CHAPTER-5

Chapter 5

Assessment of molecular mechanism of swertiamarin to elucidate it's anti-adiopogenic potentials

5.1 Introduction

Obesity is not only a cosmetic problem for about 300 million people worldwide (Waxman 2005) but is also an increasing risk for other life-threatening diseases such as TIIDM, hypertension and cardiovascular ailments (Aronne and Isoldi 2007; Shah et al. 2008). Reportedly, India has recorded 20% increase in the number of overweight individuals between 1998-2005 and 5% of the country's population is clinically obese. Currently, almost 1 in 5 men and 1 in 6 women are overweight. Urban population is at a higher risk with 40% individuals been reported to be obese (Sinha 2010). Diabesity is characterized by expansion of adipose tissue by increased adipogenesis i.e. increase in adipocytes number (hyperplasia) which is distributed in an uneven pattern, which is based on two phenomena: release of free fatty acids into liver and the subcutaneous layer has limited capacity to store excess fat and energy, therefore there is an over flow of free fatty acid into abdominal fat and other peripheral tissue that leads to loss in insulin sensitivity and hence causes peripheral insulin resistance and associated metabolic dysregulations leading to TIIDM (Klein et al. 2007; Maury and Brichard 2010; Stephens 2012).

Adipogenesis can be divided into two stages, first is adipocyte differentiation and second is maturation and its function i.e fat metabolism. It is a dynamic cellular process that gives rise to globular functionally complex cells known as adipocytes. The process of adipogenesis commences when the MSCs commit to the adipocyte lineage further, the pre-adipocytes get converted to mature adipocytes (Gesta et al. 2007; Vestergaard 2009). Live cell imaging of adipose tissue revealed that this process occurs in the angionegenic/adipogenic cell clusters, wherein, the stromal cells are the major contributors of this process (Nishimura et al. 2007). The disproportionate expansion of adipocytes decrease oxygen gas diffusion and hence leads to hypoxic condition resulting in death of the adipocytes.

The process involves array of interplay of several molecular beckons and it is a sequential event where, multiple transcriptional factors are involved, whose expressions have been found to be stage specific. There is a dramatic interplay between the cytoskeletal proteins that converts the fibroblastic cells to rounded adipocytes. At cellular level transient expressions of transcriptional factors like C/EBP-β and C/EBP-δ are observed after 24 hours when induced for adipogenesis (Ramji and Foka 2002; Tang et al. 2003; Tang et al. 2005). C/EBP-β sequentially gets phosphorylated and further activates PPAR-γ and C/EBP-α, the master transcriptional factors of adipogenesis. After the second day, C/EBP-α initiate's differentiation process by inhibiting proliferation. By the end of 8th day, 90% of the cells are differentiated into mature adipocytes (Rosen et al. 2002; Huang and Tindall 2007). PPAR-γ is the master regulator of the adipogenesis process and its action is regulated by its two isoforms: PPAR-γ1 and PPAR-γ2, where the later is expressed specifically in adipose tissue (Zhu et al. 1995; Kajimura et al. 2008). High levels of enzymes and proteins like FAS, ACC-1, aP2, leptin and adiponectin the major adipokines and the transcriptional factor PPAR-γ and C/EBP-α are found 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). Adiponectin, an important adipokine is found to be directly affected in obesity. Inverse correlation exists between adiponectin and BMI (Arita et al. 1999). This adipokine activates AMPK pathway and PPARs by AdipoR1 and AdipoR2 respectively. These stimulate energy dissipation; regulate gluconeogenesis, fatty acid oxidation and is important adipocyte maturation marker (Arita et al. 1999; Kadowaki et al. 2006; Capeau 2007; Yamauchi et al. 2007; Maury and Brichard 2010).

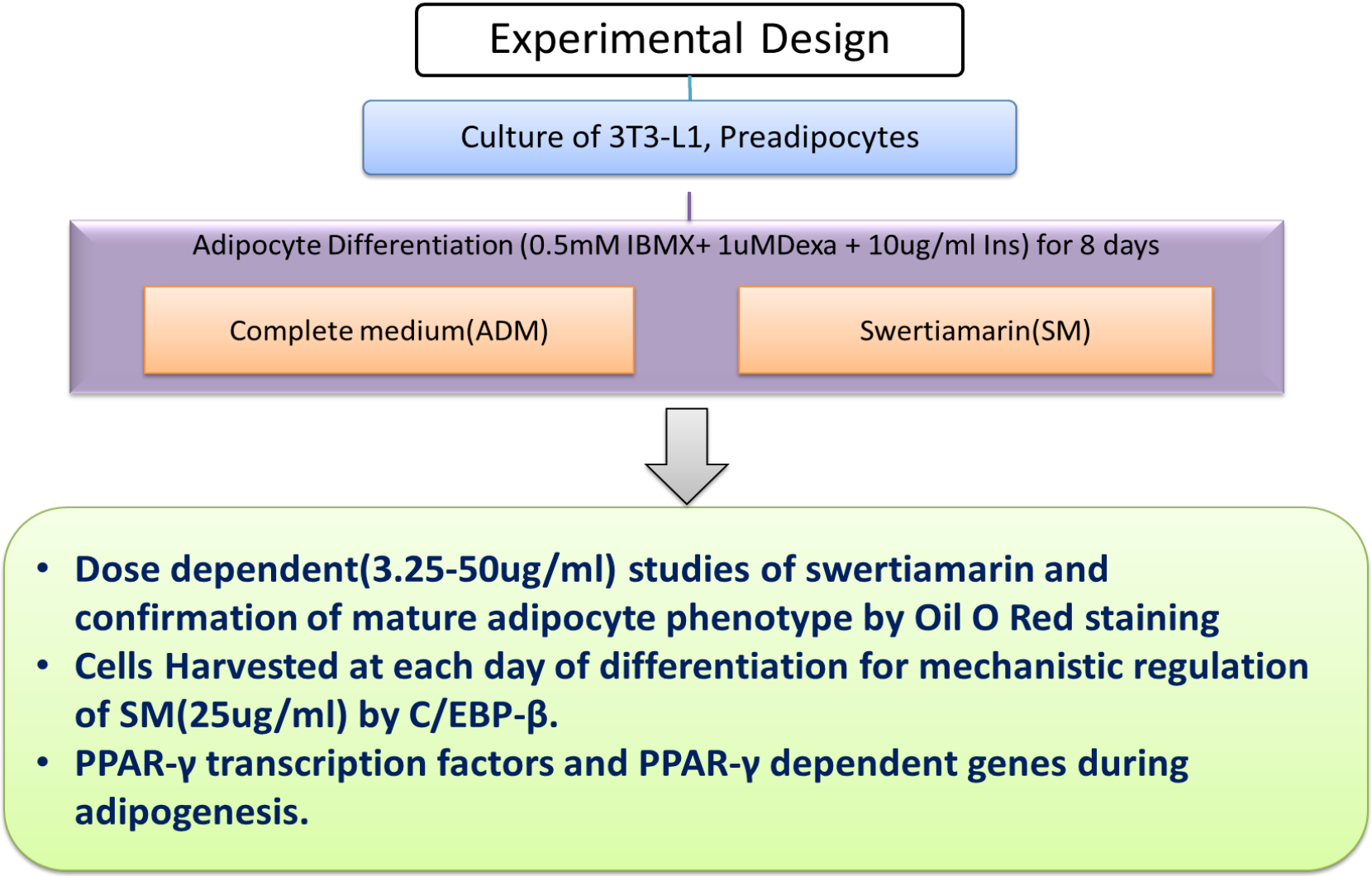
3T3-L1 cell line serves as one of the best-characterized and reliable *in vitro* model for studying the process of adipogenesis. Various stimulatory components are used in adipogenic cocktail wherein, maximum differentiation is obtained by combination of insulin and dexamethasone (elevates intracellular cAMP levels) that stimulates glucocorticoid receptor pathway, whereas, methylisobutylxanthine (IBMX), a cAMP-phosphodiesterase inhibitor, is traditionally used to stimulate the cAMP-dependent protein kinase pathway (Student et al. 1980). Insulin acts through the insulin-like growth factor 1 (IGF-1) receptor and hence, it can act as a substitute for insulin in the adipogenic cocktail (Smith et al. 1988). The mature adipocytes have an inherent ability to accumulate intracellular fat that leads to changes in the ultrastructural characteristics

that resembles to mammalian hypertrophic adipocyte (Green and Meuth 1974; Novikoff et al. 1980).

Apart from bariatric surgeries and liposuction as fat reducing therapy, various commercially marketed drugs like TZDs, GLP-1, cholestokinine etc, are used to decrease obesity and insulin resistance (Rodgers et al. 2012). But, due to adverse side effects like cardiovascular diseases, neurological interventions and (Hsu and Yen 2007) other fatal outcomes have limited the usage of these drugs (James et al. 2010). Hence, herbal compounds and their metabolites have come into limelight as an alternative safe approach for eradicating obesity mediated complications. These herbs have lipid inhibiting, better pharmacokinetic activity and no adverse or side effect. However, the mechanisms of action of these pharmaco-ingredients are still elusive (Shu 1998; He et al. 2010). Hsu and Yen indicated that phenolic compounds like rutin and O-coumaric acid efficiently inhibits adipogenesis and corrects fat metabolism (Hsu and Yen 2007).

Swertiamarin, an active and principal pharmacological compound of *Enicostemma littorale* is reported to have anti-diabetic and lipid lowering activity *in vivo* (Chapter 3) (Patel et al. 2013). Hence, this study was designed to delineate its effect at cellular and tissue specific level in order to ameliorate obese adipocyte. Results of chapters 3 and 4 showed that swertiamarin very lucidly ameliorated peripheral insulin resistance and with special reference to adipocytes it has been proved as a potent anti-obesity therapeutically viable compound. Hence, there it is necessary to understand how better is swertiamarin in inhibiting adipogenesis which leads to hyperplasia and causes insulin resistance and derailed fat metabolism. The present chapter 5 was designed with an aim to understand the anti-adipogenic activity on 3T3-L1 preadipocytes.

5.2 Experimental Design:



5.3 Material and Methods

5.3.1 Chemicals and media

All chemicals and reagents were procured as referred in Chapter 4C.3.1

5.3.2 Cell culture and adipocyte differentiation

3T3-L1 cell culture maintaince and adipocyte differentiation was carried out as per Hata et al,.2008 and detailed protocol is mentioned in Chapter 4C.3.2 & 3 (Hata et al. 2008). Cell were kept for two days after post-confluence and were differentiated by adiopogenic cocktail, insulin 10 µg/ml insulin, 0.5 mM IBMX and 1 µM dexamethasone. On day 4, complete media containing insulin was substituted till day 8. Differentiation was monitored by morphological assessment and Oil O Red staining. Anti adiopogenic activity was checked using (3.25 µg/ml to 50µg/ml) dose dependent studies (8 days) with swertiamarin .

5.3.3 Oil O Red staining and TG accumulation assay

Oil O Red staining was performed as mentioned in Chapter 4C.3.4, for visualizing lipid accumulation in adipocytes. For the quantification of the staining, the Oil O Red was extracted with 100% isopropanol from the cells and measured at 505 nm.

5.3.4 Isolation of total RNA and Semi-quantitative reverse transcriptase 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 4). 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.

5.3.5 Immunoblotting

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 4^0C overnight(Table 7). Blots were developed by ECL reagent(Chapter 4A.3.8).

5.3.6 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.

5.4 Results

5.4.1 Dose dependent anti-adiopogenic activity of swertiamarin

3T3-L1 preadipocytes were treated with ADM and after 4 days of treatment with ADM, the cells were treated with AMM for next 4 days (Chapter 4c). Dose dependent study with swertiamarin (3.25-50 µg/ml) was carried out with ADM and AMM treatment during adipocyte differentiation. Lipid accumulation was visualized and quantified by Oil O Red staining. The result suggest (Figure 5.1a & b) that swertiamarin was able to significantly (65%) inhibit adipogenesis at 25 µg/ml and more than that dose. Hence, in the present study final concentration of 25 ug/ml of swertiamarin was taken for efficient inhibition of adipogenesis.

5.4.2 Role of swertiamarin in transcriptional control of anti-adipogenesis

To explore adipogenesis inhibition with respect to transcriptional control, 3T3-L1 cells were divided into two groups, one with adiopogenic cocktail and other containing swertiamarin (25µg/ml) treated for all 8 days of adipogenesis.

The two major classes of transcriptional factors that are actively involved in adipocyte differentiation are C/EBPs and PPAR-γ. C/EBP-β and δ are the first wave of transcriptional factors during the initiation of adipogenesis whereas, C/EBP-α and PPAR-γ are the second wave of transcriptional factors appearing which are expressed at later stages. To monitor the adiopogenic process, gene and protein expression of C/EBP- β at the early phase and PPAR-γ at the late phase were analyzed for all eight days of adipogenesis. C/EBP-β gene expression starts after few hours of induction of differentiation which is a key player in commitment and

activation of late transcriptional factors. Swertiamarin treatment was able to reduce mRNA expression by 3 folds on second day and that of PPAR-γ by 2.5 fold after fourth day of adipocytes differentiation. Results found that that C/EBP-β and PPAR-γ protein expression levels showed significant difference between adipogenesis and SM treated group (Figure 5.2 & 3) in later period of differentiation.

5.4.3 Role of swertiamarin in mature adipocyte function genes

PPAR-γ is one of the key transcription factors involved in adipocyte gene expression. As adipocytes mature, they start expressing PPAR-γ2 dependent functional genes like CD36, LPL, Adiponectin and $aP_{2.}$ The expression is regulated by PPAR-γ2 promoter. SM treated group showed significant reduction in expression of PPAR-γ2 dependent gene expression of CD36, LPL, Adiponectin and aP_2 as compared to standard differentiation group. As PPAR-γ2 expression increases in later stage (4-8 days) of adipogenesis, all the dependent genes also demonstrated similar pattern and exhibited peak expression after 4 days. Swertiamarin treated group demonstrated same pattern but with much lower expression suggesting alterations in fat metabolism and TG accumulation as seen in figures (5.4a and b).

Figure 5.1

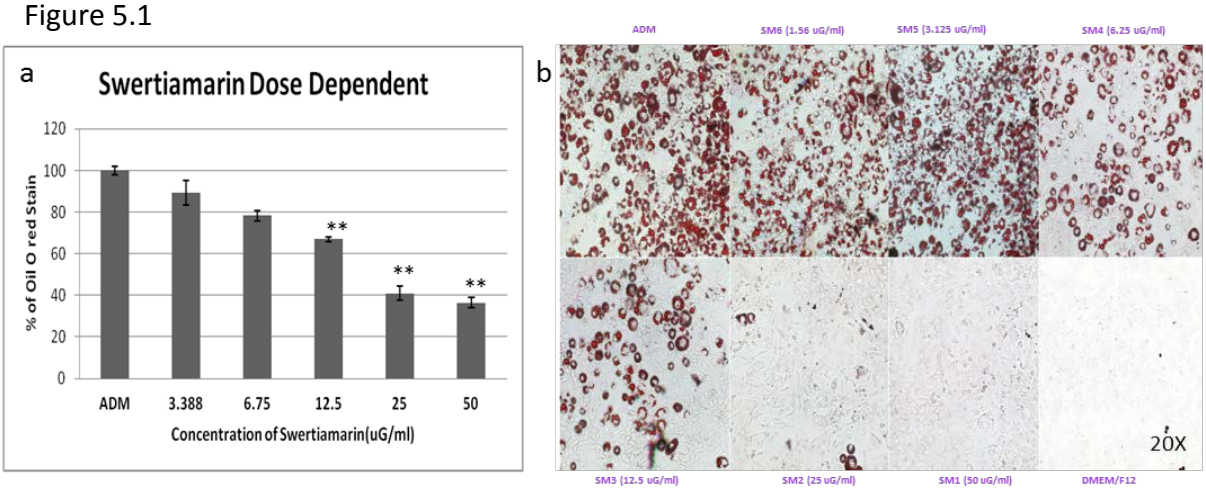


Figure 5.1 : Dose dependent studies of Swertiamarin in anti-adiopogenic phenotype as confirmed by Oil O Red staining. 3T3-L1 preadipocytes were subjected to adipocyte differentiation treated with Swertiamarin (3.35 -50 µG/ml)using adiopogenic cocktail for 8 days and were checked by the Oil O Red staining. (i) 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, n=3), (ii) Oil O Red staining under 20X magnification under Phase contrast microscope.

Figure 5.2a

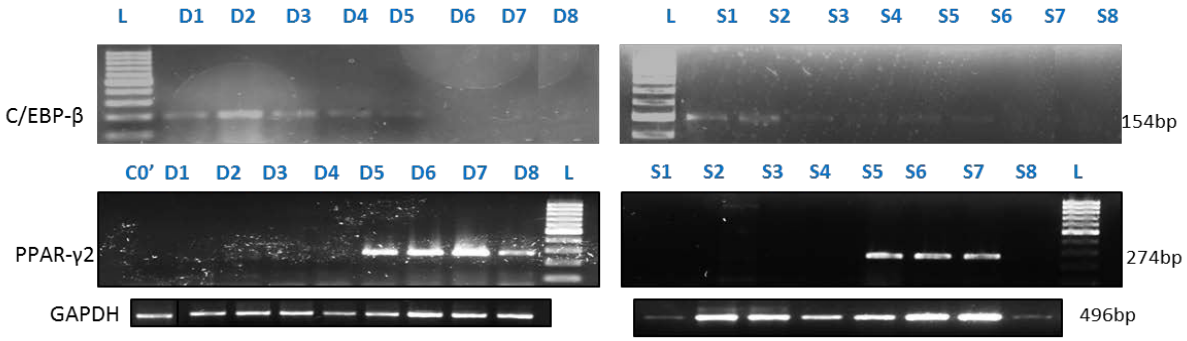


Figure 5.2b

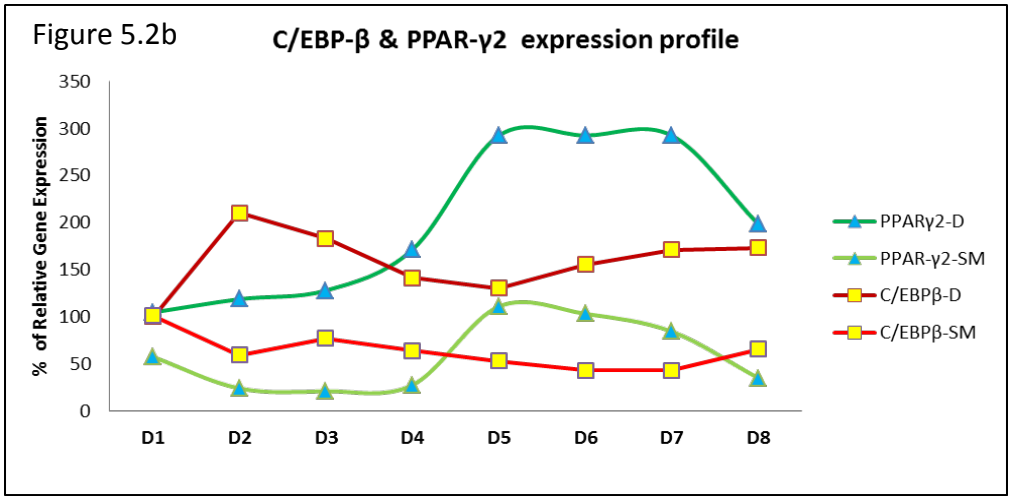


Figure 5.2: Treatment with adiopogenic cocktail and swertiamarin during adipogenesis (a) Gene expressions of C/EBP-β and PPAR-γ2 were checked using semi-quantitative RT-PCR. GAPDH taken as an internal control .
(b) Densitometric analysis of C/EBP-β and PPAR-γ2 expression profile in day dependent manner (1-8 Days). Data represented as % of relative genes expression were plotted against GAPDH, an internal control.

Figure 5.3

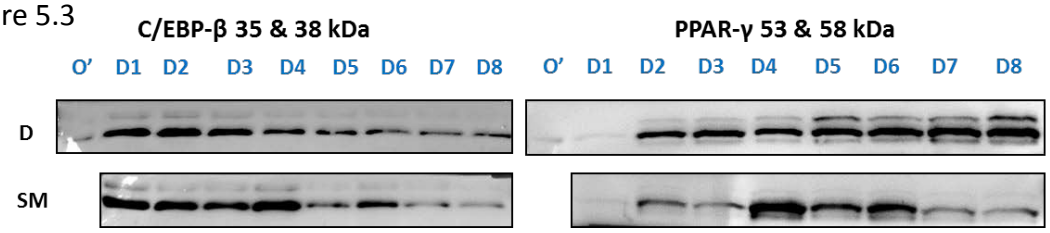


Figure 5.3 : Treatment with adiopogenic cocktail and swertiamarin during adipogenesis PPAR-γ and C/EBP-β protein expressions were examined by western blotting for all eight days (20 ug protein).

Figure 5.4a

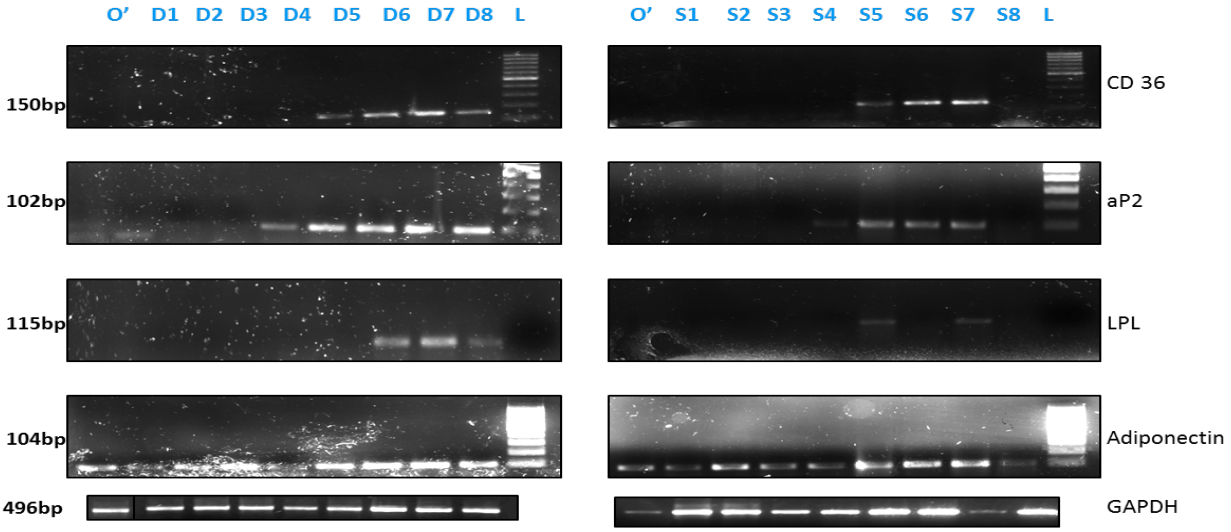


Figure 5.4a : Treatment with adiopogenic cocktail and swertiamarin during adipogenesis: Gene expressions of PPAR-γ2 dependent genes: CD36, aP2, LPL and adiponectin were checked using semi-quantitative RT-PCR. GAPDH taken as an internal control .

Figure 5.4b

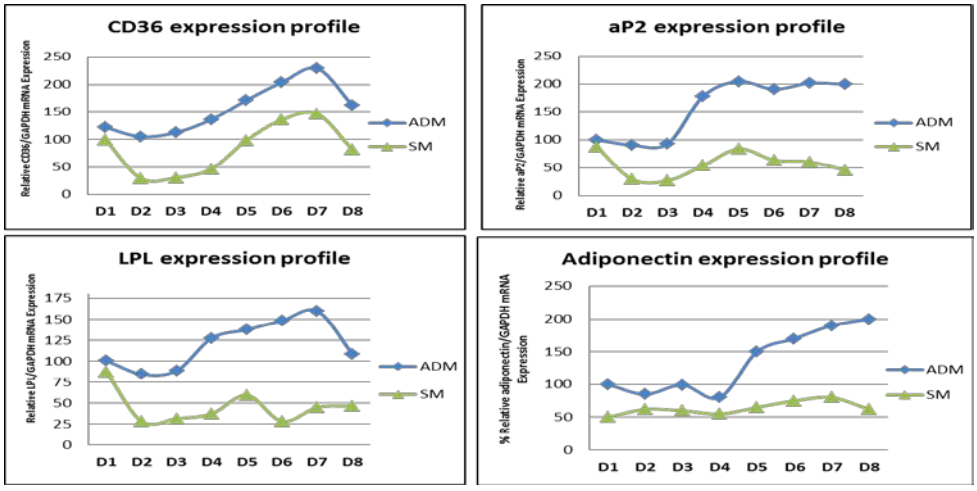


Figure 5.4b : Treatment with swertiamarin during adipogenesis and gene expressions of PPAR-γ2 dependent genes profile: CD36 ,aP2, LPL and adiponectin were checked using semi-quantitative RT-PCR. Densitometric analysis were done for all eight days of adipogenesis and % of relative expression were plotted against GAPDH as an internal control.

5.5 Discussion

Obesity in today's modern era is a serious health issue amongst urban population worldwide. Indians are now rapidly chasing the western population against obesity mediated complications like TIIDM (Misra et al. 2001; Misra and Khurana 2008; Pandya et al. 2011). In earlier chapter it has been well documented that mature adipocyte functionality is affected in insulin resistance condition leading to obesity.

The dose dependent study of swertiamarin on adipocytes showed significant reduction of triglycerides into the mature adipocytes. 25ug/ml swertiamarin was found to be an effective dose for anti-adipogenic activity in 3T3-L1 cells. Phenotypically, profoundly less adipocyte formation along with more of fibroblastic cells and less TG accumulation observed in mature adipocyte state that, this bitter glycoside reduces intracellular fat accumulating ability with decreased rate of adipogenesis.

The hormonal induction stimulated by adipogenic cocktail leads to activation of the transcriptional factors C/EBP-β and C/EBP-δ those initiating adipogensis further leading to harmonization of mitotic clonal expansion (Ramji and Foka 2002; Tang et al. 2003) as they direct the cell to re-enter the cell cycle and during their transit from resting phase to the synthesis phase, C/EBP-β is hyperphosphorylated and are activated by glycogen synthase kinase 3β (GSK3β) and MAPK (Tang et al. 2003; Tang et al. 2005), they were found to be consistently expressed and then reduced by treatment with swertiamarin till the fourth day of differentiation.

Various research groups in the past few decades highlighted the role of PPAR-γ in regulation of obesity and adipocyte differentiation (Chien et al. 2005; Kim et al. 2008; Oben et al. 2008). Further, PPAR-γ agonists and antagonists of synthetic or herbal origin have gained wide commercial popularity as therapeutic agents. Another transcription factor, SREBP-1c, regulates downstream cascade of fat metabolism by controlling the endogenous production of ligands for PPAR-γ2 (Kim and Spiegelman 1996; Brown and Goldstein 1997) and hence, higher expression of SREBP1c contributes to up-regulation of PPAR-γ2 and its downstream adipogenic factors (Kim et al. 2008). Herbal extracts of Petasites japonicas (Watanabe et al. 2010) and Momordica charantia (Nerurkar et al. 2010) appear to exert their anti-obesity potential via regulation of

PPAR-γ2 expression. The reported study noted significant up regulation of mRNA expression of both these transcriptional factors' in obese mice (Lee et al. 2010; Watanabe et al. 2010). The mRNA expression profile of PPAR-γ that started appearing on the 2nd day of adipogensis and persisted till the end of the process was markedly downregulated from 5th day of adipogensis till the end of the maturation phase. Hence, it can be inferred that swertiamarin culminated PPAR-γ 's level in differentiation and maturation stage and arrested clonal expansion and the mitogenic stimulus (Tang et al. 2003).

Along with PPAR-γ2, C/EBP-α also rises and enhances insulin sensitivity and uptake of fatty acids into the cells (Tontonoz et al. 1994), that is evidently seen by increased expression of the major fatty acid transporter CD36. It can also be presumed that activation of these transcriptional factors drastically activates the genes involved in fatty acid synthesis and fat metabolism. FAS, ACC-1, aP2, LPL and adiponectin are the major maturation markers of adipogensis. The expression profile of the genes playing role during adipogensis showed marked reduction in their levels when treated with swertiamarin. Reduced levels of FAS and ACC-1 decreased formation of adipocyte and lipogenesis, thus preventing hyperplasia and adipose tissue obesity (Schoonjans et al. 1996; Way et al. 2001; Lee et al. 2008).

Swertiamarin treatment reduced the expressions of adiponectin throughout adipogenesis which, not only prevents increase in cell mass but also the functionality of differentiated cells. Insulin stimulated glucose uptake in adipocytes is governed by mature Glut4 translocation, whose transcription factor is comprised of response elements that are regulated by C/EBP-α and PPAR-γ (Ayala-Sumuano et al. 2013). Hence, inhibition of these transcriptional factors hampers the functionality of Glut4 transporter and its translocation.

In conclusion **s**wertiamarin significantly curtailed expansion of adipocytes by controlling the activity of the master transcriptional regulator, PPAR-γ2 transcription and its target genes responsible for lipogenesis and maturation of adipocytes. Anti adiopogenic efficacy of swertiamarin thus, can be explored to develop a novel anti-obesity drug.

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