Discussion
“More commitment to prevention and early detection is desperately needed in order to complement improved treatments and address the alarming rise in cancer burden globally”...Dr. Christopher Wild, Director of IARC.

MODERNIZATION in lifestyle and extensive industrialization has contributed to the accumulation of various hazardous xenobiotics in the environment which is responsible for the rising trend of cancer incidence (Jain et al., 2013). It is estimated that as many as two-thirds of all cancer cases are linked to inadequate diet, environmental exposure of polluted air, contaminated water, radiation, smoking and toxic occupational chemicals (Anand et al., 2008; Theodoratou et al., 2014). Human beings are continuously being exposed to unknown quantities of harmful xenobiotics through these factors. Nitrosamines present a very important group in the list of numerous environmental carcinogens. Exogenous occurrence and endogenous formation of nitrosamines exaggerate its potency for carcinogenesis. For over a decade, N-Nitrosodiethylamine (NDEA) has been identified as a potent hepatocarcinogen, commonly found in tobacco, cosmetics, agricultural chemicals, pharmaceuticals, coloring agents, fixatives, and flavouring preservatives (Wang et al., 2011). In animals, activation of nitrosamine to its carcinogenic form is carried out by oxidase system that is primarily expressed in liver. Moreover, liver is an incredibly complex organ continuously filtering poisonous and unwanted chemicals from blood, hence rendering liver the most susceptible organ for cancer.

Hepatocellular carcinoma (HCC) represents the predominant histological type accounting for 85-90% of total hepatic cancers (Gomez and Lobo, 2011; Siegel et al., 2013). HCC is the most dreadful form of liver cancer ranking as the third most common cause of cancer related deaths worldwide. In other words, it can be stated that HCC is posing a major health problem worldwide because of its high incidence and extremely poor prognosis (Ferlay et al., 2013). Rising percentage of HCC in Western countries has been linked with the geographical distribution of viral hepatitis B (HBV) and hepatitis C (HCV) (Malek et al., 2014).
Understanding of many aspects of the HCC evolution, major risk factors and mechanism of 
HCC requires well designed and suitable experimental models. NDEA belongs to the family 
of carcinogenic nitrosamines acting as an environmental carcinogen and highly mutagenic to 
produce hepatic tumors (Inami et al., 2009; Glory and Thiruvengadam, 2012). NDEA in 
various experimental studies has been used as an inducer of HCC by modulating hepatic 
biochemical and molecular events (Bharati et al., 2012; Li et al., 2012).

Inadequacy of liver donors, late diagnosis, poor prognosis, liver dysfunction and high 
mortality in liver cancer patients has retarded the success rate of HCC management. 
Moreover, conventional therapeutic and radical attempts to get rid of the tumor through the 
cut, burn, and poison technique of surgery, radiation and chemotherapy have not been 
successful in managing HCC. Therefore, preventing the onset of HCC may prove more 
beneficial than curing it. Large number of evidences reveals the association of 
phytochemicals with reduced risk of developing chronic diseases, such as cancer (Mehta et 
al., 2010; Singh et al., 2014). Medicinal properties of various traditional and nutritional plants 
have attracted researchers in the field of chemoprevention. Moreover, combinatorial effects 
of phytochemicals and nutritional agents with advanced cancer therapies have proven to be a 
complementary and safe approach (Singh et al., 2014). Administration of dietary agents has 
an enhancing therapeutic efficacy as it aids in mitigating the adverse effects of conventional 
cancer treatments. Specifically phytochemicals possessing antioxidant properties have been 
suggested to be more chemopreventive. Randomized epidemiological studies and various 
clinical trials have yielded mixed results regarding the association of dietary intake of 
antioxidants and cancer incidence (Bennett et al., 2012).

Lycopene is a known nutritionally important carotenoid present in selected red-colored fruits 
and vegetables (Vogele, 1937; Egydio et al., 2010). Lycopene has been reported to have 
highest free-radical scavenging property and hence is drawing great attention worldwide for 
its health benefits (Stahl and Sies, 1996; Stahl and Sies, 2005; Palozza et al., 2012). 
Although, various reports are available in support of the chemopreventive potential of 
lycopene, its clinical recommendation is still limited. US Food and Drug Administration 
(USFDA) has designated lycopene as a ‘Generally Recognised as Safe’ (GRAS) product 
(Kavanaugh et al., 2007). However, it is still questionable if there is a conclusive evidence for 
administrating lycopene to patients in addition to a well-balanced diet. Moreover, research in 
the chemopreventive exploration of lycopene demonstrated higher protective impact of 
lycopene in phytocomplex mixture in comparison to purified lycopene (Stacewicz-
Sapuntzakis and Bowen, 2005). According to such reports the phytochemicals of tomato may work in synergism with lycopene and potentiate the protective effects and may help in maintaining the bodily homeostasis. Thus, the aim of this piece of work is to unravel the protective impact of lycopene extracted from tomatoes on NDEA induced hepatocarcinogenesis by exploring the biochemical and molecular mechanisms.

5.1 Lycopene extraction, structural characterization and determination of its \textit{in vitro} antioxidant potential

Lycopene is a tetraterpene hydrocarbon containing forty carbon atoms and fifty six hydrogen atoms. Being a lipophilic hydrocarbon, lycopene is soluble in non-polar solvents such as chloroform, hexane, benzene and ether, and insoluble in polar solvents. Lycopene is a carotenoid deeply embedded within the chromoplast membrane structures tightly bound to macromolecules (Ahrazem et al., 2010; Colle et al., 2010; Knockaert et al., 2012). The biochemistry of lycopene is quite different from carotenes as it is acyclic and lacks \(\beta\)-ionone ring and hence is devoid of pro-vitamin A activity. Lycopene in natural sources exists in \textit{all-trans} form (94-96\% of total lycopene in fruit) and is thermodynamically more stable (Clinton, 1998; Lopez-Ramirez et al., 2010). High extraction efficiencies are dependent on various factors such as solvent type, heat treatment, light exposure and duration of extraction. Solvent molecules penetrate the compact tomato matrix tissue and solubilise the lycopene pigment has to be selected for maximum yield.

In the present study hexane was used as the main extracting solvent along with acetone and ethanol. Similarly, several research groups have observed more effective yield of lycopene when hexane: acetone: ethanol in 2:1:1 was used as an extracting solvent (Lavecchia and Zuorro, 2008). A possible explanation is that hexane is the only component with a high affinity for lycopene and acetone and ethanol are two polar compounds playing auxiliary role in the overall extraction process. Addition of polar compounds with small molar volume, large basicity and greater hydrogen bonding affinity cause the swelling of the plant tissue. Acetone and ethanol possess all these characteristics and thus aid in the higher penetration of hexane in the tissue (Periago et al., 2004).

The \textit{all-trans} form of lycopene present in the fruit tissue matrix has poor bioavailability and absorption than \textit{cis}-form of lycopene (Boileau et al., 2002; Omoni and Aluko, 2005). Moreover, the tight association of lycopene with macromolecules also decreases its bioavailability. In the present extraction procedure, heat treatment at 80\degree C for an hour had
yielded higher extraction efficiency yield than without heat treatment (data not shown) which is in corroboration with the reports available in the literature (Chang et al., 2006). Actually, heat processing of tomato tissue during extraction aids in the liberation of lycopene from protein complexes and enhances its oral bioavailability through isomerization. According to Shi et al., (2008) there are several factors that can affect lycopene content during heat treatment such as degradation of isomer, isomerization of all-trans form to cis-isomer lycopene and more efficient extraction. The preferential absorption of cis-isomer of lycopene over the all-trans-form might be because of higher solubility of cis-isomer of lycopene in bile acid micelles. This enhances lycopene incorporation into chylomicrons and hence represents its bioavailability. Another, interesting factor to be considered for the efficient utilization of lycopene extracted from tomato tissue is its stability before in-vivo experiments. There are mixed observations regarding the stability of lycopene during food processing and storage. Many reports in the literature are in the agreement that lycopene remains relatively stable during extraction, except at extreme temperatures (Takeoka et al., 2001; Zanoni et al., 2003). According to others, lycopene in the plant matrix is more stable and as it is extracted from the matrix its stability decreases. Few researchers reported the half-life of 16 hr for the extracted lycopene at 4°C (Fang et al., 2003). Bioavailability and absorption of lycopene extracted from tomato tissue can be enhanced by the ingestion of dietary fat or the presence of other phyto-molecules (Shi and Le Maguer, 2000; Faisal et al., 2010). Considering all these factors regarding the extraction and stability of lycopene, the dosage of lycopene extracted from tomato (LycT) for the present in-vivo study was prepared in olive oil. LycT was reconstituted in the dietary fat (olive oil) because it aids in the uptake of lycopene into intestinal mucosal cells via stimulating the formation of bile acid micelle.

Studies characterizing lycopene extracted from tomatoes revealed the presence of lycopene in major fraction along with other non-carotenoid components. FAO/WHO (2006) also reported the occurrence of mixture of carotenoids and non-carotenoids such as fatty acids, acylglycerols, phospholipids and waxes in tomato extract. However, as reported lycopene is the primary constituent present in the carotenoid fraction. The characteristic red colored lycopene has the ability to accept energy from various electronically excited species. Techniques such as UV-VIS, NMR and FT-IR spectroscopy were used to structurally characterize the extracted lycopene i.e. LycT. In the current study, LycT showed absorbance maxima at 444, 470 and 503nm in hexane that gave the basic confirmative observation regarding lycopene extraction. The amount of lycopene was quantified at 503nm to avoid
interferences from other carotenoids present in the extract. Structural analysis was described by NMR spectroscopy, the most common research technique determining the physical and structural groups present in the compound by exploiting the magnetic resonance of protons. The NMR and FT-IR graphs confirmed the presence of -CH3 group, -CH, -CHCH, =CCH3, =CCH2, trans C=C, carbon - carbon double bond stretching, -CH2 bending, along with CH3 / CH2 stretch. Thus the spectroscopic data clearly revealed the presence of characteristic structural groups of lycopene. The NMR spectral data and FT-IR transmission value was compared with the identified values in the literature to draw the confirmatory conclusion (Johansen and Jensen, 1974; Shen et al., 2011; Konwar and Baruah, 2011).

Several studies have demonstrated the antioxidant property of various phytochemicals using DPPH radical scavenging assay, ABTS radical scavenging assay and DNA damage inhibition assay (Boileau et al., 2003; Xing et al., 2001). In DPPH* and ABTS* scavenging assay, LycT exerted dose dependent effect. DPPH* and ABTS* radicals mimics the highly reactive free radicals generated in in vivo conditions. The cellular macromolecules are highly reactive towards ROS in in vivo conditions leading to several deleterious effects including cell damage (Jomova and Valko, 2011). The present in vitro experiments showed that LycT exhibits a free radical scavenging potential and hence can be hypothesized to act similarly in in vivo conditions. Moreover, LycT has shown protective capability against DNA degradation induced by ROS. The present results also revealed that low LycT concentration exhibited high protective potential towards DNA damage as compared to high concentration. This may be due to its pro-oxidant activity at high concentrations (Lowe et al., 1999; Veeramachaneni et al., 2008). ROS in in vivo conditions can be balanced by the antioxidant action of low-molecular weight antioxidants as well as antioxidant enzymes. Among low-molecular weight antioxidants carotenoids are known to play a key role (Jomova et al., 2009). Antioxidative potentials of phytochemicals can thus be exploited to minimize oxidative damage. Phytochemicals protecting DNA in in-vitro conditions have greater probability to perform similarly inside the cell.

5.2 Chemopreventive response of LycT against NDEA induced hepatocarcinogenesis

N- Nitrosamines are a group of chemicals known to be environmental carcinogens (Mhlongo et al., 2009). Over the past decade, N-diethylnitrosamine (NDEA) was identified as a potent
hepatocarcinogen present in tobacco, cosmetics, pharmaceutical products, agricultural chemicals, colour fixatives and flavouring preservatives (Sadik et al., 2008; Wang et al., 2011). The average human intake of NDEA through food is around 1mg/day (Scanlan, 1983; Glory and Thiruvengadam, 2012). In addition to this direct exogenous exposure, humans are also exposed to endogenously produced nitrosamines (Crews, 2010). Nitration of all primary, secondary and tertiary amines generates nitrosamines; however secondary amines are the most reactive compounds. In stomach, at acidic pH dietary amines are nitrosated and hence produce nitrosamines. Human liver expresses cytochrome 2A3 and cytochrome 2E1, enzymes primarily involved in NDEA activation, thus rendering liver a target organ for its metabolism and carcinogenicity (Aiub et al., 2011a and b). Such an exposure to nitrosamines is considered to be quiet damaging since in association with other contributing factors such as micronutrient deficiency, infection of certain virus, and genetic susceptibility to nitroso carcinogens, the intake of levels of nitrosamine precursors that are considered to be normal otherwise is sufficient to cause cancer (Vermeer and van Maanen, 2001; Crews, 2010).

Cancer chemoprevention is a pharmacologic intervention with a natural or synthetic compound to reverse or suppress carcinogenesis in its early or pre-malignant stages so as to prevent or delay the development of invasive cancer. In the present study, hepatic cancer model was used to evaluate the chemopreventive potential of the LycT. Monitoring general physiological parameters was considered important in tumorigenesis study. Body weight, food and water intake were important indicators of general health of the animals. Reduction in the body weight of NDEA treated animals demonstrated the deteriorating hepatic function. And this apparent decrease has been associated with decreased food intake in the same group. NDEA being necrogenic and toxic to liver would significantly affect the functioning of liver which is expected to affect the overall status of the body (Chowdhury et al., 2012; Nohmi et al., 2012). Several studies have shown similar reduction in the body weight of animals following the administration of NDEA (Sreepriya and Bali, 2005; Bharati et al., 2012). Approximately 20% of the animals, survived in NDEA group at the end of the treatment period while in LycT + NDEA the survival of the animals increased to 60%. Further, chemopreventive response of LycT to hepatocarcinogenesis was monitored on the basis of tumor incidence, multiplicity, burden and gross morphology. At the end of the treatment period, LycT pre-treatment to NDEA challenged group had shown a decrease of 42% in tumor incidence when compared to the NDEA group. Decrease in tumor statistics upon LycT treatment indicated the chemoprotective potential of LycT in HCC development. Further, a
significant reduction in the number and size of tumor nodules was observed in LycT + NDEA group when compared to the NDEA group. The increase in hepatosomatic index in the NDEA treated group upon hepatocarcinogenesis can be correlated to the morphological and histological alterations such as increase in size of liver lobes, hyper-proliferation of cells, and tumor development.

NDEA-induced liver enlargement is attributed to initial transient hyperplasia in mice as an early event after treatment. LycT + NDEA group showed significantly reduced hepatosomatic index at 10th week but showed an increase at 20th week signifying the hyperplasia state at later stages. Gross macroscopic classification of liver tumor nodules was done according to Kai et al., (2012). The liver tumor nodules were identified with four distinctive structural patterns: single nodular type (type 1), single nodular with extranodular growth (type 2), contiguous multinodular type (type 3) and poorly demarcated nodular type (type 4). Type 1 and type 3-4 nodules correspond to pre-neoplastic and neoplastic form of HCC respectively.

The results indicated that LycT administration reduced the aggressiveness of nodule formation. The tumor nodules developed in NDEA and LycT + NDEA groups were observed to be type 3-4 and type 1 respectively. Tumor size, number and histological grading may serve as prognostic parameters for determining the chemopreventive potential of phytoagents in cancer models (Rakha et al., 2010).

In the present study, the tumors in NDEA group were of larger size and histologically higher grade which might be directly related to the high mortality observed in the same group. The histopathological investigations were carried out at 10, 16 and 24 weeks of the study and it revealed a marked delay in progression and development of HCC in LycT + NDEA group. Different stages of HCC were evident by the histopathological analysis which indicated that liver of NDEA group showed de-differentiation from well differentiated HCC to poorly/ undifferentiated HCC during the course of HCC development. Early stage of HCC cells were characterized by a large nucleus, enhanced nuclear to cytoplasmic ratio, increased cell density, pleomorphic cell and irregular border. These results were in agreement with those recorded by Roncalli et al., (2011). The tumors in NDEA group at 24th week were histologically classified as poorly differentiated to undifferentiated HCC with small and large lesions of hyperchromatic cells with scanty cytoplasm at the of the study. In our laboratory similar observations have already been reported where NDEA exposure has induced undifferentiated HCC in male Balb/c mice (Bharati et al., 2012). However, LycT + NDEA group demonstrated well differentiated HCC showing hyper proliferation of cells, cell plate thickening, stromal invasion and micro-trabecular structure at the end of the study. Moreover,
poorly to undifferentiated HCC are considered to have higher metastasizing potential than well differentiated HCC (Kojiro, 2005). Histopathological observations are in corroboration with the observed reduced tumor statistics in LycT + NDEA group indicating delay in HCC development.

Lycopene has been ascribed from the scientific literature that it bears significant potential in preventing and treating prostate cancer (Holzapfel et al., 2013). In the past decade, studies have reported the reduce risk of prostate, lung, leukemic, digestive tract and ovary cancer upon lycopene treatment (Salman et al., 2007; Scolastici et al., 2007). The anti-carcinogenic potential of lycopene as evident from several epidemiological studies has been attributed primarily to its antioxidant properties (Kong et al., 2010). It was revealed that in addition to its direct antioxidant potential, lycopene up-regulates the antioxidant response element (ARE), phase II enzymes which is associated with its anti-proliferation, anti-inflammatory and apoptotic activities (Heber and Lu, 2002). Wang et al., (2010) have reported the inhibitory effect of lycopene in non-alcoholic steatohepatitis (NASH)-promoted hepatocarcinogenesis. Recently, review reported by Ip and Wang (2014) illustrated that lycopene showed chemopreventive effects against HCC in number of in vitro, in vivo, epidemiological and clinical studies. Koul et al., (2010) had also reported the modulatory effect of lycopene against 7, 12 dimethylbenz (A) anthracene induced hepatic clastogenicity in male mice. The present observations and reports from the literature reiterate that LycT has the potential to retard the aggressiveness of carcinogenesis as was evident by the increased survival rate and decrease in tumor incidence, burden, multiplicity and histopathological grade. Based on these observations the present study was further designed to explore the mechanism behind the protective effect of LycT in HCC.

5.2.1 Scanning and Transmission Electron Microscopy

Ultrastructural examination through electron microscopic techniques is playing an essential role in both research and diagnosis of liver associated diseases (Iancu and Manov, 2011). SEM may delineate the surface ultrastructure of intrahepatic cells and other hepatic compartments. However, limited literature is available regarding the hepatic architecture and HCC. In the present study, hepatocytes were polyhedral in shape, possessing three structurally distinctive faces and were in firm contact with each other. Moreover, hexagonal hepatocytes with proper surfaces and bile canaliculi were also observed. Hepatocytes were observed predominantly in single cell thick plate. These current observations regarding
normal hepatocytes morphology are in agreement with the findings in the literature (Holz et al., 2010; Bharati et al., 2012). Development of HCC is an outcome of stepwise changes in hepatocytes and is associated with numerous alterations in inter-relationship of hepatocytes with other hepatic cells. In the current study, SEM micrographs from NDEA treated group revealed high cell density, distorted central vein, clumping of cells, and enlarged sinusoidal spaces. Rounding and altered arrangement of hepatocytes observed in the SEM may be the regions of hyper proliferation. Such observations regarding the morphological characteristics of the tumor cells have been reported from our laboratory earlier also (Bharati et al., 2012; Ganger and Koul, 2008). The profusely dividing cells gave the appearance of an outgrowth consisting of lump of tumorous cells. The possible explanation behind these observations may be speculated from the fact that rapidly dividing tumor cells attain a round contour during crowding of the cells (Paxton et al., 2000).

Cell plate thickening observed in the current LycT + NDEA group indicated well-differentiated stage of HCC. SEM of liver biopsies from LycT + NDEA also revealed numerous brighter and detached cells from hepatocytes with surface infolding. These structures may be demonstrated as apoptotic bodies with multiple blebs released from hepatocytes in the extracellular spaces. Similar bodies were also observed in the forestomach of AAILE pre-treated mice challenged with benzo(a)pyrene (Ganger and Koul, 2008). Gross changes in nuclear morphology, epigenetic regulation, chromatin packaging and overall nuclear architecture play an informative role in demonstrating the manifestation occurring during HCC.

Ultrastructural analysis using TEM contributed in revealing the morphological alterations during NDEA administration. Distorted nuclear membrane, imbalance heterochromatin, leaching of nuclear materials, pseudo-inclusion, and karyotin deposition in NDEA group as revealed in TEM analysis clearly signifies the damaging effect of the carcinogen. Dark spots in nucleus signify the condensed nucleoplasm and nucleolus was also not defined indicating damaged nucleus when compared to the normal nucleoplasm. Preferential increase of heterochromatin in different cancerous cells may indicate a close association with DNA damage or alteration in molecular machinery (Nakano et al., 1992; Di Micco et al., 2011). Recently, Carone and Lawrence (2013) reported that any compromise in heterochromatin maintenance, aids in the emergence of selective growth pattern and potential for neoplasia. Tumor cells were also accompanied by several degenerative changes in cellular organelar pattern and distribution. Decreased glycogen in tumor cells was visible in the form of white
droplets in the cytoplasm indicating high energy consumption during carcinogenesis. Abundant, enlarged and intensely stained pleomorphic mitochondria and dilated rough endoplasmic reticulum (RER) signify cytoplasmic disorganization. Transformed tumorous cells rely on the energy production during glycolysis, which might be due to the disturbance in mitochondrial respiration or hypoxia (Ismail et al., 2009). Cell membrane was disrupted and microvilli disappeared, which might be one of the reasons for the loss of cell-to-cell communication. These alterations lead to the crowding of cells and clumping of hepatocytes. These observations could be correlated to the changes in the cytoskeleton of tumorous cells (Guido et al., 2010). The possible reason behind these ultrastructural alterations may be due to the enhanced production of free radicals during NDEA metabolism which have resulted in changes in the cytoskeleton of cells. Such observations are in corroboration with the previously reported alterations in our laboratory (Bharati et al., 2012). The manifestations observed at the surface and cellular ultra-structural levels through SEM and TEM were in support of the HCC inflicted in liver of NDEA treated mice when compared with that of normal mice.

The ultrastructural analysis of LycT + NDEA group liver biopsies showed stabilized nuclear membrane, cell membrane and differentiated organelles. Abundant lysosomes and SER along with mitochondria in the liver of LycT + NDEA group suggested the existence of dismantling potential. In addition to these alterations in hepatocytes, several apoptotic cells were observed in sinusoids. Lysosomes are cytoplasmic membrane-bounded organelle consisting of hydrolytic enzymes assisting in degradation of waste products or transformed cells leading to programmed cell death (Boya, 2012). Enhanced apoptotic bodies in the liver biopsies of LycT + NDEA group showed the limiting effect against NDEA induced proliferation. Such observations were also evident in our laboratory where AAILE pre-treatment to benzo-(a)-pyrene challenged mice showed the production of apoptotic bodies in forestomach (Ganger and Koul, 2008).

5.3 Modulation of Xenobiotic Metabolizing Enzymes during NDEA induced Hepatocarcinogenesis and its Amelioration by LycT

Studying initial steps of chemical carcinogenesis might provide some possible ways for ameliorating carcinogenesis. Understanding the mechanisms involved in the initiation or stimulation of cancers during chemical carcinogenesis has been one of the prime areas for investigation and may be the effective way of controlling cancer at an early stage.
Carcinogenesis can be prevented by any of the following ways: modulating the xenobiotic metabolizing enzymes, scavenging the free radical products formed after activation, modulating DNA repair processes, altering gene expression involved in cell proliferation, apoptosis, differentiation, invasion, cell signaling etc (Anand et al., 2008). Exploring and targeting specific checkpoints along the carcinogenesis pathway might serve as a beneficial attempt in preventing this dreadful disease. Changes in liver weight or size, histological evidence of abnormal hepatocytes, pleiotropic gene expression and altered morphological changes upon NDEA treatment triggers the hepatic enzyme induction which is an adaptive response (Ennulat et al., 2010). Liver has the capability to restore its optimal mass to normal upon stimuli such as toxic insult or stress. The hepatic response may involve enzyme inductive responses to a variety of xenobiotics contributing for its altered size, morphology and biochemistry. Another objective of the present study was to reveal the modulatory effect of the LycT on xenobiotic metabolising enzymes during the process of hepatocarcinogenesis.

NDEA like any other nitrosamines is quite stable under biological conditions and is carried to the hepatic tissue via blood stream. Biotransformation of nitrosocompounds requires two phases and is carried out by enzymes of liver microsomes. It is well documented that NDEA undergoes activation to form reactive metabolites causing oxidative stress leading to cytotoxicity, mutagenicity and carcinogenicity (Oloyede et al., 2013). NDEA acts as a procarcinogen and it requires activation for its transformation into carcinogenic form aided by phase I enzymes. CYP2A3 and CYP2E1 are known isoforms of cytochrome P450 (CYP) involved in the metabolism of toxicants and carcinogens. Activation of genes regulating CYP expression might be attributable to the interaction of chemicals with the constitutive androstane receptor (CAR) (Sueyoshi and Negishi, 2001; Aiub et al., 2011a and b; Ip and Wang, 2014). Substantial proliferation and dilation of RER as shown by TEM micrographs can be linked with the microsomal hepatic enzyme induction (Maronpot et al., 2010). NDEA during the first phase of biotransformation forms a transition product (monoethyl nitrosoamine) which subsequently gets degraded to form alkyldiazonium ions (Inami et al., 2009). The diazonium ions thus formed during the biotransformation of NDEA are very reactive and are further responsible for the damaging effects and formation of other more reactive products (Inami et al., 2009). Various types of mutations may occur in the genetic material (DNA) upon covalent or non-covalent interactions of these highly reactive metabolites of NDEA if not repaired before replication. Certain mutations may eventually lead to the activation of proto-oncogenes and inactivation of tumor suppressor genes. In other
words, interaction of free (reactive metabolites) with DNA plays a crucial role in the initiation of carcinogenesis (Arora et al., 2013).

Treatment for LycT for two weeks did not cause any appreciable change in the content of phase I enzymes (CYP and cytb5) in hepatic tissue when compared to the control group. NDEA treatment in group II and group IV, for eight weeks enhanced the activities of phase I xenobiotic metabolizing enzymes. Enhanced activities of these phase I enzymes signifies metabolic activation of NDEA by CYP and cytochrome b5 enzymes. Administration of LycT to the NDEA treated animals showed an appreciable increase in phase I enzymes when compared to control group and LycT group. LycT treatment to NDEA challenged mice did not show any change in the content of cytochrome P450 and cytochrome b5 when LycT + NDEA group was compared with NDEA group. There are reports demonstrating similar observations where lycopene had shown no effect on the total CYP or CYP1A2 activity but had inhibited the activity of CYP2E1 in the rat model (Louisa et al., 2009). Phytochemicals like lycopene may quench the enzymatically generated electrophiles more efficiently as compared to the stable pro-carcinogens. No change was observed in CYPs and cytb5 content in LycT group when compared to control group at ten weeks duration.

Glutathione-S-transferases (GSTs) super family are the major phase II xenobiotic transformation enzymes that catalyse the cytosolic biotransformation of variety of exogenous and endogenous compounds. Biotransformation involves the conjugation reaction aided by GST which generally inhibits the reactive cellular metabolites from targeting cellular targets such as DNA, RNA and protein. The key principle behind the inhibition catalyzed by GST is the incorporation of non-protein thiol, GSH to reactive metabolites to form thio-ether linked glutathione conjugate. The glutathione conjugates are less toxic and possess higher hydrophilicity which facilitates their elimination via GSH-conjugate recognizing transporter (Estrela et al., 2006). GST activity thus plays a crucial role in inhibiting the initiation of carcinogenesis via removing the reactive species from cells.

In the current investigation, decreased activity of hepatic GST was observed in the NDEA challenged mice when compared to the control group at 10th week. This might be one of the possible reasons for explaining the formation of enhanced reactive metabolite. Consequently phase I induced NDEA metabolites are poorly biotransformed into non-toxic form. Decreased GST activity and enhanced ROS at 10th week in NDEA group explained the stimulation of HCC initiation. However, LycT administration to NDEA challenged showed enhanced GST
activity signifying high excretion rate of NDEA metabolites. This observation at 10\textsuperscript{th} week of the study might delineate the possible reason behind the delay in the initiation of HCC as was also evident from the histopathological observation. The boosting of phase II GST enzyme activity upon LycT administration was continued till 24\textsuperscript{th} week of the study which further explains the chemopreventive potential of LycT. The occurrence of well-differentiated HCC in LycT + NDEA group and undifferentiated HCC nodule in the NDEA challenged mice at 24\textsuperscript{th} week of the study indicates the delay in the development of HCC. Reduction in the aggressiveness of HCC upon LycT administration might be the outcome of the observed enhanced GST activity. Earlier reports from our laboratory have revealed similar observation where administration of a chemopreventive agent i.e. AAILE (neem extract) to skin tumor bearing mice enhanced the activity of GST when compared to tumors obtained in DMBA/TPA group (Koul et al., 2006). Various other research groups have also reported the positive correlation of GST activity with the treatment of some chemopreventive agents. Enhanced activity of GST in pulmonary, hepatic and forestomach tissues has been observed after the treatment with ENLE (Dasgupta et al., 2004; Subapriya et al., 2005). Thus, the above observations and reports from the literature delineate that the chemopreventive potential of LycT has been attributed through enhancing detoxification of reactive metabolites.

5.4 Oxidative stress during NDEA induced hepatocarcinogenesis and LycT mediated amelioration

Reactive oxygen species (ROS) along with reactive nitrogen species (RNS) are generally termed as oxygen-free radicals possessing “two-faced” character within cells. Free radicals are well recognised for contributing both deleterious and beneficial roles in the living system (Valko et al., 2004; Pizzimentri et al., 2010). Free radicals are involved in stimulation of number of cellular signalling cascade pathways which further contribute in physiological functioning (Valko et al., 2007). Classically, free radical/ ROS have been recognised as host defending molecules released by neutrophils for destroying bacteria. ROS also aid in maintaining the oncogenic phenotype of cancer cell, cellular senescence, and apoptosis thus acting as tumorigenic and anti-tumorigenic species. At low concentration ROS also triggers the induction of a mitogenic response. On the other hand cumulative production of free radicals through either endogenous or exogenous sources result in altered redox status. Mitochondria, CYP metabolism, peroxisomes, and inflammatory cell activation are potential endogenous sources of ROS generation (Inoue et al., 2003). Exogenous sources of free radicals such as exposure to carcinogen/ pro-carcinogen or drugs amplify the level of ROS.
inside the cells leading to altered redox status. Considering the importance of ROS in various pathogenesis, there is argument for establishing and optimizing a reliable method for ROS quantification in tissues and cells. In literature several fluorescence and chemiluminescence methods have been reported to detect ROS in tissue (Dikalov et al., 2007). 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) is a very sensitive fluorescent probe which can pass cell membranes and diacetate moiety is cleaved by intracellular esterases to DCFH. DCFH is then trapped within the cell where ROS oxidize DCFH to the fluorescent 2',7'-dichlorofluorescein (DCF) (Wrona and Wardman, 2006; Kim et al., 2006; Kalyanaraman et al., 2012). However, DCF fluorescence cannot differentiate the different types of ROS and indicate only the resultant oxidative stress (Kalyanaraman et al., 2012).

Enhanced ROS production leads to oxidative stress where free radicals can be important mediators of damage to cell structures. Cellular macromolecules such as lipids, proteins and nucleic acids are the prime targets of free radicals (Takahashi et al., 2006; Reuter et al., 2010). Generally the deleterious effects of free radicals are countered by the antioxidant action of non-enzymatic and enzymatic antioxidants. Consistent exposure of free radicals disturbs the balance of cellular oxidant and antioxidants leading to oxidative stress. Failure of antioxidant defense system and persistent oxidative stress is attributed to the free radical related cellular damage (Durackova, 2010). Moreover, free radical related damage has been proposed to play a crucial role in the onset of age-dependent diseases including cancer (Minelli et al., 2009). Persistence oxidative stress related damage to biomolecules such as DNA can alter the intrinsic cellular properties and consequently resulting in cell death (Kryston et al., 2011). Nowadays, the dual role of ROS has been exploited for targeting cancerous cell through enhancing free induced cell death and hence exhibiting anti-cancer potentials (Pizzimenti et al., 2010; Arora et al., 2013). Considering the implications of oxidative stress in pathogenesis of various diseases, there arise the needs for exploring the biomarkers of oxidative stress.

When ROS level reaches above the threshold, they target lipid and initiate the lipid peroxidation (LPO). LPO is a chain reaction that produces multiple products and takes place in both cellular and organellar membranes disturbing the cellular functioning. Under stressful conditions levels of LPO has been widely used as an indicator of ROS mediated oxidative damage to cell membranes (Niki, 2008). LPO aggravates the oxidative stress by further producing lipid-derived radicals. These lipid-derived radical more aggressively react with themselves and damage proteins and DNA. The level of LPO has been widely used as an
indicator of oxidative stress induced damage and has direct relation with the devastating effect. Malondialdehyde (MDA) is one of the end products of LPO and the increase in MDA level is considered as a hallmark of increased LPO. MDA is the most commonly measured Thiobarbituric Acid Reactive Substances (TBARS), which is the broken product of fatty acid peroxidation (Koul et al., 2007; Milei et al., 2007). The polyunsaturated fatty acids (PUFA) and ester linkage between glycerol and the fatty acids are two principle sites of ROS attack on membranes. Moreover, in last decades it has been reported that many chemopreventive agents produce numerous electrophilic species creating a pro-oxidant milieu in cell. This oxidative damage induced LPO upon persistent administration of chemopreventive agents can retard cell progression of cancer cells and so forth apoptosis (Conklin, 2004; Ganger and Koul, 2008; Arora et al., 2013).

In the current study, NDEA group showed enhanced MDA formation after 10 weeks. Observed elevated MDA values may be due to high production of free radical during NDEA metabolism which upon reaction with unsaturated lipid generates hydro-proxides or MDA, indicating enhanced LPO. This observation is in line with those previously reported in our laboratory where NDEA insults resulted in elevated MDA levels (Koul et al., 2007). Persistently elevated LPO levels was observed in NDEA group when compared to control group during hepatocarcinogenesis, however the elevation in LPO level and ROS level were less after 24th week when compared to 10th week. ROS production at earlier stage of HCC is required for stimulating the initiation of carcinogenesis. Cancerous cells maintain the increment in ROS production so as to surpass cell death, DNA mutation, replication errors, and genomic instability prior to DNA replication (Klaunig and Kamendulis, 2004; Valko et al., 2006).

On the other hand LycT administration to NDEA challenged mice exerted differential effects on the levels of LPO during hepatocarcinogenesis. Lycopene in the current experimental model had reduced the level of tissue MDA and ROS level at 10th week of the study. LycT thus exerted an antioxidant effect in NDEA challenged mice by scavenging the ROS and free radicals and hence decreasing the LPO inspite of high CYPs activity in LycT + NDEA group. However, significantly high LPO levels were observed in LycT + NDEA group after 24th week. This suggests that LycT enhanced the LPO so as to prevent the NDEA damaged cells from thriving. From our observations and reports available in the literature, it seems that an enhanced LPO level is an indication of the pro-oxidant activity of LycT. Similar observations have been previously reported demonstrating that lycopene administration has been linked to
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oxidative stress induced cell death (Zhang et al., 2003; Ford and Erdman, 2012). Antioxidant and pro-oxidant effect of lycopene depends on a number of factors such as concentration, absorption rate, site of action and duration of administration. At high concentration it has been well reported that lycopene exerts its pro-oxidant activity in cancerous tissue (Yeh and Hu, 2000). Earlier reports from our laboratory have also revealed the pro-oxidant activity of chemopreventive agent (AAILE) and have hypothesized to be responsible for its inhibitory effect on various experimental cancer models (Arora et al., 2013; Gangar and Koul, 2008).

As no significant change in LPO levels and ROS levels were observed between control and LycT group indicating that LycT selectively favours the LPO in tumorous tissue which could be one of the mechanisms for the higher apoptotic index and hence exerting chemopreventive effect. Various anti-neoplastic agents have been reported with similar kind of mechanism in targeting tissue tumors by enhancing the ROS level (Jeong and Seol, 2008; Harish et al., 2009).

5.5 Antioxidant defense system during NDEA induced hepatocarcinogenesis and its intervention with LycT

HCC is a multifactorial disorder and possible mechanisms behind this disease have not been clarified yet. Increasing evidences indicate to free radical induced oxidative stress as prime factors triggering the initiation of HCC. Cellular redox status is a balance created between pro-oxidant and antioxidant, and impairment in this equilibrium leads to deleterious effects on cell’s life. Oxidative stress is an outcome of imbalance in the cellular redox status (Klaunig et al., 2010). Decreased antioxidant mechanism can exacerbate the extent of free radical induced oxidative stress. In case endogenous antioxidants fail to counteract the reactive metabolite production, then administration of exogenous antioxidants would be primarily required to balance the redox status. This concern has resulted in an increased interest in the investigation of components of the antioxidant defense system at different stages of carcinogenesis and its modulation by LycT (after two weeks of LycT treatment, eight week and twenty two weeks of NDEA treatment). Various different observations are reported in the literature regarding the role and concentration of various non-enzymatic and enzymatic components of antioxidant defense system on carcinogenesis and their alterations during chemoprevention (Klaunig et al., 2010; Godic et al., 2014). Endogenous antioxidants mainly includes non-enzymatic antioxidants such as reduced glutathione (GSH), vitamin C and vitamin E as well as enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-s-transferase.
Multiple biochemical reactions are carried by these endogenous antioxidants for preventing the harmful oxidative damage. Genetic polymorphisms and differential expression levels of these antioxidants play a crucial role in the individual’s susceptibility to DNA damage and cancer risk.

Reduced glutathione (GSH or γ-glutamyl-cysteinyl-glycine) is a ubiquitously and unanimously recognised as thio-containing tripeptide playing a central role in a broad range of vital functions of cell biology. GSH related area has been the subject of abundant research because of its implication in the cellular detoxification of endogenously produced deleterious compounds and exogenous xenobiotics. The structural features of GSH i.e. thiol (-SH) group on the cysteinyl residue (enabling GSH to exhibit a strong reducing activity) and γ-glu linkage have attributed to its biological activities. In mammalian cells reduced form of glutathione exists in higher concentrations than oxidised glutathione (GSSG) and mixed disulfides of GSH. GSH homeostasis in healthy liver tissue is maintained in cell by the balance between biosynthesis, uptake, oxidation and export. A growing body of studies have demonstrated that hepatic GSH to be an essential actor in several human chronic diseases including cancer and cardio-vascular diseases (Traverso et al., 2013). Interest has also been arising exploring the chemopreventive agents either synergistically, activating GSH production or taking GSH during cancer treatment. GSH is a known low molecular weight scavenger of free radicals present in the cytoplasm, however during exposure of free radicals or electrophiles it shows strong reducing activity and gets converted to GSSG. Thus the conjugation of GSH with reactive electrophilic products aids in the detoxification, otherwise these toxic metabolites might attack the cellular macromolecules. The excessive concentration of GSSG altered the intracellular environment and is an indicator of oxidative stress. Oxidative stress decreases the glutathione redox ratio (GSH/GSSG) and might be responsible in triggering the initiation of carcinogenesis (Saniz et al., 2012). Beside detoxification, GSH maintains the sulphydryl groups of many proteins in their reduced form which is an essential for normal cell functioning.

In the current study NDEA treatment for eight weeks produced HCC with low levels of hepatic glutathione redox level when compared to the control group. The observed reduction might be attributed to the excess utilization of GSH to alleviate free radicals. As discussed above NDEA during metabolism in hepatic cells produces high concentration of free reactive radicals. Another explanation for the decrease in the GSH level might be related to a reduced synthesis of the tripeptide by the diseased liver and hence influencing the capability of the
liver protection system against oxidative damage (Fernandez-Checa and Kaplowitz, 2005; Czeczot et al., 2006). A similar kind of decrease in GSH levels has been observed in experimental model of various cancers such as forestomach, skin and liver. Decrease in GSH levels in forestomach of mice after benzopyrene intra-gastric instillation has been reported in our laboratory (Ganger and Koul, 2008b). There are many reports in the literature revealing the decrease in the non-enzymatic antioxidants in hepatoma bearing animals (Seufi et al., 2009; Maideen et al., 2011). In present study the observed significant increase in the hepatic GSH levels in NDEA + LycT group when compared to NDEA group might be due to the direct reaction of lycopene with ROS. Lycopene being a strong scavenger of free radicals may have decreased the hepatic ROS content in the NDEA treated mice as discussed in the above section. In other ways, it can be explained that lycopene synergistically with GSH might have played a role in lowering the hepatic ROS level. This shows the protective role of lycopene in increasing the ability of the cells to detoxify activated metabolites. As evident by literature and earlier investigations it is well documented that lycopene enhances the GSH content during hepatotoxicity (Koul et al., 2010; Meydan et al., 2011). There are reports demonstrating the increase in GSH level upon administration of chemopreventive agents like Azadirachta indica, Phyllanthus polyphyllus etc. (Dasgupta et al., 2004; Maideen et al., 2011). However, the GSH levels remained unaltered in LycT group when compared to the control group throughout hepatocarcinogenesis.

In healthy liver tissue GSH homeostasis is maintained by the reaction catalysed by glutathione-depleting (GPx, GST) and glutathione-replenishing (GR) enzymes (Traverso et al., 2013). GPx chemically detoxify hydrogen peroxide and form, GSSG (oxidized form). GR further reduces the GSSG to its reduced sulfhydryl form GSH and hence regenerate the antioxidant agent. As described earlier GST catalyzes the conjugation of GSH with electrophilic family. GPx catalyses the reduction of toxic hydroperoxides with GSH and hence associate with catalase in detoxification. However, at low H₂O₂ concentrations GPx is responsible for the detoxification and at its high concentrations catalase takes over the detoxification. GPx also reduces lipid hydroperoxides to their native alcohol. GR and NADPH is required for efficient reaction mediated by GPx. In the current study, a significant decrease in the activities of GPx and GR of NDEA group can be correlated with the decrease in production of GSH in the cancerous liver tissue. Decline in the activities of GPx and GR in NDEA treated animals further exaggerate the carcinogenic potential of reactive metabolites
of NDEA in the cells. Literature also revealed many studies reporting a decrease in the activity of GPx and GR upon NDEA treatment (Pradeep et al., 2007; Seufi et al., 2009; Maideen et al., 2011; Zhang et al., 2012). Elevated activities of GPx and GR observed in LycT + NDEA group revealed the preventive role of lycopene. Ameliorating effect of antioxidant enzymes during NDEA induced modulation might be possible mechanism behind chemopreventive effect of various agents (Seufi et al., 2009).

Antioxidant defense molecules other than glutathione and glutathione related enzymes include SOD and CAT enzymes capable of removing or neutralizing free radicals. SOD is a metalloprotein, an enzyme of prime importance as it catalyses the dismutation of superoxide (O$_2^-$) radicals into H$_2$O$_2$ and molecular oxygen (O$_2$) (McCord and Fridovich, 1988). Further, CAT and GPx efficiently converted the H$_2$O$_2$ into H$_2$O. CAT is a heme protein predominantly located in peroxisomes and inner mitochondrial membrane. SOD and CAT activities have played a very important role in the tumorigenesis in various experimental models (Jeon et al., 2007; Radenkovic et al., 2013). In the present study NDEA group at 10$^{th}$ and 24$^{th}$ week showed a significant decrease in SOD and CAT activity. Decrease activity can be correlated with the presence of enhanced ROS level in the same group. The observed reduction in enzyme activities might be attributed to NDEA metabolites (ROS). There are reports demonstrating the role of ROS in reducing the activities of enzymes. The present observation is supported by earlier studies that showed decreased activities during NDEA induced hepatocarcinogenesis (Seufi et al., 2009; Zhang et al., 2012). Administration of LycT in NDEA challenged mice enhanced the activity of SOD and CAT when compared to the NDEA challenged mice. This indicates the antioxidant potency of the LycT and so preventing the inhibition of these enzymes from ROS. Enhanced SOD has demonstrated to inhibit tumorigenesis both in vivo and in vitro (Oberley, 2005). Enhancement of SOD activity has been reported in hepatic tissue after treatment with other chemopreventive agents as reported in literature (Dasgupta et al., 2004; Seufi et al., 2009). Considering these observations antioxidant therapies against cancer are focusing on enhancing the activities of SOD and CAT (Nelson et al., 2006).

5.6 Modulatory effect of LycT on NDEA induced hepatic cytotoxicity and hepatic membrane physiological parameters during early phase of HCC
Hepatocarcinogenesis proceeds by the accumulation of karyotypic alterations resulting in mutations in number of tumor related genes (Wong et al., 2003; Zimonjic et al., 2009). Chromosomal abnormalities (CAbs) have been observed in numerous pre-neoplastic and neoplastic lesions, and are involved in initiating the development and progression of carcinogenesis (Michor, 2005; de Assumpcao et al., 2006; Aly et al., 2010). Alterations in the number of chromosomes during CAbs reflect functional loss or gain of some genes, which in turn may contribute to carcinogenesis (Feitelson et al., 2002; Rajagopalan and Lengauer, 2004). Loss or gain of chromosomes has been associated with the inactivation of tumor-suppressor genes or activation of oncogenes. Consequently CAbs provide immortality, growth advantage and invasiveness to some hepatocytes. Numerous CAbs in human HCCs, such as loss and/or gain on different loci of chromosomes have been identified by different research groups (Feitelson et al., 2002). During the early stages of HCC formation, hepatic cells acquire the chromosome instability phenotype and transformed into aberrant cells. Actually those aberrant cells that survive such clastogenic effect are thought to become cancerous. Moreover, interesting findings were deciphered on the basis of CAbs analysis i.e. genetic heterogeneity to be the characteristics parameter of hepatocarcinogenesis and HCC can be formed via different paths inspite of single etiological factors (Nishida et al., 2003). Such aberrant transformed cells are more aggressive in nature as they undergo vascular invasion, metastasis and poorly differentiated HCC (Okabe et al., 2000). Although the multistage nature of hepatocarcinogenesis has been demonstrated and studied for more than many decades, only few investigations have been carried out on unravelling the association of chromosomal structure and content during carcinogenesis.

Micronucleus is a cytoplasmic body consisting of acentric fragments or whole chromosomes which have been separated from the main nuclei of the daughter cells during abnormal cell division and are biomarkers of genotoxicity and CAbs (Fenech et al., 2011). Left out chromosomes or chromosomal fragments acquire nuclear membrane and exist as third nuclei. And during subsequent cell division micronuclei are unequally divided in daughter cells, hence lead to loss of genes. On further division, cells with reduced chromosomes either died or are phagocytised by kupffer cells. Micronuclei formation can be considered as one of the consequences of CAbs and can serve as a biomarker for evaluating genetic instability (Fenech et al., 2011). During abnormal cell proliferation in carcinogenesis there are more chances of CAbs leading to formation of micronuclei. Moreover, exposure of substances causing chromosome breakage (clastogens) as well as affecting the spindle apparatus.

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(aneugens) may act as an inducer of micronuclei formation (Ribero et al., 2008). The frequency of micronucleated cells has a direct association with the induction of carcinoma (Buajeeb et al., 2007). Thus, micronucleus assay can be used for assessing hepatic chromosomal damage induced by NDEA exposure. It may provide simple and reliable morphological criteria for measuring cytotoxicity at the level of chromosomal damage such as chromosome breakage, chromosome loss and altered cell division (Fenech, 2011).

N-Nitrosodialkylamines are known as pro-mutagens and pro-clastogens as upon metabolic activation the metabolites possess clastogenic activity leading to CAbs (Aiub et al., 2011a and b). NDEA in the present study had shown clastogenic effect at 10th week as it caused breaks in the chromosomes leading to different chromosomal rearrangements such as deletion and addition. Free radicals generated during NDEA metabolism in liver tissue are responsible for the disruption of chromosomal integrity. There are many reports in the literature demonstrating nitrosamines induced CAbs and mutagenicity in experimental carcinogenesis (Factor et al., 2000). Moreover, in the present study significantly high percentage of micronucleated cells in NDEA group indicate its hepatotoxic effect. There are many reports in the literature demonstrating similar observations where NDEA exposure has been related with high altered cell proliferation (Bursch et al., 2005; Koul et al., 2007). Moreover, the observed high LPO status in NDEA group after 10th week can be correlated with increased micronuclei formation. Mayer et al., (2000) have established a positive link between LPO status and genotoxicity as revealed by increased micronuclei score in lymphocytes. In our laboratory also there are reports indicating an increase in micronuclei score in hepatic tissue in response to intra-peritoneal injection of DMBA (Koul et al., 2010). Ganger et al., (2010) have reported an increase in micronuclei score in spleen of animals who were intragastrically treated with B(a)P.

LycT being an antioxidant had shown decreased chromosomal aberrant cells demonstrating the positive correlation between ROS production and chromosomal damage. The chromosomal analysis conducted on primary hepatocytes from 10th week study indicated that LycT had suppressed the clastogenic effect of NDEA and hence is involved in delaying the progression or development of hepatocarcinogenesis. Literature revealed similar results in primary hepatocytes cultures where vitamin E supplementation had reduced the percentage of aberrant cells by reducing ROS production (Factor et al., 2000). Cavusoglu and Yalcin (2009) had also evaluated the radioprotective role of lycopene on CAbs. Moreover pre-treatment of
LycT to NDEA challenged mice showed decreased level of hepatic micronucleated cells in the present study suggesting an anti-genotoxic effect of lycopene. Several in vivo and in vitro studies have shown the protecting effect of lycopene in terms of lowered DNA damage (Toledo et al., 2003; Koul et al., 2010). Specifically, there are number of reports on decreased hepatic genotoxicity by increasing consumption of tomato extract (Bhuvaneswari et al., 2004). Free radical scavenging ability or antioxidant property of lycopene may be contributing in decreasing the genotoxic effect.

Biological cell membrane (plasma membrane and organellar membrane) defines the cell, outlines its borders, and determines the interactions of cell with its environment. Plasma membrane is thin, fragile, flexible and semi-permeable membrane separating the internal environment of the cell from the external milieu. Plasma membrane regulates the transport of molecule in and out from the cell, hence maintaining a steady internal environment. As described in “fluid-mosaic model”, membrane structure is a phospholipid bilayer in which proteins are scattered either just outside or within the membrane. Biological membranes consist of complex assemblies of lipids and proteins allowing organized cellular compartmentalization (Raghuraman et al., 2003). The life of the cell depends on the maintenance of the normal composition and arrangement of plasma membrane. Heterogeneous distribution of the components (lipids and protein) in the membrane is responsible for the existence of unique packing and diversity in the membrane environment. Various cellular activities of the cells are governed by its physicochemical characteristics including fluidity, microviscosity, polarization, electrostatic potential and phase state.

Membrane fluidity is the most important physicochemical property of cell membrane as it plays an important role in the proper functioning of the membrane integral proteins, enzymes, receptors and ion channels. The maintenance of membrane fluidity is prerequisite for proper cell function, viability, growth and reproduction. Membrane lipid composition and lipid-protein interactions have a major influence on membrane fluidity. Membrane fluidity mainly depends upon the conformational structure of various constituents of mammalian cellular bilayers such as phospholipids and sphingolipids and membrane protein dynamics (Lenaz, 1987; Cantor, 1999). Moreover, membrane fluidity is directly associated with microviscosity, which is inversely related to the rotational and lateral diffusion rates of membrane components. In normal conditions without any constraints lipids and unrestrained integral proteins freely diffuse in the plane of membrane with high diffusion coefficients (Hollan,
Any alterations in the physiological characteristics of membranes directly or indirectly affect the crucial cellular processes, such as proliferation, differentiation, malignancy and programmed cell death (Galisteo et al., 2000). In the recent findings, various anticancer agents induce apoptosis via altering tumor cell membrane fluidity (Baritaki et al., 2007).

At present, many new macroscopic and microscopic methods are being developed for measuring translational diffusion of membrane lipids and proteins. Fluorescence spectroscopy and anisotropy measurements are the most widely used techniques in membrane research during diseased conditions. Merocyanine 540 (MC540) is a fluorescent dye having negatively charged heterocyclic chromophore and molecular weight of 570Da. MC540 is considered as a potential membrane probe (Siboni et al., 2001). Membrane electric potential and membrane packing directly influences the extent of MC540 binding and hence affect the intensity of fluorescence. Membrane electric potential has the direct relation with the membrane fluidity as MC540 binds with bilayers with wide spaced lipids. Thus, ability of MC540 to bind preferentially to biological membrane has been exploited to analyse the metabolic states of both normal and cancer cells (Chen et al., 1997; Siboni et al., 2001).

Pyrene is a UV-excited lipid probe that forms intermolecular excimers when embedded in phospholipid vesicles and biological membranes (Rebrova et al., 2013). This property of pyrene has been extended in monitoring the dynamic properties of membrane lipids revealing the alterations in microviscosity in tumor cells. Another fluorescent probe widely used to determine membrane fluidity is 1, 6-diphenyl-1,3,5-hexatriene (DPH) (Caudron et al., 2007). DPH is non-fluorescent in water but exhibits high fluorescence intensity when it gets intercalated into lipid membranes.

In the present study fluorescence spectroscopic analysis revealed that NDEA exposure to mice altered hepatic membrane physiological characteristics. After 10th week NDEA showed a rapidly increasing membrane fluidizing effect. NDEA is one of the principle nitrosamine that undergoes intercalation with membrane lipids for free radical formation. Increased ROS and LPO levels in NDEA group after 10th week may have direct relation with the current increase in the membrane fluidity. Sergent et al., (2005) had also elucidated the elevation in membrane fluidity upon ethanol metabolism and ROS formation. Moreover, LPO has been shown to perturb the bilayer structure of biomembranes and modify membrane fluidity (Chaterjee and Agarwal, 1998; Spengler et al., 2013). Similar observations have been
reported in the literature where lipid peroxides produced upon NDEA treatment have resulted in altered membrane fluidity (Gayathri et al., 2009). A rapid increase in hepatocytes membrane fluidity has been cited in literature playing a crucial role in inducing hepatotoxicity caused by several drugs (Galisteo et al., 2000, Rebillard et al., 2007). There are studies reporting the tendency of malignant cells to bind more MC540 than normal cells because of increased membrane fluidity (Siboni et al., 2001). Various parameters such as intracellular alkalinisation, increased phosphatidylethanolamine (PE), decreased cholesterol and decreased phosphatidylcholine/ phosphatidylethanolamine (PC/PE) ratio have been associated with the increased membrane fluidity (Abel et al., 2001). There are reports revealing a correlation of oxidative stress with membrane fluidity (Singh et al., 2011). However, it is very important to mention that free radicals and ROS act as double edged sword in influencing the membrane structure, fluidity and other functional properties of membranes (Remy-Kristensen et al., 2000). Altered growth pattern and progression of hepatocytes nodules during cancer development has been linked with the changes in membrane fluidity as it has a influencing role in signal transduction pathways and cellular regeneration. MRP, ABCB and OAT are membrane transporters involved in the movement of ions, small molecules, macromolecules (proteins) across the biological membrane. Membrane fluidity plays an important role in proper functioning of these transporters (Gutu et al., 2011). On the other hand, binding affinity of MC540, pyrene and DPH in isolated hepatocytes directly evaluate the alteration in the composition or structure of lipid bilayer affecting membrane physiological characteristics. In the present altered membrane fluidity is because of the oxidative products formed in the membrane or due to alterations in the phospholipid fatty acid composition. Further, detailed study showed that alterations in lipid content of hepatic membrane have a close link with the changes in membrane fluidity (Abel et al., 2001).

Pre-treatment by LycT had ameliorated the effects of NDEA by normalizing the membrane characteristics properties. The present observations regarding lycopene and maintenance of membrane characteristics are in agreement with the previously reported observations (Suwalsky et al., 2002; Gruszecki and Strzalka, 2005). The biological benefits of lycopene may be due to their potent antioxidant properties and attributed to specific physic-chemical interactions with membranes (McNulty et al., 2007). Lycopene like other carotenoids can influence membrane characteristics such as fluidity, stability, and susceptibility to oxidative damage (Kong et al., 2010). Hydrophobic carotenoids, such as lycopene and β-carotene, tend to
be solubilised in the core parallel to the membrane surface. Several research groups have designed their studies for better understanding of interactions between carotenoids and membranes using various techniques such as NMR, X-ray, fluorescence measurement, ESR and differential scanning calorimetry (Rengel et al., 2000; Socaciu et al., 2000; Kostecka-Gugala et al., 2003). Observations from different techniques showed that apolar carotenoids lie inside the hydrophobic core, perpendicular to phospholipid acyl chains. Studies have reported maintenance of membrane fluidity as a proposed ability of lycopene in response to challenges. Reduction in LPO upon LycT administration in NDEA challenged mice and decreased ROS levels could be the possible reasons behind maintaining membrane fluidity and other physico-chemical properties of membrane (Gruszecki and Strzalka, 2005).

5.7 Role of Apoptosis in Chemopreventive Action of LycT against NDEA induced hepatocarcinogenesis

Development of any cancer requires limited number of ‘mission critical’ events to propel the tumor cell into uncontrolled expansion and proliferation. Deregulated cell proliferation with obligate compensatory suppression of apoptosis provides a minimal platform supporting neoplastic transformation (Evan and Vousden, 2001; Wong, 2011). Apoptosis is a highly sensitive and orchestrated cellular process which has importance in both pathological and physiological conditions. Tumorigenesis is an outcome of excessive proliferation due to the activation of oncogenes and frequently concurrent impairment of apoptosis (Hanahan and Weinberg, 2000). Actually the alterations that may induce malignant transformations sensitize a cell to apoptosis. Thus, simultaneous persistence of defects in apoptotic pathways may protect the cell from cell death induction and hence, produces malignant transformed cells (Vousden and Lu, 2002). Defects in apoptotic pathways involve inappropriate activation of anti-apoptotic proteins or inactivation of pro-apoptotic factors (acting like tumor suppressor). Evaluation of the underlying mechanism of apoptosis during hepatocarcinogenesis is important as it plays a pivotal role in understanding the onset of hepatocarcinogenesis but may also aid in finding the clue for treating the disease. Induction of apoptotic pathway in ‘transformed cells’ may serve as potential targets for preventing and treating HCC (Choedon et al., 2006). Exploring novel chemopreventive agents targeting apoptosis has emerged as a potential measure in cancer intervention. There are many anti-apoptotic and pro-apoptotic genes well known for their important role in apoptosis. Demonstrating the variation in the expression of such genes at mRNA and protein levels may
be beneficial in determining the effectiveness of the chemopreventive agent in preventing HCC. Numerous studies have found a close association of endonuclease activation with apoptosis as evident by the observed characteristic DNA fragment pattern. Alterations in apoptosis can be analysed by various techniques such as histopathology, electron microscopy, TUNEL assay, comet assay etc. Apoptosis is characterized by various biochemical and morphological alterations including cell shrinkage, chromatin condensation, altered mitochondrial membrane potential, activation of caspases, nuclear and cell fragmentation, membrane blebbing etc. Production of free radicals during NDEA metabolism in liver may be responsible for its hepatocarcinogenic effects (Shaarawy et al., 2009). The use of potent antioxidants like lycopene seems to be a rational approach to counteract oxidative stress induced carcinogenesis. Studies point out that lycopene can modulate cellular processes such as cell proliferation, cell differentiation, cell signalling, apoptosis, etc., in several model of tumorigenesis (Wang et al., 2010; Ip et al., 2014). In-vitro studies have shown higher efficacy of lycopene phyto-complex in triggering apoptosis in phytodynamic therapy (Ettorre et al., 2010). We further extended this work to gain insight into the demonstration of apoptosis in NDEA induced hepatocarcinogenesis and its intervention using LyeT.

Various assays have been designed based on these alterations to quantify apoptosis. Electron micrographs of SEM and TEM have revealed apoptotic cells with cell shrinkage and apoptotic bodies. Another commonly used assay for the quantification of apoptosis is agarose gel DNA fragmentation assay. Apoptosis is an event accompanied by the degradation of internucleosomal DNA yielding a ladder like pattern in electrophoretic gel. Endogenous DNase cleaves the dsDNA into fragments of 180-200 base pairs (Afford and Randhawa, 2000). Small fragments of oligonucleotides are easily extracted selectively from the hepatocytes whereas the larger DNA fragments stay associated with the nuclei. Thus, small DNA fragments are visualized as ladder pattern in agarose gel using ethidium bromide (EtBr). However, genomic DNA from non-apoptotic cells would show a broad band near wells in agarose gel. In the present investigation, DNA was extracted from liver tissue from all the groups after 10th and 24th week of the study. After 10th week no visible DNA fragmentation was appeared in any of the groups. This can be explained as no significantly altered apoptosis rate was observed in any of the four groups. However, the genomic DNA from NDEA group showed a slight variation in band size and short smear with higher molecular weight. This smear can be explained due to the cytotoxic effect of NDEA. There are reports demonstrating cell death due to necrosis in tumor which potentiates tumor growth
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(Vakkila and Lotze, 2004). However, after 24th week of the study the clear DNA ladder like pattern was appeared in LycT + NDEA group indicating high apoptosis. Rest of the groups did not show any DNA fragmentation. The current observations revealed that LycT pre-treatment to NDEA challenged mice increases apoptosis rate hence may be responsible to counteract the increasing cell proliferation and mutated cells.

Similarly, terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) assay was done to identify and localize the apoptotic cells. DNA fragmentation may also be demonstrated by in situ incorporation of labelled nucleotides onto the 3’OH ends of DNA fragments using a terminal deoxynucleotidyl transferase enzyme (TdT). In the present study brominated nucleotide (BrdU) was used which get incorporated more effectively at the sites of DNA fragmentation. Biotinylated anti-BrdU antibody was used for the detection of incorporated BrdU and visualized using DAB and counter stained by methyl green. At 10th week, the hepatocytes in all groups were stained green and hence illustrate TUNEL negative cells (non-apoptotic). Few brown cells indicating TUNEL positive cells (apoptotic) were observed in the groups revealing low apoptotic index. However, at 24th week DAB generates dark brown staining in cells undergoing apoptosis and is visualized against a green counter stain in LycT + NDEA giving a significant high apoptotic index. The present observation is in agreement with the previously reported induction of apoptosis on administration of various identified chemopreventive agents in a variety of premalignant or malignant cell types in vitro and in a few animal models in vivo and in clinical trials (Sun et al., 2004; Kuno et al., 2012). NDEA group showed few brown stained cells but with high cell density. Hence, apoptotic index was found to be lower than in control group. Thus, present observations from TUNEL assay were similar to that of the DNA fragmentation assay. NDEA treatment resulted in the formation of hepatic tumors and caused no significant change in apoptotic index when compared to the control group. Studies have reported decreased rate of apoptosis with NDEA administration (Shaarawy et al., 2009).

However, another technique i.e. single cell gel electrophoresis (or COMET assay) was also carried out to demonstrate apoptosis. COMET assay is a powerful technique used for measuring both DNA strand breaks and oxidative base damage (Hao et al., 2009). The present study demonstrated an increase in number of comet shaped cells in NDEA treated mice compared to the healthy control mice. The present observations are in corroboration with the previous studies reporting NDEA induced DNA damage as indicated by comet...
shaped cells. The increment in comet formation may be due to the production of pro-mutagenic DNA lesions of NDEA bio-transformed compounds, playing an important role in inducing hepatocarcinogenesis. LycT administration to NDEA challenged mice in the present study showed a further increase in comet formation when compared to NDEA group, however statistically non-significant. Induction of apoptosis in cancer cells by lycopene has been observed in certain *in-vivo* and *in-vitro* studies. Lycopene has been shown to induce mitochondrial apoptosis in LNaCP cells and HuCC cells (Salman et al., 2007).

The modulatory effect of LycT on the mRNA and protein expression of certain apoptosis associated genes was examined during NDEA induced HCC. An enhanced expression of bel-2 and diminished expression of bax, caspase 3, caspase 9 and p53 was observed in the NDEA group when compared with the control group. The above observations are in concordance with several reported alterations in expression of apoptotic associated genes on NDEA exposure (Zhang et al., 2012). Moreover, LycT administration to NDEA challenged mice significantly down-regulated the expression of anti-apoptotic protein bel-2 and enhanced the expression of caspase 3, caspase 9 and p53 when compared to the NDEA group.

Bel-2, a major anti-apoptotic protein regulates the mitochondrial-mediated apoptosis by maintaining mitochondrial membrane permeability. Bel-2 protein inhibits apoptosis by preventing the mitochondrial release of cytochrome c and eventually resulting in inhibition of caspase activity (Kirkin et al., 2004). Bel-2 family consists of both anti-apoptotic proteins (Bel-2, Bel-X1, and Mel-1) and proapoptotic molecules (Bax, Bak, and BH3 domain). Bax, is a pore forming pro-apoptotic protein that facilitates cytochrome c release, consequently trigerring caspase mediated apoptotic cell death (Daniel et al., 2001). It has been well documented that when anti-apoptotic Bel-2 family members are over-expressed, the ratio of pro- and anti-apoptotic bel-2 family members is disturbed and apoptotic cell death can be prevented. Cysteine proteases i.e. caspases are another known key players that execute the apoptotic cascade. Caspases leads to proteolysis of specific substrates and morphological changes associated with programmed cell death. p53 is known as the first tumor suppressor gene linked to the variety of cellular outcomes, most notably cell cycle arrest and apoptosis. It has been observed that approximately fifty percent of all human cancers contain cells with mutations or deletions in both alleles of the p53 gene (Vogelstein et al., 2000). These tumors bearing mutated p53 tend to be more invasive with a high metastatic risk and are correlated with a poor prognosis. Tumor induced mutations in p53 abrogate its cardinal functions in
promoting apoptosis, cell cycle arrest and DNA repair, thereby leading to cancer development and progression. Failure of apoptosis creates a conducive environment for genomic instability resulting in accumulation of mutations that could eventually promote neoplastic transformation.

The current observations demonstrate the induction of apoptosis in LycT intervened group. The cancer-preventive effect of lycopene mediated by its ability to induce apoptosis has been previously reported (Zhang et al., 2003). The exact mechanism behind the enhanced apoptosis in cancerous cell treated with lycopene has not been elucidated. However, lycopene’s activities in causing cell death might be attributed to its metabolic products or oxidative decomposed products (Wang, 2012). Some studies inferred that lycopene metabolite product, rather than the intact molecule, may act at the gene level to modulate the expression of relevant genes and induces apoptosis in cancer cells. It has been suggested that it may depend on the intracellular formation of auto-oxidant products of lycopene rather than to lycopene itself (Zhang et al., 2003). In-vitro studies have identified lycopene oxidation products under several oxidative conditions, hence could be associated with the auto-oxidation of lycopene in biological systems during oxidative conditions (Kim et al., 2001; Zhang et al., 2003). A series of lycopene cleaved products such as apolycopenals have suggested to be produced in biological tissues under oxidative stress. Lycopene have been shown to induce mitochondrial apoptosis in several in vitro studies. Limited data exist concerning the effect of lycopene on apoptosis in experimental hepatic model. Studies have reported apoptotic effect of lycopene in gastric cancer elucidating its effect on p53-dependent apoptosis (Liu et al., 2006a). Study has shown lycopene metabolite reduced cell viability by inducing apoptosis in HL-60 cells through the activation of caspases and reduction of bcl-2 gene (Zhang et al., 2003).

Apoptosis has also been associated with the changes in cellular redox environment. Bcl-2 protects cells from oxidative stress induced cell death via different unravelled mechanisms including a reduction in the formation of ROS, the prevention of oxygen radical-mediated LPO, the inhibition of cytochrome c release from mitochondria or the alteration of the cellular glutathione pool (Lee et al., 2001). Bcl-2 has been implicated in the regulation of intracellular redox status and more interestingly Bcl-2 localizes to all sites of ROS production in cell (Droge, 2002; Chen and Pervaiz, 2009). Bcl-2 inhibits the release of GSH through cystic fibrosis transmembrane conductance regulator (CFTR), which further enhances GSH
accumulation. Combined depletion of bcl-2 and GSH has been reported and has been linked to the increased anti-tumor efficacy. Recently, direct binding of glutathione with Bcl-2 has also been described, although bcl-2 does not appear to substitute for GSH (Zimmermann et al., 2007). Down-regulation of bcl-2 gene expression in cancerous cells may contribute a protective effect leading to apoptosis. Such reports strengthen our present observations and provide explanation behind lycopene induced programmed cell death during NDEA induced hepatocarcinogenesis. Moreover, as discussed earlier lycopene administration to NDEA challenged mice showed differential effects on the levels of LPO and redox ratio during hepatocarcinogenesis. A significantly decreased LPO level and GSSG/GSH ratio after 10 weeks of treatment was presumably due to the ability of LycT to scavenge hydroxyl and peroxyl NDEA radicals. Studies have indicated a decrease in the initiation of liver pre-neoplastic foci by NDEA with dietary intake of lycopene (Astrog et al., 1997). Interestingly, marked increase in LPO and redox ratio after 24 weeks was observed in LycT + NDEA group. This present observation may be inferred to be associated with the consequences of the enhanced apoptosis with LycT administration. Similar observations have been previously reported that lycopene administration has been linked to oxidative stress induced apoptosis (Liu et al., 2006a).

5.8 Cell cycle regulation during NDEA induced hepatocarcinogenesis and its intervention with LycT

Abnormal and uncontrolled cell proliferation is considered as the prime hallmark of carcinogenesis. Cancer is a hyperproliferative disease which is the outcome of the genetic changes that arises due to the failure in precise replication of the DNA. Number of factors and proteins are involved in the proper and accurate replication of DNA and cell cycle. Chemopreventive agents can be explored acting at several points along the cell cycle and thus differentially regulating the various factors involved in progression of cells through cell cycle.

Proliferating-cell nuclear antigen (PCNA) is a multifunctional protein forming a ring around the DNA to facilitate and control DNA replication (Subapriya et al., 2006). PCNA is a ubiquitously expressed protein essential for several cellular processes such as DNA replication, DNA repair, chromosome segregation, chromatin structure maintenance and cell-cycle progression (Stoimenov and Helleday, 2009). Number of emerging evidences suggests that regardless of the origin, tumor cells express higher levels of PCNA and overexpression
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of PCNA is considered as the reliable biomarker for malignant cells (Zhong et al., 2008; Stoimenov and Helleday, 2009). Targeting PCNA’s protein interaction may serve as a useful strategy for inhibiting multiple altered pathways. In the present study, up-regulation of PCNA was observed in NDEA group indicating hyperproliferation. As discussed earlier histopathology of NDEA treated liver has also showed the increased cell density hence correlating with the enhanced mRNA and protein expression of PCNA. Several research groups have observed similar upregulation in PCNA expression during NDEA induced hepatocarcinogenesis (Subramanian and Arul, 2013). Arora et al., (2013) have also reported an enhanced PCNA expression in DMBA/TPA induced skin tumors. However, in the current study pre-treatment of LycT to NDEA challenged mice decreased the expression of PCNA indicating anti-proliferative effects. Recently, Wang et al., (2010) reported the significant reduction in PCNA positive hepatocytes upon lycopene and tomato extract consumption during DEN and NASH-promoted hepatocarcinogenesis. The lower expression of PCNA in the liver of tumor bearing experimental animals as evident in the current study and literature might be responsible for its antitumorigenic activity. Cheng et al., (2007) determined the suppressive ability of different carotenoids against PCNA in cell proliferation. Among various carotenoids (such as β-carotene, lutein, lycopene, mixed and vitamin E) lycopene had exerted higher inhibitory effect against cell proliferation than other carotenoids.

Mammalian cell cycle has evolved several checkpoints mechanisms in order to monitor and respond to different perturbations. Perturbations can be detrimental to the integrity of the genome, promote cancer development or significantly affect the efficacy of drug treatment. During perturbations these checkpoints halt the cellular progression until the defaults are fixed or the environment becomes permissible to the faithful transmission of DNA (Bartek and Lukas, 2007). The cyclin-dependent kinase inhibitor p21 plays an important role in regulating mammalian cell cycle. p21 is an important downstream mediator of p53 and regulates the function of several cell cycle proteins like cyclin D1 and cyclin dependent kinases (CDKs), consequently regulating cell cycle (Perkins, 2002). Induction of p21 leads to cell cycle arrest at G1 stage by inhibiting the activity of cyclin-CDK or –CDK4 complexes. Anti-proliferative property of p21 poised to play an important role in preventing tumor development. Biochemical and genetical evidences indicates that p21 may also act as a potent effector of multiple tumor suppressor pathways that are independent of the classical p53 mechanism. p21 is induced by number of factors including oxidative stress and by both p53-dependent and –independent mechanisms. Despite its profound role in halting proliferation
several studies have presented that p21 has both anti-proliferative and anti-apoptotic effect in number of the system (Gartel and Nyers, 2002; Roninson, 2002). In the present study, a decrease in p21 mRNA and protein expression was observed in the liver of NDEA group when compared to control group. Down regulation of p21 in NDEA group might be contributing in abnormal high proliferation as the checkpoint is perturbed. Pal et al., (2012) has also demonstrated the reduction in the expression of p21 upon NDEA treatment indicating hyperproliferation. However, in several tumors like oral SSCs or skin tumors expression of p21 has been found to be up-regulated (Kuropat, 2002; Arora et al., 2013). Treatment with LycT to NDEA challenged mice caused a significant increase in the mRNA expression of p21 when compared to the NDEA group. Increased p21 expression means enhanced anti-proliferative effect and hence may be contributing in inhibiting the NDEA induced HCC upon LycT treatment. Although it is difficult to comment how lycopene or its metabolites are inhibiting the HCC, but literature demonstrate that treatment with lycopene or metabolite increased the p21 expression and hence aid in preventing NDEA induced cancer (Ip et al., 2013). In our laboratory too other chemopreventive agents such as AAILE has shown similar results. Expression of p21 correlates with the increase in p53 expression and halt the G1 phase indicating cell cycle arrest.

Cyclin D1, an important regulator of cell cycle is a proto-oncogene playing role in the progression of G1 to S phase. Cyclin D1 is a transcriptional co-regulator forming active complexes with cyclin dependent kinases (CDK4 and CDK6) and promoting cell cycle progression. Cyclin D1 also functions as transcriptional modulator as it modulates various transcription factors and histone deacetylase. Mullany et al., (2010) reported the essential role of cyclin D1 in regenerating liver. Cancer research showed that overexpression of cyclin D1 is important for the development and progression of several cancers (Alao, 2007). Overexpression of cyclin D1 is not solely dependent on the gene amplification; rather result from the defective regulation at the post-transcriptional level. In the present study NDEA group mice showed significantly overexpressed cyclin D1 indicating uncontrolled cell cycle. Overexpression of cyclin D1 is consistent with similar findings in NDEA induced experimental tumors reported in literature (Pal et al., 2012; Nitha et al., 2014). However, administration of LycT to NDEA treated mice caused an inhibition in cyclin D1 mRNA and protein expression levels when compared to NDEA group. Several reports in literature indicate the inhibitory effect of lycopene against cyclin D1 and other proliferating factors (Liu et al., 2006a; Cheng et al., 2007). Enhanced cell proliferation and apoptotic evasion in
NDEA induced HCC was associated with the imbalance in pro-apoptotic proteins together with upregulation of PCNA and cyclin D1 and downregulation of p21, caspase-3 and 9. Inhibition of cyclin D1 and PCNA with enhanced expression of p21 and p53 by LycT suggests its regulatory effects on cell cycle progression.

5.9 Liver function marker enzymes during NDEA induced hepatocarcinogenesis and its intervention with LycT

Liver is the main site for NDEA biotransformation and uncontrolled generation of free radicals in liver cells are responsible for the oxidative stress induced cell damage. Eventually oxidative stress has been attributed in the initiation and progression of HCC as discussed earlier. The pathological changes during HCC development and its inhibition by chemopreventive agents i.e. LycT have been observed in the biochemical, histopathological, ultrastructural and molecular studies of the host system. However, the status of liver cancer can also be reflected by observing the levels of particular enzymes or factors in the serum. The activities of several enzymes such as transaminases (AST and ALT), phosphatase (ALP) and dehydrogenase (LDH) in serum reflect the status of the liver tissue.

In liver aminotransferases catalyses the reversible transfer of the amino group and participate in number of important intermediate pathways. AST and ALT transfer their oxo acid and amino acid substrates into several pathways. ALT is a pyridoxal enzyme catalyzing the conversion of L-alanine and ketoglutarate to pyruvate and L-glutamate. ALT in cytoplasm is associated with the utilization of pyruvate in glycolysis, in mitochondria involved in the conversion of alanine to pyruvate to be used in gluconeogenesis, and AST is important in transporting reducing equivalents across the mitochondrial membrane (Sakagishi, 1995). ALT and AST are not normal components of blood and have no specific function outside the organ. Routinely monitoring of the enzymes by physicians is considered as the indicator of liver cancer risk (Gowda et al., 2009). Analysis of liver function markers in blood can be considered as a potent noninvasive laboratory technique identifying the type and extent of liver damage (Solter, 2005). ALP is also another key hepatic marker enzyme. ALP increment in serum indicates the pathological alterations in bile flow.

In the present study the levels of enzymes related to liver metabolism (AST, ALT and ALP) were analyzed in serum and liver tissue. Moreover, to be more specific the study was carried out at three stages i.e. 2nd, 10th and 24th week of the experiment for better understanding the
relateion between the liver function enzymes and HCC. Significant increase in the transaminases level in serum and tissue indicates the loss of functional integrity of the hepatocytes membrane during NDEA induced HCC. NDEA metabolism in liver cells releases enormous free radicals attacking plasma membrane. As discussed earlier, NDEA group mice have altered membrane physiology and integrity. Hepatic damage with subsequent disruption in cell membranes results in the leakage of intracellular enzymes in bloodstream. Moreover, it has also been well published that exposure of hepatotoxicants or carcinogens results in the increased synthesis of aminotransferases by hepatic cells. Various clinical studies have reported comprehensive analysis of serum liver enzymes as predictors of HCC (Hann et al., 2012). Enhanced hepatic ALT in NDEA treated group may also correlated with the high glycolysis rate of tumor cell. The possible explanation may be that ALT is involved in production of pyruvate. Several epidemiological studies considered the elevations of transaminases as the most sensitive markers of HCC damage and indicator of high incidence of HCC development in patients. Among the various clinical markers for hepatic injury ALT are the important indicators for early preclinical animal testing to post marketing liver monitoring in patient (Amacher, 1998). AST in conjugation with ALT can serve as a important marker for liver mal-functioning (Kim et al., 2013). The increase found in the level of AST and ALT enzymes in the serum and tissue of mice treated with NDEA was significantly reduced by LycT treatment suggesting its protective effect against NDEA induced HCC probably by preventing membrane damage, loss of integrity and oxidative stress.

5.10 Carbohydrate metabolism during NDEA induced hepatocarcinogenesis and its intervention with LycT

Free radical induced oxidative stress generally leads to oncogenic mutation i.e. activation of proto-oncogenes and deactivation of tumor suppressor genes. Oncogenic mutation ultimately alters the metabolism in a way it support tumorigenesis. One of the essential and the necessary alteration for nearly all cancers development is the induction of aerobic glycolysis (also known as Warburg effect) (Warburg, 1956a; Koppenol et al., 2011). Recently, scientists have discovered that sustained aerobic glycolysis is linked to oncogenic mutations (Cairns et al., 2011; Koppenol et al., 2011). For many decades, the biological transformation of normal cell into malignant tumor cells has attracted the interest of researchers in this area. Aerobic glycolysis has been then assigned as the metabolic hallmark for many tumors. Tumor cell
depends on glycolytic pathways for fulfilling their ATP requirement during abnormal cell proliferation. Aerobic glycolysis and anaerobic glycolysis are similar in the way that glucose is converted into lactic acid, however aerobic glycolysis arises as an essential compensatory mechanism for damaged respiration in tumor cells and anaerobic glycolysis arises from the absence of oxygen. Several studies find the similar observations that genes for glycolysis are overexpressed in tumor cells (Altenberg and Greulich, 2004; Ortega et al., 2009). Targeting and preferential killing of tumor cell population without causing toxicity to normal cells can serve as a promising approach in cancer chemoprevention.

In the present study, the NDEA group mice showed enhanced hepatic hexokinase, phosphoglucoisomerase and aldolase enzymes activities at 24th week indicating high rate of glycolysis during HCC. Pelicano et al., (2006) reviewed several important aspects of glycolytic pathway in cancer and highlighted various glycolytic enzymes for targeting cancer. There are many studies reporting the enhanced glycolytic enzymes during chemically induced HCC in experimental animals (Pirinen et al., 2004; Langeswaran et al., 2012). Hexokinase catalyse the rate-limiting reaction in glycolysis i.e. ATP-dependent phosphorylation of glucose. Phosphorylation of glucose (non-ionic) to glucose-6-phosphate (G-6-P, anion) contributes in trapping the glucose in the cells. G-6-P serves as a substrate for glycolytic pathway or pentose phosphate pathway or glycogen synthesis. Literature supported the observation revealing the fact that rapidly growing tumors exhibits increased gene copy number of hexokinase and enhanced rate of transcription (Pirinen et al., 2004). Based on $^{18}$F-FDG uptake on PET scan of different HCC liver showed that well differentiated HCC showed low $^{18}$F-FDG uptake whereas poorly differentiated HCC showed high $^{18}$F-FDG uptake indicating increased hexokinase II expression (Lee et al., 2005). Hexokinase exists in cell in either free form in cytosol or bound form with mitochondrial membrane (Wilson, 2003). Mitochondrial bound HK II utilizes the ATP produced by oxidative phosphorylation for the reaction. Moreover, researchers have also found the relation between hexokinase activity and mitochondrial apoptotic signaling cascades. Mitochondrial membrane bound hexokinase interact with the outer membrane protein voltage dependent anion channel which contributed in suppression of intermembrane space proteins inhibiting apoptosis and thus contributing in tumor cell survival (Pastorino et al., 2002; Pastorino and Hock, 2003).

Phosphoglucoisomerase (PGI) catalysing the interconversion of G-6-P and fructose-6-phosphate is a ubiquitous cytosolic enzyme playing multiple functions. PGI is important for
both the glycolytic and gluconeogenesis pathways. Moreover, PGI has been found to be a potent mitogen/ cytokine known as autocrine motility factor (AMF) (Sun et al., 1999). Wide spectrum of malignancies, cancer progression and metastasis has been linked with the increased PGI expression (Tsutsumi et al., 2009). The elevated level of PGI has served as an excellent response of cancer condition and is taken as a marker of metastatic growth in patients. Aldolase is another key enzyme in glycolysis and catalyses the reversible cleavage of fructose-1-phosphate and fructose-1,6- (bis) phosphate (FBP) to di-hydroxyacetone and glyceraldehyde/ glyceraldehyde-3-phosphate. Aldolase exists as three isomers. Aldolase A exists pre-dominantly in muscle and red blood cells. Aldolase B exists dominantly in liver, kidney and small intestine. Aldolase C pre-dominantly exists in brain and neuronal tissues. Enhanced activity of aldolase in hepatic tissue may be linked with the enhanced increase glycolysis during HCC. Several experimental studies have observed increased levels of PGI and aldolase in hepatoma revealing high glycolytic rate and leakage from destructive neoplastic tissue (Tsutsumi et al., 2009; Langeswaran et al., 2012). However, there are reports showing aberrant expression of aldolase B in HCC patients where 57% patients have downregulation and 47% have maintained the expression (Peng et al., 2008). Supporting the current results there are also reports revealing the up-regulation of aldolase in metastatic conditions of breast tissues and HCC model (Hennipman et al., 1988; Sivalokanathan et al., 2005; El-Ashmawy et al., 2014).

Moreover, the metabolic profile of liver from NDEA group in the present study indicated significant increase in the activity of glucose-6-phosphate dehydrogenase (G6PD). The current findings are supported by other previous findings in various research laboratories. Elevated levels of G6PD have been found in multiple tumors including leukemia, gastrointestinal, renal, colon, breast, endometrial, prostate and liver cancer (Hu et al., 2013). According to the literature, cancer cells require large amount of macromolecules and lipids required during proliferation and building new cells, and hence are in continuous need of ATP and cofactors (NADPH, NAD+) production for sustaining synthetic pathways (Icard et al., 2011). G6PD is the rate limiting enzyme of the pentose phosphate pathway (PPP). The roles and regulation of G6PD in physiology and pathophysiology has been well described in literature as it was introduced in 1931 (Kornberg and Horecker, 1956). G6PD holds the central importance as it is a major source of NADPH and is highly regulated by many signals. Glucose in the cell is phosphorylated to G-6-P by hexokinase. There exist three possible pathways where G-6-P may enter i.e. glycolysis (producing energy in form of ATP and
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NADH), glycogen formation to store energy or PPP (producing various essential macromolecules such precursors of nucleic acid and NADPH). PPP consists of two stages oxidative and non-oxidative. G6PD is the first and rate limiting enzyme of initial oxidative stage of PPP where G-6-P is dehydrogenated to 6-phosphogluconolactone producing NADPH. Ectopic expression of G6PD promotes the survival of tumor cells via maintaining both extracellular pH and redox potential (Kobayashi et al., 2005). G6PD is highly regulated at various levels of transcription, translation, post translation and intracellular location. Of the most significant regulation is offered by p53. Mutated p53 (TP53) or loss of p53 leads to enhanced glucose consumption via increasing the activity of G6PD. According to the research p53 binds directly to G6PD and inhibits the formation of a G6PD molecular complex. The mechanism of tumor suppressor p53 has been recently demonstrated that p53 inhibits G6PD by catalytically converting active dimer into an inactive component. Thus, p53 is involved in suppressing glucose consumption and biosynthesis. Low expression of p53 in NDEA group thus can be correlated with the enhanced expression of G6PD in the NDEA group. Interestingly, a positive correlation has been found between G6PD and mRNA and protein expression of apoptosis inhibitory factors Bel-2 and Bel-xl (Jiang et al., 2011).

In the current study liver glycogen content was found to be decreased in HCC bearing NDEA group. Liver in normal conditions maintains the glucose homeostasis by converting dietary glucose from blood into glycogen that is stored in liver after a meal. However, after sometime in the post-absorptive state glucose is produced from glycogen or gluconeogenic precursors (Cherrington, 1999). Although enough data is available to explain high rate of laletic acid synthesis in tumors, however the reasons for the lack of glycogen accumulation has not been fully explored. As discussed above the requirement of glucose increased during hepatocarcinogenesis for energy production and macromolecule synthesis. Transformation of liver cell to tumor cell caused the loss of glucose production via gluconeogenesis. According to the literature, overproduction of a molecule called microRNA-23a is responsible in inhibiting gluconeogenesis (Wang et al., 2012). Glycogen metabolism then acts as an alternate energy source, enabling growth of the cell under metabolic stress. However, the purpose of glycogen degradation in hypoxia and cancer cell survival remained unclear. The possible explanation behind this observation is the excessive consumption of glucose and anoxic conversion to lactate. Such reduction of glycogen and depletion of glucose has also been observed in other tumor also such as human cervical tumor. In contrast there are demonstrations by Favaro et al. that in hypoxic cancer cells, depletion of liver glycogen
phosphorylase causes glycogen accumulation, leading to stress, induction of cell senescence and impaired tumor growth.

In the current study LycT administration to NDEA challenged mice ameliorated the up-regulation of glycolytic enzymes indicating anti-tumoral effects. Inhibition of ATP generating pathway i.e. glycolysis may severely abolish energy fulfilment of cancerous cells and thus attribute in killing the malignant cells (Munoz-Pinedo et al., 2003; Xu et al., 2005). Although lycopene has shown beneficial results in number of studies however there is no literature exploring the role of lycopene in maintaining carbohydrate metabolism. Ameliorating the metabolic alterations introduced by NDEA in glycolytic pathway may be one of the possible explanations behind its chemopreventive potential as elaborated by above observations. Significant reduction in the activity of hexokinase, PGI and aldolase might have abolished the energy production and eventually leading to cell death. Enhanced apoptosis in the LycT + NDEA also support the current observation. In literature there are studies where potential candidates such as allylmercaptocaptopril, kaempferol etc. have shown protective effect in experimental hepatoma via modulating these glycolytic enzymes (Sharma et al., 2011; Langeswaran et al., 2012; Wang et al., 2012 b). Lycopene played a pre-eminent role in protecting the hepatic tissue from HCC development via inhibiting the G6PD activity. In the current study enhanced p53 expression in LycT + NDEA group can serve as a possible explanation for the inhibition of G6PD activity. Although there is limited research relating the role of lycopene in regulating G6PD yet some research demonstrate the inhibitory effect of phytochemical via regulating the activity of G6PD. Edderkaoui et al., (2010) presented that synergistic effect of lycopene and ellagic acid has shown apoptosis and inhibition in proliferation in pancreatic cancer cells. This effect is due to the synergistic action on the inhibition of G6PD activity, a key step for nucleic acid synthesis. Decreased expression of Bel-2 in LycT + NDEA also is in correlation with the decreased activity of G6PD. Moreover, while observing the glycogen level in LycT + NDEA group, significant increased glycogen level was observed when compared with NDEA group. This indicates that lycopene has the potential to replenish the glycogen content either by delaying the toxic effect of NDEA and hence delayed the progression of HCC. The increase in glycogen level in mice with lycopene challenged with CCl4 supports the current observations (Omara et al., 2009).

Thus, from the above discussion it may be inferred that metabolic switch from highly efficient oxidative phosphorylation to energy inefficient increased glycolysis, ignoring
oxygen concentration is the principle biochemical characteristic of malignant cells compared to normal ones. The consequences of aerobic glycolysis in tumor cells resulted in the high levels of lactate in the tumor and this was first described by Warburg (1956b). Earlier lactate was considered as the waste product of glycolysis however, now it has been correlated with increased metastasis, tumor recurrence, and poor outcome. Doherty and Cleveland (2013) reviewed that lactate possess cell intrinsic effects on cancer metabolism and has non-tumor cell autonomous effects that help in driving tumorigenesis. Lactate in tumor cell is metabolized as an energy source and excess lactate is shuttled to neighbouring cancer cell or stromal cell or vascular endothelial cells where it induces metabolic reprogramming. Lactate has also been found to be associated with tumor promotion, inflammation and stimulating angiogenesis. The enzyme responsible for the production of this important molecule i.e. lactate is denoted as lactate dehydrogenase (LDH) which facilitates the bidirectional conversion of pyruvate and lactate. In the current study NDEA treated group demonstrated enhanced LDH level both in liver and serum at both 10th and 24th week of the study. Literature also supported the present observations as reported by Fantin et al., (2006). According to them high LDH-A protein in serum is linked with poor prognosis, greater metastatic potential and tumor maintenance and progression. These characteristics have made is as a potential target for cancer therapy (Miao et al., 2013). In various clinical studies serum LDH is considered as a useful prognostic marker for different malignancies including myeloma, haematological, lung carcinoma, and organ toxicity such as hepatotoxicity (Al-Saadoon et al., 2003; Coskun et al., 2005). Moreover, chemopreventive efficacy of several agents in hepatocarcinogenesis/ hepatotoxicity has been demonstrated via comparing serum LDH level with normal one (Koul et al., 2007; Sadik et al., 2008). LycT + NDEA group has shown a significant reduction in serum LDH level when compared to NDEA group indicating the chemopreventive potential of LycT in the current piece of work. There are many reports in literature where lycopene supplementation in diet has shown the chemopreventive potential in various cancers model (Mein et al., 2008). The reduction in LDH level in serum has been taken as the marker enzyme for assessing the chemopreventive potential. Several in vitro studies also revealed the similar observations. Lian and Wang (2008), demonstrated that lycopene metabolites have resulted in dose dependent inhibition of LDH release in BEAS-2B cells during H2O2-induced oxidative damage. According to Sheriff and Devaki (2013), lycopene help in stabilizing liver function during D-galactosamine/ lipopolysaccharide induced hepatitis in experimental rat model.
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5.11 mRNA and protein expression of hypoxia-inducible factor-1α during NDEA induced hepatocarcinogenesis and its modulation through LycT

Hypoxia (low oxygen concentration i.e. area with oxygen tension ≤2.5 mmHg) is another characteristic outcome of solid tumor development. Hypoxic areas have been found in many malignancies including cancers of the breast, uterine, head & neck, cervix, vulva, rectum, pancreas, prostate, brain, malignant melanomas, metastatic liver tumors and renal cell cancer (Vaupel and Mayer, 2007). Tumor progression including cell proliferation, invasion and metastasis is associated with various alterations in the microenvironment of tumor cells. Proliferating cells continuously require oxygen (and nutrient) supply for their metabolism however, due to inadequate vascular network there exist hypoxic or even anoxic condition within tumor (Mantovani et al., 2008; Witz, 2009). Actually in tumor biology hypoxia represents a “Janus face” i.e. it is associated with restrained proliferation, differentiation, necrosis or apoptosis and it can also lead to the development of an aggressive phenotype (Vaupel and Mayer, 2007). Hypoxia specifically stimulates a transcription factor i.e. hypoxia induced factor-1 (HIF-1). HIF-1 consists of major two subunits: HIF-1α and aryl hydrocarbon receptor nuclear transporter (ARNT). ARNT is constitutively expressed whereas HIF-1α is expressed only during hypoxic conditions. In the current study, liver from the mice of NDEA treated group showed enhanced mRNA and protein expression of HIF-1α indicating the existence of hypoxic conditions in the liver tissue. The existence of hypoxia in tissue further revealed the tumor progression as hypoxia is the characteristic outcome of carcinogenesis.

Although HCC is a hypervascular malignancy, still there exists reduced oxygen supply (Brahimi-Horn et al., 2007). Various research groups have observed the expression of HIF-1 in modulating apoptosis of HCC (Piret et al., 2005). Literature demonstrated that hypoxia enhances the expression of VEGF and decreases the ratio of Bax/Bcl-2, thus blocking apoptosis (Baek et al., 2000). Pre-treatment with LycT to NDEA challenged mice showed a significant reduction in the expression of HIF-1α at 24th week when compared to NDEA group. This observation suggests that chemopreventive effect of lycopene might be due to the inhibition of the expression of HIF-1α and hence promoting apoptosis due to hypoxic environment. There are many reports demonstrating the similar observation where lycopene has shown inhibitory response to HIF-1α both in in vivo and in vitro studies. Upadhyay et al., (2009) have done the comparative study of different antioxidants in order to assess their cancer preventive activity through inhibition of HIF-1α activity. According to their report
HIF-1α operates in the presence of free radicals and antioxidants with maximum scavenging efficiency for ROS may possess higher inhibition of HIF-1. Further there are experimental evidences that demonstrate the effect of ROS on the induction of hypoxia (Burlaka et al., 2006). Consumption of tomato and lycopene mostly inhibited the expression of HIF-1α during prostate carcinogenesis as observed by Thomas-Ahner et al., (2013).

The observations of the present study indicated the significant chemopreventive effects exerted by LycT against NDEA induced hepatocarcinogenesis. The delayed HCC development upon LycT treatment to NDEA challenged mice was observed starting from initial to later stages. During initiation of HCC, LycT interfered with the accumulation of free radicals generated during NDEA metabolism either through direct mechanism by scavenging free radicals or indirectly by boosting the antioxidant defense system. Thus, LycT aids in preventing the genotoxic and tissue damaging effects of NDEA. By maintaining cellular integrity and survival, LycT had exhibited a promising protective potential against HCC. Chromosomal aberrations, mutations and degradation were observed to be decreased after co-treatment of LycT. Enhanced apoptosis and regulated proliferation added to the beauty of LycT action against HCC. The modulatory effect of LycT in carbohydrate metabolism and energy production system during hepatocarcinogenesis served as one of the effective mechanisms underlying the chemopreventive potential of LycT. Thus, evidences from literature and the observations from the current study, suggest lycopene as a multi-targeted agent against chemically induced HCC.