Materials and Methods
2.1 Collection of materials

2.1.1 Chemicals

Routinely used organic solvents (chloroform, methanol, benzene, hexane, diethyl ether etc.) and acids (hydrochloric, sulphuric, glacial acetic, orthophosphoric acid etc.) were procured from M/s., s.d.fine-Chem. Ltd., Mumbai, India. Various other chemicals (magnesium nitrate, ammonium molybdate, sodium carbonate, copper sulphate, sodium potassium tartarate, magnesium chloride, dimethyl sulphoxide, disodium hydrogen phosphate, potassium dihydrogen phosphate, ferric chloride, sodium nitrite, sodium hydroxide, sodium citrate, aniline, diacetyl monoxime, thiosemicarbazide, bromocresol green, Brij 35, bromine, hydrogen peroxide, potassium dichromate, L-ascorbic acid, dinitrophenyl hydrazine, sodium pyruvate, urea, bovine serum albumin, nitrophenol etc.) were obtained from M/s., s.d.fine-Chem. Ltd., Mumbai, India and or M/s., Hi-Media Lab. Pvt. Ltd., Mumbai, India. Standard chemicals like cholesterol, tripalmitin etc. along with the chemicals required for enzymatic analysis (alanine, α-ketoglutaric acid, aspartate, glycine, p-nitrophenyl phosphate, glutathione, γ-glutamyl-p-nitroanilide, 5, 5'-dithiobiisnitrobenzoic acid, methionine, riboflavin etc.) were imported from M/s., Sigma-Aldrich Chemical Co., USA. D-Galactosamine used in this present study was procured from M/s., Hi-Media Lab. Pvt. Ltd., Mumbai, India.

2.1.2 Glass wares and lab wares

The required glass wares like beakers, conical flasks, test tubes, pipettes, various measuring cylinders etc., were procured from M/s., Borosil Glass Works Ltd. Mumbai, India and other lab wares like plastic reagent bottles, micro vials, centrifuge tubes, pipette stands, micro tips and appendorf tubes etc., were
obtained from M/s., Tarsons Products Pvt. Ltd., Kolkata, India. All glass wares were soaked in chromic acid and then cleaned with teepol. They were rinsed thoroughly in distilled water and dried before use. All the glasswares were sterilized by keeping in hot air oven for 2 hrs.

2.1.3 Experimental Animals

Swiss albino mice, *Mus musculus* were selected as experimental animals for the present study. Necessary approval for carrying out experimentation on animals was obtained from Animal Ethical Committee of Goa University; vide letter no- GU/ZOO/IAEC/2006/06 dated 17-08-2006. Experimental animals were procured from M/s., Sri Venkateshwara Enterprises, Bangalore, India. Two months old mice having 25-30 g body weight (including both the sexes) were used for experimentation.

2.1.4 Feed for the Experimental Animals

Commercial feed (Goldmohur Food & Feed Ltd., Mumbai, India) was used to feed the experimental animals. Maxepa, the fish oil enriched with ω-3 PUFA, as marketed by M/s., E. Merck, Mumbai, India was obtained from local pharmacy. The meat oil was extracted and concentrated in the laboratory from the fat tissue of *Capra aegagrus* as explained below. These two sources of dietary lipid are having a contrast PUFA composition and unsaturation index (Table A).

**Meat oil extraction:** 0.5g of fat tissue of goat (*Capra aegagrus*) was finely minced and placed in a clean 1000ml beaker. To this, 250ml of hexane (AR grade) was added and mixed thoroughly. The beaker was covered with a parafilm and placed in the sonicator bath (Elma transonic
Table A: Fatty acid profiles of Fish and Meat oil. Fish oil was supplied by E. Merck. Meat oil was extracted from *Capra aegagrus*.

<table>
<thead>
<tr>
<th>Type of fatty acid</th>
<th>Fish oil (Maxepa)</th>
<th>Meat oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>11.62</td>
<td>8.97</td>
</tr>
<tr>
<td>16:0</td>
<td>23.27</td>
<td>32.88</td>
</tr>
<tr>
<td>18:0</td>
<td>5.76</td>
<td>26.31</td>
</tr>
<tr>
<td>20:0</td>
<td>0.86</td>
<td>1.57</td>
</tr>
<tr>
<td>18:1</td>
<td>11.90</td>
<td>1.52</td>
</tr>
<tr>
<td>20:1</td>
<td>10.69</td>
<td>12.55</td>
</tr>
<tr>
<td>22:1</td>
<td>0.12</td>
<td>0.35</td>
</tr>
<tr>
<td>18:2n6</td>
<td>3.13</td>
<td>9.25</td>
</tr>
<tr>
<td>20:2n6</td>
<td>0.16</td>
<td>0.32</td>
</tr>
<tr>
<td>20:4n6</td>
<td>1.73</td>
<td>4.17</td>
</tr>
<tr>
<td>18:3n3</td>
<td>0.21</td>
<td>0.10</td>
</tr>
<tr>
<td>20:5n3</td>
<td>17.86</td>
<td>0.52</td>
</tr>
<tr>
<td>22:5n3</td>
<td>1.83</td>
<td>0.12</td>
</tr>
<tr>
<td>22:6n3</td>
<td>10.86</td>
<td>1.37</td>
</tr>
<tr>
<td>Sat/Unsat ratio</td>
<td>0.709</td>
<td>2.304</td>
</tr>
<tr>
<td>n-3/n-6</td>
<td>6.127</td>
<td>0.154</td>
</tr>
</tbody>
</table>
Materials and methods

Sonication was carried out for 15 minutes at a time, for one complete hour. After each sonication, the tissue was mixed well with the solvent using a clean glass rod. The same tissue was once again sonicated with 250ml of fresh hexane for another hour. The two oil extracts thus obtained were pooled together and vacuum evaporated till the solvent was completely evaporated leaving no trace of its odour. To the extracted oil, butylated hydroxy toluene (Sigma, USA) was added to make 1mM solution. The extracted oil was refrigerated in an amber coloured bottle for further use.

2.2 Maintenance of the experimental animals

Animals were maintained in the animal house attached to the Department of Zoology, Goa University. The animal house has been registered under the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Govt. of India. After the initial acclimatization for a period of one week, two months old, animals of uniform body weights i.e., 25-30 g of either sex were selected for the present study. Animals (n=12) were housed in polypropylene cage (42cm x 25cm x 15cm) with stainless steel lid, having a provision for food and water. Animals were given a bedding of paddy husk, which was changed regularly to maintain hygiene. Animals were maintained at a temperature of 29±2 °C and light 12 L: 12D (Pampori, 2003). Animals were also bred in the animal house for further experimental use. Pregnant female mice were identified and separated into
different cages provided with soft bedding, water and food *ad libitum*. The litters were weaned only after they were two months old.

2.3 Experimental Set Up

2.3.1 Effect of dietary PUFA on the well being status

To find out the effective dose of dietary lipid that would be the most beneficial, experiment was designed as follows. Experimental animals were divided into three groups viz., Control, Fish oil and Meat oil groups. Fish oil and Meat oil groups of mice were again divided into three subgroups (n= 24) each with equal male: female ratio. The control group of mice was fed *ad libitum* with standard pellet diet and water. Each sub group of F and M groups were also fed *ad libitum* the commercial pellet diet like control group but blended with 5, 10 and 20% of respective oil. The mice were maintained with control / experimental diet for 60 days and were scarified in batches of six animals from each group at interval of 15 days. Animals were sacrificed to collect blood, liver, kidney, heart and spleen tissues. Blood was used to study various hematological parameters like RBC, WBC, platelet and hemoglobin. The tissues were used to study various biochemical parameters like protein, urea, ascorbic acid, free sugars, triglycerides, cholesterol and phospholipids.

2.3.2 Effect of dietary PUFA on D-GaIN induced hepatitis

To find out the pathophysiological responses to D-Galactosamine induced hepatitis and to evaluate the effect of dietary lipid on hepatitis, the experimental set up was as follows. Experimental animals (all male) were divided into three groups (n=12) viz., Control, Fish oil and Meat oil groups. The control group
of mice were fed *ad libitum* with standard pellet diet and water. The second and third groups were fed with the same diet but were supplemented with 10% of fish oil and 10% meat oil respectively for a period of 30 days. The dose and duration of fish oil and meat oil supplementation was selected based on the result of experiment as mentioned in section 2.3.1. At the end of experimental feeding period, half the animals (n=6) from each group received 1 ml of saline by intraperitoneal injection for two consecutive days while the other half received D-Galactosamine (0.5mg/g body wt) dissolved in 1 ml of physiological saline for two consecutive days for induction of hepatotoxicity (Anandan and Devaki, 1999). After two days, animals were anesthetized to collect liver tissue and blood from heart. The effect of dietary PUFA on D-GaIN induced hepatotoxicity was studied by evaluating biochemical parameters like total protein, urea, bilirubin, ascorbic acid, tocopherol, reduced glutathione, thiobarbituric acid reactive substances and estimating the activities of various enzymes like alanine transaminase, aspartate transaminase, alkaline phosphatase, gamma glutamyl transpeptidase, glutathione peroxidase, catalase and superoxide dismutase in liver and serum. Besides, histopathological evaluation of liver was carried out with light microscopy and transmission electron microscopy (TEM). Effect of dietary supplementation of PUFA on various proinflammatory cytokine expressions was also studied by selecting four different cytokines namely, IL-1α, IL-2, TNF-α and iNOS. An attempt was also made to elucidate the lipid profile of liver upon PUFA supplementation and induction of hepatitis.
2.4 Collection of tissues

Blood was collected from *Mus musculus* by cardiac puncture with moderate suction. The needle was removed before transferring blood to the eppendorf tubes to avoid any haemolysis (Chawla, 1999) and was transferred into heparinized eppendorf tubes for haematological studies. Blood was also transferred to other eppendorf tubes containing no anticoagulant for the separation of serum. For serum collection, blood was allowed to clot completely at room temperature for a period of half an hour, eppendorf tubes were centrifuged at 4°C for 800 x g for 10 minutes and serum was separated. It was immediately stored in liquid nitrogen (-196°C) for further biochemical analysis. After opening the general viscera, various tissues like liver, kidney, heart and spleen were removed. All these tissues were washed with phosphate buffer saline (PBS, pH 7.0) and transferred immediately to ice cold bath for immediate extraction of various biomolecules or stored in liquid nitrogen for further analysis.

2.5 Routine haematology

2.5.1 Haemoglobin

Haemoglobin being a chromo protein, its content in the blood samples was determined by measuring its colour with Sahli’s hemometer. By using a Pasteur pipette, 0.1 N HCl was added to the graduated tube up to the mark 10 (the lowest mark). Blood was drawn up to the 20μl mark in the pipette and added to the acid in the tube. It was allowed to stand for at least 10 min, during which the haemoglobin was converted to acid haematin. The solution was then
diluted with distilled water by adding few drops at a time, until its colour matched with the standard glass reference blocks, against natural light. The level of the fluid was noted at its lower meniscus and the corresponding reading was recorded as g/dl haemoglobin.

2.5.2 Total count of blood cells

Total counts of blood cells like RBC, WBC and platelet counts (by using Neubauer chamber) were done by following the method of Sood (1996).

**WBC counts:** Using WBC pipette of the haemocytometer blood was drawn till the 0.5 mark. Tip of the pipette was cleaned and then WBC diluting fluid (Turke’s fluid) was drawn till the 11 mark. The dilution was 20 times. Blood was mixed with the diluting fluid gently, avoiding any bubbling. The Neubauer chamber was charged with the mixture while care was taken to see that it does not overflow. The cells were allowed to settle at the bottom of the chamber for 2 minutes. The cells were counted uniformly in the four larger corner squares using 10X or low power objective.

**RBC counts:** Blood was drawn up to the 0.5 mark in the RBC pipette. The tip was wiped clean and diluting fluid (Hayem’s fluid) was drawn up to the 101 mark. The dilution was 200 times. Blood was mixed thoroughly and the chamber was charged without allowing the fluid to overflow. Cells were counted in the 80 smallest squares using 10 X or low power objective.

**Platelet counts:** Blood was drawn up to the 0.5 mark in the RBC pipette. It was then diluted up to the 101 mark with 1% ammonium oxalate (stored at 4°C). The dilution was 200 times. The Neubauer camber was charged with the help of a Pasteur pipette. Using 40 X or high power objective, platelets were counted in the same 80 smallest squares as indicated for RBC counting.
The total number of WBC/RBC / Platelet was calculated as follows:

Total number of cells/mm$^3$

= (Cells counted \( \times \) blood dilution) / (chamber depth \( \times \) area of chamber counted).

- **Turke's fluid** - 3.0 ml of glacial acetic acid and 1ml of 1% aqueous solution of Genetian violet was made up to 100ml with distilled water. A pinch of thymol may be added to the diluting fluid to prevent growth of molds.

- **Hayem's fluid** - 0.5g mercuric chloride, 1.0 g sodium chloride and 0.5g sodium sulphate were dissolved in 200ml distilled water.

### 2.6 Serum lipid profiles

The serum lipid profiles were monitored following the method of Trinder, (1969) by using the diagnostic kit manufactured by Crest Biosystems, Coral Clinical System, Goa.

#### 2.6.1 Serum triglyceride

Lipoprotein lipase hydrolyses triglycerides to glycerol and free fatty acids. The glycerol formed with ATP in the presence of glycerol kinase forms glycerol 3 phosphate which is oxidized by the enzyme glycerol phosphate oxidase to form hydrogen peroxide. The hydrogen peroxide further reacts with phenolic compound and 4-aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of triglycerides present in the sample.

\[
\text{Triglycerides} \quad \xrightarrow{\text{lipoprotein lipase}} \quad \text{Glycerol + free fatty acids}
\]
**Materials and methods**

Glycerol kinase

\[
\text{Glycerol + ATP} \rightarrow \text{Glycerol 3 phosphate + ADP}
\]

Glycerol 3 phosphate oxidase

\[
\text{Glycerol 3 phosphate + } O_2 \rightarrow \text{Dihydroxy acetone phosphate + } H_2O_2
\]

Peroxidase

\[
H_2O_2 + 4\text{ Amino antipyrine + phenol} \rightarrow \text{Red Quinoneimine dye + } H_2O
\]

The test solution (marked as T) contained 1 ml of working reagent and 0.01 ml of serum sample. The standard solution (marked as S) contained 1 ml working reagent and 0.01 ml of triglyceride standard (conc. 200 mg/dl). The solutions were mixed properly and incubated at 37°C for 5 minutes. The absorbance of standard and test samples was measured against a suitable blank at 505 nm within 60 minutes.

Triglyceride content present in the sample was calculated using the formula:

\[
\text{Triglycerides in mg/dl} = \frac{\text{Abs T}}{\text{Abs S}} \times \text{conc. of standard.}
\]

2.6.2 Serum cholesterol:

Cholesterol esterase hydrolyses esterified cholesterol to free cholesterol. The free cholesterol is oxidized to form hydrogen peroxide which further reacts with phenol and 4-amino antipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of cholesterol present in the sample.

Cholesterol esterase

\[
\text{Cholesterol esters + } H_2O \rightarrow \text{Cholesterol + fatty acids}
\]

Cholesterol oxidase

\[
\text{Cholesterol + } O_2 \rightarrow \text{Cholestonone + } H_2O_2
\]

Peroxidase

\[
H_2O_2 + 4\text{-amino antipyrine + phenol} \rightarrow \text{Red quinoneimine dye + } H_2O
\]
The test solution (marked as T) contained 1ml of working reagent and 0.01ml of serum sample. The standard solution (marked as S) contained 1ml working reagent and 0.01 ml of cholesterol standard (200mg/dl). The solutions were mixed properly and incubated at 37°C for 5 minutes. The absorbance of standard and test samples was measured against a suitable blank at 505nm.

Cholesterol content present in the sample was calculated by using the formula: Cholesterol in mg/dl = (Abs T/ Abs S) x conc. of standard.

2.6.3 Serum HDL cholesterol

When the serum is reacted with polyethylene glycol contained in the precipitating reagent, all the VLDL and LDL are precipitated. The HDL remains in the supernatant and is assayed as a sample for cholesterol using the cholesterol reagent.

0.1 ml of precipitating reagent was added to 0.1 ml of serum sample. The contents were mixed well and incubated at room temperature for 5 minutes. The samples were then centrifuged at 800 x g for 10 minutes to obtain a clear supernatant. The clear supernatant was assayed for cholesterol as mentioned before (2.6.2) using HDL standard (25mg/dl). HDL cholesterol was calculated using the formula:

\[
\text{HDL Cholesterol in mg/dl} = (\text{Abs T/ Abs S}) \times \text{conc. of standard} \times 2
\]

(\text{where, 2 is a dilution factor due to the deproteinization step}).

2.6.4 Serum LDL and VLDL cholesterol

From the obtained data of serum triglyceride, cholesterol and HDL cholesterol, serum LDL and VLDL were calculated using Freidewald’s formula as mentioned below:

Triglyceride values divided by 5 gave the serum VLDL values. The sum of VLDL and HDL was subtracted from total cholesterol to obtain LDL values.
2.7 Extraction & Estimation of biomolecules

2.7.1 Total carbohydrate, free sugars and urea

10% tissue (liver, kidney, heart and spleen) homogenate in ice-cold water was prepared. The homogenate was deproteinized by adding equal amounts of 0.3 N barium hydroxide and 5% zinc sulphate. After centrifugation at 800 x g for 15 minutes the supernatant was stored for estimation of urea, total carbohydrates and free sugars (Roy et al., 1991). Similarly serum samples were also deproteinized by adding equal amounts of 0.3 (N) barium hydroxide and 5% zinc sulphate.

**Total carbohydrate:** Carbohydrates are dehydrated by conc. $\text{H}_2\text{SO}_4$ to form furfural. Furfural condenses with anthrone to form a blue coloured complex, which can be measured colorimetrically at 620nm.

0.1ml of deprotenized aliquot was diluted up to 1.0ml with distilled water. To this, 4ml of anthrone reagent was added and incubated for 10 minutes in a boiling water bath. The intensity of the colour developed was measured at 620nm against a suitable blank (Carroll, 1956). Quantification of the total carbohydrate content was done with the help of a standard curve of total carbohydrate (100µg of glucose/ml).

**Anthrone reagent:** 0.2gm of anthrone was dissolved in 100ml of concentrated sulphuric acid.

**Free sugars:** When sugars are heated with alkaline copper reagent it forms cuprous oxide, which gives blue coloured complex with arsenomolybdate reagent, the intensity of which can be measured at 540 nm.

To 1.0 ml deproteinized sample, 1.0 ml alkaline copper reagent was added and incubated in the boiling water bath for 20 minutes. After cooling to
Materials and methods

At room temperature 1 ml of arseno-molybdate colour reagent was added. The mixture was diluted with 7 ml distilled water. The intensity of the colour was read at 540 nm against a suitable blank (Nelson, 1944). Quantification of tissue free sugar concentration was calculated with the help of glucose standard curve, prepared by using 200 μg/ml glucose as a standard solution.

❖ Alkaline copper reagent –

A] a)- 12.0g anhydrous sodium carbonate and 6.0g sodium potassium tartarate were dissolved in 125 ml of distilled water.

b)- 2.0 g of copper sulphate was dissolved in 25ml distilled water.

Both the solutions a and b were mixed, 8.0g of sodium bicarbonate was added to it by stirring to prepare solution A.

B] 90.0g of anhydrous sodium sulphate was dissolved in 250 ml of distilled water. Boiled to expel air and then cooled to room temperature to prepare solution B.

Now both the solutions A and B were mixed and the volume was made up to 500ml with distilled water.

❖ Arsenomolybdate colour reagent – 25.0 g of ammonium molybdate was dissolved in 450 ml of distilled water. 21ml of concentrated sulphuric acid was added slowly while mixing. To this, 3.0 g disodium hydrogen arsenate (already dissolved in 25ml of water) was added. Mixed well, stored in amber coloured bottle at 37°C for 48 hours.
Materials and methods

**Urea:** In the hot acidic medium with the presence of ferric ion, diacetyl monoxime reacts with urea and gives a pink coloured substance. The intensity of the colour depends upon the amount of urea present, which can be measured at 520 nm.

1.0 ml of deproteinized sample was made up to 3 ml with distilled water. To this, 2 ml acid mixture and 0.2 ml diacetyl monoxime reagent were added. It was incubated in boiling water bath for 20 minutes and then cooled to room temperature. The intensity of the colour was measured at 520 nm against a suitable blank (Friedman, 1953). The amount of urea present in various samples was calculated by using standard curve of urea (10 μg/ml).

- **Acid mixture:** 10.0 mg ferric chloride was dissolved in 100 ml of orthophosphoric acid.
- **Diacetyl monoxime reagent:** 2.5 g of diacetyl monoxime (2, 3-butane dione monoxime) and 0.12 g of thiosemicarbazide were dissolved in 100 ml distilled water.

### 2.7.2 Protein, Ascorbic acid, Lipids and Tocopherols

10% tissue homogenate was prepared using equal amounts of ice cold distilled water and 10% PCA. The homogenate was centrifuged at 800 x g for 15 minutes. The supernatant was stored at (-) 20°C for estimating ascorbic acid concentration. The residue was re-extracted with chloroform: methanol (2:1) and then with diethyl ether. After collecting the supernatants through centrifugations at 800 x g for 15 min, the supernatants were pooled together. The pooled supernatant was treated with salt wash by adding sufficient 5% sodium chloride solution and keeping the mixture overnight at refrigerated temperature for complete phase separation. The lower phase was stored for lipid
estimation (Folch et al., 1957) and tocopherol. The residue was again treated with 10% perchloric acid at 90°C for 30 minutes and centrifuged. The supernatant was discarded and the residue was dissolved in 1 (N) sodium hydroxide solution and stored for protein estimation (Roy et al., 1991).

**Total protein:** Copper from alkaline copper reagent reacts with protein to form a protein complex. Amino acids from the complex react with tungstic acid from Folin Cio-Calteau reagent, to give a blue colour. The intensity of the blue colour is directly proportional to the amount of tyrosine and tryptophan present and can be measured at 690nm.

0.1ml of the tissue extract for protein or 20µl of serum was diluted up to 0.5 ml with distilled water. To this, 5 ml of Lowry’s reagent was added and incubated for 15 minutes at room temperature. 0.5ml of Folin Cio-Calteau (1:2 dilution) reagent was then added and the mixture was further incubated for a period of 30 minutes. The intensity of the blue coloured complex was measured against a suitable blank at 690nm (Lowry et al., 1951). Quantification of the protein content of the sample was done with the help of a standard curve of bovine serum albumin (250µg/ml BSA in 1N NaOH).

❖ **Lowry’s reagent:** To 98.0ml of 4% sodium carbonate, 1ml of each 2% copper sulphate and 4% sodium –potassium tartarate were added to make the volume up to 100 ml.

**Albumin and Globulins:** Albumin present in the sample binds specifically with bromocresol green at pH 4.1 to form green coloured complex, intensity of which can be measured colourimetrically by using 640nm.

To 50µl of tissue protein extract or serum, 5ml of albumin reagent was added. The mixture was kept at room temperature for exactly 10 minutes.
Intensity of the colour was measured at 640nm against a suitable blank (Godkar, 1994). Albumin present in the samples was quantified with the help of albumin standard curve (4.0g/dl prepared in 0.1g/dl sodium azide).

Globulins present in the samples were calculated by subtracting the albumin values from total proteins.

❖ **Albumin reagent**- 8.85g succinic acid, 0.108g of bromocresol green, 0.1g of sodium azide and 4.0 ml of Brij-35 were dissolved in 900ml of distilled water. pH of this solution was adjusted to 4.1 by using 1N sodium hydroxide. Final volume was made up to 1lt by using distilled water. This reagent can be stored at room temperature (25°C ± 5°C) for one year.

**Ascorbic acid**: Ascorbic acid in the presence of aniline and bromine forms a complex with dinitrophenyl hydrazine to form a coloured complex, intensity of which can be measured at 540nm.

0.1 ml of the tissue extract for ascorbic acid or 20 μl of serum was made up to 2 ml with distilled water. To that 0.1 ml of bromine water was added and then exactly after 10 seconds, 0.1 ml of 3% aniline was added. After adding 0.5 ml of 2% Dinitro phenylhydrazine (in 9N H₂SO₄), the samples were incubated in the boiling water bath for 5 minutes, and then kept in the ice bucket and 5ml of 70% sulphuric acid was added. The absorbance was read at 540nm against a suitable blank (Roe and Kuether, 1943). The ascorbic acid content present in the samples was quantified by using a standard curve of ascorbic acid (100μg/ml).
Materials and methods

**Total cholesterol:** In acidic medium cholesterol reacts with ferric chloride reagent to form a brownish green compound and the intensity of which can be measured at 550nm.

0.05 ml of tissue lipid extract was dried by keeping in a hot water bath. To that, 3.0 ml of glacial acetic acid and 2.0 ml of ferric chloride reagent was added and incubated at room temperature for 5 minutes. The intensity of the colour was measured at 550nm against a suitable blank (Kates, 1986). Amount of cholesterol in the samples was quantified with the help of a standard curve of cholesterol (500μg/ml in benzene).

❖ **Ferric chloride reagent** - 1.25 g of ferric chloride was dissolved in 50 ml orthophosphoric acid and stored in amber coloured bottle. This serves as a stock solution. To prepare working standard 4 ml of the stock solution was made up to 50 ml with concentrated sulphuric acid (to be freshly prepared).

**Triglycerides:** Triglycerides are alkali hydrolyzed to form glycerol. Glycerol reacts with chromotropic acid reagent in presence of iodate and arsenite ions to produce a brown coloured compound, and intensity of the colour can be measured colorimetrically at 570nm.

0.05 ml of tissue lipid extract was kept in hot water bath for complete solvent evaporation. The lipid sample was then hydrolyzed with 0.5 ml of 0.4% ethanolic sodium hydroxide at 60°C for half an hour, and then 0.5 ml of 0.25 (N) sulphuric acid was added to the mixture. After complete evaporation of the solvent over a hot water bath, 0.1 ml of 0.05 (M) sodium per iodate and 0.1 ml of 0.5 (M) sodium arsenate was added with a gap of 10 minutes. It was allowed to stand for another 10 minutes. Then, 5ml of chromotropic acid reagent was added and kept in the boiling water bath for half an hour. The triglycerides were measured at 570 nm against a suitable blank (Kates, 1986). Quantification of
Materials and methods

Triglycerides was done with the help of a tripalmitin standard curve (3.2mg/ml in benzene).

❖ Chromotropic acid reagent- 0.448 g of chromotropic salt was dissolved in 40ml of distilled water. Placed in ice bucket and ice cold mixture of 120 ml of concentrated sulphuric acid and 60ml distilled water was added to it. Stored in refrigerator.

Tissue phospholipids: The organic phosphate present in the phospholipids is converted to inorganic phosphate during the charring at 300°C with alcoholic magnesium nitrate solution. Inorganic phosphate reacts with ammonium molybdate to form ammonium phosphomolybdate which reduced by ascorbic acid to produce a blue coloured complex. The intensity of the colour can be measured at 820nm. Therefore, the amount of inorganic phosphate present in the sample is equivalent to the phospholipids present.

To 0.1ml of tissue lipid extract, 0.2 ml of 10% alcoholic magnesium nitrite was added and charred at 300°C till a completely white powder was formed with no trace of yellow colour. This powder was dissolved in 1.5 ml of 0.5 (N) hydrochloric acid. To this 3.5 ml of freshly prepared ammonium molybdate reagent was added and was kept on a water bath at 60°C for 30 minutes. The intensity of the colour developed was measured at 820 nm against a suitable blank (Kates, 1986). The quantification of phospholipids was done with the help of a standard curve of ATP (4μmole of ATP/ ml, which is equivalent to 12μmole of inorganic phosphate/ml).

❖ Ammonium molybdate reagent- 0.42% of ammonium molybdate solution in 1(N) sulphuric acid and 10% aqueous ascorbic acid solution were taken in the ratio of 6:1 in order to prepare this reagent (to be freshly prepared).
**Materials and methods**

**Free fatty acids:** Free fatty acids react with copper reagent to form copper salt of the fatty acid which further reacts with diethyl dithiocarbamate of the colour reagent to form a pale yellow coloured compound. The intensity of the colour can be measured at 440nm.

0.1ml of tissue liquid extract was kept in hot water bath for complete solvent evaporation. To that 6ml of chloroform was added and mixed well. Later 3ml of copper reagent was added. Two layers were formed and the upper layer was removed carefully using a micropipette and discarded. 0.5ml of colour reagent was added to the down layer. Intensity of the colour developed was measured at 440nm (Anstall and Trujillo, 1965). Quantification of free fatty acid content was done with the help of standard curve of palmitic acid (1mg in 6ml of chloroform).

- **Colour reagent**- 0.1% sodium diethyl dithiocarbamate in n-butanol.
- **Copper reagent**- By mixing 1(M) acetic acid, 1(M) triethanolamine and 6.5% cupric nitrate in 1:9:10 ratios.

**Tocopherols:** Ferric ions are reduced to ferrous ions by tocopherol which then reacts with 2, 2'-dipyridine to produce a red coloured complex. The intensity of the colour depends on the concentration of tocopherols and can be measured at 520nm.

To 0.5 ml of tissue or serum lipid extract, 1.5 ml ethanol and 2.0 ml of hexane were added and centrifuged. The supernatant was evaporated to dryness at 80°C, to that 0.2 ml of each 2, 2'-dipyridyl solution (0.5% in ethanol) and ferric chloride (0.2% in ethanol) were added. The mixture was kept in dark for 5 minutes and then 4 ml of butanol was added. The colour developed was read at 520 nm against a suitable blank (Baker and Frank, 1980). The concentration of tocopherol in the samples was quantified with the help of standard curve of tocopherol (0.2 μmole /ml in hexane).
2.7.3 Thiobarbituric acid reactive substances (TBARS)

Malondialdehyde forms 1:2 adduct with thiobarbituric acid to produce a coloured complex. The intensity of the colour can be measure spectrophotometrically at 535 nm. Other lipoproteins are precipitated out by trichloroacetic acid and avoided from interfering in the reaction. Only water soluble malondialdehyde reacts with thiobarbituric acid and produces a coloured complex.

The liver tissue was homogenized in Tris HCl buffer pH 7.0 to prepare a 2% homogenate. 0.1 ml of tissue homogenate or 10 µl serum was treated with 2ml of TBA-TCA-HCl reagent and placed in boiling water bath for 15 minutes, cooled and centrifuged at room temperature for 10 minutes at 500 x g. The absorbance of clear supernatant was measured against a suitable blank at 535 nm (Niehaus and Samuelsson, 1968). Thiobarbituric acid reactive substance concentration was estimated with the help of standard curve of malondialdehyde (10nmole/ml).

❖ TBA-TCA-HCl Reagent- 0.37% Thiobarbituric acid, 15% Trichloroacetic acid and 0.25(N) Hydrochloric acid were mixed in 1:1:1 ratio to prepare this reagent.

2.7.4 Reduced glutathione (GSH)

GSH reacts with 5, 5'- dithiobis, 2-nitrobenzoic acid to produce a yellow coloured compound. The intensity of the colour can be measured spectrophotometrically at 412nm.

Tissue was homogenized with 5% TCA to prepare a 2% homogenate and centrifuged at 500 x g for 5 minutes to remove the precipitate. To 1.0 ml of diluted tissue extract or serum, 2 ml of 5, 5'- dithiobis, 2-nitrobenzoic acid (DTNB) reagent was added to make the final volume 3.0 ml (Moron et. al., 1979). Absorbance was read at 412nm against a suitable blank. Reduced
Materials and methods

glutathione content of the samples was quantified with the help of a standard curve of reduced glutathione (0.2μmole/ml in 5% TCA).

❖ 5, 5'- dithiobis, 2-nitrobenzoic acid (DTNB) reagent— 19.8mg of 5, 5'- dithiobis, 2-nitrobenzoic acid was dissolved in 100ml of 0.1%sodium nitrate to prepare this reagent.

2.7.5 Bilirubin

Bilirubin exists in two forms viz. conjugated (direct) and unconjugated (indirect) form. The conjugated form is soluble in water as it is bound to glucuronic acid. Conjugated bilirubin reacts very fast with diazotized sulphanilic acid to produce a purple coloured azobilirubin. Methanol makes unconjugated bilirubin to react with diazo acid for producing azobilurubin. The intensity of the colour can be measured at 540nm.

\[
\text{Bilirubin} + \text{Diazosulphanilic acid} \rightarrow \text{Azobilirubin (Blue coloured complex)}
\]

100 mg tissue was homogenized in 2ml of diethyl ether and centrifuged at 800 x \( g \) for 15 minutes. Supernatant removes the sterols and was discarded. The residue was washed sequentially with hot water, 10% acetic acid, ethanol and hot acetic acid in order to remove bile salts, biliverdin, water etc. Finally the residue was dissolved in hot chloroform and was used for the estimation of bilirubin.

To 2.8 ml of tissue extract or 20 μl of serum 0.7 ml of diazo reagent was added and incubated at room temperature for 5 minutes. The blank was prepared by taking 2.8 ml of tissue extract or 20 μl of serum and 0.7 ml of sulphanilic acid instead of diazo reagent. Intensity of colour developed was read at 540 nm to estimate conjugated bilirubin. Now, 3.5 ml of methanol was added to all test tubes, incubated for 5 minutes. Optical density was measured again at 540 nm to
estimate total bilirubin (Malloy and Evelyn, 1937). Bilirubin present in the given sample was quantified with the help of a bilirubin standard curve (100 µg/ml in chloroform).

- **Diazot reagent**- 10 ml of 1% sulphanilic acid prepared in 0.2N HCl was mixed 0.3 ml of 0.5% sodium nitrite solution. This reagent should be freshly prepared.

### 2.8 Enzyme activity analysis

#### 2.8.1 Alanine amino transferase (ALT) [EC 2.6.1.2]

\[ \text{a-ketoglutaric acid reacts with alanine and undergoes transamination reaction. Alanine donates its amino group to a-ketoglutaric acid. As a result alanine becomes pyruvate and a-ketoglutaric acid becomes glutamic acid. Pyruvate reduces 2, 4-dinitrophenyl hydrazine to dinitrophenyl hydrazone. In alkaline medium, hydrazone produces a coloured complex. The intensity of the colour can be measured at 540nm.} \]

\[
\text{Alanine transaminase} \\
\text{Alanine + Alpha-ketoglutaric acid} \rightarrow \text{Pyruvate + glutamic acid}
\]

0.5 ml of ALT substrate prepared in phosphate buffer (0.01 M, pH 7.0) was incubated at 37°C for 5 minutes. To this 0.1 ml of serum or 5% liver tissue homogenate (in 0.01M phosphate buffer, pH 7.0) was added to assay the enzyme activity. A suitable enzyme blank was also prepared by taking 0.5ml of substrate and 0.1 ml of distilled water. They were incubated further for another 15 minutes. The reaction was stopped by the addition of 0.5 ml dinitrophenyl hydrazine reagent. They were mixed thoroughly and kept at room temperature (25°C ± 5°C) for 20 minutes. 5 ml of 0.4 N NaOH was added to develop the colour, intensity of which was read against the enzyme blank at 540 nm (Reitman and Frankel, 1957). The product formed (pyruvate) during this reaction time was quantified with the help of a standard curve of pyruvate (220
Materials and methods

µg/ml). The enzyme activity was expressed as µg of pyruvate formed / min reaction / mg of enzyme protein. Protein content in the enzyme was estimated as described earlier (Section 2.7.2.1).

2.8.2 Aspartate amino transferase (AST) [EC 2.6.1.1]

α-ketoglutaric acid reacts with aspartate and undergoes transamination reaction. By donating its amino group to α-ketoglutaric acid aspartate becomes oxaloacetic acid and α-ketoglutaric acid becomes glutamic acid. Oxaloacetic acid reduces 2, 4-dinitrophenyl hydrazine to dinitrophenyl hydrazone. In alkaline medium, hydrazone produces a coloured complex. The intensity of the colour can be measure at 540nm.

\[
\text{Aspartate transaminase} \\
\text{Aspartate + Alpha-ketoglutaric acid} \quad \rightarrow \quad \text{Oxaloacetate+glutamic acid}
\]

Using AST substrate by following the same method mentioned above (Section 2.8.1) activity of aspartate amino transferase was assayed (Reitman and Frankel, 1957). Activity of the enzyme was quantified with the help of a standard curve of sodium oxaloacetate (220 µg/ml).

- **ALT substrate**- 0.532 g alanine and 6.0 mg α-ketoglutaric acid were added to 0.1 ml of 1N NaOH prepared in phosphate buffer (0.01M, pH 7.5). Final quantity was adjusted to 20 ml with phosphate buffer (pH 7.5).

- **AST substrate**- 0.35 g aspartate and 6.0 mg α-ketoglutaric acid were added to 0.1 ml of 1N NaOH prepared in phosphate buffer (0.01 M, pH 7.5). Final quantity was adjusted to 20 ml with phosphate buffer (pH 7.5).

- **DNPH reagent**- 10 mg of dinitrophenyl hydrazine was added to 4.25 ml conc. HCl and the final quantity was adjusted to 20 ml with distilled water.
2.8.3 Alkaline phosphatase (ALP) [EC 2.6.1.2]

Paranitrophenyl phosphate is colourless. The enzyme splits off the phosphate group from it to form p-nitrophenol, which in the acid medium is also colourless. Under alkaline conditions this is converted to p-nitrophenoxide ions, which exhibit yellow colour. The intensity of the yellow colour is directly proportional to the enzyme present in the specimen and can be measured at 405nm.

\[
\text{Alkaline phosphatase} \quad 4\text{-nitrophenyl phosphate} + \text{H}_2\text{O} \rightarrow \text{Phosphate} + 4\text{-Nitrophenolate}
\]

To 2.7 ml of glycine buffer, 0.2 ml of substrate (freshly prepared) was added and incubated at 37°C for 5 minutes. To this 0.1 ml of serum or 5% liver tissue homogenate (prepared in 0.2 M glycine buffer, pH 7.0) was added to assay the enzyme activity. Simultaneously an enzyme blank was prepared by mixing 2.7 ml buffer, 0.2 ml substrate and 0.1 ml of distilled water. The reaction mixture was incubated at 37°C for 15 minutes. The reaction was stopped by adding 5 ml of 0.25 N NaOH. The intensity of the products of this reaction (p-nitrophenol) was measured at 405 nm (King and Armstrong, 1934). The amount of the p-nitrophenol released by the action of alkaline phosphatase was quantified by prepared p-nitrophenol standard curve (250μg/ml). Protein content in the enzyme was estimated as described earlier (Section 2.7.2.1). The enzyme activity was expressed as (IU/mg protein). Unit can be defined as the quantity of alkaline phosphatase that liberates 1 mg of phosphate ion from glycerol 2-phosphate in 1 hour under standard conditions.

❖ **ALP substrate** – 680 mg of p-nitrophenyl phosphate was added to 8.0 mg of MgCl₂ solution which was prepared by adding 30mg of MgCl₂ to 10 ml glycine buffer.
2.8.4 Catalase [EC 1.11.1.6]

The reaction of catalase is the decomposition of hydrogen peroxide into water and molecular oxygen.

\[
\text{Catalase} \quad 2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2
\]

The remaining \(\text{H}_2\text{O}_2\) in the samples, which was not decomposed by the enzyme, reacts with dichromate to give a blue precipitate of perchromic acid. This unstable precipitate is then decomposed by heating to give a green coloured stable compound. The intensity of the green colour can be measured at 620nm.

In duplicate sets, 1.5 ml of phosphate buffer (0.01 M, pH 7.0) and 0.4 ml of substrate (0.2 M \(\text{H}_2\text{O}_2\)) were taken and incubated at 37°C for 5 minutes. In one set 0.1 ml phosphate buffer was added to prepare enzyme blank and in another set 0.1 ml serum or 10% liver tissue homogenate (prepared in phosphate buffer) was added to assay the enzyme activity. This reaction mixture was incubated at 37°C for 15 minutes. The reaction was stopped by the addition of 2.0 ml of dichromate acetic acid reagent. The test tubes were then heated in a boiling water bath for 10 minutes. Mixed well and the intensity of the colour was measured against reference blank at 620 nm (Sinha, 1962). Protein content in the enzyme was estimated as described earlier (Section 2.7.2.1). The enzyme activity was quantified with the help of a reference curve of hydrogen peroxide (2\(\mu\)mole/ml) and expressed as \(\mu\)moles of \(\text{H}_2\text{O}_2\) consumed / min/mg protein.

**Dichromate acetic acid reagent** - 5% potassium dichromate and glacial acetic acid were taken in 1:3 ratio to prepare this reagent.

2.8.5 Glutathione peroxidase [EC 1.11.1.9]

Glutathione peroxidase catalyzes the oxidation of reduced glutathione with hydrogen peroxide to form glutathione disulfide.
Materials and methods

Glutathione peroxidase

\[
2 \text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GS-SG} + 2\text{H}_2\text{O}
\]

(where GSH represents reduced monomeric glutathione, and GS–SG represents glutathione disulfide).

5, 5'- dithiobis, 2-nitrobenzoic acid reacts with the thiol group of GSH to release 2-nitro, 5-thiobenzoate which ionises to the nitrothiobenzoate dianion at neutral pH and has a yellow colour. The intensity of this coloured complex can be measured at 412nm.

To 0.4 ml of phosphate buffer (0.4 M, pH 7.0), 0.1 ml 10 mM sodium azide, 0.2 ml of 0.5 mM glutathione, 0.1 ml of 0.2 mM hydrogen peroxide were added and incubated at 37°C for 5 minutes. To this, 0.2 ml serum sample or 5% liver tissue homogenate (prepared in 0.4 M phosphate buffer, pH 7.0) was added. At the same time, an enzyme blank was also maintained by adding 0.2 ml of phosphate buffer instead of the sample. The reaction was stopped by the addition of 0.4 ml 10% trichloroacetic acid and centrifuged at 800 x g for 5 minutes. By adding phosphate buffer, the total volume was made up to 3.0 ml. 2.0 ml of Ellman’s reagent (5,5'- dithiobis, 2-nitrobenzoic acid) was added and intensity of the colour developed was read at 412 nm, against the blank (Ellman, 1959). The enzyme activity was quantified with the help of a standard curve of reduced glutathione (2 μmole/ml). Protein content in the enzyme was estimated as described earlier (Section 2.7.2.1). The enzyme activity was expressed as IU/mg protein. One unit will catalyze the oxidation by H₂O₂ of 1.0 μmole of reduced glutathione to oxidized glutathione at pH 7.0 at 25°C.

Ellman’s reagent – 19.8 mg of 5, 5'- dithiobisnitro benzoic acid (DTNB) was dissolved in 100 ml of 0.1% sodium nitrate to prepare this reagent.
2.8.6 Gamma glutamyl transpeptidase [EC 2.3.2.2]

Gamma-glutamyl transpeptidase (GGT) is primarily a liver enzyme which catalyzes the cleavage of the gamma-glutamyl moiety of glutathione (GSH) and gamma-glutamyl related compounds

\[
GGT \quad (5\text{-L-glutamyl})\text{-peptide} + \text{an amino acid} \rightleftharpoons \text{peptide} + 5\text{-L-glutamyl amino acid}
\]

Gamma-glutamyl transpeptidase acts upon L-\(\gamma\) glutamyl p-nitroanilide. The glycyl glycine acts as an acceptor for the glutamyl moiety. The sodium nitrite in an acidic medium diazotises p-nitroaniline cleavage product to form a purple coloured product, intensity of which can be measure at 570 nm.

To 1.7 ml buffer (120 mM Tris-\(\text{HCl}\), 411 M Sodium nitrite, 12 mM MgCl\(_2\), 90 mM glycyl glycine, pH 7.0), 0.2 ml GGTsubstrate was added and warmed to 37°C for 5 minutes. To this 0.1 ml serum sample or 5% liver tissue homogenate (prepared in the above mentioned buffer) was added. To prepare enzyme blank, 0.1 ml of glycyl glycine buffer was added instead of sample. After incubation at 37°C for 15 minutes, the reaction was stopped by the addition of 2 ml glacial acetic acid. Intensity of the colour was read at 570 nm against a reference blank (Fiala et al., 1972). Enzyme activity was quantified by using L-\(\gamma\) glutamyl p-nitroanilide reference curve (10 µg/ml). Protein content in the enzyme was estimated as described earlier (Section 2.7.2.1). The enzyme activity was expressed as IU/mg protein. IU of GGT represents the amount of p-nitroaniline released in 1 min. IU for gamma glutamyl transpeptidase is equal to 0.172 times of the absorbance read at 570 nm.

- **GGT substrate**- 48 mM of L-\(\gamma\) glutamyl p-nitroanilide was prepared in 150 mM hydrochloric acid.
2.8.7 Superoxide dismutase [EC 1.15.1.1]

Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide (O$_2^-$) into hydrogen peroxide and molecular oxygen.

\[
SOD : 2O_2^{-} + 2H^+ \rightarrow H_2O_2 + O_2
\]

Illumination of riboflavin in presence of oxygen and electron donors like methionine or EDTA generates a flux of superoxide anions. The reduction of Nitroblue tetrazolium chloride by superoxide anions produces a blue formazan compound the intensity of which can be measured at 560nm.

To 2.9 ml of freshly prepared SOD substrate, 0.1 ml of serum or liver tissue homogenate (5% homogenate was prepared in ice cold 10mM of potassium phosphate buffer, pH 7.0, and it was brought down to 2.5% with 50 mM potassium phosphate buffer, pH 7.0 and then centrifuged. The clear supernatant was used for the assay) was added. Enzyme blank was prepared by adding 0.1 ml 50 mM potassium phosphate buffer, pH 7.0 instead of sample. Illumination was carried out in the aluminium foil lined box fitted with 15 V fluorescent lamp for exactly 10 minutes. The reaction was stopped by switching off the source of illumination. Intensity of the colour was read at 560 nm (Beauchamp and Fridovich, 1971). Enzyme activity was quantified with the help of a standard curve of SOD (400µg/ml). Protein content in the enzyme was estimated as described earlier (Section 2.7.2.1). SOD activity was expressed as IU/mg protein. One unit of the enzyme can be defined as the amount of SOD required to inhibit 50% of NBT reduction at standard condition.

- **SOD substrate**: To 25ml of 0.2M potassium phosphate buffer(pH 7.0), 149mg of methionine, 4.93ml of nitroblue tetrazolium chloride (1mg/ml in 50mM potassium phosphate buffer, pH 7.0) and 0.63ml of riboflavin (1mg/ml in 0.05M potassium phosphate buffer) were added and the volume was made up to 100ml with double distilled water.
2.9 Histology

2.9.1 Light microscopy studies

After opening the general viscera, liver was perfused thoroughly, by injecting phosphate buffered saline through hepatic portal vein and then drained out through pre caval vein to remove blood. The liver tissue was cut into small pieces of 1x1 mm thickness and then fixed in 10% formalin for 24hrs. After placing under running tap water overnight, the tissue was transferred to 50% alcohol for dehydration. The tissue was further dehydrated by passing through a series of ascending grades of alcohol, 70%, 80%, 90% keeping it for 1 hour in each grade and 3 changes with 100% alcohol for 1 hour each. The tissue was then placed in xylene for 15 minutes in each jar of xylene (3 changes) total 45 minutes. The tissue was then transferred to molten paraffin maintained at 68°C for 1 hour each in 3 changes. It was then made into blocks using moulds, allowed to set overnight and then transferred to refrigerator (4°C) for sectioning. After 1 day, the block was sectioned (10 μm) using a microtome and selected the good sections for the slide preparation. The slides were smeared with egg albumin and allowed to dry overnight. The slides were passed through 2 changes of xylene, and descending grades of alcohol (100%, 90%, 80%, 70%, 50%, 5 minutes in each) and then distilled water. The slides were stained with hematoxylin stain for 5 minutes. It was strengthened with acid alcohol and then stained with eosin after washing with water, passed through ascending grades of alcohol, xylene and then mounted using DPX mountant (Arora and Prakash, 1998). The slides were viewed under microscope at 40 x (Olympus BX 41) for pathological changes.
2.9.2 Transmission Electron Microscopy studies

After the liver tissue was perfused with phosphate buffer saline (pH 7.0) as mentioned in section 2.10.1, they were cut into 1x1 mm thick pieces and immediately transferred into the primary fixative i.e. 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer (pH 7.0) for 6-8 hour at 4°C. Following this fixation in primary fixative, the tissues were washed in 0.1 M buffer and then were post fixed in 1% OsO₄ for 1-2 hours at 4°C. The tissues were washed in 0.1M buffer after the post fixation procedure and then dehydrated by using 70% ethanol followed by 80% ethanol. The tissues were en-bloc stained with 2% uranyl acetate in 95% ethanol at 4°C for 1-2 hours. Two changes with a clearing agent propylene oxide were given for 15 minutes each, to facilitate infiltration. After clearing in propylene oxide, the tissues were left in a mixture of 1:1 propylene oxide: embedding medium (Araldite Cy 212) overnight on a rotator at room temperature followed by two changes in pure embedding medium, for 6 hours on a rotator at room temperature. The tissues were embedded in a flat embedding mould. The liquid embedding medium containing the tissues was polymerized in an oven at 60 °C for 48 hours. Using fresh glass knife, ultra thin sections of 60nm thickness were taken which were placed on a copper grid and stored in a grid box. The grids were stained in uranyl acetate for 1-2 hours washed well in water, dried and then stained in lead citrate for 5-7 minutes and washed immediately. The sections were viewed under the electron microscope (Decnai, G2 Bio-Dwin) and areas of interest were photographed (Frasca and Parks, 1960).
2.10 Cytokine expression studies

2.10.1 Collection of the sample

Liver tissue was collected in phosphate buffer saline (pH 7.00) prepared in RNAse free water under sterile conditions from the experimental animal *Mus musculus*.

2.10.2 RNA extraction from hepatic tissue

Liver tissue (100mg) was homogenised with 1ml of Gene Zol total RNA extraction reagent (Taurus Scientific Inc, Ohio, USA). The tissue was incubated in ice for 5 minutes and 200μl chloroform was added, mixed thoroughly, incubated for 10 minutes in ice. The homogenate was centrifuged (Hettich, Zentrifugen Universal 32 R) at 12,000 x g for 20 minutes. The aqueous phase was removed gently without disturbing other phases. To this, 400 μl of isopropanol was added, incubated at (-) 20°C for one hour. After centrifuging at 12,000 x g for 15 minutes, 400 μl of 70% alcohol was added and refrigerated at 4°C for 10 minutes. The samples were centrifuged once again at 12,000 x g for 10 minutes. The pellet thus obtained was dried on a dry bath for 10 minutes. The pellet was then dissolved in 50 μl of diethyl pyrocarbonate (DEPC) treated nuclease free water (Ausubel *et al.*, 1989). To check the quality of the RNA extract, 4 μl was mixed with the loading buffer and run on 1.2% agarose gel prepared in tris borate buffer TBE (1X) containing ethidium bromide (10 mg/ml distilled water).

2.10.3 cDNA Synthesis

cDNA was synthesized using Revert Aid™ First strand cDNA synthesis kit (Fermentas Inc. Maryland, USA). To 4μl RNA extract, 7 μl water and 1 μl
oligo dinucleotide was added and incubated for 5 minutes at 70°C. To this mixture, 4μl 5X reaction buffer, 2μl 10mM dinucleotide tri phosphate, 1μl ribonuclease inhibitor (20 units/μl) were added and incubated for 5 minutes at 37°C. After adding 1μl reverse transcriptase enzyme (Murine Maloney Lucaemia Virus -Reverse Transcriptase of strength 200 units/μl), the mixture was incubated for 1 hour at 42°C. The cDNA thus obtained was incubated at 70°C for 10 minutes to inactivate the enzymes. It was then stored at (-) 20°C (Sambrook et al., 1989). Quality of the cDNA was checked with the constitutive gene β actin. The PCR contents were as follows:

<table>
<thead>
<tr>
<th>PCR Master Mix 2X(Fermentas)</th>
<th>10μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>1μl (10pmol/μl)</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1μl (10pmol/μl)</td>
</tr>
<tr>
<td>Template cDNA</td>
<td>1μl (50ng/μl)</td>
</tr>
</tbody>
</table>

Nuclease free water was added to make the volume up to 20μl.

All reagents were mixed in 250 μl PCR tube and placed into thermal cycler (Eppendorf).
The temperature set up for PCR were as follows:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lid temperature</td>
<td>105°C</td>
</tr>
<tr>
<td>Initial denaturation</td>
<td>94°C for 5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C for 50 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>56°C for 50 sec</td>
</tr>
<tr>
<td>Polymerization</td>
<td>72°C for 50 sec</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>35 cycles</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C for 5 min</td>
</tr>
<tr>
<td>Storage</td>
<td>4°C</td>
</tr>
</tbody>
</table>

4μl of the PCR product was mixed with 2μl of loading dye (Bromophenol blue) and loaded into the wells carefully. It was run on agarose gel (1.2% prepared in TBE 1X) and observed with the help of gel doc system (Alpha Imager™ 1220 Documentation and Analysis system).

2.10.4 mRNA expressions of proinflammatory cytokines

mRNA expressions of TNF-α, IL-1α, IL-2 and iNOS were studied under the similar conditions followed for β actin gene (as mentioned in section 2.11.3) except the annealing temperatures which were as follows:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>62°C</td>
</tr>
<tr>
<td>TNF-α</td>
<td>62°C</td>
</tr>
<tr>
<td>iNOS</td>
<td>58°C</td>
</tr>
<tr>
<td>IL-2</td>
<td>56°C</td>
</tr>
</tbody>
</table>
2.10.5 Primers used for the present study

Primers used for the present study were provided by Dr. Bhaskar Sharma, IVRI, Bareily, U. P. India. The sequences of the primers were as follows:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bactin</td>
<td>B-actin/P/M/F 5' TCTAGGCACCAAGGTTTG3'</td>
<td>460bp</td>
</tr>
<tr>
<td></td>
<td>B-actin/P/M/R 5'TCATGAGGTAGTCCGTCAGG3'</td>
<td></td>
</tr>
<tr>
<td>IL1α</td>
<td>IL 1α/P/M/F 5' GATGTCAACTTCCACTTC 3'</td>
<td>250bp</td>
</tr>
<tr>
<td></td>
<td>IL 1α/P/M/R 5'ACAAACTTCTGCTGACGA3'</td>
<td></td>
</tr>
<tr>
<td>IL2</td>
<td>IL 2/M/F 5' GCAACAGAGATCGAAGCTGG3'</td>
<td>300bp</td>
</tr>
<tr>
<td></td>
<td>IL 2/M/R 5' CGTTCAACCAACAGTACAC3'</td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>TNFα/ P/M/F 5' CCCTTTACTCTGACCCCTTT3'</td>
<td>310bp</td>
</tr>
<tr>
<td></td>
<td>TNFα/ P/M/R 5' AACCTGACCACTCTCCCTTT3'</td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>iNOS /P2/M/F 5'TGCATGGACCAGTATAAGGCAAGC3'</td>
<td>533bp</td>
</tr>
<tr>
<td></td>
<td>iNOS /P2/M/R 5'CTCCTGCCAATGAGTTGC3'</td>
<td></td>
</tr>
</tbody>
</table>

❖ **TBE Buffer** - 5X Stock-To 13.5 g of tris and 6.87 g of boric acid 5 ml of EDTA was added and made up to 250 ml with distilled water to prepare TBE stock. This was diluted to 1 X as a working solution.

❖ **Loading dye** - 0.25% bromophenol blue, 0.25% xylene cynol and 40% sucrose all prepared in distilled water and taken in equal ratio.
2.11 Lipid profiling

The crude tissue lipid extract (extracted from liver as mentioned in section 2.7.2) was fractionated into neutral lipid, glycolipid and phospholipid by column chromatography. Each fraction was further separated into different fractions by thin layer chromatography using different solvent systems.

2.11.1 Column separation

Activated silica gel (100-200mesh size) was dissolved in chloroform to prepare slurry. The slurry was slowly poured into the column (1.6 x 9 cm). Enough care was taken while packing the column to avoid any air bubble. The column bed was allowed to settle for a period of two hours. The height of the column bed was maintained to 6 cm. The column was then washed thoroughly with the same solvent (chloroform) and 250μl of the total lipid sample (about 50 mg) was layered carefully over the bed. Care was taken so that column bed was never dried up or engulfed any air bubbles. About 5 bed volumes of chloroform, acetone and methanol were run to elute neutral lipid, glycolipid and phospholipids respectively (Kates, 1986). Each fraction thus collected was concentrated by using rotary evaporator and the final volume was made up to 250μl in 1 mM BHT (Butylated Hydroxy Toluene) in benzene. These fractions were stored at (-) 20°C.

2.11.2 TLC separation

Each lipid fraction (50μl) was spotted on the pre coated TLC Silica gel 60 plate (20 x 20 cm), obtained from M/s., Merck, Germany, using a Hamilton syringe. The plate was put in solvent chamber containing specific solvent mixture depending upon the fraction till a complete run (Kates, 1986). The spots were viewed by putting the dried plate in an iodine chamber. The spots were
identified against authenticated standards run along with the sample. The following solvent mixtures were used:

- Hexane: Diethyl ether: Acetic acid (80:20:1.5, v/v) for further fractionation of neutral lipid.
- Chloroform: Methanol: Water (65:25:4, v/v) for further fractionation of phospholipid.

2.11.3 Quantification of the TLC separated spots

Each one of these spots / fractions were scraped and analytically quantified. All acylglycerides, namely triglycerides, diglycerides and monoglycerides were estimated by chromotropic acid reagent (section 2.7.2.5). Free cholesterol and esterified cholesterol were estimated by ferric chloride reagent (section 2.7.2.4). Free fatty acids were assayed by sodium dithiocarbamate reagent (section 2.7.2.7). All glycolipid and phospholipid spots were estimated by anthrone reagent (section 2.7.1.1) and ammonium molybdate reagent (section 2.7.2.6) respectively.

2.11.4 Fatty acid profiles

Total lipid / total neutral lipid / total phospholipid were subjected to saponification with 1 ml of 5 N NaOH along with internal standard (C17:0) heptadecanoic acid at 80-90°C for 2 hours followed by acidification and extraction with petroleum ether (40-60°C). Fatty acid thus obtained were methylated using 2% H₂SO₄ in methanol at 70°C for 4hrs. Methyl esters thus obtained were analysed on Agilent 6890 series GLC system equipped with FID detector, using supelco SP2330 fused silica capillary column. 30m x 25mmID x
0.2μm film thickness using temperature programme. The column was initially maintained at 100 °C for 5 min, increased by 30 °C/min to 160 °C and next by 5 °C/min to 220 °C and there it was kept isothermal for 10 min. injector and detector ports were maintained at 220 °C and carrier gas (nitrogen) pressure was maintained at 18psi. (Ghafoorunissa, 1989). Fatty acids were identified by frequent comparison with authentic standards obtained from Sigma Aldrich Chemical Co., USA. The concentrations were expressed as nmole%.

2.12 Statistical Analysis

All the statistical calculations were done online (http://statpages.org). All the recorded observation was expressed in the form of arithmetic mean of six samples and the standard errors (Bailey, 1994) by using the following formula:

\[
\text{Standard Error} = \frac{\text{Standard deviation}}{\sqrt{n}},
\]

\[
\text{Standard deviation} = \sqrt{\frac{\sum (X-X')^2}{n-1}}
\]

where, \(X\) = The arithmetic mean

\(X'\) = The individual observation

\(n\) = number of observations

**Student “t” test:** Comparison test of the obtained data for each sample groups (control, treated and between the different treatment days) were calculated by using common student ‘t’ test. The formula for ‘t’:

\[
t = \frac{(X_1-X_2)}{\sqrt{\frac{(SE1)^2 + (SE2)^2}{n_1 + n_2 - 2}}}
\]

where, \(X_1, X_2\) = Mean of the two data which are under comparison.

\(SE1\) and \(SE2\) = Standard Errors of the two respective mean values.

Degree of freedom = \((n_1 + n_2) - 2\)
The calculated 't' value was tallied from the tabulated 't' values at the probability level 0.05 (at respective degree of freedom, in our case it was 10). If the calculated value was higher than the tabulated value at the probability level 0.05 then the difference was considered significant. However, if the calculated value lies between the value of probability level 0.1 and 0.05 then the difference was considered as equivocal (the difference between the two groups may or may not be statistically significant). If the calculated value was lesser than the tabulated value at probability level 0.1, it was considered as a non significant difference.

Analysis of Variance Test (ANOVA) The comparison of the different parameters between control and all the treated groups were also statistically verified by calculating 'F' value of ANOVA. This was calculated by calculating the variation between the columns and their sum of square on one hand and on another hand by calculating variation within the column and their sum of squares and finally 'F' value was calculated by calculating the values of both the variance that means (the ratio of the variance between the column and the variance within the column) at their respective degree of freedom. The calculated 'F' value was tallied from the statistical table (probability table) to find whether the variation was significant or non significant.