CHAPTER-4(A)

Chapter 4

To study the mechanism of action of swertiamarin: An active lead from *Enicostemma littorale* in insulin resistant peripheral tissue *in vitro*

4.1 Introduction

Type II diabetes mellitus (TIIDM) is a major health concern prevalent in today's present world. Modern sedentary life style with lack of exercise, dietary compositions, geographical locations, are extrinsic factors whereas, genetic makeup and adaptations, derailed metabolic functions, inflammatory responses and pathogenic infections are the intrinsic factors that are associated with its occurrence. It is identified as a cumulative effect of peripheral insulin resistance wherein, major insulin dependent tissues are affected by reduced binding efficacy with insulin receptor and glucose uptake (Qin et al. 2003).

Insulin resistance is the primary metabolic defect that leads to various complications like hypertension, cardiovascular diseases and other related peripheral ailments (Berdichevsky et al. ; Etxabe and Vazquez 1994; Bell et al. 2008; Bhat et al. 2009). *In vivo* studies (Chapter 3) showed that hyperglycemia, hyperinsulinemia and hyperlipidemia are the indicators of glucose intolerance and insulin resistance in peripheral tissues like liver, muscle and adipose tissue (Patel et al. 2013) caused due to circulating non-esterified free fatty acids (Zierath 2007) leading to ectopic fat deposition in liver and muscle. Liver is the major organ associated with carbohydrate metabolism. Excess of free fatty acids, drain into liver, hampers glucose and glycogen metabolism and a state known as non-alcoholic fatty liver is created. The immediate effect of insulin resistance is also observed in skeletal muscle, which plays a major role in glycogen storage. More than 80% of circulating glucose is stored in muscle and hence, leads to muscle insulin resistance (DeFronzo 1999; DeFronzo and Tripathy 2009). In insulin resistant adipocytes lose their ability to store fatty acids and hampers energy homeostasis due to reduced lipogenesis and lipolysis.

Various *in vivo* animal models created spontaneously or by chemical insults, dietary composition, genetic modifications and surgical manipulations are used extensively for understanding the pathophysiology of TIIDM at cellular and molecular level and understanding

the effect of various therapeutic drugs and herbal plants involved in its amelioration (Srinivasan et al. 2005). Similarly various *in vitro* models are also used for studying the insulin resistance and its related metabolic defects in peripheral tissues per se.

Treatment of TIIDM comprises of various insulin sensitizers and other drugs. However their adverse side effects have restricted their use and reinforced to search new safe, pharmacologically viable and economic approach for treating obesity, insulin resistance and allied metabolic disorders (Baby Josheph 2011).Many herbal extracts and compounds are used as therapeutics for amelioration of dyslipidemia and insulin resistance. Hsu and Yen demonstrated that phenolic compounds like rutin and O-coumaric acid efficiently inhibits adipogenesis and corrects fat metabolism. Berberine has shown anti-lipidemic activity *in vitro* in high fat fed animals. Various other phenolic and herbal plants have shown anti obesity effects (Hsu and Yen 2007; Lee et al. 2010a; Lee et al. 2010b; Ikarashi et al. 2012).

Since ages folks were using *Enicostemma littorale* (EL) therapeutically for treating diabetes, for last ten years our lab started working on this plant to unravel its potentials, as a potent antidiabetic herb.

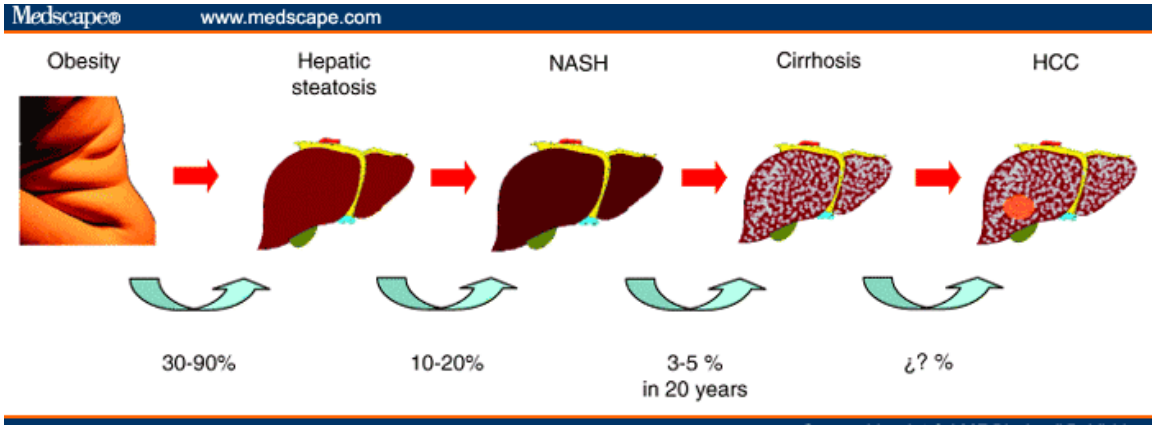
Swertiamarin is a principal compound present in EL and its administration in STZ-NA diabetic model proved it as a potent insulin sensitizer that ameliorated peripheral insulin resistance as shown in earlier **Chapter 3** (Patel et al. 2013). As it has demonstrated that insulin dependent tissues like liver, muscle and adipose tissue exhibit metabolic alterations and are responsible for hallmark metabolic syndrome changes, the present **Chapter 4** was designed to understand the mechanism of action of swertiamarin on peripheral insulin resistance *in vitro* using three model cell lines namely HepG2 (hepatocytes), L6 myocytes and 3T3-L1 preadipocytes.

Chapter-4A

To study the mechanism of action of swertiamarin in oleic acid induced model of hepatic steatosis: *in vitro*

4A.1 Introduction

Excess accumulation of fat (triglycerides) in hepatic parenchymal cells (hepatocytes) leads to a pathological condition known as non alcoholic fatty liver disease (NAFLD) (Tiniakos et al. 2010). Histopathological studies exemplified the morphological alterations prevailing in this disease that depicts moderate to gross macrovesicular changes with or without inflammation (lobular or portal, mallory bodies), fibrosis or cirrhosis (Alba and Lindor 2003). The spectrum of the disease progression commences from steatosis, steatohepatitis (NASH), NASH with fibrosis to cirrhosis and liver cancer. A large single fat vacuole with displaced nucleus having a signet ring appearance in hepatocyte is the hallmark of this ailment also, known as macrovesicular steatohepatitis. The detectable limit for lipid accumulation in liver above 5-10 % by weight is the diagnostic marker for hepatosteatosis. Microvesicular steatosis differs from the former with respect to that, the hepatocytes are submerged in the pool of fat accumulated around the cells without any observed nuclear displacement (Reddy and Rao 2006).



Etiology of this disease is very diverse. Clinically this disease is prevalent in obese, diabetics and malnutrition state such as Kwashiorkor and AIDS. Hispanic people have higher prevalence of NAFLD than whites and American-African population. The probable reason can be the difference in their fat metabolism i.e they have reduced rate of hypertriglyceridemia and low HDL. Five to twenty eight percent Indians suffer from NAFLD. The prolonged effects of

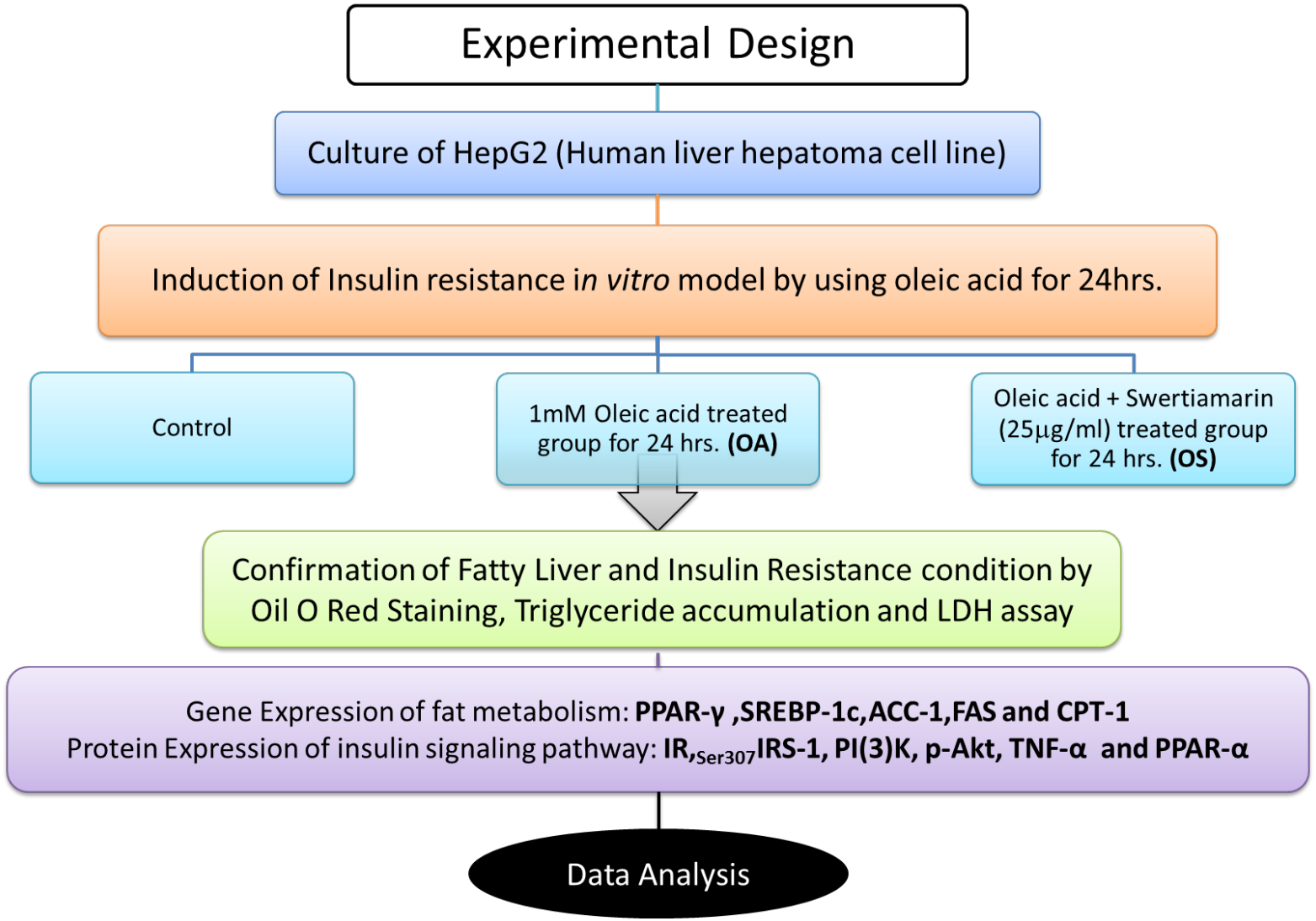
NAFLD are intrahepatic oxidative stress, lipid peroxidation and induction of inflammatory responses by provoking proinflammatory cytokines (Asrih and Jornayvaz 2013a). Excess of unconsumed and non-oxidized lipid accumulation into hepatocytes infarcts the cells and ultimately leads to liver injury (Lewis and Mohanty 2010) .

Insulin resistance, obesity and altered fat metabolism are causative factor associated with the development of NAFLD. However, many reports suggest that obesity and altered fat metabolism are responsible for pathogenesis of this disease. NAFLD is most prevalent in centrally obese people with insulin resistance condition than those of the lean insulin resistant individuals. Lower abdominal fat deposition is culprit of this disease. The activated macrophages of liver called as Kuffer cells release proinflammatory cytokines like TNF-α, IL-6, etc. PAT [perilipin, adipophilin, and tail-interacting proteins of 47kd (TIP47)] are a family of lipid droplet–associated proteins that are actively involved in droplet formation and its turnover (Anderson and Borlak 2008). They direct lipase to digest the neutral lipids and prevent lipolysis.

Extensive steatosis leads to accumulation of ballooned leaky hepatocyte that further activates stellate cells responsible for liver fibrosis. Prolonged hepatocellular progression and fibrosis leads to liver cancer (de Alvaro et al. 2004). To study the pathophysiology of the disease, various *in vivo* experiments have been carried out. Lirko mice were studied for elucidating the mechanism of action by exploring the transcriptional factors responsible for lipogenesis and lipid metabolism along with the proinflammatory cytokines involved in the inflammatory responses in hepatosteatosis. HepG2 cells (hepatoblastoma cells) are considered as the best model for understanding the pathophysiology of this disease (Srivastava et al. 2007). Various fatty acids like stearic acid (saturated), oleic acid (monounsaturated), linoleic acid (n−6 polyunsaturated) and α-linolenic acid (n−3 polyunsaturated) treated at pathophysiological dose for 24 hrs have been used for induction of steatosis in hepatocytes among which linoeic and α-linoeic showed protective effects on the cells, by inhibiting fatty acid synthesis and diminishing the enzymes involved in ROS generation. However, oleic acid contradictorily enhanced ROS, fatty acid synthesis, elevated triglyceride (TG) accumulation and hampered insulin sensitivity in the model cells which lead to hepatosteatosis (Kohjima et al. 2008).

As insulin resistance is directly associated to hepatosteatosis, increased accumulation of free fatty acids in the hepatocytes leads to altered expression of the enzymes like Carnitine palmitoyl transferase I (CPT-1) involved in mitochondrial beta oxidation which, increases oxidative stress that concomitantly elevates the inflammatory responses with activation of kuffer cells. Thus, the cell membrane integrity is lost which is detected by percent release of Lactate dehyrogenase (LDH) and caspase activation (Jadeja et al. 2010). The outcome of these events lead to alteration in insulin signaling proteins like downregulation of insulin receptor, serine phosphorylation of IRS at 307 amino acid residue, downregulation of pAkt, PI(3)K (Michael et al. 2000) and major transcriptional factors like PPAR-α responsible for carbohydrate and fat metabolism in liver. Hence, insulin resistance causes alterations in the metabolism of the fat stored in the liver cells which leads to severe injury. As discussed earlier in this study we used oleic acid induced HepG2 cells for insulin resistance condition and explored the effect of swertiamarin.

4A.2 Experimental Design



4A.3 Materials and methods

4A.3.1 Chemicals and media

Dulbecco's modified eagle's medium (DMEM) low glucose, trypsin-EDTA, oleic acid, fat free BSA and Oil O Red stain were purchased from Sigma Aldrich. Fetal Bovine Serum (FBS) and Penicillin-Streptomycin were procured from Gibco, Life Technologies and cell culture experiments were carried out in tissue culture plastic ware obtained from Nunclon. RNA extraction was performed using Trizol (Invitrogen), gene specific primers were designed from IDT and all the other reagents required for molecular biology experiments were procured from Life Technologies. TG and LDH kits were purchased from Reckon Diagnostics, Vadodara, and all proteomics grade reagents were obtained from Bio-Rad and antibodies for protein expression were obtained from CST.

4A.3.2 Cell culture

Human hepatocellular carcinoma cells (HepG2) obtained from National Centre for Cell Sciences(NCCS), Pune, India, were seeded (1×10^5cells/ T25 Flask) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) Low Glucose with 10% FBS and 1% antibiotic-antimycotic solution (10X) at 37°C with 5% CO_2 (Thermo scientific, CO_2 incubator). Cells were subsequently passaged every third day by trypsinization with 0.25 % trypsin-EDTA solution.

4A.3.3 Induction of steatotis in hepatocytes

The experiment was carried out for 24 hours which was divided into three groups: control, 1mM oleic acid were taken for induction of steatosis (Cui et al. 2010). 1mM oleic acid along with 25ug/ml swertiamarin (Chapter 5) and the positive control group cells were treated with 1mM oleic acid and 10mM metformin. As the cells are carcinogenic in nature, they underwent apoptosis when treated with metformin which possesses anti-cancer activity (Quinn et al. 2013). Hence this group was excluded from the analysis.

4A.3.4 Qualitative and quantitative analysis of *in vitro* NASH

At the end of the treatment period (24 hr), cells were fixed in buffered 4 % paraformaldehyde for 10 min at RT and were washed twice with PBS 1.0 ml of (Oil O Red) solution (1% in

isopropanol) was then added to each well and incubated at RT for 10 min. After removing the stain from each well, the cells were washed with PBS until the solution became clear. Wells were dried, mounted in glycerine and examined under phase contrast microscope, Nikon. After washing and drying completely, 1ml of isopropanol (100%) was added to each well, incubated for 10 min and then transferred to 1.5 ml tube and the absorbance were read at 520nm (Cui et al., 2010) using Microplate Reader (Multiskan ,Thermo Co.).

4A.3.5 TG accumulation assay

After 24 h of exposure of OA to the cells were washed thrice with PBS and lyzed with 1% triton in PBS. The lysates were centrifuged at 10000g for 2 min, and collected the supernatant, assayed for TG using commercially available enzymatic kit (Reckon Diagnostics, Baroda, India). Results were expressed as percentage TG. These values were normalized to total protein in the extract, measured with the Bradford reagents method (Bio-Rad).

4A.3.6 LDH release assay

Cytotoxicity was measured as the fraction of lactate dehydrogenase (LDH) released into the medium. HepG2 cells were maintained in 6 well plates for 24 hr as described above. After the collection of conditioning media, cells were washed with phosphate buffered saline (PBS) and lysed in 1% triton-X-100 in PBS. Cell lysates were collected, vortexed for 15 seconds and centrifuged at 7000 rpm (Eppendorf 5415 R) for 5 min. LDH activity was measured in the supernatant and cell lysate by a commercially available kit (Reckon Diagnostics Ltd, Baroda, India). LDH Activity was calculated for intracellular and supernatant separately from this formula = ΔA/min X F (F= 3376) and % LDH release were calculated as per the formula given below= (Media/Total LDH activity) X 100

4A.3.7 RNA extraction and Real time quantitative PCR

Total RNA was isolated from treatment groups using TRizol Reagent and 2µg of total RNA was reverse transcribed into first strand cDNA and subjected to PCR amplification for various fat metabolic genes (Table 2). cDNA, synthesized by using First Strand Reverse Transcription System Kit (Fermentas INC, USA), was used for real time analysis (Applied- Biosystem 7500-Real-Time PCR Sequence detection System) of fat metabolic gene expression. The primer sequences used for quantitative real-time polymerase chain reactions (qRT-PCR) are

listed in Table 2. Quantitative RT-PCRs were performed in two independent experiments in triplicates. 10µl of total reaction volume containing, 5µl Power SYBR-Green master mix, 10pM of each forward and reverse primers using 100ng cDNA (1/20th of total cDNA preparation) was taken. All qRT-PCR results were normalized to the level of beta-actin determined in parallel reaction mixtures to correct any differences in RNA input. Fold changes in qRT-PCR gene expression was analyzed using 7500 Real time PCR software V.2.0.6 and Data assist software (Applied Biosystems Inc.) which led to a possible estimation of the actual fold change. The qPCR results are expressed as mean ± S.E.M of RQ values versus target gene.

4A.3.8 Protein extraction and western blotting

Western blot was performed for expression of various proteins. Briefly, HepG2 cells were cultured with OA for 24 hours, and then the cell pellets were lysed with 1 ml of the lysis buffer (1mM EDTA, 50mM Tris-HCl pH 7.5, 70mM NaCl, 1% Triton, 50mM NaF) supplemented with protease inhibitor cocktail (Fermentas INC.). Protein estimation in all samples was carried out using Bradford reagent according to the manufacturer's suggestions (Bio-Rad). Cell lysates (20µg) were separated on polyacrylamide gel using Mini-tetrapod electrophoresis system (Bio-Rad) and transferred onto nitrocellulose membrane (Thermo Inc.). Blots were then incubated with blocking milk buffer (5% fat free skimmed milk with 0.1% Tween-20 in PBS). The blots were then probed for IR, pIRS, pAkt, PI(3)K, PPAR-α and TNF-α. Dilutions of primary antibodies used, against various proteins are shown in Table 7. Primary antibodies were added to blots and incubated overnight at 4^0C. Anti-rabbit and anti-mouse IgG conjugated with HRP were used as secondary antibodies and the blots were developed using Ultra-sensitive enhanced chemiluminiscence reagent (Millipore, USA) and image captured by Alliance 4.7 UVI Tec chemidoc.

4A.3.9 Statistical analysis

The results were analyzed using one-way analysis of variance (ANOVA) and students 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.

4A.4 Results:

Effects of swertiamarin on oleic acid induced hepatosteatosis are as follows:

4A.4.1 Confirmation of steatosis in oleic acid induced HepG2 cells:

HepG2 cells upon treatment with 1mM oleic acid for 24 hrs were observed to accumulate lipid which was evidently noted by morphometric changes that included transition from epithelial to round, bulky lipid accumulated morphology. The lipid accumulation was visualized and quantified by Oil O Red staining under bright filed microscope (20X). Oil O Red stain was extracted by isopropanol and quantified by measuring its optical density at 520nm, revealing that there was significant reduction in the lipid content in steatic hepatocytes treated with swertiamarin (25µg/ml) when compared to OA induced steatic hepatocytes without SM treatment. This was the primary confirmatory step for Hepatosteatosis (Figure 4A.1).

4A.4.2 Swertiamarin lowers the triglyceride (TG) content:

Fatty liver is the accumulation of excess unoxidized and unmetabolized triglycerides in hepatocytes, Which is the hallmark for hepatosteatosis. Spectrophotometric analysis of triglyceride content in steatic hepatocyte treated with swertiamarin showed significant reduction in the accumulated triglyceride content which supports the fact that swertiamarin has hypolipidemic activity and reduced ectopic fat disposition (Figure 4A.2a).

4A.4.3 Swertiamarin lowers severity of hepatosteatosis:

High LDH release is a known indicator for cytotoxicity and cellular metabolic deregulation. Swertiamarin treatment significantly reduced (50%) LDH activity as compared to HepG2 cells treated with oleic acid (Figure 4A.2b).

Figure 4A.1

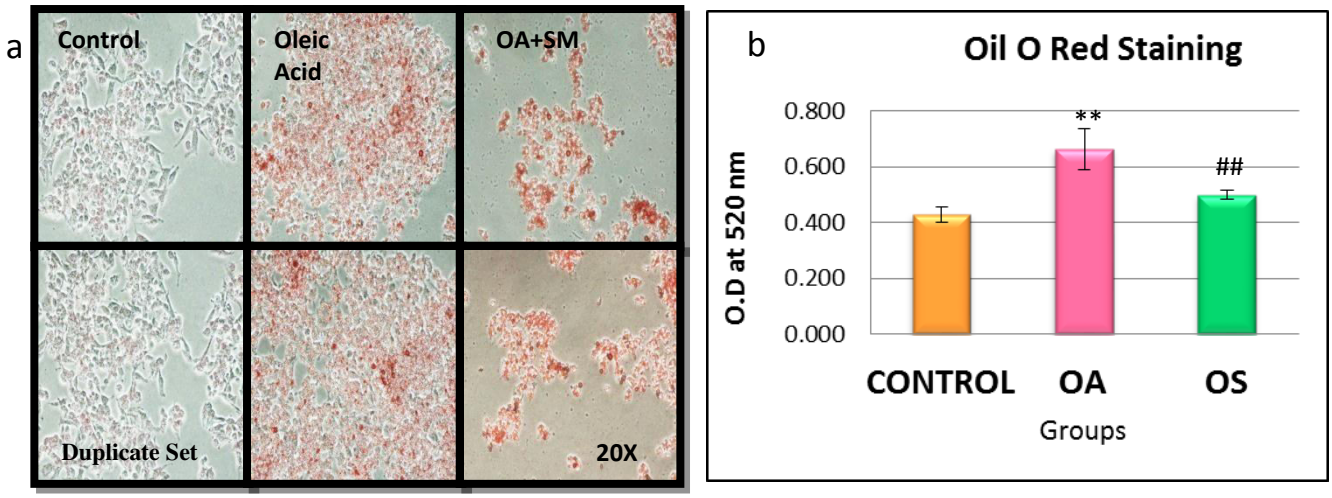


Figure 4A.1: OA-induced steatosis in HepG2 cells determined by Oil O Red staining , Quantification of Oil O Red stain after extraction procedure; (a) Oil O Red staining observed at 20X magnification under Phase contrast microscope. (b) Quantification of Oil O Red stain after extraction procedure is represented in terms of % of Oil O Red stain compared to control (p-value **≤ 0.005 as compared to control; p-value ##≤ 0.005 as compared to OA, n=3)

Figure 4A.2

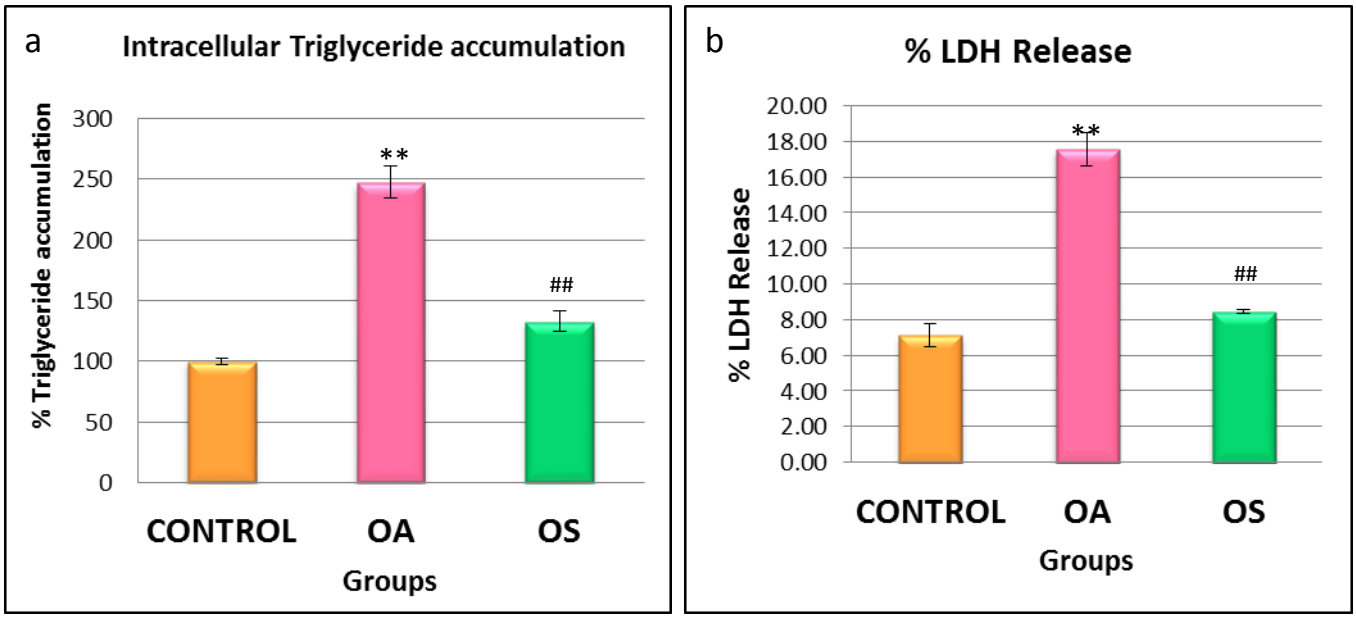


Figure 4A.2 : Effect of swertiamarin on a) Intracellular Triglyceride accumulation. b) % LDH release. p-value **≤ 0.005 as compared to control; p-value ##≤ 0.005 as compared to OA. n=3

4A.4.4 Effect of swertiamarin on insulin signaling proteins using insulin resistant HepG2:

Insulin resistance is a predominantly known causative condition for hepatosteatosis. Immunoblotting of various insulin signaling proteins revealed that swertiamarin reduced insulin resistance by decreasing the expression levels of phosphorylated ser-307 IRS-1 with concomitant increase in the levels of IR-β, PI(3)K and pAkt, proteins of insulin signaling. Swertiamarin is known for its hepatoprotective and hypolipidemic activity. There was no significant change in the levels of TNF-α as, the model cells used here are cancerous in nature express high levels of this cytokine, therefore, there was no further elevation in oleic acid treatment also, and no significant reduction was observed in swertiamarin treatment. swertiamarin reduced the expression of PPAR-α ,the master regulater which was found to be elevated in OA induced insulin resistant hepatocytes (Figure 4A.3a & b).

4A.4.5 Swertiamarin reduces fat accumulation in steatotic hepatocytes and altered fat metabolic enzymes:

As swertiamarin showed protective effect on insulin resistant hepatocytes phenotypically, at molecular lever also the expression of fat acid metabolism genes like FAS, ACC-1 were reduced significantly on treatment with swertiamarin in steatotic hepatocytes, hence decreasing fatty acid synthesis. Decreased expressions of SREBP-1c and PPAR-γ in swertiamarin +oleic acid group depicts that swertiamarin controls the transcriptional machinery and thus, reduces the progression of transition from hepatocyte to adipocyte phenotype. There was also significant decrease in the expression level of PPAR-α, the master transcriptional regulator responsible for glucose oxidation (Figure 4A.4).

Figure 4A.3a

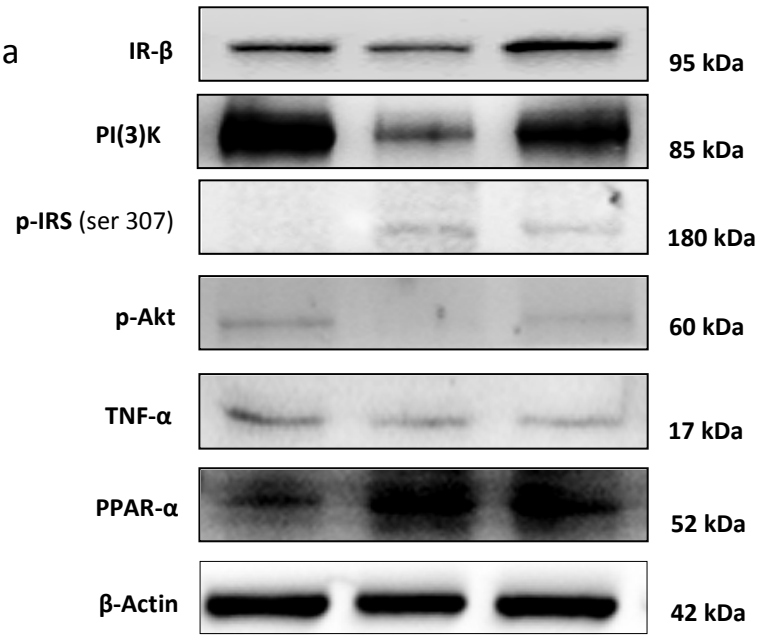


Figure 4A.3b

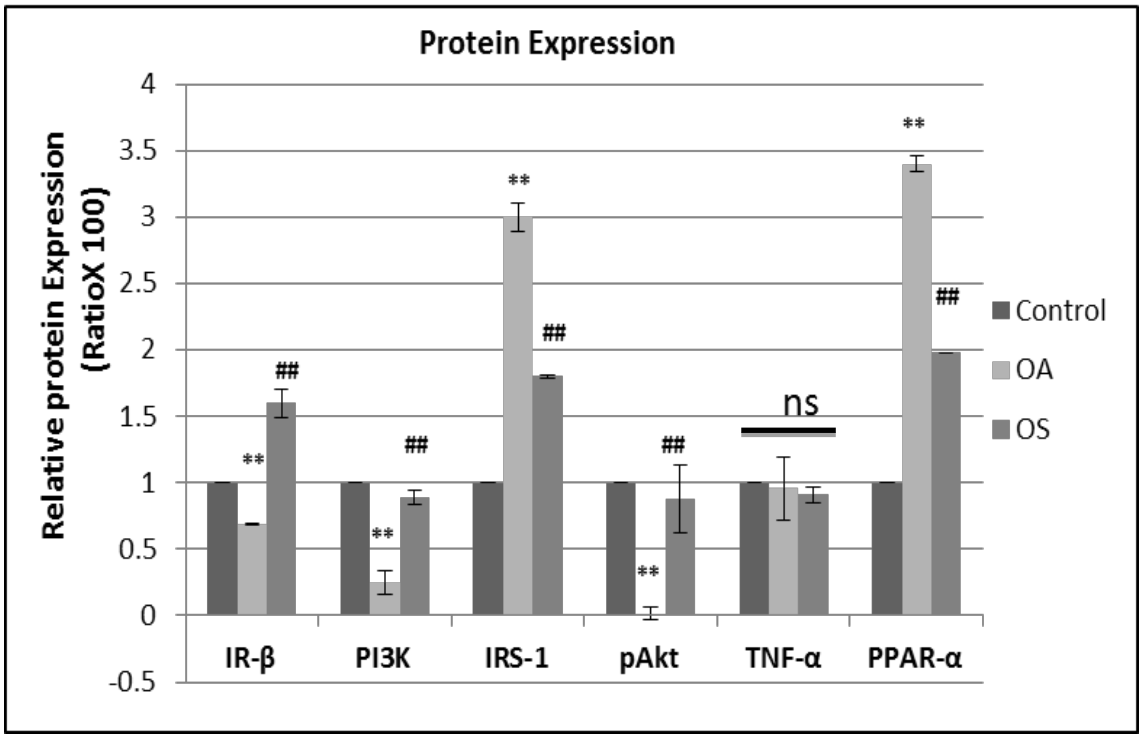


Figure 4A.3 : Effect of Swertiamarin on Signaling pathways using insulin resistant HepG2. a) Western blot study showing the effect of SM treatments on the expression of PPAR-α, TNF-α and Insulin signaling proteins: IR, p_{ser307} -IRS-1, p-Akt and PI(3)K in the HepG2 as compared to OA treated group. β-actin was taken as an internal control. b) Densitometry analysis for the above western profile using image J software, Data presented as Mean ± SEM of n=3 . p-value **≤ 0.005 as compared to control; p-value ##≤ 0.005 as compared to OA. p-value ; ns > 0.05 as compared to control & OA. (20 µg protein)

Figure 4A.4

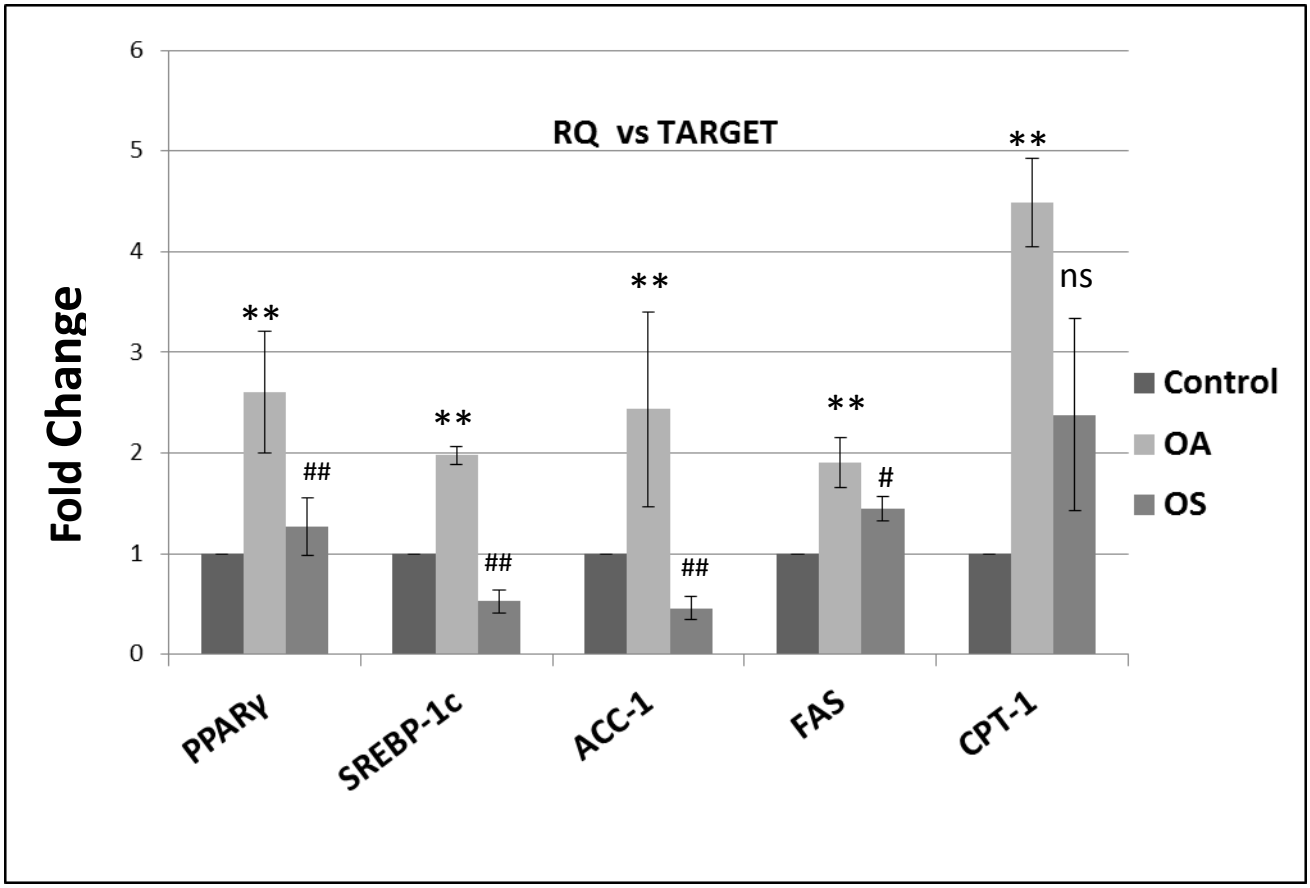


Figure 4A.4 : Effect of swertiamarin on the expression of fat metabolism genes in the liver steatosis. The expression levels of fat metabolic genes PPAR-γ, SREBP-1c, ACC-1, FAS and CPT-1 were checked using quantitative PCR. Data presented as Mean ± SEM of n=3 . p-value **≤ 0.005 as compared to control;
p-value ##≤ 0.005 as compared to OA.

4A.5 Discussion:

Increased central obesity is an alarming disease worldwide. The dysregulated adipocyte metabolism has a silent impact on liver. The stored bulky unoxidized and non-metabolised triglycerides residing in adipocytes hamper their metabolism and are infiltrated in hepatocytes, thus these hidden stored lipid creates an illusionary effect on them. Activated macrophages are infiltrated into the tissue and release proinflammatory cytokines that enhance fibrosis and liver damage (Friedman 2008). Reduced TG accumulation in hepatocytes treated with swertiamarin depicts reduced mobility of free fatty acid that decreases ectopic fat deposition and demolished insulin resistance (de Ferranti and Mozaffarian 2008; Tiniakos et al. 2010) by restricting the mobilization of free fatty acids.

Morphometric studies showed that swertiamarin treated steatotic hepatocytes were prevented from getting converted to signet ring shaped cells from transition of epithelial cell type. Due to this the adherence and functionality was also restored in swertiamarin treated group. It can be presumed that swertiamarin could restrict the transformation of hepatic stellate cells to myofibroblastic phenotype (Friedman 2008). Ectopic fat accumulation induces cell damage by diminishing membrane integrity and hence affects the cell viability which is evidently marked by LDH release (Srivastava et al. 2007). Significant reduction in LDH release provides evidence that swertiamarin prevents disruption of cell membrane and hence reduces cytotoxicity in hepatocytes.

Inflammation is known to be a key physiological feature for occurrence of hepatic steatosis. Elevated levels of TNF-α and other pro-inflammatory cytokines released by adipocytes depleted the pool of anti-inflammatory cytokines. TNF-α is associated mainly with inflammation, altered metabolism and apoptosis (Diehl 2004; Tiniakos et al. 2010). As HepG2 cells are cancerous in nature, TNF-α was already present in the study model and was not further elevated by oleic acid treatment. However, there was no significant reduction in its level when treated with swertiamarin.

PPARs are the regulatory transcriptional factors responsible for lipogenesis and fatty acid oxidation. PPAR-α predominantly expressed in liver senses fatty acid. Normal function of PPAR-α/PGC-1 pathway is to maintain fasting to feeding state by maintaining balance between fatty acid oxidation to hepatic glucose production. In TIIDM gluconeogenesis and fatty acid (FFA) oxidation are elevated through CREB-dependent induction of PPAR-α/PGC-1 pathway stimulating the mammalian tribbles homolog TRB-3 which interferes with serine-threonine kinase Akt/PKB of insulin signaling cascade. Swertiamarin treatment reduced PPAR-α that eventually restores hampered gluconeogenesis and fatty acid oxidation in hepatocytes and reducing hepatosteatosis (Koo et al. 2004).

In NAFLD, increased SREBP-1c stimulates PPAR-γ, the major transcriptional factor responsible for fatty acid synthesis by activating the enzymes. Resultant fatty acids and TGs start accumulating in the which leads to inflammatory responses and enhancing steatosis in hepatocytes (Reddy and Rao 2006). Swertiamarin treated steatotic hepatocytes showed reduced

expression of this transcription factor as compared to untreated cells. Two independent studies by Musso et al 2010 showed that, (Musso et al. 2010) apolipoprotein C3 levels directly influence SREBP-1c, hence the reduction in the expression of the later might reduce the level of apolipoprotein C3. AMPK is a key enzyme regulating the levels of SREBP-1c and ACC-1, hence it can be assumed that swertiamarin is AMPK activator and hence falls among the group of recent drugs implicated for amelioration of adiposity and insulin resistance (Musso et al. 2010). Altered SREBP-1c levels further reduced the expressions of the key enzymes like FAS and ACC-1 involved in cholesterol and fatty acid synthesis and can be presumed that there would be increased mitochondrial β-oxidation eventually reducing excessive triglyceride. These results of the present study are supported by the fact that swertiamarin demonstrated hypolipidemic activity and cholesterol reducing activity at systemic level *in vivo* (Vaidya et al. 2009; Patel et al. 2013)

The released free fatty acids from adipocytes are accumulated into liver and their metabolites are activators of various kinases involved in (ser 307) phosphorylation of IRS which leads to insulin resistance (Rui et al. 2001; Boden and Shulman 2002; Unger 2003; Tamburini et al. 2008; Tanti and Jager 2009) which is also found in oleic acid induced hepatosteatosis in hepatocytes. Western blot analysis in the current study depicted that swertiamarin significantly reduced the levels of IRS (ser 307) phosphorylation, a hallmark of insulin resistance with increase in p-AKT and PI(3)K which elevates insulin sensitivity and could probably restore glycogen storage. (Asrih and Jornayvaz 2013b). The study concomitantly relates to the *in vitro* data to that on the action of swertiamarin on TNF-α induced insulin resistance in 3T3-L1 (Chapter 5) and *in vivo* study. The increased expression of the insulin signaling proteins of the swertiamarin treated steatotic hepatocytes strengthens the evidence that shows swertiamarin as a potential insulin sensitizer.

Many key questions pertaining to the mechanistic, immunomodulatory role of swertiamarin in amelioration of hepatosteatosis are yet to be resolved. Thus, swertiamarin potentially proves to be an efficient candidate as PPAR-γ and PPAR-α dual agonist that offer its strengths as a powerful drug for the treatment of hepatosteatosis and insulin resistance.

In conclusion, swertiamarin treatment on oleic acid induced hepatosteatosis decreased the Tg accumulation along with restoration of damaged cellular membranes and reversed the altered levels of proteins involved in insulin signaling. PPAR-γ, the master transcriptional regulators of lipogenesis is controlled by swertiamarin. Hence, the overall effect of this bitter glycoside proves to be an efficient treatment for hepatosteatosis and unrevealing the mechanistic possible therapy of this disease.

4A.6 Reference:

Alba LM, Lindor K. 2003. Review article: Non-alcoholic fatty liver disease. *Aliment Pharmacol Ther* 17: 977-986.

Anderson N, Borlak J. 2008. Molecular mechanisms and therapeutic targets in steatosis and steatohepatitis. *Pharmacological Reviews* 60: 311-357.

Asrih M, Jornayvaz FR. 2013a. Inflammation as a potential link between nonalcoholic fatty liver disease and insulin resistance. *J Endocrinol* 218: R25-36.

Asrih M, Jornayvaz FR. 2013b. Inflammation as a potential link between nonalcoholic fatty liver disease and insulin resistance. *Journal of Endocrinology* 218: R25-R36.

Baby Josheph SJR. 2011. A Comparative Study on various properties of five medicinally imporant plants. *International Journal of Pharmacology* 7: 206-211.

Bell M, Wang H, Chen H, McLenithan JC, Gong D-W, Yang R-Z, Yu D, Fried SK, Quon MJ, Londos C. 2008. Consequences of Lipid Droplet Coat Protein Downregulation in Liver Cells Abnormal Lipid Droplet Metabolism and Induction of Insulin Resistance. *Diabetes* 57: 2037-2045.

Berdichevsky A, Guarente L, Bose A. Acute oxidative stress can reverse insulin resistance by inactivation of cytoplasmic JNK. *J Biol Chem* 285: 21581-21589.

Bhat M, Kothiwale SK, Tirmale AR, Bhargava SY, Joshi BN. 2009. Antidiabetic Properties of Azardiracta indica and Bougainvillea spectabilis: In Vivo Studies in Murine Diabetes Model. *Evid Based Complement Alternat Med* 2011: 561625.

Boden G, Shulman G. 2002. Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and β-cell dysfunction. *European journal of clinical investigation* 32: 14-23.

Cui W, Chen SL, Hu K-Q. 2010. Quantification and mechanisms of oleic acid-induced steatosis in HepG2 cells. *American journal of translational research* 2: 95.

de Alvaro C, Teruel T, Hernandez R, Lorenzo M. 2004. Tumor necrosis factor alpha produces insulin resistance in skeletal muscle by activation of inhibitor kappaB kinase in a p38 MAPK-dependent manner. *J Biol Chem* 279: 17070-17078.

de Ferranti S, Mozaffarian D. 2008. The perfect storm: obesity, adipocyte dysfunction, and metabolic consequences. *Clinical chemistry* 54: 945-955.

DeFronzo RA. 1999. Pharmacologic therapy for type 2 diabetes mellitus. *Ann Intern Med* 131: 281-303.

DeFronzo RA, Tripathy D. 2009. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. *Diabetes Care* 32 Suppl 2: S157-163.

Diehl AM. 2004. Obesity and alcoholic liver disease. *Alcohol* 34: 81-87.

Etxabe J, Vazquez J. 1994. Morbidity and mortality in Cushing's disease: an epidemiological approach. *Clinical endocrinology* 40: 479-484.

Friedman SL. 2008. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 134: 1655-1669.

Hsu C-L, Yen G-C. 2007. Effects of flavonoids and phenolic acids on the inhibition of adipogenesis in 3T3-L1 adipocytes. *Journal of agricultural and food chemistry* 55: 8404-8410.

Ikarashi N, Tajima M, Suzuki K, Toda T, Ito K, Ochiai W, Sugiyama K. 2012. Inhibition of Preadipocyte Differentiation and Lipid Accumulation by Orengedokuto Treatment of 3T3-L1 Cultures. *Phytotherapy Research* 26: 91-100.

Jadeja RN, Thounaojam MC, Devkar RV, Ramachandran AV. 2010. Clerodendron glandulosum.Coleb extract prevents in vitro human LDL oxidation and oxidized LDL induced apoptosis in human monocyte derived macrophages. *Food Chem Toxicol* 49: 1195-1202.

Kohjima M, Higuchi N, Kato M, Kotoh K, Yoshimoto T, Fujino T, Yada M, Yada R, Harada N, Enjoji M. 2008. SREBP-1c, regulated by the insulin and AMPK signaling pathways, plays a role in nonalcoholic fatty liver disease. *International journal of molecular medicine* 21: 507-511.

Koo S-H, Satoh H, Herzig S, Lee C-H, Hedrick S, Kulkarni R, Evans RM, Olefsky J, Montminy M. 2004. PGC-1 promotes insulin resistance in liver through PPAR-α-dependent induction of TRB-3. *Nature medicine* 10: 530-534.

Lee H, Kang R, Yoon Y. 2010a. SH21B, an anti-obesity herbal composition, inhibits fat accumulation in 3T3-L1 adipocytes and high fat diet-induced obese mice through the modulation of the adipogenesis pathway. *Journal of ethnopharmacology* 127: 709-717.

Lee M-S, Hwang J-T, Kim S-h, Yoon S, Kim M-S, Yang HJ, Kwon DY. 2010b. Ginsenoside Rc, an active component of< i> Panax ginseng</i>, stimulates glucose uptake in C2C12 myotubes through an AMPK-dependent mechanism. *Journal of ethnopharmacology* 127: 771-776.

Lewis JR, Mohanty SR. 2010. Nonalcoholic fatty liver disease: a review and update. *Dig Dis Sci* 55: 560-578.

Michael MD, Kulkarni RN, Postic C, Previs SF, Shulman GI, Magnuson MA, Kahn CR. 2000. Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Molecular cell* 6: 87-97.

Musso G, Gambino R, Cassader M. 2010. Emerging molecular targets for the treatment of nonalcoholic fatty liver disease. *Annu Rev Med* 61: 375-392.

Patel TP, Soni S, Parikh P, Gosai J, Chruvattil R, Gupta S. 2013. Swertiamarin: An Active Lead from Enicostemma littorale Regulates Hepatic and Adipose Tissue Gene Expression by Targeting PPAR- gamma and Improves Insulin Sensitivity in Experimental NIDDM Rat Model. *Evid Based Complement Alternat Med* 2013: 358673.

Qin B, Nagasaki M, Ren M, Bajotto G, Oshida Y, Sato Y. 2003. Cinnamon extract (traditional herb) potentiates in vivo insulin-regulated glucose utilization via enhancing insulin signaling in rats. *Diabetes Res Clin Pract* 62: 139-148.

Quinn BJ, Kitagawa H, Memmott RM, Gills JJ, Dennis PA. 2013. Repositioning metformin for cancer prevention and treatment. *Trends in Endocrinology & Metabolism* 24: 469-480.

Reddy JK, Rao MS. 2006. Lipid metabolism and liver inflammation. II. Fatty liver disease and fatty acid oxidation. *Am J Physiol Gastrointest Liver Physiol* 290: G852-858.

Rui L, Aguirre V, Kim JK, Shulman GI, Lee A, Corbould A, Dunaif A, White MF. 2001. Insulin/IGF-1 and TNF-α stimulate phosphorylation of IRS-1 at inhibitory Ser 307 via distinct pathways. *Journal of Clinical Investigation* 107: 181-189.

Srinivasan K, Viswanad B, Asrat L, Kaul CL, Ramarao P. 2005. Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: a model for type 2 diabetes and pharmacological screening. *Pharmacol Res* 52: 313-320.

Srivastava S, Li Z, Yang X, Yedwabnick M, Shaw S, Chan C. 2007. Identification of genes that regulate multiple cellular processes/responses in the context of lipotoxicity to hepatoma cells. *BMC Genomics* 8: 364.

Tamburini J, Chapuis N, Bardet V, Park S, Sujobert P, Willems L, Ifrah N, Dreyfus F, Mayeux P, Lacombe C. 2008. Mammalian target of rapamycin (mTOR) inhibition activates phosphatidylinositol 3-kinase/Akt by up-regulating insulin-like growth factor-1 receptor signaling in acute myeloid leukemia: rationale for therapeutic inhibition of both pathways. *Blood* 111: 379-382.

Tanti J-F, Jager J. 2009. Cellular mechanisms of insulin resistance: role of stress-regulated serine kinases and insulin receptor substrates (IRS) serine phosphorylation. *Current opinion in pharmacology* 9: 753-762.

Tiniakos DG, Vos MB, Brunt EM. 2010. Nonalcoholic fatty liver disease: pathology and pathogenesis. *Annu Rev Pathol* 5: 145-171.

Unger RH. 2003. Lipid overload and overflow: metabolic trauma and the metabolic syndrome. *Trends in Endocrinology & Metabolism* 14: 398-403.

Vaidya H, Rajani M, Sudarsanam V, Padh H, Goyal R. 2009. Swertiamarin: A lead from< i> Enicostemma littorale</i> Blume. for anti-hyperlipidaemic effect. *European journal of pharmacology* 617: 108-112.

Zierath JR. 2007. The path to insulin resistance: paved with ceramides? *Cell metabolism* 5: 161-163.

CHAPTER-4(B)

Chapter 4B

To study the mechanism of action of swertiamarin in TNF-α induced insulin resistance in L6 Myocytes

4B.1 Introduction:

Altered glucose metabolism, peripheral insulin resistance, defective insulin secretion associated with hepatic glucose production (HGP) are the major pathophysiological condition associated with TIIDM (Beck-Nielsen and Groop 1994). More than 80% of excess peripheral glucose is disposed in the skeletal muscle (DeFronzo and Tripathy 2009). Euglycemic-hyperinsulinemic clamp method is used for identifying insulin resistance in skeletal muscle which is the hallmark feature of T2DM that contributes to non-oxidative glucose metabolism and reduced glycogen storage. Insulin plays a major role in glucose uptake and its oxidation, glycogen synthesis and lipid oxidation (Lillioja et al. 1988; Eriksson et al. 1989; Gulli et al. 1992; Schalin-Jäntti et al. 1992; Vaag et al. 1995). Fasting, basal and well fed stages keep tight regulation between insulin stimulated glucose uptake, oxidation and lipid oxidation. However, it is yet to be resolved whether metabolic alterations in insulin resistance occur initially or not. This leads to the hypothesis of "metabolic inflexibility" suggesting an impaired capacity to switch between carbohydrate and fat as oxidative energy sources, a major determinant of skeletal muscle insulin resistance (Kelley and Mandarino 2000).

Not only in obese but also in lean TIIDM subjects, dyslipidemia in adipose tissue leads to increased lipid accumulation in skeletal muscle which acts as a key marker of insulin resistance (Jacob et al. 1999; Fernandes et al. 2007). Moreover, a close relationship between intramyocellular lipid concentrations (triglycerides) and insulin resistance have been reported in both healthy and type II diabetic subjects (Phillips et al. 1996) along with impaired glucose tolerance. Metabolic alterations in skeletal muscle can be associated with insulin resistance in TIIDM patients. These include 1) abnormal release of hormones from the adipose tissue (adipocytokines) such as leptin, resistin, tumor necrosis factor-α (TNF-α), and adiponectin (Saltiel 2001), 2) increased serum levels of inflammatory markers such as C-reactive protein, fibrinogen and interleukin-6 (IL-6) indicating that a chronic low-grade systemic inflammation is involved in the pathogenesis of TIIDM (Huerta and Nadler 2002), and 3) defective secretion and

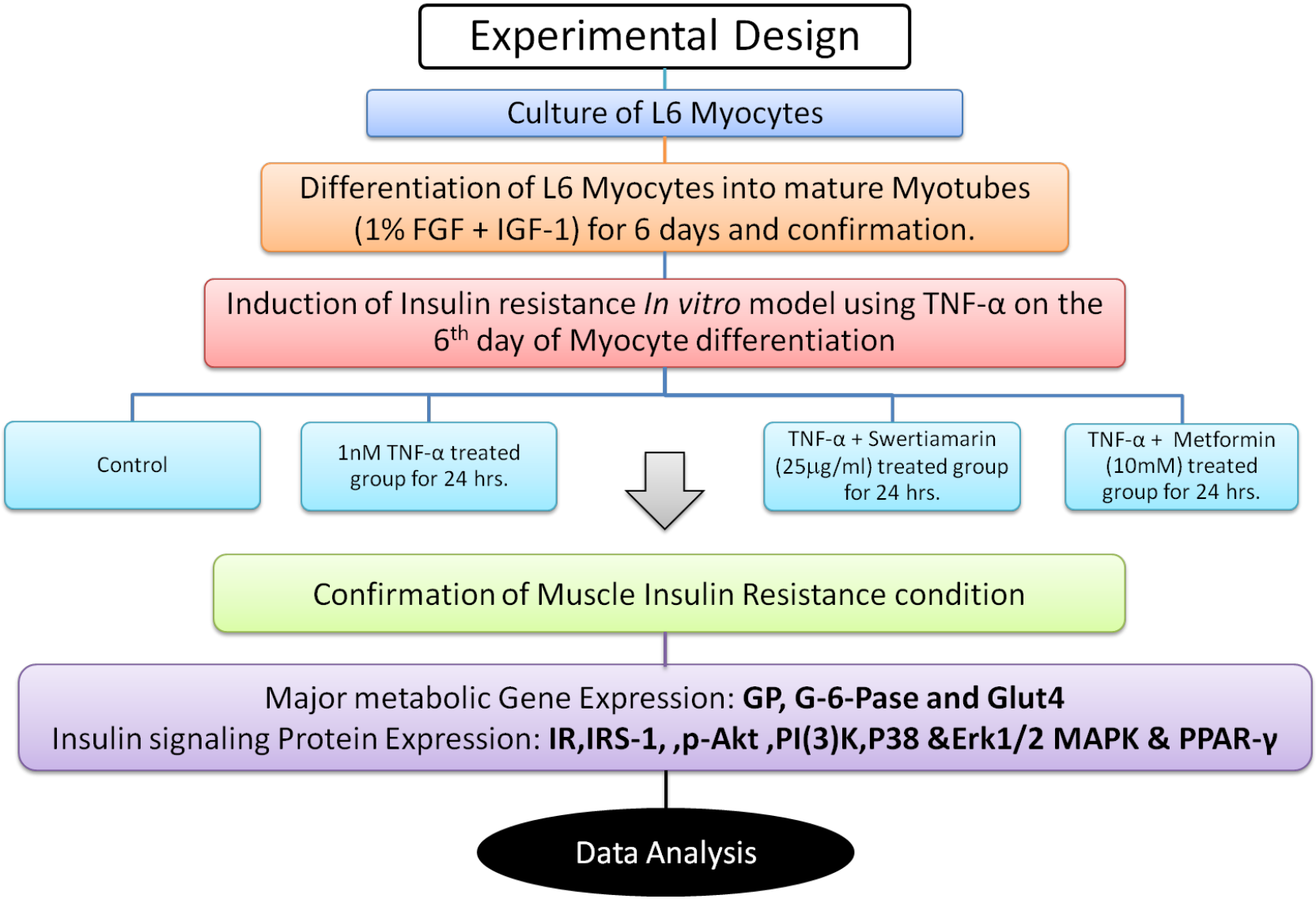
action of gut incretin hormones such as glucagon-like-peptide-1 (GLP-1), gastric-inhibitory polypeptide (GIP) and ghrelin (Blaak et al. 2012).

TNF-α is involved in the pathophysiology of insulin resistance (Antuna-Puente et al. 2008). However, Alvaro et al, have reported that the inflammatory responses generated through this cytokine activates stress kinases like P38 MAPK which leads to serine phosphorylation of IR and IRS-1,inhibits tyrosine phosphorylation(de Alvaro et al. 2004). The expression of this cytokine is slightly modified in human obesity, and it's absence in subcutaneous adipose tissue in obese and lean men has been demonstrated by direct arteriovenous balance measures. This indicates that adipose tissue is not only responsible for the increased concentrations of circulating TNF-α as seen in obesity (Antuna-Puente et al. 2008).

TNF-α is also expressed in human muscles and is found at higher levels in muscles of insulin-resistant and diabetic patients relative to normal lean control subjects. Its reducted of TNF-α activity, using a chimeric antibody or as occurs in transgenic animals lacking either TNF-α or TNF-α receptor, improves sensitivity to insulin in obese animals. Although the mechanism of action of this cyokine in these insulin-related effects is not fully understood it is known that a major component involve TNF-α mediated inhibition of upstream insulin signaling (Lorenzo et al. 2008).

Skeletal muscle is one of the main organ that is linked to dysregulation of adipose tissue metabolism. Insulin stimulated glucose uptake mechanism is same as in muscle and adipose tissue but, proportion of calorie intake and its oxidation by skeletal muscle is highest among all other organs. TNF-α induced insulin resistance in L6 myocytes are the *in vitro* model studied extensively to understand the pathophysiology of skeletal muscle insulin resistance and in this chapter 4B it was used to understand the role of swertiamarin on skeletal muscle insulin resistance.

4B.2 Experimental Design



4B.3 Materials and methods

4B.3.1 Chemicals and media

DMEM high glucose, trypsin-EDTA and IGF-1(recombinant) procured from Sigma Aldrich. Fetal Bovine Serum (FBS), Penicillin-Streptomycin were procured from Gibco, Life Technologies and cell culture experiments were carried out in tissue culture grade plastic ware from Nunclon. RNA extraction was done by Trizol (Invitrogen), Gene specific primers were designed from IDT, and all other reagents required for molecular biology experiments were procured from Life Technologies. Antibodies for protein expression were obtained from CST.

4B.3.2 Cell culture

Rat L6 myocyte cells obtained from NCCS, Pune, India, were seeded (1×10^5cells/ T25 Flask) and cultured in DMEM high Glucose with 10% FBS and 1% antibiotic-antimycotic solution (10X) at 37°C with 5% CO_2 (Thermo scientific, CO_2 incubator). Cells were subsequently passaged every third day by trypsinization with 0.25 % trypsin-EDTA solution.

4B.3.3 Differentiation of L6 myocyte to myotubes

L6 myocytes were cultured in DMEM high Glucose with 10% FBS in 6 cm^2 cell culture dish till 60-70 % confluency (2-3 days). Wash the cell with 1X PBS and treat themwith DMEM high glucose with 1% FBS and 1 nM of IGF for 6 days (De Arcangelis et al. 2003). Myocyte differentiation media was changed every second day. L6 myocyte were converted to mature myotubes (Yoon et al. 2011) which was confirmed by immunostaining of smooth muscle actin (SMA) and Desmin as maturation marker and also by immunoblotting of Desmin as per protocol given in 4B.3.6 and 4B.3.8 respectively.

4B.3.4 Induction of insulin resistance condition in mature myotube

Followed by myocyte differentiation, the experiment was divided into four groups: control, 1nM TNF-α induced cells (de Alvaro et al. 2004), 1nM TNF-α along with 25ug/ml swertiamarin and the positive control group was treated with 1nM TNF-α and 10mM metformin.

4B.3.5 Confirmation of mature myotube formation by Immunocytochemistry of SMA and Desmin

Cells were seeded for adherence on poly L-lysine coated glass coverslip for 2-3 days until it reached 60-70% confluency. Then the cells were treated with myocyte differentiation media for 6 days. They were then fixed with 4 % paraformaldehyde solution for 30 min at RT and permeablised with 0.1% Triton X-100 in PBS for 5 min and incubated in blocking buffer (1% bovine serum albumin and 4% FBS in PBS) for 1 hour at room temperature. Primary antibodies mainly Desmin (1:20) anti rabbit CF555 and α SMA FITC anti mouse (1:250) (Sigma) were prepared in blocking solution and the coverslips were incubated overnight at 4^0C in moist chamber . Cells were then washed thrice with washing buffer (1:10 dilution of blocking buffer in PBS) and incubated with secondary antibodies conjugated to CF555 fluorophores (Sigma Aldrich, USA) in dark for 60 min at RT. For negative control, the cells were incubated with normal IgG. Cells were mounted with mounting medium containing 4'6'-diamidino-2-phenylindole dihydrochloride (DAPI). Confocal imaging was carried out by using a LSM 710 microscope. All images were analyzed with the help of LSM software supplied by Zeiss (Shah et al. 2011) (Table 8).

4B.3.6 RNA extraction and Real Time quantitative PCR

Cell pellet was collected for each day of adipogenesis. RNA was isolated using the TRIzol reagent (Sigma Aldrich) as per manufacturer's instructions (Chapter 4A.3.7). A reverse-transcription reaction was performed using 2 µg RNA with MuLV reverse transcriptase in a 20 µL reaction volume containing DEPC treated water (Fermentas Kit). PCR product was amplified using gene specific primers (Table 3). β-Actin was used as an internal control. The PCR products were analyzed by electrophoresis on 2.0% agarose gels or 15% DNA-PAGE, and the gels were photographed after staining with ethidium bromide and intensities of the band were calculated by densitometric analysis using the Image J software.

4B.3.7 Protein extraction and Western blotting

Matured L6 myotubes were cultured with TNF-α and swertiamarin for 24 hours and then the cell pellets were lysed with 1 ml of the lysis buffer (1mM EDTA, 50 mM Tris-HCl pH 7.5, 70 mM NaCl, 1% Triton, 50 mM NaF) supplemented with protease inhibitor cocktail (Fermentas INC.).

total protein were separated by SDS-PAGE and transferred onto nitrocellulose blotting membrane. After blocking, the blots were probed with primary antibodies at 4^0C overnight (Table 7). Blots were developed by ECL reagent (Chapter 4A.3.8).

4B.3.8 Statistical analysis

The results were analyzed using one-way analysis of variance (ANOVA) and students 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.

4B.4 Results

4B.4.1 Confirmation of L6 myocyte differentiation

Myocyte tubes are formed by fusion of multiple nuclei at the end of differentiation process. Differentiation was further confirmed by immunofluorescence study, which showed elevated levels of the intermediate filament desmin and cytoskeleton protein smooth muscle actin. Again same thing was confirmed and quantified by immunoblotting using desmin antibody (Figure 4B.1 & 2).

4B.4.2 Swertiamarin alters the gene expression of the enzymes involved in glucose metabolism in skeletal muscle

Glucose metabolism is an essential process in skeletal muscle. Relative gene expressions of key enzymes in glucose metabolism: glycogen phosphorylase (GP) and Glucose-6 phospatase (G6Pase) showed decreased expression in model cells treated with swertiamarin along with TNF-α as compared to cells treated with TNF-α. However, there was no significant difference in mRNA expression of Glut4 present in the skeletal muscle when SM plus TNF-α group was compared to that of the TNF-α (Figure 4B.3).

4B.4.3 Proinflammatory cytokine is responsible for insulin resistance in skeletal muscle: Inflammatory responses due to proinflammatory cytokine TNF-α are very well associated with hampered insulin signaling in skeletal muscle. Western blot analysis of the key proteins involved in insulin signaling in swertiamarin and TNF-α treated group when compared to TNF-α group showed increase in the expression of IR, PI(3)K, pAkt, Glut4 and major transcriptional factor

PPAR-γ along with concomitant reduction in the phosphorylated P38 MAPK and p(ser-307)-IRS-1, a hallmark of insulin resistance. Erk1/2 which is also known as stress kinase, showed increased expression in TNF-α treated group while reduction was observed in swertiamarin and TNF-α treated group (Figure 4B.4a & b).

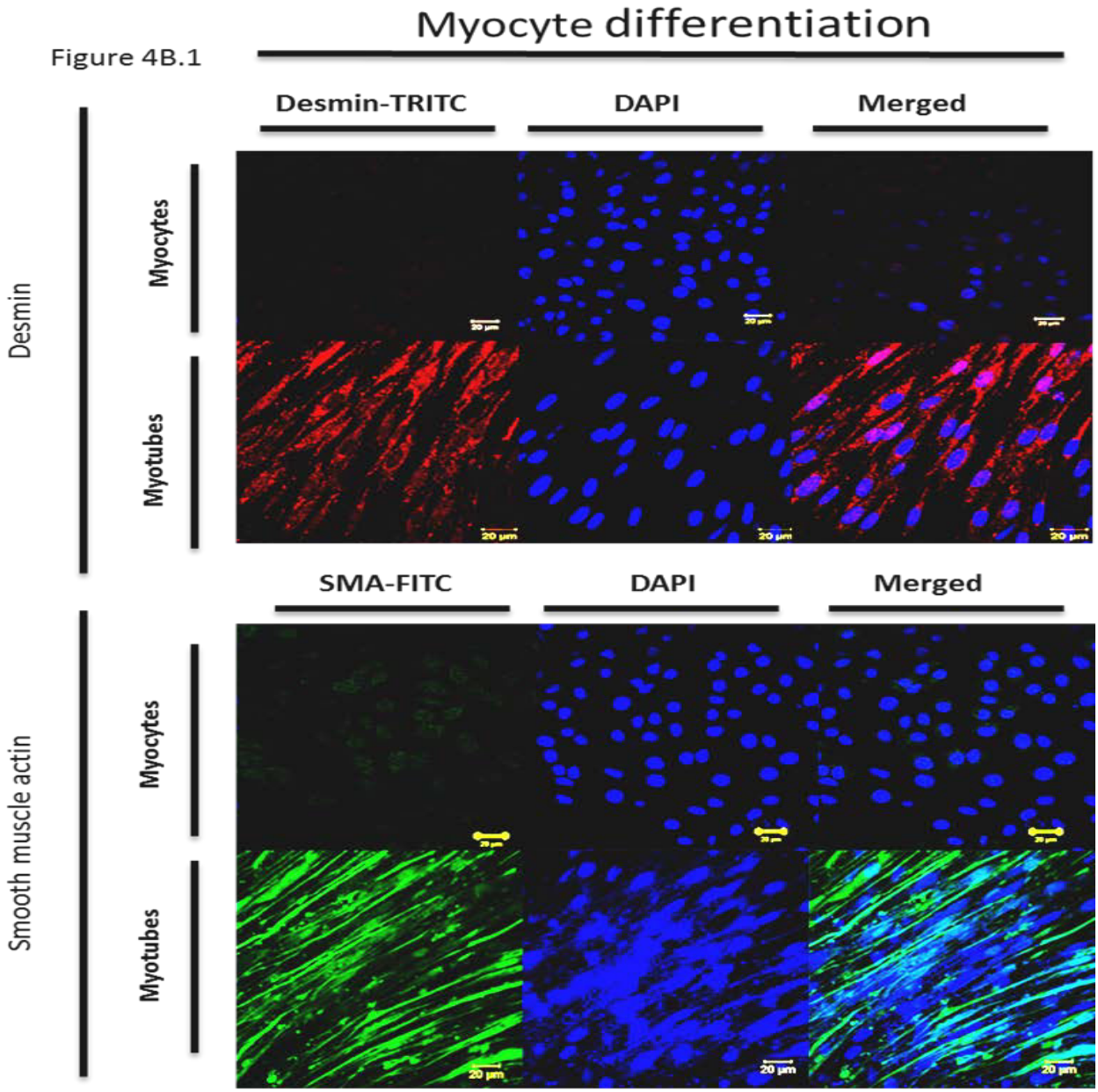


Figure 4B.1: Confirmation of myotubes by indirect immunofluorescence using smooth muscle actin (SMA) taged with FITC and Desmin labeled with CF555 captured (under 63X Objective). ICC were performed on 6th day of myocyte differentiation.

Figure 4B.2

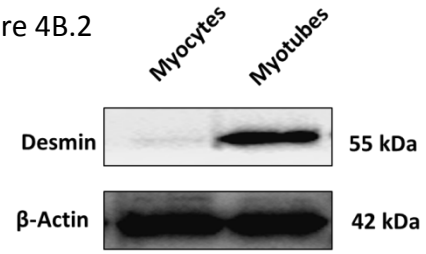


Figure 4B.2: Confirmation of myocytes to myotubes differentiation by DMEM with 1% FBS and IGF-1 for 6th days. Western blot study showing Desmin expression in differentiation group and β-actin was taken as an internal control (20 ug protein).

Figure 4B.4a

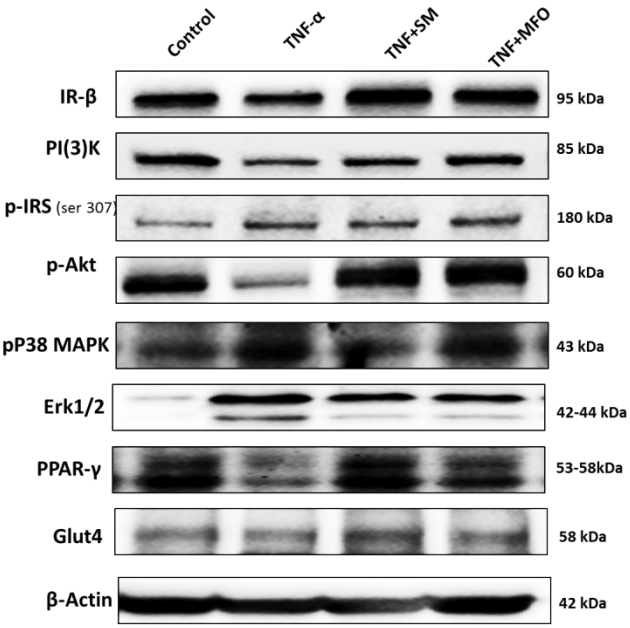


Figure 4B.4a: Effect of Swertiamarin on insulin signaling pathways using insulin resistant L6 myocytubes. Western blot study showing the effect of SM treatments on the expression of Insulin signaling proteins: IR, p_{ser307} -IRS-1, pAkt, PI(3)K ,pP38MAPK,Erk1/2 and Glut4. β-actin was taken as an internal control (20 μg protein).

Figure 4B.3

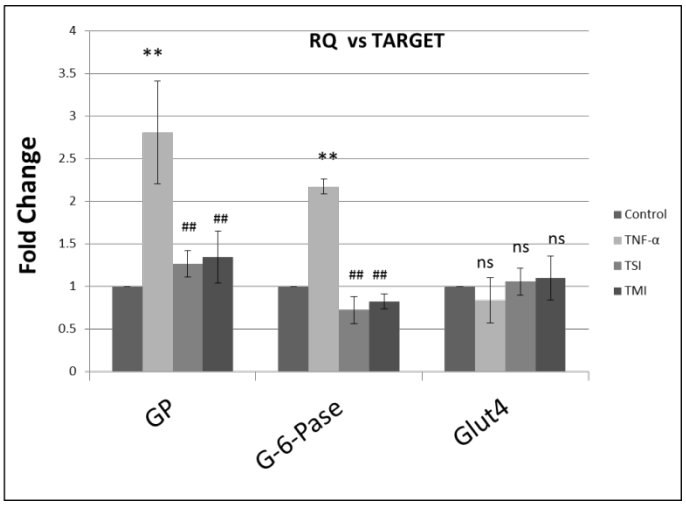


Figure 4B.3: Effect of swertiamarin on the expression of skeletal muscle metabolic genes in the TNF-α induced insulin resistant myotubes. The expression levels of metabolic genes GP, G-6-Pase and Glut4 were checked using quantitative PCR. Data presented as Mean ± SEM of n=3. p-value **≤ 0.005 as compared to control; p-value ## ≤ 0.005 as compared to TNF, p-value ; ns > 0.05 as compared to control & TNF.

Figure 4B.4b

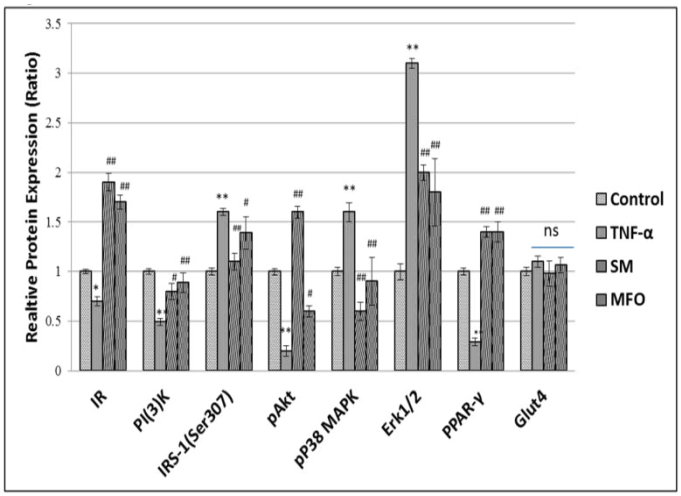


Figure 4B.4b: Effect of Swertiamarin on insulin signaling pathways using insulin resistant L6 myotubes. Densitometric analysis of western blot study showing the effect of SM treatments on the expression of Insulin signaling proteins. Ratio of target to internal control were calculated . Data presented as Mean ± SEM of n=3 . p-value **≤ 0.005 as compared to control; p-value ##≤ 0.005 as compared to TNF. n=3. p-value ; ns > 0.05 as compared to control & TNF.

4B.5 Discussion:

Inflammatory responses are directly associated with insulin resistance in various tissues where, elevated levels of proinflammatory cytokines like TNF-α and resistin were found in adipose tissue, muscles and plasma in TIIDM condition (Pradhan et al. 2001; Freeman et al. 2002; Duncan et al. 2003; Plomgaard et al. 2005). To check the effect of swertiamarin on insulin resistant myotubes, L6 myocytes were differentiated and elevated levels of desmin and smooth muscle actin protein confirmed by immunofluorescence depicted the differentiation ability of L6 myocytes to differentiate into myotubes.

Muscle is the major source for glycogen storage. Glycogen is broken down into glucose by glucose 6 phosphatase. Glycogen synthesis is hampered in insulin resistance or TIIDM due to altered insulin mediated glucose uptake. Downregulation of both glycogen phosphorylase and glucose 6 phosphatase gene in insulin resistant L6 myocytes speculate that high levels of oxidative stress due to inflammatory responses hamper the breakdown of the glycogen to glucose 1-phosphate in muscles with concomitant inhibition of gluconeogenesis, a condition similar to unexercised muscles and lowers intramuscular glycogen content hence, reduced energy substrate storage (Jensen et al. 2011; Martin et al. 2013) . These observations were reverted by the action of swertiamarin on the insulin resistant L6 myocytes hence, stating that swertiamarin enhances glucose uptake and insulin sensitivity.

There is a strong association of TNF-α with insulin resistance. Swertiamarin treated insulin resistant L6 myocyte showed increased expression of PI(3)K and Akt which concomitantly concludes that Akt activation is dependent on PI(3)K, hence enhancing the insulin signalling action. Phosphorylation of IRS-1 at ser 307, is a hallmark of insulin resistance which shows direct infusion of TNF-α into skeletal muscle. Swertiamarin treatment on TNF-α mediated insulin resistant myocytes showed reduced expression of p-Ser (307) IRS-1, which can be inferred as the ability of this bitter glycoside to demolish inflammatory responses by inhibiting various pathways like JNK and NF-kB (Plomgaard et al. 2005). Reduction of IRS-1 at ser 307 concomitantly decreases Erk1/2 the known stress kinases as observed in the swertiamarin treated group. Restored expression levels of PPAR-γ in Swertiamarin treated group showed that it regulates the insulin signalling through this transcriptional factor.

Studies on Glut4 translocation showed that insulin resistance caused in skeletal muscle is independent of it's activity on L6 myocytes when treated with leptin and resistin (Sweeney et al. 2001; Moon et al. 2003) which is concomitantly supported by our studies where the protein expression of this transporter is unaltered when treated with TNF-α as well as swertiamarin.

P38 Mitogen Activated Protein Kinase (P38 MAPK) associated inflammatory responses provoked by pro-inflammatory cytokines are very well observed in several diseases like rheumatoid arthritis, inflammatory bowel disease etc. Expression of pP38 MAPK was lowered in swertiamarin treated group. Hence, the down regulation of P38 supplements evidence that swertiamarin potentially owes anti-inflammatory activities (Perregaux et al. 1995; Johnson and Bailey 2003; Bailey et al. 2013). All the results of the current study confirm with that of the positive control metformin group, hence it potentially proves the efficacy of swertiamarin as a potent insulin sensitizer.

Swertiamarin has a positive modulatory effect on the glycogen metabolism and insulin signalling in skeletal muscle that potentially proves its ability as an effective insulin sensitizer at cellular level. The current model of TNF-α mediated insulin resistant L6 myocytes complies with the non-exercised muscles which are on the threat of insulin resistance.

In conclusion, L6 myocytes are the model cells studied widely for understanding the etiopathology of insulin resistance caused due to inflammatory responses. Swertiamarin has reduced insulin resistance when treated on TNF-α mediated insulin resistant L6 myocytes. Lowered levels of P 307-ser of IRS-1 along with increased expression of important proteins involved in insulin signaling depicts the potentials of Swertiamarin to be a better therapeutic option for correcting muscular insulin resistance.

4B.6 References:

Antuna-Puente B, Feve B, Fellahi S, Bastard J-P. 2008. Adipokines: the missing link between insulin resistance and obesity. *Diabetes & metabolism* 34: 2-11.

Bailey CJ, Aschner P, Del Prato S, LaSalle J, Ji L, Matthaei S. 2013. Individualized glycaemic targets and pharmacotherapy in type 2 diabetes. *Diab Vasc Dis Res* 10: 397-409.

Beck-Nielsen H, Groop LC. 1994. Metabolic and genetic characterization of prediabetic states. Sequence of events leading to non-insulin-dependent diabetes mellitus. *Journal of Clinical Investigation* 94: 1714.

Blaak EE, Antoine JM, Benton D, Bjorck I, Bozzetto L, Brouns F, Diamant M, Dye L, Hulshof T, Holst JJ et al. 2012. Impact of postprandial glycaemia on health and prevention of disease. *Obes Rev* 13: 923-984.

de Alvaro C, Teruel T, Hernandez R, Lorenzo M. 2004. Tumor necrosis factor alpha produces insulin resistance in skeletal muscle by activation of inhibitor kappaB kinase in a p38 MAPK-dependent manner. *J Biol Chem* 279: 17070-17078.

De Arcangelis V, Coletti D, Conti M, Lagarde M, Molinaro M, Adamo S, Nemoz G, Naro F. 2003. IGF-I-induced differentiation of L6 myogenic cells requires the activity of cAMP-phosphodiesterase. *Mol Biol Cell* 14: 1392-1404.

DeFronzo RA, Tripathy D. 2009. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. *Diabetes Care* 32 Suppl 2: S157-163.

Duncan BB, Schmidt MI, Pankow JS, Ballantyne CM, Couper D, Vigo A, Hoogeveen R, Folsom AR, Heiss G. 2003. Low-grade systemic inflammation and the development of type 2 diabetes the atherosclerosis risk in communities study. *Diabetes* 52: 1799-1805.

Eriksson J, Franssila-Kallunki A, Ekstrand A, Saloranta C, Widén E, Schalin C, Groop L. 1989. Early metabolic defects in persons at increased risk for non-insulin-dependent diabetes mellitus. *New England Journal of Medicine* 321: 337-343.

Fernandes NP, Lagishetty CV, Panda VS, Naik SR. 2007. An experimental evaluation of the antidiabetic and antilipidemic properties of a standardized Momordica charantia fruit extract. *BMC Complement Altern Med* 7: 29.

Freeman DJ, Norrie J, Caslake MJ, Gaw A, Ford I, Lowe GD, O'Reilly DSJ, Packard CJ, Sattar N. 2002. C-reactive protein is an independent predictor of risk for the development of diabetes in the West of Scotland Coronary Prevention Study. *Diabetes* 51: 1596-1600.

Gulli G, Ferrannini E, Stern M, Haffner S, DeFronzo RA. 1992. The metabolic profile of NIDDM is fully established in glucose-tolerant offspring of two Mexican-American NIDDM parents. *Diabetes* 41: 1575-1586.

Huerta MG, Nadler JL. 2002. Role of inflammatory pathways in the development and cardiovascular complications of type 2 diabetes. *Current diabetes reports* 2: 396-402.

Jacob S, Machann J, Rett K, Brechtel K, Volk A, Renn W, Maerker E, Matthaei S, Schick F, Claussen C-D. 1999. Association of increased intramyocellular lipid content with insulin resistance in lean nondiabetic offspring of type 2 diabetic subjects. *Diabetes* 48: 1113-1119.

Jensen J, Rustad PI, Kolnes AJ, Lai Y-C. 2011. The role of skeletal muscle glycogen breakdown for regulation of insulin sensitivity by exercise. *Frontiers in physiology* 2.

Johnson GV, Bailey CD. 2003. The p38 MAP kinase signaling pathway in Alzheimer's disease. *Experimental neurology* 183: 263-268.

Kelley DE, Mandarino LJ. 2000. Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes* 49: 677-683.

Lillioja S, Mott DM, Howard BV, Bennett PH, Yki-Järvinen H, Freymond D, Nyomba BL, Zurlo F, Swinburn B, Bogardus C. 1988. Impaired glucose tolerance as a disorder of insulin action. *New England Journal of Medicine* 318: 1217-1225.

Lorenzo M, Fernández-Veledo S, Vila-Bedmar R, Garcia-Guerra L, De Alvaro C, Nieto-Vazquez I. 2008. Insulin resistance induced by tumor necrosis factor-α in myocytes and brown adipocytes. *Journal of animal Science* 86: E94-E104.

Martin C, Zhang Y, Tonelli C, Petroni K. 2013. Plants, diet, and health. *Annu Rev Plant Biol* 64: 19-46.

Moon B, Kwan JJ, Duddy N, Sweeney G, Begum N. 2003. Resistin inhibits glucose uptake in L6 cells independently of changes in insulin signaling and GLUT4 translocation. *Am J Physiol Endocrinol Metab* 285: E106-115.

Perregaux DG, Dean D, Cronan M, Connelly P, Gabel CA. 1995. Inhibition of interleukin-1 beta production by SKF86002: evidence of two sites of in vitro activity and of a time and system dependence. *Molecular pharmacology* 48: 433-442.

Phillips D, Caddy S, Ilic V, Fielding B, Frayn K, Borthwick A, Taylor R. 1996. Intramuscular triglyceride and muscle insulin sensitivity: evidence for a relationship in nondiabetic subjects. *Metabolism* 45: 947-950.

Plomgaard P, Bouzakri K, Krogh-Madsen R, Mittendorfer B, Zierath JR, Pedersen BK. 2005. Tumor necrosis factor-alpha induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160 phosphorylation. *Diabetes* 54: 2939-2945.

Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. 2001. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *Jama* 286: 327-334.

Saltiel AR. 2001. You are what you secrete. *Nature medicine* 7: 887-888.

Schalin-Jäntti C, Härkönen M, Groop LC. 1992. Impaired activation of glycogen synthase in people at increased risk for developing NIDDM. *Diabetes* 41: 598-604.

Shah GM, Kandan-Kulangara F, Montoni A, Shah RG, Brind'amour J, Vodenicharov MD, Affar el B. 2011. Approaches to detect PARP-1 activation in vivo, in situ, and in vitro. *Methods Mol Biol* 780: 3-34.

Sweeney G, Keen J, Somwar R, Konrad D, Garg R, Klip A. 2001. High leptin levels acutely inhibit insulin-stimulated glucose uptake without affecting glucose transporter 4 translocation in l6 rat skeletal muscle cells. *Endocrinology* 142: 4806-4812.

Vaag A, Henriksen J, Madsbad S, Holm N, Beck-Nielsen H. 1995. Insulin secretion, insulin action, and hepatic glucose production in identical twins discordant for non-insulin-dependent diabetes mellitus. *Journal of Clinical Investigation* 95: 690.

Yoon JH, Song P, Jang JH, Kim DK, Choi S, Kim J, Ghim J, Kim D, Park S, Lee H et al. 2011. Proteomic analysis of tumor necrosis factor-alpha (TNF-alpha)-induced L6 myotube secretome reveals novel TNF-alpha-dependent myokines in diabetic skeletal muscle. *J Proteome Res* 10: 5315-5325.

CHAPTER-4(C)

Chapter 4C

To study the mechanism of action of swertiamarin on dexamethasone/TNF-α induced insulin resistance in mature adipocytes

4C.1 Introduction

Adipose tissue is not only a fat storing organ, but also plays an important role in energy and fuel homeostasis. The interplay of the cells residing in adipose tissue is responsible for insulin sensitivity and central metabolism. Adipocytes, the fat cells with lipid storage are the key cells involved in fat metabolism and insulin signaling. Various adverse conditions like high fat diet, excess of endogenous glucocorticoid level, genetic predisposition, excess sex hormones leads to increased adipogenesis and hence, the adipocytes become hypertrophic in nature and hence causes adipokine overproduction (Stephens 2012). Central obesity is the major effect of adipocyte hypertrophy. The fat storage capacity and reesterification of fatty acids in hypertrophic adipocytes are lost which leads to free fatty acid circulation and hence causes ectopic fat deposition in tissues like liver, muscle, heart etc causing peripheral insulin resistance (Raz et al. 2005). The disproportionate expansion of adipocytes decrease oxygen gas diffusion and hence leads to hypoxic condition resulting in death of the adipocytes. High levels of enzymes, proteins, adipokines and transcriptional factors like FAS, ACC-1, aP2, leptin, adiponectin, SREBP-1c and PPAR-γ responsible for fatty acid synthesis are found to be elevated in adipocytes. This increased frequency of fat development creates a state called as adipose tissue obesity and mediates insulin resistance (Xu et al. 2003).

Apart from high fat diet intake, central obesity is also an outcome of prolonged treatment of anti-inflammatory and immunosuppressant drugs called as glucorticoids. It is known that endogenous and exogenous dexamethasone induces obesity, hypertension and insulin resistance hence, leads to metabolic syndrome. It increases concentrations of amino acids by stimulating proteolysis inducing lipolysis that elevates free fatty acids, impairs glucose uptake and its disposal (Di Dalmazi et al. 2012). It inhibits glucose oxidation by inhibiting the activity of the pyruvate dehydrogenase (Berdichevsky et al. ; Etxabe and Vazquez 1994; Bell et al. 2008; Bhat et al. 2009; Di Dalmazi et al. 2012). Patients treated with dexamethasone showed elevated serum triglyceride and lowered lipoprotein lipase levels that indicated, reduced triglyceride clearance from the circulation, leading to glucose intolerance due to altered insulin secretion ultimately

hampering hepatic glucose metabolism with 24 hours of dexamethasone infusion (Nicod et al. 2003) and lipolysis is elevated by stimulating the transcription of the lipolytic enzymes like *Lipe, Mgll,* and *Angptl4* sequentially activating PKA further facilitating breakdown of lipid droplets and hence releasing free fatty acids and increasing their concentrations in circulation that leads to ectopic fat deposition in peripheral tissues (Qi et al. 2006) *In vivo* studies have shown that, there is adverse effects on adipocytes due to fatty acid accumulation in hepatocytes (Bujalska et al. 1997) and thus impaired glucose tolerance (Bujalska et al. 1997; Ayala-Sumuano et al. 2013).

As adipose tissue comprises various cells like pre-adipocytes, MSCs, pericytes, mature adipocytes etc. it is difficult to understand the effect of this glucocorticoid at cell specific level. Mature adipocytes are the key cells involved in insulin mediated glucose uptake, energy homeostasis and fat metabolism. 3T3-L1, the preadipocytes differentiated into mature adipocytes have been explored very well to understand the molecular pathophysiology of insulin resistance and associated dyslipidemia *in vitro* (Andrews and Horvath 2009). Dexamethasone hampers insulin stimulated glucose uptake by inhibiting tyrosine phosphorylation of IRS-1, further causing downregulation of PI(3)K in adipocytes (Basseri et al. ; Authier et al. 1999; Sakoda et al. 2000; Bays et al. 2004; Antuna-Puente et al. 2008; Asrih and Jornayvaz 2013a; Asrih and Jornayvaz 2013b; Bailey et al. 2013). Downstream signaling of Akt is glucose uptake, which is hampered in insulin resistance condition and also leads to lowered adiponectin and increased leptin production, which results in altered fat metabolism and causes lipid toxicity (Joslin's Diabetes Mellitus). As glucocorticoids are the nuclear receptor ligands that regulate DNA transcription, it affects expression of transcriptional factors and several proteins (Sakoda et al. 2000) involved in fat metabolism. Lipogenic genes like FAS, ACC-1, SREBP-1c, LPL and FABP4 are downregulated in insulin resistance condition. Adiponectin levels are also reduced that indicates lowered glucose breakdown hence leading to altered glucose and energy homeostasis (Ayala-Sumuano et al. 2013).

Obesity caused due to excess glucocorticoid stimulates inflammatory responses and elevates proinflammatory cytokines. TNF-α is the major secreted cytokine found to be associated with reduced lipogenesis, increased lipolysis, impaired adipocyte differentiation and induces apoptosis (Prins and O'rahilly 1997; Maury and Brichard 2010). Direct exposure of isolated cells to TNF-α inhibits insulin signaling and induces a state of insulin resistance in 3T3-L1 cells and human primary adipocytes. Excess free fatty acids activates TNF-α that triggers production of ceramide, a spingolipid and activates MAPK, NF-kB and apoptotic pathways by activating

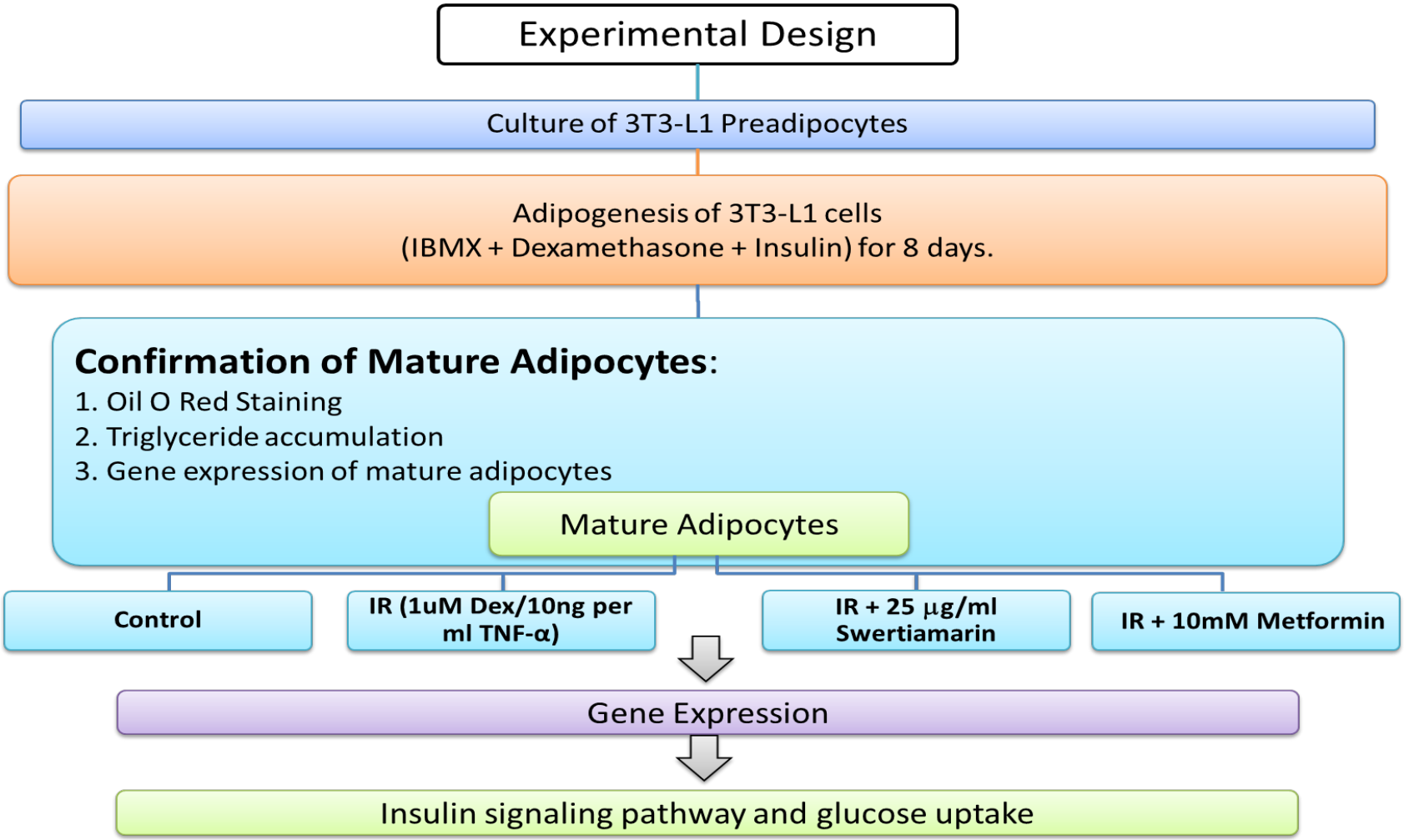
TRAF2 and TRADD resulting in activation of serine/threonine kinases P38 MAPK, Erk1/2, and JNK (Wu and Zhou 2010) which regulate insulin signaling by negative feedback mechanism mediating pSer 307 IRS-1 and inhibiting pAkt. (Lorenzo et al. 2008). Hampered PI(3)K activates various isoforms of PKCs through PDK-1 that stimulate IKK and hence causes insulin resistance in similar fashion as that of stress kinases Erk1/2 and P38 MAPK. These signaling cascades affect Glut4 translocation. After 4 days of TNF-α treatment, there was about 85-90% marked reduction in the mRNA levels of Glut4 and CCAAT/enhancer binding protein-α (C/EBP-α) *in vitro* (Stephens and Pekala 1992). Animals chronically treated with insulin showed increased Glut4 mRNA levels in adipose tissue (Charron et al. 1999). Treatment of cells with low concentrations of 0.2 nM TNF-α, repressed Glut4 gene transcription, decreased Glut4 mRNA stability and hence disrupted insulin mediated glucose uptake (Qi and Pekala 2000).

Numbers of anti-obesity drugs are currently undergoing clinical development that target peripheral episodic satiety signals (e.g. rimonabant and APD356), blockage of fat absorption (e.g.cetilistat and AOD9604), mTOR sensitizer with reduced PPAR-γ binding affinity (Colca et al. 2014) and human growth hormone fragments (Halford 2006; Melnikova and Wages 2006). As an alternative and safe approach for treating obesity and its related complications herbal extracts and compounds have been reported. Various natural compounds like flavonoids, tannic acid, procyanidins, EGCG, genistein etc. have been reported to behave antiadipogenic in nature by regulating various steps of adipogenesis (O'Brien and Granner 1991; Hwang et al. 2005; Liu et al. 2005). Vaidya et al have reported antilipidemic activity of swertiamarin and gentianine (a metabolite of swertiamarin) in *in vivo* and *in vitro* (Vaidya et al. 2009b; Vaidya et al. 2009a; Vaidya et al. 2012; Vaidya et al. 2013).

There are reports, which have highlighted the serious issue that excess glucocorticoid has 4 fold higher mortality with various metabolic disorders like CVD, obesity and insulin resistance than any other diabetic complications (Etxabe and Vazquez 1994). In adipose tissue obesity, caused due to excess glucocorticoids, adipocytes start secreting chemokines that leads to infiltration of M1 macrophages along with activated T cells in the adipose tissue and hence, enhancing release of proinflammatory cytokines like TNF-α, IL6, IL1 etc. Amongst all, TNF-α is the most potent cytokine released and causes peripheral insulin resistance. Commonly the mechanism of action of both these chemicals is through ceramide production which is abundantly held by TNF-α (Zierath 2007). Thus, these consecutive events lead us to select two different models in inflamed adipocytes, dexamethasone and TNF-α to address altered insulin resistance associated fat

metabolism and insulin resistance respectively. Thus, the present study was designed to understand the molecular mechanism of action of swertiamarin in mature insulin resistant adipocytes. Attempts were made to understand how the efficacy of swertiamarin differs in the ameliorating insulin resistance in two different *in vitro* models.

4C.2 Experimental design:



4C.3 Materials and methods:

4C.3.1 Chemicals and media:

All cell culture reagents, Dulbecco's modified eagle's medium(DMEM) high Glucose, DMEM/Ham-F12, trypsin-EDTA, Oil O Red Stain were bought from Sigma Aldrich. Fetal Bovine Serum (FBS) and Penicillin-Streptomycin were procured from Gibco, Life Technologies. All cell culture experiments were carried out in tissue culture grade plastic wares obtained from Nunclon. RNA extraction was done by Trizol and all other molecular biology kits required for gene expression experiments were procured from Life Technologies. Gene specific primers were designed from IDT and antibodies for protein expressions were obtained from CST. All proteomic grade reagents were purchased from Bio-Rad Laboratory.

4C.3.2 Cell culture:

3T3-L1 (ATCC) early passage cells was a generous gift from Katherine Cianfone, Quebec, CA. 3T3-L1 preadipocytes were maintained and cryo-preserved in DMEM high Glucose supplemented with 10% FBS and 1X penicillin-streptomycin(PS). For adipocyte differentiation preadipocytes 3T3-L1 were cultured in DMEM/Ham-F12 medium containing 10% FBS and PS. *In vitro* adipocyte differentiation was carried out as per Hata et al,. 2008 protocol with minor modifications (Hata et al. 2008).

4C.3.3 Adipocyte differentiation

Freshly sub cultured cells were seeded in culture dish at the density of 0.8 X 10^5 cell/ 3.5 cm^2 dish in DMEM/Ham-F12 containing 10 % FBS and PS. Cells were maintained for 2 days in confluent stage (to arrest cell division), which was the starting point for differentiation and considered as day zero and the culture media was replaced with adipocyte differentiation medium (ADM) containing DMEM/Ham-F12(10%) with 0.5 mM 3-isobutyl-1-methylxanthine (Calbiochem), 1 µM dexamethasone (Sigma Aldrich, USA), and 10 µg/ml insulin (Sigma Aldrich, USA). Cells were maintained for 4 days in adipocyte differentiation media (Hata et al, 2008). At the end of 4 days, culture media was replaced with adipocyte maturation media (AMM) containing DMEM/Ham-F12 with 10% FBS having 10µg/ml insulin and the cells were maintained for another 4 days. Subsequently media was replaced every 48 hrs till the end of the experiment. Differentiation was monitored by morphological assessment and Oil O Red staining.

Insulin resistance condition was induced by two chemical insults, a synthetic glucocorticoid, dexamethasone and other was inflammation mediator, TNF-α. The mature adipocytes were then treated with 1uM dexamethasone and 1uM insulin in complete media for 24 hrs (Nawano et al. 2000) to induce insulin resistance. The experiment was divided into 4 groups: 1. Control, 2.Dexamethasone induced insulin resistance, 3. Dexamethasone + 25ug/ml Swertiamarin and 4. Dexamethasone (Sakoda et al, 2000) + 10mM metformin as positive control group. Differentiated mature adipocytes were confirmed and a separate set of mature adipocyte was treated with AMM supplemented with TNF-α for 24 hrs to induce insulin resistance in mature adipocytes on 8th day of adipogenesis. The study was divided into four groups: control, insulin resistance with TNF-α (10ng/ml)., TNF-α (10ng/ml) with swertiamarin (25µg/ml) and positive control with Metformin (10mM) + TNF-α (10ng/ml)

4C.3.4 Oil O Red staining and TG accumulation assay

Oil O Red staining was performed for visualizing lipid accumulation in adipocytes. At the end of incubation, cells were washed twice with 1X PBS and fixed in 4% buffered paraformaldehyde for 10 min at RT, washed twice with milli Q water and then stained using 0.3% Oil O Red for 10 min at RT. Cells were then washed thrice with water and photographed under bright field phase contrast microscope (20X). For quantification of the extent of staining, Oil O Red stain was extracted with 100% isopropanol from the cells and OD was measured at 505nm colorimetrically. The difference in absorbance between cells with and without Oil O Red dye was calculated. Percentage adipogenesis was calculated as OD of treated cells /OD of untreated cells X 100. After 24 h of treatment, the cells were washed thrice with PBS and lysed with 1% triton in PBS. The lysates were centrifuged at 10000 g for 2 min, and the supernatant collected were assayed for TG accumulation using commercially available enzymatic kit (Reckon Diagnostics, Baroda, India). Results were expressed as microgram of TG per million cells.

4C.3.5 Western blotting of insulin signaling pathway

Insulin resistant adipocytes were collected as cell pellets, lysed with 1 ml of the lysis buffer(1mM EDTA, 50 mM Tris-HCl pH 7.5, 70 mM NaCl, 1% Triton, 50 mM NaF) supplemented with protease inhibitor cocktail l(Fermentas INC). Cell were collected for each day of adipogenesis and 20µg of total protein were separated by SDS-PAGE and transferred onto nitrocellulose blotting membrane. After blocking, the blots were probed with primary antibodies at 4^{0}C overnight (Table 7). Blots were developed by ECL reagent(Chapter 4A.3.8).

4C.3.6 RNA extraction and Real time quantitative PCR

RNA was isolated from insulin resistant mature adipocytes from all four groups The primer sequences used for quantitative real-time polymerase chain reactions (qRT-PCR) are listed in Table 4. Optimal conditions for PCR were determined by running gradient PCR. Quantitative RT-PCRs were performed in two independent experiments with triplicates. Fold changes by qRT-PCR gene expression was analyzed using 7500 Real time PCR software V.2.0.6 and Data assist software (Applied Biosystems Inc.) The qPCR results are expressed as mean ± S.E.M of RQ values versus target gene (Chapter 4A.3.7).

4C.3.7 Co-Immunoprecipitation

Cell pellets were lysed with RIPA buffer containing 1X protease inhibitor cocktail which was kept for constant agitation for 30 min at 4^{o}C. After centrifugation at 16000g 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 IR-β and PI(3)K (Table 9) was performed using Dynabeads G-protein IP kit (Invitrogen). Protein was loaded on a 10% SDS-PAGE and then electrophoretically transferred onto a Nitrocellulose membrane (GE Healthcare). The membrane containing IR-β and PI(3)K as input was then incubated for 1h at room temperature in blocking buffer (TBS-T containing 5% skimmed milk) and further probed with the primary antibodies overnight for Phospho tyrosine with slow rocking at 4°C and input with IR-β and PI(3)K antibody. Membrane was then washed four times with TBS-T, and incubated with HRP-conjugated secondary antibodies (1:2500) for 1h. Again the membrane was washed with PBS-T and PBS. Then, it was developed and visualized with Enhanced Chemiluminescence western blotting detection system (Millipore Inc. USA) (Table 7).

4C.3.8 Glucose uptake assay

Cells were seeded for adherence on glass coverslip with complete medium until confluency and then were differentiated by following adipocyte differentiation protocol. After differentiation the cells were kept in plain medium without glucose and phenol red for complete removal of D-glucose because it is known to inhibit 2-NBDG uptake. After 30 min of starvation the cells were washed with same medium. Then, the cells were treated with 100 μM 2-NBDG for 10 min. The 2-NBDG uptake reaction was stopped by removing the incubation medium and the cells were washed with pre cold KRH buffer and glucose uptake was captured at 0 and 10 min by

fluorescence microscopy (Nikon) microscope. Graphs were made by measuring fluorescence intensity by image J software.

4C.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.

4C.4 Results

4C.4.1 Adipogenesis and confirmation of mature adipocyte formation

3T3-L1, preadipocytes were treated with ADM for first four days and AMM for next four days, Pre-adipocytes were converted into mature adipocytes (Figure 4C.1a) loaded with lipid droplets (Figure 4C.1b). Intracellular triglyceride accumulation was stained with Oil O Red which was proportional to adipocyte differentiation (Figure 4C.1c). The results of gene expression (Figure 4C.1d) also showed significant up-regulation of all mature adipocyte genes such as SREBP-1c, PPAR-γ2, CD36, aP2, LPL, ACC-1 and Adiponectin as compared to undifferentiated cells, which was further confirmed by formation of mature adipocytes at molecular level.

4C.4.2 Dexamethasone induces insulin resistance in mature adipocytes

Mature adipocytes were divided into two groups. On eighth day of adipogenesis, first group was treated with AMM and second group was made diabetic by treating with AMM in presence of 1uM dexamethasone. After treatment with 1uM dexamethasone for 24 hours, mature adipocytes of the second group showed reduced expression of IR-β, PI(3)K, PPAR-γ as compared to untreated mature adipocytes (Figure 4C.2). Down regulation of insulin receptor and PI(3)K proteins indicate insulin resistance condition in mature adipocytes. Hence, these show hampered insulin signaling which resembles TIIDM condition *in vitro* and confirms insulin resistant mature adipocytes.

4C.4.3 Effect of swertiamarin on expression of fat regulating metabolic genes in dexamethasone induced insulin resistant mature adipocytes

Insulin resistant adipocytes have altered fat metabolism leading to dyslipidemia. Swertiamarin treatment on insulin resistant adipocytes showed significant increase in the expression of the genes involved in lipogenesis. Relative gene expression studies depicted that SREBP-1c was

significantly increased that further facilitates activation of PPAR-γ2 and further downstream signaling of the genes involved in fat metabolism. In insulin resistant adipocytes there was marked elevation in the levels of CD36, aP2 and ACC-1 which, clearly indicated that, swertiamarin enhances fatty acid production and increases break down of Tg and hence regulates energy homeostasis, observed by elevated LPL level, that shows clearance of free fatty acids. However, there was no significant difference in the expression of adiponectin. Swertiamarin acted in a better way when compared to Metformin treated group (Figure 4C.3).

4C.4.4 Effect of swertiamarin on insulin signaling proteins in mature adipocytes

Insulin mediated glucose uptake requires sequential expressions of the proteins involved in insulin signaling pathway. Insulin binds to insulin receptor and leads to the conformational changes inducing tyrosine phosphorylation of IR further followed by that of IRS-1 sequentially activating PI(3)K and Akt. Dexamethasone treatment increased serine phosphorylation on IRS-1 with decreased IR-β and PI(3)K expressions and hence inhibiting the activity of Akt. Densitometric analysis shows that swertiamarin treated group significantly increased IR, pAkt, PI(3)K. PPAR-γ1 and 2 both were elevated, hence increasing insulin sensitivity and glucose uptake (Figure 4C.4a and b).

4C.4.5 Swertiamarin ameliorates inflammatory (TNF-α) mediated insulin signaling defects in mature adipocytes

Insulin-induced stimulation on glucose transport in adipocyte requires IR-mediated tyrosine phosphorylation of IRS-1 and subsequent activation of downstream signaling proteins. To investigate the effects of swertiamarin on insulin signaling, differentiated 3T3-L1 adipocytes were used. We have examined different insulin signaling proteins IR-β, PI(3)K, pIRS-1, pAkt and PPAR-γ in mature insulin resistant adipocytes which were down regulated in TNF-α treated group and were restored significantly in insulin resistant adipocytes treated with swertiamarin. As cytokines are known to induce stress and activate various serine/threonine kinases, we also checked the expression of the enzymes like PKCs, Erk1/2 and P38 MAPK belonging to this family and were found to be activated in TNF-α treated group and were found to be down regulated when treated with swertiamarin (Figure 4C.5a & b).

Figure 4C.1

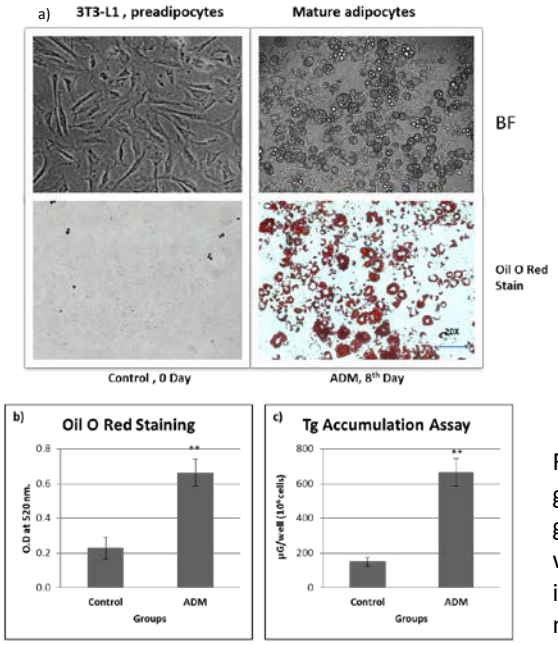


Figure 4C.1: 3T3-L1, preadipocytes were subjected to adipocyte differentiation using adiopogenic cocktail for 8 days and checked for the Oil O Red staining. (a) Oil O Red staining under 20X magnification under Phase contrast microscope (b) Quantification of Oil O Red stain after extraction procedure. (c) Cell were lysed for quantification of triglyceride accumulation. Data presented as Mean ± SEM of n=3.
p-value **≤ 0.005 as compared to control.

Figure 4C.2

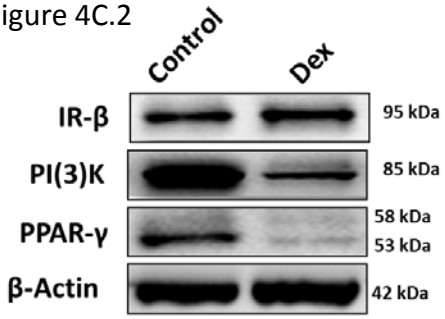


Figure 4C.2: Confirmation of dexamethasone induced insulin resistance model checked for protein content of IR, PI(3)K and PPAR-γ β-actin was taken as an internal control (20 ug protein).

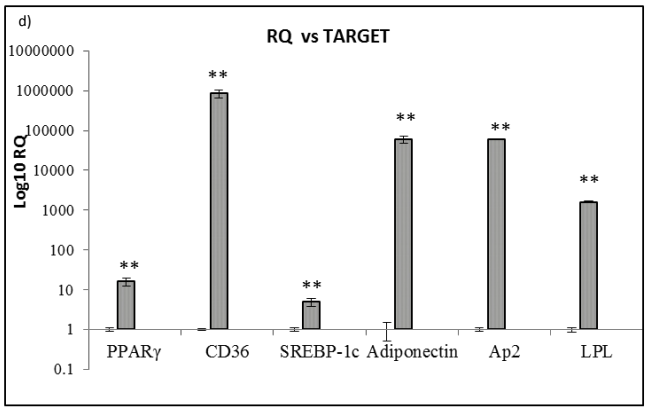


Figure 4C.1d : Confirmation of mature adipocytes functional genes expression. The expression levels of fat metabolic genes PPAR-γ, CD36, SREBP-1c, Adiponectin, aP2 and LPL were checked using quantitative PCR. β-Actin was taken as internal control. Data presented as Mean ± SEM of n=3. and normalized to p-value **≤ 0.005 as compared to control.

Figure 4C.3

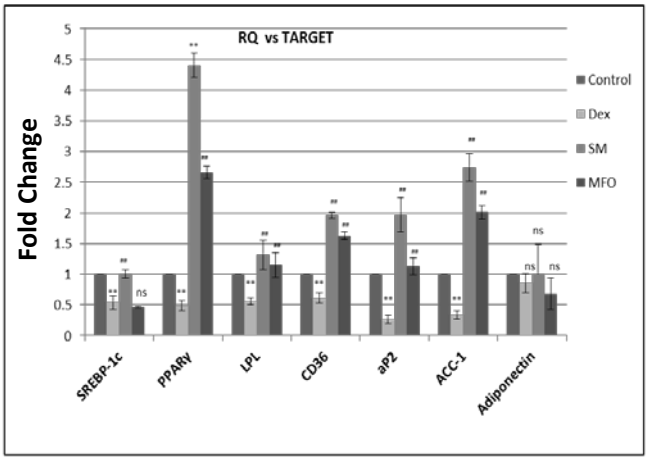


Figure 4C.3: Effect of swertiamarin treatments on the expression of fat metabolism genes in the insulin resistant mature adipocytes. The expression levels of fat metabolic genes SREBP-1c, PPAR-γ, LPL CD36, aP2, ACC-1 and Adiponectin, were checked using quantitative PCR. Data presented as Mean ± SEM of n=3. p-value **≤ 0.005 as compared to control; p-value ##≤ 0.005 as compared to Dexamethasone group.

4C.4.6 Swertiamarin enhances IR-β, PI(3)K activity and Glucose uptake

Insulin resistant mature adipocytes after treatment were harvested and 100ug of total protein was immunoprecipitated for IR-β and PI(3)K, and were analysed by immunoblotting for phospho tyrosine activity. Swertiamarin was able to potentiate tyrosine kinase activity of insulin receptor and PI(3)K. Kinase activation of PI(3)K can lead to activation of a typical protein kinase (Akt), which is mainly responsible for Glut4 translocation. Swertiamarin was able to enhance fluorescence glucose (2-NBDG) uptake at cellular levels which ensures peripheral glucose disposition in adipocytes and myocytes (Figure 4C.6 & 7a and b) .

Figure 4C.4a

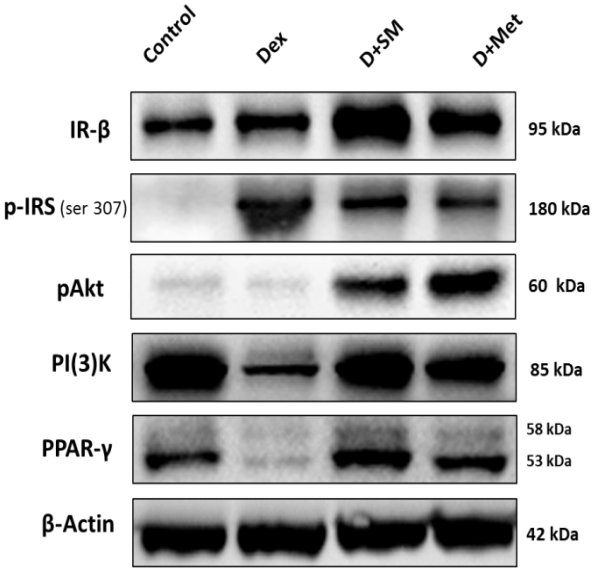


Figure 4C.4a: Effect of Swertiamarin on Signaling pathways using insulin resistant mature adipocytes. Western blot study showing the effect of SM treatments on the expression of Insulin signaling proteins: IR-β, p_{ser307}-IRS-1, pAkt, PI(3)K and PPAR-γ in the adipocyte as compared to dexamethasone treated group. β-actin was taken as an internal control (20 µg protein).

Figure 4C.4b

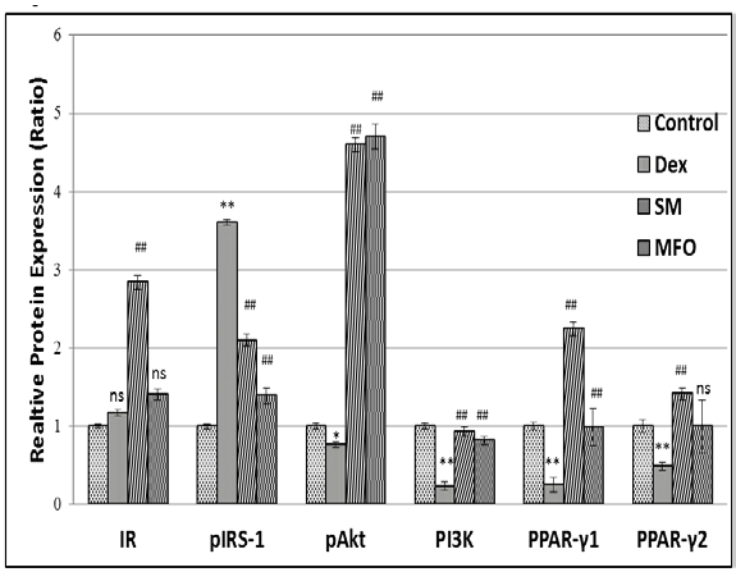


Figure 4C.4b: Effect of Swertiamarin on insulin signaling pathways using insulin resistant mature adipocytes. Densitometric analysis of western blot study showing the effect of SM treatments on the expression of Insulin signaling proteins. Ratio of target to internal control were calculated . Data presented as Mean ± SEM of n=3 . p-value **≤ 0.005 as compared to control; p-value ##≤ 0.005 as compared to TNF. n=3**.** p-value ; ns > 0.05 as compared to control & Dex group.

Figure 4C.5a

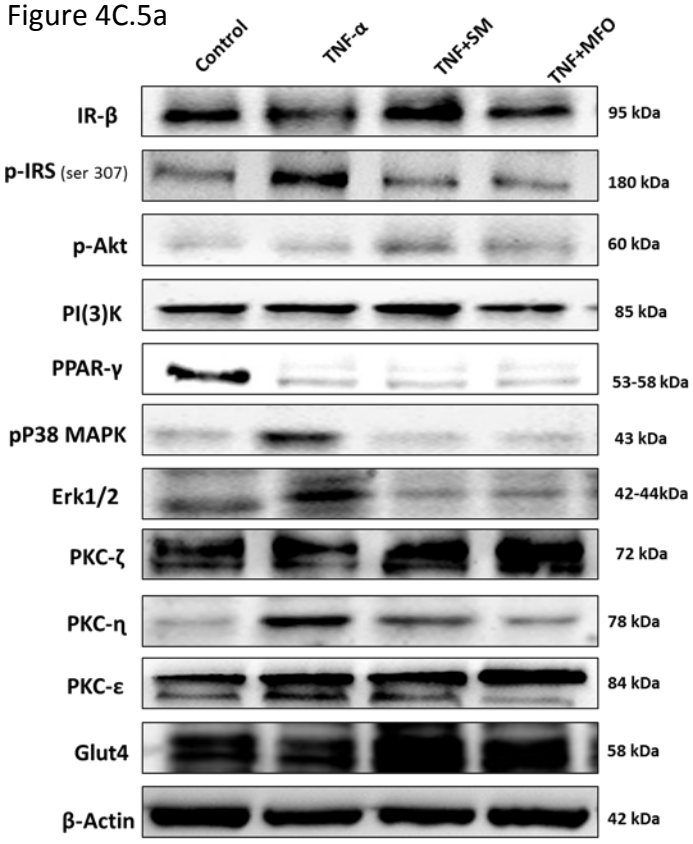


Figure 4C.5a: Effect of Swertiamarin on Signaling pathways using insulin resistant mature adipocyte: Western blot study showed the effect of SM treatments on the expression of Insulin signaling proteins: IR, p_{ser307}-IRS-1, p-Akt, PI(3)K ,Pp38 MAPK,$Erk_{1/2}$,PKCs, PPAR-γ and Glut4 in the Adipocyte as compared to TNF-α treated group . β-actin was taken as an internal control (20 μg protein).

Figure 4C.6

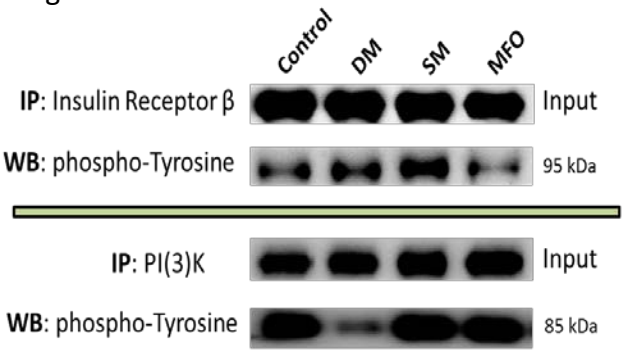


Figure 4C.6: Immunoprecipitation study showing the effect of Swertiamarin treatments on the tyrosine phosphorylation of insulin signaling proteins. Immunoprecipitation of insulin receptor & PI(3)K for their phosphorylation were checked by immunoblotting in insulin resistant mature adipocytes (100μg Protein)

Figure 4C.5b

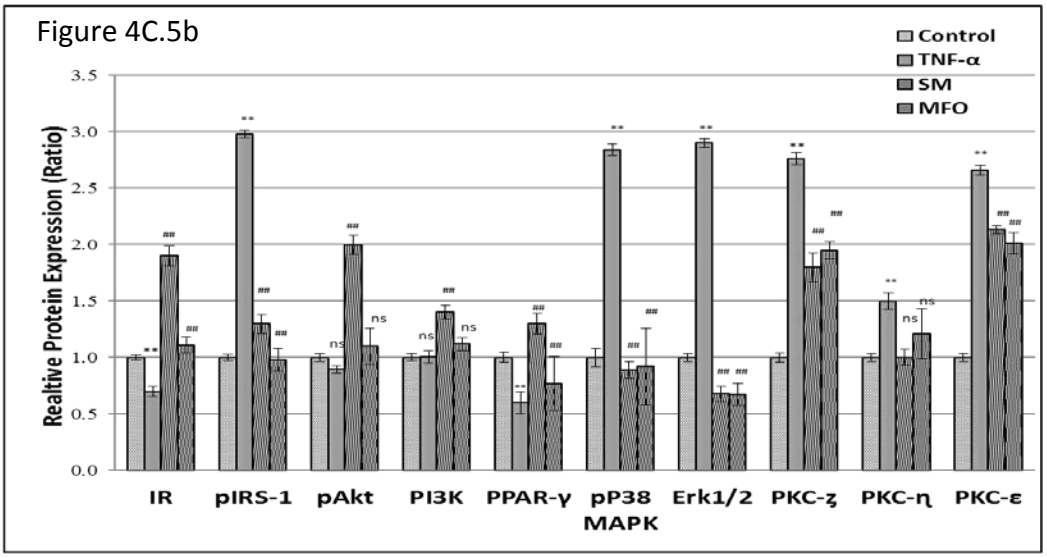


Figure 4C.5b: Effect of Swertiamarin on insulin signaling pathways using insulin resistant mature adipocytes. Densitometric analysis of western blot study showing the effect of SM treatments on the expression of Insulin signaling proteins. Ratio of target to internal control were calculated . Data presented as Mean ± SEM of n=3. p-value **≤ 0.005 as compared to control; p-value ##≤ 0.005 as compared to TNF. n=3**.** p-value ; ns > 0.05 as compared to control & TNF group.

Figure 4C.7a

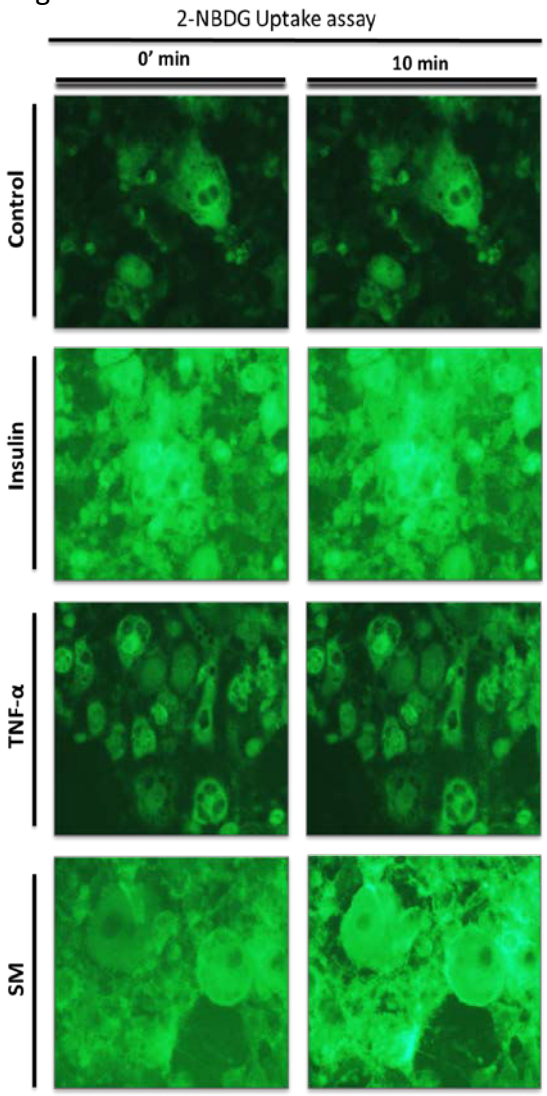


Figure 4C.7a: Effect of Swertiamarin on the insulin stimulated glucose uptake assay. 2-NBDG uptake assay were performed in mature adipocytes . After washing cells without glucose and serum . 100 µM 2-NBDG were added to differentiated adipocytes for 10 mins . Cells were washed with KRH buffer and images were captured at 10 min and 20min at excitation/emission = 485/535 nm under fluorescence microscope.

Figure 4C.7b

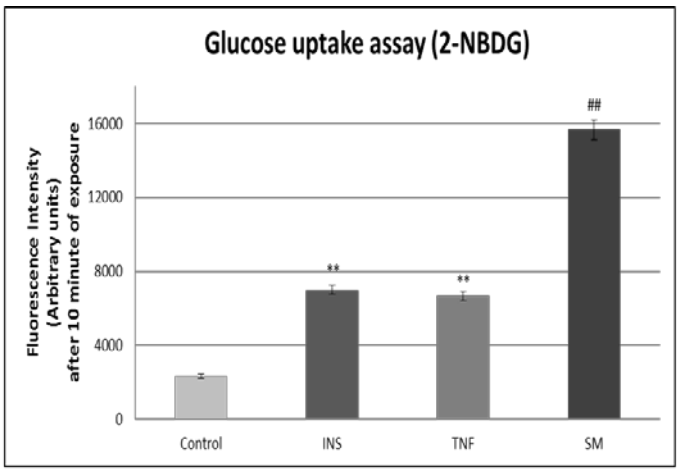


Figure 4C.7b: Effect of Swertiamarin on the insulin stimulated glucose uptake assay. 2-NBDG uptake assay were performed in mature adipocytes. Average fluorecence of intensity of cells (arbitrary unit) was calculated following 20 min of total exposure to 2-NBDG. Results were presented as Mean ± SEM of n=3. p-value **≤ 0.005 as compared to control; p-value ##≤ 0.005 as compared to INS.

4C.5 Discussion

Obesity is the most profoundly occurring disease worldwide. As TIIDM is one of the consequences of obesity caused due to visceral fat deposition, one of the conditions associated with its occurrence is the effect of dexamethasone on the adipocytes that induces insulin resistance and suppresses lipogenic genes and enhances lipolysis. As all the fat metabolic machineries are present in the mature adipocytes, it is evidently important to understand the effect of swertiamarin on mature insulin resistant adipocytes with dyslipidemia. The expression study of the genes involved in fat metabolism on swertiamarin treatment significantly restored the levels of the SREBP-1c, the transcriptional factor responsible for fatty acid synthesis by activating endogenous ligand, which further stimulates activation of PPAR-γ and the lipogenic genes under its control. Increased levels of LPL state that swertiamarin may trigger interaction between LPL and chylomicron that, would breakdown the circulating triglyceride. Restored levels of CD36, an important transporter protein that elevates nonesterified fatty acid uptake in the cells, and hence would probably regulate fat metabolism (Aggarwal 2010). (Di Dalmazi et al. 2012). The restored levels of lipogenic gene expression could regulate the levels of adipokine adiponectin and leptin that maintains glucose homeostasis by glucose uptake and fatty acid oxidation, thus regulating energy balance. Significant upregulation of ACC-1 by swertiamarin treatment on insulin resistant adipocytes, indirectly depicts elevation of malonyl Co-A and citrate levels in the mitochondria and hence, promotes fatty acid synthesis with decrease in gluconeogenesis thus, suggesting that the enzyme CPT-1 could be restored in the mitochondria of the cells and regulate energy homeostasis (Muoio and Newgard 2006). Hence, the above observations state that swertiamarin has direct role in fat metabolism and also provides a hint of its association with mitochondrial functions with respect to energy homeostasis.

Altered fat metabolism in adipocytes and dyslipidemia is associated with insulin rsistance. Therefore, the further study on antidiabetic effect of swertiamarin on dexamethasone and TNF-α mediated insulin resistance showed significant increase in insulin receptor which activates tyrosine kinase and phosphorylates tyr IRS-1. PI(3)K is a major key enzyme getting activated downstream to IRS-1. It activates Akt and inhibits the activity of serine kinases and reduces p 307 ser IRS-1 and hence, decreasing insulin resistance and increasing insulin sensitivity by translocation of Glut4 leading to insulin stimulated glucose uptake (Rui et al. 2001; Cawthorn et al. 2007). This has been very well elucidated by swertiamarin treatment on insulin resistant adipocytes and hence could reduce the activity of Janus Kinase along with its downstream

signalling pathway. Insulin resistance caused by both the chemical insults involves the action of one of the lipid moiety ceramide majorly produced by TNF-α which inactivates Akt by dephosphorylating it (Teruel et al. 2001). It is shown that production of ceramide and IRS-2 associated PI(3)K inactivation are the mandatory process for augmenting insulin resistance in adipocytes. Increased p Tyr Akt in swertiamarin treated group could probably phosphorylate FoxO1 and inhibit its downstream signaling pathway, and preventing it from binding to PPAR-γ promoter and hence increasing insulin sensitivity (Armoni et al. 2006; Tsai et al. 2014). Carnosic acid has been showed to stimulate insulin sensitivity by inhibiting IL-6 etc with increase in tyrosine phosphorylation of Akt, reduced ser 307 pIRS-1 and inhibition of FoxO1. Recent study reported that, there was one more biomolecule, heparin binding growth factor called as midkine which downregulates the activity of pIRS-1 and by phosphorylating Akt at ser 473 in TNF-α mediated insulin resistance (Fan et al. 2014). The results of the present study provides a hint that swertiamarin can suppress the activity of this inflammatory protein midkine and can reduce insulin resistance in adipocytes.

TNF-α induces insulin resistance through various inflammatory pathways by activating serine/threonine kinase and one of them is Protein kinase C (PKC) whose different isoforms are involved in initiating insulin resistance condition (Zick 2005). δ isoform of this serine/threonine kinase family was found to be associated with inhibition of Glut4 translocation by interacting with insulin receptor autophosphorylation. This enzyme is associated with oxidative stress and ROS generation. H_2O_2 treatment has shown their activation and hence, PKCs are found responsible for activation of various inflammatory responses that leads to insulin resistance condition (Konishi et al. 1997; Talior et al. 2003). Other isoform like PKC-ζ is also involved in inhibition of insulin signaling pathway (Teruel et al. 2001; Talior et al. 2003). Swertiamarin treatment reduced the levels of PKCs indicating that this bitter glycoside restricts the activity of majorly important isoforms of this enzyme.

Alike PKCs many other stress kinases like P38 MAPK including Erk1/2 are responsible for insulin resistance in adipocytes. Activation of these kinases follow typical pathway downstream to PI(3)K and as a negative feedback mechanism, they alter the expression levels of IRS, PI(3)K, Akt etc by phosphorylating the major residues present in their regulatory domains. Erk1/2 phosphorylates the regulatory residues of the key transcriptional factor PPAR-γ with reduced mRNA level and hence inactivates its activity and downregulates all the metabolic genes

responsible for fat cell development and adipogensis (Hu et al. 1996; Adams et al. 1997; Cawthorn and Sethi 2008). These cascades of events ultimately culminates Glut4 translocation to the membrane and hence, insulin mediated glucose uptake is hampered (Cawthorn and Sethi 2008).

Swertiamarin treatment has restored the levels of IR, IRS, PI(3)K with concomitant decreased expression of the stress kinases like Erk1/2 and MAPK P38. Significant reduction in the levels of stress kinases depicts the important characteristic of swertiamarin as an antiadipogenic, insulin potentiating and governing mature adipocytes functionality. Immunoprecipitation assay of tyrosine residue of PI(3)K and IR depicted increased tyrosine phosphorylation in the swertiamarin treated group when compared to TNF-α group.

Glut4 is the major glut transporter responsible for insulin mediated glucose uptake and stress levels mediated by inflammatory responses leads to its decreased translocation. Erks, PKCs, MAPKs are activated very soon by the action of TNF-α. These activated kinases affect Akt and PI(3)K by their serine phosphorylation ultimately inhibiting the translocation of Glut4 to the plasma membrane with concomitant reduction in insulin mediated glucose uptake (Zierath 2007). Glucose uptake study very well explains the enhanced insulin stimulated glucose uptake in the adipocytes in presence of swertiamarin and hence reducing hyperglycemia and hyperinsulinemia.

The elaborative profile of insulin signaling proteins in presence of the PPAR-γ agonist swertiamarin on insulin resistant adipocytes, shows its potential action as insulin sensitizer and modulator for various cellular processes like fat metabolism at the tissue and cellular specific level. It is evident to monitor this instrumental compound playing role in various physiological processes associated with PPAR-γ. To best of our knowledge this is the first report stating the potentials of swertiamarin that eradicates insulin resistance induced by two different models glucocorticoid and inflammatory cytokine that mimics TIIDM by regulating serine /threonine kinase and restores fat metabolism in adipocytes and hence reducing adipose tissue obesity and ameliorated dyslipidemia and insulin resistance and therefore reduces the burden of free fatty acids in the blood stream along with peripheral insulin resistance and making it a better therapeutic product

4C.6 References

Adams M, Reginato MJ, Shao D, Lazar MA, Chatterjee VK. 1997. Transcriptional activation by peroxisome proliferator-activated receptor γ is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site. *Journal of Biological Chemistry* 272: 5128-5132.

Aggarwal BB. 2010. Targeting inflammation-induced obesity and metabolic diseases by curcumin and other nutraceuticals. *Annu Rev Nutr* 30: 173-199.

Andrews ZB, Horvath TL. 2009. Uncoupling protein-2 regulates lifespan in mice. *Am J Physiol Endocrinol Metab* 296: E621-627.

Antuna-Puente B, Feve B, Fellahi S, Bastard J-P. 2008. Adipokines: the missing link between insulin resistance and obesity. *Diabetes & metabolism* 34: 2-11.

Armoni M, Harel C, Karni S, Chen H, Bar-Yoseph F, Ver MR, Quon MJ, Karnieli E. 2006. FOXO1 Represses Peroxisome Proliferator-activated Receptor-γ1 and-γ2 Gene Promoters in Primary Adipocytes A NOVEL PARADIGM TO INCREASE INSULIN SENSITIVITY. *Journal of Biological Chemistry* 281: 19881-19891.

Asrih M, Jornayvaz FR. 2013a. Inflammation as a potential link between nonalcoholic fatty liver disease and insulin resistance. *Journal of Endocrinology* 218: R25-R36.

Asrih M, Jornayvaz FR. 2013b. Inflammation as a potential link between nonalcoholic fatty liver disease and insulin resistance. *J Endocrinol* 218: R25-36.

Authier FJ, Chazaud B, Plonquet A, Eliezer-Vanerot MC, Poron F, Belec L, Barlovatz-Meimon G, Gherardi RK. 1999. Differential expression of the IL-1 system components during *in vitro* myogenesis: implication of IL-1beta in induction of myogenic cell apoptosis. *Cell Death Differ* 6: 1012-1021.

Ayala-Sumuano J-T, Velez-delValle C, Beltrán-Langarica A, Marsch-Moreno M, Hernandez-Mosqueira C, Kuri-Harcuch W. 2013. Glucocorticoid paradoxically recruits adipose progenitors and impairs lipid homeostasis and glucose transport in mature adipocytes. *Scientific reports* 3.

Bailey CJ, Aschner P, Del Prato S, LaSalle J, Ji L, Matthaei S. 2013. Individualized glycaemic targets and pharmacotherapy in type 2 diabetes. *Diab Vasc Dis Res* 10: 397-409.

Basseri S, Lhotak S, Fullerton MD, Palanivel R, Jiang H, Lynn EG, Ford RJ, Maclean KN, Steinberg GR, Austin RC. Loss of TDAG51 results in mature-onset obesity, hepatic steatosis, and insulin resistance by regulating lipogenesis. *Diabetes* 62: 158-169.

Bays H, Mandarino L, DeFronzo RA. 2004. Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: peroxisomal proliferator-activated receptor agonists provide a rational therapeutic approach. *J Clin Endocrinol Metab* 89: 463-478.

Bell M, Wang H, Chen H, McLenithan JC, Gong D-W, Yang R-Z, Yu D, Fried SK, Quon MJ, Londos C. 2008. Consequences of Lipid Droplet Coat Protein Downregulation in Liver Cells Abnormal Lipid Droplet Metabolism and Induction of Insulin Resistance. *Diabetes* 57: 2037-2045.

Berdichevsky A, Guarente L, Bose A. Acute oxidative stress can reverse insulin resistance by inactivation of cytoplasmic JNK. *J Biol Chem* 285: 21581-21589.

Bhat M, Kothiwale SK, Tirmale AR, Bhargava SY, Joshi BN. 2009. Antidiabetic Properties of Azardiracta indica and Bougainvillea spectabilis: In Vivo Studies in Murine Diabetes Model. *Evid Based Complement Alternat Med* 2011: 561625.

Bujalska IJ, Kumar S, Stewart PM. 1997. Does central obesity reflect "Cushing's disease of the omentum"? *The Lancet* 349: 1210-1213.

Cawthorn W, Heyd F, Hegyi K, Sethi J. 2007. Tumour necrosis factor-α inhibits adipogenesis via a β-catenin/TCF4 (TCF7L2)-dependent pathway. *Cell Death & Differentiation* 14: 1361-1373.

Cawthorn WP, Sethi JK. 2008. TNF-α and adipocyte biology. *FEBS letters* 582: 117-131.

Charron MJ, Katz EB, Olson AL. 1999. GLUT4 gene regulation and manipulation. *Journal of Biological Chemistry* 274: 3253-3256.

Colca JR, Tanis SP, McDonald WG, Kletzien RF. 2014. Insulin sensitizers in 2013: new insights for the development of novel therapeutic agents to treat metabolic diseases. *Expert opinion on investigational drugs* 23: 1-7.

Di Dalmazi G, Pagotto U, Pasquali R, Vicennati V. 2012. Glucocorticoids and type 2 diabetes: from physiology to pathology. *Journal of nutrition and metabolism* 2012.

Etxabe J, Vazquez J. 1994. Morbidity and mortality in Cushing's disease: an epidemiological approach. *Clinical endocrinology* 40: 479-484.

Fan N, Sun H, Wang Y, Zhang L, Xia Z, Peng L, Hou Y, Shen W, Liu R, Peng Y. 2014. Midkine, a Potential Link between Obesity and Insulin Resistance. *PloS one* 9: e88299.

Halford J. 2006. Obesity drugs in clinical development. *Current opinion in investigational drugs (London, England: 2000)* 7: 312-318.

Hata K, Hiwatashi K, Itoh M, Suzuki N, Watanabe T, Takahashi J, Sasaki H. 2008. Inhibitory effects of lupeol on 3T3-L1 preadipocyte differentiation. *Phytochemistry Letters* 1: 191-194.

Hu E, Kim JB, Sarraf P, Spiegelman BM. 1996. Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARγ. *Science* 274: 2100-2103.

Hwang J-T, Park I-J, Shin J-I, Lee YK, Lee SK, Baik HW, Ha J, Park OJ. 2005. Genistein, EGCG, and capsaicin inhibit adipocyte differentiation process via activating AMP-activated protein kinase. *Biochemical and biophysical research communications* 338: 694-699.

Konishi H, Tanaka M, Takemura Y, Matsuzaki H, Ono Y, Kikkawa U, Nishizuka Y. 1997. Activation of protein kinase C by tyrosine phosphorylation in response to H2O2. *Proceedings of the National Academy of Sciences* 94: 11233-11237.

Liu X, Kim J-k, Li Y, Li J, Liu F, Chen X. 2005. Tannic acid stimulates glucose transport and inhibits adipocyte differentiation in 3T3-L1 cells. *The Journal of nutrition* 135: 165-171.

Lorenzo M, Fernández-Veledo S, Vila-Bedmar R, Garcia-Guerra L, De Alvaro C, Nieto-Vazquez I. 2008. Insulin resistance induced by tumor necrosis factor-α in myocytes and brown adipocytes. *Journal of animal Science* 86: E94-E104.

Maury E, Brichard S. 2010. Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. *Molecular and cellular endocrinology* 314: 1-16.

Melnikova I, Wages D. 2006. Anti-obesity therapies. *Nature Reviews Drug Discovery* 5: 369-370.

Muoio DM, Newgard CB. 2006. Obesity-related derangements in metabolic regulation. *Annu Rev Biochem* 75: 367-401.

Nawano M, Oku A, Ueta K, Umebayashi I, Ishirahara T, Arakawa K, Saito A, Anai M, Kikuchi M, Asano T. 2000. Hyperglycemia contributes insulin resistance in hepatic and adipose tissue but not skeletal muscle of ZDF rats. *Am J Physiol Endocrinol Metab* 278: E535-543.

Nicod N, Giusti V, Besse C, Tappy L. 2003. Metabolic Adaptations to Dexamethasone-Induced Insulin Resistance in Healthy Volunteers. *Obesity research* 11: 625-631.

O'Brien RM, Granner DK. 1991. Regulation of gene expression by insulin. *Biochem J* 278 (Pt 3): 609-619.

Prins JB, O'rahilly S. 1997. Regulation of adipose cell number in man. *Clinical science* 92: 3-12.

Qi C, Pekala PH. 2000. Tumor Necrosis Factor-α–Induced Insulin Resistance in Adipocytes. *Proceedings of the Society for Experimental Biology and Medicine* 223: 128-135.

Raz I, Eldor R, Cernea S, Shafrir E. 2005. Diabetes: insulin resistance and derangements in lipid metabolism. Cure through intervention in fat transport and storage. *Diabetes Metab Res Rev* 21: 3-14.

Rui L, Aguirre V, Kim JK, Shulman GI, Lee A, Corbould A, Dunaif A, White MF. 2001. Insulin/IGF-1 and TNF-α stimulate phosphorylation of IRS-1 at inhibitory Ser 307 via distinct pathways. *Journal of Clinical Investigation* 107: 181-189.

Sakoda H, Ogihara T, Anai M, Funaki M, Inukai K, Katagiri H, Fukushima Y, Onishi Y, Ono H, Fujishiro M et al. 2000. Dexamethasone-induced insulin resistance in 3T3-L1 adipocytes is due to inhibition of glucose transport rather than insulin signal transduction. *Diabetes* 49: 1700-1708.

Stephens J, Pekala P. 1992. Transcriptional repression of the C/EBP-alpha and GLUT4 genes in 3T3-L1 adipocytes by tumor necrosis factor-alpha. Regulations is coordinate and independent of protein synthesis. *Journal of Biological Chemistry* 267: 13580-13584.

Stephens JM. 2012. The fat controller: adipocyte development. *PLoS biology* 10: e1001436.

Talior I, Yarkoni M, Bashan N, Eldar-Finkelman H. 2003. Increased glucose uptake promotes oxidative stress and PKC-δ activation in adipocytes of obese, insulin-resistant mice. *American Journal of Physiology-Endocrinology And Metabolism* 285: E295-E302.

Teruel T, Hernandez R, Lorenzo M. 2001. Ceramide mediates insulin resistance by tumor necrosis factor-α in brown adipocytes by maintaining Akt in an inactive dephosphorylated state. *Diabetes* 50: 2563-2571.

Tsai CW, Liu KL, Lin YR, Kuo WC. 2014. The mechanisms of carnosic acid attenuates tumor necrosis factor-α-mediated inflammation and insulin resistance in 3T3-L1 adipocytes. *Molecular nutrition & food research* 58: 654-664.

Vaidya H, Goyal RK, Cheema SK. 2012. Anti-diabetic Activity of Swertiamarin is due to an Active Metabolite, Gentianine, that Upregulates PPAR-gamma Gene Expression in 3T3-L1 cells. *Phytother Res*.

Vaidya H, Rajani M, Sudarsanam V, Padh H, Goyal R. 2009a. Antihyperlipidaemic activity of swertiamarin, a secoiridoid glycoside in poloxamer-407-induced hyperlipidaemic rats. *J Nat Med* 63: 437-442. 2009b. Swertiamarin: a lead from Enicostemma littorale Blume. for anti-hyperlipidaemic effect. *Eur J Pharmacol* 617: 108-112.

Vaidya HB, Ahmed AA, Goyal RK, Cheema SK. 2013. Glycogen phosphorylase-a is a common target for anti-diabetic effect of iridoid and secoiridoid glycosides. *J Pharm Pharm Sci* 16: 530-540.

Wu Y, Zhou B. 2010. TNF-α/NF-κB/Snail pathway in cancer cell migration and invasion. *British journal of cancer* 102: 639-644.

Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA. 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *The Journal of clinical investigation* 112: 1821-1830.

Zick Y. 2005. Ser/Thr phosphorylation of IRS proteins: a molecular basis for insulin resistance. *Science Signaling* 2005: pe4.

Zierath JR. 2007. The path to insulin resistance: paved with ceramides? *Cell metabolism* 5: 161-163.