Chapter 3

Clerodendron glandulosum. Coleb leaf extract retards 3T3L1 pre-adipocyte differentiation and attenuates high fat diet induced visceral adiposity in C57BL/6J mice by modulating PPARγ related genes and Leptin expression

INTRODUCTION

Obesity is not only a cosmetic problem for about 300 million people worldwide (WHO, 2003) but is also an increasing risk for other life-threatening diseases such as diabetes, hypertension and cardiovascular ailments (Aronne and Isoldi, 2007; Shah *et al.*, 2008). Reportedly, India has recorded a 20% increase in the number of overweight individuals between 1998 and 2005 and 5% of the country's population is clinically obese (The Hindu, 2007). Currently, almost 1 in 5 men and 1 in 6 women are overweight. Urban populace is at a higher risk with 40% individuals been reported to be obese (Sinha, 2010).

Currently, there are two types of anti-obesity drugs available in the market

1. Pancreatic lipase inhibitor: orlistat (Xenical) reported to reduce intestinal fat absorption through inhibition of pancreatic lipase (Drew*et al.*, 2007; Hutton and Fergusson, 2004; Thurairajah *et al.*, 2005).

2. Anorectic: Sibutramine (Reductil) is an anorectic or appetite suppressant (Lean, 2001; Poston and Foreyt, 2004; Tziomalos *et al.*, 2009).

Both drugs have side effects such as increase in blood pressure, drying of mouth, constipation, headache, and insomnia (de Simone and D'Addeo, 2008; Karamadoukis *et al.*, 2009; Slovacek *et al.*, 2008; Thurairajah *et al.*, 2005). A number of anti-obesity drugs are currently undergoing clinical development, including centrally-acting drugs (e.g. radafaxine andoleoyl-estrone), drugs targeting peripheral episodic satiety signals (e.g. rimonabant and APD356), drugs blocking fat absorption (e.g.cetilistat and AOD9604) and human growth hormone fragments (Halford, 2006; Melnikova and Wages, 2006).

Table1: Data from the 2007 National Family Health Survey (NFHS, 2006).

States	Males (%)	Males rank	Females (%)	Females rank
India	12.1	14	16	15
Punjab	30.3	1	37.5	1
Kerala	24.3	2	34	2
Goa	20.8	3	27	3
Tamil Nadu	19.8	4	24.4	4
Andhra Pradesh	17.6	5	22.7	10
Sikkim	17.3	6	21	8
Mizoram	16.9	7	20.3	17
Himachal Pradesh	16	8	19.5	12
Maharashtra	15.9	9	18.1	13
Gujarat	15.4	10	17.7	7
Haryana	14.4	11	17.6	6
Karnataka	14	12	17.3	9
Manipur	13.4	13	17.1	11
Uttarakhand	11.4	15	14.8	14
Arunachal Pradesh	10.6	16	12.5	19
Uttar Pradesh	9.9	17	12	18
Jammu and Kashmir	8.7	18	11.1	5
Bihar	8.5	19	10.5	29
Nagaland	8.4	20	10.2	22
Rajasthan	8.4	20	9	20
Meghalaya	8.2	22	8.9	26
Orissa	6.9	23	8.6	25
Assam	6.7	24	7.8	21
Chattisgarh	6.5	25	7.6	27
West Bengal	6.1	26	7.1	16
Madhya Pradesh	5.4	27	6.7	23
Jharkhand	5.3	28	5.9	28
Tripura	5.2	29	5.3	24

Due to higher costs and reported side effects of synthetic drugs, natural products are gaining increasing attention for developing as safe alternatives for treating obesity (Park*et al.*, 2005; Nakayama *et al.*, 2007; Mayer *et al.*, 2009). In this behest, traditional herbal medicines and food ingredients capable of controlling weight gain are *in vogue*. Recently, extracts of curry leaf (*Murraya koenigii* (L.), Kuwa (*Morus bombycis)*, Indian lotus (*Nelumbo nucifera*), St. John's wort (*Hypericum perforatum* L) and Japanese mugwort (*Artemisia princeps*) have been reported to possess anti-obesity potentials (Birari *et al.*, 2010; Kim *et al.*, 2010; Wu *et al.*, 2010; Husain *et al.*, 2011; Yamamoto *et al.*, 2011).

Aim: - Present work has been undertaken to decipher the influence of CG extract on the expression of key regulatory genes such as, the peroxisome proliferator-activated receptor γ2 (PPARγ2), sterol regulatory element-binding factor 1c (SREBP1c), carnitine palmitol transferase-1(CPT-1), fatty acid synthase (FAS) and leptin (LEP) in the epididymal adipose tissue of HFD induced C57BL/6J obese mouse. Further assessed is the efficacy of CG extract in controlling *in vitro* adipocyte differentiation and LEP release.

MATERIALS AND METHODS

Plant, preparation of extract and phytochemical analysis

As mentioned in chapter 1

Experimental animals: - as mentioned in chapter 2.

High fat diet induced obesity in C57BL/6J mice

The experimental set up involved 18 mice divided into three groups of six animals each. Group I (Lean; LN) consisted of mice fed with low fat diet (regular fat diet) for

20 weeks. Group II (Obese; OB) consisted of mice that received high fat diet (Chapter 2) for 20 weeks. Group III (OB+CG) consisted of mice fed with high fat diet containing 1% CG extract (Chapter 2) for 20 weeks. Our previous report on efficacy of CG extract in ameliorating HFD induced non-alcoholic steatohepatitis in C57BL/6J mice (Chapter 2) formed the basis for the selection of CG dose (1% w/w in HFD).

At the end of the experimental period, blood was collected in EDTA coated vials from overnight fasted animals under mild ether anaesthesia by the retro orbital sinus puncture technique. Plasma was obtained by cold centrifugation (4°C) of the vials for 10 min at 3000 rpm. Later, abdominal, renal and epididymal fat pads were excised from animals sacrificed by cervical dislocation under mild ether anaesthesia. Epididymal fat pad was stored in RNAlater solution (Ambion, Applied Biosystems, USA) at -80°C (Cryo Scientific Ltd, India) until analysis.

Plasma lipid profile: as mentioned in chapter 1.

Estimation of plasma leptin

Plasma leptin was assayed using anti-mouse monoclonal antibody coated 96 microtitre plate as per the instructions of the manufacturer (KRISHGEN Biosystems, Ltd) by bringing all the reagents to room temperature. Hundred 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 similar. Hundred µl of biotin antibody solution added to each well and, the 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 mentioned 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 incubated in the dark for 15 min after addition of 100 µl freshly mixed TMB substrate solution. Addition of 100 µl of 2N H_2SO_4 to each well at the end of incubation ensured stoppage of reaction. Positive wells changed colour 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 ratio of A_{260}/A_{280} >1.9 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, the 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. The tubes underwent incubation at room temperature for 10 min after the addition of 500 µl of chloroform followed by centrifugation at 12,000-x g for 8 min at 4°C. The aqueous phase formed was transferred to a fresh centrifuge tube. After addition of 500 µl of isopropanol, the tubes were incubated at room temperature for 5–10 min and then centrifuged at 12,000-x g for 10 min at 4°C. Precipitated RNA formed a gel-like white pellet on the side and bottom of the tube. The RNA pellets were washed with 1 ml of 75% ethanol and centrifuged at 7,500-x g for 5 min at 4°C. Ethanol was removed without disturbing the pellets, which were then 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 (A_{260}= 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 20µl contained 10µl Quantifast Syber green master mix (Qiagen, USA), 2µl template, 1µl of each primer and 6µl nuclease free water. The following two step 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.

Table1: 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 data obtained was analyzed by comparative cycle threshold method normalized by the GAPDH expression value and expressed as fold change. Melting curves for each PCR reaction were generated to ensure the purity of amplified product.

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

3T3L1 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 CG (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 PBS (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

3T3L1 pre-adipocytes were differentiated as described above in presence or absence of CG (10-200μg/ml). At the end of the 12th day, ORO staining was performed for visualization of adipocyte lipid accumulation 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

3T3L1 pre-adipocytes were differentiated as described above in presence of CG (10-200μg/ml) or vehicle (0.9 % NaCl) and, LEP and TG contents were assayed in the supernatant and cells respectively. On day 12, supernatant from each well was collected and LEP content 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 percentage TG.

Glycerol release assay

3T3L1 pre-adipocytes were differentiated as described above for 12 days. For glycerol release assay, differentiated adipocytes were incubated with CG (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

3T3L1 pre-adipocytes were differentiated as described above in presence of CG (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 determined as per 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, lee index and, food and water intake

High fat diet fed OB mice recorded significant weight gain (Figure 1) and higher lee index (Table 2) during 20 weeks compared to the LN mice. However, CG extract

supplementation of HFD fed mice significantly contained the HFD induced weight gain and higher lee index (Figure 1 and Table 2). There were no significant alterations in either food or water intake in control and experimental groups during the study period (Table 2)

Plasma lipids, leptin and fat pad weight

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

Quantitative RT- PCR (qPCR) analysis

High fat diet fed OB mice recorded significant increment in the expression of mRNA for PPARγ, SREBP1c, FAS and LEP and, significant decrement for CPT1 compared to lean mice (Figures 4 & 5). CG extract supplementation of OB mice prevented to a significant extent the HFD induced increment in mRNA expression for PPARγ, SREBP1c, FAS and LEP and decrement for CPT-1 (Figures 4 & 5).

Microscopic and morphometric evaluation of epididymal fat pad

Microscopic evaluation of epididymal fat pad of OB mice recorded adipocyte hypertrophy (Figure 6) characterized by significant increase in adipocyte diameter and surface area along with reduction in adipocyte number compared to LEAN mice (Figure 7). However, OB+CG mice recorded significant decrement in adipocyte

diameter and surface area along with higher number of adipocytes compared to OB mice (Figure 7).

Cytotoxicity assay in 3T3L1 pre-adipocytes

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

Qualitative and quantitative analysis of adipocyte differentiation

Oil red O staining of differentiated adipocytes at the end of 12 days revealed significant cytoplasmic lipid accumulation in the untreated differentiated adipocytes while, CG extract supplementation to differentiating 3T3L1 pre-adipocytes significantly reduced adipocyte differentiation, characterised by lesser cytoplasmic lipid accumulation (Figure 8). Quantitative analysis of Oil red O staining revealed a 30 to 70 % reduction in adipogenesis compared to untreated differentiated adipocytes (Figure 8).

Trigyceride accumulation and, leptin release from differentiated adipocytes.

Figures 9A & 9B show TG accumulation and LEP release from un-supplemented and CG supplemented differentiated adipocytes at the end of 12 days. CG extract supplementation to differentiating adipocytes recorded significant decrement in cellular TG accumulation and LEP release compared to differentiated un-supplemented adipocytes (Figures 9A & 9B).

Glycerol release and G3PDH activity assay

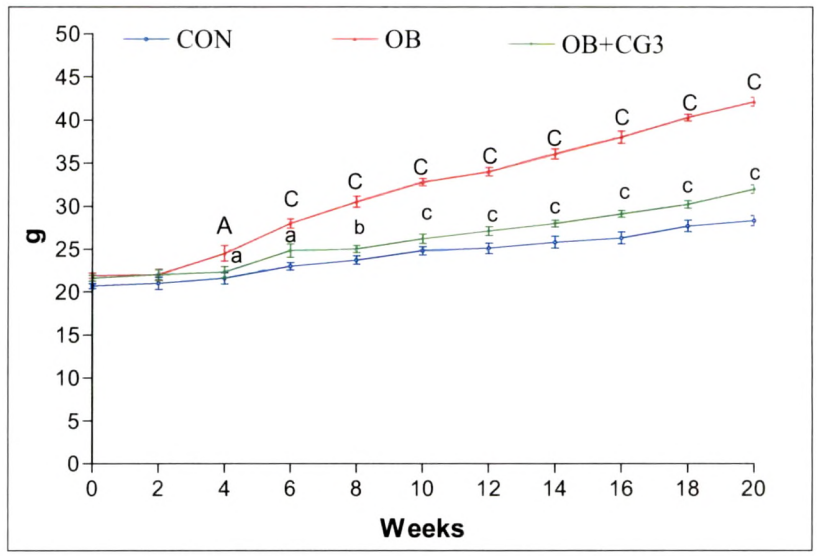
CG extract supplementation to differentiating pre-adipocytes resulted in higher indices of glycerol release and lowered cellular G3PDH activity compared to untreated differentiated adipocytes (Figures 9C & 9D).

Table 2: Effect of *C.glandulosum.Coleb* extract on plasma triglycerides, free fatty acids, leptin, food and fluid intake, lee index, and fat pad weights.

	LEAN	OB	OB+CG
Plasma			
Triglycerides (mg/dl)	46.80±3.31	191.0±8.47^{C}	52.00±5.35^{c}
Free fatty acids (mg/dl)	46.75±2.50	125.0±8.11^{C}	64.75±2.72^{c}
Leptin (ng/l)	11.83±1.44	43.33±2.69^{C}	20.00±0.96^{c}
Food intake (g/day)	2.14±0.12	1.87±0.13^{NS}	1.99±0.11^{ns}
Fluid intake (ml/day)	4.3±0.43	4.6±0.31^{NS}	4.0±0.21^{ns}
Lee index $\sqrt[3]{\frac{\text{Body weight X 10}}{\text{naso-anal length}}}$	2.94±0.07	5.50±0.07^{C}	3.01±0.03^{c}

Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to LEAN.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to OB.

Figure 1: Effect of *C.glandulosum.Coleb* extract on body weight gain.



Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to LEAN.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to OB.

Figure.2 Effect of *C.glandulosum.Coleb* extract feeding on morphological and anatomic evaluation of visceral adiposity in Lean (**A** and **D**), OB (**B** and **E**) and OB + CG (**C** and **F**) groups.

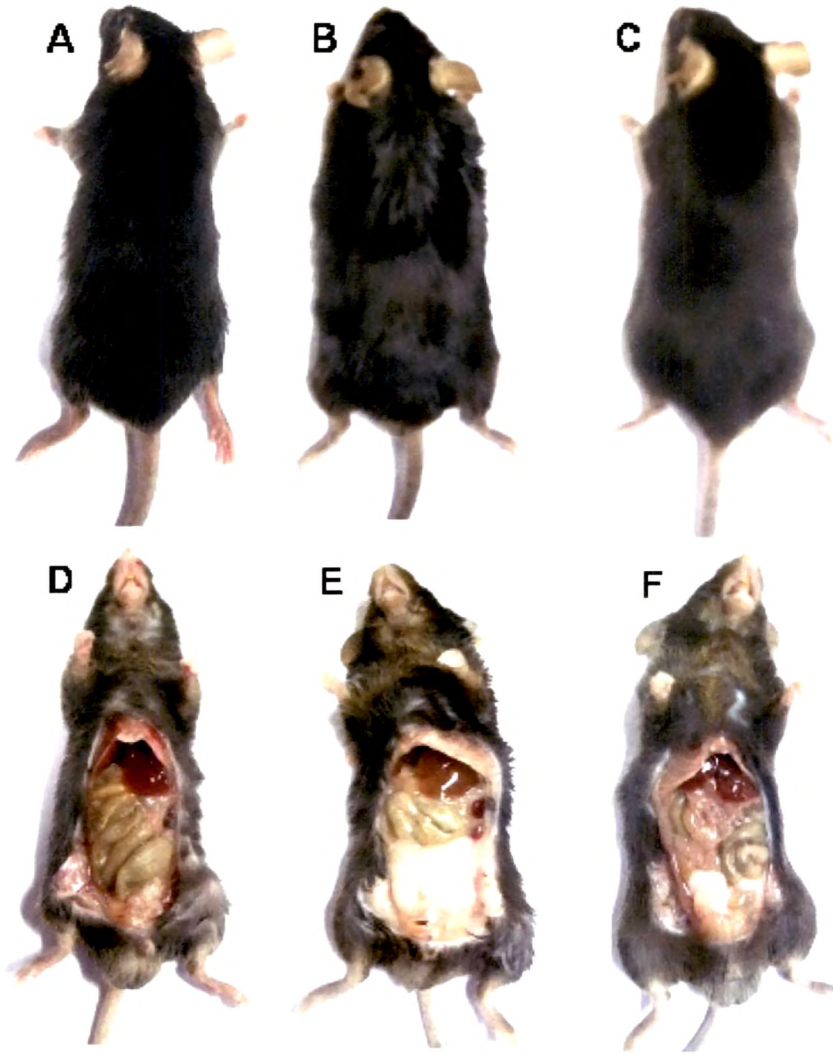
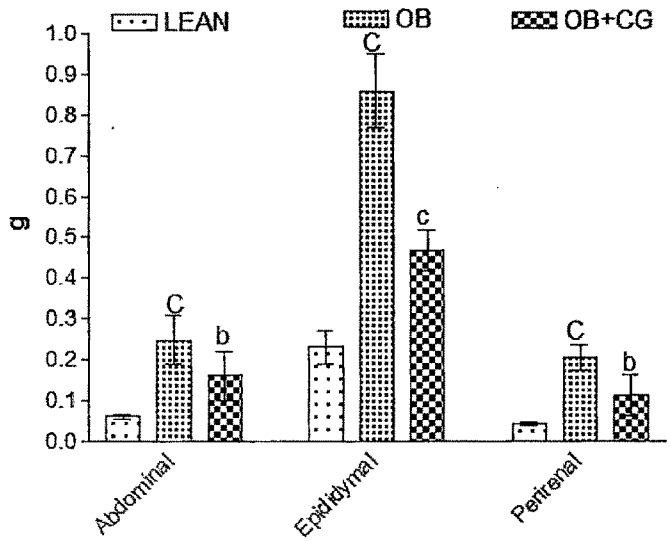


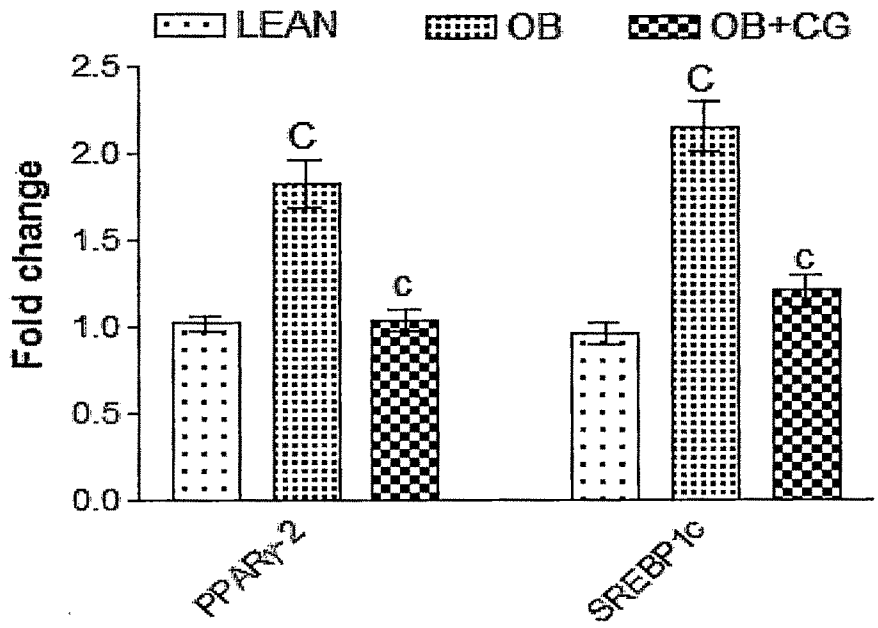
Figure.3 Effect of *C.glandulosum.Coleb* extract on fat pad weights

Fat pad weight	LEAN	OB	OB+CG
Abdominal (g)	0.05±0.006	0.25±0.06^{C}	0.16±0.05^{b}
Epididymal (g)	0.23±0.04	0.85±0.09^{C}	0.47±0.05^{c}
Perirenal (g)	0.04±0.004	0.20±0.03^{C}	0.11±0.05^{b}



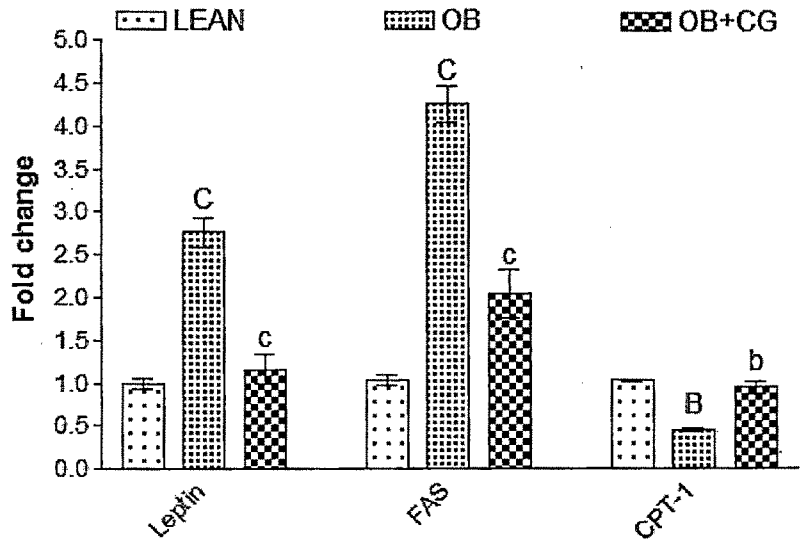
Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to LEAN.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to OB.

Figure.4 Effect of *C.glandulosum.*Coleb extract on mRNA expression of PPARγ and SREBP1c.



Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to LEAN.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to OB.

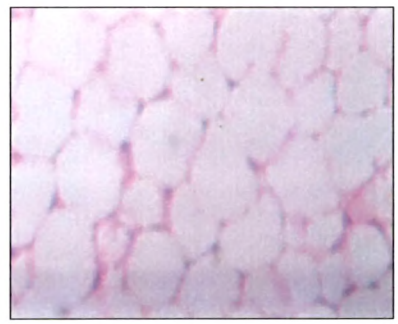
Figure.5 Effect of *C.glandulosum.*Coleb extract on mRNA expression of Leptin, FAS and CPT-1



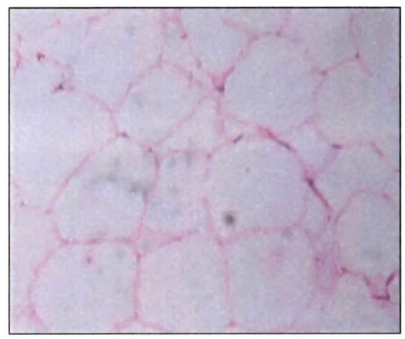
Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to LEAN.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to OB.

Figure.6 Effect of *C.glandulosum.Coleb* extract on histological changes in epididymal fat pad (200X).

LEAN



OB



OB+CG

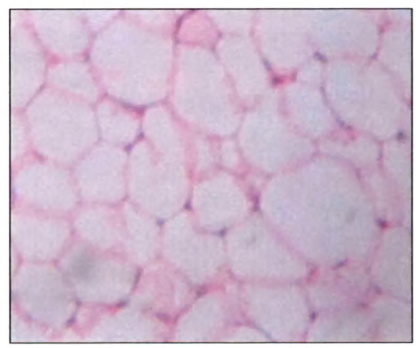
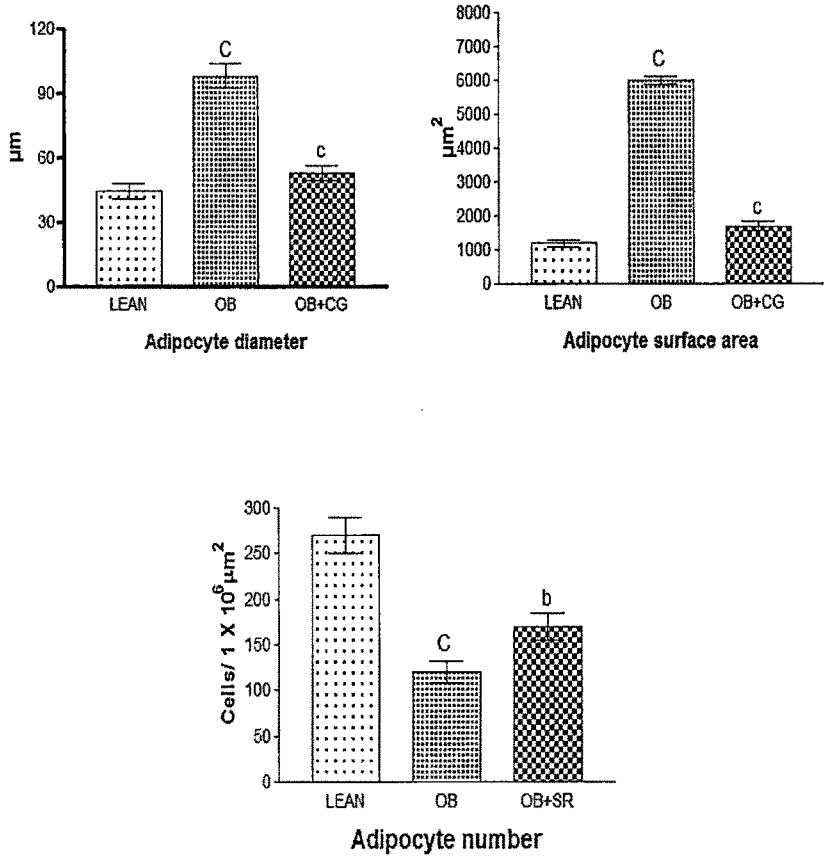
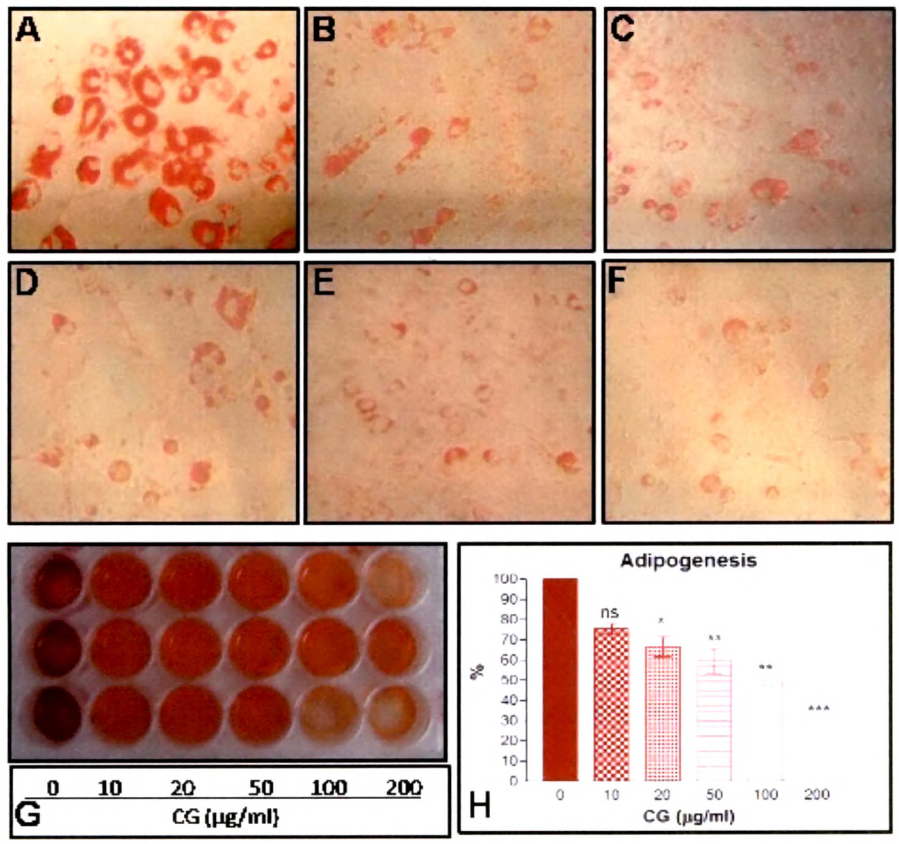


Figure.7 Effect of *C.glandulosum.Coleb* extract on adipocyte diameter, surface area and number.



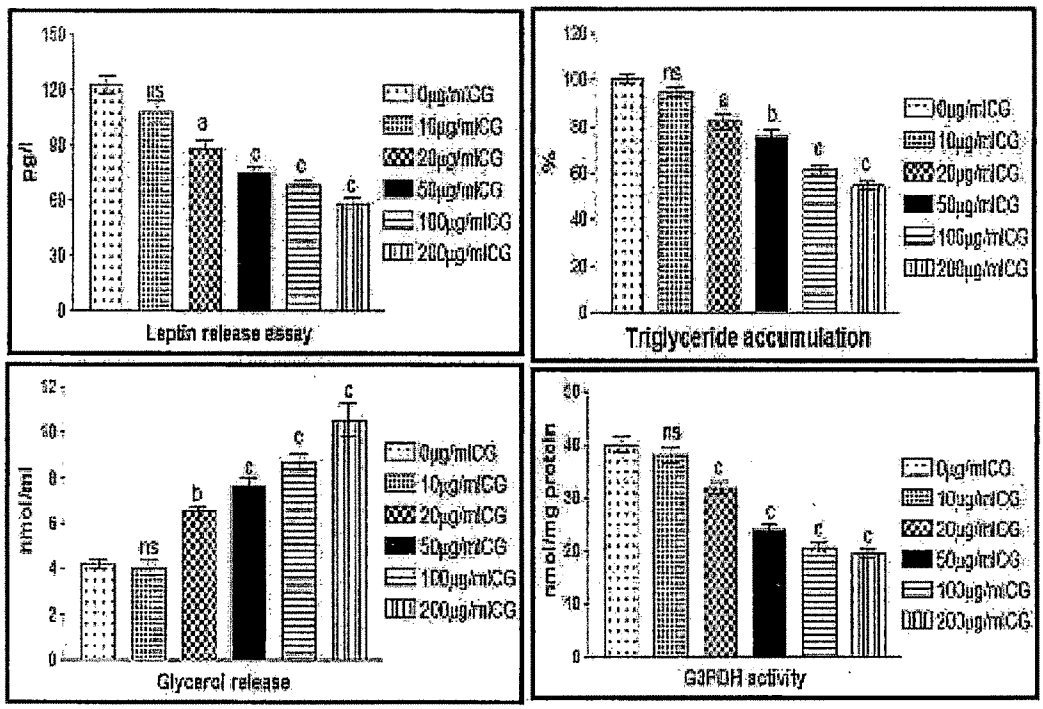
Date expressed as mean±S.E.M for n=6.
$^{A}p<0.05$, $^{B}p< 0.01$, $^{C}p< 0.001$and ^{NS}non-significant compared to LEAN.
$^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ and ^{ns}non-significant compared to OB.

Figure.8 Photomicrograph of Oil Red O stained differentiating 3T3L1 cells (**A**) untreated (**B**) treated with 10 µg/ml CG, (**C**) treated with 20 µg/ml CG, (**D**) treated with 50 µg/ml CG, (**E**) treated with 100 µg/ml CG and (**F**) treated with 200 µg/ml CG and qualitative (whole well image of Oil Red O stained adipocytes) and quantitative (% adipogenesis) evaluation of adipocyte differentiation (**G** and **H**).



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

Figure.9 Effect of *C.glandulosum.Coleb* extract on leptin release, triglyceride accumulation, glycerol release and G3PDH activity in 3T3L1 adipocytes.



Data expressed as mean±S.E.m for n=3.

$^{ns}p>0.05$, $^{a}p<0.05$, $^{b}p<0.01$ and $^{c}p<0.001$ compared to 0 µg/ml CG.

Discussion

Feeding of high fat diet apparently produce diabetes and obesity in various strains of mice (Surwit *et al.*, 1988; West *et al.*, 1992) and rats (Schemmel *et al.*, 1969). The C57BL/6J mouse serves as a good experimental model for human metabolic syndrome since it develops obesity, hyperinsulinemia, hyperglycemia, and hypertension when allowed *ad libitum* access to a high-fat diet (Surwit *et al.*, 1988), but remains lean and physically normal when restricted to low-fat diet. The development of insulin resistance, hyperglycemia, and obesity in the C57BL/6J mice resembles induction and progression of diabetes and obesity in humans. Rebuffe-Scrive *et al.* (1993) and Surwit *et al.* (1995) have shown selective deposition of fat in the mesentery, a phenomenon similar to abdominal obesity in humans in HFD induced diabetes and obesity in the C57BL/6J mice. Hyperglycemia develops within 1 month of the introduction of a high-fat diet in the C57BL/6J mouse (Surwit *et al.*, 1988, 1997). The symptoms of diabetes/obesity start appearing by 12 weeks and by 16 weeks, they develops fatty liver and obesity with leptin resistance (Black *et al.*, 1998). Like in human metabolic syndrome, obese hyperinsulinemic C57BL/6J mice also show accompaniment of hypertension and hyperactivity sympathetic nervous system (Mills *et al.*, 1993). Hence, HFD fed C57BL/ 6J mouse model is widely used to investigate anti obesity potential of various herbal extracts (Choi *et al.*, 2007; Kim *et al.*, 2008; Kim and Kim, 2009; Park and Cha, 2010). In the present study, CG supplementation to OB mice prevented HFD induced increment in body weight, lee index (obesity index) and circulating levels of plasma TG and FFA without significantly altering food and fluid intake. These results are in tune with a previous study from our laboratory reporting reduced absorption of lipids through intestine and

excretion of the same through faeces in CG treated hyperlipidemic rats (Jadeja *et al.*, 2010b). In addition, HFD fed OB mice recorded significant increment in size and mass of abdominal, renal and epididymal fat pads while, this increment was prevented in CG supplemented OB mice. Development of visceral adiposity in HFD fed OB mice was very much evident from the noted adipocyte hypertrophy, decrease in adipocyte number and increase in surface area. CG supplementation of OB mice reversed these set of changes in the histoarchitecture of adipocytes, thus further justifying the role of CG in controlling HFD induced visceral adiposity.

LEP secretion by adipose tissue, directly correlated with adipocyte TG accumulation and hence circulating LEP level, is an ideal indicator of assessing obesity in both experimental animals and humans (Maffei *et al.*, 1995; Wang *et al.*, 2010). TG accumulation and adipocyte hypertrophy are synonymous with increment in plasma LEP titres in OB mice (Kim *et al.*, 2008) and our observations are in accordance with this report. In this context, lower plasma LEP titre recorded in CG supplemented OB mice is attributable to lowered TG accumulation and prevention of adipocyte hypertrophy.

Various research groups in the past few decades have highlighted the role of PPARγ-2 in the regulation of obesity and adipocyte differentiation (Chien *et al.*, 2005; Kim *et al.*, 2008; Oben *et al.*, 2008). Further, PPARγ-2 agonists and antagonists of synthetic or herbal origin have gained wide commercial popularity as therapeutic agents. Another transcription factor, SREBP1c, regulates downstream cascade of fat metabolism by controlling the endogenous production of ligands for PPARγ-2 (Brown and Goldstein, 1997; Kim *et al.*, 1998) and hence, higher expression of SREBP1c contributes to up-regulation of PPARγ-2 and its downstream adipogenic factors (Kim

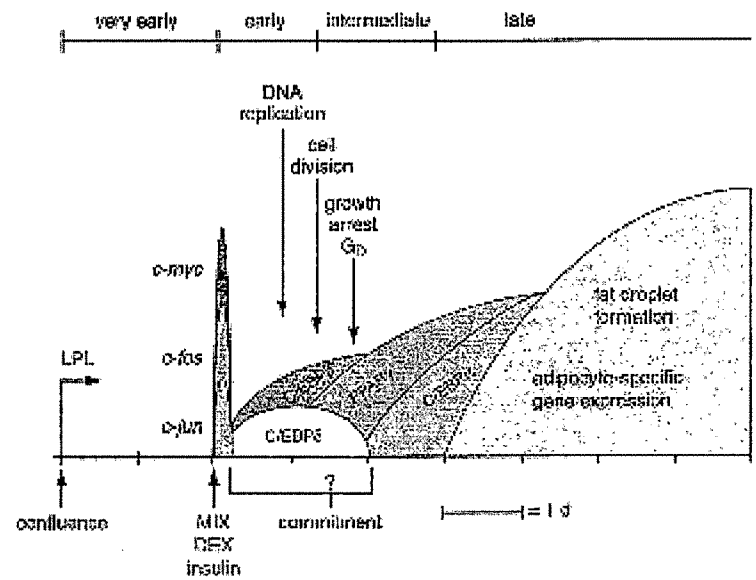
et al., 2008). Herbal extracts of *Petasites japonicas* (Watanabe *et al.*, 2010) and *Momordica charantia* (Nerurkar *et al.*, 2010) appear to exert their anti-obesity potential via regulation of PPARγ2 expression. The currently noted significant up regulation of PPARγ-2 and SERBP1c mRNA expression in OB mice is in accordance with the previous reports (Lee *et al.*, 2010; Watanabe *et al.*, 2010). CG extract apparently attenuates the up-regulatory potential of these genes as seen by the levels of transcript of these genes in OB mice supplemented with CG.

The 3T3L1 cell line serves as one of the best-characterized and reliable *in vitro* model for studying the process of differentiation of pre-adipocytes into adipocytes. In culture, differentiated 3T3L1 pre-adipocytes have an inherent ability to accumulate intracellular fat. Accumulation of intracellular fat droplets and the resultant changes in ultrastructural characteristics are similar to mammalian adipocyte hypertrophy (Novikoff *et al.* 1980; Green and Kehinde 1974). A defined adipogenic cocktail can help differentiate confluent 3T3L1 pre-adipocytes. Maximal differentiation occurs on treatment with a combination of insulin (a glucocorticoid that can elevate intracellular cAMP levels) and fetal bovine serum (Student *et al.* 1980). Insulin acts through the insulin-like growth factor 1 (IGF-1) receptor and hence, IGF-1 can be substituted for insulin in the adipogenic cocktail (Smith *et al.* 1988). Dexamethasone (DEX), a synthetic glucocorticoid agonist, is traditionally used to stimulate the glucocorticoid receptor pathway whereas, methylisobutylxanthine (IBMX), a cAMP-phosphodiesterase inhibitor, is traditionally used to stimulate the cAMP-dependent protein kinase pathway.

At the end of 24 h of sub-culture, pre-adipocytes undergo a post confluent mitosis and growth arrest (Bernlohr *et al.* 1985). The cells undergo at least one round

of DNA replication and cell division. By 2nd day of differentiation, the cells complete the post confluent mitosis and enter into an unusual growth arrest called G_D (Scott *et al.* 1982). The growth arrest is required for subsequent differentiation. The mitosis is believed necessary, to unwind DNA, allowing access for transcription factors to the regulatory response elements present in genes involved in the modulation of mature adipocyte phenotype (Cornelius *et al.* 1994). After the growth arrest, cells are committed to become adipocytes. Growth-arrested cells begin to express late markers of differentiation by 3rd day. These late markers consist of lipogenic and lipolytic enzymes, as well as other modulatory proteins. The cells then round up accumulate fat droplets and become terminally differentiated adipocytes by 5th to 7th day (Figure 10).

Figure.10 Stages of 3T3-L1 pre-adipocyte differentiation.



From:- **Ntambi J M , Young-Cheul K J. Nutr. 2000; 130:3122S-3126S.**

In the present study, plumping of adipocytes due to accumulation of red colored lipid droplets characterize adipocyte differentiation. Besides, TG

accumulation and elevated G3PDH activity characterize untreated adipocytes. Augmented release of leptin from these cells confirms the fully differentiated state of adipocytes. However, exposure to CG extract significantly reduces 3T3L1 pre-adipocyte differentiation, TG accumulation, LEP release and G3PDH activity. CG induced effective prevention of obesity *in vivo* and slower differentiation of pre-adipocytes *in vitro* are attributable to active phytochemical principle(s) that antagonise PPARγ-2 and LEP gene expressions, necessitating further in-depth analysis.

Despite the role of PPARγ-2 in adipocyte differentiation, studies have also reported its key role in lipogenesis and lipolysis via up regulated expression of lipoprotein lipase and fatty acid binding protein (Schoonjans *et al.*, 1996; Way *et al.*, 2001). This in turn, facilitates uptake of FFA into adipocytes and incorporation into TG, resulting in adipocyte hypertrophy (Anghel and Wahli, 2007). Further, PPARγ-2 mediated up-regulation of FAS orchestrates these events leading to HFD induced lipogenesis. This scenario parallels down regulation of CPT-1 expression and hence, mRNA expression of the same can serve as an indicator of PPARγ-2 induced prevention of lipolysis (Lee *et al.*, 2008). Significant down regulation of PPARγ-2 expression along with FAS and up-regulation of CPT-1 expressions recorded in CG supplemented mice provide ample testimony to the potential of the extract to abrogate the HFD induced metabolic alteration leading to obesity. The noted increment in glycerol release from CG extract exposed adipocytes *in vitro* provides further substantiation to the inferred efficacy of CG extract.

Overall, the present study helps conclude the anti-obesity potential of *C. glandulosum*.Coleb via down-regulation of PPARγ-2 expression and its downstream pathway. This study is the first scientific report that provides convincing ethnopharmacological evidence for the relevance of CG as an herb with anti-obesity properties; thus providing scientific validity to its traditional consumption by the local populace of North East India.

Summary

Present study aimed at evaluating effects of CG extract on (i) expression of genes regulating visceral adiposity and (ii) *in vitro* adipocyte differentiation and LEP release has evaluated body weight, lee index, plasma lipids and LEP together with mRNA expression of PPARγ-2, SREBP1c, FAS, CPT-1 and LEP in epididymal adipose tissue of control and experimental groups. Further assessed was the potential of CG extract on *in vitro* adipocyte differentiation and LEP release. Supplementation of CG extract to HFD fed mice significantly prevented HFD induced increment in bodyweight, lee index, plasma lipids and LEP, visceral adipocity and adipocyte hypertrophy. Moreover, CG extract supplementation resulted in down regulation of PPARγ-2, SREBP1c, FAS and LEP expression along with up-regulation of CPT-1 in epididymal adipose tissue compared to HFD fed mice. *In vitro* study recorded significant anti-adipogenic effect of CG extract that resulted in decreased adipogenesis, TG accumulation, LEP release, G3PDH activity along with higher glycerol release without significantly altering viability of 3T3L1 pre-adipocytes. *C.glandulosum*.Coleb extract prevents adipocyte differentiation and visceral adiposity by down regulation of regulation of PPARγ-2 related genes and Lep expression thus validating its traditional therapeutic use in controlling obesity.