Chapter II

Materials and Methods
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1. Materials

1.1. General Chemicals

Reduced glutathione (GSH), oxidized glutathione (GSSG), nicotinamide adenine dinucleotide phosphate reduced (NADPH), nicotinamide adenine dinucleotide phosphate oxidized (NADP), flavin adenine dinucleotide (FAD), thiobarbituric acid (TBA), trichloroacetic acid (TCA), bovine serum albumin (BSA), 1,2-dithio-bis-nitrobenzoic acid (DTNB), 1-chloro, 2,4-dinitrobenzene (CDNB), glutathione reductase, ethylene diamine tetra acetic acid (EDTA), xanthine, dichlorophenolindophenol (DCPIP), pyridoxal phosphate, phenyl methylsulfonylfluoride (PMSF), 2-mercaptoethanol, dithiothreitol, Brij 35, ethanolamine, methoxyethanol, citric acid, N,N-diethylnitrosoamine (DEN), 2-acetylaminofluorene (2-AAF), SDS, Ethidium Bromide (EtBr), phenol, chloroform and were obtained from Sigma Chemicals Co. (St. Louis, MO). Ascorbic acid, hydrogen peroxide, ferric chloride, disodium hydrogen phosphate, isoamyl alcohol, ethanol, sodium dihydrogen phosphate, formaldehyde, methanol, hydrochloric acid (HCl), sodium azide, magnesium chloride, Tween 80 and sodium hydroxide were purchased from E. Merck, India. dl-[14C] ornithine was procured from Amersham Biosciences, Chennai, India. Acetaminophen (paracetamol) was purchased from Arora chemicals, New Delhi, India. TNF-α ELISA kit were purchased from e-Bioscience, USA. Caspase-3, -7 and -9 ELISA kits were purchased from Invitrogen. Anti-phospho-p38 (dilution 1:200, Santa Cruz), anti-iNOS (dilution 1:200, Thermo scientific), anti-ki-67 (dilution 1:200, Thermo scientific), anti-COX-2 (dilution 1:200, Santa Cruz), anti-NF-κB (p65) (1:300, Biolegend) and anti-PCNA (dilution 1:200, Thermo scientific) primary antibodies were used. Poly- HRP plus one detection system (Thermo scientific).

1.2. Chemopreventive agents

Chrysin, Diosmin and Betaine were purchased from Sigma Chemicals Co. (St. Louis, MO) USA.

1.3. Plant material

*Fagonia cretica* (Dhamasa) and *Parmelia perlata* (Charila) were obtained from Khari Bowli, New Delhi, India. The identity of the plant material was verified by Prof. M. P. Sharma, Department of Botany, Hamdard University New Delhi. *Carum carvi* has been obtained from CCRUM Hyderabad, India. The rationale for using these plants for the present work is that from centuries these plants being used in alternative system of medicine in Indian folklore for the treatment of liver ailments, so in the present study we have evaluated the chemopreventive efficacy of these plants against chemically induced hepatocarcinogenesis in rats through the mechanistic approach. The methanolic extract of whole plant of *Fagonia cretica* and *Parmelia perlata* and seed of *Carum carvi* were used.
1.4. Animals
Young (6-8 weeks old), Wistar rats (male/female) were housed in plastic (polypropylene) cages in animal house facility of Hamdard University, New Delhi, India. Experiments were conducted according to protocols approved by Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), New Delhi, India, project number and date 173/CPCSEA, 28th Jan, 2000. The well ventilated animal rooms (room temperature set at 25°C) were maintained on 12 h light-dark cycles. They were acclimatized for one week before the study and had free access to standard laboratory feed (Hindustan lever Ltd, Mumbai, India) and water *ad libitum*.

1.5. Dose of Modulators used in the thesis
The below-mentioned doses of compounds were selected based on preliminary studies and previously published reports from literature. Moreover the plant extracts were assessed for acute toxicity and found non-toxic. Furthermore, in chapters IV and V, group V is give the higher dose of the modulator only in order to check the sub-acute toxicity of the modulator at higher dose.

<table>
<thead>
<tr>
<th>Modulator</th>
<th>Dose I (D1)</th>
<th>Dose II (D2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrysin</td>
<td>20 mg/kg b.wt</td>
<td>40 mg/kg b.wt</td>
</tr>
<tr>
<td>Diosmin</td>
<td>10 mg/kg b.wt</td>
<td>20 mg/kg b.wt</td>
</tr>
<tr>
<td>Betaine</td>
<td>50 mg/kg b.wt</td>
<td>100 mg/kg b.wt</td>
</tr>
<tr>
<td><em>Fagonia cretica</em></td>
<td>100 mg/kg b.wt</td>
<td>200 mg/kg b.wt</td>
</tr>
<tr>
<td><em>Parmelia perlata</em></td>
<td>100 mg/kg b.wt</td>
<td>200 mg/kg b.wt</td>
</tr>
<tr>
<td><em>Carum carvi</em></td>
<td>100 mg/kg b.wt</td>
<td>200 mg/kg b.wt</td>
</tr>
</tbody>
</table>

2. Methodology
2.1. Serum preparation
For serum preparation, blood was collected by cardiac puncture in dried centrifuge tubes that were kept at 4°C for an hour to separate the serum from cellular clot. Finally, to remove cellular clot of the serum, it was centrifuged at 800g for 10-15 min, the serum obtained was used for enzyme estimation.
2.2. Preparation of Post-Mitochondrial Supernatant (PMS), Cytosolic and microsomal fractions

Liver were removed and cleaned with ice-cold saline (0.85% sodium chloride). A 10% homogenate of liver tissues were obtained in a buffer solution containing 10mM tris-HCl, 250mM sucrose pH 7.4 using a Potter–Elvehjen homogenizer and were centrifuged at 3000 rpm for 10 min by Eltek Refrigerated Centrifuge (model RC 4100 D) to separate the nuclear debris. The aliquot so obtained was centrifuged at 12,000rpm for 20 min. by a REMI cooling centrifuge to obtain PMS, which was used as a source of various enzymes. The supernatant obtained was further ultra-centrifuged in an Ultracentrifuge (Beckman, L7-55) at 100500g (34000 rpm) for 1 hour to obtain cytosolic fraction for alcohol dehydrogenase (ADH) activity. The precipitate obtained was washed with homogenizing buffer to obtain the microsomal fraction for cytochrome P450 2E1 (CYP 2E1) activity. All the experimental were carried out at 4ºC.

2.3. DNA isolation

DNA extraction was done by standard chloroform isoamyl method. DNA was extracted from approximately 400mg of liver tissue by homogenizing the tissue in 5ml TNE buffer (50mM Trisma, 100mM EDTA, 0.5% SDS, pH 8.0) in a 2ml ground glass homogenizer. Each sample was homogenized with 10 standardized strokes of the pestle to minimize any potential effect on DNA integrity introduced by the homogenization procedure. An equal volume of buffered phenol/chloroform/isoamyl alcohol (PCI) (25:24:1, v/v/v, pH 8.0) was then added to the sample. The sample was gently mixed and allowed to settle for 5 min and then centrifuged for 5 min at 13000 rpm at 4ºC. The aqueous layer was transferred to a new micro centrifuge tube and PCI extraction was repeated. The aqueous layer was then digested by 5ml of RNase (10 mg/ mL) for 30 min at 37°C and the digest was extracted once by PCI and once by 500ml of chloroform. DNA was precipitated from the resulting aqueous layer by adding 2 volumes of absolute ethanol and 1/10 volume of 3M sodium acetate, pH 5.2. The sample was then centrifuged (13000 rpm, 15 min), and the resulting pellet rinsed with 500µl of 70% ethanol and air-dried. The amount of DNA was quantitated spectrophotometrically at 260 and 280 nm. DNA sample was resuspended in 100µL of TE buffer (10mM Trisma, 1mM EDTA) and subsequently used in the different assays.

2.4. Visualization of Isolated DNA

The quality and quantity of DNA was determined by absorbance at 260nm and 280nm in a spectrophotometer or by running on 1% gel. The sample was mixed with 10 ml of loading solution [10mM EDTA (pH 8.0), 1% (w/v) bromophenol blue and 40% (w/v) sucrose]
preheated to 70° C. The DNA samples were loaded onto agarose gel containing ethidium bromide and visualized under short-wave UV light.

3. **Estimation of in-vitro parameters**

3.1. **Total phenolic Content**

The total phenol content was determined by adding 0.5 ml of the aqueous extract to 2.5 ml, 10% Folin–Ciocalteau’s reagent (v/v) and 2.0 ml of 7.5% sodium carbonate. The reaction mixture was incubated at 45°C for 40 min, and the absorbance was measured at 765 nm in the spectrophotometer. Gallic acid was used as a standard phenol. The mean of three readings was used and the total phenol content was expressed as milligram of gallic acid equivalents/g extract (Fukumoto and Mazza, 2000).

3.2. **Estimation of lipid peroxidation**

The in-vitro inhibition of LPO by plant extracts were evaluated by the method of Buege and Aust, (1978) with some modifications. The reaction mixture in a total volume of 1.0 ml contained 0.58 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml microsome, 0.2 ml ascorbic acid (100 mM) and 0.02 ml ferric chloride (100 mM). The reaction mixture with or without plant extract was incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by the addition of 1 ml of TCA (10%). Following addition of 1.0 ml TBA (0.67%), all the tubes were placed in a boiling water bath for a period of 20 min. The tubes were shifted to ice bath and then centrifuged at 2500 x g for 10 min. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm. The results were expressed as the nmol MDA formed/h/g tissue at 37°C by using a molar extinction coefficient of 1.56 x 10^5 M⁻¹ cm⁻¹.

3.3. **Assay of DNA-sugar damage**

DNA sugar damage was assayed by the method of Halliwell and Gutteridge (1987). Briefly, the reaction mixture in a total volume of 3.0ml contained 0.5 ml calf thymus DNA (1 mg/ml of 0.15MNaCl), 0.450 ml phosphate buffer (0.1 M, pH 7.4) and 0.05 ml of FeCl₃ (100μM in final concentration) The reaction mixture was incubated with or without plant extract for 1 hr at 37°C in a water bath shaker. After the incubation was over, the reaction was stopped by the addition of 1ml of TCA (10%). 1 ml TBA (0.67%) was added to the reaction mixture and then it was kept in boiling water bath for 25 min. The TBA reacting species so generated forms an adduct showing a characteristic absorption at 535 nm which was monitored using spectrophotometer (Perkin elmer Lamda Ez 201).
3.4. **DPPH scavenging activity**

DPPH scavenging activity was done by the method of Mensor et al., 2001. The DPPH free radical method is based on the determination of the concentration of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) at steady state in a methanol solution, after adding the mixture of antioxidants. DPPH absorbs at 517nm and as its concentration is reduced by the existence of an antioxidant, the absorption gradually disappears with time. The model of scavenging stable DPPH radicals is a widely used method for evaluating antioxidant activity in a relatively short time as reported by Fenglin et al., 2004. Briefly, the reaction mixture containing 1ml of DPPH solution (0.1 mM, in 95% ethanol) incubated with different concentrations of plant extract (20, 40, 60, 80 and 100µl/ml) and allowed to react at room temperature. The mixture was shaken vigorously and allowed to stand for 30 minutes and the absorbance of the resulting solution was measured at 517nm spectrophotometer (Perkin elmer Lamda Ez 201). Lower absorbance of the reaction mixture indicated higher free-radical scavenging activity. The percent DPPH radical scavenging effect was calculated according to the following equation:

\[
\% \text{DPPH} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100
\]

3.5. **Ferric Reducing Potential**

The reducing power of methanol extract was determined according to the method of Oyaizu (1986). Sample solutions at different amounts were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide (1%). After the mixture was incubated at 50°C for 20min, 2.5ml of TCA (10%) were added and the mixture was centrifuged at 4000rpm for 10 min. Supernatant (2.5ml) was mixed with distilled water (2.5 ml) and 0.5ml of ferric chloride (0.1%), and the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicates greater reducing power.

4. **Estimation of biochemical parameters**

4.1. **Assay for catalase activity**

The catalase activity was assessed by the method of Claiborne (1985). In short, the reaction mixture was comprised of 0.05ml of PMS, 1.0 ml of hydrogen peroxide (0.019M), 1.95 ml of phosphate buffer (0.1M, pH 7.4), in a total volume of 3 ml. Changes in absorbance were recorded at 240nm, and the change in absorbance was calculated as nmol H₂O₂ consumed per min per mg of protein.
4.2. Estimation of GSH
GSH was assessed by the method of Jollow et al., (1974). A quantity of 1.0ml of 10% PMS mixed with 1.0ml of (4%) sulphosalicylic acid was taken and then incubated at 4°C for a minimum time period of 1h and then centrifuged at 4°C at 1200g for 15 min. The reaction mixture of 3.0ml was composed of 0.4 ml of supernatant, 2.2ml of phosphate buffer (0.1M, pH 7.4) and 0.4ml of DTNB (4mg/ml). The yellow color developed was read immediately at 412nm on the spectrophotometer (Perkin Elmer, lambda EZ201). The GSH concentration was calculated as nmol GSH conjugates/g tissue.

4.3. Assay for glutathione peroxidase activity
The activity of glutathione peroxidase (GPx) was calculated by the method of Mohandas et al., (1984). The total volume of 2 ml was composed of 0.1 ml of EDTA (1 mM), 0.1 ml of sodium azide (1mM), 1.44ml of phosphate buffer (0.1M, pH 7.4), 0.05ml of GR (1 IU/ml), 0.05ml of GSH (1mM), 0.1ml of NADPH (0.2mM) and 0.01 ml of H₂O₂ (0.25mM) and 0.1ml of 10% PMS. The depletion of NADPH at 340nm was recorded at 25°C. Enzyme activity was calculated as nmol NADPH oxidized/min/mg protein with the molar extinction coefficient of 6.22 × 10³ M⁻¹ cm⁻¹.

4.4. Assay for hydrogen peroxide
Hydrogen peroxide (H₂O₂) was assayed by H₂O₂ mediated horseradish peroxidase-dependent oxidation of phenol red by the method of Pick and Keisari (1981). Microsomes (2.0ml) were suspended in 1.0ml of solution containing phenol red (0.28nm), horseradish peroxidase (8.5 units), dextrose (5.5nm), and phosphate buffer (0.05M, pH 7.0) and were incubated at 37°C for 60min. The reaction was stopped by the addition of 0.01ml of NaOH (10N) and then centrifuged at 800g for 5min. The absorbance of the supernatant was recorded at 610nm against a reagent blank. The quantity of H₂O₂ produced was expressed as nmol H₂O₂ per hour per g tissue based on the standard curve of H₂O₂ oxidized phenol red (Pick and Keisari, 1981).

4.5. Glutathione reductase activity
GR activity was determined by the method of Carlberg and Mannervik (1975). The reaction mixture consisted of 1.65ml phosphate buffer (0.1M, pH 7.6), 0.1ml EDTA (0.5mM), 0.05ml GSH (1mM), 0.1ml NADPH (0.1mM) and 0.1ml 10% PMS in a total volume of 2ml. Enzyme activity was quantified at 25°C by measuring the disappearance of NADPH at 340nm and was calculated as nmol NADPH oxidized per min per mg protein using a molar extinction coefficient of 6.22× 10³ M⁻¹ cm⁻¹.

4.6. Glutathione -S- transferase activity
The reaction mixture consisted of 2.5 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml GSH (1 mM), 0.2 ml CDNB (1 mM), and 0.1 ml of the cytosolic fraction (10%) in a total volume of 3.0 ml. Changes in absorbance were recorded at 340 nm, and enzymatic activity was calculated as nmol CDNB conjugate formed per min per mg protein using a molar extinction coefficient of 9.6×10^3 M⁻¹ cm⁻¹ (Habig et al., 1974).

4.7. **Cytochrome P450 2E1 (CYP 2E1) activity**

The catalytic activity of CYP 2E1 was analyzed by measuring p-nitrophenol hydroxylation (PNPH) as described by Reinke et al., (1985). The reaction mixtures contained a 100 mM potassium phosphate buffer (pH 6.8), 1.0 mM ascorbic acid, 1 mM NADPH, 1 mg hepatic microsomes, and 100 mM p-nitrophenol in a total volume of 1.0 ml. The 4-nitrocatechol that was formed was determined spectrophotometrically at 535 nm. Data was expressed as nmol/mg/min.

4.8. **Alcohol dehydrogenase (ADH) activity**

ADH activity was determined by the method of Bonnichsen and Brink (1955). Briefly, ADH activity was measured in 50 mM glycine, pH 9.6, 0.8 mM NAD, 3 mM ethanol and 50 μl of cytosolic fraction in a final volume of 1 ml. Enzyme activity was measured at 340 nm and the activity was calculated as nmol NADH formed/min/mg protein using a molar extinction coefficient of 6.22 x 10⁶ M⁻¹ cm⁻¹.

4.9. **Estimation of Lipid Peroxidation (LPO)**

The assay of lipid peroxidation was done according to the method of Wright et al., (1981). The reaction mixture consisted of 0.58 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml microsome, 0.2 ml ascorbic acid (100 mM) and 0.02 ml ferric chloride (100 mM) in a total of 1 ml. This reaction mixture was then incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by the addition of 1 ml of TCA (10%). Following addition of 1.0 ml TBA (0.67%), all the tubes were placed in a boiling water bath for a period of 20 min. The tubes were shifted to ice bath and then centrifuged at 2500×g for 10 min. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm. The results were expressed as the nmol MDA formed/h/g tissue at 37°C by using a molar extinction coefficient of 1.56×10⁵ M⁻¹ cm⁻¹.

4.10. **Assay for serum aspartate aminotransferase and alanine aminotransferase (AST & ALT) activity**

AST and ALT activity were determined by the method of Reitman and Frankel (1957). Each substrate (0.5 ml) (2 mM α-ketoglutarate or 200 mM L-alanine or L-aspartate) was incubated for
5 min at 37 °C in a water bath. Serum (0.1 ml) was then added and the volume was adjusted to 1.0 ml with 0.1 M, pH 7.4 phosphate buffer. The reaction mixture was incubated for exactly 30 and 60 min at 37°C for ALT and AST, respectively. Then to the reaction mixture, 0.5 ml of 1 mM DNPH was added, after another 30 min at room temperature, the color was developed by addition of 5.0 ml of NaOH (0.4 N) and the product read at 505 nm.

4.11. Assay for lactate dehydrogenase activity
Lactate dehydrogenase (LDH) activity was estimated in serum by the method of Korenberg (1955). The assay mixture consisted of 0.2 ml of serum, 0.1 ml of 0.02 M NADH, 0.1 ml of 0.01 M sodium pyruvate, 1.1 ml of 0.1 M (pH 7.4) phosphate buffer and distilled water in a total volume of 3 ml. Enzyme activity was recorded at 340 nm, and activity was calculated as nmol NADH oxidized/min/mg protein.

4.12. Measurement of quinone reductase (QR) activity
The QR activity was determined by the method of Benson et al. (1980). The 3 ml reaction mixture consisted of 2.13 ml Tris–Cl buffer (25mM, pH 7.4), 0.7 ml BSA, 0.1 ml FAD, 0.02 ml NADPH (0.1 mM), and 50 μl PMS (10%). The reduction of dichlorophenolindophenol (DCPIP) was recorded calorimetrically at 600 nm and the enzyme activity was calculated as μmol of DCPIP reduced/min/mg protein using molar extinction coefficient of $2.1 \times 10^4$ M$^{-1}$ cm$^{-1}$.

4.13. Assay for xanthine oxidase activity
The activity of xanthine oxidase (XO) was assayed by the method of Athar et al. (1996). The reaction mixture consisted of 0.2 ml PMS which was incubated for 5 min at 37°C with 0.8 ml of phosphate buffer (0.1 M, pH 7.4). The reaction was started by adding 0.1 ml of xanthine (9 mM) and kept at 37°C for 20 min. The reaction was terminated by the addition of 0.5 ml of ice-cold PCA (10% v/v). After 10 min, 2.4 ml of distilled water was added and centrifuged at 4000 r.p.m. for 10 min and μg uric acid formed/min/mg protein was recorded at 290 nm.

4.14. Ornithine decarboxylase (ODC) activity
ODC was evaluated according to O'Brien et al., (1975). ODC activity was determined using 0.4 ml liver 105,000 × g supernatant fraction per assay tube by measuring release of $^{14}$CO$_2$ from DL-[14C] ornithine. The livers were homogenized in Tris–Cl buffer (pH 7.5, 50 mM) containing EDTA (0.1 mM), pyridoxal phosphate (0.1 mM), PMSF (1.0 mM), 2-mercaptoethanol (1.0 mM), dithiothreitol (0.1 mM) and Tween-80 (0.1%) at 4°C. In brief, the reaction mixture contained 400 μl enzyme and 0.095 ml co-factor mixture containing pyridoxal phosphate (0.32 mM), EDTA (0.4 mM), dithiothreitol (4.0 mM), ornithine (0.4 mM), Brig 35 (0.02%) and [14C] ornithine (0.05 /μCi) in a total volume of 0.495 ml. After adding buffer and
co-factor mixture to blank and other test tubes, the tubes were closed immediately with a rubber stopper containing 0.2ml ethanolamine and methoxyethanol mixture in the central well and kept in a water bath at 37°C. After 1h of incubation, the enzyme activity was inhibited by injecting 1.0ml citric acid solution (2.0M) along the sides of glass tubes and the incubation was continued for 1h to ensure complete absorption of 14CO2. Finally, the central well was transferred to a vial containing 2ml ethanol and 10ml toluene-based scintillation fluid was added. Radioactivity was counted in a liquid scintillation counter (LKB Wallace-1410). ODC activity was expressed as pmol 14CO2 released/h/mg protein.

4.15. Estimation of protein
The protein concentration in all samples was determined by the method of Lowry et al. (1951), Alakine copper sulphate forms complex with peptide bonds ,which gives a blue colour in presences of folins reagent.Briefly ,0.1ml (10% w/v) was diluted to 1ml water and protein precipitated to equal volume of TCA (10%),samples were kept for 2hrs at 4°C and then centrifuged at 800 X g for 5mins.The supernatant was decanted and discarded .The pellet was dissolved in 5 ml of NaOH (1N).Finally 0.1 ml of aliquot was taken for color development. 0.1ml aliquot was further diluted to 1ml with water and then 2.5ml alkaline copper sulphate reagent containing sodium carbonate (2%),Copper sulphate (1%), and sodium potassium tartrate (2%) was added. After dilution waited for 10 minutes to allow alkaline copper sulphate reagent to form complex 2.5 ml of folin’s reagent was also added. After 30 minutes blue color developed that was read at 660nm for standard Bovine serum albumin (BSA 0.1 mg/ml) was used.

4.16. Estimation of Caspase
Caspase-3, Caspase-7 and Caspase-9 activities were measured with kit (CasPASE-, Assay kit, G-Bioscience) according to the manufacturer’s directions. The results were expressed as µg/mg protein.

4.17. Estimation of TNF-alpha
Level of all the pro-inflammatory cytokines was measured with commercially available kits. Tissue was homogenized in PBS containing 0.05% Tween-20. Analysis was performed according manufacturers instruction.

4.18. Immunohistochemistry
The liver tissues were fixed in formalin and embed in paraffin. Sections of 5 µm thickness were cut onto poly-lysine coated glass slides. Sections were de-parafinize three times (5 min) in xylene followed by dehydration in graded ethanol and finally rehydrated in running tap water.
For antigen retrieval, sections were boiled in 10mM citrate buffer (pH 6.0) for 5-7 min. Sections were incubated with hydrogen peroxide for 15 min to minimize non-specific staining and then rinsed three times (5 min each) with 1X PBST (0.05% Tween-20). Blocking solution was applied for 10 min then sections were incubated with diluted rabbit polyclonal antibodies, overnight at 4°C in humid chamber. Further processing was done according to the instructions of Ultra Vision plus Detection System Anti-Polyvalent, HRP/DAB (Ready-To-Use) staining kit (Thermo scientific system). The peroxidase complex was visualized with 3, 3'-diaminobenzidine (DAB). Lastly the slides were counterstained with haematoxylin, cleaned in xylene, dehydrated with ethanol and after DPX mounting microscopic (BX 51 Olympus) analysis was done at 400x magnification.

4.19. Histopathology
Fixation, dehydration, infiltration and block preparation. The tumor from livers were excised out and fixed in Bovin’s fluid for 24-28 hrs. The tissues were then rehydrated by passing through graded series of ethyl-alcohol (50%, 70%, 90% & 100%) for one hour in each giving two changes. These were then cleared in xylene (two changes of one hour each). The cleared tissue were placed for five minutes in xylene containing molden paraffin wax at 50-60°C for infiltration.
Sections were deparaffinized by dipping in xylene and given two changes these slides were then passed through graded concentration of ethyl-alcohol (30%, 50%, 70%, 90% & 100%) with two changes of two minutes each. Then keep stained with hematoxylin for one minute and again washed in raining water thoroughly. Slides were then passed through 50% and 70% ethyl-alcohol and subsequently put in eosin stain. [Prepared in 70%, 90% & 100%, 100% + xylene (1:1)] and finally they were given two changes of xylene. All slides were mounted in DPX. They were covered with glass cover slips and kept at room temperature for drying. To avoid any type of bias, slides were coded and examined by histopathologist in blinded manner. Liver sections were evaluated at 400X magnification.

5. Statistical analysis
Differences between groups were analyzed using analysis of variance (ANOVA), followed by Dunnet's multiple comparisons test. All data points are presented as the treatment group means ± standard error of the mean (S.E.).