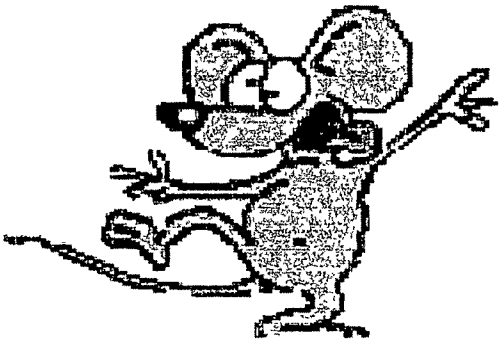
CHAPTER 3



MATERIAL AND METHODS

3.1 MATERIALS

3.1.1 Composition

(a) Each gram of **DHC-1** (manufactured by Himalaya Drug Company, Bangalore, India) contains extracts of *Bacopa monnieri* Linn. (Scrophulariaceae; Whole plant; 200mg), *Emblica officinalis* Gaertn. (Euphorbiaceae; Fruit; 200mg), *Glycyrrhiza glabra* Linn. (Papilionaceae; Roots; 200mg), *Mangifera indica* Linn. (Anacardiaceae; Bark; 200mg) and *Syzygium aromaticum* Linn. (Myrtaceae; Flower bud; 200mg).

(b) Each gram of **Activit** (manufactured by Ayur Herbals Pvt. Ltd., Baroda, India), contains extracts derived from *Mucuna pruriens* (L.) DC. (Fabaceae; Seeds; 200mg), *Withania somnifera* (L.) Dunal (Solanaceae; Roots; 200mg), *Argyreia speciosa* (L.) Sweet (Convolvulaceae; Roots; 100mg), *Centella asiatica* (L.) Urban (Araliaceae; Aerial parts; 100mg), *Tribulus terrestris* (L.) (Zygophyllaceae; Fruit; 100mg), *Asparagus racemosus* Willd. (Asparagaceae; Roots; 100mg), *Piper longum* (L.) (Piperaceae; Berries and spikes; 40mg), *Anacyclus pyrethrum* (L.) (Compositae; Roots; 40mg), *Strychnos nux-vomica* (L.) (N.O. Loganiaceae; Seeds; 20mg), *Tinospora cordifolia* Miers. (Menispermaceae; Roots; 20mg) and shring bhasma (80mg).

(c) Each gram of **Pepticare** (manufactured by Ayur Herbals Pvt. Ltd., Baroda, India) contains powders of *Tinospora cordifolia* Miers (Menispermaceae; Roots; 300mg), *Emblica officinalis* Gaertn. (Euphorbiaceae; Fruit; 200mg), *Glycyrrhiza glabra* Linn. (Papilionaceae; Roots; 300mg) alongwith Sootshekhar ras (70mg), Praval bhasma (30mg), Swarnabhasma (20mg) and Kapardi bhasma (80mg).

(d) Each gram of **Normacid** (manufactured by Ayur Herbals Pvt. Ltd., Baroda, India) contains Bhunimbadi Kwath (500mg), *Solanum nigrum* (Solanaceae; Fruit; 200mg), Mouktika bhasma (60mg), Shuddha Gairika bhasma (60mg), Kapardi bhasma (120mg), Swarnabhasma (20mg), Praval bhasma (30mg) and Shankh bhasma (10mg).

(e) **Drug-X** (manufactured by Alembic Chemicals Ltd., Baroda, India) contains powder of fruits of *Moringa pterygosperma* or *Moringa oleifera* Lam (family: Moringaceae).

3.1.2 Sources of Fine Chemicals

(a) 1,1,3,3-tetraethoxypropane, crystalline beef liver catalase, superoxide dismutase, isoproterenol and 1,1-diphenyl-2-picryl hydrazyl (DPPH) were obtained from Sigma Chemicals, St. Louis, M.O., U.S.A.

(b) Thiobarbituric acid, tris buffer, sucrose, ATP, reduced glutathione, 1-amino-2-naphthol-4-sulphonic acid, 5,5'-dithiobis (2-nitro benzoic acid), bovine serum albumin, riboflavin, nitro blue tetrazolium (NBT), ascorbic acid and pyrogallol were obtained from Hi-Media Laboratories Pvt. Ltd., Mumbai, India.

(c) Trichloroacetic acid, ammonium molybdate, citric acid monohydrate, sodium nitrate, sulphanilic acid, hydrogen peroxide, copper sulphate, sodium potassium tartarate, sodium metaperiodate, methanol, ethanol and Folin's phenol reagent were obtained from S.D.Fine Chemicals, Mumbai, India.

(d) Sodium hydroxide, sodium carbonate, sodium bicarbonate, magnesium chloride, sodium chloride, potassium chloride, calcium chloride, disodium hydrogen orthophosphate, potassium dihydrogen orthophosphate, carbon tetrachloride, chloroform, ether, hydrochloric acid and conc. sulphuric acid were purchased from Qualigens Chemicals Ltd., Mumbai, India

(e) Ethylenediaminetetraacetic acid disodium salt and epinephrine bitartarate were obtained from BDH Chemicals, Mumbai, India.

(f) Cisplatin injections (Cisplat 10) were purchased from the local market.

(g) DHC-1 was obtained from Himalaya Drug Company, Bangalore, India. Activit, Pepticare and Normacid were supplied by Ayur Herbals Pvt. Ltd., Baroda, India. Drug-X was obtained from Alembic Chemicals Ltd., Baroda, India.

All the reagents and chemicals used in the entire study were of analytical grade.

3.1.3 Animals

Albino rats of Wistar strain weighing between 150-225gms and healthy male adult rabbits weighing between 1.5-2.5 kg were used for the experiments. The animals were fed *ad libitum* with standard pellet diet and had free access to water.

The Institutional Ethical Committee approved all the protocols of the study.

3.2 METHODS (EXPERIMENTAL DESIGN)

The study was designed to evaluate the antioxidant activity of the formulations DHC-1, Activit, Pepticare, Normacid and Drug X. The study was categorized into two sections: *in vitro* and *in vivo* studies.

3.2.1 *IN VITRO* STUDIES

The free radical scavenging property of DHC-1, Activit, Pepticare and Normacid was examined *in vitro* in two systems, namely radical scavenging activity by DPPH reduction (DPPH assay) and superoxide radical scavenging activity in riboflavin/light/NBT system.

3.2.1.1 DPPH assay

Principle

DPPH assay was used as a rapid method to provide an evaluation of antioxidant activity due to free radical scavenging. Antiradical activity was measured by a decrease in absorbance at 516 nm of a methanolic solution of coloured DPPH brought about by the samples. 2,2 diphenyl-1-picrylhydrazyl radical (DPPH) a purple colored stable free radical is reduced into the yellow coloured diphenylpicryl hydrazine (Vani et al., 1997).

Reagents

1. *DPPH stock solution*

   1.3mg of 1,1-diphenyl-2-picryl hydrazyl (DPPH) was dissolved in 1ml of methanol.

2. *Methanolic extracts of drugs*

   100gms of drug (DHC-1, Activit, Pepticare and Normacid) was extracted with methanol (3 x 250 ml) by cold maceration and solvent was evaporated.

Procedure

100µl of suitably diluted stock solution of methanolic extracts of the drugs were mixed with 3ml of methanol. 75µl of DPPH solution was added and decrease in the absorbance was noted after 15 minutes at 516nm against methanol as blank. Pyrogallol was used as positive control. The percent inhibition by sample exposure was determined by comparison with a methanol-treated control group.

The obtained data was used to determine the concentration of the sample required to scavenge 50% of the DPPH free radicals (IC_{50}). The percent inhibition was plotted against the concentration and the IC_{50} was obtained. A lower IC_{50} denotes a more potent antioxidant. The results were expressed as the mean ± SEM of three replicates. IC_{50} was calculated from % inhibition.

3.2.1.2 Assay for superoxide radical scavenging activity

Principle

The assay was based on the capacity of the methanolic extract of drug to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system (Beauchamp and Fridovich, 1971).

Reagents

1. *Phosphate Buffer (50mM, pH 7.6)*

(a) 2.72gms of potassium dihydrogen orthophosphate (KH_2PO_4) was dissolved in 60 ml of distilled water. The volume was then made upto 100ml with distilled water.

(b) 800mg of sodium hydroxide (NaOH) was dissolved in 60 ml of distilled water. The volume was then made upto 100ml with distilled water.

50ml of (a) was mixed with 42.4ml of (b). The pH of the mixture was adjusted to 7.6 with 1N hydrochloric acid (HCl). The volume was then made upto 200ml with distilled water.

2. *Riboflavin*

5mg of riboflavin was dissolved in 25ml of phosphate buffer (50mM, pH 7.6).

3. *EDTA (12mM)*

402mg of EDTA was dissolved in 5ml of buffer. The volume was then made upto 10ml with phosphate buffer.

4. *Nitro blue tetrazolium (NBT) (0.1%)*

5mg of NBT was dissolved in 2ml of buffer. The volume was then made upto 5ml with phosphate buffer.

Procedure

The reaction mixture contained 2.5ml buffer, 100µl riboflavin solution, 200µl of EDTA solution, 100µl of diluted methanolic extract of drug and 100µl of NBT solution, added in the mentioned sequence. Reaction was started by illuminating the reaction mixture with different concentrations of sample extract in UV light for 5 minutes. Immediately after illumination the absorbance was measured at 290nm. Ascorbic acid was used as positive control. The percent inhibition by sample exposure was determined by comparison with a methanol-treated control group.

The obtained data was used to determine the concentration of the sample required to scavenge 50% of the superoxide radicals (IC_{50}) as mentioned above.

3.2.2 *IN VIVO* STUDIES

The formulations (a) DHC-1, (b) Activit, (c) Pepticare and (d) Normacid were tested for their antioxidant activity by using *in vivo* experimental models in which various pathological conditions, associated with the generation of free radicals, were induced. The experimental models were divided into various sets as follows:

- **SET 1: PYLORUS-LIGATION INDUCED ULCER MODEL**
- **SET 2: ETHANOL-INDUCED ULCER MODEL**
- **SET 3: ISOPROTERENOL-INDUCED MYOCARDIAL INFARCTION**
- **SET 4: CISPLATIN-INDUCED NEPHROTOXICITY MODEL**
- **SET 5: CARBONTETRACHLORIDE-INDUCED HEPATOTOXICITY.**
- **SET 6: HYPOCHOLESTEROLEMIC AND ANTIOXIDANT ACTIVITY OF DRUG-X IN NORMAL AND HYPERCHOLESTEROLEMIC RABBITS.**

SET 1: PYLORUS LIGATION- INDUCED ULCER MODEL

The animals were divided into six groups (Groups 1-6). Groups 3, 4, 5 and 6 were again divided into four subgroups, namely (a) DHC-1, (b) Activit, (c) Pepticare and (d) Normacid, each representing the respective drug treatments. Each group and subgroups consisted of six rats.

Group 1	Animals received 5% gum acacia, p.o. (5ml/kg)
(Normal control)	(Pylorus-ligation was not performed in any of the rats)
Group 2	Animals received 5% gum acacia, p.o. (5ml/kg)
(Pylorus-ligated control)	(Pylorus-ligation was performed in all the rats)
Group 3	Animals received the formulation (a), (b), (c) or (d) [125 mg/kg, p.o.] 1 hr before pylorus-ligation.
Group 4	Animals received the formulation (a), (b), (c) or (d) [250 mg/kg, p.o.] 1 hr before pylorus-ligation.
Group 5	Animals received the formulation (a), (b), (c) or (d) [500 mg/kg, p.o.] 1 hr before pylorus-ligation.
Group 6	Animals received the formulation (a), (b), (c) or (d) [1000 mg/kg, p.o.] 1 hr before pylorus-ligation.

Procedure

The method described by Shay et al. (1945) was adopted. Rats were fasted for 48 hours. The drugs were administered to the different groups of animals 1 h before pylorus-ligation. During the course of the experiment food was withdrawn. After the pretreatment period of 1 h, the animals were anaesthetised with anaesthetic ether. The abdomen was opened by a small midline incision below the xiphoid process; the pylorus portion of stomach was slightly lifted out and ligated. Precaution was taken to avoid traction to the pylorus or damage to its blood supply. The stomach was placed carefully in the abdomen and the wound was sutured by interrupted sutures. Nineteen hours after pylorus ligation the rats were sacrificed and the stomach was removed. The gastric content was collected and centrifuged (2500 rpm for 20 min). The volume, pH and total acidity of

gastric fluid was determined. Total acidity was determined by titrating 10 times diluted gastric juice with 0.01 N NaOH using phenolphthalein as an indicator. The stomach was then incised along the greater curvature and observed for ulcers. The number of ulcers was counted using a magnifying glass and the diameter of the ulcers was measured using a vernier caliper Ulcer index was determined by following the scoring method of Suzuki et al. (1976).

Score 1: maximal diameter of 1mm
Score 2: maximal diameter of 1-2mm
Score 3: maximal diameter of 2-3mm
Score 4: maximal diameter of 3-4mm
Score 5: maximal diameter of 4-5mm
Score 10: an ulcer over 5mm in diameter
Score 25: a perforated ulcer

The stomach was then weighed and homogenized in chilled Tris buffer (10mM, pH 7.4) at a concentration of 10% w/v. The homogenates were centrifuged at 10,000 X g at 0°C for 20 minutes using Remi C-24 high speed cooling centrifuge. The clear supernatant was used for the assays of lipid peroxidation (MDA content), endogenous antioxidant enzymes (superoxide dismutase and catalase) and reduced glutathione (GSH). The sediment was resuspended in ice cold Tris buffer (10mM, pH 7.4) to get a final concentration of 10% and was used for the estimation of different membrane bound enzymes (Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase) and proteins.

SET 2: ETHANOL-INDUCED ULCER MODEL

The animals were divided into six groups (Groups 1-6) Groups 3, 4, 5 and 6 were again divided into four subgroups, namely (a) DHC-1, (b) Activit, (c) Pepticare and (d) Normacid, each representing the respective drug treatments. Each group and subgroups consisted of six rats.

Group 1 (Normal control)	Animals received 5% gum acacia, p.o. (5ml/kg) for 10 days.
Group 2 (Ethanol control)	Animals received 5% gum acacia, p o. (5ml/kg) for 10 days followed by a single dose of ethanol (96%, 5ml/kg, p.o.).
Group 3	Animals received the formulation (a), (b), (c) or (d) [125 mg/kg, p.o.] mg/kg for 10 days followed by a single dose of ethanol (96%, 5ml/kg, p.o.).
Group 4	Animals received the formulation (a), (b), (c) or (d) [250 mg/kg, p.o.] for 10 days followed by a single dose of ethanol (96%, 5ml/kg, p.o.).
Group 5	Animals received the formulation (a), (b), (c) or (d) [500 mg/kg, p.o.] for 10 days followed by a single dose of ethanol (96%, 5ml/kg, p.o.).
Group 6	Animals received the formulation (a), (b), (c) or (d) [1000 mg/kg, p.o.] for 10 days followed by a single dose of ethanol (96%, 5ml/kg, p.o.).

Procedure

The formulations were administered orally to the rats of different groups for a period of 10 days. On the 10th day, 1 h after the final dose of formulation, 96% ethanol (5ml/kg, p.o.) was administered to the overnight fasted rats of groups 2 to 6. The animals were then sacrificed 1 h after the dose of ulcerogen. The stomach was then removed, incised along the greater curvature and its mucosal erosion was determined randomly by measuring the area of the lesions. The sum of the areas was expressed as ulcer index (mm^2). The stomach was then weighed and processed for assays of lipid peroxidation, superoxide dismutase, catalase, reduced glutathione, membrane bound enzymes (Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase) and proteins.

SET 3: ISOPROTERENOL-INDUCED MYOCARDIAL INFARCTION

The animals were divided into six groups (Groups 1-6). Groups 3, 4, 5 and 6 were again divided into four subgroups, namely (a) DHC-1, (b) Activit, (c) Pepticare and (d) Normacid, each representing the respective drug treatments. Each group and subgroups consisted of six rats.

Group 1 (Normal Control)	Animals received 5% gum acacia, p.o. (5ml/kg) for 30 days before injecting sterile saline (2ml/kg, s.c.)
Group 2 (Isoproterenol control)	Animals received 5% gum acacia, p.o. (5ml/kg) for 30 days followed by isoproterenol (25 mg/kg, s.c. twice at an interval of 24 hrs) administration.
Group 3	Animals received the formulation (a), (b), (c) or (d) [125 mg/kg, p.o.] for 30 days followed by isoproterenol administration.
Group 4	Animals received the formulation (a), (b), (c) or (d) [250 mg/kg, p.o.] for 30 days followed by isoproterenol administration.
Group 5	Animals received the formulation (a), (b), (c) or (d) [500 mg/kg, p.o.] for 30 days followed by isoproterenol administration.
Group 6	Animals received the formulation (a), (b), (c) or (d) [1000 mg/kg, p.o.] for 30 days followed by isoproterenol administration.

Procedure: The formulations were administered orally to the rats of different groups for a period of one month. On day 30, groups 2, 3, 4, 5 and 6 received isoproterenol (25 mg/kg, s.c. twice at an interval of 24 hours) in sterile saline. After 24 hours of the last dose of isoproterenol, blood was collected and serum was separated for estimations of creatine kinase (CK), lactate dehydrogenase (LDH), uric acid and SGOT. The animals were sacrificed and the heart was dissected out, weighed and processed for assays of lipid peroxidation, superoxide dismutase, catalase, reduced glutathione, membrane bound enzymes (Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase) and proteins. Histopathological studies on heart were also carried out.

SET 4A: CISPLATIN-INDUCED NEPHROTOXICITY MODEL
(Acute model)

The animals were divided into six groups (Groups 1-6). Groups 3, 4, 5 and 6 were again divided into four subgroups, namely (a) DHC-1, (b) Activit, (c) Pepticare and (d) Normacid, each representing the respective drug treatments. Each group and subgroups consisted of six rats.

Group 1	Animals received 5% gum acacia, p.o. (5ml/kg)
(Normal control)	1 h before sterile saline (3mg/kg, i.p.).
Group 2	Animals received 5% gum acacia, p.o. (5ml/kg)
(Cisplatin control)	followed by a single dose of cisplatin (3mg/kg, i.p.) administration after an hour.
Group 3	Animals received the formulation (a), (b), (c) or (d) [125 mg/kg, p.o.] followed by cisplatin administration after an hour.
Group 4	Animals received the formulation (a), (b), (c) or (d) [250 mg/kg, p.o.] followed by cisplatin administration after an hour.
Group 5	Animals received the formulation (a), (b), (c) or (d) [500 mg/kg, p.o.] followed by cisplatin administration after an hour.
Group 6	Animals received the formulation (a), (b), (c) or (d) [1000 mg/kg, p.o.] followed by cisplatin administration after an hour.

Procedure: The formulations were administered orally to the rats of different groups. 1 h after the drug administration a single dose of cisplatin (3 mg/kg, i.p.) was given to rats of groups 2, 3, 4, 5 and 6. On day 5, change in body weight was recorded, blood was collected and serum was separated for estimations of creatinine, urea, uric acid and blood urea nitrogen (BUN). The animals were then sacrificed and the kidneys were dissected out, weighed and processed for assays of lipid peroxidation, superoxide dismutase, catalase, reduced glutathione, membrane bound enzymes (Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase) and proteins.

SET 4B: CISPLATIN-INDUCED NEPHROTOXICITY MODEL (Chronic model)

The animals were divided into six groups (Groups 1-6). Groups 3, 4, 5 and 6 were again divided into four subgroups, namely (a) DHC-1, (b) Activit, (c) Pepticare and (d) Normacid, each representing the respective drug treatments. Each group and subgroups consisted of six rats

Group 1 (Normal control)	Animals received 5% gum acacia (5ml/kg, p.o.) and saline (3mg/kg, i.p.) every week for 28 days (days 1, 7, 14, 21 and 28).
Group 2 (Cisplatin control)	Animals received 5% gum acacia (5ml/kg, p.o.) and cisplatin (3 mg/kg, i.p.) every week for 28 days (days 1, 7, 14, 21 and 28).
Group 3	Animals received the formulation (a), (b), (c) or (d) [125 mg/kg, p.o.] and cisplatin (3 mg/kg, i.p.) every week for 28 days (days 1, 7, 14, 21 and 28).
Group 4	Animals received the formulation (a), (b), (c) or (d) [250 mg/kg, p.o.] and cisplatin (3 mg/kg, i.p.) every week for 28 days (days 1, 7, 14, 21 and 28).
Group 5	Animals received the formulation (a), (b), (c) or (d) [500 mg/kg, p.o.] and cisplatin (3 mg/kg, i.p.) every week for 28 days (days 1, 7, 14, 21 and 28).
Group 6	Animals received the formulation (a), (b), (c) or (d) [1000 mg/kg, p.o.] and cisplatin (3 mg/kg, i.p.) every week for 28 days (days 1, 7, 14, 21 and 28).

Procedure

The formulations were administered orally to the rats of different groups every week for 28 days (1, 7, 14, 21 and 28). Cisplatin was injected

1 h after the drug administration every week for 28 days (1, 7, 14, 21 and 28) to rats of groups 2, 3, 4, 5 and 6. After 24 hours of the last dose of cisplatin change in body weight was recorded, blood was collected and serum was separated for estimations of creatinine, urea, uric acid and blood urea nitrogen (BUN). The animals were then sacrificed and the kidneys were dissected out, weighed and processed for assays of lipid peroxidation, superoxide dismutase, catalase, reduced glutathione, membrane bound enzymes (Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase) and proteins. Histopathological studies on kidney were also carried out.

SET 5: CARBON TETRACHLORIDE-INDUCED HEPATOTOXICITY

The animals were divided into six groups (Groups 1-6). Groups 3, 4, 5 and 6 were again divided into four subgroups, namely (a) DHC-1, (b) Activit, (c) Pepticare and (d) Normacid, each representing the respective drug treatments. Each group and subgroups consisted of six rats.

Group 1 (Normal control)	Animals received 5% gum acacia, p.o. (5ml/kg) for 15 days followed by olive oil (2.5 ml/kg, p.o.) administration.
Group 2 (CCl_4 control)	Animals received 5% gum acacia, p.o. (5ml/kg) for 15 days followed by CCl_4 {2.5ml/kg, p.o. in olive oil (1:1)} administration.
Group 3	Animals received the formulation (a), (b), (c) or (d) [125 mg/kg, p.o.] for 15 days followed by CCl_4 administration.
Group 4	Animals received the formulation (a), (b), (c) or (d) [250 mg/kg, p.o.] for 15 days followed by CCl_4 administration.
Group 5	Animals received the formulation (a), (b), (c) or (d) [500 mg/kg, p.o.] for 15 days followed by CCl_4 administration.
Group 6	Animals received the formulation (a), (b), (c) or (d) [1000 mg/kg, p.o.] for 15 days followed by CCl_4 administration.

Procedure

The formulations were administered to animals of all groups for a period of 15 days. On the 15th day, groups 2, 3, 4, 5 and 6 received CCl_4 at the dose of 2.5ml/kg, p.o. in olive oil (1:1). After 24 hours of CCl_4 administration, blood was collected and serum was separated for estimations of SGPT, SGOT, alkaline phosphatase and total bilirubin. The animals were then sacrificed and the liver was dissected out, weighed and processed for assays of lipid peroxidation, superoxide dismutase, catalase, reduced glutathione, membrane bound enzymes (Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase) and proteins. Histopathological studies on liver were also carried out.

SET 6: HYPOCHOLESTEROLEMIC AND ANTIOXIDANT ACTIVITY OF DRUG-X IN NORMAL AND HYPERCHOLESTEROLEMIC RABBITS.

The animals were divided into four groups each consisting of six rabbits.

Group 1 (Normal control)	Animals were fed with standard laboratory diet (SLD) daily for 120 days.
Group 2	Rabbits were fed with standard laboratory diet plus *M.oleifera* powder (200mg/kg/day, p.o.) in banana pulp for 120 days.
Group 3 (Hypercholesterolemic control)	Rabbits were fed with hypercholesterolemic diet (HCD) daily for 120 days
Group 4	Rabbits were fed with hypercholesterolemic diet (HCD) plus *M.oleifera* powder (200mg/kg/day, p.o.) in banana pulp for 120 days.

During the experimental period of 120 days, the rabbits were fed with standard laboratory diet (SLD) or hypercholesterolemic diet (HCD), both provided by Alembic Ltd., Baroda, India.

Standard laboratory diet consisted of wheat bran (78%), dicalcium phosphate (1%), wheat bharda (5%), sodium chloride (1%), yeast powder (4%), Alvita M (1%) and java bharda (10%). Alvita M, manufactured by Alembic Chemical Works Ltd. (Veterinary Division), Baroda contains vitamin A, B2, B12, D3, E, K calcium pantothenate, choline chloride, calcium, phosphorus, manganese, iodine, zinc, iron and copper. Hypercholesterolemic diet contained 5% cholesterol, 5% coconut oil and 90% standard laboratory diet.

After 120 days of treatment, the change in body weight was recorded, blood was collected from the marginal ear vein and serum was separated. Serum total cholesterol, HDL-cholesterol, LDL-cholesterol, VLDL-cholesterol, total lipid, phospholipid and triglycerides were determined. The animals were then sacrificed and organs such as heart and liver were dissected out and processed for assays of lipid peroxidation, superoxide dismutase, catalase, reduced glutathione, membrane bound enzymes (Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase) and proteins.

3.2.3 BIOCHEMICAL ESTIMATIONS

The following parameters were estimated.

3.2.3.1 Tissue Parameters

1. Lipid peroxidation or malondialdehyde (MDA) formation
2. Endogenous antioxidants
   a) Superoxide dismutase (SOD)
   b) Catalase (CAT)
   c) Reduced glutathione (GSH)
3. Membrane bound enzymes, namely
   a) Na^+K^+ATPase
   b) Ca^{2+}ATPase
   c) Mg^{2+}ATPase
4. Inorganic phosphorus
5. Total proteins

was then centrifuged at 2500 rpm for 20 minutes. The serum obtained was stored at 4°C till further use.

After 120 days of treatment, blood from rabbits was collected from the marginal ear vein and serum was separated as above.

Tissues:

Reagents

1. Sucrose solution (0.25M)

85.58gms of sucrose was dissolved in 200ml of water and diluted to 1000ml with distilled water.

2. Tris hydrochloride buffer (10mM, pH 7.4)

1.21gm tris was dissolved in 900ml of distilled water and the pH was adjusted to 7.4 with 1M hydrochloric acid. The resulting solution was diluted to 1000ml with distilled water

Procedure

The animals were euthanasiously sacrificed, after blood collection, by injecting phenobarbitone (150mg/kg, i p). The organs (stomach in case of ulcer models, heart in case of isoproterenol-induced myocardial infarction model, kidneys in case of cisplatin-induced nephrotoxicity model, liver in case of CCl_4-induced hepatotoxicity model and liver and heart in case of HCD-induced atherosclerosis model) were isolated carefully in cooled petridishes The organs were blotted free of blood and tissue fluids, weighed on a Single Pan Electronic Balance (Precisa 205 ASCS). The organs were cross-chopped with surgical scalpel into fine slices, suspended in chilled 0.25M sucrose solution and quickly blotted on a filter paper. The tissues were then minced and homogenised in chilled tris hydrochloride buffer (10mM, pH 7.4) to a concentration of 10% w/v. Prolonged homogenisation under hypotonic condition was designed to disrupt, as far as possible, the structure of the cells so as to release soluble proteins. The homogenate was then centrifuged at 2500 rpm at 4°C and the part of the clear supernatant was used for the estimation of lipid peroxidation and reduced glutathione. The remaining homogenate was centrifuged at 10,000 rpm at 4°C for 25 minutes using Remi C-24

high speed cooling centrifuge. The clear supernatant was used for the determination of superoxide dismutase and catalase. The sediment was resuspended in ice-cold tris hydrochloride buffer (10mM, pH 7.4) to get a final concentration of 10% w/v and was used for the estimation of different membrane bound enzymes and proteins.

3.3 TISSUE ESTIMATIONS

3.3.1 Determination of Lipid Peroxidation (MDA content)

It was estimated using the method described by Slater and Sawyer (1971).

Reagents

1. *Thiobarbituric acid (0.67% w/v)*

   0.67gm of thiobarbituric acid was dissolved in 50ml of hot distilled water and the final volume was made upto 100ml with hot distilled water.

2. *Trichloroacetic acid (10% w/v)*

   10gms of trichloroacetic acid was dissolved in 60ml of distilled water and the final volume was made upto 100ml with distilled water.

3. *Standard Malondialdehyde stock solution (50mM)*

   A standard malondialdehyde stock solution was prepared by mixing 25µl of 1,1,3,3-tetraethoxypropane upto 100ml with distilled water. 1.0ml of this stock solution was diluted upto 10ml to get solution containing 23µg of malondialdehyde/ml. 1.0ml of this stock solution was diluted upto 100ml to get a working standard solution containing 23ng of malondialdehyde/ml.

Procedure

2.0ml of the tissue homogenate (supernatant) was added to 2.0ml of freshly prepared 10% w/v trichloroacetic acid (TCA) and the mixture was allowed to stand in an ice bath for 15 minutes. After 15 minutes, the

precipitate was separated by centrifugation and 2.0ml of clear supernatant solution was mixed with 2.0ml of freshly prepared thiobarbituric acid (TBA). The resulting solution was heated in a boiling water bath for 10 minutes. It was then immediately cooled in an ice bath for 5 minutes. The colour developed was measured at 532nm against reagent blank.

Different concentrations (0-23nM) of standard malondialdehyde (1,1,3,3-tetraethoxypropane) were taken and processed as above for standard graph. The values were expressed as nM of MDA/mg protein.

3.3.2 Assay of Endogenous Antioxidants

3.3.2.1 Superoxide Dismutase (SOD) (Superoxide: Superoxide oxido reductase, EC-1.15.1.1)

Superoxide dismutase was estimated using the method developed by Misra and Fridovich (1972).

Reagents

1. *Carbonate Buffer (0.05M, pH 10.2)*

   16.8gms of sodium bicarbonate and 22gms of sodium carbonate was dissolved in 500ml of distilled water and the volume was made upto 1000ml with distilled water.

2. *Ethylenediaminetetra acetic acid (EDTA) solution (0.49M)*

   1.82gm of EDTA was dissolved in 200ml of distilled water and the volume was made upto 1000ml with distilled water.

3. *Hydrochloric acid (0.1N)*

   8.5ml of conc. hydrocloric acid was mixed with 500ml of distilled water and the volume was made upto 1000ml with distilled water.

4. *Epinephrine solution (3mM)*

   0.99gm epinephrine bitartarate was dissolved in 100ml of 0.1N hydrocloric acid and the volume was adjusted to 1000ml with 0.1N hydrocloric acid.

5. *Superoxide Dismutase (SOD) standard (100 U/L)*

   1mg (1000 U/mg) of SOD from bovine liver was dissolved in 100ml of carbonate buffer.

Procedure

0.5ml of tissue homogenate was diluted with 0.5ml of distilled water, to which 0.25ml of ice-cold ethanol and 0.15ml of ice-cold chloroform were added. The mixture was mixed well using cyclo mixer for 5 minutes and centrifuged at 2500 rpm. To 0.5ml of supernatant, 1.5ml of carbonate buffer and 0.5ml of EDTA solution were added. The reaction was initiated by the addition of 0.4ml of epinephrine and the change in optical density/minute was measured at 480nm against reagent blank.

SOD activity was expressed as units/mg protein. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme is taken as the enzyme unit. Calibration curve was prepared by using 10-125 units of SOD.

3.3.2.2 Catalase (CAT) (Hydrogen peroxide oxido reductase, EC-1.11.1.6)

It was estimated by the method of Hugo Aebi as given by Colowick et al. (1984).

Reagents

1. Phosphate Buffer (50mmol/l, pH 7.0)

(a) 6.81gms of potassium dihydrogen orthophosphate (KH_2PO_4) was dissolved in distilled water and made upto 1000ml.

(b) 8.90gms of disodium hydrogen orthophosphate (Na_2HPO_4) was dissolved in distilled water and made upto 1000ml.

The solutions (a) and (b) were mixed in the proportion of 1: 1.5 (v/v).

Hydrogen Peroxide (30mmol/l)

0.34ml of 30% hydrogen peroxide was diluted with phosphate buffer to 100ml. This solution was prepared fresh each day.

4. Catalase standard (65,000 U/mg protein; 1mg protein/ml)

Crystalline beef-liver catalase suspension was centrifuged to isolate the crystals of the enzyme that was dissolved in 0.01M phosphate buffer (pH 7.0) to give a final concentration of 1.0mg protein/ml. Before assay, it was diluted with distilled water to obtain 1000 U/ml.

Procedure

To 2ml of diluted sample 1ml of hydrogen peroxide (30 mmol/l) was added to initiate the reaction. The blank was prepared by mixing 2ml of diluted sample (similar dilution) with 1ml of phosphate buffer (50mmol/l; pH 7.0). The dilution should be such that the initial absorbance should be approximately 0.500. The decrease in absorbance was measured at 240nm. Catalase activity was expressed as μmoles of H_2O_2 consumed/min/mg protein.

3.3.2.3 Reduced Glutathione (GSH)

Reduced glutathione was determined by the method described by Moron et al. (1979).

Reagents

1. Trichloroacetic acid (20% w/v)

20gms of trichloroacetic acid was dissolved in sufficient quantity of distilled water and the final volume was made upto 100ml with distilled water.

2. Phosphate Buffer (0.2M, pH 8.0)

0.2M sodium phosphate was prepared by dissolving 30.2gms sodium phosphate in 600ml of distilled water, the pH was adjusted to 8.0 with 0.2M sodium hydroxide solution and the final volume was adjusted upto 1000ml with distilled water.

3. DTNB reagent (0.6mM)

60mg of 5,5'-dithiobis (2-nitro benzoic acid) was dissolved in 50ml of buffer and the final volume was adjusted to 100ml with buffer.

4. Standard Glutathione

10mg of reduced glutathione was dissolved in 60ml of distilled water and the final volume was made upto 100ml with distilled water.

Procedure

Equal volumes of tissue homogenate (supernatant) and 20% TCA were mixed. The precipitated fraction was centrifuged and to 0.25ml of supernatant, 2ml of DTNB reagent was added. The final volume was made

upto 3ml with phosphate buffer. The colour developed was read at 412nm against reagent blank.

Different concentrations (10-50µg) of standard glutathione were taken and processed as above for standard graph.

The amount of reduced glutathione was expressed as µg of GSH/mg protein.

3.3.3 Assay of Membrane Bound Enzymes and Inorganic Phosphorus

3.3.3.1 Sodium-Potassium dependent adenosine triphosphatase (Na^+-K^+-ATPase) (ATP phosphohydrolase, EC-3.6.1.3)

Na^+-K^+-ATPase was assayed according to the method described by Bonting (1970).

Reagents

1. *Tris hydrochloride buffer (92mM, pH 7.5)*

   11.13gms of tris was dissolved in 900ml of distilled water and the pH was adjusted to 7.5 with 1M hydrochloric acid. The resulting solution was diluted to 1000ml with distilled water.

2. *Magnesium sulphate solution (5mM)*

   1.232gm of magnesium sulphate dissolved and made upto 1000ml with distilled water.

3. *Potassium chloride solution (5mM)*

   0.372gm of potassium chloride was dissolved in 400ml of distilled water and the final volume was made upto 1000ml with distilled water.

4. *Sodium chloride solution (60mM)*

   3.231gms of sodium chloride was dissolved in 400ml of distilled water and the final volume was made upto 1000ml with distilled water.

5. *EDTA solution (0.1mM)*

   0.372gm of EDTA was dissolved in 400ml of distilled water and the final volume was made upto 1000ml with distilled water.

6. Adenosine triphosphate (ATP) solution (40mM)

0.220gm of ATP was dissolved in 4ml of distilled water and the final volume was made upto 10ml with distilled water.

7. Trichloroacetic acid (10% w/v)

10gms of trichloroacetic acid was dissolved in sufficient quantity of distilled water and the final volume was made upto 100ml with distilled water.

Procedure

1.0ml of tris-hydrochloride buffer and 0.2ml each of magnesium sulphate, sodium chloride, potassium chloride, EDTA, ATP were added to test tube containing 0.2ml of homogenate. The mixture was incubated at 36°C for 15 minutes. The reaction was arrested by addition of 1.0ml of 10% TCA, mixed well and centrifuged. The phosphorus content of the supernatant was estimated as described in

The enzyme activity was expressed as µM of inorganic phosphorus liberated/mg protein/min.

3.3.3.2 Calcium dependent adenosine triphosphatase (Ca^{++}-ATPase) (ATP phosphohydrolase, EC-3.6.1.3)

Ca^{++}-ATPase was assayed according to the method described by Hjerken and Pan (1983).

Reagents

1. Tris hydrochloride buffer (125mM, pH 7.5)

15.12gms of tris was dissolved in 900ml of distilled water and the pH was adjusted to 7.5 with 1M hydrochloric acid. The resulting solution was diluted to 1000ml with distilled water.

2. Calcium chloride solution (50mM)

5.55gms of calcium chloride was dissolved in 400ml of distilled water and the final volume was made upto 1000ml with distilled water.

3. Adenosine triphosphate (ATP) solution (10mM)

0.551gm of ATP was dissolved in 4ml of distilled water and the final volume was made upto 100ml with distilled water.

4. Trichloroacetic acid (10% w/v)

10gms of trichloroacetic acid was dissolved in sufficient quantity of distilled water and the final volume was made upto 100ml with distilled water.

Procedure

The incubation mixture contained 0.1ml each of tris-hydrochloride buffer, calcium chloride, ATP and homogenate in a test tube. The mixture was incubated at 37°C for 15 minutes. The reaction was arrested by addition of 1.0ml of 10% TCA, mixed well and centrifuged. The phosphorus content of the supernatant was estimated as described in

The enzyme activity was expressed as µM of inorganic phosphorus liberated/mg protein/min.

3.3.3.3 Magnesium dependent adenosine triphosphatase (Mg^{++}-ATPase) (ATP phosphohydrolase, EC-3.6.1.3)

Mg^{++}-ATPase was assayed according to the method described by Ohinishi et al. (1982).

Reagents

1. Tris hydrochloride buffer (374mM, pH 7.6)

45.24gms of tris was dissolved in 900ml of distilled water and the pH was adjusted to 7.6 with 1M hydrochloric acid. The resulting solution was diluted to 1000ml with distilled water.

2. Magnesium chloride solution (25mM)

6.16gms of magnesium chloride was dissolved in 400ml of distilled water and the final volume was made upto 1000ml with distilled water.

3. Adenosine triphosphate (ATP) solution (10mM)

0.551gm of ATP was dissolved in 4ml of distilled water and the final volume was made upto 100ml with distilled water.

4. Trichloroacetic acid (10% w/v)

10gms of trichloroacetic acid was dissolved in sufficient quantity of distilled water and the final volume was made upto 100ml with distilled water.

Procedure

The incubation mixture contained 0.1ml each of tris-hydrochloride buffer, magnesium chloride, ATP and homogenate in a test tube. The mixture was incubated at 37°C for 15 minutes. The reaction was arrested by addition of 1.0ml of 10% TCA, mixed well and centrifuged. The phosphorus content of the supernatant was estimated as described in

The enzyme activity was expressed as μM of inorganic phosphorus liberated/mg protein/min.

3.3.3.4 Determination of Inorganic Phosphorus (P_i)

Inorganic Phosphorus (P_i) was estimated by the method described by Fiske and Subbarow (1925).

Reagents

1. Ammonium molybdate reagent (2.5% w/v)

2.5gms of ammonium molybdate was dissolved upto 100ml with 3M sulphuric acid.

2. 1-Amino 2-naphthol 4-sulphonic acid (ANSA) reagent

0.25% w/v of ANSA reagent in 15% w/v of sodium metabisulphite and 20% w/v of sodium sulphite.

15gms of sodium metabisulphite was dissolved in 100ml of distilled water.

Accurately weighed 20gms of sodium sulphite was dissolved in 100ml of distilled water.

250mg of ANSA was dissolved in 97.5ml of 15% w/v of sodium metabisulphite and 2.5ml of 20% w/v of sodium sulphite, mixed well and stored at room temperature.

3. Standard Phosphorus

35.1mg of potassium dihydrogen orthophosphate was dissolved in 100ml of distilled water. This contained 80µg of phosphorus/ml.

Procedure

1ml of the supernatant was taken and the volume was made upto 5.0ml with distilled water. To this, 1ml of 2.5% ammonium molybdate reagent and 0.5ml of ANSA reagent was added. The colour developed in 20 minutes was read using blank containing water instead of sample at 620nm. A standard graph was prepared taking different concentrations of standard phosphorus (16-80µg).

The values were expressed as µM of inorganic phosphorus liberated/mg protein/min.

3.3.4 Estimation of Total Protein

The method of Lowry et al. (1951) was used for the estimation of total protein.

Reagents

1. Sodium hydroxide (0.1M)

4gms of sodium hydroxide was dissolved in 400ml of distilled water and the final volume was made upto 1000ml with distilled water.

2. Lowry C reagent

a) Copper sulphate in 1% sodium potassium tartarate (1% w/v)

0.5gm of copper sulphate was dissolved in 1% sodium potassium tartarate (Prepared by dissolving 1gm of sodium potassium tartarate in 100ml of distilled water).

b) Sodium carbonate in 0.1M sodium hydroxide (2% w/v)

2gms of sodium carbonate was dissolved in 100ml of 0.1M sodium hydroxide.

2ml of solution (a) was mixed with 100ml of solution (b) just before use.

3. Standard Protein (Bovine serum albumin)

20mg of bovine serum albumin was dissolved in 80ml of distilled water and few drops of sodium hydroxide was added to aid complete dissolution of bovine serum albumin and to avoid frothing. Final volume was made upto 100ml with distilled water and stored overnight in a refrigerator.

4. Folin's phenol reagent

Folin's phenol reagent was diluted with distilled water in the ratio of 1:2. (i.e. 1ml of Folin's phenol reagent was mixed with 2ml of distilled water).

Procedure

Diluted membrane fraction aliquots (0.1ml) were taken in test tubes. To this, 0.8ml of 0.1M sodium hydroxide and 5ml of Lowry C reagent was added and the solution was allowed to stand for 15 minutes. Then 0.5ml of 1N Cio-Calteu reagent (Folin's phenol reagent) was added and the contents were mixed well on a vortex mixer. Colour developed was measured at 640nm against reagent blank containing distilled water instead of sample.

Different concentrations (40-200μg) of standard protein (Bovine serum albumin) were taken and processed as above for standard graph.

The values were expressed as mg of protein/gm of wet tissue (mg/gm).

3.3.5 Extraction and Estimation of Lipids

The procedure of Folch et al. (1957) was used for the extraction of lipids from the organs. Suitable quantity (1g) of tissue was homogenised with total 20ml of cold chloroform-methanol (2:1 v/v) mixture in a homogeniser. The contents were filtered through Whatman filter paper. The residue was re-extracted twice with small volumes of chloroform-methanol (2:1 v/v) mixture and then filtered again. The filtrates were pooled together and 0.3ml of distilled water was added, mixed well by vortexing and allowed to stand for phase separation at room temperature. The upper layer was removed with pasteur pipette and the lower phase

was washed with 0.4ml of Folch's pure upper phase solvent (consists of chloroform-methanol-water in a proportion of 3:48:47) and centrifuged at 3000rpm for 10 minutes. The upper phase was removed. The lower phase was used for the estimation of cholesterol, phospholipid and triglycerides.

3.3.5.1 Estimation of Cholesterol

Quantitative determination of cholesterol was done using enzymatic kit [Sigma Diagnostics (India) Pvt. Ltd., Baroda].

Principle

Cholesterol esterase (CHE) hydrolyses cholesterol ester. Free cholesterol is oxidised by the cholesterol oxidase (CHO) to cholest 4-ene-3-one and hydrogen peroxide. Hydrogen peroxide reacts with 4-amino antipyrine and phenol in the presence of peroxidase (POD) to produce pink coloured quinoneimine dye. The intensity of colour produced is proportional to the cholesterol concentration.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{CHE}} \text{Cholesterol} + \text{Fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHO}} \text{Cholest-4-ene-3-one} + H_2O_2$$

$$H_2O_2 + \text{4-Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + H_2O_2$$

Units were expressed as mg%

3.3.5.2 Estimation of HDL-Cholesterol

Quantitative determination of cholesterol was done using enzymatic kit [Sigma Diagnostics (India) Pvt. Ltd., Baroda].

Principle:

The VLDL and LDL fractions of serum/tissue sample are precipitated using precipitating reagent and then HDL in the supernatant is separated by centrifugation and measured for its cholesterol content. The enzyme cholesterol ester hydrolase (CHE) hydrolyses the ester cholesterol. Then cholesterol is oxidised by cholesterol oxidase (CHO) to cholest 4-ene-3-one and hydrogen peroxide. Hydrogen peroxide reacts with 4-amino antipyrine and phenol in the presence of peroxidase (POD) to produce pink coloured quinoneimine dye. The intensity of colour produced is proportional to the concentration of HDL-cholesterol.

Serum + Precipitating reagent --> Precipitate + Supernatant
(VLDL & LDL) (HDL)

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{CHE}} \text{Cholesterol} + \text{Fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHO}} \text{Cholest-4-ene-3-one} + H_2O_2$$

$$H_2O_2 + \text{4-Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + H_2O_2$$

Units were expressed as mg%

LDL and VLDL-cholesterol was calculated following the method of Friedwald et al. (1992).

3.3.5.3 Estimation of Phospholipid

Phospholipid was estimated by the modified metol method by using kit [Sigma Diagnostics (India) Pvt. Ltd., Baroda].

Principle:

Ammonium molybdate, under acidic conditions, reacts with phosphorus to form phosphomolybdate complex which is reduced to a blue coloured complex by metol. The absorbance of colour developed is proportional to the inorganic phosphorus concentration. Each mole of phosphorus contributes about 4% of the total phospholipid mass and if expressed in mg/dl, can be converted to total phospholipid mass by multiplying the phosphorus concentration by 25.

Units were expressed as mg%.

3.3.5.4 Estimation of Triglycerides

Quantitative determination of triglycerides (neutral fat) was done using enzymatic kit [Sigma Diagnostics (India) Pvt. Ltd., Baroda].

Principle:

Triglycerides in the sample are hydrolysed by microbial lipases to glycerol and free fatty acids (FFA). Glycerol is phosphorylated by ATP to glycerol-3-phosphate (G-3-P) in a reaction catalysed by glycerol kinase (GK). G-3-P is oxidised to dihydroxy acetone phosphate (DAP) in a reaction catalysed by the enzyme glycerol phosphate oxidase (GPO). In this reaction, hydrogen peroxide (H_2O_2) is produced in equimolar concentration to the level of triglycerides present in the sample. H_2O_2 reacts with 4-aminoantipyrine (4-AAP) and 3,5-dichloro-2-hydroxybenzene sulphonic acid (DHBS) in a reaction catalysed by peroxidases (HPOD). The result of this oxidative coupling is a quinoneimine red coloured dye.

The absorbance of this dye in solution is proportional to the concentration of triglycerides in the sample. The series of reaction involved in the assay is reported below:

$$\text{Triglycerides} \xrightarrow{\text{Lipases}} \text{Glycerol} + \text{FFA}$$

$$\text{Glycerol + ATP} \xrightarrow{GK} \text{G-3-P + ADPMg}^{2+}$$

$$\text{G-3-P + O}_2 \xrightarrow{GPO} \text{DAP + H}_2\text{O}_2$$

$$2\,\text{H}_2\text{O}_2 + \text{4-AAP + DHBS} \xrightarrow{HPOD} \text{Red Quinoneimine dye + 4H}_2\text{O}$$

Units were expressed as mg%.

3.4 SERUM ESTIMATIONS

3.4.1 Creatine kinase (CK)

Quantitative estimation of creatine kinase was done using ENZOPAK-CK-NAC kit [Reckon Diagnostics Pvt. Ltd., Baroda].

Principle:

(A) In this reaction, creatine kinase catalyses the formation of ATP from creatine phosphate and ADP.

$$\text{Creatine Phosphate + ADP} \xrightarrow{CK} \text{Creatine + ATP}$$

(B) Glucose is converted to glucose-6-phosphate (G-6-P) by hexokinase (HK) using ATP as a source for PO_4 moiety.

$$\text{ATP + Glucose} \xrightarrow{HK} \text{G-6-P + ADP}$$

(C) Glucose-6-phosphate is oxidised by glucose-6-phosphate dehydrogenase (G6PDH) to 6-phosphogluconate reducing NADP to NADPH. Their action after the lag phase is monitored by the increase in absorbance at 340nm and is directly proportional to the creatine kinase activity (i.e. the formation of NADPH is in equimolar amount as that of formation of creatine.

G6PDH

G-6-P + NADP ----------> 6-phosphogluconate + NADPH + H

N-acetylcysteine acts as a thiol activator and DAPP and AMP inhibit the interfering myokinase activity.

Units were expressed as U/L.

3.4.2 Lactate dehydrogenase (LDH)

Quantitative estimation of lactate dehydrogenase (LDH) was done using ENZOPAK LDH L→P kit [Reckon Diagnostics Pvt. Ltd., Baroda].

Principle:

LDH catalyses the oxidation of lactate to pyruvate accompanied by the simultaneous reduction of NAD to NADH. LDH activity in serum is proportional to the increase in absorbance due to the reduction of NAD.

LDH

Lactate + NAD ----------> Pyruvate + NADH

Units were expressed as U/L.

3.4.3 Creatinine

Quantitative estimation of creatinine was done using kit [Span Diagnostics Ltd., Surat, India].

Principle:

Creatinine in a protein free solution reacts with alkaline picrate and produced a red coloured complex, which is measured colorimetrically.

Units were expressed as mg/dl.

3.4.4 Uric acid

Quantitative estimation of uric acid was done using diagnostic kit [Span Diagnostics Ltd., Surat, India].

Principle:

Uric acid in alkaline medium reduces phosphotungstic acid to "tungsten blue" a blue coloured complex, which is measured colorimetrically. Units were expressed as mg/dl.

3.4.5 Urea and Blood urea nitrogen (BUN)

Quantitative estimation of urea and blood urea nitrogen (BUN) was done using diagnostic kit [Span Diagnostics Ltd., Surat, India].

Principle:

Urea reacts with hot acidic diacetylmonoxime in presence of thiosemicarbazide and produces a rose-purple coloured complex, which is measured colorimetrically.

Blood urea nitrogen (BUN) was calculated by multiplying serum urea concentration by 0.467.

Units were expressed as mg/dl.

3.4.6 Alanine aminotransferase (GPT) (L-alanine; L-alanine; 2-oxoglutarate aminotransferase, EC-2.6.1.2)

Quantitative estimation of Serum glutamate pyruvate transaminase (GPT) or Alanine aminotransferase (ALT) was done by the method of Reitman and Frankel using diagnostic reagent kit [Span Diagnostics Ltd., Surat, India].

Principle:

SGPT (ALT) catalyses the following reaction:

$$\alpha\text{-Ketoglutarate} + \text{L-Alanine} \xrightarrow{\text{SGPT}} \text{L-Glutamate} + \text{Pyruvate}$$

Pyruvate so formed is coupled with 2,4-Dinitrophenyl hydrazine (2,4-DNPH) to give the corresponding hydrazone, which gives brown colour in alkaline medium and this can be measured colorimetrically.

Units were expressed as units/ml.

3.4.7 Aspartate aminotransferase (GOT) (L-aspartate: L-aspartate; 2-oxoglutarate aminotransferase, EC-2.6.1.1)

Quantitative estimation of Serum glutamate oxaloacetate transaminase (GOT) or Aspartate aminotransferase (AST) was done by the method of Reitman and Frankel using diagnostic reagent kit [Span Diagnostics Ltd., Surat, India].

Principle:

SGOT (AST) catalyses the following reaction:

$$\alpha\text{-Ketoglutarate} + \text{L-Aspartate} \xrightarrow{\text{SGOT}} \text{L-Glutamate} + \text{Oxaloacetate}$$

Oxaloacetate so formed is coupled with 2,4-Dinitrophenyl hydrazine (2,4-DNPH) to give the corresponding hydrazone, which gives brown colour in alkaline medium and this can be measured colorimetrically.

Units were expressed as units/ml.

3.4.8 Alkaline Phosphatase (Orthophosphoric acid monoester phosphohydrolase, EC-3.1.3.1)

Quantitative estimation of Alkaline Phosphatase was done by the PNPP method of Szaz using diagnostic reagent kit [Span Diagnostics Ltd., Surat, India].

Principle:

At pH 10.3 alkaline phosphatase catalyses the hydrolysis of p-nitrophenyl phosphate and the change in absorbance measured at 405 nm is directly proportional to enzyme activity.

Units were expressed as IU/L.

3.4.9 Total Bilirubin

Quantitative estimation of total bilirubin was done by the method of Jendrassik and Grof. (1938)

Reagents

1. Sodium nitrite solution

Accurately weighed 500 mg of sodium nitrite was first dissolved in distilled water and then the volume was made up to 100 ml with distilled water.

2. Sulphanilic acid solution

Sulphanilic acid was (500 mg) dissolved in 1.5 ml of concentrated hydrochloric acid and then the volume was made up to 100 ml with distilled water.

3. Caffeine – Benzoate solution

7.5 gm of Sodium benzoate, 5.0 gm of Caffeine and 12.5 gm of Sodium acetate (trihydrate) were dissolved successively and volume was adjusted to 100 ml with warm distilled water.

Procedure

50 µl each of sodium nitrite, sulphanilic acid and serum were mixed with 0.5 ml of caffeine benzoate and 0.5 ml of distilled water in the order mentioned. Blank was prepared similarly by adding saline instead of sodium nitrite. Samples were then immediately incubated in dark for 5 min. and values were directly determined on RA-50 chemical analyzer.

Units were expressed as mg/dl.

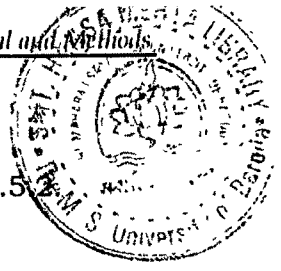
3.4.10 Lipids

3.4.10.1 Cholesterol

Total cholesterol was estimated as described in section 3.3.5.1.

3.4.10.2 HDL- cholesterol

HDL-cholesterol was estimated as described in section 3.3.5.2.



3.4.10.3 LDL- cholesterol

LDL-cholesterol was estimated as described in section 3.3.5.2.

3.4.10.4 VLDL- cholesterol

VLDL-cholesterol was estimated as described in section 3.3.5.2.

3.4.10.5 Phospholipids

Phospholipids were estimated as described in section 3.3.5.3.

3.4.10.6 Triglycerides

Triglycerides were estimated as described in section 3.3.5.4.

3.5 HISTOPATHOLOGICAL STUDIES

After the treatment period, the animals were sacrificed and the organs were excised, blotted free of blood and tissue fluids and preserved in 10% v/v formal saline solution. After a week, the tissues were washed thoroughly in repeated changes of 70% alcohol and then dehydrated in ascending grades of alcohol (70-100%). After dehydration, the tissues were cleaned in xylene and embedded in paraffin wax. Sections of 5μ thickness were cut on a microtome and taken on glass slides coated with albumin. The sections were deparaffinated in xylene and downgraded through 100, 90, 70, 50, 30% alcohol and then finally in water. The hematoxylin-stained sections were stained with eosin for 2 minutes and were then quickly passed through ascending grades of alcohol, cleaned in xylene and mounted on Canada balsam. The stained sections were examined under Olympus BX40 Photomicroscope and photographed.

3.6 STATISTICAL ANALYSIS OF DATA

Results of all the above estimations have been indicated in terms of mean ± SEM. Difference between the groups was statistically determined by analysis of variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons test, with the level of significance set at $p < 0.05$.