CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE
1.1 INTRODUCTION

Cancer is the second largest single cause of death in children and adults claiming over 7 million lives each year worldwide and more than 11 million people are diagnosed with cancer every year (WHO, 2006). It is estimated that there will be 16 million new cases every year by 2020. Cancer is a class of diseases or disorders characterized by uncontrolled division of cells and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue through invasion or by implantation into distant sites by metastasis. Cancer develops when cells no longer follow the normal pattern of the controlled growth. Genetic alteration is the fundamental underlying process that allows a normal cell to evolve into a cancerous one. Critical events in the evolution of the neoplastic disease include the loss of proliferative control, the failure to undergo programmed cell death (apoptosis), the onset of neo-angiogenesis, tissue remodeling, invasion of tumour cells into the surrounding tissue and finally metastatic determination of tumour cells to distant organs (Herzig and Christofori, 2002). Cancer risk increases with age. Most cancers that occur at advanced age are derived from cancers of epithelial cell origin of highly proliferative tissues (Finger, 2003).

The causes of cancer have been determined to be the result of genetic predisposition, environmental exposure, infection by suitable agent or a combination of these (Bishop and Schiestl, 2001). Carcinogenesis in multicellular organism can result from anyone or a combination of chemical, physical, biological and genetic insults to individual cells (Pilot, 1993).

The process of carcinogenesis may be divided into at least three stages: initiation, promotion and progression. Cancer development is now commonly recognised as a micro
evolutionary process that requires the cumulative accumulation 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 initiation of DNA mutation in a somatic cell known as initiation, stimulation of initiated cell and its clonal expansion referred as promotion and malignant conversion of benign tumour into malignant termed as progression (Athar, 2002). Several methods exist for the treatment of cancer in modern medicine, which include chemotherapy, radiotherapy, and surgery. Chemoprevention is a means of cancer control in which the occurrence of the disease is prevented by administration of one or several chemical compounds (Wattenberg, 1985).

Apoptosis has become one of the hottest areas of cell biology research, probably because of the belated realization that cell death is a biochemically-regulated process that may be as complex as other fundamental biological processes. Carl Vogt recognized the various forms of cell death involving tissues and cells as early as 1842, although it has not received primary attention for a long time (Peter et al., 1997). It is now recognised as a mechanistically driven form of cell death that is regulated in response to specific stimuli or activated in response to various forms of cell injury or stress. Apoptosis plays a central role in embryogenesis, morphogenesis and regulation of normal cell turnover in multicellular organisms. In developmental biology, apoptosis is responsible for eliminating superfluous or redundant precursor of mature cells (Meyn et al., 1994).

Defective apoptosis is one of the prime factors for promoting tumorigenesis. Normally, the induction and inhibition of apoptosis are presumably controlled by an intricate network of regulatory signals. The most direct evidence linking apoptosis with carcinogenesis was reported by Schulte-Hermann et al. (1992). Effective chemotherapy
depends on knowledge of the underlying mechanisms by which anticancer agents kill tumor cells. It is thought that many chemotherapeutic agents of diverse type act on different target molecules and will induce apoptotic cell death in neoplastic cells via a convergence of intracellular signaling pathways leading to apoptosis (Wyllie, 1997).

A complete understanding of cellular drug resistance to cancer therapy may require the elucidation of mechanisms by which anticancer agents induce cell death. Evidences also suggest that owing to various thresholds, successful treatment may depend on successful induction of apoptosis. Many toxic stimuli have been shown to induce apoptosis, even at doses or concentrations insufficient to cause general metabolic dysfunction (Dive and Hickmann, 1991). Thus, enhanced apoptosis may be responsible for reduction of many of the adverse effects of chemotherapy and for tumor regression. Conversely, insensitivity to induce apoptosis may be a major mode of drug resistance. Thus, the identification of impediments to these pathways, whether genetic or acquired, will be important in identifying potential causes of drug resistance in malignant cells and thus to revolutionise the approaches to cancer therapy. Furthermore, research for the identification of molecules that specifically induce apoptosis in cancer cells, but not in normal counterparts, have offered a specialized tool for oncologists.

Antioxidants are intimately involved in the prevention of cellular damages. The pathway is common for cancer, ageing and a variety of diseases. Antioxidants are molecules, which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Thus antioxidants are involved in fundamental metabolic and homeostatic process. Oxygen free radicals (OFR) are continuously generated in cells exposed to an aerobic environment during course of normal
metabolism. A free radical or reactive oxygen species can be defined as any atom or molecule possessing one or more unpaired electrons (Cuzzocrea et al., 2001). It is known that free radicals produce single strand and double DNA breaks in biological system (Athar, 2002). Protection against this OFR is achieved through enzymatic [superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT)] and non enzymatic antioxidant systems (vitamins A, E and C, Glutathione, uric acid, flavanoids and betacarotene). The mechanisms by which these antioxidants act at the molecular and cellular level include roles in gene expression and regulation, apoptosis and signal transduction.

Natural products have long been used as foods, fragrances, pigments, insecticides, medicines etc. In terrestrial environment, plants are the richest sources of natural products. However in marine environment, this leading position is taken up by invertebrates such as sponges, mollusks, bryozoans, tunicates, etc. (Narsinh and Werner, 2004). The marine environment may contain over 80% of world’s plant and animal species (McCarthey and Pomponi, 2004). In recent years many bioactive compounds have been extracted from various marine animals like tunicates, sponges, soft corals, etc. (Haefner, 2003). The biological diversity of marine invertebrates along the Indian coasts is well known. Among these the cephalopoda coming under the phylum Mollusca is an important constituent. The class Cephalopoda includes cuttlefish and squid (Decapoda) and octopus (Octopoda). They exhibit a wide array of behavioral patterns and are believed to be the most evolved of all marine invertebrates. Cuttlefish are also quite tasty, and prepared in every way possible, from raw to deep fried snack foods. In India, particularly in Kerala, cephalopods are exploited mainly for export. Squid and cuttlefish come under class Cephalopoda (phylum- Mollusca) which form one of the major marine
resources of India. Visceral organs form the waste material in squid and cuttlefish processing industry. The visceral organs of squid and cuttlefish mainly include liver, ink sac, gonads and the nidemental gland complex. Several bioactive compounds have been isolated from the visceral organs and ink. The ink also contains a number of bioactive substances such as antitumour agents, anti-retroviral agents and also substances, which promote phagocytic activity of murine macrophages (Takaya et al., 1994a; Rajaganapathy et al., 2000; Lu et al., 1994). The fatty acid composition of the squid liver oil was very similar to that of the cod liver oil. The predominant fattyacid in Illex illecebrus is 22: 6 acids (Hayashi and Takagi, 1979). The lipids of cephalopod flesh consisted mainly of phospholipids- lecithin and cephalin (Jangaard and Ackman, 1965).

Squid and cuttlefish eject ink from the ink sac to escape from predators. The ink act as decoy and it is believed to have an anesthetic effect as well. The melanin itself shows the property of inhibiting gastric secretion (Mimura et al., 1982a).The peptidoglycan from the ink of squid, Illex argentinus is shown to have antitumour property (Takaya et al., 1994a). The melanin free ink of the cuttlefish, Sepia officinalis is shown to have toxic effect on a variety of cell lines such asPC12,Caco2 and HPB-ALL (Russo et al., 2003). The antitumour activity of delipidated squid ink has been reported by Sasaki, et al. (1997). However no significant information is available on the medicinal properties especially the antitumour or anticancer activity of Sepia species (cuttlefish) or Loligo species (squid) from India.

The objective of the present investigation is to evaluate the antiproliferative, anticancer and antitumour activities of the ink extracts of cuttlefish and squid. The following species were selected for the studies- Sepia pharaonis, Sepiella inermis, Sepia
aculeata and Loligo duvauceli (Figure 1.1). Attempts had been made to screen the
different species of cuttlefish and squid for antitumour activity. Investigations were
carried out to isolate, purify and characterize the antitumour agent from the ink of squid
and cuttlefish. Apoptosis inducing activity of the purified antitumour agent was also
investigated using cervical cancer cell lines. The investigations envisage to identify the
clinical usefulness of ink, a waste material, as an anticancer agent. The study is thus an
attempt to project the therapeutic potential of the ink of squid and cuttlefish.
LEGEND FOR FIGURE 1.1

Photographs of dissected specimens of cuttlefish and squid showing ink sac

A-Sepia aculeata
B-Sepiella inermis
C-Sepia pharaonis
D-Loligo duvauceli
1.2. REVIEW OF LITERATURE

The marine environment covers a wide thermal range (from the below freezing temperatures in Antarctic waters to about 350°C in deep hydrothermal vents), pressure range (1-1000 atm.), nutrient range (oligotrophic to eutrophic) and it has extensive photic and non-photic zones. This extensive variability facilitated extensive speciation at all phylogenetic levels, from microorganisms to mammals. The marine environment may contain over 80% of world’s plant and animal species (McCarthey and Pomponi, 2004). Historical records have shown that humans have become aware of the venomous nature of some sea creatures for at least 4000 years (Colwell, 2002). In the 19th and early 20th centuries, cod liver oil was used as food supplements. However, it was only in middle of the 20th century that scientists began to systematically probe oceans for medicines (Narsinh and Werner, 2004). In recent years many bioactive compounds have been extracted from various marine animals like tunicates, sponges, soft corals, cephalopods, sea slugs, etc. (Donia and Hamann, 2003; Haefner, 2003). The search for new metabolites from marine organisms has resulted in the isolation of more or less 10,000 metabolites, many of which are endowed with pharmacodynamic properties (Fuesetani, 2000). In terrestrial environment, plants are the richest sources of natural products. However in marine environment, this leading position is taken by invertebrates such as sponges, mollusca, bryozoans, tunicates etc. (Narsinh and Werner, 2004). The biological diversity of marine invertebrates along the Indian coasts is well known. Among these the Cephalopoda coming under the phylum Mollusca is an important constituent. The class
Cephalopoda includes cuttlefish and squid (Decapoda) and octopus (Octopoda). They exhibit a wide array of behavioral patterns and are believed to be the most evolved of all marine invertebrates. They have a large number of glands, which produce bioactive substances such as microbicidal and antitumour agents. Also natural bioactive substances have the least quantum of side effect when compared to synthetic products as reported in the case of antibiotics isolated from the eggs of the sea snail, common dog whelk.

1.2.1 CEPHALOPOD BIOACTIVES

The phylum Mollusca comprises one of the largest and most successful phyla within the animal kingdom. Over 10,000 species of living molluscs have so far been described and rich fossil record extends back to the Cambrian. The cephalopods are unquestionably at the apex of molluscan evolution, being the largest and most highly organized of all invertebrates. The elaborate neural centres and complex behavioral pattern are expressed only by the higher vertebrates. As a group they are exclusively marine and almost all are streamlined active carnivores. In the subclass Coleidea (virtually all the cephalopods belong here) the shell is either considerably external or internal or has been completely lost (Seed, 1983).

Squid and Cuttlefish come under class Cephalopoda (phylum- Mollusca) which form one of the major marine resources of India. They are exploited mainly for export. Visceral organs form the waste material in squid and cuttlefish processing industry. The visceral organs of squid and cuttlefish mainly include liver, ink sac, gonads and the nidemental gland complex. Several bioactive compounds have been isolated from the visceral organs and ink.
Among invertebrates, cephalopods possess a well defined liver, which constitutes 7-12% of the body weight. The cuttlefish liver contains 7-32% lipid (Takahashi, 1960), which is not yet exploited. The fatty acid composition of the squid liver oil was very similar to that of the cod liver oil. The predominant fatty acid in *Illex illecebrosus* is 22:6 acids (Hayashi and Takagi, 1979). The lipids of cephalopod flesh consisted mainly of phospholipids—lecithin and cephalin (Jangaard and Ackman, 1965). Joseph *et al.* (2005) have reported that feeding a low dose of cuttlefish liver oil can stimulate the immune functions, inhibit inflammatory response and platelet aggregation in rats.

The female reproductive system in cephalopods (order: Decapoda) has a pair of large flattened glands of oval outline called Nidamental Glands (NG). The spawned eggs of cephalopods are wrapped in fragile gels, which are largely derived from the mucosubstance secreted by nidamental gland. The salt soluble component of this mucosubstance is a mucin complex (Suguira and Kimura, 1995). A pair of accessory nidamental glands (ANG) also exists in front of the nidamental glands in female cephalopods. The nidamental gland along with the accessory nidemental gland forms the nidemental gland complex. The ANG harbours a dense bacterial community and it undergoes a colour change during maturation (Branden *et al*., 1979). This colour has been attributed to the pigments of symbiotic phototropic non-sulphur bacteria (Barbieri *et al*., 1996). Because of its close association to NG and oviduct, the ANG has been thought to play a role in protecting the eggs by coating them with symbiotic bacteria to ward off pathogens. Barbieri *et al*. (1997) have reported antimicrobial activity in microbial community of the ANG and the egg cases of the Squid, *Loligo pealei*. Sherief *et al.*
(2004) and Nair et al. (2005) demonstrated antibacterial activity in the ANG extracts of squid and cuttlefish from Indian waters. A halide dependent peroxidase occurs abundantly in the accessory nidemental gland of squid, *Euprymna scolopes*. This enzyme functions not only to control pathogens, but also to modulate the interactions of host animals with their beneficial partners (Small and McFall- Ngai, 1999).

The posterior salivary gland of Octopus had long been considered to be a venomous gland containing a mixture of several toxic substances such as amines, proteins and peptides (Erspamer and Anatasi, 1962). Two novel tachykinins were isolated from the posterior salivary gland of *Octopus vulgaris* and induced immediate contraction on the carp rectum and the guinea pig ileum (Kanada et al., 2003).

### 1.2.2 CEPHALOPOD INK

Inking by cephalopods has been recognised as an adaptive response to predation and physical threat. The ink cloud produced by these animals help them retreat from a threatening situation and leave behind either a diffuse ‘smoke screen’ or a compacted, long lasting ‘decoy’ that serves as an effective visual distraction for predators. The question of the toxicity of cuttlefish ink is still up in the air, although it is clear that some cephalopod ink is indeed toxic, but again, the major reason the ink is thought to be toxic is because it coats gills of the predators, causing them to suffocate (Hanlon and Messenger, 1996). Lucero et al. (1994) postulates that in the squid, *Loligo opalascens* the ejected ink function as a warning signal or alarm substance that confuses predators and alerts conspecifics to the presence of danger. They identified two metabolites L- Dopa and dopamine as effector molecules in concentration sufficient to produce physiological effects.
Cuttlefish ink consists of melanin granules in a viscous colourless medium (Russo et al., 2003). Melanins are a group of natural black pigments. The structure of melanin macromolecules is irregular network arising from phenolic precursors in consequence of enzymatic and autooxidation (Barboi, 1999). Melanin isolated from the ink sac of *Sepia officinalis* (Sepia melanin) has been proposed as a standard for natural eumelanin (Zeise and Chedekel, 1992). The melanin pigment is manufactured in the mature cells of ink gland (Ortonne et al., 1981; Palumbo et al., 1997a, b) a highly specialized organ situated at the bottom of the ink sac and produces ink continuously. At the end of the maturation process, ink gland cells degenerate and shed their contents into the ink sac, acting as a reservoir of the exhausted material (Russo et al., 2003). Melanogenesis in the ink sac of *Sepia officinalis* in a simplified view seems to follow the general scheme of melanin formation in vertebrates (Schraermeyer, 1994). The ink gland of cuttlefish, *Sepia officinalis* has traditionally been regarded as a convenient model system for investigating melanogenesis. Both the production and ejection of the ink appear to be regulated by the glutamate/nitric oxide/cGMP signalling pathway which is localized in the ink gland (Palumbo et al., 1997b; Palumbo et al., 2000) as well as in the central nervous system and in certain neural pathways controlling the ink sac sphincters and wall muscle (Palumbo et al., 1999). The ink gland has been shown to contain a variety of melanogenic enzymes including tyrosinase (Prota et al., 1981), a dopachrome-rearranging enzyme (Palumbo et al., 1994) and peroxidase. The peroxidase was located in the rough endoplasmic reticulum and in the matrix of premelanosomes and melanosomes. The tyrosinase and dopachrome–rearranging enzyme were present in the rough endoplasmic reticulum, trans-Golgi cisternae, and coated vesicles (Palumbo et al., 1997a). Northern
blot analysis of mRNA from various tissues of *Sepia officinalis* revealed that peroxidase is exclusively expressed in the ink gland (Gesualdo *et al*., 1997). The peroxidase of ink gland serves a dual function of pigment production and immune defence. In certain species of cuttlefish the eggs are also given a coating of ink as they are shed. Moreover, the peculiar and complex organization of melanin in an invertebrate such as *Sepia officinalis* is surprising and could provide the basis for understanding the process in more evolved systems such as that of mammals (Palumbo *et al*., 1997b).

Crude melanin obtained from the ink bags of squid, *Ommastrephes bartrami* was separated into a high molecular fraction and a low molecular fraction by gel filtration on Sephadex G100 column. The second fraction which inhibited gastric secretion in rats, contained a melanoprotein, composed of melanin pigment (90%), protein (5.8%) and carbohydrate (0.8%). Chemical analysis showed that amino acids such as glycine, aspartic acid and glutamic acid were present in large amounts and there was a trace amount of sulphur containing amino acids (Mimura *et al*., 1982a). The squid ink is known to synthesize melanin from tyrosine via 3, 4 dihydroxy phenyl alanine in the ink (Matsue *et al*., 1995) and its ink has been traditionally utilized as food products commonly in Japan (Nishimoto *et al*., 1980). High molecular weight sugars were reported to exist in squid ink (Matsue *et al*., 1995). Three fucose rich glycosaminoglycans (GAG’s) have been isolated from the ink of *Illex argentinus*. The unique repeating structure of the GAGs was determined to be (-6GalNAcα1-3GlcAβ1-3Fucα1-) n (Takaya *et al*., 1994b). Squid ink is found to be rich in taurine and free aminoacids like hydroxy proline, aspartic acid, glutamic acid, alanine, leucine and arginine. A small amount of trimethylamine oxide and large amounts of homarine and glycine betaine have been
detected in squid inks (Shirai et al., 1997). Squid ink was fractionated using ion exchange and gel filtration chromatography and the peptidoglycan fraction obtained was composed of 7.8% peptide, 57% polysaccharide and 30% pigment (Takaya et al., 1994a). Squid ink has been utilized as a food material and is attracting attention in the sea food market in Japan, however little is known about the food chemistry of the extractive components. Squid ink is often utilized in raw sea food products such as salted and fermented “shiokara” products. Squid ink has been traditionally and often utilized with small amount of liquors in food products and sometimes cooked (Shirai et al., 1997).

Octopus melanin obtained from the ink juice of Octopus vulgaris were studied for its physicochemical and biological activities. Fractionation of melanin pigment revealed that it was a high molecular melanoprotein, composed of melanin pigment (79%), protein (17.5%) and sugar (1.7%). The intraperitoneal administration of the melanin fraction had no effect on peritoneal capillary permeability in mice (Mimura et al., 1982).

Melanin is a stable polyradical, containing some semiquinone radicals and accumulate some exogenous radicals and other active oxygen species, heavy metals, electrophile toxic compounds. Some of these properties determine the antioxidant, antitoxic, antiradiation and antitumour activity of melanins (Barboi, 1999). The free radical scavenging property of melanin had an important role in the protection of melanotic cells against free radical damage, particularly if the reactive radicals are generated in close proximity to the pigment granules (Rozanowska et al., 1999).

1.2.3 BIOACTIVES FROM CEPHALOPOD INK

Cuttlefish ink finds wide application in homeopathic medicine (medicinal name – Sepia). The source of this medicine is Sepia officinalis. Sepia is one of the greatest
contributions made to Homoeopathy Materia Medica by founder of homeopathy, Dr. Hahnemann. So it goes without saying that this medicine is as old as homeopathy itself and is one of the favourite medicines of homeopaths world-over. Sepia positively influences female genital organs, digestive system, liver, skin, glands and nerves. It is one of the most commonly used medicines for herpes, eczema, ring worm, hair fall, asthma and urinary tract infections. It is one of the best medicines for all possible gynecological and pregnancy related complaints. Sepia is used to treat hormonal imbalances especially in women (Boericke, 1999). Cuttlefish has a rich history in ancient Greek and Roman medicine. In Rome it was used for baldness and in Greece as a cure for kidney gravel and gonorrhea.

Natural Sepia ink is a powerful dye made from the ink of cuttlefish. Aqueous extracts of ink from cephalopod were tested against molony murine leukemia virus reverse transcriptase (MMLVRT) and have showed antiretroviral activity. Ink from young cephalopods, *S. inermis* and *L. duvauceli* showed strong inhibition of MMLVRT (Rajaganapathy *et al.*, 2000). Cuttlefish ink is shown to promote phagocytic activity of murine macrophages and increase humoral immunity. It was also showed that the level of specific antibody against Meth A sarcoma cell antigen in the serum from the mice treated with cuttlefish ink was significantly higher than that from the control, which indicated that the cuttlefish ink could increase humoral immunity (Lu *et al.*, 1994). The melanin free ink of the cephalopod, *Sepia officinalis* is shown to contain a heat labile proteinaceous component toxic to a variety of cell lines, including PC12 cells. Gel filtration chromatography indicated that the toxic component was concentrated in those
fractions eluted at a molecular weight higher than 100 kDa and exhibiting the highest tyrosinase activity (Russo et al., 2003).

Squid ink is reported to have an antibacterial activity (Mochizuki, 1979). Crude melanin obtained from squid ink has been shown to inhibit gastric secretion in rats (Mimura et al., 1982a). Takaya et al. (1994a) investigated the antitumour activity of peptidoglycan from the squid ink. They extracted squid ink using Tris-HCl buffer and further fractionated the extract by DEAE Sephacel ion exchange chromatography and sephacryl S-300 gel filtration to give peptidoglycan fraction which exhibited strong antitumour activity against Meth A fibro sarcoma in mice. The fraction had no direct cytotoxic activity on Meth A cell (Takaya et al., 1994a). The acetone delipidated squid ink was shown to have antitumour activity against Meth A fibrosarcoma in BALB/c mice. The delipidated ink enhanced the phagocytic activity of macrophages but no direct cytotoxicity was observed for the Meth A tumour cells. Hence the antitumour activity of delipidated squid ink was thought to be due to augmented cellular immunity in vivo (Sasaki et al., 1997).

Biological activity of melanin from Octopus vulgaris was studied. It was found that melanin obtained from the ink bags of Octopus vulgaris inhibited gastric secretion in rats and prevents ulcer formation in pylorus ligated rats (Mimura et al., 1982b).

1.2.4 OXIDATIVE STRESS AND CARCINOGENESIS

During the last decade, considerable attention has been focused on the involvement of oxygen free radicals (OFR) in various diseases (Athar, 2002). OFR are continuously generated in cells exposed to an aerobic environment during the course of normal metabolism. A free radical or reactive oxygen species can be defined as any atom
or molecule possessing one or more unpaired electrons (Cuzzocrea et al., 2001). They are formed when oxygen interacts with certain molecules. Once formed, these highly reactive radicals can start a chain reaction and hence they have significant biological importance. The biologically relevant free radicals derived from oxygen are the superoxide anion ($O_2^-$), the per hydroxyl radical (protonated super oxide, $HO_2$), the hydroxyl radical (OH), and free radical nitric oxide (NO) (Table 1.1).

**Table 1.1 Biologically significant free radicals.**

<table>
<thead>
<tr>
<th>Radical</th>
<th>Description</th>
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<tbody>
<tr>
<td>$O_2^-$</td>
<td>Superoxide radical</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>ROO$^-$</td>
<td>Peroxyl radical</td>
</tr>
<tr>
<td>$^1O_2$</td>
<td>Singlet oxygen</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>ONOO</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>HOCl</td>
<td>Hypochlorous acid</td>
</tr>
</tbody>
</table>

The extrinsic factors such as radiation, toxic chemical ingestion, u.v light exposure and oxygen derived free radicals that are normal consequences of the cellular metabolism of $O_2$ that generate free radicals which are therefore, also the mechanism by which they inflict their damage on DNA (Goetz et al., 1994). It is known that free radicals produce single strand and double DNA breaks in biological system (Athar, 2002).

Protection against this OFR is achieved through enzymatic [superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT)] and non enzymatic
antioxidant systems (vitamin A, E and C, Glutathione, uric acid, flavonoids and beta-carotene). Antioxidants are molecules, which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. As a defensive strategy, muscle cells are capable of inducing antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), to remove harmful ROS (Jenkins, 1988; Ji, 1998). Although there are several enzyme systems in the body that scavenge free radicals, the principal micronutrient antioxidants are vitamin E, Vitamin C and beta-carotene. The first line of defense against $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ mediated injury are antioxidant enzymes: SOD, GPx and CAT. Superoxide dismutase is an enzyme that can disarm and destroy free radicals, particularly superoxides leading to $\text{H}_2\text{O}_2$ production. In mammalian tissues, SOD exists in two main forms, dependent on the metal ion bound to its active site. Cu-Zn-containing SOD is a highly stable enzyme found primarily in the cytosol (Fridovich, 1995). Mn SOD is present in the mitochondrial matrix. The two SODs have distinct characteristics in terms of protein turnover rate. Hydrogen peroxide formed by the divalent reduction of oxygen or by the disproportionation of superoxide anions are scavenged by two classes of enzymes the catalases and peroxidases. Catalase has a double function, because it catalyses the decomposition of $\text{H}_2\text{O}_2$ to give $\text{H}_2\text{O}$ and $\text{O}_2$ and also the oxidation of H donors, for example methanol, ethanol, formic acid, phenol with the consumption of one mole of peroxide. Peroxidases are more localized in peroxisomes and are widely distributed in human saliva, adrenal medulla, liver, kidney and leucocytes (Vuillaume, 1987). Glutathione peroxidase catalyses the oxidation of GSH to GSSG at the expense of hydrogen peroxides. The mechanisms by which these antioxidants act at the molecular and cellular level include roles in gene expression and
regulation, apoptosis and signal transduction. Thus antioxidants are involved in fundamental metabolic and homeostatic process.

Despite the presence of strong antioxidant defense mechanism to counteract the OFR and minimize the plausible oxidative DNA damage (Janssen, et al., 1993; Yu, 1994), OFR dependent damage of proteins (Davies, 1993), DNA (Hussain et al., 1994) and other biomolecules accumulate during the lifetime of organisms. Condition of oxidative stress arises either from the overproduction of free radicals of oxygen or from the deficiency of antioxidant defences or repair mechanisms and results in reversible or irreversible tissue injury (Athar, 2002). It was also showed that oxidative stress from chronic inflammation favours cancer development in many organs (Weitzman and Gordon, 1990; Rosin et al., 1994; Arthur et al., 1994). It has been postulated that age dependent diseases like atherosclerosis, arthritis, neurodegenerative disorders and cancer involve oxygen free radicals (OFR) at least at some stage of their development (Halliwell et al., 1992; Ames et al., 1993). Several types of damage including base lesions, protein and DNA cross links, single stranded breaks and double stranded breaks are produced by free radical induced reactions (Simic and Jovanovic, 1986; Deng and Fridovich, 1989). Among the 20 more different products known to be formed by exposure of DNA bases to the \( \cdot \text{OH} \) radical, 8-hydroxy-2-deoxyguanosine is one of the major oxidized DNA bases (Ames, 1989). Reaction of hydroxyl and hydrogen atom with DNA bases is characterized by addition to the double bonds of these molecules to give adduct radicals of bases. An abstraction of the hydrogen atom by \( \cdot \text{OH} \) from the methyl group of thymine also occurs (Von Sonntag, 1987). Reaction of free radicals with sugar lead to the release of intact bases and result in the alterations of the sugar moiety and strand breaks (Beesk et al.,
Generally DNA damage by reactive oxygen species (ROS) can cause multiple lesions including single and double strand breaks apurine, apyrimidine (AP) sites and modified purines and pyrimidines. Some lesions in DNA are subjected to cellular repair processes, however failure of repair can have serious biological consequences (Cerutti, 1985; Cerutti et al., 1994). These structural changes manifest as point mutations and chromosomal alterations in cancer-related genes (Cerutti et al., 1994). Consequently, elderly people are predisposed to the development of cancer. Reactive oxygen species also induces protein damage. Oxidative protein damage could also affect the activity of DNA repair enzymes. It has been proposed that inflammatory bowel disease (IBD) may arise from the oxidation of proteins in the intestinal mucosa cells which thereby disrupt the critical enzyme systems that are important for the maintenance of mucosal integrity or iron transport both of which are impaired in IBD (Otamiri and Sjodahl, 1991). Another possible effect of OFR, involves their attack on lipids to initiate lipid peroxidation (Hemnani and Parihar, 1998). The only mechanism, which produces malondialdehyde (MDA) in biological systems, is lipid peroxidation. MDA is not the major product of lipid peroxidation, but a typical degradation product. MDA reacts with nitrogenous base of DNA to form DNA adducts. Many observations support the notion that lipid peroxidation plays an important role in carcinogenesis (Vuillaume, 1987).

1.2.5 CANCER

Cancer is a class of diseases or disorders characterized by uncontrolled division of cells and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue through invasion or by implantation into distant sites by metastasis (Wikipedia, 2006). Occasionally, dividing and differentiating cells deviate from their
normal genetic program and give rise to tissues called tumors or neoplasms. The process by which a cell loses its ability to remain constrained in its growth properties is called transformation. If the transformed cells stay together in a single mass, the tumor is said to be benign. If the cells of a tumor can invade and disrupt surrounding tissues, the tumor is said to be malignant and is identified as a cancer. Cells from malignant tumors can break off and move through the blood and lymphatic system, forming new tumors at other locations in the body. Malignancy can result in death due to damage to critical organs, starvation, secondary infection, metabolic problems or haemorrhage (Karp, 1996). Metastasis is defined as the stage in which cancer cells are transported through the blood stream or lymphatic system. It is the sum of all the processes, which transform normal healthy alive cells into abnormal damaged denatured cells. Cancer develops when cells no longer follow the normal pattern of the controlled growth. Genetic alteration is the fundamental underlying process that allows a normal cell to evolve into a cancerous one. Critical events in the evolution of the neoplastic disease include the loss of proliferative control, the failure to undergo programmed cell death (apoptosis), the onset of neo-angiogenesis, tissue remodeling, invasion of tumour cells into the surrounding tissue and finally metastatic distribution of tumour cells to distant organs (Herzig and Christofori, 2002). Cancer risk increases with age. Most cancers that occur at advanced age are derived from cancers of epithelial cell origin of highly proliferative tissues (Finger, 2003).

1.2.5.1 ETIOLOGY OF CANCER

The causes of cancer have been determined to be the result of genetic predisposition, environmental exposure, infection by suitable agent or a combination of
these (Bishop and Schiestl, 2001). Carcinogenesis in multicellular organism can result from anyone or a combination of chemical, physical, biologic and genetic insults to individual cells (Pilot, 1993).

1.2.5.2 CARCINOGENESIS

Carcinogens are agents, which cause cancer. They can be categorized according to the degree of certainty that they can cause cancer. Carcinogens can be genotoxic, nongenotoxic, or both. Sometimes, the distinction is arbitrary (Jackson et al., 1993; Ashby and Paton, 1993). A genotoxic agent is one for which a primary biological activity of the chemical (or a metabolite) is on the information encoded in the DNA. Nongenotoxic carcinogens are those that lack genotoxicity as a primary biological activity. While these agents may yield genotoxic events as a secondary result of other toxicity such as forced cellular growth, their primary action does not involve reactivity with DNA (Butterworth, 1990).

Carcinogenesis may result from the action of anyone or a combination of chemical (drugs, tobacco, alcohol etc.), physical (radiation-X rays, UV rays), biological (DNA & RNA viruses) and/or genetic (DNA damage or gene mutation) insults to cells.

A) CHEMICALS: The chemical origin of human malignancies was recognized by observations of unusual cancer incidences in persons in certain occupational groups. Changes in cancer frequency among migrating ethnic groups, high cancer rates associated with specific occupations and the high risk of smoking associated cancers confirmed that environmental and lifestyle exposures were major determinants of human cancer risk. Current data indicate that changing lifestyles and exposures can modify cancer risk (Wingo et al., 1999). Ethanol act through free radical mechanism. Free radicals generated
as a result of the metabolism of alcohol are shown to be responsible for augmentation of hepatic lipid peroxidation and ethanol mediated liver carcinogenesis (Mufti, 1992). Despite the diversity of chemical entities more than 95% of the various carcinogenic chemicals fall into one of the following three major categories are alkylating agents, aralkylating agents, arylhydroxylamines.

**B) PHYSICAL AGENTS:** Broadly, the term “physical carcinogens” includes a wide range of agents: electromagnetic radiations of different kinds, corpuscular (alpha and beta) radiations, low and high temperatures, mechanical traumas, and solid and gel materials. The first scientific demonstration of the carcinogenic capacity of physical agents was made by Turner, who found that Bakelite disks, implanted in rats provoked local fibrosarcomas (Anonymous, 1991). The category of hard and soft materials includes metals and metallic alloys, synthetic products, and other natural materials in the form of disks, squares, films, and foams (Minardi et al., 1990). The possible association between asbestos and cancer was suspected for the first time in 1935. Lynch and Smith (1935) described lung carcinoma in a patient with asbestosis (fibrosis of the lung due to the inhalation of asbestos dust).

**C) BIOLOGIC AGENTS:** Viruses may be the cause of at least 15% of all human cancers. The mechanisms by which viruses cause human cancers are unclear. However, direct stimulation of cell proliferation through expression of a viral oncogene, induction of a host cell response such as inflammation or regeneration, direct mutagenic effects on the host cell caused by viral integration into host DNA and consequent DNA transcription might be some of the several mechanisms by which viruses cause cancer.
1.2.5.3 STAGES OF CARCINOGENESIS

The process of carcinogenesis may be divided into at least three stages: initiation, promotion and progression. Cancer development is now commonly recognised as a micro evolutionary process that requires the cumulative accumulation 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 initiation of DNA mutation in a somatic cell known as initiation, stimulation of initiated cell and its clonal expansion referred to as promotion and malignant conversion of benign tumour into malignant termed as progression (Athar, 2002). The initiation phase appears to be irreversible and relatively easily induced by DNA damaging agents, mutagenesis has been the implied underlying mechanism responsible for this step. The promotion phase of carcinogenesis, operationally, is an interruptible process (and reversible up to a certain point). This implies that the initiated cell can be stimulated to proliferate but will not terminally differentiate. The promotion process can be implied to be an epigenetic process. Mitogenesis, rather than mutagenesis, best describe the promotion process. One of the first hypotheses concerning the mechanism of tumour promotion was derived from observations that the skin tumour promoters, phorbol esters, could block gap junctional intercellular communication, at non-cytotoxic levels, in a reversible fashion.

1.2.5.4 TUMOUR SUPPRESSOR GENES

More than a dozen tumour suppressor genes have been cloned and characterized and several more have been localized in the genome. These genes encode proteins that negatively regulate the growth of cells and just as for the proto-oncogenes function at every level in the signal transduction and cell cycle regulation. Tumour suppressor genes
associated with human tumours are shown in table 1.2. Mutation in one or more of these genes can lead to tumour formation. Unregulated growth due to defective tumour suppressor genes, unlike that due to oncogenes, is genetically recessive; tumours form if both chromosomes of a pair contain a defective gene.

1.2.5.4.1 CHARACTERISTICS OF TUMOUR SUPPRESSOR GENES

- One tumour suppressor locus is usually involved in controlling the development of several different kinds of tumours.
- Tumour suppressor genes tend to be evolutionarily conserved.
- Tumour suppressor genes are often associated with the loss of one chromosome, resulting in a reduction to homozygosity through elimination of the allele of a tumour suppressor gene as well as surrounding markers; the remaining tumour suppressor allele is inactivated by either an inherited or a somatic mutation.

**Table 1.2: Tumour suppressor genes associated with human tumours**

<table>
<thead>
<tr>
<th>Name</th>
<th>Tumour involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinoblastoma (Rb)</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>p53</td>
<td>Numerous cancers</td>
</tr>
<tr>
<td>Deleted in colon Carcinoma (DCC)</td>
<td>Colon</td>
</tr>
<tr>
<td>Wilms</td>
<td>Kidney</td>
</tr>
</tbody>
</table>

The two best understood tumour suppressor genes are the retinoblastoma gene and the gene known as p53.

1.2.5.4.2. **RETINOBLASTOMA GENE (Rb):** The study of retinoblastoma, a malignant tumour of the eye in young children led to the discovery of tumour suppressor genes in
1990. Retinoblastoma is a highly malignant tumour of the eye that as a genetic disorder is characterized by a loss of heterozygosity in chromosome 13q14. The gene implicated the Rb locus, is either missing or altered (often by one point mutation) in nearly every retinoblastoma analyzed, whether familial (germ line) or sporadic (somatic) (Friend et al., 1986). The Rb suppressor gene encodes a nuclear protein, p105, which serves as a negative regulatory factor in cell proliferation.

1.2.5.4.3. p53 GENE: The p53 gene, which is mutated in about half of human tumours at multiple organ sites, is thought to be the most frequently mutated gene in human cancer. It has been found in a variety of tumours, including those of the colon, brain, lung, breast, and in leukemias and osteosarcomas. p53 gene is located on chromosome 17p. Its action as a tumour suppressor gene is found only when it is found in the nucleus. Radiations and chemotherapy induce p53 gene, the accumulated wild type p53 binds to DNA, stimulates transcription of several genes that mediate the two major effects of p53, cell-cycle arrest in the late G1 phase and apoptosis.

1.2.6 CERVICAL CANCER

Cancer of the uterine cervix is the second most common cancer in women worldwide after breast cancer and in developing countries, the leading cause of death by cancer. It causes about 250,000 deaths annually worldwide, with women in developing countries accounting for 80% of these deaths (Suris and Dexeus, 1998). In India, nearly one-lakh women develop this cancer every year, constituting about 16% of the world’s annual incidence (Das et al., 2000). Epidemiological studies demonstrates the association of several risk factors, which include sexual promiscuity, exposure to sexual intercourse at an early age, number of pregnancies, cigarette smoking, use of oral
contraceptives, dietary and other factors with the development of cervical cancer (Zur Hausen, 1996). In addition to these epidemiological risk factors, the interaction between the genes encoded by the papillomaviruses and the host genes appears to play a crucial role during tumorigenesis. Even though surgery remains the primary mode of treatment for women with cervical cancer, chemotherapy is often administered subsequent to that to avoid metastasis/recurrence. It has been noted that cervical cancer survival rates could be increased up to 50%, when chemotherapy was also added to standard therapy (Morris et al., 1999). In advanced stages of the disease where surgery is not possible, pelvic radiotherapy has been the treatment of choice.

1.2.7. THE PHENOMENON OF APOPTOSIS

Apoptosis has become one of the hottest areas of cell biology research, probably because of the belated realization that cell death is a biochemically-regulated process that may be as complex as other fundamental biological processes. Carl Vogt recognized the various forms of cell death involving tissues and cells as early as 1842, although it has not received primary attention for a long time (Peter et al., 1997). In 1970, Wyllie and Kerr formalized the existence of a human form of cell death distinct from necrosis that they termed as apoptosis. They coined this word from classical Greek to mean “falling off” like leaves from a tree in autumn, where cell death occurs without the death of the organism (Kerr et al., 1972). It is now recognised as a mechanistically driven form of cell death that is regulated in response to specific stimuli or activated in response to various forms of cell injury or stress. Apoptosis plays a central role in embryogenesis, morphogenesis and regulation of normal cell turnover in multicellular organisms. In
developmental biology apoptosis is responsible for eliminating superfluous or redundant precursor of mature cells (Meyn et al., 1994).

The molecular events that trigger and accompany the process of cellular suicide has been the area of research in cell biology for the past few years. Several hypothesis have been put forward indicating the intricate and complex steps involved in apoptosis (Dive and Hickman, 1991; Steller, 1995; Evan and Littlewood, 1998; Hengartner, 2000). Apoptosis is a complex network of biochemical pathways with fine regulatory mechanisms controlling death events in the cell. Apoptosis is an essential and fundamental phenomenon occurring during various biological processes, including growth, differentiation, tissue remodeling and immunological development (Ravi et al., 2000). Apoptosis is a cell suicide mechanism, which is of critical importance for maintenances of homeostatic balance in cell populations of organisms, wherein growth and specializations are achieved through process of deliberate self–elimination. Apoptosis is utilized by the organism to maintain spatial, temporal organization and optimum function of its cell populations. Besides its important involvement in development, apoptosis play a critical role in the response of organisms to a number of external agents such as radiation, drugs, bacteria, viruses etc.

It is important to distinguish apoptosis from the other major form of cell death, necrosis. First, at tissue level, apoptosis produces little or no inflammation, since shrunken portions of the cells are engulfed by the neighbouring cells, especially macrophages, rather than being released into the extracellular fluid (Figure 1.2). In contrast, in necrosis, cellular contents are released into the extracellular fluid and thus have an irritant effect on the nearby cells causing inflammation (Table 1.3). Apoptosis
mechanism of cell death is fundamental to the normal development of tissues and organisms. In contrast, cell death by necrosis does not have such significance.

Morphological features of apoptosis include cell shrinkage, membrane blebbing, chromatin condensation, nuclear and cytoplasmic condensation and partition of cytoplasm and nucleus into membrane bound vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material (Schlte-Hermann et al., 1992). In vivo, these apoptotic bodies are rapidly recognized and phagocytosed by either macrophages or adjacent epithelial cells. Due to this efficient mechanism for the removal of apoptotic cells in vivo, no inflammatory response is elicited. In vitro, apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyse. This terminal phase of in vitro cell death has been termed ‘secondary necrosis’.

1.2.7.1 NECROSIS

It is also called accidental or pathological cell death and occurs when cells are exposed to extreme variance from physiological conditions (eg. hyperthermia and hypoxia), which may result in damage to the plasma membrane. Under physiological conditions, agents like complement and lytic viruses evoke direct damage to the plasma membrane. Necrosis apparently begins with an impairment of the cell’s ability to maintain homeostasis, leading to the influx of water and extracellular ions. Intracellular organelles, most notably the mitochondria and the entire cell swell and rupture (cell lysis) due to the ultimate break-down of plasma membrane, the cytoplasmic contents, including lysosomal enzymes are released into the extracellular fluid. Therefore, in vivo necrotic cell death is often associated with extensive tissue damage resulting in an inflammatory response.
Figure 1.2: Salient features of apoptosis and necrosis

Table 1.3: Major differences between apoptosis and necrosis

<table>
<thead>
<tr>
<th>NECROSIS</th>
<th>APOPTOSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss of membrane integrity</td>
<td>Membrane blebbing, but no loss of membrane integrity</td>
</tr>
<tr>
<td>Cellular swelling and lysis</td>
<td>Cellular condensation (cell shrinkage)</td>
</tr>
<tr>
<td>No vesicle formation</td>
<td>Formation of membrane bound vesicles</td>
</tr>
<tr>
<td></td>
<td>(Apoptotic bodies)</td>
</tr>
<tr>
<td>Mitochondrial swelling</td>
<td>Mitochondria intact</td>
</tr>
<tr>
<td>Inflammatory reaction</td>
<td>No inflammatory reaction</td>
</tr>
<tr>
<td>No energy requirement</td>
<td>Energy (ATP) dependent</td>
</tr>
<tr>
<td>Random digestion of DNA</td>
<td>Mono and oligo nucleosomal fragmentation of DNA</td>
</tr>
</tbody>
</table>
1.2.7.2. SIGNIFICANCE OF APOPTOSIS IN TUMOR DEVELOPMENT

The normal regulation of cell proliferation and cell death is critical in maintaining the total number of cells in an organism nearly constant. Earlier studies regarding cancer have shown that uncontrolled proliferation is a major contributor in the development of a malignancy. However, researchers in the last decade have shown that cellular propensity to undergo programmed cell death (PCD)/ apoptosis could be a factor as important as proliferation in the context of cancer development (Schulte-Hermann et al., 1992). Up regulation or down regulation of any of these events results in abnormalities in cellular homeostasis. The number of cells at a transitional stage of differentiation depends on the events that increase cell numbers, such as input from stem cells and proliferation or events that decrease cell numbers such as differentiation and apoptosis. The homeostatic events may be broken by alterations of genes that regulate apoptosis, proliferation and differentiation leading to neoplasia.

Defective apoptosis is one of the prime factors for promoting tumorigenesis. Normally, the induction and inhibition of apoptosis are presumably controlled by an intricate network of regulatory signals. When these signals become deregulated/blocked, they lead to illegitimate cell survival. Such events along with a gain of function of any oncogenes, can give the cell a selective advantage leading to uncontrolled expansion of cell populations. The most direct evidence linking apoptosis with carcinogenesis was reported by Schulte-Hermann et al. (1992). They were able to show that in a rat hepatic tumor induction model, preneoplastic foci, although showing high proliferation rate, did
not grow because the rate of apoptosis was also high and therefore, there was sufficient
cell death to counterbalance the increased number of cells. Normal organ growth as well
as the process of carcinogenesis are affected by both proliferative and apoptotic rates.

Therefore, defective expression of molecules that induce apoptosis at critical
stages of differentiation, may contribute to the development of neoplasia by prolonging
cell survival (Figure 1.3). Conversely, over expression of molecules that suppress
apoptosis has also been associated with the development of neoplasia.

**Figure 1.3: A model for neoplasia**

**Homeostasis**

![Homeostasis Diagram]

**Neoplasia**

![Neoplasia Diagram]
1.2.7.3 ROLE OF CASPASES IN APOPTOSIS

The caspases, first discovered almost a decade ago, are considered essential for almost all forms of metazoan programmed cell death (PCD) (Chang et al., 2000). Apoptosis is a process that is mediated by caspases; a ubiquitous family of cysteine proteases that includes both upstream (initiator) and downstream (effector) caspases (Nunez et al., 1998; Chen and Wang, 2002). Caspases derived their name from their characteristic property of being cysteine proteases with aspartic acid residue specificity. Mature caspases has a heterotetrameric structure with two active sites that may function independently. Based on their substrate specificities and DNA sequence homologies, the 14 currently identified mammalian caspases can be divided into three groups-apoptotic initiators, apoptotic executioners and inflammatory mediators (Strasser, 2000; Chang et al., 2000). They are synthesised as procaspases and are converted to active proteases during apoptosis through an intricately regulated proteolytic cascade with upstream and downstream caspases. Caspases are synthesised in an inactive proform that is activated by proteolytic cleavage at two or more sites. Cleavage at one site generates the large and small subunits of the mature, active proteases where as cleavage at a second site removes the prodomain (Nunez et al., 1998; Thornberry and Lazebnik, 1998; Green, 2000). The initiator caspases, typically caspase 8 and 9 are activated by two alternative pathways, both of which lead to apoptotic cell death. One pathway is triggered by cellular stresses that induce changes in mitochondrial function and is primarily associated with the activation of caspase 9 (“Intrinsic” apoptotic pathway) (Green, 1998; Sun et al., 1999). The second (“Extrinsic”) pathway activates caspase 8 and proceeds via the formation of a
DISC (death inducing receptor signalling complex) at the cell surface, which provides a mechanism for aggregation and autocleavage (autoactivation) of the caspase 9 (Ashkenazi and Dixit, 1998). In both pathways the initiator caspases cleaves and thereby activates downstream effector caspases, such as caspase 3, caspase 9 and others. This caspase cascade ultimately leads to proteolytic cleavage of a variety of cellular proteins and induces the broad range of morphological changes that are characteristic of cells undergoing apoptosis (David and Pamela, 2003).

1.2.7.4 MITOCHONDRIAL CELL DEATH PATHWAY

In stress induced cell death, signals received by mitochondria stimulate mitochondrial membrane permeabilisation and release several apoptotic factors into the cytosol (Green, 1998; Van Loo et al., 2002; Lemasters et al., 2002). Key mitochondrial factors released in this manner include cytochrome C (Slee et al., 1999), certain caspases (Susin et al., 1999a), AIF, which induces chromatin condensation and DNA fragmentation (Susin et al., 1999b; Joza et al., 2001) and smac/Diablo, which neutralises IAP (inhibitor of Apoptosis) proteins and allows caspase activation to proceed (Verhagen et al., 2000; Salvesen and Duckett, 2002). Mitochondrial release of cytochrome c triggers the formation of the apoptosome, an oligeric multiprotein complex comprising cytochrome C, ATP, caspase 9 and the scaffold protein Apaf-1, which stimulates the activation of caspase 9 and downstream apoptotic events (Chinnaiyan, 1999; Cain et al., 2002).

1.2.7.5 RECEPTOR MEDIATED CELL DEATH PATHWAY.

Receptor mediated cell death is initiated by the binding of a death inducing ligand to a cysteine rich repeat region in the intracellular domain of a death receptor. This in
turn leads to activation of trimerized death receptor at the cell surface and activation of caspase 8 dependent cell death. Death receptor ligands include TNF-alpha (tumour necrosis factor alpha), Fas ligand and TRAIL (TNF-related apoptosis inducing ligand), each associated with its own specific death receptor. These death receptor activating ligands are expressed in both membrane bound and soluble forms and share a homologous 150-aminoacid region that interacts with, and may serve aggregate, the death receptor (Baker and Reddy, 1998). Each death receptor contains a cytoplasmic tail “death domain” that binds the corresponding COOH- terminal death domain of an adaptor protein such as FADD (Fas associated death domain containing protein) (Micheau et al., 1999). The adaptor protein additionally contains a death effector domain (Tibbetts et al., 2003) that binds to NH₂ terminus of the caspase 8 prodomain, thus facilitating DISC formation and proteolytic autoactivation of caspase 8.

1.2.7.6 CASPASE INDEPENDENT PATHWAY

Neurons and perhaps other cells, have another way to self destruct that unlike the above two pathways does not require caspases. Apoptosis – inducing factor (AIF) is a protein that is normally located in the intermembrane space of mitochondria. When it receives signals (triggered by dangerous reactive oxygen species) it is released from mitochondria and migrate into nucleus and triggers the destruction of DNA and cell death (Susin et al., 1999b).

1.2.7.7 APOPTOSIS: RELEVANCE TO CANCER CHEMOTHERAPY

Effective chemotherapy depends on knowledge of the underlying mechanisms by which anticancer agents kill tumor cells. It is thought that many chemotherapeutic agents
of diverse type act on different target molecules and will induce apoptotic cell death in neoplastic cells via a convergence of intracellular signaling pathways (Wyllie, 1997).

A complete understanding of cellular drug resistance to cancer therapy may require the elucidation of mechanisms by which anti-cancer agents induce cell death. During the past decade, the understanding of apoptosis has unveiled the mechanisms by which tumor cells can acquire or lose sensitivity to drugs. Evidences also suggest that owing to various thresholds, successful treatment may depend on successful induction of apoptosis. Many toxic stimuli have been shown to induce apoptosis, even at doses or concentrations insufficient to cause general metabolic dysfunction (Dive and Hickman, 1991). Thus, enhanced apoptosis may be responsible for reduction of many of the adverse effects of chemotherapy and for tumor regression. Conversely, insensitivity to induce apoptosis may be a major mode of drug resistance. Thus, the identifications of impediments to these pathways, whether genetic or acquired, will be important in identifying potential causes of drug resistance in malignant cells and thus to revolutionise the approaches to cancer therapy. Furthermore, research for the identification of molecules that specifically induce apoptosis in cancer cells, but not in normal counterparts, have offered a specialized tool for oncologists. Drugs of differing structure and specificity induce the characteristic morphological changes associated with apoptosis, and it is now believed that apoptotic pathways contribute to the cytotoxic action of most chemotherapeutic drugs.

There are different classes of agents that are currently used as cancer chemotherapeutic agents. Some of them are antimetabolites, alkylating agents, topoisomerase inhibitors, antibiotics, hormone antagonists and cancer
immunotherapeutics. The other miscellaneous agents include signal transduction agents, apoptosis inducers, metalloproteinase inhibitors, angiogenesis inhibitors, microtubule antagonists and differentiation inducers.

1.2.8 TREATMENT OF CANCER

Several methods exist for treatment of cancer in modern medicine, which include chemotherapy, radiotherapy and surgery. Surgery is the oldest treatment of cancer and until recently, was the only treatment that could care patients with cancer. Radiotherapy is also used to effectively reduce the initial tumour load. It is common to combine treatment such as surgery with irradiation or chemotherapy. Successful treatment of tumours with drugs and radiation depends upon the greater sensitivity of neoplastic cells to the treatment than that of normal cells. Proliferating cells such as neoplastic cells are more sensitive to these agents than are quiescent cells (Enrique et al., 2003).

However, when cisplatin therapy was included with radiotherapy, there was a longer progression free survival (Morris et al., 1999; Rose et al., 2001). While cancers with advanced stages are rare in western countries, it is on the increase in most of the developing countries. Therefore, chemotherapeutic agents play an important role in the management of this disease. More recently, studies on some cell lines have indicated that, inhibitors of tyrosine kinase and modulators of signal transduction potentiate the effects of the common cancer chemotherapeutic agents (Nakagawa et al., 2000; Park et al., 2001).

1.2.8.1 ANTICANCER DRUGS

Classically, antitumour drugs were grouped as chemotherapy, hormonal therapy and immunotherapy. Chemotherapy included number of families defined by both their
chemical structure and mechanism of action; alkylating agents, antibiotics, antimetabolites, topoisomerase I & II inhibitors, mitosis inhibitors, platinum compounds and others (Enrique et al., 2003).

Classical classification of anticancer drugs

Chemotherapy  alkylators

Antibiotics

Antimetabolites

Topoisomerases inhibitors

Mitosis inhibitors

Others

Hormonal therapy  Steroids

Anti-estrogens

Anti-androgens

LH- RH analogues

Anti – aromatase agents

Immunotherapy  Interferon

Interleukin 2

Vaccines

I) DRUGS DIRECTED AGAINST TUMOUR DNA

The drugs may act on DNA either by breaking the helix itself, interfering with DNA related proteins or modifying the expression of specific genes. Most classical anticancer agents have one of these mechanisms of action, and new drugs are being incorporated every year.
A) DNA HELIX

Alkylating agents were the first compounds identified to be useful in cancer. Alkylators belong to one of several families; nitrogen mustards, nitrosoureas, triazenes, platinum compounds and antibiotics. They form a variety of interstrand cross links called adducts, that alter DNA structure and function. The most common site of alkylation is the N-7 position of guanine, but it varies depending on the family of drugs (Hurley, 2002). Some antibiotics also belong to the group of alkylators; bleomycin and mitomycin C.

B) DNA RELATED PROTEINS

Topoisomerase I & II inhibitors, antimetabolites and eetinascidin could be grouped together as drugs directed at protein DNA complexes, because they do not bind directly to DNA (Hurley, 2002). The antracyclins (doxorubicin and their analogs epirubicin and idarubicin) inhibit topoisomerase I and form free radicals. On the other hand antimetabolites interfere with enzymes that contribute to DNA synthesis. A marine derivative, eetinascidin or ET-743, has a unique mechanism of action. Formerly thought to be an alkylator, recent investigations have shown that it blocks transcriptional factors (Scotto, 2002). Ecteinascidin has been used in patients with refractory sarcomas (Delaloge et al., 2001).

C) SPECIFIC GENES

The classical representatives in this group are hormonal agents. Steroids, anti-hormones and retinoids share a common mechanism of action because they modify the expression of specific genes. Steroid hormones, such as glucocorticoids, bind to receptor proteins in the cytoplasm or nucleus to form a hormone receptor complex. This complex has the capacity to activate regulatory sequences in DNA (Enrique et al., 2003). The
antitumour activity of interferon alpha appears to be due to a combination of direct antiproliferative as well as indirect immune mediated effects. It has also antiangiogenic effects mediated through interferon gamma (Jonasch and Haluska, 2001).

II) DRUGS DIRECTED AGAINST TUMOUR RNA

A number of anticancer drugs such as the fluoro-pyrimidines and platinum compounds interfere with RNA synthesis. However, they mainly act by binding to DNA. The major representatives in this group are antisense oligonucleotides. These molecules are directed against specific mRNAs. The mRNAs of bcl-2, myb, p53, mdm2, Her-2 and methyl transferase-I have been targeted with these oligonucleotides (Tolcher, 2001; De Bufalo et al., 1996; Strasberg et al., 2001; Wang et al., 2002). The synthesis of antisense oligonucleotide is complex and improved methods to deliver the compound in the target are needed (Goffin and Eisenhauer, 2002; Henry et al., 2001; Jansen and Zangemeister, 2002).

III) DRUGS DIRECTED AGAINST PROTEINS IN THE TUMOUR CELL

In the last decade, a great number of compounds have joined this group, mainly monoclonal antibodies and small molecules. They are all very specific and their effect is cytostatic rather than cytotoxic. They can bind to membrane receptors or cytoplasmic proteins.

A) RECEPTORS IN THE TUMOUR MEMBRANE

Two groups may be distinguished; monoclonal antibodies and small molecules. The former block the extracellular domain of the receptor, where as the latter cross the membrane and inhibit the intracellular domain, usually a tyrosine-kinase. The first antitumour antibodies were directed against lymphoid antigens, such as CD20 and CD52.
Some of them combine the antibody with an isotope to increase efficacy (Davis et al., 2000; Vose et al., 2000; Rai et al., 2002; Lundin et al., 2002). Small molecules bind to receptors of the epidermal growth factor family. Some of them are specific for EGFR (Her-1), such as genfitinib (ZD-1839) (Herbst and Kies, 2002; Ranson, 2002).

**B) INTRACELLULAR PATHWAYS IN TUMOUR CELLS**

A number of metabolic pathways carry proliferation signals to the nucleus. These pathways are activated by growth factors and a few of them have been targeted with specific drugs. The better known drug in this group is imatinib, which inhibits the tyrosine kinase of bcr/abl and c-kit (Cohen et al., 2002; Joensuu et al., 2002).

**C) TUBULIN**

Tubulin contributes to the maintenance of cell shape, intracellular transport and mitosis, so drugs interfering with tubulin are grouped here in the present classification. The vinca alkaloids bind to specific sites on tubulin and prevent polymerization of tubulin dimmers, thereby disrupting the formation of microtubules. The taxanes have a different binding site and stabilize microtubules; this unusual stability inhibits the normal organization of the microtubule network. Oral formulations of taxanes will improve convenience if they prove to be active as the parent drugs (Rose et al., 2001).

**IV) DRUGS ACTING ON THE ENDOTHELIUM AND EXTRACELLULAR MATRIX**

Compounds directed against the endothelium inhibit either endothelial growth factors or the receptors of such factors. On the other hand, most drugs acting in the extracellular matrix inhibit metalloproteinases (MMPs). They all have antiangiogenic effects.
A) ENDOTHELIUM

The main endothelial growth factors- vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)-are inhibited by thalidomide (Neben et al., 2001; Raje and Anderson, 2001). Cyclooxygenase 2 may stimulate endothelial growth, hence one of the possible mechanisms of action of COX-2 inhibitors (Wang et al., 2002).

B) EXTRACELLULAR MATRIX

Activation of matrix metallo proteinases (MMPs) in tumours facilitates invasion and is an essential step in angiogenesis. MMPs may stimulate the release of VEGF, bFGF and insulin growth factor. A number of MMP inhibitors are currently under clinical investigation (Hoekstra et al., 2001). Tetracycline derivatives such as neovastat also down regulate the production, inhibit the activation and increase the degradation of MMPs (Falardeau et al., 2001; Batist et al., 2002).

A successful anticancer agent should kill cancer cells without causing excessive damage to normal cells. Most of the anticancer drugs are highly expensive. So appropriate technologies should be developed to find out cheaper drugs and investigations may be done for the same. A number of squid and cuttlefish processing plants operate along the cost of kerala and they give out an enormous amount of waste leading to environmental pollution. The waste arising from squid and cuttlefish processing is mainly composed of the visceral organs like liver, ink sac and nidemental gland complex. Of the visceral organs, the ink sac in limited quantity is sometimes exported to Japan where it is doubted to be used for developing biomedical research material. Cuttlefish nidemental gland under the trade name cuttlefish ‘roe’ is also exported to European countries where it is used as salad material (Santhosh Kumar et al., 2002).
Despite considerable interest since time immemorial, the biochemical and biological properties of squid and cuttlefish ink are still little defined, nor is there any concerted attempt at systematically screening their bioactive components. The present study intends to screen squid and cuttlefish ink for bioactive components especially anticancer agents.