Materials & Methods
3. MATERIALS AND METHODS

3.1. Material: Plant


Fresh plant material for the study was locally obtained, during the month of December-January. Pharmacognostic study was also conducted for identification of the plant by Dr. Sitesh Das, (Botanist), Regional Research Institute on Ayurveda, Calcutta.

3.1.1 Extraction of the plant material, *Cajanus indicus*

Fresh leaves *Cajanus indicus* was extracted with water. The water insoluble part was extracted with petroleum ether, the residue of which was again extracted with 70% alcohol in a Soxhlet apparatus. The alcoholic extract was evaporated to dryness in a rotary evaporator and the residue was taken in diethylether. The ethereal solution was extracted with 2% HCl and 2% NaOH, respectively. The compounds were regenerated by adding 2% NaOH to the acidic part and 2% HCl to the basic part. The acidic, basic and neutral parts were separately extracted with ether. The ethereal solution was distilled and the residue was taken in 70% alcohol for screening hepatoprotective activity.

As hepatoprotective activity was not found in the basic, acidic and neutral part, attention was focused on the aqueous extract of the plant material. Both the protein and the non-protein constituents of the aqueous extract has been studied. Only the protein fraction was found to possess hepatoprotective activity. Further detailed study was made to identify and purify the protein responsible for the above physiological activity.

Fresh leaves of *Cajanus indicus* was taken and thoroughly washed with distilled water and air dried on filter paper. The dry weight of the leaves were taken and crushed in cyclomixer with double distilled water. The aqueous extract of the dried leaves were filtered in starch free cheese cloth and the homogenous filtrate was centrifuged at 10,000 rpm for 30 mins at 4°C in a (Hitachi CR20B2) cold centrifuge. The resultant clear supernatant was then brought to 60% ammonium sulphate saturation and kept overnight in cold at 4°C with gentle stirring. This preparation was then centrifuged at 10,000 rpm for 30 mins and the supernatant obtained was further brought to 90% ammonium sulphate saturation and similarly processed. The
pellet collected after centrifugation at 10,000 rpm for 30 mins at 4°C, was reconstituted in 50 mM Tris HCl buffer, pH-7.2. It was further taken for ice cold acetone precipitation at 4°C to obtain acetone powder in order to remove excess chlorophyll. The acetone powder was then dissolved in 50 mM Tris HCl buffer, pH-7.2, dialysed against double distilled water (Millipore, USA) and concentrated by lyophilization at -48°C in Alpha 1-4 (Christ) 6824 model.

3.1.2 Protein estimation

The crude protein concentration was estimated according to the method of Bradford. A standard curve was prepared using crystalline BSA, 1 mg/ml (Sigma, USA) (figure ). The crude protein at a dose of (i.p., conc. 50-100 μg/ml) were injected in mice and rats for testing its hepatoprotective activity.

3.1.3 Purification of the crude protein

The crude protein was taken for gel filtration through Sephadex G-75 (Pharmacia, USA) column (81 cm x 1.8 cm) which was equilibrated with 50 mM Tris HCl buffer, pH-7.2. The flow rate of the column was maintained at 16.23 ml/hr and volume eluted per fraction was 4.5 ml. Active fractions from tube (36-58) were pooled, concentrated by lyophilization and subsequently applied on to a Sephadex G-50 (Sigma, USA) column (75 cm x 1.6 cm), pre equilibrated with 50 mM Tris HCl buffer, pH-7.2 and 3.5 ml of fractions were collected at a flow rate of 14.2 ml/hr. The protein content of each chromatographic fraction was determined by measuring the optical density at 280 nm in UV Spectrophotometer (Hitachi U2000). Sephadex G-50 column chromatography showed 3 main peaks. All the 3 fractions were tested separately at a dose of (i.p.; conc. 50-60 μg/ml). The fraction 2 showing hepatoprotective activity was further purified.

The active fractions from tube (39-54) were pooled, concentrated by lyophilization to reduce the volume to 10 ml. Subsequently, it was applied onto a DEAE cellulose (Sigma, USA) column (14 cm x 2.2 cm) pre-equilibrated at 20 mM Tris HCl buffer pH-6.8-6.9, having a capacity to bind to 8-10 mg protein. About 5-6 mg of protein was applied to the column at a
time. A linear gradient of a total (100-120 ml) of (0.0 M - 0.5 M NaCl) in the equilibrating buffer was applied to the column at a flow rate of 22-25 ml/hr. 2.0 ml of fractions were collected and protein content was determined by measuring the optical density of 280 nm in UV Spectrophotometer (Hitachi U2000). The protein of the pooled fraction was estimated by Bradford’s method, using BSA as standard\textsuperscript{134}. DEAE cellulose, ion exchange chromatography showed 2 main peaks. Fractions 27-34, in the 1st peak (active fraction) displaying peak hepatoprotective activity, was designated as CI-1.

3.1.4 Native and SDS polyacrylamide gel electrophoresis

The homogeneity of the purified active fraction, CI-1 was checked by native and sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis according to the method of Blackshear and Hames (1984) and Laemmli (1970), using Bio Rad Mini Protean II electrophoresis chamber\textsuperscript{138,139}. 15%, 12% and 10% non denaturing gel (Native Gel) and SDS-PAGE at both alkaline and acidic pH was done.
To run an SDS-PAGE, the following chemicals and stock solution were used:

Table 3.1 Stock solutions for Tris glycine (Laemmli)\textsuperscript{13} denaturing (SDS) discontinuous electrophoresis.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide-Bisacrylamide mix.</td>
<td>29.2 g acrylamide and 0.8 g of bis-acrylamide to 100 ml; stored at 20°C</td>
</tr>
<tr>
<td>Separating gel buffer; 1.5 M Tris-HCl pH 8.8</td>
<td>Tris, 18.15 g. Bring the pH to 8.8 and the volume to 100 ml; stored at 4°C</td>
</tr>
<tr>
<td>Stacking gel buffer; 0.5 M Tris HCl</td>
<td>Tris, 6.05 g. Bring the pH to 6.8 with concentrated HCl and the volume to 100 ml; stored at 4°C</td>
</tr>
<tr>
<td>SDS, 10% (w/v)</td>
<td>SDS, 10 g in 100 ml. distilled water. Filter through Whatman No. 1 before use; stored at 20°C</td>
</tr>
<tr>
<td>10% Ammonium persulphate</td>
<td>Ammonium persulphate, 0.5 g in 5 ml. distilled water; made freshly.</td>
</tr>
<tr>
<td>Electrophoretic Buffer, 1 litre</td>
<td>Tris, 3 g. glycine, 14.4 g and SDS, 1 g. The volume made to 1000 ml.</td>
</tr>
<tr>
<td>Sample buffer, 5X. 10 ml.</td>
<td>1(M) Tris HCl; 0.6 ml, 50% glycerol; 5 ml, 10% SDS (w/v); 2 ml, 2-mercapto ethanol; 0.5 ml, 1% bromophenol blue; 1 ml, 0.9 ml distilled water, stored at 20°C.</td>
</tr>
</tbody>
</table>
Table 3.2 Gel composition for Tris-glycine (Laemmli) denaturing (SDS) discontinuous electrophoresis

<table>
<thead>
<tr>
<th>Final % of acrylamide desired</th>
<th>10%</th>
<th>12%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Lower separating gel, 10 ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed first</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30% Acrylamide bis-acrylamide (ml)</td>
<td>3.35</td>
<td>4.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Separating gel buffer; 1.5 M Tris (ml)</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>10% SDS; w/v (ml)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Degassed water (ml)</td>
<td>4.0</td>
<td>3.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Added to polymerise</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% APS (µl)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>TEMED (µl)</td>
<td>5-6</td>
<td>5-6</td>
<td>5-6</td>
</tr>
<tr>
<td>B. Upper Stacking gel, 10 ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed first</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30% Acrylamide bis-acrylamide (ml)</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stacking gel buffer; 0.5 M (ml)</td>
<td>1.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% SDS; w/v (ml)</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degassed water (ml)</td>
<td>6.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Added to polymerise</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% APS (µl)</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEMED (µl)</td>
<td>8-10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Normally, the separating gel takes 45 min to get set and the stacking gel about 30 min. After pipetting the separating gel mixture into the gel-plate upto a certain height, waited for 45 min before pipetting the stacking gel in between the pair of gel plates. A comb was placed at the top of the stacking gel during setting. After waiting for 30 min the plates with the gel is placed in the gel apparatus, and the buffer chambers above and below were flooded with the
electrophoretic buffer. The gel faces the buffer at the top. Next the comb was lifted catiously. Extreme care was taken not to trap any air bubble within the gel or at those places where the buffer comes in contact with the gel.

Now for preparing the samples for running an SDS-PAGE, 4 vol of a sample (or sample + stack tris buffer, pH 6.8) and 1 vol of the sample denaturing buffer was taken in an eppendorf tube and heated over boiling water-bath for 10 min, cooled, and loaded into the lanes on the stacking gel. Appropriate standard protein solutions were similarly diluted, heat treated and run along with the sample solutions. At least four standard proteins were necessary for standardizing mobilities with respect to their molecular weights.

When all the sample and standard proteins had been appropriately loaded into the lanes, connections from an electrophoresis power supply to the gel electrophoresis apparatus was done, and the setting adjusted to 2-3 volts/cm. The protein with the dye began to move downwards, (i.e., + pole) and when the dye-front reaches the separating gel boundary, the voltage was increased to 4-5 volts/cm. The proteins continued to move downwards, and when the dye had reached the end of the glass plate or the gel, power supply was switched off, the plates were separated and the proteins in the gel were fixed.

For Coomassie-staining, done for detecting proteins ≥ 1 μg in the gel, five gel volume of the staining solution (0.25% Coomassie brilliant blue R-250 in 50% MeOH and 10% acetic acid) was added. Fixing and staining of the gel was continued overnight. After that, the gel was destained with successive changes of 5% MeOH, 7.5% AcOH at room temperature until the background was clear. In an alternative method, proteins could also be stained by 0.25% Coomassie brilliant blue R-250 in 25% isopropanol and 10% acetic acid for 1 hour and destained by 10% acetic acid.

For silver-staining, the gel was fixed overnight with Fix-I (50% MeOH, 12% AcOH), then 2 x 15 mins with Fix-II (10% EtOH, 5% AcOH), then 2 x 15 min with Fix-III (10% distilled Ethanol). It was then kept for 55-60 sec in solution A (100 ml of double distilled water + 50 μl of 37% formalin + 42 μl of 43% sodium thiosulphate (Na2S2O3) and washed 3 times x 60 sec with double distilled water. Next, the gel was kept for 10 min in Solution B (100 ml of double distilled water + 200 mg of AgNO3 + 50 μl of 37% formalin) and washed again for 2 x 60 sec with double distilled water. Next, the gel was kept in solution C (100 ml of
double distilled water + 6 g of Na₂CO₃ + 50 µl of 37% formalin + 1 µl of 43% Na₂S₂O₃). The gel was allowed to develop for 30 sec to 10 min, watching constantly until the desired intensity had reached for the protein bands. Generally, 30 sec for 500 ng protein or 90 sec for 50 ng protein is necessary. After the desired intensity, solution C was poured off and 5% AcOH was added for a few min to stop the reaction. The gel was washed with double distilled water and kept in 5% glycerine + 1% AcOH for storage.

For running a Native PAGE, the procedure followed was same as in the case with denaturing gel electrophoresis (SDS-PAGE) except that the addition of SDS was completely omitted, and 20% glycerol was uniformly present in all buffers (the sample buffer, the stacking gel buffer, running gel buffer) to keep the protein in active state. In addition, the sample buffer was not heated with protein solution and the electrophoresis was carried out in the cold room. The lanes in the gel was cut into different slices, proteins were eluted in an appropriate gel-elution buffer and the protein activity was studied in a preparative native-gel electrophoresis procedure for protein purification138-139.

3.1.5 Molecular weight determination

SDS-PAGE was used to determine the molecular weight of a protein since protein migration is generally proportional to the mass of the protein142,143. A standard curve was generated with protein of known molecular weight, and the molecular weight of the protein of Cl-I was interpolated from this curve.

Following gel electrophoresis and staining, the distance of migration of the proteins as well as that of a tracking dye (bromophenol blue) were measured. Distance of migration was measured from beginning of the separating gel to the leading edge of the protein band.

Calculation of Rf values:

\[
R_f = \frac{\text{distance of the protein migration}}{\text{distance of tracking dye migration}}
\]
The logarithm to base 10 of the known protein molecular weights were plotted as a function of their Rf. The molecular weight of the unknown protein, CI-1 was read from the graph based on its Rf values.

3.1.6 Isoelectric focusing

Isoelectric pH of the protein, CI-1 was determined on a 5% polyacrylamide gel using, ATTO IEF Apparatus, Japan (Model AE-3230) as per manufacturer's recommendation. The pl of CI-1 was compared with the known isoelectric pH of the standard protein, using isoelectric focusing calibration kit, ranging from pH 3-10. 25 μgs of CI-1 was absorbed in absorbent paper (3 x 3 mm), and was directly placed on the gel. 0.04 M DL-Aspartic acid was used as anode solution and 1 M NaOH as cathode solution. The focusing of the gel was done for about 2 hours at 900 V in a cold laboratory at 4°C. On completing focusing the gel was taken out for staining and destaining.

The gel was first immersed in the fixative solution (5% Sulphosalicylic acid, 10% Acetic acid) for 0.5 hours to precipitate the focused proteins, then in the destaining solution (30% Methanol, 10% Acetic acid) for 0.5 hours to diffuse the carrier ampholytes. The gel was stained in a staining solution (0.2 Coomassie brilliant blue G-250) for 2 hours to stain the proteins. It is then immersed in a destaining solution until the background was cleared.

3.1.7 Anthrone Reaction

The test was performed to assess the carbohydrate moiety in the protein. The total amount of hexose unit in a sample can be determined by converting the hexoses to the furfural derivatives in strong acid and reacting them with anthrone with which they form coloured complexes that can be estimated spectrophotometrically. Soluble and insoluble carbohydrates all yield coloured complexes like free hexoses in strong sulphuric acid. The following method is essentially that of Yamamoto and Rouser.
Reagent:

(i) A stock solution of 2% Anthrone in 98% H$_2$SO$_4$ (10 ml), stable for 3 weeks if refrigerated, was diluted with 87.5% H$_2$SO$_4$ (90 ml), (ii) Carbohydrate sample (20-200 µg), (iii) Dimethyl formamide and (iv) glucose-free-galactose (200 µg/ml) prepared in distilled water.

Procedure:

(20-200 µl) hexose or equivalent amount of glycoprotein (CI-1, sample) in 1 ml dimethyl formamide were added to 4 ml of Anthrone reagent, and the mixtures were heated on a boiling water-bath (100°C) for 15 minutes in pyrex test tubes (test-tubes capped with marbles). The tubes were removed from water bath and rapidly cooled and their absorbances were measured at 625 nm in UV Spectrophotometer (Hitachi U2000) against a reagent blank. The amount of carbohydrate (hexose) present in a sample (w/w or w/v) are determined from a calibration curve, obtained ideally with a pure glycolipid/glycoprotein standard or with a standard stock solution of glucose/ galactose (200 µg/ml). In the present case galactose was used (Fig. 3.2).

3.1.8 Periodic acid - silver staining

A SDS polyacrylamide electrophoresis was done. The gel was fixed overnight in 25% isopropanol (v/v) and 7% acetic acid (v/v). It was then kept in Periodic acid solution (Periodic acid, 1.05 g dissolved in 150 ml deionized water) for 5 mins. Then the solution was drained off. The gel was washed with deionized water 8 x 30 mins. It was then stained with ammonical silver nitrate solution for 10 mins (28 ml of 0.1 (N) NaOH; 1.2 ml 25% Ammonia solution volume made to 200 ml with distilled water, to it 5 ml of 20% AgNO$_3$ solution was added slowly until the brown precipitate vanishes). Then the gel was washed with deionized water 4 x 10 mins. The stain was developed with a developer solution containing (50 mg of citric acid; 0.5 ml of 37% formaldehyde, volume made to 1 litre with distilled water) for 10-20 mins. Stop solution (7% acetic acid, v/v) was added to stop the reaction and the gel was kept for 1 hour at room temperature. The gel was washed with double distilled water and kept in 5% glycerol and 1% Acetic acid for storage.

The carbohydrate moiety will produce black colour and it will be stained faster than proteins$^{148,149}$.
3.1.9 Circular Dichroism

Circular Dichroism is used to measure the optical activity of asymmetric molecules in solution. It gives information about the unequal absorption of left and right handed circular polarised light by optically active molecules. The CD spectra of the protein, CI-1 (1 mg/ml) was taken in sodium phosphate buffer (degassed and filtered), pH 7.5 and both far and near UV-CD spectra were taken. The concentration of protein used in near UV-region was approximately four times than the concentration required in the far UV-region. This was obvious as protein absorbs more strongly in the far UV-region.

Instrumental parameters:

1. Instrument used: JASOC Spectropolarimeter (Model J-600)
2. Band width = 1.0 mm (for both near and far UV-region)
3. Sensitivity = 5 m deg
4. Wave length range = 200-250 for far UV and 250-300 for near UV-region
5. Scan speed = 100 mm/min
6. No. of accumulations = 3

3.1.10 Chemicals and Reagents

Tris-HCl, tris, ammonium sulphate, ammonium persulphate, glycine, sodium dodecyl sulphate, 2-mercaptoethanol, sodium thiosulphate, silver nitrate, glycerol were purchased from SRL, India; Merck, India. Electrophoretic dyes like Brilliant R-250, G-250, Bromophenol blue were procured from SRL, India. Protein purification chemicals like SephadeX G-75, SephadeX G-50, SephadeX G-10, DEAE-cellulose were obtained from Pharmacia, USA. Bovine serum albumin, ovalbumin, acrylamide, bisacrylamide, standard molecular weight markers, TEMED, collagenase type IV for hepatocyte isolation were purchased from Sigma, USA. Silymarin procured from Aldrich Chemicals. Spectroscopic grade of CCl4, ethanol from BDH, India. Paracetamol (Aldrich, USA), β-galactosamine (Sigma, USA); Diagnostic Reagent Kits for different enzymes like transaminase, phosphatase, dehydrogenase, serum total protein/albumin; triglycerides, bilirubin and plasma prothrombin time were obtained from Span Diagnostics, India.

Microscopic stains, paraffin (Std. fine Chemicals, India) Cedar wood oil (Daniel, UK), Electron Microscopic Reagents viz., osmium tetroxide, CY212 araldite, dodecenyl succinic
anhydride (DDSA), tridimethyl amino phenol (DMP 30), uranyl acetate, lead acetate and glutaraldehyde from Agar Scientific Ltd., UK.

Immunochemicals viz., Freund’s complete/incomplete adjuvant from Difco Lab., USA.

Cell culture media e.g., RPMI-1640, CPSR-2, Heps buffer, PBS, Percoll, Trypan Blue from Himedia Lab., Mumbai. Horse raddish peroxidase, HRP anti-immunoglobulin conjugate, micro
ELISA plates were purchased from Geni Bangalore Pvt. Ltd., India and Gibco, USA.

All other chemicals were of analytical grade purchased locally from Qualigen, SRL, Merck, India.

3.2 Animal experiments

Inbred strain of swiss albino mice (male, body wt. 30 g ± 2) were maintained in Bose Institute, Animal House. The animals were acclimatize under laboratory conditions for one week before the experiment and provided with standard pellet diet (Lipton India Ltd.), water ad libitum.

Inbred strain of Charles Foster adult rats (male; body wt. 90 g ± 10) were maintained in Bose Institute, Animal House and housed under conditions of controlled temperature (23 ± 1°C); standard pellet diet (Lipton India Ltd.) and water ad libitum. The animals were left to acclimatized under laboratory conditions for one week before the experiment.

The animals were divided into several groups of six to ten (n = 6-10 in each group) in different models of experiments.

3.2.1 Acute liver damage models

a) Carbon tetrachloride induced hepatotoxicity:

(i) Charles Foster rats (male, body wt. 90 g ± 10) were treated with CCl₄ at a dose of 0.3 ml/100g body wt.; orally, twice at a interval of 7 days along with corn oil as vehicle.¹⁵⁰,¹⁵¹

(ii) Swiss albino mice (male; body wt. 30 ± 2 g) were administered intraperitoneally with CCl₄ at a dose of 0.1 ml/100g of body weight, twice a week along with liquid paraffin as vehicle.¹⁵²
b) **Acetaminophen/Paracetamol induced toxicity**: Swiss albino mice (male body wt. 30 g ± 2) were fasted overnight and injected intraperitoneally with a saturated solution of paracetamol 35 mg/ml in warm (45-50°C) normal saline at a single dose of 300-500 mg/Kg.

c) **β-galactosamine induced hepatotoxicity**: Swiss albino mice (male; body wt. 30 g ± 2) were subcutaneously injected with an aqueous solution of β-galactosamine HCl at a single dose of 0.5 ml; 500 mg/Kg.

d) **Ethanol induced hepatotoxicity**: Swiss male albino mice (body wt. 30 g ± 2) were orally fed with 40% ethanol (w/v) 2 ml/100g of body wt. for 21 days; (0.6 ml, p.o. 21 days)

Experimental studies on both male albino swiss mice and Charles Foster rats were performed in our laboratory. A comparative effect of the four hepatotoxicants induced liver damage were studied to evaluate their effect on the liver cell.

36-48 hours after the second dose of CCl₄ administration the animals were sacrificed. 24 hours after paracetamol, β-galactosamine HCl injection and 21 days after ethanol toxicity, the experimental animals were sacrificed by ether anaesthesia and cervical dislocation.

### 3.2.2 Treatment by the protein, CI-1

The animals were treated with purified protein CI-1, at a varying dose of 1.0, 1.5, 3.0, 4.0 and 6.0 mg/Kg i.p. once daily for 7, 14 and 21 days in different set of animals in different models of experiments.

The pre-treated group of animals were first treated with the active protein fraction prior to hepatotoxin administration. The post-treated group of animals were first intoxicated by the hepatotoxin and then separately treated with the active protein.

### 3.2.3 Toxicity or LD₅₀ estimation of CI-1

Acute toxicity studies provide the first indication of the safety/ toxicity of a new drug. The initial studies were conducted in mice and rats. The major objective of these studies was to
establish what dose level of the drug would be lethal to the test animals. The most common measurement of acute toxicity is the median lethal dose i.e., the dose which kills 50% of the animals\textsuperscript{156,157}.

Swiss albino mice (male; body wt. 30 g ± 2); Charles Foster rats (male; body wt. 90 g ± 10) were used as experimental animals for toxicity test of CI-1 protein. The purified fraction CI-1 from the leaves of *Cajanus indicus* were introduced in varying concentration in experimental animals via intraperitoneal routes. LD\textsubscript{50} estimation were made for a period of 24 hr., in each batch of experimental animals consisted of 8-10, and each experiment was repeated 4-5 times. The values noted represent mean observation from repetitions.

### 3.3 Biochemical study

#### 3.3.1 Estimation of serum glutamate pyruvate transaminase

Biochemical estimation of *in vitro* glutamate pyruvate transaminase, GPT (also called Alanine transaminase, ALT) activity in serum according to the method of Reitman and Frankel (1957)\textsuperscript{158} using Diagnostic Reagent kit, Span Diagnostics Ltd., India, Code No. 25912.

**Reagents supplied in the kit:**

Reagent 1 : Buffered alanine-α-ketoglutarate substrate, pH 7.4.
Reagent 2 : 2,4-dinitrophenyl hydrazine (2,4-DNPH) colour Reagent.
Reagent 3 : Sodium hydroxide, 4N.
Reagent 4 : Working pyruvate standard, 2mM.

**Preparation of working solutions:**

Solution 1 : Diluted 1 ml of Reagent 3 in 10 ml with distilled water. The reagents 1, 2 and 4 were ready for use.

**Preparation of a standard curve:**

The standard curve was plotted initially when the first test was performed with the working pyruvate standard, 2 mM.

**Experimental Procedure:**

0.25 ml of Buffered substrate, pH-7.4 was incubated at 37°C for 5 minutes. 0.05 ml of serum was added and mixed well, incubated at 37°C for 30 minutes. 2,4 DNPH colour reagent (0.25 ml) was added, mixed well and allowed to stand at room temperature (RT) for 20 minutes.
2.5 ml of solution was added, mixed well and allowed to stand at RT for 10 minutes. The optical density (OD) was measured at 505 nm against distilled water in UV Spectrophotometer (Hitachi U2000).

### 3.3.2 Estimation of serum glutamate oxaloacetate transminase

Biochemical estimation of \textit{in vitro} glutamate oxaloacetate transaminase, GOT (also called Aspartate transaminase, AST) activity in serum by the method of Reitman and Frankel (1957) using Diagnostic Reagent kit, Span Diagnostic Ltd., India, Code No. 25913.

**Reagents supplied in the kit:**
- Reagent 1: Buffered aspartate-o-ketoglutarate substrate, pH-7.4.
- Reagent 2: 2,4 DNPH colour reagent
- Reagent 3: Sodium hydroxide, 4N
- Reagent 4: Working pyruvate standard, 2 mM.

**Preparation of working solution:**
- Solution 1: Diluted 1 ml of Reagent 3 in 10 ml with distilled water.
- Reagents 1, 2 and 4 were ready for use.

**Preparation of Standard Curve:**
- A standard curve was plotted initially when the first test was performed with the working standard pyruvate solution, 2 mM.

**Experimental Procedure:**
- 0.25 ml of Buffered Aspartate-α-KG Substrate, pH-7.4 was incubated at 37°C for 5 minutes. 0.05 ml of serum was added to it, mixed well and incubated at 37°C for 60 minutes.
- 0.25 ml of 2,4 DNPH colour reagent was added, mixed well and allowed to stand at room temperature for 20 minutes. 2.5 ml of Solution 1 was added after 20 minutes, vortexed and allowed to stand at RT for 10 minutes. The optical density at 505 nm was measured against distilled water in UV Spectrophotometer (Hitachi U2000).

### 3.3.3 Estimation of liver tissue GPT and GOT

Livers of the experimental animals were quickly removed and placed in chilled 0.25 M Sucrose; blotted dry, weighed, minced and homogenized (1:4) w/v in ice cold Tris HCl buffer
pH-7.4, containing 1.5% KCl. The tissue homogenate was centrifuged at 2000 rpm, for 10 min. at 4°C in Hitachi CR 20B2 cold centrifuge. The supernatant was collected and finally suspended in ice cold Tris HCl buffer, pH-7.4 and biochemical estimation of GPT and GOT were done according to the method of Reitman and Frankel (1957) 158.

3.3.4 Estimation of serum alkaline phosphatase


Reagents supplied in the kit:
Reagent 1: Buffered substrate, pH-10.
Reagent 2: Chromogen reagent
Reagent 3: Phenol standard, 10 mg

Preparation of working solution:
One vial of Reagent 1, was reconstituted with 4.5 ml of distilled water. Reagents 2 and 3 were ready for use.

Experimental procedure:

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Control (C)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Buffered Substrate</td>
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<td>0.5 ml.</td>
<td>0.5 ml.</td>
<td>0.5 ml.</td>
</tr>
<tr>
<td>Distilled Water</td>
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<td>1.5 ml</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol Standard 10 mg%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed well and incubated for 3 minutes at 37°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromogen Reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol Standard 0.05 ml</td>
<td>0.05 ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed well and incubated for 15 minutes at 37°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All the test tubes were mixed well after the addition of each reagent and the optical density was measured at 510 nm in UV Spectrophotometer (Hitachi U2000).
Calculation of serum alkaline phosphatase activity in KA units:

\[
\frac{\text{OD Test} - \text{OD control}}{\text{OD Standard} - \text{OD Blank}} \times 10
\]

3.3.5 Estimation of Serum Lactate Dehydrogenase

Biochemical determination of the *in vitro* lactate dehydrogenase (LDH) activity in serum by 2,4 DNPH method (King, 1959)\(^{160,161}\); Diagnostic Reagent Kit, Span Diagnostics Ltd., India; Code No. 25903.

Reagents supplied in the kit:

- Reagent 1: Buffered lactate substrate, pH-10.0
- Reagent 2(A): NAD, Nicotinamide adenine dinucleotide for test
- Reagent 2(B): Nicotinamide adenine dinucleotide (NAD) for standard curve.
- Reagent 3: 2,4 DNPH colour agent
- Reagent 4: NADH
- Reagent 5: Sodium hydroxide, 4N
- Reagent 6: Working pyruvate standard, 1 mM.

Preparation of working solution:

- Solution I(A): Each vial of Reagent 2(A) was reconstituted with 0.3 ml of distilled water
- Solution I(B): Each vial of Reagent 2(B) was reconstituted with 1.4 ml of distilled water
- Solution II: Each vial of Reagent 4 was reconstituted with 1.2 ml of Reagent 1 water
- Solution III: 1 ml Reagent 5 was diluted to 10 ml with distilled water.

Preparation of Standard Curve:

Standard Curve was plotted initially when the first test was performed with working pyruvate standard, 1 mM and with Solution I (B).
**Experimental Procedure:**

**Lactate Dehydrogenase (LDH)**

<table>
<thead>
<tr>
<th></th>
<th>Control (C)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 1 : Buffered Lactate Substrate</td>
<td>0.5 ml.</td>
<td>0.5 ml.</td>
</tr>
<tr>
<td>Serum</td>
<td>-</td>
<td>0.05 ml.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.1 ml.</td>
<td>-</td>
</tr>
</tbody>
</table>

Mixed and incubated at 37°C for 5 mins

<table>
<thead>
<tr>
<th>Solution I(A)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1 ml.</td>
</tr>
</tbody>
</table>

Mixed and incubated at 37°C for 15 mins

<table>
<thead>
<tr>
<th>Reagent 3 : DNPY Color Reagent</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>0.5 ml.</td>
</tr>
<tr>
<td></td>
<td>0.05 ml.</td>
</tr>
</tbody>
</table>

Mixed and incubated at 37°C for 15 mins

<table>
<thead>
<tr>
<th>Solution III</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.0 ml.</td>
</tr>
<tr>
<td></td>
<td>5.0 ml.</td>
</tr>
</tbody>
</table>

Mixed well by inversion, allowed to stand at RT for 5 minutes. The optical density of Test (T) and Control (C) were measured against distilled water at 440 nm in UV Spectrophotometer (Hitachi U2000).

**Calculations:**

The nett. optical density of Test (Tn) was calculated: \( Tn = OD (Test) - OD (C) \). The nett OD of Test (Tn) was marked on Y axis of the Standard Curve and the corresponding enzyme activity on X axis was extrapolated.
3.3.6 Estimation of serum bilirubin

In vitro determination of Bilirubin in serum according to the modified Diazo Method (Malloy and Evelyn, 1937) by using Diagnostic Reagent Kit, Span Diagnostics Ltd., India; code No. 25923.

Reagents supplied in the kit:

- Reagent 1: Total bilirubin reagent
- Reagent 2: Direct bilirubin reagent
- Reagent 3: Sodium nitrite reagent
- Reagent 4: Artificial standard (10 mg % Bilirubin)

Preparation of working solutions:

Solution I: 10 ml of Reagent 1 was mixed with 0.05 ml of Reagent 3 and allowed to stand for 15 minutes at room temperature before use.

Solution II: 10 ml of Reagent 2 was mixed with 0.05 ml of Reagent 3 and allowed to stand for 15 minutes at room temperature before use.

Experimental Procedure:

<table>
<thead>
<tr>
<th></th>
<th>Total (T)</th>
<th>Total Blank (TB)</th>
<th>Direct (D)</th>
<th>Direct Blank (DB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>0.15 ml.</td>
<td>0.15 ml.</td>
<td>0.15 ml.</td>
<td>0.15 ml.</td>
</tr>
<tr>
<td>Solution I</td>
<td>3.0 ml.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reagent 1</td>
<td>-</td>
<td>3.0 ml.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Solution II</td>
<td>-</td>
<td>-</td>
<td>3.0 ml.</td>
<td>-</td>
</tr>
<tr>
<td>Reagent 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.0 ml.</td>
</tr>
</tbody>
</table>

Mixed well and incubated at 37°C for 5 minutes.

The optical density was measured against distilled water at 540 nm in UV Spectrophotometer (Hitachi U2000). OD of Reagent 4 (Artificial standard = 10% mg bilirubin) against distilled water was taken.
Calculations:

Bilirubin concentration in mg/100 ml

\[
\text{Total (A)} = \frac{\text{OD } T - \text{OD TB}}{\text{OD Std.}}
\]

\[
\text{Direct (B)} = \frac{\text{OD D - OD DB}}{\text{OD Std.}}
\]

Indirect = (A) - (B)

3.3.7 Estimation of serum triglyceride

In vitro estimation of serum triglyceride by Acetylacetone Method (Fletcher, 1968)\textsuperscript{163} using Diagnostic Reagent Kit, Stanbio, India.

Reagents:

Heptane, Isopropanol, Sulphuric acid, Potassium hydroxide, Periodate reagent, Ammonium acetate, Acetylacetone, Triglyceride standard.

Preparation of working solution (Chromogen):

0.1 ml of Acetylacetone was added to 14 ml of Ammonium acetate solution and mix. Chromogen is stable at least for two weeks when stored at 2-8°C.
Experimental Procedure:

Three test tubes were marked as Blank (B), Standard (S), and Test (T) and proceeded as follows:

**STEP - I**

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum/Plasma</td>
<td>-</td>
<td>-</td>
<td>0.5 ml.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.5 ml.</td>
<td>0.5 ml.</td>
<td>-</td>
</tr>
<tr>
<td>Triglyceride standard</td>
<td>-</td>
<td>0.5 ml.</td>
<td>-</td>
</tr>
<tr>
<td>Heptane</td>
<td>2.0 ml.</td>
<td>2.0 ml.</td>
<td>2.0 ml.</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>3.5 ml.</td>
<td>3.0 ml.</td>
<td>3.5 ml.</td>
</tr>
<tr>
<td>Sulphuric Acid</td>
<td>1.0 ml.</td>
<td>1.0 ml.</td>
<td>1.0 ml.</td>
</tr>
</tbody>
</table>

Mixed on a vortex mixture, and allowed to stand at room temperature for 10 minutes for the clear separation of two layers.
Another set of test tubes marked Blank (B), Standard (S) and Test (T) were arranged and proceeded as follows:

**STEP - II**

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top Solvent layer (STEP - I)</td>
<td>0.4 ml.</td>
<td>0.4 ml.</td>
<td>0.4 ml.</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>2.0 ml.</td>
<td>2.0 ml.</td>
<td>2.0 ml.</td>
</tr>
<tr>
<td>Potassium Hydroxide</td>
<td>1 drop</td>
<td>1 drop</td>
<td>1 drop</td>
</tr>
</tbody>
</table>

The contents of the test tubes were mixed well and closed tightly with cotton plug. They were kept in a hot water bath or oven at 60-65°C for 10 minutes and cooled to room temperature. Then added:

- Periodate Reagent: 0.2 ml. 0.2 ml. 0.2 ml.
- Chromogen: 1.0 ml. 1.0 ml. 1.0 ml.

The contents were mixed well, cooled and kept again at the same temperature for another 10 min.

The optical density of Test (T), Standard (S) and Blank (B) were measured in a UV Spectrophotometer (Hitachi U2000), at 420 nm against distilled water.

**Calculation:**

\[
\text{OD of Test - OD of Blank} \\
\text{OD of Standard - OD of Blank} \\
\]

Serum Triglyceride in mg/dl = \[\text{\ldots\ldots\ldots\ldots\ldots\ldots}\] x 200

3.3.8 **Estimation of Plasma prothrombin time**

Biochemical determination of *in vitro* one stage prothrombin time of plasma by Quick’s Method, (Quick, A.J., 1932; Quick, A.J.; 1939)\textsuperscript{164,165} (Thomson, 1980)\textsuperscript{166} using Diagnostic Reagent Kit, Span Diagnostics Ltd., India, Code No. 20419B.
Reagent supplied in the Kit:

Reagent 1: Brain thromboplastin
Reagent 2: Reconstituting solution

Preparation of working solution:

Each vial of Reagent 1 (Brain thromboplastin) was reconstituted with 1 ml of Reagent 2 and gently rotated to mix the contents on a vortex mixer to obtain a homogenous suspension.

The working solution was incubated at 37°C for 30 min before use.

Experimental procedure of the Test Sample:

3.8% sodium citrate solution was used as an anticoagulant. The ratio of anticoagulant to blood, collected from the experimental animals was 1:10 (0.25 ml of sodium citrate + 1.8 ml of blood). Plasma was separated by centrifugation and was immediately taken for estimation of plasma prothrombin time.

0.1 ml of plasma was taken in a test tube and kept at 37°C in a water bath for 5 min. 0.2 ml of pre-incubated thromboplastin working solution was mixed by gentle rotation. The stop-watch was stopped as soon as fibrin clot was seen and the time was recorded for coagulation of plasma as “one stage prothrombin time”. The experiment was repeated 5 times and the mean prothrombin time of the experimental animals was determined.

3.3.9 Estimation of Serum total protein and albumin

In vitro estimation of total proteins and albumin in serum by modified Biuret and Dumas Method (Dumas, 1971) (Peters et al., 1982), using Span Diagnostic Reagent Kits, India; Code No. 25931

Reagent supplied in the Kit:

Reagent 1: Biuret reagent (modified)
Reagent 2: Buffered dye reagent
Reagent 3: Protein standard
**Experimental Procedure:**

**Total Proteins**

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>0.05 ml.</td>
</tr>
<tr>
<td>Reagent 3 : Protein Standard</td>
<td>-</td>
<td>0.05 ml.</td>
<td>-</td>
</tr>
<tr>
<td>Reagent 1 : Biuret Reagent (Modified)</td>
<td>2.5 ml.</td>
<td>2.5 ml.</td>
<td>2.5 ml.</td>
</tr>
</tbody>
</table>

Mixed well and allowed to stand at RT for 5 mins. The optical density of standard (S) and Test (T) were measured in UV spectrophotometer Hitachi (U2000) at 550 nm against Blank (B).

**Albumin**

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 2 : Buffered Dye Reagent</td>
<td>3.0 ml.</td>
<td>3.0 ml.</td>
<td>3.0 ml.</td>
</tr>
<tr>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>0.02 ml.</td>
</tr>
<tr>
<td>Reagent 3 : Protein Standard</td>
<td>-</td>
<td>0.02 ml.</td>
<td>-</td>
</tr>
</tbody>
</table>

Mixed well and allowed to stand at RT for 1 min. The optical density of standard (S) and Test (T) were measured in UV spectrophotometer Hitachi (U2000) at 600 nm against Blank (B).
Calculation:

\[
\text{Serum total protein in g/100 ml, } X = \frac{\text{OD Test}}{\text{OD Standard}} \times \text{Conc. of total protein}
\]

\[
\text{Serum albumin in g/100 ml, } Y = \frac{\text{OD Test}}{\text{OD Standard}} \times \text{Conc. of Albumin}
\]

3.3.10 Estimation of urine bilirubin and urobilinogen

Biochemical estimation of bilirubin\(^{169}\) and urobilinogen\(^{170}\) in urine by Ames, Multiple Reagent Strips, Bayer Diagnostics, India. Bilirubin, the test is based on the coupling of bilirubin with diazotised dichloroaniline in a strongly acid medium. Urobilinogen, is based on a modified Ehrlich reaction, in which \(p\)-diethylaminobenzaldehyde in conjunction with a colour enhancer reacts with urobilinogen in a strongly acid medium to produce a pink-red colour (Free & Free, 1972, Talib, 1978)\(^{169,170}\).

Experimental procedure:

Few drops of fresh urine specimen of the experimental animals was taken on a clean, dry slide. One strip from the bottle was taken and the reagent areas of the strip was immersed in urine specimen and was removed immediately to avoid dissolving out reagents. The strip was held in the horizontal position to prevent possible mixing of chemicals from adjacent reagent areas. Bilirubin at 30 secs and Urobilinogen at 60 secs after dipping were read visually, comparing with the darkest colour to appropriate colour chart.

3.4 Histopathological Study

3.4.1 Light Microscopical Study

Small pieces of liver were removed after autopsy to Bouin’s fluid (Picric acid, 75 ml; Formalin, 25 ml; Acetic acid, 5 ml) for 24 hours. The tissues were dehydrated in graded concentration of ethanol; first 50% ethanol and then progressively in 70%, 80%, 90%, 95-96% and absolute ethanol. The tissues were rinsed in xylene for 10 min and kept in cedar wood oil (Daniel, UK) for clearing.
Embedding of tissues were done in commercially available paraffin wax, 50-52°C; 58-60°C for 3-4 hours with 2 changes to speed up the impregnation of wax. After embedding the tissues along with the molten paraffin were poured in the metallic L pieces or in paper boats to form paraffin blocks. Paraffin sections of 5-8 μm were prepared in the rotatory microtome (WESWOX, MT 1090) and stained in hematoxylin and eosin (Harris, 1900) and mounted in neutral DPX medium.171,172

3.4.2 Electron Microscopical Study

A simplified method for tissue processing for Transmission Electron Microscopy (TEM).173

The animals were sacrificed by ether anaesthesia and cervical dislocation. Small pieces of liver were taken and rinsed in 0.1M phosphate buffer, pH 7.2. Approximately 1 mm³ liver pieces were trimmed and immediately fixed into 3% ice cold glutaraldehyde in 0.1M phosphate buffer and kept overnight at 4°C. The tissue pieces were washed 3 times for 15 min in 0.1M phosphate buffer pH 7.2 to remove traces of free glutaraldehyde.

Then the tissue pieces were further fixed in 1% osmium tetraoxide in 0.1M phosphate buffer for 5-6 hours at 4°C. After post fixation the tissues were washed again in 0.1M phosphate buffer; 3 times and were kept in buffer at 4°C overnight; to remove free osmium tetraoxide.

The tissues were dehydrated using ascending grades of acetone.

<table>
<thead>
<tr>
<th>Acetone Concentration</th>
<th>Time and Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acetone in distilled water;</td>
<td>10 mins. x 2 changes</td>
</tr>
<tr>
<td>50% acetone in distilled water;</td>
<td>10 mins. x 2 changes</td>
</tr>
<tr>
<td>70% acetone in distilled water;</td>
<td>10 mins. x 2 changes</td>
</tr>
<tr>
<td>80% acetone in distilled water;</td>
<td>10 mins. x 2 changes</td>
</tr>
<tr>
<td>90% acetone in distilled water;</td>
<td>10 mins. x 2 changes</td>
</tr>
<tr>
<td>95% acetone in distilled water;</td>
<td>10 mins. x 2 changes</td>
</tr>
<tr>
<td>100% acetone in distilled water;</td>
<td>10 mins. x 2 changes</td>
</tr>
</tbody>
</table>
Infiltration:

100 g of CY212 araldite; 75 g of dodecenyl succinic anhydride (DDSA); 2 g of tridimethyl amino methyl phenol (DMP 30) were mixed thoroughly. The air bubbles were removed by keeping the resin mixture in vacuum oven. The tissues were kept in the embedding mixture for infiltration as follows:

- 1 part of embedding mixture and 3 parts of Acetone for 4 hours in a vacuum oven.
- 1 part of embedding mixture and 1 part of Acetone at room temperature overnight.
- 3 parts of embedding mixture and 1 part of Acetone for 3 hours with 2 changes in a vacuum oven.

The tissues were kept only in the pure embedding mixture for 1 hour for block preparation. Polymerization of the tissues were done at 48°C for overnight, then kept at 60°C for 48 hours in an oven.

Ultrathin sections of 800 Å were cut using ultramicrotone (LKB Ultratome, NOVA). The grids containing sections were stained with 2% uranyl acetate and 0.2% lead acetate. The sections were examined on a Transmission Electron Microscope, JEOL GEM 200 CX, Japan at Regional Sophisticated Instrumentation Centre (RSIC, Bose Institute, Calcutta).

3.5 In vitro studies on rat hepatocytes

3.5.1 Isolation of hepatocytes from rat liver

The procedure, involving in situ enzymatic perfusion of a rat liver to procure a population of viable hepatocytes, is adapted from the technique of Seglin (1975) with slight modifications174,175.

Adult male, Charles Foster strain albino rats weighing (120-150 g) were maintained under laboratory conditions and provided with standard pellet diet (Lipton India Ltd.) and water ad libitum.

The rats were anaesthetised with sodium pentobarbital (0.5 mg/Kg. i.p.). The portal vein and inferior venacava were exposed and an 18-gauge needle was inserted into the former vessel, while a thermostat clamped off the latter vessel. Using a peristaltic pump delivering 10-20 ml solution/min., the first perfusing solution [containing 0.5 mM EGTA in Hepes-buffered HBSS (Hank’s Blank Salt Solution), pH-7.4] was delivered slowly (around 10 ml/min) via the portal
vein, gradually swelling and blanching the organ. The thoracic inferior vena cava was cannulated
with a catheter near the heart for the escape of the perfusate. A total of 100 ml of the first
perfusing solution was administered.

A total of 300 ml second perfusing solution (containing 100 units/ml collagenase IV,
630 U/mg) in Hepes buffered HBSS, pH-7.4) was introduced (flowrate at 20 ml/min). At the end
of the perfusion with the second solution, the liver was transferred to a petridish containing
HBSS. The clean liver was next transferred to a dish containing 30 ml second perfusing solution.
After stripping away the capsule, hepatocytes were gently released into the liquid with a blunt
metal comb. The cell suspension was poured through gauge and centrifuged at 75 g for 5 min at
25°C, then cells were resuspended in 5 ml HBSS. The cell suspension was layered onto cold
38% percoll in 0.14 M NaCl, and centrifuged at 20,000 g for 10 mins at 4°C. Hepatocytes,
located in the bottom layer were collected and transferred to a tube containing 10 ml HBSS.

3.5.2 Trypan blue exclusion test / Viability test

One drop of hepatocyte stock suspension was mixed with three drops of Trypan Blue
solution (0.02%). The unstained viable cells were counted under microscope using Neubauer
hematocytometer and could be easily distinguished from the damaged cells stained blue. The
present viable cells were calculated accordingly (Patterson, 1979).

3.6 Immunological Study

3.6.1 Immunization of animals

Male large rabbits were taken for immunization schedules. Fresh sheep red blood cells
(SRBC) was collected in Alsevier’s solution (containing dextrose 20.5 g; sodium citrate 8.0 g;
citric acid 0.55 g; NaCl 4.2 g; volume made upto 1 litre with double distilled water pH 6.0-6.2)
and washed thoroughly in phosphate buffer saline (PBS); pH 7.0. 10% SRBC solution in PBS,
(0.5 ml x 10^9 cells/ml) was subcutaneously injected and immunization procedure was repeated
after 2 weeks. In another large male rabbit, 500 µg of CI-1 (antigen) in Freund’s complete
adjuvant was injected intramuscularly into the thigh muscle. The inoculation was repeated after
two weeks, a booster dose of 300 µg of CI-1 was injected. Blood was collected about 7 days
after the booster dose, by bleeding the marginal ear vein. The serum was isolated and antibody
tire to SRBC immunized and CI-1 immunized rabbits were analyzed by Haemagglutination, Radial immunodiffusion (RID) and enzyme linked immunosassay (ELISA) techniques. Albino swiss mice (male, body wt. 30 g ± 2) were immunized with 10% SRBC in PBS, subcutaneously. Approximately 25 x 10⁶ cells were administered in the primary and 50 x 10⁶ cells were administered in the secondary immunization. Blood samples were collected from retro orbital plexus using microcapillary techniques on the 7th day after primary immunization and 5th day after secondary immunization. The serum was separated at 20°C and kept at 4°C until analysed. Purified protein, CI-1 was injected intraperitoneally for 7-10 days at a varying dose of 50-150 µg in swiss albino mice, male body wt. 30 g ± 2. This group was immunized with 10% SRBC; keeping unimmunized CI-1 treated controls. Similarly, another group of mice were immunized with a standard protein, BSA (200-300 µg) keeping unimmunized CI-1 treated controls. Blood was collected from retro orbital plexus using microcapillary techniques or by cardiac puncture and the serum was isolated.

3.6.2 Studies on cell mediated immunity

3.6.2.1 Systemic anaphylaxis reaction

Male swiss albino mice (body wt. 30 g ± 2) were sensitized by intraperitoneal or subcutaneous injection with 1 mg of BSA in 0.2 ml aluminium hydroxide at day 0 and were shocked by intravenous injection with 1 mg of BSA in 0.2 ml of PBS at day 17. For negative control, 1 mg of ovalbumin in 0.2 ml of PBS was injected instead of BSA at the time of shocking injection. To examine the immunomodulatory activity of CI-1, mice in the experimental groups, were intraperitoneally injected with 6.0 mg of CI-1/Kg body wt. twice a week for a total of seven times including two injections before BSA sensitization. In experiment 2, mice in one experimental group received the shocking injection combined with 150 µg of CI-1 and 1 mg BSA in 0.2 ml of PBS at day 17. The systemic anaphylaxis reaction was observed within 30 min after the shocking injection and rated in the following way: positive reaction, mouse died or rendered stationary for at least 1 min; negative reaction, no changes observed and movement remained normal.
3.6.2.2 Delayed type of hypersensitivity (DTH)

Foot pad thickness test (FPT): Swiss albino mice (male; body wt 30 g ± 2; n = 10 per group) were sensitized with 10% sheep red blood cells (SRBC) (1 x 10^8 cells) at day 0 and day 7 subcutaneously. They were divided into two groups - one group were treated with CI-1 (6.0 mg/Kg body wt.; i.p.) administered seven times, at days -6, -3, 0, 3, 6, 9, 12, while the control group received PBS. On the 14th day, both groups were challenged with 1 x 10^8 SRBC cells, intradermally into the left foot pad of each mouse whereas PBS was injected into the right hind paw. The increase in foot pad thickness was measured 24 hours after SRBC challenge by dial caliper. The degree of DTH reaction was expressed as the percentage increase in foot pad thickness (left-right) over the control values.

3.6.2.3 Leucocyte migration inhibition test (LMTT):

Swiss albino mice (male, body wt 30 g ± 2; n = 6 per group) were sensitized with 10% SRBC (1 x 10^8 cells) at day 0 and day 7 subcutaneously. They were divided into two groups - one group were treated with CI-1 (6.0 mg/Kg body wt.; i.p.) administered seven times, at days -6, -3, 0, 3, 6, 9, 12, while the control group received PBS. On the 14th day the animals of both groups were bled by cardiac puncture.

The blood was added to 2 ml 3% dextran in 0.15 M NaCl, mixed well, incubated for 1 hour at 37°C and centrifuged at 400 g for 10 min. The leucocyte rich pelleting was washed thrice with Hanks blank salt solution (HBSS) at 400 g. Cell suspension containing 15 x 10^6 leucocytes/ml was prepared in tissue culture medium RPMI 1640 with 10% fetal calf serum (FCS). Microcapillary tubes were filled with cell suspension and centrifuged at 250 g for 5 min. after sealing one end with plasticine. The capillaries were cut with a file cutter at cell-fluid interphase and cell “explants” were mounted in migration chambers of 1.5 ml capacity with sterile silicon grease. The chambers were filled with RPMI 1640 enriched with 10% FCS. To one set of chambers, antigen (10% SRBC) was incorporated in the medium and to another (control chamber) only medium was added. The chambers were closed with cover slips and incubated at 37°C in a humid condition for 20 hours. The area of migration of leucocytes in
control and test chambers were recorded, by following the method of Bloom with some modifications and the percentage of leucocyte migration inhibition was calculated

3.6.2.4 Macrophage migration inhibition test (MMIT)

Peritoneal exudate cells from SRBC sensitized and control groups were centrifuged at 400 g for 10 mins. The cell pellet thus obtained was washed three times with tissue culture medium RPMI-1640 pH-7.2 and a final suspension containing 15 x 10^6 cells/ml was prepared. The cells were used in the direct migration inhibition assay as described above for LMIT.

3.6.3 Studies on humoral immune response

Swiss albino mice (male bodywt. 30 g ± 2, n = 6 per group) were sensitized with 10% SRBC (1 x 10^8 cells) at day 0 and day 7 subcutaneously. Similarly, another group of mice were immunized with 1 mg BSA in 0.2 ml aluminium hydroxide at day 0 and day 7 subcutaneously. The experimental immunized groups were treated with CI-1 at a different doses of 50-150 µgs/25 g of b.wt. i.p. for seven times, at days -6, -3, 0, 3, 6, 9 and 12, while the immunized control group received PBS. On the 14th day, blood was collected from retro-orbital plexus using micro capillary technique.

Quantitation of serum IgM and IgG was carried out by single radial immunodiffusion in 0.9% agarose slides containing respective anti IgM and IgG. The serum antibody titre to SRBC and BSA were estimated by ELISA and haemagglutination assay. The antibody titre were expressed as log 2 of the reciprocal of the highest dilution of the test serum showing three times or more absorbance as compared with normal mice serum.

3.6.4 Hematological Study

3.6.4.1 Differential count of WBC and estimation of neutrophil phagocytic index

Blood films of sacrificed mice were fixed in methanol and stained by May Grunwald Giemsa stain. One hundred WBC were counted and percentage was calculated.
Percentage of large and small lymphocytes were counted on the basis of nucleus size. Large sized lymphocytes showed clear blue cytoplasm on the margin of the nucleus, and in the small lymphocytes dark violet coloured nucleus almost filled the entire cell and had a rim of clear cytoplasm.

Neutrophil phagocytic index was estimated by myeloperoxidase staining method. The cytoplasm of neutrophils and some monocytes, staining for myeloperoxidase showed black dots. One hundred WBC were counted and percentage of neutrophil (cells staining for +ve myeloperoxidase versus -ve cells) was determined.

3.6.4.2 Bone marrow cell culture

Unfractioned mouse bone marrow cells, flushed from the femurs with RPMI 1640, pH-7.3 preincubated at 37°C, were repeatedly aspirated and ejected from a syringe to obtain a single cell suspension. The bone marrow cells (at a conc. of 3 x 10^6 cells/ml) were incubated at 37°C in serum free medium for 24 hr. supplemented with CPSR-2 (Sigma, USA). At the end of the incubation period the cells were separately pelleted by centrifugation (500 rpm, 10 min.) at 4°C and the supernatant fluid was collected.

The cell free soup was then subjected to 0.22 membrane filtration under N_2 pressure with continuous stirring. The proteins of the crude filtrate were precipitated by 60% ammonium sulphate saturation at 4°C. The protein concentration was estimated by Bradford’s method using BSA as standard. The crude protein was applied to a Sephadex G-10 (Sigma, USA) column (1.8 cm x 27 cm) pre-equilibrated with 50 mM Tris HCl buffer, pH-7.2. The flow rate of the column was maintained at 8.0 ml/hr and 2 ml fractions were collected. The protein of the collected fractions was measured at 280 nm in spectrophotometer (Hitachi U2000) and the concentration was estimated by Bradford’s method. The present study deals with the pooled fractions (8-12) under the first peak, designated as bone marrow (BM) secretory protein.

3.6.4.3 Thymus cell culture

As in bone marrow cell culture, thymus cell culture was also performed in all the set of experimental animals. The thymus glands were isolated and teased in RPMI-1640 pH-7.3,
preincubated at 37°C, and repeatedly syringed to obtain a single cell suspension. The thymus cells (at a conc. of $1 \times 10^6$ cells/ml) were incubated at 37°C in serum free medium; supplemented with CPSR-2 (Sigma, USA) for 24 hr. At the end of the incubation period, the cells were separately pelleted by centrifugation (500 rpm, 10 min) and the supernatant fluid was collected.

The cell free soup was then subjected to 0.22 membrane filtration under N₂ pressure with continuous stirring, as mentioned in bone marrow cell culture. The proteins of the crude filtrate were precipitated by 60% ammonium sulphate saturation at 4°C. The protein concentration was estimated by Bradford's method using BSA as standard. The crude protein was applied to a Sephadex G-10 (Sigma, USA) column (1.5 cm x 26 cm); pre-equilibrated at 50 mM Tris HCl Buffer, pH-7.2. The flow rate of the column was maintained at 9.0 ml/hr and 1.5 ml fractions were collected. The protein of the collected fractions was measured at 280 nm in UV Spectrophotometer (Hitachi U2000) and the concentration was estimated by Bradford's method.

The present study deals with the pooled fractions (9-14), designated as thymus secretory protein (Thy SP).

3.6.4.4 Trypan blue exclusion test

Cell suspension of bone marrow (at a conc. of $3 \times 10^6$ cells/ml) and thymus (at a conc. of $1 \times 10^6$ cells/ml) was taken separately in a test tube. 0.9 ml of cell suspension was mixed with 0.1 ml trypan blue solution (0.4%). After 5 min, the unstained viable cells were counted under microscope using Neubauer hematocytometer and the percentage calculated accordingly.
Fig. 3.1: A Standard Curve of BSA, Bovine Serum albumin (1 mg/ml)
Fig. 3.2: A Calibration Curve with Standard stock solution of galactose (200 μg/ml)