CHAPTER II
REVIEW OF LITERATURE

Amid the types of cancers, Hepatocellular carcinoma (HCC) is the fifth most common existing and lethal malignancy in the world, accounting for 5,00,000 to 10,00,000 new cases per year and causing 6,00,000 deaths globally (Jemal et al., 2007). Because of its poor prognosis, the number of deaths almost matches the number of cases being diagnosed each year (Parkin and Bray, 2005). This heterogeneous cancer show great geographical variation, predominantly in the Asia Pacific region, where the incidence of HCC has been static over recent decades at over 20 cases/1,00,000 population (Kamangar et al., 2006; Yuen et al., 2009). Hepatocellular carcinoma (HCC) is among the most common malignancy in both underdeveloped and developing countries, leading to 6,00,000 deaths annually worldwide. HCC is especially frequent in Asia due to a high prevalence of chronic HBV and HCV infections (Raza et al., 2007). Lacking effective biomarkers, many patients diagnosed at the advanced stage miss the best opportunity for anti-cancer therapy, including liver resection or transplantation. Furthermore, the post-operative five years survival is relatively low at 30% to 40%, since the patients who were resected often suffer a high frequency of tumour metastasis/recurrence (Hwang, 2006). As is the case with tumours in general, HCC is believed to develop following multi-pathogenetic steps, initially with premalignant lesions, through hyperplasia to dysplasia, then carcinoma in situ, and finally invasive carcinoma (El-Serag and Rudolph, 2007).

2.1. Distribution of hepatocellular carcinoma

The incidence of HCC is geographically variable due to the prevalence of specific etiological factors and ethnicities. HCC almost always develops in patients with chronic hepatitis B and C viral infection (HBV and HCV), alcohol consumption or dietary exposure to aflatoxins (Badvie, 2000). The highest frequencies (>20/1,00,000 annually) are seen in South-East Asia, China, Hong Kong, Taiwan, and sub-Saharan Africa, where HBV infection is highly endemic. In low-incidence areas (<5/1,00,000 annually) including North and South America, Northern and Western Europe and Oceania, HCC is highly associated with HCV infection and liver diseases induced by alcoholism (Bosch et al., 1999). HCC receives a great deal of clinical attention because of its growing worldwide incidence.
2.1.1. National status of hepatocellular carcinoma

In India, HBV is the main etiological factor associated with HCC (Sarin et al., 2001; Kar et al., 1997). In the West, majority of HCC are diagnosed incidentally during routine evaluation. However, in India, most of the patients in clinical practice present at an advanced stage, ruling out curative treatment in most cases. There is paucity of published literature on profile of HCC patients in India, making formulation of a proper health care strategy difficult. In order to study the characteristics of HCC in India, comprehensive analyses of these patients especially with regard to their clinical, etiological, radiological and cytohistological profile have been carried out.

2.1.2. International status of hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is a major global health problem. In the USA, the incidence of HCC has risen in recent years and this increase is expected to continue over the next two decades, equaling that currently experienced in Japan (Tanaka et al., 2002). HCC is now the leading cause of death among cirrhotic patients. HCC develops in a cirrhotic liver in 80% of cases and this pre-neoplastic condition is the strongest predisposing factor (Bosch et al., 1999). Hepatitis B virus (HBV) infection is the main risk factor in Asia and Africa (Brechot et al., 2001). Chronic carriers have a 100-fold relative risk for developing HCC, with an annual incidence rate of 2 to 6% in cirrhotic patients (Fattovich et al., 1995). Aflatoxin B$_1$ exposure further enhances the risk (Sun et al., 1999). In Western countries and in Japan, Hepatitis C Virus (HCV) infection is the main risk factor, together with other causes of cirrhosis including hemochromatosis (Tsukuma et al., 1993). Approximately 20 to 30% of the estimated 170 million HCV-infected individuals worldwide will develop cirrhosis. Once cirrhosis is established, the annual incidence of HCC is of 3 to 5%. One-third of all cirrhotic patients develop HCC over their lifetime (WHO, 1997). The multifactorial etiology of HCC may explain its complex molecular pathogenesis.
2.2. Progression of hepatocarcinogenesis in multiple phases

Carcinogenesis is a complex, multistep process with molecular events corresponding to each stage of disease progression in which a normal cell evolves through precancerous condition of cell hyperplasia and dysplasia and finally into a cancerous lesion. This malignant transformation of a normal cell into a cancer cell is thought to be caused by aberrant gene expression critical to cellular processes such as cell cycle control, cell growth, differentiation, apoptosis, cell adhesion and other functions at the cellular, molecular and genetic levels. Malignant hepatocytes result from step-by-step changes that accumulate in mature hepatocytes or can be derived from stem cells (Bruix et al., 2004). The most widely accepted hypothesis describes a sequential process in which this stepwise transformation begins in the liver tissue undergoing chronic hepatitis or cirrhosis caused by external stimuli (HBV or HCV infection, alcohol or metabolic diseases), progresses through a series of hyperplastic and dysplastic stages (low and high-grade dysplastic) stages, and progresses further to become more malignant, making metastases possible.

2.3. Chemical carcinogenesis

Carcinogenesis in human and laboratory animals is a complex, multistep process and may result from the action of any one or a combination of chemical, physical, biological and genetic insult to cells (Dean, 1998). Carcinogenesis by small molecular weight chemicals involves either a direct action of the chemical on cellular DNA or metabolism of the parent chemical to produce a permanent chemical change in the DNA structure. Epidemiological and laboratory studies indicate that chronic inflammation and hyperplasia induced by viral or nitroso compounds are closely associated with the development of HCC (Ahn et al., 1999).

Experimental hepatocarcinogenesis can be induced by various chemical carcinogens such as diethyl nitrosamine (DEN), Aflatoxin B$_1$ etc. Diethyl nitrosamine is a potent hepatocarcinogen in rats influencing the initiation stage of carcinogenesis and induces DNA base modifications, DNA strand breaks and inter hepatocellular carcinomas without cirrhosis, through the development of putative preneoplastic focal lesions (Tatematsu et al., 1988). N-Nitrosodiethylamine (DEN) is a potent hepatocarcinogenic nitrosamine present in tobacco smoke, water, cheddar cheese, cured and fried meals, occupational settings, cosmetics,
agricultural chemicals and pharmaceutical agents (Brown, 1999; Reh and Fajen, 1996). It has been suggested that, on metabolic activation, it produces the pro-mutagenic products, O$_6$-ethyl deoxy-guanosine and O$_4$ and O$_6$-ethyl deoxy-thymidine in liver which are responsible for its carcinogenic effects (Verna et al., 1996). It is also reported that the generation of reactive oxygen species (ROS) by DEN causes carcinogenic effects. ROS are potentially dangerous by-products of cellular metabolism that have direct effect on cell development, growth and survival. Oxidative stress caused by ROS has been reported in membrane lipid peroxidation, DNA damage and mutagenesis associated with various stages of tumor formation process (Parola and Robino, 2001). Hence the model of DEN-induced HCC is considered as one of the most accepted and widely used experimental models to study hepatocarcinogenesis. Administration of DEN to animals has been shown to cause cancer in liver and at lower incidences, in other organs (Lijinsky et al., 1981). The DEN model of experimental hepatocarcinogenesis provides a unique tool for studying cellular changes resulting from the administration of carcinogen to the development of premalignant phenotype of the cell, mechanism of cell growth, differentiation and cell death (Farber, 1984).

2.4. HCC invasion and metastasis

HCC invasiveness is associated with the ability of tumor cells to invade the capsule and portal vein (El-Assal et al., 1997). The intrahepatic and extra hepatic metastases occur in >50% of patients after resection of HCC, with intrahepatic metastasis occurring more frequently. Common sites of extra hepatic metastasis include the lung, bone, peritoneum, spleen and lymph nodes (Poon et al., 2000). The occurrence of venous infiltration in the liver and extra hepatic metastases are classic signs for advanced HCC and metastasis is undoubtedly the most significant cause of liver failure and mortality in HCC patients. Yet there is no promising curative therapy targeting metastatic HCC. HCC can be varied in appearance but it is mostly a soft tumor with areas of necrosis and appears different in color from yellow or grey to green, indicating the presence of bile. Moreover, hepatic vein, portal vein and vena-cava are the most common blood vessels of vascular invasion by HCC. Microscopically, HCC cells grow in the pattern that resemble to normal hepatocytes in their early stage but differently in the late stage. Edmondson and Steiner (1954) have proposed a grading system of HCC based on this observation. Edmondson-Steiner grading system is divided into four scales from I to IV with
grade IV HCC cells having the highest nuclear irregularity, hyperchromatism and nuclear to cytoplasmic ratio (Mac Sween et al., 2007).

2.5. Clinical features of HCC

The prognosis of HCC is poor, partially attribute to the absence of definite symptoms in its early stage. As a result, patients with the tumors are usually presented in the advanced stage when first diagnosed. Their median survival time is often shorter than one year, if the tumor is left untreated. In the past, liver ultrasonography provided an inexpensive way for the detection of HCC, but the sensitivity of detecting small nodule was low and thus not reliable. With the aid of current medical imaging technologies, computed tomography (CT) and Magnetic Resonance Imaging (MRI) provide a much greater sensitivity to detect small nodules down to 1-2 cm with precision over 80% (Llovet et al., 2003). Upper abdominal pain, malaise, fatigue, weight loss and abdominal swelling are often the chief complaints of HCC patients. Signs, which could be observed by a medical practitioner, include palpable abdominal mass, ascites, splenomegaly, fever and jaundice. Other manifestations caused by metastasis such as Budd-Chiari syndrome, bone pain, cough and dyspnea are rarely reported (Carr, 2005). Alpha-fetoprotein (AFP), a distinct serum marker produced by HCC, was used as the primarily laboratory screening for HCC long time ago. Around 70% to 80% of HCC patients were found to have an elevated AFP concentration greater than 500μg/L in their serum (Kasper and Harrison, 2005). However, due to the limited specificity and sensitivity of AFP, hepatic imaging technique usually accompanies to confirm the diagnosis (Mac Sween et al., 2007).

2.6. Current therapeutical options for HCC

Treatments for HCC are simply divided into two categories namely surgical and non-surgical treatments. Surgical treatments refer to the therapeutical options that require large and invasive operation including liver resection and liver transplantation. Non-surgical treatments refer to non-invasive or minimally invasive methods including ablation, chemoembolization, radiation and molecular target therapy. Different therapeutical options have also been classified into curative and palliative treatments, in which curative treatment aims to remove the tumour completely whereas palliative treatment aims to slow down the progression of disease and improve patient’s quality of life (Llovet et al., 2003). The choice of suitable
therapeutical options for each HCC patient depends on many factors such as tumor stage, presence of underlying liver disease, availability of expertise and appropriate surgical equipments (El-Serag et al., 2008).

2.6.1. Surgical treatments

2.6.1.1. Liver resection

Liver resection offers one of the best curative treatments for HCC with five years survival rate of 60% to 70%. Yet, a vast number of HCC patient’s (70% to 80%) are ineligible to perform this operation because of underlying liver cirrhosis, advanced tumor stage or insufficient functional reserve of the liver (Kassahun et al., 2006). With the improvement of surgical techniques, the mortality rate after operation for those who are eligible for partial hepatectomy, is about 5% to 7% when compared to 15% two decades ago (Poon and Fan, 2004). Although liver resection offers a hope for cure, the disease-free survival rate remains unsatisfactory due to 10 major concern of possible intrahepatic recurrence. Once the tumor is recurred, a more aggressive treatment is needed to lengthen the survival of patients (Poon et al., 1999).

2.6.1.2. Liver transplantation

Liver transplantation offers an effective treatment for HCC patient, if tumor recurrence or metastasis is neglected. It can eliminate both the tumors and underlying liver diseases simultaneously so that the five years survival rate is over 70% with only 15% of tumor recurrence rate (Mazzaferro et al., 1996). The current protocol adopted by the United States in selecting patients for liver transplantation is based on tumour size and Classification of Malignant Tumors cancer staging system (Koller et al., 2007). They include: i) a solitary tumor less than 5 cm in diameter ii) fewer than three tumor nodules with each one less than 3 cm in diameter and iii) no tumor metastasis and no vascular or lymph node invasion. Nevertheless, this procedure is often not feasible because the availability of hepatic allograft donors cannot meet the demands and the patient selection criteria are very strict (Mc Cormack et al., 2005).
2.6.2. Non-surgical treatments

2.6.2.1. Radiofrequency ablation (RFA)

The use of radiofrequency ablation (RFA) is gaining its popularity as a first line therapeutical option for unresectable HCC patients these days (Ng and Poon, 2005). It is performed by the application of electromagnetic energy to tumor with frequency operated in the range of 375 to 500 kHz through an electrode probe (Nikfarjam et al., 2005). Electrode probe can be placed within the tumor percutaneously under the image guidance of ultrasound, CT or MRI or in open surgical approach (Forner et al., 2006). Alternating electric current generated at the tip of the probe creates frictional heating between molecules within tissue, causing a localized rise in tissue temperature and therefore capable of forming necrotic tumor residue above 60°C (Nikfarjam et al., 2005). The advantage of this treatment is that it has fewer complications than surgical resection and the response rate is satisfactory, with two year survival over 50% reported in several literatures (Ng and Poon, 2005). As a result, more clinical studies should be established to explore the potential of RFA in HCC management.

2.6.2.2. High intensity focused ultrasound (HIFU)

In the last century, there was already a brief introduction of the therapeutical significance of ultrasound in medicine (Wood and Loomis, 1927). The early application of high intensity focused ultrasound (HIFU) in the late 1950’s was in the field of neurosurgery to treat Parkinson’s disease and other neurological deficits. After that, some studies suggested that HIFU can be also used to treat cancer with regard to its tissue destruction ability (Fry and Johnson, 1978). Basically, ultrasound is a kind of longitudinal sound wave with frequency beyond the upper limit of normal human hearing, which is approximately 20 kHz (Takeda et al., 1992). In clinical setting, the frequency of HIFU machine is set between 0.8 to 3.5 MHz. If both the correct focal length and amount of energy are set, ultrasound will penetrate skin tissue harmlessly and reach the liver tissue of interest which then turns mechanical energy into heat energy to induce focal hyperthermia (Leslie and Kennedy, 2006).
2.6.2.3. Microwave ablation

Like other electromagnetic energy ablation methods, microwave ablation achieves focal hyperthermia using the principle similar to microwave oven heating, but operates on a lower frequency of 2450 MHz. Although microwave device of 915 MHz is also commercially available, 2450 MHz model remains the most popular and conventional applicator for microwave ablation. Electric dipoles such as water molecules create rotational motion as they try to line up with the oscillating electric field of microwave. This rotational motion, as a result, collides with other molecules and creates frictional heating (Liang and Wang, 2007). In a study, microwave ablation achieves a slightly higher rate of complete ablation than radiofrequency ablation, in which around 90% of HCC can be completely ablated by microwave (Dong et al., 2003 and Xu et al., 2004). In addition, the five years cumulative survival rate of HCC patients was reported to be 56.7% with mean tumor size of 4.1 cm. Despite the fact that microwave ablation is a safe and effective treatment for HCC patients, high tumor recurrence rate remains the major concern of all (Dong et al., 2003).

2.6.2.4. Percutaneous ethanol and acetic acid ablation

Percutaneous ethanol injection (PEI) was one of the mainstream therapeutical options for HCC in Japan and Europe a decade ago (Shiina et al., 1993). In most cases, absolute ethanol (99.5%) is directly injected into the tumor nodules through fine needle under the guidance of CT or ultrasound. Because of the cellular dehydrating properties of ethanol, tumor nodules degenerate and give rise to coagulative necrotic tissues (Clark, 2007). PEI can obtain a 70 to 80% of necrosis with tumor size equal or smaller than 3 cm in diameter and the five years survival rate is 50% (El–Serag et al., 2008; Forner et al., 2006). PEI is an inexpensive procedure, well tolerated by patients and with few complications (Clark, 2007). Therefore, it is often used as an adjuvant therapy with thermal ablation and chemotherapy. Acetic acid ablation is first proposed by Ohnishi et al. (1994) as a substitute for PEI because of its greater infiltration in tissue than ethanol. Their randomized controlled trial suggested that percutaneous acetic acid ablation using 50% acetic acid obtained a better response rate and lower local recurrence rate than PEI (Ohnishi, 1998).
2.6.2.5. Tran-catheter arterial chemoembolization (TACE)

TACE is used extensively as a primary treatment for unresectable HCC patients because of its promising results (Guan and Liu, 2006). This method is based on the rationale that liver tumors receive blood supply solely from hepatic artery, rather than the hepatic portal vein (Carr, 2005). As the name suggested, TACE is the combination of injecting chemotherapeutic agents and embolization agents to the hepatic artery. While arterial embolization can effectively cut off the supply of nutrient to tumor, the cytotoxic effect of anticancer drug will create tumor necrosis without affecting peripheral liver function (Lau and Lai, 2008). There are neither 15 standard choices of anticancer drugs nor embolization agents. And yet, Gelfoam, metallic coils and polyvinyl alcohol sponge are the common choices of embolization materials whereas doxorubicin, mitomycin and cisplatin are the most common anticancer drugs (Forner et al., 2006). TACE is performed with interventional radiology techniques under the guidance of angiogram to reach hepatic artery via celiac axis, where femoral artery is chosen to be the site of access by catheter (Guan and Liu, 2006). Significant tumor response to TACE varies a lot between patients, from 17% to 61.9% but almost none of them achieve complete tumor response since there are still remaining viable cells. Therefore, the reported five years survival rate was only 1% to 8% (Jansen et al., 2005).

2.6.2.6. Radiation therapy

Because of the numerous choices of therapeutical strategies for HCC and the potentiality that high dose of radiation may as well damage the surrounding normal liver parenchyma, the use of whole-liver radiation therapy to treat HCC has been limited in the past (Carr, 2005). Recently, yttrium-90 intra-arterial radiation therapy has been introduced in clinical trials to alleviate the symptoms caused by the liver metastases from neuroendocrine tumors with invigorating results (Cunningham et al., 2007). Therefore, more systematic clinical studies should be carried out to explore the therapeutical efficacy of this new technology.

2.6.2.7. Molecular target therapy

With the better understanding of molecular pathogenesis of HCC, several classes of drugs have been evolved to alter the activity of molecular signaling pathways. Based on the fact that
growth of HCC depends on oxygen and nutrients supplied from blood vessels, inhibition of angiogenesis thus becomes one of the main strategies to treat HCC (Forner et al., 2006). Sorafenib is an oral inhibitor of multiple kinases, including Raf and vascular endothelial growth factor (VEGF) receptor 2 and 3, in which these kinases are responsible for tumour cell proliferation and angiogenesis respectively. This drug is now undergoing phase III randomized controlled trial and is proving to be effective in improving the survival and quality of life in advanced stage HCC patients (El–Serag et al., 2008; Furuse, 2008). Another Angiogene inhibitor called Avastin, is a commercially available humanized recombinant monoclonal antibody which also targets VEGF. In view of its low response rate (< 20%) and short median progression-free survival (< 10 months), this drug is often used with other anti-cancer drugs in TACE to increase the survival chance of HCC patients (Zhu, 2008).

2.7. Natural remedies for HCC

2.7.1. Bioactive nature of flavonoids

Numerous preclinical and some clinical studies suggest that flavonoids have potential for the prevention and treatment of several diseases. Some epidemiological studies support a protective role of diets rich in foods with flavonoids and a reduced risk of developing cancer and cardiovascular diseases (Neuhouser, 2004 and Maron, 2004). Preclinical in vitro and in vivo investigations have shown plausible mechanisms by which flavonoids may confer cancer and cardiovascular protection. In addition to their preventive potential, certain flavonoids may be useful in the treatment of several diseases. Some evidence supporting the therapeutic potential of flavonoids comes from the study of plants used in traditional medicine to treat a wide range of diseases, which has shown that flavonoids are common bioactive constituents of these plants (Middleton et al., 2000; Ren et al., 2003).

Flavonoids have a variety of biological and chemical properties. These compounds, widely distributed in the plant kingdom are strong antioxidants (Letan, 1966) and have antimicrobial (Nishino et al., 1987), anti-inflammatory/anti-allergic (Middleton and Kandaswami, 1992), antimitogenic (Edenharder et al., 1993), anticlastogenic (Heo et al., 1992) and anticarcinogenic properties (Verma et al., 1988). Due to their function as antioxidants, flavonoids are included together with β-carotene and vitamins C and E, among the radio-
protective molecules present in human diets that are rich in fruits and vegetables (Shimoi et al., 1996). These antioxidants may play an important role in scavenging free radicals, such as the highly reactive DNA-damaging hydroxyl radicals induced by ionizing radiation as a result of the radiolysis of water. Nevertheless, these reports of the beneficial effects of flavonoids are counterbalanced somewhat by studies indicating that flavonoids can induce DNA damage, mutations and apoptosis (Salti et al., 2000).

Luteolin (3, 4, 5, 7-tetrahydroxyflavone), an important member of the flavonoid family, is present in various fruits and vegetables and has contributed to the antioxidant activity of artichoke leaf extract on reactive oxygen species in human leucocytes (Perez-Garcia et al., 2000). Luteolin is also reported to have anti-inflammatory properties and mediates its action by inhibiting of nitric oxide production. A report by Kimata et al. (2000) has established luteolin as a potent inhibitor of human mast cell activation through the inhibition of protein kinase C activation and Ca\(^+\) influx. Luteolin exerts growth inhibitory effects on NK Lymphoma ascites-tumour cell cultures (Molnar et al., 1981). The growth and metabolism of human leukaemic CEM-C\(_1\) and CEM-C\(_7\) cell lines are also inhibited by luteolin (Post and Varma, 1992). The Flavonoids, luteolin and quercetin are also reported to arrest cell cycle in the G\(_1\) phase of human melanoma cells (Casagrande and Darbon, 2001). Luteolin abundant in celery, green pepper, parsley, perilla leaf and chamomile tea, is one of the most common flavones (Shimoi et al., 1998). It is thought to play an important role in the human body as an antioxidant, a free radical scavenger, an agent in the prevention of inflammation, a promoter of carbohydrate metabolism and an immune system modulator. These characteristics of luteolin are also believed to play an important part in the prevention of cancer. Multiple research experiments describe luteolin as a biochemical agent that can dramatically reduce inflammation and the symptoms of septic shock (Jang et al., 2008). But the low solubility of luteolin in oil results in its poor permeation across the intestinal epithelial cells and the gastrointestinal tract. Phospholipids are an important component of cell membranes, keeping cell membrane fluidity and for treating hepatic disorders. Bombardelli et al. (1991) have studied the preparation and the biological activity of complexes of several natural principles such as silymarin and aescin with phospholipids. The complexes exhibited pharmacological activities significantly greater than those observed for the free constituents. It is expected that luteolin combined with phospholipids
might result in an improvement of the lipophilic properties of luteolin. In this study, a complex of luteolin and phospholipid were prepared and the physicochemical properties and antioxidant activities of the complex have been investigated (Wu et al., 1993).

2.7.2. Pharmacological mechanisms of luteolin

Luteolin is one of the major flavonoids in *Chrysanthemum*. As an ubiquitous flavonoid, luteolin has been extensively studied for its various biological effects, such as estrogenic and anti-estrogenic activity, anti-oxidant activity, anti-inflammation, anti-proliferation, anti-carcinogenesis and anti-tumor effects. Many of these activities effects on proliferation, cell cycle, apoptosis, topoisomerase and several protein kinases (Fig. 1).

![Fig 1: Biological activity and possible mechanism action of Luteolin](image)

2.7.3. Estrogenic and anti-estrogenic activity of luteolin

Estrogens are hormones involved in the proliferation and differentiation of target cells. In response to estrogens, estrogen receptor (ER) will be activated and it then stimulates DNA synthesis and cell proliferation (Colditz, 2005). Flavonoids are naturally occurring phytoestrogens because they can bind to ER and activate its signaling pathway
(Collins-Burow et al., 2000). So, it is suggested that these groups of natural compounds may be used to replace conventional hormones in therapy of menopause disorder. Luteolin possesses potent estrogenic activity at very low concentration (Zand et al., 2000), suggesting that it may be useful in hormone replacement therapy. However, there were also reports about the anti-estrogenic effects of luteolin, similar to genistein, a well studied soy isoflavone with both estrogenic and anti-estrogenic properties (Han et al., 2002). The mechanism behind this still remains controversial. A possible explanation is that flavonoids are estrogenic because they have a high affinity towards ER and thus activate ER, if the estrogen is deficient. Nevertheless, their estrogenic activity is relatively weak, 103 to 105 fold less than 17 \( \beta \)-estradiol (Zand et al., 2000). Thus, in the presence of estradiol, flavonoids could possibly inhibit estrogen by competing for its receptors. Since ER is one of the major risk factors in breast cancer, the anti-estrogenic activity of flavonoids has been suggested to be closely related to their anti-proliferation activity and potential in breast cancer therapy and prevention.

Luteolin, as well as other flavonoids such as daidzein, genistein and quercetin, is able to inhibit the proliferation-stimulating activity in Michigan Cancer Foundation (MCF) 7 cells, caused by environmental estrogens such as diethylstilbestrol, clomiphene and bisphenol (Han et al., 2002). The suppressive effect of flavonoids suggests that these compounds have anti-estrogenic and anti-cancer activities. Wang et al. (1998) also found that luteolin inhibits estradiol-induced DNA synthesis. In an in vivo test, Holland and Roy (1995) proved that luteolin reversed the estrogen-stimulated proliferation of mammary epithelial cells in female Noble rats, suggesting that it may play a preventive role in estrogen-induced mammary carcinogenesis. It is however important to point out that the anti-estrogenicity of flavonoids does not always correlate with their ER binding capacity, suggesting that alternative signaling mechanisms could have been involved in their antagonistic effects (Collins-Burow et al., 2000). Mammalian cells contain two classes of estradiol binding sites viz., type I (Kd \~1.0 nM) and type II (Kd \~20 nM), named according to their affinity. Luteolin was found to compete for estradiol binding to cytosol and nuclear type II sites but it did not interact with estrogen receptors. In an in vivo study, injection of luteolin blocked estradiol stimulation of nuclear type II sites in the immature rat uterus and this correlated with an inhibition of uterine growth. Further studies also
showed that luteolin could bind to nuclear type II sites irreversibly due to covalent attachment (Markaverich et al., 1988).

2.7.1.1. Antioxidant activity of luteolin

Flavonoids are well known antioxidants and there were also many reports about the antioxidant effects of luteolin. Robak et al. (1998) found that luteolin inhibits lipoxygenase activity, cyclooxygenase activity and ascorbic acid-stimulated malonaldehyde formation in liver lipids. Luteolin also inhibits DNA damage induced by hydrogen peroxide or singlet molecular oxygen in human cells (Devasagayam et al. (1995); Noroozi et al. (1998)). The glycosylated form of luteolin, luteolin-7-O-glucoside, demonstrates a dose-dependent reduction of LDL oxidation, although it is less effective than luteolin. Studies of the copper-chelating properties of luteolin-7-O-glucoside and luteolin suggest that both of them act as hydrogen donors and metal ion chelators (Brown and Rice-Evans, 1998). Since oxidative stresses is closely related to mutagenesis and carcinogenesis, luteolin, as an anti-oxidant, may act as a chemo preventive agent to protect cells from various forms of oxidant stresses and thus prevent mutagenesis and carcinogenesis.

Although the ability of flavonoids to protect cells from the oxidative stress has been demonstrated, there is also increasing evidence for their pro-oxidant property (Galati and O'Brien, 2004). Flavonoids could behave as antioxidants or pro-oxidants, depending on the concentration and the source of the free radicals. The pro-oxidant activity of flavonoids may be related to the ability of flavonoids to undergo autoxidation catalyzed by transition metals to produce superoxide anions (Hanasaki et al., 1994). Alternatively the phenol rings of flavonoids are metabolized by peroxidase to form pro-oxidant phenoxy radicals, which are sufficiently reactive to co-oxidize glutathione (GSH) or nicotinamide-adenine hydrogen (NADH) accompanied by extensive oxygen uptake and reactive oxygen species formation (Galati et al., 2002). One important understanding is that the pro-oxidant properties of flavonoids could contribute to their ability in the induction of tumor cell apoptosis and cancer chemoprevention (Ueda et al., 2002). Exposure of mammalian cells to flavonoids is accompanied by an increase in intracellular ROS levels and lipid peroxidation, which lead to apoptotic or necrotic cell death (Shen et al., 2004).
Structure-activity relationship study on pro-oxidant cytotoxicity of flavonoids showed that flavonoids containing a phenol ring are generally more bioactive than that containing a catechol ring (Galati et al., 2002). Further studies showed that an increase in cytotoxicity is correlated with an increase in ease of electrochemical oxidation of flavonoids and their lipophilicity (Sergediene et al., 1999). Although luteolin has been shown to induce apoptosis in several cancer cells, it remains to be determined whether the pro-oxidant activity of luteolin is part of the mechanisms causing apoptotic cell death.

2.7.1.2. Anti-inflammatory activity of luteolin

Inflammation is a defense mechanism to guard against infection and help heal injury. During an inflammation, monocytes and macrophages become activated by various immune molecules such as cytokines or endotoxin such as lipopolysaccharide (LPS), an outer membrane component of gram-negative bacteria. The activated macrophages will vigorously produce inflammatory molecules such as TNFα (Tracey and Cerami, 1994), ILs (Akira et al., 1993), free radicals and nitric oxide (NO) etc. (Nathan and Xie, 1994), which will lead to inflammation and turn on a deadly cascade of events. Production and release of inflammatory cytokines by LPS depends on inducible gene expression mediated by the activation of transcription factor NF-κB (Beuvink et al., 2005). The signals from LPS converge upon the IκB kinase (IKK) complex, which phosphorylates the inhibitor of NF-κB (IκB), causing its ubiquitination and degradation. Removal of IκB liberates NF-κB proteins such as p65 for nuclear translocation, binding to κB-promoter elements and induction of gene transcription.

Macrophages participate in host defense and are main targets for the action of LPS. Pretreatment of murine macrophages RAW 264.7 with luteolin or luteolin-7-glucoside inhibits both the LPS-stimulated TNFα and IL-6 release. Furthermore, luteolin abolishes the LPS-induced phosphorylation of Akt, which may link LPS activation to NF-κB activation (Xagorari et al., 2001). However, over-expression of a dominant negative form of AKT does not alter LPS-induced TNF-α release, suggesting that inhibition of this kinase does not mediate the inhibitory action of luteolin. It is possible that luteolin interferes with LPS signaling by reducing the activation of MAPK family members ERK and p38, but not c-Jun N-terminal kinase (JNK) (Xagorari et al., 2002). The active anti-inflammatory components of Glossogyne tenuifolia were
identified as oleanolic acid and luteolin-7-glucoside. Both of them inhibited LPS-stimulated inflammatory mediator production and NF-κB activation (Wu et al., 2004).

Similar effects and mechanisms of luteolin on innate immunity were found in intestinal epithelial cells and dendritic cells. Luteolin significantly blocks LPS-induced IκB phosphorylation and degradation, NF-κB transcriptional activity and intercellular adhesion molecule-1 (ICAM-1) gene expression in rat IEC-18 cells (Kim and Jobin, 2005). This effect is by directly inhibiting the LPS-induced IKK activity. Interestingly, although luteolin shows potent inhibition on LPS-stimulated NF-κB transcriptional activity in Rat-1 fibroblasts, it does not inhibit either IκBα degradation, NF-κB nuclear translocation, or DNA binding induced by LPS (Kim et al., 2003). Rather, luteolin prevents LPS-stimulated interaction between the p65 subunit of NF-κB and the transcriptional co-activator CBP, suggesting that the effect of luteolin on NF-κB signaling varies depending on the cell types.

Luteolin not only inhibits LPS stimulated release of pro-inflammatory cytokines such as TNF and ILs, but also directly inhibits the signaling triggered by TNF or ILs. Intercellular adhesion molecule-1 (ICAM-1) is an immunoglobulin super-family expressed on endothelial cells and important for adhesion of leukocytes and trans-endothelial migration. Luteolin inhibits TNF-α-stimulated ICAM-1 expression by inhibiting IKK activity, IκBα degradation, NF-κB DNA-protein binding and NF-κB luciferase activity in respiratory epithelial cells (Hubbard and Rothlein, 2000). The inhibitory effects of luteolin on ICAM-1 expression are also mediated by the sequential attenuation of the three MAPKs activities, the c-fos and c-jun mRNA expressions, and the activator protein-1 (AP-1) transcriptional activity (Chen et al., 2004). Through a similar mechanism, luteolin can inhibit TNF-alpha-induced IL-8 production in human colonic epithelial cells (Kim et al., 2005a).

Another important inflammation mediator, NO is synthesized by inducible NO synthase (iNOS), which is activated by LPS. Luteolin and its glycoside, luteolin-7-O-glucoside, suppress the production of NO and prostaglandin E₂ (PGE₂) in LPS activated-mouse macrophage RAW264.7. The inhibitory effect is attributed to the suppression of both iNOS and cyclooxygenase-2 (COX-2) protein expression by luteolin, without affecting the enzymatic
activity directly (Hu and Kitts, 2004). It should be pointed out that it seems unlikely that the inhibitory action of luteolin on pro-inflammatory cytokine production is the result of antioxidant properties. This is based on observations that some flavonoids with strong antioxidant properties are completely ineffective in reducing LPS-stimulated Tumour Necrotic Factor (TNF) production (Devasagayam et al., 1995). A structure-activity study has shown that the presence of a double bond at position C2-C3 of the C ring with oxo-function at position 4, along with the presence of the OH groups at positions 3’ and 4’ of the B ring is required for optimal inhibition of LPS-stimulated TNF- release (Xagorari et al., 2001). The anti-inflammatory ability of luteolin has been also evaluated in vivo. Mice receiving LPS exhibited high mortality after the LPS challenge. On the contrary, mice that had received luteolin (0.2 mg/kg, intraperitoneally) before LPS showed an increased survival (Kotanidou et al., 2002). Luteolin pretreatment also reduces LPS-stimulated TNF-α release in serum and ICAM-1 expression in the liver (Kotanidou et al., 2002), which is in agreement with many in vitro observations. The effect of luteolin was also tested in an acute Chlamydia pneumoniae infection model in C57BL/6J mice. Luteolin was found to suppress inflammation in lung tissue that was caused by Chlamydia pneumonia. However, luteolin treatment had no effect on iNOS but significantly decreased the expression of constitutive NOS enzyme (Tormakangas et al., 2005).

2.7.1.3. Anti-carcinogenesis activities of luteolin

Carcinogenesis is a long-term and multi-stage process that results from accumulation of mutation and dysfunction of important molecules regulating cell proliferation and cell death. The process of chemical carcinogenesis may be divided into three stages: initiation, promotion and progression. During initiation, a potential carcinogen is transformed into a mutagen by phase I enzymes such as cytochrome P450. The mutagen may react with cellular molecules such as DNA and result in genetic mutation. During the promotion stage, the genetic alterations will lead to enhanced cell proliferation and/or reduced cell death. During the promotion stage, the mutations are enhanced and the cells are proliferating in an uncontrolled manner (Pitot, 1993).

The inhibitory effects of flavonoids, including luteolin on carcinogenesis have been well documented. In an in vivo study, it was observed that luteolin significantly decreased the
incidence of fibrosarcoma induced by 20-methylcholanthrene (20-MC), a strong carcinogen, in male Swiss albino mice (Elangovan et al., 1994). Other studies showed that, to prevent tumor development, different stages of carcinogenesis can be targeted by luteolin. In the initiation stage, luteolin were found to inhibit the metabolism of carcinogens in isolated liver microsomes (Buening et al., 1981). In another study, it has been reported that luteolin inhibits the mutagenic activity resulting from the metabolic activation of benzo-pyrene and trans-7,8-dihydroxy-7,8-dihydrobenzo-pyrene in rat liver microsomes (Huang et al., 1983). Later in Oguri et al. (1998) proved that luteolin suppresses formation of mutagenic and carcinogenic heterocyclic amines.

Luteolin targets the enzymes involved in DNA synthesis, for example, DNA topoisomerases, to suppress tumor promotion. DNA topoisomerases are the essential enzymes that catalyze the interconversion of topological isomers of DNA molecules. Acting by sequential breakage and reunion strands of DNA, two topoisomerases (topoisomerase I and topoisomerase II) are involved in many vital cellular processes such as DNA replication, transcription, recombination, integration and chromosomal segregation (Corbett and Berger, 2004). The dysfunction of these vital enzymes will result in DNA damage that may induce cell cycle arrest or apoptosis. Several flavonoids have been shown to exert their action by interacting with DNA topoisomerases and promoting site-specific DNA cleavage. Luteolin inhibits topoisomerase II activity of HL-60 cells by forming a luteolin- topoisomerase II-DNA ternary complex and then induces apoptosis in the cells (Yamashita and Kawanishi, 2000). By inhibiting DNA synthesis and promoting topoisomerase-II-mediated cleavage of kinetoplast DNA mini-circles, luteolin inhibits the growth of Leishmania donovani pro-mastigotes and arrest its cell cycle progression, leading to apoptosis (Mitra et al., 2000). In addition, luteolin also strongly inhibits the catalytic activity of eukaryotic DNA topoisomerase I (Chowdhury et al., 2002). Luteolin intercalates directly with the enzyme as well as the substrate DNA to stabilize the topoisomerase-DNA covalent complex and thus to block the subsequent rejoining of the DNA breaks.

2.7.1.4. Inhibition on cell proliferation

One character of cancerous cells is that they are undergoing rapid and unlimited proliferation. Proliferation requires the success of DNA synthesis and then cell division, which is controlled by signaling pathways triggered by growth factors, such as epidermal growth factor
receptor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF). Many flavonoids, including luteolin were found to be able to inhibit the proliferation of cancer cells derived from nearly all tissues such as human breast cancer cells MCF-7, human neuroblastoma cells SHEP and WAC2 (Han et al., 2002), lymphoma cells (Ramanathan et al., 1994), pancreatic cancer cells MiaPaCa-2 (Lee et al., 2002), human leukemia cells HL-60 (Ko et al., 2002), hepatic stellate cells (Zhao, 2002), human thyroid carcinoma cell lines (Yin et al., 1999), human epidermoid carcinoma A431 (Huang et al., 1999) and human prostatic tumor cells (Knowles et al., 2000).

The anti-proliferation mechanisms of luteolin have been explored extensively in several aspects. Firstly, it is controversial whether the anti-proliferation effect of luteolin is dependent on endoplasmic reticulum (ER) (Ross and Kasum, 2002). Luteolin, as well as several other flavonoids, suppresses the proliferation of human prostatic tumor cells (PC-3), androgen-independent cells, indicating that flavonoids show their antiproliferation activity in an androgen-independent manner (Knowles et al., 2000). On the other hand, it was found that luteolin inhibits estradiol-induced DNA synthesis in both estrogen-dependent MCF-7 and estrogen-independent MDA-MB-231 human breast cancer cells (Han et al., 2002). In a separate study, luteolin was shown to inhibit the proliferation of several human thyroid carcinoma cell lines, UCLA NPA-87-1 (with estrogen receptor), UCLA RO-82W-1 (with anti-estrogen binding site) and UCLA RO-81A-1 (lacking both estrogen receptor and anti-estrogen binding site), suggesting that the inhibitory activity of luteolin on cancer cell proliferation is not dependent on estrogen receptor or androgen receptor but via other mechanisms (Yin et al., 1999). Secondly, since luteolin is able to inhibit the activity of topoisomerases, which is critical for DNA synthesis (Mitra et al., 2000; Chowdhury et al., 2002), it has been suggested that luteolin inhibits cell proliferation by inhibiting topoisomerases and DNA synthesis (Makino et al., 2001). Thirdly, the inhibitory effect of luteolin on cancer cell proliferation is related to its effects on various growth factors and their signaling pathways, including EGF, PDGF and VEGF. Luteolin was found to inhibit the proliferation of pancreatic cancer cell MiaPaCa-2 and its effect is closely related to the inhibition of the activity of EGF receptor, but not protein synthesis (Lee et al., 2002). The same group found that luteolin inhibits proliferation of human epidermoid carcinoma A431 cells.
via a similar mechanism (Huang et al., 1999). PDGF is one of the principal regulators of proliferation and migration of vascular smooth muscle cells (VSMCs). PDGF binding to its receptor leads to its phosphorylation on multiple tyrosine residues. The activated PDGF receptor is associated with a number of proteins including the phosphatidylinositol 3'-kinase (PI3K), which mediates Raf-MEK-ERK transduction (Claesson-Welsh, 1994).

Luteolin inhibits PDGF-induced proliferation and DNA synthesis of rat aortic VSMCs by inhibiting PDGF receptor phosphorylation. As a consequence, luteolin significantly inhibits PDGF-induced ERK, Akt and phospholipase C (PLC)-γ1 activation as well as c-fos gene expression (Kim et al., 2005c). These results suggest that the inhibitory effect of luteolin on the PDGF-induced proliferation of rat aortic VSMCs may be mediated by blocking phosphorylation of PDGF receptor (Kim et al., 2005b). Another important growth factor is VEGF, which is one of the most important factors regulating key angiogenic responses of endothelial cells. They include proliferation, migration and differentiation, as well as protection from apoptosis (Ferrara and Gerber, 2001). In a murine xenograft model, luteolin was demonstrated to inhibit tumor growth and angiogenesis. Furthermore, it was found that luteolin inhibits proliferation of human umbilical vein endothelial cells by inhibiting VEGF-induced PI3K activity and activation of Akt, a downstream target of PI3K. However, luteolin does not affect VEGF-induced ERK activation, which is considered important for the mitotic effects of VEGF (Bagli et al., 2004).

2.7.1.5. Inhibition on cancer metastasis

In addition to rapid and continuous division and proliferation, another important feature of cancer cells is their ability to spread from the primary site to other more distant sites. This process, called metastasis, contributes to over 90% of human cancer mortality. The deadly process involves several sequential steps, migration, invasion and adhesion, which are driven by growth factors such as EGF, and MMPs (Brinckerhoff and Matrisian, 2002). Trans-activation of the epidermal growth factor receptor (EGFR) tyrosine kinase activity is proposed to stimulate cell migration by regulating MMP expression. By blocking of the EGFR-signaling pathway, luteolin is able to reduce the level of phosphorylated FAK as well as the secreted MMP, which may lead to the suppression of cell invasion and metastasis (Huang et al., 1999 and Lee et al., 2004). In addition to many growth factors, cytokines also control MMP expression.
For example, interleukin 6 (IL6) is known as a cytokine that induces MMP-1 expression. Luteolin is potent in inhibiting the production of IL-6 and suppressing the expression of MMP-1 (Kim et al., 2004). Since ILs production is regulated by NF-κB, it is possible that the inhibitory effect of luteolin on NF-κB may play a role in suppressing IL production and MMP expression. Interestingly, luteolin as well its glycoside can directly inhibit the activity of MMP-2 and MMP-9, in a non-competitive manner (Ende and Gebhardt, 2004). On the other hand, luteolin is a potent inhibitor of in vitro invasion of human PC-3 prostate cancer cells (Lansky et al., 2005). Since elevation of Focal adhesion kinase (FAK) activity in human carcinoma cells is associated with increased invasive potential, the inhibitory effect of luteolin on FAK phosphorylation may contribute to suppression of cell invasion ability (Huang et al., 2005).

Luteolin has been proved to be a potent anticancer agent in a variety of cancer cells in vitro as well as in a number of in vivo animal models. Firstly, it can inhibit carcinogenesis at different stages: initiation, promotion and progress. Secondly, it can inhibit cancer cell proliferation, or modulate cancer cell cycle progression, or induce apoptotic cell death or suppress angiogenesis. Lastly, luteolin possesses strong anti-metastasis effect, an important property in cancer therapy to restrict the mortality of cancer. It is possible that a number of common mechanisms are involved in the diverse activity of luteolin on cancer. For example, inhibition on receptor tyrosine kinases and topoisomerases contributes to its inhibitory effects on carcinogenesis, proliferation, cell cycle and apoptosis. The inhibition on NF-κB could contribute to its anti-inflammatory, anti-carcinogenesis, anti-angiogenesis and anti-metastasis activities.

2.8. Antioxidants and prevention against human disease

There are a number of epidemiological studies that have shown inverse correlation between the levels of established antioxidants/phytonutrients present in tissue/blood samples and occurrence of cardiovascular disease, cancer or mortality due to these diseases. However, some recent meta-analysis show that supplementation with mainly single antioxidants may not be that effective (Vivekananthan et al., 2003), a view that contrasts with those of preclinical and epidemiological studies on consumption of antioxidant-rich foods. Based on the majority of epidemiological and case control studies, recommendations were made for the daily dietary intake of some established antioxidants like vitamin E and C as well as others.
Requirement for antioxidants in Indian conditions differ from that of industrialized western countries due to the nutritional differences. There are also a number of dietary supplements rich in antioxidants tested for their efficacy. There are many laboratories from India working on the antioxidant effect of plant compounds, mainly derived from natural sources that are capable of protecting against such damage. Such studies show that compounds with potent antioxidant activity include carotenoids, curcumin from turmeric, flavonoids, caffeine present in coffee, tea, etc., orientin, vicenin, glabridin, glycyrrhizin, emblicanin, punigluconin, pedunculagin, 2-hydroxy-4-methoxy benzoic acid, dehydrozingerone, picroliv, withaferin, yakuchinone, gingerol, chlorogenic acid, vanillin (food flavouring agent) and chlorophyllin (a water-soluble analogue of chlorophyll).

Increased levels of glycoprotein such as hexose, hexosamine and sialic acid in serum, hepatoma and surrounding liver tissues of DEN-treated rats as well as altered levels of protein bound carbohydrate are well documented during neoplastic diseases. Racheshky et al., (1982) cited that an increased glycoprotein level were observed in animals treated with carcinogen, dimethylnitrosamine. This change in surface carbohydrate during cellular differentiation and neoplastic transformation suggests their importance in physiology and behavior of the cells. Such changes have long been implicated in malignant transformation (Hynes, 1976). Sialic acid is an acylated derivative of neuraminic acid and exists as a terminal component of the non-reducing end of carbohydrate chains of glycoprotein in mammals. Their implications in a variety of surface-related vital cell functions in numerous tissues are well documented (Olden et al., 1982). Liver is the major site involved in the synthesis of sialic acid and also other glycoproteins. The synthesized glycoproteins are made to circulate in the blood. Hence, there is pronounced increase in serum rather than in other organs (i.e., liver). About 36% increase in serum sialic acid content in tumor-bearing rats has been reported by Srinivasa Rao and Sirsi (1970) and it has been concluded that sialic acid content of glycoprotein can be taken as a very useful tool in the confirmation of hepatic tumors.

Lysosomal proteolytic activity in liver cells plays a decisive role in protein metabolism, with cathepsin-A and cathepsin-D being the main enzymes involved in catabolic processes (Marzo et al., 2002). Tumor cells require specific proteolytic enzymes for invasion and metastasis, including lysosomal peptidasescathepsin. Recently, it was noticed that proteases play
a critical role not only in tumor cell invasion, but also in the earliest stages of carcinogenesis and its associated changes viz., angiogenesis and metastasis. Proteases are also signaling molecules that modulate other molecules by underlying pathways in addition to their degradative role (Flores-Reséndiz et al., 2009).

2.9. Other compounds with anti-tumour activities

Jigrine is a polypharmaceutical herbal formulation containing aqueous extracts of 14 medicinal plants used for liver ailments. Few studies are reported for its formulation (Najmi et al., 2002), safety evaluation (Valecha et al., 1990), mechanism of hepatoprotective action (Karunakar et al., 1997a; Aftab et al., 1999) and anti-inflammatory activity (Karunakar et al., 1997b). Hepatoprotective and anti-inflammatory effects of some of the individual ingredients of jigrine are also reported in literature (Sultana et al., 1995; Zafar and Ali, 1998).

Morin (3,5,7,2,4 pentahydroxyflavone) is a kind of flavonoid belonging to the group of flavonols, found in almonds (P. guajava L.), mill (Prunus dulcis), Chlorophora tinctoria and other Moraceae which are used as dietary agents and also as herbal medicines (Xie et al., 2006). It has been shown to act as a potent antioxidant, xanthine oxidase inhibitor, protein kinase C inhibitor, cell proliferation inhibitor, apoptosis inducer, modulator of lipoxygenase and cyclooxygenase activities in the arachidonic acid cascade (Laughton et al., 1991). Furthermore, morin exhibits an anti-tumor promotion effect by significantly inhibiting the 12-O-tetradecanoylphorbo13-acetate (TPA)-induced Epstein-Barr virus early antigen activation and the two-stage skin tumor promotion. It also exhibits inhibition of TPA-induced hepatocellular transformation (Hsiang et al., 2005). Morin also acts as a chemopreventive agent against oral carcinogenesis, in vitro and in vivo (Kawabata et al., 1999).

Vanadium, a group VB, first transition series, ultra-trace element (molecular weight 50.942) with various oxidation states ranging from −1 to +5, is an endogenous constituent of plants, animals and most mammalian tissues (Hopkins and Mohr, 1971). This dietary micronutrient is believed to have a regulatory role in biological systems and is very probably an essential element, just like other 40 essential micronutrients, requiring small amount for normal cell metabolism as well as for proper growth and development of mammals (Nielsen and Uthus, 1980). It influences the behaviour of enzymes, regulates the activities of
second messengers, signal transduction cascades and carbohydrate metabolism, mimics insulin and growth factor activities, stimulates protein tyrosine kinase and inhibits phosphotyrosine phosphatases and modulates gene expression. This nutritional element has further been considered as a potential agent owing to its ability to prevent regular wear and tear of the genome and accordingly, it is involved in various DNA maintenance reactions and thereby may prevent genomic instability leading to cancers (Evangelou, 2002). Vanadium compounds have been found to be potentially effective against murine leukaemia, fluid and solid Ehrlich ascites tumour (Kopf-Maier and Kopf, 1988), murine mammary adenocarcinoma and HEp-2 human epidermoid carcinoma cells and human carcinomas of lung, breast, and gastrointestinal tract (Kopfmaier, 1994). Furthermore, *in vivo* and *in vitro* antitumour activities of different vanadium compounds have been documented by several workers. Sakurai et al. (1995) have found strong antitumour chemopreventive activities of vanadyl complexes of 1,10-phenanthroline and related derivatives against human nasopharyngeal carcinoma and the observed effects were found to be superior than the chemotherapeutic drug, *cis*-diaminedichloroplatinum. Recently, organometallic vanadocene compounds have been found to be potent anti-proliferative agents disrupting bipolar mitotic spindle formation and inducing cell cycle growth arrest in cancer cell lines (Navara et al., 2001). Bis (4,7-dimethyl-1,10-phenanthroline) sulfatooxovanadium (IV) or Metvan is equally the most promising multitargeted antitumour vanadium complex with apoptosis-inducing property against human leukemia cells, multiple myeloma cells and a number of solid tumours derived from cancer patients (D’Cruz and Uckun, 2002).

2.10. DNA Fragmentation

DNA fragmentation is a vital characteristic in apoptosis. With respect to the mechanism, it has been known that there are plenty of species of endogenous DNases existing as a status of zymogen in the cytoplasm. DNAase molecules can recognize a specific sequence between adjacent chromosomes. When the zymogen of a given DNase is activated, DNA molecules within cells will be chopped up into various fragments with different lengths, thus leading to DNA fragments with 180-200 bp and their integral times, where a typical DNA laddering can be seen in agarose-gel after electrophoresis, which has been regarded as a vital marker in apoptosis that is distinctively different from necrosis. DNA fragmentation are directly linked through the caspase-3 mediated cleavage of DNA fragmentation factor (DFF) (also known as inhibitor of
caspase-activated deoxyribonuclease, ICAD), a cytosolic factor which binds to and inhibits the activity of an endonuclease (caspase-activated nuclease, CPAN or caspase-activated deoxyribonuclease, CAD) directly responsible for DNA fragmentation during apoptosis. This endonuclease, CAD/CPAN, is only activated during apoptosis and is therefore believed to be responsible for DNA fragmentation (Halenbeck et al., 1998; Sakahira et al., 1998).

2.11. Argyrophilic nucleolar organizer region

The silver-stained Argyrophilic nucleolar organizer region (AgNOR) proteins are a group of proteins that are associated with the nucleolar organizer regions and are selectively stained by silver methods (Howell, 1982). During interphase, they are located in the fibrillar nucleolar components (Hernandez-Verdun, 1983). The AgNOR proteins are necessary for ribosomal RNA transcription. Several studies performed at light microscopic level on cytological samples, silver-stained for AgNOR proteins have shown that the quantity of these proteins progressively increases when the cells enter the mitotic cycle from G1 to the end of S-phase (Pession et al., 1991). Mean number of AgNORs may reflect the cellular kinetics in rat hepatocarcinogenesis (Tanaka et al., 1989). Moreover, prolonged administration DFMO after DEN exposure markedly suppressed the development of liver cell foci and neoplasms.

2.12. Mast cells

Tumor infiltrating inflammatory cells in the microenvironment plays very important functions in tumor angiogenesis (Peng et al., 2005). Additionally, the synthesis of prostaglandins by inflammatory cells expressing the inducible cyclooxygenase-2 (COX-2) may also participate in metastatic progression (Minn et al., 2005). Mast cells (MCs) play an important role in the inflammatory component of a developing neoplasm. Recent research indicates that mast cells are a novel target for therapeutic intervention in the treatment of cancer (Gounaris et al., 2007). MCs circulate in blood as progenitors and undergo terminal differentiation into mature cells only when they enter the tissues (Rodewald et al., 1996). There is considerable circumstantial evidence implicating mast cells in the pathogenesis of several cancers including hepatocellular carcinoma (Grizzi et al., 2003). Mast cells can secrete several proangiogenic factors, which can jump-start tumor angiogenesis switch (Coussens et al., 1999) and they have been shown to accumulate in tissues undergoing angiogenesis during tumor growth (Heissig et al., 2005).
Decrease in mast cell density (MCD) upon silymarin treatment might be due to its strong anti-inflammatory property (DeLaPuerta et al., 1996) via decreasing the expression of COX-2 in liver cancer (Ramakrishnan et al., 2008) because most of the anti-inflammatory drugs act via inhibiting the expression of COX-2. Other explanation for the decrease in MCD by silymarin is due to its ability to inhibit transforming growth factor-β1 (TGF-β1) (Jia et al., 2001) because mast cell infiltration in DEN induced rat hepatoma may be due to the chemotactic effect of TGF beta expressed in tumor cells (Zheng et al., 2000). Mast cells are derived from pluripotent haematopoietic stem cells in the bone marrow that leave and circulate as immature cells only to mature once they reach their destination (Ishizaka et al., 1993). This is distinctly different from basophils that mature in the bone marrow before being released into circulation (Yong, 1997). Once released, mast cells undergo a maturation process that involves numerous factors including the specific cytokine, stem cell factor (SCF). The SCF receptor, c-Kit, is abundantly expressed in mature mast cells and plays a critical role in the maturation, development and secretory action of mast cells (Galli, 1993). The role of inflammatory cells especially mast cells (MCs) in the activation of MMP and angiogenesis were very well established (Heissig et al., 2005).

2.13. Transmission Electron Microscopy

Transmission electron microscopic studies potentiate the histological observations showing the degeneration of the hepatocytes. Ultrastructure of rat hepatocytes showed that they contained swollen mitochondria (Ernster and Schatz, 1981) which are ultimately responsible for the enhancement of mitochondrial membrane permeability leading to the alteration in the mitochondrial matrix enzymes activity. These alterations might affect the redox state of the mitochondrial thiol groups by modifying the mitochondrial NAD reduction level (Balázs and Halmos, 1985) which ultimately disturbs the intracellular free calcium level and its function. It leads to change in the permeability of the mitochondrial membrane and as a result membranes damages, cytoskeleton disassembly, chromatin condensation and decreasing of ATP.

2.14. Docking

The centrality of binding sites to medicinal chemistry has led to a multitude of different approaches for their identification in a protein structure and their subsequent exploitation, including methods based on structure (Wangikar et al., 2003 and Amitai et al., 2004), sequence
conservation (Dean et al., 2001) and interaction energies. In most cellular processes, proteins interact with other molecules to perform their biological functions. The knowledge about these interaction sites helps us to understand protein functions. Knowing the location of the functional sites (e.g., substrate or ligand-binding sites of enzymes or receptor proteins) on protein surfaces makes it possible to design inhibitors or antagonists and to introduce targeted mutations aimed at improving the protein function. The protein surface can form pockets that are binding sites of small molecule ligands. Therefore, the identification of pocket sites on the protein surface is often the starting point for protein function annotation and structure-based drug design. Also, proper ligand-binding site detection is a prerequisite for protein–ligand docking. In recent decades, many computational methods have been developed to predict protein–ligand binding sites based on detection of cavities on protein surface. These methods include POCKET (Levitt and Banaszak, 1992), LIGSITE (Hendlich et al., 1997), LIGSITEcs (Huang and Schroeder, 2006), SURFNET (Laskowski, 1995), CAST (Liang et al., 1998), PASS (Brady and Stouten, 2000), and PocketPicker (Weisel et al., 2007).