Chapter Four  
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4.4.5 Study of Curative Effect of *Cichorium intybus* against CCl₄ induced liver cell damage in rats.
**Part of the plants used**: Whole plant

**Solvent used for extraction**: 50% ethanol

**Drugs and Chemicals used**

The following drugs and chemicals were used in the present study.

<table>
<thead>
<tr>
<th>Drug/Chemical</th>
<th>Supplier/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde Solution</td>
<td>Nice Chemicals Pvt. Ltd, Cochin-682024</td>
</tr>
<tr>
<td>Alcohol</td>
<td>Bengal Chemicals &amp; Pharmaceuticals</td>
</tr>
<tr>
<td>Acacia</td>
<td>Central Drug House(P), Ltd. Asif Ali Road, New Delhi-110002</td>
</tr>
<tr>
<td>Sodium Chloride Solution</td>
<td>do</td>
</tr>
<tr>
<td>Sulphuric acid (A.R)</td>
<td>do</td>
</tr>
<tr>
<td>Sulphuric acid (L.R)</td>
<td>do</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>do</td>
</tr>
<tr>
<td>Potassium dichromate</td>
<td>do</td>
</tr>
<tr>
<td>Carbon Tetrachloride</td>
<td>MERCK, Merck Limited, Worli, Mumbai-400018</td>
</tr>
<tr>
<td>Cichorium intybus / Taraxacum officinale whole Plant</td>
<td>Collected from Kashmir University Campus</td>
</tr>
<tr>
<td>Rat feed</td>
<td>Supplied by RRL Jammu</td>
</tr>
<tr>
<td>Acesure SGOT Reagent Kit. (DNPH METHOD)</td>
<td>ACE DIAGNOSTICS &amp; BIOTECH LIMITED. Huda, Gurgaon (Haryana)-122001.</td>
</tr>
<tr>
<td>SGPT Kit</td>
<td>do</td>
</tr>
<tr>
<td>HDL-CHOLESTROL Kit (AUTOPAK)</td>
<td>Bayer Diagnostic, 589 Sayajipura, Ajwa Road, Baroda, Gujarat, 390019.</td>
</tr>
<tr>
<td>p-Nitrophenyl phosphate</td>
<td>Indian Drugs and Pharmaceuticals Ltd. Hyderabad</td>
</tr>
<tr>
<td>Magnesium Chloride</td>
<td>do</td>
</tr>
<tr>
<td>Hydrochloric Acid</td>
<td>do</td>
</tr>
</tbody>
</table>
(a) **ANIMALS**

Albino rats (wistar strain) and mice, were most of the time purchased from Central Animal House of Regional Research Laboratory Jammu and were kept in the Animal House of the Department of Pharmaceutical Sciences (801/03/CPCSEA).

(b) **INSTRUMENTS**

- Centrifuge **REMI**
- Spectrophotometer **ERBA CHEMISTRY ERBA-CHEM-5 PLUS**
- Weighing Balance **5 kg and 2 kg capacity**
- Hot water Bath
- Micropipettes
SCHEME DEPICTING THE COURSE OF STUDY.

Collection of Plant Materials

Identification of the plants

Drying

Coarsely powdering of the plant materials

Extraction (Cold maceration) with 50% ethanol

Filtration of the extract (1st Filtrate)

Addition of 50% ethanol to the residue

Filtration of the extract (2nd Filtrate)

Combining the 1st and 2nd Extract

Recovery of the solvent (ethanol) under vacuum

Drying of the extract to a semi solid mass

Hepatoprotective studies of ethanolic extract in different models.
A. PHYTOCHEMICAL STUDIES

I. Cichorium intybus

a) Identification and collection

The whole plant of *Cichorium intybus*, family Compositae, was collected from the University Campus and also purchased from the local market. The identification was done on the basis of characters described by Kirtikar and Basu, 1935 and by Dr. Naqshi (Consultant Plant Taxonomist). The plant material was dried in a well ventilated room with outside temperature ranging between 18 to 32°C.

b) Extraction of *Cichorium intybus* with 50% Ethanol

The dried plant of *Cichorium intybus* was coarsely powdered and 500g was allowed to macerate for 48 hrs with 50% ethanol, with occasional shaking. After 48 hrs, the ethanolic extract was filtered through *Whatman’s* filter paper under vacuum. The plant material was then macerated again with fresh 50% ethanol and the combined filtrate obtained from the first and second maceration was then distilled under vacuum, the temperature of distillation being in the range of 33-44°C. After the distillation of alcohol, the extract was then evaporated to dryness and the yield was noted.
II. *Taraxacum officinale*

a) **Identification and collection**

The *Taraxacum officinale* whole plant, family Compositae, was collected from the fields of University Campus, Hazratbal Srinagar, and identified on the basis of characters described by Kirtikar and Basu (1933) and by Dr. Naqshi (Consultant Plant Taxonomist). The plant material was dried in a well ventilated room with outside temperature ranging between 18 to 32°C.

b) **Extraction of the whole plant of *Taraxacum officinale***

After cleaning the *Taraxacum officinale* whole plant, 500g of it was macerated twice for 48 hours, with 50% ethanol. The combined ethanolic extract obtained from first and second maceration was filtered through Whatman’s filter paper under vacuum at reduced temperature, the temperature being in the range of 42-48 °C and allowed to air dry. The yield was noted down.
B. PHARMACOLOGICAL STUDIES:

50% ethanolic extracts of *Cichorium intybus* whole plant and *Taraxacum officinale* whole plant were screened for prophylactic and curative effect against liver damage.

TEST ANIMALS

Rats and mice of either sex were used in the experimental studies. They were procured from central Animal House, RRL, Jammu (67/100/CPCSEA) after proper approval. They were randomly distributed according to age, weight, sex and were housed in clean polypropylene cages and fed with rat feed (Hindustan Lever, India) and water ad-libitum. The animals were kept under identical conditions of food, water, temperature, and degree of nursing care. They were exposed to 12 hr. light-dark cycle and the relative humidity was in the range of 61-76 %.

All procedures were performed in accordance with the *Institutional Animal and Ethics Committee* (IAEC) at the Deptt. of Pharmaceutical Sciences, University of Kashmir (801/03/CPCSEA).
HEPATOTOXICITY was produced by the following methods.

(I) Paracetamol was administered orally to mice in a single dose of 500 mg /kg body weight, 48 hrs., before the administration of drugs and in the prophylactic study 48 hrs. after the administration of extracts.

(II) Carbon tetrachloride (CCL₄) was given by forced oral administration to rats at the dose of 2.0 ml/kg body weight daily for 14 days along with liquid paraffin (1:1).

50% ethanolic extracts were administered as suspensions (in 1% gum acacia) in distilled water as vehicle.

Both chronic and acute, hepatoprotective studies were conducted on the ethanolic extracts of Cichorium intybus and Taraxacum officinale, in different sets of experiments. In the first two sets of experiments, Paracetamol was used as acute hepatotoxic model in rats and mice. In the next experiment, Carbon tetrachloride (CCL₄) was used for inducing hepatocellular damage in rats as a chronic model.
PARACETAMOL INDUCED ACUTE HEPATOCELLULAR DAMAGE IN RATS

I) Study of Curative Effect of 50% Ethanolic Extract of *Taraxacum officinale* whole plant against Paracetamol induced acute hepatocellular damage in rats.

**Experimental Animal:**
- Strain: Wistar rats
- Sex: Male/Female
- Weight: 150-230g

**Experimental Groups:**
- Group I: Vehicle Control
- Group II: Paracetamol (500mg/kg), p.o, Single dose.
- Group III: Paracetamol (500mg/kg), p.o, Single dose. + *Taraxacum officinale* (100mg/100g/day) Daily for five days.

**Treatment method:**
- Drug treatment: Vehicle control On 1st day
- Paracetamol On 1st day
- *Taraxacum officinale* after 48 hrs
- Enzyme estimations On Day 7

In this experiment, effect of *Taraxacum officinale* whole plant against paracetamol induced liver cell damage was studied. Healthy albino rats
(Wistar strain) of either sex, weighing between 150-230 g, were divided into three groups of 8 animals each.

Group I served as vehicle control and received only 1% gum acacia suspension. Paracetamol solution prepared by dissolving paracetamol powder in distilled water by heating was administered in a single oral dose of 500 mg/ kg to animals of Groups II and III. 48hrs. after paracetamol administration, Group III received 50% ethanolic extract of *Taraxacum officinale* whole plant at the dose of 100 mg/100g daily for 5 days in a single oral dose. Group II served as negative control and did not receive any further treatment for the next five days.

Rats were weighed every alternate day, during one week experimental study and the dose of the drug was calculated accordingly. Also the average body weight of each group of rats was calculated after one week of experimental study.

After the administration of last dose of drugs (after 5 days), the animals were fasted overnight. Blood samples were withdrawn from the retinobulbar venous plexus of the eyes of the rats with the help of glass capillary under light anaesthesia. The blood samples were kept at room temperature for a minimum of 1-1 1/2 hour and allowed to clot. They were then centrifuged and the serum thus separated was subjected to various biochemical estimations like SGOT, SGPT, SAP and Cholesterol. The animals were then sacrificed by stunning on the head; their livers were dissected out, washed under tap water and dried gently between the folds of filter paper. The weight of the liver of each rat was noted and then average liver weight per 100 g body weight of each group was calculated. The livers were then preserved in 10% formalin solution for histopathological studies.
II. Study of Prophylactic effect of extracts of *Taraxacum officinale* and *Cichorium intybus* against Paracetamol induced hepatotoxicity in Mice.

Experimental animal :-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Wistar mice.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex-</td>
<td>Male/Female.</td>
</tr>
<tr>
<td>Weight-</td>
<td>16-23g.</td>
</tr>
</tbody>
</table>

Experimental Groups :-

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Vehicle Control (1% gum acacia).</td>
</tr>
<tr>
<td>II</td>
<td>Toxic agent only P (Paracetamol 500mg/kg b.w), single dose.</td>
</tr>
<tr>
<td>III</td>
<td><em>T officinale</em> (50 mg/100g/day) for 5 days +P.</td>
</tr>
<tr>
<td>IV</td>
<td><em>T officinale</em> (100 mg/100g/day) for 5 days +P.</td>
</tr>
<tr>
<td>V</td>
<td><em>T officinale</em> (200 mg/100g/day) for 5 days +P.</td>
</tr>
<tr>
<td>VI</td>
<td><em>C intybus</em> (50mg/100g/day) for 5 days + P.</td>
</tr>
<tr>
<td>VII</td>
<td><em>C intybus</em> (100mg/100g/day) for 5 days + P.</td>
</tr>
<tr>
<td>VIII</td>
<td><em>C intybus</em> (200mg/100g/day) for 5 days + P.</td>
</tr>
</tbody>
</table>

Treatment method:-

Drug treatment: Paracetamol (P) was administered in a single oral dose of 500mg/kg, on Day 6\textsuperscript{th} to mice of Group II, III, IV, V, VI, VII & VIII.

Enzyme estimations: On Day 7\textsuperscript{th}. 
In the Second set of experiment, healthy albino mice (Wistar strain) of either sex weighing between 16-23 g were divided into eight groups of 6 animals each. They were housed under uniform animal husbandry conditions, in polypropylene cages, fed with proper diet and water ad-libitum.

First Group served as vehicle control. Groups III, IV, V received 50% ethanolic extract of *Taraxacum officinale* whole plant at the dose of 50 mg, 100 mg and 200 mg/100g/day respectively, and Groups VI, VII and VIII received 50, 100, 200 mg/100g/day of 50% ethanolic extract of *Cichorium intybus* daily for 5 days in single oral dose. After five days, i.e. on day 6th Groups II, III, IV, V, VI, VII, and VIII were administrated the hepatotoxic agent i.e Paracetamol solution, (500mg/kg body weight), in a single oral dose (Pandey & Shrivastava, 1990). The animals of Group I received 1% gum acacia suspension in distilled water daily for 6 days.

Mice were weighed on every alternate day during the one week prophylactic study and the doses of the drugs were calculated accordingly. At the end of the experiment, i.e on day 7th, blood was collected from the retino-bulbar venous plexus of mice of all the Groups I-VIII and serum thus separated was estimated for ALT levels. Livers were dissected out from mice & after weighing, were preserved in 10% formalin for histopathological studies.
III. Study of Curative effect of 50% Ethanolic Extracts of *Taraxacum officinale* and *Cichorium intybus* against Carbon tetrachloride induced hepatotoxicity in Rats:

Experimental Animal :-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Wistar rats.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male/Female</td>
</tr>
<tr>
<td>Weight</td>
<td>140-170g.</td>
</tr>
</tbody>
</table>

Experimental Groups :-

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>CCl₄ (Only Toxic agent).</td>
</tr>
<tr>
<td>II</td>
<td>CCl₄ + <em>T officinale</em> (50mg/100g/day)</td>
</tr>
<tr>
<td>III</td>
<td>CCl₄ + <em>T officinale</em> (100mg/100g/day)</td>
</tr>
<tr>
<td>IV</td>
<td>CCl₄ + <em>C intybus</em> (25mg/100g/day)</td>
</tr>
<tr>
<td>V</td>
<td>CCl₄ + <em>C intybus</em> (50mg/100g/day)</td>
</tr>
</tbody>
</table>

Treatment Method :-

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Treatment</th>
<th>CCl₄ was administered at the dose of 2ml/kg, b.w, twice a week for 14 days.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme estimations</td>
<td>On Day 15.</td>
<td></td>
</tr>
</tbody>
</table>

In this study, effect of *Taraxacum officinale* and *Cichorium intybus* against Carbon tetrachloride induced Liver Cell Damage was studied for 15
days. Healthy albino rats (Wistar strain) of either sex weighing between (140-170g) were divided into six groups of 10 animals each.

Group I received, 2.0ml/kg b.w of carbon tetrachloride in liquid paraffin (1:1), subcutaneously twice a week for 14 days. Groups II & III received 50% ethanolic extract of *Taraxacum officinale* at the doses of 50 and 100mg/kg/day respectively for 14 days along with CCl₄ (2.0ml/kg twice a week). Groups IV and V received 50% ethanolic extract of *Cichorium intybus* at the doses of 25 and 50mg/100g/day, respectively, for 14 days along with CCl₄ (2.0ml / kg, s.c, twice a week).

*Taraxacum officinale* and *Cichorium intybus* extracts were administrated as suspensions orally for 14 days. The weights of the rats were recorded every alternate day. The temperature and humidity of the room, in which the animals were kept, was recorded daily during the experimental study.

After 14 days of drug treatment and CCl₄ administration, the rats were fasted overnight, blood samples were collected from the retino-bulbar venous plexus. Serum was separated and subjected to biochemical estimations and the livers of the rats were excised out, washed under tap water, dried between the folds of the filter paper; weighed and then preserved in 10% formalin for histopathological studies.
C) BIOCHEMICAL ASSESSMENT OF LIVER FUNCTION

Conventional tests are used to screen for liver disease, to confirm its presence and to estimate severity.

i) Serum Enzyme Tests

The following tests were performed for the Biochemical Assessment of the liver function.

a) Aspartate amino transferase (AST) or SGOT.
b) Alanine amino transferase (ALT) or SGPT.
c) Serum Alkaline Phosphatase (SAP)
d) HDL-Cholesterol

Aminotransferases (transaminases)

Serum aspartate aminotransferase (AST or SGOT) and serum alanine aminotransferase (ALT or SGPT) were estimated by the method of Reitman and Frankel (1957) using Acesure Reagent Kit.

Test Principle

\[
\text{L-aspartate} + 2\text{-oxaloacetate} \rightarrow \text{Oxaloacetate} + \text{L-Glutamate}
\]

SGOT (AST) catalyses the transfer of amino group from aspartic acid to 2-oxalocarboxylate to form oxaloacetate and L-Glutamate. The oxaloacetate thus formed reacts with 2,4-dinitrophenylhydrazine to form a corresponding
hydrazone, a brownish red coloured complex in an alkaline medium. The colour intensity is measured photometrically at 505 nm or with green filter.

Reagent Composition

<table>
<thead>
<tr>
<th>Contents</th>
<th>Active Ingredients Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent -1 Substrate Reagent</td>
<td>L-aspartic acid 33mM/L</td>
</tr>
<tr>
<td></td>
<td>2-Oxalo glutarate 5mM/L</td>
</tr>
<tr>
<td>Reagent -2 SGOT Colour</td>
<td>2,4-dinitrophenyl 1.0mM/L</td>
</tr>
<tr>
<td></td>
<td>hydrazine</td>
</tr>
<tr>
<td>Reagent -3 Calibrator</td>
<td>Sodium pyruvate Value on label</td>
</tr>
<tr>
<td>Reagent -4 Alkali reagent</td>
<td>Sodium hydroxide 4N</td>
</tr>
</tbody>
</table>

Reagent Preparation and Storage

Dilute reagent-4 (Alkali reagent) 1:10 with deionised water. All other reagents are ready to use. The reagents are stable up to expiry date stated on the label when stored at 2-8°C.

Specimen:

Clear, unhaemolysed serum was used. Serum should be removed from clot as soon as possible after collection because of the approximately of 10-fold greater concentration of GOT in erythrocytes than in serum. Haemolysis of the serum must be avoided.
**Test Procedure**

<table>
<thead>
<tr>
<th>Pipette into test tube labelled as</th>
<th>Blank</th>
<th>Calibrator</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate Reagent</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5ml</td>
</tr>
<tr>
<td>Deionised Water</td>
<td>0.1 ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1ml</td>
</tr>
<tr>
<td>Calibrator</td>
<td>-</td>
<td>0.1 ml</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Mix and incubate at 37°C for 60 minutes.

| SGOT Colour Reagent              | 0.5 ml| 0.5 ml | 0.5 ml | 0.5ml|
| Sample                           | -     | -      | 0.1 ml | -    |

Mix and incubate for 20 minutes at room temperature.

| Alkali Reagent (Diluted)         | 3.0 ml| 3.0 ml | 3.0 ml | 3.0ml|

Read absorbance of all these tubes against distilled water at 505 nm or with Green filter.

**CALCULATION**

SGOT (AST) activity in U/L = \[
\frac{\text{Abs. Test} - \text{Abs. Control}}{\text{Abs. Calibrator} - \text{Abs. Blank}} \times \text{Conc. Of Calibrator}
\]

SGPT (ALT) activity in U/L = \[
\frac{\text{Abs. Test} - \text{Abs. Control}}{\text{Abs. Calibrator} - \text{Abs. Blank}} \times \text{Conc. Of Calibrator}
\]
Serum Alkaline Phosphatase (SAP)

Estimation was carried out by Bessey- Lowry Brock method (1946).

Test Principle

In buffered alkaline medium, alkaline phosphatase hydrolysis p-nitrophenyl phosphate to phosphate and p-nitrophenol which produces a yellow colour. Intensity of the colour so produced is directly proportional to alkaline phosphatase activity and is measured photometrically at 405 nm.

\[
\text{Phosphatase} \\
p\text{-nitrophenyl phosphate } + H_2O \rightleftharpoons \text{p-nitrophenol } + H_3PO_4
\]

(Colourless in solution) (Colourless in acid but yellow in alkaline solution)

Reagent preparation and storage

i). Alkaline buffer, 0.1 M glycine, pH-10.5
Weigh 7.5 g of glycine and 95 mg of MgCl\(_2\) and transfer to 1 litre volumetric flask containing 750 ml of water. Mix thoroughly and store in a refrigerator.

ii) p-Nitrophenyl phosphate Stock Substrate, 0.4%
Dissolve 100 mg of p-nitrophenyl phosphate in 25 ml water.

iii) Alkaline buffered Substrate (pH 10.3- 10.4)
Mix equal volumes of (i) & (ii).

iv) Sodium Hydroxide, 0.02N
Pipet 20 ml of 1 N-NaOH into a 1 litre volumetric flask and dilute to volume with water. Mix well.

v) Hydrochloric acid, concentrated
Test Procedure

1. Into the reagent blank and test tubes, pipette 1.0 ml of alkaline buffered substrate.
2. Place the tubes (blank and tests) into 37°C controlled water bath for 5 minutes, for the tubes and the reagent to come to water bath temperature.
3. Set a timer for 30 minutes, upon addition of water (0.1 ml) to the blank, start the timer and every $\frac{1}{2}$ minute thereafter, add the serum (0.1 ml) to its respective tube in the water bath.
4. Allow the tubes to incubate until exactly 30 minutes after the water is added to the blank.
5. Exactly 30 minutes by the timer, remove the blank from the water bath and add 10 ml of 0.02 N NaOH. Continue at $\frac{1}{2}$ minute interval until the reaction has stopped in all the test tubes.
6. Stopper the tubes with clean stoppers and mix by inverting.
7. Record the absorbance (A) of the tests against the reagent blank at 410 nm.
8. Add 0.1 ml (2 drops) of conc. HCl to each tube and mix by shaking tube. This removes the colour due to p-nitrophenol.
9. Reread each tube against the reagent blank at 410 nm.
10. Subtract the second reading from the first reading. The value of this corrected absorbance is then read.

Cholesterol

The HDL-Cholesterol was determined by Phosphotungstate method (AUTOPAK Kit method). This reagent kit is used for in vitro quantitative
determination of HDL-Cholesterol (High Density Lipoprotein-Cholesterol) in liver or plasma.

Test Principle

Chylomicrons, VLDL (Very Low Density Lipoproteins) and LDL fractions in serum or plasma are separated from HDL by precipitating with Phosphotungstic acid and Magnesium Chloride. After centrifugation, the Cholesterol in the HDL fraction, which remains in the supernatant, is assayed with enzymatic cholesterol method, using cholesterol Esterase, Cholesterol Oxidase, Peroxidase and the chromogen 4-Aminoantipyrine/Phenol.

Sample Collection, Storage and Stability

Serum is preferred. EDTA or heparinised plasma can also be used. As far as possible, use samples on the same day. Samples are stable for a week when stored tightly capped at 2-8 C.

Reagents

**Reagent 1 (Enzymes/Chromogen):**

- Cholesterol Esterase \( \geq 200 \text{ U/L} \)
- Cholesterol Oxidase \( \geq 250 \text{ U/L} \)
- Peroxidase \( \geq 1000 \text{ U/L} \)
- 4-Aminoantipyrine 0.5 mmol/L

**Reagent 1A (Buffer):**

- Pipes buffer, pH 6.90 50 mmol/L
- Phenol 24 mmol/L
- Sodium Cholate 0.05 mmol/L
Reagent 2 (Precipitating Reagent):
Phosphotungstic Acid 2.4 mmol/L
Magnesium Chloride 39 mmol/L

Standard (HDL Cholesterol 50mg/dL):
Cholesterol 0.5 g/L

Storage & Stability of the Reagents

When stored at 2-8°C and protected from light, the reagents are stable until the expiry dates stated on the labels.

Reagent Reconstitution

Allow the reagents to attain room temperature. Dissolve the contents of one bottle of reagent 1 into one bottle of reagent 1A. Mix by gently swirling till completely dissolved.

Wait for 5 minutes before using.

The reconstituted reagent is stable for 3 months when stored at 2-8°C.

Test Procedure

The samples, the precipitating reagent 2 and the reconstituted reagent should be brought to room temperature prior to use.
I  Precipitation

Dispense into Centrifuge Tube:

<table>
<thead>
<tr>
<th>Test</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>0.20 ml (200 μL)</td>
</tr>
<tr>
<td>Precipitating Reagent 2</td>
<td>0.20 ml (200 μL)</td>
</tr>
</tbody>
</table>

Mix well. Centrifuge at 1500 g or 3500-4000 rpm. For 10 min. Separate the clear supernatant immediately and determine the cholesterol content.

The 50 mg/dL HDL-Cholesterol standard should not be subjected to the precipitation step.

II  Cholesterol Assay

The following general system parameters are to be used with this kit:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Type</td>
<td>Endpoint</td>
</tr>
<tr>
<td>Reaction Slope</td>
<td>Increasing</td>
</tr>
<tr>
<td>Wavelength</td>
<td>500 nm (492-550 nm)</td>
</tr>
<tr>
<td>Flow cell Temp.</td>
<td>30 C</td>
</tr>
<tr>
<td>Incubation</td>
<td>5 min 37 C</td>
</tr>
<tr>
<td>Sample Vol. (Supernatant)</td>
<td>20 μL</td>
</tr>
<tr>
<td>Reagent Volume</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Standard Concentration</td>
<td>100 mg/dL</td>
</tr>
<tr>
<td>Zero setting with</td>
<td>Reagent Blank</td>
</tr>
</tbody>
</table>

Set the instrument using above system parameters.
Dispense into test tubes:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reconstituted Reagent</td>
<td>1 mL</td>
<td>1 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>20 μL</td>
<td>-</td>
</tr>
<tr>
<td>Supernatant</td>
<td>-</td>
<td>-</td>
<td>20 μL</td>
</tr>
</tbody>
</table>

Incubate for 5 minutes at 37 °C. Mix and read.
ii) Histopathology

After preserving the livers in 10% formalin, prepared in Normal Saline, the sections were dehydrated with acetone three times after every two hours. Next, clearing of the tissue was done with benzene making 2 changes at two hours intervals. After embedding in paraffin wax blocks, sections were cut serially at 4-6 (microns) by rotatory microtone. The sections were then passed, in ascending and descending series, through acetone and then stained using Haematoxylin Eosin (H&E). After fixing, the sections were examined microscopically.

Data and Statistical Analysis:

Values are expressed as mean ± SE from the number of replications described in the test. Total variation present in a set of data has been estimated by analysis of Variance (ANOVA). The t-value was also calculated for two sided test. P values < 0.05 were considered significant and P < 0.01 were considered highly significant.

RESULTS:

Biochemical studies:
Tables 1-20.
Figures a-t.

Histopathology:
Fig 1-16.