CHAPTER III
MATERIALS AND METHODS
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Experimental Animal

Male Sprague Dawley rats (body weight 120-150 gm.) were taken as experimental animal in the present investigation. The animals were randomly selected and reared in the animal culture laboratory of Institute of Advanced Study Science and Technology, Khanapara at room temperature 25±2°C. The rats were housed individually.

The rats were fed with basal diet (Table 3.1) in the proportion of 12 gm/day/rat and provided with water *ad libitum*. To evaluate the effect of aqueous extract of *Terminalia chebula* on rat liver. The experiment were conducted into two phase. In first phase, healthy rat were selected for the experiment in different days of treatment. upto 28th days and findings are correlate with their respective control group. In second phase, the study was further extended to evaluate the hepatoprotective activity of aqueous extract of *Terminalia chebula* upto 7th days through a standard model by administering CCl₄ in intraperitoneal injection.

Phase - I

The following groups of rat were made for the study in first phase of experiment.

(a) Control: received basal diet expended to 28th days.
(b) Test Set(s): received basal diet and oral dose of aqueous extract of Terminalia Chebula (TC) 100 mg/kg b.w./day for a period of 7th, 14th, 21st and 28th days accordingly and studied separately in four different sets.

After the stipulated period each animal was sacrificed and samples of blood (from Jugular vein) and liver tissue were collected from control and test groups on 7th, 14th, 21st and 28th day of treatment to assess the level of different biochemical parameters: viz.

a) Blood Glucose (BG),

b) Serum Protein level; Total Protein (STP) and Albumin (SALB)

c) Serum Bilirubin (SRBN) both direct and total.

d) Serum Alkaline Phosphatase (SALP)

e) Transaminase of Serum (SGOT and $GPT$)

f) Transaminase of liver (LGOT and LGPT)

**Phase - II**

In the second phase of study, male, young and healthy Sprague Dowley rats (body weight 120 - 150 gm) were taken as experimental animal. The rats were kept in separate cage for each group. They were feed with basal diet with water *ad libitum*. In each set of experiment six numbers of rat were kept for the study and divided as follows:

i) Control Group

Received basal diet with liquid paraffin through i.p. (2 ml/mg body weight) in the 3rd and 6th day.
ii) CCl₄ treated group
Received CCl₄ 2 ml/mg body weight on 3rd and 6th day of experimentation.

iii) Test Group TC₁
Received aqueous extract of *Terminalia chebula* 200 mg/kg body weight/day upto the 7th day. On 3rd and 6th day of experimentation two dose of CCl₄ i.p.

iv) Test group TC₂
Received aqueous extract of T.C. in the dose of 500 mg/kg/body weight for 7 day, on 3rd and 6th day, CCl₄ injection in dose of 2 ml/kg/body weight through intraperitonial route.

The *Terminalia chebula* extract were fed through oral root by rubber catheter upto 7th consecutive day.

The following bio-chemical parameters were estimated in Serum for assessment of liver function:

a) Serum Total Protein (STP)

b) Serum Bilirubin (SRBN)

c) Serum Alkaline Phosphatase (SALP)

d) Serum glutamic oxalacetic transaminase (SGOT)

e) Serum glutamic pyruvic transaminase (SGPT)

**Composition of Basal Diet**

Each Individual animal was given 12 gms of diet with the following compositions (Farrais 1950).
Table 3.1 Composition of basal diet in (gms).

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Whole wheat</td>
<td>67.5</td>
</tr>
<tr>
<td>2</td>
<td>Yellow corn.</td>
<td>62.5</td>
</tr>
<tr>
<td>3</td>
<td>Barley</td>
<td>37.5</td>
</tr>
<tr>
<td>4</td>
<td>Anik Spray</td>
<td>37.5</td>
</tr>
<tr>
<td>5</td>
<td>Bone meal</td>
<td>2.5</td>
</tr>
<tr>
<td>6</td>
<td>Calcium chloride</td>
<td>2.5</td>
</tr>
<tr>
<td>7</td>
<td>Salt</td>
<td>2.5</td>
</tr>
<tr>
<td>8</td>
<td>Oil</td>
<td>37.5</td>
</tr>
<tr>
<td>9</td>
<td>Vitamin B₁₂</td>
<td>1 tab</td>
</tr>
</tbody>
</table>

From the slaughtered animal blood was taken from the jugular vein in centrifuged tube and Serum was collected for estimation of stated different biochemical parameters.

Collection of Liver

Liver from the sacrificed animal immediately removed into a beaker containing precold distilled water and one gm from it weighted and homogenized with 10 ml precold Phosphate buffer in a glass homogenizer plunged in ice. The homogenate was centrifuged in cold, the supernatant were collected for estimation of liver transaminase group of enzymes following the procedure described later.

Collection of Termination Chebula Retz.

The fruit materials of *Terminalia chebula* Retz. was collected from Mandakata Medicinal Plant Garden, Assam located 25 km. north from Guwahati city (Plate 1 - 3) and was authenticated by Pharmacognosy Laboratory of Govt. Ayurvedic College, Guwahati.
Preparation of Aqueous Extract of *Terminalia chebula* Retz.

Measured quantity of coarse powder of dry *Terminalia chebula* fruit was mixed with four times its quantity of distilled water for 8 hours in a mechanical shaker working at a speed of 102 strokes per minute. The liquid extract was strained and discarded the residue. The extract was clarified by filtration. The filtrate was used in all the experiments (Gaind K.N. *et al* 1962).

Standardization of Aqueous Extract of *Terminalia chebula* Retz.

To standardize the extract a Physico chemical and quantitative analysis was carried out at the Pharmaceutical Chemistry Laboratory of Institute of Post Graduate Teaching and Research, Jamnagar.

Table 3.2  **Physico-Chemical and Quantitative Analysis.**

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Name of Test</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Loss of drying at 110°C</td>
<td>08.72% W/W</td>
</tr>
<tr>
<td>2.</td>
<td>Ash Value</td>
<td>04.99% W/W</td>
</tr>
<tr>
<td>3.</td>
<td>Acid Insoluble Ash</td>
<td>0.44% W/W</td>
</tr>
<tr>
<td>4.</td>
<td>Water Soluble Extract</td>
<td>57.82% W/W</td>
</tr>
<tr>
<td>5.</td>
<td>Volatile Oil Content</td>
<td>Nil</td>
</tr>
<tr>
<td>6.</td>
<td>pH of 10% (W/W) Aqueous Solution</td>
<td>04.05</td>
</tr>
<tr>
<td>7.</td>
<td>Test for Tannin</td>
<td>Positive</td>
</tr>
<tr>
<td>8.</td>
<td>Assay Tannins by USSRP</td>
<td>49.88%</td>
</tr>
</tbody>
</table>
METHODOLOGY

The following bio-chemical parameters were estimated using autoanalyser (Micro-Lab 100, E. Merck) and Stangen kit for estimation of various enzyme.

Estimation of Blood Glucose (GOD-PAP Method)

Principle

Glucose oxidase (GOD) Catalyzes the oxidation of Glucose in accordance with the following equation

Glucose + O₂ + H₂O ⇌ Gluconic Acid + H₂O

The Hydrogen Peroxidase formed in this reaction reacts with 4 aminoantipymine and 4 - hydroxbenzoic acid in the presence of Peroxidase (POD) to form N-(4-antiphryl)-P. banzaquione imine. The addition of mutarotase accelerators the reaction. The amount of dye formed is proportional to the Glucose Concentration (Barham et al. 1972).

Specimen - Serum

The Glucose Concentration remain unchanged for 5 days at room temperature and 4°C in whole blood which has been deproteminized and centrifuged immediately after withdrawal Glucose remains stable for 24 hours at 4°C in serum and plasma obtained within 30 minutes of blood sampling (Teuscher et al. 1971).
Reagents

1. Buffer Solution (4 x 100 ml.)
2. Enzyme Solution (1.25 ml.)
3. Standard Solution (100 mg/dl. = 5.55 mm. 0/1 (30 ml.)

Concentration of the reaction solutions PIPES pH 7.2 - 0.05 m 0/1

1. Phenyl-2, 3-diethyl-4-amino Pyrazolone (s) - 1.00 mm 0/1
   4-Hydroxybenzoic acid. 6.00 mm 0/1
   Glucose Oxidase 6.00 KU/1
   Peroxidase 3.20 KU/1
   Bacterial Mutrotase
   Detergents, Stabilizers. 0.05 KU/1

Preparation of Reaction Solution

Added 250 ml of the bottle (2) to the contents of bottle (1). Stable for 4 weeks at 2°C to 3°C and for 1 week at 15°C to 25°C.

Procedure

Absorption maximum - 510 mm.
Measuring wave length - 546 mm.
Layer thickness - 1 cm.

Without deproteinnisation, pipetted into test tubes sample blank

Serum - 10 µl.

Reaction Solution (2) 1000 µl. - 1000 µl.
Mixed and incubated measured after that the absorbance of the analytical sample (AS) against the blank after 15-40 minutes at 37°C.

Calculation

Determination with factor

Glucose Concentration = AS x F (F = 405 nm)

Estimation of Total Protein and Albumin (Biuret & BCG Dye Binding Method.)

Principle

The Proteins bind with copper ions in an Alkaline medium of the Biuret reagent and produce a purple coloured complex, whose absorbance is proportional to the Protein Concentration.

Albumin in a buffered medium binds with Bromocresol green (BCG) and produce a green colour; whose absorbance is proportional to the Albumin Concentration. (Anino, J.B. 1976; Webster - D. 1977; Doumas B. J. 1978)

Reagents

1. Biuret reagent - 125 ml.
2. Buffered Dye Reagent - 125 ml
3. Protein Standard (Box No - 2) - 3 ml.
Specimen - Serum

Procedure

Total Protein Assay

Three test tubes were taken in labelled blank, Std. and test, 1 ml. Biurel Reagent (1) was taken to each tube following the addition of 2 ml distilled water in blank std and test 0.05 ml. Protein in Std. (3) and 0.05 ml. Serum were added to Standard end and test tube respectively. Mix well and incubated all the three tubes at 37°C for 10 minutes. The tubes were measured absorbance of standard and test against blank on a spectrophotometer at 555 mm.

Albumin Assay

Pipetted into three test tubes labelled blank, Standard and test. 1 ml. Buffered dye reagent (2) was taken to each tube following the addition of 2nd distilled water in all the tube. Protein Standard (3) 0.01 ml and Serum 0.01 ml. were added to standard and test tube respectively. Mix well and measured immediately absorbance of standard and test against blank on a spectrophotometer at 630 mm.

Estimation of Total Bilirubin and Direct Bilirubin (Modified Jenderassik and Grof's Method).

Principle

Bilirubin reacts will diazoties sulfanilic acid in acidic medium to form azobilirubin, a purple coloured complex whose absorbance is proportional to bilirubin concentration. The total bilirubin (Direct and Indirect) the diazotization is carried out
in the presence of an activator. (Jendrassik et al. 1938). Direct bilirubin, being water soluble is allowed to react with diazotized sulfuric acid in the absence of an activator.

**Reagents**

1. Diazo A - 1 x 100 ml.
2. Diazo B - 1 x 5 ml.
3. Activator - 1 x 50 ml.
4. Artificial Standard (10 mg%) - 2 x 5 ml.

**Specimen - Serum**

**Procedure**

Four test tubes were taken labelled T1, T2 (for total bilirubin) and D1 and D2 (for direct bilirubin) respectively. 1.0 ml Diazo A reagent added to the all the tube. Diazo B 0.1 ml added to the T1 and D1 tube.

1.0 ml. activator (3) added to the tube T1 and T2 and distilled water 2.5 ml., 2.6 ml., 3.5 ml and 3.6 ml. was added T1, T2, D1 and D2 respectively. Lastly, 0.2 ml Serum Mixed in all the tubes and kept in dark at room temperature for 5 minutes. The T1 and T2 read on spectrophotometer at 540 nm. against distilled water within 30 minutes. The D1 and D2 read on spectrophotometer at 540 nm against distilled water just after one minute of mixing with the serum.
Estimation of Serum Alkaline Phosphatase (Kind and Kings Method) (E.C. 3.1.3.1)

Principle

Serum ALP hydrolyzes phenylphosphate into phenol and disodium hydrogen phosphate at pH 10.0. The phenol so formed acts with 4-Aminoantipyrine in alkaline medium in presence of oxidizing agent potassium ferricyanide to form a red colored complex whose absorbance is proportionate to the enzyme activity. (Kind and King, 1954; King et al. 1959; Varley, 1975).

\[
\text{Disodium Phenyl + H}_2\text{O} \xrightarrow{\text{ALP}} \text{Disodium Phenol + Hydrogen Phosphate pH 10.0}
\]

\[
\text{Phenol} \xrightarrow{\text{Alkaline medium}} \text{Red Colored Complex}
\]

Reagents

1. Buffered Substrate 20 x 4.5 ml.
2. Color Reagent 2 x 100 ml.
3. Phenol Standard 10 mg% 1 x 3 ml.

Specimen - Serum.

ALP in serum is stable for at least 7 days at 2.8°C.

Working Reagent Preparation

Reconstituted Buffered Substrate (1) with 4.5 ml. of distilled water / deionized water and mixed well.
Procedure

Pipetted into the test tubes labelled Blank (B), Standard (S) Control (C), and Test (T) as follows.

Table 3.3:

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>S</th>
<th>C</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Buffered Substrate</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Deionized water</td>
<td>3.1 ml</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
</tr>
</tbody>
</table>

Incubated for 3 minutes at 37°C

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th>0.1 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Phenol Standard (3)</td>
<td>-</td>
<td>0.1 ml</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Incubated for 15 minutes at 37°C

<table>
<thead>
<tr>
<th></th>
<th>2.0 ml</th>
<th>2.0 ml</th>
<th>2.0 ml</th>
<th>2.0 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color Reagent(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>0.1 ml</td>
<td>-</td>
</tr>
</tbody>
</table>

Mixed well after each addition of reagent and measured absorbance (A) for blank (B), standard (S), control (C) and Test (T) against deionized water on spectrophotometer at 510nm.

Estimation of Transaminase

GPT - Glutamate Pyruvate Transaminase (E.O. 2.6.1.2)

Principle

The SGPT catalyzes the transfer of aminogroup from L - Alamine to L - Katogthta rate with the formation of Pyruvate and glutamate. The pyruvate so formed
is allowed to react with 2, 4 D W P H to produce 2 : 4 dinitron Phenyle hydrazone derivative which is brown coloured in alkaline Medium. The absorbance of this hydrozone derivative is correlated to SGPT activity by plotting a Calibration curve using Pyruvate Standard. Retiman and Frankol’s (1957) and Tictz, N.W. (1970).

\[ \text{L-alamine} \quad \text{&} \quad \text{Oxeloglutarate} \quad \xrightarrow{\text{SGPT}} \quad \text{pH 7.4} \quad \xrightarrow{\text{Pyruvate}} \quad \text{&} \quad \text{L. Glutamate} \]

\[ \text{Pyruvate} \quad \& \quad 2, 4 \text{DNPH} \quad \xrightarrow{\text{Alkaline Medium}} \quad 2, 4 \text{ dinitrophenyl hydrozone} \quad \text{(brown coloured)} \]

**Reagents**

1. Buffered Substrate pH 7.4 2 x 12.5 ml.
2. DPNH Colour Reagent 2 x 12.5 ml.
3. Sodium Hydroxide & N. 1 x 25.0 ml.
4. Pyruvate Standard 2 mM 1 x 3.0 ml.

**Specimen** - Fresh Unhemolysed Serum.

**Reagent Preparation**

Diluted Sodium Hydroxide(s) 1 : 10 with distilled water before use.

**Procedure**

Pipetted into clean dry test tubes numbered 1, 2, 3, 4, and 5 as below
Table 3.4:

<table>
<thead>
<tr>
<th></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>
<tr>
<td>Buffered Substrate (1)</td>
<td>0.5 ml</td>
<td>0.45 ml</td>
<td>0.40 ml</td>
<td>0.35 ml</td>
<td>0.30 ml</td>
</tr>
<tr>
<td>Pyruvate Standard (4)</td>
<td>-</td>
<td>0.05 ml</td>
<td>0.10 ml</td>
<td>0.15 ml</td>
<td>0.20 ml</td>
</tr>
<tr>
<td>Distilled water 0.1 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>DNPH Colour Reagent (2)</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Working Sodium Hydroxide</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

Mixed well and allowed to stand at R.T. for 20 minutes and then read absorbances of tubes 2 to 5 respectively against tube 1 (Reagent Blank) on spectrophotometer at 505 nm.

Then plotted the graph of absorbance of test tube 2, 3, 4 and 5 on Y axis, Versus corresponding Enzyme activity on X-axis.

Assay

Pipetted into clean dry test tubes and labelled Blank (B) & Test (T) as shown below:

Table 3.5:

<table>
<thead>
<tr>
<th></th>
<th>(B)</th>
<th>(T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffered Substrate</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Serum</td>
<td>-</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>DNPH Colour Reagent(s)</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.1 ml</td>
<td>-</td>
</tr>
<tr>
<td>Working Sodium Hydroxide</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

Incubated at 37°C for 3 minutes

Mixed well and incubated at 37°C for 20 minutes

Mixed well and allowed to stand at RT for 20 minutes
Mixed well and allowed to stand at RT for 10 minutes. The absorbance of Test (T) against Blank (B) on Photocolorimeter using Spectro Photometer at 505 nm. On Calibration Curve the absorbance was read, Test (T) on Y axis & corresponding enzyme activity on X-axis.

**GOT - Glutamate Oxaloacetate Transaminase (E.C. 2.6.1.1)**

**Principle**

SGOT Catalyzes transfer of amino group from L. asparate to L - Kalogluterate with formation of Oxaloacetate and glutamate. The Oxaloacetate so formed, is allowed to react with 2, 4, DNPH to form 2 : 4 dinitro Phenyle hydrazone derivative which is brown coloured is alkaline medium. The absorbance of this hydrazone derivative is correlated to SGOT activity by plotting a calibration curve using pyruvate standard. Retiman, S. and Frankel, S. (1957); Tietz, N.W. (1970)

\[
\begin{align*}
\text{L - asparate} & \quad \text{SGOT} \\
\quad + & \quad \text{L-Kalogluturate} \\
\quad + & \quad \text{Oxaloacetate} \\
\quad + & \quad \text{L. Glutamate}
\end{align*}
\]

\[
\begin{align*}
\text{Oxaloacetate} & \quad \text{Alkaline} \\
\quad + & \quad \text{2, 4 dinitrophenyl} \\
\quad + & \quad \text{(brown coloured)}
\end{align*}
\]

**Reagents**

1. Buffered Substrate pH 7.5 - 2 x 12.5 ml.
3. Sodium Hydroxide 4 N. - 1 x 25.0 ml.
4. Pyruvate Standard 2 nM - 1 x 3.0 ml.
Specimen - Fresh unhamolysed Serum. Serum was separated as soon as possible. The specimen is stable for 3 days at 2.8°C.

Reagent Preparation

Diluted Sodium Hydroxide (3) 1:10 with distilled water before used.

Procedure

Calibration Curve

Pipetted into test tubes and numbered 1, 2, 3, 4, & 5 as shown below:

<table>
<thead>
<tr>
<th>Table 3.6:</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>
<tr>
<td>Buffered Substrate (1)</td>
<td>0.5 ml</td>
<td>0.45 ml</td>
<td>0.40 ml</td>
<td>0.35 ml</td>
<td>0.30 ml</td>
</tr>
<tr>
<td>Pyruvate Standard (4)</td>
<td>-</td>
<td>0.05 ml</td>
<td>0.10 ml</td>
<td>0.15 ml</td>
<td>0.20 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.10 ml</td>
<td>0.10 ml</td>
<td>0.10 ml</td>
<td>0.10 ml</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>DNPH Colour Reagent (2)</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

Mixed well and allowed to stand at RT for 20 minutes

Working Sodium Hydroxide | 5.0 ml | 5.0 ml | 5.0 ml | 5.0 ml | 5.0 ml |

Mixed well and allowed to stand at RT for 10 minutes and then read absorbances of tubes 2 to 5 respectively against tube 1 (Reagent Blank) on photocolormeter using a green filter or on spectrophotometer at 505 nm. Then plotted the graph of absorbance of test tubes 2, 3, 4, & 5 on Y axis versus corresponding enzyme activity on X-axis.
Assay

Pipetted into two clean dry test tubes labelled Blank (B) & Test (T) as shown below.

Table 3.7:

<table>
<thead>
<tr>
<th></th>
<th>(B)</th>
<th>(T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffered Substrate</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td></td>
<td>Incubated at 37°C for 3 minutes.</td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>-</td>
<td>0.1 ml</td>
</tr>
<tr>
<td></td>
<td>Mixed well and incubated at 37°C for 60 minutes</td>
<td></td>
</tr>
<tr>
<td>DNPH Colour Reagent (2)</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td></td>
<td>Mixed well and allowed to stand at RT for 20 minutes.</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.1 ml</td>
<td>-</td>
</tr>
<tr>
<td>Working Sodium Hydroxide</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

Mixed well and allowed to stand at RT for 10 minutes and measured the absorbance of Test (T) against Blank (B) on photocolorimeter using a green filter or on spectrophotometer at 505 nm. On calibration curve read the absorbance of Test (T) on Y-axis and corresponding enzyme activity on X-axis.

Liver Weight and Volume

After the animal were sacrificed, the abdomen cut open and the liver was taken out. The ratio of wet liver weight and volume to animal body weight was computed and recorded Reddy et al. (1993).
Histopathological Studies

On the eight day surviving animals from each group were sacrificed by decapitation and the histopathological techniques. Luna L.G. (1968). The liver was carefully dissected out, extraneous tissue was cleaned off and then wet weight and volume were noted. The Part of the liver was fixed in formol-saline and processed for microtome sectioning at 5 micron thickness, stained with harmatoxylin and eosin.

The damaged produced in the liver structure in the form of degeneration, necrosis and fibrosis was graded Shirwalkar et al. (1992).

a) Degeneration

O : On degeneration.

+ : Few vacuolated cells per lesion.

++ : More than ten cells per lesion.

+++ : One or two rows of vacuolated cells, around necrotic zone per lesion with haemorrhagic areas.

++++ : More than two rows of vacuolated cells around necrotic zone per lesion.

b) Necrosis

O : No Necrosis.

+ : Focal Necrosis of one two cells per lesion.

++ : Focal Necrosis of more than two cells per lesion.

+++ : Massive Cetrilobular necrosis with haemorrhages.

++++ : Massive centrilobular necrosis with necrotic tissue bridging the central veins.
c) Fibrosis

+: Normal appearance of liver

+: Central necrosis, hydropic, degeneration, accumulation of mesenchymal fibrosis.

++: Presence of fibrous tissue in portal tract area only, along with other changes mentioned for Grade I.

+++: Fibrous tissue insinuating surrounding hepatic parenchyma.

++++: Formation of pseudolobules by insinuated fibrous tissues.

Statistical Methods Employed

To arrive at meaningful conclusion the data were analysed by standard statistical procedures Snedecor and Cochran (1967). Average and standard errors were calculated at different days after treatment and also in different treatment groups, for each of the parameter studied. In order to investigate the effect of days of treatment (0 - 28 days) as well as the effect of the treatment groups (control, CCl₄, TC₁ and TC₂) on different biochemical constituents and enzymes one way analysis of variance (ANOVA) was carried out. Whenever an effect was found to be significant the critical difference (CD) test were carried out in order to compare the subclass means for significance.