CHAPTER-3

Chapter 3

To assess effect of *Enicostemma littorale* Blume bioactive ingredient in regulation of candidate metabolic gene expression in insulin sensitive peripheral tissue of experimental NIDDM rat model

3.1 Introduction

Diabetes mellitus is the third most prevalent fatal disease in the world. Epidemiology shows that it is one of the major global health problems in current scenario, targeting 8.3% of total world population (Zimmet et al. 2001; Nam Han Cho et al. 2013). Among all the types of diabetes Type II diabetes mellitus (TIIDM) contributes to about 85-95%. Around 61.5 million people are diabetic in Indian subcontinent; hence, India leads globally in this disease (Shaw et al. 2010; Nam Han Cho et al. 2013). Indian population is more prone to diabetes than western population as their metabolism is quite different with lots of epigenetic modifications. Constant migration of people from rural to urban areas has contributed significantly in availability of food, calorie intake and physical activities which, has major impact on the metabolic programming of an individual (Yajnik and Ganpule-Rao 2010).

To understand the physiological and metabolic alterations of this disorder, many animal models are used. NA-STZ (Nicotinamide -Streptozotocin) rat is a non-obese type II diabetes model that reflects a majority of pathophysiological conditions of diabetic patients among Asian races (Nakamura et al. 2006). Thus, in the present study, NA-STZ model was used to mimic the experimental NIDDM conditions in adult rat. STZ, a cytotoxin for β- cells was administered along with NA which partially protected the cells from STZ. NA-STZ yields 75-80 % of diabetic animals with 40% reduction of pancreatic β-cell mass, stable non-fasting hyperglycaemia of 150–180 mg/dl, glucose intolerance, and significant alterations in glucose stimulated insulin secretion and *in vivo* responsiveness to tolbutamide. NA-STZ NIDDM model resembles lean Type II diabetes than other available chemical models (Masiello et al. 1998).

Type II Diabetes Mellitus is a heterogeneous metabolic disorder. Liver, adipose tissues and skeletal muscles being insulin sensitive tissues show significant metabolic changes (Srinivasan

and Ramarao 2007). The pathogenesis of TIIDM, resulting from defects in insulin receptor (IR) function, IR-signal transduction, glucose transport, glycogen synthesis, glucose oxidation and dysregulation of fatty acid metabolism contributes to insulin resistance in target tissues and impairment of pancreatic insulin secretion (Saltiel and Kahn 2001). Insulin, apart from governing glucose uptake and metabolism, also influences the expression levels of many genes related to energy metabolism (O'Brien et al. 2001).

Liver is the master organ where, metabolism of all the biomolecules takes place. It is the main organ apart from skeletal muscles and adipocytes which maintains the concentration of glucose and even fatty acids in the blood, wherein, insulin governs the process of glycolysis, gluconeogenesis, glycogenesis and glycogenolysis (Harvey 2011). It not only changes the rate of reactions catalyzed by enzymes like Glucokinase, Glucose-6-phosphatase, PEPCK, Fructose-1, 6-bisphosphatase and Glycogen phosphorylase, but also alters their gene expression levels (Sun et al. 2002).

Glut2 plays a major role in glucose uptake and its metabolism in hepatic tissue, whose gene expression is influenced by glucose and insulin concentrations hence, remarkable alterations in glucose, would lead to significant change in the expression profile of this transporter. The next key player is the Glucokinase (GK) enzyme which functions as a rate limiting step in glycolysis. In diabetic condition the expression of this enzyme is down regulated which eventually leads to insulin resistance and hyperglycemia (O'Brien and Granner 1991; Postic et al. 2004). PEPCK is a rate limiting enzyme in gluconeogenesis, hence its elevated expression leads to increased hepatic glucose production (HGP). The processes of glycolysis, gluconeogenesis, glycogenesis and glycogenolysis is governed by insulin action which are hampered due to altered action of insulin in TIIDM (Collier and Scott 2004).

Adipose tissue, the storehouse of body fat, plays a key role in controlling glucose and fat homeostasis in the entire body. Hence, being obese with an abnormal accumulation of fat in it disturbs their normal functions, which leads to development of TIIDM (Kadowaki et al. 2003). The balanced secretion of various adipokines plays a key role in controlling glucose and fat homeostasis of the entire body. PPAR-γ plays a major role in regulation of transcription; and is responsible for adipogenesis, mature adipocyte function, insulin signaling, carbohydrate

metabolism, fat metabolism and secretion of various adipokines like adiponectin, leptin etc. Adiponectin positively and leptin negatively regulate insulin signaling in liver and muscle tissues (Ferre 2004). Looking towards the importance of this regulator most studies have been directed toward developing synthetic PPARs ligands for insulin resistance and dyslipidemia in amelioration of TIIDM complications (Sheng et al. 2008).

There are many treatments available ranging from synthetic drugs like Metformin, Thiazolidinediones, GLP-1, DPP-4 etc. to herbal formulations like *Momordica charantia, Artemisia dracunculus, Gymnema sylvestre,* etc in amelioration of obesity and TIIDM (Cefalu et al. 2008). In recent years, there has been renewed interest in the treatment of diabetes using herbal drugs, as World Health Organization (WHO) recommended evaluation of the effectiveness of plants due to side effects of modern drugs (Baby Josheph 2011).

Enicostemma littorale (EL) belonging to family Gentianaceae is being used by rural folk for treatment of diabetes and our lab has been working on its antidiabetic potential since long. Previous studies in the lab showed its hypoglycemic, hypolipidemic and antioxidant potentials in alloxan-induced diabetic rats (Vijayvargia et al. 2000; Maroo et al. 2002; Maroo et al. 2003) as well as in newly-diagnosed NIDDM patients (Vasu et al. 2003). Studies with isolated rat pancreatic islets showed glucose-induced insulin release indicating insulin secreatogouge activity of EL (Sarita Gupta 2010), along with cytoprotective effect against H_2O_2 induced apoptosis. Apart from antidiabetic activity, islet neogenic potential from tissue specific stem/progenitors of swertisin and normoglycemia in diabetic rat is also reported (Sarita Gupta 2010; Dadheech et al. 2013). Insulin resistance and glucose intolerance was ameliorated by EL in high fructose fed rats along with reduction in serum triglyceride levels (Bhatt NM et al. 2009; Bhatt NM et al. 2011). The aqueous extract of EL and its isolated compound, Swertiamarin (SM) is the major compound found in EL Blume and its pharmacokinetic study suggest that, its rapid distribution in most of the tissues with high concentrations were found in liver and kidney, indicating swertiamarin is absorbed and eliminated through these organs (Li HL et al. 2011). Swertiamarin demonstrated increased HDL levels, decreased serum cholesterol, triglyceride levels and significantly reduced Hepatic HMG-CoA reductase activity in cholesterol fed rats as well in Zucker Fatty Rats (Vasu et al. 2005a; Vaidya et al. 2009b). EL increases insulin sensitivity and
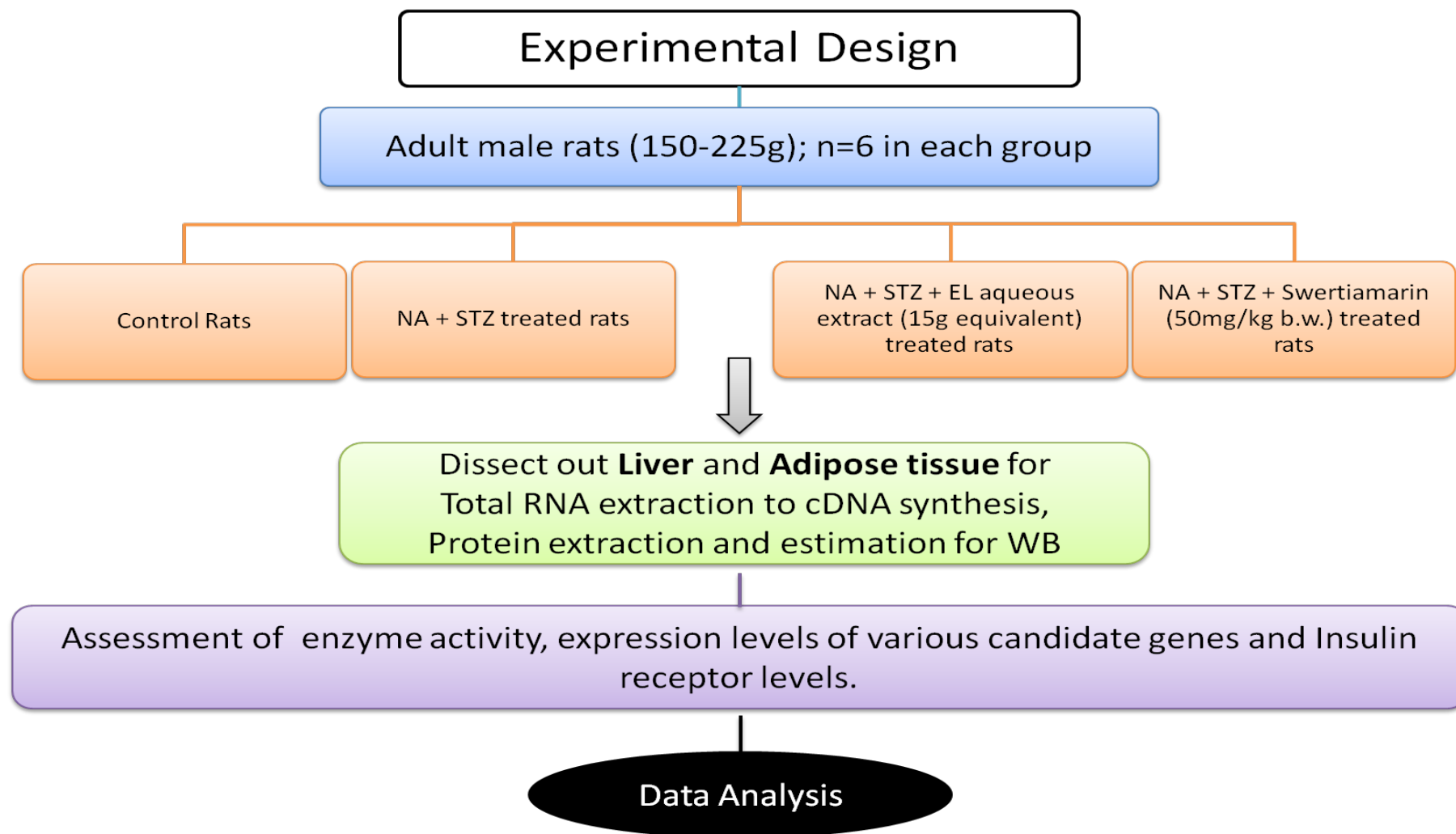
normalizes dyslipidaemia in NIDDM rat model (Murali et al. 2002). Amongst the already known drug targets for TIIDM, PPAR's lower triglyceride levels, increases HDL levels and insulin sensitivity via Glut4 translocation leading to peripheral glucose uptake.

Hence, it can be presumed that the EL extract potentially owes varied beneficiary activities due to the presence of many compounds within it. There is only single report which explored the mechanistic action of swertiamarin derivatives on TIIDM on 3T3-L1, preadipocyte metabolism (Vaidya et al. 2013). Also other groups have focused on physiological and biochemical studies in neonatal-STZ models to understand SM's role in antilipidemic and dyslipidemia. However, the mechanism of action of swertiamarin has not been explored at systemic level (Vaidya et al. 2009a; Vaidya et al. 2012b).

Therefore, for the first time in this study we aim to assess the antidiabetic efficiency of swertiamarin in treating nicotinamide-streptozotocin (NA-STZ) diabetic rats and to elucidate its probable mechanisms of action. The current study was designed to answer the key question, 'What is the mechanism of swertiamarin in regulating the expression levels of the candidate genes involved in carbohydrate, fat metabolism and insulin signaling in the liver and adipose tissue in TIIDM ?'

3.2 Experimental Design

Experimental Design

Adult male rats (150-225g); n=6 in each group

| Control Rats | NA + STZ treated rats | NA + STZ + EL aqueous extract (15g equivalent) treated rats | NA + STZ + Swertiamarin (50mg/kg b.w.) treated rats |

Dissect out **Liver** and **Adipose tissue** for Total RNA extraction to cDNA synthesis, Protein extraction and estimation for WB

Assessment of enzyme activity, expression levels of various candidate genes and Insulin receptor levels.

Data Analysis

3.3 Materials and methods

3.3.1 Chemicals and media

1. Streptozotocin (Sigma, Aldrich # S0130-1G)
2. 0.1M Citrate buffer (pH=4.5)
3. Nicotinamide (Duchefa Biochemia # N0610.0100)
4. GOD-POD kit (Reckon Diagnostics)
5. Insulin Elisa kit (Mercodia)
6. Cholesterol Kit (Reckon)
7. Triglyceride Kit (Enzopak)
8. TRIzol reagent (Invitrogen)
9. Chloroform and Isopropanol (Sisco Research Laboratory)
10. Primers (IDT)
11. PCR master mix (Sigma)
12. DEPC water: 0.1% (v/v)
13. Agarose (Invitrogen)
14. 30% Acrylamide (Biorad)
15. TEMED (Amresco)
16. 10%v(w/v) APS
17. 5X TBE (Amresco)

3.3.2 Plant material, preparation of aqueous EL extract and isolation of the Swertiamarin

The plant material of dry *E. littorale* was procured from Saurashtra region, Gujarat, India during the month of August. Specimen was authenticated at Botany Department with Voucher Specimen number [Oza 51, 51(a)] deposited at the Herbarium of Botany Department, The M. S. University of Baroda. Whole plant material was cleaned and dried. The fine powder of 40-60 mesh particle size was prepared in electric grinder. The powder was soaked in thrice the amount of water for 2 hours and then boiled for 30 minutes. Three such extractions were done on each batch. Residue was removed by filtration and water-soluble filtrate was pooled and evaporated to obtain extract concentration of 1g dry plant weight equivalent per ml as per the method described above (Maroo et al. 2002). The yield of dry EL extract was found to be 28% (w/w). Isolation and

characterization of swertiamarin from *E. littorale* was carried out by recording melting point and UV spectrometry with the standard sample of swertiamarin (Figure 1a). Purity of the sample was checked by HPTLC on ethyl acetate: methanol:water (0.7:0.2:0.1) as solvent system (Vishwakarma 2004).

3.3.3 Animals and housing

Male *Charles Foster* rats housed at animal house facility of Department of Biochemistry were used for study with *ad libitum* access to water and commercial chow (Pranav Agro Industries Ltd, Pune, India) in a well-ventilated animal unit (26 -28^{0}C, humidity 60 %, 12 h light–12 h dark cycle). Care and procedures adopted for the present investigation were in accordance with the approval of the Institutional Animal Ethics Committee (938/a/06/CPCSEA, BC/14/2009-10). NIDDM rat model was developed by intra-peritoneal injection of NA dissolved in normal saline at a concentration of 230mg/kg body weight 15 minutes before giving an intra-peritoneal injection of STZ (Sigma, Aldrich) which was dissolved in 0.1M citrate buffer (pH 4.5) at a concentration of 65mg/kg body weight (Masiello et al. 1998). Hyperglycemia was confirmed by the elevated glucose level in fasting and Post Prandial Blood Sugar (PP$_2$BS) after 15-20 days of STZ-NA injection.

3.3.4 Dosing of swertiamarin and aqueous extract in NIDDM rats

Swertiamarin and aqueous extract were orally administered for 40 days and fasting serum glucose levels, OGTT profiles and serum triglyceride levels were monitored. Rats were divided into five groups having six rats in each group, Group I: normal control (NC), Group II: NIDDM, Group III: NIDDM + Aqueous extract (15 grams dried plant equivalent extract / kg b.w/day, p.o.), Group IV: NIDDM+ Swertiamarin (50 mg/kg/day, p.o.), Group V: NIDDM+ Metformin (500 mg/kg b.w/day, p.o.).

3.3.5 Biochemical Parameter

3.3.5.1 Oral Glucose Tolerance Test (OGTT)

Rats were kept for overnight (10-12 hrs) fasting; blood was collected from retro orbital sinus and serum was separated for estimation of fasting blood sugar. To measure OGTT of the rats, 2 gms/kg body weight of glucose was given orally and blood was collected at regular intervals of

every 30 min. till 2 hours. Serum was separated and glucose level was estimated using GOD-POD method by commercially available kit (Enzopak, India).

3.3.5.2 Determination of serum insulin by ELISA

Fasting serum insulin was estimated by rat Insulin ELISA kit (Mercodia, Sweden). Mercodia Rat Insulin ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies which is bound to microtiter well. A simple washing step removes unbound enzyme labeled antibody. The bound conjugate is detected by reaction with 3,3', 5, 5'-tetramethylbenzidine. The reaction is stopped by adding acid to give a colorimetric endpoint that is read at 450nm spectrophotometrically. Calibrator curve was run along with samples and concentration of unknown samples was Calculated.

3.3.5.3 Lipid profile

Total cholesterol, HDL-cholesterol and TG were estimated using commercially available kits (Enzopak, India) and then the values of LDL-cholesterol and VLDL-cholesterol was derived from Friedewald's formula.

3.3.5.3.1 Serum total cholesterol (Kit Method) and high density lipoprotein(HDL)

Serum cholesterol level is an indicator of liver function, biliary function, intestinal absorption, propensity towards coronary artery disease & adrenal disease. Decreased HDL cholesterol levels associated with increased risk of developing coronary artery disease and other atherosclerotic complications.

Values of LDL and VLDL was derived from Friedewald's formula as follows:

$$[\text{LDL-chol}] = [\text{Total chol}] - [\text{HDL-chol} + (\text{TG}/5)] \quad \& \quad [\text{VLDL-chol}] = [\text{TG}/5]$$

3.3.5.3.2 Serum triglyceride Estimation (McGowan et al. 1995)

Lipase hydrolyses triglycerides sequentially to di- and mono-glycerides and finally to glycerol. Glycerol kinase phosphorylates glycerol to glycerol-3-phosphate using ATP as a source of phosphate which is oxidized to DHAP and H_2O_2 is simultaneously liberated by glycerol-3-

phosphate oxidase. H_2O_2 is reduced by peroxidase while 4-aminoantipyrine and ADPS are oxidized to purple colored complex which is read at 546nm that is directly proportional to the serum triglyceride concentration.

3.3.6 Determination of Liver Enzymes

3.3.6.1 Glucose-6-phosphatase

Glucose-6-phosphatase was assayed according to the method of Koida and Oda and the inorganic phosphorus (Pi) liberated was estimated by Fiske and Subbarow method.

Glucose-6-Phosphatase is an enzyme exclusively present in the liver, localized in the ER lumen. Its activity was estimated by microsomal fraction of the cells. Microsomal fraction preparation requires ultracentrifugation, i.e., centrifugation of liver homogenate at 105,000g for 60 minutes. Another method of preparing a crude microsomal fraction is to centrifuge 10% liver homogenate prepared in 0.25M sucrose at 10000g for 30 minutes.

Data was represented in specific activity = Units of enzyme/ mg of protein.

3.3.6.2 HMG-CoA reductase activity (Vasu et al. 2005b)

Hepatic HMG-CoA reductase activity was indirectly measured in terms of the ratio between HMG-CoA and mevalonate. HMG-CoA was determined by its reaction with hydroxylamine at alkaline pH and subsequent colorimetric measurement of the resulting hydroxylamine acid by formation of complexes with ferric salts at 540nm. Mevalonate was estimated by reaction with the same reagent but at pH 2.1. At this pH, the lactone form of mevalonate readily reacts with hydroxylamine to form the hydroxyamate. The ratio between HMG-CoA and mevalonate is inversely proportional to HMG-CoA reductase activity, i.e. an increase in ratio indicates decreased activity.

3.3.7 RNA isolation & semi-quantitative PCR

Animals from each group were sacrificed and tissues (liver and adipocytes) were pooled. RNA was isolated from the homogenized liver and adipose tissue using the TRIzol reagent (Sigma Aldrich) as per manufacturer's instructions. A reverse-transcription reaction was performed using 2μg RNA with MuLV reverse transcriptase in a 20μL reaction volume containing DEPC treated water (Fermentas Kit). PCR product was amplified using gene specific primers (Table 1).

β-Actin was used as an internal control and the PCR amplicon were analyzed by electrophoresis on 2.0% agarose gels or 15% DNA-PAGE, and images were captured after staining with ethidium bromide and intensities of the band were calculated by densitometric analysis using the Image J software

3.3.8 Immunoprecipitation & immunoblotting for insulin signaling proteins

Tissue were collected, suspended in lysis buffer containing 1X protease inhibitor cocktail and homogenized. After centrifugation at 16000 g for 15 min. at 4°C, the supernatant was collected. Total protein content was quantified using Bradford assay (Bio-Rad Bradford Solution, USA). Immunoprecipitation with Insulin receptor (anti-IRβ 1:50) was performed using Dynabeads G-protein IP kit (Invitrogen). Protein was loaded on a 10% SDS-Polyacrylamide gel and then electrophoretically transferred onto a Nitrocellulose membrane (GE Healthcare). The membrane was then incubated for 1h at room temperature in blocking buffer (TBS-T containing 5% skimmed milk) and further incubated overnight with the primary antibodies for insulin receptor (1:1000), p-Tyr (1:1000) and PI(3)K (1:1000) at 4°C. Membrane was then washed four times with PBS-T, and incubated with HRP-conjugated secondary antibodies (1:2500) for 1h. Finally, membrane was developed and visualized with Enhanced Chemiluminescence western blotting detection system (Millipore Inc. USA) by Chemidoc (Uvitec Alliance 4.7) (Table 7).

3.3.9 Statistical analysis

The results were analyzed using one-way analysis of variance (ANOVA) and student's t-test to determine the level of significance. $p < 0.05$ was considered to be significant. Results were expressed as mean ± SEM. The statistical analysis was carried out by using the Graph Pad Prism 3.0 software.

3.4 Results

3.4.1 Isolation and confirmation of swertiamarin from *Enicostemma Littorale* Blume

The *n*–butanol fraction yielded 7.31% w/w of swertiamarin. HPTLC densitogram confirmed identity of compound with standard swertiamarin as well as established purity of the same. Ultraviolet absorption spectrum showed λmax in the range of 240-245 nm. Melting point of the compound was 190-192 ℃. The mass fragmentation pattern of compound represented base peak m/z of 374 representing molecular weight and m/z of 212 (M-162) a characteristic peak after removal of sugar moiety from the compound. TLC profile observed under UV 254 nm; and Densitograms of tracks (Figure 3.1a,b,c & d).

Figure 3.1a
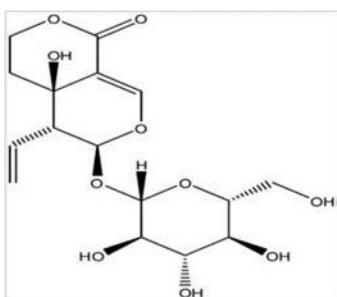
Figure 3.1a: Chemical structure of Swertiamarin

Figure 3.1b
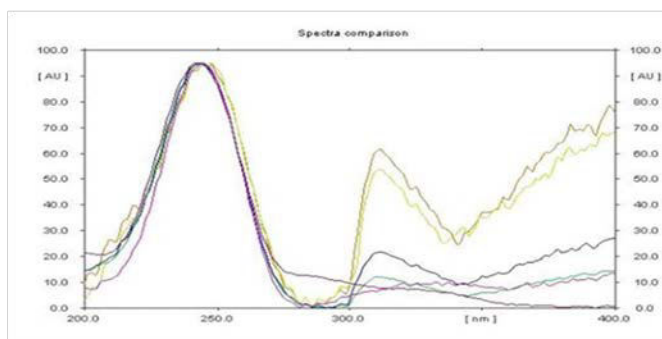
Figure 3.1b: Overlay of ultraviolet absorption spectrum of Swertiamarin isolated in lab and reference standard (λmax :240-245 nm)
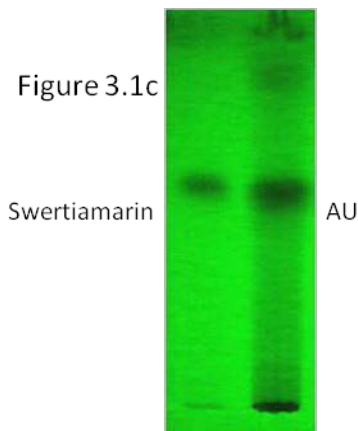
Figure 3.1c

Figure 3.1c: TLC profile observed under UV 254 nm

Figure 3.1d
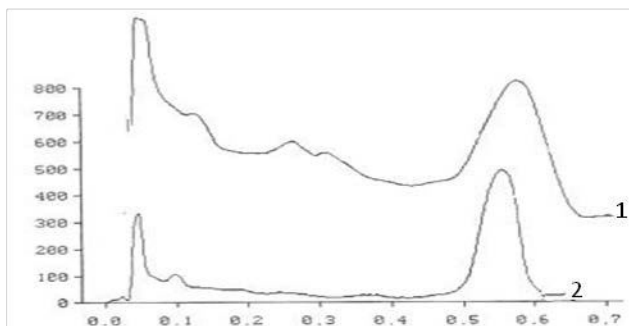
Figure 3.1d
1d: Densitograms of tracks 1: *Enicostemma littorale* extract, 2: swertiamarin.

3.4.2 Swertiamarin positively regulates various physical and biochemical parameters in TIIDM

Decrease in body weight is a characteristic hallmark of TIIDM that happens due to the loss of the stored energy reserves. A classical way of determining the efficacy of drug treatment is the ability to restore body weight. As expected, we found a drastic decrease in the body weight in NA-STZ-induced diabetic rats as compared to the controls. The animals treated with standard drug metformin (MFO), aqueous extract and SM showed significant increase in the body weight which, thus, indicated reversal of DM condition (Figure 3.2).

Further, in an attempt to confirm the NIDDM condition in the animal model, oral glucose tolerance test was performed to ascertain severity of the diabetic condition. Our observation is in agreement with the known facts: the diabetic rats have a high PP_2BS and showed glucose intolerance as compared to the control rats. MFO effect, as expected in Type II diabetics, reduced the PP_2BS in our rats (Figure 3.3a & b). The aqueous extract and SM treated diabetic rats were observed to be normoglycemic.

Hyperinsulinemia is a characteristic feature of TIIDM. However in our DM group of rats, the serum insulin levels were lower than those in the control rats which matches with the reported model where insulin content is reduced up to 40% (Masiello et al. 1998) (Figure 3.3c) and which mimics a later stage of TIIDM .The EL extract and SM treatments were capable of significantly ameliorating the hypoinsulinemic condition, by increasing the serum insulin levels.

3.4.3 Swertiamarin reduces Glucose 6 Phosphatase activity

Changes due to diabetes are not only seen at the mRNA level but also at the protein levels. Many of enzyme activities are altered in the peripheral tissues of diabetics. G-6-Pase is the key enzyme of gluconeogenesis in hepatic tissue. Its activity increases under diabetic condition due to deficiency of insulin or of insulin action. The EL extract and SM restored elevated specific activity of G-6-Pase to normal levels (Figure 3.4).
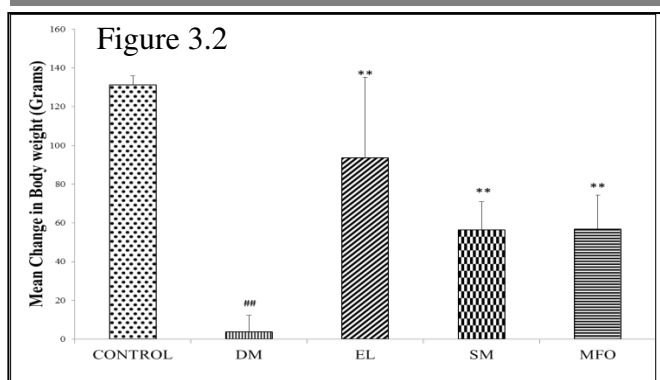
Figure 3.2: Change in Body weight upon induction of diabetes and treatment of diabetic rats with EL, SM and standard drug MFO. Data presented as Mean ± SEM of 6 independent observations. ## p < 0.05 vs. control rats; ** p< 0.05 vs. Diabetic rats
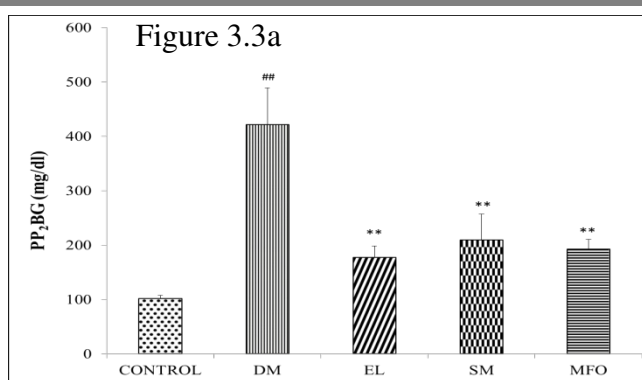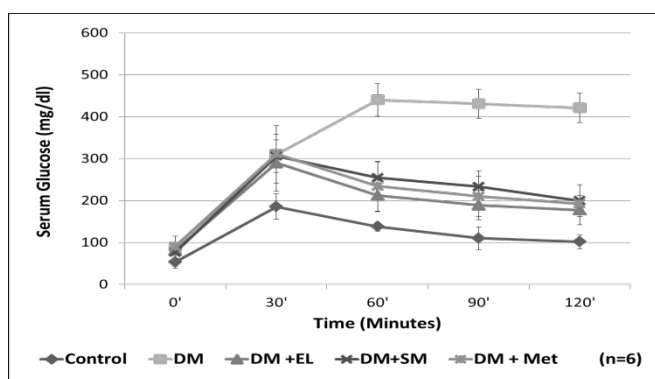

Figure 3.3a: Effect of EL extract, SM and MFO treatments for 40 days on the Post-prandial serum glucose levels in diabetic conditions. Serum glucose levels were measured using GOD-POD. Data presented as Mean ± SEM of n=6 independent observations. ## p <0.05 vs. control rats; ** p<0.05 vs. Diabetic rats

Figure 3.3b: Effect of EL extract, SM and MFO treatments for 40 days on the OGTT profile in diabetic conditions. Serum glucose levels were measured using GOD-POD. Data presented as Mean ± SEM of n=6 independent observations.
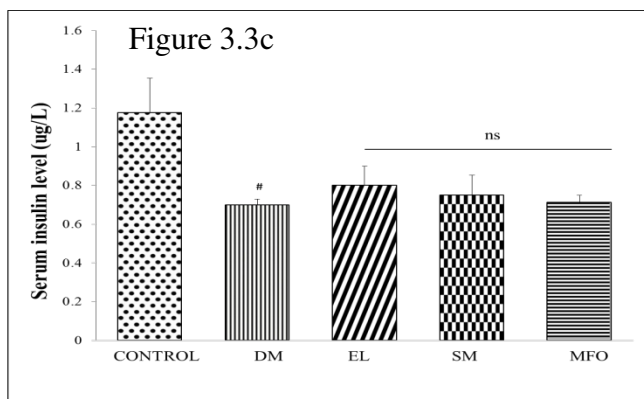

Figure 3.3c: Effect of EL extract, SM and MFO treatments for 40 days on the Serum insulin levels in diabetic conditions. Serum insulin levels were measured using ELISA kit. Data presented as Mean ± SEM of 4 independent observations. # p< 0.05 vs. control rats. p-value ns vs. Diabetic rats
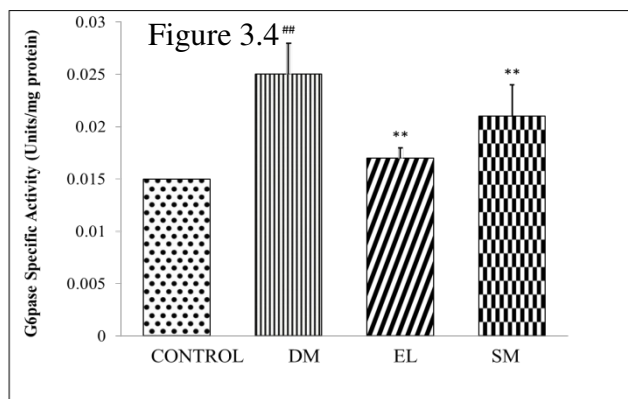

Figure 3.4: Effect of EL extract, SM and MFO treatments for 40 days on the specific activity of G-6-Pase enzyme from hepatic tissue in diabetic conditions. It was assayed according to Koida and Oda method and released Pi was estimated using Fiske-Subbarao Method. Data presented as Mean ± SEM of 5 independent observations. ## p< 0.05vs. control rats; **p< 0.05vs. diabetic rats.

3.4.4 Swertiamarin regulates the expression levels of candidate genes of carbohydrate metabolism in TIIDM

Diabetes affects the expression of many candidate genes in the insulin dependent peripheral tissues like liver, adipose and skeletal muscles. Glut transporters are the main glucose transporters in different peripheral tissues. Glut2 is present in liver and is insulin independent. In diabetic rats, Glut2 expression decreased significantly as compared to the control rats. We observed that the two treatments: EL extract and SM rescue this decrease in expression and the extract was more efficacious then the compound. PEPCK and Glucokinase are the main enzymes of gluconeogenesis and glycolysis respectively. They are regulated by insulin at the transcriptional level. In the diabetic condition, PEPCK has increased expression, while GK has decreased expression in liver. Treatments for diabetes should thus, decrease the expression of PEPCK and increase the expression of GK, which was seen in EL extract and SM group (Figure 3.5a & b)

3.4.5 Swertiamarin regulates the altered expression of Insulin Signaling proteins in Liver

The liver homogenate was subjected to immunoprecipitation and immunoblotting with anti-phospho tyrosine and anti-insulin receptor antibody. There was a decrease in the protein expression of Insulin receptor in the diabetic group as compared to control. However, treatment with SM and EL extract restored not only level of insulin receptor protein but also increased its phosphorylation. PI(3)K is a molecule downstream to insulin receptor which gets recruited via IRS signaling pathway (Figure 3.6a & b).

3.4.6 Lipid profile

We observed a significant increase in the serum triglyceride levels in the diabetic rats as compared to the control rats. This observation assertively showed that the serum triglyceride levels increase due to the insufficient action of insulin in the diabetic condition at the peripheral tissues. Metformin(MFO) did not bring down the serum triglycerides levels significantly. But EL extract and its compound, SM, had a higher efficacy and reduced the serum triglyceride near to control levels, thus making them out to be a safer alternative than the available anti-diabetic drugs. Aqueous extract and SM, both were able to decrease serum cholesterol, serum LDL, VLDL levels and increase HDL- cholesterol (Table 3.1).
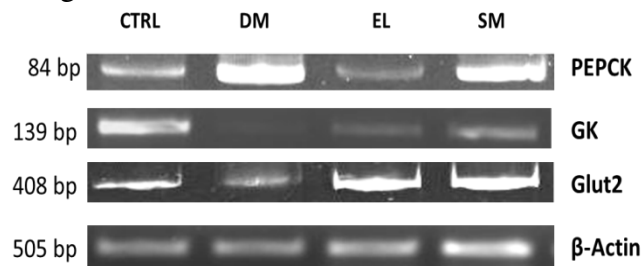
Figure 3.5a

Figure 3.5a: Effect of EL extract and SM treatments on the mRNA expression of PEPCK, GK, Glut2 and β-Actin in the hepatic tissue as compared to diabetic rats .
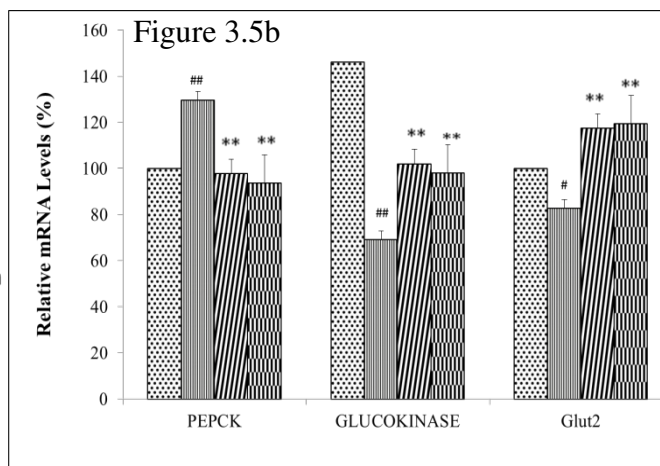

Figure 3.5b: Effect of EL extract and SM treatments on the expression of PEPCK,GK and Glut2 in the hepatic tissue as compared to diabetic rats. The expression levels were checked using semi-quantitative RT-PCR and densitometric analysis. Data presented as Mean ± SEM of 4 independent observations. ## $p < 0.05$ vs. control rats; **$p < 0.05$ vs. diabetic rats. ▒ = Control Rats, ▦ = DM, ▨ = EL and ▥ = SM.
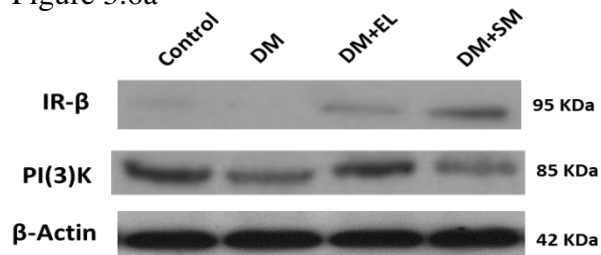
Figure 3.6a

Figure 3.6a: Western blot study showing the effect of EL extract and SM treatments on the expression of insulin signaling proteins: IR and PI(3)K in the hepatic tissue as compared to diabetic rats. β-actin was taken as an internal control.
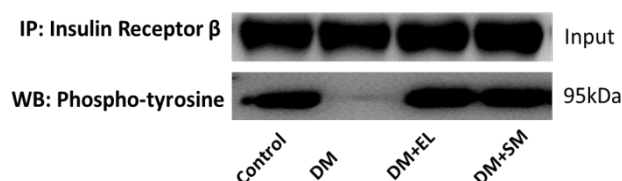
Figure 3.6b

Figure 3.6b: Immunoprecipitation study showing the effect of EL extract and SM treatments on the tyrosine phosphorylation of insulin signaling proteins: IR in the hepatic tissue as compared to diabetic rats (200ug Protein).
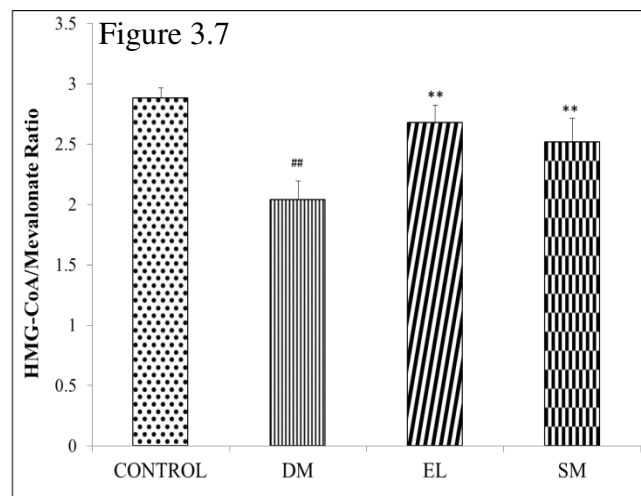

Figure 3.7 : Effect of EL extract and SM treatments for 40 days on the ratio of (absorbance of HMG CoA/absorbance of Mevalonate) was taken as an index of the of HMG CoA reductase activity from hepatic tissue in diabetic conditions. Data presented as Mean ± SEM of 5 independent observations. ## $p < 0.05$ vs. control rats;** $p < 0.05$ vs. diabetic rats.

Table 3.1. Lipid profile of control and treated diabetic rats.

Groups	Control	DM	DM+EL	DM+SM	DM+Metformin
Triglyceride #	55.86 ±4.69	110.94 ±14.32*	49.63 ±6.37[a]	63.34 ±7.86[b]	94.54 ±19.31
Total cholesterol#	88.55 ±7.28	123.26 ±15.80*	95.15 ±10.88[a]	86.75 ±13.64[b]	106.78 ±12.54
HDL-C #	55.86 ±4.66	27.44 ±8.27*	44.63 ±5.49[a]	40.94 ±6.77[b]	41.89 ±4.69
LDL-C #	21.52 ±1.93	73.63 ±3.2*	40.59 ±4.37[a]	33.1 4 ±5.86[b]	45.99 ±5.64[c]
VLDL-C #	11.17 ±0.69	22.19 ±4.76*	9.93 ±1.3[a]	12.67 ±1.01[b]	18.90 ±2.31

Units: mg/dl. Values are given as mean ± SEM from 6 rats in each group, * P < 0.005 compared to Control and [a,b & c] compared to Diabetic group .

3.4.7 Swertiamarin regulates activity of HMG-CoA Reductase enzyme of cholesterol biosynthesis

HMG-CoA reductase is the major regulatory enzyme of cholesterol biosynthesis in the liver. An estimate of the enzyme activity can be used as a measure of the severity of the diabetic condition. The results of the present study showed inhibition of the HMG-CoA reductase activity in the diabetic rats treated with SM and EL extract as observed by higher HMG-CoA/mevalonate (substrate/product) ratio compared to that of the diabetic control rats (Figure 3.7), thus supporting earlier reported hypolipidemic activity of SM.
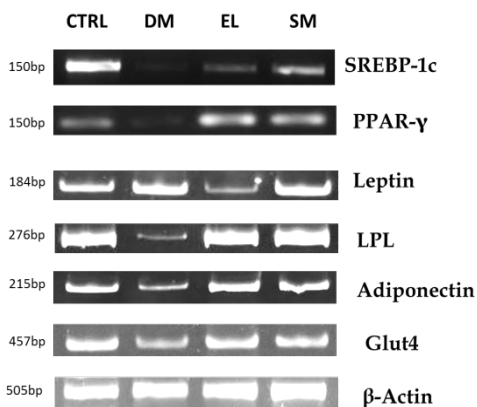
Figure 3.8a

Figure 3.8a : Effect of EL extract and SM treatments on the mRNA expression of SREBP-1c, PPAR-γ, Leptin, LPL, Adiponectin, Glut4 and β-actin in the adipose tissue as compared to diabetic rats.
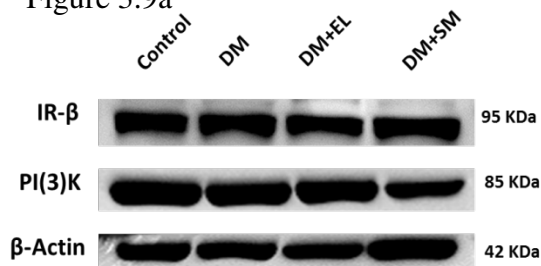
Figure 3.9a

Figure 3.9a: Western blot study showing the effect of EL extract and SM treatments on the expression of Insulin signaling proteins: IR and PI(3)K in the adipose tissue as compared to diabetic rats. β-actin was taken as an internal control.
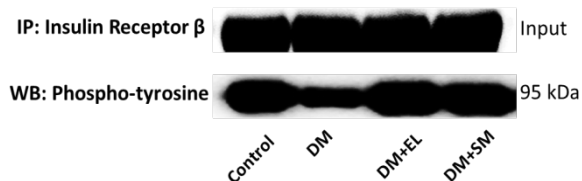
Figure 3.9b

Figure 3.9b: Immunoprecipitation study showing the effect of EL extract and SM treatments on the Phosphorylation of Insulin signaling proteins: IR in the adipose tissue as compared to diabetic rats (100μg Protein).
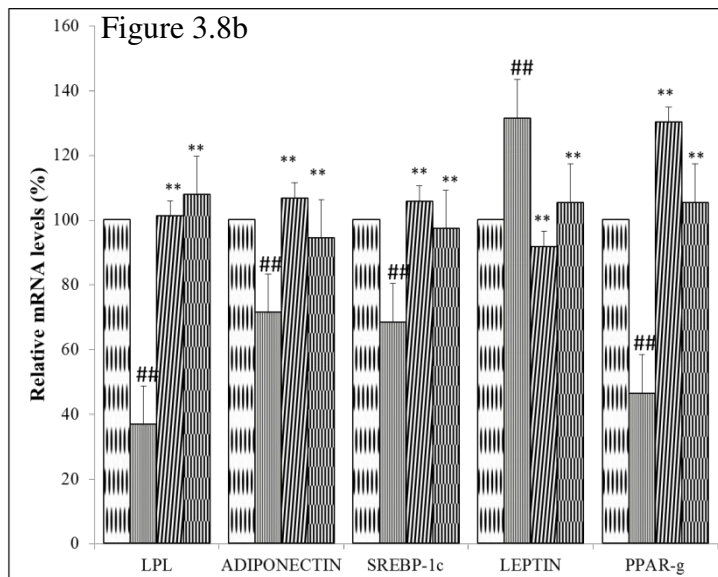

Figure 3.8b : Effect of EL extract and SM treatments on the expression of the major genes regulating the fat metabolism in the adipocytes as compared to diabetic rats. The expression levels were checked using semi-quantitative RT-PCR and densitometric analysis. Data presented as Mean ± SEM of 4 independent observations.## $p < 0.05$ vs. control rats ; ** $p < 0.05$ vs diabetic rates .

▦ = Control Rats, ▥ = DM, ▨ = EL and ▥ = SM.

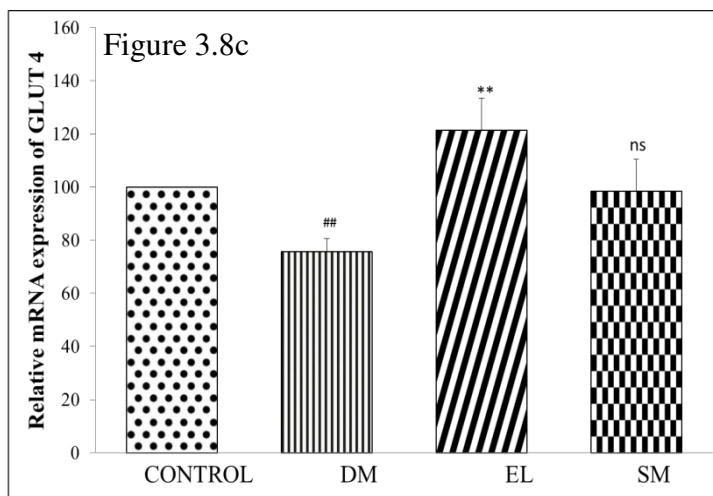Figure 3.8c: Effect of EL extract and SM treatments on the expression of Glut 4 in the adipocytes as compared to diabetic rats. The expression levels were checked using semi-quantitative RT-PCR and densitometric analysis. Data presented as Mean ± SEM of 4 independent observations. ;## $p < 0.05$ vs. control rats;**$p < 0.05$ vs diabetic rates p value ns vs. Diabetic rats.

▦ = Control Rats, ▥ = DM, ▨ = EL and ▥ = SM.

3.4.8 Swertiamarin regulates the expression levels of various key enzymes of lipid metabolism and glucose transporter

Glut4, an insulin dependent glucose transporter present in adipocytes and skeletal muscles, has decreased expression in diabetic rats due to increased insulin resistance. The key regulators of fat metabolism like adiponectin, SREBP-1c and PPAR-γ and Lipoprotein Lipase (LPL) are also found to be down-regulated, while leptin is up-regulated. The treatments with EL extract and SM helped in overcoming the insulin resistance by restoring the above gene expressions to normal levels (Figure 3.8a,b & c).

3.4.9 Expression of insulin signaling proteins in adipose tissue

The adipose tissue homogenate was subjected to immunoprecipitation and immunoblotting with anti phospho-tyrosine and anti-insulin receptor antibodies. There was decrease in the protein expression of insulin receptor and PI(3)K in the diabetic group as compared to control (Figure 3.9a). Treatment with SM and aqueous extract restored the level of insulin receptor, IR phosphorylation and PI (3)K protein (Figure 3.9b).

3.5 Discussion

Obesity and insulin resistance are major causes of TIIDM. Multiple problems in diabetes lead to a cascade of complications in peripheral tissues. For controlling hyperglycemia, dyslipidemia and insulin resistance, many synthetic drugs have been used. Also, the beneficiary effects of herbal extracts and compounds have been exploited (Baby Josheph 2011).

Enicostemma litorrale blume belonging to *Gentianaceae* family has been evaluated for its hypoglycemic, antioxidant and hypolipidemic activities (Vasu et al. 2003; Vasu et al. 2005b). Further, the author's research group is continuously involved in exploring the wide spectrum hidden potentials of this plant for islet neogenesis and various diabetic complications (Bhatt NM et al. 2009; Bhatt NM et al. 2011; Dadheech et al. 2013). Qualitative analysis demonstrated the presence of flavonoids and secoiridoid glycosides. Swertiamarin, a secoiridoid glycoside is one of the most valuable compound that is present in abundance and possesses various therapeutic activities: antidiabetic, antinociceptive, antilipidemic, and anti-inflammatory (Jaishree et al. 2009; Vaidya et al. 2009a). Hence, it is interesting to unravel the mechanism of this compound's action against the development and progression of TIIDM.

Various animal models are available for studying TIIDM. NA-STZ non-obese NIDDM rat model was selected for this study that best mimics the non-insulin dependent diabetes condition prevalent in humans (Masiello et al. 1998). Glucose intolerance, altered insulin content and skewed lipid profile of the experimental animals resembles the hallmarks of this model that actually persists in later stage of human TIIDM patients. Effect of SM in restoration of body weight, OGTT profile and hypolipidemic activity on the experimental animals potentially proves the reported characteristics of this compound in the present study. Many herbal compounds are reported in regulating the expressions of the candidate genes involved in metabolic pathways and thus ameliorating TIIDM. This led to our interest in unraveling the molecular mechaniswertiamarin of aqueous extract and swertiamarin in restoring the altered expressions of the candidate genes involved in TIIDM.
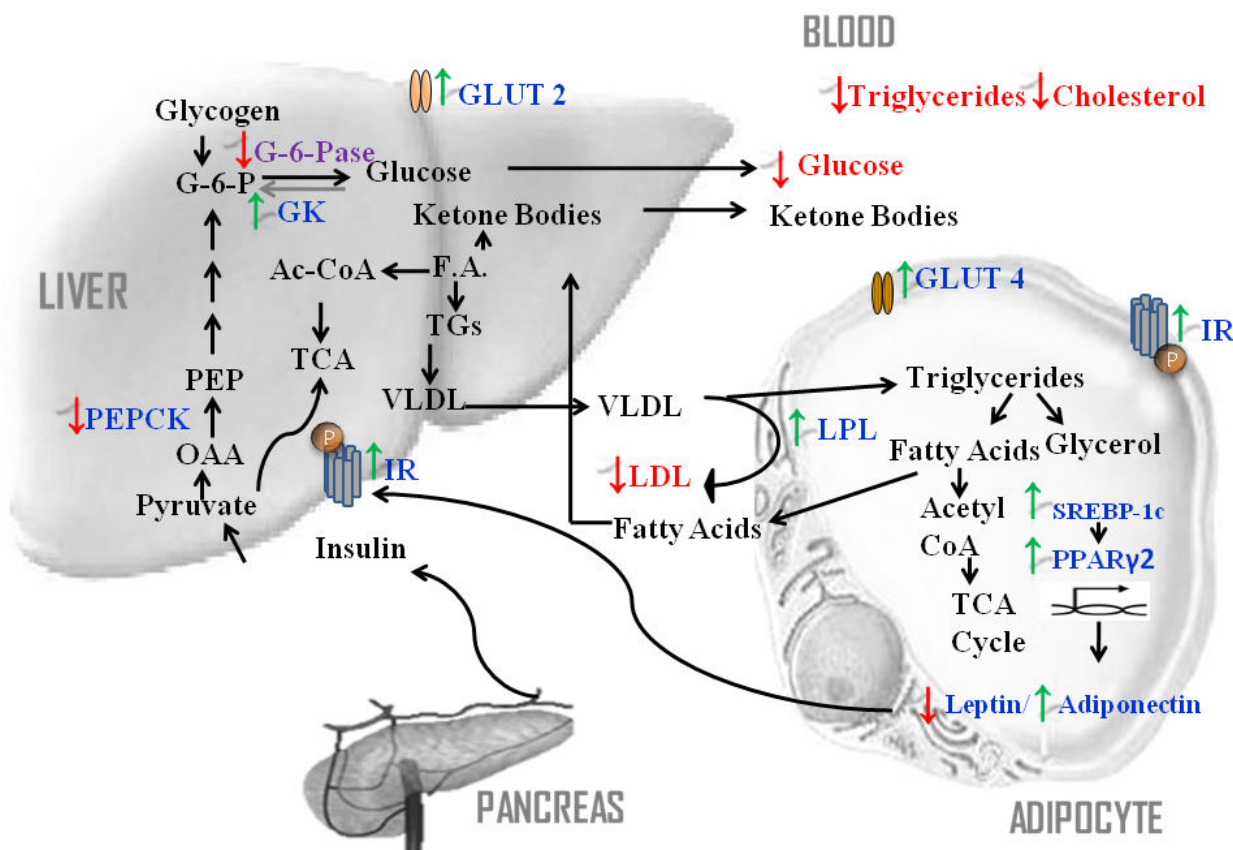
Figure 3.10: Schematic representation of swertiamarin in amelioration of insulin resistance and TIIDM. Figure shows carbohydrate and fat metabolic pathways and candidate genes which are altered during diabetes. Swertiamarin treatment modulates not only the expression of these target genes which is marked in blue but also the metabolite levels in blood marked in red.

Carbohydrate and fat metabolism regulation is governed mainly in insulin sensitive peripheral tissues like liver, muscle and adipose tissue. Liver is the master organ in metabolism where Glucokinase, PEPCK, glycogen 6-phosphatase are rate limiting enzymes in glycolysis, gluconeogenesis, glycogenesis and glycogenolysis respectively (Collier and Scott 2004).

Decreased activity of G-6-Pase was observed in swertiamarin treated diabetic rats, which correlate to the results reported by us previously in aqueous extract treated diabetic rats. Reduction in the PEPCK gene expression was observed when diabetic rats were treated with swertiamarin in the current study. PEPCK expression restoration reflects increased insulin

sensitivity (Hanson and Reshef 1997; Vihas T. Vasu et al. 2003). Increased activity of this limiting enzyme leads to more hepatic glucose production (HGP), which worsens the diabetic condition. Glucose concentration increases binding of SREBP-1c on promoter of Glut2 increasing its transcription, which is regulated by glucose and insulin. Expression levels of Glucokinase(GK) and Glut2 have been shown to be decreased in the hepatocytes of the diabetic rats (Ribnicky et al. 2006). In agreement with earlier reports, our results show that diabetic rats have decreased GK and Glut2 expressions, which are reversed upon treatment with EL aqueous extract and swertiamarin.

It is well documented that diabetic patients exhibit dyslipidemia. Our lab previously reported decrease in serum triglycerides, cholesterol, LDL, VLDL with increased HDL level in aqueous extract treated cholesterol fed rats (Vasu et al. 2005b). Swertiamarin is beneficial in bringing the lipid profile in noenatal-STZ rats to normal. (Vaidya et al. 2012b). HMG-CoA reductase is a key enzyme involved in the cholesterol biosynthesis in the hepatic tissue which increases the free fatty acid level that leads to insulin resistance. Swertiamarin and aqueous extract corrected lipid profile and HMG-CoA reductase activity (Vaidya et al. 2009b).

Adipose tissue plays an important role in fat metabolism. In TIIDM, increased lipolysis and decreased lipogenesis occurs in liver and adipose tissues. Obesity decreases expression in lipogenic genes like SREBP-1c, PPAR-γ and aP2 which causes increase in hepatic lipogenesis hence leading to fatty liver (Suh et al. 2005). PPAR-γ is a key transcriptional factor regulating the expression of SREBP-1c, leptin, adiponectin and LPL. Low adiponectin and high leptin levels can cause insulin resistance in adipocytes thus leading to diabetes (Huang et al. 2008). In the present study, aqueous extract and SM both regulate PPAR-γ mRNA levels in NA-STZ induced diabetic rat model along with induced expression of adiponectin, LPL and SREBP-1c suggesting it as a potent modulator of diabetes related modification in adipocytes and thus, corrects overall lipid metabolism, which can correct dyslipidemia by increasing insulin sensitivity (Vaidya et al. 2012a).

Insulin sensitivity depends on the binding of insulin to its receptor which autophosphorylates and further leads to downstream signaling cascade. Treatment of diabetic animals with aqueous extract and swertiamarin showed increased insulin receptor protein synthesis and its auto

phosphorylation in liver and adipose tissues, which improves insulin sensitivity in TIIDM. Phosphorylation of PI(3)K is mainly responsible for insulin stimulated glucose uptake by Glut4, which is responsible for peripheral glucose disposition in muscle and adipose tissue. It has been reported that Cinnamon extract improves insulin action and glucose uptake by enhancing the insulin-signaling pathway in skeletal muscle (Qin et al. 2003).

 The results of the current study prove that swertiamarin activates PPAR-γ and its regulatory genes, which improves fat metabolism in adipose tissue. By controlling PPAR-γ, swertiamarin can maintain status of small adipocytes that reduces expression of Leptin and TNF-α and increases expression of Adiponectin. Increased Adiponectin secretion acts in an autocrine and paracrine manner, which improves expression of insulin receptor, its autophosphorylation and downstream insulin signaling in liver as well as in adipose tissue (Kadowaki et al. 2003). This is the need of the hour, a drug which is able to maintain a balance between all the players involved in the carbohydrate and fat metabolism in the peripheral tissues.

In conclusion the NA + STZ treated rats show glucose intolerance, increased serum TG and decreased serum insulin levels, indicating NIDDM-like condition. Treatment with aqueous EL extract and swertiamarin has been found to reduce the glycemic burden as is monitored by OGTT profile. In diabetic rats it enhances insulin sensitivity resulting in restoration of altered gene expression of glucose metabolism in liver. In dyslipidemic condition swertiamarin plays a crucial role in lowering surplus cholesterol by inhibiting HMG-CoA reductase activity. This is the first report *in vivo* that highlights a significant role of swertiamarin as a regulator of gene expression under the control of transcriptional factors like PPAR-γ, hence suggesting that swertiamarin improves insulin sensitivity and modulates carbohydrate and fat metabolism by regulating PPAR-γ (Figure 3.10). Present results thus, strongly suggest that swertiamarin can be a potent therapeutic agent against TIIDM.

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