CHAPTER 3

RESULTS

The hypoglycemic agents available today for the treatment of diabetes were developed from \textit{in vivo} anti-diabetic drug discovery approach on animal models. The conventional methods are slow and time consuming. The present work aims towards a new approach to develop rapid \textit{in vitro} screens that are simple, and help to identify new bioactive molecules from plant materials involved in traditional diabetes treatment. These \textit{in vitro} screens also help to establish interplay between traditional medicine and modern science. In recent years there is a growing awareness in the screening of medicinal plants and the potential of plant products to develop into new therapeutic agents. Advancements in cell biology and molecular biology have enabled scientists to understand the possible mechanisms of cell function and to design target specific screens.

The main objective of this thesis is to develop \textit{in vitro} screens based on novel cellular markers to validate the anti-diabetic activity of traditional Indian plant materials used in diabetes treatment. \textit{In vitro} screens are developed to detect cellular and molecular markers known to play a role in glucose metabolism like i. glucose uptake into peripheral tissues ii. the expression of glucose transporters in different tissues iii. transporter protein level iv. rates of whole-body glucose metabolism and glucose-6-phosphate (G-6-P) concentrations (Cline \textit{et al.}, 1999). v. glycogen synthase activity. The above factors are known to be decreased in diabetics. The plant extracts are
used to modulate these activities by increasing the uptake of glucose, concentrations of glucose metabolites (G-6-P) and expression levels of glucose transporters.

Thus using above parameters, the objective was to develop \textit{in vitro} screens using appropriate cell lines or primary cultures and monitor whether these parameters are altered in the presence of plant extracts or not. This will provide a quick and reliable information. Further, once a particular plant is found to show such a property, it will be subjected to fractionation protocols and each fraction monitored at different levels of extraction. The purity of each compound in each fraction is identified by using the chromatographic techniques like TLC, HPLC and NMR. Suitable solvent systems were established initially by extracting at small scale in test tubes and individually evaluated for the bioactivity. Once anti-diabetic activity was established, large scale extraction was carried out by rotaevaporation, silica gel column chromatography to purify the compound for structural elucidation. This work led to the identification of bioactive molecules from plant materials and may help in anti-diabetic drug development programme. The scheme of bioassays and structure elucidation is shown in Fig. 3.

\section*{3.1 EXTRACTION OF BIOACTIVE COMPOUND}

The first step in the development of a new drug is the isolation of an active compound with therapeutic value, which might be advanced to clinical trials and possibly to commercial use (Grever \textit{et al.}, 1992). Extraction is very important and time-consuming activity involves isolating, separating and purifying bioactive compounds. Before going to extraction of active compound the plant materials were subjected to physical characterisation to assess the
Fig. 3  Scheme showing extraction, bioassays and identification of structure
genuine, purity of material i.e. from which part the material is collected as it plays an important role, since all parts in the plant are not of medicinal value.

Plant materials were extracted with organic solvents starting from non-polar solvents to polar solvents. The extracted material was reduced under vacuum through rotaevaporator. The reduced material was passed through silica gel column for purification. From the purified compound, thin layer chromatography was carried out. High performance liquid chromatography (HPLC), mass spectroscopy, nuclear magnetic resonance (NMR) were employed for structural elucidation.

### 3.1.1 Physical characterisation of materials

Initially plant samples of venthyam, naval, sirukuringi, neem and vilvam in powder form weighing 400-500 grams each were collected to yield enough extract for the isolation of active constituent for *In vitro* bioassays and structural elucidation of the pure component.

Physical characterisation of the plant material was done from which part it was collected i.e. leaf, root, seed etc. Other parameter such as location, season etc. were noted as these play an important role in the medicinal property. The five plants selected in the study were already in use for the treatment of diabetes by traditional healers of South India. Hence the knowledge from traditional practice was considered as the first screen for the selection of plants prior to the application on *In vitro* screens of a specific bioactivity.

Medicinal plant compounds are mostly heat sensitive. Care was taken that the active constituents in the plant material are retained by not exposing it to direct sunlight. They were dried under shade only to preserve active
component. Plant parts like leaf, seed, root etc. were separated without contamination and ground into fine powdered form to get the smallest possible size to yield more compound. The material was sieved through fine muslin cloth and stored in air tight containers as moisture contamination may spoil the material. The parameters like colour, texture and taste were recorded in (Table.3.1).

<table>
<thead>
<tr>
<th>S. No</th>
<th>Local name</th>
<th>Scientific name</th>
<th>Plant Part</th>
<th>Colour</th>
<th>Taste</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Venthyam</td>
<td>Trigonella foenum graecum</td>
<td>Seed</td>
<td>Cream</td>
<td>Bitter</td>
</tr>
<tr>
<td>2.</td>
<td>Naval</td>
<td>Syzygium cumini</td>
<td>Leaf</td>
<td>Green</td>
<td>Characteristic</td>
</tr>
<tr>
<td>3.</td>
<td>Sirukuringi</td>
<td>Gymnema sylvestre</td>
<td>Leaf</td>
<td>Green</td>
<td>Characteristic</td>
</tr>
<tr>
<td>4.</td>
<td>Neem</td>
<td>Azadiracta indica</td>
<td>Leaf</td>
<td>Green</td>
<td>Bitter</td>
</tr>
<tr>
<td>5.</td>
<td>Vilvam</td>
<td>Aegle marmelos</td>
<td>Leaf</td>
<td>Green</td>
<td>Characteristic</td>
</tr>
</tbody>
</table>

### 3.2 EXTRACTION

#### 3.2.1 Small scale extraction for screening purposes

Small scale extraction in micro tubes was found to be more suitable for 100 mg of plant powder dissolved in 1ml of non-polar to polar solvents i.e. hexane, dichloromethane (DCM), ethyl acetate, methanol, water etc. Small scale extraction helped in establishing the specific bioactivity of the plant material before going into large scale extraction. By small scale
extraction wastage of plant material was minimised. It was found that 1.5 ml micro tubes are best suited for small scale extraction.

3.2.2 Solubility in different solvents

Solubility of the plant material in which the active compound is soluble has been carried out in different organic solvents starting from nonpolar solvents like hexane, chloroform and dichloromethane to polar solvents like ethyl acetate, methanol and water. Nonpolar solvents did not show solubility of the plant materials, as the active compounds were not extracted in them. Whereas polar solvents showed solubility of the compound which is confirmed in bioassays. Out of the polar solvents it was found that methanol is the best solvent system followed by ethyl acetate.

3.2.3 Better resolution with mobile phase, 50% hexane in ethyl acetate for thin layer chromatography (TLC)

The crude extracts of venthyam, naval, sirukuringi, neem and vilvam are subjected to TLC to find the best mobile phase for thin layer chromatography. TLC is the oldest and most useful technique to separate organic compounds based on affinity. The compounds having affinity towards mobile phase moved faster along with it and those having affinity towards solid phase moved slowly. Crude plant extracts showed different spots corresponding to the number of compounds present in the mixture. Different solvent combinations of non polar and polar solvent systems were studied. The best resolution was with 50% hexane in ethyl acetate. This is confirmed with maximum number of spots and better separation on TLC plates (Fig 3a).
Different solvent systems like various percentages of solvent systems were used for TLC to check the purity of the compound. Less polar solvents did not show proper separation where as in high polar solvents system, the compound moved along with mobile phase. Best resolution was obtained with 50% ethyl acetate in hexane. The pure compound was seen as a single spot on TLC.

1=Venthayam, 2=Naval, 3=Neem, 4=Sirukuringi, 5=Vilvam.
A=Hexane in dichloromethane, B=Ethyl acetate in methanol,
C=hexane in ethyl acetate, D=Sirukuringi pure compound
3.2.4 TLC developed under UV, Iodine vapours and developer

After finding the best mobile phase for the crude extracts the TLC plates were first observed under UV. The fluorescent compounds were seen under UV. They were recorded and further developed in iodine vapours. More number of compounds were observed and these were marked and recorded. Ninhydrin did not show much compounds as there was affinity of the compounds towards Ninhydrin. The TLC plates developed in developing solution showed permanent spots. The developer solution showed more affinity to the compounds indicated by the number of spots on TLC.

3.3 BIOASSAYS

3.3.1 Glucose uptake measurement as In vitro screen

Cells utilise glucose during growth. As the cells grow the glucose in the medium is depleted. Under normal circumstances glucose levels gradually fall in the medium. This was used as an effective In vitro screen to determine whether in the presence of specific plant extracts, the glucose level in the medium is rapidly diminished in comparison to the untreated controls. HEp-2 cells, lymphocytes, muscle or adipose tissue were tested for similar effects. If these plant extracts are capable of reducing glucose levels in the medium, it suggests activation of the transporters and increased glucose metabolism.

In the glucose uptake bioassay, crude extract of plant materials in hexane, dichloromethane, ethyl acetate, methanol and water were added to HEp-2 cells. Hexane and water fractions did not show significant glucose uptake from the medium. Dichloromethane and chloroform were toxic to the
cells as the cells were lysed and there was no glucose uptake. Ethyl acetate fraction showed moderate glucose uptake.

There was maximum glucose uptake with methanol fraction. The other extracts with different solvents were stored for subjecting it to other screens at later date. All methanol fractions of the different plants extracts were subjected to glucose uptake screens. Maximum glucose uptake was seen in the plant extracts from vilvam and sirukuringi whereas other plant extracts did not exhibit differences in glucose uptake (Fig. 3.1).

The different solvent extracts of five plants were tested for glucose uptake studies. In this study we focused mainly on one plant (sirukuringi) for all further purification and structural elucidation. The various fractions of sirukuringi were tested for glucose uptake and maximum uptake was obtained in methanol fractions (Fig. 3.2).

3.3.2 Cytotoxicity and optimum dose

The cytotoxic effect of the methanol fraction of sirukuringi was tested on HEp-2 cells. Different doses from 5 μg to 40 μg / ml were added to cultures and the cytotoxic effect was monitored using (3H) thymidine assays (Fig 3.3). Doses above 40 μg / ml were found to be toxic, while doses 15 and 20 μg / ml were considered to be optimum. For all further studies of glucose uptake and metabolism a dose of 20μg / ml was used.

3.3.3 Time course analysis of glucose uptake

Methanol extract of sirukuringi was added to HEp-2 cells and the glucose uptake was monitored from 2h to 24h and compared with the untreated
In the initial experiments, methanol fraction of different plant extracts were applied at a dose of 20 µg/ ml medium showed varied degrees of glucose uptake in HEp-2 cells. The untreated control (C) cells and methanol solvent control (SC) cells did not show glucose uptake. Venthyam (Ven), Naval (Nav) and Neem (Nm) treated cells showed little glucose uptake. Although there was glucose uptake with sirukuringi (Sir) and vilvam (Vil) treated cells when compared to untreated control (C) cells, it was not significant as HEp-2 cell line is not a model to study glucose uptake but it gave indication that these plant extracts can modulate glucose uptake in treated cells.

**Fig. 3.1 Glucose uptake in HEp-2 cells with different plant extracts**

In the initial experiments, methanol fraction of different plant extracts were applied at a dose of 20 µg/ ml medium showed varied degrees of glucose uptake in HEp-2 cells. The untreated control (C) cells and methanol solvent control (SC) cells did not show glucose uptake. Venthyam (Ven), Naval (Nav) and Neem (Nm) treated cells showed little glucose uptake. Although there was glucose uptake with sirukuringi (Sir) and vilvam (Vil) treated cells when compared to untreated control (C) cells, it was not significant as HEp-2 cell line is not a model to study glucose uptake but it gave indication that these plant extracts can modulate glucose uptake in treated cells.
Sirukuringi plant extract in different solvent systems applied at 20 μg/ml medium showed glucose uptake in lymphocytes. The maximum uptake was seen with methanol fraction when compared to untreated control and other solvent systems. Further experiments were continued with sirukuringi methanol fraction only.

C=Control, HEX=Hexane, DCM=Dichloromethane, EA=Ethyl acetate, METH=Methanol, AQUA=water

Fig. 3.2 Methanol- Effective solvent system
Out of five different doses, 5\,\mu g, 10\,\mu g, 15\,\mu g, 20\,\mu g and 40\,\mu g/ml of medium applied (stock solution = 1\,\mu g/\,ml) on HEp-2 cells, the doses 5\,\mu g and 10\,\mu g did not show any activity. Whereas 15\,\mu g and 20\,\mu g showed optimum activity. The dose 40\,\mu g was toxic to cells. The optimum dose has been established as 20\,\mu g/\,ml of medium and further experiments carried out with same dose.
controls. Differences in glucose uptake was noted from the 8th hour. By 12h maximum glucose uptake was noted in the treated samples (Fig. 3.4). Glucose level in the medium dropped significantly after 12th hour.

3.3.4 Glucose uptake in lymphocytes

HEp-2 cells showed that the plant extracts tested were capable of modulating glucose uptake, however, maximum glucose uptake was observed with plant extracts of sirukuringi and vilvam. Further glucose uptake studies on normal PBMCs (Peripheral Blood Mononuclear cells), although these are not target cells for diabetes, were conducted to detect whether these plant extracts are capable of modifying glucose transport in these cells. Such a study will be useful since these cells are easily accessible (Daneman, 1992), and also PBMCs could be obtained from different classes of diabetic patients. This may help us to postulate a clinical validation and capability of improving any impairment in glucose uptake and metabolism that is often seen in diabetic patients.

Glucose uptake was monitored on PBMCs isolated from normal individuals. The different methanolic fractions that were tested on HEp-2 cells were added to the PBMCs. Maximum glucose uptake was noted in methanolic fraction of sirukuringi. Naval and vilvam also demonstrated notable glucose uptake (Fig. 3.5). Similar studies carried out on PBMCs from diabetic patients (NIDDM) also showed that sirukuringi methanolic extract demonstrated maximum glucose uptake (Fig.3.6).

3.4 GLUCOSE UPTAKE IN TISSUES

Glucose uptake studies were done on rat muscle explants and rat adipose tissue. Muscle and adipose tissues are insulin dependent and were used for glucose uptake and metabolism studies.
Fig. 3.4  Time course analysis of glucose uptake with plant extract

Sirukuringi methanol fraction applied at 20 μg/ml medium on lymphocytes at different time points showed glucose uptake. The difference in glucose uptake by the cells was effective from 12h to 24h. Maximum glucose being taken up by the treated cells resulted in rapid decrease in glucose levels in the medium as compared to untreated cells.
Fig. 3.5  Glucose uptake in normal lymphocytes with different extracts

Lymphocytes were isolated from normal humans and *invitro* cultured in multiwell plates. Cells treated with plant extracts at a dose of 20 μg/ml medium showed glucose uptake at 24h. Naval (Nav) and Vilvam (Vil) showed little glucose uptake over untreated control (C), Venthyam (Ven) and Neem (Nm). Cells treated with Sirukuringi (Sir) extract showed more glucose uptake in normal lymphocytes when compared to other plant extracts.
Fig. 3.6  Glucose uptake in lymphocytes in NIDDM patients with different plant extracts

Different plant extracts applied on lymphocytes from NIDDM patients at 20 µg/ml medium showed varied degrees of glucose uptake. Venthyam (Ven), Naval (Nav) and Neem (Nm) treated cells showed same levels of glucose uptake. Cells treated with Sirukuringi (Sir) showed maximum glucose uptake followed by vilvam (Vil) when compared to untreated control (C) cells.
3.4.1 Glucose uptake in muscle tissue

Glucose uptake in muscle tissue was compared between the two methanolic fractions of sirukuringi and vilvam. Maximum glucose uptake was noted by 12 h. in sirukuringi fraction (Fig. 3.7).

3.4.2 Glucose uptake in adipose tissue

The methanolic extracts of sirukuringi and vilvam were tested on explants of adipose tissue for glucose uptake. Maximum glucose uptake was noted in the methanolic fraction of sirukuringi (Fig. 3.7)

3.5 LARGE SCALE EXTRACTION AND PURIFICATION

After having established that the methanolic fractions of sirukuringi showed maximum glucose uptake, large scale extraction of plant material was done. One of the objectives of this thesis was to characterise the active molecule in the methanolic extract that was capable of eliciting the increased glucose uptake in the cells. Large scale extractions were conducted as indicated in materials and methods and standard column purification procedures were adopted. Briefly, it involved three step column purification followed by monitoring the bioactivity of each fraction on glucose uptake and also following the characteristic pattern of each fraction on thin layer chromatography (TLC).

3.5.1 1st step Column Fractionation

Twenty five fractions of 10 ml volume hexane in 2%, 5%, 10%, 15% and 25% ethyl acetate were collected and subjected to thin layer chromatography (TLC).
Muscle explants and adipose tissue from rat were cultured \textit{in vitro} and treated with Sirukuringi (Siru), Vilvam (Vil) extracts. There was significant glucose uptake in treated tissues over positive control (Pos) and untreated control (C). The glucose uptake was maximum in muscle tissue at 12h treatment as it is the main site of glucose utilisation.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{glucose_uptake.png}
\caption{Glucose uptake in rat muscle and adipose tissues}
\end{figure}

C(M) = Control (Muscle), C(A) = Control (Adipose tissue)
3.5.1.1 TLC profile of Column-I fractions

In TLC, many spots were seen as the fraction size was large (10 ml). Some fractions showed the same \( R_F \) value. These fractions were pooled into four groups and named as sirukuringi column one fractions (SR-1, 2, 3 and 4). The TLC profile is shown in Fig. 3.8A.

3.5.1.2 Bioassay of Column-I fractions

Glucose uptake studies on lymphocytes showed that SR2 fraction demonstrated maximum glucose uptake followed by other fractions. The order of increased glucose uptake was SR2 > SR4 > SR3 > SR1. (Fig. 3.8B)

3.5.2 2nd Step Column purification

The fraction SR2 was further purified in second silica gel column and 35 fractions of 5 ml volume were collected. These fractions were further tested by TLC to check for purity.

3.5.2.1 TLC profile of column-II fractions

The TLC profile of column II fractions showed different spots of various \( R_F \) values. Fractions of same values were pooled into three groups and named as SR II-1, 2, 3. The TLC profile is shown in Fig. 3.9A.
Methanol fraction of sirukuringi was subjected to silica gel column chromatography and obtained 25 fractions of 10ml each. These fractions were pooled into four groups according to the TLC profile and bioassay was performed.

**B Glucose uptake studies with sirukuringi column-1 fractions**

Lymphocytes treated with sirukurigi column-1 fractions at a dose of 20μg/ml medium showed different levels of glucose uptake. Maximum glucose uptake was seen with SR2 fraction over untreated control.

Cont= Control, Pos=Positive, SR=Sirukuringi crude extract 
SR1 to SR4= Column-I fractions (1-4)
Sirukuringi column-II fractions were added to cultured lymphocytes at a dose of 20 μg/ ml. Decrease in glucose level in the medium with SR II-3 fraction was maximum over the untreated controls.

Cont= Control, Pos=Positive, SRII-1 to 3= Column-II fractions (1-3)
3.5.2.2 Bioassay of column-II fractions

Among the three pooled fractions, SRII-3 showed maximum glucose uptake by 24 h. The order of increased glucose uptake can be arranged as SRII-3 > SRII-1 > SRII-2. All fractions were stored for further analysis. (Fig. 3.9B).

3.5.3 3rd column Purification

SR II-3 fraction was further purified at third level with silica gel column and 50 fractions of 2 ml volume were collected. These fractions are further confirmed for purity with TLC.

3.5.3.1 TLC profile of column-III fractions

Better separation was shown in thin layer chromatography as the fraction size was small. All 50 fractions of similar TLC profiles were grouped into five groups (Fig.3.10A). These fractions were denoted as sirukuringi column III fractions (SR III. 1-5). SR III-5 showed single spot. This pure fraction was further subjected to Mass and NMR studies.

3.5.3.2 Bioassay column-III fractions

Glucose uptake was maximum with SR III-5. (Fig.3.10B). The order of glucose uptake was SRIII-5 > SRIII-3 > SRIII-2 > SRIII-4 > SRIII-1. The fraction SRIII-5 contained only one spot on TLC. This fraction also showed the ability to induce glucose uptake. Hence, it can be said that major TLC spot that appears in all the types of fractions can be implicated for glucose uptake.
The fraction, SR II-3 was purified on silica gel column and 50 fractions of 2ml each were collected. These fractions a pooled into five groups and a single spot was obtained in SR III-5 fraction as shown in TLC profile.

Lymphocytes treated with SR III-5 fraction showed maximum decrease of glucose from the medium than the control untreated cells.

Cont= Control, Pos=Positive, III-1 to 5=Column-III fractions (1-5)
Fig. 3.11 RT-PCR: Expression of GLUT-4 mRNA in rat muscle tissue

Muscle explants were treated with extracts of venthyam (ven), Naval (Nav), sirukuringi (Siru), Neem (Nm) and vilvam (Vil). There was an increased expression of GLUT-4 mRNA but not GS. The untreated control (Cont) did not show any expression whereas Sirukuringi treatment expressed more GLUT-4 mRNA.

M= 100 bp marker, 1=GS, 2=GLUT-4, 3=GLUT-1.
It is this fraction, which was further subjected to purification by HPLC and used for structural elucidation by mass spectrophotometer and NMR analysis.

3.6 MECHANISM OF GLUCOSE UPTAKE: RT-PCR

Transport of glucose across plasma membrane of non-epithelial cells is mediated by a family of facilitative glucose transporters (Longo et al., 1990).

The plasma membrane is impermeable to polar molecules such as glucose, the cellular uptake of this important nutrient is accomplished by membrane-associated carrier proteins that bind and transfer it across the lipid bilayer. Glucose uptake into the cells is carried out through specific transporters (GLUTs). For the majority of tissues, glucose represents the main fuel source. Muscle and adipose tissues are primarily dependent on insulin and are responsible for converting glucose into energy and storing excess glucose as glycogen by the activity of glycogen synthase (GS).

The availability of specific primers have enabled to detect the levels of these transporters. If glucose uptake studies indicate that glucose is being rapidly taken up by the cells, then the transporters are active. This strategy was applied to monitor specific transporters on specific cell population in the presence and absence of plant extracts. Both the fractions that showed maximum glucose uptake were studied by RT-PCR.

3.6.1 GLUT-4 expression in Muscle

The expression of GLUT-4 in muscle explants treated with different crude methanol plant extracts of venthyam, naval, neem, sirukuringi and
vilvam at a dose 20 μg/ml medium was monitored. Increased expression of transporter-4 (GLUT-4) at 2 h time point was noted. Out of all plant extracts sirukuringi showed maximum expression of GLUT-4 mRNA over untreated control (Fig.3.11). Since muscle tissue is insulin dependent, GLUT-4 expression was maximum whereas GLUT-1 was expressed to a lesser extent and Glycogen synthase (GS) was not expressed at 2h treatment.

3.6.2 Decreased expression of GLUT-4 in muscle with time

The expression levels of GLUT-4 in muscle were monitored at 2 h, 4 h and 24 h of treatment with crude methanol extracts of sirukuringi and vilvam. The expression of GLUT-4 in rat muscle explants treated with crude methanol extracts decreased along with time duration. It was observed to be highest at 2h, moderate at 4h and diminished by 24h (Fig. 3.12). The mRNA level was highest at early hours treatment. Sirukuringi treatment showed more expression than vilvam.

3.6.3 Increased expression of GLUT-4 with SR III-5 fraction

Each of the column purified fractions, SR2, SRII-3 and SRIII-5 that demonstrated maximum glucose uptake was added to rat muscles explants and GLUT-4 expression was monitored. The purified SRIII-5 that showed a single compound in TLC did elevate the expression levels of GLUT-4. This was suggestive that the purified compound that was obtained from the methanolic extract was responsible for modulating glucose transport (Fig.3.13).

3.6.4 Expression of GLUT-1 in lymphocytes

Glucose uptake studies demonstrated that lymphocytes were modulated in the presence of plant extracts. This served as a direct correlation
Fig. 3.12  RT-PCR: Expression of GLUT-4 mRNA at 2h, 4h and 24h in muscle

Muscle explants were treated with sirukuringi (siru) and vilvam at 2h, 4h and 24h. Expression of GLUT-4 mRNA was higher at 2h followed by 4h. The expression was diminished at 24 h. Expression was higher with sirukuringi treatment when compared to Vilvam. While control showed minimal or no detectable levels of expression.

M = Marker (100bp), P = Positive, C = Control, S = Sirukuringi, V = Vilvam
Fig. 3.13 RT-PCR: Expression of GLUT-4 mRNA with different column fractions at different stages of purification on muscle explants

Muscle explants from rat was treated *in vitro* with sirukuringi crude methanol fraction (SRC), silica gel column-I fraction (SR-2), column-II fraction (SRII-3) and column-III fraction (SR III-5). The untreated Control (C) did not show any expression whereas in positive (+ve) there was GLUT-4 mRNA expression. There was an increased expression in SR III-5 treated muscle followed by SR2 treated explants.

M = Marker, C = control (untreated), + = Positive control, SRC = siru crude, SR-2 = Col.II fraction, II-3 = Col.II fraction, III-5 = Col.III fraction.
of the data with the clinical relevance. GLUT-1 is the transporter that was identified to be expressed in lymphocytes.

Lymphocytes from normal and diabetic individuals expressed glucose transporter-1 (GLUT-1) when treated with crude methanol extract of sirukuringi and vilvam. Lymphocytes from diabetic patients expressed more GLUT-1 mRNA in response to sirukuringi when compared to vilvam (Fig. 3.14).

In normal lymphocytes sirukuringi extract elevated the mRNA levels of GLUT-1 to a greater extent, when compared to vilvam extract. The increased expression of GLUT-1 in the presence of plant extracts is suggestive that glucose uptake increased because of the activation of transporters, which gets impaired in diabetics specifically NIDDM. Thus, it suggests the modality on how these medicinal plant extracts work at the cellular level.

3.6.5 Expression of GLUT-1 in lymphocytes with purified fractions

The crude extract of sirukuringi showed an elevated expression of GLUT-1 in lymphocytes. The purified fractions were added to the lymphocytes at the same concentration. All the fractions showed elevated levels of GLUT-1 expression than the untreated controls. Among the three fractions SR2, SRII-3 and SRIII-5, the fraction SRIII-5 showed maximum enhancement of the expression of GLUT-1 (Fig. 3.15).

3.7 GLUCOSE METABOLISM

The first step in glycolysis is the rapid phosphorylation of glucose to glucose-6-phosphate (Coffee, 1998), which allosterically activates glycogen
Lymphocytes from normal subjects and diabetic patients were treated with sirukuringi (S) and vilvam (V) for 2 h. In normal lymphocytes sirukuringi treatment only showed GLUT-1 mRNA expression whereas in lymphocytes from diabetic patients sirukuringi treatment showed high expression than vilvam treated cells. The expression level was higher in lymphocytes from diabetic patients than in normal controls.

Abbreviated: M = Marker(100bp), P = Positive, C = Control, S = Sirukuringi, V = Vilvam
Fig. 3.15  RT-PCR: Expression of GLUT-1 mRNA in lymphocytes isolated from diabetic patients

Lymphocytes from diabetic patients were treated with sirukuringi crude methanol fraction (SRC), silica gel column-I fraction (SR-2), column-II fraction (SRII-3) and column-III fraction (SR III-5). The expression of GLUT-1 mRNA increased with the treatment of SR III-5 when compared to other fractions than controls.

M = Marker, Pos = Positive control, SRC = siru crude C = control (untreated), SR-2 = Col.I fraction, II-3 = Col.II fraction III-5 = Col.III fraction
synthase. The cytosolic concentration of glucose-6-phosphate is dependent on the glucose uptake by the tissues.

3.7.1 Increased levels of G-6-P in muscle

Results with glucose uptake studies showed that the highest uptake of glucose was with crude sirukuringi methanol extract and SR III-5 column fraction. Once the level of glucose is increased in the muscle it is phosphorylated to glucose-6-phosphate. The level of G-6-P in rat muscle was elevated with the treatment of sirukuringi fractions over untreated controls (Fig.3.16). The level of G-6-P was highest with the treatment of SR III-5 fraction, which also showed maximum glucose uptake. The second highest level of G-6-P was observed with SR2 fraction of sirukuringi. Increased glucose uptake with sirukuringi fraction SR III-5 facilitated the elevated levels of G-6-P in muscle, which is the main site of glucose metabolism and in turn elevated glucose-6-phosphate intracellular levels.

3.7.2 Expression of G-6-P in liver

Similar results were observed in liver with the treatment of sirukuringi column fraction SR III-5. Elevated levels of glucose conversion into G-6-P was noted. Sirukuringi fraction SR2 showed second highest level of glucose conversion into G-6-P. In untreated controls the phosphorylation rate was slow as indicated by the concentrations of G-6-P in liver tissue (Fig.3.17).

3.7.3 Glycogen synthase(GS) expression in muscle (24h)

Glycogen synthase (GS) is the key enzyme in glycogen synthesis. The activity of GS decreases in diabetic conditions. Using RT-PCR it was
Fig. 3.16  Effect of sirukuringi column purified fractions on glucose metabolism in rat muscle explants

Muscle explants were treated with Sirukuringi crude methanol fraction (SR), first column fraction (SR-2), second column fraction (SR II-3), third column fraction (SR III-5) and positive control (Pos) showed increased glucose metabolism over untreated control (Cont.) tissue by converting glucose to Glucose-6-Phosphate (G-6-P). The glucose metabolism was maximum with column three fraction (SR III-5) followed by column one fraction (SR2).

C=control (untreated), SR=siru crude, SR-2= Col.I fraction, II-3 = Col.II fraction, III- 5=Col.III fraction
Liver tissue of rat treated with Sirukuringi crude methanol fraction (SR), silica gel first column -I fraction (SR-2), column-II fraction (SR-II-3) and column-III fraction (SR III-5) increased the conversion of glucose to Glucose-6-Phosphate over untreated control. The level of Glucose-6-Phosphate (G-6-P) was maximum with column-III fraction (SR III-5) compared to positive control (Pos), crude methanol fraction and other column fractions.

C=control (untreated), Pos=Positive control SR=siru crude, SR-2=Col.I fraction, II-3=Col.II fraction, III-5=Col.III fraction
found that GS was not expressed in muscle at early hours (2h) treatment with plant extracts (Fig. 3.18). There was an increase in the expression of GS at the late hours of treatment (24h). In the early hours, the glucose uptake was not significant. Once the glucose level increased in the cell it was phosphorylated to glucose-6-phosphate, which in turn activated glycogen synthase and converted glucose into glycogen by the action of glycogen synthase. The traditional anti-diabetic plants, vethyam, naval, sirukuringi, neem and vilvam extracts that were tested showed varying degrees of GS expression. Maximum GS expression was seen with sirukuringi treatment (Fig. 3.18).

3.8 WESTERN BLOTTING OF GLUT PROTEINS

3.8.1 Expression in muscle

In insulin dependent cells like muscle glucose is transported into the cells through GLUT-4 glucose transporter protein. The GLUT-4 proteins are present in the cytosol of the cell and expressed when stimulated. Western blot analysis confirmed high level of protein expression in the cells treated with SRIII-5 fraction. (Fig. 3.19).

3.8.2 Expression in adipose tissues

In the adipose tissue, which is also insulin dependent like muscle, similar results were obtained. The glucose transporter protein GLUT-4 was expressed. Western blot analysis showed high level of GLUT-4 protein expression when treated with SRIII-5 fraction (Fig.3.20).
Fig. 3.18  RT-PCR: Expression of GS mRNA with different plant extracts

Plant extracts of venthyam (ven), Naval(Nav), sirukuringi (Siru), Neem (Nm) and vilvam (Vil) were applied on muscle explants from rat at 24h showed increased expression of GS mRNA but not GLUT-4 or GLUT-1. There was no GS expression in untreated control (Cont). Sirukuringi treated GS expression was high followed by vilvam. Ventyham and naval treatment showed moderate expression whereas the expression with neem was minimum.

1= GS (Glycogen Synthase), 2=GLUT-4, 3=GLUT-1.
Fig. 3.19  Western blot analysis of GLUT-4 Protein in rat muscle explants

Total protein extracts from rat muscle explants treated with sirukuringi crude methanol fraction (SR), silica gel column-I fraction (SR-2), column-II fraction (SR-II-3) and column-III fraction (SR III-5). The untreated Control (C) showed minimal or no expression of GLUT4 protein, whereas in positive control (+ve) there was GLUT-4 expression. SR III-5 treated GLUT-4 protein expression was higher followed by SR 2 fraction.

M =Rainbow marker, C=control (untreated)
+ve= Positive control, SRC=siru crude, SR-2= Col.I fraction, SRII-3 = Col.II fraction SR III- 5=Col.III fraction
Fig. 3.20  Expression of GLUT-4 protein in adipose tissue

Adipose tissue of rat was treated with sirukuringi crude methanol fraction (SRC), silica gel first column fraction (SR-2), second column fraction (SRII-3) and third column fraction (SR III-5). Maximum expression levels of GLUT-4 proteins was seen in SRC and SR III-5 treated cells.

M= Rainbow marker, C=control (untreated) +ve= Positive control, SRC=siru crude, SR-2= Col.I fraction, SRII-3 = Col.II fraction SR III- 5=Col.III fraction
3.9 IMPLICATIONS OF DIABETES AND IMMUNE RESPONSE

Type-1 (IDDM) is an autoimmune disease caused by the infiltration of T-lymphocytes in the islets of Langerhans. Th2 cells secrete IL-4, IL-6, IL-10 and IL-13. The levels of protective cytokines IL-4 and IL-13 have been reported to be low in individuals with diabetes. Experiments were conducted to study the levels of IL-4 expression in lymphocytes isolated from diabetic patients treated with plant extracts.

3.9.1 Expression of IL-4

Lymphocytes isolated from diabetic patients were treated with sirukuringi extract and column fractions. The expression of IL-4 mRNA was measured using RT-PCR. SRIII-5 fraction treated lymphocytes showed increased expression levels of IL-4 mRNA (Fig. 3.21).

3.9.2 Expression of interferon gamma

Lymphocytes from diabetic patients were treated with the extract and different fractions of sirukuringi and expression of mRNA level of IFN-γ was measured. The expression in lymphocytes treated with sirukuringi extracts and column fractions was lower, whereas in untreated control the level was higher. The expression level was lowest in sirukuringi column fraction III-5 followed by SR2 fraction (Fig.3.22). The results indicate the bioactive compound might be present in this fraction, which showed lowest expression of interferon gamma expression. These studies indicated that the plant extracts can protect the lymphocytes by secreting protective cytokines like IL-4 and suppressing the levels of inflammatory cytokines like interferon gamma (IFN-γ).
Lymphocytes isolated from diabetic patients were treated with sirukuringi crude extract (SRC), column-I fraction (SR-2), column-II fraction (SRII-3) and column-III fraction (SR III-5). The expression of IL-4 mRNA increased with the treatment of SR III-5 when compared to other fractions and untreated control.

M = Marker, C = control (untreated), Pos = Positive control, SRC = sirukurigi extract, SR-2 = Col.I fraction, II-3 = Col.II fraction, III-5 = Col.III fraction
Fig. 3.22 RT-PCR: Expression of IFN-γ mRNA in lymphocytes isolated from diabetic patients

The lymphocytes isolated from diabetic patients were treated with sirukuringi crude extract (SRC), column-I fraction (SR-2), column-II fraction (SRII-3) and column-III fraction (SR III-5). The expression of IFN-γ mRNA decreased with sirukuringi treatment and maximum decrease was with SR III-5 when compared to other fractions. There was maximum expression in untreated control.

M = Marker, C = control (untreated), Pos = Positive control, SRC = sirukurigi extract, SR-2 = Col.I fraction, II-3 = Col.II fraction, III-5 = Col.III fraction
Lymphocytes were obtained from diabetic patients and cultured in *in vitro* condition. Crude methanol extract of *sirukuringi* was added. Glucose uptake was monitored at 12 and 24 hrs. In all cases there was an enhanced uptake of glucose in the cells compared to untreated controls.

Cont= Untreated controls, P=Patients
3.10 GLUCOSE UPTAKE IN CLINICAL SAMPLES

Blood samples were collected from 12 diabetic patients. Lymphocytes were isolated and *In vitro* studies were carried out to detect whether the extracts of *sirukuringi* are capable of elevating glucose uptake or not. Glucose uptake was observed at a dose of *sirukuringi* extract 20 μg/ml culture medium from 12 h and continued till 24 h in a single dose treatment. In all lymphocytes isolated from all diabetic patients there was an increase in glucose uptake compared to the untreated control. These studies suggest that *sirukuringi* extract stimulated glucose uptake in lymphocytes from diabetic patients (Fig. 3.23).

3.11 DIABETIC ANIMAL MODEL

Diabetes was induced by injecting alloxan at a dose of 90mg/kg body weight to fasted rats. Diabetes was established after a week. The glucose level in blood was monitored and the diabetic rats (blood glucose levels above 250mg/dL) were selected. In each group there were five animals. *Sirukuringi* extract at a dose of 50mg/kg body weight was orally administered for 2-3 weeks to diabetes induce rats whereas to untreated rats normal saline was given. In control untreated (CUT) rats there was no change in glucose concentration. In control treated (CT) rats also there was no change in blood glucose.

In diabetes induced untreated rats (DUT) rats the glucose level was higher and did not decrease. Whereas in diabetic rats treated with *sirukuringi* extract (DSR) and column fraction (DCF), the blood glucose level reduced to normal (Fig.3.24). The blood glucose reduced to normal in three weeks of daily
Fig. 3.24 Effect of sirukuringi plant extract and purified column fraction in alloxan induced diabetic animal model

Diabetes was induced in wistar rats through intraperitoneal injection of alloxan. Diabetic animals having blood sugar level above 200mg/dl were selected and divided into three groups (n=5). Two groups of normal rats (n=5) were selected as control untreated [C(UT)] and control treated [C(T)]. Diabetic untreated rats [D(UT)] were not given any plant extract whereas other two groups were treated with diabetic sirukuringi methanol fraction [D(SR)] and sirukuringi column fraction [D(CF)]. These diabetic groups showed decrease in blood sugar. In normal treated rats with sirukuringi extract no decrease in glucose was observed.
administration with sirukuringi extract whereas with column fraction, normal levels of glucose was observed after ten days. These results suggest the antihyperglycemic activity of sirukuringi. The histological studies also correspond with the animal model experiments and regeneration of β-cells are also seen in treated pancreas tissue (3.24a),

3.12 STRUCTURAL ELUCIDATION

The column fraction of sirukuringi (SR III-5), which showed biological activity, was subjected to analytical studies through MASS and NMR to find the structure of the compound. Based on the mass of the compound and the shift in bonding the structure of the compound was predicted.

3.12.1 Mass spectroscopy studies

The mass of the compound was studied in the range of 300-1500 mass and three peaks were observed. The mass of the compound was found as 490. The results were monitored through the computer screen and recorded. The results were correlated with NMR data (Fig. 3.25).

3.12.2 NMR studies

The pure fraction was studied for solubility and it was found that the sample dissolved completely in dichloromethane (DCM). There was no crystal formation. The DCM solvent was completely removed and dissolved in CDCl₃ and transferred to NMR tube. The NMR readings were recorded as indicated by the peaks (Fig. 3.26). The shift in bonding and the mass of the compound were analysed and predicted the structure as gymnestrogenin one of the compounds of the family of sirukuringi as gymnestrogenin (Fig. 3.27).
Fig. 3.24a Histological study of pancreas from alloxan induced diabetic rats and the effect of sirukuringi treatment on β-cells

Pancreas from the alloxan induced diabetic rats and normal rat was collected and histological studies were done. In control untreated [C(UT)] and control treated [C(T)] rats there was no effect on β-cells. In diabetic untreated rats [D(UT)] the β-cells were completely destroyed where as in diabetic rats treated with sirukuringi extract [D(SR)] and sirukuringi column fraction [D(CF)] regeneration of β-cells was observed.

A= Normal controls showing β-cells cells in pancreas
B= Diabetic rats treated with sirukuringi extract
C= Diabetic rats treated with column fraction showing regeneration of β-cells cells.
D= Diabetic untreated rats showing complete destruction of β-cells cells.
Fig. 3.25  Mass spectroscopy studies of sirukuringi SR III-5

The column purified fraction of sirukurungi SRIII-5 was studied for mass analysis in the range of 300-1500. The mass of the peaks was analysed and it was found that the mass of SR III-5 was 490. This was compared with the mass of corresponding compounds of Gymnema. The purified fraction was further studied through NMR analysis and the structure of the compound was established.
Fig. 3.26  NMR analysis of sirukuringi purified compound

Sirukuringi column fraction SR III-5 was purified through silica gel chromatography and the pure compound was subjected to NMR studies for structural elucidation. The peaks corresponding to the carbon atoms and other atoms helped to predict the structure of the compound.
The purified fraction from sirukuringi leaves was isolated and the structure was established as Gymnesticogenin belonging to one of the Gymnema compounds. It is a triterpinoid and showed anti-diabetic activity both in vitro and in vivo bioassays.