Materials & Methods
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Plant Material

The present investigation was carried out in plant *Wattakaka volubilis* (L) that were collected from Jamal Mohamed College garden, Tiruchirappalli, district in Tamil Nadu (Plate – 1).

Plant parts used in traditional medicine:

Leaves, roots and whole herb

Systematic Position

Kingdom : Plantae
Division : Angiospermae
Class : Dicotyledons
Sub Class : Gamopetalae
Series : Bicarpellatae
Order : Gentinales
Family : Asclepiadaceae
Genus : *Wattakaka*
Species : *volubilis*
Plate 1
Morphology of *Wattakaka volubilis*

a. Habit; b. Stem; c. Inflorescence; d. Flower; e. Fruit
Methods

Tissue Culture Techniques

The culture technique involves five steps. They are

1. Sterilization
2. Preparation of media
3. Inoculation
4. Incubation
5. Hardening.

1. Sterilization

Sterilization of glass wares

The glasswares used in tissue culture tubes, conical flasks, beakers, pipettes, standard flasks, autoclavable screw, cub bottles, petridishes and measuring cylinders are sterilized. Microorganisms like bacteria and fungi can get into the culture medium through any of these sources and therefore they need to be sterilized. The sterilization of the glass wares involves the following procedure. The glasswares were first soaked in 50% sulphuric acid for four hours and then washed well under a jet of tap water. They were again soaked in detergent and again washed under the jet of running tap water and dried in oven at (120°C). They were thus autoclaved at 15 pSi pressure and (120°C) for 20 minutes.

Surface Sterilization of the explants

Explants from the plants were collected from the field itself without removing the plant from habitat. The leaves, nodes, internodes, and shoot apex were used as explants. They also contain sources of contamination. The explants
were first washed with running tap water for half an hour to remove the soil particles and other extraneous fine particles. Explants were surface sterilized with Bavistin 5% for 15 minutes to remove the fungal contamination. They were rinsed in distilled water twice or thrice and were then taken to the laminar air flow chamber where they were surface sterilized with 0.1% HgCl₂ for 4 minutes. They were again washed twice or thrice using sterile distilled water. Explants were obtained from the field plants, transferred immediately to the lab, surface sterilized immediate as described above and inoculated for various studies. Importance was given to complete the work in the minimum possible time from the collection to the inoculation.

2. Preparation of MS medium:

MS medium was used for the present work. The chemicals used for the experiment, the macro and micro nutrients were obtained from Hi-media Laboratories, Mumbai. The vitamins, amino acids and hormones were obtained from Sigma Company, USA. The best quality agar (Bacteriological grade) was purchased from Glaxco Laboratories, Mumbai. Preparation of MS medium involves three steps as below:
Preparation of Stock Solution

Table 1. Constituents of MS Medium (Murashige and Skoog, 1962)

<table>
<thead>
<tr>
<th>Macro nutrients</th>
<th>DH$_2$O</th>
<th>For 800 ml</th>
<th>For 400 ml</th>
<th>Per lit media</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH$_4$NO$_3$</td>
<td>33.0 g</td>
<td>16.5 g</td>
<td>40 ml</td>
<td></td>
</tr>
<tr>
<td>KNO$_3$</td>
<td>38.0 g</td>
<td>19.0 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>3.4 g</td>
<td>1.7 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>7.4 g</td>
<td>3.7 g</td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>CaCl$_2$</th>
<th>DH$_2$O</th>
<th>For 200 ml</th>
<th>For 100 ml</th>
<th>Per lit media</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>8.8 g</td>
<td>4.4 g</td>
<td>10 ml</td>
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<table>
<thead>
<tr>
<th>Micro Nutrients</th>
<th>DH$_2$O</th>
<th>For 200 ml</th>
<th>For 100 ml</th>
<th>Per lit media</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_3$BO$_3$</td>
<td>1240 mg</td>
<td>620 mg</td>
<td>1 ml</td>
<td></td>
</tr>
<tr>
<td>Na$_2$.MoO$_4$.2H$_2$O</td>
<td>50 mg</td>
<td>25 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COCl$_2$.6H$_2$O</td>
<td>5 mg</td>
<td>2.5 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>5 mg</td>
<td>2.5 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>1720 mg</td>
<td>860 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MnSO$_4$.4H$_2$O</td>
<td>4460 mg</td>
<td>2230 mg</td>
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<td></td>
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</table>

<table>
<thead>
<tr>
<th>KI</th>
<th>DH$_2$O</th>
<th>For 200 ml</th>
<th>For 100 ml</th>
<th>Per lit media</th>
</tr>
</thead>
<tbody>
<tr>
<td>KI</td>
<td>166 mg</td>
<td>83 mg</td>
<td>1 ml</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Glycine</th>
<th>DH$_2$O</th>
<th>For 20 ml</th>
<th>For 10 ml</th>
<th>Per lit media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>40 mg</td>
<td>20 mg</td>
<td>1 ml</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3 Vitamins</th>
<th>DH$_2$O</th>
<th>For 100 ml</th>
<th>For 50 ml</th>
<th>Per lit media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine HCl</td>
<td>10 mg</td>
<td>5 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>50 mg</td>
<td>25 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>50 mg</td>
<td>25 mg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Iron Source</th>
<th>FeSO$_4$.7H$_2$O</th>
<th>–</th>
<th>586</th>
<th>200</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na$_2$.EDTA.2H$_2$O</td>
<td>–</td>
<td>746</td>
<td>200</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vitamins and Amino Acids</th>
<th>Glycine</th>
<th>0.10</th>
<th>–</th>
<th>50</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nicotinic Acid</td>
<td>0.50</td>
<td>–</td>
<td>50</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Pyridoxine - HCl</td>
<td>0.50</td>
<td>–</td>
<td>50</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Thiamine - HCl</td>
<td>0.01</td>
<td>–</td>
<td>50</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Myoinositol</td>
<td>–</td>
<td>100</td>
<td>–</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Preparation of Growth Hormones

Table-2: Growth Hormones Preparation

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Hormones</th>
<th>Dissolving Agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2,4-Dichlorophenoxyacetic acid (2,4-D)</td>
<td>50% Ethyl alcohol</td>
</tr>
<tr>
<td>2.</td>
<td>6-Benzyl aminopurine (6-BAP)</td>
<td>1N NaOH</td>
</tr>
<tr>
<td>3.</td>
<td>Indole-3 Butyric Acid (IBA)</td>
<td>1N NaOH</td>
</tr>
<tr>
<td>4.</td>
<td>Naphthalene Acetic Acid (NAA)</td>
<td>1N NaOH</td>
</tr>
<tr>
<td>5.</td>
<td>Indole Acetic Acid (IAA)</td>
<td>1N NaOH</td>
</tr>
</tbody>
</table>

10 mg of the above hormones were dissolved in 10 ml of their respective dissolving agent. Now, 1 ml of the solution contains 1 mg of that hormone. Hormone stocks were added to the medium during preparation according to the following formula:

\[
\text{Hormone stock solution to be added in ml} = \frac{\text{ml of the medium to be prepared} \times \text{Required hormone concentration}}{100}
\]

Preparation of MS medium

- Stock solutions of macro nutrients, micro nutrients and supplements were prepared as per the Table-1.
- About 500 ml of double distilled water was taken in the one litre standard flask and various constituents were added as shown in Table-1. Iron was added after chelating with Na₂ EDTA.
- Sucrose was added to a final concentration of 3% (30 gm/l).
- The solution was made up to one litre using double distilled water.
The made up solution was divided into necessary aliquots according to the hormone proportions planned.

Each aliquot was added with appropriate amount of hormones. The pH was adjusted at 5.6 to 5.8 using 0.1 N NaOH or 0.1 N HCl.

The medium was solidified with 0.8% bacteriological grade agar.

The melted media was distributed to the culture tubes at the rate of 10 ml/tube, plugged with cotton and bundled as 10 cultures tubes per bundle (each bundle is considered to be of a particular concentration of combination).

The culture tubes with media were autoclaved for 15 minutes at 120° C and 15 pSi pressure.

The sterilized media were cooled for a minimum period of 24 hours before any inoculation work.

**Carbon and Energy Source**

The most preferred carbon source in plant tissue culture is sucrose. Sucrose, while autoclaving the medium, is converted into glucose and fructose. In the process, first glucose is used and then fructose. It is usual to add 3% of sucrose. Phytohormones or plant growth regulators are required to induce callus tissue and to promote the growth of many cell lines.

i. **Nutrient medium for callus induction**: MS medium with different concentration of IAA, NAA, 2,4-D + BAP was used for callus inductions.
ii. **Culture media for shoot elongation**: MS medium supplemented with 0.5 to 1 mg/l concentration of NAA and 2,4-D used for multiple shoot proliferation.

iii. **Culture media for direct regeneration**: MS medium with different concentration of IAA and BAP was used for direct regeneration.

iv. **Culture media for indirect regeneration**: MS medium with different concentration of NAA and 2,4-D was used for indirect regeneration.

v. **Culture media for rooting**: MS medium supplemented with 1 to 3.5 mg/l concentration of IBA and NAA were used for rooting.

**pH Adjustment**

The pH was adjusted with a single electrode electronic pH meter at 5.6 to 5.8. Adjustment is done with 0.1 N HCl and 0.1 N NaOH. The pH affects the uptake of ions and so it is to be maintained in its optimal value. After adjusting the pH, 0.8% agar was added to the medium and was heated (60°C) to dissolve the agar. Later it was dispensed in pre-sterilized culture tubes (10 ml/tube).

**Media Sterilization**

The media were sterilized by autoclaving. It is a method of sterilization with water vapour under higher pressure. The media were kept in an autoclave for 15 minutes at 15 pSi. The autoclaved media after solidification was used for inoculation of explants.
3. **Inoculation**

There is a high risk of contamination of the nutrient medium at the time of transfer of the explants into the culture medium. Therefore inoculation has to be carried out especially. The aseptic inoculation of the explants into the culture medium involves three steps. They are:

a) Sterilization of the explants
b) Sterilization of the transfer area
c) Transfer of the explants.

**a) Sterilization of the Explant**

The plant previously sterilized by Bavistin and were then taken to the lamina air flow chamber where they were surface sterilized with 0.1% HgCl₂ for 4 minutes. They were again washed twice or thrice using sterile distilled water.

**b) Sterilization of the Transfer Area.**

A laminar air flow chamber was used as a transfer area. The laminar air flow chamber was sterilized by mopping the floor of the chamber with spirit. The sterile petridishes, forceps and knife holder with sterile blade were kept inside the chamber and were flame sterilized. The whole chamber and the instrument were exposed to the UV light for about 30 minutes. The sufficient time was also given to all the heat sterilized equipment to get cooled before inoculation.
c) **Transfer of the explants**

Minimum precaution was taken during the time of transfer of the explants. The hands were thoroughly washed with detergents and wiped with spirit. The surface sterilized explants were cut into required size and inoculated on pre-sterilized media in the presence of a spirit lamp. Non-absorbent cotton plug wrapped with gauze cloth was used to plug the culture tubes.

4. **Incubation**

Cultures were maintained at 20 ± 2°C with a photoperiod of 16 hours white light and 8 hours dark per day of fluorescent light (2000-3000 lux) for all treatments subculture was made on the same medium after 15th day.

5. **Hardening**

The rooted plantlets were removed from the culture tubes and washed in running tap water. Then they were transplanted into plastic cups containing sterilized vermiculite. The plants need 95-100% humidity and they were covered with plastic bags with perforation or holes. After 15 days the plantlets in the plastic cups were transferred to a shadow for about 30 days and then transferred to the soil.

**Gas Chromatography – Mass Spectrometry (GC-MS)**

In this study to identify the compounds from the plant extract GC – MS technique is used.
Plant Sample Extraction

Twenty grams of powdered plant leaf material is soaked in 50 ml of absolute alcohol overnight and then filtered through Whatman filter paper No.41 along with 2 gms sodium sulphate to remove the sediments and traces of water in the filtrate. Before filtering the filter paper along with sodium sulphate is wetted with absolute alcohol. The filtrate is then concentrated by bubbling nitrogen gas into the solution and reduces the volume to one ml. The extract contains both polar and non-polar phytocomponents of the plant material and the plant extract is injected in the Gas Chromatography – Mass Spectrometer.

One micro litre of the filtrate was injected into the Gas Chromatography column. The sample gets evaporated and carried away by gas helium. It gets segregated into individual components. The sample fraction coming out of the column was led into mass detector and the mass spectrum of each component was recorded. The mass spectrum of unknown component was compared with the known spectrum of NIST library and the components were identified.

Principle and application of GC – MS Detector in Phytochemical analysis

GC – MS plays a key role in the analysis of unknown components of plant origin. GC-MS ionizes compounds and measures their mass numbers. Ionization is typically, the C.I. (Chemical ionization) and E.I. (Electron ionization). The E.I. method provides good results for quantitative analysis as well of the compounds and it is a highly selective method for interfering components. Gas Chromatography technique involves the separation of volatile components in a test sample using suitable capillary column coated with polar and non-polar or intermediate polar, chemicals.
Elite-1 column (100% Dimethyl poly siloxane) is a non-polar column used for analysis of phytocomponents in medicinal plants and pesticide residues. Elite-5 column (5% phenyl and 95% methyl polysiloxane) is an intermediate column used for the estimation of pesticide residues in soft drinks and food grains. Elite wax (polyethylene glycol) is a polar column used in the estimation of fragrances in rice, alcohol, flowers and fatty acid profile of edible oils. An inert gas such as hydrogen or nitrogen or helium is used as a carrier gas.

The components of test sample is evaporated in the injection port of the GC equipment and segregated in the column by adsorption and adsorption technique with suitable temperature programme of the oven controlled by software. Different components are eluted from the column based on the boiling point of the individual components. The GC column is heated in the oven between 60 to 270 °C. The time at which each component eluted from the GC column is termed as retention time (RT).

The eluted component is detected in the mass detector. The spectrum of the unknown component is compared with the spectrum of the known components stored of the NIST library and ascertains the name, molecular weight and structure of the components of the test materials and GC-MS study. Food grain fragrances, floral fragrances, pesticide residues, Therpenes, steroids, alkaloids and fatty acids are some of the useful components analyzed in the GC-MS study.
Antidiabetic Activity

Animals

Healthy male swiss albino rats (6-8 weeks old) were used throughout the study. They were maintained in a controlled environmental condition of temperature and humidity. All animals were fed standard pellet diet (Gold mohar rat feed; Ms Hindustan lever Ltd., Mumbai) and ad libitum.

Source of Chemicals

Alloxan chemical analytical grade, reduced glutathione, bovine serum albumin, NAD, ATP was purchased from Sigma chemicals Company, St Louis U.S.A. All other chemicals used were of analytical grade obtained from Ranbaxy Research Laboratories also laboratories, Reddy’s laboratories, and nice pharmaceutical Company. Qualigen chemicals, SDS Chemical India.

Experimental Groups

The animals were divided in to four groups.

Group - I Normal Healthy Control
Group – II Diabetic control
Group – III Alloxan with plant extract
Group - IV Plant extract only.

Sample collection

The animals were carefully Mani forced everyday and weighted every week and urine, sugar and blood glucose of all rats were determined. Animals described as fasted were deprived of food for at least 12 hours out allowed free
access to drink water. After 6 weeks of treatment the rats were sacrificed by cervical dislocation blood was collected for biochemical estimation.

**EXTRACTS PREPARATION**

**Collection of Plant Materials**

The plant material was collected from Jamal Mohamed college garden in Tiruchirappalli district which was carefully identified with the help of regional floras.

**Preparation of Leaf Powder**

The plant was collected, washed and cut into small pieces and dried at room temperature for two weeks and made into powder for further analysis.

**Extraction of plant material**

Aqueous and alcoholic extracts were prepared according to the methodology of Indian Pharmacopoeia (Anonymous, 1966). The shady dried plants materials were subjected to pulverization to get coarse powder. The coarse powder material was subjected to soxhlet extraction separately and successively with alcohol and distilled water. These extracts were concentrated to dryness in flash evaporator under reduced pressure and controlled temperature (40-50°c). The aqueous and alcohol extracts put in air tight containers stored in a refrigerator.
CHEMICAL PREPARATION

Estimation of serum glutamate oxaloacetate transferase (Sgot) Activity (Bergmeyer, HU, 1974).

Principle

Under controlled condition the oxaloacetic acid is converted to pyruvic acid and the pyruvate is made to react with DNPH. The hydrazine formed is coupled to NaOH to give brown colour and the OD is read using a green filter.

Reagents

Buffered substrate

- DNPH reagent: 99 mg of DNPH is dissolved 50ml conc HCl and made up to 500ml with water
- 0.4N NaOH.

Stock pyruvate standard

- 220 mg of sodium pyruvate is dissolved in 102 ml of phosphate buffer.

Working standard

- Dilute 1ml stock to 10 ml with phosphate buffer.

Procedure

0.5ml of buffered substrate is added and incubated at room temperature for few minutes and to the test tube 0.1ml of sample is added and incubated for 60 minutes, at 37°C. Add 0.5ml of DNPH, mix well and incubate at room temperature for 20 mins, then add 5ml of 0.4N NaOH, wait for 10 min. Then the OD is read using a green filter.
ESTIMATION OF SGPT ACTIVITY (Reitman and Frankel, 1957)

**Principle**

Under controlled conditions the pyruvic acid, is made to react with DNPH. The hydrazone formed is coupled to NaOH to give brown colour and the O.D is read using a green filter.

**Reagents**

Phosphate buffer (pH - 7.4)

**Buffered substrate**

Dissolve 9gms of alanine in 90ml of water and 2.5 ml of 1N NaOH and add 0.146gms of ketoglutaric acid and dissolve it by adding few drops of 1N NaOH adjust the pH to 7.4 and make up the volume to 500 ml with phosphate buffer.

**DNPH reagent**

- 1.99 mg of DNPH is dissolved in 50ml con. HCL and made up to 500 ml with water.
- 0.4N NaOH

**Stock pyruvate standard**

- 1.220mg of sodium pyruvate is dissolved in 100ml of phosphate buffer.

**Working pyruvate standard**

Dilute 1ml stock to 10ml with phosphate buffer.
Procedure

0.5ml of buffered substrate is added and incubated at room temperature for few minutes and to the test tube 0.1ml of sample is added and incubated for 60 minutes at 37 °C. Add 0.5ml of DNPH, mix well and incubate at room temperature for 20 minutes, then add 5ml of 0.4N NaOH, wait for 10 minutes and read O.D using a green filter at 540 nm.

ESTIMATION OF ALKALINE PHOSPHATASE (Lowrey et al., 1954)

The enzyme phosphatase catalyzes the splitting off of phosphoric acid from certain monophosphoric esters. The filtrate obtained after dephosphorylation by TCA is treated with acid molybdate which reacts with inorganic phosphatate to form phosphomolybdc acid. The hexavalent molybdenum in phosphomolybdic acid is reduced by ANSA to give blue coloured compound which is estimated colorimetrically.

REAGENTS

Disodium phenyl phosphate (0.01M)

Sodium carbonate

Dissolve 3.18 gm of anhydrous sodium carbonate and 1.68gms of sodium bicarbonate in distilled water and made upto 500ml with distilled water

Buffered substrate

It is prepared by mixing equal volumes of solution (1) and (2), the pH is adjusted to 10.

- 20% TCA
Acid molybdate

Dissolve 5 gm of ammonium molybdate in 100ml of 5N H$_2$SO$_4$

- 5N H$_2$SO$_4$

Add 14ml con H$_2$SO$_4$ slowly to 86ml of distilled water.

- ANSA

0.25% ANSA is prepared by adding 0.5gms of dry powder of ANSA to 195ml of 15% sodium bisulphite and 5ml of 20% sodium sulphite. Mix well until it dissolves.

Stock phosphate

Dissolve 0.2194 gm of pure K H$_2$PO$_4$ in GDW and make upto 50ml and add few drop of chloroform.

Working standard

Dilute 0.2ml of stock solution to 50 ml with GDW.

Procedure

6ml buffered substrate is taken in a test tube and is placed in a water bath at 37°C for few mins. Add 0.3ml of serum to the test tube and mixwell. Incubate the test tube in a water bath at 37°C for 15mins. Remove the test tube and add 1.2ml of 20% TCA. Shake well and filter. Take 0.5ml of the filtrate &add 0.8ml of acid molybdate reagent followed by 0.2ml of ANSA. Mixwell and allow to stand for 10mins.

The results are expressed as KA Units/ 100 ml.
ESTIMATION OF GLUTATHIONE PEROXIDASE (Rotruck et al., 1972)

Glutathione peroxidase catalyses the following reactions.

\[ 2 \text{GSH} + 2 \text{H}_2\text{O} \rightarrow \text{GSSG} + \text{H}_2\text{O} \]

The enzyme was assayed with serum according to the method of Rotruck et al. (1972).

Reagents

- PO\textsubscript{4} Buffer pH 7.0 (0.32 M)
- EDTA – 0.8 mM
- Sodiumazide – 10 mM
- Glutathione (reduced) – 4 mM
- H\textsubscript{2}O\textsubscript{2} – 2.5 mM
- TCA – 10%
- Na\textsubscript{2}HPO\textsubscript{4} – 0.3 m
- DTNB – 40 mg of 55’ disthibis 2 nitrobenzoic acid in a sodium citrate.
- Standard glutathione having concentration of 10 mM was prepared.

Procedure

An incubation mixture containing 0.4 ml of buffer, 0.2 ml each EDTA, sodiumazide, GSH and H\textsubscript{2}O\textsubscript{2} was pre-incubated at 37 °C for 10 min. 0.1 ml of serum was added and the incubation was carried out at 37 °C for 10 minutes. The reaction was terminated by the addition of 0.5 ml of TCA. The reaction mixture was centrifuged and 0.5 ml of supernatant was taken and 3.0 ml phosphate solution and 1.0 ml of DTNB was added and the colour developed was read immediately at 412 min. Suitable aliquots of standard solution were
taken titrated in the similar manner to obtain a standard curve for comparison. glutathione peroxide is expressed as activity of GSH utilized / min/mg.

ESTIMATION OF BLOOD SUGAR (Ortho Toluidine Method)

Principle

Glucose reacts with ortholuidine in glacial acetic acid at 100 °C to form blue to green colored gel cosamine. The intensity of the colour developed is read at 620 nm.

Reagents Required

1. 10% trichloro acetic acid (TCA)
2. Orthotuluidine reagent
3. Acetic acid (glacial)
4. Distilled water

The reagent 2, 3 and 4 are mixed in the ratio of 15 : 75:10 and to this 2.5 gms of boric acid and 25 gms of thio urea are added and mixed well. The solution is preserved in a brown bottle.

5. Stock standard glucose : 100 mg of glucose in 100 ml of distilled water.
6. Working Standard : 100 ml dilution of stock standard gives working standard (100 µg/ml).

Normal Level : 80 – 120 mg / 100 ml of blood.

Procedure

To 0.1 ml of blood, 2 ml of 10% TCA is added to precipitate proteins. Then it is centrifuged at 3000 rpm for 5 minutes.
To 1 ml of supernatant (filtrate) 4 ml of ortho toluidine reagent is added and is heated in a boiling water bath for 15 mins. After incubation the tube is cooled and the optical density measured at 620 mm.

A blank is prepared by taking distilled water instead of the supernatant.

A standard graph is prepared by taking 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard is made upto 1 ml with distilled water and proceeded for test.

**ESTIMATION OF CATALASE ACTIVITY (Luck, 1974)**

Catalase catalyses the following reaction.

\[ 2 \text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \]

This enzyme is assayed by measuring the unreacted \( \text{H}_2\text{O}_2 \) by its ability to reduce dichromate in acetic acid solution.

**Reagents**

1. Dichromate acetic acid solution : 5% potassium dichromate in glacial acetic acid diluted 1:3 by volume.
2. \( \text{H}_2\text{O}_2 \) – 0.2 M
3. Phosphate buffer, pH 7.0, 0.01 M

**Procedure**

To 0.1 ml of serum, 1 ml of buffer and 0.4 ml of \( \text{H}_2\text{O} \) was added. The reaction was initiate the addition of 0.5 ml of \( \text{H}_2\text{O}_2 \) to the reaction mixture and was incubated at 37 °C for 15 minutes. The reaction was terminated by the
addition of 2 ml of dichromate acetic acid reagent. Standard H₂O₂ solution in
the range of 4 – 20 µM were taken and treated in the same manner. The tubes
were treated in boiling H₂O bath for 15 minutes. After incubation the tube is
cooled and the optical density measured at 620 nm.