CHAPTER I

1. INTRODUCTION

Cancer is a growing public problem whose estimated worldwide new incidence is about 6 million cases per year. It is the second major cause of deaths after cardiovascular diseases. Cancer is a general term applied of series of malignant diseases that may affect different parts of the body. These diseases are characterized by a rapid and uncontrolled formation of abnormal cells, which may mass together to form a growth or tumor or proliferate throughout the body, initiating abnormal growth at other sites. If the process is not arrested, it may progress until it causes the death of the organism. These cells are born due to imbalance in the body and by correcting this imbalance the cancer may be treated (Siegel and Zhu, 2009).

1.1. Causes of cancer

Modern medicine attributes most cases of cancer to changes in DNA that reduce or eliminate the normal controls over cellular growth, maturation and programmed cell death. These changes are more likely to occur in people with certain genetic backgrounds as illustrated by the finding of genes associated with some cases of cancer and familial prevalence of certain cancers and in persons infected by chronic viruses e.g., viral hepatitis may lead to liver cancer and HIV may lead to lymphoma. The ultimate cause, regardless of genetic propensity or
viruses that may influence the risk of cancer, is often exposure to carcinogenic like chemicals those found in nature and the natural cosmic radiation are coupled with a failure of the immune system to eliminate the cancer cells at an early stage in their multiplication. The immunological weakness might arise years after the exposure to chemicals or radiation. Other factors such as tobacco smoking, alcohol consumption, excess use of caffeine and other drugs, sunshine, infections from such as the oncogenic viruses like cervical papillomaviruses, adenoviruses, Karposi’s sarcoma (KSHV) are exposure to asbestos. They obviously are implicated as causal agents of mammalian cancers. However, a large population of people is often exposed to these agents. Consequently, cancer cells continue to divide even in situation and in which normal cells will usually wait for a special chemical transduction signal. The tumor cells would ignore such stop signals that are sent out by adjacent tissues. A Cancer cell also has the character of immortality even in in vitro whereas normal cells stop dividing after 50–70 generations and undergoes a programmed cell death called as Apoptosis. Cancer cells continue to grow invading nearby tissues and metastasizing to distant parts of the body. Metastasis is the most lethal aspect of carcinogenesis (McNutt, 1995).
1.1.1. Types of cancers (Sakarkar and Deshmukh, 2011)

1. Cancers of Blood and Lymphatic Systems:
   (a) Hodgkin's disease (b) Leukemias (c) Lymphomas (d) Multiple myeloma, (e) Waldenstrom's disease

2. Skin Cancers:
   (a) Malignant Melanoma

3. Cancers of Digestive Systems:
   (a) Esophageal cancer (b) Stomach cancer (c) Cancer of pancreas
   (d) Liver cancer (e) Colon and Rectal cancer (f) Anal cancer

4. Cancers of Urinary system:
   (a) Kidney cancer (b) Bladder cancer (c) Testis cancer (d) Prostate cancer

5. Cancers in women:
   (a) Breast cancer (b) Ovarian cancer (c) Gynecological cancer
   (d) Choriocarcinoma

6. Miscellaneous cancers:
   (a) Brain cancer (b) Bone cancer (c) Carcinoid cancer (d) Thyroid cancer
   (e) Nasopharyngeal cancer (f) Retroperitoneal sarcomas (g) Soft tissue cancer

Among the various cancers, hepatocellular carcinoma (HCC) is one of the most common and deadly cancers worldwide. It accounts for about 90% of all
liver cancer and it represents more than 4% of all cancer cases worldwide (El-Serag and Rudolph, 2007).

1.2. Xenobiotics

Many xenobiotics drugs and environmental chemicals are capable of causing some degree of liver injury. In Asia, xenobiotic-induced liver toxicity is implicated in 2–5% of hospitalizations for jaundice, an estimated 15–30% of the cases of fulminant liver failure and 40% of the acute hepatitis cases in individuals older than 50 (Bass and Ockner, 1996; Lewis and Zimmerman, 1989). Fortunately, most drug-induced liver injuries resolve once the offending agent is withdrawn, but morbidity may be severe and prolonged as recovery ensues. The overall mortality rate for drug-induced liver injury is 5% (Werth et al., 1993).

The liver is prone to xenobiotic-induced injury because of its central role in xenobiotic metabolism, its portal location within the circulation and its anatomic and physiologic structure (Jones, 1996). The liver is divided into multiple lobules, each centered around a terminal hepatic central venule and surrounded peripherally by six portal triads. Afferent blood is supplied by the portal venules and hepatic arterioles of the portal triads, flows through the hepatic venous sinusoids and empties into the terminal hepatic venule. The regional pattern of hepatocellular necrosis observed with some xenobiotic-induced liver injuries can be understood by dividing the liver into functional subunits referred
to as acini (Rappaport and Wanless, 1993). Each liver acinus is divided into three concentric zones of hepatocytes radiating from a portal triad and terminating at one or more adjacent terminal hepatic venules. Hepatocytes closest to the portal triad zone one receives blood most enriched with oxygen and other nutrients and are most resistant to injury. Hepatocytes more distal to the blood supply receive a lower concentration of essential nutrients, making them more susceptible to ischemic or nutritional damage. Most important for xenobiotic-induced hepatic damage the centrilobular zone three hepatocytes are the primary sites of cytochrome P450 enzyme activity, which frequently makes them most susceptible to xenobiotic-induced liver injury (Thurman et al., 1986).

1.2.1. Metabolism of xenobiotic-induced liver carcinoma

Most drugs are not intrinsically toxic to the liver but can cause injury secondary to the production of a hepatotoxic drug metabolite, a process known as bioactivation (Vessey, 1996). Because gastrointestinal absorption is enhanced by lipid solubility, most xenobiotics are highly lipophilic compounds, which are poorly excreted by the kidneys. The liver plays a critical role in promoting excretion of these compounds by transforming them into metabolites of greater water solubility.

Metabolic reactions are two types, phase I and phase II (Vessey, 1996). Phase I (oxidation, reduction or hydrolysis) reactions typically occur first and
enhance water solubility by generating hydroxyl, carboxy or epoxide functional groups in the parent compound. These functional groups in turn facilitate phase II reactions, conjugation with glucuronate, sulfate, acetate or glutathione moieties. Conjugation reactions generally serve to further enhance water solubility and renal excretion. Phase II reactions also play a role in the prevention of xenobiotic-induced liver injury because most conjugates are biologically inactive (Lee, 1995). Disruption of normal phase II processes can lead to accumulation of hepatotoxic phase I metabolites.

Phase I oxidation and reduction reactions are primarily catalyzed by cytochrome P450 enzymes, a supergene family of heme-containing, mixed-function oxidase enzymes found in greatest concentration in the smooth endoplasmic reticulum of centrilobular hepatocytes (Watkins, 1992). These enzyme reactions have the potential to induce cellular injury via several mechanisms of toxicity. The cytochrome P450 enzyme-catalyzed oxidation of xenobiotics such as bromobenzene or acetaminophen generates a highly electrophilic intermediate capable of forming covalent adducts with critical cellular macromolecules such as thiol-containing membrane proteins that regulate calcium homeostasis (Bernhardt, 1995; Bellomo and Orrenius, 1985). The induction of increased intracellular calcium concentrations may be the common pathway leading to cell death. Cytochrome P450 enzyme mediated reduction of halogenated hydrocarbons such as carbon tetrachloride or halothane can also
generate free radical intermediates, which can directly damage cell membranes via lipid peroxidation or can target nucleophilic DNA residues (De Groot and Noll, 1983). Similar cellular damage can result from the generation of reactive oxygen species such as hydrogen peroxide and hydroxyl free radical during a process known as redox cycling (Recknagel et al., 1989).

1.3. Diethylnitrosamine (DEN)

Diethylnitrosamine (Fig. 1) is an N-nitroso alkyl compound, categorized as a potent hepatotoxin and hepatocarcinogen in experimental animals, producing reproducible tumors after repeated administration. The main cause for concern is that diethylnitrosamine is found in a wide variety of foods like cheese, soybean, smoked, salted and dried fish, cured meat and alcoholic beverages (Liao et al., 2001). Metabolism of certain therapeutic drugs is also reported to produce diethylnitrosamine (Akintonwa, 1985). It is also found in tobacco smoke at a concentration ranging from 1 to 28 ng/cigarette and in baby bottle nipples at a level of 10 ppb (IARC, 1972). Diethylnitrosamine is reported to undergo metabolic activation by cytochrome P450 enzymes to form reactive electrophiles, which cause oxidative stress leading to cytotoxicity, mutagenicity and carcinogenicity (Janani et al., 2010). The detection of diethylnitrosamine in commonly consumed food products makes the human population vulnerable to its exposure. This constraint underscores the need for the development of novel
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hepatoprotective drug with potent antioxidant activity. Various plants and plant derived products have been tested and found to be effective against diethylnitrosamine induced hepatocarcinogenesis and hepatotoxicity (Jayakumar et al., 2012).

Diethylnitrosamine (DEN), one of the most important environmental carcinogens, which is known to cause perturbations in the nuclear enzymes involved in DNA repair/ replication and normally used as a carcinogen to induce liver cancer in animal models (Ramakrishnan et al., 2006). N-nitroso compounds are known hepatocarcinogenic agents and have been implicated in the etiology of several human cancers (Bansal et al., 2005). These compounds are considered to be effective health hazards to man. The presence of nitroso compounds and their precursors in the environment, in certain occupational settings used as solvent in fiber industry, as a softener for copolymers and as additive for lubricants in condensers and also due to the use of tobacco products, cosmetics, pharmaceutical products as well as their endogenous formation in the human body from dietary components are considered as potential risk factors for the development of cancer (Bartsch and Montesano, 1984).
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Fig.1.1 Shows the structure of Diethylnitrosamine (DEN)

1.3.1. Metabolic Activation of DEN

DEN became metabolically active by the action of cytochrome P450 enzymes to produce reactive electrophiles, which increase hepatocarcinogenesis level leading to cytotoxicity, mutagenecity and carcinogenicity. It has been shown that on primary metabolic activation, DEN produces the promutagenic adducts, O6-ethyl deoxy guanosine and O4- and O6-ethyl deoxy thymidine that can produce DNA chain damage, depurination or binding to DNA and often generating a miscoding gene sequence, paving way to initiation of liver carcinogenesis through free radical mechanisms (Fig. 2). DEN is producing a reproducible tumor after repeated administration and also it serves as a standard model to study the beneficial effects of many drugs and treatments on hepatocarcinogenesis (Ueno et al., 2005). Chemoprevention is one of the strategies by which we can revert or delay the response of carcinogen.
1.4. Multistep carcinogenesis

The induction of cancer by chemicals involves a multistage and multistep process. While this process involves multiple molecular and cellular events that transform a normal cell to a malignant neoplastic cell, evidence in recent years has defined multiple stages or steps in the chemical carcinogenesis process. Two of the early steps in the process are initiation and promotion. Initiation results when a normal cell sustains a DNA mutation, when preceded by a round of DNA synthesis, results in fixation of the mutation, producing an initiated cell. Production of the initiated mutated cell can occur through interaction with physical carcinogens such as UV light and radiation, as well as chemical carcinogens (Klaunig and Kamendulis, 2007). The term genotoxic agent is applied to both physical and chemical carcinogens that directly damage DNA and possess mutagenic properties. In addition, recent evidence has suggested that during normal cell proliferation and DNA synthetic processes, mutations may be acquired through misrepair of damaged DNA resulting in spontaneous initiated mutated cells. Following formation of initiated cells, chemicals as well as endogenous physiological events can influence the subsequent selective clonal growth of initiated cell populations through the process of tumor promotion. Tumor promotion in the liver involves the expansion of initiated cells to histologically defined focal lesions. While the promotion process does not involve DNA reactivity or damage, a hallmark of this stage is the modulation of gene
expression resulting in increased cell number either through cell division and/or decrease in apoptotic cell death (Klaunig and Kamendulis, 2007). Following additional chemical insults or through multiple divisions and acquisitions of mutations in the preneoplastic focal lesions, the formation of benign and/or malignant neoplasms can occur during the progression stage. While this multistep process has been established and defined in rodent systems, increasing evidence has shown that a similar process occurs in nonhuman primates and humans.

1.5. Hepatic cancer mode of action

The mechanisms by which carcinogens induce their effects have been studied extensively for over half a century. Using the rodent liver model as an example, the modes of action by which carcinogens induce hepatic cancer can be categorized based upon molecular targets and cellular effects that include genotoxic (DNA reactive) and nongenotoxic mechanisms. Genotoxicity reflects the interaction of the agent or its metabolite with genomic DNA resulting in mutational events. Agents that act through genotoxicity usually function at the initiation or progression stage of the cancer process. Besides genotoxicity, a number of nongenotoxic modes of action have been ascribed to a variety of chemical agents that induce cancer following chronic exposure in rodents. Nongenotoxic agents can be cytotoxic or mitogenic and may act through specific receptor-mediated pathways. Cytotoxicity, in the case of the liver, is produced by
Fig. 1.2 shows the mechanism of hepatocarcinogenesis induced by diethylnitrosamine (DEN)
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compounds such as chloroform, nitrosamine and carbon tetrachloride, which at relatively high doses will induce hepatocyte necrosis and damage resulting in a compensatory hyperplasia enabling the liver to replace damaged cells. Spontaneous mutations can be acquired as a result of enhanced cell proliferation or the clonal expansion of initiated cells, events that may ultimately lead to tumor formation. Compounds that work through cytotoxic mechanisms thus function at the promotion stage where they either enhance already formed spontaneous initiated cells and/or can induce new initiated cells through compensatory hyperplasia (Klaunig and Kamendulis, 2007).

1.6. Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS)

In recent years there is an increasing awareness among people in prevention of disease especially the role of free radicals in health and disease. The theory of oxygen-free radicals has been known about fifty years ago (Valko et al., 2006). However, only within the last two decades, has there been an explosive discovery of their roles in the development of diseases and also of the health protective effects of antioxidants. Free radicals are continuously produced by the body's normal use of oxygen (Velavan, 2011). Oxygen is an element indispensable for life. When cells use oxygen to generate energy free radicals are produced by the mitochondria. These by-products are generally reactive oxygen
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species (ROS) as well as reactive nitrogen species (RNS) that result from the cellular redox process (Tiwari, 2004).

ROS and RNS are the terms collectively describing free radicals and other non-radical reactive derivatives also called oxidants. Radicals are less stable than non-radical species, although their reactivity is generally stronger. A molecule with one or more unpaired electron in its outer shell is called a free radical (Lian et al., 2008). Free radicals are formed from molecules via the breakage of a chemical bond such that each fragment keeps one electron, by cleavage of a radical to give another radical and also via redox reactions. Free radicals include hydroxyl (OH\textsuperscript{•}), superoxide (O\textsubscript{2}\textsuperscript{•}) nitric oxide (NO\textsuperscript{•}), nitrogen dioxide (NO\textsubscript{2}\textsuperscript{•}), peroxyl (ROO\textsuperscript{•}) and lipid peroxyl (LOO\textsuperscript{•}), also, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), ozone (O\textsubscript{3}), singlet oxygen (\textsuperscript{1}O\textsubscript{2}), hypochlorous acid (HOCl), nitrous acid (HNO\textsubscript{2}), peroxynitrite (ONOO\textsuperscript{–}), dinitrogen trioxide (N\textsubscript{2}O\textsubscript{3}), lipid peroxide (LOOH), are not free radicals and generally called oxidants, but can easily lead to free radical reactions in living organisms. Biological free radicals are thus highly unstable molecules that have electrons available to react with various organic substrates such as lipids, proteins, DNA (Velavan, 2011).

1.6.1. Generation of free radicals

Formation of ROS and RNS can occur in the cells by two ways: enzymatic and non-enzymatic reactions. Enzymatic reactions generating free radicals
include those involved in the respiratory chain, phagocytosis, prostaglandin synthesis and the cytochrome P450 system (Bandyopadhyay, 1999). For example, the superoxide anion radical (O$_2$$^-\cdot$) is generated via several cellular oxidase systems such as NADPH oxidase, xanthine oxidase, peroxidases. Once formed, it participates in several reactions yielding various ROS and RNS such as hydroxyl radical (OH*), peroxynitrite (ONOO$^-$), hypochlorous acid (HOCl), etc. H$_2$O$_2$ (a non radical) is produced by the action of several oxidase enzymes, including aminoacid oxidase and xanthine oxidase. The last one catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid (Slater, 1985).

Hydroxyl radical (OH*), the most reactive free radical in vivo, is formed by the reaction of O$_2$$^-\cdot$ with H$_2$O$_2$ in the presence of Fe$^{2+}$ or Cu$^{2+}$ (catalyst). This reaction is known as the Fenton reaction. Hypochlorous acid (HOCl) is produced by the neutrophil-derived enzyme, myeloperoxidase, which oxidizes chloride ions in the presence of Hydrogen Peroxide (H$_2$O$_2$). Nitric oxide radical (NO*) is formed in biological tissues from the oxidation of L-arginine to citrulline by nitric oxide synthase. Free radicals can be produced from non-enzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing radiations. The non enzymatic process can also occur during oxidative phosphorylation (i.e., aerobic respiration) in the mitochondria (Halliwell, 2007).
ROS and RNS are generated from either endogenous or exogenous sources. Endogenous free radicals are generated from immune cell activation, inflammation, mental stress, excessive exercise, ischemia, infection, cancer, aging. Exogenous ROS/RNS result from air and water pollution, cigarette smoke, alcohol, heavy or transition metals (Cd, Hg, Pb, Fe, As), certain drugs (cyclosporine, tacrolimus, gentamycin, bleomycin), industrial solvents, cooking (smoked meat, used oil, fat), radiation. After penetration into the body by different routes, these exogenous compounds are decomposed or metabolized into free radicals.

1.6.2. ROS in normal physiology

Typically, low concentration of ROS is essential for normal physiological functions like gene expression, cellular growth and defense against infection. Sometimes they also act as the stimulating agents for bio-chemical processes within the cell. ROS exert their effects through the reversible oxidation of active sites in transcription factors such as nuclear factor-kappa B (NF-kB) and activator protein-1 (AP-1) leading to gene expression and cell growth. ROS can also cause indirect induction of transcription factors by activating signal transduction pathways. One example of signal transduction molecules activated by ROS is the Mitogen Activated Protein Kinases (MAPKs). ROS also appear to serve as secondary messengers in many developmental stages. Apart from these ROS also
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participate in the biosynthesis of molecules such as thyroxin, prostaglandin that accelerate developmental processes. It is noteworthy that in thyroid cells, regulation of H\textsubscript{2}O\textsubscript{2} concentration is critical for thyroxine synthesis, as it is needed to catalyze the binding of iodine atoms to thyroglobulin. ROS are also used by the immune system. For example, ROS has been shown to trigger proliferation of T cells through NF-KB activation. Macrophages and neutrophils generate ROS in order to kill the bacteria that they engulf by phagocytosis. Further more, tumor necrosis factor (TNF-α) mediates the cytotoxicity of tumor and virus infected cells through ROS generation and induction of apoptosis (Valko et al., 2006; Tiwari, 2004).

1.6.3. ROS induced oxidative damages

Depending upon their nature, ROS (for e.g., OH\’ radicals) reactions with biomolecules such as lipid, protein and DNA, produce different types of secondary radicals like lipid radicals, sugar and base derived radicals, amino acid radicals and thyl radicals. These radicals in presence of oxygen are converted to peroxyl radicals. Peroxyl radicals are critical in biosystems, as they often induce chain reactions. The biological implications of such reactions depend on several factors like the site of generation, nature of the substrate, activation of repair mechanisms and redox status among many others. Cellular membranes are vulnerable to the oxidation by ROS due to the presence of high concentration of
unsaturated fatty acids in their lipid components. ROS reactions with membrane lipids cause lipid peroxidation, resulting in formation of lipid hydroperoxide (LOOH) which can be further de-compose to an aldehyde such as malonaldehyde (MDA), 4-hydroxy nonenal (4-HNE) or form cyclic endoperoxide, isoprotans and hydrocarbons. The consequences of lipid peroxidation are cross linking of membrane proteins, change in membrane fluidity and formation of lipid-protein, lipid-DNA adduct, which may be detrimental to the functioning of the cell (Cully et al., 2002).

1.7. Lipid peroxidation (LPO)

Lipids are heterogeneous groups of compound having significant role in various functions of body, when molecular oxygen reacts with unsaturated lipids catalyzed by free radicals (non-enzymatic LPO) or enzymes (enzymatic LPO) turning them rancid due to oxidative deterioration without releasing energy known as lipid peroxidation leads to cell damage by disturbance of fine structures, alteration of integrity, fluidity and functional loss of biomembranes and modifies Low Density Lipoprotein (LDL) to proatherogenic and proinflammatory mediated potentially toxic products (Halliwell B, Gutteridge, 1990).
1.7.1. Cellular damage and lipid peroxidation

The study of lipid peroxidation (LP) is attracting much attention in recent years due to its role in disease processes. Membrane lipids are particularly susceptible to LP due to the presence of polyunsaturated fatty acids. Since membranes form the basis of many cellular organelles like mitochondria, plasma membranes, endoplasmic reticulum, lysosomes, peroxisomes, etc. the damage caused by LP is highly detrimental to the functioning of the cell and its survival. It has been implicated in the pathogenesis of a number of diseases and clinical conditions. These include atherosclerosis, cancer, adult respiratory distress syndrome, Alzheimer’s disease, Parkinson’s disease, ischaemia-reperfusion injury of various organs, chemical and radiation-induced injury, diabetes, etc. Experimental and clinical evidence suggests that aldehyde products of LP can also act as bioactive molecules in physiological and pathological conditions. It is now generally accepted that LP and its products play an important role in liver, kidney and brain toxicity (Usyal et al., 1989; Gutteridge, 1995).

1.8. Protein Oxidation

Proteins can undergo direct and indirect damage following interaction with ROS resulting into peroxi-dation, changes in their tertiary structure, proteolytic degradation, protein-protein cross linkages and fragmentation. The side chains of all amino acid residues of proteins, in particular tryptophan, cysteine and...
methionine residues are susceptible to oxidation by ROS. Protein oxidation products are usually carbonyls such as aldehydes and ketones (Stadtman and Levine, 2000).

1.9. DNA Oxidation

Although DNA is a stable, well-protected molecule, ROS can interact with it and cause several types of damage such as modification of DNA bases, single and double strand DNA breaks, loss of purines (apurinic sites), damage to the deoxyribose sugar, DNA-protein cross-linkage and damage to the DNA repair system. Not all ROS can cause DNA damage and OH$^\cdot$ radical is one of the potential inducers of DNA damage. A variety of adducts are formed on reaction of OH$^\cdot$ radical with DNA (Fraga et al., 1990). The OH$^\cdot$ radical can attack purine and pyrimidine bases to form OH$^\cdot$ radical adducts, which are both oxidizing and reducing in nature. This induces base modifications and some-times release of bases. Some of the important base modifications include 8-hydroxydeoxyguanosine (8-OHdG), 8-or 4-, 5-hydroxyadenine, thymine peroxide, thymine glycols and 5-hydroxymethyl uracyl. Free radicals can also attack the sugar moiety, which can produce sugar peroxyl radicals and subsequently inducing strand breakage. The consequence of DNA damage is the modification of genetic material resulting into cell death, mutagenesis, carcinogenesis and ageing (Dandona et al., 1996).
1.1. Free radicals and diseases

Free radicals and oxidants play a dual role as both toxic and beneficial compounds, since they can be either harmful or helpful to the body. They are produced either from normal cell metabolisms in situ or from external sources, when an overload of free radicals cannot gradually be destroyed their accumulation in the body generates a phenomenon called oxidative stress. This process plays a major part in the development more than hundred diseases (Halliwell and Gutteridge, 1999).

1.1.1. Cancer and oxidative stress

The development of cancer in humans is a complex process including cellular and molecular changes mediated by diverse endogenous and exogenous stimuli. It is well established that oxidative DNA damage is responsible for cancer development. Cancer initiation and promotion are associated with chromosomal defects and oncogene activation induced by free radicals. A common form of damage is the formation of hydroxylated bases of DNA, which are considered an important event in chemical carcinogenesis. This adduct formation interferes with normal cell growth by causing genetic mutations and altering normal gene transcription. Oxidative DNA damage also produces a multiplicity of modifications in the DNA structure including base and sugar lesions, strand breaks, DNA-protein cross-links and base-free sites (Diplock et al., 1994).
1.10.2. Cardiovascular disease and oxidative stress

Cardiovascular disease (CVD) is of multifactorial etiology associated with a variety of risk factors for its development including hypercholesterolaemia, hypertension, smoking, diabetes, poor diet, stress and physical inactivity amongst others. Recently, research data has raised a passionate debate as to whether oxidative stress is a primary or secondary cause of many cardiovascular diseases. Further *in vivo* and *ex vivo* studies have provided precious evidence supporting the role of oxidative stress in a number of CVDs such as atherosclerosis, ischemia, hypertension, cardiomyopathy, cardiac hypertrophy and congestive heart failure (Vijaykumar *et al*., 2010).

1.10.3. Neurological disease and oxidative stress

Oxidative stress has been investigated in neurological diseases including Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, Amyotrophic Lateral Sclerosis (ALS), memory loss and depression. In a disease such as Alzheimer’s, numerous experimental and clinical studies have demonstrated that oxidative damage plays a key role in the loss of neurons and the progression to dementia. The production of β-amyloid, a toxic peptide often found present in Alzheimer’s patients’ brain, is due to oxidative stress and plays an important role in the neurodegenerative processes.
1.10.4. Rheumatoid arthritis and oxidative stress

Rheumatoid arthritis is an autoimmune disease characterized by chronic inflammation of the joints and tissue around the joints with infiltration of macrophages and activated T cells. The pathogenesis of this disease is due to the generation of ROS and RNS at the site of inflammation. Oxidative damage and inflammation in various rheumatic diseases were proved by increased levels of isoprostanes and prostaglandins in serum and synovial fluid compared to controls (Geronikaki et al., 2006).

1.10.5. Nephropathy and oxidative stress

Oxidative stress plays a role in a variety of renal diseases such as glomerulonephritis and tubulointerstitial nephritis, chronic renal failure, proteinuria, uremia. The nephrotoxicity of certain drugs such as cyclosporine, tacrolimus (FK506), gentamycin, bleomycin, vinblastine are mainly due to oxidative stress via lipid peroxidation. Heavy metals (Cd, Hg, Pb, As) and transition metals (Fe, Cu, Co, Cr)-induced different forms of nephropathy and carcinogenicity are strong free radical inducers in the body (Galle, 2001).

1.10.6. Diabetes

Experimental evidence suggests that destruction of islets of pancreas due to accumulation of free radicals is one of the causes for the pathogenesis of...
insulin dependent diabetes mellitus. Excess generation of mitochondrial ROS due to hyperglycemia initiates a vicious circle by activating stress-sensitive pathways such as NF- B, p38 MAPK and JAK/STAT, polyol (sorbitol) and hexosamine pathways, PKC and AGES. Enhanced production of AGES, sorbitol and proinflammatory cytokines exerts a positive feedback on ROS and RNS synthesis and potentiates PKC-mediated vascular dysfunction by altering gene expression as well as vascular function and structure (Johansen et al., 2005).

1.10.7. Male Infertility

Free radicals are known to reduce sperm motility and viability and thus may contribute to male infertility. The lipid composition of plasma membrane of mammalian spermatozoa is markedly different from mammalian somatic cells. They have very high levels of phospholipids, sterols, saturated and polyunsaturated fatty acids. Therefore sperm cells are particularly susceptible to the damage induced by excessive ROS release. Lipid peroxidation plays a major role in the etiology of defective sperm function. This may lead to the onset of male infertility via the mechanism involving the induction of peroxidative damage to plasma membrane (Yadav et al., 2006).

1.10.8 Ageing process

Mitochondrial ROS production and oxidative damage to mitochondrial DNA results in ageing. The most recent review on free radicals and ageing by
Barja emphasizes that caloric restriction is the only known experimental manipulation that decreases rate of mammalian ageing (Barja, 2004).

Cancer chemopreventive agents are able to reduce the incidence of tumorigenesis by intervening in one or more stages of carcinogenesis initiation, promotion or prolongation. In developing countries about 35% of prescribed drugs are derived from natural products. The reduced cancer risk and lack of toxicity associated with high intake of natural products suggest that specific concentrations of phytochemicals from these plant sources may produce cancer chemopreventive effects without causing significant levels of toxicity. These natural products are believed to suppress the inflammatory process that lead to neoplastic transformation, hyperproliferation, promotion and progression of carcinogenic process and angiogenesis.

1.11 Antioxidants

An antioxidant has been defined as “a substance that when present at low concentrations compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate”. Antioxidants may exert their activity by several mechanisms, like by suppressing the production of active species by reducing hydroperoxides and \( \text{H}_2\text{O}_2 \), by sequestering metal ions, termination of chain reaction by scavenging active free radicals and also caused repairing and/or clearing damage of cell. Biosynthesis of other antioxidants or
defense enzymes also induced by some antioxidants (Halliwell B, Gutteridge, 1990).

A variety of components act against free radicals to neutralize them from both endogenous and exogenous in origin (Jacob, 1995). These include: Endogenous enzymatic antioxidants, Non enzymatic, metabolic and nutrient antioxidants, Metal binding proteins like ferritin, lactoferrin, albumin and ceruloplasmin and Phytoconstituents and phytonutrients.

1.1.1. Classification of antioxidants

Antioxidant classified based on the solubility and defensive mechanism. They are two types.

1.1.2. A. Based on solubility:

(a) Hydrophilic antioxidants: They are soluble in water. Water soluble antioxidants react with oxidants react with oxidants in the cell cytoplasm and blood plasma.

(b) Hydrophobic antioxidants: They are soluble in lipids. Lipid soluble antioxidants protect cell membranes from lipid peroxidation.

1.1.3. B. Based on line of defense:

(a) First line defense (Preventive antioxidant)
Enzymatic antioxidants complement the nonenzymatic antioxidants and either catalyze reactions to remove ROS or to regenerate (reduce) oxidized antioxidants. These are enzymes like Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx), Glutathione Reductase (GR) and some minerals like Se, Mn and Cu etc.

SOD functions in the cell as one of the primary enzymatic antioxidant defenses against superoxide radicals. Increases in SOD enzyme activity corresponds with enhanced resistance to oxidative stress. Superoxide Dismutase (SOD) is primarily located in mitochondria and the cytosol of cells and neutralizes superoxide radicals. Two isoforms of SOD are known to exist in skeletal muscle in mammals ie, Cu, Zn SOD, which is located primarily in the cytosol and MnSOD, which is located primarily in mitochondria. SOD activity in muscle is highest in high oxidative muscle fibers (ie, types I and IIa). An extracellular form of SOD also exists that requires Cu and Zn and is found in both plasma and tissues (Christopher et al., 2003).

The decomposition of hydrogen peroxide to form water and oxygen is accomplished in the cell by catalase. This antioxidative enzyme is widely distributed in the cell, with the majority of the activity occurring in the mitochondria and peroxisomes. CAT requires iron as a cofactor and similar to
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GSHPx and SOD, its activity is highest in highly oxidative muscle fibers (Bandyopadhyay et al., 1999).

GPx is another antioxidant enzyme with a much greater affinity for hydrogen peroxide than catalase. Glutathione peroxidase (GSHPx) is found in mitochondria, the cytosol and the membranes of cells and reduces H₂O₂ and organic hydroperoxides using reduced glutathione (GSH) as the electron donor. GSHPx activity is selenium dependent and its activity is highest in type I muscle fibers (ie, highly oxidative fibers). Glutathione Reductase (GR) is essential for conversion of Oxidized Glutathione (GSSG) back to the reduced form (GSH) using NADPH as the reductant (Halliwell and Gutteridge, 1999).

(b) Second line defense (Radical scavenging antioxidant)

These are glutathione, Vit C, Vit E, uric acid, albumin, bilirubin, carotenoid, flavonoid etc. β-carotene is an excellent scavenger of singlet oxygen.

Vitamin C is essential for life and must be ingested for survival in human. It is a powerful water soluble antioxidant. Vitamin C is an electron donor and therefore a reducing agent. All known physiological and biochemical actions of vitamin C are due to its action as an electron donor. Ascorbic acid donates two electrons from a double bond between the second and third carbons of the 6-carbon molecule. Vitamin C is called an antioxidant because, by donating its electrons, it prevents other compounds from being oxidized. However, by the
very nature of this reaction, vitamin C itself is oxidized in the process (Nishikimi and Yagi, 1996).

Glutathione is a ubiquitous thiol-containing tripeptide, which plays a central role in cell biology. It is implicated in the cellular defense against xenobiotics and naturally occurring deleterious compounds, such as free radicals and hydroperoxides. Glutathione status is a highly sensitive indicator of cell functionality and viability. GSH provides a first line of defense against ROS, as it can scavenge free radicals and reduce H2O2. Glutathione-dependent enzymes provide a second line of defense, as they primarily detoxify noxious byproducts generated by ROS and also help to prevent propagation of free radicals (Pastore et al., 2003). The glutathione status is used as a biological marker of aging (Nokata et al., 1996).

The main protective roles of glutathione against oxidative stress are: (i) glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g., glutathione peroxidase (GPx) and others; (ii) GSH participates in amino acid transport through the plasma membrane; (iii) GSH scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase and (iv) glutathione can regenerate the most important antioxidants, vitamins C and E, back to their active forms;
glutathione can reduce the tocopherol radical of vitamin E directly or indirectly, via reduction of semidehydroascorbate to ascorbate (Pastore et al., 2003).

Vitamin E is lipid soluble vitamin in the body and derived from diet. It is thought to be an important chain-breaking antioxidant and directly scavenge reactive oxygen members (ROMs). It is the major lipid-soluble antioxidant present in all cellular membranes, which protects against lipid peroxidation. Vitamin E can directly act with a variety of oxyradicals, including the perxy radical (ROO$^\cdot$), OH$^\cdot$, O$_2$$^\cdot$ and singlet oxygen (Machlin and Bendich, 1987). The major function of vitamin E is its role as a physiological membrane bound antioxidant, protecting cell membrane lipids from oxidative damage initiated by ROMs. Vitamin E donates hydrogen from the 6th position of its chromonal ring to free radical to form oxidized vitamin E (tocopheroxyl radical). The oxidized vitamin E can be reduced by glutathione or ascorbic acid.

(c) **Third line defense (Repair and de-novo enzymes)**

These are a complex group of enzymes for repair of damaged DNA, protein, oxidized lipids and peroxides and also to stop chain propagation of peroxyl lipid radical. These enzymes repair the damage to biomolecules and reconstitute the damaged cell membrane.
1.12. Cancer Therapeutic Agents

Anticancer therapy is one of the biggest challenges in medicine. The main forms of treatment for cancer in humans are surgery, radiation and drugs (cancer chemotherapeutic agents). Combination therapy is a usable tool to improve the response of therapy and outcome. The critical function of drug classification serves to comprehend the mechanism of action that is important for the design of combination therapy, since multidrug regimens usually include drugs belonging to different groups to increase efficacy and decrease toxicity. Classically, anticancer drugs were grouped as chemotherapy, immunotherapy, hormonal therapy and radiotherapy (Espinosa et al., 2003).

1.12.1. The mechanism on cancer therapy (Sakarkar and Deshmukh, 2011)

1. Inhibiting cancer cell proliferation directly by stimulating macrophage phagocytosis, enhancing natural killer cell activity.

2. Promoting apoptosis of cancer cells by increasing production of interferon, interleukin-2 immunoglobulin and complement in blood serum.

3. Enforcing the necrosis of tumor and inhibiting its translocation and spread by blocking the blood source of tumor tissue.

4. Enhancing the number of leukocytes and platelets by stimulating the hemopoietic function.
5. Promoting the reverse transformation from tumor cells into normal cells.

6. Promoting metabolism and preventing carcinogenesis of normal cells.

7. Stimulating appetite, improving quality of sleep, relieving pain, thus benefiting patient’s health.

Cancer chemotherapeutic agents can often provide temporary relief of symptoms, prolongation of life and occasionally cures. In recent years, a lot of effort has been applied to the synthesis of potential anticancer drugs. Many hundreds of chemical variants of known class of cancer chemotherapeutic agents have been synthesized but have a more side effects. Chemotherapy agents can be divided into several categories: alkylating agents (e.g., cyclophosphamide, ifosfamide), antibiotics which affect nucleic acids (e.g., doxorubicin, bleomycin), platinum compounds (e.g., cisplatin), mitotic inhibitors (e.g., vincristine), antimetabolites (e.g., 5-fluorouracil) and camptothecin derivatives (e.g., topotecan). Hormonal therapy such as steroids, anti-estrogens, anti-androgens, LH–RH analogs and anti-aromatase agents. Immunotherapy such as Interferon, Interleukin 2 and Vaccines (Espinosa et al., 2003). A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. The agents most noted for creating cellular damage by initiating free radical oxidants are the alkylating agents, the tumor antibiotics and the platinum compounds. The agents in these categories demand definition concerning
interactions with antioxidants which might reduce effectiveness of chemotherapy. There is also the possibility of adverse interaction between antioxidant treatment and agents that do not act via an oxidative mechanism (e.g., 5-fluorouracil or tamoxifen) (Wu, 2006).

In addition to the idea that chemotherapy must create a lethal injury to DNA to produce malignant cell death is the mechanism of apoptosis. A dose of chemotherapy which does not produce necrosis can trigger apoptosis, either immediate or delayed. Additionally, anti-apoptotic mutations can result in drug resistance in human tumors. At least one antioxidant (quercetin) has been demonstrated to overcome such an anti-apoptotic blockage (Yoshida et al., 1990).

Radiotherapy uses ionizing radiation to produce cell death through free radical formation. Two mechanisms are involved. The apoptosis mechanism results in cell death within a few hours of radiation. The second mechanism is radiation-induced failure of mitosis and the inhibition of cellular proliferation, which kills cancer cells. Currently, the principal target of radiation is considered to be cellular DNA. However, studies show the signal for apoptosis can be generated by the effect of radiation on cell membranes, apparently through lipid peroxidation. This suggests an alternate mechanism to the hypothesis that DNA damage is required for cell death (Holland et al., 1997).
Chemotherapy remains the treatment of choice in many malignant diseases. However, the emergence of resistance to anticancer drugs, in particular multidrug resistance (MDR), has made many of the available anticancer drugs ineffective (Borowski et al., 2005). MDR is a complex multifactorial phenomenon that can result from a number of biochemical mechanisms, including a decreased drug uptake or an increased drug efflux; the perturbed expression of target enzymes or altered target enzymes; the altered metabolism of drugs; the increased repair of drug-induced DNA damage or a failure to undergo apoptosis. The enhanced activity of various members of the family of adenosine triphosphate binding cassette (ABC)-transporters was associated with different types of MDR (Lage, 2008). These membrane-embedded transport proteins act as energy-dependent drug extrusion pumps that decrease the intracellular concentration of multiple anticancer agents of different chemical structures and mode of action. The most significant mechanism of MDR, referred to as typical or classical MDR, is that resulting from the over expression of ABC-transporter proteins. The most important and widely studied members of ABC transporters are MDR1/P-glycoprotein (MDR1/P-gp, ABCB1), multidrug resistance associated protein (MRP1/ABCC1) and breast cancer resistance protein (BCRP, ABCG2) (Lage, 2008).

Synthesis of modifications of known drug continues as an important aspect of research. However, a waste amount of synthetic work has given relatively
small improvements over the prototype drugs. There is a continued need for new prototype-new templates to use in the design of potential chemotherapeutic agents: natural products are providing such templates. Recently, there have been considerable efforts to search for naturally occurring substances for the intervention of carcinogenesis. Recent studies of tumor-inhibiting compound of plant origin have yielded an impressive array of novel structures. Many of these structures are extremely complex and it is most unlikely that such compounds would have been synthesized in empirical approaches to new drugs (Tyler, 1994). Many components derived from dietary or medicinal plants have been found to possess substantial chemopreventive properties (Surh, 2003).

1.13 Computer models

Computers can help to understand the various basic principles of biology. Specialized computer models and software programs help to design new medicines. Computer generated simulations are used to predict the various possible biological and toxic effects of a chemical or potential drug candidate without animal dissection. Only the most promising molecules obtained from primary screening are used for in vivo experimentation. For example, to know the receptor binding site of a drug, in vivo experimentation is necessary. Software known as Computer Aided Drug Design (CADD) is used to predict the receptor binding site for a potential drug molecule. CADD works to identify probable binding site and hence avoids testing of unwanted chemicals having no biological
activity. Also, with the help of such software programs we can tailor make a new drug for the specific binding site and then in final stage animal testing is done to obtain confirmatory results (Vedani, 1991).

Another popular tool is the Structure Activity Relationship (SARs) computer programs. It predicts biological activity of a drug candidate based on the presence of chemical moieties attached to the parent compound. Quantitative Structure Activity Relationship (QSAR) is the mathematical description of the relationship between physicochemical properties of a drug molecule and its biological activity (Knight et al., 2006). The activities like carcinogenicity and mutagenicity of a potential drug candidate are well predicted by the computer database. The recent QSAR software shows more appropriate results while predicting the carcinogenicity of any molecule. The advantages of computer models over conventional animal models are the speed and relatively inexpensive procedures (Matthews and Contrera, 1998).

1.15. Medicinal plants with anticancer activity

Medicinal plants are assuming greater importance in the primary health care of individuals and communities in many developing countries. There has been an increase of demand in international trade because of very effective, cheaply available, supposedly have less or no side effects and used as alternative to allopathic medicines. Medicinal plants are believed to be much safer and
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proved elixir in the treatment of various ailments. Medicinal plants are a major source of biodynamic compounds of therapeutic values (Ashis, 2003).

Plant and plant products are being used as a source of medicine since long. According to World Health Organization (WHO) more than 80% of the world’s population, mostly in poor and less developed countries depend on traditional plant-based medicines for their primary healthcare needs (WHO, 1993). Medicinal plants are the nature’s gift to human being to make disease free healthy life. It plays a vital role to preserve our health. India is one of the most medico-culturally diverse countries in the world where the medicinal plant sector is part of a time-honored tradition that is respected even today. Here, the main traditional systems of medicine include Ayurveda, Unani and Siddha. The earliest mention of the use of plants in medicine is found in the Rigveda, which was written between 4500 and 1600 BC. During British period due to Western culture our Traditional art of natural healing is disappeared. Now it is reappearing due to realization of its importance in curing diseases without any side effect. Owing to the global trend towards improved ‘quality of life’, there is considerable evidence of an increase in demand for medicinal plant. (Kotnis et al., 2004).

Use of plants for treating various ailments of both man and animal is as old practice as man himself. India is richly endowed with a wide variety of plants having medicinal value. These plants are widely used by all sections of the
society whether directly as folk remedies or indirectly as pharmaceutical preparation of modern medicine. (Uniyal, 2003). In recent times, focus on plant research has increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants used in various traditional systems (Ayurveda, Siddha and Unani).

1.16 *Bryonopsis laciniosa*

*Bryonopsis laciniosa* is genus of about 6 species of annual slender plants in the family cucurbitaceae, native to throughout India from the Himalayas to Ceylon, tropical Africa, Australia and is one of the most versatile medicinal plants having a wide spectrum of biological activity *Bryonopsis laciniosa* is a highly valuable medicinal cucurbit commonly known as lollipop climber and it is called as “Shivlingi” in India (Acharya, 2007; Panda, 2004). It has been included in Vrishya rasayana category in Ayurvedic texts. Gond and Bharia tribes of patalkort vally worship this plant. According to them, this herb is boon for the childless parents. *Bryonopsis laciniosa* was proved as remedy by the German founder of Homeopathy, Dr. Samuel Hahnemann in 1834. (Alekhya et al., 2014).

1.16.1 Taxonomic Classification (Kirtikar and Basu, 1987)

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
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<tbody>
<tr>
<td>Phylum</td>
<td>Tracheophyta</td>
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<tr>
<td>Class</td>
<td>Magnoliopsida</td>
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<tr>
<td>Subclass</td>
<td>Rosida</td>
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</tbody>
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Anticancer activity of *Bryonopsis laciniosa* on Nitrosodiethyl amine induced liver cancer in male albino rats
Order : Cucurbitales
Family : Cucurbitaceae
Genus : Bryonia
Species : B. laciniosa.
Botanical name : Bryonopsis laciniosa

1.16.2 Vernacular Name (Reddy et al., 2010)

Tamil : Aiveli, Aiviral kovai, Aivirali, Iviralikovai, Ivoralikovai

Hindi : Bajguriya, Shivlingi,

Marathi : Kovdoli,

Sanskrit : Baja, Citraphalah, lingini,

Figure 1.3 Bryonopsis laciniosa Plant with Fruit
1.16.3 Morphology

Stem is much branched, slender, grooved and glabrous. Tendrils are slender, striate and scabrous. Leaves are membranous 10-15cm long and broad, green and scabrid above, paler and smooth or nearly so beneath. Deeply cordate at base. Five lobed and the lobes are oblong, lanceolate, midrib sometimes subserrate. Petioles are 2.5-7.5cm long striate and slender. Calyx is glabrous, 205 long and teeth subulate. Corolla is 3-4 mm long, segment ovate, oblong, acute, pubescent and Female-solitary are few, or many peduncles and shorter than males.

Flowers monoeicious, often male and female clustered together. Male flowers are with small fascicles of 3-6, penduncle 5-20 mm long filiform glabrous, margin denticulate, undulate or subcrenulate. Fruits berries, spherical yellowish-green red or green-white (Kirtikar and Basu, 1987). Fruits are subsessile 13-205cm in diameter, globose, smooth, streaked with broad vertical lines and having seeds with 5-6 mm long, yellowish brown. Seeds ovoid, with thickened, corrugated, margins. It is bitter and aperients, and is considered to have tonic properties (Yadavalli et al., 2012). Plant flowers during the period from August to December.
1.16.4 Therapeutic Uses of *Bryonopsis laciniosa*

*Bryonopsis laciniosa* is one of the most versatile plant having wide spectrum of biological activity (Bhatia *et al.*, 2012). Plant leaves and fruits are cooked as vegetables so they are edible in nature. (Bandyopadhyay, 2009). The juices of leaves are used in treatment of jaundice (Mosaddik and Haque, 2003; Vhotracharcho, 1987). Leaves and seeds are anti inflammatory (Gupta *et al.*, 2003), antimicrobial, (Singh *et al.*, 2009) febrifuge and release the stress (Gabrielian and Gevorgovich, 1997). They are used to treat flatulence, fever and reduce inflammation (Sivakumar, *et al.*, 2005; Moghe *et al.*, 2011). The seeds are used in Homeopathy and Ayurveda as a tonic for females and they rejuvenate female reproductive system and promotes conception of child. In males the seeds promote spermatogenesis and increase sperm count (Chauhan and Dixit 2010). The seeds are also used as antidote for snake bite (Bonyadi *et al.*, 2009). Seeds are antibacterial and anti-fungal (Saxena *et al.*, 2004).

In Homeopathy, a tincture made from the roots is the lollipop plant is prescribed for the treatment of inflammation of uterus, vaginal disorders and other urinary genital problems (Mosaddik *et al.*, 2003). A juice made from the leaves can be applied for pains and joints. Whole plant is used to treat ailments such as diabetes (Singh and Malviya, 2006), asthma, cough and bronchitis. Fruits are used as aphrodisiac tonic, Sharp cutting, lancinating or tearing pain, serous
inflammation; pain in serous cavities, with muscular tension (Wallis, 2006). Bryonopsis laciniosa is widely employed as a herbal drug for the treatment of gastrointestinal, analgesic, (Sivakumar et al., 2004; Reddy, 2010), respiratory, rheumatic and metabolic disorders, as well as for liver and infectious diseases (Acharya, 2007).
1.19. Objectives of the present investigation

The following are the main objectives of the present study

- To select the effective dose of plant extract
- To analyse Antioxidants in tissue and serum
- To determine Lipid peroxides in tissue and serum
- To examine Diagnostic markers in serum
- To study Tumor marker enzymes in serum
- To Monitor the glycoproteins and membrane ATPases
- To Analyse the glucose metabolizing enzymes
- To examine the Morphometric and Histopathological status.