5. **Materials and Methods**

5.1. **Materials**

HPLC standardized TSE, FURA-2-acetoxy methyl ester (FURA 2/AM) [Merck, Germany, catalog # 344905], insulin ELISA kit (Mercodia, Sweden, catalogue 10-1250-01), nitric oxide assay kit (Biovision, USA, catalog # 262-200), collagenase type V (Sigma Aldrich, Germany), culture medium RPMI 1640 (Sera Laboratories International Ltd, UK), benzylpenicillin (Sigma Aldrich, Germany), streptomycin (Sigma aldrich, Germany), L-glutamine, and heat-inactivated fetal calf serum (Sigma Chemical Co.), Apo-BrdU-IHC™ *In Situ* DNA Fragmentation Assay Kit (Biovison, USA, Cat#K403-50), TNF-α (Raybiotech Inc. cat#ELR-TNF-α-001), streptozotocin (Sigma Chemicals U.S.A), metformin (Ranbaxy Ltd, Gudgaon, India), glimepiride (Batch no. P010743397) manufacturer: Hetero Labs Ltd. which, was provided by: Panacea Biotech Ltd. Malpur, Baddi, Solan (India), α-glucosidase (Bioassay, USA), adiponectin (Mediagnost, Germany), HDL and LDL/VLDL cholesterol quantification Kit (Catalog #K613-100; Biovision, USA), Taq DNA polymerase (Bioline Ltd, Catalog-A5209, 0200), horseradish peroxidase conjugated IgG (Jackson Immunoresearch Laboratories, USA), anti-GLUT antibodies (Abcam, UK), deoxycorticosterone acetate (DOCA, HiMedia, India), superoxide dismutase (SOD), glutathione, and catalase (Biovision, USA).

5.2. **Collection and authentication of crude drugs**

Seeds of *T. indica* were collected from Kharibauli, New Delhi in the month of May and its authentication was done by Dr. Roshini Nayar, Scientist at the National Bureau of Plant Genetic Resources, New Delhi, India. (Voucher No. NHCP/NBPGR/2010-52.)

5.3. **Preparation of the extract**

Accurately weighed amount (10 g) of the powdered drug was added to 100 ml of solvent in a round bottom flask. The contents of the flask were refluxed for 1 hour on water bath and filtered through Whatman filter paper. Filtrate was concentrated under reduced pressure to obtain a residue. The residue called as extract was used to perform the chemical tests.

5.4. **Chemical tests for the identification of active constituents**

Chemical tests were performed on aqueous and alcoholic extracts of drugs to identify the constituents (Evans, 2002; Kokate, 2006).
5.4.1. Test for Carbohydrates

**Molisch test:** to small quantity of the extract, 2-3 drops of α-naphthol was added and 1 ml of concentrated sulphuric acid was added dropwise along the walls of the test tube. Appearance of purple-violet ring at the junction of two layers indicates the presence of carbohydrates.

5.4.2. Test for Steroids

**Lieberman- Burchard’s test:** The extract was treated with few drops of acetic acid, boiled and cooled. Few drops of concentrated sulphuric acid were added along the walls of the test tubes. A brown ring appears at the junction of the two layers. Presence of green colour in the upper layer indicated the presence of steroids, whereas, deep red colour in the lower layer indicates the presence of tri-terpenoids.

5.4.3. Test for alkaloids

**Mayer’s test:** To the extract, few drops of Mayer’s reagent (Potassium mercuric iodide) were added. Formation of white creamy precipitate indicates the presence of alkaloids.

**Dragendorff’s test:** To the extract, few drops of Dragendorff’s reagent (Potassium bismuth nitrate) were added. Formation of orange-yellow precipitate indicates the presence of alkaloids.

**Test for coumarin glycosides:** The extract was made alkaline with 10% solution of potassium hydroxide. Appearance of blue-green fluorescence indicates the presence of coumarins.

5.4.4. Test for flavonoids

**Shinoda test:** To the extract, 5 ml of 95% ethanol was added. To this, few drops of concentrated hydrochloric acid and 0.5 g of magnesium turnings were added. Appearance of orange, pink, red colour indicates the presence of flavonoids.

5.4.5. Test for tannins and phenolic compounds

**Ferric chloride test:** To the extract, few drops of 5% ferric chloride were added. Formation of deep blue-black colour indicates the presence of tannins and phenolic compounds.

5.4.6. Test for saponin glycosides

**Foam test:** The extract were taken in test tubes with small quantities of distilled water and shaken well. Formation of rich lather indicates the presence of saponins.
5.4.7. Test for proteins

Millon’s test: To 2 ml of the test solution (extract), 2 ml of Millon’s reagent was added. Formation of white precipitate which turns brick-red upon heating indicates the presence of proteins.

5.4.8. Test for starch

Iodine test: To the extract, weak aqueous iodine solution was added. Appearance of blue colour indicates the presence of starch.

5.4.9. Test for amino acids

Ninhydrine test: To the test solutions, Ninhydrine solution was added and boiled. Appearance of violet colour indicates the presence of amino acids.

5.5. Determination of percentage (%) of tannins in drugs

5.5.1. Preparation of 0.1N KMnO4 (Indian Pharmacopoeia 1996)

About 0.3 gm of KMnO4 was weighed and was dissolved in sufficient water to produce 100 ml.

5.5.2. Standardization of 0.1N KMnO4 using sodium oxalate

Some analytical grade sodium oxalate was dried at 105-110°C for 2 hours, and it was then allowed to cool in a covered vessel in a desiccator. From this about 0.3 g of the dried sodium oxalate was weighed out into a 600 ml beaker. To it, 240 ml of recently prepared distilled water was added, and 12.5 ml of concentrated sulphuric acid or 250 ml of 1M sulphuric acid was carefully added. It was cooled to 25-30°C and was stirred until the sodium oxalate dissolved. 90-95% of the required quantity of permanganate solution was added from a burette at the rate of 25-35 ml/minute while it was stirred slowly. It was heated to 55-60°C (using thermometer as stirring rod), and the titration was completed by adding permanganate solution until a faint pink colour persisted for 30 seconds. The last 0.5-1.0 ml was added dropwise with particular care which allowed each drop to become decolorized before the next was introduced.

5.5.3. Procedure for determination of tannins (%) in crude drugs (Samant, 1971)

A weighed quantity (1 gm) of the sample of drug powder was accurately weighed and transferred to a 250 ml glass stoppered glass. 100 ml of water was added to it and the flask was shaken for 1 h and was left overnight. The solution was filtered through Whatman filter
paper no.1 and first 20 ml of the filterate was discarded. Rest 10 ml of the filterate was transferred to a 1 litre conical flask. To it 750 ml water and 25 ml of indigosulphuric acid was added. It was then titrated with 0.1 N KMnO4 and shaken till a golden yellow end point was reached (T2). Blank titration (T1) was also performed.

Preparation of indigosulphuric acid indicator: - 1.2 gm of indigo carmine AR was dissolved in 10 ml concentrated sulphuric acid and was diluted to 250 ml with distilled water.

Each ml of 0.1 normal KMnO4 = to 0.004157 gm of tannins.

\[
\text{% of tannins} = \frac{(T2 - T1) \times \text{actual normality} \times 0.004157 \times 100}{W \times 0.1}
\]

Where W=weight of material taken.

### 5.6. Determination of heavy metals (Mukherjee, 2005; Indian Herbal Pharmacopoeia, 1999).

#### 5.6.1. Processing of plant material for the determination of lead, cadmium and mercury by Nitric acid digestion method (Hseu 2004).

This approach was partly modified from that of Zheljazkov and Nielson (1996). One gram of sample was placed in a 250 ml digestion tube and 10 ml of concentrated HNO3 was added. The sample was heated for 45 min at 90°C, and then the temperature was increased to 150°C at which the sample was boiled for at least 8 h until a clear solution was obtained. Concentrated HNO3 was added to the sample (5 ml was added at least three times) and digestion occurred until the volume was reduced to about 1 ml. The interior walls of the tube were washed down with a little distilled water and the tube was swirled throughout the digestion to keep the wall clean and prevent the loss of the sample. After cooling, 5 ml of 1% HNO3 was added to the sample. The solution was filtered with Whatman No. 42 filter paper and <0.45 lm Millipore filter paper. It was then transferred quantitatively to a 25 ml volumetric flask by adding distilled water.

The concentrations of Cd, and Pb in the final solutions were determined by an atomic absorption spectrometer (AAS) (Elementar).
5.6.2. Processing of plant material for determination of arsenic

A weighed quantity (50 g) of powdered drug was taken into a 1 liter Kjeldhal flask. 25 ml of double distilled water, 50 ml of nitric acid and 20 ml of sulfuric acid were added to the flask. Contents of the flask were heated slowly and carefully to avoid excess foaming. Nitric acid equivalent to 100 g/l was added drop by drop until all the organic matter got destroyed. This was identified by no darkening of the solution by further addition of nitric acid and continued heating. The flask was then allowed to cool. 75 ml of distilled water and 25 ml of ammonium oxalate (25 g/l) were added to the flask to expel nitrogen oxide from the solution. Heated again and cooled. The contents were transferred into a 250 ml volumetric flask and made-up the final volume with double distilled water. The solution was filtered through membrane filters. The processed sample was subjected to AAS for determination of Arsenic. Three determinations were done under each sample to get concordant results.

5.5. Determination of microorganisms

(WHO/PHARMA/92.559/rev. 1, 1992; Indian Herbal Pharmacopoeia, 1999)

5.7.1. Test for Escherichia coli

A weighed quantity (10 g) of powdered drug was diluted upto 100 ml with lactose broth and incubated at 37°C for 4 h. 1 ml of this stock solution was diluted with 9 ml of Mac Conkey broth to get 100 mg, 10 mg and 0.1 mg of drug concentration in culture media. Diluted sample tubes were incubated at 37°C for 24 to 48 h. 1 ml of all the diluted samples were sub-cultured on a plate containing Mac Conkey agar media and incubated at 37°C for 18 to 24 h. Growth of well developed reddish colonies indicates the presence of Escherichia coli.

5.7.2. Test for Salmonella typhi

A weighed quantity (10 g) of powdered drug was suspended and made upto 100 ml with lactose broth and incubated at 37°C for 5 h for enrichment of culture. 10 ml of this was made upto 100 ml with tetrathionate bile brilliant green broth and incubated at 37°C for 24 h. 1 ml of this sample was plated on xylose deoxycholate agar media and incubated at 37°C for 48 h. Small transparent organisms surrounded by pink zone indicates the presence of Salmonella.

5.7.3. Test for Pseudomonas aeruginosa

A weighed quantity (10 g) of powdered drug was suspended and made upto 100 ml with nutrient agar broth and incubated at 37°C for 4 h. 1 ml of this was diluted upto 100 ml with soybean-casein digest broth and incubated at 37°C for 48 h. 1 ml of this sample was plated on
centrimide agar media and incubated at 37°C for 48 h. Gram –ve bacilli usually appears in green fluorescence indicates the presence of Pseudomonas aeruginosa.

5.7.4. Test for *Staphylococcus aureus*

A weighed quantity (10 g) of powdered drug was suspended and made up to 100 ml with nutrient agar broth. It was incubated at 37°C for 4 h. 1 ml of this sample was plated on Baird-Parker agar media and incubated at 37°C for 48 h. Black colonies of gram +ve cocci often surrounded by clear zones indicates the presence of *Staphylococcus aureus*.

5.8. Preparation of aqueous extract of seeds of *Tamarindus indica*

Plant material (seed) was spread as a layer on stainless steel trays and dried at a temperature less than 40°C. Aqueous extract of the seeds of *T. indica* was prepared using the method mentioned by National Institute of Health and Family Welfare, India (Khillare, 2000). After incubation for 2 days at 40°C, the seeds of *T. indica* were powdered in a grinder. 100 g powder suspended in 500 ml redistilled water and the extraction was performed in Soxhlet apparatus for 18 h. A deep brown aqueous extract was obtained which was filtered using a coarse sieve filter paper. The filtrate was then dried under reduced pressure and finally lyophilized. Phytochemical screening and standardization of the extract was done before commencement of the in vivo study (Maiti et al. 2005). The aqueous extract yielded 2.8 g lyophilized powder (0.28%) from 1 kg of Tamarind seeds.

5.9. HPTLC profile of drug

As per shown in Table 1, the HPTLC profiling of the herbal drugs was carried out at 254 densitometry. (Mallavadhani and Sahu, 2003; Indian Herbal Pharmacopoeia, 1999). HPTLC plate, pre-coated with silica gel G F254 (E.Merck) (10X10 cm) of uniform thickness (0.2 mm) was activated at 110°C for 20 minutes. Two tracks each of sample and standard were spotted on HPTLC plate using Linomat 5 (Camag, Switzerland) applicator. The spotted plate was placed in developing chamber saturated with mobile phase. When the plate was developed approximately 3/4th of its length, it was taken out of the chamber and allowed to dry. The details regarding mobile phase and detection wavelength are mentioned in Table 1.
Table 1

<table>
<thead>
<tr>
<th>DRUG</th>
<th>SAMPLE PREPARATION</th>
<th>STANDARD PREPARATION</th>
<th>SOLVENT SYSTEM</th>
<th>DETECTION</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tamarindus indica</em> L.</td>
<td>1 g sample with 30 ml ether was sonicated, evaporated and the residue dissolved in 10 ml methanol</td>
<td>2 mg catechin &amp; epicatechin dissolved in 10 ml methanol to obtain 0.2 mg/ml</td>
<td>CHCl₃: methanol: acetic acid (8:2:0.08)</td>
<td>Densitometry at 254 nm</td>
</tr>
</tbody>
</table>

5.10. **Analytical high performance liquid chromatography (HPLC)**

5.10.1. **Standard solutions and sample preparation**

Standard stock solutions were prepared by accurately weighing 4.8 mg of catechin and epicatechin each into separate 100 ml volumetric flasks and dissolved in 100 ml of 0.2% aqueous acetic acid with the aid of sonication. Working standard solutions, 2.4-19.2 μg/ml, were prepared by dilution with water from the stock standard solutions. The calibration curve of Concentration vs. Area was plotted. The solutions were stored at 4°C. In this study, Freeze-dried material (TSE 5 g) was extracted with petroleum ether in a Soxhlet apparatus (3 h) to remove the lipid content. After drying, the solids were extracted with methanol (3 x 3 h). The dry powder (0.1 g) of TSE was sonicated with 10 ml methanol for 30 min. The extracted solution was then centrifuged at 2500 rpm at room temperature for 10 min. The supernatant was filtered through a 0.45 μm membrane filter prior to HPLC analysis (Owen et al. 2003).

5.10.2. **HPLC analysis**

HPLC was carried out on a Hitachi liquid chromatograph model 665-II equipped with an Autosampler (model 655A-40, Hitachi Ltd., Tokyo, Japan) fitted with a C-18 (250 x 4, 6.5 μ) column. For the separation of individual compounds, the mobile phase consisted of phosphoric acid dissolved in double distilled water (A) and acetonitrile (B) utilizing the following gradient: 95% A for 0.01 min, 70% A for 35 min, 70% A for 36 min, and 95% A for 40 min. The flow rate of the mobile phase in both the cases (A and B) was 1.0 ml/min. The column temperature and injection volume was maintained at 30°C and 10 μl,
respectively. The chromatogram was scanned up to 20 min, which was detected at 280 nm, followed by washing and reconditioning of the column. The analysis was done in triplicate.

5.11. Animals

To induce NIDDM, STZ (sigma chemicals, USA, 90 mg/kg) was administered i.p. to a group of 2 days old pups. The pups were weaned for 21 days, and 6 weeks after the injection of STZ, the animals were checked for fasting glucose level (FPG) ≥ 160 mg/dl were considered as diabetic. All rats were housed under conventional conditions with controlled temperature, humidity and light (12 h light–dark cycle), and were provided with a standard commercial diet and water (ad libitum). All experimental procedures were conducted according to the Institutional Animal Ethical Committee (Protocol No.2/2010/ IAEC/DIPSAR) and CPCSEA guidelines.

5.12. Study Design

A total of 40 male rats were used and were divided into five groups (n=8):

- **Group 1**: normal untreated rats/ normoglycemic control
- **Group 2**: diabetic control rats/ hyperglycemic control
- **Groups 3**: diabetic rats treated with TSE extract (120 mg/kg)
- **Group 4**: diabetic rats treated with TSE (240 mg/kg)
- **Group 5**: diabetic rats treated with metformin (100 mg/kg).

The treatment period was for 4 weeks. In the morning, after administration of last dose, blood samples were collected under fasting conditions and body weight was measured.

5.13. Body weight determination

Every week during the treatment the body weight of the animals in each group was recorded.


Every week, during the treatment the blood glucose of the animals in each group was measured by Accu-check Active glucose strips by pricking the tail of the rats. This is based on Glucose Oxidase- Peroxidase (GOD-POD) method.

5.15. Collection of blood sample

At the end of drug treatment, blood samples were withdrawn by retro-orbital sinus under mild
ether anaesthesia. The blood samples were collected into centrifuge tubes for serum and vacutainer which are precoated with EDTA as anti coagulant for plasma.

5.16. Separation of blood plasma and serum

**Serum.** The Blood samples were allowed to clot for 30 minutes before centrifugation at 3000 rpm for 15 min. Serum was removed and assayed immediately or samples were aliquoted or stored at -20°C. The sample was centrifuged again after thawing before the assay. Avoid repeated freeze-thaw cycles were avoided.

**Plasma.** Plasma was collected using heparin as an anticoagulant, which was followed by centrifuged at 3000 rpm for 15 min within 30 min of collection. The samples are assayed immediately or taken aliquot and stored at -20°C. The sample was centrifuged again after thawing before the assay. But repeated freeze-thaw cycles were avoided.

5.17. Collection of tissue

At the end of the experiments, animals were sacrificed after treatment by an overdose of anaesthetic ether. The pancreas was perfused by injecting 5-6 ml of 10% buffered formalin in the left ventricle of heart. The tissue was immediately excised and was washed with 10% formaldehyde in phosphate buffer and was preserved in the same and was stored at 4°C until processed for histopathology and the pH was maintained at 7.2.

5.18. Isolation and culture of islets

Animals were killed by decapitation and pancreatic islets of Langerhans isolated as previously described (Lacy and Kostianovsky., 1967), using digestion with collagenase obtained from Clostridium histolyticum (Sigma aldrich, Germany). Digestion and sedimentation of islets were carried out in Hanks’ solution containing 5.5 mM glucose. Islets were then handpicked under a stereomicroscope and transferred to Petri dishes containing culture medium RPMI 1640 (Sera Laboratoriesinternational Ltd, UK) supplemented with 100 U/ml benzylpenicillin (Sigma Aldrich, Germany), 0.1 mg/ml streptomycin (Sigma Aldrich, Germany), 2 mM L-glutamine, and 10% (vol/vol) heat-inactivated fetal calf serum (Sigma Chemical Co.). Islets were cultured free floating at 37°C, with an atmosphere of 5% CO₂/95% air. The time period of culture was 48 h except in time course studies of induction and reversibility.

5.19. Determination of Ca^{2+} oscillations

Pancreatic islet cells were prepared from adult rats and cultured overnight in RPMI 1640 culture medium supplemented with 10% (v/v) fetal calf serum, 100 i.u./ml penicillin, 100
µg/ml streptomycin and 60 µg/ml gentamycin. The cells were loaded with Ca\textsuperscript{2+} indicator by incubation with 2 µM fura-2/AM (Merck, India) for 45 min in the culture medium (which contained 11 mM glucose). They were then washed in medium containing 140 mM NaCl, 5.9 mM KCl, 1.28 mM CaCl\textsubscript{2}, 1.2 mM MgCl\textsubscript{2}, 25 mM HEPES, pH 7.4, and 1 mg/ml BSA. The cells (about 4×10\textsuperscript{6}) were suspended in 2.5 ml of fresh washing medium in a stirred cuvette placed in a spectrofluorometer (Perkin Elmer 50 B) for monitoring [Ca\textsuperscript{2+}], changes by 340 or 340/380 nm excitation ratio and emitted fluorescence 510 nm respectively at 37 °C. Intracellular Ca\textsuperscript{2+} levels could not be directly calibrated, and the [Ca\textsuperscript{2+}]i changes presented are relative changes in fluorescence. After a stable baseline was reached, glucose (20 mM) was added. The time that the cells spent in the absence of glucose, that is, the time needed for washing, suspending and establishment of baseline, was 10-15 min. Each experiment used a separate preparation of islets (Thomas et al., 1991).

5.20. Immunocytochemistry

Whole pancreas from rats was removed under anesthesia and fixed in 10% buffered formalin for 24 h. Tissues were dehydrated in graded series of alcohol, embedded in paraffin, sectioned at 5 µ thickness and used for immunostaining. The tissue sections were stained with haematoxylin and eosin while the remaining serial sections were used for immunostaining. Serial sections of the rat pancreas were immunostained by strepavidin-biotin peroxidase method using pre-diluted polyclonal antibodies. All sections were de-paraffinized in xylene bath to remove the excess wax. The slides were placed in two changes of absolute alcohol for 3 min each. The same procedure was repeated with 90% alcohol. The slides were placed in blocking reagent in order to block the endogenous peroxidase activity for 5 min, which was pre-diluted with 5 volumes of 100% ethanol. The slides were placed in two changes of 70% alcohol for 3 min each. The excess alcohol around the sections was removed and the slides were quickly immersed in Tris buffer, pH 7.6 for 5 min. Two drops of tissue conditioner was added and the sections were incubated for 5 min and then rinsed in buffer solution. Pre-diluted primary polyclonal anti-guinea pig antibody to insulin (1:1,000) [Genetex, USA] raised against human insulin was added to the sections and incubated for 1 h. The secondary antibody for insulin was anti-rabbit polyclonal antibodies. After incubation for half an hour, the sections were rinsed with Tris buffer, peroxidise solution was added, incubated for 30 min and later rinsed with the buffer. AEC (3-amino, 9-ethyl carbazole) chromogen substrate was added to the sections and was incubated for 15 min and rinsed with distilled water. The
sections were counter-stained with Harris haematoxylin for 45 sec to facilitate nuclear identification (Hsu et al., 1981).

5.21. DNA Fragmentation Assay

For detection and localization of apoptosis in the pancreas, we used the technique of TUNEL. Briefly, sections were deparaffinized, hydrated, and digested with proteinase K (20 μg/ml), and then added biotinylated dUTP to the 3’ end of DNA fragments by incubating sections in 0.05 mol/l Tris–HCl buffer (pH 7.6) with 0.03 U/μl TdT and 0.04 nmol/μl biotin-11-dUTP at 37°C for 1 h. The sections were rinsed in PBS. Endogenous peroxidase was blocked with 0.3% H₂O₂ in distilled H₂O. The sections were rinsed with PBS and covers with 2% blocking solution in 0.1 mol/l sodium maleate to reduce background staining. The sections were then incubated with avidin-peroxidase complexes in PBS (1:50) for 30 min and rinsed with PBS (3×5 min). Peroxidase activity was visualized with 3,3-diaminobenzidine until the brown product was clearly visible. The sections were then counterstained with methyl green. The positive apoptotic cells were the cells with brown nucleus (Matsuno, 1997).

5.22. RNA isolation and Real-time PCR analysis

Real-time PCR amplifications for GLP-1(Gene ID: 25051); GLUT–4 (Gene ID: 25139) and SREBP-1c (Gene ID: 78968) was conducted using Light-Cycler® 480 SYBR Green I Master (Roche) according to the manufacturer's instructions.

GLUT – 4:

5’-AGCGTAGGTACCAACACTTTC-3’ - Forward primer

&

5’-CCGCCCTTAGTTGGTCAGAAG-3’ – Reverse primer

GLP-1:

5’- CAACCGGACCTTTGATGAC-3’ - Forward primer

&

5’- GCTGTGCAGAACC GGTTACAC-3’ - Reverse primer

SREBP-1c:

5’- GGAGCCATGGATTGCACATT-3’ - Forward primer

&
Materials and Methods

5'- AGGAAGGCTTCCAGAGAGGA-3' - Reverse primer

β-actin:

5’-TCACCCACACTGTGCCCCATCTACGA-3’ - Forward primer

&

5’ CAGCGGAACCGCTCATTGCCAATGG-3’ - Reverse primer

Reaction conditions were 10X PCR buffer [20 mM Tris (pH 8.4), 50 mM KCl and 2.5 mM MgCl₂], 20 pM oligonucleotide, 300 μM dNTPs, 1:1000 SYBR Green I nucleic acid stain, 0.5 U Taq DNA polymerase (recombinant), and 50 ng templates cDNA in a 25 ml reaction volume. Thermal cycling conditions were 95ºC for a 3 min denaturation step, followed by 40 PCR cycles (94ºC for 30 s, 56ºC for 30 s and 72ºC for 1 min) and reactions were performed in a Light Cycler 480 (Roche) instrument. Fluorescence was detected at the end of the 56ºC segment in the PCR step. PCR products of β-actin primers gene, were used as internal standards. All assays were carried out in triplicate. Real-time PCR analysis and subsequent calculations were performed on Light-Cycler® 480 software (Roche, version LCS480 1.2.0.169).

5.23. Melting curve analysis

After 40 amplification cycles, a melting analysis was carried out to verify the correct product by its specific melting temperature (Tm). The thermal profile for melting curve analysis consisted of a denaturation for 1 min at 95°C, lowered to 55°C for 30 s and then increased to 95°C with continuous fluorescence readings.

5.24. GLUT-2 expressions in liver and pancreas; and GLUT-4 expressions in soleus muscle

Western Blot Analysis: 0.3 g of each liver, muscle and pancreas slices were homogenized with ice-cold 10mM Tris–HCl buffer (pH 7.4) containing 1mM EDTA 2Na, 250mM sucrose, 1mM phenylmethyl sulfonyl fluoride and 1000 U/ml aprotinin, the homogenates were centrifuged at 600 rpm for 10 min at 4°C, and the supernatant was then centrifuged at 11000 rpm for 15 min at 4°C. In this study, the precipitate was designated as the total membrane fraction of the liver. The protein concentration of each sample was determined using BCA Protein Assay Kit.

For determination of GLUT-2 protein expressions in pancreas and liver; and GLUT-4 protein expressions in skeletal muscle; each sample prepared was mixed with 1% sodium
dodecyl sulfate and 50 mM dithiothreitol, and the mixture was subjected to electrophoresis with 10% polyacrylamide gel and molecular standard markers according to the method of Laemmli, 1970. The separated proteins on the gel were electrotransferred to a polyvinylidene difluoride membrane. After blocking with 5% skim milk solution including 0.05% Poly(Oxyethylene) sorbitan monolaurate (Tween 20) overnight at 4°C, the membrane was reacted with anti-GLUT-2 antibody (Abcam, UK) & anti-GLUT-4 antibody (Abcam, UK) for 2 h. Subsequently, it was incubated with horseradish peroxidase conjugated IgG (diluted 1: 2000) [Jackson immunoresearch laboratories, USA] for 2 h at room temperature. The blots were detected with chemiluminescence reagents (Western Blot) and quantitation was performed using a densitometry system (Yoshihiko et al., 2007).

5.25. Gastrointestinal functions in diabetic rats

Either the vehicle or the test compound was administered to the diabetic rats that had been fasted overnight. 30 min later (compound treatment examination), glucose solution (0.2 g/ml glucose, 0.25% methylcellulose, 1 mg/ml phenol red and 10 mg/ml charcoal) was orally administered at a volume of 15 ml/kg. Under ether anesthesia, the stomach was ligated and removed, after which it was transferred to a tube and cryopreserved. The entire length of the small intestine (between the pylorus of the stomach and the end of the ileum) and the distance to the charcoal front was measured. The rats in the control group were given vehicle solution in order to measure the total amount of glucose solution injected into the stomach. At 15 min after administration, the pylorus of the stomach was ligated under ether anesthesia; after which the stomach was immediately removed, and small intestinal transit was checked. To measure the gastric emptying rate, 0.1 mol/l NaOH solution (5 ml) was added to the stomach sample and the mixture was homogenized. After centrifugation (3000 rpm, 10 min), 20% TCA solution (50 ml) was added to a 500-µl aliquot of the supernatant. The mixture was then stirred and centrifuged (15,000 rpm, 10 min). A 100 µl aliquot of the supernatant was then dispensed into a 96-well assay plate and to it, 0.5 mol/l NaOH solution (50 µl) was added. After stirring, phenol red concentration in the sample was determined using the phenol red (0–1000 µg/ml) calibration curve. The gastric emptying rate (%) was calculated using the following equation: [(Mean value of the control group) – (Mean value of the sample value)]/ (Mean value of the control group). The small intestinal transit rate (%) was calculated using the following equation: (Distance travelled by the charcoal front)/ (Entire length of the small intestine) (Matsuyama et al. 2008).

The α-glucosidase inhibitory activity was determined according to Earnst et al. (2005), by measuring the release of 4-nitrophenol from 4-nitrophenyl α-D-glucopyranoside (4-NPGP). The assay procedure was according to the protocol of a micro-well kit. TSE samples were prepared at dilutions of 1, 10, 100, 1000, 10000 µg/ml concentrations in 50 mM phosphate buffer (pH 7). The assay media contained 200 µl (1 mM) α-NPG substrate, 20 µl samples and 200 µl of calibrator. Absorbance of the reactants was measured at 405 nm using a microplate reader (Model 550, BIO-RAD Lab, Japan). The rate of reaction was directly proportional to the enzyme activity.

5.27. Determination of biochemical metabolic parameters

The quantitative measurement of all the following biomarkers studied in this investigation was done by ELISA technique and the standard operating procedures followed according to manufacturer’s protocol for the respective ELISA kits.

5.27.1. Serum nitric oxide

NO plays an important role in neurotransmission, vascular regulation, immune response and apoptosis. NO is rapidly oxidized to nitrite and nitrate which are used to quantitate NO production. BioVision’s Nitric Oxide Colorimetric Assay Kit used in present study to measure total nitrate/nitrite in a simple two-step process. The first step converts nitrate to nitrite utilizing nitrate reductase. The second step uses Griess Reagents to convert nitrite to a deep purple azo compound. The amount of the azochromophore accurately reflects nitric oxide amount in samples. The detection limit of the assay was approximately 0.1 nmole nitrite/well, or 1 µM.

5.27.2. Tumor necrosis factor α

TNF-α is secreted by macrophages, monocytes, neutrophils, T-cells, NK-cells following their stimulation by bacterial lipopolysaccharides, or any inflammatory reaction. TNF-α shows a wide spectrum of biological activities. It causes cytolysis and cytostasis of many tumor cell lines in vitro. Within hours after injection TNF-α leads to the destruction of small blood vessels within malignant tumors. TNF-α also enhances phagocytosis and cytotoxicity in neutrophilic granulocytes and also modulates the expression of many other proteins. The RayBio® Rat TNF-α ELISA (Enzyme-Linked Immunosorbent Assay) kit used in this study for the quantitative measurement of rat TNF-α in plasma/serum samples. This assay utilized an antibody specific for rat TNF-α coated on a 96-well plate. Standards and samples were
pipetted into the wells and TNF-α present in a sample was made bound to the wells by the immobilized antibody. The wells were washed and biotinylated anti-Rat TNF-α antibody was added. After washing away unbound biotinylated antibody, HRP conjugated streptavidin was pipetted to the wells. The wells were again washed, a TMB substrate solution was added to the wells and colour developed in proportion to the amount of TNF-α bound. The Stop Solution changes the colour from blue to yellow, and the intensity of the colour was measured at 450 nm.

5.27.3. Adiponectin

Adiponectin was described for the first time in the early 90th of the last century as an endocrine factor produced by adipocytes. Adiponectin is involved in regulation of energy- and fat metabolism. So its concentration in the circulation is said to reflect the risk of atherosclerosis and the degree of insulin resistance. Based on the high incidence of these diseases, adiponectin was and still is object of intensive research regarding the underlying biological mechanisms and regarding its value as biomarker. The influence of Tamarind seeds on adiponectin concentration in diabetic rats was determined by the Medignost ELISA kit which was based on the principle of Sandwich-Assay using two specific and high affinity antibodies. The adiponectin in the samples binds to the first antibody coated on the microtiter plate. In the following step the second specific anti-adiponectin-antibody binds in turn to the immobilised adiponectin. The second biotinylated antibody applied in a mixture with a Streptavidin-Peroxidase-Enzyme Conjugate. In the closing substrate reaction the turn of the colour was catalysed quantitatively depending on the adiponectin-level of the samples.

5.27.4. Insulin

The insulin concentration in the plasma samples was determined by Mercodia rat insulin ELISA assay, where solid phase two-site enzyme immunoassay based sandwich technique was used, in which two monoclonal antibodies were directed against separate antigenic determinants on the insulin molecule. Insulin in the sample reacts with anti-insulin antibodies bound to microtitration wells and peroxidase-conjugated anti-insulin antibodies in the solution.

5.27.5. Hemoglobin A1c

The HbA1c quantification by Biosystems (Spain) utilized the principle of turbidometric assay. After preparing the hemolysate using Tetradecyltrimethylammonium bromide (TTAB) as the detergent, the Hemoglobin A1c (HbA1c) concentration was quantified by a
turbidimetric inhibition immunoassay. First, by the addition of the sample to a reagent with antibodies against a specific site of HbA1c, soluble complexes formed by the union of two molecules appeared. After the addition to the reaction mixture of a second reagent formed by polihaptens, the excess anti-HbA1c antibodies form insoluble complexes, antibody-polyhapten, which was then determined turbidimetrically. The estimation of the HbA1c in percent was made by the measure of total hemoglobin concentration by spectrometry.

5.27.6. HDL/LDL

Regulation of HDL (high-density-lipoprotein)-cholesterol and LDL (low-density-lipoprotein)-cholesterol plays a central role in various disease developments. It is well known that low levels of HDL and high level of LDL are associated with an increased risk of diabetes and cardiovascular events. BioVision’s HDL and LDL/VLDL cholesterol quantification kit provided a simple quantification method of HDL and LDL/VLDL after a convenient separation of HDL from LDL and VLDL (very low-density lipoprotein) in serum samples. In the assay, cholesterol oxidase specifically recognizes free cholesterol and produces products which react with probe to generate colour ($\lambda = 570$ nm) and fluorescence (Ex/Em = 538/587 nm). Cholesterol esterase hydrolyzes cholesteryl ester into free cholesterol, therefore, cholesterol ester and free cholesterol can be detected separately in the presence and absence of cholesterol esterase in the reactions.

5.28. Statistical analysis

All values are means ± SEM. Data analysis was done with one-way analysis of variance (ANOVA) followed by Dunnet’s multiple test where a diabetic group was considered a positive control (Sigma Plot, USA 11). Group means were considered to significantly differ at $P < 0.05$, as determined by Dunnet’s multiple range analysis. In case of Islets Ca$^{2+}$ $P < 0.05$ and ** $P < 0.01$ for difference from 0 mM glucose, * $P < 0.05$ and ### $P < 0.001$ for difference from 20 mM glucose.