CHAPTER 2

REVIEW OF LITERATURE

2.1 Liver and Structure

Medical terms related to the liver often start in *hepato-* or *hepatic* from the Greek word for liver.

Human liver development begins during the third week of gestation and does not achieve mature architecture until about 15 years of age. It reaches its largest relative size, 10% of fetal weight, around the ninth week. It is about 5% of body weight in the healthy neonate. The liver is about 2% of body weight in the adult. It weighs around 1400 g in an adult female and about 1800 g in the adult male.

2.1.1 Anatomy

It is both the largest internal organ (the skin being the largest organ overall) and the largest gland in the human body. It is located in the right upper quadrant of the abdominal cavity, resting just below the diaphragm.

Figure 2.1 Posterior and inferior surfaces of the liver. (Right lobe labeled at upper right.)
The liver lies to the right of the stomach and overlies the gallbladder. It is connected to two large blood vessels, one called the hepatic artery and other called the portal vein.

The hepatic artery carries blood from the aorta, whereas the portal vein carries blood containing digested nutrients from the entire gastrointestinal tract and also from the spleen and pancreas. These blood vessels subdivide into capillaries, which then lead to a lobule. Each lobule is made up of millions of hepatic cells which are the basic metabolic cells. The liver is further subdivided into four lobes as right, left, caudal and quadrate. The right lobe is considered by many anatomists to include an inferior quadrate lobe and a posterior caudate lobe. However, on the basis of internal morphology, primarily the distribution of blood, the quadrate and caudate lobes more appropriately belong to the left lobe (Tortora et al., 2008).

2.1.2 Microanatomy of the liver

The lobes of the liver are made up of many functional units called lobules which are polyhedral in shape. A lobule consists of specialized epithelial cells, called hepatic (liver) cells or heptocytes, arranged in irregular, branching, interconnected plates around a central vein. Rather than capillaries, the liver has larger spaces lined by endothelium called sinusoids, through which the blood passes. The sinusoids are also partly lined with stellate reticuloendothelial (Kupffer’s) cells. These phagocytes destroy worn out white and red blood cells, bacteria, and toxic substances. Bile, secreted by hepatic cells, enters bile capillaries or canaliculi that empty into small bile ducts. These small ducts eventually merge to form the larger right and left hepatic ducts, which unite and exit the liver as the common hepatic duct. Hepatocytes, the most common site of toxicity account for 60% in rats and 85% in human of the total number of liver cells.
Hepatocytes constitute the major cell type in the parenchyma and are arranged into cell plates separated by narrow sinusoids lined with endothelial cells. The lumen of these channels is narrow and is penetrated by resident macrophages called Kupffer cells and liver associated natural killer lymphocytes or Pit cells that sit on the lumenal surface of the sinusoidal endothelial cells. The sinusoids allow oxygenated, nutrient rich blood arriving from the portal tract (which is composed of a branch of the hepatic artery, a portal vessel and a bile duct) to percolate slowly past the hepatocyte cell plates, allowing maximal exchange of material before the blood leaves the liver through the central vein branches that run to the hepatic vein (Fig. 5). Thus, leukocytes entering the liver can arrive through vascular endothelial cells lining the portal vein in the portal tract, or through the microvascular endothelial cells that line the sinusoids or through the terminal hepatic veins (Lalor and Adams, 2002).
**Figure 2.3 Changes in the hepatic architecture during chronic liver injury** (A) Associated with advanced hepatic fibrosis (B). Following chronic liver injury, inflammatory lymphocytes infiltrate the hepatic parenchyma. Some hepatocytes undergo apoptosis, and Kupffer cells activate, releasing fibrogenic mediators. HSCs (hepatic stellate cells) proliferate and undergo a dramatic phenotypical activation, secreting large amounts of extracellular matrix proteins. Sinusoidal endothelial cells lose their fenestrations, and the tonic contraction of HSCs causes increased resistance to blood flow in the hepatic sinusoid (Bataller and Brenner, 2005).

**2.1.3 Contents of Hepatocytes**

1. Nucleus 2. Mitochondria (energy providing process and enzymes) 3. Rough endoplasmic reticulum (synthesize proteins, triglycerides etc) 4. Smooth endoplasmic reticulum (vesicles which contain microsomes, site of bilirubin conjugation and detoxication) 5. Lysosomes (intracellular scavengers) 6. Golgi apparatus (packing sites). Apart from this hepatocyte the other cells in liver are endothelial cells, Kupffer cells (Mobile macrophases) and fat storing cells (Tortora et al., 2008)
2.1.4 Functions

Various functions of the liver are carried out by the liver cells or hepatocytes. Currently, there is no artificial organ or device capable of emulating all the functions of the liver. Some functions can be emulated by liver dialysis, an experimental treatment for liver failure. The liver is thought to be responsible for up to 500 separate functions, usually in combination with other systems and organs.

❖ Synthesis

➢ A large part of amino acid synthesis.

➢ The liver performs several roles in carbohydrate metabolism:
  • *Gluconeogenesis* (the synthesis of glucose from certain amino acids, lactate or glycerol)
  • *Glycogenolysis* (the breakdown of glycogen into glucose)
  • *Glycogenesis* (the formation of glycogen from glucose)(muscle tissues can also do this)

➢ The liver is responsible for the mainstay of protein metabolism, synthesis as well as degradation

➢ The liver also performs several roles in lipid metabolism:
  • *Cholesterol synthesis*
  • *Lipogenesis*, the production of triglycerides (fats).
    • A bulk of the lipoproteins is synthesized in the liver.

➢ The liver produces coagulation factors I (fibrinogen), II (prothrombin), V, VII, IX, X and XI, as well as protein C, protein S and antithrombin.

➢ In the first trimester fetus, the liver is the main site of red blood cell production. By the 32nd week of gestation, the bone marrow has almost completely taken over that task.
- The liver produces and excretes bile (a yellowish liquid) required for emulsifying fats. Some of the bile drains directly into the duodenum, and some is stored in the gall bladder.

- The liver also produces insulin like growth factor 1 (IGF-1), a polypeptide protein hormone that plays an important role in childhood growth and continues to have anabolic effects in adults.

- The liver is a major site of thrombopoietin production. Thrombopoietin is a glycoprotein hormone that regulates the production of platelets by the bone marrow.

- **Breakdown**
  - The breakdown of insulin and other hormones.
  - The liver glucoronidates bilirubin, facilitating its excretion into bile.
  - The liver breaks down or modifies toxic substances (e.g., methylation) and most medicinal products in a process called drug metabolism. This sometimes results in toxication, when the metabolite is more toxic than its precursor. Preferably, the toxins are conjugated to avail excretion in bile or urine.
  - The liver converts ammonia to urea (urea cycle)

- **Other functions**
  - The liver stores a multitude of substances, including glucose (in the form of glycogen), vitamin A (1–2 years supply), vitamin D (1–4 months supply), vitamin B\textsubscript{12} (1–3 years supply), iron, and copper.
  - The liver is responsible for immunological effects-the reticuloendothelial system of the liver contains many immunologically active cells, acting as a 'sieve' for antigens carried to it via the portal system.
  - The liver produces albumin, the major osmolar component of blood serum.
➢ The liver synthesizes angiotensinogen, a hormone that is responsible for raising the blood pressure when activated by renin, an enzyme that is released when the kidney senses low blood pressure.

The gall bladder produces bile salts necessary for lipid solubilization and transport and together these organs may be referred to as the hepatobiliary system.

2.2 Hepatocellular carcinoma (HCC)

Hepatocellular carcinoma (liver cancer) is a cancer arising from the liver. It is also known as primary liver cancer or hepatoma. The liver is made up of different cell types (for example, bile ducts, blood vessels, and fat-storing cells). However, liver cells (hepatocytes) make up 80% of the liver tissue. Thus, the majority of primary liver cancers (over 90%-95%) arises from liver cells and is called hepatocellular cancer or carcinoma. The liver is known as a favourable site for malignant seed second only to the skin, presumely because of its rich blood supply, liver is common site for the spread of malignant disease. Primary malignant hepatic tumors may arise from any constituent cells of the liver, but the only two common liver cell cancers are hepatocellular carcinoma and carcinoma of the biliary epithelium (cholangiocarcinoma). Other rare tumors viz., Fibrolamellar carcinoma, squamous carcinoma, epithelial hemangio endothelioma, Angiosarcoma, Kaposi’s sarcoma and hepatocellular adenoma may also arise from the liver.

The classification present in table 2.1 is based on the definitions and nomenclature recommended by the World Health Organisation (Mac Sween et al., 1979).
Table 2.1 Classification of HCC

<table>
<thead>
<tr>
<th>Origin</th>
<th>Benign</th>
<th>Malignant</th>
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<tbody>
<tr>
<td>Epithelial tumours</td>
<td>Liver – cell adenoma</td>
<td>Liver cell (hepatocellular) carcinoma</td>
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<td></td>
<td>Bile – duct adenoma</td>
<td>Bile-duct carcinoma (Cholangiocarcinoma)</td>
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<td></td>
<td>Bile – duct cystadenoma</td>
<td>Bile–duct cystadenocarcinoma Hepatoblastoma</td>
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<tr>
<td>Non– epithelial Tumour</td>
<td>Haemangioma</td>
<td>Haemangiosarcoma</td>
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<td></td>
<td>Infantile haemangio- endothelioma</td>
<td>Embryonal rhabdomyosarcoma, Leiomyo and other sarcoma</td>
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<tr>
<td>Miscellaneous</td>
<td>Focal nodular hyperplasia partial nodular transformation</td>
<td></td>
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<tr>
<td></td>
<td>Nodular regenerative hyperplasia</td>
<td></td>
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<tr>
<td></td>
<td>Mesenchymal haematoma</td>
<td></td>
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<tr>
<td></td>
<td>Cysts</td>
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<tr>
<td></td>
<td>Peliosishepatis, Teratoma</td>
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2.2.1 Stepwise development of the carcinocenic process

Cancer is a slow multi-stage, multi-step process which involves the appearance of discrete cell populations at different stages in the process (Farber and Carneron, 1980). Each stage signifies a distinct biological endpoint which is relevant to the process and permits both the proposal of a hypothesis and the design of experiments to test the hypothesis. Although all the steps involved in the development of cancer have yet to be elucidated, seminal studies using the mouse skin carcinogenesis mode (Berenblurn and Shubik, 1947) have identified three major stages - initiation, promotion and progression (Figure 2.6). These stages have now been extended to many other organs such as liver, colon, mammary gland and urinary bladder.
(Farber and Cameron 1980). This discussion will give emphasis to studies of these three stages as they pertain to liver carcinogenesis.

**Figure 2.4** Schematic representation of the multi-step development of liver cancer in the rat initiated with genotoxic carcinogens.

**2.2.1.1 Initiation**

Initiation is defined as the process by which a given organ or target tissue acquires the ability to be promoted or selected to develop focal lesions, one or more of which can act as sites of origin for the subsequent development of malignant neoplasia (Farber and Sarma, 1987). The essential features of initiation are that, it is an irreversible process which occurs rapidly usually after a brief exposure to a small dose of a carcinogen and affecting a small population of cells (1 in \(10^5-10^6\) hepatocytes) (Farber and Cameron, 1980). It is induced by the carcinogen and in general not by agents which subsequently are used to promote their focal proliferation. In the liver, at least two known obligatory steps are required for the formation of an initiated cell. The first major event involves the generation of a biochemical/molecular lesion(s) and the second a fixation or "making permanent" of the lesion(s) by at least one round of cell proliferation (Ying et al., 1982; Farber and Sarma, 1987). Although the nature of the lesion(s) is
not completely known, DNA has been strongly implicated as the major target of the carcinogen during the initiation process (Farber and Sarma, 1987). The result of such alterations in DNA include formation of adducts, translocations, deletions and the activation of proto-oncogenes among others. The second important step in the formation of an initiated cell is the requirement of cell proliferation. Experiments have shown that a single non-necrogenic dose of a hepatocarcinogen rarely results in hepatocellular carcinoma in an adult animal. Nonetheless, liver cell cancer can be induced in situations where there is high cell proliferation, as in neonatal animals (Peraino et al., 1984) or when the carcinogen is given in association with a liver cell proliferative stimulus such as 2/3 partial hepatectomy (PH) as demonstrated by Craddock (Craddock, 1971). Several investigators (Columbano et al., 1981; Ying et al., 1982; Kaufmann et al., 1991) subsequently substantiated these initial observations and showed that some chemicals which were not carcinogenic became carcinogenic when their administration was coupled with PH. Delaying the proliferative stimulus after carcinogen administration resulted in a lack of initiation (Ishikawa et al., 1980; Ying et al., 1982). It was proposed that the lack of initiation in these cases was due to the repair of the carcinogen induced critical lesion(s) in DNA by one of several DNA repair mechanisms. Since replication of the carcinogen damaged DNA results in a transfer of the damage to daughter cells, initiation is referred to as an irreversible step.

The type of cell proliferative stimulus has now been identified as an important factor for initiation. The compensatory type of cell proliferation such as that induced by 2/3 PH (Craddock, 1971; Pitot, 1979; Columbano et al., 1981) or liver cell necrosis induced by cytotoxic agents such as CCl₄ (Ying et al., 1982) supports initiation. Direct mitogenic type of cell proliferation induced by compounds such as lead nitrate, nafenopin, cyproterone acetate and ethylene dibromide however, do not support
initiation (Columbano et al., 1981). The nature of the differences between compensatory and direct mitogenic cell proliferation in their ability to support initiation still remains obscure. It must be highlighted that initiated cells are by no means capable of independent, autonomous growth, invasion or metastasis unless they undergo clonal expansion and possibly other alterations during the tumor promotion and progression phases of carcinogenesis.

2.2.1.2 Promotion

Promotion is operationally defined as the selective, clonal amplification of the initiated cells into focal proliferations such as papillomas in the skin and urinary bladder, nodules in the liver and polyps in the colon, one or more of which may act as precursors for the development of cancer (Farber and Sarma, 1987). Tumor promoters by definition are not carcinogenic and there is no current implication of any biochemical and/or molecular event(s) associated with them. Many aspects of tumor promotion were first described in the skin model of carcinogenesis in mice. Whereas initiation usually occurs in response to a single brief exposure to a given carcinogen, promotion is dependent on repeated or longterm exposure to the promoting agent to induce focal proliferations in the skin (Berenblurn and Shubik, 1947), liver (Farber and Cameron 1980; Laconi et al., 1997) and other organs (Dragan and Pitot 1992). In comparison to initiation, promotion is a relatively slow process, requiring anywhere from a few weeks to a few months for the generation of grossly visible focal lesions. Further, unlike initiation it is a reversible process, at least in the early stages since most papillomas in the skin, polyps in the colon and nodules in the liver have been shown to regress or redifferentiate into normal looking cells upon withdrawal of the promoter. The fact that initiated cells per se can grow to form focal proliferations, albeit very slowly, suggests that they have a growth advantage over the surrounding normal tissue. However, in the
presence of promoting regimens they grow faster. Thus the fundamental requirement in tumor promotion appears to be the creation of a selective environment or differential, which permits greater amplification of initiated cells over the non-initiated ones (Farber, 1982). Several working hypotheses define how a differential can be achieved for the development of focal proliferations. These include, differential stimulation (Dragan and Pitot, 1992), differential killing, differential recovery (Yuspa and Morgan, 1981), and most pertinent to this dissertation, differential mitoinhibition (Farber, 1982).

2.2.1.3 Progression

Progression is defined as the process through which one or more focal proliferations, such as papillomas, polyps or nodules undergo a slow, but increasingly malignant cellular evolution to cancer without any external stimulus or intervention (Farber and Sarma, 1987). This definition also includes a series of changes involved in the final processes of invasion and metastasis by the neoplasm. Although there is no clear demarcation between where promotion ends and progression begins, in the rat liver, this process is commonly characterized by the appearance of nodules within nodules (Farber, 1982) with cancer eventually occurring inside these nodules (Solt et al., 1977). This material continuity between the nodules and the end point cancer establishes that hepatic nodules are one precursor lesion for hepatocellular carcinoma. During progression several important changes occur within the persistent nodules. Foremost, there is an increase in the proliferating cell population and an increased rate of cell loss. However, the overall balance favours cell proliferation, thus accounting for a slow rate of enlargement of the nodules (Rotstein et al., 1986). The basis for progression is not clearly known at either the biochemical and/or the molecular level. However, production of growth factors, chromosomal changes, genetic instability hormones and dietary influences are all postulated to play an
important role during progression (Dragan and Pitot, 1992). One of the challenges presented by the carcinogenic process is to understand the mechanism by which initiated hepatocytes grow to form focal proliferations, foci and nodules. Several experimental models have been designed to answer this question. These models offered different perspectives and contributed to an understanding of the carcinogenic process.

2.2.2 Causative Factors of Hepatocellular Carcinoma (HCC)

The distribution pattern of HCC shows geographical variation and its pathogenesis is multifactorial. Environmental, infectious, nutritional, metabolic and endocrine factors are contributed directly or indirectly to hepatocarcinogenesis. The role in carcinogenesis of prolonged cellular damage such as viral or bacterial infection related chronic inflammations has become widely recognised. Major risk factors for HCC include cirrhosis, Albino exposure, hepatitis B virus (HBV) and hepatitic C virus (HCV) infection (Byeongwoo et al., 1999). Hepatocellular carcinoma is a major problem not only in developed countries but also in most underdeveloped countries. Since liver is the major site in the body that metabolises ingested material, it is more susceptible to carcinogenic insult. Moreover, due to the high tolerance of liver, hepatocellular carcinoma is seldom detected at the early stage and once detected treatment has a poor prognosis in most cases.

2.2.2.1 HCC and Cirrhosis

Hepatitis is associated with liver cell necrosis, inflammation, regeneration and fibrosis, which may proceed to cirrhosis. Chronic hepatitis is characterised by repetitive cycles of necrosis and regeneration, which facilitate successive acquisition of genomic alterations. These may escape repair mechanisms, which ultimately lead to the development of HCC through monoclonal expansion (Ueno et al, 2001).
2.2.2.2 **HCC and Infectious Hepatitis B and C Virus**

Epidemiological studies have convincingly demonstrated that chronic infection with hepatitis B virus (HBV) is a major risk factor for the development of HCC. Integration of HBV-DNA into cellular DNA of HCC and the development of chronic hepatitis are frequently encountered and are considered to be a hallmark of HBV-related hepatocarcinogenesis. In HBV mediated HCC, first the viral DNA insertion occurs during liver cell proliferation, secondary to necrosis or apoptosis of adjacent hepatocytes and may induce chromosomal rearrangements, including deletion and translocation (Rocken and Mc Grath, 2001). Despite, the genome of human HBV does not contain oncogenes and it may probably exert its effect on hepatocarcinogenesis through trans-activation of HBV related gene products (Rocken and Mc Grath, 2001). Approximately 20% of hepatitis C Virus develops HCC, whereas 5% in the case of HBV. Patients suffering from chronic hepatitis C have increased risk of HCC to 2.7 fold compared with patients suffering from chronic hepatitis B (Colombo, 1999). Generally, inflammation in chronic viral hepatitis is related to clearance of hepatocytes by cytotoxic T cells and mononuclear cells. During inflammation, hepatocytes may be exposed to genotoxic agents such as oxygen radicals, and perforin leading to DNA damage, which initiate hepatocellular carcinoma (Rocken and Mc Grath, 2001).

2.2.2.3 **Hereditary Liver Disease**

Individuals with hereditary diseases are reported to be at high risk for HBV and HCV infection, which may further increase their risk for developing HCC. The development of cirrhosis is a feature of many hereditary disorders that affect the liver, such as hemochromatosis, α1-antitrypsin deficiency, type 1 glycogen storage disease, hypercitrullinemia, porphyria, tyrosinemia and Wilson’s disease (Polio et al., 1989).
2.2.2.4 Ethanol Ingestion

Habitual alcohol consumption plays a prominent role in causes of HCC. It is possible that habitual alcohol intake accelerates the development of cirrhosis and increases the risk for HCC in hepatitis B surface antigen (HBsAg) carriers. Reports reveal that 100% of patients with alcoholic cirrhosis and HCC had HBV detected in neoplastic liver cells (Brechot et al., 1982).

2.2.2.5 Exogenous Hormonal Intake

The association between prolonged use of oral contraceptives and hepatic adenoma is well established (Greer, 1989). Prolonged use of long term oral contraceptives for ten years have an increased risk of developing HCC (Palmer et al., 1989). The putative role for androgenic hormones increasing the risk for HCC has been used to explain the high male to female ratio of HCC in high and low incidence populations (Di Bisceglie, 1989).

2.2.2.6 Obesity

Obesity - defined as a body mass index (BMI) ≥ 30 - has become a worldwide epidemic. In USA the proportion of obese people in the population reaches even 30% (Low et al., 2009). Recently several epidemiological studies have confirmed obesity as an independent risk factor for different cancers like endometrium cancer, breast cancer or colon cancer (Qian and Fan, 2005). There are also epidemiological data, which suggest a role of obesity in incidence and mortality of hepatocellular carcinoma (Wolk et al., 2001). The key link between obesity and liver cancer may be non alcoholic fatty liver disease (NAFLD). NAFLD is a clinicopathological term that comprises a disease spectrum ranging from fat accumulation in hepatocytes (fatty liver, hepatic steatosis) to hepatic steatosis with inflammation (non alcoholic steatohepatitis, NASH), fibrosis and cirrhosis (Browning and Horton, 2004). The prevalence of NAFLD in developed countries is 20 – 30%; 10 % percent of these cases suffer from the more
severe form NASH (Yeh and Brunt, 2007). The prevalence of steatosis in obese people is estimated between 65% and 75% and is correlated with an increase of BMI (Preiss and Sattar, 2008). The relevance of obesity and associated NAFLD in hepatocarcinogenesis was demonstrated in a recent study demonstrating that 13% of HCC patients suffered from NAFLD (Marrero et al., 2002).

2.2.2.7 Chemicals

Extensive evidence of chemical carcinogenesis has come from studies of people whose occupations bring them into contact with various substances (Table II). Epidemiological and experimental evidence indicate that exposure to chemicals also contributes to the development of HCC. Hepatotoxic chemical may be divided into genotoxic and nongenotoxic. Genotoxic chemicals directly interact with DNA, forming covalent adducts and induces genetic changes upon cell replications viz (N-nitrosodiethylamine (NDEA), N-nitrosodimethylamine (NDMA), DAB (Dimethlyaminoazobenzene), Benzopyrene, 2-FAA (acetylaminofluorene), NMU (Nitrosomethylurea), Albino (AFB1).

Non-genotoxic chemicals viz (CCl4, Phenobarbital, phorbor ester, Dichlorodiphenyltrichloroethane (DDT) stimulate tumour formation by altering kinetics of cell proliferation, cell death, and cell differentiation through a variety of epigenetic pathways (Wogan, 2000). The early liver lesion in animals is one of the periportal necrosis proceeding to fibrosis and at a later stage liver tumour occurs (Murray Lyon, 1983). Recent studies have suggested that half of the HCC found in China and South Africa have guanine-thymidine mutation in the third nucleotide on codon 249 at the p53 allele, a tumour suppressor gene (Hsu et al., 1991). This type of DNA base change has been associated with high Albino ingestions (Haydon and Hayes, 1995).
Table: 2.2 Examples of occupational exposure to chemicals that cause cancer (Tomatis, 1990)

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Chemical</th>
<th>Site/Type of Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insecticide spraying</td>
<td>Arsenic</td>
<td>Skin</td>
</tr>
<tr>
<td>Nickel refining</td>
<td>Nickel</td>
<td>Paranasal sinuses</td>
</tr>
<tr>
<td>Chromium plating</td>
<td>Chromium</td>
<td>Lung</td>
</tr>
<tr>
<td>Shale oil production</td>
<td>Polynuclear aromatic hydrocarbons</td>
<td>Scrotum</td>
</tr>
<tr>
<td>Gas retort work</td>
<td>2-Napthylamine</td>
<td>Bladder</td>
</tr>
<tr>
<td>Tyre manufacture</td>
<td>Benzene</td>
<td>Leukaemia</td>
</tr>
<tr>
<td>Vinyl chloride production, N-nitrosodiethlyamine production</td>
<td>Vinyl chloride, N-nitrosodiethlyamine</td>
<td>Liver</td>
</tr>
</tbody>
</table>

2.2.2.8 **Genetics of Hepatocellular Carcinoma**

Cancer proceeds through accumulation of mutations in the genes that govern cell proliferation and death, and it is believed that this process accounts for hepatocarcinogenesis. Thus, the absence of an obviously inherited predisposition to liver cancer has hampered the identification of specific critical genes in hepatocarcinogenesis. HCC shows various genomic alterations, including DNA rearrangements, loss of heterozygosity, chromosomal amplification and loss of imprinting and mutations. Different genes have been implicated in the pathogenesis of HCC, and may be divided into four major groups such as genes regulating DNA damage response (p53 pathway-p53), genes involved in cell cycle control (RB1 pathway-RB1, P16 INK4A, Cyclin D), genes involved in growth inhibition and apoptosis (TGF-β pathways – M6P/1GF2R, SMAD2, SMAD4) and genes responsible for
cell-cell interaction and signal transduction (APC/ β- catenin pathway-APC, E-cadherin) (Rocken and Mc Grath, 2001).

2.2.3 Signs and symptoms

HCC may present with jaundice, bloating from ascites, easy bruising from blood clotting abnormalities or as loss of appetite, unintentional weight loss, abdominal pain, especially in the upper – right part, feeling very full after a small meal, an enlarged liver, felt as a mass under the ribs on the right side, itching, swelling or fluid build-up in the abdomen, Enlarged veins on the belly that become visible through the skin, Yellowing of the skin and eyes, nausea, emesis, or fatigue.

2.2.4 Pathology of Hepatocellular carcinoma

The gross and microscopic anatomical features of HCC have been widely recognized and described in detail, from autopsy specimens and more recently in hepatectomy specimens (Ishak et al., 1994). Grossly, three main forms of HCC are encountered such as the massive, multinodular, and diffuse. The massive type of HCC is mostly seen in the upper part of the right lobe of the liver, rarely in the left lobe and has several satellite tumour nodules of variable sizes close to or somewhat away from the main mass. In multinodular HCC, several sharply demarcated, relatively rounded tumour nodules of variable sizes are widely dispersed over a large part of the liver. It occurs again commonly in the right lobe but without a large tumour mass. The diffuse form of HCC on the other hand, has small ill-defined nodules of tumour tissue distributed diffusely throughout the liver lobes. The colour and consistency of tumour is similar to those of the background (Nayak, 2003).

2.2.4.1 Microscopic identification of HCC

Several microscopic patterns have been observed. The commonest pattern is the trabecular (plate-like/sinusoidal) in which, tumour cells are arranged in trabeculae or plates. The plates are thick cell layer and are
separated by irregular, variably wide sinusoidal blood spaces, flat endothelial cells (Nayak, 2003). Fibrous tissue is not seen in between or within the trabeculae but may be present between tumour nodules or when the tumour is invading portal tracts or the capsule. The next frequent pattern is the pseudo glandular, adenoid, or acinarin, which as the name indicates tumour cell plates are arranged around central space. This space is empty or has a granular or light staining hyaline material, all of which result from degeneration and slow disappearance of the centrally placed tumour cells. The rare pattern is the compact or solid pattern, which the tumour cells are present in sheets (Nayak, 2003).

2.2.4.2 Macroscopic identification of HCC

The tumour may be a solitary mass, multiple nodules, or may diffusely infiltrate the liver. There is a tendency for nodules to become necrotic centrally. The liver harbouring the tumour will usually be cirrhotic. The right lobe is more commonly affected than the left. There may be infraction of tumour, haemorrhage (or) rapture (Ryley et al., 1996).

2.2.5 Diagnosis of hepatocellular Carcinoma

Liver Function Tests and others – Tumour cells have certain properties that help to distinguish them from normal cells for example; some tumour cells show similarity to embryonic cells. These tumours make certain proteins, which are similar to those, made during embryonic development. The occurrence of such proteins in patient’s blood is used to diagnose different cancers. AFP (alpha fetoprotein) is a diagnostic test for liver cancer. There are tumour marker enzymes viz, serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvate transaminase (SGPT), lactate dehydrogenase (LDH), 𝛾- glutamyl transpeptidase (GGT) and 5’-nucleotidase are often elevated. Isoenzymes of alkaline phosphatase like variant alkaline phosphatase (ALP) (Portugal et al., 1970) and increased
level of vitamin B<sub>12</sub> binding protein, serum carcino embryonic antigen and human chorionic gonadotrophin are associated with complication in HCC patients (Murry-Lyon, 1983). As association between elevated serum AFP levels and HCC, measurement of serum AFP levels has been the most useful laboratory test to suggest a diagnosis of HCC in a patient with a liver mass. More recently, des γ-carboxy prothrombin (DCP) has been shown to be tumour specific in human HCC (Okuda, 1990). Apart from that some other parameters are useful to diagnose the liver cancer like Ultrasound, Computed tomography (CT), Magnetic resonance imaging (MRI), Angiography, Bone scan, Laparoscopy, Biopsy (Needle biopsy, Laparoscopic biopsy, Surgical biopsy), Blood clotting tests, Tests for viral hepatitis, Kidney function tests, Complete blood count (CBC) and Electrolytes and blood chemistry tests.

2.2.6 Prevention

Since hepatitis B or C is one of the main causes of hepatocellular carcinoma, prevention of this infection is key to prevent hepatocellular carcinoma. Thus, childhood vaccination against hepatitis B may reduce the risk of liver cancer in the future. In the case of patients with cirrhosis, alcohol consumption is to be avoided. Also, screening for hemochromatosis may be beneficial for some patients.

2.2.7 Treatment and management of HCC

Inspite of the spectacular advances made by medical sciences during the present century, treatment of cancer remains an enigma. What makes neoplastic cells stop growing is a question still unanswered in the medical world. Cancer treatment has evolved greatly in last decades where improved therapies have managed to increase the survival rates to greater than 50% of diagnosed patients. Besides, a much better knowledge of different novel strategies based on it, is still the most efficient ways for treatment than the classical methods such as surgery, radiotherapy and chemotherapy. It has
been proposed that the clinical efficacy in cancer prevention and therapy depends on their ability to modulate cell growth differentiation and apoptosis in premalignant and malignant cells by regulating gene expression (Lotan, 1995). The development of very expensive new technology for treating cancer like surgery, intensive chemotherapy, endocrine, immunotherapy, gene therapy and inhibition of angiogenesis and radiation therapy with appropriate supportive drugs including analgesics, antibiotics, and blood products probably will improve survival in some patients, but at a substantial increase in health care costs, patients morbidity and disability will have little effect on overall cancer incidence (Reizeustein et al., 1994). Cancer cells cannot be killed easily with chemical drugs, because cancer cells are cells of our own body (only the regulatory mechanisms have gone haywire). Hence, it is much more difficult to prepare drugs that will selectively kill cancer cells without harming the normal cells. Therefore, it becomes very important to detect a cancer early enough when it is localised and has not formed metastasis. In that case, it can be removed completely by surgery. Radiation therapy and chemotherapy are considered to be treatments of second choice except in some specific cases where they are preferred.

2.2.7.1 Surgery

It is most effective and the oldest form of single treatment of cancer. The most crucial limitation of surgery is that this method cannot treat wholly a localized cancer or that has metastasized to different, distant and various organ systems, widely throughout the body. Surgery is of seldom value for hepatic metastases (Murry-Lyon, 1983). The use of X-ray and gamma ray radiation to treat disease often used alone or in combination with surgery to eradicate malignant tumours is another trend.

2.2.7.2 Liver transplantation

To replace the diseased liver with a cadaveric liver or a living donor graft. Historically low survival rates (20%-36%). During 1996–2001 the rate
had improved to 61.1%, likely related to adoption of the Milan criteria at US transplantation centers (Fan et al., 2009). Expanded Shanghai criteria in China resulted in overall survival and disease-free survival rates similar to the Milan criteria. Studies from the late 2000 obtained higher survival rates ranging from 67% to 91%. If the liver tumor has metastasized, the immunosuppressant post-transplant drugs decrease the chance of survival. Considering this objective risk in conjunction with the potentially high rate of survival, some recent studies conclude that: "LT can be a curative approach for patients with advanced HCC without extrahepatic metastasis" (Obed et al., 2009). For those reasons, and others, it is considered nowadays that patient selection is a major key for success.

2.2.7.3 Radiofrequency ablation (RFA)

It uses high frequency radio-waves to destroy tumor by local heating. The electrodes are inserted into the liver tumor under ultrasound image guidance using percutaneous, laparoscopic or open surgical approach. It is suitable for small tumors (<5 cm). A large randomised trial comparing surgical resection and RFA for small HCC showed similar 4 years-survival and less morbidities for patients treated with RFA (Chen et al., 2006).

2.2.7.4 Focused External Beam Radiation

Stereotactic Radiotherapy (SRT) is a technique of using highly focussed radiation to small target volume. SRT has been tried successfully in the liver for treatment of metastases, and currently clinical studies are underway to evaluate its efficacy in treating Hepatocellular Carcinoma. The early results are promising. With the advent of modern computer technology, it is now possible to treat directly to affected areas of the liver, while sparing normal healthy liver tissue.

2.2.7.5 Selective internal radiation therapy

This can be used to destroy the tumor from within (thus minimizing exposure to healthy tissue). There are currently two products available, SIR-
Spheres and TheraSphere The latter is an FDA approved treatment for primary liver cancer (HCC) which has been shown in clinical trials to increase survival rate of low-risk patients. SIR-Spheres are FDA approved for the treatment of metastatic colorectal cancer but outside the US SIR-Spheres are approved for the treatment of any non-resectable liver cancer including primary liver cancer. This method uses a catheter (inserted by a radiologist) to deposit radioactive particles to the area of interest.

2.2.7.6 Cryosurgery

Cryosurgery is a new technique that can destroy tumors in a variety of sites (brain, breast, kidney, prostate and liver). Cryosurgery is the destruction of abnormal tissue using sub-zero temperatures. The tumor is not removed and the destroyed cancer is left to be reabsorbed by the body. Initial results in properly selected patients with unresectable liver tumors are equivalent to those of resection. Cryosurgery involves the placement of a stainless steel probe into the center of the tumor. Liquid nitrogen is circulated through the end of this device. The tumor and a half inch margin of normal liver are frozen to -190°C for 15 minutes, which is lethal to all tissues. The area is thawed for 10 minutes and then re-frozen to -190°C for another 15 minutes. After the tumor has thawed, the probe is removed, bleeding is controlled, and the procedure is complete. The patient will spend the first post-operative night in the intensive care unit and typically is discharged in 3 – 5 days. Proper selection of patients and attention in performing the cryosurgical procedure are mandatory in order to achieve good results and outcomes. Frequently, cryosurgery is used in conjunction with liver resection as some of the tumors are removed while others are treated with cryosurgery. Patients may also have insertion of a hepatic intra-arterial catheter for post-operative chemotherapy. As with liver resection, the surgeon should have experience with cryosurgical techniques in order to provide the best treatment possible.

2.2.7.7 Cancer chemoprevention
Chemotherapy can affect cure of certain cancers and may provide palliation for others. The classification of the conventional chemotherapeutic agents is listed below in table 2.3 (Goodman and Gilman, 2001).

Epidemiological studies indicate that approximately 80% of human cancer is caused by exposure to chemical carcinogens in tobacco smoke, in the diet or in the work place (Manfred, 1980). Based on this observation, three approaches to the prevention of cancer can be envisioned. First, reduce human exposure to environmental carcinogens through careful monitoring of the work place and through education approaches to encourage changes in life style. Second, identify individuals at high risk for cancer development through predisposing genetics or biochemical factors, followed by appropriate clinical follow up. Third, provide chemoprevention by dietary or synthetics means. The use of chemotherapy to treat cancer began in 1943 following the observation of leukopenia (reduction in number of leukocytes) in military personnel exposed to mustard gas after explosion of a battleship in Bari harbour. Cancer chemoprevention has been defined as a process facilitated by blocking induction of neoplastic process or preventing transformed cells from progression to malignant phenotypes (Sporn and Hong, 1997) by administration of one or more chemical entities, either as individual drugs or naturally occurring constituents of the diet.

Chemotherapy is the standard and accepted treatment tool for cancer, alone or in conjunction with elective surgery and radiation, as the case may be. Chemotherapeutic drugs are cytotoxic by design, and thus most of the formulation drugs that are available can effectively kill the cancerous cell, they also damage the DNA of normal cells and are able to cause serious dose limiting adverse effects at therapeutics doses. The side effects include hematopoietic toxicity, hepatotoxicity, gastrointestinal toxicity, gonadal toxicity, mouth toxicity, teratogenicity, cardiac toxicity and nephrotoxicity.
Other side effects include nausea, vomiting, mutagenicity and carcinogenicity. In this way methotrexate inhibits dihydrofolate reductase, thereby limiting synthesis of reduced folate, which is necessary for production of purine and pyrimidines. Similar agents include 5FU (fluorouracil) and cytarabine. Some drugs cause structural damage to mature DNA, as exemplified by alkylating agents (cyclophosphamide, chloroambucil and procarbazine) and platinum derivatives (cisplatin and carboplatin). Functioning of mitotic spindle is variously affected by vinca alkaloids (vincristine and vinblastin) and the taxanes (paclitaxel and related compounds). Adequate therapeutics interventions may reduce the overall toxicity mediated by classical chemotherapeutic agents and thereby improve their efficacy. Since the pioneering work of Wattenberg (1966), a number of experiments with humans in the early stages of the drug discovery process, under all circumstances, even those in which the loss of human life is near certain, model test systems come into play. As a result, we rely on model systems to provide an indication of potential human efficacy. Accordingly, it is certainly legitimate to scrutinize the validity of model systems, and a great deal of work is required to devise suitable model systems. Besides ultimate test system, the human, animal models have traditionally been regarded as being capable of providing the most salient indication of human efficacy. The approaches have developed for the discovery of natural product cancer chemopreventive agents is distinctive (Pezzuto, 1995).

The drugs, which are most often used to treat liver cancer, are doxorubicin, cisplatin, methotrexate and 5FU (fluorouracil). Thus, an ideal drug is one, which should be highly selective for tumour tissue in that it can kill or incapacitate tumour cells, while not affecting normal tissues.
### Table 2.3 Chemotherapeutic agents used in Neoplastic disease

<table>
<thead>
<tr>
<th>CLASS</th>
<th>TYPES OF AGENTS</th>
<th>NONPROPRIETARY NAMES (OTHER NAMES)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alkylating agents</strong></td>
<td>Nitrogen mustard</td>
<td>Mechlorethamine</td>
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<tr>
<td></td>
<td></td>
<td>Cyclophosphamide, Ifosfamide, Melphalan (L-sarcolysin), Chlorambucil</td>
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<tr>
<td></td>
<td>Ethylenamines and methylmelamines</td>
<td>Hexamethylmelamine</td>
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<td></td>
<td></td>
<td>Thiotepa</td>
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<td></td>
<td>Nitrosoureas</td>
<td>Lomustine (CCNU)</td>
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<td></td>
<td></td>
<td>Semustine (methyl-CCNU)</td>
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<td></td>
<td></td>
<td>Streptozocin (streptozotocin)</td>
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<tr>
<td></td>
<td>Triazenes</td>
<td>Dacarbazine (DTIC., dimethyltriazenoimid azolecarboxamide)</td>
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<td></td>
<td>Alkyl Sulfonate</td>
<td>Busulfan</td>
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<tr>
<td><strong>Antimetabolites</strong></td>
<td>Folic acid analogs</td>
<td>Methotrexate (amethopterin)</td>
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<td></td>
<td>Pyrimidine analogs</td>
<td>Fluorouracil (5-fluorouracil, 5-FU)</td>
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<td></td>
<td></td>
<td>Flouxuridine (fluorodeoxyuridine., FUdR) cytarabine (cytosine arabinoside)</td>
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<tr>
<td></td>
<td>Purine analogs and related inhibitors</td>
<td>Mercaptopurine (6-mercaptopurine., 6-MP), Thioguanine (6-thioguanine, TG), Pentostatin (2'deoxycoformycin)</td>
</tr>
<tr>
<td><strong>Natural products</strong></td>
<td>Vinca alkaloids</td>
<td>Vinblastine (VBL)</td>
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<td></td>
<td></td>
<td>Vincristine</td>
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<tr>
<td></td>
<td>Epipodophyllotoxin</td>
<td>Etoposide teniposide</td>
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<td></td>
<td>Antibiotics</td>
<td>Dactinomycin (actinomycin D)</td>
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<td></td>
<td></td>
<td>Daunorubicin (daunomycin,rubidomycin)</td>
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<td></td>
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<td>Bleomycin</td>
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<td></td>
<td></td>
<td>Plicamycin (mithramycin)</td>
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<td></td>
<td></td>
<td>Mitomycin (mitomycin C)</td>
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<tr>
<td></td>
<td>Enzymes</td>
<td>L-Asparaginase</td>
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<tr>
<td></td>
<td>Taxanes</td>
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<td></td>
<td></td>
<td>Paclitaxel</td>
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<td><strong>Immunomodulating</strong></td>
<td>Interferons</td>
<td>Interferon (α-2a)</td>
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<td>Interferon (α-2b)</td>
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### Table of Agents

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<th>Category</th>
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<th>Aldesleukin</th>
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</thead>
<tbody>
<tr>
<td><strong>Miscellaneous agents</strong></td>
<td>Platinium co-ordination complexes</td>
<td>Cisplatin (cis–DDP)</td>
</tr>
<tr>
<td></td>
<td>Anthracenedione</td>
<td>Mitoxantrone</td>
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<td></td>
<td>Substituted urea</td>
<td>Hydroxyurea</td>
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<td></td>
<td>Methylhydrazine derivative</td>
<td>Procarbazine (N-methylhydrazine, MIH)</td>
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<tr>
<td></td>
<td>Adrenocortical suppressant</td>
<td>Mitotane (O, P'-DDD)</td>
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<tr>
<td></td>
<td></td>
<td>Aminoglutethimide</td>
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<tr>
<td><strong>Hormones and antagonists</strong></td>
<td>Adrenocorticosteroids</td>
<td>Prednisone</td>
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<tr>
<td></td>
<td>Progestins</td>
<td>Hydroxyprogesterone caproate</td>
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<td></td>
<td></td>
<td>Medroxyprogesterone acetate</td>
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<td></td>
<td></td>
<td>Megestrol acetate</td>
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<td></td>
<td>Estrogens</td>
<td>Diethyl stilbestrol</td>
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<td></td>
<td></td>
<td>Ethinyl estradiol</td>
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<td></td>
<td>Antiestrogens</td>
<td>Tamoxifen</td>
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<tr>
<td></td>
<td>Androgens</td>
<td>Testosterone propionate</td>
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<td></td>
<td></td>
<td>Fluoxymesterone</td>
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<tr>
<td></td>
<td>Antiandrogens</td>
<td>Flutamide</td>
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<tr>
<td></td>
<td>Gonadotropin releasing hormone analog</td>
<td>Leuprolide</td>
</tr>
</tbody>
</table>

### 2.2.8 Experimental hepatocarcinogenesis

#### 2.2.8.1 Background

The liver has received considerable attention as a target for chemical carcinogenesis since the discovery by Sasaki and Yoshida of liver cancer induction with o-amino-azotoluene in 1935. Because of its susceptibility to cancer induction under a variety of conditions and because of a large and growing body of knowledge about its cellular and molecular biology and pathology, the liver has been examined many times with many carcinogens for biochemical, physiological and morphological alterations as a function of time during the development of cancer. Such studies have consistently found many cellular and tissue changes involving hepatocytes and other types of cells in the liver before the development of unequivocal hepatocellular
carcinoma, the commonest form of liver cancer. Carcinogenesis with chemicals appears to consist of a series of episodes or sequences each one consisting of a discrete discontinuous change, the rare event, followed by the selective growth of one or more types of altered cells. In solid organ, such as liver, this produces ‘nodules within nodules’, an indication of the continuing development of neoplasia (Emmanuel Farber, 1980)

2.2.8.2 N-nitrosocompound as hepatocarcinogens

A variety of undesirable compounds may be present in foods consumed by human being. These may range from the naturally occurring cyanogenic glycosides to contaminants such as pesticide residues or nonessential trace elements (e.g Pb, Cd). The presence of mycotoxin (produced by molds), marine toxins (produced by microorganisms), and polynuclear aromatic hydrocarbons (produced as a result of food processing, e.g., roasting etc.) can be cited as further examples of potentially harmful substances that have shown to be present in certain foods of both animals and plant origin (Concon, 1988). In 1937, Freund reported (Freund, 1937) two cases of toxic parenchymatous hepatitis in chemists who were preparing dimethylnitrosamine (DMN). Nitrosamines are powerful hepatotoxin and hepatocarcinogens and they have long been the focus of intense interest among scientist and lay public (Sharpley, 1976) concern for some years as contaminants of food. Magee and Barnes discovered DMN to be a powerful hepatocarcinogen in rats that were being studied for chronic toxicity. All the animals under study developed liver tumours in 26-40 weeks (Manfred, 1980). This observation opened up an entirely new class of chemical carcinogens, the N-nitroso compounds. There have been two reasons for concern. Firstly, a majority of these compounds are potent carcinogens in laboratory animals. Second, many of the NOCs occur at trace levels in a wide variety of foods and beverages. N-nitroso compounds can be divided
into two major categories: nitrosamines and nitrosamides. Nitrosamines fall into three subcategories depending on the nature of the R-groups attached to the N-nitroso moiety. As increasing numbers of these N-nitroso compounds are synthesized and tested for carcinogenicity, it becomes apparent that there are wide variations in their carcinogenic specificity in terms of species and tissue susceptibility and the degree of carcinogenic potency. The reasons for this variance most likely lie in the relative chemical stability of the compounds under physiological conditions (Mirvish, 1975) and the level and inducibility of the metabolic activation enzymes in the tissue being tested.

Nitrosamines require metabolic activation by the microsomal mixed function oxidases and require oxygen and reduced pyridine nucleotides as cofactors. Unlike Polycyclic hydrocarbon (PAH), nitro amines require only one activation step to form a hydroxylated intermediate, which by itself is sufficiently unstable chemical to rapidly decompose and generate the reactive carbonium, which rapidly alkylates target macromolecules in the cell. In contrast, the nitrosamides (e.g., nitrosomethylurea) do not require metabolic activation. This is due to inherent chemical instability in aqueous solution.

Nitrosamides decompose non enzymatically at physiological pH to produce the same class of reactive electrophiles as the nitrosamines. This marked difference in chemical stability between nitrosamines and nitrosamides clearly parallels the in vivo activity of these compounds. Since nitrosamines require metabolic activation, they persist in the body for relatively long periods, where they are carried by the circulation to the liver, yielding severe hepatotoxicity and tumourigenicity with relatively little pathology at the injection site. However nitrosamides cause severe toxicity at the application site because of their almost immediate decomposition to the
reactive carbonium ion. Therefore they cause relatively little hepatotoxicity (Manfred, 1980).

A high degree of organ specificity of many N-nitroso compounds suggest that the relative susceptibility of a given tissue is most likely a function of the ability of the cellular enzymes to activate the compound for decomposition in the proximity of target site for malignant transformation. Kennaway, (1994) impressed by report of experimental hepatocarcinogenicity of butter yellow and α-aminoazotoluene and intrigued by the epidemiology of cancer of liver in humans, suggested that environmental carcinogens might play an important role in the etiology of the neoplasm.

2.2.8.3 N-nitrosodiethylamine (NDEA) as a model in experimental hepatocarcinogenesis

The basic goal in developing experimental tumour is to simplify and stimulate major factor affecting human cancer leading to test the tumour inducing potential of chronic tissue damage (Byeongwoo et al., 1999). Hepatocarcinogenesis induced by various carcinogens is a multistep and complex process and is a favoured model in the rat that facilitates the study of the mechanism of transformation from a normal cell to a malignancy (Mitali Basu et al., 2004). The liver is often the first organ to be affected by metastasizing cancer (Mitali Basu et al., 2004). N-nitrosodiethlyamine (NDEA), also known as diethlynitrosamine or DEN, is slightly yellow liquid with a boiling point of 175-177°C and a specific gravity of 0.94. It is soluble in water, ethanol, diethyl ether, and organic solvents. An NDEA model was used because nitrite and nitrosamine synthesis is increased in viral hepatitis (Liu et al., 1992).

Therefore, an NDEA model may be useful as a model of hepatocarcinogenesis due to viral hepatitis. DEN has found widespread use
as an experimental model in the carcinogenesis process and in chemoprevention (Lee and Lee, 1999). Diethylnitrosamine (DEN) is a well-known liver carcinogen in rats, forming DNA adducts in the liver and inducing hepatocellular carcinoma without cirrhosis through the development of putative preneoplastic enzyme-altered focal lesions (Mitali Basu et al, 2004).

Chemical induction of hepatic carcinoma may be regarded as a special form of hepatotoxicity that is mediated by alteration of the informational molecules of the hepatocytes. It has been the subject of intense interest for a half century. A major part of that period chemically induced hepatic carcinoma appeared to be of greater interest as a model of carcinogenesis in general than as usefully relevant to liver disease of human. However, with the demonstration of potent natural and man made hepatocarcinogens in the environment (Bannasch and Zerban, 1986) it has created widespread interest in experimental hepatocarcinogenesis as a model of hepatic carcinoma in humans. Hepatic chemical carcinogenesis is a multistep process in experimental animals (Bannasch et al, 1980). Carcinogens initiate the process, which is followed by regenerative growth and clonal proliferation, eventually leading to preneoplastic foci and carcinoma. NDEA has been found in a variety of products that would result in human exposure, including mainstream and sidestream tobacco smoke (Hoffman et al, 1980), meat and whiskey (Sen et al, 1980). Diethylnitrosamine (DEN) is a widely occurring nitrosamine which is present in tobacco and various processed foods. These nitroso compounds can also be formed in vivo in physiological conditions (Coker et al, 1991). Mechanism that have been proposed to play roles in NDEA carcinogenicity in hepatocytes include DNA adduct formation followed by gene mutation, cytolethality following regenerative proliferation and oxidative stress or damage by impairment of mitochondrial respiration by free radicals (Byeongwoo et al, 1999).
The biotransformation of NDEA and mechanism of DNA-adduct formation has been illustrated that nitrosamine require only one activation step to form a hydroxylated intermediate, it is sufficiently unstable chemically to rapidly decompose and generate the reactive carbonium and it rapidly alkylates target macromolecules in the cell. The activation pathway begins with the hydroxylation occurring at the $\alpha$-carbon forming a labile hydroxyl group having a proton that is attracted by the nitroso oxygen. This is most likely to result in a concerted shift that produces an unstable diazohydroxide and eliminates an aldehyde. The diazo intermediate in turn forms an alkyl diazo salt that decomposes to molecular nitrogen and the carbonium ion that serves as the active alkylating group, which interacts with cellular target sites and causes tumorigenesis.

**Fig. 2.5** Mechanism for the Activation of DEN (Manfred, 1980)
Steps involved in mechanism of action of N-nitrososdiethylamine-induced hepatocellular carcinoma.

Nitrosamine forms a hydroxylated intermediate, which is chemically unstable and generates the reactive carbonium, which alkylates target molecule in the cell.

- Activation begins with the hydroxylation occurring at the $\alpha$- carbon forming labile hydroxyl group having a proton which is attracted by nitroso oxygen (Step-I).
- Due to the concerted shift an unstable diazohydroxide and an aldehyde are produced (Step-II).
- The diazo intermediate forms an alkyl diazo salt that decomposes to molecular nitrogen and the carbonium ion (Step-III).
- Carbonium ion that serves as the active alkylating group (Step-IV) interaction with cellular targets and result in tumorigenesis.

2.2.8.4 Liver tumour promoters

The rodent liver has been used as an animal model of carcinogenesis since 1930s and the multi stage nature of hepatocarcinogenesis has been demonstrated with various initiators and promoters since the pioneering work of (Peraino et al, 1984). Extensive research in experimental animals during the past 3 decades has shown that chemical carcinogenesis frequently involves separate steps on initiation and promotion. Numerous studies have been carried out during the past 30 years on basic mechanisms operative in carbon tetrachloride hepatotoxicity. Carbon tetrachloride is a classic hepatotoxin, and it illustrates broad aspects of hepatotoxicity. It provides the prototype for several toxic phenomena. Potent hepatotoxicity agents that lead to zonal necrosis, agents that lead to renal and hepatic injury (Hardin, 1954) and agents that lead to hepatic injury mainly by a directly destructive effect.
of free radical metabolite that lead to peroxidation and other physicochemical damage to hepatocytes membrane (Zimmerman, 1968).

Carbon tetrachloride has been the subject of study during most of this century. During the first quarter of this century carbon tetrachloride was found to produce hepatic injury in human and laboratory animals (Meyer and Pessoa, 1993). These studies have been comprehensively reviewed (Recknagel and Glende, 1988) that led to widely accepted view that CCl₄ hepatotoxicity depends on the reductive dehalogenation of CCl₄ catalyzed by cytochrome P-450 in the liver cell endoplasmic reticulum (ER). It has also become clear that a cascade of secondary mechanisms is evoked by the initial events of CCl₄ metabolism, and that the secondary mechanism is responsible for ultimate plasma membrane disruption and death of the cell (Recknagel and Glende, 1988). CCl₄ causes fatty degeneration of the liver and centrilobular necrosis. CCl₄ is metabolised by the monoxygenase system of endoplasmic reticulum (ER). The key step is a one–electron reductive dehalogenation catalyzed by a specific isoenzyme of cytochrome P-450 (Noguchi et al, 1982). Thus, the initial reductive dehalogenation of CCl₄ catalyzed by cytochrome P-450, yields CCl₃, some of which is immediately converted to OOCCl₃, with subsequent rapid formation of a variety of chemically reactive species. In summary, damage occurs due to activation of CCl₄ by hepatic cytochrome P-450 to free radicals, which induce lipid peroxidation, covalent binding to macromolecules and inhibition of the calcium pump of microsomes, leading to liver cell necrosis.

2.2.9 Antioxidant and Disease

It is increasingly being realized that a majority of the present day diseases are due to the shift in the balance of the pro-oxidant and the antioxidant homeostatic phenomenon in the body. Pro-oxidant conditions dominate either due to the increased generation of the free radicals caused by
excessive oxidative stress of the current life, or due to the poor scavenging/quenching in the body caused by depletion of the dietary antioxidants (Dringen, 2000).

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and our metabolism. They are continuously produced by body’s use of oxygen such as in respiration and some cell-mediated immune functions. They are also generated through environmental pollutants, cigarette smoke, automobile exhaust, radiation, air-pollution, pesticides, etc. (Li and Trush, 1994). Normally there is a balance between the amount of free radicals generated in the body and the antioxidant defense systems that scavenge/quench these free radicals preventing them from causing deleterious effects in the body (Nose, 2000).

The antioxidant defense systems in the body can only protect the body when the amount of the free radicals is within the normal physiological level. But when this balance is shifted towards more of free radicals, increasing their burden in the body, either due to environmental condition or due to their production within the body, it leads to oxidative stress, which may result in tissue injury and subsequent diseases (Finkel and Holbrook, 2000). Since free radicals play an important role in the disease scenario of an individual, a thorough understanding of the various physiologically significant free radicals is of paramount importance before the search of the radical scavengers or the antioxidant principles to treat the physiological disorders caused by them.
2.2.10 Review on antitumor against N-nitrosodiethylamine (NDEA) induced- hepatocarcinogenesis

2.2.10.1 Phytoconstituents used in HCC

❖ Glycyrrhizin

Glycyrrhizin showed inhibition of hepatocellular carcinoma in diethylnitrosamine treated mice by showing the normalised morphology of liver tissue and levels of marker enzymes indicating that these offered protection against chemical carcinogenesis (Goshi et al., 1999).

❖ Lycovin

Aqueous extract of Lycovin has been found to be a potent inhibitor of lipid peroxide formation, scavenger of hydroxyl radical and superoxide radical in vitro. Lycovin syrup 1.5 and 7.5 ml/kg body wt administered orally reduced the development of sarcoma induced by 20 MC by 35% and 70% respectively. Lycovin syrup was found to inhibit the hepatocarcinogenesis induced by NDEA. The tumor incidence was 100% in control group. Liver weight, GGT, GST, reduced gluthathione (GSH) and aniline-4-hydroxylase in liver was elevated in NDEA alone treated animals. The serum parameters indicative of liver injury such as bilirubin, lipid peroxides, alkaline phosphatase and glutamate pyruvate transaminase were also elevated by NDEA administration. These elevated levels were significantly reduced in animals treated with lycovin syrup along with NDEA in a dose dependent manner (Joy et al., 1999).

❖ Apigenin

Apigenin, a dietary plant derived flavone subclass of flavonoid play a role in cancer chemoprevention and cancer chemotherapy. Here experiment establish treatment of apigenin (25 mg/kg body weight) for 14 consecutive days to (N-nitrosodiethylamine) DEN induced (200 mg/kg body weight by single ip. injection) and phenobarbital promoted (0.05% through drinking water for 14 successive weeks) rats to provide protection against the
oxidative stress caused by the carcinogen. The level of lipid peroxidation (LPO) increased markedly in carcinogen administered animals, which was brought back to near normal by apigenin treatment. In contrast the activities/levels of the antioxidant status both in liver and kidney were decreased in carcinogen administered animals, and it was recouped back to near normal upon apigenin administration. Apigenin prevents LPO and protects antioxidant system in DEN induced and phenobarbital promoted hepatocellular carcinogenesis (Prince et al., 2004).

**Embelin and curcumin**

The effects of embelin (50 mg/kg/day), a benzoquinone derivative of *Embelia ribes*, and the effects of curcumin (100 mg/kg/day), the active principle of *Curcuma longa*, against N-nitrosodiethylamine (DENA)-initiated and phenobarbital (PB)-promoted hepatocarcinogenesis were studied in Wistar rats. Embelin and curcumin were able to prevent the induction of hepatic hyper plastic nodules, body weight loss, increase in the levels of hepatic diagnostic markers, and hypoproteinemia induced by DENA/PB treatment (Sreepriya and Bali, 2005).

**Silymarin**

The efficacy of silymarin on the antioxidant status of *N*-nitrosodiethylamine (NDEA) induced hepatocarcinogenesis in Wistar albino male rats were assessed. Starting 1 week prior to NDEA administration group animals were treated with silymarin in diet for 16 weeks, 10 weeks after NDEA administration group animals were treated with silymarin and continued till the end of the experiment period (16 weeks). In contrast, silymarin + NDEA treated groups animals showed a significant decrease in the number of nodules with concomitant decrease in the lipid peroxidation status. The levels of GSH and the activities of antioxidant enzymes in both haemolysate and liver were improved when compared with hepatocellular carcinoma induced group 2 animals. The electron microscopy studies were
also carried out which supports the chemopreventive action of the silymarin against NDEA administration during liver cancer progression. Silymarin suppresses NDEA induced hepatocarcinogenesis by modulating the antioxidant defense status of the animals (Ramakrishnan et al., 2006).

❖ **Morin**

Morin (3,5,7,2,4-pentahydroxyflavone), a plant-derived flavonoid belonging to the subclass of flavonol is believed to play a role in chemoprevention and cancer chemotherapy. The cotreatment of morin (500 ppm in diet) for 16 weeks to N-nitrosodiethylamine-induced (200 mg/kg bodyweight in drinking water) rats provides protection against the oxidative stress caused by the carcinogen and thereby prevents hepatocellular carcinogenesis. The morin prevents lipid peroxidation, hepatic cell damage and protects the antioxidant system in N-nitrosodiethylamine-induced hepatocellular carcinogenesis (Sivaramakrishnan et al., 2008).

2.2.10.2 **Plants used in HCC**

❖ **Embilica officinalis**

Extracts of *Embilica officinalis*, significantly inhibited hepatocarcinogenesis induced by N-nitrosodiethlyamine (NDEA) in a dose dependent manner. The anticarcinogenic activity of these extracts was evaluated by their effect on tumor incidence, levels of carcinogen metabolising enzymes, levels of liver cancer markers and liver injury markers. Treatment of extract significantly reduced the drug metabolising enzymes, which was elevated in NDEA treated groups. Levels of liver cancer marker, which was elevated in NDEA treated, were reduced in extract treated group and also in liver injury markers. Morphology of liver tissue and levels of marker enzymes indicated that these extracts offered protection against chemical carcinogenesis (Jose Jeena et al., 1999).


- **Phyllanthus amarus**

  Extracts of *Phyllanthus amarus* significantly inhibited hepatocarcinogenesis induced by N-nitrosodiethlyamine (NDEA) in a dose dependent manner. The anticarcinogenic activity of these extracts was evaluated by their effect on tumour incidence, levels of carcinoen metabolising enzymes, levels of liver cancer markers and liver injury markers. Treatment of extract significantly reduced the drug metabolising enzymes, which was elevated in NDEA treated groups. Levels of liver cancer marker, which was elevated in NDEA treated, were reduced in extract treated group and also in liver injury markers. Morphology of liver tissue and levels of marker enzymes indicated that these extracts offered protection against chemical carcinogenesis (Jose Jeena et al., 1999).

- **Picrorhiza kurroa**

  Extracts of *Picrorhiza kurroa* significantly inhibited hepatocarcinogenesis induced by N-nitrosodiethlyamine (NDEA) in a dose dependent manner. The anticarcinogenic activity of these extracts was evaluated by their effect on tumour incidence, levels of carcinoen metabolising enzymes, levels of liver cancer markers and liver injury markers. Treatment of extract significantly reduced the drug metabolising enzymes, which was elevated in NDEA treated groups. Levels of liver cancer marker, which was elevated in NDEA treated, were reduced in extract treated group and also in liver injury markers. Morphology of liver tissue and levels of marker enzymes indicated that these extracts offered protection against chemical carcinogenesis (Jose Jeena et al., 1999).

- **Phyllanthus amarus**

  The effect of *Phyllanthus amarus* extract administration after induction of hepatocellular carcinoma (HCC) by N-nitrosodiethlyamine (NDEA) was studied in Wistar rats. Administration of an aqueous extract of *P. amarus* was found to significantly increase the survival of hepatocellular
carcinoma harbouring animals. *P. amarus* administration was found to be ineffective in controlling the liver weight, elevation of tissue GGT, serum alkaline phosphatase and serum glutamate pyruvate transaminase of HCC harbouring animals (*Rajeshkumar and Kuttan, 2000*).

- **Berberis aristata**
  Berberine, an alkaloid isolated from the plant *Berberis aristata* (10, 25 or 50 mg/kg) was administered simultaneously with NDEA, the markers of liver injury (liver weight, GGT activity and glutathione S-transferase levels) were reduced significantly compared with animals treated with NDEA only, which resulted in all the values being elevated. A similar decrease was noted in the serum levels of lipid peroxide, bilirubin and glutamate pyruvate transaminase. Morphology of liver tissue and levels of marker enzymes indicated that berberine offered protection against chemical carcinogenesis (*Anis et al., 2001*).

- **Orange oil**
  The effect of orange oil on the suppression of preneoplastic hepatic lesions during N-nitrosodiethylamamine (DEN) induced hepatocarcinogenesis was studied electron microscopically. Orange oil administration following DEN treatment showed decreased liver weights, increased intracellular gap junctional complexes; cell density and polarity when compared with only the DEN treated rats. Chemopreventive effect of orange oil on DEN - induced hepatic preneoplasia in rats, and it’s association with the restoration of the normal phenotype and upregulation of junctional complexes, has been demonstrated (*Bodake et al., 2002*).

- **Rhodotorula glutinis**
  *Rhodotorula glutinis* NCIM 3353 showed significant effect on the prevention of liver tumour development. It showed quite effective in prevention of liver tumour development especially when administered after
DEN treatment, indicating possible preventive effects at the promotional stages (Prakash et al., 2002).

- **Agaricus blazei**

  The modifying potential of prior administration of an aqueous extract of the mushroom *Agaricus blazei* Murrill (Agaricaceae) (Ab) on hepatotoxicity induced by different doses of diethylnitrosamine (DEN) in male Wistar rats was evaluated. After DEN-treatment, ALT levels, PCNA labeling index, and the number of GST-P positive hepatocytes were lower in rats that received *A. blazei* treatment and were exposed to 100 mg/kg of DEN. Findings suggest that previous treatment with *A. blazei* exerts a hepatoprotective effect on both liver toxicity and hepatocarcinogenesis process induced by a moderately toxic dose of DEN (Barbisan et al., 2002).

- **Phellinus rimosus**

  Aqueous extract of macrofungus *Phellinus rimosus* against N-nitrosodiethlyamine induced hepatocellular carcinoma has been studied. The serum parameters indicative of liver injury marker, tumour marker enzymes and drug metabolizing enzymes were reverted to normal against NDEA treated group. This shows the chemopreventive action of aqueous extract of macrofungus *Phellinus rimosus* against N-nitrosodiethlyamine induced Hepatocellular carcinoma (Ajith et al., 2003).

- **Allium sativum**

  The protective effects of garlic (*Allium sativum*) were investigated against N-nitrosodiethylamine (NDEA) induced hepatic tumourigenesis by assessing tumour marker enzymes, gamma glutamate transpeptidase (GGT), Glutathione S-transferase (GST), glucose 6-phosphatase (G6Pase), Alkanline phosphatase (ALP), 5’-nucleotidase, aspertate (AST) and alanine transminases (ALT). Oral administration of garlic extract normalised the activities of tumour marker enzymes (Sundaresan and Subramanian, 2002). Diallyl sulfide (DAS) can act, as a promoter in rat liver but exerts no co-
promoting effect. Conversely, DADS (Diallyl disulfide) was found to have promotion – inhibiting ability, suggesting that DADS has greater value than DAS as a chemopreventive agent (Guyonnet et al., 2004).

- **Curcuma longa**

  The modulatory effect of turmeric on nitrosodiethylamine (NDEA)-induced hepatocarcinogenesis in female wistar rats. NDEA-treated rats receiving 1% or 5% turmeric before, during and after carcinogen exposure showed significant decrease in number of γ glutamyl transpeptidase (GGT) positive foci measuring > 500 or >1000 µm and decrease in the incidence of NDEA-induced focal dysplasia (FD) and hepatocellular carcinomas. Decrease in the number of GGT positive foci measuring > 1000 µm was also observed in NDEA-treated rats receiving 0.2% turmeric, although no decrease in tumour incidence was noted. On the other hand, similar levels of turmeric treatment (0.2, 1 and 5%) after exposure to NDEA did not show any protective effects (Thapliyal et al., 2003).

- **Terminalia arjuna**

  Sivalokanathan et al., (2006) evaluate the antioxidant nature of ethanolic extract of *Terminalia arjuna* bark (EETA) on N-nitrosodiethylamine (DEN) induced liver cancer in male Wistar albino rats. Liver cancer was induced by single intraperitoneal injection of DEN (200 mg/kg). After 2 weeks of DEN administration, Phenobarbital (PB) was given to promote the cancer for up to 14 successive weeks. EETA extract (400 mg/kg) was given post-orally for 28 days to hepatocellular carcinomabearing rats. A significant increase in LPO levels were observed while the levels of enzymic and non-enzymic antioxidants were decreased, when subjected to DEN induction. These altered enzyme levels were ameliorated significantly by administration of EETA at the concentration of 400 mg/kg in drug-treated animals. The protective effect of EETA was associated with inhibition of LPO induced by DEN and to maintain the antioxidant enzyme levels. Their
results show an antioxidant activity of *T. arjuna* bark against DEN-induced liver cancer.

**Acacia nilotica**

Chemopreventive potential of *Acacia nilotica* bark extract (ANBE) against single intraperitoneal injection of *N*-nitrosodiethylamine (NDEA, 200 mg/kg) followed by weekly subcutaneous injections of carbon tetrachloride (CCl₄, 3 ml/kg) for 6 weeks induced hepatocellular carcinoma (HCC) in rats was studied. At 45 day after administration of NDEA, 100 and 200 mg/kg of ANBE were administered orally once daily for 10 weeks. The results strongly support that *A. nilotica* bark prevents lipid peroxidation (LPO) and promote the enzymatic and non-enzymatic antioxidant defense system during NDEA-induced hepatocarcinogenesis which might be due to activities like scavenging of oxy radicals by the phytomolecules in ANBE (Singh et al., 2009)

**Achyranthes aspera**

Kartik et al., (2010) assessed the antioxidant potential and suppressive effects of *Achyranthes aspera* by evaluating the hepatic diagnostic markers on chemical-induced hepatocarcinogenesis. At 20 weeks after the administration of NDEA and CCl₄, treated rats received *A. aspera* extract (AAE) at a dose of 100, 200, and 400 mg/kg once daily route. Administration of AAE suppressed hepatic diagnostic and oxidative stress markers as revealed by decrease in NDEA and CCl₄-induced elevated levels of SGPT, SGOT, SALP, GGT, bilirubin, and LPO. There was also a significant elevation in the levels of SOD, CAT, GPx, GST, and GSH as observed after AAE treatment. The liver and relative liver weight was decreased after treatment with AAE in comparison to positive control group. The architecture of hepatic tissue was normalized upon treatment with extract at different dose graded at 100, 200, and 400 mg/kg. b.w. in comparison to positive control group. *A. aspera* significantly alleviate
hepatic diagnostic and oxidative stress markers which signify its protective effect against NDEA and CCl\textsubscript{4}-induced two-stage hepatocarcinogenesis.

- **Ginkgo biloba and Silybum marianum**

  To evaluate the potential chemopreventive activities of *Ginkgo biloba* extract (EGb) and *Silybum marianum* extract (silymarin) against hepatocarcinogenesis induced by N-nitrosodiethylamine (NDEA) in rats were designed. In NDEA group, MDA level was elevated with subsequent decrease in GSH level and SOD, GPx and GR activities. In addition, NDEA group revealed a significant increase in serum ALT, AST and GGT activities and VEGF level. Furthermore, NDEA administrated animals showed a marked increase in comet assay parameters. These biochemical alterations induced by NDEA were confirmed by the histopathological examination of rat livers intoxicated with NDEA that showed an obvious cellular damage and well differentiated HCC. In contrast, silymarin+NDEA treated groups (3&5) and EGb+NDEA treated groups (4&6) showed a significant decrease in MDA level and a significant increase in GSH content and SOD, GPx and GR activities compared to NDEA group. Silymarin and EGb also beneficially down-regulated the increase in serum ALT, AST, GGT activities and VEGF level induced by NDEA. In addition, silymarin and EGb significantly decreased comet assay parameters. Histopathological examination of rat livers treated with either silymarin or EGb exhibited an improvement in the liver architecture compared to NDEA group. Their findings suggested that silymarin and EGb may have beneficial chemopreventive roles against hepatocarcinogenesis through their antioxidant, antiangiogenic and antigenotoxic activities *(El Mesallamy et al., 2011)*

- **Rubia cordifolia**

  The ameliorative effect of the *Rubia cordifolia* methanol extract was evaluated against *N*-nitrosodiethylamine-induced experimental
hepatocellular carcinogenesis in rats. Upon *Rubia cordifolia* methanol extract co-treatment (250, 500 and 750 mg/kg body weight) in group III alone levels of serum marker enzymes and antioxidants increased significantly in a dose-dependent manner. The levels of hydroxyl radicals and lipid peroxidation decreased. Mitochondrial enzymes and respiratory chain enzymes, which were decreased in *N*-nitrosodiethylamine-induced rats, increased significantly in RC treated rats. These findings demonstrated that *Rubia cordifolia* can be a source of potent antioxidants for treatment of diseases such as cancer (Shilpa et al., 2012).

### 2.2.11 Silymarin as standard drug

Silymarin is obtained from *Silybum marianum* (milk thistle), an edible plant that has been used medicinally for centuries as an herbal medicine for the treatment of liver-related disorders. It is widely prescribed by herbalists and has almost no known side effects (Nitin et al., 2007). Silymarin is a polyphenolic flavonoid, extracted using 95% ethanol, from the seeds of the milk thistle. The plant consists of approximately 70-80% of the Silymarin flavonolignans and approximately 20-30% of a chemically undefined fraction, comprising mostly polymeric and oxidized polyphenolic compounds. The most prevalent component of the silymarin complex is silybin (50-60% of silymarin), which is the most active photochemical and is largely responsible for the claimed benefit of the silymarin. Besides silybin, which is a mixture of two diastereomers (A and B) in approximately 1:1 proportion, considerable amounts of other flavonolignans are present in the silymarin complex, namely silychristin (20%), silydianin (10%), isosilybin (5%), dehydrosilybin, and a few flavonoids, mainly taxifolin. The seeds also contain betaine, trimethylglycine, and essential fatty acids that may contribute to silymarin's hepatoprotective and anti-inflammatory effects (Nitin et al., 2007).
Figure 2.6 Structure of silybin and its analogs.

- **Pharmacokinetics**

  Silymarin is not soluble in water and is usually administered in an encapsulated form. Silymarin is absorbed when given orally. Peak plasma concentration is achieved in 6-8 h. The oral absorption of silymarin is only
about 23-47%, leading to low bioavailability of the compound; it is administered as a standard extract (70-80% silymarin). After oral administration the recovery in bile ranges from 2-3%. Silybin and the other components of silymarin are rapidly conjugated with sulfate and glucuronic acid in the liver and excreted through the bile. The poor water solubility and bioavailability of silymarin led to the development of enhanced formulations; e.g., silipide (Siliphos\textsuperscript{B}), a complex of silymarin and phosphatidylcholine that is ten times more bioavailable than Silymarin; an inclusion complex formed between silymarin and b-cyclodextrin, which is approximately 18 times more soluble than Silymarin (Nitin et al., 2007). There have been reports of silybin glycosides that have better solubility and stronger hepatoprotective activity (Morazzoni et al., 1994).

\begin{itemize}
  \item **Toxicity**
  
  Studies on the acute toxicity of silymarin after intravenous infusion have been carried out in mice, rats, rabbits, and dogs. The LD\textsubscript{50} values were 400 mg/kg (mice), 385 mg/kg (rats), and 140 mg/kg (rabbits and dogs). Depending on the infusion rate these values vary. With slow infusion (over 2-3 h) the LD\textsubscript{50} was 2 g/kg in rats and after oral administration it was 10 g/kg. Intravenous bolus dose of silymarin as the hemisuccinate sodium salt has also been used to carry out acute toxicity studies in beagle dogs, rabbits, Wistar rats, and NMRI mice. The LD\textsubscript{50} was 1050 mg/kg (male mice), 970 mg/kg (female mice), 825 mg/kg (male rats), 920 mg/kg (female rats), and 300 mg/kg (rabbits, dogs) (Nitin et al., 2007). These data demonstrate that the acute toxicity of silymarin is very low. Similarly, its subacute and chronic toxicity are also very low (Morazzoni et al., 1994).

\item **Pharmacology of Silymarine**
  
  \begin{itemize}
    \item **Hepatoprotective activities**
    
    Various experimental studies using compounds that directly or indirectly cause liver damage have been carried out to demonstrate the
hepatoprotective action of silymarin in xenobiotic intoxication and fungal intoxication such as Carbon tetrachloride, Phenylhydrazine, tert-Butyl hydroperoxide, Ethanol, Halothane, Thioacetamide, Galactosamine, Paracetamol, Erythromycin estolate, Microcystin, Amanita phalloids toxin (Nitin et al., 2007).

Silymarin's hepatoprotective effects are purportedly accomplished via several mechanisms; these include:

- Antioxidation.
- Inhibition of lipid peroxidation.
- Stimulation of ribosomal RNA polymerase and subsequent protein synthesis, leading to enhanced hepatocyte regeneration.
- Enhanced liver detoxification via inhibition of phase I detoxification.
- Enhanced glucuronidation and protection from glutathione depletion.
- Anti-inflammatory effects, including inhibition of leukotriene and prostaglandin synthesis, Kupffer cell inhibition, mast cell stabilization, and inhibition of neutrophil migration.
- Slowing or even reversing of fibrosis by reduction of the conversion of hepatic stellate cells into myofibroblasts.
- Anticarcinogenesis by inhibition of cyclin-dependent kinases and arrest of cancer cell growth.
- Silymarin is also found to have immunomodulatory effects on the diseased liver.

**Therapeutic indication**

#### Amanita mushroom poisoning

The most remarkable use of silymarin is in the treatment of mushroom poisoning caused by *Amanita phalloides* (death cap). Many of the *Amanita* species are highly toxic and ingestion results in severe liver damage and death. This mushroom is known to possess two powerful hepatotoxins, namely, amanitin and phalloidin. Silybin given along with benzyl penicillin
has been shown to be effective against amanitin poisoning (Pradhan et al., 2006). In animal studies, silymarin given within 10 min after Amanita toxin ingestion completely counteracted the toxic effects and if given within 24 h of toxin ingestion it prevented death and greatly reduced liver damage.

Carducci et al., (1996) in a case study, reported that silybin hemisuccinate given by the intravenous route had a favourable effect on a family of four suffering from severe liver damage caused by Amanita mushroom (amatoxin) compared to when they were treated with the standard therapy. Moreover, investigations done after 2 months showed an absence of any morphological alteration in the hepatobiliopancreatic echography, suggesting that silybin may play a significant role in hepatic tissue protection.

➢ **Hepatitis**

Studies have shown that silymarin is effective in the treatment of both acute and chronic hepatitis. In acute viral hepatitis, administration of silymarin shortened treatment time and lowered the elevated serum bilirubin, AST, and ALT. In patients with acute hepatitis who were given either silymarin (140 mg) or placebo three times daily for three weeks, the proportion of patients whose AST was normalized was much higher in the treated group (82%) than in controls (52%). In patients with chronic hepatitis, 420 mg silymarin per day for six months also yielded improved serum liver enzyme levels (Magliulo et al., 1978).

➢ **Alcoholic liver disease and cirrhosis**

Silymarin administration has demonstrated normalization of serum liver enzyme and total bilirubin levels in patients with alcoholic liver disease; there was also improvement in liver tissue histology (Fehr et al., 1989). In patients with cirrhosis, long-term (41 months) administration of silymarin at 420 mg per day resulted in a significant increase in survival compared to a placebo group.
- **Hypercholesterolemia**

  In an animal study conducted by Kreeman et al., (1998) silymarin given to rats with diet-induced hypercholesterolemia demonstrated an anticholesterolemic effect similar to probucol, with an increase in HDL cholesterol and a decrease in total and biliary cholesterol.

- **Psoriasis**

  Abnormally high levels of cAMP and leukotrienes have been observed in patients with psoriasis and normalization of these levels may improve the condition. The effectiveness of silymarin in the treatment of psoriasis may be due to its ability to improve endotoxin removal by the liver, inhibit cAMP phosphodiesterase, and inhibit leukotriene synthesis (Kock et al., 1985).

- **Newer application**

  - **Silybin/silymarin as chemoprotective and anticancer agents**

    The chemopreventive action of silymarin helps it inhibit the carcinogenic action of many chemicals. The incidence of urinary bladder neoplasms and preneoplastic lesions induced by N-butyl-N-(4-hydroxybutyl) nitrosamine were significantly reduced.

![Figure 2.7 Mechanism of action of Silymarin](image-url)
Silymarin also significantly inhibited azoxymethane-induced colon carcinogenesis in rats (Kohno et al., 2002). Skin carcinogenesis induced by benzoyl peroxide or 12-0-tetradecanoylphorbol-13-acetate was also inhibited by Silymarin (Lahiri-Chatterjee et al., 1999; Agarwal et al., 1994 and Zhao et al., 1999).

 ➢ **Neuroprotective and neurotropic activities of silybin/Silymarin**

Due to its antioxidative activity, silymarin has been found to be useful in treatment and prevention of many neurodegenerative and neurotoxic processes. Wang et al., (2002) demonstrated that silymarin could effectively protect dopaminergic neurons against lipopolysaccharide-induced neurotoxicity by inhibiting the activation of microglia which represents macrophage-like population of brain cells and which act in host defense and tissue repair in the CNS.

 ➢ **Silybin/silymarin in treatment and prevention of gastrointestinal problems**

Many gastrointestinal problems can be treated and/or prevented by silybin/silymarin preparations. In the pancreas, silybin acts mainly as chemoprotectant and can stimulate recovery after intoxication. Alloxane, which causes necrosis of b-pancreatic cells and lack of insulin secretion, causes production of H$_2$O$_2$ which produces cellular damage followed by cell death. Silymarin, due to its antioxidant action, has been found to prevent a rise in both plasma glucose and pancreatic lipid peroxidation in the hyperglycemic rats (Soto et al., 1998 and Soto et al., 2003).

 ➢ **Silybin/silymarin in treatment and prevention of cardiopulmonary problem**

During cancer therapy, the use of cardioprotective drugs, e.g. doxorubicin, is limited by the cardiotoxicity that is known to be mediated by oxidative stress and induction of apoptosis. Silybin, due to its antioxidant effect, can be very effective in such cardioprotective applications. In the
study conducted by Chlopňková et al., the cell membrane stabilizing and radical scavenging potency of silymarin and its isolated components helped to protect cardiomyocytes (rat) against doxorubicin-induced oxidative stress (Chlopečková et al., 2004).

➢ **Silybin/silymarin in skin protection**

Silymarin has been shown to exhibit preventive effects against photocarcinogenesis in various animal tumor models. Topical application of silymarin to mouse skin reduced UVB-induced tumor incidence, multiplicity, and size compared to that in nontreated animals (Katiyar et al., 1997). Silybin inhibited photocarcinogenesis in mice whether applied topically or administered in the diet (Mallikarjuna et al., 2004).

❖ **Adverse effects**

As described earlier in the section on toxicity, silymarin has very low toxicity and has been shown to possess a good safety profile. At high doses, a laxative effect is observed due to increased bile secretion and bile flow. Adverse effects related to the GI tract such as dyspepsia, bloating, nausea, and diarrhoea were reported in 2-10% of patients in a clinical trial (Jacobs et al., 2002) Serious adverse effects, which are rare, include gastroenteritis associated with collapse and allergy.

2.3  **Rational for selection of the plants of this study**

The Indian Ayurvedic system treasures a host of medicinal formulations from which numerous valuable medicines have been derived and recently some Ayurvedic herbal drugs have been shown to possess cytotoxic and cytostatic effects in tumour cell in culture (Smit, 1995). Although there is an important local ethnobotanical bibliography describing the most frequently used plants in the treatment of conditions consistent with cancer symptomatology, there are few experimental studies, which validate the possible antitumour properties of the plants. The whole plant of *Fumaria indica* pugsley (pitpapda, Fumariaceae) has long been used as a common
household remedy and forms a constituent of many common Ayurvedic, Unani medicinal preparations and polyherbal liver formulations. *Fumaria indica* is a safe hepatoprotective agent against various hepatotoxins (carbon tetrachloride, paracetamol, rifampicin and D-galactosamine) in albino rats and act similar to that of silymarin as hepatoprotective and is more effective inducer of biochemical enzymes indicating the anticarcinogenic properties (Kishore, 2009).

*Tephrosia purpurea* is used as herbal medicine for hepatitis and also reported for several hepatoprotective activity (Khatria et al., 2009; Murthy and Srinivasan, 1993., Girish et al., 2009) including antioxidant activity (Gunjegaonkar et al., 2010), wound healing (Santram et al., 2006), antiulcer activity (Despande et al., 2003), immunomodulatory activity (Damre et al., 2003), anticancer activity against a human nasopharyngeal epidermoid tumor cell line (KB) (Santram et al., 2006) and also exhibiting anticancer activity against human breast cancer (MCF-7) cell line (Gulechan and Sivakuma, 2011). Though *Tephrosia purpurea* has been studied on various cancer models its effect on hepatocellular carcinoma (HCC) is not yet documented.

In view of these facts, the present study is framed to evaluate the effectiveness of selected medicinal plants against hepatocarcinogenesis activity. Considering the antitumor, hepatoprotective and antioxidant effects and the present study is aimed to evaluate the therapeutic efficacy in experimental animal models of these medicinal plants against N-nitrosodiethylamine induced hepatocellular carcinoma. The rat liver responses to chemical carcinogen like N-nitrosodiethlyamine (NDEA) provide useful model for the study of carcinogenic process. The carcinogenic action of NDEA in animals and man, as well as underlying molecular mechanism *in vivo* has been well documented (Archer, 1989; Lee and Lee, 1999).
2.4 Review of plant (*Fumaria indica*)

Botanical name : *Fumaria indica* Pugsely  
Family : Fumariaceae  

**Vernacular Names**

<table>
<thead>
<tr>
<th>Language</th>
<th>Name</th>
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<tbody>
<tr>
<td>Bengali</td>
<td>Shotara pipapapra</td>
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<tr>
<td>English</td>
<td>Common fumitory</td>
</tr>
<tr>
<td>Hindi &amp; Marathi</td>
<td>Pitpapara</td>
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<td>Chata- rashi</td>
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<td>Sanskrit</td>
<td>Parpata</td>
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**Part Used** : Whole Plant

**Distribution:**

It is distributed over the greater part of India up to 8000 ft. on Himalayas, Baluchistan, Afganistan, Turkistan, Pakistan, Persia, and Mongolia. An annual herb suberect or diffuse, probably scarcely scandent, leaves multifid, more or less glaucouse, flower pink or white with purple tips (Kartikar and Basu., 1985).

**Description:**

An annual herb suberect or diffuse, probably scarcely scandent, leaves multifid, more or less glaucose.

- Leaves : 3-10cm long, 2-5cm broad  
- Flower : 5-6mm long (usually white or pale pinkish)  
- Fruit : 2-2.5mm in diam. (rugose when dried)  
- Seed : 1-1.5mm in diam. (single, brownish)
Chemical constituents:


Other parts of the plant show:

Aerial parts : Papracine, paprazine, sitosterol, stigmasterol, campesterol.

Root : Protopine, octacosanol, alkaloid–narcumine, narlumidine, adlumidine, orsanguinartine.

Leaves & stem : Narlumicine, protopine, narlumidine, nonacosanol, alcohol, n-alkanes.

Figure 2.8 Whole Plant of Fumaria indica
Seed : Fumariline, dihydrocoptisine, tetrahydrocoptisine, bicuculine, oxysanguinarine, narceimicine

**Medicinal Properties & Uses**

The plant is reported to be diuretic, diaphoretic and laxative. It is used in fever and influenza. It is also used to purify blood and in obstruction of lever. The plant is used as one of the component of many Ayurvedic, Unani and liver formulations (Rao and Mishra, 1998). The plant has reputation as anthelmentic, anti-dyspeptic, cholagogue, stomachic, sedative and tonic along with it is also considered useful to treat abdominal cramps, diarrhoea, fever, jaundice, leprosy and syphilis [John et al., (1981); Anonymous et al., (1956); Haq et al., (1993); and Nadkarni et al., (1976)].

2.4.1 Chemical review

Pandey et al., (1971) isolated seven isoquinolene alkaloid such as protopine, tetrahydro coptisine, fumariline, bicuculine, narlumidine, fumarilicine and narceimine from the alcoholic extract of whole plant of *Fumaria indica*.

Satish and Bhakuni, (1972) isolated protopine, quanternary salt of protopine, nonacosanol and sitosterol from the stem and leaves of *Fumaria indica*.

Pandey et al., (1974) reported that protopine content of the seeds is about double that of whole plant, the yield of tetrahydro coptisine is 50 times more in seeds than in whole plant.

Pandey et al., (1979) isolated three alkaloids fumariline, 8 - methoxy dihydro sanguinarine and oxysanguinarine from *Fumaria indica*.

Tripathi et al., (1988) isolated seco-phalidi isoquinoline alkaloid narceimine from *Fumaria indica* seeds.

Atta-ur-Rahman et al., (1991) isolated a new isoquinoline base papracine along with six known bases (fumaritine N-oxide, parfumine,
lastourvilline, feruloyl tyramine, fumariflorine and N-methyl corydaldine) which have been isolated from *Fumaria indica*.

*Atta-ur-Rahman et al.,* (1992) isolated two new spirobeanzyl isoquinoline alkaloids such as papracinine and paprazine together with six other known alkaloids identified as fumaritine N-oxide, parfumine, lastourvilline, feruloyl tyramine, fumariflorine and N-methyl corydaldine from aerial parts of *Fumaria indica*.

*Tripathi et al.,* (1992) isolated new seco-phalidi isoquinoline alkaloid narlumicine from stem of *Fumaria indica*.

*Atta-ur-Rahman et al.,* (1995) isolated three new seco-phalidi isoquinoline alkaloids such as paprafumine, paprarine and papraline along with three other known alkaloids as cryptopine, raddeanine and oxocoptisine from the aerial part of *Fumaria indica*.

### 2.4.2 Pharmacological review

*Pandey et al.,* (1971) reported smooth muscles relaxant and hydrocholeretic effect of *Fumaria indica* is due to protopine which is present as major alkaloid in the plant. As a smooth muscles relaxant protopine was found to be slightly weaker than papaverine.

*Rao and Mishra, (1997)* reported that whole plant of *Fumaria indica* has hepatoprotective activities against carbon tetrachloride, paracetamol and rifampicin-induced hepatotoxicities in albino rats. The petroleum ether extract against carbon tetrachloride, total aqueous extract againsts paracetamol and methanolic extract against rifampicin - induced hepatotoxicities showed similar reductions in the elevated levels of some of the serum biochemical parameters in a manner similar to that of silymarin indicating its potential as a hepatoprotective agent.

*Rao and Mishra, (1998)* isolated monomethyl fumarate from methanolic extract of the whole plant of *Fumaria indica*, it was characterized and screened for its antihepatotoxic activity in albino rats. The compounds
showed significant antihepatotoxic activity against thioacetamide in vitro, and against hepatotoxicities induced by carbon tetrachloride, paracetamol and rifampicine in vivo to an extent almost similar to that of silymarine, a known antihepatotoxic agent.

Nimbkar and Juvekar, (2000) studied on hepatoprotective activity against anti-tubercular drug induced hepatotoxicity in Albino rats. Ethanolic extract of *Fumaria indica* showed normalization of biochemical parameters alanine and aspartate aminotransferase, alkaline phosphatase. Cholesterol levels were found to be slightly raised. Plasma levels of rifampicin were found to drop in *Fumaria indica* treated group as compared to anti-tubercular drug treated group.

Gilani et al., (2005) reported that crude extract of *Fumaria indica* whole plant (Fi.Cr) & its fractions were studied in vitro for spasmogenic & spasmolytic effects to rationalize some of the traditional uses. Fi.Cr (1.0-5.0 mg/ml.) caused a moderate degree of atropine sensitive spasmogenic effect in guinea pig ileum. These finding indicate that the presence of cholinergic and CCB (calcium channel blocked) constituents in Fi.Cr may explain the respective traditional use of the *Fumaria indica* in constipation and diarrhoea.

Rathi et al., (2008) demonstrated the hepatoprotective potential of 50% ethanolic water extract of whole plant of *Fumaria indica* and its three fractions viz., hexane, chloroform and butanol against d-galactosamine induced hepatotoxicity in rats.

Singh and Kumar, (2011) evaluated the acute and sub-chronic toxicity of a 50% ethanolic extract of FI in mice and rats respectively. There was no mortality or abnormal behaviour, observed in acute toxicity study in mice at all the three (1, 2.5 and 5 g/kg) dose levels. In sub-chronic toxicity study, FI did not produce any significant change in body weight, daily food and water
intake of rats when compared to vehicle treated rats. Further, haematological and biochemical parameters were also found normal.

Singh et al., (2012) evaluated the anti-stress activity of standardized extract of *Fumaria indica* (FI) through validated behavioral models of rodents. For comparison, *Panax ginseng* (PG) extract was used as standard adaptogen stress. FI and PG significantly decreased chronic unpredictable stress induced elevation of corticosterone, and both extracts normalized also the abnormal oxidative status of the brain observed in stressed rats. FI treatment also sup-pressed the elevated level of IL-1β, TNF-α and IL-10 in stressed animals. These finding indicated that FI could be another adaptogenic herb and fumaric acid and its conjugates are possibly involved in observed bioactivity of its extract.

2.5 **Review of plant (**Tephrosia purpurea**)**

<table>
<thead>
<tr>
<th>Botanical name</th>
<th>Tephrosia purpurea Linn.</th>
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<tbody>
<tr>
<td>Family</td>
<td>Leguminosae</td>
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**Vernacular Names**

<table>
<thead>
<tr>
<th>Bengali</th>
<th>Sarphotika</th>
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<tr>
<td>English</td>
<td>Wild indigo</td>
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<tr>
<td>Hindi &amp; Marathi</td>
<td>Sarphonka</td>
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<tr>
<td>Kannada</td>
<td>Empali</td>
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<tr>
<td>Tamil</td>
<td>Kolingi</td>
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<td>Telugu</td>
<td>Vempali</td>
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<td>Sanskrit</td>
<td>Sharapunkha</td>
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<tr>
<td>Malayalam</td>
<td>Kolinnil</td>
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</table>

**Part Used**

Whole Plant
Figure 2.13 Plant of *Tephrosia purpurea*

- **Distribution and habitat**

  *T. purpurea* is native to tropical Asia and it is a common weed found throughout India to an altitude of 1850 m in the Himalayas. *T. purpurea* occurs naturally in grassy fields, waste places, on ridges and roadsides. It prefers dry, gravelly or rocky and sandy soils, but in Madras (India) it grows well on loamy soils. Sometimes cultivated as a green manure crop in paddy fields, in coconut and banana plantations in south India.

- **Description**

  A polymorphic, much-branched, suberect, perennial herb usually 30-60 cm tall, branches spreading, glabrous or sparsely pilose. Leaves imparipinnate, 5-15 cm long, petioles 6-12 mm long, stipules linear subulate, nerved, erect or reflexed, leaflets 7-21, narrow, elliptic to oblanceolate, apex obtuse or retuse, mucronate, base acute or cuneate, 2-2.8 cm long and 0.8-1.3 cm wide, glabrous above, silky pubescent beneath, with numerous closely
parallel veins, petiolules of lateral leaflets 1.5-2.5 mm long, those of the terminal leaflets 3-4.5 mm long. Flowers pink, red or purple, 4-9 mm long, thinly silky, teeth triangular subulate, as long as the tube, corolla twice as long as the calyx, standard pubescent on the back. Fruit (pod) slightly curved, compressed, glabrescent, dehiscing by both sutures, 3-5 cm long, containing 4-10 greenish-grey, smooth, ovoid seeds. Flowers between July and November and fruits from September to January in northern and central India, flowering and fruiting occur year-round in southern India.

**Phytochemical contents**

Phytochemical investigation on *T. purpurea* has revealed the presence of glycosides, rotenoids, isoflavones, flavanones, chalcones, flavanols, flavones and sterols. It also contains long chain-saturated ketones, rotenoids (Harper, 1939), flavanols (Rangaswami and Ramasastry, 1955), tephrosin (Carison et al., 1973), pongaglabol (Talapatra et al., 1980), semiglabrin (Waterman and Khalid, 1980). The roots, leaves and seeds contain tephrosin, deguelin and quercetin, the roots also contain isotehrnosin and rotenone. 2.5% rutin is found in roots and leaves (Krewson and Naghski, 1953). Rotenoids are produced by in vitro tissue cultures of the plant parts (Sharma and Khanna, 1975). Purpurin, a flavonone has been isolated from the seeds, as also 8-substituted flavonoid and 3-substituted oxygenated chalcones (Venkata Rao and Ranga Raju, 1984).

**2.5.1 Chemical review**

Two flavonoids viz. Purpurenone, a new β-hydroxychalcone; (+)-purpurin, a diastereoisomer of (−) purpurin; dehydroisoderricin, and (−)-maackiain have been isolated from the roots of *Tephrosia purpurea* (Rao and Raju, 1984).

An isoflavone, and a chalcone both novel compounds, as well as six constituents of known structure were obtained as active compounds from *T.*
purpurea, using a bioassay based on the induction of quinone reductase (QR) activity with cultured Hepa 1c1c7 mouse hepatoma cells. Additionally, three inactive compounds were isolated and identified. (Chang et al., 1997).

The flavonoids like tephrosin, pongaglabol, and semiglabrin from Tephrosia purpurea aerial parts have been isolation by Ahmad et al., (1999).

Chemical investigations of aerial parts of Tephrosia purpurea yielded rare prenylated flavonoids, tephropurpulin A and isoglabratephrin, in addition to a previously identified flavonoid, glabratephrin. Structures were established by 1D and 2D NMR spectroscopy, as well as by HR-MS analysis (Mohamed-Elamir et al., 2009).

The stem extract of Tephrosia purpurea showed antiplasmodial activity against the D6 (chloroquine-sensitive) and W2 (chloroquine-resistant) strains of Plasmodium falciparum. A new prenylated flavone, named terpurinflavone, along with the known compounds lanceolatin A, semiglabrin and lanceolatin B have been isolated from this extract. The new compound, terpurinflavone, showed the highest antiplasmodial activity. (Juma et al., 2011)

2.5.2 Pharmacological review

In Ayurvedic system of medicine, the whole plant has been used to cure tumours, ulcers, leprosy, allergic and anti-inflammatory conditions such as rheumatism, asthma and bronchitis (Kirtikar and Basu, 1975).

The plant is used as a digestive, diuretic and anti tussive in ayurvedic practice. It has undergone clinical trials in viral hepatitis and is claimed to improve liver function (Watt and Breyer-Brandwijk, 1962).

Studies have been carried out to demonstrate the usefulness of T.purpurea in acute and chronic inflammatory liver diseases, and in cases of various hepatocellular and hepatocanicular conditions (Upadhyaya, 1965).
The whole plant of *T. purpurea* was evaluated for its efficacy in rats by inducing hepatotoxicity with D-galactosamine HCl (acute) and carbon tetrachloride (chronic). The administration of *T. purpurea* along with the hepatotoxins offered a protective action in both acute (D-galactosamine) and chronic (CCl₄) models (Murthy and Srinivasan, 1993).

*T. purpurea* is widely used in the traditional Indian system of medicine as an anti-inflammatory agent and also used in various liver, spleen and kidney disorders. The plant has also been reported to be useful in cough, asthma, inflammatory conditions, and in enlargement-obstruction of the liver, spleen and kidneys (Nadkarni, 1976).

The benzene extract of the plant was evaluated for its action on the CNS of different laboratory animals and found that *T. purpurea* can abrogate the tumor-promoting effect of croton oil (phorbol ester) in murine skin (Saleem et al., 2001).

The alcohol extract of *T. purpurea* showed a significant hydroxyl radical scavenging activity in vitro. Using a Trypan blue exclusion assay. The hydroxyl radical scavenging effect of the extract was enhanced with increases in the concentration of drug, suggesting the role of free radical scavengers in minimizing gentamicin-induced kidney cell damage (Prashanth et al., 2001).

Prophylactic treatment of rats with *T. purpurea* at doses of 5 mg/kg body weight and 10 mg/kg body weight prevented N-diethylnitrosamine-initiated and KBrO₃ promoted renal oxidative stress and toxicity. All the antioxidant enzymes were recovered dose-dependently. The data indicate that *T. purpurea* besides a skin antioxidant can be a potent chemopreventive agent against renal oxidative stress and carcinogenesis induced by N-diethylnitrosamine and KBrO₃ (Khan et al., 2001).
Effect of oral administration of flavonoid fraction of *T. purpurea* (10-40 mg/kg) on sheep red blood cells (SRBC)-induced delayed-type hypersensitivity reaction has been studied. The results showed ability of the flavonoidal fraction of *T. purpurea* to modulate both the cell-mediated and the humoral components of the immune system (Damre et al., 2003).

In ayurvedic literature *T. purpurea* is given the name of ‘sarwa wranvishapaha’, which means that it has the property to cure all type of wounds. Alcoholic extract of *T. purpurea* possesses significant antiulcer property, which could be either due to cytoprotective action of the drug or by strengthening of gastric and duodenal mucosa and thus enhancing mucosal defence (Deshpande et al., 2003).

The ethanolic extract of *Tephrosia purpurea* (aerial part, in the form of simple ointment using) was evaluated against three types of wound models in rats as incision wound, excision wound and dead space wound. The results were comparable to standard drug Fluticasone propionate ointment, in terms of wound contraction, tensile strength, histopathological and biochemical parameters such as hydroxyproline content, protein level, etc. Histopathological study showed significant (*P* < 0.05) increase in fibroblast cells, collagen fibres and blood vessels formation (Santram et al., 2006).

The methanolic extract of *Tephrosia purpurea* has been evaluated against clinical isolates and standard strains of *Helicobacter pylori*, including metronidazole-resistant strains. Fractionation of the extract revealed the *n*-hexane and chloroform fractions possess marked inhibitive activity. Apolar fractions of *Tephrosia purpurea* may have therapeutic potential in combating *Helicobacter pylori* mediated gastroduodenal disorders (Chinniah et al., 2009).
Hepatoprotective activity of the aerial parts of *Tephrosia purpurea* (500 mg/kg, po) against thioacetamide-induced hepatotoxicity was evaluated. A significant reduction has been reported in serum aspartate aminotransaminase (35%), alanine aminotransaminase (50% respectively), gamma glutamyl transpeptidase (56%), alkaline phosphatase (46%), total bilirubin (61%) and liver MDA levels (65%), and significant improvement in liver glutathione (73%) when compared with thioacetamide damaged rats. (Khatri et al., 2009).

The fractions of *Tephrosia purpurea* (TP) has been tested for *in vitro* anticancer activity using human MCF 7 cell line by trypan blue exclusion method. The result shows that among all these fractions of TPI, TPIII, FRI and FRIII shows better anticancer activity compared to other fractions. The anticancer potential of TP and FR fractions were found in MCF 7 cell line (Gulecha and Sivakuma, 2011).

*Tephrosia purpurea* methanol extract (METP) was investigated for diuretic potential in male wistar rats. The powdered plant material was extracted with methanol by hot extraction. METP at various dose levels exhibited significant diuretic activity as evidenced by increased urine volume, electrolyte concentration, and alkaline pH in comparison to control group of animals. The present study provides a quantitative basis for explaining the folkloric use of *Tephrosia purpurea* as a diuretic agent in Indian traditional system of medicine (Ashokkumar et al., 2012).