3 Methodology

3.1 Screening of diabetes and prediabetes

A cross sectional study was carried out in an urban and rural areas of Gwalior-Chambal region, Madhya Pradesh, Central India, an urban area lying between 26°12'19.8” of the northern latitudes, 78°11'57.9”eastern longitudes and rural areas lies between 26°29'41.1” of the northern latitudes, 78°36'52.6”eastern longitudes and 25°56'33.9”of the northern latitudes, 78°22'01.3”eastern longitudes (Details of area are given Figure 3.1 and 3.2). Study was conducted during May 2014 to September 2016, with approval of Institutional human ethical committee (No.JU/IHEC/2013 –A/08).

3.1.1 Sample methods and sample size

Non-probability convenience sampling method was chosen with door to door approach to collect the samples. The subjects/households screened were given prior counseling regarding the purpose and benefits of the study. Also, they were advised to be on minimum 10 hours fasting before blood sampling. As per the consent of the volunteers, their fasting blood glucose level in finger prick whole blood was measured using Glucometer (Accu-Chek®, Roche Diagnostics Australia). In 2011 Anjana et al reported that, the Indian prevalence of T2DM was 14.2 % based on that the sample size was calculated by using the formula 4pq/l². P=14.2 q=100-p, l relative error 6% and 12% non-responder. So the final target sample size was 7530 (Figure 3.3).

The subjects were categorized into diabetes and prediabetes following WHO Criteria (WHO, 2006). Those with fasting blood glucose levels between 110–125 mg/dL (i.e. 6.1–6.9 mmol/L) were considered as prediabetic and those ≥126 mg/dL (i.e. ≥7.0 mmol/L) after an overnight fast were categorized diabetic. Further, demographical details and personal information about each subject, such as age, physical activity, occupation, family history, personal habits like smoking & alcohol consumption and food habits were recorded.
Figure 3.1: Geographical representation of urban areas surveyed
Figure 3.2: Geographical representation of rural areas surveyed
Table 3.1: Selection of study subjects – Criteria used

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Subjects aged ≤79 or ≥20 years of either gender.</td>
<td>1. Subjects aged &lt; 20 or ≥80 years of either gender.</td>
</tr>
<tr>
<td>2. Participants who gave the consent for the fasting blood glucose test.</td>
<td>2. Participants who did not give their consent / who avoided the fasting blood glucose test.</td>
</tr>
<tr>
<td>3. Participants who were ready to share the personal information.</td>
<td>3. Participants who disagreed to share their personal information.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total population surveyed = 7530</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urban population visited</td>
</tr>
<tr>
<td>5530 No.s</td>
</tr>
<tr>
<td>Subjects not Participated</td>
</tr>
<tr>
<td>74 (1.3 %)</td>
</tr>
<tr>
<td>Urban population screened</td>
</tr>
<tr>
<td>5456 (98.7 %)</td>
</tr>
<tr>
<td>Rural population visited</td>
</tr>
<tr>
<td>2000 No.s</td>
</tr>
<tr>
<td>Subjects not participated</td>
</tr>
<tr>
<td>34 (1.7 %)</td>
</tr>
<tr>
<td>Rural population screened</td>
</tr>
<tr>
<td>1966 (98.3 %)</td>
</tr>
<tr>
<td>Total population monitored for fasting blood glucose: 7422 (97.6 %)</td>
</tr>
</tbody>
</table>

Figure 3.3: Outline of population surveyed

3.2 Screening of diabetic subjects with metabolic syndrome

The metabolic syndrome was diagnosed in subjects who attended the weekend diabetes clinic run by Center for translation research, SOS in Biochemistry, Jiwaji University. Subjects were categorized as MetS positive or negative by using Harmonized
criteria (Alberti et al., 2009). The study was carried out in between May 2014 to Nov 2016. A total of 1190 subjects (819 males and 371 females) from 20 to 79 years of age were included in this study. All participants had given the written consent for participation. Details of Harmonized criteria are given in Table 2.4.

3.2.1 Chemicals and glassware

The chemicals used in the present study were procured from Hi-Media Laboratories Pvt. Ltd. (Bombay, India), Sisco Research Laboratories Pvt. Ltd. (SRL Mumbai India) and Sigma-Aldrich (USA). The kits for biochemical estimation were procured from Coral Clinical Systems (Goa India) and the glassware used for experimental work was procured from Borosil glass works Ltd. (Mumbai India) or JSIL (Agra).

3.2.2 Anthropometric parameters

Details of age, gender were recorded; waist circumference was measured (cm) at the level of the umbilicus with the patient standing and breathing normally, while weight (kg) with weighing balance and height (cm) were measured in light clothing without footwear. After 10-15 minutes rest, blood pressure was measured on the dominant arm in a sitting position, using a sphygmomanometer.

3.2.3 Collection and processing of blood samples

Two ml of blood samples were collected in ETDA tube from each subject after 10-12 hours overnight fast. Plasma was separated in a cooling centrifuge at 2000 rpm × 10 minute × 4°C. The plasma was aliquoted and used for analysis of all biochemical parameters and the remaining sample was frozen at -20°C. After the removal of plasma and the buffy coat, the red cells were washed thrice with normal saline. Haemolysate was prepared by mixing 1.9 ml of cold distilled water to 0.1 ml of red cell suspension. Haemolysate was used for all antioxidant markers.
3.2.4 Estimation of biochemical parameters

Various biochemical parameters estimated included: fasting & postprandial blood glucose, HbA1c and lipid profile.

3.2.4.1 Estimation of blood glucose

**Principle:** Fasting and postprandial of blood glucose was estimated by glucose oxidase-peroxidase method (Raabo and Terkildsen, 1960). Glucose was oxidized to gluconic acid and hydrogen peroxide (H$_2$O$_2$) in the presence of glucose oxidase (GOD). In subsequent enzymatic reaction, H$_2$O$_2$ reacted with phenol and 4-aminobipyrine by the catalytic action of peroxidase (POD) to form a red colored quinoneimine dye complex. Intensity of the color formed was directly proportional to the amount of glucose present in the sample.

**Procedure:** The estimation was done as per the instructions of the manufacturer of the kit. To 1 ml of GOD/POD buffer, 10µl of plasma sample/standard was added. Mixed well, and incubated for 15 minutes at room temperature (RT) and absorbance was taken within 30 minutes against the buffer blank at 505 nm.

3.2.4.2 Estimation of Glycosylated haemoglobin

**Principle:** Glycosylated Hemoglobin (GHb) was estimated using Ion Exchange Resin method by Abraham *et al.*, (1978). GHb has been defined operationally as the fast fraction hemoglobins HbA1 which elute first during the column chromatography. The non-glycosylated hemoglobin, which consists of the bulk of haemoglobin has been designated as HbAo. A hemolysed preparation of the whole blood is mixed continuously for 5 minutes with a weakly binding cation exchange resin. The labile fraction is eliminated during the hemolysate preparation and during the binding. During this mixing, HbAo binds to the ion exchange resin leaving GHb free in the supernatant. The percent glycosylated haemoglobin is determined by measuring absorbance of the glycosylated haemoglobin (GHb) fraction and the total haemoglobin (THb) fraction. The ratio of absorbance of the glycosylated haemoglobin and the total haemoglobin fraction of the control and the test is used to calculate the percent Glycosylated Hemoglobin of the sample.
**Methodology**

**Procedure:**

a. **Hemolysate preparation:** In a test tube 0.5 ml lysing buffer, 0.1 ml blood sample/control were added, mixed well.

b. **Glycosylated haemoglobin preparation:** 0.1 ml of hemolysate was taken in a clean test tube and resin separator was inserted into tube, the rubber sleeve was approximately 1 cm above the liquid level of the resin suspension, vortexed for 5 minutes. The resin was allowed to settle and then pushed the resin separator into tubes until the resin was firmly packed. Each supernatant was poured directly into the cuvette and the absorbance was observed at 415 nm.

c. **Total Hemoglobin (THb) fraction:** 0.5 ml of distilled water was dispensed into tubes, 20 µl of the hemolysate was added and mixed well. The absorbance was read against water at 415 nm.

   After calculation, conversion of GHb to HbA1C was done by the charts provided in the kit.

**3.2.4.3 Estimation of lipid profile parameters**

**3.2.4.3.1 Total cholesterol**

**Principle:** Total cholesterol was estimated by CHOD-PAP method according to Stockbridge *et al.*, (1989). Cholesterol esters were hydrolysed to free cholesterol. The free cholesterol is oxidised to form hydrogen peroxide which further reacts with phenol and 4-amino anti pyrine by the catalytic action of peroxidase to form a red colored quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of cholesterol present in sample.

**Procedure:** The estimation was done as per the instructions of the manufacturer of the kit. To 1 ml of buffer, 10µl of plasma sample/standard was added. Mixed well, and incubated for 15 minutes at RT and absorbance was taken within 30 minutes against the buffer blank at 505 nm.
3.2.4.3.2 Estimation of triglycerides

**Principle:** Triglyceride was estimated by GPO-PAP method (Fossati *et al.*, 1982). Triglyceride (a form of lipid), by the action of lipase were hydrolyze to glycerol and fatty acid. Glycerol by the enzymatic reaction of Glycerol Kinase was converted into Glycerol-3-phosphate. In the subsequent enzymatic oxidation by Glycerol-3-phosphate oxidation (GPO), H₂O₂ was formed. Then, H₂O₂ reacted with aminoantipyrine and chlorophenol in the presence of peroxidase (POP) to form a colored quinonimine complex. Intensity of the colour formed is directly proportional to the amount of triglyceride present in sample.

**Procedure:** The estimation was done as per the instructions of the manufacturer of the kit. To 1 ml of buffer, 10µl of plasma sample/standard was added. Mixed well, and incubated for 15 minutes at RT and absorbance was taken within 30 minutes against the buffer blank at 505 nm.

3.2.4.3.3 Estimation of HDL-cholesterol

**Principle:** HDL – Cholesterol was estimated using phosphotungstate method by Lopes-Virella *et al.*, (1977). Chylomicrons, VLDL and LDL fraction in serum separated from HDL by precipitating with phosphotungstic acid and magnesium chloride, after centrifugation the cholesterol in HDL fraction, which remained in the supernatant, was assayed with cholesterol (CHOD/PAP) method.

**Procedure:** The estimation was done as per the instructions of the manufacturer of the kit. To 200 µl of precipitating reagent and 200 µl of plasma sample was added. Mixed well and centrifuged for 10 minutes at 3000 rpm. The clear supernatant was used to estimate the HDL cholesterol. To 1 ml of buffer, 50µl of clear supernatant sample/standard was added. Mixed well, and incubated for 15 minutes at RT and absorbance was taken within 30 minutes against the buffer blank at 505 nm.

3.2.4.3.4 LDL-Cholesterol & VLDL- Cholesterol

LDL-C and VLDL-C were calculated from total cholesterol, triglycerides and HDL cholesterol by using Friedwald’s formula (Friedewald *et al.*, 1972).
Methodology

\[
\text{Triglycerides} = \frac{\text{VLDL Cholesterol}}{5}
\]

\[
\text{LDL Cholesterol} = \text{Total cholesterol} - (\text{HDL} + \text{VLDL})
\]

3.3 Therapeutic potential of *G. sylvestre* therapies in T2DM subjects with MetS.

3.3.1 Registration of subjects for study

A total of 40 subjects those who have T2DM with MetS were randomly selected from the above study for *G. sylvestre* therapy with approval of Institutional human ethical committee (No.JU/IHEC/2013–A/13).

3.3.2 Taxonomical identification of *G. sylvestre*

Dried leaves of *G. sylvestre* were kindly provided by the M/S Deendayal Aushadhi Pvt. Ltd. India and it was identified by Prof. A.K. Jain, SOS Botany, Institute of Ethnobiology, Jiwaji University, Gwalior. Voucher specimens were prepared and deposited at the centre with accession number IOE-501. Two forms of *G. sylvestre*, leaf aqueous extract (GSAE) and ethanolic extracts (GSEE) were studied.

3.3.2.1 Preparation of *G. sylvestre* extracts

3.3.2.1.1 Preparation of *G. sylvestre* aqueous extract

*G. sylvestre* aqueous extract was prepared by patient himself in their home. Six grams of *G. sylvestre* powder was soaked in 200 ml of drinking water overnight and boiled to reduce volume up to one third (70 ml approx.); it was then cooled to room temperature and filtered. The filtrate was divided in to two parts for consumption (one half in morning and another half in evening).

3.3.2.1.2 Preparation of *G. sylvestre* ethanolic extract

*G. sylvestre ethanolic extracts* was prepared by suspending *G. sylvestre* powder in 80% ethanol (1:10 ratio) overnight in shaking incubator. After overnight shaking, ethanol extract was filtered with screen cloth (pore size 0.3mm). The extract was concentrated under reduced pressure below 50°C in vacuum rotary evaporator. Powder was made with
the help of spray dryer and filled up in capsules 13% w/w yield obtained after ethanolic extraction.

3.3.3 Parameters monitored

1. Anthropometric parameters: Waist circumstance, BMI and Blood pressure
2. Parameters for anti-hyperglycemic: Fasting & PP blood glucose, HbA1c (Ion exchange resin method), C-peptide by ELISA method (for ELISA section 3.5.2.5)
3. Parameters for lipid profile: Total cholesterol, Triglyceride, HDL cholesterol, LDL-C and VLDL-C

The protocols are explained in section 3.2

4. Parameters for antioxidant potential: SOD, Catalase, GSH and TBARS
5. Parameters for kidney function: Urea, uric acid and creatinine
6. Parameters for liver function: Bilirubin, SGOT and SGPT

Table 3.2: Details of *G. sylvestre* therapies administered

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Group I</th>
<th>Group II</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. sylvestre leaf aqueous extract</td>
<td>G. sylvestre leaf ethanolic extract in capsule form</td>
<td></td>
</tr>
<tr>
<td>Dose</td>
<td>70 ml aqueous extract (in two doses) obtained from 6 gm dry powder.</td>
<td>800 mg/day, 400 mg BDS (Equivalent to 6 grams of dry powder.)</td>
</tr>
<tr>
<td>Time of administration</td>
<td>After food</td>
<td>After food</td>
</tr>
<tr>
<td>Duration</td>
<td>3 months</td>
<td>3 months</td>
</tr>
<tr>
<td>Number subjects 0 day</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Number of subjects on 90th day</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Discontinued subjects</td>
<td>Seven subjects</td>
<td>Five subjects</td>
</tr>
</tbody>
</table>

Note: The drug was administered orally under the supervision of an Ayurvedic physician. The subjects were kept away from any other type of anti-diabetic medication.
3.3.3.1 Estimation of oxidative stress markers

Reduced glutathione (GSH) was estimated in whole blood whereas, Thiobarbituric acid reactive substances (TBARS), Superoxide dismutase (SOD) and catalase were estimated from haemolysate.

3.3.3.1.1 Reduced glutathione

**Principle:** This method was based on the fact that, DTNB (5,5'-dithio bis (2- nitro benzoic acid)) is reduced by sulphydryl group (SH) present in reduced glutathione to form one mole of 2-nitro -5- mercapto benzoic acid per mole of SH according to Ellman, (1959). The reaction is:

\[
\text{DTNB} + \text{R-SH} \rightarrow \text{Nitro-Mercaptobenzoic acid}
\]

The nitromercaptobenzoic acid anion has an intense yellow colour and can be used to measure SH groups. Development of maximum colour intensity occurred immediately after the addition of DTNB but, absorbance decreases rapidly with time.

**Reagents:** Sulphosalicylic acid - 4%, Dithionitrobenzoic acid (DTNB) - 10mM, Phosphate buffer 0.2 M; pH- 7.4

**Procedure:** Whole blood 200 µl, 1.8ml D/W(chilled), 3 ml of 4% sulphosalicylic acid were added to a glass tube, vortexed and centrifuged at 6000rpm for 15 minutes in 4°C. Then 0.5ml of clear supernatant was added to 1 ml of phosphate buffer and 200 µl DTNB. Absorbance was read after 5 minutes at 412 nm.

3.3.3.1.2 Thiobarbituric acid reactive substances

**Principle:** The estimation was done as per Ohkawa et al., (1979). Malonaldehyde (MDA), a decomposition product of lipid hydro peroxides reacted with thiobarbituric
Methodology

Acid (TBA) in the acidic medium to generate a colored product by the reaction shown below.

\[
\begin{align*}
\text{TBA} & \quad \text{CHO} \\
\text{OH} & \quad \text{CH}_2
\end{align*}
\]

The intensity of the colored obtained was proportional to the lipid peroxidation. In acid solution, the product absorbs light at 532 nm and readily extractable into organic solvents such as n-butanol.

**Reagents:** Thiobarbituric acid (TBA) -0.8%, Sodium hydroxide (NaOH) - 1N, Acetic acid -30%; pH- 3.5, Sodium dodecyl sulphate (SDS) - 8.1%, n-Butanol : pyridine - 15 : 1

**Procedure:** The assay system contained 200 µl hemolysate, 200 µl of 8.1% SDS and 1.5ml of 30% acetic acid. The pH was adjusted to 3.5 with NaOH and then 1.5ml of 0.8% of TBA was added. The mixture was made up to 4 ml with D/W and incubated in water bath for 60 minutes at 95°C. After cooling 1 ml of D/W and 5 ml of n-Butanol : pyridine were added and shaken vigorously. There after the mixture was centrifuged at 4000rpm for 10 minutes at RT. The orange layer was taken and read at 532 nm.

3.3.3.1.3 Superoxide dismutase (SOD)

**Principle:** The estimation was performed as per Winterbourn (1993). Oxidation of Phenazine methosulphate in the presence of Nicotinamide generated superoxide anion. Nitrobluetetrazolium then reacted to form a stable NADH-phenazine methosulphate-nitroblue tetrazolium formazan complex. Superoxide dismutase present in the sample inhibited the reduction of NBT by NADH and PMS. This inhibition was detected as reduction in the intensity of the colour developed due to formazan formation.

**Reagents:** Sodium pyrophosphate buffer- 0.052M, Phenazine methosulphate (PMS) -180 µM, Nitrobluetetrazolium (NBT) - 300µM, Nicotinamide adenosine dihydride (NADH)- 780µM
**Procedure:** 250 µl ethanol, 150 µl chloroform was added to 1 ml of hemolysate, vortexed and centrifuged at 3000 rpm for 10 minutes in 4°C to get clear liquid, then 200µl of clear liquid was added to 1.2 ml of sodium pyrophosphate, 300µl PMS, 300µl NBT, 800µl D/W, 200µl NADH. Violet colour appeared incubated at 37°C for 90 seconds. Reaction stopped by adding 1 ml acetic acid, read at 560nm after 10 minutes.

### 3.3.3.1.4 Catalase

**Principle:** The estimation was done as per Aebi (1984). The potassium dichromate reacted with acetic acid when heated in presence of hydrogen peroxide to form chromic acetate. Presence of catalase split hydrogen peroxide and the redaction was stopped. Chromic acetate was measured spectrophotometrically at 570nm, since dichromate had no absorbance in this region, the presence of the dichromate in the assay mixture did not interfere at all with the colorimetric determination of chromic acetate.

**Reagents:** Acetic Acid reagent (5% solution of K₂Cr₂O₇ was mixed with glacial acetic acid in the ratio of 1:3), Hydrogen peroxide (H₂O₂)- 0.2M, Phosphate buffer: 0.01M.

**Procedure:** In a glass test tube, 1ml of hemolysate, 1 ml of 0.01 M Phosphate buffer (pH-7), 0.4ml of D/W, 0.5ml of 0.2M H₂O₂ were pipette d, incubated at 37°C for 1 minute in dark, reaction was stopped by adding 2 ml of acetic acid reagent. Boiled in water bath for 15 minutes and cooled, absorbance was read at 570nm.

### 3.3.3.1.5 Protein

**Principle:** Protein reacts with the Folin- Ciocalteau reagent to give a colored complex (Lowry et al., 1951). The color was formed due to the reaction of alkaline copper with the protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The intensity of color depended on the amount of these aromatic amino acids present and thus varies for different proteins.

**Reagents:** Solution 1: Alkaline sodium carbonate solution (2% Na₂CO₃ in 0.1 N NaOH) Solution 2: 0.5% Copper sulphate (CuSO₄.5H₂O) in 1% Sodium potassium tartarate Reagent 1: Freshly prepared mixture of solution 1and solution 2 in the ratio 1 :49 Reagent 2: Standard BSA (Bovine serum albumin) 1mg/ml
Reagent 3: Folin- Ciocalteau reagent

**Procedure:** To BSA standard/sample was added D/W to make up the volume to 1 ml each. Then 5 ml of reagent 1 was added, reaction mixture was vortexed and incubated at RT for 10 minutes, then 0.5 ml of Folin- Ciocalteau reagent was added and left for 30 minutes at dark, absorbance was read at 660 nm.

### 3.3.3.2 Estimation of kidney function markers

#### 3.3.3.2.1 Estimation of urea

**Principle:** Urea hydrolyses ammonia and CO2 in the presence of urease by modified Berthelot method (Chaney & Marbach, 1962). The ammonia formed further combines with a phenolic chromogen and hypochlorite to form a green colored complex. Intensity of the color formed was directly proportional to the amount of urea present in the sample.

**Procedure:** The estimation was done as per the instructions of the manufacturer of the kit. To 1 ml of buffer, 10µl of plasma sample/standard was added. Mixed well, and incubated for 10 minutes at RT. Then 200 µl of chromogen reagent was added, mixed well and incubated for 10 minutes at RT. Absorbance was taken within 30 minutes against the working reagent as blank at 570 nm.

#### 3.3.3.2.2 Estimation of creatinine

**Principle:** Picric acid in an alkaline medium reacted with creatinine to form an orange colored complex with the alkaline picrate (Mod. Jaffe’s kenetic method) by Bowers and Wong (1980). Intensity of the colour formed during the fixed time is directly proportional to the amount of creatinine present in the sample.

**Procedure:** In a clean test tube, 0.5 ml of picric acid reagent and 0.5ml of buffer reagent was dispensed. Mixed well and then 100µl of plasma /standard was added. Mixed well and initial absorbance A1 was read exactly 30 seconds. Another absorbance A2 was read exactly 60 seconds later against the reagent blank at 492 nm.

#### 3.3.3.2.3 Estimation of Uric Acid

**Principle:** Uric acid was oxidized to allantion and hydrogen peroxide by uricase method (Watts, 1974). In a subsequent enzymatic reaction, the hydrogen peroxide formed further
reacts with 4 aminoantipyrine and phenolic compound (2,4,6-tribromo 3- hydrobenzoic acid) in the presence of peroxidase to form a red colored quinoeimine dye complex. Intensity of the colour formed is directly proportional to the amount of uric acid present in the sample.

**Procedure:** The estimation was done as per the instructions of the manufacturer of the kit. To 1 ml of buffer, 20µl of plasma sample/standard was added. Mixed well, and incubated for 15 minutes at RT and absorbance was taken within 30 minutes against the buffer blank at 520 nm.

### 3.3.3.3 Estimation of liver function markers

#### 3.3.3.3.1 Estimation of serum glutamate pyruvate transaminase (SGPT)

**Principle:** Alanine aminotransferase (ALT) catalysed the transfer of amino group between L- Alanine and α Ketoglutaratate to form pyruvate and glutamate by modified IFCC method (Bergmeyer *et al* 1986). The pyruvate reacted with NADH in the presence of lactate dehydrogenase to form NAD. The rate of oxidation of NADH to NAD was measured as a decrease in absorbance which was directly proportional to the ALT activity in the sample.

**Procedure:** The estimation was done as per the instructions of the manufacturer of the kit. To 0.8 ml of enzyme reagent, 0.1 ml serum was added. Mixed well and incubated at RT for 1 minute. 0.2 ml of starter reagent was added. The contents were mixed well and incubated for 60 seconds and the decrease in absorbance was read after every minute, for 3 consecutive minutes against enzyme reagent blank at 340 nm.

#### 3.3.3.3.2 Estimation of serum glutamate oxaloacetate transaminase (SGOT)

**Principle:** L-Aspartate and α- Ketoglutarate reacted to give L- glutamate and oxaloacetate in the presence of asparatate aminotransferase (AST) by modified IFCC method (Bergmeyer *et al* 1986). The oxaloacetate produced, then reduced by NADH in the presence of malate dehydrogenase to yield L malate and NAD+. The rate of NADH oxidation of NADH to NAD+ was measured as a decrease in absorbance which was directly proportional to the AST activity in the sample.
Methodology

**Procedure:** The estimation was done as per the instructions of the manufacturer of the kit. To 0.8 ml of enzyme reagent, 0.1 ml serum was added. Mixed well and incubated at RT for 1 minute. 0.2 ml of starter reagent was added. The contents were mixed well and incubated for 60 seconds and the decrease in absorbance was read after every minute, for 3 consecutive minutes against enzyme reagent blank at 340 nm.

### 3.3.3.3 Estimation of bilirubin

**Principle:** Bilirubin reacted with diazotized sulphanilic acid to form a colored azobilirubin compound by Modified Jendrassik Grof method (Simmons, 1968).

The uncoupled bilirubin coupled with sulphanilic acid in the presence of caffeine benzoate accelerator. Intensity of the colour formed was directly proportional to the amount of Bilirubin present in the sample.

**Procedure:** The estimation was done as per the instructions of the manufacturer of the kit. In a glass test tube, 80 µl of sulphanilic acid, 20 µl of nitric acid, 100 µl of serum and 500 µl of accelerator was added. The contents were mixed well and incubated for 30 minutes. Then 500 µl of Fehling solution II was added to it. Mixed well and incubated for 15 minutes at RT. Absorbance was read at 546 nm.

### 3.4 In silico studies

#### 3.4.1 Ligand preparation

The structures of reported bioactive compounds of *G. sylvestre* and other known anti-diabetic drugs were downloaded as SDF format from Pubchem/ ChemSpider (http://www.chemspider.com/), energy were minimized using Avogadro software (Hanwell *et al.*, 2012) and converted into PDB format.

#### 3.4.2 Protein preparation

The protein/ enzymes involved in the insulin signalling pathway were downloaded from the protein databank (www.rcsb.org/pdb) and the hetero atoms were removed by using discovery studio visuvilizear before the docking analysis.
Methodology

Table 3.3: G. sylvestre molecules used for in silico study

<table>
<thead>
<tr>
<th>Molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gymnemic acid I (A)</td>
</tr>
<tr>
<td>Gymnemic acid II (B)</td>
</tr>
<tr>
<td>Gymnemic acid III (C)</td>
</tr>
<tr>
<td>Gymnemic acid IV (D)</td>
</tr>
<tr>
<td>Deacyl Gymnemic Acid (DGA) (E)</td>
</tr>
<tr>
<td>Gymnemagin (GMG) (F)</td>
</tr>
</tbody>
</table>

Note: Structural illustration is given in Figure 2.9.

Table 3.4: Molecular targets explored in docking study

<table>
<thead>
<tr>
<th>Protein used for this study</th>
<th>PDB id</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein tyrosine phosphatase 1B (PTP1B)</td>
<td>1C83</td>
</tr>
<tr>
<td>Insulin receptor (IR)</td>
<td>1IRK</td>
</tr>
<tr>
<td>Insulin receptor substrate (IRS)</td>
<td>1IRS</td>
</tr>
<tr>
<td>Phosphor inositide –depended protein kinase-1 (PDK1)</td>
<td>2PE1</td>
</tr>
<tr>
<td>Glycogen synthase kinase 3 beta (GSK-3β)</td>
<td>3F7Z</td>
</tr>
<tr>
<td>Serine/threonine protein kinase PKB/ Akt</td>
<td>3MV5</td>
</tr>
</tbody>
</table>

3.4.3 Docking analysis

**PatchDock:** The bioactive compounds of *G. sylvestre* were molecularly docked with ligands in silico by submitting the structures to the PatchDock server (http://bioinfo3d.cs.tau.ac.il/ PatchDock/), which is based on shape complementarity principles (Schneidman-Duhovny, *et al.*, 2005).

**FireDock:** The PatchDock results were resubmitted for refinement using the FireDock server (http://bioinfo3d.cs.tau.ac.il/FireDock/), which rearranges the interface side chains.
and adjusts the relative orientation of the molecules (Andrusier et al. 2007; Mashiach et al. 2008).


### 3.5 Cell line studies

#### 3.5.1 Chemicals

Alpha MEM, Fetal bovine serum, Trypsin, Antibiotic/antimycotic solution and TRIZOL reagent were from Gibco, USA. O-phenylenediamine dihydrochloride, protease inhibitor cocktail and all other chemicals unless otherwise noted were from Sigma Chemical (St. Louis, MO). Antibodies against primary polyclonal anti-myc, monoclonal anti-actinin-1, phosphor-IRS-1, phosphor-Akt, and phosphor-gsk were from Cell Signalling Technology (USA).

#### 3.5.2 Cell culture

L6 skeletal muscle cells stably expressing rat GLUT4 with a myc epitope inserted in the first exofacial loop were cultured in Dulbecco Modified Eagle’s Medium supplemented with 10% FBS, blasticidin S (2 μg/ml), and 1% antibiotic/antimycotic solution (10,000 U/ml penicillin G, 10 mg/ml streptomycin, 25 μg/ml amphotericin B) in a humidified atmosphere of air and 5% CO2 at 37 °C. Differentiation was induced by switching confluent cells to medium supplemented with 2% FBS. Experiments were performed in differentiated myotubes 6–7 days after seeding.

#### 3.5.2.1 Preparation of Test Solutions

For cytotoxicity studies, GSEE was dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% FBS to obtain a stock solution of 1mg/ml concentration and sterilized by filtration. Different dilutions were prepared from this for carrying out cytotoxic studies.
3.5.2.2 *Determination of cell viability*

The compounds were dissolved in DMSO for application to cell cultures. The cytotoxicity was checked by MTT assay and was used for the determination of cell viability. L6 skeletal muscle cells were seeded and incubated for 24 hours. Cells were treated with various concentrations (10, 25, 50 and 100 µg/ml) of GSEE and incubated for 24 hours. Then, cell viability in L6 skeletal muscle was evaluated. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated.

3.5.2.3 *GLUT4 translocation*

The translocation of GLUT4 from the cytoplasm to the plasma membrane was measured by an antibody-coupled colorimetric assay using GLUT4myc myotubes (Tamrakar *et al.*, 2010). It relies on GLUT4 antibodies directed to an external epitope of the transporter that binds to it as soon as it is exposed to the extracellular medium after translocation to the plasma membrane. After the indicated treatments, cells were washed in ice-cold PBS supplemented with 1 mM CaCl2 and 1 mM MgCl2 (pH 7.4). The cells fixed by adding 3% para formaldehyde to each well, without shaking the cells. Incubated for 10 minutes at 4°C and for 20 minutes at RT and quenched in 100 mM glycine for 10 min, all at 4°C. Cells were blocked in 5% FBS for 15 min and then incubated with anti-myc antibody solution for 1 hour at 4°C.

After labeling, excess antibodies were removed by extensive washing in ice-cold PBS. Cell surface GLUT4-bound antibodies were probed by HRP-conjugated secondary antibodies followed by detection of bound HRP by O-phenylenediamide assay. The fractions of GLUT4 at the cell surface were measured as fold induction with respect to unstimulated cells.

3.5.2.4 *Western blotting*

After the GSEE treatments, L6 cells were lysed in 1% triton X-100 in PBS supplemented with NaOV3 (1 mM), NaF (1 mM) and protease inhibitor cocktail (1:1000). Cells lysates were centrifuged 1000 rpm for 10 minutes at 4°C and the protein
concentration was measured by BCA method. Lysates containing equal amount of protein were heated at 65°C for 10 minutes in 1x Laemmli sample buffer (sigma Aldrich) supplemented with 10% β-mercaptoethanol. After heating samples, they were vortexed on high for 20s to shear DNA and reduce viscosity. Proteins are then separated by 10% or 12 % SDS-PAGE according to the molecular weight and transferred to polyvinylidene difluoride membrane. Membranes were blocked with 5% blocking solution (skimmed milk or BSA) for 1hr. After blocking, membranes were incubated over night with monoclonal anti-actinin-1, phospho-Akt, and phospho-gsk and followed by incubation with appropriate HRP-conjugated secondary antibodies.

Immunoreactive bands were visualized by enhanced chemiluminescence according to manufacturer's instructions (GE Healthcare, UK). Immunoblots were exposed to X-ray film to produce bands in a linear range, and then quantified using the National Institute of Health (NIH) Image J software. Actinin-1 was used as an invariant control for equal loading. Densities of bands in western blotting analyses were normalized with the internal invariable control. Variations in the density were expressed as fold changes compared with the control in the blot.

### 3.5.3 Inflammatory markers

Inflammatory markers (IL10, IL6, TNF-α and MCP-1) were measured in supernatant of treated cells by BD optEIA™ ELISA kit (BD Biosciences-USA) according to manufacturer’s instruction. Briefly 96 well plates was coated with 100µl per well capture antibody diluted in coating buffer for overnight at 4°C. All the further experiments were performed at RT. Next day, plate was aspirated and washed 3 times with wash buffer and complete removal of buffer at each step. Plate was blocked with 200 µl of 3% BSA for 1 hour. After blocking, plate was washed and incubated with 100 µl of sample and standard with appropriate dilution and again incubated for 2 hours. After the incubation, the cells were washed 4-5 times with buffer and 100 µl of detection antibody was added. Plate was incubated for 1 hour and the plate was washed 5 times with wash buffer. Plate was incubated for 30 minutes in enzyme reagent diluted in assay diluents. Plate was again washed 7 times with buffer and 100 µl of substrate solution was added, plate was
incubated for 30 minutes and then reaction was stopped with 50 µl of stop solution. Absorbance of plate was read at 492nm. Standard plot was created and value of sample was calculated.

3.6 Statistical analysis

Statistical Package for the Social Sciences (SPSS) version 20 was used for regression analysis to calculate the risk ratio of the every attributing factor. Paired t test and one way ANOVA (Tukeys multiple comparison test) were used to compare the groups (GraphPad Prism version 6). P<0.05 was considered statistically significant.