Scope and Plan of Present work
SCOPE AND PLAN OF PRESENT WORK

According to the recent estimates, the human population worldwide appears to be in the midst of an epidemic of diabetes in particular type 2 diabetes mellitus and insulin resistance. Despite the great strides that have been made in the understanding and management of type 2 diabetes, insulin resistance and diabetes related complications are increasing unabated. Various models of experimental diabetes are though present for investigators to explore numerous physiological and biochemical defects observed during the progression of disease. Moreover, there will always be physiological, pathological and morphological differences among different animal models of diabetes. The purpose of this study was to standardize/develop suitable animal models for type 2 diabetes and insulin resistance which can mimic the disease present in the human being. However, an unavoidable reality is this that none of models are perfectly equivalent to the human disease state. Parallel to this, recent developments in understanding the pathophysiology of disease process have opened up several new avenues to identify and develop novel therapies to combat the diabetic plague and insulin resistance by developing plant based insulin resistant reversal agents. Concurrently, identification of phytochemicals from natural sources provide an exciting opportunity for the development of new types of management therapeutics for diabetes and insulin resistance. This has accelerated the global efforts to harness and harvest those terrestrial flora and marine flora and fauna that bear substantial amount of potential compounds showing multiple beneficial effects in combating insulin resistance, type 2 diabetes and diabetes related complications like cataract, neuropathy, nephropathy etc. Therefore, as the problem of diabetes and insulin resistance are spreading unabated, there is an urgent need of identifying indigenous natural resources in order to procure them and study in depth, their potential on different antidiabetic in vitro targets and in vivo animal models for type 2 diabetes and insulin resistance in order to develop them as antidiabetic drugs.

The present study is an attempt to identify few target based antidiabetic compounds (like α-glucosidase, aldose reductase and protein tyrosine phosphatase 1 B inhibitors) standardize/develop animal models for type 2 diabetes and insulin
Scope and Plan of Present Work

resistance by manipulation in the diet or chemical treatment fructose-enriched diet fed rats and preclinical development of identified herbal and marine extracts

Chapters I details the antihyperglycaemic effects in some terrestrial flora and marine flora and fauna in STZ-induced diabetic rats. This chapter also deals the results of few target based designed (α-glucosidase, aldose reductase and protein tyrosine phosphatase inhibition) synthetic compounds both for diabetes and insulin resistance. Identification of few lead compounds is one of the main achievements of this section.

Chapter II deals with antidiabetic effects of one standardized marine preparation CDR-134 and one herbal extract CT-1, their effect on regulatory enzymes of carbohydrate metabolism (pyruvate kinase, phosphofructokinase, lactate dehydrogenase, glycogen phosphorylase, glucose-6-phosphatase and protein tyrosine phosphatase). Lowering the activities of glucose-6-phosphatase and protein tyrosine phosphatase by the above said preparations under in vivo conditions are the novel findings.

Chapter III deals with the studies about the measurement of insulin resistance index using HOMA in the animal models for non-insulin dependent diabetes mellitus and insulin resistance. This chapter provides new chemical entities for the development of insulin resistant reversal agents from herbal source.

Overall the studies of present dissertation will help in selecting the antidiabetic plants and marine substances for further study, clinical development of CT1 and CDR-134 for diabetes as well as insulin resistance and the optimization of leads for best synthetic antidiabetic compounds hitting the novel target Protein tyrosine phosphatase-1B.
Materials & Methods
MATERIALS AND METHODS

ANIMALS

Male/Female inbred/outbred Charles Foster/Sprague Dawley/Wistar strain of albino rats (8-10 weeks of age: body weight 160±20 gm) were procured from the animal colony of Central Drug Research Institute, Lucknow, India. Rats were always placed in groups of three to five in polypropylene cages. The following norms were always followed for animal room environment: Temperature 23±2°C; Humidity 50-60%; Light 300 Lux at floor level with regular 12h light cycle; Noise level 50 decibel; ventilation 10-15 air changes per hour. The animals had free access to pellet diet (Lipton, India) and tap water unless stated otherwise.

CHEMICALS

Folin ciocalataeu's phenol reagent, fructose, sodium pyruvate, oxaloacetate, tris HCl, potassium chloride, magnesium chloride, adenosine triphosphate, adenosine diphosphate, glucose-6-phosphate were procured from Sisco Research Laboratory (SRL), Bombay. Streptozotocin, bovine serum albumin, adenosine monophosphate, NADH, NADPH, glycogen, PEP, LDH, MDH, glucose-1-phosphate, fructose-6-phosphate, sodium fluoride, EDTA, aldolase, triosephosphate isomerase were purchased from Sigma Chemical Company, St. Louis, USA. Casein, starch, sucrose, glucose were purchased from Himedia, Bombay. Ethanol, methanol, chloroform, sodium acetate, sulphuric acid, hydrochloric acid, acetic acid, lithium sulphate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, citric acid, sodium citrate were purchased from E-Merck, Germany. The Radioimmunoassay kits for serum insulin measurements were purchased from Bhabha Atomic Research Centre (BARC), Bombay, India. All other chemicals and solvents used were of highest purity grade.
Structure of Standard Antidiabetic Drugs:

Glyburide

Metformin

Rosiglitazone

Gliclazide

Acarbose
Acarbose

\[ o-4,6\text{-dideoxy-4\(-[1s-(1\alpha,4\alpha,5\beta,6\alpha)]\)-4,5,6-trihydroxy-3-(hydroxymethyl)-2-cyclopyranosyl-(1\(-4\)-D-glucose}; \]

\[ 4",6"\text{-dideoxy-4\"-[(1s)-(1,4,6/5)-4,5,6-trihydroxy-3-hydroxymethyl-2-cyclohexenylamino]maltotriose}; \]

Bay g 5421; Glucobay; Prandase; Precose

C\textsubscript{25}H\textsubscript{43}NO\textsubscript{18} MW 645.60

C 46.51%; H 6.71%; N 2.17%; O 44.61%

Pseudotetrasaccharide containing an unsaturated cyclitol moiety. An \(\alpha\)-glucosidase inhibitor that reduces sugar absorption in the gastrointestinal tract.

Amorphus powder \([\alpha]_D^{18} +165^\circ (c=0.4 \text{ in water})\)

Glibenclamide

\[ 5\text{-Chloro-N-[2-[4-[[((cyclohexylamino) carbonyl] amino] sulfonyl] phenyl] ethyl]-2-methoxybenzamide}; \]

\[ 1\text{-[p-[[2-(5-chlor-O-anisamido)ethyl]-phenyl] sulfonyl]-3-cyclohexylurea}; \]

\[ N^-\text{[4-I\(\beta\)-(2-methoxy-5-chlorobenzamido) ethyl] benzosulfonyl]-N^1\text{-cyclohexylurea};} \]

\[ N^1\text{-[4-[\beta-(2-methoxy-5-chlorobenzoxylamino) ethyl]benzenesulfonyl]-N^2\text{-cyclohexylurea};} \]

Glybenclamide; glyburide; HB-419; U-26452; Adiab; Azuglucon; Bactiverit; Dibasam; Diabeta; Daonil; Duraglucon; Euglucon; Gilemal; Gliben-Puren N; Gli-diabet; Glimidstada; Glucomed; Gluco-Tablinen; Glycolande; Lederglib; Libanil; Lisaglucon; Malix; Maninil; Micronase; Praeciglucon.

C\textsubscript{23}H\textsubscript{28}ClN\textsubscript{3}O\textsubscript{5}S MW 494.01

C 55.92%; H 5.71%; Cl 7.18%; N 8.51%; O16.19%; S 6.49%

Second generation sulfonurea with hypoglycaemic activity.
Gliclazide

N-[[Hexahydrocyclopenta [c] pyrrol-2 (14)-yl amino] carbonyl] -4-methylbenzenesulfonamide;

1-(hexahydrocyclopenta[c] pyrrol-2 (14)-yl)-3-(p-tolylsulfonyl) urea;

N-(4-methylbenzenesulfonyl)-N'-(3-azabicyclo [3.3.0]-oct-3-yl)-urea

S-1702; Diamicron; Glimicron; Nordialex;

\[ \text{C}_{15}\text{H}_{21}\text{N}_3\text{O}_3\text{S} \quad \text{MW 323.42} \]

C 55.714%; H 6.55%; N 12.99%; O 14.84%; S 9.91%

Crystal from anhydrous ethanol mp 180-182°C, LD₅₀ orally in mice >3 gm/kg (Duhault).

Metformin

N,N-Dimethylimidodicarbonimidic diamide

1,1-dimethylbiguanide

N,N- dimethylbiguanide

N', dimethylguanylguanidine (DMGG)

LA-6023

\[ \text{C}_4\text{H}_{11}\text{N}_5 \quad \text{MW 129.16} \]

C 37.2%; H 8.58%; N 54.22%

Oral hypoglycaemic agent

Hydrochloride

Diabetosan; Diabex; Glucophage; Metiguanide;

\[ \text{C}_4\text{H}_{11}\text{N}_5\text{HCl} \quad \text{MW 165.63} \]

Prisms from water, mp 232° (Werner, Bell). Crystal from propanol, mp 218-220° (uncover) (Shapiro).
Materials and Methods

Soluble in water, 95% alcohol. Practically insoluble in ether, chloroform.

LD$_{50}$ in rats (mg/kg): 1000 orally, 300 s.c. (*Rx Bulletin*).

**Roziglitazone**

5-[[4-[2-(methyl-2-pyridinylamino)ethoxy]phenyl]methyl]-2,4-thiazolidinedione

5-[[4-[2-[N-methyl-N-(2-pyridinyl) amino] ethoxy] benzyl]-2, 4, - thiazolidinedione

BRL- 49653

C$_{18}$H$_{19}$N$_3$O$_3$S  MW 357.43

C 60.49%; H 5.36%; N 11.76%; O 13.43%; S 8.97%

Insulin sensitizer; binds to peroxisome proliferator activated receptor (PPAR-γ).

**Standardized Preparations:**

CT-1

1. **Background:** This plant was randomly collected for general biological screening programme. Whole plant turned out to be a promising antihyperglycaemic agent.

2. **Distribution:** It is available in temperate regions of Himalayas from Kashmir to Bhutan and in Nepal also.

3. **Availability:** This plant is available throughout the year.

4. **Storage:** Dried whole plant is powered in disintegrator. Powder is charged in hot extraction unit and submerged in deionised water. Allow to stand for 10 minutes and then drained off. Material soaked again with similar quantity of deionised water and temperature is raised to 98-100° C and maintained for 2h. Heating discontinued and allowed to stand for 10 min.

4. **Processing:** Whole plant extract is strained through muslin and collected separately. The concentrated extract is spread in trays and dried in a vacuum-shelves drier (temp>60° C) for about 6-7 hrs. The dried residue is collected
and powered in a pulveriser to give a blackish brown power of uniform particle size.

CDR-134

1. **Background:** The mangrove was randomly collected for general biological screening programme. Interestingly epicarp of fruit turned out to be a promising antihyperglycaemic agent.

2. **Distribution:** In tidal forests along the east and west coastal areas up to Maharastra and Andaman islands.

3. **Availability:** The fruits are available throughout the year.

4. **Collection devices:** Motorboats and Dongies are required for bulk collection of this material.

5. **Storage:** Various parts of fruit are collected, chopped into small pieces for shade drying. After proper drying the material is stored in cold room for further work.

6. **Physical Characteristics:** Mangrove trees upto 10 m tall with bijugate leaves, leaflets obovate, entire rounded at apex, tapering at base, fruits large, globose, splitting tardily into 4 valves, seeds 10-15 in number, pyramidal shaped with a corky testa.

7. **Quality control specifications:**
   
<table>
<thead>
<tr>
<th>Specification</th>
<th>Specification Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical status</td>
<td>Dark brown solid material</td>
</tr>
<tr>
<td>Ash content</td>
<td>not more than 35.0%</td>
</tr>
<tr>
<td>Acid insoluble ash</td>
<td>not more than 3.0%</td>
</tr>
<tr>
<td>Ethyl acetate extractive</td>
<td>not less than 2.5%</td>
</tr>
<tr>
<td>Butanol extractive</td>
<td>not less than 10.0%</td>
</tr>
<tr>
<td>HPLC-fingerprinting</td>
<td>HPLC chromatogram at λ=230 nm (gradient) with one identified marker (Gedunin) (Rt. ~ 10 min)</td>
</tr>
</tbody>
</table>
Materials and Methods

Evaluation of Plant extracts/fractions for Antihyperglycaemic activity
Sucrose loaded rat model: Single dose protocol

Male albino rats of Charles Foster strain of average body weight 160±20 g were selected for study. The blood glucose of each animal was checked after 16 hours starvation using glucostrips (Boehringer Mannheim). Animals showing blood glucose between 60 to 80 mg/dl (3.33 to 4.44 mM) were divided into groups of five to six animals in each. Rats of experimental group were administered suspension of the desired test extract orally (made in 1.0% gum acacia) at 250mg/kg-body weight. Animals of control group were given an equal amount of 1.0% gum acacia. A glucose load (2.0 gm/kg) was given to each animal orally exactly after 30 min post administration of the test sample/vehicle. Blood glucose profile of each rat was again determined at 30, 60, 90 and 120 min post administration by glucostrips. Food but not water was withheld from the cages during the course of experimentation. Quantitative glucose tolerance of each animal was calculated by Area under curve method. Comparing the AUC of experimental and control groups determined the percentage antihyperglycaemic activity. Statistical comparison was made by Dunnett's test.

Streptozotocin-induced diabetic rats: Single dose protocol

Male albino rats of Sprague Dawley strain of body weight 160±20g were selected for this study. Streptozotocin (Sigma, USA) was dissolved in 100 mM citrate buffer pH 4.5 and calculated amount of the fresh solution was injected to overnight fasted rats (60 mg/kg) intraperitoneally. Blood was checked 48 h later by glucometer (Boehringer Mannheim) and animals showing blood glucose value between 250 to 450 mg/dl (13.9 to 25 mM) were included in the experiments and termed diabetic. The diabetic animals were divided into groups consisting of three to six animals in each group. Rats of experimental groups were administered suspension of the desired test extract orally (made in 1.0% gum acacia) at 250 mg/kg-body weight. Animals of control group were given an equal amount of 1.0% gum acacia. Blood glucose level was again checked at 1, 2, 3, 4, 5, 6, 7 and 24 hours, respectively post administration of gum acacia/test extract. Food but not water was withheld from the cages during the
experimentation. The average % fall in blood glucose values from 1 to 7 hours by test extracts were calculated according to the area under curve (AUC) method. Comparing the AUC of experimental and control groups determined the percent antihyperglycaemic activity. Statistical analysis was done by Dunnett's test.

In-vitro determinants for the evaluation of antidiabetic activity

Preparation of α-Glucosidase from Rat Intestinal Mucosa

This was done according to a slight modification of the procedure reported by Cogoli et al. (1972). Intestine of male albino rats (CF strain, average body weight 200±20 g) were excised opened and the mucosa was collected and pooled. A 10% homogenate was prepared in 150mM KCl using Potter Elvejhem glass homogenizer fitted with Teflon pestle. The homogenate was centrifuged at 1,000xg for 15 min and the supernatant was decanted and stored at 4°C. The supernatant was dialyzed at 4°C against 50 mM Tris-HCl buffer pH 7.0 with two to three changes of buffer. The dialyzed supernatant was saturated with ammonium sulphate to a final concentration of 30%. The sample was kept at 4°C overnight and then centrifuged to collect the precipitate and the supernatant separately. The 30% ammonium sulphate saturated supernatant was further saturated to 60% with ammonium sulphate. Again the precipitate and supernatant were separated by centrifugation. Finally the 60% ammonium sulphate saturated supernatant was further saturated to 100% with further addition of ammonium sulphate. The precipitate and supernatant was once again separated and all the samples were analysed for α-glucosidase activity using p-nitrophenyl-α-D-glucopyranoside (PNPG) as substrate. The enzyme activity was found maximum in 60-100% ammonium sulphate precipitate (Table-1) and this fraction was stored at 4°C and used as a source of enzyme for studying the effect of test compounds.
Materials and Methods

Table-1: Purification steps of α-Glucosidase from Rat Intestinal mucosa

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total Protein mg/ml</th>
<th>Total Activity (nmol/min)</th>
<th>Specific activity (nmol/min/mg protein)</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1.50±0.03</td>
<td>433.4±0.30</td>
<td>288.94±10.1</td>
<td>1.0</td>
</tr>
<tr>
<td>1000 x g supernatant</td>
<td>0.74±0.09</td>
<td>358.02±8.07</td>
<td>483.81±89.7</td>
<td>1.68</td>
</tr>
<tr>
<td>0-30% ammonium sulphate precipitate</td>
<td>0.18±0.02</td>
<td>99.48±0.78</td>
<td>552.69±38.9</td>
<td>1.91</td>
</tr>
<tr>
<td>30-60% ammonium sulphate precipitate</td>
<td>0.31±0.03</td>
<td>224.24±2.83</td>
<td>723.36±94.2</td>
<td>2.50</td>
</tr>
<tr>
<td>60-100% ammonium sulphate precipitate</td>
<td>0.60±0.06</td>
<td>822.55±3.23</td>
<td>1370.91±53.8</td>
<td>4.74</td>
</tr>
<tr>
<td>100% saturated ammonium sulphate supernatant</td>
<td>0.12±0.02</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
</tbody>
</table>

Determination of α-Glucosidase Inhibitory Activity

100 µl of purified α-glucosidase (0.1 mg/ml) and 25µl of glutathione (1.0 mg/ml) were added and the total volume was made up to 1 ml by adding 0.67 mM phosphate buffer (pH 6.8). The reaction mixture was incubated at room temperature for 10 min with the desired test compound (10 mM) dissolved in 100% DMSO. Reaction was started by the addition of 50 µl p-nitrophenyl-α-D-glucopyranoside (3 mg/ml) and increase in absorbance was recorded at 400 nm for a period of 5 min at the interval of 30 sec (Lebovitz, 1997; Chiba, 1997).

Aldose Reductase

Preparation of Aldose reductase from rat erythrocyes

10 ml blood was withdrawn from overnight fasted male SD (Sprague Dawley) rats in heparinized vials and was incubated with 50 mM D-glucose at 37°C for 6 hours in a Dubnoff metabolic shaker with gentle shaking. Subsequently the blood was
centrifuged at 1500xg for 10 min at 4°C and the erythrocyte were separated and washed three times with Phosphate Buffered Saline (PBS) [0.145 M NaCl/0.1 M Potassium Phosphate pH 7.4, 9:1 v/v]. Washed erythrocytes were hemolyzed in 4 volumes of Buffer A (10 mM Potassium Phosphate pH 6.0/5 mM 2-mercaptoethanol). The hemolysate was centrifuged at 10,000xg for 1 hour in cold and dialyzed overnight against 100 volumes of the same buffer A. Dialyzed hemolysate was again centrifuged at 10,000xg for one hour at 4°C and then supernatant was used as source of enzyme (Srivastava et al., 1986).

Assay mixture for the determination of aldose reductase activity in a 1.0 ml final volume contained 0.1 mM NADPH, 50 mM Potassium Phosphate buffer pH 6.0, 10 mM DL-glyceraldehyde, 5 mM 2-mercaptoethanol, 0.4 M LiSO₄ and an appropriate amount of the enzyme. The effect of library compounds was studied by incubating 250 μg of library compounds in the reaction system and determining the residual enzyme activity. The reaction was started by the addition of 0.1 mM NADPH (Srivastava et al., 1986; Saab et al., 1999). The decrease in absorbance at 340 nm was measured at 37°C for 4 min. One unit of enzyme activity was defined as μmol of NADPH utilized/min at 37°C.

Protein Tyrosine Phosphatase (PTPase)

Preparation of Protein Tyrosine Phosphatase

Animal was sacrificed by cervical dislocation; tissues like liver, kidney and muscle were excised, washed in cold saline and tissues were homogenised in buffer containing HEPES 25 mM pH 7.0, EDTA 1 mM, DTT 1 mM, PMSF 0.0025% (w/v) and Aprotinin 1 U/ml using a Potter Elvejhem homogeniser. The homogenates were centrifuged at 600xg for 10 min. Pellet was discarded and supernatant was again centrifuged at 10,000xg for 20 min to separate mitochondria. The post mitochondrial supernatant was then centrifuged at 100,000xg for 45 min. The final supernatant was designated as the cytosolic fraction and the pellet was designated as the particulate fraction. The particulate fraction was solubilized before assay by stirring at 4°C for 45 min. in 25 mM HEPES pH 7.0, 2.5% PMSF and 1% Triton X-100.
PTPase assay was performed according to Goldstein et al. (2000); assay mixture was made in a final volume of 1.0 ml containing 10 mM pNPP in 50 mM HEPES buffer pH 7.0, with 1 mM DTT and 2 mM EDTA. The reaction was stopped by the addition of 500 µl of 0.1 N NaOH and the absorbance was determined at 410 nm. A molar extinction coefficient of $1.78\times10^4$ M$^{-1}$cm$^{-1}$ was utilized to calculate the concentration of the p-nitrophenolate ions produced in the reaction mixture. One unit of enzyme activity was defined as the amount of enzyme required to form 1 µmol p-nitrophenolate ion per min during the initial reaction period.

**Measurement of insulin resistance**

The steady state basal plasma glucose and insulin concentrations are determined by their interactions in a feed back loop. A computer-solved method has been used to predict the homeostatic concentrations, which arise from varying degrees of β-cell deficiency and insulin resistance. Comparison of fasting values of glucose and insulin with model's predictions allows a quantitative assessment of the contributions of insulin resistance and β-cell deficiency to the fasting hyperglycaemia (Mathews et al., 1985). A method was devised for the measurement of insulin resistance, known as Homeostasis Model Assessment (HOMA), which was based on the relationship between fasting blood glucose and fasting insulin level. This measure had been used in various population trials including UKPDS. According to this model, insulin resistance was measured by the formula:

$$\text{Insulin resistance (HOMA)} = \text{Insulin/ 22.5 } \times \text{e}^{-\text{ln glucose}}$$

$$= I_0 \times \ln G_0 / 22.5$$

β-cell dysfunction = $(20 \times I_0) / (G_0 - 3.5)$

Where $G_0$ was fasting glucose concentration (mM) and $I_0$ was fasting insulin concentration (µU/ml)
Development of animal models for evaluation particularly type II diabetes mellitus and insulin resistance

Neonatal STZ rat model

One-day old pups of Wistar strain rats were taken along with mother. Streptozotocin was given subcutaneously to these pups at 120 mg/kg dose level (Portha et al., 1974; Weir et al., 1982). Weaning was done on 4th week; males and females were separated on 5th week. OGTT was performed on 6th, 8th, 11th, 13th, 15th, 18th, 20th and 22nd weeks post STZ-injection; OGTT was found to be maximally impaired and insulin level was high as compared to normal control on 22nd week, therefore this week was selected to start the treatment of CDR-134/ CT-1 once daily for 5 weeks and 3 weeks respectively.

Fructose-enriched diet fed rats

Control group of male Sprague Dawley rats were fed either with standard diet (59% Starch, 29% Casein and 4% Fat) and experimental group of fructose-enriched diet (66% Fructose, 22% Casein and 12% Fat) (Yagi et al., 1995). This feeding was continued for 10 weeks and during feeding Glucose Tolerance Test was performed on 2, 4, 6, 8 and 10 week. On 11th week animals of Fructose fed group showed a maximum level of glucose intolerance as depicted in the graph (p<0.05). However, glucose tolerance curve of standard diet group was found to be normal. Further animals were selected from the fructose fed group (n=12) and divided into groups each containing six animals, were termed as Fructose-enriched Control and Fructose-enriched + CDR-134/ CT-1. One more group was made of Standard diet fed rats, termed as a Normal Control.

Protocol for the evaluation of the effect of CDR-134 and CT-1 in animal models for type 2 diabetes and insulin resistance

CDR-134, a marine based antidiabetic agent, was fed to fructose fed rats and neonatal rats for five weeks once daily. Body weight was recorded on alternate days. An oral glucose tolerance test was performed weekly and on the last week of feeding after OGTT, blood was collected from retro orbital plexus; each animals were
Materials and Methods

sacrificed using cervical dislocation and tissues like liver, kidney and muscle were kept at \(-80^\circ\text{C}\) for biochemical studies.

CT-1, a plant based antidiabetic agent, was given orally to fructose fed rats and neonatal rats to see the efficacy of this agent in animal model for type 2 diabetes and insulin resistance. This test drug was fed for three weeks once daily. Oral glucose tolerance test (OGTT) was performed weekly and body weight was recorded on alternate days. Finally on the last day of treatment with CT-1, blood was collected and each animal were sacrificed using cervical dislocation method and tissues like liver, kidney and muscle were stored at \(80^\circ\text{C}\) for further studies.

Preparation of Homogenate

The overnight-starved animals were sacrificed by cervical dislocation. The liver, kidney and muscle were quickly excised, washed thoroughly with chilled normal saline and kept at \(-80^\circ\text{C}\). Tissues were taken out and thawed at the time of enzymatic studies. A 10\% homogenate was prepared of liver, kidney and muscle in 150 mM KCl with the help of Potter Elevejham homogenizer with Teflon pestle. This homogenate was termed as crude homogenates. These crude homogenates were centrifuged at 3000\(\times\)rpm for 20 min in cold, cell and debris free supernatant was used for determining the activity of Glycogen phosphorylase, Glucose-6-phosphatase, Pyruvate kinase, Phosphofructokinase, Phosphoenolpyruvate Carboxykinase, Lactate dehydrogenase and Protein tyrosine phosphatase.

Blood Glucose

The glucose content of the blood was determined enzymatically using glucose oxidase and peroxidase according to the method of Bergmeyer and Benut (1963). To 0.2 ml aliquot of deproteinized blood was added 1.25 ml buffer enzyme mixture containing phosphate buffer (120 mM, pH 7.0), peroxidase (40 \(\mu\)g/ml), glucose oxidase (250 \(\mu\)g/ml) and the chromogen, O-dianisidine-di-HCl (10 \(\mu\)g/ml). The light orange colour of the complex was read after 20 min at 436 nm in Shimadzu UV-190 double beam spectrophotometer. Glucometers were also used using glucostrips (Boehringer Mannheim).
Materials and Methods

Serum Insulin

Serum insulin was estimated using the radioimmunoassay kit (RIAK-1) provided by BARC, Mumbai, by the following method. EDTA-phosphate buffer 0.3 ml (0.01 M EDTA, 0.04 M phosphate, pH 7.4), containing 0.5% BSA (w/v) was mixed with 0.1 ml of standard insulin or unknown samples and 0.1 ml of antibody (antiinsulin guineapig serum diluted 1:1,000,00 times) and kept overnight at 4°C. 0.1 ml of 1125-insulin (about 1×10^6 cpm) was then added to the sample mixture and was again kept for 5 min at 4°C. Subsequently, 0.1 ml of the second antibody (anti-guineapig-globulin raised in rabbit) was added. Antibody bound reactivity was separated from that of unbound one by precipitation in 12% polyethylene glycol 6000 and counted in Minnigamma counter (Wallac, LKB) according to Wilson and Miles (1977).

Pyruvate kinase (ATP: Pyruvate phosphotransferase, EC 2.7.1.40)

It was assayed according to Bucher and Pfeiderer (1955). Their procedure was modified by using tris-HCl buffer in place of triethanolamine. The reaction mixture contained tris-HCl buffer 200 mM, pH 7.4; KCl 100 mM; MgCl₂ 10 mM; ADP 5 mM; PEP 5 mM; LDH 4 units; NADH₂ 0.24 mM and enzyme protein. Optical density was measured at 340 nm at 30 sec interval for 3 min.

PEP Carboxykinase (GTP: Oxaloacetate carboxylase (phosphorylating), EC 4.1.1.32)

It was assayed according to Ward et al., (1969). The reaction mixture contained tris-HCl buffer 200 mM, pH 7.4; MnCl₂ 10 mM; NaHCO₃ 20 mM; GDP 1 mM; MDH 9 units; PEP 5 mM; NADH₂ 0.24 mM and enzyme protein. Optical density was measured at 340 nm at 30 sec interval for 3 min.

Glucose-6-phosphatase (D-glucose-6-phosphate phosphorylase; EC 3.1.3.9)

Glucose-6-phosphatase activity was determined according to the method of Hubscher and West (1965). The assay system contained 0.3 M citrate buffer pH 6.0; EDTA 28 mM; NaF 14 mM, 400 µl of glucose-6-phosphate 200 mM in a total volume of 1.0 ml. The reaction was started with an appropriate amount of enzyme.
The mixture was incubated at 37°C for 30 min after which the reaction was stopped by addition of 1.0 ml of 10% TCA. Protein free supernatant was taken after centrifugation at 1000xg for 15 min for the estimation of Pi according to the method of Taussky and Shorr (1953).

**Glycogen phosphorylase (α-1,4, glucan: orthophosphate glucosyl transferase, EC 2.4.1.1)**

It was assayed according to Rall et al. (1957). Assay mixture (0.4 ml) contained 0.2 ml mixture A [containing glycogen (57 mg); G-1-P (188 mg); NaF (42 mg) and 5-AMP (4 mM) in 10.0 ml distilled water] and 0.1 ml B, enzyme protein (80-100 μg). It was incubated at 37°C for 30 min; the reaction was stopped by 0.1 ml TCA (5% w/v) and 0.4 ml sodium acetate (100 mM) was added to prevent spontaneous hydrolysis of G-1-P present in the incubation mixture. Protein free supernatant was taken after centrifugation at 1000xg for 15 min for the estimation of Pi according to the method of Taussky and Shorr (1953).

**Phosphofructokinase (ATP: F-6-P-1-phosphotransferase, EC 2.7.1.11)**

It was assayed by the method of Racker (1947). The reaction mixture contained tris-HCl buffer (pH 8.6) 200 mM, MgCl₂ 10 mM, F-6-P 10 mM, ATP 10 mM, NADH₂ 0.24 mM, aldolase 0.6 units, TPI 17 units, GDH 2 units, and enzyme protein (250-350 mg). Optical density was measured at 340 nm at 30 sec interval for 3 min.

**Lactate dehydrogenase (L-lactate: NAD oxido reductase, EC 1.1.1.27)**

Lactate dehydrogenase activity was determined according to Kornberg (1955). The assay mixture contained 200 mM tris HCl buffer pH 7.4, 100 mM KCl, 5 mM sodium pyruvate and enzyme protein. The reaction was started by the addition of 0.24 mM NADH and change in absorbance was recorded at 340 nm for 3 min at 30 sec interval.

**Protein tyrosine phosphatase**

Modifying Goldstein et al. method (2000), assay was performed in a final volume of 1.0 ml at 37°C for 30 min in reaction buffer containing 10 mM pNPP in 50
mM HEPES buffer pH 7.0 with 1 mM DTT and 2 mM EDTA. The reaction was stopped by the addition of 500 µl of 0.1 N NaOH and the absorbance was determined at 410 nm. A molar extinction coefficient of $1.78 \times 10^4$ M$^{-1}$cm$^{-1}$ was utilized to calculate the concentration of the p-nitrophenolate ion produced in the reaction mixture.

**Expression of enzyme units**

One unit of the enzyme is the amount required to convert the transformation of one mmol of substrate or the formation of one mmol of product per min under specified experimental conditions. The extinction for oxidation or reduction of pyridine nucleotides was measured at 340 nm using silica cuvettes of 1 cm path length and extinction coefficient of $6.22 \times 10^6$ cm$^2$/mole (Horecker and Kornberg, 1948) was used to calculate reduced NAD/NADP.

Activity of PK, PFK, PEPCK, LDH and AR were expressed as nmol of NAD formed/min/mg of protein.

Blood Glucose values are expressed as mM.

Serum insulin values are expressed as µU/ml

Glucose-6-phosphatase and glycogen phosphorylase activities were expressed as nmol Pi released/min/mg protein.

Protein tyrosine phosphatase activity was expressed as nmol of p-nitrophenol formed/min/mg protein

**Statistical analysis**

Each biochemical parameter was expressed in terms of Mean±SD. Groups were compared by analysis of variance with student ‘t’ test. Significance difference were set at * - p<0.05; ** - p<0.01 or *** - p<0.001.