Materials & Methods
4. MATERIAL AND METHODS

4.1. PHARMACOGNOSTICAL AND PHYTOCHEMICAL STUDIES

4.1.1. Collection and identification of fruits of *H. isora*

The fruits of *Helicteres isora* were bought from the local market of Ahmedabad. Fruit was identified by comparing its morphological characteristics described in different standard text and floras (Kirtikar and Basu, 1935). The fruits were also identified and authenticated by Dr M. B. Shah, Department of Pharmacognosy and Phytochemistry, L. M. College of Pharmacy, Ahmedabad, India. A specimen was also deposited in the same department (voucher no. LM-138).

4.1.2. Determination of Ash values

Determination of total ash

Accurately weighed 1 g of powdered drug was taken in a tared silica dish and it was incinerated at a temperature 450 °C until free from carbon. The sample was cooled and weighed. If carbon free ash can not be obtained in this way, the charred mass was exhausted with hot water. The residue was collected on an ashless filter paper and the residue and the filter paper were incinerated the filtrate was evaporated to dryness, and ignited at a temperature not exceeding 450 °C. the percentage of ash was calculated with reference of the air dried drug.

Determination of acid-insoluble ash

The ash obtained as described above was boiled for 5 min. with 25 ml of dilute hydrochloric acid. The insoluble matter was collected in a Gooch crucible or on an ashless filter paper and washed with hot water and ignited to constant weight. The percentage of acid-insoluble ash was calculated with reference to the air dried drug.

Determination of water-soluble ash

The ash obtained as described above was boiled for 5 min. with 25 ml of water and insoluble matter was collected in a Gooch crucible, or on an ashless filter paper, washed with hot water and ignited for 15 min. at a temperature not exceeding 450 °C. Weight of the insoluble matter was subtracted from the weight of the ash. The
difference in weight represents the water-soluble ash. The percentage of water-soluble ash was calculated with reference to the air-dried drug.

4.1.3. Phytochemical analysis

Dried fruit powder of *H. isora* was screened qualitatively for the major groups of chemical constituents using standard reagents like alkaloids, flavonoids, steroids, glycosides, phenols, anthraquinones, and tannins.

**Test for Alkaloids**

1 g of dried powder was extracted with 20 mL alcohol (95%) by refluxing for 15 minutes and filtered and the filtrate was evaporated to dryness. The residues were dissolved in 15 mL of H₂SO₄ (2N) and filtered. After making alkaline the filtrate was extracted with chloroform. The residue left after evaporation, when tested for the presence of alkaloids with dragendorff's reagent did not give orange color.

**Test for flavonoids**

(a) Shinoda test

1 g powder was extracted with 10 mL of ethanol (95%) for 15 min on a boiling water bath and filtered. To the filtrate was added a small piece of magnesium ribbon and 3 to 4 drops of concentrated sulphuric acid. Formation of red colour indicated the presence of flavonoids.

(b) Fluorescence test

1 g powder was extracted with 15 mL methanol for 2 min on a boiling water bath, filtered while hot and evaporated to dryness. To the residue was added 0.3 mL of boric acid solution (3 % w/v) and 1 mL oxalic acid solution (10 % w/v). The mixture was evaporated to dryness and the residue was dissolved in 10 mL ether. The ethereal layer showed greenish fluorescence under UV indicating presence of flavonoids.

**Tests for saponins**

0.1 of powder was vigorously shaken with 5 mL of distilled water in a test tube for 30 seconds and was left undisturbed for 20 min. Persistent froth indicated presence of saponins.
Test for sterols

Liberman Burchadt test

To 1 g dried powder moistened with 1.0 ml of acetic anhydride on a clean tile, was added 2 drops of sulphuric acid. The powder was mixed well and the color gained by the powder was observed. Formation of green-blue-purple-red colour indicated presence of sterols.

Test for tannins

Aqueous extract of the dried powder was prepared by refluxing 10 g powder with 50 ml of water for about 1 h and was used for the following tests.

(a) Test with gelatin

To 2-3 ml of aqueous extract, was added 1 % gelatin solution containing NaCl. Heavy white precipitate indicated presence of tannins.

(b) Reaction with lead acetate

Tannins were precipitated from the aqueous extract by adding 2 ml of 10 % solution of lead acetate. Precipitates obtained were partially soluble in 1 ml of 10 % acetic acid indicating presence of condensed tannins.

(c) Reaction with FeCl₃

1 ml of aqueous extract was treated with 0.5 ml of 5 % FeCl₃. A green colour formed indicated presence of pyrocatechol partial structures (whereas a blue colour is characteristics for partial pyrogallol structure).

(d) Reaction with bromine water

To 2 ml of aqueous extract, 0.5 ml of freshly prepared bromine water was added. Precipitate formed indicated presence of condensed tannins.

(e) Reaction with formaldehyde

To 2 ml of the aqueous extract, 1 ml of formaldehyde and HCl were added. Formation of red precipitate of phlobaphenes upon heating indicated presence of condensed tannins.

(f) Reaction with vanillin-HCl

2 ml of aqueous extract upon treatment with 1 % vanillin in alcohol followed by hydrochloric acid gave pinkish red colour suggesting presence of condensed tannins.
(g) Matchstick’s test

A wood portion of matchstick was dipped into the aqueous extract, dried and was moistened with HCl. The matchstick was warmed by bringing near to the flame. Pink stain developed on wood indicated presence of condensed tannins.

Estimation of phenolics

Preparation of extract

0.1 g of air dried rootstocks powder was extracted with 100 ml methanol by maceration for 24 h and filtered. The final volume of the filtrate was adjusted 100 ml using ethanol. 5 ml of this extract was diluted with an equal volume of methanol and was used for the estimation of phenols.

Method

To 1 ml of the methanolic extract was added 10 ml of distilled water and 1.5 ml of diluted (1:2) Folin ciocalteu reagent and the mixture was kept aside for 5 min. After adding 4 ml of 20 % Na2CO3 solution the final volume was adjusted to 25 ml using distilled water. The absorbance was measured at 765 nm at an interval of 30 min up to 2 h using distilled water as a blank. The total phenol content was measured using following formula:

\[ C = (A \times 282.6) - 8.451 \]

\[ A = \text{Absorbance} \]

Estimation of flavones and flavonols

Preparation of extract

*H. isora* extract (HE) was prepared as follows. One gram powder was extracted with 25 ml of 95 % v/v ethanol for 24 h at 37 °C and the filtrate was adjusted to 25 ml with 80 % v/v ethanol.

Method

Flavones and flavonols in *H. isora* were expressed as rutin equivalent. The standard solution and HE (0.5 ml) were mixed with 1.5 ml 95 % v/v ethanol, 0.1 ml 10 % m/V aluminium chloride, 0.1 ml of 1 mol L⁻¹ potassium acetate and 2.8 ml water. The volume of 10 % m/V aluminium chloride was substituted by the same
volume of distilled water in blank. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm.

4.1.4. Isolation and fractionation

The dried fruits of *H. isora* were crushed, powdered and shifted from 40 # sieve. Dried powder of *H. isora* was extracted with 50 % alcohol. The extract was concentrated under reduced pressure to yield dry solid.

In another set of extraction, dried powder first extracted with pet-ether then ethyl acetate, and N-butanol successively. Thus obtained pet-ether, ethyl acetate, and N-butanol extracts were concentrated under reduced pressure and air dried to remove the solvents completely. The obtained alcoholic, pet-ether, ethyl acetate, and N-butanol extracts were studied in different experimental animal models.

**Scheme for fractionation**

```
Fruit powder
   ↓ Pet-ether
   ↓ Dry powder
   ↓ Ethyl acetate
   ↓ Dry powder
   ↓ N-butanol
   ↓ Dry powder
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Pet-ether soluble fractions

Ethyl acetate soluble fractions

N-butanol soluble fractions
4.1.5. HPTLC analysis of extracts

Pet-ether, ethyl acetate and N-butanol extracts were dissolved in methanol (HPLC grade) to get 5 mg/ml solution of each. 10 μl of each solution were employed on precoated aluminium sheets of silica gel 60 F<sub>254</sub> (0.25 mm thick, Merck) using CAMAG automatic sample spotter. 10 μl of lupeol, β-sitosterol, and α-amyrin were used as a reference standard. The chromatograms were developed using mobile phase containing toluene: methanol (9.6: 0.4). The developed plate was scanned on CAMAG TLC scanner at 540 nm after derivatization with anisaldehyde reagent.

During HPTLC of extracts, Rf values of different spots of sample extracts were compared with standard for the presence of lupeol, β-sitosterol, and α-amyrin. While area under curve (AUC) was determined for quantifying the phytochemicals present in the extracts by comparing with AUC of reference standard.
4.2. PHARMACOLOGICAL EVALUATION

4.2.1. Antiulcer activity

Animals

Wistar albino rats of either sex (150-200 g) were selected for the study. The animals were housed under standard conditions of 12 h dark: light cycle, 60 ± 10% humidity and temperature of 21.5 ± 1°C. Experiment complied with the guidelines of ethics committee of our laboratory for animal experimentation. Rats were fasted for 24 h with water ad libitum. Rats were kept in cages with grated floor to prevent coprophagy.

4.2.1.1. Ethanol-induced gastric ulcers in rats (Robert et al, 1979)

Group-1: Only 1% Na CMC as vehicle
Group-2: Pantoprazole (20 mg/kg, p.o.)
Group-3: Alcoholic extract (100 mg/kg, p.o.)
Group-4: Pet ether extract (100 mg/kg, p.o.)
Group-5: Ethyl acetate extract (100 mg/kg, p.o.)
Group-6: N-butanol extract (100 mg/kg, p.o.)

Gastric lesions in rats were induced by oral administration of 1 ml of 95% alcohol. All the treated animals were given the test extracts 1 h before the alcohol administration. Two hrs after the alcohol administration, rats were sacrificed, abdomen opened, and stomachs were removed. Stomachs were opened through the greater curvature and ulcer indices were determined (Ganguly and Bhatanagar, 1973). Stomach tissues were processed to measure the gastric wall mucus content (Corne et al, 1974), lipid peroxidation (Okhawa et al, 1979), catalase activity (Aebi et al, 1974), super oxide dismutase activity (SOD) (Misra et al, 1972), and reduced glutathione (Beutler et al, 1963).

4.2.1.2. Ethanol-induced gastric mucosal lesion in indomethacin-pretreated rats (Arrieta et al, 2003)

Group-1: Indomethacin + 1% Na CMC
Group-2: Indomethacin + alcoholic extract (100 mg/kg, p.o.)
Group-3: Indomethacin + N-butanol extract (100 mg/kg, p.o.)
To investigate the involvement of endogenous prostaglandins for the gastroprotective activity of alcoholic and N-butanol extracts of H. isora, indomethacin (70 mg/kg, s.c.) injected 75 min before the treatment of groups with 1% Na CMC, alcoholic extract (100 mg/kg, p.o.), N-butanol extract (100 mg/kg, p.o.). One hour after drug treatment, 1 ml of 95% alcohol was administrated orally. Two hours after alcohol treatment animals were sacrificed and gastric mucosal lesions were measured (Bhatanagar and Ganguly, 1973).

4.2.1.3. Ethanol-induced gastric mucosal lesions in \textit{N}^\text{G}-\text{nitro-L-arginine methyl ester} pretreated rats (Arrieta et al, 2003)

Group-1: L-NAME + 1% Na CMC
Group-2: L-NAME + alcoholic extract (100 mg/kg, p.o.)
Group-3: L-NAME + N-butanol extract (100 mg/kg, p.o.)

To investigate the involvement of endogenous nitric oxide (NO) for the gastroprotective activity of alcoholic and N-butanol extracts of H. isora, \textit{N}^\text{G}-\text{nitro-L-arginine methyl ester} (L-NAME, 70 mg/kg, dissolved in 0.9% w/v NaCl solution) was intraperitoneally injected 30 min before the treatment of groups with 1% Na CMC, alcoholic extract (100 mg/kg, p.o.), N-butanol extract (100 mg/kg, p.o.) respectively. One hour after drug treatment, 1 ml of 95% alcohol was administrated orally. Two hours after alcohol treatment animals were sacrificed and gastric mucosal lesions were measured (Bhatanagar and Ganguly, 1973).

4.2.1.4. Ethanol-induced gastric mucosal lesion in N-ethylmaleimide-pretreated rats (Arrieta et al, 2003)

Group-1: N-ethylmaleimide + 1% Na CMC
Group-2: N-ethylmaleimide + alcoholic extract (100 mg/kg, p.o.)
Group-3: N-ethylmaleimide + N-butanol extract (100 mg/kg, p.o.)

To investigate the involvement of endogenous sulfhydryl compounds for the gastroprotective activity of alcoholic and N-butanol extracts of H. isora, N-ethylmaleimide (10 mg/kg, dissolved in 0.9% w/v NaCl solution) was subcutaneously injected 30 min before the treatment of groups with 1% Na CMC, alcoholic extract (100 mg/kg, p.o.), N-butanol extract (100 mg/kg, p.o.) respectively.
One hour after drug treatment, 1 ml of 95% alcohol was administrated orally. Two hours after alcohol treatment animals were sacrificed and gastric mucosal lesions were measured (Bhatanagar and Ganguly, 1973).

4.2.1.5. Ethanol-induced and pylorus ligated gastric mucosal lesions in rats

(Sener et al., 2004)

The animals of various groups received treatment as follows.

Group-1: Pylorus ligation + 1% Na CMC
Group-2: Ethanol + Pylorus ligation + 1% Na CMC
Group-3: Ethanol + Pylorus ligation + pantoprazole (20 mg/kg, p.o.)
Group-4: Ethanol + Pylorus ligation + alcohol extract (100 mg/kg, p.o.)
Group-5: Ethanol + Pylorus ligation + N-butanol extract (100 mg/kg, p.o.)

Animals of various groups were treated as above one hour before the alcohol administration (1 ml/rat, p.o.). Pylorus of anesthetized rat was ligated one hour after the alcohol administration. Two hours after pylorus ligation, rat was sacrificed by ether anesthesia, and esophagus was clamped. The stomach was removed, opened along the greater curvature and the ulcer index was determined (Bhatanagar and Ganguly, 1974). The gastric content was centrifuged at 2500 rpm for 10 min. The volume of the supernatant was measured. The juice was subjected to biochemical analysis for total acidity, total acid output (Hawk, 1965), pepsin activity (Debnath et al., 1974), Total carbohydrates (TC) (Nair, 1976), and protein content (PR) (Lowry et al., 1951). TC/PR ratio was calculated which reflects the mucin activity.

4.2.2. Hepatoprotective activity of *H. isora*

Wistar albino rats of either sex weighing 150-200 g were selected for the study. Animals were fed a standard chow diet and water, which was freely available under standard conditions of a 12 h dark:light cycle, 60 ± 10 % humidity and a temperature 21 ± 1 °C. This experiment complied with guidelines of ethics committee of our laboratory for animal experimentation.

The animals were randomly divided into following groups of six animals each.

Group-1: Control group (Only vehicle given)
Group-2: CCl₄ + Vehicle treated group
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Group-3: CCl₄ + Silymarin (50 mg/kg, p.o.) treated group
Group-4: CCl₄ + Alcohol extract (100 mg/kg, p.o.) treated group
Group-5: CCl₄ + Pet-ether extract (100 mg/kg, p.o.) treated group
Group-6: CCl₄ + Ethyl acetate extract (100 mg/kg, p.o.) treated group
Group-7: CCl₄ + N-butanol extract (100 mg/kg, p.o.) treated group

Liver injury was produced in the animals of group 2-7 giving 1 ml/kg CCl₄:liquid paraffin (1:1) on alternate days for one week as mentioned above. On the 8th blood samples were collected from retro orbital plexus, and the serum was separated. Serum samples were analyzed for biochemical parameters like SGPT, SGOT, and alkaline phosphatase by standard diagnostic kits (Span Diagnostic Ltd.). Liver from each animal was dissected and washed with phosphate buffer (pH=7). 10% of homogenate of liver tissue in phosphate buffer was used for the determination of SOD (Misra et al, 1972), catalase activity (Aebi et al, 1974), reduced glutathione (Beutler et al, 1963), lipid peroxidation measured as thiobarbituric acid reacting substances (TBA-RS) (Okhawa et al, 1979), and tissue protein (Lowry et al, 1951). Liver tissues were preserved in 10% formalin solution for histological study.

4.2.3. Antihyperlipidemic activity of H. isora

Sprague Dawley rats of either sex weighing 200-250 g were used. Rats were maintained on a standard diet and water ad libitum. The rats were divided into seven groups, one of which i.e. control group received a standard chow diet whereas other groups were fed with a high cholesterol diet (HCD). Except normal and HCD control groups, all other groups received treatment as shown below for seven days along with high cholesterol diet. On 8th day blood samples were collected from retro orbital plexus under light ether anaesthesia from over night fasted rats. Blood samples were allowed to clot and serum was separated by centrifugation at 3000 for 30 min. Serum samples were analyzed for serum cholesterol, triglyceride, HDL using standard diagnostic kits (Span Diagnostics Ltd.) VLDL and LDL were calculated as per Friedevelad’s equation.

\[
VLDL = \frac{\text{Total Serum Triglycerides}}{5}
\]
Materials and methods

\[
\text{LDL} = \frac{\text{Total Cholesterol} - \text{Total Serum Triglycerides} - \text{HDL}}{5}
\]

\[
\text{Atherogenic Index} = \frac{\text{Total Serum Triglycerides}}{\text{Total Serum HDL}}
\]

The high cholesterol diet (HCD) includes a standard diet mixed with 2% cholesterol, 1% sodium taurocholate, and 10% coconut oil.

The animals were randomly divided into following groups of six animals each.

Group-1: Control group (Only vehicle given)
Group-2: HCD + Vehicle treated group
Group-3: HCD + Atorvastatin (1.5 mg/kg, p.o.) treated group
Group-4: HCD + Alcohol extract (100 mg/kg, p.o.) treated group
Group-5: HCD + Pet-ether extract (100 mg/kg, p.o.) treated group
Group-6: HCD + Ethyl acetate extract (100 mg/kg, p.o.) treated group
Group-7: HCD + N-butanol extract (100 mg/kg, p.o.) treated group

4.2.4. Antidiabetic activity of *H. isora*

Wistar albino rats of either sex weighing 150-200 g were selected for the study. Animals were fed a standard chow diet and water ad libitum, under standard condition of a 12 h dark-light cycle, 60 ± 10% humidity and a temperature 21 ± 1°C. This experiment complied with guidelines of ethics committee of our laboratory for animal experimentation. Diabetes was induced with alloxan monohydrate (65 mg/kg) dissolved in normal saline, administered as a single intravenous tail vein injection under light ether anesthesia. Control animals were injected with an equivalent volume of normal saline. Animals were checked for glucosuria 48 h after alloxan monohydrate treatment. Animals showing blood sugar level more than 250 mg/dL were included in the study. The selected animals were divided into six groups of six animals each. They received the treatment for fifteen days as follows;

Group-1: Control animals treated with 1% Na CMC
Group-2: Diabetic animals treated with 1% Na CMC
Group-3: Diabetic animals treated with alcoholic extract (300 mg/kg, p.o.)
Group-4: Diabetic animals treated with Pet-ether extract (300 mg/kg, p.o.)
Group-5: Diabetic animals treated with Ethyl acetate extract (300 mg/kg, p.o.)
Group-6: Diabetic animals treated with N-butanol extract (300 mg/kg, p.o.)

Blood samples were collected at the end of 15th day of treatment from the retroorbital plexus under light ether anesthesia and were allowed to clot for 30 min at room temperature. Serum was separated by centrifugation at 3000 rpm and stored at -20 °C until the analysis was carried out. Serum samples were analyzed for glucose, cholesterol, triglyceride, HDL, creatinine, urea, serum glutamate oxaloacetate (SGOT), serum glutamate pyruvate transaminase (SGPT). VLDL and LDL were calculated as per Friedevald's equation mentioned earlier. Changes in body weight, food intake, and water intake were also recorded throughout the study.

Determination of ulcer index (Ganguly & Bhatanagar, 1973)

Each linear lesion was measured along its greater length and width. For circular lesion, diameter (d) was measured and finally area was calculated. 5 patches were considered to be equivalent to 1 mm² of ulcerated area. The ratio (X) of total area of stomach mucosa and that of ulcerated mucosa was calculated.

\[
X = \frac{\text{Total ulcerated area}}{\text{Total stomach mucosa}} \\
\text{Area of circular lesion} = \pi d^2 / 4 \quad (d=\text{diameter of circular lesion})
\]

Area of linear lesion = Length x Width

\[
\text{Area of stomach area} = \pi d^2 / 8 \quad (d=\text{diameter of stomach})
\]

Volume of gastric content (Hawk, 1965)

The total amount of gastric secretion was collected and measured. The volume of gastric content was expressed in ml/100 g body weight.

Total acidity & Total acid output (Hawk, 1965)

Gastric contents were assayed for total acidity by titration against 0.01 N NaOH to pH 8.0 using phenolphthalein as indicator. Phenolphthalein will change color from colorless to pink indicating the total acidity. The amount of HCl in gastric
content was calculated and expressed as mEq/L. The total acid output was expressed as μEq/100 g body weight.

\[
\text{Acidity} = \frac{\text{ml NaOH} \times N \times 100}{0.1} \text{mEq/L}
\]

\[
\text{Total acid output} = \frac{(\text{Total acidity} \times \text{Volume of gastric content}) \times 100}{\text{Body weight}}
\]

**Estimation of pepsin activity (Debnath et al, 1974)**

The determination of pepsin activity was carried out as described earlier by Anson (1938) and modified by Debnath et al, 1974.

**Reagents**

- **0.01 & 0.05 N HCl:** It was prepared by dilution in distilled water from 1 N HCl at the time of use.
- **Haemoglobin solution:** 2% haemoglobin solution was prepared freshly in 0.05 N HCl, filtered, and used.
- **Trichloroacetic acid:** 10% solution of TCA in distilled water, some amount of charcoal was added, thoroughly mixed and kept for 10 min. It was then filtered and kept in glass stoppered bottle.
- **Ciocalteau folin's reagent:** 1 N C.F. reagent was prepared by 2 times dilution with distilled water.
- **Alkaline mixture:** 2% solution of Na₂CO₃ in 0.1 N NaOH. This solution was always prepared fresh before use.
- **Alkaline reagent:** 100 ml of alkaline mixture was mixed with 1 ml of 4% of aqueous solution of potassium tartarate and 1 ml of 2% aqueous copper sulphate.

**L-tyrosine:** It was used as standard in estimation of digested substrate to indicate pepsin activity.

**Procedure**

0.4 ml of the diluted gastric juice (1:250) and 1 ml of haemoglobin solution in 0.05 N HCl were taken in separate test tubes. Both the test tubes were kept in the incubator at 37 °C for 10 min. The haemoglobin solution was then added to the diluted
gastric juice and this mixture will be incubated at 37 °C for 20 min. Digestion was stopped by adding equal volume i.e., 1.4 ml of ice cold trichloroacetic acid and kept in ice bath for 15 min. The mixture was then filtered out to separate the precipitated undigested protein. 0.4 ml of filtrate was taken to determine the concentration of liberated amino acid tyrosine by first adding 4 ml of alkaline reagent. It was followed after 10 min by addition of 0.4 ml of diluted Ciocalteau-Folin reagent as per the method of Lowry et al (1951). The optical density was determined with UV-Spectrophotometer set at 610 nm against the blank prepared similarly using 0.01 N HCl instead of diluted gastric juice after 10 min of adding Ciocalteau-Folin reagent. The peptic activity was expressed in terms of μg/ml of tyrosine liberated per ml of gastric juice.

**Total carbohydrates (Nair, 1976)**

**Reagents**
Phenol (5 %): 5 % solution of phenol was prepared freshly in distilled water and filtered.
Sulphuric acid (95 %)
Glucose: Used as a standard for estimation of total carbohydrates

**Procedure**

1 ml of 5 % phenol was pipetted out into test tubes containing 0.15 ml of gastric juice and blank containing 0.15 ml of distilled water and mixed thoroughly. 5 ml of 95 % sulphuric acid was added and mixed slowly. After 10 min, the test tubes were shaken and placed in water bath kept 20-30 °C temperature for 20 min. The optical density of the developed yellow-orange chromophore was read in UV-spectrophotometer at 482 nm. Several concentrations of glucose standard solutions were run to prepare a standard curve. Total carbohydrates were expressed in terms of μg/ml of gastric juice.

**Determination of free radical scavenging activity of *H. isora***

Tissue homogenate (10 % w/v) was prepared homogenizing the sample tissue in ice-cold 50 mM phosphate buffer (pH=7). The homogenate was then centrifuged at 800 g for 10 min followed by centrifugation of the supernatant at 12,000 g for 15 min and the obtained mitochondrial fraction was used for the estimations of lipid
peroxidation (measured as TBA-RS), catalase activity, superoxide level, and reduced glutathione level.

**Determination of lipid peroxidation (Okhawa et al, 1974)**

Lipid peroxidation was measured as per method described by Okhawa et al (1979) hereinafter. Lipid peroxidation product MDA (malondialdehyde) was estimated. 1.0 ml sample was mixed with 0.2 ml sodium dodecyl sulfate (4 % w/v), 1.5 ml 20 % acetic acid in 0.27 M hydrochloric acid (pH=3.5) and 1.5 ml 0.5 % thiobarbituric acid (TBA, pH= 7.4). The mixture was heated in a hot water bath at 85 oC for 1 h. The intensity of pink color developed was read against blank at 532 nm following centrifugation at 1200 g for 10 min. The amount of malondialdehyde (thiobarbituric acid reacting substances, TBA-RS) was calculated using molar extinction coefficient 1.56 x 10\(^{-5}\) M\(^{-1}\) cm\(^{-1}\) and was reported as μmoles of MDA/mg protein.

**Determination of super oxide dismutase level (Misra et al, 1972)**

Super oxide dismutase level was measured by as per method described by Misra et al (1972). 0.1 ml of sample was mixed with 0.1 ml EDTA (1 x 10-4 M), 0.5 ml of carbonate buffer and 1 ml of epinephrine (3 x 10-3 M). The optical density of formed adrenochrome was measured at 480 nm for 3 min at an interval 30 sec. Results were expressed as units/mg protein. One unit of enzyme activity was defined as the enzyme concentration required to inhibit the chromogen production by 50 % in 1 min under the defined assay conditions.

**Determination of catalase activity (Aeibi, 1974)**

**Reagents**

- 0.05 M Potassium phosphate, pH 7.0
- 0.059 M hydrogen peroxide in 0.05 M potassium phosphate, pH 7.0

The catalase level was measured as per method described by Aeibi et al (1974). Decomposition of H\(_2\)O\(_2\) in presence of catalase was measured at 240 nm. A 0.1 ml of sample was added to the cuvette containing reagent grade water (1.9 ml) and buffered substrate (1.0 ml) and made total volume 3 ml. The decrease in absorbance was
recorded at 37 oC for 2-3 min at an interval of 15 sec. The activity was calculated using extinction coefficient of H$_2$O$_2$ (0.081 M$^{-1}$ cm$^{-1}$) at 240 nm and results have been expressed as μmoles of H$_2$O$_2$ utilized/min/mg protein.

**Determination of reduced glutathione (GSH) (Beutler et al, 1963)**

**Reagents**

- Trichloroacetic acid: 10 % w/v
- Disodium hydrogen phosphate: 0.3 M
- 5, 5'-dithiobis-2-nitrobenzoic acid: 40 mg dissolved in 100 ml of 1 % w/v sodium citrate

The reduced glutathione level was measured as per method described by Beutler et al (1963). GSH contents in tissue homogenates were measured after precipitation of protein with chilled trichloroacetic acid (10 % w/v). Samples were kept in ice bath and were centrifuged after 30 min at 1000 g for 10 min at 4 °C. GSH levels were measured in the supernatant. Supernatant (0.5 ml) was mixed with 2.0 ml 0.3 M disodium hydrogen phosphate solution and 0.25 ml 5, 5'-dithiobis-2-nitrobenzoic acid was added just before measuring the absorbance at 412 nm. Different concentrations of GSH were also processed similarly to prepare a standard curve simultaneously. Results were expressed as μmoles of GSH/mg protein.

**Gastric wall mucus content (Corne et al, 1974)**

**Reagents**

- HCl: 1 M
- Sucrose solution: 0.25 M
- Magnesium chloride: 0.5 M
- Alcian blue solution (1 % w/v): Alcian blue 8 GX (Sigma) in 0.16 M sucrose solution buffered with 0.05 M sodium acetate and finally adjusting the pH to 5.8 with 1 M HCl.

**Procedure**

The glandular segment of stomach after opening the greater curvature was removed and weighed. Each segment was transferred immediately to 10 ml of 0.1 % w/v alcian blue solution. Tissues were allowed to stain for 2 h in alcian blue solution. Excess dye was removed by rinsing the tissue twice with 10 ml of 0.25 M sucrose,
first for 15 min and then for 45 min. Dye complexed with the gastric wall mucus was
extracted from the glandular tissue with 10 ml of 0.5 M magnesium chloride that was
intermittently shaken for 1 min every 30 min for 2 h. 4 ml of this blue colored extract
solution was then shaken vigorously with an equal volume of diethyl ether. The
resulting emulsion was centrifuged at 3600 rpm for 10 min and aqueous layer
separated. Concentration of alcian blue was determined in this layer. Absorbance was
recorded by using an UV spectrophotometer at 598 nm. The quantity of alcian blue
extracted per gram of wet glandular tissue was then calculated from standard curve
prepared which obeyed the Beer-Lambert law at the concentration of dye used.

Determination of protein (Lowry et al, 1951)

Reagents

Reagent A: 2 % NaCO₃ in 0.1 N NaOH.
Reagent B: 0.5 % CuSO₄ 5 H₂O in 1% sodium or potassium tartrate.
Reagent C: Alkaline reagent solution, mix 50 ml of reagent A with 1 ml of
reagent B. discard after 1 day.
Reagent D: 1 N Folin reagent (diluted about 2 fold)
Bovine albumin: Standard solution

Procedure

To a sample of 5 to 500 γ of protein in 0.2 ml or less in a 3 to 10 ml test-tube,
1 ml of reagent C is added. Mix well and allow to stand for 10 min or longer at room
temperature. 0.1 ml of Reagent D is added very rapidly and mixed within a second or
two. After 30 min or longer, the sample is read in a colorimeter or spectrophotometer.
For the range of 5 to 25 γ of protein per ml of final volume, it is desirable to make
readings at or near λ = 750 μ, the absorption peak. For stronger solutions, the
readings may be kept in a workable range by reading near λ = 500 μ. Calculate the
concentration from a standard curve.

Determination of serum glucose (GOD/POD method)

Principle

Glucose is oxidized by the enzyme glucose oxidase (GOD) into gluconic acid
and hydrogen peroxide. Hydrogen peroxide in the presence of enzyme peroxidase
(POD) oxidizes the chromogen 4-aminoantipyrine/phenolic compound to a red
colored compound. The intensity of the color produced is proportional to glucose concentrations in the sample and is measured at 505 nm. This final color is stable for two hours.

\[
\text{GOD} \\
\text{Glucose} + \text{O}_2 \rightarrow \text{Gluconic acid} + \text{H}_2\text{O}_2
\]

\[
\text{POD} \\
\text{H}_2\text{O}_2 + \text{Phenolic Compound} + 4\text{-amino antipyrine} \rightarrow \text{Red compound} + 2\text{H}_2\text{O}
\]

**Preparation of working solution**

One buffer/enzyme chromogen tablet was gently dissolved in 20 ml of distilled water in a clean beaker, with continuous stirring.

**Procedure**

One ml of the working solution was added to test tube containing 10 µl of serum sample. Similarly standard and blank were prepared by using 10 µl of glucose standard (provided in the kit) and distilled water respectively. They were then mixed and incubated at room temperature for 30 min. Absorbance of test and standard was measured against blank at 505 using UV-visible spectrophotometer (UV-1601 Shimadzu, Japan).

The concentration of glucose in test sample was calculated using following formula.

\[
\text{Serum glucose (mg/dl)} = \frac{\text{O. D. of test}}{\text{O. D. of std}} \times 100
\]

**Determination of Cholesterol**

**Principle**

\[
\text{Cholesterol esterase} \\
\text{Cholesterol ester} + \text{O}_2 \rightarrow \text{Cholesterol} + \text{Fatty acids}
\]

\[
\text{Cholesterol Oxidase} \\
\text{Cholesterol} + \text{O}_2 \rightarrow \text{Dehydroacetone phosphate} + \text{H}_2\text{O}_2
\]

\[
\text{peroxidase} \\
2\text{H}_2\text{O}_2 + \text{Phenol} + 4\text{-aminoantipyrine} \rightarrow \text{Red quinine} + 4\text{H}_2\text{O}
\]
The intensity of the red complex (red quinone) formed during the reaction is directly proportional to the cholesterol concentration in the sample and is measured at 500 nm.

Procedure
Reagents are reconstituted as described in the leaflet supplied along with the kit. 10 μl of serum samples, distilled water serving as control and standard triglyceride (200 mg/dl) serving as standard were mixed well with 1.0 ml reconstituted reagent i.e. enzyme/chromogen mixture. They were incubated at 37 oC for min and absorbance was read against blank at 500 nm.

Calculations

Serum cholesterol (mg/dl) = \( \frac{O. \ D. \ of \ test}{O. \ D. \ of \ std} \times 200 \)

Triglycerides

Principle
Triglycerides are enzymatically hydrolyzed to glycerol according to the following reactions.

\[
\begin{align*}
\text{Tiglycerides} + H_2O & \rightarrow \text{Glycerol} + \text{Free fatty acids} \\
\text{Glycerol} + \text{ATP} & \rightarrow \text{Glycerol-3-Phosphate} + \text{ADP} \\
\text{Glycerol-3-Phosphate} + \text{O}_2 & \rightarrow \text{Dehydroacetone Phosphate} + \text{H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 + 4\text{-} \text{aminoantipyrine} + \text{ADPS} & \rightarrow \text{Red quinone} + 4\text{H}_2\text{O}
\end{align*}
\]

GPO = Glycerol-3-Phosphate Oxidase
ADPS = N-Ethyl-N-Sulfopropyl-n-anisidine
The intensity of the red complex (red quinone) complex formed during the reaction is directly proportional to the triglyceride concentration in the sample and is measured at 546 nm. The final color is stable for at least 30 min.

**Procedure**

Reagents are reconstituted as described in the leaflet supplied along with kit. 10 µl of serum samples, distilled water serving as control and standard triglyceride (200 mg/dl) serving as standard were mixed well with 1 ml reconstituted reagent 1 i.e. enzyme/chromogen mixture. They are incubated at 37 oC for min and absorbance was read against blank at 546 nm.

**Calculations**

\[
\text{Serum triglyceride (mg/dl)} = \frac{\text{O. D. of test}}{\text{O. D. of std}} \times 200
\]

**HDL-Cholesterol**

**Principle**

Chylomicrons, VLDL, and LDL fractions in serum or plasma are separated from HDL by precipitating with phosphotungstic acid and magnesium chloride. After centrifugation, the cholesterol in HDL fraction, which remains in the supernatant is assayed with enzymatic cholesterol method, using cholesterol esterase, cholesterol oxidase, peroxidase and the chromogen 4-amino antipyrine/phenol.

**Procedure**

Reagents are reconstituted as described in the leaflet supplied along with the kit. 0.2 ml of serum sample was mixed well with 0.2 ml of precipitating reagent (Reagent 2) and centrifuged at 3500-4000 for 10 min. supernatant 20 µl and 1 ml of reconstituted reagent 1 was added. In case on blank 1 ml reconstituted reagent 1 was taken. Absorbance of test samples was measured against reagent blank at 500 nm.

**Calculations**

\[
\text{Serum HDL-C (mg/dl)} = \frac{\text{O. D. of test}}{\text{O. D. of std}} \times 50
\]
Creatinine

Principle
Creatinine in a protein free solution reacts with alkaline picrate and produces a red colored complex, which is measured colorimetrically at 520 nm.

Procedure
Deproteinization of test sample
0.5 ml of serum sample was mixed well with 0.5 ml distilled water and 3 ml picric acid (Reagent 1). It was kept in boiling water bath exactly for one minute and cooled immediately under running tap water and centrifuged.
2.0 ml of the supernatant from the above step is mixed with 1.0 ml sodium hydroxide solution (Reagent 2). 0.5 ml of distilled water and working creatinine standard mixed with 1.5 ml picric acid and 0.5 ml sodium hydroxide solution served as blank and standard respectively. All the tubes were allowed to stand at room temperature after thorough mixing for 20 min. The absorbance of blank, standard and samples were measured immediately against distilled water at 520 nm.

Calculations
Serum creatinine concentration was calculated using following formula.

\[
\text{Serum creatinine (mg/dl)} = \frac{\text{O. D. test} - \text{O. D. blank}}{\text{O. D. std} - \text{O. D. blank}}
\]

Urea

Principle
The test is based on the Berthelot's reaction. Urease splits urea into ammonia and carbon dioxide. The ammonia reacts with phenol in the presence of hypochlorite to form an indophenol which with alkali gives a blue colored compound. The intensity of the color is proportional to the concentration of urea in the sample and is measured at 546 nm. The color of the reaction is stable for 8 h.

Procedure
Reagents are reconstituted as described in the leaflet supplied along with the kit. 10 µl of serum sample, urea standard (40 mg/dl) and distilled water as blank were mixed with 100 µl of urease solution (Reagent 1). Contents are mixed and incubated
at 37 °C for 10 min. 1.5 ml of phenol (Reagent 2) and sodium hypochlorite solution (Reagent 3) were added to all test tubes and mixed well. The absorbance was read at 540 nm in UV-Visible spectrophotometer (UV-1601 Shimadzu, Japan). The final color developed is stable for at least 30 min.

The concentration of urea was calculated by using following formula

\[
\text{Serum Urea (mg/dl)} = \frac{\text{O. D. of test}}{\text{O. D. of std}} \times 40
\]

**Serum glutamate pyruvate transaminase (SGPT)**

**Principle**

SGPT catalyses transfer of amino group from L-alanine to α-ketoglutarate with formation of pyruvate and glutamate. The pyruvate so formed, is allowed to react with 2, 4-DNPH to produce 2, 4-dinitrophenyl hydrazone derivative which is brown colored in alkaline medium. The absorbance of this hydrazone derivative is correlated to SGPT activity by plotting a calibration curve using pyruvate standard.

\[
\text{SGPT (pH= 7.4)}
\]

\[
\text{L-arginine + α-ketoglutarate} \rightarrow \text{pyruvate + L-glutamate}
\]

\[
\text{Pyruvate + 2, 4-DNPH} \rightarrow \text{2, 4-dinitrophenyl hydrazone (Blue colored)}
\]

**Procedure**

**Calibration curve**

In five clean test tubes buffered substrate, pyruvate standard distilled water and DNPH color reagent were added as per mentioned in the leaflet supplied with the kit. Contents were mixed well and incubated at room temperature for 20 min. 5 ml of working sodium hydroxide was added to all the tubes, mixed and allowed to stand at room temperature for 10 min. absorbance of tubes 2 to 5 was measured against tube 1 reagent blank at 505 nm. A graph of absorbance was plotted against enzyme activity (supplied in leaflet).
Materials and methods

Assay

0.5 ml buffered substrate was incubated at 37 oC for 3 min. 0.1 ml serum sample was added to buffered substrate and incubated at 37 oC for 60 min. To this DNPH color reagent was added and allowed to stand at room temperature for 20 min. Finally 5.0 ml working sodium hydroxide solution was added to all the tubes. After mixing well the tubes were allowed to stand at room temperature for 10 min. In case on blank similar procedures was followed except that instead of serum 0.1 ml distilled water was added. Absorbance of test samples was measured against reagent blank at 505 nm and was read on calibration curve to find out enzyme activity (supplied in leaflet).

Serum glutamate oxaloacetate transaminase (SGOT)

Principle

SGOT catalyses transfer of amino group from L-aspartate to α-ketoglutarate with formation of oxaloacetate and glutamate. The oxaloacetate so formed, is allowed to react with 2, 4-DNPH to produce 2, 4-dinitrophenyl hydrazone derivative which is brown colored in alkaline medium. The absorbance of this hydrazone derivative is correlated to SGOT activity by plotting a calibration curve using pyruvate standard.

\[
\text{L-aspartate} + \alpha\text{-ketoglutarate} \rightarrow \text{Oxaloacetate} + \text{L-glutamate}
\]

\[
\text{Oxaloacetate} + 2, 4\text{--DNPH} \rightarrow 2, 4\text{--dinitrophenyl hydrazone (Brown colored)}
\]

Procedure

Calibration curve

In five clean test tubes buffered substrate, pyruvate standard distilled water and DNPH color reagent were added as per mentioned in the leaflet supplied in with the kit. Contents were mixed well and incubated at room temperature for 20 min. 5 ml of working sodium hydroxide was added to all the tubes, mixed and allowed to stand at room temperature for 10 min. Absorbance of tubes 2 to 5 was measured against
tube I reagent blank at 505 nm. A graph of absorbance was plotted against enzyme activity (supplied in leaflet).

**Assay**

0.5 ml buffered substrate was incubated at 37 °C for 3 min. 0.1 ml serum sample was added to buffered substrate and incubated at 37 °C for 60 min. To this DNPH color reagent was added and allowed to stand at room temperature for 20 min. Finally 5.0 ml working sodium hydroxide solution was added to all the tubes. After mixing well the tubes were allowed to stand at room temperature for 10 min. In case on blank similar procedure was followed except that instead of serum 0.1 ml distilled water was added. Absorbance of test samples was measured against reagent blank at 505 nm and was read on calibration curve to find out enzyme activity.

**Alkaline phosphatase (ALP)**

**Principle**

Alkaline phosphatase (ALP) from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10.0. Phenol so formed reacts in alkaline medium with 4-aminoantipyrine in presence of the oxidizing agent potassium ferricyanide and forms an orange-red colored complex, which can be measured calorimetrically. The color intensity is proportional to the enzyme activity.

The reaction can be represented as:

\[ \text{ALP (pH=10.0)} \]

\[ \text{Phenyl Phosphate} \quad \text{Phenol + Pi} \]

\[ \text{Phenol + 4-aminoantipyrine} \quad \text{Pot. Ferricyanide} \quad \text{Orange-red colored complex} \]

**Procedure**

Reagents are reconstituted as described in the leaflet supplied along with the kit. 100 µl of serum samples, distilled water serving as control and standard triglyceride (10 mg %) serving as standard were mixed well with 3.0 ml reconstituted absorbance was read against blank at 510 nm.
Determination of liver glycogen content (Carroll et al, 1956)

Reagents

Anthrone reagent: A solution containing 0.05% anthrone, 1% thiourea, and 72% by volume H$_2$SO$_4$ is used. For each liter of reagent, place in a suitable flask 280 ml of distilled water and add cautiously 720 ml of concentrated H$_2$SO$_4$. Place in a flask 500 mg of purified anthrone, 10 g of highest purity thiourea, and 1 liter of the 72% H$_2$SO$_4$. Warm the mixture to 80-90 °C occasionally shaking the flask to mix the contents.

Tichloroacetic acid: 5% w/v

Ethanol: 95% v/v

Glucose standard: Stock solution: Dissolve 100 mg of dry, highest purity glucose in 100 ml of saturated benzoic acid solution. Working standard: Place 5 ml of the stock solution in a 100 ml volumetric flask and make up to volume with saturated benzoic acid solution. 2 ml of this solution, containing 0.1 mg of glucose, are used as a standard.

Procedure

Place the tissue sample in an efficient blender under an appropriate volume of TCA and homogenize for 3 min. Pour the homogenate into a suitable centrifuge tube or bottle. Centrifuge and decant the supernatant fluid upon an acid-washed filter paper placed in a funnel draining into a graduated cylinder. Transfer the residue quantitatively to the blender with an appropriate volume of TCA and homogenize again for 1 min. Centrifuge the mixture and pour the supernatant fluid through the same filter. Two more extractions may be made in the same manner if it is desired to extract better than 97% of the glycogen present. Make up to the desired volume with 5% TCA and mix thoroughly. The final volume should be a quantity that will contain 10 to 200 γ of glycogen per ml. One ml of the trichloroacetic acid filtrate is pipetted into a 15 ml Pyrex centrifuge tube. To obtain the most reliable results, duplicate samples of each unknown are analyzed. To each tube are added 5 volumes of 95% ethanol with careful blowing to effect thorough mixing. This should be checked by noting the absence of an interface. The tubes are capped with clean rubber stoppers and allowed to stand overnight at room temperature (alternatively, placing the tubes in a water bath at 37-40 °C for 3 h may be carried out). After precipitation is complete,
the tubes are centrifuged at 3000 rpm for 15 min. The clear liquid is gently decanted from the packed glycogen and the tubes are allowed to drain in an inverted position for 10 min.

The glycogen is dissolved by addition of 2 ml of distilled water, the water being added in a manner that will wash down the sides of the tube. If the glycogen does not dissolve instantly, agitate the tube until solution is complete. A reagent blank is prepared by pipetting 2 ml of water into a clean centrifuge tube. A standard blank is prepared by pipetting 2 ml of standard glucose solution, containing 0.1 mg of glucose, into a similar tube.

At this point 10 ml of anthrone reagent are delivered into each tube with vigorous, but consistent, blowing. The stream of anthrone reagent is directed into the center of the tube and should be sufficient to insure good mixing. As each tube receives anthrone reagent, it is tightly capped with an air condenser and placed in a cold tap water bath. The air condenser is prepared by cutting off the small end of a size 0 rubber stopper and inserting a 4 inch length of glass tubing, 3 to 4 mm in diameter. This serves to prevent water from entering the tube from the water bath. After all tubes have reached the temperature of the cold water, they are immersed in a boiling water bath to a depth a little above the level of the liquid in the tubes for 15 min and then removed to a cold water bath and cooled to room temperature. The tubes and stoppers are wiped dry and the contents of each tube are transferred to a colorimeter tube and read at 620 μm after adjusting the colorimeter with the reagent blank. Care is taken to avoid introduction of lint or contaminating carbohydrate into the anthrone reaction.

\[
\text{Glycogen (mg/100 gm tissue)} = \frac{DU}{DS} \times 0.1 \times \frac{\text{volume of extract}}{\text{g of tissue}} \times 100 \times 0.9
\]

Where, \( DU = \) optical density of the unknown, \( DS = \) optical density of the standard, \( 0.1 = \) mg of glucose in 2 ml of standard solution, \( 0.9 = \) factor for converting glucose value to glycogen value.
4.2.4. Estimation of glucose-6-phosphatase activity (Parker et al, 1998)

Microsome preparation:

Microsomes were prepared by the method of Nordie and Arion modified as follows: Sprague Dawley rats were fasted overnight and euthanized by decapitation. Livers were removed, placed in an ice-cold buffer at pH 7.4 containing 250 mmol/l sucrose, 25 mmol/l HEPES-KOH, 2.5 mmol/l EDTA, and 0.1 mmol/l phenylmethylsulfonylfluoride and homogenized. The homogenate was centrifuged at 12000 g for 10 min. and the resulting supernatant was centrifuged for 1 h at 100,000 g. The pellet was resuspended at a protein concentration at ~40 mg/ml in the homogenization buffer and stored as single use aliquots at -80°C.

Glucose-6-Phosphatase assay:

Glucose-6-Phosphatase activity was measured by monitoring the release of phosphate from glucose-6-phosphate. Microsomes (0.5 µl of the preparation described above) were incubated at room temperature in 100 µl of a buffer at pH 7.2 containing 50 mmol/l HEPES, 100 mmol/l KCl, 2.5 mmol/l EGTA, 2.5 mmol/l MgCl₂, and 1.0 mmol/l Glucose-6-Phosphatase. The released phosphate was measured by adding 150 µl of 1 N HCl containing 1 mg/ml ammonium molybdate and 0.38 mg/ml malachite green. After a 15 min incubation at room temperature, the absorbance was measured at 620 nmol/l. Test compounds were added before the addition of the enzyme.

4.2.5. Glucose uptake by isolated rat diaphragm

Glucose uptake by rat diaphragm was estimated by the methods described by Walaas & Walaas, 1952; Chattopadhyay et al, 1992. Four sets containing six numbers of graduated test tubes (n=6) each, were taken as follows:

Group-1: 2 ml of Tyrode solution with 2 % glucose.
Group-2: 2 ml of Tyrode solution with 2 % glucose + 0.5 ml of 0.5 units per ml solution.
Group-3: 2 ml of Tyrode solution with 2 % glucose + 1.5 ml of alcoholic extract of
      H. isora (100 µg/ml)
Group-4: 2 ml of Tyrode solution with 2 % glucose + 1.5 ml of alcoholic extract of
      H. isora (100 µg/ml) + 0.5 ml of insulin solution (0.5 units/ml)
Materials and methods

The volumes of all the test tubes were made up to 4 ml with distilled water to match the volume of the test tubes of Group 4. Twelve albino rats were fasted overnight and killed by decapitation. The diaphragms were dissected out quickly with minimal trauma and divided into two halves. Two diaphragms form the same animals were not used for the same set of experiment. Six numbers of diaphragms were used for each group. The hemi-diaphragms were placed in test tubes and incubated for 30 min at 37°C in an atmosphere of 100% oxygen with shaking at 140 cycles/min. Glucose uptake per gram of tissue was calculated as the difference between the initial and final glucose content in the incubated medium.

Statistical analysis

Results are presented as Mean ± SEM. Statistical differences between the means of the various groups were evaluated using one-way analysis of variance (ANOVA) followed by student’s ‘t’ test. Data were considered statistically significant at P < 0.05.