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ORIGINAL ARTICLE

Triphala, Regulates Adipogenesis through Modulation of Expression of Adipogenic Genes in 3T3-L1 Cell Line

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ABSTRACT

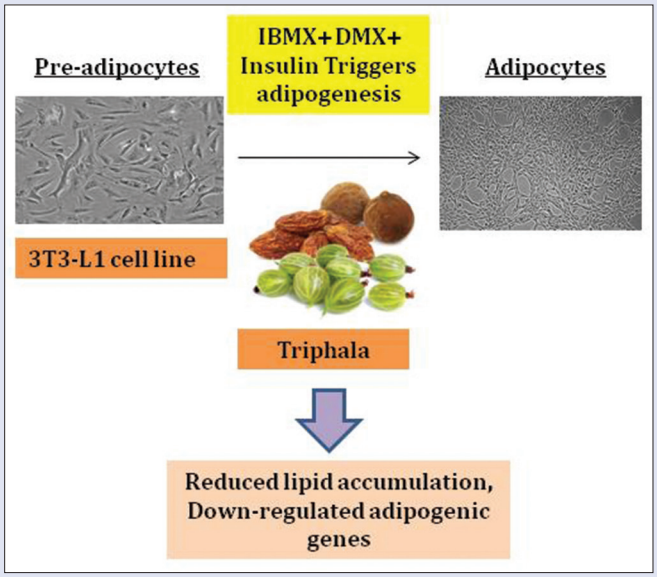
Background: Triphala, an Ayurvedic polyherbal formulation, is used for the treatment of various diseases including obesity. **Objective:** The present study was planned to evaluate the anti-adipogenic potential of aqueous extract of Triphala (TP_{aq}) using 3T3-L1 adipocyte cell line model. **Methods:** The effect of aqueous extract of Triphala (TP_{aq}) was tested on the viability of 3T3- L1 cells by MTT assay. The cells were treated with a cocktail of dexamethasone (DEX), isobutylmethylxanthine (IBMX) and insulin to induce adipogenesis. The cells were treated either with the induction cocktail or with the cocktail containing different concentrations (1, 10 and100 µg/ml) of TP_{aq}. Intracellular lipid content was analyzed using Oil O Red stain and was quantified after extracting with isopropanol at 500 nm wavelength. The expression of early (PPAR-γ and C/EBP-α) and late (GLUT4 and FAS) phase adipogenic genes was studied by real time PCR. **Results:** TP_{aq} did not affect the viability of 3T3-L1 cell line. Interestingly, TP_{aq} induced a concentration dependant decrease in the intracellular lipid content and expression of both early and late phase adipogenic genes. This decrease was statistically significant compared to cells treated with only induction cocktail. **Conclusion:** These results suggested that Triphala regulated lipid accumulation by down regulating expression of adipogenic genes, resulting into prevention of adipogenesis.

Keywords: Adipocyte, Obesity, Lipid accumulation, 3T3-L1

SUMMARY

- The purpose of this study was to evaluate the effect of an ayurvedic polyherbal drug Triphala on adipogenesis using 3T3-L1 cell line. The results suggested that Triphala regulated lipid accumulation by downregulating expression of adipogenic genes, resulting into the prevention of adipogenesis.

Abbreviations used: TP_{aq}: Aqueous extract of Triphala; DMEM: Dulbecco's Modified Eagle's medium; FBS: Fetal Bovine Serum; IBMX: Isobutyl methylxanthine; DMX: Dexamethasone; MTT: [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay; PPARγ: Peroxisome proliferator-activated receptor; C/EBP:Enhancer binding protein α, FAS:Fatty acid synthase; Glut-4: Glucose phosphate transporter 4.



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INTRODUCTION

The cause of obesity is related to formation of adipocytes (fat cell) through adipogenesis which leads to enhanced lipid accumulation. Adipogenesis involves differentiation of preadipocytes into mature adipocytes by clonal cell expansion.[1] The process is highly regulated by various transcriptional factors from early to terminal phase of adipogenesis.[2] 3T3-L1 cell line is a well-defined *in vitro* model for studying adipogenesis and glucose uptake mechanism. The differentiation of 3T3-L1 cell requires synergistic activity of multiple transcription factors and adipogenic modulatory factors including peroxisome proliferator-activated receptor-gamma, CCAAT/enhancer-binding protein (C/EBP), fatty acid synthase, and so on.[3]

Various medicinal plants have been studied for their inhibitory effect on adipogenesis. In the present study, we selected Triphala, an ayurvedic polyherbal formulation comprising of equiproportional fruit parts of *Terminalia chebula*, *Terminalia bellerica*, and *Phyllanthus emblica*. Our previous report on drug usage of Triphala by ayurvedic practitioners and the survey of marketed antiobesity herbal formulations have demonstrated wide use of Triphala in the management of

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obesity.[4] The ingredients of Triphala have been studied individually or in combination with other medicinal plants for antioxidant, antidiabetic, antihypercholesteremic, and antiobesity activities.[5] *Phyllanthus emblica* has been reported to induce minimal adipocyte differentiation and stimulate glucose uptake.[6] *T. bellerica* and *T. chebula* have been found to be effective in reducing lipid accumulation in adipocytes.[7] However, the whole formulation has not yet been studied for its effect on adipogenesis. With this background, we have evaluated the antiadipogenic activity of aqueous extract of Triphala (TP_{aq}) by studying its effect on lipid accumulation and expression of early- and late-phase adipogenic genes in 3T3-L1 fibroblast cell line.

MATERIALS AND METHODS

Materials

3T3-L1 cells were obtained from National Centre for Cellular Science (NCCS, Pune, India). Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other chemicals used in study such as trypsin, insulin, isobutylmethylxanthine (IBMX), dexamethasone, isopropanol, and oil O red stain were purchased from Sigma-Aldrich, US. The primers and reagent used for gene expression study were purchased from Invitrogen, Waltham, Massachusetts, USA.

Cell culture

3T3-L1 cell line was maintained in DMEM containing 10% FBS in a humidified atmosphere of 5% CO_2 at 37°C. Once the cells were 80% confluent, differentiation to adipocytes was induced using a cocktail of 0.57 µg/ml of insulin, 0.5 mM of IBMX, and 0.25 µM of dexamethasone for 48 h. This was followed by culturing of cells in DMEM supplemented with 10% FBS with or without aqueous extract of Triphala (TP_{aq}) and the media was changed after every 2 days up to 8 days.

Drug

Standardized TP_{aq} was procured from Pharmanza Herbal Pvt. Ltd. Petlad, Gujarat, India [Appendix 1]. Different concentrations of TP_{aq}, namely, 1, 10, and 100 µg/ml were prepared by dissolving the powdered extract (5 mg/µg) in sterile distilled water. The doses were prepared in DMEM according to the concentration of the stock solution.

Cell viability

The effect of TP on viability of 3T3-L1 cells was studied using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. 3T3-L1 preadipocytes were grown in a 96-well plate with 1, 10, and 100 µg/mL concentrations of TP_{aq} for 24 h. Cell viability was also determined on day 4 and on day 8 of the experiments in 3T3 cells treated with induction cocktail and 1, 10, and 100 µg/mL concentrations of TP_{aq}. Cells were incubated with MTT for 24 h[8] to develop crystals. Insoluble formazan crystals were then dissolved in isopropanol, and absorbance was measured between 570 and 630 nm using microplate reader (FLUOstar Omega, BMG LABTECH GMBH Ortenberg, German).

Cell morphology

Cell differentiation and formation of lipid droplets was seen under inverted microscope from day one onward. On the last day of experiment, cells were washed gently with Phosphate Buffered Saline (PBS) twice, fixed with 10% fresh formaldehyde in PBS for 30 min at room temperature, and stained by oil O red solution with 70% isopropanol and water (1:1) for 1 h. After staining of lipid droplets with water, the oil O red solution was removed and the plates were rinsed with water and dried. Images were taken on fluorescent microscope (FLoid cell imaging station, life technologies, India).

Quantification of lipid accumulation

The intracellular lipid accumulation of the control and treated cells was analyzed using ImageJ software, and the density histograms of images were plotted. Further, the dye retained in the cells was eluted with isopropanol and quantified by measuring optical absorbance at 500 nm using microplate reader (FLUOstar Omega, BMG LABTECH GMBH Ortenberg, German).

Quantitative real-time polymerase chain reaction

For studying the effect of TP_{aq} on expression of adipogenic genes, 2 early-phase genes, namely, peroxisome proliferator-activated receptor-gamma (PPARγ) and C/EBP-α and 2 late-phase genes, namely, glucose transporter receptor 4 (GLUT4) and fatty acid synthase (FAS) of adipogenesis were selected. Total RNA was isolated on the 8th day of the experiment using standard laboratory protocol. Two µg of total RNA were used to synthesize cDNA using reverse transcriptase. After reverse transcription, samples were examined by SYBR premix Ex Taq using Takara Thermal Cycler Dice Real Time System (Otsu, Shiga, Japan). Each cDNA was amplified (95°C for 5 s, 58°C–64°C for 10 s, and 72°C for 20 s for 40 cycles) using gene-specific primers designed from sequences acquired from NCBI nucleotide sequence database [Table 1]. All reactions were done in triplicates and the gene expressions were normalized using glyceraldehyde-3-phosphate dehydrogenase as reference gene.

Statistics

The data were shown as mean ± standard deviation and analyzed using one-way ANOVA test with Tukey's multiple comparison test. $P < 0.05$ was considered as level of significance. The data were analyzed using GraphPad Prism 6 Software, San Diego, California, USA.

RESULTS

Cell viability studies with aqueous extract of Triphala

Initially, 3T3-L1 cells were treated with different concentrations (1–100 µg/mL) of TP_{aq} for a period of 24 h to test its effect of on cell viability. TP_{aq} *per se* was found to be nontoxic to the cells at the tested concentrations [Figure 1a]. Further, the effect TP_{aq} on the cell viability of 3T3-L1 was observed after the 4th and 8th days of treatment with the induction cocktail (containing insulin, IBMX, and dexamethasone) alone [Figure 1b and c]. The viability of the cells was not affected after treatment with induction cocktail containing TP_{aq}. However, at 100 µg/ml, there was a significant ($P < 0.001$) reduction in the percentage of viable cells (74.71 ± 3.86%) after 8 days.

Changes in cell morphology with aqueous extract of Triphala

The control cells revealed spindle-like appearance of fibroblasts under inverted microscope. Contrarily, the cells treated with induction cocktail alone and with different concentrations of TP_{aq} showed the presence of mature spherical-shaped adipocytes with lipid droplets. The extent of lipid droplets was less in TP_{aq}-treated cells compared to induction cocktail-treated cells [Figure 2].

Aqueous extract of Triphala reduced lipid accumulation

On day 8 of experiment, cells treated with induction cocktail showed ~2.2-fold increase in lipid content as compared to control cells ($P < 0.001$) [Figure 3a]. The cells treated with TP_{aq} showed ~1.43-,

Table 1: Primers sequence used for reverse transcription-polymerase chain reaction

Genes	Forward primer	Reverse primer	Temperature (°C)
PPARγ	CTTGTGAAGGATGCAAGGGT	ATACAAATGCTTTGCCAGGG	62
C/EBP-α	TTGAAGCACAATCGATCCATCC	GCACACTGCCATTGCACAAG	64
GLUT4	GCTTTGTGGCCTTCTTTGAG	CAGGAGGACGGCAAATAGAA	60
FAS	AGCACTGCCTTCGGTTCAGTC	AAGAGCTGTGGAGGCCACTTG	62
GAPDH	TGAACGGGAAGCTCACTGG	TCCACCACCCTGTTGCTGTA	58-64

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; FAS: Fatty acid synthase; GLUT4: Glucose transporter receptor 4; PPARγ: Peroxisome proliferator-activated receptor-gamma; C/EBP- α: Enhancer-binding protein-alpha

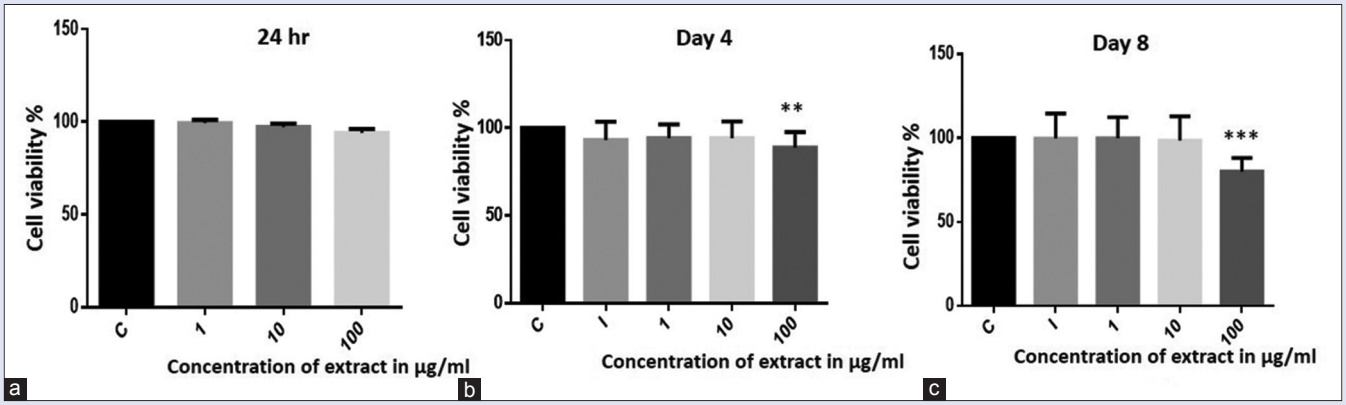

Figure 1: Effect of true positive on viability of 3T3-L1 preadipocytes cells (a) Only true-positive treatment (b) on day 4 when incubated with induction cocktail (c) on day 8 when incubated with induction cocktail *P < 0.01, **P < 0.01, and ***P < 0.001 as compared to control using one-way ANOVA followed by Tukey's posttest

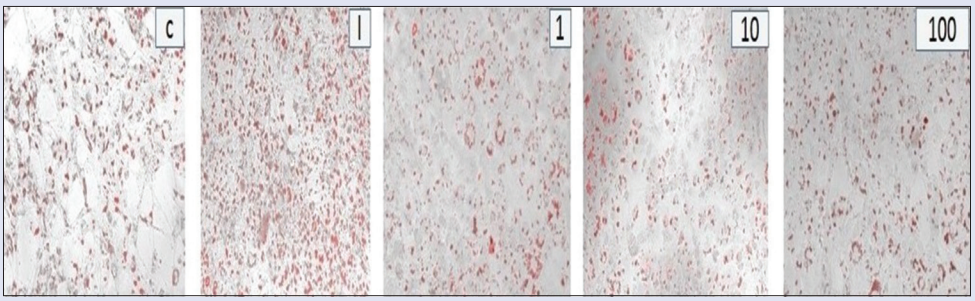

Figure 2: Cell morphology of control (c), induction (i), concentration of Triphala 1, 10, and 100 µg/ml

1.67-, and 2.5-fold decreases in lipid content at 1, 10, and 100 µg/ml concentrations, respectively, compared to the cells treated with induction cocktail alone. The decrease was statistically significant (P < 0.001).

The intracellular lipid accumulation was quantified further. The cells treated with induction cocktail resulted in ~2-fold increase in lipid content compared to untreated control cells (P < 0.001). On the other hand, the cells treated with TP_{aq} showed ~1.33-, 1.45-, and 1.78-fold decrease in lipid content at 1, 10, and 100 µg/ml concentrations, respectively, which was statistically significant (P < 0.001) compared to the cells treated with induction cocktail alone [Figure 3b].

Aqueous extract of Triphala downregulated adipogenic gene expression

The mechanism of TP_{aq} was studied using relative mRNA expression of genes involved in adipogenic process. All the four studied genes, namely, PPAR-γ, C/EBP-α, GLUT4, and FAS showed ~20-fold increase in mRNA expression after 8 days of treatment with induction cocktail compared to the untreated control cells (P < 0.001). The treatment with TP_{aq} decreased the mRNA expression of all the genes in a concentration-dependent manner.

The expression of PPAR-γ was decreased by ~5, 11, and 30 folds at 1, 10, and 100 µg/ml concentrations, respectively, compared to cells treated with induction cocktail alone [Figure 4a]. Interestingly, expression of C/EBPα was decreased by ~8, 12, and 60 folds at 1, 10, and 100 µg/ml concentrations, respectively. This decrease was significant statistically (P < 0.001) at all studied concentrations [Figure 4b].

TP_{aq} treatment reduced mRNA expression of GLUT4 by ~3, 16, and 45 folds [Figure 4c] whereas expression of FAS was found to be decreased by ~5, 10, and 30 folds at 1, 10, and 100 µg/ml concentrations, respectively [Figure 4d]. This reduction was statistically significant (P < 0.001).

DISCUSSION

The present study reports the antiadipogenic potential of TP_{aq} in 3T3-L1 cell line model. The cells treated with TP_{aq} reduced lipid accumulation and downregulated expression of adipogenic genes. Various plants

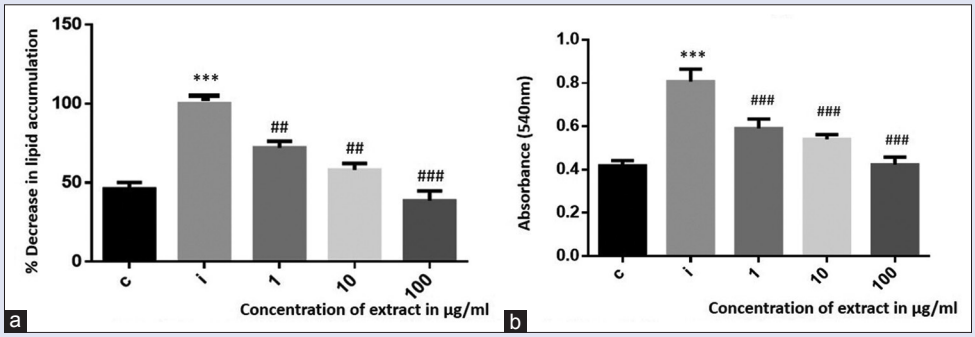

Figure 3: Intracellular lipid accumulation using (a) ImageJ software density histogram (b) isopropanol extraction method. ***$P < 0.001$ as compared to control, ##$P < 0.01$, ###$P < 0.001$ as compared to induction using one-way ANOVA followed by Tukey's posttest

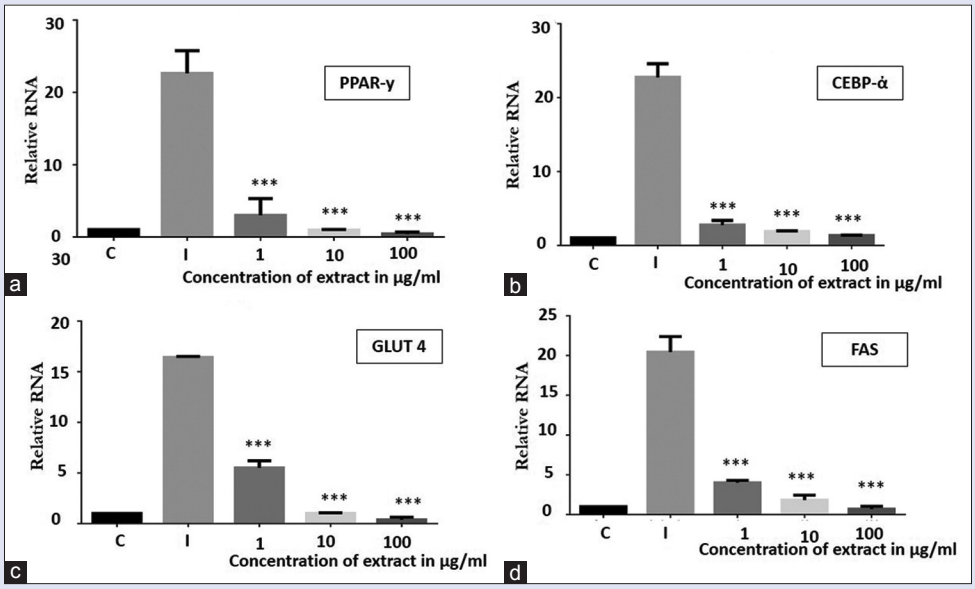

Figure 4: Relative mRNA expression of (a) peroxisome proliferator-activated receptor-gamma, (b) C/enhancer-binding protein-alpha, (c) glucose transporter receptor 4 and (d) fatty acid synthase. ***$P < 0.001$ as compared to induction using one-way ANOVA followed by Tukey's posttest

such as *Curcuma longa*, *Moringa oleifera*, *Murraya koenigii*,[9] D-seco limonoids of *Swietenia mahogani*,[10] Platyphylloside isolated from *Betula platyphylla*,[11] and yanggyuksanhwa-tang[12] have been reported to exhibit antiadipogenic activity in 3T3 cell line model. It has been proposed that different phytoconstituents that target different stages of the adipocyte life cycle might prove beneficial for decreasing lipid accumulation, inducing apoptosis, or by inhibiting adipogenesis or both.[13]

Triphala is the most common formulation used by ayurvedic physicians and is marketed as one of the formulations against obesity. A previous study using high-fat diet-induced obesity in animal model has reported lipid-lowering activity of Triphala.[14] Even though the individual ingredients of Triphala have been studied for their antiadipogenic potential *in vitro*, the whole formulation has not been tested for its effect on adipogenesis.

The programmed differentiation of preadipocytes to fully differentiated adipocytes with increased lipid accumulation throughout the adipogenic process is accompanied by an increase in the expression of various transcription factors and adipocyte-specific genes.[15]

During differentiation, the action of adipogenic genes, which include members of the C/EBP family (C/EBP-α, -β, and -δ) and PPARγ, induces adipogenesis.[16] C/EBP-β is expressed early in the adipocyte differentiation program, and it initiates mitotic clonal expansion. In response to an adipogenic induction, C/EBP-β and-δ are first activated to promote PPARγ and C/EBPα expression.[17] The transcription factor PPARγ is a master regulator of adipocyte differentiation, and its activation is both necessary and sufficient for adipocyte differentiation.[18] The activation of C/EBPα and PPARγ leads to terminal differentiation by inducing the transactivation of many adipocyte genes encoding proteins and enzymes responsible for maintaining the adipocyte phenotype such as FAS.[19] In the present study, TP_{aq} downregulated mRNA expression of both PPARγ and C/EBPα. *Phyllanthus emblica*, one of the ingredients of Triphala, has been shown to inhibit PPARγ.[20] Interestingly, the whole formulation modulates these two important genes involved in regulation of adipogenesis.

A number of genes are involved in adipocyte lipid accumulation. FAS regulates *de novo* lipogenesis from acetyl-CoA, malonyl-CoA, and nicotinamide adenine dinucleotide phosphate and is expressed at high levels in adipose tissue, liver, and lung.[21] GLUT4 is the insulin-regulated glucose transporter found primarily in adipose tissues and striated muscle (skeletal and cardiac). Its expression is increased during adipocyte differentiation, and it maintains glucose homeostasis in

differentiated and insulin-responsive cells.[22] We demonstrated that TP_{aq} downregulated mRNA expression of GLUT4 and FAS which are responsible for lipid accumulation in adipocytes.

All these data suggest that Triphala could be explored as potential drug candidate in regulating obesity.

CONCLUSION

Triphala significantly decreased the adipogenesis in 3T3-L1 cells by reducing lipid accumulation and inhibiting the expression of adipogenic genes. These results confirm the antiobesity potential of Triphala.

Acknowledgment

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Cristancho AG, Lazar MA. Forming functional fat: A growing understanding of adipocyte differentiation. Nat Rev Mol Cell Biol 2011;12:722-34.
2. Sargis RM, Johnson DN, Choudhury RA, Brady MJ. Environmental endocrine disruptors promote adipogenesis in the 3T3-L1 cell line through glucocorticoid receptor activation. Obesity (Silver Spring) 2010;18:1283-8.
3. Ntambi JM, Young-Cheul K. Adipocyte differentiation and gene expression. J Nutr 2000;130:3122S-6.
4. Banjare JB, Bhalerao S. A survey of marketed ayurvedic/herbal anti-obesity products. Int J Pharm Pharm Sci 2016;8:384-6.
5. Hamid KS, Ranjbar SH, Esfehani MM, Mohammad K, Larijani B. A systematic review of the antioxidant, anti-diabetic, and anti-obesity effects and safety of triphala herbal formulation. J Med Plants Res 2013;7:831-44.
6. Kalekar S, Karve A, Munshi R, Bhalerao S. Evaluation of the adipogenic potential and glucose uptake stimulatory activity of *Phyllanthus emblica* and *Tinosporacordifolia*: An *in Vitro Study*. Int J Pharm Bio Sci 2012;3:231-2.
7. Yang MH, Vasquez Y, Ali Z, Khan IA, Khan SI. Constituents from *Terminalia* species increase PPARα and PPARγ levels and stimulate glucose uptake without enhancing adipocyte differentiation. J Ethnopharmacol 2013;149:490-8.
8. Koppikar SJ, Choudhari AS, Suryavanshi SA, Kumari S, Chattopadhyay S, Kaul-Ghanekar R, *et al*. Aqueous cinnamon extract (ACE-c) from the bark of *Cinnamomum cassia* causes apoptosis in human cervical cancer cell line (SiHa) through loss of mitochondrial membrane potential. BMC Cancer 2010;10:210.
9. Krishanu S, Trimurtulu G, Venkateswara Rao C, Ajit Kumar M. An herbal formula LI85008F inhibits lipogenesis in 3T3-L1 adipocytes. Food Nutr Sci 2011;17:2011.
10. Yang H, Choi M, Lee DY, Sung SH. Anti-differentiation effect of B, D-Seco limonoids of *Swietenia mahogani*. Pharmacogn Mag 2017;13:293-9.
11. Lee M, Sung SH. Platyphylloside isolated from *Betula platyphylla* inhibit adipocyte differentiation and induce lipolysis via regulating adipokines including PPARγ in 3T3-L1 cells. Pharmacogn Mag 2016;12:276-81.
12. Jeong SJ, Yoo SR, Seo CS, Shin HK. Traditional medicine yanggyuksanhwa-tang inhibits adipogenesis and suppresses proliferator-activated receptor-gamma expression in 3T3-L1 cells. Pharmacogn Mag 2015;11:502-8.
13. Rayalam S, Della-Fera MA, Baile CA. Phytochemicals and regulation of the adipocyte life cycle. J Nutr Biochem 2008;19:717-26.
14. Gurjar S, Pal A, Kapur S. Triphala and its constituents ameliorate visceral adiposity from a high-fat diet in mice with diet-induced obesity. Altern Ther Health Med 2012;18:38-45.
15. Otto TC, Lane MD. Adipose development: From stem cell to adipocyte. Crit Rev Biochem Mol Biol 2005;40:229-42.
16. Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM. Transcriptional regulation of adipogenesis. Genes Dev 2000;14:1293-307.
17. Linhart HG, Ishimura-Oka K, DeMayo F, Kibe T, Repka D, Poindexter B, *et al*. C/EBPalpha is required for differentiation of white, but not brown, adipose tissue. Proc Natl Acad Sci U S A 2001;98:12532-7.
18. Farmer SR. Regulation of PPARgamma activity during adipogenesis. Int J Obes (Lond) 2005;29 Suppl 1:S13-6.
19. MacDougald OA, Lane MD. Transcriptional regulation of gene expression during adipocyte differentiation. Annu Rev Biochem 1995;64:345-73.
20. Sato R, Buesa LM, Nerurkar PV. Anti-obesity effects of Emblica officinalis (Amla) are associated with inhibition of nuclear transcription factor, peroxisome proliferator-activated receptor gamma (PPARγ). FASEB J 2010;241 1 Suppl: 661-4.
21. Ranganathan G, Unal R, Pokrovskaya I, Yao-Borengasser A, Phanavanh B, Lecka-Czernik B, *et al*. The lipogenic enzymes DGAT1, FAS, and LPL in adipose tissue: Effects of obesity, insulin resistance, and TZD treatment. J Lipid Res 2006;47:2444-50.
22. Abel ED, Peroni O, Kim JK, Kim YB, Boss O, Hadro E, *et al*. Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. Nature 2001;409:729-33

APPENDIX

Appendix 1: Chemical analysis of Triphala procured from authentic source

Chemical analysis	Specification	Test method	Result
Total tannin	Report	By titration	60.25%
Total gallic acid	Report	By HPLC	9.26%
Loss on drying	NMT 6%	USP <731>	3.95%
Herb extract ratio	5:1	In-house specification	5:1
Moisture content	NMT 4%	USP <921>	2.65%
pH	3-5	USP <791>	4.12%
Acid insoluble ash	NMT 8%	USP <281>	2.85%
Solubility in water	Soluble	Indian pharmacopoeia	Soluble
Solubility in alcohol	Partially soluble	Indian pharmacopoeia	Partially soluble
Water soluble extractive	NLT 65%	Indian pharmacopoeia	71.85%
Alcohol soluble extractive	NLT 35%	Indian pharmacopoeia	52.87%
Solvent	Water	In-house specification	Confirms
Carrier	None	In-house specification	None
Excipient	None	In-house specification	None

HPLC: High-performance liquid chromatography