CHAPTER 5

ANTIHEPATOTOXIC ACTIVITY
5. OBJECTIVES:

Antihepatotoxic activity of *Lychnis coronaria*, *Marrubium vulgare* and *Berberis pachycantha* extracts and that of isolated compounds was performed with the following objectives.

(a) To ascertain that the plant materials possessed antihepatotoxic activity.

(b) To study the potential of these extracts for their claim as antihepatotoxic agents against the liver damage in an experimental rat model of hepatic lesions caused by carbon tetrachloride in rats.

(c) To perform the pharmacological screening of these extracts *in vivo*.

(d) To compare their antihepatotoxic effects with the standard drug i.e. silymarin.
To ascertain the antihepatotoxic activity of the pure isolated compounds by performing biochemical parameters and histopathological studies.

5.1. Techniques and Models Employed In Screening of Antihepatotoxic Activity.

Antihepatotoxic effect was measured in the experimental animals (Wells, 1988). Carbon tetrachloride was used to induce liver damage and an attempt was made to counteract this damage with the extracts and their isolated compounds. The magnitude of the repair was evaluated by measuring the enzymes activities and histopathological examination of the liver tissues.

Other substances which are used more frequently used for producing liver injury or toxicity are CCl₄, paracetamol, D-galactoamine, ethyl alcohol, thioacetamide etc. (Harbone, 1973). These substances produce liver damage by different mechanisms, but the parameters employed to monitor the magnitude of the damage is usually common. Different hepatotoxic substances and different kinds of test models are chosen for successful determination of the antihepatotoxic effect, namely in vitro and in vivo methods. For the present investigation CCl₄ was used to produce damage.

In vivo tests

The activity of the crude plant extracts and that of the isolated compounds was determined by keeping the whole hepatic tissue intact within the animal (Shukla et al, 1992).

5.2. TOXICOLOGICAL PROFILE

The different plant extracts and their pure isolated constituents were evaluated for pharmacological activity.

5.2.1. Experimental animals

Male Wistar rats weighing 150–200 gm were employed for assessing the antihepatotoxic activity. They were procured from the Central Animal House of Jamia Hamdard, New Delhi (173/CPCSEA), after approval under the project proposal number-
They were fed with a standard pellet diet and water *ad libitum*. The animals were maintained at 25°C to 28°C with 40-70% RH and 12 hr light/dark cycles and were fastened for 12 hours prior to the experiment.

5.2.2. Administration of hepatotoxins

Carbon tetrachloride was mixed with liquid paraffin in a ratio of 1:1 and 1 mg/kg body weight of this mixture was given intraperitoneally to each rat. The effect of the toxicant was ascertained by measuring biochemical parameters.

5.2.3. Administration of treatment agents

The drug treatment was followed for 7 days after CCl₄: paraffin administration. The treatment was given orally in the form of suspension (1% gum acacia). On the last day, two rats from each group were sacrificed and serum from 5 animals was taken for biochemical evaluation. The blood taken out from the retro-orbital sinus was allowed to coagulate at 37°C for 30 min and serum was separated by centrifugation at 3500 rpm.

5.2.4. Assessment of liver function

Biochemical parameters like serum SGOT, SGPT (Reitzmann and Frankel, 1957), ALKP (Kind & King, 1954) and TP (Wooton, 1964) were carried out by reported methods.

There is no single biochemical parameter to assess the liver function and thus three to four tests must be carried out as such serum bilirubin, proteins, lipids, urea, enzymes (alkaline phosphatase, aspartate and alanine transaminase, glutathione, glutathione-s-transferase, gamma glutamyl transpeptidase, malonaldehyde and less frequently lactic dehydrogenase, ornithine carbonyl transferase, ether dehydrogenase and cholinesterase) galactose estimation capacity, bromosulphaljie clearance rate etc, are carried out. The most common and economical parameters are serum glutamic oxaloacetetic transaminase (SGOT) or AST, serum glutamic pyruvate transaminase (SGPT) or ALT, alkaline phosphatase (ALKP) and total proteins.
5.2.5. Statistical analysis

The data of biochemical estimations were reported as ± S.E, where n = 5. For determining the statistical significance one way analysis of variance (ANOVA) and Dunnett's test was employed. P-values of less than 0.05 were considered significant (Dunnett's, 1964).

5.3. TREATMENT SCHEDULE

5.3.1. Antihepatotoxic activity of *Lychnis coronaria*.

**Group – I (Normal control)**: This group was given neither CCl₄ nor treatment. The livers of two animals were taken for histopathology at the end of study. The blood sample of five animals was taken for liver function tests.

**Group – II (Toxic control)**: The animals were given CCl₄ diluted with liquid paraffin in a ratio of (1:1) (0.7ml/kg b.w, i.p) on the first day only to produce toxicity in liver (Ahmed et al., 2001; Vogel et al., 2002) and thereafter no treatment of extracts.

**Group – III (Standard control)**: The animals were given single dose of CCl₄ diluted with liquid paraffin in a ratio of (1:1) (0.7ml/kg b.w, i.p) on the first day and thereafter received treatment with standard drug silymarin (Silybon-70) at a dose of 10 mg/kg body weight, p.o for 7 days.

**Group – IV (Petroleum ether fraction)**: The animals were given single dose of CCl₄ diluted with liquid paraffin in a ratio of (1:1) (0.7ml/kg b.w, i.p) on the first day and thereafter received treatment with petroleum ether fraction at a dose of 500 mg/kg body weight, p.o for 7 days.

**Group – V (Chloroform fraction)**: The animals were given single dose of CCl₄ diluted with liquid paraffin in a ratio of (1:1) (0.7ml/kg b.w, i.p) on the first day and thereafter received treatment with chloroform fraction at a dose of 500 mg/kg body weight, p.o for 7 days.

**Group – VI (M ethanol fraction)**: The animals were given single dose of CCl₄ diluted with liquid paraffin in a ratio of (1:1) (0.7ml/kg b.w, i.p) on the first day and thereafter
received treatment with methanol fraction at a dose of 500 mg/kg body weight, p.o for 7 days.

On the 8th day the blood samples were withdrawn by puncturing the orbital plexus and the rats were sacrificed by decapitation. The blood samples were allowed to clot for 30-40 min. at room temperature. Serum was separated by centrifugation at 2500 rpm for 15 min. and various biochemical parameters were estimated.

The serum from five animals was taken for the estimation of biochemical parameters and the livers of the two animals were taken for the histopathology at the end of study.

5.3.2. Antipatotoxic activity of Marrubium vulgare

Group – I (Normal control): This group was given neither CCl₄ nor treatment. The livers of two animals were taken for histopathology at the end of study. The blood sample of five animals was taken for liver function tests.

Group – II (Toxic control): The animals were given CCl₄ diluted with liquid paraffin in a ratio of (1:1) (0.7ml/kg body weight, i.p) on the first day to produce toxicity in liver (Ahmed et al, 2001; Vogel et al, 2002) and thereafter no treatment of extracts.

Group – III (Standard control): The animals were given single dose of CCl₄ diluted with liquid paraffin in a ratio of (1:1) (0.7ml/kg b.w, i.p) on the first day and thereafter received treatment with standard drug silymarin (Silybon-70) at a dose of 10 mg/kg body weight, p.o for 7 days.

Group – IV (Petroleum ether fraction): The animals were given single dose of CCl₄ diluted with liquid paraffin in a ratio of (1:1) (0.7ml/kg body weight, i.p) on the first day and thereafter received treatment with petroleum ether fraction at a dose of 500 mg/kg body weight, p.o for 7 days.

Group – V (Chloroform fraction): The animals were given single dose of CCl₄ diluted with liquid paraffin in a ratio of (1:1) (0.7ml/kg body weight, i.p) on the first day and thereafter received treatment with chloroform fraction at a dose of 500 mg/kg body weight, p.o for 7 days.
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Group - VI (Methanol fraction): The animals were given single dose of CCl₄ diluted with liquid paraffin in a ratio of (1:1) (0.7ml/kg body weight, i.p) on the first day and thereafter received treatment with methanol fraction at a dose of 500 mg/kg body weight, p.o for 7 days.

On the 8th day the blood samples were withdrawn by puncturing the orbital plexus and the rats were sacrificed by decapitation. The blood samples were allowed to clot for 30-40 min. at room temperature. Serum was separated by centrifugation at 2500 rpm for 15 min. and various biochemical parameters were estimated.

The serum from five animals was taken for the estimation of biochemical parameters and the livers of the two animals were taken for the histopathology at the end of study.

5.3.3. Antihepatotoxic activity of Berberis pachycantha

Group - I (Normal control): This group was given neither CCl₄ nor treatment. The livers of two animals were taken for histopathology at the end of study. The blood sample of five animals was taken for liver function tests.

Group - II (Toxic control): The animals were given CCl₄ diluted with liquid paraffin in a ratio of (1:1) (0.7ml/kg body weight, i.p) on the first day to produce toxicity in liver (Ahmed et al, 2001; Vogel et al, 2002) and thereafter no treatment of extracts.

Group - III (Standard control): The animals were given single dose of CCl₄ diluted with liquid paraffin in a ratio of (1:1) (0.7ml/kg body weight, i.p) on the first day and thereafter received treatment with standard drug silymarin (Silybon-70) at a dose of 10 mg/kg body weight, p.o for 7 days.

Group - IV (Petroleum ether fraction): The animals were given single dose of CCl₄ diluted with liquid paraffin in a ratio of (1:1) (0.7ml/kg body weight, i.p) on the first day and thereafter received treatment with petroleum ether fraction at a dose of 500 mg/kg body weight, p.o for 7 days.

Group - V (Chloroform fraction): The animals were given single dose of CCl₄ diluted with liquid paraffin in a ratio of (1:1) (0.7ml/kg body weight, i.p) on the first day and
thereafter received treatment with chloroform fraction at a dose of 500 mg/kg body weight, p.o for 7 days.

**Group – VI (Methanol fraction):** The animals were given single dose of CCl₄ diluted with liquid paraffin in a ratio of (1:1) (0.7ml/kg body weight, i.p) on the first day and thereafter received treatment with methanol fraction at a dose of 500 mg/kg body weight, p.o for 7 days.

**Group – VII (Compound BP-3):** The animals were given single dose of CCl₄ diluted with liquid paraffin in a ratio of (1:1) (0.7ml/kg body weight, i.p) on the first day and thereafter received treatment with Compound BP-3 at a dose of 50 mg/kg body weight, p.o for 7 days.

On the 8th day the blood samples were withdrawn by puncturing the orbital plexus and the rats were sacrificed by decapitation. The blood samples were allowed to clot for 30-40 min. at room temperature. Serum was separated by centrifugation at 2500 rpm for 15 min. and various biochemical parameters were estimated.

The serum from five animals was taken for the estimation of biochemical parameters and the livers of the two animals were taken for the histopathology at the end of study.

5.4.1. Estimation of Serum Glutamic Oxaloacetic Transaminase (SGOT) or Aspartate Transaminase (AST).

It is a mitochondrial enzyme present in larger quantities in the liver, heart, skeletal muscles and kidneys. Wherever these tissues are destroyed, these enzymes are released from damaged cells. It is estimated by using Reitzman and Frankel’s method. (Reitzman and Frankel, 1957; Nobert, 1970; Toro and Ackermann, 1975).

Principle:

GOT (AST) catalyses the following reaction:

\[ \alpha - \text{Ketogluterate} + \text{L - Aspartate} \rightarrow \text{L - Glutamate} + \text{Oxaloacetate}. \]

Oxaloacetate so formed is coupled with 2, 4- dinitrophenyl hydrazine (2,4-DNPH) to give corresponding hydrazine, which gives colour that can be estimated colorimetrically. As the reaction proceeds with time, more amounts of products are formed and since end products inhibit the enzyme, there is more of inhibition. This is the major problem with the colorimetric method for the estimation of this enzyme. On the other hand in kinetic methods, since the enzyme activity is measured during the initial few minutes, the amount of products formed during that short time are negligible to cause any inhibition. Because of the above problem, it is necessary to standardize any calorimetric method against a standard kinetic method. In the kit purchased from Span Diagnostics this standardization is done against the standard Karmen Unit Assay (Kinetic) and is extrapolated to different amount of pyruvate and this has been thoroughly rechecked. At this point it is important to note that the standard graph of enzyme activity (in units/ml) on X-axis vs. O.D, Y-axis is not linear one, which shows that O.D. increases with increase in enzyme activity at a decreasing rate.
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Reagents:

Reagent - 1: Buffered Aspartate a - Ketoglutarate substrate (PH 7.4)
Reagent - 2: DNPH colour reagent
Reagent - 3: Sodium hydroxide (4N)

Preparation of working solutions

Solution - 1: 1 ml of reagents 3 was diluted upto 10 ml with distilled water.
Solution 1 is quite stable at room temperature
Reagents 1, 2 and 4 were ready for use.

Procedure (Standard Curve)

The reagents were added in test tubes as shown in table no. 13

Table No. 13: Procedure for the Preparation of Standard Curve of Serum

Aspartate Transaminase (AST) or (GOT).

<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>
<tr>
<td>Reagent - 1</td>
<td>0.5</td>
<td>0.45</td>
<td>0.4</td>
<td>0.35</td>
<td>0.30</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 (DNPH)</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 solutions are mixed well and allowed to stand at room temperature for 20 minutes.

Solution - 1 (0.4 N NaOH) | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
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After addition of solution 1 the tubes were vortexed and allowed to stand for 10 min. at room temperature. Then O.D. was measured at 505 nm against distilled water. A standard graph was plotted by taking enzyme activity on X - axis and O.D. on the Y - axis.

The O.D. recorded for the standard curve was as follows:

<table>
<thead>
<tr>
<th>Tube number</th>
<th>Absorbance</th>
<th>Enzyme activity Units/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.35</td>
<td>0</td>
</tr>
<tr>
<td>2.</td>
<td>0.43</td>
<td>24</td>
</tr>
<tr>
<td>3.</td>
<td>0.54</td>
<td>61</td>
</tr>
<tr>
<td>4.</td>
<td>0.63</td>
<td>114</td>
</tr>
<tr>
<td>5.</td>
<td>0.72</td>
<td>190</td>
</tr>
</tbody>
</table>

Procedure (Test serum samples)

For analyzing the levels of transaminase, 0.1 ml of serum was taken in a test tube containing 0.5ml of reagent - 1. Mixed well and incubated at 37°C for 60 min. Then 0.5 ml of reagent - 2 (DNPH) was added and allowed to stand for 20 min. Later on 5.0 ml of solution 1 was added each test tube and mixed well and allowed to stand for 10 min. at room temperature and the absorbance at 505 nm was measured against distilled water. The sequence of addition is also shown in table no. 15

<table>
<thead>
<tr>
<th>Reagent - 1</th>
<th>Buffered Aspartate</th>
<th>0.5 ML</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

Mixed well and incubate for 60 min. at 37°C

<table>
<thead>
<tr>
<th>Reagent - 2</th>
<th>DNPH</th>
<th>0.5 ml</th>
</tr>
</thead>
</table>
Mixed well and allowed to stand for 20 min. at room temperature

<table>
<thead>
<tr>
<th>Solution - 1</th>
<th>5.0 ml</th>
</tr>
</thead>
</table>

Mixed well and allowed to stand for 10 min. at room temperature and measured O.D. using green filter.

Calculations
The O.D. of test (t) was marked on the Y-axis of the standard curve and extrapolated it to the corresponding enzyme activity on the X-axis.

5.4.2. Estimation of Serum Glutamic Pyruvate transaminase (SGPT) or Alanine transaminase (ALT)
It is a cytosolic enzyme present abundantly in liver cells. The serum levels of ALT are elevated in the liver disease. This is considered one of the most and sensitive indications of liver damage particularly in viral hepatic necrosis e.g. viral hepatitis or toxin induced liver injury. Lesser degree of elevations is seen in mild acute viral hepatitis, chronic hepatitis, cirrhosis and hepatic metastasis. It is determined by using Reitzman and Frankel method. (Karmen, 1995; King, 1965; Steinberg and Baldwin, 1965).

Principle
SGPT (ALT) catalyses the following reaction:
\[ \alpha\text{-Ketoglutarate} + L\text{-Alanine} \rightarrow L\text{-Glutamate} + \text{Pyruvate.} \]
Pyruvate formed is coupled with 2, 4 dintrophenyl hydrazine to give the corresponding hydrazone which gives a brown colour in alkaline medium and is measured colorimetrically.

Reagents
Reagent - 1 : Buffered Alanine- \( \alpha \) -ketoglutarate substrate (PH 7.4)
Reagent - 2 : DNPH colour reagent
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Reagent – 3 : Sodium hydroxide (4N)
Reagent – 4 : Working Pyruvate Standard, 2mM

Preparation of working solutions:

Solution – 1 : 1 ml of reagents 3 was diluted upto 10 ml with distilled water. Reagents 1, 2 and 4 were ready for use.

Procedure (Standard Curve)

The reagents were added in test tubes as shown in table no. 13 for plotting the standard curve for recording SGPT.

Table No. 16: Serial Dilution to plot Standard Curve 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>
<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>0.05</td>
<td>0.1</td>
<td>0.15</td>
<td>0.2</td>
<td></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 DNPH (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>

All the solutions were mixed well and allowed to stand at room temperature for 20 min.

| Solution – 1 (0.4 N NaOH) | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
After the addition of Solution - 1 (0.4 N NaOH) each test tube was mixed well again and allowed to stand for 10 min. at room temperature. Then absorbance was measured at 505 nm against distilled water.

<table>
<thead>
<tr>
<th>Tube number</th>
<th>Absorbance</th>
<th>Enzyme activity units/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.36</td>
<td>0</td>
</tr>
<tr>
<td>2.</td>
<td>0.45</td>
<td>28</td>
</tr>
<tr>
<td>3.</td>
<td>0.56</td>
<td>57</td>
</tr>
<tr>
<td>4.</td>
<td>0.63</td>
<td>97</td>
</tr>
<tr>
<td>5.</td>
<td>0.74</td>
<td>150</td>
</tr>
</tbody>
</table>

A standard graph was plotted by taking enzyme activity on X - axis and O.D. on the Y - axis.

After plotting the standard curve the different serum samples from the test animals were taken for determining the optical density. The method for preparation of the serum for estimation is as summarized in the table no. 18.

<table>
<thead>
<tr>
<th>Reagent - 1 Buffered Aspartate</th>
<th>0.5 ML</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubate at 37°C for 5 min.</td>
<td></td>
</tr>
<tr>
<td>Serum added</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Mixed well and incubated for 30 min at 37°C</td>
<td></td>
</tr>
<tr>
<td>Reagent - 2 DNPH</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

Table No. 18: Measurement of the O.D. of Samples for SGPT
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<table>
<thead>
<tr>
<th>Mixed well and allowed to stand for 20 min at room temperature.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution – I added 0.5 ml</td>
</tr>
</tbody>
</table>

The resulting solutions were mixed well and allowed to stand for 10 minutes at room temperature and the optical density was observed at 505nm against distilled water.

Calculations

The O.D. of test (t) was marked on the Y- axis of the standard curve and extrapolated to the corresponding enzyme activity on the X-axis.

5.4.3. Estimation of Alkaline Phosphatase (ALKP)

Principle.

Alkaline phosphatase from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10.0. Phenol so formed reacts in alkaline medium with 4–amino antipyrine in presence of the oxidizing agent potassium ferricyanide and forms an orange–red coloured complex, which can be measured colorimetrically. The colour intensity is proportional to the enzyme activity (King & King, 1954; King and Jagatheesan, 1956; Varley, 1975).

The reaction can be represented as:

Phenyl phosphate \( \xrightarrow{\text{Alk. Phosphatase}} \) Phenol + Pi

Pot. Ferricyanide

Phenol + 4-Aminoantipyrine \( \xrightarrow{\text{OH}^-} \) Orange-red complex

\( (\lambda_{\text{max}} = 510-520 \text{ nm}) \)

Test Sample

Serum: Various blood groups are collected aseptically in a clean dry tube.
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Reagents:

Reagent 1 : Buffered substrate, pH 10.0
Reagent 2 : Chromogen Reagent
Reagent 3 : Phenol standard, 10 mg%

Preparation of working solution

Reconstituted one vial of reagent 1, Buffered substrate with 4.5 ml of distilled water, reagent 2 & 3 was ready for use.

Procedure

The experimental procedure for estimation of Alkaline Phosphatase colorimetrically is summarized below in table no. 19.

Table No. 19: Experimental Procedure for Estimate of Alkaline Phosphatase

<table>
<thead>
<tr>
<th></th>
<th>Back (B)</th>
<th>Standard (S)</th>
<th>Control (C)</th>
<th>Test (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>Distilled 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>

Mixed well and incubated for 3 minutes at 37°C

<table>
<thead>
<tr>
<th></th>
<th>Back (B)</th>
<th>Standard (S)</th>
<th>Control (C)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Phenol standard 10 mg%</td>
<td>-</td>
<td>0.1 ml</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Mixed well and incubated for 15 minutes at 37°C

<table>
<thead>
<tr>
<th></th>
<th>Back (B)</th>
<th>Standard (S)</th>
<th>Control (C)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromogen reagent</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

The solutions were mixed well after the addition of each reagent and O.D. of Blank (B), Standard (S), Control (C) and Test (T) was measured against distilled water using green filter i.e. 520 nm.
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Calculations

The enzyme activity was calculated by using following formula

\[ \text{Serum alkaline phosphatase} = \frac{\text{O.D. Test} - \text{O.D. Control}}{\text{O.D. std.} - \text{O.D. Blank}} \times 10 \]

(activity in KA Units)

5.4.4. Estimation of Total Proteins Principle

Total proteins:

Proteins and peptides containing at least 2 adjacent peptide bonds react with cupric complex ions of Biuret reagent in alkaline medium to form a blue purple complex with absorption maximum at 540 nm (Dumas, 1971)

Procedure

Total Proteins: Colorimetrically

For estimating the total proteins content in the serum sample, the solutions were prepared according to the table no. 20.

Table No. 20: Preparation of Solutions for Estimating the Total Proteins content of Serum Sample.

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein reagent</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total protein standard</td>
<td>-</td>
<td>50 µl</td>
<td>-</td>
</tr>
<tr>
<td>Specimen (serum)</td>
<td>-</td>
<td>-</td>
<td>50 µl</td>
</tr>
</tbody>
</table>
Mix by lateral shaking and incubate all the tubes at 37°C for exactly 10 minutes or at room temperature for 15 min. Measure optical density (O.D) of all the tubes at 540 nm against blank adjusted to zero.

Calculation

The concentration of total protein in each unknown is determined by following calculation:

Serum total Proteins in gm/100ml = RT/RS X 6gm/dl.

Where, RT = O.D of unknown.

RS = O.D of standard.
5.5. HISTOPATHOLOGICAL STUDIES

After blood collection, all the animals were sacrificed by decapitation under light ether anesthesia and livers were dissected out. Liver tissues were washed with normal saline and were preserved in 10% neutral buffered formalin. The techniques that were employed include fixation, processing of liver tissue, preparation and cutting the section, preparation and staining of slides etc. as described by Lunas (Luna, 1968).

Procedure

Preparation of neutral formalin solution:

4.0 gm of sodium phosphate monobasic and 6.5 gm of sodium phosphate dibasic (anhydrous) were added to the 100 ml of 37-40% formalin and then volume was made up to 1000 ml with distilled water.

1. Fixation of tissue

Tissues were placed in the fixative solution immediately upon removal from the body to preserve the relation of the tissue elements as they were in life. Care was taken to ensure that hardening process was of such degree that the tissue components and architecture were minimally affected by any subsequent procedure. Tissues were grossly cut to pieces of 1 mm thickness to enable the fixing fluid to penetrate the tissue in a reasonably short time. Tissues were immersed in twenty times their volume inside the fixative solution. Ten percent neutral buffered formalin was used as fixative. Fixation was done for 36 hours. After fixation the tissues were washed in running water before it was dehydrated cleared and embedded.

2. Processing of tissues

The specimens were marked with an identification tag kept with block throughout processing. The surface, from which sections were cut, was indicated by notching the opposite surface. When the tissue was embedded, the marked surface of the block was kept upper most. Paraffin was used for embedding (because embedding in paraffin is
achieved rapidly and gives the best results when thin sections of soft tissues are desired. Since paraffin is not miscible with water, the tissues were dehydrated by passing in different grades of ethanol (70-100%) and then cleared by using chloroform. Tissues were changed twice. The solutions were kept within one inch of the top of the beakers on the processor.

3. Embedding in paraffin

Embedding was done using brass L-moulds. Block size: 2 cm × 2 cm × 1 cm. Paraffin was filtered before use.

4. Preparation and cutting of sections

The results produced by histopathological studies greatly depend upon the type of knives used. A perfect edge for a microtome knife may be defined in simple terms as the junction of two smooth plane surfaces at an angle of about 14 degrees. After a paraffin block was mounted on the object holder, cleaned of excess paraffin clamped in the block holder on the microtome, the knife clamp was adjusted towards the paraffin block and sectioned slowly. The facilitate sectioning, wet cotton was applied to the surface of the block after rough cutting.

5. Preparation of Slides

Folds and bubbles were removed by pulling the ribbon very gently across the long edge of glass slide half below the surface in the water bath. After the section was mounted on the slide bubbles in the tissues are removed by gently brushing with a fine camel’s hair brush.
6. Resealing Block

After the needed sections were cut from a paraffin block, it was resealed to prevent the drying of the tissue or destruction by insects. This makes subsequent cutting easier. The block sealer provides a continuous supply of molten paraffin with which to seal cut specimen block as they are removed from the microtome.

7. Staining

Sections were picked on aluminized slides dried and wound paraffin wax was removed by successive passage 2-3 times in xylene, different grades of ethanol 100-50% and running water. Then the slides were stained with hematoxylin and eosin.
5.6. ANTIHEPATOTOXIC INVESTIGATION

The whole plant of *Lychnis coronaria*, *Marrubium vulgare* and rhizomes of *Berberis pachycantha* were successively fractionated with petroleum ether, chloroform and methanol to get the respective extracts. These fractions and the compounds isolated from them were given to the rats for 7 days to assess the antihepatotoxic activity. On the 8th day the animals were sacrificed. The biochemical parameters recorded for the three plants are given in the tabular form and histopathological slides of liver are given as plates.
Chapter 5

Antihepatotoxic activity

**Graph-1: Standard Curve for SGOT**

**Graph-2: Standard Curve for SGPT**
Table No. 21: Effect of various fractions of *Lychnis coronaria* whole plant on serum enzymatic activity in CCl₄ induced liver damage in rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Dose</th>
<th>SGOT units/ml</th>
<th>SGPT units/ml</th>
<th>ALKP units/ml</th>
<th>TP gm/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal control</td>
<td>---</td>
<td>36.47 ± 1.45</td>
<td>25.52 ± 0.70</td>
<td>27.58 ± 0.56</td>
<td>7.36 ± 0.16</td>
</tr>
<tr>
<td>II</td>
<td>Toxic control</td>
<td>1 mg/kg (s.c.)</td>
<td>119.19 ± 4.05</td>
<td>94.70 ± 4.03</td>
<td>55.08 ± 2.39</td>
<td>4.49 ± 0.16</td>
</tr>
<tr>
<td>III</td>
<td>Silymarin (standard drug)</td>
<td>10 mg/kg (p.o.)</td>
<td>55.97 ± 1.61***</td>
<td>41.70 ± 2.90**</td>
<td>35.32 ± 1.05**</td>
<td>7.15 ± 0.12***</td>
</tr>
<tr>
<td>IV</td>
<td>Petroleum ether fraction</td>
<td>500 mg/kg (p.o.)</td>
<td>94.73 ± 4.99*</td>
<td>63.69 ± 3.40*</td>
<td>37.61 ± 1.82*</td>
<td>5.56 ± 0.10*</td>
</tr>
<tr>
<td>V</td>
<td>Chloroform fraction</td>
<td>500 mg/kg (p.o.)</td>
<td>84.05 ± 2.09*</td>
<td>67.28 ± 4.11*</td>
<td>48.73 ± 2.78*</td>
<td>6.30 ± 0.15**</td>
</tr>
<tr>
<td>VI</td>
<td>Methanol fraction</td>
<td>500 mg/kg (p.o.)</td>
<td>75.57 ± 3.87**</td>
<td>56.62 ± 3.10**</td>
<td>43.95 ± 2.05*</td>
<td>6.64 ± 0.10**</td>
</tr>
</tbody>
</table>

SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvate transaminase; ALKP, alkaline phosphate; TP, total protein; s.c subcutaneous; p.o.

**P < 0.01; *P < 0.05 vs CCl₄.

Values are mean ± S.E. of five animals. One way analysis and Dunnett’s test.
Graph-3: SGOT values after treatment with different fractions of Lychnis coronaria against toxic control.

Graph-4: SGPT values after treatment with different fractions of Lychnis coronaria against toxic control.
Graph-5: ALKP values after treatment with different fractions of *Lychnis coronaria* against toxic control.

Graph-6: Total Protein values after treatment with different fractions of *Lychnis coronaria* against toxic control.
### Table No. 22: Effect of various fractions of *Marrubium vulgare* whole plant on serum enzymatic activity in CCl₄ induced liver damage in rats.

<table>
<thead>
<tr>
<th>Groups (n=5)</th>
<th>Treatment</th>
<th>Dose</th>
<th>SGOT units/ml</th>
<th>SGPT units/ml</th>
<th>ALKP units/ml</th>
<th>TP gm/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal (control)</td>
<td>---</td>
<td>36.47 ± 1.45</td>
<td>25.52 ± 0.70</td>
<td>27.58 ± 0.56</td>
<td>7.36 ± 0.16</td>
</tr>
<tr>
<td>II</td>
<td>Toxic (control)</td>
<td>1 mg/kg (s.c.)</td>
<td>119.19 ± 4.05</td>
<td>94.70 ± 4.03</td>
<td>55.08 ± 2.39</td>
<td>4.49 ± 0.16</td>
</tr>
<tr>
<td>III</td>
<td>Silymarin (standard drug)</td>
<td>10 mg/kg (p.o.)</td>
<td>55.97 ± 1.61***</td>
<td>41.70 ± 2.90**</td>
<td>35.32 ± 1.05**</td>
<td>7.15 ± 0.12***</td>
</tr>
<tr>
<td>IV</td>
<td>Petroleum ether fraction</td>
<td>500 mg/kg (p.o.)</td>
<td>98.10 ± 3.57*</td>
<td>76.49 ± 2.39*</td>
<td>45.54 ± 2.48*</td>
<td>5.50 ± 0.32*</td>
</tr>
<tr>
<td>V</td>
<td>Chloroform fraction</td>
<td>500 mg/kg (p.o.)</td>
<td>111.21 ± 3.23*</td>
<td>73.57 ± 3.02*</td>
<td>39.66 ± 2.73*</td>
<td>5.92 ± 0.34*</td>
</tr>
<tr>
<td>VI</td>
<td>Methanol fraction</td>
<td>500 mg/kg (p.o.)</td>
<td>82.44 ± 3.52**</td>
<td>57.49 ± 3.37**</td>
<td>35.59 ± 2.34**</td>
<td>6.03 ± 0.28**</td>
</tr>
</tbody>
</table>

SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvate transaminase; ALKP, alkaline phosphatase; TP, total protein; s.c., subcutaneous; p.o., per oral.

**P < 0.01; *P < 0.05 vs CCl₄; P > 0.05 ns.**

Values are mean ± S.E. of five animals. One way analysis and Dunnett's test.
Graph-7: SGOT values after treatment with different fractions of *Marrubium vulgare* against toxic control.

Graph-8: SGPT values after treatment with different fractions of *Marrubium vulgare* against toxic control.
Graph-9: ALKP values after treatment with different fractions of *Marrubium vulgare* against toxic control.

Graph-10: Total Protein values after treatment with different fractions of *Marrubium vulgare* against toxic control.
Table No. 23: Effect of methanol fraction of *Berberis pachycantha* rhizomes on serum enzymatic activity in CCl₄ induced liver damage in rats.

<table>
<thead>
<tr>
<th>Groups (n=5)</th>
<th>Treatment</th>
<th>Dose</th>
<th>SGOT units/ml</th>
<th>SGPT units/ml</th>
<th>ALKP units/ml</th>
<th>TP gm/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal (control)</td>
<td>---</td>
<td>36.47 ± 1.45</td>
<td>25.52 ± 0.70</td>
<td>27.58 ± 0.56</td>
<td>7.36 ± 0.16</td>
</tr>
<tr>
<td>II</td>
<td>Toxic (control)</td>
<td>1 mg/kg (s.c.)</td>
<td>119.19 ± 4.05</td>
<td>94.70 ± 4.03</td>
<td>55.08 ± 2.39</td>
<td>4.49 ± 0.16</td>
</tr>
<tr>
<td>III</td>
<td>Silymarin (standard drug)</td>
<td>10 mg/kg (p.o.)</td>
<td>55.97 ± 1.61**</td>
<td>41.70 ± 2.90**</td>
<td>35.32 ± 1.05**</td>
<td>7.15 ± 0.12**</td>
</tr>
<tr>
<td>IV</td>
<td>Methanol extract</td>
<td>500 mg/kg (p.o.)</td>
<td>91.32 ± 2.30**</td>
<td>71.91 ± 3.37**</td>
<td>35.59 ± 2.34**</td>
<td>6.03 ± 0.28**</td>
</tr>
<tr>
<td>V</td>
<td>Compound (BP – 3)</td>
<td>50 mg/kg (p.o.)</td>
<td>69.13 ± 3.74**</td>
<td>55.23 ± 3.05**</td>
<td>41.53 ± 2.35**</td>
<td>6.83 ± 0.12**</td>
</tr>
</tbody>
</table>

SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvate transaminase; ALKP, alkaline phosphatase; TP, total protein; s.c., subcutaneous; p.o., per oral.

** P < 0.01; *P < 0.05 vs CCl₄; P > 0.05 ns.

Values are mean ± S.E. of five animals. One way analysis and Dunnett’s test.
Graph-11: SGOT values after treatment with methanol fraction of *Berberis pachycantha* and compound BP-3 against toxic control.

Graph-12: SGPT values after treatment with methanol fractions of *Berberis pachycantha* and compound BP-3 against toxic control.
Graph-13: ALKP values after treatment with methanol fraction of *Berberis pachycantha* and compound BP-3 against toxic control.

Graph-14: Total Protein values after treatment with methanol fraction of *Berberis pachycantha* and compound BP-3 against toxic control group.
5.7. HISTOPATHOLOGY

Plate No. I. Liver of normal control rat on 8th day. PT = Portal Triad and CV = Central vein. Low power (HE x 10X).

Plate No. Ia. Liver of normal control rat on 8th day. High power (HE x 40X)
Plate No. II. Low power photomicrograph of toxic control rat liver on 8\textsuperscript{th} day (HE x 10X).

Plate No. IIa. High power photomicrograph of toxic control rat liver on 8\textsuperscript{th} day (HE x 40X).
Plate No. III. Low power photomicrograph of standard control treated rat liver on 8<sup>th</sup> day (HE x 10X).

Plate No. IIIa. High power photomicrograph of Standard drug treated rat's liver on 8<sup>th</sup> day (HE x 40X).
Plate No. IV. Low power photomicrograph of *Lychnis coronaria* petroleum ether fraction treated rat liver on 8th day (HE x 10X).

Plate No. IVa. High power photomicrograph of *Lychnis coronaria* petroleum ether fraction treated rat liver on 8th day (HE x 40X).
Plate No. V. Low power photomicrograph of *Lychnis coronaria* chloroform fraction treated rat liver on 8\textsuperscript{th} day (HE x 10X).

Plate No. Va. High power photomicrograph of *Lychnis coronaria* chloroform fraction treated rat liver on 8\textsuperscript{th} day (HE x 40X).
Plate No. VI. Low power photomicrograph of *Lychnis coronaria* methanol fraction treated rat’s liver on 8th day (HE x 10X).

Plate No. VIa. High power photomicrograph of *Lychnis coronaria* methanol fraction treated rat’s liver on 8th day (HE x 40X).
Plate No. VII Low power photomicrograph of *Marrubium vulgare* petroleum ether fraction treated rat liver on 8\(^{th}\) day (HE x 10X).

Plate No. VIIa. High power photomicrograph of *Marrubium vulgare* petroleum ether fraction treated rat liver on 8\(^{th}\) day (HE x 40X).
Plate No. VIII. Low power photomicrograph of *Marrubium vulgare* chloroform fraction treated rat liver on 8\(^{th}\) day (HE x 10X).

Plate No. VIIIa. High power photomicrograph of *Marrubium vulgare* chloroform fraction treated rat liver on 8\(^{th}\) day (HE x 40X).
Plate No. IX. Low power photomicrograph of *Marrubium vulgare* methanol fraction treated rat liver on 8\textsuperscript{th} day (HE x 10X).

Plate No. IXa. High power photomicrograph of *Marrubium vulgare* methanol fraction treated rat liver on 8\textsuperscript{th} day (HE x 40X).
Graph 1: Biochemical parameters of *Lychnis coronaria* L..

Graph 2: Biochemical parameters of *Berberis pachycantha* L.

Graph 3: Biochemical parameters of *Berberis pachycantha* Koehne.
Conclusion

The above mentioned biochemical and histopathological results of these plants revealed that different plant fractions possessed varied degree of antihepatotoxic activity. The methanolic fractions from the three plants were found to possess significant antihepatotoxic activity as compared to their petroleum ether and chloroform extracts. We have also tested the antihepatotoxic activity of pure chemical compound BP-3, which has been characterized as 7-methyl-1-methylene-9,10-dimethoxy-5,6,7-trihydroisoquinolino [3,2-a] isoquinoline-3-ol, isolated from rhizomes of Berberis pachycantha. It showed significant antihepatotoxic potential against CCl₄ as compared to standard silymarin.

Further, the extracts as well as the isolated compound can be studied for their toxic effects, if they were found safe (as in our study devoid of mortality and no behavioral changes in the experimental animals), they may be used for formulating some preparations for the treatment of different diseases of liver. The compound BP-3, an isoquinoline, besides showing potent antihepatotoxic activity has been reported for the first time and has been designated as pachycanthine. The compound may also be chemically modified so as to enhance its pharmacological activity and suppressed toxicological effects (if any). Also compound MV-3 has been reported for the first time and has been characterized as p-Menthane-5,6-dihydroxy-3-carboxylic acid.