Chapter 2 MATERIALS AND METHODS

Materials

2.1 List of chemicals and equipments:

1. Autoanalyzer, (Qualisystems AR-601, Glaxo Smithkline Pharmaceutical Ltd, Mumbai),
2. Microcentrifuge, 3. Homogenizer (Remi motors, Mumbai), 4. Digital pH meter (Micropro labmete),
5. UV/Vis Spectrophotometer (Lab India UV-3000), 6. Electronic balance,
7. Metabolic cages.

2.2 List of chemicals:

1. DTNB - gentamicin, cisplatin, cyclosporine, formalin, hydroxylamine, nitro blue tetrazolium, hydrogen peroxide, petroleum ether, estimation kits (Erba and Swan), methanol,
sodium chloride, sodium hydroxide, sodium metabisulphate, sulphosalicylic acid, thiobarbituric acid.
2. Gentamicin (KAPL), Hydrogen peroxide, Potassium dihydrogen ortho phosphate, Na₂PO₄ 2H₂O (Na₂ HPO₄ 2H₂O), Na₂H₂PO₄- Himedia Labs Pvt. Ltd. Mumbai.
Sodium carbonate (Na₂CO₃), Formaldehyde solution- S.D. Fine chemicals Pvt Ltd. Mumbai.

2.3 Experimental animals:

Adult male albino rats (150-200g) and Swish albino mice were selected for the study. These animals were kept in groups of six as per the CPSCEA guidelines. The animals were procured from the reliable supplier and provided with free access of water. The animals are housed in separate groups (six rats in each cage) in clean sanitized with standard animal cages containing dry paddy husk as bedding material. The bedding materials of the cages were changed every day.

The distribution of animals each group is made in a randomized way and the is also followed as per CPCSEA guidelines. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC.Clear/15/2009-10).

2.4 Collection of Plant material- The stem barks of *Ficus racemosa* were collected during April 2010, from Kikkeri village ranges, in K.R.Petaluk of Mandya district, Karnataka. The collected barks were identified and authenticated by a qualified Botanist.

Methods
2.5 Extraction procedures: The collected stem barks were shaded in the shade. The shade dried stem barks were cleaned and they were ground to get course powder. The shade dried and powdered stem bark of this plant was used for the extraction using a Soxhlet apparatus. 3-4 boiling chips were added into the round bottom solvent vessel. The solvent vessel was dried in a drying oven (about 1h) at 109± 2°C. The course ground powder of *Ficus racemosa* bark was transferred into the extraction glass and the plant material was loaded into the main chamber of the Soxhlet extractor. A cotton swab, moistened with ethanol was used to wipe the sample into the extraction storage part. Then this part of the extractor is connected into the round bottom flask containing extraction solvent. The ground course powder was packed in the tightly in the Soxhlet extractor and ethanol solvent was used for the extraction of the *Ficus racemosa* stem bark powder. In this extraction process 250 ml solvent was used and was carried for about 6h. The extract was filtrated and re-extracted under the same conditions to ensure complete extraction. The condenser was fixed to the Soxhlet extractor and the solvent containing round bottom flask was heated using heating mantle. The vapor of the solvent enters the distillation arm. The ethanol was filled into the solvent vessel and extracted at a temperature of 55°C for 25-30 extraction cycles for 6 h. The solvent was drained into a beaker by opening the spigot on the Soxhlet extractor. The solvent was removed from the extractor and dried by means of rotary drier.

After the extraction the extract was separated and kept for evaporation under reduced pressure at 60°C to get a dried solid product and was stored in dry air tight bottles for the pharmacological studies. The portion of the extract which is non-soluble remains in the thimble and it was discarded. The ethanol extraction of the stem bark powder of *Ficus racemosa* was followed by the aqueous extraction using hot water bath (70°C, 3-4h). The ethanol and water extracts were concentrated and then evaporated to dryness. These extracts were kept in air tight containers.

2.6 Preliminary phytochemical screening (Khandenwal KR et al., 2002)

**Tabel No 4** Preliminary phytochemical screening of EFR and AFR.
<table>
<thead>
<tr>
<th>SN</th>
<th>Phytoconstituents</th>
<th>Name of the test</th>
<th>Procedure</th>
<th>Observation</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td>Dragendorff’s test</td>
<td>Extract+ dil HCl + Dragendorff’s reagent</td>
<td>Orange brown</td>
<td>Indicates the presence of alkaloids.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mayer’s test</td>
<td>Extract+ dil HCl + Mayer’s reagent</td>
<td>White ppt</td>
<td>Indicates the presence of alkaloids.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hager’s test</td>
<td>Extract+ dil HCl + Hager’s reagent</td>
<td>Yellow ppt</td>
<td>Indicates the presence of alkaloids.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wagner’s test</td>
<td>Extract+ dil HCl + Wagner’s reagent</td>
<td>Reddish brown ppt</td>
<td>Indicates the presence of alkaloids.</td>
</tr>
<tr>
<td>2</td>
<td>Glycosides</td>
<td>Keller-killiani test</td>
<td>Extract+ glacial acetic acid+ FeCl₃ + H₂SO₄</td>
<td>Reddish brown color at the junction</td>
<td>Indicates the presence of glycosides.</td>
</tr>
<tr>
<td>3</td>
<td>Saponins</td>
<td>Foam test</td>
<td>Extract+ water + vigorously shaken</td>
<td>Foam formation</td>
<td>Indicates the presence of saponins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Haemolytic test</td>
<td>Extract + one drop of blood</td>
<td>Haemolytic zone</td>
<td>Indicates the presence of saponins</td>
</tr>
<tr>
<td>4</td>
<td>Steroids</td>
<td>Salkowski reaction</td>
<td>Extract + chloroform + Con H₂SO₄</td>
<td>Chloroform layer-red and acid layer –yellow</td>
<td>Indicates the presence of steroids</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Libermann-Burchard reaction</td>
<td>Extract+ chloroform + acetic anhydride + H₂SO₄</td>
<td>First- red, then blue and finally green</td>
<td>Indicates the presence of steroids</td>
</tr>
<tr>
<td>5</td>
<td>Tannins and phenolic compounds</td>
<td>-</td>
<td>Extract+ lead acetate sol.</td>
<td>No white ppt</td>
<td>Indicates the presence of steroids</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Extract + 5% FeCl₃</td>
<td>No deep blue-black color</td>
<td>Indicates the presence of steroids</td>
</tr>
<tr>
<td>6</td>
<td>Flavonoids</td>
<td>Shinoda test</td>
<td>Extract+ 95% ethanol, con H₂SO₄ + 0.5g of magnesium turnings</td>
<td>Pink color</td>
<td>Indicates the presence of flavonoids</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Extract + NaOH</td>
<td>Yellow color</td>
<td>Indicates the presence of flavonoids</td>
</tr>
</tbody>
</table>

**2.7 Dose fixation** - The doses of stem bark extracts of *Ficus racemosa* 200 and 400 mg /kg b.w. were selected for the rats and mice as per the acute toxicity studies conducted by...
Sachan, et al., (2009), and Jaykaran, et al., (2009). As per their studies these doses not produced any toxic effects in rats and mice.

2.8 Protective effect of stem bark ethanol and aqueous extracts of *Ficus racemosa* against gentamicin induced nephrotoxic rats.

2.8.1 Selection of animals: The animals were selected in such a way that they were free from illness, injury and diseases. Before administration animals were kept in their cages for about 5 days and were allowed for acclimatization to the laboratory conditions. Only those animals which are healthy having weights 150-200g were selected and were maintained at standard laboratory conditions.

2.8.2 Preparation and dosing schedule: The selected doses of 200 and 400mg/kg b. for the rats. They are given by oral route using oral gavage.

2.8.3 Observations: Rats are watched for the observations like changes in skin, reddening of the eyes and mucous membrane and other CVS, respiratory, ANS and CNS effects. The motor activity and behavioral pattern were also observed. The rats were also observed for the convulsions and tremors if any during the treatment.

2.8.4 Experimental design: The animals were kept for acclimatization and then they were divided randomly into 6 groups, each consisting of 6 rats. To the group gentamicin was administered at a dose of 80 mg/kg ip for eight days for the induction of nephrotoxicity. For the first three days these animals are treated with normal saline. Three days before the administration of gentamicin, EFR and AFR at a dose of 200 and 400mg/kg. b.w. are administered to group III, IV, V, and VI respectively and this treatment was continued for eight days together with gentamicin administration. The normal rats are administered with vehicle alone throughout the course.
Table No 5 Experimental design in studying the protective effects of EFR and AFR in gentamicin induced nephrotoxicity in rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Trea, dose and rout</th>
<th>Schedule</th>
<th>Studied parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal (vehicle treated)</td>
<td>3 + 8 = 11 days</td>
<td>Kidney weight, urine glucose, urine Na(^+), urine K(^+), urine volume, urinary urea, urinary creatinine, serum urea, serum creatinine, lipid peroxidation, SOD, catalase GSH and histopathology.</td>
</tr>
<tr>
<td>II</td>
<td>Vehicle Gentamicin (80mg/k.g.b.w) i.p.</td>
<td>3 days 8 days</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>EFR (200mg/k.g.b.w) p.o. Gentamicin (80mg/k.g.b.w) i.p.</td>
<td>3 + 8 = 11 days 8 days</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>EFR (400mg/k.g.b.w) p.o. Gentamicin (80mg/k.g.b.w) i.p.</td>
<td>3 + 8 = 11 days 8 days</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>AFR (200mg/k.g.b.w) p.o. Gentamicin (80mg/k.g.b.w) i.p.</td>
<td>3 + 8 = 11 days 8 days</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>AFR (400mg/k.g.b.w) p.o. Gentamicin (80mg/k.g.b.w) i.p.</td>
<td>3 + 8 = 11 days 8 days</td>
<td></td>
</tr>
</tbody>
</table>

2.8.5 Collection and analysis of urine:
Metabolic cages were cleaned to prevent contamination. The experimental animals were transferred to the separate metabolic cages after the last day administration. Twenty-four-hour urines were collected. The collected urine samples were transferred to clean containers and mixed with suitable quantity of purified water.

A drop of conc.HCl was added to the collected urine. This prevents the growth of microbes and also prevents metal hydrolysis. The collected urine was measured and transferred to a cleaned airtight container and used for the urine analysis. From the collected urine samples of rats, urine glucose, sodium, potassium, urinary creatinine and urinary urea were estimated using autoanalyzer and diagnostic kits. (Erba and span diagnostic kits).

2.8.6 Collection of serum:--
The anesthesia was induced with the intraperitoneal administration of ketamine (60mg/kg) and xylazine (5mg/kg) after 24h of the last dose administration. The retro orbital sinus puncture technique was followed for the collection of blood samples of experimental animals. After the blood collection, the blood was transferred to the clean container. The blood samples containers were kept at 37°C for 40min for blood coagulation. The clot was removed from the container and the remaining serum of the experimental animals was added to the centrifuge tube. The centrifuge tubes containing serum were subjected to centrifugation for ten min at 3000RPM. The resultant clear serum was transferred into the clean container and was kept in the refrigerator. From the serum, creatinine and urea were measured. Erba diagnostic kit and semi-auto analyzer are used for these estimations.

2.8.7 Measurement of kidney weight: After the collections of blood samples, the rats were sacrificed for the separation of kidneys. The isolated kidneys are washed with normal saline and weighed.

2.8.8 Preparation of PMS and assessment invivo antioxidant activity-
The collected kidneys of the rats were processed in cold KCl (1.17% w/v) for the homogenization using homogenizing apparatus. The homogenate was transferred to the centrifuge tube after the filtration and then it was centrifuged for 20min at 10,000 rpm, using cold centrifuge maintained. The clear superficial liquid was a taken into clean container. This sample was used for the estimation of thiobarbituric acid reactive substances (TBARS), SOD, catalase, and GSH.

2.8.9 Histopathological estimations: After sacrificing the rats the kidneys were isolated. From each groups two kidneys are selected and these were kept in 10% neutral buffered formalin and were processed for the section cuttings. Each kidney was sliced longitudinally in to two halves. These pieces were again transferred to the container containing 10% formalin solution. The halves of each kidney were kept in the paraffin wax, and using a microtome thin sections of the kidney halve were taken. Hematoxylin and eosin staining agents are used for the staining of these thin sections of the kidney. After staining the thin sections were examined under microscope.
2.8.10 **Principle and procedure for the estimation of glucose from the rat urine.**

(Trinder's method): Glucose in the sample gets oxidized in presence of glucose oxidase. The products of this oxidation are gluconic acid and hydrogen peroxide. The enzyme peroxidase catalyzes the oxidative coupling of 4-amino antipyrine with phenol to give a colored complex known as quinone imine. The absorbance developed after this reaction is depended on the quantity of the glucose the glucose present.

Glucose + O$_2$ + H$_2$O $\rightarrow$ glucose oxidase $\rightarrow$ Gluconic acid + H$_2$O$_2$

H$_2$O$_2$ + Phenol + 4-AAP $\rightarrow$ peroxidase $\rightarrow$ Quinone imine dye + 2H$_2$O

**Clinical significance:** Accurate measurements of glucose in body fluids are important in the diagnosis and management of diabetes, hypoglycaemia, adrenal dysfunction and various other conditions. Elevated levels of glucose appear in the urine as an indication of nephrotoxicity.

**Table- 6 Procedure: Serum glucose assay procedure (end point)**

<table>
<thead>
<tr>
<th>Pipette into tube marked as</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>sample</td>
<td>_</td>
<td>_</td>
<td>10 µl</td>
</tr>
<tr>
<td>Standard</td>
<td>_</td>
<td>10 µl</td>
<td>_</td>
</tr>
<tr>
<td>Enzyme reagent</td>
<td>1ml</td>
<td>1ml</td>
<td>1ml</td>
</tr>
</tbody>
</table>

**Normal value:** Glucose (fasting)- 65-110 mg/dl, Glucose (fasting)- 90-130 mg/dl

2.8.11 **Principle and procedure for the estimation of sodium and potassium in the rat urine.**

**For sodium:** (colorimetric method) Sodium and proteins are precipitate with magnesium and uranyl acetates to form uranyl magnesium sodium acetate. The precipitates are separated by centrifugation. The excess of uranyl ions in supernatant will react with potassium ferrocyanide to produce a brownish color in acidic medium. The absorbance of the color was estimated at 550. The absorbance developed in the sample is inversely proportional to the content of sodium ions present in the sample.
For Potassium:

Potassium is measured turbid metrically. Potassium reacts with sodium tetra phenyl boron, resulting in an insoluble turbid suspension. The extent of turbidity was estimated 630 nm. The concentration of the potassium present in the sample is dependent on the turbidity developed in this reaction.

Clinical significance: Sodium: The extracellular fluid mainly consists of cations like sodium ions. The osmotic pressure, water balance and electrolyte balances of the body fluids like extracellular fluid is very important and this is mainly achieved by the optimum concentration of caions like Na\(^+\) in the tissue fluid. The transportation of these sodium ions across the cell membranes is mainly carried by the membrane bound called sodium pump or Na\(^+\)K\(^+\) ATPase pump. This sodium pump utilizes the ATP for the active transport of cations like sodium across the cell membrane. The transportation of sodium and potassium ions across these pump play a vital role in maintaining the neuronal transmission across the nerve ending and this helps for the transmission of nerve impulses across the nerve endings. The transportation of electrolytes is also important in maintaining the muscle contraction.

The cations like Na\(^+\) ions across the membrane are also responsible for various electrolyte dependent transportation processes. Examples- transportation of sugar molecules across the intestinal cells of the GIT, transportation of sodium and protons across the membrane of the heart. It is the major extracellular cation. Sodium with its associated anion gives the osmotic pressure to the blood plasma. A loss these ions from body results in a decrease of extra cellular fluid volume affecting circulation, renal function and nervous system. Elevated sodium levels are associated with dehydration, CNS trauma or during hyper adrenalism. Decreased sodium level are associated with metabolic acidosis, while decrease in which sodium reabsorption is defective and Addison’s disease.

Potassium: Potassium makes up 70% of the positive ions in the cells. The role of cations like potassium is important in maintaining the neuronal activity. The neuronal membrane expresses Na\(^+\)K\(^+\) ATPase, the osmotic pressure between the cells and interstitial fluid is maintained by the active transport of potassium across the neuronal membrane. For this active process, the Na\(^+\)K\(^+\) ATPase uses energy in the form of ATP. By using ATPs the pump transports 3 sodium ions out of the cells and 2 potassium ions in to the cells. Electrochemical gradient is developed across the cell membrane. The opening of potassium channel across the
neuronal membrane is important in the cell membrane hyperpolarization. It is one of the important positive bearing ions present in the extracellular space of the cells.

An increase in serum potassium may occur in renal failure, acidosis, while decrease in serum potassium is seen in starvation, vomiting, diarrhea, malabsorption syndrome, hyperaldosteronism, metabolic alkalosis, renal tubular defects, testosterone administration etc.

Reference range- Sodium: 135-145mEq/L, Potassium: 3.5-5.1mEq/L

**Table 7 Assay parameters: sodium and potassium**

<table>
<thead>
<tr>
<th>Mode</th>
<th>End Point for Na</th>
<th>End Point for K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>530-550nm</td>
<td>600-630nm</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
<td>37°C</td>
</tr>
<tr>
<td>Optical path length</td>
<td>1 cm</td>
<td>1 cm</td>
</tr>
<tr>
<td>Blanking</td>
<td>Reagent blank</td>
<td>Purified Water</td>
</tr>
<tr>
<td>Sample volume</td>
<td>20 µL</td>
<td>20 µL</td>
</tr>
<tr>
<td>Reagent volume</td>
<td>1100 µL</td>
<td>1000 µL</td>
</tr>
<tr>
<td>Incubation time</td>
<td>5 min</td>
<td>5 min</td>
</tr>
<tr>
<td>Concentration of std</td>
<td>150 mEq/L</td>
<td>5 mEq/L</td>
</tr>
<tr>
<td>Stability of final color</td>
<td>15 min</td>
<td>15 min</td>
</tr>
<tr>
<td>Linearity</td>
<td>200 mEq/L</td>
<td>8 mEq/L</td>
</tr>
<tr>
<td>Units</td>
<td>mEq/L</td>
<td>mEq/L</td>
</tr>
</tbody>
</table>

**Table 8 Sodium estimation procedure: Step-1 Precipitation**

<table>
<thead>
<tr>
<th>Pipette</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample serum</td>
<td>------</td>
<td>20 µL</td>
</tr>
<tr>
<td>Reagent No 5</td>
<td>20 µL</td>
<td>------</td>
</tr>
<tr>
<td>Reagent No 1</td>
<td>1000 microliter</td>
<td>1000 microliter</td>
</tr>
</tbody>
</table>

The contents were mixed and kept at (15-30°C) for 5 min, centrifuged for 10 min at 3000 rpm. The clear supernatant solution was used for the measurement of absorbance.

**Step-II Sodium estimation**

<table>
<thead>
<tr>
<th>Pipette</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear solution from step 1</td>
<td>------</td>
<td>20 µL</td>
<td>20 µL</td>
</tr>
</tbody>
</table>
Reagent No 1 | 20 microliter | ----- | -------
---|---|---|---
Reagent No 2 | 1000 µL | 1000 µL | 1000 µL
Reagent 3 | 100 µL | 100 µL | 100 µL

Mixed well. Incubated at room temperature (15-30°C) for 5 min. Measured the absorbance and concentration directly from the auto analyzer.

2.8.12 Estimation of rat urinary and serum urea

**Theory:** The urea is also known as carbamide is an organic compound and is produced by the metabolism of proteins. Hence it is one of the metabolite and chemically it consists of two amino groups, which are held by a carbonyl functional group. In human and animals urea is the main organic metabolite and is appearing in the urine. Urea is the nitrogen containing metabolite, hence it is also known as blood urea nitrogen. If amino acid content increases then the formation of urea also increases. After the deamination of the amino acids, ammonia is formed, this ammonia combines with carbon dioxide from the respiration and urea is formed.

The urea synthesis takes place by the hepatocytes of the liver. Then the urea transported by the hepatic vein of the liver in to the inferior vena cava. This vein carries urea into the heart and from the heart the pulmonary arteries carries the urea into the lungs. After the purification of blood the oxygenated blood the urea returned to the heart through the pulmonary veins. From the left auricle the urea containing blood pumped to the left ventricle. The left ventricle pumped the blood into the aorta. The aorta branches into the renal artery which delivers blood with urea in to the kidney. The blood enters the glomeruli through the afferent vessel, then to the Bowman’s capsule, through the glomerular filtrate the filtered urea enters the lumen of the PCT cells, then in to the lumen of the loop of Henley, and then to the DCT and finally it is transported in to the collecting duct of the nephron. Urea flows through the urethra out of the body as urine.

**Principle:** The estimation of urea in serum involves the following enzyme catalyzed reactions:

$$\text{Urea} + H_2O \rightarrow 2 \text{NH}_3 + \text{CO}_2$$

$$\text{NH}_3 + \alpha\text{-KETOGLUTARATE} + \rightarrow \text{NADHGLUTAMATE} + \text{NAD}$$
Glutamate dehydrogenase

The absorbance rate was monitored at 340nm. The rate of absorbance is depended on the concentration of urea present in the sample.

**Clinical significance:** Urea is one of the organic molecules formed in the liver after the deamination of the amino acids. Urea is the main component of the non-protein nitrogenous substance of the blood plasma. Mainly the urea is excreted by the nephrons of the kidney and it is eliminated via urine. The serum urea concentration depends on the protein intake, breakdown of proteins and excretion via kidney. Normal blood urea level is 15 to 45 mg/100ml. It is slightly higher in males. There will be slow increase of blood urea with age. Urea concentration depends on the protein content of the diet. Increase in blood urea disorders of kidney.

In pre-renal most of the blood urea is excreted into urine by glomerular filtration. When glomerular filtration rate is decreased, blood urea level is elevated. When plasma volume is diminished as in diarrhea, vomiting, shock, severe burns and in hemorrhage, glomerular filtration decreases and blood urea is elevated. In renal cases blood urea levels are increased in all forms of kidney diseases like acute and chronic glomerulonephritis, late stages of nephritis, polycystic kidney, malignant hypertension and hydro-nephrosis.

In renal cases any type of obstruction in the lower urinary tract diminishes glomerular filtration resulting in elevated blood urea levels. Enlargement of the prostate gland, stones in the urinary tract and bladder, tumors of the bladder are some of the post-renal causes for increased blood urea levels. Elevated serum urea concentrations are observed in impaired kidney function, liver disease, CCF, diabetes, which impairs kidney function. Normal value: 13-45 mg/dL.

**Procedure:** As per the dosage schedule the treatments were made. The last day treatments followed by twenty four hours, the rats were subjected to the anesthesia with the intraperitoneal administration of ketamine (60mg/kg) and xylazine (5mg/kg). The retro orbital sinus puncture technique was followed for the collection of blood samples from the rats. The blood was transferred to clean container and the blood was kept for about 40min at 37°C for the blood coagulation. After the separation of clot the serum was transferred to another container and the serum was taken into a clean centrifuge tubes. The blood sample containing centrifuge tubes were subjected to centrifugation for ten minutes at 3000RPM.
Then the clear supernatant serum was transferred to a clean container and was stored in cool place. The serum urea was estimated from this sample of serum using Erba diagnostic kit.

**Table No 9 Urea estimation assay procedure**

<table>
<thead>
<tr>
<th>Pipette</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000µl</td>
<td>1000µl</td>
</tr>
<tr>
<td>Standard</td>
<td>100µl</td>
<td>-</td>
</tr>
<tr>
<td>Test</td>
<td>-</td>
<td>100µl</td>
</tr>
</tbody>
</table>

The contents were mixed well and the final absorbance and concentrations were recorded after 80 sec of mixing.

2.8.13 Estimation of rat urinary and serum creatinine:

**Theory:** Creatine and creatinine are not same. The creatine is also known as creatinine citrate. The kidney, liver and pancreas produce creatine and through blood circulation creatine reaches the muscles. The muscle needs creatine in the form of creatine phosphate. The creatinine is the end product of creatine phosphate. Hence creatine is required by the muscles. Depending on the muscle mass constant formation of creatinine takes place. Creatine is a type of amino acid, but it is not found in the proteins and chemically creatine is an organic acid containing nitrogen.

The enzyme creatine kinase present in the muscles helps in transferring high energy phosphate group ATP to the creatine molecule. This results in the formation of creatine phosphate. Creatine phosphate, helps in converting ADP to ATP, thus involved in the formation of ATP molecules. This helps in providing additional ATP at the time of severe anaerobic exercise. Therefore creatine phosphate has a major importance for energy production in muscles. Creatine synthesized by the liver, kidneys and pancreas. An additionally 1-2g per day is provided by the diet food containing fish and meat. The kidney has the capacity to excrete creatinine, which is a waste product of creatinine phosphate. Each day kidney excrete about 1 to 2 g creatinine get filtered by the glomerulus of the kidney. About two percent of total creatinine gets converted to creatinine each day.
Through blood circulation the creatinine is transported renal glands. These glands remove most of the creatinine and this gets excreted in the urine. The afferent arteriole carries the creatinine into the glomerulus. In case of damaged kidney the glomerular filter fails to filter the creatinine normally. Less than the normal creatinine gets filtered by the glomerular filter. The excretion of the creatinine through urine decreases, the serum concentration of creatinine gets increases. Hence by the estimation of urine or serum creatinine is one of the important tools for the evaluation of the kidney function.

In gentamicin induced acute renal failure loses its capacity to excrete creatinine through urine. This increases the serum creatinine level. In renal failure, the filtering (GFR) of the kidney is deficient, blood levels of some substances will rise and urine levels will decrease.

The amount of creatinine produced is nearly constant and the production of creatinine normally is a normal function of muscle mass. The excess is transported to the kidney through renal blood vessels. It is then removed from the blood plasma by glomerular filtration and then excreted through urine without any appreciable absorption by the cells of the nephrons. It is one of the biomarker determine the functioning of the kidney. Rats induced with prolonged doses of gentamicin (8 days) will cause kidney damage leading to nephrotoxicity. In this condition creatinine will not be excreted completely from urine and hence the concentration of creatinine in serum will increase.
Principle:

Creatinine reacts with alkaline picrate to produce an orange yellow color and this follows the Jaffe’s reaction. A reddish orange color of sodium picrate is formed due to the reaction between creatinine and picrate in presence of alkaline medium such as sodium hydroxide. The absorbance of the orange yellow color formed depended on creatinine content.

Procedure: The collected urine was measured and transferred to a cleaned airtight container and used for the urine analysis. From the collected urine samples of rats, urine creatinine was measured using autoanalyzer and diagnostic kits. The rats were anesthetized with the intraperitoneal administration of ketamine (60mg/kg) and xylazine (5mg/kg).

For the biochemical estimations the blood samples from the rats are obtained by retro orbital sinus puncture technique. The blood samples are collected into well cleaned containers and these containers were kept for 40min at 37°C for the development of coagulation of blood. Then the serum was separated and it is then was transferred in to well cleaned centrifuge tubes. The serum samples of the rats were subjected to centrifugation using cold centrifuge at 3000 RPM and for 40min. Then the serum was transferred to another clean container after the filtration and was stored in the cool place. This is used for the measurement of serum urea using Erba diagnostic kit. The working reagent, standard and test solutions (rat urine/ serum) were prepared. The absorbance and concentrations (mg/dL) of the creatinine were measured directly from the autoanalyzer.

Table No 10 Creatinine estimation assay procedure

<table>
<thead>
<tr>
<th>PIPETTE</th>
<th>STANDARD</th>
<th>TEST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000µl</td>
<td>1000µl</td>
</tr>
<tr>
<td>Standard</td>
<td>100µl</td>
<td>-</td>
</tr>
<tr>
<td>Test</td>
<td>-</td>
<td>100µl</td>
</tr>
</tbody>
</table>

The contents were mixed well, the absorbance and concentrations (mg/dl) were recorded 80 sec after mixing. Normal serum creatinine values: 0.7 -1.4 mg/dL

2.8.14 Estimation of lipid peroxidation (LPO)

Mechanism and Principle: The lipids are the important components of the cell. The lipid peroxidation reaction causes oxidative deterioration of lipids of the cell. The carbon-carbon
double bonds present in the lipids get affected by the lipid peroxidation. The structural integrity of the cell gets affected by the lipid peroxidation. The oxidative reactions of unsaturated fatty acids, glycolipids, cholesterol esters, cholesterol leads to the release of non-radical intermediates called as lipids peroxides. These lipid peroxides produces damaging effects on the cell. The lipid peroxides formation leads to the generation of various enzymatic and non-enzymatic reactions and these reactions responsible for the various ROS.

The ROS exert toxic effects in the tissues of the body by various pathways. Various ROS are generated by this reaction are responsible for the injurious effect of the nephron. These includes hydroxyl radicals, lipid peroxyl, etc. Peroxinitrite is also formed from nitric oxide. All these ions exert injurious effect on the cells and these ions are considered as a unit and are now named ‘free radicals’.

Superoxide radical (O$_2^-$) (ROS) is generated in the mitochondrial electron transport mechanism in the cell. Its formation is dependent on the exogenous components of the cell. The catalytic enzymatic reactions lead to the production of hydrogen peroxide. The enzymes responsible for the release of hydrogen peroxide are present on the microsomes, peroxisomes and mitochondria of the cells. The hydrogen peroxide is also produced in plant or animal cells by the enzyme super oxide dismutase. The dismutation of O$_2^-$ by superoxide dismutase causes the release of H$_2$O$_2$. The formation of H$_2$O$_2$ leads to the lowering of oxidative reactions. The H$_2$O$_2$ is scavenged by another enzyme present in the cells called catalase. Hence the dual function of dismutation and catalase is responsible for removal of H$_2$O$_2$ and as well as the maintenance of true cellular antioxidant activity.

The mechanism involved in the lipid peroxidation is explained by three mechanisms like

The self-oxidation is 3 sequences: Initiation, propagation and termination. The lipid peroxidation initiation in a cell which is free from peroxide lipid system includes the entry of ROS. This entry leads to the generation of free radicals from fatty acids of poly-saturated type. The hydroxyl ion is the important ROS responsible for this initiation and the O$_2^*$ is insufficiently reactive species.

\[
\text{CH}_2 + \text{OH} \rightarrow \text{CH} + \text{H}_2\text{O}
\]

The mannitol, formate and tocopherol are the antioxidant substances inhibit the lipid peroxidation.
The conjugated diene is formed by the molecular rearrangement and under aerobic conditions. These conjugated form of the dienes are combine with oxygen and peroxyl radicals, $\text{ROO}^\cdot$, are formed.

Lipid peroxidation is one of the important oxidative reaction occur in the cells and causes the pathological changes in the cell due to the cell processes like cancer, aging and blood vessel disorders like atherosclerosis.

Lipid Peroxidation brings about several changes in biological membrane. Lipid peroxidation is the important oxidative destructive reaction occurs in cellular organelles. The impact of lipid peroxidation is the alteration of biochemical functions of the cell and structural components of the cell. This may be responsible of the severe damage of the components of the cell and this may also leads to the necrosis of the cell. The elevation of lipid peroxides and the aldehydes in the biological fluids indicates the oxidative stress. The anti-oxidants help in reducing the levels of TBARS in the body fluids. The anti-oxidation depends on the quantity of the antioxidants in the biological fluids.
**Procedure:**
The assay was performed according to the procedure shown in the article Tajdar Hussain Khan AB et. al. 2011. The reaction mixture was prepared by using 0.58ml phosphate buffer (0.1M, pH 7.4), 0.2ml PMS, 0.2ml ascorbic acid (1mM) and 0.02ml ferric chloride (100mM) in a total volume of 1 ml.

The mixture was incubated at 37°C in a shaking water bath for 1h. Then 1ml 10% w/v trichloroacetic acid and 1ml of 0.67% of TBA was transferred to the tubes. For about 20 min all these tubes were kept in the water baths. The absorbance was measured at 535nm. The results were expressed as nM of MDA formed /100g. of tissue. The extinction coefficient of 1.56×105 M−1cm−1 was used to calculate the amount of MDA.

**Calculation**
The amount of serum MDA was calculated by using the formula as

\[
\text{Conc. of MDA} = \frac{\text{Absorbance of test}}{\text{Molar extinction co-efficient}} \times \frac{\text{Total volume}}{\text{Sample volume}}
\]

2.8.15 Measurement of reduced GSH.

**Theory**

The glutathione is a tripeptide molecule. The glutamate peptide molecule is formed by a gamma peptide linkage cysteine and carboxylic acid of the glutamate. As it acts as an antioxidant it protects the cellular components from the damaging effects caused by ROS and lipid peroxides.

In animal cells thio groups act as reducing agents, existing in a concentration of approximately 5mM. In this reaction, the glutathione is oxidized to glutathione disulfide. Once oxidized, glutathione get reduced by the action of the enzyme called as glutathione reductase by utilizing an electron donor known as NADPH. The balance between reduced and oxidized forms alters the cellular functions altered and this is an indicative measure of cellular damage. Glutathione is the important thiol available in excess and the ratio between glutathione and glutathione disulfide is the important redox in the animal cells.
The deficiency of glutathione is responsible for the oxidative stress. This leads to the processes of the cell like aging and etiological, and pathological development of number of diseases.

**Principle**

The sulfhydryl groups present in glutathione forms a colored product with DTNB, the color developed was estimated at 412 nm.

**Procedure:**

Reduced glutathione (GSH) in the kidneys was measured by precipitating PMS (10%) with 1ml sulphosalicylic acid (4%). The kidney PMS samples were stored at refrigerator temperature (4°C) for about1h. The sample was centrifuged c (1200RPM for 15min at 4°C). The clear sample of 0.1ml was added with 2.7ml of phosphate buffer (total 3ml). The absorbance of the developed yellow color was read at 412nm using spectrophotometer. The GSH content in the kidney tissue was measured using molar extinction coefficient 1.36 x 10^4 in mcg/mg of protein. The amount of serum GSH was calculated by using the formula.

2.8.16 Estimation of superoxide dismutase (SOD)-These enzymes are essential for the antioxidant activity, they scavenge the stress mediating substances released in the body as they increases the reaction of dismutation caused by the interaction between superoxide ions and \( \text{H}_2\text{O}_2 \). Hence they act as body defense by preventing damaging effects caused by these superoxide ions and \( \text{H}_2\text{O}_2 \). Different types of SOD are exists in different tissues of the body. Some are located in the cytoplasm (SOD₁), some are located in the mitochondria (SOD₂) and some are located in the ECF. The drugs like gentamicin, cyclosporine and cisplatin increases the damaging effect in the kidney cells by decreasing the levels of SOD. Thus they exert damaging effects in the nephron tissue leading to the development of nephrotoxicity. This SOD helps in preventing damaging effects of the cells from the oxidative stress.

The normal cellular processes lead to the generation of superoxide radicals, especially high energy yielding reactions and must be broken down regularly.

**Procedure**- 100 µl of serum was taken in Beckman quartz cuvette of one cm path length. 1 ml of sodium carbonate and NBT (0.4 ml) was taken in the cuvette and the zero min reaction was performed at 560 nm. This reaction was preceded by the addition of 0.4 ml of
hydroxylamine HCl to the test tube. The reaction mixture were then incubated at 37° C for 5 min, the reduction of nitro blue tetrazolium was read at 560 nm.

2.8.17 Estimation of catalase (CAT)

Theory: Catalase is one of the important antioxidant enzymes. Normally it is located in most cells of the most of the organism. Catalase decomposes the formed hydrogen peroxide by the other oxidative pathway. The released hydrogen peroxide also exerts damaging effect on the normal tissues. The main function of the catalyze enzyme is the decomposition of H₂O₂, thereby preventing the damaging effects caused by this oxidative product. The products of this decomposition are water and oxygen. The catalase is structurally a protein together with an iron moiety. As the nephrotoxicants releases more number of oxidative stress molecules, more catalase get utilized for the decomposition of increased levels of H₂O₂. Hence the decreased level of catalase is an indicative of renal injury. Catalase enzyme is located in the cell organelle like peroxisomes. Catalase accelerates chemical reactions, as otherwise the reactions proceed slowly. The catalase is the enzyme, which acts as a catalyst.

Principle

It is a known metal enzyme, which catalyze one of the most efficient protease catalysis known to promote the redox reaction. \(2\text{H}_2\text{O}_2 \rightarrow \text{Catalase} \rightarrow 2\text{H}_2\text{O} + \text{O}_2\). Hydrogen peroxide is an intracellular precursor of more reactive oxidants such as OH·, although catalase is rather specific for \(\text{H}_2\text{O}_2\). In oxidative stress the catalase level are reduced. Elevation of the enzyme activity indicates the effectiveness of the drug. Estimation of the catalase activity was done by determining the decomposition of hydrogen peroxide at 240 nm in an assay mixture containing phosphate buffer pH 7.0.

Reagents

Sodium phosphate buffer (0.3 M, pH 7.2): 4.6803 g of disodium hydrogen phosphate and 4.2588 g of sodium hydrogen phosphate was separately dissolved in 100 ml of distilled water and mixed. Hydrogen-peroxide (0.042ml=12.3 M): 0.14 ml of 30% hydrogen-peroxide was diluted to 1000 ml with distilled water.

Procedure

100 µl of serum was added to 1.9 ml of phosphate buffer and the absorbance was estimated at 240 nm. This mixture is added with 1 ml of \(\text{H}_2\text{O}_2\)was added and the absorbance noted after standing for 1 min at 240 nm using phosphate buffer as blank. The molar extinction coefficient of hydrogen peroxide was found to be 43.6 M cm⁻¹ and this was utilized for the
determination of the catalase activity and was expressed as μmol/mg protein.. The amount of serum catalase was calculated by using the formula.

2.9 Study of protective effects of ethanol and aqueous stem bark extract of *Ficus racemosa* in cisplatin induced nephrotoxicity in mice.

2.9.1 Experimental design-

Swiss albino mice (25-30g) were used for the present study. The mice were kept in well ventilated AC rooms at a temperature of 24±2 °C and had free access to water and food. The animal care and experimental protocols were made in accordance with CPCSEA/ IAEC.

### Table No 11 Experimental design in studying the protective effects of EFR and AFR in cisplatin induced nephrotoxicity in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment, dose and route of administration</th>
<th>Schedule</th>
<th>Studied parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal (vehicle treated)</td>
<td>1(^{st}) h, 24(^{th}) h and 48(^{th}) h.</td>
<td>Serum urea, serum creatinine, LPO, GSH and catalase,</td>
</tr>
<tr>
<td>II</td>
<td>Vehicle Cisplatin (12mg/k.g.b.w) i.p.</td>
<td>1(^{st}) h, 24(^{th}) h and 48(^{th}) h. 1(^{st}) day</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>EFR (200mg/k.g.b.w) p.o. Cisplatin (12mg/k.g.b.w) i.p.</td>
<td>1(^{st}) h, 24(^{th}) h and 48(^{th}) h. 1(^{st}) day</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>EFR (400mg/k.g.b.w) p.o. Cisplatin (12mg/k.g.b.w) i.p.</td>
<td>1(^{st}) h, 24(^{th}) h and 48(^{th}) h. 1(^{st}) day</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>AFR (200mg/k.g.b.w) p.o. Cisplatin (12mg/k.g.b.w) i.p.</td>
<td>1(^{st}) h, 24(^{th}) h and 48(^{th}) h. 1(^{st}) day</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>AFR (400mg/k.g.b.w) p.o. Cisplatin (12mg/k.g.b.w) i.p.</td>
<td>1(^{st}) h, 24(^{th}) h and 48(^{th}) h. 1(^{st}) day</td>
<td></td>
</tr>
</tbody>
</table>

2.9.2 Methodology-

The mice were selected and randomized into 6 groups, each group consisting of 6 mice. Nephrotoxicity in mice was induced by the administration of cisplatin (12mg/kg). The stem bark ethanol and aqueous extracts of *Ficus racemosa* (EFR and AFR) were administered (200, 400mg/kg) to mice orally 1 h before the administration of cisplatin and at 24h and 48h after cisplatin injection.
The parameters were studied 72 h after cisplatin administration. The normal control group was not administered with either extract or cisplatin. The mice were selected and randomized into 6 groups, each group consisting of 6 mice. After 72h of last treatment the mice were anaesthetized by chloroform. Blood was then collected by cardiac puncture and kidneys were dissected out immediately. The blood sample was allowed to clot at 37°C for 40min and the clot was removed and the serum was taken in a cleaned centrifuge tube. The sample serum was centrifuged at 3000 RPM for 10min. the serum sample was kept in the refrigerator in a cleaned airtight container and was used for the determination of creatinine and urea levels in the serum sample using autoanalyzer and Erba diagnostic kits.

2.9.3 Post mitochondrial supernatant (PMS) preparation- Kidneys were removed immediately from the sacrificed mice. They were kept in a container containing ice cold normal saline. The kidneys are homogenized using homogenizer after adding chilled KCl (0.17%). The resultant homogenate was subjected for the centrifugation at 800RPM for a period of 4°C. The supernatant liquid so obtained was again centrifuged at 10,500 RPM for 20min. The PMS so obtained was used for the study of various biochemical parameters like SOD, LPO, glutathione and catalase and catalase (Terky N et.al , 2009).

2.9.4 Estimation of serum urea-

The liver produces ammonia at the time of metabolism of proteins. The formed ammonia, then converted into another product called urea. The renal glands have the ability to filter urea in the glomerular filter. The urea is also excreted through sweat. Some urea remains in the blood and is termed serum urea. Serum urea concentration is important to determine as it gives the information about the health of the kidney. Kidney excretes excess amount of urea, if there is defect in the kidney.

Urea is also known as carbamide. It has two amine groups and joined by a ketone group. The metabolism of nitrogenous substances is aided by urea present in the blood. The normal serum urea is 8-20mg/dL of blood plasma. The serum urea present in the mice blood samples was estimated using semi autoanalyzer and Erba diagnostic kit.

2.9.5 Serum creatinine estimation-

Creatinine produces reddish yellow color when it reacted with picric acid in presence of sodium hydroxide solution. The alkaline medium is provided by sodium hydroxide. In Jaffe’s
reaction, the sodium salt of picric acid, sodium picrate is formed in the first phase. The sodium picrate reacts with creatinine to form reddish yellow crystals of creatinine picrate and upon acidification with HCl these crystals are dissolved. The depth of the color intensity is directly proportional to the amount of creatinine present in the sample (Nigam et al. 2007). Creatinine present in the mice serum was estimated using semi-automatic analyzer.

2.9.6 Lipidperoxidation (LPO) estimation-Lipid peroxidation occurs in the cell leading to the release of oxygen free radicals. These radicals cause oxidative damage of cell membrane by oxidation of cellular lipids. The reactive hydrogens are present in the fats containing polyunsaturated fatty acids. The lipid peroxidation occurs in three stages namely-initiation, propagation and termination. Finally the lipid peroxidation leads to the formation of lipid peroxide. The antioxidants like vitamin E prevent the lipid peroxidation, thereby protecting the cell membrane. The lipid peroxidation end products are also injurious to cells. The end products of this reaction may cause cancer or alteration of the gene. The end product of lipid peroxidation like malonialdehyde may react with components of the DNA, leading to cellular toxicity. The diagnostic test for the determination of lipid peroxidation in the animal cells involves the quantification of the malonialdehyde. The thiobarbiturate acid reactive substance assay is one of the methods for the estimation of extent of lipid peroxidation in the tissue cells. In this assay method the thiobarbituric acid reacts with melonaldehyde to form a colored complex. By measuring the extent of absorbance at 586nm, the extent of lipid peroxidation may be calculated.

The MDA content was assayed by TBARS method. The reaction mixture was prepared by mixing 0.2ml of 8.1% w/v sodium lauryl sulphate, 1.5 ml of 20% v/v acetic acid. The pH was adjusted by adding NaOH, to this 1.5ml of 0.8% w/v aqueous solution of thiobarbituric acid and 0.2ml of 10w/v of PMS were added. The resultant was made up to 4ml with distilled water. The mixture was heated at 95°C for 60min. It was cooled with tap water and 1ml distilled water and 5ml mixture of n-butanol and pyridine (15:1) were added and centrifuged. The organic layer was separated and its absorbance measured at 532nm.

2.9.7 Glutathione estimation- (GSH) Reduced glutathione is important antioxidant occurring in the living cells. The xenobiotics are detoxified by the reduced glutathione. It also helps the living cells by scavenging the excess hydroperoxides, thereby protecting the cell by
injury caused by the stress oxidative radicles. This antioxidant also helps in maintaining the oxidation status of the protein sulfhydryl.

Reduced glutathione was determined using the modified method of Elliman (1951). To 1 ml of the kidney homogenate 0.5 ml of Elliman’s reagent and the volume make up to 3 ml with phosphate buffer and the resultant solution was kept aside and its absorbance was measured at 415 nm. The percentage inhibition of GSH was calculated using the equation:

\[
\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100
\]

**2.9.8 Estimation of SOD –**

These enzymes are essential for the antioxidant activity, they scavenge the stress mediating substances released in the body as they increase the reaction of dismutation caused by the interaction between superoxide ions and \( \text{H}_2\text{O}_2 \). Hence they act as body defense by preventing damaging effects caused by these superoxide ions and \( \text{H}_2\text{O}_2 \). Different types of SOD exist in different tissues of the body. Some are located in the cytoplasm (SOD\(_1\)), some are located in the mitochondria (SOD\(_2\)) and some are located in the ECF. The drugs like gentamicin, cyclosporine and cisplatin increases the damaging effect in the kidney cells by decreasing the levels of SOD. Thus they exert damaging effects in the nephron tissue leading to the development of nephrotoxicity. Thus SOD helps in preventing damaging effects of the cells from the oxidative stress. The normal cellular processes lead to the generation of superoxide radicals, especially high energy yielding reactions and must be broken down regularly.

100 µl of serum was taken in Beckman quartz cuvette of one cm path length. 1 ml of sodium carbonate and NBT (0.4 ml) was taken in the cuvette and the zero min reaction was performed at 560 nm. This reaction was preceded by the addition of 0.4 ml of hydroxylamine HCl to the test tube. The reaction mixture were then incubated at 37° C for 5 min, the reduction of nitro blue tetrazolium was read at 560 nm. A parallel control without serum was run.

**2.9.9 Catalase estimation –**

**Procedure**

100 µl of serum was added to 1.9 ml of phosphate buffer and the absorbance was estimated at 240 nm. 1 ml of \( \text{H}_2\text{O}_2 \) was added to this mixture and the absorbance noted after standing for 1 min at 240 nm using phosphate buffer as blank. The molar extinction coefficient of hydrogen peroxide was found to be 43.6 M cm\(^{-1}\) and this was utilized for the determination of the
catalase activity and was expressed as µmol/mg protein. The amount of serum catalase was calculated by using the formula.

2.10 Study of protective effects of ethanol and aqueous stem bark extract of *Ficus racemosa* in cyclosporine induced nephrotoxicity in rats.

2.10.1 Experimental design: The cyclosporine (50mg/kg p.o.) given for 21 days for the induction of nephrotoxicity in rats of group II.

The EFR and AFR are given to the rats of group III and IV for a period of 24 days including 3 days pretreatment, but cyclosporine was given to these rats for 21 days. Only vehicle was administered to the normal control rats of group I. The grouping of rats was made randomly and six animals were selected for each group. The bark extracts and cyclosporine were administered orally.
Table No-12 Experimental design in studying the protective effects of EFR, AFR in cyclosporine induced nephrotoxicity.

<table>
<thead>
<tr>
<th>Group n=6</th>
<th>Treatment, dose and route of administration</th>
<th>Schedule</th>
<th>Studied parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal (vehicle treated)</td>
<td>3 + 21 = 24days</td>
<td>Lipid peroxidation, GSH, SOD, serum urea, serum creatinine, catalase.</td>
</tr>
<tr>
<td>II</td>
<td>Vehicle</td>
<td>3 days 21 days</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclosporine (50mg/k.g.b.w) p.o.</td>
<td>3 days 21 days</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>EFR (200mg/k.g.b.w) p.o.</td>
<td>3 + 21 = 24 days 21 days</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclosporine (50mg/k.g.b.w) p.o.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>EFR (400mg/k.g.b.w) p.o.</td>
<td>3 + 21 = 24 days 21 days</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclosporine (50mg/k.g.b.w) p.o.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>AFR (200mg/k.g.b.w) p.o.</td>
<td>3 + 21 = 24 days 21 days</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclosporine (50mg/k.g.b.w) p.o.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>AFR (400mg/k.g.b.w) p.o.</td>
<td>3 + 21 = 24 days 21 days</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclosporine (50mg/k.g.b.w) p.o.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

I. Normal control. II. Cyclosporine (50mg/kg. p.o.) for 21 days, III. Cyclosporine (50mg/kg. p.o.) for 21 days + EBFR (200mg/kg ) for 24 days IV. Cyclosporine (50mg/kg. p.o.) for 21 days+ EFR (400mg/kg ) for 24 days V. Cyclosporine (50mg/kg. p.o.) for 21 days+ AFR (200mg/kg ) for 24 days VI.

Cyclosporine (50mg/kg. p.o.) for 21 days+ ABFR (400mg/kg) for 24 days. At the end of the study the treatment was made on 24th day. Next day after the last treatment, the blood samplings were collected by cardiac puncture method. The blood was processed and the serum was transferred into a clean container and it was used for the estimation of serum creatinine, and urea using semi-automatic analyzer and Erba diagnostic kit.

2.10.2 Post mitochondrial supernatant (PMS) preparation- Kidneys were removed immediately from the sacrificed rats. They were kept in a container containing ice cold normal saline. The kidneys are homogenized using homogenizer after adding chilled KCl (0.17%). The resultant homogenate was subjected for the centrifugation at 800RPM for a period of 4°C. The supernatant liquid so obtained was again centrifuged at 10,500 RPM for
20min. The PMS so obtained was used for the study of various biochemical parameters like SOD, LPO, glutathione and catalase and catalase (Terky N et.al , 2009).

2.10.3 Serum creatinine and urea estimation-
Creatinine produces reddish yellow color when it reacted with picric acid in the presence of sodium hydroxide solution. The alkaline medium is provided by sodium hydroxide. In Jaffe’s reaction, the sodium salt of picric acid, sodium picrate is formed in the first phase. The sodium picrate reacts with creatinine to form reddish yellow crystals of creatinine picrate and upon acidification with HCl these crystals are dissolved. The depth of the color intensity is directly proportional to the amount of creatinine present in the sample (Nigam et.al. 2007). Creatinine present in the rats serum was estimated using the semi-automatic analyzer. Creatinine present in the mice serum was estimated using semi-automatic analyzer.

During the metabolism of protein in the body, the ammonia one of the metabolic waste is formed in the liver. This gets broken down into another byproduct called urea. Serum urea concentration is important to determine if the kidneys are working normally. If the serum urea level is elevated indicates that the kidneys are not filtering properly.

The liver produces ammonia at the time of metabolism of proteins. The formed ammonia, then converted into another product called urea. The renal glands have the ability to filter urea in the glomerular filter. The urea is also excreted through sweat. Some urea remains in the blood and is termed serum urea. Serum urea concentration is important to determine as it gives the information about the health of the kidney. The kidney excretes excess amount of urea if there is a defect in the kidney.

Urea is also known as carbamide. It has two amine groups and joined by a ketone group. The metabolism of nitrogenous substances is aided by urea present in the blood. The normal serum urea is 8-20mg/dL of blood plasma. The serum urea present in the rat blood samples was estimated using semi autoanalyzer and the Erba diagnostic kit.
2.10.4 LPO assay

Lipid peroxidation occurs in the cell leading to the release of oxygen free radicals. These radicals cause oxidative damage of cell membrane by oxidation of cellular lipids. The reactive hydrogens are present in the fats containing polyunsaturated fatty acids. The lipid peroxidation occurs in three stages namely-initiation, propagation and termination. Finally the lipid peroxidation leads to the formation of lipid peroxide. The antioxidants like vitamin E prevent the lipid peroxidation, thereby protecting the cell membrane. The lipid peroxidation end products are also injurious to cells. The end products of this reaction may cause cancer or alteration of the gene. The end product of lipid peroxidation like malonaldehyde may react with components of the DNA, leading to cellular toxicity. The diagnostic test for the determination of lipid peroxidation in the animal cells involves the quantification of the malonaldehyde. The thiobarbiturate acid reactive substance assay is one of the methods for the estimation of the extent of lipid peroxidation in the tissue cells. In this assay method the thiobarbituric acid reacts with melonaldehyde to form a colored complex. By measuring the extent of absorbance at 586nm, the extent of lipid peroxidation may be calculated.

The MDA content was assayed by TBARS method. The mixture was prepared by mixing 0.2ml of 8.1%w/v sodium lauryl sulphate, 1.5 ml of 20% v/v acetic acid. The pH was adjusted by adding NaOH, to this 1.5ml of 0.8% w/v aqueous solution of thiobarbituric acid and 0.2ml of 10w/v of PMS were added. The resultant was made up to 4ml with distilled water. The mixture was heated at 95°C for 60min.

\[
\text{Conc. of MDA} = \frac{\text{Absorbance of test}}{\text{Molar extinction co-efficient}} \times \frac{\text{Total volume}}{\text{Sample volume}}
\]

2.10.5 Glutathione estimation-

Reduced glutathione is an important antioxidant occurring in the living cells. The xenobiotics are detoxified by the reduced glutathione. It also helps the living cells by scavenging the excess hydroperoxides, thereby protecting the cell from injury caused by the stress oxidative radicals. This antioxidant also helps in maintaining the oxidation status of the protein sulphhydryl.

Reduced glutathione was determined using the modified method of Elliman (1951). To1ml of the kidney homogenate 0.5ml of Elliman’s reagent and the volume make up to 3ml with
phosphate buffer and the resultant solution was kept aside and its absorbance was measured at 415nm. The percentage inhibition of GSH was calculated using the equation:

\[
\text{Percentage Inhibition} = \left(1 - \frac{A_{	ext{sample}}}{A_{	ext{control}}}\right) \times 100
\]

2.10.6 Catalase estimation- 100 µl of serum was added to 1.95 ml of phosphate buffer and the absorbance was estimated at 240 nm. This mixture is added with 1 ml of H2O2 was added and the absorbance noted after standing for 1 min at 240 nm using phosphate buffer as blank. The molar extinction coefficient of hydrogen peroxide was found to be 43.6 M cm-1 and this was utilized for the determination of the catalase activity and was expressed as µmol/mg protein.

2.11 Study of protective effects of ethanol and aqueous stem bark extract of *Ficus racemosa* in lithium induced nephrogenic diabetes insipidus.

2.11.1 Methodology-

Nephrogenic diabetes insipidus (NDI) in rats is to developed by feeding lithium carbonate containing rat chow (60 mmol/kg) dry food for one month. Normal diets without lithium was given to the controls were given food without lithium. All rats were allowed free access to drinking fluid to prevent volume depletion. The rats were selected and randomly they were divided into six groups and each group consisting of six rats. The bark extract was administered orally.

The minimum and maximum doses of ethanol (EFR) and aqueous (AFR) stem bark extract of *Ficus racemosa* were selected. The rat doses of EFR and AFR were selected as 200, 400mg/kg b.w, p.o. respectively. The EFR and AFR were administered three days before the lithium administration and continued for 4 weeks.

I.Normal control. Normal diet. II.Lithium diet. III.Lithium diet + EFR (200mg/kg bw p.o.)

IV.Lithium diet + EFR (400mg/kg bw p.o.), V.Lithium diet + AFR (200mg/kg bw p.o.), VI.Lithium diet + AFR (400mg/kg bw p.o.).

Parameters studied.

Serum sodium and potassium, serum creatinine, BUN, urine volume and specific gravity of urine.
Study of protective effects of stem bark extracts of Ficus racemosa in drug induced diabetes insipidus and nephrotoxicity in animal models

Table No 13: Study design for the study of the protective effect of EFR and AFR in lithium induced nephrogenic diabetes insipidus.

<table>
<thead>
<tr>
<th>Group n=6 rats</th>
<th>Treatment, dose and route of administration</th>
<th>Schedule</th>
<th>Studied parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal (Normal diet treated)</td>
<td>3 + 28 = 31 days</td>
<td>Urine flow, specific gravity of urine, Body weight, BUN, serum sodium, blood potassium, and creatinine.</td>
</tr>
<tr>
<td>II</td>
<td>Vehicle&lt;br&gt;Iithium diet 60 mmol/kg dry food</td>
<td>3 days&lt;br&gt;28 days</td>
<td></td>
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<tr>
<td>III</td>
<td>EFR (200mg/k.g.b.w) p.o.&lt;br&gt;Iithium diet 60 mmol/kg dry food</td>
<td>3 + 28 days&lt;br&gt;28 days</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>EFR (400mg/k.g.b.w) p.o.&lt;br&gt;Iithium diet 60 mmol/kg dry food</td>
<td>3 + 28 days&lt;br&gt;28 days</td>
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<td>AFR (200mg/k.g.b.w) p.o.&lt;br&gt;Iithium diet 60 mmol/kg dry food</td>
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</table>

2.11.2 Urine Collection and analysis-

After one of the last dose administration, the blood samplings are collected by retro-orbital technique under light anesthesia. The collected blood was kept for 40 min at 370°C to get clot. The clot was removed and the serum was separated and transferred to another clean centrifuge tube. The serum containing centrifuge tubes were subjected for the centrifugation at 3000 RPM for ten min. The clear supernantant serum was separated and used for the biochemical estimations.

Metabolic cages were cleaned to prevent contamination. Additionally, food and water consumption was supervised every day. The urine was collected throughout a day.

2.11.3 Urine flow and specific gravity measurement: A drop of conc. HCl was added to the collected urine. This prevents the growth of microbes and also prevents metal hydrolysis. The collected urine was measured and transferred to a cleaned airtight container and used for the urine analysis. The daily urine out flow and the specific gravity was measured. Before sacrificing the rats they were weighed and recorded.
2.11.4 Serum collection - The anesthesia was induced by the intraperitoneal administration of ketamine (60mg/kg) and xylazine (5mg/kg) after 24h of the last dose administration. The retro orbital sinus puncture technique was followed for the collection of blood samples of experimental animals. After the blood collection, the blood was transferred to the clean container. The blood sample containers were kept at 37° C for 40min for blood coagulation. The clot was removed from the container and the remaining serum of the experimental animals was added to the centrifuge tube. The centrifuge tubes containing serum were subjected to centrifugation for ten min at 3000RPM. The resultant clear serum was transferred into the clean container and was kept in the refrigerator. From the serum, creatinine and urea were measured. Erba diagnostic kit and semi-auto analyzer are used for these estimations.

2.11.5 Measurement of serum sodium and potassium:

For sodium: (colorimetric method) Sodium and proteins are precipitate with magnesium and uranyl acetates to form uranyl magnesium sodium acetate. The precipitates are separated by centrifugation. The excess of uranyl ions in supernatant will react with potassium ferrocyanide to produce a brownish color in acidic medium. The absorbance of color is measured at 550 nm and is inversely proportional to the concentration of Sodium in the sample.

For Potassium: Potassium is measured turbid metrically. Potassium reacts with sodium tetraphenyl boron, resulting in an insoluble turbid suspension. The extent of turbidity is measured at 630 nm and is proportional to the concentration of potassium in the sample.
2.11.6 Measurement of Blood Urea Nitrogen (BUN)

During the metabolism of protein in the body, the ammonia one of the metabolic waste is formed in the liver. This gets broken down into another byproduct called urea. Serum urea concentration is important to determine if the kidneys are working normally. If the serum urea level is elevated indicates that the kidneys are not filtering properly.

The liver produces ammonia at the time of metabolism of proteins. The formed ammonia, then converted into another product called urea. The renal glands have the ability to filter urea in the glomerular filter. The urea is also excreted through sweat. Some urea remains in the blood and is termed serum urea. Serum urea concentration is important to determine as it gives the information about the health of the kidney. Kidney excretes excess amount of urea, if there is defect in the kidney.

Urea is also known as carbamide. It has two amine groups and joined by a ketone group. The metabolism of nitrogenous substances is aided by urea present in the blood. The normal serum urea is 8-20mg/dL of blood plasma. The serum urea present in the ratblood samples was estimated using semi auto analyzer and the Erba diagnostic kit.

2.11.7 Measurement of serum creatinine: Creatinine produces reddish yellow color when it reacted with picric acid in the presence of sodium hydroxide solution. The alkaline medium is provided by sodium hydroxide. In Jaffe’s reaction, the sodium salt of picric acid, sodium picrate is formed in the first phase. The sodium picrate reacts with creatinine to form reddish yellow crystals of creatinine picrate and upon acidification with HCl these crystals are dissolved. The depth of the color intensity is directly proportional to the amount of creatinine present in the sample (Nigam et.al. 2007). Creatinine present in the rats serum was estimated using semi-automatic analyzer. Creatinine present in the mice serum was estimated using semi-automatic analyzer.