ORIGINAL ARTICLE

Anti-adipogenic Activity of 3-Hydroxyflavone on 3T3-L1 Pre-adipocyte Differentiation

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Abstract

Objective: The study proposed to explore the anti-adipogenic activity of 3-Hydroxyflavone on 3T3-L1 pre-adipocyte differentiation. **Method:** 3T3-L1 pre-adipocytes were differentiated into adipocytes in the presence of (3, 3×10^2 and 3×10^4 ng/well) 3-Hydroxyflavone for 6 days, the untreated 3T3-L1 pre-adipocytes served as control. After differentiation, the adipocytes were subjected to Oil Red O staining and the m RNA expression of leptin, peroxisome proliferator activated receptor (PPARγ), and the Sterol regulatory element-binding transcription factor 1 (SREBP-1) were studied. **Results:** A round-shaped adipocytes were appeared in the cultured medium on the 6th day of the study period. The untreated adipocytes showed intense accumulation of the red dye; however, a dose-dependent reversal of red dye accumulation was seen in the adipocytes on 3-Hydroxyflavone exposure. The m RNA expressions of leptin, PPAR γ and SREBP in the untreated adipocytes were significantly up regulated. However, on 3-Hydroxyflavone treatment, the m RNA expressions of leptin, PPAR γ and SREBP in the adipocytes were down regulated in a dose-dependent manner. The high dose (3×10^4 ng per well) of 3-Hydroxyflavone appreciably reduced the oil accumulation and also significantly down regulated the expression of leptin, PPAR γ and SREBP in the adipocytes. **Conclusion:** The 3-Hydroxyflavone displayed the anti-adipogenic activity in the adipocytes by reducing the oil accumulation and also by down regulating the m RNA expression of leptin, PPAR γ and SREBP, which are need for the adipogensis.

Key words: Obesity, 3-hydroxyflavone, 3T3-L1 pre-adipocyte and adipogenesis

INTRODUCTION

Obesity is a condition in which due to an abnormal amount of fat deposition in the body, with reference to body mass index value, which is determined by dividing the whole body weight with height in meter (Kg/M^2).[1] The cause for the development of obesity is multi- factorial, which may due to any one or associated contributing factors namely excessive feeding, lack of physical activity, sleepiness, and endocrine problem, genetic and social economic issues.[2,3] Over the period of a past decade, the prevalence of obesity in childhood and adolescents has increased significantly. According to the world health organization (WHO) report (2016), globally 7% of children and 13 % of adults were found to be obese.[1] The consistent growth of the obsessed individual in the arena is the major setback for the public health sector, and also for the exponential economic growth activity. Moreover, 4 million deaths and 120 million disabilities have been reported worldwide due to obesity.[4] Obesity is one of the contributing risk factors for cardiovascular abnormalities, type 2 diabetes, renal, muscular, and psychological disorders.[5] Obesity is one of the treating causes for coronavirus disease-2019 outcome.[6] In fact, expression of angiotensin-converting enzyme 2 was significantly unregulated in the adipocytes of people with obesity and diabetes, which makes the obese peoples as potential viral reservoir.[7] In India, more than 135 people were found to be obese.[8] Forecasts suggest that the prevalence of overweight will be doubled in Indian adults between 2010–2040 and the prevalence of obesity will be

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tripled.[9] Adipogenesis is a process in which adipocytes (fat-laden cell) are developed and accumulated in the form of adipose tissue at various sites of the body. Adipocytes, store the engery in form of fat, when energy in take is exceeding the expenditure, and it also mobilizes the fat, when it is vice versa. Obesity always favors for hyperplasia and hypertrophy, a condition in which, there is increase in size and number of adipocyte.[10] The fat deposition in the cytoplasm of adipocytes is possible only with the involvement of fatty acid synthase (FAS), lipoprotein lipase (LPL), and adipocyte fatty acid-binding protein (aP2). However, these proteins are carefully regulated by the protein hormone leptin and transcription factors peroxisome proliferator activated receptor (PPARγ) and sterol regulatory element binding protein (SREBP-1c). The possible inhibitions of these regulatory proteins are directly reflected in the size and count of the adipocytes.[11] Hence, the study was proposed to explore the anti-adipogenic activity of 3-Hydroxyflavone on 3T3-LI pre-adipocytes. 3-Hydroxy Flavone belongs to the family of flavanoids and is used as a fluorescent probe to study the membranes and intermembrane proteins. It exhibits anti-aging, anti-inflammatory, anti-cancer, and anti-viral activity.[12]

METHODOLOGY

Chemicals

The chemical and kits used for the study were procured from HiMedia Laboratories.

Culture and maintenance of 3T3-L1 pre-adipocytes

3T3-L1 pre-adipocyte was procured from the National Centre for Cell Science (Pune, India). The cells were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% FBS and penicillin/streptomycin (100 IU, 100 mg/ml, respectively) at 37°C in 5% CO_2. Once confluence was attained, the cells were used for the adipogenesis process.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

MTT assay was performed as per the protocol of Mosmann[13] with slight modifications. In brief, pre-confluent 3T3-L1 pre-adipocytes were seeded (8000 cells/well) in 96 well plates, incubated overnight at 37°C in humidified atmosphere with 5% CO_2. Following incubation, cells were exposed to 3-Hydroxyflavone at concentrations ranging from (3 - 3 × 10^4 ng per well) and incubated for 20 hours. After incubation period, 10µl of MTT (5 mg/ml) were added to each well and incubated for 4 hours. After an incubation period, the medium was aspired carefully from each well and 100 µl of Dimethyl sulfoxide (DMSO) was added to all the wells. The color intensity was read at 570 nm with 630 as reference wavelength in Thermo scientific Multiskan spectrum, USA.

Adipogenesis

Adipogenesis assay was performed based on Anne *et al.* 2001[14] with slight modification. 3T3-L1 pre-adipocytes were seeded at a density of 200,000 cells in a plate and allowed to adhere to the flask overnight. For induction and differentiation, 2-day post-confluent 3T3-L1 pre-adipocytes (day 0) were stimulated for 24 h by adding 51.8 mM 3-isobutyl-1-methylxanthine, 0.25 µM dexamethasone, and 0.1% insulin (MDI) to the DMEM with 10% FBS culture medium. Subsequently, on day 3, the MDI medium was replaced with DMEM with 10% FBS containing 1µM insulin. On day 4, the MDI medium was replaced with DMEM with 10% FBS and refreshed for 2 days until analysis was performed on day 6.

3-Hydroxyflavone exposure to 3T3-L1 pre-adipocytes

3-Hydroxyflavone was dissolved in sterile distilled water and filtered (0.22 µ). The filtrate containing 3-Hydroxyflavone at concentration of 3, 3×10^2 and 3×10^4 ng per well was premixed with the culture medium from day 0 to day 5 for the induction of 3T3-L1 pre-adipocytes to differentiated adipocytes. The undifferentiated cells (3T3-L1 pre-adipocytes) were used as normal control, whereas differentiated adipocytes treated with vehicle (0.1% DMSO) were used as positive control. After day 6 3T3-L1 adipocytes were subjected for Oil Red O Staining assay to study the oil droplet accumulation in the cell lines.

Oil red o staining

The formation of oil droplets in treated and untreated differentiated 3T3-L1 pre-adipocytes cells was analyzed by Oil Red O staining based on the method of Harmon *et al.* 2001[14] with minor modifications. Subsequent to differentiation of the 3T3-L1 pre-adipocytes, the media was removed; the cells were washed once with phosphate-buffered saline (PBS), and then fixed for at least 1h with pre-chilled 10% formaldehyde in PBS. Cells were stained with Oil Red O solution (a mixture of three parts of 0.5% (w/v) Oil Red O in isopropanol and two parts of water) for 2h at room temperature. Following staining procedure, cells were washed with PBS twice, ethanol once and water twice. Oil Red O staining was visualized using inverted epifluorescence microscope (Motic, China). The oil content in the adipocytes was measured by dissolving the Oil Red O-stained lipids with 100 % isopropanol and the absorbance of the each sample were measured at 490nm.

Isolation of total RNA

Total RNA was extracted using TRIzol Reagent (Sigma, USA). After homogenizing the cells with TRIzol reagent, the tubes were incubated for 10min and centrifuged at

1000 rpm for 5 min. 200 ml of chloroform was added to the supernatant, allowed to incubate for 5 min at room temperature and centrifuged at 12000 rcf for 20 min. Then, 500 ml of isopropyl alcohol was added to the supernatant to precipitate the total RNA and centrifuged at 12000 rcf for 15 min following the incubation period of 10 min. The supernatant was decanted carefully; the pellet was washed thrice with 75% ethanol, centrifuged at 12000 rcf for 15 min. The pellet was air dried and re-suspended in 20 ml of RNase free water and stored at −80°C until use.

Reverse transcriptase polymerase chain reaction (RT –PCR) mRNA expression for PPARγ, leptin and SREBP1

RT- PCR was performed to determine the mRNA expression of leptin, PPARg, SREBP The annealing temperature of the primers was optimized prior to mRNA expression study. mRNA from normal control was reverse transcribed to cDNA using Prime RT-PCR premix (2×) master mix [Genet Bio, Korea] and further used for optimization of the primer annealing temperature. The primers were used at a concentration of 20 rmol/ml for cDNA synthesis. After optimization of annealing temperature, RT-PCR was carried out using gradient PCR [Eppendorf, Germany] and semi-quantified using Bio1D software [Vilber Loumart, France]. The isolated RNA was amplified using reverse transcription and polymerization reaction to get cDNA using gradienet PCR [Eppendorff, Germany]. Amplified PCR product was loaded in agarose gel, electrophoresed at 80 V for 30 min and the gene expression was analyzed using band intensity. 200 ng of RNA was used for RT-PCR (according to the manufacturer's instructions) [Genet Bio, Korea]. The following sequence was performed for each PCR reaction: 42°C for 30 s, 94°C for 5 min (1 cycle); 94°C for 1 min, optimized annealing temperature for each primer for 1 min, 72°C for 1 min (with 34 cycles); and a final extension phase at 74°C for 10 min. β actin was used as internal control.

The mRNA primer sequences for the RT-PCR of different transcription factors are:

PPARγ

Forward 5'-TTT TCA AGG GTG CCA GTT TC-3'
Reverse 5'-AAT CCT TGG CCC TCT GAG AT-3'.

SREBP1

Forward 5'-TGT TGG CAT CCT GCT ATC TG-3'
Reverse 5'-AGG GAA AGC TTT GGG GTC TA-3'.

Leptin

Forward 5'-GGA TCA GGT TTT GTG GTG CT-3'
Reverse 5'-TTG TGG CCC ATA AAG TCC TC-3'.

RESULTS

MTT assay for 3-hydroxy flavone

MTT assay was carried in order to ascertain the IC50 value of 3-Hydroxyflavone in 3T3-L1 pre-adipocytes, the data obtained are depicted in Figure 1. The 3T3-L1 pre-adipocytes were exposed to different concentrations of 3-Hydroxyflavone ranging from 3 to 3×10^4 ng/well, the amount of formazan complex released from the cell lines was analyzed, the data obtained were subjected to regression equation analysis, and the IC50 value of the test drug was calculated on the basis of 50% cytotoxicity or cell death. The IC]50 value of 3-Hydroxyflavone in 3T3-L1 pre-adipocytes was found to be 37.05 ng. Based on IC50 value 3, 3×10^2 and 3×10^4 ng of 3-Hydroxyflavone were used to evaluate the anti-adipogenic activity.

Adidopegenesis

The 3T3-L1 pre-adipocytes were made to differentiate in to adipocytes in the MDI medium for 6 days. The morphological changes on differentiation of adipocytes were observed from day 1 to 6th. Various morphological changes at different stages of adipocyte differentiation are illustrated in Figure 2. On 1st day spindle shaped fibroblast cells were observed. On 2nd day appearance of oval shaped cells was inferred, whereas on day 3rd and 4th days round shaped adipocytes appeared. On 5th and 6th day, the pre-adipocyte was completely differentiated, with appearance of clear round shaped adipocyte in the culture medium.

Oil red stain analysis

Oil Red O staining assay is one of the realistic tools to screen the oil droplet presence in the adipocyte. After

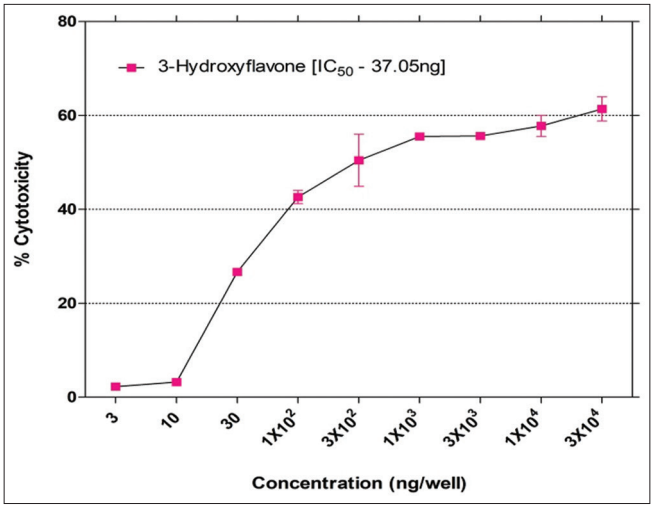

Figure 1: The Cytotoxicity Potential of 3-Hydroxyflavone in 3T3-L1 pre-adipocytes. Cytotoxic effect of 3-Hydroxyflavone on 3T3-L1 pre-adipocytes

adidopegenesis, the adipocytes were stained with oil red O dye, and it was visualized under epifluorescence microscope. The captured images are shown in Figure 3. The amount of oil accumulation in the differentiated adipocytes was enumerated; the data obtained are depicted in the Figure 4. The pre-Adipocytes (control) not stain deeply and the oil accumulation was found to 26%. However, untreated adipocytes showed high accumulation of fat (84.6%) content in their cytosol and stained intensely. However, on treatment with 3- Hydroxy]favones, the reserve accumulation of fat content in the cytoplasm of adipocyte was observed in a dose-dependent manner. On high dose treatment of 3-Hydroxyflavone to adipocytes, showed a significant reduction of staining and with less accumulation of fat (34 %) was seen. From this it is clearly understood that, the high dose (3×10^4 ng) of 3-Hydroxyflavone significantly prevented the fat accumulation in the cytoplasm of the adipocyte.

Gene expression analysis

After differentiation, the m RNA expression of leptin, PPAR γ and SREBP were studied using RT-PCR, the data obtained are depicted in Figure 5. The leptin, PPAR γ and SREBP mRNA expressions in the adipocytes were quantified; the data obtained are illustrated in Figures 6. The untreated adipocytes showed a significant rise ($P \geq 0.01$) of leptin, PPAR γ and SREBP mRNA expression, when compared to control pre adipocyte. Whereas low dose (3 ng) treated adipocytes, showed a reduction in the m RNA expression; however, it is not at a significant mark. However, moderate (3×10^2 ng)

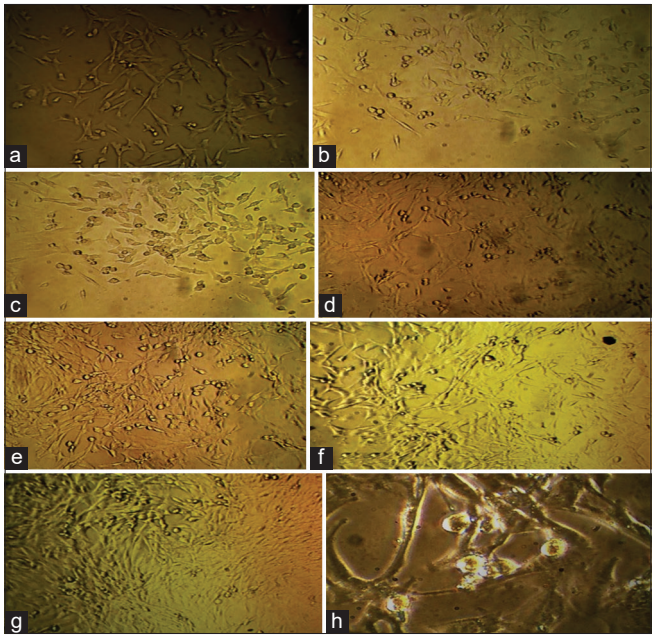

Figure 2: Differentiation of Adipocyte from 3T3-L1 Pre-adipocytes. (a) Day 1 - 10× development spindle shaped fibroblast Day (0) (b) Day 2- 10× Development of slightly oval shaped cells (Day 2) (c) 10× Growth increased in oval shaped cells (Day 2) (d) 10× Development of spherical shaped cell (Day 3) (e) 10× Appearance of round shaped cells (Day 4) (f) and (g) Dense growth and appearance of fat droplet in the adipocytes (Day 5& 6) (h) 40× Appearance of the visible spherical shaped adipocytes (Day 6)

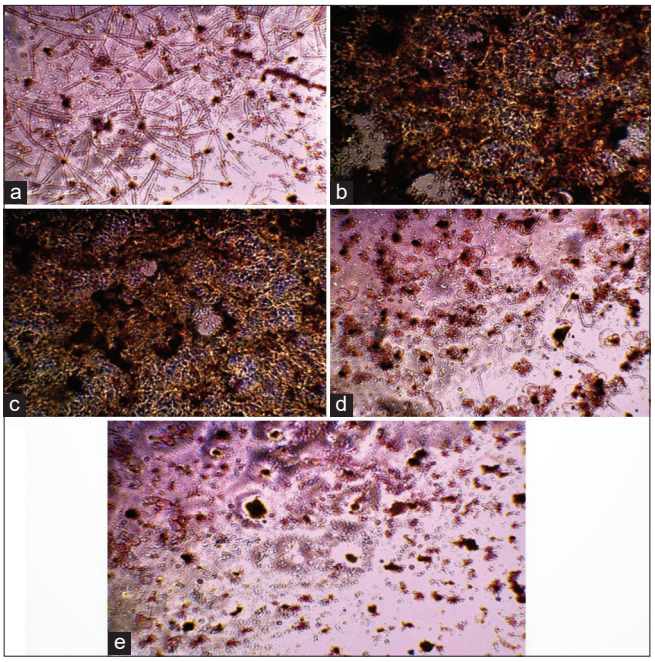

Figure 3: Effects of 3- Hydroxyflavone on differentiated Adipocytes in Oil red O dye staining. (a) 10 × Pre-Adipocytes (control) with a no evidence of fat droplet accumulation and staining (b) 10× untreated adipocytes showing intense staining with more fat accumulation (c) 10× low dose 3- Hydroxyflavone (3 ng/well) treated cells showing a marked decrease of staining with low a fat accumulation. (d) 10× moderate dose of 3- Hydroxyflavone (3×10^2 ng/well) treated adipocyte showing drastic reduction in staining and fat accumulation (e) 10× high dose treated adipocytes (3×10^4 ng/well) with no evidence of stain and oil accumulation

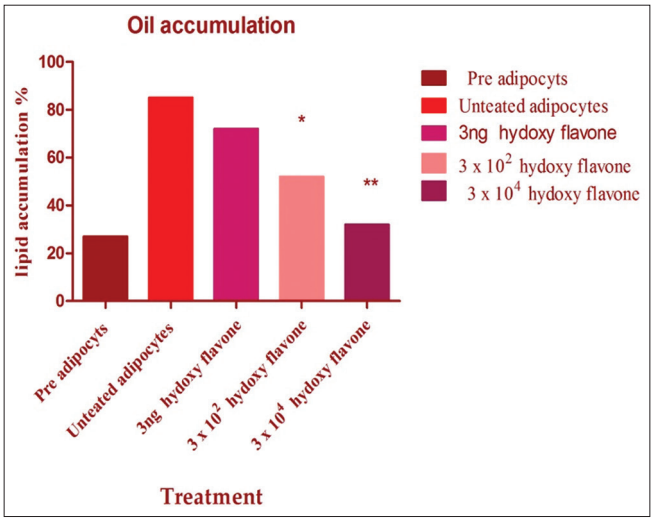

Figure 4: The effect of 3- Hydroxyflavone in the differentiated Adipocytes on oil accumulation. Percentage of oil accumulation in the differentiated adipocytes. The numerical data are expressed in the percentage. * and ** indicates the P ≥ 0.05 and P ≥ 0.0 significance respectively when compare to untreated adipocytes.

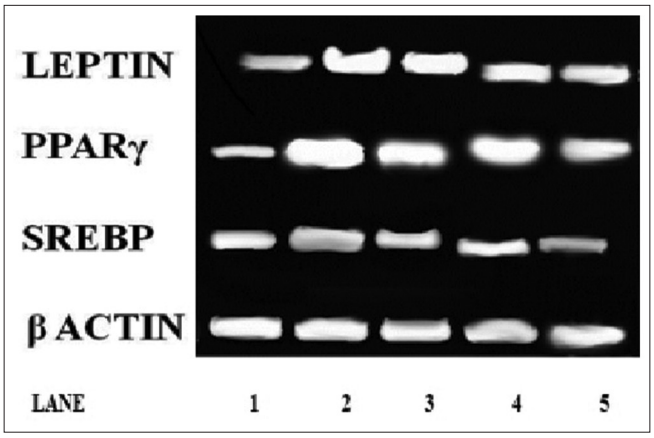

Figure 5: The leptin, PPAR γ and SREBP m RNA expressions in the differentiated Adipocytes. Lane 1 – Pre-adipocyte, Lane 2 – Differentiated adipocytes, Lane 3- 3-Hydroxyflavone (Low dose: 3 ng), Lane 4- 3-Hydroxyflavone (Mid dose: 3×10^2 ng), Lane 5- 3-Hydroxyflavone (High dose: 3×10^4 ng). Un treated adipocytes showed an increased m RNA expression of leptin, PPAR γ and SREBP. The treated adipocytes were showed a significant reduction in the expression of m RNA (Leptin, PPAR γ and SREBP) in a dose-dependent manner

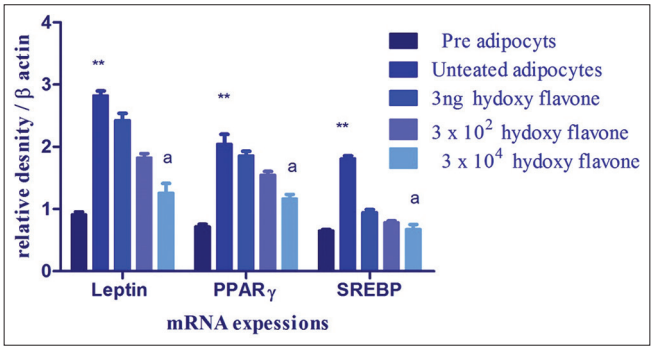

Figure 6: Effects of 3-Hydroxyflavone on leptin, PPAR γ and SREBP m RNA expressions in the differentiated Adipocytes. The numerical data are expressed as mean ± SD, where $n = 3$. The superscript[a] means $P \geq 0.05$ significance when compared to untreated adipocytes. ** indicates the $P \geq 0.01$ significance when compare to pre adipocytes

and high dose (3×10^4 ng) treatment of 3-Hydroxyflavone to the adipocytes showed a significant reduction in the m RNA expression of leptin ($P \geq 0.05$), PPAR γ ($P \geq 0.05$) and SREBP (High dose $P \geq 0.05$), when compared to untreated adipocytes in the toxic control. The high dose treatment (3×10^4 ng) of 3-Hydroxyflavone was found to be effective when compared to moderate and low dose treatments, with respect to m RNA expressions (leptin, PPAR γ and SREBP) in the adipocyte.

DISCUSSION

3-Hydroxyflavone or 3-Hydroxy-2-phenyl-4H-chromen-4-one is a type of flavanoid, which showed an inhibitory activity against the epidermal growth factor -induced proliferation in the A431 cells.[15] The 3-Hydroxyflavone has two pharmacophores in their structure, namely catechol group in ring B and hydroxyl (OH) group at the 3-position. The pharmacophores present in the 3-Hydroxyflavone have been reported to protect the cells against the peroxynitrite toxicity.[16] However, the anti-adipogenic activity of 3-Hydroxy Flavone has not been explored yet. Hence, the present study was proposed to evaluate the role of 3-Hydroxyflavone on adipogenesis using the 3T3-L1 pre-adipocyte cell lines.

3T3-L1 pre adipocyte is one of the preferred or suitable experimental model to study the anti-adipogenic activity for the given test drug, which is primarily developed from murine Swiss 3T3 cells.[17] In the present study, 3T3-L1 pre adipocytes were exposed to different concentration 3-Hydroxyflavone ranging from 3 to 3×10^4 ng/well to determine the cytotoxic potential on exposure. On assessment, the IC 50 value of 3-hydroxy flavone on 3T3-L1 pre adipocyte was found to be 37.05 ng. Based on the IC 50 index value, namely different concentrations of 3, 3×10^2 and 3×10^4 ng of 3-Hydroxyflavone were taken to study the anti-adipogenic activity on 3T3-L1 pre adipocytes.

In the present study, 3T3-L1 pre adipocytes were differentiated in to adipocytes in the presences of 3-Hydroxyflavone for 6 days. Up on differentiation of 3T3-L1 pre adipocytes attained the various morphological stages, such as spindle shaped fibroblast cells on 1st day, appearance of oval shaped cell on day 2nd, a round shaped adipocyte on 3rd and 4th day and a clear round shaped adipocytes appeared on day 5th and 6th. The various stages attained during the differentiation of 3T3-L1 pre adipocytes in the present study are in par with the kong *et al*., 2017 report.[18]

Oil red O dye staining method is extensively used to distinguish the differentiation of preadipocytes from adipocytes and the degree of staining is directly proportional to the extent the adipocytes had differentiated in the medium.[19] This method (Oil red O dye staining) also gives the realistic picture about the triglyceride accumulation in the cytoplasm of the cell.[20] In the present study, the untreated adipocytes were stained deeply, which indicates the high accumulation of fat content in their cytosol and abundant in mass. However on treatment with 3-Hydroxyflavone, the fat accumulation in the cytosol of adipocytes was in reverse trend. The low and moderate dose of 3-Hydroxyflavone to the adipocyte on fat accumulation showed only a partial recovery. However, high dose treatment of 33-Hydroxyflavone on adipocytes showed a considerable reduction of triglyceride in their cytosol and the majority of adiocytes were found to be undifferentiated. The adiposity is mainly associated with increase in the number of adipocytes and the lipid content of the adipocytes.[21] The high dose exposure of 3-Hydroxyflavone inhibited the adpiogeneis, so that adipocytes were not able to differentiate completely to attain enough number of adipocytes and oil accumulation also considerably reduced. Hence, the oil red O dye was not

able to stain even on high exposure. From this it is clearly demonstrated that 33-Hydroxyflavone has inhibited adiposity as a result enough matured adipocytes were not present in the medium to acquire the oil red O dye.

Proliferator-activated receptor gamma PPAR γ or PPARG is a type of type II nuclear receptor, encoded by PPARG gene.[22] PPARG is mainly found in adipose tissue; however, it is also present in colon and macrophages. Two iso forms of PPARG have been identified namely PPAR-γ1, which is present almost in all the tissues except muscle and PPAR γ2 is mainly present in the adipose tissue and the intestine.[23] PPAR γ plays a key role in the regulation of genes involved in the adipocyte differentiation, glucose homeoststsis and the development of adipose tissue.[24] It is also proved that PPAR γ regulates the transcription of LPL, adipocyte protein 2, phosphoenolpyruvate carboxykinase, fatty acid transporters and uncoupling protein-2 in the adipocytes.[25,26] In the present study, the PPAR γ m RNA expression on high dose exposure of 3-Hydroxyflavone in adipocytes was down regulated significantly. However, the low and moderate doses of 3-Hydroxyflavone have not showed any considerable reduction in the PPAR γ expression. From this it is clearly demonstrated that 3-Hydroxyflavone has regulated the expression of PPAR γ on adiposity. As a result, in the present study, the differentiation of preadipocytes to adipocytes was in negative correlation mode. SREBPs are the type of transcription factors, which regulate lipid homeostasis, by inducing the genes responsible for the synthesis and consumption of cholesterol, fatty acid and triglycerides in the adipocytes.[27] In fact, it also regulates the transcription of genes involved in the synthesis of acetyl CoA carboxylase and FAS,[28] which are the key enzymes involved in the synthesis of fatty acids.[29] It has been already reported that SREBP also regulate the transcriptional expression of FAS by binding to the promoter region.[30] In the present study, the SREBP expression in the adipocytes was significantly reduced on high dose exposure of 3-Hydroxyflavone. However, the moderate and low dose of 3-Hydroxyflavone has not revealed any significant down regulation of SREBP expression. From this it is clearly understood that, the 3-Hydroxyflavone has modulated the expression of SREBP as result it was down regulated. Leptin is a protein, secreted by adipocytes plays a vital role in the regulation of body weight by controlling the size and the number of the adipocytes.[31] The leptin m RNA expression can be directly used to correlate the adiposity and the body weight changes.[32] In the present study, the leptin mRNA expression was significantly down regulated on high dose treatment of 3-Hydroxyflavone to the adipocyte. The moderate and low dose of 3-hydroxy Flavone has not revealed any productive effect on leptin expression in the adipocytes. The down regulated mRNA expression of SREBP, PPAR γ and the leptin in the adipocytes on 3-Hydroxyflavone exposure resulted in reduced accumulation of fat and may be effective in treatment of obesity.

CONCLUSION

3-Hydroxyflavone showed anti-adipogenic activity in the 3T3-L1 pre-adipocytes by down regulating the m RNA expression of SREBP, PPAR γ and leptin on differentiation and there by reduced the fat accumulation in the cytosol.

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