"The work described in this thesis was carried out by me under the supervision of Dr. (Mrs.) S.M.D.N. Wickramasinghe and Professor (Mrs.) P. Angunawela and a report on this has not been submitted to any University for another degree"

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Effects of garlic (*Allium sativum*) on chemically-induced hepatocarcinogenesis in normal and hypercholesterolaemic Wistar rats

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ABSTRACT

Hepatocellular carcinoma is one of the most common cancers in the modern world and it is prevalent in Asia. Environmental and dietary factors play an important role in the development of many cancers. Dietary modification can also play an important role in reducing the risk of cancer. Garlic (*Allium sativum*) is a well-known medicinal herb and a food item that has been used all over the world since prehistoric times. Previous studies using cancer cell lines and animal models have indicated that garlic and some of its sulfur compounds are potential anticarcinogens.

The present study was carried out to investigate the effects of a therapeutic dose (20mg /kg body wt./day) of garlic on chemically-induced hepatocarcinogenesis in normal and hypercholesterolaemic Wistar rats. In the first phase of this study the inhibitory effects of a therapeutic dose (20mg /kg body wt./day) of garlic on diethylnitrosamine (DEN)-induced neoplasia of the liver were examined in normal male Wistar rats. Medium-term Bio assay system of Ito based on the two step model of hepatocarcinogenesis was used as the assay method.

In phase II, the effects of garlic on hepatocarcinogenesis in hypercholesterolaemic Wistar rats were investigated. The selection of hypercholesterolaemic model was based on the available evidence for the relationship between dietary fat and the development of cancer. Hypercholesterolaemic model was developed by feeding rats with a 0.5% cholesterol-enriched diet for a period of two weeks. Rats having serum cholesterol level > 120 mg/dl were considered as hypercholesterolaemic (normal range 75±10 mg/dl) and the cholesterol-enriched diet was continued throughout the
experiment. Carcinogenic potential was scored by comparing the number and area of induced Glutathione S-transferase placental form positive (GST-P⁺) liver foci as well as histopathological examination of liver sections.

Daily treatment with garlic markedly reduced the number and area of GST-P⁺ foci (48% inhibition and 49% inhibition respectively) as compared with the control group of animals receiving distilled water. Significant inhibition of induction of GST-P⁺ foci (34% inhibition in number and 44% inhibition in area) due to garlic treatment was also observed in the hypercholesterolaemic group of rats. However, percentage inhibition was higher in garlic treated normal rats than garlic treated hypercholesterolaemic rats. Also, it was evident from this study that hypercholesterolaemic rats are more susceptible to induction of liver GST-P⁺ foci.

Results of GST-P⁺ expression were supplemented by histopathological examination of liver sections of garlic treated normal as well as hypercholesterolaemic Wistar rats. Granular and vacuolar degeneration were used as parameters in assessing histopathological alterations. In normal rats, garlic treatment reduced the pathological alterations in liver sections caused by DEN. Similar results were obtained from experiments with hypercholesterolaemic rats.

Hence, the results of this study provide strong supportive evidence for the anticarcinogenic activity of garlic.
During the past decade, traditional systems of medicine have become a topic of global importance. Current estimates suggest that, in many developing countries, a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs. Although modern medicine may be available in these countries, herbal medicines (phytomedicines), have often maintained popularity for historical and cultural reasons. Concurrently, many people in developed countries have begun to turn to alternative or complementary therapies, including medicinal herbs.

(WHO Monographs on selected Medicinal Plants, Volume 1, 1999)
1. INTRODUCTION

1.1 BACKGROUND AND JUSTIFICATION

Cancer has become an important topic in medicine since it is a major cause of death in developing countries and it is now only secondary to that of myocardial infarction (Gruddy, 1991). Increasing mortality rate of cancer may be due to continuously changing environment and life style of people. Great majority of human cancers (about 80-90%) are attributable to environmental factors (Doll and Peto, 1981; Epstein, 1974). While modern surgery has significantly reduced cancer mortality, the use of additional treatments such as radiotherapy and chemotherapy have resulted in no more than 5% reduction in the number of deaths (Benjamin et al., 1990). Therefore, people search for better control and preventive methods in order to reduce cancer mortality and related side effects. Hepatocellular carcinoma is one of the top five common cancers in the world and it is the most prevalent malignant neoplasm in Asia. Hepatocellular carcinoma is responsible for at least 250,000 deaths world wide annually (Hie-Won, 1998).

It is apparent, that the most economical means of cancer control lies in prevention (Benjamin et al., 1990). It has been estimated that a high percentage of cancer in humans is related to dietary factors (Willet et al., 1984; Cairns, 1985). Dietary factors, which may lower cancer risk, have also been identified. Examples include dietary fiber, cruciferous vegetables, antioxidant vitamins, minerals (Benjamin et al., 1990; Thomas et al., 1998). Thus dietary modification has been considered as a powerful and cost effective method for reducing the risk of cancer (Boyd et al., 1982).
Garlic (*Allium sativum* of family liliaceae) has been used as a food item as well as a herbal remedy since antiquity. Literature on clinical and animal studies have confirmed that garlic contains many biologically and pharmacologically active compounds which are beneficial to human health (Agarwal, 1996; Lea, 1996). Therefore it is important to investigate the effects of garlic on carcinogenesis. According to epidemiological reviews, numerous studies have indicated that garlic reduces the blood cholesterol (total and HDL) and triglyceride level in hyperlipidaemic and hypercholesterolaemic subjects including humans and experimental animal models (Kaul and Prasad, 1990; Mirahadi, 1991; Sheela *et al.*, 1995). As a result, different commercial forms of garlic preparations such as tablets and capsules are marketed all over the world for the treatment of hypercholesterolaemia. Since the introduction of commercial garlic for the treatment of hypercholesterolaemia, anti-carcinogenic activity of garlic has received considerable attention from research groups all over the world.

Recent epidemiological studies as well as studies using cancer cell lines and animal models have indicated that garlic and some organo sulfur compounds of garlic are potential anticarcinogens (Benjamin, 1990; Agarwal, 1996; Takada *et al.*, 1997). However no proper scientific investigation has so far been reported on the effects of a therapeutic dose (for a adult, 900mg/day; Silagy and Neil, 1994) of garlic on chemically induced carcinogenesis in normal and hypercholesterolaemic rats.

Therefore this study was undertaken to find out the effects of a therapeutic dose of garlic on chemically-induced hepatocarcinogenesis in normal and hypercholesterolaemic Wistar rats. Hypercholesterolaemic condition was selected because garlic is used for the treatment of hypercholesterolaemia and also there is
evidence that excessive dietary fat is a risk factor for the development of cancer (Howard et al., 1991; Carrol, 1980).

1.2 SCOPE OF THE STUDY

The major objective was to find out the effects of garlic on chemically induced hepatocarcinogenesis in Normal and Hypercholesterolaemic Wistar rats.

1.2.1 Specific Objectives

1. To investigate the effects of garlic on chemically induced hepatocarcinogenesis in normal Wistar rats.

In the study, “Modified method of the medium term bioassay system” of Ito based on the two step model of hepatocarcinogenesis was used as an assay system. This system was initially introduced in order to screen environmental and naturally occurring carcinogens (Ito et al., 1988). However later, it was satisfactorily used for identifying different anti-carcinogens (Tadashi et al., 1990; Ito et al., 1989).

Diethylnitrosamine (DEN) was used as a carcinogen to initiate hepatocarcinogenesis because it is a proven and specific carcinogen for hepatocarcinogenesis. Many researchers have used it with much satisfaction to induce hepatocarcinogenesis (Takahashi, 1992; Ito et al., 1988; Matsuda, 1994).

Glutathione S-transferase placental form positive (GST-P+) foci, an enzyme marker, was used to assess the modifying effects of garlic on hepatocarcinogenesis.
2. To investigate the effects of garlic on chemically induced hepatocarcinogenesis in hypercholesterolaemic Wistar rats.

According to the available literature, the studies which have been carried out so far have investigated only the effects of garlic on carcinogenesis in normal (normocholesterolaemic) subjects. There has not been a single reported study on the effects of garlic on carcinogenesis in hypercholesterolaemic subjects.

Therefore, in the second phase of this study, hypercholesterolaemic rats were used as the experimental model. Induction of hepatocarcinogenesis and assessment of carcinogenicity were carried out by methods described in specific objective I.
2. LITERATURE REVIEW

2.1 GARLIC (*Allium sativum*) of family Liliaceae

English: Garlic, Sinhala: Sudulunu, Tamil: Vellavengayam, Vellaippundu.

"Garlic then has power to save from death.
Bear with it though it maketh the unsavoury breath.
And scorn not garlic, like some that think
It only maketh men wink, drink and stink"

(Sir John Harington, 1809)

Plate 2.1: Bulbs of garlic (*Allium sativum*)
2.1.1 Botanical Aspects

A bulbous herb with a short, flat axis giving off slender very thin, papery scales which are enlarged and dilated below and bear at their axils large, oblong-ovoid, sessile bulbs pressed together with the outer ones curved to form collectively a lobed white tapering bulb; Flowering stem terminal, solid from the centre of the bulb, 45-60cm long, lower half surrounded by leaf-sheaths; Flowers regular, bisexual, very long stalked, projecting beyond the bulbil; perianth 6, dirty-white, strongly imbricate in two rows and membranous; anther introrse, dorsifixed; ovary superior, globular, trigonous, 3-locular with a single persistent style. (Jayaweera, 1981).

2.1.2 Major Chemical Constituents

The most important chemical constituents reported from bulbus *Allium Sativum* are the sulfur compounds (Farnsworth, 1992; Reuter and Sendl, 1994; Sendl, 1995). It has been estimated that cysteine sulfoxides (e.g. alliin and the non-volatile γ-glutamylcysteine peptides make up more than 82% of the total sulphur content of garlic (Sendl, 1995). Allicin (1) and its precursor Alliin (2) have been considered as marker components of garlic. Alliin lyase is the enzyme, which catalyses the cleavage of alliin into allicin, pyruvate, and ammonia, thereby demonstrating that alliin is the precursor of the major flavour compounds of garlic (Sendl, 1995). When the garlic bulb is crushed, minced or otherwise processed, alliin is released from compartments and interacts with the enzyme allinase.

At elevated temperatures, or especially in fatty oil, allicin appear to be unstable. Several transformation products, such as ajoenes (3), vinylthiins (4), and sulfides have been
formed and they play a major role in the action of garlic as a medicinal agent (Vernin, 1986; Wagner, 1987). Sulfur chemical profiles of bulbus Alli sativi products reflected the processing procedure: bulb mainly alliin, allicin; dry powder, mainly alliin, allicin; volatile oil, almost entirely diallyl sulfide, diallyl disulfide (5), diallyl trisulfide, and diallyl tetrasulfide; oil macerate, mainly 2-vinyl-[4H]-1,3-dithiin, 3-vinyl-[4H]-1,3-dithiin, E-ajoene, and Z-ajoene (Ziegler and Sticher, 1989; Iberl et al., 1990; Lawson et al., 1991; Reuter and Sandl, 1994).
2.1.3 Medicinal and other Uses of Garlic (*Allium Sativum*)

2.1.3.1 Introduction: The use of garlic began in prehistoric times. Garlic has been used as a food item in many different cultures. By 3000 B.C. garlic was being used as a medicine by Egyptians, Chinese, Babylonians, Indians, Romans and Vikings. The Ebers Papyrus contains 22 therapeutic formulae in which garlic is prescribed for heart disease, headache, worms and tumours (Wickramanayake, 1996). *Allium Sativum* is a bulbous herb, which has an acrid biting, somewhat bitter taste and a strong pungent odour. All parts of the plant have similar though less powerful taste and odour (Bently and Triman, 1880; Kirtikar and Basu, 1933). It is widely used as a condiment and a flavouring agent.

Medicinally it is a stimulant, carminative, anthelmintic, diphoretic, diuretic, expectorent, tonic etc. when taken internally.

2.1.3.2 Carminative properties: Garlic is involved in digestion and absorption of food and act as a gastric stimulant. It also improves appetite; hence help in gaining weight and renders sleep regularly. Clears the voice and improves complexion (Bently and Triman, 1880; Kirtikar and Basu, 1933). The efficacy of Bulbus Allii Sativi as a carminative has been demonstrated in human studies. A clinical study of 29 patients taking two tablets daily (~ 1000 mg/day) of a dried garlic preparation demonstrated that garlic relieved epigastric and abdominal distress, belching, flatulence, cholic and nausea, as compared with placebo. It was concluded that garlic sedated the stomach and intestines, and dispersed gas (Damrau *and* Ferguson, 1949).
2.1.3.3 **Expectorant properties:** As an expectorant it has a special influence over the bronchial and pulmonary secretion and is useful in dilating bronchi, diminishing cough, reducing expectoration and in old chronic catarrhs (Jayaweera, 1981).

2.1.3.4 **Hypolipaemic and hypocholesteroleamic properties:** As reported, in humans and in animal models, this property is mainly due to decrease in hepatic cholesterol synthesis by (a) inhibition of HMG Co A reductase (Gebhardt et al., 1994:) and lipogenic enzymes. (b) stimulatory effect on plasma Lecithine Cholesterol Acyl Transferase (L CAT) and (c) faecal excretion of sterols and bile salts. Lowering of serum lipid by garlic may decrease atherosclerosis and hence reduce the risk of cardiovascular disease (Kaul and Prasad, 1990; Mirhad, 199; Sheela et al., 1995).

2.1.3.5 **Anti-thrombotic properties:** This is another major beneficial effect of garlic. Several garlic constituents have been shown to possess anti platelet aggregation activity in vivo and in vitro. It is said that raw garlic is more effective than cooked garlic in preventing thrombosis. Effect is due to inhibition of cyclooxygenase activity which decreases the level of serum thromboxane (Kirtikar and basu, 1933; Ali M., 1995; Bordia et al., 1996). An increase in fibrinolytic activity in the serum of patients suffering from atherosclerosis was also observed after administration of aqueous garlic extracts, essential oil, and garlic powder (Bordia et al., 1977; Harenberg et al., 1988).
2.1.3.6 Anti-hypertensive properties: The results of several studies that investigated the effects of garlic on systolic and diastolic blood pressure have led to the conclusion that garlic may have some clinical usefulness in mild hypertension, but there is still insufficient evidence to recommend the drug as a routine clinical therapy for the treatment of hypertension (Silagy and Neil, 1994).

2.1.3.7 Anti-microbial properties: Bulbous garlic and its extracts have been shown to possess a broad range of antibacterial and antifungal activity (Medicinal plants in VietNam, WHO Regional Publications, 1990). Reported studies indicate that it contains several constituents such as diallyl thio sulphinate (allicin), methylallyl thio sulphinate which showed significant anti-bacterial and anti-fungal activities (Hughes et al., 1991). Aqueous extracts of garlic and allicin both possessed significant antibacterial activity against multiple drug resistant strain of shigella (Chowdhry et al., 1991).

2.1.3.8 Anthelmintic properties: Garlic has been used in the treatment of roundworm (Ascaris strongyloides) and hookworm (Ancylostoma caninum and Necator americanus) (Soh et al., 1960) infestations. Allicin appears to be the active anthelminthic constituent.

2.1.3.9 Anti-tuberculosis activity: Water extract of garlic and its two protein fractions inhibited the growth of *Mycobacterium tuberculosis* at mid log phase. Supplementation of minced garlic with honey for cases of *Lupus Vulgaris* in addition to the usual antituberculosis treatment showed accelerated healing. Results also have indicated that the
primary mechanism of anti-tuberculosis activity is by inhibition of protein synthesis (Kirtikar and Basu, 1933; Ratnakar and Murthy, 1996; Sami et al., 1989).

2.1.3.10 Anti-oxidative properties: Sulfide fraction, fractionated from aged garlic has been found to have high inhibitory activity towards lipid peroxidation in rat liver microsomes. Another reported study has indicated that S-allyl cysteine sulfoxide controlled lipid peroxidation. Hence garlic contains effective anti-oxidant properties (Horie et al., 1992, Augusti et al., 1996).

2.1.3.11 Hypoglycaemic activity: Oral administration of extracts of garlic has lowered blood glucose levels in rabbits and rats (Jain and Vyas, 1975; Reuter and Sendl, 1994). However some studies have reported negative results (Swanston-Flatt et al., 1990). Allicin administered orally to alloxan-diabetic rats lowered blood glucose level and increased insulin activity in a dose dependent manner (Reuter and Sendl, 1994). Oral administration of garlic powder (800 mg/day) to 120 patients for 4 weeks in a double-blind, placebo-controlled study has decreased the average blood glucose by 11.6% (Kisewetter et al., 1991). In another study no such activity was found after dosing non-insulin-dependent patients with 700 mg/day of a spray-dried garlic preparation for 1 month (Sitprija et al., 1987).

2.1.3.12 Malignant diseases (anti-carcinogenic/anti-tumour): Epidemiological studies have indicated that garlic and its organosulphur constituents inhibit the induction and growth of cancer, hence reducing the deaths caused by malignant diseases (Benjamin, 1990). Several studies have indicated that garlic contains several potentially
important agents that possess anti-tumour and anti-carcinogenic properties in humans and experimental animal models (Belman, 1983; Lea Ma, 1996). In vitro incubation of Ehrlich ascites tumour cells with fresh garlic extracts rendered these cells non-tumourigenic (Fujiwara et al., 1967). A China-U.S. collaborative investigation has shown that a person who consumes 20g garlic/ day may inhibit nitrate production by bacteria in stomach. Subsequently, gastric nitrite, which is a precursor for nitrosamine ill be lowered and hence, reduce the risk of developing stomach cancer (Mei et al., 1982).

In a study of mammary cancer induced by 7,12 dimethylbenz(a)anthracene (DMBA), it has been found that Se-enriched garlic, garlic powder and its sulphur containing constituents such as diallyl disulfide, diallyl sulfide and allyl methyl sulfide can act as cancer preventive agents. Action of inhibition is possibly by reducing DMBA-DNA binding (Liu, 1992). Another study has demonstrated that naturally occurring organosulphur compounds diallyl sulfide S-allyl cysteine at a dose of 200mg/ kg body weight inhibited of DMH-induced colonic nuclear toxicity and carcinogenesis in mice. Diallyl disulfide has also exhibited chemopreventive activity against 4 (methyl nitrosamine)-1-(3-pyridyl)-1- butanone (NNK) induced lung tumourigenesis (Hong, 1992; Sumiyoshi and Wargovich, 1990).

Two organosulphur compounds, methyl propyl disulfide(MPD) and propeline sulfide (PS) from garlic and onions have been studied for their modifying effects on hepatocarcinogenesis in F344 rats using medium term bioassay system of Ito and it has been shown that both of these organosulphur compounds significantly reduce the
number and area of GST-P foci in treated rats compared with controls (Matsuda et al., 1994). A recent study indicates that S-methyl cysteine (SMC) from Allium sativum and cysteine significantly inhibit the induction of Glutathione S-transferase placental form positive (GST-P+) foci in Diethylnitrosamine (DEN)-induced hepatocarcinogenesis in F344 rats when given during initiation and promotion stages (Takada et al., 1997).

Food plants may contain anti-carcinogens as well as carcinogens (Ames, 1983). A few reported studies have indicated that some of organosulphur compounds of garlic also have enhancing effects on hepatocarcinogenesis. DAS exerts an enhancing effect on development of GST-P+ foci in DEN-induced liver carcinogenesis at doses of 200mg and 50mg / kg body weight (Takahashi et al., 1992). In another study Takada et al., (1994) investigated the modifying effects of ten organosulphur compounds on DEN-induced neoplasia of liver in male F344 rats using the bioassay system of Ito. It was found that high doses of diallyl disulfide, diallyl trisulfide, allyl Me sulfide, and allyl Me trisulfide, dipropyl sulfide enhance the GST-P+ focus formation, while high doses of Me propyl disulfide and propyl sulfide significantly decreased the number of GST-P+ foci. However, it has been reported (Hong, 1992; Sumiyoshi and Wargovich, 1990) that DAS exerts chemopreventive action against carcinogenesis in some of the other organs for example, in NNK-induced lung carcinogenesis and DMH-induced colonic carcinogenesis.

2.1.3.13 Other medicinal uses: Garlic oil is often applied in rheumatic pain to reduce inflammation of joints. Indians who regularly use garlic as a food are free from beri-beri and influenza. Use of garlic as an antiseptic is very common (Kirtikar and Basu, 1933).
In children it is used as a remedy in atonic deafness; few drops of juice is applied in to the ear (Bently and Triman, 1880; Kirtikar and Basu, 1933). The effect of garlic extracts against enzymatic glycosylation of rat lens protein (important in pathogenesis of cataracts) has been found to be important. It decreases the glycosylation process by inhibiting the formation of hydroxy methyl furfural (Srivastara and Afaq, 1990).

According to "Unani" traditional medicine, garlic is a remedial agent in the treatment of inflammation, paralysis, pain in the joints, troubles of the spleen, liver and lungs. It clears the voice, and is good for lumbago, chronic fever, thirst, caries of the teeth and leucoderma (Kirtikar and Basu, 1933).
2.2 CARCINOGENESIS

The crab, shown in plate 2.2 is a 15th century illustration for an Italian book of prayers, gave mankind’s dreaded disease its name. The hard center and clawlike projection of a spreading tumour seemed to Hippocrates, the ancient-Greek physician, to resemble the crustacean, and he named the disease "karkinoma", after the Greek word for “crab”.

“Cancer” is the Latin translation.

(William and Phyllis, 1981)

Plate 2.2: The Crab and Cancer
2.2.1 Introduction to Carcinogenesis

Clonal theory of carcinogenesis
Cancers derive from an original, transformed cell. This transformed cell, or clone, is a normal cell whose genetic material has been altered such that the cell loses those regulatory functions that govern cell replication and death. By an evolutionary, multistep process, cells derived from the initially modified cell begin to multiply, uncontrolled by the usual local inhibitory system, often invading other parts of the body (Sager, 1983).

2.2.2 Carcinogen
A carcinogen is a substance or a physical force, which can change normal cells into neoplastic or transformed cells. At present, epidemiologists believe that 60% to 90% of human cancers are caused by environmental agents (Miller, 1978). The main environmental agents which are thought to cause cancers can be chemical, physical, and biological (Pitot, 1986).

2.2.2.1 Chemical carcinogens
Chemical carcinogens include a multitude of organic and inorganic substances. A large number of organic chemicals belong to polycyclic aromatic hydrocarbons. Aromatic amines, nitrosamines and various drugs are also important organic carcinogens. Inorganic chemicals such as As, Ni, Be, Cd, Cr, etc are involved in carcinogenesis (Robert, 1996). Naturally occurring carcinogens include aflatoxin-B1 and dactinomycin (Robert, 1996).
These carcinogens can be further classified as

(a) Direct carcinogens: Interact directly with DNA to induce carcinogenesis.

(b) Procariogns: These require prior metabolism to become an active carcinogen. Metabolic activation of procariogns by one or more enzyme reactions gives rise to chemically active ultimate carcinogens. (Miller, 1978) Enzymes responsible for this metabolism are principally species of cytochrome P 450 located in the endoplasmic reticulum, especially in the liver cells.

2.2.2.2 Physical carcinogens

These are the forms of radiation energy and mechanical agents. UV-rays, α, β, γ, and x-rays are known to induce cancer. The damage may be direct or indirect. Formation of apeurinic or apyrimidinic sites, single or double strand breakage’s, formation of pyrimidine dimers and cross linkage of strands may be induced by radiation energy (Folkman and Klagsbrun, 1987).

2.2.2.3 Biological carcinogens

A large number of animal tumours are known to be caused by viruses. This is due to viral vectors carrying oncogenes in their genomes. Oncogenes arise from mutation of proto-oncogenes, which they acquired from a viral infected cell. Infection of another cell by this virus can transfer this abnormal gene to its genome, thus inducing cancer. Most retroviruses have this action (Wyllie, 1992).
2.2.3 Mechanism of Carcinogenesis

2.2.3.1 Two step carcinogenesis

In certain organs such as skin, mammary gland, bladder, pancreas and liver it has been shown that mechanism of carcinogenesis can be divided at least into two stages. Hence this is known as two stage carcinogenesis. Stages include initiation and promotion (Rous and Kidd, 1941; Berenblum and Shubik, 1947; Pitot et al., 1980).

Initiation: This stage appears to be rapid and irreversible. It is presumed to involve modification of DNA perhaps resulting in one or more mutations. Substances, which induce initiations, are known as initiators (Pitot and Srca, 1980).

Promotion: Second, much slower stage of carcinogenesis, resulting from application of promoter. This is by the alteration of the expression of genetic information in the cell (Pitot and Srca, 1980).

Most carcinogens are capable of acting both as initiator and promoter.

2.2.3.2 Multi stage carcinogenesis

This is progressed through four obligatory phases: an induction phase, an in situ phase, an invasion phase and a dissemination phase (Moolgavkar and Knudson, 1981; Bernard and Per Winkel, 1994).

Induction Phase: during this phase, the cells are exposed to one or more carcinogens. Additional factors such as genetic or tissue susceptibility, the presence of other carcinogens or cocarcinogens, the site at which the carcinogen may act, the duration of exposure, and obviously the nature, amount or concentration of carcinogen play a role...
in deciding which individuals may get cancers. The time between the induction phase and the clinically apparent cancer can be as long as 20 years.

**In situ phase:** followed by the induction phase. This phase represent the time during which the transformed cell actually develops into a cancer. Cancer remains localized in the original site and does not invade other tissues.

**Invasion phase:** During this phase malignant cells multiply and invade into the deeper tissues through the basement membrane, thereby gaining access to blood vessels and lymphatic channels.

**Dissemination phase:** which last 1 to 5 years, the invading cancer spreads through blood and lymphatic systems.

### 2.2.4 Dietary Fat and Carcinogenesis

#### 2.2.4.1 Introduction

The literature concerning correlation between dietary items and cancer report that there is a strong relationship between death rates of certain cancers and the per capita consumption of total fat. Numerous studies including animal experiments as well as case-control studies over the past decades of years have shown that subjects on high fat diets develop certain types of cancers more rapidly than do their counterparts fed low fat diets. This effect has been observed with several malignancies (Freedman et al., 1990; Kolonel. *et al.*, 1999; Van Kranen *et al.*, 1998; Black, 1998; Willet, 1998). However, some studies are not in agreement with the effects of dietary fat on carcinogenesis (Cave, 1991; O'Conner *et al.*, 1989). It was also evident that the n-3 PUFA series can inhibit growth of human breast cancer cells both *in vitro* and *in vivo* (Stoll, 1998).
2.2.4.2 Hepatocarcinogenesis

Studies have shown that increasing dietary fat, leads to the high incidence of hepatocellular carcinomas in rodents with chemically-induced hepatocarcinogenesis. In rats, increasing the fat content of the diet enhances the development of 2-acetylaminofluorine (AAF)-induced tumours and GGT-positive foci in the liver (Sugai et al., 1962; Baldwin and Parker, 1986). However a number of other experiments have shown that there were no effects of dietary fat on hepatocarcinogenesis if it is administered after the initiation of carcinogenesis (Glauert and Pitot, 1986). Recently it has been found that when rats are fed diets high in PUFA, before receiving the hepatocarcinogen DEN, they develop more GGT-positive foci in the liver (Glauert et al., 1991).
2.3 TUMOUR MARKERS

2.3.1 Definition

A tumour marker is a substance present in or produced by a tumour or by the tumour’s host in response to the tumour’s presence (Sell, 1992).

2.3.2 Features of a Tumour marker

It can be used to diagnose or monitor the growth or presence of cancer. Such a substance can be found in cells, tissues or body fluids. It can be measured qualitatively or quantitatively by chemical, immunological, or molecular biological methods to determine the presence of cancer (Sell, 1992).

An ideal tumour marker should be both specific for a given type of cancer and sensitive enough to detect small tumours for early diagnosis or during screening. Few markers are specific for a single individual tumour (tumour-specific marker), most are found with different tumours of the same tissue type (tumour-associated marker). They are present in higher quantities in cancer tissues or in blood from cancer patients than in benign tumours or in the blood of normal subjects. Few tumour markers are specific for the organ where the tumour resides (Chu, 1992). Tumour markers are used in monitoring of treatment and progression of cancer. They are also used to detect the recurrence of cancer and as targets for localisation and therapy (Sell, 1991).
2.3.3 Classification

Tumour markers include enzymes and isoenzymes, oncofetal antigens, hormones and hormone receptors, carbohydrate epitopes recognised by monoclonal antibodies, and genetic markers.

2.3.3.1 Enzymes

2.3.3.1.1 Introduction: Enzymes were one of the first group of tumour markers identified. Their elevated activities were used to indicate the presence of cancer. (Gutman et al., 1938) Measurements of enzymes was relatively easy using spectrophotometric determination of enzymatic activities. With the introduction of antibody techniques, some enzymes, such as prostate specific antigen (PSA), are measured as protein antigen (based on mass concentration) instead of its catalytic activity. Some of enzyme markers and their associated types of malignancy, that are commonly used are given in table 2.1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Type of Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>Bone, liver, leukaemia, sarcoma</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>Liver</td>
</tr>
<tr>
<td>Creatine kinas-BB</td>
<td>Prostate, lung (small cell), etc.</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>Liver, lymphomas, leukaemia, various, etc.</td>
</tr>
<tr>
<td>Prostatic acid phosphatase</td>
<td>Prostate</td>
</tr>
<tr>
<td>Prostate specific antigen (PSA)</td>
<td>Prostate</td>
</tr>
<tr>
<td>Galactosyltransferase</td>
<td>Colon, bladder, gastrointestinal, various</td>
</tr>
<tr>
<td>Glutathione S-transferase (GST)</td>
<td>Liver</td>
</tr>
</tbody>
</table>

It should be mentioned that, not all patients with a particular cancer type have elevated enzyme levels, and many non-cancer diseases are associated with elevation of many of these enzymes (Bernard and Per Winkel, 1994).

2.3.3.1.2 Glutathione S-transferase (GST; EC.2.5.18):

2.3.3.1.2.1 Introduction

Glutathione S-transferase (GST) is a complex family of cytosolic isoenzymes involved in detoxification and excretion of a wide range of physiological and xenobiotic substances including environmental pollutants, carcinogens, mutagens and toxic products (Hayes and Palford, 1995; Jakoby, 1978). They catalyse reactions towards a large number of structurally diverse substrates, including alkyl and arylhalides, lactones, epoxide, quinones, esters and activated alkenes (Mannervik and Danielson, 1988). All these substrates possess the common feature that they are hydrophobic and contain an electrophilic centre. GSTs appear to be ubiquitous among aerobic organisms. They have also been purified from plants, fish, insects, fungi, yeast and recently from bacteria (Mathew and Michael, 1994).

2.3.3.1.2.2 Classification

Initial studies of GSTs have demonstrated that there were only three distinct families of mammalian GSTs namely Alpha (α), Mu (μ) and Pi (π), (Mannervik et al., 1985). Subsequent studies have identified an additional class of cytosolic GSTs termed Theta (θ) (Meyer, 1991). It is characterised by the presence of an active-site serine residue, which contrasts with an active-site tyrosine in Alpha, Mu and Pi. Recently, a soluble mitochondrial specific GST termed as Kappa (κ) has been identified (Board et al.,
1997), although little is known about the structure, function or evolution of this class. Non-mammalian GSTs such as Sigma ($\zeta$), which is well represented in cephalopods and Delta ($\delta$) in insects are examples of the additional diversities of GSTs (Buetler and Eaton, 1992). More recently new evolutionary class of GST has been identified in a range of species from plant to human, and named Zeta ($\zeta$)(Board et al., 1997) These classes of GSTs differ from each other on the basis of substrate and inhibitor specificities, sequencing data and immunohistochemistry.

2.3.3.1.2.3 Structure

GST exist as either homo or hetero dimer forms. Members within any class exhibit similar monomer sizes (about 24 to 28 KDa). They share high amino acid sequence identity (typically 60-80%) and have distinctive but overlapping substrate specificities. (Malthew and Michael, 1994).

The cytosolic enzymes have two active sites per dimer, which behave independently of one another (Mannervik et al., 1985). Each active site consists of at least two ligand-binding regions. GSH binding site is very specific for glutathione, whereas the binding site for the electrophilic substrate is less specific. This is important in keeping with the ability of GST to react with wide variety of toxic agents (Malthew and Michael, 1994).

2.3.3.1.2.4 Functions

2.3.3.1.2.4.1 As a Tumour Marker: Human GST $\pi$ has been reported as a marker protein in hepatocarcinogenesis (Tatematsu, 1987), renal carcinoma, lung carcinoma,
cervical lesions and in several other carcinomas (Tsuchida and Sato, 1992; Mulder et al., 1995).

Different models of hepatocarcinogenesis in the rat involve the quantification of altered hepatic foci. This has been used for the characterisation of effects of modifiers (stimulators/ inhibitors) on initiation and promotion phase of multistage hepatocarcinogenesis (Ito et al., 1988, 1989; Pitot et al., 1978; Howard et al., 1991; Nobuyasu et al., 1997).

Rat GST-P (Glutathione S-transferase placental form), which is related to human GST π in enzymatic and immunological properties is used by many researches as a reliable marker for preneoplastic lesions, since it is strongly and specifically expressed in very early phase of chemically induced hepatocarcinogenesis but not in normal hepatocytes (Morimura et al., 1993). It was also reported that GST-P would be the one of the best single marker for altered hepatic foci if only one marker is used (Suzann et al., 1987). It is assumed that in hepatocarcinogenesis all hepatocytes are susceptible and single hepatocytes that express GST-P are the initiated cell population for focal lesions. Therefore GST-P+ have been proposed as putatively initiated cell population for the preneoplastic lesions in hepatocarcinogenesis (Ito et al., 1982; Farber, 1984).

It is now generally accepted that quantitative data of two dimensional (2D) or three dimensional (3D) data for preneoplastic foci can be used to assess indices of initiating and promotion potential in hepatocarcinogenesis (Farber, 1980; Ricardo et al., 1991; Suzann et al., 1987). However it has been clearly concluded that 2D values are
adequate to determine the modifying potential (inhibitory/ stimulating) of the chemicals in medium term bioassay for liver carcinogens and promoters (Katsumi et al., 1989; Tadashi et al., 1990).

2.3.3.1.2.4.2 Detoxification properties: Glutathione S-transferase (GST) is involved in detoxification and excretion of a wide range of physiological and xenobiotic substances including environmental pollutants, carcinogens, mutagens and toxic products (Jakoby, 1978; Hayes and Palford, 1995). They catalyse the nucleophilic addition of thiol of reduced glutathione (γ-glutamyl cysteinyl glycine) to electrophilic centres of above substances. The glutathione conjugates so formed are more water soluble, less reactive and easily excreatable (Matthew and Michael, 1994).

2.3.3.1.2.4.3 Other functions: In addition to their catalytic activity, GSTs also acts as intracellular transporters of various non-substrate hydrophobic compounds such as bile acids, steroids, heme, bilirubin, thyroid hormone, neurotransmitters and a wide variety of drugs (Mannervik et al., 1985; Suxing et al., 1998). These will cause inhibition of the enzyme’s catalytic activity.

GSTs protect against accumulation of cellular phospholipid hydroperoxide. This action is important when status of the other antioxidant become low (Rachel, 1998).

2.3.3.2 Oncofetal antigens

2.3.3.2.1 Introduction: They are proteins produced during foetal life. These proteins are present in high concentration in the sera of foetuses and decrease to low levels or disappear after birth. In cancer patients, these proteins reappear. The production of these
proteins demonstrate that certain genes are reactivated as the result of the malignant transformation of cells (Daniel and Stewart, 1994). However these proteins are not recommended for cancer screening (Bernard and Per Winkel, 1994). Several oncofetal antigens are listed in table 2.2.

Table 2.2: Oncofetal antigens as tumour markers

<table>
<thead>
<tr>
<th>Oncofetal protein</th>
<th>Type of cancer associate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-fetoprotein (AFP)</td>
<td>Hepatocellular, germ cell (nonseminoma)</td>
</tr>
<tr>
<td>Carino embryonic antigen (CEA)</td>
<td>Colorectal, gastrointestinal, pancreatic, etc.</td>
</tr>
<tr>
<td>Human chorionic gonadotrophin (hCG)</td>
<td>Placenta and testis</td>
</tr>
<tr>
<td>β-Oncofetal antigen</td>
<td>Colon</td>
</tr>
<tr>
<td>Squamous cell carcinoma antigen (SCC)</td>
<td>Cervical, lung, skin, head and neck</td>
</tr>
</tbody>
</table>


2.3.3.3 Hormones and hormone receptors:

2.3.3.3.1 Introduction: The production of hormones in cancer involves two separate routes. First, there can be an excess production of hormone by the endocrine tissue that normally produces it. Second, a hormone may be produced at a distant site by a non-endocrine tissue that normally does not produce hormone. They were used for the detection and monitoring of cancer. The introduction of specific RIA method for a particular hormone that has very little cross reactivity with other similar hormones made it possible to monitor the treatment of cancer patients. However elevation of the level of a given hormone is not a basis of diagnosis of specific tumour (Daniel and Stewart, 1994). List of hormones used as tumour markers is given in table 2.3.
Table 2.3: Hormones and Hormone receptors as tumour markers

<table>
<thead>
<tr>
<th>Hormones</th>
<th>Type of cancer associate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenocorticotrophic hormone (ACTH)</td>
<td>Cushing's syndrome, lung (small cell)</td>
</tr>
<tr>
<td>Human Chorionic Gonadotropin (hCG)</td>
<td>Embryonic, choriocarcinoma, testicular (nonseminomas)</td>
</tr>
<tr>
<td>Calcitonin (CT)</td>
<td>Medullary thyroid</td>
</tr>
<tr>
<td>Growth hormone</td>
<td>Pituitary adenoma, renal, lung</td>
</tr>
<tr>
<td>Parathyroid hormone</td>
<td>Liver, renal, breast, lung, various</td>
</tr>
<tr>
<td>Breast estrogen and progesterone receptors</td>
<td>Breast</td>
</tr>
</tbody>
</table>


2.3.3.4 Carbohydrate markers:

2.3.3.4.1 Introduction: Carbohydrate-related tumour markers are either antigens on the tumour cell surface or those that are secreted by the tumour cells (Hakomori, 1992). These markers represent a new generation of clinically useful tumour markers. They tend to be more specific. They are high molecular weight mucin or blood group antigens. Some of them are listed in table 2.4.

Table 2.4: Carbohydrate markers as tumour markers

<table>
<thead>
<tr>
<th>Carbohydrate marker</th>
<th>Type of cancer associate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA 15-3</td>
<td>Breast</td>
</tr>
<tr>
<td>CA 125</td>
<td>Ovarian, endometrial</td>
</tr>
<tr>
<td>Mucinlike carcinoma-associated antigen</td>
<td>Breast, ovarian</td>
</tr>
<tr>
<td>CA 19-9</td>
<td>Colorectal, pancreatic, hepatic</td>
</tr>
<tr>
<td>CA 72-4</td>
<td>Gastrointestinal tract, breast, and ovary</td>
</tr>
</tbody>
</table>

2.3 3.5 Genetic Markers:

2.3.3.5.1 Introduction: Cancer is an inheritable characteristic of cells and must be the outcome of genetic changes (Soloman et al., 1991). Multiple genetic changes may be necessary for the transformation of a cell from a normal state to a cancerous one and, finally, for metastasis. Genetic markers are emerging with great potential for diagnostic application, particularly with the advent of the polymerase chain reaction (PCR) technique. Two classes of genes are implicated in the development of cancer: oncogenes and suppressor genes (listed in table 2.5).

2.3.3.5.2 Oncogenes: They are derived from proto-oncogene. Proto-oncogenes are normal cellular genes and their activation is found to be associated with cancer. Amplification of the oncogene will lead to abnormal cell growth, resulting in malignancy. Few oncogenes are shown to be useful as tumour markers (Sell, 1990).

2.3.3.5.3 Suppressor gene: These genes are normally expressed in nontransformed cells and appear to play a role in chromosome stability or in the regulation of cellular differentiation or cellular proliferation (Sager, 1989). Certain types of human tumours, such as retinoblastoma, involve the loss of specific genes. The loss of tumour suppressor genes may involve point mutation in the specific gene or deletion of part of the chromosome containing the specific gene. Mutation and allelic losses of the p53 tumour suppressor gene have been observed in several human tumours (Smith et al., 1991; Hsu et al., 1991).
Table 2.5: Genetic markers as tumour markers

<table>
<thead>
<tr>
<th>Genetic marker</th>
<th>Type of cancer associate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oncogenes</strong></td>
<td></td>
</tr>
<tr>
<td>N-ras gene</td>
<td>Acute myeloid leukaemia, neuroblastoma</td>
</tr>
<tr>
<td>c-myc gene</td>
<td>B- and T-cell Lymphoma, lung (small cell)</td>
</tr>
<tr>
<td><strong>Suppressor gene</strong></td>
<td></td>
</tr>
<tr>
<td>Detected colon carcinoma (DCC)</td>
<td>Colon cancer</td>
</tr>
<tr>
<td>P53</td>
<td>Liver and several other</td>
</tr>
</tbody>
</table>

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1 Plant Material
Garlic (*Allium sativum* Linn. of family Liliaceae) bulbs were oven dried at 60°C and powdered. (kindly supplied by Aushada Lanka (Pvt.) LTD., Katuwawala, Sri Lanka).

3.1.2 Animal Model
Male Wistar rats (6 weeks old) were obtained from Medical Research Institute (M.R.I.), Colombo, Srilanka. Rats were housed (3 animals / cage) in temperature controlled room (25 ±1°C) under a 12hr light-dark cycle and fed with a standard laboratory diet, made at Medical Research Institute (M.R.I.), Colombo (Carbohydrate 61%, Protein 19%, Fat 5.5%, Cholesterol 0.1%, Fiber 3.4%, Moisture 10%) (Feed ingredients in standard diet are shown in table 3.1) and water ad libitum. All the experiments were carried out at the M.R.I., Sri Lanka.

3.1.3 Chemicals
Cholesterol: powdered form purchased from Sigma Chemical Company Ltd U.S.A. Diethylnitrosamine (DEN): purchased from Sigma Chemical Company, U.S.A. Rabbit polyclonal anti-GST-P antibody: purchased from Medical and Biological Laboratories (MBL) Co. LTD., Japan. Diagnosis kit consists of skim milk, biotin labeled goat anti rabbit IgG (1:200), Avidine Biotin Peroxidase complex (ABC), and diaminobenzidine (DAB) obtained from Dako, Netherland.
Ethanol, xylene, formaline, paraffin wax (b.p. 58°C), haematoxylin and eosin were purchased from Sigma Chemical Company, U.S.A.
<table>
<thead>
<tr>
<th>Feed ingredient</th>
<th>Breeding feed</th>
<th>0.5% cholesterol</th>
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<tbody>
<tr>
<td>Maiz</td>
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<td>1990</td>
</tr>
<tr>
<td>Brown Rice</td>
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<td>500</td>
</tr>
<tr>
<td>Rice Bran</td>
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</tr>
<tr>
<td>Weat Bran</td>
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<td>100</td>
</tr>
<tr>
<td>Weat Flour</td>
<td>675</td>
<td>675</td>
</tr>
<tr>
<td>Fish Meal</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>Soya Meal</td>
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<td>400</td>
</tr>
<tr>
<td>Milk Powder</td>
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<td>300</td>
</tr>
<tr>
<td>Molasses</td>
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<td>125</td>
</tr>
<tr>
<td>Soya oil</td>
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<td>89</td>
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<tr>
<td>Grass powder</td>
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<td>150</td>
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<td>Bone Meal</td>
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<tr>
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<tr>
<td>Betamix E50</td>
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<td>1.0</td>
</tr>
<tr>
<td>DL Methionine</td>
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<td>B complex (tbs)</td>
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<td>25</td>
</tr>
<tr>
<td><strong>Total quantity</strong></td>
<td><strong>5000</strong></td>
<td><strong>5000</strong></td>
</tr>
</tbody>
</table>

Table 3.1: Feed formulae for rat
3.2 METHODS

3.2.1 Development of a Hypercholesterolaemic Rat Model

Animals were fed with cholesterol-enriched diets containing different concentration of cholesterol and blood samples were collected on day 0 and at the end of each week for the determination of total serum cholesterol. Values were compared with a control group on a normal isocaloric diet (Carbohydrate 61%, Protein 19%, Fat 5.5%, cholesterol 0.1%, Fiber 3.4%, Moisture 10%).

3.2.1.1 Collection of blood samples and separation of serum

Blood (0.50ml) was collected from the lateral vein of the tail using 1ml syringes and 21 gauge needles and serum was separated using centrifugation at 2500rpm for 15 minutes.

3.2.1.2 Quantitative determination of total serum cholesterol

This method involves enzymatic, colorimetric assay of cholesterol. Cholesterol esterase hydrolyzes cholesterol esters to cholesterol and free fatty acids. The cholesterol produced from this reaction plus free cholesterol present in the sample is oxidized by cholesterol oxidase to form cholest-4-en-3-one and hydrogen peroxide. Peroxidase catalyzes the hydrogen peroxide oxidation of 4-aminoantipyrine with subsequent coupling to p-hydroxybenzoate. The end product is a quinoneimine dye which has maximum absorbance at 500nm (Allain et al., 1974). A diagnostic kit obtained from Sigma chemical company, U.S.A. was used for the determination of serum total cholesterol. Standard curve (Figure3.1) was plotted to cover the range of 0-200mg/dl.
Standard was analyzed along with other samples as a quality control estimation to note any day to day variations in the estimation.

3.2.1.2.1 Preparation of cholesterol reagent

The amount of deionized water to each vial as stated on the label was added and swirled gently to dissolve the contents. Allowed to stand 10 minutes at room temperature before use.

3.2.1.2.2 Procedure

For each sample, 1.0ml cholesterol reagent and 1.0ml of deionized water were dispensed into labeled test tubes. 0.02ml of each standard, control and sample was added to their respective test tubes, mixed gently and incubated for 8 minutes at room temperature. 0.02ml deionized water was used as reagent blank. Absorbances of all samples were read at 500nm (Elico SL 150, UV-VIS spectrophotometer).
Figure 3.1: Standard curve for assay of total serum cholesterol

Data are Mean ± SD of six values

R² = 0.9931
3.2.2 Liver Medium-Term Bioassay Protocol

This system was initially introduced by Ito et al. in order to identify the environmental carcinogens which are specific to liver (Ito et al., 1988). Later on, this system has been successfully used for the detection of inhibitors of hepatocarcinogenesis as well (Ito et al., 1989). Protocol consisting of initiation with a carcinogen, diethylnitrosamine (DEN) (Shirai et al., 1985), 6 weeks test chemical administration (orally, via sonde needle), partial hepatectomy during this period (Hasegawa et al., 1986) and quantification of glutathione S-transferase placental form positive (GST-P*) foci which are widely accepted as indicators of neoplastic development (Ricardo et al., 1991).

3.2.2.1 Experimental design

Is shown in Figure 3.2. The rats were randomly divided into three groups. Group I was given a single intraperitoneal injection of DEN dissolved in 0.9% NaCl at 200 mg/kg body weight to initiate hepatocarcinogenesis. After two weeks on basal diet, they received test compound (powdered garlic, 20mg/ kg body weight/day) orally, and subjected to partial hepatectomy (PH) at week 3. Group II was given DEN and subjected to PH in the same manner as Group I but without the test compound. Rats in Group III were injected with 0.9% NaCl (saline) alone and were then administered the test compound as in group I. PH was performed at week 3 in all groups of rats to maximize any interaction between proliferation and the effects of the compound tested. Treatment with test compound was continued until the experiment was terminated at week 8.
Figure 3.2: Assay method. Group I. Drug / Test chemical alone; Group II. Carcinogen alone; Group III. Carcinogen + Drug / Test chemical. All rats were subjected to PH at week 4 and sacrificed at week 10.

3.2.2.2 Partial hepatectomy

Removal of median and left lateral lobes (70%) is classically recognized as partial hepatectomy and this is done on rats according to the technique describe by Higgins and Anderson (Higgins and Anderson 1931). This is a surgical procedure, performed under anesthesia with sodium phenobarbital. Rats tolerate this procedure well and over the past few years, the average mortality rate following PH has been 0-5% (Ricardo et al., 1991).
3.2.2.2.1 Procedure (plate 3.1(A3.1-G3.1))

Anaesthetized (intra venous (i.v.) injection of pentobarbital sodium; 3mg / 100g body weight of rat) rat was laid on it's back and with its tail towards the investigator. A midline ventral abdominal skin incision was made (extending from just above the xiphoid cartilage to about half way towards the base of the tail) (A3.1). A similar incision was made in the abdominal muscle layer (extending from immediately below the xiphoid cartilage posteriorly to just beyond the liver). Small bolster was placed under the thorax (causing the liver to fall slightly forwards away from the diaphragm). Falciform ligament, which is transparent and attaching convex face of the liver to diaphragm was cut. Median and left lateral lobes were moved out and placed on a piece of dry gauze (B3.1). Two other suspensory ligaments were revealed (C3.1). Ligature tie was put at the base of two lobes to interrupt the blood flow (D3.1). Several cuts were made (to allow it to bleed on to the gauze and not into the abdominal cavity when they are finally transected) (E3.1). Two lobes were severed as near the ligature as possible (F3.1). Muscle and Skin incisions were closed (4.0 nylon suture material with eye less needle was used) (G3.1).
A 3.1. Making a midline ventral abdominal skin and muscle incision.

B 3.1  Placing a small bolster

C 3.1  Cutting ligaments.

Plate 3.1  Procedure of partial hepatectomy in rat.
D 3.1 Putting ligature tide at the base of two lobes.  E 3.1 Making several cuts on the liver.

F 3.1. Removal of two lobes.

G 3.1. Closing the muscle and skin incision.

Plate 3.1. Procedure of partial hepatectomy in rat.
3.2.2.3 Collection and fixation of liver tissues
At autopsy, livers were excised and slices 2-3mm thick (six slices of liver, two each
from the right posterior, right anterior and caudate lobes) were cut with a surgical blade,
fixed in 10% phosphate-buffered formalin (12-24 hrs).

3.2.2.3.1 10% Buffered formalin (PH = 7)
This was prepared by mixing 37-40% formaldehyde (100ml), distilled water (900ml),
sodium phosphate monohydrate (4.0g) and disodium phosphate monohydrate (dibasic
anhydrous) (6.5g).

3.2.2.4 Processing, embedding, and cutting of liver tissues
Hand processing was done on fixed tissue sections. Tissue sections were washed with
tap water for 4 hrs and processed sequentially through 70% alcohol (overnight ), 95%
alcohol(2x2 hr), 100% alcohol(2x2 hrs), 100% alcohol(overnight). Excess ethanol was
removed from tissue blocks and they were transferred to xylene and kept until tissue
became clear (if necessary another xylene bath was used) then transferred into paraffin
wax ( 58°C) (2 x1.5 hrs). Finally they were embedded in molted wax ( 58°C) to made
tissue blocks. (Graeme & Keith, 1996) tissue sections (6μ thick) were cut using a
rotator microtome and used for the GST-P immunohistochemistry and haematoxylin &
Eosin staining.

3.2.2.5 Glutathione S-transferase-P (GST-P) Immunohistochemistry
The Avidin Biotin-peroxidase Complex (ABC) method described by Hsu et al. (1981)
was used to demonstrate GST-P liver foci. Use of this immunoenzymatic technique
provides a simple and sensitive method to localize antigen in formaline-fixed tissues.
Among the several procedures available, the Avidin-Biotin-Peroxidase Complex (ABC) method, which involves an application of biotin-labeled secondary antibody followed by the addition of avidin-biotin-peroxidase complex gives high staining intensity as well as increased sensitivity (Geesdon et al., 1979). This property is attributed to the formation of a large complex containing multiple peroxidase molecules referred to as lattice-like structure.

3.2.2.5.1 Procedure
Paraffin-embedded, 5µm thick sections were deparaffinized, pretreated with 3% hydrogen peroxide and treated sequentially with skim milk (1%), rabbit polyclonal anti-GST-P antibody (1:2000; MBL Medical and Biological Laboratories Co. LTD., Japan), biotin labelled goat anti rabbit IgG (1:200) and ABC. Preimmune rabbit serum was used instead of antiserum in the negative control for the specificity of anti-GST-P antibody binding. After each step sections were washed thrice with phosphate buffered saline (PBS).

The sites of peroxidase binding was visualized using the diaminobenzidine (DAB). Sections were then counterstained with hematoxylin for microscopic examination. The number and area of the GST-P positive foci and total area of the liver sections were measured using a research microscope (Olympus BX 50 microscope, Tokyo, Japan).

3.2.2.5.2 Preparation of Phosphate buffered saline (PBS) (0.01M)
NaH₂PO₄.2H₂O (0.45), Na₂HPO₄.2H₂O (3.23g) and NaCl (8g) were dissolved in distilled water and was made up to 1L.
3.2.2.6 Haematoxylin and Eosin (H & E) staining

3.2.2.6.1 Procedure

The method described by Alan & Ian was employed in making haematoxylin & eosin stained sections (1996). Paraffin-embedded, mounted 5µm thick sections were deparaffinized in xylene and rehydrated in descending grades of alcohol, sequentially through absolute alcohol, 90% alcohol, 70% alcohol, 50%, alcohol and then in distilled water (2 minutes in each). The sections were stained in Mayer's Haematoxylin (2-5 minutes) and washed in running tap water (2-3 minutes or until the sections turn blue). Slides were then immersed in Eosin (30 s) with agitation and washed in running tap water (30 s). Sections were then dehydrated in 70%, 90%, and two changes of absolute ethanol with agitation (1 minute in each). Finally sections were cleared in xylene and mounted in DPX. The sections were examined under a light microscope (Multihead,) and photographed by using an Olympus( model ) Camera with a Kodak Film ASA 100.

3.2.2.7 Histopathological Examination

H & E stained liver tissues were used. For each slide, five high power fields (random) were examined. Degree of degeneration (vacuolation and granularity) of livers were measured according to the severity of degeneration. Each slide was graded as negative, mild, moderate and severe according to the severity. Decision was made as indicated below.

<table>
<thead>
<tr>
<th>% of damaged cells</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 70</td>
<td>Sever</td>
</tr>
<tr>
<td>70 &gt;&gt; 40</td>
<td>Moderate</td>
</tr>
<tr>
<td>40 &gt;&gt; 15</td>
<td>Mild</td>
</tr>
<tr>
<td>no/ &lt; 15</td>
<td>negative</td>
</tr>
</tbody>
</table>
3.2.2.8 Dosage and administration of Garlic (*Allium sativum*)

Dosage: 20mg of powdered garlic / 1 kg of body weight of rat / day.

Powdered garlic was dissolved in distilled water (2mg/2.5ml) and was administered via a sondi needle (stomach tube).

3.2.2.9 Induction of Carcinogenicity

Dosage: 200mg of Diethylnitrosamine (DEN) / kg of body weight of rat.

Diethylnitrosamine was dissolved in normal saline (100mg/1ml) and was administered using a syringe with fixed needle. A single dose of DEN was given by intraperitoneal (i.p.) injection to initiate hepatocarcinogenicity.

3.2.2.10 Statistical Analysis

The results were expressed as the Mean ± Standard Error of the Mean (SEM). The significance of the difference in the number and area of foci between the control (DEN alone) and the garlic treated group was analyzed by student’s t-test using Microsoft Excel version 5.
3.3 EXPERIMENTAL

3.3.1 Development of a Hypercholesterolaemic Rat Model

3.3.1.1 Experiment I: Effects of cholesterol-enriched diets (0.25%, 0.5%, 1%) on the level of serum cholesterol in Wistar rats

In an attempt to find out the optimum (effective) dose of cholesterol, 48 rats were randomized into four groups (group I, II, III and IV).

Group I (control group) was given a standard diet (Carbohydrate 61%, Protein 19%, Fat 5.5%, Cholesterol 0.1%, Fiber 3.4%, Moisture 10%) for two weeks while groups II, III and IV were fed with 0.25%, 0.5% and 1% cholesterol-enriched diets respectively for two weeks. Body weights were recorded once a week and blood samples were collected from the rats at day 0, day 7 and day 14 for the determination of serum total cholesterol as described in section 3.2.1.2.2.

3.3.1.2 Experiment II: Effects of treatment with 0.5% cholesterol-enriched diet for six weeks on the level of serum cholesterol level in male Wistar rats

This experiment was carried out to determine the effects of long term feeding with 0.5% cholesterol-enriched diet on the level of total serum cholesterol. Twenty four rats were assigned into two groups (group I and II).

Group I was continued on the standard diet for six more weeks and group II was fed with the most effective cholesterol-enriched diet (0.5% cholesterol-enriched) for the
same period as group I. Body weights were recorded once a week and blood samples were collected at day 0 and after each week for the determination of serum total cholesterol. Assay method was the same as that of experiment I (section 3.2.1.2.2).

3.3.2 Effects of Garlic on Chemically Induced Hepatocarcinogenesis in Wistar Rats

3.3.2.1 Experiment III: Effects of garlic on chemically induced hepatocarcinogenesis in normal Wistar rats

3.3.2.1.1 Animal treatments

Thirty six (36) rats were randomly assigned into three groups (group I, II and III) and treated according to the modified method of liver medium-term bioassay protocol as shown in figure 3.3.

Modified Liver Medium-Term Bioassay Protocol

<table>
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<tr>
<th>Weeks</th>
<th>2</th>
<th>4</th>
<th>10</th>
</tr>
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<tbody>
<tr>
<td>Group I</td>
<td>![Group I Diagram]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>![Group II Diagram]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>![Group III Diagram]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Group I Diagram]

- Diethylnitrosamine (DEN) 200mg/kg body wt.(i.p.);
- Partial hepatectomy (PH);
- Sacrifice;
- : Saline;
- Garlic 20mg/kg body wt./day(i.g.);
- : No treatments.

**Figure 3.3:** Assay method. Group I. Garlic alone;
Group II. DEN alone; Group III. DEN + Garlic.
All rats were subjected to PH at week 4. Sacrifice at week 10.
Group I: After two weeks on a standard diet and treatment with a therapeutic dose of garlic (20mg/kg body wt./day), rats in group I were given a single intra peritoneal (i.p.) injection of DEN (200mg/kg body wt) to initiate hepatocarcinogenesis. Garlic treatment was continued for two more weeks and animals were subjected to two-third partial hepatectomy (PH) to maximize any interaction between proliferation and the effects of garlic. Garlic treatment was continued for 6 more weeks. Rats in group II were given DEN and subjected to PH in the same manner as group I. Distilled water was given to this group in place of garlic. Group III was treated similarly to group I except for DEN administration. Saline was administered in place of DEN.

Body weights were recorded at day 0 and after each week. Surviving rats in each group were sacrificed for examination at week 10. At autopsy, liver sections were collected and they were used for immunohistochemical examination of GST-P positive foci and the H & E staining as described in chapter 3 (sections 3.2.2.5 and 3.2.2.6).

3.3.2.2 Experiment IV: Effects of garlic on chemically induced hepatocarcinogenesis in hypercholesterolamic Wistar rats

3.3.2.1.2 Animal treatments
Rats (male, 6 weeks old and Wistar) were fed with the 0.5% cholesterol-enriched diet for a period of two weeks to induce hypercholesterolaemia and rats having serum cholesterol levels > 120mg/dl were considered as hypercholesterolaemic (normal range 76 ± 10, Wayne and Flack, 1989). Twenty four hypercholesterolaemic rats were
randomly assigned into three groups (group I, II, and III) and treated the same as in experiment II, and shown in figure 3.3 (Chapter 3 section 3.2.2).

After 2 weeks of treatment with a therapeutic dose (20mg/Kg body wt.) of garlic rats in group I were given a single i.p. injection of DEN (200mg/Kg body wt./day) to initiate hepatocarcinogenesis. Garlic treatment was continued for 2 more weeks and animals were subjected to two third partial hepatectomy (PH) to promote hepatocarcinogenesis. Garlic treatment was continued for six more weeks. Rats in group II were given DEN and subjected to PH in the same manner as group I, and distilled water was given in place of garlic. Group III was treated similarly to group I except for the administration of DEN. Blood (0.5ml) of blood was collected from each surviving rat for the determination of total serum cholesterol and they were killed for examination at week ten. The livers were examined immunohistochemically for GST-P expression and hematoxyline and eosin stained sections were used for histopathological examination as described in chapter 3 section 3.2.2.5 and 3.2.2.6. All rats were continued on the 0.5% cholesterol-enriched diet throughout the experiment.
4. RESULTS

4.1 Development of a hypercholesterolaemic rat model.

4.1.1 Effects of cholesterol-enriched diets (0.25%, 0.5% and 1%):

4.1.1.1 On the serum cholesterol level

The procedure used for the development of the hypercholesterolaemic rat model is described in section 3.2.1 in chapter 3. The mean serum cholesterol concentration of the test groups (cholesterol-enriched diet) and the control group (normal diet) are shown in table 4.1. It is evident that the concentration of serum cholesterol in the 1% cholesterol-enriched diet fed group is significantly higher (98% increase) than that of the other 2 groups (73% and 46% increase in 0.5% cholesterol-enriched and 0.25% cholesterol-enriched diets respectively) after one week. However, the difference in serum cholesterol concentration between 0.5% and 0.25% cholesterol fed groups (P<0.003) was not as high as between 0.5% and 1% cholesterol-enriched diet groups (P<0.009). At the end of two weeks highest % increase was found in the 1% cholesterol-enriched diet fed group.

4.1.1.2 On average body weights

Mean body weights are reported in table 4.2. Weights of the four groups were not significantly different from each other (P> 0.04).
Table 4.1: Effects of cholesterol-enriched diets containing different amounts of cholesterol on the level of serum cholesterol in Wistar rats.

Cholesterol-enriched diets containing different amount of cholesterol were fed to male Wistar rats for two weeks and blood samples were collected at day 0, day 7 and day 14 for estimation of serum total cholesterol. All diets were isocaloric and containing the same amount of proteins.

Values are expressed as the Mean ± S.E.M. NS-Not significant. a, b, c indicate results significantly different from the corresponding immediate low concentrated cholesterol-enriched diet group $p^a < 0.009$, $p^b < 0.003$, $p^c < 0.02$. 
<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 0</td>
<td>day 7</td>
<td>day 14</td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>218.75</td>
<td>243.52</td>
<td>257.95</td>
<td></td>
</tr>
<tr>
<td>(control)</td>
<td>± 15.81</td>
<td>± 17.75</td>
<td>± 12.75</td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>217.34</td>
<td>250.60</td>
<td>265.80</td>
<td></td>
</tr>
<tr>
<td>(0.25% cholesterol-enriched diet)</td>
<td>± 17.50</td>
<td>± 15.54</td>
<td>± 14.67</td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>221.74</td>
<td>247.63</td>
<td>262.50</td>
<td></td>
</tr>
<tr>
<td>(0.5% cholesterol-enriched diet)</td>
<td>± 12.57</td>
<td>± 19.00</td>
<td>± 17.51</td>
<td></td>
</tr>
<tr>
<td>Group IV</td>
<td>210.54</td>
<td>237.85</td>
<td>249.65</td>
<td></td>
</tr>
<tr>
<td>(1% cholesterol-enriched diet)</td>
<td>± 20.04</td>
<td>± 20.10</td>
<td>± 20.75</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.2**: Effects of cholesterol-enriched diets containing different amounts of cholesterol on mean body weights of Wistar rats.

Each value is the Mean ± S.E.M. When examined by the student's t-test the values obtained from the cholesterol-enriched diets fed groups were not significantly different from the control group. (p>0.04)
4.1.2 Effects of treatment with 0.5% cholesterol-enriched diet for six weeks:

4.1.2.1 On the serum cholesterol level

The effects of 0.5% cholesterol-enriched diet on the serum cholesterol level for six weeks is shown in figure 4.1. Data indicates, that the initial increase is more rapid (figure 4.1) and maximum cholesterol level (around 140mg/dl) was reached in two weeks time.

4.1.2.2 On average body weights

Mean body weights of the 0.5% cholesterol-enriched diet (isocaloric to standard diet) fed group didn't show any significant difference when compared to the control group on the standard diet. Data are reported in table 4.3.
Figure 4.1: Effects of a control diet and 0.5% cholesterol-enriched diet on level of serum total cholesterol in Wistar rats.

Cholesterol-enriched diet was fed for six weeks for one group while the other group was continued on a normal diet for the same period and blood samples were collected at day 0 and after each week for the estimation of serum total cholesterol. Each point is the Mean ± S.E.M.
<table>
<thead>
<tr>
<th></th>
<th>Group I (control)</th>
<th>Group II (0.5% cholesterol-enriched diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 0</td>
<td>251.30 ± 15.85</td>
<td>247.10 ± 14.96</td>
</tr>
<tr>
<td>week 1</td>
<td>280.7 ± 15.70</td>
<td>276.20 ± 16.20</td>
</tr>
<tr>
<td>week 2</td>
<td>295.00 ± 18.67</td>
<td>291.60 ± 15.60</td>
</tr>
<tr>
<td>week 3</td>
<td>305.30 ± 18.52</td>
<td>299.10 ± 16.79</td>
</tr>
<tr>
<td>week 4</td>
<td>322.10 ± 20.99</td>
<td>316.3 ± 17.26</td>
</tr>
<tr>
<td>week 5</td>
<td>336.70 ± 21.04</td>
<td>332.10 ± 20.35</td>
</tr>
<tr>
<td>week 6</td>
<td>343.60 ± 31.07</td>
<td>340.13 ± 14.38</td>
</tr>
</tbody>
</table>

Table 4.3: Effects of 0.5% cholesterol-enriched diets on mean body weight of Wistar rats. Values are expressed as the Mean ± S.E.M. When examined by the student's t-test. The values obtained from the cholesterol-enriched diets fed groups were not significantly different from the control group (p>0.05).
4.2 Effects of garlic on DEN-induced hepatocarcinogenesis in normal Wistar rats

4.2.1 Inhibition of glutathione S-transferase placental form positive (GST-P⁺) foci.

The effects of garlic on DEN-induced liver GST-P⁺ positive foci in normal Wistar rats are summarized in table 4.4. It is evident that daily administration of a therapeutic dose of garlic markedly reduces the number and area of GST-P positive liver foci (48% reduction (P<0.0006) and 49% reduction (P<0.0005) respectively). This indicates that there is an inhibitory effect of garlic on DEN-induced hepatocarcinogenesis in normal Wistar rats. No GST-P⁺ foci were detected in the control group which was not treated with carcinogen, but treated only with garlic (group III). Microphotographs showing immunohistochemical staining of GSTP⁺ foci of garlic treated group (A₄₁) and DEN control (B₄₁) are given in plate 4.1.
<table>
<thead>
<tr>
<th></th>
<th>Number of GST-P⁺ foci / cm² of liver section</th>
<th>Area of GST-P⁺ foci (mm²) / cm² of liver section</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>(Drug control / Garlic treated, No DEN)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>32.53 ± 9.44</td>
<td>0.89 ± 0.21</td>
</tr>
<tr>
<td>(Carcinogen control / DEN treated, No Garlic)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>16.12 ± 4.62*</td>
<td>0.46 ± 0.15**</td>
</tr>
<tr>
<td>(Garlic treated / DEN treated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Reduction of induction of foci</td>
<td>48%</td>
<td>49%</td>
</tr>
</tbody>
</table>

**Table 4.4:** Effects of Garlic on GST-P⁺ liver foci in DEN-induced hepatocarcinogenesis in normal (normo-cholesterolaemic) Wistar rats.

Group I: Treated with garlic (20mg/Kg body weight/day) throughout the experiment.  
Group II: Given single dose of DEN (200mg/Kg body weight) at week 2.  
Group III: Given single dose of DEN (200mg/Kg body weight) at week 2. Garlic treatment was started 2 weeks before giving DEN and continued throughout the experiment.  
All rats were subjected to partial hepatectomy at week 4 and sacrificed at week 10. GST-P foci were demonstrated by Immunohistochemical method (ABC method) and slides were examined under research microscope.  
Values are expressed as the Mean ± S.E.M. Results statistically different from DEN control are indicated by asterisks. p* < 0.0006, p** < 0.0005, N.D.-Not detected.
Plate 4.1: Immunohistochemical staining of DEN-induced glutathione S-transferase placental form positive (GST-P') foci (hematoxylin counterstain) in normal Wistar rats (magnification *200).
4.2.2 Histopathological evidence

Representative microphotographs showing histopathological changes and data of granular and vacuolar degeneration of liver tissue sections are shown in plate 4.2 (Fig. A₄.2-C₄.2) and table 4.5 respectively.

The livers of garlic control (no DEN) group of rats showed the typical mammalian liver architecture (Fig. C₄.2). Further microscopic examination revealed that central vein and portal tracts are normal. Limiting plates are intact and most of the hepatocytes are normal but occasional foci have shown very mild degree of granular degeneration. No other obvious pathological effects seen. Fig. A₄.2 shows the liver section of carcinogen control (DEN only/no garlic) group of rats. In that almost all hepatocytes show severe and diffused granular degeneration and they are swollen because the sinusoids are compressed. Nuclei are enlarged, open and vesicular with prominent nucleoli. However central vein and portal tracts are normal, no cellular infiltration also limiting plates are intact. Liver architecture of group III (DEN+ garlic treated) is shown in Fig. B₄.2. Some of the hepatocytes show very minimal granular degeneration while some show moderate degree of degeneration. Degree of degeneration or alterations in normal liver architecture is much less when compared to carcinogen only group. Central vein and portal tracts are normal. Limiting plates are intact.

4.2.3 Body weights

Mean body weights taken at four stages (initial, after DEN, after pH and final) are presented in table 4.6. No significant inter group variation was observed.
Plate 4.2: Representative microphotographs showing the effects of garlic treatment on histopathology of liver tissues of normal Wistar rats (magnification *200).
Plate 4.2 continued...

C4.2 - Garlic control (Group I/No DEN)
Vacuolation of hepatocytes
(Number of slides with vacuolation / total number of slides per group)

<table>
<thead>
<tr>
<th></th>
<th>Strong</th>
<th>Moderate</th>
<th>Week</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>0</td>
<td>0</td>
<td>6/30</td>
<td>24/30</td>
</tr>
<tr>
<td>(Garlic control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>27/33</td>
<td>6/33</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(DEN control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>0</td>
<td>27/33</td>
<td>9/33</td>
<td>0</td>
</tr>
<tr>
<td>(Garlic + DEN)</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Table 4.5.1: Effects of garlic on vacuolation of hepatocytes.

Granularity of hepatocytes
(Number of slides with granulation / total number of slides per group)

<table>
<thead>
<tr>
<th></th>
<th>Strong</th>
<th>Moderate</th>
<th>Week</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>0</td>
<td>0</td>
<td>6/30</td>
<td>24/30</td>
</tr>
<tr>
<td>(Garlic control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>30/33</td>
<td>3/33</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(DEN control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>0</td>
<td>27/33</td>
<td>6/33</td>
<td>0</td>
</tr>
<tr>
<td>(Garlic + DEN)</td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>

Table 4.5.2: Effects of garlic on Granulation of hepatocytes

Table 4.5 (4.5.1 & 4.5.2): Effects of Garlic treatment on the histopathalogy of livers of normal (normo-cholesterolaeemic) Wistar rats.

Group I: Treated with garlic (20mg/Kg body weight/day) throughout the experiment.
Group II: Given single dose of DEN (200mg/Kg body weight) at week 2.
Group III: Given single dose of DEN (200mg/Kg body weight) at week 2.

Garlic treatment was started 2 weeks before giving DEN and continued throughout the experiment. All rats were subjected to partial hepatectomy at week 4 and sacrificed at week 10. H & E stained sections were examined under light microscope.
<table>
<thead>
<tr>
<th></th>
<th>Group I (Garlic control)</th>
<th>Group II (DEN control)</th>
<th>Group III (Garlic + DEN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>101.50 ± 15.85</td>
<td>103.91 ± 13.26</td>
<td>105.33 ± 11.16</td>
</tr>
<tr>
<td>After DEN</td>
<td>189.16 ± 12.44</td>
<td>186.00 ± 11.00</td>
<td>189.33 ± 17.33</td>
</tr>
<tr>
<td>After PH</td>
<td>253.60 ± 24.08</td>
<td>238.54 ± 13.58</td>
<td>238.54 ± 21.48</td>
</tr>
<tr>
<td>Final</td>
<td>330.4 ± 22.40</td>
<td>316.90 ± 20.82</td>
<td>315.81 ± 19.83</td>
</tr>
</tbody>
</table>

**Table 4.6:** Effects of garlic on mean body weight of normal Wistar rats in DEN-induced hepatocarcinogenesis.

Values are expressed as the Mean ± S.E.M. When examined by the student's t-test the values obtained from the garlic treated group were not significantly different from the control (DEN only) group throughout the experiment (p > 0.05).

DEN: Diethylnitrosamine

PH: Partial hepatectomy
4.3 Effects of garlic on DEN induced hepatocarcinogenesis in hypercholesterolaemic Wistar rats:

4.3.1 Inhibition of GST-P$^+$ foci induction

The number and area of DEN-induced liver GST-P$^+$ Foci in hypercholesterolaemic Wistar rats are shown in table 4.7. Garlic treatment markedly reduces the number and area of DEN-induced liver GST-P$^+$ perneoplastic foci in hypercholesterolaemic Wistar rats (34% reduction in number of GST-P$^+$ foci (P<0.0008) and 44% reduction in area of GST-P$^+$ foci(P<0.002)). It is evident that the garlic treatment significantly inhibits the carcinogenesis of DEN in hypercholesterlaemic Wistar rats. GST-P$^+$ foci given positive immunohistochemical staining are presented in plate 4.3.
<table>
<thead>
<tr>
<th>Group</th>
<th>Number of GST-P&lt;sup&gt;+&lt;/sup&gt; foci / cm&lt;sup&gt;2&lt;/sup&gt; of liver section</th>
<th>Area of GST-P&lt;sup&gt;+&lt;/sup&gt; foci (mm&lt;sup&gt;2&lt;/sup&gt;) / cm&lt;sup&gt;2&lt;/sup&gt; of liver section</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Drug control / Garlic treated, No DEN)</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>II (Carcinogen control / DEN treated, No Garlic)</td>
<td>58.39 ± 5.25</td>
<td>1.67 ± 0.14</td>
</tr>
<tr>
<td>III (Garlic treated / DEN treated)</td>
<td>37.46 ± 5.73&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.86 ± 0.21&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

% Reduction of induction of foci

34% 44%

Table 4.7: Effects of Garlic on GST-P<sup>+</sup> liver foci in DEN-induced hepatocarcinogenesis in hypercholesterolaemic Wistar rats.

All rats were made hypercholesterolaemic by feeding with 0.5% cholesterol-enriched diet for two weeks and same diet was continued throughout the experiment.

Group I: Treated with garlic (20mg/Kg body weight/day) throughout the experiment.
Group II: Given single dose of DEN (200mg/Kg body weight) at week 2.
Group III: Given single dose of DEN (200mg/Kg body weight) at week 2. Garlic treatment was started 2 weeks before giving DEN and continued throughout the experiment.

All rats were subjected to partial hepatectomy at week 4 and sacrificed at week 10. GST-P foci were demonstrated by Immunohistohemical method (ABC method) and slides were examined under research microscope.

Values are expressed as the Mean ± S.E.M. Results statistically different from DEN control are indicated by asterisks. p < 0.002, p < 0.0008, N.D.-Not Detected.
Plate 4.3: Immunohistochemical staining of DEN-induced glutathione S-transferase placental form positive (GST-P') foci (heamatoxylin counterstain) in hypercholesterolaemic Wistar rats (magnification*200).
4.3.1 Histopathological evidence

Summarized data for two histopathological parameters, granularity and vacuolation are shown in table 4.8.1 and table 4.8.2. Representative microphotographs showing histopathological changes are shown in plate 4.4 (A₄₄₄ - C₄₄₄).

Representative liver section of the garlic control group of rats which are hypercholesterolaemic is shown in C₄₄₄. In most of the hepatocytes there is mild degree of microvesicular fatty changes but other pathological features such as granularity, swollen nuclear enlargement etc remain normal. In hepatocytes of the carcinogen control, (A₄₄₄) almost all hepatocytes show severe degree of microvesicular fatty changes with occasional macrovesicular fatty degeneration. Nuclear enlargement with prominent nucleoli are also evident. Portal tracts and central vein still remain normal. B₄₄₄ shows the liver architecture of group III (DEN+ garlic treated). As reported in table 4.8.1 and 4.8.2 only a certain percentage show microvesicular fatty changes (classified as moderate) which is of markedly low degree compared to livers of carcinogen control group of rats.
Vacuolation of hepatocytes
(Number of slides with vacuolation / total number of slides per group)

<table>
<thead>
<tr>
<th>Group</th>
<th>Strong</th>
<th>Moderate</th>
<th>Week</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (Garlic control)</td>
<td>0</td>
<td>0</td>
<td>9/24</td>
<td>15/24</td>
</tr>
<tr>
<td>Group II (DEN control)</td>
<td>24/24</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group III (Garlic + DEN)</td>
<td>3/24</td>
<td>21/24</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.8.1: Effects of garlic on vacuolation of hepatocytes.

Granularity of hepatocytes
(Number of slides with granulation / total number of slides per group)

<table>
<thead>
<tr>
<th>Group</th>
<th>Strong</th>
<th>Moderate</th>
<th>Week</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group IB (Garlic control)</td>
<td>0</td>
<td>0</td>
<td>9/24</td>
<td>15/24</td>
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<tr>
<td>Group II (DEN control)</td>
<td>24/24</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group III (Garlic + DEN)</td>
<td>3/24</td>
<td>21/24</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.8.2: Effects of garlic on Granulation of hepatocytes

Table 4.8 (4.8.1 & 4.8.2): Effects of Garlic treatment on the histopathalogy of livers of hypercholesterolaemic Wistar rats.

All rats were made hypercholesterolaemic by feeding with 05% cholesterol-enriched diet for two weeks and same diet was continued throughout the experiment.

Group I: Treated with garlic (20mg/ Kg body weight /day) throughout the Experiment.

Group II: Given single dose of DEN (200mg/Kg body weight) at week 2.

Group III: Given single dose of DEN (200mg/Kg body weight) at week 2.

Garlic treatment was started 2 weeks before giving DEN and continued throughout the experiment. All rats were subjected to partial hepatectomy at week 4 and sacrificed at week 10.

H & E stained sections were examined under light microscope.
Plate 4.4: Representative microphotographs showing the effects of garlic treatment on histopathology of liver tissues of hypercholesterolaemic Wistar rats (magnification ×200).
Plate 4.4 continued...

C_{4,4} - Garlic control (Group I/No DEN)
4.3.3 Body weights

Data for mean body weight are given in 4.9. There was no significant difference between these groups at any stage of the experiment.

4.3.4 Level of mean total serum cholesterol

Values for initial and final cholesterol levels are summarized in table 4.10. Treatment with therapeutic doses of garlic (20mg/kg body weight/day) for 10 weeks didn't show a significant difference in cholesterol level in group II (DEN + garlic) when compared to DEN control group (No garlic/group II).
Table 4.9: Effects of garlic on mean body weight of hypercholesterolaemic Wistar rats in DEN-induced hepatocarcinogenesis.

Values are expressed as the Mean ± S.E.M. When examined by the student's t-test values obtained from the garlic treated group were not significantly different from the control (DEN only) group throughout the experiment (p > 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Group I (Garlic control)</th>
<th>Group II (DEN control)</th>
<th>Group III (Garlic + DEN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>157.00 ± 8.50</td>
<td>156.37 ± 7.37</td>
<td>159.37 ± 6.48</td>
</tr>
<tr>
<td>After DEN</td>
<td>234.25 ± 10.87</td>
<td>216.37 ± 8.87</td>
<td>214.25 ± 7.31</td>
</tr>
<tr>
<td>After PH</td>
<td>237.75 ± 13.62</td>
<td>228.42 ± 10.01</td>
<td>233.00 ± 10.25</td>
</tr>
<tr>
<td>Final</td>
<td>308.87 ± 18.08</td>
<td>304.14 ± 10.97</td>
<td>308.62 ± 10.78</td>
</tr>
<tr>
<td>Group</td>
<td>Initial</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>---------------</td>
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<td></td>
</tr>
<tr>
<td>I (Garlic)</td>
<td>138.65 ± 2.31</td>
<td>146.73 ± 6.16</td>
<td></td>
</tr>
<tr>
<td>(Garlic treated, No DEN)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II (DEN)</td>
<td>140.37 ± 9.44</td>
<td>151.89 ± 2.80</td>
<td></td>
</tr>
<tr>
<td>(DEN treated, No Garlic)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III (Garlic + DEN)</td>
<td>141.12 ± 5.66</td>
<td>145.46 ± 7.28</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.10**: Effects of garlic on total serum cholesterol in hypercholesterolaemic Wistar rats in DEN-induced hepatocarcinogenesis.

Values are expressed as the Mean ± S.E.M. When examined by the student's t-test the values obtained from garlic treated group (with DEN) were not significantly different from DEN control as well as garlic control (p>0.05).
4.4 Comparison of GST-P+ foci induction in normal and hypercholesterolaemic Wistar rats.

4.4.1 Number and area of GST-P+ foci induction

Data were obtained from two separate experiments (Experiment I: Effect of garlic on DEN induced hepatocarcinogenesis in normal rats and Experiment II: Effect of garlic on DEN induced hepatocarcinogenesis in hypercholesterolaemic rats) carried out under identical conditions were used in this study. It is clearly shown in results (table 4.7) that number of GST-P+ foci (for Garlic treated group with DEN 37.46±5.73 and DEN alone group 58.39±5.25) and area (for Garlic treated group with DEN 0.87±0.21 and DEN alone group 1.67±0.14) are two fold higher in DEN administered hypercholesterolaemic groups when compared to the normal rats. These are clearly shown in figure 4.2 and figure 4.3 respectively. This increase in Marker enzyme expression is attributed to the hypercholesterolaemic condition.
Figure 4.2: Effects of Cholesterol-enriched diet on number of DEN-induced liver GST-P+ foci in Wistar rats.

Assay Method: For all rats, DEN was given at week 2. Rats were subjected to PH at week 4 and sacrificed at week 10 and liver sections were collected at autopsies. Cholesterolaemic diet group was fed on 0.5% cholesterol-enriched diet, started two weeks before DEN administration and continued throughout the experiment while normal diet group was continued on standard diet.

*Note: Data shown in figure 4.2 & 4.3 are taken from two experiments, experiment III (Effects of garlic on hepatocarcinogenesis in normal Wistar rats) and experiment IV (Effects of garlic on hepatocarcinogenesis in hypercholesterolaemic Wistar rats). Except for the diet all other conditions were identical for both experiments.
Figure 4.3: Effects of Cholesterol-enriched diet on area of DEN-induced liver GST-P⁺ foci in Wistar rats.

Assay Method: For all rats, DEN was given at week 2. Rats were subjected to PH at week 4 and sacrificed at week 10 and liver sections were collected at autopsies. Cholesterolaemic diet group was fed on 0.5% cholesterol-enriched diet, started two weeks before DEN administration and continued throughout the experiment while normal diet group was continued on standard diet.
5. DISCUSSION

Development of a hypercholesterolaemic rat model

Serum cholesterol concentration of rats can be increased by dietary supplementation of cholesterol (Xizhong et al., 1990). Data shown in table 4.1 clearly indicates that, the feeding of cholesterol-enriched diets increases the level of cholesterol in Wistar rats. Also it was indicated that the percentage increase in the serum concentration of cholesterol depends on the amount of cholesterol supplied by the diet.

Many reported studies on making hypercholesterolaemic models have used different types of diets containing several ingredients/ components in addition to ingredients present in the normal diet. For example, in many previous studies investigators have used high concentration of cholesterol or cholesterol plus sucrose, sodium cholate, pork fat etc. to induce hypercholesterolaemia. (Farias et al., 1996; Vasquez-Freire et al., 1996). In this study the purpose of developing a hypercholesterolaemic model was to use it for studies on chemically induced carcinogenesis. Therefore it was decided to minimize chemical ingredients used to make the hypercholesterolaemic model. Only powdered cholesterol was used to make the model.

Serum cholesterol $>120\text{mg/dl}$ was considered as hypercholesterolaemic. Since increase in level of serum cholesterol is adequate and also % increase is maximum between 0.25% and 0.5% cholesterol-enriched diet Groups. 0.5% cholesterol-enriched diet was selected for our investigations on the effects of garlic on chemical carcinogenesis.
Initial increase is more rapid with 0.5% cholesterol-enriched diet (figure 4.1) and maximum cholesterol level (around 140mg/dl) was reached in two weeks time. Therefore rats were treated with 0.5% cholesterol-enriched diet for two weeks to make them hypercholesterolaemic and same diet was continued throughout the experiment.

Effects of Garlic on DEN-induced hepatocarcinogenesis in normal Wistar rats

In this experiment medium-term bioassay protocol, which involves GST-P form as an enzyme marker was used as the assay system, since it has been shown to be a specific assay system for preneoplastic lesions which occur during the early phase of hepatocarcinogenesis. (Ito et al., 1982; 1988; Ricardo et al., 1991) This system has been initially developed to replace the long term liver bioassay protocol, that has been used for the prediction of carcinogenic potential of environmental carcinogens in rodents with relation to humans (IARC, 1980). Later on, it has been proven that this system also possesses the advantage of allowing detection of inhibitory agents in hepatocarcinogenesis. (Ito et al., 1988, 1989; Tadashi et al., 1990; Nobuyasu et al., 1997).

GST-P, appears in rapidly growing preneoplastic lesions (Suzanne, 1987). The sequential development of hepatocellular carcinoma from preneoplastic lesions and therefore reliability of preneoplastic enzyme altered liver foci as indicators of carcinogenicity has been well established (Ito et al., 1982). Since these GST-P foci are preneoplastic and induced specifically during the early phase of chemically induced hepatocarcinogenesis, it is a valid marker of liver carcinogenesis (Ricardo, 1991;
Morimura et al., 1993; Muramatsu et al., 1992). Hence GST-P is a very useful marker in the quantitative assessment of initiation and promotion potential of chemical carcinogens and modifiers in hepatocarcinogenesis (Farber, 1980; Pitot and Campbell, 1987).

In this study inhibitory effects of garlic on chemically-induced hepatocarcinogenesis is reflected by the reduction in GST-P expression in both parameters, namely the number and the area (table 4.4). Results of the histopathological examination of the livers (table 4.5 & plate 4.2) indicate that there are only moderate changes in liver normal architecture in garlic treated rats (with DEN) whereas the untreated group (DEN only) showed major changes. The above findings are in good agreement with an earlier study on inhibitory effects of some organosulfur compounds (OSC) of garlic on chemically induced hepatocarcinogenesis in Wistar rats by Takada et al. and Matsuda et al. (1997; 1994) (section 2.1.3.12 in chapter 2).

Takahashi et al. (1992) have also reported that there is a proportional correlation between ODC/SAT (Onithine decarboxylase / Spermidine / Spermine N1-acetyltransferase) activities and induction of GST-P positive foci, which are generally considered as preneoplastic lesions in hepatocarcinogenesis. It has also been demonstrated that organosulfur compounds (OSC) from garlic prevented elevation of ODC when administered before initiation of carcinogenesis (Takada et al., 1997). Since ODC and SAT are biomarkers of cell proliferation, garlic can exert its inhibitory effects on hepatocarcinogenesis by OSCs such as S-methylcystein (SMC), methyl propyl disulfide etc. The results of this study also provide supportive evidence for the findings
of previous investigations of anti-carcinogenic activity of garlic and organosulfur compounds of garlic on several other cancer models. These cancers include stomach, mammary, lung etc. as detailed in chapter 2, section 2.1.3.12. (Mei et al., 1982; Liu et al., 1992; Sumiyopshi and Wagowich, 1990; Tong, 1992; Wargowich et al., 1985).

Takada et al. (1994) and Thakahashi et al. (1992) have reported that some organosulfur compounds of garlic enhance chemically-induced hepatocarcinogenesis in rats. However it should be mentioned that in those studies, purified organosulfur compounds in extremely high doses (200 or 100 mg/kg body wt.) have been employed. Such serum concentrations will never be reached under physiological conditions due to garlic therapy. The normally recommended therapeutic dose is 20mg of powdered garlic / kg body wt./day, which is comparable to the dosage prescribed by physicians for an adult (900mg/day) (Silagy and Neil, 1994).

Since it was thought that pretreatment may be important for protection against chemical carcinogenesis, rats were pretreated with garlic for 2 weeks before initiating carcinogenesis by DEN and treatment was continued throughout the experiment (figure 3.2). Except for the day that DEN was administered, all rats were active, no effect was seen on relative body weights throughout the experiment and normal liver architecture was evident in garlic control (no DEN/ plate C42). Accordingly, the results indicate that there is no toxic effect due to daily treatment of garlic in this study.

The exact mechanism(s) underlying this inhibitory effect of garlic on hepatocarcinogenesis is not known and a wide range of mechanisms may be responsible for these effects (Matsuda et al., 1994).
One possibility is that garlic may be involved in detoxification of the carcinogen. There are some indications that garlic alters the activity of drug metabolizing enzymes and inhibit the initiation of carcinogenesis. thereby modulating the formation of enzyme altered liver foci. These foci are considered as initial cell population for hepatocellular carcinoma. Detoxification systems such as GSTs can minimize carcinogenicity by phase II conjugation reaction which add functional group(s) to the carcinogen, thereby lowering the biological activity and increasin their excreatability (Sparnins et al., 1982)

As suggested by Marsh et al (1987) immune-stimulating properties may also be responsible for the therapeutic effects of garlic on chemically-induced hepatocarcinopgenesis.

Administration of garlic has been found to reduce lipid peroxidation of the liver (Imai, 1993). This action has been attributed to the anti-oxidant properties as well as free radical scavenging properties of garlic and its OSCs.

It appears that garlic may exert its protective effects in three ways.

1. a direct inhibition of tumor cell metabolism. This is useful only when the tumor size or number of cells is small.

2. Inhibition of the initiation and/or promotion phases of carcinogenesis.

3. Modulating the host immune response (useful in all phases of cancer control) (Benjamin et al., 1990)
Effects of garlic on DEN-induced hepatocarcinogenesis in hypercholesterolaemic Wistar rats.

It is evident from the results of this study that garlic treatment significantly inhibits the carcinogenicity of DEN in hypercholesterolaemic Wistar rats. Results obtained on the immunohistochemical expression of GST-P foci as well as histopathological examination (table 4.7, table 4.8, plate 4.3 and plate 4.4) agree very well. However the % inhibition in both parameters (number and area of GST-P foci) is lower in hypercholesterolaemic Wistar rats (34% and 44% respectively), compared to normal rats. Another important finding from this study is that in the hypercholesterolaemic group of rats there is a marked increase in GST-P foci expression in the control group receiving only DEN. Nearly two fold higher expression of GST-P foci is seen in DEN administered hypercholesterolaemic group compared to the normal rats (figure 4.2 and figure 4.3). These markedly increased values in marker enzyme expression might be attributed to the effects of hypercholesterolaemia.

Preneoplastic foci were not seen in the drug control (garlic only/ no DEN) group in hypercholesterolaemic rats. Therefore it can be clearly stated that dietary cholesterol itself doesn’t initiate carcinogenesis.

This is the first reported study on the effects of dietary cholesterol on hepatocarcinogenesis and the effects of garlic on hepatocarcinogenesis in hypercholesterolaemic subjects.
There is a strong possibility that dietary cholesterol enhances hepatocarcinogenesis. Literature indicates that, increasing the fat content of the diet enhances the development of neoplasms induced by different carcinogens such as 2-acetylamino-fluorene (2-AAF), P-dimethylaminoazobenzene (DAB), diethylnitrosamine (DEN) (Sugai et al., 1962; Howard et al., 1991).

The effects of dietary fat on hepatocarcinogenesis could be explained by different mechanisms. Increasing the fat content of the diet increases cytochrome p-450 and related activities which is important for carcinogen activation (Wade et al., 1978; Rutten and Flake, 1987).

Caloric effects coming from consumption of high fat diet also may be a contributory factor for the enhancing effects of carcinogenesis in hypercholesterolaemic rats. As suggested by Kritchevsky et al. the enhancement of carcinogenesis by dietary fat may be caused at least in part by high caloric intake (1987). Furthermore caloric restriction has been found to inhibit carcinogenesis in many tissues in experimental animals (Boissonneault, 1991). High caloric consumption may have effects on several factors including growth factors, immunity, neuroendocrine functions, and metabolic regulation. Any of the above effects could lead to the alteration in gene expression (Boissonneault, 1991) thereby effecting carcinogenesis.

The decreased inhibition of DEN-induced hepatocarcinogenesis by garlic in hypercholesterolaemic Wistar rats may be attributed to the hypercholesterolaemic condition.
Detoxification enzymes such as GSTs act as transporters for non-substrate molecules such as cholesterol, heme, bilirubin etc. (Mannervik et al., 1985) thereby decreasing their detoxification activity. Hypercholesterolaemic animals therefore develop more preneoplastic positive foci, which has been observed in this study.

It is well known that environmental factors are responsible for the development of a majority of human cancers. Human diet contains a large number of precarcinogens (Ames, 1983) apart from carcinogens. Our diet also contains a variety of compounds which inhibit mutagenesis and/or carcinogenesis in laboratory animal models (Ames, 1983; Carr, 1985; Hayatsu, 1988). Therefore naturally occurring anticarcinogens can be included in the human diet to prevent the incidence of cancer. Results of this study supports the view that garlic is one such chemopreventive agent and regular incorporation of garlic into the daily diet may be important for cancer prevention. Findings of this study also suggest that the use of garlic for treatment of hypercholesterolaemia has an additional benefit of reducing the risk of developing cancer.
6 CONCLUSIONS

(1) Daily treatment of a therapeutic dose of garlic (20mg/kg body weight/day) inhibits DEN-induced hepatocarcinogenesis in normal and hypercholesterolaemic male Wistar rats. Higher % inhibition on carcinogenesis was found among normal rats as compared with hypercholesterolaemic rats. Results of this study provide strong supportive evidence for the anti-carcinogenic activity of garlic.

(2) Hypercholesterolaemic rats are more susceptible to chemical hepatocarcinogenesis induced by DEN. Though cholesterol is not an initiator of carcinogenesis by itself it possibly enhances the deleterious effects of other carcinogens.
7 SUGGESTIONS FOR FURTHER STUDIES.

(1) Since clinical trials are not feasible due to ethical reasons, other animal models can be used to confirm the findings of this study.

(2) Effects of garlic on hepatocarcinogenic models induced by other carcinogens can be suggested for further studies.

(3) Effects of garlic on other carcinogenic models including lung, mammary, skin etc can be studied.

(4) Further studies are needed to investigate the exact mechanism of inhibition.
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