Chapter 3

Sida rhomboidea.Roxb leaf extract down-regulates expression of PPARγ and leptin genes in high fat diet fed C57BL/6J mice and retards *in vitro* 3T3L1 pre-adipocyte differentiation

The focus of this study is on the mRNA expression PPARγ-2, SREBP1c, CPT-1, FAS and LEP in the epididymal adipose tissue of a high fat diet fed C57BL/6J mice and, the efficacy of SR in controlling *in vitro* adipocyte differentiation and LEP release.

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INTRODUCTION

Leading onto this century, the world has witnessed a polarity trend of of diagonally opposite trends in terms of human health and nutritional perspectives. Malnutrition and obesity are equally prevalent current problems with 1 billion people suffering from extreme poverty and malnourishment and close to 1 billion affected by significant overweight caused by environmental factors and/or inheritance. Obesity, a fast spreading epidemic, is a major contributor to the global burden of chronic disease and disability. Currently, more than one billion adults worldwide are overweight and at least, 300 million of them are clinically obese (WHO, 2003). Induction of obesity in humans is either 'genetic' or 'lifestyle' related. The latter is however, a complex intermix of sedentary lifestyle and a high calorie diet amounting to nutritional overload (Barsh *et al.*, 2000, Bråkenhielm *et al.*, 2004).

Obesity starts to develop in humans and mammal as the dietary intake starts exceeding energy expenditure. Obesity, best defined as a condition of accumulation of fat in various tissues, result in specific health disorders and increased mortality. Excessive body weight generally predisposes individuals to various diseases, particularly cardiovascular diseases such as atherosclerosis, type II diabetes mellitus, sleep apnea, osteoarthritis, reduced fertility and many more. Apart from being an individual clinical condition, obesity also poses as a serious public health problem (Bonadonna *et al.*, 1990, Rexrode *et al.*, 1996). A balanced adipose tissue homeostasis is of great importance, which includes a well-regulated synthesis of TG in the postprandial state (lipogenic pathway) and degradation of TG during food deprivation (lipolytic pathway) with both processes required to be in a state of physiological equilibrium. The necessity to screen natural/herbal products to treat obesity has

become the need of the hour as the available synthetic anti-obesity drugs have not only proven to be costly but also untenable due to the undesirable side effects manifested by most of them (Yun, 2010). Many traditional herbal preparations in recent times have therefore been scrutinized to explore their anti-obesity potential and the underlying mechanism of action (Ahn *et al.*, 2006). The present study in this context evaluates the effects of SR on the expression of genes associated with adipogenesis, lipolysis and lipogenesis in HFD fed C57BL/6J mice. The focus of this study is on the mRNA expression PPARγ-2, SREBP1c, CPT-1, FAS and LEP in the epididymal adipose tissue of a high fat diet fed C57BL/6J mice and, the efficacy of SR in controlling *in vitro* adipocyte differentiation and LEP release.

MATERIALS AND METHODS

Plant material and preparation of extract: - as mentioned in chapter 1

Experimental animals: - as mentioned in chapter 2

High fat diet induced obesity in C57BL/6J mice

A total of 18 mice were randomly allocated to 3 groups of 6 animals each.

- Group I (LEAN) consisted of mice fed with low fat diet (regular fat diet).
- Group II (Obese; OB) consisted of mice fed with high fat diet (Chapter 2).
- Group III (OB+SR) consisted of mice fed with high fat diet containing 1% SR (Chapters 2; Thounaojam *et al.*, 2010c).

All animals were fed with their respective diets for 20 weeks. At the end of the experimental period, overnight fasted animals were given mild ether anesthesia and blood was collected by retro orbital sinus puncture in EDTA coated vials. Plasma was obtained by cold centrifugation (4°C) of the vials for 10 min at 3000 rpm. Later, animals were sacrificed by cervical dislocation under mild ether anesthesia and

various fat pads were excised and, the epididymal fat pad was stored in RNAlater solution (Ambion) until analysis at -80°C (Cryo Scientific Ltd, India).

Plasma lipid profile: as mentioned in chapter 1.

Estimation of plasma leptin

Plasma leptin was assayed using anti-mouse monoclonal antibody coated 96 microtiter plate as per the instructions of the manufacturer (KRISHGEN Biosystems, Ltd) by bringing all the reagents to room temperature. 100 µl/well of standards (31.2, 62.5, 125, 250, 500, 1000 or 2000 pg/ml) or samples were added to the 96 well coated plates. The plate was aspirated and washed 4 times with wash buffer and blot residual buffer by firmly tapping plate upside down on absorbent paper. All subsequent washes were performed similarly. 100 µl of biotin antibody solution was added to each well and plate was sealed and incubated at room temperature for 2 hr with shaking. The plate was aspirated and washed 4 times with wash buffer as mention earlier. Streptavidin-HRP conjugate was diluted (1:1000) and 100 µl of diluted streptavidin-HRP solution was added to each well. Plate was sealed and incubated at room temperature for 30 min with shaking. Again the plate was aspirated, washed 4 times and 100 µl freshly mixed TMB substrate solution was then added followed by incubation in the dark for 15 min. The reaction was stopped by adding 100 µl of 2N H_2SO_4 to each well. Positive wells changed color from blue to yellow. The absorbance was read at 450nm within 30 min of stopping reaction.

Isolation of total RNA from epididymal adipose tissue

Total RNA was isolated from the epididymal fat pad of control and experimental mice using Tri-reagent (Sigma Aldrich, USA). Quantity and quality of isolated RNA were assessed using nanodrop spectrophotometer (Thermo scientific, Ltd) and samples with

a ration of A_{260}/A_{280} >1.9 was processed for cDNA synthesis. A lobe of epididymal fat pad from each experimental group was homogenized in 1 ml of TRI Reagent solution using liquid nitrogen and allowed to stand at room temperature for 5 min. At the end of incubation, vials were centrifuged at 12,000 x g for 10 min at 4°C and the lower aqueous phase was transferred to a fresh tube. Later, 500 µl of chloroform was added, tubes were incubated at room temperature for 10 min and centrifuged at 12,000 x g for 8 min at 4°C and aqueous phase was separated in a fresh centrifuge tube. Later, 500 µl of isopropanol was added, incubated at room temp for 5–10 min and centrifuged at 12,000 x g for 10 min at 4°C. Precipitated RNA forms a gel-like or white pellet on the side and bottom of the tube. 1 ml of 75% ethanol was added to each sample to wash the RNA pellets and centrifuged at 7,500 x g for 5 min at 4°C and ethanol removed without disturbing the pellet and RNA pellets were air dried for 3-5 min. The concentration of RNA in solution was determined by measuring its absorbance at 260 nm using Nano Drop spectrophotometer. To determine the RNA concentration in µg/ml, the A260 was multiplied by the dilution factor and the extinction coefficient (1 A260= 40 µg RNA/ml).

Total RNA = A260 X dilution factor X 40 = µg RNA/ml

Analysis of gene expression by quantitative RT- PCR (qPCR)

Complementary DNA (cDNA) synthesis was carried out using Omniscript cDNA synthesis kit (Qiagen, USA). A reaction mixture of 20 µl contained, 2 µg total RNA, 10 × RT buffer, dNTP mixture (5mm each), 10 × random hexamer, RNase inhibitor (10 U/µl), Omniscript RT (4 U/rxn) and RNase free water. The cDNA synthesis was carried out at 37°C for 1 hr using a Veriti 96 well thermal cycler (Applied Biosystems, USA). Real-time PCR assays were performed in 96-well plates in ABI

7500 Fast real-time PCR machine (Applied Biosystems, USA). Primer sequences for qPCR analysis are shown in Table.1. Syber Green reaction mixture of 20 µl contained 10µl Quantifast Syber green master mix (Qiagen, USA), 2 µl template, 1 µl of each primer and 6µl nuclease free water. The following two steps thermal cycling profile was used for qPCR analysis; Step I (cycling step): 95°C for 10 min, 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec for 40 cycles and step II (Melt Curve step): 60°C for 15 sec, 60°C 1 min and 95°C for 30 sec.

Table. 1 Primer sequence and list of genes used for qPCR analysis

Accession Number	Gene	Forward primer/ Reverse primer	Product length
NM_011146	**PPARγ2**	5'TCACAAGAGCTGACCCAATG3' 5'GCATCCTTCACAAGCATGAA3'	230 bp
NM_011480	**SREBP-1c**	5'GATCAAAGAGGAGCCAGTGC3' 5'TAGATGGTGGCTGCTGAGTG3'	191 bp
NM_007988	**FAS**	5'GGGTCTATGCCACGATT 3' 5'CACAGGGACCGAGTAATG3'	217 bp
NM_013495	**CPT-1**	5'CTCAGTGGGAGCGACTCTTCA3' 5'GGCCTCTGTGGTACACGACAA3'	105 bp
NM_008493	**LEPTIN**	5'GACACCAAAACCCTCAT3' 5'CAGAGTCTGGTCCATCT3'	150 bp
NM_008084	**GAPDH**	5'AGGCCGGTGCTGAGTATGT3' 5'GTGGTTCACACCCATCACAA3'	146 bp

The fold change in the expression of genes mentioned herein was done by normalizing the values of threshold cycle (CT) of target gene with the CT value of Housekeeping gene (GAPDH) so as to obtain the ΔCT value. These values were further normalized with ΔCT values of CON ($\Delta CT_{OB \text{ or } OB+SR} - \Delta CT_{LEAN}$) so as to obtain ΔΔCT values. The fold change in expression was then obtained as log2 – ΔΔCT (Kumar *et al.*, 2010).

2.8. Microscopic and morphometric examination of epididymal fat pad

Epididymal fat pad was fixed in 4% buffered paraformaldehyde, dehydrated in graded alcohol series and embedded in paraffin wax using automated tissue processor. Five µm sections stained in hematoxyline-eosin were observed under a Leica microscope. Photographs of adipocyte were taken with a Canon power shot S70 digital Camera at 400 X magnification and adipocyte number per 1000 mm was calculated using image analysis software.

Maintenance of 3T3L1 cells

3T3-L1 mouse pre-adipocytes (Obtained from National Centre for Cell Sciences, Pune, India) were maintained in DMEM containing 10% FBS (Himedia Pvt ltd, Mumbai, India) and 1% antibiotic-antimycotic solution (10X; (Himedia Pvt ltd, Mumbai, India) and sub cultured every 3rd day using 0.25 % trypsin-EDTA solution (Himedia Pvt ltd, Mumbai, India).

In vitro Cytotoxicity assay

Pre-confluent pre-adipocytes (5.0 X 10^3cells /well) were maintained in 96 well plates (Tarson India Pvt Ltd) for 72 hr in presence of SR (10-1000µg/ml) or vehicle (0.9 % Nacl). At the end of incubation period, 10 µl of MTT (5 mg/ml) was added to wells and the plates were incubated at 37°C for 4 h. At the end of incubation, culture media were discarded and the wells washed with phosphate buffer saline (Himedia Pvt Ltd, Mumbai, India). 150 µl of DMSO was added to all the wells and incubated for 30 min. Absorbance was read at 540 nm in ELX800 Universal Microplate Reader (Bio-Tek instruments, Inc, Winooski, VT) and % cell viability was calculated (Chapter 3).

In vitro adipocyte differentiation protocol

In vitro adipocyte differentiation was carried out as per the protocol of Hata *et al.* (2008) with minor modifications. Freshly sub cultured cells were seeded on 12 well cell culture plates at the density of 1.0X 10^5cell/ well in DMEM containing 10 % FBS and allowed to become confluent. Cells were maintained for 2 days in confluent stage (to arrest cell division). Later (at day 0), culture media were replaced with DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine (Sigma Aldrich, USA), 0.25 µM dexamethosone (Sigma Aldrich, USA), and 10 µg/ml insulin (Sigma Aldrich, USA) and cells were maintained for 4 days. At the end of 4 days, culture media were replaced with maturation media containing complete DMEM with 10 µg/ml insulin and the cells were maintained for another 8 days; subsequently media were replaced every 48 hours until the end of the experiment.

Qualitative and quantitative analysis of adipocyte differentiation

3T3-L1 pre-adipocytes were differentiated as describe above in presence or absence of SR (10-200µg/ml). At the end of the 12^{th} day, ORO staining for adipocyte lipid accumulation was performed as mentioned in Chapter. 3. At the end of incubation, cells were washed twice with PBS and fixed in a 4% buffered paraformaldehyde for 10 min, washed twice with Milli Q water (Millipore India Pvt Ltd) and then stained using 0.5% ORO for 15 min at room temperature. Excess ORO dye was washed with Milli Q water and photographs were taken in Leica DMIL inverted microscope using Canon power shot S70 digital camera.

In another set of experiment, the stained adipocytes were treated with 100% isopropanol (to extract intracellular oil red O stain) and then the absorbance (Optical density; OD) of the extracts was measured at 490 nm. Reagent blank and cell blank

assays were also performed simultaneously to minimize non-specific staining. The difference in absorbance between cells with and without ORO dye was calculated. Percentage adipogenesis was calculated as: OD of treated cells ÷ OD of untreated cells X 100.

Leptin release and triglyceride accumulation assays

3T3-L1 pre-adipocytes were differentiated as described above in presence of SR (10-200μg/ml) or vehicle (0.9 % Nacl) and LEP and TG contents were assayed in the supernatant and the cells respectively. On day 12, supernatant from each well was collected and LEP content was analyzed using mouse specific LEP ELISA kit (Krishgen, Biosystems) as per the instructions of the manufacturer. After removal of supernatants, cells were washed twice with PBS and solublized in 100 μl of 1% Triton X 100 (in PBS) and, assayed for total TG using commercially available enzymatic kit (Reckon Diagnostics, Baroda, India) using Merck Micro lab L300 Semi-autoanalyzer. Results were expressed as % TG.

Glycerol release assay

3T3-L1 pre-adipocytes were differentiated as described above for 12 days. For glycerol release assay, differentiated adipocytes were incubated with SR (10-200μg/ml) or vehicle (0.9 % NaCl) for 48 hr. At the end of incubation, supernatant was collected from each well and glycerol content determined by the method of Sturgeon *et al.* (1979)`.

Glyceraldehyde-3-phosphate dehydrogenase activity assay

3T3-L1 pre-adipocytes were differentiated as described above in presence of SR (10-200μg/ml) or vehicle (0.9 % NaCl). On day 12 after removal of supernatants, cells were washed twice with ice-cold PBS and lysed in Tris-EDTA buffer (25 mM Tris/1

mM EDTA, pH 7.5) and G3PDH activity was determined according to the procedure of Wise and Green. (1979). Protein content in the cell lysate was determined by the method of Lowry *et al.* (1951).

Statistical analysis

Statistical evaluation of the data was done by one way ANOVA followed by Bonferroni's multiple comparison test .The results were expressed as mean ± S.E.M using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California USA.

RESULTS

Bodyweight gain and food intake

As shown in Figure.1, HFD fed OB mice recorded significant weight gain after 20 weeks compared to the lean control. However, HFD induced weight gain was significantly controlled by SR supplementation of OB mice. There was also time dependent decrement in food intake in SR supplemented OB mice (Figure.1).

Plasma lipids, leptin and fat pad weight

As shown in Table 2, OB mice recorded significant increase in plasma TG, FFA and LEP compared to lean mice while, SR supplementation to HFD fed mice resulted in minimal increment in plasma TG, FFA and LEP compared to OB mice (Table 1). As shown in Figure.2, visceral adiposity was evident in the form of increased fat pad weights (Renal, abdominal and epididymal) in OB mice after 20 weeks of HFD feeding (Figure.3). OB mice supplemented with SR extract showed significant attenuation in HFD induced visceral adiposity and increment in fat pad weights (Figures. 2 & 3).

Microscopic and morphometric evaluation of epididymal fat pad

Microscopic evaluation of epididymal fat pad of OB mice recorded adipocyte hypertrophy that was marked by significant reduction in adipocyte number per 1000mm area (Figure. 4). However, OB+SR mice recorded significantly higher number of adipocytes per 1000mm area.

Quantitative RT PCR analysis

HFD fed OB mice recorded significantly higher levels of mRNA expression for PPARγ, SREBP1c, FAS and LEP, while CPT-1 expression was significantly lowered compared to lean mice. However, these set of changes were significantly prevented in SR supplemented OB mice (Figure. 7).

Cytotoxicity assay

Cytotoxicity analysis of SR in pre adipocyte cells registered non significant alteration in the cell viability in the dose range of 10-1000 μg/ml (Data not shown).

Qualitative and quantitative analysis of adipocyte differentiation

ORO staining of differentiated adipocytes at the end of 12 days revealed significant cytoplasmic lipid accumulation in the untreated differentiated adipocytes whereas, SR extract supplementation to differentiating 3T3L1 pre-adipocytes significantly reduced adipocyte differentiation, characterized by lesser cytoplasmic lipid accumulation (Figures. 8 B-F). Quantitative analysis of ORO staining recorded 30 % to 75 % inhibition in adipogenesis in the SR supplemented 3T3L1 cells.

Trigyceride accumulation and leptin release in differentiated adipocytes.

Figures. 9A & 9B show TG accumulation and LEP release in the untreated and SR supplemented differentiated adipocytes at the end of 12 days. Whereas, untreated adipocytes showed higher levels of cellular TG accumulation and LEP release

compared to pre-adipocytes (Figures. 9A & 9B), SR supplementation to differentiating adipocytes recorded significant decrement in the cellular TG accumulation and LEP release compared to differentiated untreated adipocytes (Figures. 7A & 7B).

Glycerol release and G3PDH activity assay

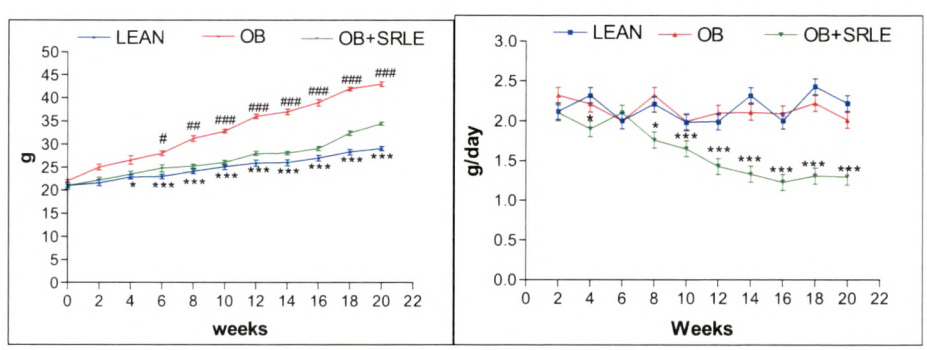
SR supplementation to differentiating pre-adopocytes resulted in higher indices of glycerol release and lowered cellular G3PDH activity compared to untreated differentiated adipocytes (Figures. 9C & 9D)

Table. 2 Effect of *S.rhomboidea.* Roxb extract on plasma triglycerides, free fatty acids, leptin and lee index.

	LEAN	OB	OB+SR
Plasma			
Triglycerides (mmol/l)	0.53±0.02	2.16±0.05 $^{###}$	0.61±0.04 ***
Free fatty acids (mmol/l)	1.80±0.07	4.68±0.09 $^{###}$	2.33±0.08 ***
Leptin (ng/l)	12.00±1.98	46.24±2.13 $^{###}$	25.01±1.89 ***
Lee index $\sqrt[3]{\frac{\text{Body weight} \times 10}{\text{naso-anal length}}}$	2.88±0.06	5.00±0.05 $^{###}$	2.98±0.06 ***

Results are expressed as means ± S.E.M., n = 6. Where, $^{\#}$ P < 0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with LEAN, *P < 0.05, **P<0.01 and ***P<0.001 and ^{ns} non significant compared with OB.

Figure 1. Effect of *S.rhomboidea.*Roxb leaf extract feeding on (A) body weight gain and (B) food intake.



Results are expressed as means ± S.E.M., n = 6. Where, $^{\#}$ P < 0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 and ^{NS} non significant compared with LEAN, *P < 0.05, **P<0.01 and ***P<0.001 and ^{ns} non significant compared with OB

Figure. 2 Morphological and anatomic evaluation of visceral adiposity in Lean (A & D), OB (B & E) and OB+SR (C & F) groups.

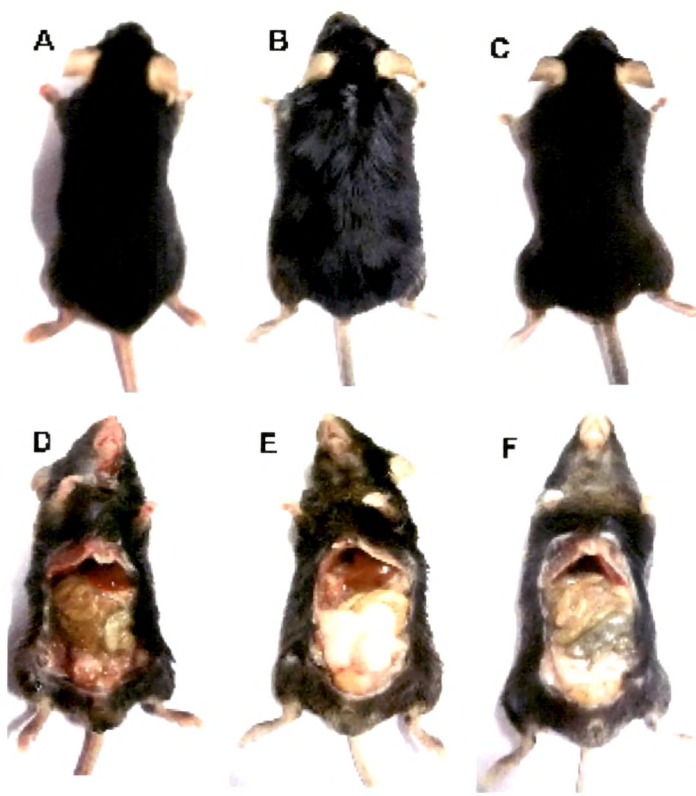


Figure.3 Effect of *S.rhomboidea*.Roxb leaf extract feeding on morphological and anatomic evaluation of visceral adiposity in Lean (A & D), OB (B & E) and OB+SR (C & F) groups and abdominal, epididymal and perirenal fat pad weights.

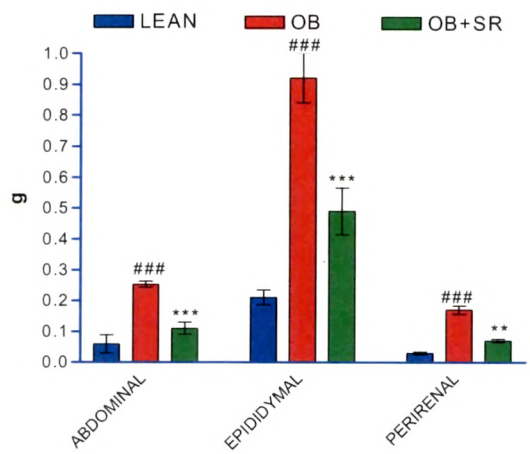
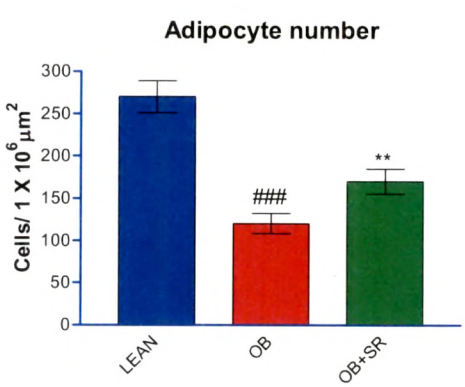
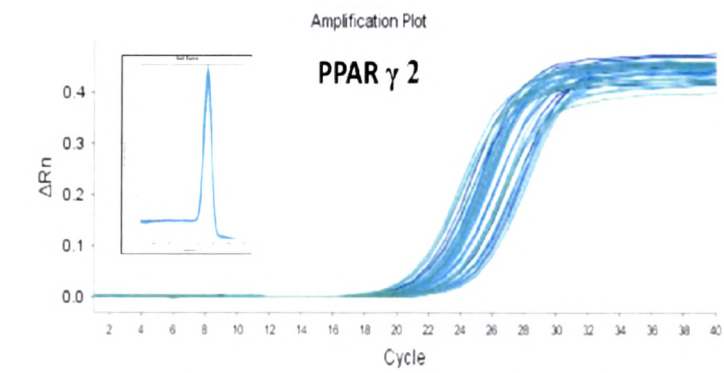


Figure.4 Effect of *S.rhomboidea*.Roxb leaf extract feeding on adipocyte number.



Results are expressed as means ± S.E.M., n = 6. Where, $^{\#}$ P < 0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 and ^{NS} non significant compared with LEAN, *P < 0.05, **P<0.01 and ***P<0.001 and ^{ns} non significant compared with OB.

Figure. 5 Amplification plot and melting curve for PPARγ2 and SREBP1C during qPCR analysis.



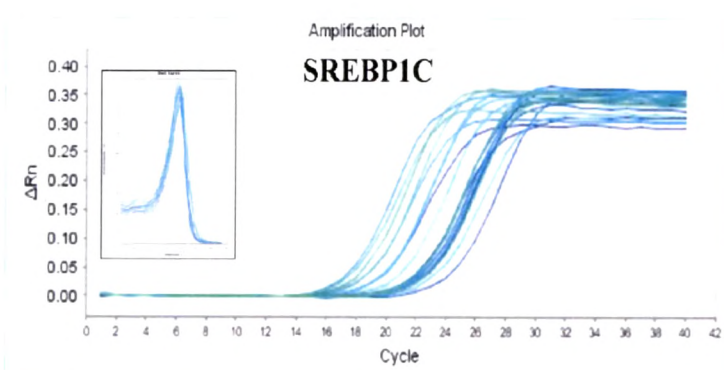


Figure. 6 Amplification plot and melting curve for FAS,CPT-1 and leptin during qPCR analysis.

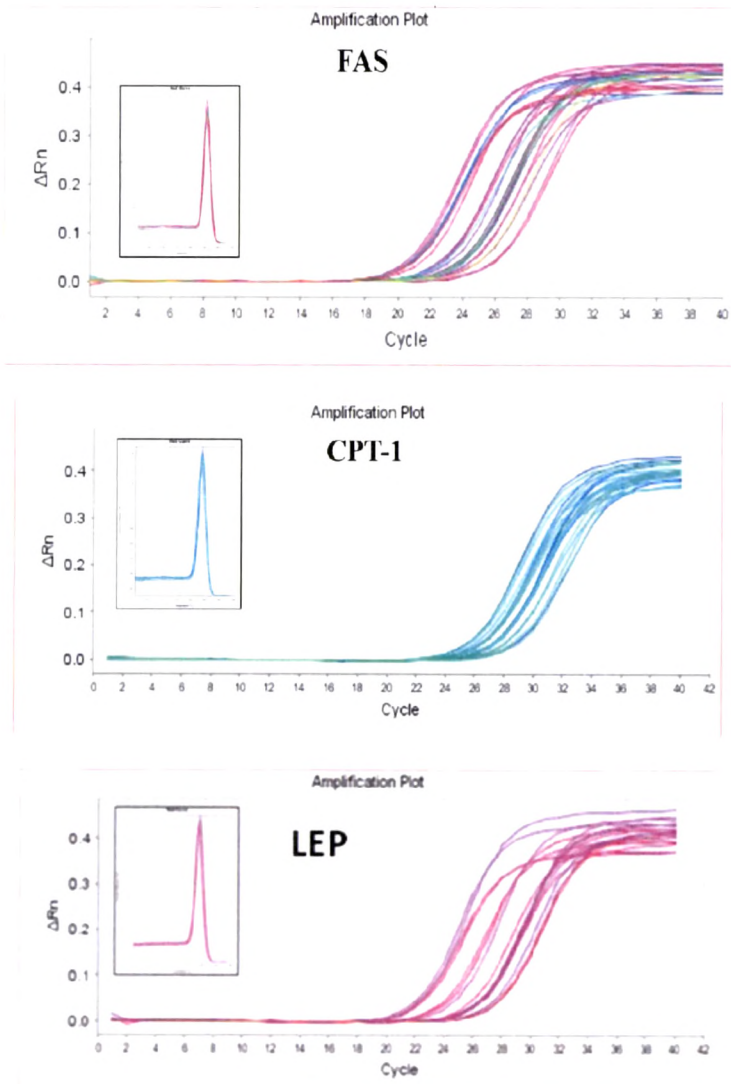
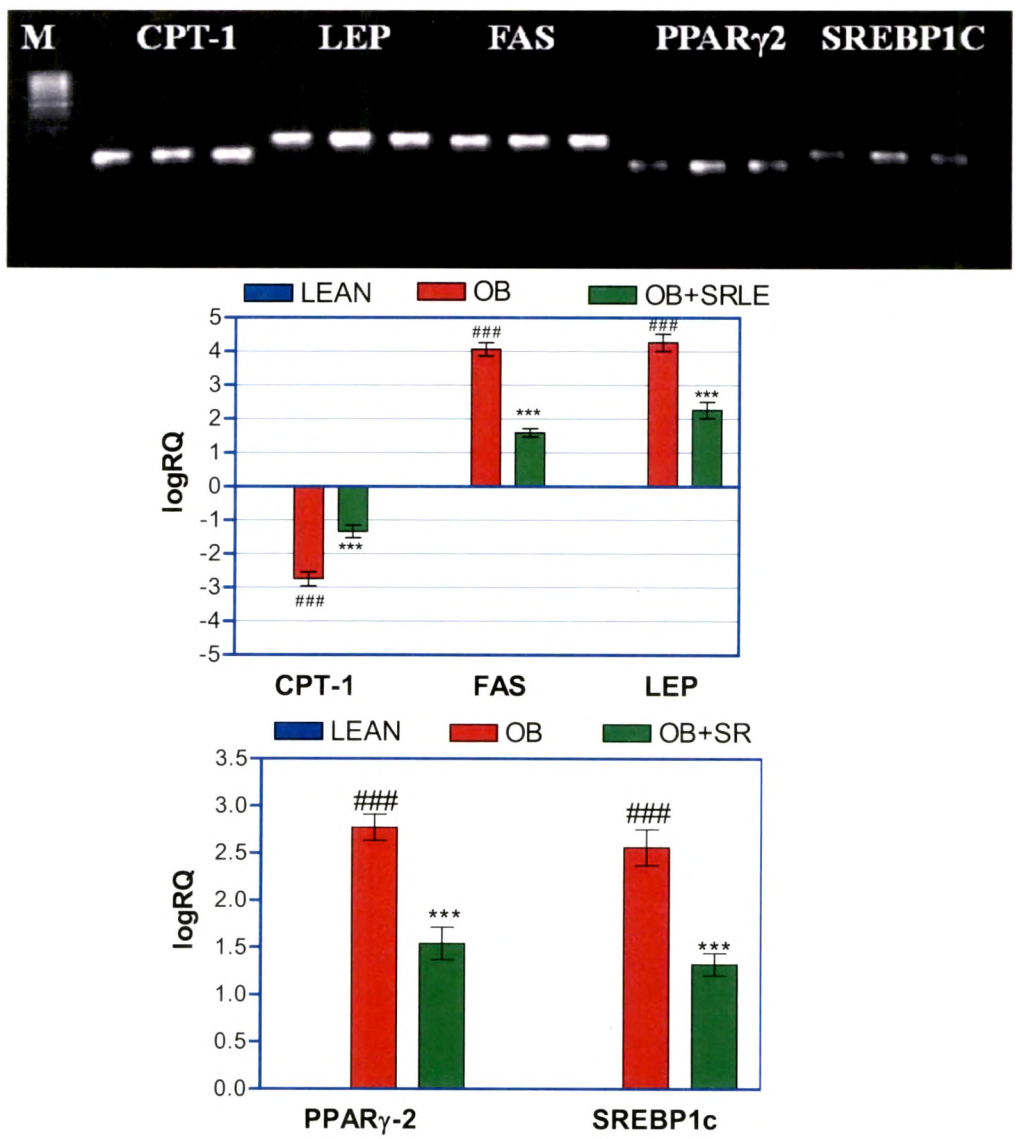
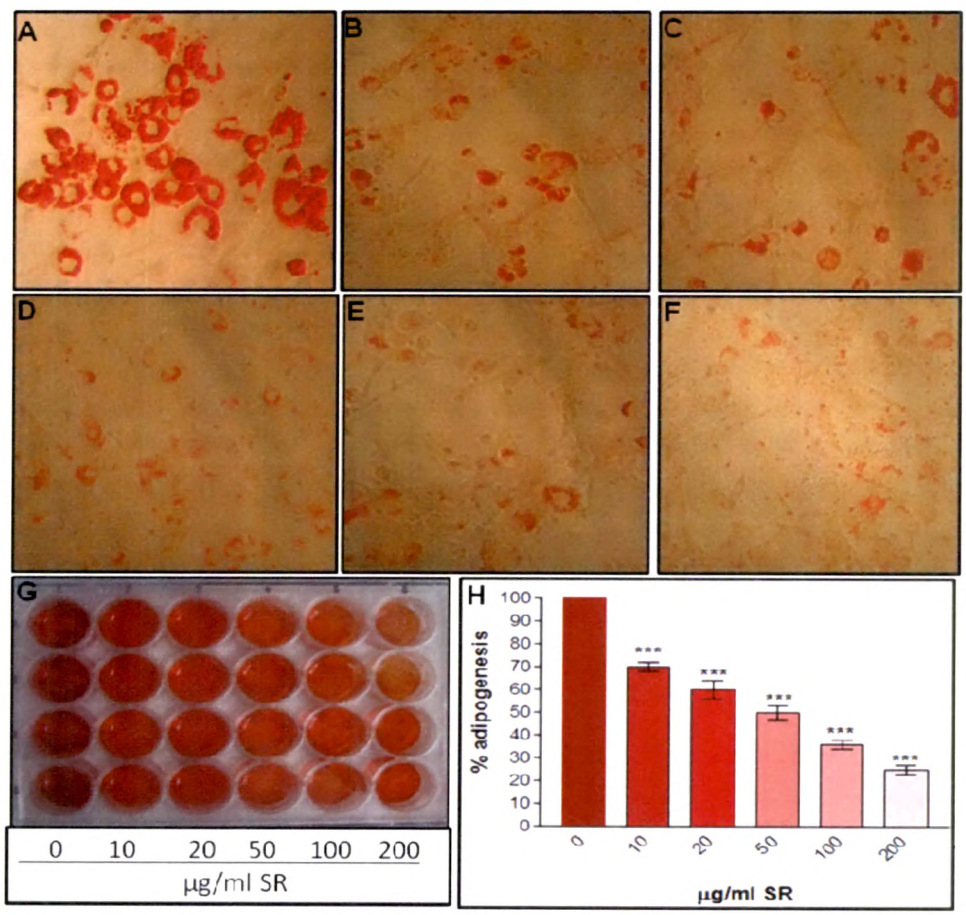


Figure.7 Effect of *S.rhomboidea*.Roxb leaf extract feeding on quantitative RT-PCR analysis of PPARγ2 and SREBP1c, CPT-1 FAS and LEP mRNA expression.



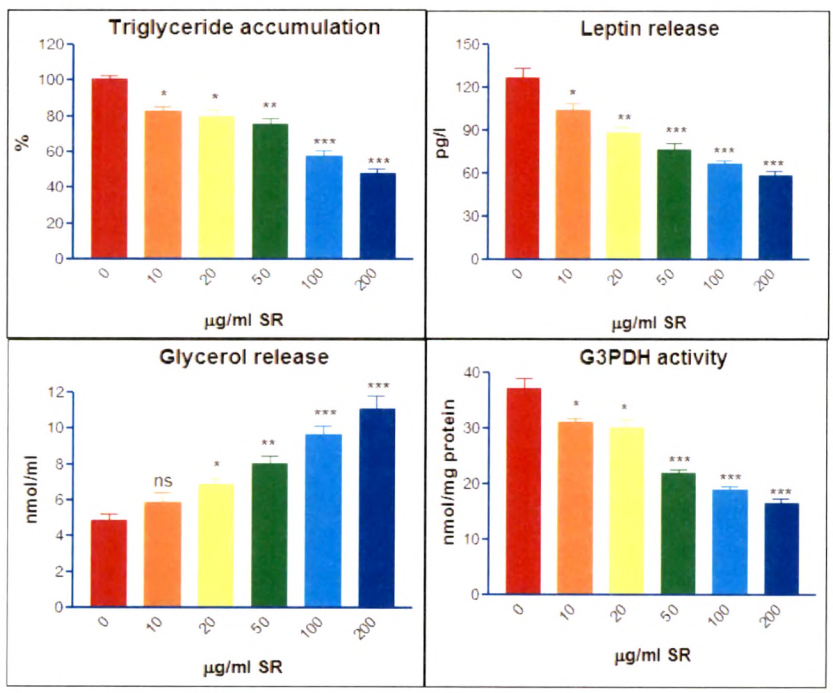
Results are expressed as means ± S.E.M., n = 6. Where, $^{\#}$ P < 0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 and ^{NS} non significant compared with LEAN, *P < 0.05, **P<0.01 and ***P<0.001 and ^{ns} non significant compared with OB.

Figure. 8 Photomicrograph of oil red o stained differentiating 3T3L1 cells (A) untreated (B) treated with 10µg/ml SR, (C) 20 µg/ml SR, (D) 50 µg/ml SR, (E) 100 µg/ml SR and (F) 200 µg/ml SR and effect of SR extract on adipogenesis (G & H).



Results are expressed as means ± S.E.M., n = 3.Where, $^{*}P < 0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ and ^{ns} non significant compared 0 µg/ml SR.

Figure.9 Effect of *S. rhomboidea*.Roxb extract on *in vitro* (A) leptin release, (B) triglyceride accumulation, (C) glycerol release and (D) G3PDH activity.



Results are expressed as means ± S.E.M., n = 3.Where, $^{*}P < 0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ and ^{ns} non significant compared 0 µg/ml SR.

DISCUSSION

The onset and progression of HFD induced experimental obesity in BL6 mice have physiological resemblance to high calorie diet induced obesity in humans (West and Kalbfleisch, 1971). The high fat diet induced obesity in BL6 mouse characterized by selective deposition of fat in the mesentery, is comparable to visceral adiposity in humans (Rebuffé-Scrive *et al.*, 1993; Surwit *et al.*, 1995). Hence, this experimental model is widely used to investigate anti-obesity potential of herbal formulations (Kim *et al.*, 2008; Hong *et al.*, 2009). Significant reduction in body weight gain and food intake caused due to SR supplementation of HFD fed mice recorded in the present study are in agreement with our previous observations (Chapter.2). SR induced decrement in food intake could be attributed to the presence of ephedrine and pseudo-ephedrine in various species of *Sida* (Anon, 1972), including *Sida rhomboidea.*Roxb (Prakash *et al.*, 1981; Khatoon *et al.*, 2005). Hence, it is assumable that, ephedrine induced appetite suppression is the key aspect that inhibits body weight gain in SR supplemented OB mice. Ephedrine content of SR is < 0.01 % and, in the present study, it amounts to 50μg/kg bodyweight, a dose that is almost 400 times lesser than the upper limit toxic dose (24mg) set by USFDA (Cristin, 2010). Further, the safety evaluation of SR reported from our laboratory had shown that a dose of 3000 mg/kg body weight is non-toxic in *Swiss* albino mice (Thounaojam *et al.*, 2010d).

Adipogenesis is a complex process in which both adipocyte growth and differentiation contribute to overall adipose mass (Garaulet *et al.*, 2006).Therefore, adipocyte differentiation is crucial in the maintenance of adipose tissue integrity. The net balance between the three processes determines the onset of visceral obesity. There are several visceral (vis) and subcutaneous (sc) fat depots, such as white

adipose tissue (WAT) and brown adipose tissue (BAT) (Cinti, 2005) of which, WAT is abundant and widely dispersed and is a major site of energy storage, important for energy homeostasis. It stores energy in the form of triglycerides during calorie abundance and releases it as free fatty acids and glycerol during deprivation (Gibbons, 2005). Although, WAT provides a survival advantage in times of starvation, excess WAT is now linked to obesity-related health problems. The ability to carry out these functions efficiently is dependent on changes in the expression of genes that carry out the lipogenic or lipolytic programs.

Adipose tissue represents a dynamic endocrine organ that not only maintains energy balance but also controls lipid homeostasis mainly by two reciprocal processes; lipogenesis and lipolysis (Marques *et al.*, 1998). The adipose depot secretes molecules such as leptin, that limit obesity through effects on food intake and energy expenditure (Anghel and Wahli, 2007). Leptin (from the Greek *leptos*, meaning thin) is a small 16 kDa polypeptide of 167 amino acids produced mainly by adipocytes in direct proportion to adipose tissue mass. It is important in regulating body fat mass through actions on food intake and energy expenditure (Prolo *et al.*, 1998). Circulating plasma leptin concentrations increase in proportion to adipose mass and decrease rapidly during early fasting. The action of leptin on energy balance is mainly mediated through a central effect at the hypothalamus wherein, leptin receptors are abundant (Tartaglia *et al.*, 1995). However, leptin also has an autocrine effect on adipose tissue by induction of lipolysis in WAT (Wang *et al.*, 1999).

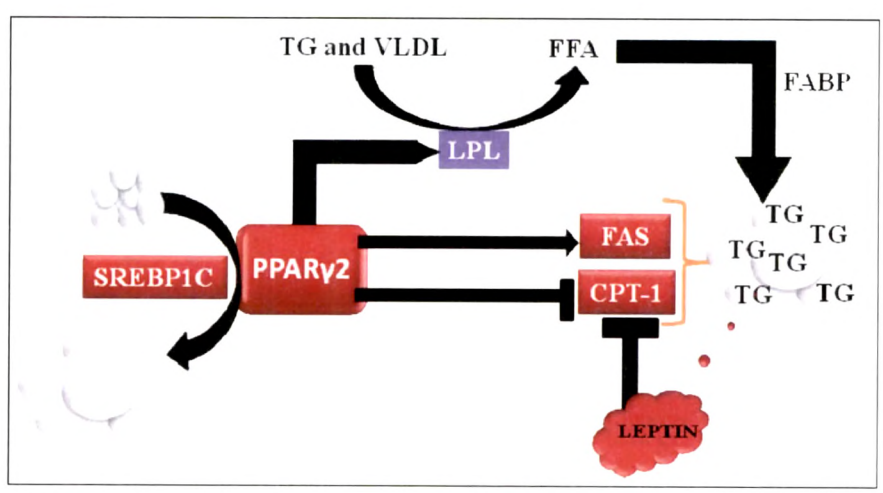
In the present study, OB mice recorded significantly increased mass of various fat pads. These results stand further substantiated by the observation of concomitant decrement in adipocyte number due to augmented TG deposition and adipocyte

hypertrophy. The observed changes in epididymal fat pad of OB mice are in agreement with the recorded elevation in plasma leptin titre and the upregulated expression of leptin mRNA in adipose tissue. However, SR supplementation of HFD fed mice show higher number of non-atrophid adipocytes, decrement in fat pad weights and plasma TG levels coupled with lower plasma leptin titre and down regulation of epididymal leptin mRNA expression compared to OB mice. Since plasma leptin and its mRNA expression are synonymous with obesity (Maffei *et al.*, 1995) and TG induced adipocyte hypertrophy (Staiger *et al.*, 2003), the results recorded herein are indicative of the mitigative nature of SR against HFD induced visceral adiposity, essentially by altered leptin gene expression in adipocytes.

The sequence of events beginning with adipocyte differentiation and ending with obesity is marked by up-regulation of prominent adipogenic genes (Nerurkar *et al.*, 2010).The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily that play a crucial role in stimulating differentiation of pre-adipocytes and progenitor cells into adipocytes (Rousseau *et al.*, 1997). To date, three isoforms have been identified, encoded by separate genes: PPARα, PPARδ and PPARγ. PPARγ is expressed as three isoforms; PPARγ1 and 3 encode the same protein product, whereas PPARγ2 differs due to the addition of 28 amino acids at its N-terminus. PPARγ1 is the ubiquitous isoform found in all PPARγ-expressing tissues such as WAT, BAT, macrophages, liver, skeletal muscle, kidney, colon and vascular endothelium. PPARγ2 has a 30 (rodents) or 28 (human) residue N-terminal extension over that of PPARγ1 and is expressed primarily in adipose tissue (Lehrke and Lazer, 2005). Recent studies have shown that some of the lipid lowering plant extracts act as PPARγ2 antagonists that prevent fat accumulation and adipocyte differentiation

(Christensen *et al.*, 2009, Freise *et al.*, 2010). SREBP1c is another adipogenic transcription factor that controls the production of endogenous ligands for PPARγ2 (Brown and Goldstein, 1997) thus regulating the downstream cascade of lipid homeostasis and adipogenesis (Hsuy and Huang, 2006). SREBP1c mediated lipogenesis in hepatic and adipose tissues is differential (Kersten, 2001). PPARγ2 reportedly mediates lipogenesis in adipose tissue either directly or via modulation of SREBP1c (Figure.10; Kim *et al.*, 1998; Farmer, 2005). In the present study, OB mice recorded significant up-regulation of PPARγ2 and SREBP-1c expression in the epididymal adipose tissue. However, SR supplementation not only prevented the upregulated expression of these genes but also attenuated adipogenesis.

Figure.10 Role of PPARγ in modulation of lipid metabolism.

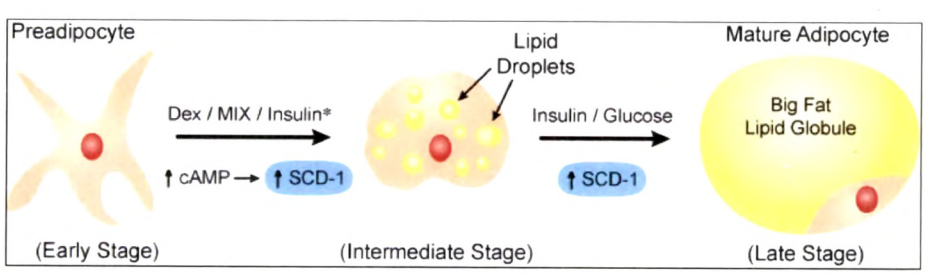


FAS gene expression governed by PPARγ2 is involved in the facilitated conversion of FFA into TG in adipocytes thus contributing to adipocyte hypertrophy and visceral adiposity in HFD fed OB mice (Schachtrup *et al.*, 2004). This physiological condition is paralleled by elevated plasma levels of TG and FFA.

However, the recorded relatively lowered plasma TG and FFA levels in SR supplemented HFD fed mice is attributable to the reduced food intake of these animals. Further, the down regulation of FAS in adipocytes can again be correlated with lower expression of PPARγ2 in SR supplemented OB mice and the resultant anti-adipocyte hypertrophy. The anti-lipogenic role of down-regulated PPARγ2 expression by SR can further be accounted by the higher expression of CPT-1 gene.

In the present study, HFD fed OB mice recorded significant up-regulation of FAS expression along with down-regulation of CPT-1 expression thus shifting the overall balance towards lipid anabolism. However, up-regulation of CPT-1 expression observed in SR supplemented OB mice along with down-regulation of PPARγ2 and FAS provide ample evidence for the anti-lipogenic potential of SR. It is inferable from the results observed herein that, the anti-obesity property of SR is a two-pronged process that acts via reduction of dietary intake and down regulation of PPARγ2.

Figure.11 Schematic representation of adipocyte differentiation



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The differentiation of preadipocytes to adipocytes is arbitrarily divisible into four steps. First, the preadipocytes withdraw from the cell cycle and, down regulation of genes responsible for the "preadipocyte phenotype" occurs. The second step, is

called the "mitotic clonal expansion", which allows a last round of cell division. Then, the cells start to acquire the "early adipocyte phenotype" after 48 hours, followed by "differentiated adipocytes" whence, genes involved in energy storage and fat metabolism such as PPARγ express maximally (Ntambi, 2000). The *in vivo* monitoring of progression of preadipocyte differentiation in an experimental model is cumbersome. The 3T3-L1 preadipocytic cells undergo *in vitro* differentiation, to acquire morphological and biochemical characteristics of adipocytes when treated with IBMX, dexamethasone and insulin (Figure.12; Holm, 2003). Hence, it is the most commonly used cell line for assessing *in vitro* anti-obesity potential of any therapeutic agent. Anti-obesity potential of herbal extracts is also assessed *in vitro* using 3T3L1 pre-adipocytes as, these cells accumulate TG and release LEP when they differentiate into adipocytes (Lee *et al.*, 2006; Rayalam *et al.*, 2009). In the present study, experimentally induced adipocyte differentiation is characterized by plump appearance of adipocytes due to accumulation of red colored lipid droplets and higher percentage of adipocyte differentiation. Moreover, TG accumulation and G3PDH activity level are elevated in untreated adipocytes. The fully differentiated state of adipocytes stands confirmed by the augmented released of LEP from these cells. However, in presence of SR, differentiating adipocytes reveal an overall decrement in the qualitative and quantitative indices. An admixture of undifferentiated and differentiated cells and minimal lipid accumulation mark the inhibitory effect of SR on adipocyte differentiation qualitatively. These observations further substantiate the dose dependent reduction in TG content, G3PDH activity and LEP titer. Further, treatment of differentiated adipocytes with SR results in dose dependent increment in glycerol release. These findings provide unequivocal evidence for the potential of SR

in preventing adipocyte differentiation, lipid accumulation and LEP release and promoting lipolysis.

It can be concluded from the present study that, the mechanism governing the anti-obesity potential of SR is a two-pronged process that involves (i) attenuation of food intake and (ii) down regulation of PPARγ2 and related lipogenic genes that control visceral adiposity. Substantiation for these contentions comes from the demonstrated SR induced prevention of *in vitro* adipocyte differentiation and leptin release. However, detailed scrutiny of the active phyto-ingredients of SR along with clinical investigations is warranted to develop a herbal anti-obesity formulation.

SUMMARY

In this study, we report effect of SR on (i) *in vivo* modulation of genes controlling HFD induced obesity and (ii) *in vitro* 3T3L1 pre-adipocyte differentiation and LEP release. Supplementation with SR significantly prevented HFD induced increment in bodyweight, lee index, plasma lipids, LEP, visceral adiposity and adipocyte hypertrophy. Further, SR supplementation reduced food intake, down regulated PPARγ, SREBP1c, FAS and LEP expressions and, up-regulated CPT-1 in epididymal adipose tissue compared to OB mice. *In vitro* adipogenesis of 3T3L1 pre-adipocytes seems significantly retarded in presence of SR extract. Further recorded are decreased TG accumulation, LEP release and G3PDH activity along with higher glycerol release without significant alteration in viability of 3T3L1 pre-adipocytes. Our finding suggest that prevention of HFD induced visceral adiposity is primarily by down regulation of PPARγ and LEP gene expressions coupled with attenuation of food intake in C57Bl/6J mouse model. SR induced prevention of pre-adipocyte differentiation and LEP release further substantiate these findings and scientifically validates the potential for application of SR as a therapeutic agent against obesity.

Schematic summary