CHAPTER – V
EXPERIMENT - IV

The preventive role of selected protein isolates on induced hepatocellular carcinoma in mice

5.1. Introduction

Cancer is characterized by excessive, uncontrolled growth of abnormal cells, which invade and destroy other tissues. Cancer develops in almost any organ or tissue of the body, but certain types of cancer are more lethal than others. A healthy human body is composed of 30 trillion cells, most of which are in constant turnover as cells die and others reproduce to replace them in an orderly fashion. Healthy cells of the skin, hair, lining of the stomach, and blood, for example, regularly reproduce by dividing to form two daughter cells. This cell cycle proceeds under the regulation of the body's intricately tuned control system. Among other functions, this control system ensures that cells only divide when needed, so that organs and tissues maintain their correct shape and size. If this system fails, a variety of backup safety mechanisms prevent the cell from dividing uncontrollably. In order for a cell to become cancerous, every one of these safety mechanisms must fail.

Cancer has become one of the major health problems in most nations of the world, and it was predicted that there will be new cases of cancer every year and millions of people would die due to several types of cancer (American Cancer Society, 2005). Although cancer occurs in every country around the world, there are wide geographic variations in the incidence of cancer. The incidences of hormone-related cancers including breast, prostate, endometrium, and ovarian cancers are much higher in the U.S. and European countries compared to Asian countries such as Japan and China (Arai et al., 2000). It has been believed that the difference in the incidences is due to the environmental and lifestyle factors including dietary habits. The Japanese and the Chinese consume a traditional diet high in soy products and consecutively, epidemiological studies have revealed that high consumption of soybean is inversely associated with the risk of hormone-related cancers, as isoflavones in soy aid in prevention and inhibition of cancer progression (Lee et al., 1991; Aldercreuetz et al., 1995; Jacobsen et al., 1998). Cancer is one of the major killers of human populations
worldwide. The rise of cancers has diverse reasons and varied causative agents, however most cancers that are major killers, including colorectal and hepatocellular cancer are major forms of cancer.

Cancers are further identified according to the type of cell affected. For example, squamous cells are flat, scale-like cells found in epithelial tissue. Cancers that originate in these cells are called squamous cell carcinomas. Adenomatous cells are glandular or ductal cells, and carcinomas that originate in these cells are called adenocarcinomas. Sarcomas that develop in fat cells are called liposarcomas, and those that develop in bone cells are called osteosarcomas.
5.2. REVIEW OF LITERATURE

5.2.1. Hepatocellular Carcinoma

Hepatocellular carcinoma is a malignant tumor that arises from hepatocytes, the major cell type in liver tissues. HCC is the most common primary hepatic tumor and the fifth most common tumor worldwide (Daniel et al., 2006). Hepatocarcinoma is a major problem not only in developed countries, but also in most undeveloped countries. While toxic industrial chemicals, air and water pollutants are a source of hepatocarcinogenicity in the former, food additives and fungal toxins are a major source of liver disease in the latter (Peers and Linsell, 1973). Since liver is the major site in the body that metabolizes ingested material, it is more susceptible to carcinogenic insult (Jeena, 1999).

Hepatocarcinogenesis is the result of interactions between hereditary and environmental factors. Inheritance determines the susceptibility of individuals to cancer, and the environment determines which susceptible individuals go on to express cancer. Studies directed at unraveling the genetic and epigenetic mechanisms of hepatocarcinogenesis suggested that HCC development is a complex process involving polygenic factors and multiple pathways, through the activation of proto-oncogenes and the inactivation of tumor suppressor genes induced by genetic and epigenetic alterations, which are core biological processes of hepatocarcinogenesis; RB1 and p53 pathways are commonly affected in HCCs of different etiologies, which reflect common pathogenetic and pathologic sequence of HCC: chronic liver injury, cirrhosis, atypical hyperplastic nodules, and HCC of early stages (Xue, 2005). The molecular pathogenesis of HCC development is very complex and involves alterations in the structure or in the expression of several tumor suppressor genes, oncogenes and, possibly; mechanisms leading to attainment of genetic instability due to mismatch-repair deficiency or chromosomal instability, and aneuploidy due to defective chromosomal segregation. Central to the molecular pathogenesis of HCCs are mutations of various genes and a genetic instability, which in most cases results from chronic liver disease and the subsequently associated enhancements in liver cell regeneration and hepatocellular mitotic activity.
5.2.2. Epidemiology

Hepatocellular carcinoma represents approximately 6 per cent of all malignancies. It is the fifth most common malignancy in men and the ninth one in women, with an estimated 500,000 to 1 million new cases annually reported from around the world. Its prevalence in young people has risen in recent years due to environmental risk factors at their birth (Elserag, 2001).

5.2.3. Risk factors, predisposing conditions and pathogenesis

Documentation of the wide geographical variations in the incidence of hepatocellular carcinoma has led to the clear identification of several risk factors. Chronic alcohol consumption and cirrhosis are cofactors that increase the development of HCC in patients with chronic viral infection. These include chronic infection with hepatitis B and/or hepatitis C and dietary exposure to hepatotoxic contaminants such as aflatoxin. All these agents may cause hepatocellular carcinoma, but manner by which they interact and their relation to cirrhosis; which underlies most cases, remains an area of active research (Johnson, 1996). HCC etiology varies depending on the geographical location. In countries where HCC is endemic (sub-Saharan Africa, Asia and Alaska), the most common cause is HBV infection, but in low-risk countries the most common HCC cause is cirrhosis caused by chronic viral infection or alcohol consumption (Bailey, 2002).

5.2.4. Cirrhosis

Independent of its cause, cirrhosis is considered to be a major clinical and histopathological risk factor for HCC development. Five per cent of all cirrhotic patients develop HCC (Bailey, 2002).

5.2.5. Precursor of histological injuries

Hepatocarcinogenesis is the development and progression of a HCC chronic liver disease, and is usually a multistep process characterized by poorly understood interacting genetic alterations. HCC coexists with a number of microscopically distinct lesions that are thought to be its precursors. Regenerative nodules are characteristic lesions of the cirrhotic liver. They exhibit a lack of bile ducts and
usually represent poorly organized hepatocytes, which are surrounded by fibrosis and proliferating cholangiocytes. Regenerative nodules may present dysplastic foci, which are smaller than 1 mm and can only be recognized by microscopic studies.

There are two types of dysplastic foci in cirrhotic livers; the small cell-dysplasia and the large cell-dysplasia, according to the nucleocytoplasmic ratio of each one (high in cell-dysplasia and normal in large cell-dysplasia). Small cell-dysplasia are thought to be HCC precursor lesions that result from the proliferation of hepatocytes and oval cells. On the other hand, large cell-dysplasia apparently arise from persistent necroinflammation-induced senescent hepatocytes, and are therefore not considered to be HCC precursor lesions, although patients with large cell-dysplasia are at an increased risk of HCC (Libbrecht, 2005). Dysplastic nodules (DN) are macroscopically recognizable lesions that show atypical features microscopically, such as increased nucleocytoplasmic ratio, nuclear contour, thickness of hepatocellular plates and compression of adjacent hepatocytes. DN represents parts of a spectrum that is arbitrarily divided for the purposes of clinical utility into low-grade or high-grade DN, according to the presence of cytological or structural atypia or both (Hytiroglou, 2004). The risk of HCC in patients with high-grade DN is four-fold higher. By contrast, patients with only low-grade DN are not at a significantly increased risk of HCC (Borzio, 2003).

5.2.6. Hepatitis B infection

Chronic HBV infection is well established as a risk factor for HCC development. Sixty to ninety per cent of patients with HBV-related HCC have cirrhosis, but cirrhosis development is not necessary for HCC development (Bailey, 2002). Chronic HBV infection raises HCC risk because of different factors. Genetic alterations in hepatocytes because of viral DNA; virally-induced chronic inflammation with high cellular proliferation and replication errors with low DNA restoration, eventually produce premalignant cells; and HBV-mediated low activity of intrahepatic Natural Killer cells induces low immunological surveillance. Gender is important in these patients because there is an association between high testosterone levels and HCC in early tumors (Kaplan, 2003).
5.2.7. Hepatitis C virus infection

HCV infection is recognized as a significant risk factor for HCC development, with 6–75 per cent of HCC cases showing the presence of antibodies positive for HCV (Zein et al., 1996; Bailey, 2002). A number of studies have demonstrated a direct relationship between HCC incidence and advanced stages of hepatic fibrosis in chronic active hepatitis (Kaplan, 2003). Because of a HCV-related nonspecific inflammatory process that induces hepatocyte proliferation associated with a rise in alanine-aminotransferase levels, patients with high inflammatory and proliferation activity are more prone to the progression to HCC (Tarao, 2002). High incidence of HCC is seen in people with HCV infection and high alcohol consumption (Kaplan, 2003).

5.2.8. HCV/HBV infection

Co-infection of HBV in people with HCV infection elevates HCC development risk. The mechanisms that cause this high incidence include augmented fibrosis, and inflammation and high cellular re-change (Kaplan, 2003).

5.2.9. Aflatoxins

Aflatoxin is a toxin produced by *Aspergillus flavus* and *A. parasiticus*, which grow in foods like peanuts (Bailey, 2002). It causes alterations in the hepatocyte DNA. It is related to HCC in countries where infestation of crops and animal feed is common (Bosch, 2005). Aflatoxin metabolism produces aflatoxin B1-8, 9-epoxide, which is a toxic product that induces a G to T mutation of the p53 gene at codon 249 up-regulating insulin-like growth factor II that leads to a reduction of apoptosis and HCC formation (Lee, 2000).

5.2.10. Hereditary hemochromatosis

Hereditary hemochromatosis (HH) is an autosomal, recessive disease in which an alteration in iron absorption occurs; finally inducing its deposition in the liver and other organs. HH is a significant risk factor for HCC development. Its presence is associated with a 200% major risk for HCC. Iron toxicity in the liver is produced by free radical formation, lipid peroxidation of cell organs causing cell death with fibrosis and cirrhosis (Bailey, 2002).
5.2.11. Genetic alterations

Some genetic alterations have been associated with HCC development. p53, a protein localized in chromosome 17, is found to be mutated in 30 per cent of HCC cases worldwide. This mutation primarily occurs either because of aflatoxins or HCV, HBV chronic infection (Ozturk, 1999). A protein is produced by p53 that recognizes injured DNA and controls cell replication. B1-8, 9-epoxid-aflatoxin is a toxic product of aflatoxin metabolism and it is metabolized by the epoxide hydrolase and glutathione-S-transferase. If this toxin is not metabolized, it combines with genomic structures to create mutations in p53, producing toxic accumulation (Macdonald, 2001).

5.2.12. Soy protein

The scientific association between soy protein consumption and the reduction of risk of some kinds of tumours is clearly increasing. Several epidemiological and animal studies suggest that consumption of diets containing soybeans and soybean-based products reduces the risk of developing certain types of cancer including breast, prostate and colon cancer (Billings et al., 1990; Herbert et al., 1998; Reza et al., 2001) and lung metastases (Li et al., 1999). The results so obtained have been substantial to account for the therapeutic effects of soy rich diet. Soy which is rich in proteins, saponins, phytoestrogens (genistein and daidzein) and isoflavones have been shown to guarantee protective action against number of diseases.

Soybeans and soybean-based foods are a good source of several phytochemicals, including phytoestrogens such as genistein and daidzein, which are mainly present as the glycoside conjugates, genistin and daidzin. Genistein has been investigated as a potential chemopreventive agent in an azoxymethane induced colon carcinogenesis model using aberrant crypt foci or colon tumour formation in two published studies. In the first study, genistein was administrated in a diet at levels of 75 or 150 mg/kg from 1 week before the azoxymethane to 4 weeks after the first azoxymethane dose for a total of 5 weeks. At both doses, the mean number of foci per colon was significantly reduced (Steele et al., 1995). In a second study, an increase in tumors (mainly non-invasive) was reported in azoxymethane treated rats fed genistein
(Rao et al., 1997). Thus, conflicting results have been reported for the effects of dietary genistein.

In a study Hakkak et al. (2000) have demonstrated that American Institute of Nutrition (AIN)-93G-based diets, with soy protein isolate as the protein source, reduced the incidence of chemically-induced mammary tumors by 16-23% compared with diets with casein as protein source in female Sprague Dawley rats. In fact, isoflavones have an empirical formula similar to estrogens can bind to the same receptors, blocking their action. Genistein has been shown to possess several properties such as antioxidant protection, inhibition of arachidonic acid metabolism, modulation of the cellular integration signals, inhibition of hormone activity, suppression of neoplastic cell growth and of oncogenes. There are other biologically factors present in soy protein such as saponins, phytoesterols, protease inhibitors, and myoinositol hexaphosphate have been also implicated in cancer prevention (Adlercreutz and Mazur, 1997).

5.2.13. Garlic protein

Consumption of more vegetables, especially onion and garlic probably reduces the risk of intestinal and stomach cancer. Allium-containing vegetables such as garlic have been used throughout history for their medicinal properties and treating infections. Several studies showed a decreased risk of gastric cancer with increase in consumption of allium vegetables, possibly by imposing an effect on Helicobacter pylori, as this organism is notoriously associated with gastric cancer.

Inhibition of hepatocellular proliferation in DEN-induced rat liver carcinogenesis is a possible mechanism by which aged garlic extract prevents hepatocarcinogenesis. Daily intake of aged garlic extract could exert a chemopreventive effect on hepatocarcinogenesis in addition to carcinogenesis of the bladder, mammary gland, colon, esophagus, lung, skin, and stomach (Frohlich, 1997).

Garlic contains a limited number of characteristic S-containing precursor peptides including S-allyl cysteinesulfoxide (alliin), which are enzymatically converted to alkyl thiosulfimates such as allylthiosulfinate (allicin). The resultant thiosulfimates are then rapidly transformed during processing to various volatile S-compounds, including allylmercaptocysteine, allylsulfides, and vinylthiin, by
nonenzymatic chemical cascade reactions. Dehydrated garlic powder retains S-containing precursor peptides such as γ-glutamyl-s-allylcysteine and alliin (Kim et al., 1997; Tsao and Yin, 2001).

Diethylnitrosamine treatment in rats was accompanied by a significant decrease in the levels of β-carotene, ascorbic acid, vitamin E, reduced glutathione, glutathione peroxidase, superoxide dismutase and catalase. Garlic administered rats experienced a significant reduction in lipid peroxidation with a simultaneous elevation in antioxidant levels. Researchers hypothesize that the presence of organosulphur compounds could have decreased lipid peroxidation and increased antioxidant levels in DEN administered rats (Sivapatham and Perumal, 2003).

Morioka et al. (1993) demonstrated that a protein fraction (Fraction 4) isolated from aged garlic extract, enhanced cytotoxicity of human peripheral blood lymphocytes against both natural killer sensitive K562 and NK-resistant M14 cell lines and showed that F4 is a very efficient immunopotentiator.

5.2.14. Milk proteins

Milk possesses a protein system constituted by two major families of proteins: caseins (insoluble) and whey proteins (soluble). Caseins account for 80% (w/w) of the whole protein. Whey proteins have been claimed by Tsuda et al. (2000) to prevent cancer; examples include breast and intestinal cancers in female rats, when included in their diet (MacIntosh et al., 1995; Badger et al., 2007). The impact of whey proteins upon cancer prevention has been thoroughly reviewed by Gill and Cross (2000).

The whole whey protein system apparently protects against colon and mammary tumours that had been chemically induced in vivo (Hakkak et al., 2000; Rowlands et al., 2001). In vivo experiments have also unfolded the anticarcinogenic and anticancer activities ascertained to whey protein concentrate (WPC), via their effect upon increase of GSH concentration in relevant tissues; stimulation of immunity via the GSH pathway actually originates anti-tumour effects in low-volume tumours (Bounous, 2000).

On the other hand, published evidence (Parodi, 1998) suggests that WPC can deplete tumour cells which possess a concentration of GSH higher than normal cells,
and in addition render them more vulnerable to chemotherapy; Hydrolyzed WPI protected against oxidant-induced cell death in a human prostate epithelial cell line, again because of the increment of GSH synthesis effected thereby (Kent et al., 2003). WPI may also protect from cancer by acting as a coadjuvant of baicalein- an anticancer drug; the cytotoxicity of this molecule is enhanced by inducing more apoptosis in the human hepatoma cell line Hep G2, which is in turn associated with depletion of GSH (Tsai et al., 2000).

Whey proteins suppressed in vitro lymphocyte mitogenesis and alloantigen-induced proliferation, when included in mature murine lymphocytes solutions (Barta et al., 1991). Modified WPC also suppressed the mitogen-stimulated secretion of α-interferon, as well as the surface expression of interleukin-2 receptor, when added to T and B lymphocyte cultures (Cross and Gill, 1999). On the other hand, Mercier et al. (2004) claimed that addition of whey proteins from micro filtered WPI to cell culture media, at a concentration of 100 mg/ml, stimulates in vitro proliferation of murine spleen lymphocytes.

5.2.15. Diethylnitrosamine

Diethylnitrosamine is a potent carcinogenic dialkylnitrosoamine used to induce liver cancer in animal models. DEN belongs to the group of N-nitrosamines, causing a wide range of tumors in all animal species and suspected to be health hazards in man (Loeppky, 1999; IARC, 2000). Exposure of man to preformed N-nitrosamines occurs through the diet and in certain occupational settings and is also because of the use of tobacco products, cosmetics, pharmaceutical products and agricultural chemicals.

The formation of reactive oxygen species results in oxidative stress, which may be one of the key factors in the etiology of liver cancer (Loeppky, 1999). Diethylnitrosamine is present in a majority of important dietary sources like cured meat, salami, millet flour and dried cuttle fish. DEN is metabolized primarily in the liver by cytochrome P450 to ethyl-acetoxyethyl-nitrosamine. This intermediate is conjugated by the phase II enzymes to a nontoxic compound, or it can alternatively
produce ethyl-diazonium ion, which directly ethylates cellular macromolecules (Muzio et al., 1999). Metabolic activation of DEN by cytochrome P450 enzymes is responsible for its cytotoxic, mutagenic and carcinogenic effects (Archer, 1989). The increase in ROS production caused by DEN is instrumental in bringing about HCC (Bansal et al., 2000).

In experimental carcinogenesis, a multipotential element called oval cell proliferates in the early stages. The cellular events are accompanied by increased expression of several growth factors that enhance the survival of carcinogen-activated cells by suppressing apoptosis and increasing elements entering the cell cycle. Hepatic carcinogenesis is a complex process associated with accumulation of genetic and epigenetic changes that run through steps of initiation, promotion and progression. Activation of oncogenes of the "ras" family and others has been detected during chemically-induced HCC in rodents.

The high incidence of carcinoma, especially hepatocellular carcinoma due to exposure to carcinogens as a result of pollution and use of various chemicals in industry and day to day life, is alarming. Sensing the need to prevent the spread of hepatocellular carcinoma, the present study is taken up with the objective of finding a cancer preventive neutraceutical from the common food. Therefore, we hypothesized that protein isolates from soy, garlic, coconut, whey and casein will have therapeutic value in checking the onset of hepatocellular carcinoma and this hypothesis is tested experimentally in the mice model.
5.3. MATERIALS AND METHODS

5.3.1. Chemicals

Diethylnitrosamine were obtained from M/s. Sigma Chemical Company, St. Louis. MO, USA. Other chemicals used in this study were obtained from Himedia laboratories, Mumbai, India and were of analytical grade.

5.3.2. Animals

Male Swiss albino mice (*Mus musculus*) of body weight in the range of 10-11 g were used for the present study. Mice were obtained from National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India. The animals were housed individually in polypropylene cages with wire mesh bottom and maintained at 25°C ± 2, under standard lighting conditions (12 -hrs light/dark cycle).

All animal experimental procedures were carried out in accordance with the ‘principles of laboratory animal care’ (NIH publication no.85-23, revised 1985) and with the approval of the “Institute’s ethical committee on animal experiments” (Registration no: 738/03/abc/CPCSEA) at St. Joseph’s College (Autonomous), Tiruchirapalli, Tamil Nadu, India.

5.3.3. Diet composition and preparation

The diet fed to mice in these experiments were formulated according to AIN-93G guidelines (Reeves *et al.*, 1993 and Reeves, 1997) and the composition of the diet used is given in Table 5.1.

Specific protein isolate diet was prepared by mixing the protein (20%) with salt and vitamin mixtures (as shown in Table 5.1). The diets were prepared once in 2 weeks and was stored in airtight containers in a refrigerator and used.

5.3.4. Preparation of protein isolates

i) *Isolation of garlic protein (Biju and Augusti, 1996)*

Fresh garlic (*Allium sativum*) cloves were purchased from the local market and were washed thoroughly and freed from contaminants. The garlic cloves were then blended at high speed for 5-10 minutes with 200 ml of distilled water/kg of cloves. Slurry was transferred into a filter bag made of closely woven fabric and pressed. A
Table - 5.1: Dietary composition of various protein source diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Soy protein (gm)</th>
<th>Garlic protein (gm)</th>
<th>Coconut protein (gm)</th>
<th>Whey protein (gm)</th>
<th>Casein (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein*</td>
<td>240</td>
<td>240</td>
<td>240</td>
<td>240</td>
<td>240</td>
</tr>
<tr>
<td>Starch</td>
<td>635</td>
<td>635</td>
<td>635</td>
<td>635</td>
<td>635</td>
</tr>
<tr>
<td>Groundnut oil</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Mineral mix •</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>L.Cystine</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Preparation of protein isolates explained in 5.3.4.

• Preparation given in Table 5.2
Table 5.2: Mineral mix that supplies the recommended concentrations of elements (Patrick, 1983)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>gm/kg mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salt mixture A#</td>
<td>16.7</td>
</tr>
<tr>
<td>Calcium citrate</td>
<td>308.2</td>
</tr>
<tr>
<td>Ca (H₂PO₄)₂ H₂O</td>
<td>112.8</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>218.7</td>
</tr>
<tr>
<td>KCl</td>
<td>124.7</td>
</tr>
<tr>
<td>NaCl</td>
<td>77</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>68.5</td>
</tr>
<tr>
<td>MgSO₄ (anhydrous)</td>
<td>38.3</td>
</tr>
<tr>
<td>3MgCO₃ . Mg (OH)₂ . 3H₂O</td>
<td>35.1</td>
</tr>
<tr>
<td><strong>Salt mixture A#</strong></td>
<td><strong>gm</strong></td>
</tr>
<tr>
<td>FeNH₄ citrate, USP</td>
<td>91.41</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>5.98</td>
</tr>
<tr>
<td>NaF</td>
<td>0.76</td>
</tr>
<tr>
<td>MgSO₄.2H₂O</td>
<td>1.07</td>
</tr>
<tr>
<td>KAl (SO₄).12 H₂O</td>
<td>0.54</td>
</tr>
<tr>
<td>KI</td>
<td>0.24</td>
</tr>
<tr>
<td><strong>100.00</strong></td>
<td><strong>100.00</strong></td>
</tr>
</tbody>
</table>
thick residue as well as an extract was obtained as a result. The residue was double extracted and the residue to water ratio was kept as 1:10 for 10 hrs and the mixture was then centrifuged at 6000 rpm for 30 minutes. This process led to the formation of a supernatant (Supernatant II) and yet another residue, which was discarded later on.

On the other hand, the extract formerly obtained was taken separately and centrifuged at 6000 rpm for 30 minutes. This process gave off an extract (supernatant I and a residue), i.e., residue I, which was discarded. Both supernatants were then taken and combined together, and this mixture was acidified to a pH of 4.5 with 1N HCl (At pH of 4.5, about 80% of the water-soluble proteins precipitated out, indicating that this is the isoelectric pH of major storage proteins) allowed to stand for 6 hrs at 4°C, and later on, centrifuged at 10,000 rpm for 30 minutes. An acidified precipitate of protein was obtained. This precipitate was redissolved in water, dialyzed extensively against distilled water at 4°C and then freeze-dried. The total yield was in the range of 50-60 g protein/kg wet garlic cloves.

ii) Isolation of coconut protein

Fresh coconuts (*Cocus nucifera*) were purchased from the local market and the kernel was peeled carefully and defatted with solvent n-hexane. Later the extracts were air dried to remove all solvent n-hexane. Then the contents were homogenized in a high speed blender using phosphate buffer (Mepba and Achinewhu, 2003). Ammonium sulphate precipitation was carried out to concentrate the protein present in the supernatant obtained after centrifugation of the coconut homogenate.

Typically, the supernatant, whose pH has been brought to neutrality by addition of ammonium hydroxide, was stirred gently in an ice-water bath, whilst finely powdered solid ammonium sulphate were added in small portions, with periodic checking of the pH, until 95-100 % saturation was reached (650-697 g in 1 L of solution). After the suspension has been stirred gently for 30 minutes, the precipitate was removed by centrifugation, typically at 10,000 g for 10 minutes at 0-4°C Celsius.

The supernatant was carefully removed by suction, and the precipitated pellet was resuspended in a small quantity of buffer. Strips of dialysis tubing (Himedia make- LA 402) of 15 cm length were cut and boiled twice in 0.1M NaHCO₃
containing 10 mM EDTA to remove heavy metal ions and thoroughly rinsed (inside and outside) with distilled water, stored at 4°C in 20% (v/v) ethanol and used.

The protein sample was introduced and the free ending of the tubing was closed by knotting. The filled dialysis bag was placed in dialysis buffer in a beaker and stirred with magnetic stirrer. Periodically the dialysis buffer was changed to accelerate the process (Pingoud et al., 2002). The dialyzed sample was lyophilized and stored at -20 °C and used.

iii) Soy protein, Whey protein and Casein

Soy protein and whey protein were obtained from Warkem Biotech Pvt. Limited, Thane, India. Casein protein powder (code no-RM497) was obtained from Himedia laboratories, Mumbai, India.

5.3.5. Experimental setup

Weaned mice of 10-11 g of body weight were used for the study. The animals were divided into the following seven groups of six mice each.

Group I- Control
Group II- DEN (Experimental control)
Group III- Soy protein
Group IV- Garlic protein
Group V- Coconut protein
Group VI- Whey protein
Group VII- Casein

The first group, the control and the second group DEN (Experimental control) were fed with the normal protein diet and water ad libitum throughout the study. The groups from III to VII were given protein from different sources as given above, but equal in amount to the normal diet.

All the seven groups were acclimatized in their respective diets for 60 days.

On the 60th day, all the animal in groups, II to VII, DEN were given intraperitoneally as a single dose (120 mg/kg body weight) in saline. This was followed by weekly subcutaneous injections of carbon tetrachloride for four weeks at individual doses of 3 mL/kg body weight to induce hepatocellular carcinoma.
control and DEN animals were continued on the normal diet and the groups III to VII were fed their respective diets till the 120\textsuperscript{th} day. Thus Group II consisted of animals that were HCC-induced with DEN. Animals belonging to groups III-VII were pre and post treated with different protein isolates from various sources. A schematic representation is given in Figure 5.1.

At the end of the experimental period, which is on day 120, the mice were sacrificed and blood was collected. The serum was separated from blood and used for the determination of various parameters. The liver tissue was excised immediately and thoroughly washed with physiological saline. Alpha-fetoprotein, ornithine decarboxylase, tumor necrosis factor-\(\alpha\), \(\gamma\)-glutamyl transpeptidase, aspartate transaminase, alanine transaminase, alkaline phosphatase, superoxide dismutase, catalase, and glutathione peroxidase were determined both in serum and liver homogenate. In addition to the above mentioned parameters histological evaluation was carried out.

5.3.6. Alpha-Fetoprotein

AFP was estimated in serum and liver tissue by ELISA using a kit purchased from Monobind, Inc., (USA), as per manufacturer’s instructions.

Principle

AFP calibrator, sample / control are first added to a streptavidin-coated well. Biotinylated monoclonal and enzyme labeled antibodies (directed against distinct and different epitopes of AFP) are added and the reactants are mixed. Reaction between the various AFP antibodies and native AFP forms a sandwich complex that binds with the streptavidin coated to the well. After the completion of the required incubation period, the enzyme-AFP antibody bound conjugate is separated from the unbound enzyme-AFP conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well is quantitated by reaction with a suitable substrate to produce colour.

Procedure

The immobilization of AFP takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well with
Figure 5.1: Schematic representation of the study using different protein sources on induced hepatocellular carcinoma

Groups

I. Control
II. DEN
III. Soy protein
IV. Garlic protein
V. Coconut protein
VI. Whey protein
VII. Casein

Diet from day 1 to 120

I. Normal
II. Normal
III. Soy protein
IV. Garlic protein
V. Coconut protein
VI. Whey protein
VII. Casein

Day 60

I. Saline
II. DEN
III. Soy protein
IV. Garlic protein
V. Coconut protein
VI. Whey protein
VII. Casein

Weekly dose of CCl4 (4 weeks)

I. Saline
II. CCl4
III. CCl4
IV. CCl4
V. CCl4
VI. CCl4
VII. CCl4

Day 120

I. Sacrificed
II. Sacrificed
III. Sacrificed
IV. Sacrificed
V. Sacrificed
VI. Sacrificed
VII. Sacrificed
exogenously added biotinylated monoclonal anti-AFP antibody. Upon mixing monoclonal biotinylated antibody, the enzyme labeled antibody and serum containing the native antigen, a reaction results between the native antigen and the antibodies; without competition or steric hindrance, to form a soluble sandwich complex. The interaction is illustrated using the following equation:

\[
\text{EnzAb} + \text{Ag}_{\text{AFP}} + \text{BnAb}^{(m)} \rightarrow \text{EnzAb} - \text{Ag}_{\text{AFP}} - \text{BnAB}^{(m)}
\]

\[\text{BnAb}^{(m)} = \text{Biotinylated Monoclonal Antibody (Excess Quantity)}\]

\[\text{Ag}_{\text{AFP}} = \text{Native Antigen (Variable Quantity)}\]

\[\text{EnzAb} - \text{Ag}_{\text{AFP}} - \text{BnAb}^{(m)} = \text{Antigen-Antibodies Sandwich Complex}\]

\[K_a = \text{Rate Constant of Association}\]

\[K_a = \text{Rate Constant of dissociation}\]

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

\[
\text{EnzAb} \cdot \text{Ag}_{\text{AFP}} \cdot \text{BnAb}^{(m)} + \text{Streptavidin}_{\text{c.w.}} \Rightarrow \text{Immobilized complex}
\]

\[\text{Streptavidin}_{\text{c.w.}} = \text{Streptavidin immobilized on well}\]

\[\text{Immunobilized complex} = \text{sandwich complex bound to the well}\]

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration.

**Reagents**

1. Alpha- fetoprotein
2. Anti- AFP Enzyme Reagent: contains enzyme-labeled antibody, biotinylated monoclonal IgG in buffer.
3. Streptavidin- Coated Microplate
4. Wash Solution Concentrate (containing a surfactant in buffered saline)
5. Substrate A - containing tetramethylbenzidine in buffer.
**Test Procedure**

The microplate wells were formatted for each serum reference, control and specimen to be assayed in duplicate. 25 µl of the appropriate serum reference, control or specimen was pipetted out into specifically assigned wells. 100 µl of the anti-AFP enzyme reagent was added to each well. The microplate was swirled gently for 20-30 seconds to mix and later, covered. The mixture was incubated for 60 minutes at room temperature. The contents of the microplate were discarded by decantation or aspiration. While decanting, the plate was tapped and blotted dry with absorbent paper. 300 µl of wash buffer was added, decanted (by tapping and blotting). The procedure was repeated twice for a total of three washes. The wash was decanted and the same procedure was repeated twice. Later 100 µl of working substrate solution was added to all the wells. Incubation was carried out for a period of fifteen minutes at room temperature. Then 50 µl of stop solution was added to each well and mixed gently for 15-20 seconds. Precautionary measures were taken in ensuring the addition of reagents in the same order, to minimize reaction time differences in between the wells. The absorbance in each well was read at 450 nm (using a reference wavelength of 620-630 nm to minimize well imperfections) in a microplate reader. The results were obtained within thirty minutes of adding the stop solution.

**5.3.7. TNF-α**

TNF-α was estimated in serum and liver tissue by ELISA using a kit purchased from BioSource Kit, (USA), as per manufacturer’s instructions.

**Principle**

Samples, including standards of known mouse (Ms) TNF-α content, and control specimens are pipetted into Ms TNF-α coated wells followed by the addition of a biotinylated monoclonal secondary antibody. During the first incubation, the Ms TNF-α antigen binds simultaneously to the immobilized (captured) antibody on one site, and to the solution phase-biotinylated antibody on a second site. After removal of excess second antibody, streptavidin-peroxidase (enzyme) is added. This enzyme binds itself to the biotinylated antibodies to complete the four-member sandwich. After a second incubation and washing to remove the entire unbound enzyme, a
substrate solution is added; which is acted upon by the bound enzyme to produce colour. The intensity of this colored product is directly proportional to the concentration of Ms TNF-α present in the samples.

Reagents
1. Mouse TNF-α standard: recombinant Ms TNF-α
2. Standard diluent buffer: 15 mM sodium azide
3. Incubation buffer: 8 mM sodium azide
4. Mouse TNF-α high and low controls: recombinant Mouse TNF-α
5. Mouse TNF-α Antibody coated wells
6. Mouse TNF-α biotin conjugate (Biotin-labeled anti-TNF-α): contains 15 mM sodium azide.
7. Streptavidin-Peroxidase containing 3.3 mM thymol
8. Wash buffer concentrate
9. Stabilized chromogen; Tetramethylbenzidine
10. Stop solution.

Procedure
To obtain a standard curve, 100 µl of standards was added to the appropriate microtiter wells and 100 µl of standard diluent buffer was added to zero wells. Serum samples were diluted two-fold using incubation buffer. Later 200 µl of incubation buffer was added to each well, followed by the addition of 50 µl of the sample. 100 µl buffered solution and control was added to appropriate microtitre wells. 50 µl of biotinylated anti-TNF-α (Biotin conjugate) solution was added into each well except that for the chromogen blank. The wells containing standard aliquots and samples were incubated for 90 minutes at room temperature. Later, the contents were aspirated and wells were washed for roughly about four times. 100 µl of Streptavidin-HRP working solution was added to each well except the chromogen blank. Once again, the wells were incubated for 30 minutes at room temperature. The contents of the wells were aspirated and the washing step was repeated again for around four times. Then 100 µl of stabilized chromogen was added to each well, incubated for 30 minutes at room temperature in the dark and finally; 100 µl of stop solution was added
to all wells. The absorbance of each of the wells was read at 450 nm having calibrated the plate reader against a chromogen blank composing 100 µl each of both stabilized chromogen and the stop solution.

5.3.8. Ornithine decarboxylase

Ornithine decarboxylase activity was measured in serum and liver tissue according to the method of Badolo et al. (1999).

Principle

Putrescine is measured directly in terms of the H₂O₂ generated by reaction with soybean amine oxidase.

Reagents

1. Horse radish peroxidase, type II
2. Diaminohexane
3. 4-aminoantipyrin
4. Tris-(hydroxymethyl) aminomethane.
5. Phenol
6. Putrescine
7. 5’’-pyridoxal phosphate
8. Ornithine monohydrochloride

Purification of soybean amine oxidase (Nikolov et al., 1999)

Briefly soybean (Glycine max) seeds were germinated at room temperature for 7 days in the dark. The seedlings were blended in an equal volume of water. The macerate was filtered and the supernatant thus obtained was fractionated by using different concentrations of ammonium sulphate. The precipitate was dissolved in a minimum of phosphate buffer (pH 7.8) and applied to a sephacryl S-200 column eluted with buffer solution. Fractions were collected and the most active ones were pooled out and desalted by passing through a column packed with sephadex G-25. The purified, crystalline enzyme was stored at -25°C and was used when carrying out the assay.
Enzymatic assay of putrescine

Putrescine was measured by a colorimetric quantification of the H$_2$O$_2$ released after its oxidation by soybean-amine oxidase (SAO). The assay was performed according to the method of (Emerson, 1943) adapted for the microplate (96 wells) assay and absorbance was read using a microplate reader. Each well contained a 230 µl reaction mixture composed of 50 µmol Tris buffer of pH 9.5, 50 µg phenol, 40 µg 4-aminoantipyrin, 0.11 U SAO, 3.5 U horse radish peroxidase and different amounts of putrescine (from 2.5 to 50 nmol). Samples were added to spiked samples to simulate a biological medium. The reaction starts with the addition of the substrate. The oxidation of 1 mol putrescine by SAO usually generates 1 mol H$_2$O$_2$, which reacts with 4-aminoantipyrin and phenol by the means of horse radish peroxidase to yield a coloured complex which shows absorption maxima at 492 nm (reference 690 nm). The absorbance was determined every 10 minutes. The absorbance was stopped at the end of the oxidation reaction, indicated by the end of the change in absorbance values.

Ornithine decarboxylase assay

ODC (0.222 mg) (4.8 mu-1U release 1 µ mol CO$_2$ min$^{-1}$ from ornithine) in 100 µl buffer (20 mM sodium phosphate 0.2 mM EDTA and 0.1 mM 5”-pyridoxal phosphate, pH 7.4) was incubated at 37°C for 5 minutes, followed by which, 50 µl of water was added and then the mixture was further incubated for 15 minutes. Ornithine (50 µl, 5 mM) was added and the reaction was allowed to proceed for 30 minutes. The assay was put to an end by heating the mixture at 90°C for 1 minute to denature the ODC protein. The mixture (25 µl) was directly used for the putrescine assay.

5.3.9. Gamma-Glutamyl Transpeptidase

GGTP activity was assayed in serum and liver tissue using a kit purchased from Biosystems (Spain), as per manufacturer’s instructions.

Principle

Gamma-glutamyltransferase (γ-GT) catalyzes the transfer of the γ-glutamyl group from γ-glutamyl-3-carboxy-4-nitroanilide to glycylglycine, liberating 3-
carboxy-4-nitroaniline. The catalytic concentration is determined from the rate of 3-carboxy-4-nitroaniline formation.

\[
gamma\text{-Glutamyl-3-carboxy-4-nitroanilide} + \text{Glycylglycine} \xrightarrow{\gamma\text{-GT}} \text{Glutamyl-glycylglycine} + 3\text{-carboxy-4-nitroaniline}
\]

**Reagents**

A. Glycylglycine 206.25 mmol/L, sodium hydroxide 130 mmol/L, pH 7.9  
B. \(\gamma\)-Glutamyl-3-carboxy-4-nitroanilide 32.5 mmol/L

Working reagent was prepared by mixing reagent A and B.

100 µL of sample and 1.0 mL of working reagent was taken in a cuvette, and then the contents were mixed, following which, the initial absorbance was taken at 1 minute intervals thereafter for 3 minutes using 405 nm. The difference between consecutive absorbance readings and the average absorbance difference per minute was calculated.

**5.3.10. Alanine Transaminase**

ALT activity was assayed in serum and liver tissue using a kit purchased from Qualigens, as per manufacturer’s instructions.

**Principle**

Alanine transaminase catalyzes the transfer of an amino group from L-alanine to \(\alpha\)-ketoglutarate with the formation of pyruvate and glutamate as products of the reaction. The pyruvate thus formed is allowed to react with 2, 4 dinitrophenyl hydrazine (DNPH) to produce 2, 4 dinitrophenyl hydrazone derivative which is brown coloured in alkaline medium. The absorbance of hydrazone derivative is correlated with alanine transaminase activity by plotting a calibration curve using the values obtained for pyruvate standard.

\[
\text{Pyruvate} + 2, 4\text{DNPH} \xrightarrow{\text{alkaline medium}} 2, 4\text{dinitrophenyl hydrazone (Brown coloured)}
\]
Reagents

1. Buffered substrate (pH 7.4): 1.78 g of DL-alanine and 30 mg of α-ketoglutarate were dissolved in 20 ml of phosphate buffer containing 1.25 ml of 0.4 M NaOH and the solution was made up to 100 ml with buffer and adjusted to pH 7.4 upon sensing the necessity for doing so.

2. DNPH colour reagent: 200 mg of 2, 4 dinitrophenylhydrazine (2, 4 DNPH) was dissolved in hot 1M HCl and made up to 1 liter with the same.

3. Sodium hydroxide (4 N): 16 g of sodium hydroxide was dissolved in about 800 ml of distilled water and then accurately made up to 1 liter with distilled water. (1:10 dilution with distilled water before use).

4. Pyruvate standard (2 mM): 220 mg of sodium pyruvate was dissolved in phosphate buffer and made up to 100 ml. 10 ml of this solution was diluted to 100 ml with phosphate buffer to obtain a working standard solution containing 2 mol of pyruvate per ml.

Procedure

500 µl of buffered substrate was taken, both in blank test tube and sample test tubes and then, the mixture was incubated at 37°C for 3 minutes. 100 µl of serum/tissue homogenate was taken in sample tubes. The contents were mixed well and the test tubes were incubated at 37°C for 30 minutes. 500 µl of DNPH colour reagent was added to all test tubes, and they were mixed well and allowed to stand at room temperature for 20 minutes. 0.1ml of distilled water was added to the blank following 500 µl of working sodium hydroxide, which was added to all tubes, mixed well and allowed to stand at room temperature for 10 minutes and then, the absorbance was taken for each of the tubes at 505 nm. Suitable aliquots of standard solution were taken and treated in a similar manner to obtain a standard curve for comparison.

5.3.11. Aspartate Transaminase

AST activity was assayed in serum and liver tissue using a kit purchased from Qualigens, as per manufacturer’s instructions.
**Principle**

Aspartate transaminase catalyzes the transfer of an amino group from L-alanine to α-ketoglutarate, which culminates in the formation of oxaloacetate and glutamate as products of the reaction. The oxaloacetate so formed, is allowed to react with 2, 4 dinitrophenyl hydrazine (DNPH) to produce a derivative of 2, 4 dinitrophenylhydrazone, which is brown-coloured in an alkaline medium. The absorbance of hydrazone derivative is correlated with aspartate transaminase activity by plotting a calibration curve using pyruvate standards.

\[ \text{L-alanine} + \alpha\text{-ketoglutarate} \xrightarrow{\text{AST, pH 7.4}} \text{oxaloacetate} + \text{L-glutamate} \]

\[ \text{Oxaloacetate} + 2,4 \text{DNPH} \xrightarrow{\text{alkaline medium}} 2,4 \text{dinitrophenylhydrazone (Brown coloured)}} \]

**Reagents**

1. Buffered substrate pH 7.4: 2.66 g DL-aspartic acid and 30 mg α-keto glutarate were both dissolved in 20.5 ml of 1 M NaOH. The pH was adjusted to 7.4 by adding 1 M NaOH drop-wise while stirring the solution.

2. DNPH colour reagent: 200 mg of 2, 4 dinitro-phenylhydrazine (2, 4 DNPH) was dissolved in hot 1M HCl and made up to 1 litre with 1M HCl.

3. Sodium hydroxide 4 N: 16 g sodium hydroxide was dissolved in about 800 ml of distilled water and made up to 1 litre using the same.

4. Pyruvate standard 2 mM: 220 mg of sodium pyruvate was dissolved in phosphate buffer and made up to 100 ml. 10 ml of this solution was further made up to 100 ml with phosphate buffer to obtain a working standard solution containing 2 mmol of pyruvate per ml.

**Procedure**

500 µl of buffered substrate was taken, both in blank test tube and sample test tubes and then, the mixture was incubated at 37°C for 3 minutes. 100 µl of serum/tissue homogenate was taken in sample tubes. The contents were mixed well and the test tubes were incubated at 37°C for 30 minutes. 500 µl of DNPH colour reagent was added to all test tubes, and they were mixed well and allowed to stand at room temperature for 20 minutes. 0.1 ml of distilled water was added to the blank
following 500 µl of working sodium hydroxide, which was added to all tubes, mixed well and allowed to stand at room temperature for 10 minutes and then, the absorbance was taken for each of the tubes at 505 nm. Suitable aliquots of standard solution were taken and treated in a similar manner to obtain a standard curve for comparison.

5.3.12. Alkaline Phophatase

Alkaline phosphatase was estimated according to the method of (Moss et al., 1971).

Reagents

A. Sodium phenyl phosphate, 100 mmol/l. 2.18 g in a liter of water. The reagent was boiled, cooled and then, a little amount of chloroform was added.

B. Sodium carbonate-sodium bicarbonate, 100 mmol/l. 6.36 g anhydrous sodium carbonate and 3.36 g sodium bicarbonate was made up to 1 liter.

1. Buffered substrate (pH 10) - Prepared by mixing equal volumes of solution A and B.

2. Folin - Ciocalteau’s reagent.

3. Sodium carbonate solution: 150 g of anhydrous sodium carbonate in 1 litre of water.

4. Stock standard phenol reagent, (1 g/l): 1gm of pure, crystalline phenol was dissolved in 100 mmol/l hydrochloric acid and made up to 1 liter with the same.

5. Working standard solution containing phenol reagent: 100 ml of diluted phenol reagent and 5 ml of stock standard was made upto 500 ml with distilled water.

Procedure

4 ml of buffered substrate was pipetted out into a test tube and placed in a water bath at 37°C for few minutes, followed by which; 0.2 ml of serum was added and incubation was continued for another 15 minutes in the water bath. Immediately, 1.8 ml of diluted phenol reagent was added to it. At the same time, a control containing 4 ml buffered-substrate and 0.2 ml of serum along with 1.8 ml diluted phenol reagent was prepared, mixed well and centrifuged. 2 ml of sodium carbonate
was added to 4 ml of the supernatant. At the same time, a standard was prepared by adding 2 ml of sodium carbonate to 4 ml working standard containing phenol reagent. The tubes were placed at 37˚C for 15 minutes and absorbance was read using 700 nm against the blank containing 3.2 ml of water, 0.8 ml of diluted phenol reagent and 2 ml of sodium carbonate reagent.

Alkaline phosphatase activity

\[
\frac{\text{Reading of unknown} - \text{Reading of control}}{\text{Reading of standard} - \text{Reading of blank}} = \frac{40}{10^3} \times \frac{6}{4} \times \frac{100}{0.2} = \text{U/L.}
\]

5.3.13. Super Oxide Dismutase

SOD activity was assayed in serum and liver tissue using a kit purchased from Cayman (USA), as per manufacturer’s instructions.

Superoxide dismutases are metalloenzymes that catalyze the dismutation of the superoxide anion to yield molecular oxygen and hydrogen peroxide, and thus form a crucial part of the cellular antioxidant defense system.

\[
2\text{O}_2^- + 2\text{H}^+ + \text{SOD} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

The Cayman chemical kit for superoxide dismutase assay utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of the enzyme needed to exhibit 50% dismutation of the superoxide radical.

Pre-assay preparation

1. Assay buffer (10X): 3 ml of assay buffer concentrate was diluted with 27 ml of HPLC grade water. This assay buffer (50 mM Tris-HCL, pH 8.0, containing 0.1 mM diethylenetriaminepenta-acetic acid and 0.1 mM hypoxanthine, was used to dilute the radical detector.
2. Sample buffer (10X): sample buffer concentrate was diluted with 18 ml of HPLC grade water. This final sample buffer (50 mM Tris-HCL, pH 8.0) was used for the preparation of SOD standards and for diluting xanthine oxidase.
3. Radical detector: Solution of tetrazolium salt.
4. SOD standard: bovine erythrocyte SOD.
5. Xanthine oxidase: 50 µl of enzyme source was diluted with 1.95 ml of the sample buffer.
Sample preparation

Tissue homogenate: Liver tissue was perfused with phosphate-buffered saline, pH 7.4, containing as little as 0.16 mg/ml heparin to remove any RBC and clots that may interfere with the assay. The tissue was homogenized in 5-10 ml of cold 20 mM HEPES buffer, pH 7.2, containing 1 mM EDTA, 210 mM mannitol and 70 mM sucrose per gram tissue. The homogenate was centrifuged at 1,500 x g for 5 minutes at 4°C. Supernatant was used for the assay.

Procedure

20 µl of SOD standard was diluted with 1.98 ml of sample buffer and aliquots comprising a series of working standard in the range 20-200 µl were prepared and used. 10 µl of standards /serum sample and 200 µl of diluted radical detector were added to the designated wells on the plate. Reactions were initiated by the addition of 20 µl of diluted xanthine oxidase to all the wells that were used. The plate was incubated at room temperature for 20 minutes and finally, the absorbance was read at 450 nm using a plate reader.

Super oxide dismutase is expressed as U/mg tissue.

5.3.14. Glutathione Peroxidase

Glutathione peroxidase enzyme activity was assayed according to the method of Rotruck et al. (1973).

Principle

Glutathione peroxidase catalyses the following reaction

\[ 2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + \text{H}_2\text{O} \]

Reagents

1. Phosphate buffer (pH 7.0); 0.3 M
2. EDTA 0.8 mM
3. Sodium azide 10 mM
4. Glutathione (reduced) : 4 mM
5. Hydrogen peroxide
6. Trichloroacetic acid 10%
7. Disodium hydrogen phosphate: 0.3 M
8. DTNB: 40 mg of 5, 5’ dithio bis (2-nitro benzoic acid) in 1% sodium citrate.
9. Standard glutathione (reduced): 10 mM.

**Procedure**

An incubation mixture containing exactly 0.4 ml of buffer, 0.2 ml each of EDTA, sodium azide, GSH and H₂O₂ was pre-incubated at 37°C for 10 minutes. 0.1ml of sample was added and the incubation was carried out at 37°C for 10 minutes. The reaction was terminated with the addition of 0.5 ml of TCA. The reaction mixture was centrifuged and 0.5 ml of supernatant was taken. Following this, 3.0 ml of phosphate solution and 1.0 ml of DTNB was added and the colour developed was read at 412 nm. Suitable aliquots of standard solution were taken and treated in a similar manner to obtain a standard curve for comparison.

Glutathione peroxidase is expressed as mg GSH utilized /min/mg protein

**5.3.15. Catalase**

Catalase (EC. 1.11.1.6) enzyme activity was assayed according to the method of (Sinha, 1972).

**Principle**

This enzyme catalyses the reaction

\[ H₂O₂ \rightarrow 2H₂O + O₂ \]

Unreacted H₂O₂ is measured by its ability to reduce dichromate in acetic acid solution.

**Reagents**

1. Dichromate acetic acid solution: 5% potassium dichromate in glacial acetic acid.
2. Hydrogen peroxide 0.2 M.
3. Phosphate buffer (pH 7.0): 0.01 M.
Procedure

To 0.1 ml of sample, 1 ml of buffer and 0.4 ml of distilled water was added. The reaction was initiated by the addition of 0.5 ml of H₂O₂, and the reaction mixture was incubated at 37°C for 1 minute. The reaction was terminated with the addition of 2.0 ml of dichromate-acetic acid reagent. Standard H₂O₂ solution in the range of 4-20 µ moles was taken and treated in the same manner. The tubes were heated in a boiling water bath for 15 minutes, cooled and then the absorbance was read using a 510 nm filter.

Catalase activity is expressed as µ moles of H₂O₂ utilized/min/mg protein.

5.3.16. Histology

Liver tissue was fixed in 10% formalin, routinely processed and embedded in paraffin. Paraffin sections (5 µm) were cut on glass slides and stained with hematoxylin and eosin (H and E) and examined under light microscope.

5.3.17. Statistical Analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Bonferroni’s test using Statistical Program for the Social Sciences (SPSS) software version 13.0. Results were expressed as mean for six mice in each group. Values of (P <0.05) were considered significant.
5.4. RESULTS AND DISCUSSION

In our results and discussion the following order is followed, first the body weight is analyzed; based on the body weight the growth of the animals before and after treatment with DEN is discussed. The biochemical parameters are measured both in the serum and liver tissue. In our analysis of results, the data on the cancer marker growth regulator (AFP) and cytokine (TNF-α) pertaining to the serum and liver tissue are first taken and analyzed and discussed. This is followed by an analysis and discussion of the data on the cancer marker enzymes (ODC and GGTP). Thirdly the liver function enzymes and the antioxidant enzymes are dealt with separately.

5.4.1. Body weight

The weaned mice, just introduced to solid food were taken for the present study. As already explained they were divided into groups and provided with protein isolates from different dietary sources. Their growth was recorded in terms of body weight at regular time intervals. Animal groups from III to VII were treated with five different protein isolates for 60 days and at the end of it DEN was injected into the animal in all groups. They were continued on their respective diets for 120 days. The control and DEN alone administered group were on normal diet through out.

i) Growth Rate

The body weights of mice fed with control and the five different source proteins isolate diets are presented in Table 5.3 and Figure 5.2. The growth rate, as calculated by a gain of body weight was similar in all groups, upto 3 weeks; the start of the study. From the 4\textsuperscript{th} week onwards, variations in their growth rates was observed, such that the body weight gain seen in garlic protein, coconut protein and casein-fed mice was found to be higher than that for soy and whey protein-fed mice.

The body weight of whey protein fed animals was 75\% and that of soy protein fed animals was 93\% of the control. A significant (P<0.05) decrease was observed in the whey protein-fed group when compared to control and DEN-induced pro-hepatocellular carcinoma animals. Royle \textit{et al.} (2008) showed that whey protein diet fed animals had low body weight gain (-21\%) relative to casein fed animals. Whey
Table - 5.3: The body weight of mice fed the various protein diets

<table>
<thead>
<tr>
<th>Age in weeks</th>
<th>Control</th>
<th>DEN</th>
<th>Soy protein</th>
<th>Garlic protein</th>
<th>Coconut protein</th>
<th>Whey protein</th>
<th>Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3</strong></td>
<td>Initial body weight in (g)</td>
<td>10.37 ± 0.79</td>
<td>10.67 ± 0.42</td>
<td>10.62 ± 1.06</td>
<td>10.06 ± 1.02</td>
<td>10.45 ± 1.30</td>
<td>10.78 ± 0.96</td>
</tr>
<tr>
<td><strong>12</strong></td>
<td>Body weight on 60(^{th}) day (g)</td>
<td>22.03 ± 1.09 (112 %)</td>
<td>22.55 ± 0.51 (111 %)</td>
<td>20.5 ± 1.16 (93 %)</td>
<td>23.14 ± 1.34 (130 %)</td>
<td>23.91 ± 2.20 (128 %)</td>
<td>18.90 ± 1.14 (75 %)</td>
</tr>
<tr>
<td><strong>DEN</strong></td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>20</strong></td>
<td>Body weight on 120(^{th}) day (g)</td>
<td>29.31(^{b}) ± 1.15 (33 %)</td>
<td>25.95(^{a}) ± 0.64 (15 %)</td>
<td>27.14 ± 1.18 (32 %)</td>
<td>31.73(^{b}) ± 0.82 (37 %)</td>
<td>32.13(^{a}) ± 1.93 (34 %)</td>
<td>24.67(^{a}) ± 1.26 (30 %)</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD for 6 animals.

\(^{a}\) P<0.05 significantly different compared with control animals.

\(^{b}\) P<0.05 significantly different compared with DEN alone treated animals.

(%) As percent of control group.
Figure 5.2: Body weight of mice in different groups at various stages of the study
protein has proved to be effective in curbing body fat accumulation, owing to the presence of glycomacropeptide.

A significant (P<0.05) increase in the body weight of casein-fed animals when compared to soy protein and whey protein-fed groups has been observed. Similar observations were reported by (Hakkak et al., 2001). Thus, the casein protein-fed animals and the control diet-fed animals were almost similar in their efficiency in promoting growth.

Body weight gain in garlic protein and coconut protein-fed animals was higher than that in soy-protein fed animals. Torre et al. (2008) showed that rats fed with soy diets gained less weight, due to an increase in the thermogenic capacity mediated by uncoupling protein-1 expression in liver, which plays a key role in body weight regulation through its effects on thermogenesis and body protein composition. The utilization of dietary proteins for energy in high proportions limits weight gain in the body (Westerp and Plantenga, 2003).

ii) POST DEN status

At the end of 120 days of pretreatment, followed by the administration of DEN, it was observed that the final body weight of garlic protein-fed mice was higher than that of the other four groups. Reza, (2000) had previously reported that the body weight of casein-fed rats was slightly greater than that of soy protein-fed or whey protein-fed rats. Similarly, in our study, the final average body weight of animals pretreated with soy protein was found to be lower than that in the control, garlic protein, coconut protein and casein fed mice, but higher than that in DEN-induced animals.

It was observed that whey protein fed mice and DEN administered mice had lower body weight gain when compared to all the other groups. However, body weights did not differ between DEN treated and whey protein-treated groups. The administration of the hepatocarcinogen DEN to rats damages the liver and this could be primary reason for changes in body weight (Subramanian et al., 2007).

Whey as the primary source of proteins leads to reduction in body weight due to its effective inhibition of fat accumulation in adipocytes. Casein being a complete
protein well maintained the growth rate. A thermogenic utilizing effect caused a reduction in body weight of the soy protein fed animals.

**Cancer Markers**

The cancer markers are evaluated to measure the intensity of the hepatocarcinoma in the animals treated with DEN as well as in those animals pretreated with different protein isolates and then with DEN. The parameters measured in this study may be categorized into, one, the cancer markers, namely the growth regulator (AFP and TNF-α) and marker enzymes (ODC and GGTP) and two, the liver function marker enzymes (AST, ALT and ALP) and three, the antioxidant enzymes (SOD, CAT and GPx).

**5.4.2. Alpha fetoprotein - Results**

The data on the levels of α-fetoprotein in serum and liver tissue of control and experimental animal are presented in Table 5.4 and Figure 5.3. Serological markers for HCC are important for the diagnosis and monitoring of tumor aggressiveness, treatment responsiveness and survival.

The most common marker is AFP. The AFP gene, a growth regulator, is normally expressed in the fetal liver and expressed only at very low levels in the normal liver. Diethylnitrosamine is a known carcinogen (Loeppky, 1999) and causes hepatocarcinoma.

**Serum AFP:** The serum AFP level was the highest in the DEN administered but untreated animals. The level was four times higher than the control animals. However, we observed a reduction in AFP in the animals pretreated with various protein isolates; the extent of reduction varied with different protein isolates. The serum concentration of AFP was, of course higher than the control, in all the groups. Among the various groups the least concentration of AFP was recorded in the soy protein (123% as compared to control), and it gradually increased in the following order; coconut protein (135%), garlic protein (171%) and whey protein (318%). The AFP level of soy protein and coconut protein treated mice, though higher than control, was not significantly different.
Table-5.4: Levels of alpha-fetoprotein in serum and liver tissue of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Alpha-fetoprotein (ng/ml)</th>
<th>Liver Alpha-fetoprotein (ng/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.71 ± 1.0^b</td>
<td>84.73 ± 1.06^b</td>
</tr>
<tr>
<td>DEN</td>
<td>50.83 ± 1.29^a (399%)</td>
<td>203.38 ± 5.81^a (240%)</td>
</tr>
<tr>
<td>Soy protein*</td>
<td>15.75 ± 1.07^b (123%)</td>
<td>89.00 ± 2.89^b (105%)</td>
</tr>
<tr>
<td>Garlic protein*</td>
<td>21.76 ± 1.07^a,b (171%)</td>
<td>105.66 ± 5.16^a,b (124%)</td>
</tr>
<tr>
<td>Coconut protein*</td>
<td>17.27 ± 0.76^b (135%)</td>
<td>95.83 ± 5.11^a,b (113%)</td>
</tr>
<tr>
<td>Whey protein*</td>
<td>40.52 ± 5.86^a,b (318%)</td>
<td>125.5 ± 5.39^a,b (148%)</td>
</tr>
<tr>
<td>Casein*</td>
<td>44.28 ± 4.27^a,b (348%)</td>
<td>157.5 ± 7.84^a,b (185%)</td>
</tr>
</tbody>
</table>

* Pretreated and DEN administered
Results are expressed as mean ± SD for 6 animals.
^a P<0.05 significantly different compared with control animals.
^b P<0.05 significantly different compared with DEN alone treated animals.
(%) As percent of control group.

Figure 5.3
The level of serum AFP in soy protein, coconut protein and garlic protein ranged between 15.75 ng/ml to 21.76 ng/ml, concentration close to the control level of 12.71 ng/ml. On the contrary whey protein treated animals (40.52 ng/ml) and casein treated animal (44.28 ng/ml) had a level closer to the DEN administered untreated animals (50.83 ng/ml).

**Liver AFP:** Animals treated with diethylnitrosamine alone had a significant ($P<0.05$) increase in the level of liver AFP when compared to control mice. The level was twice higher than the control mice. A significant ($P<0.05$) decrease in AFP was observed in all groups treated with various protein isolates compared with DEN alone treated; but the percentage of decrease varied within groups in the following order; soy protein treated (105%), coconut protein (113%), garlic protein (124%) ,whey protein (148%) and casein (185%) compared to control animals. The AFP of soy protein treated mice, though higher than control, was not significantly different.

**5.4.3. AFP - Discussion**

It is well known that liver cancer is one of the most important cancers affecting humans world over, with more than 1 million patients being affected by it, and causing over 260,000 deaths annually (Liu *et al*., 2006); which therefore substantiates the need to find ways and means to prevent as well as to treat it. Tumour markers are generally used to identify and evaluate hepatocellular carcinoma.

Tumour markers are molecular products metabolized and secreted by neoplastic tissues and can be characterized biochemically in cells and body fluids. They are indicators of tumour stage and grade, and are useful for monitoring responses to treating and predicting the recurrence of disease. These markers may be: hormones, antigens, aminoacids and nucleic acids, enzymes, polyamines and specific cell membrane proteins and lipids. Tumor markers are helpful in diagnosis and most of these are commonly elevated in a broad spectrum of malignancies, whereas some are highly tissue specific (Hall *et al*., 1998).

In this study, we evaluated the following parameters: growth regulator (AFP), cytokine (TNF-α), cancer marker enzymes (ODC and GGTP), liver function marker enzymes (AST, ALT, and ALP) and antioxidant enzymes (SOD, CAT and GPx) as
indicators to assess the severity of the disease status in different pretreatment situations.

Serological markers for HCC are important for the diagnosis and monitoring of tumor aggressiveness, treatment responsiveness and survival. The most common marker is AFP. AFP is a large, 591 aminoacid-containing glycoprotein, with a weight of 70 KD (Ryder, 2003) belonging to the intriguing class of onco-developmental proteins.

It is a member of the albuminoid gene super family (Terentiev and Moldogazieva, 2006) and the most important protein component of fetal serum. It is synthesized in the visceral endoderm of the vitelin sac in the first phase of fetal development, after which it is synthesized in the liver.

The AFP gene is normally expressed in the fetal liver and expressed only at very low levels in the normal liver. The level of AFP in the normal adult mice in our studies is 12.71 ng/ml in the serum and 84.73 ng/g of liver tissue (Table 5.4). However, variations in the level of AFP are possible due to differences in the strain of the animal. Other reports have given a background level of AFP that normally persists in the serum of approximately 200 ng/ml in mice, 50 ng/ml in rats and 5-10 ng/ml in humans (Abelev and Elgort, 1982).

AFP expression is induced in regenerating liver and liver tumors. AFP levels eventually diminish after birth to virtually undetectable levels, and only are seen to be elevated under pathological conditions. Its high serum level is useful clinically as an important marker for the detection of hepatocellular carcinoma.

Investigation of the biological function of AFP indicates that it has a close association with the occurrence of hepatocellular carcinoma (Mengsen et al., 2007). It has been firmly established that the oncoembryonal protein, AFP, functions as growth regulator both during ontogenic growth and tumor progression (Mizejewski et al., 1995). Therefore alpha fetoprotein along with other markers such as ODC and TNF alpha are widely used as valuable references in animal studies to diagnose and observe the development of hepatocarcinogenesis (Thirunavukkarasu et al., 2005).

In the present study, a sharp and significant increase in the level of AFP in serum and liver tissue was observed in the DEN treated group of mice compared to the control group (Table 5.4). A wave like short term increase of AFP level is
reported after liver injury or hepatotoxin is administered (Abelev and Elgort, 1982). Similar observations were made by Nermin et al. (2008) in the DEN treated animals indicating the onset of hepatocellular carcinoma.

Liver injury induces AFP (Abelev and Elgort, 1982). Chemical liver injury by carbon tetrachloride (CCl₄) and other hepatotoxins induced AFP production. AFP reappeared in a one cell layer surrounding the necrotic area, independent of size and localization of necrosis. Any hepatocyte located in this region resumed AFP synthesis (Abelev, 1980).

A number of studies (Baranov and Engelhardt, 1987; Gleiberman et al., 1989) have demonstrated that AFP synthesis in mature hepatocytes is reversibly reexpressed under the influence of intercellular interactions. The nature of this crucial interaction and the pathway from intercellular contact to specific gene need to be further studied.

Alpha-fetoprotein, known largely to be a growth-promoting agent, possesses a growth-inhibitory motif, termed as Growth Inhibitory Peptide (GIP) which is a 34-amino acid fragment. It has a proven record of growth suppression in both fetal and tumor cells, but not in normal adult cells. Even though the exact mechanism of action by which it asserts this influence has not been completely elucidated, GIP participates in various biological activities such as endocytosis, angiogenesis, and cytoskeleton-induced/cell shape changes. GIP has been reported to inhibit various cytoplasmic enzyme activities, modulate apoptotic events, and regulate cytoplasmic signal transduction (MAP kinase) cascades (Mizejewska and Butterstein, 2006).

Specific binding sites for AFP are found on the surface of monocytes and phagocytes. It has been postulated that AFP binding to these receptors modulates immune responses (Meng et al., 2002).

As already discussed the concentration of AFP in serum and liver tissue was the highest in the DEN administered group, and significantly declined in the five groups pretreated with different protein isolates. However, the decrease in AFP was not uniform but the results varied within the groups. A dramatic decline in the concentration of AFP both in serum and liver of the soy, coconut protein and garlic-fed groups was observed. It may be emphasized here that the levels of AFP were near normal in these three groups.
Thus the soy protein, coconut protein and garlic protein seem to have the highest ameliorative effect compared to other proteins used. The plausible mechanism by which soy protein would have prevented the HCC formation is by the presence of isoflavones capable of activating the cytotoxic T lymphocytes and thus influencing immune reactions and preventing cancer formation.

Coconut kernel protein is found to have high content of arginine. It is tempting to attribute the curative effect to the high content of arginine present in coconut protein. It is known that arginine is a potent immunomodulator and has been shown to have marked effects in fighting cancer and alleviating the metabolic and biochemical chaos brought about by cancer. Animals fed arginine rich diets (5%) had considerably fewer tumors when later treated with the carcinogen dimethylbenzanthracene (DMBA) (Takeda et al., 1975). Therefore, coconut protein being rich in arginine may have prevented the deleterious effect of hepatocellular carcinoma by interfering with the immune system. The exact mechanism by which this is modulated needs to be further studied.

Garlic is a known anticarcinogen. It is believed to induce CYP 2E1 activity and also to activate immune system, thereby preventing the formation of cancers in various animal models. A protein fraction (F4) from aged garlic extract enhanced cytotoxicity and acted as immuopotentiator. Garlic diets inhibited pre-initiation, post-initiation and promotion stages of DEN induced hepatocarcinogenesis (Kyung et al., 2007). Thus in our study the level of AFP in serum and liver tissue in these groups which were more or less brought to near normal levels indicate the anticancer activity of these proteins.

The whey and casein protein-fed animals had, although had a significant decrease in serum and liver AFP values, when compared with the induced HCC group, this decline was in no way closer to the animals treated with soy and coconut protein.

Whey protein is known for its high content of glutathione which is a potent antioxidant. By virtue of this character whey protein has been found to prevent the formation of cancers. Animal studies have shown that whey protein was protective against carcinogen induced colon tumour (Belobrajdic et al., 2003). However, in the
liver the anticarcinogenic influence of whey and casein protein was not efficient as the other three proteins.

Casein is composed of biologically active peptides, which manifest immunomodulatory functions by influencing the activity of the immune system and displays antitumour activity in mice. Recent studies have shown that peptides with antioxidant properties are released from food sources such as casein (Suetsuna et al., 2000). The administration of various proteins reverted DEN induced alterations in AFP levels in liver and circulation.

5.4.4. Tumor necrosis factor - α - Results

The levels of TNF-α in serum and liver tissue of control and experimental animals are shown in Table 5.5 and Figure 5.4. TNF-α is an inflammatory cytokine that has been associated with cancer related inflammation, hence used as a marker. It is expressed in high levels in neoplastic tissues. TNF-α possesses both growth promoting and inhibiting properties and induces cell death in many types of tumour cells.

**Serum TNF- α:** A significant ($P <0.05$) increase in the level of serum tumor necrosis factor-α was observed in diethylnitrosamine alone administered mice when compared to that of control mice. Pretreatment with soy protein, garlic protein, coconut protein, whey protein and casein for a period of 120 days significantly ($P <0.05$) decreased the tumor necrosis factor-α level in both the serum and liver tissue. The serum concentration of TNF-α was the highest in untreated group (337% as compared to control). Among the various groups treated with different protein isolates, the least concentration of TNF-α was observed in soy protein (181%) as compared to control animals. Percentage of TNF-α decrease varied within groups treated with five protein isolates. Next highest concentration after the DEN induced group was recorded in the casein fed animals (287 %). The other three groups, namely garlic protein (239 %), coconut protein (231 %) and whey protein (241%) had almost similar level of TNF-α in the serum.

**Liver TNF-α:** The pattern was similar to serum TNF-α. The level of increase in TNF-α expressed as percent of control value, was less in the liver tissue of protein treated groups than in the serum. The elevation of the increase in TNF-α as percent of
Table-5.5: Levels of tumor necrosis factor α in serum and liver of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Tumor necrosis factor α (pg/ml)</th>
<th>Liver tumor necrosis factor α (pg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>37.58 ± 2.21^b</td>
<td>133.54 ± 3.24^b</td>
</tr>
<tr>
<td>DEN</td>
<td>126.76 ± 3.87^a (337%)</td>
<td>482.48 ± 14.40^a (361%)</td>
</tr>
<tr>
<td>Soy protein*</td>
<td>68.35 ± 2.33^a,b (181%)</td>
<td>239.41 ± 2.92^a,b (179%)</td>
</tr>
<tr>
<td>Garlic protein*</td>
<td>89.95 ± 2.31^a,b (239%)</td>
<td>251.81 ± 5.06^a,b (188%)</td>
</tr>
<tr>
<td>Coconut protein*</td>
<td>86.95 ± 3.70^a,b (231%)</td>
<td>249.71 ± 2.89^a,b (186%)</td>
</tr>
<tr>
<td>Whey protein*</td>
<td>90.77 ± 6.30^a,b (241%)</td>
<td>294.36 ± 3.58^a,b (220%)</td>
</tr>
<tr>
<td>Casein*</td>
<td>108.20 ± 1.95^a,b (287%)</td>
<td>314.38 ± 2.71^a,b (235%)</td>
</tr>
</tbody>
</table>

* Pretreated and DEN administered
Results are expressed as mean ± SD for 6 animals.
^a P<0.05 significantly different compared with control animals.
^b P<0.05 significantly different compared with DEN alone treated animals.
(%) As percent of control group.

Figure 5.4
control is compared between the serum and liver. In the serum, it was less than the liver in DEN induced group, almost similar elevation in the soy protein treated, but higher than the liver in all other groups.

5.4.5. TNF-α - Discussion

Tumor necrosis factor-α is an inflammatory cytokine and also a trimeric protein encoded within the major histocompatibility complex and exists as the 27 kd form. This cytokine is produced by several kinds of cells, among which, stimulated macrophages are noteworthy in their ability to produce 27kd TNF-α (Perez et al., 1990).

This cytokine possesses both growth stimulating properties and growth inhibitory properties. It induces neutrophil proliferation during inflammation and also induces neutrophil apoptosis (Murray et al., 1997). TNF interacts with tumor cells to trigger cytolysis or cell death.

Many organs of the body appear to be affected by TNF-α. It serves a variety of functions, which includes its role in the immune response to bacterial, fungal, viral, parasitic invasions and necrosis of specific tumours. In leukocytes and fibroblasts, TNF-α induces the release of superoxide radicals by the activation of membrane-bound NADPH oxidases. This process induces either proliferation or cell death, which usually depends on the condition of the ROS-producing cell (Shalaby et al., 1985).

TNF-α induces cell death in many types of tumor cells and has been used in model systems for studies of the molecular mechanisms by which it governs cell death. The mechanisms and means by which the ROS contribute to the induction of cell death depend on the signaling and execution pathways that are subsequently activated by their release. Cell death induced by TNF-α can either be necrosis or apoptosis, depending on the cell types under consideration. Multiple intracellular pathways may be involved in the course of TNF-α-induced cell death through complex signal transduction cascades initiated by the activation of TNFR1. Mitochondrial-dependent ROS production is the major signaling cascade involved in TNF-α-induced apoptosis (Sidoti et al., 1998).
Excessive production of oxygen-derived free radicals or reactive oxygen species creates a condition known as oxidative stress, participating in various diseases and syndromes (Erkilic et al., 2003). In transformed cell lines, TNF-α induces endogenous ROS production in the mitochondria of transformed cell lines (Schulze et al., 1992). The ROS interacts with nucleic acids, proteins and lipids; thus enhancing TNF production, and thereby inducing cellular dysfunction and cell death.

In an advanced, recent study to delve into the mechanisms of apoptosis, it was shown that the initial binding of TNF-α to TNFR1 causes a rapid recruitment of TRADD, RIP1 and TRAF2 (Figure 5.5). Then, TRADD and RIP1 associate with FADD to form death-inducing signaling complex (DISC). DISC subsequently activates caspase-8 to execute the death signals (Micheau and Tschopp, 2003; Benjamin et al., 2007).

In the present study a significant increase in TNF-α levels in the serum and liver tissue of DEN administered animals was recorded (Table 5.5 and Figure 5.4). The increase was more than thrice the concentration of the control level. A chemical like CCL4 and diethylnitrosamine cause liver injury and induces inflammation. Hepatic macrophages secrete the TNF-α and the other potent mediators of inflammatory response such as ROS and nitric oxide and thus control the early phase of liver inflammation. Similar to our observation Porta et al. (2007) reported a cancer related inflammation and a high expression of the cytokine, TNF-α in neoplastic tissues.

Prolonged over production of TNF-α would result in complications such as anorexia, weight loss and anemia during pathological conditions like cancer and AIDS (Beutler et al., 1985). The reduced body weight of DEN administered mice may be attributed to over production of TNF-α causing anorexia and weight loss.

Similarly, in our study, elevated TNF-α level were observed in serum and liver tissue of DEN administered group alone; and in groups pretreated with protein isolates, there was a significant decrease in TNF levels, but the results varied within the several groups. The restoration of TNF levels to near normal range was highest following pretreatment with soy protein, and a little lesser in coconut protein and garlic protein fed animals.
Figure 5.5: Model for signaling via TNFR 1 induced apoptosis
(Benjamin et al., 2007)

TRADD-TNF receptor- associated death domain, DD-Death Domain,
NF-κB-Nuclear factor kappa B, TRAF-TNF receptor-associated factors,
FADD-Fas-Associated Death Domain
In our study AFP and TNF-α are considered as hepatocellular carcinoma marker molecules which enable us to understand the impact of dietary protein isolates in ameliorating the carcinogenic effect of DEN.

Since AFP and TNF-α are interlinked and closely involved in the immunoregulatory process, the values of AFP and TNF-α as % increase over the control are analyzed. These values are presented in (Table 5.6).

The AFP / TNF-α ratio in serum is considerably reduced in garlic (0.71), soy (0.67) and coconut protein (0.58) administered mice (Table 5.7). This reduction is primarily due to a low synthesis of AFP.

In the whey and casein administered animals, the ratio is high. This is because the AFP is not reduced as much as in garlic, soy and coconut protein, although TNF-α is lowered.

The picture of AFP / TNF-α in the liver tissue is different such that there is a proportionate synthesis of AFP and TNF-α. Hence, there is not much of variation among the animals. However, the lowest value was observed in soy protein fed animals and the highest in casein fed animals. This may be attributed primarily to the synthesis of AFP, the lowest being in soy and the highest in casein, in proportion to TNF-α.

These facts led to the consideration of half life and degradation of AFP. Serum AFP disappeared rapidly after birth in infants. The half life AFP degradation was estimated to be 5.5 days between birth and second week, 11 days between second week to 2 months of age. Although synthesis occurs after birth, the rate of synthesis decreased with age.

Any factor affecting the rate of synthesis may influence the serum AFP level. The wide range of variants in serum AFP levels suggests that there must be more than one unknown factor affecting the serum AFP level (James et al., 1981).

Table 5.7 provides a picture of the rate of synthesis of AFP and TNF-α in the liver and their retention in serum. The synthesis and retention of AFP is the lowest in soy and coconut and high in whey and casein fed animals among the protein isolate administered groups.

The ratio of the tumour necrosis factor α in serum/liver is almost identical in all the protein isolate fed groups and lowest in DEN administered animals.
Table-5.6: Pattern of the increase over the normal values of AFP and TNF-α in response to dietary protein isolates

<table>
<thead>
<tr>
<th>Groups</th>
<th>SERUM</th>
<th>LIVER</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Values as % of control</td>
<td>AFP/TNF-α Ratio</td>
<td>Values as % of control</td>
<td>AFP/TNF-α Ratio</td>
</tr>
<tr>
<td></td>
<td>AFP</td>
<td>TNF-α</td>
<td></td>
<td>AFP</td>
<td>TNF-α</td>
</tr>
<tr>
<td>DEN administered</td>
<td>399</td>
<td>337</td>
<td>1.18</td>
<td>240</td>
<td>361</td>
</tr>
<tr>
<td>Soy protein</td>
<td>123</td>
<td>181</td>
<td>0.67</td>
<td>105</td>
<td>179</td>
</tr>
<tr>
<td>Garlic protein</td>
<td>171</td>
<td>239</td>
<td>0.71</td>
<td>124</td>
<td>188</td>
</tr>
<tr>
<td>Coconut protein</td>
<td>135</td>
<td>231</td>
<td>0.58</td>
<td>113</td>
<td>186</td>
</tr>
<tr>
<td>Whey protein</td>
<td>318</td>
<td>241</td>
<td>1.31</td>
<td>148</td>
<td>220</td>
</tr>
<tr>
<td>Casein</td>
<td>348</td>
<td>287</td>
<td>1.21</td>
<td>185</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td>AFP (As % of control)</td>
<td>Serum/Liver Ratio</td>
<td>TNF-α (As % of control)</td>
<td>Serum/Liver Ratio</td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>----------------------</td>
<td>-------------------</td>
<td>-------------------------</td>
<td>-------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>Liver</td>
<td></td>
<td>Serum</td>
<td>Liver</td>
</tr>
<tr>
<td>DEN administered</td>
<td>399</td>
<td>240</td>
<td>1.66</td>
<td>337</td>
<td>361</td>
</tr>
<tr>
<td>Soy protein</td>
<td>123</td>
<td>105</td>
<td>1.17</td>
<td>181</td>
<td>179</td>
</tr>
<tr>
<td>Garlic protein</td>
<td>171</td>
<td>124</td>
<td>1.37</td>
<td>239</td>
<td>188</td>
</tr>
<tr>
<td>Coconut</td>
<td>135</td>
<td>113</td>
<td>1.19</td>
<td>231</td>
<td>186</td>
</tr>
<tr>
<td>Whey protein</td>
<td>318</td>
<td>148</td>
<td>2.15</td>
<td>241</td>
<td>220</td>
</tr>
<tr>
<td>Casein</td>
<td>348</td>
<td>185</td>
<td>1.88</td>
<td>287</td>
<td>235</td>
</tr>
</tbody>
</table>
The lower level of AFP and the high level of TNF-α may be attributed to the influence of peptide fragments from the protein isolates. Thus, there is necrosis of cancer cells through immunomodulation.

AFP and TNF-α are functionally interconnected. AFP prevents from TNF-α cell killing. AFP inhibits TNF-α induced cell death of murine HCC through association with TNF-α and inhibition of TNFR signaling (Cavin et al., 2004). Therefore, silencing AFP would enhance TNF-α mediated toxicity. AFP may hence function as a decoy for TNF-α in the extracellular environment.

NF-κB is required for cell survival in hepatocarcinoma. Repression of IKK2 (inhibitor of nuclear factor kappa β kinase) activity in hepatocarcinoma promotes down regulation of AFP gene expression (Cavin et al., 2004).

NF-κB mediated regulation of AFP gene expression during liver tumour formation and embryonic development of the liver constitutes a potential novel mechanism to evade immune surveillance (Cavin et al., 2004). IKK2 (1 kappa β kinase 2) is an enzyme that serves as a protein component of a cytokine activated intracellular signaling pathway involved in triggering immune responses. Its activity causes activation of nuclear transcription factor kappa-B. Activated IKK2 phosphorylates a protein called inhibitor of NF-κB, IκB, which binds NF-κB to inhibit its function. Phosphorylated IκB is degraded via ubiquination pathway freeing NF-κB and allowing its entry into the nucleus of the cell where it activates various genes involved in inflammation and other immune responses.

NF-κB is widely used by eukaryotic cells as regulator of genes that control cell proliferation and cell survival. As such, many different types of tumours have misregulated NF-kB that is; NF-κB is constitutively active. Active NF-κB turns on the expression of genes that keep cell proliferating and protect cell from conditions that would cause it to die via apoptosis.

This is because NF-κB regulates anti apoptotic genes especially TRAF1 and TRAF2 and thereby checks the activities of caspase family of enzymes which are centers to apoptotic processes (Sheik and Huary, 2003). TNF-α is an agent that causes apoptosis. AFP inhibits TNF-α induced cell death of murine HCC thus associating with TNF-α and inhibiting of TNFR1 signaling.
5.4.6. Ornithine decarboxylase - Results

Ornithine decarboxylase (EC 4.1.1.17) is the first enzyme of the polyamine biosynthetic pathway. This enzyme decarboxylates L-ornithine to form putrescine. This enzyme plays an important role in embryonic development, cell proliferation, differentiation and cell death. ODC activity is elevated during tumorigenicity. High ODC activity has also been positively correlated with being a marker of carcinogenesis and tumor progression (Liu et al., 2005). Hence it is used as a marker to measure HCC.

Table 5.8 presents the activity of ornithine decarboxylase in serum and liver tissue of control and experimental animals. The putrescine formation was taken as an indicator of ODC activity.

**Serum ODC:** ODC activity was the highest in the DEN administered group. The level of activity was thrice more than the control animals. ODC activity was significantly less in the animals pretreated with various protein isolates. Among the different groups the percentage of decrease in ODC activity as compared to DEN alone group is as follows; soy protein treated and coconut protein showed maximum decrease of ODC activity (108%) followed by garlic protein (120%), whey protein (143%) and casein (178%) treated animals. The ODC activity of soy protein and coconut protein treated mice was comparable to that of the control.

**Liver ODC:** A significant \( P < 0.05 \) increase in liver ODC activity was observed in DEN alone treated mice. The level was two and a half time (254%) higher than the control animals. However, we observed a reduction in ODC activity in the animals pretreated with various protein isolates; the extent of reduction varied with the different protein isolates. The ODC activity of soy protein treated mice, though slightly higher than control, was not significantly different. The ODC activity of soy protein and coconut protein was found to be 23.82 and 25.02 (nmoles of putrescine oxidized/min/mg tissue), and thus was close to the control level of 21.82 (nmoles of putrescine oxidized/min/mg tissue). Although the garlic treated animals had the next higher ODC activity above these two groups, it was significantly higher than control (Figure 5.6).
Table 5.8: Levels of ornithine decarboxylase in serum and liver of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ornithine decarboxylase (n moles of putrescine liberated/ml)</th>
<th>Ornithine decarboxylase (n moles of putrescine oxidized/min/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.40 ± 0.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.82 ± 1.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DEN</td>
<td>27.17 ± 2.31&lt;sup&gt;a&lt;/sup&gt; (289%)</td>
<td>55.61 ± 1.84&lt;sup&gt;a&lt;/sup&gt; (254%)</td>
</tr>
<tr>
<td>Soy protein&lt;sup&gt;*&lt;/sup&gt;</td>
<td>10.18 ± 0.33&lt;sup&gt;b&lt;/sup&gt; (108%)</td>
<td>23.82 ± 1.30&lt;sup&gt;b&lt;/sup&gt; (109%)</td>
</tr>
<tr>
<td>Garlic protein&lt;sup&gt;*&lt;/sup&gt;</td>
<td>11.36 ± 0.36&lt;sup&gt;a,b&lt;/sup&gt; (120%)</td>
<td>38.42 ± 1.60&lt;sup&gt;a&lt;/sup&gt; (176%)</td>
</tr>
<tr>
<td>Coconut protein&lt;sup&gt;*&lt;/sup&gt;</td>
<td>10.22 ± 0.71&lt;sup&gt;b&lt;/sup&gt; (108%)</td>
<td>25.02 ± 1.27&lt;sup&gt;a,b&lt;/sup&gt; (114%)</td>
</tr>
<tr>
<td>Whey protein&lt;sup&gt;*&lt;/sup&gt;</td>
<td>13.50 ± 0.66&lt;sup&gt;a,b&lt;/sup&gt; (143%)</td>
<td>41.63 ± 1.80&lt;sup&gt;a,b&lt;/sup&gt; (190%)</td>
</tr>
<tr>
<td>Casein&lt;sup&gt;*&lt;/sup&gt;</td>
<td>16.82 ± 0.21&lt;sup&gt;a,b&lt;/sup&gt; (178%)</td>
<td>45.03 ± 1.82&lt;sup&gt;a,b&lt;/sup&gt; (206%)</td>
</tr>
</tbody>
</table>

* Pretreated and DEN administered

Results are expressed as mean ± SD for 6 animals.

<sup>a</sup> P<0.05 significantly different compared with control animals.

<sup>b</sup> P<0.05 significantly different compared with DEN alone treated animals.

(%) As percent of control group.

Figure 5.6
5.4.7. Ornithine decarboxylase - Discussion

The enzyme, ornithine decarboxylase catalyzes the formation of putrescine and \( \text{CO}_2 \) from ornithine. Ornithine decarboxylase is a key regulating enzyme in the biosynthesis of polyamines in mammalian cells and plays an important role in the control of a variety of biological processes including cellular metabolism, differentiation, membrane function, and proliferation.

Ornithine decarboxylase activity increases markedly during the initial stage of cell proliferation and differentiation, and elevated activities are normally present in rapidly growing tissues (Lin et al., 1998). This enzyme is induced very rapidly and many-fold by various growth-promoting stimuli, including hormones, drugs and toxins. Increased ODC activity leads to increased intracellular concentrations of polyamines.

ODC and polyamines are known to play a pivotal role in cell proliferation and to contribute to the development of cancer. In normal cells, the activity of ODC shows a rapid, transient increase upon stimulation by growth factors (Heby and Persson, 1990; Pegg, 1988), whereas the cells transformed by chemical carcinogens and oncogenes such as \( v-src \) and \( \text{ras} \) seem to have a constitutively elevated ODC activity (Gilmour et al., 1986; Holta et al., 1994). Most tumor promoters induce ornithine decarboxylase \textit{in vivo} (Kitchin and Brown, 1989).

In our study, ODC activity was significantly increased in serum and liver tissue of DEN administered animals (Table 5.8 and Figure 5.6). Actively dividing mammalian cells contain higher levels of polyamine than that of initially developing or quiescent cells. A significant increase in the levels of putrescine, spermidine and spermine was reported in hepatoma and surrounding liver tissues of cancer bearing animals (Seiler, 1991). Increased intracellular concentrations of polyamines reflected to show the rate of tumor proliferation in the induced group.

An elevated ODC activity has been assumed to be associated with tumorigenicity (Liu et al., 2005). The results obtained from the present study for evaluating the levels of ornithine decarboxylase is in accordance with Akihiro et al. (1994) who showed that ornithine decarboxylase activity, polyamines (putrescine, spermidine and spermine) and ornithine decarboxylase mRNA levels were
significantly (P<0.05) higher in hepatoma tissues than in their non-cancerous counterparts.

In a recent study, the over expression of ODC promoted the survival of human gastric cancer cells under stressful conditions such as hydrogen peroxide, cisplatin, doxorubicin, paclitaxel, 5-flourouracil and ionizing radiation. These mechanisms of anti-apoptosis seem to be multi-factorial; however, one of the witnessed anti-apoptotic effects is believed to be associated with the increase in the production of polyamines.

Soy protein and coconut protein fed animals showed the lowest ODC activity in serum and liver tissue, followed by the garlic, whey and casein protein fed animal groups. The soy protein seems to have an anticancer effect because it was reported in studies elsewhere that the activity of ornithine decarboxylase and polyamine concentrations in the rat mammary epithelium were significantly lower in the soy protein treated group (Hawrylewicz et al., 1995).

The fundamental concept of polyamine metabolism in tumours is that the transformed tumor cells have a greater requirement for polyamine synthesis for continuous growth, when compared to normal tissues, which have a lesser requirement for polyamines, because of their controlled growth behavior.

Regulation of the abnormality of polyamine biosynthesis forms a main strategy for cancer prevention. The DNA injury caused by a carcinogen exposure occurs at initiation phase of tumourigenesis and subsequently, at the promotion phase of tumourigenesis. Initiated cells begin to proliferate in response to growth stimulation, finally leading to the development of tumours (Kumar, 1990).

Thus, it is considered that agents (blocking agent) inhibiting the initiation phase block the formation of the DNA injury and that agent suppressing the promotion phase (suppressing agent) lowers the proliferation of the initiated cells (Tanaka et al., 1993).

Also, reports show the possibility that the cancer preventive agent acts, as blocking and suppressing agents, which is more essential component in cancer prevention, that protein isolates could suppress/reduces the induction of polyamines and subsequent cell proliferation.
The concentrations of intestinal lumenal polyamine and the activity of ODC are modulated by the administering dietary protein sources (Meziani et al., 1999). The present study results showed that protein isolates form various sources suppressed the development of DEN initiated HCC by regulating cell proliferation through the inhibition of polyamine biosynthesis.

5.4.8. Gamma glutamyl transpeptidase - Results

The level of the activity of gamma glutamyl transpeptidase in serum and liver tissue of control and experimental animals is given in Table 5.9. Gamma glutamyl transpeptidase exhibits a tissue-specific pattern of expression which is modified under various physiologic and pathogenic conditions such as those seen during fetal development and carcinogenesis. It is a widely distributed enzyme that has been studied extensively in relation to hepatocarcinogenesis, hence used as a marker enzyme to study HCC.

 Serum GGTP: A significant ($P < 0.05$) increase in serum GGTP activity was observed in DEN treated mice. However, we observed a reduction in GGTP activity in the animals pretreated with various protein isolates; the extent of reduction varied with the different protein isolates. The GGTP activity of soy protein and coconut protein treated were respectively 13.26 (U/L) and 16.68 (U/L) which were close to the control level of 11.48 (U/L). The GGTP activity of soy protein treated mice, though slightly higher than control, was not significantly different. On the contrary whey protein treated 25.35 (U/L) and casein treated 28.49 (U/L) showed activity closer to the DEN administered animals 35.68 (U/L).

 Liver GGTP: A significant ($P < 0.05$) increase in liver GGTP activity was observed in DEN treated mice. Following pretreatment with various protein isolates, the GGTP activity was significantly ($P < 0.05$) less in all protein isolates treated animals. Among the various groups the least activity of GGTP was observed in the soy protein treated group (118% as compared to control), and it gradually increased in the following order; coconut protein (166%), garlic protein (235%), whey protein (252%) and casein (284%). The GGTP activity in serum and liver of soy protein treated mice, though slightly higher than control, was not significantly different (Figure 5.7).
Table 5.9: Activity of gamma glutamyl transpeptidase in serum and liver of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Gamma glutamyl transpeptidase (U/L)</th>
<th>Liver Gamma glutamyl transpeptidase (µmol of p-nitroaniline liberated/min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.48 ± 2.04(^{b})</td>
<td>12.03 ± 1.22(^{b})</td>
</tr>
<tr>
<td>DEN</td>
<td>35.68 ± 1.83(^{a}) (310%)</td>
<td>44.97 ± 2.01(^{a}) (373%)</td>
</tr>
<tr>
<td>Soy protein*</td>
<td>13.26 ± 0.68(^{b}) (115%)</td>
<td>14.26 ± 2.60(^{b}) (118%)</td>
</tr>
<tr>
<td>Garlic protein*</td>
<td>23.61 ± 1.77(^{a,b}) (205%)</td>
<td>28.34 ± 2.13(^{a,b}) (235%)</td>
</tr>
<tr>
<td>Coconut protein*</td>
<td>16.68 ± 2.45(^{a,b}) (145%)</td>
<td>20.02 ± 2.94(^{a,b}) (166%)</td>
</tr>
<tr>
<td>Whey protein*</td>
<td>25.35 ± 2.93(^{a,b}) (220%)</td>
<td>30.42 ± 3.52(^{a,b}) (252%)</td>
</tr>
<tr>
<td>Casein*</td>
<td>28.49 ± 3.03(^{a,b}) (248%)</td>
<td>34.19 ± 3.63(^{a,b}) (284%)</td>
</tr>
</tbody>
</table>

* Pretreated and DEN administered

Results are expressed as mean ± SD for 6 animals.

\(^{a}\) P<0.05 significantly different compared with control animals.

\(^{b}\) P<0.05 significantly different compared with DEN alone treated animals.

(%) As percent of control group.

Figure 5.7
5.4.9. Gamma glutamyl transpeptidase - Discussion

Gamma glutamyl transpeptidase (EC. 2.3.2.2) is a membrane-bound enzyme which catalyses the degradation of gamma glutamyl compounds by hydrolyzing the \( \gamma \)-glutamyl moiety or by transferring it to a suitable acceptor. GGT is a heterodimeric glycoprotein. This enzyme exhibits a tissue-specific pattern of expression which is modified under various physiologic and pathogenic conditions such as those seen during fetal development and carcinogenesis (Tang et al., 1999; Yao et al., 2004).

The levels of circulating GGTP are highest in the fetal liver, and it plunges to the lowest values soon after birth. It is a widely distributed enzyme that has been studied extensively in relation to hepatocarcinogenesis.

In the present study, we observed a significant increase of GGTP activity in serum and liver tissue of DEN administered animals when compared to control (Table 5.9). Chemically induced hepatomas have been shown to contain considerably higher levels of GGTP, which is involved in the detoxification of carcinogens through mercapturic acid formation.

Tang et al. (1999) and Plebani et al. (2005) showed that total GGTP activity in patients with liver diseases and extra-hepatic tumors was abnormally high.

Even though we have not studied GSH activity, Sadik et al. (2008) have reported a significant (P<0.05) increase in the levels of hepatic GSH in the DEN administered group of animals. Increased GSH concentration in DEN treated rat liver is probably due to the simultaneously observed, parallel increase in the activities of GGTP and \( \gamma \)-glutamylcysteine synthetase, the two key enzymes involved in the biosynthesis of GSH (Marinho et al., 1997). The ubiquitous manner of elevation in the levels of GGTP prominently observed in many rodent, human carcinomas, both hepatic and extrahepatic (Hanigan and Pitot, 1985) has led to the hypotheses that increase in GGTP expression favors the growth of focal cells during carcinogenesis.

A plausible hypothesis is that the advantage offered in bringing about growth may be due to the role of GGTP in the transport of GSH constituents, leading to increase in cellular GSH. The latter is required for proliferation and resistance (Komlosh et al., 2002). Thus, GGTP may act by circumventing the rate-limiting \( \gamma \)-glutamylcysteine synthetase and its feedback inhibition by GSH, and may thus participate in the promotion of growth.
Previous studies have shown that an increased GSH level was associated with an early proliferative response and was essential for the cell to enter the S-phase. The requirement for increased GSH or thiols prior to DNA synthesis may be in relation to the fact that proliferating cells require increased amounts of pentoses and thiols. DNA synthesis depends almost completely on the formation of pentoses and on their conversion to deoxyribose by ribonucleotide reductase.

The activity of this rate-limiting enzyme in DNA synthesis requires reduced glutaredoxin or thioredoxin, which is maintained by GSH. An increase in the cellular GSH content may change the thiol-redox status of the cell, which leads to the activation of genes necessary to bring about cellular G1 to S transition (Holmgren, 1981; Lu, 1999).

Total GGTP activity in patients with liver diseases and extrahepatic tumors was found to be abnormally increased. Several studies have demonstrated that these increases are often associated with structural changes in the sugar chains of the enzyme. GGTP is re-expressed during the development of HCC (Deng et al., 2007).

As already discussed the activity of GGTP in serum and liver tissue was the highest in the DEN administered group, and significantly declined in the five groups pretreated with different protein isolates. However, the decrease in GGTP activity was not uniform but the results varied within the groups. A dramatic decline in GGTP activity both in serum and liver of the soy, coconut protein and garlic protein fed groups was observed (Figure 5.6).

The whey and casein protein-fed animals had, although had a significant decrease in serum and liver GGTP values, when compared with the induced HCC group, this decline was in no way closer to the animals treated with soy and coconut protein. Pretreatment with protein isolates brought the activities of these enzymes to normal, indicating its protective effect during cancer.

Liver Function Marker Enzymes (AST, ALT and ALP)

The anticarcinogenic activity of various protein isolates were evaluated by the levels of liver function marker enzymes namely AST, ALT and ALP. The parameters were measured both in serum and liver tissue. In our analysis, results of AST, ALT and ALP are analyzed separately and they are finally discussed.
5.4.10. Aspartate transaminase - Results

Table 5.10 depicts the activity aspartate transaminase in serum and liver of control and experimental animals. Aspartate transaminase catalyses the transfer of amino group from L-alanine to α-ketoglutarate with the formation of oxaloacetate and glutamate. AST is found in every tissue of the body, including red blood cells, liver and is particularly high in the cardiac muscle. AST measurements are useful in the diagnosis and monitoring of patients with hepatocellular disease.

Serum AST: DEN alone treated mice showed a significant (P<0.05) increase in serum AST enzyme activity when compared to those from the control group. The level was two and a half times higher than the control animals. A significant (P<0.05) decrease in the enzyme activity was observed in groups pretreated with various protein isolates. However, the extent of reduction varied with the different protein isolates. The serum concentration of AST in soy protein treated group was (96% as compared to normal), and it gradually increased in the following order; coconut protein (103%), garlic protein (142%), whey protein (156%) and casein (165%). The serum level of AST in soy protein, coconut protein and garlic protein treated ranged between 16.15 U/L to 23.91 U/L, activity close to the normal control animals. However, the AST activity of soy protein treated mice, though slightly higher than control, was not significantly different.

Liver AST: Diethylnitrosamine alone treated mice showed a significant (P<0.05) increase in liver AST activity when compared to control mice. The level was two and a half time higher than the control mice. A significant (P<0.05) decrease of AST activity was observed in all groups treated with various isolates but the percentage of decrease varied within groups in the following order; soy protein treated (113%), coconut protein (125%), garlic protein (160%) whey protein (188%) and casein (198%) compared to control animals. However, the AST activity of soy protein treated mice, though slightly higher than control, was not significantly different (Figure 5.8).

5.4.11. Alanine transaminase - Results

Table 5.11 depicts the levels of aspartate transaminase in serum and liver of control and experimental animals.
Table 5.10: Levels of aspartate transaminase in serum and liver of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Aspartate transaminase (U/L)</th>
<th>Liver aspartate transaminase (nm) of pyruvate liberated/min/mg/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.81 ± 0.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.34 ± 1.71&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DEN</td>
<td>40.56 ± 1.38&lt;sup&gt;a&lt;/sup&gt; (241%)</td>
<td>80.98 ± 2.91&lt;sup&gt;a&lt;/sup&gt; (250%)</td>
</tr>
<tr>
<td>Soy protein*</td>
<td>16.15 ± 0.75&lt;sup&gt;b&lt;/sup&gt; (96%)</td>
<td>36.67 ± 3.70&lt;sup&gt;b&lt;/sup&gt; (113%)</td>
</tr>
<tr>
<td>Garlic protein*</td>
<td>23.91 ± 2.19&lt;sup&gt;a,b&lt;/sup&gt; (142%)</td>
<td>52.02 ± 3.92&lt;sup&gt;a,b&lt;/sup&gt; (160%)</td>
</tr>
<tr>
<td>Coconut protein*</td>
<td>17.44 ± 0.73&lt;sup&gt;a,b&lt;/sup&gt; (103%)</td>
<td>40.56 ± 1.38&lt;sup&gt;a,b&lt;/sup&gt; (125%)</td>
</tr>
<tr>
<td>Whey protein*</td>
<td>26.31 ± 1.03&lt;sup&gt;a,b&lt;/sup&gt; (156%)</td>
<td>60.85 ± 1.00&lt;sup&gt;a,b&lt;/sup&gt; (188%)</td>
</tr>
<tr>
<td>Casein*</td>
<td>27.75 ± 0.78&lt;sup&gt;a,b&lt;/sup&gt; (165%)</td>
<td>64.06 ± 6.63&lt;sup&gt;a,b&lt;/sup&gt; (198%)</td>
</tr>
</tbody>
</table>

* Pretreated and DEN administered

Results are expressed as mean ± SD for 6 animals.

<sup>a</sup> <i>P</i>&lt;0.05 significantly different compared with control animals.

<sup>b</sup> <i>P</i>&lt;0.05 significantly different compared with DEN alone treated animals.

(%) As percent of control group.

Figure 5.8
Serum ALT: A significant (P<0.05) increase in serum ALT enzyme activity was observed in DEN alone treated animals when compared to those from the control group. The level was twice higher than the control animals. The level of ALT in soy protein, coconut protein and garlic protein ranged between 11.4 U/L to 16.8 U/L, concentration close to the control level of 10.29 U/L. On the contrary whey protein (18.57 U/L) and casein treated (19.6 U/L) had a level closer to the DEN administered animals (23.55 U/L). The ALT activity of soy protein treated mice, though slightly higher than control, was not significantly different.

Liver ALT: A significant (P<0.05) increase in liver ALT activity was observed in DEN alone treated animals when compared to those from the normal control group. The level was twice higher than the control animals. A significant (P<0.05) decrease of ALT activity was observed in all groups treated with various isolates but the percentage of decrease varied within groups in the following order; soy protein treated (111%), coconut protein (112%), garlic protein (125%), whey protein (149%), and casein (179%) compared to control animals. However, the ALT activity of soy protein and coconut protein treated mice, though higher than control, was not significantly different (Figure 5.9).

5.4.12. Alkaline phosphatase - Results

Table 5.12 depicts the levels alkaline phosphatase in serum and liver of control and experimental animals.

Serum ALP: A significant (P<0.05) increase in serum ALP activity was observed in DEN alone treated animals when compared to those from the control group. The activity was nearly three times higher than the control animals. However, we observed a reduction in ALP activity in the animals pretreated with various protein isolates; the extent of reduction varied with different protein isolates. The serum concentration of ALP was, of course higher than the control, in all the groups. Among the various groups the least concentration of ALP was recorded in the soy protein group (120% as compared to control), and it gradually increased in the following order: coconut protein (121%), garlic protein (142%) and whey protein (151%) and casein treated (156%).
Table-5.11: Levels of alanine transaminase in serum and liver of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Alanine transaminase (U/L)</th>
<th>Liver Alanine transaminase (nm) of pyruvate liberated/min/mg/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.29 ± 0.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.47 ± 1.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DEN</td>
<td>23.55 ± 1.00&lt;sup&gt;a&lt;/sup&gt; (228%)</td>
<td>41.75 ± 0.97&lt;sup&gt;a&lt;/sup&gt; (203%)</td>
</tr>
<tr>
<td>Soy protein*</td>
<td>11.40 ± 0.52&lt;sup&gt;b&lt;/sup&gt; (110%)</td>
<td>22.82 ± 1.21&lt;sup&gt;b&lt;/sup&gt; (111%)</td>
</tr>
<tr>
<td>Garlic protein*</td>
<td>16.89 ± 1.55&lt;sup&gt;a,b&lt;/sup&gt; (164%)</td>
<td>25.66 ± 2.75&lt;sup&gt;a,b&lt;/sup&gt; (125%)</td>
</tr>
<tr>
<td>Coconut protein*</td>
<td>12.41 ± 0.50&lt;sup&gt;a,b&lt;/sup&gt; (120%)</td>
<td>23.06 ± 0.72&lt;sup&gt;b&lt;/sup&gt; (112%)</td>
</tr>
<tr>
<td>Whey protein*</td>
<td>18.57 ± 0.72&lt;sup&gt;a,b&lt;/sup&gt; (180%)</td>
<td>30.68 ± 1.08&lt;sup&gt;a,b&lt;/sup&gt; (149%)</td>
</tr>
<tr>
<td>Casein*</td>
<td>19.60 ± 0.51&lt;sup&gt;a,b&lt;/sup&gt; (190%)</td>
<td>36.72 ± 2.76&lt;sup&gt;a,b&lt;/sup&gt; (179%)</td>
</tr>
</tbody>
</table>

* Pretreated and DEN administered

Results are expressed as mean ± SD for 6 animals.
<sup>a</sup> P<0.05 significantly different compared with control animals.
<sup>b</sup> P<0.05 significantly different compared with DEN alone treated animals.

(%) As percent of control group.

Figure 5-9
Table 5.12: Levels of alkaline phosphatase in serum and liver of control and experimental animals

<table>
<thead>
<tr>
<th></th>
<th>Serum Alkaline phosphatase (KAU/dl)</th>
<th>Liver Alkaline phosphatase (µmol of phenol liberated/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>14.66 ± 0.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.70 ± 0.95&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DEN</td>
<td>42.33 ± 1.92&lt;sup&gt;a&lt;/sup&gt; (288%)</td>
<td>72.77 ± 1.71&lt;sup&gt;a&lt;/sup&gt; (183%)</td>
</tr>
<tr>
<td>Soy protein*</td>
<td>19.68 ± 2.21&lt;sup&gt;a,b&lt;/sup&gt; (134%)</td>
<td>47.78 ± 2.21&lt;sup&gt;a,b&lt;/sup&gt; (120%)</td>
</tr>
<tr>
<td>Garlic protein*</td>
<td>27.99 ± 1.24&lt;sup&gt;a,b&lt;/sup&gt; (190%)</td>
<td>56.54 ± 1.35&lt;sup&gt;a,b&lt;/sup&gt; (142%)</td>
</tr>
<tr>
<td>Coconut protein*</td>
<td>20.02 ± 2.56&lt;sup&gt;a,b&lt;/sup&gt; (136%)</td>
<td>48.15 ± 2.59&lt;sup&gt;a,b&lt;/sup&gt; (121%)</td>
</tr>
<tr>
<td>Whey protein*</td>
<td>31.97 ± 3.45&lt;sup&gt;a,b&lt;/sup&gt; (218%)</td>
<td>60.07 ± 3.45&lt;sup&gt;a,b&lt;/sup&gt; (151%)</td>
</tr>
<tr>
<td>Casein*</td>
<td>34.04 ± 2.26&lt;sup&gt;a,b&lt;/sup&gt; (232%)</td>
<td>62.14 ± 2.26&lt;sup&gt;a,b&lt;/sup&gt; (156%)</td>
</tr>
</tbody>
</table>

* Pretreated and DEN administered

Results are expressed as mean ± SD for 6 animals.

<sup>a</sup> P<0.05 significantly different compared with control animals.

<sup>b</sup> P<0.05 significantly different compared with DEN alone treated animals.

(%) As percent of control group.

Figure 5.10

![Figure 5.10: Serum-Alkaline phosphatase](image1)

![Figure 5.10: Liver-Alkaline phosphatase](image2)
**Liver ALP:** Diethylnitrosamine alone treated mice showed a significant ($P<0.05$) increase in liver ALP activity when compared to control mice. The level was thrice higher than the control mice. A significant ($P<0.05$) decrease of ALP activity was observed in all groups treated with various isolates but the percentage of decrease varied within groups in the following order; soy protein (134%), coconut protein (136%), garlic protein (129%) whey protein (218%), and casein treated (232%) compared to control animals (Figure 5.10).

### 5.4.13. AST, ALT and ALP - Discussion

Aspartate transaminase also called glutamic oxaloacetic transaminase (EC 2.6.1.1) is similar to alanine transaminase in that it is another enzyme associated with liver parenchymal cells. Two isoenzymes are present in humans. They have high similarity. GOT1, the cytosolic isoenzyme derived mainly from red blood cells and heart. GOT2, the mitochondrial isoenzyme is predominantly present in liver. It facilitates the conversion of aspartate and alpha-ketoglutarate to oxaloacetate and glutamate, and vice-versa. AST is commonly measured clinically as a part of diagnostic liver function tests, to determine liver health. The level is raised in acute liver damage.

Alanine transaminase is a transaminase enzyme (EC 2.6.1.2). It is also called glutamic pyruvic transaminase or alanine aminotransferase. ALT is found in serum and in various bodily tissues, but is most commonly associated with the liver. It catalyzes the transfer of an amino group from alanine to $\alpha$-ketoglutarate, the products of this reversible transamination reaction being pyruvate and glutamate. It is commonly measured clinically as a part of a diagnostic liver function test, to determine liver health. Significantly elevated levels of ALT often are observed in liver damage.

Alkaline phosphatase (EC 3.1.3.1) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. In humans, alkaline phosphatase is present in all tissues throughout the entire body, but is particularly concentrated in liver, bile duct, kidney, bone, and the placenta. ALP measurements are useful in the diagnosis and monitoring
of patients with hepatocellular disease. The assay of alkaline phosphatase enzyme is of great value in the detection of lesions in liver (Cameron, 1971).

In the present study, a pattern of sharp increase in the values of AST, ALT and ALP was seen in the serum and liver of DEN administered HCC group when compared with the trend that was seen in the control group (Table 5.10 to 5.12). Elevated activities of AST and ALT are indicative of DEN induced hepatic damage and subsequent leakage of these enzymes into the blood circulation. Elevated serum and liver AST, ALT and ALP levels were observed in DEN induced hepatocarcinogenesis (Subramanian et al., 2007).

Alanine transaminase, aspartate transaminase and alkaline phosphatase are valuable references, widely used in animal studies to diagnose and observe the development of hepatocarcinogenesis (Thirunavukkarasu et al., 2005).

Elevation of serum enzymes by tumors may be due to leakage from the tumor itself or from an adjacent normal tissue damaged by the neoplasm. Serum levels of hepatic enzymes released from damaged tissue have been widely used for the detection of metastatic spread to the liver (Sreepriya, 2005).

It has been postulated that the assay of aminotransferases together with alkaline phosphatase gives a higher percentage of positive results in metastatic liver disease than either of these carried out separately (Zein and Discombe, 1970). We observed a significant (P<0.05) increase in alkaline phosphatase activity in serum and liver of diethyl nitrosamine treated group.

The animals fed with soy protein and coconut protein showed the highest decrease in the DEN induced elevation of liver function enzyme levels followed by garlic, whey and casein-fed groups of animals. The data obtained from our present study showed that pretreatment with soy protein, garlic protein, coconut protein, whey protein and casein significantly decreased the activities of liver function marker enzymes, ameliorated DEN induced changes and offered protection to the liver (Figures 5.7 to 5.9).

As already discussed the peptide fragments from these proteins act upon the immunoregulatory systems and these animals therefore are immunologically protected against the onset of hepatocellular carcinoma. Due to this protective influence of these
protein isolates, the deleterious effect of DEN is neutralized or reduced in these animals according to the protective strength of each these proteins.

**Antioxidant Enzymes**

Carcinogenic substances inhibit the activities of antioxidant enzymes. SOD, CAT and GPx constitute a mutually protective set of enzymes against the damaging effects of ROS. As a measure of tissue damage caused by DEN, we studied the activity of these enzymes in serum and liver. In our analysis, the results of SOD, CAT and GPx are dealt separately and they are finally discussed.

### 5.4.14. Superoxide dismutase - Results

The levels of superoxide dismutase in serum and liver tissue of control and experimental animals are shown in Table 5.13.

**Serum SOD:** A significant decrease (P<0.05) in serum SOD activity was observed in DEN alone-treated mice when compared to control animals. The level was nearly three times lower than the control animals. Following pretreatment with soy protein, garlic protein and coconut protein isolates for 120 days a significant (P<0.05) increase in the activity of SOD was observed in these three groups when compared to DEN alone treated mice. The extent of increase varied with different protein isolates. Among the various groups the highest activity of SOD was recorded in the soy protein group (98% as compared to control), and it gradually decreased in the following order; garlic protein (88%) and coconut protein (78%), whey protein (48%) and casein (43%). There was no significant increase in SOD activity in groups treated with whey protein and casein compared to DEN alone treated mice.

**Liver SOD:** A significant decrease (P<0.05) in liver SOD activity was observed in DEN alone-treated mice when compared to control animals. Following pretreatment with soy protein, garlic protein, coconut protein and whey protein isolates for 120 days a significant (P<0.05) increase in the activity of SOD was observed in these four groups when compared to DEN alone treated mice. The extent of increase varied with different protein isolates. Among the various groups the highest activity of SOD was recorded in the soy protein (97% as compared to control), and it gradually decreased in the following order; garlic protein (86%),
Table 5.13: Levels of superoxide dismutase in serum and liver of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Superoxide dismutase (Units/ml)</th>
<th>Liver Superoxide dismutase (U/mg) protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.05 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.0 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DEN</td>
<td>0.84 ± 0.03&lt;sup&gt;a&lt;/sup&gt; (40%)</td>
<td>1.78 ± 0.11&lt;sup&gt;a&lt;/sup&gt; (44%)</td>
</tr>
<tr>
<td>Soy protein*</td>
<td>2.02 ± 0.11&lt;sup&gt;b&lt;/sup&gt; (98%)</td>
<td>3.88 ± 0.12&lt;sup&gt;b&lt;/sup&gt; (97%)</td>
</tr>
<tr>
<td>Garlic protein*</td>
<td>1.82 ± 0.04&lt;sup&gt;a,b&lt;/sup&gt; (88%)</td>
<td>3.44 ± 0.36&lt;sup&gt;a,b&lt;/sup&gt; (86%)</td>
</tr>
<tr>
<td>Coconut protein*</td>
<td>1.61 ± 0.03&lt;sup&gt;a,b&lt;/sup&gt; (78%)</td>
<td>2.91 ± 0.09&lt;sup&gt;a,b&lt;/sup&gt; (72%)</td>
</tr>
<tr>
<td>Whey protein*</td>
<td>0.97 ± 0.04&lt;sup&gt;a&lt;/sup&gt; (48%)</td>
<td>2.36 ± 0.45&lt;sup&gt;a,b&lt;/sup&gt; (59%)</td>
</tr>
<tr>
<td>Casein*</td>
<td>0.90 ± 0.04&lt;sup&gt;a&lt;/sup&gt; (43%)</td>
<td>2.21 ± 0.16&lt;sup&gt;a&lt;/sup&gt; (55%)</td>
</tr>
</tbody>
</table>

* Pretreated and DEN administered
Results are expressed as mean ± SD for 6 animals.
<sup>a</sup> P<0.05 significantly different compared with control animals.
<sup>b</sup> P<0.05 significantly different compared with DEN alone treated animals.
(%) As percent of control group.

Figure 5.11
coconut protein (72%) and whey protein treated (59%). There was no significant increase in SOD activity in casein treated compared to DEN alone treated mice (Figure 5.11).

5.4.15. Catalase - Results

The levels of catalase in serum and liver tissue of control and experimental animals are shown in Table 5.14.

**Serum CAT:** A significant decrease (P<0.05) in serum CAT activity was observed in DEN alone-treated mice when compared to control animals. The level was nearly twice lower than the control animals. Following pretreatment with soy protein for 120 days a significant (P<0.05) increase in the activity of CAT was observed in this group when compared to DEN alone treated mice. The extent of increase observed in soy protein group was (94% as compared to control), garlic protein and coconut protein (72%), whey protein (65%) and casein (52%) respectively. The level of CAT in garlic protein and coconut protein treated ranged between 4.21(IU/L) to 4.26 (IU/L), activity close to the control level of 5.84 (IU/L). On the contrary whey protein 3.81(IU/L) and casein treated 3.07 (IU/L) had a level of activity closer to DEN administered animals 2.90 (IU/L).

**Liver CAT:** A significant decrease (P<0.05) in liver CAT activity was observed in DEN alone-treated mice when compared to control animals. We observed an increase of CAT activity in the animals pretreated with various protein isolates; the extent of increase in level of activity varied with different protein isolates. The maximum level of CAT activity was recorded in soy protein group (89% as compared to control), and it gradually decreased in the following order: coconut protein (82%), garlic protein (80%), whey protein (75%) and casein treated (70%) (Figure 5.12).

5.4.16. Glutathione peroxidase - Results

The activity glutathione peroxidase in serum and liver tissue of control and experimental animals are shown in Table 5.15.

**Serum GPx:** A significant decrease (P<0.05) in serum GPx activity was observed in DEN alone treated mice when compared to control animals. Following pretreatment with soy protein, garlic protein, coconut protein and whey protein
**Table 5.14: Levels of catalase in serum and liver of control and experimental animals**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Catalase (IU/L)</th>
<th>Liver Catalase (µmol of H₂O₂ consumed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.84 ± 0.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50.21 ± 0.78&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DEN</td>
<td>2.90 ± 0.72&lt;sup&gt;a&lt;/sup&gt; (49%)</td>
<td>30.62 ± 1.24&lt;sup&gt;a&lt;/sup&gt; (61%)</td>
</tr>
<tr>
<td>Soy protein*</td>
<td>5.52 ± 1.20&lt;sup&gt;b&lt;/sup&gt; (94%)</td>
<td>45.01 ± 1.04&lt;sup&gt;a,b&lt;/sup&gt; (89%)</td>
</tr>
<tr>
<td>Garlic protein*</td>
<td>4.21 ± 0.55&lt;sup&gt;a&lt;/sup&gt; (72%)</td>
<td>40.50 ± 0.88&lt;sup&gt;a,b&lt;/sup&gt; (80%)</td>
</tr>
<tr>
<td>Coconut protein*</td>
<td>4.26 ± 0.80&lt;sup&gt;a&lt;/sup&gt; (72%)</td>
<td>41.37 ± 0.84&lt;sup&gt;a,b&lt;/sup&gt; (82%)</td>
</tr>
<tr>
<td>Whey protein*</td>
<td>3.81 ± 0.32&lt;sup&gt;a&lt;/sup&gt; (65%)</td>
<td>37.84 ± 0.56&lt;sup&gt;a,b&lt;/sup&gt; (75%)</td>
</tr>
<tr>
<td>Casein*</td>
<td>3.07 ± 0.66&lt;sup&gt;a&lt;/sup&gt; (52%)</td>
<td>35.60 ± 0.75&lt;sup&gt;a,b&lt;/sup&gt; (70%)</td>
</tr>
</tbody>
</table>

* Pretreated and DEN administered

Results are expressed as mean ± SD for 6 animals.

<sup>a</sup> P<0.05 significantly different compared with control animals.

<sup>b</sup> P<0.05 significantly different compared with DEN alone treated animals.

(%) As percent of control group.

**Figure 5.12**

![Graph of Serum-Catalase](image1)

![Graph of Liver-Catalase](image2)
Table 5.15: Levels of glutathione peroxidase in serum and liver of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Glutathione peroxidase (IU/L)</th>
<th>Liver Glutathione peroxidase (nmol of GSH oxidized / mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$4.58 \pm 0.36^b$</td>
<td>$23.71 \pm 0.84^b$</td>
</tr>
<tr>
<td>DEN</td>
<td>$2.30 \pm 0.34^a$ (50%)</td>
<td>$11.51 \pm 0.76^a$ (48%)</td>
</tr>
<tr>
<td>Soy protein*</td>
<td>$4.15 \pm 0.25^b$ (90%)</td>
<td>$21.32 \pm 0.54^{a,b}$ (89%)</td>
</tr>
<tr>
<td>Garlic protein*</td>
<td>$3.93 \pm 0.28^{a,b}$ (85%)</td>
<td>$18.04 \pm 0.80^{a,b}$ (76%)</td>
</tr>
<tr>
<td>Coconut protein*</td>
<td>$3.82 \pm 0.36^{a,b}$ (83%)</td>
<td>$19.04 \pm 0.50^{a,b}$ (80%)</td>
</tr>
<tr>
<td>Whey protein*</td>
<td>$3.31 \pm 0.25^{a,b}$ (72%)</td>
<td>$16.89 \pm 0.45^{a,b}$ (71%)</td>
</tr>
<tr>
<td>Casein*</td>
<td>$2.84 \pm 0.19^a$ (62%)</td>
<td>$14.70 \pm 0.75^{a,b}$ (61%)</td>
</tr>
</tbody>
</table>

* Pretreated and DEN administered
Results are expressed as mean ± SD for 6 animals.
$^a P<0.05$ significantly different compared with control animals.
$^b P<0.05$ significantly different compared with DEN alone treated animals.
(%) As percent of control group.

Figure 5.13
isolates for 120 days a significant (P<0.05) increase in the activity of GPx was observed in these four groups when compared to DEN alone treated mice. The extent of increase varied with different protein isolates. Among the various groups the highest activity of GPx was recorded in the soy protein treated group (90% as compared to control), and it gradually decreased in the following order; garlic protein (85%), coconut protein (83%) and whey protein (72%). There was no significant increase in GPx activity in group treated with casein compared to DEN alone-treated mice. However, the GPx activity of soy protein treated was not significantly different from that of control.

**Liver GPx:** A significant decrease (P<0.05) in liver GPx activity was observed in DEN alone-treated mice when compared to control animals (Figure 5.13). We observed a significant (P<0.05) increase in GPx activities in all groups treated with various protein isolates. However, the extent of increase varied with different protein isolates. Among the various groups the highest activity was recorded in soy protein (89%), followed by coconut protein (80%), garlic protein (76%), whey protein (71%) and casein treated (61%).

**5.4.17. SOD, CAT and GPx - Discussion**

Free radicals and reactive non-radical species derived from radicals exist in biological cells and tissues at low, but rather measurable concentrations. Their concentrations are determined by the balance between their rates of production and their rates of clearance by various antioxidant compounds and enzymes (Halliwell and Gutteridge, 1989). SOD, CAT and glutathione peroxidase are involved in the clearance of superoxide and hydrogen peroxide. SOD catalyses the diminution of superoxide in (H₂O₂) which is eliminated by glutathione peroxide and/or catalase (Rushmore *et al*., 1993).

Experimental studies have shown that oxygen-derived free radicals are involved in the onset of various kinds of liver injury. Most of the data that have been published concerning free oxygen radical scavenger enzymes in the diseased liver have been obtained from studies using animal models (Keen *et al*., 1985). Free radicals are mainly derived from a univalent sequential reduction of molecular oxygen. Mitochondria are the main sites for intracellular production of these free
radicals, which may result from auto-oxidation of small molecules or by the functioning of some enzymes.

The free radical theory of cell dysfunction is basically founded on three main observations (1) free radicals are extremely reactive species; (2) the production of oxy free radicals, mainly O$_2$ is a constant phenomenon in the living organism with beneficial and damaging effects at the cellular and molecular levels; and (3) natural defense or control mechanisms occur by enzymatic antioxidants.

The excessive influences of free radicals are checked by certain control mechanisms involving antioxidants. Antioxidants are substances that are able, at relatively low concentrations, to compete with other oxidizable substrates and, thus significantly delay or inhibit the oxidation of the substrates. This includes the enzymes such as SOD, glutathione peroxidase, and catalase, as well as non-enzymatic compounds such as α-tocopherol (vitamin E), β-carotene, ascorbate (vitamin C), and glutathione.

Therefore, an imbalance between their production and control mechanisms is supposed to result in the continuous or progressive accumulation of deleterious changes throughout the cells and tissues, thus generating intense functional disorder at each level of organization of the ultra structures of cells and organs (Crastes, 1990). Superoxide dismutase and CAT constitute a mutually protective set of enzymes against the damaging effects of ROS.

We have observed a significant (P<0.05) decrease of SOD, CAT and GPx activities in serum and liver of DEN treated mice compared to control animals (Table 5.13 to 5.15). This decreased antioxidant enzymes may be attributed to increased production of free radicals leading to the formation of lipid peroxides (Beckman and Amnes, 1998). The activities of antioxidant enzymes, especially SOD, are reduced when malignancies are present and the manganese-containing isoform of SOD may function as a tumor suppressor (Safford et al., 1994).

Carcinogenic substances inhibit SOD and CAT activities by a direct mechanism while anticarcinogens increase the activities of these detoxifying enzymes (Subramanian et al., 2007). Furthermore, it has been reported that a decrease in SOD activity in Ehrlich ascites carcinoma-affected mice was due to the combined loss of Mn$^{2+}$- containing SOD activity and the loss of mitochondria, leading to a decrease in
total SOD activity in the liver (Sun et al., 1989). An inhibition of SOD and CAT activities as a result of tumor growth, which is similar to our results, has been reported in other studies (Marklund et al., 1982).

In addition, there are compounds that have a relatively low specific antioxidative activity, i.e., on a molar basis, but, when present at high concentrations, can contribute significantly to the overall ROS scavenging activity. The most prominent examples of such high-level, low-efficiency antioxidants are free amino acids, peptides, and proteins (Halliwell and Gutteridge, 1989). Although, in the present study, the carcinogen DEN significantly reduced the level of these antioxidant enzymes, the group of animals pretreated with soy protein, garlic protein and coconut protein had an elevated activity of SOD, CAT and GPx.

Of the three proteins mentioned, in the soy protein treated group the activity of SOD, CAT and GPx was the highest and their serum level was almost identical and statistically insignificant to that of the control level, thus making soy protein the anticarcinogenic premium protein. Garlic and coconut protein pretreated animals also had significantly higher activity of these antioxidant enzymes (Figure 5.11 to 5.13).

An elevated level of antioxidant enzymes SOD, CAT and GPx in the soy protein pretreated mice, inspite of the exposure to the carcinogenic DEN may be attributed to the antioxidant activity of the protein and its constituents itself. For it has been recorded that apart from the regular antioxidant enzymes as well as non enzymatic compounds such as α-tocopherol, β-carotene and ascorbic acid which carry out ROS scavenging activity, there are compounds that have relatively low specific antioxidative activity i.e., in a molar basis. But when these low antioxidant molecules are present at high concentrations, can contribute significantly to the overall ROS scavenging activity.

Practically all amino acids serve as targets for oxidative attack by ROS. Some amino acids such as tryptophan, tyrosine, histidine, and cysteine are particularly sensitive to ROS (Stadtman, 1993). Since free amino acids have a cumulative intracellular concentration in the order of $10^{-1}$ M, they are quantitatively important ROS scavengers.

It is acknowledged by some workers that the treatment of human skeletal muscle cells with proteasome inhibitors causes a substantial increase in intracellular
Figure 5.14: Mechanisms of redox homeostasis. Balance between ROS production and various types of scavengers (Wulf, 2002)
ROS levels and that this increase is reportedly reversible by the addition of free amino acids. The relatively high intracellular concentration of glutathione and other antioxidative compounds provides a strong basal scavenging capacity to tissues containing them. The ROS-mediated oxidation of proteins and the resulting increase in proteolytic degradation brings about a cumulative increase in the intracellular capacity to scavenge ROS and thereby aids in contributing to the maintenance of redox homeostasis.

A wide variety of living organisms have the capacity to respond to increased levels of ROS with an increase in intracellular glutathione concentration or with increased expression of proteins/enzymes with potentiality to scavenge ROS. The resulting increase in the capacity to bring about ROS clearance allows cells and tissues to maintain redox homeostasis (Figure 5.14).

Higher activities of antioxidant enzymes SOD, CAT and, GPx was reported by (Chunmei et al., 2008) in soy bean protein-fed mice than those fed with casein, which is attributed to the antioxidant activities of soybean protein itself. It is thus evident from the present study that the proteins and their liberated amino acid constituents may have exerted their influence by acting as antioxidants and in particular, some protein sources might have boosted cellular glutathione levels and thereby, protected the liver from incurring further DEN induced oxidative damage.

5.4.18. Hepatic histology - Results

Histological findings of liver sections from control, DEN administered and protein isolate-pretreated group of animals are shown in Plate 5-11. Histological examination of liver sections from control animals revealed normal tissue pattern and integrity (Plate 5); whereas in DEN administered group (Plate 6) the liver had lost its characteristic architecture compared to that of the control group.

Diethylnitrosamine treated mice showed partial effacement of the liver architecture and large spaces filled with proteinaceous fluid; nuclei showed nucleoli and nuclear pleomorphism. The nucleus: cytoplasmic ratio is higher when compared to normal hepatocytes.

Restoration of most of the normal hepatocyte architecture with regular dark nuclei was observed in groups treated with soy protein, garlic protein and coconut
protein (Plate 7-9) and few hepatic vacuolations, dilated sinusoids and dysplasia were seen in groups pretreated with whey protein and casein (Plate 10 and 11).

5.4.19. Hepatic histology - Discussion

Histology was carried out, considering it to be a conclusive, end point biomarker for viewing the extent of tissue damage. In this study, we observed that the normal untreated group revealed normal liver parenchymal cells with granulated cytoplasm and small uniform nuclei radially arranged around the central vein (Plate 5).

In the DEN administered group, gross structural alterations were seen with predominantly basophilic, eosinophilic and clear cell foci. Extensive vacuolation was observed in the cytoplasmic region encircling the nucleus with masses of acidophilic materials. Some nuclei in the cells were large and hyperchromatic with prominent and centrally located nucleoli resembling a condition known as pre-neoplastic condition. Phenotypically altered hepatocyte populations in the form of altered liver cell foci and nodules, to a varying extent were noticeable throughout the hepatic parenchyma. A similar finding in DEN induced animals was reported (Nermin et al., 2008; Subramanian et al., 2007).

Soy protein-pretreated group showed more or less normal architecture of hepatocytes with granulated cytoplasm and sinusoids, almost parallel to our findings observed in control tissues. In addition, the cytoplasmic vacuoles were less in number and the nuclei of the hepatocytes appeared to be normal (Plate 7). Histopathologic evaluation of the mammary tumors revealed more benign fibroadenomas and lower-grade adenocarcinomas in the soy protein group (Hawrylewicz et al., 1995).

In the coconut pre-treated group (Plate 9), the cellular architecture of hepatic lobules seemed to be normal. The sinusoidal spaces were highly obliterated. In garlic protein pre-treated group (Plate 8), the nucleocytoplasmic ratio was decreased considerably, when compared to the induced group. However; improvement in hepatic cellular architecture was observed in these groups, when comparing sections from these groups with the DEN induced counterparts. Next to soy protein, pretreatment of animals with garlic protein and coconut protein was shown to be effective in restoring deleterious effects produced by DEN administration in
maintaining the activities of marker proteins and antioxidant enzymes. Thus the results of various biochemical parameter study parallels with the histological findings.

Liver sections from whey protein and casein pretreated group (Plate 10 and 11) appeared with inflammatory cellular infiltration, vacuolated hepatocytes, dilated sinusoids, and the hepatic vacuolations were found to be extensively increased; the cytoplasm appeared to be highly vacuolated with irregular, darkly stained nuclei. The histological observations of garlic protein treated and coconut protein treated group coincides with the results of biochemical findings in which growth regulator (AFP and TNF-α), cancer marker enzymes and antioxidant enzymes were restored comparable to control animals with few exceptions.

From the present study, it is clear that soy protein, garlic protein and coconut protein pretreated groups elicited the maximum protection against DEN induced hepatocarcinogenesis. Liver cells from these groups were found to contain compact cytoplasmic material with only clear cell foci.

Even though pretreatment with whey protein and casein showed marginal improvement, their liver sections appeared to have inflammatory cellular infiltration, vacuolated hepatocytes, and dilated sinusoids with occurrence of mild dysplasia. The results of biochemical studies are thus, confirmed by the histological observations.
Plate 5: Liver section of control mice
(Stained with hematoxylin and eosin)

CV-Central Vein, N-Nucleus, NH-Normal Hepatocyte

Highlights
1) Uniform nuclei, radially arranged around the central vein
2) Normal hepatocyte architecture
Plate 6: DEN administered mice liver section

CV-Central Vein, V-Vacuole

Highlights
1) Altered hepatocyte architecture
2) Nucleoli and nuclear pleomorphism
3) Higher nucleus: Cytoplasmic ratio
4) Extensive vacuolation
Plate 7: Soy protein treated mice liver section

CV-Central vein, N-Nucleous, NH-Normal Hepatocyte

Highlights
1) Likeness to normal hepatocyte architecture
2) Normal nucleus
Plate 8: Garlic protein treated mice liver section

CV-Central vein, N-Nucleus, NH-Normal Hepatocyte

Highlights
1) Improved hepatic architecture
2) Normal nucleus
3) Decreased cytoplasmic ratio
Plate 9: Coconut protein treated mice liver section

Highlights
1) Normal cell architecture
2) Obliterated sinusoidal spaces
3) Decreased cytoplasmic ratio
Plate 10: Whey protein treated mice liver section

CV-Central vein, N-Nucleus, V-Vacuole

Highlights
1) Vacuolated hepatocytes
2) Dilated sinusoids
3) Darkly stained nuclei
4) Mild dysplasia
Plate 11: Casein treated mice liver section

N-Nucleus, V-Vacuole

Highlights
1) Vacuolated hepatocytes
2) Dilated sinusoids
3) Darkly stained nuclei
4) Mild dysplasia
5.4.20. Candidate Proteins - A General discussion

The struggle against cancer is one of mankind’s greatest challenges. Cancer has been shown to be present for many centuries, but has become unprecedented in its growth since the later half of the twentieth century. Factors such as the presence of pollutants, toxins, high stress lifestyles, intake of poor quality and junk food, and food contaminated by the excessive use of pesticides, irradiated and genetically modified diets, pathogens, and electromagnetic radiation, contribute to its induction, progression and the fatal manifestations associated with it. Dietary factors play a key role in the development of various human diseases (Sanjay, 2002). Good nutrition have been suggested to play a major role in averting cancer, since it has been estimated that 30-40% of all tumors can be forestalled by leading a healthy life style and intake of a healthy balanced diet on a daily basis (Duilio et al., 2006).

All cancers manifest a condition known as cachexia, showing increased protein degradation and lack of protein sequestration in cells. Supplementation of proteins in the diet may be one measure to counter protein loss witnessed during the progression of most cancers. In addition, recent reports suggest that protein diets offer significant protection against various diseases. Hence, in this study, the therapeutic effect of protein isolates (from soy, garlic, coconut, whey and casein) on induced hepatocellular carcinoma was investigated in mice.

In our study, a significant increase in the levels of growth regulators (AFP and TNF-α), HCC marker enzymes (GGTP and ODC) and liver function enzymes (AST, ALT and ALP) were observed in serum and liver tissue of DEN alone treated animals. Similarly a significant decrease in the activity of antioxidant enzymes (SOD, CAT and GPx) were observed in serum and liver tissue of DEN administered mice. Pretreatment with five protein isolates mainly brought about significant alterations for the betterment in the levels of these marker enzymes and improved antioxidant status in these protein fed groups.

Dietary proteins play an important role in the maintenance of health and in the prevention of disease. Thus, protein in the diet significantly influences tumorigenesis either through enhancement of metabolic systems that prevent activation of carcinogens or by acceleration of carcinogen inactivation. They influence the metabolism of several classes of environmental carcinogens such as polycyclic
aromatic hydrocarbons, aflatoxins, nitrosamines and hydrazines and also prevent carcinogen activation. Dietary proteins may modify carcinogenesis by modifying phase I enzymes for carcinogen activation and enhance the detoxification of carcinogen through Phase II enzymes and scavenge DNA agents, thus suppressing the proliferation of early, preneoplastic lesions and by the inhibition of certain cancers by antiangiogenic activity. Thus antiangiogenic compounds inhibit tumour growth by preventing the generation of new blood vessels (neo-angiogenesis) to support tumour expansion and metastasis (Claudine and Jeffrey, 2002).

Of the five groups treated with different protein isolates, pretreatment with soy protein significantly restored the cancer marker enzymes to near normal levels. Soy protein feeding in male rats was shown to induce phase I carcinogen inactivation by significantly activating CYPIA2 enzymes in the liver through the reduction of aryl hydrocarbon receptor protein levels post-translationally; which reduces procarcinogen-induced CYP1A1 induction and its subsequent metabolic activation (Badger et al., 1999).

Soy protein isolate is rich in phytate, a powerful antioxidant, and a few associated isoflavonoids. Two soy peptides with strong antioxidative activity has been reported by Suetsuna (1999). In addition its amino acid and peptide composition contribute to the antioxidant activity of soy protein (Hisa et al., 2002; Sin et al., 2007). Antioxidative activity of histidine-containing peptides (Chen et al., 1998) and Arginine richness (Wallner et al., 2001) in soy protein (Table 2.1) may further contribute to its antioxidative character.

Appelt and Reicks (1997) reported that soy protein isolate and soy flour increased GST activity in rat liver. Soy isoflavones modulate the signal transduction processes of macrophages and phagocytic cells and enhance the activity of cytotoxic T lymphocytes, thus influencing both non-specific and specific immune responses (Rumsey et al., 1994; Stewart and Hoskin, 1997). Thus soy protein as a rich source of arginine and having a number of antioxidative peptides may have prevented the DEN induced changes in animal groups fed the same diet.

Next to soy protein, garlic protein isolate was the most effective protein in preventing the DEN induced changes and bringing about the restoration of various parameters more or less to normal control levels. The garlic powder extract reportedly
contains many biologically and pharmacologically active compounds, which are beneficial to human health against a number of diseases, including anticancer action (Yogeshwer and Neetu, 2007). Animal experiments have established the effectiveness of garlic powder (Agarwal, 1996) in inhibiting the initiation of carcinogenesis at a variety of sites and with variety carcinogens.

Several mechanisms have been proposed by which garlic could exert its cancer preventive effects. A protein fraction (F4) isolated from aged garlic extract showed enhanced cytotoxicity of human peripheral blood lymphocytes against natural killer cells - sensitive K562 and NK - resistant M14 cell lines. It was also suggested that F4 was a very efficient immunopotentiator, thereby obviating for its use in immunotherapy (Morioka et al., 1993).

It is suggested that compounds in garlic modify common metabolic events leading to suppression in cancer development. The compounds of garlic powder were shown to selectively inhibit tumour proliferation by a number of factors, e.g., controlling DNA repair mechanisms, chromosomal stability and cell cycle regulation (Yogeshwer and Neetu, 2007). Other action include inhibition of DNA adduct formation, free radical scavenging, and effects on cell proliferation and tumour growth. Human lymphatic leukemia formation was prevented by garlic powder through its antiproliferative activity (Seki et al., 2000).

Although not many studies have been carried out in coconut protein, we observed it to be almost equal to garlic protein in its effectiveness in preventing DEN induced changes and bringing about the restoration of cancer marker enzymes and antioxidant enzymes in serum and liver tissue to near normal levels to that of garlic protein fed group.

Coconut protein is reported to have high content of arginine. Arginine addition to diet caused a significant reduction of aberrant crypt foci preneoplastic biomarkers of colon cancer (Wargovich et al., 1996).

Our survey of aminoacids (Experiment-Ia) in candidate proteins has shown the presence of high arginine content in soy, garlic and coconut protein. And other analytical studies (Wallner et al., 2001) have established the same. The ameliorative effect of arginine in connection with cancer therapy may be five fold: Inhibition of cellular replication of tumors, efficient release of growth hormones from their sites of
synthesis, enhancement of immune responsiveness, increased rate of wound healing and apoptosis.

L-arginine has been shown to retard tumor formation, growth and incidence in cancer patients, which has been correlated with its ability to down regulate ODC (Ornithine decarboxylase). Also, it may decrease the production of polyamines, which are unusually elevated in cancers (Critselis et al., 1977). Animals fed arginine rich diets (5%) had considerably fewer and more benign tumors when treated with the carcinogen DMBA (Takeda et al., 1975). When added to drinking water in animals, it was able to inhibit subcutaneous tumor growth (Pryme, 1978), and when added to diet of mice (5% of wt) it produced fewer tumors, slower growing tumors, and twice the mean survival time as compared to untreated mice (Milner et al., 1979). Arginine supplementation in tumor-bearing mice provided enhanced T-cell function, increased response to autologous tumors, retarded tumor growth, and prolonged median survival time (Reynolds et al., 1990). In mice with neuroblastomas, arginine supplementation provided significant tumor retardation in the immunogenic group (Reynolds, 1988).

Cancers involve cachexia, whereby protein loss affects thymus size, thus deteriorating the activities of the immune system. Arginine in free form has been shown to act as precursor for the synthesis of growth hormone. The size and the health of the thymus gland seemed to improve from supplementation of free L-arginine in the diet in cancer patients. This increases the production of T-cells and assists overall in T-cell maturation process, determining the release of an abundance of mature T-cells for fighting cancer (Critselis et al., 1977).

Arginine supplementation may stimulate lymphocyte immune response (Barbul et al., 1981). Arginine supplements in mice provided significant enhancement of cytotoxic T-lymphocytes, natural killer cell activity, interleukin-2 receptors and general immune improvements (Reynolds et al., 1988).

Arginine also dramatically improves wound healing. Drawing from animal studies, it is speculated arginine may be essential in the body for the synthesis of reparative collagen in wound recovery and in decreasing some of the negative aspects of metabolic responses to injury (Seifter et al., 1978).

Shayanthan et al. (2009) demonstrated that arginine inhibited human gastric cancer cell growth and was associated with increased apoptosis given at (2 mM)
concentrations. Furthermore, the study acknowledged a change in expression of a number of genes involved in key cancer pathways. In particular an increase in expression of caspase 8 was seen at mRNA and protein level. Caspase 8 is an important protein which is involved in apoptosis triggered by the extrinsic pathway (Figure 5.5). Binding of FasL and TNF to their corresponding receptor domains at the surface of the cell leads to the activation of caspase 8. Subsequently, this initiates a cascade of caspase activation that ultimately results in apoptosis (Cohen, 1997). Thus, it is affirmative that arginine’s tumoricidal abilities go beyond its protein sparing abilities or immune stimulation functions.

Whey and casein proteins although protective against cancer, their efficiency is, however, less than the other proteins studied.

Whey protein suppressed tumour development by several mechanisms, which included: induction of apoptosis, inhibition of angiogenesis and by the modulation of carcinogen metabolizing enzymes (Parodi, 2007) through increasing the antioxidant potential. As a rich source of cysteine and γ-glutamylcysteine, whey protein provides precursors for the synthesis of glutathione (Goldman et al., 1990).

Glutathione is a multifunctional tripeptide, which prevents oxidative damage and eliminates endogenous, exogenous mutagens as well as carcinogens. Graeme et al. (1995) reported that elevation of glutathione concentration in liver tissue of whey protein-fed rats influenced xenobiotic deactivation via the glutathione /glutathione S-transferase activity and was found to retard tumour growth. Whey protein, besides acting as a source for glutathione synthesis, improved both the immune response and the inflammatory response against cancer (Grimble et al., 1998; McIntosh, 2001). We also have recorded based on our analysis that sulphur amino acid content is high in whey protein and low in soy protein isolate, and may have accounted for the cancer preventive effect (Table 2.1).

Dietary casein protein may enhance the immune system and promote host protection against the development of cancer. Like whey proteins, casein proteins are composed of biologically active peptides, particularly β and κ-casein fractions, which are released by enzymatic hydrolysis of milk. They manifest immunomodulatory functions by influencing the activity of the immune system and by displaying antitumor activities in animals.
Several of the biologically active peptides released by enzymatic hydrolysis of milk proteins affect the cells of the immune system (Coste and Tome, 1991; Kayser and Meisel, 1996). In addition, they contain immunologically active peptides in biologically active form. Evidence for immunoregulatory activity has been derived from both in vitro studies of the direct effects of these peptides on cells of the immune system, and in vivo studies of the effects of peptides delivered by dietary or parenteral routes (Werner et al., 1986; Jolles et al., 1992; Schlimme and Meisel, 1995). α\textsubscript{S1}-casein was reported to significantly inhibit the proliferative responses of murine splenic lymphocytes and rabbit Peyer’s patch cells (Otani and Hata, 1995).

Pepsin/trypsin hydrolysis of α\textsubscript{S1}-caseins has been shown to significantly suppress mitogen-induced proliferation of human peripheral blood mononuclear cells in vitro (Kayser and Meisel, 1996). Peptide residues derived from the pepsin/trypsin hydrolysis of β-caseins were also found to significantly suppress the mitogen-induced proliferation of human lymphocytes (Kayser and Meisel, 1996). β\textsubscript{-}casein hydrolysis results in the production of hexapeptide Pro-Gly-Pro-Ile-Pro-Asn (residues 63–68) and the tripeptide Leu-Leu-Tyr (LLY, residues 191–193). Both peptides have been shown to promote antibody formation and accelerated phagocytosis by murine peritoneal macrophages in vitro (Migliore and Jolles, 1988; Jolles and Migliore, 1992).

Likewise β-casomorphins fragments of the β-casein sequence 60-70, was found to have opiate-like properties and have been shown to modulate the immune system. Depending on the concentration, β-casomorphin-7 (residues 60–66 of β-casein) and β-casokinin-10 (residues 193–202 of β-casein) exhibit bipolar modulatory effects on human peripheral blood lymphocyte proliferation. In in vitro culture with mitogen-stimulated T lymphocytes, both peptides have been shown to suppress proliferation at low concentrations (Kayser and Meisel, 1996).

As discussed above proteins in the diet help in the prevention of cancer and give sufficient protection through various mechanisms. However, the efficiency of protein in this protective process varies according to the source protein as observed in our studies. Summing up soy, followed by coconut and garlic protein are highly effective and whey and casein are a little less in efficiency.
Thus, we may infer that protein pretreatment decisively inhibits weight loss, restore the near normal level of HCC markers of the liver and liver-specific enzymes, and prevents the fall of antioxidant serum and tissue enzymes. Soy, coconut and garlic protein-feeding are more protective than whey or casein administration.

Annually, the nutraceutical market is flooded with potentially useful products with an ever increasing demand for such commodities. Our studies suggest the role of these protein isolates, possibly as nutraceutical products in future, in preventing the biochemical events leading to the initiation and progression of liver cancer in humans. As a follow up study, a survey may be carried out to correlate the intake of these proteins in normal diet in certain packets among the human population of our country and the prevalence of cancer among them. Since these are harmless and normally used in food, clinical trials can be carried out in human volunteers to get more conclusive data than that seen in experimental animal models. Our present study partly fills the lacuna of data currently available in addressing the effectiveness of dietary proteins as nutraceuticals in prevention of hepatocellular carcinoma.