CHAPTER SIX

SPECIFIC BIOMARKER ANALYSIS: THE INDUCTION OF REDUCED GLUTATHIONE BY THE ACETONE FRACTION

6.1 INTRODUCTION

6.1.1 BIOMARKERS

Clinical cancer prevention studies using disease as an endpoint are large, time–consuming and very costly. Development in cancer chemoprevention is being accelerated by the application of intermediate markers to preclinical and clinical trials (Kensler *et al.*, 1998).

The concept of using biological markers in studies of human disease is not new. These endpoints, termed biomarkers, have received increasing attention and the field of molecular epidemiology is based entirely upon them. Molecular epidemiology is designed to study chronic diseases with environmental components. A prime example of such a disease is cancer (Albertini, 1999).

A large number of potential chemopreventive agents have been identified from epidemiological studies, experimental pre–clinical and clinical observations and structural homology with known chemopreventive agents. Before these agents can be evaluated in clinical trials however, multiple experimental systems are required to screen and analyse their efficacy. Pre–screening of agents using biomarkers of the carcinogenic process via *in vitro* assay models utilising cell cultures is particularly useful (Sharma *et al.*, 1994).

6.1.2 Reactive Oxygen Species

During normal metabolism in higher eukaryotes, oxidising equivalents or reactive oxygen species (ROS) are generated during respiration in the mitochondrion. This occurs as electrons leak out of the electron transport chain in the course of molecular oxygen reduction. Alternative sources of ROS generation include endogenous enzyme systems and organellar sources. Reactive oxygen species have been implicated in regulating diverse cellular functions, intracellular signalling, transcriptional activation, proliferation and apoptosis, amongst others (Davis *et al.*, 2001).

The reduction–oxidation (redox) state of the cell results from the precise balance between the levels of ROS and reducing equivalents. Elevation in ROS exceeding the capacity of buffers and enzymatic systems designed to modulate ROS levels results in potentially cytotoxic oxidative stress. Under these conditions, the reactive radicals can interact with nucleic acids, proteins and lipid components, which may result in damage and ultimately cell death. Cells have developed two important defence mechanisms to counteract the effects of oxidative stress, namely a thiol reducing buffer system and enzymatic systems (Davis *et al.*, 2001).

6.1.3 The Thiol Redox Buffer

The thiol redox buffer consists of small proteins containing redox–active sulphydryl moieties. The largest component of the endogenous thiol buffer is the glycine, γ–glutamic acid, cysteine tripeptide glutathione (Davis *et al.*, 2001). Glutathione (Figure 6.1) is widely distributed among living cells and is the most prevalent non–protein thiol and the main low molecular weight thiol compound found in living plant cells (Hissin and Hilf, 1976).

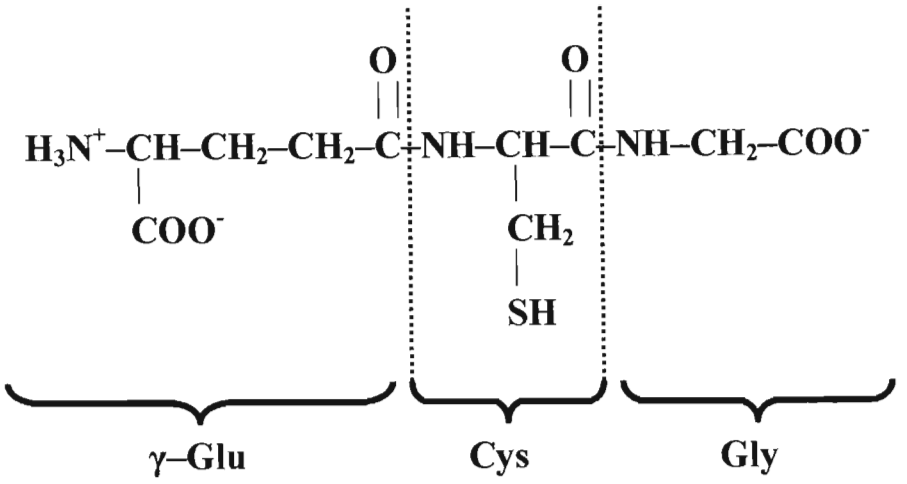


Figure 6.1: Chemical structure of reduced glutathione (Matthews and van Holde, 1996).

Glutathione is present in all mammalian cells at concentrations ranging from 0.1mM to 10mM (Baker *et al.*, 1990). It is present in both the reduced (GSH) and oxidised (GSSG) states. Oxidised glutathione is reduced to two GSH by glutathione reductase (Figure 6.2), an NADPH–dependant enzyme. As GSH is the active form, it is present in a much higher concentration in the cell. The intracellular ratio of GSH to GSSG is approximately 500:1 (Mathews and van Holde, 1996).

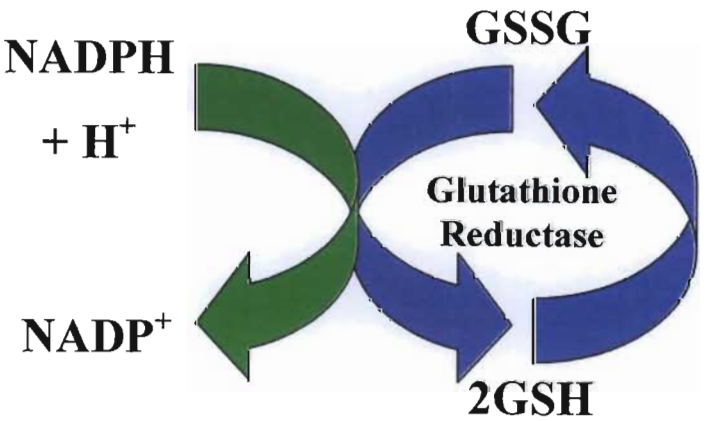


Figure 6.2: Generation of reduced glutathione from oxidised glutathione by the NADPH–dependent enzyme glutathione reductase (Adapted from: Mathews and van Holde, 1996).

Cellular redox changes following environmental stress induced by cytotoxic challenge may not be exclusively due to generation of ROS, but also to the extrusion of GSH from the cells. Glutathione has a role in the modulation of apoptotic signalling, evidenced by the effect of the pro–apoptotic protein Bcl–2 on GSH metabolism. Overexpression of this protein results in the redistribution of GSH from the cytosol to the nucleus, altering nuclear redox and blocking caspase activity. Thus, compartmentalisation of GSH within the cell impacts on the activity of proteins responsible for the promotion of cell survival (Davis *et al.*, 2001).

6.1.4 GLUTATHIONE

Free radicals are causative factors in premature cell death and contribute to premature ageing as well as over 50 diseases including emphysema, eczema and heart disease (Skin and Its' Antioxidant Defence System, 2000). The production of free radicals is increased when the body is subjected to stresses including disease. Antioxidants neutralise excess free radicals, preventing the chain reaction that contributes to various diseases and premature ageing, thereby combating oxidative stress (Geriatric Diseases (Diseases of old age), 2000).

The body's primary antioxidant, L–glutathione, is synthesised in the liver and can be transported to most cells in the body via the bloodstream (Skin and Its' Antioxidant Defence System, 2000). It maintains an intracellular reducing environment, preventing intracellular protein thiols from oxidising to disulphides and non–enzymatically reduces a number of substances, such as free radicals and peroxides, which accumulate in cells under oxidising conditions. Through the action of GST's, GSH is involved in the detoxification of many substances, including xenobiotics and electrophiles produced through the activity of cytochrome P_{450}–linked oxidases (Mathews and van Holde, 1996).

Glutathione helps reduce the development of wrinkles and other signs of ageing and together with synergistic antioxidants may reduce the occurrence of skin cancer (Skin and Its' Antioxidant Defence System, 2000). Studies have shown that free radicals are involved in many "old age" diseases, including neurodegenerative disorders, such as Alzheimer's and Parkinson's disease. This could be linked to the fact that glutathione levels are shown to decrease with age (Geriatric Diseases (Diseases of old age), 2000). Deficiency in GSH has also been observed in acquired immunodeficiency syndrome patients at all stages of the disease (Oe *et al.*, 1998).

As glutathione is a potent antioxidant, it can protect the cell during both the initiation and promotion phases of carcinogenesis. Electrophiles conjugate with the nucleophilic thiol of GSH, causing a decrease in the availability of reactive electrophiles to bind to DNA. This decreases the possibility of the initiation of transformation within the cell. During the promotion phase, glutathione limits oxidative free radical attack, thus protecting the cell (Sharma *et al.*, 1994). Depletion of GSH can result in a 25–fold increase in the sensitivity of AFB_1–DNA adduct formation in the murine liver (Kelly *et al.*, 2000). Elevation of GSH is associated with increased cellular proliferation and impairment of apoptosis. Lowering of intracellular GSH via an efflux mechanism has been reported during apoptosis and although this can be caspase–dependent, it has been proposed that GSH efflux is a critical early event in the initiation of apoptosis (Pullar *et al.*, 2004).

Epidemiological studies have demonstrated that regular dietary consumption of fruit and vegetables is associated with decreased incidence of many cancers, particularly those of the gastrointestinal tract. Short–term dietary interventions with cruciferous vegetables in humans have been shown to influence drug metabolism and decrease oxidative DNA damage. Similar effects with vegetable extracts have been noted in rodents. Therefore, the ability of edible plants to confer resistance against neoplasia can be attributed partly to the stimulation of cytoprotective mechanisms in the host (McMahon *et al.*, 2001).

Glutathione possesses no UV–chromophores and does not fluoresce. However, it reacts with *ortho*–phthaldialdehyde (OPA) to form a fluorescent product that is activated at 350nm with an emission peak at 420nm. This reaction forms the basis for the analysis used in this study (Sharma *et al.*, 1994).

6.1.5 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High Performance Liquid Chromatography (HPLC) is a powerful analytical tool that can be used for the qualitation and quantitation of many compounds. All forms of liquid chromatography are based on the same basic principle, that sample molecules partition between a moving liquid phase (mobile phase) and a stationary (liquid or solid) phase. The extent to which the molecular types separate depends largely on the adjustment of the separation conditions so that the different molecules have different separation coefficients. Unlike gas chromatography, the character of both the mobile and stationary phases can be manipulated (Willard *et al.*, 1988).

Of the many liquid chromatographic methods available, the reverse–phase technique is the most widely used, comprising almost half of all liquid chromatographic methods published. It is most likely to provide optimum retention and selectivity for compounds with no hydrogen–bonding groups or those that are predominantly aliphatic or aromatic in nature (Willard *et al.*, 1988).

6.1.6 FLUORESCENCE SPECTROPHOTOMETRY

Two analytical photoluminescence methods exist, namely phosphorescence and fluorescence spectrophotometry, with the latter being the more widely used. A major analytical role has been assumed by fluorescence spectrophotometry, especially concerning the determination of trace contaminants in our environments. This is due to the high sensitivity and specificity provided by the method. Sensitivity ranges in the low parts per trillion result from the difference between the excitation and emission wavelengths. The high specificity is due to reliance on the two different spectra, namely the excitation and emission spectra (Willard *et al.*, 1988).

6.1.7 OBJECTIVES

This study was undertaken to assess the inducive effects, if any, of the AF on GSH production in the SNO cell line, due to the importance of this compound and it's usefulness as a biomarker of chemoprevention. Two methods of GSH analysis were employed, namely HPLC and fluorescence spectrophotometry. This was done to validate the results and evaluate the most sensitive method for analysis of samples.

6.2 MATERIALS AND METHODS

6.2.1 MATERIALS

Trichloroacetic acid (TCA) and *o*–phthaldialdehyde (OPA) were purchased from Sigma Chemical Company, SA and the reduced glutathione (GSH) was obtained from Roche Diagnostics. Disodium hydrogen orthophosphate (anhydrous), ethylenediaminetetra–acetic acid (EDTA) disodium salt and sodium acetate were purchased from Merck, SA.

6.2.2 METHODS

6.2.2.1 Sample Preparation

The SNO cells were maintained as described in Chapter 4 until confluence was attained, at which time the assay was conducted. The ability of the AF to induce reduced glutathione production in the SNO cells was tested by treatment with two millilitres of the AF or ethanol control dilutions. The final concentrations of the AF used for this experiment were 0.02µg/ml, 0.2µg/ml, 2µg/ml and 20µg/ml. Equivalent ethanol solvent control dilutions were prepared.

Following an incubation period of 24 hours in the culture environment the media was decanted, the cells washed twice with cold PBS (pH 7.2) and trypsinised. One millilitre of 0.25M sucrose buffer containing 5% TCA was then added to each flask, the cells were removed and lysed via sonication. The supernatant obtained from the subsequent ultracentrifugation at 12 000 x g for 30 minutes at 4°C was stored at –70°C until analysis.

This experiment was conducted five times using fresh cells and reagents. Supernatants from each experiment were kept separate and not pooled according to treatment type. All samples were analysed using both HPLC and fluorescence spectrophotometry and the results are represented as mean values for each treatment type.

6.2.2.2 The Induction of Reduced Glutathione Assay

The cell supernatants prepared above, were thawed slowly on ice before being used in the assay. Cell supernatant (100µl) was added to phosphate–EDTA buffer (1.8ml; pH 8.0; Appendix 3). The samples were derivatised by the addition of OPA (100µl; 1mg/ml in methanol). The reaction was allowed to proceed at room temperature for exactly two hours before the amount of fluorescent product was analysed via HPLC or fluorescence spectrophotometry.

6.2.3 THE ANALYSIS OF REDUCED GLUTATHIONE

6.2.3.1 High Performance Liquid Chromatography (HPLC)

Each sample was injected (20µl) at a flow rate of 0.4ml/minute using a mobile phase of 0.1M sodium acetate buffer (pH 3.8; Appendix 4): methanol (HPLC grade; 1:1). A Luna C18 [2] column with a 3µm particle size and dimensions of 150 x 3mm was used for the analysis. The fluorescence detector was set at 230nm (ex) and 440nm (em) and the UV detector was set at 336nm. A Thermo Separation Products HPLC system was used. The system consisted of a Spectra Physics P2000 pump, AS3000 autosampler, FL2000 fluorescence detector and a Spectra Focus UV detector. Data was processed using PC1000 software (Thermo Separation Products, SMM Instruments).

6.2.3.2 Fluorescence Spectrophotometry

For this method of analysis, the sample volumes were doubled, as a minimum of four millilitres of liquid is required for the beam of light to pass through the sample. The Varian DMS 100 UV Visible Spectrophotometer with fluorescence attachment was used. Both the UV and visible lamps were used and the analysis was conducted at the excitation wavelength of 350nm.

Percentage transmission was read in single beam mode with a slit width of 4nm and a time constant of 5 seconds. Square, quartz cuvettes with a 10mm path length were used. All four sides of the cuvettes had to be clear, due to the path of the light in fluorescence work.

The fluorescence attachment was inserted in place of the cuvette holder and maximum gain set. The most concentrated standard was inserted and used to zero the instrument. With this standard still in the holder, the gain was adjusted to give a reading of between 80 and 90% transmission (%T). All remaining standards and samples were read.

6.2.4 STANDARD CURVE

The concentration of GSH present in each sample was calculated using standard curves. This was performed for both methods of analysis. Each time samples were analysed, selected standards were also analysed to verify the accuracy of the instrumentation and the reproducibility of the reaction. A stock solution of GSH (Appendix 5) was prepared fresh daily in buffer and a working stock (Appendix 5) was prepared from this solution. The standards were prepared from the working stock solution of GSH (Table 6.1).

Table 6.1: Preparation of reduced glutathione standards.

TUBE NUMBER	[GSH] (µg/ml)	VOLUME GSH STOCK (ml)*	VOLUME BUFFER (ml)*
1	1.0	1.0	0.9
2	0.9	0.9	1.0
3	0.8	0.8	1.1
4	0.7	0.7	1.2
5	0.6	0.6	1.3
6	0.5	0.5	1.4
7	0.4	0.4	1.5
8	0.3	0.3	1.6
9	0.2	0.2	1.7
10	0.1	0.1	1.8

* The remaining 100µl of the final volume (2ml) was made up by the addition of the OPA derivatising reagent.

6.2.5 STATISTICAL ANALYSIS OF DATA

The Student's T–Test was performed on all data to determine significant differences between AF and EtOH control treatments and between the EtOH control samples and the untreated control. The level of significance was set at $p < 0.05$ and all calculations were performed using Microsoft Excel for Windows 98.

6.3 RESULTS

6.3.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The graphs obtained from HPLC analysis are referred to as chromatograms and display elution peaks representing the solutes traversing the detector. The peak area generated is proportional to the concentration of the solute (GSH–OPA derivative) and is used to derive standard curves and quantify the amount of GSH in the samples. Examples of chromatograms obtained for a standard solution of GSH as well as a selected test sample are depicted in Figures 6.3 and 6.4.

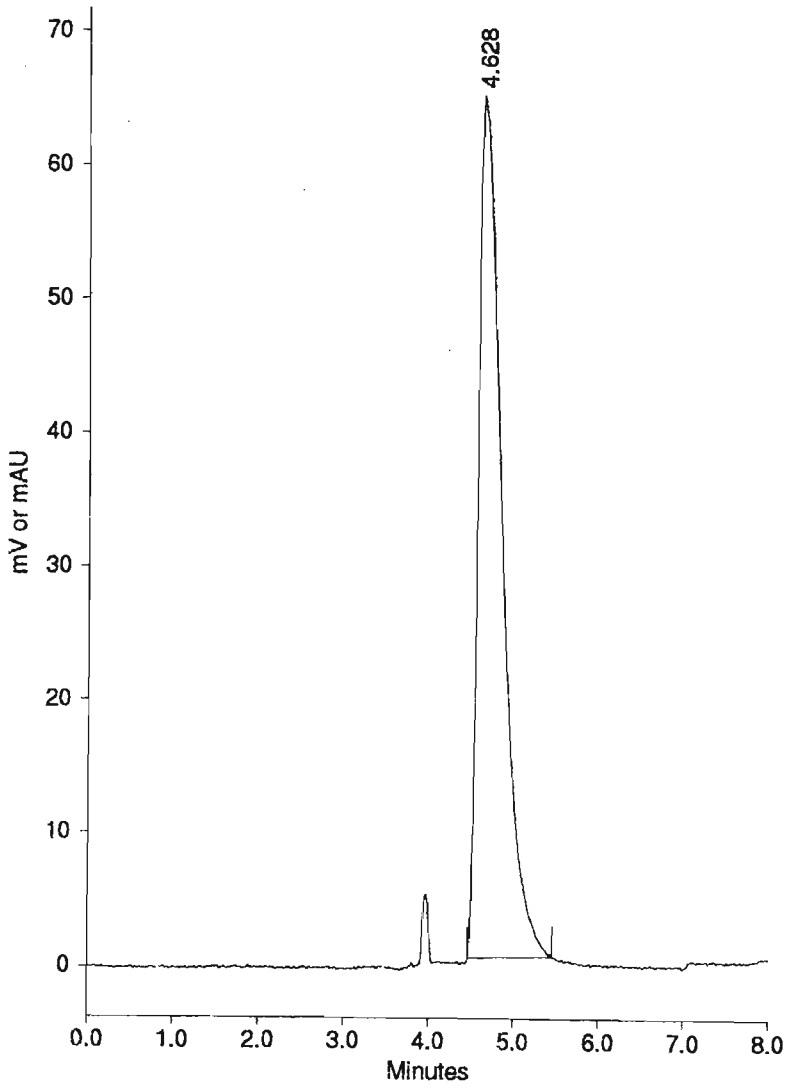


Figure 6.3: Chromatogram obtained for the 1µg/ml reduced glutathione standard.

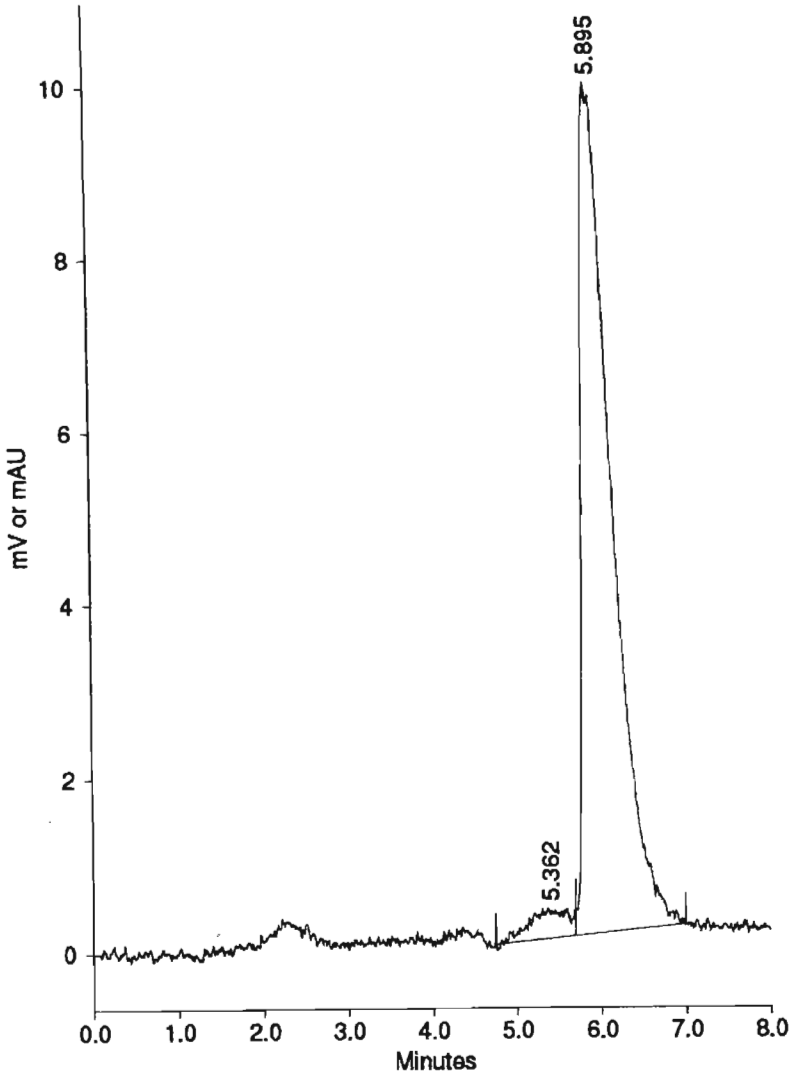


Figure 6.4: Chromatogram obtained for a sample of SNO cells treated with the acetone fraction (0.2µg/ml).

The standard curves generated for the calculation of GSH concentration in untreated SNO cells and SNO cells treated with AF and the ethanol control are shown in Figures 6.5 and 6.6 respectively.

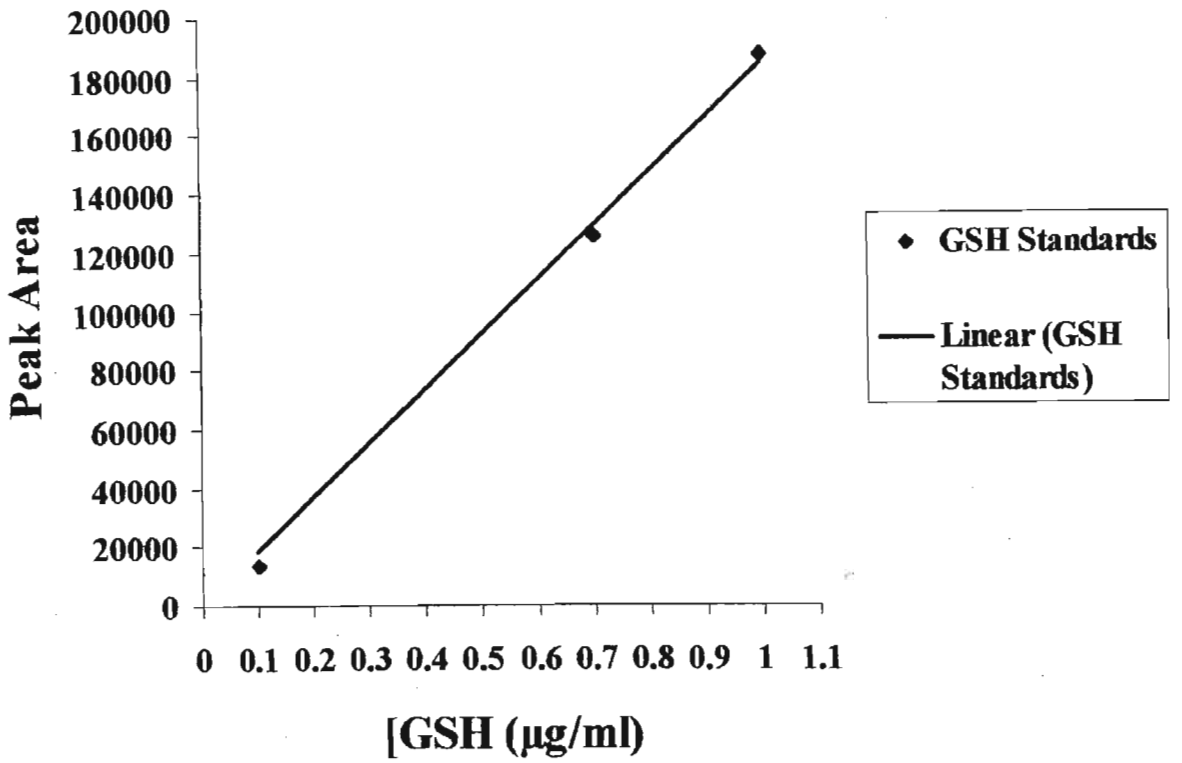


Figure 6.5: **Standard curve used for the analysis of reduced glutathione in untreated SNO cells using high performance liquid chromatography.**

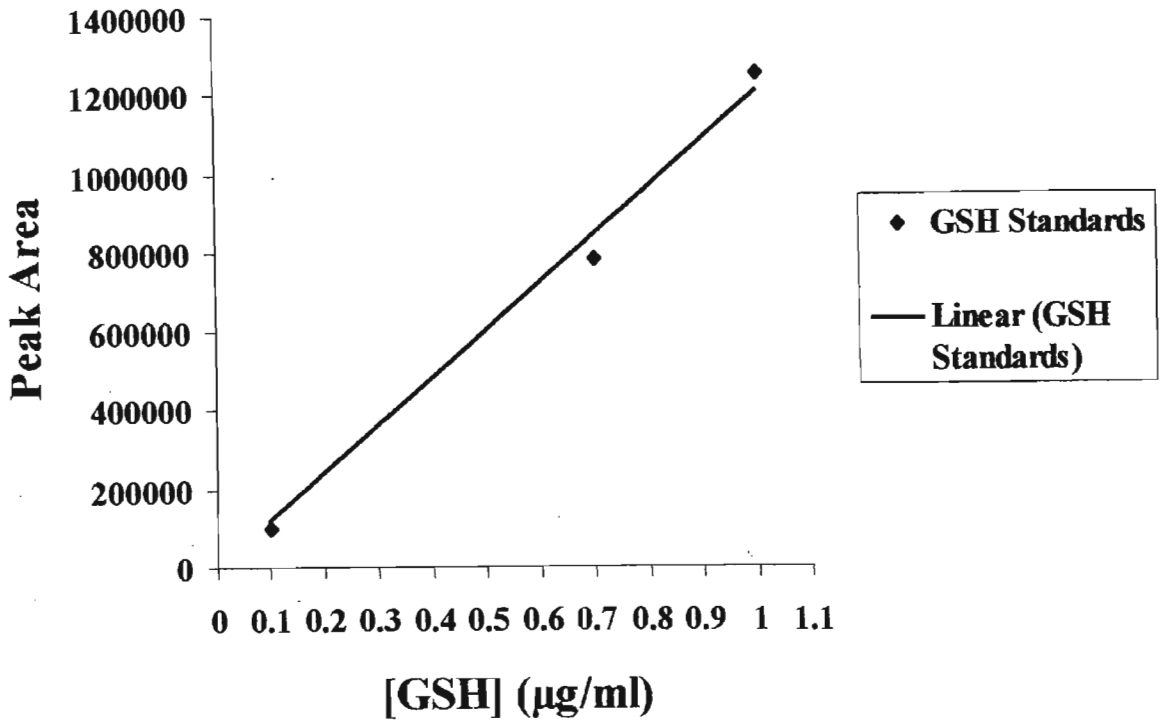
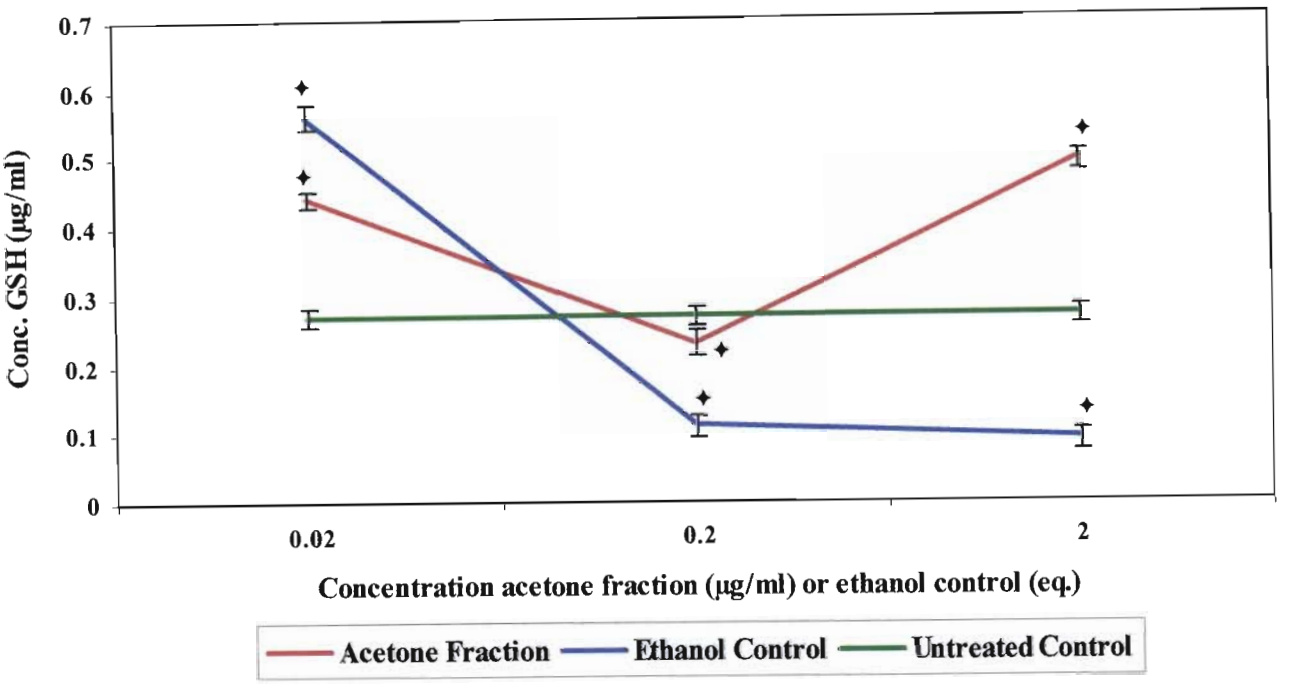


Figure 6.6: Standard curve used for the analysis of reduced glutathione in SNO cells treated with the acetone fraction and ethanol control using high performance liquid chromatography.

The concentration of GSH in the samples was determined from these standard curves. The untreated SNO cells were found to contain approximately 0.27µg GSH/ml and this was referred to as the GSH baseline level for the SNO cells. Figure 6.7 represents the levels of GSH in cells treated with the AF and ethanol control.



✦ Student's T-Test: $p < 0.05$ (Significance to control)

Figure 6.7: Effect of the acetone fraction and ethanol control on reduced glutathione concentration in SNO cells, as determined by high performance liquid chromatography.

The concentration of GSH in cells treated with the 20µg/ml AF and ethanol control concentrations was below the detection limit of this method of analysis.

The 0.02µg/ml (eq.) ethanol control concentration was the most effective inducer of GSH in the SNO cells and at the same concentration, the AF was seen to decrease GSH production. However, the remaining concentrations for the AF were seen to stimulate GSH production with regards to the ethanol control. The 2µg/ml AF concentration in particular not only counteracted the detrimental effects of the ethanol control at this concentration, but also almost doubled the GSH baseline concentrations.

6.3.2 FLUORESCENCE SPECTROPHOTOMETRY

The standard curves obtained for the spectrophotometric analysis of GSH are shown in Figures 6.8 to 6.10.

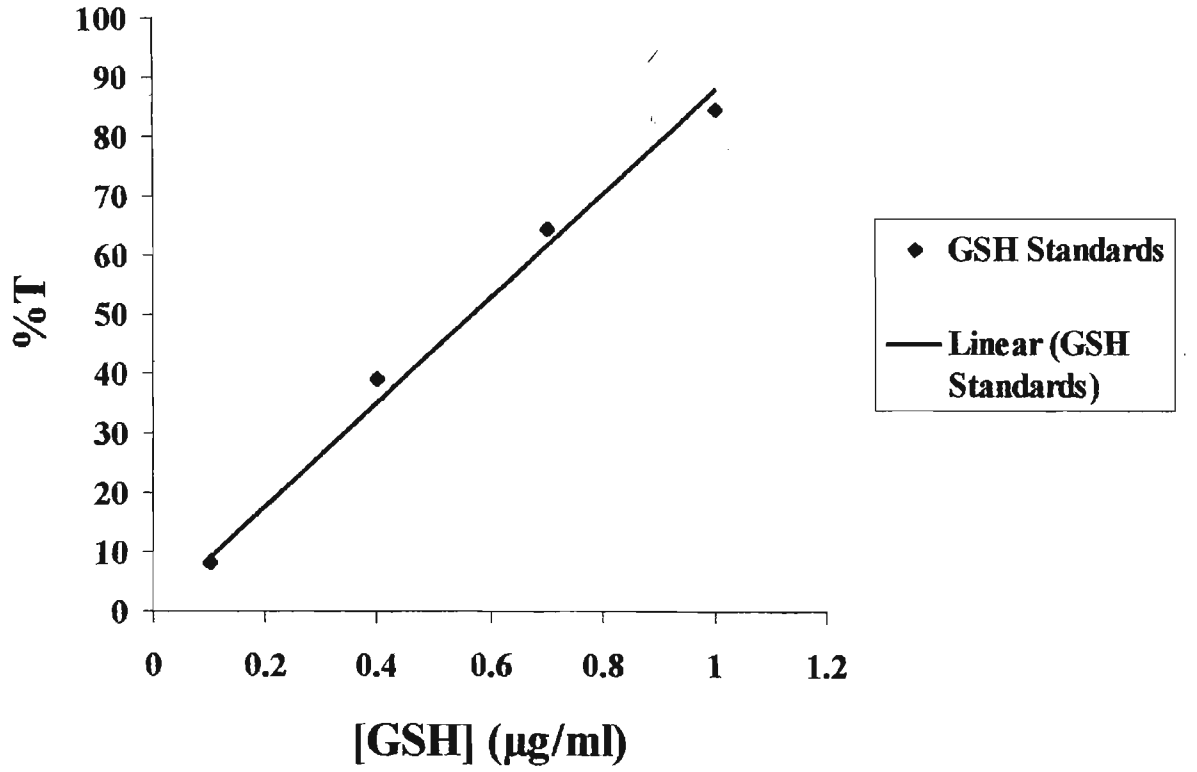


Figure 6.8: Standard curve used for the spectrophotometric analysis of reduced glutathione in untreated SNO control cells.

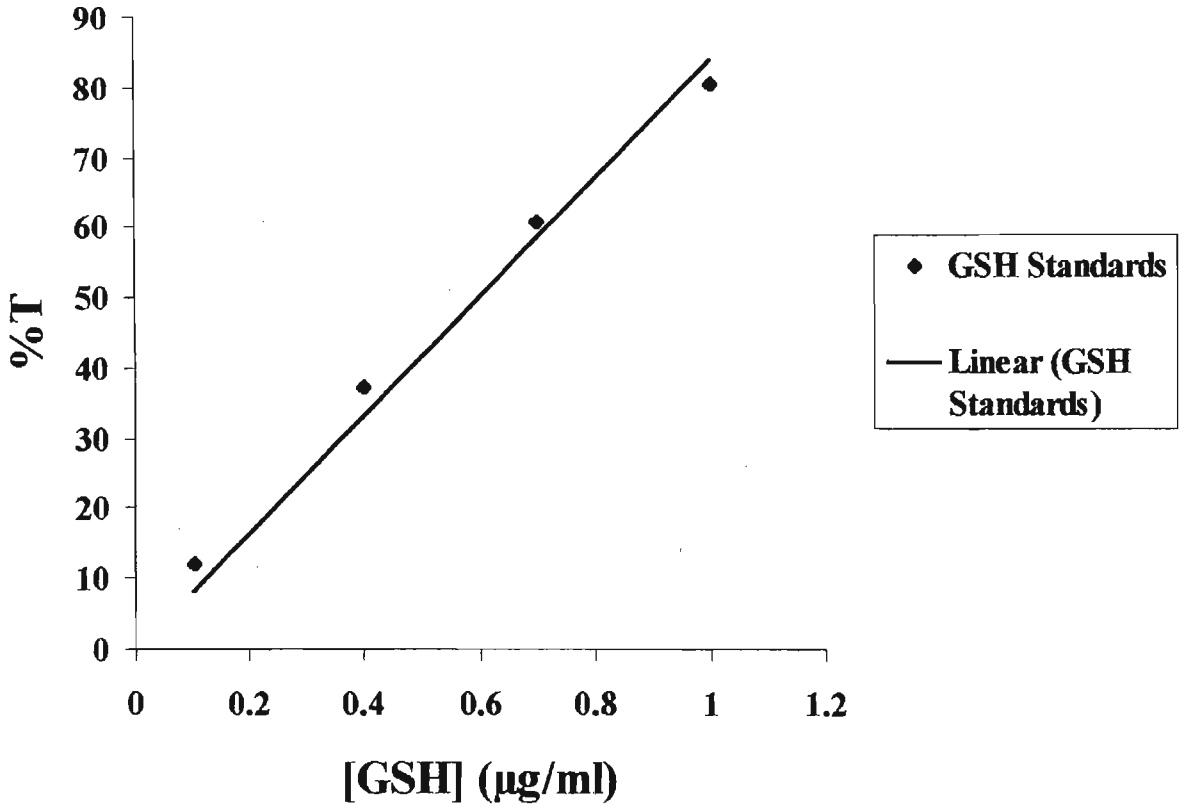


Figure 6.9: **Standard curve used for the spectrophotometric analysis of reduced glutathione in SNO cells treated with the acetone fraction.**

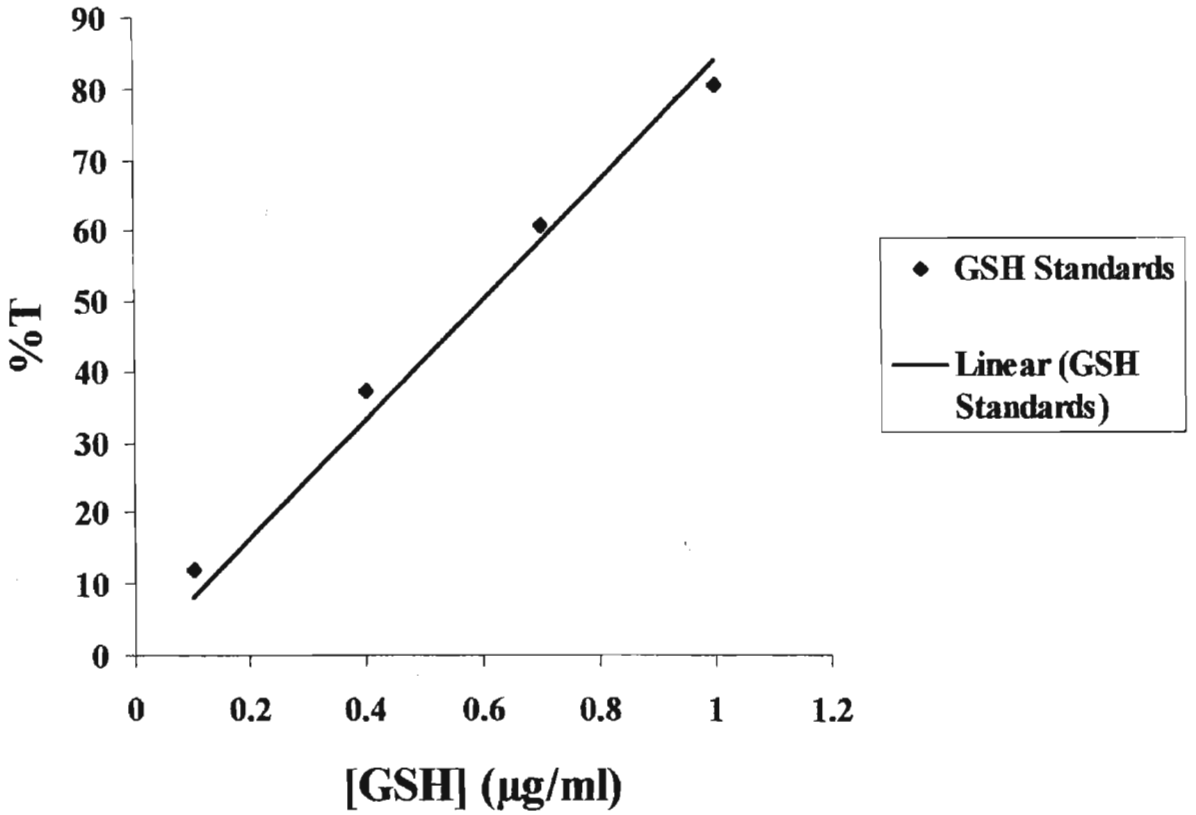
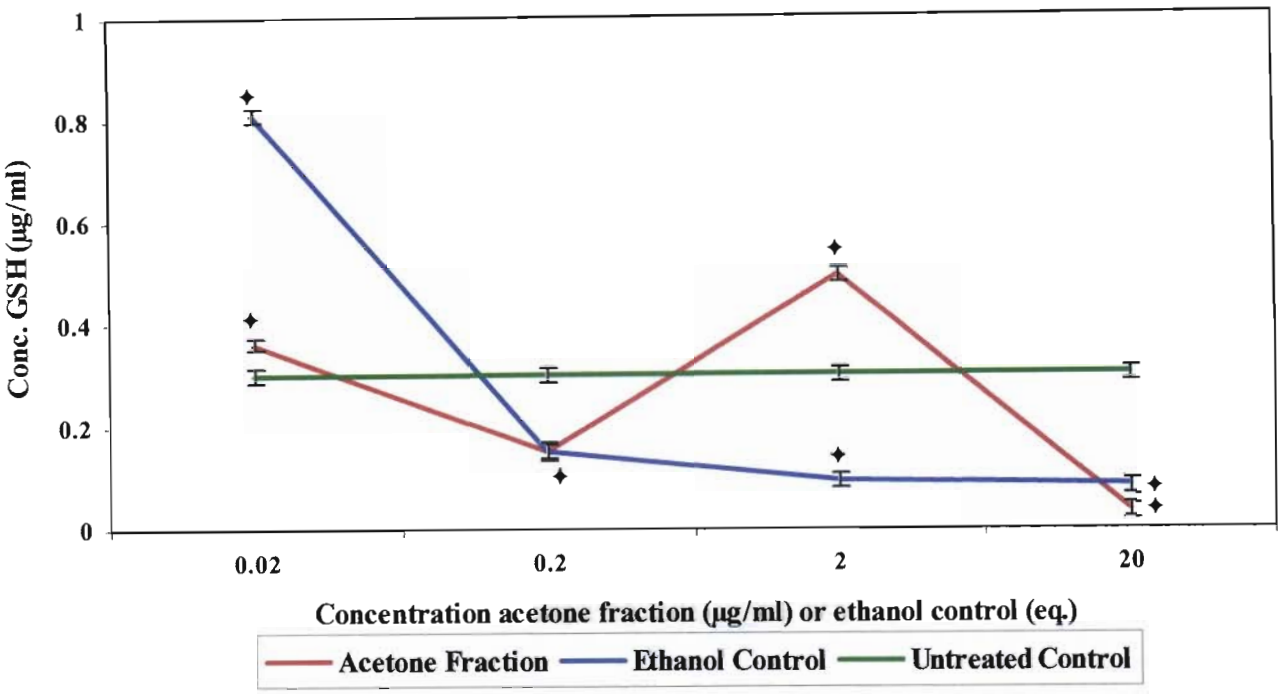


Figure 6.10: Standard curve used for the spectrophotometric analysis of reduced glutathione in SNO cells treated with the ethanol control.

The levels of GSH in the samples were determined from these standard curves. The baseline level for the untreated SNO cells was determined to be approximately 0.3µg GSH/ml. Figure 6.11 represents the levels of GSH in cells treated with the AF and ethanol control.



✦ Student's T-Test: $p < 0.05$ (Significance to control)

Figure 6.11: Effect of the acetone fraction and ethanol control on reduced glutathione concentration in SNO cells, as determined by fluorescence spectrophotometry.

As for the HPLC results, the 0.02µg/ml (eq.) ethanol control concentration was most effective in inducing GSH in the SNO cells, with the AF decreasing GSH production. The readings for the 0.2µg/ml concentration were identical for the AF and ethanol control. This indicates no effect on the part of the fraction, as the decrease in GSH levels can be entirely attributed to the effect of the ethanol solvent at this concentration.

Again, the 2µg/ml AF concentration was the most effective in the induction of GSH in the SNO cells. However, the 20µg/ml AF concentration was ineffective, almost completely depleting cellular GSH.

6.4 DISCUSSION

The 2μg/ml AF concentration induced reduced glutathione in the SNO cells, as compared to the ethanol control as well as the baseline GSH levels for the SNO cells. The 0.2μg/ml AF concentration slightly inhibited GSH as compared to the baseline levels, but not as drastically as the ethanol control at this concentration. Thus, the AF was seen to overcome some of the negative effects induced by the ethanol at this concentration. The 0.02μg/ml (eq.) ethanol control concentration showed the greatest degree of induction, with more than double the baseline level being detected by both methods of analysis. Thus, at this concentration the AF is seen to inhibit GSH production, as the levels detected were lower than those of the ethanol control (Figures 6.7 and 6.11).

The results obtained using high performance liquid chromatography and fluorescence spectrophotometry correlated regarding the observed trends governing the effects of the AF and ethanol control. There were slight variations in some of the readings, as is to be expected when using two different techniques. However, the spectrophotometric method was more sensitive than HPLC, having a lower detection limit (Figure 6.6). The overall level of GSH recovered from the untreated SNO cells was well below baseline levels described in the literature (Baker *et al.*, 1990). However, cancerous oesophageal tissue has been found to be depleted of GSH (Levy *et al.*, 1999) and this may be the case in culture. Alternatively, it could be due to losses incurred during sample preparation or incomplete derivitisation of GSH with the OPA reagent.

It must also be borne in mind that in the third world situation advanced instrumental analysis, such as HPLC, is not always a viable analytical option. This is due to drawbacks such as costly equipment, specialised sample preparation methodologies and unsuitability of the technique for on–site analysis (Szurdoki *et al.*, 1996).

The current study utilised two different methods for the analysis of intracellular GSH, namely HPLC and fluorescence spectrophotometry. Additional advantages to the lower detection limit of the spectrophotometric method mentioned above are the simplicity of the instrumentation used, as well as much lower cost. Spectrophotometers are relatively common laboratory instruments and require minimal training for operation. Conversely, HPLC instrumentation is quite advanced and very costly, especially the modern, computerised machines, which require specialised training for the correct operating of the equipment. Although HPLC is a more time–efficient option, especially for large sample numbers, financial considerations as well as the level of skill required for its operating often preclude its use.

Several other options are available for the analysis of GSH, including commercially available ELISA kits and other ELISA based methods, such as that described by Baker *et al.* (1990). However, although these methods are also time–efficient, the kits are extremely cost prohibitive for use and only provide enough reagent for the analysis of one 96 well microtitre plate. This would only allow for the analysis of 32 different samples in triplicate (excluding standards) or 24 different samples (with standards). Thus, such kits are not suitable for large–scale undertakings, such as the screening of many plant extracts at various concentrations for GSH inducing capacity. Although the method described by Baker *et al.* (1990) is appropriate for large–scale use, its employment is dependent on the availability of an ELISA plate reader with the appropriate wavelength filter, as are the commercial kits.

Glutathione is conjugated to the highly reactive AFBO and the resultant conjugate is then excreted from the cell. This is one way of greatly reducing the levels of AFB_1 in the body, leading to a reduced risk of carcinogenesis. Using chemical agents to prevent AFB_1–induced hepatocellular carcinoma represents a valuable approach to improved public health in regions where the producing mould is encountered (Kelly *et al.*, 2000).

The risk of developing liver cancer from AFB_1 exposure is greatest in economically developing countries. Financial constraints may limit the use of prophylactic agents in these regions. Therefore, the identification of naturally occurring foodstuffs capable of preventing AFB_1 hepatocarcinogenesis would be extremely advantageous, as these foods could be incorporated into the human diet at minimal cost (Kelly *et al.*, 2000).

A large–scale study conducted by Sharma and colleagues (1994) identified numerous agents as potent or moderate inducers of GSH in a normal rat liver cell line. These included sulphur–containing compounds, such as N–acetyl–L–cysteine, as well as phenolic type compounds, including curcumin. Also among the moderate inducers were the lipid–soluble vitamin D, retinol and α–tocopherol succinate. The AF may contain these types of compounds and this may account for the inductive activity observed for several concentrations of this fraction.

6.5 CONCLUSION

Of the two analytical methods employed for the determination of GSH levels in both treated and untreated SNO cells, fluorescence spectrophotometry was deemed superior to HPLC. The spectrophotometric method possessed greater sensitivity and was more time– and cost–effective than HPLC, although the latter method has the advantages of smaller sample volume. In addition, the spectrophotometric method is ideally suited for use in the third world situation as the equipment is readily available and easy to use.

The glutathione assay indicated that certain acetone fraction concentrations were inducive to reduced glutathione production. This is very promising from a chemopreventive point of view.

CHAPTER SEVEN

INVESTIGATION INTO THE APOPTOTIC PROPERTIES OF THE ACETONE FRACTION ON NORMAL HUMAN LYMPHOCYTES

7.1 INTRODUCTION

7.1.1 APOPTOSIS: A BRIEF OVERVIEW

Cell death can be due to injury or programmed in a multicellular organism. Apoptosis is one of two common forms of cell death described for vertebrate tissues, with the other being necrosis (Cohen, 1993). Necrosis is a pathological form of cell death that results from overwhelming cellular injury (Steller, 1995) and is sometimes referred to as accidental cell death. Some features of necrosis are early changes in mitochondrial shape and function and the inability of the cell to maintain homeostasis. Typically, the plasma membrane loses its osmoregulatory ability, causing swelling and eventually lysis of the cell. This results in the cellular contents spilling into the intracellular spaces and this elicits an inflammatory response. The inflammatory response is desirable, as cell injury of this type is often extensive and cellular debris must be efficiently cleared away so that repair may begin (Cohen, 1993).

Apoptosis is a widespread and morphologically distinct process of cell death that is involved in many physiological and pathological processes (Buttke and Sandstrom, 1994). In contrast to necrosis, the apoptotic cell shrinks exhibiting condensation of the nucleus and cytoplasm. The plasma membrane becomes ruffled and blebbed resulting in the formation of apoptotic bodies (Cohen, 1993).

The cell eventually fragments into many such bodies, which are phagocytosed and digested by neighbouring cells or macrophages, resulting in the rapid removal of dead cells and the prevention of any leakage of noxious and possibly dangerous contents (Steller, 1995). Therefore, apoptosis is an advantageous mechanism of cell death.

Although there is a large amount of variation in the signals and metabolic events necessary for the induction of apoptosis in diverse cell types, the morphological features associated with apoptosis are highly conserved. This suggests the co–existence of multiple signalling pathways converging upstream of a common sequence of events that ultimately result in apoptosis. Many agents that induce apoptosis either are oxidants or stimulate cellular oxidative metabolism. Alternatively, many apoptotic inhibitors enhance intrinsic cellular antioxidant defences or have antioxidant properties. Thus, it could be possible that eukaryotic cells invoke oxidative stress as a mediator of apoptosis (Buttke and Sandstrom, 1994).

One of the earliest changes associated with apoptosis is alteration of the plasma membrane. The translocation of phosphatidylserine (PS) from the inner membrane leaflet to the outer layer is one such change, resulting in the exposure of PS at the external surface of the cell. Annexin V is a Ca^{2+} dependent phospholipid–binding protein with a high affinity for PS with minimal binding to other phospholipid species, such as phosphatidylcholine and sphingomyeline, which are constitutively parts of the outer plasma membrane (Figure 7.1). Thus, annexin V can be used as a sensitive probe for PS exposure on the outer membrane (Vermes *et al.*, 1995).

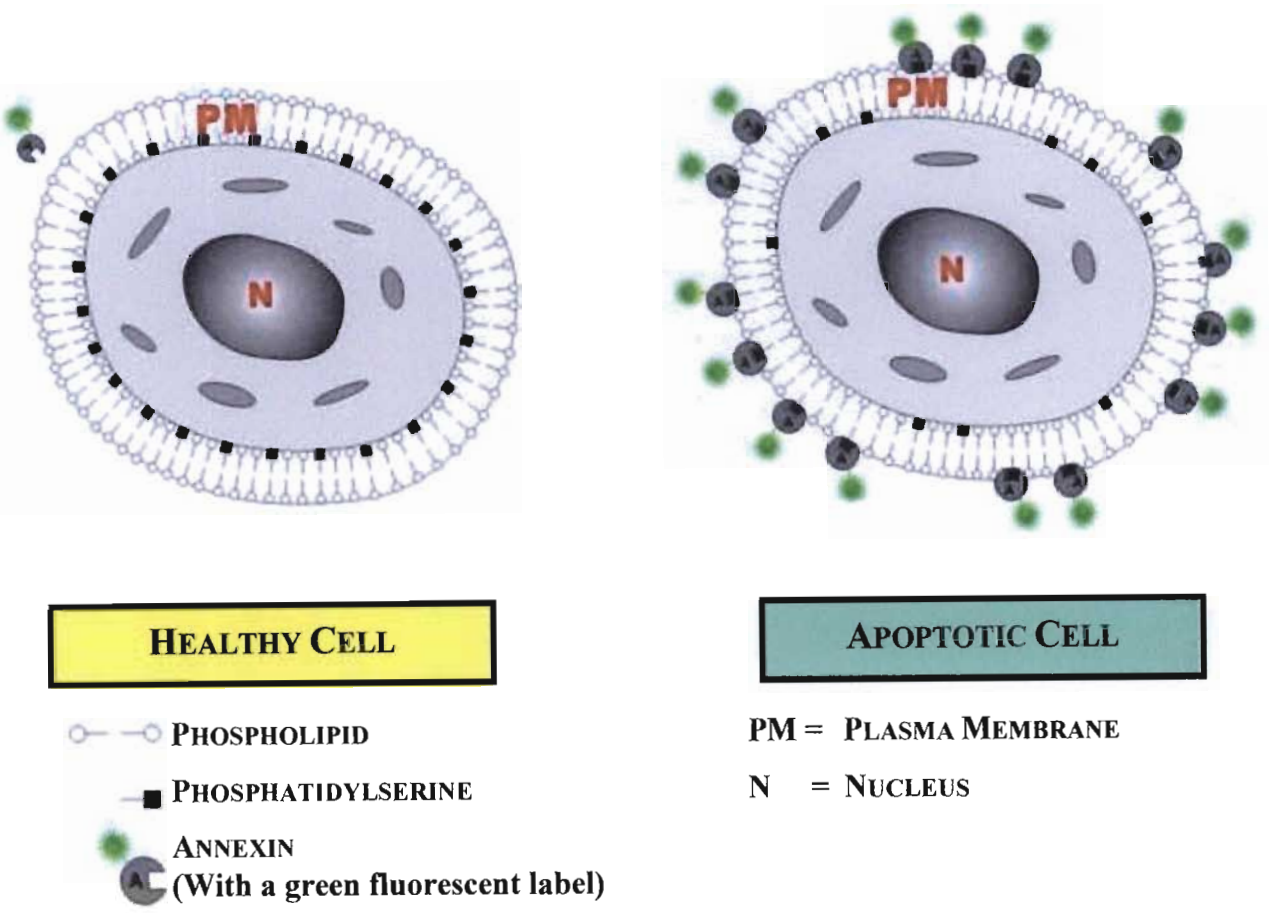


Figure 7.1: A schematic representation of the principle of detecting apoptosis by staining plasma membranes with annexin (Applications of confocal microscopy: Detection of apoptosis, 2004).

However, this translocation of PS is not unique to apoptosis, but also occurs during necrosis and thus a secondary method is needed to differentiate between necrotic and apoptotic cells. The difference between the two forms of cell death is that the cell membrane remains intact during the initial stages of apoptosis, while membrane integrity is lost very early in the onset of necrosis. Thus, the inclusion of a dye exclusion test, such as propidium iodide (PI) uptake, into the annexin V assay allows for the discrimination between apoptotic cells and necrotic cells utilising flow cytometry (Vermes *et al.*, 1995).

7.1.2 FLOW CYTOMETRY

Flow cytometry is a laser–based technology used to measure the characteristics of biological particles, including whole cells and cellular constituents such as nuclei and organelles. Single particles or cells are scanned as they flow in a liquid medium past an excitation light source. The underlying principle of flow cytometry is that light is scattered and fluorescence is emitted as the light from the excitation source encounters the moving cells. Light scattering and fluorescence is measured for each individual cell passing the excitation source. These data are then used to examine a variety of biochemical, biophysical and molecular aspects of the cell (Jaroszeski and Heller, 1998).

Flow cytometry is a powerful tool in many scientific disciplines, including cancer research, cell biology and clinical diagnostics. It is particularly useful in biological investigations as it allows both qualitative and quantitative examination of whole cells, or constituents thereof, that have been labelled with a wide range of commercially available reagents, including dyes. Additionally, large quantities of cells can be analysed in a matter of seconds, allowing for the quantitative analysis of many samples daily (Jaroszeski and Heller, 1998).

7.1.3 OBJECTIVES

The purpose of this investigation was to ascertain whether the AF was able to induce apoptosis in normal human lymphocytes that had been exposed to the mycotoxins AFB_1 and FB_1, whilst not adversely affecting these cells on its own.

7.2 MATERIALS AND METHODS

7.2.1 MATERIALS

The Annexin–V–Fluos kit was purchased from Roche, SA. The blood vials were supplied by Endomed, SA. All other reagents were obtained from suppliers as previously described.

7.2.2 METHODS

7.2.2.1 Sample Preparation

Normal human lymphocytes were isolated from freshly drawn whole blood via the Histopaque method described in Chapter 4 and used immediately.

The cells were treated overnight (12 hours) in the culture environment with selected AF (100μg/ml), AFB_1 (50μM) and FB_1 (5μM) concentrations, individually and in combination. In addition, the ethanol and AFB_1 solvent vehicle controls were performed individually and in combination. Untreated lymphocytes were used as a control to assess the amount of endogenous apoptosis and necrosis occurring in the samples. All dilutions were prepared as described in previous chapters and the experiment was conducted in triplicate.

Following incubation, the lymphocytes were pelleted via centrifugation in a bench top centrifuge for 10 minutes at room temperature and the media was discarded. The pellets were washed with PBS (1000μl) and recentrifuged for 10 minutes at room temperature. The supernatant was discarded and lymphocyte pellets were resuspended in fresh PBS (700μl).

7.2.2.2 The Annexin–V–Fluos Assay

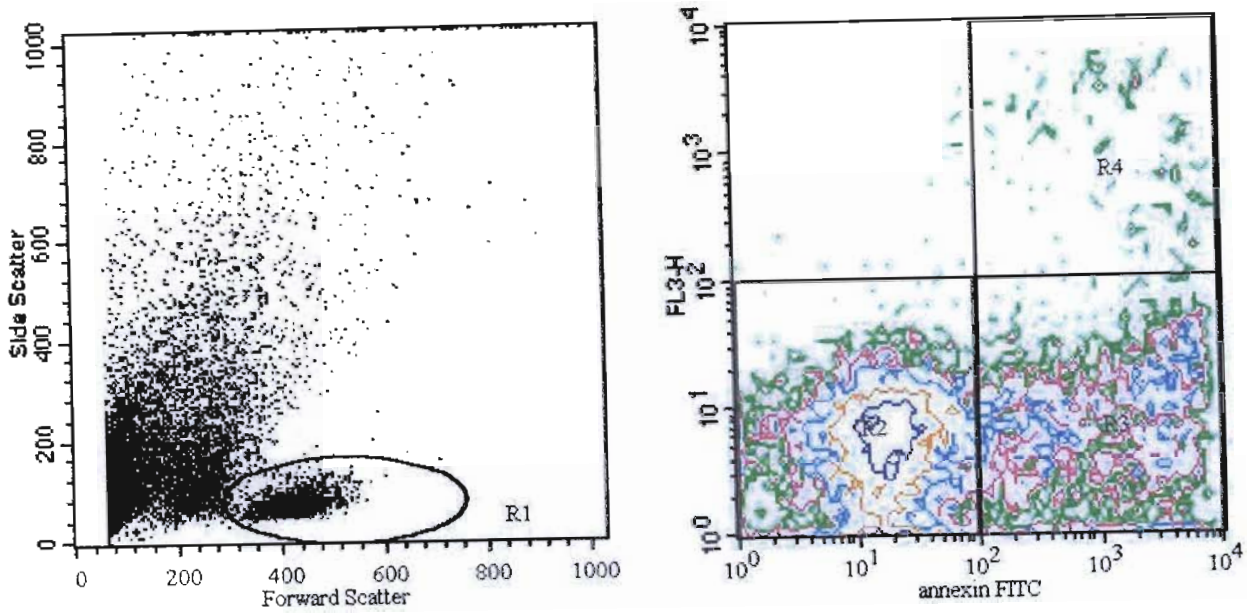
The lymphocytes were treated with the Annexin–V–Fluos labelling solution (100µl; Appendix 6) and incubated in the dark for 15 minutes at room temperature. Following this, the samples were vortexed and incubation buffer (400µl) was added to each to increase the volume available for analysis. Flow cytometric analysis of all samples was conducted using a BD FACSCalibur flow cytometer (BD Biosciences, SA). The excitation wavelength was set at 488nm. A 515nm bandpass filter was utilised for fluorescein detection and a filter greater than 560nm was used for PI detection.

As mentioned previously, the PI was included in the labelling solution to allow for the discrimination between apoptotic and necrotic cells. Apoptotic cells will stain positive for annexin only, whilst necrotic cells will stain both annexin and PI positive and dead cells will take up only the PI stain.

Data was acquired using Cell Quest Pro software. For each treatment, 100 000 events were recorded and the percentage of necrotic cells, percentage of apoptotic cells and percentage of viable cells was determined. The Student's T–Test was performed on all data to determine significant differences between test samples and their corresponding controls. The level of significance was set at $p < 0.05$ and all calculations were performed using Microsoft Excel for Windows 98. The results shown are means of the three separate experiments.

7.3 RESULTS

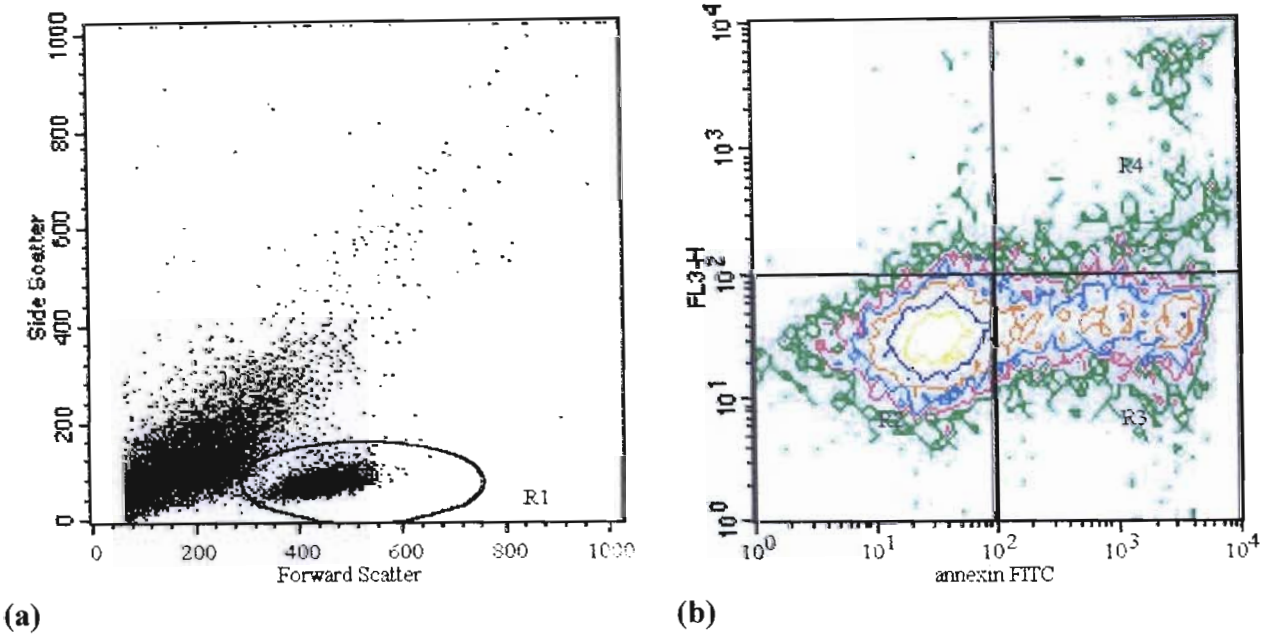
The data generated by flow cytometric analysis is represented as graphs known as histograms. Examples of the histograms obtained for the untreated lymphocyte control as well as a selected sample are shown in Figures 7.2 and 7.3.



(a) (b)

Figure 7.2: **a) Dot plot obtained for untreated lymphocytes indicating the gated population selected for analysis (R1).**

b) Contour plot obtained for untreated lymphocytes showing the distribution of cells according to their staining pattern.



(a) (b)

Figure 7.3: **a) Dot plot obtained for lymphocytes following overnight incubation with the acetone fraction (100µg/ml) indicating the gated population selected for analysis (R1).**

b) Contour plot obtained for lymphocytes following overnight incubation with the acetone fraction (100µg/ml) showing the distribution of cells according to their staining pattern.

In Figures 7.2a and 7.3a, R1 represents the population of cells that was gated for analysis. In Figures 7.2b and 7.3b, the R2 quadrant represents cells that were annexin negative and PI negative and these were referred to as viable cells or cells that were alive. The apoptotic cells are represented in the R3 region, as these cells only took up the annexin stain and not the PI. The R4 area indicates the population of cells that were PI positive and annexin positive and these are therefore classed as necrotic cells.

The response of the lymphocytes to overnight treatment with the AF (100μg/ml), AFB_1 (50μM) and FB_1 (5μM), as well as the EtOH and EtOH+AFB_1 solvent controls, individually and in combination are summarised in Table 7.1. The results are represented as the percentage of necrotic, apoptotic and viable cells in each of the treatment groups.

Table 7.1: Effect of the acetone fraction, ethanol control, aflatoxin B_1 and fumonisin B_1 individually and in combination on the levels of apoptosis and necrosis in normal human lymphocytes following overnight incubation.

TREATMENT TYPE	% NECROTIC	% APOPTOTIC**	% VIABLE
UNTREATED CONTROL	2.27	25.1	72.22
AF ONLY	5.31✦	25.84✦	66.83✦
AFB_1 ONLY	2.16	28.6✦	68.77✦
AF+AFB_1	7.45✦	25.2✦	65.23
ETOH ONLY	2.83	27.27✦	69.51✦
AFB_1 (S)* ONLY	2.06	30.15✦	67.12✦
EtOH+AFB_1 (S)*	2.68	30.56✦	65.92✦
FB_1 ONLY	2.31	28.9✦	68.2✦
AF+FB_1	6.3✦	26.54✦	65.34✦

** The % apoptosis levels throughout all treatments are above average. This was due to the lymphocyte donor being on medication for influenza infection for the duration of the experiment

* (S) = EtOH+DMSO as described in Chapter 5

✦ Student's T-Test: $p < 0.05$ (Significance to control)

The AF (100µg/ml) induced increased necrotic cell death in the lymphocytes with a corresponding decrease in the level of viable cells and little deviation from the level of apoptosis observed in the untreated control. This effect was not due to the solvent vehicle, as the ethanol control gave a similar response to the untreated control, exhibiting the second highest level of viable cells after the untreated control (Table 7.1).

The AFB_1 only treatment demonstrated increased apoptosis (28.6%) and decreased necrosis (2.16%) as compared with the untreated control (25.1% and 2.27% respectively). This trend was observed for the AFB_1 solvent vehicle control also, however, the induction of apoptosis was higher (30.15%) than seen in the toxin only treatment, with a resultant decrease in viable and necrotic cells (Table 7.1).

The treatment with the AF and AFB_1 together (Table 7.1) demonstrated a dramatic increase in necrotic cell death (7.45%), an effect noted to be additive when observing the values for the individual treatments (5.31% and 2.16% respectively). This resulted in a decreased number of viable cells (65.23%), with levels of apoptosis (25.2%) remaining largely unchanged from the untreated control. Compared with the combination solvent control, the values for viable cells were similar. The increased apoptosis evident in the combination solvent treatment can be attributed to the AFB_1 solvent only. Levels of necrotic cells were greatly reduced in the combination solvent treatment compared to the AF and AFB_1 treatment, due to the increased apoptosis (Table 7.1).

The two FB_1 treatments (individual and in combination with the AF) exhibited increased apoptosis compared to the untreated lymphocytes (Table 7.1). The FB_1 only treatment showed the highest levels of apoptosis (28.9%) and the treatment in combination with the AF demonstrated increased apoptosis compared to the AF only control (26.54% and 25.84% respectively).

The AF and FB_1 treatment also exhibited increased necrotic cell death (6.3%), induced by the AF, although the effect was not as pronounced as for AF with AFB_1 (7.45%; Table 7.1). These effects were accompanied by a decrease in the percentage of viable cells, due to the combined increase in apoptosis, induced by the FB_1, and necrosis, induced by the AF.

7.4 DISCUSSION

The AF (100μg/ml) was observed to increase necrotic cell death in the lymphocytes. This trend was continued in the treatments with both AFB_1 and FB_1, with the effects being additive in the case of aflatoxin. This effect was independent of ethanol solvent effects, as the values for this treatment were similar to those of the untreated control. The AFB_1 solvent vehicle control demonstrated increased apoptosis, which may be attributed to the DMSO constituent of this solvent system, as the ethanol alone did not demonstrate such effects (Table 7.1).

Although the AF induced necrosis rather than apoptosis in the lymphocytes, the level of induction was not severe enough to raise concern regarding its potential use as a chemotherapeutic agent, as these treatments displayed acceptable levels of viable cells. Indeed, the observation that the AF treatments in combination with the toxins yielded additive effects is promising. Apoptosis is a reversible process and thus cells may recover from the initial stages, allowing affected cells to progress through the cell cycle and proliferate. Necrosis, however, is not reversible and the affected cell will be killed and removed from the body.

7.5 CONCLUSION

The AF acted to remove cells that have been damaged by the effects of two mycotoxins with different mechanisms of toxicity, one a known tumour initiator and the other a tumour promoter. This activity warrants further investigation to elucidate the mechanisms of action of the active compounds in the AF.

CHAPTER EIGHT

CONCLUSION

This study was undertaken as a pilot project to determine whether the *Amaranthus lividus* imifino plant warranted investigation with regards to possible chemopreventive activity. The selection of this plant was based on epidemiological observations. It is a dietary component of many populations at risk of exposure to dietary carcinogens. The plant is robust and grows readily in the wild or in domestic gardens without requiring specialised agricultural tending hence, it is readily availability to rural and lower income groups at risk.

The results of the initial cytotoxic profiling showed that the plant fractions did not exhibit high cytotoxicity towards the cancerous SNO oesophageal cell line *in vitro*. The majority of chemopreventive studies consider cancerous cell death induced by potential agents to be indicative of chemopreventive activity. However, this is not a pre–requisite for potential agents and the lack of toxicity with regards to the normal human lymphocytes was very encouraging. Fractions that were highly toxic towards the normal cells would not have been suitable for further testing.

The results of the co–treatment of the cancerous cells with the plant fractions and the two important dietary carcinogens AFB_1 and FB_1 showed that both the water fraction and the acetone fraction counteracted the toxic activity of the aforementioned toxins. This is seen as a significant finding and deserving of further investigation, especially since the toxins possess differing mechanisms of action and the effects were noted in cancerous cell lines derived from two different tissue types.

In light of the anti–mycotoxigenicity results obtained for the co–treatment of SNO cells with the water fraction and FB_1, the interaction between FB_1 and water–soluble nutrients, such as sugars and amino acids, should be investigated further. The results of the present study raise questions concerning the ability of these substances to modify FB_1 into inactive derivatives. It may be hypothesised that these reactions occurred in the growth medium, either preventing FB_1 from accessing the cells or rendering it inactive if entry was gained. An alternative hypothesis is that these reactions are enzyme–mediated and occur intracellularly.

Long–term studies using non–transformed (normal) cell lines to elucidate the anti–neoplastic or inhibitory effects of the plant fractions would be preferable to studies utilising cancerous cells. However, non–transformed cells are more difficult to culture for prolonged periods and have specialised, costly growth requirements. Ideally, the elucidation of the full chemopreventive capacity of the *Amaranthus lividus* plant would require studies in appropriate animal models. This would incorporate the entire physiology of the animal and would additionally allow for the initial evaluation of potential deleterious side effects of the active fractions.

The MTT assay is suitable for rapid pre–screening of the crude extracts for cytotoxic as well as anti–mycotoxigenic activity. However, further testing to confirm the results obtained should be conducted using other methods, such as the trypan blue dye exclusion assay or the brdU incorporation assay.

In addition, fractionation of the water and acetone fractions with subsequent testing of the resultant sub–fractions would allow for the isolation and identification of the active compounds in these crude fractions. These compounds could then be utilised in further studies to elucidate the precise mechanisms of chemoprevention and permit a more rigorous evaluation of the overall chemopreventive capacity of this plant.

Glutathione is the largest component of the endogenous thiol buffer system and the body's primary antioxidant. It protects cells from oxidative damage and is involved in detoxification reactions with xenobiotics, including AFB_1. As a potent antioxidant, GSH can protect the cell during both the initiation and promotion phases of carcinogenesis. In addition, increased intracellular GSH levels have been associated with increased cell proliferation, while GSH efflux from the cell has been reported as an early event during apoptosis. Thus, the induction of this important antioxidant by several acetone fraction concentrations is viewed as a significant result, worthy of further analysis.

Due to financial and time constraints, only the acetone fraction was evaluated using the GSH assay. However, Sharma *et al.* (1994) found that water–soluble compounds, such as nicotinic acid and vanillin induced GSH production. Thus, the water fraction should also be investigated in this regard, especially in view of the positive results obtained for the anti–mycotoxigenic analysis.

Research is currently being conducted with regards to the molecular mechanisms of chemoprevention, especially the modulation of phase I and II metabolism. Such research would shed further light on the mechanisms of activity exerted by these plant fractions. However, this approach is unsuited to the third world scenario due to the exorbitant costs involved.

In conclusion, the results presented in this manuscript indicate that the indigenous herb, *Amaranthus lividus*, has chemopreventive properties. Although this plant is already widely consumed, many people are not aware of the potential of this plant with regards to improving health. Thus, the community at large needs to be educated about the benefits of consuming this herb on a regular basis.

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APPENDICES

APPENDIX 1

COMPLETE CULTURE MEDIUM

Adherent Cell Lines (SNO and A549)

Eagles Minimal Essential Medium (EMEM) was supplemented with 2% foetal calf serum (FCS), 1% L–Glutamine and 1% antibiotic (PenStrep–Fungizone). Heat inactivated foetal calf serum was purchased and was filtered into the media using a 0.45μm millipore filter. In addition, the EMEM purchased contained HEPES buffer, as the incubator used did not have a CO_2 facility. All media and components were purchased from Adcock Ingram, SA.

Normal Human Lymphocytes

Roswell Park Memorial Institute (RPMI–1640) medium was supplemented with 10% heat inactivated FCS, 1% L–Glutamine and 1% PenStrep–Fungizone. The additional FCS was required to stimulate cell growth, as normal cells do not survive as readily as cancerous cell lines do. Again, the medium contained HEPES buffer, due to the lack of a CO_2 incubator.

APPENDIX 2

PHOSPHATE BUFFERED SALINE (pH 7.4)

Preparation of Stock Solutions

A. 0.2M Sodium Dihydrogen Orthophosphate

NaH_2PO_4

Molecular weight: 156.01g/mol (MERCK)

Dissolved 3.12g sodium dihydrogen orthophosphate in 100ml distilled water.

B. 0.2M Disodium Hydrogen Orthophosphate Anhydrous

Na_2HPO_4

Molecular weight: 141.96g/mol (MERCK)

Dissolved 2.84g disodium hydrogen orthophosphate anhydrous in 100ml distilled water.

C. Dissolve 8.5g sodium chloride (MERCK) in 1000ml distilled water.

Preparation of 0.1M PBS (pH 7.2)

Stock A:	16ml
Stock B:	34ml
Stock C:	50ml
Total =	**100ml**

APPENDIX 3

PHOSPHATE-EDTA BUFFER

A. 0.1M Disodium Hydrogen Orthophosphate Anhydrous

Na_2HPO_4

Molecular weight: 141.96g/mol (MERCK)

Weigh out 7.098g Na_2HPO_4 for 500ml.

B. 0.005M Ethylenediaminetetra-Acetic Acid Disodium Salt

$[(CH_2.NCCH_2.COOH)CH_2COONa]_2.2H_2O$

Molecular weight: 372.24g/mol (MERCK)

Weigh out 0.9306g EDTA for 500ml.

The appropriate amount of each component was weighed out, transferred into in a volumetric flask and made up to just below the mark with double distilled water. Once the components had completely dissolved via mixing with a magnetic stirrer, the pH was adjusted to approximately 8.0 and the volume made up to the mark with double distilled water. The buffer was stored in the refrigerator, but was brought to room temperature before use. The pH was analysed before each use and adjusted if necessary.

APPENDIX 4

MOBILE PHASE USED FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

0.1M Sodium Acetate Buffer

Molecular weight: 136.08g/mol

The appropriate amount was weighed out, transferred into a volumetric flask and half the volume of double distilled water was added. The pH was adjusted to pH 3.8 by the drop–wise addition of glacial acetic acid. Thorough mixing was maintained throughout the adjustment using a magnetic stirrer and the adjustment was conducted in the fume cupboard. Double distilled water was then added up to the mark.

This solution was filtered using a millipore vacuum system and the pH was re–analysed, before the final mobile phase of one part buffer, one part HPLC grade methanol was made up. The mobile phase was degassed via sonication for 20 minutes prior to use.

APPENDIX 5

REDUCED GLUTATHIONE STOCK SOLUTIONS

Glutathione Stock Standard

A 200µg/ml stock solution was prepared fresh daily in room temperature phosphate–EDTA buffer. The reduced glutathione was purchased from Roche, SA.

Working Glutathione Standard

A 1:100 dilution in phosphate–EDTA buffer was performed on the stock GSH solution to provide the 2µg/ml working standard solution used for the preparation of the standards in Table 6.1.

APPENDIX 6

ANNEXIN–V–FLUOS LABELLING SOLUTION

The labelling solution comprised annexin–V–fluorescein solution (20µl), propidium iodide solution (20µl) and incubation buffer (1000µl), as per manufacturers instructions.

All solutions were supplied with the Annexin–V–Fluos Staining kit, purchased from Roche, SA.