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ORIGINAL ARTICLE

Anti-adipogenic Effect of *Terminalia chebula* Fruit Aqueous Extract in 3T3-L1 Preadipocytes

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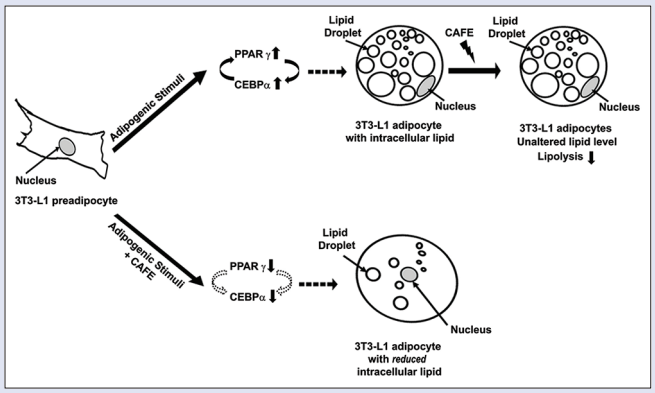
ABSTRACT

Background: Phytoextracts, due to its complex nature of formulations yet little or no side effects, have been pursued as alternative medicine for the treatment of complex metabolic disorders such as obesity. One of the appealing strategies to achieve this is the modulation of adipocyte development and function with the treatment of phytoextracts. The current study explored the activity of *Terminalia chebula* fruit, a component of Ayurveda formulation "*Triphala*" on these aspects of adipogenesis. **Materials and Methods:** The effect of *T. chebula* aqueous fruit extract (CAFE) on the process of adipocyte development and function was investigated. To test the effect of CAFE on adipocyte development, 3T3-L1 preadipocytes were differentiated in the presence and absence of CAFE followed by estimation of lipid content and expression of adipogenic genes. To test its effect on adipocyte function, mature 3T3-L1 adipocytes were treated with the extract followed by estimation of lipolysis. **Results:** Treatment of 3T3-L1 preadipocytes with this extract had efficiently inhibited differentiation and lipid accumulation in these cells. Gene expression of key adipogenic regulators, peroxisome proliferative-activated receptor γ and C/CAAT enhancer-binding protein α, was suppressed due to the treatment with CAFE. Preadipocytes exposed to CAFE also showed suppressed expression of important adipogenic effector genes such as perilipin 1 and fatty acid synthase. Treatment of differentiated adipocytes with CAFE did not affect total lipid contents of the cells. However, CAFE treatment reduced lipolysis to a small extent. **Conclusion:** CAFE is an anti-adipogenic and anti-lipolytic agent which inhibits adipocyte differentiation by downregulating expression of key adipogenic genes.

Key words: 3T3-L1 differentiation, adipogenesis, lipolysis, obesity, *Terminalia chebula*

SUMMARY

- This study revealed that the aqueous extract of *Terminalia chebula* fruit "CAFE" had significant inhibitory effect on 3T3-L1 adipogenesis, and it lowered lipid accumulation in differentiating 3T3-L1 cells. The extract also reduced lipolysis in differentiated mature 3T3-L1 adipocytes to a small extent without changing the lipid contents of the cells.



Abbreviations used: A + AD: Adenosine + Adenosine deaminase; ATGL: Adipose triglyceride lipase; C/EBP: CAAT enhancer-binding protein; FABP4: Fatty acid-binding protein 4; FAS: Fatty acid synthase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; HSL: Hormone-sensitive lipase; IBMX: 3-Isobutyl-1-methyl xanthine; ISO: Isoproterenol; LPL: Lipoprotein lipase; ORO: Oil red O; PLN.1: Perilipin1; PPARγ: Peroxisomeproliferative-activated receptor γ; TAG: Triacylglycerol.

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INTRODUCTION

Adipogenesis and its modulation have been an active area of investigation due to the central role played by adipocytes (fat cells) in a number of metabolic disorders. Imbalance in the adipocyte formation and functioning due to genetic, epigenetic, or lifestyle-related factors has been implicated in disorders and diseases such as obesity, type 2 diabetes, cardiovascular problems, and cancer.[1] Hypertrophy and hyperplasia are the two adverse adipocyte scenarios in the adipose tissue, which arise due to failure of intricate regulation of adipogenesis. Hypertrophy is the excess accumulation of triacylglycerol (TAG) in each adipocyte whereas hyperplasia arises upon excess generation of new adipocytes. Increase in adipogenesis beyond basal level may lead to both excess number of adipocytes and enlarged size of adipocytes.[2] Thus, excess adipogenesis is the major determinant of overweight and obesity.[3] Hypertrophic adipocytes also contribute to the adverse changes in the adipose tissue microenvironment causing insulin resistance.

Adipogenesis like many other cell differentiation processes is a two-step process. In the first step, an adipogenic competent cell upon receiving adipogenic stimuli undergoes a clonal expansion phase. In the second stage, adipocytes are formed from those cells by terminal differentiation and maturation. Mature adipocytes accumulate TAG as an intracellular lipid droplet. These cells are highly responsive to action of the hormone

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insulin. Mature adipocytes express a number of factors required for TAG synthesis and lipid droplet formation as well as lipid breakdown. Fatty acid synthase (FAS), perilipin 1 (PLN1), glucose transporter type 4, fatty acid-binding protein 4 (FABP4/aP2), lipoprotein lipase, adipose tri-acyl glycerol lipase, and hormone sensitive lipase are some of the major factors that govern adipocyte functioning. While expression of many of these genes is directly under control of peroxisome proliferator activated receptor γ (PPARγ), others are indirectly regulated by the PPARγ initiated adipogenic program. Thus, PPARγ is at the center of adipogenic regulations and is thought to be essential and sufficient factor for fat cell formation.[4] However, C/CAAT enhancer-binding protein α (CEBPα), which appears after the induction of PPARγ, is a crucial assistant to PPARγ.[5] CEBPα along with PPARγ sets the complete program through which preadipocytes differentiate into adipocytes and start accumulation of lipid. CEBPα knock-out cells differentiate into adipocytes but show insulin resistance.[6] CEBPα deficiency also leads to lesser lipid accumulation in PPARγ expressing adipocytes.[7]

Herbal formulation to modulate adipocyte development and function has been an active area of research to tackle health problems such as obesity and type 2 diabetes. One such herbal formulation with anti-obesity activity is "*Triphala,*" which contains fruits of three endemic plants of India: *Terminalia chebula, Terminalia bellirica*, and *Phyllanthus emblica*. "*Triphala*" is used in Ayurveda since ancient time with two different formulations by mixing *T. chebula, T. bellirica,* and *P. emblica* in the ratio of 1:1:1 or 1:2:4.[8] Previously, it was shown that treatment with "*Triphala*" significantly reduced obesity in high-fat-diet-induced obese mouse.[9] In a recent study, it was found that aqueous extract of "*Triphala*" inhibits lipid accumulation in 3T3-L1 preadipocytes during adipogenic differentiation.[10] However, several studies suggest that the different extracts of the components of "*Triphala*" individually have variable effects on adipocyte differentiation. The hot water extract of the fruit of *T. bellirica* was shown to be pro-adipogenic during 3T3-L1 adipogenic differentiation.[11] On the other hand, organic or aqueous extracts of *T. chebula* and *P. emblica* fruits in animal models showed anti-obesity and hypolipidemic effect, suggesting a possible anti-adipogenic activity of these extracts.[12-16] While a pro-adipogenic agent can increase hyperplasia, an anti-adipogenic agent can resolve it. Thus, a clear understanding of the effects of individual components of "*Triphala*" on adipocyte differentiation and function can lead to the development of a potent anti-obesity formulation. Hence, the current study analyzed the effect of crude aqueous extract of *T. chebula* fruit pulp (named as CAFE) on 3T3-L1 adipogenic differentiation. Results showed that CAFE is anti-adipogenic in nature during 3T3-L1 differentiation. The extract also reduced lipolysis to a small extent without affecting lipid content in mature adipocytes.

MATERIALS AND METHODS

Preparation of the extract CAFE

Fresh, undamaged fruit of *T. chebula* was collected and then washed thoroughly with tap water followed by distilled water. The fruits were deseeded, and then, pulps were washed again with distilled water. Fruit pulps were broken into small pieces and shade-dried. Dried fruit pulps were ground to powder using a domestic mixture grinder. Five grams of the powdered material was mixed with 100 ml of distilled water. The slurry was mixed continuously for 4–5 h and then incubated at 4°C overnight. It was then centrifuged and the supernatant was syringe filtered and lyophilized to obtain the extract named "CAFE" for "*Chebula* aqueous fruit extract." The material was dissolved at desired concentration with sterile water and aliquots were stored at −20°C until used. Each aliquot was thawed once only and further syringe filtered before adding to cell culture.

Cell culture

3T3-L1, L6, RAW 264.7, and HEK293T cells were cultured in medium containing Dulbecco's modified Eagle's medium (DMEM) (HiMedia, AL007A) with 10% fetal bovine serum (FBS) (HiMedia, RM10409) and 1X antibiotic and antimycotic (Invitrogen, 15240062) in a humidified 5% CO_2 incubator at 37°C.

Cell survivability assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to check cell survivability of cultured 3T3-L1 cells on exposure to CAFE as described previously.[17,18] Briefly, 20,000 cells/well was plated in groups and in triplicate in a 96-well plate. Cells were cultured in medium containing DMEM, 10% FBS, and 1X antibiotic and antimycotic (maintenance media) and kept in a 5% CO_2 incubator at 37°C for 24 h. For RAW, L6, and HEK cells 5000 cells/well were seeded. CAFE was introduced to the cells (when confluence is around 70%) at doses of 0–500 μg/ml for 24 h. 20 μL of MTT (5 mg/ml) (Sigma) was added in each group, and then, medium with MTT was replaced carefully not disturbing the cells with 1:1 solution of isopropanol and 0.2M HCl and 11% sodium dodecyl sulfate. The plate was shaken for 15 min and the absorbance was taken at 590 nm.

Differentiation of 3T3-L1 into adipocytes

3T3-L1 cells were induced to 8 days of differentiation assay by standard protocol with few modifications.[19,20] Differentiation was started after 2 days of postconfluency (Day 0) with cocktail containing 1 μM dexamethasone (Sigma D4902), 0.5 mM isobutylmethylxanthine (Sigma I7018), and 5 μg/ml insulin (Sigma I5500) in maintenance media and cultured for 2 days at 37°C with 5% CO_2. Thereafter, the media were replaced with insulin-containing maintenance media and cultured for another 2 days. On day 4, media were changed to maintenance media and cells were cultured for 4 days at 37°C with 5% CO_2 with the change of media after every 2 days.

Oil red O staining

The culture medium of differentiated 3T3-L1 cells was discarded carefully and cells were fixed with 10% formalin for 45 min at room temperature. Intracellular lipid droplets were then stained with method mentioned earlier.[21] Excess stain was removed and then washed with distilled water, followed by 60% isopropanol. This washing was repeated three times, and sterile water was added and quickly images of cells were captured. Finally, the water was discarded, intracellular oil red O (ORO) stain was extracted with 100% isopropanol, and the absorbance was read at 500 nm.

RNA isolation and semiquantitative reverse transcriptase-polymerase chain reaction

TRIzol (Invitrogen) was used to isolate total RNA from the cells as per the manufacturer's instructions. Gene expression study was done by performing reverse transcriptase-polymerase chain reaction (RT-PCR) using specific primer sequences [Table S1] and cDNA was prepared from the total RNA using cDNA synthesis kit (Clontech, Otsu, Japan; 6110A). GelQuant.NET software (BiochemLabSolutions, Wayne, PA, USA) was used for analysis of agarose gel bands. Expression level of all genes was normalized by glyceraldehyde 3-phosphate dehydrogenase.

Glycerol release assay

3T3-L1 cells were seeded at a density of 75,000 cells/well of 96-well plate and differentiated to mature adipocytes. Thereafter, media were discarded and Krebs Ringer buffered with 4% fatty acid-free bovine

serum albumin and 5 mM D-glucose (lipolysis buffer) with or without CAFE (100 μg/ml) or 10 μM isoproterenol (ISO) and incubated for 3 h in CO_2 incubator. In another set of the same treatment groups, to remove adenosine-mediated background inhibition,[22] cells were first treated with 10 μM adenosine (Sigma) for 1 h followed by 3 h of ISO or CAFE treatment in the presence of 1U/ml adenosine deaminase (Sigma). Estimation of glycerol release was performed using glycerol assay kit (Sigma MAK117).

Phytochemical assay

Biochemical assays for the identification of phytochemicals in the extract was performed following methods described before.[23] CAFE was screened for phenolics, flavonoids, saponins, tannins, steroids, and reducing and non-reducing sugars.

Statistical analysis

Unpaired *t*-test was used to find statistical significance wherever applicable.

RESULTS

Tolerable doses of CAFE in selected cultured cell lines

Any drug or extract administered in the body not only interacts with its target tissue but also interacts with other tissues in the body. While the extract should have a desired effect on the target tissue, it should not have any undesired toxicity on the target as well as other tissues. Thus, cytotoxicity of the crude aqueous extract of *T. chebula* fruit pulp (CAFE) was evaluated in cultured cell lines of different tissue origin. The cell lines which were selected to test cytotoxicity are mouse preadipocytes (3T3-L1), rat muscle cells (L6), mouse macrophage cells (RAW 264.7), and human kidney cells (HEK). MTT assay showed that CAFE treatment on 3T3-L1 cells did not affect its survivability [Figure 1a] at the tested doses, i.e., 100–500 μg/ml with an exposure of 24 h [Figure 1a]. CAFE was also found to be noncytotoxic to L6 rat muscle cells at tested does of 100–1000 μg/ml [Figure 1b]. However, the extract was noncytotoxic only till the dose of 250 μg/ml in other two cell lines HEK and RAW 264.7 [Figure 1c and d]. Higher dose of CAFE in these cells showed some toxicity leading to cell survivability below 80% [Figure 1c and d].

Effect of CAFE on lipid accumulation in differentiating 3T3-L1 cells

Lipid accumulation in differentiating 3T3-L1 cells was studied using ORO staining of the intracellular lipids. CAFE was introduced in non-toxic doses (50, 75, 150, and 250 μg/ml) to 3T3-L1 cells undergoing adipogenic differentiation (with use of adipogenic cocktail) for the first 4 days of differentiation. Afterward, cells were allowed to grow in maintenance medium for another 4 days in the absence of the extract. After completion of 8 days of differentiation, intracellular lipids were stained with ORO. Microscopic observation of CAFE treated and untreated cells showed a dose-dependent reduction of lipid accumulation in the treatment groups [Figure 2a]. The reduction in lipid accumulation was as high as 80% at the highest dose of 250 μg/ml. This was confirmed by

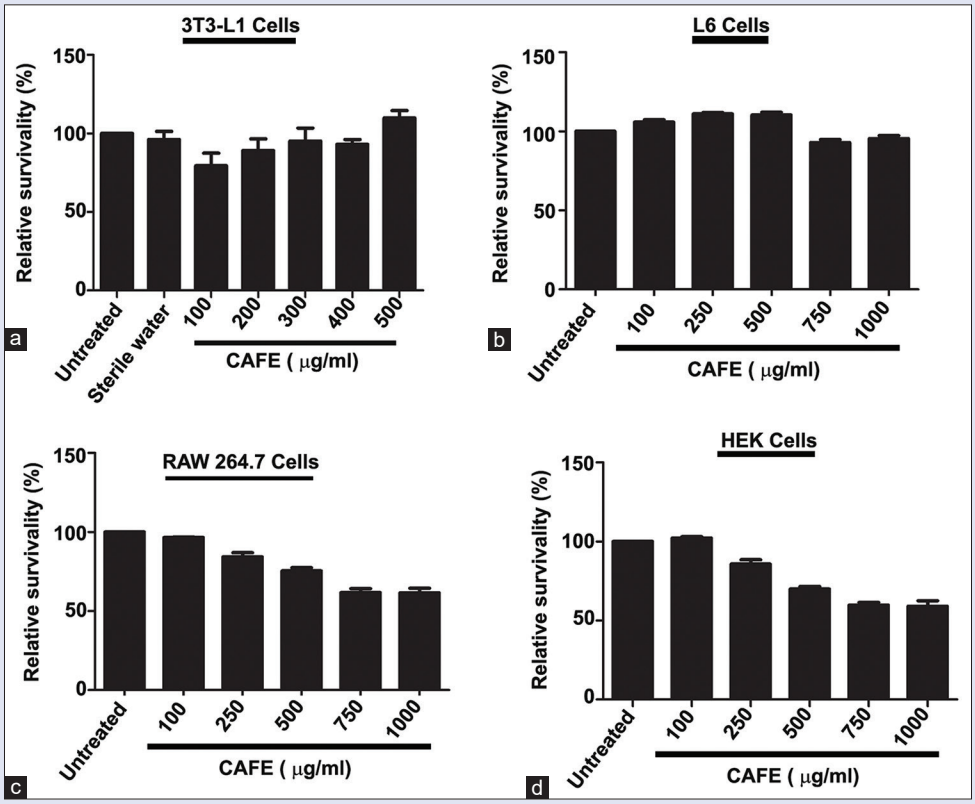

Figure 1: Effect of CAFE on cell survivability of 3T3-L1, L6, RAW 264.7, and HEK 293T cells. (a) CAFE at tested dose of 100–500 μg/ml did not affect cell survivability of cultured 3T3-L1 cells. (b) CAFE did not affect survivability of L6 cells at concentration range of 100–1000 μg/ml. (c) CAFE was noncytotoxic to RAW 264.7 cells till 250 μg/ml of treatment. However, at higher dose of 500–1000 μg/ml, the extract showed negative impact on cell survival. (d) CAFE was noncytotoxic to HEK 293T cells till 250 μg/ml of treatment. Treatments with higher doses showed reduced survivability of HEK 293T cells. $n = 3$. Bars represent means ± standard error of the mean

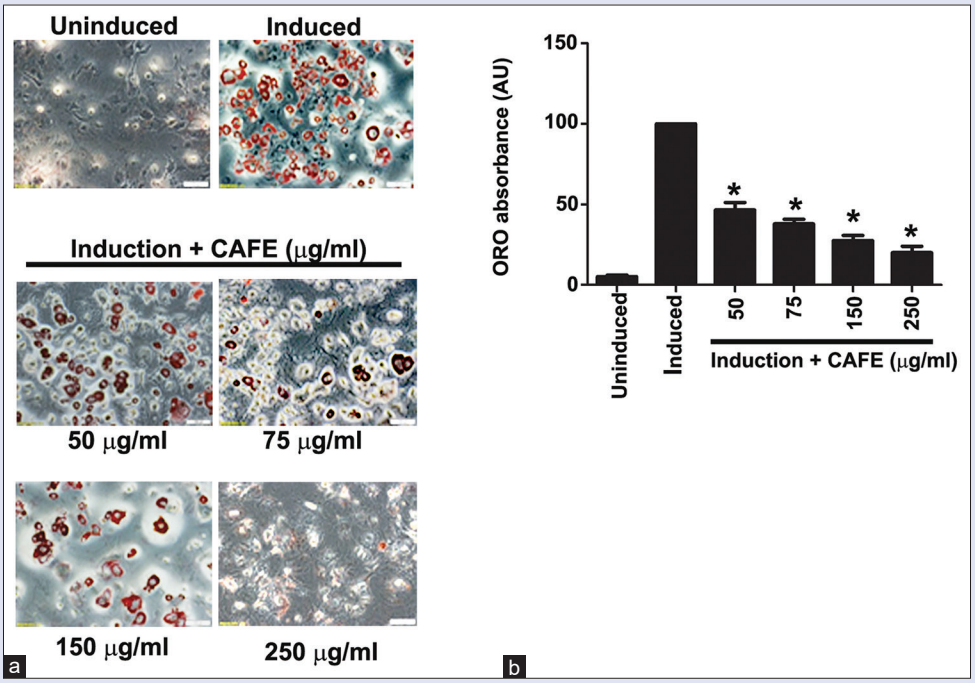

Figure 2: CAFE inhibited lipid accumulation in differentiating 3T3-L1 cells. (a) 3T3-L1 cells were differentiated in the presence or absence of CAFE at mentioned doses for 4 days and then maintained in maintenance medium for another 4 days. Thereafter, oil red O staining was performed. Images represent micrographs of oil red O stained cells. Scale bar: 100 μm. (b) Graph represents absorbance of extracted oil red O from different groups of cells represented in panel A. $n = 3$. Bars represent means ± standard error of the mean. P values are from t-test. $^*P < 0.05$

estimating lipid dissolved ORO stain by eluting and reading the ORO absorbance at 510 nm [Figure 2b]. This showed a significant reduction of lipid accumulation in all the treated groups.

Effect of CAFE on peroxisome proliferator-activated receptor γ and C/CAAT enhancer-binding protein α mRNA expression in differentiating 3T3-L1 cells

PPARγ isoforms and CEBPα are the transcription factors which act as master regulators of the adipocyte differentiation and maturation. Effect of CAFE on the mRNA expression of these transcription factors was tested. 3T3-L1 cells undergoing adipogenic induction was introduced to 50 and 100 μg/ml CAFE in the first 4 days of adipogenic induction, and then, the cells were allowed to grow in maintenance medium for another 4 days. Thereafter, the cells were harvested and expression of PPARγ isoforms and CEBPα was measured by semi-quantitative RT-PCR analysis [Figure 3a]. Dose-dependent and significant reduction in the mRNA content of both the isoform of PPARγ, namely PPAR γ1 and PPAR γ2 [Figure 3c and d], was observed in CAFE-treated cells. The extract also significantly reduced expression of CEBPα, a co-regulator of PPARγ, in a dose-dependent manner [Figure 3b].

Effect of CAFE on perilipin 1, fatty acid-binding protein 4, and fatty acid synthase mRNA expression in differentiating 3T3-L1 cells

During adipogenic differentiation, PPARγ isoforms and CEBPα directly or indirectly regulate the induction of several genes which are crucial for lipid biogenesis and accumulation. PLN1, FABP4, and FAS are three such genes which play a very important role in this process. 3T3-L1 cells received the induction and CAFE treatment in initial 4 days of adipogenic differentiation and then allowed to mature for another 4 days. After 8 days of adipogenic differentiation, the mRNA expression of the genes was measured by semi-quantitative RT-PCR analysis [Figure 4a]. CAFE treatment reduced a significant amount of mRNA expression of FAS at higher dose of 100 μg/ml [Figure 4b]. CAFE treatment reduced the expression of lipid droplet coat protein PLN1 significantly. This decrease was dose dependent [Figure 4c]. However, the expression of adipocyte FABP4/aP2 was not much affected by CAFE treatment [Figure 4d].

Effect of CAFE on maturation of differentiated 3T3-L1 cells

After differentiation of preadipocytes into adipocytes, these cells mature by continuous accumulation of lipids and formation of large lipid droplets in the cytoplasm. To test the effect of CAFE in the post-differentiation maturation phase of adipogenesis, fully differentiated 3T3-L1 adipocytes at 8 days post-differentiation were treated with CAFE followed by measurement of lipid content. However, 4 days treatment of fully differentiated adipocytes with CAFE showed no decrease in the accumulated lipids in tested doses of 50, 75, 150, and 250 μg/ml [Figure 5a]. Lipid content was measured by elution and spectrophotometric measurement of ORO from the stained cells and was found unchanged in CAFE-treated cells at all the doses [Figure 5b]. Effect of CAFE on lipolysis was also measured by estimating glycerol released in the cell culture medium. Fully mature adipocytes were treated with 100 μg/ml CAFE for 3 h, and thereafter, glycerol released was measured. While the known lipolytic inducer ISO (10 μM) increased glycerol release significantly beyond the basic level of lipolysis, CAFE treatment did not show such increase [Figure 5c]. Instead, the extract somewhat reduced the glycerol release [Figure 5c]. Even though the reduction was less, it was consistent. Similar scenario was also observed when the background lipolysis was eliminated with the use of adenosine followed by adenosine deaminase. CAFE did not stimulate glycerol release from adipocytes [Figure 5d] rather reduced it consistently. This suggested that CAFE treatment may not increase lipolysis in mature adipocytes rather can decrease it.

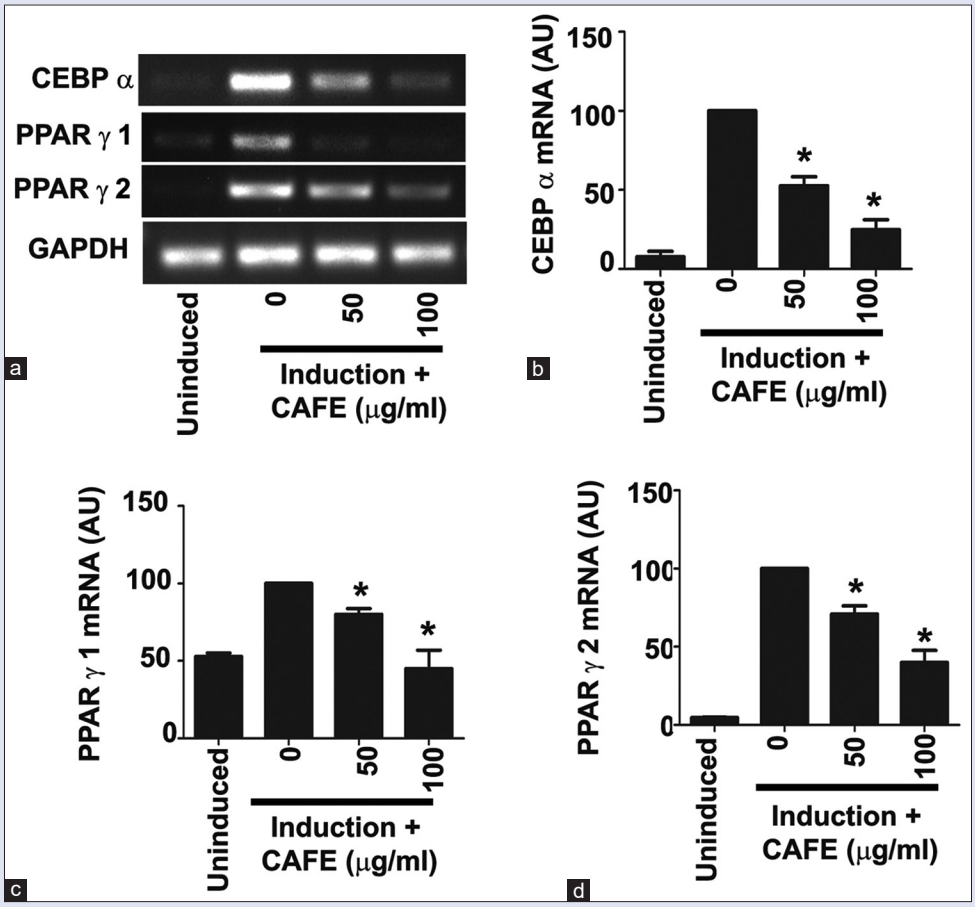

Figure 3: CAFE-reduced expression of key adipogenesis regulators. (a) Expression of peroxisome proliferator-activated receptor γ 1, peroxisome proliferator-activated receptor γ 2, and C/CAAT enhancer-binding protein alpha were analyzed by semiquantitative reverse transcriptase-polymerase chain reaction. Cells were induced for differentiation ± CAFE (50 and 100 μg/ml) in the same manner as described in Figure 2. (b) Bars represent normalized expression of C/CAAT enhancer binding protein alpha expression after 8 days of adipogenic induction. (c) Bars represent normalized expression of peroxisome proliferator-activated receptor γ 1 after 8 days of induction. (d) Normalized mRNA expression of peroxisome proliferator-activated receptor γ 2 after 8 days of induction. Bars represent mean ± standard error of the mean $n = 2$. P values are from t-test. *$P < 0.05$

Phytochemical study of the extract CAFE

Phytochemical analysis of the extract CAFE showed the presence of saponins, cardiac glycosides, and phenolics. All the tests for flavonoids showed negative results, suggesting the absence of these groups of molecules in the extract. Total phenolic content of CAFE was found to be 317.53 μg of phenolic content of gallic acid equivalent/mg of extract. Estimated total tannin content in the CAFE was 235.38 μg tannin/mg of lyophilized extract [Table 1].

DISCUSSION

In search of a phytoextract with adipogenesis modulating activity, the current study explored the crude aqueous extract of medicinal herb *T. chebula* fruit pulp (CAFE). The non-toxic doses of the extract could inhibit differentiation of 3T3-L1 preadipocytes very effectively. While at the lowest tested dose of 50 μg/ml, the inhibition was over 50%, at the highest tested dose of 250 μg/ml, 80% inhibition of 3T3-L1 differentiation was observed. *T. chebula* is also one of the components of well-known Ayurvedic formulation "*Triphala*" which contains fruits of three medicinal plants: *T. chebula*, *T. bellirica,* and *P. emblica* in the ratio of 1:1:1 or 1:2:4.[8] A recent report showed that the aqueous extract of "*Triphala*" is an efficient inhibitor of 3T3-L1 differentiation, and at the dose of 10 μg/ml itself, adipocyte differentiation was inhibited.[10] However, an important difference in the treatment regimen of that study and the current study exists. While in the earlier study, cells were treated with the "*Triphala*" extract throughout differentiation and maturation of adipocytes, in the current study, the treatment was done only during the induction of the adipogenesis (first 4 days). The current study also showed that unlike organic extraction of *T. chebula* (which led to the isolation of compounds such as chebulagic acid from *T. chebula*) which showed pro-adipogenic effect,[24] aqueous extraction from fruit pulp of *T. chebula* provides anti-adipogenic activity. This could be due to the presence of higher amount of tannic acid in CAFE. *T. chebula* is reportedly rich in tannic acids.[25] Earlier reports showed as high as 32% of tannic acid in *T. chebula*.[26] Tannic acid was reported to reduce adipogenesis in 3T3-L1 cells.[21,27] Tannic acid also reduces lipolysis in mature adipocytes.[21,28] The total tannin content in CAFE was estimated at 235.38 μg tannin per mg of CAFE. High tannin content in the CAFE suggested that tannins like tannic acid could be the probable bioactive molecules in the extract responsible for its anti-adipogenic activity.

Gene expression pattern observed in CAFE-treated cells strongly supported the observed phenotype of suppressed adipogenesis. PPARγ and CEBPα are the two very important transcription factors which are considered as the master regulators of the adipogenic program and show strong induction during the process of adipogenesis.[21] Inhibited mRNA expression of both the transcription factor genes in CAFE-treated

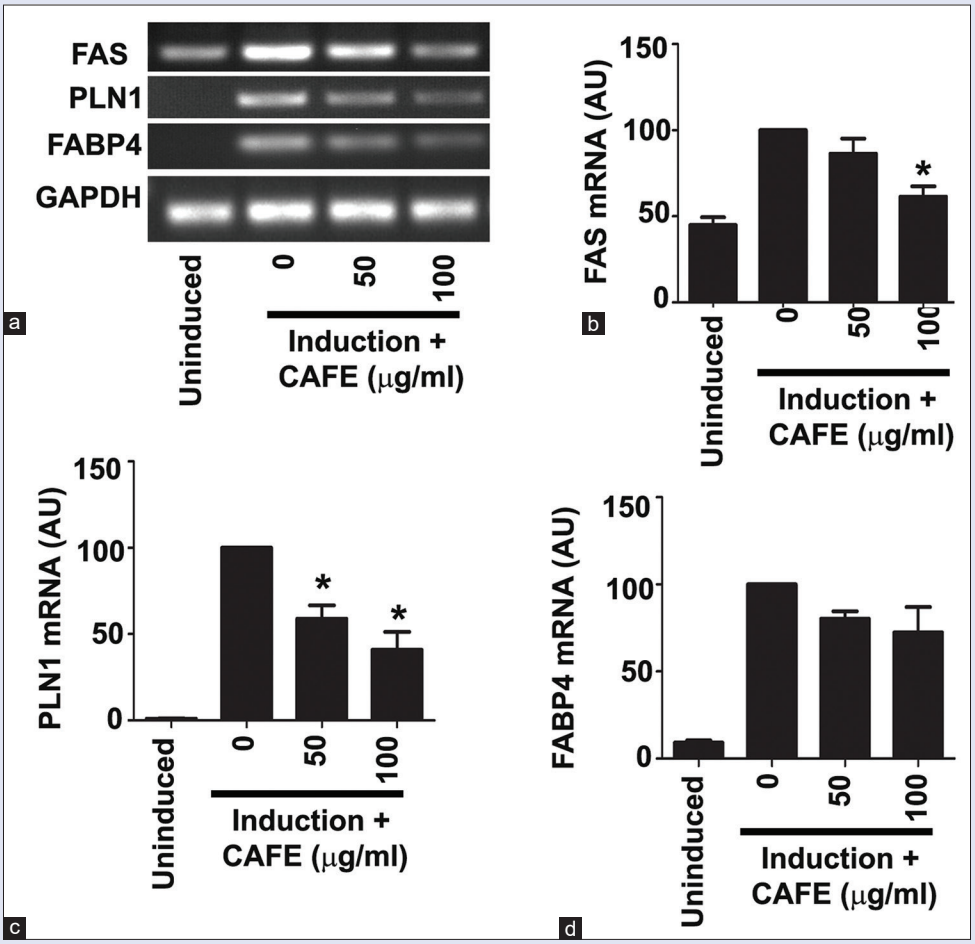

Figure 4: CAFE reduced expression of key adipogenic effector genes, perilipin 1, and fatty acid synthase. Expression of perilipin 1 and fatty acid synthase transcripts were reduced in CAFE-treated group of induced 3T3-L1 cells. However, CAFE had no significant effect on the mRNA expression of fatty acid-binding protein 4. Cell differentiation and treatments were same as in Figure 3. (a) Representative gel images of amplified adipogenic effector gene transcripts by semi-quantitative reverse transcriptase-polymerase chain reaction. (b) Bars represent normalized expression of fatty acid synthase after 8 days of induction. (c) Normalized expression of perilipin 1 after 8 days of induction. (d) Bars represent normalized expression of fatty acid-binding protein 4 after 8 days of induction. Values in the bar diagrams represent mean ± standard error of the mean $n = 2$. P values are from t-test. *$P < 0.05$

Table 1: Phytochemicals in *chebula* aqueous fruit extract (CAFE)

Test name	Test for	CAFE
Foam test	Saponins	Positive
Phosphomolybdic acid test	Phenolics	Positive
Ferric chloride test	Phenolics	Positive
NaOH test	Flavonoids	Negative
Shinoda test	Flavonoids	Negative
Zn HCl test	Flavonoids	Negative
Braemer's test	Tannins	Positive
Salkowski test	Steroids	Negative
Fehling test	Reducing sugars	Positive
Iodine test	Nonreducing sugars	Negative
Bradford	Proteins	Negative
Legal test	Cardiac glycosides	Positive
Total phenolic content		317.53 μg of phenolic content of gallic acid equivalent/mg of lyophilized extract
Total tannin content		235.38 μg tannin/mg of lyophilized extract

preadipocytes suggested that CAFE regulates adipogenesis by regulating PPARγ and CEBPα gene expression. Earlier reports showed that while aqueous extracts of "*Triphala*" and *P. emblica* fruits had inhibited expression of these two transcription factors, gallic acid from *T. bellirica* had no significant effect on the expression of these two transcription factors.[11] As a consequence of downregulation of these two transcription factors, CAFE treatment inhibited expression of important downstream target genes of these two transcription factors, PLN1,

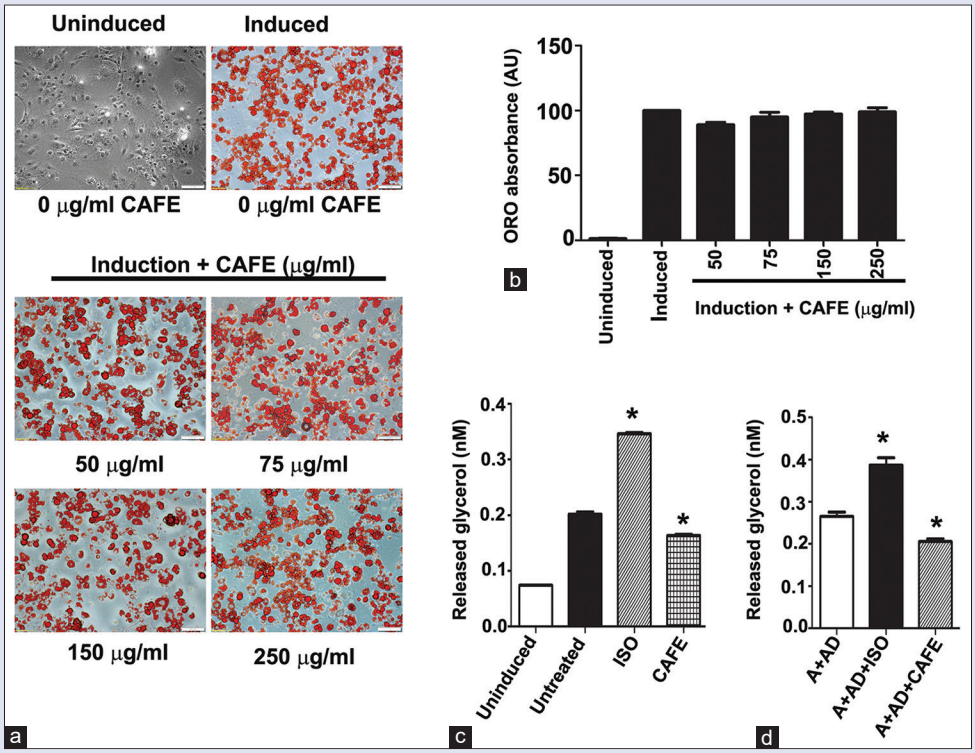

Figure 5: CAFE did not reduce accumulated lipid in mature 3T3-L1 adipocytes, but reduced lipolysis to a small extent. (a) Analysis of lipid content by oil red O staining in fully differentiated 3T3-L1 adipocytes treated with CAFE. 3T3-L1 cells were differentiated to mature adipocytes followed by treatment with CAFE (50–250 μg/ml) for 4 days. Images represent micrographs of oil red O stained cells. Scale bar: 100 μm. (b) Graph represents absorbance of extracted oil red O from different groups of cells represented in panel A. (c and d). CAFE consistently reduced glycerol release from mature adipocytes to a small extent. Mature adipocytes were treated with CAFE or isoproterenol with (panel D) or without (panel C) adenosine (panel A) and adenosine deaminase treatment. Thereafter, glycerol release in the medium was assayed. Bars represent means ± standard error of the mean $n = 3$. P values are from t-test. *$P < 0.05$

which is required for lipid accumulation in the adipocytes.[29] PLN1 is a protein that coats lipid droplets in adipocytes. It protects triglycerides from lipase-mediated degradation and hence regulates the process of lipolysis.[30] Reduced mRNA expression of PLN1 in the CAFE-treated cells can have adverse effect on lipid droplet biogenesis and hence reduce adipogenesis. Adipogenesis requires induced expression of FAS, which synthesizes fatty acids required for the production of triglycerides and biogenesis of lipid droplets.[31] FAS was downregulated to a significant extent by treatment with higher concentration of CAFE. Reduction of FAS expression may lead to reduced production of triglycerides and reduced biogenesis of lipid droplets. Fatty acids produced by FAS activity also act as ligands for PPARγ.[31] Thus, the activity of the PPARγ may also be inhibited by CAFE treatment. Expression of FABP4, which is involved in intracellular transport of fatty acids and triglyceride metabolism, is also induced strongly during adipogenesis.[30] However, CAFE did not change the mRNA expression of FABP4. Intriguingly, the expression of FABP4 was inhibited by aqueous extract of "*Triphala.*" This is possibly due to the effect of other two fruits in the formulation.

Treatment of mature adipocytes with CAFE had consistently reduced lipolysis to a small extent without affecting the overall lipid contents of mature adipocytes. Elevated free fatty acids generated due to lipolysis are known to cause insulin resistance in obesity-associated type 2 diabetes conditions.[32] Earlier studies showed that partial reduction of lipolysis can reduce the level of free fatty acids and thus can improve glucose homeostasis without affecting body mass.[33] Thus, it will be important to investigate the effect of CAFE on glucose homeostasis.

Excess adipogenesis beyond basal level can lead to excess number of adipocytes (hyperplasia) as well as excess accumulated fat in the adipocytes (hypertrophy). Both of these conditions exist in obese adipose tissue. The inhibition of adipocyte differentiation can reduce the formation of new adipocytes and thus reduce hyperplasia. However, the reduction of lipid in mature adipocytes happens through lipolysis. However, recent studies have clearly shown an inverse correlation of lipolysis with insulin sensitivity.[32,34] Among the components of "*Triphala,*" while aqueous extract of *T. bellirica* is pro-adipogenic, combined extract of *P. embilica* and *T. chebula* is anti-adipogenic.[11,35] However, the combined extract also increases lipolysis, which may cause undesired insulin resistance.[35] Effect of "*Triphala*" aqueous extract on lipolysis yet to be tested. Thus, the unique combinations of anti-adipogenic and anti-lipolytic property of CAFE make it a good candidate alone or in combination with other extracts to be developed into an effective agent for the management of obesity and associated disorders.

CONCLUSION

CAFE is an anti-adipogenic and anti-lipolytic agent which inhibits adipocyte differentiation by downregulating the expression of key adipogenic genes.

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Conflicts of interest

There are no conflicts of interest.

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