# DURBAN INSTITUTE OF TECHNOLOGY

# CHEMOPROTECTIVE ACTION OF NATURAL PRODUCTS ON CULTURED HUMAN EPITHELIAL CELLS EXPOSED TO AFLATOXIN $B_1$

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# CHEMOPROTECTIVE ACTION OF NATURAL PRODUCTS ON CULTURED HUMAN EPITHELIAL CELLS EXPOSED TO AFLATOXIN $B_1$

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## AUTHOR'S DECLARATION

This study presents the original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology, Faculty of Engineering, Science, and the Built Environment, Durban Institute of Technology, South Africa, under the supervision of Professor B. Odhav.

_____

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Professor B. Odhav (Ph.D.) Date

Supervisor

## DEDICATION

*I dedicate this work to:*

the sources of my inspiration, my parents, my husband, my two sons and my beloved Guru and God Bhagawan Sri Sathya Sai Baba.

## PUBLICATIONS ARISING FROM THIS DISSERTATION

**Peer-reviewed articles:**

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## ABBREVIATIONS

| | |
|---|---|
| AFB | aflatoxin B |
| $AFB_1$ | aflatoxin $B_1$ |
| $AFB_2$ | aflatoxin $B_2$ |
| $AFG_1$ | aflatoxin $G_1$ |
| $AFG_2$ | aflatoxin $G_2$ |
| $AFH_1$ | aflatoxin $H_1$ |
| AFL | aflatoxicol |
| $AFM_1$ | aflatoxin $M_1$ |
| $AFP_1$ | aflatoxin $P_1$ |
| AFQ | aflatoxin quercetin |
| $AFQ_1$ | aflatoxin Q1 major *in vitro* microsomal metabolite produced by human and monkey liver |
| AIDS | acquired immune-deficiency syndrome |
| ATCC | American Type Culture Collection |
| ATP | adenosine triphosphate |
| $A_w$ | water activity |
| BHT | butylated hydoxytoluene |
| BSA | bovine serum albumin |
| CCM | complete culture medium |
| DMEM | Dulbecco's modified eagle's medium |
| DMFO | α-difluoromethylornithine |
| DMSO | dimethylsulphoxide |
| EGFR | epidermal growth factor receptor |

| | |
|---|---|
| ELISA | enzyme-linked immunosorbent assay |
| ER | endoplasmic reticulum |
| FAO | Food and Agriculture Organisation |
| FAPY | formamidopyrimidine |
| FCS | foetal calf serum |
| GCE | glycine cleavage enzyme |
| GC-TA | guanine cytosine-thymine adenine |
| GGPP | geranylgeranyl phosphate |
| GST | glutathione S-transferase |
| G-T | guanine-thymine |
| HBSS | Hank's balanced salt solution |
| HBV | hepatitis B virus |
| HCC | hepatocellular carcinoma |
| HCI | hydrochloric acid |
| HIV | human immuno-deficiency virus |
| HPLC | high performance liquid chromatography |
| HRP | horseradish peroxidase |
| IARC | International Agency for Research on Cancer |
| Ip | intraperitonial |
| MTT | (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) |
| NADPH | nicotinamide dinucleotide phosphate (reduced form) |
| NK | natural killer |
| NRBM | National Repository for Biological Materials |
| NSAID | non-steroidal anti-inflammatory drug |
| NSL | nuclear location sequence |

| | |
|---|---|
| OH | hydroxyl |
| OTA | ochratoxin A |
| PBS | phosphate buffered saline |
| PLC | primary liver cancer |
| PNPD | p-nitrophenyl phosphate in diethanolamine |
| Q | quercetin |
| QPA | quercetin pentaacetate |
| ROS | reactive oxygen species |
| SSB | single strand breaks |
| TdT | terminal deoxynucleotidyl tranferase |
| TEM | transmission electron microscopy |
| THF | tetrahydrofuran |
| TLC | thin layer chromatography |
| TNFα | tumour necrosis factor-alpha |
| UV | ultraviolet |
| XTT | (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) |

## ABSTRACT

Aflatoxins are mycotoxins that are produced by some species of *Aspergillus* which contaminate agricultural crops. Exposure to aflatoxin $B_1$ ($AFB_1$) has been associated with primary human hepatocellular carcinoma (HCC). Cancer is caused by alterations to DNA in one or more forms and these relate to disturbances in the normal processes of cell division. Some of these alterations include the inhibition of DNA synthesis, DNA fragmentation indicative of apoptosis, and DNA adducts, all of which have been shown to occur both *in vivo* and *in vitro*. Chemotherapeutic products from natural sources have novel mechanisms of action, and some are potential inhibitors of cancer formation. One group of natural products, carotenoids, occurring in abundance in fruits and vegetables are powerful natural antioxidants, and have been shown to inhibit various types of cancers.

This study was undertaken to investigate the cellular and molecular mechanisms by which beta-carotene and lycopene prevent the $AFB_1$-induced toxic changes in human hepatocytes. Initial experiments involved optimizing an *in vitro* system to test the chemoprotective effects of lycopene and beta-carotene on HepG2 cells exposed to different concentrations of $AFB_1$. Mitochondrial activity, cell viability and reactive oxygen species (ROS) were measured using enzyme assays and flow cytometry. Morphological changes were examined using phase contrast light and transmission electron microscopy. The mechanism of cell death, i.e. apoptosis was determined using the TdT-FragEL DNA fragmentation kit to detect apoptotic bodies as genomic markers. Genetic changes were studied by measuring $AFB_1$-$N^7$-guanine adduct formations using an indirect competitive enzyme-linked immunosorbent assay (ELISA) and the levels of *p53* tumour suppressor gene protein was measured using a pantropic ELISA.

Results showed that in the presence of $AFB_1$ the cells exhibited a significant decrease in mitochondrial activity and an increase in reactive oxygen species (ROS) was generated as compared to normal untreated cells. Ultrastructurally these cells showed mitochondrial damage, nuclear condensation and a loss in cell-to-cell contact indicating dysfunctional gap junctions and a loss in cell-to-cell communication. At the genomic level, $AFB_1$ produced $AFB_1$- $N^{7-}$guanine adducts, caused cell death due to apoptosis and a suppression in the level of *p53* protein. In cells pretreated with lycopene and beta-carotene, protection from the toxic effects of $AFB_1$ at both the cellular and molecular levels was observed.

The toxicity of $AFB_1$ in HepG2 cells is was prevented by both lycopene and beta-carotene by the inhibition of $AFB_1$-protein binding as indicated by the relative increase in mitochondrial activity and cell viability and a decrease in ultrastructural damage. The carotenoids also showed their powerful antioxidant by reducing the levels of ROS in the cells. Protection against $AFB_1$-induced carcinogenicity, i.e. $AFB_1$-DNA binding, was indicated by a decrease in apoptosis, an increase in p53 protein, and a decrease in $AFB_1$-guanine adducts.

In keeping with the mitochondrial activities measured earlier it appeared that the cells go into apoptosis along the mitochondrial pathway. Beta-carotene is converted to retinal in the cytosol and thus may compete with $AFB_1$, thereby reducing the chance of $AFB_1$ activation and thus inducing apoptotic cell death. The p53 protein measurements showed that both beta-carotene and lycopene upregulated the p53 *gene* product. The *p53* tumour suppressor gene can induce cell cycle arrest to allow DNA repair or undergo apoptosis. Beta-carotene and lycopene exert their influence by diverting the highly reactive electrophilic $AFB_1$-8,9-epoxide to undertake an alternate pathway.

# CHAPTER 1

# BACKGROUND AND AIM OF THE STUDY

Previous studies indicate that a mutation in the non-oncogenic *p53* gene is epidemiologically linked to human HCC (Ozturk, 1991; Chan *et al*., 2003). Hsu *et al*. (1991) found this link in Chinese, South African and Asian patients and Hollstein *et al*. (1993) found the same gene mutation in Taiwanese patients. The incidence of these aberrations is reported to be about 20-50% in HCC's (Kishimoto *et al*., 1997).

There is sufficient evidence to indicate that carotenoids in addition to their well known antioxidant properties (Paiva and Russel, 1999), also affect intercellular communication, immune responses, neoplastic transformations and growth control, and cellular levels of enzymes that detoxify carcinogens (Zhang *et al*., 1991; Brockman *et al*., 1992; Pryor *et al*., 2000). To date studies carried out have used the rat (Foote *et al*., 1970; Gradelet *et al*., 1998) and the mule duckling model (Cheng *et al*., 2001) to show the protective effect of these carotenoids against $AFB_1$ exposure. Of the well known carotenoids, lycopene and beta-carotene occur in abundance in fruits and vegetables and are safe for human consumption.

Aflatoxin $B_1$ frequently induces mutations of the *p53* gene which is linked to HCC. Although there is much evidence from epidemiological studies linking the beneficial aspects of carotenoids to the prevention of cancer, the cellular and molecular mechanisms need to be understood in order to implement large scale intervention strategies to prevent $AFB_1$ induced carcinoma.

The use of chemical or dietary interventions to alter the susceptibility of humans to the actions of carcinogens and to block, retard or reverse carcinogenesis is an emerging chemoprotective strategy for disease prevention (Abdulla and Gruber, 2000; Kensler *et al*., 2003; Bingham and Riboli, 2004). Chemoprotection by natural products involves maintaining

cellular integrity, preventing DNA alterations, activation of p53 suppressor protein and apoptosis.

The aim of this study was thus to investigate the cellular and molecular mechanisms by which beta-carotene and lycopene may prevent the $AFB_1$-induced toxic changes in human hepatocytes. In order to achieve this aim, the following objectives were set out:

i. To optimise an *in vitro* system for the evaluation of $AFB_1$ damage to cultured hepatocytes.
ii. To determine the biochemical protection offered by beta-carotene and lycopene to $AFB_1$-exposed hepatocytes, by measuring the mitochondrial activity, cell viability and ROS levels using appropriate enzyme assays and flow cytometry.
iii. To determine the cellular protection offered by beta-carotene and lycopene to $AFB_1$-exposed hepatocytes, by studying the morphological changes at the structural and ultrastructural levels using phase contrast light and electron microscopy respectively.
iv. To determine the molecular protection offered by beta-carotene and lycopene to $AFB_1$-exposed hepatocytes, by detecting apoptotic bodies as genomic markers and measuring the levels of p53 protein and $AFB_1$-$N^7$-guanine adducts produced.

This study design may prove useful in determining the safety and medicinal value of the numerous indigenously grown plants of Southern Africa.

The limitations of this study are the following:

i. The study was carried out *in vitro* and therefore the results have to be extrapolated to human *in vivo* effects.

ii. The HepG2 cell line used in the study is a transformed cell line (human hepatocellular carcinoma) and may thus not exactly resemble the activity and structure of primary human hepatocytes, although it has most of the important enzyme systems intact.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 INTRODUCTION TO AFLATOXINS

### 2.1.1 Production of aflatoxins

Aflatoxins are a group of mycotoxins belonging to the aromatic polyketides group (secondary metabolites of fungi) that were first discovered with the outbreak of the Turkey X disease in 1960 in Europe (Eaton and Groopman, 1994). Over the past thirty years more than 8 000 articles describing the exposure, toxic effects and mechanisms of action of aflatoxins have been published. From this information, the International Agency for Research on Cancer (IARC) classified $AFB_1$ as a group 1 human carcinogen (IARC, 1987). Aflatoxins may enter the human dietary system by direct or indirect contamination of foods. Direct contamination occurs when the food becomes infected with a toxigenic fungus that produces the toxin. Indirect contamination occurs by the consumption of animal products which are themselves contaminated.

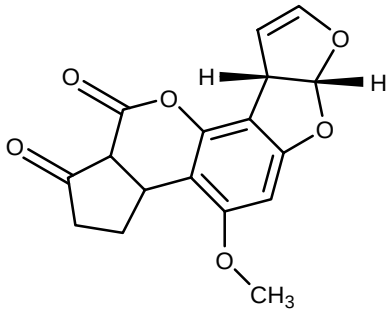
Aflatoxins are produced by two species of fungi, namely, *Aspergillus flavus* and *Aspergillus parasiticus*. Both species grow at a wide range of temperatures (10°C to 43°C) with an optimum temperature of 33°C. The optimum water activity ($a_w$) for growth and aflatoxin production is near 0.99 and the optimum pH is near 6 (McLean and Dutton, 1995).

*A. flavus* produces $AFB_1$ and aflatoxin $B_2$ ($AFB_2$) only, while *A. parasiticus* produces all four naturally occurring aflatoxins, namely, $AFB_1$, $AFB_2$, aflatoxin $G_1$ ($AFG_1$) and aflatoxin $G_2$ ($AFG_2$). These are structurally represented in Figure 2.1. Of these aflatoxins, $AFB_1$ has been found to be the most potent toxin as it poses a quadruple threat: as a potent toxin, mutagen, a teratogen and a group 1 carcinogen (IARC, 1987; Ueno and Ueno, 1978). According to Ames

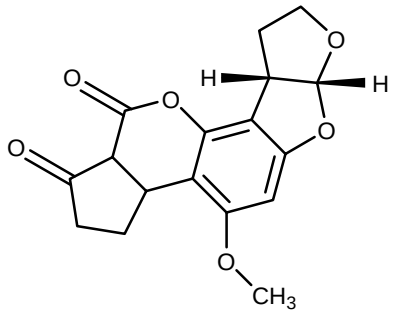
*et al*. (1990) only dioxins significantly exceed $AFB_1$ in its potency. Using $TD_{50}$ parameters $AFB_1$ is found to be 1 000 times more potent as a carcinogen than benzo pyrene (Eaton *et al*., 1994).

Aflatoxin $B_1$ has a molecular weight of 312 g/mol, a melting point of 267°C and is freely soluble in moderately polar solvents like dimethylsulphoxide (DMSO), chloroform and methanol (Betina, 1989). This compound is stable at high temperatures with little or no disruption occurring under ordinary cooking conditions or during pasteurization. The presence of the lactone ring in its structure makes the aflatoxin molecule susceptible to alkaline hydrolysis (McLean and Dutton, 1995).

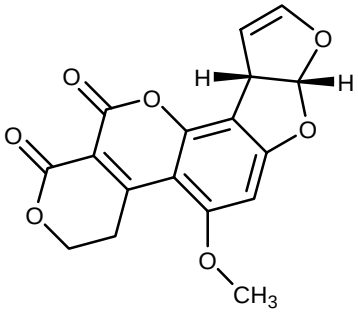
The aflatoxin structures contain a bisfuran unit fused to an aromatic ring, and their remarkably complex biosynthetic origin begin with a poly-β-keto chain. After undergoing Baeyer-Villiger oxidation (involving the migration of an alkyl group from the ketone *via* cytochrome P450 or FAD-dependent enzymes to form esters), versicolorin A and B are produced as intermediates. A second Baeyer-Villiger oxidation produces a dihydrobisfuran system, sterigmatocystin. Aflatoxin $B_1$ formation requires oxidative cleavage of an aromatic ring in sterigmatocystin. Aflatoxin $G_1$ is derived by further modification of $AFB_1$, cleaving the cyclopentenone ring and forming a lactone, perhaps *via* a further Baeyer-Villiger reaction (Dewick, 2002).



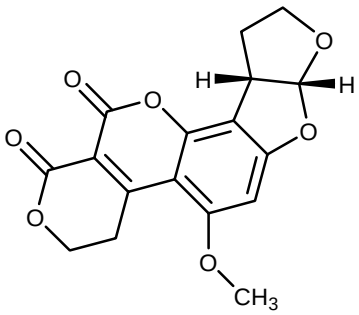
AFLATOXIN B1



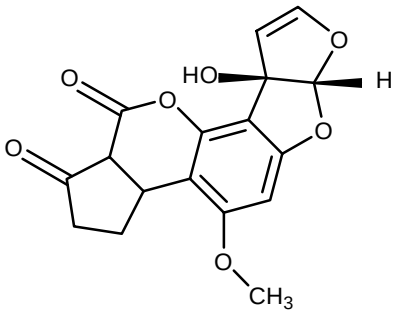
AFLATOXIN B2



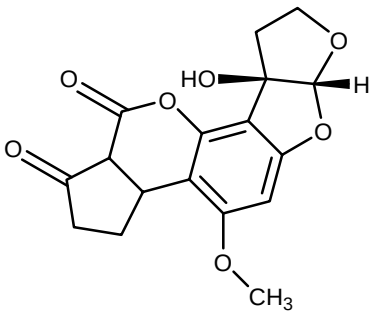
AFLATOXIN G1



AFLATOXIN G2



AFLATOXIN M1



AFLATOXIN M2

**Figure 2.1 Structures of naturally-occurring aflatoxins ($B_1$, $B_2$, $G_1$ and $G_2$) and their metabolites $AFM_1$ and $AFM_2$.**

### 2.1.2 Epidemiology

#### 2.1.2.1 Incidence

Aflatoxicosis is poisoning that results from ingestion of aflatoxins in contaminated food or feed. Food products susceptible to aflatoxin contamination are cereals (maize, sorghum, pearl millet, rice and wheat), oilseeds (groundnut, soybean, sunflower and cotton), spices (chilli, black pepper, turmeric, coriander and ginger), nuts (almond, pistachio, walnut, coconut and peanuts) milk and milk products (FAO Food and Nutrition Paper, 2001). The most common food type contaminated is vegetable oil and fat followed by peanuts and peanut products. **Figure 2.2** below indicates the incidence of aflatoxins from 1998 to 2000 in the USA.

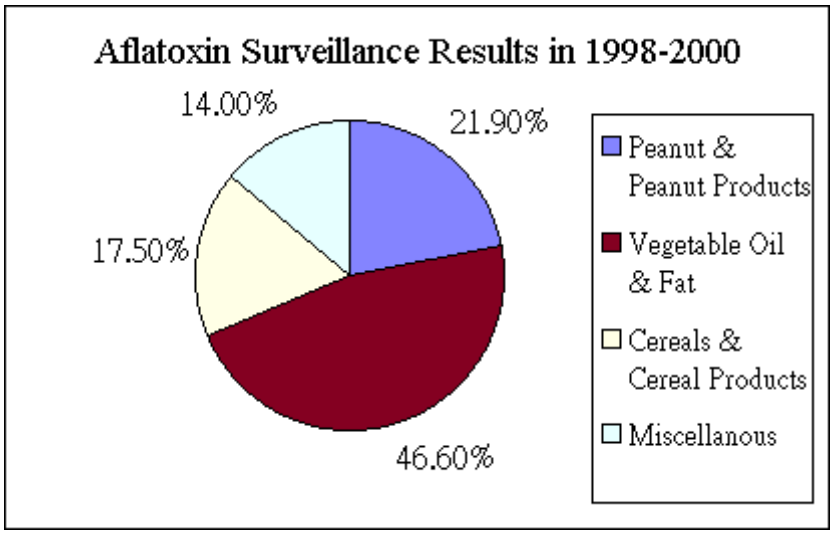



**Figure 2.2 Incidence of aflatoxin in plants during 1998-2000 in USA (Food and Environmental Hygiene Department, 2001).**

The historical incidence of aflatoxin diseases in plants, animals and humans has been recored since since 1959 (**Table 2.1)**. Aflatoxin poisoning is reported from all parts of the world in

almost all domestic and non-domestic animals like cattle, horses, rabbits, and non-human primates. Aflatoxicoses is reported in humans in many parts of the world and is also associated with other serious diseases. In 1974, 379 people died in north west India of 55 μg/kg body weight daily intake of $AFB_1$ (Krishnamachari *et al*., 1975). In 1982, human mortality resulted from 38 μg/kg body weight intake of $AFB_1$ in Kenya (Ngindu et al., 1982). Experiments conducted in high-risk areas, such as China and Africa, show a high incidence of hepatitis B virus infection where dietary exposure to aflatoxins is prevalent. Subsequent research proved that both aflatoxins and hepatitis B virus act synergistically in the aetiology of liver cancer (Groopman & Kensler 1996; Montesano *et al*., 1997). Other studies show a significant correlation among the aflatoxin exposure during neonatal stages, and stunted growth in children of Africa (Gong *et al*., 2002). Apart from this, the capacity of aflatoxins to cross the placental barrier and cause genetic defects at foetal stages has been described by Maxwell and others (1998). In low and intermediate risk areas, including Italy and India, alcohol and childhood cirrhosis are the most important risk factors for liver cancer. Aflatoxin causes malnutrition, loss of weight and contaminates breast milk (Groopman *et al*., 1988;McLean and Dutton, 1995). Lack of certain nutritional factors, e.g. protein or vitamin A, may predispose an individual to the toxic or even carcinogenic effects of $AFB_1$ (Decoudu *et al*., 1992). Aflatoxicoses has also been found to predispose other serious illnesses such as lung and breast cancer, human immuno-deficiency virus (HIV), acquired immune-deficiency syndrome (AIDS) and Reye's syndrome.

The seriousness of aflatoxin is clearly illustrated by experiments described in CAST (2003). Six rat livers (**Figure 2.3**) were injected with increasing doses of $AFB_1$. The one at the upper left corner received no aflatoxin (control), while the one at the lower right corner received the highest dose. The livers show distinct colour changes indicating degeneration.

**Table 2.1 Historical incidences of aflatoxin diseases in plants, animals and humans.**

| Outbreak | Country | Plant | Animal | Human | Reference |
|---|---|---|---|---|---|
| 1956-1960 | Great Britain, USA, Brazil | Peanut meal | Poisoning of ducklings, pheasants, partridge poults and turkey; Turkey X disease | | Blount, 1961; Newberne *et al*., 1966 Smith and Moss, 1985; Eaton and Groopman, 1994 |
| 1960's-1970's | Southern and North-Western USA; India; Germany | Pre-harvest contamination of maize; processed foods: milk, dry milk, cheddar cheese | | Acute aflatoxin poisoning; lung cancer | Shank *et al.*, 1972; Van Rensberg *et al.*, 1974; Krishnamachari *et al.*, 1975; Peers *et al.*, 1976; Bhat and Krishnamachari, 1977; Dvorackova and Pichova, 1986; Bullerman, 1981; Ngindu *et al.*, 1982; Payne, 1998; Dvorackova *et al.*, 1981 |
| 1980's | South Africa; Mozambique, Transkei, Uganda, Swaziland, Kenya, China, Thailand, Philippines, Taiwan, India, South-East USA, New Zealand, Czechoslovakia, UK, Canada, France | Processed foods: peanut butter, peanut candies, roasted peanuts, corn products, spaghetti, wheat flour | | Reye's syndrome, primary liver cancer (PLC), HCC, colon cancer, kwashiorkor, breast-milk contamination, | Smith and Moss, 1985; Apeagyei *et al*., 1986; Jelanek, 1987; Zhu *et al*., 1988; Hendrickse and Maxwell, 1989; Wood, 1989; DeVries, 1989; Forrester *et al.*, 1990; Ozturk, 1991; Hsing *et al.*, 1991; Zhang *et al.*, 1991; Ross *et al.*, 1992; Kolars, 1992; Yap *et al.*, 1993; Stoloff, 1996; Maxwell *et al*., 1998; Galvano *et al.*, 2001; |

| | | | | | |
|---|---|---|---|---|---|
| | | | | reduced baby weight, intoxication during pregnancy, neonatal jaundice, prenatal death, HCC linked with HBV | Gong *et al.*, 2002; CAST, 2003 |
| 1990's | Africa, USA, South-East Asia | Pistachios, cottonseed, copra, corn, sorghum, wheat, rice, peanuts, oil-seed crops, groundnuts | Ducks, chickens, swine (pigs), quail, cattle, (pregnant sows), fish, dogs, trout, sheep, rats | Immuno-suppression linked to HIV infection, breast cancer | Harrison *et al.*, 1993; Denning *et al*., 1995; Dutton and Kinskey, 1995 |
| 2000-2004 | USA, Brazil | Corn, Brazil nuts | | | Wang *et al.*, 2001;White and Rocheford, 2003 |

HBV, Hepatitis B virus; HCC, hepatocellular carcinoma; HIV, human immuno-deficiency virus; PLC, primary liver cancer

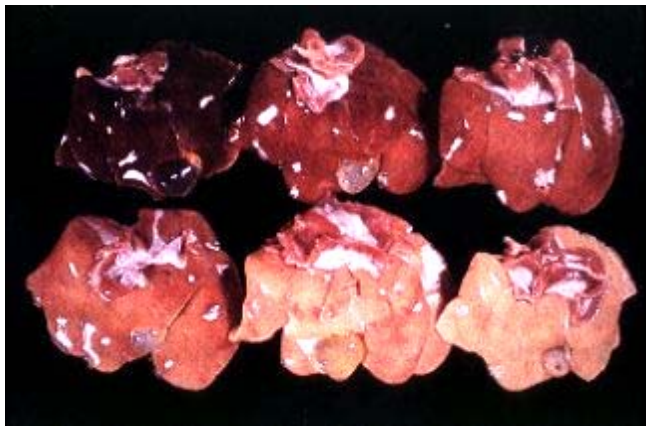


**Figure 2.3 Rat livers exposed to increasing doses of $AFB_1$ (CAST, 2003).**

2.1.2.2 Prevention and control of aflatoxins

Aflatoxin concentration in foodstuffs have been regulated by the United States since 1965 and since then of the 140 countries surveyed, 77 countries have regulated aflatoxins, 13 have no regulations and no regulatory information is available for 50 countries (CAST, 2003). Permitted levels appear to range from 2 ng/g to 35 ng/g in foodstuffs and up to 200 ng/g in animal feeds worldwide. In South Africa, the regulation limit imposed on all foods for total aflatoxins is 10 ng/g and for $AFB_1$ is 5 ng/g. The European Union regulation limit for $AFB_1$ is 2 ng/g in foods (CAST, 2003).

There are several possible methods that are used to control aflatoxins as described below. However, most of them are not practical or commercial. Aflatoxins can be controlled by physical methods such as mechanical separation, density segregation, thermal inactivation, irradiation and solvent extraction. Chemical methods of detoxification include ammoniation, treatment with bisulphate and ozonization (Park *et al*., 1988; Kubena *et al.*, 1991). Decreased bioavailability of aflatoxins has been achieved using clay and zeolitic minerals (Phillips *et al*., 1990). There are also reports on the inactivation of the toxin by biological competition

rendered by non-toxigenic strains of *Aspergillus* and bacteria (Bhatnagar *et al*., 1994). Chemoprotection of the toxin by certain nutrients in the diets is also reported.

In animals and humans, there are metabolic pathways that detoxify the aflatoxins and thereby prevent the activation of the toxins into harmful molecules. These metabolic pathways are discussed in section 2.2.2.

## 2.2 BIOCHEMICAL MECHANISMS AND IMPLICATIONS OF THE TOXICITY OF AFLATOXINS AS RELATED TO AFLATOXIN CARCINOGENESIS

### 2.2.1 Acute and chronic toxicity

Aflatoxin is acutely toxic to most animal species at high levels. The $LD_{50}$ value of $AFB_1$ for most young animals (ducklings, dogs, rabbits, guinea pigs, rainbow trout, etc.) is about 0.5 mg/kg of body weight. If this quantity is consumed, death of the animal will occur in about 72 h. If such animals are examined, it will be evident that they suffered from liver damage and hemorrhaging in the intestinal tract and peritoneal cavity (Eaton and Groopman, 1994). It is also reported (Marth, 1997) that most animals develop some resistance to the toxin, as they grow older. There is no toxicity value for humans, but epidemiological, clinical, and experimental studies reveal that exposure to large doses (>6.0 mg) of aflatoxin may cause acute toxicity with lethal effect (Eaton and Groopman, 1994). In the most severe case of acute poisoning of aflatoxin reported (Bhat and Krishnamachari, 1977) 25% of the exposed population died after ingestion of the moulded maize with aflatoxin levels ranging from 6.25 mg/kg to 15.6 mg/kg.

Following ingestion, the aflatoxins rapidly reach the liver via the porto-hepatic circulation. As a result the liver becomes the primary target organ. The symptoms of acute hepatic toxicity are described as follows:

i) After the invasion of aflatoxins into the liver, lipids infiltrate hepatocytes.
ii) Aflatoxin metabolites react negatively with different cell proteins.
iii) This leads to inhibition of carbohydrate and lipid metabolism and protein synthesis (decrease in liver function).
iv) There is a derangement of the blood clotting mechanism, icterus (jaundice), and a decrease in essential serum proteins synthesized by the liver.
v) This leads to necrosis or liver cell death.
vi) Other general signs are oedema of the lower extremities, abdominal pain, and vomiting.

Chronic toxicity is due to long-term exposure of moderate to low levels of aflatoxin. The symptoms include decrease in growth rate, lowered milk or egg production, and immuno-suppression. Sometimes, carcinogenicity is also observed. The liver is the main target organ and damage manifests itself as colour that is characteristic of jaundice. The gall bladder appears swollen. Immuno-suppression is due to the reactivity of aflatoxins with T-cells, decrease in vitamin K activities, and a decrease in phagocytic activity in macrophages. These immuno-suppressive effects of aflatoxins predispose the animals to many secondary infections due to other fungi, bacteria and viruses (Robens & Richard, 1992; McLean and Dutton, 1995).

### 2.2.2 Metabolism of aflatoxin in the liver

Aflatoxin $B_1$ requires metabolic activation to exert its toxicity. This activation takes place in the liver. The toxicity of the metabolite may be modified by the induction or inhibition of the mixed function oxidase system, such as the cytochrome P450 (CYP450) group of enzymes. There are several competing pathways in the target hepatocyte that influence its toxicity (McLean and Dutton, 1995). The fate of aflatoxin in the liver is illustrated in **Figure 2.4**. The aflatoxin may be detoxified by hydroxylation, in which case it is excreted in the urine or bile. It may be activated into a highly reactive $AFB_1$-8,9-epoxide that can induce toxicity or cancer (Niranjan and Avadhani, 1980).

***Detoxification***

Detoxification reactions **(Figure 2.4)** involve conjugation of the toxin to glucuronic acid, sulphate or glutathione (Hsieh, 1987). The majority of $AFB_1$ is detoxified by conjugation of the reactive epoxide to glutathione (mediated by glutathione S-transferase, GST) [Degen and Neuman, 1981]. The $AFB_1$ glutathione conjugate may be excreted primarily through the bile (Hsieh, 1987), hydrolysed by intestinal microflora to release the $AFB_1$ for reabsorption and enterohepatic circulation (Hsieh and Wong, 1982) or may be detoxified by other epoxide hydrolases (Hayes *et al*., 1991). One exception is the conversion of $AFB_1$ to aflatoxicol (AFL).

The metabolism in the liver of $AFB_1$ to AFL and *via* aflatoxin quercetin (AFQ) to aflatoxin $H_1$ ($AFH_1$) is catalyzed by a cytoplasmid nicotinamide adenine dinucleotide phosphate (NADP)-dependent dehydrogenase (Eaton *et al*., 1994). The conversion of $AFB_1$ to AFL is a

reversible reaction and may be inhibited by 17-keto-steroid sex hormone. The reversibility of this reaction may provide a store of intracellular $AFB_1$, thus enhancing its aflatoxin effects.

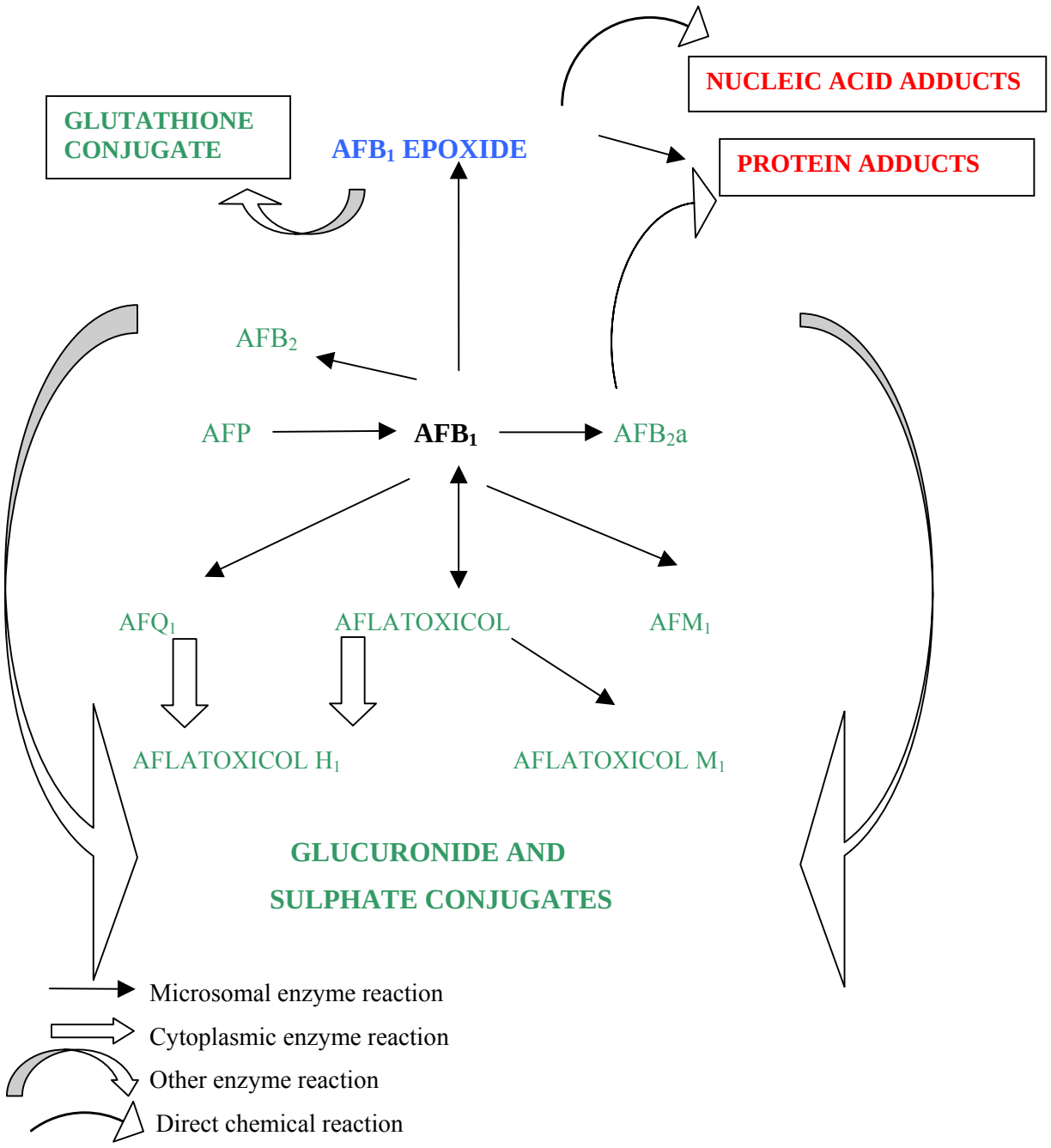



**Figure 2.4 Metabolism of aflatoxins in the liver**.

***Activation***

During metabolism, certain products have the capacity to react covalently with various nucleophilic centers in cellular macromolecules such as DNA, RNA, and protein. Along with the above, another metabolite, aflatoxin 8,9-epoxide, is also formed. The amount of this metabolite decides the species susceptibility as this can induce mutations by intercalating into DNA or by forming an adduct with guanine moiety in the DNA (Smela *et al.,* 2001). Figure 2.5 emphasizes the toxicity and/or carcinogenicity by activation of the epoxide.

Following transport across the plasma membrane, the $AFB_1$ molecule is activated by microsomal (smooth/tubular endoplasmic reticulum (ER)-associated) mixed function oxidases (requiring CYP450, NADP and molecular oxygen) to form the highly reactive $AFB_1$-8,9-epoxide (Swenson *et al*., 1974). In the rat hepatocyte, the nuclear envelope is also reported to contain all the enzymes necessary for the metabolic activation of $AFB_1$ (Kasper and Gonzalez, 1982). The $AFB_1$-epoxide may interact with nuclear DNA resulting in nuclear damage or may bind to sex-linked sites on the endoplasmic reticulum (ER). This binding to the ER may result in ribosomal detachment and polysomal degradation. Aflatoxin $B_1$ may also be reversibly converted by an NADP-reductase to AFL. The mixed function oxidases may also transform $AFB_1$ into various metabolites that may be eliminated by the hepatocyte (Swenson *et al*., 1974). Aflatoxin $B_2$, thought to be a hydrolytic product of $AFB_1$, binds to nucleic acids and proteins and may form the carcinogenic Schiff bases. This may then cause acute toxic effects of $AFB_1$ (Hsieh *et al*., 1977).

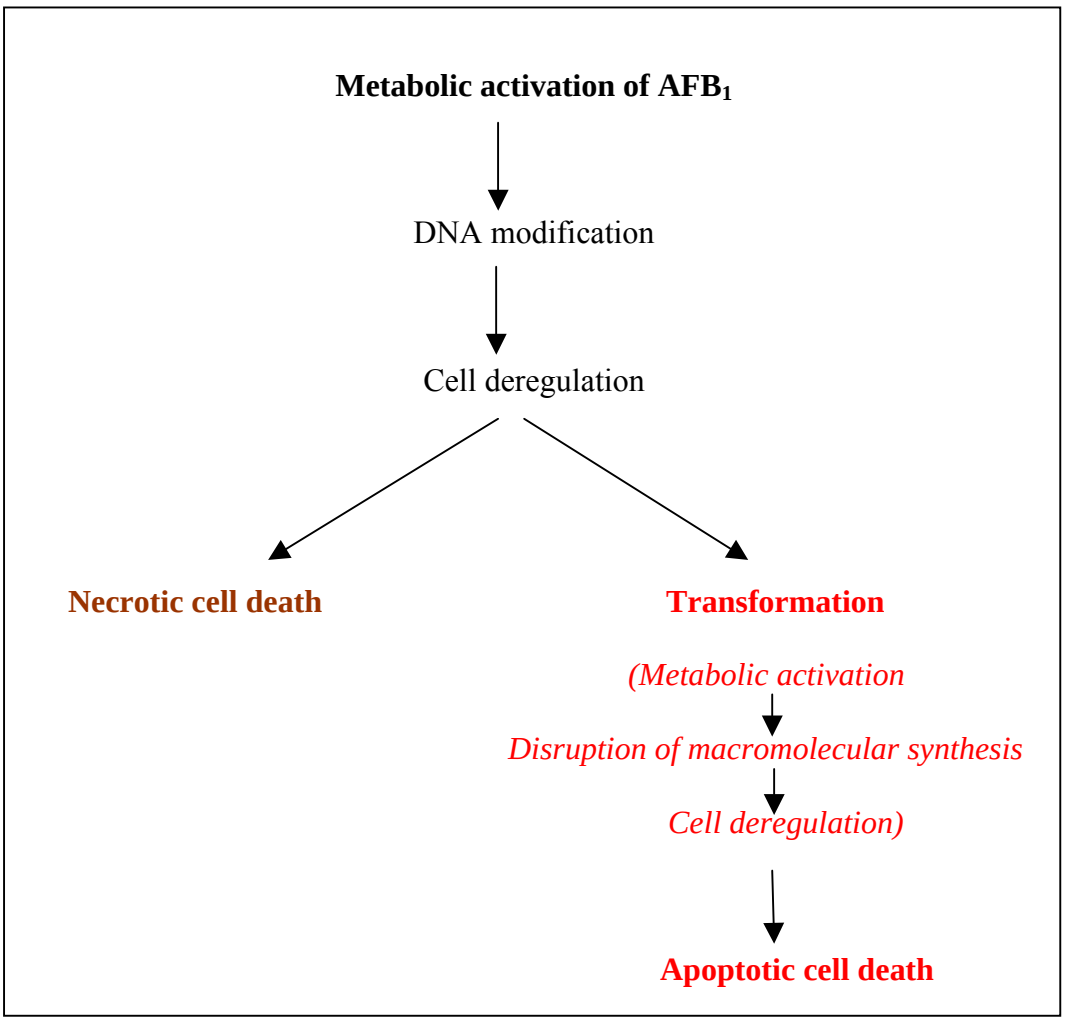



**Figure 2.5** **Probable primary biochemical lesions and the early cellular events in the cascade of cellular events leading to toxic cell injury or cellular deregulation of $AFB_1$ (Eaton and Gallager, 1994; Ueno *et al.*, 1995).**

Aflatoxin $B_1$ may exert its genotoxic effect by an indirect mechanism: through membrane-active, via the intermediacy of active oxygen, lipid hydroperoxydases and small aldehydes. In a study carried out by Amstad *et al.* (1984), sister chromatid exchanges were produced in human lymphocytes at very low levels of $AFB_1$-DNA adducts, which could not be explained entirely in terms of a direct genotoxic action.

Activation of $AFB_1$ to aflatoxin B (AFB)-8,9-epoxide appears to be mediated by several CYP450s including CYP1A2 and CYP3A4. Substantial evidence indicates that CYP1A2 is relatively more active at low substrate concentrations, but with limited capacity, whereas at higher substrate concentrations CYP3A4 activity predominates (Eaton and Groopman, 1994). Aflatoxin $Q_1$ ($AFQ_1$) appears to be formed predominantly, if not exclusively, by CYP3A family enzymes, whereas aflatoxin $M_1$ ($AFM_1$) is formed by CYP1A enzymes (either or both 1A1 and 1A2) at low substrate concentrations, and also perhaps by CYP2A family enzymes.

Aflatoxin $B_1$-epoxide has been shown to exist in two stereomeric forms. The *exo* form appears to be primarily responsible for AFB-DNA adduct formation, and the ratio of endo- to exo- epoxide formed may very depending on the specific CYP450 involved (Raney *et al.,* 1992). Different glutathione S-transferases also appear to possess different catalytic activities toward these two epoxides (Raney *et al.,* 1992).

### 2.2.3 Molecular effects

The covalent incorporation of the $AFB_1$ moiety into nucleic acids and proteins is considered to be an important mechanism by which toxicity and carcinogenicity are initiated. Aflatoxin $B_1$ albumin adducts are found in peripheral blood after exposure to $AFB_1$ and the measurements of these adducts are a potentially useful tool in the aetiology of liver cancer and aflatoxicosis.

***Nucleic acids***

Nitrogen and oxygen in the organic bases of nucleic acids are susceptible to electrophilic attack by metabolites of aflatoxins, forming covalent adducts. Any alteration in nucleic acid

(both DNA and RNA) structure effected by these adducts will impair DNA and RNA activity, resulting in inhibition of protein synthesis. The possible point mutations may lead to the manufacture of non-functional molecules and adduct formations may lead to cell transformations or even cell death, depending on the severity of impairment of template activity (McLean and Dutton, 1995).

Both $AFB_1$-epoxide and, to a lesser extent, its hydration product (dihydrodiol form of $AFB_1$) react with nucleic acids. The epoxide specifically makes an electrophilic attack on the $N^7$ position of guanine of DNA and RNA (Vidyasagar *et al*., 1997), while the dihydrodiol forms a Schiff base with amino groups of the bases (Hsieh, 1987). The dihydrodiol is highly reactive and binds with proteins at the site of its formation (Neal and Colley, 1979). The formation of the adducts are represented in **Figure 2.6.** Aflatoxin $M_1$ and aflatoxin $P_1$ ($AFP_1$) also form adducts with the $N^7$ position of guanine (Essigmann *et al*., 1982). The $N^7$ guanyl adduct is unstable and may undergo spontaneous depurination to form pyrimidyl adducts ($AFB_1$- formamidopyrimidine [FAPY] and $AFB_1$ dihydrodiol). These adducts are not lost spontaneously and are detected in the urine of exposed individuals. Depurination at guanine residues could lead to guanine/cytosine-thymine/adenine (GC-TA) conversion during replication while the FAPY derivatives are repair resistant and may result in mutations if present at the time of DNA replication.

One of the first measurable effects of $AFB_1$ on cells and tissues is inhibition of DNA synthesis. It would appear that $AFB_1$ blocks the initiation step in DNA replication rather than the elongation process. Inhibition may result from covalent binding of $AFB_1$ to DNA and proteins, leading to modification of DNA template activity and/or inactivation of certain enzymes in DNA synthesis (Hsieh, 1987).

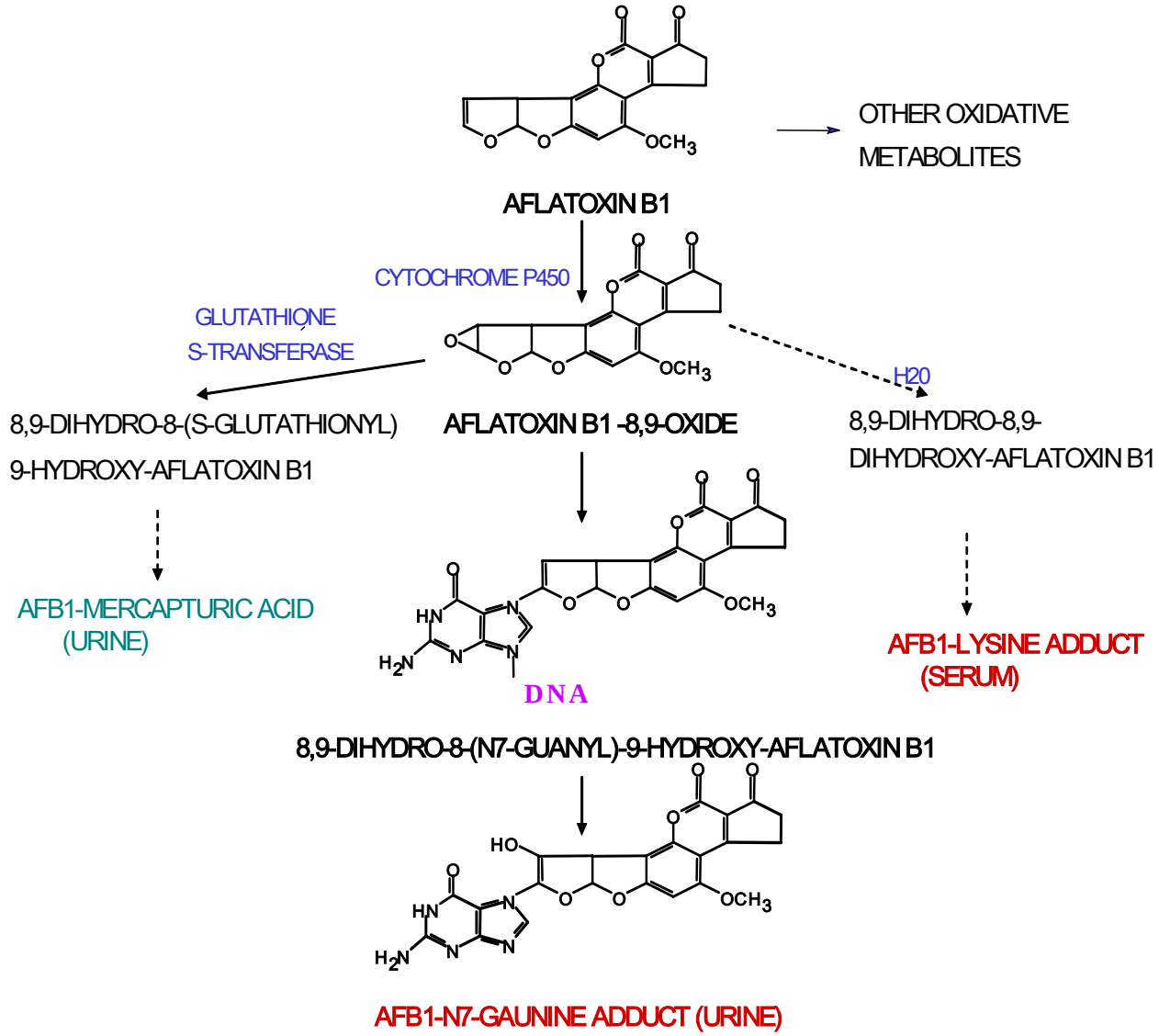



**Figure 2.6 Biotransformation of $AFB_1$**

Synthesis of rat liver RNA is inhibited rapidly by $AFB_1$ (Sporn *et al*., 1966; Yu, 1981), especially nucleolar RNA synthesis, related to formation of rRNA. Yu (1983) found that after *in vivo* and *in vitro* activation, $AFB_1$ binds preferentially to the physiologically active regions of the nucleolar chromatin of rat liver cells, possibly explaining the measured decreases in RNA synthesis. Aflatoxin $B_1$ disrupts post-transcriptional processing of nuclear RNA for the manufacture of rRNA from nucleolar RNA precursors. Alteration in nuclear and nucleolar morphology are prominent effects of aflatoxin in treated animals (Terao and Ueno, 1978). Ultrastructural changes include a gradual redistribution of nucleolar components (macrosegregation), resulting in segregation of granular and fibrillar components, fragmentation and the development of ring-shaped nucleoli. These observations indicate alterations in RNA synthesis (Yu, 1981).

Aflatoxin $B_1$ caused marked, rapid (1 h) inhibition of RNA synthesis and subsequent cytotoxic response in isolated and primary cultured hepatocytes, from control rats, which are known to metabolize $AFB_1$ (Metcalfe and Neal, 1983). A rat liver-derived cell line, BL8L, was found to be less susceptible. These cells showed no detectable $AFB_1$ metabolizing capacity, but a less potent, anti-mitotic action of $AFB_1$ was observed. Thus, $AFB_1$ seems to require metabolism to exert its acute cytotoxic action, which is found at very low $AFB_1$ concentrations, although a direct antimitotic effect, independent of metabolism, is seen in dividing cells. *In vivo* studies also show (Kensler *et al*., 1994) that phenobarbitone and 3-methylcholanthrene (known inducers of $AFB_1$ metabolism) results in reducing $AFB_1$ inhibition of RNA synthesis and cytotoxicity in hepatocytes, but only at lower concentrations of $AFB_1$ used, whereas cells from $AFB_1$ fed rats were much less susceptible to $AFB_1$ toxicity at all concentrations used. This resistance to cytotoxicity of $AFB_1$ appears to involve

detoxification mechanisms, primarily the formation of polar conjugates of $AFB_1$ metabolites (particularly glutathione conjugates).

***Proteins***

The nucleophilic nitrogen, oxygen and sulphur hetero-atoms in proteins make them important cellular receptors. Non-specific irreversible covalent interaction (denaturation or blocking of binding sites) and specific reversible non-covalent interactions (competitive interaction) may occur with aflatoxins. Proteins interacting covalently act as reservoirs of the toxin, prolonging toxin exposure or they may serve as carriers in the transport of reactive metabolites (McLean and Dutton, 1995). Cytoplasmic interacting proteins destined for the nucleus translocate to microsomes. The majority of the epoxide is detoxified and is removed rapidly from the cell as water-soluble polar metabolites. A portion of the activated $AFB_1$ is translocated to various sub-cellular sites such as the rough ER, mitochondria and nucleus. Recently, cellular proteins were found to interact with $AFB_1$ while the presence of a nuclear location sequence (NSL) markedly increased nuclear translocation and activation of $AFB_1$ in the nucleus. Mycotoxin interaction may inhibit protein activity such as those involved in biosynthetic pathways, neurotransmission, membrane transport, hormone functions and immune mechanisms.

Ultrastructurally, studies show degranulation (detachment of ribosomes) from ER . This may be due to disruptive changes including: direct damage to the ER membranes, interference with the ribosome interaction sites on the membrane, interference with the ribosomal cycle, inhibition of the release of newly synthesized proteins, or a suppression of mRNA synthesis. As a result of this dissociation of ribosomes, ER-mediated protein synthesis is likely to be disrupted. Aflatoxin $B_1$-induced alterations also occur in the phosphoprotein patterns in soluble and insoluble rat liver fractions (Viviers and Schabort, 1985).

*In vitro* studies of $AFB_1$ binding with plasma albumin suggest a possible *in vivo* transport mechanism for this toxin as an albumin complex (Eaton *et al*., 1994). The frequency of chromosomal aberrations and micronuclei in the bone marrow was measured and compared to the level of $AFB_1$ bound covalently to albumin in the peripheral blood of rats and mices. The results also suggest that $AFB_1$-albumin may reflect the level of genetic alterations resulting from the initial interaction of this carcinogen to cellular DNA. Thus, the adduct used as a biomarker in studies of human exposure to aflatoxins may provide information not only on exposure but also on the risk of genetic alterations consequent to that exposure (Vidyasagar *et al*., 1997).

***Inhibition of ATP generation***

Exposure to acute levels of mycotoxins affects cellular energy production. Aflatoxins inhibit the electron transport chain between cytochromes b and c (Doherty and Campbell, 1973) and also act at the cytochrome oxidase level (Betina, 1989). Further investigations by Niranjan and Avadhani (1980) indicate that the presence of the CYP450 mixed function oxidases in rat liver mitochondria is capable of generating electrophilic reactive metabolites that bind covalently to mitochondrial DNA, RNA and proteins. Uncoupling of oxidative phosphorylation results in depletion of cellular adenosine triphosphate (ATP). As a result, sodium and potassium gradients within the cell as well as mitochondria are affected (Hsieh, 1987). Aflatoxin $B_1$ also inhibits ATPase and ATP synthase activity of the inner mitochondrial membrane (McLean and Dutton, 1995).

***Immunocytochemical localization of $AFB_1$***

Immunocytochemical investigation in plant tissue confirm that $AFB_1$ acts directly on the nucleic acids, particularly the DNA. It is probable that several of the measured decreases (e.g. RNA and protein syntheses) following $AFB_1$ exposure will then be secondary manifestations resulting from $AFB_1$-DNA binding. **Figure 2.7** summarizes the sites of $AFB_1$ action in the cell.

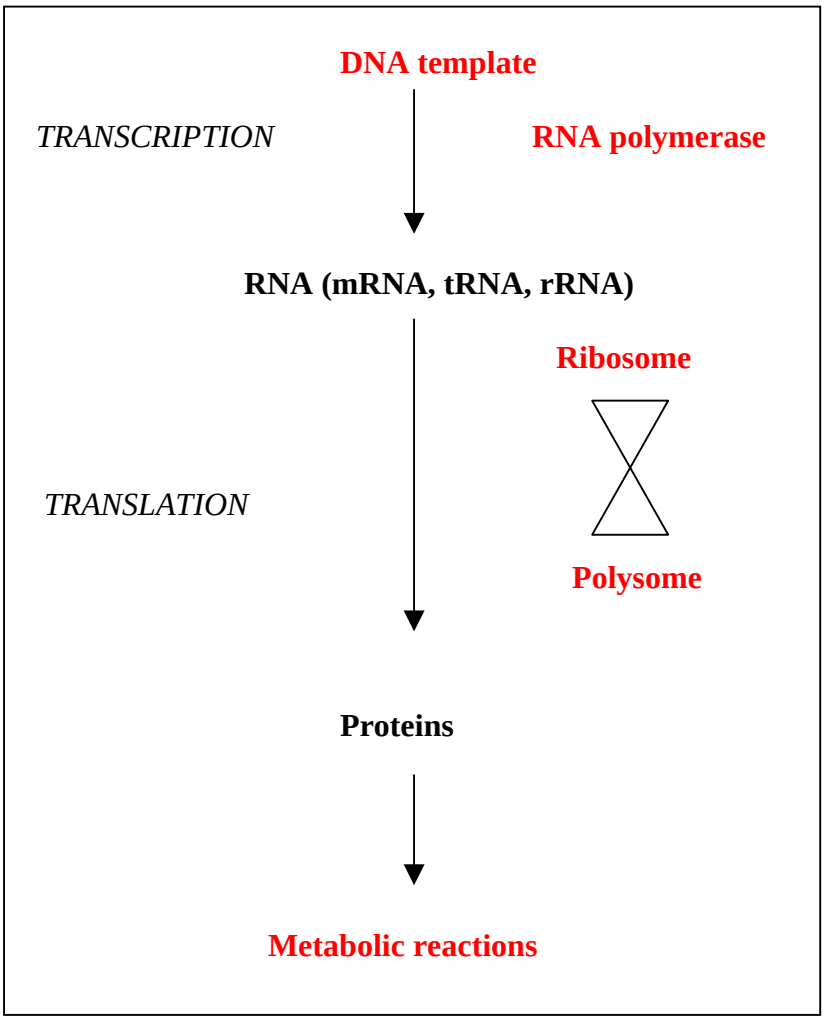



**Figure 2.7 Sites of $AFB_1$ action in the cell (Target sites are highlighted in red).**

***Carbohydrate and lipid metabolism***

Dietary levels of $AFB_1$ are known to cause lipid accumulation in the liver (Hamilton, 1975) through impaired lipid transport. Damage to mitochondria in $AFB_1$-treated cells is observed which may be the result of reduced oxidation of fats or increased lipid synthesis.

Several animal species administered with $AFB_1$ exhibit reduced hepatic glycogen levels and elevated serum glucose levels (Kiessling, 1986). These biochemical effects may arise from either: an inhibition of glycogenic enzymes (e.g. glycogen synthase), an inhibition of glyconeogenesis, a decrease in glucose transport into hepatocytes, or an increase in the activity of enzymes metabolizing glycogen precursors.

### 2.2.4 Systemic effects

Generally, an inhibition of protein synthesis could result in an alteration of various systems in the body. Sub-acute doses of $AFB_1$ in several experimental animals have produced alterations in non-specific and specific immune systems (Reddy *et al*., 1987; Pestka and Bondy, 1990). Acute doses have lowered immunoglobulin levels and reduced the efficacy of vaccinations. In human lymphocytes, low doses of $AFB_1$ caused mitotic aberrations in a dose-dependent manner (Amstad *et al*., 1984).

Aflatoxin $B_1$ is known to reduce the nuclear acceptor sites for the hormonal systems such as glucocorticoid-cytosol receptor complex in rat liver and the uterine cytosol receptor site (McLean and Dutton, 1995).

The $AFB_1$-epoxide, however, creates more serious damage as a potent mutagen causing chromosomal aberrations and DNA breakages in animal cells (Smith and Moss, 1985). As potent inhibitors of protein synthesis, $AFB_1$ is expected to cause impairment of development of primordial and differentiation in the foetus (McLean and Dutton, 1995).

Imaoka and co-workers (1990) found that rat hepatic microsomes displayed the greatest mutagenic activation as compared to other tissues of the body. It confirmed that the bio-activation of $AFB_1$ is dependent on the specific classes of CYP450's. Ames *et al*. (1990) showed that $AFB_1$ is activated by liver homogenates to form potent frame shift mutagens and suggested that these carcinogens cause cancer by somatic mutation. They used rat liver homogenate for carcinogen activation in their study as well as a set of *Salmonella* histidine mutants for mutagen detection. The *in vitro* interaction has a strict requirement for NADPH and oxygen, suggesting that it has basic functions in common with the mixed oxidase system responsible for the N- and C-oxygenation of aromatic amines.

Aflatoxin $B_1$, $AFG_1$ and $AFM_1$ has been found to be both initiators and promoters in the cancer process. As discussed earlier, $AFB_1$ selectively and covalently binds to mitochondrial DNA, perhaps reflecting a lack of appropriate excision repair mechanisms in this organelle. As a result, mitochondrial transcription and translation may be persistently inhibited by these lesions, contributing to neoplastic transformation of the cell (McLean and Dutton, 1995).

Another aspect to consider is that DNA methylation may be inhibited by $AFB_1$-DNA binding thereby altering gene expression and cellular differentiation. Oncogenes may then be activated, precipitating oncogenic transformation of mammalian cells. Aflatoxin $B_1$ has been demonstrated to activate the Ki-*ras* gene in rat liver. In this regard, in the final stages of

$AFB_1$-induced rat liver (HCC), two activating mutations in the codon 12 region of Ki-*ras* genes GGT-GAT (McMahon *et al*., 1987; Soman and Wogan, 1993) and GGT-TGT (Sinha *et al*., 1988) have been identified. This phenomenon has not been seen in human HCC (Mc Lean and Dutton, 1995).

The *p53* tumour suppressor gene and most specifically codon 249 of this gene have been linked epidemiologically to human HCC. Hsu *et al.* (1991) found a striking mutational specificity in the third base position of codon 249 resulting primarily in a guanine-thymine (G-T) substitution in Chinese patients. The same transversion was found in 50% of the analyzed HCC tumours in Southern Africa and Asia. The *p53* gene mutations also occurred in 30% of advanced HCC cases in Japan. Hollstein *et al.* (1993) found an AGG-AGT transversion at codon 249 and an ATC-AAC transversion at codon 254 in 15 Taiwanese HCC patients. They were also positive for $AFB_1$ liver adducts and $AFB_1$ serum albumin adducts. The genotype GST was also absent in 12 of the 15 patients (McLean and Dutton, 1995). Ozturk (1991) showed a high correlation between hepatitis B virus (HBV) infection and HCC in Mozambique and Transkei but a low correlation between *p53* mutation and HCC.

## 2.3 THE ROLE OF PHYTOCOMPOUNDS IN CANCER

Cancer chemoprevention can be defined as the prevention, inhibition, or reversal of carcinogenesis by the administration of one or more chemical entities, either as individual drugs, immunotherapy, vaccines or as naturally-occurring constituents of the diet. Knowledge of phytocompounds and their role in chemopreventive science and its application in clinical studies have been growing rapidly over the last decade and is well documented in reviews (Stoner *et al*., 1997; Manson *et al*., 2000; Reddy *et al*., 2003).

These phytochemicals act at various levels, preventing cancer by acting as blocking agents during the initiation stages **(Table 2.2)** or they act as suppressing agents in the latter stages of malignancy **(Table 2.3).**

*In vitro* assessments of potential anticarcinogenesis efficacy include measurements of an agent's antioxidant activity, induction of phase 2 metabolizing enzymes and effects upon cellular proliferation and apoptotic control pathways. *In vivo* efficacy is assessed primarily in rodent models of carcinogenesis that are specific for a given organ target (Stoner *et al*., 1997). Clinical assessment of the efficacy of a preventive agent comprises a multi-step process of identification. It includes an optimal preventive agent (phase 1), demonstration of efficacy in humans through the modulation of reversal of a tissue, biochemical, and molecular surrogates for neoplastic transformation and invasion (phase 2) and cancer risk reduction in large cohort trials (phase 3). Opportunities and future needs to include: the development of reliable, predictive *in vivo* models of carcinogenesis, careful exploration of the preventive pharmacology of therapeutic agents that are being used for non-cancer prevention indications, and the incorporation of genetic risk cohorts to define cancer chemopreventive efficacy.

**Table 2.2 Categories of blocking agents (Stoner *et al*., 1997).**

| Mechanism | Examples |
|---|---|
| Inhibition of cytochrome P450 | Dithiocarbamates, ellagic acid, diallyl sulfide, isothiocyanates |
| Induction of cytochrome P450 | Indole-3-carbinol, β-naphthoflavone |
| Induction of phase 2 enzymes: | |
| Glutathione *S*-transferase | Allyl sulfides, dithiolethiones, isothcyanates |
| UDP-glucuronyltransferase | Poly phenols |
| Glutathione peroxidase | Selenium |
| Scavenge electrophiles | Ellagic acid, *N*-acetylcysteine |
| Scavenge free radicals | Sodium thiosulfate, polyphenols, vitamin E |
| Increase overall levels of DNA repair | Vanillin |
| Increase poly (ADP-ribosyl) transferase | *N*-Acetylcysteine |
| Suppress error-prone DNA repair | Protease inhibitors |

ADP, adenosine diphosphate

**Table 2.3 Categories of suppressing agents (Stoner *et al*., 1997).**

| Mechanism | Examples |
|---|---|
| Inhibit polyamine metabolism | DFMO, polyphenols, substituted putrescines |
| Induce terminal cell differentiation | Calcium, retinoids, vitamin $D_3$ |
| Modulate signal transduction | Glycyrrhetinic acid, NSAID's, polyphenols retinoids |
| Modulate hormonal/growth factor activity | NSAID's, retinoids, tamoxifen |
| Inhibit oncogene activity | Genistein, NSAID's, monoterpenes |
| Promote intracellular communication | Carotenoids, polyphenols, retinoids |
| Restore immune response | NSAID's, selenium, vitamin E |
| Induce apoptosis | Butyric acid, genistein, selenium, sulindac sulfone, retinoids. |
| Correct DNA methylation imbalances | Folic acid, choline, methionine |
| Inhibit basement membrane degradation | Protease inhibitors |
| Inhibit arachidonic acid metabolism | Glycyrrhetinic acid, *N*-acetylcysteine, NSAID's, polyphenols |

NSAID, non-steroidal anti-inflammatory drug; DFMO, α-difluoromethylornithine

## 2.4 SOURCES OF NATURAL PRODUCTS AS CHEMOPROTECTIVE AGENTS

Natural chemoprotective products work mainly as antioxidants and are found in vegetables and fruits **(Table 2.4)** and in plant extracts and herbs **(Table 2.5)**. Although the mechanisms of the protective effect of some of these products are not always well defined, the fact that the consumption of fruits and vegetables lowers the incidence of carcinogenesis at a wide variety of sites is broadly supported. Epidemiological evidence suggests protection against a wide array of cancers, particularly those of the respiratory and digestive tracts and, to a lesser extent, the hormone–related cancers (Langset, 1995; Bingham and Riboli, 2004). Latest research (Techen *et al*., 2004; Lai and Roy, 2004; Kensler *et al*., 2004) suggest targeted chemoprevention might be the most appropriate for the present generation of individuals at risk.

**Table 2.4 Chemoprotective products from fruits and vegetables.**

| Source | Active component | Mechanism of action | Cancer inhibited (References) |
|---|---|---|---|
| Olives | Polyphenols | Antioxidant | Various cancers (Langset, 1995) |
| Apples | | Antioxidant | Various cancers (Eberhardt *et al*., 2000) |
| Strawberries, raspberries, cantaloupe melon | Vitamin C, bioflavonoids, chalcones | Antioxidant | Various cancers (Nixon, 1999; Paiva & Russell, 1999) |
| Leafy greens, cabbage, broccoli, cauliflower | Vitamin C, lutein, zeaxanthin | Scavenger of ROS, antioxidant, suppresses promotion of lung tumours in mice | Various cancers; crypt foci in SD rat colon (Ceruti *et al*., 1986; Rauscher *et al*., 1998; Abdulla & Gruber, 2000; Nishino *et al*., 2000; Stahl *et al*., 2000) |
| Vegetable oils, cold –pressed seed oils, wheat germ. | Vitamin E | Protects against lipid peroxidation | Skin cancer (Paiva & Russell, 1999; Stahl *et al*., 2000) |
| Yellow-orange vegetables and fruits | Beta-carotene | Antioxidant | Various cancers (Paiva & Russell, 1999; Stahl *et al*., 2000) |
| Carrots | Alpha- and beta-carotene, phenolic compounds | Antioxidant, pS2 gene expression, alpha-carotene more effective, inhibits tumours in rats and mice | Pancreatic, colon, breast cancer, liver cells; rat, mice colon and liver cancer (King *et al*., 1997; Eberhardt *et al.,* 2000; Nishino *et al*., 2000; Cheng *et al*., 2001). |
| Tomatoes | Lycopene, vitamin C | Strong antioxidant, inhibits DNA damage in lymphocyte | Leukaemia, lung cancer; mice tumours (Giovannucci, 1999; Hecht *et al.*, 1999; Watzl *et al.*, 1999; Kim *et al*., 2000b) |

| | | | |
|---|---|---|---|
| Grapes, red wine | Phenols, catechins | Antioxidant | Various cancers (Abdulla & Gruber, 2000) |
| Citrus Fruits | β-cryptoxanthin, bioflavonoids, chalcones, vitamin C | Antioxidant, stimulates expression of RB gene and p73 gene (a *p53* related gene) | Rat tumours, various cancers (Nishino *et al*., 2000) |
| Garlic, onions, leeks, chives | Allicin, flavonoids, vitamin C, selenium, sulphur | Detoxifies carcinogen, inhibits *H. pylori,* cell cycle arrest from S to G2M boundary phase | Stomach cancer (Barch *et al*., 1996; Zheng *et al*., 1997) |
| Common bean | Phenolic compounds | Anti-mutagenic | Aflatoxin-induced cancer (Galvano *et al*., 2001; Cardador-Martinez *et al*., 2002) |

**Table 2.5 Chemoprotective products found in plant extracts and herbs.**

| Source | Active component | Mechanism of action | Cancer inhibited (References) |
|---|---|---|---|
| *Gymnosporia rothiana Laws* | GCE/chloroform ether extract | DNA/RNA and protein synthesis inhibited after treatment for 12-36 hours | Leukaemia in mice (Chapekar & Sahasrabudhe, 1981) |
| *Rhizoma zedoariae* | Beta-elemene | Cell cycle arrest from S to G2M phase | (Zheng *et al*., 1997; Yuan *et al*., 1999) |
| *Pinus pinaster, P. maritime* | Polyphenolic fraction, ferrulic acid, bioflavonoids, proanthocyanidins, Procydin (S.A.), Pycnogenol (Europe) | Antioxidant, improves blood circulation, increases cytokine levels, increases activity of NK cells, modulates mitogenic signalling and induction of G1 arrest and apoptosis | DU145 cells, prostrate, skin cancer (Agarwal *et al*., 2000; Saija *et al*., 2000) |
| *Viscum album var, Viscum var. coloratum* (Korean mistletoe) | Lectin-alkaloids | Caspace 3 activation, lectin 11 induced apoptosis, inhibition of telomerase via mitochondrial controlled pathway independent of *p53*, enhancement of cytokine release | U937, HL-60, lymphoblastoid cells, hepatocarcinoma cells, Human colon carcinoma LoVo cells, leukaemic cells (Ribereau-Gayon *et al*., 1997; Kim *et al*., 2000a; Duong *et al*., 2001; Park *et al*., 2001 |
| | Hexamethylene bioacetamide | p53-dependent apoptosis, induction with telomerase activity | Zhang *et al*., 2000) |
| *Azadirachta indica Juss* (Neem leaf) | polyphenolic | Cytotoxic | Various cancers (Gogate, 1991) |

| | | | |
|---|---|---|---|
| Muscadene berries | Resveratrol | Antioxidant | Lung tumour in mice (Hecht *et al*., 1999) |
| | Myo-inositol, dexamethasone | Antioxidant | Lung tumour in mice, liver cancer (Hecht *et al*., 1999; Witschi *et al.*, 1999) |
| *Curcuma longa L* turmeric | Curcumin | Antioxidant | Prostate, lung tumour in mice (Hecht *et al*., 1999; Dorai *et al*., 2001; Li & Lin-Shia, 2001) |
| | Esculetin | Antioxidant | Lung tumour in mice (Hecht *et al*., 1999) |
| *Acanthopanax gracilistylus* (Chinese herb) | | Antioxidant | Liver cancer cells (Shan *et al*., 2000) |
| *Cylopia intermedia* Honeybush tea | Polyphenolic compounds | Antioxidant, antimutagenic, interferes with CYP450-mediated metabolism | Various cancers (Marnewick *et al*., 2000) |
| *Undaria pinnantifida* (seaweed) | (viva -Natural) | Prophylactic | Lewis lung cancer in mice (Furusawa & Furusawa, 1985) |
| Rosemary, sage, other spices | Carnosic acid, rosemary acid | Antioxidants | Various cancers (Lai and Roy, 2004) |
| *Rubia cordifolia* | RC-18 | Forms DNA adducts | P388 and L1210 cells, B16 Melanoma (Adwankar & Chitnis, 1982; Poginsky *et al.*, 1991) |
| | Valepotrites | Cytotoxic | (Bounthanah *et al*., 1981) |

| | | | |
|---|---|---|---|
| *Scutellariae radix, S. indica* | Flavonoids | Prostaglandin $E_2$ production | Rat C6 glioma cells (Nakahata *et al*., 1998) |
| Soybeans | Isoflavones, phenolic acids, genistein (piperazine complex) | Protein tyrosine kinase inhibitor, diverse EGFR and p21 ras expression phenotypes, dependent on epidermal cell growth factor receptor, oestrogen-like action | Jurkat T-leukaemia cells, bladder cancer (Theodorescu *et al*., 1998; Abdulla & Gruber, 2000; Polkowski *et al*., 2000) |
| Various plants | Quercetin, kaempferol, rutin, hesperidin | OH scavenger | B16 melanoma (Day *et al*., 2000; Drewa *et al*., 2001; Elattar and Virji, 2000; Snyckers and Salemi, 1974) |
| *Camellia sinensis* green tea, black tea | Polyphenols, epigallocatechin-3-gallate | Apoptosis induction, cell cycle arrest | Tumour cells (Ahmad *et al*., 1997) |
| *Aspalathus linearis* Unfermented rooibos tea | Phenols | Antioxidants | Various cancers (Von Gadow *et al*., 1997) |
| *Coriolus versicolor* (Chinese herb) | Bisbenzylisoalkal-oids, bufalin, berberine, tetrandrine | Apoptosis induction, complexes with DNA | HL60, U937 cells (Jing *et al*., 1994; Kuo *et al*., 1995; Dong *et al*., 1997) |
| *Uncaria tomentosa* | | Apoptosis induction | Tumour cells (Sheng *et al*., 1998) |
| *Eucalyptus grandis* | Euglobal -G1 | | Various cancers (Takasaki *et al*., 2000) |
| *Ornithogalum* | Cholestane glycoside | Apoptosis induction | HL60 cells (Hirano *et al*., 1996) |
| *Cucurbita andreana* | cucurbitacins | | Various cancers (Jayaprakasam *et al*., 2003) |

EGFR, epidermal growth factor receptor; OH, hydroxyl; NK, natural killer; GCE, glycine cleavage enzyme

## 2.5 NATURAL PRODUCTS AND AFLATOXINS

Galvano and colleagues (2001) reviewed various dietary strategies to contain the toxic effects of mycotoxins using various groups of natural products. These include antioxidant compounds, food components, medicinal herbs, plant extracts, minerals and biological interaction. Available data are primarily from *in vitro* studies. Compounds with antioxidant properties are potentially efficacious because of their ability to act as superoxide anion scavengers. Interesting results have been obtained from components contained in coffee, strawberries, tea, pepper, grapes, turmeric, garlic, cabbage and onions. Some of these herbs and plant extracts also show promise as chemoprotectors against the effect of $AFB_1$ and fumonisin $B_1$.

### *Trace elements*

Since some mycotoxins, including $AFB_1$, are known to produce membrane damage through increased lipid peroxidation (Galvano *et al* 2001), the protective properties of antioxidant substances have been extensively investigated. Selenium, some vitamins (A, C, and E), and their precursors have marked antioxidant properties that act as superoxide anion scavengers. In an *in vivo* study on rats, Kensler *et al* (1994) demonstrated that selenium inhibits $AFB_1$-DNA interaction and adduct formation. The same authors (Kensler *et al*., 1994) found that sodium selenite and selenium-enriched yeast extract protect hamster ovary cells from $AFB_1$ cytotoxicity but not from mutagenicity.

### *Vitamins*

Vitamin C is reported to protect guinea pigs from $AFB_1$ hepatotoxicity (Kensler *et al*., 1994). Analogous protective actions have also been attributed to vitamin E and vitamin A toward

exposure to both ochratoxin A (OTA) and $AFB_1$ (Sinha *et al*., 1988). Vitamin A status in rats is strongly related to the hepatocarcinogenicity of $AFB_1$. An enhancement of $AFB_1$-induced DNA damages during vitamin A deficiency and these damages were reversed on vitamin A supplementation. Soni *et al*. (1993) demonstrated that vitamin supplementations exceeding up to 25% of the standard requirements could reduce the negative effects of $AFB_1$ and environmental stress on turkeys. In addition, riboflavin has a potential chemopreventive action against $AFB_1$-induced DNA damages in rats. The group of precursors of vitamin A, namely, the carotenoids is discussed in the next section (2.6).

***Phenolic compounds***

Ellagic acid is a phenolic compound that occurs naturally in some foods, such as strawberries, raspberries, and grapes. It has both antimutagenic and anticarcinogenic activity as demonstrated in a wide range of *in vitro* and *in vivo* assays. Ellagic acid inhibited $AFB_1$ direct-acting mutagenicity in *Salmonella* cells, particularly when incubated with metabolic enzymes. The result of sequential incubation indicated that the formation of an $AFB_1$-ellagic acid chemical complex is involved in mechanism of inhibition.

Further studies by Sahoo and Mukherjee (2003) emphasize the role of phenolic compounds in the activation and detoxification processes, hence in modulating the carcinogenicity of $AFB_1$. In tests performed on rats fed on a synthetic diet containing various food-associated phenolic compounds each at the 0.5% level, the authors observed a marked decrease in the ability of liver microsomes to catalyze reactions of $AFB_1$, leading to its activation and DNA adduct formation. The phenolic compounds tested were several flavonoids (fisetin, kaempferol, morin, naringin, and catechin), phenolic acids (caffeic acid and chlorogenic acid), and other phenolic (eugenol and vanillin) and synthetic phenolic (butylated hydroxyanisole and

butylated hydroxytoluene) antioxidants. Some phenolic compounds (naringin, catechin, eugenol, vanillin, and butylated hydroxyanisole) were also found to induce cytosolic glutathione S-transferase activity that stimulated the formation of specific $AFB_1$-glutathione conjugate. Kensler *et al*. (1994) found that eugenol does not modify the unscheduled DNA synthesis in hepatocytes exposed to $AFB_1$.

Williams *et al*. (1986) reported that butylated hydroxyanisole and butylated hydroxytoluene inhibited the initiation of hepatocarcinogenesis by $AFB_1$ in rats. However, the permitted dose of butylated hydroxytoluene, added to processed food as preservative, plays no role in the biotransformatin of $AFB_1$. Soni *et al*. (1993) confirmed that ellagic acid and butylated hydroxyanisole ameliorated aflatoxin-induced mutagenicity and carcinogenicity. Two flavonoids, alpha- and beta-naphtoflavone, strongly inhibit microsome-catalyzed $AFB_1$-DNA interacting in trout.

Natural phenolics such as quercetin, keaempferol, ellagic acid, and curcumin generally reduced the *in vitro* enzyme activity consequent to $AFB_1$ treatment (Dorai *et al*., 2001). According to the authors, the suppression of protein kinase C activity by phenolic compounds could be a way to control $AFB_1$ carcinogenicity.

A range of natural dietary constituents, including garlic oil, ethoxyquine, indole-3-carbinol, and phenethy1 isothiocynate, have *in vitro* chemoprotective actions toward $AFB_1$ (Manson *et al*., 2000; Wattenberg, 1985). Some natural polyphenolic compounds, i.e., polyhydroxylated flavonoids and phenolic acids, where found to be effective in reducing $AFB_1$-DNA adduct.

S-methyl methanethiosulfonate, a compound present in the juice of cabbage and onion, has been demonstrated to have a suppressive effect on $AFB_1$- or methy1 methanesulfonate-induced chromosome aberrations in rat bone marrow cells. In addition, the precursor of S-methyl-L-cysteinesulfoxide significantly suppressed $AFB_1$- or methyl methanesulfonate-induced chromosome aberrations.McLean and Dutton (1995), although other mechanisms are not excluded, the protective properties of S-methyl methanethiosulphate may result from its ability to modify -SH groups in proteins.

Cavin *et al*. (2001) identified two diterpenes, cafestol and kahweol, present in green and roasted beans as potentially chemoprotective agents against the covalent interaction of $AFB_1$ metabolites to DNA of rats. It has been postulated that these compounds may act as blocking agents by producing a co-ordinated modulation of multiple enzymes involved in carcinogen detoxification. Manson *et al*. (2000) found that caffeic acid also has *in vitro* chemoprotective actions toward $AFB_1$. On the contrary, caffeine has been demonstrated to potentiate the *in vitro* genotoxicity of $AFB_1$. The afulvins, compounds isolated from black tea aqueous infusions, despite their overall ability in reducing the mutagenicity of other food carcinogens, enhanced the mutagenicity of $AFB_1$. Propionic acid and potassium sorbate used as preservatives in bread making in France destroyed up to 71% of the $AFB_1$.

***Coumarins***

More than 300 coumarins with general pharmacological and biochemical properties have been identified from green plants. Coumarin (1,2-benzopyrone), a natural food constituent especially present in *Fava tonka*, has a chemoprotective action against $AFB_1$. As demonstrated by Goeger *et al*. (1988) in *in vitro* studies on hamster ovary cells, liver cells from rats, and chick embryos, coumarin decreased cytotoxicity and mutagenicity of $AFB_1$,

although with marked specie differences in chemoprotection. Different oxygenated substituents on 4-methylcoumarins (i.e. acetoxy>hydroxy>methoxy, in order of inhibition ability) also have chemopreventive properties on $AFB_1$-DNA interaction *in vitro*. However, it must be considered that coumarin also has toxic properties and, because of their structural similarity, counteracts vitamin K absorption.

Quercetin pentaacetate (OPA) (unlike quercetin) demonstrated time-dependent inhibition of liver microsome catalyzed $AFB_1$ epoxidation. The action of QPA is similar to that of the acetoxy 4-methylcoumarins in that they are acted upon by microsomal transacetylase, which lead to modulation of catalytic activities of certain enzymes [such as P450 enzymes, NADPH cytochrome C reductase and glutathione s-transferase (GST)] possibly by way of protein acetylation. Kohli *et al.* (2002) documented the transacetylase-mediated action of QPA in preventing genotoxicity due to $AFB_1$.

***Chlorophyll and its derivatives***

Dashwood *et al*. (1998) demonstrated that chlorophylline (a food grade, water-soluble derivative of the green plant pigment chlorophyll) has chemopreventive properties against wide classes of mutagens, including $AFB_1$. Chlorophyllin acts as an interceptor molecule by forming a strong noncovalent complex with $AFB_1$, reducing hepatic $AFB_1$-DNA adducts and liver tumours (Bonn, 2002). In particular, it has been demonstrated that the complex formation occurs between the porphyrin-like structure of chlorophyllin and the planar molecular surface of $AFB_1$. Chlorophyllin mixed with chitosan, a polyglucosamine, can form an insoluble, salt-like material able to trap $AFB_1$. The chemoprotective effect of chlorophyllin was confirmed in an *in vivo* study, where 2000 to 4000 ppm of chlorophylline reduced $AFB_1$-DNA adduction up to 77% in rainbow trout (Egner *et al*., 2003).

***Sulphide compounds***

Dietary administration of diallyl sulfide and allyl mercaptan to rats strongly reduced hepatic DNA breaks induced by $AFB_1$ and, to a lesser extent, its mutagenicity (Kensler *et al*., 1994).

***Herb and plant extracts***

Some studies highlighted the capability of several extracts from medicinal herbs and plants to counteract the $AFB_1$ toxicity. Ethanol extract from a concentrate of *Cassia senna* (a medicinal herb commonly used as vegetable laxative) inhibits the *in vitro* mutagenic effect of $AFB_1$ at low concentrations but not at higher ones. Anthraquinone aglycones and naphtopyrone glycosides, two compounds detected in methanol extract of *Cassia tora*, showed a marked *in vitro* antimutagenic activity against $AFB_1$. *Semecarpus anacardium* nut extract was demonstrated to be effective in reducing $AFB_1$-induced hepatocarcinoma. The administration of a methanol extract of the leaves of *Piper argyrophyllum* normalized the genotoxic effect of $AFB_1$ on rat cells (Zheng *et al*., 1997).

A study on rats showed that an extract from *Thanninga sanguinea* is able to protect against acute hepatotoxicity caused by $AFB_1$. Carnosol and carnosic acid, two natural polyphenols found in *Rosmarinus officinalis L*., are potent inhibitors of *in vitro* $AFB_1$-induced DNA adduct formation.

A reduction of *in vitro* metabolic activation of $AFB_1$ by *Azadirachta indica var. siamensis* and *Momordica charantia L*. was observed by Lai and Roy *et al*. (2004). In a study on rats, that a lignin-enriched extract of the fruit of *Schisandra chinensis* provided hepatoprotective action against $AFB_1$ by enhancing the hepatic antioxidant and detoxification system.

Piperine (1-piperoylpiperidine), the major alkaloid constituent of pepper (*Piper nigrum*), is potentially a protective agent against the carcinogenic effect of $AFB_1$. It is well known that the $AFB_1$ toxicity is bioactivated by the CYP450. In rat cultured cells, piperine reduced dramatically CYP450 2B1-mediated toxicity of $AFB_1$, thus offering a chemopreventive effect against procarcinogens activated by CYP450 2B1.

Soni and others (1993) found that certain food additives and active ingredients with general antioxidant properties [such as turmeric (*Curcuma longa*), curcumin (diferlolyl methane), and garlic] ameliorate $AFB_1$-induced mutagenicity and carcinogenicity. Dorai *et al.* (2001) demonstrated that curcumin reduces the formation of $AFB_1$-DNA adducts by modulating CYP450 function.

## 2.6 CAROTENOIDS AS CHEMOPROTECTIVE AGENTS

Carotenoids belong to the chemical group, tetraterpenes. They are a group of powerful, natural antioxidants found in fruits and vegetables that have been shown to inhibit various types of cancers. These compounds play a role in photosynthesis, but are also found in non-photosynthetic plant tissues, fungi and bacteria. Formation of the tetraterpene skeleton, e.g. phytoene, involves tail-to-tail coupling of two molecules of geranylgeranyl phosphate (GGPP) in a sequence essentially analogous to that seen for squalene and triterpenes [in plants and fungi, this double bond has the Z (*cis*) configuration whilst the bacteria have the E (*trans*) configuration]. Removal of pairs of hydrogens alternately from each side of the triene system gives lycopene, the simplest form of carotenoid **(Figure 2.8).** Depending on which proton is lost from the cyclized cation, three different alkene cyclic systems can arise at the

end of the chain, described as β-, γ-, or ε-ring systems. The β-ring system, known as beta-carotene, displays additional cyclization of the chain ends using oxygen and NADPH, giving rise to other carotenoids (zeathanthin, fucoxanthin, violaxanthin and capsanthin). The ε-ring system similarly may produce lutein (Dewick, 2002).

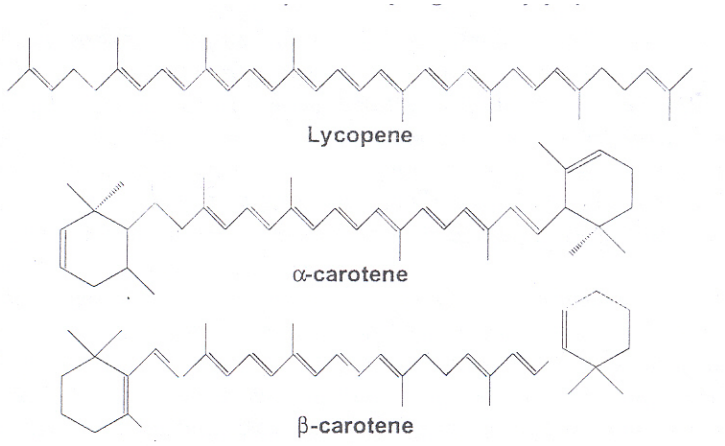



**Figure 2.8 Chemical structure of lycopene, alpha-carotene and beta-carotene (Kim *et al.*, 2000b).**

The extended π-electron system confers colour to the carotenoids, and accordingly they contribute yellow, orange and red pigmentations to plant tissues. Lycopene is the characteristic carotenoid pigment in ripe tomato fruit (*Lycopersicon esculente*; Solanaceae). The orange colour of carrots (*Daucus carota*; *Umbeliferrae/Apiaceae*) is due to beta-carotene **(Figure 2.8).** Carotenoids function along with chlorophylls in photosynthesis as accessory light-harvesting pigments, effectively extending the range of light absorbed by the photosynthetic apparatus. They also serve as important protectants for plants and algae against photo-oxidative damage, quenching toxic oxygen species. Some herbicides (bleaching herbicides) act by inhibiting carotenoid biosynthesis, and the unprotected plant is subsequently killed by photo-oxidation. Carotenoids are important antioxidant molecules in

humans, quenching singlet oxygen and scavenging peroxyl radicals, thus minimizing cell damage and affording protection against some forms of cancer (Dewick, 2002). The most significant dietary carotenoid in this respect is lycopene, with tomatoes and processed tomato products featuring as a predominant source. The extended conjugated system allows free radical addition reactions and hydrogen abstraction from positions allylic to carbon chain. Foote *et al*. (1970) described the carotenoids as powerful free radical quenchers and thus their importance in biological protection.

The vitamin A group is another important metabolites of carotenoids. Vitamin $A_1$ (retinol) effectively has a diterpene structure, but is derived in mammals by oxidative metabolism of tetraterpenoid, mainly beta-carotene, taken in the diet. Cleavage occurs in the mucosal cells of the intestine, and is catalyzed by an $O_2$–dependant dioxygenase, probably via an intermediate peroxide. This can theoretically yield two molecules of the intermediate aldehyde, retinal, which is subsequently reduced to the alcohol, retinal. Although beta-carotene cleaved at the central double bond is capable of giving rise to two molecules of retinal, there is evidence that cleavage can also occur at other double bonds, so-called excentric cleavage. Further chain shortening then produces retinal, but only one molecule is produced per molecule of beta-carotene. Vitamin $A_2$ (dehydroretinol) is an analogue of retinal containing a cyclohexadiene ring system; the corresponding aldehyde, and retinal, are also included in the A group of vitamins. The structure of these metabolites is shown in **Figure 2.9.** Retinal and its derivatives are found only in animal products and these provide some of our dietary needs. Cod-liver oil and halibut-liver oil are rich sources used as dietary supplements.

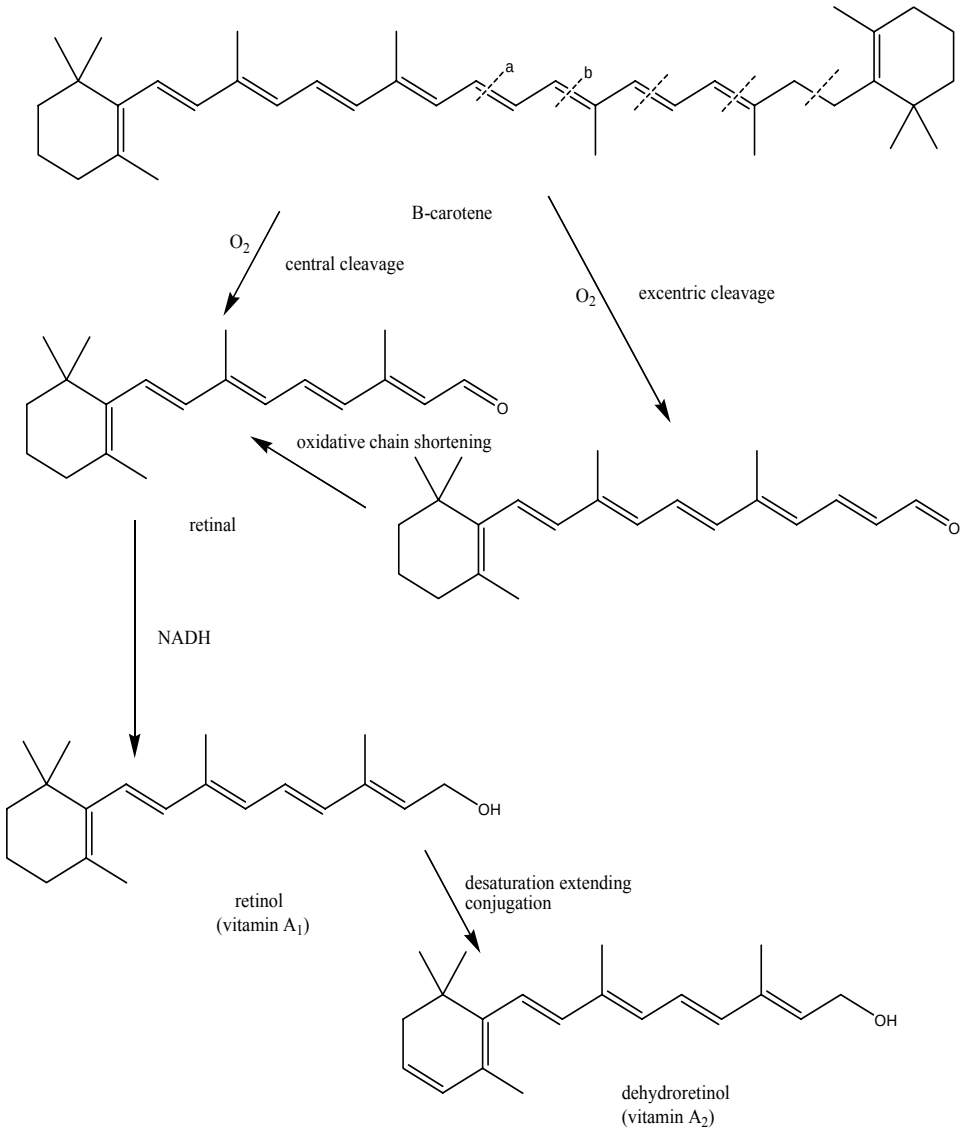



**Figure 2.9 Cleavage of beta-carotene in the body to produce vitamin A (Dewick, 2002)**

One of the most important causes of cancer is oxidative damage to DNA. If a cell containing damaged DNA divides before the DNA can be repaired, the results are likely to be a permanent genetic alteration leading to carcinogenesis. Body cells that divide rapidly are more susceptible to carcinogenesis because there is less opportunity for DNA repair before cell division (Colic and Pavelic, 2000).

Although the number of dietary carotenoids in fruits and vegetables is in excess of 40, only 13 from selected classes are absorbed, metabolized, and utilized by the human body. Vitamin A active carotenoids, such as alpha- and beta-carotene are absorbed intact and in part, converted to vitamin A. In contrast, metabolic transformations of other major carotenoids such as lutein, zeaxanthin, and lycopene involve a series of oxidation–reduction reactions (Dewick, 2002).

Although there has been considered enthusiasm for the potential anti-carcinogenic properties of beta-carotene, research findings suggest that several different carotenoids are likely to be associated with reduced cancer risks. In two intervention trials that investigated the potential protective effects of beta-carotene against cancer, an unexpected significantly higher incidence of lung cancer was found among men taking vitamin supplements compared with those not taking additional beta-carotene. These men were long-time heavy smokers, and therefore the study was inappropriately designed (Michaud *et al*., 2000). Such complex studies do not invalidate the primary importance of antioxidant nutrients but do underline the need for properly designed trials, to examine the efficacy of a single antioxidant nutrient (as distinct from complex mixtures of antioxidants in foods), with sub-objectives to examine interactions between the effects of smoking, each and several antioxidant nutrients and disease progression.

Many *in vitro* studies report that beta-carotene inhibits the growth of cancer cells, such as human colon carcinoma cells *in vitro*, by induction of apoptosis in proliferating cells (Briviba *et al*., 2001; Brockman *et al*., 1992). Beta-carotene at levels just above those seen in human plasma was shown to induce apoptosis in human adenocarcinoma cells *in vitro via* a free-

radical-mediated mechanism. The risk of lung cancer has been significantly reduced by the intake of beta-carotene and lycopene (Michaud *et al*., 2000).

Lycopene has been associated with the possible prevention of prostrate cancer and cardiovascular disease in humans. Another carotenoid, lutein was found to produce a remarkable inverse association with colon cancer in both men and women especially in subjects where the disease was diagnosed when they were young. Erythema is greatly suppressed by beta-carotene intake (Stahl *et al*., 2000).

Eight clinical trials have shown that beta-carotene and vitamin E produces regression of oral leukoplakia, but comprehensive studies in oral leukoplakia have limitations. All available evidence supports a significant role for antioxidant nutrients in preventing oral cancer. Smokers have lower beta-carotene levels in plasma (Birringer *et al*., 2003).

## 2.7 THE ROLE OF CAROTENOIDS IN AFLATOXICOSIS

Various studies in animals have indicated the promise of carotenoids as potential protectors against $AFB_1$-induced liver DNA damage (Gradelet *et al*., 1998). They concluded that beta-apo-8' carotenal, canthaxanthin, and astaxanthin exert their protective effect through the deviation of $AFB_1$ metabolism toward detoxification pathways, leading to the formation of $AFM_1$, a less toxic metabolite. Regarding beta-carotene, since it does not alter metabolism, its protective action should be mediated by other mechanisms, whereas no protective effects were observed by administration of supplementary lycopene and excess of vitamin A.

Vitamin A status is strongly related to the hepatocarcinogenicity of $AFB_1$ in rats. In fact, Nishino *et al.* (2000) observed an enhancement of $AFB_1$-induced DNA damages during vitamin A deficiency, whereas damages were reversed on vitamin A supplementation. Vitamin supplementations exceeding up to 25% of the standard requirements can reduce the negative effects of $AFB_1$ and environmental stress on turkeys.

Okotie-Eboh *et al*. (1997) conducted a study on broilers to confirm the protective properties of beta-carotene and canthaxanthin against $AFB_1$. The authors concluded that beta-carotene was not effective in ameliorating aflatoxicosis in broiler chickens, whereas canthaxanthin was shown to be somewhat effective with respect to certain blood chemistry indicators.

An overall inhibition of biochemical and cellular events thought to be precursors of $AFB_1$-induced hepatocarcinogenesis in rats was observed by as a consequence of dietary administration of carotenoid extracts. Another study on solvent-extracted carotenoids (i.e. alpha-carotene, beta-carotene and lycopene) and xanthophylls (beta-cryptoxanthin from carotenoid-rich foods) showed both *in vitro* and *in vivo* ability to reduce mutagenic effects of $AFB_1$ (Rauscher *et al*., 1998). In a study on antimutagenic activity of natural xanthophylls against $AFB_1$ on *Salmonella typhimurium*, Gonzalez *et al.* (1999) was reported that xanthophylls inhibited the mutagenicity of $AFB_1$ in a dose-dependent manner.

To study the effects of carotenoids on the initiation of liver carcinogenesis by $AFB_1$ male weanling rats were fed beta-carotene, beta-apo-8-carotenal, canthaxanthin, astaxanthin or lycopene (300 mg/kg diet) or an excess of vitamin A or were injected intraperitonial (ip) with 3-methylcholanthrene (3-mc) [6 × 20 mg/kg body wt] before and during ip treatment with $AFB_I$ (2 × 1 mg/kg body wt). The rats were later submitted to 2-acetylamiofluorene treatment

and partial hepatectomy. Placental GST-positive liver foci were detected and quantified. The *in vivo* effects of carotenoid or of 3-mc on $AFB_1$–induced liver DNA damage were evaluated using different endpoints: liver single-strand breaks (SSB) induced by $AFB_1$ and *in vivo* interaction of [$^3$H] $AFB_1$ to liver DNA and plasma albumin. Finally, the modulation of $AFB_1$ metabolism by carotenoids or by 3-mc was investigated *in vitro* by incubating [$^{14}$C] $AFB_1$ with liver microsomes from rats that had been fed with carotenoids or by treated by 3-mc. The metabolites formed were analyzed by high performance liquid chromatography (HPLC). In contrast to lycopene or to an excess of vitamin A, both of which had no effect, beta-carotene, beta–apo-8'carotenal, astaxanthin and canthaxanthin as well as 3-mc were very efficient in reducing the number and size of the liver preneoplastic foci. In a similar way as 3-mc, the P4501A-a inducers carotenoid beta-apo-8' carotenal astaxanthin and canthaxanthin, decreased *in vivo* $AFB_1$-induced DNA SSB and the interaction of $AFB_1$ to liver DNA and plasma albumin and increased *in vitro* $AFB_1$ to liver DNA and plasma albumin, and increased *in vitro* $AFB_1$ metabolism to $AFM_1$, a less genotoxic metabolite. It is concluded that these carotenoids exert their protective effects through the deviation of $AFB_1$ metabolism towards detoxication pathways. In contrast beta-carotene did not protect hepatic DNA from $AFB_1$–induced alterations, and caused only minor changes of $AFB_1$ metabolism: seemingly, its protective effects against the initiation of liver preneoplastic foci by $AFB_1$ is mediated by other mechanisms (Gradelet *et al*., 1998).

Yu *et al*. (1994), using woodchuck hepatocytes as a model to investigate the effects of vitamins A, C, and E and beta-carotene on $AFB_1$-DNA adducts, reported contrasting results. In fact, they showed that vitamin C and, particularly, vitamin A were effective in reducing $AFB_1$-DNA interaction, whereas vitamin E and beta-carotene enhanced it. The authors

concluded that additional studies are needed to understand the mechanism of enhanced adduct formation (Gradelet *et al*., 1998).

Two vitamin A2 compounds (3-dehydroretinol and 3-dehydroretinyl palmitate) mainly present in freshwater fish have been demonstrated to be very effective in inhibiting the microsome-catalyzed formation of DNA-$AFB_1$ adduct (Sahoo and Mukherjee, 2003). The inhibition should be due to modulation of microsomal enzymes, which activate the carcinogen, hence suggesting a potential chemopreventive role of these compounds against carcinogenesis induced by $AFB_1$.

The suppressive effects of the plant product, crocetin (a carotenoid) on hepatocellular lesions induced by $AFB_1$ were investigated in male rats fed with $AFB_1$ and crocetin for ten days. Thirty-five weeks later, the group that had received $AFB_1$ and crocetin, exhibited a 40% reduction in liver lesions as compared with the $AFB_1$-alone group (McLean and Dutton, 1995). The crocetin-only group exhibited no lesions. The protective effects of crocetin may result from elevated GST activity and decreased formation of hepatic $AFB_1$-DNA adducts.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 ETHICAL APPROVAL

This study involved *in vitro* tests using cell cultures. Ethical approval for the use of human cell lines for the *in vitro* experiments carried out in this study was obtained from the Durban Institute of Technology's (former M L Sultan Technikon) Ethics Committee in July 2000. The Ethics Clearance number for this study is 2000:08.

## 3.2 CHEMICALS

Most of the media and chemicals used were of tissue culture grade and were obtained from Highveld Biological (South Africa), Merck (Germany) and Sigma (USA). Details of media and reagents are included in the Appendix (section A) at the end of the dissertation.

## 3.3 CELL CUTURE AND MAINTAINANCE

### 3.3.1 Source of human cell lines

Four human carcinoma epithelial cell lines were used in this study. These comprised a lung epithelial cell line A549 (NRBM No. 0102; ATCC CCL-185), a larynx epidermoid cell line Hep2 (NRBM No. 0177 and ATCC CCL-23), an oesophageal cell line SNO (NRBM No. 0010) and a hepatocellular cell line HepG2 and (NRBM No. 0158 and ATCC 8065). They were purchased from the National Repository for Biological Materials (NRBM) in Modderfontein, Gauteng, South Africa. The cells were received in an active state and incubated immediately at 37°C in a 5% $CO_2$ atmosphere. When the cells were 80% confluent, they were subcultured, and stock cultures were stored at -70°C until required.

### 3.3.2 Cell maintenance

Cells were grown aseptically according to established tissue culture techniques (Freshney, 1987; Odhav, 1996) in 250 ml tissue culture flasks (Greiner, Germany) containing complete culture medium (CCM) comprising Dulbecco's modified eagle's medium (DMEM) with 10% sterile foetal calf serum (FCS) and 1% penicillin/streptomycin solution. The cultures were incubated at 37°C in a humidified incubator (Heraeus, Germany) with a 5% $CO_2$ atmosphere. They were examined on a daily basis for colour and turbidity changes of the growth medium. Cell growth was monitored using an inverted light microscope (Nikon, USA). The cells were harvested when they about 80% confluent.

### 3.3.3 Cell harvesting

When cells reached 80% confluency, the medium was removed and the attached cells were washed twice with pre-warmed (37°C) phosphate buffered saline (PBS), pH 7.2 or Hank's balanced salt solution (HBSS) without any calcium or magnesium. They were trypsinized by adding 5 ml of trypsinizing solution [0.25% trypsin with 0.1% EDTA (1:1 v/v) and 0.1% glucose] and incubated at 37°C for 5 min to allow cells to detach. Upon cell rounding and detachment from the flask, 2 ml of CCM was added to the cell suspension to stop the action of trypsin. The cell suspension was centrifuged (Medifuge, Heraeus, Germany) at 2000 rpm for 5 min. The cell pellet was used for further studies.

### 3.3.4 Freezing and storage of cells

The cell pellet was resuspended in 0.5 ml FCS and cooled on ice. A 20% DMSO in DMEM (1:4 v/v) solution was prepared as the cryoprotecting agent and also placed on ice. Equal aliquots (0.5 ml) of the cell suspension and the cryoprotective agent were added to a cryotube (Nunc, Japan). The tubes were left overnight at -20°C and subsequently stored in a biofreezer (Former Scientific, UK) at -70°C.

### 3.3.5 Regeneration of cells

Cryotubes containing the cells were removed from the biofreezer, swabbed with 70% ethanol and rapidly thawed in a warm water bath. The cells were transferred to 20 ml of pre-warmed CCM in flasks and incubated as previously described. The unattached and dead cells were removed by replacing the medium with fresh CCM after 6 h.

### 3.3.6 Cell enumeration

The cell concentrations used in the various experiments were determined by the dye-exclusion method of Freshney (1987) using a Neubauer counting chamber (haemocytometer). This method determines the proportions of living and dead cells per cell suspension. The test was performed in duplicate as follows:

- Fifty microlitres of the cell suspensions was mixed with 50 µl of the 0.2% trypan blue solution (Biowhittaker, Walkersville, USA) in eppendorf tubes and incubated for 1 min at RT.

- With the cover-slip in place, a micro-capillary tube was used to transfer a small amount of the trypan blue cell suspension to both chambers of the haemocytometer.
- Starting with chamber 1 of the haemocytometer, all the cells in the centre square and the four 1 mm corner squares were counted. Viable cells appeared translucent whilst non-viable cells stained blue.
- The above step was repeated for chamber 2.
- The total number of viable cells were enumerated using the following formulae:

  Each square with the cover slip in place, represented a total volume of $10^{-4}$ ml

  Cells/ml = average number of cells per primary square x $10^4$ (dilution factor)

- Percentage cell viability was calculated as follows:

$$\text{Percentage cell viability} = \frac{\text{Total viable cells (unstained)}}{\text{Total cells (stained and unstained)}} \times 100$$

- The average of the viable cells (test was done in duplicate) was calculated.

## 3.4 OPTIMIZATION STUDIES

To determine the effect of the carotenoids (lycopene and beta-carotene) on the $AFB_1$-treated epithelial cells, an optimized *in vitro* system had to be established. This involved: (1) choosing a cell line that was susceptible to $AFB_1$ and was not harmed by lycopene and beta-carotene, (2) finding a suitable assay to determine cell viability, (3) ascertaining the dose and time response of the cell line to $AFB_1$, lycopene and beta-carotene and (4) determining the $AFB_1$ uptake by the cells.

### 3.4.1 Cell lines selection

***Principle***

Four human epithelial cell lines were cultured as described (3.3). They were exposed to varying concentrations of $AFB_1$ to determine the cell line sensitivity to the toxin.

***Methodology***

The cell lines, namely, A549, SNO, Hep2 and HepG2 were subcultured onto 6 cm tissue culture plates (Greiner, Germany). Three ml of CCM containing 2 x $10^3$ cells/ml was incubated at 37°C in a 5% $CO_2$ atmosphere. The cultures were then treated with three different concentrations of $AFB_1$ prepared in DMSO (10 μg/ml, 20 μg/ml and 40 μg/ml) (Appendix, Section C) and incubated for a further 12 h. Untreated cells and 1% DMSO-treated cells were used as controls. The effect of $AFB_1$ on the cell lines was observed with an inverted microscope and the number of viable cells was determined by the trypan blue exclusion test as described in section 3.3.

***Calculations and statistics***

The number of viable cells were counted and expressed per milliliter of cells. Percentage cell viability was calculated as follows:

$$\% \text{ Cell viability} = \frac{\text{Number of viable cells in sample}}{\text{Number of viable cells in control (untreated cells)}} \times 100$$

Light micrographs were used to confirm the cell viability in each case. The student's t-test was used to determine the significance of the results.

### 3.4.2 Selection of suitable assay to measure cell viability

***Principle***

Four quantitative methods were used to determine the most sensitive method for measuring cell viability. These included the trypan blue dye exclusion assay (Freshney, 1987), crystal violet assay (Odhav, 1997), (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) XTT assay (Scudiero *et al*., 1988) and the (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) MTT assay (Hanelt *et al*., 1994). The test principles and the parameters that were measured are outlined in **Table 3.1.** The above assays were compared for reliability, repeatability, speed and ease of use.

***Methodology***

The assays were carried out in 96-well, flat-bottomed microtitre plates (Cellstar, Greiner, Germany). HepG2 cells (200 µl containing 1.2 x $10^3$ cells/ml) were cultured overnight and treated with a three different concentrations of $AFB_1$ (10 µg/ml, 20 µg/ml and 40 µg/ml). The untreated cells represented the control and was used to determine cell viability of the treated cultures. The cultures were all incubated for 12 h at 37°C in 5% $CO_2$ atmosphere.

Each assay was carried out in triplicate and standard deviations were used to determine the reliability of the assay. To determine the variability of the results over different periods, each assay was carried out at 10 different time periods using 20 µg/ml $AFB_1$.

**Table 3.1 Principles and test parameters of cell viability assays.**

| Assay | Substrate | Parameter measured | Test principle | Quantitation |
|---|---|---|---|---|
| Trypan blue | 0.2% trypan blue | Viable cells exclude trypan blue dye | Viable cells impermeable to trypan blue | Viable cell counts using Neubauer chamber |
| Crystal violet | 0.25% crystal violet in distilled water | Cell membrane integrity | Dissolution of crystal violet that binds to intact cell membranes and is solubilized in 33% glacial acetic acid | Spectrophoto metric, $A_{590}$ |
| XTT | (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide | Cell viability by mitochondrial dehydrogenase | XTT is converted to a water-soluble, coloured formazan derivative | Spectrophoto metric, $A_{450}$, |
| MTT | (3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide | Cell viability by mitochondrial dehydrogenase | MTT is converted to an insoluble, coloured formazan derivative which is then solubilized in acidic isopropanol | Spectrophoto metric, $A_{570}$, |

***Trypan blue exclusion assay***

This assay was used to determine the number of viable cells after trypsinization as described in section 3.3. Percentage cell viability was calculated as follows:

$$\% \text{ Cell viability} = \frac{\text{Number of viable treated cells}}{\text{Number of viable untreated cells}} \times 100$$

***Crystal violet dye elution assay***

The media was removed from the wells and the remaining cells were washed with 100 µl PBS (pH 7.4). The cells were fixed with 1% glutaraldehyde (in $dH_2O$) for 12 min at RT. The

cells were subsequently stained with 20 µl of 0.25% (w/v) aqueous crystal violet for 20 min. The staining solution was removed and the remaining attached cells were washed thoroughly with tap water and air-dried. Thereafter, 100 µl of 33% (v/v) glacial acetic acid (Merck NT, South Africa) was added to each well and mixed thoroughly to allow for the maximum dissolution of the crystal violet. The amount of dye released was measured at 590 nm using an ELISA plate reader (Digital and Analog Systems, Rome, Italy).

***XTT assay***

To each culture well (200 µl), 40 µl of XTT reagent (Sigma, St. Louis, USA) was added (the preparation of XTT reagent is described in Appendix, Section C). The plates were then incubated for 2 h at 37°C in 5% $CO_2$ atmosphere. Mitochondrial dehydrogenase (MDH) converts XTT into a water-soluble coloured formazan derivative. The amount of MDH in the culture was read spectrophotometrically at 450 nm with a reference wavelength of 690 nm using the ELISA plate reader. The percentage cell viability was then calculated.

***MTT assay***

To each culture well (200 µl), 20 µl of MTT reagent (Sigma, St. Louis, USA) was added (Appendix 4.3). The plates were then incubated for 4 h at 37°C in 5% $CO_2$ atmosphere. Mitochondrial dehydrogenase converts MTT into a water-insoluble, coloured formazan derivative. At the end of the incubation, 100 µl DMSO was added to each well, mixed thoroughly and incubated for a further 1 h to dissolve the dark formazan crystals. The amount of MDH in the culture was read spectrophotometrically at 570 nm with a reference wavelength of 630 nm using the ELISA plate reader.

***Calculations and statistics***

The percentage viability was determined for all assays using the formula below:

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

The student's t-test was used to determine the significance of the results.

### 3.4.3 Determination of the optimum concentrations of $AFB_1$, lycopene and beta-carotene

***Principle***

The concentration of $AFB_1$ that causes the death of 50% of the HepG2 cells and the concentrations of lycopene and beta-carotene that causes minimal damage to HepG2 cells was determined by exposing the cells to various concentrations of the above chemicals.

***Methodology***

Three 96 well microtitre plates containing 1.2 x $10^4$ cells/ml/well were incubated overnight until they were 80% confluent. They were then exposed to two-fold serially diluted test compounds: $AFB_1$ (2 µg/ml to 128 µg/ml in DMSO) and lycopene and beta-carotene (12.5 µg/ml to 0.1 µg/ml in tetrahydrofuran) (Appendix, Section C). To determine 100% viability, 2 µl of each of DMSO, tetrahydrofuran (THF) and CCM was used. The cells were incubated for 18 h at 37°C in 5% $CO_2$ atmosphere. After incubation, cell viability was measured by the MTT assay procedures as described in section 3.4. Each treatment was carried out in triplicate.

***Calculations and statistics***

The $IC_{50}$ was established from standard curves. An additional microtitre plate was also set up for each treatment to observe the effect of various doses on cell morphology.

### 3.4.4 Determination of optimum exposure time of $AFB_1$, lycopene and beta-carotene

***Principle***

The concentration ($IC_{50}$) of $AFB_1$ that causes cell death and the concentrations of lycopene and beta-carotene that causes minimal damage ($IC_{80}$) to HepG2 cells had to be determined in order to set up a cell culture system that would give a measure of the chemoprotective effect of the carotenoids on $AFB_1$ exposed cells.

***Methodology***

HepG2 cells ($1.2 \times 10^4$ cells/ml) were cultured overnight in 96-well microtitre plates until they were 80% confluent. They were then treated with 2 µl of test molecules, namely $AFB_1$ (11 µg/ml); lycopene (0.5 µg/ml) and beta-carotene (1 µg/ml). Undiluted DMSO and untreated cells were treated as negative controls. The cultures were incubated at 37°C in 5% $CO_2$ atmosphere over time periods of 2 h to 24 h. Each treatment was carried out in triplicate. After incubation, cell viability was measured by the MTT assay procedures as described in section 3.3.2.

***Calculations and statistics***

The optimum time period for the treatments was established from standard curves.

### 3.3.5 Determination of the amount of $AFB_1$ taken up by the HepG2 cell line

***Principle***

To determine the amount of $AFB_1$ actually absorbed by the HepG2 cells, the thin-layer chromatography (TLC) densitometry method described by Fried and Sharma (1996) was used. HepG2 cells were treated with different concentrations of $AFB_1$, and the amount of $AFB_1$ absorbed by the cell was determined by subtracting the amount left in the culture medium from the original treatment amount. The extracted $AFB_1$ was analysed using TLC – Digital image based fluoro densitometry.

***Methodology***

HepG2 cells (3 ml cultures at 1.2 x $10^4$ cells/ml) were cultured in tissue culture dishes and treated with three different concentrations of $AFB_1$ (11 µg/ml, 1.1 µg/ml and 0.1 µg/ml) and incubated for 18 h as described in section 3.2. The toxin was subsequently extracted from the culture medium (440 µl). An equal volume (440 µl) of 1% NaCl (w/v) in chloroform was added to the culture medium and vortexed. This was centrifuged at 3 500 rpm at RT for 10 min. The bottom chloroform layer was collected and passed through anhydrous $NaSO_2$. The sample was dried in an oven at 70°C and subjected to TLC analysis.

Firstly $AFB_1$ standards (220 ng and 110 ng) made up in Benzene/Acetonitrile (98:2 v/v) (Merck, Germany) were spotted onto the TLC plate. Thereafter each sample extract (50 µl) was dissolved in 450 µl Benzene/Acetonitrile (98:2 v/v) and 10 µl of sample was spotted onto a 10 x 10 $cm^2$ silica gel G25 coated on polyester TLC sheet (Merck, Germany). Spots were dried in an oven at 70°C and placed in a solvent system (mobile phase) of toluene/ethyl

acetate/formic acid (6:3:1 v/v/v) for 10 min. The TLC plates were viewed under an ultraviolet (UV) light (365 nm) to detect blue fluorescent spots.

To quantitate the $AFB_1$, the TLC plate was placed inside an Uvi Tech Image Analyzer (Cambridge, UK) and an image was captured using a Digital Change-coupled device camera with an attenuation of 0.8.

***Calculations and statistics***

The data was analyzed by Uvi Soft Image Acquisition Software. The concentration of $AFB_1$ in the samples was calculated using a log plot and regression analysis. The volume, height and area of each spot was obtained from the programme. The concentration of $AFB_1$ in the samples was calculated from the $AFB_1$ standard data. Volume of each spot was plotted against concentration of $AFB_1$. A log standard curve and regression analysis was drawn using the Sigma Plot Statistics package.

## 3.5 BIOCHEMICAL EFFECTS OF LYCOPENE AND BETA-CAROTENE ON $AFB_1$-EXPOSED CELLS

Using the optimum doses and times for $AFB_1$, lycopene and beta-carotene, the biochemical changes on the selected HepG cells were investigated by determining the mitochondrial activity, cell viability and ROS in the treated cells.

### 3.5.1 Mitochondrial activity

***Principle***

The mitochondrial activity of HepG2 cells was measured spectrophotometrically using the MTT assay (Reubel *et al*., 1987) as described previously in section 3.4. The principle of this reaction was based on the reduction of the yellow-coloured MTT salt to purple formazan crystals by the succinate tetrazolium reductase system which belongs to the respiratory chain of mitochondria in viable cells showed that MTT is reduced by microsomal enzymes that require nucleotides, but agree that succinate can also act as an electron donor in MTT reduction through mitochondrial succinate dehydrogenase. An increase in the number of viable cells result in an increase in an overall enzyme activity in the sample. This augmentation leads to a greater quantity of formazan dye being produced which correlate with the number of metabolically active cells (Cole, 1986).

***Methodology***

HepG2 cell cultures (3 ml/plate of a 8 X $10^3$ cells/ml culture) were prepared as previously described (section 3.4) and incubated overnight. The cells were exposed to (a) 11 µg/ml $AFB_1$ for 18 h; (b) 0.5 µg/ml lycopene for 22 h; (c) 1 µg/ml beta-carotene for 22 h; (d) pretreated for 4 h with 0.5 µg/ml lycopene and subsequently treated with 11 µg/ml $AFB_1$ for 18 h; and (e) pretreated for 4 h with 1 µg/ml beta-carotene and subsequently treated with 11 µg/ml $AFB_1$. Untreated cells (control) were used to indicate 100% activity. The treatments were carried out in triplicate.

***Calculations and statistics***

The percentage mitochondrial activity was calculated in the same way as calculated for percentage cell viability. One-way ANOVA was used to calculate the significance of the results.

### 3.5.2 Measurement of cell viability

***Principle***

Cell viability was measured using the trypan blue dye exclusion method, which involved counting of viable translucent cells as described in section 3.3. A confirmatory test for cell viability was carried out with propidium iodide (Promega, USA) dye exclusion and quantitated using flow cytometry (O'Brien *et al.,* 2000). The above test stained only the dead HepG2 cells treated with $AFB_{1,}$ lycopene and beta-carotene.

*Methodology*

The preparation and treatment of HepG2 cell cultures were described in section 3.5.1. The cells were subsequently harvested and the pellet was resuspended in 50 µl PBS (pH 7.5) and added to 10 µl of 0.04 mg/ml PI.

HepG2 cells were gated according to forward/side scatter (Appendix) on the FACSCalibur$^{TM}$ Flow Cytometer (Becton Dickinson Immunocytometry Systems, USA). The cells were gated at 1 x $10^4$ cells per sample. The relative fluorescence intensity of viable cells (PI neg) in treated HepG2 cultures was measured by the flow cytometer. Dead cells took up the PI dye (PI pos).

***Calculations and statistics***

Fluorescence readings distinguished between viable (M1) and non-viable cells (M2) (see Appendix. Section D). The percentage cell viability was calculated. The student's t-test was used to calculate the significance of the results.

### 3.5.3 Measurement of reactive oxygen species

***Principle***

2',7'-dichlorofluoroscin diacetate (DCF-DA, Promega, USA) readily stains ROS (Shen *et al*., 1995) and is easily detected and quantitated on flow cytometry. Thus this method was employed to give sensitive analysis of the amount of ROS left in the $AFB_1$ -exposed HepG2 cells which were treated with lycopene and beta-carotene.

***Methodology***

The preparation and treatment of HepG2 cell cultures were described in section 3.5.1. The pellet was resuspended in 50 µl PBS (pH 7.4) and added to 10 µl of a fluorophore DCF-DA (5 µm) solution. The amount of ROS produced was measured using flow cytometry. The flow cytometer was set as previously described (section 3.5.2).

***Calculations and statistics***

The percentage ROS was calculated using the formula below:

$$\% \text{ ROS} = \frac{\text{fluorescence of treated cells}}{\text{fluorescence of untreated cells}} \times 100$$

Student's t-test was used to calculate the significance of the results.

## 3.6 MORPHOLOGICAL CHANGES OF LYCOPENE AND BETA-CAROTENE ON $AFB_1$-EXPOSED CELLS

To determine the chemoprotective potential of lycopene and beta-carotene on $AFB_1$-treated HepG2 cells, morphological changes were compared using phase contrast light microscopy and the ultrastructural changes were investigated by transmission electron microscopy (TEM).

### 3.6.1 Phase contrast light microscopy

***Principle***

Morphological features such as the cell number, cell shape and cell membrane integrity of the treated cells were studied at 100-1000X total magnification using phase contrast light microscopy.

***Methodology***

HepG2 cells were cultured in tissue culture dishes (3 ml cultures, 3 x $10^6$ cells/ml) and treated as it in previous section 3.5.1. Untreated cells were used to indicate normal morphology. After incubation, the cells were harvested and the pellet was washed in eppendorfs with PBS (pH 7.4).

The pellet was resuspended in 1 ml PBS and a smear was made on a light microscope slide. The cells were fixed with 1% glutaraldehyde for 15 min at RT. The smear was then stained with toluidene blue and a cover slip was attached. The cells were viewed under oil immersion at 1000X using the phase contrast microscope (Nikon, USA) which was connected to an

image-capturing device. Using the API software, images were captured and stored as JPEG files.

### 3.6.2 Transmission electron microscopy

***Principle***

The ultrastructural features such as the cytoplasm to nucleus ratio, the nuclear material, the mitochondria, and other organelles and the cell to cell contact the treated cells were studied at 17 000-45 000 X total magnification using lead-citrate staining and TEM.

***Methodology***

HepG2 cells were cultured in tissue culture dishes (3 ml cultures, 3 x $10^6$ cells/ml) and treated as it in previous section 3.5.1. Cells were processed for TEM as described in **Table 3.2** and sections were examined using a JEOL 1210 TEM. Digital images and negatives were captured for examination.

**Table 3.2 Processing of cells for TEM**

| | STEPS | Temperature/ time |
|---|---|---|
| 1 | **Primary fixation** | |
| | Cell pellet was fixed with 0.5% glutaraldehyde | 3 h |
| | They were then washed twice with PBS (pH 7.4) | 5 min |
| 2 | **Secondary fixation** | |
| | The samples were further fixed with 1% osmium tetroxide | 30 min |
| | They were then washed twice with PBS (pH 7.4) | 5 min |
| 3 | **Dehydration** | |
| | The samples were dehydrated in an ascending graded series of ethyl alcohol solutions: | |
| | 50% (v/v),<br>70% (v/v),<br>90% (v/v),<br>100% (v/v) alcohol and<br>5% propylene oxide in Spurr's resin | 10 min<br>10 min<br>10 min<br>10 min<br>35°C/30 min |
| 4 | **Infiltration** | |
| | The cells were drained of most of the propylene oxide.<br>A 1:1 (v/v) mixture of 100% ethyl alcohol and Spurr's resin | RT/30 min |
| 5 | **Embedding** | |
| | The mixture was replaced with 100% Spurr's resin | RT/30 min |
| 6 | **Polymerization** | |
| | The resin was replaced and cured in oven with caps on. | 60°C/24 h |
| | The cells were polymerized further with caps off. | 60°C/24 h |
| 7 | **Cutting** | |
| | Sections (50nm) were cut on a Reichert ultracut microtome | |
| 8 | **Staining** | RT/25min |
| | Thin sections were picked up on copper grids and double stained with Uranyl acetate and Lead citrate | |

## 3.7 GENOMIC EFFECTS OF LYCOPENE AND BETA-CAROTENE ON $AFB_1$-EXPOSED CELLS

The molecular changes due to $AFB_1$ and the effect of lycopene and beta-carotene were investigated by: ascertaining the mechanism of cell death (necrotic or apoptotic), by measuring the expression of the *p53* gene, and by measuring the amount of $AFB_1$-DNA adducts formed.

### 3.7.1 Determination of the mechanism of cell death by DNA fragment end labelling for apoptotic bodies

***Principle***

Cell death has been shown to occur by two major mechanisms, necrosis and apoptosis. Classical necrotic cell death occurs due to noxious injury or trauma while apoptosis takes place during normal cell development, regulating cellular differentiation and number. While necrotic cell death results in cell lysis, cellular apoptosis is characterized morphologically by cell shrinkage, nuclear pyknosis, chromatin condensation, and blebbing of the plasma membrane. Apoptosis is induced by external stimuli such as Fas ligand, tumour necrosis factor (TNF-α) and deficiency in survival factors or by internal stimuli such as DNA damage, signalling "imbalance" or other stresses such as viral infections. Endogenous endonucleases are also activated, which generate free 3'-OH groups at the ends of DNA fragments which can be labeled for the detection of apoptotic cells using a histochemical or cytochemical technique (Willingham, 1999).

In this study, the Tdt-FragEL™ DNA Fragmentation Detection Kit (Oncogene Research Products, San Diego, USA), a non-isotopic system, was used for the labelling of DNA breaks in apoptotic nuclei in cell preparations fixed on slides. This kit not only enabled measuring the DNA modification, but also allowed the observation of histological changes in apoptotic cells by the identification of the well characterized morphological changes.

The principle steps in the FragEL™ DNA Fragmentation assay were as follows: (1) the terminal deoxynucleotidy1 transferase (TdT) binds to exposed 3'-OH ends of DNA fragments generated in response to apoptotic signals and catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides, (2) biotinylated nucleotides are detected using a streptavidin-horseradish peroxidase (HRP) conjugate, (3) diaminobenzidine reacts with the labeled sample to generate an insoluble, coloured substrate at the site of DNA fragmentation, and (4) counterstaining with methy1 green aids in the morphological evaluation and characterization of normal and apoptotic cells. A schematic representation of the principle steps in the assay are shown in **Figure 3.1.**

***Methodology***

HepG2 cells were prepared overnight in tissue culture dishes (3 ml, 3 x $10^4$ cells/ml) and treated with 30 µl of (1) 11 µg/ml $AFB_1$, (2) 33 µg/ml $AFB_1$, (3) 11 µg/ml $AFB_1$ and 0.5 µg/ml lycopene, (4) 33 µg/ml $AFB_1$ and 0.5 µg/ml lycopene, (5) 11 µg/ml $AFB_1$ and 1 µg/ml beta-carotene, (6) 33 µg/ml $AFB_1$ and 1 µg/ml beta-carotene. The seventh culture dish was left untreated for comparison. All cultures were treated and incubated as before (section 3.3). After incubation, the media was aspirated and the cells were prepared for labelling as described in **Table 3.3.** All experiments were carried out in triplicate.

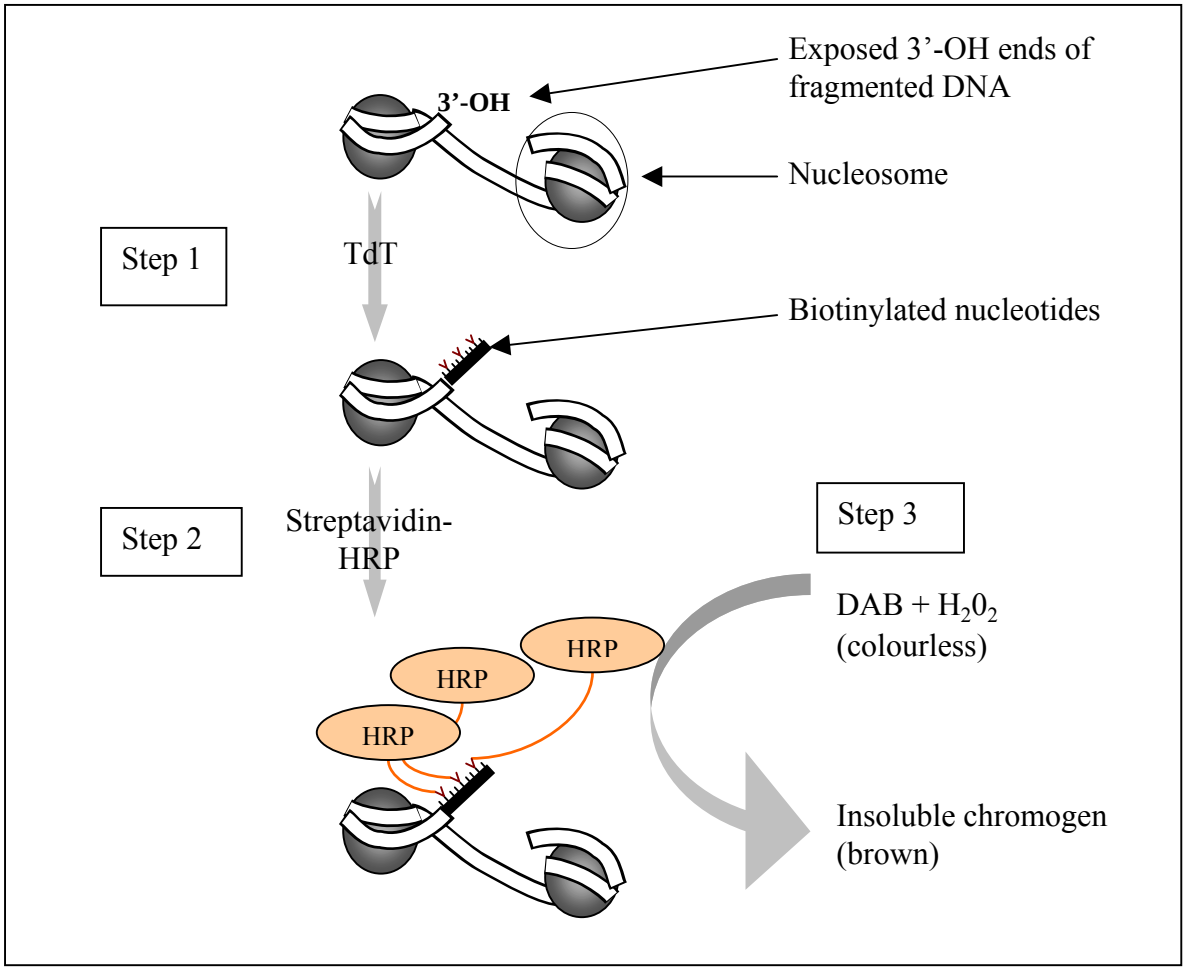



**Figure 3.1 The principle steps involved in the labelling of DNA fragments in HepG2 cells.**

The cells were viewed under light microscopy and the digital images were captured.

**Table 3.3 Histochemical labelling of DNA fragments in HepG2 cells.**

| | SAMPLE PREPARATION | Time, Temperature |
|---|---|---|
| 1. | **Cell harvesting**<br>Trypsinize and collect pellet by centrifugation @ 1500 rpm (in eppendorfs) | 10 min, RT |
| 2. | **Slide preparation**<br>Dot onto Poly L-Lysine coated slides (20 µl each; spread with coverslip) Air dry | 30 min |
| 3. | **Fixative**<br>Use 4% para-formaldehyde (PFA) in PBS (250 ml in slide holder)<br>Wash in PBS (in slide holder)<br>*For labelling at a later stage: Dry & Store in fridge* | 10 min |
| | **DNA FRAGMENTATION LABELLING** | |
| 1. | **Permeabilize**<br>1 X TBS<br>Proteinase K *(1 µl PK + 99 µl 0.01M Tris)*<br>Wash in 1 X TBS | 15 min |
| 2. | **Endogenous Peroxidase**<br>3% $H_2O_2$ in methanol *(10 µl 30% $H_2O_2$ + 90 µl methanol)*<br>Wash in 1 X TBS | 5 min |
| 3. | **Labelling**<br>Equilibrium Buffer *(20 µl 5xTdT Buffer + 80 µl $dH_2O$)*<br>Prepare TdT Reaction Mixture:<br>- vortex TdT Reaction Mix, Pulse-spin TdT enzyme<br>*(57 µl TdT L.R.Mix + 3 µl TdT enzyme)*<br>Blot off Buffer<br>60 µl TdT L.R. Mix (cover with parafilm) | 10-30 min<br><br><br><br>1.5 h 37°C |
| 4. | **Terminate**<br>Wash slide in 1 X TBS<br>(Pre-warm Stop buffer to $37^0$C)<br>100 µl Stop Solution<br>Wash slide in 1 X TBS | 5 min, RT |
| 5. | **Detection**<br>100 µl Blocking Buffer<br>Blot, no washing<br>*Add Conjugate (2 µl 50x Conjugate + 98 µl Blocking Buffer, in humid chamber)*<br>DAB (1 tablet to 100 µl; then 10 µl + 90 µl $dH_2O$)<br>Wash in $dH_2O$ | 10 min, RT<br><br>RT, 30 min<br>10-15 min, RT |
| 6. | **Counterstain**<br>100 µl Methyl Green<br>Blot off<br>Remove Wells | 3 min, RT |
| 7. | **Dehydration**<br>80% Ethanol<br>100% Ethanaol<br>Xylene<br>Mount in Entellen | dip 2-4 times |

***Observation of Results***

The dark brown apoptotic cell bodies were observed on the pictures and compared to the counterstained green untreated cells.

### 3.7.2 Measurement of p53 protein

***Principle***

The p53 protein product inhibits mitosis thereby suppressing tumour formation. The p53 is a recessive gene that is, as long as the cell contains one normal allele, tumour suppression continues. Oncogenes, by contrast, behave as dominants; one defective allele can predispose the cell to tumour formation. The product of the tumor suppressor gene *p53* is a protein of 53 kilodaltons. The p53 protein prevents a cell from completing the cell cycle if its DNA is damaged or the cell has suffered other damage. It does this by binding to a transcription factor called E2F. This prevents E2F from binding to the promoters of such proto-oncogenes as c-*myc* and c-*fos*. Transcription of c-*myc* and c-*fos* is needed for mitosis so blocking the transcription factor needed to turn on these genes prevents cell division. If the damage is minor, p53 halts the cell cycle (hence cell division) until the damage is repaired. If the damage is major and cannot be repaired, p53 triggers apoptosis. These functions make p53 a key player in protecting humans against cancer; hence it is an important tumour suppressor gene. More than half of all human cancers do, in fact, harbour p53 mutations and have no functioning p53 protein. The *p53* gene mutations have been closely-linked to the development of $AFB_1$-induced liver cancer.

***Methodology***

HepG2 cells were cultured overnight in tissue culture dishes (3 ml, 3 x $10^5$ cells/ml) and treated as in section 3.5.1. The principle steps involved in the assay are presented in **Figure 3.2.**

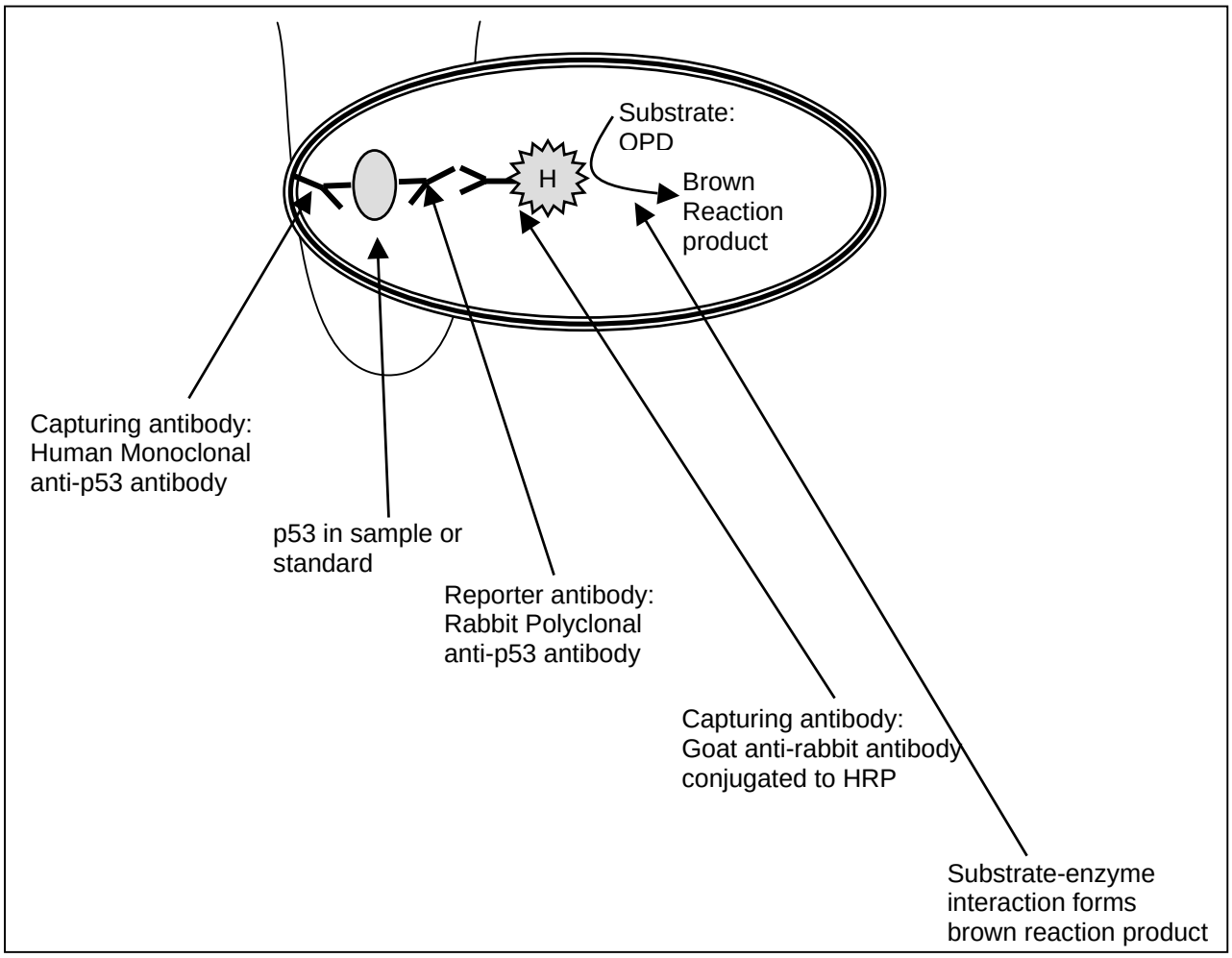



**Figure 3.2 Schematic representation of the principle steps of the p53 ELISA assay.**

The pantropic p53 ELISA kit (Oncogene Research Products, San Diego, USA) was used to quantitate the total amount of p53 (mutant and wild type) protein in the treated and untreated cells. **Table 3.4** outlines the protocol of the assay procedure.

**Table 3.4 Protocol of the pantropic p53 ELISA assay.**

| | SPECIMEN | Time, Temperature |
|---|---|---|
| 1. | **Sample preparation: Cells in tissue culture**<br>Trypsinize and collect cell pellet<br>Centrifuge @ 2 000 rpm (in eppendorfs)<br>Resuspend the pellet in 20x cell pellet volume cell wash buffer<br>Wash three times<br>Resuspend pellet in 10x cell pellet volume of lysis buffer<br>Incubate with occasional mixing<br>Add 100 µl antigen extraction agent per 500 µl cell suspension<br>Incubate with occasional mixing<br>Centrifuge @ 2 000 rpm (in eppendorfs)<br>Collect and retain supernatant.<br>Store at -70°C for future analysis | <br>5 min<br><br><br>30 min<br><br>5 min<br><br>5 min<br>10 min |
| 2. | **ELISA**<br>**Preparation of standards**<br>Prepared p53 stock standard solution (1 500 pg/ml) as follows:<br>Add 1 677 µl standard diluent provided to lyophylised p53 protein<br>Prepared a 2-fold dilution series of p53 standards (1 500 pg/ml to 93.75 pg/ml) in standard diluent. | <br><br><br><br>keep on ice<br>15 min |
| 3. | **Wetting of anti-p53 antibody in wells of 96 well plate provided**<br>Prepare wash buffer (50 ml 10x wash buffer provided diluted in d.$H_2O$<br>Add 200 µl wash buffer to each well and remove the buffer by shaking out onto paper. | |
| 4. | **Addition of standards and samples**<br>Pipette 100 µl of standards and samples into wells.<br>Cover plates with cling wrap. | <br><br>12 h, $4^0$C |
| 5. | Shake out well contents onto paper.<br>Wash 4x with 200 µl wash buffer. | |
| 6. | **Addition of reporter antibody**<br>Prepare Reporter antibody as follows:<br>- Add 125 µl reporter antibody provided to 2 375 µl assay buffer<br>Pipette 100 µl reporter antibody to each well Incubate. | <br><br><br>2 h, RT |
| 7. | Shake out well contents onto paper.<br>Wash 4x with 200 µl wash buffer. | |
| 8. | **Addition of peroxidase conjugate**<br>Prepare peroxidase conjugate as follows:<br>- 12.5 µl peroxidase conjugate provided to 2 487.5 µl assay buffer<br>Add 100 µl 1x peroxidase conjugate to each well. Incubate. | <br><br><br>1 h, RT |
| 9. | Shake out well contents onto paper.<br>Wash 4x with 200 µl wash buffer per well. | |
| 10. | **Addition of substrate solution**<br>Dissolve 1 o-phenyldiamine (OPD) tablet in 4 ml substrate diluent. (Use within 30 min and avoid exposure to light)<br>Add 100 µl substrate solution to each well. Incubate. | <br><br>30 min, RT in the dark |
| 11. | Shake out well contents onto paper.<br>Wash 4x with 200 µl wash buffer per well. | |
| 12. | Add 100 µl stop solution to each well in the same order as the substrate solution. | |
| 13. | Read absorbance at 490/630 nm within 30 min of adding stop solution. | |

***Calculations and statistics***

Absorbance readings of the p53 protein standard were used to draw up the standard curve. The amount of protein in the samples were calculated using the standard curve. The student's t-test was used to calculate the significance of the results.

### 3.7.3 Measurement of $AFB_1$-$N^7$-guanine DNA adducts

***Principle***

Aflatoxin $B_1$ adduct formation occurs *via* the biotransformation pathway in liver cells. This occurs through the microsomal mixed function oxidases and leads to the formation of a highly reactive electrophilic intermediate: $AFB_1$-8,9-epoxide, which reacts with nucleophilic sites in cellular macromolecules to form adducts. The major adducts of $AFB_1$ are $AFB_1$-$N^7$-guanine and $AFB_1$-albumin. Traditionally, $AFB_1$-$N^7$-guanine adducts have been measured in urine as they are quickly excreted, reflecting relatively recent exposure to $AFB_1$, whilst $AFB_1$-albumin adducts are measured in serum and may last for a few weeks. Analytical methods used to detect and quantitate $AFB_1$-$N^7$-guanine adducts include multiple monoclonal antibody affinity chromatography and reverse-phase liquid chromatography used for rat urine (Eaton and Groopman, 1994). In this study, hepatocytes were exposed to low levels of $AFB_1$ in the presence of carotenoids. Aflatoxin $B_1$-$N^7$-guanine adducts were measured using the indirect competitive ELISA method developed by Vidyasagar *et al*. (1997). A schematic representation of the principle steps is given in **Figure 3.3.**

The principle steps involved, (1) coating the well with antigen (CT-DNA-$AFB_1$), (2) blocked for non-specific interaction, (3) incubating with antibody and standard $AFB_1$ adduct, (4)

incubating with probe (alkaline phosphatase-IgG), (5) developing the probe with substrate p-nitrophenyl phosphate in diethanolamine (PNPD), and (6) reading the absorbance at 405 nm.

***Methodology***

The HepG2 cells were cultured overnight as described in 3.3. The sample size was four with an individual culture size of 3 ml. The treatments included, (1) $AFB_1$ (33 μg/ml), (2) $AFB_1$ (33 μg/ml) and 4 h pre-treatment with lycopene, and (3) $AFB_1$ (33 μg/ml) and 4 h pre-treatment with beta-carotene. An untreated culture dish was also maintained. Cultures were incubated for 22 h at 37ºC with 5% $CO_2$ atmosphere. Each treatment was carried out in triplicate.

After treatment, the cells were centrifuged at 2 000 rpm for 10 min at 4°C. The cell pellet was resuspended in PBS (pH 7.4) in 1.5 ml sterile eppendorf tubes and centrifuged at 2 000 rpm in an Eppendorf table top centrifuge (Germany) at 4°C for 5 min. Thereafter, 20 μl of lysis buffer (20 mM EDTA, 100 mM Tris, pH 8.0), and 0.8% SDS (w/v) was added to the pellet and mixed by stirring with a sterile pipette tip, before adding 10 μl RNase A (500 units/ml) (Roche, USA). The lysed cells were mixed well by flipping the tip of the tube. Vortexing was avoided. The cells were incubated at 37°C for 2 h. Proteinase K (10 μl, 20 mg/ml, Ambion, USA) was thereafter added and mixed (as above) and incubated in a 50°C waterbath for 12 h. The DNA from the cell samples were quantitated at 260 nm and 280 nm using a Cary spectrophotometer (Varian, USA) to ensure sufficient DNA was present for quantitation of $AFB_1$-DNA adducts.

The DNA-adduct formed were quantitated as follows:

1. Microtitre plates (Microlon-600 flat-bottomed polystyrene, 96-well plates, Greiner, Germany) were coated with 50 µl coating buffer (0.01M sodium phosphate buffer, pH 7.2 with 0.01% sodium azide) per well containing 750 ng antigen CT-DNA-$AFB_1$ (equivalent to 16 ng equivalent of $AFB_1$) (Osmania University, Hyderbad, India).
2. The plate was dried overnight at 37°C in an incubator.
3. Antigen-coated plate was washed 3 times with washing buffer (0.01M sodium phosphate buffer, pH 7.2 with 0.05% Tween 20, 0.85% NaCl and 0.01% sodium azide.
4. Wells were blocked for non-specific binding with 50 µl blocking buffer (0.01M sodium phosphate buffer, pH 7.2 with 0.85% NaCl and 0.01% sodium azide containing 0.1% gelatin) per well, for 30 min at 37°C.
5. The plate was washed three times with washing buffer. Thereafter 25 µl of antisera diluted in 2 X diluent buffer (0.02M sodium phosphate buffer, pH 7.2 with 1.7% NaCl and 0.04% BSA, radioimmunoassay grade). The antisera was diluted (1:3 500 v/v) and added to each well along with different concentrations of standard $AFB_1$-$N^7$-guanine (Osmania University, India) in 25 µl distilled water. This was incubated at 37°C for 2 h.
6. The wells were washed three times and incubated at 37°C for 1 h with 50 µl per well of 1:5000 diluted alkaline-phosphatase-labeled anti-rabbit IgG (raised in goat) (Osmania University, India) in PBS (0.01 M sodium phosphate buffer with 0.85% NaCl, pH 7.2).
7. After 1 h, the plate was washed three times and 150 µl substrate buffer [1.25 mg/ml p-nitrophenyl phosphate in 10% diethanolamine in HCl buffer (pH 9.6) containing 0.05 mM $MgCl_2$]was added per well.

8. The reaction was terminated after 45 min by adding 100 µl stopping reagent (5 N NaOH) per well.
9. The absorbance at 405 nm for sample and reagent blank was recorded with an ELISA reader.

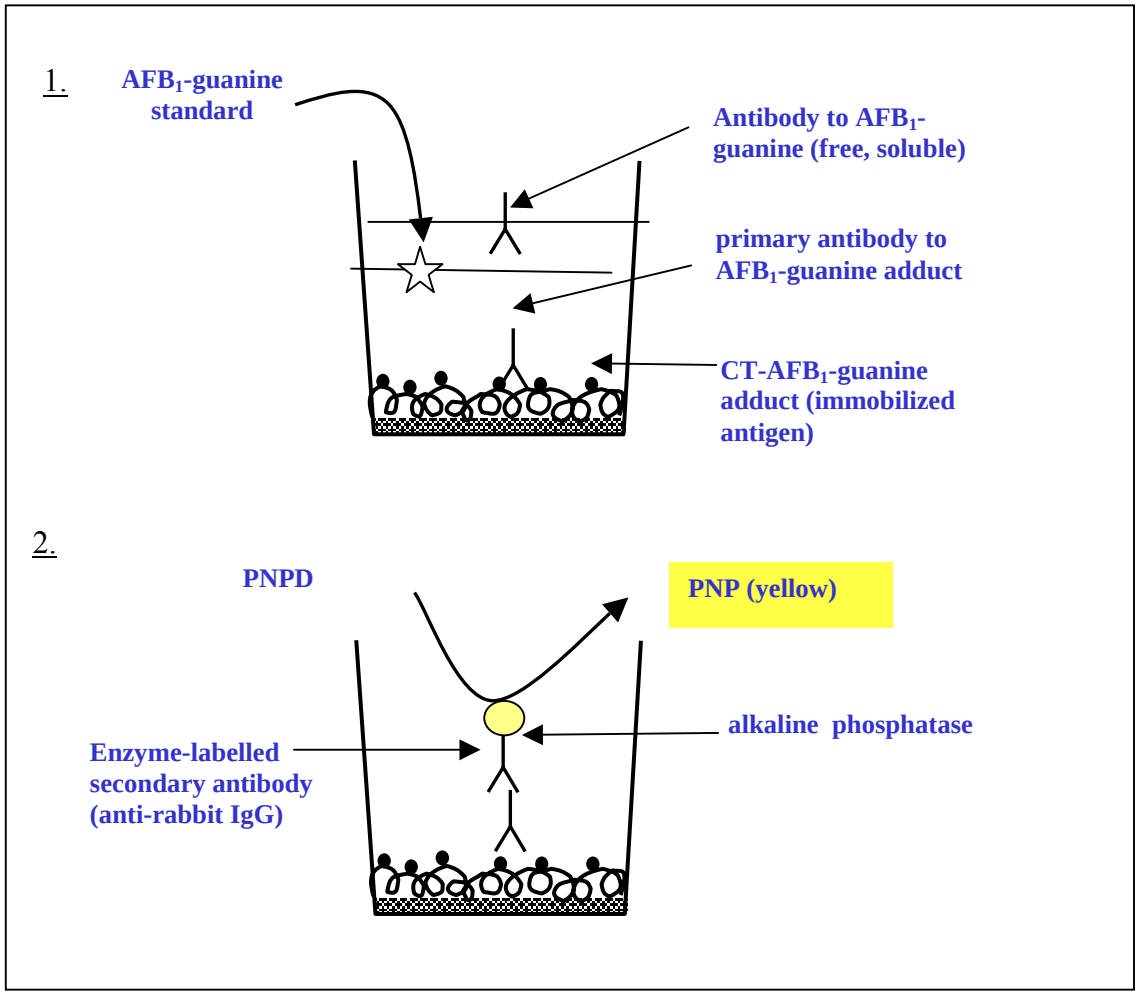



**Figure 3.3 Schematic representation of the principle steps in indirect competitive ELISA assay for the measurement of $AFB_1$-DNA adducts.**

***Calculations and statistics***

Mean and coefficient of variation were calculated using the SPSS 11 version software.

# CHAPTER 4

# RESULTS

## 4.1 OPTIMISATION STUDIES

### 4.1.1. Selection of cell line

A comparison of the susceptibility of lung (A549), larynx (Hep2), oesophageal (SNO) and liver (HepG2) epithelial cell lines to $AFB_1$ showed no significant difference in viability ($p \leq 0.05$) between the Hep2 cell line and A549 cell line. The cell viability of the SNO and the HepG2 cells was lower as shown in **Table 4.1**. The cell viability at different concentrations of $AFB_1$ (**Figure 4.1)** was best observed with the HepG2 and A549 cells. The HepG2 cells showed the biggest difference between the control and treated cells and the least toxicity to the 1% DMSO (solvent control). Light microscopic observation (**Figure 4.2 - Figure 4.5**) of the cell lines showed degenerating cells when exposed to $AFB_1$ and the unexposed cells appeared as translucent rounded, and healthy cells.

**Table 4.1 Number of viable cells (A549, SNO, Hep2 and HepG2) exposed to $AFB_1$.**

| **$AFB_1$** | **CELL LINES** | | | |
|---|---|---|---|---|
| | **A549** | **SNO** | **Hep2** | **HepG2** |
| Untreated | $3.6 \times 10^3 \pm 1.7 \times 10^2$ | $4.0 \times 10^3 \pm 2.0 \times 10^2$ | $3.5 \times 10^3 \pm 1.0 \times 10^2$ | $3.9 \times 10^3 \pm 1.5 \times 10^2$ |
| 1% DMSO | $3.4 \times 10^3 \pm 1.5 \times 10^2$ | $4.0 \times 10^3 \pm 1.9 \times 10^2$ | $3.2 \times 10^3 \pm 0.8 \times 10^2$ | $4.1 \times 10^3 \pm 2.0 \times 10^2$ |
| 10 µg/ml | $1.7 \times 10^3 \pm 0.6 \times 10^2$ | $2.6 \times 10^3 \pm 1.2 \times 10^2$ | $2.8 \times 10^3 \pm 1.3 \times 10^2$ | $1.8 \times 10^3 \pm 0.7 \times 10^2$ |
| 20 µg/ml | $1.3 \times 10^3 \pm 0.5 \times 10^2$ | $2.4 \times 10^3 \pm 1.0 \times 10^2$ | $2.7 \times 10^3 \pm 1.1 \times 10^2$ | $1.6 \times 10^3 \pm 0.7 \times 10^2$ |
| 40 µg/ml | $1.2 \times 10^3 \pm 0.4 \times 10^2$ | $2.3 \times 10^3 \pm 0.7 \times 10^2$ | $2.5 \times 10^3 \pm 0.9 \times 10^2$ | $1.2 \times 10^3 \pm 0.5 \times 10^2$ |

Mean ± SD are given of three replicates

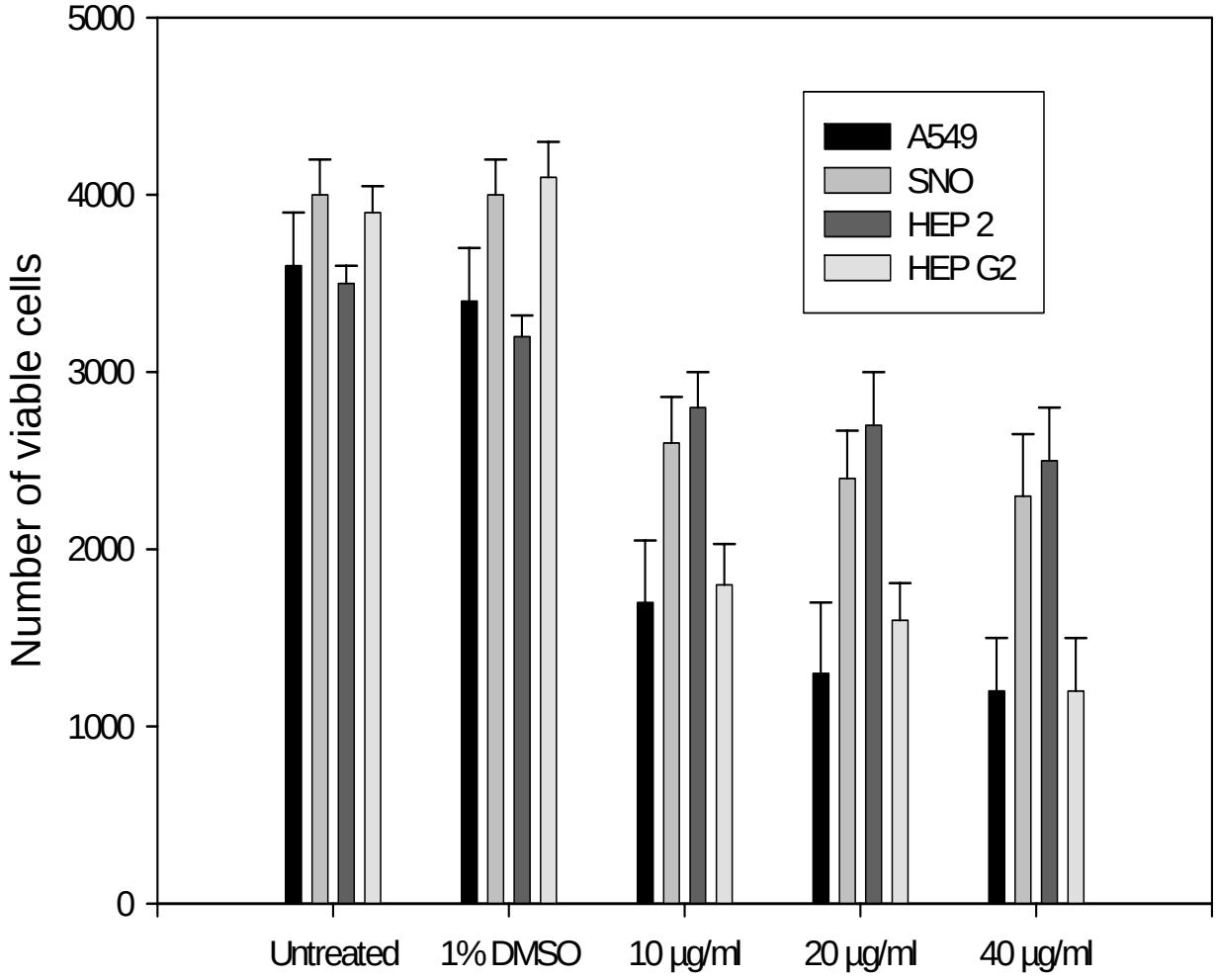
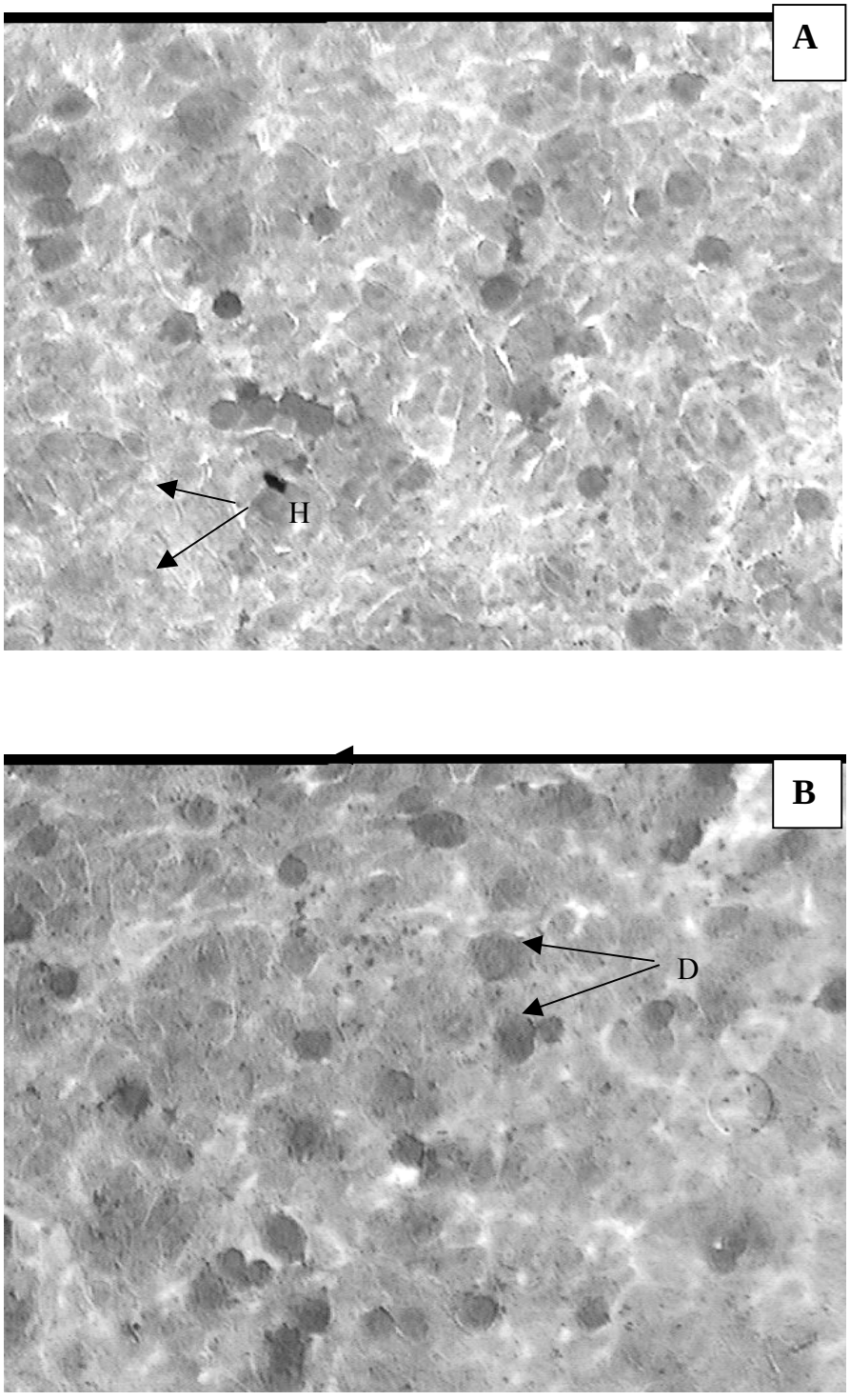
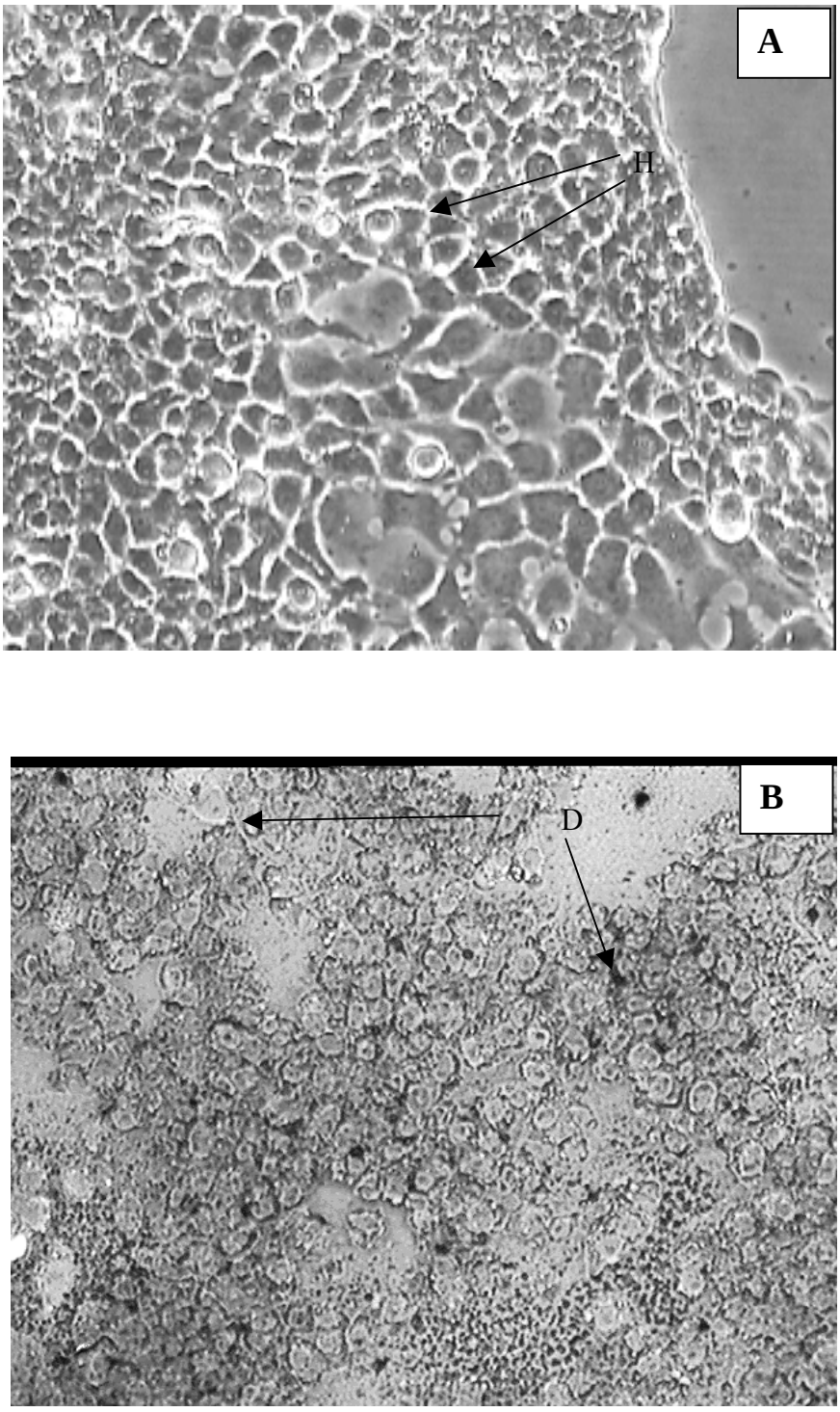



**Figure 4.1 Concentration effect of $AFB_1$ on four epithelial cell lines.**



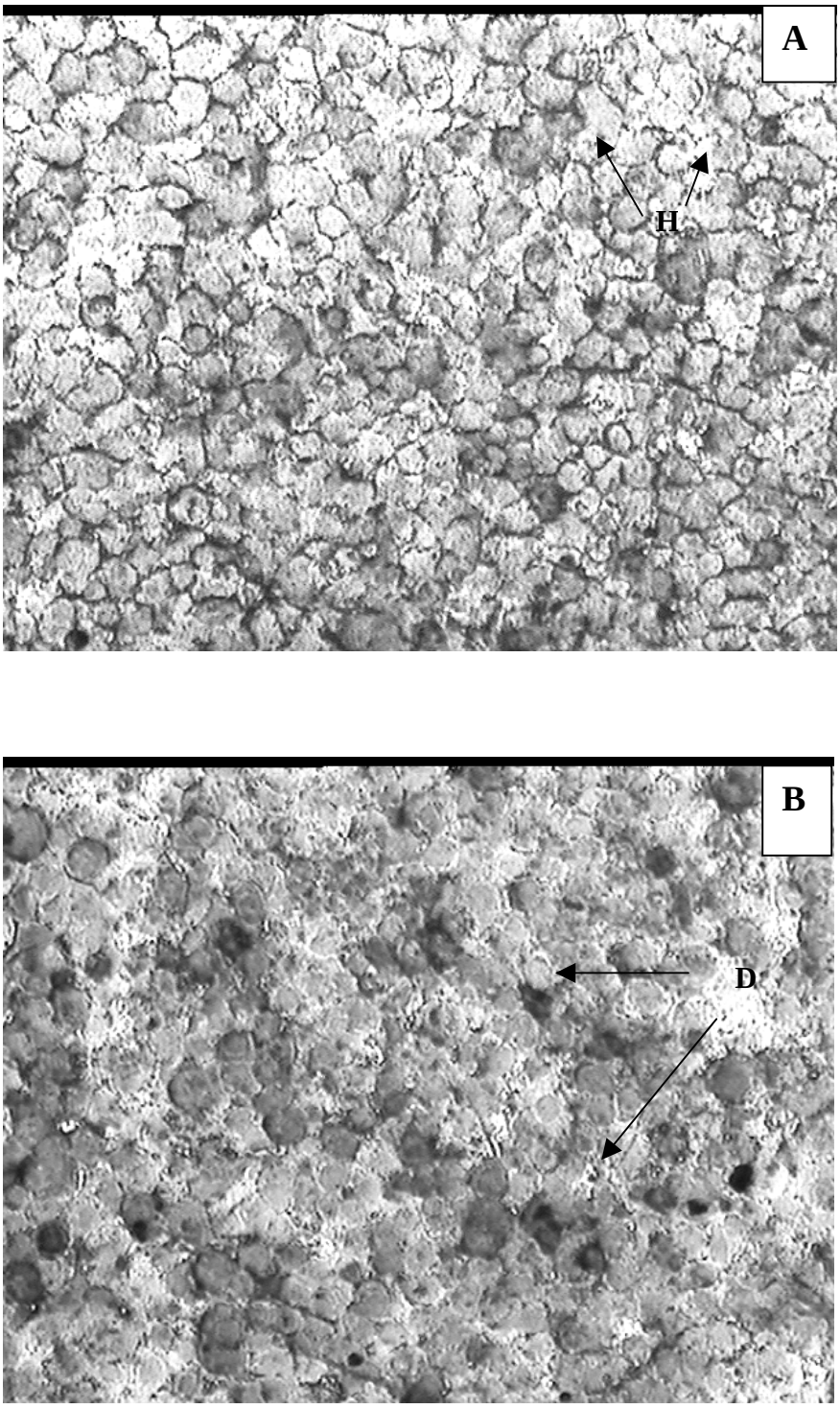

[H = healthy cells, D = degenerating cells]

**Figure 4.2 Phase contrast micrographs of A549 cells exposed to 10 µg/ml $AFB_1$ (B) and untreated cells (A); (mag. 200X).**



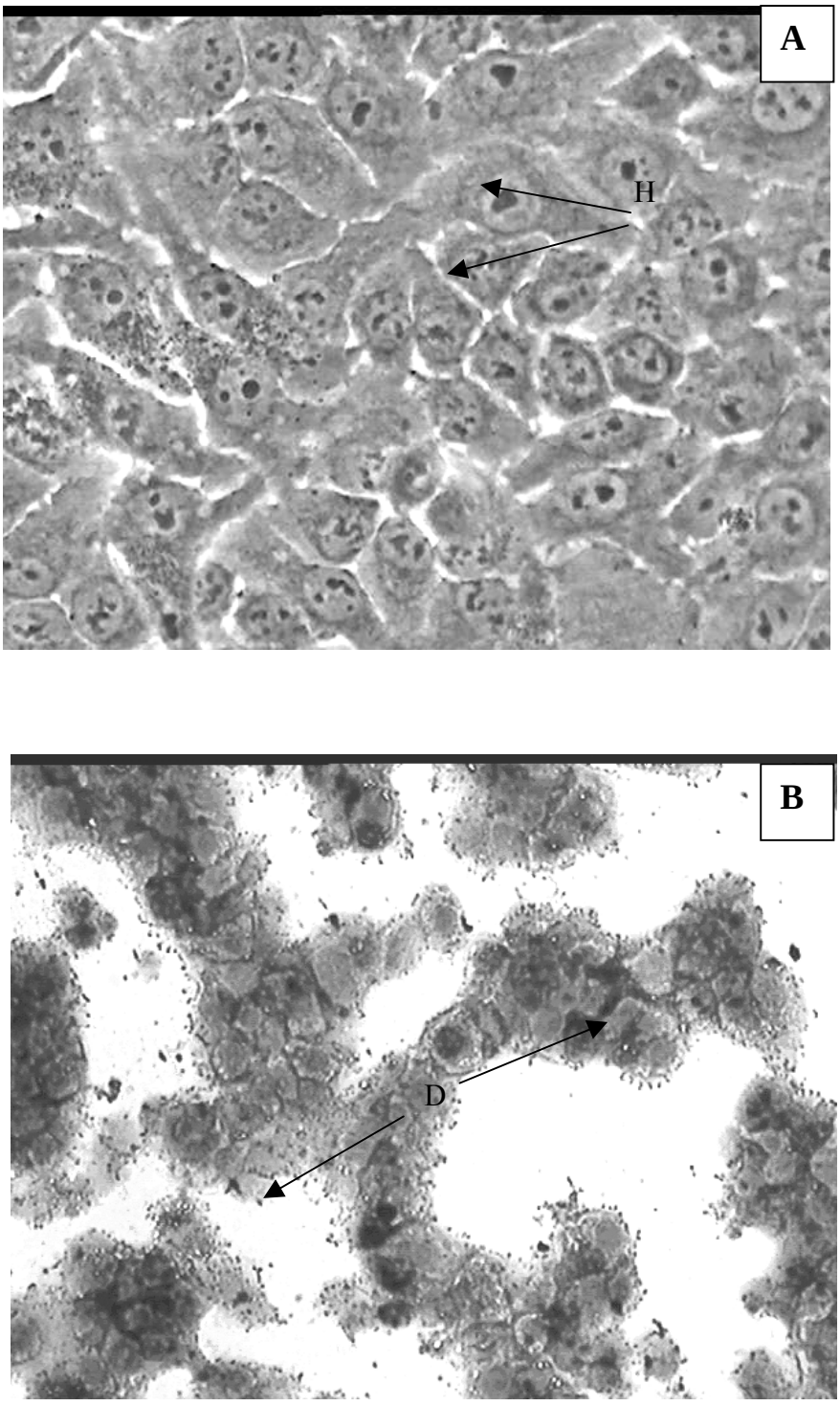

[H = healthy cells, D = degenerating cells]

**Figure 4.3 Phase contrast micrographs of SNO cells exposed to 20 µg/ml $AFB_1$ (B) and untreated cells (A) (mag. 200X).**




[H = healthy cells, D = degenerating cells]

**Figure 4.4 Phase contrast micrographs of Hep2 cells exposed to 20 µg/ml $AFB_1$ (B) and untreated cells (A) (mag. 200X).**




[H = healthy cells, D = degenerating cells]

**Figure 4.5 Phase contrast micrographs of HepG2 cells exposed to 20 µg/ml $AFB_1$ (B) and untreated cells (A) (mag. 200X).**

### 4.1.2 Selection of cell viability assay

The comparison of the viability measurements of Crystal Violet, XTT and MTT assays to the trypan blue assay showed that were not significantly different with $p < 0.05$ . The % viability is given in **Table 4.2**. The reliability between triplicate samples showed that the MTT assay was more consistent amongst the three replicates. The MTT assay wasfound to be easy to carry out, although compared to the XTT assay it took one hour longer and had an additional step (dissolving of the formazan crystals).

**Table 4.2 Comparison of percentage cell viability of HepG2 cells exposed to different concentrations of $AFB_1$.**

| $AFB_1$ (µg/ml) | % Cell Viability | | | |
|---|---|---|---|---|
| | **Trypan Blue Dye exclusion assay** | **Crystal Violet Staining assay** | **XTT assay** | **MTT assay** |
| **10** | 46 | 47 | 49 | 52 |
| **20** | 25 | 28 | 30 | 35 |
| **40** | 21 | 25 | 28 | 30 |

### 4.1.3 Determination of $IC_{50}$ for $AFB_1$ and $IC_{80}$ for lycopene and beta-carotene

An $IC_{50}$ dose of $AFB_1$ (50% toxicity) was established by treating cells with $AFB_1$ concentrations ranging from 2 µg/ml to 128 µg/ml. This $IC_{50}$ value was found to be 11 µg/ml (**Figure 4.6**). Above 17 µg/ml all the cells were killed by $AFB_1$ hence, the figure only represents the concentration interval 0 to 18 µg/ml $AFB_1$.

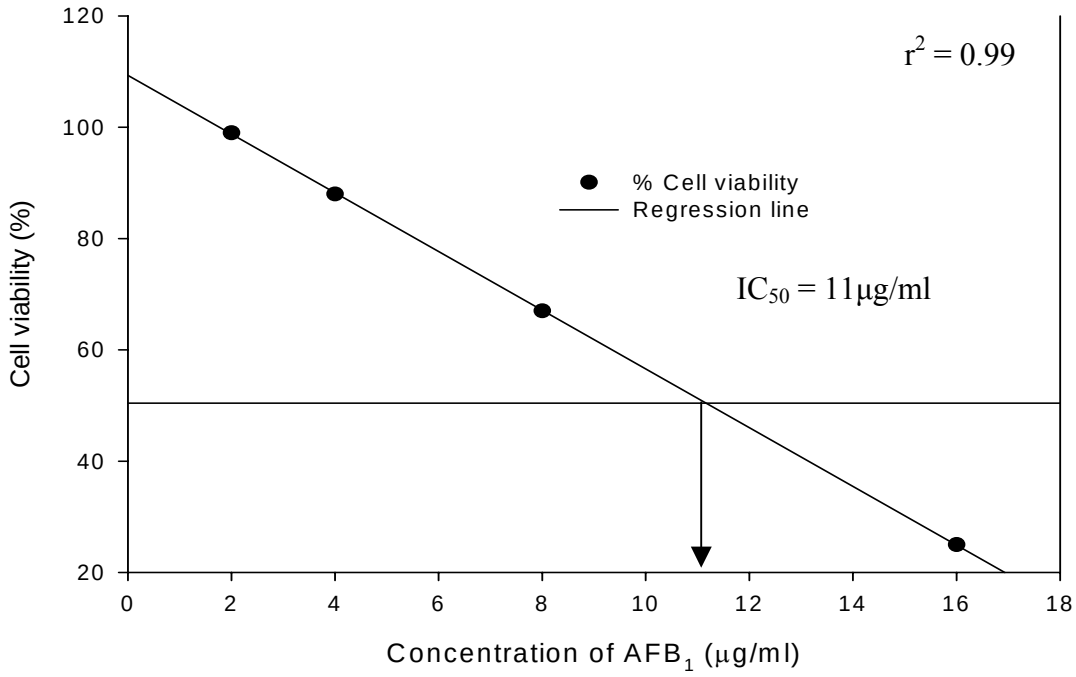



**Figure 4.6 Dose-response curve of HepG2 cells treated with $AFB_1$.**

The protective effect of lycopene and beta-carotene was determined by establishing the least damaging dose on the HepG2 cells; hence the $IC_{80}$ was determined using a dose response curve (**Figure 4.7**). The $IC_{80}$ dose-response for lycopene was 0.5 µg/ml and for beta-carotene it was found to be 1.0 µg/ml.

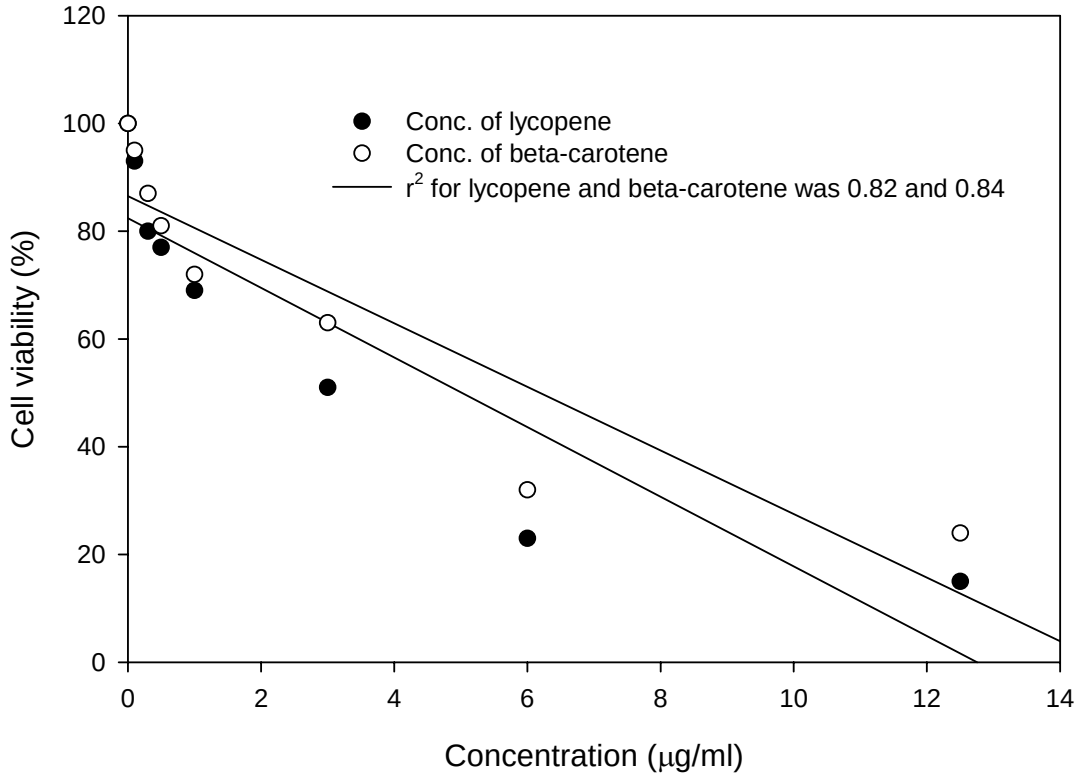



**Figure 4.7 Dose-response curve of HepG2 cells treated with lycopene and beta-carotene.**

The period taken to obtain 50% cell viability when cells were exposed to $AFB_1$ at the $IC_{50}$ dose (11 μg/ml) was 18 h. The maximum cell viability on exposure to lycopene and beta-carotene was found to be 4 h (greater than 80% cell viability) (**Figure 4.8**). Thereafter the viability gradually decreased, but did not induce toxicity of more than 70%. Thus for all subsequent experiments the cells were pre-incubated with lycopene or beta-carotene for 4 h.

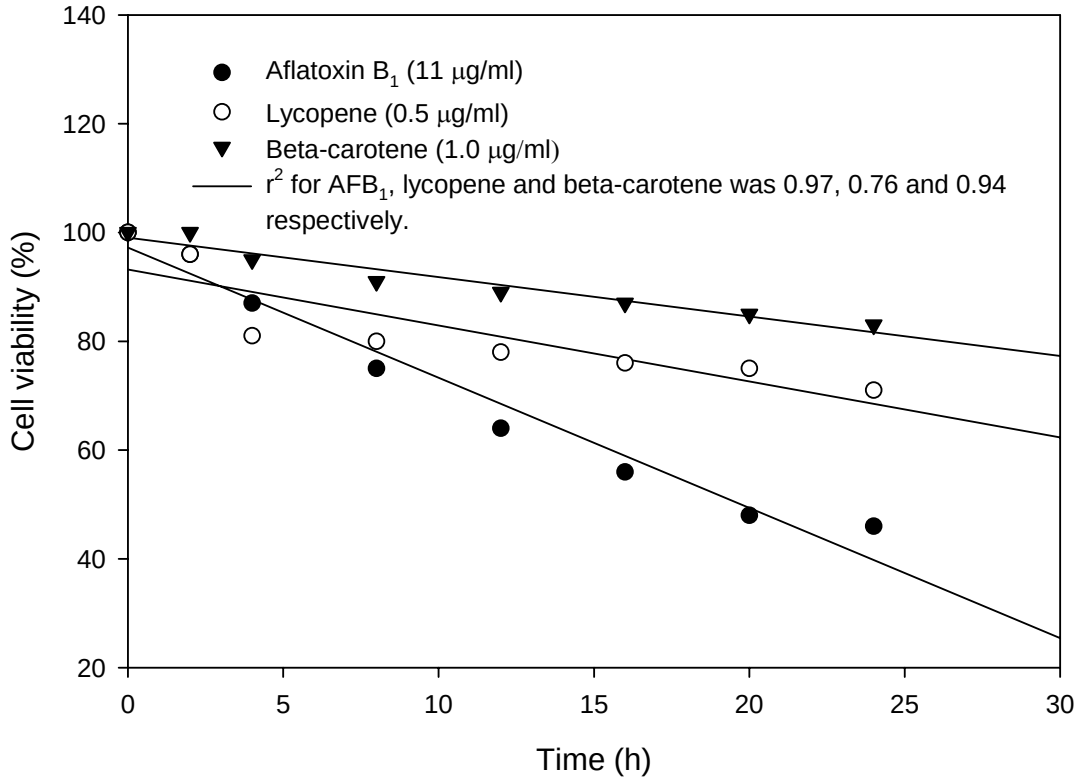



**Figure 4.8 Cell viability of HepG2 cells treated with $AFB_1$, lycopene and beta-carotene over 24 h.**

Morphological changes using the $IC_{50}$ of $AFB_1$ and $IC_{80}$ of lycopene and beta carotene show the protective effect induced by the pretreatment of the cells for four hours. This The $AFB_1$ treatment caused severe cell damage and at higher concentration of $AFB_1$ there was almost complete cell death (**Figure 4.9 B and C**). When the cells were pre-treated with either lycopene or beta carotene there was a decrease in cells damage (**Figure 4.9 C, D, E** and **F**). A comparison of the effect of lycopene and beta-carotene showed that the cells that were exposed to beta carotene resemble the untreated control HepG2 cells.

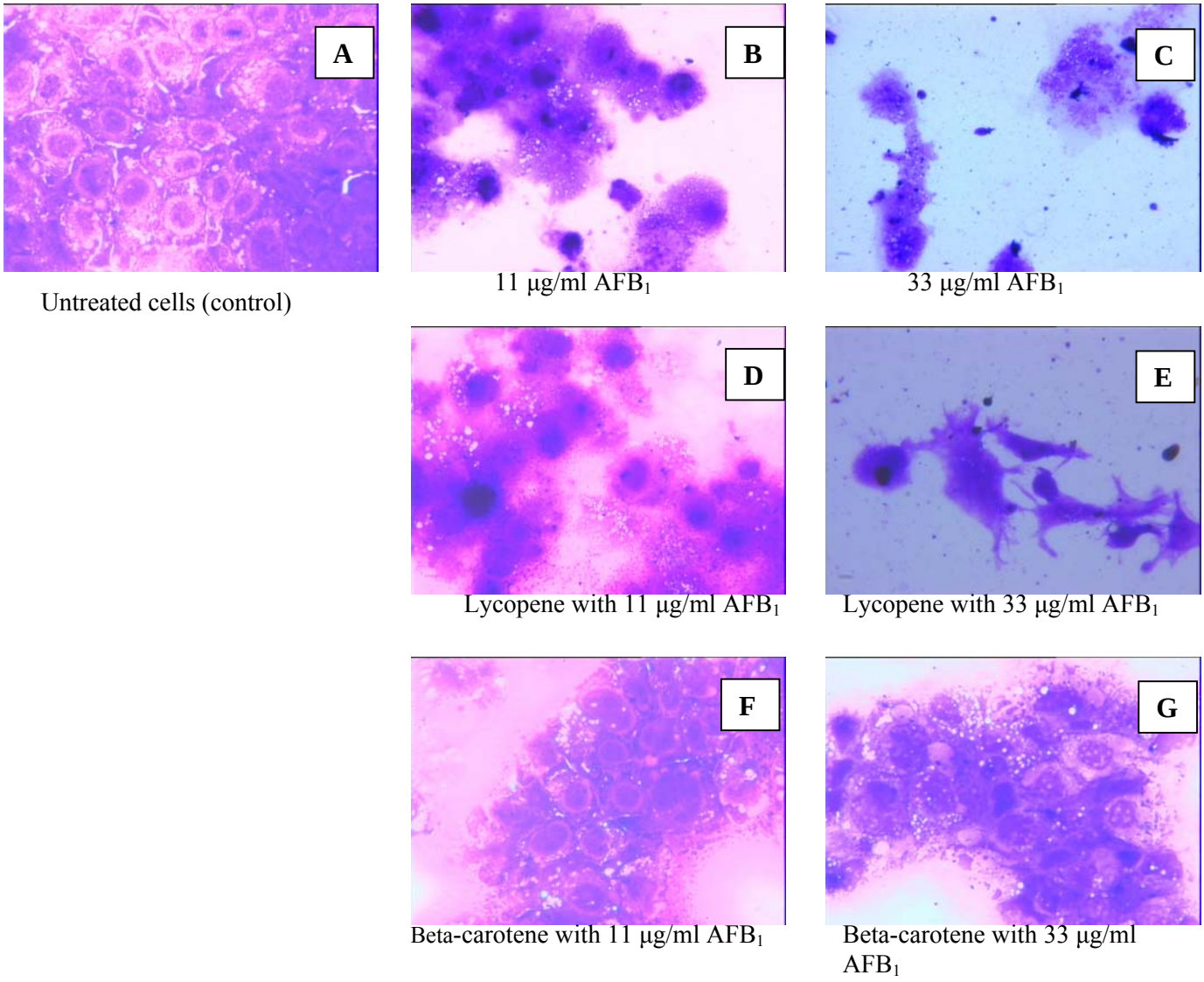



**Figure 4.9 Light micrographs of HepG2 cells pretreated with lycopene and beta-carotene for 4 h and exposed to $AFB_1$ (mag. 200 X).**

### 4.1.4 Percentage of $AFB_1$ taken up by the HepG2 cells

The amount of $AFB_1$ absorbed by the HepG2 cells was determined using TLC fluorodensitometry. The concentration of $AFB_1$ extracted from the treated HepG2 samples was determined using the standard curve for $AFB_1$ (**Figure 4.10**). The $AFB_1$ extracted from the culture medium (thus not taken up by the cell) was calculated by comparing the peak volume units of sample $AFB_1$ spots with standard $AFB_1$ spots. This amount was subtracted from the original concentration of $AFB_1$ used to treat the cells. The difference was expressed as a ratio, giving the percentage uptake of $AFB_1$ by the cell. The percentage of $AFB_1$ was calculated to be 63% of the initial dose.

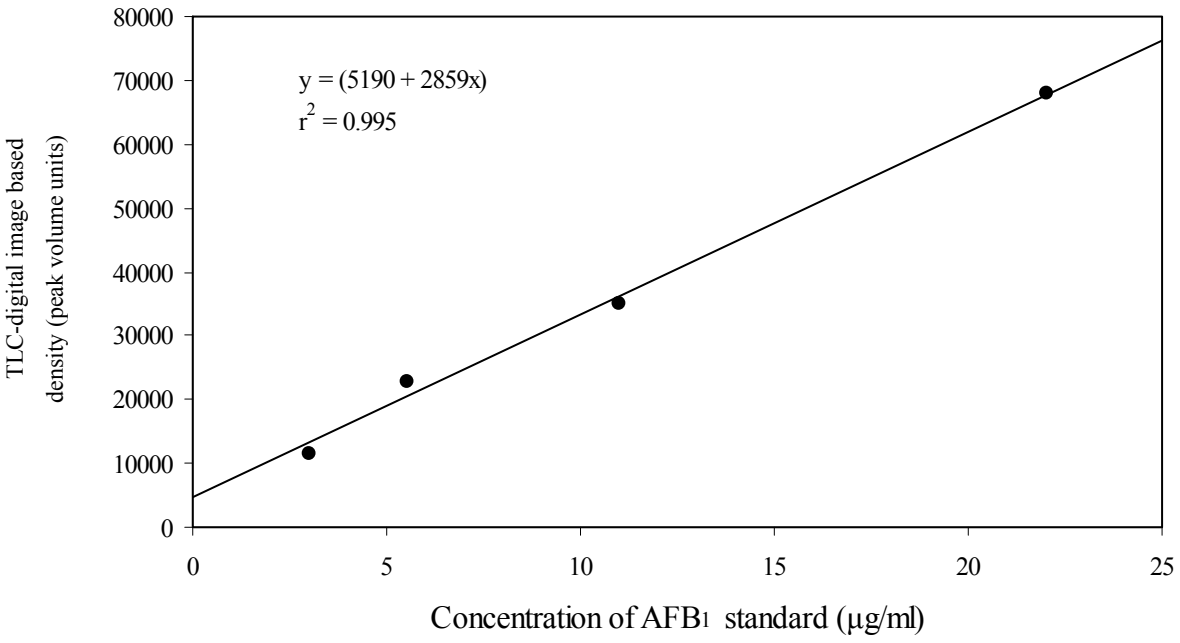



**Figure 4.10 Standard curve of $AFB_1$ by TLC densitometry.**

## 4.2 BIOCHEMICAL MEASUREMENTS OF CHEMOPROTECTION IN HepG2 CELLS

### 4.2.1 Mitochondrial activity

Mitochondrial dehydrogenase levels were used to determine the mitochondrial activity in the HepG2 cells. The results are shown in **Figure 4.11**. Cells treated with $AFB_1$ showed only 50% decrease in activity. Cells treated with lycopene or beta-carotene showed 81% and 87% activity respectively. Cells exposed to $AFB_1$ and lycopene had a 75% activity and those exposed to $AFB_1$ and beta-carotene had an 85% activity.

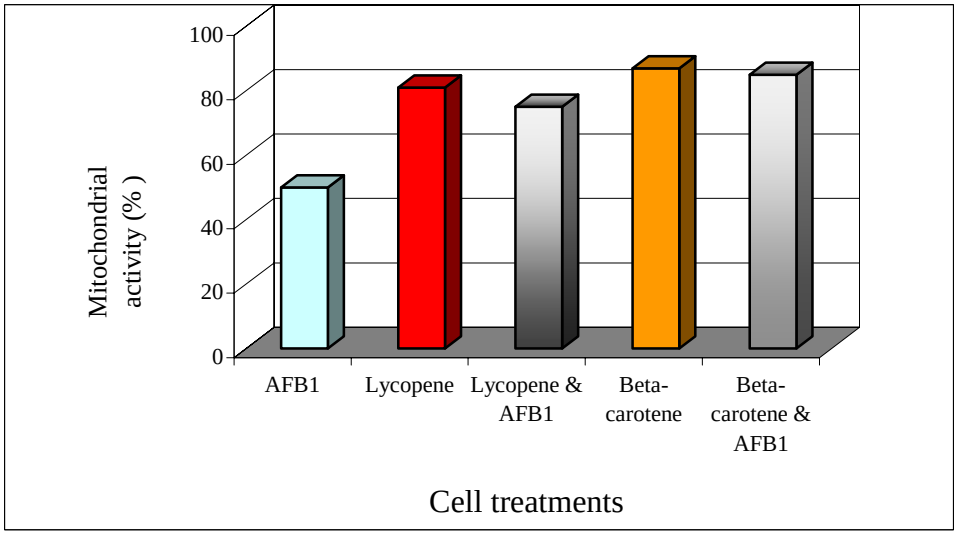



**Figure 4.11 Mitochondrial activity in HepG2 cells exposed to $AFB_1$ to lycopene and beta-carotene and subsequently exposed to $AFB_1$.**

One-way ANOVA revealed a significant difference between treatment with $AFB_1$ (11 µg/ml) alone as compared with pretreatment with lycopene and $AFB_1$ ($p < 0.05$), and pretreatment with beta-carotene alone and subsequent treatment with $AFB_1$ ($p < 0.05$). Lycopene and beta-carotene alone showed 19% and 13% cell mortality respectively.

### 4.2.2 Cell viability

Cell viability was measured using two methods, namely the crystal violet dissolution assay and flow cytometry. A comparison of the effects of lycopene and beta-carotene on $AFB_1$ exposed cells (11 and 33 µg/ml) as measured by the crystal violet dissolution assay is shown in **Figure 4.12.** In the case of the 33 µg/ml $AFB_1$ treatments lower cell viability was observed as compared to the 11 µg/ml $AFB_1$ treatments, with and without pretreatment with lycopene or beta-carotene. In both cases however, beta-carotene protected the cells more than lycopene by 21% (with 33 µg/ml $AFB_1$) and 54% (with 11 µg/ml $AFB_1$) respectively.

To confirm the protective trends described above, flow cytometry (with PI labelling) results of cell viability were analysed. **Table 4.3** indicates the amount of cells that showed no PI labelling (viable cells), the percentage cell viability as compared to the control and the percentage protection offered by lycopene and beta-carotene when cells were exposed to $AFB_1$. Beta-carotene protected the cells from $AFB_1$ damage by 24% and lycopene by 30% when cells were exposed to 11 µg/ml $AFB_1$ ($p < 0.001$). The greater protective effect of beta-carotene as compared to lycopene was confirmed by a 6% increase in cell viability (with 11 µg/ml $AFB_1$) in the case of beta-carotene treatment.

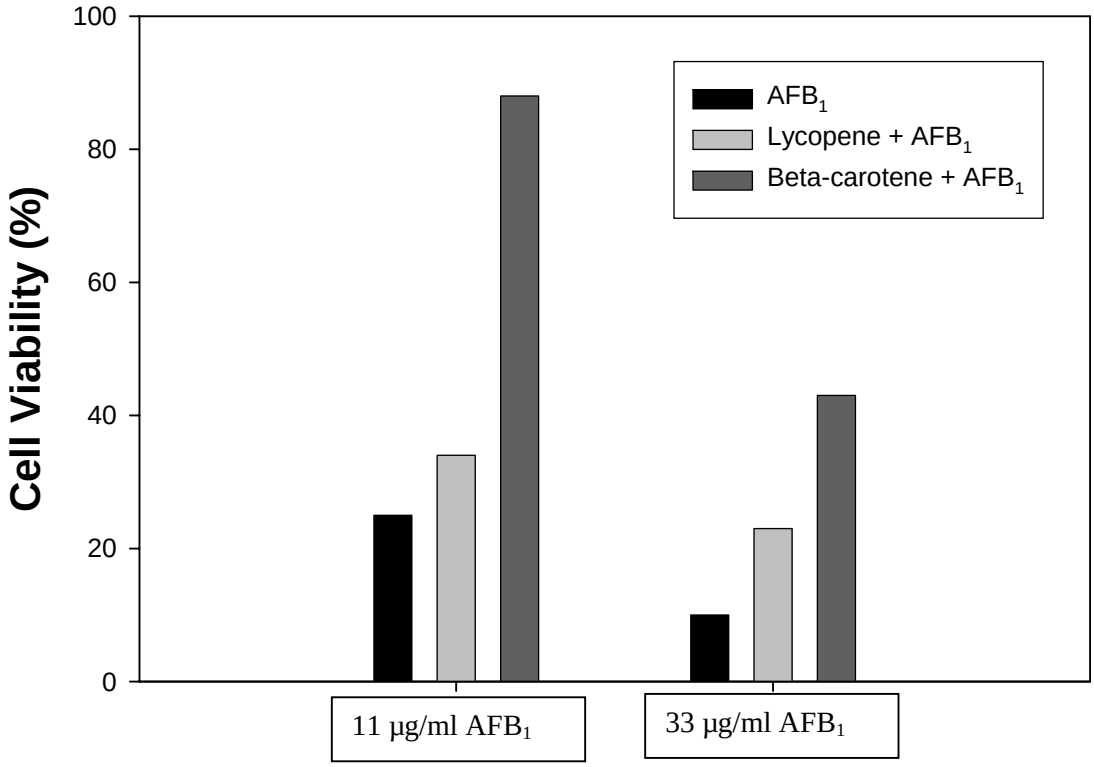



**Figure 4.12 Comparison of cell viability at chosen concentrations and time of exposure using the crystal violet dissolution assay.**

**Table 4.3 Cell viability of HepG2 cells pre-treated with lycopene and beta-carotene and subsequently exposed to $AFB_1$ for 18 h.**

| Cell treatments | Untreated | $AFB_1$ | Lycopene | Lycopene and $AFB_1$ | Beta-carotene | Beta-carotene and $AFB_1$ |
|---|---|---|---|---|---|---|
| **Fluorescence (PI –ve)** | 63 | 49 | 62 | 64 | 61 | 68 |
| **Cell viability (%)** | 100 | 78 | 98 | 102 | 97 | 108 |
| **Protection (%)** | - | - | - | 24 | - | 30 |

The treatments used in the above experiments were the optimum concentrations and times calculated in section 4.1. They are as follows: $AFB_1$ (11 µg/ml), lycopene (0.5 µg/ml), lycopene (0.5 µg/ml) + $AFB_1$ (11 µg/ml), beta-carotene (1 µg/ml), beta-carotene (1 µg/ml), + $AFB_1$ (11 µg/ml).

(PI –ve): propidium iodide negative.

### 4.2.3 Measurement of ROS

The proportions of ROS labeled in the HepG2 cells after treatments were quantitated by flow cytometry and are shown in **Table 4.4.** The percentage ROS as compared to the untreated (control) cells for all the trearments was calculated and is represented in **Figure 4.13**. There was a significant decrease ($p < 0.001$) in ROS in the HepG2 cells treated with lycopene and beta-carotene at optimum levels. Both lycopene and beta-carotene pre-incubated HepG2 cells showed a 41% reduction in ROS as compared to $AFB_1$ treated cells. The lowest level of ROS was observed in the cells treated with only lycopene.

**Table 4.4 ROS of HepG2 cells pre-treated with lycopene and beta-carotene and subsequently exposed to $AFB_1$ for 18 h.**

| Cell treatments | Untreated control Cells | $AFB_1$ | Lycopene | Lycopene and $AFB_1$ | Beta-carotene | Beta-carotene and $AFB_1$ |
|---|---|---|---|---|---|---|
| **DCF – labelling (fluorescence units)** | 223 ± 12 | 256 ± 16 | 182 ± 13 | 163 ± 11 | 188 ± 9 | 163 ± 13 |

Values represent the Mean +- SD.

The treatments used in the above experiments were the optimum concentrations and times calculated in section 4.1. They are as follows: $AFB_1$ (11 µg/ml), Lycopene (0.5 µg/ml), Lycopene (0.5 µg/ml) + $AFB_1$ (11 µg/ml), Beta-carotene (1 µg/ml), Beta-carotene (1 µg/ml), + $AFB_1$ (11 µg/ml).

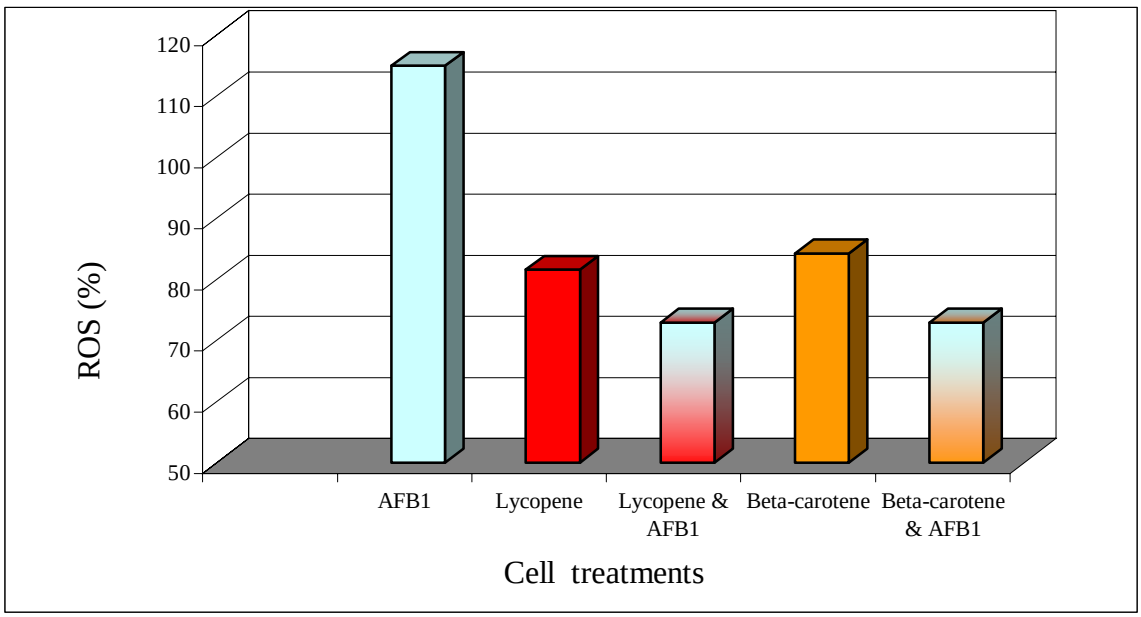
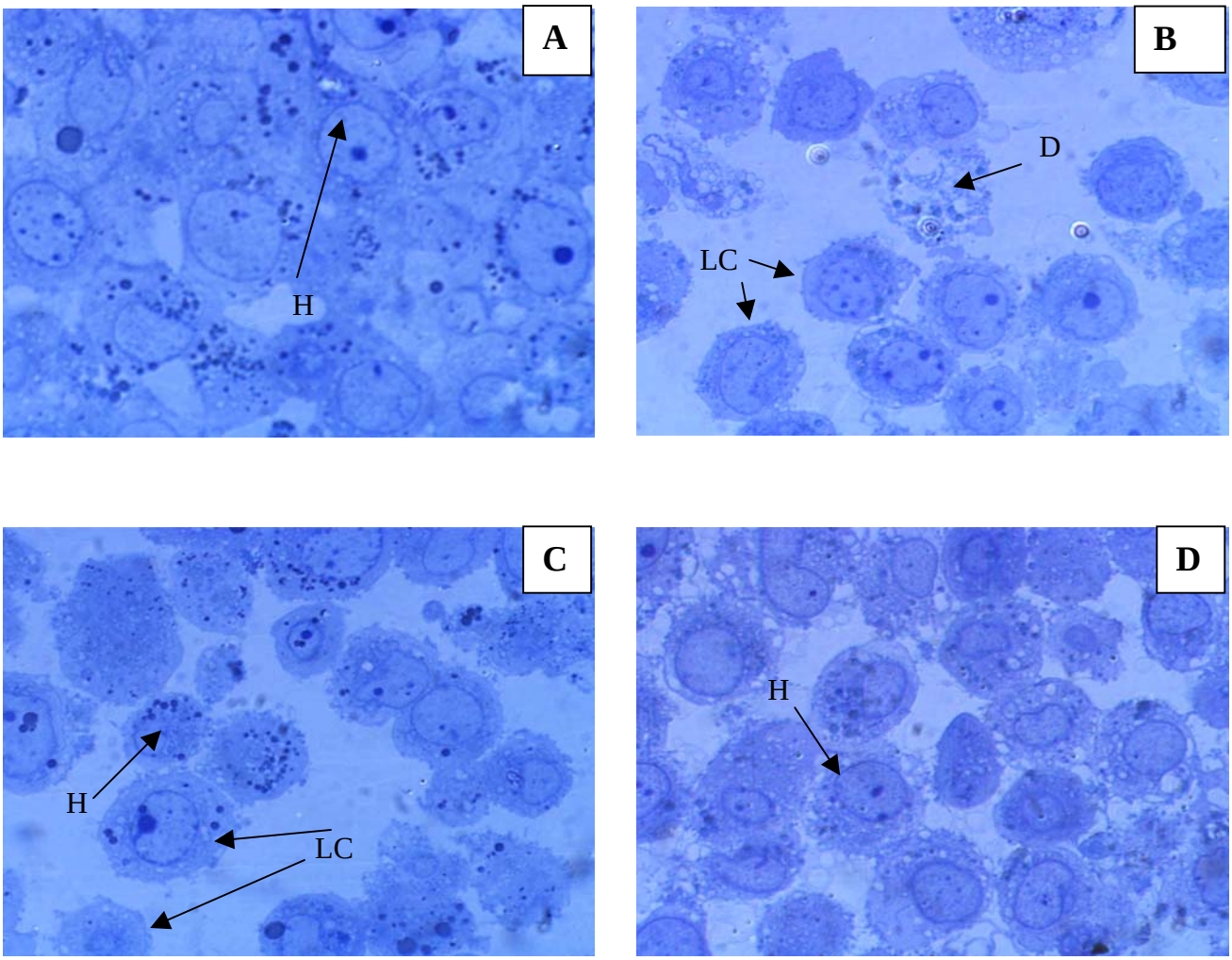



**Figure 4.13 ROS in HepG2 cells pre-treated with lycopene and beta-carotene and subsequently exposed to $AFB_1$.**

## 4.3. CYTOPATHOLOGY

### 4.3.1 Structural changes using phase contrast microscopy

The morphological changes in cell cultures exposed to $AFB_1$ showed a distinct decrease in cell number. The cells appeared irregular in shape, there was a loss of cell-to-cell contact and many cells showed signs of disintegration (**Figure 4.14 B**). Cells pretreated with lycopene and subsequently treated with $AFB_1$(**Figure 4.14 C**) appear to be intact with little loss in cell morphology as compared to the normal untreated cells, but there was a decrease in the number of cells. Cells pretreated with beta-carotene (**Figure 4.14 D**) were similar to the normal untreated cells. Cytoplasmic vacuolation was seen in normal untreated cultures (**Figure 4.14 A**) and in all the treated cells.




[mag. 1000X, H = healthy cells, LC = loss in cell-to-cell contact, D = disintegration of cells]

**Figure 4.14 Light micrographs of HepG2 cells stained with toluidine blue, showing normal untreated cells (A), $AFB_1$ treated cells (B), cells pretreated with lycopene and subsequently exposed to $AFB_1$ (C) and cells pretreated with beta-carotene and subsequently exposed to $AFB_1$ (D).**

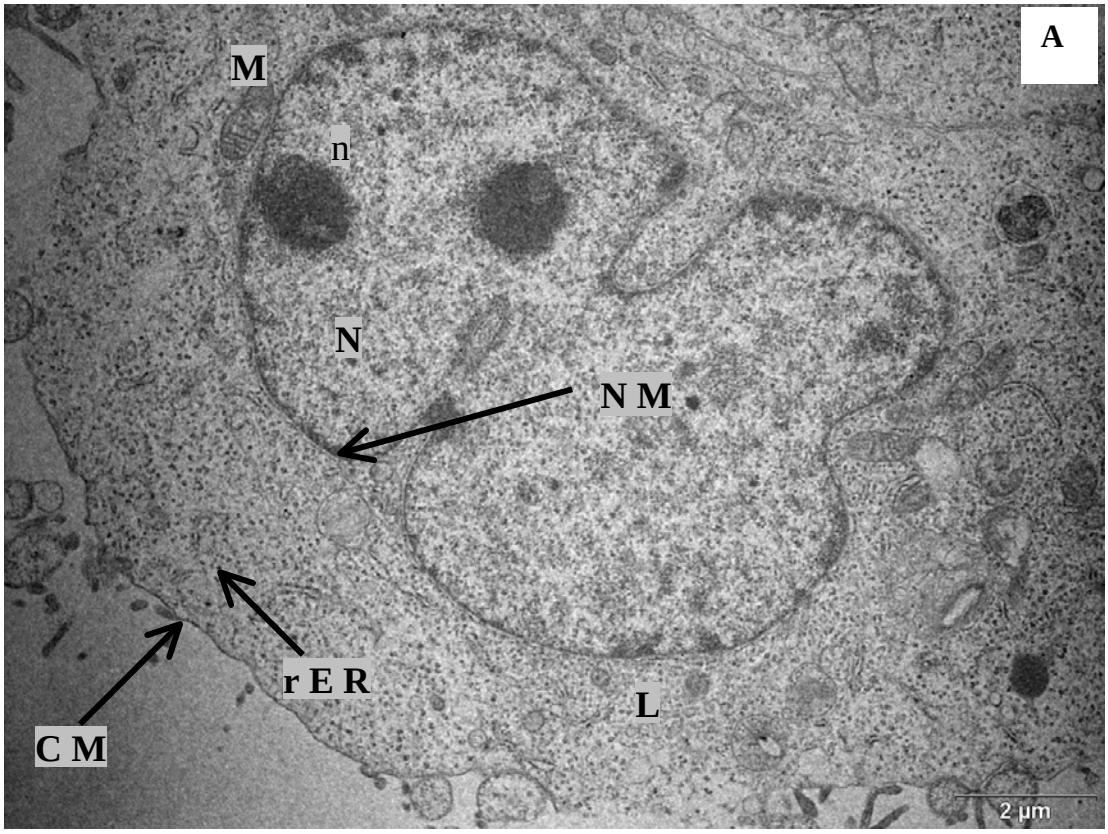
### 4.3.2 Ultrastructural changes using TEM

Normal untreated HepG2 cells **(Figures 4.15 A and B)** show an intact cell membrane with no breakages. The cytoplasm is of even density and there are no large vacuoles present. Cytoplasmic organelles, namely the mitochondria, endoplasmic reticulum, ribosomes and lysosomes are intact. The nucleus show an intact, smooth nuclear membrane with an even distribution of the nuclear density.

Cells exposed to 11 μg/ml $AFB_1$ **(Figure 4.16 A and B)** show an intact cell membrane with an increased number of podocytes. The cytoplasm shows vacuolation and a slight enlargement of lysosomal vesicles. The mitochondria are enlarged and the structures within are disrupted. In some images the membrane does not seem to be intact (cellular function badly compromised). The ER are swollen with patchy ribosomes. Cell-to-cell communication is disrupted as the cells pull away from one another, and many cells are lost. The nucleus is enlarged (nuclear cytoplasmic ratio changed) and the nucleolemma appear swollen.

**Figure 4.15** **Transmission electromicrographs of HepG2 cells stained with 2% uranyl acetate and 0.3% lead citrate showing untreated cells at 17000X magnification (A) and at 6800X magnification (B).**

**[M = mitochondria, n = nucleolus, N = nucleus, Nm = nuclear membrane, rER = endoplasmic reticulum with ribosomes, L = lysosomes, CM = cell membrane]**


A
M
n
N
N M
r E R
L
C M
2 µm

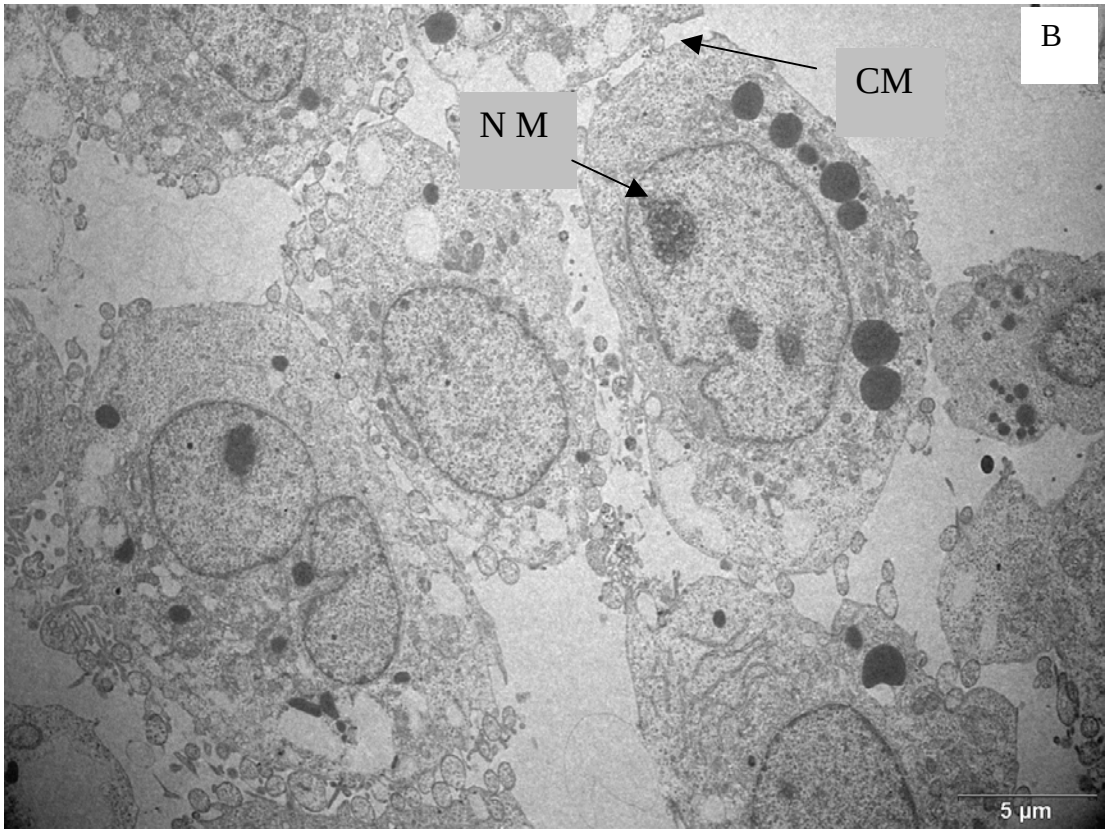
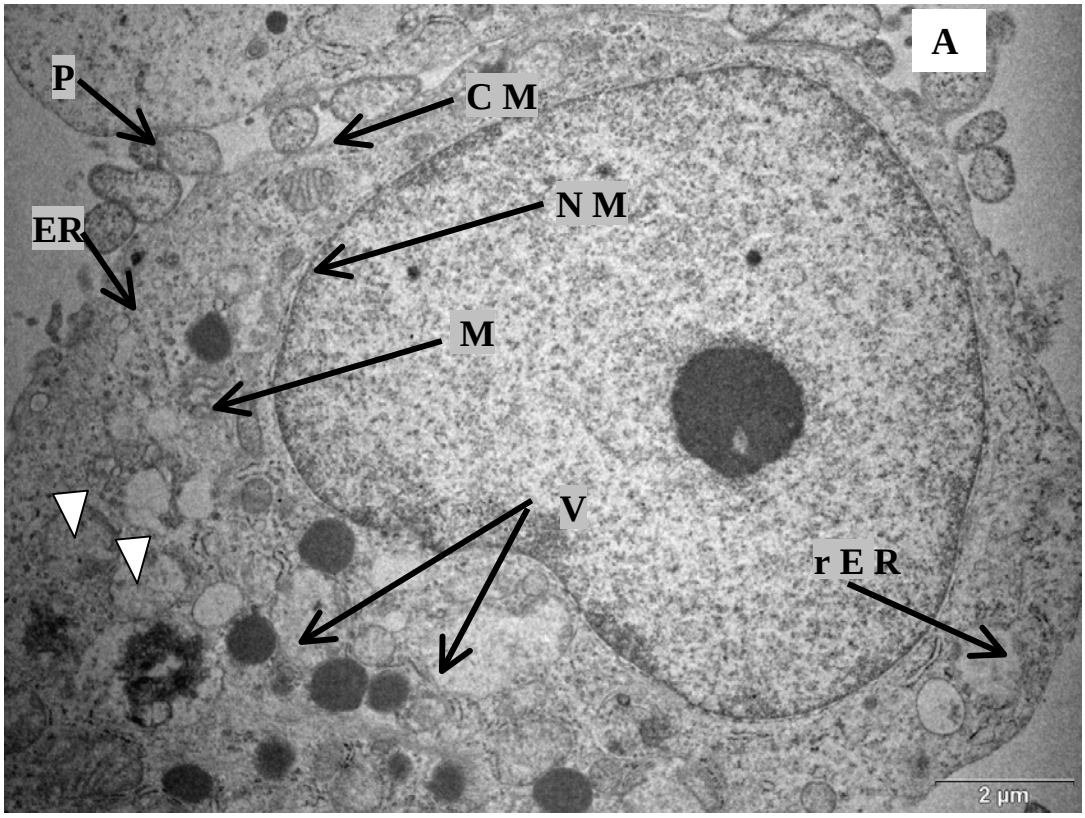
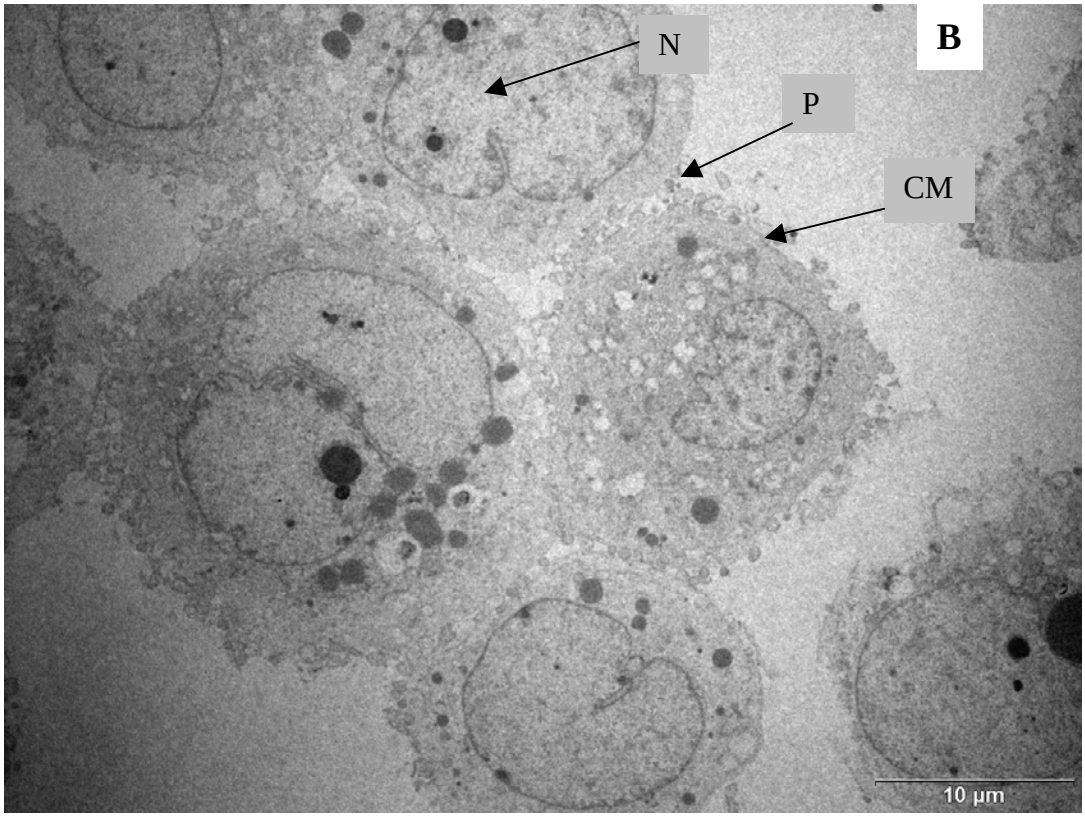

B
CM
N M
5 µm

**Figure 4.16** **Transmission electromicrographs of HepG2 cells stained with 2% uranyl acetate and 0.3% lead citrate showing $AFB_1$ treated cells (11 µg/ml) at 17000X magnification (A) and at 4900X magnification (B).**

**[P = podocytes, M = mitochondria, Nm = nuclear membrane, ER = endoplasmic reticulum, rER = endoplasmic reticulum with ribosomes, L = lysosomes, V = vacuoles, CM = cell membrane, ◁ = enlarged structures, N = nucleus]**


A
P
C M
N M
ER
M
V
r E R
2 µm

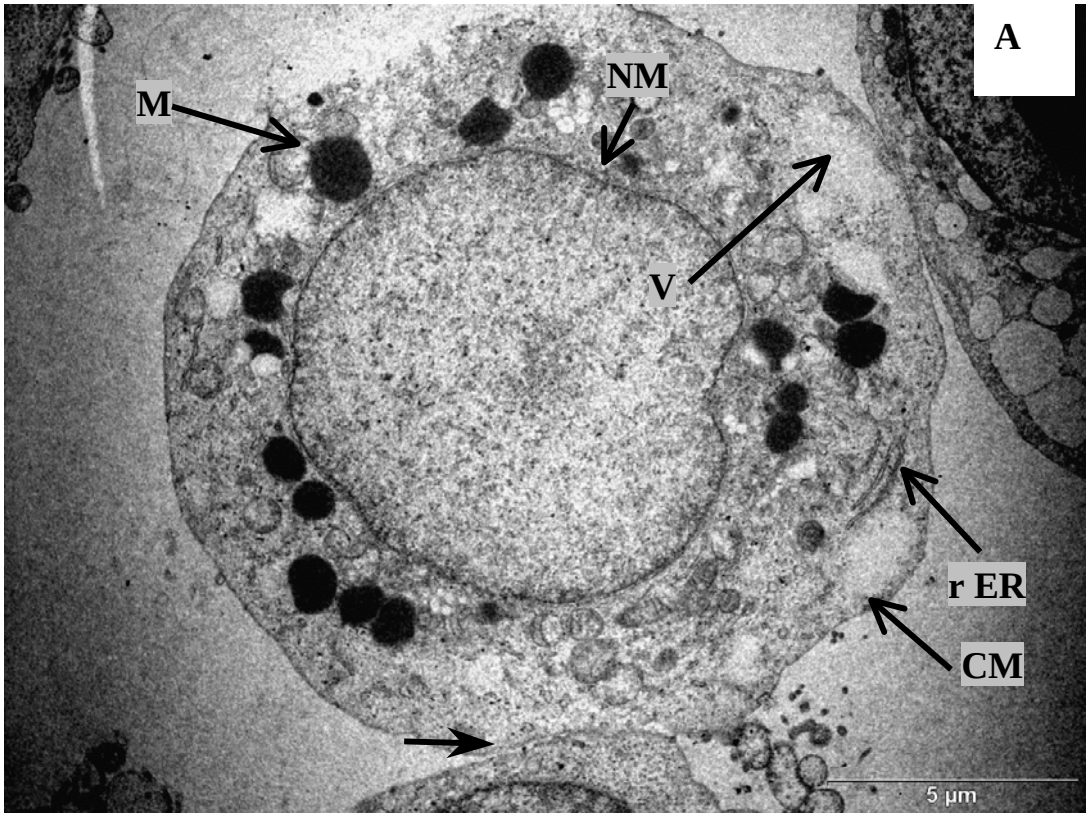

B
N
P
CM
10 µm

Cells pre-treated with lycopene and subsequently exposed to $AFB_1$ **(Figure 4.17 A** and **B)** show an intact cell membrane with no breakages. The cytoplasm showed increased vacuolization. The mitochondria although impaired appear better than those treated with $AFB_1$ alone. Cell-to-cell communication is also better and is and there are a number of podocytes. The nucleolemma appeared hazy but was similar to $AFB_1$ treated cells. There was no difference in the ER in all the treated cells.

Cells pre-treated with beta-carotene and subsequently exposed to $AFB_1$ **(Figure 4.18 A** and **B)** show an intact cell membrane with no breakages. The cytoplasm showed increased vacuolization. The mitochondrial structure is severely impaired. There are very few ER visible and ribosome were not visible. Cell-to-cell communication shows less podocytes than those with $AFB_{1.}$ The nucleolemma is damaged (hazy).

**Figure 4.17** **Transmission electromicrographs of HepG2 cells stained with 2% uranyl acetate and 0.3% lead citrate showing cells pretreated with lycopene (0.5 µg/ml) and subsequently exposed to $AFB_1$ (11 µg/ml) at 12200X magnification (A) and at 6800X magnification (B).**

**[M = mitochondria, Nm = nuclear membrane, rER = endoplasmic reticulum with ribosomes, N = nucleus, V = vacuoles, CM = cell membrane, ⟶ = cell-to-cell communication]**


A
M
NM
V
r ER
CM
5 µm

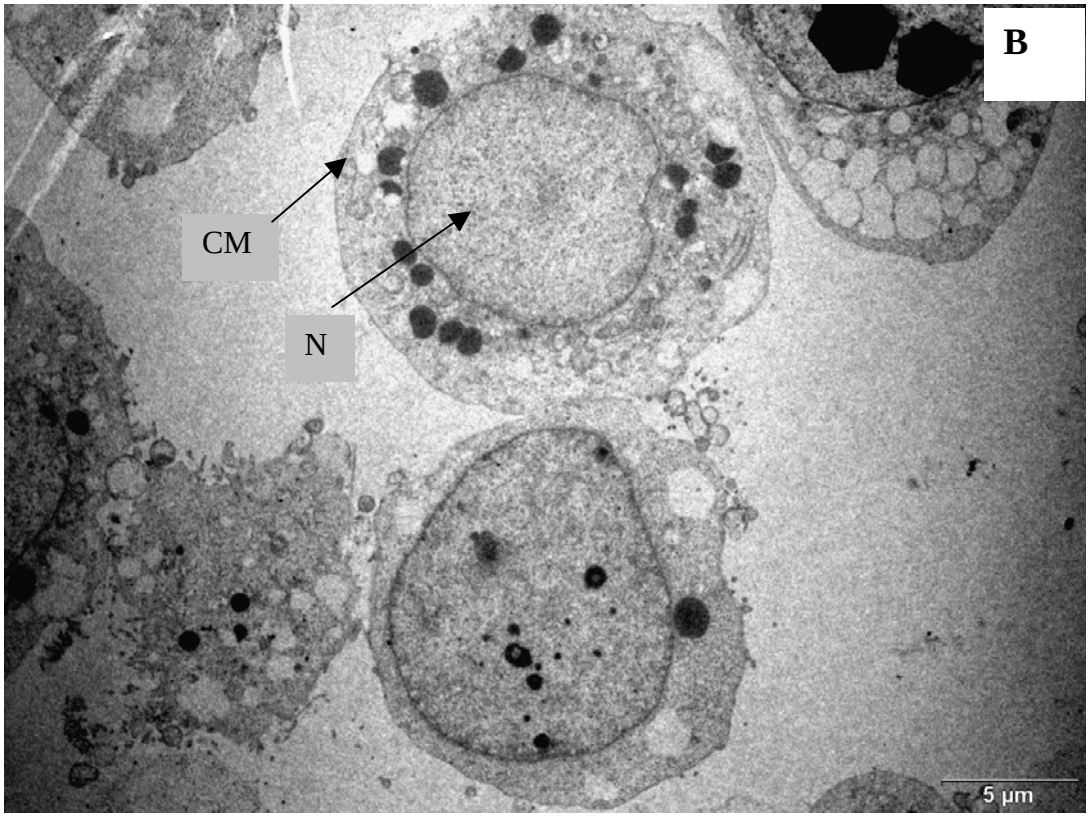
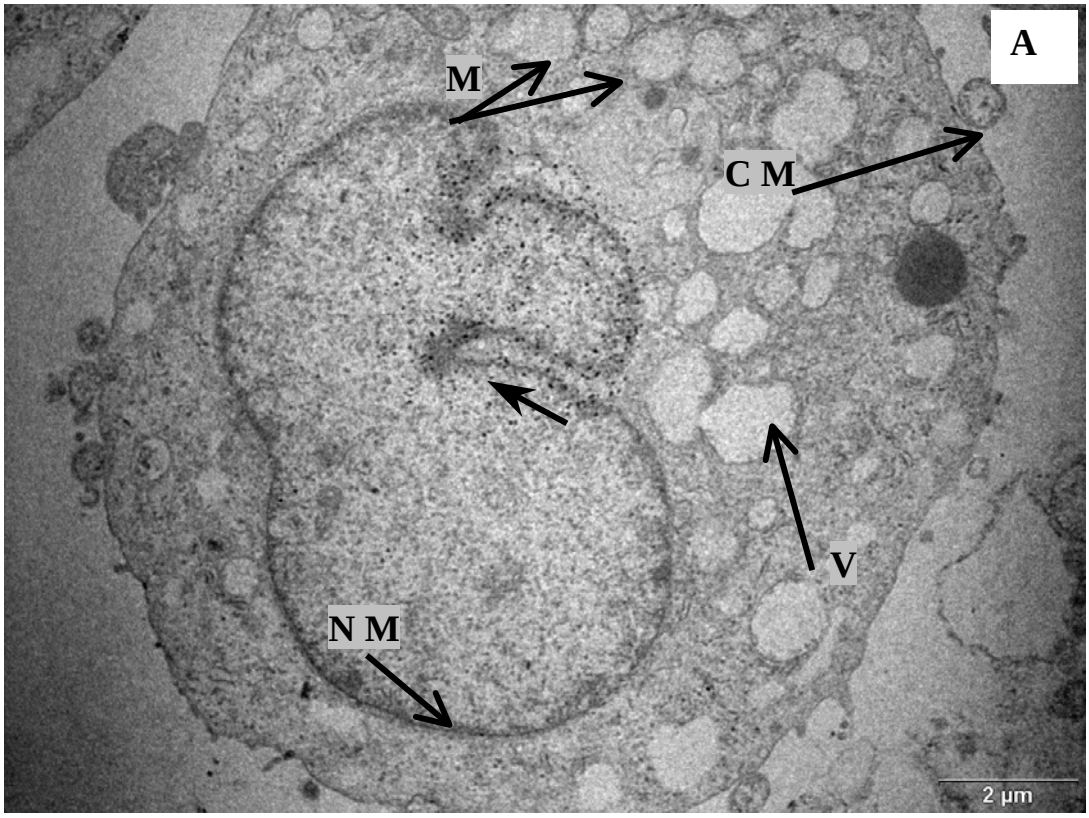
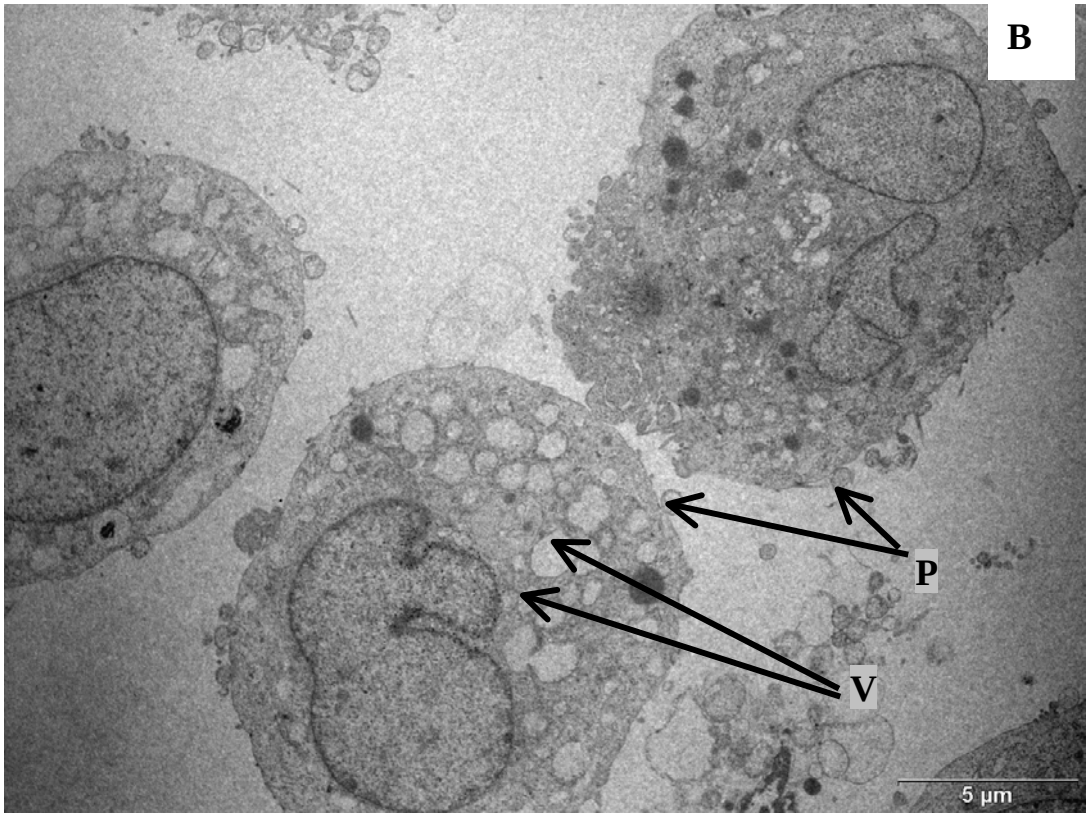

B
CM
N
5 µm

**Figure 4.18** **Transmission electromicrographs of HepG2 cells stained with 2% uranyl acetate and 0.3% lead citrate showing cells pre-treated with beta-carotene (1.0 µg/ml) and subsequently exposed to $AFB_1$ (11 µg/ml) at 17000X magnification (A) and at 9800X magnification (B).**

**[P = podocytes, M = mitochondria, Nm = nuclear membrane, V = vacuoles, CM = cell membrane, → = indentation of nucleus]**


A
M
C M
V
N M
2 µm


B
P
V
5 µm

## 4.4 GENOMIC EFFECTS

### 4.4.1 Apoptosis

The influence of beta-carotene and lycopene on nuclear changes is observed by end- labelling TdT which binds to the exposed ‘3-OH ends of the DNA (giving a brown colour). The $AFB_1$ treated cells **(Figure 4.19 B)** showed an increase in fragment end-labelling as compared to the untreated cells (**Figure 4.19 A**). Further confirmation of this effect is evidenced by an increase in labelling with 33 µg/ml $AFB_1$ **(Figure 4.19)**.

When cells were pretreated with lycopene there was a decrease in the labelling as evidenced in the cells treated with 33 µg/ml $AFB_1$ (**Figure 4.20 C**). This effect is not very clear with the cells treated with 11 µg/ml $AFB_1$ **(Figure 4.20 B)** due to insufficient cell numbers.

When cells were pretreated with beta-carotene and subsequently exposed to 11 µg/ml these cells appeared similar to normal (**Figure 4.21 B**). At a higher $AFB_1$ exposure (33 µg/ml) (**Figure 4.21 C**) there was an increase in end-labelling.

The effect of beta-carotene and lycopene $AFB_1$ exposed cells showed labelling in beta-carotene and lycopene. A comparison of labelling between lycopene pretreated **(Figure 4.22 C)** and beta-carotene **(Figure 4.22 D)** showed less labelling with beta-carotene.

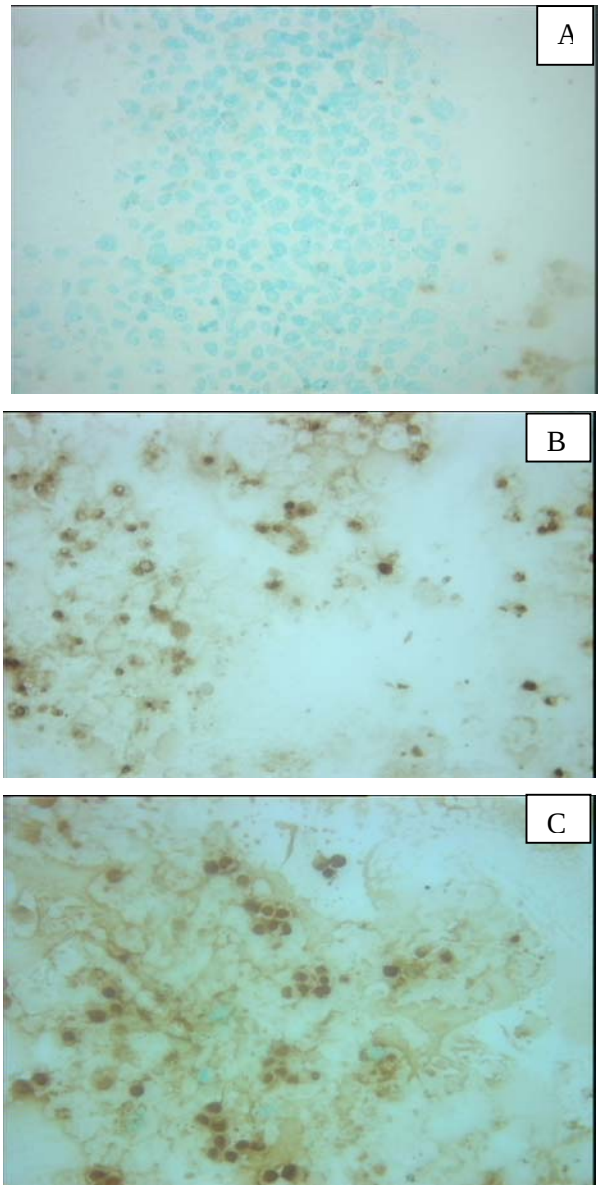



**Figure 4.19** **Fragment end-labelling of apoptotic bodies in HepG2 cells, untreated (A), $AFB_1$ treated (11 µg/ml) (B) and $AFB_1$ treated (33 µg/ml) (C); (400X magnification**).

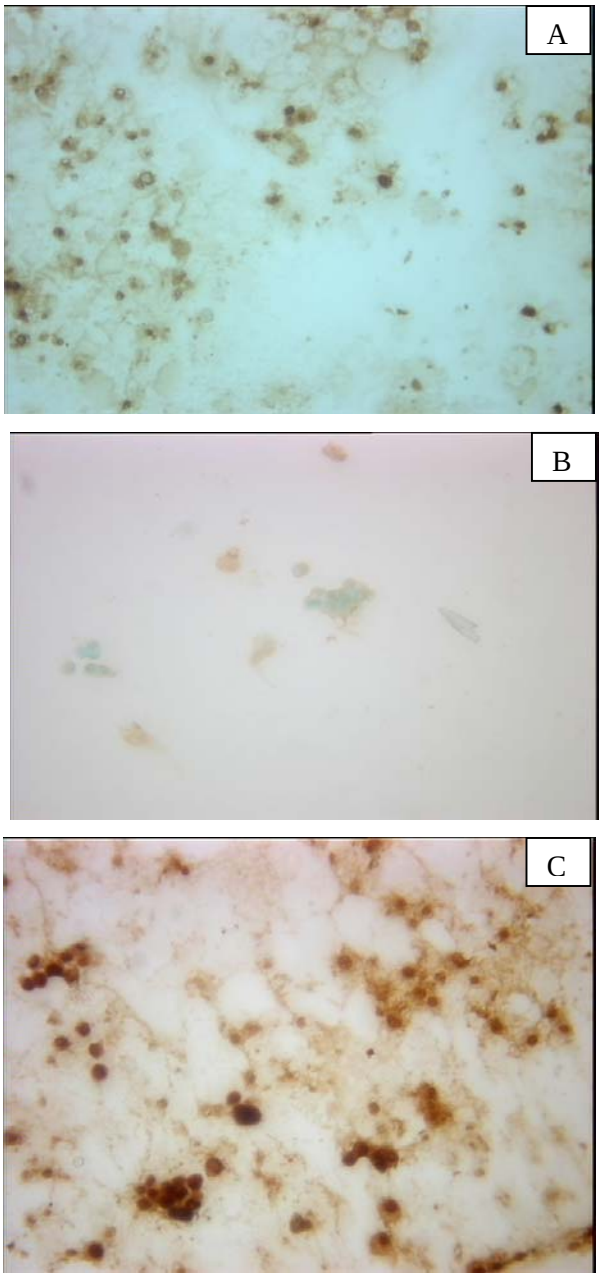



**Figure 4.20** **Fragment end-labelling of apoptotic bodies in HepG2 cells treated with $AFB_1$ (11 µg/ml) (A), pretreated with lycopene (0.5 µg/ml) and subsequently exposed to 11 µg/ml $AFB_1$ (B) and pretreated with lycopene (0.5 µg/ml) and subsequently exposed to 33 µg/ml $AFB_1$ (C); (400x magnification).**

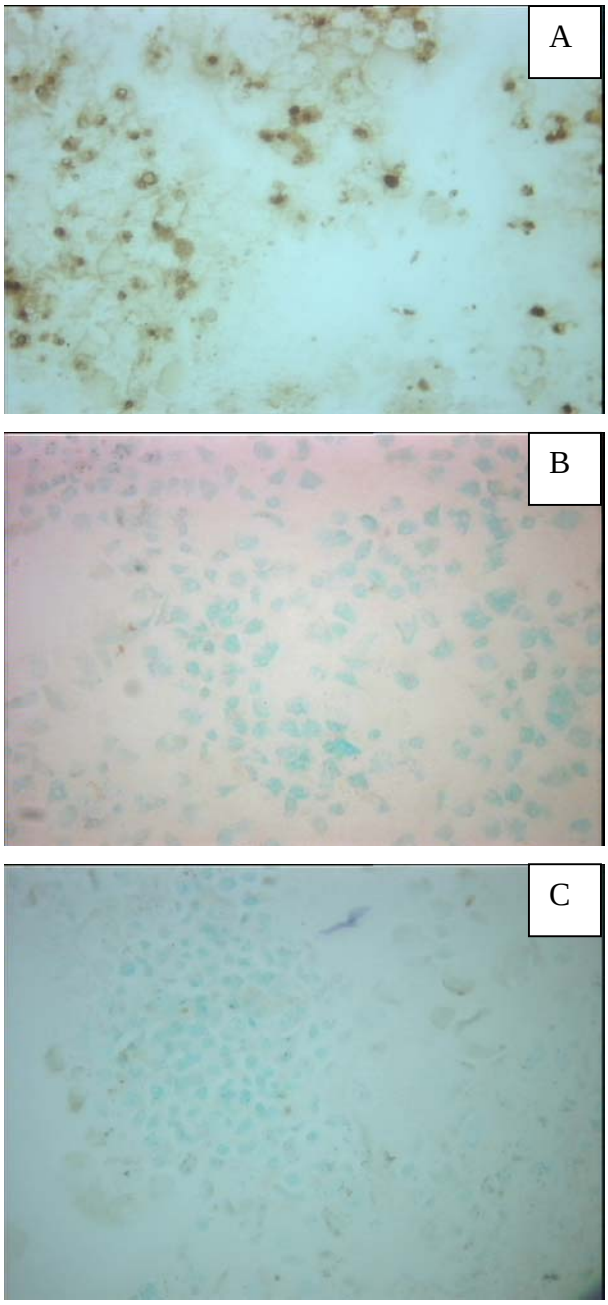



**Figure 4.21** **Fragment end-labelling of apoptotic bodies in HepG2 cells treated with $AFB_1$ (11 µg/ml) (A), pretreated with beta-carotene (1.0 µg/ml) and subsequently exposed to 11 µg/ml $AFB_1$ (B) and pretreated with lycopene (1.0 µg/ml) and subsequently exposed to 33 µg/ml $AFB_1$ (C); (400X magnification).**

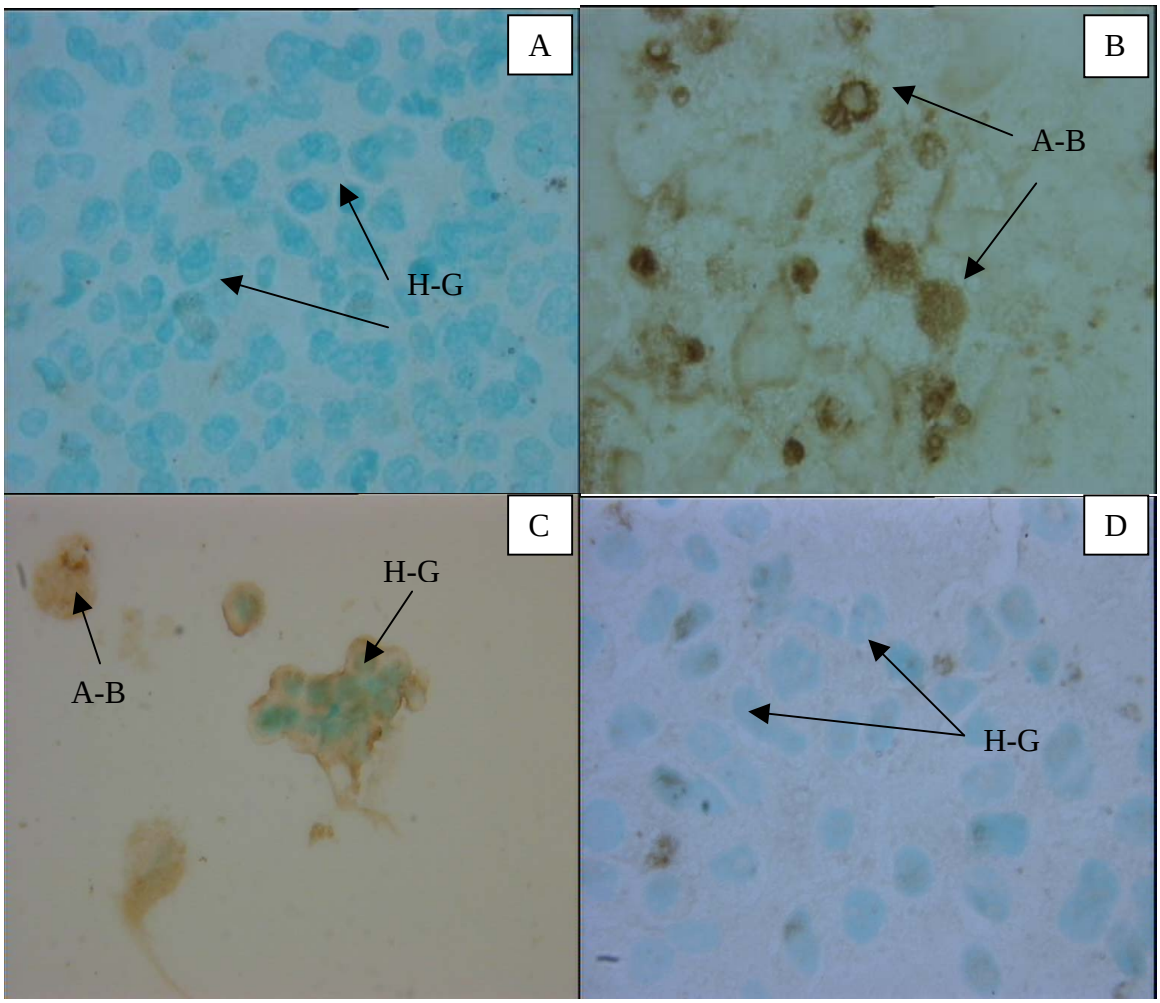



**Figure 4.22** **Fragment end-labelling of apoptotic bodies in HepG2 cells, untreated (A), $AFB_1$ treated (11 µg/ml) (B), pretreated with lycopene (0.5 µg/ml) and subsequently exposed to 11 µg/ml $AFB_1$ (C) and pretreated with beta-carotene (0.5 µg/ml) and subsequently exposed to 11 µg/ml $AFB_1$ (D); (1000X magnification).**

**[H-G = healthy green counterstained cells, A-P = apoptotic bodies]**

### 4.4.2 p53 ELISA assay

The p53 protein levels in HepG2 cells were measured using the pantropic ELISA. **Figure 4.23** shows a standard curve of the p53 protein standards. The sample absorbance values were all within the absorbance range of the standards.

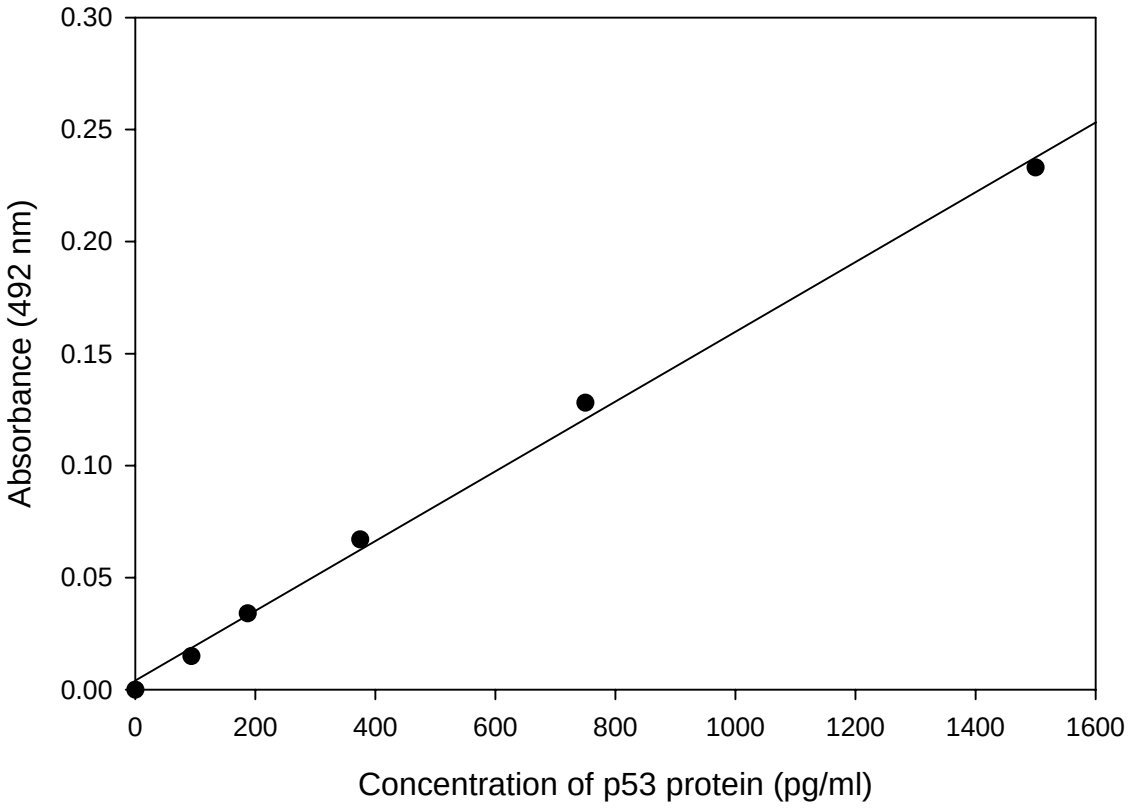



**Figure 4.23 Standard curve of p53 protein.**

**Table 4.5** shows the concentration of p53 protein in the cells after the various treatments. It is noted that $AFB_1$ showed a decrease in p53 protein concentration as compared to the control (untreated) cells. There was a significant difference ($p < 0.001$) in the p 53 concentration in cells exposed to lycopene or beta-carotene relative to $AFB_1$ treated cells. Cells pretreated with lycopene or beta-carotene, and subsequently exposed to $AFB_1$, also showed a significant

difference ($p < 0.001$). In the presence of the aflatoxin p53 protein concentration is reduced. Beta-carotene and lycopene cause an increase in the p53 protein concentration in the presence of $AFB_1$ and without $AFB_1$, there is an increase in the p53 levels.

**Table 4.5 $AFB_1$ exposed to p53 protein in HepG2 cells were pretreated with lycopene or beta-carotene.**

| | **Untreated** | **$AFB_1$** | **Lycopene + $AFB_1$** | **Beta-carotene + $AFB_1$** | **Lycopene** | **Beta-carotene** |
|---|---|---|---|---|---|---|
| **Absorbance (490 nm)** | 0.042 ± 0.003 | 0.038 ± 0.002 | 0.082 ± 0.002 | 0.100 ± 0.006 | 0.111 ± 0.004 | 0.117 ± 0.002 |
| **Concentration (pg/ml)** | 231.6 | 209.56 | 452.20 | 551.47 | 612.13 | 645.22 |

The treatments used in the above experiments were the optimum concentrations and times calculated in section 4.1. They are as follows: $AFB_1$ (11 µg/ml), lycopene (0.5 µg/ml) + $AFB_1$ (11 µg/ml), beta-carotene (1 µg/ml), + $AFB_1$ (11 µg/ml).

### 4.4.3 $AFB_1$-DNA adduct measurements

Data pertaining to $AFB_1$-DNA adduct formed in control and experimental groups of hepatocyte cell line, as determined by ELISA method is presented below **(Table 4.6)**. The standard curve utilized for the calculation of the concentration of adducts.

**Table 4.6 DNA-$AFB_1$ adducts in HepG2 cells pretreated with lycopene and beta-carotene.**

| Treatment | Concentration of DNA-$AFB_1$ adduct formed (μg/ 4 x $10^5$ cells) | % Protection against aflatoxin toxicity |
|---|---|---|
| $AFB_1$ (33 μg/ml ) (+ solvent THF ) | 3.88 ± 0.125 | _ |
| Lycopene (0.5 μg/ml) + $AFB_1$ (33 μg/ml) | 2.10 ± 0.180 * | 46 |
| Beta-carotene (1.0 μg/ml) + $AFB_1$ (33 μg/ml) | 2.22 ± 0.161 * | 43 |

Values = based on triplicate analysis (Mean ± SD). * $p$ = < 0.05

1. HepG2 cell line was pre-incubated with lycopene or beta-carotene for four h, before they were exposed to $AFB_1$ for 18 h under normal incubation conditions ($37^0$C, 5% $CO_2$ in humid atmosphere).

Using students to t-test there was a significant difference ($p \leq 0.05$) between the aflatoxin treated cells and cells pretreated with lycopene and beta-carotene. There was a 46% protection with lycopene and a 43% protection with beta-carotene in the presence of $AFB_1$.

# CHAPTER 5

# DISCUSSION

Aflatoxin $B_1$ is epidemiologically linked to HCC and as such has the potential to cause DNA oxidation (a potential cause of cancer). Carotenoids, found in fruits and vegetables, have been found to protect against cancer, in part because they contain antioxidants, which decrease the level of oxidation of DNA and also because they may possess other possible mechanisms, such as an enhancement of cellular repair of this DNA damage (Collins *et al*., 2003). Carotenoids have been shown to effectively reduce liver preneoplastic foci by deviating the $AFB_1$ metabolism towards detoxification pathways (Gradelet *et al*., 1998). The results of this study focus on the chemoprotective potential or the nutritional modulation of lycopene and beta-carotene, in maintaining cellular integrity and preventing DNA alterations to the hepatocytes exposed to $AFB_1$.

## 5.1 OPTIMISATION STUDIES

All the chemoprotection protocols tested to date have involved concomitant exposure to $AFB_1$ and the chemoprotector, in animal models. This study focused firstly on developing an optimized *in vitro* system that would yield information on the toxic effects of $AFB_1$ that would be used as a reference to evaluate the chemoprotective potential of the test agents, lycopene and beta-carotene, on human cells. To this end a suitable human cell line was selected to represent the human liver, and effective methods to measure and view these changes were employed.

All four cell lines showed morphological changes when exposed to $AFB_1$ compared with the untreated cells. The structural changes to A549 (**Figure 4.2 B**), SNO (**Figure 4.3 B**) and Hep2 (**Figure 4.4 B**) cells were not as extensive as observed with the HepG2 cells (**Figure**

**4.5 B**). The HepG2 cells showed a distinct decrease in cell number, extensive granulation and loss of morphology.

Initial experiments to optimize an effective *in vitro* system to study the chemoprotective effects of beta-carotene and lycopene provided a convenient controlled system in which to compare biochemical, morphological and genomic changes of the $AFB_1$-treated HepG2 cells. A decrease in cell numbers, structural damage and extrinsic granulation was noticed in the A549 and HepG2 cells indicating that they are the most susceptible cell lines to $AFB_1$ in a dose response manner. This is not surprising as the A549 cells originate from the lung and the HepG2 cells from the liver, these being the organs found to be most susceptible to $AFB_1$ toxicity (Eaton and Groopman, 1994). The main factors seem to be the ability of the toxin to bind to cellular receptors and/or penetrate the cell membranes and this is dependent on the size, structural conformation of the toxin molecules and the polarity of the compound (Hanelt *et al*. 1994). The SNO and Hep2 cell lines appeared to be unaffected by the $AFB_1$ in the concentration range of 10 μg/ml to 40 μg/ml. Varying cytotoxic responses of different cell types could be due to the molecular and genetic profile of the target cells. In particular, $AFB_1$ exerts toxicity mainly by activation *via* the CYP450 enzyme system which converts $AFB_1$ into its reactive epoxide. The CYP450 enzyme system was intact in the HepG2 cell line (hepatocyte) and was able to activate $AFB_1$ into its epoxide. Since $AFB_1$ is primarily regarded as a hepatotoxin (McLean and Dutton, 1995), the HepG2 cell line was chosen as the *in vitro* system for all subsequent investigations.

The MTT assay was selected as the most suitable assay to measure cell viability as opposed to the trypan blue dye exclusion assay, the crystal violet dissolution assay and the XTT assay. The MTT assay was found to offer the highest sensitivity despite the longer processing time

that was required to ensure complete dissolution of the formazan crystals. This assay was also found to be the most repeatable and reproducible in comparison to the other techniques. Furthermore, this test is widely used as a cytotoxicity assay in many *in vitro* cell culture systems (Freshney, 1987; Odhav 1997). There is also a report (Hanelt *et al*., 1994) stating that cytotoxicity measured with the MTT assay closely resembles dermal toxicity in animals; such findings suggest that trends of cytotoxicity found in *in vitro* studies may be compared to trends in organs. The MTT assay provided an analysis system by which the chemoprotective actions of lycopene and beta-carotene against $AFB_1$-induced cell damage could be determined.

There are no established cell toxicity values for humans, but epidemiological, clinical, and experimental studies reveal that exposure to large doses (>6000 mg) of aflatoxin may cause acute toxicity with lethal effect (Eaton and Groopman, 1994). The optimum concentration and time of exposure of $AFB_1$ on HepG2 cells was found to resemble that used in other *in vitro* studies (O'Brien *et al*., 2000). It should be noted that only 63% of the 11 µg/ml $AFB_1$ was absorbed by the cells and this is similar to the circulatory levels of the toxin in the human body (McLean and Dutton, 1995). The lycopene and beta-carotene concentrations (0.5 µg/ml and 1.0 µg/ml respectively) and time of exposure chosen were those which produced minimal damage to the hepatocyte (<20%). These very low concentrations resembled physiological levels of these carotenoids in the body (Khachlik *et al*., 1997). Beta-carotene and lycopene at high doses have been reported to be toxic (Leo and Lieber, 1999) and thus over-supplementation of these carotenoids in the diet should be cautioned. Studies using rats indicate that a high concentration (100-1000 fold greater) of the lycopene accumulates in the liver relative to that remaining in the serum upon intake (Khachlik *et al*., 1997). The site of beta-carotene metabolism to vitamin A is also the liver (Dewick, 2002). At low

pharmacological doses, retinoids have been shown to be effective anti-cancer agents (Sun and Lotan, 2002).

Untreated (control) cells appeared healthy and normal (**Figure 4.9 A**). These grew abundantly and maintained their shape and integrity. In contrast, results indicated that cells exposed to $AFB_1$ showed a decrease in mitosis, detached cells, vacuolization, nuclear condensation and giant cell formation (**Figure 4.9 B**). Stick-like formations indicating cell death was also observed (**Figure 4 C**). Cells pre-incubated with lycopene and then treated with 11 µg/ml $AFB_1$, resulted in increased number of cells. However, these cell did exhibit broken cell membranes as well as giant cell formation (**Figure 4.9 D**). When carotenoid treated cells were treated with 33 µg/ml $AFB_1$ (**Figure 4.9 E**) the morphology of the cells was similar to that produced by only $AFB_1$ (**Figure 4.9 C**). Cells pre-incubated with beta-carotene and then treated with 11 µg/ml $AFB_1$ remained healthy (**Figure 4.9 F**), similar to the control cells. Cells pre-incubated with lycopene and then treated with 33 µg/ml $AFB_1$, also remained healthy, with some patches of clustered viable cells and a few cells with condensed nuclei (**Figure 4.9G**).

## 5.2 BIOCHEMICAL EFFECTS

Aflatoxin interaction may inhibit protein activity such as those involved in biosynthetic pathways, neurotransmission, membrane transport, hormone functions and immune mechanisms. Exposure to acute levels of mycotoxins affects cellular energy production since they inhibit the electron transport chain between cytochromes *b* and *c* (Doherty and Campbell, 1973). This part of the study was undertaken to determine the effects the

carotenoids would have in preventing changes in cell death, mitochondrial activity and their action on free radical oxidative species.

An assessment of cell viability using flow cytometry with PI labelling showed the protection of cells from $AFB_1$ damage (**Table 4.3**). Lycopene protected the cells from $AFB_1$-induced damage by 24% whilst beta-carotene protected the cells from $AFB_1$-induced damage by 30%.

Measurements of the mitochondrial activity of HepG2 cells clearly show a significant increase in levels of mitochondrial dehydrogenase with carotenoid pre-treatment of $AFB_1$ exposed cells. Cells that were pretreated with only lycopene and exposed to 11 μg/ml $AFB_1$, were protected from $AFB_1$ toxicity by 10%. With 33 μg/ml $AFB_1$ exposure, 14% of the cells were protected by lycopene. Beta-carotene protected 61% of the cells from 11 μg/ml $AFB_1$ toxicity and 33% of the cells from to 33 μg/ml $AFB_1$.

Aflatoxin $B_1$ clearly showed a higher level of ROS in the cells. This indicates its potential to causes DNA damage (Collins *et al*., 2000). There was a 41% reduction in ROS ($p < 0.001$) in the HepG2 cells treated with lycopene and beta-carotene at optimum levels. These results are supported by various studies on the antioxidant potential of these natural products (Cheng *et al*., 2001; Galvano *et al*., 2001).

Cells constantly generate ROS as a part of their normal cellular activity. Oxidizing pollutants such as aflatoxins and many viruses also induce ROS production in normal cells. The ROS are highly reactive and can destroy cellular membranes, cellular proteins and nucleic acids and excessive activity can lead to ROS-induced tissue damage. As a defense mechanism, the body produces a number of endogenous antioxidants capable of scavenging these harmful

ROS molecules to maintain an optimal oxidant:antioxidant balance, thereby maintaining normal cellular function and health. However, under conditions of high oxidative stress, the ability of these antioxidants to eliminate ROS is often exceeded and, therefore, dietary sources of antioxidants or drugs are required. The most widely used dietary antioxidants include vitamin E, vitamin C, carotenoids, flavanoids, zinc and selenium (Galvano *et al*., 2001).

Mitochondrial activity, cell viability and ROS measurements indicate that beta-carotene (1 μg/ml) and lycopene (0.5 μg/ml) protect cells against damage in the presence of $AFB_1$. However, it seems that beta-carotene behaves differently in different systems (Pryor *et al.*, 2000). The most valuable dietary carotenoid with respect to chemoprotective potential is lycopene. It has been shown to be the most efficient singlet oxygen quencher among biological carotenoids. Tomatoes, and processed tomato products feature as the predominant source of lycopene (Nishino *et al*., 2000). Beta-carotene is also a powerful carotenoid (Packer *et al*., 1991). One property that seems to be agreed upon is that it is a powerful antioxidant in tumour cells with low oxygen levels, as is the case in the cell line under investigation in this study.

Recently it was shown that CYP4501A1 also catalyses the conversion of retinal to retinoic acid. Thus it is now increasingly apparent that microsomal CYP450 plays a role not only in detoxification of foreign compounds such as $AFB_1$, but also contributes to important physiological processes, including (pro-vitamin A) beta-carotene metabolism and maintenance of vitamin A homeostasis (Rock, 1997). Retinol (stored in liver stellate cells) on the other hand is an alcohol, and as such should compete with ethanol for the same enzymatic

pathways. Thus the insufficiency as well as the excess of beta-carotene in smokers and alcoholics should be cautioned (Leo and Lieber, 1999).

## 5.3 MORPHOLOGICAL EFFECTS

Ultrastructurally, degranulation (detachment of ribosomes) from ER frequently has been reported in $AFB_1$- treated cells (Terao and Ueno, 1978). Such damage may arise as a result of disruptive changes, including direct damage to the ER membranes, interference with the ribosome binding sites on the membrane, interference with the ribosomal cycle, inhibition of the release of newly synthesized proteins and a suppression of mRNA synthesis (Terao and Ueno, 1978).

In this study many of the morphological changes seen in cell cultures exposed to $AFB_1$ may be seen in dying cells. Pycnosis, karyorrhexis and cytoplasmic vacuolation are seen in normal cultures, and an increase in their incidence reflects a cytotoxic effect of $AFB_1$ resulting in cell death and a decrease in number of cells seen in the culture. These changes have been observed earlier (Legator *et al.,* 1965). Aflatoxin $B_1$ treated cells show an increased number of podocytes, indicating a loss in cell-to-cell communication as described by (Viviers and Schabort, 1985). Degranulation (detachment of ribosomes) from ER also occurred. Other disruptive changes included direct damage to the ER membranes (such as swelling), interference with the ribosome interaction sites on the membrane. The implication of all of these cellular changes, would indicate an interference with the ribosomal cycle, inhibition of the release of newly synthesized proteins, or a suppression of mRNA synthesis which leads a disruption of protein synthesis. All of these changes were inhibited to some extent when the cells were pretreated with the carotenoids, indicating their protection.

Severe mitochondrial swelling characteristically shown in $AFB_1$-treated cells was reduced in the lycopene and the beta-carotene treated cells. This is supported by Doherty and Campbell (1973) who linked $AFB_1$ toxicity with mitochondrial DNA damage and thus the risk of carcinogenecity. Beta-carotene has shown an inhibition of these changes to an extent. Pryor *et al*. (2000) found that beta-carotene influences cell function by regulating membrane fluidity and gap-junction communication that an apoptotic cell undergoes. Other changes observed in the $AFB_1$-treated cells were characteristic of apoptosis. These were chromatin condensation, DNA fragmentation, nuclear breakdown, membrane blebbing and cell fragmentation (apoptotic bodies). All of the above were reduced in the beta-carotene-treated cells. Lycopene was found to up-regulate intercellular gap-junctional communication by inducing connexin 43 mRNA expression (Kim *et al*., 2000b).

## 5.4 GENOMIC EFFECTS

Aflatoxin may be activated into a highly reactive $AFB_1$-8,9-epoxide that can induce toxicity or cancer (Niranjan and Avadhani, 1980). This $AFB_1$-epoxide reacts with nucleic acids and specifically makes an electrophilic attack on the $N^7$ position of guanine of DNA and RNA (Vidyasagar *et al*., 1997). It is reported (Hsu *et al.,* 1991 and Hollstein *et al.,* 1993) that there is a mutational specificity in the third base position of codon 249 which results in a guanine-thymine (G-T) substitution . The *p53* tumour suppressor gene and most specifically codon 249 of this gene have been linked epidemiologically to human HCC. The biomarker in biological fluids for AFB1 exposure has been $AFB_1$-lysine and $AFB_1$-$N^7$-guanine adducts (Vidyasagar *et al*., 1997). Thus in this part of the study looked at the effect of caretenoids on apoptosis, $AFB_1$-$N^7$-guanine adducts, and the expression of the p53 protein.

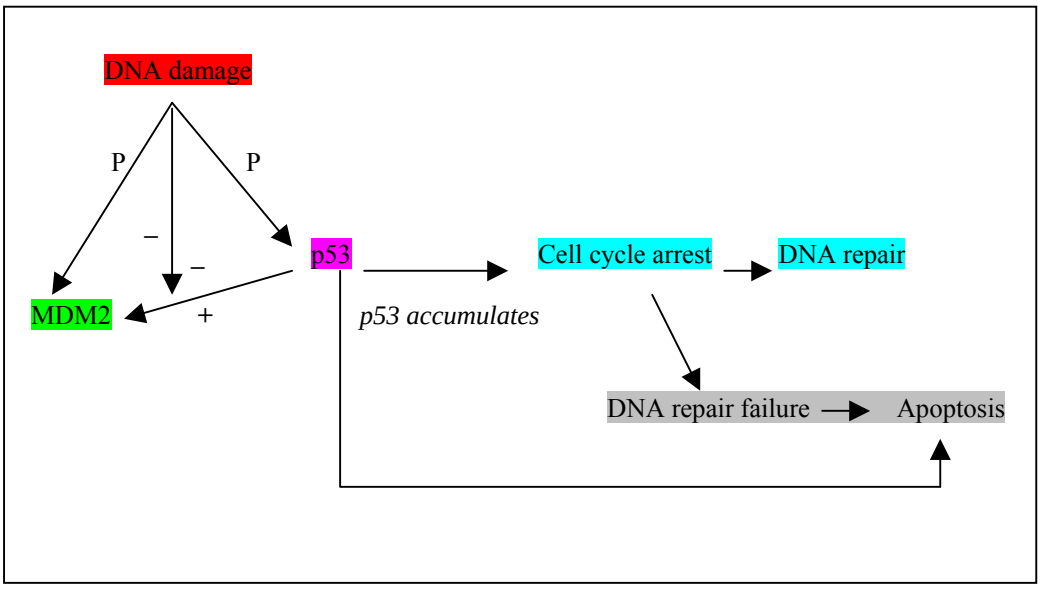
O'Brien *et al*. (2000) found that the mechanism of cell death in the presence of $AFB_1$, is apoptosis. In this study, low concentrations of lycopene and beta-carotene reduced the degree of apoptosis due to $AFB_1$ treatment. **Figure 5.2** describes the possible apoptotic pathways through which the cells may be affected by $AFB_1$ and beta-carotene may be inhibiting apoptosis at some point. Kuo *et al*. (2002) also found this effect using berberine and reveratrol. In keeping with the mitochondrial activities measured earlier it appears as if the cells go into apoptosis along the mitochondrial pathway *via* the production of cytochrome *c*. This suggests that beta-carotene converted to retinal in the cytosol competes with $AFB_1$, thereby reducing the chance of $AFB_1$ activation and subsequently apoptosis.

Studies in cats, dogs, cattle and pigs reported uptake of beta-carotene by all lymphocyte subcellular fractions, especially mitochondria. The mitochondrial electron transport system utilizes ~85% of the oxygen consumed by the cell to generate ATP; therefore, they are the most important source of ROS. Cytochrome *c* located between the inner and outer mitochondria membranes plays a critical role in the apoptotic process. Release of cytochrome *c* is regulated by the pro-apoptotic proteins BAX, BID and BIM, and by the anti-apoptotic proteins Bcl-2, Bcl-XL and BFL-1. Therefore, the mitochondria is likely to be a key player in immunity and disease, and the localisation of the carotenoids in the mitochondria is therefore of particular relevance. The presence of these carotenoids in subcellular organelles can protect the immune cells against oxidative injury, and ensure optimal cellular functions, including apoptosis, cell signalling and gene regulation. Carotenoids can also influence immune function through their ability to regulate membrane fluidity, and gap-junction communication. All these actions are most likely interrelated in their modulation of an immune response. Evidence has suggested that the action of carotenoids on aflatoxicoses may be mediated, at least in part, by their ability to quench ROS (Shen *et al*., 1995). However, the

action of ROS is multifaceted: on the one hand, they are toxic to cellular components, but on the other hand, intracellular and extracellular ROS are important signalling molecules involved in the regulation of gene expression, cell growth and cell death. The action of carotenoids on immune response hangs upon a delicate balance within the intra- and extracellular milieu, the outcomes of which depend not only on the type and concentration of the carotenoid but also on the cell type and animal species involved. Even though studies to date have provided evidence for a specific action of carotenoids, much has yet to be done to truly understand their molecular action. The use of molecular bioscience techniques can provide the necessary research tool to probe the complex interaction of carotenoids within cell systems.

Cells pretreated with lycopene and beta-carotene showed an increase in p53 protein content as compared to cells treated with only $AFB_1$. This illustrated their potential to act as cell guardians and halting cell division whilst DNA is being repaired as depicted in **Figure 5.1**. Other tumour cell lines have shown similar results (Kim *et al*., 2000a; Kim *et al*., 2000b; Nishino *et al*., 2000) indicating the crucial role of *p53* gene regulation.

The tumour suppressor p53 is a transcription factor with powerful antitumour activity that is controlled by its negative regulator mouse double minute 2 (MDM2) through a feedback mechanism (Klein and Vassilev, 2004). The MDM2 molecule, which is overproduced in many tumours, binds p53 and inhibits its function by modulating its transcriptional activity and stability.




P = phosphorylation

**Figure 5.1 Regulation of p53 function and expression levels after DNA damage.**

Aflatoxin $B_1$ has been found to cause a point mutation in the *p53* gene, resulting in a decrease in the levels of p53 protein in the cells (Mace *et al*., 1997; Chan *et al*., 2003), as compared to the untreated cells (control). This was confirmed by this study. On the other hand, p53 protein measurements in the HepG2 cells, showed that lycopene and beta-carotene upregulated the *p53* gene as shown by comparatively higher p53 protein levels in the cells. Studies show that dietary lutein (another carotenoid) decreased mammary tumour growth, increased the mRNA expression of the proapoptotic genes *p53* and BAX, decreased the expression of the anti-apoptotic gene Bcl-2, and increased the BAX:Bcl-2 ratio in tumours. Measurements of the other genes mentioned above should be carried out as future work to further elucidate a pathway for beta-carotene. The p53 tumour suppressor gene can induce cell cycle arrest to allow DNA repair or apoptosis. Its pathway is independent of the mitochondria, and therefore

of ROS. On the other hand, Bcl-2 functions as a suppressor of apoptotic death and is negatively regulated by wild type *p53*. The predominance of BAX over Bcl-2 accelerates apoptosis. Bcl-2 resides in the outer mitochondria membrane and prevents cytochrome *c* release. BAX is inactive until it is translocated to the mitochondria where it binds to Bcl-2 to induce cytochrome *c* release. Once released, cytochrome *c* activates caspases to bring about apoptosis. Apoptosis or programmed cell death is important in normal development and health. Uncontrolled cell proliferation can lead to cancer and autoimmune diseases whereas excessive cell death can lead to neurodegenerative diseases and AIDS.

Both phosphorylated-p53 and total p53 were substantially increased in the lungs of these groups. In contrast, low-dose beta-carotene greatly attenuated the smoke-induced phosphorylation of JNK, p38, c-Jun, p53, and total p53, accompanied by upregulated MKP-1.

This is the first report of $AFB_1$-$N^7$-guanine adducts in the cultured cells. Aflatoxin $B_1$-treated HepG2 cells exposed to low levels of beta-carotene and lycopene showed a significant decrease ($p<0.05$) $AFB_1$-$N^7$-guanine adducts. This means that beta-carotene diverted the highly reactive electrophilic $AFB_1$-8,9-epoxide to undertake an alternate pathway. This illustrated the potential of beta-carotene as an anticarcinogenic agent. Adduct measurements, as biomarkers, showed a similar pattern with male F344 rats fed with limonene and perillyl alcohol and then exposed to $AFB_1$ (Elegbede and Gould, 2002). There was a significant inhibition ($p<0.05$) in $AFB_1$-DNA adducts formation in rat hepatocytes. They have postulated that these monoterpenes may have potential as chemoprotective agent against aflatoxin-induced liver cancer.

The overall protective effect of lycopene and beta-carotene is clearly demonstrated in this study. The results of the carotenoid pretreatment on the hepatocytes at the metabolic, ultrastuctural and genomic levels are summarized in **Table 5.1.**

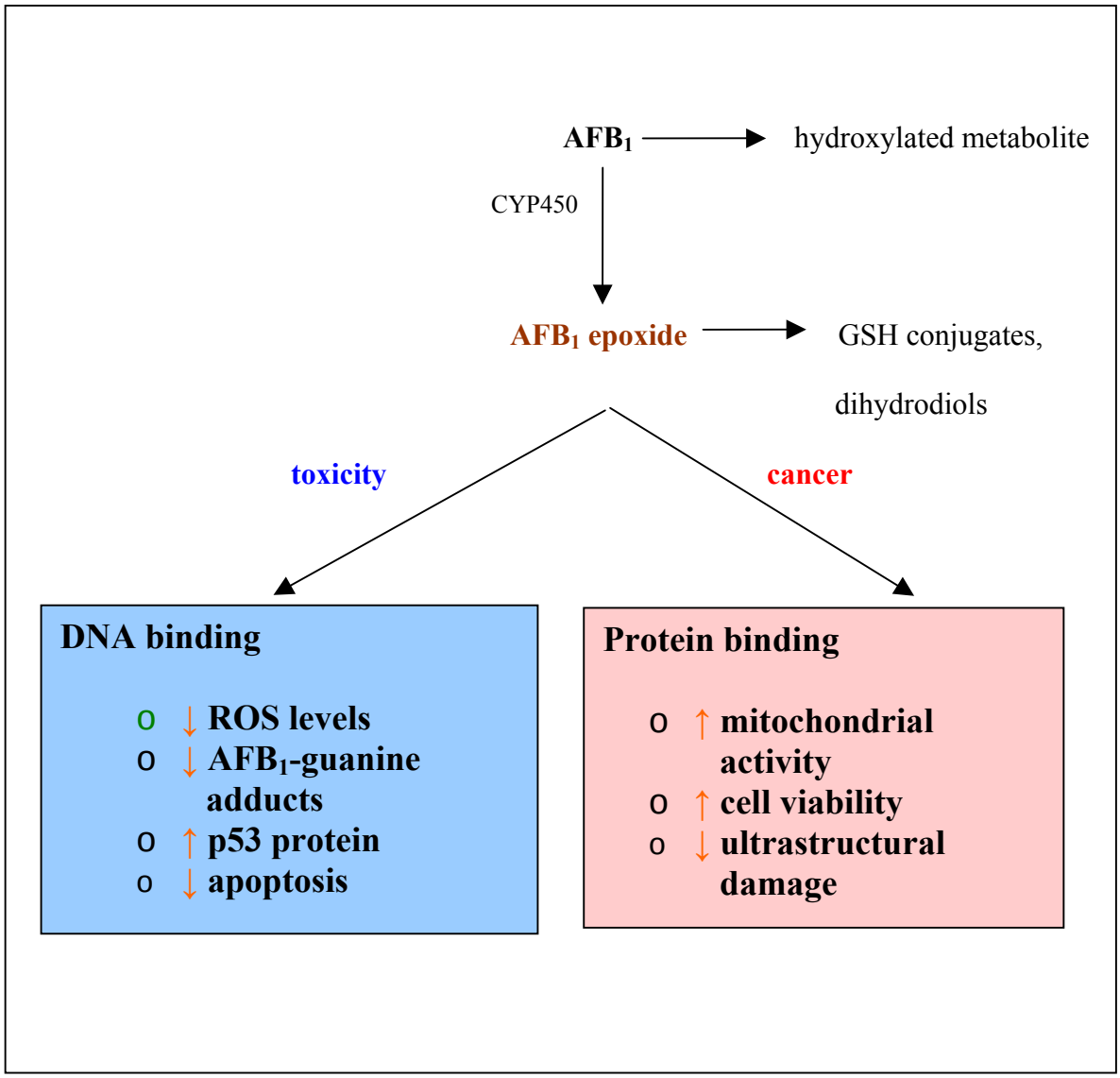
**Table 5.1 Overall protective action of lycopene and beta-carotene on $AFB_1$ exposed HepG2 cells.**

| | $AFB_1$ | Lycopene and $AFB_1$ | Beta-carotene and $AFB_1$ |
|---|---|---|---|
| **Biochemical effects** | | | |
| Mitochondrial activity | ↓ | ↑ | ↑ |
| Cell viability | ↓ | ↑ | ↑ |
| Reactive oxygen species | ↑ | ↓ | ↓ |
| **Morphology** | | | |
| Cell number | ↓ | ↑ | ↑ |
| Cell-to-cell contact | ↓ | ↑ | ↑ |
| Mitochondrial damage | ↑ | ↑ | ↓ |
| Rough endoplasmic reticulum | ↓ | ↑ | ↑ |
| Nuclear membrane damage | ↑ | ↓ | ↓ |
| Nuclear condensation | ↑ | ↓ | ↓ |
| **Molecular effects** | | | |
| Apoptosis | ↑ | ↓ | ↓ |
| p53 protein levels | ↓ | ↑ | ↑ |
| $AFB_1$-guanine adducts | ↑ | ↓ | ↓ |

↑ = increase , ↓ = decrease

Based on these observations, a mechanism is proposed for the protective effect of lycopene and beta-carotene (**Figure 5.2)**. These carotenoids prevent the toxic effects caused by $AFB_1$-protein binding by sustaining mitochondrial activity, preventing cell death and decreasing ultrastructural damage. Lycopene and beta-carotene also indicate their potential to protect against $AFB_1$-induced carcinogenicity, i.e. $AFB_1$-DNA binding, as indicated by a decrease in ROS, $AFB_1$-$N^7$-guanine adducts and apoptosis; and an increase in p53 protein. Furthermore

in inhibiting the formation of $AFB_1$-$N^7$-guanine adducts by the epoxide, the carotenoids prevent mutation of the *p53* gene.




Chemoprotective effects of lycopene and beta-carotene effects are shown by the orange arrows:

↑ = increase and ↓ = decrease

**Figure 5.2 Inhibition of toxicity and cancer by lycopene and beta-carotene in $AFB_1$-exposed cells.**

A trial on dietary intake of fruits and vegetables has recently shown how increased dietary intake of fruits and vegetables has protected against breast cancer in the USA (Pierce *et al*., 2004). It appears that carotenoids can bring about a host of changes at the levels of both gene expression and protein activity in the cells (Palozza, 2004). In particular, some evidences are shown that carotenoid molecules may interfere in cancer related molecular pathways and change the expression of many proteins involved in: 1) cell proliferation, differentiation, apoptosis and angiogenesis; 2) carcinogen detoxification; 3) DNA damage and repair; 4) immunosurveillance. Carotenoids seem to affect gene expression either directly by interference with the control apparatus of the gene expression machinery or by virtue of metabolites or metabolic conditions induced (hormonal status, cellular redox status, etc.) that, in turn, alter cell functions implicated in the cancer process. The suppression as well as the induction of cancer by carotenoids raises issues about possible doses of carotenoid administration, possible synergy as well as antagonistic interactions between carotenoids and other dietary components.

# CHAPTER SIX

# SUMMARY AND CONCLUSIONS

This study was undertaken to investigate whether lycopene and beta-carotene could prevent the metabolic, structural and genomic effects of $AFB_1$ in an *in vitro* system. Aflatoxin $B_1$ is known to cause its harmful effects through the activation of $AFB_1$ into its epoxide which may form $AFB_1$ adducts, cause mutation of the *p53* suppressor gene and lead to cancer. Cultured hepatocytes therefore provided a suitable *in vitro* system to test the chemoprotective potential of the carotenoids (lycopene and beta-carotene) against $AFB_1$ damage. The changes in $AFB_1$ cells that have been previously reported, were observed.

Both lycopene and beta-carotene protected the hepatocytes from the toxic and carcinogenic effects of $AFB_1$ at normal dietary levels. As indicated in Figure 6.1, the results in this study confirm that the carotenoids act at various target points which include: preventing cell death due to mitochondrial damage, reducing the levels of ROS, protecting the cells from structural damage, preventing apoptotic cell death, upregulating the *p53* tumour suppressor gene and inhibiting the formation of $AFB_1$-$N^7$-guanine adduct formation. It is possible that these carotenoids (lycopene and beta-carotene) exert their protective effect through the deviation of $AFB_1$ metabolism towards detoxification pathways.

Hsu *et al*. (1991) showed a 50% transversion in codon 249 in the *p53* tumour suppressor gene which was epidemiologically linked to the incidence of HCC in Southern Africa. Thus clinical application of lycopene and beta-carotene as therapy or as a diet rich in the carotenoids are highly recommended for persons at risk of $AFB_1$ intoxication in this region. However caution must be taken against over-supplementation of these carotenoids.

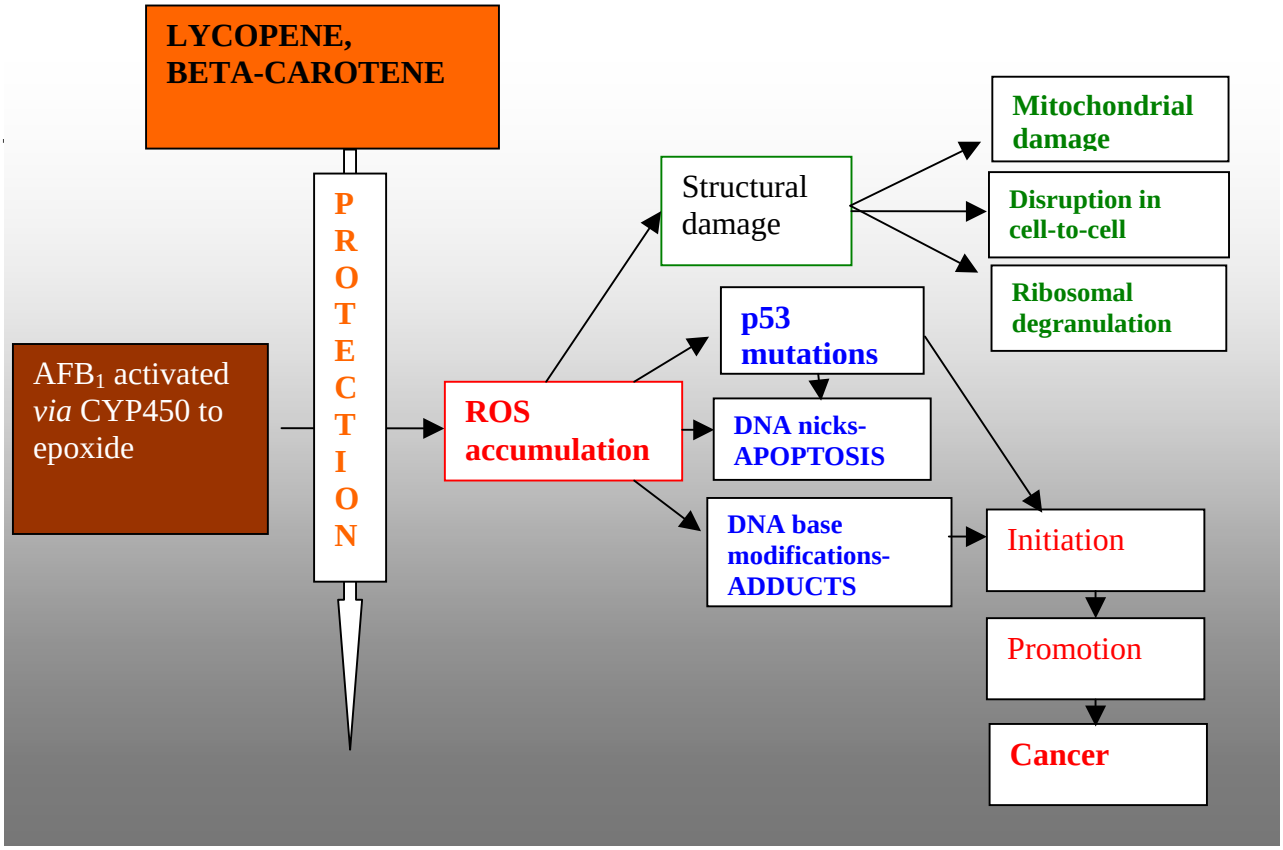



**Figure 6.1 Possible target points at which lycopene and beta-carotene protect hepatocytes from $AFB_1$ damage.**

## 6.1 FUTURE WORK

This study focused on the preventative aspects of natural products, that is, pretreated cells were exposed to the $AFB_1$. Future work would involve determining the therapeutic aspects of the natural products by first exposing the cells to $AFB_1$ and subsequently treating the cells with the natural products, lycopene and beta-carotene.

Although measurements of caspases were attempted, to elucidate the apoptotic pathway, the techniques use did not give conclusive results and hence are not reporte in this study. This will be re- investigated in the future.

It is suggested by many workers (Kensler *et al*., 1994; Dorai *et al*., 2001; Sahoo and Mukherjee, 2002) that other natural products can modulate the balance between activation (toxicity and carcinogenecity) and detoxification. It is possible that lycopene and beta-carotene can also do the same. Future work should therefore measure the levels of detoxification products of $AFB_1$ such as hydroxylated metabolites of $AFB_1$, GSH conjugates and dihydrodiols in lycopene and beta-carotene treated cells exposed to $AFB_1$, to confirm this.

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# APPENDIX

# PREPARATION OF REAGENTS, BUFFERS AND STANDARD TEST CHEMICALS

## SECTION A

### MEDIA AND REAGENTS FOR CONTINUOUS CELL LINE CULTURE

1. Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/l glucose, 0.110 /l sodium pyruvate with L-glutamine (Highveld Biological, South Africa).
2. Foetal calf serum (FCS) was filter sterilized and gamma irradiated at 25 to 28 kGy under conditions which preserve the biological integrity of the serum. The serum was aliquoted into 50 ml sterile flasks and kept frozen (-20˚C) until use. (Highveld Biological, South Africa)
3. Hank's Balanced Salt Solution (HBSS) (Highveld Biological, South Africa)
4. 0.25% (w/v) Trypsin in Ca++ and Mg++ free phosphate buffered saline (PBS) (Highveld Biological, South Africa).
5. 0.1% (w/v) EDTA in Ca++ and Mg++ free PBS (Highveld Biological, South Africa).
6. Glucose (Saarchem- UNIV**AR,** South Africa).
7. Penicillin/streptomycin mixture contained 100 mg/ml penicillin G sodium and 100 mg/ml streptomycin sulphate in double reverse osmosis (DRO) water. One ml aliquots were frozen (-20˚C) until use (Highveld Biological, South Africa).
8. Phosphate buffered saline contained 8 g NaCl, 0.2 g KCl, 0.12 g $KH_2PO_4$ and 0.91 g $Na_2HPO_4$ in one litre of distilled water. The pH was adjusted to 7.2 and the solution

was filter sterilized, dispensed into 500 ml aliquots and autoclaved (Merck NT, South Africa).

9. Dimethyl sulphoxide (DMSO) was tissue culture grade (Highveld Biological, South Africa).
10. 70% ethanol contained 70 ml Analar grade ethanol and 30ml distilled water. This was used as a general tissue culture disinfectant (Merck).
11. Complete culture medium (CCM) contained 40 ml FCS and 4 ml penicillin/streptomycin mixture in 356 ml DMEM and was stored in a sterile bottle at 4˚C.
12. Trypsinizing solution contained 0.25% Trypsin/0.1% EDTA (v/v 1:1) with 0.1 mg/ml glucose.

## SECTION B

## REAGENTS FOR CYTOTOXICITY ASSAYS

1. 3-{4,5-dimethylthiazol-2-yl}-2,5 diphenyl tetratrazolium bromide (MTT) reagent was prepared by dissolving 5 mg MTT in 1 ml PBS (pH 7.5). The solution was filter sterilized and stored in the fridge.
2. 0.25 % (w/v) crystal violet reagent was prepared in (v/v) ethanol : 1% ammonium oxalate (1:4) (Merck, Germany).
3. 2,3-bis[2-metoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carbonyanilide inner salt (XTT) reagent was prepared by dissolving 5 mg XTT (with 1% phenazine methosulphate) in 5 ml DMEM (Sigma, St Louis, USA).
4. 0.2% (w/v) trypan blue solution (Biowhittaker, Walkersville, USA) was made in PBS (pH 7.4).

## SECTION C

## PREPARATION OF TEST CHEMICALS

1. Aflatoxin $B_1$ ($AFB_1$) (Sigma, St Louis, USA) standard solution was prepared by adding 1mg to 0.25 ml DMSO to give a 4 mg/ml $AFB_1$ standard and kept in a freezer (-20˚C) until use.

   *NB.* $AFB_1$ is a toxin, carcinogen, teratogen, and a mutagen.

2. Lycopene (LC) (Sigma, St Louis, USA) was prepared by adding 1 mg to 1 ml tetrahydrofuran (THF) (Merck NT, South Africa) and kept in the freezer until use.

   *NB.* Lycopene is light and oxygen sensitive and should be stored at -70˚C.

3. Beta-carotene (Sigma, St. Louis, USA) was prepared by adding 1 mg to 1 ml THF and kept in freezer until use.

   *NB.* Beta-carotene is light and oxygen sensitive and should be stored at -70˚C.

## SECTION D

## FLOW CYTOMETRY CALIBRATION READINGS

***SEE NEXT PAGE***