Review of literature

2.1. Induction of hepatocarcinogenesis

Tumor development has generally been considered to be composed of three distinct steps, initiation, promotion, and progression, based on the carcinogenesis studies in rodents (**Trosko, 2001).** Each stage results in unique biological and morphological changes occurring in cells. Initiation, an irreversible multistage carcinogenesis, and short-term event, was ascribed to DNA damage leading to mutagenesis **(Campos *et al.*, 2007).** Promotion, an interruptible or reversible and long term process, was believed to be caused by an epigenetic mechanism, which resulted in the expansion of damaged cells to form an actively proliferating multicellular premalignant tumor cell population. Progression, an irreversible process, was believed to be caused by the genetic instability that leads to mutagenic and epigenetic changes, and is related to the production of a new clone of tumor cells with increased proliferative capacity, invasiveness, and metastatic potential **(Klaunig and Kamedulis, 2004).**

Primary liver cancer, also known as hepatocellular cancer or hepatocellular carcinoma (HCC), remains a disease with an extremely poor prognosis, grim prospects and a 5-yr survival rate below 9% **(Sherman, 2005).**

The key risk factors for HCC occurrence include contamination of foods with mycotoxins, hepatitis viral infections and exposure to genotoxic and

cytotoxic chemicals, high levels of alcohol consumption, all of which cause chronic liver injury and inflammation **(Gao *et al*., 2012).**

A causal relationship between inflammation and cancer has long been suspected**.** It has been described that in this inflammatory process, the vast majority of cellular events require the nuclear factor-kappa B (NF-*κ*B) activation **(Pan *et al*., 2010).** However, the molecular mechanisms linking chronic inflammation and cancer are not well known yet. NF-*κ*B is a transcription factor that regulates inflammation, immunity, apoptosis, cell proliferation, and differentiation after binding to DNA and activating gene transcription **(Naugler and Karin, 2008; Guo *et al*., 2009).**

Animal models of liver cancer using hepatocarcinogens have been extensively used in the preclinical investigation of compounds with potential anti-hepatocarcinogenic activity. Chemical models have also been useful in the elucidation of the prominent biochemical mechanisms which play an important role in the etiology, pathophysiology as well as progression of liver cancer **(Darvesh and Bishayee, 2013).** The rodent hepatocarcinogenesis model employs diethylnitrosamine (DENA), which is a potent genotoxic carcinogen and used as an initiating agent **(Kato *et al*., 1993).**

Hepatocellular cancer occurs in a milieu of oxidative stress and inflammation. Oxidative and inflammatory processes, such as production of cytokines and chemokines as well as generation of free radicals, such as reactive oxygen and nitrogen species, due to viral infections, hepatitis, hepatic cirrhosis, and hepatocarcinogens represent the early malignant steps

in the pathogenesis of liver cancer. Cellular proliferation, in response to tissue injury, in the background of continued oxidative stress and inflammation is also a major driving force in hepatic tumorigenesis **(Klaunig and Kamedulis, 2004; Mantovani *et al*., 2008; Nejak-Bowen and Monga, 2011).** Besides oxidative stress and the inflammatory cascade, various signaling pathways have been implicated in the pathogenesis of HCC **(Whittaker *et al*., 2010).**

Current studies have shown that the irregular modulation of various signaling pathways involved in cell proliferation, survival, differentiation, inflammation, angiogenesis along with several others remains the primary causes of both the development and progression of HCC. Identification of cellular pathways implicated in liver tumor development benefits the understanding of disease pathophysiology, aids in diagnosis and development of diagnostic markers, and also is a valuable tool in the design of effective preventive and intervention strategies. Several excellent reviews discuss the current understanding of the signaling mechanisms implicated in HCC development and progression as well as the novel molecular targets for HCC therapy **(Whittaker *et al*., 2010; Simile *et al.*, 2011).**

Diethylnitrosamine (DENA) is a carcinogen that, at low doses, acts only as an initiating agent in the rat liver **(Goldsworthy and Pitot, 1985)**. In rat liver, it can initiate development of altered hepatic foci, which are preneoplastic lesions that develop in hepatic carcinogenesis **(Pitot *et al*., 1988),** and it can reflect clonal development from individual initiated hepatocytes **(Weinberg *et al*., 1987).** Neonatal rats provide a more sensitive

model than adult rats for study of altered hepatic foci **(Schulte-Hermann *et al*., 1986).**

Diethylnitrosoamine (DENA) either used alone or in combination with a promoting agent such as phenobarbital has found considerable use in the development and progress of experimental liver cancer as reported in the literature in the past 2 decades **(Bishayee and Chatterjee , 1995; Bishayee and Dhir , 2009).** Diethylnitrosamine (DENA) is known to cause perturbations in the nuclear enzymes involved in deoxyribonucleic acid (DNA) repair/replication and is normally used as a carcinogen to induce liver cancer in animal models **(Newell *et al.*, 2008).** The dose of DENA has been shown to influence its organotropism in rat **(Schmahl and Habs, 1980).**

Diethylnitrosamine is reported to undergo metabolic activation by cytochrome P450 enzymes to form reactive electrophiles which cause oxidative stress leading to cytotoxicity, mutagenicity and carcinogenicity **(Archer, 1989).** DENA has been shown to be metabolized to its active ethyl radical metabolite, and the reactive product interacts with DNA causing mutation, which would lead to carcinogenesis **(Chakraborty *et al*., 2007).** Moreover, experimental, clinical and epidemiological studies have provided evidences supporting the role of reactive oxygen species in the etiology of cancer. Diethylnitrosamine has been suggested to cause oxidative stress and cellular injury due to the enhanced formation of free radicals **(Valko *et al*., 2006).**

Diethylnitrosamine is found in a wide variety of foods like cheese, soybean, smoked, salted and dried fish, cured meat and alcoholic beverages **(Liao *et al*., 2001).** Metabolism of certain therapeutic drugs is also reported to produce diethylnitrosamine **(Akintonwa, 1985).**

Nitrate and nitrite are added to meat and fish for the purpose of preservation, as color fixatives and as flavouring. Ingestion of nitrite and nitrate can result in the endogenous formation of nitroso compounds, particularly in the presence of nitrosatable precursors, such as primary amines, in the acidic condition of the stomach **(Lin *et al*., 2002).**

Phenobarbital (PB), a sedative and antiepileptic drug (**Kwan and Brodie**, **2004),** is a well characterized rodent nongenotoxic carcinogen which causes an increase in the incidence of liver tumors after long term exposure. This follows a reversible increase in liver weight, attributed to a combination of hyperplasia and hypertrophy **(Carthew *et al*., 1998).**

Phenobarbital is a well-known hepatopromoter and inducer of cytochrome P-450s and is effective for promotion in terms of hepatic foci and tumor development. It is known to increase the number and size of foci and neoplasms when fed after initiation by a single dose of DENA as quantified by enzyme-altered foci **(Morita *et al*., 2013a).**

Initiation and promotion using phenobarbital have been linked to prostaglandin (PG) biosynthesis **(Kroll *et al*., 1999)** and free radical formation **(Buko and Sadovnichy, 1996).** Growth of some types of normal and neoplastic cells can be regulated by PGs**.** The role of PGF2a

demonstrated in previous studies supports the theory that PGF2a has a pivotal role in the promotion phase of carcinogenesis **(Hendrich *et al*., 1991).**

Phenobarbital, one of the cytochrome P450 family 2 subfamily B (CYP2B) inducers, is a liver tumor promoter related to the nuclear translocations of constitutive active/ androstance receptor (CAR) and to oxidative DNA damage resulting from the generation of microsomal reactive oxygen species (ROS). It has been reported that treatment with PB increased mRNA expression and protein levels of CYP2B in a 2- stage liver carcinogenesis bioassays in rats **(Waxman and Azaroff, 1992; Kinoshita *et al*., 2003).** The nuclear translocation of CAR induced by PB stimulates the expression of various genes that enhance hepatic tumor promotion **(Kawamoto *et al*., 1999; Deguchi *et al*., 2009).** In addition, it has been shown that microsomal ROS production and production of thiobarbituric acid reactive substances (TBARS) and 8-hydroxydeoxyguanosine (8-OH dG) are induced by PB with increasing the CYP2B **(Morita *et al*., 2011).**

Previous studies showed that the short term sole administration of Phenobarbital had a limited influence on the induction of neoplasia. It was found that Fischer F344 rats exposed to PB in the diet for two years showed no changes in the incidence of neoplastic nodules at 500 or 600 ppm **(Hagiwara *et al*., 1999)**.

In the liver, initiation can be induced by a single dose of any carcinogen only if associated with a regenerative stimulus (i.e., physiological developmental growth, necrosis induced by the carcinogen itself or by

chemicals, or viral infections in humans) **(Farber and Sarma, 1987; Stuver *et al*., 1996)**. The liver cell necrosis induced by DENA is known to be the proliferative stimulus essential for the initiating event **(Farber and Sarma, 1987).** Surprisingly, it was found that a subnecrogenic dose of diethylnitrosamine (DENA) induced the development of focal lesions in rats refed after complete food withdrawal, but not in regularly fed animals **(Tessitore *et al*., 1996).**

Among several protocols of rat hepatocarcinogenesis, the resistant hepatocyte (RH) model distinguishes early and easily persistent lesions from remodeling ones through the use of histochemical staining procedures **(Tatematsu *et al*., 1983).** Consequently, the preneoplastic lesions can be easily identified, quantified, and their number and size determined. In addition, using the RH model, investigators have assessed the effects of compounds that are potentially able to modulate an ongoing carcinogenic process **(Sampaio *et al*., 2007).** The model consists in initiating hepatocarcinogenesis by a single dose of diethylnitrosamine (DENA) or other carcinogens and in selecting hepatocytes resistant by a brief exposure to 2-acetylaminofluorene (2-AAF) followed by a mitogenic stimulus such as partial hepatectomy (PH) (**Tatematsu *et al*., 1983).**

Induction of hepatocarcinogenesis using DENA was performed by several protocols. Some research work relied solely on the use of multiple intraperitoneal injection of DENA as reported by **Zhang *et al*. (2013)** who performed an intraperitoneal injection of DENA (100 mg/kg body weight/week) once a week for a period of 3 weeks. Also intragastric

administration of 1% aqueous solution of DENA (70 mg/kg) once a week, consecutively for 14 weeks was used **(Liu *et al*., 2009).**

Hepatocellular carcinoma in rats was also induced by providing 0.01% DENA through drinking water for 15 weeks **(Arul and Subramanian, 2012)** or at a concentration of 40 ppm in drinking water from one to five weeks of experiment **(Okuno *et al*., 1998)**

On the other hand, a single intraperitoneal injection of 200 mg DENA/kg body weight dissolved in saline to initiate hepatocarcinogenesis followed by a promoting substance such as Orphenadrine or Phenobarbital after 2 weeks of injection was mostly conducted **(Morita *et al*., 2013a, Morita *et al*., 2013b).** Similarly, **Bishayee *et al.* (2011)** used a single intraperitoneal DENA injection at a dose of 200 mg/kg body weight but it was mixed with peanut oil. Then after a 2-week recovery period, the promoter PB was incorporated into the drinking water at the concentration of 0.05% for 18 successive weeks. In some protocols, PB (0.05 % in drinking water) was given after one week of DENA I/P injection for 19 weeks (**Jagadeesh *et al*. 2011).**

In the model using both DENA and Phenobarbital, the numbers and areas of placental glutathione S transferase (GST-P) positive foci were significantly increased after 6 weeks of treatment with phenobarbital at doses of 60 ppm and over. Interestingly treatments with low doses tended to decrease development of GST-P positive foci, and particularly their numbers at 2 ppm and the areas at 1 and 2 ppm were significantly lowered. Thus, the induction curve of GST-P positive foci showed a J-shape, indicating a

hormesis phenomenon **(Ito *et al*., 2003).** Additional data showed phenobarbital treatment at 2 ppm inhibited cell proliferation within GST-P positive foci in clear contrast to the enhancement observed with 500 ppm **(Kinoshita *et al*., 2003).**

Some other protocols used also a single intraperitoneal DENA injection at a dose of 200 mg/kg body weight dissolved in saline for initiation but used carbon tetrachloride after 2 weeks in a single dose (2 mL/kg) by gavage as 1:1 dilution in corn oil to stimulate liver cell proliferation and regeneration (**Al-Rejaie *et al*., 2009).** Moreover, liver preneoplasia was initiated by intraperitoneal injection of diethylnitrosamine (once at 100 mg/kg body weight) or by 2-nitropropanel (6 times at 100 mg/kg body wt). After three week, rats received a diet containing 50 mg/kg 2-acetylaminofluorene during the two following weeks, in the middle of which two-thirds partial hepatectomy was performed on all rats **(Astorg *et al*., 1997).**

Moreover, 2-acetylaminoflorene has been used as a promoting agent as it was indicated in a previous study. In this study, the initiation was performed at 42 days of life by means of a single intraperitoneal dose of DENA (200 mg/kg body weight) dissolved in saline solution. Seventeen days after the initiation, the animals received, during 4 consecutive days through gavage, 2-AAF (20 mg/kg body weight) dissolved in propyleneglycol. On the fifth day after beginning of 2-AAF administration, a two-thirds Partial hepatectomy was performed as a mitogenic stimulus. In the second and fourth days after hepatectomy, an additional dose of 2-AAF (20 mg/kg body

weight) was administered. All animals were sacrificed at 110 days of life (**Silva-Oliveira *et al*., 2010)**.

Gamma benzene hexachloride (γ –BHC) which after metabolism induces microsomal cytochrome P-450 (CYP-450) enzymes in the liver, has also been used as a promoting agent after DENA I/P injection **(Gupta *et al*., 2011).**

Partial hepatectomy in rats has also been conducted in several protocols in order to induce regenerative cell replication. This was performed at 4 or 3 weeks after DENA I/P injection **(Gupta *et al*., 2011; Morita *et al*., 2013)**

In a previous study, five doses ranging from a cumulative total of 0.5 to 4 mmol DENA per kg body weight were given as weekly I/P injections for 10 weeks. This was followed by 4 weeks recovery, after which the groups were maintained on either a basal diet or 0.05% phenobarbital (PB) to promote liver tumor development. It was shown that 2.0 mmol DENA/kg has produced substantial cytotoxicity, i.e. approximately 30%, and cell proliferation was increased approximately 4-fold over the next lower exposure. At this exposure, tumors were induced in 100% of rats even without PB promotion and accordingly, this exposure was strongly carcinogenic. Up to this exposure, the enhancement of cell proliferation, the induction of hepatocyte altered foci (HAF) and the increment in tumorigenicity were non-linear, indicating a synergism of effects. Presumably, the enhanced cell proliferation resulted in more cells being transformed because of conversion of promutagenic DNA damage to permanent mutations during replication (**Williams *et al*., 1996).**

2.2. Biochemical changes in hepatocarcinogenesis

DENA causes alterations in some enzymes found in the serum and tissues. When the liver cells are injured, several types of liver-specific enzymes, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phospatase (ALP) and γ-glutamyltransferase (GGT), are elevated**.** Studies indicated that DENA elevates the activities of these enzymes and also total bilirubin **(Al-Rejaie *et al*., 2009).** It was found that a single intraperitoneal administration of diethylnitrosamine (200 mg/kg) to rats resulted in significantly elevated levels of serum aspartate transferase (AST) and alanine transferase (ALT) **(Pradeep *et al*., 2007).** AST, ALT and ALP are the group of enzymes, which are used to evaluate the status of liver damage and are considered more sensitive parameters to assess liver injury in rodent species **(Limdi and Hyde, 2003).**

G-GT is an enzyme embedded in the hepatocyte plasma membrane, mainly in the canalicular domain, and its liberation into serum indicates damage to the cell and thus injury to the liver (**Bulle *et al*., 1990; Sivaramakrishnan *et al*., 2008)**. It is important to point out that serum G-GT activity is considered to be one of the best indicators of liver damage **(Bulle *et al*., 1990).**

Moreover, DENA significantly increased total nitrate/nitrite (NOx) and thiobarbituric acid reactive substances (TBARS) and decreased reduced glutathione (GSH), glutathione peroxidase (GSHPx) and catalase (CAT) in liver tissues, suggesting that reactive oxygen and nitrogen species induced

by DENA play an important role in DENA-induced hepatic carcinogenesis **(Al-Rejaie *et al*., 2009).**

It has been reported that reactive oxygen species (ROS) play a major role in tumor promotion through interaction with critical macromolecules including lipids, DNA, DNA repair systems, and other enzymes (**Kensler and Trush, 1984).** There was an increased generation of reactive oxygen species and decreased antioxidant enzymes in liver tissues in many models of DENA induced hepatocellular carcinoma (**Sivaramakrishnan *et al*., 2008**).

Diethylnitrosamine induced oxidative stress was confirmed by elevated levels of lipid peroxidation and decreased levels of superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase (GR) and glutathione-S-transferase (GST) in the liver tissue. The status of non-enzymic antioxidants like, vitamin-C, vitamin-E and reduced glutathione (GSH) were also found to be decreased in diethylnitrosamine administered rats. Further, the status of membrane bound ATPases was also altered indicating hepatocellular membrane damage **(Pradeep *et al*., 2007).**

It was also observed that DENA increased the plasma level of nitrite oxide which is one of the major mediators in chronic inflammatory processes **(Wang *et al*., 2002; Atakisi *et al*., 2013).** This increase may be due to the enzymatic or chemical break down of N-NO bound found in the chemical structure of DENA, which leads to the release of NO **(Atakisi *et al*., 2013).** It was speculated that DENA could potentially serve as NO/NO+ donor. The break down of α-hydroxy-N-nitroso compounds could indirectly

result in DNA damage via the generation of alkylating agents that are produced from N-nitrosamines **(Wang *et al*., 2002).** Nitric oxide can interact with superoxide (O2·) to form peroxynitrite (ONOO-) which is also a powerful oxidant capable of oxidizing potentially dangerous reactions including thiol groups of proteins that lead to cellular damage. Other than its own oxidizing effect, breakdown of peroxynitrite could give rise to the production of hydroxyl radical (·OH) which is a potent oxidant leading to cell injury **(Iraz *et al*., 2005).** It was found that Uncontrolled, prolonged and/or massive production of NO by inducible NOS (iNOS) may cause liver damage, inflammation and even tumor development **(Ahn *et al.*, 1999).**

It was reported that the enhancement of Gap junction intercellular communication (GJIC) and the inhibition of inflammation, Ornithine decarboxylase (ODC) activity, cell proliferation, and Matrix metalloproteinases (MMPs) production, among others, are considered as important biomarkers, which are involved in blocking tumor promotion and tumor progression processes in multistage carcinogenesis **(Lee *et al*., 2004).**

Absolute and relative liver weights in rats which received 0.2%, 1% and 5% turmeric diet after DENA treatment were comparable with those in DENA-treated group and significantly higher when compared with untreated control **(Thapliyal *et al*., 2003).** Further, the final body weights of the rats receiving the carcinogen (DENA) were slightly lower than those of rats in the normal control group (**Zhang *et al*., 2013).**

2.3. Pathological findings of hepatocellular carcinoma

Numerous studies have focused on a series of microscopic lesions called "foci" and "nodules" that have been designated "preneoplastic" or "premalignant" **(Farber, 1984).** These preneoplastic lesions can be easily identified, counted, and their number and size can be determined by morphometry to measure multistage hepatocarcinogenesis **(Farber, 1984; Pitot, 1990).**

The histological changes during the hepatocarcinogenesis in DENA-treated rat models were similar to those seen in humans, including non-specific damage, fibrosis, cirrhosis, dysplastic nodules, early tumorous nodules, progression and metastasis, which appeared to be sequential events. **(Liu *et al*., 2009).**

In the experimental hepatocarcinogenesis, foci of altered hepatocytes appear as small microscopic collections during or shortly after initiation with different carcinogens **(Tsuda *et al*., 1980).** After further exposure to carcinogens or to other environmental tumor promoting, foci of altered hepatocytes grow to become grossly visible nodules **(Tatematsu *et al*., 1983).** These lesions have been designated as preneoplastic **(Farber, 1984).** However, most of the phenotypically altered hepatocyte nodules (93–98%) remodel to a normal appearing liver through a complex process involving cell structure and architecture, blood supply, and biochemical properties; whereas a small subgroup of these lesions persists, some of which may progress into hepatocellular carcinoma **(Tatematsu *et al*., 1983; Farber and Rubin, 1991).**

The animals submitted to the resistant hepatocyte (RH) model of hepatocarcinogenesis presented persistent and remodeling lesions that varied in number, size, and staining intensity. The lesions classified as persistent were those sharply delimited from the surrounding parenchyma of the liver, presenting uniform negative staining for Glucose-6-phosphatase. Remodeling lesions were those exhibiting irregular boundaries with the surrounding parenchyma and non uniform G6Pase negative staining (**Silva-Oliveira *et al*., 2010**).

Histological sections of the liver stained by hematoxilin and eosin showed partial disruption in the hepatic architecture due to the presence of nodules. The nodules were generally rounded, heterogeneous in size, and sometimes delimited from the surrounding parenchyma by thin layers of conjunctive tissue. The hepatocytes inside these lesions presented enlarged nuclei and nucleoli and different size and color (eosinophilic or basophilic) from the normal cell. The sinusoids in the inner portion of the nodules were, at times, compressed by the enlarged hepatocytes (**Silva-Oliveira *et al*., 2010)**.

The histological changes of livers of the DENA-treated rats (intragastric administration of 1% aqueous solution of DENA (70 mg/kg) once a week, consecutively for 14 weeks) can be divided into three stages. Initially, from the 2nd to 8th week, non-specific injury occurred such as cellular swelling, fatty changes, necrosis, inflammatory infiltration and hepatocyte regeneration. On the 10th to the 14^{th} week, significant liver fibrosis occurred. At the 10th week, the livers showed a quantitative increase in connective tissue, and encapsulation of regenerative nodules, while at the

end of the 12th week, nodular cirrhosis could be seen macroscopically. At the 14th week, gray-white nodules, 3 mm to 5 mm in diameter, could be distinguished from the surrounding reddish brown cirrhosis nodules in the livers of 2/10 rats. These were histologically diagnosed as dysplastic nodules. From the 16th to the 20th week the number of nodules increased significantly. At the 16th week, nodules, 5 mm to 1.5 cm in diameter, could be distinguished in the livers of 8/10 rats, while at the 18th and the 20th week, gray-white nodules were present in the livers of all 20 rats. In addition, by the 20th week, abdominal cavity and lung metastases were observed in 2/10 rats **(Liu *et al*., 2009).**

At 6 weeks from Phenobarbital or orphenadrine administration, histopathological examination of liver samples from rats given intraperitoneal injection of diethylnitrosamine showed centrilobular hepatocyte hypertrophy with eosinophilic cytoplasm and diffuse vacuolar degeneration in the Phenobarbital and/ or orphenadrine treated groups. Eosinophilic hepatocyte altered foci were also observed in the high Phenobarbital, high orphenadrine and Phenobarbital plus orphenadrine groups (**Morita *et al*., 2013b).**

At 8 weeks from DENA injection, the histological examination of liver revealed disorganization of normal hepatic architecture and the presence of oval- or irregular-shaped hepatocytes in the DENA-treated rats with or without Gamma benzene hexachloride (γ -BHC) treatment **(Gupta *et al*., 2011).**

At 15 weeks after 0.01 % DENA oral administration, liver showed loss of architecture and tumour cells were smaller than normal cells with granular cytoplasm and large hyperchromatic nuclei. The ultrastructural studies of the liver cells in DENA group showed presence of multiple nuclei close to each other with irregular cytoplasm which demonstrates dysplasia **(Arul and Subramanian, 2012).**

The Gross visible persistent nodules (PNs) represent focal proliferating, gamma glutamyltranspeptidase- positive hepatic lesions with a low tendency to spontaneous regression. The persistent nodules include altered hepatocyte foci (AHF) /hyperplastic foci (areas of cellular alterations) and hyperplastic nodules (neoplastic nodules). Hyperplastic foci are the lesions smaller than a liver lobule in size and are mainly visible microscopically. The hyperplastic nodules are generally spherical lesions that usually occupy an area equivalent in size to that of several liver lobules with architectural distortion **(Farber, 1984a; Farber, 1984b).**

Phenotypically altered hepatocyte populations including persistent nodules and hyperplastic foci of cellular alteration (AHF) representing small to large aggregates of tinctorially distinct hepatocytes within the hepatic parenchyma being considered as preneoplastic lesions were found scattered in the livers of all 2-AAF-treated groups The H&E stained sections of liver slices revealed hepatocellular focal lesions that were clearly distinguishable from the non nodular surrounding parenchyma (NNSP) (**Chakraborty *et al*., 2007).**

A gross alteration in hepatocellular architecture was noticed with different types of focal lesions. Basophilic foci with irregular boundaries in 2-AAF-control rats were seen. Basophilic foci were frequently comprised of hepatocytes that are smaller than the surrounding hepatocytes. Some nuclei in the cells were large and hyperchromatic (basophilic) with prominent and centrally located nucleoli. A large and prominent eosinophilic focus of cellular alteration was observed that has a well demarcated border. Hepatocytes comprising eosinophilic foci typically have an increased cytoplasm that stains more eosinophilic than the cytoplasm of surrounding hepatocytes. A vacuolated focus was comprised of a sharply demarcated collection of hepatocytes containing clear spaces. The altered hepatocytes of foci were found to be consistently enlarged with more than 1 nucleus, and hepatocytes appeared oval or irregular in shape. (**Chakraborty *et al*., 2007)**.

Moreover, occasional mitosis and binucleation was detected in individual hepatocytes of altered hepatocellular foci. Oval cell hyperplasias as well as bile duct hyperplasia have been recorded in the livers of rats treated with DENA **(Farag *et al*., 2014).**

2.4. Immunohistochemical findings in HCC

The induction of **glutathione S-transferase placental form (GST-P)** positive foci in the liver is considered as an early biomarker of hepatocarcinogenesis and is used as an alternative method for the long-term carcinogenicity testing **(Ito *et al*., 2003)**. GST-P is involved in the detoxification of xenobiotics and its expression is increased in response to the toxic effects of DENA **(Parody *et al*., 2007). Van Gijssel *et al*. (1997)**

have reported that GST-P expression in a single hepatocyte promotes cell proliferation as well as its own expression in the adjacent cells.

It was found that at the end of the recovery period (14 weeks), hepatocellular altered foci, which expressed the placental form of glutathione S-transferase, were induced by all exposures to DENA , with an increase of -4-fold between the exposures of 1.0 and 2.0 mmol/kg **(Williams *et al*., 2006).**

It was shown that reducing the single initiating dose of DENA to 100mg/kg induced as many GST-P-positive foci as the higher dose **(Astorg *et al*., 1994)** however, it induces fewer gamma glutamyl transpeptidase (γ-GT) positive foci, and the foci of both types are smaller. The GST-P-positive foci were 8-20 times more numerous than the γ-GT-positive foci but were two to four times smaller **(Astorg *et al*., 1997).**

γ-Glutamyl transpeptidase and placental glutathione S-transferase-positive foci also known as enzyme altered foci were detected in frozen-cut liver sections by histochemical and immunohistochemical techniques, respectively **(Astorg *et al*., 1997).** Serial cryostat liver sections were stained for γ-GT according to the histochemical procedure of **Rutenburg and co-workers (1969).**

Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) as a cell proliferation marker and protein expression of the apoptosis inducer Bax and the apoptosis repressor Bcl-2 were carried out for

evaluating antihepatocarcinogenic effect of some plant extracts as described in previous studies (**Bishayee *et al*., 2011).**

2.5. Camel milk

CM exhibits a wide range of biological activities; antimicrobial, antioxidative, antithrombotic, antihypertensive, and immuno-modulatory effect **(FitzGerald and Meisel, 2000; Kohonen and Pihlanto, 2003; Saltanat *et al*., 2009).** In addition, the treatment with camel's milk was found to alleviate alcohol-associated hazards and protects hepatic tissue from alcohol-induced toxicity **(Darwish *et al*., 2012).**

Recent studies have reported that camel milk is the most effective milk among other species against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, and rotavirus (**Conesa *et al*., 2008).** In addition, it has been demonstrated that camel milk, in addition to secretory IgA and IgM, also contains numerous non-antibody components which possess antiviral activity, including lactoferrin **(Redwan and Tabll, 2007).**

Camel milk is often used as an adjuvant treatment for several chronic diseases, such as diabetes mellitus, or in allergic patients **(Cardoso *et al*., 2010; Ehlayel *et al*., 2011).** However, scientific basis for the positive effect of camel milk improving the health status of those patients especially those suffering from hepatitis is not yet understood.

Interestingly, A recent work have shown the ability of camel milk to significantly inhibit the induction of the cytochrome P4501A1 (*Cyp1a1)*, a

cancer-activating gene, and to induce the NAD(P)H : quinone oxidoreductase 1 *(NQO1)*, cancer chemopreventive gene in murine hepatoma Hepa 1c1c7 cells at the transcriptional and posttranscriptional levels **(Korashy *et al*., 2012a).** Camel milk significantly inhibited human hepatoma (HepG2) and human breast cancer cells (MCF7) proliferation through the activation of caspase-3 mRNA and activity levels, and the induction of death receptors in both cell lines. In addition, Camel milk enhanced the expression of oxidative stress markers, heme oxygenase-1 and reactive oxygen species production in both cells. Mechanistically, the increase in caspase-3 mRNA levels by camel milk was completely blocked by the transcriptional inhibitor, actinomycin D; implying that camel milk increased *de novo* RNA synthesis. Furthermore, inhibition of the mitogen activated protein kinases differentially modulated the camel milk-induced caspase-3 mRNA levels. Taken together, camel milk inhibited HepG2 and MCF7 cells survival and proliferation through the activation of both the extrinsic and intrinsic apoptotic pathways **(Korashy *et al*., 2012b).**

Camel's milk (CM) is an excellent source of well balanced nutrients and also exhibits a range of biological activities that influence digestion, metabolic responses to absorbed nutrients, growth and development of specific organs and resistance to diseases. These biological activities are mainly due to the presence of peptides and protein in milk **(Korhonen and Pihlanto, 2001).** CM is low in fat, high in protein and vitamin C than cow's milk. It also contains fat with a relatively large amount of polyunsaturated fatty acids and linoleic acids, which are essential for human nutrition (**Gorban and Izzeldin, 2001**).

CM is different from other ruminant milk; it is lower in cholesterol, protein and sugar, but higher in minerals, vitamins, and insulin **(Yousef, 2004).** It has no allergic properties and can be consumed by lactose-intolerant individuals **(Cardoso *et al*., 2010).**

Camel milk possesses a protein system constituted by two major classes of proteins: caseins and whey proteins. Caseins account for 80 % (w/w) of the total milk protein content **(Shah, 2000)** and whey contains numerous proteins such as immunoglobulins, α-lactalbumin, lactoperoxidase, lysozyme and lactoferrin, among other proteins with biological functions **(El-Agamy, 2000).**

Moreover, camel milk contains PP3 fraction, also called lactophorin, as a major whey protein fraction. The PP3 component of camel milk belongs to the glycosylation-dependent cell adhesion molecule (GlyCAM-1) family **(Girardet *et al*., 2000)** and could, therefore, play an immunological role in camel or its suckling young. The number and sequence of amino acid residues (137 residues) of camel PP3 differ from that of cow milk (123 residues), particularly in the N-terminal part of the molecule **(Girardet *et al*., 2000).**

The antimutagenic potential of casein was investigated by **Van Boekel *et al*. (1993)** using several mutagens. They found that preincubation increased the antimutagenic potential of casein towards N-nitroquinoline-1-oxide (NQO). They added that the antimutagenic potential of casein increased with pepsin hydrolysis. They postulated that this increase was due to the peptides formed and might be explained by a better accessibility of casein peptides

for interaction with mutagens. In addition, **Bosselaers *et al.* (1994)** studied the possible antimutagenic effect of five different proteins including casein. They found that casein significantly inhibited 4-nitroquinoline 1-oxide (4-NQO) and 1-methyl-1-nitroso-3-nitroguanidine (MNNG) induced sister chromatid exchange (SCE). They also reported that pepsin-hydrolysed casein inhibited SCE induction by 4NQO and MNNG. Therefore, they concluded that casein and its pepsin hydrolysis products may protect mammalian cells against certain genotoxic compounds. They added, although the mechanism of antimutagenicity is unknown, it seems believable that the protein acts as a blocking agent by chemical or physical interaction with the mutagens. They added that the accessibility of protein molecules and the presence of nucleophilic binding sites may be significant factors in determining the antimutagenic properties of proteins. Moreover, **Goeptar *et al*. (1997)** presented that enzymatic digestion of sodium caseinate greatly improved its antimutagenicity potential. They suggested that the molecular structure of a protein determines the protective effect against mutagens. They added that a stronger protection appears to correspond with a lack of secondary and tertiary structure. Their findings shed new light on the possible prevention of mutagenesis and/or carcinogenesis by food proteins, with a unique role for milk proteins.

It was proven that Camel casein induced apoptosis and that the viability of Huh7.5 cells was greatly reduced **(EL-Fakharany *et al*., 2012).** The apoptosis caused by camel casein is in accordance with the results published in the study of **Håkansson *et al*. (1995)** in which it was reported that human casein (α-lactalbumin bound to casein was implicated in its apoptotic activity) and bovine α-lactalbumin unfolded and forming a complex with

oleic acid called "human α- lactalbumin made lethal to tumor cells" (HAMLET), induced differential and significant apoptosis in several cancer lines **(Hallgren *et al*., 1998).** It was also described in a previous study that camel casein initiated apoptosis in HepG2 cells, reducing their viability and consequently, the detection of HCV RNA **(Almahdy *et al*., 2011)**.

Lactoferrin plays an important and multifunctional role in innate and specific host defense against infection by microorganisms, alone or with other milk proteins such as lysozyme and immunoglobulins **(Farnaud and Evans, 2003).**

Lactoferrin is an iron-binding glycoprotein of the transferrin family **(Al-Majali *et al*., 2007).** This relatively recent known protein has a number of properties such as antibactericidal activity, antiviral, antifungal, anticarcinogenic, anti inflammatory activity, antioxidant and analgestic properties (**Konuspayeva *et al*., 2004).** A comparative survey of lactoferrin concentrations in different milks showed that camel's milk contains the greatest amount of lactoferrin **(Konuspayeva *et al*., 2004).** Lactoferrin inhibit the HCV infectivity starting from 0.25 mg/ml. However, α-lactalbumin, human IgGs and casein failed to demonstrate any activity against HCV infectivity **(EL-Fakharany *et al*., 2012).**

The protective character of lactoferrin has been demonstrated, on numerous occasions, on chemically induced tumors in laboratory rodents. Lactoferrin has even been reported to inhibit the development of experimental metastases in mice **(Wolf *et al*., 2003)**. Lactoferrin- mediated inhibition of tumor growth might be related to apoptosis of these cells,

induced by the activation of the Fas signaling pathway. Nevertheless, the exact mechanism of this function has not been discovered so far **(Fujita *et al*., 2004).**

Lactoferrin was thought to support cell proliferation due to its ability to transport iron into cells. However, lactoferrin has later been proven to act as a growth factor activator. The effect of lactoferrin alone on small intestine epithelial cells is more potent than that of the epidermal growth factor. Lactoferrin alone (without the presence of any other cytokines and factors) is able to stimulate the proliferation of endometrium stroma cells. Lactoferrin has also been identified as a transcription factor. It can penetrate a cell and activate the transcription of specific DNA sequences **(Adlerova *et al*., 2008)**

Camel milk lactoferrin exerted antioxidant activity through scavenging nitric oxide radical (NO_) and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, as well as the capability to furnish reducing power as evident by the ferric-reducing/antioxidant power (FRAP) and the total antioxidant assays. lactoferrin also inhibited DNA damage most likely through binding catalytic iron. Moreover, Lactoferrin inhibited the growth of colon cancer cells **(Habib *et al*., 2013)**

α-Lactalbumin is a low molecular weight acidic protein (14.2 KDa) present in the whey fraction of milk. Recently, the capacity of defending the newborn from pathogenic microorganisms has been putatively ascribed to α-lactalbumin **(Pellegrini *et al*., 1999; Salami *et al*., 2009).** α-Lactalbumin itself does not possess any antimicrobial activity; however, when it changes

to a particular conformation it acquires antimicrobial and antitumoral properties **(Pellegrini *et al*., 1999).**

Camels have a unique and special class of antibodies which were referred to as Heavy-chain antibodies (HCAbs) because they lack the classical light-chain and are composed of a homodimer of heavy-chains **(Hamers-Casterman *et al*., 1993).** It was found that Camel milk naïve polyclonal IgGs isolated from camel milk could inhibit the HCV infectivity and demonstrated strong signal against its synthetic peptides **(EL-Fakharany *et al*., 2012).**

The value of camel milk is due to its high content of volatile acids espicielly linoleic acid and polyunsaturated fatty acid which are essential for human nutrition **(Konuspayeva *et al*., 2008).** Conjugated linoleic acid ‘‘CLA’’ was identified as a component of milk and dairy products for over 30 years ago. It is formed as an intermediate in the course of the conversion of linoleic acid into oleic acid in the rumen **(Kritchevsky, 2000).** CLA compounds could serve as useful food antioxidants and provide additional value because of their potential bioactivity in disease prevention **(Badr El-Din and Omaye, 2007).** The results obtained by **Liew *et al*. (1995)** supported a mechanism involving the inhibition of carcinogen activation by CLA, as opposed to direct interaction with procarcinogen, scavenging of electrophiles or selective induction of phase I detoxification pathways. Moreover, **Yang *et al*. (2001)** concluded that CLA modulate phIP (2-amino-1-methyl-6-phenylimidazol [4, 5-b] pyridine) induced mutagenesis in a tissue-specific manner.

Farah *et al*. (1992) reported that the vitamin C content in camel's milk is about three times higher than that in cows' milk. This high value contributes to a consideration that camel milk has a stimulating effect on the human immune system **(Elkhidir, 2002).**

The anticytotoxic and antigenotoxic effects of most of the Camel Milk constituents against the genotoxic effects of chemicals were investigated (e.g. vitamin C: **Rao *et al*., 2001,** Selenium: **Hassan *et al*., 2006,** Zinc: **Hurna´ and Hurna´, 2000**; Casein: **Goeptar *et al*., 1997**; Lactoferrin: **Konuspayeva *et al*., 2004**).

Camel's milk was found to contain high concentrations of vitamins A, B2, C and E and is very rich in magnesium and other trace elements **(Yousef, 2004).** These vitamins act as antioxidants and have been useful in preventing toxicant- induced tissue injury.

2.6. Curcuma longa

Polyphenolic phytoconstituents of dietary origin have been shown to possess potent preventive and therapeutic properties. Dietary polyphenols such as anthocyanins, curcumin, resveratrol, as well as catechins and theaflavins—the tea polyphenols have been critically evaluated for their pharmacological effects in several serious illnesses **(Bishayee and Darvesh, 2010; Catalgol *et al.*, 2012).** Dietary polyphenols owe their therapeutic effects to their potent antioxidant, antiinflammatory properties as well as their ability to modulate several enzymes and signaling pathways **(Izzi *et al*., 2012).**

Curcuma longa is a rhizomatous perennial herb that belongs to the family Zingiberaceae, native to South Asia and is commonly known as turmeric. Turmeric (*Curcuma longa* L.) has been used as a food additive in curries to improve palatability and storage stability. Curcuminoid, a natural coloring agent, is recognized as a rich source of phenolic compounds, consisting of three different compounds: curcumin, demethoxycurcumin and bisdemethoxycurcumin (**Surojanametakul *et al*., 2010)**. It also has potential as a pharmaceutical expient, since it possess antioxidant, anti-inflammatory, antimutagenic and anti HIV properties and can reduce blood glucose **(Du *et al*., 2006)** and LDL **(Fan *et al*., 2006).**

In addition, turmeric plant is used as herbal remedy due to the prevalent belief that the plant has medical properties. In Ayurveda, turmeric has been used internally as a stomachic, tonic and blood purifier and externally in the prevention and treatment of skin diseases **(Anon, 2001).** Traditional Indian medicine claims the use of its powder against biliary disorders, anorexia, coryza, cough, diabetic wounds, hepatic disorder, rheumatism and sinusitis **(Ammon *et al*., 1992).** It was reported that orally administered curcuminoids enter into the general blood circulation in rats, and are present exclusively as glucuronide and glucuronide-sulfate conjugate forms **(Asai & Miyazawa, 2000).**

In folk medicine, the rhizome juice from *C. longa* is used in the treatment of many diseases such as anthelmintic, asthma, gonorrhea and urinary, and its essential oil is used in the treatment of carminative, stomachic and tonic **(Phansawan and Poungbangpho, 2007).**

Curcumin is one of the most attractive compounds to be explored for chemoprevention of cancers because of its nontoxic nature and multiple beneficial clinical effects **(Ammon and Wahl, 1991).**

Turmeric and curcumin(s), the yellow phenolic compounds in turmeric, have shown their efficacy in preventing chemical-induced experimental carcinogenesis in various tissues including skin **(Limtrakul *et al*., 1997)**, colorectal **(Pereira *et al*., 1996)**, forestomach **(Deshpande *et al*., 1997)**, and mammary glands **(Deshpande *et al*., 1998).**

A significant reduction in tumor incidence when turmeric was administered during initiation phase as opposed to the post-initiation phase is suggestive of antiinitiating effects of turmeric/ curcumin in vitro **(Deshpande and Maru, 1995)** as well as in vivo **(Deshpande *et al*., 1998)**. The anti-initiation effects of turmeric/curcumin in experimental systems have mostly been established employing representative polycyclic aromatic hydrocarbon(s) (PAHs) which require metabolic activation. Turmeric/ curcumin(s) have been shown to inhibit isozymes of CYP 450s in vitro as well as in vivo **(Thapliyal and Maru, 2001).**

DENA-treated rats receiving 1 or 5% turmeric before, during and after carcinogen exposure showed significant decrease in number of gamma glutamyl transpeptidase (GGT) positive foci measuring 500 or _ 1000 mm and decrease in the incidence of DENA-induced focal dysplasia (FD) and hepatocellularcarcinomas. Decrease in the number of GGT positive foci measuring 1000 mm was also observed in DENA-treated rats receiving 0.2% turmeric, although no decrease in tumor incidence was noted. On the other

hand, similar levels of turmeric treatment (0.2, 1 and 5%) after exposure to DENA did not show any protective effects **(Thapliyal *et al*., 2003).**

The curcuminoids are poorly soluble in the hydrocarbon solvents, therefore alcohol and acetone are a good extractants and the yields can also be expected to be high because of extraction of non-flavor components. Soxhlet extraction of turmeric powder with acetone gave a yield of about 4.1% containing in 3 hours. Acetone as solvent was slightly superior to alcohol and ethyl acetate, the curcuminoids content also is on the high side, suggesting selective extraction. The results of extraction with acetone have, however been reported to give high yields of curcuminoids than alcoholic and remaining extraction **(Popuri and Pagala, 2013).**

Numerous analytical methods have been reported by some researchers for quantitative analysis of curcuminoids. Some of the methods are based spectrophotometric techniques, expressed as the total color content of the sample. However, using this technique it is not possible to separate and to quantify the curcuminoids individually **(Jayaprakasha *et al*., 2002).** For this reason, chromatographic based techniques and electrophoresis are among the methods of choice for determination of curcuminoids attributed to their separation capacities.

Turmeric extract was found to possess in vitro free radical scavenging, ROS scavenging ability and cell proliferation activity in cell line **(Suhit *et al*., 2010).** Moreover, the previous studies have shown that the aqueous extract of *C. longa* has hepatoprotective activity against carbon tetrachloride toxicity in mice. It was suggested that *C. longa* has an immunotherapeutic

properties along with its ability to ameliorate hepatotoxicity **(Sengupta *et al*., 2011).**

Similarly, it was said that the progression of liver cirrhosis could be inhibited by the antioxidant and anti-inflammatory activities of Curcuma longa rhizome ethanolic extract (CLRE) and the normal status of the liver could be preserved **(Salama *et al*., 2013).**

The main constituents of CLRE extract are the flavonoid curcumin and various volatile oils, including tumerone, atlantone, and zingiberene. The hepatoprotective effects of turmeric and curcumin might be due to direct antioxidant and free radical scavenging mechanisms, as well as the ability to indirectly augment glutathione levels, thereby aiding in hepatic detoxification **(Girish *et al*., 2009).** The volatile oils and curcumin of C. longa exhibit potent anti-inflammatory effects **(WHO, 2002).**

It was reported that CLRE induced apoptosis, inhibited hepatocytes proliferation but had no effect on hepatic CYP2E1 levels **(Salama *et al*., 2013).** A significant increase in the serum level of Bax protein and decrease in Bcl-2 protein in CLRE- treated animals was recorded compared with the cirrhosis group animals. This was confirmed by the ratio Bax/Bcl-2 which was high in the treated groups compared with the cirrhosis group and the large number of Bax positive-stained hepatocytes together with few Bcl2 positive-stained hepatocytes at both doses of CLRE-treated animals, compared with the cirrhosis Group) indicating the susceptibility of these cells to apoptosis and the role of curcuminoids in inducing apoptosis **(Wang *et al*., 2012; Salama *et al*., 2013).**

Curcumin suppresses the growth of several tumor cell lines, including drug-resistant lines **(Mukhopadhyay *et al*., 2002).** It suppresses the expression of cyclin D1, which is deregulated in a wide variety of tumors. Cyclin D1 is a component subunit of cyclin-dependent kinases (CDK) 4 (Cdk4) and 6 (Cdk6), which are rate limiting in progression of cells through the cell cycle **(Mukhopadhyay *et al*., 2002).**

Curcumin also suppresses the activation of several transcription factors that are implicated in carcinogenesis **(Aggarwal *et al*., 2003).** It suppresses the activation of nuclear factor kappa B (NF-_B), activator protein 1 (AP-1), and at least two of the signal transducer and activator of transcription proteins (STAT3, STAT5), and modulates the expression of early growth response protein 1 (Egr-1), peroxisome proliferator associated receptor gamma (PPAR-_), _-catenin, and Nrf-2. Curcumin also modulates expression of genes involved in cell proliferation, cell invasion, metastasis and angiogenesis **(Aggarwal *et al*., 2003).**

Curcumin suppresses the activation of NF-_B induced by various tumor promoters, including phorbol ester, TNF, and hydrogen peroxide (**Singh and Aggarwal, 1995).** Subsequently, others showed that curcumin-induced downregulation of NF-_B is mediated through suppression of I_B_ kinase (IKK) activation **(Plummer *et al*., 1999).**

It has been shown to downregulate the expression of Bcl-2, *Bcl*XL, cyclooxygenase 2 (COX-2), matrix metalloproteinase (MMP)-9, tumor necrosis factor (TNF), cyclin D1, and the adhesion molecules (**Shishodia *et***

***al.*, 2005).** Numerous studies in animals have demonstrated that curcumin has potent chemopreventive activity against a wide variety of tumors.

Curcumin, was reported to enhance apoptosis of damaged hepatocytes which might be the protective mechanism whereby curcumin down-regulated inflammatory effects and fibrogenesis of the liver **(Wang *et al*., 2012).** Curcumin seems to regulate the immune function of mice in a dose-dependent fashion as curcumin treatment enhanced the phagocytosis of peritoneal macrophages and differentially regulates the proliferation of splenocytes **(Li and Liu, 2005).**

Curcumin has been found to modulate the growth and cellular response of various cell types of the immune system. Numerous lines evidence suggests that curcumin can modulate both the proliferation and the activation of T-cells. Curcumin inhibited the proliferation induced by concanavalin A (Con A), phytohemagglutinin (PHA) and Phorbol-12-myristate-13-acetate (PMA of lymphocytes derived from fresh human spleen **(Ranjan *et al*., 2004).** The curcumin can suppress the PHA-induced proliferation of human peripheral blood mononuclear cells (PBMCs) and inhibit IL-2 expression and NF-AB **(Joe and Lokesh, 2000).**

Soni *et al*. (1992) reported a protective effect of curcumin on aflatoxin-induced hepatic preneoplastic focus formation in rats. Similarly in another study, dietary curcumin has been shown to effectively inhibit DENA-induced hepatocellular carcinoma formation and to modulate alterations in the levels of several representative cellular markers including p21ras, PCNA, and CdC2 in mouse **(Chuang *et al*., 2000).**

The curcumin administration (by oral intubation after 5 days of initiation at a dose of 200 mg/kg of body weight) was found to restore the normal levels of the enzymes glutathione S-transferase and γ-glutamyl transferase in rat liver following DEN-AAF exposure. Similarly, a significant protection was provided by curcumin in the enzyme-deficient foci for the adenosine triphosphatase-, alkaline phosphatase-, and glucose-6-phosphatase-treated groups in comparison to the DEN-AAF-treated group. These results showed that curcumin can effectively suppress the DEN-induced development of altered hepatic foci (AHF) in rat liver (**Shukla and Arora, 2003)**.

Pre- and co-treatment with Curcumin and Embelin was found to protect the liver against the carcinogenic effects of DENA/PB. This protection was due to their ability to prevent changes in the levels of elements studied such as calcium, potassium and sodium in addition to chromium, copper, magnesium, molybdenum and zinc and by the statistically significant decrease in the activity of Lactate dehydrogenase (LDH) that increased in DENA/PB treated rats**.** Notably**,** animals were given curcumin orally (100 mg/kg/day) in Tween-20, for 14 weeks **(Jagadeesh *et al*. 2011).**

Remarkably, curcumin with cisplatin administration was also shown to modulate certain tumor marker indices, namely, aminotranferase, GGT, and ALP in experimental fibrosarcoma **(Navis *et al*., 1999)**. It has also been found to protect cisplatin-induced clastogenesis by acting as free radical scavenger **(Antunes *et al*., 2000).**

The suppressing effect of curcumin on the development of AHF may result either from its ability to scavenge free radicals thereby acting as a

potent antioxidant **(Subramanian *et al*., 1994)**, or by inhibiting genetic damage through the modulation of phase I and phase II enzymes **(Manson *et al*., 1997).** The antimutagenic potential of curcumin may also account for its protective effect on AHF formation. Curcumin is known to be antimutagenic in micronucleus assay, *Salmonella typhimurium* assay, and in recombinant mutation assay in Drosophila **(Hamss *et al*., 1999).** Previous work has shown the antimutagenic potential of curcumin on chromosomal aberrations in Wistar rats **(Shukla *et al*., 2002).** The anticarcinogenic and antimutagenic potential of curcumin has been reported at comparatively high doses, i.e., up to 0.5% in diet or 500 mg/kg of body weight administered orally **(Ireson *et al*., 2001; Perkin *et al*., 2002).** This may be attributed to its avid metabolism and poor absorption. The systemic bioavailability of curcumin is reported to be low and its pharmacological activities may be mediated in part by its metabolites **(Ireson *et al*., 2001).** Hydroxylated derivatives of curcumin are also known to be antimutagenic and its *p*-hydroxy group has been shown to be essential for its chemopreventive effect **(Nagabhushan and Bhide, 1992).**

2.7. Cisplatin

Cisplatin developed into one of the most widely used drugs in cancer chemotherapy. Cisplatin is the prototype of the chemotherapy class of platinum drugs that include carboplatin and oxaliplatin. They cause cell death by binding to DNA to form DNA adducts, preventing further replication **(Arnesano and Natile, 2009).** The biochemical mechanisms of cisplatin cytotoxicity involve the binding of the drug to DNA and non- DNA targets and further induction of cell death through apoptosis, necrosis or both

within the heterogeneous population of cells that forms a tumoral mass **(Cvitkovic, 1998)**. Activation of the Fas pathway is a common mechanism by which cytotoxic drugs such as cisplatin induce apoptosis in tumor cells **(Friesen *et a.l*, 1999)**

It is generally accepted that binding of *cis*-DDP to genomic DNA (gDNA) in the cell nucleus is the main event responsible for its antitumor properties **(González *et al*., 2001).** Cisplatin exerts its anticancer effects mainly through a direct chemical reaction with N-7 of guanine or adenine forming intra and inter strand covalent bonds with DNA. Thus, the damage induced upon binding of cisplatin to gDNA may inhibit transcription, and/or DNA replication mechanisms. Subsequently, these alterations in DNA processing would trigger cytotoxic processes that lead to cancer cell death **(Baik *et al*., 2003).**

Cisplatin is considered to be a potent antitumor drug. Currently, Cisplatin is a key drug in standard regimens for the treatment of various cancers, including those of the respiratory, digestive and genitourinary systems **(Go and Adjei, 1999).** Cisplatin-based combination chemotherapy regimens are currently used as front-line therapy in the treatment of testicular cancer, ovarian germ cell tumors, epithelial ovarian cancer, head and neck cancer, advanced cervical cancer, bladder cancer, mesothelioma, endometrial cancer, non-small cell lung cancer, malignant melanoma, carcinoids, penile cancer, adrenocorticol carcinoma and carcinoma of unknown primary **(Langerak and Dreisbach, 2001).** It is used as consolidation therapy for many types of solid tumors that have failed standard treatment regimens. The therapeutic effects of cisplatin are significantly improved by dose

escalation. However, high-dose therapy with cisplatin is limited by its cumulative nephrotoxicity and neurotoxicity **(O'Dwyer *et al*., 1999).**

Hepatic arterial infusion using cisplatin (CDDP-HAI) is especially preferable, as CDDP is mainly excreted from the kidneys, and the dose-limiting toxicities are nephrological, neurological and hematological in nature and, as such, rarely severely burden hepatocytes **(Go and Adjei, 1999). Court *et al.* (2002)** reported that approximately half of the 195mcisplatin, which was intravenously injected in six HCC cases, was taken up by liver cancers according to first-pass kinetics. It was also found that Genistein, a soy-derived isoflavone, reinforced the inhibitory effect of cisplatin on HCC cell proliferation and tumor recurrence and metastasis after curative hepatectomy in nude mice, possibly through mitigation of cisplatin-induced MMP-2 (extracellular matrix metalloproteinase) upregulation **(chen *et al*., 2013).**

It was found that cisplatin induced serious side effects such as nephrotoxicity, neurotoxicity, ototoxicity, nausea, and vomiting **(Giaccone, 2000).** With cumulative doses of cisplatin, a peripheral neuropathy can develop, which can be irreversible. Anaphylactic type reactions, which are a class effect, have also been described **(Katzenstein *et al*., 2009).**

Reports of accidental overdoses, all of which have led to renal failure, confirm the potency of cisplatin as a renal toxin in humans **(Chu *et al*., 1993).** Nephrotoxicity is an unusual side effect of chemotherapy in general. Most chemotherapy drugs target pathways that are essential to dividing cells. The rapidly dividing cells in the bone marrow are sensitive to these agents.

The dose-limiting toxicity of carboplatin is bonemarrow suppression with cumulative anemia **(McKeage, 2000).**

Using an established mouse model of cisplatin nephrotoxicity (intraperitoneal injection of cisplatin 20 mg/kg body weight), the appearance of apoptotic epithelial cells by Tunel assay was first shown predominantly in the distal tubular and collecting duct **(Megyesi *et al*., 1998)**. Subsequent studies have confirmed and extended these findings. Apoptosis in this model has now been documented by a variety of additional methods (including hematoxylin-eosin staining, DNA laddering, and electron microscopy) to occur in both distal and proximal tubular cells, predominantly in the outer medullary region **(Tsuruya *et a.l*, 2003).** Apoptosis was evident within 3 days of cisplatin injection, temporally correlating with the onset of renal dysfunction. Several analogous studies have also been completed in a rat model (cisplatin 5 mg/kg intraperitoneally), with comparable results **(Huang *et al.*, 2001; Chang *et al.*, 2002).**

A recurrent theme gleaned from these works is that cisplatin induces apoptosis in a dose- and duration-dependent manner, and that while this agent activates programmed cell death at lower (10-100 mM) doses, it can also result in necrotic cell death at higher (200-800 mM) concentrations (**Hanigan and Devarajan, 2003).** Cisplatin induces renal epithelial cell apoptosis at least in part via activation of death receptor pathways (**Hanigan and Devarajan, 2003).**

Studies of tumor cell lines have shown a correlation between increased levels of intracellular glutathione (GSH) and resistance to cisplatin **(Chen *et al*., 1995).** Glutathione-s-transferases (GSTs) are a family of enzymes that catalyze the conjugation of glutathione (GSH) to a variety of substrates. GSTs can also bind the electrophilic substrates and thereby inactivate them **(Oakley *et al*., 1999).** Several isoforms of GST have been shown to bind cisplatin *in vivo* **(Sadzuka *et al*., 1994).** There are conflicting data in the literature as to which GST isozymes correlate with cisplatin resistance **(Nishimura *et al*., 1998; Kigawa *et al*., 1998).**

Rats injected with 0.4 mg of cisplatin per kg I/P in saline daily for the period of 8 weeks exhibited acute tubular necrosis in the kidney, dilation of proximal convoluted tubules with desquamtiom of almost entire tubular epithelium. Ultrastructurelly, cisplatin regimens induced a total absence of microvilli, with several rounded cisternae of smooth endoplasmic reticulum (SER) and presence of dense chromatin in the nucleus of the tubular lining and intact but highly increased mitochondrial population were also observed. Lumen showed presence of degenerative debris along with increased lysosomal bodies which was noticeable effect observed after cisplatin treatment (**Kim *et al*., 1995; Ravindra *et al*., 2010).**

Rats injected with cisplatin intraperitoneally (0.5 mg/kg/day or 1 mg/kg/day) exhibited no drug-related histopathology in kidneys or livers at 24 h after injection. However, in the 7-day cisplatin-treated kidneys, necrosis occurred in the proximal tubular epithelial cells of the S3 segment located in the outer stripe of the outer medulla. Necrosis was also accompanied by scattered apoptosis, as evidenced by diminished cell size, reduced

cytoplasmic volume with condensed eosinophilic cytoplasm, condensed nuclear chromatin, and karyorrhexis. These changes were accompanied variably by formation of apoptotic bodies in individual tubular epithelial cells. Variable interstitial lymphoplasmacytic inflammatory infiltrates were observed. Moreover, some dilated tubules were lined with flattened, hyperchromatic epithelium. Mitotic figures were occasionally noted in regenerative epithelium. In contrast to the nephrotoxicity, no hepatotoxicity was found in cisplatin-treated livers **(Huang *et al*., 2001).**

Cisplatin at a dose of 5mg/kg B.W, I/P have shown to cause severe tubular necrosis. The stoma was edematous with separation of tubules. The tissue was densely infiltrated by chronic inflammatory cells composed of small lymphocytes. Many of the glomeruli showed diffused eosinophilic sclerosis. Tubular atrophy and tubular dialation were also present confirming the nephrotoxic effect of cisplatin at the dose used **(Khanam *et al*., 2011).**

Rats treated with 7 mg/kg cisplatin and sacrificed 1 week later showed a good significant correlation between normalized kidney weight and logarithmic scale of BUN and Creatinine. Relationship between BUN, Creatinine or normalized kidney weight and pathology damage score was significant **(Nematbakhsh *et al*., 2013).**

In rats treated with 10 mg/kg cisplatin intraperitoneally I/P showed the presence of serious cast formation in the kidney tubules as well as a significant swelling and severe desquamation of tubular epithelial cells. Moreover significant tubular necrosis and many apoptotic bodies were observed. Mild interstitial inflammation and mild glomerular degeneration were seen as well as loss of microvillus **(Sener *et al*., 2012).**

The sections of kidney of rats treated with a weekly intraperitoneal injection of cisplatin 2 mg/kg body weight revealed moderate congestion and marked intertubular hemorrhages on day 14. Few sections revealed marked tubular dilatation. Sections on day 28 revealed moderate hemorrhages with disrupted tubular epithelium with few tubules showing moderate degenerative changes. Few glomeruli revealed disappearance of capillary network while few with shrunken glomeruli. The sections of kidney in the group treated with cisplatin and administered 0.05 ppm turmeric by oral gavage needle showed moderate tubular dilatation with mild degenerative changes on day 14 where as sections on day 28 showed mild dilatation of tubules **(Ramya *et al*., 2013).**

Light microscopic observations revealed that higher doses of cisplatin (1mg/kg) and doxorubicin caused massive hepatotoxicity including dissolution of hepatic cords, focal inflammation and necrotic tissues. Interestingly, low doses (0.2mg/kg) also exhibited abnormal changes, including periportal fibrosis, degeneration of hepatic cords and increased apoptosis. These changes were confirmed at ultrastructural level, including vesiculated rough endoplasmic reticulum and atrophied mitochondria with ill-differentiated cisternae, dense collection of macrophages and lymphocytes as well as fibrocytes with collagenous fibrils manifesting early sign of fibrosis, especially in response to cisplatin and doxorubicin – treatment **(El-Sayyad *et al*., 2009).**

Light Microscopic studies of liver after cisplatin treatment (0.4 mg/kg of CDDP I/P daily, for a period of 8 weeks) revealed damage to parenchymal

tissue of liver and large vacuolations in the perinucleus region. Perilobular connective appeared larger than normal and portal spaces were expanded. Almost all the hepatocytes showed abnormal vacuolations and irregular morphology. Nevertheless few hepatocytes appeared normal **(Kamble and Bhiwgade, 2011).**

Histological examination of livers showed that cisplatin (6 mg / kg body weight single dose) produced pathological changes after 4 days manifested as severe fatty changes, congestion and dilatation of portal blood vessels and central veins, dilatation and congestion of sinusoids, with multiple necrotic foci and apoptotic cells **(Ahmad and Al-Jawary, 2012).** There is a suggestion that the drug accumulates in significant amounts in hepatic tissue particularly when injected in high doses **(Liu *et al*., 1998).** Despite its excellent anticancer activity **(Park *et al*., 2009),** the clinical use of cisplatin is often limited by its undesirable severe toxic side effects that interfere with its therapeutic efficacy **(Dank *et al*., 2008).** Extensive investigations have been conducted on the hepatotoxicity of this anticancer drug **(Kim *et al*., 2004).**

On the other hand, another study showed that after one week of a single dose of cisplatin (6 mg/kg), the induced toxicity was observed clearly in kidney tissue. However, no damage and other abnormality were detected in the liver tissue **(Nematbakhsh *et al*., 2012).**