Chapter 4

Materials and Methods
4. MATERIALS AND METHODS

4.1 Collection and Identification of *Ganoderma lucidum*

Fresh isolates of *Ganoderma lucidum* from different host trees and floor of Kolli hills were collected from various places. They were identified using Simon and Schuster's Guide to Mushrooms and microscopic examination after cotton blue mount.

4.2 Microscopic examination- Slide culture technique

Slide culture technique is used to identify the hyphae structure of fungi. For this technique Lacto phenol Cotton blue stain is used. Potato Dextrose agar and Rose Bengal Agar was prepared aseptically. A piece of medium was cut by using a sterile blade and placed on a glass slide and kept in a petri dish. The mycelia growth of *Ganoderma lucidum* was placed on the for corners of the medium. Then a sterile cover slip was placed over the medium. Wet cotton (Sterile) was kept in the Petridish nearby the slide for moisture and incubated at room temperature for 3-4 days. After incubation period, the slides were stained with Lactophenol cotton blue stain. Then the cover slip which containing mycelia growth of *Ganoderma lucidum* was placed on the stain without air bubbles. This slide was observed under the 10x and 40x magnifications. By using this method, the wild type *Ganoderma lucidum* were compared with the MTCC culture.

4.3 Tissue culture technique

The fruiting bodies of identified *Ganoderma lucidum* were used for tissue culture technique. Mycelia was collected by cutting the Ganoderma longitudinally after surface sterilization with ethanol. This soft tissue was placed in the centre of the sterilized Potato Dextrose Agar and Rose Bengal Agar and incubated at room temperature (28±2°C) for 4-5 days. The grown mycelium around the placed tissue, without contamination was considered as positive growth. The pure culture was collected and stored in slants at 4°C for a period of a month.

4.4 Preparation of mother spawn

Spawn preparation was done in *Sorghum vulgare* grains. The *Sorghum vulgare* grains (1 kg) were placed in a trough of water to remove the chaff grains. Then it was half cooked (Approximately 30 min). The excess water was drained and spread over a
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clean sac or Hessian cloth. For every kg of grains, 30 g calcium carbonate was mixed so as to maintain the pH for the growth of the fungus. Moreover, the calcium carbonate coating prevents the grains from sticking. These grains were filled in clean empty glucose drip bottles (300g/bottle).

Then the bottle were tightly plugged with nonabsorbent cotton and wrapped with paper, tied with a thread and placed in an autoclave for sterilization (20 lbs pressure for 2 hours). After cooling the bottles were ready for inoculation. With the help of cork borer a 10 mm diameter disc was made from the petridishes having fully grown pure mycelium and transferred. The bottles were incubated at a room temperature. It about 15 days the white colored mycelia growth is spread over the bottle, during the incubation period indicates positive results. From this mother spawn, 30 first generation spawn were prepared. Each first generation bottle having 10g of mother spawn and from this second generation spawn were prepared.

4.5 Preparation of Mushroom bed

For the preparation of mushroom bed various substrates were used. Viz.,

6. Saw dust
7. Rice chaff
8. Mixer of saw dust and rice chaff
9. Agro wastes from industry and

The wooden chips of various plants were used to cultivate the *Ganoderma lucidum*. The wood chips were cut into 1-2 cm bits and soaked in water trough for about 10-12 hours. The water was drained and the presoaked wood chips were transferred to any metallic vessel and water was added. The wood chips were boiled for 30 min. Again the water was drained. And the wood chips were spread on a clean sac or hessian cloth which is previously soaked in fungicide solution or potassium...
permanganate solution. The wood chips were shade dried and it should contain optimum moisture. Low moisture content in the wood chips will not permit proper spawn run. Polythene bag of 30x15 cm size taken and the bottom of the bag was tied with the thread to provide a flat circular bottom of the mushroom beds. After cooling, the wooden chips were filled in polythene bags along with previously prepared spawn. The spawning was done in 4-5 layers. 1 bottle of spawn was sufficient for two such bags. The bags were plugged with nonabsorbent cotton. These polythene bags were kept in dark room at 28-30°C. Humidity of the room was maintained at 70-90% after 15-20 days. The pinheads of *Ganoderma lucidum* emerged from the mouth of the polythene bag. The growth rate, weight of fruiting bodies, number of fruiting bodies, number of generations of production which were grown on various substrates were measured and were compared periodically and tabulated.

4.6 Analysis of growth rate

After regeneration, the growth rate of Ganoderma mushroom which is grown on various substrates were measured at different time intervals and then the size of the Ganoderma are measured in millimeter. The food consumption by the *Ganoderma lucidum* from the substrates was also calculated by the weight of the mushroom bed before and after cultivation. The gross conversion efficiency (GCE) is an indication for the growth performance of any living things. The food conversion ratio (FCR) and Gross conversion efficiency (GCE) were calculated by the following formula.

\[
\text{FCR} = \frac{\text{Food consumption}}{\text{Weight gain}} \times 100
\]

\[
\text{GCE} = \frac{\text{Weight gain}}{\text{Food consumption}} \times 100
\]

4.7 Determination of Moisture content

The moisture content of the sporophore was estimated by drying 2 gm of fresh sporophore in an oven at 80°C for three consecutive days. It was cooled in a
desiccators and weighed. The moisture content was calculated by the following formula.

\[
\text{Moisture content(\%) =} \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fresh weight}} \times 100
\]

4.8 Collection and Harvesting of fruiting bodies

The final stage of growth of *Ganoderma lucidum* was identified by the brown color growth of fruiting body. That then the fruit bodies were harvested by sterile blade. The weight of Ganoderma was measured in gram and results were tabulated.

4.9 Water extract preparation

The harvested fruit bodies of *Ganoderma lucidum* (250 gm) was made into small pieces and shade dried. The it was made into powder and homogenized in a warring blender with 500ml of distilled water. The extraction was carried out in a cold room (20°C±1°C) with constant stirring overnight. The homogenate was then squeezed through cheese cloth and centrifuged at 2000 rpm for 10 min at 0-4°C. The supernatant was concentrated and used as the *Ganoderma lucidum* aqueous extract. It was kept at 4°C until use.

4.10 Experimental animals

Wistar albino rats (200-250gm) of either sex bred in Animal house, Muthayammal College of Arts and Science, Rasipuram, Namakkal District, India were used. Animals were housed in an air-conditioned animal room at 23±2°C with 12 h light/dark photoperiod and maintained with free access to water and ad libitum feeding. All animal experiments were approved by the ethical committee, and were in accordance with the guidelines of the National Institute of Health Guide (1985).

4.11 Chemicals

Streptozotocin was obtained from Sigma Chemical Co., Banglore. All other chemicals were of analytical grade.

4.12 Induction of diabetes

Rats were made diabetic by single intra – peritoneal administration of Streptozotocin (55mg/kg/i.p) dissolved in 0.1M citrate buffer, PH 4.5. Forty-eight hours later, blood samples were collected and glucose level was determined to
confirm the development of diabetes. Those animals with hyperglycemia (blood glucose level > 240 mg/dl) were used in the experiment.

4.13 Animal Groups for the study

Two normal rat group of 6 animals each was considered as Group I and Group II. Group II received *Ganoderma lucidum* aqueous extract. The diabetic rats were divided into 4 groups of 6 animals each. Group III from first batch of diabetic rats served as diabetic control. Group VI received *Ganoderma lucidum* aqueous extract (10 mg / kg / p.o) dissolved in 0.1 m – citrate buffer. Group V & Group VI received the *Ganoderma lucidum* aqueous extract (20 mg, 30 mg / kg p.o) respectively suspended in vehicle.

4.14 Biochemical analysis

After 45 days treatment, Blood samples were collected by cervical dislocation. The plasma was separated and used to determine the biochemical parameters. Blood glucose level was measured by glucose oxidase method (Triender, 1969)


**Principle**

Glucose is oxidized by the enzyme glucose oxidase (GOD) to give D-gluconic acid and hydrogen peroxide. Hydrogen peroxide in presence of enzyme peroxidase (POD) oxidizes phenol with combines with 4 – aminoantipyrene to produce a red coloured quinoneimine dye. The intensity of the colour developed is proportional to glucose concentration in the sample. It was measured photometrically at 505 nm (500-550 nm)

\[
\text{D – Glucose + H}_2\text{O+O}_2 \rightarrow \text{D-Gluconic acid + H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + 4\text{-aminoantipyrene + Phenol } \rightarrow \text{Red quinoneimine dye + H}_2\text{O}
\]

**Reagent requirements**

1. Enzyme reagents  
2. Buffer solution  
3. Glucose standard

**Reagent preparation**

Dilute one vial of the enzyme reagent (1) in one bottle of buffer solution (2). Mix gently to dissolve. The prepared working enzyme reagent is stable for at least a month at 2-8C.
Procedure:

<table>
<thead>
<tr>
<th>Working enzyme reagent</th>
<th>B</th>
<th>S</th>
<th>T</th>
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<tr>
<td>Distilled water</td>
<td>1.0 ml</td>
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<td>Distilled water</td>
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<td>Glucose standard</td>
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<td>Serum / plasma</td>
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Pipette into clean dry test tubes labeled blank (B), Standard (S) and test (T). Mix well and incubate at 37°C for 15 minutes. Then measure the absorbance of test (T) and standard (S) against blank (B) on a photo-colorimeter with a green filter or on a spectrophotometer at 505 nm. The calculation of glucose is in mg% = \( \frac{A_{(T)}}{A_{(S)}} \times 100 \)

4.14.2. Biochemical analysis for analyzing the antioxidant property of *Ganoderma lucidum* aqueous extract on STZ induced diabetic rats

After 45 days treatment, blood was collected into heparinized tubes. The plasma was separated by centrifugation and the biochemical parameters were determined by following different methods: Blood glucose level by glucose Oxidase method (Triender 1969), plasma TBARS content by the method of Nichans and Samuelson (1968), hydroperoxide (Jiang *et al.*, 1992), reduced glutathione (Beutler and Kelley, 1963), and ascorbic acid (Rao and Kuether, 1943). Percent of glycemic changes was calculated as a time function, by applying the formula: \( \% \) Glycemia changes = \( \frac{G_x - G_o}{G_o} \). Where \( G_o \) - initial glycemia values, \( G_x \) - Glycemia values at \( x \) minutes time interval.

4.14.2.1 Estimation of plasma TBARS (Nichans and Samuelson, 1968)

**Principle**

Lipid peroxidation was estimated as evidenced by the formation of thiobarbituric acid reactive substances (TBARS). Plasma was deproteinised with phosphotungstic acid and the precipitate was treated with thiobarbituric acid at 90°C
for 1 hour. The pink colour formed gives a measure of the thiobarbituric acid reactive substances (TBARS) which was read at 530nm.

Reagents

1. 0.083 N sulphuric acid
2. 10% phosphotungstic acid
3. Thiobarbituric acid (TBA): 670mg was dissolved in 100ml water. To this, 100ml of glacial acetic acid was added.

Procedure

To 0.5 ml of plasma, 4.0 ml of 0.083N sulphuric acid was added. To this mixture, 0.5ml of 10% phosphotungstic acid was added and mixed. After standing at room temperature for 5 minutes, the mixture was centrifuged at 3000 g for 10 minutes. The supernatant was discarded. The sediment was mixed with 2.0 ml of sulphuric acid and 0.3 ml of 10% phosphotungstic acid. The mixture was shaken well and centrifuged at 3000 g for 10 minutes. The sediment was suspended in 4.0 ml of distilled water and then 1.0 ml of TBA reagent was added. The reaction mixture was heated at 95°C for 60 min. After cooling, 5.0 ml of n-butanol was added and the mixture was shaken vigorously and centrifuged at 3000 g for 15 min. The colour extracted in the butanol layer was read at 530 nm. Tubes containing standard malondialdehyde 1 to 5 nmoles were treated in a similar manner along with a blank containing 4.0 ml of distilled water. The result of TBARS level was expressed as n mol/ml plasma.

4.14.2.2 Estimation of hydroperoxide (Jiang et al., 1992)

Principle

Oxidation of ferrous ion (Fe²⁺) under acidic conditions in the presence of xylenol orange leads to the formation of a chromophore which has no absorbance maximum at 560nm.

Reagents

For reagent: Butylated hydroxyl toluene (BHT) – 88 mg, Xylenol orange- 7.6 mg, Ammonium iron (II) sulphate were added to 90 ml methanol and 10 ml of H₂SO₄.
Procedure

Accurately 0.9 ml of fox reagent were mixed with 0.1 ml of plasma or tissue homogenate and incubated for 30 min at room temperature. The absorbent was read in a colorimeter at 560 nm. The amount of hydroperoxide produced was calculated by using the molar extinction co-efficient of $4.6 \times 10^4$ (moles/1/cm). The results of hydroperoxide were expressed as m mol/mg protein in tissues.

4.14.2.3 Estimation of reduced glutathione (Beutler and Kelley, 1963)

Principle

This method was based on development of yellow colour when 5, 5'–dithiobis-2, nitrobenzonic acid (DTNB) was added to compound containing sulphhydryl groups.

Reagents

1. 10% TCA
2. 1% Sodium citrate
3. Ellman's reagent: 34 mg of DTNB in 10 ml of 1% sodium citrate.
4. 0.3M Disodium hydrogen phosphate (pH 8.0)
5. Standard solution: 10 mg of reduced glutathione in 100 ml distilled water.

Procedure

To 0.5 ml of plasma or erythrocytes, 1.5 ml of TCA and 1.0 ml of distilled water were added and centrifuged. 2.0 ml of supernatant along with 2.0 ml of blank containing distilled water also taken. A set of standards (20-100 μg) were taken and made upto 2.0 ml with water. To all the tubes 4.0 ml of 0.3M disodium hydrogen phosphate and 1.0 ml of DTNB reagent were added and colour developed was read at 412nm. The values were expressed as mg/dl for plasma and mg/dl for erythrocytes and mg/100 mg tissues for lever and kidney.

4.14.2.4 Estimation of α-tocopherol (Baker et al., 1980)

Principle

Tocopherol can be estimated based on the reduction of ferric to ferrous ions by tocopherol, which forms a red colour with 2,2' dipyridyl. Tocopherols and carotenes are first extracted with xylene and the extinction was read at 460nm to measure carotenes. Correlation is made for these after adding ferric chloride and reading at 520nm.

Studies on the Effect of Artificially Cultivated *Ganoderma lucidum* on Streptozotocin induced Diabetic rats.
Reagents

1. Absolute alcohol
2. Xylene
3. 2,2’ dipyridyl-1.2g in 1 litre of n-propanol
4. Ferric chloride solution-1.2g of FeCl3.6H2O in one litre of ethanol. Stored in a brown bottle
5. Standard solution of D,L-α- tocopherol acetate

Extraction of animal tissues

The tissue sample was homogenized in a warring blender. Accurately 2.5 g of the homogenized tissue was weighed into a conical flask. 50 ml of 0.1N sulphuric acid was added slowly without shaking. Stoppered and allowed to stand overnight. The next day, content of the flask was shook vigorously and filtered through Whatman No.1 filter paper, discarding the initial 10-15ml of the filtrate. Aliquotes of the filtrate were used for the estimation.

Procedure

Three stoppered centrifuge tubes were taken as test, standard and control and 1.5ml of tissue sample, 1.5 ml of the standard and 1.5 ml of water were pipette into them. Again 1.5 ml of ethanol was added to the test and blank, 1.5 ml of water to the standard. 1.5ml of xylene was added to all the above tubes, stoppered, mixed well and centrifuged.

One ml of xylene was transferred into another stopper tube, taking care that ethanol or protein no included.1ml of 2,2’-dipyridyl reagent was added to each tube, stoppered and mixed. 1.5ml of the mixture was pipette into colorimeter cuvettes and the extinction of test and standard were read against the blank at 460nm. Separately 0.33ml of ferric chloride solution was added to the blank and mixed well.After 15 minutes incubation test and standard were read against the blank at 520nm. The amount of tocopherol was calculated as mg/dl.

4.14.2.5 Estimation of Ascorbic acid (Rao and Kuether, 1943)

Principle

Ascorbate is converted to dehydroascorbate by treatment with activate charcoal or bromine. Dehydroascorbic acid then reacts with 2, 4-dinitrophenyl
hydrazine to form osazones, which dissolves in sulphuric acid to give an orange coloured solution whose absorbance can measured spectrophotometrically at 540 nm.

Reagents
1. 4% trichloracetic acid
2. 9N sulphuric acid
3. 2% 2, 4-dinitrophenyl hydrazine reagent (DNPH) –dissolved 2 g DNPH in 100 ml of 9N sulphuric acid
4. 10% thiourea solution
5. 85% sulphuric acid
6. Stock standard solution: dissolved 10mg ascorbic acid in 100 ml of 4% TCA
7. Working standard: diluted 10 ml of the stock solution to 100 ml with 4% TCA

Procedure
1 g of tissue sample was ground and homogenized in 4% TCA up to 10 ml. Centrifuged at 2000 rpm for 10 minutes. The supernatants obtained were treated with a pinch of activated charcoal. Shaken well and kept for 10 minutes. Centrifuged again to remove the charcoal residue. The volume of the clear supernatants obtained was noted.

0.5 & 1 ml aliquots of these supernatants were taken for the assay. The assay volumes were making up to 21.0 ml with 4% TCA. 0.2 ml to 1 ml of the working standard solution containing 20-100 μg of ascorbate respectively were pipetted out into clean dry test tube, the volume of which were also made up to 2.0 ml with 4% TCA. Added 0.5 ml of DNPH reagent to all the tubes, followed by 2 drops of 10% thiourea solution. Incubated at 37° C for 3 hrs. the osazones formed were dissolved in 2.5 ml of 85% sulphuric acid, in cold, drop by drop, with no appreciable rise in temperature. To the blank alone, DNPH reagent and thiourea were added after the addition of sulphuric acid. After incubation for 30 min at room temperature, the absorbance was read spectrophotometrically at 540 nm. The content of ascorbic acid in the tissue sample using the standard graph was plotted.

4.14.3. Estimation of enzyme antioxidants
Superoxide dismutase (SOD) activity was determined in the tissue homogenates according to Madesh and Balasubramanium (1998). Catalase (CAT)
activity was measured in the homogenates by the method of Maehly and Chance (1954). Lipid peroxidation in tissue homogenate was studied by the quantification of malonaldehyde (MDA) levels according to Ohkawa et al., (1979).

**Preparation of Tissue Homogenate**

Tissue samples from animals were washed with ice cold saline and dried between folds of filter paper, weighed and homogenized using appropriate buffer in an all glass homogenizer with Teflon pestle. The homogenate was centrifuged at 1000g for 5 minutes and the supernatant was then used for the biochemical estimations.

**4.14.3.1 Estimation of Catalase (Maehly and Chance 1954)**

**Principle**

Dichromate in acetic acid was reduced to chromic acetate, when heated in the presence of hydrogen peroxide with the formation of perchromic acid as an unstable intermediate. The chromic acetate formed was measured at 590 nm. Catalase was allowed to split H2SO4 for different periods of time. The reaction was stopped at different time intervals by the addition of dichromate–acetic acid mixture and the remaining H2O2 was determined by measuring chromic acetate colorimetrically after heating the reaction mixture. The activity of catalase was assayed by the method of Maehly and Chance (1954).

**Reagents:**

1. Phosphate buffer, 0.01 M, pH 7.0
2. Hydrogen peroxide 0.2 M
3. Potassium dichromate, 5%
4. Dichromate–acetic acid reagent: Potassium dichromate and glacial acetic acid were mixed in the ratio of 1:3. From this 1 ml was diluted again with 4 ml of acetic acid.
5. Standard hydrogen peroxide, 0.2 mM.

**Procedure**

To 0.9 ml phosphate buffer, 0.1 ml plasma or hemolysate or tissue homogenate and 0.4 ml H2O2 were added. The reaction was arrested after 15, 30, 45 and 60 seconds by adding 2.0 ml of dichromate–acetic acid mixture. The tubes were
kept in a boiling water bath for 10 minutes, cooled and the color developed was read at 530 nm. Standards in the concentration range of 20-100 μ volumes were processed as for the test.

The activity of catalase was expressed as U/ ml for plasma, U / mg Hb for erythrocytes and U/ mg protein for tissues (U - μ moles of H2O2 utilized / sec.).

4.14.3.2 Estimation of Superoxide dismutase (Madesh and Balasubramanium, 1998)

Principle

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

Reagents required

1. 50 mM phosphate buffer pH 7.4
2. 20 mM L-Metheonine
3. 1% (v/v) Triton X100
4. 10mM hydroxylamine hydrochloride
5. 50μM EDTA
6. 50μM riboflavin
7. Griess reagent : 1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine hydrochloride

Procedure

In a test tube, 1.4 ml from the aliquot of reaction mixture was pipette in. 100 μl of the sample was added followed by preincubation at 37°C for 5 min. 80μl of riboflavin was added and the tubes were exposed for 10 min to 200W Philips fluorescent lambs. The control tube had equal amount of buffer. The sample and its respective control were run together. Tube and the absorbance of the colour formed were 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.
4.14.3.3 Estimation of tissue TBARS (Ohkawa et al., 1979)

Reagents

1. 8.1% Sodium dodecyl sulphate
2. 20% Glacial acetic acid.
3. 8% Thiobarbituric acid
4. N-Butanol and Pyridine (5:1 v/v)
5. Standard solution: 1,1,3,3'-tetra methoxy propane-0.5 nmol/ml

Procedure

To 0.2 ml of tissue homogenate, 0.2 ml of 8.1% sodium dodecyl sulfate and 1.5 ml of 20% acetic acid were added. The pH was adjusted to 3.5 with sodium hydroxide, then 1.5 ml of 0.8% aqueous solution of TBA was added to the mixture and the volume was made up to 4ml with distilled water. The reaction mixture was heated in an oil bath at 95°C for 60 minutes. After cooling by tap water, 1 ml of distilled water and 5 ml of n-butanol pyridine mixture were added and shaken vigorously. After centrifugation at 4000rpm for 10 minutes, the organic layer was removed and absorbance was read at 535 nm. The level of TBARS in tissue was expressed as n mol/100mg protein.

4.15 Hypolipidaemic and cholesterol lowering activity of Ganoderma lucidum aqueous extract on STZ induced Diabetic rats

Blood and tissues collection

After 45 days of treatment, the animals were killed by cervical dislocation. Blood was collected in heparinized tubes, and then serum was separated by centrifugation. The liver, kidney, and cardiac tissues were quickly removed, washed in ice cold, isotonic saline and blotted individually on ash-free filter paper. The tissues were then homogenized in 0.1 M Tris–HCl buffer, pH 7.4. The homogenate was used for the estimations of lipid profile and other parameters.

4.15.1 Biochemical analysis

The extraction of serum phospholipid was estimated by Zilversmit and Davis (1950). The total cholesterol is measured by Zak’s method. The triglycerides were estimated by the method of Foster and Dunn (1973) and free fatty acid was estimated by the method of Falhot et al., 1973.
4.15.1.1 Estimation of Phospholipid (Zilversmit and Davis, 1950)

Principle

The phosphorous reacts with acid molybdate and forms phosphomolybdic acid. This acid is reduced by the addition of ANSA reagent to produce blue colour (molybdenum blue) which is probably a mixture of oxides of molybdenum. The intensity of the blue colour is directly proportional to the phosphorous content.

Reagents requirements

1. 5 N H$_2$SO$_4$
2. Con. Nitric acid
3. 2.5% Ammonium molybdate
4. ANSA: 0.2 g of ANSA was mixed 1.2 g of sodium bisulphate and 1.2 g of sodium sulphite. 0.25g was taken from this mixture and dissolved in 100 ml of water.

Procedure

An aliquot of the liquid extract was pipette out in to Kjeldahal flask and evaporated to dryness. 1 ml of 5 N H$_2$SO$_4$ was added and digested in a digestion rack till light brown. To these 2-3 drops of con. Nitric acid was added and the digestion continued till become colourless. The Kjeldahal flask was cooled. 1 ml of water added and heated in a boiling water bath for about 2 min then 1 ml of 2.5 % Ammonium molybdate and 0.1 ml of ANSA were added. The volume was then made up to 5 ml with double distilled water and absorbance was measured at 680nm with 10 min. The amount of phospholipids was expressed as mg / dl

4.15.1.2 Estimation of free fatty acids (Falhot et al., 1973)

Principle

Non esterified free fatty acid were estimated by this method using copper soap formation in the presence of phosphate buffer, the extract was shaken with a high density copper reagent (pH 8.1). The copper soap examined in the organic layer which was determined colorimetrically with diphenyl carbazide.

Reagents Required

1. Extraction solvent: Chloroform, heptanes, methanol (5:5:1)
2. Phosphate buffer (pH 6.4): 4.539 g/l of potassium dihydrogen phosphate and 5.938 g/l disodium hydrogen phosphate (2:1 v/v)
3. Stock copper solution: 50 mM copper nitrate / l
4. Triethanolamine 1 M
5. Sodium hydroxide 1 M
6. Copper reagent: 10 ml of copper solution, 10 ml of triethanolamine and 6 ml of NaOH were mixed well and diluted to 100 ml with water. To this 33 g of NaCl were mixed well and diluted to 100 ml with water. To this 33 g of NaCl was added with pH was adjusted to 8.1.
7. Diphenyl carbazole solution: 40 mg is 10 ml of ethanol with 0.1 ml of triethanolamine.
8. Palmitic acid 2 mM/l

Procedure

The lipid extract (0.1 ml) was evaporated to dryness and 1 ml phosphate buffer, 6 ml extraction solvent & 2.5 ml of copper reagent were added. The blank standard (10-60 mg) was also treated in the same manner. The tubes were shaken vigorously for 90 sec and allowed to stand for 15 min and centrifuged at 4000 g for 5 min. 3 ml of upper layer was transferred to a tube containing 0.5 ml diphenyl carbazole solution mixed carefully and read at 550 nm. The amount of free fatty acid is expressed as μeq of pyruvate liberated / dl.

4.15.1.3 Estimation of Triglycerides (Foster and Dunn, 1973)

Principle

The triglycerides contained in an isopropanol of serum are hydrolyzed to glycerol (i.e. saponification), which is then oxidized to formaldehyde. This reacts with acetyl acetone and ammonia to give a yellow compound, whose absorbance is measured. Phospholipids which would also be hydrolyzed to glycerol are removed by absorption on to an alumina mixture.

Reagents

1. Isopropanol
2. alumina
3. Saponification reagent: 5 gm of KOH is dissolved in 60 ml of distilled water and 40 ml of isopropanol is added. it can be stable for 6 months at room temperature.
4. acetyl acetone reagents: 0.75 ml of 2, 4 pentaedione (acetyl acetone) is added to 20 ml of isopropanol and mixed well. 80 ml of distilled water is added and mixed well. It is stable for 6 months at room temperature.

5. Sodium metaperiodate reagent: 19.25 gm of anhydrous ammonium acetate is dissolved in about 100 ml of distilled water. 15 ml of glacial acetic acid and 162.5 mg of sodium metaperiodate are added and dissolved and diluted to 250 ml with distilled water. It can be stable for at least 6 months at room temperature.

6. Stock standard: 1 gm of tripalmitin is dissolved in 100 ml of isopropanol (800 mg / 100 ml)

**Procedure**

To appropriately labeled 15 x 125 mm stoppered glass tubes, 0.1 ml serum is added. 4 ml of isopropanol is added and mixed well. 0.4 gm alumina (a calibrate scoop may be used) is added to all tubes and the tubes are placed on a mechanical rotator for 15 min and centrifuged, 2 ml of supernatant is transferred to properly labeled 15 x 125 mm tubes. 0.6 ml of saponification reagent is added and placed in a water bath at 65°C for 30 min. The tube is centrifuged and the OD is read against a reagent blank. For blank 0.1 ml of distilled water is used instead of serum. For standard 0.1 ml of working standard is taken and treated as the test. The amount of triglycerides expressed in mg / dl.

**4.15.1.4 Estimation of total cholesterol (Friedwald, 1972)**

**Principle**

The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4 – amino – antipyrene in the presence of phenol and peroxides.

\[
\begin{align*}
\text{Cholesterol esterases} & \\
\text{Cholesterol ester} + H_2O & \longrightarrow \text{Cholesterol} + \text{fatty acid} \\
\text{Cholesterol oxidases} & \\
\text{Cholesterol ester} + H_2O & \longrightarrow \text{Cholestene – 3 – one} + H_2O \\
\text{Peroxidases} & \\
H_2O_2 + \text{Phenol} + 4 – \text{amino – anti} & \longrightarrow \text{quinoneimine} + H_2O
\end{align*}
\]
The absorbance at 500nm wavelength is determined and total cholesterol is calculated.

Cholesterol concentration in the sample = $\frac{\Delta \text{Sample}}{\Delta \text{Standard}} \times \text{Conc. of standard}$

4.16 Antidiabetic effect of *Ganoderma lucidum* aqueous extract on STZ induced diabetic rats

Fasting blood glucose was estimated by Glucose oxidase method (Triender, 1969), haemoglobin was estimated by cyanmethaemoglobin method (Drabkin and Austin, 1932). Glycosylated haemoglobin which was estimated by the method of Sudhakar and Pattabiraman (1981) was modified by Bannon (1982). Plasma insulin level was assayed by enzyme linked immune sorbent assay (ELISA) kit (Boehringer Mannheim kit). Hexokinase, glucose-6-phosphatase and fructose-1,6-phosphatase were assayed according to the method of Brandstup *et al.*, (1957), Baginsky *et al.*, (1974) and Gancedo and Gancedo (1971) respectively. Estimation of bilirubin was by Pearlman and Lee method and the inorganic phosphate (Pi) liberated was estimated by the method of Fiske and Subbarow (1925). Estimation of reduced glutathione was by Ellman (1959) method, Protein estimation was by Lowry *et al.*, (1951) method. Montgomery’s method was employed for the estimation of glycogen, King (1965) method was applied for the estimation of SGOT and SGPT. Lowry’s method was used to estimate alkaline phosphatase and Habig *et al.*, (1974) method was applied to estimate Glutathione-S-transferase.

4.16.1 Estimation of Haemoglobin by Cyanmethaemoglobin Method (Drabkin and Austin, 1932)

Principle

The haemoglobin is treated with a reagent containing potassium ferricyanide, potassium cyanide and potassium dihydrogen phosphate. The ferricyanide forms methaemoglobin which is converted to cyanmethaemoglobin by the cyanide.

Chemicals required

- Haemoglobin reagent and haemoglobin powder
- Ferric cyanide . . . . . . . 200 mg
Materials and Methods

Potassium cyanide .... 50 mg
Distilled water ...... 1 lit

The standard was prepared from haemoglobin powder.

Procedure
1. 0.5 ml of EDTA treated blood samples and standard were taken in separate tubes.
2. 2.5 ml of Haemoglobin reagent was added and mixed well.
3. Kept at room temperature for 30 minutes.
4. The optical density was measured at 540 nm.

\[
Hb \text{ (gm / 100)} = \frac{\text{O.D. of sample}}{\text{O.D. of standard}} \times \frac{\text{Conc. Of standard} \times 10}{\text{Vol. of sample}}
\]

Test procedure
1. Bring regent and all samples to room temperature before testing.
2. Take two test tubes and make them as “Test” and “Blank”.
3. Pipette into each test tube 5 ml of hemocor – D
4. To the test tube and 0.02 ml of well mixed fresh whole blood.
5. Mix and allow the tubes to stand for at least three minutes.
6. Measure the optical density of the test against “blank” at 540 nm.
7. Calculate haemoglobin concentration of the test tube using calibration curve.

Calibration curve
Haemoglobin standard is used for calibration. The value of haemoglobin in mg / dl is given as the haemoglobin standard vial label. Pipette into three clean and dry test tubes. Hemocor – D and haemoglobin standard as follows.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Hemocor – D reagent</th>
<th>Haemoglobin standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.0 ml</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

Studies on the Effect of Artificially Cultivated *Ganoderma lucidum* on Streptozotocin induced Diabetic rats.
Measure the optical density of tube number 2 and 3 against number 1 which serves as a reagent blank. Calculate the equivalent of g / dl haemoglobin concentration on undiluted and 1.1 diluted haemoglobin standard as shows below.

The equivalent g / dl haemoglobin = haemoglobin value of undiluted standard \times \text{dilution factor}

Plot a graph of optical density against haemoglobin concentration. Alternatively if a single standard reading is taken then following formula should be used for calculation. Haemoglobin concentration (g / dl)

\[
\text{Haemoglobin concentration (g / dl)} = \frac{\text{OD of the sample} \times \text{Concentration of standard}}{\text{OD of the haemoglobin standard}} \times \text{dilution factor}
\]

\[
\frac{\text{OD of the sample}}{1000}
\]

4.16.2 Estimateion of Glycosylated haemoglobin

(Sudhakar and Pattabiraman, 1981 was modified by Bannon (1982)

Procedure

1. Lyophilized calibrators and controls should be prepared before starting the assay. After reconstitution allow for 10 min to complete dissolution of lyophilized materials.

2. Add 1.5 ml of haemolytic reagent to each haemolytic tube.

3. Add 100 μl of well mixed whole blood sample to appropriate haemolytic tube mixing the pipette tip to completely deliver the sample. Allow to stand for 15 min.

4. Label and prepare resin column for sample. Prepare the columns about 3-4 at a time, by shaking them up an down until the resin is completely resuspended. Immediately remove the column caps. Then snap off the tips. Place the columns in a vertical position in rack and allow them to drain the waste contained. Do not allow the resin is allowed to settle in the columns before removing the caps and snapping off the tips. If a resin is allowed to settle first, the column flow late will be retarded.

5. After draining the column carefully pipette out 100 μl of sample hemolysate against the side of the column wall to prepare, the resin bed, tapping the wall couple of times to draw the test hemolysate on the wetted surface.

6. Add 1.5 ml of elution buffer and allow to drain.
7. After that the transferred column tubes a fresh tube and adds 4 ml of elution buffer. Allow to drain. To this elutes A – add 4 ml of elution buffer 2 in to fresh tube and 20 μl of sample hemolysate is added into the tube. This is read at 405 nm. The serum glycosylated haemoglobin level was expressed.

**Preparation of Tissue Homogenate**

Tissue samples from animals were washed with ice saline and dried between folds of filter paper, weighted and homogenized using appropriate buffer in an all glass homogenizer with Teflon pestle. The homogenate was centrifuged at 1000g for 5 minutes and the supernatant was then used for the biochemical estimations.

**4.16.3 Estimation of inorganic phosphorus (Fiske and Subbaraow, 1925)**

**Principle**

The inorganic phosphorus present in the solution reacts with molybdic acid to produce phosphomolybdic acid. This is reduced by the addition of 1, 2, 4 – aminonapthol sulphonic acid (ANS) reagent giving a blue colour, the intensity of which is proportional to the amount of phosphorus present.

**Reagent**

1. Ammonium molybdate reagent – 0.5% ammonium molybdate in 1N H2SO4
2. ANSA reagent – 0.2% ANSA
3. 1.2% Sodium sulfite
4. 1.2% Sodium metabisulfite
5. Standard phosphate solution – 44 mg of potassium dihydrogen phosphate was dissolved in water, 1.0 mo of 10 N H2SO4 was added and made up to 100 ml in a standard flask. 1 ml of this solution contains 100 μg phosphate.

**Procedure**

Standard solution was pipetted out in concentrations ranging from 10-50μg and 1ml aliquot from Na+–K+ ATPase assay and CA2+ ATPase assay into a series of test tubes and made up to 1 ml with water. These tubes were added with 250 μl of ammonium molybdate reagent and 50 μl of ANSA reagent mixed well and incubated at 37°C for 30 min. Colour developed was read at 660nm against blank containing only reagents. From the standard graph, amount of inorganic phosphorous present in aliquot was calculated and expressed in micrograms.
4.16.4 Determination of Hexokinase (Brandstrup et al., 1957)

The hexokinase assay is based on the reduction of NAD+ through a coupled reaction with glucose-6-phosphatase dehydrogenase. The excised liver tissue homogenate was prepared in saline. To 0.1 ml of homogenate were added 2.28 ml of Tris (200mmol l-1)-MgCl2 buffer (20 mol l-1), pH 8, 0.5 ml of 0.67 M glucose, 0.1 ml of 16 mM ATP, 0.1 ml of 6.8 mM NAD and 0.01 ml of 300 U ml-1 glucose-6-phosphate dehydrogenase. The solution was mixed thoroughly, and absorbance was measured at 340 nm.

4.16.5 Determination of Glucose-6-phosphatase activity (Baginsky et al., 1974)

Principle

The enzyme glucose-6-phosphatase acts on the substrate glucose-6-phosphatase and release the inorganic phosphorous. The phosphorous content is estimated by Fiske and Subbarao method (1925). The phosphorous reacts with acid molybdate and forms phosphomolybdic acid. This acid reduced by the addition of ANSA reagent to produce blue colour (molybdenum blue) which is probably a mixture of oxides of molybdenum. The intensity of the blue colour is directly proportional to the phosphorous content.

Extraction of enzymes

The known weight of the tissue was homogenized in 0.1 M Tris HCl buffer, pH 7.4 at 4°C in a homogenizer (6000 rpm/3 min). Homogenize was centrifuged at 3000g for 10 min at 4°C. The soup was used for the enzyme assay.

Reagents

1. Ammonium molybdate solution: Dissolve 25 g of ammonium molybdate in 200 ml of distilled water in 1 litre volumetric flask. Place 500 ml of 10N sulfuric acid. Add the molybdate solution and dilute to 1 litre.
2. 1,2,4-aminonaphthol sulfonic acid (ANSA): Place 195 ml of 15% sodium bisulfate in a glass stoppered cylinder. Add 0.5 g ANSA and 5 ml of 20% sodium sulfite (if the powder is not completely dissolved add more sulfite).
3. Standard phosphate solution: 35.1 ml of mono potassium dihydrogen phosphate was dissolved in water. Add 10 ml of 10N sulfuric acid. Dilute 100 ml with water.

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4. Working standard: 10 ml of standard solution was diluted with 100 ml of distilled water. 1 ml of this contains millimoles.
5. 0.1 M Tris HCl buffer (pH 7.4)
6. 0.01M glucose-6-phosphate in distilled water
7. 0.1 M citrate buffer (pH 6.5)

**Procedure**

Two test tubes labeled test and control were taken. To that 0.3 ml of buffer, 0.5 ml of substrate were added. To the test alone 0.2 ml of enzyme extract was added. The two tubes were incubated at 37°C for 60 min. After the incubation period 1 ml of 10% TCA was added to both the tubes. 0.2 ml of the extract was added to the control after TCA and the tubes were centrifuged and the phosphorous content was estimated along with the standards.

A set of standards in the range of 1-5 ml was taken and 1 ml of ammonium molybdate, 0.4 ml of ANSA was added. The volume of those tubes were made up to 10 ml with distilled water and allowed to stand at room temperature for 10 min. A blank was prepared using 1 ml of ammonium molybdate, 0.4 ml of amino naphthol and 8.6 ml of water was added. The colour developed was read at 640 nm spectrophotometrically. A standard graph was drawn by plotting the OD against the concentration in μmoles. The amount of phosphorous liberated is directly proportional to the enzyme activity.

**4.16.6 Determination of Fructose-1,6-bis phosphate (Gancedo and Gancedo, 1971)**

Fructose 1,6-bisphosphatase activity was measured by Gancedo and Gancedo (1971). The assay mixture in a final volume of 2 ml contained 1.2 ml of Tris-HCl buffer (0.1 M, pH 7.0), 0.1 ml of substrate (0.05 M), 0.25 ml of magnesium chloride (0.1 M), 0.1 ml of potassium chloride (0.1 M), 0.25 ml of EDTA (0.001 M) and 0.1 ml of liver homogenate. The incubation was carried out at 37°C for 15 min. The reaction was terminated by adding 1 ml of 10% TCA. The suspension was centrifuged and the supernatant was used for phosphorus estimation by the method of Fiske and Subbarrow (1925) as described previously.
4.16.7 Estimation of bilirubin (Pearlman and Lee method)

Principle

Vanden Bergh developed a colorimetric method for the estimation of serum bilirubin. The underlying principle is the conversion of bilirubin to the purple colored Azobilirubin. When coupled with Diazotized Sulphanilic acid. The water soluble bilirubin glucurnides (conjugated bilirubin) react fast (within 1 min) with the Diazo reagent (direct reaction) on the other hand the free billirubin (Unconjuaged bilirubin) which is present in serum complexed with albumin reacts very slowly and requires on accelerator or solubilizer such as methanol (Indirect reaction). It was found that when a reaction was carried out in Methanolic (50%) solution, both types of bilirubin react fast with Dizotized reagent. Indirect reaction gives total bilirubin value, i.e, conjugated and Unconjugated bilirubin and direct reaction give conjugated(Directed) bilirubin.

Reagents

Diazo reagent

(i) Diazo Reagent – Make freshly before use by mixing 10 ml solution A and 0.3ml of solution B.

Solution A: 1 gm of Sulphanilic acid and 1.5 ml of con.Hcl/litre in water. This solution can be kept indefinitely at room temp.

Solution B: 0.5 gm Sodium Nitrite/100 ml in water. This solution should be kept in Regrigerator.

(ii) Diazo Blank: 1.5 ml of conc. Hcl/ liter in water.

(iii) Bilirubin Standard (10 mg per 100 ml). weigh 10 mg into 100 ml volumetric flask. Working away from bright light, dissolve the bilirubin in a minimum (about 5 ml) of 0.1N sodium Carbonate solution as quickly as possible, since it is unstable in alkaline solution. Make volume with methanol or with Human citrated Plasma obtained from blank Bank from outdated bottles. It must not be hemolysed or lipaemic. It should be stored at 4°C and not frozen. Plasma is left in sunlight for some hours before used to destroy bilirubin present. This is to be kept frozen in small aliquots.
Determination of direct bilirubin
Label the test tubes. Allow the test tube to stand for 30 min and read the OD using a green filter (540nm)
Calculation
Direct bilirubin = \(\frac{OD \ of \ Test - OD \ of \ Blank \times \ amount \ of \ standard}{OD \ of \ Standard - OD \ of \ Blank \times Volume \ of \ Serum}\) x 100
Total Bilirubin = \(\frac{OD \ of \ Test - OD \ of \ Blank \times amount \ of \ standard}{OD \ of \ Standard - OD \ of \ Blank \times Volume \ of \ Serum}\) x 100

4.16.8 Estimation of reduced glutathione (Ellman, 1959)
Principle
The reduced glutathione level was determined by the method of Ellman, 1959). This method was based on development of yellow colour when 5, 5'-dithio-bis-2-nitrobenzoic acid (DTNB) is added to compound containing sulphhydryl groups. The colour developed was read at 412nm.
Reagents
1. 0.3 M diasodium hydrogen phosphate.
2. 0.1% disodium salt of EDTA
3. Precipitating reagent: 1.67 g of metaphosphoric acid, 0.2 g EDTA disodium salt, 30 g sodium chloride in one litre of distilled water.
4. 5,5' dithio (bis) -2-nitrobenzoic acid (DTNB) reagent: 40 mg of DTNB in 100 ml of 1% sodium citrate.
5. Standard solution: 10 mg of reduced glutathione in 100 ml distilled water.
Procedure
0.2 ml of sample (erythrocytes or tissue homogenate) was mixed with 1.8 ml of EDTA solution. To this 3.0 ml of precipitating reagent was added, mixed thoroughly and kept for 5 minutes before centrifugation. To 2.0 ml of the filtrate, 4 ml of 0.3 M disodium hydrogen phosphate solution and 1 ml of DTNB reagent were added and the colour developed was read at 412 nm. A set of standard solution containing 20-100µg of reduced glutathione was treated similarly. The values were expressed as mg/dl for erythrocytes and mg/100mg tissues for tissues.

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4.16.9 Estimation of Protein (Lowry et al., 1951)

Principle

The peptide bonds (CO-NH) in polypeptide chain react with copper sulphate in the alkaline medium to give a blue coloured complex. In addition, tyrosine and tryptophan residues of protein cause reduction of the phosphomolybdate components of the Folin-ciocalteau reagent to give bluish products, which contributes towards enhancing the sensitivity of this method.

Reagents

1. Alkaline copper sulphate
   Solution A: 2% sodium carbonate (Na2CO3) in 0.1N sodium hydroxide (NaOH)
   Solution B: 0.5% copper sulphate in water
   Solution C: 1% sodium potassium tartarate in water.
   50 ml of solution A was mixed with 0.5 ml of solution B and 1.0 ml of solution C just before use.

2. Folin — ciocalteau reagent: the reagent was diluted in the ratio of 1:2 with double distilled water just before use.

3. Standard bovine serum albumin (BSA): 100mg of BSA was dissolved in 100 ml dissolved in 100 ml of water in a standard flask. A small quantity of alkali was added to make complete dissolution of BSA. 10 ml of the stock was diluted to 100 ml to get a working standard containing 100 g.

Procedure

To 0.05 ml of the sample 0.95 ml of 10% TCA was added to precipitate the proteins and centrifuged. The precipitate was added with 1 ml of 0.1 N sodium hydroxide. 4.5 ml of the alkaline copper reagent was added to 0.1 ml of the above aliquot and left at room temperature for 10 min. 0.5 ml of diluted foil phenol reagents was added to that. The blue colour developed was read at 640 nm after 20 min. standards in the range of 20-100 µg were treated similarly together with a blank containing only the reagent.
4.16.10 Estimation of glycogen (Montomery’s method)

Principle

Glycogen is released from the tissue by digesting it with strong alkali and precipitate in ethanol, sodium sulphite is added as a co precipitate to give a quantitative yield of glycogen. The polysaccharides are then hydrolyzed in acid and glucose released is estimated.

Isolation

1 ml of liver extract is taken in a test tube with 2 ml of 30% KOH. The sample is digested on boiling water bath for 20 min with occasional shaking. Then the tube cooled in ice. About 0.2 ml of saturated sodium sulphite is added and mixed thoroughly. The glycogen is precipitated by adding 5 ml of ethanol and then centrifuged at 3000 rpm for 5 min. Then the end of 2 hours 1 drop of phenol red and neutralize slowly with drops of 0.1M NaOH. It turns from pink to orange finally to yellow. The mixture is made up to 10 ml. the glucose release is estimate by anthrone method.

Estimation

About 4 ml of anthrone reagent is added to 1ml of sample mixture. the mixture is kept in ice for 15 min. the optical density is read at 650 nm.

Standard glucose:

Working standard is prepared by dissolving 10 mg of glucose is 100 ml of distilled water. Series of glucose concentration ranging from 10-15 mg is taken in different tubes. The final volume in each tube is made up to 1 ml with distilled water. Amount of anthrone reagent is added to each tube the tubes are incubated in ice over for 15 minutes. The blank is prepared with 1 ml of water and 4 ml of anthrone. The colour develop is read at 650nm.

4.16.11 Estimation of SGOT activity (King, 1965)

Principle

The enzyme SGOT (serum glutamate oxaloacetate Transaminase or Aspartate Transaminase) catalyses the reaction. Under controlled conditions the oxaloacetate is converted to pyruvate and pyruvate is made to react with DNPH. The hydrazone fromed is coupled to NaOH to give brown colour and the OD is read using green filter.

Aspartate +α -ketoglutarate → glutamate + oxaloacetate
Reagent

1. SGOT buffered substrate: dissolve 13.3 g of Aspartic acid in 90 ml of 1 N NaOH, adjust the pH to 7.4 and make up the volume to 500 ml with phosphate buffer.

2. DNPH Reagent: 99 Gram of DNPH in 50 ml conc. HCl and make up to 500 ml with water.

3. 3. 0.4 N NaOH

4. Stok pyruvate standard: 220 mg of sodium pyruvate is dissolved in 100 ml of phosphate buffer.

5. Working standard: dilute 1 ml of stock to 100 ml with phosphate buffer.

Procedure

Two tubes named test and blank are taken and to each tube 0.5 ml of SGOT buffered substrate is added and incubated at room temperature for few minutes and to the test tube 0.1 ml of serum is added and incubated at 37 C. add 0.5 l of DNPH, mix well and incubate at room temp for 20 min, then add 5 ml of 0.4 N NaOH, wait for 10 minute. Then the OD is read using a Green filter.

4.16.12 Estimation of SGPT activity (King, 1965)

Principle

The enzyme SGPT (serum glutamate pyruvate transaminase or alanine transaminase) catalyses the reaction. Under controlled conditions the Pyruvic acid is made to react with DNPH. The hydrazone formed is coupled to NaOH to give Brown colour. The OD is taken using Green filter.

Alanine + - ketoglutarate ---- glutamate + pyruvate

Reagents

(i) Phosphate buffer (ph 7.4); 11.3 g of dry Anhydrous Na2HPO4 and 2.7 g of dry anhydrous Potassium Dihydrogen Phosphate (KH2PO4) are taken and overall volume is made up to litre by using water.

(ii) SGPT buffered substrate: dissolve 9gms of Alanine in 90 ml of water with addition of about 2.5 ml of 1N NaOH to adjust the ph 7.4. Add 0.146 gm of - keto glutaric acid dissolve it by adding few drops of NaOH and adjust to pH 7.4. make up the volume to 500 ml with phosphate buffer.
(iii) DNPH reagent; 99 mg of DNPH in 50 ml of HCl and make up the volume with 500 ml of water.
(iv) 0.4 N NaOH
(v) Stock pyruvic acid Standard: 200 mg of sodium pyruvate is dissolved in 100 ml of phosphate buffer.
(vi) Working standard: dilute 1 ml of the stock to 10 ml of phosphate buffer.

Procedure

Two test tubes named test and blank are taken to both tubes. 0.5 ml of SGPT substrate is taken both the test tubes are incubated at room temp for few min. now to the tube 0.5 ml of serum is added hen the tubes are incubated ofr 30 min. at 37 C. add 0.5 ml of DNPH, mix well and incubated at room temp for 20 min. then add 5 ml of 0.4N NaOH, wait for 10 min. the OD is read using a Green filter.

4.16.13 Estimation of serum alkaline phosphatase (Lowry’s method)

Principle

Phosphatases are enzymes which catalyze the splitting of phosphoric acid from certain mono phosphoric esters, a reaction of considerable importance with several body processes. In this method Disodium phenyl phosphate is hydrolyzed with the liberation of phenol and formation of sodium phosphate. The amount of phenol so formed is estimated colorimetrically at 650 nm.

Reagents

(i) Disodium phenyl phosphate – (0.01 m). dissolve 1.09 gmsof Disodium phenyl phosphate in water and make up to 500 ml bring quickly to he boil cool and added little chloroform is added and keep it in the refrigerator.
(ii) Sodium carbonate: NaHCO3 buffer (0.1 M) – dissolve 3.18 gm of anhydrous Na2CO3 and 1.68 gms of NaHCO3 is water and make up to 500 ml.
(iii) Buffered buffered for use:- Prepare by mixing equal volume of solution (1) and (2) this has pH of 10
(iv) Tris chloro acetic acid (TCA) 20%(w/v)
(v) Acid molybdate Reagent: Dissolve 5g of Ammonium molybdate in 5N H2SO4 (add 14ml of conc. H2SO4 slowly to 86 ml of water to prepare acid)

(vi) 1,2,4, Amino napthal sulphanilic acid (ANSA): 0.25% of ANSA is prepared by adding 0.5 gm of the dry powder ANSA to 195 ml of 15 % NaHSO4 and 5 ml of the 20% Na2SO3 stopper and shake well until it is dissolved.

(vii) Stock Solution: Dissolve 2.194gms of pure KH2PO4 in water and make up to 500 ml and add few drops of chloroform.

(viii) Working standard: Dilute 2 ml of stock standard to 500 ml (5ml is equal to 0.02mg)

Procedure

Six ml of buffered substrate was measured into a test tube and the test tube was placed in a water bath at 37°C for few minutes, 0.3 ml of serum was added, mixed well and incubated for 15 min. after incubation 1.2 ml of 20% TCA was added, shook well, and filtered. At the same time control and blank were set. 0.3 ml of water and 6 ml of buffered substrate was added to the blank.0.3 ml of serum and 6 ml of distilled water was added to the control. 1.2 ml of 20% TCA was added to both blank and control, mixed well and filtered. 5 ml of filtrate from the test, blank and control were taken and 0.8 ml of acid molybdate was added followed by 0.2 ml of ANSA. It was mixed well and allowed to stand for 10 minutes and read at 680 nm.

4.16.14 Estimation of glutathione – S-transferase (Habig et al., 1974)

Principle

GST activity was measured by increase in absorbance at 340 nm using 1-chloro 2,4 dinitro benzene (CDNB) as the substrate.

Reagents

phosphate buffer 0.3M, pH 6.5
GSH 30 mM
30 Mm CDNB in 95% ethanol.
Materials and Methods

Procedure

In a test tube 1 ml of phosphate buffer, 0.1 ml of CDNB and 0.1 ml of tissue homogenate were taken the volume was adjusted to 2.9 ml with water. The reaction mixture was preincubated at 37°C for five minutes and the reaction was started by the addition of 0.1 ml of 30 mM glutathione. The absorbance was followed for 5 minutes at 540 nm. A system devoid of enzyme served as the blank. The specific activity of GST was expressed as μmoles of CDNB – GSH conjugate formed/min/mg protein.

4.17 Statistical analysis

Values were expressed as mean ± SEM. Unpaired student t- test was used for statistical comparison. Incase of Invivo studies comparison were made between normal and diabetic, diabetic verses diabetic treated animals. Changes were considered significant when the p-value was less than 0.05.

Two – way analysis of variance (ANOVA) with interaction effects was employed for analyzing the initial dose response data , and one – way analysis of variance was employed for analyzing the antioxidant status and general parameter. Inter group comparisons were done using Ducan’s Multiple Range Test (DMRT) with 95% confidence intervals. The SPSS package was used for analysis.

4.18 Histopathology

4.18.1 Assessment of histopathological response of extract in STZ diabetic rats

The diabetic rats were divided into 5 groups of 6 animals each. Group I received vehicle alone and served as control. Group II received STZ (60 mg / kg / i.p) dissolved in 0.1 M – citrate buffer. Group III & Group IV received the aqueous extract of fruit of Trichosanthes dioica (25mg, 50 ml / kg / p.o) suspended in vehicle followed by single intra-peritonial administration of STZ. Group V received tolbutamide (250 mg / kg / p.o), suspended in vehicle followed by single intra – peritoneal administration of STZ.

4.18.2 Tissues collection

After 45 days of treatment, the animals were killed by cervical dislocation. The pancreas, skeletal muscle, liver, and kidney tissues were quickly removed, washed in ice cold, isotonic saline and blotted individually on ash – free filter paper and organ weights were measured (Plate 4). Organ slices fixed for 48 hr in 10%
formosaline were processed for paraffin embedding following the standard micro technique sections (5 mm) of livers stained with Haemotoxyline and eosin were evaluated for histopathological changes under a light microscope. Histopathological findings were graded for degree of liver cell damage.

4.18.3 Micro Techniques

Micro technique is a technique, which provides disciplined, channelized laboratory process for the preparation of the material (slide) from the tissues for study under the microscope. It involves the use of some methods borrowed from other sciences. The science of micro technique may be called a social art practiced with other laboratory workers and always in cooperation with other laboratory technicians everywhere. The essential features of micro techniques are collection and preparation of material, fixation, dehydration and clearing of material, embedding of material in wax and block making, microtomy, staining and mounting the sections mounted on a slide.

Fixation

The term “fixation” means to immobilize. Fixation is a process in which the cell is fixed in such a meaner so as to preserve them in the shape they had during life and hardened in older to render them of consistency suitable to subsequent manipulations. Fixing and hardening agents are usually combined into one solution known as fixative. Fixative normally used ar Clarke’s fixative, Bovin’s fixative and 10% neutral formalin.

(a) Clarke’s Fixative

It is reduced, caused great shrinkage and hardening. It is mixture of ethanol and acetic acid in the ratio 3:1. Normally alcohol hardens the tissues and acid softens the tissues. Fixative form bonding with macromolecule and brings about denaturing of proteins. This fixative doesn’t interfere with subsequent digestion of pepsin; it requires no special washing out and gives a very characteristic appearance.

(b) Bovin’s Fixative

It is composed of the following chemicals saturated aqueous solution of picric acid 75 ml, 40% formaldehyde – 25 ml, Glacial acetic acid – 5 ml. The advantage of this is that the object can be left in it without becoming hard. Its demerits are that,
picric acid forms water-soluble compounds with many substances as a result. Section show large vacuoles. Even small traces of Picric acid interfere with staining. Picric acid precipitates all proteins as protein picrates.

(c) Neutral Formalin

It consists of 10% formalin with calcium carbonate, manganese carbonate or lithium carbonate to neutralize formalin to pH – 7.0. This is usually done for protein staining. It is reduced and hardened greatly. The composition of Buffer Sodium phosphate is monobasic – 100 mg, dibasic – 650 mg Distilled water – 90 ml.

Dehydration

After fixation the tissues are washed thoroughly over night with tap water and then dehydrated. Dehydration is a stepwise process to remove water from the tissue. In this process – graded dehydration 30%, 50%, 70% 90% and absolute alcohol are used as grades. These grades are prepared accordingly and stored in large bottles, which constitute the stock. Care should be taken to see that the tissues were carried out for 5-10 min.

Clearing

After absolute alcohol treatment cleaning of the tissue is done using xylene. This is a good clearing agent, which brings about quick removal of alcohol from the tissues, and speeds up the infiltration of paraffin into them. Dehydrated tissues were first treated with alcohol. Xylene (1:1 ratio) and then with 2 changes of xylene.

Cold Infiltration

In this step powdered paraffin is added to xylene containing the processed tissue and each tissue is allowed to dissolve, and made to saturate by mixing it well.

Hot Infiltration

For hot infiltration the paraffin is made to melt in a wax-embedding chamber maintained at 60°C. Three chambers named 1, 2 and 3 are used to give these changes of the tissue in molten wax. Precaution was taken not to char the tissue. Hot infiltration at each charge of components are present which may be distinguished by their ability to retain dyes of contrasting colors when stained in 2 or 3 different types of stains during differential staining. When specifically desired to study the cells at

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cytochemical or histochemical levels. The common dyes differentiate the nucleus and the cytoplasm.

**Embedding**

Klebs introduced the method of embedding or re-enforcement to the fixed tissue in 1869 using wax having a melting point of 58°C - 60°C. This has several advantages like the process is quick, easy, thin sections are taken and they can be kept permanently.

**Sectioning**

The blocks are trimmed using a blade or scalpel, fixed to the block holder and sections are taken at 8-10 microns using a rotary microtome.

**Spreading**

The slides, on which the ribbons are to be mounted, are first smeared with any adhesive such as Meyer’s albumin. After that small pieces of ribbon are placed on a slide and flood with water by a dropper.

**Staining**

Staining is a process by which the objects are impregnated with a color stain, which renders them visible. In many tissues of assortment is kept for relatively less period in alcohol as this hardens the tissue.

(a) **Natural Stains**

These are the dyes, which obtained from natural source like plants and animals.

e.g. Hematoxylin.

(b) **Synthetic Stains**

These are usually the artificial chemical compounds of an acid and a base. It may be acid stains, basic and neutral stains. Any one of the above mentioned stain is added to the tissue until the correct depth of colour is reached the correct intensity of color is taken in the tissue, and then the sections are thoroughly washed in running water.

**Counting**

A transparent mounting medium (DPX) is applied that does not alter the colour of the stain the sections are covered with cover slip without any air bubble. Press the cover slip gently in place and allow it to dry.
4.18.4 Histology

Tissues fixed in Bovin’s are embedded in paraffin, sectioned, spread over clean slides, deparaffinized and dehydrated were used for histological staining and observation. Haematoxylin and eosin are dyes commonly used in histological study. Haematoxylin stains nuclei, some cytoplasmic components like ergastoplasm and the matrix of cartilage. Eosin stains the cytoplasm, cytoplasmic material and extracellular fibers. Eosin generally used as potassium Eosinate, in which the base is colorless. Below isoelectrical point proteins will react with acid dyes like Eosin.

Preparation of Stains

Haematoxylin (Ehrlic reagent)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>30 ml</td>
</tr>
<tr>
<td>95% alcohol</td>
<td>3 ml</td>
</tr>
<tr>
<td>Glycerin</td>
<td>30 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>3 ml</td>
</tr>
<tr>
<td>Haematoxylin</td>
<td>0.7 Grams</td>
</tr>
</tbody>
</table>

Ammonium alum is excessively used as a mordant. Make up the stain by dissolving haematoxylin in the acid and alcohol then dissolve 1 gm of ammonium alum in the water and add this together with glycerin, shake well and add about 10 gm of ammonium alum to the bottle. Allow remaining for months, making sure that there are always a few crustals of ammonium alum in the bottom of the bottle. This stain can be kept for 10 years and works best after it reaches a year old.

EOSIN 0.5 %

Method

Bring sections to water is Ehrlich acid haematoxylin for 2-5 min wash in tap water. Destain in Picric acid for one to 5 min wash in running tap water to differentiate nucleus from cytoplasm. Nucleus should be blue in colour and cytoplasm colourless. If over stained (red colour) give a acid wash (Drop of Conc. HCl in distilled water) counter stain in Eosin, wash in distilled water, dehydrate clear in xylene and mount in DPX. The nucleus stained dark purple with haematoxylin and the cytoplasm stained red with Eosin.
## Materials and Methods

### Procedure for staining

<table>
<thead>
<tr>
<th>Procedure</th>
<th>time taken (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Deparaffinization</strong></td>
<td></td>
</tr>
<tr>
<td>Xylene - I</td>
<td>-10</td>
</tr>
<tr>
<td>Xylene - II</td>
<td>-10</td>
</tr>
<tr>
<td>Xylene: Alcohol</td>
<td>-5</td>
</tr>
<tr>
<td><strong>Down Grade Series (Hydration)</strong></td>
<td></td>
</tr>
<tr>
<td>Alcohol 100%</td>
<td>-3.5 min</td>
</tr>
<tr>
<td>Alcohol 90%</td>
<td>-3.5 min</td>
</tr>
<tr>
<td>Alcohol 70%</td>
<td>-3.5 min</td>
</tr>
<tr>
<td>Alcohol 50%</td>
<td>-3.5 min</td>
</tr>
<tr>
<td>Alcohol 30%</td>
<td>-3.5 min</td>
</tr>
<tr>
<td><strong>Staining</strong></td>
<td></td>
</tr>
<tr>
<td>Haematoxylin</td>
<td>-5-15 min</td>
</tr>
<tr>
<td>Running water</td>
<td>-5 min</td>
</tr>
<tr>
<td>Acid – alcohol dip</td>
<td>-5 min</td>
</tr>
<tr>
<td>Picric acid</td>
<td>-5 min</td>
</tr>
<tr>
<td>Distilled water</td>
<td>-5 min</td>
</tr>
<tr>
<td><strong>Counter Staining</strong></td>
<td></td>
</tr>
<tr>
<td>Eosin</td>
<td>-3 min</td>
</tr>
<tr>
<td><strong>Up Grade Series (dehydration)</strong></td>
<td></td>
</tr>
<tr>
<td>Alcohol 70%</td>
<td>-3 min</td>
</tr>
<tr>
<td>Alcohol 90%</td>
<td>-3 min</td>
</tr>
<tr>
<td>Alcohol 100%</td>
<td>-3 min</td>
</tr>
<tr>
<td>Alcohol: Xylene</td>
<td>-3 min</td>
</tr>
<tr>
<td>Xylene I</td>
<td>-3 min</td>
</tr>
<tr>
<td>Xylene II</td>
<td>-3 min</td>
</tr>
</tbody>
</table>

**Mounting**

The slide is mounted with DPX mounting (Diphthalate stere xylene)

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Studies on the Effect of Artificially Cultivated *Ganoderma lucidum* on Streptozotocin induced Diabetic rats.