Methanolic extract of *Momordica cymbalaria* enhances glucose uptake in L6 myotubes *in vitro* by up-regulating PPAR-γ and GLUT-4

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**ABSTRACT** The present study was undertaken to evaluate the influence of the methanolic fruit extract of *Momordica cymbalaria* (MFMC) on PPARγ (Peroxisome Proliferator Activated Receptor gamma) and GLUT-4 (Glucose transporter-4) with respect to glucose transport. Various concentrations of MFMC ranging from 62.5 to 500 μg·mL⁻¹ were evaluated for glucose uptake activity *in vitro* using L6 myotubes, rosiglitazone was used as a reference standard. The MFMC showed significant and dose-dependent increase in glucose uptake at the tested concentrations, further, the glucose uptake activity of MFMC (500 μg·mL⁻¹) was comparable with rosiglitazone. Furthermore, MFMC has shown up-regulation of GLUT-4 and PPARγ gene expressions in L6 myotubes. In addition, the MFMC when incubated along with cycloheximide (CHX), which is a protein synthesis inhibitor, has shown complete blockade of glucose uptake. This indicates that new protein synthesis is required for increased GLUT-4 translocation. In conclusion, these findings suggest that MFMC is enhancing the glucose uptake significantly and dose-dependently through the enhanced expression of PPARγ and GLUT-4 *in vitro*.

**KEY WORDS** *Momordica cymbalaria*; Diabetes; Glucose transport; PPARγ; L6 myotubes; Antidiabetic activity

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**Introduction**

Medicinal plants with their natural phytoconstituents form a base for the discovery of new chemical entities in modern drug discovery. A significant number of plant-derived active principles having therapeutic use in the treatment of type 2 diabetes mellitus have been reported [1]. In this context, the various plant parts of *Momordica cymbalaria* Fenzl ex Naudin (Cucurbitaceae) have been demonstrated to have antidiabetic and hypoglycemic activity [2-4]. However, the rate limiting step in glucose utilization is glucose transport, especially in insulin-targeted skeletal muscle, mediated by major glucose transporters (GLUT proteins, GLUT-4 and GLUT-1) [5]. Cloning and characterization of insulin responsive glucose transporters has revealed that GLUT-1 and GLUT-4 are expressed in human skeletal muscle [6]. Insulin resistance in type 2 diabetes is characterized by decreased insulin-stimulated glucose transport and weakened metabolism in adipocytes and skeletal muscle resulting in down-regulation of the major insulin responsive GLUT’s [7].

Weakened GLUT-4 translocation, and reduced expression of peroxisome proliferator activated receptor gamma (PPARγ) have been studied under diabetic conditions. Studies have shown the role of PPARγ in insulin signaling, and also enumer-
ated the up-regulation of insulin-dependent glucose transport and GLUT-4 translocation in cultured L6 myotubes [3].

PPARγ, a transcription factor belonging to the nuclear receptor superfamily [8], is essential for adipocyte differentiation [10], and PPARγ agonists directly enhance insulin signalling and glucose uptake in the muscles by binding with the PPARγ receptors [8].

Previously, various plant parts of *Momordica cymbalaria*, were extensively studied for their hypoglycemic and antidiabetic activity. However, no studies related to its mechanism of action have been carried out, and hence the molecular aspects of the mechanism behind the hypoglycemic and antidiabetic effects of *M. cymbalaria* need to be explored. With this background, the present study was carried out to exemplify the molecular mechanistic action of MFMC, at the cellular level, by evaluating the influence of MFMC on glucose transport (GLUT-4), and PPARγ expression using L6 myotubes in vitro.

**Materials and Methods**

**Reagents, chemicals, and cell lines**

All cell culture supplements were purchased from Sigma-Aldrich (St. Louis, MO, USA). All fine chemicals, TRI reagent, and Avian Moloney Leukemic virus reverse transcriptase (AMLV), dNTP, and Taq polymerase were also purchased from Sigma-Aldrich. All other reagents and chemicals purchased were of molecular biology grade and were purchased from Sigma-Aldrich.

**Collection of plant material**

The fruits of *Momordica cymbalaria*, were collected from Hospete (Bellary District, Karnataka, India), in the period of May–June 2012, considered the seasonal conditions for obtaining maximum phytoconstituents. The collected plant material was authenticated and certified by Prof. K. P. Srinath, Department of Botany, Bangalore University, Bangalore, a voucher specimen of the plant material is preserved in the department with specimen no. 2012-13/MC/BT-01.

**Plant material preparation**

The fruits of *Momordica cymbalaria*, were shade-dried and powdered. The fruit powder (50 g) was extracted sequentially with hexane, dichloromethane, ethyl acetate, and methanol, in a Soxhlet apparatus. Initially, the plant material was extracted with hexane (1 L) at 60 °C for 24 h, the marc obtained was completely dried, and extracted with dichloromethane (1 L) for 24 h, and then the marc was extracted with ethyl acetate (1 L), followed by methanol (1 L).

Extracts were concentrated in a rotor evaporator (Remi Instruments), under reduced pressure at room temperature, and dried extract (1 mg) was reconstituted to 1 mL with respective solvents, and diluted to attain a final concentration of 500 μg·mL⁻¹ from the stock solution of 500 μg·mL⁻¹. Further concentrations were prepared by serial dilution (250, 125, 62.5 μg·mL⁻¹), for glucose uptake studies.

**Propagation and maintenance of L6 cells**

L6 cell cultures (rat skeletal muscle), was procured from the National Centre for Cell Sciences (NCCS), Pune, India. L6 cells were cultured and maintained in Dulbecco’s modified Eagle’s medium (DMEM), with 10% inactivated Fetal Bovine serum (FBS), along with penicillin (100 IU·mL⁻¹), streptomycin (100 μg·mL⁻¹), and amphotericin B (5 μg·mL⁻¹), in a humidified atmosphere of 5% CO₂ at 37 °C, until confluence. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, and 0.05% glucose in PBS). The stock cultures were grown in 25 cm² culture flasks.

**Glucose uptake assay**

Completely differentiated myotubes demonstrates a high response to insulin, and rosiglitazone mediated glucose uptake. The differentiated myotubes were serum-starved overnight, and washed with HEPES in Kreb’s Ringer phosphate solution (KRP buffer), and incubated with KRP buffer with 0.1% BSA (Bovine serum albumin) for 30 min at 37 °C. The myotubes were treated with various concentrations of plant extract, and standard along with vehicle control, in 60 mm Petri plates. Subsequently, D-glucose solution was added to all the plates, and incubated for 30 min at 37 °C. Subsequently, the liquid medium was aspirated from all the plates, to terminate the glucose uptake, and the cells were washed three times with ice-cold KRP buffer solution. Further, the cells were lysed with 0.1 mol·L⁻¹ NaOH solution, and aliquots of the cell lysate was used to measure the cell-associated glucose. Glucose uptake was estimated by using Biovision glucose estimation kit (Biovision Kit Inc, USA). Three independent experimental values, in duplicates were taken to determine the percentage enhancement of glucose uptake compared with control [11-12].

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

RT-PCR was performed as published [10]. In short, after the completion of incubation, the cells were lysed in TRI Reagent, the protein content was extracted with chloroform, and the total RNA was precipitated with isopropanol. The RNA precipitate was washed with 70% ethanol, and resuspended in DEPC-treated water (50 μL). Reverse transcription was performed using 200 units of avian reverse transcriptase, and 200 ng·μL⁻¹ oligo (T)₁₈. The primers used were as follows, GLUT-4: sense, 5'-CGG GAC GTG GAG CTG GCC (318-bp) [12]; PPARγ: 5'-GCC CCT CGA GTG A-3'; anti-sense, 5'-CCC CCT CGA GTG A-3' (318-bp) [12]; PPARγ: sense, 5'-GGC TTC ATG ACC ACC AGG (318-bp) [12]; anti-sense, 5'-GCC TTC ATG ACC AGG (318-bp) [12]; glyceraldehydes-3-phosphate dehydrogenase (GAPDH); sense, 5'-CCA CCC ATG GCA AAT TCC ATG CTC-3'; anti-sense, 5'-CCC CCT CGA GTG A-3' (155-bp) [12]. For PCR reaction, cDNA mixture (1 μL) was added to a PCR reaction mix containing 10 × PCR buffer, 2 mmol·L⁻¹ dNTP, 10 pmol·L⁻¹ of paired primers, and 2 units of Taq polymerase. PCR products were run on 1.5% agarose gels, stained with ethidium bromide, and the gel was photographed by scanning densitometer.

**Statistical analysis**

All the values were expressed as X ± SEM. The results
were analyzed statistically using one way ANOVA followed by Tukey’s multiple comparison test using Graph Pad Prism version 4.03 for Windows, Graph Pad Software, San Diego CA, USA. The minimum level of significance was fixed at $P < 0.05$.

**Results**

**Extraction of the plant material**

The fruits powder of *M. cymbalaria* was successively extracted with hexane, dichloromethane, ethyl acetate, and methanol. The extractive values of hexane, dichloromethane, ethyl acetate, and methanol were found to be 0.6%, 0.45%, 2.3%, and 3.2% $W/W$, respectively.

**Evaluation of glucose uptake**

The glucose uptake activity of MFMC was evaluated using a 50 $\mu$g·mL$^{-1}$ concentration of rosiglitazone as reference standard, based on a pilot study (unpublished data). The MFMC at concentrations ranging from 62.5 to 500 $\mu$g·mL$^{-1}$ showed dose-dependent stimulation of glucose uptake, at 500 $\mu$g·mL$^{-1}$ concentration it offered approximately two-fold increase in glucose uptake from the basal concentration of glucose, similarly rosiglitazone also showed a significant increase in glucose uptake. The results are given in Fig. 1.

**Discussion**

The abnormal glucose transport associated with deficient GLUT-4 translocation, and/or faulty insulin signaling cascade are manifested as among the dominant defects in insulin resistance in type 2 diabetes. The intense rise in GLUT-4 and

Fig. 2  Effect of the methanolic fruit extract of *Momordica cymbalaria* (MFMC) on Glut-4 transcripts in L6 myotubes ($\bar{x} \pm$ SEM, $n = 3$)
M: Ikbp marker Lane 1: Indicates 500 µg·mL$^{-1}$ MFMC, Lane 2: indicates 250 µg·mL$^{-1}$ MFMC, Lane 3: 125 µg MFMC, Lane 4: Rosiglitazone, Lane 5: control
Means of various groups were statistically compared by one way ANOVA followed by Tukey’s multiple comparison test using Graph Pad version 4.03. *P* < 0.05, **P** < 0.01 vs control

Fig. 3  Effect of methanolic fruit extract of *Momordica cymbalaria* (MFMC) on PPARγ transcripts in L6 myotubes ($\bar{x} \pm$ SEM, $n = 3$)
M: Ikbp marker Lane 1: indicates 500 µg·mL$^{-1}$ MFMC, Lane 2: indicates 250 µg·mL$^{-1}$ MFMC, Lane 3: 125 µg MFMC, Lane 4: Rosiglitazone, Lane 5: control
Means of various groups were statistically compared by one way ANOVA followed by Tukey’s multiple comparison test using Graph Pad version 4.03. *P* < 0.05, **P** < 0.01 vs control

P13 kinase, mRNA levels in euglycemic and hyperinsulinemic clamp in the presence of insulin refined the role of GLUT-4, and P13 kinase in insulin-mediated glucose uptake.

Fig. 4  Effect of cycloheximide on methanolic fruit extract of *Momordica cymbalaria* (MFMC) intervening glucose uptake.
Note : ns, Not significant ($\bar{x} \pm$ SEM, $n = 3$)
Means of various groups were statistically compared by one way ANOVA followed by Tukey’s multiple comparison test using Graph Pad version 4.03. †*P* < 0.001 corresponds to Rosiglitazone vs control; *P* < 0.05, **P** < 0.01, ***P** < 0.001 corresponds to MFMC vs control. "P" > 0.05 corresponds to MFMC + CHX vs MFMC 62.5 µg·mL$^{-1}$
transport 13. Apart from insulin, PPARγ agonists like rosiglitazone and pioglitazone have been shown to facilitate glucose transport in type 2 diabetes by acting as insulin sensitizers 14. Hence the up-regulation of such molecular switches reinforced their role in glucose transport. In the present study, an attempt was made to identify the mechanism behind the glucose uptake activity of MFMC in in vitro using L6 myotubes.

In this context, the L6 muscle cell line was a suitable in vitro model to study glucose transport activity. Moreover, skeletal muscle is the major site for primary glucose clearance and glucose utilization 15. Earlier reports of L6 myotubes evidenced the maximum glucose uptake in the presence of troglitazone and rosiglitazone at 10 and 100 μmol·L−1 concentrations, respectively 16. In addition, the enhanced glucose uptake in L6 cells is mostly mediated through elevated GLUT-4 levels 8, 15. In similar terms, the findings in the present study are in agreement with the literature reports where there is a concomitant increase in glucose uptake along with the enhanced GLUT-4 levels in L6 myotubes, the MFMC has shown enhanced glucose uptake, and assisted the enhanced GLUT-4 expression. Furthermore, cycloheximide, a protein synthesis inhibitor, has completely blocked troglitazone-mediated glucose uptake on chronic exposure, suggesting the importance of new protein synthesis for glucose transport 8. Similarly, in the present study the glucose uptake activity of MFMC was completely abolished by cycloheximide, which clearly confirmed the role of new protein synthesis relevant to glucose transport.

Together, the above considerations established that glucose uptake is dependent on increased or decreased expression of GLUT-4; Moreover, the enhanced glucose uptake in the present study was concomitant with the increased expression of GLUT-4 encoding mRNA in L6 myotubes upon incubation with MFMC 16.

Additionally, the expression of PPARγ was also increased, together with increased expression of GLUT-4, and enhanced glucose uptake in L6 myotubes when incubated with MFMC. In support of these findings, PPARγ agonists are known to increase the GLUT-4 transcription by acting through PPARγ receptors 17.

Interestingly, the findings of the present study have convincing inference to understand the mechanism governing the activation of glucose transport associated with MFMC. All the findings in the present study are in agreement to support the glucose uptake enhancing property of MFMC. Based on the gene expression study outcomes it was hypothesized that methanolic fruit extract of M. cymbalaria could activate the glucose transport by up-regulation of GLUT-4 and PPARγ by acting as PPARγ agonist. Duplication of the above study in 3T3 L1 adipocyte cell-line could help to better understand the mechanism of action of M. cymbalaria.

**Conclusion**

In the current study, the unifying approach of medicinal chemistry and in vitro screenings assays was manifested, which assured the evidence of a set of targets on glucose transport. Also this study demonstrated the importance of GLUT-4, and PPARγ up-regulation by the methanolic fruit extract of M. cymbalaria in enhancing glucose transport. Its role in augmenting glucose uptake by modulating a battery of targets like GLUT-4, PPARγ and their significance in such processes has been studied. Purification of the above plant extract towards the isolation of the active principle(s) is in progress.

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