MATERIALS AND METHODS

4.1 Sources of chemicals and drugs

All solvents, chemicals, solutions and reagents used in the study were of analytical grade.

**Table 4.1:** List of chemicals and drugs

<table>
<thead>
<tr>
<th>Chemical and drugs</th>
<th>Name of company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid (pure drug)</td>
<td>Ranbaxy Laboratories Limited, New Delhi</td>
</tr>
<tr>
<td>Rifampicin (pure drug)</td>
<td>Lupin Drug Laboratory Limited , Pune</td>
</tr>
<tr>
<td>Silymarin (pure drug)</td>
<td>Sigma Aldrich Chemicals Pvt. Ltd.</td>
</tr>
<tr>
<td>Petroleum ether (60°-80°C)</td>
<td>SD Fine Chemicals Pvt. Ltd., Mumbai.</td>
</tr>
<tr>
<td>Methanol</td>
<td>SD Fine Chemicals Pvt. Ltd., Mumbai.</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Merck Ltd., Mumbai.</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>Natural Remedies, Bangalore.</td>
</tr>
<tr>
<td>Rutin</td>
<td>Natural Remedies, Bangalore.</td>
</tr>
<tr>
<td>Ammonia</td>
<td>Merck Ltd., Mumbai.</td>
</tr>
<tr>
<td>Acetone</td>
<td>Merck Ltd., Mumbai.</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>Merck Ltd., Mumbai.</td>
</tr>
<tr>
<td>Formic acid</td>
<td>S.D. fine Chem. Ltd., Mumbai.</td>
</tr>
<tr>
<td>Toluene</td>
<td>S.D. fine Chem. Ltd., Mumbai.</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Merck Ltd., Mumbai.</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>Merck India Ltd., Mumbai.</td>
</tr>
</tbody>
</table>
Table 4.2: List of equipments

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double beam UV spectrophotometer 160A</td>
<td>Shimadzu, Japan.</td>
</tr>
<tr>
<td>Melting point apparatus</td>
<td>Remi equipments, India.</td>
</tr>
<tr>
<td>Electronic balance</td>
<td>Sartorius, India.</td>
</tr>
<tr>
<td>Micropipettes</td>
<td>Tarsons, India.</td>
</tr>
<tr>
<td>Incubator</td>
<td>Scientific laboratory, India.</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Remi equipments, Delhi.</td>
</tr>
<tr>
<td>Deep freezer</td>
<td>Westfrost, India.</td>
</tr>
</tbody>
</table>

4.2 Apparatus, Chemicals and Instruments

- Ultraviolet spectra of extracts and isolated compounds were recorded on Spectrophotometer (160A UV-Vis, Shimadzu, Japan) in methanol carried out Faculty of Pharmacy Integral University, Lucknow.

- Infrared spectra were recorded on Bio-Red FTIR Spectrophotometer using KBr pellets; $\nu_{\text{max}}$ values are given in cm$^{-1}$. It was carried out at Sophisticated Analytical Instrument Facility CSIR- Central Drug Research Institute (CDRI), Lucknow.

- $^1$H NMR spectra were screened on Bruker spectrospin 400 MHz instrument using CDCl$_3$ as solvent and TMS as an internal standard. Chemical shift values are presented in ppm scale and coupling constants ($J$) in Hz. Notations used throughout as $s$ = singlet, $dd$ = double-doublet, $t$ = triplet, $m$ = multiplet, and brs = unresolved broad singlet. It was carried out at Sophisticated Analytical Instrument Facility CSIR-Central Drug Research Institute, Lucknow.
- $^{13}$C NMR spectra were recorded on Bruker Spectrospin 400 MHz in 5 mm spinning tubes at 27 °C at Sophisticated Analytical Instrument Facility CSIR-Central Drug Research Institute (CDRI), Lucknow.

- Mass spectra were scanned by effecting FAB ionization at 70 eV on a JEOL-JMS-DX 303 instruments equipped with direct inlet probe system. The $m/z$ values of the more intense peaks were mentioned and the figures in parenthesis to each $m/z$ value indicate relative intensities with respect to the base peak at Sophisticated Analytical Instrument Facility CSIR-Central Drug Research Institute (CDRI), Lucknow.

- Silica gel (Merck), 60-120 mesh for column packing; silica gels G (Merck) for TLC spots were visualized by exposure to iodine vapours and UV radiation.

4.3 Collection and identification of plant materials

The fresh leaves of Adenanthera pavonina (A. pavonina) and Erythrina indica (E. indica) were collected from Pallavaram, Chennai, Tamil Nadu in the month of June 2010 and 2011 respectively. The plant specimen was authenticated by National Institute of Herbal Science, Plant Anatomy Research Center, Chennai, Tamil Nadu (References no. PARC / 2011/ 954 & 955).

4.4 Preparation of extracts

Freshly collected leaves of A. pavonina and E.indica were washed with distilled water to remove dirt and soil and dried under shade in a ventilated room at temperature. Coarsely powdered drug (500 g) was packed in muslin cloth and subjected to soxhlet extraction for continuous hot extraction with methanol for 72 h at 50°C. Methanolic extracts of A. pavonina and E.indica
were filtered through Whatman paper no. 42 and filtrates were concentrated under reduced pressure and at temperature below 40 °C. For animal studies the extracts was prepared in methanol (50%) by cold percolation method at room temperature. The yield of methanolic extract of A. pavonina and E.indica was 11.2 % and 12.5% respectively. Extracts were stored in freezer and was used for phytochemical and pharmacological studies.

4.5 Preparation of Column chromatography

4.5.1 Preparation of slurry
Methanolic extracts of each plant was concentrated on the water bath and dried under reduced pressure to get green brown mass extracts and 115 g (A. pavonina) and 80 g (E. indica) extracts was used for the slurry. The concentrated extract of the drug was taken in a China dish and heated continuously on a water bath by gradually adding methanol in small portions with constant stirring, till desired consistency was obtained. A weighed quantity of silica gel for column chromatography was then added slowly with continuous mixing with a steel spatula until the whole methanolic solution of plant extract adsorbed on silica gel particles. It was dried in the air; the larger lumps were broken by rubbing between hands and finally passed through a sieve (No. 8) to get uniform particle size.

4.5.2 Packing of column & Isolation of phytoconstituents
A column of 3.0 feet, height and 16 mm internal diameter was taken, cleaned properly and dried. The lower end of the column was plugged with non-absorbent cotton wool. The column was clamped and fitted in a vertical position on a stand. The column was then half filled with petroleum ether (b.p.
60-80 °C). Silica gel (for column, 60-120 mesh) was then poured in small portions and allowed to settle down and the dried plant extract slurry was loaded over the column and then eluted successively with different solvents, in their order of increasing polarity. The developments and elution of the column were carried out with successive series of different solvents in various combinations, such as petroleum ether (100), petroleum ether: chloroform (75:25, 50:50, 25:75,), chloroform (100), chloroform: methanol (99:1, 98:2, 97:3, 95:5, 90:10, 80:20) and methanol to isolate the compounds:

### 4.5.3 Homogeneity of the fractions
The fractions collected were subjected to thin layer chromatography (TLC) to check homogeneity of various fractions. Chromatographically identical fractions (having same Rf values) were combined together and concentrated. Isolated compounds were crystallized with suitable solvent system.

### 4.6 Preliminary phytochemical screening
The preliminary phytochemical screening was carried out using different plant extracts for content of different classes of compounds. The extract obtained was subjected to qualitative chemical tests for identification of various constituents present in the crude drug. The extract was subjected to preliminary phytochemical investigation for detection of following [1-3]
Table 4.3: Chemical test for alkaloids

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Experiments</th>
<th>Inference</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sample with Dragendorff’s reagent</td>
<td>Formation of orange yellow precipitate.</td>
<td>Presence of alkaloids</td>
</tr>
<tr>
<td>2.</td>
<td>Sample with Hager’s reagent</td>
<td>Formation of yellow precipitate.</td>
<td>Presence of alkaloids</td>
</tr>
<tr>
<td>3.</td>
<td>Sample with Mayer’s reagent</td>
<td>Formation of white or cream color precipitate.</td>
<td>Presence of alkaloids</td>
</tr>
</tbody>
</table>

Table 4.4: Chemical test for Carbohydrates

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Experiments</th>
<th>Inference</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sample with Molisch reagent</td>
<td>Formation of violet colored ring at the interface.</td>
<td>Presence of Carbohydrates</td>
</tr>
<tr>
<td>2.</td>
<td>Sample with Fehling reagent</td>
<td>Formation of brick-red colored ppt.</td>
<td>Presence of reducing sugar</td>
</tr>
</tbody>
</table>

Table 4.5: Chemical test for glycosides

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Experiments</th>
<th>Inference</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sample with Killer-Killani reagent</td>
<td>Formation of reddish brown color at the junction of two layers and the upper layer turned bluish green.</td>
<td>Presence of glycoside</td>
</tr>
<tr>
<td>2.</td>
<td>Sample with Borntrager’s reagent</td>
<td>Formation of reddish pink color.</td>
<td>Presence of glycosides</td>
</tr>
<tr>
<td>3.</td>
<td>Sample with Legal reagent</td>
<td>Formation of blue color.</td>
<td>Presence of glycosides</td>
</tr>
<tr>
<td>4.</td>
<td>Sample with sodium picrate reagent</td>
<td>Formation of orange and yellow color.</td>
<td>Presence of glycosides</td>
</tr>
</tbody>
</table>
Table 4.6: Chemical test for phenolic compounds

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Experiments</th>
<th>Inference</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sample with ferric chloride solution</td>
<td>Formation of green and blue color.</td>
<td>Presence of phenolic compounds</td>
</tr>
<tr>
<td>2.</td>
<td>Sample with lead acetate solution</td>
<td>Formation of ppt.</td>
<td>Presence of phenolic compounds</td>
</tr>
<tr>
<td>3.</td>
<td>Sample with Legal reagent</td>
<td>Formation of blue color.</td>
<td>Presence of phenolic compounds</td>
</tr>
<tr>
<td>4.</td>
<td>Sample with sodium picrate reagent</td>
<td>Formation of orange and yellow color.</td>
<td>Presence of phenolic compounds</td>
</tr>
</tbody>
</table>

Table 4.7: Chemical test for flavonoids

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Experiments</th>
<th>Inference</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Shinoda test</td>
<td>Formation of pink color.</td>
<td>Presence of flavonoids</td>
</tr>
</tbody>
</table>

Table 4.8: Chemical test for proteins

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Experiments</th>
<th>Inference</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Million’s test</td>
<td>Formation of red ppt.</td>
<td>Presence of proteins</td>
</tr>
<tr>
<td>2.</td>
<td>Xanthoprotein test</td>
<td>Formation of yellow color.</td>
<td>Presence of proteins</td>
</tr>
<tr>
<td>3.</td>
<td>Biuret test</td>
<td>Formation red and violet color.</td>
<td>Presence of proteins</td>
</tr>
</tbody>
</table>

Table 4.9: Chemical test for sterols

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Experiments</th>
<th>Inference</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Salkowski reaction</td>
<td>Formation of yellow ring at junction which turns red after one minute.</td>
<td>Presence of sterols</td>
</tr>
<tr>
<td>2.</td>
<td>Libermann reaction</td>
<td>Formation of blue color.</td>
<td>Presence of sterols</td>
</tr>
</tbody>
</table>
Table 4.10: Chemical test for saponin

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Experiments</th>
<th>Inference</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Foam test</td>
<td>Formation of honeycomb like frothing</td>
<td>Presence of saponin</td>
</tr>
</tbody>
</table>

Table 4.11: Chemical test for terpenoids

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Experiments</th>
<th>Inference</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sample with Noller’s reagent</td>
<td>Formation of pink color.</td>
<td>Presence of terpenoids</td>
</tr>
</tbody>
</table>

Table 4.12: Chemical test for resins

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Experiments</th>
<th>Inference</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sample with acetone and HCl heated.</td>
<td>Formation of pink color.</td>
<td>Presence of resin</td>
</tr>
</tbody>
</table>

4.7 Ash value

Ash value was determined by the method described by Choudhry, (1996). The ash remaining following ignition at 450°C of crude drugs was determined by three different methods, which measure total ash, acid-insoluble ash and water-soluble ash [4].

4.7.1 Total ash

1. Indian Pharmacopoeia 1996 and WHO prescribes methods for determination of ash values.

2. About 2-3 gm of air dried crude drug was placed in the tarred silica crucible and was incinerated at a temperature not exceeding 450 °C until free from carbon, cooled and weighed to get the total ash content.
4.7.2 Acid insoluble ash

1. Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth.
2. Ash was boiled with 25 ml of hydrochloric acid for 5 minutes. The insoluble matter was collected on ash less filter paper, washed with hot water and ignited at a temperature not exceeding 450°C to a constant weight.

4.7.3 Water soluble ash

Water soluble ash is the difference in weight between the total ash and the residue after treatment of total ash with water. It is a good indicator of either previous extraction of water-soluble salts in the drug or in corrected preparation. Ash was dissolved in distilled water and the insoluble part collected on an ash less filter paper and was ignited at 450°C to a constant weight. By, subtracting the weight of insoluble part from that of ash, the weight of the soluble part of ash was obtained.

4.8 Extractive values

According to Indian Pharmacopoeia 1996, British Pharmacopoeia 1980 and WHO guideline the determination of water soluble and alcohol soluble extractives is used as a means of evaluating crude drugs which are not readily estimated by other means. The extraction of any crude drug with particular solvent yields a solution containing different phytoconstituents that is such extractive value provides an indication of the extent of polar, medium polar and non polar components present in the plant material [4-5].
4.9 Loss on drying

This parameter determines the amount of moisture as well as volatile components present in a particular sample. The powdered drug sample (10 gm) was placed on a tarred evaporating dish and dried at 105 °C for 6 hours and weighed. The drying was continued until two successive reading matches each other or the difference between two successive weighing was not more than 0.25% of constant weight [6].

4.10. In-vitro antioxidant property of plant extract.

4.10.1 Estimation of total phenols

The total phenolic content was determined according to the method described by Singleton [7]. A suitable aliquot of the methanolic extract of *E. indica* and *A. pavonina* was placed in test tubes and made up to 1 ml with distilled water.

**Reagents** - Folin-Ciocalteu (F.C) 10 % reagent in distilled water, Na$_2$CO$_3$ (1M) in distilled water and Gallic acid (Standard) 1mg/ml solution in methanol was prepared. Different dilutions of gallic acid (5 μg/ml to 75μg/ml) were made in methanol.

**Samples preparation** – Prepared 10 mg/ml solution of drug in methanol followed by addition of 5 ml F.C. reagent and 4 ml Na$_2$CO$_3$ solution. Absorbance was taken at 725 nm after 40 minutes.

**Preparation of standard**- 0.5 ml of each standard dilution was taken and added ml F.C. reagent and 4 ml Na$_2$CO$_3$ solution. Absorbance was taken at 725 nm after 40 minutes.

**Blank solution**– Methanol (0.5 ml) and 5 ml F.C. reagent taken and 4 ml Na$_2$CO$_3$ solution was added.
Taking the absorbance of standard dilutions as mentioned above, calibration curve was plotted (Fig. 4.1). Phenolic contents in extract were calculated by using standard calibration. Total phenolic content in the methanolic extract of *A. pavonina* and *E. indica* were 55.43 ±1.07µg/mL and 53.44±7.1µg/mL respectively.

(Fig. 4.1) Calibration curve of gallic acid for total phenolic content

**Table 4.13:** Content of total phenolic in methanolic extract of *A. pavonina.*

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Absorbance</th>
<th>Intercept</th>
<th>Slope</th>
<th>Conc. (µg/mL)</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.688</td>
<td>0.128</td>
<td>0.0098</td>
<td>57.2</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>0.672</td>
<td>0.128</td>
<td>0.0098</td>
<td>55.6</td>
<td>55.43 ±1.07</td>
</tr>
<tr>
<td>3.</td>
<td>0.652</td>
<td>0.128</td>
<td>0.0098</td>
<td>53.5</td>
<td></td>
</tr>
</tbody>
</table>
**Table 4.14:** Content of total phenolic in methanolic extract of *E.indica.*

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Absorbance</th>
<th>Intercept</th>
<th>Slope</th>
<th>Conc. (µg/mL)</th>
<th>Mean ± SEM (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.783</td>
<td>0.128</td>
<td>0.0098</td>
<td>66.90</td>
<td>53.44±7.1</td>
</tr>
<tr>
<td>2.</td>
<td>0.620</td>
<td>0.128</td>
<td>0.0098</td>
<td>50.21</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>0.551</td>
<td>0.128</td>
<td>0.0098</td>
<td>43.23</td>
<td></td>
</tr>
</tbody>
</table>

**4.10.2 Estimation of total flavonoids**

Total flavonoids content was estimated by aluminium chloride colorimetric method [8]. From the methanolic extract, concentration of stock solution of 1mg/ml was prepared. Then 0.5 ml of the stock solution (1mg/ml) of extract was taken in 1.5 ml of methanol and 0.1 ml of potassium acetate (1M) was added to reaction test tubes and volume was made up 5ml with distilled water. It was incubated at room temperature for 30 min with intermittent shaking and absorbance was measured at 514 nm. The calibration curve was prepared by using rutin solutions as standard at concentrations of 10-125 µg/ml in methanol.

**Preparation of Standard**– 0.5 ml of standard dilution of rutin was taken and 1.5 ml methanol added. To this added 0.1 ml of aluminium chloride (AlCl₃) and 0.1 ml of sodium acetate (CH₃COONa) reagents were mixed followed by 2.8 ml distilled water and kept for 30 minutes after that absorbance was taken at 514 nm.

The concentration of total flavonoids in the extract was obtained by extrapolating the absorbance of rutin through graph. The total flavonoid content was expressed as rutin equivalent to µg/mL extracts.
**Figure 4.2:** Calibration curve of rutin for total flavonoid content

### Table 4.15: Total flavonoids content in methanolic extract of *A. pavonina*

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Absorbance</th>
<th>Intercept</th>
<th>Slope</th>
<th>Conc. (µg/ml)</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.255</td>
<td>0.055</td>
<td>0.0058</td>
<td>53.45</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.267</td>
<td>0.055</td>
<td>0.0058</td>
<td>55.67</td>
<td>52.87±1.8</td>
</tr>
<tr>
<td>3</td>
<td>0.232</td>
<td>0.055</td>
<td>0.0058</td>
<td>49.50</td>
<td></td>
</tr>
</tbody>
</table>

### Table 4.16: Total flavonoids content in methanolic extract of *E. indica*

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Absorbance</th>
<th>Intercept</th>
<th>Slope</th>
<th>Conc. (µg/ml)</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.347</td>
<td>0.055</td>
<td>0.0058</td>
<td>69.39</td>
<td>51.10±9.4</td>
</tr>
<tr>
<td>2</td>
<td>0.208</td>
<td>0.055</td>
<td>0.0058</td>
<td>45.50</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.169</td>
<td>0.055</td>
<td>0.0058</td>
<td>38.70</td>
<td></td>
</tr>
</tbody>
</table>

### 4.10.3 Assay for DPPH radical scavenging capacity

The assay was performed according to the method of Lim [9]. The radical scavenging activities of the leaves extract of *A. pavonina* and *E. indica*...
against 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) were determined by UV spectrophotometer at 517 nm. An aliquot of (0.05, 0.1, 0.5, 1.0, 1.0 mg/ml) of extract was mixed in a test tube containing 3 ml of methanol and 0.5 ml of 1 mM DPPH. Ascorbic acid was used as the standard at same concentrations of 0.05 - 1.50 mg/ml. A blank solution (control) was prepared containing the same amount of methanol and DPPH. The reaction mixture was incubated at 37 °C for 30 min. The radical scavenging activity was calculated using the following equation:

\[
\% \text{ Scavenging} = \frac{[\text{Abs. control} - \text{Abs. Sample}]}{\text{Abs. Control}} \times 100
\]

### 4.10.4 Nitric oxide anion scavenging activity.

The procedure was based on the principle that sodium nitroprusside solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitric ion that was estimated by using Geiss reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthylenediamine dihydrochloride) [10]. Scavenger of nitric oxide competes with oxygen leading to reduce production of nitric ion. For the experiment, an aliquot (1ml) of different concentrations (0.05-1.5 mg/ml) of methanolic extracts of *A. pavonina* and *E. indica* leaves were dissolved in phosphate buffer solution (PBS) and added 1ml of sodium nitroprusside (10 mM) and incubated at room temperature for 150 min. The reaction without the extracts sample but equivalent amount of methanol served as control. After incubation period, 0.5ml of Greiss was added. Same concentration of ascorbic acid was used for same experiment. The absorbance was determined by UV spectrophotometer at 546 nm. The radical scavenging activity was calculated using the following equation:

\[
\% \text{ Scavenging} = \left[ \frac{\text{Absorbance Control} - \text{Absorbance Sample}}{\text{Absorbance Control}} \right] \times 100
\]
4.10.5 Reducing power

The total reducing power of the extract was measured according to Oyaizu method (1986). Extract (10, 25, 50 and 100 mg/ml) in 1 ml of distilled water was mixed with 2.5 ml phosphate buffer (0.2 mM, pH 6.6) and 1% of 2.5 ml potassium ferricyanide. The mixture was incubated at 55°C for 25 min. Subsequently 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 3000 rpm. The upper layer of the solution (1.5 ml) was mixed with 1.5 ml distilled water and ferric chloride (0.3 ml, 0.1%) and the absorbance were measured at 680 nm using UV spectrophotometer. Elevated absorbance of the reaction mixture indicated better reducing power [11].

4.11 Pharmacological studies.

4.11.1 Animals.

Male Sprague-Dawley (SD) rats (150-200 g) and Swiss albino mice (25-30 g) were procured from the National Laboratory Animal Centre (NLAC), Central Drug Research Institute (CDRI) Lucknow, India. The animals were housed separately in polypropylene cage at temperature of 25 ± 2°C and 50-60% relative humidity, 12 h light/dark cycle respectively, for one week before and during the commencement of experiment. Animals were allowed to access standard rodent pellet feed (Dayal Industries Limited, Barabanki, U.P) and drinking water. Food was withdrawn 18-24 hours before the experiment, though water was allowed *ad libitum* and allocated to different experimental groups. All experimental procedures involving animals were conducted in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The study protocols were
Chapter 4  

Materials and methods

approved by Institutional Animal Ethics Committee (IAEC) of Integral University, Faculty of Pharmacy, Lucknow, India (Reg. No. IU/Pharm/Ph.D/CPCSEA/IU/12/04).

4.11.2 Acute toxicity study.

Acute toxicity study of the 50% methanolic extract of *Adenanthera pavonina* and *Erythrina indica* were estimated in Swiss albino mice (25-30 g) according to OECD guidelines (Organization for Economic Co-operation and development, guideline, No. 423, 2001 [12] . Drug was administered orally in mice. Twenty four animal were divided equally into four groups (n=6) as per protocol for each plant extract. The extract was administered in 1% carboxy methyl cellulose (CMC) at doses of 50, 300 and 2000 mg/kg body weight while the control received the CMC (1%) suspension only. Food and water withheld for 1-2 h after drug administration. Mice were observed for the initial 4 h after the administration of drugs, after that once daily during the following days. The behavioural changes observed for hyperactivity, ataxia tremors convulsion salivation, diarrhoea, sleep, and coma. The total observation period for eventually mortality was 14 days. No mortality was observed up 2000mg/kg. One tenth and one twentieth of the maximum tolerated dose (2000mg/kg) of both plants extracts was selected for study. Therefore biological study was carried out at doses of 100mg and 200mg/kg.

4.11.3 Isoniazid and Rifampicin induced hepatotoxicity

Rifampicin (R) and Isoniazid (I) ((50 mg/kg body wt. each, p.o) suspension were prepared separately in carboxy methyl cellulose (CMC) (1%). Rats were treated with isoniazid (I), co-administered with rifampicin (R) for 28 days orally to produce hepatotoxicity [13].
4.11.4 Treatment protocol design.

The Male Sprague-Dawley (SD) rats were divided into five groups (for each plant). Each group of six animal (n=6) for the study duration 28 days. Group I served as a normal control and treated as 1% CMC only with distilled water. Groups II, III, IV and V were treated with isoniazid (I) and rifampicin (R) (50mg/kg each). After administration of (I+R), test groups group III and IV were administered orally 100mg and 200mg/kg body wt. with methanolic extract of A. pavonina (MEAP)/methanolic extract of E.indica (MEEI) respectively in the form of aqueous suspension. Group V served as standard control and received silymarin (100mg/kg body wt.).

- Group I: Animals served as normal control for 28 days.
- Group II: Animals were treated with selected dose of anti-tubercular drugs R+I (50 mg/kg body wt. each, p.o) for 28 Days.
- Group III: Animals were treated with (R+I) + MEAP/MEEI (100 mg/kg for 28 days.
- Group IV: Animals were treated with (R+I) + MEAP/MEEI (200 mg/kg) for 28 days.
- Groups V: Animals were treated with (R+I) + standard drug Silymarin (100 mg/kg) for 28 days.

All the treatments were given orally in CMC (1%) in distilled water by means of orogastric cannula for 28 days.

4.11.5 Biochemical determinations

Twenty four hours after administration of the last dose of the treatment schedule with drugs and extracts. Isolation of liver for enzyme estimation and histopathology was carried out by under ether anesthesia. Whole blood was
withdrawn from the rats by sino retro-orbital puncture after an overnight fast with light anesthesia. The blood was allowed to coagulate at room temperature for 30 min and then centrifuged at 2000 rpm for 15 min for separation of serum. The serum was further used for estimating the biochemical parameters viz. aspartate transaminase (AST/SGOT), alanine transaminase (ALT/SGPT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), albumin (ALB), total protein (TP), serum bilirubin (SBL) and cholesterol (CHL) were assayed with standard kits methods. The supernatant of the liver homogenate was subjected for the estimation of enzymes anti-oxidants like catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) by a colorimetric method for determination of total antioxidant activity.

4.12 In-vivo antioxidant activity.

4.12.1 Tissue reduced glutathione (GSH)

Principle
This spectrophotometric procedure was based on the method of Ellman[14]. DTNB, 5, 5'-dithiobis-(2-nitrobenzoic acid), is reduced by –SH groups to form one mole of 2-nitro-5- mercaptobenzoic acid per mole of –SH. The nitromercapto benzoic acid anion has an intense yellow color that is determined spectrophotometrically at 412 nm.

Preparation of the Reagents
1. EDTA (0.2 M): 22.3 gm of EDTA was dissolved in 300 ml of warm double distilled water.
2. EDTA (0.02 M): 20 ml of the above solution was diluted to 200 ml with double distilled water.
3. DTNB (0.01M): 99 mg of DTNB was dissolved in 25 ml of absolute methanol.

4. Tris buffer (0.4M, pH 8.9): 24.2 of tris buffer was dissolved in 100 ml of double distilled water, 50 ml 0.2 M EDTA was added to it and the volume of the solution was made up to 500 ml with double distilled water. The pH of the solution was adjusted to 8.9 with 6 N HCl.

5. TCA (50%): 50 gm of TCA was dissolved in 100 ml of double distilled water.

Procedure

A known weight of liver tissue ranging from (300-600 mg) was homogenized in 5-8 ml of 0.02 M EDTA and then 4.0 ml of cold distilled water was added to it. After mixing it well, 1 ml of 50 % trichloroacetic acid (TCA) was added and shaken intermittently for 10 minutes using a vortex mixer. After 10 minutes the contents was transferred to centrifuge tubes (rinsed in EDTA) and centrifuged at 6000 rpm for 15 minutes. Following centrifugation, 2 ml of the supernatant was mixed with 4.0 ml of 0.4 M tris buffer (pH 8.9). The whole solution was mixed well and 0.1 ml of 0.01M DTNB was added to it. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank.

Blank: The method was same as for the test except that 0.02 M EDTA was added in place of tissue homogenate.

Calculation

GSH (tissue) was calculated from the following equation and expressed as µg/mg of protein.

\[
GSH (\mu g/mg \text{ of protein}) = \frac{\text{O.D. at 412 nm} \times 50 \times 3.5 \times 2.25 \times 1}{0.337 \times 2 \times \text{mg of protein}}
\]
4.12.2 Catalase)

Principle
In the UV range $\text{H}_2\text{O}_2$ shows a continuous increase in absorption with decreasing wavelength. The decomposition of $\text{H}_2\text{O}_2$ can be followed directly by the decrease in absorbance at 240 nm. The difference in absorbance ($\Delta A$) per unit time is a measure of the catalase activity [15].

Preparation of working reagents:
1. Potassium dihydrogen phosphate ($\text{KH}_2\text{PO}_4\cdot2\text{H}_2\text{O}$): 6.81 gm of $\text{KH}_2\text{PO}_4$ was dissolved in double distilled water and volume was made up to 1000 ml with double distilled water.
2. Disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4\cdot2\text{H}_2\text{O}$): 8.9 gm of $\text{Na}_2\text{PO}_4\cdot2\text{H}_2\text{O}$ was dissolved in double distilled water and volume was made up to 1000 ml with double distilled water.
3. Potassium phosphate buffer (50 mM; pH 7.4): It was prepared by mixing the above solution (i) and (ii) in the ratio of 1:1.55
4. Hydrogen peroxide 30% (19 mM/L): 187µl of 30% $\text{H}_2\text{O}_2$ was dissolved in 100 ml phosphate buffer.

Procedure
Liver tissue was homogenized in 50 mM/L potassium phosphate buffer with a ratio of 1:10 w/v. The homogenate was centrifuged at 10,000 rpm at 4$^\circ$ C in a cooling centrifuge for 20 minutes. Catalase activity was measured in supernatant obtained after centrifugation. Supernatant (50 µl) was added to cuvette containing 2.95 ml of 19 mM/L solution of $\text{H}_2\text{O}_2$ prepared in potassium phosphate buffer. The change in absorbance was monitored at 240 nm
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wavelength at 1-minute interval for 3 minutes. Presence of catalase decomposes H₂O₂ leading to a decrease in absorbance.

Calculation
Catalase activity was calculated as
\[ \eta \text{ moles of } H_2O_2 \text{ consumed/minute/mg protein} = \frac{\Delta A/\text{minute} \times \text{volume of assay}}{0.081 \times \text{volume of homogenate} \times \text{mg of protein}} \]

4.12.3 Superoxide Dismutase (SOD)

Principle
Pyrogallol auto-oxidizes rapidly in aqueous solution; higher the pH faster is auto-oxidation and several intermediate products are formed. Thus the solution first becomes yellow-brown with a spectrum showing should between 400-425 nm. After a number of minutes the color begins to turn green and finally after a few hours, a yellow color appears. So the auto-oxidation is studied essentially during the first step and the rate is taken from the linear increase in absorbance at 420 nm, which is seen for a number of minutes after an induction period of some 10 seconds. Super oxide anion radical (O₂⁻) catalyses the auto-oxidation of pyrogallol. A simple and rapid method for assay of SOD is described, based on the ability of the enzyme to inhibit the auto-oxidation of pyrogallol [16].

Preparation of reagents
1. Tris HCl buffer (pH 8.5):788 mg of Tris HCl buffer and 186 mg of EDTA were dissolved in 100 ml double distilled water and the and the pH was adjusted to 8.5 using 1 N NaOH.
2. Potassium di hydrogen phosphate (KH₂PO₄.2H₂O):6.81 gm of KH₂PO₄ was dissolved in double distilled water and volume was made up to 1000 ml with double distilled water.
3. Disodium hydrogen phosphate (Na$_2$HPO$_4$·2H$_2$O): 8.9 gm of Na$_2$PO$_4$·2H$_2$O was dissolved in double distilled water and volume was made up to 1000 ml with double distilled water.

4. Potassium phosphate buffer (50 mM; pH 7.4): It was prepared by mixing the above solution (i) and (ii) in the ratio of 1:1.55.

5. Pyrogallol (24 mM): 15.1 mg of pyrogallol was dissolved in 5 ml of 10 Mm HCl. The solution was prepared freshly at the time of assay.

**Procedure**

The supernatant was assayed for Superoxide Dismutase (SOD) activity by following the inhibition of pyrogallol autoxidation. 100 μl of cytosolic supernatant was added to tris HCl buffer (pH 8.5). The final volume of 3 ml was adjusted with the same buffer. At least 25 μl of pyrogallol was added and changes in absorbance at 420 nm are recorded at 1 minute interval for 3 minutes. The increase in absorbance at 420 nm after the addition of pyrogallol was inhibited by the presence of SOD.

**Calculation**

1 unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation per 3 ml of assay mixture and given by the formula

\[
\text{Unit of SOD per ml of sample} = \frac{(A - B) \times 100}{A \times 50}
\]

Where,

A is the difference of absorbance in 1 minute in control

B is the difference of absorbance in 1 minute in test sample.

Data was expressed as SOD units per mg of protein.
4.12.4 Estimation of Thio Bariatric Acid Reactive Substances (TBARS).

Principle

Lipid peroxidation is a free radical mediated event. The primary products of such damage are a complex mixture of peroxides which then breakdown to produce carbonyl compounds. The malondialdehyde (MDA) is one such carbonyl compound, which forms a characteristic chromogenic adduct with two molecules of TBA. The colorimetric reaction of TBA with MDA, a secondary product of lipid peroxidation has been widely accepted for measuring lipid peroxidation (LPO).

The dissected out liver samples were washed immediately with ice cold saline to remove as much blood as possible. Liver homogenized (5%) in ice cold 0.9% NaCl with a glass homogenizer. The homogenate was centrifuged at 800 for 10 min and the supernatant was again centrifuged at 12,000 for 15 min and the obtained mitochondrial fraction was used for the estimation Malondialdehyde [17]. A volume of the homogenate (0.2ml) was transferred to a vial and was mixed with 0.2 ml of a 8.1% (w/v) sodium dodecyl sulphate solution, 1.5 ml of a 20% acetic acid solution (adjusted to pH 3.5 with NaOH) and 1.5 ml of a 0.8% (w/v) solution of thiobarbituric acid (TBA) and the final volume was adjusted to 4.0 ml with distilled water.

Each vial was tightly capped and heated in a boiling water bath for 60 min. The vials were then cooled under running water. Equal volumes of tissue blank or test samples and 10% trichloroacetic acid were transferred into a centrifuge tube and centrifuged at 1000g for 10 min. The absorbance of the supernatant fraction was measured at 532 nm (Spectrophotometer, 160A UV-Vis, and Shimadzu, Japan). Control experiment was processed using the same experimental procedure except the TBA solution was replaced with distilled
Malondialdehyde (MDA) is an end product of lipid peroxidation, which reacts with thiobarbituric acid to form pink chromogen thiobarbituric acid reactive substance. 1, 1, 3, 3-tetraethoxypropan was used as standard for calibration of the curve and is expressed as n mole/mg protein.

4.13. Assessments of Liver Function Test (L.F.T):

Serum alanine transaminase (ALT), aspartate transaminase (AST), Alkaline Phosphate (ALP), Bilirubin, Total Cholesterol, Albumin (ALB), total Protein and lactate Dehydrogenase (LDH) was determined by using standard kits from Span diagnostic Ltd, Surat, India. Serum lactate dehydrogenase (LDH) was estimated by using standard kits from Accurex biomedical Pvt. Ltd, Mumbai, India. All estimation was carried out using UV spectrophotometer (Shimadzu, India) as per standard kit methods. The estimation procedure is obtained in detail from leaflets provided by the commercially available kits are as follows.

4.13.1 Alanine aminotransferase/ Alanine transaminase (ALT/SGPT) test kit

Modified UV (IFCC), kinetic assay, Span Diagnostic Ltd. (Liquid Gold), Surat.

Alanine aminotransferase (ALT) also known as glutamate pyruvate transaminase (GPT) is a transaminase. The highest levels are found in the liver and in kidneys, and in smaller amount in heart and skeletal muscle.

Assay Principle

Alanine transaminase (ALT) catalyses the transamination of L-Alanine and α-ketoglutarate to form Pyruvate and L-Glutamate. In subsequent reaction, Lactate Dehydrogenase (LD) reduces Pyruvate to Lactate with simultaneous oxidation of Nicotinamide Adenine Dinucleotide [reduced] (NADH) to
Nicotinamide Adenine Dinucleotide (NAD). The rate of oxidation of NADH is measured kinetically by monitoring the decrease in absorbance at 340 nm. LD rapidly and completely reduces the endogenous sample Pyruvate during the initial incubation period, so that it does not interfere with the assay.

\[ \text{L- Alanine} + \alpha \text{-Ketoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{L-Glutamate} \]

\[ \text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LD}} \text{Lactate} + \text{NAD} \]

**Working Reagent Preparation**

Added reagent 2 to reagent 1 in 1:4 ratio i.e., 1 mL of Reagent 2 + 4 mL of Reagent 1.

**Assay Parameters**

- **Mode**: Kinetic
- **Reaction direction**: Decreasing
- **Wavelength**: 340 nm
- **Flow- cell temperature**: 37°C
- **Optical path length**: 1 cm
- **Blanking**: Purified water
- **Sample volume**: 100 µL
- **Reagent volume**: 1000 µL
- **Delay**: 60 seconds
- **Interval**: 30 seconds
- **Number of reading (s)**: 4
- **Permissible Reagent Blank absorbance**: >1.0 AU
- **Kinetic factor**: 1768
- **Maximum ΔA/minute**: 0.26
- **Linearity**: 450 IU/L
- **Units**: IU/L

**Procedure**

- **Pipette into tube marked Test**
  - Serum/Plasma: 100 µL
  - Working ALT Reagent: 1000 µL
Mix well and aspirate immediately for measurement.
Programme the analyser as per assay parameters.
1. Blank the analyser with Purified Water.
2. Read absorbance after 60 seconds. Repeat readings after every 30 seconds i.e., upto 120 seconds at 340 nm wavelength.
3. Determine the mean absorbance change per minute (ΔA/minute).

**Calculation**
ALT Activity (IU/L) = ΔA/minute x kinetic factor

Where: ΔA/minute = change in absorbance per minute
Kinetic factor (K) = 1768

Kinetic factor is calculated by using following formula

\[ K = \frac{1}{M} \times \frac{TV}{SV} \times \frac{1}{P} \times 10^6 \]

M = Molar extinction coefficient of p-Nitrophenol and is equal to 1.8x10³ lit/mol/cm at 405 nm
TV = Sample volume + Working Reagent volume
SV = Sample volume
P = Optical path length
10⁶ = Constant

**Clinical Significance**
Serum AST and ALT levels are elevated in viral and other forms of liver diseases associated with Hepatic necrosis. ALT is more liver specific enzyme.

Increased activity: Serum activity of ALT is increased in liver diseases, in trauma or skeletal disease after Renal infract and in various haemolytic conditions. In Viral Hepatitis associated with necrosis the elevation would be 20 to 50 fold, peak values reaching between 7th and 12th day returns to normal levels in 3 to 5 weeks in uneventful recovery. In Alcoholic Hepatitis there is moderate elevation. In Viral Hepatitis ALT levels are increased even before...
appearance of Jaundice. Moderate increase may be seen in Cirrhosis, Extra hepatic Cholestaticis and Carcinoma of Liver.

4.13.2. Alkaline phosphatase test kit

pNPP-AMP (IFCC), Kinetic Assay, Span Diagnostic Ltd. (Liquid Gold), Surat.

Assay Principle

At pH 10.3, alkaline Phosphatase (ALP) catalyses the hydrolysis of colourless p-Nitrophenyl Phosphate (pNPP) to yellow coloured p-Nitrophenol and phosphate. Change in absorbance due to yellow colour formation is measured kinetically at 405 nm and is proportional to ALP activity in the Sample.

\[
p-\text{Nitrophenyl Phosphate} + \text{H}_2\text{O} \xrightarrow{\text{ALP}} \text{p-Nitrophenol} + \text{Phosphate}
\]

Assay Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mode</td>
<td>Kinetic</td>
</tr>
<tr>
<td>Wavelength</td>
<td>Increasing</td>
</tr>
<tr>
<td>Flow-cell temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Optical path length</td>
<td>1 cm</td>
</tr>
<tr>
<td>Blanking</td>
<td>Purified water</td>
</tr>
<tr>
<td>Sample volume</td>
<td>20 µL</td>
</tr>
<tr>
<td>Reagent volume</td>
<td>1000 µL</td>
</tr>
<tr>
<td>Delay</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Interval</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Number of reading(s)</td>
<td>4</td>
</tr>
<tr>
<td>Permissible reagent Blank</td>
<td>&lt;0.1 AU</td>
</tr>
<tr>
<td>Kinetic factor</td>
<td>2712</td>
</tr>
<tr>
<td>Maximum ∆A/minute</td>
<td>0.36</td>
</tr>
<tr>
<td>Linearity</td>
<td>Upto 1000 IU/L</td>
</tr>
<tr>
<td>Units</td>
<td>IU/L</td>
</tr>
</tbody>
</table>
**Procedure**

**Pipette into tube marked**

<table>
<thead>
<tr>
<th>Test</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum/ Plasma</td>
<td>20 µL</td>
</tr>
<tr>
<td>Working ALP Reagent</td>
<td>1000 µL</td>
</tr>
</tbody>
</table>

Mix well and aspirate immediately for measurement

Programme the analyser as per assay parameters.

1. Blank the analyser with Purified Water

2. Read absorbance after 30 seconds. Repeat reading after every 30 seconds i.e., upto 120 seconds at 405 nm wavelength.

3. Determine the mean absorbance change per minute (ΔA/minute).

**Calculations**

ALP activity (IU/L) = ΔA/minute x Kinetic factor

**Where**

ΔA/minute = Change in absorbance per minute

Kinetic factor (K) = 2712

**Kinetic factor is calculated using following formula**

\[ K = \frac{1}{M} \times \frac{TV}{SV} \times \frac{1}{P} \times 10^6 \]

M = Molar extinction coefficient of p-Nitrophenol and is equal to 1.8x10³ lit/mol/cm at 405 nm

TV = Sample volume + Working Reagent volume

SV = Sample volume

P = Optical path length

10^6 = Constant

**Clinical Significance**

Serum ALP measurement is of particular interest in the hepatobiliary disease and is bone diseases. The main site of synthesis of this enzyme is hepatocytes adjacent to biliary canaliculi and active osteoblast. However, it is known that
response of a liver to any form of biliary tree obstruction is to synthesize more ALP.

**Increased activity:** Serum ALP is increased in diseases of bone including Metastasis, Rickets, Paget’s disease and in healing fractures, Intrahepatic or extrahepatic obstruction in Liver. Elevated levels are seen in growing Children due to new bone formation (Osteoblastic activity). Increase in ALP activity may often be the first indication of hepatotoxic action of therapeutic drugs. Marked elevation in the absence of Jaundice but in the presence of primary source may be indicative of Metastasis.

**Decreased activity:** Low levels of ALP are found in a rare Congenital Defect, Hypophosphatasemia and in Pernicious Anemia.

4.13.3. Aspartate Transaminase (AST/SGOT) test kit

**Kinetic Assay. Span Diagnostics ltd. (Liquid Gold) Surat.**

The assay procedure for AST is similar to that of ALP and ALT as mentioned above. Therefore, the kinetic assay was performed accordingly.

4.13.4. Lactate dehydrogenase (LDH)- Infinite LDH UV Kinetic

**Accurex Biomedical Pvt. Ltd. Mumbai.**

**Assay Principle**

Lactate dehydrogenase (LD or LDH) catalyzes the reduction of private by NADH to form lactate and NAD\(^+\). The catalytic concentration is determined from the rate of decrease of NADH measured at 340 nm.
Assay Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction type</td>
<td>UV-Kinetic</td>
</tr>
<tr>
<td>Reaction direction</td>
<td>Decreasing</td>
</tr>
<tr>
<td>Wavelength</td>
<td>340 nm</td>
</tr>
<tr>
<td>Flowcell temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Zero setting with</td>
<td>Distilled water</td>
</tr>
<tr>
<td>Delay time</td>
<td>60 seconds</td>
</tr>
<tr>
<td>No. of readings</td>
<td>4</td>
</tr>
<tr>
<td>Interval</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Blank absorbance limit</td>
<td>≥1.000 Abs.</td>
</tr>
<tr>
<td>Sample volume</td>
<td>0.02 ml (20 µl)</td>
</tr>
<tr>
<td>Working solution volume (4 R₁ : 1 R₂)</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Factor</td>
<td>8109</td>
</tr>
<tr>
<td>Linearity</td>
<td>2000 IU/L</td>
</tr>
</tbody>
</table>

**Preparation of Working Solution**

Prepare working solution by mixing Reagent R₁ and Reagent R₂ in the ratio 4:1 as per requirement.

**Procedure**

<table>
<thead>
<tr>
<th>Pipette into tube marked</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum / Plasma</td>
<td>0.02 ml (20 µl)</td>
</tr>
<tr>
<td>Working solution</td>
<td>1.0 ml (800 µl R₁ + 200 µl R₂)</td>
</tr>
</tbody>
</table>

Mix and aspirate. After the initial delay of 60 seconds, record the absorbance of the test at an interval of 30 seconds for the next 90 seconds at 340 nm. Determine the mean change in absorbance per minute.

**Calculation**

Activity of LDH in IU/L = ΔAbs./min. x 8109

**Clinical Significance**

Lactate dehydrogenase (LDH) can be found in nearly all cells of the body with highest activities in myocardium, liver, kidney and skeletal muscle.
Consequently, elevations of LDH in the serum have been considered nonspecific (for any disease disorder). LDH increases in case of acute myocardial infarction, hepatic disorders (vital Hepatitis, Cirrhosis), muscular dystrophy, cancer, metastasis, anemia, kidney diseases and in numerous other diseases involving tissue damage.

4.13.5. Albumin test kit

**Bromocresol Green, End Point Assay, Span Diagnostic Ltd. (Liquid Gold), Surat.**

**Assay Principle**

At pH 3.68, albumin acts as a cation and binds to the anionic dye Bromocresol Green [BCG], forming a green coloured complex. The absorbance of final colour is measured at 630 nm. The colour intensity of the complex is proportional to Albumin concentration in the Sample.

\[
\text{Albumin + BCG} \quad \rightarrow \quad \text{pH = 3.68} \quad \rightarrow \quad \text{Green coloured Complex}
\]

**Assay Parameters**

- **Mode**: End Point
- **Wavelength**: 630 nm (600-630 nm)
- **Flow- cell temperature**: 37°C
- **Optical path length**: 1 cm
- **Blanking**: Reagent Blank
- **Sample volume**: 10 µL
- **Reagent volume**: 1000 µL
- **Incubation time**: 1 minute
- **Concentration of standard**: 4.0 g/dL
- **Stability of final colour**: 2 hours
- **Permissible reagent Blank absorbance**: <0.1 AU
- **Linearity**: Upto 6 g/dL
- **Units**: g/dL
Procedure

<table>
<thead>
<tr>
<th>Pipette into tube marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum/Plasma</td>
<td>-</td>
<td>-</td>
<td>10 µL</td>
</tr>
<tr>
<td>Reagent 2</td>
<td>-</td>
<td>10 µL</td>
<td>-</td>
</tr>
<tr>
<td>Reagent 1</td>
<td>1000 µL</td>
<td>1000 µL</td>
<td>1000 µL</td>
</tr>
</tbody>
</table>

Mix well. **Incubate at Room temperature (15-30°C) for 1 minute.**

Programme the analyzer as per above assay parameter.

1. Blank the analyzer with Reagent Blank
2. Measure absorbance of the Standard followed by the Test.

**Calculation**

\[
\text{Albumin (g/dL)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 4
\]

Globulins = Total Protein – Albumin

**Conversion factor**

Albumin concentration in g/dL = Albumin concentration in g/dL \times 10

**Clinical Significance**

Albumin is one of the few Plasma Proteins without carbohydrate side chain. Albumin is primarily synthesized by hepatic parenchymal cells except in early fetal life. After digestion albumin transports the absorbed Amino acids to tissues.

**Increased concentration:** Hyperalbuminemia is rare and is seen in presence of acute dehydration or shock. An increase in albumin concentration will only be temporarily as intestinal water is drawn into the vascular bed by increased osmotic forces.
Decreased concentration: Over hydration, Protein loss through Kidneys (Nephrotic syndrome), from Skin (severe Burns), Starvation and Protein Malnutrition, Severe Non Viral Liver cell damage.

4.13.6 Determination of serum total protein

Serum protein was determined by the method of Lowry (1951) using Span diagnostics kit [18].

Principle:
Protein reacted with cupric ions in alkaline medium to form a violet colored complex. The intensity of the complex was measured at 530nm against reagent blank 0.01ml of the standard solution, which was treated in the same way.

Procedure:
The reagents used were from Span diagnostics kit. 1ml of working reagent was mixed with 0.01 ml of serum and absorbed at 530nm. The reagent blank, 0.01ml of standard solution was treated in same way.
Serum total protein (g/dl) = \[ \frac{\text{O.D. Treated} \times 6}{\text{O.D. Standard}} \]

The total protein level was expressed as g/dl.

4.13.7. Cholesterol test kit

Total Cholesterol: CHOD-PAP, Span diagnostic Ltd. (Liquid Gold), Surat.
Cholesterol is both coming from food and synthesized by the human body, mainly in hepatic and intestinal cells. Cholesterol is a component of cells. It is a metabolic precursor of bile acids, vitamin D and steroid hormones. Cholesterol, insoluble molecule, circulates associated with lipoproteins (HDL, LDL and VLDL).
Quantification of total cholesterol allows the detection of hypercholesterolemia isolated or associated with hypertriglyceridemia. High cholesterol concentration is associated with a high risk for vascular accident and apparition of atherosclerosis. The LDL/HDL ratio should be taken in consideration for evaluating the risk of cardiovascular diseases.

**Assay Principle**

Cholesterol esters are hydrolyzed by Cholesterol Esterase (CE) to give free Cholesterol and Fatty acids. In subsequent reaction, Cholesterol Oxidase (CHOD) oxidizes the 3-OH group of free Cholesterol to liberate Cholest-4-en-3-one and Hydrogen Peroxide. In presence of Peroxidase (POD), Hydrogen Peroxide couples with 4- Aminoantipyrine (4-AAP) and Phenol to produce Red Quinoneimine dye. Absorbance of coloured dye is measured at 505 nm and is proportional to amount of Total Cholesterol concentration in the Sample.

\[
\begin{align*}
\text{Cholesterol esters} & \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol + fatty acids} \\
\text{Cholesterol + O}_2 & \xrightarrow{\text{CHOD}} \text{Cholest-4-en-3-one + H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 + \text{Phenol + 4 AAP} & \xrightarrow{\text{POD}} \text{Quinoneimine dye + H}_2\text{O}
\end{align*}
\]

**Assay Parameters**

- **Mode**
  - End point
- **Wavelength**
  - 505 nm (490-510 nm)
- **Flowcell temperature**
  - 37°C
- **Optical path length**
  - 1 cm
- **Blanking**
  - Reagent blank
- **Sample volume**
  - 10 µL
- **Reagent volume**
  - 1000 µL
Incubation time  10 minutes at 37°C or 30 minutes at room temperature (15-30°C)
Concentration of Standard  200 mg/dL
Stability of final colour  1 hour
Permissible Reagent Blank  <0.2 AU absorbance
Linearity  750 mg/dL
Units  mg/dL

Procedure

<table>
<thead>
<tr>
<th>Pipette into tubes marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum/ Plasma</td>
<td>-</td>
<td>-</td>
<td>10 µl</td>
</tr>
<tr>
<td>Reagent 2</td>
<td>-</td>
<td>10 µl</td>
<td>-</td>
</tr>
<tr>
<td>Reagent 1</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
</tbody>
</table>

Mix well. Incubate at 37°C for 10 minutes or at Room Temperature (15-30°C) for 30 minutes.

Programme the analyser as per assay parameters.

1. Blank the analyser with Reagent Blank.
2. Measure absorbance of Standard followed by the Test.

Calculation

Cholesterol concentration (mg/dL) = Absorbance of Test / Absorbance of standard x 200

Conversion factor

Cholesterol concentration in mmol/L = Cholesterol concentration in mg/dL x 0.0259
Clinical Significance

Serum cholesterol serves as an indicator of propensity towards Coronary Heart Disease (CHD), Liver function, biliary function, Intestinal absorption, Thyroid function and Adrenal disease. Primarily, two lipoprotein classes transport the Cholesterol. HDL transports Cholesterol from tissues to the Liver for catabolism while LDL transports Cholesterol from sites of origin to deposition in tissues.

**Increased concentration**: Increased Cholesterol concentration is found in Idiopathic Hypercholesterolemia, Hyperlipoproteinemia, Nephrotic syndrome, Hypothyroidism, Nephrosis and Diabetes Mellitus. Hypercholesterolemia is known to be associated with an increased risk of Coronary Heart Disease (CHD). Increased HDL-Cholesterol concentration reduces the risk of cardiovascular disease. Moderate to vigorous exercise, estrogens and moderate consumption of alcohol may increase Serum HDL-Cholesterol.

**Decreased concentration**: Decreased Cholesterol concentration is found in Hepatocellular disease, Hyperthyroidism, Chronic Anaemia, Starvation and Hypobetalipoproteinemia. Serum Cholesterol concentration is very low in rare genetic disease like Abetalipoproteinemia. Decreased HDL-Cholesterol concentration increases the risk of cardiovascular diseases. It is lowered in Tangier disease, heavy cigarette smoking, Obesity, very high carbohydrate diets, uncontrolled Diabetes Mellitus and in male Sex hormone therapy.

4.13.8. Bilirubin test kit

**Jendrassik and Grof, Span Diagnostic Ltd. (Liquid Gold), Surat.**

**Assay principle**

Total Bilirubin consists of both indirect (Unconjugated) and Direct (Conjugated) Bilirubin. Indirect Bilirubin is insoluble in water and hence
requires an activator i.e., Caffeine to dissolve, where as Direct Bilirubin is water soluble and does not require any activator.

Sulphanilic Acid present in the reagent reacts with Sodium Nitrite to form Diazotised Sulphanilic Acid (DSA). Direct Bilirubin reacts with DSA formed to give a coloured complex called azobilirubin, whereas, indirect bilirubin reacts with DSA in presence of caffeine to give coloured azobilirubin. The absorbance of colour is measured at 546 nm (546- 630 nm in bichromatic mode) and is directly proportional to the concentration of Total Bilirubin in the sample.

\[
\text{Sulphanilic Acid} + \text{Sodium Nitrite} \rightarrow \text{DSA} \\
\text{Bilirubin (Direct)} + \text{DSA} \rightarrow \text{Azobilirubin} \\
\text{Bilirubin (Total)} + \text{DSA} + \text{Caffeine} \rightarrow \text{Azobilirubin}
\]

**Assay Parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mode</td>
<td>End Point</td>
</tr>
<tr>
<td>Primary Wavelength</td>
<td>546 nm</td>
</tr>
<tr>
<td>Secondary wavelength</td>
<td>630 nm</td>
</tr>
<tr>
<td>Flow- cell temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Optical path length</td>
<td>1 cm</td>
</tr>
<tr>
<td>Blanking</td>
<td>Purified water</td>
</tr>
<tr>
<td>Incubation time</td>
<td>5 minutes at R.T. (15-30°C)</td>
</tr>
<tr>
<td>Sample volume</td>
<td>50 µL</td>
</tr>
<tr>
<td>Reagent volume</td>
<td>For Total Bilirubin 1100 µL</td>
</tr>
<tr>
<td></td>
<td>For Direct Bilirubin 1100 µL</td>
</tr>
<tr>
<td>Factor</td>
<td>26.312</td>
</tr>
<tr>
<td>Stability of final colour</td>
<td>15 minutes for Total Bilirubin</td>
</tr>
<tr>
<td></td>
<td>10 minutes for Direct Bilirubin</td>
</tr>
<tr>
<td>Permissible reagent Blank absorbance</td>
<td>&lt;0.1 AU</td>
</tr>
<tr>
<td>Linearity</td>
<td>25 mg/dL</td>
</tr>
<tr>
<td>Units</td>
<td>mg/dL</td>
</tr>
</tbody>
</table>
**Procedure**

Note: For icteric sample, it is essential to take sample blank

<table>
<thead>
<tr>
<th>Pipette into tube marked</th>
<th>Total bilirubin</th>
<th>Direct bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample Blank</td>
<td>Test (AT1)</td>
</tr>
<tr>
<td>Reagent 1</td>
<td>--</td>
<td>50 µL</td>
</tr>
<tr>
<td>Reagent 2</td>
<td>100 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>Mix properly</td>
<td>1000 µL</td>
<td>--</td>
</tr>
<tr>
<td>Working reagent</td>
<td>1000 µL</td>
<td>1000 µL</td>
</tr>
<tr>
<td>Serum/ Plasma</td>
<td>50 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>Normal saline</td>
<td>--</td>
<td>1000 µL</td>
</tr>
</tbody>
</table>

Mix well. Incubate at R.T. for 5 minutes.

Programme the analyser as per assay parameters.

1. Blank the analyser with purified water.

2. Measure the absorbance of the sample blank and test at 546 nm (λ1) and 630 nm (λ2).

**For Standardization of Instrument**

Take 1 ml of Artificial Standard in test tube, measure absorbance of the Standard against purified water at 546 nm and 630 nm. Do not pour back the used Standard to the Artificial Standard (reagent 4) bottle.

**Calculation**

**With Factor**

Total Bilirubin (mg/dL) = (AT1-AB1) x factor

Direct Bilirubin (mg/dL) = (AT2-AB2) x factor
Where,

$AB_1 = \text{Absorbance of sample blank for Total Bilirubin}$

$AB_2 = \text{Absorbance of Sample blank for Direct Bilirubin}$

$AT_1 = \text{Absorbance of test for Total Bilirubin}$

$AT_2 = \text{Absorbance of test for Direct Bilirubin}$

**With Standard**

Bilirubin concentration (mg/dL) = Abs. Of test - Abs of sample blank/ Abs. of Standard x conc. of Standard

**Clinical Significance**

Bilirubin is the main bile pigment which is formed from the breakdown of heme of red blood cells by reticuloendothelial system. This Bilirubin is free and lipid soluble, which needs to be converted into water soluble form for excretion. The Free/ Unconjugated/ Indirect Bilirubin is carried by Albumin to the Liver, where it is conjugated with glucuronic acid and becomes water soluble in the presence of an enzyme glucuronyl transferase. Direct Bilirubin is excreted via bile salts to Intestine where it is converted to urobilinogen and later stercobilinogen. Urobilinogen is excreted in urine where as stecobilinogenin is excreted through faces. Bilirubin is helpful in measuring the severity of Liver disease.

**Increased concentration:** Total Bilirubin concentration is increased mildly in chronic hemolytic disease, moderately to severely in Hepatocellular disease and markedly in Cholestasis. There is a predominant increase in Direct Bilirubin in Obstructive Jaundice, due to regurgitation, while in Hepatic Jaundice, both direct as well as indirect Bilirubin concentration increase. In hemolytic Jaundice as well as in neonatal Jaundice, Indirect Bilirubin level increases without corresponding increase in Direct Bilirubin.
4.14 Histopathological studies

Histopathological studies, the slices of liver from each of the six animals in all groups were preserved in 10% buffered neutral formalin (pH 7.4). The tissues were mounted in the laboratory by embedding paraffin sections of 5-10 µ size. These sections were then stained with haemotoxyline eosin dye. The degree of liver damage was examined by a pathologist of R. S. Diagnostic Centre, Lucknow, for observation under a low power microscope for any pathological changes. Centrilobular necrosis, fatty infiltration, fibrosis, lymphocyte infiltration, etc. was noted.

4.5 Statistical analysis

A result of biochemical estimation has been expressed as mean ± Standard of Error of Mean (S.E.M). The values were subjected to One Way Analysis of Variance (ANOVA) using Graph Prism version 3.0. The variance in a set of data has been estimated by Tukey multiple compare test. The values of p<0.05 was considered statistically significant.
References


