List of chemicals procured from different companies

Sigma chemicals Co. St. Louis, Mo, USA
Bovine serum albumin (BSA)
Isoproterenol (ISO)

SISCO Research Laboratories Private Ltd., Maharashtra, India
L-Aspartic acid
α-Ketoglutaric acid

Qualigens, Maharashtra, India
Cholesterol
Dipotassium hydrogen ortho phosphate
Ethylene diamine tetra acetic acid disodium salt (EDTANa₂)
Folin & Ciocalteu reagent (2N)
Hydrogen peroxide
Magnesium sulphate
Potassium dihydrogen ortho phosphate
Sodium carbonate
Sodium chloride
Sodium dihydrogen ortho phosphate
Trichloroacetic acid
Trisodium citrate
Sd-fine Chemicals, Maharastra, India

2,4-Dinitro phenyl hydrazine (DNPH)
Ortho phosphoric acid

Merck AG, Darmstadt, Germany

Sodium disulphite
Sodium sulphite
Potassium sodium tartarate

All other chemicals used for the estimations were of analytical grade (AR).

*Tribulus terrestris fruit aqueous extract (TTFAEf)*

Tribulus terrestris fruit aqueous extract was received as a gift from Chemiloids (manufactures and exports of herbal extracts) Vijayawada, Andhra Pradesh (India). The extract was suspended in distilled water prior to use.
2.1 Animal ethical clearance

Local Institutional Animal Ethical Committee of our University, obtained ethical clearance for conducting experiments on animals from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (Regd. no. 470/01/a/CPCSEA). The present work was carried out with a prior permission from Local Institutional Animal Ethical Committee.

2.2 Procurement of animals and maintenance

Male albino Wister rats of body weight 130-150 g were procured from Sri Venkateswara enterprises, Bangalore. Animals were maintained as per the guidelines of NIN animal user's manual. Animals were acclimatized for 7 days to the conditions of animal house, which was maintained at temperature of 22 to ± 2° C. The animal room was regulated by a 12h light: 12h dark schedule. Two to three animals were housed per cage, sized 41cm length, 28 cm width and height of 14 cm. Paddy husk was used for bedding and on very alternative day bedding was changed and washed thoroughly with water along with Domex, a disinfectant and detergent. The rats were fed on a standard pellet diet purchased from Sai Durga Feeds and Foods, Bangalore, and water ad libitum.

2.3 Induction of Cardiac stress in rats

Cardiac stress was induced in rats by intra peritoneal injection of Isoproterenol at a concentration of 85mg kg⁻¹ body weight. ISO solution was given once a day for two days with an interval of twenty four hours. ISO was first weighed individually in eppendorf tubes for each animal according to the weight and then solubilized, just prior to injection in 0.2ml of physiological saline.
2.4 Experimental Design

In the present study, a total of 24 rats were used. The rats were divided into four groups of six rats each.

Group 1: Normal control rats.

Group 2: Rats treated with *Trichosanthes cucumerina* Fruit aqueous Extract (TTFAEt) (50 mg kg\(^{-1}\) body weight, for 40 days).

Group 3: ISO administered rats (85 mg kg\(^{-1}\) body weight, once a day for two days).

Group 4: TTFAEt pretreated (50 mg kg\(^{-1}\) body weight) for 40 days and then i.p. injected with ISO (85 mg kg\(^{-1}\) body weight, once a day for two days). The dose was fixed according to previous literature. Incubation was accomplished by means of a slightly bend steel incubation needle with ball like thickening terminal, attached to a syringe and extract was infused down the food pipe.

2.5 Animal sacrifice and organ collection

After the experimental period of 40 days, animals from all three groups were sacrificed by cervical dislocation and immediately hearts were removed and washed thoroughly with ice-cold saline (0.9 % NaCl. Hearts of every animal was suspended in 0.15M potassium chloride in polypropylene containers, sealed with parafilm, labeled carefully and frozen at -80 °C until assays were carried out.

2.6 Total lipid extraction

Total lipid was extracted from the heart tissue according to the method of Folch et al (1951). A known volume of suspension was mixed with 10 ml of chloroform – methanol mixture (2:1 v/v) and shaken vigorously. Then it was filtered through Whatmann filter paper (No.42) into a separating funnel. The filtrate was mixed with 0.2 ml of physiological saline and the mixture was kept overnight undisturbed.
2.6.2 Total cholesterol

**Principle:** Cholesterol esterase hydrolyses cholesterol esters into free cholesterol and fatty acids. Cholesterol oxidase converts cholesterol to cholest-4-en-3-one and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide oxidatively couples with 4-aminoantipyrine and phenol to produce red quinoneimine dye which has absorbance maximum at 510 nm. The intensity of the red colour is proportional to the amount of total cholesterol, (Allain, 1974).

**Reagents:**
1. Accurex Infinite cholesterol liquid: 50mM buffer pH 6.8, 100 IU/L Cholesterol oxidase, 150 IU/L Cholesterol esterase, 500 IU/L Peroxidase, 0.5 mM 4-amino antipyrene, 10mM Phenol and stabilizers are the components of this reagent.
2. Cholesterol Standard: 200 mg %.

**Procedure:** To 0.01 ml of Cardiac homogenate, 1.0 ml of the reagent was added, mixed and incubated at 37 °C for 10 min. Cholesterol standard and water blank were also treated in a similar manner. After incubation, absorbance was read at 510 nm and values are expressed as mg/dL.

2.6.3 Triglycerides

**Principle:** Glycerol released from hydrolysis of triglycerides by lipoprotein lipase is converted by glycerol kinase into glycerol-3-phosphate, which is oxidized by glycerol phosphate oxidase to Dihydroxyacetone phosphate and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide oxidizes phenolic chromogen to a red coloured compound which is measured at 510nm. (Foosati et al., 1982).

**Reagents:**
1. Accurex Infinite triglyceride liquid;
2. Lipase \( \geq 4KU/L \)
Glycerol Kinase \( \geq 40\text{U/L} \)
Glycerol phosphate oxidase \( \geq 5\text{KU/L} \)
Peroxidase \( \geq 820\text{U/L} \)

2. Triglyceride Standard: \(200\text{mg\%}\)

**Procedure:** To 0.01ml of serum cardiac homogenate, 1.0ml of the reagent was added, mixed and incubated at 37°C for 10 min. Triglyceride standard and water blank were also treated in a similar manner. After incubation, absorbance of the standard and serum was read at 510 nm against and values are expressed as mg/dL.

### 2.6.4 High density lipoprotein cholesterol (HDL)

Phosphotungstate/Mg\(^{2+}\) precipitates chylomicrons and low density lipoprotein (LDL) fractions. High density lipoprotein (HDL) fraction remains unaffected in supernatant. Cholesterol content of HDL fraction is assayed using Autozyme cholesterol diagnostic kit (Assmann, 1983).

**Reagents:**
1. Precipitating reagent: 2.4 mM Phosphotungstic acid and 40 mM magnesium chloride
2. Autozyme HDL cholesterol working solution.

**Procedure** To 0.2 ml of cardiac homogenate, 0.2 ml of precipitant reagent was added, mixed and centrifuged at 4,000 rpm for 10 min to obtain a clear supernatant. To 0.05 ml of supernatant, 1.0 ml of Autozyme of HDL cholesterol working solution was added, incubated for 10 min at 37°C and colour developed was read at 510 nm against a blank and a standard (50 mg\%) was run simultaneously. Values are expressed as mg/dL.

### 2.6.5 Very Low Density Lipoprotein (VLDL) and Low Density Lipoprotein (LDL)

VLDL and LDL were calculated using the Friedewald et al (1972) formulas.
As follows:

\[ \text{VLDL} = \frac{\text{TG}}{5} \]

\[ \text{LDL} = \text{Total CHL} - \frac{\text{TG}}{5} + \text{HDL} \]

2.7 Sample collection and preparation for biochemical estimations and assays

Blood collection and serum separation

Blood was collected in eppendorf tubes from 12 h fasted rats by means of capillary tube through retinobital flexus after second dose of ISO administration. The blood collected without added anticoagulant was allowed to clot for 30 min and the serum was separated by centrifugation. Serum was used for the analysis of electrolytes, minerals and estimation of albumin and globulin.

2.7.1 Sodium

Serum sample was analyzed for sodium by magnesium uranyl acetate using the method of Trinder (1951). Sodium is precipitated as the uranyl zinc acetate. The uranyl radical is then converted to uranyl potassium ferrocyanide and the colour developed read at 480 nm. To 0.1 ml of serum, 5 ml of magnesium uranyl acetate reagent (8 g uranyl acetate, 30 g magnesium acetate, 30 ml of glacial acetic acid in 150 ml water boiled and made up to 1000 ml with absolute ethanol , mixed with 1 ml 1% NaCl, stored in brown bottle) was added, stoppered and allowed to stand for 5 min. The tube was shaken vigorously for 30 sec and centrifuged .To the two ml of the supernatant in to a 100 ml volumetric flask to which 80 ml of acetic acid (1%) and 4 ml of 10 % potassium ferrocyanide were added and made up with acetic acid. It was mixed thoroughly and intensity of the colour read with in 10 min at 480 nm, against blank reagent. Standardard sodium (200 mmol/L) was also treated similarly.
2.1.2 Potassium

The serum sample was analyzed for potassium by the method of Jacobs and Hoffmann (1931).

Potassium is precipitated as the sodium potassium cobaltinitrite. The precipitate is decomposed and the nitrite determined by the sulphonic acid naphthylamine reaction.

To 1 ml of serum, 1.2 ml of sodium cobaltinitrite regent [solution containing 25 g cobaltous nitrate, 50 ml water and 12.5 ml glacial acetic mixed with 120 g sodium nitrite in 180 ml water] was added with shaking and allowed to stand for 45 min at a moderate speed. The supernatant was removed and washed with water. Then 2 ml of 70 % ethanol was added and the precipitate was mixed, centrifuged for 5 min, inverted and drained. The washing with ethanol was repeated. After the final draining, 2 ml of water was added and placed in a water bath for 10 min and shaken frequently to dissolve the precipitate, allowed to cool and 1 ml of 1 % chlorine hydrochloride and 2 % sodium ferrocyanide were added and made up to 6 ml mark. At the same time, 2 ml of cobalt standard (5 mEq/L) was treated similarly. The intensity of the colour was red at 620 nm against a blank.

2.7.1.3 Calcium

Serum calcium was estimated by OCPC method as described by Stem and Lewis, (1957). Calcium in alkaline medium reacts with O-creasolthalein complexone to form a purple colour complex whose absorbance is proportional to the calcium concentration. Interference due to magnesium and iron is eliminated by using 8- hydroxyquinoline. To 0.02 ml standard / serum 2 ml of buffer solution was added and mixed well and incubated at room temperature for 5 min. The absorbance of the standard and test was measured against blank at 570 nm within 30 min.
2.7.1.4 Iron

Serum iron content was estimated by the method of Ramsay (1957). Equal volumes of serum (50μl) were mixed in glass stoppered centrifuge tubes. The tubes were heated in a boiling water bath for 5 min. the content were cooled and 12 ml of chloroform was added to each tube. The tubes were stoppered and mixed vigorously or 30s and centrifuged for 5 min at 1000 rpm. The colour intensity was measured at 520 nm.

2.7.2 Albumin and globulin

Serum albumin and globulin were estimated by the method described by Wotton, (1964). To 5.5 ml of 18% sodium sulphite solution (28%), 100μ of serum was added, mixed by inversion and was followed by the addition of 1ml of ether. The tube was stoppered and gently inverted for 20 min and the contents were centrifuged for 10 min. The globulin disc was pushed aside and 3 ml of BSA solution (50 mg BSA in 10ml of 9% NaCl) to 3ml water (blank), to 4ml supernatant; 5ml of biuret reagent was added and the intensity of the colour developed was measured after 10 minutes at 540 nms.

Globulin (g/100ml) = Total protein – Albumin.

2.8 Preparation of liver homogenate

For 10 % liver tissue homogenate, liver was weighed accurately and homogenized with help of Teflon homogenizer in 0.01 ml of tris Hcl buffer (pH 7.5) in ice cold condition. The homogenate was centrifuged at 250 g and the clear supernatant solution was used for the estimation of liver tissue marker enzymes.
2.8.1 Alanine aminotransferase (ALT)

**Principle:** The enzyme alanine aminotransferase is widely distributed in a variety of tissue sources. The major source of ALT is of hepatic origin, and elevated levels are found in hepatitis, obstructive jaundice and in myocardial infarction. The enzymatic reaction sequence in the assay is as follows (Expert panel, 1976).

\[
\text{\(\alpha\)-keto glutarate + L-alanine} \leftrightarrow \text{L-glutamate + pyruvate.}\]

**Reagents:** Reconstituted reagent: \(\alpha\)-keto glutamate 13mM

- L-Alanine 400mM
- NADH 0.2 mM
- LDH 800mM
- Buffer 100mM, (pH 7.5)

**Procedure:** To 100 µl of Liver homogenate, 1ml of reconstituted regent was added, mixed and incubated at 37 °C for 1 minute. After incubation, change in the optical density was measured up to 3 min at an interval of 1 min against reagent blank at 340nm. The activity of glutamate oxaloacetate transaminase is expressed as IU/L of Liver homogenate.

2.8.2 Aspartate aminotransferase (AST)

Aspartate aminotransferase (AST) is also known as glutamic oxaloacetic transaminase is a tissue enzyme that catalyses the transfer of an amino and keto group between \(\alpha\)-keto acids. It is widely distributed in tissues mainly heart, liver, muscle and kidney, and injury to these tissues results in the release of AST into the circulation (Expert panel, 1976).

\[
\text{\(\alpha\)-keto glutarate + L-aspartate} \leftrightarrow \text{L-glutamate + oxaloacetate \rightarrow Pyruvate}\]

**Reagents:** Reconstituted reagent: 2-oxoglutarate 12mM
L. Aspartic acid -200mM
NADH-0.19mM
LDH - 800 U/L
MDH - 600 U/L
Buffer -100mM (pH-7.8)

Procedure: To 100 μl of liver homogenate, 1ml of reconstituted regent was added, mixed
and incubated at 37 °C for 1 minute. After incubation, change in the optical density was
measured upto 3 min at an interval of 1 min against reagent blank at 340 nm. The activity
of glutamate oxaloacetate transaminase is expressed as IU/L of Liver homogenate.

2.8.3 Lactate Dehydrogenase (LDH)

Principle: The enzyme LDH is distributed in tissues particularly in heart, muscle and
kidney. LDH catalyses the oxidation of lactate to pyruvate in the presence of NAD which
is subsequently reduced to NADH. The rate of NADH formation Measured at 340nm is
directly proportional to serum LDH activity (Dawson, et al., 1965).

Working reagents: L-Lactate 75 mM
NAD 5.5 mM
Buffer 8.0 mM (pH-9.0).

Procedure: The activity of liver homogenate LDH was assayed by the Teco Diagnostic
Kit Method. 1 ml working reagent was added to 25 ul of sample, mixed and incubated at
37°C for 1 min. After incubation, change in the optical density was measured for 3 min at
an interval of 1 min against reagent blank at 340 nm. The activity of serum LDH is
expressed as IU/L of serum.
2.9 Histopathological studies

After sacrificing the rats, liver were removed and cut into small pieces and preserved in buffered formalin for histomorphological examination.

2.9.1 Fixation

Tissue blocks with 3 mm thickness were cut from small pieces of hepatic tissue and were placed in a fixative solution (pH 7.0), prepared by adding 100ml of 37-40% formaldehyde, 900ml of distilled water, in which 4g of Na$_2$HPO$_4$ and 6.5g of NaH$_2$PO$_4$ were dissolved.

2.9.2 Tissue processing

The tissue block was processed through a series of solvents: alcohol 80%-1hr, alcohol 95%-1hr, alcohol 95%-2 changes-1hr each, isopropyl alcohol-1hr, acetone-2 changes-1hr each, chloroform-3 changes-1hr each and paraffin-2 changes-1hr each as per scheduled for dehydration, clearing and paraffin infiltration. This block was then ready for embedding. During the process of embedding, the tissue blocks were oriented so that sections were cut in the desired plane of the tissue. Two L-shaped metal moulds were laid on metal plate so as to enclose a rectangular or square space. This was then partly filled with melted paraffin and the tissue was placed in it in the desired position. The container was then filled with melted paraffin and allowed to cool until reasonably firm so that the set block of paraffin with the tissue can be removed from the moulds. The block was trimmed to a suitable size and fixed on a metal objects holder. The block was further trimmed so that paraffin overlaying the piece of tissue is excluded and an adequate area of the tissue facing the knife is exposed. The block was then kept for cooling at 0°C.
2.9.3 Section Cutting

The sections were cut at 5μm thickness and floated in a water bath between 38-49°C. The sections from the water were mounted on clean glass slides, which have been smeared with a drop of Mayer’s egg albumin. They were then dried on a hot plate at 50°C for 30 min and the sections on the slides were then subjected to staining.

2.9.4 Staining

The slide containing the section was processed serially as follows; xylol 1-3 min, xylol II-3 min, acetone-3 min, 95% alcohol-3 min, running water-3 min, hematoxylin stain-20 min, wash in running tap water-20 min, eosin working solution-2 min-15 sec, 95% alcohol-2 to 3 dips, 95% alcohol-2 changes 1 to 2 min each, acetone-2 changes-3 min each, xylol-2 changes-3 min each and mounted in D.P.X. and viewed under microscope. The nuclei were stained with blue and cytoplasm in various shades of pink (Raghuramulu et al., 1983).

2.10 Statistical analysis

All the results were expressed as mean ± SE of a six individual observations. Duncan’s Multiple Range (DMR) test was performed to know the level of significance among all the experimental groups (Duncan, 1955).