1. INTRODUCTION

Drug development programme for the identification of new chemotherapeutic agents involve extensive pre-clinical evaluation of vast number of chemicals for detection of antineoplastic activity. The safety evaluation of chemicals necessitates the screening of even greater numbers of chemicals. The safety evaluation of chemicals involves an extensive range of studies on mutagenicity, carcinogenicity and chronic toxicity. The major application of *in vitro* cultures is currently with analyzing toxicity and chronic toxicity testing.

Carbamate insecticides, like baygon, mesurol, carbaryl, carbofuran, aldicarb, primicarb etc., are widely used in the agriculture fields resulting in the contamination of food, feed and fodder, which is being continuously consumed by man and his animals, causing continuous loss of body weight, growth parameters, reproduction and other abnormalities. These insecticides though consumed in minute quantities, have shown to be mutagenic, carcinogenic and teratogenic. However, the degree of their effect on internal tissues like hepatic tissue, lymphatic tissue, immune system and others is not expressed phenotypically within the living organism. Cell and tissue culture techniques provide a novel way to study the effect of these carbamate insecticides on various tissues. Cell and tissue culture has been excessively used in the study of toxic effect of various metabolites on different tissues. However, less work has
been done to see the toxic effects of carbamate insecticides on lymphocytes, spleenocytes, hepatocytes and erythrocytes cultured in vitro.

Cancer is a difficult disease to cure. If a tumor is discovered in time, it can often be removed by an operation. If the tumor is discovered too late, there's a risk that cancer has spread to other tissues. In such cases, other methods must be used to destroy the cancer cells. Radiation can be used to kill cancer cells. The rays destroy the cell's DNA so they can't survive. The disadvantage of radiation therapy is that it's difficult to avoid radiating healthy tissue. Cancer cells can also be attacked with chemical poisons that attack the cell's development processes which is called as chemotherapy in therapeutic terms. The poison used can't differentiate normal cells from cancer cells, thus limiting the use of such treatment.

Cancer patients are often under a great deal of pressure to make decisions about the type of treatment to take, at a time when they are shocked and frightened over a diagnosis of cancer and probably feeling unwell in addition. Whatever the cancer or the circumstances may be, a collapse in the aspects of the immunity is the fundamental cause of all cancers and it is important to reverse this as soon as possible to offer the best chance of healing, hence it is advisable to immediately start on a course of a powerful antioxidant.
Attempt is being made in this study to see the effect of carbamate insecticides on few representative animal cells cultured *in vitro* and to explore the possibility of using these carbamates for the treatment of cancer. Among various carbamates, the commonly used carbamates viz., baygon, carbaryl and carbofuran formed a subject for the present study.

1.1. CARBAMATE INSECTICIDES

Carbamate insecticides are a group of compounds closely related to the Organophosphate insecticides in chemical structure, mode of action and many other properties. These compounds inhibit the enzyme acetylcholine esterase (AChE) in the neuromuscular junction and cause the death of the organism by neuromuscular paralysis. The carbamates are mainly used in agriculture, as insecticides, fungicides, herbicides, nematocides, or sprout inhibitors. In addition, they are used as biocides for industrial or other applications and in household products. A potential use is in public health vector control. Thus, these chemicals are part of the large group of synthetic pesticides that have been developed, produced, and used on a large scale industrial basis.

1.1.1. Classes and Properties

Three classes of carbamate pesticides are known. (1) The carbamate ester derivatives, used as insecticides (and nematocides), are generally stable and have a low vapour pressure and low water solubility. (2) The carbamate
herbicides (and sprout inhibitors) have the general structure $R_1\text{NHC(O)}R_2$, in which $R_1$ and $R_2$ are aromatic and / or aliphatic moieties. (3) Carbamate fungicides contain a benzimidazole group.

1.1.2. Effects on experimental animals and in vitro test systems

The acute toxicity of the different carbanates ranges from highly toxic to only slightly toxic or practically non-toxic. The LD50 for the rat ranges from less than 1 mg/kg to over 5000 mg/kg body weight. The acute dermal toxicity of carbanates is generally low to moderate. Carbanates produce slight to moderate skin and eye irritation, depending on the vehicle used, duration of contact, and on whether the substance is applied to the abraded or intact skin. Apart from the anticholinesterase activity, the following changes can be found: an influence on the haemopoietic system, an influence on the functioning of and at higher dosages degeneration of the liver and kidneys and degeneration of testes. These abnormalities in the different organ systems depend on the animal strain and on the chemical structure of the carbanate. A considerable number of reproduction and teratogenicity studies have been carried out with different carbanates and various animal species. Different types of abnormalities were found, i.e., increase in mortality, disturbance of the endocrine system, and effects on the hypophysis and its gonadotrophic function. These effects were mainly seen at high dose levels. Generally, the fetal effects included an increase in mortality, decreased weight gain in the first few weeks after birth,
and induction of early embryonic death. All these effects can be summarized as embryotoxic effects. Certain carbamates also induce teratogenic effects, mainly at high dose levels applied by stomach tube. When the same dose level was administered with the diet, no effects were seen.

1.1.3. Mutagenicity and related end-points

The well-known carbamates have been tested for their mutagenic activity in different test systems. Some induce mutagenic effects, others are negative. In general, the methyl carbamates are negative in mammals tested, while compounds such as carbendazim, benomyl, and the 2 thiophanate derivatives showed a positive effect with very high dose levels in certain systems. The benzimidazole moiety may act as a base analogue for DNA and as a spindle poison. They are antimitotic agents and cause mitotic arrest, mitotic delay, and a low incidence of chromosome damage. Sometimes, the results are contradictory or cannot be reproduced, but positive results for point mutation and chromosome aberrations are well documented. These benzimidazole derivatives can be considered as weak mutagenic compounds.

1.1.4. Carcinogenicity

Ethyl carbamate (urethane) is a well-known carcinogen, and it seems that its chemical structure is optimal for such an effect. Any change in the molecule seems to decrease the carcinogenic potency, particularly when the ethyl group is
replaced by larger side chains. Alkyl groups on the nitrogen also reduce this activity. However, no clear indications of carcinogenic effects have been found in the available long-term carcinogenicity studies with different carbamates. The carcinogenicity studies with benzimidazole derivatives showed either positive or equivocal results.

1.1.5. Effects on man

Health hazards for man occur mainly from occupational over-exposure to carbamate insecticides resulting in poisoning characterized by cholinergic symptoms caused by inhibition of the enzyme AChE. Various cases of intoxication have been described. Most of them were spraymen applying insecticides inside houses in the tropics to control mosquito vectors of malaria, or plant protection workers. The main routes of exposure are inhalation and skin. From controlled human studies, it is clear that poisoning symptoms can be seen a few minutes after exposure, and can last for a few hours. Thereafter, recovery starts and within hours, the symptoms disappear, and the choline esterase (ChE) activity in erythrocytes and plasma returns to normal, because the carbamate is rather rapidly metabolized and the metabolites excreted. Other signs and symptoms induced by certain carbamates are skin and eye irritation, hyperpigmentation, and influence on the function of testes (slight increase of sperm abnormalities). The antidote for carbamate poisoning is Atropine. Three representative carbamates viz., baygon, carbaryl and carbofuran were used in the present study.
1.2. BAYGON

The technical name of baygon is propoxur, which is a non-systemic insecticide, introduced in 1959 (Hayes and Laws, 1990). Pure propoxur is a white crystalline solid with a faint characteristic odor. Technical propoxur is about 95% pure and is a white to cream colored crystalline powder with a milk-phenol odor. It is formulated as emulsifiable concentrates, 50% wettable powder, baits, and 1 and 2% dusts. It is unstable in alkaline media, as it is hydrolyzed by strong alkalis. It has a half-life of 40 minutes at pH 10. Propoxur is dangerous if it is heated to decomposition, as it emits highly toxic fumes.

Propoxur is not used on food crops. It is used against mosquitoes in outdoor areas, for flies in agricultural settings, for fleas and ticks on pets, as an acaricide, on lawns and turf for ants, on flowering plants, and in private dwellings and public buildings. It is also used as a molluscicide, a chemical that kills snails. It is effective against cockroaches, aphids and leafhoppers (TOXNET, 1986). Propoxur is one of the chemicals that has replaced DDT in the control of black flies and mosquitoes (McEwen and Stephenson, 1979). It is a nonsystemic insecticide with contact and stomach action that has longstanding residual poisonous, or toxic activity when it is in direct contact with the target pest (Hartley and Kidd, 1983). Trade names include Baygon spray, Bayer 39007, Arprocarb, UNDEN, Suncide, Sendran, Invisigard, Bay 9010, Bifex, Bolfo, Blattanex, Rhoden, Propogon, Propyon, Sendra, Tendex and Undene.
1.2.1. Toxicological effects

1.2.1.1. Acute toxicity

Propoxur is classified as highly toxic to humans (American Conference of Governmental Industrial Hygienists, Inc. 1986).

The amount of a chemical that is lethal to one-half (50%) of experimental animals fed the material is referred to as its acute oral lethal dose fifty, or LD50. The LD50 for propoxur in rats ranges from 83 mg/kg to 150 mg/kg (Berg, 1986).

1.2.1.2. Chronic toxicity

Prolonged or repeated exposure to propoxur may cause symptoms similar to acute effects. Propoxur is very efficiently detoxified, or made into nonpoisonous forms, thus making it possible for rats to tolerate daily doses approximately equal to the LD50 of the insecticide for long periods, provided that the dose is spread out over the entire day, rather than ingested all at once.

1.3. CARBARYL

Carbaryl is a wide-spectrum carbamate insecticide which controls over 100 species of insects on citrus fruits, cotton, forests, lawns, nuts, ornamentals, shade trees, and other crops, as well as on poultry, livestock and pets. It is also used as a molluscicide and an acaricide. Carbaryl exerts toxic effects both through ingestion or absorbed through direct contact. The chemical name for
carbaryl is 1-naphthol N-methylcarbamate. Product names include Carbamine, Denapon, Dicarbam, Hexavin, Karbaspray, Nac, Ravyon, Septene, Sevin, Tercyl, Tricarnam, and Union Carbide 7744.

Carbaryl is formulated as a solid which varies from colorless to white to gray, depending on the purity of the compound. The crystals are odorless. This chemical is stable to heat, light and acids under storage conditions. It is non-corrosive to metals, packaging materials, or application equipment. It is found in all types of formulations including baits, dusts, wettable powder, granules, oil, molasses, aqueous dispersions and suspensions. Carbaryl is being used regularly as a pesticide.

1.3.1. Toxicological effects

1.3.1.1. Acute toxicity

Carbaryl is moderately to highly toxic. It can produce adverse effects in humans by skin contact, inhalation or ingestion. Direct contact of the skin or eyes with moderate levels of this pesticide can cause burns. Inhalation or ingestion of very large amounts can be toxic to the nervous and respiratory systems resulting in nausea, stomach cramps, diarrhea and excessive salivation. High doses of the chemical cause sweating, blurring of vision, incoordination, and convulsions.
The oral LD50 of carbaryl ranges from 250 mg/kg to 850 mg/kg for rats, and from 100 mg/kg to 650 mg/kg for mice. The inhalation LD50 for rats is 0.005 to 0.023 mg/kg. Low doses can cause minor skin and eye irritation in rabbits (Baron, 1991).

1.3.1.2. Chronic toxicity

Although it may cause minor skin and eye irritation, carbaryl does not appear to be a significant chronic health risk at or below occupational levels.

1.4. CARBOFURAN

Carbofuran is an odorless, white crystalline solid. It is used against soil and foliar pests of field, fruit, vegetable and forest crops. Carbofuran is available in liquid and granular formulations. Trade names include Furadan, Bay 70143, Curaterr, D 1221, ENT 27164, Yaltox, Furacarb and Carbogran 3G.

1.4.1. Toxicological effects

1.4.1.1. Acute toxicity

Carbofuran is highly toxic by inhalation and ingestion and moderately toxic by dermal absorption (Hayes, 1982). Risks from exposure to carbofuran are especially high for persons with asthma, diabetes, cardiovascular disease, mechanical obstruction of the gastrointestinal or urogenital tracts, or those in vagotonic states. Carbofuran may cause contact burns to the skin or eyes.
As with other carbamate compounds, cholinesterase inhibiting effect of carbofuran is short-term and reversible (Kearney and Kaufman, 1975). Symptoms of carbofuran poisoning include: nausea, vomiting, abdominal cramps, sweating, diarrhea, excessive salivation, weakness, imbalance, blurring of vision, breathing difficulty, increased blood pressure or hypertension, and lack of control of urine or feces release, referred to as incontinence. Death may result from respiratory system failure associated with carbofuran exposure. Complete recovery from an acute poisoning by carbofuran, with no long term health effects, is possible if exposure ceases and the victim has time to reform the normal level of cholinesterase and to recover from symptoms. The oral LD50 for rats is 5 mg/kg, for mice is 2 mg/kg, and for dogs is 19 mg/kg. The dermal LD50 for rabbits is 885 mg/kg.

1.4.1.2. Chronic toxicity

Prolonged or repeated exposure to carbofuran may cause the same symptoms as an acute exposure. The Environmental Protection Authority (EPA) has established a Lifetime Health Advisory (LHA) level of 40 ppb of carbofuran in drinking water. Consuming carbofuran at high levels well above the LHA level over a long period of time has caused damage to the testes and uterus of test animals. It has also caused cholinesterase inhibition in both humans and test animals.
1.5. MAMMALIAN ORGAN SYSTEMS

The effect of carbamates on mammalian organs like liver and spleen and cells viz. lymphocytes and erythrocytes were studied in the present work. Hence if forms a basic input to describe such organs and cells in brief.

1.5.1. Liver

Liver is an important organ, which performs complex functions including metabolism, synthesis and detoxification. Loss of liver function due to toxic substances, not only results in the metabolic derangement of the body, but also affects the functions of other vital organs, like brain, heart, kidney, lungs and vascular tissues (Ganong, 1989; Guyton and Hall, 1996). Hence it is the need of the hour to study the effects of toxic substances on the liver. Toxic substances reaching liver may take a long route before they affect the organ hence in vitro systems provides a novel way to study the effect of these toxins directly on the organ.

Hepatocytes are epithelial cells found in the liver. They perform important functions, such as helping to detoxify blood, and to synthesize transport proteins, such as lipoprotein, albumin and transferrin. Primary and secondary cultures of hepatocytes are useful for studying the mechanisms of liver regeneration and differentiation. Historically, primary hepatocytes have exhibited a limited replicating lifespan in culture. In addition, when stimulated to divide in culture they have generally lost differentiated functions such as the
ability to synthesize and secrete albumin and transferrin. While substantial progress has been made in understanding the factors which affect these characteristics of hepatocytes in culture, primary and secondary hepatocytes are still not suitable for use in some emerging medical technologies and applications. Among the emerging potential applications for cultured hepatocytes are gene therapy, bio-artificial organs, cell transplants, drug production, and drug and chemical testing. These technologies may benefit from the use of cloned and immortalized hepatocytes. Hence, there is a significant effort to develop hepatocyte cell lines for this purpose.

1.5.2. Spleen

Spleen is an essential organ which functions as filtering organ, removal of effected plates and cell and an important organ for immune response. The function of spleen also include pitting i.e. repairing of red cells, iron reutilization, pooling of platelets, reservoir of blood during sleeping and haematopoiesis.

1.5.3. Lymphocytes

Lymphocytes arise from the bone marrow stem cells. Depending on the site of subsequent maturation, they develop into two classes that cannot be distinguished by light microscopy. Lymphocytes are important immunological cells which are responsible for humoral immune response through B-lymphocytes and cell-mediated immune response through T-lymphocytes.
1.5.4. Erythrocytes

Erythrocytes, or red blood cells, are round disks, concave on two sides, and approximately 7.5 thousandths of a millimeter in diameter. In humans, and most other mammals, the mature red blood cell contains no nucleus; in some vertebrates, it is oval, and nucleated. Hemoglobin, a protein in the red blood cells, gives blood its red color and transports oxygen from the lungs to the body cells, where it picks up carbon dioxide for transport back to the lungs to be expired. Hemoglobin also transports nitric oxide, which regulates blood pressure by expanding or contracting blood vessel walls. Red blood cells are formed in the bone marrow. After an average life of 120 days, red blood cells are broken down and removed by the spleen.

1.6. CANCER

Cancer is not one particular disease but rather a group of diseases in which abnormal cells grow in an irregular way. The body is made of billions of normal cells that are like the bricks and mortar of a building. Each tiny cell has a specific job to do, whether it's relaying messages to the brain, digesting food or pumping blood. Normal cells grow and divide in an orderly fashion and respect the boundaries of neighboring cells. A cancer usually develops from a normal cell that has changed or mutated. The change may have occurred because of a virus, chemical or radiation injury, a family predisposition or unknown causes. This abnormal cell grows without following the rules that
directed it. Some cancers grow quickly; others grow slowly. As the cancer grows, it can invade normal organs causing normal body functions to be compromised or stopped. Tumors can squeeze organs or block passages. This will eventually cause symptoms or problems. Cancer also tends to spread (metastasize) to other parts of the body. This spread is accomplished in one of three ways:

1. By directly moving from the tumor to other body parts. Projections from the main tumor can be seen.
2. By crossing into the blood vessels and floating along until the cancer cell takes root in another area.
3. By crossing into the lymph system which normally acts as a filtering system for bacteria and debris. Eventually the lymph system empties into the blood. A cancer cell may be trapped in a lymph node (filtering station) and grow there or it may find its way into the blood stream and the rest of the body.

1.6.1. Cancer Stages

Cancers are often "staged" or rated on how extensive they are before treatment. By determining the correct stage, a physician can plan the best therapy and evaluate the results. Many systems are used to stage or grade tumors. The earlier the stage when diagnosed, the greater the opportunity for cure or improvement. Typically, the stages are -
1. Stage I, the simplest form, indicates that the tumor is small and confined to the organ or tissue where it began. The best chance for cure is when the tumor is still in Stage I.

2. Stage II means the tumor has spread to surrounding tissues.

3. Stage III means the tumor has spread into surrounding tissues and lymph nodes.

4. Stage IV signifies more extensive spread, often to many organs or parts of the body.

1.6.2. Therapy

There are five major types of cancer therapy:

- surgery
- radiation therapy
- chemotherapy
- immunotherapy
- blood and marrow transplant

The types of therapies that are used depend on several factors -

- Types of cancer - Some tumors are more sensitive to radiation; others respond better to chemotherapy.
- Location of the tumor - Some tumors can be removed surgically.
- Extent or stage of the disease - whether it has spread from the original spot.
1.6.4.1. Chemotherapy

Chemotherapy is the use of chemicals or medicines to treat cancers. Because chemotherapy is generally transported through the blood, it can treat cancers that have spread or are suspected of spreading some distance from the starting place. The chemotherapy includes a vast range of chemicals such as Alkylating Agents, Anti-estrogens, Antimetabolites, Antineoplastic Antibiotics, Antineoplastic Hormones, Interleukins, Mitotic Inhibitors, various antibiotics (cyclophosphamide, doxorubicin or Adriamycin), herbs (Chamomile, St Mary's Thistle, Alfalfa, Rosehips, Thuja, Parsley, Fennel, Blue Flag, Ginger and Liquorice, Bach Flowers; Walnut, Wild Oat, Sceranthus, Honeysuckle and Crab Apple), Ayurvedic preparations (*kushen*, *baihuasheshcao*, *yujin*, *ezhu*) drugs (vinblastine, vincristine, methotrexate and 5-fluorouracil), toxins (Coley’s toxins, dimethyl sulfoxide, hydrazine sulfate, podophyllotoxin) and to the specific poisonous compound like cyanide derivatives such as phenethyl isothiocyanate (PEITC) in the prevention of lung cancer (Hecht, 2003).

1.6.4.2. Immunotherapy

Immunotherapy is a form of treatment that enhances the body's ability to recognize cancer cells and destroy them. The body's immune system can usually destroy or help destroy most infections or other invaders like cancer cells. It can be given intravenously or by subcutaneous injection.
1.7. Scope and objectives of the present investigation

Controversial reports on the effects of carbamate insecticides on animal systems and non-availability of data on the effects of these insecticides in the \textit{in vitro} animal organ systems increased the interest to test the effect of some of the representative carbamate insecticide preparations (formulations) available in the market. Three representative carbamates closely related in chemical properties and biological action viz., baygon, carbaryl and carbofuran were selected for the present study.

These carbamate insecticides have shown to be toxic and carcinogenic to human and animals. As evidenced by the literature, a good carcinogen is also a potent anti-cancer agent. Keeping this in view an attempt was made to explore the possibility of using these carbamate insecticides in the treatment of cancer, in addition to study the effects on normal animal cells. Further, in the event of these carbamate insecticides being capable of destroying the cancer cells, the target of the present study was to evaluate how safe or harmful will this treatment be to other cells of the vital organs of the body. There was no available literature on such study. The present study will open a new vistas towards the treatment of cancer and open doors for vast variety of research in cancer chemotherapy.

\textit{In vitro} cell culture techniques provide a noval way to screen a vast variety of chemicals for the chemotherapy of various diseases. Hence screening
of these carbamate insecticides either for organ systems or tumor cells was done *in vitro*.

Considering the importance of carbamates on the host cells and its activity as an antitumor agent, the present study was undertaken with the following objectives.

1) *In-vitro* culture of hepatocytes, lymphocytes and spleenocytes

2) To study the effect of commercial formulations of the carbamate insecticides, baygon (Baygon spray), carbaryl (Sevin) and carbofuran (Carbogran 3G) on hepatocytes, lymphocytes and spleenocytes cultured *in vitro*.

3) To study the effect of technical grade baygon, carbaryl and carbofuran on hepatocytes, lymphocytes and spleenocytes cultured *in vitro*.

4) Identification of the toxic concentration range of carbamate insecticides and establishment of relationship of concentration to exposure time.

5) To explore the possibility of treating cancer with Carbamate insecticides viz., baygon, carbaryl and carbofuran.

6) To study the effect of carbamate treatment on Erythrocytes (Indirect measure of hypoxia) in addition to the above mentioned animal cells (systems) viz., liver (detoxicating organ), lymphocytes and Spleenocytes (immune system).