Chapter 2

MATERIALS AND METHOD
Chemicals

All the chemicals and biochemicals used in this study were either of analytical grade or of highest purity grade available commercially. Reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), bovine serum albumin (BSA), 1,2-dithio-bis-nitrobenzoic acid (DTNB), 1-chloro-2, 4-dinitro benzene (CDNB), nitrilotriacetic acid (NTA), Diethyl nitrosamine, reduced nicotinamide adenine dinucleotide phosphate (NADPH), glucose-6-phosphate, tween-20, 2,6-dichlorophenolindophenol (DCPIP), thiobarbituric acid (TBA), flavin adenine dinucleotide (FAD), dithiothreitol, horseradish peroxidase, hydrogen peroxide, tween-80, 2-mercaptoethanol, pyridoxal-5-phosphate, brij 35, L-ornithine, Calf thymus deoxyribonucleic acid (DNA), ethylene diamine tetra acetic acid (EDTA) were obtained from Sigma chemicals Co (St Louis, MO, USA). DL \[^{14}C\] ornithine (specific gravity 56nmCi mmol) and \[^{3}H\] thymidine (specific gravity 82mCi mmol) were purchased sodium dihydrogen phosphate, sodium hydroxide and paraffin were purchased from E. Merk, India. Napthalene, sodium azide, sodium bicarbonate, chloroform, acetone, hydrochloric acid, methoxyethanol, formaldehyde, glycerol, nitric acid were obtained from S. D. Fine Chemicals, India. Hematoxylin, eosin, sodium carbonate, dextrose, Leishman's stain were purchased from Qualigen, India.

Animals

Four to six weeks old male Wistar rats (130-150g) of Wistar strain were obtained from Central Animal House of Hamdard University, New Delhi, India. They were housed in polypropylene cages in groups of six rats per cage and were kept in a room maintained at 25±2°C with a 12h light/dark cycle. They were allowed to acclimatize for one week before the experiments and were given free access to standard laboratory feed (Hindustan Lever Ltd., Bombay, India) and water ad libitum.

Plant Material

Total extract of Acorus calamus and Terminalia chebula in semi-solid form were purchased from Saiba Industries, Mumbai, India. The extract is claimed to possess all the active ingredients of the plant. Methanolic fraction was used for the present study after preliminary in vitro tests whereas Morus indica was collected from the herbal garden of Jamia Hamdard, New Delhi. Professor Mohammed Iqbal, Medicinal Plant Division, Department of Environmental Botany, Hamdard University, New Delhi verified the
identity of plant material. Freshly collected plant material was chopped, shade dried and coarsely powdered to a mesh size of 1 mm as described by Antonio & Brito (1998).

**Preparation of extract**

The extraction procedure was exactly the same as described by Didry et al (1998). Powdered plant material was repeatedly extracted in 4000 ml round bottom flask with 2000 ml solvents of increasing polarity starting with petroleum ether, benzene, ethyl acetate, acetone, methanol and double distilled water. The reflux time for each solvent was 4 hr. The methanolic extracts was cooled at room temperature, filtered, and evaporated to dryness under reduced pressure in a rotatory evaporator (Buchi Rotavapor, Switzerland). The residues yield for each solvent was stored at 4°C. The concentrated methanolic fraction prepared was kept in fridge and freshly prepared whenever required for dosing.

**Preparation of Fe-NTA solution**

Fe-NTA was prepared fresh immediately before its use by the method of Awai et al. 1979. To prepare Fe-NTA, ferric nitrate (0.16 mmol/kg body weight) solution was mixed with four fold molar excess of disodium salt of NTA (0.64 mmol/kg body weight) and the pH was adjusted to 7.4 with sodium bicarbonate solution.

**Treatment protocols**

Treatment protocols that include selection of dose regimen, mode of administration, and time period at which animals were sacrificed etc. has been described here.
(I) Treatment protocol for renal oxidative stress, toxicity and ODC induction

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of animals</th>
<th>Treatment schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>6</td>
<td>Saline i.p. (0.85% NaCl at a dose of 10 ml/kg body w.t)</td>
</tr>
<tr>
<td>Group II</td>
<td>6</td>
<td>Toxicant (NiCl₂/Fe-NTA)</td>
</tr>
<tr>
<td>Group III</td>
<td>6</td>
<td>Plant extract/compound by gavage once daily for seven consecutive days (D1) + Toxicant (NiCl₂/Fe-NTA).</td>
</tr>
<tr>
<td>Group IV</td>
<td>6</td>
<td>Plant extract/compound by gavage once daily for seven consecutive days (D2) + Toxicant (NiCl₂/Fe-NTA).</td>
</tr>
<tr>
<td>Group V</td>
<td>6</td>
<td>Plant extract/compound by gavage once daily for seven consecutive days (D2)</td>
</tr>
</tbody>
</table>

Nickel chloride (NiCl₂): Animals were given single subcutaneous injection of NiCl₂ at a dose level of 250μmol/kg body weight/2ml and were killed by cervical dislocation 16 h after NiCl₂ administration.

Ferric nitrilotriacetic acid (Fe-NTA): Animals were given Fe-NTA at a dose level of (9mg/kg b.wt) and were sacrificed by cervical dislocation 12h after its administration.

(II) Treatment protocol for \(^{3}\text{H}\) incorporation into renal DNA

The grouping of animals and schedule for prophylaxis were same as described above. One hour after the last treatment with Plant extract/compound, the animals of group II, III and IV received only a single subcutaneous injection of NiCl₂ at a dose level of 250μmol/kg body weight/2ml and Fe-NTA at a dose level of 9mg/kg body weight. Eighteen hours after the treatment with NiCl₂/Fe-NTA the rats were given \(^{3}\text{H}\) thymidine (30μCi/animal) by intraperitoneal injection. Two hours later, they were sacrificed by cervical dislocation and their kidneys were quickly removed.
(III) Treatment protocol for two stage renal carcinogenesis

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of animals</th>
<th>Treatment schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>20</td>
<td>Saline i.p. (0.85% NaCl at a dose of 10 ml/kg body weight)</td>
</tr>
<tr>
<td>Group II</td>
<td>20</td>
<td>A single i.p. injection of DEN (200 mg/kg body weight) + Fe-NTA (9 mg Fe/kg body weight) twice a week for 16 weeks</td>
</tr>
<tr>
<td>Group III</td>
<td>20</td>
<td>Fe-NTA (9 mg Fe/kg body weight) twice a week for 16 weeks</td>
</tr>
<tr>
<td>Group IV</td>
<td>20</td>
<td>A single i.p. injection of DEN (200 mg/kg body weight) + oral treatment of Plant extract/compound by gavage (D1), twice a week one hour before the treatment with Fe-NTA, i.p. (9 mg Fe/kg body weight) twice a week for 16 weeks</td>
</tr>
<tr>
<td>Group V</td>
<td>20</td>
<td>A single i.p. injection of DEN (200 mg/kg body weight) + oral treatment of Plant extract/compound by gavage (D2), twice a week one hour before the treatment with Fe-NTA, i.p. (9 mg Fe/kg body weight) twice a week for 16 weeks</td>
</tr>
</tbody>
</table>

At the end of 24 weeks, the animals were sacrificed by cervical dislocation and their kidneys were quickly removed and preserved in 10% neutral buffered formaldehyde for histopathological studies. Haematoxylin and eosin preparations of preserved sections were prepared for microscopic examination.
Dose Regimen for plant extract/compound

<table>
<thead>
<tr>
<th>Modulator</th>
<th>Dose I (D1)</th>
<th>Dose II (D2)</th>
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</thead>
<tbody>
<tr>
<td><em>Terminalia chebula</em></td>
<td>25mg/kg b.wt</td>
<td>50 mg/kg b.wt</td>
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<tr>
<td><em>Morus indica</em></td>
<td>50mg/kg b.wt</td>
<td>100mg/kg b.wt</td>
</tr>
<tr>
<td><em>Acorus calamus</em></td>
<td>100mg/kg b.wt</td>
<td>200mg/kg b.wt</td>
</tr>
<tr>
<td>Luteolin</td>
<td>10 μmol/kg b.wt</td>
<td>20 μmol/kg b.wt</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>10 mg/kg b.wt</td>
<td>20 mg/kg b.wt</td>
</tr>
</tbody>
</table>

METHODS

Serum Preparation

Serum was prepared according to the routine method. Briefly, blood was taken out from the retroorbital sinus using heparinized capillary tubes in dried test tubes that were kept in tilted position for ten minutes at room temperature and then for an hour at 4°C to get serum separated from cellular clot. To remove cellular contamination from the serum, it was centrifuged for 5 min at 800-x g.

Tissue preparation

The preparation of tissues for all the biochemical estimations was done following the standard procedure as described by Athar and Iqbal (1998). The control and treated groups animals were sacrificed by cervical dislocation after the desired time period. The animals were immediately dissected, with tissue excised and washed in ice-cold saline (0.85%) and extraneous material was removed. All subsequent operations were carried out in ice at a temperature not above 4°C. The washed tissues were blotted between the folds of a filter paper and weighed on a meter balance. A portion of a tissue was cut into pieces of 2-3 mm thickness and preserved in 10% neutral buffered formalin for histopathological studies. For biochemical studies, a known amount of tissue was taken, minced properly and homogenized in KCl-phosphate buffer.
Materials and Method

Homogenization

The animals were killed by cervical dislocation and their kidneys were quickly removed, cleaned free of extraneous material and washed with ice-cold saline (0.85%NaCl). Kidneys were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17%) using a potter Elvehjen homogenizer.

Subcellular fractionation

The homogenate was filtered through a muslin cloth and was centrifuged at 800 x g for 5 min at 4°C to separate the nuclear debris. The aliquot so obtained was centrifuged in an Eltek refrigerator centrifuged (Model RC 4100 D) at 105, 00 x g for 20 min at 4°C to get post mitochondrial supernatant (PMS) which was used as a source of enzymes. A portion of the PMS was centrifuged in an ultracentrifuge (Beckman L7-55) at 105,000 x g for 60 min at 4°C. This pellet was considered to be the microsomal fraction and was suspended in phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17%).

BIOCHEMICAL DETERMINATIONS

For all biochemical estimations, post mitochondrial supernatant, cytosol or microsomes of kidney were used. Serum samples were usually utilized.

Estimation of reduced glutathione

Reduced glutathione was determined by the method of Jollow et al. (1974). 1.0 ml sample of PMS was precipitated with 1.0 ml of sulfosalicylic acid (4%). The samples were kept at 4 °C for 1 h and then centrifuged at 1200 x g for 20 min at 4 °C. The assay mixture contained 0.1 ml filtered aliquot, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml DTNB (100 mM) in a total volume of 3.0 ml. The yellow color developed was read immediately at 412 nm on a spectrophotometer (Milton Roy Model-21 D).

Estimation of lipid peroxidation

The assay for microsomal lipid peroxidation was done following the method of Wright et al. (1981) as modified by Khan et al. (2001). 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 microsomes, 0.2 ml ascorbic acid (100 mM), 0.02 ml ferric chloride (100 mM). The reaction mixture
was incubated at 37 °C in a shaking water bath for 1 h. The reaction was stopped by addition of 1.0 ml 10% trichloroacetic acid (TCA). Following addition of 1.0 ml 0.67% thiobarbituric acid (TBA), all the tubes were placed in boiling water-bath for 20 min and then shifted to crushed ice-bath before centrifuging at 2500 × g for 10 min. The amount of malondialdehyde formed in each of the samples was assessed by measuring optical density of the supernatant at 535 nm using spectrophotometer (Milton Roy 21 D) against a reagent blank. The results were expressed as nmol MDA formed/h/g tissue at 37 °C using molar extinction coefficient of 1.56 × 10²/M/cm.

**Estimation of blood urea nitrogen**

Estimation of blood urea nitrogen was done by diacetyl monoxime method of Kanter (1975). Protein free filtrate was prepared. To 0.5 ml of protein free filtrate, were added 3.5 ml of distilled water, 0.8 ml diacetylmonoxime (2%) and 3.2 ml sulphuric acid–phosphoric acid reagent (reagent was prepared by mixing 150 ml 85% phosphoric acid with 140 ml water and 50 ml of concentrated sulphuric acid). The reaction mixture was placed in a boiling water-bath for 30 min and then cooled. The absorbance was recorded at 480 nm.

**Estimation of creatinine**

Creatinine was estimated by the alkaline picrate method of Hare (1950). Protein free filtrate was prepared. To 1.0 ml serum were added, 1.0 ml sodium tungstate (5%), 1.0 ml sulfuric acid (0.6 N) and 1.0 ml distilled water. After mixing thoroughly, the mixture was centrifuged at 800 × g for 5 min. The supernatant was added to a mixture containing 1.0 ml picric acid (1.05%) and 1.0 ml sodium hydroxide (0.75 N). The absorbance at 520 nm was recorded exactly after 20 min.

**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). 2.0 ml of microsomes was suspended in 1.0 ml of solution containing phenol red (0.28 nm), horseradish peroxidase (8.5 units), dextrose (5.5 nm) and phosphate buffer (0.05 M, pH 7.0) and was incubated at 37 °C for 60 min. The reaction was stopped by the addition of 0.01 ml of NaOH (10 N) and then centrifuged at 800 × g for 5 min. The absorbance of the
supernatant was recorded at 610 nm against a reagent blank. The quantity of \( \text{H}_2\text{O}_2 \) produced was expressed as nmol \( \text{H}_2\text{O}_2 \)/g tissue/h based on the standard curve of \( \text{H}_2\text{O}_2 \)-oxidized phenol red.

**Assay for glutathione-S-transferase activity**

Glutathione-S-transferase activity was assayed by the method of Habig et al. (1974). The reaction mixture consisted of 1.475 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml reduced glutathione (1 mM), 0.025 ml CDNB (1 mM) and 0.3 ml PMS (10% w/v) in a total volume of 2.0 ml. The changes in the absorbance were recorded at 340 nm and enzyme activity was calculated as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of \( 9.6 \times 10^3 \)/M/cm.

**Assay for glutathione peroxidase activity**

Glutathione peroxidase activity was assayed by the method of Mohandas et al. (1984). The reaction mixture consisted of 1.49 ml phosphate buffer (0.1 M, pH 7.4), 0.1 ml EDTA (1 mM), 0.1 ml sodium azide (1 mM), 0.05 ml glutathione reductase (1 U ml\(^{-1}\)), 0.05 ml GSH (1 mM), 0.1 ml NADPH (0.2 mM), 0.01 ml \text{H}_2\text{O}_2 (0.25 mM) and 0.1 ml 10% PMS in a total volume of 2 ml. The disappearance of NADPH at 340 nm was recorded at 25 °C. Enzyme activity was calculated as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of \( 6.22 \times 10^3 \)/M/cm.

**Assay for glutathione reductase activity**

Glutathione reductase activity was determined by method of Carlberg and Mannervik (1975). The reaction mixture consisted of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml oxidized glutathione (1 mM), 0.1 ml NADPH (0.1 mM) and 0.1 ml 10% PMS in a total volume of 2 ml. Enzyme activity was quantitated at 25 °C by measuring disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of \( 6.22 \times 10^3 \)/M/cm.

**Assay for catalase activity**

Catalase activity was assayed by the method of Claiborne (1985). The reaction mixture consisted of 1.95 ml phosphate buffer (0.1 M, pH 7.4), 1.0 ml hydrogen peroxide.
(0.019 M) and 0.05 ml 10% PMS in a final volume of 3 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated as nmol H$_2$O$_2$ consumed/min/mg protein.

**Assay for glucose-6-phosphate dehydrogenase activity**

The activity of glucose-6-phosphate dehydrogenase was determined by the method of Zaheer et al. (1965). The reaction mixture consisted of 0.3 ml Tris–HCl buffer (0.05 M, pH 7.6), 0.1 ml NADP (0.1 mM), 0.1 ml glucose-6-phosphate (0.8 mM), 0.1 ml MgCl$_2$ (8 mM), 0.3 ml PMS (10%) and 2.1 ml distilled water in a total volume of 3 ml. The changes in absorbance were recorded at 340 nm and enzyme activity was calculated as nmol NADP reduced/min/mg protein using a molar extinction coefficient of 6.22 × 10$^3$/M/cm.

**Assay for xanthine oxidase activity**

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

**Assay for quinone reductase activity**

The activity of quinone reductase was determined by the method of Benson et al. (1980). The 3 ml reaction mixture consisted of 2.13 ml Tris–HCl buffer (25 mM, pH 7.4), 0.7 ml BSA, 0.1 ml FAD, 0.02 ml NADPH (0.1 mM) and 50 µL (10%) PMS. The reduction of dichlorophenolindophenol (DCPIP) was recorded calorimetrically at 600 nm and enzyme activity was calculated as nmol of DCPIP reduced/min/mg protein using molar extinction coefficient of 2.1 × 10$^3$/M/cm.

**Estimation of protein**

The protein concentration in all samples was determined by the method of Lowry et al, 1964. Peptide bonds form a complex with alkaline copper sulphate reagent, which gives
a blue colour with Folins reagent. Briefly, a 0.1 ml (10% w/v) was diluted to 1 ml water and protein precipitated with equal volume of TCA (10%) samples were kept overnight 4°C and centrifuged at 8000xg for 5 minutes. The supernatant was decanted and discarded. The pellet was dissolved in 5 ml of NaOH (1 N). Finally 0.1 ml of diluted aliquot was taken for colour development. 0.1 ml of aliquot was further diluted to 1 ml with water and then 2.5 ml of alkaline copper sulphate reagent containing sodium carbonate (2%) copper sulphate (1%) and sodium potassium tartarate (2%) was added. Following 10 minutes after addition of alkaline copper sulphate reagent to allow complex formation, 0.25 ml of Folin's reagent was added. After 30 minutes blue colour developed that was read at 660 nm for standard Bovine serum albumin (BSA 0.1 mg/ml) was used.

**ESTIMATION FOR TUMOR MARKERS**

**Assay for ornithine decarboxylase activity**

ODC activity was determined using 0.4 ml renal 105,000xg supernatant fraction per assay tube by measuring release of $^{14}$CO$_2$ from DL-$^{14}$C ornithine by the method of O'Brien et al. (1975). The kidneys were homogenized in Tris-HCl 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 $^{14}$C 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 0.2 ml ethanolamine and methoxyethanol mixture in the central well and kept in a water bath at 37°C. After 1 hr of incubation, the enzyme activity was arrested by injecting 1.0 ml citric acid solution (2.0 M) along the sides of glass tubes and the incubation was continued for 1 hr to ensure complete absorption of $^{14}$CO$_2$. Finally, the central well was transferred to a vial containing 2 ml ethanol and 10 ml toluene based scintillation fluid was added. Radioactivity was counted in a liquid scintillation counter (LKB Wallace-1410). ODC activity was expressed as pmol $^{14}$CO$_2$ released/h/mg protein.
Materials and Method

Assay for renal DNA synthesis

The isolation of renal DNA and assessment of incorporation of $[^3]H$ thymidine into DNA were carried out by the method of Smart et al. (1986). The rat kidneys were quickly removed and cleaned free of extraneous material and homogenate (10% w/v) was prepared in ice-cold water. The precipitate thus obtained was washed with cold TCA (5%) and incubated with cold PCA (10%) at 4 °C overnight. After this, incubation mixture was centrifuged and the precipitate was washed with cold PCA (5%). The precipitate was dissolved in warm PCA (10%), incubated in a boiling water bath for 30 min, and filtered through Whatman 50 paper. The filtrate was used for $[^3]H$ counting in a liquid scintillation counter (LKB Wallace-1410) after adding scintillation fluid. The amount of DNA in filtrate was estimated by diphenylamine method of Giles and Myers (1965). The amount of $[^3]H$ thymidine incorporated was expressed as dpm/μg DNA.

Preparation of Scintillation fluid

Napthalene (52g), 2, 5-diphenyloxazole (3.25g), 1, 4 bis (5-phenyl-2-oxazolyl) benzene (0.065g) were dissolved in a mixture containing 1,4 dioxane (250 ml), toluene (aldehyde free, 250 ml) and methanol (150 ml) and stored in dark bottle. Scintillation fluid was prepared immediately before its use.

HISTOPATHOLOGY

Fixation, dehydration, infiltration and block preparation

The tumors from kidney were excised out and fixed in Bovin's fluid for 24-48 hours. The tissues were then dehydrated by passing through graded series of ethyl alcohol (50 %, 70%, 90% and 100%) for one hour in each giving two changes. These were then cleared in xylene (two changes of one hour each). The cleared tissues were placed for 5 minutes in xylene containing molten paraffin wax at 50-60 °C for infiltration. After giving two changes of molten paraffin (1 hour each) the tissues were embedded in fresh paraffin wax. Embedded tissues was sectioned (5-6 μ thickness) on a microtome and spread on albumin coated glass slides. These were then dried at 35-40°C.
Staining Procedure

Sections were deparaffinised by dipping in xylene and were given two changes. These slides were then passed through graded concentrations of ethyl alcohol (100%, 90%, 70%, 50%, and 30%) with two changes of two minutes each, then kept stained with hematoxyline for one minute and again washed in running water thoroughly. Slides were then passed through 50% and 70% ethyl alcohol and subsequently put into eosin stain (repared in 70% ethyl alcohol). These were then passed through graded series of xylene. All slides were mounted in D.P.X. They were covered with glass cover slips and kept at room temperature for drying.

Statistical analysis

Differences between groups were analyzed using Dunnet's t-test followed by analysis of variance (ANOVA).

**RENAL TUMORS**

**DEN+ Fe-NTA treated group**

Terminology Used for Histopathology

<table>
<thead>
<tr>
<th>AC</th>
<th>Adenocarcinoma,</th>
<th>DC</th>
<th>Dense chromatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCT</td>
<td>Distal convoluted tubule,</td>
<td>DT</td>
<td>Distal convoluted tubule</td>
</tr>
<tr>
<td>G</td>
<td>Glomerulus,</td>
<td>GT</td>
<td>Ghost tissue</td>
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<td>LIC</td>
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<td>PCT</td>
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