Introduction & Review of literature
INTRODUCTION

Cancer as a disease in the human population is becoming a larger health problem, and the medicines used as treatments have clear limitations. In some areas of the world, cancer has become or shortly will become the leading disease, released cause of death of human population. For example, in United States cancer has become the second leading cause of death within few years. According to a recent report cancer causes death of six million peoples within in one year, globally. In India, the cancer registry data estimated that half a million new cancer cases are reported per year in the country (Sanghvi, 1994). So over the years a number of cancer patients are increasing significantly. As the plateu of cancer death continues the need for new approaches to prevent this hazardous diseases become imperative.

The main curative therapies for cancer – surgery and radiation – are generally only successful if the cancer is found at an early-localized stage. Once the disease has progressed to locally advanced cancer or metastatic cancer, these therapies are less successful. Chemotherapy is another mode emerged in the 1940s from toxicological studies of nitrogen mustard – based war gas (Chabner et al, 1996). In the development of new chemotherapeutic agents, several issued need to be addressed, including improved and durable antitumour efficiency, reduction of toxicities that can prevent effective dosing of potentially efficacious drugs, and prevention of drug resistance caused by the interest in genomic instability of tumours (Jackson, 2000). Intensive efforts to discover new anticancer drugs are continuing and laboratory and clinical studies have suggested methods for the more effective use of the available agents.

During the early development of cancer chemotherapy, the use of multiple drugs was considered confusing and inappropriate. The available anticancer drugs have selective mechanism of action, which may vary at different drug concentrations and in their effects, on different types of normal and cancer cells. While not selectively lethal to cancer cells, in many instances these drugs produce more extensive injury to certain cancer cells than to normal tissues, presumably because of quantitatively altered metabolic processes in the cancer cells or slower recovering of cancer cells than
of normal cells. In many cases, initially, responsive cancers recur in a form resistant to the previously effective problems, there is a great deal of information on how anticancer drugs act at the cellular level to inhibit the growth of, or to destroy susceptible cells. Generally the normal cells process and receive a number of different signals from the immediate environment during Go-G1 phase to determine whether nutritional or hormonal conditions are appropriate for G1 traverse to DNA synthesis. This transit is a highly ordered process in which individual replicons are synthesized at precise times and only one per cell cycle. Thus G1-S transition and the S period itself present major regulatory challenges to the cell. These transition points of the cell cycle are referred to as cell cycle check points and believed to play a major role in maintaining the integrity of the genome (Moller and Walin, 1998).

The formation of cancer is a multistage process in which multiple genetic alterations occur usually over the span of years to derail sufficiently the control of cell growth, division and differentiation. As in cancer predisposing syndromes, these genetic alterations are some time carried in the germline. Among human tumours, most of the genetic alterations are acquired in the form of chromosomal translocations, deletions, inversions, amplifications and point mutations. Certain oncogenic viruses also play important roles in a few human tumours (Devitta et al., 1993).

Cancer arises from a stepwise accumulation of genetic changes that liberates neoplastic cells from the homeostatic mechanisms that govern normal cell proliferation. Despite the apparent complexity of the cancer phenotype, early studies indicated that cancer might be the result of very few changes - perhaps as few as one - in the genome (Hahn and Weinberg, 2002).

Plants have always been a common medicament either in the form of traditional preparations or pure active principles. In a survey done by WHO it has been estimated that 30% of more than 4,000 million inhabitants of the world relay chiefly on traditional medicines for their primary healthcare needs and it can safely be presumed that a major part of traditional therapy involves the use of plant extracts or their active principles. (Fransworth et al., 1985). In the developed countries also plant-derived remedies are important. In USA, for example, 25% of the all the prescriptions
dispensed from community pharmacies, contain plant extracts or active principles prepared from higher plants (Fransworth, 1976; 1984). So plants are invaluable in the search for new drugs. There is a tremendous historical legacy in folklore use of plant preparations in medicine (Suffiness and Douros, 1982). Scientific studies on plants used in ethnomedicine led to the discovery of many valuable drugs. Some examples of plant-derived drugs are taxol, camptothecin, vincristin and vinblastin (Devitta et al., 1993). Some of the indigenous plants have cytotoxic and antitumour property in experimental animal models (Shylesh and Padikkala, 2000).

Plant produces a broad variety of chemical compounds that have large economical importance. First of all, these compounds are connected with important traits of the plant itself eg. colour or fragrance of flowers, taste and color of food, and resistant against pest and diseases (Harborne 1978; Harborne and Tomas Barberan 1991); but also for the production of fine chemicals such as drugs, antioxidants, flavors, fragrance, dyes, insecticides and pheromones. In past years there has been a rapidly increasing interest in plant secondary metabolism (Verpoorte et al., 1999). So many pharmaceuticals and other industrial products are based on plants, are now available (Tabata, 1977; Constabel et al., 1982; Berlin, 1984; Balandrin et al., 1985; Staba, 1985). It is important to note that approximately 60% of medicinal plants are used in the traditional systems of medicine (Ayurveda, Siddha, Unani). It is estimated that more than 90% of the plant species used by industry is collected from the wild and more than 70% of the plant drugs involved destructive harvesting and very few are in cultivation. Development of biotechnological methods such as micro-propagation, tissue/cell /organ culture is major solutions to circumvent these problems. On this line, development of fast growing root culture systems offers unique opportunities for providing root drugs in the laboratory, without resorting to field and cultivation (Sudha and Seeni, 2001). Secondary products are biosynthetically derived from primary metabolites, but more limited in distribution, usually being restricted to a particular taxonomic group. In terms of cellular economy they are metabolically expensive to produce and accumulate and are therefore present in plants in small quantities. Also in contrast to primary metabolites they are tend to be biosynthesized
in specialized cell types at distinct stages of development (Balandrin and Klocke, 1998).

The present thesis comprises seven chapters.

Chapter I covers introduction and review of literature. Chapter II involves the detailed materials and methods. Chapter III discuss about Ophiorrhiza rugosa var. decumbens- a novel source for camptothecin. Chapter IV focuses the attention on biotechnological production of camptothecin- a feasible approach. Chapter V deals with the bioactive secondary metabolite gossypin from Hibiscus furcatus. Chapter VI is pharmacological activity of gossypin; has four sections, Section 1 describes about the toxicity studies of gossypin, Section 2 deals with antioxidant and hepatoprotective activity. Section 3 discusses the antitumour and anticarcinogenic activity of gossypin. Section 4 pays attention on anti-inflammatory and gastric cytoprotective activity of gossypin.

Bibliography is included at the end of the thesis.
REVIEW OF LITERATURE

The cancer phenotype encompasses a broad collection of characteristics that together create the clinical entity of cancer. By comparing cells and tissues that are derived from cancer patients with those of normal individuals, it is possible to catalogue their many differences in molecular, cellular and biological properties. Indeed, the recent application of transcriptional profiling to cancer had documented changes in the expression of thousands of genes, as normal cells undergo transformation into their neoplastic derivatives. (Golub, 1999; Poron 2000). Several types of cancer cells share some of the changes in the expression, whereas others seem to be specific to one or a small subset of cancer cell types that are encountered in the oncology clinic. Observations such as these have led some to rationalize in terms of a small number of underlying principles that govern countless changes in cancer cell genotype and phenotype (Li et al, 1997).

1.1. THE HALLMARKS OF CANCER

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 tumour 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 and Weinberg, 1996).

Cancer research developing into a logical science, where the complexities of the disease, described in the laboratory and clinic, will become understandable in terms of a small number of underlying principles. Several lines of evidence indicate that tumourigenesis in humans is a multistep process and these steps reflect genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives. Many types of cancers are diagnosed in the human population with an age dependent incidence implicating four to seven rate-limiting,
stochastic events (Renan, 1993). Pathological analyses of a number of organ sites reveal lesions that appear to represent the intermediate steps in a process through which cells evolve progressively from normalcy via a series of premalignant states into invasive cancers (Foulds, 1954). Different acquired capabilities of cancer cells were shown in Fig.1.1.

1.1.1. Self Sufficiency in growth signals

Normal cells require mitogenic growth signals (GS) before they can move from a quiescent state into an active proliferative state. These signals are transmitted into the cell by transmembrane receptors that bind distinctive classes of signaling molecules: diffusible growth factors, extracellular matrix components, and cell-to-cell adhesion/interaction molecules. No type of normal cell can proliferate in the absence of such stimulator signals. Many of the oncogenes in the cancer catalogue act by mimicking normal growth signaling in one way or another (Hanahan and Weinberg, 2000).

Cancer cells can also switch the types of extracellular matrix receptors (integrins) they express, favoring ones that transmit progrowth signals (Lukashev and Werb, 1998; Giancotti and Ruoslahti, 1999). Both ligand-activated growth factor (GF) receptors and progrowth integrins engaged to extracellular matrix components can activate the SOS-Ras-Raf-MAP kinase pathway (Aplin et al., 1998; Giancotti and Ruoslahti, 1999).

1.1.2. Insensitivity to antigrowth signals

Within a normal tissue, multiple antiproliferative signals operate to maintain cellular quiescence and tissue homeostasis; these signals include both soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the surfaces of nearby cells.

Antigrowth signals can block proliferation by two distinct mechanisms. Cells may be forced out of the active proliferative cycle into the quiescent (G0) state from which they may reemerge on some future occasion when extracellular signals permit.
Fig. 1.1. Different acquired capabilities of cancer
Incipient cancer cells must evade these antiproliferative signals if they are to prosper. Much of the circuitry that enables normal cells to respond to antigrowth signals is associated with the cell cycle clock, specifically the components governing the transit of the cell through the G1 phase of its growth cycle. Cells monitor their external environment during this period and on the basis of sensed signals, decide whether to proliferate, to be quiescent, or to enter into a postmitotic state. At the molecular level, many and perhaps all antiproliferative signals are funneled through the retinoblastoma protein (pRb) and its two relatives, p107 and p130 (Weinberg, 1995).

1.1.3. Evading apoptosis

The ability of tumour cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of cell attrition. Programmed cell death – apoptosis – represents a major source of this attrition. The evidence is mounting, principally from studies in mouse models and cultured cells, as well as from descriptive analyses of biopsied stages in human carcinogenesis, that acquired resistance toward apoptosis, is a hallmark of most and perhaps all types of cancer.

The apoptotic machinery can be broadly divided into two classes of components – sensors and effectors. The sensors are responsible for monitoring the extracellular and intracellular environment for conditions of normality or abnormality that influence whether a cell should live or die. These signals regulate the second class of components, which function as effectors of apoptotic death. Intracellular sensors monitor the cell’s well-being and activate the death pathway in response to detecting abnormalities, including DNA damage, signaling imbalance provoked by oncogene action, survival factor insufficiency, or hypoxia (Evan and Littlewood, 1998). Further, the life of most cells is in part maintained by cell-matrix and cell-cell adherence based survival signals whose abrogation elicits apoptosis (Ishizaki et al., 1995; Giancotti and Ruoslahti, 1999).

Many of the signals that elicit apoptosis converge on the mitochondria, which respond to proapoptotic signals by releasing cytochrome C, a potent catalyst of apoptosis (Green and Reed, 1998). Members of the Bcl-2 family of proteins, whose
members have either proapoptotic (Bax, Bak, Bid, Bim) or antiapoptotic (Bcl-2, Bcl-XL, Bcl-W) function, act in part by governing mitochondrial death signaling through cytochrome C release.

New technologies will be able to display the apoptotic pathways still operative in specific types of cancer cells and that new drugs will enable cross talk between the still intact components of parallel apoptotic signaling pathways in tumour cells, resulting in restoration of the apoptotic defense mechanism, with substantial therapeutic benefit.

1.1.4. Limitless replicative potential

The early work of Hayflick demonstrated that cells in culture have a finite replicative potential (Hayflick, 1997). Once such cell populations have progressed through a certain number of doublings, they stop growing - a process termed senescence. The senescence of cultured human fibroblasts can be circumvented by disabling their pRb and p53 tumour suppressor proteins, enabling these cells to continue multiplying for additional generations until they enter into a second state termed crisis. The crisis state is characterized by massive cell death, karyotypic disarray associated with end-to-end fusion of chromosomes, and the occasional emergence of a variant \((1 \times 10^7)\) cell that has acquired the ability to multiply without limit, the trait termed immortalization (Wright et al., 1989).

Provocatively, most types of tumour cells that are propagated in culture appear to be immortalized, suggesting that limitless replicative potential is a phenotype that was acquired \textit{in vivo} during tumour progression and was essential for the development of their malignant growth state (Hayflick, 1997).

1.1.5. Sustained angiogenesis

The oxygen and nutrients supplied by the vasculature are crucial for cell function and survival, obligating virtually all cells in a tissue to reside within 100 mm of a capillary blood vessel. During organogenesis, this closeness is ensured by coordinated growth of vessels and parenchyma. Once a tissue is formed, the growth
of new blood vessels - the process of angiogenesis - is transitory and carefully regulated. Because of this dependence on nearby capillaries, it would seem plausible that proliferating cells within a tissue would have responding to a distinct programme of angiogenesis that has been developed by a specific class of human tumours (Folkman, 1993).

1.1.6. Tissue invasion and metastasis

Sooner or later during the development of most types of human cancer, primary tumour masses spawn pioneer cells that move out, invade adjacent tissues, and thence travel to distant sites where they may succeed in founding new colonies. These distant settlements of tumour cells – metastases – are the cause of 90% of human cancer deaths (Sporn, 1996). The capability for invasion and metastasis enables cancer cells to escape the primary tumour mass and colonize new terrain in the body where, at least initially, nutrients and space are not limiting. The newly formed metastases arise as amalgams of cancer cells and normal supporting cells conscripted from the host tissue. Like the formation of the primary tumour mass, successful invasion and metastasis depend upon all of the other five acquired hallmark capabilities.

Invasion and metastasis are exceedingly complex processes, and their genetic and biochemical determinants remain incompletely understood. At the mechanistic level, they are closely allied processes, which justifies their association with one another as one general capability of cancer cells. Both utilize similar operational strategies, involving changes in the physical coupling of cells to their microenvironment and activation of extracellular proteases.

1.2. CELL CYCLE AND GROWTH OF CANCER CELLS

In every population of cells, there are three sub-populations. The first group is the cycling cells that continuously proliferate giving from one mitosis to next one. The second is composed of terminally differentiated cells that irrevocably leave the cell growth cycle and are destined to die without dividing again. A third sub-population of non-dividing cells is not cycling and do not divide but can re-enter the cell cycle if an appropriate stimulus is applied (Baserga, 1985). Also shows that cycling cells go
through four different phases that are defined as G1, S, G2 and M phase:

Most of the bone marrow stem cells are in G0, a fortunate occurrence because these cells are often protected from chemotherapeutic agents used to treat leukemia or metastatic cancer. The bone marrow depletion caused by chemotherapeutic agents stimulated the protected stem cells to re-enter the cell cycle and eventually re-populate the bone marrow.

In any population, cells can grow by any one of the three following mechanisms: shortening the length of the cell cycle, resulting in more cells being produced per unit time, decreasing the rate of cell death and moving G0 cells into the cell cycle again resulting in more cells produced per unit time. In most of the tumours, all the three mechanisms are important in determining the aggressiveness of the tumour, which is best characterised by its doubling time (Bresciani et al., 1974). The doubling time ranges from as little as 17 days for Ewing Sarcoma to more than 600 days for certain adenocarcinomas of the colon and rectum (Steel, 1977).

1.3. METASTASIS AND CANCER

Cancer cells detach from the primary tumour, translate to distant sites, and grow as secondary colonies at the new anatomic locations. The establishment of the secondary tumours, no longer contiguous to the primary tumour is known as metastasis. (Mc Kinnell et al., 1998). It is a cascade of interrelated stepwise process and disruption of any of the events in the cascade; at least in theory restraints the establishment of disseminated cancer (Bastida 1998; Stracke and Liotta 1992). Cancer cells disseminate via capillary and lymphatic vessels. The thick wall of an artery is rarely penetrated. It is believed that cancer cells can enter in newly formed capillaries more easily than pre-existing capillaries because of defects in the new blood vessels, such as gaps between endothelial cells and a discontinuous or absent basement membrane (Ausprunk and Folkman 1977; Folkman, 1993).
1.4. MOLECULAR BASIS OF CANCER

1.4.1. Chromosomes and cancer

Boveri (1914) speculated that malignant tumours might be a result of a certain abnormal condition of the chromosomes. The role of aneuploidy in embryonic development has been amply sustained in plants (Blakeslee, 1934) and in animals (Fankhauser, 1945). More recently, the abnormal development of certain unclear transplant embryos (Di Berardino, 1979; 1987; 1997) and the progeny of animals exposed to mutagens (Mc Kinnell, 1979; 1980) have been shown to have chromosomal basis. In a remarkably short time, a consistent chromosomal aberration was found in the cells of patients with chronic myeloid leukemia (CML). A minute (tiny) chromosome fragment was found to “replace” one of the four small chromosomes (19,20,21 and 22) in some cells of seven patients with CML (Nowell and Hungerford, 1980). A cellular proto-oncogene known as Abelson (c-abl) is known to be located on the long arm of chromosome 9 in normal human cells. Chromosome changes occur in other leukemias including acute non-lymphomatic leukemia, chromic lymphocytic leukemia and acute lymphoblastic leukemia. Gains, losses and rearrangements of chromosomes in these hematologic malignancies are reviewed by Verma (1990) Mitelman (1991) and Le Beau (1997).

1.4.2. Oncogenes and cancer

The discovery of both oncogenes and oncosuppressor genes will almost certainly prove pivotal role in our understanding of the mechanisms of carcinogenesis and this finally link the details of carcinogen activation, adduct formation and repair and neoplastic conversion and metastasis with definable molecular events.

An oncogene is an altered form of a normal cellular gene called a proto-oncogene. It encodes a regulating protein with dominant transforming properties. The first oncogenes were identified in studies of cancer causing retroviruses. An important step in the retrovirus infection cycle is the stable yet random integration of the provirus into the host chromosome, which can alter expression of the region of the host chromosome into which it inserts, if the locus is a proto oncogene, the provirus
infection can contribute significantly to tumourigenesis (Weiss et al., 1982; Hayward et al., 1982; Blair et al., 1981).

Non random chromosomal abnormalities have been invaluable for identifying genes involved in organogenesis and for providing diagnostic clues for certain tumours. A curious and significant correlation exists between the type of chromosomal abnormality and the histopathologic type of tumour in which it is found, suggesting that certain lineages are susceptible to the transforming effects caused by deregulation of the particular gene at that translocation.

1.4.3. Tumour suppressor genes

Proto-oncogene and oncogenes are classified primarily according to their functional role and position in pathways of signal transduction and sub categorised as growth factors, receptors, non-receptors, tyrosine kinases, GTP-binding proteins, serine/threonine kinases or nuclear proteins and transcription factors.

It is a very diverse group of genes and the product of these genes negatively regulate the growth of cancer cells. More than a dozen suppressor genes have been cloned and characterised and several more have been localized in the genome. (Holywood et al., 1995). These genes encode proteins that negatively regulate the growth of cells and just as for protooncogenes function, at a variety of levels in signal transduction and cell cycle.

1.4.4. Tumor suppression and p53

p53 first identified as a tumour antigen in SV40 – transformed cells and later as a cellular protein involved in SV40 transformation. p53 has emerged in recent years as a central player in many human tumours. Alterations in the p53 locus on chromosome 17, p have been found in a large percentage and wide variety of human tumours and are the most common alterations in human cancers (Levine et al, 1991; Hollstein et al., 1991). Indeed 75-80% of color tumours show abnormalities at both p53 alleles. One allele is often deleted and other has point mutations. Analysis of p53 may prove as a powerful marker of prognosis and may emerge as a part of a
routine patient work up.

The length of the cell cycle, the growth fraction, and the rate of the cell death regulate growth in cell number. The wild type p53 induces apoptosis; a mutant p53 loses a property of regulating cell death (Yonish-Ronach et al, 1991). Another candidate for regulating the rate of the cell deaths Bcl-2 prevents apoptosis (Williams, 1991).

1.5. GROWTH FACTORS AND CANCER

Growth factors are polypeptide molecules that regulate cell growth and function by binding with high affinity to specific receptor molecules in the plasma membrane and stimulated the receptor-mediated activation of intracellular signal transduction pathways.

When growth factors bind to and activate their specific receptors, the event causes activation of a cascade of biochemical reactions collectively known as signal transduction pathways. (Aaronson, 1991; Ullrich and Schlessinger, 1990). Many converging lines of evidence strongly suggest that growth factors play a central role in malignancy. Many oncogenes are analogues of cellular proto-oncogenes that code for growth factors, their receptors, soluble tyrosine protein kinases, or biochemical pathways mediated by tyrosine kinase activation (Cross and Dexter, 1991; Varmus, 1989). Experiments using recombinant molecular technology have demonstrated that constitutive over expression of cellular oncogenes encoding growth factors or their receptors can cause non-malignant cells to display a malignant phenotype. Examination of primary specimens of human lung cancer tissue using immunohistochemical techniques have demonstrated that high levels of endothelial growth factor receptors (EGFR) are displayed on the most squamous cell carcinomas, most adeno carcinomas and none of the small lung cancers (Ozanne et al, 1986).

1.6. CARCINOGENESIS

A carcinogen is any substance or agent that significantly increases tumour incidence and the process is called carcinogenesis. Carcinogenesis is a multistage
process driven by genetic damage and epigenetic changes (Cohen and Ellwein, 1991; Harris et al., 1992). It is often characterized by four sequential stages: initiation, promotion, progression and malignant conversion. In this process, initiation occurs when a carcinogen interacts with DNA, producing a stand break or more often an altered nucleotide called an adduct. Then if the genome is replicated before the repair enzyme can correct the damage, a DNA polymerase may misread the damaged sequence and permanently fix a heritable error in the genome. The vast majority of such mis-incorporations are probably neutral to the cell. If the alteration occurs in a sequence that encodes growth regulatory protein, provide the cell with a selective growth advantage.

The promoters are believed to preferentially select or stimulate proliferation of initiated cells to form multiple benign tumours or hyperplastic lesions, and they represent the second stage of carcinogenesis. This event or series of events is proposed that allows some permanent selective growth advantage to initiated cells or increase the probability of a cell will become neoplastic. This stage called progression provides the impetus for conversion from benign adenomas to infiltrative and finally metastasizing neoplasms.

1.6.1. Chemical carcinogenesis

The English Surgeon Percinall Pott is generally regarded as the father of carcinogenesis studies for his astute recognition that an environmental agent was responsible for the tumour induction in chimney sweeps in London (Pott, 1775). Despite the diversity of chemistries, more than 95% of the various carcinogenic chemicals fall into one of three major categories: alkytating agents, aralkylating agents and aryl hydroxylamines. They are either intrinsically react with DNA or can be metabolically activated to a stable DNA – reactive form. These so called electrophiles bond with electron sharing atoms of the DNA nucleotides, such as ring nitrogen or exocyclic oxygen atoms to form stable altered nucleotide or adducts (Hemminki, 1994). Most frequently studies on carcinogens include N-nitrozo compounds, and aflatoxins, polycyclic aromatic hydrocarbons (aralkylating agents) and aromatic
amines and aminoazodyes (aryl-hydroxylic amines).

1.6.2. Organic compounds

1.6.2.1. Alkylating agents

Alkylating agents are the chemicals that transfer alkyl groups, often methyl, or ethyl groups to nucleotides to from DNA adducts. This group includes N-nitroso compounds, especially the nitrosamines, are the most incidious and therefore potentially most hazardous of the various carcinogenes. The potent carcinogenicity of several of these compounds was tested including non-human primates (Magee and Barnes, 1956; Kelly et al., 1966). Activation of these compounds often requires biotransformation either enzymatically by oxidation or directly by alkali-mediated hydrolysis. In either case a methyl group (CH₃) or an ethyl group (CH₃ CH₂) depending on the chemical, is available for the modification of a DNA base.

Aflatoxin is a serious problem in the areas of the world where methods of food preservation are deficient. Its carcinogenicity for humans is suggested in epideomeologic evaluations of African populations with a high incidence of liver tumours (Alpert et al., 1968) and in the mutational spectrum of genetic lesions found in the p53 suppressor gene from hepato cellular cancers (Hsu et al, 1991).

1.6.2.2. Aralkylating agents

These are the chemicals that transfer aromatic or multiringed compounds to a nucleotide to form an adduct. Polycyclic aromatic hydrocarbons (PAH), the principle group of aralkylating agents, remain an occupational problem in several industries. The exposure to hydrocarbons in soot increased the incidence scrotal cancer in British Chimney sweeps (Heller, 1930). Benzo(a)pyrene and the potent 7,12-Dimethyl benz(a)anthracene (DMBA), which are generated in the cigarette smoke and on the charcoal grilled meats (Lijinsky and Shubik, 1964). These compounds readily induce tumours in laboratory animals, causing rapid tumourigenesis in rat mammary tissue following ingestion (Huggins et al., 1961).
1.6.2.3. Aryl hyroxyl amines

These chemicals transfer aromatic amines to nucleotides to form adducts. Occupational exposure to aniline dyes in dyestuff industry caused high incidence of bladder cancer (Rehn, 1985). In the study of workers involved in the distillation of 2-naphthyl amine, nearly all heavily exposed 2-naphthylamine was implicated in the high incidence of bladder tumours in the manufactures of rubber (Case and Hosker, 1954).

1.6.3. Inorganic compounds and asbestos

Certain inorganic metals and minerals exhibit carcinogenic activities or are associated with elevated risk for cancer in humans. These include arsenic, nickel, chromium and asbestos. The various mineral forms of asbestos generally reflect differences in fiber structure, differences that affect the ability of the fiber to be retained in the lungs upon inhalation (McKinnell et al., 1998).

1.6.4. Physical agents

Chronic exposure to UV light as a consequence of increased leisure time or to radon because of improved standards of house hold insulation have refocused attention on the various forms of radiation. UV-radiation includes wavelengths 200-400nm and is often subdivided into three regions UV-A, 320-400nm; UV-B-280-320nm, UV-C, 200 – 280nm. The biologic effects are elicited primarily with UV-B radiation, which induces the acute symptoms of sunburn and the adaptive responses to exposure of hyper pigmentation and skin thickening. There are however, indications that, UV-A radiation effect tissue injury in conjugation with UV-B radiation or some photo sensitizing chemicals (Urbach, 1993).

UV radiation catalyses the formation of pyrimidine cyclobutane dimers (Beukers and Berends 1960) and 6-4' photoproducts, both of which formed between adjacent thymine bases and can cause GC to AT transition mutations in DNA if not repaired. The involvement of dimer formation in carcinogenesis is strongly supported
by studies of the genetic defect Xeroderma pigmentosum, a complex of disorders characterized by deficient excision repair of UV induced pyrimidine dimers and a high skin cancer incidence (Kraemer et al., 1987).

1.6.5. Radiation

Ionising radiation is a well-established human carcinogen and has been clearly linked to the excess cancer cases in populations exposed to nuclear detonation (Shimizu et al., 1989). The mechanism of carcinogenesis from radiation is believed to involve indirect formation of mutagenic oxygen free radicals. Due to the tissue penetrance of certain types of ionizing radiation, oxygen free radicals can be generated at the DNA and by ionizing the shell of hydration surrounding the DNA, thus making it a readily available target for these highly reactive and extremely short linked molecules. Once formed the reactive oxygen species (OH [hydroxyl radicals]) H2O2 [Hydrogen Peroxide], O2 [singlet oxygen], or O2− [superoxide radicals] can induce more than 30 different DNA adducts as well as DNA protein cross links (Feig et al., 1994).

1.6.6. Viral carcinogenesis

Each of the viruses associated with human cancers is thought to be involved at an early stage in carcinogenesis. Subsequent cellular genetic events such as somatic mutations are thought to be important at each step in the multistep process of malignant progression. Among the tumour viruses, the retroviruses have been a primary subject of research by virologists, oncologists and molecular biologists. Considerable evidences indicate an etiologic involvement of Hepatitis B Virus (HBV) with human hepatocellular carcinoma (HCC). This evidence stems primarily from epidemiologic studies. There is a striking correlation between the worldwide geographic incidence of HCC and prevalence of HBsAg chromic carriers (Sgmuness, 1978).
1.7. REACTIVE OXYGEN SPECIES AND CANCER

During the last decade, considerable attention has been focused on the involvement of oxygen free radicals (OFR) in various diseases (Halliwell et al., 1992; Aemes et al., 1993; Cerutti and Trump, 1991; Guyton and Kensler, 1993). OFR are continuously generated in cells exposed to an aerobic environment during the course of normal metabolism. Despite the presence of strong antioxidant defence mechanism to counteract the OFR and minimize the plausible oxidative damage (Janssen et al., 1993; Yu, 1994; Davies, 1993); OFR dependent damage of proteins (Hussain et al., 1994), DNA another biomolecules accumulate during the lifetime of organisms. It has been postulated that age-dependent diseases as atherosclerosis, arthritis, neuro-degenerative disorders and cancer involve OFR at least at some stage of their development.

Cancer development is now commonly recognized as a micro-evolutionary process that requires the cumulative action of multiple events. These events may occur in a single cell clone and can be explained by a simplified three-stage model. These stages include (a) the induction of DNA mutation in a somatic cell known as initiation, (b) stimulation of the initiated cell and its clonal expansion referred as promotion, and (c) malignant conversion of the benign tumour into cancer termed as progression. OFR have been shown to stimulate cancer development by playing a role at all the three stages namely, initiation, promotion and progression (Cerutti, 1994; Pryor, 1987).

1.7.1. Etiology of oxygen-free radicals

The term OFR refers to the forms of oxygen exhibiting high reactivity and having at least one unpaired electron. However, other reactive forms of oxygen are non-free radicals. Both of these forms are collectively referred as reactive oxygen species (ROS) and include singlet oxygen, superoxide anion, hydrogen peroxide, hydroxyl radical, etc. Singlet oxygen is formed by the transfer of radiant energy to the oxygen molecule which is triplet and paramagnetic at ambient temperatures. The stepwise univalent reduction of oxygen leads to the formation of superoxide anion, hydrogen peroxide and hydroxyl radical (Fig. 1.2). The oxidation potential and
Fig. 1.2. Different ways in which singlet oxygen can be generated.
reactivity of various ROS may be given in the following order (Fridovich, 1978).

\[ \cdot \text{O}_2 < \text{H}_2\text{O}_2 < ^1\text{O}_2 < \cdot \text{OH} \]

OFR are short-lived species that are generated \textit{in situ} in normal cells under pathological conditions. In addition, the metabolism of xenobiotics on exposure to ionizing radiation also generates these species. An important feature of free radical reactions with non-radical species is the formation of new radical species. The free radical driven reactions are usually chain reactions (Halliwell and Gutteridge, 1984).

1.7.2. Oxidative stress in carcinogenesis

In aerobic biota, the OFR are formed in normal cell metabolism from molecular oxygen. Despite tight antioxidant defences, these OFR cause constant damage to oxidizable molecules, which are repaired or replaced in a dynamic equilibrium. The condition of cellular oxidative stress arises either from the overproduction of OFR or from the deficiency of antioxidant defences or repair mechanism(s) and results in reversible tissue injury. Examples of short-term oxidative stress are the ischemia reperfusion injury syndrome, acute inflammation and hyperoxia (due to exposure to hyperbaric oxygen). In addition, important endogenous manifestation of chronic oxidative stress is inflammatory disorders (Cerutti and Trump, 1991).

The role of OFR in different stages of carcinogenesis is described as follows.

1.7.3. Role of OFR in initiation

Evidences have accumulated to suggest that ROS play an important role in tumour initiation by enhancing or facilitating the metabolic activation and/or initiating effects of carcinogens. Out of many mechanisms described for the chemical initiation of tumourigenesis, a number of them may involve free radicals in the cascade of reactions (Pryor, 1987). Initiators are in general metabolized to an ultimate carcinogen, which usually forms adduct(s) with DNA. A number of initiators have been shown to produce free radicals by themselves (Floyd et al., 1978; Aemes, 1983; Nakayama et al., 1983). DNA is the potential target for initiators of carcinogenesis.
Free radicals cause DNA base damage, DNA single strand breaks, cross-linking between DNA and proteins or DNA and chromosomal aberration. One of the major products of base damage in DNA is thymine glycol (Kaneko and Leadson, 1987). For example, N-hydroxy-2-naphthyl amine and benzo(a)pyrene have been shown to produce thymine glycol in cultured fibroblasts or DNA in solution (Leadson, 1987). Superoxide anion generated from hypoxanthine-xanthine oxidase system induces chromosomal aberration in cultured Chinese hamster cells (Sofuni and Ishidate, 1984) and V79 cells (Iwata et al., 1984). In addition, it has also been shown to act as a weak complete carcinogen in other model systems (Zimmerman and Cerutti, 1984). Hydrazine and its derivatives dimethyl hydrazine and isoniazid, which produce ROS have been shown to induce sister chromatid exchange in Chinese Hamster ovary (CHO) cells (Macrae and Stich, 1979). Chromosomal breakage, rearrangement and sister chromatid exchange are also formed as a result of photochemical or enzymatic generation of superoxide radicals. It has been implicated that H2O2 and hydroxy1 radical, which are formed within the cells of cultured human (IMR-90) fibroblasts by the exposure to fluorescent light were responsible for the induction of DNA damage (Parshad et al., 1980). H2O2 has also been implicated as a causative agent in the induction of chromosomal damage (Bradely and Erickson, 1981) and found associated with the induction of cancer in animal model system (Shamberger, 1972; Ito et al., 1981). H2O2 induces molecular damage leading to induction of transformation in the normal cells in vitro (Kennedy et al., 1984).

Strong evidence for the involvement of free radicals in free radicals-induced DNA damage and consequent carcinogenesis comes from the fact that these processes can be abolished by free radical scavengers/antioxidants (Yi sun, 1990; Machlin and Bendich, 1987). Potent inhibitors of skin tumour initiation in mice include antioxidants butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and selenium, flavones (7, 8-benzoflavone and quercetin) and vitamins (A, C and E) etc. Some of the flavones and antioxidants appear to inhibit skin carcinogenesis by inhibiting the metabolism of a procarcinogen to its ultimate carcinogenic from (Hocman, 1988). The antioxidant, BHA is widely used as food preservative and has been shown to inhibit
chemically induced lung, liver, mammary, fore stomach, colon and liver cancer in experimental animals. Similarly, inhibitory effects of selenium and vitamins E and C have been demonstrated (Ito and Hirose, 1987; Katiyar et al., 1993) have shown that the possible inhibition of N-nitrosodiethylamine and benzo(a)pyrene induced forestomach and lung tumourigenesis by some polyphenolic fraction isolated from green tea, is due to the induction of antioxidant enzymes by these fractions.

1.7.4. Free radical – generating compounds as tumour promoters

There is a long list of oxidants with tumour promoting properties. H₂O₂, peroxypivalic acid, chlorobenzoic acid, benzoyl peroxide, decanoyl peroxide, cumene hydroperoxide, lauryl peroxide, dicumyl peroxide, p-nitroperbenzoic acid and periodic acid are demonstrated to be tumour promoters in cultured mouse epidermal cell and in mouse skin models (Gimenez-Conti et al., 1991). H₂O₂ has also been reported to be a complete promoter in rat duodenum (Hirota and Yokayama, 1981). Promoting potentials of these peroxides is attributed to their ability to generate ROS within the biological system. Keratinocytes and epidermal cells exposed to hydroperoxide tumour promoters have been shown to generate free radicals (Taffe and Kensler, 1986; Taffe et al., 1987; Timmins and Davies, 1993).

The generation of free radicals from a variety of oxidant tumour promoters has also been studied in other biological systems such as liver microsomes and cytosolic fraction (Davies, 1989; Davies, 1993; Greenley and Davies, 1993). Antipsoriatic agent, anthrone derivatives such as anthralin and chrysarobin that can generate directly free radical by the simple oxidation in presence of air and light, have been shown to be mouse skin tumour promoters. The ultimate promotional ability of these compounds has been suggested to be due to the production of superoxide radical (Block and Burns, 1963; Ashton et al., 1983; Kruszewski et al., 1985). The metabolism of benzo(a)pyrene has been shown to be accompanied initially by the generation O₂ and subsequently by H₂O₂ and OH, and the involvement of these radicals in tumour promotion has also been suggested (Lesko et al., 1975).
1.7.5. Activation of reactive oxygen by tumour promoters

It is now known that tumour promoters act at least in part by inducing cellular prooxidant state. Many tumour promoters accentuate the elaboration of ROS by the endogenous sources to create a cellular prooxidant state. 12-tetradecanoyl phorbol 13-acetate (TPA) stimulate leukocytes and macrophages for the increased consumption of oxygen and in turn generate ROS. It has been suggested that ROS derived from these pro-inflammatory cells are critical component of the tumour promotion processes (Keisari et al., 1984). Another potential source ROS in some target tissues is xanthine oxidase. It has observed that a 2-3 fold increase in xanthine oxidase activity in keratinocytes following TPA treatment (Reiners et al., 1987). Keratinocytes exposed to hydroperoxide tumour promoters generate free radicals (Perchellet et al., 1988; Perchellet and Perchellet, 1989; Gali et al., 1992). The levels of hydroperoxides are increased gradually by successive applications of TPA until a plateau is reached (Perchellet and Perchellet, 1989). In vivo, TPA stimulates the infiltrations of neutrophils and as a result, myeloperoxidase activity in dermis is enhanced. It is known that it also enhances the formation of O$_2$ and oxidised DNA bases in epidermis (Wei and Frenkel, 1991; Wei et al., 1993). Tumour promotion process is mediated through the interaction of TPA with the protein kinase C (PKC) receptor. The tumour promoting activity of different analogues of TPA was found to be in concordance with their ability to act as stimulators of PKC (O’ Brian et al., 1988; Gopalakrishna and Andeson, 1989; Larsson and Cerutti, 1989). The other classes of epidermal tumour promoters such as mezerein and indole alkalodis, which are chemically different from TPA, also activate PKC and stimulate O$_2$ production. The weak endogenous skin tumour promoter, diacylglycerol also stimulates superoxide generation from neutrophils (Fujita et al., 1984). Some tumour promoters such as anthralin, indolacetic acid and tween 60, which do not activate PKC, do not evoke this response. Further, other tumour promoters such as polytoxin and thapsigargin also evoke oxidative burst in neutrophils but apparently not through the PKC, other protein kinases have been shown to get activated under the predisposed conditions of oxidative stress. For example, oxidants have been found to activate the rat insulin receptor kinase (Chan et
al., 1986; Hayes and Lockwood, 1987) and stimulate phosphorylation of the ribosomal protein through Ca^{2+}-dependent event (Larson and Cerutti, 1988).

1.7.6. Modulation of cellular antioxidant defense systems by tumour promoters

In addition to the production of free radicals, many tumour promoters have been shown to modulate the cellular antioxidant mechanism. Solanki et al (1981) have shown that in mouse epidermis TPA, anthralin, non-phorbol-ester tumour promoter, etc. cause a rapid and sustained decrease in the activities of superoxide dismutase and catalase. Similarly, the TPA-mediated changes in glutathione peroxidase activity were found to be time-dependent and are transiently increased within 30 min of TPA treatment, which is followed by a depression between 1 to 12 h, glutathione reductase (GPx) activity was also depressed throughout the multiple exposure of TPA (Taffe and Kensler, 1986). Consistent with these enzymatic changes, a four-fold increase in the levels of oxidized glutathione was observed (Perchellet and Perchellet, 1989). In mouse epidermal JB6 cells also showed that TPA treatment reduces superoxide dismutase (SOD), catalase (CAT) and (GPx) activities (Kolde et al, 1976). A known liver tumour promoter, clofibrate modulates antioxidant enzyme activities. CAT activity per unit volume of peroxisome has been shown to decrease continuously during the treatment of clofibrate in the male rat liver. Ciriolo et al., (1982) have reported that clofibrate and other peroxisome proliferator tumour promoter, fenofibrate lowered the activities of GPx and SOD.

1.7.7. Antioxidant as antipromoters

Kensler et al (Nakamura et al., 1985) have postulated that if tumour promotion is related to the increase in the intracellular free radical level, the application of free radical scavenger or antioxidant could conceivably modulate the tumour growth. Indeed, exogenous addition to mouse skin of a lipophilic biomimetic SOD, Cu (II) (3, 5-dispporpil salicylic acid), Cu DIPS, inhibits TPA-induced ornithine decarboxylase (ODC) activity and tumour formation (Engler and Kensler, 1985;
Antioxidants such as GSH, cysteine, and α-tocopherol were shown to prevent the TPA-mediated decrease in the ratios of reduced to oxidized glutathione in mouse epidermal cells. In the same system, these antioxidants inhibit TPA-induced ODC activity as well as tumour growth (Perchellet and Perchellet, 1989). Oberley and Oberley (1988) have demonstrated the role of antioxidant enzymes in cell immortalization, transformation and carcinogenesis. Furthermore, the free radical scavengers such as BHA and BHT, which are known inhibitors of lipid peroxidation, were shown to inhibit TPA- and BPO-induced ODC activity and tumour promotion in mouse skin (Kozumbo et al., 1983). Additionally, these compounds and their analogues were found positive for their inhibitory effects of TPA-stimulated chemiluminescence in PMNs. Various antioxidants were found to prevent ferric nitrate triacetate (Fe-NTA)-mediated induction of ODC and [3H] thymidine incorporation both in liver and kidney cells (Athar and Iqbal, 1998; Iqbal et al., 1995). There is also evidence that contrary to their antioxidant properties, these antioxidants under certain circumstances may act tumour promoter. For example, BHA has been shown to promote forestomach and urinary bladder tumourigenesis, while it was found to inhibit liver and mammary gland carcinogenesis. BHT on the other hand, promoted carcinogenesis of urinary bladder, thyroid and esophagus, while it inhibited ear duct and mammary gland carcinogenesis. It has been reported that BHT and vitamin E inhibited the promoting action of polyunsaturated fatty acids in mammary gland carcinogenesis in rats initiated with DMBA the effect has been proposed to be caused by their ability to block prostaglandin synthesis or by inhibition generation of oxidative products of fatty acids, which may play a role in tumour promotion by blocking oxidative metabolism of polyunsaturated fatty acids. The naturally occurring plant products such as diallyl sulfide having antioxidant properties abrogate the tumour promoting effects of BPO in murine skin (Athar et al, 1990). Ascorbic acid (Smart et al., 1987) and α-tocopherol have been shown to have inhibitory effects on the mouse skin tumour promotion by TPA. Sarcophytol, a marine product has been reported to inhibit the two-stage carcinogenesis in DMBA initiated telocidile/TPA
promoted mouse skin (Fujiki et al., 1989). The anti-tumour promotional activity is proposed to be due to their ability to suppress the PMN infiltration, ROS generation and oxidative DNA damage. Several free radical scavengers have been shown to inhibit chyrosarobin-and anthralin-induced tumour promotion. The known renal tumour promoter, potassium bromate-mediated DNA damage is inhibited by glutathione, cysteine and vitamin C (Sai et al., 1992). The dietary antioxidant, tannic acid suppresses DMBA-induced skin and benzo(a)pyrene-induced lung and forestomach tumourigenesis (Athar et al., 1989).

1.7.8. Role of ROS in tumour progression

Rotestein et al (1987) have shown that low frequency of conversion of papillomas to carcinomas can be increased by generating compound, benzoyl peroxide. Theses results promoted many other researchers to further investigate the role of ROS in the progression stage of carcinogenesis. It has been shown that Benzo (a) pyrene epoxide (BPO) enhances the invasiveness of mouse epidermal carcinoma cell lines (Athar et al., 1989; Warren et al., 1993). Many initiators also have potential to enhance the conversion of papilloma to carcinoma (Hennings et al., 1983). In a two-stage carcinogenesis model, exposure to ionizing radiation as source of free radicals augmented the malignant conversion although; it did not alter the incidence of papillomas. Athar et al (1989) have demonstrated the efficacy a number of different free radical generating compounds to enhance the malignant conversion of benign papillomas into carcinoma. They have also suggested that the effectiveness of these compounds may be related to the type of radicals produced in the biological system. Further, it has been shown that organic hydroperoxides are metabolized into free radicals by normal mouse skin keratinocytes and by the human carcinoma keratinocytes. These observations suggest that pro-oxidant compounds having ability to be metabolized into free radicals may enhance the rate of progression of benign tumours to malignant neoplasms involving free radicals. These findings suggest that tumour progression rate may be sensitive to the ROS-mediated genetic alterationas. It was also postulated that progression of cancer may

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involve free radical-induced clastogenic changes leading to the activation and/inactivation of various cellular genes in the experimental carcinogenesis. Ruggeri et al (1991) showed the inactivation of p53 tumour suppressor gene during the progression stage. This inactivation may lead to onset of genes coding metalloproteases, which are involved in the invasion/metastasis (Matrisian et al., 1986). Athar et al (1991) have reported the protective effect of all trans-retinoic acid (RA) in the free radicals-mediated conversion of chemically induced and UV-B radiation-induced skin papillomas to carcinomas.

1.8. CANCER THERAPY

There are different strategies used in cancer treatment are surgery, radiation and chemotherapy. The physical removal of tumour mass is the foundation of surgery. Radiotherapy and chemotherapy are exposure to toxic ionizing radiation or cytotoxic chemicals respectively to destroy cancer cells without having to find and remove them.

1.8.1. Surgery

Surgery is the oldest treatment for cancer and until recently, was the only treatment that could cure patients with cancer. Surgical procedures are used to remove malignant tissues physically, and it remains one of the important modality of treatment for malignant tumours.

1.8.2. Radiation therapy

Ionizing radiation continues to be a curative option for many patients alone or in combination with other modalities. Radiation therapy for cancer originated in finding that X-rays sterilize germs by killing the proliferating germ cells in the tests that maintain spermatogenesis. Radiation is most toxic to proliferating cells, higher doses are required to kill cells that are capable of proliferating, but are not actively dividing (quiescent cells) at the time of exposure. Mammalian cells are most sensitive
to radiation induced damage in the late G2 and M-phase of the cell cycle, cellular
damage produced by radiation therapy is an indirect result of ionizing chemicals in
the cell to very reactive compounds. Cytotoxicity is primarily caused by Oxygen
derived free radicals such as H₂O₂, superoxide and hydroxyl radicals. Synthesis of
radio sensitizers, which augment the amount of injury and induced by radiation to
hypoxic cells that are relatively resistant, has increased the effectiveness of radiation
therapy.

1.8.2.1. Radioprotectors

Radiotherapy is the most common modality for treating human cancers. Eighty percent of cancer patients need radiotherapy at some time or other, either
creative or palliative purpose. To obtain optimum results, a judicious balance between
the total dose of radiotherapy delivered and the threshold limit of the surrounding
normal critical tissues is required. In order to obtain better tumour control with a
higher dose, the normal tissues should be protected against radiation injury. Thus the
role of radio protective compounds is very important in clinical radiotherapy (Nair
et.al, 2001).

Ionizing radiation causes damage to living tissues through a series of molecular
events, such as photoelectric, Compton and Auger effects, depending on the radiation
energy. Because human tissues containing 80% of water, the major radiation damage
is due to the aqueous free radicals generated by the action of radiation on water. The
major free radicals resulting from aqueous radiolysis are OH, H, Caq, HO₂, H₂O⁺, etc.
These free radicals react with cellular macromolecules, such as DNA, RNA, proteins,
membrane etc., and cause cell dysfunction and mortality. These reactions take place in
tumour as well as normal cells when exposed to radiation.

Radioprotecting agents can be classified into three groups, (a) radioprotectors
(b) adaptogens and (c) absorbents. These include several myelo, entero and cerebro
protectors. Adaptogens act as stimulators of radio resistance. These are natural
protectors, which offer chemical protection under low levels of ionizing radiation.
These are generally extracted from cells of plants and animals and have least toxicity.
Absorbents protect the organisms from internal radiation and chemicals. These include drugs which prevent the incorporation of radioiodine by thyroid gland and absorption of radio nucleides, \(^{137}\text{Cs}\), \(^{90}\text{Sr}\), \(^{239}\text{Pu}\) etc. Different Radioprotectors and their mechanisms of action were shown in Table 1.1.

1.8.3. Chemotherapy

Cancer chemotherapy had its roots in the work of Paul Ehrlich, who coined the word Chemotherapy. The era of modern chemotherapy may be started in 1948 with the introduction of nitrogen mustard. Most agents currently in use appear to exert their effects primarily on the cell multiplication and tumour growth. Because cell multiplication is characteristic of normal cells as well as cancer cells, most of the chemotherapeutic agents also have toxic effects on the normal cells. Inhibition of cell multiplication and tumour growth occurring at several levels within the cells are: (1) macromolecular synthesis and function, (2) cytoplasmic organization and (3) cell membrane synthesis and function. Most chemotherapeutic agents particularly those that affect macromolecular synthesis can be grouped according to whether they depend on the cell being in cycle. (Wilson, 1975; Teng, 1977).

1.8.3.1. Chemotherapeutic agents

Chemotherapeutic agents can be classified into two groups based on their source/origin:

1. Non-plant derived anticancer agents
   (a) Alkylating agents, (b) Antimetabolites, (c) Antitumour antibiotics, (d) Enzymes

2. Plant derived anticancer agents

Since the dawn of industry, plant material has been used in the treatment of illness referred today as tumours and cancers. In recent years, extensive research for antitumour agents from plants has been undertaken. National Cancer Institute (NCI) in USA carried out one of the most extensive of such projects out. From these...
Table 1. Different Radioprotectors and their mechanism of action

<table>
<thead>
<tr>
<th>Radioprotectors</th>
<th>Mechanism of action</th>
</tr>
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<tbody>
<tr>
<td><strong>A. Sulphydryl compounds</strong></td>
<td>Free-radical scavenging, donation of H atom</td>
</tr>
<tr>
<td>Cysteine, Cysteamine, Glutathione, AET etc.</td>
<td></td>
</tr>
<tr>
<td><strong>B. Antioxidants</strong></td>
<td>Free-radical scavenging</td>
</tr>
<tr>
<td>Tempase, Hoechst 33342, Vitamin A, E, &amp; C, TMG etc.</td>
<td></td>
</tr>
<tr>
<td><strong>C. ACE inhibitors</strong></td>
<td>Protease inhibition (through rennin angiotens system), anti-oxidation, Collagen synthesis inhibition.</td>
</tr>
<tr>
<td>Captopril, Elnopril, Pencillamine etc.</td>
<td></td>
</tr>
<tr>
<td><strong>D. Cytoprotective agents</strong></td>
<td>Reduced toxicity of chemotherapeutic drugs, decrease of urothelial toxicity</td>
</tr>
<tr>
<td>Mesna, Dextrazoxane</td>
<td></td>
</tr>
<tr>
<td><strong>E. Metalloelements</strong></td>
<td>Metallothionine induction</td>
</tr>
<tr>
<td>Manganese chloride, Cadmium salts, Bismuth</td>
<td></td>
</tr>
<tr>
<td><strong>F. Immunomodulators</strong></td>
<td>Immune stimulation, increased production of cytokines</td>
</tr>
<tr>
<td>Gamma-interferon, Polysaccharides AM5,</td>
<td></td>
</tr>
<tr>
<td><strong>G. Lipopolysaccharides and prostaglandins</strong></td>
<td>Prostaglandin synthesis, DNA repair &amp; elevated levels of cyclic AMP.</td>
</tr>
<tr>
<td><strong>H. Plant extracts and isolated compounds</strong></td>
<td>Free-radical scavenging, anti-oxidation</td>
</tr>
<tr>
<td>Curcmin, Orientin, Vicinin</td>
<td></td>
</tr>
<tr>
<td><strong>I. DNA binding ligands</strong></td>
<td>Electron transfer, free-radical scavenging</td>
</tr>
<tr>
<td>Hoechst 33342</td>
<td></td>
</tr>
<tr>
<td><strong>J. Other compounds</strong></td>
<td>Free-radical scavenging, Antioxidant, free-radical scavenging</td>
</tr>
<tr>
<td>Melatonin, Carnosin, Tempase, Tempol</td>
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screening projects vincristin, vinblastin, podophyllotoxins, taxol and recently camptothecins were selected, used for clinical treatment for cancer. The most important plant derived drugs are listed in table 1.2.

(a) Vinca alkaloids: Vinca alkaloids are naturally occurring or semisynthetic nitrogenous bases that are present in minute quantities in the pink periwinkle plant *Crateranthes roseus* G. Don. Until 1994, only two vinca alkaloids, vincristine and vinblastine were approved for the treatment of malignant diseases in United States. Other Vinca alkaloids with antitumour activity include vinleurosine and vinorisidine. Recently two semisynthetic derivatives of vinblastine, vinorelbine and vinzolidine, have undergone clinical evaluation (Budman, 1993). Vinca alkaloids induce cytotoxicity by interacting with tubulin (Correia, 1991). Despite the diverse biochemical and biologic properties, the cytotoxic activity of the vinca alkaloids is primarily due to their ability to disrupt microtubules, especially microtubules comprising the mitotic spindle apparatus, thereby inducing metaphase arrest in dividing cells. The vinca alkaloids bind to the sites on tubulin that is similar to the binding sites for maytansine, another complex plant alkaloid (Correia, 1991).

(b) Taxanes: Taxanes are an important class of anticancer agents that exert their cytotoxic effects on microtubules by a unique mechanism of action. Both plaxitaxel, the prototypical taxane, which has been approved world wide for the treatment of several malignancies, and docetaxel, a potent semisynthetic analogue, have significant activity in a broad range of tumour types that are generally refractory to conventional therapies including chemotherapy resistant epithelial ovarian cancer, advanced breast cancer, small and non small cell lung cancer, bladder cancer, and head and neck cancer. The binding site for plaxitaxel on microtubules is different from the binding sites for exchangeble GTP, colchicine, podophyllootoxins and vinblastine (Schiff et al., 1979). Taxanes inhibit proliferation of cells by inducing a sustained mitotic block at the metaphase-anaphase boundary at much lower concentrations, than those required to increase microtubule polymer mass and
<table>
<thead>
<tr>
<th>Compound</th>
<th>Plant</th>
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<tbody>
<tr>
<td>Baccharin</td>
<td>Babris mega</td>
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<tr>
<td>Burceatin</td>
<td>Brreca antidsentrica</td>
</tr>
<tr>
<td>Ceasline</td>
<td>Cesalpinia gilliseri</td>
</tr>
<tr>
<td>3-deoxycolchicine</td>
<td>Colchicum speciosum</td>
</tr>
<tr>
<td>Ellipticine, 9-methoxy elliptine</td>
<td>Ochorosia moorei</td>
</tr>
<tr>
<td>Fagaronine</td>
<td>Fagara zanthoxyloids</td>
</tr>
<tr>
<td>Harringtonine, Homoharringtonine</td>
<td>Cephalotaxus barringtonia</td>
</tr>
<tr>
<td>Holacanthone</td>
<td>Holacanthe emoryi</td>
</tr>
<tr>
<td>Indicine N-oxide</td>
<td>Heitropyum indicum</td>
</tr>
<tr>
<td>Maytansine</td>
<td>Maytenus bachanaii, Verrucosa &amp; puttericka</td>
</tr>
<tr>
<td>Podophyllotoxin</td>
<td>Podophyllum peltatum</td>
</tr>
<tr>
<td>Thalicarpine</td>
<td>Thalictrum dasycarpum</td>
</tr>
<tr>
<td>Tripdiolide, triptolide</td>
<td>Trypterygium wilfordi</td>
</tr>
<tr>
<td>Vinblastin, vincristin</td>
<td>Catharantbus roseus</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>Camptoheca acuminrta, Nothapodytes Foetida</td>
</tr>
</tbody>
</table>
Fig. 1.4. a) Mechanisms of DNA strand breakage and religation by topoisomerase I
b) Action of Camptothecin to stabilize the DNA-topoisomerase I cleavable complex, with result in cytotoxic DNA damage
microtubule bundle formation (Jordan et al., 1993).

Following the disruption of microtubules and other cellular processes by the taxanes, the precise means by which cell death occurs are not clear. Morphologic features and DNA fragmentation pattern that are characteristic of programmed cell death, or apoptosis, in plaxitaxel treated human myeloid leukemia cells, indicate that the taxanes may trigger apoptosis similar to many other chemotherapeutic agents (Tishler et al., 1992; Steren et al., 1993; Choy et al., 1992).

(c) Epipodophyllotoxins: Podophyllotoxins, an antimitotic agent that binds to a site on tubulin distinct from that occupied by the vinca alkaloids, was identified as the main constituent possessing cytostatic activity as early as the 1940s from extracts of the mandrake plant (Podophyllum peltatum). The two glycosidic forms of podophyllotoxins are etoposide (VP-16) and teniposide (UM 26) have demonstrated highly significant, clinical activity against a wide variety of neoplasms, including nonhodkins lymphoma, germ cell malignancies, leukemias and small cell lung carcinoma (O'Dwyer et al., 1985).

By cell cycle analysis, the epipodophyllotoxins were found to arrest in late S or early G2 phase of the cell cycle, rather than the G2/M border that would have been expected of an anti microtubule agent (Krishan et al., 1975). Epipodophyllotoxins most likely exert their cytotoxic effects by interfering with the session-reunion reaction of enzyme topoisomerase II by stabilizing the putative cleavable complex – DNA complex in a cleavable state (Yang et. al, 1985). The enzyme covalently binds to DNA, forming single stranded, protein associated breaks.

(d) Taxol: The clinical development of taxol began in 1983 and for the first few years, proceeded slowly. It was isolated for the first time from Taxus brevifolia by Wall and co-workers, identified taxol as the active constituent of the bark extract. (Arnold and White house, 1984). Taxol binds preferentially to microtubules rather than to tubulin dimers with a binding constant of approximately 1μmol (Horwitz et al., 1986). Unlike other microtubule agents such as vinca alkaloids and colchicines, that induce
microtubule disassembly, taxol shifts the equilibrium towards microtubule assembly and stabilized the microtubules at concentrations as low as 0.05 µmol/l, which can be easily achieved by the patients. Although taxol related cells shows evidence of entry into mitosis as manifested by chromosomal condensation and break down of the nuclear membrane, they lack normal mitotic spindle apparatus. Instead of forming two mitotic spindle asters enucleated by bipolar centrioles, large numbers of abnormal asters that do not require centrioles for enucleation are formed. These distinct morphologic effects suggest that taxol may adversely affect critical microtubule functions during interphase and mitosis, but the precise reasons for cell death are nuclear.

(e) Homoharringtonin: Homoharringtonin, a class of cephalotoxin esters was first isolated from the bark of the species of *Cephalotaxus*. Cephalotoxins include harringtonine, isoharringtonine and deoxyharringtonine. It acts on ribosome to inhibit the protein synthesis.

(f) Camptothecin (CPT)

\[ \text{Fig. 1.3.} \]

Camptothecin (CPT) analogues are a promising family of anticancer agents
with a unique mechanism of action, the inhibition of DNA unwinding enzyme topoisomerase I. The parent compound CPT, is a naturally occurring alkaloid formed in the bark and wood of the Chinese tree, *Camptotheca acuminate*. In 1966 Wall et al, identified CPT as the active agent. Because of the promising preclinical activity, the drug entered clinical trials in the early 1970s under National Cancer Institute sponsorship. Because of its insolubility in aqueous solutions, CPT was formulated as its sodium salt (NSC-100880). CPT are the only well characterized inhibitors of topoisomerase I. The elucidation of this novel mechanism of action led to successful attempts to develop more soluble, less toxic topoisomerase I inhibitors with even greater preclinical anticancer activity. The other CPT derivatives are irinotecan and topotecan have clinical anticancer activity (Slichenmyer et al., 1993; Potmesil, 1994; Burris and Fields, 1994). Four CPT analogues are currently undergoing clinical evaluation, including irinotecan, topotecan, 9-aminocamptothecin and GG 211.

In the presence of CPT, the topoisomerase reaction is altered, resulting in a drug induced stabilization of the cleavable complex (Hsiang et al., 1985). CPT interact non-covalently with the DNA bound topoisomerase I and inhibit the realization step of the reaction. So there is an accumulation of stabilized cleavable complexes and persistence of single stranded DNA breaks (Fig. 1.4). This DNA damage alone is not toxic to cell because the lesions are highly reversible and can be repaired rapidly once the drug is removed (Covey et al., 1989).

1.8.3.2. Toxicity of chemotherapeutic agents

Effective chemotherapeutic agents are limited in their clinical use by the effect they produce on normal cells. The ratio of the doses at which therapeutic effect and toxicity occur is referred to as the therapeutic index. Ideally one would like to have at his disposal drugs that have higher therapeutic index, a maximum therapeutic benefit with virtually no toxicity. Alkylating agents generally produce nausea and vomiting of varying degrees. Generally, less the dose administered, less severe the effect. Some combination programmes that utilise oral agents produce less nausea and vomiting in comparison with equivalent doses given by intravenous bolus

1.8.3.3. Combined modalities

As new chemotherapeutic agents with efficacy against a variety of epithelial neoplasms become available, it seems probable that combined modality therapy involving combinations of radiotherapy or of surgery, radiotherapy and chemotherapy will come to increasingly widespread uses. Such combined modality therapy has a number of potential advantages. It may permit effective eradication of primary tumour by either surgery or radiotherapy coupled with the effective treatment of micro metastases by adjunctive chemotherapy. In addition where chemotherapy is effective at least in part against the primary neoplasm it may permit substantial reductions of radiotherapeutic dose and thus sharply decrease the hazard of late tissue injury and of significant complications.

1.8.3.4. Combination of chemotherapy and radiotherapy

The combined use of chemotherapy and radiation therapy in cancer treatment would seem to be a logical and reasonable approach. Local control of the primary tumour mass has been achieved by high dose radiation therapy combined with systematic chemotherapy in place of surgery with the hope to control metastatic diseases (Steel and Peckam, 1971; Newlands, 1978; Fu and Philips, 1991). It is widely recognised that the combined effects of antitumour agents and radiation are markedly influenced by variety of factors including not only the specific tumour type and normal tissues involved, the selected drugs, their dosage, schedules and the sequence of administration of the two modalities, but also the radiation dose, dose rate and fractionation schedules utilized (Bellamy and Hill, 1984; Goffman et al., 1990). Most of the experimental studies have been to determine whether the effectiveness of radiation can be modified by the drug themselves or may potentiate the effects of radiation. It is reported that the combination of radiation and drug may be more effective in chemotherapeutic trials (Philips, 1988; Vokes and Weichselbaum 1990).
<table>
<thead>
<tr>
<th>Class</th>
<th>Basic skeleton</th>
<th>Example</th>
<th>Main source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic acids</td>
<td>C6-C1</td>
<td>Gallic acid, vanillic acid, syringic acid, tannic acid</td>
<td>Common among higher Plants and ferns</td>
</tr>
<tr>
<td>Hydroxycinnamic acid</td>
<td>C6-C3</td>
<td>Ferulic acid, p-coumaric acid, caffeic acid</td>
<td>Common in higher plants, often as components of plant cell walls</td>
</tr>
<tr>
<td>Coumarins, isocoumarins</td>
<td>C6-C3</td>
<td>Umbelliferone, aesculetin, scopoletin</td>
<td></td>
</tr>
<tr>
<td>Stilbenes</td>
<td>C6-C2-C6</td>
<td>Resveratrol</td>
<td>Grape skins an especially good source</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>C6-C2-C6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavonoids</td>
<td>C6-C3-C6</td>
<td>Apigenin, EGCG, genistein, kaempferol, Myricetin, rutin,quercetin</td>
<td>Components of certain plant cell walls (Dietary fibre)</td>
</tr>
<tr>
<td>Lignins</td>
<td>(C6-C3)&lt;sub&gt;n&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.9. PLANT POLYPHENOLS AS A CLASS OF NATURAL THERAPEUTIC DRUGS.

Polyphenols are ubiquitous in plant foods eaten within human and animal diets and, apart from known vitamins and minerals, may be one of the widest marketed groups of dietary supplement. This class of plant metabolites contains more than 8000 known compounds, ranging from simple phenols such as phenol itself, are materials of complex and variable composition such as tannins (Bravo, 1998; Harborne, 1993).

More recently interest has been rekindled with the recognition that, many polyphenols, although now nutrients, show antioxidant, anti-inflammatory, anti-oestrogenic, anti-mutagenic and anticarcinogenic effects (Table. 1.3), at least in *in vitro* or in animal systems (Harborne, 1993).

1.9.1. Flavanoids

The term 'flavanoids' covers a large group of naturally occurring phenolic compounds in which two benzene rings are linked by a propane bridge. (Geissman, 1962). Since the biologic and pharmacologic functions of flavanoids are many and varied. The biological functions of flavanoids in man and animal was first suggested by Szent Gyorgi, who reported that the flavanoids present in citrus peels are more effective in preventing capillary bleeding and fragility associated with Scurvy (Kamil, 1993). Cytotoxic flavanoids and flavones have been isolated from *Eupetorium semiserratum* and *Baccharis* sps (Kupchan et al., 1969). Structure activity relationships among the flavanoids suggested that strong bacterial mutagenicity required a double bond between positions 2 and 3 and a hydroxyl group at position 3 (Nagao et al., 1981). There are mechanisms by which polyphenols can act as oxidants. Oshima et al, 1998 reported that a number of flavanoids are considered as antioxidants. A number of flavones and flavanoids have been identified as topo I and II poisons. (Austin et al., 1992; Kashiwada et al., 1993; Finlay et al., 1994). Flavanoids are the only group of
polyphenols to have detailed structure activity relationships published for topo II inhibition, other polyphenols such as ellagic acid can also interact with topo I or topo II enzymes (Constantinou et al., 1995). Those polyphenols that affect topo II enzymes may be of particular concern. Topo II enzymes play an essential role in chromosome condensation, and disruption of their function prevents accurate chromosome segregation, as well as increasing recombination (Holm et al., 1989).

Certain polyphenols may directly influence the activity of DNA repair enzymes through modulating gene expression as in the case of the flavanoid myricetin, which appeared to enhance the removal of highly mutagenic oxidation products from DNA in hepatocytes, treated with an iron salt. Northern blot analysis of DNA polymerase betagene expression showed that this enzyme was induced by myricetin in a dose dependent manner (Abalea et al., 1999).

Several flavanoids including tangeretin, chrysin, apigenin, naringenin, genistein, and quercetin were gavaged over 2 consecutive weeks to female rats, which were then treated with 2 -amino 1-methyl 6-phenylimidazo (4,5,6) pyridine (ph1P). Ph1P - DNA adduct formation in colour was slightly, but significantly, inhibited by quercetin, genistein and tangeretin (Breinhold et al., 1999). It was reported that DNA adduction by dibenzo(a)pyrene could be strongly inhibited by ellagic acid and genistein (Smith et al., 1988).

In course of continuing search for tumour inhibitor from plant origin, two more cytotoxic flavanol were isolated from the alcoholic extracts of the leaves of Bacharis sorothroides (Kupchan et al., 1971). Antimicrobial activity of naturally occurring flavanoids was studied and quercetin was found to completely inhibit the growth of Staphylococcus aureus at a concentration of 0.1 mg/ml. Fistin completely inhibited the growth of S.albus at a concentration of 10 mg/ml and S.aureus at 40 mg/ml in liquid broth media. (Miiischer et al., 1980). Several flavanoids were found to exhibit prophylactic action against fixed rabies virus in mice. Of this quercetin showed significant activities. Recently synthesis and protein tyrosine kinase inhibitory activities of flavonoid analogues have been studied. A topical application of chalcone derivative 4,2,4’ trihydroxychalcone inhibited epidermal ODC induction and ear
oedema formation and inflammation caused by topical application of TPA in CD-1 mice and inhibited DMBA initiated TPA promoted skin papilloma formation (Yamamoto et al., 1994).

1.10. PRODUCTION OF ANTI NEOPLASTIC SECONDARY METABOLITES THROUGH PLANT BIOTECHNOLOGY

Plant secondary metabolism is very important for traits such as flower color, flavor of food and resistance against pests and diseases. Moreover, it is the source of many fine chemicals such as drugs, dyes, flavours and fragrance. It is thus interesting to engineer the secondary metabolite production of the plant cell factory (Verpoorte et al., 2000) for the production of phytochemicals, the use of large-scale plant cell cultures in bioreactors has been extensively studied (Verpoorte et al, 1991, 1998, 1999).

In order to achieve the end product we need to know the plant secondary metabolite pathways, as well as the role of the secondary metabolites for the plant. After having established the pathways at the level of intermediates, the next step is to establish the pathway at the level of enzymes. Determining the enzyme selectivity is an important aspect of pathway mapping. A simplified model for network of secondary metabolite biosynthesis was given in Fig. 1.5.

The production of plant cell cultures is economically feasible for certain compounds provided that cell cultures do produce them. In fact, this turns out to be the major bottleneck. Most economically important natural products are produced only at very low levels or not at all (eg. quinine, morphine, vinblastine, vincristine). As a result some products of plant cell biotechnological processes have found their way to the market are stikonin (Fujita and Tabata, 1987) and ginseng roots and certain polysaccharide mixtures from cell cultures and rosmarinic acid. Taxol is found a success through biotechnology.
Fig. 1.5. Simplified model for network of secondary metabolite biosynthesis; $E =$ Enzyme, $S =$ Product.
Some of the advantageous of *in vitro* production over *ex vitro* plants are:

- In cultures large-scale production of natural compounds can be carried out throughout the year.
- Unaffected by seasonal variation.
- Risk of crop failure due to natural hazards and danger of extinction of some species due to their mass extraction from natural population can be eliminated.
- Production of high value secondary metabolites is possible by bioconversion of low value compounds.
- Easy growth under controlled nutrient environmental condition could ensure sustained supply and constant quality of the drug material free of unwanted products.
- Moreover some novel compounds produced in cell culture are not produced in the intact plants.

Over the past 15 years, plant cell cultures have enabled a better understanding of the complex organization of secondary metabolism at cell and tissue levels. Since many pharmaceuticals and other industrial products are based on the plant products, much effort has been invested in the biotechnological production of secondary metabolites by plant cell cultures. Over 60 cell culture systems, which are better producers than the respective plants, are now available (Tabata, 1977; Constabel et al., 1982; Curtin, 1983; Berlin, 1984; Balandrin et al., 1985; Staba, 1985; Wink, 1988).

### 1.10.1. Sites of secondary metabolic biosynthesis

#### 1.10.1.1. Tissue and organ specificity

Secondary metabolites are usually not distributed uniformly within the whole plant. Some are restricted to specific organs eg. roots or seeds, others to specify tissues such as epidermis. The storage of a compound in a specific cell or cell layer does not necessarily imply that the compound being synthesized by these cells. For eg. lupine alkaloids are accumulated in the epidermal cells (Wink, 1985) but they are formed in
the chloroplasts of leaf cells.

It has been observed that the plant cells resumes the metabolite production, when an unorganized structure is induced to undergo organogenesis or embryogenesis. For, undifferentiated calli of Atropa balladona do not produce the tropane alkaloid hyoscyamine, however where roots form on the callus, alkaloid formation resumes. A comprehensive review of the role of morphological and cellular differentiation in the synthesis of secondary metabolites has been presented by Raj Bhandary et al (1969). A schematic representation of compartmentalization of alkaloid biosynthesis in Catharanthus roseus is given in Fig.1.6.

1.10.2. Root culture as an experimental system

Roots are considered as the metabolic factories and have been economically valuable as a source of food. Since potatoes and cassava are common staples in many parts of the world. The medicinal properties of gentian roots (Gentiana sp) were used as a stimulant in the treatment of nervous disorders since before Christian era.

Root cultures obtained from normal plants, however, have a major disadvantage that they grow rather slowly. In recent years, root cultures has been developed as an experimental system making use of the natural genetic engineering ability of the bacterial plant pathogen Agrobacterium rhizogenes (Flores et al, 1987). These gram-negative soil microbes infect plant cells by transferring a segment of DNA contained in its root inducing (Ri) plasmid into the plant cell nucleus. In its new locale, this piece of transferred DNA (T-DNA) expresses bacterial genes, which cause the infected plant cell to behave like a root cell. Fast growing so called hairy roots arise at the site of inoculation and can be excised and established in culture after antibiotic treatment to kill the remaining bacteria. A.rhizogenes is known to induce on wounded explants, the formation of the transformed roots of a clonal origin (David et al, 1984). The transformed root cultures are characterized by a high degree of genetic stability (Aird et al., 1988). There has been growing interest in genetic manipulation of plants in order to enhance the secondary metabolite production in vitro (Hamill et al, 1987; Constabel, 1990; Yamada and Hashimoto, 1990; Ronald et al, 1991; Constabel and
Fig. 1.6. Compartmentalization of alkaloid biosynthesis in *Catharanthus roseus*; all steps are shown as occurring in a single cell.
Taylor, 1994; Ian, 1994). Polysaccharides with antitumour properties were isolated from hairy root cultures of *Althea officinalis* by Ionkova et al, 1991 employing *A. rhizogene* strains.