CHAPTER 5

HEPATOPROTECTIVE ACTIVITY
HEPATOPROTECTIVE ACTIVITY

The liver holds a unique position in the body because of its anatomical connection and varied functions. The liver has a double blood supply and it has large capacity for metabolic conversions and is continuously exposed to different kinds of xenobiotics and therapeutic agents. Alteration in structure may result in portal hypertension, ascites, jaundice, increased bleeding tendency and complex metabolic changes affecting most body constituents (Sherlock et al., 1987).

The rapidly growing morbidity and mortality rate from liver diseases due to drugs and chemicals in industrial nation is largely attributed to the increasing numbers of noxious medical agents and environmental pollutants. Alcohols, Carbon tetrachloride (CCl₄), Paracetamol (PCM), Halothane etc, are the examples of foreign substances which are known to cause hepatic dysfunction in man and in experimental animals (Edward et al., 1975; Kail et al., 1995; Ravindra et al., 1994; Handa et al., 1995).

An increase in lipid peroxidation in the liver has been demonstrated as a frequent feature after poisoning with hepatotoxic substances. Increased lipid peroxidation has been found however, also in nontoxic fatty liver as can be caused by dietary changes such as choline deficiency, feeding on orotic acid rich diet, alcohol treatment or in cases of Kwashiorkor disease, thus lipid peroxidation cause degradation of biomembranes which occurs in alcohol induced fatty liver and carbon tetrachloride induced liver injury, is one of the principle cause of hepatotoxicity (Charles et al., 1975).

Thioacetamide and Its Toxicity

Thioacetamide (TAA) is a crystalline compound used as a laboratory reagent in place of hydrogen sulfide. It is unstable in acid or alkaline solution undergoing decomposition to liberate H₂S, it is rapidly metabolized after administration and converted to acetate via the acetamide pathway. TAA is a potent hepatocarcinogen, the striking nucleolar changes induced by TAA have led to the hypothesis that it interferes with movement of RNA from nucleus to cytoplasm, although the relevance of this change to the genesis of necrosis is unclear. The membrane stabilizing compounds such as strophanthin can
Hepatoprotective Activity

protect against the necrosis suggests that the necrogenic effect of TAA may be affected by membrane injury. The apparent selective injury to plasma membrane adenosine triphosphatase supports this view. There is evidence that a metabolite of TAA, formed by action of the anine oxidase of Ziegler (Flavoprotein monooxidase) rather than by P-450 dependent enzyme system is responsible for the hepatic injury; TAA is apparently converted to acetate, which is presumably converted to an active toxic metabolite that binds covalently to tissue molecules provoking necrosis. TAA administration down regulated the enzymes of primary metabolic pathways such as fatty acid β-oxidation, branched chain amino acids and methionine breakdown. This phenomenon is suggestive of the depletion of succinyl-CoA which effects heme and iron metabolism (Gary, 1997; Hyman, 1999; Teck, 2004).

5 (i) To evaluate hepatoprotective activity of different extracts of leaves, roots and compounds CI-3, CI-4 isolated from roots of *C. intybus*

**Materials and methods**

<table>
<thead>
<tr>
<th>Equipments</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Photometric colorimeter</td>
<td>Systronics 103, Ahmedabad.</td>
</tr>
<tr>
<td>4. Rotary microtome</td>
<td>Yorko Scientific Indus., Delhi.</td>
</tr>
<tr>
<td>6. pH meter</td>
<td>Control Dynamics, Bangalore.</td>
</tr>
</tbody>
</table>

**Diagnostic Kits**

| 1. AST kit                                      | Span Diagnostics, Udhna, Surat. |
| 2. ALT kit                                      | Span Diagnostics, Udhna, Surat. |
| 4. Total Protein kit                            | Techno Pharmchem, Bahadurgarh. |
Animals used

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Albino rats</td>
<td>Obtained from Central Animal House, Hamdard University.</td>
</tr>
<tr>
<td>2.</td>
<td>Sex</td>
<td>Male</td>
</tr>
<tr>
<td>3.</td>
<td>Weight</td>
<td>150-200 g</td>
</tr>
<tr>
<td>4.</td>
<td>Strain</td>
<td>Wistar</td>
</tr>
<tr>
<td>5.</td>
<td>Animal diet</td>
<td>Amrut, Rat Feed, Maharatra.</td>
</tr>
</tbody>
</table>

Drugs and Chemicals

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Silymarin</td>
<td>Ranbaxy, Indore.</td>
</tr>
<tr>
<td>2.</td>
<td>Thioacetamide</td>
<td>CDH, Mumbai.</td>
</tr>
<tr>
<td>4.</td>
<td>Diethyl ether</td>
<td>CDH, Mumbai.</td>
</tr>
<tr>
<td>5.</td>
<td>Formalin</td>
<td>Qualigens, Mumbai.</td>
</tr>
</tbody>
</table>

Preparation of drug solutions

1. Preparation of test solutions

Preparation of plant extracts: Fifty gram of dried leaves and roots powders were extracted with ethanol (99%), hydroalcoholic mixture (ethanol:water::50:50 ratio) and with water, the extracts were filtered, evaporated to dryness in the rotary vacuum evaporator. Dried extracts were used for making different doses.

200 mg and 300 mg of dried extract of *C. intybus* leaves and roots were suspended in 2% w/v, gum acacia in distilled water.

Compound CI-3 and Compound CI-4 (245 mg) isolated from roots of *C. intybus* were suspended in 2% w/v, gum acacia in distilled water.

Thioacetamide (13.72 gm) solution in water for injection was prepared.

Silymarin suspension (3.22 gm) of standard silymarin powder obtained from capsule sivylar (140 mg) [Marketed by Ranbaxy, Indore] was weighed and dissolved in 2% w/v, gum acacia in distilled water.
2. Experimental set up

Animals: One hundred two Male Wistar albino rats (150-200 g) were obtained from the Central Animal House of Jamia Hamdard (Animal proposal no. 314). Animal study was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and conducted according to the regulations of Institutional Animal Ethics Committee, Jamia Hamdard.

They were divided into 17 groups, each group consisting of 6 rats and housed in plastic cages under standard animal house condition; the room temperature was maintained at 25 ± 1 °C. Animals were fed *ad libitum* standard pellet diet and had free access to distilled water.

Treatment plan

The animals were treated as per the given schedule

Group-I: Normal control group; the rats were fed standard pellet diet, received 2% w/v, gum acacia solution and distilled water for all seven days.

Group-II: Toxic control group; the TAA group of animals received distilled water for all seven days and single subcutaneous injection of TAA (100 mg/kg, s.c.) as a 2% w/v solution in water for injection and sacrificed 48 h after the administration of thioacetamide (Ahmed *et al*., 2002).

Group-III: Standard group; animals of this group received silymarin suspension (25 mg/kg/day, p.o.) for seven days and single dose of TAA solution in water for injection (100 mg/kg s.c.) on 7th day.

Group-IV: Alcoholic leaf extract of *C. intybus* (200 mg/kg/day p.o.) for seven days (L Al-1).

Group-V: Alcoholic leaf extract of *C. intybus* (400 mg/kg/day p.o.) for seven days (L Al-2).

Group-VI: Hydroalcoholic leaf extract of *C. intybus* (200 mg/kg/day p.o.) for seven days (L Hy-1).

Group-VII: Hydroalcoholic leaf extract of *C. intybus* (400 mg/kg/day p.o.) for seven days (L Hy-2).

Group-VIII: Aqueous leaf extract of *C. intybus* (200 mg/kg/day p.o.) for seven days (L Aq-1).
Group-IX: Aqueous leaf extract of *C. intybus* (400 mg/kg/day p.o.) for seven days (L Aq-2).

Group-X: Alcoholic root extract of *C. intybus* (200 mg/kg/day p.o.) for seven days (R Al-1).

Group-XI: Alcoholic root extract of *C. intybus* (400 mg/kg/day p.o.) for seven days (R Al-2).

Group-XII: Hydroalcoholic root extract of *C. intybus* (200 mg/kg/day p.o.) for seven days (R Hy-1).

Group-XIII: Hydroalcoholic root extract of *C. intybus* (400 mg/kg/day p.o.) for seven days (R Hy-2).

Group-XIV: Aqueous root extract of *C. intybus* (200 mg/kg/day p.o.) for seven days (R Aq-1).

Group-XV: Aqueous root extract of *C. intybus* (400 mg/kg/day p.o.) for seven days (R Aq-2).

Group-XVI: Animals of this group received compound CI-3 suspension (25 mg/kg/day p.o.) for seven days (Compd - CI-3).

Group-XVII: Animals of this group received compound CI-4 suspension (25 mg/kg/day p.o.) for seven days (Compd - CI-4).

Animals of group III to group XVII received a single dose of thioacetamide (100 mg/kg s.c.) on 7th day. At the end of experimental regimen, the rats were fasted overnight. On 9th day, the blood samples were collected from the retro-orbital plexus for biochemical studies and rats were sacrificed for histopathological studies on the same day.

**Separation of serum:** About 3-5 ml of blood was collected in a sterile centrifuge tube and left undisturbed for one h at 37 °C, till the formation of clot. The clot was dislodged using a sterile loop and refrigerated at 2-8 °C for 3-4 h. During this period serum exuded and the clot retracted. The serum was aspirated using a sterile pipette after centrifugation at 3000 rpm for 15 min. Serum collected was analyzed for enzyme levels immediately or within 24 h after storing at 0-4 °C. The collected serum was estimated for the following biochemical parameters:

1. Serum Glutamate Oxaloacetate Transaminase (SGOT or AST).
2. Serum Glutamate Pyruvate Transaminase (SGPT or ALT).
3. Serum Total and Direct Bilirubin (TB and DB).
4. Serum Total Proteins (TP).
5. Serum Alkaline Phosphatase.

5 (i) Biochemical investigations in serum

1. Determination of serum glutamate oxaloacetate transaminase (SGOT)

Principle

GOT catalyses the following reaction

\[ \alpha - \text{Ketoglutarate} + \text{L - Aspartame} \rightarrow \text{L - Glutamate} + \text{Oxaloacetate}. \]

Oxaloacetate so formed was coupled with 2, 4-dinitro phenyl hydrazine (2,4 - DNPH) to give corresponding hydrazone, which given brown color in alkaline medium and that was measured on photometer at 505 nm.

Reagents (supplied in kit)

Reagent 1: Buffered aspartateKG substrate, pH 7.4
Reagent 2: DNPH color reagent
Reagent 3: Sodium hydroxide, 4 N
Reagent 4: Working pyruvate standard 2 mM.

Preparation of working solution

Solution 1: Dilute 1ml of reagent 3 to 10 ml with distilled water. Reagents 1 and 2 were ready to use.

Storage and stability

All the reagents were stable at 2-8 °C till the expiry.

Precautions

Precaution was taken to obtain non-haemolysed serum.
Procedure
Preparation of standard curve: Five clean dried tubes were taken and to this were added different volumes of reagents 1, 2 and 4 as given in Table 5.1.

**Table 5.1. Different volumes of reagents were used for preparation of standard curve**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Tube 1</th>
<th>Tube 2</th>
<th>Tube 3</th>
<th>Tube 4</th>
<th>Tube 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 1 (ml)</td>
<td>0.5</td>
<td>0.45</td>
<td>0.4</td>
<td>0.35</td>
<td>0.3</td>
</tr>
<tr>
<td>Reagent 4 (ml)</td>
<td></td>
<td>0.05</td>
<td>0.1</td>
<td>0.15</td>
<td>0.2</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Reagent 2 (ml)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The above solutions in five tubes were allowed to stand at room temperature for 20 min and the volume of each tube was made to 5 ml with solution 1. Table 5.2 given the reported enzyme activity in units/ml for these 5 tubes.

**Table 5.2. Showing the reported enzyme activity (as given in the kit) for different volumes of reagent (as given above) prepared to plot standard graph for SGOT.**

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme activity Units/ml</td>
<td>0</td>
<td>24</td>
<td>61</td>
<td>114</td>
<td>190</td>
</tr>
</tbody>
</table>

The absorbance for all the five tubes was taken at 505 nm. The absorbances for the five tubes are recorded in Table 5.3.

**Table 5.3. Absorbance for different volume of reagents at 505 nm to prepare standard curve**

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance at 505 nm</td>
<td>0.325</td>
<td>0.475</td>
<td>0.538</td>
<td>0.667</td>
<td>0.784</td>
</tr>
</tbody>
</table>
Procedure for test

0.25 ml of reagent 1 was taken and incubated at 37 °C for 5 min, serum 0.05 ml was added. This was mixed well and incubated at 37 °C for 60 min. To this 0.25 ml of reagent 2 was added. The solution was mixed well and allowed to stand for 20 min at room temperature. The total volume was made to 2.5 ml with solution 1. This was again mixed well and allowed to stand for 10 min at room temperature. The absorbance was recorded at 505 nm and the enzyme activities for corresponding absorbance were calculated from the standard graph.

Calculations

The O.D of the test (T) was marked on the Y-axis of the standard curve and extrapolated it to the corresponding activity on X-axis, the results of SGOT estimation shown by histogram representation (Fig. 5.1) (Reitmann and Frankel, 1957).

Observations

It was observed that aqueous extract of leaves, roots (400 mg/kg), compound CI-3 and CI-4 (25 mg/kg) isolated from roots showed 68.88%, 72.80%, 76.24% and 80.10% reduction in serum SGOT levels respectively. The % reduction in serum SGOT levels by standard silymarin was 80.92%. So it was concluded that compound CI-4, comparable to that of silymarin (Fig. 5.2).

2. Determination of serum glutamate pyruvate transaminase (SGPT)

Principle

\[ \alpha \text{-Ketoglutate} + L\text{-Alanine} \rightarrow L\text{-Glutamate} + \text{Pyruvate.} \]

Pyruvate so formed was coupled with 2, 4-dinitrophenyl hydrazine (2, 4-DNPH) to give the corresponding hydrasone, which given brown color in alkaline medium.

Reagents: supplied in kit

Reagent 1: Buffered alanine \(\alpha\)-KG substrate.

Reagent 2: DNPH color reagent

Reagent 3: Sodium hydroxide, 4N

Reagent 4: Working pyruvate standard, 2 mM
Preparation of working standard

Solution I: Dilute 1 ml of reagent 3 to 10 ml with distilled water. Reagents 1 and 2 were ready to use.

Storage and stability

All the reagents were stable 2-8 °C till the expiry.

Precautions

Precaution was taken to obtain non-haemolysed serum.

Procedure

Preparation of standard curve: Five clean dried tubes were taken and to this were added different volume of reagent 1 and reagent 4 as given in Table 5.4.

Table 5.4. Different volumes of reagents were used for preparation of standard curve.

<table>
<thead>
<tr>
<th>Reagents (ml)</th>
<th>Tube 1</th>
<th>Tube 2</th>
<th>Tube 3</th>
<th>Tube 4</th>
<th>Tube 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 1</td>
<td>0.5</td>
<td>0.45</td>
<td>0.4</td>
<td>0.35</td>
<td>0.3</td>
</tr>
<tr>
<td>Reagent 4</td>
<td>-</td>
<td>0.05</td>
<td>0.1</td>
<td>0.15</td>
<td>0.2</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Reagent 2</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

All the five test tubes as prepared in the above Table 5.4 were mixed well and allowed to stand at room temperature and the volume of all the five test tubes was made 5 ml with working solution 1. Table 5.5 gives the reported enzyme activity in units/ml for these 5 tubes. The test tubes were allowed to stand at room temperature and there absorbance taken with spectrophotometer at 505 nm as given in Table 5.5.
Table 5.5. Showing the reported enzyme activity (as given in the kit) for different volumes of reagent (as given above) prepared to plot standard graph for SGPT.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme activity Units/ml</td>
<td>0</td>
<td>28</td>
<td>57</td>
<td>97</td>
<td>150</td>
</tr>
</tbody>
</table>

The absorbance for all the five tubes was taken at 505 nm. In Table 5.6 recorded absorbance for the five tubes.

Table 5.6. Absorbance for different volumes of reagents at 505 nm to prepare standard curve

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance at 505nm</td>
<td>0.261</td>
<td>0.335</td>
<td>0.451</td>
<td>0.536</td>
<td>0.609</td>
</tr>
</tbody>
</table>

Procedure for test
0.25 ml of reagent 1 was taken and incubated at 37 °C for 5 min, serum 0.05 ml was added. This was mixed well and incubated at 37 °C for 30 min. To this 0.25 ml of reagent 2 was added. The solution was mixed well and allowed to stand for 20 min at room temperature. The total volume was made to 2.5 ml with solution 1. This was again mixed well and allowed to stand for 10 min at room temperature. The absorbance was recorded at 505 nm and the enzyme activities for corresponding absorbance were calculated from the standard graph.

Calculations
The O. D of the test (T) was marked on the Y-axis of the standard curve and extrapolated it to the corresponding activity on X-axis, results of SGPT estimation shown by histogram representation (Fig. 5.3) (Reitmann and Frankel, 1957).
Observations
It was observed that aqueous extract of leaves, roots (400 mg/kg), compound CI-3 and CI-4 (25 mg/kg), isolated from roots showed 77.32%, 79.86%, 82.48 and 86.29% reduction in serum SGPT levels respectively. The % reduction in serum SGPT levels by standard silymarin is 83.62%. So it was concluded that compound CI-4, comparable to that of silymarin (Fig. 5.4).

3. Determination of total bilirubin

Principle
Direct (Conjugated): Bilirubin couples with diazotized sulfanillic acid and formed azobilirubin, a red purple colored product in acidic medium.
Indirect (Un-conjugated): Bilirubin was diazotized only in the presence of its dissolving solvent (methanol). Thus red purple colored azobilirubin produced in the presence of methanol originates from both direct and indirect fractions and that represents total bilirubin concentration. The difference and total gives (Un-conjugated) bilirubin. The intensity of the red purple color so developed above was measured at 540 nm.

Bilirubin diazotized sulfanillic acid azobilirubin
Red-purple color at 540 nm
Sample
0.4 ml serum from all 102 animals was used.
Reagents: supplied in kit
Reagent 1: Diazo-A
Reagent 2: Diazo-B
Reagent 3: Diazo blank
Reagent 4: Methanol
Reagent 5: Artificial standard (10 mg % bilirubin)

Preparation of working solutions
Diazoreagent: Just before use mix 1ml of reagent 1 with 0.03 ml of reagent 2.

Procedure
Four test tubes of each sample were made as given in Table 5.7.
### Table 5.7. Sample preparation for bilirubin

<table>
<thead>
<tr>
<th>Contents</th>
<th>T1</th>
<th>T2</th>
<th>D1</th>
<th>D2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (ml)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Distilled water (ml)</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Reagent 3 (ml)</td>
<td>-</td>
<td>0.25</td>
<td>-</td>
<td>0.25</td>
</tr>
<tr>
<td>Diazo reagent (ml)</td>
<td>0.25</td>
<td>-</td>
<td>0.25</td>
<td>-</td>
</tr>
<tr>
<td>Distilled water</td>
<td>-</td>
<td>-</td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td>Reagent 4: methanol (ml)</td>
<td>1.25</td>
<td>1.25</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The contents in D1 and D2 were mixed well and their absorbance was recorded at 540 nm.

Tubes T2 and T1 were mixed well in dark at room temperature for 30 min and their absorbance were recorded at 540 nm.

The following calculations were done:

Total Bilirubin concentration in mg/100ml (A) = \( \text{Absorbance of } T1 - \text{Absorbance of } T2 \times 10 \) Absorbance of Standard

Direct Bilirubin (B) = \( \frac{\text{Absorbance of } D1 - \text{Absorbance of } D2 \times 10}{\text{Absorbance of Standard}} \)

Fig. 5.5 and 5.6 were showing the results of total and direct bilirubin (Malloy and Evelyn, 1937).

**4. Determination of serum alkaline phosphatase by Principle**

Alkaline phosphatase from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10.0. Phenols so formed reacts in alkaline medium with 4-aminoantipyrine in presence of oxidizing agent potassium ferricyanide and formed orange red colored complex which was measured by UV at 510 nm.
Alkaline phosphatase
Phenyl phosphate phenol + phosphate
pH 10.0
Potassium ferricyanide
Phenyl + 4-Aminoantipyrine orange red colored complex OH⁻ 510-520 nm
Reagents: Supplied in kit
Reagent 1: Buffered substrate, pH 10
Reagent 2: Chromogen reagent
Reagent 3: Phenol standard, 10 mg %
Preparation of working solution
Solution 1: Reconstitute one vial of reagent 1, buffered substrate with 2.2 ml of purified water.
Procedure
Four tubes for each sample were taken and marked as blank (B), standard (S), control (C) and test (T). The contents of the tubes are given in Table 5.8.
Table 5.8. Preparation of blank, standard, control and test samples for the determination of alkaline phosphatase

<table>
<thead>
<tr>
<th>Contents</th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Control (C)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution I (ml)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Distilled water (ml)</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

This was mixed well and incubated for 3 min at 37 °C

| Serum (ml)                | -         | -            | -           | 0.05     |
| Phenol standard 10mg % (ml)| -         | 0.05         | -           | -        |

This was mixed and incubated for 15 min at 37 °C

| Chromogen Reagent (ml)    | 1.0       | 1.0          | 1.0         | 1.0      |
| Serum (ml)                | -         | -            | 0.05        | -        |

Four tubes as mentioned in the Table 5.8, for the entire sample were mixed well and Absorbance recorded at 510 nm.
Calculations

Serum alkaline phosphatase activity in KA units =
\[ \frac{\text{Absorbance of Test} - \text{Absorbance of control \times 10}}{\text{Absorbance of Standard} - \text{Absorbance of Blank}} \]

Fig. 5.7 was showing the results of serum alkaline phosphatase (King and King, 1954).

5. Determination of total proteins in serum

Principle

Total Protein: Proteins in serum reacts with copper of biuret reagent alkaline medium to form blue purple complex with absorbance maxima at 550 nm

Serum

Serum required for total protein was 0.02 ml.

Reagents: Supplied in kit

Reagent 1: Biuret reagent

Reagent 2: Buffered bromocresol dye reagent

Reagent 3: Protein standard.

Procedure

Biuret reagent was taken as blank for total protein.

Standard: To 3.0 ml of biuret reagent and 0.05 ml of reagent 3 was added, used as standard.

Test samples: To 3.0 ml of biuret reagent and add 0.05 ml of serum from each sample was added, used as test samples.

All the above blank, standard and test samples were mixed well and allowed to stand at room temperature. The absorbance of the above mentioned blank, standard and test samples were taken at 550 nm.

Calculations

Serum total protein in g/100ml (X) =
\[ \frac{\text{Absorbance of Test} \times \text{concentration of standard protein (7 %)}}{\text{Absorbance of Standard}} \]

Fig. 5.8 was showing the result of total protein (Biuret and Dumas, 1971).

Statistical analysis: For determination of significant intergroup differences each parameters was analyzed separately and one way Analysis of Variance (ANOVA) was carried out. Dunnett’s test was use for individual comparisons.
### Table 5.9. Effect of alcoholic, hydroalcoholic and aqueous extracts of leaves, roots and compound CI-3, CI-4 isolated from roots of *C. intybus* on various biochemical parameters.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SGOT (u/ml)</th>
<th>SGPT (u/ml)</th>
<th>Total Bilirubin (mg/100ml)</th>
<th>Direct Bilirubin (mg/100ml)</th>
<th>Total Protein (g/100ml)</th>
<th>Alkaline Phosphatase (u/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group- I (control)</td>
<td>61 ± 6.50</td>
<td>52.50 ± 4.55</td>
<td>0.53 ± 0.133</td>
<td>0.201 ± 0.038</td>
<td>8.03 ± 0.515</td>
<td>22.83 ± 1.62</td>
</tr>
<tr>
<td>Group- II (Toxic control)</td>
<td>305.83 ± 11.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>380.66 ± 12.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.33 ± 0.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.83 ± 0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.35 ± 0.190&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68.66 ± 4.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group- III (Standard)</td>
<td>58.33 ± 6.01**&lt;sup,a&lt;/sup&gt;</td>
<td>62.33 ± 5.35**&lt;sup,a&lt;/sup&gt;</td>
<td>1.01 ± 0.15&lt;sup,a,b&lt;/sup&gt;</td>
<td>0.23 ± 0.02**&lt;sup,a&lt;/sup&gt;</td>
<td>7.34 ± 0.56**&lt;sup,a&lt;/sup&gt;</td>
<td>29.66 ± 3.106**&lt;sup,a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group- IV (L Al -1)</td>
<td>207.16 ± 11.86**&lt;sup,a,b&lt;/sup&gt;</td>
<td>242.50 ± 10.22**&lt;sup,a,b&lt;/sup&gt;</td>
<td>3.90 ± 0.07&lt;sup,a,b&lt;/sup&gt;</td>
<td>1.80 ± 0.18&lt;sup,a,b&lt;/sup&gt;</td>
<td>3.08 ± 0.24&lt;sup,a&lt;/sup&gt;</td>
<td>58.16 ± 5.74&lt;sup,a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group- V (L Al -2)</td>
<td>171.66 ± 10.22**&lt;sup,a,b&lt;/sup&gt;</td>
<td>201.66 ± 16.43**&lt;sup,a,b&lt;/sup&gt;</td>
<td>2.90 ± 0.05&lt;sup,a,b&lt;/sup&gt;</td>
<td>1.41 ± 0.03**&lt;sup,a,b&lt;/sup&gt;</td>
<td>4.06 ± 0.33**&lt;sup,a,b&lt;/sup&gt;</td>
<td>46.33 ± 11.27**&lt;sup,a&lt;/sup&gt;</td>
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<tr>
<td>Group- VI (L Hy -1)</td>
<td>167.33 ± 5.07**&lt;sup,b&lt;/sup&gt;</td>
<td>164.83 ± 5.77**&lt;sup,b&lt;/sup&gt;</td>
<td>3.28 ± 0.04&lt;sup,b&lt;/sup&gt;</td>
<td>1.65 ± 0.02**&lt;sup,b&lt;/sup&gt;</td>
<td>3.54 ± 0.17&lt;sup,b&lt;/sup&gt;</td>
<td>52.16 ± 7.27&lt;sup,b&lt;/sup&gt;</td>
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<tr>
<td>Group- VII (L Hy - 2)</td>
<td>143.33 ± 13.39**&lt;sup,b&lt;/sup&gt;</td>
<td>138.83 ± 4.38**&lt;sup,b&lt;/sup&gt;</td>
<td>2.40 ± 0.10&lt;sup,a,b&lt;/sup&gt;</td>
<td>1.36 ± 0.06**&lt;sup,b&lt;/sup&gt;</td>
<td>4.24 ± 0.23**&lt;sup,b&lt;/sup&gt;</td>
<td>41.33 ± 4.52**&lt;sup,a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group- VIII (L Aq-1)</td>
<td>142.50 ± 9.01**&lt;sup,b&lt;/sup&gt;</td>
<td>125.83 ± 8.18**&lt;sup,b&lt;/sup&gt;</td>
<td>2.80 ± 0.04&lt;sup,b&lt;/sup&gt;</td>
<td>1.22 ± 0.08**&lt;sup,b&lt;/sup&gt;</td>
<td>5.2 ± 0.18**&lt;sup,b&lt;/sup&gt;</td>
<td>44.66 ± 4.15**&lt;sup,a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group- IX (L Aq-2)</td>
<td>95.16 ± 5.26**&lt;sup,b&lt;/sup&gt;</td>
<td>86.33 ± 4.07**&lt;sup,a&lt;/sup&gt;</td>
<td>2.03 ± 0.03&lt;sup,a&lt;/sup&gt;</td>
<td>0.90 ± 0.03&lt;sup,b&lt;/sup&gt;</td>
<td>6.19 ± 0.07**&lt;sup,a&lt;/sup&gt;</td>
<td>38.32 ± 4.51**&lt;sup,a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
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Fig. 5.1. Standard curve of SGOT estimation

Fig. 5.2. Results of SGOT estimation.
Fig. 5.3. Standard curve of SGPT estimation.

Fig. 5.4. Results of SGPT estimation.
Fig. 5.5. Results of total bilirubin estimation.

Fig. 5.6. Results of direct bilirubin estimation.
Fig. 5.7. Results of total protein estimation.

Fig. 5.8. Results of alkaline phosphatase estimation.
5 (ii) Histopathological studies of the liver tissues

The rats were sacrificed under light ether anesthesia and their livers were removed and washed with normal saline small pieces of liver section (tissues) were collected in 10% buffered formal saline for proper fixation. Fixed tissues were then subjected to staining procedure as follow:

Routine Hematoxylin and Eosin staining: various steps involved using in this staining were

1. **Dehydration:** The water content of the tissues was replaced using increasing concentrations of ethanol.
   - 80% alcohol – 1 h
   - 95% alcohol – 1 h (2 changes)
   - 100% alcohol – 1 h

2. **Clearing:** The reagent used for clearing must be miscible with dehydrant and paraffin, when dehydrant was removed, the tissue cleared and become translucent signifying the completion of the process.
   - Xylene was used as clearing agent.

3. **Impregnation:** Complete removal of clearing agents by substitution was done by paraffin wax as it penetrates the tissue. Impregnation was done with 3 paraffin bath for 3 h. Paraffin with melting point 56-58 °C was used. Precaution was taken so that heating 5 °C above the melting point of paraffin was avoided, which might shrink and harden the tissue.
   - The tissues were then cast into block of paraffin wax.

4. The blocks were kept for freezing. The frozen blocks were then carefully taken and sections of the tissue, 5-6 μ thickness were cut with the help of a rotary microtome.

5. The section ribbons were made and floated on warm water and then placed on glass slides to remove wrinkles slightly warmed and dried.

6. **Hydration:** The sections were hydrated with xylene for 2 min and 70% alcohol for 5 min they were then rinsed with distilled water.

7. **Staining:** Section were stained with 1% hematoxylin (3 min) rinsed with distilled water and then 1% eosin in 90% alcohol was then added for 1 min and slides were dried.
8. The stained section were then covered with DNP mounting agent and cover slip was placed carefully on the section, taken care that no air bubble could enter the permanent slides were prepared, which were observed under various magnifications in Olympus microscope (Olympus Vanoz-s-AH-2, Japan) and photograph were taken with the help of camera attached to the microscope (Luna, 1968).

**Histopathological observation on male Wistar Albino rat liver tissue**

**Control group:** Liver sample showed normal architecture without any degeneration or necrosis. Normal hepatocytes were seen in cord pattern with portal triad (PT) and central vein (CV) (Fig. 5.9 and 5.10).

**Toxic control group:** Liver sample showed centrizonal necrosis and sinusoidal dilatation around the central vein area. The portal tract and the periportal zone did not show necrosis or inflammation (Fig. 5.11 and 5.12).

**Standard group:** Liver sample showed almost normal appearance both in architecture and in morphological appearance of hepatocytes; with mild sinusoidal dilatation in the centrizonal areas. A focal area of bile pigment deposits was seen near the central vein (Fig. 5.13 and 5.14).

**Alcoholic leaf extract, 200 mg (L- Al-1):** Sections from the liver of alcoholic leaf extract treated group animals showed liver tissue with centrizonal necrosis, inflammation and sinusoidal dilatation. The portal tract and the periportal zone did not show necrosis or inflammation (Fig. 5.15 and 5.16).

**Alcoholic leaf extract, 400 mg (L- Al-2):** Sections from the liver of alcoholic leaf extract treated group animals showed liver tissue with moderate centrizonal necrosis, inflammation and sinusoidal dilatation. The portal tract and the periportal zone did not show necrosis or inflammation (Fig. 5.17 and 5.18).

**Hydroalcoholic leaf extract, 200 mg (L- HyAl-1):** Sections from the liver of hydroalcoholic leaf extract treated group animals showed liver tissue with centrizonal sinusoidal dilatation. Necrotic foci were few. The portal tract and the periportal zone did not show necrosis or inflammation (Fig. 5.19 and 5.20).
Hydroalcoholic leaf extract, 400 mg (L- HyAl-2): Sections from the liver of hydroalcoholic leaf extract treated group animals showed liver tissue with centrizonal sinusoidal dilatation. Necrotic foci were small. The portal tract and the periportal zone did not show necrosis or inflammation (Fig. 5.21 and 5.22).

Aqueous leaf extract, 200 mg (L- Aq-1): Sections from the liver of aqueous leaf extract treated group animals showed liver tissue with centrizonal necrosis and inflammation. The portal tract and the periportal zone did not show necrosis or inflammation (Fig. 5.23 and 5.24).

Aqueous leaf extract, 400 mg (L- Aq-2): Sections from the liver of aqueous leaf extract treated group animals showed liver tissue with a focus of necrosis and inflammation in the centrizonal area. The portal tract and the periportal zone did not show necrosis or inflammation (Fig. 5.25 and 5.26).

Alcoholic root extract, 200 mg (R- Al-1): Sections from the liver of alcoholic root extract treated group animals showed liver tissue with centrizonal necrosis, inflammation and sinusoidal dilatation. The necrotic zone was infiltrated with inflammatory cells. The portal tract and the periportal zone did not show necrosis or inflammation (Fig. 5.27 and 5.28).

Alcoholic root extract, 400 mg (R- Al-2): Sections from the liver of alcoholic root extract treated group animals showed liver tissue with a lesser degree of centrizonal necrosis, inflammation and sinusoidal dilatation. The portal tract and the periportal zone did not show necrosis or inflammation (Fig. 5.29 and 5.30).

Hydroalcoholic root extract, 200 mg (R- HyAl-1): Sections from the liver of hydroalcoholic root extract treated group animals showed liver tissue with pronounced centrizonal necrosis and inflammation. The portal tract and the periportal zone did not show necrosis or inflammation (Fig. 5.31 and 5.32).

Hydroalcoholic root extract, 400 mg (R- HyAl-2): Sections from the liver of hydroalcoholic root extract treated group animals showed liver tissue with pronounced centrizonal necrosis and inflammation. There was significant inflammatory cell infiltration and necrosis seen. The portal tract and the periportal zone did not show necrosis or inflammation (Fig. 5.33 and 5.34).
Aqueous root extract, 200 mg (R- Aq-1): Sections from the liver of aqueous root extract treated group animals showed liver tissue with pronounced centrizonal necrosis and inflammation. The portal tract and the periportal zone did not show necrosis or inflammation (Fig. 5.35 and 5.36).

Aqueous root extract, 400 mg (R- Aq-2): Sections from the liver of aqueous root extract treated group animals showed liver tissue with minimal amount of centrizonal necrosis and inflammation. The portal tract and the penportal zone did not show necrosis or inflammation (Fig. 5.37 and 5.38).

Compound-CI-3, 25 mg (Compd-CI-3): Sample from the protection group (compound-CI-3) animals showed liver tissue with scattered foci of centrizonal necrosis and inflammation. The portal tract and the periportal zone did not show necrosis or inflammation (Fig. 5.39 and 5.40).

Compound-CI-4, 25 mg (Compd-CI-4): Sample from the protection group (compound-B) animals showed liver tissue with only a mild dilatation of sinusoids in the centrizonal areas. A focal area of bile pigment deposits was seen near the central vein (Fig. 5.41 and 5.42).
Table 5.10. Observation of histopathological studies

<table>
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<th>Slide No.</th>
<th>Experiment Group</th>
<th>Architecture</th>
<th>Periportal Zone</th>
<th>Centizonal Area</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td>Hepatocyte Necrosis</td>
<td>Inflammation Cell</td>
</tr>
<tr>
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<td>Control</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>Compd-CI-4</td>
<td>WNL</td>
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</table>
Fig. 5.9. Low power photomicrograph of liver from animal in normal control group showing normal arrangement of cells in the liver lobule (HE x 100 X).

Fig. 5.10. High power photomicrograph of liver from animal in normal control group showing normal structures within the portal triad (HE x 400X).
Fig. 5.11: Low power photomicrograph of liver from group receiving thioacetamide (TAA) only, shows liver tissue with centrizonal necrosis and sinusoidal dilatation around the central vein area. The portal tract and the periportal zone did not show necrosis or inflammation (HE x 100).

Fig. 5.12. High power photomicrograph of liver from group receiving thioacetamide (TAA) only, shows liver tissue in the centrizonal area. The necrotic zone was infiltrated with inflammatory cells (HE x 400).
Fig. 5.13. Low power photomicrograph of section of liver from group receiving TAA and silymarin showing only a mild dilatation of sinusoids in the centrizonal areas, PT = portal triad and CV = central vein (HE x 100X).

Fig. 5.14. High power photomicrograph of section of liver from group receiving TAA and silymarin showing sinusoidal dilatation in the centrizonal areas. A focal area of bile pigment deposits was seen near the central vein (HE x 400X).
Fig. 5.15. Low power photomicrograph of section of liver from group receiving TAA and alcoholic leaf extract (200 mg/kg b.w) showing liver tissue with centrilobular necrosis, inflammation and sinusoidal dilatation. The portal tract and the periporal zone did not show necrosis or inflammation, PT = portal triad and CV = central vein (HE x 100).

Fig. 5.16. High power photomicrograph of section of liver from group receiving TAA and alcoholic leaf extract (200 mg/kg b.w.) showing liver tissue in the centrilobular area. The necrotic zone was infiltrated with inflammatory cells (HE x 400).
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Fig. 5.17. Low power photomicrograph of section of liver from group receiving TAA and alcoholic leaf extract (400 mg/kg b.w.) showing liver tissue with moderate centrizonal necrosis, inflammation and sinusoidal dilatation. The portal tract and the periportal zone did not show necrosis or inflammation, PT = portal triad and CV = central vein (HE x 100).

Fig. 5.18. High power photomicrograph of section of liver from group receiving TAA and alcoholic leaf extract (400 mg/kg b.w.) showing liver tissue in the centrizonal area. The necrotic zone was infiltrated with inflammatory cells (HE x 400).
Fig. 5.19. Low power photomicrograph of section of liver from group receiving TAA and hydroalcoholic leaf extract (200 mg/kg b.w.) showing liver tissue with centrilobular sinusoidal dilatation. Necrotic foci were few. The portal tract and the periportal zone did not show necrosis or inflammation, PT = portal triad and CV = central vein (HE x 100).

Fig. 5.20. High power photomicrograph of section of liver from group receiving TAA and hydroalcoholic leaf extract (200 mg/kg b.w.) showing a necrotic focus in the centrilobular area. The necrotic zone was infiltrated with inflammatory cells (HE x 400).
Fig. 5.21. Low power photomicrograph of section of liver from group receiving TAA and hydroalcoholic leaf extract (400 mg/kg b.w.) showing liver tissue with centrilobular sinusoidal dilatation. Necrotic foci were small. The portal tract and the periportal zone did not show necrosis or inflammation, PT = portal triad and CV = central vein (HE x 100).

Fig. 5.22. High power photomicrograph of section of liver from group receiving TAA and hydroalcoholic leaf extract (400 mg/kg b.w.) showing a necrotic focus in the centrilobular area. The necrotic zone was infiltrated with inflammatory cells (HE x 400).
Fig. 5.23. Low power photomicrograph of section of liver from group receiving TAA and aqueous leaf extract (200 mg/kg b.w.) showing liver tissue with centrilobular necrosis and inflammation. The portal tract and the periportal zone did not show necrosis or inflammation, PT = portal triad and CV = central vein (HE x 100).

Fig. 5.24. High power photomicrograph of section of liver from group receiving TAA and aqueous leaf extract (200 mg/kg b.w.) showing liver tissue in the centrilobular area. The necrotic zone was infiltrated with inflammatory cells (HE x 400).
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Fig. 5.25. Low power photomicrograph of section of liver from group receiving TAA and aqueous leaf extract (400 mg/kg b.w.) showing liver tissue with a focus of necrosis and inflammation in the centrizonal area. The portal tract and the periportal zone did not show necrosis or inflammation, PT = portal triad and CV = central vein (HE x 100).

Fig. 5.26. High power photomicrograph of section of liver from group receiving TAA and aqueous leaf extract (400 mg/kg b.w.) showing liver tissue in the centrizonal area. The necrotic zone was infiltrated with inflammatory cells (HE x 400).
Fig. 5.27. Low power photomicrograph of section of liver from group receiving TAA and alcoholic root extract (200 mg/kg b.w.) showing liver tissue with centrizonal necrosis, inflammation and sinusoidal dilatation. The portal tract and the periportal zone did not show necrosis or inflammation, PT = portal triad and CV = central vein (HE x 100).

Fig. 5.28. High power photomicrograph of section of liver from group receiving TAA and alcoholic root extract (200 mg/kg b.w.) showing liver tissue in the centrizonal area. The necrotic zone was infiltrated with inflammatory cells (HE x 400).
Fig. 5.29. Low power photomicrograph of section of liver from group receiving TAA and alcoholic root extract (400 mg/kg b.w.) showing liver tissue with a lesser degree of centrizonal necrosis, inflammation and sinusoidal dilatation. The portal tract and the periportal zone did not show necrosis or inflammation, PT = portal triad and CZ = centrizonal (HE x 100).

Fig. 5.30. High power photomicrograph of section of liver from group receiving TAA and alcoholic root extract (400 mg/kg b.w.) showing liver tissue in the centrizonal area. The necrotic zone was infiltrated with inflammatory cells (HE x 400).
Fig. 5.31. Low power photomicrograph of section of liver from group receiving TAA and hydroalcoholic root extract (200 mg/kg b.w.) showing liver tissue with pronounced centrizonal necrosis and inflammation. The portal tract and the periportal zone did not show necrosis or inflammation, CV = central vein (HE x 100).

Fig. 5.32. High power photomicrograph of section of liver from group receiving TAA and hydroalcoholic root extract (200 mg/kg b.w.) showing liver tissue in the centrizonal area. There was significant inflammatory cell infiltration and necrosis seen (HE x 400).
Fig. 5.33. Low power photomicrograph of section of liver from group receiving TAA and hydroalcoholic root extract (400 mg/kg b.w.) showing liver tissue with pronounced centrizonal necrosis and inflammation. The portal tract and the periportal zone did not show necrosis or inflammation, PT = portal triad and CV = central vein (HE x 100).

Fig. 5.34. High power photomicrograph of section of liver from group receiving TAA and hydroalcoholic root extract (400 mg/kg b.w.) showing liver tissue in the centrizonal area. There was significant inflammatory cell infiltration and necrosis seen (HE x 400).
Fig. 5.35. Low power photomicrograph of section of liver from group receiving TAA and aqueous root extract (200 mg/kg b.w.) showing liver tissue with pronounced centrilobular necrosis and inflammation. The portal tract and the periportal zone did not show necrosis or inflammation, PT = portal triad and CV = central vein (HE x 100).

Fig. 5.36. High power photomicrograph of section of liver from group receiving TAA and aqueous root extract (200 mg/kg b.w.) showing liver tissue in the centrilobular area. There was some inflammatory cell infiltration and necrosis seen (HE x 400).
Fig. 5.37. Low power photomicrograph of section of liver from group receiving TAA and aqueous root extract (400 mg/kg b.w.) showing liver tissue with minimal amount of centrizonal necrosis and inflammation. The portal tract and the periportal zone did not show necrosis or inflammation, PT = portal triad and CV = central vein (HE x 100).

Fig. 5.38. High power photomicrograph of section of liver from group receiving TAA and aqueous root extract (400 mg/kg b.w.) showing liver tissue in the centrizonal area. There was some inflammatory cell infiltration (HE x 400).
Fig. 5.39. Low power photomicrograph of section of liver from group receiving TAA and compound-CI-3 (25 mg/kg b.w.) showing liver tissue with scattered foci of centrizonal necrosis and inflammation. The portal tract and the periportal zone did not show necrosis or inflammation, PT = portal triad and CV = central vein (HE x 100).

Fig. 5.40. High power photomicrograph of section of liver from group receiving TAA and compound-CI-3 (25 mg/kg b.w.) showing liver tissue in the centrizonal area. There was minimum inflammatory cell infiltration and necrosis seen (HE x 400).
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Fig. 5.41. Low power photomicrograph of section of liver from group receiving TAA and compound-CI-4 (25 mg/kg b.w.) showing significant recovery except mild dilatation of sinusoids in the centrizonal areas, PT = portal triad and CV = central vein (HE x 100X).

Fig. 5.42. High power photomicrograph of section of liver from group receiving TAA and compound-CI-4 (25 mg/kg b.w.) showing liver tissue in the centrizonal area with mild sinusoidal dilation. Necrosis and inflammation were absent, CV = central vein (HE x 400).
References


