APPENDICES

APPENDIX -1 ESTIMATION OF SUPEROXIDE DISMUTASE (SOD)
(Das et al., 2000)

Principle

The method involves generation of superoxide radical of riboflavin and its
detection by nitrite formation from hydroxylamine hydrochloride. The nitrite reacts
with sulphanilic acid to produce a diazonium compound which subsequently reacts
with naphthylamine to produce a red azo compound whose absorbance is measured at
543 nm.

Reagents

- 50 mM Phosphate buffer, pH 7.4
- 20 mM L-Methionine
- 1 % (v/v) Triton X-100
- 10 mM Hydroxylamine hydrochloride
- 50mMEDTA
- 50 uM Riboflavin
- Greiss reagent: 1 % sulphanilamide, 5 % phosphoric acid and 0.1
  % naphthylethylene diamine dihydrochloride.

Procedure

Pipetted out 1.4 ml aliquot of the reaction mixture in a test tube. 100 [U of the
sample was added followed by a preincubation at 37°C for 5 min. 80 μl of riboflavin
was added and the tubes were exposed for 10 min to 200 W Philips fluorescent lamps.
The control tube contained equal amount of buffer instead of sample. The sample and
its respective control were run together. At the end of the exposure time, 1.0 ml of
Greiss reagent was added to each tube and the absorbance of the color formed was
measured at 543 nm.

One unit of enzyme activity was defined as the amount of SOD capable of
inhibiting 50 % of nitrite formation under assay condition.

APPENDIX - 2
ESTIMATION OF CATALASE (CAT)
(Sinha, 1972)

Principle

Catalase causes rapid decomposition of hydrogen peroxide to water.

\[
\text{Catalase} \quad 2\text{H}_2\text{O}_2 \longrightarrow 2\text{H}_2\text{O}+\text{O}_2
\]

The method was based on the fact that dichromate in acetic acid reduced to
chromic acetate when heated in the presence of H\text{2}O\text{2} with the formation of perchloric
acid as an unstable intermediate. The chromic acetate thus produced was measured
colorimetrically at 610 nm. Since dichromate has no absorbance in this region, the
presence of the compound in the assay mixture did not interfere with the colorimetric
determination of chromic acetate. The catalase preparation was allowed to split H\text{2}O\text{2}
for different periods of time. The reaction was stopped at specific time intervals by
the addition of dichromate / acetic acid mixture and the remaining H\text{2}O\text{2} was
determined by measuring chromic acetate colorimetrically after heating the reaction.

Reagents

- 0.01M Phosphate buffer, pH 7.0
- A: 0.1M Monobasic sodium phosphate
- B: 0.1M Dibasic sodium phosphate
Mixed 39 ml of A and 61 ml of B that is diluted to a total of 200 ml. 10 ml of this solution is further diluted to 100 ml with distilled water.

0.2 M Hydrogen peroxide

Stock dichromate / acetic acid solution: Mixed 5% potassium dichromate with glacial acetic acid (1:3 by volume).

Working dichromate/acetic acid solution: The stock was diluted to 1:5 with water to make the working dichromate / acetic acid solution.

Procedure

The assay mixture contained 0.5 ml of t^Oi, 1.0 ml of buffer and 0.4 ml of water. 0.2 ml of the enzyme was added to initiate the reaction. 2.0 ml of the dichromate / acetic acid reagent was added after 0, 30, 60, 90 seconds of incubation. To the control tube the enzyme was added after the addition of the acid reagent. The tubes were then heated for 10 min. and then color developed was read at 610 nm.

The activity of catalase was expressed as umoles of H2O2 decomposed / min / mg protein.

APPENDIX - 3

ESTIMATION OF GLUTATHIONE S-TRANSFERASE (GST)
(L, 1973)

Principle

Glutathione-S-transferase catalyses the reaction of 1-chloro 2,4 dinitrobenzene (CDNB) with the sulphhydryl group of glutathione.

\[ \text{CDNB} + \text{GSH} \xrightarrow{\text{GST}} \text{CDNB-S-glutathione} \]

The conjugate, CDNB-glutathione absorbs light at 340 nm and the activity of the enzyme can therefore be estimated by measuring the increase in absorbance.
Reagents

- 0.5 M phosphate buffer, pH 6.5
- 30mMCDNB in 95% ethanol (30mg/5mlH₂O) <-* 30mM reduced glutathione (14mg/l .5ml H₂O) Procedure

To 1.0ml of buffer, 0.1ml of sample, 1.7ml of water and 0.1ml of CDNB were added and incubated at 37°C for 5 min. After incubation, 0.1ml of reduced glutathione were added. The increase in optical density of the enzyme was measured against that of the blank at 340nm. The enzyme activity is calculated in terms of umoles of CDNB conjugate formed/min/mg protein.

APPENDIX - 4

ESTIMATION OF GLUCOSE 6 PHOSPHATE DEHYDROGENASE (G6PD)
(Balinsky and Bernstein, 1963) Principle

Glucose 6-phosphate dehydrogenase is assayed by measuring the increase in absorbance, which occurs at 340nm. This reaction takes place when electrons are transferred from glucose 6 phosphate to NADP in the reaction catalyzed by glucose - 6-phosphate dehydrogenase.

Reagents

- 0.1MTrisHCl buffer ,pH 8.2
- Sol A: 0.1M solution of Tris (12.1g/1000ml water)
- SolB:0.1MHCl
- Mixed 50 ml of solution A and 21.9ml of B and diluted to a total of 200ml
- 0.2mMNADP
- 0.1M Magnesium chloride
6mM glucose-6-phosphate

**Procedure**

0.4ml of Tris-HCl buffer, 0.2ml of NADP, 0.2ml of magnesium chloride, 1.0ml water and 0.2ml of enzyme were taken in a cuvette. The reaction was started by the addition of 0.2ml of glucose-6-phosphate and the increase in OD was measured at 340nm.

The activity was expressed in terms of units/mg protein, in which one unit is equal to the amount of enzyme that brought about a change in OD of 0.01/mnin.

**APPENDIX - 5**

**ESTIMATION OF GLUTATHIONE PEROXIDASE (GPx)**

*(Rotruck et al., 1973)*

**Principle**

A known amount of enzyme preparation were allowed to react with hydrogen peroxide in the presence of GSH for a specified time period. Then the remaining GSH were measured by the method of Ellman.

**Reagents**

- 0.4 MTris buffer, pH 7.0
- 10 mM Sodium azide
- 2.5 mM Hydrogen peroxide
- 4-mM Reduced glutathione
- 10%TCA
- 0.3 M Phosphate solution
Procedure

To 0.2 ml of tris buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide and 0.5 ml of tissue homogenate were added. To this mixture, 0.2 ml of glutathione and 0.1 ml of hydrogen peroxide were added. The contents were mixed well and incubated at 37°C for 10 minutes along with a tube containing all the reagents except sample. After 10 minutes the reaction were arrested with the addition of 0.5 ml of 10% TCA, centrifuged and the supernatant were assayed for glutathione by Ellman's method.

To 2.0 ml of the supernatant, 3.0 ml disodium hydrogen phosphate solution and 1.0 ml of DTNB reagent were added. The colour developed were read at 412 nm. Standards in the range of 200-1000 jag were taken and treated in the similar manner. The activity was expressed in term of fig of glutathione consumed/min/mg protein.

APPENDIX - 6
ESTIMATION OF TOTAL REDUCED GLUTATHIONE (GSH)
(Bonye and Ellman, 1972) Principle

The method was based on the reaction of reduced glutathione with DTNB to give a compound that absorbs at 412 nm.

Reagents

Metaphosphoric acid : 1.67 g of glacial metaphosphoric acid, 0.2 g of EDTA and 30 g of NaCl in 100 ml of distilled water.

- 0.4MNa$_2$HP0$_4$.

- DTNB reagent: 40 mg of DTNB in 100 ml of 1% trisodium citrate.
Standard glutathione: 20 mg of reduced glutathione was dissolved in 100 ml of distilled water.

Procedure

1.0 ml of 10 % tissue homogenate was precipitated with 4.0 ml of metaphosphoric acid. The precipitate was removed by centrifugation. To 2.0 ml of the supernatant, 2.0 ml of disodium hydrogen phosphate and 1.0 ml of DTNB reagent was added. The absorbance was read, within 2 mins at 412 nm against a reagent blank. A set of standards was also treated in the above manner.

The amount of glutathione is expressed as uM / mg protein.
ESTIMATION OF ASCORBIC ACID (vit C)  
(Sadashivam and Manickam, 1997)

Principle:

Ascorbic acid is first dehydrogenated by bromination. The dehydro ascorbic acid is then reacted with 2,4 dinitro phenyl hydrazine to form ozazone and dissolved in sulphuric acid to give an orange red colored solution which is measured at 540nm.

Reagents:

- 4% oxalic acid
- 0.5N sulphuric acid
- 2% 2, 4 Dinitro phenyl hydrazine reagent: dissolved by heating, 2g of DHPH in 100ml of 0.5N sulphuric acid. Filter and use.
- 10% thiourea
- 80% sulphuric acid
- Bromine water: dissolved 1 to 2 drops of liquor bromine in approximately 100ml of cool water.
- Ascorbic acid stock standard: the dissolved 100 mg of ascorbic acid in 100 ml of 4% oxalic acid solution in a standard flask.
- Working standard: diluted 10 ml of stock standard solution to 100 ml with 4% oxalic acid. Converted into dehydro form by bromination. The concentration of working standard is 100ug/ml.
Extraction: ground 5g of sample either mechanically or using a pestle and mortar in 100 ml of 4% oxalic acid solution. Centrifuged at 1000 g for 20 minutes or filtered and collected the liquid. Transferred 10 ml aliquot to a conical flask and added bromine water dropwise with constant mixing. The enolic hydrogen atoms in ascorbic acid are removed by bromine. When the extract turned orange yellow due to excess bromine expelled it by blowing in air. Made upto 100 ml with 4% oxalic acid solution. Similarly converted 10 ml of stock ascorbic acid into dehydro form by bromination.

Procedure:

Pipetted out 0.2 - 1 ml of working standard-ascorbic solution corresponding to fig values 20 - 100. Similarly pipetted out 1 ml of brominated sample extract in 1 ml of unknown solution. Made up the volume in each tube to 3 ml by adding distilled water. Added 1 ml of dinitro phenyl hydrazine reagent followed by 1 - 2 drops of thiourea into each tube. A blank was set as above but with water in place of ascorbic acid solution. Mixed the contents of the tube thoroughly and incubated at 37°C for three hours. After incubation the tubes were kept in the ice bath. Dissolved the orange red ozazone crystals formed by adding 7 ml of 80% sulphuric acid drop wise while the tubes were still in the water bath. The tubes in ice bar were removed and allowed to stand for 30 minutes at room temperature and measured the absorbance at 540 nm.

Mixed thoroughly and incubated the mixture at 37°C for 15 min. Read in a spectrophotometer at 540 nm or in a photocolorimeter with green filter (520-560 nm), setting instrument to zero with reagent blank. The color is stable for 60 min so absorbance should be read within that period.

APPENDIX-8
ESTIMATION OF VITAMIN E (Vit E)
(Varleye/fli, 1981) Principle

Tocopherol can be estimated using Emmerie-Engel reaction, which is based on the ferric to ferrous ions by tocopherols, which then forms a red color with 2,2'-dipyridyl. Tocopherols and carotenes are first extracted with xylene and the extinction
read at 460 nm to measure carotenes. A correlation is made for these after adding ferric chloride and reading at 520 nm.

**Reagents**

- Absolute ethanol
- Xylene
- 2,2'-dipyridyl: 1.2 g/L of n-propanol
- Ferric chloride solution: 1.2 g of FeCl3-6H2O in one litre of ethanol.
- Standard solution D, L-a-tocopherol: 10 mg/L in absolute ethanol 0.91 mg of a-tocopherol is equivalent to 100 mg of tocopherol acetate.
- Tissue extraction: Weighed 1.0 g the tissue and were homogenised in a blender and transferred to a conical flask. Added 50 ml of 0.1 N sulphuric acid slowly without shaking. Stoppered and allowed to stand overnight. The next day, the contents of the flask were shaken vigorously and filtered through Whatmann No.1 paper, discarding the initial 10-15 ml of the filtrate. Aliquot of the filtrate was used for the estimation.

**Procedure**

Into 3 stoppered centrifuge tubes (test, standard and blank) pipetted out 1.5 ml of each liver tissue extract, 1.5 ml of the standard and 1.5 ml of water respectively. To the test and blank added 1.5 ml of ethanol and to the standard added 1.5 ml of water. Added 1.5 ml of xylene to all the tubes, stoppered, mixed well and centrifuged.

Transferred 1.0ml of xylene layer into another stoppered tube, taking care not to include any ethanol or protein, added 1.0ml of 2,2'-dipyridyl reagent to each tube, stoppered and mixed. Pipetted out 1.5 ml of the mixtures into spectrophotometer cuvettes and read the absorbance of test and standard against the blank at 460 nm. Then in turn beginning wit the blank, added 0.33 ml of ferric chloride solution. Mixed
well and after exactly 15 min read test and standard against the blank at 520 nm. The amount of vitamin E can be calculated using the formula.

\[(\text{AA}_{520\text{nm}} - \text{AA}_{460\text{nm}} \times \text{cone [s] x 0.29)} \times \text{Total volume}\]

\[
\text{Vitamin E (u.g/g) = (AA}_{520\text{nm} \times \text{Vol for experiment} \times \text{wt of sample}}
\]
APPENDIX-9
ESTIMATION OF PROTEIN
(Lowry et al., 1951)

Principle

The blue colour developed by the reduction of the phosphomolybdic phosphotungstic components in the Folin Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein along with the color developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured at 660 nm.

Reagents

- 2 % sodium carbonate in 0.1 N NaOH (Reagent A)
- 0.5 % Copper sulphate in 1 % potassium sodium tartarate (Reagent B)
- Alkaline copper reagent: Mixed 50 ml of A and 1.0 ml of B prior to use
- Folin-Ciocalteau reagent: Mixed 1 part of reagent with 2 parts of water.
- Stock standard: Weighed 50 mg of bovine serum albumin and made up to 50 ml in a standard flask with saline.
- Working standard: Diluted 10 ml of the stock of 50 ml with distilled water. 1.0 ml of this solution contains 200 ug of protein.

Procedure

Pipetted out 0.2 to 1.0 ml working standard solution, 0.1 ml of the sample was taken. The volumes in all the tubes were made up to 1.0ml with distilled water. Added 5.0 ml of alkaline copper reagent to each tube. Mixed well and allowed to stand for 10 mins. Then added 0.5 ml of Folin-Ciocalteau reagent. Mixed well and
incubated at room temperature for 30 minutes. A reagent blank was also prepared. After 30 minutes, the blue color developed was read at 660 run.
APPENDIX - 10
DPPH RADICAL SCAVENGING ACTIVITY
(Makris et al., 2007) Principle

DPPH radical is scavenged by antioxidants through the donation of a proton forming the reduced DPPH. The colour change from purple to yellow after reduction can be quantified by its decrease in absorbance at wavelength 517 nm.

Reagents

- 0.2 mM DPPH
- 80% Methanol
- Butylated Hydroxyl Anisole

Procedure

Various concentrations of ethanol extract of the sample (4.0 ml) were mixed with 1.0 ml of methanolic solution containing DPPH radicals, resulting in the final concentration of DPPH being 0.2 mM. The mixture were shaken vigorously and left to stand for 30 min, and the absorbance was measured at 517 nm. BHA were used as control. The percentage of DPPH decolorization of the sample were calculated according to the equation: % decolorization - [1-(ABS sample/ABS control)] x 100

APPENDIX- 11
ABTS RADICAL SCAVENGING ACTIVITY
(Reef et al., 1999) Principle

ABTS decolonisation assay involves the generation of the ABTS$^+$ chromophore by the oxidation of ABTS with ammonium persulphate. It is applicable for both hydrophilic and lipophilic compounds. The scavenging activity of the plant extracts on ABTS radical cation were measured at 734 nm.
Reagents

- TmMABTS
- 2.45 mM Ammonium per sulphate
- ABTS solution: 7 mM of ABTS were mixed with 2.45 mM ammonium per sulphate and the mixture was allowed to stand in dark at room temperature for 12-16 hours before use. ABTS solution was diluted to an absorbance of 0.7 ± 0.05 with ethanol at 734 nm.
- Ethanol

Procedure

Samples were diluted to produce 0.2 to 1.0 mg/ml. The reaction were initiated by the addition of 1.0 ml of diluted ABTS"" to 10 \( \mu l \) of different concentration of ethanolic extract of the sample or 10 ul of ethanol as control. The absorbance was read at 734 nm and the percentage inhibitions were calculated. The inhibition were calculated according to the equation \( I = A_0 - A_i/A_0 \times 100 \), where \( A_0 \) is the absorbance of control reaction, \( A_i \) is the absorbance of test compound.

APPENDIX - 12
FRAP ASSAY
(Ozgen, 2006) Principle

The total antioxidant potential of sample were determined using ferric reducing ability of plasma FRAP assay as a measure of antioxidant power. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue coloured Fe II-tripyridyl triazine compound from colourless oxidized Fe III form by the action of electron donating antioxidants.

Reagents

- 10 mM 2,4,6-tripyridyl-s-triazme (TPTZ)
❖ 40 mM HCl

❖ 20 mM Ferri chloride

❖ 0.3 M Acetate buffer, pH 3.6

❖ Trolox

❖ FRAP reagent: It contains 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution and 25 ml acetate buffer. It were freshly prepared and warmed to 37°C.

**Procedure**

The stock solution of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, 20 mM FeCl₃.6H₂O and 0.3 M acetate buffer (pH 3.6) were prepared. The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution and 25 ml acetate buffer. It was freshly prepared and warmed to 37°C. Then, 900 pi FRAP reagent were mixed with 90 pi water and 30 pi test sample/methanol/distilled water/standard antioxidant solution. The reaction mixture was then incubated at 37°C for 30 minutes and the absorbance was recorded at 593 nm. An intense blue coloured complex were formed when ferric tripyridyl triazine (Fe³⁺-TPTZ) complex were reduced to ferrous (Fe²⁺) form. The absorption at 540 nm was recorded. The calibration were plotted with absorbance at 593 nm vs concentration of ferrous sulphate in the range 0.1 mM both aqueous and ethanol solutions). The concentrations of FeS0₄ were in turn plotted against concentration of standard antioxidants L-ascorbic acid or Trolox.

**APPENDIX-13**

**p- CAROTENE BLEACHING ASSAY**

*(Lim and Quah, 2007) Principle*

Anti-autooxidation was measured using the p-carotene bleaching method.

**Reagents**
- p- carotene
- Chloroform
- Linoleic acid
- Tween 20

Procedure

1.0 nil of p-carotene solution (0.2 mg/ml chloroform) was pipetted into a round bottom flask (50 ml) containing 0.02 ml of linoleic acid and 0.2 ml 100% Tween 20. The mixture was then evaporated at 40°C for 10 minutes by means of a rotary evaporator to remove chloroform. After evaporation the mixture was immediately diluted with 100 ml of distilled water. The distilled water was added slowly to the mixture with vigorous agitation to form an emulsion.

5.0 ml aliquots of the emulsion were transferred into different test tubes containing 0.2 ml of samples in 80% methanol at 1 mg/ml. The tubes were gently mixed and place at 45°C in a water bath for 2 hours. Absorbance of the samples was measured at 470 nm. Standards at the same concentration with samples were used as comparison. 0.2 ml of 80% methanol in 5.0 ml of the above emulsion was used as the control. The measurement was carried out at 15 min interval. The percentage inhibition of the sample was calculated.

APPENDIX -14
CHELATING ABILITY ON FERROUS IONS
(Dastmalchi et al., 2008) Principle

Iron (II) chelating activity were measured by the inhibition of the formation of iron (II) - ferrozine complex after preincubation of the sample. The Fe$^{2+}$ was monitored by measuring the formation of ferrous iron - ferrozine complex against ethanol blank at 562 nm.
Reagents

- 2 mM Ferric chloride
- Methanol
- 5 mM ferrozine

Procedure

The reaction mixture contained 1.0 ml of various concentrations of the extract, 0.1 ml of 2 mM FeCl₂ and 3.7 ml methanol. The control contained all the reaction reagents except sample. The reactions were initiated by the addition of 0.2 ml of 5 mM ferrozine. After 10 mins at room temperature, the absorbance of the mixture were determined at 562 nm against a blank. A lower absorbance of the reaction mixture indicated a higher Fe²⁺ chelating ability. The capacity to chelate the ferrous ion were calculated by % chelation = [1-(ABS_{sample}/ABS_{control})] x 100.

APPENDIX-15
REDUCING POWER
(Barroso/a/; 2007) Principle

Reducing power were measured by direct electron donation in the reduction of Fe³⁺(CN)₆ to Fe²⁺(CN)₆. The product were visualized by the addition of of free Fe³⁺ ions after the reduction reaction, by forming the intense Prussian blue colour complex, Fe⁺[Fe²⁺(CN)₆]₃ and quantified by absorbance at 700 nm.

Reagents

- 0.2 M Phosphate buffer, pH 6.6
**Procedure**

The reaction mixture contained 2.5 ml of various concentrations of methanol extract of the sample, 2.5 ml of 1% potassium ferricyanide and 2.5 ml of 0.2 M sodium phosphate buffer. The control contained all the reagents except the sample. The mixture were incubated at 50°C for 20 min. and were terminated by the addition of 2.5 ml of 10% (w/v) of trichloroacetic acid, followed by centrifugation at 3000 rpm for 10 min. 5.0 ml of the supernatant upper layer were mixed with 5.0 ml of deionized water and 1.0 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm against blanks that contained distilled water and phosphate buffer. Increased absorbance indicates increased reducing power of the sample.

**APPENDIX -16**

**HYDROXYL RADICAL SCAVENGING ASSAY**

(Rajeshwar et al., 2005) **Reagents**

- 2.8 mM Deoxyribose
- 0.1 mM FeCl₃ diluted with 100 ml of distilled water. The distilled water was added slowly to the mixture with vigorous agitation to form an emulsion.

5.0 ml aliquots of the emulsion were transferred into different test tubes containing 0.2 ml of samples in 80% methanol at 1 mg/ml. The tubes were gently mixed and place at 45°C in a water bath for 2 hours. Absorbance of the samples was measured at 470 nm. Standards at the same concentration with samples were used as comparison. 0.2 ml of 80% methanol in 5.0 ml of the above emulsion was used as the control. The measurement was carried out at 15 min interval. The percentage inhibition of the sample was calculated.
Iron (II) chelating activity were measured by the inhibition of the formation of iron (II) - ferrozine complex after preincubation of the sample. The Fe⁺ was monitored by measuring the formation of ferrous iron - ferrozine complex against ethanol blank at 562 nm.

Reagents

- 2 mM Ferric chloride
- Methanol
- 5 mM ferrozine

Procedure

The reaction mixture contained 1.0 ml of various concentrations of the extract, 0.1 ml of 2 mM FeCl₂ and 3.7 ml methanol. The control contained all the reaction reagents except sample. The reactions were initiated by the addition of 0.2 ml of 5 mM ferrozine. After 10 mins at room temperature, the absorbance of the mixture were determined at 562 nm against a blank. A lower absorbance of the reaction mixture indicated a higher Fe⁺ chelating ability. The capacity to chelate the ferrous ion were calculated by % chelation = \[1-(ABS_{sample}/ABS_{control})\times 100\].

Procedure

The reaction mixture contained EDTA (6uM), with 3p,g NACN, riboflavin (2uM), NBT (2uM), KH₂PO₄ - Na₂HPO₄ buffer (67 mM, pH 7.8) and various concentrations of the extracts in a final volume of 3.0ml. The tubes were illuminated under incandescent lamp for 15 minutes. The optical density at 560 nm was measured before and after illumination. The inhibition of superoxide radical was determined by
comparing the absorbance values of the control with those of the treatments. Ascorbic acid was used as a standard.

APPENDIX -18
NITRIC OXIDE RADICAL SCAVENGING ASSAY
(Madanefaf., 2005) Principle

The interaction of ethanolic extract of *Grewia umbellifera* and *Gmelina arborea* with nitric oxide was assessed by the nitride detection method. Nitric oxide was generated from sodium nitro prusside and measured by Griess illosvoy reaction. Sodium nitro prusside in aqueous solution at physiological pH spontaneously generated nitric oxide, which interacts with oxygen to produce nitrite, which can be estimated by the use of Griess illosvoy reagent, in the present experiment, nitrite ion was measured by using Griess illosvoy reagent, which is modified by using naphthyl ethylene diamine dihydrochloride instead of 1-naphthylamine.

Reagents

Sodium nitro prusside solution (10mM): 0.2998 g of sodium nitro prusside was accurately weighed and dissolved in distilled water to make up the volume to 100ml in a volumetric flask.

- Napthyl ethylene diamine dihydrochloride (NEDD) (0.1 %): Weighed accurately 0.1 g of NEDD and dissolved in 60ml of 50% glacial acetic acid by heating and made up the volume to 100ml in a volumetric flask with distilled water.

- Sulphanilic acid (0.33% w/v) reagent: 0.33 g of sulphanilic acid was dissolved in 100ml 20% glacial acetic acid by heating.

- Phosphate buffer saline (PBS) pH 7.

- Dimethyl sulfoxide (DMSO), distilled.

Procedure
Nitric oxide generated from sodium nitro prusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions which were measured at 540 nm. The reaction mixture (6.0 ml) containing sodium nitro prusside (4.0 ml), phosphate buffer saline (PBS, 1.0 ml) and extract (1.0 ml) in DMSO was incubated at 25°C for 15 minutes after incubation, 0.5 ml of the reaction mixture containing nitrite was removed, 1.0 ml of sulphanilic acid reagent was added, mixed well and allowed to stand for 5 minutes for completion of diazotization and 1.0 ml of naphthyl ethylene diamine dihydrochloride was added, mixed well and allowed to stand for 30 minutes in diffused light. A pink coloured chromophore was formed. The absorbance of these solutions was measured at 540 nm against corresponding blank solutions. Ascorbic acid was used as a standard.
APPENDIX -19

MTT Assay (Rabkin and Kong, 2000)

Principle

The principle involved in the cleavage of tetrazolhim salt MTT (3-(4,5 dimethyl thiazole 2yl) - 2,5- diphenyl tetrazolium bromide) into a blue coloured (formazon) by mitochondrial enzyme succinate dehydrogenase. The number of cells are found to be proportional to the extent of formazon production by the cell used.

Methodology

- The monolayer cell culture was trypsinized and the cell count was adjusted to 1 X 10 cells /ml using medium containing 10% newborn calf serum.

- To each of the 96 well microtitre plate 0.1 nil of the diluted cell suspension (10,000 cells) were added.

- After 24 hrs, when a partial monolayer was formed the supernatant was flicked off, washed once with PBS and 100 of different extract concentration was added to the cells in microtitre plates.

- The plates were incubated at $37^{\circ}$C for 3 days in 5 % CC>2 atmosphere and microscopic examination was carried out and observation recorded every 24 hours.

- After 72 hrs the extract solution in the well was discarded and 50 1 of MTT was added to each well.

- The plates were gently shaken and incubated for 3hrs at $37^{\circ}$C in 5% CO2 atmosphere.

- The supernatant was removed and 50ul of propanol was added and gently solubilize the formed forrnazon.
The absorbance was measured using a microtitre plate reads at a wavelength of 540nm. The percentage growth inhibition was calculated. Using the formula below:

\[
\text{% growth inhibition} = \frac{100 - \text{Mean OD of test group}}{\text{Mean of OD control group}} \times 100
\]

APPENDIX - 20
DNA FRAGMENTATION ASSAY
(Hyunet al., 1997) DNA fragmentation buffer

- % Triton x 100
- 5mM Tris HC1 (pH-8.0)
- 20mm EDTA
- Methodology
  - 100μl of DNA fragmentation buffer was added into the medium with 5 X 1/f cells.
  - To this 2.5% PEG (Polyethylene glycol) and 1M NaCl was added and kept on ice for 10 minutes.
  - Spin at 9000 rpm for 20 minutes at room temperature.
  - Supernatant was taken and to this 2 volumes of absolute alcohol was added and kept on ice "for 15 minutes.
  - Spin at 9000 rpm for 5 minutes and pellet was dissolved in TE buffer.
  - Loaded on 1.5% Agarose gel and run with 1 X TAB buffer.
Agarose gel electrophoresis

This is a standard method used to separate and identify DNA fragmentation and also to detect their size. 0.8% (w/v) of agarose was weighed out and added to 1X TAB (Tris acetate in EDTA buffer) and melted in a microwave oven. Cooled the melted agarose to 55°C and poured onto the gel casting platform, whose ends had been sealed with cellotape. Before the agarose solidified a gel was last in a tuff bound by tape. A comb was made to stand with 2mm gap between the bottom of the gel and the comb tip. Allowed it to solidify. Soon the comb was carefully removed. The platform with the gel was placed in an electrophoresis tank containing 1X TAB buffer to lower the gel to a depth of 1mm. The apparatus was loaded along with indicator dye into the well, stained the gel in 0.5 mg/ml of Ethidium Bromide at room temperature and visualized the DNA on a UV trans illuminator.

APPENDIX-21
ESTIMATION OF HEMOGLOBIN
(Drabkin and Austin, 1932)

Principle

In alkaline medium, hemoglobin and its derivatives are oxidized in the presence of potassium ferricyanide and get converted to methemoglobin which then reacts with potassium cyanide to form purple red coloured cyanmethemoglobin complex, the intensity of which is measured at 546 nm.

Reagents

- Drabkin's solution
- Cyanmethemoglobin standard: 65 mg/dl

Procedure

Pipetted out 0.02 ml of serum and 5.0 ml of Drabkin's solution into a test tube.
Simultaneously, a blank was set up with Drabkin's solution and distilled water. Mixed well the above tubes and allowed to stand at room temperature for 5 minutes. Measured the absorbance of test at 546 nm. Take the absorbance of cyanmethemoglobin standard was taken directly without adding working reagent against blank at 546 nm. The results were expressed as g/dl in serum.

APPENDIX-22

ESTIMATION OF RED BLOOD CELLS
(Chesbrough and McArthur, 1972) Principle

The blood specimen is diluted (usually 200 times) with red cell diluting fluid which does not remove the white cells but allows the red cells to be counted under 400X magnification in a known volume of the fluid. Finally the number of cells in undiluted blood is calculated and reported as the number of red cells /mm$^3$.

Reagents

RBC diluting fluid: (Trisodium citrate-3g, distilled water~99ml, and formalin-1ml).

Procedure

The whole blood was taken into the RBC pipette exactly up to the 0.5 mark (Thoina pipette mark 101) and the diluting fluid (formal citrate solution) was immediately drawn up to the mark 101. The pipette was rotated between the thumb and the forefinger. This gave a dilution of 1:200.

The cover glass was placed in position over the ruled area using gentle pressure. The suspension was mixed thoroughly by rotating the pipette for about a minute, holding it in horizontal position, and finally shook it sidewise. The fluid was expelled from the stem of the pipette and filled the chamber immediately by holding the pipette at an angle of 45° and slightly touching the tip against the edge of the cover glass. There should not be any bubbles under the cover glass. Then the red corpuscles were allowed to settle for 2 to 3 minutes. The number of RBCs was
counted in 180 small squares (4 squares of 16 at each four corners and one of 16 at centre). The cells touching the lower and right hand lines were not counted, but the cells touching the upper and left hand lines were counted. The cells counted are expressed as million cells /nm³ blood.

**Calculation**

\[
\text{Number of RBCs/mm}^3 = \text{Number of cells counted in 5 squares} \times 10000.
\]
APPENDIX-23
ESTIMATION OF WHITE BLOOD CELLS
(Chesbrough and McArthur, 1972)

Principle

Blood is diluted with acid solution which removes the red cells by hemolysis and also accentuates the nuclei of the white cells, counting is done with a microscope under the low power (100X magnification) and knowing the volume of the fluid examined and dilution of the blood, the number of white cells in undiluted whole blood is calculated and reported as the number of WBCs/ mm$^3$.

Reagents

WBC diluting fluid (Turk solution): (Acetic acid-3ml, distilled water-97ml).

Procedure

The whole blood was taken up to the mark 0.5 in WBC pipette and diluted up to the mark 11 with WBC fluid as described in RBC counting and filled the counting chamber in the same manner. Then the cells area was allowed to settle for 3 minutes. The neubaur counting chamber was used to count the cells in the four comers and each of these 4 sq mm. areas is subdivided into 16 squares by using the low power objective and a medium ocular. While counting, the cells included were those touching the lines on the left and bottom. The difference between the two squares millimeter area as thousand cells /mm blood.

Calculation

Number of WBCs/mm$^3$ = Number of cells counted x 50.
APPENDIX - 24
ESTIMATION OF ASPARATE TRANSAMINASE (AST) (Reitman and Frankel, 1957)

Principle

The enzyme catalyses the following reaction

\[
\text{AST} \ L - \text{Aspartate} + \ \text{a-ketoglutarate} \rightarrow \ ^\text{Oxaloacetate} + \ L - \text{Glutamate}
\]

The oxaloacetate is measured by the reaction with 2,4 dinitrophenyl hydrazine giving a brown colored hydrazine after the addition of sodium hydroxide. The color developed is read at 520 nni.

Reagents

- **Phosphate buffer**: 0.1M, pH 7.5.
- **Sol A**: 0.1M solution of monobasic sodium phosphate.
- **Sol B**: 0.1M solution of dibasic sodium phosphate. Mixed 16ml of A and 84ml of B, diluted to a total of 200ml.
  - **Substrate**: Dissolved 146mg of a-ketoglutarate and 13.3gm of aspartic acid in 1N NaOH with constant stirring. Adjusted the pH to 7.4 and made upto 1000ml with phosphate buffer.
  - **Standard Pyruvate, 2mM**: Dissolved 22mg of sodium pyruvate in 100ml of phosphate buffer 0.2ml of standard contain 0.4mM of sodium pyruvate.
  - **Dinitrophenyl hydrazine reagent, Immol / L**: 200mg/ L in Imol / L HCl.
0.4N NaOH : Dissolved 16gm of NaOH in 1L distilled water.

**Procedure**

0.2ml of sample and 1.0ml of the buffered substrate was incubated for 60mins at 37°C. To the control tubes, enzyme was added after arresting the reaction with 1.0ml of DNPH and the tubes were kept at room temperature for 20mins. Then 10ml of 0.4N NaOH was added. A set of standard pyruvate was also treated in a similar manner. The color developed was read at 520nm.

The enzyme activity in serum was expressed as μmol of pyruvate liberated / L and in liver homogenate as nM of pyruvate liberated / min / mg protein.

**APPENDIX - 25**

**ESTIMATION OF ALANINE TRANSAMINASE (ALT)**

*(Reitman and Frankel, 1957)*

**Principle**

The enzyme catalyses the following reaction:

\[
\text{ALT} \quad \text{L-Alanine} + \text{a-ketoglutarate} \quad \rightarrow \quad \text{Pyruvate} + \text{L-Glutamate}
\]

The pyruvate is measured by the reaction with 2,4-dimtrophenylhydrazine giving a brown colored hydrozone after the addition of sodium hydroxide. The color developed is read at 520nm.

**Reagents**

- Phosphate buffer: 0.1M, pH 7.5.
Substrate: Dissolved 146mg of a-ketoglutarate and 17.8gm of L-alanine in IN NaOH with constant stirring. Adjusted the pH to 7.4 and made up to 1000ml with phosphate buffer.

Standard pyruvate, 2mM: Dissolved 22mg of sodium pyruvate in 100ml of phosphate buffer. 0.2ml of standard contains 0.4mM of sodium pyruvate.

Dinitrophenyl hydrazine reagent, Immol/L: 200mg/L in Imol/L HC1.

0.4N NaOH: Dissolved 16gm of NaOH in 1L distilled water.

Procedure

0.2ml of Sample and 1.0ml of the buffered substrate were incubated for 30 mins at 37°C. To the control tubes, enzyme was added after arresting the reaction with 1.0ml of DNPH and the tubes were kept at room temperature for 20 mins. Then 10ml of 0.4N NaOH was added. A set of standard pyruvate was also treated in a similar manner. The color developed was read at 520nm.

The enzyme activity in serum was expressed as umoles of pyruvate liberated/L and in liver homogenate as nM of pyruvate liberated/min/mg protein.

APPENDIX - 26
ESTIMATION OF UREA
(Natelson et al., 1951)

Principle

Diacetyl monoxime in the presence of acid, hydrolyse to produce the unstable compound diacetyl. This reacts with urea to produce a yellow diazone derivative. The color of this product becomes pink by addition of thiosemicarbazide which is measured colorimetrically at 540nm.

Reagents
❖ TCA, 10%

❖ Diacetylmonoxime: 1.56 g of diacetyl monoxime is dissolved in 250 ml of distilled water.

❖ Thiosemicarbazide: 41 mg of thiosemicarbazide is dissolved in 250 ml of distilled water and stored in a brown bottle.

❖ Ferric chloride solution: 324 mg of ferric chloride is dissolved in 10 ml of 56% orthophosphoric acid and stored in a brown bottle.

❖ Acid reagent: To 1 L of 20% sulphuric acid added 1 ml of ferric chloride reagent.

❖ Stock standard: 100 mg of urea/100 ml.

❖ Working standard: 2.0 ml of stock standard was diluted to 100 ml. 1 ml of this solution contains 20 p.g / ml.

Procedure

To 0.5 ml of supernatant, 1.0 ml of diacetylmonoxime and 1.0 ml of thiosemicarbazide and 3.0 ml of acid reagent was added. Kept in a boiling water bath for 30 mins. A blank was set up with water. A series of standard were put up simultaneously and treated as for test. Cooled and read at 540 nm. The result was expressed as mg/dl in serum.

APPENDIX - 27

ESTIMATION OF LIPID PEROXIDATION (LPO)

(Huge and Aust, 1978)

Principle

Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red color absorbing at 535 nm.
Reagents

- Stock TCA-TBA-HCl reagent: 15% w/v trichloroacetic acid, 0.375 w/v thiobarbituric acid and 0.25 N HCl. The solution was heated mildly to assist the dissolution of the TBA.

Procedure

To 1.0 ml of the sample, 2.0 ml of TCA-TBA-HCl reagent was added and mixed thoroughly. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1,000 g for 10 min. The absorbance was determined at 535 nm against a blank that contains all the reagents except the sample.

The results were expressed as nmoles of MDA formed/min/mg protein using an extinction coefficient of the chromophore 1.56 x 10^5 Mem and expressed as nmoles of MDA formed/min/mg protein.

APPENDIX - 28

ESTIMATION OF CREATINE KINASE
(Okinaka, 1961)

Reagents

- Tris-HCl buffer: 0.1 mM, pH 9.0
- ATP: 18.5 mM in Tris HCl buffer
- Magnesium- cysteine reagent: 24.65 mg magnesium sulfate and 15.76 mg cysteine HCl were dissolved in 10 ml of distilled water.
- Creatine: 240 mM Procedure:

The incubation mixture containing 0.75 ml of double distilled water, 0.05 ml of serum, 0.1 ml of ATP solution, 0.1 ml of magnesium - cysteine reagent and 0.1 ml of
creatine was incubated at 37°C for 20 mins. The tubes were centrifuged and the supernatant was used for the estimation of phosphorus.

The enzyme activity is expressed as IU/L.

APPENDIX - 29

ESTIMATION OF CREATINE KINASE- MB ACTIVITY

(Neumeir, 1981)

Principle

The sample is incubated in the CK-MB reagent which includes the anti-CK-M antibody. The activity of the non-inhibite CK-B is then determined using the following series of reactions:

\[
\text{CK ADP + Creatine Phosphate} \rightarrow \text{Creatinine + ATP}
\]

\[
\text{HK}
\]

\[
\text{ATP + Glucose} \rightarrow \text{ADP + Glucose-6-phosphate/}
\]

\[
\text{G6PDH G-6-P + NAD}^+ \rightarrow 6\text{-Phosphogluconate + NADH + H}^+
\]

CK-B catalyzes the reversible phosphorylation of ADP, in the presence of creatine phosphate, to form ATP and creatine. The auxiliary enzyme hexokinase (HK) catalyzes the phosphorylation of glucose by the ATP format, to produce ADP and glucose-6-phosphate (G-6-P) is oxidized to 6-phosphogluconate with the concomitant production of NADH. The rate of NADH formation, measured at 340nm, is directly proportional to serum CK-MB activity.

Reagent

- CK-MB Reagent:
Creatine Phosphate 30mM Adenosine-5-Phosphate 2mM NAD

2mM Hexokinase (Yeast) > 3000U/L G-6-PDH (Bacterial) >
2000U/L Anti Human CK-M antibody (Goat) - sufficient amount to inhibit up to 1500
U/L ofCK-MM.

**Procedure**

To the test tubes added 1000ul of the reagent and 50ul of the sample. The
mixture was mixed and incubated at 37 C. The absorbance was measured after 300
seconds. Two additional absorbance was taken at 1 minute interval. The mean
absorbance change/minutes (AA/min) was calculated. The change in absorbance/
minute was multiplied by factor 3376 that is equal to CK-MB.
APPENDIX-30
TROPONIN T ASSAY

Materials

- Deionized water for diluting Read Buffer.
- Phosphate buffered saline + 0.05% Tween-2Q (PBIJ-T) for plate washing.
- Adhesive plate seals.
- Microtiter plate shaker.
- Liquid handling equipment, or other efficient multi-channel pipetting equipment that must accurately dispense 25 and 150 uL into a 96-well microplate.
- Automatic plate washer or multi-channel pipette for washing 96-well plates

Procedure

Begin with a MULTI-SPOT 96-well 4 Spot Human cTnT plate. Add 25 
\(\mu L/well\) of Detection Antibody Solution. Add 25 jL/well Calibrator or sample, cover with an adhesive plate seal, and incubate at room temperature with shaking for 1 hour. Prepare SECTOR Imager so that the plate can be read immediately after Read Buffer addition. Wash plates 3 times with PBS-T. Add 150 uL/well IX Read Buffer T. Avoid bubbles. The use of an electronic multi-pipettor at moderate speed setting is recommended. Analyze immediately with SECTOR Imager.

APPENDIX - 31
ESTIMATION OF ALKALINE PHOSPHATASE (ALP)
(King and Armstrong 1934)

Principle
The method used was that of King and Armstrong in which disodium phenyl phosphate is hydrolysed with the liberation of phenol and inorganic phosphate. The liberated phenol is measured at 700nm with Folin-ciocalteau reagent

**Reagents**

- **Sodium Carbonate - Sodium bicarbonate buffer, 100mmol/L**: Dissolved 6.36g anhydrous sodium carbonate and 3.36g sodium bicarbonate in water and made to a litre.

- **Disodium phenyl phosphate, 100mmol/L**: Dissolved 1g in water, heated to boil, cooled and made to a litre. Added 1.0ml of chloroform and stored in the refrigerator.

- **Buffered - Substrate**: Prepared by mixing equal volume of the above two solution. This has a pH of 10.

- **Folin - ciocalteau reagent**: Mixed 1.0ml of reagent with 2.0ml of water.

- **Sodium carbonate solution, 20%**: Dissolved 20g of anhydrous sodium-carbonate in 100ml of water.

- **Standard phenol solution, 1g/L**: Dissolved 1 g pure crystalline phenol in 100mmol/L HC1 and made to litre with the acid.

- **Working standard solution**: Added 100ml of dilute phenol reagent to 5.0ml of stock standard and diluted to 500ml with water. This contained 10 μg phenol/ml.

**Procedure**

Pipetted 4.0ml of the buffered substrate into a test tube and incubated at 37°C for 5 mins. Added 0.2ml of sample and incubated further for exact 15 mins. Removed
and immediately added 1.8ml of diluted phenol reagent. At the same time a control was set up containing 4.0ml buffered substrate and 0.2ml sample, to which 1.8ml phenol reagent was added immediately. Mixed well and centrifuged. To 4.0ml of the supernatant added 2.0ml of sodium carbonate. Took 4.0ml of working standard solution and for blank taken 3.2ml of water and 0.8ml of phenol reagent. Then added 2.0ml of sodium carbonate. Incubated all the tubes at 37°C for 15 min. Read the colour developed at 700 nm. The activity of serum alkaline phosphatase was expressed in umoles of phenol liberated/ L. The activity in tissue homogenate was expressed as umole of phenol liberated/ min/ mg protein.

**APPENDIX - 32**

**ESTIMATION OF LACTATE DEHYDROGENASE (LDH)**

*(King, 1965b)*

**Principle**

The lactate is acted upon by lactate dehydrogenase to form pyruvate in the presence of NAD. The pyruvate forms pyruvate phenyl hydrazone with 2, 4 dinitrophenyl hydrazine. The color developed is read in a spectrophotometer at 440nm.

**Reagents**

- Glycine buffer, 0.1 M, pH 10: 7.505 g of glycine and 5.85 g of sodium chloride were dissolved in 1 litre of water.

- Buffered substrate: 125ml of glycine buffer and 75ml of 0.1N NaOH were added to 4 g of lithium lactate and mixed well.

- Nicotinamide Adenine Dinucleotide: 10mg of NAD was dissolved in 2ml of water.

- 2, 4 - Dinitrophenyl hydrazine: 200 mg of DNPH was dissolved in 100ml of IN HCl.
0.4 N NaOH.

Standard pyruvate, lumol/ml: 11 mg of sodium pyruvate was dissolved in 100ml of buffered substrate (1 umole of pyruvate /ml).

NADH solution, lumol/ml: 8.5 mg/lOml buffered substrate.

Procedure

Placed 1.0ml buffered substrate and 0.1ml sample into each of two tubes.

Added 0.2ml water to the blank. Then to the test added 0.2ml of NAD. Mixed and incubated at 37°C for 15 mins. Exactly after 15 mins, 1.0ml of dinitrophenyl hydrazine was added to each (test and control). Left for further 15 mins. Then added 10ml of 0.4N Sodium hydroxide and the color developed was read immediately at 440 nm. A standard curve with sodium pyruvate solution with the concentration range 0.1 -1.Oumole was taken.

LDH activity in serum was expressed as umoles of pyruvate liberated / L and in liver homogenate as nmoles of pyruvate liberated / minute / rng protein.

APPENDIX-33
ESTIMATION OF GLUTATHIONE REDUCTASE (GR)
(Goldberg and Spooner, 1983)

Principle

Glutathione reductase catalyses the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) and is assayed by measuring the decrease in absorbance at 340nm.

\[
\text{GR} \quad \text{NADPH (NADH)} + \text{GSSG} \geq \quad \text{NADP}^+(\text{NAD})^+ + 2\text{GSH}
\]
Reagents

- 0.3 M phosphate buffer, pH 6.8.
- 25mMEDTA.
- 12.5μM oxidized glutathione.
- 3mM NADPH.

Procedure

0.2 ml of sample, 1.5 ml of buffer, 0.5 ml EDTA, 0.2 ml GSSG and 0.1 ml NADPH was added. The decrease in optical density of the enzyme was measured against that of the blank at 340 nm.

The enzyme activity is calculated in terms of umoles of NADPH oxidized / min / mg protein.

APPENDIX-34
ESTIMATION OF VITAMIN C (Vit G)
(Omayerfol, 1979)

Principle

Ascorbic acid was oxidised by copper to form dehydroascorbic acid and diketogluutaric acid. These products were treated with 2,4-dinitrophenyl hydrazine to form the derivative of bis 2,4-dinitrophenyl hydrazine. This compound, in strong sulphuric acid undergoes a rearrangement to form a product with an absorption band that is measured at 520 nm. The reaction was run in the presence of thiourea to provide a mildly reducing medium, which helps to prevent interference from non-ascorbic acid chromogens.

Reagents
5% TCA

65% sulphuric acid

DTCS reagent: 3g of 2,4-dinitrophenyl hydrazine, 0.4 g of thiourea and 0.05 g of copper sulphate were dissolved in 9 N sulphuric acid and made upto 100 ml with the same.

Standard solution: standard in the range of 4-20 ug/ml were prepared in 5% oxalic acid.

Procedure

1.0 ml of 10% homogenate was precipitated with 5% ice-cold TCA and centrifuged for 20 mins at 3,500 rev/min. 1.0 ml of the supernatant was mixed with 0.2 ml of DTCS reagent and incubated for 3 hours at 37°C. Then 1.5 ml of ice-cold 65% sulphuric acid was added mixed well and the solutions were allowed to stand at room temperature for an additional 30 mins. Absorbance was determined at 520 nm.

The results are expressed as jag/mg protein.

APPENDIX - 35
ESTIMATION OF ACID PHOSPHATASE (ACP)
King (1965a)

Principle

The method used was that of King and Armstrong in which disodium phenyl phosphate is hydrolyzed with the liberation of phenol and inorganic phosphate. The liberated phenol is measured at 500nm with Folin-ciocalteau reagent.

Reagents

Citrate buffer: 0.1M, pH 5.
Sol A: Citric acid (21.01g in 1000ml)

Sol B: Sodium citrate (29.41g in 1000ml)

20.5ml of A and 29.5ml of B, diluted to a total of 100ml.

Disodium phenyl phosphate, 100mmol/L: Dissolved 2.18gm in water, heated to boil, cooled and made to a litre. Added 1.0ml of chloroform and stored in the refrigerator.

Buffer - substrate: Prepared by mixing equal volume of the above two solution.

This has a pH of 5.0.

- Folin-ciocalteau reagent: Mixed 1.0ml of reagent with 2.0ml of water.

- Sodium carbonate solution, 15 %: Dissolved 15g of anhydrous sodium carbonate in 100ml of water.

- Standard phenol solution, Ig/L: Dissolved 19 pure crystalline phenol in 100mmol/L HCL and made to a litre with the acid.

- Working standard solution: Diluted 10ml of stock standard to 100ml with water. This contains 100ug phenol/ml.

Procedure

Pipetted 4.0ml of the buffer substrate into a test tube and incubated at 37°C for 5 mins. Added 0.2ml of sample and incubated further for exact 60 mins. Removed and immediately added 1.8ml of diluted phenol reagent. At the same time a control was set up containing 4.0ml buffer substrate and 0.2ml sample which 1.8ml phenol reagent was added immediately. Mixed well and centrifuged. To 4.0ml of the supernatant added 2.0ml of sodium carbonate. Took 4.0ml of working standard solution and for blank taken 3.2ml water and 0.8ml of phenol reagent. Then added
2.0ml of sodium carbonate, Incubated all the tubes at 37°C for 15 mins. Read the colour developed at 700 nm.

The activity of serum acid phosphatase was expressed in jimoles of phenol liberated / L. The activity in tissue homogenate was expressed as nmoles of phenol liberated / min / mg protein.

APPENDIX-36
ESTIMATION OF CATHEPSIN D
(Sapolsky et al., 1973)

Reagents

- Sodium acetate buffer 0.1 M, pH 3.6: 92.5 ml of 0.1 M acetic acid was mixed with 7.5 ml of 0.1 M sodium acetate solution.

- Substrate 1.5 %: 1.5 gm of haemoglobin was dissolved in 100 ml of sodium acetate buffer.

- TCA5%: 5 g of TC A was dissolved in 00ml of distilled water.

- Folin's phenol reagent:

- NaOH5%: 5 g of NaOH was dissolved in 100ml of distilled water.

- Alkaline copper reagent (Lowry's Reagent) was prepared.

- Standard: A solution of tyro sine in the concentration of 10 mg / 100 ml was prepared with 0.1 N HC1.

Procedure
0.9 ml of buffered substrate was mixed with 0.1 ml of enzyme preparation and incubated for 2 hours at 37 °C. The reaction was stopped with 1.0 ml of 5 % TCA and the samples were centrifuged for 10 minutes. To the control tubes, the enzymes preparation was added after the addition of TCA. To 1.0 ml of supernatant, 1.0 ml of 5 % sodium hydroxide and 4.5 nil of alkaline copper reagent were added. After 10 minutes, 0.5 ml of Folin's phenol reagent was added and the colour developed was read at 640 nm after 15 minutes. The standards were treated similarly.

Enzyme activity is expressed as umoles of tyrosine liberated / hour / mg protein at 37°C.

APPENDIX-37

ESTIMATION OF N-ACETYL-D-GLUCOSAMINIPASE ACTIVITY (NAG)

(Maruhn, 1976)

Reagents:

- Citric acid - sodium citrate buffer, 0.1 M, pH 4.2
- Solution A: 2.1% of citric acid
- Solution B: 2.94% of sodium citrate
- 10.8 ml of solution A was mixed with 9.2 ml of solution B before use.
- Glycine buffer, 0.2 M, pH 10.7.
- Glycine buffer was prepared by mixing equal volumes of 0.2 M glycine, 0.125 M sodium carbonate and 0.1 M sodium chloride.
- Substrate: 3.42 mg of 4-nitrophenyl-N-acetyl glucosaminide was dissolved in 1 ml of citrate buffer.
- Standard: 5 mg of p-nitrophenol in 100 ml of water.
**Procedure:**

To 0.2 ml of dialysed urine, 0.2 ml of buffered substrate was added and incubated at 37°C for 40 minutes. At the end of incubation period, the reaction was an-ested by the addition of 2.2 ml of 0.2 M glycine buffer and the color was read at 420 nm.

The activity is expressed as fi moles of p- nitrophenol liberated / minute /mg-protein in tissue sample, hi serum and urine the activity is expressed as a moles of p-nitrophenol liberated/ minute / L of sample.

**APPENDIX - 38**

**ESTIMATION OF MALATE DEHYDROGENNASE (MDH)**

*et al., 1948*)

**Principle**

Malate dehydrogenase is one of the enzymes involved in TCA cf'Cle. It catalyses the reversible conversion of oxaloacetic acid to malic acid.

\[
\text{Mg}^{2+} - \text{Oxaloacetic acid} + \text{NADH} \rightarrow \text{Malic acid} + \text{NAD}^+
\]

The decrease in absorbance due to oxidation of NADH is measured at 340nm.

**Reagents**

- 75uM phosphate buffer, PH 7.4
- 0.76 uM oxaloacetate (15.4 mg/5ml)
- 0.15uM NADH(9.1mg/5ml) Procedure

The reaction mixture contained the following reagents and enzyme in a total volume of 3.0ml. 75 uM of phosphate buffer, 0.15uM of NADH and 0.76 uM of oxaloacetate .The reaction was carried at 25 °C and was started by the reagents by the
addition of enzyme preparation. The control tubes contained all reagents except NADH. The change in OD at 340nm was measured for 2min at interval of 1.5secs.

The activity of the enzyme was expressed as micromoles of NADH oxidized/min/mg protein using the extinction coefficient of NADH as $6.22 \times 10^3$.

APPENDIX -39

ESTIMATION OF SUCCINATE DEHYDROGENASE (SDH)
(Slater and Bonner, 1952)

Principle

The rate of oxidation reaction is followed by a coupling reaction to a redox dye. The dye di-chloro-phenol indophenol acts as a hydrogen acceptor from FDH2 and gets reduced. Thus by following the decrease in blue color of the dye. The rate of oxidation of succinate of fumarate.

Reagents

- 0.3M phosphate buffer, pH 7.6
- 0.03 MEDIA
- 0.03M potassium cyanide
- 0.4M sodium succinate, pH 7.6
- 3%BSA(w/v)
- 75nM potassium ferricyanide

Procedure

Added 1.0ml of phosphate buffer, 0.1ml of EDTA, 1.0ml of KCN and made up to 2.9ml with water. Note the extinction at 455nm, then started the reaction by the
addition of enzyme and followed the change in extinction during the first two min. Initial rates were taken as a measure of activity. A blank rate (all reagents except succinate) must be determined separately.

In this determination, 1 mole of succinate reduces 2 moles of potassium ferricyanide. Concentration of potassium ferricyanide rates can be measured by following the reaction at 420 nm (\(s =1.03 \times 10\) cm).

The enzyme activity is expressed as micromoles of succinate produced/min/mg protein under incubation condition.

APPENDIX-40
ESTIMATION OF ISOCITRATE DEHYDROGENASE (IDH)
(Slater and Bonner, 1952)

The enzyme activity was assayed according to the method of Bell and Barren (1960).

Reagents

- 150mmol Sodium chloride solution: 9g of sodium chloride dissolved in 1 litre of distilled water.

- Tris-HCl buffer (100mmol, pH 7.5): 12.14 g tris was dissolved in 80 ml of distilled water and the pH was adjusted to 7.5 with few drops of concentrated HCl.

- 0.15 M saline: 1.75 g of sodium chloride was dissolved in 200 ml of distilled water.

- Substrate: 294 mg of trisodium DL-isocitrate was dissolved in 0.15 M saline.

- Manganese chloride: 29.6 mg of manganese chloride was dissolved in 0.15 M saline.
Co-enzyme (NADP): 7.87mg of NADP was dissolved in 0.15 M saline.

Sodium salt of ethylene diamine tetraacetate (EDTA) 5% solution in distilled water.

Sodium hydroxide: 1.6 g of sodium hydroxide dissolved in 100ml of distilled water.

2,4-Dinitrophenyl hydrazine (DNPH): 4.95 mg of DNPH was dissolved in IN HCl.

Standard solution: 1.5 g of α-ketoglutarate in 50 ml of potassium salt was dissolved.

**Procedure**

0.3 ml of buffer solution was taken in a test tube and 0.2 ml of substrate, 0.3ml of manganese chloride and 0.2 ml of the mitochondrial suspension were added. A control tube was also prepared simultaneously. 0.2 ml of co-enzyme solution was added to the test and 0.2 ml of saline was added to control tubes. After mixing well, both the tubes were incubated for 60 mins. 1 ml of colour reagent (DNPH) was added to both the tubes followed by 0.5 ml of EDTA. The tubes were kept at room temperature for 20 mins and 10 ml of 0.4 N NaOH was added to the tubes. A blank was run simultaneously. The colour was measured at 390 nm. A standard curve was prepared using α-ketoglutarate.

The activity of isocitrate dehydrogenase is expressed as nanomoles of α-ketoglutarate liberated / min / mg of total protein under incubation curve.

**APPENDIX-41**

**ESTIMATION OF Na⁺ K⁺ ATPase**

*(Bonting, 1970)*

**Principle**
Na¹ K ATPase, transports Na⁺ K against concentration gradient at the cost of ATP molecule liberating inorganic phosphate (Pi). Na⁺ K^ ATPase activity was estimated from the amount of Pi liberated by the method of Bonting (1970). The inorganic phosphorous liberated is estimated by Fiske and Subbarow method.

Reagents

- 184mM Tris-HCl buffer, pH 7.5 -
- 50mM MgSO₄
- 50mM KCl
- 60mM NaCl
- ImM EDTA
- 40mM ATP

Procedure

1.0 ml of Tris buffer and 0.2 ml of each of the above reagents were mixed, together. Thus the assay medium in a final volume of 2.0 ml, contained 92mM tris buffer, 50mM MgSO₄, 60mM NaCl, ImM EDTA and 4mM ATP. After 10 minutes, equilibrium at 37°C in an incubator, reaction was started by the addition of 0.1 ml of homogenate. The assay medium was incubated for 15 minutes. After incubation, the reaction was arrested by the addition of 1.0 ml of 10% TCA. The phosphorus content in the supernatant was estimated by the method of Fiske and Subbarow.

The enzyme activity is expressed as micromoles of Pi liberated/min/mg protein.

APPENDIX-42

ESTIMATION OF Mg²⁺ ATPase
(Ohnishie et al., 1982) Principle

The activity of the enzyme was estimated according to the method of Ohnishi et al., (1982) The inorganic phosphorus liberated is estimated by Fiske and Subbarow method.

Reagents

- 75 mMTris-HCl buffer pH 7.6
- 5MmMgCl₂
- 2mMATP

Procedure

The assay was initiated by the addition of 0.1 ml of homogenate to an incubation medium containing 0.1 ml of water and 0.1 ml of each of the above reagents. The final concentration of tris buffer, MgCl₂ and ATP were 75mM, 5mM and 2mM respectively in total incubation volume of 0.5 ml. The reaction was terminated after 15 minutes by the addition of 1.0 ml of 10% TCA. The liberated Pi was estimated by the method of Fiske and Subbarow.

The enzyme activity is expressed as u. moles of Pi liberated/min/mg protein.
Principle

The activity of the enzyme was estimated according to the method of Ohnishi et al., (1982). The inorganic phosphorus liberated is estimated by Fiske and Subbarow method.

Reagents

- 75mM Tris-HCl buffer pH 7.6
- 5mM CaCl₂
- 2mM ATP

Procedure

The assay was estimated by the addition of 0.1ml of homogenate to an incubation medium containing 0.1ml of water and 0.1ml of each of the above reagents. The final concentration of Tris buffer, CaCl₂ and ATP were 75mM, 5mM, 2mM in total incubation volume of 0.5ml. The reaction was terminated after 15 minutes by the addition of 1.0ml of 10% TCA. The liberated Pi was estimated by the Fiske and Subbarow method.

The enzyme activity is expressed as micromoles of Pi liberated / min / mg protein.
ESTIMATION OF PHOSPHORUS

(Fiske and Subbarow, 1925)

Principle

Phosphorus reacts with molybdic acid to form phosphomolybdic acid. On treatment with 1, 2, 4 amino napthol sulphonic acid (ANSA), phosphomolybdic acid is reduced to produce a deep color (molybdenum blue) which is a mixture of lower acids of molybdenum. The blue color is measured spectrophotometrically at 660 nm.

Reagents

- 2.5% ammonium molybdate: Dissolved 25 g of ammonium molybdate in 200 ml water and transferred to 1 L flask containing 300 ml of 10 N H2SO4 and diluted to 1 L with water.

- Amino Napthol Sulphonic acid Reagent (ANSA): Added 0.5 g of ANSA to 195 ml of 15% Sodium bisulphite and 5 ml of 20% Sodium sulphite in a conical flask. Shook until it is dissolved. If the solution is not complete, added more sodium sulphite, 1.0 ml at a time with shaking but avoiding excess. This reagent should be prepared freshly.

- Stock standard phosphate solution: Weighed 35.1 mg of mono potassium dihydrogen phosphate (KH2PO4) dissolved in water and added 1.0 ml of 10 N sulphuric acid and made up to 100 ml with water. 1.0 ml of solution contains 80 ug of phosphorus.

- Working standard: 10 ml of the stock standard was diluted to 100 ml with distilled water. 1.0 ml of the solution contains 8(ig of phosphorus.

Procedure
Into a series of test tubes pipetted out 1.0-5.0 ml of working standard solution corresponding to u.g values 8-40. 1.0 ml of the sample solution was taken in separate test tubes. The volume in all the tubes was made up to 8.6 ml with distilled water. Set up a blank with 8.6 ml of distilled water. Added 1.0 ml of 2.5% ammonium molybdate and 0.4 ml of ANSA to all the tubes. Mixed well and allowed to stand for 10 minutes. The blue color developed was read at 660 nm in a spectrophotometer.

APPENDIX - 45
EXTRACTION OF LIPIDS
(Folchriflf/, 1975)

Reagents

- 0.1NKC1

- Floch reagent - 0.1 N KC1: Methanol: Chloroform (10:10:1 v/v)

Procedure

The tissues were washed with saline and dried between filter paper. A weighed amount of tissue (500 mg) was homogenized with 7.0 ml of methanol in a Potter-Elvehjem homogeniser and filtered through a Whatman No 1 filter paper into a conical flask. The residue after filtration was scraped and homogenized in 14 ml chloroform. The residue was once again scraped from the filter paper and ground with 10 ml of chloroform-methanol mixture and the resulting filtrate was evaporated to dryness.

The dried lipid residue after evaporation was dissolved in 5.0 ml of chloroform-methanol mixture. The redissolved lipid extract was mixed with 1.0 ml of 0.1 N KC1 and the contents were shaken well. The upper aqueous phase containing gangliosides and other water soluble compounds were separated. The lower chloroform phase, containing neutral and phospholipids was again washed 3 times with 2.0 ml of Folch's reagent and the upper aqueous phase was aspirated. The lower
chloroform phase was made up to known volume (2.0 ml) and aliquots were taken for the analysis of cholesterol, triglycerides, free fatty acids and phospholipids.
Principle

Cholesterol reacts with ferric chloride in the presence of concentrated sulphuric acid to give a pink color. The intensity of the color developed is directly proportional to the amount of cholesterol present and is read at 540nm in a colorimeter.

Reagents

- Stock ferric chloride: 840mg of pure dry ferric chloride was weighed and dissolved in 100ml of glacial acetic acid.

- Ferric chloride precipitating reagent: 1 Oml of stock ferric chloride reagent was taken in 100ml of standard flask and made up to the mark with pure glacial acetic acid.

- Ferric chloride diluting reagent: 8.5ml of stock ferric chloride was diluted to 100ml with pure glacial acetic acid.

- Standard cholesterol solution: 100mg of cholesterol was dissolved in 100ml with glacial acetic acid. The concentration of working standard is 100µg/ml.

- Working standard: 10ml of stock was dissolved in 0.85ml of stock ferric chloride reagent and made up to 100ml with glacial acetic acid. The concentration of working standard is 100µg/ml.

Procedure

To 0.1ml of serum, added 4.9ml of ferric chloride precipitating reagent.
Centrifuged and to 2.5ml of supernatant added 2.5ml of ferric chloride diluting reagent. Added 4.0ml of concentrated sulphuric acid. A blank was prepared simultaneously by taking 5.0ml of diluting reagent and 4.0ml of concentrated sulphuric acid. A set of standards (0.5 - 2.5 ml) were taken and made up to 5.0ml with FeCl₂ diluting reagent. Then added 4.0ml of con. HFeSO⁴ After 30min. the intensity of the color developed was read at 540nm against reagent blank.

The amount of cholesterol in the serum was expressed as mg / dl. The amount of cholesterol in tissue was expressed as mg / g tissue.

APPENDIX - 47
ESTIMATION OF PHOSPHOLIPIDS
(Rouser, 1970)

Principle

The organic phospholipid phosphorus is converted to inorganic phosphorus, which reacts with ammonium molybdate to form phosphomolybdic acid, which on reduction and reaction with ANSA forms a stable blue color and has absorption at 710nm

Reagent

- 70% perchloric acid
- 3%ammonium molybdate
- 3%ascorbic acid
- Standard: 35.1 mg of KH₂O₄ was dissolved in 100ml of water. This contains 80(ig of phosphorous/ml.

Procedure
To O.1ml of serum or lipid extract, 1.0ml of perchloric acid was added and digested on a sand bath until it becomes colorless. The volume was made up to 5.0ml with water. Standards in the range 5-20 jig were also taken and 0.8ml of perchloric acid was added and made up to 5.0 ml with water. To all tubes, 0.5ml of ammonium molybdate was added followed by 0.5ml of ascorbic acid solution and mixed well. The tubes were heated in a boiling water bath for 6 mins and the color developed was read immediately at 710nm. Phosphorus content was multiplied by a factor 25. which gave the weight of phospholipids. Phospholipids are expressed as mg/100ml in serum and mg/g in tissues.

**APPENDIX - 48**

**ESTIMATION OF TRIGLYCERIDES**

*(Rice, 1970)*

**Principle**

The glycerol moiety is oxidised to formaldehyde and the latter condensed with ammonia and 2,4-.pentanedione (acetyl acetone) to produce 3,5-diacetyl 1,4-dihydrotoludine which is yellow in colour and has absorption at 405 run.

**Reagents**

- Chloroform-methanol mixture (2:1).
- Activated silicic acid: Silicic acid washed with 4 N or 2 N HC1 and then with water until the washings become neutral. After drying ether was added and stirred well. The supernatant was discarded, silicic acid was then dried at 60°C and activated at 100°C over night prior to use.
- \(0.2\text{NH}_2\text{SO}_4\)
- Saponification reagent: 5.0 g KOH/60 ml water and added 40 ml isopropanol.
- Sodium metaperiodate reagent: To 77 g of anhydrous ammonium acetate in 700 ml water, added 60 ml acetic acid and 650 rug of sodium metaperiodate. Dissolved and diluted in 1000 ml with distilled water.

- Acetyl acetone reagent: Added 0.75 ml of acetyl acetone to 20 ml of isopropanol and mixed well. Added 80 ml of distilled water and mixed.

- Tripalmitin standard containing 100 (ig/ml in chloroform.

**Procedure**

Took 0.1 ml of the serum or dried lipid extract. Made up the volume to 4.0 ml with isopropanol. Mixed well and added 400 mg of silicic acid. Placed them in a mechanical shaker and centrifuged.

To 2.0ml of the supernatant added 0.6 ml of saponification reagent and incubated at 60-70°C for 15 min. After cooling added 1.0 ml of sodium metaperiodate and mixed well. Then added 0.5 ml of acetyl acetone reagent and mixed again. Incubated the tubes at 50°C for 30 mins. After cooling read the colour at 405 nm. Standard tripalmitin(20-1 00 jig) were taken in tubes and treated similarly. Triglycerides were expressed as mg/100 ml in serum and mg/g in tissues.

**APPENDIX - 49**

**ESTIMATION OF FREE FATTY ACID**

*(Horn and Mehanan, 1981)*

**Principle**

The free fatty acids were extracted from lipids by CHM mixture. The free fatty acids form a complex with cupric ions when mixed with copper reagent. The coloured complex formed with copper is soluble in chloroform and diethyl dithiocarbamate and is used as a color developer. The color developed was read at 430nm.

**Reagent**
Chloroform -heptane-methanol mixture (CHM mixture), the mixture was prepared in the ratio of 200:150:7(v/v)

Activated silicic acid

Copper nitrate-triethanolamine solution: 9 volumes of aqueous 1M triethanolamine, 1 volume of 1 N acetic acid and 10 volumes of 6.45% Cu (NO₃)₂ H₂O were mixed with 33g of sodium chloride. The pH was adjusted to 8.1.

0.1% diethyl dithiocarbamate in n-butanol

Standard: A solution containing 200mg/100ml of palmitic acid was prepared in CHM mixture. The solution was diluted 10 times for use (200µg/ml).

Procedure

To 0.2ml serum or lipid extract, 6.0ml of CHM mixture and 200mg of activated-silicic acid were added, mixed well and centrifuged. The supernatant was transferred to another tube. Standard were also made up to 6.0ml with CHM mixture. Blank contained 6.0ml of CHM mixture. To all these tubes, 2.0ml of copper nitrate-TEA solution was added and mixed on a mechanical shaker for 20min. They were then centrifuged to give two separate phases. 2.0ml of the upper phase was transferred to another tube; 1.0ml of the color reagent was then added and shaken well. The color developed was read at 430nm against a reagent blank.

Free fatty acids are expressed as mg/100ml in serum and mg/g in tissues.

APPENDIX-50

ESTIMATION OF HDL CHOLESTEROL
(KIT METHOD) et al., 1985)
Principle

Cholesterol reacts with hot solution of ferric per chlorate, ethyl acetate and sulphuric acid (Cholesterol reagent) and gives a lavender coloured complex which is measured at 560nm.

High density lipoproteins (HDL) are obtained in the supernatant after centrifugation. The cholesterol in the HDL fraction is also estimated by this method.

Procedure

(i) HDL - Cholesterol Separation

<table>
<thead>
<tr>
<th>Pipette into centrifuge tube</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Precipitating reagent</td>
<td>0.2 ml</td>
</tr>
<tr>
<td></td>
<td>0.2ml</td>
</tr>
</tbody>
</table>

Mix well, keep at room temperature for 10 minutes and then centrifuged at 2000 rpm for 15 minutes to obtain a clear supernatant. Proceed to step II.

(n) HDL - Cholesterol Estimation

<table>
<thead>
<tr>
<th></th>
<th>Blank(B)</th>
<th>Standard(S)</th>
<th>Test(T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 1 : Cholesterol reagent</td>
<td>3.0ml</td>
<td>3.0ml</td>
<td>3.0ml</td>
</tr>
<tr>
<td>Reagent 2: Working cholesterol standard, (200mg %)</td>
<td>-</td>
<td>0.015ml</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(15ul)</td>
<td>0.12ml</td>
</tr>
<tr>
<td>Supernatant from step - 1</td>
<td>-</td>
<td>-</td>
<td>(120uJ)</td>
</tr>
</tbody>
</table>

Mix well and keep the tubes immediately in the boiling water bath exactly for 90 seconds. Cool them immediately to room temperature, under running tap water. Measure the O.D of Standard (S) and Test (T) against Blank (B) on a colorimeter with a yellow green filter or on a spectrophotometer at 560 nm.
The LDL, VLDL and Atherogenic index were calculated using the Friedewald's formulae:

\[ \text{LDL} = \text{TC} - \text{HDL} - \text{VLDL}. \]

\[ \text{VLDL-TG IS} \]
ESTIMATION OF HEXOSE
(Niebes, 1972)

Reagents

- Orcinol- sulphuric acid reagent

- Reagent A: 60 ml concentrated sulphuric acid were mixed with 40 ml of water.

- Reagent B: 1.6 g of orcinol in 100 nil of distilled water

Reagents A and B we're mixed in the ratio of 15:2 (v/v) just before use.

- Standard: 5 mg of galactose and 5 mg of mannose were dissolved in 100 ml of distilled water (100 Jag/ ml).

- Sample: A known amount of delipidated residues of liver and kidney were hydrolysed with 2.0 ml of 3 N HC1 at 80°C for 4 hr. The hydrolysed material were neutralised and used for the estimation of protein bound carbohydrates.

Procedure

To 0.5 ml neutralized sample were made up to 1.0 ml with distilled water. To this 8.5 ml of ice-cold orcinol-sulphuric acid were added. The contents were mixed well and the tubes were heated at 80°C for 15 minutes. Cooled and left in the dark for 25 min for colour development. The absorbance was read at 540 nm against a reagent blank. The hexose content was expressed as mg/100 mg of defatted tissue.
ESTIMATION OF HEXOSAMINE
(Wagner, 1979)

Reagents

- Acetyl acetone reagent 3.5%
- Reagent A: Trisodium phosphate 1 M
- Reagent B: Potassium tetraborate 0.5 M
- 3.5 ml of acetyl acetone were added to a mixture containing reagent A and B in the ratio of 98:2 (v/v).
- Ehrlich's reagent: 320 mg of p-dimethylaminobenzaldehyde were dissolved in 21 ml of isopropanol and 3.0 ml of concentrated HCl.
- Standard: 10 mg of galactosamine were dissolved in 100 ml of distilled water which gave a concentration of 100 u-g/ml.

Procedure

0.5 ml of the neutralized sample was made up to 1.0 ml with distilled water. Standard galactosamine (in the range of 10-40 jag) were also made up to 1.0 ml. Blank comprised of 1.0 ml distilled water, 0.6 ml of acetyl acetone reagents were added to all the tubes and heated in a boiling water bath for 30 minutes. After cooling, 2.0 ml of Ehrlich's reagent were added and then contents were shaken well. The pink colour developed was read at 540 nm against a reagent blank. Hexosamine content is expressed as mg/g of defatted tissue.

APPENDIX-53
FUCOSE
**Reagent**

- Sulphuric-acid reagent: this was prepared by mixing 6 volume of concentrated sulphuric acid with 1 volume of distilled water (v/v).

- Cysteine solution: (0.3%) 0.3 g of cysteine hydrochloride was dissolved in 10 ml water (w/v)

- Standard: 20 mg of methyl pentose was dissolved in 100 ml of distilled water.

**Procedure**

To a weighed amount of defatted tissue, 5 ml of sulphuric acids: water mixture was added and heated in boiling water bath for 10 minutes. After cooling the tubes, 0.1ml of cysteine reagent was added. The colour developed after 150 minutes was at 396 nm and 430nm. The standard was also treated in a similar manner. The difference between the two wavelength was calculated for standard, test and blank and this was used to calculate the fucose content.

The fucose level in mitochondria is expressed as jpg/mg of defatted mitochondria.

**APPENDIX-54**

**ESTIMATION OF SIALIC ACID**

*(Warren, 1959)*

**Reagents**

- 0.25 M Periodate: 53.4mg of sodium periodate was dissolved in 100ml of 0.1N H2SO4. 4% Sodium Meta arsenite: 4g of Sodium Meta arsenite was dissolved in 100ml of O,INHCl.
Thiobarbituric acid (TEA) 0.1M: 140mg of TBA was dissolved in 10ml of distilled water. The pH was adjusted to 9.0 with 6N NaOH and the reagent was prepared fresh.

Acidified butanol: 95ml of n-butanol was mixed with 5.0ml of cone. HC1.

Standard: 10mg of N- acetyl neuraminic acid was dissolved in 100ml of distilled water which has a concentration of 100ug/ml.

Procedure

To 0.5ml of neutralized sample, 0.25 ml of periodate was added and incubated at 37°C for 30 minutes. After incubation, the reaction was arrested by the addition of 0.25ml of arsenite. The tubes were shaken well and 2.0ml of TBA was added and the tubes were heated in a boiling water bath for 6 minutes. After cooling, 5.0ml of acidified butanol was added and the butanol phase was separated after shaking well. The absorbance was read at 540nm against a blank treated similarly using a Photochem colorimeter. Standard solutions containing 10-50ug of N-acetyl neuraminic acid were also treated similarly.

APPENDIX-55
ESTIMATION OF GLYCOGEN
(Raghuramulu et al., 1983)

Principle

Glycogen is treated with 45% alcohol to remove glucose. Glucose is dehydrated by sulphuric acid to furfural derivative which then complexes with anthrone to give a green colored complex, which is read at 620nm.

Reagents
Extraction of glycogen: 100mg of the liver tissue was mixed thoroughly in a beaker kept at 0°C. The mixed liver was then homogenized with 5% TCA (2.0 to 3.0ml/gm tissue). The homogenate was centrifuged at 3000g for 10mins in cold. The supernatant was collected. Rehomogenised the sediment with half the volume of TCA and centrifuge in cold. Pooled the supernatants, to this added twice the volume of 45% ethanol, and kept overnight for glycogen precipitation. The precipitate was collected by centrifugation and dissolved in minimal volume of water. This was then reprecipitated as before by adding twice the volume of ethanol. The precipitate obtained was again washed with ethanol and dissolved in 5.0ml of water.

- Anthrone reagent: 0.2% anthrone in concentrated sulphuric acid.
- Stock standard: 100mg of glucose was dissolved in 100ml of distilled water.
- Working standard: 10ml of stock standard was made up to 100ml with distilled water. 1.0ml of the solution contains 100jig of glucose.

**Procedure**

To 0.5ml of sample, 0.5ml of distilled water was added. Glucose standards were prepared by taking 0.2-1.0ml and made up to 1.0ml with distilled water. Added 4.0ml of anthrone reagent to all tubes and heated in a boiling water bath for 8 mins, cooled and read at 620nm.

**APPENDIX-56**

**ESTIMATION OF ALBUMIN AND GLOBAL**

(Wolfson, 1948)

**Principle**

The blue color developed by the aminoacids tyrosine and tryptophan present in the protein by the biuret reaction of the protein with the alkaline cupric tartarate are measured at 555nm.

**Reagents**
Sulphate - Sulphite solution: Weighed 208gm of sodium sulphate (anhydrous) and 70gm of sodium sulphite (anhydrous) and dissolved with stirring in about 900ml of distilled water, to which 2.0ml of concentrated sulphuric acid was added. Transferred to 1L volumetric flask and made up to the mark with distilled water. The pH as adjusted to 7.0.

- Stock biuret reagent

  Dissolved 45gm of Rochelle salt in 400ml of 200mmol/L NaOH and added 15gm of copper sulphate and mixed. 5gm of potassium iodide was added and made up to a litre with 200mmol/L NaOH.

- Working biuret reagent Diluted 20ml of stock reagent to 100ml with 200mmol/L NaOH.

- Ether

- Stock standard solution 1 OOmg of bovine serum albumin was dissolved in 100ml of saline.

- Working standard 1ml of stock was diluted to 5ml with distilled water. Therefore 1ml of this solution contains 200ug of protein.

Procedure

Total protein

Pipetted out 6ml of sulphate-sulphite solution in a test tube and onto it layered 0.4ml of serum and mixed well. From the mixture, 2.0ml were taken and to it 5.0ml of biuret reagent was added.

Albumin

Added about 3.0ml of ether to the rest of the serum-sulphate mixture and shaken 40 times, twice each second for 20 seconds. The tube was centrifuged for 5
mins. After centrifuging, the tube was tilted and inserted a pipette into the clear solution below the globulin layer and pipetted out 2.0ml. To this, 5.0ml of biuret reagent was added.

A set of standards were taken, to this 6.0ml of sulphate-sulphite solution was added and mixed well. To 2.0ml of this mixture, 5.0ml of biuret reagent was added. All the tubes were warmed at 37°C for 10mins. Allowed to cool for 5 mins at room temperature and color was read at 555nm.

The difference between the amount of total protein and albumin gives the globulin. The values in serum were expressed as gm/dl and in liver as mg/gm tissue.
ESTIMATION OF URIC ACID

(Caraway, 1963)

Principle

Uric acid is oxidized to allantoin and carbon dioxide by phosphotungstic acid reagent in alkaline solution. Phosphotungstic acid is reduced in this reaction to tungsten blue, which is measured at 640 nm.

Reagent

- Phosphotungstic acid reagent.
- 10% Sodium carbonate.
- Standard uric acid: 100 μg of uric acid and 60 mg of lithium carbonate were taken in a beaker and about 50ml of water was added. This was heated to about 60°C to dissolve the uric acid completely. After cooling, the solution was finally made upto 100ml with water.

  - Working standard: Dilute 1.0ml of the stock standard to 10ml with water. 1.0ml of this solution contains 20 μg of uric acid.

Procedure

0.1ml of the sample was taken and to this 2.9ml of water was added followed by 0.6ml each of phosphotungstic acid and sodium carbonate. A blank was set up with 3.0 ml water. Standard were also treated in the same manner. The color was read at 640 run alter 10 minutes. The result was expressed as mg/dl in serum.
ESTIMATION OF CREATININE  
(Owen etal, 1954)

Principle

Creatinine forms a colored complex with picrate in alkaline medium. The rate of formation of the complex is measured at 490nm.

Reagents

- Picric acid: 8.02g/L
- Sodium hydroxide: 12.8g/L
- Standard creatinine: Dissolved 100 mg of Creatinine in 100ml with distilled water.
- Working standard: Diluted 2.0 ml of stock solution to 100 ml with distilled water. This contains 20ug of creatinine / ml.
- Reagent mixture: Mixed one part by volume of diluted NaOH with one part by volume of picric acid at least 30 minutes before the assay.

Procedure

Pipetted out 0.2ml of serum and 2.0ml of the reagent mixture into a cuvette. Simultaneously, a blank was set up with the reagent mixture and distilled water. Mixed well and the change in absorbance was measured after 30sec, which was taken as A1 and exactly after 2 min, the absorbance was read as A2 at 490nm. Sets of standards were also treated in the same manner. A1-A2 gives the change in absorbance, which was the measure of the creatinine present in the sample.

The result was expressed as mg/dl in serum.
APPENDIX - 59

SEPARATION OF SERUM LDH ISOENZYME BY ELECTROPHORESIS

(McKenzie and Henderson, 1983)

Agarose gel (1%) was prepared and applied immediately to the glass slide. After the agar gel sets properly, serum samples were applied into a well. After the run, the gels were removed and stained by the following method. The staining solution contained 1.0 ml of 1.0M lithium lactate, 1.0 ml of 0.1M sodium chloride, 1.0 ml of 5.0mM magnesium chloride, 2.5 ml of 0.1% (w/v) nitro blue tetrazolium (NBT), 0.25 ml of 0.1% phenazine methosulphate, 2.5 ml of 0.5M phosphate buffer, pH 7.5 and 10 mg of NAD in a total volume of 10 ml. The gels were incubated with the staining solution at 37°C in the dark for a suitable period. The separated LDH isoenzymes appeared as purple bands. The gels were washed with 7.5% acetic acid, preserved in 5% acetic acid and scanned using densitometer.

APPENDIX - 60

SEPARATION OF SERUM SOD ISOENZYME BY ELECTROPHORESIS

(Beauchamp and Fridovich, 1971)

Extracts containing 40/g mitochondrial proteins were separated on a 12% native polyacrylamide gel. The separated SOD was stained with Riboflavin - 1.52 mg, nitroblue tetrazolium - 20.4 mg, EDTA - 37.2 mg, TEMED - 324 µl in 100 ml of 50mM potassium phosphate buffer (pH 7.8). The activity of MnSOD was identified by blue colour bands.

APPENDIX-61

HISTOPATHOLOGICAL EXAMINATION

(Dunn, 1974)
The liver and spleen samples were preserved in 20% commercial formalin immediately on removal from the animal.

**Tissue Processing**

Heart tissue was placed in 10% formal saline (10% formalin in 0.9% sodium chloride) for one hour to rectify shrinkage due to higher concentration of formalin. The tissue was dehydrated by ascending grades of Isopropyl alcohol by immersing in 80% isopropanol overnight, 100% Isopropyl alcohol for one hour. The dehydrated tissues were cleared in two changes of xylene, one hour each. Then the tissues were impregnated with histology grade paraffin wax (melting point 58-60°C) at 60°C for one hour. The wax-impregnated tissues were embedded in paraffin blocks using the same grade wax. The paraffin blocks were mounted and cut with rotary microtome at three micron thickness. The sections were floated on a tissue floatation bath at 40°C and taken on glass slides and smeared with equal parts of egg albumin and glycerol. The sections were then melted in an incubator at 60°C and after five minutes the sections were allowed to cool.

**Tissue Staining**

The sections were deparaffinised by immersing in xylene for 10 minutes in horizontal staining jar. The deparaffinised sections were washed in 100% Isopropyl alcohol and stained in Ehrlich's hematoxylin for three minutes in horizontal staining jar. After staining in hematoxylin, the sections were washed in tap water and dipped in acid alcohol to remove excess stain (8.3% HCl in 70% Alcohol). The sections were men, placed in running tap water for 10 minutes for blueing (slow alkalization). The sections were counter stained in 1% aqueous eosin (1g in 100ml tap water) for one minute and the excess stain was washed in tap water and the sections were allowed to dry. Complete dehydration of stained sections was ensured by placing the sections in the incubator at 60°C for five minutes. When the sections were cooled, they were mounted in DPX mount having the optical index of glass (the sections were wetted in xylene and inverted on to the mountant placed on cover slip).
The architecture was observed at low power objective. The liver cell injury and other aspects were observed under high power dry objective.
APPENDIX - 62

QUALITATIVE ANALYSIS OF ETHANOLIC EXTRACT OF

*Grewia umbellifera* and *Gmelina arborea* LEAVES (Paech and Tracey, 1955)

IDENTIFICATION OF ALKALOIDS AND FLAVONOIDS

Alkaloids

**Dragendorff’s Test** 8 g of Bi (NO\(_3\))\(_3\) \(5\text{H}_2\text{O}\) was dissolved in 20 ml of Nitric acid and 2.72g of Potassium iodide in 50 ml, of water. They were mixed and allowed to stand when Potassium nitrate crystals out. The supernatant was decanted off and made upto 100ml with distilled water. The alkaloids were regenerated from the precipitate by treating with sodium carbonate followed by extraction of the liberated base with ether.

To 0.5 ml of alcoholic solution of *Grewia umbellifera* and *Gmelina arborea* leaves 2.0 ml of HCl was added. To this acidic medium 1.0 ml of reagent was added. An orange red precipitate produced immediately indicates the presence of alkaloids.

**Wagner's Test (Iodine-Potassium-Iodide Solution)**

1.0g of iodine and 2.0 g of potassium iodide solution was diluted to 100 ml. 10 ml of alcoholic extract of *Grewia umbellifera* and *Gmelina arborea* leaves was acidified by adding 1.5% v/v of HCl and a few drops of Wagner's reagent. Formation of yellow or brown precipitate confirmed the presence of alkaloids.

**Meyer's Test (Potassium Mercuric Iodide)**

1.36 g Mercuric chloride was dissolved in 60 ml of distilled water and 5 g of potassium iodide in 10 ml of water. The two solutions were mixed and diluted to 100 ml with distilled water.

To 1 ml of acidic aqueous solution of *Grewia umbellifera* and *Gmelina arborea* leaves few drops of reagent was added. Formation of white or pale precipitate showed the presence of alkaloids.
Flavonoids

In a test tube containing 0.5 ml of alcoholic extract of the *Grewia umbellifera* and *Gmelina arborea* leaves 5-10 drops of dilute Hydrochloric acid and small piece of Zn or Mg were added and the solution was boiled for few minutes. In the presence of flavonoids, reddish pink or dirty brown colour was produced.

IDENTIFICATION OF TANNINS AND SAPONINS

Tannins

Ferric chloride test

To 1.2 ml of an aqueous solution of *Grewia umbellifera* and *Gmelina arborea* leaves few drops of 1% solution of lead acetate was added. A yellow or red precipitate was formed, indicating the presence of tannins.

Saponins

In a tube containing about 5.0 ml of an aqueous extract of *Grewia umbellifera* and *Gmelina arborea* leaves a drop of sodium bicarbonate was added. The mixture was shaken vigorously and kept for 3 minutes. A honey comb like froth was formed and it showed the presence of saponins.
IDENTIFICATION OF PHENOLS

Phenols

Ferric Chloride test

1 ml of alcoholic solution of *Grewia umbellifera* and *Gmelina arborea* leaves was diluted with water followed by the addition of a few drops of 10% aqueous ferric chloride solution. Formation of blue or green colour indicates the presence of phenols.

Lead acetate test

1 ml of alcoholic solution of *Grewia umbellifera* and *Gmelina arborea* leaves was diluted to 5 ml with distilled water and to this few drops of 1% aqueous solution of lead acetate was added. A yellow precipitate was formed to indicate the presence of phenols.

Libermann's test

A small quantity of alcoholic extract of the *Grewia umbellifera* and *Gmelina arborea* leaves was dissolved in 0.5 ml of 20% sulphuric acid solution followed by the addition of a few drops of aqueous sodium nitrate solution. A red colour was obtained on dilution and it turned blue when made alkaline with aqueous sodium hydroxide solution.

IDENTIFICATION OF GLYCOSIDES

GLYCOSIDES

A small amount of alcoholic extract of *Grewia umbellifera* and *Gmelina arborea* leaves was dissolved in 1.0 ml of water and then aqueous sodium hydroxide solution was added. Formation of a yellow colour indicates the presence of glycosides.
IDENTIFICATION OF STEROIDS

Libermann-Burchard’s test

To 1.0 ml of *Grewia umbellifera and Gmelina arborea* leaves petroleum ether extract in chloroform, 1ml of concentrated sulphuric acid was added followed by the addition of 2.0 ml of acetic anhydride solution. A greenish colour developed and it turned blue. It indicated the presence of steroids.

Salkowski reaction

To 2.0 ml of chloroform extract of *Grewia umbellifera and Gmelina arborea* leaves, 1.0 ml of concentrated sulphuric acid was added carefully along the sides of the test tube. A red colour was produced in the chloroform layer.

APPENDIX-63

ESTIMATION OF TOTAL ALKALOIDS

*(Harborne, 1973)*

Procedure

10 mg of plant material was homogenized in a mortar and pestle. Added around 20ml of methanol : ammonia (68:2). Decanted the ammonical solution after 24 hrs and added fresh methanolic ammonia. Repeated the procedure thrice and pooled the extracts evaporated the extracts using a flash evaporator. Treated the residue with IN HCl and kept it overnight. Extracted the acidic solution with 20ml of CHCls thrice, pooled the organic layers and evaporated to dryness, basic fraction (prserpine). Basified the acidic layer with cone. NaOH to pH-12 and extracted with CHCU (20 ml) thrice, pooled the CHCls layers, dry over absorbent cotton and evaporated to dryness.

Weighed the fraction that contains ajmalicine and serpentine expressed as mg/100 g. *(Harborne, 1973)*
APPENDIX - 64
EXTRACTION AND ESTIMATION OF FLAVONOIDS
(Cameron et al, 1993)

Extraction

A Portion of the ground plant material was weighed out and the extraction was carried out in two steps, firstly with MeOH : H2O(1:1). At each step, sufficient solvent was added to make liquid slurry and the mixture was left for 6-12 hrs. Filtration to separate the extract from the plant material was carried out rapidly by using a glass wool or cotton wool plugged in the neck of a filter funnel. The two extracts were then combined and evaporated to about one third the original volume or until most of the MeOH has been removed. The resultant aqueous extract was cleared of low polarity contaminants such as fats, terpenes, chlorophylls and xanthophylls by extraction (in a separating funnel) with hexane or chloroform. This was repeated several times and the extracts were obtained. The solvent-extracted aqueous layer containing the bulk of the flavonoids was then concentrated.

Reagents

- Vanillin reagent: 1% vanillin in 70% cone. H₂SO₄
- Catechin standard: 110 jig/ml

Procedure

An aliquot of the extract was pipetted into a test-tube and evaporated to dryness. Then added 4 ml of vanillin reagent and heated for 15 minutes in a boiling water bath. A standard was also treated in the same manner. Then the optical density was read at 340 or 360 run.
EXTIMATION OF TANNINS (Bray and Thorpe, 1954)

Principle

The vanillin reagent will react with any phenol that has an unsubstituted resorcinol or phloroglucinol nucleus and forms a coloured substitution product which is measured at 500 nm.

Reagents

Vanillin hydrochloride reagent: Mixed equal volume of 8% hydrochloric acid in methanol and 4% vanillin in methanol. The solution must be prepared freshly.

- Catechin stock standard: 1 mg/ml
- Working standard: 1 Oml of stock to 100ml with water
- Extract: Extracted 1 g of sample in 50ml methanol. After 20 - 28 hrs,
- Centrifuged and collected the supernatant.

Procedure

Pipetted out 1.0ml of supernatant and added 5.0ml of hydrochloride reagent.

Read in a spectrometer at 500 nm after 20 minutes. Prepared a blank with reagent alone. Tannins in sample is expressed as catechin equivalents.
APPENDIX-66

ESTIMATION OF TOTAL PHENOLS

(Bray and Thorpe, 1954)

Principle

Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium and produce a blue coloured complex (molybdenum blue) that can be estimated colorimetrically at 650 nm.

Reagents

- 80% ethanol
- Folin-Ciocalteau reagent
- 20% Sodium carbonate
- Stock standard: Dissolved 100mg of catechol in 100ml distilled water
- Working Standard: Diluted 10ml of stock to 100ml with distilled water. 1.0ml of the solution contains 100µg of catechol.
- Sample Preparation: Weighed 0.5 - 1 g of sample and ground it with a mortar and pestle in 10X volume of 80% ethanol. Centrifuged the homogenate at 10,000 rpm for 20 minutes. Saved the supernatant. Re-extracted the residue with 5 times the volume of 80% ethanol, centrifuged and pooled the supernatant to dryness. Dissolved the residue in a known volume of distilled water.

Procedure

Pipetted out 0.1ml of sample in two test tubes. Made up the volume in all tubes to 3.0ml with distilled water and added 0.5ml of Folin-Ciocalteau reagent. After
3 minutes, added 2.0ml of 20% sodium carbonate. Mixed thoroughly, placed the tubes in boiling water bath for exactly 1 minute, cooled and read the absorbance at 650 nm against a reagent blank. A set of standards were also treated in the above manner.

**APPENDIX - 67**

**ESTIMATION OF TOTAL CARBOHYDRATE**

*(Hedge and Hofreiter, 1962)*

**Principle**

Carbohydrate is first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to Hydroxymethyl furfural. This compound forms with anthrone a green coloured product with absorption maximum at 630 nm.

**Reagents**

- Glucose stock standard: 100 mg of glucose was dissolved in 100 ml of water in a standard flask.

- Working standard: 10 ml of the stock was diluted to 100 ml, 1.0 ml of this solution contains 100 u.g of glucose.

- Anthrone reagent: 0.2% anthrone was dissolved in ice cold concentrated sulphuric acid. Prepared fresh before use.

- 2.5NHC1.

**Procedure**

Weighed 100 mg of the sample into a boiling tube. Hydrolysed by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N HCl and cooled to room temperature. Neutralised it with solid sodium carbonate until the effervescence ceases. Made up the volume to 100 ml and centrifuged. Collected the supernatant and take 0.2 to 1.0 ml was taken for analysis. Prepared the standards by taking 0.2-1.0 ml of the
working standards. 1.0 ml of water serves as a blank. Made up the volume to 1ml in all the tubes with distilled water. Then added 4 ml of anthrone reagent. Heated for eight minutes in a boiling water bath. Cooled rapidly and read the green to dark green colour at 630 nm.

Calculation

A standard graph was drawn by taking the concentration of glucose along X axis and spectrophotometer reading along Y axis. From the graph the concentration of glucose in the sample was calculated.

APPENDIX-68
ESTIMATION OF STEROIDS
(Zak's, 1977)

Principle

Steroids react with ferric chloride in the presence of concentrated sulphuric acid to give a pink color. The intensity of color developed is directly proportional to the amount of Steroids present and is read at 540 nm in a colorimeter.

Regents

- Stock ferric chloride: 840 mg of pure dry ferric chloride was weighed and dissolved in 100ml of glacial acetic acid.

- Ferric Chloride precipitation reagent: 10ml of stock ferric chloride reagent was taken in 10 ml of standard flask and made up to the mark with pure glacial acetic acid.
Ferric chloride diluting reagent: 8.5 ml of stock ferric chloride was diluted to 100ml with pure glacial acid.

Standard solution: 100 mg of Steroids was dissolved in 100 ml of glacial acetic acid.

Working standard: 10 ml of stock was dissolved in 0.85 ml of stock ferric chloride reagent and made up to 100 ml with glacial acetic acid. The concentration of working standard is microgram/ml.

Procedure

To 0.1 ml of plant extract, added 4.9 ml of ferric chloride precipitating reagent. Centrifuged and to 2.5ml of supernatant, added 2.5 ml of ferric chloride diluting agent. Added 4.0ml of concentrated sulphuric acid. A blank was prepared simultaneously by taking 5.0 ml of diluting reagent and 4.0ml of concentrated sulphuric acid. A set of standards (0.5-2.5ml) were taken and made up to 5.0 ml with ferric chloride diluting reagent. Then added 4.0 ml of concentrated sulphuric acid. After 30 minutes, the intensity of color developed was read at 540 nm against a reagent blank. The amount of Steroids in the sample is expressed as mg/dl.
## Table 1, Levels of total cholesterol in serum and heart of control and experimental rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total Cholesterol</th>
<th>Triglycerides</th>
<th>Free Fatty Acids</th>
<th>Phospholipids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum (mg/dl)</td>
<td>Heart (mg/g)</td>
<td>Serum (mg/dl)</td>
<td>Heart (mg/g)</td>
</tr>
<tr>
<td>Group I</td>
<td>94.17±4.10</td>
<td>5.75 + 0.06</td>
<td>144.52 + 4.37</td>
<td>3.95 + 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>127.93 + 2.51</td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>135.53 ±11.48&quot;</td>
<td>7.22 + 0.09a</td>
<td>184.94 ±4.36&quot;</td>
<td>5.66±0.05a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>161.03 + 2.76a</td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>125.25 + 13.62&quot;b</td>
<td>6.83±0.31b</td>
<td>173.12 + 4.61&quot;b</td>
<td>5.27 ± 0.04b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>154.40±3.41b</td>
<td></td>
</tr>
<tr>
<td>Group IV</td>
<td>116.15 ±7.47c</td>
<td>6.49 ± 0.27c</td>
<td>165.45 ±4.25c</td>
<td>4.88 ± 0.04c</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>145.37 + 2.85c</td>
<td></td>
</tr>
<tr>
<td>Group V</td>
<td>110.81 ± 7.63d</td>
<td>6.26 + 0.21d</td>
<td>158.60 ±3.68d</td>
<td>4.41+0.04d</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>139.96 ±2.95d</td>
<td></td>
</tr>
<tr>
<td>Group VI</td>
<td>101.41 ±7.26e</td>
<td>5.90±0.20e</td>
<td>151.20 + 3.67e</td>
<td>4.20+0.04e</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>133.51 + 2.72e</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SD of six samples

**Comparisons are made between:**

- a - Group I and Group II
- c - Group IV and Group II
e - Group VI and Group II

**Statistical significance:** a,b,c,d and e significant at P<0.05.

- b - Group III and group II
d - Group V and Group II
Table 2. Biochemical parameters in serum of control and experimental rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Protein (mg/dl)</th>
<th>A/G ratio (mg/dl)</th>
<th>Glycogen (mg/dl)</th>
<th>Uric acid (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>6.41 ± 0.30</td>
<td>1.67 ± 0.13</td>
<td>20.48 ± 1.41</td>
<td>2.86 ± 0.03</td>
</tr>
<tr>
<td>Group II</td>
<td>4.79 ± 0.16</td>
<td>0.84 ± 0.16(^a)</td>
<td>12.32 ± 1.46(^a)</td>
<td>7.28 ± 0.05(^a)</td>
</tr>
<tr>
<td>Group III</td>
<td>5.16 ± 0.09(^b)</td>
<td>0.99 ± 0.07(^b)</td>
<td>14.89 ± 1.42(^b)</td>
<td>6.6 ± 0.05(^b)</td>
</tr>
<tr>
<td>Group IV</td>
<td>5.33 ± 0.04(^c)</td>
<td>1.24 ± 0.09(^c)</td>
<td>16.18 ± 1.44(^c)</td>
<td>5.72 ± 0.03(^c)</td>
</tr>
<tr>
<td>Group V</td>
<td>5.48 ± 0.06(^d)</td>
<td>1.39 ± 0.14(^d)</td>
<td>17.54 ± 1.40(^d)</td>
<td>4.4 ± 0.04(^d)</td>
</tr>
<tr>
<td>Group VI</td>
<td>5.75 ± 0.10(^e)</td>
<td>1.48 ± 0.08(^e)</td>
<td>18.7 ± 1.42(^e)</td>
<td>3.67 ± 0.03(^e)</td>
</tr>
</tbody>
</table>

Values are mean ± SD of six samples

**Comparisons are made between:**
- \(^a\) - Group I and Group II
- \(^c\) - Group IV and Group II
- \(^e\) - Group V and Group VI

**Statistical significance:**
- \(^a\),\(^b\),\(^c\),\(^d\) and \(^e\) significant at P<0.05.
- \(^b\) - Group III and group II
- \(^d\) - Group IV and Group VI
Table 3. Levels of Glycoproteins parameters in control and experimental rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hexose</th>
<th>Hexosamine</th>
<th>Fucose</th>
<th>Sialic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum (mg/dl)</td>
<td>Heart (mg/g)</td>
<td>Serum (mg/dl)</td>
<td>Heart (mg/g)</td>
</tr>
<tr>
<td>Group I</td>
<td>128.34 ±9.56</td>
<td>142.39 ±4.98</td>
<td>26.82 ±1.40</td>
<td>14.89 ±1.41</td>
</tr>
<tr>
<td>Group II</td>
<td>243.28 ±6.43[a]</td>
<td>199.63 ±5.32[a]</td>
<td>39.34 ±1.41[a]</td>
<td>18.63 ±2.79[a]</td>
</tr>
<tr>
<td>Group III</td>
<td>218.36 ±7.28[b]</td>
<td>183.96 ±5.97[b]</td>
<td>35.21 ±2.84</td>
<td>16.84 ±1.43[b]</td>
</tr>
<tr>
<td>Group IV</td>
<td>187.49 ±6.37[c]</td>
<td>172.21 ±5.74[c]</td>
<td>32.68 ±1.44[c]</td>
<td>16.31 ±1.47[c]</td>
</tr>
<tr>
<td>Group VI</td>
<td>147.28 ±7.98[e]</td>
<td>158.24 ±5.71[e]</td>
<td>28.24 ±1.36[e]</td>
<td>15.36 ±1.54[e]</td>
</tr>
</tbody>
</table>

Values are mean ± SD of six samples

**Comparisons are made between:**

- a- Group I and Group II
- c - Group IV and Group II
- e- Group VI and Group II
- b - Group III and group II
- d - Group V and Group II
Statistical significance: a, b, c, d, and e significant at $P<0.05$. 
Table 4. Level of lipid peroxides (LPO) in the serum and heart of control and experimental group rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>LPO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
</tr>
<tr>
<td>Group I</td>
<td>3.85 ±0.09</td>
</tr>
<tr>
<td>Group II</td>
<td>4.81 ±0.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group III</td>
<td>4.70±0.15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group IV</td>
<td>4.44±0.10&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group V</td>
<td>4.34 ±0.30&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group VI</td>
<td>4.20±0.13&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SD of six samples

Units

LPO - mg/dl (serum) nmoles/mg protein (heart)

Comparisons are made between: a- Group I and Group II  
c -Group IV and Group II  e- Group VI and Group II

Statistical significance: a,b,c,d and e significant at P<0.05

b - Group III and Group II  d - Group V and Group II
Table 5. The activities of marker enzymes namely AST and ALT in the serum of control and experimental rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST</th>
<th>ALT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
<td>Heart</td>
</tr>
<tr>
<td>Group I</td>
<td>41.88 ± 1.24</td>
<td>18.30 ± 0.33</td>
</tr>
<tr>
<td>Group II</td>
<td>134.77 ± 0.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.28 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group III</td>
<td>92.22 ± 1.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.29 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group IV</td>
<td>84.02 ± 0.78&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.66 ± 0.20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group V</td>
<td>73.15 ± 2.33&lt;sup&gt;d&lt;/sup&gt;</td>
<td>15.43 ± 0.44&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group VI</td>
<td>57.85 ± 2.30&lt;sup&gt;e&lt;/sup&gt;</td>
<td>16.73 ± 0.35&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SD of six samples Units
(Serum) - AST and ALT - IU/L;  (Heart) - AST and ALT- nmoles of pyruvate liberated /min / mg protein

Comparisons are made between:

- a- Group I and Group II
- b- Group III and Group II
- c- Group IV and Group II
- d- Group V and Group II
- e- Group VI and Group II

Statistical significance: a,b,c,d and e significant at P<0.05.
Table 6. Activities of lysosomal enzymes in serum and heart of control and experimental rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>ACP</th>
<th></th>
<th>Cathepsin D</th>
<th></th>
<th>NAG</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
<td>Heart</td>
<td>Serum</td>
<td>Heart</td>
<td>Serum</td>
<td>Heart</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>79.95 ±0.30</td>
<td>124.87 ±1.99</td>
<td>15.37 + 0.37</td>
<td>25.34 + 0.56</td>
<td>20.50 + 0.62</td>
<td>46.86±0.33</td>
</tr>
<tr>
<td>Group II</td>
<td>111.33 ± 1.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>165.25 + 3.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.45 ±0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.34 + 0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.85 + 0.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58.21 ±0.46&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group III</td>
<td>103.97 ±8.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>154.32 ±2.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.59 ±0.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.01 ±0.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.82 ±0.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55.32 ± 0.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group IV</td>
<td>94.23 ±1.65&lt;sup&gt;c&lt;/sup&gt;</td>
<td>144.77 ±2.73&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.53 ±0.46&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29.38 + 0.56&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25.87 + 0.28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>53.77 ±0.32&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group V</td>
<td>89.51 ±0.55&lt;sup&gt;d&lt;/sup&gt;</td>
<td>139.71 ±2.03&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18.63 ±0.45&lt;sup&gt;d&lt;/sup&gt;</td>
<td>28.28 + 0.56&lt;sup&gt;d&lt;/sup&gt;</td>
<td>24.17 + 0.53&lt;sup&gt;d&lt;/sup&gt;</td>
<td>51.85 + 0.45&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group VI</td>
<td>85.21 ±0.44&lt;sup&gt;e&lt;/sup&gt;</td>
<td>134.54 + 2.65&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17.30 ±0.56&lt;sup&gt;e&lt;/sup&gt;</td>
<td>27.19 + 0.42&lt;sup&gt;e&lt;/sup&gt;</td>
<td>22.27 ± 0.59&lt;sup&gt;e&lt;/sup&gt;</td>
<td>49.93 ± 0.34&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SD of six samples.

les
Units:

ACP - (Serum) - 1U/L Cathepsin D - (Serum) - IU/L NAG - (Serum) - IU/L

(Heart) - nmoles of phenol liberated/min/mg protein (Heart) — mmoles of tyrosine liberated/h/mg protein (Heart) - umoles of p-nitrophenol formed/h/mg protein.

Comparisons are made between:

a- Group I and Group II
c- Group IV and Group II
e- Group VI and Group II

b- Group III and group II
d- Group V and Group II

Statistical significance: a, b, c, d, e significant at P<0.05.
Table 7. Activities of mitochondrial enzymes in heart of control and experimental rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDH</th>
<th>SDK</th>
<th>IDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>340.31 ±17.84</td>
<td>254.39 + 20.37</td>
<td>768.60+13.79</td>
</tr>
<tr>
<td>Group II</td>
<td>247.27 + 13. 10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>151.00 ±17.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>537.57 + 20.74&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group III</td>
<td>269.60 ±15.99&lt;sup&gt;b&lt;/sup&gt;</td>
<td>177.00 +13.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>609.89 ±17.34&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group IV</td>
<td>292.26 ±15.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>195.69 ±11.93&lt;sup&gt;c&lt;/sup&gt;</td>
<td>658.81±183&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group V</td>
<td>311.69+ 19.18&lt;sup&gt;d&lt;/sup&gt;</td>
<td>225.97 ±12.16&lt;sup&gt;d&lt;/sup&gt;</td>
<td>688.57±17.92&lt;sup&gt;1f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group VI</td>
<td>330.86 + 17.66&lt;sup&gt;e&lt;/sup&gt;</td>
<td>244.02 ±11.73&lt;sup&gt;e&lt;/sup&gt;</td>
<td>710.89 ±H70&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SD of six samples

**Units:**

MDH - (imoles of NADH oxidized/min/mg protein. SDH - umoles of NADH oxidized/min/mg protein. IDH - umoles of NADH oxidized/min/mg protein.

**Comparisons are made between:**

- a- Group I and Group II
- c - Group IV and Group II e- Group VI and Group II
- b - Group III and group II d - Group V and Group II

**Statistical significance**

: a,b,c,d and e significant at P<0.05.
Table 8. Activities of membrane bound enzymes in heart of control and experimental rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Na⁺ K⁺ ATPase</th>
<th>Ca²⁺ ATPase</th>
<th>Mg²⁺ ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>0.56 ± 0.04</td>
<td>0.69 ± 0.27</td>
<td>0.46 ± 0.05</td>
</tr>
<tr>
<td>Group II</td>
<td>0.29 ± 0.03ᵃ</td>
<td>1.68±0.06ᵃ</td>
<td>0.91 ±0.05ᵃ</td>
</tr>
<tr>
<td>Group III</td>
<td>0.36±0.04ᵇ</td>
<td>1.50±0.04ᵇ</td>
<td>0.79 ± 0.04ᵇ</td>
</tr>
<tr>
<td>Group IV</td>
<td>0.42 ± 0.04ᶜ</td>
<td>1.32 ±0.04ᶜ</td>
<td>0.68±0.04ᶜ</td>
</tr>
<tr>
<td>Group V</td>
<td>0.47 ± 0.04ᵈ</td>
<td>1.13±0.06ᵈ</td>
<td>0.57±0.05ᵈ</td>
</tr>
<tr>
<td>Group VI</td>
<td>0.52 ± 0.03ᵉ</td>
<td>1.04±0.06ᵉ</td>
<td>0.51 ± 0.04ᵉ</td>
</tr>
</tbody>
</table>

Values are mean ± SD of six samples

**Units:**

Na⁺K⁺ ATPase - (Heart) - mmoles of inorganic phosphorus / mg protein
Ca²⁺ ATPase - (Heart) - mmoles of inorganic phosphorus / mg protein
Mg²⁺ ATPase - (Heart) - mmoles of inorganic phosphorus / mg protein

**Comparisons are made between:**

- Group I and Group II
- Group IV and Group II
- Group VI and Group II
- Group III and Group II
- Group V and Group II
- Group VI and Group II

**Statistical significance:**

- a, b, c, d and e significant at P<0.05
Table 9. Free radical scavenging activities of *Grewia umbellifera* and *Gmelina arborea*

<table>
<thead>
<tr>
<th>% inhibition</th>
<th>Sample concentration (mg/ ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>DPPH</td>
<td>27.63 ± 1.4</td>
</tr>
<tr>
<td>ABTS</td>
<td>33.23 ± 1.7</td>
</tr>
<tr>
<td>Hydroxyl radical</td>
<td>25.54 ± 1.9</td>
</tr>
<tr>
<td>Superoxide radical</td>
<td>20.27 ± 1.4</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>19.31 ± 0.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% inhibition</th>
<th>Sample concentration (mg/ ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>P - carotene</td>
<td>14.12 ± 0.7</td>
</tr>
<tr>
<td>Absorbance</td>
<td>Sample concentration (mg/ ml)</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>FRAP (593 nm)</td>
<td>0.305 ±0.02</td>
</tr>
<tr>
<td>Reducing power (700nm)</td>
<td>0.178± 0.009</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Absorbance</th>
<th>Sample concentration (mg/ ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Chelating ability (562 nm)</td>
<td>0.1 77 ±0.02</td>
</tr>
</tbody>
</table>