Chapter- 9

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9.4. Discussions
9.1. Introduction

Aflatoxins are one of the most potent toxic substances that occur naturally, which is produced by the fungi Aspergillus flavus and A. parasiticus. Aflatoxin poisoning in human is reported from all parts of the world and almost all domestic and nondomestic animals. Diet is the major way through which humans as well as animals are exposed to aflatoxins. They are stable in food and resistant to degradation under normal cooking procedures. Aflatoxins are detected occasionally in milk, cheese, corn, peanuts, cottonseed, nuts, almonds, figs, and a variety of other foods and feeds. Epidemiological, clinical, and experimental studies reveal that exposure to large doses of aflatoxin may cause acute toxicity with lethal effect, whereas exposure to small doses for prolonged periods is carcinogenic. Aflatoxin B1, the most toxic of the aflatoxins, is the most potent naturally occurring chemical liver carcinogen known (Bumrela et al., 2012) and major cause for the high prevalence of hepatocellular carcinoma in Asia and Africa (Jeena et al., 1999). It has been estimated that more than 5 billion people in developing countries worldwide are at risk of chronic exposure to aflatoxins through contaminated foods. In addition to liver cancer, aflatoxin has also been linked to stunted growth in children and immune system disorders.

Aflatoxins, which are well-known to be potentially mutagenic, hepatocarcinogenic, teratogenic, hepatotoxic, genotoxic and immunosuppressive, also inhibit several metabolic systems (Towner et al., 2003 and Thusu et al., 1991). The carcinogenic mechanism of aflatoxin B1 has been extensively studied. It has been shown that aflatoxin B1 is metabolically activated by hepatic cytochrome P450 enzymes to produce a reactive intermediate, aflatoxin B1-8,9-epoxide, which consequently binds to nucleophilic sites in DNA and the major adduct 8,9-dihydro-8-(N7 guanyl)- 9-hydroxy aflatoxin B1is formed (Koss et al., 1982, Hogberg et al., 1974 and Schamhart et al., 1979). The formation of aflatoxin-DNA adducts is considered as a key step in the initiation of aflatoxin induced hepatocarcinogenesis (Baggetto, 1992).

Essential oils which are present in the spices possess many significant biological activities. Spices like turmeric, ginger and black pepper were traditionally used as food additives. These spices have a significant role in system of alternative therapy and are
used in the preparation of Ayurveda, Siddha, Unani and Chinese medicines. Recently, natural foods and food derived antioxidants and phenolic phytochemicals in essential oils have received growing attention, because they are known to function as chemopreventive agents against oxidative damage and genotoxicity.

The rhizome of *Curcuma longa* L. belonging to the family Zingiberaceae is extensively and traditionally used in many Asian countries to treat a myriad of diseases and ailments as well as to enhance the food quality. The turmeric essential oil (TEO) is prepared from the rhizome of turmeric by steam distillation and the major constituent is reported to be ar-turmerone (Roy, 1968). Some of the medicinal and pharmacological properties such as antifungal, insect repellent, anti-bacterial, anti-platelet, neuroprotective activity, anti-arthritis and anti-mutagenic activities of TEO has been reported (Roy, 1968, Sweeney et al., 1998, Soni et al., 1992 and Soni, 1997). Our previous work on turmeric essential oil has shown that TEO possess *in vitro* and *in vivo* antioxidant, anti-inflammatory and antinociceptive activity. Studies have also shown that turmeric essential oil acts as a chemopreventive agent in human oral submucous fibrosis and also act against DNA damage *in vitro* in oral mucosal cells (Deepa et al., 2010 and Hastak et al., 1997). The mechanism of action of TEO was found mainly due to its antioxidant potential. In the present chapter we describe the role of turmeric essential oil on aflatoxin B1 production and aflatoxin B1 induced toxicity, mutagenicity and hepato carcinogenesis.

9.2. Materials and Methods

9.2.1. Animals

Wistar rats (Average weight 130 g) and 1 day old ducklings were purchased from the Small Animal Breeding Station, Kerala Veterinary and animal Sciences University, Mannuthy, Thrissur, Kerala, India (Chapter 2, section 2.1.5).

9.2.2. *Aspergillus flavus* (MTCC 2799)

*Aspergillus flavus* was supplied by Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chadigarh. (Chapter 2, section 2.1.4)
9.2.3. *Salmonella typhimurium* strains

Auxotrophic *Salmonella* strains, TA-98 and TA-100 were kindly supplied by Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chadigarh, India and were used for antimutagenic studies. (Chapter 2, section 2.1.3)

9.2.4. Effect of TEO on the growth of *Aspergillus flavus*

*Aspergillus flavus* was usually grown on Sabouraud's dextrose agar. After seven days of growth at 37°C the cultures were uniformly suspended in glucose (5%) ammonium nitrate (0.24%) medium (5 ml). A 100 µl quantity of the suspension was inoculated into 2 ml of glucose ammonium nitrate medium containing zinc sulphate (26 mg/l) and cobalt nitrate (2.6 mg/l) as mineral supplements (Brain *et al*., 1961). Various concentrations of TEO (20µl, 10µl, 5µl, 2µl and 1µl) were added to the above mineral solution and incubated for 6 days. After 6 days incubation, the mycelium was taken from each tube and dried in the oven. The dry weight of mycelium, which corresponds to the growth of the fungus, was determined separately and the difference in the weights from that of control (without TEO) was recorded. The percentage of inhibition of fungal growth was calculated using the equation:

\[
\% \text{ inhibition} = \left( \frac{C - T}{C} \right) \times 100
\]

C is the dry weight of mycelium in control tubes (without TEO).

T is the dry weight of mycelium in treated samples (in the presence of various concentrations of TEO).

9.2.5. Effect of TEO on aflatoxin production by *Aspergillus flavus*

*Aspergillus flavus* cultures originally grown on Sabouraud’s Dextrose Agar were uniformly suspended in 5ml of sterile glucose ammonium nitrate medium. This suspension (100µl) was inoculated into a test tube containing 2ml of sterile glucose ammonium nitrate medium and different concentrations of TEO (20µl, 10µl, 5µl, 2µl and 1µl). Tubes were incubated for 6 days at 37°C and aflatoxin was extracted from mycelium using modified Pon’s method (Rati *et al*., 1987).
9.2.5.1. Extraction of aflatoxin

The aflatoxin was extracted using modified Pon’s method. The mycelium of *Aspergillus flavus* was crushed in the same medium and a uniform suspension was made. This suspension (2 ml) was mixed with 11 ml of acetone and vortexed for 15 minutes. The mixture was filtered using Whatman filter paper and 2 ml of the filtrate was mixed with equal volume of water, followed by extraction with chloroform twice, (2 ml each). Chloroform was evaporated to dryness and the extract was redissolved in 1 ml of chloroform.

9.2.5.2. Estimation of aflatoxin

The quantification of aflatoxin in the TEO treated and control samples were estimated using fluorospectrometer (ND-3300). The total amount of aflatoxin production by *Aspergillus flavus* in the medium was calculated by comparing with the standard. All the experiments were repeated thrice and average values were taken. The percentage of inhibition of aflatoxin production by TEO was calculated by comparing with control using the equation:

\[
\text{% inhibition} = \left( \frac{C - T}{C} \right) \times 100
\]

C is the amount of aflatoxin produced in control tubes (without TEO treated).
T is the amount of aflatoxin produced in TEO treated samples

9.2.5.3. Detection of aflatoxin in test samples using TLC (Thin Layer Chromatography)

Aflatoxin standard mixture solution, control and TEO treated sample solutions were spotted on imaginary line 1 cm from the bottom edge of TLC plate. The plate was then placed in a chromatographic chamber containing a mixture of acetone-chloroform (5:95) for 10 minutes. TLC plate was then removed from the chamber and allowed to dry at room temperature, and illuminated from above by placing its flat, coated side up, using a long wave ultraviolet cabinet. The fluorescent spots were observed.
9.2.6. Evaluation of antimutagenic potential of TEO against aflatoxin B1 after metabolic activation by S9 mixture

Rat liver microsomal enzyme was used for metabolic activation of mutagen in vitro (Jayaprakash et al., 2002). Microsome P450 enzymes was induced in rat liver by oral administration of 0.1 % phenobarbital dissolved in water for 4 days. The animals were sacrificed on the 5th day (Garner et al., 1972). Livers were excised aseptically and microsomal S9 fraction was prepared by centrifuging the homogenate at 9000 g for 15 min. Activation mixture was prepared by mixing S9 mix (500 μl) with sodium phosphate buffer (0.2M, pH 7.4), NADP (0.1 M), glucose 6 phosphate (1 M, pH 7.4), MgCl2–KCl (10 μl) in presence of mutagen, aflatoxin B1 (1 μg/plate) or different concentrations of TEO (500, 250, 100 and 50 μg), and bacterial strains TA 98 and TA 100 and incubated at 37°C for 45 min (Ames et al., 1973). This mixture was then added to 2 ml of melted top agar and 0.2 ml of 0.5 mM histidin/biotin solution gently mixed and overlaid onto the minimal glucose agar plates. After solidification, the plates were inverted and incubated for 48 h at 37°C. The number of revertant colonies were counted using colony counter. All the plates were prepared in triplicate. The percentage inhibition of mutagenicity was calculated using the formula:

\[
\text{Percentage inhibition} = \frac{[(C-SR) - (T-SR)]}{(C-SR)} \times 100
\]

where ‘C’ is the number of revertants in the presence of mutagen alone, ‘T’ is the number of revertants in the presence of TEO with mutagens, ‘SR’ is the spontaneous revertants.

9.2.7. Effect of TEO on aflatoxin induced toxicity in ducklings

Seven day old ducklings (10 nos/group) were used for the experiment. Ducklings were divided into three groups as follows,

- **Group I**: Untreated
- **Group II**: Aflatoxin B1 alone
- **Group III**: Aflatoxin B1 + TEO (500 mg/kg b. wt.)

Aflatoxin B1 was dissolved in acetone and 0.2 ml of the solution containing 5μg of aflatoxin was mixed with hot duckling feed (boiled wheat in milk), so that the solvent was evaporated immediately. Each animal received aflatoxin B1 at a concentration of 100
µg/day for 2 weeks. 500 mg/kg body weight TEO was mixed with the feed containing aflatoxin and given for the entire period of experiments. After two weeks all the ducklings were sacrificed, blood collected by heart puncture method. Liver was removed and frozen immediately (Soni et al., 1992).

9.2.7.1. **Body weight and relative organ weight of ducklings**

The weight of each duckling was recorded on the first day and at 3 days intervals throughout the course of the study and mean body weights were calculated. The weight of the brain, liver, intestine, kidney, thymus, pancreas, bursa of fabricius and spleen were recorded and expressed in relation to the final body weight.

9.2.7.2. **Hematological and biochemical analysis**

Blood was analyzed for haematological parameters such as haemoglobin, RBC, platelet, total WBC and differential count. Indicators of hepatic function such as AST, ALT, ALP (Mc Comb et al., 1979), renal function markers and GGT (Tate and Meister, 1974) in serum were analyzed.

9.2.7.3. **Histopathological analysis of organs**

A portion of the selected organs (brain, liver, intestine, kidney, thymus, pancreas, bursa of fabricius and spleen) of control and treated group was fixed in 10% neutral buffered formalin (Chapter 2, Section 2.2.6).

9.2.8. **Effect of TEO against aflatoxin B1 induced liver carcinogenesis.**

Male Wistar rats, weighing approximately 100g were used in the study (Soni et al., 1997). The animals were divided into different groups (10 nos/group) as follows.

<table>
<thead>
<tr>
<th>Group I</th>
<th>Untreated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group II</td>
<td>Aflatoxin B1 alone (Control)</td>
</tr>
<tr>
<td>Group III</td>
<td>Aflatoxin B1 + paraffin oil (Vehicle control)</td>
</tr>
<tr>
<td>Group IV</td>
<td>Aflatoxin B1 + 250 mg/kg b. wt. TEO</td>
</tr>
<tr>
<td>Group V</td>
<td>Aflatoxin B1 + 500 mg/kg b. wt. TEO</td>
</tr>
</tbody>
</table>
TEO was administered to the rats at different doses in 1 ml paraffin oil. Dosage required was prepared fresh each time. Animals group II-V received aflatoxin B1 by oral administration thrice weekly for 20 weeks in single doses of 25 µg/kg body weight (total dose of 1.5 mg/kg body weight). The amount of DMSO received per dose of aflatoxin was 0.5 µl. The TEO was given, starting one week prior to the carcinogen exposure (20 weeks) and for two weeks thereafter. After this period all animals were maintained in the basal diet. All the animals were sacrificed 32nd week to quantify the hepatocellular carcinoma induced by aflatoxin. Liver was excised and washed with ice cold saline. A small piece of liver was kept in 10% formalin for histopathological analysis.

9.2.8.1. Morphology and weight of the liver

Liver from each animal were 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.

9.2.8.2. 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 (1974). Total bilirubin was determined by Jendrassik-Diazotized sulphathic acid method (1938). Alkaline phosphatase (ALP) was estimated by p-nitrophenyl phosphate (PNPP) hydrolysis (Mc Comb et al., 1979) and alanine amino transferase (ALT) as well as aspartate aminotransferase (AST) by kinetic method using commercially available kits (Span Diagnostics, India).

9.2.8.3. 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 H₂O₂ in the presence of GSH (Hafman 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) (Habig et al., 1974). Lipid peroxidation (malondialdehyde) was done by the method of Ohkawa et al., (1979). Catalase activity in tissue was determined according to the method of Beer and Sizer (1952).

9.2.8.4. Mitochondrial and respiratory marker enzymes

9.2.8.4.1. Preparation of mitochondrial fraction

Immediately after sacrifice, liver for analysis of mitochondrial enzymes and glycolytic enzymes was removed and all the blood vessels and connective tissues were trimmed off. Wash the liver tissue well and cut in to small fragments and homogenize in buffer containing 0.25 M sucrose and 1 mM EDTA. Centrifuge the suspension in a refrigerated centrifuge (Susin, 2000).

The homogenate was centrifuged at 1000g for 10 min: the supernatant was transferred in to test tubes. The pellet was dissolved in sucrose buffer and centrifuged for 10 min at 1000g. The supernatants were pooled and centrifuged for 10 min at 10000g, the pellet collected represents mitochondrial fraction. Each fraction was resuspended in sucrose and the washings combined with the supernatants. This has the advantage of producing purer fractions. Carefully the mitochondrial pellet was resuspended in about 2ml of sucrose as the enzyme source and store on ice until required.

9.2.8.4.2. Effect of TEO on mitochondrial marker enzymes (TCA cycle enzymes)

9.2.8.4.2.1. Determination of activity of the Isocitrate dehydrogenase (ICDH) activity

Principle

ICDH activity was determined from the rate of reduction of NAD⁺ in the presence of trisodium isocitrate at 340 nm.

Reagents

\[
\begin{align*}
\text{Trisodium citrate} & : 100 \text{ mM/L} \\
\text{MnCl}_2 & : 15 \text{ mM/L}
\end{align*}
\]
NAD : 100 mM/L

Procedure
One ml of the reaction mixture containing 100 millimol/l trisodium isocitrate, 15 millimol/MnCl₂, 100 millimol/l NAD, mitochondrial protein (approximately 40 µg) and 100 mmol/l Tris-HCl (pH 7.5) was monitored at 340 nm for 2 min with an interval of 30 sec after the addition of NAD. The activity was expressed as µmols of NAD reduced/min/mg protein using extinction coefficient 6.3 mM⁻¹cm⁻¹.

\[
\text{ICDH} = \frac{\Delta A/\text{Min.} \times 1000}{2.2 \times \text{mg protein}}
\]

9.2.8.4.2.2. Determination of succinate dehydrogenase (SDH) activity
SDH activity was estimated by the method of Nulton-Persson and Szweda (2001).

Reagents
- Succinate dehydrogenase : 10 mM/L
- BSA in 1% KCN : 3%
- KCN : 0.9 mM/L
- 2,6-dichlorophenol indophenol : 80 µmol/L
- Sodium phosphate buffer (pH 7.4) : 0.1M

Principle
The activity was determined from the rate of decrease in absorbance at 600nm after treating the mitochondria with the reaction mixture containing sodium succinate in the presence of electron acceptor DCPIP (2,6-dichlorophenol indophenol) which is converted to its reduced form.

Procedure
The reaction mixture contained 10 mM sodium succinate, 0.5 mg BSA, 0.9 millimol/l KCN, mitochondrial protein (approximately 20 µg), 80 µM DCPIP (2, 6-dichlorophenolindophenol) and 100 mM phosphate buffer (pH 7.4) in a final volume of 1 ml. The reaction was monitored after the addition of DCPIP at 600 nm for 2 min at 30
sec interval. The activity was calculated using the extinction coefficient of DCPIP (19.1 mM$^{-1}$ cm$^{-1}$) and expressed as micromoles of DCPIP reduced/min/mg protein.

\[
\text{SDH} = \frac{\Delta A/\text{min} \times 1000 \times 1}{19.1 \times \text{mg protein}}
\]

9.2.8.4.2.3. *Determination of malate dehydrogenase (MDH) activity*

MDH activity was estimated by the method of Mehler *et al.*, (1948).

**Reagents**

- Tris HCl : 0.25M, pH 7.4
- NADH : 0.015M
- Oxaloacetate : 0.0076M, pH 7.4

**Principle**

The activity was determined from the rate of decrease in absorbance at 340 nm after treating the mitochondria with the reaction mixture containing oxaloacetate and NADH and the activity was calculated from the rate of oxidation of NADH.

**Procedure**

The reaction mixture contained 1.5 millimol/l NADH, 7.6 millimol/l oxaloacetate, approximately 40 µg of mitochondrial protein, Tris HCl buffer (0.1 M, pH 7.5) and distilled water in a final volume of 3 ml. The reaction was started with the addition of NADH and was monitored at 340 nm for 2 min at 30 sec interval. The activity was expressed as µmoles of NADH oxidized/min/mg protein using the extinction coefficient of NADH 6.3 mM$^{-1}$ cm$^{-1}$.

9.2.8.4.2.4. *Determination of NADH dehydrogenase activity*

The activity of NADH dehydrogenase was assayed according to the method of Minakami *et al.*, (1962).

**Reagents**

- Phosphate buffer : 0.1 M, pH 7.4
- 2 NADH : 0.1%
Potassium ferricyanide : 0.03 M solution

Procedure
The reaction mixture contained 1ml of phosphate buffer, 0.1 ml of potassium ferricyanide, 0.1 ml of NADH and 1.6 ml of distilled water in a total volume of 3 ml. The temperature was brought to 30°C and NADH was added just before the addition of the sample. A suitable aliquot of mitochondrial solution was added and change in absorbance was measured at 420 nm as function of time 3 min at intervals of 15 seconds in a Shimadzu-UV spectrophotometer. A control containing all the reagents except NADH was also treated similarly. The activity of NADH-dehydrogenase is expressed as per µ moles of NADH oxidized per minute per mg of protein.

\[
\text{NADH dehydrogenase} = \frac{\Delta \text{A/min.} \times 1000 \times 3}{6.3 \times \text{mg protein}}
\]

9.2.8.4.3. Glycolytic enzymes

9.2.8.4.3.1. Assay of hexokinase activity
Hexokinase was assayed by the method of Brandstrup et al., (1957)

Reagents
- Glucose solution : 0.005M
- ATP : 0.72M
- MgCl₂ : 0.05M
- H₂PO₄ : 0.0125M
- KCL : 0.1M
- Sodium fluride : 0.5M
- Tris HCl buffer (pH 8) : 0.01M

Principle
Hexokinase converts glucose to glucose-6-phosphate in the presence of ATP. The residual glucose in the supernatant was estimated by the glucose oxidase method.
**Procedure**

The reaction mixture in a total volume of 5.0 ml contained the following: 1.0 ml of glucose solution, 0.5 ml ATP solution, 0.1 ml of Magnesium chloride, 0.4 ml of dipotassium hydrogen phosphate solution, 0.4 ml of potassium chloride, 0.1 ml of sodium fluoride solution and 2.5 ml of tris HCl buffer (pH 8.0). The mixture was preincubated at 37°C for 5 min. The reaction was initiated by the addition of 2.0 ml of the tissue homogenate. 1.0 ml aliquot of the reaction mixture was taken immediately (zero time) to tubes containing 1.0 ml of 10% TCA. A second aliquot was removed after 30 min of incubation at 37°C. The precipitated protein was removed by centrifugation and the residual glucose in the supernatant was estimated by the o-toluidine method of Sasaki and Matsui (1972). As described previously. A reagent blank was run with each test. The difference between the two values gives the amount of glucose phosphorylated. The enzyme activity is expressed as n moles of glucose-6-phosphate formed/min/mg protein.

9.2.8.4.3.2. **Assay of glucose-6-phosphate dehydrogenase activity**

Glucose-6-phosphate dehydrogenase activity was determined by the method of Ells and Krikman (1961).

**Reagents**

- Tris-HCl buffer : 0.05M, pH 7.5
- Magnesium chloride : 1.0 M
- NADP : 1.0 M
- Phenazine methosulphate (PMS) : 0.005%
- 2,6-dichloro phenol indophenols (DCPI) : 0.01%
- Glucose-6-phosphate : 0.02 M

**Principle**

The blue dye, dichlorophenol indophenol reduces in the presence of NADPH to the colorless form. It is estimated in the presence of phenazine methosulfate (PMS). The rate at which color visually disappear in the reaction mixture is proportional to the glucose-6-phosphatedehydrogenase content of sample and is expressed as units/mg protein.
Procedure
The incubation mixture in a total volume of 5.5 ml contained the following: 1.0 ml of Tris-HCl buffer, 0.5 ml of PMS, 0.4 ml of DCPI solution, 0.1 ml of magnesium chloride, 0.1 ml of NADP and a indispensable amount of enzyme preparation. The mixture was allowed to stand at room temperature for 10 minutes to permit the oxidation of endogenous materials. The reaction was initiated by the addition of 0.5 ml of glucose-6-phosphate. The optical density was read at 640 nm against a water blank in a UV spectrophotometer. The enzyme activity is expressed as units/mg protein.

9.2.8.4.4. Gluconeogenic enzymes
9.2.9.4.4.1. Glucose-6-phosphatase activity
Glucose-6-phosphatase was assayed according to the method of King (1965).

Reagents

<table>
<thead>
<tr>
<th>buffer</th>
<th>: 0.1 M, pH 6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>: Glucose-6-phosphate, 0.1M</td>
</tr>
<tr>
<td>TCA</td>
<td>: 10%</td>
</tr>
</tbody>
</table>

Principle
Glucose-6-phosphate is dephosphorylated to free glucose and inorganic phosphate in the presence of glucose-6-phosphatase.

Procedure
The incubation mixture in a total volume of 1ml contained 0.3 ml of buffer, 0.5 ml of substrate and 0.2 ml of the enzyme. Incubation was carried out at 37°C for 60 min. The reactions arrested by the addition of 1 ml of TCA and centrifuged. The enzyme activity is expressed as µM of Pi liberated/min/mg protein.

9.2.9.4.4.2. Fructose-1, 6-diphosphatase activity
Fructose-1, 6-diphosphatase was assayed by method of Gancedo and Gancedo (1971).

Reagents

<table>
<thead>
<tr>
<th>buffer</th>
<th>: 0.1 M , pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>: Fructose-1, 6-diphosphate 0.05 M</td>
</tr>
</tbody>
</table>
MgCl$_2$: 0.1 M  
KCl: 0.1 M  
EDTA: 0.001 M  
TCA: 10%  
Ammonium molybdate, ANSA

**Principle**  
Fructose-1,6-bis phosphate converted to fructose-6-phosphate and inorganic phosphate in the presence of to fructfructose1,6- bisphosphatase.

**Procedure**  
The assay medium in a final volume of 2 ml contained 1.2 ml of buffer 0.1 ml of substrate 0.25 ml of magnesium chloride, 0.1 ml of potassium chloride, 0.25 ml of EDTA and 0.1 ml of tissue homogenate. The incubation was carried out at 37°C for 15 minutes. The reaction was terminated by addition of 1 ml of 10% TCA. The suspension was centrifuged and the phosphorous content of the supernatant was estimated. The enzyme activity is expressed as nM of Pi liberated/min/mg protein.

9.2.8.5. **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).

9.2.9. **Effect of TEO on aflatoxin B1 metabolism and adduct formation in liver.**

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

- **Group I**: Untreated
- **Group II**: Aflatoxin 250 µg/kg b. wt. i.p./3day/week (Control)
- **Group III**: Aflatoxin 250 µg/kg + paraffin oil (vehicle control),
- **Group IV**: Aflatoxin 250 µg/kg + TEO 100 mg/kg b. wt.
- **Group V**: Aflatoxin 250 µg/kg + TEO 500 mg/kg b. wt.
Different doses of TEO were administered once daily for 21 days orally, and AFB1 (250 µg/kg b. wt., i.p. 3 day/week). The rats were sacrificed 24 h after the last dose of AFB1. The livers of all the animals were excised quickly and washed thoroughly in ice-cold saline and kept at -80°C. 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).

9.2.9.1. Estimation of Cytochrome p450 enzymes

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-ethylation (EROD), CYP1A1 (Nerurkar et al., 1993) were done (Chapter 7, Section 7.2.6.4).

9.2.9.2. Protein carbonyl assay

Protein carbonyls content will be determined as described by Mercier et al (2004) in liver homogenates (25%, 0.1M phosphate buffer, pH 7.4) by measuring the reactivity of carbonyl groups with 2,4- dinitrophenylhydrazine (2,4-DNPH). Thus, 100 µl of supernatant of liver will be placed in glass tubes. 800 µl of 10 mM DNPH in 2.5 M HCl were added. Tubes were left for 1 h of incubation at room temperature in the dark. Samples will be vortexed every 15 min. Then 1 ml of 20% TCA will be added to samples, and the tubes were left in ice bucket for 10 min and centrifuged for 5 min at 4000 rpm to collect the protein precipitates and the supernatants will be discarded. Next, another wash is performed using 1 ml of 10% TCA, and protein pellets are broken mechanically with the aid of glass rod. Finally, the pellets are washed with 1 ml of ethanol-ethyl acetate (1:1, v/v) to remove the free DNPH. The final precipitates are dissolved in 500 µl of guanidine hydrochloride 6 M and are left for 10 min at 37°C with general vortex mixing. Any insoluble materials are removed by additional centrifugation. Protein carbonyls concentration will be determined from the absorbance at 370 nm. The
amount of protein carbonyl was determined from the mM extinction coefficient value 6.364.

9.2.9.3. Estimation of DNA adduct in liver tissue

The AFB1-DNA adducts formation will be carried out by using aflatoxin DNA adduct competitive ELISA kit (CELL BIOLABS, INC. Catalog Number: AKR-351). Liver tissue will be incubated on 37°C for 1h with 700 µl lysis buffer (.2% Triton X-100, 10 mM Tris HCL, pH 7.4, 10 mM EDTA), 10 µl RNase (10 mg/ml), and 10 µl proteinase K (20 mg/ml). After incubation 700 µl of Phenol/chloroform/isomylalcohol (25:24:1) will be added and mix well and centrifuge at 13000 RPM at 4°C for 10 min (repeat this step 2 times). After centrifugation aqueous phase will be collected and add equal volume of chloroform to the sample and briefly mix. Centrifuge at 13000 RPM at 4°C for 10 min (repeat this step 2 times). Remove the top aqueous layer and place in a new tube. Precipitate the DNA by adding 1/10 volume of sodium acetate (3M) and double the volume of ice cold 100% ethanol. After keeping one day at incubation at -70°C, centrifuge the sample at 13000 RPM for 30 min at 4°C remove supernatant. Wash the sample with 1 ml 70 % ethanol and centrifuge at 13000 RPM for 10 min. Remove the ethanol completely and air dry the pellet for couple of minute to drain off ethanol. DNA precipitate will be dissolved in TE buffer (Tris-EDTA buffer). The DNA from the cell samples will be quantitated at 260 and 280 nm using a nano-spectrophotometer to ensure sufficient DNA will be present for quantitation of AFB1-N7-guanine adducts.

Cell Biolabs’ aflatoxin DNA adduct competitive ELISA kit provides a convenient method for the detection of total aflatoxin B1-DNA adducts (ring-opened and ring closed forms). First, the TEO treated AFB1-DNA samples or AFB1-DNA standards are added to the AFB1-DNA conjugate preabsorbed ELISA plate. After a brief incubation, an anti-AFB1-DNA antibody is added, and followed by an HRP conjugated secondary antibody. The total content of AFB1 DNA in unknown samples is determined by comparison with a predetermined AFB1-DNA standard curve.

9.2.10. Statistical analysis

(Chapter 2, section 2.2.11.)
9.3. Results

9.3.1. Effect of TEO on the growth of *Aspergillus flavus*

The effect of TEO on the growth of *Aspergillus flavus* was shown in Figure 1. Dry weight of mycelium was taken as a measure of fungal growth. The results showed that TEO significantly inhibited the mycelia growth of *Aspergillus flavus*. Maximum inhibition of mycelia growth was observed at 20µl TEO (Table 9.1). The increasing concentration of TEO leads to significant decrease of fungal growth (Fig. 9.1).

9.3.2. Effect of TEO on aflatoxin production by *Aspergillus flavus*

The effect of TEO on aflatoxin production by *A. flavus* is shown in Table 9.2. The level of aflatoxin production without TEO in the medium under the conditions was 16.02 ± 2.8 µg/ml. TEO (20µl/ml) inhibited the aflatoxin production by 95.19% (0.77 ± 0.19 µg/ml). The concentration of the TEO needed for 50% inhibition of aflatoxin production was found to be approximately 0.7 µg/ml. The result indicated that TEO showed a significant inhibition of aflatoxin production by *Aspergillus flavus* at all concentrations tested in a concentration dependent manner.

9.3.3. Detection of aflatoxin in test samples using thin layer chromatography (TLC)

TLC analysis of aflatoxin showed a blue fluorescing spot under long wavelength UV. The blue fluorescing spots indicated the presence of different types aflatoxin in test samples (Fig.9.2). Results revealed that there was a significant decrease in the fluorescence of treated sample compared with the control. It indicates that TEO significantly inhibiting the aflatoxin production by *Aspergillus flavus*.

9.3.4. Evaluation of antimutagenic potential of TEO against aflatoxin B1 after metabolic activation by S9 mixture

Antimutagenic activity of TEO against the aflatoxin B1 was evaluated using *S. typhimurium* TA 98 and TA 100. TEO significantly (P<0.001) inhibited the mutagenicity induced by aflatoxin in a concentration dependent manner for both strains. TEO inhibited
aflatoxin B1 induced mutagenicity by 89.4% (TA98) and 90.2% (TA100) at a concentration of 500 µg/plate, (Table 9.3).

9.3.5. Effect of TEO on aflatoxin induced toxicity in ducklings

9.3.5.1. Body weight and relative organ weight of ducklings

Administration of aflatoxin for 14 days decreased the body weight of ducklings when compared to the untreated group (Figure 9.3). But TEO showed significant increase in body weight when compared with the aflatoxin alone group. There were no morphological changes in the organ in any of the treated groups. Organ weight (liver, pancreas and thymus) was decreased by aflatoxin administration when compared with untreated ducklings. TEO (500 mg/kg body weight) treated groups significantly restored aflatoxin induced altered organ weight and body weight in ducklings (Fig. 9.4).

9.3.5.2. Haematological analysis of duckling’s blood after oral administration of aflatoxin and TEO.

The levels of HB, platelet, RBC and total count were decreased in serum of aflatoxin alone groups when compared with untreated groups (Table 9.4). Gamma-glutamyl-transferase (GGT) which is a marker of cell proliferation in the liver and biliary obstructions was significantly increased by 14 days aflatoxin administration in ducklings (Fig. 9.5). TEO treated group (500 mg/kg body weight) significantly (p<0.001) restored all the haematological parameters and GGT levels.

9.3.5.3. The effect of TEO on hepatic and renal function parameters in serum of aflatoxin B1 induced toxicity in ducklings

The levels of urea, creatinine, AST, ALT and ALP were increased significantly in serum of aflatoxin alone treated animals compared with untreated group. TEO treated group significantly normalize these hepatic and renal parameters (Table 9.5).
9.3.5.4. Histopathology analysis

Histopathological analysis of brain tissue shows minimal edema in some area. Thymus tissues shows areas of necrosis and a few keratin pearls. Liver shows loss of normal architecture and portal area show dilated vascular spaces. Hepatocytes appear larger, pleomorphic, and cytoplasmic vacuolation. There are some areas showing necrosis and sinusoidal spaces appear congested. Spleen shows congested blood vessels, areas of haemorrhage, sinusoidal congestion, lymphastasis and proliferation. Kidney section shows less damage with narrow Bowman’s and interstitial tissue appears haemorrhagic in some places. In the case of pancreas Islets cells show hyperplasia and there are groups of large oval cells containing granules in the cytoplasm. The small intestinal section shows some scattered lymphocytes and plasma cells in the mucosa and submucosa. Moreover the bursa of fabricius exhibited many areas of columnar epithelium show formation of papillary structures and mucin secretion. But TEO significantly restored almost normal architecture of brain, liver, kidney, intestine, thymus, bursa of fabricius and pancreas when compared with aflatoxin alone treated mice (Fig. 9.6).

9.3.6. Effect of TEO against aflatoxin B1 induced liver carcinogenesis.

The animals were not showing any abnormal changes during the aflatoxin induced hepatocarcinoma study and little liver weight changes were observed in any of the treated groups when compared with untreated animals. Morphology of the aflatoxin treated liver showed no nodule formation nevertheless light color changes and roughness were observed

9.3.6.1. Effect of TEO on biochemical analysis of serum

The levels of urea, creatinine, bilirubin, AST, ALT and ALP were increased significantly in serum of aflatoxin alone and vehicle control animals when compared with the untreated group. TEO treated groups (100 and 500 mg/kg body weight) significantly (p<0.001) normalize these hepatic parameters in a concentration dependent manner (Table 9.5). The serum levels of GGT in control and vehicle control groups were significantly (p ≤ 0.05) elevated by the administration of aflatoxin, when compared to
untreated group. The treatment with TEO (100 and 500 mg/kg) showed a dose depended decrease (p ≤ 0.05) in GGT levels (Table 9.6)

9.3.6.2. Effect of TEO on antioxidant enzymes in the liver

The activities of catalase, GST and GSH were significantly decreased in control and vehicle control groups when compared with untreated animals. In TEO treated group (100 and 500 mg/kg body weight), there was a significant increase in the activities of catalase, GST and GSH when compared with control and vehicle control groups in a concentration dependent manner. The value of GPx was decreased after aflatoxin B1 treatment and restored GPx activity after treatment with TEO. Different concentration of TEO (100 and 500 mg/kg body weight) significantly reduced aflatoxin induced lipid peroxidation level when compared with control group (Table 9.7).

9.3.6.3. Effect of TEO on mitochondrial marker enzymes (TCA cycle enzymes) on aflatoxin induced carcinogenesis in rats

There was significant decrease (P<0.01) in the levels of TCA cycle enzymes such as Isocitrate dehydrogenase, succinate dehydrogenase, NADH-dehydrogenase and malate dehydrogenase in control and vehicle control animals when compared to the untreated animals (Table 9.8). TEO administration significantly restored TCA cycle enzymes near to normal level.

9.3.6.4. Effect of TEO on glycolytic enzymes in aflatoxin induced carcinogenesis in rats

The activity of hexokinase and glucose-6-phosphate dehydrogenase enzymes in control and vehicle control group was increased when compared with untreated groups. TEO treated rats showed a significant decrease in the hexokinase and glucose6-phosphate dehydrogenase activity nearer to the normal level, when compared with control and vehicle control group (Table 9.9).
9.3.6.5. The effect of TEO on gluconeogenic enzymes in aflatoxin induced carcinogenesis in rats.

There was a decrease in the activity of gluconeogenic enzymes such as glucose-6-phosphatase and fructose 1,6-bisphosphatase in control and vehicle control groups when compared with the untreated group. In the case of different concentrations of TEO treated groups the levels of gluconeogenic enzymes were elevated when compared to aflatoxin induced rats and were approximately nearer to untreated groups in a concentration dependent manner (Table 9.10).

9.3.6.6. Histopathological analysis

Aflatoxin alone and vehicle control rat liver section shows tissue with complete effacement of normal architecture. Hepatocytes are pleomorphic large polyhedral cells, many of them are showing vacuolated cytoplasm and they have vesicular nuclei. Some areas show necrosis, haemorrhage and inflammatory cells infiltrate (Figure 9.7). But TEO treated section shows liver tissue with normal structure. Portal triads are with normal bile ductules. Hepatic vein appear normal. Hepatocytes also appear normal and they are arranged in cords. Kupffer's cells and sinusoidal spaces appear normal.

9.3.7 Effect of TEO on aflatoxin B1 metabolism and adduct formation in liver.

9.3.7.1. Estimation of Cytochrome p450 enzymes

Cytochrome P450 enzymes (Phase I enzymes) were found to be significantly increased following AFB1 administration. Significant inhibition (P<0.001) of Phase I enzymes was observed after TEO administration as seen from the hepatic MROD (CYPIA2), PROD (CYP2B1/2) and EROD (CYP1A1) levels (Table 9.11). Oral administration of TEO significantly inhibited (P<0.001) MROD levels in a concentration dependent manner and maximum inhibitory effect was seen in 500 mg/kg body weight (74.75%). TEO significantly decreased the level of EROD and PROD to 59.43 and
Table 9.1. Effect of turmeric essential oil (TEO) on growth of mycelium of *Aspergillus flavus*

<table>
<thead>
<tr>
<th>Concentrations of TEO</th>
<th>Dry weight of mycelium (µg)</th>
<th>Percentage inhibition of growth of mycelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>58.8 ± 2.61</td>
<td>-</td>
</tr>
<tr>
<td>1 µl</td>
<td>53.3 ± 2.40&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>9.35</td>
</tr>
<tr>
<td>2 µl</td>
<td>48.4 ± 3.41&lt;sup&gt;**&lt;/sup&gt;</td>
<td>17.69</td>
</tr>
<tr>
<td>5 µl</td>
<td>42.6 ± 2.82&lt;sup&gt;***&lt;/sup&gt;</td>
<td>27.55</td>
</tr>
<tr>
<td>10 µl</td>
<td>33.5 ± 2.12&lt;sup&gt;***&lt;/sup&gt;</td>
<td>42.03</td>
</tr>
<tr>
<td>20 µl</td>
<td>27.2 ± 1.41&lt;sup&gt;***&lt;/sup&gt;</td>
<td>53.74</td>
</tr>
</tbody>
</table>

Values are mean ± SD of triplicate.

<sup>ns</sup> P>0.05; <sup>**</sup> P<0.01; <sup>***</sup> P<0.001
Fig: 9.1. Effect of TEO on growth of mycelium and fruiting body development of

*Asperillus flavus*

A: *A. flavus* alone
B: *A. flavus*+1μl TEO
C: *A. flavus*+2μl TEO
D: *A. flavus*+5μl TEO
F: *A. flavus*+10μl TEO
F: *A. flavus*+20μl TEO.

Fig: 9.2 TLC analysis of aflatoxin detection after treated with TEO
Table 9.2. Effect of TEO on aflatoxin production by *Aspergillus flavus*.

<table>
<thead>
<tr>
<th>Concentration of TEO</th>
<th>Aflatoxin production (µg/ml)</th>
<th>Percentage inhibition of aflatoxin production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>16.02 ± 2.84</td>
<td>-</td>
</tr>
<tr>
<td>1 µl</td>
<td>3.75 ± 0.98***</td>
<td>76.56</td>
</tr>
<tr>
<td>2 µl</td>
<td>2.75 ± 0.35***</td>
<td>82.83</td>
</tr>
<tr>
<td>5 µl</td>
<td>2.00 ±0.33***</td>
<td>87.51</td>
</tr>
<tr>
<td>10 µl</td>
<td>1.50 ±0.40***</td>
<td>90.63</td>
</tr>
<tr>
<td>20 µl</td>
<td>0.77 ± 0.19***</td>
<td>95.19</td>
</tr>
</tbody>
</table>

Values are mean ± SD of triplicate.

ns P>0.05; *** P<0.001
Table 9.3. The effect of TEO against aflatoxin B1 induced mutagenicity on *Salmonella typhimurium* TA 98 and TA 100 strains.

<table>
<thead>
<tr>
<th></th>
<th>Aflatoxin-B1 alone</th>
<th>SR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TEO (50 µg/plate)</th>
<th>TEO (100 µg/plate)</th>
<th>TEO (250 µg/plate)</th>
<th>TEO (500 µg/plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. typhimurium TA 98</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of colonies</td>
<td>366 ± 15.4</td>
<td>56 ± 3.4</td>
<td>223 ± 9</td>
<td>195 ± 6</td>
<td>165 ± 9.9</td>
<td>89 ± 6.8</td>
</tr>
<tr>
<td>Percentage inhibition</td>
<td>-</td>
<td>-</td>
<td>46.3%</td>
<td>55.3%</td>
<td>64.95%</td>
<td>89.4%</td>
</tr>
<tr>
<td><strong>S. typhimurium TA 100</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of colonies</td>
<td>471 ± 15.63</td>
<td>155 ± 9.2</td>
<td>315 ± 11.4</td>
<td>287 ± 7.5</td>
<td>214 ± 8.99</td>
<td>185 ± 7.7</td>
</tr>
<tr>
<td>Percentage inhibition</td>
<td>-</td>
<td>-</td>
<td>49.2%</td>
<td>58%</td>
<td>81.4%</td>
<td>90.2%</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation of the 3 test.

*P<0.05; **P<0.01; ***P<0.001, <sup>a</sup>Spontaneous revertant
Fig: 9.3. Body weight of ducklings after treatment with aflatoxin B1 and TEO

Fig: 9.4. Relative organ weight of ducklings after oral administration of aflatoxin B1 and TEO
Table 9.4. Hematological parameters of ducklings treated with aflatoxin B1 and TEO

<table>
<thead>
<tr>
<th>Groups</th>
<th>HB (g/dl)</th>
<th>RBC ($\times 10^6$)</th>
<th>Platelet ($\times 10^6$)</th>
<th>WBC (mm$^3$)</th>
<th>Neutrophils (%)</th>
<th>Lymphocytes (%)</th>
<th>Eosinophils (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>13.1±0.3</td>
<td>3.1±0.2</td>
<td>830.10±127.26</td>
<td>8310±756</td>
<td>67.2±4.1</td>
<td>30.1±4.5</td>
<td>2.7±1.2</td>
</tr>
<tr>
<td>Aflatoxin B1</td>
<td>10.7±0.6</td>
<td>2.6±0.2</td>
<td>295.10±24.47</td>
<td>4060±207</td>
<td>58.7±4.3</td>
<td>38.7±4.5</td>
<td>2.6±1</td>
</tr>
<tr>
<td>Aflatoxin+TEO (500mg/kg b.wt)</td>
<td>13.7±0.3***</td>
<td>3.0±0.2***</td>
<td>788.10±86.73***</td>
<td>7280±507***</td>
<td>65.8±6.1</td>
<td>30.7.8±5.5</td>
<td>3.5±1.6</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation of 10 ducklings/group.

*** P<0.001
Table 9.5. Effect of TEO on Aflatoxin B1 induced hepatic and renal toxicity in ducklings

<table>
<thead>
<tr>
<th>Groups</th>
<th>Urea (mg/dL)</th>
<th>Creatinine (mg/dL)</th>
<th>Bilirubin (mg/dL)</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>ALP (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>11 ± 3.94</td>
<td>0.11 ± 0.03</td>
<td>0.37 ± 0.15</td>
<td>39.6 ± 15.2</td>
<td>35.3±6.7</td>
<td>972.9±45.38</td>
</tr>
<tr>
<td>Aflatoxin B1</td>
<td>32 ± 1.25</td>
<td>0.22 ± 0.12</td>
<td>0.4 ± 0.07</td>
<td>78.9 ± 7.64</td>
<td>53.3±10.7</td>
<td>1354.4±62.7</td>
</tr>
<tr>
<td>Aflatoxin B1+TEO (500mg/kg b.wt.)</td>
<td>14.5 ± 1.06***</td>
<td>0.13 ± 0.03***</td>
<td>0.38 ± 0.05</td>
<td>36.8 ± 9.04***</td>
<td>34.9 ± 5***</td>
<td>1182.2±63.72***</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation of the 10 ducklings/group.

*** P<0.001
Fig: 9.5. Gamma glutamyl transferase (GGT) analysis of aflatoxin induced toxicity in ducklings after oral administration of TEO
Fig: 9.6. Effect of TEO on aflatoxin B1 induced toxicity in ducklings- histopathological analysis of organs
Table 9.6. Effect of TEO on liver function parameters during aflatoxin B1 induced liver carcinogenesis in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Total bilirubin (mg/100ml)</th>
<th>ALT (IU/L)</th>
<th>AST (IU/L)</th>
<th>ALP (IU/L)</th>
<th>GGT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0.55 ± 0.14</td>
<td>36.86 ± 12.81</td>
<td>184.09 ± 6.14</td>
<td>55.37 ± 17.92</td>
<td>7.26 ± 2.77</td>
</tr>
<tr>
<td>Aflatoxin B1 alone (control)</td>
<td>1.36 ± 0.38</td>
<td>110.1 ± 16.19</td>
<td>220.68 ± 35.07</td>
<td>85.44 ± 6.58</td>
<td>21.37 ± 8.37</td>
</tr>
<tr>
<td>Aflatoxin B1+paraffin oil*</td>
<td>1.22 ± 0.40</td>
<td>117 ± 20.33</td>
<td>210.67 ± 26.37</td>
<td>95.54 ± 21.08</td>
<td>20.64 ± 7.11</td>
</tr>
<tr>
<td>TEO 100 mg/kg b.wt.</td>
<td>0.71 ± 0.49</td>
<td>59.6 ± 20.06***</td>
<td>170.74 ± 39.52*</td>
<td>62.95 ± 9.63***</td>
<td>8.21 ± 5.06***</td>
</tr>
<tr>
<td>TEO 500 mg/kg b.wt.</td>
<td>0.58 ± 0.25**</td>
<td>48.72 ± 11.71***</td>
<td>157.44 ± 67.37***</td>
<td>60.38 ± 11.58***</td>
<td>5.75 ± 3.35***</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation of the 10 rats/group.

*P<0.05; **P<0.01; ***P<0.001, * Vehicle control
Table 9.7. Effect of TEO on aflatoxin induced liver carcinogenesis in rats (In vivo antioxidant enzymes and GSH analysis in liver tissue)

<table>
<thead>
<tr>
<th>Group</th>
<th>GPx (U/mg protein)</th>
<th>GST (nmol of NADPH consumed/min/mg protein)</th>
<th>Catalase (U/mg protein)</th>
<th>Lipid peroxidation (MDA nmoles/mg protein)</th>
<th>GSH (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>57.62 ± 13.34</td>
<td>86.15±28.71</td>
<td>8.38±3.59</td>
<td>0.23±0.07</td>
<td>30.5 ± 7.77</td>
</tr>
<tr>
<td>Aflatoxin alone (control)</td>
<td>31.43 ± 4.51</td>
<td>29.56±11.56</td>
<td>3.98±1.90</td>
<td>0.52±0.12</td>
<td>20.5 ± 2.04</td>
</tr>
<tr>
<td>Aflatoxin B1+paraffin oil (Vehicle control)</td>
<td>29.59 ± 6.77</td>
<td>27.79±8.78</td>
<td>4.22±1.19</td>
<td>0.49±0.13</td>
<td>21.92 ± 1.77</td>
</tr>
<tr>
<td>TEO 100 mg/kg b.wt.</td>
<td>51.34 ± 8.35***</td>
<td>68.88±23.79***</td>
<td>7.77±2.42***</td>
<td>0.34±0.02***</td>
<td>26.2 ± 4.6*</td>
</tr>
<tr>
<td>TEO 500 mg/kg b.wt.</td>
<td>54.04 ± 5.00***</td>
<td>76.24±21.98***</td>
<td>8.01±1.54***</td>
<td>0.29±0.05***</td>
<td>28.17±3.18***</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation of the 10 rats/group; *P<0.05; **P<0.01; ***P<0.001
Table 9.8. Effect of TEO on liver mitochondrial marker enzymes on aflatoxin treated rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Untreated</th>
<th>Aflatoxin alone (Control)</th>
<th>Aflatoxin + paraffin oil (Vehicle control)</th>
<th>Aflatoxin + TEO 100 mg/lg b.wt.</th>
<th>Aflatoxin + TEO 500 mg/lg b.wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Isocitrate dehydrogenase (ICDH)</em> (µmoles of NAD⁺ reduced/min/mg protein)</td>
<td>34.89±9.94</td>
<td>5.13±1.55</td>
<td>5.60±2.26</td>
<td>29.41±12.31***</td>
<td>32.21±14.14***</td>
</tr>
<tr>
<td><em>Succinate dehydrogenase (nmoles of NADH oxidized/min/mg protein)</em></td>
<td>4.99±2.09</td>
<td>3.0±1.27</td>
<td>2.83±1.61</td>
<td>3.98±2.16*</td>
<td>4.77±1.24**</td>
</tr>
<tr>
<td><em>Malate dehydrogenase (SDH) (nmoles of NADH oxidized/min/mg protein)</em></td>
<td>912.99±56.06</td>
<td>552.67±136.34</td>
<td>597.6±12.36</td>
<td>649.2±44.60*</td>
<td>906.16±57.02***</td>
</tr>
<tr>
<td><em>NADH-dehydrogenase (µmoles of NADH oxidized/min/mg protein)</em></td>
<td>8.06±1.72</td>
<td>4.7±1.1</td>
<td>4.5±1.9</td>
<td>6.19±2.15*</td>
<td>7.83±2.64**</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation of the 10 rats/group; *P<0.05; **P<0.01; ***P<0.001
Table 9.9. Effect of TEO on glycolytic enzymes in aflatoxin treated rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Untreated</th>
<th>Aflatoxin alone (Control)</th>
<th>Aflatoxin + paraffin oil (Vehicle control)</th>
<th>Aflatoxin + TEO 100 mg/lg b.wt.</th>
<th>Aflatoxin + TEO 500 mg/lg b.wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphosphate-dehydrogenase (μmoles of DCIP reduced /min/mg protein)</td>
<td>33.95±1.21</td>
<td>44.64±3.82</td>
<td>43.76±1.72</td>
<td>36.02±0.58**</td>
<td>34.71±1.83**</td>
</tr>
<tr>
<td>Hexokinase (nmoles of glucose-6-PO4 liberated/min/protein)</td>
<td>4.04±1.01</td>
<td>7.08±2.01</td>
<td>8.07±2.2</td>
<td>5.06±1.1**</td>
<td>4.54±0.81***</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation of the 10 rats/group; *P<0.05; **P<0.01; ***P<0.001
Table 9.10. Effect of TEO on gluconeogenic enzymes in aflatoxin induced carcinogenesis in rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Untreated</th>
<th>Aflatoxin alone (Control)</th>
<th>Aflatoxin + paraffin oil (Vehicle control)</th>
<th>Aflatoxin + TEO 100 mg/lg b.wt.</th>
<th>Aflatoxin + TEO 500 mg/lg b.wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphatase (µg of inorganic PO₄ liberated/ mg protein/minute)</td>
<td>35.72±7.64</td>
<td>9.45±4.38</td>
<td>9.23±1.41</td>
<td>27.62±3.09***</td>
<td>31.53±7.84***</td>
</tr>
<tr>
<td>Fructose1,6-bisphosphatase (n moles of organic phosphate liberated/mg protein/minute)</td>
<td>6.52±0.65</td>
<td>1.57±0.70</td>
<td>1.54±0.44</td>
<td>6.08±0.66***</td>
<td>6.30±0.87***</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation of the 10 rats/group; *P<0.05; **P<0.01; ***P<0.001
Table 9.11. Effect of administration of TEO on AFB1 induced P450 enzymes in rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>MROD (nmol/min/mg protein)</th>
<th>PROD (nmol/min/mg protein)</th>
<th>EROD (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.16 ± 0.29</td>
<td>0.68 ± 0.15</td>
<td>1.82 ± 0.34</td>
</tr>
<tr>
<td>AFB1 alone</td>
<td>6.73 ± 2.38</td>
<td>3.67 ± 0.64</td>
<td>6.07 ± 2.71</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>5.98±1.71</td>
<td>3.94±0.73</td>
<td>6.36±2.33</td>
</tr>
<tr>
<td>TEO 100 mg/kg b.wt.+ AFB1</td>
<td>2.77 ± 1.13***</td>
<td>1.72 ± 0.43***</td>
<td>3.91 ± 1.14*</td>
</tr>
<tr>
<td></td>
<td>(53.67%)</td>
<td>(56.35%)</td>
<td>(38.52%)</td>
</tr>
<tr>
<td>TEO 500 mg/kg b.wt.+ AFB1</td>
<td>1.51 ± 0.44***</td>
<td>0.94 ± 0.20***</td>
<td>2.58 ± 0.73**</td>
</tr>
<tr>
<td></td>
<td>(74.75%)</td>
<td>(76.14%)</td>
<td>(59.43%)</td>
</tr>
</tbody>
</table>

Vehicle control: Paraffin oil + AFB1.
MROD: 7-methoxyresorufin-O-demethylase.
PROD: 7-pentoxyresorufin-O-depentylase.
EROD: 7-ethoxyresorufin-O-deethylase.
Each value represents the mean ± SD (n=6). *p<0.05, **p<0.01, ***p<0.001 compared with vehicle control.
Fig: 9.7 Effect of TEO on aflatoxinB1 induced liver carcinoma-Histopathological analysis.

(A) Untreated
(B) Aflatoxin B1 alone
(C) Aflatoxin B1+ Paraffin oil
(D) Aflatoxin B1+ 500 mg/kg b.wt. TEO
Fig: 9.8. Effect of TEO on AFB1 induced protein carbonyl in rats liver tissue

\[ \text{Values are mean ± standard deviation of the 6 animals/group.} \]

Fig: 9.9. Effect of TEO on DNA adduct formation in the liver of AFB1 treated rats.

\[ \text{Values are mean ± standard deviation of the 6 animals/group.} \]
76.14% respectively. These results indicated that oral administration of TEO inhibited the AFB1 induced elevation of MROD, PROD and EROD levels.

9.3.7.2. Estimation of effect of TEO on AFB1 induced protein carbonyl in rats liver tissue (Protein carbonyl assay)

There was significant increases in the levels of protein adduct formation in control and vehicle control animals when compared to the untreated animals (Fig. 9.8). TEO administration reduced the protein adduct formation near to normal level.

9.3.7.3. Estimation of DNA adduct formation in rat liver tissue

Development of DNA adducts in liver of Aflatoxin B1 (250 µg/kg) treated rats was found to be increased to 11.42 ± 4.9 nanomole/µg DNA. It was significantly decreased to 3.2±0.29 and 3.047 ± 0.65 nanomole/µg DNA by TEO administration at doses 100 and 500 mg/kg b.wt. respectively (Fig. 9.9).

9.4. Discussions

Aflatoxin B1 is produced by many strains of Aspergillus species is the most potent naturally occurring mycotoxin/carcinogenic substance. Aspergillus produced different types of aflatoxin such as B1, B2, G1 and G2 as well as B 1 is the most toxic aflatoxin and present in food and feed. (Sweeney and Dobson, 1998; Vaamonde et al., 2003). Afatoxin contaminated maize and peanut causes severe health problems, aflatoxicosis and death cases in Kenya and other places of the world (Strosnider et al., 2006). Liver is the primary target organ of metabolic action of aflatoxin and it is highly sensitive to liver as well as induce hepatic carcinoma. Aflatoxin may modulate the immune system and it leads to stunted growth in children (Williams et al., 2004 and Gong et al., 2002). It is already reported that aflatoxin in the diet causes bile ductile proliferation in trout, tissue necrosis, hepatic cirrhosis and biliary epithelial cell proliferation. Ducklings are the most sensitive organism towards aflatoxin. Present study highlights the effects of TEO on the growth of Aspergillus flavus, production of aflatoxin, toxicity in ducklings and its histopathological changes. Moreover, we discussed the amelioration of TEO on aflatoxin induced liver carcinogenesis in rats.
The results indicate that turmeric essential oil has a significant inhibition of growth and aflatoxin production by *Aspergillus flavus*. TEO treatment exhibited concentration dependent gradual inhibition of growth and aflatoxin production. As a result, TEO may be used as food preservative instead of synthetic food additives to control the aflatoxin contamination as well as it will be highly recommended in food industry. Mutation is the preliminary steps of cancer in cells. Administration of aflatoxin B1 induces DNA adduct and mutation in the liver and it will lead to hepato cellular carcinoma. The metabolism of aflatoxin B1 was done by the cytochrome p450 enzymes present in liver and convert to active forms. *In vivo* mutagenicity study revealed that aflatoxin B1 induced mutations in auxotropic *Salmonella* strains, TA 100 and TA 98 after metabolic activation by S9 fraction isolated from phenobarbitone induced cytochrome p450 enzymes rich liver (Ames test). Nevertheless the TEO treatment significantly reverses the aflatoxin induced mutations of *Salmonella* strains in a concentration dependent manner. Hence we can assess that TEO can act as an antimutagenic agent against aflatoxin B1.

The toxicity study revealed that aflatoxin administration in ducklings exhibited toxicity related properties. Aflatoxin showed significant changes in hematological and serum biochemical parameters as well as histological examinations of selected organs, tissue also supported the toxicity of aflatoxin. Moreover TEO can successfully suppress the toxic effects of aflatoxin. Organ related weight and body weight measurement are significant toxicity evaluation method. Oral administration of aflatoxin reduces the body weight of ducklings. TEO treated group showed any significant body weight changes when compared with the untreated group indicated that TEO can defeat the aflatoxin toxicity in ducklings. There was no significant relative organ weight change in any of the organs of duckling. Liver and pancreas showed minor organ weight reduction when compared with untreated group, but there was no variation between TEO treated and untreated group. Analysis of hematopoietic parameters is one of the profound methods to measure the toxicity of drugs in human and animals. HB, RBC and platelet count were altered by aflatoxin and TEO administration almost restored all these parameters.
Serum hepatic biochemical enzyme parameters are associated with state of health and used for assessment of liver damage by drugs. Elevation of ALT, ALP, AST, urea and creatinine in serum is an indicator of liver, kidney and heart damage as well as specific enzyme markers of necrotic injury and cholestasis in liver (Wasan et al., 2001; Spech and Liehr, 1983). ALT, ALP, AST, Creatinine and urea level are significantly elevated in aflatoxin B1 alone group and restored by TEO administration. TEO significantly increased aflatoxin induced lowering of GGT in the liver. Results showed that oral administration of TEO can suppress aflatoxin induced liver damage. Treatment related changes or microscopic lesions were observed in histopathology of brain, liver, intestine, kidney, thymus, pancreas, bursa of fabricius and spleen tissue demonstrated the aflatoxin toxicity in ducklings. But TEO treatment showed normal histopathology when compared with the untreated group.

TEO showed significant activity against aflatoxin B1 induced hepatocarcinogenesis in rats. The animals were little changes of liver weight exhibited in control animals when compared with aflatoxin B1 treated animals. Morphology of the aflatoxin treated liver showed no nodule formation nevertheless light color changes and roughness were observed. AST and ALP are related to carbohydrate and protein metabolism. These enzymes are released abnormally into the blood circulatory system by cell injury and may be indicative of liver damage. The increase in the activities of AST, ALT, bilirubin and ALP in serum of aflatoxin treated rats might be due to the increased permeability of the plasma membrane or cellular necrosis leading to leakage of the enzymes to the blood stream (Bumrela et al., 2012). In the present study the level of AST, ALP, bilirubin and ALT were found to be higher in the aflatoxin alone group of duckling serum suggesting the hepatic tissue damages and necrosis. But TEO restored all these parameters indicates its protective effects.

Gamma-GT enzyme is the best indicator of liver damage and it is present in the plasma membrane of liver (Jeena et al., 1999). It has an important role in metabolism of foreign substances, cell growth and differentiation (Thusu et al., 1991). A parallel increase in γ-GT activity with alkaline phosphatase activity is frequent in hepatocellular carcinoma. It is due to the tumor progression along with synthesis of γ-GT (Koss et al.,
The elevated level of γ-GT is predominantly reduced with turmeric essential oil treatment in a dose dependent manner.

Histopathological studies of the liver also proved the significant activity of TEO against aflatoxin toxicity in ducklings. Aflatoxin B1 is metabolized by cytochrome P450 enzymes to produce highly reactive intermediate aflatoxin B1 8, 9-epoxide along with the generation of reactive oxygen species known to cause hepatocellular carcinoma (Towner et al., 2003). The mechanism of action of mycotoxins on the cell is mediated through the production of free radicals and reactive oxygen species (ROS). Lipid peroxidation is considered as one of the basic mechanisms of tissue damage caused by free radicals. The aflatoxin B1 treated rats significantly increased the lipid peroxidation, which was measured by estimating its end product MDA levels. Aflatoxin B1 might induce the generation of oxygen free radicals attack the cell membrane rich in polyunsaturated fatty acids, initiating a chain reaction leading to peroxidation of fatty acids. GST is found in the cytosol and microsomes of liver cells that detoxify the unstable reactive metabolites and inhibit adduct formation (Hogberg et al., 1974). There is a significant decrease in GST levels in aflatoxin B1 treated rats, indicating reduced conjugation of forming AFB1 epoxide which leads to develop hepatocellular carcinoma in rats which was significantly reduced by oral administration of TEO. We found that TEO significantly increased aflatoxin induced lowering of antioxidant enzymes GSH, GPx, catalase and GST.

Mitochondria play a central role in oxidative metabolism and apoptosis, and also the primary target of reactive oxygen species. Free radical production reduces the ability to meet the energy demands of the cell by reducing the levels of mitochondrial TCA cycle enzymes. TCA cycle enzymes are involved in sugar, amino acids and lipid metabolism. In cancerous cells, TCA cycle enzymes producing mutated genes have been exposed (Gimenez-Roqueplo et al., 2001). The citric acid cycle enzymes were decreased under oxidative stressed and Roy (1968) reported mitochondrial swelling during aflatoxicosis. In the present study, the activities of citric acid cycle enzymes such as isocitrate dehydrogenase, succinate dehydrogenase, NADH-dehydrogenase and malate dehydrogenase were found to be decreased in long term aflatoxin B1 administered animals. This may be due to the mitochondrial damage caused by aflatoxin B1 induced
oxidative stress. Glucose-6-phosphatase and fructose-1,6-diphosphatase are marker enzymes for liver microsomal activity and it is greatly inhibited in cancer bearing animals. It reported that in adenocarcinoma bearing mice liver glucose-6-phosphatase enzyme was significantly lower than the normal mouse liver (George and Irvine, 1954). The decreased activities of glucose-6-phosphatase reveal the progressive failure of gluconeogenesis in cancerous conditions (Baggetto, 1979; Schamhart, 1979). On TEO treated animals revealed these enzymes were significantly (P<0.01) brought back to near normal levels when compared with aflatoxin alone treated animals.

Altered energy metabolism like glycolysis is one of the key characters of cancer cells (Hanahan et al., 2011). Hexokinase and 3 gluco-6-phospho-dehydrogenase are the enzymes involved in energy liberation from carbohydrate by glycolysis. In cancer cells, the rate of energy liberation by carbohydrate metabolism is very high for the growth and development of cancerous tissues since the hexokinase and 3 gluco-6-phospho-dehydrogenase enzymes activities also increased (Warburg et al., 1956 and Geschwind et al., 2004). The glycolytic activity in tumor cells has been shown to endorse resistance to chemotherapeutics (Lu et al., 2008). Here the aflatoxin B1 significantly induced the hexokinase and 3 gluco-6-phospho-dehydrogenase activities in liver when compared with untreated animals. The level of hexokinase and 3 gluco-6-phospho-dehydrogenase are increased due to the long term administration of aflatoxin B1 was significantly ameliorated by the TEO administration in rats. Hence the glycolytic levels were significantly decreased by the oral administration of TEO rats. The inhibition of glycolysis is one of the best targets of the treatment of cancer because by this way tumor tissues become sensitive to immunotherapy.

Cytochrome p450 enzymes are crucial enzymes involved in cancer development and treatment. Hence it is much important that inhibition or activation of these cytochrome p450 enzymes in development of potential cancer chemopreventive or therapeutic drugs. The carcinogenic mechanism of aflatoxin B1 has been extensively studied. It has been shown that aflatoxin B1 is metabolically activated by hepatic cytochrome P450 enzymes to produce a reactive intermediate, aflatoxin B1-8,9-epoxide, which consequently binds to nucleophilic sites in DNA and the major adduct 8,9-
Dihydro-8-(N7 guanyl)- 9-hydroxy aflatoxin B1 is formed (Koss et al., 1982, Hogberg et al., 1974 and Schamhart et al., 1979). The metabolic product of aflatoxin by cytochromes P450 was covalently bound with cellular DNA or protein leading to mutation or cancer. The formation of aflatoxin-DNA adducts is considered as a key step in the initiation of aflatoxin induced hepatocarcinogenesis (Baggetto, 1992). Our study revealed that TEO can significantly decrease AFB1 induced DNA adducts and protein carbonyl in rat’s liver by TEO in a concentration dependent manner. Moreover the aflatoxin metabolic cytochrome p450 enzymes such as CYP1A2, CYP2B1/2 and CYP1A1 were also significantly decreased by the TEO treatment in rats. From this it can be concluded that TEO prevent or decreased the aflatoxin metabolism and reduced the DNA adduct and protein carbonyl formation in liver. And thereby TEO have potential protect effect against aflatoxin induced hepatocarcinogenesis and toxicity.

During the present study, TEO was evaluated for its antimutagenic, toxicity and anticancer activity against aflatoxin B1 induced hepatocellular carcinoma. We conclude that TEO can used to food preservative against fugu since significantly prevent the growth and production of A. flavus and aflatoxin B1. There is a significant restoration of altered abnormal serum and tissue biochemical parameters, antioxidant enzymes, GSH and GGT level indicating the protective effect of TEO against aflatoxin toxicity and hepatocellular carcinoma. Histological observations are also support the protective ability of TEO. Moreover TEO showed significant antimutagenic activity against aflatoxin B1. Besides TEO significantly decreased the aflatoxin metabolic enzymes and reduced the DNA/protein adduct formation in liver. From these in vivo and in vitro studies we can conclude that the TEO has a prominent role in showing protective activity against A. flavus and its toxin.