ORIGINAL ARTICLE

Effect of selected Indian Medicinal Plants on PPARγ expression using 3T3 L1 Adipocytes as an *In Vitro* model

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Abstract

Introduction: *Azadirachta indica, Gymnema sylvestre, Momordica charantia, Syzygium cumini,* and *Trigonella foenum-graecum* are known for their antidiabetic effects; however, molecular mechanism/s have not been evaluated. We have attempted to elucidate a possible mechanism, namely, effect on peroxisome proliferator-activated receptor-g expression of these plant extracts using 3T3-L1 adipocytes. **Materials and Methods:** 3T3 L1 fibroblasts were initially differentiated into adipocytes, following which RNA was extracted and reverse transcribed to cDNA for evaluation of PPARγ expression. Relative expression was normalized using GAPDH. Relative messenger RNA expression level was calculated according to ΔΔCT method and compared with Pioglitazone, a known insulin sensitizer. **Results:** *M. charantia, S. cumini,* and *G. sylvestre* showed an increase in PPARγ expression as compared to Control cells. Pioglitazone also showed ~2.5-fold increase in PPARγ expression. Of these three plant extracts, hydroalcoholic extract of *M. charantia* showed maximum PPARγ expression with ~4.3-fold increase which was statistically significant compared to control and reference standards. **Discussion:** Activation of PPARγ leads in improvement of insulin sensitivity through binding of synthetic drugs resulting in a decrease in insulin and glucose levels in patients with diabetes. **Conclusion:** Our data thus demonstrates that *M. charantia, G. sylvestre,* and *S. cumini* can be considered as PPAR modulators, acting through the PPAR signaling pathway resulting in enhanced transcription during adipocyte differentiation. This could be beneficial in the management of diabetes and its long-term complications with lesser toxicities.

Key words: Adipocytes, Diabetes mellitus, Indian medicinal plants, PPAR gamma expression, Thiazolidinediones

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease that affects millions of people globally and is associated with multiple comorbidities and complications. Insulin, a potent anabolic hormone, is essential for appropriate tissue development, growth, and maintenance of whole-body glucose homeostasis.[1] Glucose hemostasis regulated by insulin acts by decreasing hepatic glucose output and enhancing the rate of glucose uptake mainly into adipose tissue and skeletal muscle. The clearance of circulating glucose in muscle and fat cells depends on the translocation of GLUT4 glucose transporter to the cell surface.[2] Therefore, therapies that enhance peripheral insulin sensitivity and mimic the action of insulin on target tissues, that is, induce glucose uptake in cells, would be beneficial in the long-term management of diabetic patients.

A relatively new classes of drugs used to increase the sensitivity of the body to insulin are the thiazolidinedione (TZD) agents such as pioglitazone which is widely used as a treatment modality in the management of hyperglycemia induced through insulin resistance in patients with diabetes.[3] TZDs function as agonists for the nuclear receptor peroxisome proliferator-activated receptor-g (PPARγ).[4] PPARγ is a member of a superfamily of transcription factors that also include the closely related members, PPARα and PPARδ. TZDs act by potentiating the actions of insulin in the liver, adipose tissue, and skeletal muscle, increasing peripheral

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glucose disposal and decreasing glucose output by the liver. Given that PPARγ is predominantly expressed in adipose tissue, the effects of PPARγ agonists seen in the liver and skeletal muscle may be exerted through endocrine signaling from adipocytes.[5]

However, the undesirable side effects lower the risk benefit ratio of these compounds, and hence, there is a constant demand for new compounds for the treatment of diabetes, especially in the prevention or delay in the occurrence of complications. This has served as a motivating factor to increase the efforts to search for new and better drugs from alternative sources. Research conducted over the past several decades has shown that plant-based therapies have a high potential to treat and control diabetes mellitus[6-8] and its complications.[9] Thus, the search for novel anti-diabetic drugs advocates the utilization of plants as a potential source which is apparently effective, with minimal side effects and is of relative lower cost compared to oral synthetic antihyperglycemic agents. Ayurveda, our traditional medicinal system, provides an immense plethora of potential medicinal plants which are been extensively used by traditional medicinal practitioners for the treatment of diabetes.[10] As per literature, *Azadirachta indica, Momordica charantia, Trigonella foenum-graecum, Gymnema sylvestre,* and *Syzygium cumini* are plants known for their anti-diabetic, anti-oxidant, and anti-inflammatory activities. However, the potential mechanism/s of action behind the activity of these plants has not been clearly elucidated at the molecular level.

Hence, in this study, we have attempted to elucidate the possible mechanism/s of action of these five medicinal plants on glucose uptake, an essential process of insulin action, in 3T3-L1 adipocytes using 3T3 L1 (fibroblast) cell line for PPARγ expression.

MATERIALS AND METHODS

Chemicals

3-isobutyl-1-methylxanthine (IBMX), dexamethasone, insulin, Oil Red O stain solution, and Pioglitazone were purchased from Sigma Aldrich (Chemicals Co., St. Louis Mi, USA), Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Invitrogen corporation, USA). Antibiotic mixture, fetal bovine serum (FBS), and dimethyl sulfoxide (DMSO) were obtained from HiMedia laboratories (Mumbai, India).

Collection of plant material

The leaves of *A. indica* and *G. sylvestre,* fruit of *M. charantia* and *S. cumini,* and seed *of T. foenum-graecum* were collected from Nashik district, which were authenticated and certified.

Preparation of plant extracts

The aqueous (Aq), hydroalcoholic (HA), and alcoholic (Alc) extracts were prepared using the selected part of these plants. These parts were washed with water to remove the impurities and further air dried and finely grounded into powder. This dried powder was extracted successively with water as a solvent for preparing Aq extract using Soxhlet extractor. The powder was charged into thimble of Soxhlet apparatus and extracted using water as a solvent by maintaining a temperature at 100°C. For the HA extract, the dried powder of the extract was extracted successively with a mixture of alcohol and water in a proportion of (70:30) by maintaining a temp of 80–90°C. Whereas for the Alc extract, the dried powder was extracted successively with alcohol as a solvent by maintaining a temp of [80–90°C]. All the extractions were continued until a colorless solvent appeared from the siphon tube. All three extracts of the five plants were freeze-dried and used for PPARγ expression. The HA and Alc extracts of all the plants except *A. indica* dissolved in DMSO.

Cell culture

3T3-L1 fibroblast cell line was procured from National Center for Cell Sciences, Pune and maintained in DMEM supplemented with 100 units/ml of penicillin, 100 µg/ml streptomycin, and 10% FBS maintained in humidified incubator in the presence of 5% CO_2 at 37°C. Cells were cultured to 80% confluence before use for the glucose uptake assay.

Cell viability assay

Cell viability was performed using MTT assays[11] using concentrations ranging from 10 to 200 µg/ml of the plant extracts to select non-toxic concentration of the plant extracts under study. 3T3 L1 cells of 2×10^6 were seeded in 96-well microplate. (Complete medium used DMEM with 10% FBS). The plate was incubated at 37°C overnight. After 24 h, cells were treated with varying concentrations of the plant extracts. After 72 h, the cells were treated with MTT dye (5 mg/ml) and plate was, further, incubated for 4 h at 37°C. The plate was removed after 4 h and 1N HCL: Isopropanol (1:24) was added in each well to dissolve purple formazan crystals. The absorbance was measured using ELISA reader at 550 nm.

Evaluation of PPARγ expression

Glucose uptake-stimulatory activity of the extracts of the selected medicinal plants was assessed *in vitro* using the 3T3-L1 adipocytes model. 3T3 L1 fibroblasts were first differentiated into adipocytes and then used for evaluation of PPARγ expression.

Cell culture and adipocyte differentiation

On attaining confluency, the cells at a concentration of 1 × 10^4 cells/ml were seeded in 24 well plates. Differentiation was induced by supplementing the media with a cocktail[12] of 1 mg/L insulin, 100 mg/L IBMX, and 0.1 mg/L dexamethasone for 48 h followed by insulin alone for an additional 48 h. The media was then replaced with fresh culture medium (DMEM supplemented with 10% FBS) after 2 days and then every 2 day thereafter for 12 days. During differentiation, plant extracts and pioglitazone were added to the cell culture medium at the concentration selected from the viability studies followed by addition of the samples after every 2 days along with the media replacement. Pioglitazone (6 µg/ml) treated cells group was used as a standard control group, where the untreated differentiated cells served as the Control group.

Oil Red O staining[13]

On day 12, the medium from the wells of plates was removed and 3T3-L1 adipocytes were rinsed with phosphate-buffered saline (PBS) and then fixed with 10% formalin for 20–30 min at room temperature. The formalin was removed, and the cells were washed with 60% isopropanol and allowed to dry completely. Oil Red O working solution was added and the plate was incubated at room temperature. The cells were, then, rinsed with PBS and accumulation of lipid droplet was seen under a microscope.

Evaluation of select five medicinal plants on PPARγ transcriptional level

On the 12th day after differentiation, total RNA was extracted using Qiagen RNA kit according to the manufacturer's instructions from the differentiated adipocytes. cDNA was synthesized with the Revert Aid cDNA Synthesis Kit (Fermentas, Austin, TX) using 2.0 µg of total RNA. SYBR Green chemistry was used to perform the quantitative determination of the relative expression of PPAR gene using the Quant studio 3 RT-PCR (Thermo fisher). The primers used in the experiments are shown in Table 1. All results were obtained from at least three independent experiments. Transcript levels were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) levels. The relative messenger RNA (mRNA) expression levels were calculated according to the comparative CT (ΔΔCT) method and were also compared with Pioglitazone, a known insulin sensitizer. The target quantity was normalized to endogenous control, that is, untreated differentiated cell control group and vehicle control.

RESULTS

Cell viability assay

To evaluate the effect of plants *per se* on the 3T3 L1 cells, viability studies were carried out to eliminate the cytotoxic doses of plant extracts and thereby find out the precise range of dose of the extracts for further PPAR gene expression. The concentrations studied ranged from 5 to 200 µg/ml. As evident from the Figure 1, all the three extracts of five plants did not affect the viability of 3T3 L1 cells when compared to untreated cells. Hence, the concentrations of the plant extracts selected for PPARγ gene expression were 50 µg/ml, 100 µg/ml, and 200 µg/ml.

Oil Red O staining

3T3 L1 fibroblasts were differentiated into adipocytes using a cocktail of IBMX-Dexamethasone and Insulin. The differentiation of pre-adipocytes into adipocytes induced by these agents was evaluated by microscopic observation of lipid granules stained with Oil Red O staining on 12th day of treatment. As shown in Figure 2, microscopic observation revealed that the control cells showed spindle-like appearance of fibroblast, whereas cocktail treated cells revealed the presence of mature spherical-shaped adipocytes with lipid droplets when stained with Oil Red O. The lipid granules in the differentiated cells took up the stain and appeared red indicating adipocytes. These differentiated adipocytes were further used for PPARγ gene expression.

Effect of plant extracts on PPARγ expression

In the present study, we determined the effect of various plant extracts on PPARγ mRNA expression by RT-PCR and the values were compared with standard control, pioglitazone, and cell control. As depicted in Figure 3, *M. charantia, S. cumini,* and *G. sylvestre* showed an increase in PPARγ expression as compared to the Control. As expected, Pioglitazone also showed ~2.5-fold increase in the PPARγ expression. Among these three plants, Aq, HA, and Alc extracts of *M. charantia* showed significant upregulation in PPARγ expression. The HA extract of *M. charantia* showed the maximum upregulation of PPARγ expression with ~4.3-fold increase and was statistically significant as compared to control and the reference standard. The Aq extract of *M. charantia* showed significant dose-dependent upregulation of PPARγ expression in comparison to control and the increase was also comparable to that of Pioglitazone. Its Alc extract at 100 µg/ml and 200 µg/ml concentrations showed ~2-fold increase in PPARγ expression. Thus, all three extracts of *S. cumini* showed an increase

Table 1: Primer sequences used for PCR amplification

Genes	Accession No.	Forward (5'-3')	Reverse (5'-3')
GAPDH	BC083080	GTATGACTCCACTCACGGCAAA	GGTCTCGCTCCTGGAAGATG
PPARγ	NM_011146	TGTGGGGATAAAGCATCAGGC	CCGGCAGTTAAGATCACACCTAT

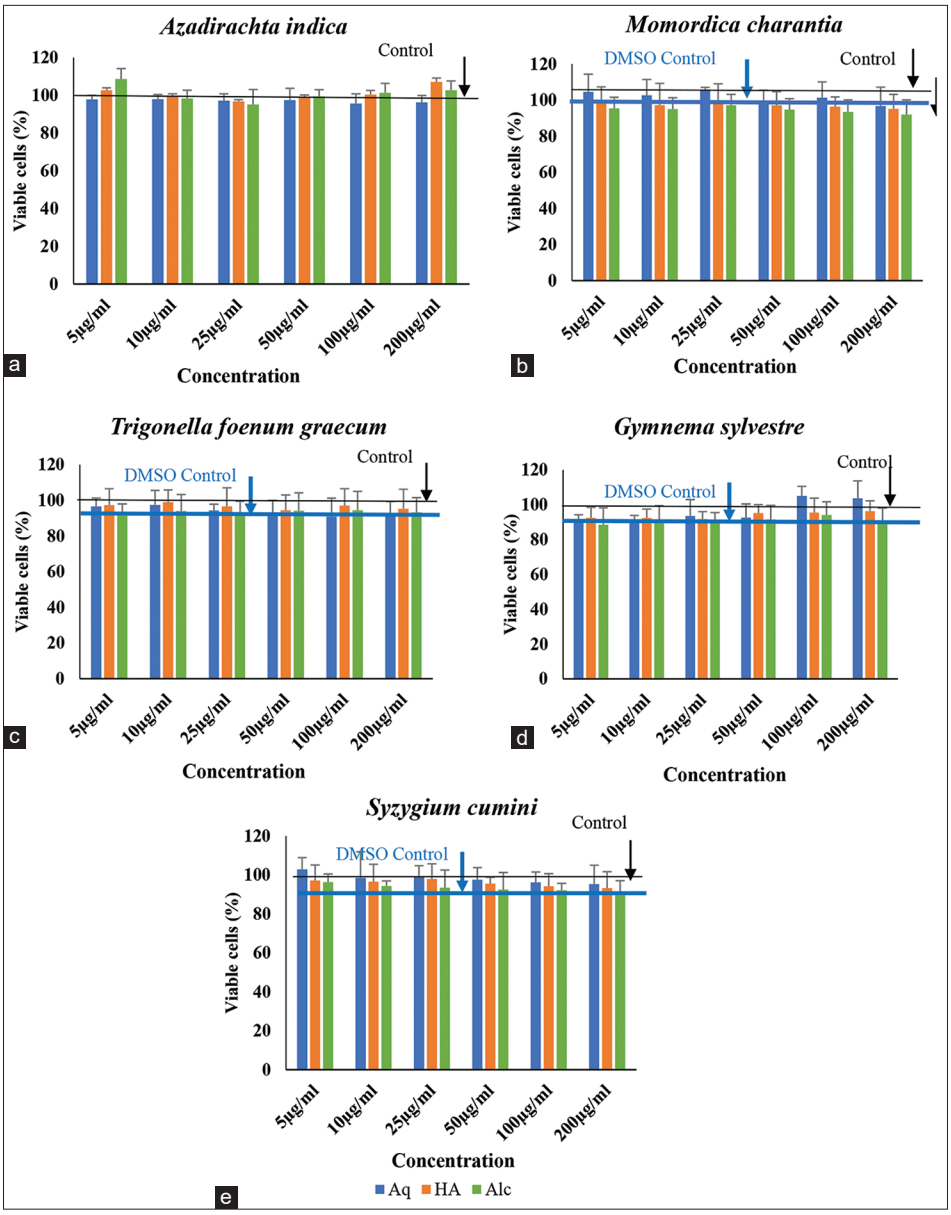

Figure 1: (a-e) Effect of selected five plant extracts on 3T3L1 cell viability assay, (a) *Azadirachta indica*; (b) *Momordica charantia*; (c) *Trigonella foenum-graecum*; (d) *Gymnema sylvestre*; and (e) *Syzygium cumini*. $P > 0.05$, Not significant (NS) as compared to control cells and compared by one-way ANOVA followed by *post hoc* Tukey test. Data expressed as Mean ± SD of three experiments. Error bars indicate ± SD. Aq: Aq extract, HA: HA extract, Alc: Alcoholic extract

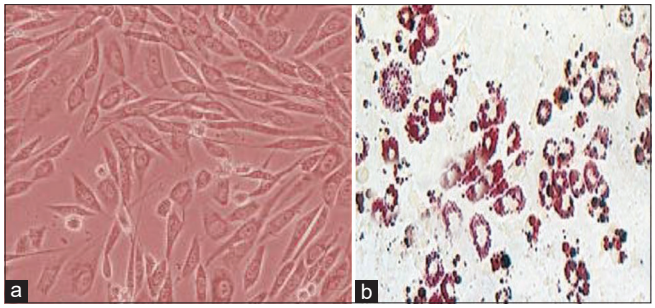

Figure 2: Photomicrograph of Oil Red Staining, (a) fibroblast spindle-shaped cells when differentiated with cocktail showed the (b) presence of mature spherical-shaped adipocytes with lipid droplets when stained with Oil Red O. Images were taken at ×40

in PPARγ expression, but the maximal significant effect was observed with the HA extract in comparison to the cell control and the increase was comparable to the Standard drug, Pioglitazone. All the three extracts of *G. sylvestre* at 200 µg/ml concentration also showed maximum increase in PPARγ expression. A significant increase in PPARγ expression was observed at 100 µg/ml concentration of *T. foenum-graecum* as compared to the cell control.

DISCUSSION

Adipocytes are an important site for insulin activity and play a significant role in glucose digestion just as in the regulation of

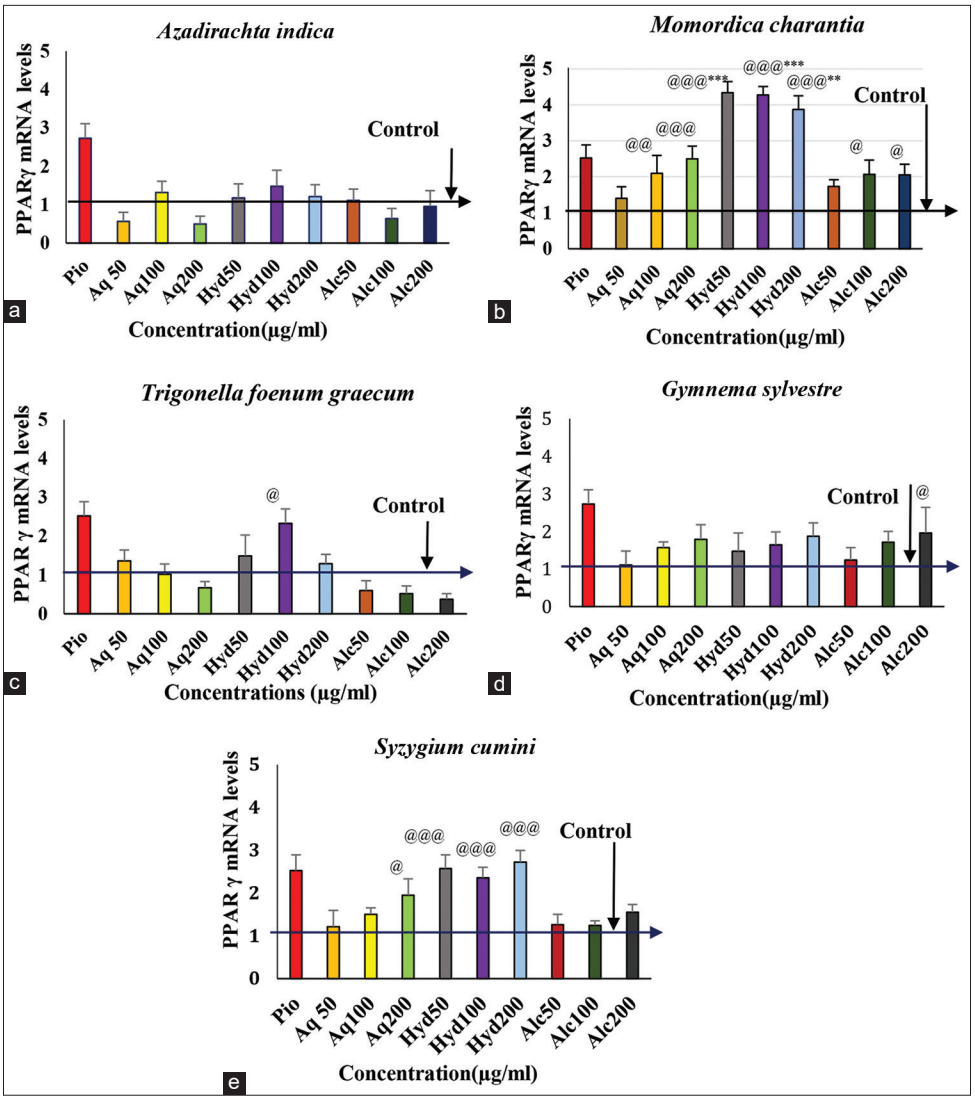

Figure 3: Effect of selected five plant extracts on PPARγ expression, (a) *Azadirachta indica*; (b) *Momordica charantia*; (c) *Trigonella foenum-graecum*; (d) *Gymnema sylvestre*; and (e) *Syzygium cumini*. $^{@}P < 0.05$, $^{@@}P < 0.01$, $^{@@@}P < 0.001$ as compared to control; $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to standard drug (Pioglitazone) and compared by one-way ANOVA followed by *post hoc* Tukey test. Data are expressed as Mean ± SD of three experiments. Error bars indicate ± SD. Aq50: Aq extract at concentration 50 µg/ml, Aq100: Aq extract at concentration 100 µg/ml, Aq200: Aq extract at concentration 200 µg/ml, HA50: HA extract at a concentration 50 µg/ml, HA100: HA extract at a concentration 100 µg/ml, HA200: HA extract at a concentration 200 µg/ml, Alc50: Alcoholic extract at a concentration 50 µg/ml, Alc100: Alcoholic extract at a concentration 100 µg/ml, and Alc200: Alcoholic extract at a concentration 200 µg/ml

whole-body glucose homeostasis. The adipogenesis process involves the differentiation of preadipocytes precursor cells to mature adipocytes and is usually accompanied by a series of gene expressions regulated by various transcriptional factors. PPAR gamma is a nuclear transcriptional factor that induces adipogenesis and is required for maintenance of adipocyte functions. PPAR gamma has a crucial role in the insulin receptor signaling cascade in glucose uptake and adipocyte differentiation. The chemical class of TDZ is insulin-sensitizing drugs. Pioglitazone widely used anti-diabetic drugs and potent PPARγ agonists used in the management of T2DM to improve insulin sensitivity[14] and glucose tolerance but are usually associated with unwanted side effects of weight gain and hypoglycemia.[15]

In comparison to various organs, PPARγ is highly expressed in adipose tissue. Several hormones are released by the adipose tissue that regulates the lipid and glucose metabolism and energy hemostasis. Imbalance in the production of these hormones leads to impaired insulin secretion or resistance. Thus, adipocytes are now a major drug target for diabetes and obesity-related metabolic syndrome. 3T3-L1 adipocytes fill in as a great *in vitro* model and are viewed as a significant implement in the understanding of glucose metabolism. It has been documented in various studies that TZDs treated adipocyte showed increased glucose uptake and glucose transporter expression and similar results may be evident in muscle too.[16]

Thus, we decided to use 3T3-L1 adipocytes as a model to evaluate the effect of 5 Indian medicinal plant extracts, namely, *A. indica, M. charantia, T. foenum-graecum, G. sylvestre,* and *S. cumini* on the insulin sensitivity activity with regard to PPARγ expression. First, the MTT assay was performed to rule out non-toxic concentration of the selected plant extracts and the viability was not affected until 200 µg/ml concentration. Hence, the plant extract at concentrations of 50, 100, and 200 µg/ml was selected for PPARγ gene expression studies. Second, all the plant extracts were tested for its adipogenesis activity by performing Oil Red O staining and it was observed that almost all the plant extracts were effective in differentiating into mature adipocytes.

Further to understand the mechanism of selected plant extracts, we determined the PPARγ expression in the 3T3L1 cell line. Among the various plant extracts, *M. charantia, G. sylvestre,* and *S. cumini* showed significant increase in PPARγ mRNA levels as compared to the control (undifferentiated cells). All the three extracts of *M. charantia* demonstrated the maximum up-regulation of PPARγ expression of about 4.3-fold in HA extract and 2.5-fold in Aq extract, thereby leading to an increase in glucose uptake. Similarly, up-regulation of PPARγ and GLUT4 expression was observed by Kumar *et al.,*[17] in which the fruit extract of *M. charantia* showed 2.8-fold increase and 2.3-fold increase was also observed by Baek *et al.,*[18] wherein *M. charantia* in combination with *Ligularia fischeri* leaves effect was measured *in vitro.* Raman and Lau[19] documented that *M. charantia* exhibits adipogenesis activity which could be attributed to the occurrence of active constituent charantin and polypeptide in the extract. *G. sylvestre* and *S. cumini,* in our study, showed 2.0-fold and 2.6-fold increase in PPARγ expression, respectively, and our findings are *in lieu* with the available literature,[20,21] wherein the increase in PPARγ expression was also reported. As expected, pioglitazone, a potent PPAR agonist, showed an increase in PPARγ gene expression indicating enhanced expression of adipocytes specific genes. Activation of PPARγ leads in improvement of insulin sensitivity through binding of synthetic drugs resulting in a decrease in insulin and glucose levels in patients with diabetes. It has been observed that various Indian medicinal plants have shown marked improvement in metabolic marker in *in vivo* models with lesser side effects in comparison to TZD agonists.[22]

CONCLUSION

Our data demonstrates that *M. charantia, G. sylvestre,* and *S. cumini* can be considered as PPAR modulators acting through the PPAR signaling pathway leading to increased PPARγ expression resulting in enhanced transcription during adipocyte differentiation. This data could be beneficial in the management of diabetes and its long-term complications with lesser toxicity. However, these findings need to be confirmed with the isolation of active constituents in these plant extracts and also to identify other genetic transcriptional factors involved in insulin signaling pathway to confirm the mechanism of action.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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