Chapter 7

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**7.4. Discussion**
7.1. Introduction

Cancer is considered as a disease caused by gathered alterations of genes in somatic cells. It may be caused due to the exposure of chemicals and biological carcinogenic agents such as aflatoxin, nitrosamines and polycyclic aromatic hydrocarbons in foods, heterocyclic amines in fried meat and fish, and pyrrolizidine alkaloids from plants (Sugimura, 1982 and Ames, 1983). Liver cancer is the third most common and lethal cancer in the world. According to American Cancer Society, there were more than 7 lakhs fresh cases of primary liver cancer worldwide. Liver cancer consists of different hepato malignancies and the most common type is hepatocellular carcinoma (HCC) (70-85%). Animal models for HCC are the best way to recognize the mechanisms underlying its pathogenesis. Diet is responsible for more than 50% of the hepatic cancers in the world (Hislop, 1990). N-nitrosodiethylamine (NDEA) is a well-known compound that initiates liver cancer. It is associated with carcinogenesis in several animal species including primates. Induced neoplastic alterations are similar to those found in human hepatic cancer. NDEA occurs extensively in cheese, soybean, processed meats, alcoholic beverages, tobacco products, cosmetics and agriculture chemicals. NDEA is metabolized by cytochrome P450 enzymes to from an ultimate reactive form which makes DNA adducts through electrophilic as well as nucleophilic reactions (Miller et al., 1977). Reactive form of NDEA is esterified by phase II enzymes and eliminated from the body. Methylcholanthrene (3-MC) and DMBA are metabolized by the action of isoforms of cytochrome P450 enzymes (Phase I enzymes) and converted into potent carcinogenic compounds. Methylcholanthrene induced sarcoma is a type of cancer that generally arises in the connective tissues of the body.

*Curcuma longa* L, is a well-known spice with several medicinal properties, cultivated in most of the Asian countries. Its rhizome is widely used as a coloring agent as well as used for food preservation. Turmeric essential oil (TEO) extracted from the rhizome by steam distillation has many pharmacological properties. Turmeric essential oil contains ar-turmerone as its main constituent which has the ability to prevent cancer by the induction of apoptosis in cancer cells (Cheng et al., 2012). It has been reported to have antioxidant, anti-inflammatory and anti-mutagenic activity (Jayaprakasha et al.,
The chemopreventive efficacy of TEO has been reported against submucous fibrosis in humans (Deepa et al., 2010) and was found to reduce benzo[a]pyrene induced DNA damage \textit{in vitro} in oral mucosa cells (Hastak et al., 1997). TEO prevented the growth of \textit{Aspergillus flavus} species and significantly reduced aflatoxin B1 production which is a potent liver carcinogen (Ferreira et al., 2013). It could also improve the bioavailability of curcumin after oral administration in humans (Antony et al., 2008). Food and drug administration (FDA) has accepted TEO as a food additive and is Generally Recognized As Safe (GRAS). It is safe in rats (NOAEL) up to an oral dose of 500 mg/kg body weight (Chapter 3).

The protective effects of TEO as a chemopreventive agent against NDEA induced hepatocellularcarcinoma in male Wistar rats and two-stage mouse skin papilloma development induced by DMBA as initiator and croton oil as promoter is also being reported in this study. In order to assess the anticarcinogenic efficacy of TEO we have also analyzed 3-methyl cholantherene (3-MC) induced sarcoma in mice. To determine the possible mechanism of action of TEO we have evaluated the inhibition of different cytochrome P450 enzymes (Phase I enzymes) by TEO \textit{in vitro} and \textit{in vivo}. Moreover, we have studied the levels of the drug metabolizing enzymes, glutathione-S-transferase (GST) and UDP-glucuronyl transferase after TEO administration in rats.

7.2. Materials and methods

7.2.1. Animals

Male Wistar rats, Male Balb/C mice and male Swiss albino mice were used for this study (Chapter 2, section 2.1.5).

7.2.2. Preparation of turmeric essential oil (TEO)

TEO prepared from the rhizome of \textit{Curcuma longa} L., was purchased from Kancore Ingredients Limited., Angamali, Kerala, India. TEO was dissolved in paraffin oil for animal studies. For \textit{in vitro} study TEO was dissolved in DMSO.
7.2.3. Effect of TEO on N-nitrosodiethylamine induced hepatocarcinogenes is in rats

Six groups of male Wistar rats, each group consisting of 8 animals were used for this study.

- **Group I**: Untreated
- **Group II**: Control receiving (NDEA alone).
- **Group III**: Vehicle control (NDEA and paraffin oil).
- **Group IV**: NDEA + 20 mg/kg b. wt. TEO
- **Group VI**: NDEA + 100 mg/kg b. wt. TEO
- **Group VI**: NDEA + 500 mg/kg b. wt. TEO

Animals were treated with 0.02% NDEA (2.5 ml/animal/dose) for 20 weeks (6 days/week) and thereafter observed for 9 more weeks. At the end of 29th week, all the animals were sacrificed by ether anaesthesia. Liver were excised and washed with ice cold saline. A small piece of liver was kept in 10% formalin for histopathological analysis.

7.2.3.1. Morphology and weight of the liver

Liver from each animal was washed in ice-cold saline (0.9%) and observed for tumour nodules and other morphological abnormalities. Weight of each liver was recorded and expressed in relation to the body weight.

7.2.3.2. Biochemical analysis of serum

Blood was collected by direct heart puncture in non-EDTA tubes and serum was separated after centrifugation at 5000 rpm for 10 minutes and used for the following investigations. Gamma-glutamyl transferase (γ-GT) activity was assayed by the method of Tate and Meister (Tate et al., 1975). Total bilirubin was determined by Jendrassik-diazotized sulphanilic acid method (Garber et al., 1981). Alkaline phosphatase (ALP) was estimated by p-nitrophenyl phosphate (PNPP) hydrolysis (McComb et al., 1979) and alanine amino transferase (ALT) as well as aspartate aminotransferase (AST) by kinetic method using commercially available kits (Span Diagnostics, India).
7.2.3.3. Effect of TEO on antioxidant enzymes in the liver

Liver homogenate (25%) was prepared in Tris-HCl buffer (0.1 M, pH-7.4), centrifuged at 1000 rpm for 10 minutes at 4°C to remove the cell debris. The supernatant was used for assessing the activity of GSH by its reaction with 5,5-dithiobis-2-nitrosobenzoic acid (Moron et al., 1979). Glutathione peroxidase (GPx) activity was checked from the degradation of H$_2$O$_2$ in the presence of GSH (Hafeman et al., 1974). Glutathione-S- transferase (GST) was measured based on the rate of increase in conjugate formation between GSH and 1-chloro -2, 4-dinitrobenzene (CDNB) (Moron et al., 1979).

7.2.3.4. Histopathological analysis

A portion of the liver was cut, washed in PBS and fixed in 10% neutral buffered formalin (Chapter 2, Section 2.2.6).

7.2.4. Activity of TEO on DMBA induced two-stage skin papilloma formation in mice

Male Balb/c mice were used for the DMBA induced skin papilloma study. DMBA, croton oil and TEO was applied on the shaved dorsal side (2 cm diameter) of the mice at least two days before the application of chemicals. Animals having no hair growth after 2 days were selected for the experiment. Balb/C mice were divided in to eight groups and each group having 8 animals.

Groups  | I    : DMBA + croton oil  
Groups  | II   : DMBA + croton oil + Paraffin oil  
Groups  | III  : DMBA + croton oil + 10% TEO  
Groups  | IV   : DMBA + croton oil + 25% TEO  
Groups  | V    : DMBA + croton oil + 50 % TEO  
Groups  | VI   : DMBA alone  
Groups  | VII  : Croton oil alone  
Groups  | VIII : DMBA+ TEO

Single dose of DMBA (470 nmol/mouse dissolved in 200 µl acetone) was used for the study. All groups were applied with 1% croton oil in acetone (200 µl/ animal)
twice weekly for six weeks except group VI and VIII. TEO dissolved in paraffin oil was applied twice weekly for 6 weeks 30 minutes before 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 papilloma formation was recorded weekly and the tumor growth greater than 1 mm in diameter was included in the cumulative total if they persisted for 2 weeks or more. Formation of the onset papilloma and number of papilloma per cage in various groups were recorded every week.

7.2.5. Effect of TEO against 3-methylcholanthrene (3-MC) induced sarcoma development in mice

Balb/C mice weighing 20-25 g was divided into 5 groups and each group consisting of 15 animals.

| Group   | Description                              |
|---------|------------------------------------------|
| I       | Untreated                                |
| II      | 3-MC alone (Control)                     |
| III     | 3-MC + Paraffin oil (Vehicle control)    |
| IV      | 3-MC + TEO 20 mg/kg b. wt.               |
| V       | 3-MC + TEO 100 mg/kg b. wt.              |
| VI      | 3-MC + TEO 500 mg/kg b. wt.              |

Methylcholanthrene (3-MC) was administered on the shaven dorsal surface of each mouse (200 µg/animal/dose). Oral administration of TEO was started 24 h before the injection of 3-MC and 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 30 weeks.

7.2.6. Effect of TEO on Phase I enzymes

7.2.6.1. Determination of effect of TEO on MROD, PROD and EROD in vitro

Rats were administered with phenobarbitone continuously for 4 days (60 mg/kg body weight, once daily) and sacrificed 24 h after the last dose of phenobarbitone. The livers of all the rats were excised quickly, washed thoroughly in ice-cold saline and kept at -70°C. Liver homogenate (25%) was made in cold phosphate buffer (pH 7.4, 0.1 M).
Homogenate was initially centrifuged at 14000 g for 20 min in a cold centrifuge (Remi) and supernatant was then further centrifuged at 10, 5000 g for 1 h in an ultracentrifuge (Sorvall). The microsomes obtained from liver homogenate were washed and resuspended in cold phosphate buffer (pH 7.4, 0.1 M) and used for determination of the effect of TEO on the dealkylation of methoxy resorufin by 7-methoxyresorufin-O-demethylase (MROD), CYPIA2, pentoxy resorufin by 7-pentoxyresorufin–O-depentylase (PROD), CYP2B1/2 and ethoxy resorufin by 7-ethoxyresorufin-O-deethylase (EROD), CYP1A1 (Pohl and Fouts, 1980; Nerurkar et al., 1993).

Reaction mixture containing sodium phosphate buffer (0.1 M, pH 7.4), 6.25 mM MgSO$_4$, 60 μM EDTA, 5 μM (ER, MR or PR), 100 μg microsomal protein, and various concentrations of turmeric essential oil (50, 100 and 200 μg/ml) in a final volume of 1 ml was incubated for 5 min at 37°C. The reaction was stopped by the addition of 2 ml of chilled methanol. The precipitated protein was centrifuged and supernatant was used for the estimation of enzyme activity by measuring resorufin formed using a fluorescent spectrophotometer (Nanodrop ND-3300, Thermo Scientific, USA.) at the excitation wavelength of 550 nm and the emission wavelength of 585 nm. Blanks were set without addition of NADPH. The percentage inhibition was calculated by the following formula:

\[
\frac{C-T}{C} \times 100.
\]

Where ‘C’ is the optical density of control without essential oil; ‘T’ is the optical density with essential oil.

**7.2.6.2. Evaluation of the effect of TEO on aniline hydroxylase**

Inhibition of aniline hydroxylase activity (an indicator of CYP2E1 activity) was measured by the method of Mazel (1971).
**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 (630 nm) the blue color formed when p-aminophenol reacts with phenol.

**Procedure**

The total volume of the reaction mixture is 1.5 ml containing the microsomal fraction (1 – 1.5 mg protein) phosphate buffer (150 mM, pH 7.4), MgCl₂ (5 mM), aniline (32 mM in ethanol) and various concentration of TEO (50, 100 and 200 μg/ml) 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 3000 rpm for 10 minutes. Supernatant (1.5ml) was mixed with 750μl of 10% Na₂CO₃, 1.5ml of phenol (2% in 0.2M NaOH) and mixture was incubated at 37°C for 30 minutes. The absorbance was measured at 630 nm against the phosphate buffer. The percentage of inhibition was calculated.

7.2.6.3. *Aminopyrene-N-demethylase activity*

Aminopyrene-N-demethylase activity (an indicator of CYP 1A, 2A, 2B, 2D and 3A activity) was measured by the method of Mazel, (1971).

**Principle**

Aminopyrene was dealkylated by microsomal enzymes to form 4-aminoantipyrine and formaldehyde. Formaldehyde so formed was measured by condensation with Nash reagent. The colour development and absorbance was measured at 412 nm.

**Procedure**

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), MgCl₅ (5 mM), aminopyrene (320 mM), semicarbazide hydrochloride (120 mM) and various concentrations of TEO (50, 100 and 200 μg/ml) were incubated at 37°C for 5 min. NADPH (33.3 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 60ºC for 30 minutes for colour development and absorbance was measured at 412 nm against distilled water. The percentage of inhibition was calculated.

7.2.6.4. Effect of TEO on various isoforms of cytochrome P450 enzymes in vivo

Wistar rats (120-150 g) were divided into the following groups consists of six animals per group.

| Group  | Description                                      |
|--------|--------------------------------------------------|
| I      | Untreated                                        |
| II     | Phenobarbitone alone (60 mg/kg b. wt., i.p./day) (Control) |
| III    | Phenobarbitone + paraffin oil (vehicle control), |
| IV     | Phenobarbitone + TEO 250 mg/kg b. wt.            |
| V      | Phenobarbitone + TEO 500 mg/kg b. wt.            |
| VI     | Phenobarbitone + TEO 1000 mg/kg b. wt.           |

Different doses of TEO were administered once daily for 15 days orally, and phenobarbitone (60 mg/kg body weight, i.p./day) was started on day 12 and continued for 4 days. The rats were 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 -70ºC. Liver homogenate (25%) was prepared in cold phosphate buffer (pH 7.4, 0.1M). Homogenate was initially centrifuged at 14000 g for 20 min in a cold centrifuge (Remi) and supernatant was then further centrifuged at 10,5000 g for 1 h in an ultracentrifuge (Sorvall) and microsomes obtained were washed and resuspended in cold phosphate buffer (pH 7.4, 0.1 M) and used for determination of the activities of methoxyresorufin by 7-methoxyresorufin-O-demethylase (MROD), CYP1A2, pentoxyresorufin by 7-pentoxyresorufin-O-depentylation (PROD), CYP2B1/2 (20) and ethoxyresorufin by 7-ethoxyresorufin-O-de-ethylase (EROD), CYP1A1 (Nerurkar et al., 1993). Assay mixture
consisted of sodium phosphate buffer (0.1M, pH 7.4), 6.25 mM MgSO4, 60 µM EDTA, 5 µM MR/PR/ER, 100 µg microsomal protein from treated and untreated animals and (100 µM) NADPH in a final volume of 1 ml. The reaction time was 5 min with a preincubation period of 5 min without the addition of NADPH at 37°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 wavelength of 550 nm and the emission wavelength of 585 nm. Blanks were set without any NADPH. Calibration curves were built by determining the fluorescence of known amounts of the authentic resorufin. Concentration of protein in each sample was measured. Results were expressed as pmols of resorufin formed /min/mg protein for in vivo studies.

7.2.7. Assessment of the effect of TEO on phase II enzymes in vivo.

Five groups of (120-150 g) Wistar rats, with each group consisting of 6 animals were divided into the following groups.

Group I : Control (Untreated)
Group II : Vehicle control (Paraffin oil),
Group III : TEO 250 mg/kg b. wt.
Group IV : TEO 500 mg/kg b. wt.
Group V : TEO 1000 mg/kg b. wt.

Different concentrations of TEO were administered orally once daily for 15 days. Animals were sacrificed on 15<sup>th</sup> day and liver homogenate was prepared and used for analysis of phase II enzymes Urudine 5’-diphospho-glucuronyltransferase (UDP-GT) (Hollman <i>et al.</i>, 1962) and glutathione-S-transferase (Habig <i>et al.</i>, 1974) (Chapter 2, Section 2.2.5.6).

7.2.7.1. UDP glucuronyl transferase activity

The UDP glucuronyl transferase was estimated by the method of Issalbacher <i>et al.</i> (1962) modified by Hollman and Touster (1962).
Reagents

Tris HCl buffer (1M, pH 7.4) was dissolved in dist. water.
UDP glucuronic acid (30 mM) was dissolved in dist. water.
P- nitro phenol (5mM) was dissolved in dist. water.
MgCl$_2$ (50 mM) was dissolved in water.

Triton X-100 : 0.25%
TCA : 5%
NaOH : 2 M

Procedure

The incubation mixture, containing 0.5ml buffer (0.2 ml Triton X-100, 0.05 ml MgCl$_2$, 0.05 ml 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. Supernatant (1 ml) and NaOH (0.25ml) was added and read at 450 nm using a photochemical colorimeter. The activity of UDP glucuronyl transferase was expressed as n moles/min/mg protein.

7.2.7.2. Glutathione-S-transferase

The glutathione-S-transferase was estimated by the methods of Habig et al. (1974) (Chapter 2, Section 2.2.5.6).

7.2.8. Statistical analysis

The values are expressed as mean ± SD. The statistical significance was compared between control and experimental groups by one way analysis of variance (ANOVA) followed by appropriate post hoc test (Dunnet multiple comparison test) using Graph pad in Stat software (version 3.05). Data of essential oil treated animals were compared with control animals.
7.3. Results

7.3.1. Effect of TEO on n-nitrosodiethylamine induced hepatocarcinogenesis in rats

7.3.1.1. Morphology and liver weight

Morphology of NDEA alone treated rats liver showed numerous tumour nodules on the surface with variable shapes, colour and normal morphology of liver was completely lost in most of the animals. Irregular protuberances were seen in most of the liver of NDEA treated animals (Fig. 7.1. A, B, C). Liver of animals treated with TEO (20 and 100 mg/kg body weight) showed fewer incidences of tumour nodules and retained its normal morphology of the liver with small necrotic masses seen in some animals. In animals treated with 500 mg/kg body weight TEO normal morphology of liver was seen and did not show any significant tumour incidence. Liver weight was increased in both control (NDEA alone) and vehicle control (NDEA+paraffin oil) groups. TEO treated groups (20, 100 and 500 mg/kg body weight) showed decreased liver weight when compared to control groups (Table 7.1). The present study revealed that TEO significantly restored NDEA induced altered liver weight and morphology in a concentration dependent manner.

7.3.1.2. Biochemical analysis of serum

The levels of bilirubin, AST, ALT and ALP were drastically elevated in serum of control and vehicle control animals. TEO treated groups (100 and 500 mg/kg body weight) showed significant (p<0.001) decrease in these hepatic parameters when compared with control group. The elevated level of γ-GT as seen a marker of cell proliferation was decreased significantly (p<0.001) by TEO administration (Table 7.2).

7.3.1.3. Effect of TEO on antioxidant enzymes in the liver

NDEA administration suppressed the GSH, GPx and GST level in liver tissue, which was significantly (p<0.001) increased by the TEO administration in a concentration dependent manner (Table 7.3).
7.3.1.4. Histopathological analysis

Untreated rat liver section showed normal portal triads, bile ductules and hepatocytes. Sinusoidal spaces and Kupffer cells appeared normal. Both NDEA alone and vehicle control group showed the liver tissue with complete effacement of normal architecture. Hepatocytes were pleomorphic large polyhedral cells, many of them showing vacuolated cytoplasm and they had vesicular nuclei. Some area showed necrosis, haemorrhage, and inflammatory cell infiltrate. However, in TEO treated animals (500 mg/kg body weight) normal architecture of the liver was seen with normal portal triads and bile ductules. Portal triads are normal in bile ductules, hepatic vein and hepatic artery. Hepatocytes also appeared normal and they are arranged in cords. Kupffer cells and sinusoidal spaces appeared normal (Fig. 7.1.D,E,F). TEO treated groups retained the normal architecture of liver at higher concentration.

7.3.2. Effect of TEO on DMBA induced two-stage papilloma development in mice

Papilloma development in mice was started 6th week in control and vehicle control group. Onset of papilloma was found to be significantly delayed or prevented by TEO treatment. In TEO (10 and 25%) treated groups, formation of papilloma were started in the 12th week after DMBA application. DMBA alone, croton oil alone and DMBA + croton oil + 50% TEO treated groups did not produce any papilloma on mice. At the end of 20th week the average number of papilloma per mouse was 7 ± 1.06 and 6.86 ± 1.04 in control and vehicle control group respectively and this number was reduced to 1.2 ± 0.5 and 1 ± 0.3 by topical application of TEO 10% and 25% respectively (Fig. 7.2). Number of animals which developed papilloma developed was 2 out of 8 animals and 5 out of 8 animals in 25% and 10% TEO treated groups (Table 7.4). Inhibition of development of papilloma was 82.9, 85.7 and 100% in 10, 25 and 50% TEO treated groups respectively (Fig. 7.3).
7.3.3. Effect of TEO against 3-methyl cholangrene (3-MC) induced carcinogenesis in mice

Sarcoma developed at the site of injection i.e at the subcutaneous region of the neck in mice. The animals in the control and vehicle control groups (3-MC alone) started to develop fibrosarcoma by 8th week and 100% animals developed sarcoma at 15th week (Fig. 7.4). TEO treated groups (20 and 100 mg/kg body weight) started to develop sarcoma only at 10th week and 500 mg/kg body weight at 12th week (Table 7.5). TEO elevated the survival rate of the animals harboring sarcoma. All the animals in control and vehicle control animals died by the end of 17th week. In the case of TEO treated group (500 mg/kg body weight) 6 out of 15 animals and in 100 and 20 mg/kg body weight TEO treated group 5 out of 15 animals were found to be alive by the end of 17th week (Fig. 7.5). These results indicated that TEO could significantly inhibit and delay the development of the 3-MC induced sarcoma in mice.

7.3.4. Effect of TEO on Phase I enzymes in vitro

7.3.4.1. Effect of TEO on MROD, PROD and EROD in vitro

Administration of phenobarbitone increased the level of cytochrome P450 enzymes EROD, MROD and PROD in rats, when compared with untreated group. Percentage inhibition of EROD at various concentration of TEO such as 50, 100 and 200 µg/ml was 78.7%, 80.6% and 83.8% respectively (Fig. 7.6). TEO significantly inhibited MROD activity (P<0.001) and 80% inhibition was shown at 200 µg/ml. Similarly activity of PROD was also inhibited by TEO at various concentrations which were 63.5%, 66.4% and 81.1%. IC50 values of MROD, PROD, EROD by TEO is given in (table 7.6).

7.3.4.2. Effect of TEO on aniline hydroxylase (CYP2E1) in vitro

TEO significantly inhibited (p<0.001) aniline hydroxylase activity in a dose dependent manner. Concentration needed for 50% inhibition (IC50) of aniline hydroxylase was 62.5 µg/mL (Table 7.6). Inhibition of aniline hydroxylase activity at the concentration of 50, 100 and 200 µg/ml TEO was 46.6%, 62.2% and 73.3% respectively (Fig. 7.7A).
Table 7.1: Effect of TEO on relative liver weights and nodule incidence of rats treated with NDEA.

| Groups                               | Liver weight (g)/100g body weight | Nodule incidence |
|--------------------------------------|-----------------------------------|------------------|
| Untreated                            | 3.34 ± 0.46                       | 0/6              |
| Control (NDEA alone)                 | 6.17 ± 0.91                       | 8/8              |
| Vehicle control (NDEA + Paraffin oil)| 6.41 ± 1.52                       | 8/8              |
| 20 mg/kg b.wt. TEO + NDEA           | 4.08 ± 0.71***                    | 4/8              |
| 100 mg/kg b.wt. TEO + NDEA          | 3.61 ± 0.49***                    | 3/8              |
| 500 mg/kg b.wt. TEO + NDEA          | 3.44 ± 0.68***                    | 2/8              |

NDEA: N-nitrosodiethylamine.
Data are expressed as the mean ± standard deviation (n=8 per group).
(P value) *** p<0.001.
Fig: 7.1. Effect of turmeric essential oil on liver morphology and histopathology of NDEA treated rats

Liver Morphology

A) Untreated liver morphology
B) Treated with NDEA alone
C) Treated with NDEA and TEO

Liver Histopathology

D) Untreated liver histopathology
B) Treated with NDEA alone
C) Treated with NDEA and TEO
Table 7.2: Effect of TEO on serum Bilirubin, ALP, AST, ALT and γ-GT in NDEA treated rats.

| Group                        | Bilirubin (mg/100ml) | ALP (KA Units) | AST (IU/L) | ALT (IU/L) | γ-GT (IU/L) |
|------------------------------|----------------------|----------------|-------------|------------|-------------|
| Untreated                    | 0.54 ± 0.1           | 23.89 ± 1.9    | 098.88 ± 17.6 | 61.58 ± 11.7 | 12.71 ± 3.10 |
| Control (NDEA alone)         | 1.44 ± 0.2           | 39.83 ± 2.9    | 188.35 ± 37.0 | 104.80 ± 12.9 | 118.8 ± 15.2 |
| Vehicle control              | 1.45 ± 0.1           | 39.57 ± 8.2    | 164.43 ± 50.3 | 102.43 ± 15.4 | 108.0 ± 13.9 |
| 20 mg/kg b.wt. TEO           | 0.77 ± 0.2<sup>a</sup> | 26.77 ± 4.3<sup>a</sup> | 127.92 ± 17.6<sup>a</sup> | 78.26 ± 11.9<sup>b</sup> | 23.22 ± 6.3<sup>a</sup> |
| 100 mg/kg. b.wt. TEO         | 0.65 ± 0.1<sup>a</sup> | 25.03 ± 2.3<sup>a</sup> | 121.40 ± 24.9<sup>a</sup> | 70.38 ± 16.7<sup>a</sup> | 17.41 ± 5.2<sup>a</sup> |
| 500 mg/kg b.wt. TEO          | 0.58 ± 0.1<sup>a</sup> | 23.45 ± 2.8<sup>a</sup> | 112.69 ± 23.7<sup>a</sup> | 64.81 ± 12.4<sup>a</sup> | 13.02 ± 3.2<sup>a</sup> |

ALP : Alkaline phosphatase.  
AST : Aspartate aminotransferase.  
ALT : Alanine amino transferase.  
γ-GT : Gamma-glutamyl transferase.  
NDEA : N-nitrosodiethylamine.  
Vehicle control : Paraffin oil+NDEA.  
Data are expressed as the mean ± standard deviation (n=8 per group).  
(P value)<sup>a</sup> p<0.001.  <sup>b</sup> P<0.01.
Table 7.3: Effect of TEO on oxidative stress in the liver of NDEA treated rats.

| Groups               | GSH     | GST     | GPx     |
|----------------------|---------|---------|---------|
|                      | (nmol/mg protein) | (nmol/mg protein) | (U/mg protein) |
| Untreated            | 29.49 ± 4.7 | 22.04 ± 3.75 | 345.13 ± 32.4 |
| NDEA alone           | 12.55 ± 1.76 | 10.32 ± 2.13 | 187.32 ± 27.71 |
| Vehicle control      | 13.37 ± 2.22 | 9.78 ± 1.94  | 173.42 ± 46.95 |
| 20 mg/kg b. wt TEO   | 22.4 ± 2.3 b  | 14.69 ± 1.97 b | 238.30 ± 16.95 b |
| 100 mg/kg b. wt TEO  | 24.64 ± 6.83 a | 17.05 ± 1.6 a  | 252.16 ± 22.15 a |
| 500 mg/kg b. wt TEO  | 26.6 ± 4.82 a  | 20.33 ± 3.27 a  | 293.86 ± 45.49 a |

NDEA : N-nitrosodiethylamine.

GSH : Glutathione.

GST : Glutathione-S-transferase.

GPx : Glutathione peroxidase.

Vehicle control : Paraffin oil+NDEA.

Data are expressed as the mean ± standard deviation (n=8 per group).

(P value) a p<0.001. b p<0.01.
Table 7.4: Effect of TEO on skin papilloma formation in mice using DMBA as initiator and croton oil as promoter

| Groups                        | No. of mice developed Papillomas/groups | No. of papillomas per mice | % inhibition of papilloma development |
|-------------------------------|----------------------------------------|----------------------------|--------------------------------------|
| DMBA+ Croton oil              | 7/7                                    | 7 ± 1.3                    |                                      |
| DMBA+ Croton oil + Paraffin oil | 7/7                                    | 6.9 ± 1.04                 |                                      |
| DMBA alone                    | 0/6                                    | Nil                        |                                      |
| Croton oil alone              | 0/6                                    | Nil                        |                                      |
| DMBA + TEO                    | 0/8                                    | Nil                        |                                      |
| DMBA + Croton oil + TEO 10%   | 5/8                                    | 1.2 ± 0.5<sup>a</sup>      | 82.9                                 |
| DMBA + Croton oil + TEO 25%   | 2/8                                    | 1.0 ± 0.3<sup>a</sup>      | 85.7                                 |
| DMBA + Croton oil + TEO 50%   | 0/8                                    | Nil                        | 100                                  |

(P Value)<sup>a</sup> p<0.001
Fig. 7.2. Figure showing effect of TEO on DMBA induced two-stage papilloma development in mice

A: Control mice showing papilloma development
B: TEO 50% treated mice without papilloma development

Fig. 7.3. Effect of TEO on DMBA induced two-stage papilloma development in mice

DMBA: 7, 12-dimethylbenz (a) anthracene
TEO: Turmeric essential oil
Table 7.5: Effect of TEO on development of onset of 3-MC induced sarcoma in mice.

| Number of days | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 120 | 130 | 140 | 210 |
|---------------|----|----|----|----|----|-----|-----|-----|-----|-----|-----|
| **Number of mice developed sarcoma** |    |    |    |    |    |     |     |     |     |     |     |
| 3-MC alone (Control) | 4  | 8  | 11 | 13 | 14 | 15  |
| 3-MC + Vehicle control<sup>a</sup> | 5  | 7  | 9  | 13 | 14 | 14  | 15  |
| 3-MC + 500 TEO mg/kg b.wt | 0  | 0  | 0  | 1  | 5  | 6   | 9   | 9   | 9   | 11  | 11  |
| 3-MC + 100 TEO mg/kg b.wt | 0  | 0  | 2  | 4  | 6  | 9   | 9   | 11  | 11  | 12  | 12  |
| 3-MC + 20 TEO mg/kg b.wt  | 0  | 0  | 4  | 5  | 9  | 9   | 12  | 13  | 13  | 14  | 14  |

3-MC: 3-methyl cholanthrene.

<sup>a</sup>Paraffin oil.

TEO: turmeric essential oil.
Fig: 7.4. Figure showing effect of TEO against 3-MC induced sarcoma development in mice

3-MC alone 3-MC + TEO

Fig: 7.5. Effect of turmeric essential oil on the survival of 3-MC induced sarcoma bearing mice

Survival of animals

Number of Days

- Control
- Vehicle control
- 500 mg/kg b.wt. TEO
- 100 mg/kg b.wt. TEO
- 20 mg/kg b.wt. TEO
Table 7.6: Inhibition of cytochrome P450 enzymes by TEO (*in vitro*)

| Cytochrome P450 enzymes                                                                 | IC$_{50}$ Values of TEO (µg/ml) |
|----------------------------------------------------------------------------------------|---------------------------------|
| CYP1A2 (MROD)                                                                           | 66                              |
| CYP2B 1/2 (PROD)                                                                        | 36                              |
| CYP1A1 (EROD)                                                                           | 31                              |
| CYP 1A, 2A, 2B, 2D and 3A (Aminopyrene-N-demethylase)                                   | 45                              |
| CYP2E1 (Aniline hydroxylase)                                                            | 62.5                            |

MROD: 7-methoxyresorufin-O-demethylase;

PROD: 7-pentoxyresorufin-O-depentyllase;

EROD: 7-ethoxyresorufin-O-deethylase.
Table 7.7: Effect of administration of TEO on phenobarbitone induced P450 enzymes in rats.

| Group                          | MROD (nmol/min/mg protein) | PROD (nmol/min/mg protein) | EROD (nmol/min/mg protein) |
|-------------------------------|----------------------------|-----------------------------|----------------------------|
| Untreated                     | 0.9 ± 0.33                 | 0.27 ± 0.42                 | 1.3 ± 0.2                  |
| Phenobarbitone alone          | 7.73 ± 2.16                | 11.0 ± 3.1                  | 7.85 ± 1.2                 |
| Vehicle control + phenobarbitone | 8.15 ± 1.8                | 10.85 ± 0.9                 | 7.31 ± 2.0                 |
| TEO 1000 mg/kg b.wt. + phenobarbitone | 0.31 ± 0.04\(^a\) (95.9%) | 0.94 ± 0.17\(^a\) (91.5%)  | 0.60 ± 0.10 (91.8%)        |
| TEO 500 mg/kg b.wt. + phenobarbitone | 0.55 ± 0.05\(^a\) (92.7%) | 1.64 ± 0.20\(^a\) (85.1%)  | 1.73 ± 0.20 (76.3%)        |
| TEO 250 mg/kg b.wt. + phenobarbitone | 0.62 ± 0.11\(^a\) (91.97%)| 2.37 ± 0.24\(^a\) (78.5%)  | 2.52 ± 0.45 (65.5%)        |

Vehicle control: Paraffin oil.
MROD: 7-methoxyresorufin-O-demethylase.
PROD: 7-pentoxyresorufin-O-depentylase.
EROD: 7-ethoxyresorufin-O-deethylase.

Data are expressed as the mean ± standard deviation (n=6 per group).
(P value) \(^a\) P<0.001.
Fig: 7.6. Effect of turmeric essential oil on the inhibition of cytochrome P450 enzymes (Phase I enzymes-in vitro)

EROD : 7-methoxyresorufin-O-demethylase
PROD : 7-pentoxyresorufin-O-depentyllase
MROD : 7-methoxyresorufin-O-demethylase

Fig: 7.7. Effect of turmeric essential oil on the inhibition of aniline hydroxylase and aminopyrene-N-demethylase activity

(A) Aniline hydroxylase
(B) Aminopyrene-N-demethylase
Table 7.8: Effect of TEO on Phase II enzyme activity in rats

| Groups               | UDP-Glucuronyl transferase (nmol/min/mg protein) | Glutathione –S-Transferase (nmol of CDNB conjugate formed/min/mg protein) |
|----------------------|-------------------------------------------------|--------------------------------------------------------------------------|
| Untreated            | 33.34 ± 3.90                                    | 46.1 ± 6.01                                                              |
| Vehicle control      | 31.37 ± 6.41                                    | 46.6 ± 11.2                                                              |
| 1000 mg/kg b.wt. TEO | 62.91 ± 7.92<sup>a</sup>                        | 167.5 ± 27.61<sup>a</sup>                                               |
| 500 mg/kg b.wt. TEO  | 49.94 ± 4.17<sup>b</sup>                        | 134.5 ± 17.54<sup>a</sup>                                               |
| 250 mg/kg b.wt. TEO  | 43.02 ± 5.31<sup>c</sup>                        | 127.47 ± 31.42<sup>a</sup>                                              |

Vehicle control: Paraffin oil.

Data are expressed as the mean ± standard deviation (n=6 per group).

(P value): <sup>a</sup>P<0.001. <sup>b</sup>P<0.01. <sup>c</sup>P<0.05.
7.3.4.3. Effect of TEO on aminopyrene-N-demethylase activity (in vitro)

Aminopyrene-N-demethylase activity is an indicator of CYP 1A, 2A, 2B, 2D and 3A. TEO inhibited the activity of aminopyrene-N-demethylase significantly (P<0.001) (Fig. 7.7B). IC_{50} value of aminopyrene-N-demethylase by TEO is 45 \mu g/mL. (Table 7.6).

7.3.5. Effect of TEO on Phase I enzymes in vivo

Cytochrome P450 enzymes (Phase I enzymes) were found to be significantly increased following phenobarbitone administration. Significant inhibition (P<0.001) of Phase I enzymes was observed after TEO administration as seen from the hepatic MROD (CYP1A2), PROD (CYP2B1/2) and EROD (CYP1A1) levels (Table 7.7). Oral administration of TEO significantly inhibited MROD levels in a concentration dependent manner and maximum inhibitory effect was seen at 1000 mg/kg body weight (95.9%). TEO significantly decreased the level of EROD and PROD to 91.8 and 91.5% respectively. These results indicated that oral administration of TEO inhibited the phenobarbitone induced elevation of MROD, PROD and EROD levels.

7.3.6. Effect of TEO on phase II enzymes.

The level of glutathione-S-transferase in the liver was found to be significantly increased (P<0.001) after oral administration of TEO. Similarly TEO administration was found to increase the level of UDP-glucuronyl transferase in a concentration dependent manner (Table 7.8).

7.4. Discussion

Chemical analysis of essential oil from C. longa indicate the presence of 13 compounds of which ar-turmerone (61.79%) and curlone (12.48%) are found to be the most prominent. Ar-turmerone present in TEO has been reported to have significant pharmacological and anticancer potential (Hislop, 1990; Sugimura, 1970 and Ames, 1983). It is already reported that ar-turmerone (12.66%) and curlone (6.82%) exhibited prolonged and systemic bioavailability as well as plasma elimination half-life were 7.2 and 6.8 h respectively (Prakash et al., 2011). Present study was investigated for
chemopreventive potential of TEO against N-nitrosodiethylamine (NDEA) induced hepatocarcinogenesis in rats and DMBA induced two-stage skin papilloma development in mice. The effect of TEO on 3-methylcholanthrene induced sarcoma development in mice was also evaluated. Moreover, we have studied the levels of cytochrome P450 (Phase I enzymes) and phase II enzymes (glutathione-S-transferase and UDP-glucuronyl transferase) to elucidate the mechanism of action of anticancer potential of TEO.

The chemopreventive effect of TEO indicated that it can restore NDEA induced alteration in liver weight, morphology and hepatic enzyme parameters in a concentration dependent manner. NDEA induced liver nodule incidence was significantly reduced by oral administration of TEO. Increase in the levels of ALT, AST and ALP in serum are general indicators of liver injury and liver cell damage (Hilaly et al., 2004). Serum GGT levels are considered as biomarker of preneoplastic lesions and liver cell proliferation. Increase in the level of GGT indicates the release of enzymes from the cytoplasm of the cells into the blood circulation after cellular damage. There was close connection between GGT activation and carcinogenesis (Shaarawy et al., 2009). Results showed a decrease in the level of these enzymes after oral administration of TEO indicating that it can suppress NDEA induced liver damage.

Reactive oxygen species is believed to cause oxidation and damage to DNA and other macromolecules. NDEA metabolism takes place in liver and the generation of free radicles is recognised as its reason for carcinogenic effect. Free radicals are continuously generated after induction of NDEA which causes oxidative stress in tissues and biological systems of the body that leads to tissue injury, alteration of biochemical compounds, chromosomal mutations, all of which are involved in carcinogenesis (Shaarawy et al., 2009). Antioxidant enzyme markers such as GSH, GPx, and GST play an important role in suppression of liver carcinogenesis induced by NDEA (Saydam et al., 1997; Bansal et al., 2005). GSH can effectively scavenge free radicals of oxygen species through non-enzymatic and enzymatic process by conjugation with GPx and GST (Ramakrishnan et al., 2006; Zeng et al., 2008 and Lie, 2002). GST is located in cytosol and plays an important role in detoxification and excretion of xenobiotics (Rao et al., 2006). We have found that simultaneous administration of TEO significantly increased NDEA induced liver damage.
lowering of antioxidant enzymes, GSH, GPx and GST. Our previous study revealed that oral administration of TEO increased the antioxidant enzymes in normal mice (Chapter 4, section 4.3.1 and 2). The elevation of these antioxidant enzymes and GSH in vivo may be one of the reasons for protective activity of TEO against NDEA induced hepatocarcinogenesis. Histological observations of liver tissues also support the potential of TEO against hepatocellular carcinoma.

DMBA is a polycyclic aromatic hydrocarbon and mostly used for studies in carcinogenic animal model experiment (Hamizah et al., 2012). DMBA is metabolized by CYP1A1 and CYP1B1, which convert DMBA to the ultimate carcinogen 1, 2-epoxide-3, 4-diol DMBA (Sharma et al., 2012; Kawajiri and Ikuta, 1999; Anqus et al., 1999; Buters et al., 1999), which form adducts with DNA leading to carcinogenesis (Cheng et al., 1988). Suppression of CYP1A1 enzyme activity by TEO can reduce the effect of DMBA induced carcinogenesis. In present study we have shown that the incidence and number of skin papillomas formation after treated with 7, 12 dimethylbenz[a]anthracene and promoted by croton oil was significantly decreased and absolutely prevented (50% TEO treated group) by treatment with TEO in mice. This implies that TEO may have either inhibited the metabolism of DMBA to its active form, or delayed the promotion phase of carcinogenesis (Agrawal and Sonam, 2009). In order to evaluate the chemopreventive potential of TEO we used a polynuclear aromatic hydrocarbon (3-Methylcolantherene), a potent carcinogenic agent which elicits mutagenic and carcinogenic responses in laboratory animals. TEO delayed and prevented 3-MC induced sarcoma development in mice. The activity of 3-MC metabolizing CYP1A1 (EROD) enzyme was found to be inhibited by oral administration of TEO, which may be its possible mechanism of action.

The carcinogenic activity of these compounds depends up on its metabolic activation to reactive intermediates. The activation and elimination of the chemical carcinogens are done by phase I and phase II reactions. Cytochrome P450 enzymes are most extensively studied biological proteins, which are present in liver and plays an important role in the metabolism of drugs as well as endogenous and exogenous chemicals. Some of natural products from plants prevent cancer by inhibiting phase I enzymes (Cytochrome P450) and stimulating phase II enzymes such as glutathione-S-
transferase (GST) and uridine diphosphate glucuronyl transferase (UDP-GT) enzymes. Present study indicated that phenobarbitone induced increase in the level of P450 enzymes which are involved in the activation of various known chemical carcinogens was decreased by oral administration of TEO. Cytochrome P450 enzymes such as CYP1A2, CYP2B1/2, CYP1A1, CYP2A, CYP2D and CYP3A were significantly reduced after treated with TEO in a concentration dependent manner. GST can catalyze the conjugation of toxic and carcinogenic molecules with GSH and thus protect cellular damage. UDP glucuronyl transferase which are localized in the endoplasmic reticulum, are responsible for elimination of many endobiotics and xenobiotics by glucuronidation reaction. Glucuronidation means conversion of hydrophobic substrates into more polar ‘glucuronides’, which can be eliminated from the body by excretion (Desai et al., 2003). UDP glucuronyl transferase facilitates the excretion of potentially carcinogenic compounds and may reduce cancer risk (Saracino et al., 2007). Present study in rats indicated that the oral administration of TEO can increase the liver Phase II enzymes (GST and UDP glucuronyl transferase) levels.

Dose dependent inhibition (P<0.001) in the levels of hepatic CYP450 enzymes were observed after oral administration of TEO. CYP2E1 is the main enzyme present in liver which is involved in the conversion of NDEA into active mutagens. Moreover, CYP3A and CYP2B1/B2 enzymes are also known to be good catalysts of NDEA metabolism (Aiub et al., 2011). NDEA increases the liver mRNA expression of CYP2B2, CYP2E1, CYP2B1, CYP4A3 and CYP3A1 to several folds (Funae et al., 1993). The uncoupling of electron transfer and oxygen reduction by these cytochrome P450 enzymes could result in the release of O2 and H2O2, and consequently an increase in the metabolites which can induce DNA damage in both hepatic cells and non-parenchymal cells, which leads to HCC. (Zhang et al., 2012 and Aiub et al., 2011). Significant inhibition in the levels of hepatic MROD (CYP1A2), EROD (CYP1A1) and PROD (CYP2B1/2) enzymes were observed in a dose dependent manner after oral administration of TEO. Moreover, the activity of aniline hydroxylase, indicator of CYP2E1 was significantly reduced by TEO. These results suggest that TEO can restore
the phenobarbitone induced levels of cytochrome P450 enzymes significantly and reduce hepatocarcinoma in rats.

Detoxification and elimination of DMBA are done by phase I and phase II enzymes. Inhibition of cytochrome p450 enzymes (Phase I enzymes) and promotion of phase II enzymes in liver would inhibit the metabolic activation of DMBA and prevent cancer formation. These results suggest the role of TEO as chemopreventive and anticancer agent. 3-MC elicits CYP1A1 and 1A2 expression by sustained transcriptional activation of the corresponding promoters (Fazili et al., 2010). Cytochrome P450 1A1 and 1B1 enzyme present in liver plays a main role in the metabolic activation of polycyclic aromatic hydrocarbons like 3-MC which leads to the formation of highly reactive intermediates that can bind with DNA and forming adducts, which may be the reason for the sarcoma development in mice (Thorgeirsson et al., 1977). Our study revealed TEO significantly inhibited the 3-MC metabolizing cytochrome enzymes CYP1A1 and significantly reduces sarcoma development.

It can be concluded that oral administration of TEO significantly reduced the NDEA induced tumor nodule formation in the liver and attenuated the levels of hepatic marker enzymes and GGT. Moreover TEO could protect, NDEA induced oxidative stress by restoration of antioxidant enzyme levels. Moreover TEO significantly delayed and prevented the skin papilloma development in DMBA and croton oil treated mice. 3-Methylcholanthrene induced sarcoma development was also delayed and there was significant increase in the life span of mice after oral administration of turmeric essential oil. We also conclude that amelioration of NDEA, DMBA and 3-MC induced carcinogenesis by TEO could be due to its antioxidant activity and inhibition of metabolic activation of NDEA by inhibiting phase I cytochrome P450 enzymes as well as enhancing the elimination and detoxification of the mutagenic substance in liver by inducing phase II enzymes such as UDP-glucuronyl transferase and GST.