Chapter 5
Anticarcinogenic and antitumor activity of lutein and its mechanism of action
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5.4. Discussion
5.1. Introduction

Cancer is the second largest cause of death which killed 7.6 million people worldwide in 2005 (Danaei et al., 2005). The number is believed to become 9 million in 2015 and 11.5 million in 2030 (World Health Organization, 2007). Presently various treatment modalities available, cancer still continue to be second leading cause of death among human beings (Jemal et al, 2007). Radiation therapy, chemotherapy and surgery fail many times even in combination therapies. Genotoxic damage is the crucial initiating event in carcinogenesis (De Flora et al., 1996). Previous studies have shown that there is an accumulation of genetic changes that occur during the process of tumorigenesis induced by genotoxic carcinogens. These changes, which include DNA mutations, deletions, and chromosome aberrations, cause an imbalance between cell proliferation and cell death, leading to a loss of cellular homeostasis that can lead to neoplastic transformation (Park et al., 1998). Many genotoxic carcinogens require metabolic activation to an electrophilic form that can react with DNA. This process commonly occurs in the liver. Poly aromatic hydrocarbons, which become metabolically active by the action of cytochrome P450 enzymes, produce reactive electrophiles leading to cellular toxicity, mutagenecity and carcinogenicity (Archer, 1989, Vitaglione et al., 2004).

Chemoprevention of cancer may be defined as the prevention of various forms of cancers by the use of chemical-pharmaceutical agents that inhibit, delay or reverse the process of carcinogenesis (Kruger et al., 2002). Half of the drugs which have been approved recently are from natural sources (Kim & Park, 2002; Newman & Cragg, 2007). Plants have been extensively used as natural sources to develop anticancer drugs because of their active constituents (Schwartsmann et al., 2002). A number of epidemiological studies have consistently shown an inverse relationship between human cancer incidence and intake of foods rich in carotenoids. Carotenoids are part of a vast spectrum of natural pigments and have long been hypothesized to reduce the risk of many chronic diseases, including certain forms of cancer, heart disease and certain eye diseases (Johnson, 2002). Carotenoid-rich diets from fruits and vegetables have been associated with a decreased risk of lung, stomach and prostate cancers (Flag et al., 1995). Carotenoids may reduce lung carcinogenesis because of their antioxidant properties (Michaud et al., 2000). Lutein and zeaxathin are important carotenoid components in the human diet and several investigators have suggested that elevated intake of food rich in lutein is related to decreased risk of many degenerative diseases.
Lutein possesses pronounced free radical scavenging activity both *in vitro* and *in vivo* due to its polarity and conjugated double bonds. In fact lutein, zeaxanthine and meso-Zeaxanthine are the only carotenoids present in the macula where it protects the retina from the oxidative damage induced by singlet oxygen produced by ultra violet light. Recently administration of lutein has also been shown to have significant protective effect against atherosclerosis, affording cardiac protection.

There is an urgent need for developing an effective chemo preventive dietary compound to reduce the incidence of cancer. Lutein is non toxic and is considered as GRAS (generally regarded as safe) by Food and Drug Administration as a nutritional supplement. In the present study we have looked in to the antimutagenic activity of carotenoid lutein in animal models. This study was aimed at the evaluation of the cytotoxicity and antitumor activity of lutein and its mechanism of action. We have also looked into the possible mechanism of action of lutein as a chemo preventive agent.

5.2. Materials and methods

5.2.1. Lutein preparation

Lutein was dissolved in sunflower oil (5%) for oral administration of animals as well as external application in DMBA induced skin papilloma studies. Lutein was dissolved in ethanol for the *in vitro* cytotoxic assay. Free lutein was dissolved in hexane (10mg/10 mL) and 10µL of triton X 100 was added and further evaporated to dryness, vigorously shaken and finally made up the volume to 10 mL with distilled water and was used to detect the inhibition of *in vitro* cytochrome P450 enzymes.

5.2.2. Effect of lutein on nitrosodiethyl amine (NDEA) induced hepatocellular carcinoma in rats

Thirty two male Wistar rats were divided into four groups and each group contained eight animals.

- **Group I**: Without any treatment (Normal)
- **Group II**: NDEA + sunflower oil (Vehicle control) (0.5 mL)
- **Group III**: NDEA + lutein (50 mg/kg b.wt.)
- **Group IV**: NDEA + lutein (250 mg/kg b.wt.)

Group I was kept as normal. Groups II-IV were treated with 0.02% NDEA in distilled water (2.5 mL/day) for 20 weeks. Lutein dissolved in sunflower oil (0.5 mL) was given through oral gavage to group III and IV daily during the entire period. All the animals
were observed for nine more weeks without any treatment (Rajeshkumar & Kuttan, 2000). At the end of 29th week, the animals were sacrificed, their blood was collected by cardiac puncture, serum was separated and used to determine the activities of alanine transaminase (ALT), aspartate transaminase (AST) (Reitman & Frankel, 1957) alkaline phosphatase (ALP) (Kind & King, 1954) gamma glutamyl transferase (GGT) (Schumann et al., 2002) and bilirubin (Malloy & Evelyn, 1937) using commercially available kits. Liver was excised, tumor incidence was noted in the liver and morphological changes and liver weight were recorded. The excised liver was washed in ice-cold Tris-HCl buffer (0.1 M, pH 7.4), and homogenized (25%) and cytosolic samples were prepared by centrifuging at 10,000 rpm for 30 min at 4°C. Liver homogenate was used for the estimation of GSH (Moron & Ames, 1983) and hepatic marker liver enzymes, using commercially available kits. Estimation of tissue protein was done by the method of Lowry et al. (1951).

5.2.3. Determination of the effect of lutein on 3-Methyl cholangthrene (3-MC) induced sarcoma in mice

Male Balb/c mice were used for the study. Animals were divided into four groups, each group contained 20 animals.  
Group I- 3-MC alone (Control)  
Group II-3-MC+ sunflower oil (Vehicle control)  
Group III- 3-MC+ lutein (50 mg/kg b.wt.)  
Group IV- 3-MC+ lutein (250 mg/kg b.wt.)  
3-MC was dissolved in DMSO. To the shaven ventral surface of each mouse 3-MC was administered subcutaneously at a dose of 200μg/animal. Oral treatment with lutein was started 24 hours after the injection of 3-MC and was continued for 6 days in a week for 20 consecutive weeks. The animals were then observed for the onset of sarcoma and the survival time for thirty weeks (Joy et al., 1999).

5.2.4. Determination of the effect of lutein on 7,12 dimethyl benz[a] anthracene (DMBA) and croton oil induced papilloma in mice

Male Balb/c mice were used for the study. They were kept as 4 animals per cage. Aggressive males were removed and kept separately. The dorsal side (2 cm diameter) of the mice was shaved with a razor at least two days before the application of chemicals. The animals in the resting phase of hair growth cycle were selected for
the experiment. They were divided into seven groups, each group contained six animals.

Group I- DMBA+ croton oil (Control)
Group II-DMBA+ croton oil+ sunflower oil (Vehicle control)
Group III- DMBA+croton oil+0.1% lutein
Group IV- DMBA+croton oil+0.2% lutein
Group V- DMBA+croton oil+ 1% lutein
Group VI- DMBA alone
Group VII-croton oil alone.

A single dose of DMBA (470 n mol/mouse in 200 µl acetone) was used for the study. All the animals except group VI were applied with 1% croton oil in acetone (200 µl/animal). Lutein dissolved in sunflower oil was applied topically two weeks after DMBA topical application. Lutein was applied twice weekly for six weeks, 30 minutes before each croton oil application. The animals in all groups were watched for food intake as well as any apparent toxicity such as weight loss or mortality during the entire period of the study. Skin tumor formation was recorded weekly and the tumor growth greater than 1 mm in diameter was included in the cumulative total if they persisted for two weeks or more. Delays in the onset of tumors and number of papilloma per animal in various groups were recorded up to twenty weeks (Reddy & Fa1kow, 1983).

5.2.5. Determination of the \textit{in vitro} cytotoxic activity of lutein to DLA cells

Dalton’s lymphoma ascites (DLA) cells were aspirated from the peritoneal cavity and washed 3 times with PBS. 1 million cells were incubated for 3 hours at 37°C with various concentrations of lutein dissolved in ethanol in a final volume of 1ml. After incubation, the viability of the cells was determined by the trypan blue exclusion method (Gupta & Bhattacharya, 1978) (Detailed procedure given in chapter 2, section 2.2.2.1.).

5.2.6. Determination of the cytotoxicity of lutein to L929 cells in culture.

Cytotoxicity of lutein in culture was determined using L929 cells. The cells (5000 cells/ well) were plated in 96 well flat bottom titer plates. After 24hrs different concentrations of lutein were added and further incubated for 48 hrs. 20µL MTT (5mg/ml) was added 4hr before the completion of incubation (Cole, 1986; Campling et al, 1991). The plates were centrifuged and the supernatant removed and then 100 µL
DMSO was added and the intensity of the blue color was read at 570nm using ELISA plate reader (Detailed procedure given in chapter 2, section 2.2.2.2).

5.2.7. Determination of lutein on ascites tumor bearing animals.
Dalton’s lymphoma ascites (DLA) cell line was used for the experiment. Ascites tumor was induced to 6 groups of BALB/c mice (6 animals/group) by injecting 1 million cells/animal in the peritoneal cavity. Drug administration was started after 24hrs of tumor inoculation and continued for 10 consecutive days. The animals were grouped as follows:
Group I: DLA cells alone (control)
Group II: DLA cells + sunflower oil (vehicle control)
Group III: DLA cells + 50mg/kg b.wt lutein in sunflower oil (oral)
Group IV: DLA cells + 100 mg/kg b.wt lutein
Group V: DLA cells + 250 mg/kg b.wt lutein
Group VI: DLA cells + Cyclophosphamide (15 mg/kg, b.wt, ip)
The death pattern of the animals due to tumor burden was noted everyday and the percentage of increase in lifespan was calculated using the formula T-C/C X 100 were ‘T’ and ‘C’ are the number of days that treated and control animals survived respectively (Kuttan et al, 1985).

5.2.8. Determination of the effect of lutein on the solid tumor development
Dalton’s lymphoma ascites (DLA) cells (1x10^6 cells/animal) was injected subcutaneously on the right hind limb of 6 groups of BALB/c mice (6 animals/group).
Group I: DLA cells alone (control)
Group II: DLA cells + sunflower oil (vehicle control)
Group III: DLA cells + 50mg/kg b.wt lutein in sunflower oil (oral)
Group IV: DLA cells + 100mg/kg b.wt lutein
Group V: DLA cells + 250 mg/kg b.wt lutein
Group VI: DLA cells + Cyclophosphamide (15 mg/kg, b.wt, ip)
Drug administration was started 24hrs after tumor inoculation and continued for 10 consecutive days. Initial diameter of the hind limb was noted using vernier calipers. Tumor diameter was measured on every 3rd day from 7 days after tumor inoculation for 31 days. The tumor volume was calculated using the formula \( V = \frac{4}{3} \pi r_1^2 r_2 \).
5.2.9. Determination of the effect of lutein on inhibition of *in vitro* drug metabolizing enzymes (cytochrome P450)

5.2.9.1. Inhibition of cytochrome P450 enzymes by lutein

Male Wistar rats were administered phenobarbitone continuously for 4 Days (60 mg/kg b.wt, i.p., once daily) and sacrificed 24 h after the last dose of phenobarbitone. The livers of all the animals were excised quickly and washed thoroughly in ice cold saline and kept at -70°C. 20% of homogenate was prepared in cold phosphate buffer (pH 7.4, 0.1M). Homogenate was initially centrifuged at 10000 g for 30 minutes in a cooling centrifuge (Remi) and supernatant was separated. The supernatant was then further centrifuged at 105000 g for 1 h in an ultracentrifuge (Sorvall ultracentrifuge) and microsomes obtained were washed and resuspended in cold phosphate buffer.

5.2.9.2. Effect of lutein on the inhibition of *in vitro* cytochrome P450 enzymes

The effect of lutein on the dealkylation of methoxyresorufin by 7-methoxy resorufin-O-methylase (MROD) an indicator of CYP1A2, pentoxyresorufin by 7-pentoxy resorufin-O-depentylase (PRD) an indicator of CYP2B1/2 and ethoxyresorufin by 7-ethoxy resorufin-O-deethylase (EROD) an indicator of CYP1A1 were studied (Pohl & Fouts, 1980, Nerurkar *et al.*, 1993). The assay mixture contained sodium phosphate buffer (0.1M, pH 7.4), 6.25 mM MgSO₄, 60μM EDTA, 5μM ER, MR or PR, 100μg microsomal protein (from phenobarbitone treated animals) 100μM NADPH and various concentrations of lutein in a final volume of 1 ml. The reaction time was 5 minutes with a pre incubation period of 5 minutes (without NADPH) at 37º C. The reaction was stopped by the addition of 2 ml of chilled methanol. The precipitated protein was centrifuged down and supernatant was used for the estimation of enzyme activity using a fluorescent spectrophotometer (Nanodrop – ND 3300) at the excitation wave length of 550 nm and the emission wave length of 585 nm. Blanks were set without any NADPH. The percentage inhibition was calculated following the formula C-T/ C × 100, where C is the optical density of control without lutein; T is the optical density with lutein.
Inhibition of aniline hydroxylase activity (an indicator of CYP 2E1 activity) and aminopyrene-N-demethylase activity (an indicator of CYP 1A, 2A, 2B, 2D and 3A activity) were measured by the method of Mazel.

5.2.9.3. Determination of the activity of aminopyrene-N-demethylase

**Principle**

Aminopyrene was dealkylated by microsomal enzymes to form 4-amoantipyre and formaldehyde. Formaldehyde so formed was measured by condensation with Nash reagent.

**Protocol**

Total volume of the reaction mixture was 1.5 ml containing microsomal fraction (1-1.5 mg protein), phosphate buffer (150 mM, pH 7.4), MgCl₂ (5 mM), aminopyrene (32 nM), semicarbazole hydrochloride (120 mM) and various concentrations of lutein were incubated at 37°C for 5 min. NADPH (0.33 mM in buffer) was added to the mixture and incubation continued for 2 h at 37°C. After incubation, reaction was stopped by the addition of a mixture at 10% ZnSO₄ (500 μl) and saturated Ba(OH)₂ solution (500 μl), the mixture was centrifuged at 3000 rpm for 10 min, the supernatant was mixed with 500 μl Nash reagent (Nash reagent is a mixture of ammonium acetate (30 g) and acetyl acetone (400 μl) in a total volume of 100 ml distilled water). The tubes were then placed in a water bath at 60 °C for 30 min for colour development and absorbance was measured at 412 nm against distilled water. The percentage of inhibition was calculated.

5.2.9.4. Determination of aniline hydroxylase activity in the microsomal fraction

**Principle**

Aniline hydroxylase catalyses the hydroxylation of aniline to p-aminophenol in the presence of NADPH. The activity of aniline hydroxylase was determined by measuring the quantity of p-aminophenol formed. P-aminophenol formed was allowed to react with phenol in an alkaline medium to form the blue colored which can be measured at 630 nm.

**Protocol**

The total volume of the reaction mixture is 1.5ml containing the microsomal fraction (1 – 1.5 mg protein) phosphate buffer (150 mM, pH 7.4), MgCl₂ (5mM) aniline (32 mM in ethanol) and various concentration of extract was incubated at 37°C for 5 minutes. NADPH (0.33 mM in buffer) was added to the mixture and incubation contained for 2 hours at 37°C. After incubation 500μl of 20% TCA was added to the
mixture and was centrifuged at 3000rpm for 10 minutes. 1.5ml of the supernatant was mixed with 750µl of 10% Na₂CO₃, 1.5ml of phenol (2% in 0.2M NaOH) and mixture is incubated at 37°C for 30 minutes. The absorbance was measured at 630nm against the buffer. The percentage of inhibition was calculated.

5.2.9.5. Effect of lutein on the inhibition of in vivo cytochrome P450 enzymes

Thirty male Wistar rats were randomly divided into the following five groups having six animals per group.

Group I- Without any treatment (Normal)
Group II- Phenobarbitone alone (Control)
Group III- Phenobarbitone+ sunflower oil (Vehicle control)
Group IV- Phenobarbitone+ lutein (50 mg/kg b.wt)
Group V- Phenobarbitone+ lutein (250 mg/kg b.wt.)

Lutein and sunflower were administered once daily for 15 days. Administration of phenobarbitone was started on 12th day and continued for 4 days (60 mg/kg. b. wt., intra peritoneally, once daily). The liver microsomal fraction from lutein and sunflower oil treated animals and from untreated animals was prepared as given above were used to determine the activities of EROD, MROD and PROD. Calibration curves were constructed by determining the fluorescence of known amounts of authentic resorufin (Harikumar & Kuttan, 2006). Concentration of protein in each sample was measured by the method of Lowry et al. Results were expressed as n mols of resorufin formed per minute per mg protein for in vivo studies.

5.2.10. Induction of Phase 2 enzymes in vivo by lutein

Eighteen male Wistar rats were randomly divided into four groups having six animals per group as follows,

Group I- Normal (without any treatment)
Group II- Sunflower oil alone
Group III- Lutein (50 mg/kg b.wt)
Group IV- Lutein (250 mg/kg b.wt.)

Lutein and sunflower were administered daily once for 15 days and on 15th day all the animals were sacrificed and the liver tissue homogenate was prepared as given above. The method of Habig et al was followed to assay the activity of glutathione-S-transferase (GST) which is based on the rate of increase in conjugate formation.
between GSH and 1-chloro-2, 4-dinitrobenzene (CDNB) (Habig et al., 1974). UDP glucuronyltransferase was estimated by the method of Issalbacher et al. (1962) modified by Hollman & Touster (1962).

5.2.10.1. Determination of UDP glucuronyl transferase activity

The UDP glucuronyl transferase was estimated by the methods of Issalbacher et al., (1962) modified by Hollman and Touster (1962).

Reagents

1. Tris HCl buffer (1M, pH 7.4) was dissolved in 100ml of water and pH was adjusted to 7.4 with HCl, 2. Triton X-100 (0.25%), 3. MgCl\(_2\) (50mM) of 47.5mg was dissolved in 10 ml of water. 4. P- nitro phenol (5mM) of 0.84 mg was dissolved in 1ml of water 5. UDP glucuronic acid (30mM) of 19.38 mg was dissolved in 1ml of water, 6. TCA (5%), 7. NaOH (2M)

Protocol

The incubation mixture, containing 0.5ml buffer (0.2ml triton X-100, 0.05ml MgCl\(_2\), 0.05ml. p- nitro phenol, 0.18 ml water and 0.1ml enzyme was incubated at 37°C for 2 minutes. Then 0.1ml of UDP glucuronic acid was added. Then 0.1ml of aliquot of this mixture was arrested at 0, 10 and 15 minutes with TCA and centrifuged. To 1ml of the supernatant 0.25ml of NaOH was added and read at 450nm using a photochemical colorimeter.

The activity of UDP glucuronyl transferase was expressed as nmoles/min/mg protein

5.3. Results

5.3.1. Effect of lutein on the induction of hepatocellular carcinoma by NDEA

The morphological analysis of the livers of control animals showed a number of tumor nodules on the surface with variable shapes. The normal morphology was completely lost in all the animals. Necrotic mass was seen at some places, and some tumor nodules showed irregular protuberances from their surfaces (Fig. 5.1.). The liver of animals treated with 50 mg/kg b. wt. lutein showed fewer incidences of tumor nodules. It retained the normal morphology of the liver with small necrotic masses seen in the large lobe of some animals. The liver tissue of animals treated with 250 mg/kg b.wt. lutein showed normal morphology and did not show any tumor incidence.

Administration of NDEA significantly elevated the liver weight of the vehicle control animals as compared with normal untreated animals. Increased liver weight was
Figure 5.1: Morphology of liver during NDEA induced HCC

Normal

Vehicle control (sunflower oil + NDEA)

Lutein treated (250 mg/ kg. b. wt)
found to be reduced by the administration of lutein (250 mg/kg. body weight) (Table 5.1.).

The activity of the enzyme γ-glutamyl transpeptidase, a marker of cellular proliferation, was found to be elevated in the serum and the liver of the vehicle control group when compared to normal group. GGT levels in serum were significantly reduced in lutein treated groups. Similarly the increased activity of GGT in the liver tissue in NDEA treated group was significantly lowered by lutein treatment. Elevated levels of three major marker enzymes of hepatic function like ALP, GPT and GOT which were observed in the serum of control group were significantly decreased by the treatment of lutein (Table 5.2.). The activities of ALP, ALT and AST in liver tissue were also found to be significantly elevated in control group and these activities were significantly decreased by lutein treatment (Table 5.3). There was no significant change observed in control group compared with normal. The level of GSH in liver tissue found to be 4.61 ± 1.92 nmol/mg protein in normal animals was reduced to 1.37 ± 0.17 in NDEA and sunflower oil treated animals. The level of GSH in 50 mg/kg b. wt. lutein treated group was found to be increased to 2.82 ± 1.47 and in 250 mg/kg b. wt group it was increased to 3.99 ± 0.91 nmol/mg proteins.

Histopathological analysis confirmed the protection of liver by lutein from the actions of NDEA induced hepatocellular carcinoma. The liver tissue of control and vehicle control rats showed carcinomatous growth composed of groups of large pleomorphic polyhedral or oval cells having hyperchromatic nuclei. There were many degenerating cells and mitotic cells. Stroma showed areas of necrosis and inflammatory reaction. Many congested blood vessels and areas of haemorrhage were also seen. Liver tissue of lutein treated (250 mg/kg b. wt) rats had normal structure; their portal areas and central venous system appeared normal. Hepatocytes showed normal morphology. Kupffer cells and sinusoidal spaces appeared normal and no specific lesion was identified (Fig. 5.2). These results clearly indicated that lutein could protect rats from NDEA induced hepatocellular carcinoma.

5.3.2. **Effect of lutein on 3-MC induced sarcoma in mice**

Administration of lutein significantly delayed the sarcoma development induced by 3-MC. The onset of sarcoma on 7th and 8th week in control and vehicle control groups was delayed to 13 weeks and 16 weeks in 50 and 250 mg/kg. b. wt lutein treated groups respectively. All the animals in the control and vehicle control groups
Table 5.1: Effect of lutein on tumour incidence and liver weight in NDEA induced hepatocellular carcinoma in rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tumour incidence</th>
<th>Liver weight (g) /100 g.b.wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0/6</td>
<td>3.44 ± 0.48</td>
</tr>
<tr>
<td>Vehicle control (Sunflower oil + NDEA)</td>
<td>8/8</td>
<td>4.40 ± 0.42***</td>
</tr>
<tr>
<td>50 mg/kg.b.wt. Lutein + NDEA</td>
<td>4/8</td>
<td>4.34 ± 0.25ns</td>
</tr>
<tr>
<td>250 mg/kg b.wt. Lutein + NDEA</td>
<td>0/8</td>
<td>3.88 ± 0.54ns</td>
</tr>
</tbody>
</table>

Values are Mean ± S.D, n= 8 for treated animals, n= 6 for normal animals. * P < 0.05, ** P <0.01, *** P< 0.001, significance was calculated against vehicle control group, lutein and NDEA treatment continued for 20 weeks, ns-not significant.
Table 5.2: Effect of lutein on bilirubin and serum hepatic enzymes in NDEA induced hepatocellular carcinoma.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total Bilirubin mg/dl</th>
<th>ALP IU/L</th>
<th>ALT IU/L</th>
<th>AST IU/L</th>
<th>GGT IU/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.40 ± 0.03</td>
<td>48.43± 9.59</td>
<td>64.33± 4.88</td>
<td>99.60±5.42</td>
<td>26.80±1.66</td>
</tr>
<tr>
<td>Vehicle Control</td>
<td>0.52±0.04***</td>
<td>281.71±26.06 ***</td>
<td>181.12±13.48 ***</td>
<td>200.14±17 ***</td>
<td>107±10.74 ***</td>
</tr>
<tr>
<td>50 mg/kg b.wt. lutein+ NDEA</td>
<td>0.49±0.03</td>
<td>169.13 ±21.7 ***</td>
<td>93.85 ±11.95 ***</td>
<td>144.25 ±8.96 ***</td>
<td>38.46±6.08 ***</td>
</tr>
<tr>
<td>250 mg/kg. b.wt lutein + NDEA</td>
<td>0.41 ±0.07***</td>
<td>111.5±8.28 ***</td>
<td>78.28 ±8.17 ***</td>
<td>114.41 ±9.74 ***</td>
<td>28.21±2.45 ***</td>
</tr>
</tbody>
</table>

Values are Mean ± S.D, n= 8 for treated animals, n= 6 for normal animals. * P < 0.05, ** P < 0.01, *** P< 0.001, Significance was calculated against vehicle control group, lutein and NDEA treatment continued for 20 weeks.
Table 5.3: Effect of lutein on hepatic enzymes in NDEA induced hepatocellular carcinoma

<table>
<thead>
<tr>
<th>Group</th>
<th>ALP KA/mg protein</th>
<th>AST IU/mg protein</th>
<th>ALT IU/mg protein</th>
<th>GGT IU/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5.90 ±2.49</td>
<td>210 ± 5.16</td>
<td>274.25 ±19.69</td>
<td>22.01 ±4.08</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>23.93±5.70***</td>
<td>339.75 ± 27.22***</td>
<td>581.75 ±90.80***</td>
<td>66.32 ±4.82***</td>
</tr>
<tr>
<td>50 mg/kg b.wt. (lutein NDEA)</td>
<td>11.31 ± 5.18***</td>
<td>225.66 ±5.77***</td>
<td>289.75 ± 23.80***</td>
<td>36.99 ±6.83***</td>
</tr>
<tr>
<td>250 mg/kg.b.wt. (lutein + NDEA)</td>
<td>5.17 ± 2.92***</td>
<td>195.25 ±20.1***</td>
<td>245.8 ± 20.38 ***</td>
<td>28.45 ±2.42***</td>
</tr>
</tbody>
</table>

Values are Mean ± S.D, n= 8 for treated animals, n= 6 for normal animals.  * P < 0.05, ** P < 0.01, *** P< 0.001, Significance was calculated against vehicle control group, lutein and NDEA treatment were continued for 20 weeks.
Figure 5.2: Histopathology of rat liver during NDEA induced hepatocellular carcinoma

Normal

Vehicle control (sunflower oil + NDEA)

Lutein treated (250 mg/ kg. b. wt)
developed sarcoma by the end of 9th week. In the case of lutein treated group (50 mg/kg b. wt.), only 4 out of 15 and only 2 animals in the case of 250 mg/kg b. wt. lutein treated group developed sarcoma by the end of 9th week. Lutein elevated the survival rate of the animals harbouring sarcoma. All the animals in control and vehicle control groups died by the end of 19th week. In 50 mg/kg. b.wt lutein treated group only 6 out of 15 animals and in 250mg/kg. b. wt lutein treated group, 11 out of 15 animals were found to be alive by the end of 19th week (Fig. 5.3.). These results indicated that lutein could significantly inhibit the 3-MC induced sarcoma in mice.

5.3.3. Effect of lutein on DMBA and croton oil induced skin papilloma

The onset of papilloma in control group was on 5th week and in vehicle control group it was found to be on 6th week. The onset of papilloma was found to be significantly delayed by lutein treatment and in 0.1% lutein treated group it was on 8th week and in 0.2% lutein group it was found to be on 9th week respectively (Table 5.4.). In 1% lutein treated group there was no papilloma formation. Normal animals, DMBA alone and croton oil alone treated groups did not develop any papilloma growth on their skin. At the end of 20th week the average number of papilloma per mouse was found to be 5.63 ± 1.06 and 5.75 ± 1.04 in control and vehicle control groups and this number was significantly reduced to 0.5 ± 1.06 and 0.25 ± 0.70 by 0.1% and 0.2% lutein treatment (Fig. 5.4). These observations support the chemo preventive role of lutein against skin papilloma.

5.3.4. Cytotoxicity of lutein on DLA cells and L929 cells

Lutein was tested in vitro for their toxic effect on Daltons lymphoma ascites tumour cells. The 100% cytotoxicity was attained at 14µg/ml (Table 5.5). The cytotoxic effect appeared to be concentration-dependent, increasing as the concentration of lutein increased. In MTT assay using L929 cells with 48 hour incubation there was 100% cytotoxicity at 50 µg/ml concentration (Table 5.6). Higher concentration of lutein was needed for 100% cytotoxicity in MTT assay may be due to the decreased solubility of lutein in culture media while incubated with cancer cells.

5.3.5. Effect of lutein on life span of ascites tumor bearing animals

Life span of ascites tumor bearing animals induced by DLA cells was found to be increased by lutein treatment. In all lutein treated groups, the lifespan was increased in a concentration dependent manner. In 250mg/kg b.wt group, there was a significant
Figure 5.3: Effect of lutein on the survival rate of 3-MC induced sarcoma in mice.

Figure 5.4: Effect of lutein on number of papilloma in DMBA induced papilloma bearing mice
Table 5.4: Effect of lutein on papilloma bearing mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Average number of papilloma per group (20th week)</th>
<th>Onset of papilloma per mouse (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Croton oil alone</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>DMBA alone</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>DMBA+Croton oil</td>
<td>5.63 ± 1.06**</td>
<td>5</td>
</tr>
<tr>
<td>DMBA+Croton oil + Sunflower oil</td>
<td>5.75 ± 1.04**</td>
<td>6</td>
</tr>
<tr>
<td>DMBA+Croton oil + 0.1% lutein</td>
<td>0.5 ± 1.06***</td>
<td>8</td>
</tr>
<tr>
<td>DMBA+Croton oil + 0.2% lutein</td>
<td>0.25 ± 0.70***</td>
<td>9</td>
</tr>
<tr>
<td>DMBA+Croton oil + 1% lutein</td>
<td>Nil</td>
<td>Nil</td>
</tr>
</tbody>
</table>

Values are mean ± SD from 8 animals in each group.* p< 0.05, ** p < 0.01, *** p< 0.001, Significance was calculated for treated groups against vehicle control group.
**Table 5.5: In vitro cytotoxicity of lutein to DLA cells**

<table>
<thead>
<tr>
<th>Concentration of lutein (μg/ml)</th>
<th>% of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.8</td>
</tr>
<tr>
<td>1</td>
<td>2.3</td>
</tr>
<tr>
<td>4</td>
<td>9.09</td>
</tr>
<tr>
<td>6</td>
<td>28.57</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
</tr>
<tr>
<td>10</td>
<td>46.40</td>
</tr>
<tr>
<td>12</td>
<td>89.06</td>
</tr>
<tr>
<td>14</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table 5.6: Cytotoxicity of lutein to L929 cells in vitro (MTT assay)**

<table>
<thead>
<tr>
<th>Concentration of lutein (μg/ml)</th>
<th>% of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>54</td>
</tr>
<tr>
<td>25</td>
<td>76</td>
</tr>
<tr>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>
increase in lifespan of 47.14% whereas the cyclophosphamide treated group was 51.93% (Table 5.7, Figure 5.5).

5.3.6. Effect of lutein on solid tumor induced by DLA cells

Lutein treatment on DLA induced mice showed significant reduction in tumor volume when compared with control and vehicle control animals. Significant reduction in tumor was also found in DLA induced solid tumor bearing animals treated with different concentrations of lutein. On 31th day the mean tumor volume of the control animals and vehicle control animals were 4.23±1.70 and 4.18±1.62 cm$^3$. But in 50mg/kg b.wt, 100mg/kg b.wt, and 250mg/kg b.wt, the mean tumor volume were found to be 1.77±0.63 cm$^3$, 1.54±0.09 cm$^3$ and 0.92±0.08 cm$^3$ (p<0.001) respectively (Figure 5.6). The mean tumor volume of cyclophosphamide induced tumor volume was found to be 0.85±0.06 cm$^3$. This indicates that there was a dose dependent reduction in the tumour volume in mice with lutein treatment (Table 5.8).

5.3.7. Effect of lutein on the inhibition of in vitro drug metabolizing enzymes (cytochrome P450)

Using microsomal preparation we have tested the effect of lutein on the inhibition of P450 enzymes in vitro. Since the P450 enzymes are a super family of more than 150 isoforms with diverse substrate specificity, it is not possible to analyse the effect on all the enzymes. We had checked their effect on the isoforms of the genes CYP1A1, CYP2A, CYP2B1/2, CYP2E1, CYP 1A, 2A, 2B, 2D and 3A. It is known that CYP1A1 family metabolize the poly aromatic hydrocarbons and CYP2E1 metabolizes the ethanol while CYP1A2 family metabolizes aromatic amines. It was found that activity of P450 enzymes were significantly inhibited by lutein in a concentration dependent manner. Concentration needed for 50% inhibition of CYP1A1, CYP1A2 and CYP2B1/2 was almost similar (<9 µg/mL) while that of aniline hydroxylase was 5µg/mL and aminopyrine-N-demethylase was 12 µg/mL. Results were indicated that there was a significant inhibition of P450 enzymes by lutein in vitro (Fig. 5.7).
Table 5.7: Effect of lutein on the survival rate of DLA induced ascites bearing mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of animals developed tumour</th>
<th>Percentage increase of life span</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6/6</td>
<td>---</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>6/6</td>
<td>---</td>
</tr>
<tr>
<td>Cyclophosphamide (15 mg/kg b.wt)</td>
<td>6/6</td>
<td>51.93</td>
</tr>
<tr>
<td>50 mg/kg b.wt lutein</td>
<td>6/6</td>
<td>28.85</td>
</tr>
<tr>
<td>100 mg/kg b. wt lutein</td>
<td>6/6</td>
<td>34.62</td>
</tr>
<tr>
<td>250 mg/kg b. wt lutein</td>
<td>6/6</td>
<td>47.14</td>
</tr>
</tbody>
</table>

Table 5.8: Effect of lutein on inhibition of DLA induced tumor volume in Swiss Albino mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Percentage inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (DLA alone)</td>
<td>---</td>
</tr>
<tr>
<td>Vehicle control (sunflower oil)</td>
<td>---</td>
</tr>
<tr>
<td>Lutein (50 mg/kg.b.wt)</td>
<td>60.46</td>
</tr>
<tr>
<td>Lutein (100 mg/kg.b.wt)</td>
<td>66.06</td>
</tr>
<tr>
<td>Lutein (250 mg/kg.b.wt)</td>
<td>81.63</td>
</tr>
<tr>
<td>Cyclophosphamide (15 mg/kg b.wt)</td>
<td>84.18</td>
</tr>
</tbody>
</table>
Figure 5.5 Effect of lutein on the survival rate of ascites tumor bearing mice

Figure 5.6 Effect of lutein on DLA induced solid tumor in mice
Figure: 5.7 Effect of lutein on the inhibition of Cytochrome P450 enzymes *in vitro*
5.3.7.1. Effect of lutein on inhibition of \textit{in vivo} drug metabolizing enzymes (cytochrome P450)

Cytochrome P 450 enzymes were significantly elevated after four days of phenobarbitone treatment in rats liver microsomes. The levels of EROD (CYP1A1), MROD (CYP1A2) and PROD (CYP2B1/2) were elevated significantly in control and vehicle control groups. These enzyme levels were reduced significantly by the treatment of lutein (Table 5.9). These results indicated that lutein treatment could effectively reduce the level of cytochrome P450 enzymes in rats compared with untreated animals.

5.3.7.2. Effect of lutein on the induction of phase 2 enzymes

UDP glucuronyl transferase was elevated by 31.74 and 45.78\% in 50 and 250 mg/kg. b. wt lutein treated groups. Glutathione S transferase was significantly elevated by 36.29 and 50.22\% in 50 and 250 mg/kg. b wt lutein treated groups (Table 5.10.). These results indicated that lutein could significantly inhibit phase 1 enzymes and elevate the activity of Phase 2 enzymes in rats.

5.4. Discussion

Reactive oxygen species plays an important role in carcinogenesis and can scavenge oxygen radicals and cause degenerative diseases including cancer (Bagchi & Puri, 1998). Many carcinogens are metabolized by CYP enzymes to either biologically inactive metabolites or to chemically reactive electrophilic metabolites that covalently bind to DNA thereby producing mutation and hence carcinogenicity (Conney, 2003).

Carotenoid lutein has been implicated in the prevention of several human health disorders including molecular degeneration, cancer and heart disease. In the present study we have explored the inhibition of carcinogenesis by lutein using three different animal models. This included NDEA induced hepatocellular carcinoma in rats, 3-MC induced sarcoma and DMBA induced papilloma in mice. N-Nitrosodiethylamine is present in a variety of foods, including cheeses, soybeans, soybean oil, various fish, salt-dried fish, meats and alcoholic beverages. It is also detected in tobacco smoke condensate at concentrations of 1.0 to 28 ng/ cigarette per day (Brunnemann & Hoffmann, 1978, Verna \textit{et al}., 1996). It has been suggested that on metabolic activation, it produces the pro-mutagenic products, O6-ethyl deoxyguanosine and O4 and O6-ethyl deoxy thymidine in liver which are responsible for its carcinogenic effects.
Table 5.9: Effect of lutein on inhibition of Phase 1 enzymes *in vivo*.

<table>
<thead>
<tr>
<th>Groups</th>
<th>7-ethoxy resorufin-O-deethylase (EROD)</th>
<th>7-methoxy resorufin-O-methylase (MROD)</th>
<th>7-pentoxyresorufin-O-depentylase (PROD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.42±0.09</td>
<td>1.22±0.22</td>
<td>0.88±0.19</td>
</tr>
<tr>
<td>Control</td>
<td>3.20±0.42***</td>
<td>3.68±0.29***</td>
<td>4.48±0.582***</td>
</tr>
<tr>
<td>Sunflower oil treated</td>
<td>3.08±0.39***</td>
<td>3.60±0.54***</td>
<td>4.80±0.23***</td>
</tr>
<tr>
<td>Lutein 50 mg/kg wt</td>
<td>2.51±0.19***</td>
<td>1.20±0.15***</td>
<td>1.60±0.34***</td>
</tr>
<tr>
<td>Lutein 250 mg/kg wt</td>
<td>1.08±0.08***</td>
<td>0.84±0.20***</td>
<td>0.86±0.17***</td>
</tr>
</tbody>
</table>

Values expressed in nmol/ml of resorufin formed/minute/mg protein.

Values are Mean ± S.D, statistical significance of the treatment were done by using one way ANOVA followed by by Tukey test (post-hoc), n= 6  *** P< 0.001, significance was calculated against normal to control and vehicle control group, treated with vehicle control group.

Table 5.10: Effect of lutein on activation of Phase 2 enzymes *in vivo*

<table>
<thead>
<tr>
<th>Groups</th>
<th>UDP glucuronyl transferase (n mol/ml/min/mg protein)</th>
<th>Glutathione Transferase (n mol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.48±0.82</td>
<td>41±9.51</td>
</tr>
<tr>
<td>Sunflower oil treated</td>
<td>1.67±0.16</td>
<td>44.62±12.06</td>
</tr>
<tr>
<td>Lutein 50 mg/kg wt</td>
<td>2.20±0.21**</td>
<td>70±8.92**</td>
</tr>
<tr>
<td>Lutein 250 mg/kg wt</td>
<td>3.08±0.19**</td>
<td>89.63±11.44***</td>
</tr>
</tbody>
</table>

Values are Mean ± S.D, statistical significance of the treatment were done by using one way ANOVA followed by by Tukey test (post-hoc), n= 6 for normal animals.  * P < 0.05, ** P < 0.01, *** P< 0.001, significance was calculated against normal with vehicle control group and treated with vehicle control group.
Generation of reactive oxygen species (ROS) by NDEA causes carcinogenic events. Oxidative stress caused by ROS has been reported to produce tumor formation process (Ahmedin Jemal et al., 2007). Liver damage caused by NDEA produces instability of liver cell metabolism which leads to distinctive changes in the serum enzyme activities (Parola & Robino, 2001). Serum transaminases, ALP and GGT are markers of liver function; their increased levels are indicators of liver damage (Plaa & Hewitt, 1989). Present study indicates that lutein could effectively reduce these elevated enzymes and thus prevent hepatocellular carcinoma.

Lutein delayed the onset of 3- MC induced sarcoma effectively in treated groups. It was observed the increased survival rate in mice. In the case of DMBA and croton oil induced skin papilloma formation, the tropical application of lutein delayed the incidence and number of papilloma in mice. Poly aromatic hydrocarbons like DMBA need to be metabolically activated to its active carcinogenic and tumor initiating properties in mice. Croton oil or its active constituent, 12-O-tetradecanoylphorbol-13-acetate (TPA), acts as promoter to the initiated skin (Boutwell, 1974). The epidermal target cells of mouse skin contain a variety of enzymes capable of metabolizing polyaromatic hydrocarbons (PAH). The major enzymes catalyzing the reaction are mono oxygenases (cytochrome P-450-dependent), peroxidases (cyclooxygenase), and hydrolases (epoxide hydratase). Cytochrome P-450-dependent enzyme complex responsible for oxidizing PAH, is highly active and inducible by a variety of PAH substrates (Manil et al., 1981). It has been reported that mutations induced by numerous mutagens were reduced by scavengers of reactive oxygen species (Kim et al., 1991, Goud et al., 1993). Dietary supplementation of antioxidants present in fruits and vegetables are thought to decrease free radical attack on DNA and hence protect against mutation that causes cancer (Duthie et al., 1996).

The cytotoxicity analysis on mouse tumour cell lines revealed high killing ability of lutein, with minimum concentration of drug. Present study also revealed the in vivo tumour reducing ability of lutein, as the administration of lutein simultaneous with tumour inoculation, as determined for its tumour preventive ability, showed a remarkable decrease in tumor volume in mice. In the ascites tumour model, lutein showed a significant increase in the survival of mice which is comparable to that of standard drug cyclophosphamide.
Disruption in the balance between the cell-generating process of mitosis and apoptotic cell death can lead to the development of cancer. Blocking cell proliferation and inducing apoptosis are thus considered as important properties of chemopreventive and chemotherapeutic agents (Sun et al., 2004). Hallmarks of apoptosis include cell shrinkage, chromatin condensation, nuclear fragmentation and exposure of phosphatidyl serine on the surface of cells at the early stages (Elmore, 2007).

Present study proves that lutein could effectively inhibit the phase 1 enzymes both \textit{in vitro} and \textit{in vivo} and hence could inhibit carcinogen activation. Phase II drug metabolizing enzymes are responsible for conjugating a xenobiotic to its excretable metabolites (Low & Castagnoli, 1982) were activated by lutein such as UDP glucuronyl transferase as well as GST. Lutein was found to be non toxic to rats up to a concentration of 5 gm/kg. body weight (Harikumar et al., 2008). The results are suggesting that lutein may be developed as an effective chemopreventive agent.