PHARMOCOLOGICAL EVALUATION

EXPERIMENTAL MODELS USED FOR DIABETIC STUDIES

Different models for evaluation of anti-diabetic studies are available and are as follows

A] PANCREATECTOMY IN DOGS:

Dysfunction of the visceral tract has been considered for a long time to be the cause of diabetes mellitus. Bomskov (1910) reported severe diabetic symptoms in dogs after cannulation of the ductus lymphaticus. The final proof for the existence of a hormone in the pancreas was furnished by Banting and Best (1992) who could reduce the elevated blood sugar levels in pancreatectomized dogs by injection of extracts of the pancreatic glands. The role of the pituitary gland in development of diabetes has first been elucidated by Houssay (1930, 31) in pancreatectomized dogs.

B] ALLOXAN DIABETES:

Diabetes can be produced in various species of animals by the intravenous injection of alloxan. The dose varies with species, age and route of administration. In the dog the dose is about 50 mg/kg of weight given intravenously. In the rabbit it is 150 mg/kg intravenously. In rats 60 mg/kg body weight intravenously and is successively produced in rats by 120 mg/kg body weight subcutaneously. Alloxan destroyed the beta cells in the islet of langerhans.

C] STREPTOZOTOCIN INDUCED DIABETES:

Rakieten et al. (1963) reported the diabetogenic activity of the antibiotic streptozotocin. The compound turned out to be specifically cytotoxic to beta-cells of the pancreas.

D] OTHER DIABETOGENIC COMPOUNDS:

Several other compounds have been found to induce symptoms of diabetes and/or obesity such as dithizone or goldthioglucose.

E] HORMONE INDUCED DIABETES:

- Growth hormone induced diabetes: Cotes et al. (1949) described the diabetogenic action of pure anterior pituitary growth hormone in cats. In intact adult dogs and cats the repeated administration of growth hormone induces an intensively diabetic condition with all symptoms of diabetes including severe ketonuria and ketonemia.
• Corticosteroid induced diabetes: Ingle (1941) described hyperglycemia and glycosuria in forced fed rats treated with cortisone. In the guinea pig and in the rabbit, experimental corticoid diabetes could be obtained without forced feeding.

F] INSULIN ANTIBODIES:
A transient diabetic syndrome can be induced by injection of a guinea pig anti-insulin serum in various species.

G] VIRUS INDUCED DIABETES:
Juvenile-onset (type-1) diabetes mellitus may be due to virus infections and β-cell specific autoimmunity. The D-variant of encephalomyocarditis virus (EMC-D) selectively infects and destroys pancreatic β-cells in susceptible mouse strains similar to human insulin dependent diabetes(Vogel, 1997).

Alloxan
1. Chemistry:
Alloxan is called mesoxalylurea, mesoxalylcarbamide, 2,4,5,6 – tetra-oxohexahydropyrimidine or pyrimidinetetrones. It crystallizes with four parts of water, three of which can be removed successively by drying over sulphuric acid, to yield the form usually employed, the monohydrate, which is most probably hydrated at position 5 of the molecule. Heating under vacuum yields the anhydrous compound which in contrast to the colorless hydrates shows a strong yellow color due to the presence of three adjacent oxo-groups. Structurally similar to alloxan are the following substances, which differ only with regard to the substitution at position 5 : barbituric acid ; dialuric acid ; uramil ; violuric acid (=N-OH). Alloxan has a slight oxidizing effect and yields alloxantin (uroxin), which may be regarded as a condensation product of two molecules of alloxan upon reduction. Further reduction yields dialuric acid, which may be regarded as 5-hydroxy barbituric acid. Dialuric acid and alloxantin may easily be reconverted into alloxan. In aqueous solution as well as in plasma alloxan undergoes a spontaneous change into alloxanic acid, which is a structural isomer of alloxan monohydrate. Oxidation of alloxan yields parabanic acid and carbon-dioxide. Alloxan can be obtained by direct oxidation of uric acid with nitric acid. Synthesis may be achieved with uric acid, alloxantin; or benzylidene-barbituric acid as starting material.
2. Route of administration, distribution, dose and sensitivity:

Alloxan diabetes has been produced after intravenous, intramuscular, intraperitoneal, subcutaneous, oral and intrapulmonary administration. When a certain route was chosen deliberately it was to see whether still untried routes were effective or in order to perform a direct comparison of different routes.

Alloxan has been reported to pass the placenta. At doses diabetogenic to the mother it did not cause diabetes in the offspring. This suggested that the alloxan never had reached a diabetogenic concentration in the embryo.

In homeothermic animals the distribution of alloxan in the body is difficult to assess because of the extremely short half-life of the substance at the prevailing pH and temperature. A radio-autographic study of tissues in rats treated with diabetogenic doses of $^{14}$C-labelled alloxan led to the conclusion that alloxan is not selectively accumulated in pancreatic islet tissue. However, in mice treated with much lower doses, i.e. 4 to 5% of those used for production of diabetes, radioactivity accumulation in the islets far exceeded that in any other tissue expect initially in the kidney. It is uncertain from the data whether alloxan entered the cells or was concentrated at the cell membranes.

With regard to differences in sensitivity to the diabetogenic effect of alloxan, at least three sources of variation should be clearly distinguished:

1) The variation in mean sensitivity between species,

2) The variation in mean sensitivity within species between strains or laboratories and

3) The variation in individual sensitivity within a given experiment under the prevailing laboratory conditions which may, as a first approximation, be assumed to follow a log normal distribution.

An exact comparison of the sensitivity to alloxan between and even within species would require dependable determinations of the ED$_{50}$, the dose rendering half of the animals diabetic.

Factors influencing the size of the diabetogenic dose are the route of administration and in case of I.V. application, the speed of injection. The mean
sensitivity to alloxan in a given species of a group of animals may depend upon
the special laboratory conditions at hand, the animals state of nutrition and the
composition of the diet. Alloxan has a very short half-life in blood ($t_{0.5} < 1\text{min}$).
The ideal situation would be to inject or infuse it into the pancreatic artery. This
would minimize the amount of alloxan and therefore yield a maximal protection
against general toxic effects.

These theoretical considerations do agree well with the data from the
literature showing that the doses for diabetogenes is usually are the lowest when
given I.V. In rats diabetes could be produced with 60mg/kg body weight of
alloxan intravenously,(Singe et al,1989) whereas to obtain the same result after
intraperitoneal injection the dose had to be increased at least 5-fold. The per oral
route which ordinarily would not be expected to be effective, was shown to yield
diabetes only under special conditions, i.e., if high doses were given (0.5 to
1g/kg), if the animals were starved and if the alloxan food mixture was eaten
rapidly. Hence the I.V. route is the only rational one and the least toxic one. It is
obvious that the production of diabetes also depends upon the speed of the I.V.
injection at a given dose level, the slower injection rates causing less effect
(Claus, 1970).

Alloxan is producing diabetes possibly due to following:
i) Since glucose like shape of alloxan molecules, allows it to be taken up by the plasma
membrane of β-cells (Fisher, 1975).
ii) Inhibitory action on the adenyl cyclase, thus blocking at some point in Kreb’s cycle (Gaunerson,1973).
iii) Shown to increase concentration of hydrogen peroxide, superoxide anions, which may
be damaging to the pancreatic beta cells (Cohen,1974).

3. Mechanism of diabetogenic action:
1) Strecker reaction:
   The strecker reaction proceeds slowly and to provide an appreciable amount of the
products in the β-cell, this reaction should take place intracellularly. In view of the short
half-life of alloxan in blood and the finding that it follows the distribution of mannitol
being confined to the extracellular space. Diabetogenesis with the fact that several non-
diabetogenic substances yields a positive strecker reaction.
2. **Reaction with Sulphydryl Groups:**

The hypothesis that alloxan may act by occupation or inactivation of –SH groups was advocated by Lazarow who showed that substances containing free sulphydryl groups protect against alloxan diabetes. These substances reduce alloxan to dialuric acid, which is non-diabetogenic unless it is reoxidized to alloxan. Furthermore, glutathione may react with alloxan to form an addition compound. Free sulphydryl groups in the islet β-cells are necessary for insulin synthesis and they are supplied by cysteine and glutathione. The depletion of glutathione in the blood after injection of alloxan is in keeping with this concept.

3. **Chelating action:**

Kadota has suggested that alloxan diabetogenesis may be due to a combination of alloxan with zinc in the islet β-cell, which in turn may cause cell necrosis. This effect would be analogous to the mode of action of the chelating agents oxine and dithizone.

4. **Other Hypothesis assuming enzymatic or metabolic alterations:**

The possibilities of a selective islet β-cell damage by enzymatic or metabolic alterations within, at the membrane of or outside the β-cell have recently been presented. Possible actions include the following: inhibition of hexokinase or other enzymes inactivation of Co-enzyme A; reaction with β-cell membranes and release of trypsin from the exocrine tissue. Hexokinase inactivation would require the alloxan to enter the cell and as for the reaction of alloxan with sulphydryl groups.

By the study of all the above given hypothesis we arrive at some conclusions with a reasonable degree of certainty:

1) The site of action of alloxan for diabetogenesis is the islet β-cell membrane.
2) After i.v. injection the binding of alloxan to its site of action is completed within a few minutes.
3) The histological and most biochemical changes observed later than 5min after i.v. alloxan injection are secondary changes and are not due to a direct alloxan effect. (Gaunerson 1973, Cohen 1974).
4. **Mechanisms responsible for changes in blood glucose during induction of diabetes.**

   *A) Early hyperglycemia:* The mechanism of the early hyperglycemia is not generally agreed upon. Sudden cessation of insulin release together with liberation of epinephrine result in the high blood sugar values recorded. Supporting evidence for this is that the hyperglycemia could be imitated by injection of epinephrine into normal rabbits and that adrenodemedullation attenuated or abolished the early hyperglycemia.

   Binding of released insulin by means of I.V. overdoses of anti-insulin serum in mice was followed by a much slower rise of blood glucose than after alloxan. An anti-insulin serum overdose would however, be expected to yield a faster rise of blood sugar than a sudden cessation of endogenous insulin release. Since the antibody would even combine with circulating insulin and probably with some insulin in the tissues.

   *B) Hypoglycemic Phase:* There is general agreement that the hypoglycemic phase is brought about by insulin. Evidence in favour of this view is that plasma immunoreactive insulin is high during this phase anti-insulin serum treatment abolishes the hypoglycemia.

   The occurrence of the hypoglycemic phase shows that alloxan does not inactivate the insulin present in the β-cell and it has been suggested that this phase is the consequence of an uncontrolled leakage of insulin from the damaged cells. If the integrity of the islet β-cell should depend upon intact insulin releasing mechanism located in the cell membrane the destruction of this functional part of the cell by alloxan would fit most of the experimental evidence.

   *C) Chronic Diabetic Phase:* The mechanism of the third phase is uniformly agreed to be the consequence of insulin lack. Histologically only a few β-cells if any are detectable in animals with fully developed alloxan diabetes. Plasma immunoreactive insulin is very low or unmeasurable. Exogenous insulin readily restores normal blood glucose levels. Insulin sensitivity persists during the whole period of alloxan diabetes. If a spontaneous remission is observed, as shown by decrease of mean blood sugar values with times towards normal, immunoreactive plasma insulin is again detectable after injection of an insulin-releasing substance (Claus, 1970).
Acute oral toxicity – Acute toxic class method: (OECD "Guidelines for the testing of chemicals", 2000)

The acute oral toxicity study was carried out as per the guidelines set by Organization for Economic Cooperation and Development (OECD), received draft guidelines, received from Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India

Principle of test:

It is the principle, which is based on a stepwise procedure with the use of a minimum number of animals per step; sufficient information is obtained on the acute toxicity of the test substance to enable its classification. The substance is administered orally to a group of experimental animals at one of the defined doses. The substance is tested using a stepwise procedure, with the initial dose being selected as the lower fixed dose, depending on the presence of mortality, until the study objective is achieved i.e. the classification of the test substance based on the identification of doses causing mortality, when there are no effects at the highest fixed dose.

DESCRIPTION OF THE METHOD

1. Selection of animal species:

Healthy young albino mice of either sex weighing between 25 to 40g were used for acute toxicity study to determine LD$_{50}$ of various extracts.

2. Housing and feeding condition:

The temperature in the experimental room was around $25^\circ$C ± 1. Lightning was artificial, the sequence being 12 hours dark, 12 hours light. The conventional laboratory diet was fed, with drinking water ad libitum.

3. Preparation of animals:

The animals were randomly selected, marked to permit individual identification and kept in their cages for five days prior to dosing to allow for acclimatization to the laboratory condition.

4. Preparation of doses:

The pet. ether, benzene, chloroform, acetone extracts were suspended in 5% tween-80 in water. The alcoholic and aqueous extracts dissolved in water.
5. **Administration of doses:**

The test substances were administered orally by oral feeding tube. Animals were fasted prior to dosing, following the period of fasting the animals were weighed and test substance was administered. After the dose was administered, food was withheld for a further 3-4 hrs in rats.

6. **Number of animals and dose levels:**

In each steps six animals were used in each group. Starting dose was 300mg/kg body weight up to 5000mg / kg body weight.

1/10\(^{th}\) of this lethal dose was taken as effective dose (therapeutic dose) for subsequent antidiabetic activity.

7. **Observations:**

Animals were observed after dosing at least once during the first 30 minutes then periodically during the first 24 hours. In all cases death was observed within first 24 hours. Additional observations like changes in skin, fur, eyes and mucous membranes, respiratory, circulatory, autonomic central nervous systems, somatomotor activity and behaviour pattern. Attention was also given to observation of tremors and convulsions.

Toxicity study is also conducted and confirmed by Miller and Tainter method (Miller and Tainter, 1994).

**EVALUATION OF ANTI-DIABETIC ACTIVITY**

The acclimatized animals were kept fasting for 24 hrs. with water *ad libitum*. Animals were separated according to their body weight. Freshly prepared alloxan monohydrate in normal saline solution was injected intraperitoneally (i.p.) at a dose of 120 mg kg\(^{-1}\) b.w. (Vijayavargia,2000; Geetha,2001; Raghavan,2006;Jothivel 2007). After one hour of alloxan administration, animals were given feed *ad libitum* and 1 ml of (100 mg/ml) glucose orally to combat ensuring severe hypoglycemia. After 72 hrs of the alloxan injection, the animals were tested for the evidence of diabetes by estimating their blood glucose level by using Glucometer (Accu-chek active, Roche Diagnostics GmbH, Germany). The blood glucose level more than 200 mg/dl of blood was the criteria (Tenpe, 2008).
The animals were segregated into ten groups of six rats each, one group was normal control and others were diabetic control, petroleum ether, benzene, chloroform, acetone, ethanol (successive), aqueous, alcoholic extracts and standard glibenclamide group. To the animals, the test extracts and standard drug glibenclamide were administered by suspended in 5% Tween-80/ water. The blood samples were obtained through the tail vein puncturing with Lancet.

The animals were divided into Ten groups (n = 6).

**Table.13:** Grouping scheme for antidiabetic study

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control rats given normal saline.</td>
</tr>
<tr>
<td>II</td>
<td>Alloxanized diabetic rats.</td>
</tr>
<tr>
<td>III</td>
<td>Alloxanized diabetic rats were given pet. ether extract (Successive) orally.</td>
</tr>
<tr>
<td>IV</td>
<td>Alloxanized diabetic rats were given benzene extract (Successive) orally.</td>
</tr>
<tr>
<td>V</td>
<td>Alloxanized diabetic rats were given chloroform extract (Successive) orally.</td>
</tr>
<tr>
<td>VI</td>
<td>Alloxanized diabetic rats were given acetone extract (Successive) orally.</td>
</tr>
<tr>
<td>VII</td>
<td>Alloxanized diabetic rats were given ethanolic extract (Successive) orally.</td>
</tr>
<tr>
<td>VIII</td>
<td>Alloxanized diabetic rats were given aqueous extract orally.</td>
</tr>
<tr>
<td>IX</td>
<td>Alloxanized diabetic rats were given alcoholic extract orally.</td>
</tr>
<tr>
<td>X</td>
<td>Alloxanized diabetic rats were given glibenclamide orally.</td>
</tr>
</tbody>
</table>

**Estimation of blood sugar:**

The blood samples were obtained through tail vein by puncturing with Lancet. A drop of blood so obtained was placed on glucostrip, which was kept in the Glucometer (Accu-chek active, Roche Diagnostics GmbH, Germany). The glucometer was kept on, then after 5 sec. glucomonitor reading was recorded.

**ACUTE STUDY**

The measurement of blood sugar level at 0, 2, 4, 6 hrs. were done after administration of dose orally.
**CHRONIC STUDY**

The extracts were administered for a period of 21 days and blood sugar level and body weights of individual animals were observed at 1, 7, 14 and 21 days.

The statistical analysis was carried out by one way ANOVA followed by Dunnett’s test (Kulkarni, 1993, Remington 1995).

Oral glucose tolerance test (OGTT):

The OGTT is the only form of glucose tolerance testing recommended for the diagnosis of diabetes. The relationship between blood glucose levels after an external load of glucose can be studied using OGTT.

At the end of 21 days of administration of the extracts, the animals were fasted overnight and water was provided *ad libitum* for the performance of OGTT. At the day of OGTT, the animals were given an oral dose of glucose at a concentration of 2gm/kg of body weight. At 0 min. blood samples were taken from the normal, diabetic control, alcoholic extract group, Pet. ether extract and glibenclamide group. Then blood samples were also collected subsequently at 30, 60, 90, 120 minutes and noted the blood glucose level. (Shrinivasan, 2005; Gandhipuram, 2006).
Other biochemical parameters.

At the end of the treatment blood was collected by direct cardiac puncture and serum was separated by centrifugation at 2500 rpm. The rats were sacrificed by cervical dislocation and pancreas were excised immediately and thoroughly washed with ice cold physiological saline. The serum collected was used for biochemical estimations.

Estimation of total cholesterol and HDL cholesterol (Wybenga and Pileggi Method):

Total cholesterol and HDL cholesterol were estimated by using standard kit obtained from Biolab Diagnostics (I) Pvt. Ltd. Tarapur, Maharashtra.

Principle:

In hot acidic medium, cholesterol oxidises ferric ions to a brown coloured complex which absorbs at 530 nm and is directly proportional to cholesterol concentration.

Procedure:

The kit contents were brought to room temperature.

Table 14: Procedure for estimation of total cholesterol.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank (B) (ml)</th>
<th>Standard (S) (ml)</th>
<th>Test (T) (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol reagent No. 1</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.05</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard reagent No. 2</td>
<td>-</td>
<td>0.05</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>0.05</td>
</tr>
</tbody>
</table>

The test tubes were labelled as B (Blank), S (Standard) and T (Test). Serum of test sample was added to test tube labelled T.

These were mixed well by gently shaking the test tube and kept in boiling water bath for 90 seconds. Cooled for 5 minutes under running tap water. Absorbance of the standard (S) and test sample (T) were measured against the reagent blank (B) with the help of colorimeter at 530 nm.
The total cholesterol was calculated using the formula

\[
\text{Total Cholesterol(mg/dl)} = \frac{O.D.(\text{Test})}{O.D.(\text{Standard})} \times 200
\]

**Estimation of HDL cholesterol:**

This procedure consists of two steps:

**Step 1:**

In a glass tube 0.2 ml serum was added with HDL reagent No. 3. After mixing, the tubes were incubated for 10 minutes at room temperature and then centrifuged. The clear supernatant obtained was taken for the HDL cholesterol estimation.

**Step 2:**

Five ml of cholesterol reagent No. 1 was put in test tubes labelled B, S and T. 0.2 ml of HDL reagent No. 3 was added to test tube labelled B and S. Then 0.2 ml of clear supernatant obtained by step 1 was added to test tube labelled T, while cholesterol standard was added to standard tube (S).

**Table 15: Procedure for estimation of HDL cholesterol.**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank HDL (B) (ml)</th>
<th>Standard HDL (S) (ml)</th>
<th>Test HDL (T) (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol reagent No. 1</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>HDL reagent No. 3</td>
<td>0.2</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>Supernatant from step 1</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
</tr>
<tr>
<td>Cholesterol standard (200mg)</td>
<td>-</td>
<td>0.02</td>
<td>-</td>
</tr>
</tbody>
</table>

These were mixed well by gently shaking the test tube and kept in boiling water bath for 90 seconds, cooled for 5 minutes under running tap water. Absorbance of the standard (S) and test sample (T) were measured against the reagent blank (B) with the help of colorimeter at 530 nm.

The HDL cholesterol was calculated as follows:

\[
\text{HDL Cholesterol(mg/dl)} = \frac{O.D.(\text{Test HDL})}{O.D.(\text{Standard HDL})} \times 40
\]
• Estimation of triglycerides (GPO-PAP Method):

Triglycerides estimation kit consists of enzyme reagents, triglyceride standard and diluent buffer, was obtained from Biolab Diagnostics (I) Pvt.Ltd. Tarapur, Maharashtra.

Principle:

Triglycerides are split into glycerol and fatty acids in the presence of lipoprotein lipase. In the presence of ATP and glycerol-kinase, glycerol is converted into glycerol-3-phosphate and ADP. Glycerol-3-phosphate oxidase dissociates glycerol-3-phosphate into dihydro-acetone phosphate and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide reacts with 4-aminoantipyrine and ESPAS (N-Ethyl-N-Sulfopyropyl-n-methoxyaniline) to form a red coloured quinoneimine as indicator.

Preparation of working reagent:

The lyophilized material is dissolved with 1.5 ml buffer. A uniform solution takes place after 5 minutes which is ready to use.

Procedure:

One ml of working reagent was added to test tubes labelled B, S and T. Blank test tube was added with 0.05 ml distilled water, while 0.05 ml of standard was added to test tube labelled S and 0.05 ml of sample (serum) was added to test tube labelled T.

These were mixed well by gently shaking the test tube and incubated for 10 minutes at 37°C.

Absorbance of the standard (S) and test sample (T) were measured against the reagent blank (B) with the help of colorimeter at 500 nm within 30 minutes.
Table 16: Procedure for estimation of triglycerides.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank HDL (B) (ml)</th>
<th>Standard HDL (S) (ml)</th>
<th>Test HDL (T) (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>0.05</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Triglycerides standard reagent No. 3</td>
<td>-</td>
<td>0.05</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>0.05</td>
</tr>
<tr>
<td>Working reagent</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The Triglycerides concentration was calculated as follows:

\[
\text{Triglycerides (mg/dl)} = \frac{O.D_{(\text{Test})}}{O.D_{(\text{Standard})}} \times 200
\]

Study of mechanism of actions

\(\alpha\)-glucosidase inhibitory activity

Normal healthy rats fasting for 20 hrs were sacrificed by cervical dislocation. The small intestine obtained was flushed several times with ice-cold NaCl, and 50 mM (pH 7.0) sodium phosphate buffer. The mucosa was scraped with glass slide on ice-cold glass surface. The obtained material was centrifuged and pellet homogenized in phosphate buffer containing 1% Triton X 100, further cold butanol was added to remove Triton and sample subjected to overnight dialysis. The enzyme thus obtained was used after proper dilution (Lee, 1980).

5µmol P-Nitrophenyl-\(\alpha\)-D-Glucopyranoside (PNPG), enzyme solution (0.1 µl), in 900µl of sodium phosphate buffer (50 mM), pH 6.8 in the final volume of 1ml. Each extract 100 µg was dissolved in 20µL of distilled water and added to the test mixture before adding the substrate. Blank sample contained whole test mixture and the extract without enzyme solution. Distilled water added to the control sample (20 µl) and in the positive control 20 µL acarbose (100 µl) was enhanced. The mixture incubated at 37\(^\circ\)C for 30 mins, the reaction terminated by adding 3 volumes of NH\(_4\)OH solution (0.05 M). The absorbance at 405 nm was determined by Spectrophotometer. The inhibitory activity calculated using following formula

\[
\text{Inhibitory activity(\%)} = \frac{O.D_{(\text{Control})} - O.D_{(\text{Test})}}{O.D_{(\text{Control})}} \times 100
\]
Each test performed 3 times and the mean value used for the inhibitory activity of plant extracts (Ahmad, 2008).

**HISTOPATHOLOGICAL STUDIES:**

At the end of the study, rats were sacrificed and the pancreas were collected. The tissue was fixed in 10% formalin immediately after removal from the animal to avoid decomposition.

**Preparation of histopathological slides**

1) **FIXATION**

Fixatives were used to preserve tissue from degradation, to maintain the structure of cells including sub-cellular components such as cell organelles (e.g. nucleus, endoplasmic reticulum, mitochondria etc.).

**Example of Fixative:** - Formalin (4 % Formaldehyde in Phosphate buffered saline).

**Mechanism:** - This aldehyde fixative preserved tissue or cells mainly by producing cross-linking of amino groups in proteins through the formation of CH₂ (methylene) linkage.

**Procedure:** - The tissue samples were isolated and rinsed with distilled water. Any adherent fatty contents were removed and preserved in formalin.

2) **DEHYDRATION**

In this process water from biological tissue was removed.

(A) **Cutting of Tissue:**

Tissue sample sections were taken, wrapped separately in filter paper and tied with thread (Wrapping should be kept up to the step of Infiltration and opened before the step of embedding with paraffin).

(B) **Ethanol Treatment:**

Wrapped tissues were placed in a bottle containing ethanol for 15 min. Then the wrapped tissue were removed from the bottle and dried.

(C) **Acetone Treatment:**

Now wrapped tissue were placed in a series of bottles containing acetone as follow:

i. **Acetone I:** Wrapped tissue was placed in a bottle labelled as Acetone I containing acetone for 30 min. Wrapped tissue was then removed from the bottle, dried it and placed in a 2nd bottle labelled as Acetone II containing acetone.

ii. **Acetone II:** Same as above.

iii. **Acetone III:** Same as above.
iv. **Acetone IV:** Same as above.

v. **Acetone V:** Same as above.

**D) Xylene treatment:**
After giving $5^{th}$ acetone treatment wrapped tissue were removed from bottle and dried. Then transferred to bottle labelled as Xylene I as follows:

i. **Xylene I** : - Wrapped tissues were placed in a bottle labelled as **Xylene I** containing xylene for 45 min. Then the wrapped tissues were removed from the bottle, dried it and placed in a $2^{nd}$ bottle labelled as **Xylene II** containing xylene.

ii. **Xylene II** : - Same as above.

iii. **Xylene III** : - Same as above.

**3) INFILTRATION:**
After giving $3^{rd}$ xylene treatment, wrapped tissues were removed from the bottle and dried. After drying wrapped tissues were kept in a tissue capsule/cassettes and placed in a paraffin bath maintained at $50-60^\circ C$ for 2 hrs. During this period temperature was strictly maintained at $50-60^\circ C$. Overheating may damage the tissue. This process was called as ‘Infiltration’. The capsule from paraffin bath was removed after 2 hr and wrapped tissue were removed from the capsule. Wrapping over the tissue were opened. Tissue was sufficient hard to cut.

4) **EMBEDDING:**
Tissue samples were placed on plane surface. The mould size was adjusted. The melted paraffin wax was then poured having temperature $50^\circ C$ in mould and allowed to cool until solidify. The moulds were removed carefully. The blocks were ready for taking sections/cut on microtome.

**5) CUTTING AND PREPARATION OF SLIDES:**

a. Wooden block was attached to the opposite side of the tissue.

b. Blocks were kept in ice-bath for 10 min.

c. The blocks were kept on microtome in proper way and the size of the sections was adjusted.

d. Ice was applied to block and knife/cutter.

e. Required thin sections of the tissue sample were taken in the form of ribbon. These thin ribbons (sections) were transferred in cold water for 2-3 min with the help of brush and then again transferred to hot water ($50-60^\circ C$) with the help of test tube.
f. A drop of egg albumin was applied and spread over the glass side. Then the glass side was inverted at an angle of 90° near the ribbon in hot water and these ribbons were taken on side.

6) STAINING:-
   a. The slides were kept in staining rack/cradle in proper direction.
   b. This rack containing slides was placed in paraffin oven at 60°C until paraffin present on slide melts.
   c. Now this hot rack was placed in plastic box containing xylene for 10 min to dissolve the melted paraffin and leaves only thin section of tissue sample on slide. After 10 min the rack was removed from xylene and washed under running tap water for 2-5 min.
   d. The rack was kept in plastic box containing alcohol for 5-10 min. Then removed the rack from alcohol and washed under running tap water for 2-5 min.
   e. The rack containing slides was kept in Hematoxylin for 10 min. Then removed the rack and washed under running tap water for 5 min.
   f. This rack was again kept in acidic alcohol for 2-3 min to develop blue colour. Then the rack was removed and washed under running tap water for 5 min.
   g. Now the rack containing slides were placed in eosin for 4 min. Then rack was removed and washed under running tap water for 5 min.
   h. The slides were removed from rack one by one, cleaned with muslin cloth and dried at room temperature, kept aside for 1 day to dry it completely.
   i. A drop of DPX mount was applied on slide and a coverslips were placed carefully on these slides. Again kept aside for 1 day to dry completely.
   j. Now the slides were permanent and ready for examination under light microscope.

ANTI-CANCER ACTIVITY

Cell lines and culture

MCF-7 (Brest cancer cell line) was obtained from the National Centre for Cell Science (NCCS), PUNE. These cell lines were maintained in MEM (minimum essential medium) supplemented with 10 % (v/v) fetal bovine serum.

MTT-Microculture tetrazolium assay

Cells were seeded at a concentration of $1.5 \times 10^5$ cells/ml in a 96 well plates. After overnight incubation, serial concentrations of the compounds were added. Serial concentrations of test sample were prepared by dissolving the
compound in dimethyl sulfoxide (DMSO) followed by dilution with RPMI-1640 medium to yield the final DMSO concentration in the assay well as 0.2%. Each concentration was repeated three times. These cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C for 1 day. Then 20µl MTT solution (5 mg/ml) was added to each well and incubated at 37 °C for 4 h. The medium was removed and formazan was dissolved in DMSO and the optical density was measured at 570 nm using an Elisa plate reader. The growth inhibition was determined using:

\[
\text{Percentage cell viability} = \frac{\text{Absorbance of drug treated cells} \times 100}{\text{Absorbance of control cells}}
\]

And IC₅₀, which is the drug concentration resulting in a 50% inhibition of cell growth, is calculated from dose–inhibition curves (Perera,2008).
References

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