CARCINOGENESIS RESPONSE MODULATION BY GELONIN ENCAPSULATED IN LIPOSOME

By

K. S. Nakhuru
Department of Biochemistry

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Dedicated

To

My Beloved Mom, Dad & Teachers

THE NORTH-EASTERN HILL UNIVERSITY

Shillong, India

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I, **K. S. Nakhuru**, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University or Institute.

This is being submitted to the North-Eastern Hill University for the degree of Doctor of Philosophy in Biochemistry.

(K. S. Nakhuru)

PROF. ANIS ALAM
Supervisor,
Department of Biochemistry

PROF. R. LALTHANTLUANGA 27/9/2006
Head, Deptt. Biochemistry
NEHU-Shillong-22
Department of Biochemistry

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ABBREVIATIONS

RIPs	Ribosome inactivating proteins
CMC	Carboxymethyl cellulose
DNA	Deoxyribonucleic acid
BMV	Brome mosaic virus
GGT	γ- Glutamyl transpeptidase
AChE	Acetylcholine esterase
GST	Glutathione-S-transferase
GSH	Glutathione (reduced)
BrdU	5-Bromo-2'-deoxyuridine
PBS	Phosphate Bufferred Saline
DTNB	5, 5'-dithiobis-(2-nitrobenzoic acid)
CDNB	1-chloro-2, 4-dinitrobenzene
EDTA	Ethylene diamine tetra-acetic acid
TCA	Trichloroacetic acid
SDS	Sodium Dodecyl Sulfate
DMEM	Dulbecco's Modified Eagle Medium
FCS	Fetal Calf Serum
POD	Peroxidase
DPX	Diesterene Plasticizer Xylene
DBN	N-dibutyl nitrosoamine
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEMED	N, N, N', N' -tetramethylethylenediamine
APS	Ammonium Persulfate
ELISA	Enzyme Linked Immunosorbent Assay
DTT	Dithiothretol
PE	Phosphatidyl ethanolamine
DPPC	Dipalmitoyl phosphatidylcholine
DCP	Dicetyl phosphate
PG	Phosphatidyl glycerol
Chol	Cholesterol

CHAPTER -1

INTRODUCTION

1.0.0 INTRODUCTION

Cancer is an ancient disease and has been known since human societies first learnt to record their activities. Although diseases of the heart and blood vessels are still the main cause of death in our ageing population, cancer is a major problem. Nearly everyone's life has been directly or indirectly affected by cancer. Therefore, its control or even better prevention by making use of improved systemic cancer therapies is urgently required.

Most scientists involved in cancer research believe that many cancer cases may be associated with the environment in which we live and work. In this context, the environment is anything that people interact with including lifestyle choices such as what we eat, drink, or smoke, natural and medical radiation, including exposure to sunlight, workplace exposures, drugs, socioeconomic factors that effect exposures and susceptibility and substances in the air, water and soil (Office of Technology and Assessment, 1981). Other factors that play a major role in cancer development are infectious diseases, aging and individual susceptibility such as genetic predisposition. We rarely know what environment factors and conditions are responsible for the onset and development, especially for cancers related to certain occupational exposures or the use of specific drugs.

Many experts firmly believe that much of the cancer associated with the environment may be avoided (Tomatis, L. *et al,* 1997). By changing our habits,

therefore, we should, in principle, be able to reduce drastically our chance of developing almost any given type of cancer. This is demonstrated most clearly by a comparison of cancer incidence in different countries; for almost every cancer that is common in a country, there is another country where the incidence is several times lower; and migrant population tend to take on the pattern of cancer incidence typical of the host country, implying that the differences are due to the environmental, not genetic factors. From such data it is estimated that 80-90% of cancer should be avoidable. Unfortunately, different cancers have different environmental risk factors, and a country that happens to escape on such danger is no more likely than other countries to escape the rest; thus the incidence of all cancers combined (among individuals of a given age) is similar from country to country. There are, however, some subgroups whose abstinent way of life does seem to reduce the total cancer death rate: the incidence of cancer among strict mormous in Utah, for example, is only about half that among Americans in general.

While such epidemiological observations indicate that cancer can be avoided, it remains difficult to identify the specific environmental risk factors to establish how they act. Some certainly operate as mutagenic tumour initiators, directly provoking genetic changes; others presumably serve as tumour promoters that help to enlarge the population of cells liable to progress, through further mutation, to full-blown cancer. The carcinogen in tobacco smoke, like the aflatoxin on tropical peanuts, probably belong mostly in the first category, while the reproductive hormones that curculates in a woman's body at different stages of her life may belong in the second category. The

importance of these hormones is indicated by the strictly correlations that exist between a woman's reproductive history and her risks of developing breast cancer; the hormones presumably affect cancer incidence through their influence on cell proliferation in the breast. It is possible that some factors act in still other ways, for example, by causing heritable epigenetic changes. Of course, it is not necessary to understand how cancer causing agents act in order to identify them and show how to avoid them. In this task cancer epidemiology has had some notable success and promises more to come. Simply by revealing the role of smoking, it has shown a way to reduce the total cancer death rate in North America and Europe by as much as 30%. The prevention of cancer is not only better than cure but seems also, given our present state of knowledge to be much more readily attainable.

Normal body cells grow, divided and die under control mechanisms. In early years of one's life, normal cells grow and divide more rapidly until an adult is attained and then body cells divide only to replace worn-out or dying cells and to repair the injuries. Cancerous cells result when a normal cell escaped normal growth-regulating mechanisms and continue to grow and divide thereby giving rise to clones of cells that can be expanded to a considerable size producing tumor.

A tumor, which does not invade the neighbouring tissue or spread to other part of the body, with very rare exceptions, are not life threatening, is benign. Malignant is one that started in one part of the body gets into blood stream or lymph vessels and spread and invades other parts of the body where they may replicate and result in the death of the patient. The term cancer refers to the full-blown malignant tumour.

Malignant tumors are broadly classified according to the embryonic origin of the tissue from which the tumour is derived. Malignant neoplasms arising from endodermal or ectodermal are called carcinomas, which constitute the major form. Malignant neoplasms that originate from connective tissue, muscle, cartilage, fats or bone are sarcomas. Leukemias and lymphomas are malignant tumours of hematopoitic cells of bone marrow.

After a quarter century of rapid advances, cancer research has generated a rich and complex body of knowledge, revealing cancer to be a disease involving dynamic changes in the genome. The foundation has been set in the discovery of mutations that produce oncogenes with dominant gain of function and tumor suppressor genes with recessive loss of function; both classes of cancer genes have been identified through their alteration in human and animal cancer cells and by their elicitation of cancer phenotypes in experimental models (Bishop, J. M. and Weinberg, R. A., 1996). The changes involved in the structure and function of cellular DNA by one of the following mechanisms: (a) direct interaction with DNA or RNA, which results in changes that are inheritable from cell line to cell line, (b) interference with enzymes that control DNA repair, replication, or transcription and, (c) interference with normal control mechanisms such as the histone that may contribute to DNA replication/synthesis.

Oncogenes; capable of causing cancer arise from the mutation of proto-oncogenes. 'Cancer genes' are component of the normal cellular genome whose activity is unleashed or augmented by carcinogens of various kinds and is then responsible for sustaining the undisciplined behaviour of cancer cells. Thus cancer genes are not alien

intruders but normal, indeed essential genes run amok; the damage done by a carcinogen turns friend to foe, perhaps by acting directly on cancer gene or perhaps by crippling a second gene that normally polices the activity of the cancer gene (Bishop, J. M. 1982).

1.2.0 CARCINOGENESIS

Carcinogenesis initiates and also can be induced experimentally by well characterized biological, chemical and physical agents (carcinogens). Some of the known carcinogenic agents are also natural causes of cancer in man and animals. Chemical carcinogenesis by tobacco smoke products is a major cause of common lung cancers. Physical carcinogenesis by ionizing radiations poses a potential world-wide threat in this nuclear age. Skin carcinogenesis by solar ultraviolet radiation is expected to increase even above its present high incidence as the ozone layer of the atmosphere undergoes depletion. Nevertheless, the specific causes of most common human cancers of breast, colon, rectum, lymph nodes, uterus, bladder, pancreas, bone marrow, stomach, and so on- remain unknown. Most types of cancer are difficult to eradicate and some, like hepatocellular carcinoma, are almost always fatal.

1.2.1 CHEMICAL CARCINOGENESIS

The discovery of chemical carcinogenesis was made by Sir Percival Pott (1713-1788), an English surgeon, who related the cause of scrotal skin cancer in a

number of his patients to a common history of occupational exposure to a large amount of coal soot as chimney sweeper when they were boys. Chemical carcinogens include a high diverse collection of chemicals substances both organic and inorganic chemicals, solid state materials, hormones, and immunosupressants (Miller, E. C. *et al*, 1981). Chemical carcinogens of synthetic (man made) or natural origin are extremely diverse in structure without any common feature, and are classified into two categories: genotoxic and non-genotoxic.

Direct-acting or DNA-reactive, activation independent carcinogens that bind covalently to cellular genomic DNA is genotoxic and are mutagens. However, there are other carcinogens that required prior metabolism (activation dependent) to become carcinogenic (procarcinogens). The process whereby one or more enzyme–catalyzed reactions convert procarcinogens to active carcinogens is called metabolic activation. Any intermediate compounds formed are proximate carcinogens (there may be one or more), and the final compound that reacts with cellular components (e.g. DNA) is the ultimate carcinogen. A possible sequence can be displayed as follows:

Procarcinogens→ Proximate carcinogens→ Ultimate carcinogen

The procarcinogen itself is not a chemically reactive species, whereas the ultimate carcinogen is often highly reactive. An important generalization is that ultimate carcinogens are usually electrophiles (i.e. molecules deficient in electrons), which readily attack nucleophilic groups in DNA, RNA and proteins.

The metabolism of carcinogens and other xenobiotics involves monooxygenase and transferases. The enzymes responsible for metabolic activation of procarcinogens are principally species of cytochrome P450, located in the endosplamic reticulum.

Carcinogens have been found to interact with the purine, pyrimidine, or phosphodiester groups of DNA. The most common site of attack is guanine, and the addition of various carcinogens to the N_2, N_3, N_7, O_6 and O_8 atoms of this base has been observed.

One of the characteristics of chemical or physical carcinogenesis is the usually extended period of time (latent period) between the contact with the carcinogens and the appearance of the tumour. Repeated exposures to the active agents are often, although not always, required which is another characteristic. The latency between exposure to a carcinogen and cancer formation is divisible into stages: initiation caused by agents that irreversibly and heritably alters the cell genome, promotion, the mechanism of which is not well understood, the latent period can be shortened and the tumour yield increased by treatment with certain 'promoting agents' which are not carcinogenic in themselves, or very weakly so. Progression, the third definable stage of neoplastic development, is separated from promotion in which high incidence of carcinomas can be produced by subsequent applications of a different initiating agent, suggesting a second event "second hit" in the induction of cancer, Thus, it appears those of the three stages of carcinogenesis- initiation, promotion and progression- initiation, most certainly, and progression most likely involve molecular genetic changes.

The actions previously described are those of agents, which react with cellular DNA and cause genomic alterations. As more and more chemicals are tested for carcinogenicity, a number are now being recognized as "non-genotoxic". The chemicals do not form stable covalent bonds with cellular DNA or other macromolecules. Possible mechanism for epigenetic effects include chronic tissue injury, hormonal imbalance, immunological effects, or promotional activity on cells that are either genetically abnormal or have been independently altered by genotoxic carcinogens (Weisburger, J. H., *et al*, 1984). This category contains cytotoxic agents, solid-state carcinogens, hormones, immunosuppressants, and promoters. Examples of epigegntic carcinogens include nitrolotriacetic acid, asbestos, phorbol esters, estradiol, azothioprine, etc.

1.2.2 NITROSOAMINE

N-Nitrosamines constitute one of the most interesting classes of chemical carcinogens. Early interest in the study of these compounds stemmed from findings that they present industrial occupational hazards. In recent years it has become increasingly evident, however, that N-Nitroso compounds are of great concern, not only to the industrial workers, but also to the population at large. With the recognition that N-Nitroso compounds can be readily formed from the precursors widely present in the environment, N-Nitroso compounds have emerged as one of the most important classes of the environment carcinogens. Experimentally, N-Nitroso compounds are among the most potent and versatile agents inducing tumors in every animal species tested; this renders these compounds highly useful tools for the study of chemical carcinogenesis.

They induced tumors in a wide variety of organs and tissues in a large number of animal species. The potency and targets specificities of an N-Nitroso compound depends not only on the structure of the chemical, but also on the dosage, treatment schedule, the route of administration, the animal species, and various other factors such as the age, sex and diet of the animals(Arcos, J. C., *et al* , 1982).

Nitrosoamines have been investigated for their carcinogenic and toxic properties since 1956 when the simplest alkylnitrosoamine, N-nitrosodimethylamine, at the time an important industrials intermediate, was shown to produce liver tumors in rodents. Since then more than 100 nitrosoamines have been shown to be carcinogens in the experimental animals, causing tumors mainly in the liver and also in a number of other tissues. The nitrosoamines require metabolic activation mediated by the cytochrome P 450 and flavin dependent oxidases. Such biotransformation yields electrophiles that readily alkylate nucleophilic sites in DNA (Arcos, J. C. *et al,* 1982).

1.2.3 MECHANISM OF ACTION

The main site of alkylation in nucleic acids is the N-7 position of guanine; N-1, N-3 and N-7 position of adenine, and N-3 position of cytosine, thymine and uracil have also been observed. It was hypothesized that O-6-alkylation of guanine leads to the inability of the guanine residue to undergo normal base pairing with cytocine and thus may lead to "transition" mispairing, resulting in mutation. The inability of the system to remove O-6-alkyl guanine from DNA, and therefore to reduce the level of this abnormal

purine in DNA before DNA replication, may be of critical importance for determining susceptibility to carcinogenesis by N-Nitroso compounds (Arcos, J. C. *et al*, 1982).

In addition to O-6-alkylation of guanine, the following reactions in nucleic acids have been suggested as playing an important role in carcinogenesis and mutagenesis by Nitroso compounds: (a) alkylation of phosphate moieties forming phosphodiesters, which in DNA are chemically stable, and (b) alkylation at the N-3 position of guanine, the O - 4 position of thymine, the N-7 position of adenine, and the N-3 position of cytosine.

1.2.4 N-DIBUTYLNITROSOAMINE (DBN)

N-Dibutylnitrosoamine is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals (International Agency for Research on Cancer: 1974, 1978, 1982, 1987). Interest in the study of the structure-activity relationships of DBN and related compounds arose mainly because of the somewhat unusual ability of DBN to induce bladder tumors. It was noted that dibutyl nitrosoamine induces tumors of the liver, esophagus, and bladder after oral administration. A shift of organ specificity was observed after sub cutaneous injections,

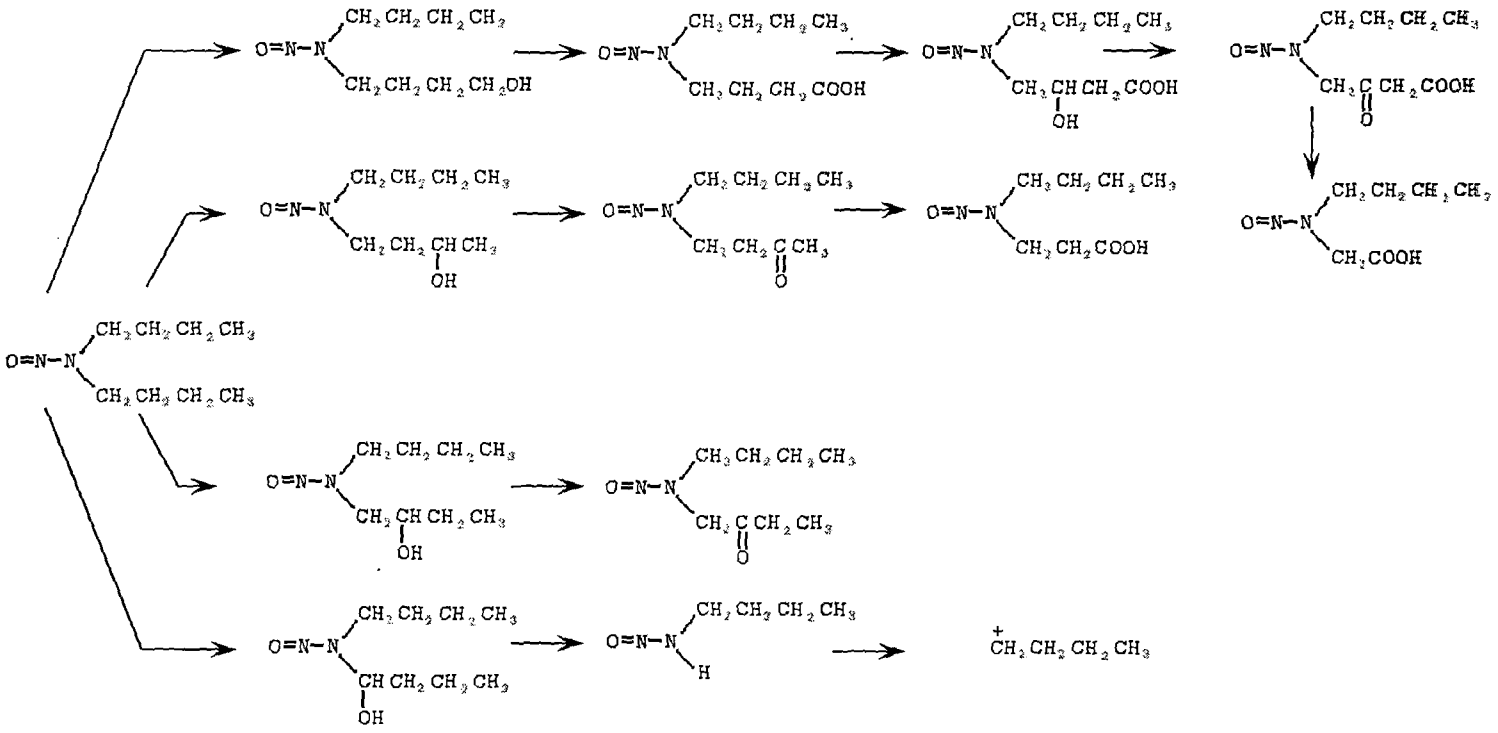


Fig. 1.1 Proposed metabolic pathways of N, N-dibutylnitrosoamine (Arcos, J. C. *et a., 1982)*

with the bladder as the main target organ. An extensive series of synthesis and carcinogenesity studies of various compounds related to DBN and its metabolites have revealed that they have markedly different carcinogenic effects. The metabolism of DBN is complex, because hydroxylation can take place at each of the four carbon atoms of the butyl chain.

N-Nitrosodibutylamine is pale yellow oil with a characteristic odour. It is soluble in water and miscible with hexane, dichloromethane, and many other organic solvents. It is sensitive to light, especially to ultraviolet light, and undergoes relatively rapid photolytic degradation. When heated to decomposition, it emits toxic fumes of nitrogen oxides (Hazardous Substances Data Bank, 2000; International Agency for Research on Cancer, 1978).

N-Nitrosodibutylamine has been detected in a variety of products as a result of the nirosation of amines present in these products. It is present in soya bean oil at a concentration of 290 g / kg, and in smoke or cured meat at 0.2 to 3.9 g / kg (International Agency for Research on Cancer, 1978). It has also been detected in tobacco smoke at a concentration of 3 ng /cigarette. N-Nitrosodibutylamine may be formed from secondary or tertiary –butylamines and quaternary ammonium salts by reaction with nirosation agents, such as nitrite, in the stomach or during cooking processes. The computer estimated half-life of N-Nirosodibutylamine in various phases is 2.8 days.

1.3.0 CHEMOTHERAPY OF CANCER

Nearly more than five decades, chemotherapy has been the main modality for systemic treatment of advanced or metastatic cancer (Chabner, B., 1990; Frei. E. III *et al,* 1997). Cancer chemotherapy is limited by intrinsic or acquired multi-drug resistance of tumor cells and toxicity to normal cells (Chabner, B., 1990; Frei. E. III *et al*, 1997). Although the effectiveness of chemotherapy can be increased by escalating the doses, the option for dose elevation is always restricted by toxicity of chemotherapeutic drugs.

The majority of clinically-used anticancer drugs are systemic anti-proliferative agents (cytotoxins) that preferentially kill dividing cells, primarily by attacking their DNA at some level such as synthesis, replication or processing. These cytotoxins have many advantages as anticancer drugs, especially the ability to kill large number of tumor cells. However, these drugs are not truly selective for cancer cells, and their therapeutic efficacy is limited by the damage they also cause to proliferating normal cells such as those in the bone marrow and gut epithelia. This is particularly true in the treatment of solid tumor, when the majority of the tumor cells themselves are not dividing rapidly. A more selective delivery of the cytotoxic agents to the primary tumors and their metastases would allow a dose escalation and reduce the peripheral toxicity (Magrath, T., 1994).

The concept of delivering cytotoxic drugs through a carrier such as tumor specific monoclonal antibodies, hormones, liposomes etc. to certain tumors has opened a new era in the development of potential therapeutic agents for cancer treatment. In the recent years, many ribosome-inactivating proteins (RIPs) from plants that catalytically

inactivate eukaryotic ribosome have been extensively used as therapeutic agents in cancer treatment (Chabner, B., 1990; Frei. E. III *et al*, 1997).

1.4.0 RIBOSOME-INACTIVATING PROTEINS (RIPs)

Ribosome-inactivating proteins (RIPs) are a group of naturally occurring plant proteins with a RNA-*N*-glycosidases activity which depurinate rRNA at a specific universally conserved position (i.e. cleavage of N-glycosidic bond of a specific adenine of 28S rRNA). The first two RIPs known were ricin and abrin. Dixon in 1887 (Dixon, T., 1887) was the first to suspect that the toxic principle of the caster beans was a protein. Shortly afterwards, Stillmark in Kobert's laboratory in Dorpat purified the protein, for which he proposed the name ricin, and attributed its toxicity to the property of agglutinating erythrocytes (Stillmark, H., 1888, 1889). Hellin also in Kobert's laboratory discovered the agglutinating properties of abrin (Hellin, H., 1891).

Interest in these toxins was revived as late as 1960, when Lin *et al.* reported that they were more toxic to tumor (Ehrlich ascites) than to normal cells (Lin, J., *et al,* 1970). These typical plant proteins receive a lot of attention in the biological and biochemical research because of their unique biological activities towards animal and human cells. In fact, for a long time the interest in RIPs focused on possible medical and therapeutical applications because several of these proteins were found to be more toxic to tumor cells than to normal cells, and hence offered a theoretical opportunity to develop antitumor drugs that selectively target tumor cells (Lin, J., *et al,* 1970). With the development of monoclonal antibodies as tools for identifying and targeting cell

surface markers, researchers gained the ability to couple antibodies to RIPs and thus deliver the toxic protein directly to specific cells. The potential for using RIPs as cell destructive agent in immunotoxins stimulated intense efforts to isolate and characterize such proteins from many different plant sources (Frankel, A. E, *et al,* 1996; Pastan, I., *et al,* 1991). Unfortunately, RIP-derived immunotoxins are not perfect clinical tools. For example, they are generally highly antigenic and promote immune responses in animals receiving prolonged treatment with the immunotoxins (Barbieri, L, *et al,* 1993). A second problem is with vascular leak syndrome, a deleterious side effect that limits clinical efficacy as a cancer therapy (Kreitman, R. J. 1999). Nevertheless refined approaches to inhibit toxicity are showing promises (Baluna, R. *et al,* 1999; Hirao, I., *et al.* 2000) and a number of clinical trials are ongoing (Frankel, A.E., *et al* 1996; Kreitman, R. J., 1999).

1.4.1 TYPES OF RIPs

RIPs are classified into three groups based on their physical properties (Mundy, J., *et al,* 1994). Type 1 RIPs, such as pokeweed antiviral protein (PAP), saporina from *Saporinia officialinis*) and barley (*Hordeum vulgare*) are monomeric enzymes, each with an approximately Mr of 30,000 (Asano, K., 1984; Barbieri, L., *et al,* 1993; Irvin, J. D., 1975). Type 1 RIPs were discovered in 1925 when Duggar and Armstrong (Duggar, B. M., *et al,* 1925) observed that the so-called *Phytolacca americana* antiviral protein (PAP) inhibits the transmission of tobacco mosaic virus (TMV) in plants. However, only in 1978 was PAP recognized as an inhibitor of protein synthesis (Dallal, J. A. *et al,*

1978). Many, but certainly not all, type 1 RIPs are antiviral proteins. Type 1 RIPs are not cytotoxic and do not behave as toxins because they are not able to cross the cell membrane on their own. Some specialized animal cells, however, can import type 1 RIPs by endocytosis and subsequently become sensitive to the RIP activity. To date, most RIPs that have been characterized fall into the type 1 class (Barbieri, L., *et al,* 1993).

Type 2 RIPs were discovered more than a century ago when Stillmark isolated the toxic principle from castor bean seeds. Ironically, the high toxicity of ricin was attributed to its agglutining activity, which means that the carbohydrate binding activity of type 2 RIPs was recognized long before their enzymatic activities and their inhibitory activity on protein synthesis. Type 2 RIPs, like ricin and abrin, are highly toxic heterodimeric proteins with enzymatic and lectin properties in separate polypeptide subunits, each of approximate mw of 30,000 (Olsnes, S., *et al,* 1973; Olsnes, S., *et.al.* 1982; Stripe, F., *et al,* 1978). One polypeptide with RIP activity (A-chain) is linked to a galactose binding lectin (B-chain) through a disulfide bond (Olsnes, S., *et al,* 1973; Olsnes, S., *et al,* 1982; Stripe, F., *et al,* 1978). The lectin chain can bind to galactosyl moieties of glycoproteins and /or glycolipids found on the surface of eukaryotic cells (Lehar, S. M., *et al,* 1994; Olsnes, S., *et al.,* 1988; Sandvig, K., *et al,* 1976; Steeves, R. M., 1999; Swimmer, C., *et al,* 1992) and mediate retrograde transport of the A-chain to the cytosol (Beaumelle, B., *et al,* 1993; Olsnes, S., *et al,* 1982; Sandvig, K. *et al,* 1994;Van Deurs, B., *et al,* 1986). Once it reaches the cytosol, the RIP has access to the translational machinery and readily disrupts protein synthesis.

Type 2 RIPs owes their carbohydrate binding activity to the B-chain, which contains two or possibly three binding sites (Frankel, A. E., *et al,* 1996; Steeves, R. M., *et al,* 1999). Though the B- chain of different type 2 RIPs share high sequence similarity and virtually identical 3-dimensional structures, there are pronounce differences in sugar binding specificity. These differences in lectin activity and specificity are important because the toxicity and cytotoxicity of type 2 RIPs is (partly) determined by the binding of the B-chain to a sugar-containing receptor on the cell surface. Due to the extreme toxicity of ricin and abrin, type 2 RIPs are usually associated with highly toxic proteins (Barbieri, L., *et al,* 1993). However, type 2 RIPs show marked differences in (cyto) toxicity. Ricin, for example, causes 50% cell death at concentration below 1ng/ml, whereas some elderberry type 2 RIPs show no effect at mg/ml (Battelli, M. G., *et al,* 1997)

Type 3 RIPs are synthesized as inactive precursors (proRIPs) that require proteolytic processing events to occur between amino acids involved in formation of the active site (Mundy, J., *et al,* 1994). These RIPs are much less prevalent than type1 or type 2 RIPs. To date, type 3 RIPs have been characterized only from maize and barley (Bass, H. W., *et al,* 1992; Chaudhry, B., *et al,* 1994; Reinbothe, S., *et al,* 1994).

1.4.2 ACTIVITY OF RIPs

Along with the effort to develop some RIPs into anticancer compound, attempts were made to find out what RIPs do and how they act. This led to the finding that RIPs

are RNA *N*-glycosidases that inactivate ribosomes through a site-specific deadenylation of the large ribosomal RNA (Endo *et al,* 1987; Endo, 1987; Endo, Y., 1988).

Once it turned out that the so-called single chain protein synthesis inhibitors share a substantial sequence similarity with the A-chain of ricin, the first functional linked between typel and type 2 RIPs becomes obvious and the search for a common working mechanism started. This search soon revealed that ricin, abrin and PAP inhibit cell-free protein synthesis by irreversibly inactivating the ribosomes in such a way that the function of elongation factors EF-1 and EF-2 is blocked (Barbirie, L., *et al,* 1993; Sperti, S., *et al,* 1975). Ricin recognizes a highly conserved region in the large 28S rRNA and cleaves a specific N-C glycosidic bond between adenine and the nucleotide on the RNA whereby the adenine residue is removed. Due to the removal of this adenine, the deadenylated (or abasic) site becomes unstable and a β elimination reaction can occur after the RNA is treated with acidic aniline, whereby the 3' – end of the rRNA is cleaved and can be detected by electrophoresis. For the most often used substrate rat liver ribosome the specific site is A_{4324} in 28S rRNA. This site is usually depicted as being present in a single-stranded loop, called the sarcin / ricin loop. It is located in domain VII some 400 nucleotides from the 3' end of the RNA. Subsequent work revealed that this particular site-specific RNA *N*-glycosidase activity is a common property of all identified type 1 and type 2 RIPs.

Although all RIPs exhibit RNA *N*-glycosidase activity towards ribosomes, there are marked differences in substrate specificity. For example, ricin is highly active towards mammalian and yeast ribosomes but poorly active or even inactive on plant and

Escherichia coli ribosomes (Barbieri, L., *et al,* 1993). In contrast PAP depurinates ribosomes from plants, bacteria, yeasts, and lower and higher animals. Most type 1 RIPs have a rather broad specificity whereas type 2 RIPs have a preference for animal ribosomes. Both RIPs and ribosomes contribute to the apparent substrate specificity. Since the rRNA target structure is universally conserved, differences in sensitivity between ribosomes most likely reside within the ribosomal proteins, which may either allow or prevent access of the RIPs to the sarcin /ricin loop. Vater *et al.* identified rat liver ribosomal proteins L9 and L10e as the binding target of the ricin A-chain (Vater, C. A., *et al,* 1995), whereas yeast ribosomal protein L3 was identified as the binding factor of PAP (Hudak, K. A., *et al,* 1999). The specific interaction between PAP and L3 probably explains the broad spectrum activity of PAP towards ribosomes from species of different taxonomic groups because L3 is highly conserved in ribosomes. Differences in activity and ribosome substrate specificity are also due to differences in the structure of different RIPs. This was demonstrated by an approach in which PAP-ricin A-chain protein hybrids were created and examined for activity on rabbit reticulocyte and E. coli ribosomes. According to the results of these experiments, the amino-terminal half of the hybrid proteins determine the substrate activity. Structurally dissimilar surface polypeptide loops apparently do not play a role (Chaddock, J. A., *et al,* 1996).

The mechanism of entry onto the cells of protein toxins with intracellular sites of action has been of many studies in recent years (Olsnes, S., *et al,* 1985 and 1988). Nevertheless, it is not yet completely understood. The interest in toxin internalization is growing because this mechanism is a key point in a possible therapeutic utilization of

them or of their derivatives, and also because these studies give important insights in the intracellular transport and sorting of physiological ligands. In fact, much evidence currently suggests that toxin entry and routing inside cells are not toxin-specific but mimic pathways of physiological molecules.

RIPs can be taken up by cells through two mechanisms: the endocytosis dependent on the binding of RIPs to either the galactosyl residue (type 2 RIPs) or the mannose receptors on the cell membrane, and the fluid-phase endocytosis which allows the internalization of molecule without a receptor-mediated mechanism (Van Deurs, B., *et al,* 1989). The entry mechanism of type 1 RIPs is not well understood and only hypotheses can be formulated. Glycosyl residues are present in most of the type 1 RIPs, which could be internalized after binding to carbohydrate receptor on cell membrane. Peritoneal exudes cells (PEC) internalize gelonin by mannose receptors in a saturable manner. Mannan inhibites the specific binding and changes the pattern of gelonin uptake by PEC to the non-saturable pattern observed in cells lacking mannose receptors in spite of the gelonin to PEC (Madan, S., *et al,* 1992). The lower toxicity of the type 1 RIPs as compared to that of type 2 RIPs, suggests that the receptor-mediated internalization is not very efficient for type 1 RIPs. However, a considerable amount of evidence suggests that type 2 RIPs do not cross directly the plasma membrane but enter the cytosol by endocytic pathway. Electron microscopic studies show that a ricin-ferritin conjugate clusters first at the cell surface and appears in the endocytic vessels 60 minutes latter (Nicolson, G.L., 1974). Endocytosis is temperature dependent process. Thus, very little intoxication can be observed if cells are exposed to RIPs at 0 ^{0}C and

then washed with the competing ligand before restoring a physiological temperature (Sandvig, K., *et al,* 1979). Metabolic inhibitors protect the cells from toxin activity by preventing endocytosis, which is an energy consuming process (Sandvig, K., *et al,* 1982). The time lag of 30 minutes observed between exposition to ricin and inhibition of protein synthesis correlates with the time required for the intracytoplasmic visualization of the toxin linked to horseradish peroxidase (Gonatas, J., *et al,* 1980). Therefore, endocytosis appears to be a necessary step in the internalization of RIPs. Gelonin, and possibly other type of RIPs, could be internalized by the fluid-phase endocytosis into cells lacking the receptors for the glycosyl residues present in the RIP molecule (Madan, S., *et al,* 1992).

Covalently cross-linked RIPs to specific antibodies or cytokines (immunotoxins) have successfully been used in cancer immunotherapy. Immunotoxins, combines the respective benefits of the toxicity and specificity of monoclonal or polyclonal antibodies which recognize antigenic determinants on the cell surface of the tumor cells as vehicle for delivery of toxic agents (Lambert, J. M., *et al,* 1988; Singh, V., *et al,* 1989 and 1992; Laemmeli, U.K., 1969). Although immunotoxins were hailed as a new class of anticancer agent in the early 1980s, they have not measured up to their full potential (Singh, V., *et al,* 1992). Despite some bright spots, response rates generally have been low, and immunotoxins limited by their toxicity and narrow therapeutic window. The major disadvantage of immunotoxins is their toxicity to non target organs, which has limited the progress in the field of immunotoxin research.

Like antibodies, hormones are quite specific in their interaction with their receptors, therefore, analogous to immunotoxins, hormonototoxins (so called because the hormone component of the construct provided specificity for the toxin action) were constructed by conjugating gonadotropin hormones (such as oLH or hCG) with the RIP to target the hybrid complex to selective cells in the gonads (Carlsson, J., *et al,* 1978; Stripe, F., *et al,* 1980; Singh, V., *et al,* 1989; Srinivasan, Y., *et al,* 1985).

Physiological barriers hinder the effective delivery of drugs to tumors (Jain, R. K., 1998; Pluen, A. *et al,* 2001). To target cancer cells, a blood borne therapeutic agent must first cross the vasculature and then travel through the interstitium. Recent strategies have attempted to avoid these barriers by attacking the blood supply instead of cancer cells, either by suppressing the formation of new vessels (antiangiogenic therapy) or by abolishing established vascular network (antivascular therapy). This approach has the advantage that the delivery vehicle, once in the blood stream, should have direct access to the target endothelial cells. Recent studies have shown that cationic liposomes have a propensity for localizing in tumor vessels, but the mechanism behind this selectivity and the optimal formulation to maximize this effect have not been defined. Delivery of endogenous macromolecules to the cytosol is a fundamentally inefficient process. This difficulty arises from the fact that cells have an obligation to maintaining homeostasis; hence the need for strict control over what is allowed passage, into the cells. Because of their hydrophilicity and large hydrodynamic volumes, macromolecules such as DNA and protein are effectively impermeant to the cell's plasma membrane. Those that are taken by cells via, for example, fluid phase or

receptor mediated endocytosis are ultimately degraded within the endosomal/lysosomal pathway, or in some cases are returned to the extracellular environment (Mellman, I., 1996; Mukherjee, S., *et al,* 1997). For many bio-molecules with therapeutic potential, direct interaction with an intracellular target may be a prerequisite for efficacy. This condition is particularly true for many plant-derived toxins that have cytostatic or cytotoxic activities and thus have potential as anti-cancer therapy. Gelonin, a 30 kDa type I toxin obtained from the seeds of *Gelonium multiflorum,* is an ideal candidate for therapeutic delivery due to its minimal toxicity when added extracellularly (Stripe, F., *et al,* 1980). Numerous strategies have been employed to efficiently deliver gelonin to the cytosol of cancer cells including conjugation of folate, hormones or monoclonal antibodies, and entrapment into liposomes or polymers (Alam, A., *et al,* 1992; Atkinson, S. F., *et al,* 2001; Betler, M. 1996; Lambert, J. M., *et al,* 1985; Mclntosh, D. P., *et al,* 1982; Pattrick, .N. G., *et al,* 2001; Rosenblum, M. G., *et al,* 1999; Singh, V., *et al,* 1989 and Veenendal, L. M., *et al,* 2002). These approaches are predominantly aimed at increasing cell-type specific binding and uptake and in some cases augmenting delivery into the cytosol.

1.5.0 LIPOSOMES AS TOXIN CARRIERS

Liposomes are versatile vehicles in terms of structural characteristics and mode of drug incorporation. This creates a wide range of options for the design of effective liposomal drug formulations to induce tumoricidal effector mechanisms.

Liposomes are phospholipids vesicles where lipids are arranged in a concentric bilayer enclosing an aqueous space. These structures form spontaneously when lipids are suspended in aqueous solution. Water soluble material can be accommodated in the aqueous space of liposomes, whereas lipophilic substances get associated in the lipid part. Size and charge of liposomes could be varied according to the requirement.

Liposomes are commonly prepared by ultrasonic irradiation of water dispersion of phosphatidyl choline (lecithin). However, this method suffer from the drawbacks that it provides a wide range of size heterogenicity and also results in degradation of phospholipids. If macromolecules like DNA, protein, enzyme etc, are present in the sonication mixture, these macromolecules undergo denaturation or degradation during sonication. To overcome these problems, quite a few alternative methods have been described (Bangham, A. D., *et al*, 1965; Batzri, S. and Korn, E. D., 1973; Deamer, D. and Bangham, A. D., 1976; Kagawa, Y. and Racker, E., 1971; Szoka, F. Jr. and Papahadjopoulos, D., 1978).

Phospholipids solution in organic solvent when injected in buffer gives rise to formation of liposomes. One of these methods which makes use of chloroform and methanol mixture as organic solvent was originally developed by Papahadjopoulos (Papahadjopoulos, D., 1978) has recently been modified to give large size unilamallar liposomes, which retain the entrapped protein intact (Alam, A., *et al*, 1992)). The method is simple and highly reproducible even at large scale preparation.

Structural versatility, innocuous nature, and easy biodegradability, in addition to other properties, have made liposomes a more potent biological carrier in drug delivery

and drug targeting. Most of its applications have been based on the ability of the liposomes to preferentially migrate to reticuloendothelial system (RES) or tissues such as liver and spleen (Vertut-Doi, *et al,* 1996). However, avoiding degradation of liposomes by mononuclear phagocytic system has been a problem (Vertut-Doi, *et al,* 1996) Nonetheless, in principle, liposome as drug carrier for targeted delivery has potentials of application in chemotherapy of cancer.

The successful application of liposomes as carrier for drugs and enzymes in therapy is heavily dependent on their stability in circulation, tissue distribution and also on their mode of interaction with target cell(s). Size and surface charge of the liposomes appear to control the rate of liposomes clearance from blood. Large liposomes are cleared more rapidly than smaller one (Juliano, R. L., *et al,* 1975; Hinkle, G. H., *et al,* 1978). Liposomal preparation of mixed sizes possesses biphasic rates of clearance, whereas liposomes of homogeneous size exhibit a simple exponential clearance (Gregoriadis, G., *et al,* 1974). The surface charge of liposome is also an important determinant of cellular clearance. Neutral and positively charged liposomes were cleared less rapidly than were unilamellar negatively charged one (Gregoriadis, G., *et al,* 1974). Survival of liposomes in circulation is also affected by the choice of its constituents. Incorporation of increased amount of cholesterol in liposomes promotes their stability in blood (Kirby, C., *et al,* 1980).

Liposomes have served dual role: as a valuable experimental tool for membrane research and in addition, as an *in vivo* delivery system for enhancing the efficacy of various biologically active molecules (Papahadjopoulos, D., 1978; Knight,

C.G., 1981; Ostro, M. J., 1987; Gregoriadis, G., 1988). Animal studies have shown that liposomes can decrease the toxicity of several anti-tumor and antifungal drugs (Rahman, A., *et al*, 1986; Forssen, E. A., *et al*, 1981; Olson, F., *et al*, 1982; Gabizon, A., *et al*, 1986; Lopez-Berestein, G., 1983). Moreover, liposomes have also been shown to be an efficient carriers for anti-parasitic drugs for treating intracellular infections such as Leshmaniasis (Black, E. D., *et al*, 1977; New, R. R., *et al*, 1978; Alving, C. R., *et al*, 1978), of macrophage activators for increasing tumoricidal activity in models of metastasis (Koss, W. C., *et al*, 1985), and of various antigens and adjuvants for enhancing the immune response (Alving, C. R., *et al*, 1990; Gregoriadis, G., 1989). These studies have led to several promising chemical trials (Sculier, J. P., *et al*, 1988; Gabizon, A., *et al*, 1989; Treat, J., *et al*, 1990; Lopez-Berestein, G., *et al*, 1985; Presant, C. A., *et al*, 1988).

Liposomes have been found not to accumulate appreciably in tissues such as heart, kidneys and gastrointestinal tract. This results in lowering of the toxicity of encapsulated agent to these tissues (Szoka, F. C., 1981). Major proportions of liposomes from circulation of injected animals are captured by liver and spleen (Gregoriadis, G., *et al*, 1972 and Gregoriadis, G., 1979). Other tissues such as lung, kidney, and skeletal muscle etc. uptake are rather modest and seldom exceed 2-5 % of the dose per gram of tissue (Gregoriadis, G., *et al,* 1974 and 1977).

Targeting of liposomes to specific cells in bio-phase does not guarantee the delivery of liposomal content to cells. The delivery is mainly controlled by the mode of interaction between liposomes and cells. Liposome-cell interaction may proceed by one

(or more) of the following modes: (a) stable adsorption, (b) lipid transfer, (c) fusion, and (d) endocytosis. Fusion and endocytosis are the two useful interactions which ensure delivery of liposomal contents to target cells (Pagono, R. E., *et al*, 1980).

Conventional liposomes are best studied for delivery to cells of reticuloendothelium system (RES), localizing their contents mostly in the liver and spleen within a minutes after intravenous administration. However, liposomes specifically targeted to tissues other than RES require the presence of special ligands on their surface for specific binding to cells or tissues, and in addition also require a long enough circulation time to be able to reach their target. These findings prompted us to investigate the cytotoxic effect of liposome encapsulated gelonin on liver cancer.

The present investigation is an attempt for the following objectives:

- ☞ Isolation and purification of gelonin from the seeds of *Gelonium multiflorium.*
- ☞ Determination of biological activity of purified gelonin.
- ☞ Induction of hepatocarcinogenesis in Swiss albino mice by intravenous administration of DBN.
- ☞ Encapsulation of gelonin into liposomes.
- ☞ Study the effect of liposome encapsulated gelonin on cancer regression by *in vitro* and *in vivo* experiments.

CHAPTER -2

MATERIALS

2.0.0 MATERIALS

2.1.0 RIBOSOME INACTIVATING PROTEIN, GELONIN

The seeds of *Gelonium multiflorum* were obtained from United Chemicals and Allied Products, Kolkata. Gelonin was isolated from the dry deshelled seeds using aqueous extraction, cation-exchange chromatography and finally gel-filtration chromatography as described in methods section (Stripe, F., *et al,* 1980; Singh, V. and Sairam, M. R., 1989; Singh, V., *et al,* 2001).

2.2.0 CHEMICALS AND REAGENTS

SIGMA:- N, N-dibutyl nitrosoamine (DBN), γ-Glutamyl-p-nitroanilide, Glycylglycine, Trizma base (Tri [hydroxymethyl] aminomethane), Acetylcholine chloride, 5, 5' –dithiobis-(2-nitrobenzoic acid) (DTNB), Triton X-100, Glutathione(GSH), Trypsin, Fetal Calf Serum (FCS), Sodium Dodecyl Sulfate (SDS), N, N'–bis methylene-acrylamide, N, N, N' N'-tetramethylelene diamine (TEMED), Acrylamide, Ammonium per sulfate (APS), Coomassie brilliant blue R-250, Coomassie brilliant blue G, Dulbecco's Modified Eagle Medium (DMEM), Trypan blue, L-glutamine, L-arginine, L-asparagine, Dipalmitoylphosphatidylcholine, Diacetylphosphate, Cholesterol, Phosphatidylcholine, Phosphatidyl ethanolamine, Phosphatidyl serine, Phosphatidyl glycerol.

ROCHE APPLIED SCIENCE, MANNHEIM:-. 5-Bromo-2'-deoxy-uridine Labeling and Detection Kit III, Penicillin/Streptomycin.

AMERSHAM INTERNATIONAL LTD.:- C^{14} labelled cholesterol

HI-MEDIA: - 1-Chloro-2, 4-dinitrobenzene (CDNB), Polyethylene glycol,

QUALIGENS:- Sodium dihydrogen phsosphate (NaH_2PO_4), di-sodium hydrogen phosphate (Na_2HPO_4), Sodium hydroxide(NaOH), Sodium chloride(NaCl), Hydrochloric acid (HCl), Trichloroacetic acid, Tri-sodium citrate, Formalin, Acetic acid, Xylene, DPX, Eosin, Haematoxylin, Methonol, Chloroform, Ethylenediamine tetraacetic acid (EDTA), Paraffin wax, Glutathione (GSH), Sucrose.

BANGLORE GENEI: - Protein molecular markers (PMW-M), Proteinase k

SPECTROCHEM: - Scintillation fluid (Cocktail 'T').

2.3.0 INSTRUMENTS/APPARATUS

HOMOGENIZER: - Remi Motors Type RQ-127A, H.P 1/8, R.P.M. 800.

CENTRIFUGE: - Remi C24, Spinwin model MC -01.

SPECTROPHOTOMETER: - Hitachi Model U - 2001 UV/ Vis

LIQUID SCINTILLATION COUNTER: - System 1400TM Waalac 1409.

ELECTROPHORESIS UNIT:-

(a) Bio-Rad Model Mini-PROTEAN ®II CELL,

(b) Bio-Rad Mini transilluminator.

MICROSCOPE: - Carl Zeiss JENA 30 –GO603.

MICROTOME: - WESWOX Rotary Microtome.

CHROMATOGRAPHIC UNITS:-

(a) BioRad Biologic LP,

(b) Pharmacia.

WEIGHING BALANCES: - Mettler Toledo Model AB54, Sartorius Type 1409 and 2405.

LYOPHILYZER: - LSL, SECFRIOD laboratory freeze dryer Lyolab BII

2.4.0 EXPERIMENTAL ANIMALS

Swiss albino mice (BALB/c), bred at the animal house of the department by random inbreeding were kept on basal diet *ad libitum* and housed in plastic cages in a temperature controlled animal room (24 ± 2 °C) with a 12 h light and dark cycle. The mice weighed 20-25 g at the start of the experiment.

CHAPTER -3

METHODS

3.0.0 METHODS

3.1.1 EXTRACTION OF GELONIN

Gelonin was isolated from the dry seeds of *Gelonium multiflorum* by the method described below:

Procedure: This method was originally described by Stripe *et al,* (Stripe, F., *et al,* 1980) with minor modification as described earlier (Singh, V. and Sairam, M. R., 1989; Singh, V., *et al,* 2001). In Brief, the deshelled seeds were blended with ten volumes of the extraction buffer (50 mM sodium phosphate buffer, pH 7.2 containing 100 mM NaCl). The smooth slurry was stirred overnight at 4 °C and centrifuged at 10000 xg for 60 min in cold. The precipitate was re-extracted with same buffer and again centrifuged. The supernatants of both the extractions were pooled and dialyzed extensively against dialyzing buffer (50 mM sodium phosphate, pH 6.0, without NaCl). The dialyzed supernatant was again centrifuged to remove the precipitate and then passed through a CMC-52 column, pre-equilibrated with dialyzing buffer. The column was extensively washed with the equilibration buffer until the eluent showed absorbance less than 0.05 at 280 nm. The material bound to the column was eluted by applying a linear gradient of 0 mM – 300 mM NaCl in the same buffer at a flow rate of 30 ml/hr at 4 °C. Fractions were read at 280 nm. The protein eluting at around 150 mM – 200 mM gradient was pooled together, concentrated and subjected to gel-filtration chromatography on a Sephacryl S200HR (factionation range, Mw ~ 1 – 80 kDa) column. The column was developed with 50 mM ammonium bicarbonate and run at the flow rate of 20 ml / hr.

The proteins eluted read at 280 nm. Fractions 51- 57 containing maximum amount of desired protein were pooled, concentrated and stored at -20 °C.

3.1.2 SDS-PAGE ANALYSIS OF GELONIN

Requirements:

The following reagents were prepared as stock solution for gel casting:

A. Acrylamide stock solution: was made by dissolving 29.2 g of acrylamide and 0.8 g of bis-acrylamide to a final volume of 100 ml in double distilled water.

B. 1.5 M Tris-HCl buffer, pH 8.8,

C. 0.5 M Tris-HCl buffer, pH, 6.8,

D. 10% ammonium persulphate (APS),

E. 10% sodium dodecyl sulphate (SDS),

F. Electrode reservoir buffer: a solution containing of 7.2g glycine, 1.5g tris and 5ml of 10% SDS in 500ml and pH of which was adjusted at 8.3 ,

G. sample buffer (5 ×): containing 5 ml of buffer C, 5 g sucrose, 0.25 ml mercaptoethanol, 1 ml of 0.5% bromophenol blue made up to a final volume of 10 ml with doubled distilled water, and

H. TEMED.

Procedure: Acrylamide discontinuous gels (resolving gel - 10%, stacking gel - 4%) were prepared according to the procedure described by Laemmli, U. K. (Laemmli, U. K., 1970). Briefly, a mixture of 2 ml double distilled water, 1.7 ml acrylamide

solution A and 1.3 ml tris- HCl (pH, 8.8) solution B was degassed and to it 0.05 ml of 10% SDS solution E, 0.05 ml of APS solution D and 0.002 ml TEMED was added immediately. The solution was mixed gently and carefully poured between the glass plates assembled for the electrophoretic run and left the preparation for polymerization. A space of 3 cm was left from the top of the gel for casting the stacking gel. The space provided for stacking gel was fitted with comb. Stacking gel was made by mixing with 1.4 ml double distilled water, 0.33 ml acrylamide solution A and 0.25 ml of 0.5 M tris-HCl buffer, pH 6.8 solution C. The mixture was degassed and 0.02 ml 10% SDS solution E, 0.02 ml 10% APS solution D and 0.002 ml TEMED was added and poured over the polymerized separating gel. Comb was removed after polymerization of the stacking gel to which sample is to be loaded. The whole set up was then transferred on to the electrophoretic chamber, and then an appropriate amount of the reservoir buffer solution F was poured slowly. 20 µl of the protein sample was mixed with 5 µl of sample buffer solution G and heated in boiling water for about 3 minutes. Sample was then loaded onto the wells. Molecular weight markers were also loaded on to one of the cornermost wells. Gel was run at 60 mA and at a constant voltage of 200 V for 45 minutes. The gel was fixed in methanol/acetic acid, stained with Coomassie blue R 250 and destained with methanol/acetic acid for 24 hr.

3.1.3 *IN VITRO* CELL FREE TRANSLATION ASSAY

The inhibitory activity of purified gelonin preparations on cell-free protein synthesis was determined using a system consisting of nuclease treated rabbit

reticulocyte lysate. The extent of protein synthesis was determined by measuring incorporation of [^{3}H]-leucine into the TCA insoluble protein fraction. Briefly, to a total of 15 µl reaction mixture, 2 µl of test sample was incubated with 1 µl BMV mRNA (0.5 µg/l), 2 µl each amino acid mixture (minus leucine) and tritiated leucine, 0.5 µl each 500 mM potassium chloride and 200 mM magnesium chloride, 7 µl nuclease treated rabbit reticulocyte lysate. The positive control was incubated with mRNA (no test sample) while negative control received equal volume of RNAse free water as shown in the table-1 given below. After incubation at 30 °C for 60 min. 5µl reaction mixture was spotted at 10 -15 places on a 3 mm Whatman filter paper and dipped in cold 5% TCA containing 0.2% leucine. Three more washing of two minutes each was carried out with 5% cold TCA. The paper was heated at 90 °C in 5% TCA for 2 min then quickly washed with cold ethanol and dried in the air. The protein precipitated was counted in a Scintillation counter (Waalac 1409) after adding 8 ml scintillation cocktail. Percentage of protein synthesis was calculated on the basis of the radioactivity incorporated in the precipitate. The inhibition of protein synthesis (in percentage) was expressed as: $ED_{50} = (B/B_0) \times 100$, where B is the radioactivity incorporated in the TCA precipitate in the presence of inhibitor and B_0 is in absence of any inhibitor. ED_{50} is the dose of inhibitor required for 50% inhibition of protein synthesis.

Table: 3.1. *In vitro* Cell Free Translation Assay (RIP Activity Determination)

Requirements	With RNA	Without RNA	
		Positive Control	Negative control
RN ase Free Water	-	2 µl	3 µl
Test Sample	2 µl	-	-
mRNA	1 µl	1 µl	-
Tritiated Leucine	2 µl (100 Ci/m mol)	2 µl	2 µl
Amino Acid Mixture (without Leucine)	2 µl	2 µl	2 µl
200 mM Magnisium Chloride	0.5 µl	0.5 µl	0.5 µl
500 mM Potassium Chloride	0.5 µl	0.5 µl	0.5 µl
Rabbit Reticulocyte Lysate	7 µl	7 µl	7 µl
Total Volume	**15**	**15**	**15**

3.2.0 CANCER INDUCTION

Healthy mice, 5-6 weeks old were administered an aqueous solution of DBN (10 mg kg^{-1} body weight) intravenously at weekly intervals for 12 weeks. After completion of the DBN exposue, the experimental mice were sacrificed to monitor the indction of tumour. Various parameters such as maker enzymes (GGT, AChE, and GST), GSH level, histology and electrophoretic banding of genomic DNA were studied to monitor the progress of tumourigenesis. Sham-treated, age-matched normal mice served as controls.

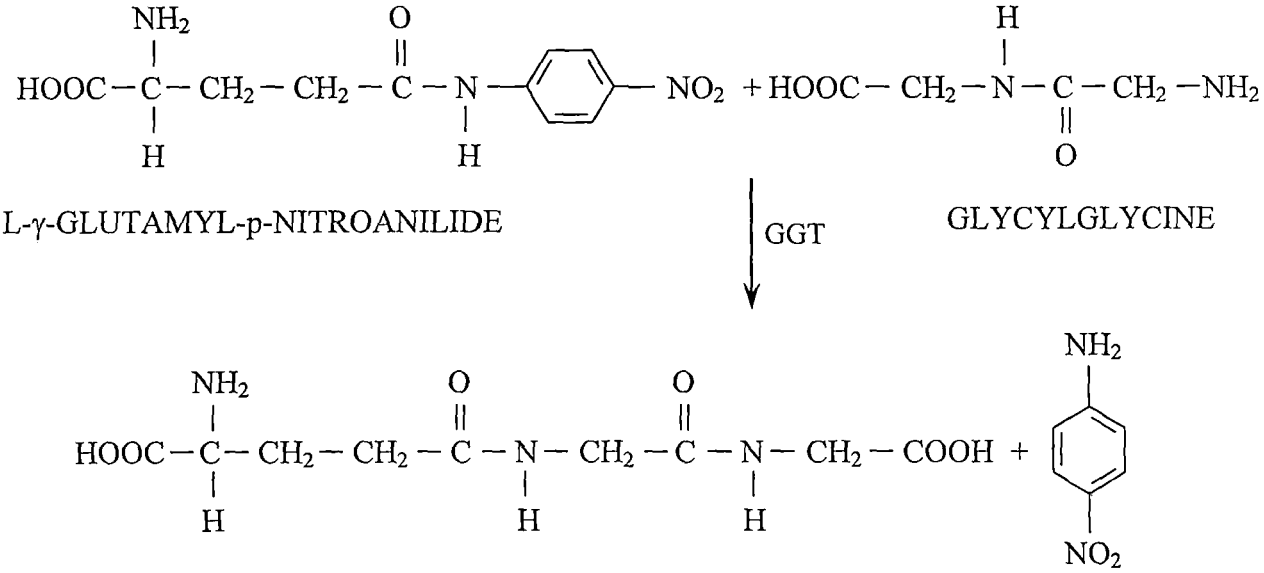
3.2.1 TISSUE PREPARATION FOR ENZYMATIC ASSAYS

After complete DBN treatment mice were killed by cervical dislocation and the liver was quickly excised and removed, rinsed in chilled normal saline (0.9% NaCl), blotted dry and weighed. A 10% homogenate was prepared in chilled 0.25 M sucrose solution and then centrifuged at 20,000 × *g* for 30 min at 4 °C. The resulting supernatant was used for the enzyme assays as described below. The total protein content in the supernatant was estimated using Bradford method (Bradford, M. A., 1976).

3.2.2 GGT ACTIVITY ASSAY

γ-Glutamyl transpeptidase (GGT) activity was assayed according to the method described by Meister *et al.* (Meister, A. *et al,* 1981) with slight modifications.

Reaction mechanism:



Assay principle: GGT catalyzes the transfer of L-γ-glutamyl moiety of L-γ-glutamyl-p-nitroanilide to glycylglycine forming L-γ-glutamyl-glycylglycine and releases p-nitroaniline as shown below, which absorbs at 410 nm. The amount of p-nitroaniline formed was measured by reading the absorbance at 410 nm.

Requirements: Reagents prepared for the assay were:

A. 5 mM L- γ-glutamyl-p-nitroanilide, pH 8.0,

B. 0.1 M glycylglycine, pH 8.0, and

C. 0.1 M tris-HCl buffer, pH 8.0.

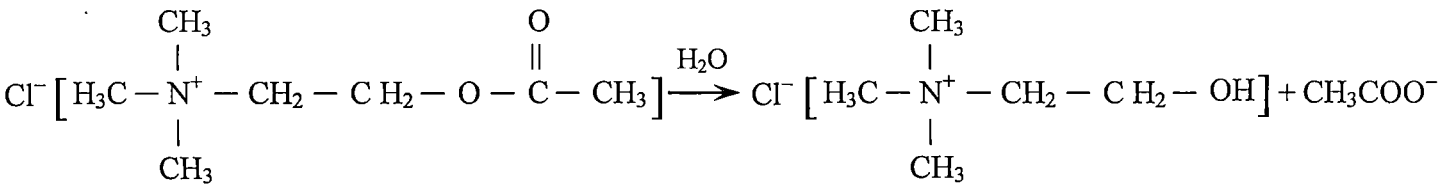
Procedure: 0.2 ml of 5 mM L-γ-glutamyl-p-nitroanilide (pH, 8.0), 0.2 ml of 0.1 M glycylglycine (pH, 8.0), and 0.6 ml of 0.1 M tris-HCl buffer (pH 8.0) were taken in 3 ml cuvette and placed it in the cuvette holder of spectrophotometer. The reaction was initiated by adding 0.2 ml of the above prepared sample and mixed well. P-nitroanilde, released during transpeptidation was determined by measuring the absorbance at 410nm for 3 minutes at 10 seconds intervals.

3.2.3 AChE ACTIVITY ASSAY

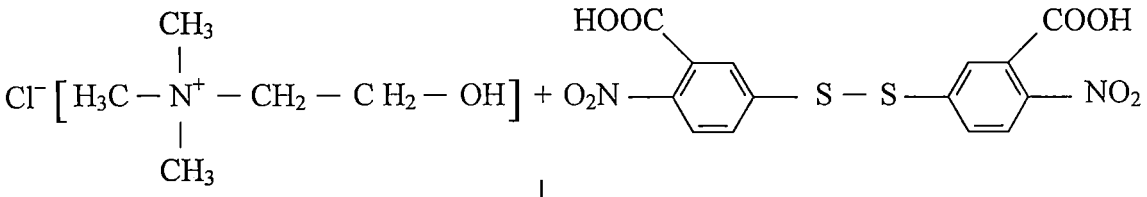
Acetylcholine esterase activity was assayed according to the method previously described by Ellman *et al.* (Ellman, G. L., *et at,* 1961), and Wright & Plummer (Wright, D. L. and Plummer, D. T., 1972) with slight modifications.

Assay principle: Acetylcholine chloride, the ester of choline and acetic acid used as substrate. Acetylcholine esterase (AChE) hydrolyses acetylcholine chloride to choline and acetate. Choline reacts with an oxidizing agent, 5, 5′- dithiobis-(2-nitrobenzoic acid) (DTNB), which subsequently breaks up into two products, 5-thio-2-nitrobenzoate that absorbs at 412 nm and choline-thiobenzoate..

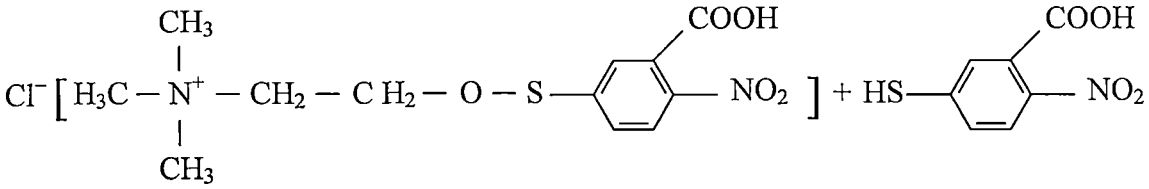
Reaction mechanism:



ACETYLCHOLINE CHLORIDE CHOLINE ACETATE



CHOLINE 5, 5'-DITHIOBIS (2-NITROBENZOIC ACID)



CHOLINE - THIOBENZOATE 5-THIO-2-NITROBENZOATE ($A_{412\,nm}$)

Requirements: Following are the requirements prepared for the assay:

A. 0.2 M phosphate buffer saline (PBS), pH 8.0,

B. 6.0 mM acetylcholine chloride,

C. 0.75 mM dithionitrobenzoic acid (DTNB) in PBS, and

D. 0.3 % triton X-100 in PBS.

Procedure: 1 ml of 0.2 M phosphate buffer saline (PBS), 0.5 ml of 6.0 mM acetylcholine chloride, 0.5 ml of 0.75 mM DTBN and 0.5 ml of 0.3% triton X-100 in PBS were taken in a cuvette and mixed them well. Finally, 0.5 ml of the prepared supernatant was then added. Absorbance was measured at 412 nm at 10 seconds intervals for 3 minutes.

3.2.4 GST ACTIVITY ASSAY

Glutathione S-transferase (GST) activity in the liver was determined by the method described by Habig and Jacoby (Habig, W. H. and Jakoby, W. B., 1981) with slight modification.

Assay Principle: Glutathione S-transferase catalyses the conjugation of CDNB with GSH. The conjugate of GSH and CDNB thus formed was measured spectrophometrically at 340 nm.

Reaction mechanism:

1-CHLORO-2, 4-DINITROBENZENE GLUTATHIONE GLUTATHIONE CONJUGATE

Requirements: The reagents required for the assay are given below:

A. 0.2 M sodium phosphate buffer saline of pH 6.5,

B. 20 mM GSH in deionised water, and

C. 20 mM CDNB in 95% ethanol.

Procedure: The following reagents were added to 1 ml cuvette: 500 µl of sodium phosphate buffer saline (0.2 M), 50 µl of GSH (20 mM), 250 µl of deionized water and 50 µl of the prepared supernatant to be assayed. These were mixed thoroughly. The reaction was initiated by addition of 50 µl of CDNB (20 mM). The increase in absorbance at 340 nm was recorded at 10 seconds intervals for 3 minutes.

3.2.5 GLUTATHIONE ASSAY

Glutathione (GSH) level was determined by the method described by Ellman (Ellman, G. L., 1959) with slight modifications.

Principle: The assay was based on the non-enzymatic reduction of 5, 5′-dithiobis-[2-nitrobenzoic acid] by GSH to 2-nitro-5-mercaptobenzoic acid, a yellow coloured complex. The yellow colour product formed was then spectrophotometrically measured at 412 nm.

Requirements: Reagents required for preparation of the assay sample were:

A. 25 mM EDTA in normal saline (pH 7.5),

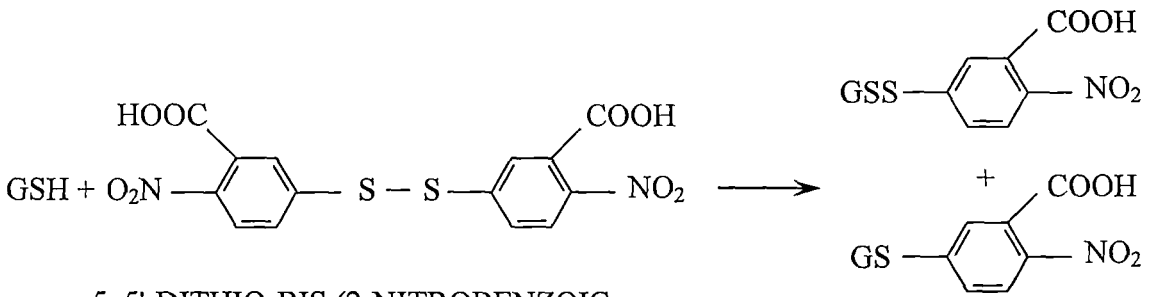
B. 1% tri-sodium citrate,

C. 20% TCA in reagent B,

D. 0.04% DTNB in reagent B, and

E. 0.3 M disodium hydrogen phosphate.

Reaction mechanism:



5, 5'-DITHIO-BIS (2-NITROBENZOIC ACID) (DTNB)

MERCAPTURIC ACID ($A_{412\ nm}$)

Procedure: 0.2 gm of the perfused liver was taken in 2 ml of solution A and homogenized. Equal volume of reagent C was added to the homogenate and vortexed. The precipitated proteins were then spun down at 11,000 x g for 30 minutes at 4 °C. The supernatant, which contains acid soluble GSH, was then used to estimate GSH level.

1 ml of reagent A, 1 ml of reagent D, 0.5 ml of reagent E and 0.5 ml of the sample were taken in a cuvette and mixed thoroughly. This was allowed to stand for 10 minutes after which the absorbance was read at 412 nm. The GSH content was calculated from a standard graph of GSH concentrations.

3.2.6 TOTAL PROTEIN ESTIMATION

The concentration of protein at any step was determined by Bradford's method (Bradford, M. A., 1976).

Principle: This method is based on the capacity of a protein to bind dye (Coomassie brilliant blue G 250) quantitatively.

Requirements: Reagents prepared were:

A. 100 mg of Coomassie brilliant blue G 250 was dissolved in 50 ml of 95% ethanol. The final volume was made up to 100 ml with 85% phosphoric acid. Thus prepared solution was stored freeze in amber bottle.

B. working solution: 15 ml of stock solution was made up to 100 ml with double distilled water prior to estimation of protein. The solution was mixed thoroughly and filter through whatmann filter No.1.

Procedure: 10 µl of the test sample was taken in triplicate and the final volume was made to 100 µl. 5 ml of the fresh working solution was added to each test tube and vortex carefully so as to avoid frothing. The reaction mixture was left for incubation at room temperature for 5 minutes for blue color development. The absorbance was read at 595 nm. Total protein in the supernatant of the liver homogenate was then calculated using bovine serum albumin (BSA) as standard.

3.2.7 PREPARATION OF HISTOLOGICAL SECTIONS

The histological sections of the liver tissue of the normal and DBN treated animals were prepared following the method described by Ratcliffe (Ratcliffe, N. A., 1983).

Requirements:

A. Fixative: - The fixative used was Bouin's fluid. It had the following composition:

Picric acid (saturated aqueous solution)	75 ml
Formalin (40% aqueous)	25 ml
Acetic acid (glacial)	5 ml

B. Graded series of alcohol: - 30%, 50%, 70%, 90% and absolute alcohol,

C. Xylene,

D. Wax,

E. DPX,

F. Haupts Adhesive:

1.0g of gelatin dissolved in 100ml distilled water at 30°C. When completely dissolved, add 2 g phenol crystal and 15 ml glycerol, and mixed them well.

G. 4% aqueous solution of formalin for disinfecting the slides

H. Stains: - haematoxylin and eosin, and

F. Glass slides and coverslips.

Procedure: - Liver of 12 weeks DBN treated mouse was used for this study. Freshly excised liver was cut into pieces and fixed in the freshly prepared Bouin's fluid for 24 hours. Excess fixative was washed overnight under running tap water. Fixed tissue was then dehydrated by placing in an increasing alcoholic grade of 30%, 50%, 70%, 90% and 100% successively. The changes were performed at an interval of 2 hours in each grade and with two changes each in 90% and absolute alcohol. Alcohol was then cleared by placing the tissue in xylene for about 5 hours till it become translucent. Wax infiltration steps were carried out at 55 °C by transferring the tissue onto a 1:1 mixture of xylene and wax and left it for 1 hour. Again, the tissue was transferred to 100% molten wax for 1-2 hours and the same step was repeated twice. Then tissue was allowed to embed in wax by cooling it gradually. The embedded tissue was cut in ribbons of thin sections (7 μm thick) in a microtome. The ribbons were mounted on slides smeared with Haupt's adhesive, which was disinfected with 4% formalin. The sections were warmed. Slides were then left overnight for drying. Mounted slides were immersed in xylene to de-wax. Rehydration was done by dipping successively in decreasing grades of alcohol: 100%, 90%, and 70%. The sections were then stained with haematoxylin for 10-15 minutes followed by washing the excess under running tap water. Slides were then dipped in 70% alcohol for few seconds and counterstained with eosin for 5-10 min. Excess stain was again washed off with running tap water. The stain sections were now successfully treated with 70%, 90% and 100% alcohol, and the alcohol was cleared in xylene. A drop of DPX was allowed to drop on the slide and cover slip was placed carefully on it. Thus prepared slides were left to dry for two days

after which they were evaluated under phase contrast microscope and got them photographed.

3.2.8 PREPARATION OF HEPATOCYTES

Intact hepatocytes were isolated by the application of the collagenase treatment of the perfused liver. The method followed has been described earlier by Fry and Seglen (Fry, J. R., 1981; Seglen, P. O., 1994). This method involved a two-step procedure, in which the mouse liver is perfused first with a Ca^{2+}- free buffer to separate the desmosomal cell contacts, then with collagenase and Ca^{2+} to dissolve the extracellular matrix.

Requirements: Reagents prepared for perfusion were:

A. Perfusion buffer concentrate: 207.5g NaCl, 12.5 g KCl, 60 g Hepes, and 6 g solid NaOH; water to 1000 ml.

B.Collagenase buffer concentrate: 1.25 g collagenase (Sigma type IV) was dissolved in 200 ml water, and 1.75 g $CaCl_2.2H_2O$ was added to it. 10 g NaCl, 1.25 g KCl, 60 g Hepes, and 6.6 g solid NaOH was dissolved in 250 ml water. The two solutions were mixed and water was added to 500 ml. The solution was filtered through a Millipore serum filter.

C. Calcium concentrate: 4.5 g $CaCl_2.2H_2O$ to a final volume of 500 ml in water.

D. Ca / Mg concentrate: 1.3 g $MgCl_2.6H_2O$, and 1.8 g CaCl. $2H_2O$ dissolved to a final volume of 500 ml in water.

E. Suspension buffer concentrate: 40 g NaCl, 4.0 g KCl, 1.5 g KH_2PO_4, 1 g Na_2SO_4, 72 g Hepes, 65 g tricin, and 21 g solid NaOH; water to1000 ml.

Working solutions:

F. Perfusion buffer: To 20 ml perfusion buffer concentrate water was added to 500 ml and pH adjusted to 7.4 at 37 °C. This was stored overnight at 37 °C covered with a parafilm in which a small puncture was made for the outlet of excess gas on heating.

G. Collagenase buffer: 10 ml collagenase buffer concentrate was made up to a final volume of 50 ml with double distilled water and pH adjusted to 7.6 at 37 °C. This was stored at 4 °C and warmed to 37 °C for 15 minutes prior to use.

Reagents for liver dissociation and hepatocytes purification:

(i). Suspension buffer: 20 ml suspension buffer concentrate was mixed with 10 ml Ca / Mg concentrate and water was added to 200 ml; pH was adjusted to 7.6 at 37 °C.

(ii). Wash buffer: 20 ml perfusion buffer concentrate was mixed with 10 ml calcium concentrate, and water was added to 500 ml, pH was adjusted to 7.4 at 37 °C.

Procedure: Mouse was anesthetized with chloroform. Abdominal cavity sterilized thoroughly with alcohol soaked cotton and then cut open to locate the portal vein through which perfusion was done with perfusion buffer followed by collagenase treatment, both of which were pre-warmed at 37 °C for about 10 minutes each. During

this period, circulating blood was flushed out and the liver increases approximately double the original size.

After perfusion the liver was chopped into pieces and transferred to a wide petridish containing 80-100 ml of ice-cold suspension buffer. The liver was held in the portal connective tissue with forceps and the cells were carefully raked out. The suspension was incubated at 37 °C for 30 minutes and hepatocytes are purified by repeated centrifugation at 4 °C for 3 min. each in flat bottomed centrifugation tubes by gentle resuspending the cells in ice-cold wash buffer each time. The yield of viable cells was checked by trypan blue exclusion method. This preparation was used for BrdU incorporation and cell viability assays.

3.2.9 DETERMINATION OF VIABLE CELL NUMBER

The hepatocytes prepared as described above were tested for viability using the trypan blue exclusion method using Neubaur haemocytometer. This is based on the principle that only the non-viable cells will take up the dye, when the cells are exposed to it.

Requirements: Trypan blue solution (0.6%, isotonic):- 150 mg trypan blue and 120 mg NaCl was dissolved in 25 ml of warmed double distilled water. The solution was filtered and stored in freeze.

Procedure: 1: 2 volumes of cell suspension (as prepared above) and trypan blue solution were taken in a test tube. The mixture was incubated at 37 °C for 10 minutes

after which a small amount was transferred onto the counting chamber with the cover slip on. Ten squares in total (five squares, four corner most and the middle one from each chamber) were counted and thereafter the total cell population of the cell suspension was determined.

Calculations: Add the total number of cells in a total of ten chambers (five from one side and five from the other) to give the total number of cell in 1×10^{-3} ml (1×10^{-4} ml/ squares = a volume of 10^{-3} ml). Multiply the total number of cells by 1000 and dilution factor to give the total number of cells/ ml in the sample counted.

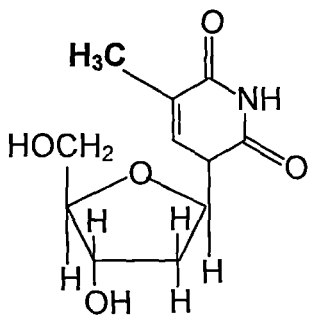
Cell viability (%) = total viable cells (unstained) / total cells (stained and unstained cell) ×100.

From the prepared cell suspension, the required volume of cell suspension that would give the required number of cells was calculated and used for BrdU assay.

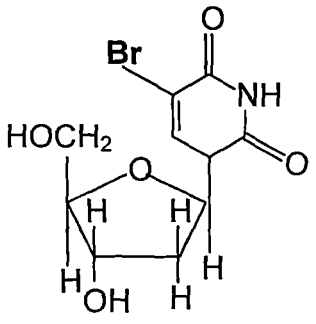
3.2.10 BrdU INCORPORATION ASSAY

Molecular structure of thymidine and BrdU .

Thymidine
(5-methyluracil-2'-deoxyribose)



5-Bromo-2'-deoxyribose
(5-Bromouracil-2'-deoxyribose)



Cell proliferation and DNA synthesis were studied by monitoring the incorporation of BrdU, an analog of thymidine, as described by Gratzner (Gratzner, H. G., 1982). 5-Bromo-2'-deoxy-uridine Labelling and Detection Kit III from Roche Allied Scientific, Mannheim was used for the assay.

Principle: The assay is a cellular immunoassay, which uses a mouse monoclonal antibody directed against BrdU based on the ELISA principle. Cells cultured in a microtiter plate are incubated with BrdU, which gets incorporated into newly synthesized DNA of proliferating cells in place of thymidine. Incorporated BrdU is detected with the monoclonal anti-BrdU-POD, Fab fragments, and the bound conjugate is visualized with the soluble chromogenic substrate ABTS and measured spectrophotometrically at 405 nm using an ELISA reader.

Requirements: The following reagents were supplied in the kit.

A. BrdU labeling reagent (10 mM 5-bromo-2'-deoxyuridine in PBS), which was prepared by diluting the stock in the ratio of 1:100 with sterile culture medium (final concentration 100 μM),

B. Fixdenat (ready to use),

C. Anti-BrdU POD stock solution: dissolve Anti-BrdU POD in 1.1 ml double distilled water for 10 min and mix thoroughly,

D. Anti-BrdU POD working solution: dilute Anti-BrdU POD stock solution in ratio of 1:100 with antibody dilution solution (supplied in the kit),

E. Washing solution: dilute washing buffer concentrate 1: 10 with double distilled water,

F. Substrate solution: tetramethyl-benzidine (TMB) (ready to use).

Additional requirements were:

a. Sterile cell culture medium, supplemented with 10% FCS, L-agenine, L glutamine, and L-aspartate,

b. Sterilized double distilled water, and

c. Hepatocytes: prepared as described in section 2.1.7

Procedure: 1×10^6 (approx.) cells were taken in 200 µl culture medium and incubate for 10-12 hours under humid condition at 37 °C. Add 20 µl / well BrdU labeling solution (final concentration: 20 µM BrdU) and reincubate the cells for 2 - 24 h at 37 °C. Centrifuged the cells at 300 x g for 10 minutes and remove the labeling medium by flicking off. Cells were dried using hair-dryer for about 15 minutes. Add 200 µl / well FixDenat to the cells. Incubate for 30 minutes at 15 – 25 °C. Remove FixDenat solution thoroughly by flicking off and tapping. Add 100 µl/ well anti-BrdU-POD working solution. Incubate for approximately 30 minutes at 15 - 25 °C. Remove antibody conjugate by flicking off and rinse wells three times with 200 µl -300 µl / well washing solution. Remove washing solution by tapping. Add 100 µl / well Substrate solution. Incubate at 15 - 25 °C until color development is sufficient for photometric detection (5-30 min). The color developed was read at 405 nm. This amount of color product

measured was directly proportional to the amount of BrdU, which was incorporated during the incubation period.

3.2.11 GENOMIC DNA ANALYSIS

Genomic DNA was extracted using a method originally described by Blin and Stafford (Blin, N. and Stafford, D.W., 1976) with slight modification.

Requirements:

A. Phosphate buffered saline

10 mM, pH 7.4

B. Digestion buffer

100 mM NaCl

10 mM Tris –Cl (pH, 8.0

25 mM EDTA, pH, 8.0

0.5 (w/v SDS)

(Proteinase K was added fresh for each use at a concentration of 0 .1mg/1ml)

C. Phenol/Chloroform/isoamylalcohol

(25:24:1v/v)

D. Ammonium acetate (7.5 mM)

E. 70% and absolute ethanol

H. TE buffer (pH, 7.6)

10 mM Tris.Cl

1 mM EDTA

I. 1 % agarose in TAE

J. Sample dye (6x)

40 % sucrose

0.2 % bromophenol blue

K. Running buffer (TAE), pH 8.4

40 mM Tris acetate

1 mM EDTA

L. Ethidium bromide Staining solution

0.3g / ml H_2O

M. Destaining (dd H_2O)

Procedure: 100 mg of liver tissue was chopped and suspended in ice-cold PBS. The tissue debris was removed by spinning at low rpm and cells were collected by further spinning at 3000 rpm for 5 minutes. Digestion buffer (1.2 ml / 0.1 g tissue) was added to it and incubated at 50°C for 12–18 hrs. It was then extracted by equal volume of solution C. Vortex vigorously for 10 sec and let it settled for 2-3 hrs. The top aqueous extract was carefully collected in a new test tube. To 1.0 ml of the extract, 0.5 ml of ammonium acetate and 2.0 ml of absolute ethanol was added and centrifuged at 3000 rpm for 2 minutes. The pellet was rinsed thrice with 70 % alcohol by centrifugation at 10000 rpm for 2 minutes each and dried it in incubator at 37 °C. DNA thus isolated was dissolved in TE buffer, which was then electrophoresed on 1 % agarose at 100V for an hour. Gel was stained for 15 min and destained for another 15 min. The DNA

intercalated ethidium bromide fluorescence was visualized on a transilluminator and photographed.

3.3.1 GELONIN ENTRAPMENT INTO LIPOSOMES

Dry film method was chosen for the preparation of liposomes. This method is simple and highly reproducible. Also the biomolecules entrapped in liposomes retains its biological activity after the entrapment procedure though its entrapment efficiency is low. Two different combinations of lipids and phospholipids i.e. DPPC: Cholesterol: DCP, DPPC: Cholesterol: PG were tried out by this method for encapsulating the gelonin.

Requirements: Requirements are as follows:

A. DPPC (75 mg in 5 ml of chloroform and methanol in the ratio of 1: 1),

B. Cholesterol (37.5 in 5ml 1:1 of chloroform and methanol),

C. DCP (15 mg in 5 ml 1: 1 of chloroform and methanol),

D. PG,

E. Nitrogen gas ($N_2\uparrow$), and

F. Tris buffered saline (10 mM), pH 7.4).

Procedure: -DPPC: Cholesterol: DCP and DPPC: Chol: PG in the molar ratio of 1.0: 0.9: 0.25 and 0.9: 0.25: 0.5 respectively were mixed in chloroform and methanol (1:1) in test tube. The solvents used were evaporated by flushing with nitrogen gas ($N2\uparrow$)

through the lipid mixture, alternately dipping the test tube in a water bath, whose temperature was maintained at 30 °C – 35 °C, till a fine film was formed around the inner wall of the test tube. The lipid film was solubilized again in minimum amount of $CHCl_3$ (~250 µl) followed by vortexing. Solvent used wasagain flushed with nitrogen gas and while doing so the tube was rotated thereby making thin film around the inner wall of the tube. Gelonin prepared in tris-buffered saline at a concentration of 2 mg/ml was added followed by vortexing for about 15 – 20 minutes until milky suspension was formed. The resultant suspension was subjected to centrifugation at 20,000 x *g* for 30 min. at 4 °C. The supernatant was collected while the pellet was resuspended in tris buffered saline and re-centrifuged under the same conditions. The resultant supernatants were pooled. The concentration of unentrapped (free) gelonin was determined in the pooled supernatant, and then the percent entrapment efficiency of gelonin into the liposomes was determined. The pellet containing liposome encapsulated gelonin was resuspended in tirs buffered saline and used for *in- vitro* and *in- vivo* studies.

3.3.2 DETERMINATION OF LIPOSOME UPTAKE BY VARIOUS ORGANS

Uptake of liposomes by various organs *in vivo* was determined by measuring the incorporation of radioactivity into the tissues upon administration of liposomes containing ^{3}H-cholesterol.

Requirements: Requirements are as follows:

A. DPPC (75 mg in 5 ml of chloroform and methanol in the ratio of 1: 1),

B. Cholesterol, [1, 2-^{3}H (N)]-,

C. DCP (15 mg in 5 ml 1: 1 of chloroform and methanol),

D. PG,

E. Nitrogen gas ($N_2\uparrow$), and

F. Tris-buffered saline (10 mM), pH 7.4), and

G. Scintillation cocktail.

Procedure: Lipsomes were prepared as described above, except that the Cholesterol was replaced with ^{3}H-Cholesterol. From the prepared liposomal preparation, 100 µl of which was was drawn and administered into the mouse through intravenous route. After 30 minutes, animals were sacrificed by cervical dislocation. Tissues such as liver, kidneys and spleen were quickly excised, rinsed, weighed and a homogenate (50%) was made in the tris-buffered saline which was then subjected to centrifugation at 18000 rpm for 30 minutes. The resultant supernatant was separated from the pellet. Radioactivity was counted both in the supernatant and pellet after adding scintillation cocktail and the total radioactivity was calculated.

3.4.1 *IN-VITRO* INHIBITION EFFECTS OF FREE AND LIPOSOME ENCAPSULATED GELONIN

Hepatocytes (~1 x 10^6) were incubated in the presence of free gelonin and liposome encapsulated gelonin in a culture medium in a humidified atmosphere for 16 hr. The inhibition effects were monitored by determination of the number of viable cells as described in method section 3.2.9.

Requirements: The requirements are as follows:

A. Culture medium (DMEM) supplement with 10% FCS, L-argenine, L-glutamine, and L-aspartate,

B. Hepatocytes suspension (as described above in method section 3.2.8),

C. Free and liposome encapsulated gelonin preparation above,

D. PEG (10 mg/ml), and

E. Trypan blue (as prepared above)

Procedure: Assay was performed in triplicate. Isolated hepatocytes ($\sim 1\times10^6$) as discussed above in the method section 3.2.8 were suspended in each culture tube with 1 ml of culture medium (solution A). Four sets of three tubes each were made. In the first set 100 μg of free gelonin, in second and third sets liposome encapsulated gelonin (100 μg equivalent) was added. In addition to that 100 μg PEG was also added to the third set. The fourth set of tubes containing hepatocytes only served as control. Final volume of each tube was made equal with tris-bufferred saline (10 mM, pH 7.4). Incubation was carried out for 16 hrs at 37 ^{0}C in a humidified atmosphere of 5% CO_2 and rest air. After incubation, the hepatocytes were tested for their viability using the Trypan blue exclusion method as mentioned in the section 3.2.9.

3.4.2 *IN-VIVO* INHIBITION EFFECT OF LIPOSOME ENCAPSULATED GELONIN

Healthy mice, 6 - 8 weeks old, were selected for this study. The mice were administered aqueous solution of DBN (10 mg kg-^{1} body weight) by intravenous

route at weekly interval for a period of 12 weeks. Upon complete exposure, the experimental mice were divided in two groups. The first group received liposome encapsulated gelonin (100 μg equivalent) thrice by intravenous route at weekly intervals. Second group of mice without any treatment served as control. Mice from both the groups were sacrificed one, two and four weeks after gelonin administration to study the effect of free and liposome encapsulated gelonin on tumor regression.

Experimental, control and normal mice were killed by cervical dislocation and the liver was quickly excised and removed, rinsed in chilled normal saline (0.9 % NaCl), blotted dry and weighed. A 10% homogenate was prepared in chilled 0.25 M sucrose solution and then centrifuged at 20,000 × *g* for 30 min at 4 °C. The resulting supernatant was used for the enzyme assays i.e. GGT, AChE, and GST as discussed in method sections 3.2.2 - 3.2.4. Also glutathione level measured as described in method section 3.2.5. In addition to the above parameters, histology, viability assessment, DNA synthetic index and electrophoretic study of genomic DNA were also carried out in liver cells using the methods described in the method sections 3.2.7, 3.2.9, 3.2.10 and 3.2.11.

CHAPTER 4

RESULTS

4.1.1 PURIFICATION OF GELONIN

Single chain RIPs like luffin, gelonin etc. are normally basic proteins. Based on this property, soluble proteins extract from seeds were initially purified by cation-exchange chromatography. The elution profiles of CMC-52 chromatography of the two different batches of gelonin are depicted in Figure 4.1. Slight variations in the profiles may be attributed to the age of the seeds and seasons of harvesting. Two peaks were observed. The major peak (fraction 52-100) contained gelonin. The major peak fractions were pooled, concentrated and subjected to gel-filtration on Sephacryl S-200HR column.

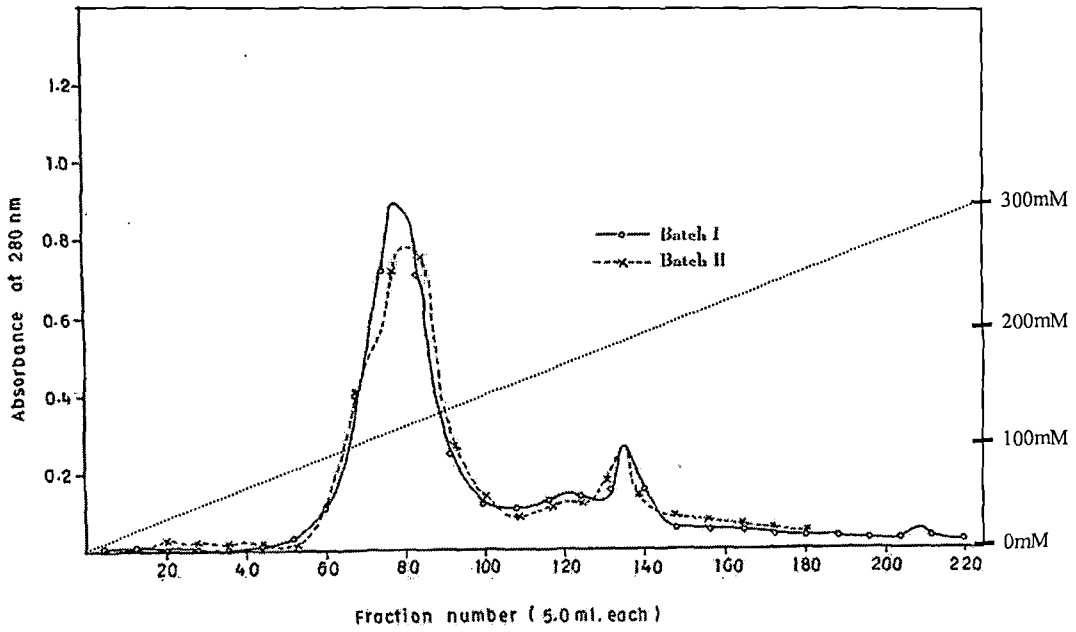


Fig.4.1: Elution profile of CMC-52 column. The bound protein was eluted with 0.0mM – 300 mM Nacl at a flow rate of 25 ml/hr at 4°C and fractions were read at 280 nm. The major protein peak (fraction 52-100) were pooled concentrated and further subjected to gel filtration chromatography on Sephacryl S-200HR column.

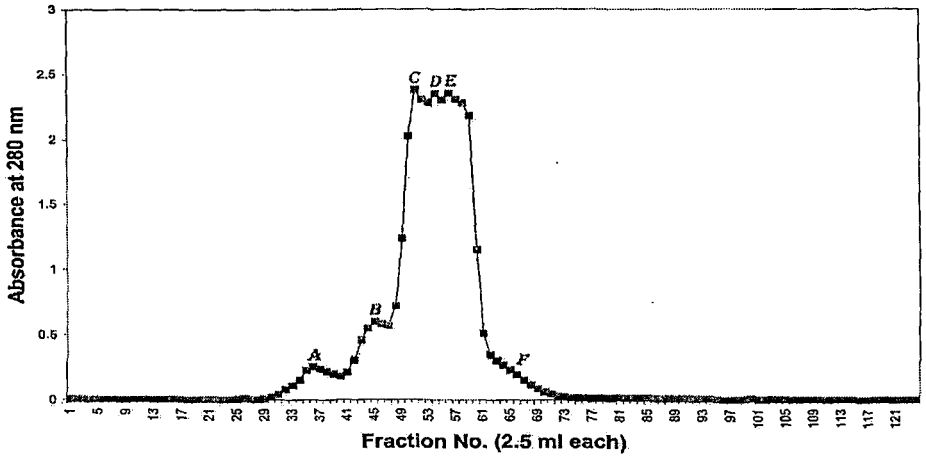


Fig. 4.2: The elution profile of gelonin (batch-I) on Sephacryl S-200HR column. The fractions (52-100) as indicated in Fig.4.1 were pooled, concentrated and loaded on Sephacryl S-200HR column (2.5 x 120 cm). Six peaks were identified. The maximum amount of gelonin was eluted out in the fractions corresponding to peaks C, D and E.

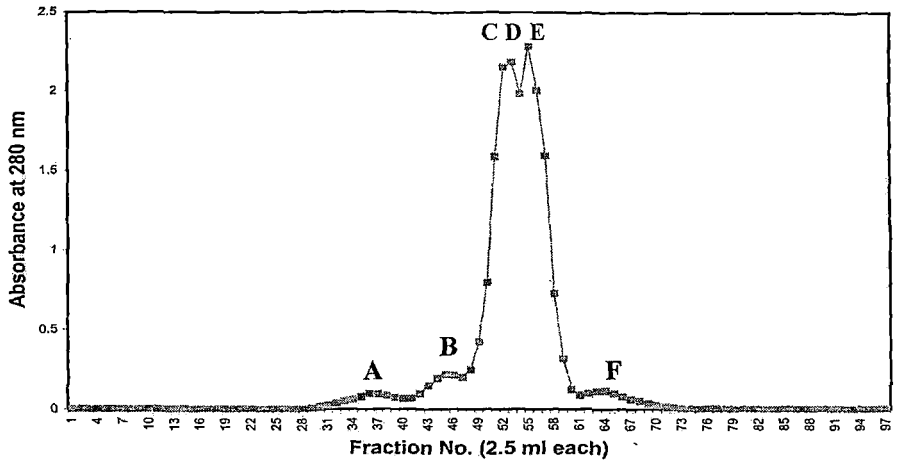


Fig. 4.3: The elution profile of gelonin (batch-II) on Sephacryl S-200HR column. The fractions (52-100) as indicated in Fig.4.1 were pooled, concentrated and loaded on Sephacryl S-200HR column (2.5 x 120 cm). Six peaks were identified. The maximum amount of gelonin was eluted out in the fractions corresponding to peaks C, D and E.

Elution profiles of gelonin on Sephacryl S-200HR of batch-I & II are shown in Figures 4.2 and 4.3 respectively. Six peaks marked as A, B, C, D, E and F corresponding to fraction number 31-40, 42-47, 48-50, 51-57, 58-60 and 61-70 respectively appeared in the elution profile. Fractions corresponding to each peak were pooled together and concentrated to run on SDS-PAGE.

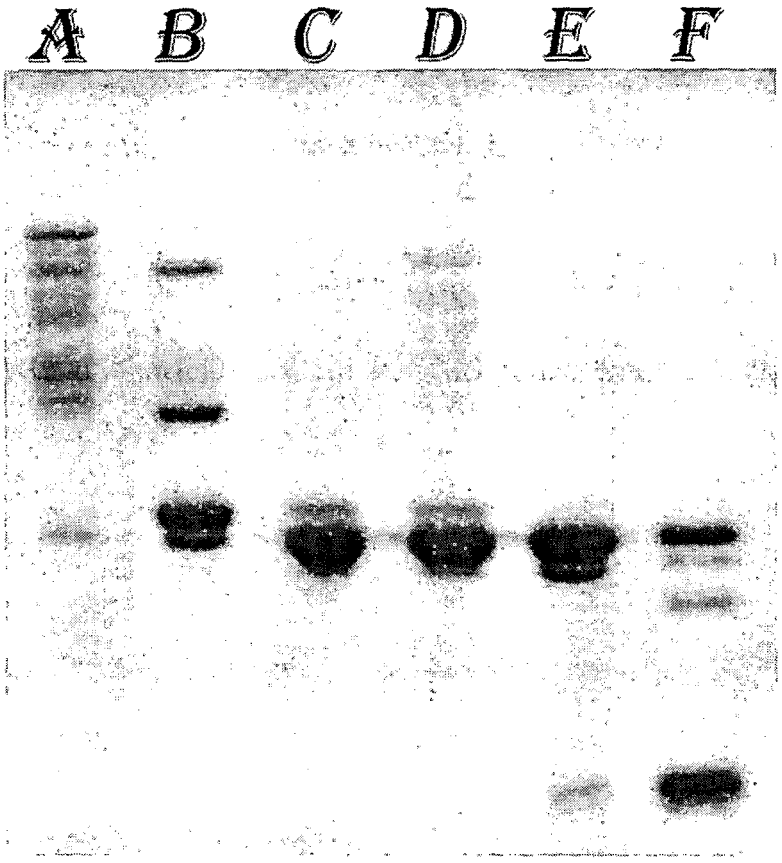


Fig. 4.4: A representative SDS-PAGE analysis of gelonin. The protein was run in a gel consisting of 10% acrylamide. The gel was fixed in methanol/acetic acid; stained with Coomassie Brilliant Blue and destained with methanol/acetic acid for 24 hr. Lanes A to F correspond to the six peaks as obtained in Fig. 4.2 & 4.3.

A representative SDS-PAGE analysis of peaks A-F performed under reducing condition is shown in Fig. 4.4. Peaks C, D and E showed high intensity bands that correspond to gelonin. The homogeneity and molecular weight of gelonin were determined by SDS-PAGE analysis which was run along with the appropriate molecular weight markers under reducing condition.

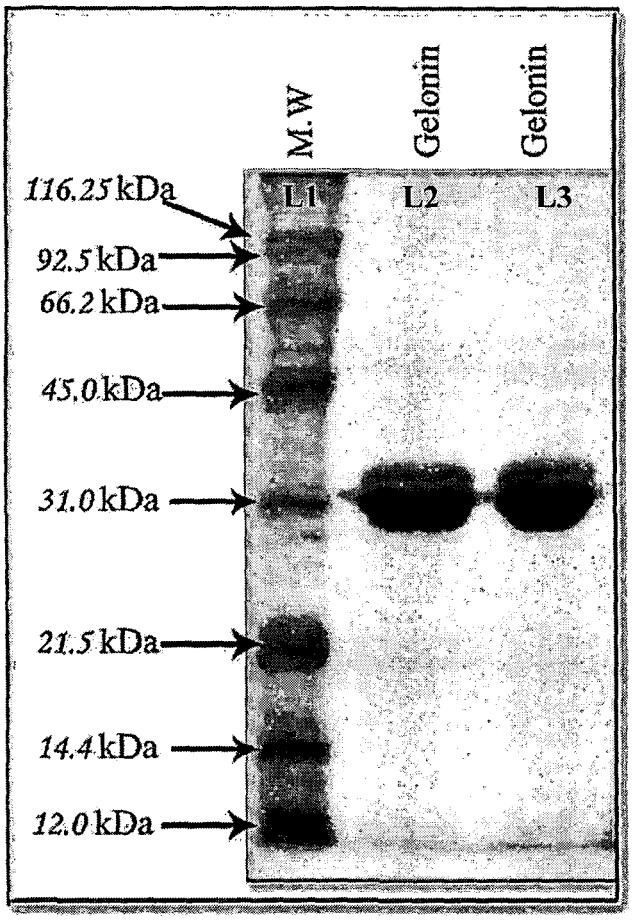


Fig. 4.5: SDS-PAGE analysis of gelonin purified on Sephacryl S 200HR. The protein was run on 10% acrylamide gel. The gel was fixed in methanol/acetic acid, stained with Coomassie Brilliant Blue and destained with methanol/acetic acid for 24 hr. Lane-1, molecular weight markers. Lane 2 and 3 are gelonin from two different batches purified on Sephacryl S 200HR.

The results are shown in Figure 4.5. Molecular weight of gelonin was found to be ~30 kDa in both the preparations.

4.1.2 INHIBITORY ACTIVITY OF GELONIN ON PROTEIN SYNTHESIS

The cell free translation experiment was carried out in presence of purified preparation of gelonin. Figure 4.6 shows a relationship between the percentage protein synthesis inhibition with the different doses of gelonin and luffin. The quantitative data are recorded in Table 4.1. The percentage of ribosome-inactivating

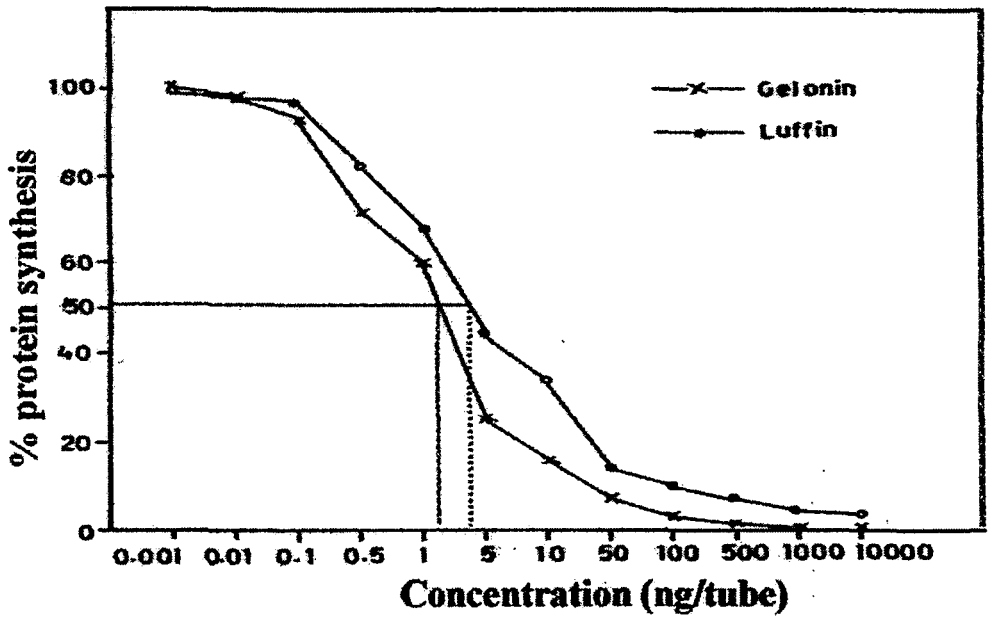


Fig. 4.6: The figure shows the protein synthesis inhibition curve of gelonin and luffin. Protein synthesis was measured by counting the ^{3}H-Leucine incorporated into TCA protein precipitate. Percent protein synthesis inhibition was calculated by measuring ED_{50} of gelonin and luffin. ED_{50} of gelonin = 2.25 ng and ED_{50} of luffin = 4 ng.

activity was determined by measuring ED_{50} i.e. the dose of test sample required for 50% inhibition of protein synthesis. The RIP activity of luffin was also measured and compared with that of gelonin. Gelonin ED_{50} was found to be two fold higher than that of luffin when calculated from the inhibition curve Figure 4.6. This observation is of great importance as eventually this would be used *in vivo* cytotoxicity.

Table 4.1: Ribosome-inactivating activity of various doses gelonin and luffin obtained from the seeds of *Gelonium multiflorum* and *Luffa cylindrica* respectively.

Concentration (ng/ml)	Protein synthesis (%)	
	Gelonin (mean ± SD), n = 3	Luffin (mean ± SD), n = 3
0.001	100 ± 0.00	100 ± 0.00
0.01	97.70 ± 1.33	97.30 ± 1.01
0.1	93.26 ± 0.72	95.20 ± 0.40
0.5	72.20 ± 1.54	81.86 ± 1.10
1.0	61.26 ± 2.93	68.43 ± 1.10
5	24.76 ± 0.96	43.83 ± 2.14
10	16.20 ± 0.30	34.26 ± 0.75
50	7.43 ± 0.21	14.00 ± 0.72
100	3.26 ± 0.31	10.20 ± 0.30
500	1.16 ± 0.15	7.16 ± 0.34
1000	0.63 ± 0.15	4.16 ± 0.15
10,000	0.03 ± 0.005	3.50 ± 0.10

n = number of experiments

4.2.0 CARCINOGENESIS INDUCTION

DBN, a *N*-nitroso compound, is a well established hepatocarcinogen over a wide range of doses with or without promoters in rodents. Interest in the study of this

compound stemmed from the findings that it presents industrial occupational hazards. Carcinogenesis was induced in experimental mice upon chronic exposure of DBN administered by intravenous route.

4.2.1 GENERAL OBSERVATIONS

Mice exposed to DBN showed signs of lethargy and sluggishness. Slight decrease in body weight was also observed. However, when the liver was excised and weighed it was found to be slightly heavier than those of the control age-matched mice (data not shown). The other visible lesions on the liver were formation of nodules, which were grayish-white in appearance in some cases. However, in most of the cases the tissues were found to be swollen and hardened and appeared almost pale in color

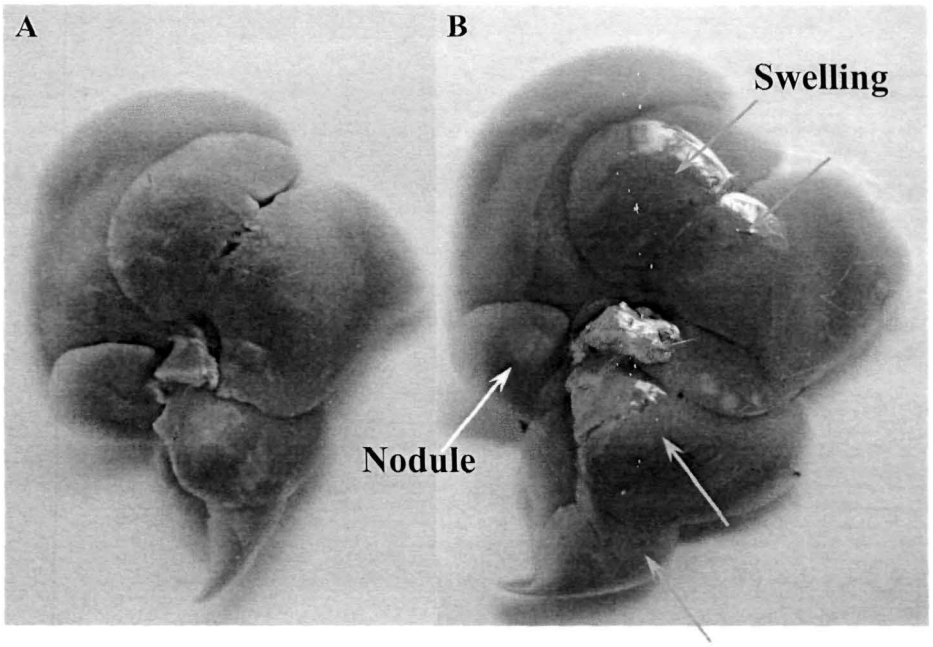


Fig. 4.7: Photographs showing livers. A. Normal, B. DBN-exposed. Arrows show the nodules formation and swelling of the tissue in the DBN-exposed mouse.

whereas in control age-matched mice no such signs were noticed. Preneoplastic lesions in the liver have shown to be suitable markers of hepatic cellular carcinoma (HCC) in rodents (Farber, E. *et al,* 1980; Pitot, H.C. 1979). In other tissues such as spleen and kidneys no such lesions were visible however the weight increases in case of exposed ones (data not given).

4.2.2 GGT ACTIVITY

The GGT activity in the supernatant fractions of liver tissue homogenates was

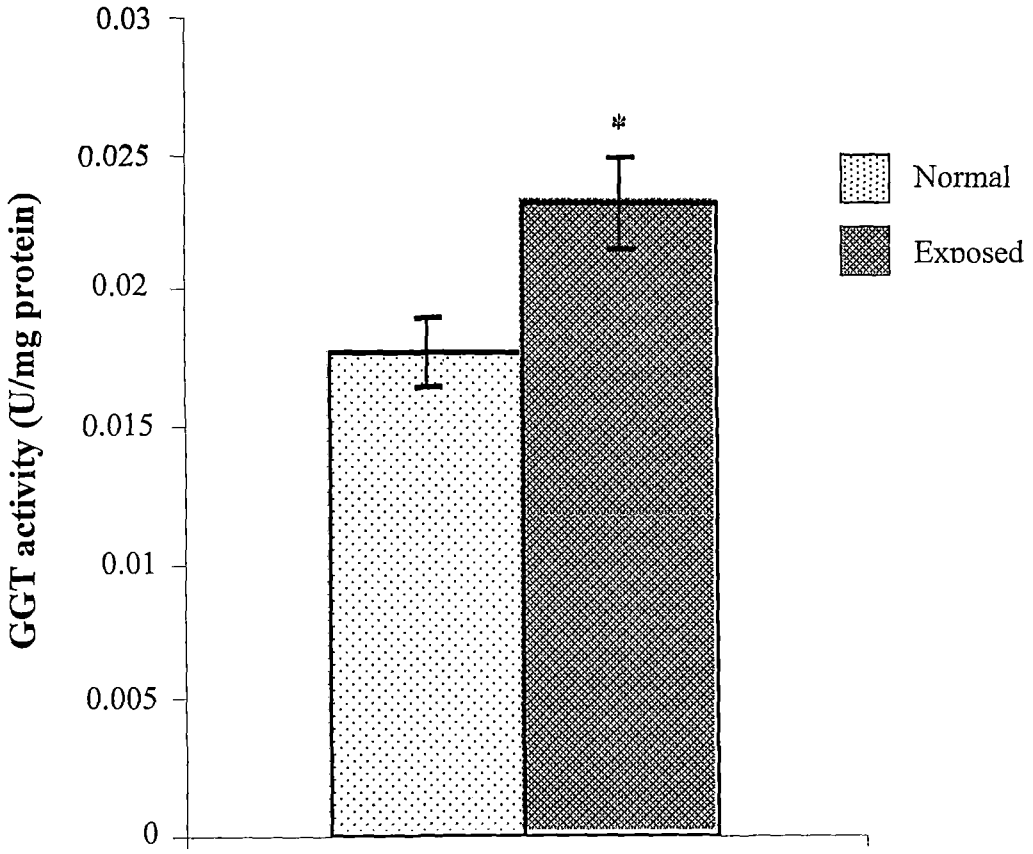


Fig. 4.8: GGT activity in the supernatant fractions of liver tissues of the age-matched control and DBN-exposed mice. The activity shown here was recorded after 12 weeks of DBN exposure. * Statistically significant $P < 0.05$

monitored separately for DBN-exposed and age-matched control mice. The level of GGT activity in the DBN-treated mice was found to be significantly higher as compared to that of the normal control group as shown in Fig. 4.8 & Table 4.2.The activity in the former was elevated from the eighth week of treatment onwards (data not shown). The level increases progressively with successive weeks of DBN exposure.

4.2.3 AChE ACTIVITY

AChE activity was monitored in the supernatant fractions of liver tissue homogenates obtained from both the normal control and DBN-exposed mice. The level

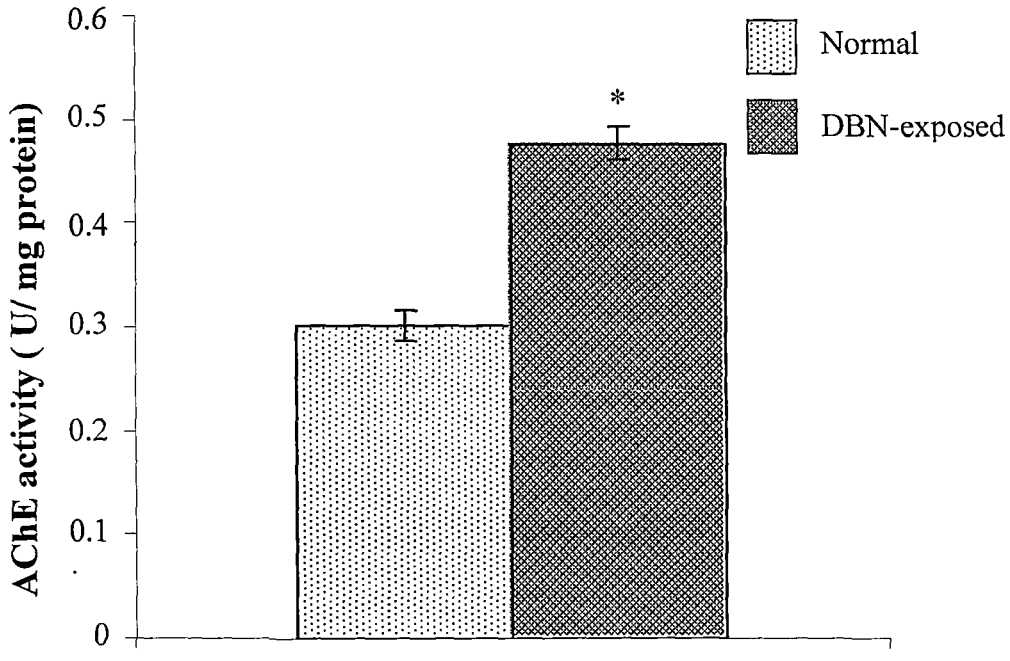


Fig.4.9: Liver AChE activity in the supernatant fractions of the age-matched normal and those of DBN exposed mice. The activity determined in the liver tissue supernatant fractions after 12 weeks of DBN exposure.* Statistically significant, $P < 0.05$

of the enzyme activity was higher in DBN-exposed mice than those of the normal control mice. Enzyme activities recorded after 12 weeks of treatment are shown in Fig.4.9 & Table 4.2. Elevation in the enzyme activity in DBN exposed mice was statistically significant as compared to that of the control group.

4.2.4 GST ACTIVITY

GST activity was determined in DBN-treated and compared with that of the control group of mice. A significant alteration in GST activity was observed as shown

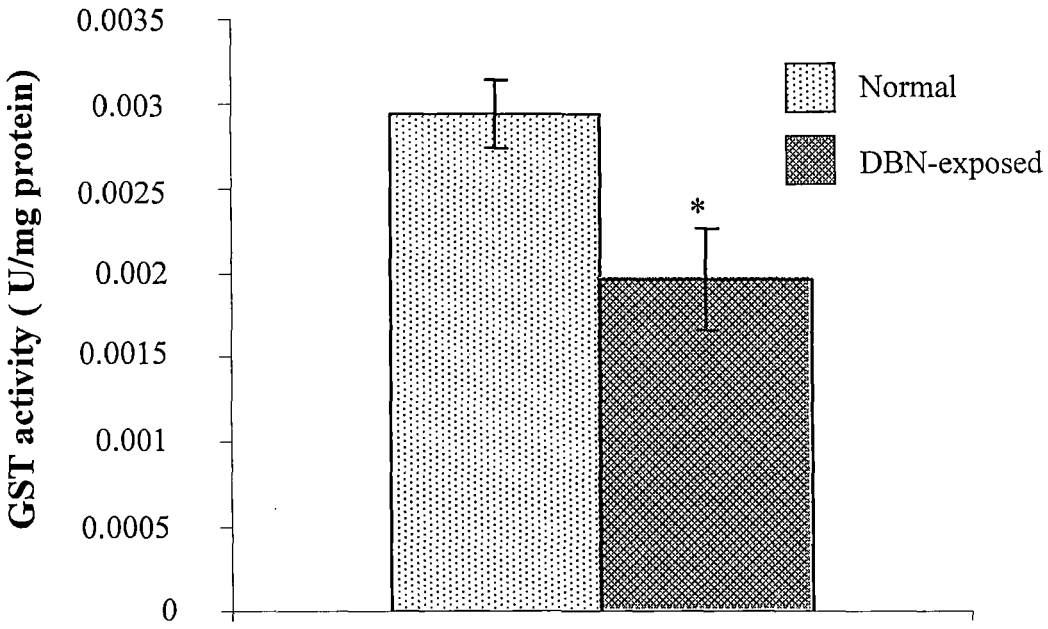


Fig. 4. 10: GST activity in the age-matched normal control and DBN-exposed mice. The activity was determined in the liver tissue supernatant fractions after 12 weeks of DBN exposure. * Statistically significant ($P < 0.05$).

in Figure 4.10 & Table 4.2. GST activity showed a decrease by 1.5 folds after 12 weeks of DBN-exposure.

4.2.5 GSH LEVEL

The GSH level was found to be higher in the DBN-exposed mice than that of the normal control. (Figure 4.11 & Table 4.2). GSH level was monitored after 12 weeks of treatment and compared with those of the age-matched normal control mice. The difference observed in the experimental mice was 1.5 folds higher in case of the exposed group.

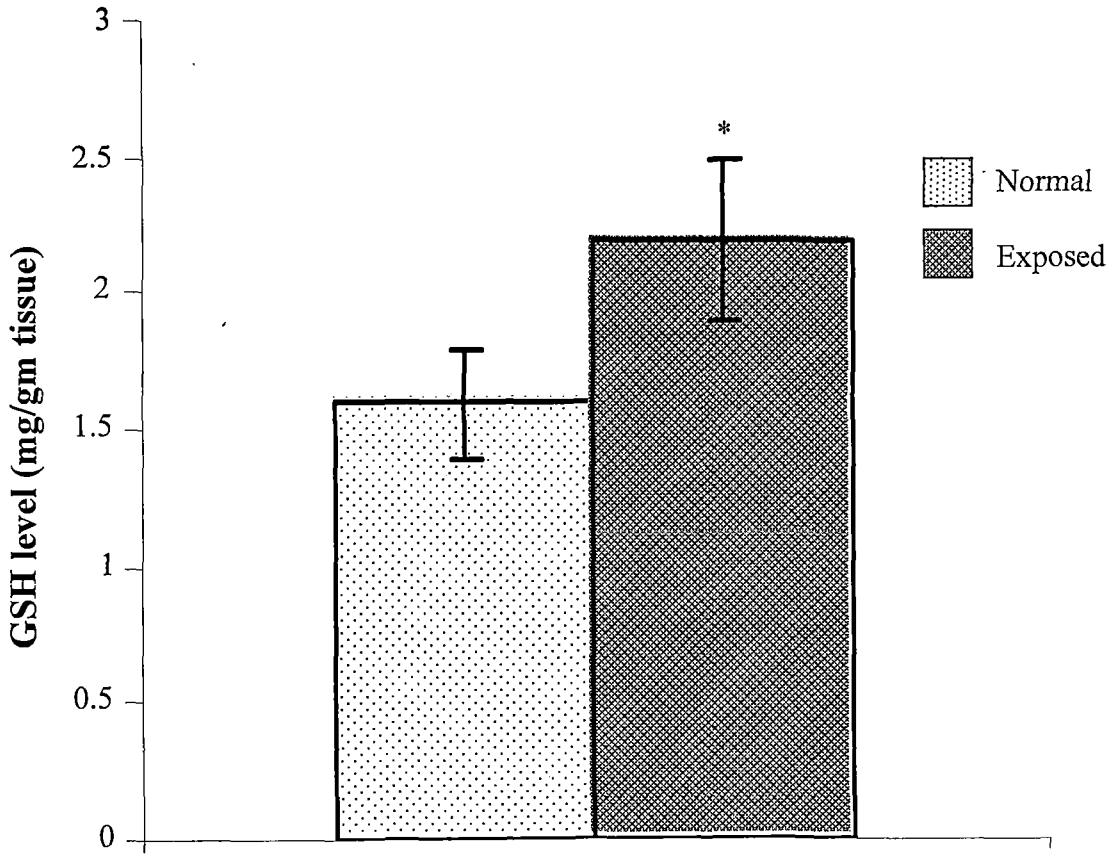


Fig.4.11: GSH levels in the age-matched normal control and DBN-exposed mice. The GSH levels were determined in the liver tissue supernatant fraction after 12 weeks of DBN exposure.* Statistically significant ($P < 0.05$).

Table 4.2: Marker enzymes activities and GSH levels in normal and DBN-exposed mice.

Groups	GGT activity (U/mg protein)	AChE activity (U/mg protein)	GST activity (U/mg protein)	GSH level (mg/g tissue)
Normal Control	0.018 ± 0.002	0.302 ± 0.014	0.003 ± 0.0002	1.624 ± 0.051
DBN-exposed (12 weeks)	0.023 ± 0.003*	0.478 ± 0.016*	0.002 ± 0.0003*	2.398 ± 0.048*

Values are present as mean ± SEM, number of observations,n=10

* *P*, significance, <0.05 as compared to that of the age-matched normal control,

4.2.6 DNA SYNTHETIC INDEX

BrdU incorporation *in vitro* was determined in the hepatocytes of DBN-exposed and age-matched control mice. The results are represented in the bar diagram (Fig. 4.12) and the experimental data are given in Table 4.3. Liver cells from DBN-exposed mice

Table 4.3. BrdU incorporation into hepatocytes of normal control and DBN-exposed mice. Each sample analysed for BrdU incorporation contained ~10^6 viable cells. Absorbance was measured at 405 nm

Groups	Absorbance at 405 nm (Mean ± SEM), n = 8
Age-matched normal	0.183 ± 0.009
DBN-exposed (12 weeks)	0.534 ± 0.019

n = Number of observations

exhibited a marked difference in BrdU incorporation as compared to that of the normal control. The incorporation of BrdU was approximately three folds higher in case of the exposed group.

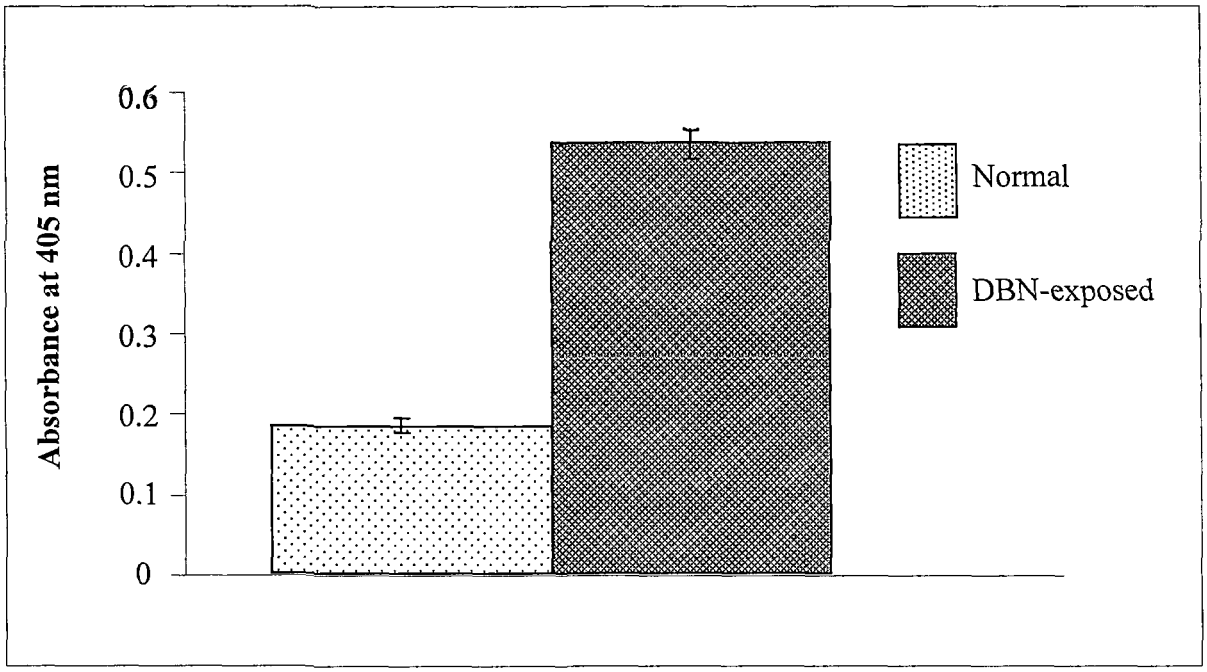


Fig.4.12: BrdU incorporation into hepatocytes of age-matched normal control & DBN-exposed mice.

4.2.7 ELECTROPHORETIC STUDY OF GENOMIC DNA

Genomic DNA extracts from the liver tissues of normal control and DBN-exposed mice were run on 1% agarose gel. DBN-exposed liver cell extract exhibited a more intense and broad band of DNA as compared to that of the normal control as depicted in Fig. 4.13. It was also observed that the DBN-exposed band was lagging to that of the normal control.

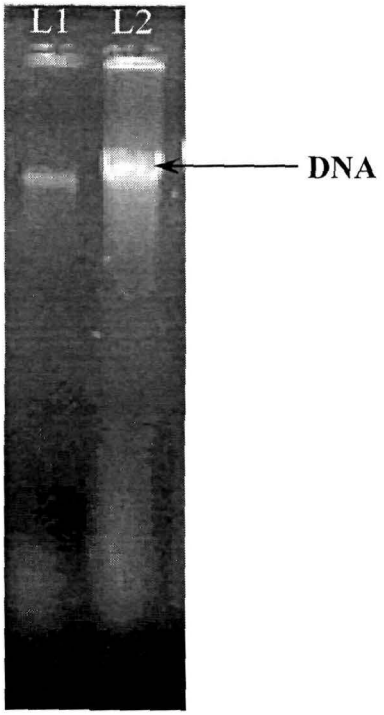


Fig. 4.13: Genomic DNA extracts analysed on 1% agarose gel. The gel was stained with ethidium bromide. L1 = normal control lane and L2 = DBN–treated lane.

4.2.8 HISTOLOGICAL SECTIONS EXAMINATION

The histological sections of liver tissue of DBN-exposed and normal control mice showed distinct differences when examined microscopically (Fig.4.14). Hepatocytes of normal mice had well-defined outlines. The cells were of mono- and bi-nucleated with a regular morphology and appeared to be tightly packed. However, the hepatocytes of DBN–exposed mice lost its regular arrangement showing variations in shape and size. No proper outlines were visible and cells seemed to have lost contact

with their neighboring cells. It was also observed that most of the cells became multi-nucleated.

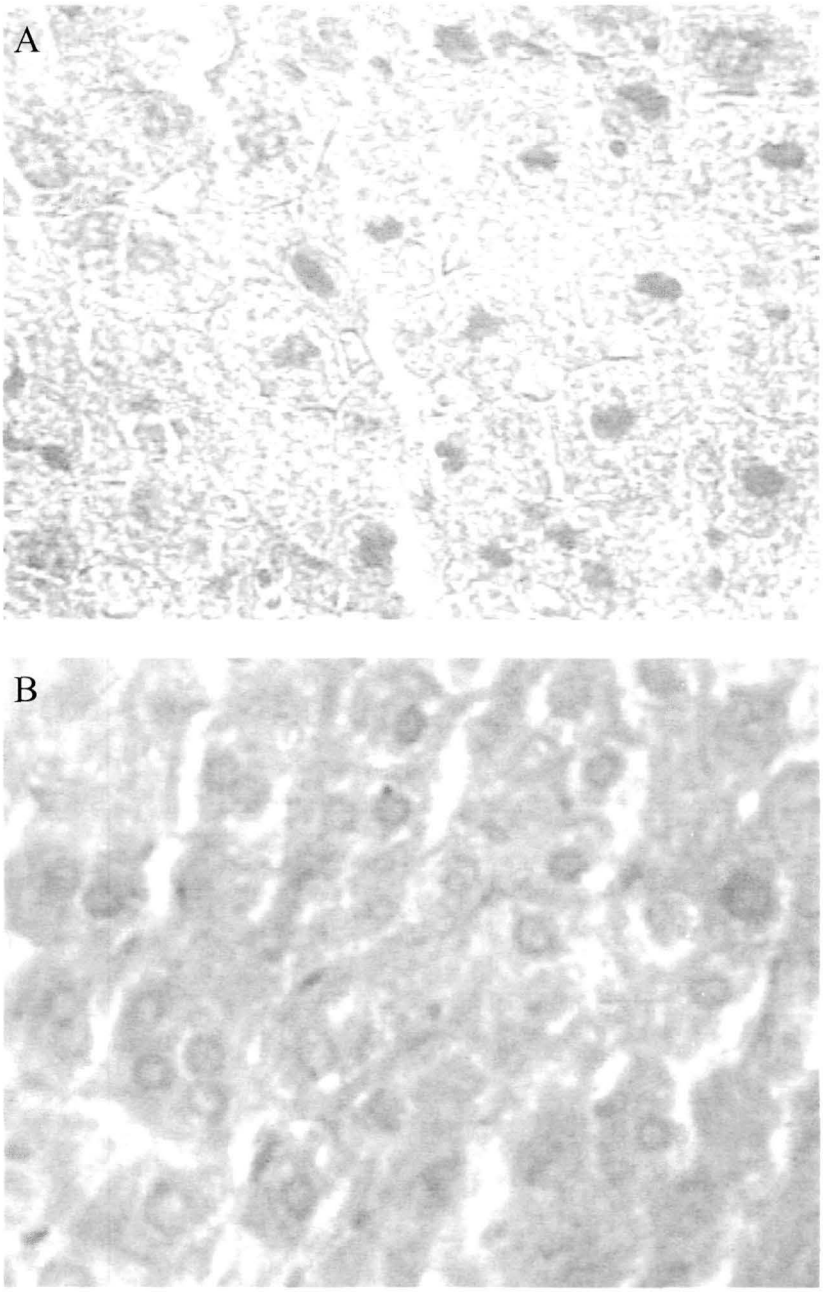


Fig. 4.14: Microphotograph of histological sections of liver tissue from age-matched normal control mouse (A) and DBN-exposed mouse (B). The sections were 7μm thick and stained with haematoxylin and eosin. Magnification: 10 x 40.

4.3.0 CARCINOGENESIS RESPONSE MODULATION BY GELONIN ENCAPSULATED IN LIPOSOME

Ribosome-inactivating proteins (RIPs) are a group of naturally occurring plant and fungal proteins that have been widely studied for their potential applications especially as anticancer agents (Peumans, W. J., *et al*, 2001). Gelonin, a protein extracted from the seeds of *Gelonium multiflorum,* is one of the most interesting RIPs and has been widely used in preparation of conjugates directed against tumor epitopes (Roseblum, M. G. *et al,* 1999; Pagliaro, L. C. *et al,* 1998). One favorable aspect of using gelonin is its low systemic toxicity (LD_{50} 40 mg/Kg body weight) and its low toxicity on intact cells ($IC_{50} \geq 33$ μM on HeLa) due to its incapability of penetrating cell membranes. Furthermore, it is rapidly removed from the blood stream by kidney filtration (Barbieri, L., *et al*, 1990).

Numerous strategies have been employed to effectively deliver gelonin to the cytosol of cancer cells including conjugation to folate, hormones or monoclonal antibodies, etc. (Atkinson, S. F., *et al,* 2001; Singh, V. *et al*, 1989; Rosenblum, M. G. *et al,* 1999). These approaches are predominantly aimed at increasing cell-type specific binding and uptake and in some cases augmenting delivery into the cytosol. In the present investigation our approach has been to encapsulate gelonin into conventional liposomes and study the cytotoxic effect of gelonin on transformed hepatocytes *in vitro* and upon administration *in vivo*. Mice chronically exposed to DBN for 12 weeks were used for this study. The parameters employed to monitor tumor induction were followed to study the tumor regression too.

4.3.1 GELONIN ENTRAPMENT INTO LIPOSOMES

Liposomes were made using two different lipid compositions in the molar ratio as shown in Table 4.4. A total of 8.5 – 9.0 mg of lipids was taken to entrap 2.0 mg of gelonin. The preparations were found to be heterogeneous and multilamellar in nature when observed by phase contrast-microscopy (data not shown). The percentage entrapment effiency gelonin into liposomes was calculated after separating

Table 4.4: Percentage entrapment efficiency of gelonin into liposomes.

Lipid compositions	Molar ratio	Gelonin (µg)	(Mean ±SD) Free (µg)	Entrapped (µg)	%entrapment	n
DPPC:Chol :DCP	1.0:0.9:0.25	2000	1370 ± 0.025	630 ± 0.023	31.5 ± 1.30	6
DPPC : Chol : PG	0.9:0.25:0.5	2000	1535 ± 0.021	465 ± 0.021	23.3 ± 1.06	6

n=Number of observations

free from entrapped protein by centrifugation. Liposome consists of DPPC, Cholesterol and DCP exhibited a better entrapment efficiency as shown in Table 4.4.

4.3.2 LIPOSOME UPTAKE *IN VIVO* BY RETICULO ENDOTHE--LIAL SYSTEM

Uptakes of liposomes by Reticulo Endotheleial System (RES) of mice were determined. Liposomes consisting of different lipid compositions as shown in table 4.5

were administered by intravenous route and sacrificed by cervical dislocation after 30 miniutes. Radioactivity was monitored in liver, kidneys and spleen. The results of this

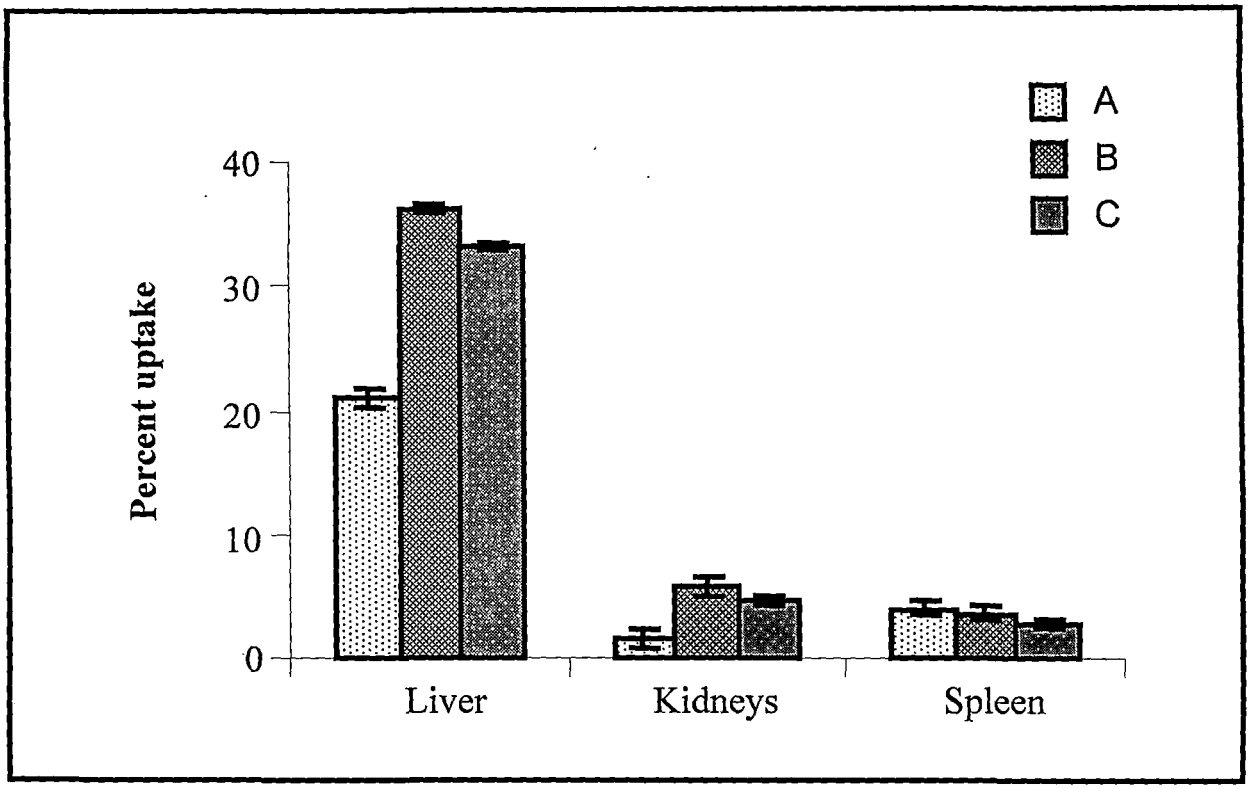


Fig.4.15. Liposome uptake by Reticulo Endothelial System (RES) *in vivo*. Liposomes made up of different lipid compositions with ^{3}H Cholesterol were administered by intravenous route. Mice were sacrificed by cervical dislocation after 30 minutes of administration. Radioactivity monitored in liver, kidneys and spleen and the Percent uptake was calculated.
Lipid compositions: A= DPPC+ DCP+ ^{3}H Cholesterol, B= DPPC + PG +^{3}H Cholesterol, and C= DPPC + PE + ^{3}H Cholesterol

study as shown in Fig. 4.15 and Table 4.5 indicate that the uptake of ^{3}H-labelled liposomes by the liver was invariably significantly very high as compared to that of kidneys and spleen in all the three liposomal preparations. Moreover, the maximum

uptake by liver was observed when the liposomes were prepared from DPPC, PG, ^{3}H Cholesterol and DPPC, PE, ^{3}H Cholesterol respectively.

Table 4.5: Liposomes uptake *in vivo* by Reticulo Endothelial System.

Lipid compositions	% Uptake of liposomes (Mean ± SD)		
	Liver	Kidneys	Spleen
DPPC + DCP + ^{3}H Cholesterol	21.00 ±0.773	3.96 ± 0.089	1.50 ± 0.178
DPPC + PG + ^{3}H Cholesterol	36.25 ±0.818	5.82 ± 0.704	3.58 ±0.429
DPPC + PE + ^{3}H Cholesterol	33.23 ± 0.669	4.43 ±0.536	2.73 ± 0.102

Total Count ^{3}H Chol (10 µl) = 544515 CPM
Number of observations, n =10

4.3.3 *IN VITRO* INHIBITION EFFECTS OF FREE AND LIPOSOME ENCAPSULATED GELONIN

No detectable cell death was observed when transformed hepatocytes culture was incubated with free gelonin (~100 µg). However, when the culture was incubated with same amount of gelonin encapsulated in different liposomal compositions (Table 4.6) cell death was observed in the range of ~ 13- 16%. Further, when the culture was supplemented with PEG (indicated by + sign in the Table 4.6), a slight increase in cell death was observed (~19%).

Table 4.6. *In vitro* inhibition effects of free and encapsulated gelonin on transformed hepatocytes.

Liposomal compositions	Free gelonin (μg)	Liposome Encapsulated gelonin (μg)	PEG (1mg/ml)	% cell death (Mean ± SD)
-	100	-	-	N D
DPPC : Chol : DCP	-	100	-	16.00 ± 0.46
DPPC : Chol : DCP	-	100	+	19.00 ± 0.45
DPPC : Chol : PG	-	100	-	16.00 ± 0.59
DPPC : Chol : PG	-	100	+	18.50 ± 0.30
PC : Chol : PE	-	100	-	13.46 ± 0.64
PC : Chol : PS	-	100	-	13.60 ± 0.46

number of observations, n =8
ND = Not detectable

4.3.4 POST-TREATMENT GGT STATUS

The results of GGT activity of age-matched normal control mice, DBN-exposed mice and those of DBN-exposed mice followed by treatment with liposome-encapsulated gelonin are represented in Fig. 4.16. Liposome-encapsulated gelonin treatment resulted in a downfall of the enzyme activity which reached close to the normal enzyme level (Fig.4.16 C-E). The decrease was more pronounced one week after the treatment. The enzyme activity was monitored up to four weeks after the

treatment. However, a marginal increase in GGT activities was seen after two and four weeks of treatment.

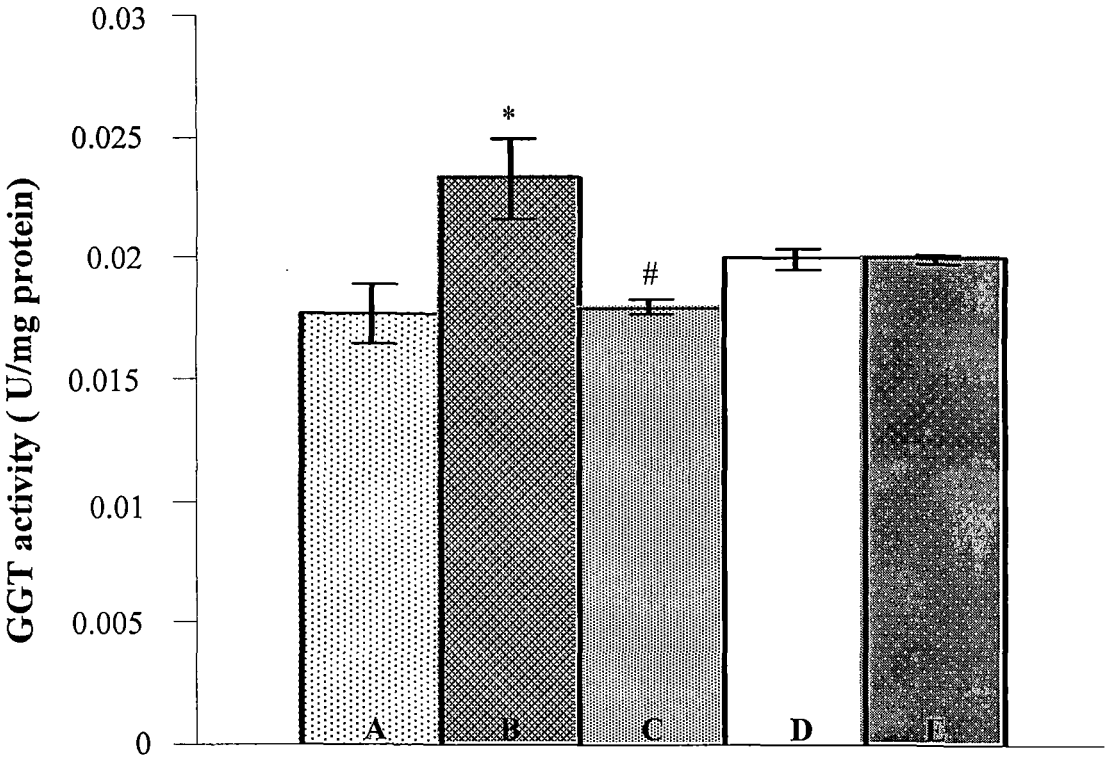


Fig.4.16: GGT activity in the supernatant fractions of the liver homogenate of the normal control (A), DBN-exposed (B) and DBN-exposed followed by treatment with liposome encapsulated gelonin (C-E). Bar C-E represent the enzyme activity at 1, 2 and 4 weeks respectively after the treatment.*statistically significant ($P < 0.05$) in comparison to (A), #statistically significant ($P < 0.05$) in comparison to (B).

4.3.5 POST-TREATMENT AChE STATUS

Liposome–encapsulated gelonin treatment also suppressed the elevated AChE levels observed upon DBN-exposure (Fig. 4.17 C-E). The reduction in the enzyme activity was significant ($P< 0.05$) and was found closed to that of normal control. No

further elevation in the enzyme activity was observed even after four weeks of treatment.

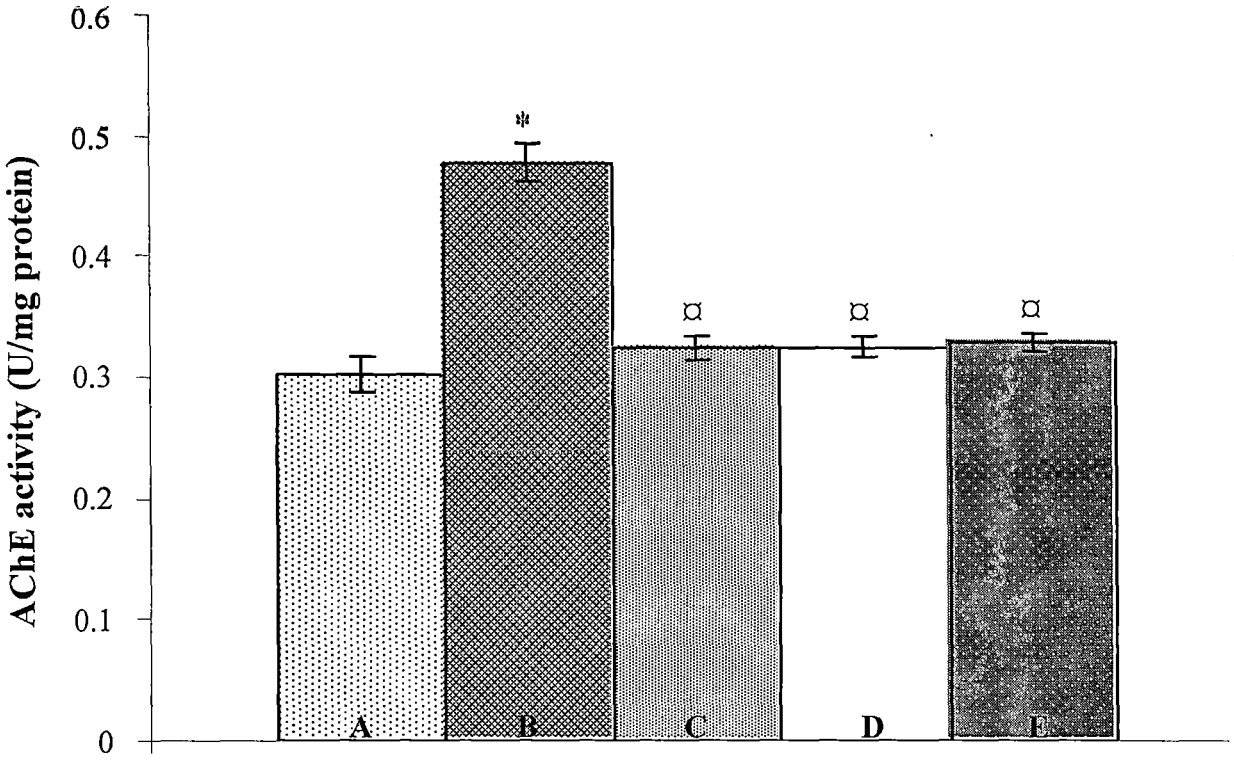


Fig.4.17: AChE activity in the supernatant fractions of the liver homogenate of the normal control (A), DBN-exposed (B) and DBN-exposed followed by treatment with liposome-encapsulated gelonin (C-E). Bar C-E represent the enzyme activity at 1, 2 and 4 weeks respectively after the treatment.
¤, statistically significant ($P < .05$) in comparison to (B),*, statistically significant ($P < .05$) in comparison to (A).

4.3.6 POST-TREATMENT GST STATUS

DBN-exposed mice upon treatment with liposome-encapsulated gelonin showed a marked elevation (~2 fold) in the GST activity as compared to those of DBN-exposed mice as shown in Fig.4.18 A-E. It was then subsequently decreased at 2 and 4 weeks after the treatment. However, a drastic decrease in the enzyme activity was seen at 4th

week, in which case the level went down significantly below to the normal level (Fig.4.18 E).

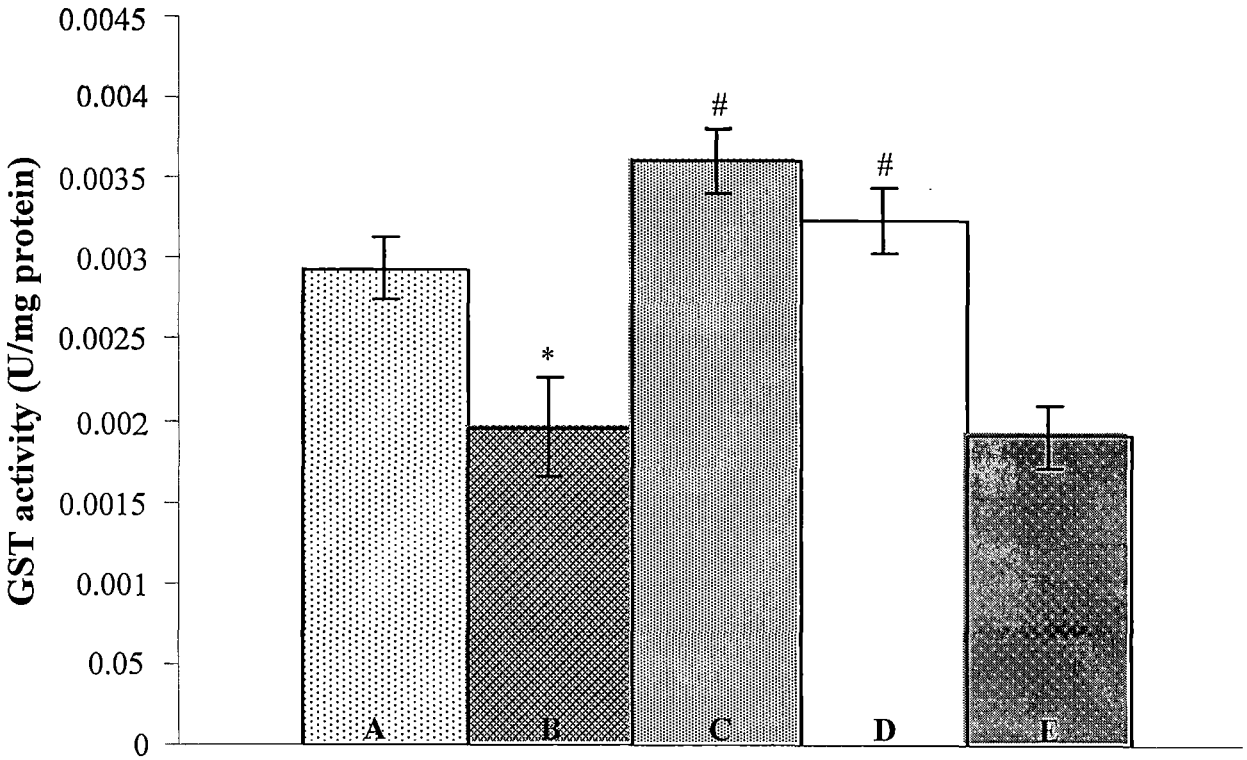


Fig.4.18: GSTactivity in the supernatant fractions of the liver homogenate of the normal control (A), DBN-exposed (B) and DBN-exposed followed by treatment with liposome-encapsulated gelonin (C-E). Bar C-E represent the enzyme activity at 1, 2 and 4 weeks respectively after the treatment. #, statistically significant ($P < .05$) as compared to (B), * , statistically significant ($P < 0.05$) as compared to (A).

4.3.7 POST-TREATMENT GSH LEVEL

The liver GSH level of the DBN-exposed mice significantly decreased upon treatment with liposome-encapsulated gelonin. It reached close to the GSH level in the normal control one week after the treatment period. The values of GSH level in normal

control, DBN-exposed and treated animals are shown in Fig.4.19 A-E. Even after four weeks of treatment no further significant increase in the GSH level was observed in gelonin treated mice.

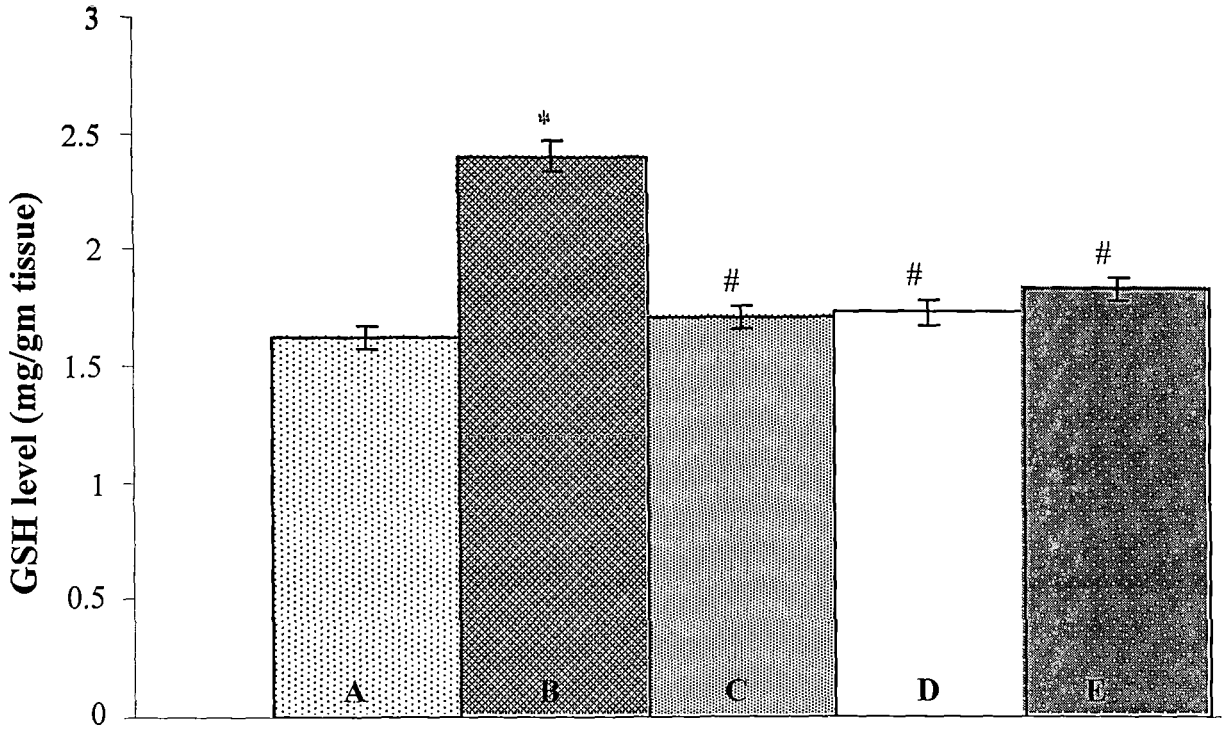


Fig.4.19: GSH level in the normal control (A), DBN-exposed (B) and DBN-exposed followed by treatment with liposome-encapsulated gelonin (C-E). Bar C-E represent the GSH level at 1, 2 and 4 weeks respectively after the treatment. *, statistically significant ($P < .05$) as compared to (A), #, statistically significant ($P < .05$) as compared to (B).

Post treatment datas for the marker enzymes activities (GGT, AChE, and GST), and the GSH levels given in Table 4.7. The results of these values in age-matched normal control, DBN-exposed mice are summarized.

Table.4.7: Values of enzymes activities and GSH levels determined in the supernatant fractions of liver tissue homogenate of normal control, DBN exposed and DBN-exposed mice followed by treatment with liposome-encapsulated gelonin.

Groups	GGT activity (U/mg protein)	AChE activity (U/mg protein)	GST activity (U/mg protein)	GSH level (mg/g tissue)
Normal control	0.018 ± 0.002	0.302 ± 0.014	0.003 ± 0.0002	1.624 ± 0.051
DBN-exposed	0.023 ± 0.003*	0.478 ±0.016*	0.002 ± 0.0003*	2.398 ± 0.048*
Gelonin treated (1 week)	0.018 ± 0.0003#	0.324 ±0.009#	0.004 ± 0.0002#	1.712 ± 0.028#
Gelonin treated (2 weeks)	0.020 ± 0.0004	0.325±0.007#	0.003±.0002#	1.730±0.030#
Gelonin treated (4weeks)	0.020 ± 0.0002	0.329 ±0.008#	0.002 ± 0.0002	1.833±0.029#

Activities (U//mg protein) and GSH level (mg/g tissue), Values are expressed as mean ± SEM, number of observations, n = 10
P =Level of significnce,
*, statistically significant (P<.05) against (A),
#, statisically significant (P<.05) as compared to that of (B).

4.3.8 CELL VIABILITY ASSESSMENT

Liver cell viability was assessed upon administration of liposome-encapsulated gelonin in mice previously exposed to DBN. The percent cell viability was determined at 1, 2 and 4 weeks post treatment and was compared with untreated DBN-exposed mice. Results of this study as shown in Fig. 4.20 indicate that initially the % cell viability decrease significantly upon treatment. However, at two and four weeks post

treatment the cell viability increased and attained a level equivalent to that of DBN exposed animals.

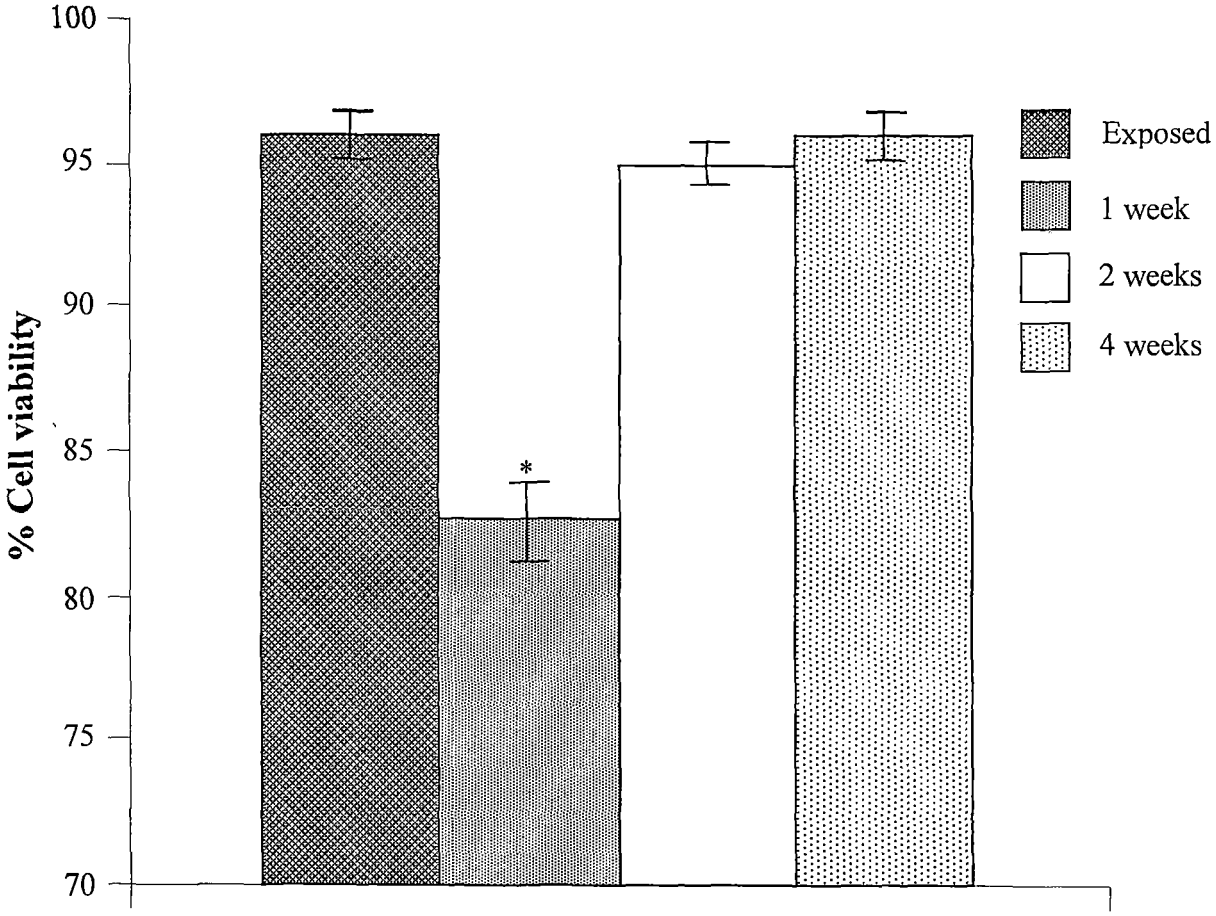


Fig.4.20: Percent viability of DBN-exposed hepatocytes (A) and the DBN-exposed mice after treatment (1,2 and 4 weeks) with liposome encapsulated gelonin (B-D respectively). Cell viability was assessed by trypan blue exclusion method.
* Statistically significant ($P<.05$) against (A), Values are given as means ± SD
No of observations, n = 8

4.3.9 POST-TREATMENT GENOMIC DNA

Genomic DNA extracts from liver tissue of the normal control and liposome-encapsulated gelonin treated DBN-exposed mice was electrophoresed on 1 % agarose gel and stained with ethidium bromide. The results of the genomic DNA for both

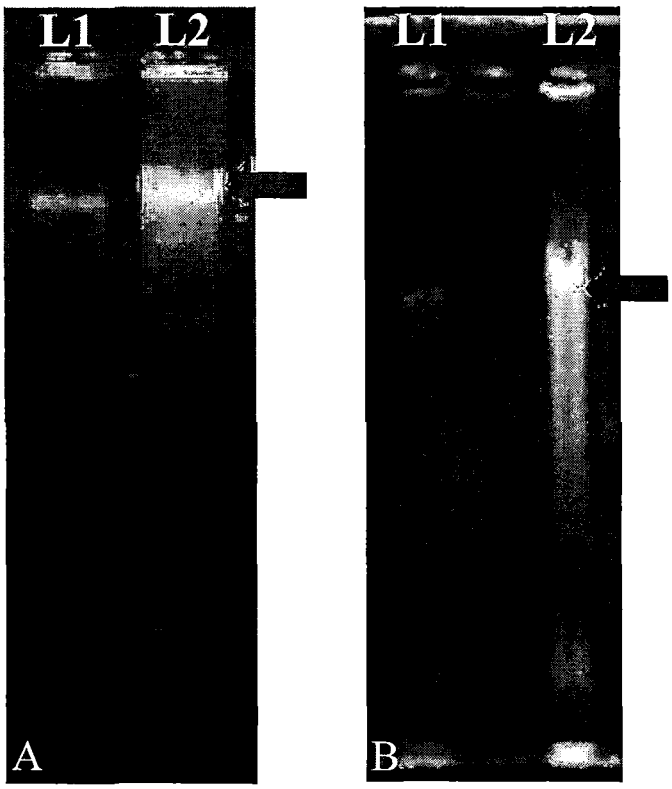


Fig.4.21: Genomic DNA extracts from normal control and liposome-encapsulated gelonin treated DBN- exposed mice analysed on 1% agarose gel. The gel was stained with ethidium bromide. Lane L1-normal control and lane L2-treated.

groups i.e. normal as well as gelonin treated mice are shown in Fig.4.21. Upon treatment the hepatic DNA (Fig.4.21-L2) showed shearing of DNA whereas no such shearing was observed with normal hepatic DNA (Fig.3.21-L1).

4.3.10 POST-TREATMENT DNA SYNTHETIC INDEX

The rate of DNA synthesis was carried out by monitoring BrdU incorporation in the hepatocytes of age-matched control animals, mice exposed to DBN and DBN-exposed mice followed by treatment with liposome-encapsulated gelonin. The results

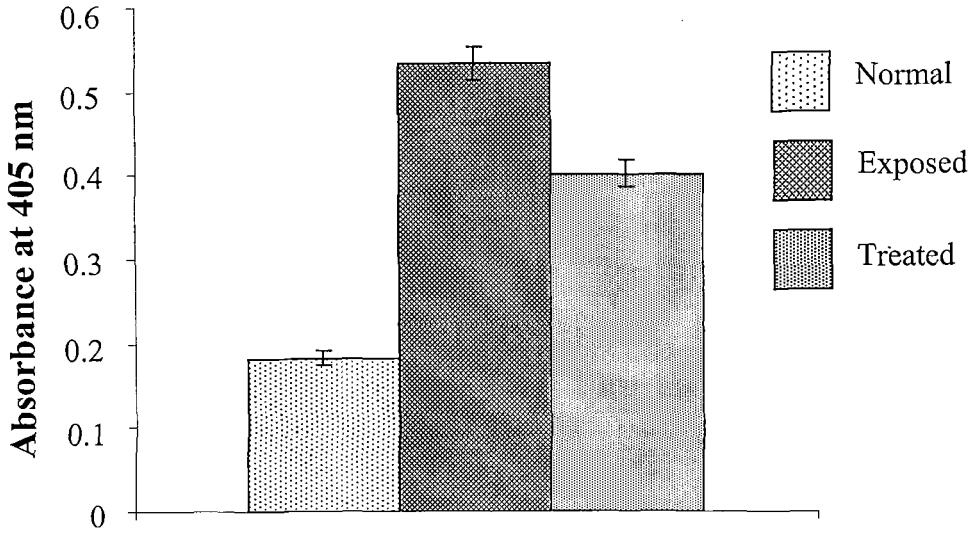


Fig.4.22: DNA synthetic index in hepatocytes of normal control, DBN-exposed and those of DBN-exposed mice treated with liposome-encapsulated gelonin. Each sample analysed for BrdU incorporation contained $\alpha 10^6$ viable cells. Incorporation of BrdU into cellular DNA was determined spectrophotometrically.

Table 4.8: DNA synthesis index measurement by BrdU labeling assay.

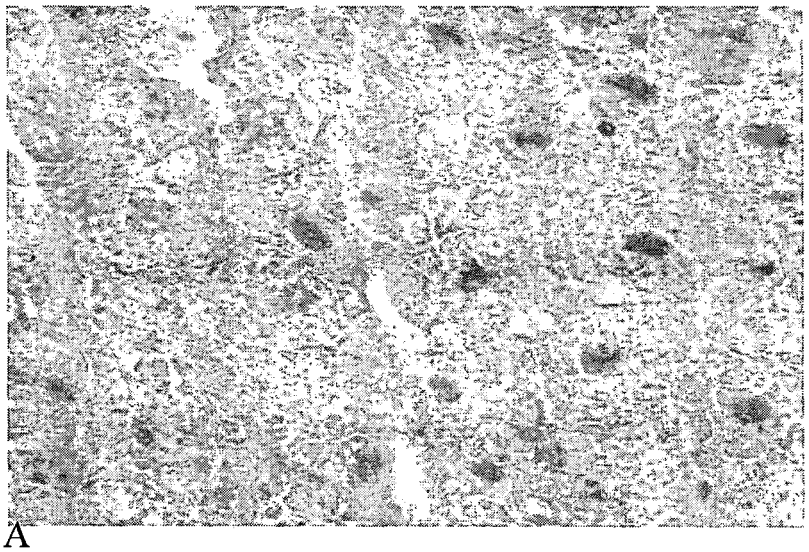
Groups	**Gelonin (μg)**	**Absorbance at 405nm (Mean ± SEM), n = 6**
Normal Control	-	0.183 ± 0.009
DBN-exposed	-	0.534 ± 0.019
Encapsulated gelonin treated	100	0.402 ± 0.016

n = Number of observations

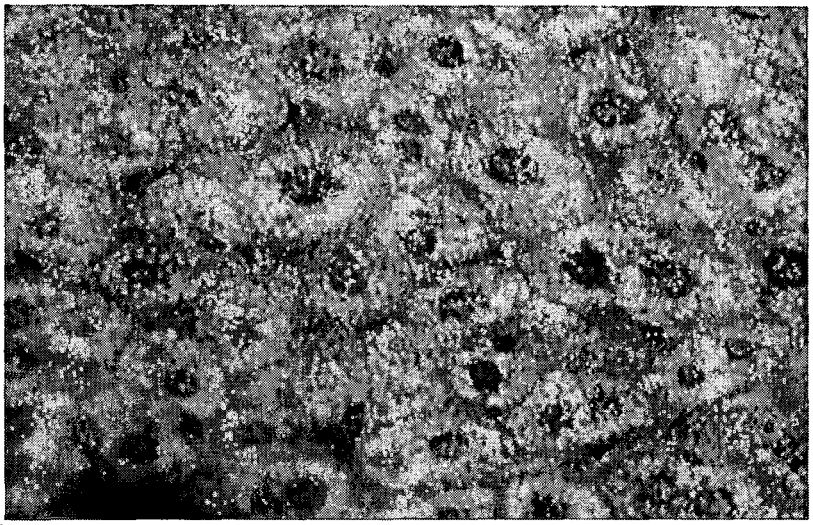
are shown in Fig. 4.22. Mice upon DBN-exposure showed significant elevation (~3 folds) in DNA synthesis compared to that of the normal control. However, these mice

after treatment with liposome-encapsulated gelonin exhibited decrease in the index values by showing less incorporation of BrdU. Data are given in Table 4.8.

4.3.11 POST-TREATMENT HISTOLOGICAL SECTION



A



B

Fig.4.23. Micrographs of histological sections of liver tissue from normal control mouse (A) and Liposome-encapsulated gelonin treated DBN-exposed mouse (B). The sections were 7μm thick and stained with eosin and haematoxylin. Magnification: 10 x 40.

The histological sections of liver tissues of DBN-exposed mice treated with liposome-encapsulated gelonin was prepared and studied. A comparison was made Between DBN-exposed and normal liver sections. Liver cells of the treated mice were still seen without much regularity in shapes and sizes and cells seemed to have poor contact with their neighboring cells. Besides, a good number of cells were found to be mono and bi-nucleated which were also seen in DBN-exposed tissues (Fig. 4.23 B). The only major difference observed in the treated tissue was that the multinucleated cells disappeared and the chromatins become more condensed compared to that of the DBN-exposed liver tissue.

CHAPTER-5

DISCUSSION

RIPs occur in many, but certainly not all plant species. This implies that RIPs are not ubiquitous and do not play a universal role in the growth, development, or defense of plants. Type 1 RIPs have been isolated from over 50 plants both monocots and dicots, indicating more general occurrence compared with type 2 RIPs (only six with potent toxicity) reported so far. Type 1 and type 3 RIPs are not cytotoxic and exhibit no documented oral toxicity towards higher animals or invertebrates. However, type 1 RIPs have shown to possess great potential as effective anti-tumor agents by virtue of its enzymatic capacity to inactivate ribosomes and arrest protein synthesis thereby effectively limiting the growth of cancer cells.

Gelonin, a type-1 RIP was isolated and purified from the seeds of an Indian plant, *Gelonium multiflorum*. The protein was extracted in two batches. Based on the nature of the protein it was purified by cation-exchange and gel filtration chromatography respectively. The CMC-52 elution profiles of gelonin obtained from the two batches are given in Figure 4.1. The profile showed a broad major peak and a small peak. Gelonin was eluted out in the major peak. The slight variation observed in the profile may be due to the age of the seeds and the seasons of harvesting which might also attribute to such variations. All the fractions comprising the major peak were pooled, concentrated and run on Sephacryl S-200HR column for further purification (Fig. 4.2 & 4.3). Six peaks marked as A, B, C, D, E and F appeared in the elution profile of Sephacryl S-200HR column. Fractions corresponding to each peak were pooled, concentrated and analyzed by SDS-PAGE (Fig. 4. 4). Peaks C, D and E contained maximum amount of gelonin as evident from the high intensity bands.

Homogeneity and molecular weight of gelonin was determined by SDS-PAGE under reducing conditions along with the appropriate molecular weight markers (Fig.4.5). The SDS-PAGE analysis revealed a homogeneous protein of Mw ~30 kDa which was in agreement the molecular weight of gelonin reported earlier (Singh, V. & Kar, S. K., 1992). A minor band which appeared just above the gelonin band (Fig.4.5) could be due to the presence of its variant. This band was unaffected after treatment either with DTT or mercaptoethanol. Thus, it may be concluded that each of these components represented a single polypeptide chain. Further, since, the gelonin contains 2-3% carbohydrate it is likely that the difference in glycoprotein may result in minor variation. However, such predictions demand further detailed investigations.

The gelonin purified on Sephacryl S-200HR column was subjected to determine its inhibitory activity of protein synthesis. The *in vitro* cell-free translational experiments using nuclease-treated rabbit reticulocyte lysate were carried out with gelonin and luffin (obtained from the seeds of *Luffa cylindrica*) in order to compare the relative biological activity of gelonin. Figure.4.6 shows a relationship between the percentage protein synthesis inhibition with the different doses of gelonin and luffin. The quantitative data are recorded in Table.4.1. The percentage of ribosome-inactivating activity was determined by calculating ED_{50} i.e. the effective dose of test sample required for 50% inhibition of protein synthesis. Sephacryl S-200HR column purified gelonin showed ED_{50} 2.5 ng whereas ED_{50} of luffin was found to be 4.0 ng. A two fold difference in ED_{50} of gelonin and luffin has earlier been reported, the former showed lower ED_{50} than the latter (Singh, V. & Kar, S. K., 1992). The ED_{50} value of

gelonin extracted by using different methods and purified on Sepharose S column has been reported between 1.16 - 3.5 ng (Singh, V. & Kar, S. K., 1992). Thus, the ED_{50} value of the gelonin we purified falls well within the range reported in literature. It also indicates that gelonin retained the biological activity upon purification. This observation is of great importance as eventually this would be used for *in vivo* cytotoxicity after encapsulating it into liposomes.

In the present investigation DBN at a dose of 10 mg kg^{-1} body weight was used to induce carcinogenesis in Swiss albino mice. The carcinogen was administered by intravenous route at weekly intervals for a period of 12 weeks. This protocol triggered the initiation process of carcinogenesis. Further, the induction of carcinogenesis was achieved without making use of any promoter with DBN. The nodules formation, swelling and enlargement of liver tissue of DBN-exposed mice (Fig.4.7) support our finding. However, the available reports state that carcinogenesis in liver is induced only when the carcinogens are coupled with a promoter (Ying, T. S. *et al,* 1882). The major metabolic pathway of DBN is the ω-hydroxylation of the butyl chain (Bellec, G. *et al.*, 1997, Suzuki, E. *et al.*, 1981, Hootsmark, J. *et al.*, 1983). These electrophillic metabolites ultimately alkylate specific bases in DNA which bring about mutagenic effects on DNA leading to hepatocellular transformation and subsequent cell division. This may likely be the reason for pronounced carcinogenesis induction by DBN.

Mice were sacrificed after being received the complete DBN treatment protocol and the liver tissues of animals were examined for any visible changes. The liver of some DBN-treated mice showed nodules formation and swelling at some places

(Fig.4.7). However, these observations were not found uniformly. No such nodules and swellings were seen in the liver of normal control mice.

The abnormal production of enzymes, proteins and hormones is generally associated with the initiation of carcinogenesis. Alterations (decrease or increase) in the enzyme activity are common findings in neoplastic tissues. Thus, to monitor cancer induction three marker enzyme assays were carried out. Upon DBN exposure GGT activity was significantly elevated in experimental mice when compared with the age-matched normal control mice (Fig.4.8). Elevation in GGT activity in mice upon DBN treatment has also been reported earlier (Alam, A. *et al.,* 2005). A marked elevation in GGT activity provides strong evidence of neoplastic transformation in the hepatocytes (Naoyuki, T. *et al,* 1974 and Fiala, S. *et al,* 1970). Elevated GGT activity is also observed in most liver cancers in the rat, mouse and human. This enzyme plays a role in the detoxification of carcinogens. The histochemical GGT test has been widely used to characterize preneoplastic foci in rat liver. The preneoplastic foci are also characterized by an increase in other enzymes involved in GSH metabolism and phase II drug metabolizing enzymes. Thus, GGT is used as an early marker of preneoplastic cells, which will expand into GGT + nodules from which tumors are believed to arise. All these changes define a resistant phenotype to xenobiotics, which is an adaptive response to the stress induced by the carcinogenic treatment; it is not necessarily linked to the appearance of transformed cells since most of the nodules will not develop into tumors. However, this new phenotype might confer a selective advantage to these cells and facilitate their clonal expansion (Chikhi, N. *et al,* 1999).

Alteration in plasma membrane characteristics is a known feature of cellular transformation. AChE activity was chosen as the second marker enzyme because it is a membrane bound enzyme and thus, any cellular transformation brought upon under the influence of the administration of DBN could therefore be monitored by assaying AChE activity. The assay showed that the liver tissue of the DBN-exposed mice have higher activities than the normal control (Fig.4.9). This elevation of the AChE activity indicates that DBN exposure had brought about some changes in the membrane of hepatocytes. It also provides evidence for some loss of membrane integrity.

Acetylcholine esterase, a serine hydrolase, plays a key role in cholinergic neurotransmission. By rapid hydrolysis of the transmitter acetylcholine, the enzyme effectively terminates the chemical impulse, thereby setting the basis for rapid, repetitive responses and enabling the reuptake (and recycling) of choline.

Another marker enzyme studied to monitor the cellular transformation in DBN-exposed mice was GST. The GST activity in DBN-treated mice was found to be lower than in normal control mice (Fig.4.10). Alteration in GST activity has been reported in a number of tumor cases (Kilty, C. *et al,* 1998 and Singh, S. V. *et al,* 1990). The GSTs play important roles in the metabolism of drugs and xenobiotics, as well as in antibiotic resistance and the biosynthesis of leukotrienes. In vertebrates, the biological role of these enzymes appears to involve the detoxification of electrophilic substances. The GST supergene family includes several loci that demonstrate well characterized polymorphism. These enzymes play critical roles in cellular protection from cytotoxic and mutagenic effects of electrophiles so that alleles associated with impaired

detoxification may confer an increased susceptibility to a wide range of diseases (Strange, R. C., *et al,* 1998).

Glutathione (GSH) is the most important non-protein thiol in living systems and is of widespread occurrence in the intracellular milieu of animals, plants and microorganisms. GSH protects cells from the toxic effects of reactive oxygen metabolites and is an important component of the system that uses reduced pyridine nucleotide to provide the cell with its reducing properties (Meister, A. 1988). Therefore, monitoring the level of GSH in normal and DBN-exposed mice may provide further evidence for cellular transformation. The liver GSH level in the DBN-exposed mice was found to be statistically high as compared to that of the normal control (Fig. 4.11). An increase in GSH immediately prior to nuclear division has been reported (Kosower, E. S. and Kosower, E. M., 1978). It is also reported that in mammalian cells, the non-protein -SH content rises upon passage of cells from G_1 (or G_0) to S in growth cycle. GSH increases in parallel with mitotic activity in regenerating liver (Kosower, E. S. and Kosower, E. M., 1978).

Cancer is a state where the cells do not respond to normal growth controls and hence uncontrolled proliferation of cells occur in such conditions. In order to keep up with the uncontrolled cell division, nucleic acids of proliferating cells have to step up their rates of synthesis that result in an increase in their synthetic indices. In the present investigation, this was monitored by quantitating BrdU incorporation into the DNA of replicating hepatocytes. The assay results showed that the BrdU incorporation was three folds higher in the hepatocyte of DBN-exposed mice than those of the normal

hepatocytes, as was inferred from the higher absorbance in the former (Fig.4.12). This clearly indicates the increase in the nucleic acid synthetic indices in liver cells upon DBN-exposure. This finding was further confirmed by analysis of genomic DNA in the liver cell extracts of normal control mice and that of DBN-exposed mice. The total genomic DNA extracts were run on 1% agarose gel. The results are shown in (Fig.4.13). The DNA band corresponding to DBN-exposed mouse was much more intense in comparison to the normal control mouse. The high DNA content signifies increased replication of DNA in the proliferating cell upon DBN-exposure.

It has been demonstrated that carcinogens, either by themselves or after being activated, interact with DNA (Singer, B., 1979). Evidence was also obtained which indicates that it is the DNA replication during cell proliferation, which is important for the induction of the initiated hepatocyes. In addition, it has also been shown that liver DNA with carcinogen-induced lesions replicate *in vivo* and that such newly made DNA synthesized on a carcinogen-damaged template is stable (Zahner, A. J. *et al.,* 1977). Although the mechanism by which cell proliferation exerts its unique effect in the initiation phase of carcinogenesis is not clearly understood, replication of DNA with carcinogen-induced lesion prior to repair offers an alternative mechanism by which carcinogen-induced critical damage may be appropriately transcribed and fixed into the newly made DNA and thus result in an initiated cell (Columbano, A. *et al.,* 1980).

Clinically, cancer is confirmed based on biopsy and histological verification of the disease. Therefore, histological study was also taken up for monitoring the induction process. The histological section of the liver of DBN-exposed mouse showed a lot of

differences and alterations in comparison to that of the normal. The liver section from DBN-exposed mouse showed a loss of several features such as the heptocytes had ill-defined boundaries, cells were irregular in shape and were less well packed with cells seeming to have lost contact with the neighboring cells. Some cells were found to be multinucleated. The nuclei appeared more densely stained that may be due to more condensed chromatin (Fig.4.14). However, in the normal control mouse liver section hepatocytes could be seen with well–defined cell membrane, a regular shape and a compact tissue structure containing cells with one or two nuclei each. The loss of regular shape indicates changes in membrane structure and composition, which may be related to a change in function. These changes may also be related to the perturbation in enzyme activities, especially those of GGT and AChE and the level of GSH as these being cellular components and associated with membrane function directly or indirectly. The densely stained nuclei showed condensed chromatin as it is normally observed in dividing cells. Thus, the morphological changes observed in the liver cells of DBN-exposed mice clearly indicate that the cells are in a high state of division.

Thus, the parameters monitored to follow carcinogenesis induction i.e. GGT, AChE, and GST marker assays, GSH level, cell proliferation assay (BrdU incorporation assay), Genomic DNA analysis and the histological sections examination of liver confirmed the induction of hepatocellular carcinogenesis by DBN (10 mg kg^{-1} body weight) at a weekly interval for a period of 12 weeks.

After having established that cellular transformation occurs in mice upon chronic exposure of DBN, regression studies were carried out in such DBN-exposed

mice upon treatment with liposome encapsulated gelonin. The studies were carried out both by *in vitro* and *in vivo* experiments. Cell viability assay parameter was used to determine the cytotoxic effects of gelonin in *in vitro* study. However, the parameters monitored to study regression effects in *in vivo* study were marker enzyme activities, GSH level, nucleic acid synthetic index, cell viability assay, genomic DNA analysis in liver and the histological examination of microsections of liver tissues. Before performing the regression studies gelonin was encapsulated into liposmes and the percent entrapment efficiency of the liposome for gelonin was determined.

Liposomes were made by conventional dry film method using different lipid compositions as shown in Table 4.4. At present there are several methods of preparing liposomes, each having their own advantages and disadvantages (Mitchell, M. S. *et al.,* 1988). One of the major disadvantages of these methods is that the material to be entrapped into liposomes requires exposure to the organic solvent or detergent, which may lead to denaturation. Dry film method is simple and highly reproducible which involves extremely mild conditions so that the biological activity of the entrapped material remains unchanged. The entrapment efficiency of gelonin into liposomes produced by this dry film method ranged between 23-32% (Table 4.4). Although, the entrapment efficiency has been significantly low as compared to other methods reported in literature, this method was found highly suitable for entrapment of gelonin. Encapsulation of gelonin into liposomes using a modified reverse phase evaporation method has earlier been reported (Alam, A. *et al.,* 1992) with a very high entrapment efficiency of ~70-75%. Although the method was simple and highly reproducible but

the biological activity of gelonin decreased slightly after its encapsulation. Therefore, the dry film method was preferred over any other conventional methods as it does not alter the biological activity of the entrapped material. Low entrapment efficiency by this method is known.

The successful application of liposomes as carrier for drugs and enzymes in therapy is heavily dependent on their stability in circulation, tissue distribution and also on their mode of interaction with target cell(s). Study on liposome uptake by the tissues such as liver, kidneys andspleen was carried out. For this purpose ^{3}H cholesterol was used along with other lipids to prepare liposomes (Table 4.5). Mice were sacrificed by cervical dislocation 30 minutes after the intravenous administration of liposomes. The radioactivity was counted in the tissues which were excised immediately. Major portion of liposomes (~25-40%) from circulation of injected mice were captured by liver (Fig.4.15). Kidneys and spleen exhibited little uptake in comparison to liver. High uptake of liposomes from circulation by liver is well documented. It was further observed that liposomal preparation containing PG showed maximum uptake by all the three tissues. This is because, the inclusion of PG in liposomal composition is known to increase the circulation time of liposomes that may results in increase uptake of the same by these tissues. It was further observed that liposomes prepared by incorporation of phosphatidyl ethanolamine (a positively charged lipid) showed better uptake by liver in comparison to the conventional liposmes made from DPPC, Cholesterol and DCP (Fig.4.15). Reports are available about the fast clearance and fast uptake of charged liposomes by macrophages of RES. Therefore, the high uptake of positively charged

liposomes may be due to its fast uptake by the kupffer cells in liver. These observations, therefore, clearly indicate that sufficient amount of gelonin can be targeted to liver tissue by its encapsulation into liposomes without putting any additional specific ligand (such as monoclonal antibody or hormone etc.) on its surface.

Two important observations were made and reported in the 1980s regarding the fate of intravenously administered liposomes. First, it was found that the endothelial lining of 'healthy' blood vessels form an efficient barrier to liposomal escape from the blood circulation upon intravenous administration. Only in sinusoidal tissue is escape possible for small liposomes. But in general, for conventional liposomes removal from the circulation is too fast to benefit from this escape mechanism. Thus, long circulation times of liposomes were required to take full advantage of this 'leaky endothelium effect'. This problem was overcome by the development of 'stealth liposome", which reduces the rate of uptake by macrophages and leads to a prolonged presence of liposomes in the circulation and consequently provides ample time for these liposomes to escape from circulation through leaky endothelium (Papahadjopoulos, D. *et al.,* 1995 and Allen, T.M. *et al., 1994*). Such liposomes are generally useful in targeting of the drugs to specific tissue.

Being a hydrophilic macromolecule, gelonin has limited access to its target sub-cellular compartment, the cytosol; it is effectively plasma membrane-impermeant and subject to rapid degradation within endosomes and lysosomes upon cellular uptake as it lacks the membrane translocating capability that is typically provided by a disulfide-linked B polypeptide found in the type 2 toxins (*e.g.* ricin). These inherent

characteristics generate the need for the development of specialized cytosolic delivery strategy for gelonin as an effective anti-tumor therapeutic agent.

The successful entrapment of gelonin in the liposomes with retention of ribosome-inactivating property prompted us to investigate its effectiveness on tumor regression. The studies were carried out both in *in vitro* and *in vivo*. In *in vitro* experiments, unencapsulated gelonin upon treatment with DBN-exposed viable hepatocytes exhibited no detectible cytotoxicity (Table4.6). However, upon treatment with equivalent amount of liposome encapsulated gelonin (of different liposomal compositions) showed significant killing of hepatocytes (Table4.6). A total of 14-16% killing was observed by conventional (DPPC:Chol:DCP) and the non-conventional [DPPC:Chol:PG and DPPC:Chol:PE(PS)] liposomes (Table 4.6). A slight increase in cytotoxicity was observed when the culture was supplemented with poly ethylene glycol (PEG of MW=2000). PEG has widely been used as a membrane fusion agent. Since, gelonin exhibits cytotoxic effect only upon its cellular uptake. It was thought that addition of PEG to culture medium will enhance cellular uptake of liposomal gelonin, which will ultimately lead to an increase in cell death. Although, we could not achieve any significant improvement in cytotoxicity by addition of PEG in our preliminary *in vitro* study, however, a further detailed investigation with other potent membrane fusion agents may be of significant importance.

After studying the *in vitro* cytotoxicity of liposome encapsulated gelonin on transformed hepatocytes, its effects on cancer regression *in vivo* was also carried out. Liposome encapsulated gelonin was administered in DBN-exposed mice at weekly

intervals by intravenous route as described in method and material (section 3.4.2). Mice were sacrificed at 1, 2 and 3 weeks after treatment. The parameters monitored to study regression were marker enzyme activities, GSH level, nucleic acid synthetic indices, electrophoretic study of cellular DNA and cell viability in liver tissues and the histological examination of liver sections.

Post treatment GGT status is shown in Figure-4.16. After one week of treatment, a significant decrease in the GGT activity was observed in comparison to DBN-exposed mice. The activity was found close to that of normal control (Fig.4.16-A and C). However, a small rise in the enzyme activity was observed in mice that were sacrificed after 2 and 4 weeks of treatment, but it was still less than the level of enzyme activity in untreated DBN-exposed mice (Fig.17 - D, E & B). Low GGT activity in liver of gelonin treated mice signifies the cytotoxic effect of gelonin on hepatocytes. This elevation in GGT activity may be because still a lot of transformed cell were present in liver and multiplying.

A similar trend was observed with the activity of AChE upon treatment. The AChE activity with a significant decrease approached towards the value observed in normal control animals. No further change in the enzyme activity was found even after three weeks of treatment (Fig.4.17-A-E). Attenuation of the marker enzyme activity towards the normal value provides evidence for some gain in integrity of cellular membrane that may be attributed to the regression effects.

GST was another marker enzyme studied to monitor the regression effect in DBN-exposed mice upon treatment with liposome encapsulated gelonin. The GST

activity initially elevated significantly then it decreased subsequently after two and four weeks of treatment (Fig. 4.18-C-E). Alteration in GST activity was found to be inversely related to GGT activity (Fig.4.16 & 4.18). This observation supports our earlier finding that in a carcinogenic condition an increase in GGT activity acompanied by a decrease in GST (Alam, A. *et al.,* 2005). The reason for this could not be explained on the basis of these preliminary observations. However, from the level of marker enzymes (GGT & GST) activities in the treated mice, it may be concluded that the cytotoxic effect of gelonin was more pronounced up to one week after the treatment. These initial significant alterations in marker enzymes activities are likely due to killing of significant number of cells in liver. Since, the regeneration is fast in liver, the further change in the level of these enzymes may be related to this effect.

GSH level decreased significantly upon treatment and it approached to the level observed in normal control mice. The level was maintained without any further significant alterations (Fig. 4.19). The low GSH level in treated mice may be due to the decrease in cell proliferation, which is caused by the cytotoxic effects of gelonin. The cytotoxic effect of liposome encapsulated gelonin was further supported by the observations obtained in cell viability assay (Fig. 4.20). Significant decrease in the number of viable cells one week after the treatment clearly indicates that there was large cell destruction in liver following administration of liposome encapsulated gelonin. The cell viability later increased and it became equivalent to that of normal control by second weeks of treatment. This effect may be due to regeneration of liver tissue.

Post treatment genomic DNA analysis (Fig 4.21 B-L2) showed the appearance of a broad but highly diffused DNA band as compared to that of the normal control however it was of lower intensity in comparison with DBN-exposed liver cells. Upon comparison the following conclusion may be drawn. The high intensity of DNA band in treated hepatocytes in comparison to normal may be due to increase in DNA synthesis, which accompanies liver regeneration that takes place to replace the cells destructed by gelonin induced cytotoxicity. However, when the same DNA band was compared with that of DBN-exposed mice it was found to be of lower intensity and highly diffused. The decrease in intensity may likely be due to decrease of actively dividing transformed cells. However, the appearance of diffused band may also be attributed to various reasons such as DNA fragmentation and changes in genomic DNA because of DBN exposure followed by gelonin treatment. The loss in cell cycle check points, which is seen in tumour cells, results in daughter cells with incompletely formed genomic DNA or fragmented chromosomes. This may results in generation of low molecular weight DNA fragments than that of the intact genomic DNA of normal cells.

BrdU incorporation into the DNA of replicating liver cells was also monitored after administration of liposome encapsulated gelonin in the DBN-exposed mice. The assay showed less BrdU incorporation in hepatocytes of gelonin treated mice than that of the DBN-exposed hepatocytes, as was inferred from the lesser absorbance in the former (Fig. 4.22). This shows a decrease in the DNA synthetic index in liver cells upon gelonin treatment. This observation indicates that the rate of DNA replication had decreased in accordance with the decrease in tumour cell proliferation.

The histological sections studied did not show significant changes in liver tissue following treatment with liposome encapsulated gelonin when compared with DBN-exposed liver tissue (Fig.4.23). In treated tissue, the cells were seen with a small regularity in shapes and sizes, but still having a poor contact with their neighboring cells. The only noticeable change which we observed was that multinucleated cells disappeared. The chromatin was still condensed as the nuclei found to be densely stained as compared to that of the normal liver tissue. These small morphological changes in liver cells of the treated animals may be considered a positive indication of cancer regression caused by liposome encapsulated gelonin.

Thus, all the tests carried out viz. enzyme marker assays (GGT, AChE and GST), estimation of liver GSH, cell proliferation assay (BrdU incorporation assay), genomic DNA analysis, cell viability assessment and the histological examination of liver microsections give a positive indication of cancer regression, which is caused by the cytotoxic effects of liposome encapsulated gelonin upon its administration, in the mice bearing cancer.

CHAPTER-6

CONCLUSION

The study carried out can be summarized as follows:

- Gelonin was purified from the dry seeds of *Gelonium multiflorum* using cation-exchange and gel filtration chromatography.
- The protein after getting in its purified form was subjected to different analyses to determine its homogeneity, molecular weight and protein synthesis inhibition activity.
- The molecular weight of gelonin obtained from two different batches was found to be ~30 kDa.
- Sephacryl S-200HR column purified gelonin exhibited high protein synthesis inhibition activity in cell free translation system.
- Hepatocarcinogenesis was induced in mice by intravenous administration of DBN (10 mg kg-1 body weight) at weekly interval up to 12 weeks.
- Elevation in GGT and AChE activities, increase in GSH level, lowering of GST activity and increase in nucleic acid synthetic index in mice upon DBN exposure signifies hepatocellular transformation.
- DBN exposure resulted distinct changes in the nature of the hepatocytes such as variation in the cell shape and size, appearance of more densely stained nuclei and multinucleated cells as elucidated in the histological section. These observations further support induction of carcinogenesis in liver.
- Liposomes prepared by the conventional dry film method exhibited a reproducible entrapment efficiency of gelonin (23.25-31.5 %).

- In *in vitro* experiments, liposome-encapsulation enabled liposomal gelonin to kill about ~15-19 % of hepatocytes of DBN-exposed mice. By contrast, cells treated with equivalent concentrations of unencapsulated gelonin exhibited no detectable cytotoxicity.
- Liver exhibited the predominant uptake of liposomes when administered by intravenous route.
- In *in vivo* experiments, DBN-exposed mice upon treatment with liposome encapsulated gelonin showed that the level of marker enzymes (GGT, AChE & GST), level of GSH and the rate of nucleic acid synthesis approaching close to the normal levels. These observations signify the regression effects.

RIPs have proven a potent anticancer agent for therapy. Our preliminary observations, therefore, indicate that the effectiveness of RIPs can further be improved by its encapsulation into liposomes, especially when liver is the target tissue for cancer therapy.

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CURRICULUM VITAE

K. S. Nakhuru
Scientist 'B',
Defence Research Laboratory,
Defence R & D Organisation,
Tezpur - 784001,
Assam, India.
E-mail: snakhuru@yahoo.com

Academic Qualifications

Degree	Conferred	University
B.Sc. (Biotechnology), 1st Div., 8th position	1998	North Eastern Hill University
M.Sc. (Biochemistry), 1st Div., 6th position	2000	North Eastern Hill University

Other Achievements

Qualified "Graduate Aptitude Test in Engineering - GATE, 2001"

Qualified "Joint CSIR-UGC National Eligibility Test (NET), 2001"

Seminar / Symposia / Conferences attended

72nd Annual Session of the National Academy of Sciences, India, October 25 to 27, 2002, North Eastern Hill University ,Shillong, Meghalaya.

Regional Symposium on Research Thrust in Animal Sciences in N. E. region - An appraisal, March 24-25, 2006, Department of Zoology, North Eastern Hill University, Shillong-793022, Meghalaya.

CME on Cancer Challenges in India with Particular Reference to North East, July 13-14, 2006, Department of Radiation Oncology, NEIGRIHMS, Shillong, Meghalaya.