Materials and Methods
2. MATERIALS AND METHODS

2.1 Animals

Wistar albino male rats weighing around 180 to 220 gm purchased from Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Chennai, India, were used in this study. They were fed pellet feed, purchased from TANUVAS and were provided water *ad libitum*. They were housed in polypropylene cages over husk bedding and a 12 h light and dark cycle was maintained throughout the experimental period. All animal experiments were performed after getting prior approval from the Institutional Animal Ethical Committee (IAEC).

2.2 Collection and authentication of plant (*Cassia fistula* Linn.) materials

Fresh leaves of *Cassia fistula* Linn. were collected from Tamil Nadu Medicinal Plant Farms and Herbal Medicine Corporation Ltd. (TAMPCOL), Chennai, India, during the month of August - December, 2001 - 2003. The plant and its leaves were authenticated by Dr. S. Narayanappa, Chief Botanist, TAMPCOL. A voucher specimen is deposited in Presidency College, Chennai (Herbarium No. 507/2002), Botanical Survey of India, Coimbatore (Herbarium No B.S.I/C.F./001/2002) and in the Department of Pharmacology and Environmental Toxicology, University of Madras, Taramani, Chennai (Specimen No. PET/C.F./001/2002).
2.3 Ethanolic leaf extract (ELE) of *Cassia fistula* Linn.

Immediately after collection, the leaves were washed in tap water twice and in distilled water once to remove all the external dust, dirt and unwanted materials. The leaves were then dried under shade for 72 h. Small bits of plant materials, the petioles, midribs and twigs were removed after shade drying. The dried leaves were then crushed by hand into coarse powder. 100 gm of this powder was soaked in 1 liter of 95 percent ethanol for 30 days. The soaked leaves were allowed to undergo natural percolation under occasional shaking, twice a day at an interval of 6 to 8 h during day time. The leaf extract was filtered using Whatmann no. 1 filter paper and the filtered extract was evaporated to dryness at 60°C over a water bath. The yield of the ethanolic leaf extract (ELE) was between 18 to 20 percent. This extract was found to be sparingly soluble in water and a suspension of ELE in distilled water was used for this study.

2.4 Phytochemical screening of ELE

The ELE was subjected to standard phytochemical screening as described by Harbone (1973). The qualitative tests performed for the identification of various plant principles and the results obtained thereon are tabulated as follows:
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Test</th>
<th>Test Methods</th>
<th>Test Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Tests for flavonoids</td>
<td>1. Sodium hydroxide test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Ferric chloride test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Lead acetate test</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>Tests for glycosides</td>
<td>1. Molisch test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Benedict’s test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Fehling’s test</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Tests for alkaloids</td>
<td>1. Dragendorff’s test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Wagner’s test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Mayer’s test</td>
<td>+</td>
</tr>
<tr>
<td>4.</td>
<td>Tests for tannins</td>
<td>1. Lead acetate test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Ferric chloride test</td>
<td>+</td>
</tr>
<tr>
<td>5.</td>
<td>Tests for saponins</td>
<td>1. Foam test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Liberman’s Test</td>
<td>+</td>
</tr>
</tbody>
</table>

+ - Positive

The qualitative phytochemical screening of ELE showed positive colour development for the presence of flavonoids, alkaloids, glycosides, tannins and saponins.

2.5 Drugs and Chemicals

Isonicotinic acid hydrazine (Isoniazid, INH) was purchased from Loba Chemie, India. Rifampicin (RIF) and Pyrazinamide (PYR) was obtained as
gratis from Medi Pharm, Chennai, India. Silymarin was obtained as gratis from Central Drug Research Institute (CDRI), Lucknow, India. All other chemicals used in this study were of analytical grade and were purchased locally.

2.5.1 Criteria for dosage selection of antitubercular drugs (INH, RIF and PYR) for evaluation of hepatotoxicity

In conventional clinical practice, INH, RIF and PYR are the recommended as the “drug of choice” for the treatment of tuberculosis. The National Tuberculosis Control Programme recommends 300, 600 and 1500 mg/day for INH, RIF and PYR respectively as the adult human dose (for a period of 9 months) for the chemoprophylaxis of tuberculosis. In this study, the recommended adult human dose was extrapolated to rats for their administration, by applying the conversion table, which was calculated based on the body surface area of common laboratory species and human as detailed by Ghosh (1984). Accordingly, on extrapolation of the above recommended human dose to rats, the dosage for INH, RIF and PYR was derived as 29.66, 54.33, and 148 mg/kg respectively for administration in rats. This dosage was corrected as 25, 50 and 140 mg/kg for INH, RIF and PYR respectively for their administration in rats as described previously by Kale et al. (2003).

2.6 Animal experimentation

2.6.1 Evaluation of acute toxicity of ELE

In this study protocol, ELE was administered to rats orally (p.o.) as a suspension in water at a dose of 250, 500, 1000, 1500, 2000 and 2500 mg/kg
b.w., to evaluate its acute toxicity. For doses $\geq 2000$ mg/kg b.w., ELE was administered at two equal installments at an interval of 3 h. Four nos. of animals were used per test dose to evaluate the mortality and $LD_{50}$ of ELE for a period of 24 h as detailed by Weil (1952). The animals were also observed for other toxic symptoms, if any, for a further period, upto 72 h after administration of ELE.

### 2.6.2 Evaluation of hepatoprotective and antioxidant properties of ELE on its simultaneous treatment against CCl$_4$ induced hepatotoxicity

In this model, the efficacy of ELE for its hepatoprotective and antioxidant properties during its simultaneous treatment against CCl$_4$ induced liver damage was evaluated in rats. The study protocol, the no. of animals used and the dosage schedule is given in the following table:

<table>
<thead>
<tr>
<th>Groups (n=6)</th>
<th>Treatment (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group – I</td>
<td>Normal control - Liquid paraffin treated daily for 7 days</td>
</tr>
<tr>
<td>Group – II</td>
<td>CCl$_4$ in liquid paraffin daily for 7 days (1:1 ratio)</td>
</tr>
<tr>
<td>Group – III</td>
<td>CCl$_4$ in liquid paraffin (1:1 ratio) followed by ELE daily for 7 days</td>
</tr>
<tr>
<td>Group – IV</td>
<td>ELE alone for 7 days</td>
</tr>
</tbody>
</table>

**Dosage**

- Liquid paraffin : 0.1ml/100gm b.w., s.c.
- CCl$_4$ : 0.1ml/100gm b.w., s.c. in liquid paraffin (1:1 ratio)
- ELE : 500mg/kg p.o. in distilled water
2.6.3 Evaluation of hepatoprotective and antioxidant properties of ELE and SIL on INH alone induced hepatotoxicity

In this model, the efficacy of ELE and SIL for hepatoprotective and antioxidant properties against sub acute INH alone induced hepatotoxicity in rats was evaluated. In addition, studies on the alterations in vital lipid parameters (TL, TG, CHO, PL and FFA) in plasma, liver and adipose tissue induced by INH alone treatment and its prevention by ELE and SIL was also investigated. The treatment schedule for this investigation, the no. of animals used in each group and the dosage schedule is given in the following table:

<table>
<thead>
<tr>
<th>Groups (n=6)</th>
<th>Treatment (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group – I</td>
<td>Normal control - Saline treated (30 days)</td>
</tr>
<tr>
<td>Group – II</td>
<td>INH alone for 30 days</td>
</tr>
<tr>
<td>Group – III</td>
<td>Simultaneous treatment of INH and ELE for 30 days</td>
</tr>
<tr>
<td>Group – IV</td>
<td>Simultaneous treatment of INH and SIL for 30 days</td>
</tr>
<tr>
<td>Group – V</td>
<td>ELE alone for 30 days</td>
</tr>
<tr>
<td>Group – VI</td>
<td>SIL alone for 30 days</td>
</tr>
</tbody>
</table>

**Dosage**

Saline : 0.25ml/100gm b.w., p.o.
INH : 50mg/kg b.w., i.p. in saline
ELE : 500mg/kg b.w., p.o. in distilled water
SIL : 50mg/kg b.w., p.o. in propylene glycol
2.6.4 Evaluation of hepatoprotective and antioxidant properties of ELE and SIL on combined treatment of antitubercular drugs (INH+RIF+PYR) induced hepatotoxicity

In this model, the efficacy of ELE and SIL for hepatoprotective and antioxidant properties against combined treatment of antitubercular drugs (INH+RIF+PYR) induced hepatotoxicity in rats was evaluated. In addition, studies on the alterations in vital lipid parameters (TL, TG, CHO, PL and FFA) in plasma, liver and adipose tissue induced by combined treatment of antitubercular drugs (INH+RIF+PYR) and its prevention by ELE and SIL was also investigated. The treatment schedule for this investigation, the no. of animals used in each group and the dosage schedule is given in the following table:

<table>
<thead>
<tr>
<th>Groups (n=6)</th>
<th>Treatment (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group – I</td>
<td>Normal control - Saline treated for 45 days</td>
</tr>
<tr>
<td>Group – II</td>
<td>INH+RIF+PYR alone for 45 days</td>
</tr>
<tr>
<td>Group – III</td>
<td>Simultaneous treatment of INH+RIF+PYR and ELE for 45 days</td>
</tr>
<tr>
<td>Group – IV</td>
<td>Simultaneous treatment of INH+RIF+PYR and SIL for 45 days</td>
</tr>
<tr>
<td>Group – V</td>
<td>ELE alone for 45 days</td>
</tr>
<tr>
<td>Group – VI</td>
<td>SIL alone for 45 days</td>
</tr>
</tbody>
</table>
Dosage

Saline : 0.25ml/100gm b.w., p.o.
INH : 25mg/kg b.w., p.o. in saline
RIF : 50mg/kg b.w., p.o. in 0.001N HCl (Thomas and Zeitz, 1980)
PYR : 140mg/kg b.w., p.o. in saline
ELE : 500mg/kg b.w., p.o. in distilled water
SIL : 50mg/kg b.w., p.o. in propylene glycol

2.7 Collection of samples for biochemical and histological analysis

At the end of the experimental period, all the animals were anaesthetized under mild ether anesthesia and blood was collected by retro-orbital vein puncture. The animals were subsequently sacrificed by cervical decapitation and the liver tissue was excised quickly. Adipose tissue was collected from the epididymal fat pads. The tissues were washed in physiological saline to remove blood clot and other tissue materials. For histopathological studies, a piece, approximately of 1 cm$^3$ of liver tissue was cut and placed immediately in phosphate buffered formal saline (pH - 7.4).

2.7.1 Collection of blood sample and separation of serum

The blood samples were collected in plain centrifuge tubes and were kept in inclined position to allow complete clotting of blood. The tubes were then centrifuged at 2500 rpm for 30 min. The resultant clear supernatant was pipetted out and preserved in small vials in the freezer for the purpose of biochemical investigations.
2.7.2 Collection of blood sample and separation of plasma

Blood was also collected in separate tubes containing EDTA (1mg/ml) as anticoagulant. The whole blood containing EDTA was subjected to centrifugation at 2500 rpm for 30 min. The resultant clear supernatant plasma was pipetted out and preserved in small vials in the freezer for the assay of various lipid parameters.

2.7.3 Preparation of liver tissue for enzyme assay

Within 3 h after sacrifice, liver samples were blotted to dryness. From this, a piece weighing about 100 mg was taken and homogenized at 4°C in Tris - HCl buffer (0.1M; pH - 7.4). The tissue homogenates were centrifuged at 2500 rpm for 30 min. The resultant supernatant was kept under refrigeration until further biochemical analysis. All the assay procedures were carried out within 48 h after sample collection.

2.8 Biochemical Assays

Various biochemical assay procedures carried out for this study in serum/plasma, liver and adipose tissue samples are detailed here under.

2.8.1 Estimation of activity of Aspartate Transaminase (AST) in serum and liver tissue

The activity of Aspartate Transaminase (L - Aspartate: \( \alpha \) - oxoglutarate amino transferase – EC. 2.6.1.1.) was estimated by the method of Wooten (1964).
Principle

The activity of AST was estimated by measuring the amount of pyruvate formed by the enzyme when subjected to the following reaction:

\[
\alpha - \text{oxoglutaric acid} + \text{DL-aspartic acid} \xleftrightarrow{} \text{Oxaloacetate} + \text{Glutamic acid} \xrightarrow{} \text{Pyruvate}
\]

The liberated oxaloacetate is quickly converted into pyruvate complex, on treatment with 2,4 - dinitrophenylhydrazine and sodium hydroxide yields a colour, which was measured spectrophotometrically.

Reagents

1. *Phosphate buffer (0.1M; pH - 7.4)*: 420 ml of 0.1M disodium hydrogen phosphate was mixed with 0.1M potassium dihydrogen orthophosphate until the pH was adjusted to 7.4.

2. *AST - substrate*: 2.66 gm of DL - aspartic acid and 29.2 mg of \(\alpha\) - ketoglutarate were dissolved in 50 ml of phosphate buffer and its pH was adjusted to 7.4 using 1N NaOH. The resultant solution was made upto 100 ml with phosphate buffer.

3. *2,4 - Dinitrophenyl hydrazine (DNPH)*: 19.8 mg of DNPH was dissolved in 100 ml of 1N HCl.

4. *Sodium hydroxide (NaOH - 0.4N)*: 16 gm of NaOH pellet was dissolved and made upto a liter with distilled water.

5. *Standard pyruvate solution (2\(\mu\)mole/ml)*: 22 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer.
Procedure

0.5 ml of AST substrate was incubated with 0.05 ml of serum or liver tissue homogenate along with the standard tubes having standard pyruvate, taken at a concentration ranging from 0.2 to 0.8 μmoles in separate tubes, at 37°C for 1 h. The reaction was arrested by the addition of 1.0 ml of DNPH and was left at room temperature for 20 min. Simultaneously, a control, each for serum and liver tissue homogenate was prepared separately. These tubes contained AST substrate alone and the serum/liver tissue homogenates were added after addition of DNPH. Finally, the colour was developed in all the above tubes by the addition of 5 ml of 0.4N NaOH and their absorbance was measured at 540 nm against blank using spectrophotometer. (ECIL, India Ltd).

The activity of Aspartate Transaminase in serum is expressed as IU/L (International Units/liter) and in tissue as IU/gm wet tissue.

2.8.2 Estimation of activity of Alanine Transaminase (ALT) in serum and liver tissue

The activity of Alanine Transaminase (L - Alanine: α - oxoglutarate amino transferase – EC. 2.6.1.2.) was estimated by the method of Wooten (1964).
Principle

The activity of Alanine Transaminase was estimated by measuring the amount of pyruvate formed by the enzyme when subjected to the following reaction:

$$\alpha$$-oxoglutaric acid + DL-alanine $\rightleftharpoons$ Glutamic acid + Pyruvate

The liberated pyruvate complex, on treatment with 2,4 - dinitrophenylhydrazine and sodium hydroxide yields a colour, which was estimated spectrophotometrically.

Reagents

1. *Phosphate buffer (0.1M; pH - 7.4)*: 420 ml of 0.1M disodium hydrogen phosphate was mixed with 0.1M potassium dihydrogen orthophosphate until the pH was adjusted to 7.4.

2. *ALT - Substrate*: 1.78 gm of DL - Alanine and 29.2 mg of $\alpha$-ketoglutarate were weighed and dissolved in phosphate buffer and the pH was adjusted to 7.4 using 1N NaOH. This was made upto 100 ml with phosphate buffer.

3. *2,4 - Dinitrophenyl hydrazine (DNPH)*: 19.8 mg of DNPH was dissolved in 100 ml of 1N HCl.

4. *Sodium hydroxide (NaOH - 0.4N)*: 16 gm of NaOH pellet was dissolved and made upto a liter with distilled water.

5. *Standard pyruvate solution (2μmole/ml)*: 22 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer.
Procedure

0.5 ml of ALT substrate was incubated with 0.05 ml of serum or liver tissue homogenate along with standard tubes having standard pyruvate, taken at a concentration ranging from 0.2 to 0.8 μmoles in separate tubes, at 37°C for 30 min. The reaction was arrested by the addition of 1.0 ml DNPH and was left at room temperature for 20 min. Simultaneously, a control each for serum and liver tissue homogenate was prepared separately. These tubes contained ALT substrate alone and the serum and liver tissue homogenates were added after addition of DNPH. Finally, the colour was developed in all the above tubes by the addition of 5 ml of 0.4N NaOH and their absorbance was measured at 540 nm against blank using spectrophotometer.

The activity of Alanine Transaminase in serum is expressed as IU/L and in tissue as IU/gm wet tissue.

2.8.3 Estimation of activity of Alkaline Phosphatase (ALP) in serum and liver tissue

The serum and tissue Alkaline Phosphatase activity (Ortho-phosphoric monoester phosphohydrolase - EC. 3.1.3.1.) measured by the method of King (1965a).

Principle

Phenol liberated by enzymatic hydrolysis at alkaline pH 10 from disodium phenyl phosphate under definite condition of time and temperature was measured spectrophotometrically.
Reagents

1. *Sodium carbonate - Sodium bicarbonate buffer (0.1M, pH-10):* 3.18 gm of anhydrous sodium carbonate and 1.68 gm of sodium bicarbonate were dissolved in 500 ml of distilled water. This solution has a pH - 10.

2. *Disodium phenylphosphate substrate (0.01M):* 25 mg of Disodium phenylphosphate was dissolved in 10 ml of distilled water. This was prepared just before use.

3. *Magnesium chloride (MgCl₂ - 0.1M):* 1.310 gm of crystalline MgCl₂ was dissolved in 100 ml of distilled water.

4. *Folin and Ciocalteu’s phenol reagent:* Commercially purchased Folin and Ciocalteu’s phenol reagent (SRL, India) was diluted 1:2 with distilled water. This solution was prepared just before use.

5. *Sodium carbonate (Na₂CO₃ - 20%):* 20 gm of Na₂CO₃ was dissolved and made upto 100 ml with distilled water.

6. *Stock phenol solution (1mg/ml):* 100 mg of analar phenol was weighed quickly and made upto 100 ml with distilled water and stored in cold.

7. *Working Standard Solution:* The stock standard phenol was diluted suitably with distilled water to get a final concentration of 1 μmole/ml.

Procedure

To the blank, standard tubes containing phenol, taken at a concentration ranging from 0.05 to 0.2 μmole and the test containing 0.05 ml of serum or liver homogenate, taken in separate tubes, 1.5 ml of sodium carbonate - sodium bicarbonate buffer, 1.0 ml of disodium phenylphosphate and 0.1ml of MgCl₂ was added and vortexed well. After adjusting the final volume to 3.0 ml with distilled water, this mixture was incubated at 37°C for
15 min. 1.0 ml of Folin and Ciocalteu’s phenol reagent was added to arrest the reaction after incubation. Simultaneously, a control without serum and liver homogenate was also subjected to the above treatment and incubation, excepting that serum and liver homogenate were added after arresting the reaction. The precipitate formed after addition of Folin and Ciocalteu’s phenol reagent was made to settle at the bottom of the tubes by centrifugation. The clear supernatant was transferred to fresh tubes and 1.0 ml of 20% Na₂CO₃ was added to this and left for 20 min for the blue color to develop. The absorbance was measured at 640 nm against the blank using spectrophotometer.

The activity of Alkaline Phosphatase in serum is expressed as IU/L and in tissue as IU/gm wet tissue.

2.8.4 Estimation of activity of Lactate Dehydrogenase (LDH) in serum and liver tissue

The serum and tissue Lactate dehydrogenase activity (L - Lactate: NAD⁺ oxidoreductase - EC. 1.1.1.27.) were measured by the method of King (1965b).

Principle

Lactate is converted to pyruvate in the presence of enzyme and NAD⁺. The liberated pyruvate on treatment with DNPH and NaOH yields a colour, which was measured spectrophotometrically. The reaction of pyruvate formation catalyzed by the enzyme and NAD⁺ is as follows:
\[
\begin{align*}
\text{CH}_3\text{CHOH.COO}^- + \text{NAD}^+ & \iff \text{CH}_3\text{CO.COO}^- + \text{NADH} + \text{H}^+ \\
\text{Lactate} & \iff \text{Pyruvate}
\end{align*}
\]

**Reagents**

1. *Glycine buffer (0.1M; pH - 9):* 7.505 gm of glycine and 5.85 gm of NaCl was dissolved in distilled water and made up to a liter.

2. *LDH substrate:* 75 ml of glycine buffer and 25 ml of 0.1N NaOH and 2.0 gm of lithium lactate was added and mixed well. This was prepared just before use.

3. *Nicotinamide Adenine Dinucleotide (NAD\(^+\)):* 10 mg of NAD\(^+\) was dissolved in 2 ml of distilled water. This solution was prepared just before use.

4. *2,4 - Dinitro phenylhydrazine (DNPH):* 200 mg of DNPH was dissolved in hot 1N HCl and made up to a liter.

5. *Sodium hydroxide (NaOH - 0.4N):* 16 gm of sodium hydroxide pellet was dissolved and made up to a liter with distilled water.

6. *Phosphate buffer (0.1M; pH - 7.4):* 420 ml of 0.1M disodium hydrogen phosphate was mixed with 0.1M potassium dihydrogen orthophosphate until the pH was adjusted to 7.4

7. *Standard pyruvate Solution (2\(\mu\)mole/ml):* 22 mg of sodium pyruvate was dissolved in 100 ml of 0.1M phosphate buffer.

**Procedure**

0.1 ml of serum or tissue homogenate and 0.2 ml of NAD\(^+\) was placed in 1.0 ml of glycine buffer and incubated at 37\(^\circ\)C for 15 min. 1.0 ml of DNPH reagent was added to arrest the reaction and left for further 15 min. Simultaneously, a control without serum and liver homogenate was also
subjected to the above treatment and incubation, excepting that serum and liver homogenate were added after arresting the reaction. Then, 10 ml of 0.4 N NaOH was added to all these tubes and the colour developed was read at 440 nm against blank using spectrophotometer.

The activity of Lactate Dehydrogenase in serum is expressed as IU/L and in tissue as IU/gm wet tissue.

2.8.5 Estimation of activity of γ - Glutamyl Transpeptidase (γ - GT) in serum and liver tissue

The activity of γ - Glutamyl Transpeptidase (γ - Glutamyl - peptide: aminoacid γ - glutamyl transferase - EC. 2.3.2.1.) was estimated by the method of Rosalki and Rau (1972).

Principle

The p - nitroaniline liberated by the enzyme in the presence of substrate i.e., L - γ - glutamyl - p - nitroanilide, produces a yellow colour, which was measured spectrophotometrically.

Reagents

1. *Tris - HCl (0.1M) - Glycylglycine (0.05M) buffer (pH - 9.0)*: 1.57 gm of Tris - HCl and 660 mg of glycylglycine was dissolved in distilled water and the pH was adjusted to 9.0 with 1N NaOH and made upto 100 ml.

2. *Tris - HCl Buffer (0.1M; pH - 8.5)*: 1.57 gm of Tris - HCl was dissolved in 100 ml of distilled water and the pH was adjusted to 8.5 with 1N NaOH.
3. **L - γ-glutamyl - p - nitroanilide substrate (6.25 mM):** 190 mg of L - γ-glutamyl - p - nitroanilide was dissolved in 100 ml of pre-warmed Tris- HCl - glycylglycine buffer.

4. **Acetic acid (10%):** 10 ml of glacial acetic acid was made upto 100 ml with distilled water.

5. **Standard p - nitroaniline (1 μmole/ml):** 13.8 mg of p - nitroaniline was dissolved in 100 ml of Tris-HCl buffer.

**Procedure**

0.05 ml of serum or tissue homogenate was made upto 0.5 ml by the addition of L - γ-glutamyl - p - nitroanilide substrate and was incubated at 37°C for 30 min. The standard tubes taken at a concentration ranging from 0.1 to 0.4 μmole were also incubated as above. The reaction was arrested by the addition of 2.5 ml of 10% acetic acid. Simultaneously, a control without serum and liver homogenate was also subjected to the above treatment and incubation excepting that serum and liver homogenate were added after arresting the reaction. The yellow colour developed after the addition of acetic acid was measured at 410 nm against the blank using spectrophotometer.

The activity of γ - Glutamyl Transpeptidase in serum is expressed as IU/L and in tissue as IU/gm wet tissue.

2.8.6 **Estimation of Bilirubin in serum**

The Bilirubin content in the serum was estimated according to the method of Malloy and Evelyn (1937).
Principle

The serum, diluted with water and methanol was added in an amount sufficient to precipitate the protein and sufficient to permit all the bilirubin to react with the diazo reagent (by Vanden - Berg reaction) to form a purple compound i.e., azobilirubin. This coloured complex was measured spectrophotometrically.

Reagents

1. Absolute Methanol
2. Hydrochloric acid (HCl - 1.5%)
3. Diazo reagent (Vanden - Berg reagent): This reagent was prepared freshly before use by adding 0.3 ml of solution B to 10 ml of solution A.
4. Solution A: 1.0 gm of sulphanilic acid was dissolved in 15 ml of conc. HCl and made upto a liter with water.
5. Solution B: 0.5 gm of sodium nitrite was dissolved in water and made upto 100 ml with the same.
6. Bilirubin standard (0.1 mg/ml): 10 mg of bilirubin was dissolved in 100 ml of chloroform. This solution was prepared fresh just before use.

Procedure

0.2 ml of serum, taken in clean tubes was made upto 2.0 ml with distilled water. 0.5 ml of 1.5% HCl was made upto 2.0 ml with distilled water and this constituted the blank. The standard bilirubin taken at a concentration ranging from 5 to 20 μg was made upto 2.0 ml with methanol. To all the
tubes, 0.5 ml of diazo reagent was added. Finally, 2.5 ml of methanol was added to each of these tubes and allowed to stand for 30 min for purple colour to develop, which was measured at 540 nm against blank using spectrophotometer.

The total Bilirubin content thus estimated was expressed as mg/dL of serum.

2.8.7 Estimation of activity of Glutathione Reductase (GR) in the liver tissue

The activity of Glutathione Reductase (NADPH: oxidized glutathione oxidoreductase - EC. 1.6.4.2.) was estimated in the liver tissue by the method of Mize and Langdon (1962).

Principle

The reduction of oxidized glutathione in the presence of NADPH in a reaction mixture containing phosphate buffer and EDTA, upon addition of the enzyme results in the decrease in absorbance, which was measured spectrophotometrically.

Reagents

1. *Phosphate buffer (0.1M; pH - 7.6):* 420 ml of 0.1M disodium hydrogen phosphate was mixed with 0.1M potassium dihydrogen orthophosphate until the pH was adjusted to 7.6.

2. *Ethylene diamine tetra acetic acid (EDTA - 0.5mM):* 14.61 mg of disodium salt of EDTA was dissolved in 100 ml of distilled water.
3. *Glutathione oxidized (GSSG - 1mM) substrate*: 4.90 mg of GSSG was dissolved in 8 ml of distilled water.

4. *Nicotinamide adenine dinucleotide phosphate reduced (NADPH - 0.1mM)*: 8.35 mg of NADPH was dissolved in 100 ml of distilled water.

**Procedure**

The reaction mixture containing 2.0 ml of phosphate buffer, 0.5 ml of EDTA and 0.2 ml of GSSG substrate, taken in clean tubes was incubated at 37°C for 10 min. The reaction was started by addition of 0.1 ml of tissue homogenate and 0.1 ml of NADPH. The measurement of decrease in optical density per min after the addition of tissue homogenate and NADPH was measured at 340 nm in spectrophotometer. The molar absorbency index of NADPH at 340 nm was assumed to be $6.22 \times 10^3 \text{cm}^{-1} \text{M}^{-1}$.

One unit of enzyme activity is defined as the quantity of enzyme, which catalyses the oxidation of 1μmole of NADPH/min under the above condition. The activity of Glutathione Reductase in the tissue sample is expressed as μmoles of GSSG reduced/min/mg protein.

**2.8.8 Estimation of activity of Catalase (CAT) in the liver tissue**

The activity of Catalase (Hydrogen peroxide: Hydrogen peroxide oxidoreductase - EC. 1.11.1.6.) in the liver tissue was estimated by the method of Sinha (1972).
Principle

The method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of hydrogen peroxide ($\text{H}_2\text{O}_2$), resulting in the formation of perchromic acid as an unstable intermediate. The chromic acetate, thus produced was measured spectrophotometrically.

Reagents

1. *Phosphate buffer (0.01M, pH - 7.0)*: 84 ml of 0.01M disodium hydrogen phosphate was mixed with 0.01M of potassium dihydrogen orthophosphate until the pH was adjusted to 7.

2. *Potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$, 7$\text{H}_2\text{O}$ - 5%)*: 5 gm of $\text{K}_2\text{Cr}_2\text{O}_7$ was mixed with distilled water and made up to 100 ml.

3. *Dichromate - Acetic acid reagent (1:3 v/v)*: This reagent was prepared by mixing one volume of 5% potassium dichromate solution with 3 volumes of glacial acetic acid.

4. *Hydrogen peroxide ($\text{H}_2\text{O}_2$ - 0.2M)*: 0.02 ml of $\text{H}_2\text{O}_2$ (S.G. - 1.01) was taken and immediately mixed in 1 liter of distilled water. This solution was used for initiation of reaction with tissue homogenate.

Procedure

0.1 ml of triplicate samples of tissue homogenate taken in separate tubes was mixed with 1.0 ml of phosphate buffer. 0.5 ml of $\text{H}_2\text{O}_2$ was added to these tubes and the reaction was arrested immediately by addition of 2.0 ml of dichromate - acetic acid reagent at 0, 30 and 60 second intervals. The reagent blank was prepared by the addition of 1.6 ml of buffer and 2.0 ml
of dichromate acetic acid reagent taken in separate tubes. The test and blank tubes were heated in boiling water bath for 10 min for the green colour to develop. The tubes were cooled to room temperature and the intensity of colour was measured at 570 nm using spectrophotometer against the blank.

The activity of Catalase in the tissue homogenate is expressed as μmoles of H₂O₂ utilized/min/mg protein.

2.8.9 Estimation of activity of Superoxide Dismutase (SOD) in the liver tissue

The activity of Superoxide dismutase (Superoxide: Superoxide oxidoreductase - EC. 1.15.1.1.) in the liver tissue was estimated according to the method of Marklund and Marklund (1974).

Principle

Pyrogallol autooxidizes rapidly in aqueous solution at a faster rate at a higher pH (8.0) to produce several intermediate products. The inhibition of pyrogallol autooxidation by the enzyme present in the sample is employed in the quantification of activity of Superoxide Dismutase. The inhibition of autooxidation brought about by the addition of enzyme is evaluated at the early stage as an increase in absorbance at 420 nm.

Reagents

1. Tris - HCl Buffer (0.1M; pH - 8.2): 1.576 gm of Tris-HCl was dissolved in distilled water and the pH was adjusted to 8.2 using 1N NaOH and made upto 100 ml with distilled water.
2. *Pyrogallol (0.2mM)*: 126 mg of pyrogallol was dissolved in 100 ml of Tris - HCl buffer. This solution was prepared just before use.

3. *Ethylene diamine tetra acetic acid (EDTA - 1mM)*: 29.22 mg of EDTA was dissolved in 100 ml of distilled water.

4. *Diethylenetriamine pentaacetic acid (DTPA - 1mM)*: 19.67 mg of DTPA was dissolved in 50 ml of distilled water.

**Procedure**

A mixture containing 2.5 ml of Tris - HCl buffer, 0.1 ml of EDTA and 0.5 ml of DTPA was prepared. To this mixture, 0.5 ml of pyrogallol was added and the increase in absorbance was read at 420 nm against the blank for 3 min to determine the rate of autooxidation of pyrogallol. 0.1 ml of tissue homogenate taken in separate tube was mixed with 2.5 ml of Tris - HCl buffer, 0.1 ml of EDTA and 0.5 ml of DTPA. To this mixture, 0.5 ml of pyrogallol was added and the increase in absorbance was read at 420 nm using a spectrophotometer against the blank for a period of 3 min. This measurement constituted the rate of inhibition of autooxidation of pyrogallol brought about by the enzyme present in the tissue homogenate. The reagent blank contained a mixture of 3.1 ml of Tris - HCl buffer, 0.1 ml of EDTA and 0.5 ml of DTPA and this was used to set 100% absorbance.

The activity of Superoxide Dismutase was expressed as Units/mg of tissue protein and it is defined as the 50% inhibition of autooxidation of pyrogallol per min by the enzyme.
2.8.10 Estimation of activity of Glutathione - S - Transferase (GST) in the liver tissue

The activity of Glutathione - S - Transferase (1 - chloro - 2,4 dinitrobenzene: reduced glutathione transferase - EC. 2.5.1.18.) in the liver tissue was estimated by the method of Habig et al. (1974).

Principle

The change in absorbance in unit time produced by the addition of the enzyme in the presence of a mixture containing the substrate 1 - chloro - 2, 4-dinitrobenzene and glutathione was measured spectrophotometrically.

Reagents

1. *Phosphate buffer (0.1M; pH - 6.5)*: 420 ml of 0.1M disodium hydrogen phosphate was mixed with 0.1M potassium dihydrogen orthophosphate until the pH was adjusted to 6.5.

2. *Reduced glutathione (GSH - 5mM)*: 15.36 mg of GSH was dissolved in 10 ml of distilled water.

3. *1 - chloro 2, 4 - dinitrobenzene (CDNB) substrate (1mM)*: 10 mg of CDNB was dissolved in 50 ml ethanol.

Procedure

0.1 ml of the enzyme (liver tissue homogenate) was mixed in a mixture containing 1.5 ml of phosphate buffer, 1.0 ml of CDNB and 0.5 ml of GSH. The rate of change in absorbance on addition of tissue homogenate was measured at 340 nm using a spectrophotometer against a blank, containing
3.1 ml of phosphate buffer. A control containing the complete assay mixture without enzyme was also measured spectrophotometrically. The rate of change in enzyme activity per min was calculated.

The activity of Glutathione - S - Transferase was expressed as μmoles of CDNB utilized/min/mg of protein.

2.8.11 Estimation of Reduced Glutathione (GSH) in the liver tissue

The Reduced Glutathione content in the liver tissue homogenate was estimated by the method of Ellman (1959) with little modification (Beutler et al., 1963).

Principle

5 - 5'dithio bis (2 - nitrobenzioc acid; DTNB) reacts with aliphatic thiol compounds at pH - 8 to produce 1 mole of p - nitrothiophenol anion/mole of thiol. The reaction is as follows: (Ellman, 1959)

![Chemical reaction diagram]

The release of p - nitrothiophenol produces an intense yellow colour with a molar extinction co-efficient of \( \frac{1}{(C \log I/I_o)} \) of 13,600/cm M⁻¹ at 412 nm. This property is used to measure the thiol concentration of reduced glutathione.
Reagents

1. **Phosphate buffer (0.2M; pH - 8.0):** 94.7 ml of 0.2M disodium hydrogen phosphate was mixed with 5.3 ml of 0.2M sodium dihydrogen orthophosphate. This solution has pH-8.

2. **5'-dithiobis (2-nitrobenzoic acid) substrate (DTNB - 0.6mM):** 2.37 mg of DTNB was dissolved in 100 ml of 0.2M phosphate buffer.

3. **Trichloroacetic acid (TCA - 5%):** 5 gm of TCA was dissolved in 100 ml of distilled water.

4. **Glutathione (GSH) standard (200 μg/ml):** 20 mg of GSH was dissolved in 100 ml of distilled water.

Procedure

0.2 ml of tissue homogenate was made upto 1.0 ml by addition of 5% TCA and the protein flocculate formed in the sample was precipitated by centrifugation. 0.2 ml of the protein free supernatant was used for the assay. 2.0 ml of DTNB was mixed with 0.2 ml of the supernatant and the final volume was made upto 3.0 ml with phosphate buffer and its optical density was measured at 412 nm in a spectrophotometer within 60 seconds, against the blank. The blank contained 0.2 ml of TCA and 2.0 ml of DTNB, which was made upto 3.0 ml with phosphate buffer. The standard glutathione prepared in separate tubes at a concentration range of 5 to 20 μg were treated with 2.0 ml of DTNB and their volume was made upto 3.0 ml with phosphate buffer. The blank and the standard were also measured at 412 nm.

The amount of Reduced Glutathione in the liver tissue was expressed as μg/gm wet tissue.
2.8.12 Estimation of activity of Glutathione peroxidase (GPx) in the liver tissue

The activity of Glutathione peroxidase (Glutathione: Hydrogen peroxide oxidoreductase - EC. 1.11.1.9.) in the liver tissue was estimated by the methods of Rotruck et al. (1973) and Beutler et al. (1963).

Principle

Reduced Glutathione (GSH) is converted into oxidized Glutathione (GSSG) in the presence of the enzyme Glutathione Peroxidase (GPx) and its reaction is as follows:

\[ 2\text{GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{GPx}} \text{GSSG} + 2\text{H}_2\text{O} \]

In this assay procedure, the amount of GSH utilized was estimated by the principle as detailed under section 2.8.11.

Reagents

1. Phosphate buffer (0.1M; pH - 7.0): 420 ml of 0.1M dosodium hydrogen phosphate was mixed with 0.1M of potassium dihydrogen orthophosphate until the pH was adjusted to 7.0.

2. Sodium azide (10mM): 65 mg of Sodium azide was dissolved and made upto 100 ml with distilled water.

3. Glutathione (GSH - 4mM): 125 mg of glutathione was dissolved and made upto 100 ml with distilled water.
4. *Ethylene diamine tetraacetic acid (EDTA - 1mM):* 29.2 mg of EDTA was dissolved and made up to 100 ml of distilled water.

5. *Hydrogen peroxide (H₂O₂ - 2.5mM):* 0.03 ml of H₂O₂ was placed in 100 ml of distilled water.

6. *Trichloro acetic acid (TCA - 10%):* 10 gm of TCA was dissolved and made up to 100 ml with distilled water.

7. *Phosphate solution (0.3M):* 4.25 gm of disodium hydrogen phosphate was dissolved and made up to 100 ml with distilled water.

8. *5′-5′ dithiobis (2-nitrobenzio acid) substrate (DTNB - 0.6mM):* 2.37 mg of DTNB was dissolved in 100 ml of 1% trisodium citrate.

9. *Trisodium citrate (1%):* 1 gm of trisodium citrate was dissolved and made up to 100 ml with distilled water.

10. *Glutathione (GSH) standard (200 μg/ml):* 20 mg of GSH was dissolved in 100 ml of distilled water.

**Procedure**

0.1 ml of triplicate samples of liver tissue homogenate, taken in separate tubes was mixed with 0.4 ml of phosphate buffer, 0.1 ml each of sodium azide, EDTA and H₂O₂. Then, 0.2 ml of GSH was added to all these tubes and the reaction was arrested by the addition of 10% TCA at 0, 1.5 and 3 min intervals. The tubes were then centrifuged and 1.0 ml of the supernatant was transferred to fresh tubes. The blank contained 1 ml of distilled water. The standard glutathione was prepared in separate tubes at a concentration range of 5 to 20 μg in a final volume of 1 ml. To all the above tubes 4 ml of
phosphate solution was added followed by 0.5 ml of DTNB and the colour developed was read at 412 nm in a spectrophotometer against the blank.

The activity of Glutathione Peroxidase in the liver tissue is expressed as Units/mg protein. One unit of enzyme activity is the amount of the enzyme that converts 1 µmole of GSH to GSSG in the presence of H₂O₂/min.

2.8.13 Estimation of Lipid Peroxidation (LPO) in liver tissue

The status of Lipid Peroxidation in the liver tissue homogenate was estimated according to the method of Ohkawa et al. (1976).

Principle

Malondialdehyde, a secondary product of lipid peroxidation reacts with thiobarbituric acid to form a pink chromogen (Thiobarbituric acid - 2 malondialdehyde adduct), which was measured spectrophotometrically.

Reagents

1. *Thiobarbituric acid (TBA - 0.8%)*: 800 mg of TBA was dissolved in 100 ml of warm distilled water.

2. *Sodium dodecyl sulphate (SDS - 8.1%)*: 8.1 gm of SDS was dissolved in 100 ml of distilled water.

3. *Acetic acid (20% v/v)*: 20 ml of glacial acetic acid was made upto 100 ml with distilled water.
4. \(1,1,3,3, \textit{tetraethoxypropane (malondialdehyde - MDA) standard (50mM/ml):}\) 1.197 ml of MDA was made up to 100ml in distilled water.

5. \emph{Working standard:} The stock standard solution was suitably diluted to get a final concentration of 6 nmoles/ml.

**Procedure**

0.2 ml of liver tissue homogenate, 0.8 ml of distilled water (reagent blank), standard tube containing MDA taken at a concentration range of 3 to 12 nmoles was mixed with 0.2 ml of 8.1% SDS, 1.5 ml of acetic acid and 1.5 ml of TBA. This mixture was made up to 4 ml with distilled water and was kept in boiling water bath at 90°C for 1 h. After cooling to room temperature using tap water, 1 ml of distilled water was added. The pink colour formed was measured at 532 nm against blank.

The Thiobarbituric Acid Reactive Substance (TBARS) thus measured was expressed as nmoles of MDA formed/min/mg protein.

**2.8.14 Estimation of Vitamin - C (Vit. C) in liver tissue**

The Vitamin - C content in the liver tissue sample was determined according to the method of Omaye \textit{et al.} (1979).

**Principle**

Ascorbic acid is oxidized by copper to form dehydroascorbic acid and diketogluconic acid. These products are then treated with DNPH to form a derivative i.e., bis - 2, 4-dinitrophenylhydrozone. This compound, in strong
sulphuric acid undergoes a rearrangement to form a yellowish orange product, which was measured spectrophotometrically.

**Reagents**

1. *Trichloroacetic acid (TCA - 10%)*: 10 gm of TCA was dissolved in 100 ml of distilled water.

2. *Dinitrophenylhydrazine/Thiourea/Copper sulphate (DTC) reagent*: 0.4 gm of thiourea, 0.05 gm of copper sulphate and 3.0 gm of DNPH was mixed and made upto 100 ml with 9N H₂SO₄.

3. *Sulphuric acid (H₂SO₄ - 9N)*: 25 ml of conc. H₂SO₄ was made upto 100 ml with distilled water.

4. *Sulphuric acid (H₂SO₄ - 65%)*: 65 ml of conc. H₂SO₄ was made upto 100 ml with distilled water.

5. *Ascorbic acid standard (1 mg/ml)*: 100 mg of DL - Ascorbic acid was dissolved in 100 ml of distilled water.

**Procedure**

1.0 ml of tissue homogenate was made upto 3.0 ml by addition of 10% TCA and the precipitated protein was cleared off by centrifugation. 1.0 ml of the supernatant was placed in 0.5 ml of DTC reagent and was incubated at 37°C for 3 hours. After incubation, 2.5 ml of 65% H₂SO₄ was added, mixed and kept for 30 min. The yellowish orange colour formed was measured at 520 nm in a spectrophotometer, against blank. The standard tubes containing 10 to 40 µg / ml of ascorbic acid taken in separate tubes were also treated similarly and the colour was measured against the blank. The reagent
blank was prepared by taking 1.0 ml of water, 2.0 ml of 10% TCA, 0.5 ml of DTC reagent and 2.5 ml of 65% H$_2$SO$_4$, which was treated on par with the test samples.

The amount of Vitamin - C in the liver sample was expressed as mg/gm wet tissue.

2.8.15 Estimation of Vitamin - E (Vit. E) in liver tissue

The liver tissue $\alpha$ - Tocopherol (Vitamin - E) content was estimated as detailed in Varley et al. (1976).

Principle

Tocopherol in the liver tissue was initially extracted into xylene and its content was measured using Emmeric - Eugel reaction. This reaction is based on the reduction of ferric ions (Fe$^{3+}$) to ferrous ions (Fe$^{2+}$) by the tocopherols in the presence of $\alpha$, $\alpha'$-dipryidyl, resulting in the formation of red colour, which was measured spectrophotometrically.

Reagents

1. Absolute alcohol
2. Xylene
3. $\alpha$, $\alpha'$-dipryidyl solution: 1.2 gm of $\alpha$, $\alpha'$-dipryidyl was dissolved in 1 liter of n-propanol.
4. **Ferric chloride (FeCl₃, 6H₂O) solution**: 1.2 gm of FeCl₃ was dissolved in 1 liter of ethanol and was kept in brown bottle until use.

5. **DL - α - Tocopherol standard (1 mg/ml)**: 100 mg of DL - α - tocopherol was dissolved in 100 ml of ethanol.

**Procedure**

To 1.5 ml of liver tissue homogenate, 1.5 ml of standard α - tocopherol taken at a concentration of 1.5 mg, and 1.5 ml of water (blank) taken separately in centrifuge tubes, 1.5 ml of ethanol was added and mixed well. To all these tubes 2.0 ml of xylene was added and vortexed. The protein precipitate formed was cleared off by centrifugation. 1.5 ml of xylene layer was removed and to this 1.5 ml of α, α' - dipyridyl reagent was added to each of these tubes and their optical density was measured at 460 nm using a spectrophotometer. Then 0.66 ml of FeCl₃ solution was added to these tubes and exactly 1.5 min after the addition the optical density of all the tubes i.e., standard and the test was once again read at 520 nm against the blank. The difference in optical density measured at 520 nm and 460 nm was taken for the calculation of α - tocopherol content in the liver tissue.

The amount of Vitamin - E present in the liver tissue was expressed as μg/gm wet tissue.

2.8.16 **Estimation of activity of Mg²⁺ ATPase in liver tissue**

The activity of Mg²⁺ ATPase (ATP: Phosphohydrolase - EC. 3.6.1.3.) in the liver tissue was assayed by the methods of Ohnishi et al. (1982) and Fiske and Subbarow (1925).
Principle

The liberation of inorganic phosphorous on incubation of the tissue extract in a medium containing Tris - HCl buffer, MgCl₂, and ATP was measured spectrophotometrically.

Reagents

1. **Tris - HCl Buffer (75 mM; pH - 7.5):** 591 mg of Tris-HCl was dissolved in 50 ml of distilled water and its pH was adjusted to 7.5 using 1N NaOH.

2. **Magnesium Chloride (MgCl₂ - 5mM):** 25.4 mg of MgCl₂ was dissolved in 25 ml of distilled water.

3. **Adenosine triphosphate substrate (ATP - 3mM):** 8.26 mg of ATP was dissolved in 5.0 ml of distilled water.

4. **Trichloroacetic acid (TCA - 30%):** 30 gm of TCA was dissolved and made upto 100 ml with distilled water.

5. **Ammonium Molybdate (2.5%):** 2.5 gm of ammonium molybdate was dissolved in 100 ml of 5N H₂SO₄.

6. **Sulphuric acid (H₂SO₄ - 5N):** 6.9 ml of conc. H₂SO₄ was made upto 50 ml with distilled water.

7. **1 - amino - 2 - naphthol - 4 - sulphonic acid (ANSA):** 500 mg of ANSA was dissolved in a solution containing 195 ml of 15% sodium bisulphate and 5 ml of 20% sodium sulphite and stored in brown bottle.

8. **Sodium bisulphate (15%):** 15 gm of anhydrous sodium bisulphate was dissolved and made upto 100 ml with distilled water.
9. **Sodium sulphite (20%)**: 20 gm of anhydrous sodium sulphite was dissolved and made upto 100 ml with distilled water.

10. **Standard inorganic Phosphate (80 \(\mu\)g/ml)**: 35.1 mg of potassium dihydrogen orthophosphate was dissolved in 100 ml of distilled water and this has a concentration of 80 \(\mu\)g/ml of inorganic phosphorous (Pi).

**Procedure**

0.1 ml of tissue homogenate taken in centrifuge tubes was incubated in a medium containing 1.5 ml Tris - HCl buffer, 0.1 ml MgCl\(_2\) and 0.1 ml ATP for 30 min at 37\(^0\)C. The reaction was arrested by the addition of 1.0 ml of 30% TCA. The tissue control taken separately contained the buffer, MgCl\(_2\) and ATP was also incubated on par with the test. 0.1 ml of homogenate was added in this tube after addition of 1.0 ml of TCA. The precipitate formed on addition of TCA in both the test and tissue control tubes was removed by centrifugation and the supernatant was transferred to fresh tubes. The reagent blank contained 1.8 ml of Tris - HCl buffer. The standard tubes containing Pi taken at a concentration range of 2 to 10 \(\mu\)g/ml was placed in distilled water and was made upto 1.8 ml with Tris - HCl buffer. To all the above tubes 0.5 ml of ammonium molybdate and 0.2 ml of ANSA was added and left for 20 min for the blue colour to develop, which was read at 620 nm against the reagent blank using spectrophotometer.

The activity of Mg\(^{2+}\) ATPase in the liver tissue was expressed as \(\mu\)moles of phosphorus liberated/h/gm wet tissue.
2.8.17 Estimation of activity Ca\textsuperscript{2+} ATPase in liver tissue

The activity of Ca\textsuperscript{2+} ATPase (ATP: Phosphohydrolase - EC. 3.6.1.3.) in the liver tissue was assayed as described by Hjerten and Pan (1983) and Fiske and Subbarow (1925).

Principle

The liberation of inorganic phosphorus on incubation of the tissue extract in a medium containing Tris - HCl buffer, CaCl\textsubscript{2} and ATP was measured spectrophotometrically.

Reagents

1. Tris - HCl Buffer (25mM; pH - 8.0): 98.5 mg of Tris - HCl was dissolved in 50 ml of water and its pH was adjusted to 8 using 1N NaOH.

2. Calcium chloride (CaCl\textsubscript{2} - 10mM): 36.75 mg of CaCl\textsubscript{2} dihydrate was dissolved in 25 ml of distilled water.

3. Adenosine triphosphate substrate (ATP - 2mM): 5.5 mg of ATP was dissolved in 5.0 ml of distilled water.

4. Trichloroacetic acid (TCA - 20%): 20 gm of TCA was dissolved and made upto 100 ml with distilled water.

The other reagents i.e., ammonium molybdate (2.5%), 5N H\textsubscript{2}SO\textsubscript{4}, ANSA, sodium bisulphate (15%), sodium sulphite (20%) and standard inorganic phosphate were prepared as described in the estimation of Mg\textsuperscript{2+} ATPase (Section 2.8.16).
Procedure

0.1 ml of tissue homogenate taken in centrifuge tubes was incubated in a medium containing 1.5 ml Tris - HCl buffer, 0.1 ml CaCl<sub>2</sub> and 0.1 ml ATP for 30 min at 37<sup>0</sup>C. The reaction was arrested by the addition of 1.0 ml of 20% TCA. The tissue control taken separately contained the buffer, CaCl<sub>2</sub> and ATP was also incubated on par with the test. 0.1 ml of homogenate was added in this tube after addition of 1.0 ml of TCA. The precipitate formed on addition of TCA in both the test and tissue control tubes was removed by centrifugation and the supernatant was transferred to fresh tubes. The reagent blank contained 1.8 ml of Tris - HCl buffer. The standard tubes containing Pi taken at a concentration range of 2 to 10 µg was placed in distilled water and was made up to 1.8 ml with Tris - HCl buffer. To all the above tubes, 0.5 ml of ammonium molybdate and 0.2 ml of ANSA was added and left for 20 min for the blue colour to develop, which was read at 620 nm against the reagent blank using spectrophotometer.

The activity of Ca<sup>2+</sup> ATPase in the liver tissue is expressed as µmoles of phosphorus liberated/h/gm wet tissue.

2.8.18 Estimation of activity of Na<sup>+</sup>/K<sup>+</sup> ATPase

The activity of Na<sup>+</sup>/K<sup>+</sup> ATPase (ATP: Phosphohydrolase - EC. 3.6.1.3.) in the liver tissue was assayed by Bonting (1970) and Fiske and Subbarow (1925).
Principle

The liberation of inorganic phosphorus on incubation of the tissue extract in a medium containing Tris-HCl buffer, NaCl, KCl and ATP was measured spectrophotometrically.

Reagents

1. *Tris - HCl Buffer (75 mM; pH - 7.5)*: 591 mg of Tris - HCl was dissolved in 50 ml of distilled water and its pH was adjusted to 7.5 using 1N NaOH.

2. *Magnesium Sulphate (MgSO₄ - 5mM)*: 150.37 mg of MgSO₄ was dissolved in 25 ml of distilled water.

3. *Potassium chloride (KCl - 50mM)*: 93.18 mg of KCl was dissolved in 25 ml of distilled water.

4. *Sodium chloride (NaCl - 600mM)*: 876.6 mg of NaCl was dissolved in 25 ml of distilled water.

5. *Ethylene diamine tetra acetic acid (EDTA - 1mM)*: 7.30 mg of EDTA was dissolved in 25 ml of distilled water.

6. *Adenosine Triphosphate substrate (ATP - 3mM)*: 8.26 mg of ATP was dissolved in 5.0 ml of distilled water.

7. *Trichloroacetic acid (TCA - 10%)*: 10 gm of TCA was dissolved and made upto 100 ml with distilled water.

The other reagents i.e., ammonium molybdate (2.5%), 5N H₂SO₄, ANSA, sodium bisulphate (15%), sodium sulphite (20%) and standard inorganic phosphate were prepared as described in the estimation of Mg²⁺ ATPase (Section 2.8.16).
Procedure

0.1 ml of tissue homogenate taken in centrifuge tubes was incubated in a medium containing 1.5 ml Tris - HCl buffer, 0.1 ml each NaCl, KCl, MgSO₄, EDTA and 0.1 ml ATP for 30 min at 37°C. The reaction was arrested by the addition of 1.0 ml of 10% TCA. The tissue control taken separately contained the buffer, NaCl, KCl, MgSO₄, EDTA and ATP was incubated on par with the test. 0.1 ml of homogenate was added in this tube after addition of 1.0 ml of TCA. The precipitate formed on addition of TCA in both the test and tissue control tubes was removed by centrifugation and the supernatant was transferred to fresh tubes. The reagent blank contained 1.8 ml of Tris - HCl buffer. The standard tubes containing Pi taken at a concentration range of 2 to 10 μg/ml were placed in distilled water and was made up to 1.8 ml with Tris - HCl buffer. To all the above tubes, 0.5 ml of ammonium molybdate and 0.2 ml of ANSA was added and left for 20 min for the blue colour to develop, which was read at 620 nm against the reagent blank using a spectrophotometer.

The activity of Na⁺/K⁺ ATPase in the liver tissue was expressed as μmoles of phosphorus liberated/h/gm wet tissue.

2.8.19 Estimation of Total Protein in serum and liver tissue

The total protein in serum and liver tissue sample was estimated by the method of Lowry et al. (1951).
Principle

Peptide bonds of protein was made to react with Cu\textsuperscript{2+} under alkaline condition to produce Cu\textsuperscript{+}, which reacts with phospho molybdotungstate of Folin and Ciocalteu’s phenol reagent to produce hetero polymolybdenum blue by the copper catalyzed oxidation of aromatic aminoacids i.e., tryptophan and tyrosine to produce a dark blue colour, which is measured spectrophotometrically.

Reagents

1. *Solution A*: 2.0 gm of sodium carbonate was dissolved in 0.1N NaOH and made upto 100 ml.

2. *Solution B*: 1.0 gm of sodium potassium tartarate was dissolved in distilled water and to this solution 500 mg of copper sulphate was added and made upto 100 ml.

3. *Solution C (Alkaline copper reagent)*: 50 ml of solution A was mixed with 1 ml of solution B, just before use.

4. *Folin and Ciocalteu’s phenol reagent*: Commercially purchased Folin and Ciocalteu’s phenol reagent (SRL, India) was diluted 1:2 with distilled water. This solution was prepared just before use.

5. *Protein Standard*: 10 mg of Bovine Serum Albumin (BSA) was dissolved in 10 ml of 0.1N NaOH. This solution was suitably diluted to get a final concentration of 200 \( \mu \text{g/ml} \).

Procedure

0.1 ml of serum (1 in 100 times diluted sample) or tissue homogenate and standard bovine serum albumin taken at a concentration ranging from
10 to 40 μg was made upto 1.0 ml with distilled water. To each of this tube 5.0 ml of alkaline copper reagent was added and allowed to stand at room temperature for 15 min. 0.5 ml of Folin and Ciocalteu’s phenol reagent was added and kept at room temperature for 30 min and the blue colour formed was measured at 660 nm against blank using spectrophotometer.

The protein level in the serum sample was expressed as gm/dL and in tissue homogenate as mg/gm wet tissue.

2.8.20 Extraction of lipids from tissue samples

The liver and adipose tissue lipids were extracted by the method of Folch et al. (1957).

Principle

This method involves two successive operations. In the first step, the tissues were homogenized with a mixture of chloroform : methanol (2:1 v/v) and the homogenate was filtered through a Whatmann No.1 fat free filter paper. In the second step, the filtrate containing the tissue lipids as well as non-lipid substances were placed in contact with the mineral i.e., potassium chloride. By doing so, a biphasic system without any interphasial fluff was obtained. The upper phase containing all the non-lipid substances, namely strandins was decanned, while the lower phase containing essentially all the tissue lipids was taken for assays.
Reagents

1. Chloroform
2. Methanol
3. Chloroform : Methanol mixture, 2:1 (v/v)
4. Potassium chloride (KCl - 0.37%): 370 mg of KCl was dissolved in 100 ml of distilled water.

Extraction Procedure

The adipose tissue and the liver tissue was defrosted and blotted to dryness using fat free filter paper. 100 to 150 mg of the tissue was weighed separately and was minced well with scissors. This was homogenized at cold using chloroform : methanol mixture and was filtered through a fat free filter paper into a side arm suction flask. The residue in the filter paper was scrapped off and again homogenized in 10 ml of chloroform : methanol mixture and was filtered as before. The filtrate thus collected in the suction flask was evaporated to dryness by suction using a motor type suction machine. At this point, the weight ($W_1$) of the dry suction flask was taken. To the residue in the flask 5ml of chloroform : methanol mixture was added and was shaken well. This content was transferred to a stoppered graduated centrifuge tube. The dry weight ($W_2$) of the suction flask was taken again. The difference in weight ($W_1 - W_2$) was taken as the amount of total crude lipid extracted from the liver or adipose tissue samples. A 0.1 ml of aliquot of this crude extract was stored in separate vial for the estimation of total lipids by spectrophotometric method as described in section 2.8.25. To the remaining
extract, 1 ml of 0.37% KCl was added and vortexed well. This was centrifuged at 2,500 rpm for 10 min to get a clear separation of two phases without any interphasic fluff. The clear upper phase containing non-lipid substances was aspirated. This procedure of washing and aspiration was repeated twice. The volume of final lower lipid phase was recorded for the purpose of calculation. From this lipid phase, an aliquot of 0.1 ml each was taken for the estimation of free fatty acid, triglyceride and cholesterol. The remaining lipid phase was mixed with 5 ml of chloroform, vortexed and then transferred to a side arm suction flask and was evaporated to dryness by suction. To this residue, 1 ml of chloroform was added and whole of the residue was collected into chloroform by gentle shaking. Then, the content in the flask was transferred to a clean glass vial. An aliquot of 0.1 ml of extract was taken for the estimation of phospholipids.

2.8.21 Estimation of Triglyceride (TG) in plasma and tissue samples

The Triglyceride contents of plasma and tissue extracts were estimated as described by Varley et al. (1980).

Principle

The TG present in the sample was extracted by using a mixture of isopropanol and alumina. The TG after being adsorbed into the alumina, and extracted into isopropanol, are subjected to saponification with potassium hydroxide to liberate glycerol, which was treated with acetyl acetone to give yellow colour, the intensity of which was read spectrophotometrically.
Reagents

1. Isopropanol

2. Alumina: Activity grade aluminium hydroxide was washed with distilled water to remove fine particles. The fine particle free wet aluminium hydroxide was dried overnight in an oven and stored.

3. Saponification reagent: 50 gm of potassium hydroxide was dissolved in 600 ml of distilled water and was mixed with 40 ml of isopropanol.

4. Sodium metaperiodate reagent: 77 gm of anhydrous ammonium acetate was dissolved in about 700 ml of distilled water containing 60 ml of glacial acetic acid. 650 mg of sodium metaperiodate was added to this mixture and when dissolved completely, it was made upto 1 liter with distilled water. When stored at cold in amber coloured bottle, this reagent was stable for atleast 6 months.

5. Acetyl acetone reagent: 7.5 ml of acetyl acetone was mixed with 200 ml of isopropanol and made upto a liter with distilled water. This reagent was stable for 6 months when stored in amber coloured bottle.

6. Triolein standard (1 mg/ml): 100 mg of triolein was mixed with 100 ml of isopropanol.

7. Working Standard: The triolein standard was serially diluted with isopropanol to get a final concentration at a range of 50 to 200 µg/ml, which was used for standard preparations.

Procedure

0.1 ml of aliquot of plasma or tissue lipid extract was taken in a stoppered centrifuge tube, to which 3.9 ml of isopropanol was added while shaking. Then 400 mg of alumina was added and vortexed vigorously for 10 min. To prepare plasma and tissue blank separately, 0.1ml each of
physiological saline and chloroform was taken in separate centrifuge tubes and was subjected to the above treatment. Simultaneously, the standard tubes containing working standard taken in the concentration range of 50 to 200 μg/ml were treated similarly. All the tubes were centrifuged at 2,500 rpm for 10 min. 2 ml of clear supernatant was taken and transferred to clean stoppered tubes. To this 0.6 ml of saponification reagent was added and incubated at 65°C for 15 min. After cooling, 1ml of sodium metaperiodate reagent and 0.5 ml of acetyl acetone reagent was added and mixed well. The tubes were then incubated at 50°C for 30 min to get yellow colour, the intensity of which was read at 405 nm after cooling, against the blank using spectrophotometer.

The plasma Triglyceride concentration is expressed as mg/dL and of tissue extracts as mg/gm wet tissue.

2.8.22 Estimation of Cholesterol (CHO) in plasma and tissue extracts

The total Cholesterol in plasma and tissue extracts were estimated according to the method of Varley (1967).

Principle

Zlakakis et al. (1953) used the pink colour, which cholesterol in acetic acid solution gives with ferric chloride and sulphuric acid for cholesterol estimation. This reaction was adapted here for cholesterol estimation as described by Varley (1967).
Reagents

1. Acetic acid - aldehyde free (suitable for cholesterol estimation)

2. *Ferric chloride (FeCl₃.6H₂O) - acetic acid reagent*: 50 mg of FeCl₃ was dissolved in 100 ml of aldehyde free glacial acetic acid. This solution was stable for 6 months at room temperature when stored in amber coloured bottle.

3. Sulphuric acid (conc. H₂SO₄)

4. *Cholesterol standard (1 mg/ml)*: 100 mg of cholesterol powder was dissolved in 100 ml of aldehyde free glacial acetic acid.

5. *Working standard*: The standard cholesterol was diluted suitably to give a final concentration of 25 to 100 μg/ml with ferric chloride acetic acid reagent.

Procedure

a) **Estimation in plasma**

To 0.1 ml of plasma, 10 ml of FeCl₃ - acetic acid reagent was added slowly while vortexing. This was allowed to stand for few minutes for proteins to flocculate and then centrifuge at 2500 rpm for 10 min. An aliquot of 5 ml of clear supernatant was transferred to fresh tubes. The tubes containing the working standard at concentration ranging from 25 to 100 μg/ml and separate tubes containing 0.1 ml of physiological saline (blank) was made upto 5 ml with FeCl₃ - acetic acid reagent. To all the above tubes, 3 ml of conc. H₂SO₄ was added and vortexed well for the pink colour to develop. After allowing the tubes to stand for 20 to 30 min, the tubes were read at 540 nm against blank using spectrophotometer.
b) Estimation in tissue samples

0.1 ml of aliquot of liver or adipose tissue extracts, 0.1 ml of chloroform (for blank preparation) and standard tubes containing working standard at a concentration range of 25 to 100 μg/ml were initially made up to 5 ml with FeCl₃ - acetic acid reagent. Then, 3 ml of conc. H₂SO₄ was added to each of this samples and vortexed well for colour development. After allowing the tubes to stand for 20 to 30 min, they were read at 540 nm against the blank using spectrophotometer.

The plasma Cholesterol concentration is expressed as mg/dL and that of tissue extracts as mg/gm wet tissue.

2.8.23 Estimation of Phospholipids (PL) in plasma and tissue samples

The Phospholipid content of plasma and tissue extracts were estimated by the methods of Ziversmitd and Davis (1950) and Fiske and Subbarow (1925).

Principle

The protein precipitate, obtained after addition of trichloroacetic acid to the plasma, liberates phosphorus following digestion with perchloric acid (Ziversmit and Davis, 1950). This phosphorus on treatment with acid molybdate reagent forms phosphomolybdicacid. The hexavalent molybdenum of the phosphomolybdicacid, after reduction with 1 - amino - 2 - naphthol - 4 - sulphonic acid to give a blue coloured complex, which was read at 640 nm spectrophotometrically.
Reagents

1. **Trichloro acetic acid (TCA - 5%)**: 5 gm of TCA was dissolved and made up to 100 ml with distilled water.

2. Perchloric acid

3. **Ammonium molybdate reagent (2.5%)**: 2.5 gm of ammonium molybdate was dissolved and made up to 100 ml with 5N H₂SO₄.

4. **Sodium bisulphate (15%)**: 15 gm of sodium bisulphate was dissolved and made up to 100 ml with distilled water.

5. **Sodium sulphite (20%)**: 20 gm of sodium sulphite was dissolved and made up to 100 ml with distilled water.

6. **1 - amino - 2 - naphthol - 4 - sulphonic acid (ANSA) reagent**: 500 mg of ANSA was dissolved in 195 ml of 15% sodium bisulphate and 5 ml of 20% sodium sulphite with mild heating. This was filtered through a Whatmann No.1 filter paper to remove suspended particles. The filtrate was stored in an amber coloured bottle.

7. **Phosphate standard (1 mg/10 ml)**: 0.439 gm of potassium dihydrogen ortho phosphate was dissolved and made up to a litre with distilled water. This solution contains 100 mg of phosphorus/litre.

8. **Working standard**: The standard stock was diluted serially to give a final concentration in the range of 0.5 to 2 μg/ml and was used for standard preparation.

Procedure

a) **Estimation in plasma**

To 0.1 ml of plasma, 5 ml of 5% trichloro acetic acid was added while vortexing and the precipitate formed was centrifuged at 2,500 rpm for 10 min.
The supernatant was discarded and to the tightly packed residue, 1 ml of 70% perchloric acid was added and digested in a hot sand bath till the precipitate become colourless. After cooling, it was made upto 5 ml with distilled water. To the standard tubes containing phosphorous in the concentration range of 0.5 to 2 µg/ml and to blank containing 0.1 ml of physiological saline, 1 ml of perchloric acid was added and the volume was made upto 5 ml with distilled water. All the tubes were then treated with 0.5 ml of 2.5% ammonium molybdate and 0.2 ml of ANSA reagent. After vortexing, the tubes were kept in boiling water bath for 5 min for the blue colour to develop. After cooling to room temperature, the tubes were read at 640 nm against the blank using spectrophotometer.

The Inorganic Lipid Phosphorus, thus estimated was converted to phospholipid (while calculating) by multiplying the phosphorus level with factor 25, adapting the method of Frings and Dunn (1972). The phospholipid levels were expressed as mg/dL of plasma.

b) Estimation in tissue samples

To 0.1 ml of the tissue lipid extract and to 0.1 ml of chloroform (for blank preparation), 0.5 ml of perchloric acid was added and digested in hot sand bath till the preparation become colourless. After cooling, the tubes were made upto 5 ml with distilled water. The standard tubes containing phosphorous in a concentration ranging from 0.5 to 2 µg/ml were made upto 5 ml with distilled water. The tubes were then treated with 0.5 ml of 2.5% ammonium molybdate and 0.2 ml of ANSA reagent. After vortexing, the
tubes were left at room temperature for 20 min for colour development. The blue colour developed was read against the blank at 640 nm using spectrophotometer.

The PL levels in liver tissue are expressed as mg/gm wet tissue.

2.8.24 Estimation of Free Fatty Acid (FFA) in plasma and tissue samples

The plasma and tissue Free Fatty Acid content were estimated by the method of Chromy et al. (1977) and Regouw et al. (1971).

Principle

The tissue and plasma free fatty acid were extracted into organic solvents using stable copper reagent to form free fatty acid - copper soap (FFA - Cu soap) (Chromy et al., 1977). The FFA - Copper soap was then coloured with sodium diethyl dithio carbamate (Ragouw et al., 1971) and estimated spectrophotometrically.

Reagents

1. *Extraction solution (Chloroform : Heptane : Methanol - CHM):* 280 ml of chloroform was mixed with 210 ml of heptane and 10 ml of methanol. This was prepared just before use.

2. *Stable copper reagent:* 0.75 gm of sodium citrate dihydrate, 6.75 gm of triethanolamine, 3.25 gm of copper nitrate trihydrate and 25 gm of sodium chloride were dissolved in distilled water and made upto 100ml. While stored at cold, this reagent remains stable for atleast for 6 months.
3. *Diethyl dithio carbamate reagent (DDC - 0.1%)*: 100 mg of DDC was dissolved in 100 ml of n-butanol. This was prepared just before use.

4. *Palmitic acid standard (1 mg/ml)*: 100 mg of palmitic acid was dissolved in 100 ml of CHM.

5. *Working standard*: The stock standard was diluted serially with CHM to give a final concentration in the range of 5 to 20 µg/ml and was used for standard preparation.

**Procedure**

0.1 ml aliquot of plasma or tissue extract was taken in stoppered centrifuge tube and the volume was made upto 3.5 ml with CHM. To this 0.5 ml of stable copper reagent was added and vortexed vigorously for 5 min. A blank containing 0.1 ml of chloroform (tissue extract blank), physiological saline (plasma blank) and standard tubes containing the working standards in a range of 5 to 20 µg/ml were also treated similarly. The tubes were then centrifuged at 2,500 rpm for 10 min. 2 ml of the clear supernatant was carefully removed and transferred (without touching on the sides or bottom of the tube) to fresh tubes. The colour was developed by the addition of 1 ml of 0.1% DDC and was read against the blank at 437 nm using spectrophotometer.

The plasma Free Fatty Acid concentration was expressed as mg/dL and that of tissue as mg/gm wet tissue.
2.8.25 Estimation of Total Lipids (TL) in plasma and tissue extracts

The Total Lipid concentration of plasma and tissue extracts were estimated by the method of Frings and Dunn (1970).

Principle

Drevon and Smith (1964) reported on the colour reaction given by lipids with vanillin in a medium of sulphuric acid and phosphoric acid. Frinks and Dunn (1970) improved the sulpho - phospho - vanillin reaction for the total lipid estimation, which was adapted here for the total lipid estimation of plasma and tissue extracts.

Reagents

1. Sulphuric acid (conc. H₂SO₄)

2. *Vanillin (0.6%)*: 600 mg of vanillin was dissolved in 100 ml of distilled water.

3. *Phospho - Vanillin reagent*: 800 ml of conc. phosphoric acid was added to 200 ml of 0.6% vanillin slowly while stirring. When stored in amber coloured bottle at room temperature this reagent was stable for atleast 6 months.

4. *Standard total lipid (1 mg/ml)*: The total volume (3 to 3.5 ml) of the lipid extract obtained from 100 to 150 mg of liver and/or adipose tissue taken from untreated control animals using extraction procedure (as detailed under section 2.8.20 for extraction of lipids) was diluted suitably to get a final concentration of 1 mg/ml and this preparation was used as stock standard.

5. *Working standard*: The stock standard was diluted suitably with chloroform to get a final concentration of 50 to 200 μg/ml, which was used for standard preparation.
Procedure

0.1 ml aliquot of plasma or liver tissue extract was made upto 2 ml with conc. H$_2$SO$_4$ and was vortexed well. Standard tubes containing the working standards taken in a concentration range of 50 to 200 µg/ml, 0.1 ml of physiological saline (plasma blank) and 0.1ml of chloroform (tissue blank) taken in separate tubes were also treated similarly. These tubes were kept in boiling water bath for 5 min. After cooling to room temperature 0.1 ml of this aliquot was transferred to fresh tubes and to this was added 5 ml of phospho-vanillin reagent while vortexing. The tubes were then incubated at 37°C for 5 min. After cooling to room temperature the pink colour formed was measured at 540 nm against the blank within 10 min using a spectrophotometer.

The Total Lipids levels in the plasma is expressed as mg/dL and that of tissue as mg/gm wet tissue.

2.9 Histopathology of liver tissue

To investigate the histopathological changes in the liver tissue of various treatments, permanent mounts of the liver tissue were prepared as detailed by Bancroft and Cook (1984).

Principle

The formalin fixed tissues were washed and subjected to dehydration in ascending grades of alcohol. The tissues were blocked in paraffin wax,
sectioned into ribbon and fixed on glass slides. After staining and destaining with hematoxylin and eosin, they were permanently mounted with DPX mount for histopathological studies.

Reagents

1. *Phosphate buffered formalin (0.1M; pH - 7.4)*: 420 ml of 0.1M disodium hydrogen phosphate was mixed with 0.1M potassium dihydrogen orthophosphate until the pH was adjusted to 7.4. A 10% (v/v) solution of formalin was prepared with this phosphate buffer.

2. *Hematoxylin stain*: 1 gm of hematoxylin stain was dissolved in 10 ml of absolute alcohol and mixed with a solution containing 20 gm of potassium alum, previously dissolved in hot distilled water. After bringing this mixture to boiling point, 0.5 gm of mercuric oxide was added. This mixture was cooled rapidly under tap water. This was then filtered and left to ripe for 10 to 15 days.

3. *Eosin stain (0.5%)*: 500 mg of eosin yellow powder was dissolved in 100 ml of distilled water and stored.

Chemicals

1. Absolute alcohol
2. 30, 50, 70 and 90% alcohol
3. Xylene
4. Paraffin wax
5. Egg albumin
Procedure

Approximately, 5 to 10 mm$^3$ of liver tissue, fixed in formal saline was washed thoroughly in running water. It was dehydrated by placing the fixed tissues in an increasing order of alcohol (30, 50, 70, 90 and 100%) for 30 min. The tissue was then transferred to xylene for clearing and subjected to cold infiltration at room temperature in a mixture containing paraffin wax dissolved in xylene for 30 min. The tissues were then hot infiltrated in oven at 58 to 60°C in molten paraffin wax for 1 h. The hot infiltrated tissues were removed and blocked quickly using metal blocks. The solidified blocks were trimmed to small size and sectioned using microtome to get ribbons of 8 to 10 micron thickness. The ribboned sections were placed on glass microscopic slides coated with egg albumin. The microscopic slides were then exposed to the decreasing order of alcohol (100, 90, 70, 50 and 30%) for 5 min each. The sections were then dipped in hematoxylin stain for 5 min and washed in running tap water for 3 to 5 min. After drying, the tissues were exposed to 0.5% eosin stain for 5 min and washed again in running tap water to remove the excess stain. The slides were dried and dehydrated in an increasing order of alcohol (30, 70, 90) for few seconds. After drying, the slides were cleared in xylene twice for 10 min. The tissue sections were then permanently mounted with DPX mountant. The permanently mounted sections of liver tissue were observed under light microscope for histopathological evaluations.
2.10 Assessment of hepatoprotective and antioxidant properties of ELE and SIL against CCl₄, INH and antitubercular drugs (INH+RIF+PYR) induced hepatotoxicity in rats

The percentage (%) hepatoprotective and antioxidant properties of ELE and SIL against CCl₄, INH alone and antitubercular drugs (INH+RIF+PYR) induced hepatotoxicity was calculated by applying the following formula as detailed by Rajesh and Latha (2004).

\[
% = 1 - \frac{(HC - N)}{(C - N)} \times 100
\]

where,

\[
HC : \text{Mean values of the parameter in hepatotoxic agents and ELE/SIL treated groups.}
\]

\[
N : \text{Mean value of the parameter in normal control group.}
\]

\[
C : \text{Mean value of the parameter in hepatotoxicity induced group.}
\]

This formula was applied to the corresponding parameters of various markers of liver toxicity as well as antioxidant parameters for the assessment of % of hepatoprotection and % of antioxidant activity exerted by ELE and SIL in serum and liver tissue of rats.

2.11 Statistical analysis

The data were subjected to One-way Analysis Of Variance (ANOVA) and Tukey’s Multiple Comparison Test was done to evaluate the significance of difference of means of various treatment groups, using SPSS statistical package (Version: 7.5). The values are presented as mean ± S.E. and P value < 0.05 was considered significant.