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INFLUENCE OF 5,7-DIMETHOXY COUMARIN ON ADIPOGENESIS IN HIGH GLUCOSE INDUCED INSULIN RESISTANT 3T3-L1 ADIPOCYTES

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ABSTRACT

Insulin resistance in adipocytes disrupts whole-body metabolic homeostasis contributing to various complications. This study investigates the adipogenic potential of 5,7-dimethoxy Coumarin (5,7-DMC) in high glucose-induced insulin resistance in 3T3-L1 cell lines. Insulin resistance was induced in 3T3-L1 adipocytes by exposing with 25 mM glucose and 0.6 nM insulin for 24 hours. The IC_{50} value of 5,7-DMC was determined using the MTT assay. The cells were divided into four groups: Group I – Normal control (3T3-L1 adipocytes), Group II – Diabetic control (IR-3T3-L1), Group III – Diabetic cells treated with 5,7-DMC (60 µM), and Group IV – Diabetic cells treated with rosiglitazone (0.1 µM). Glucose uptake, intracellular triglyceride levels, and glycerol-3-phosphate dehydrogenase activity were evaluated in experimental groups. IR-3T3-L1 adipocytes exhibited reduced glucose utilization, lower triglyceride content and decreased dehydrogenase activity. Treatment with 5,7-DMC significantly improved these parameters, indicating enhanced insulin sensitivity. Oil Red O staining further confirmed increased triglyceride accumulation in treated cells. Molecular docking revealed a strong interaction between 5,7-DMC and Peroxisome Proliferator-Activated Receptor gamma (PPARγ), with a binding energy of –6.6 kcal/mol, comparable to rosiglitazone. These results suggest that 5,7-DMC improves insulin sensitivity in adipocytes offering therapeutic value in the management of diabetes mellitus.

INTRODUCTION

Adipose tissue is an extraordinarily flexible endocrine organ that plays a central role in energy storage and metabolic homeostasis (Luo & Liu., 2016). Beyond its function as an energy reservoir, it secretes numerous adipokines that regulate the activities of several organs including brain, skeletal muscle and liver (de Oliveira dos Santos *et al.*, 2021). Chronic hyperglycemia induced dysfunction of adipocytes leads to whole body insulin resistance contributing to chronic complications of diabetes mellitus (Zatterale *et al.*, 2020).

Insulin resistance in adipose tissue is characterized by impaired glucose uptake, reduced lipid accumulation with dysregulated adipogenesis (Imi *et al.*,2023). These alterations compromise the ability of adipocyte to store triglycerides and causes lipid spillover into non-adipose tissues, aggravating insulin resistance and systemic metabolic disorders (Porro *et al.*,2021). Current pharmacological agents such as thiazolidinediones target PPARγ to improve insulin sensitivity, but their long-term use is associated with side effects including weight gain, fluid retention, and cardiovascular risks (Kounatidis *et al.*, 2025). This highlights the urgent need for novel and safer therapeutic agents that can improve insulin sensitivity and restore adipocyte function.

Natural compounds with insulin-sensitizing and adipogenic properties have gained attention as alternative therapeutics (Jugran *et al.*, 2021). In this context, 5,7-dimethoxy coumarin (5,7-DMC), a cyclic polyphenolic compound in the essential oils of several plants, such as *Citrus limon*, *Citrus bergamia*, *Citrus*, and *Carica papaya* (Rathnayake, *et al.*, 2021) exhibits diverse pharmacological properties including antioxidant, anti-inflammatory, antimicrobial, and anticancer activities (Zhou *et al.*, 2019; Liu *et al.*, 2018; Kim *et al.*, 2016; Wang *et al.*, 2021). The present study was designed to investigate the adipogenic potential of 5,7-DMC in high glucose-induced insulin-resistant 3T3-L1 adipocytes, a well-established *in vitro* model for studying adipose tissue dysfunction.

METHODOLOGY

Chemicals

Foetal bovine serum (FBS), 5,7-DMC, and media were obtained from Sigma Aldrich Pvt. Ltd., (St. Louis, MO, USA). Analytical grade chemicals were used in this study.

Cell Culture and differentiation

3T3-L1 preadipocytes procured from National Centre for Cell Science (NCCS), Pune, India was cultured in Dulbecco's Modified Eagle Medium (DMEM) with glucose, supplemented with 10% FBS, 100U/mL penicillin, and 100µg/mL streptomycin and maintained at 37°C in a humidified atmosphere containing 5% CO_2. On reaching confluence, differentiation was initiated using a medium containing 1 µM dexamethasone,0.5 mM IBMX, and 1 µg/mL insulin in DMEM with 10% FBS. The differentiation medium was replaced every two days until mature adipocytes were formed.

MTT Assay and establishment of IR-3T3-L1adipocytes

MTT assay was carried out to determine IC_{50} of 5,7-DMC. In short, 3T3-L1 cells cultivated in 96-well plates were exposed to 5,7-DMC (10-100 µM) for 24 h. Cell viability was assessed by dissolving the violet-Formosan crystals with 100 µL DMSO after 4 h of incubation with MTT solution in DMEM at 37^{0}C. The absorbance was measured at 570 nm (Mosmann 1983). 3T3-L1 adipocytes were made insulin resistant on exposure with glucose (25 mM) and insulin (0.6 nm/L) for 24 h. To determine the onset of insulin resistance, the residual glucose in all the experimental groups were measured.

Experimental design

3T3-L1 and IR-3T3-L1 were grouped as follows:

Group I- Normal control (3T3-L1 adipocytes)

Group- II- Diabetic control (IR-3T3-L1) (Gao *et al.*, 2015)

Group-III- Diabetic cells treated-IR-3T3-L1 treated with 5,7-DMC (60 µM)

Group IV- Diabetic cells treated - IR-3T3-L1 treated with rosiglitazone (0.1 µM) (Harada *et al.*, 2020)

Glucose uptake assay

Glucose uptake assay was performed by the method of (Yin *et al.*, 2008). Briefly, adipocytes were seeded ($5x10^4$) in 96 well plates. A part of adipocytes was made insulin resistant as described previously and treated as per experimental design. The supernatant from each group was collected and the residual glucose levels was measured and expressed in percentage.

Assay of Glycerol-3-phosphate dehydrogenase

Glycerol-3-phosphate dehydrogenase activity in experimental cell lines was determined according to the method of Wise &Green (1979). Briefly, cells from each experimental group were washed twice with ice-cold PBS and harvested using 300 µL of ethanolamine / Hcl buffer (pH 7.5) and EDTA. After sonication (10 sec) and centrifugation (13000xg for 5 min at 4^{0}C), supernatant was collected. The reaction mixture contained tri-ethanolamine / Hcl buffer, EDTA, 2- mercapto ethanol and NADH. The reaction was initiated by the addition of dihydroxyacetone phosphate and the rate of NADH oxidation was read at 340 nm. Enzyme activity was expressed as percentage against control.

Intracellular TG content and Oil red O Staining

The intracellular TG content was determined by the method of Mayerson *et al.*, (2002) using TG assay kit. Briefly, cells washed in ice cold PBS was sonicated after the addition of 1ml of triglyceride extraction reagent. Supernatant were collected from cell lysate after centrifugation at 4^{0}C for 5 min and TG was measured. Similarly, lipid droplets were visualized using Oil red O staining according to Zacharies *et al.*, (1992). Cells washed in PBS were fixed in formalin (10%) for 15min. After incubation with Oil Red O staining for 20 min at 37 ^{0}C, cells were rinsed with 50% ethanol for 3 sec to remove excess stain. Images were photographed and the dyes retained in experimental groups were eluted with isopropanol for an hour and the absorbance was measured at 510 nm.

In silico analysis

The interaction of 5,7-DMC with the target protein PPARγ was examined using Pyrx software's AutoDock (V.4.0). Docking analysis was displayed using the Biovia Discovery Studio Visualizer 2020.The binding energy (kcal/mol) was used to compute the molecular interaction.

Statistical Methods

Experimental values expressed as mean ± standard deviations of three experiments. One-way ANOVA was carried out and values with $P<0.05$ are considered statistically significant. All calculations were done using the SPSS 26.0 version.

RESULTS AND DISCUSSION

Effect of 5,7-DMC on the viability of 3T3-L1 Adipocytes

The effect of 5,7-DMC on the viability of 3T3-L1 adipocytes were determined by MTT assay and the results were shown in Fig.1 Mature 3T3-L1 cells exposed to various concentration of 5,7-DMC (10-100µM) for 24h exhibited a dose-dependent response on cell viability and the inhibitory concentration (IC_{50}) was found to be 60 µM. Further studies are carried out with this dose.

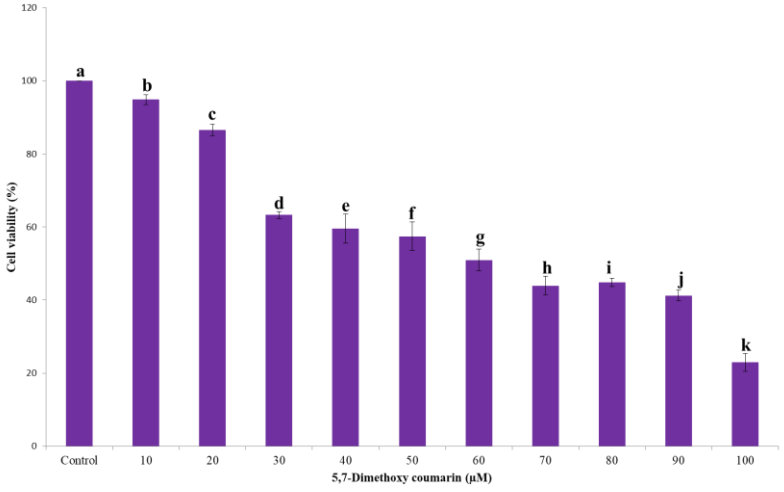


Fig. 1. Effect of 5,7-DMC on the viability of 3T3-L1 adipocytes

Values are expressed as the mean ± SD (n=3). Difference between the groups were evaluated by one-way ANOVA followed by Duncan's Post hoc test $P<0.05$.

Influence of 5,7-DMC on glucose uptake in 3T3-L1 cell lines

The results of glucose uptake in experimental groups were depicted in Fig. 2. A significant decline in glucose uptake was observed in IR-3T3-L1 cells compared to normal control. Treatment with 5,7 DMC enhanced glucose uptake in a manner similar to that of rosiglitazone treated cell lines. In adipose tissue, glucose plays a critical role by providing the glycerol phosphate backbone required for fatty acid esterification and contributing to *de novo* lipogenesis. On interaction with its receptor, insulin activates the PI3K/AKT signaling pathway which facilitates glucose uptake by the translocation of GLUT4 to membrane. In the current study, IR-3T3-L1 adipocytes exhibited a significant reduction in glucose uptake, indicative of impaired insulin responsiveness. Remarkably, treatment with 5,7-DMC (60 µM) led to a substantial increase in glucose uptake in insulin-resistant cells. These

findings support the potential role of 5,7-DMC in ameliorating insulin resistance by promoting glucose uptake in adipocytes. These observations are consistent with previous findings by Menget *al.*, (2020), who reported that a *Morus alba* L. leaf extracts promoted glucose uptake in IR-3T3-L1 adipocytes by modulating the PI3K/AKT signaling pathway.

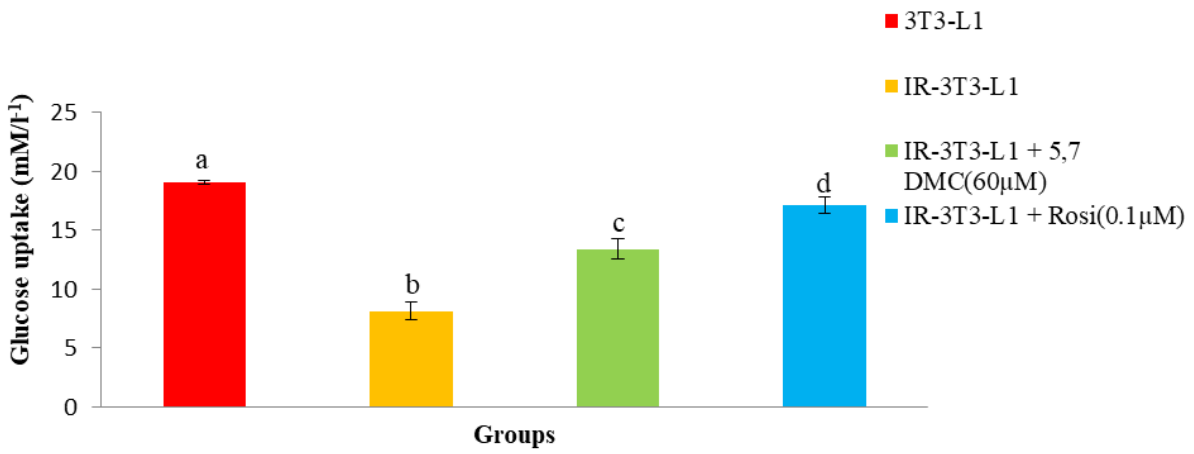


Fig. 2. Effect of 5,7-DMC on glucose uptake in 3T3-L1 cell lines.

Values are expressed as the mean ± SD (n=3). Difference between the groups were evaluated by one-way ANOVA followed by Duncan's Post hoc test P<0.05.

Effect on Glycerol-3-phosphate dehydrogenase activity in 3T3-L1 cells

In the present study, G3PDH activity was decreased in IR-3T3-L1 adipocytes compared to the normal control group, indicating compromised adipogenic function. However, treatment with 5,7-DMC (60 µM) for 24 h resulted in a marked increase in G3PDH activity in insulin resistant cells (Figure 3). Liver and adipose tissues are the sites of lipogenesis, encompassing the synthesis of fatty acids and triglycerides. G3PDH is a key enzyme in triglyceride biosynthesis and adipocyte differentiation, serving as a crucial link between glycolysis and lipid formation. G3PDH catalyzes the reversible conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate, which provides the glycerol backbone required for triglyceride assembly (Sears *et al.*, 2009). As the activity of G3PDH is modulated by insulin, resistance to insulin action typically leads to reduced G3PDH activity with impaired adipocyte function and disruption of lipid metabolism. The elevation in G3PDH activity following 5,7-DMC treatment was comparable to that observed in rosiglitazone treated cells, implying that 5,7-DMC may exert similar regulatory effects on adipocyte differentiation by enhancing insulin signaling and promoting the expression of adipogenic enzymes. These results are consistent with those reported by Seethalakshmi et al. (2023), who demonstrated that α-phellandrene enhanced G3PDH activity in insulin-resistant 3T3-L1 adipocytes.

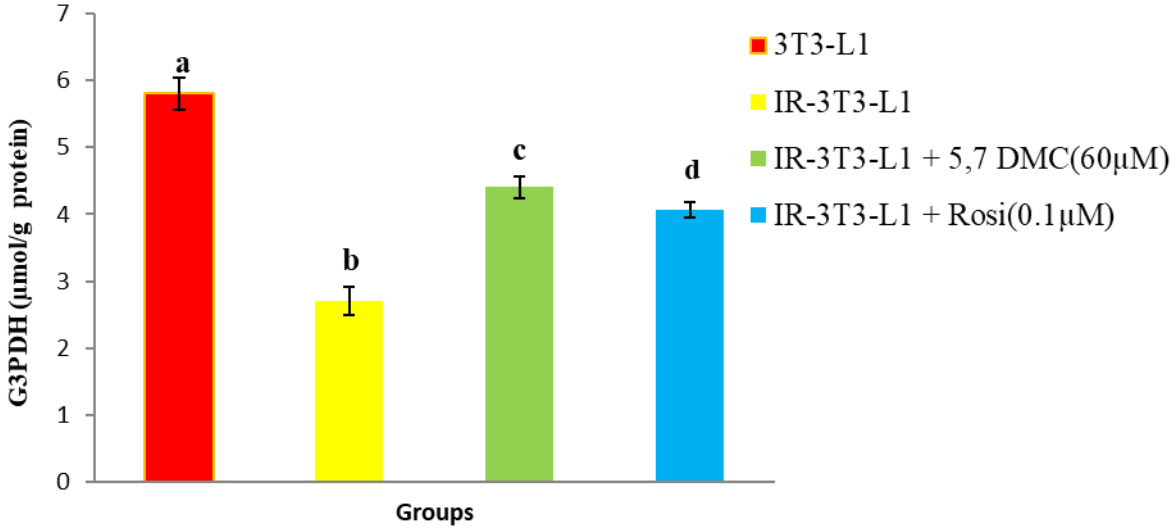


Fig. 3. Effect of 5,7-DMC on G3PDHactivity in IR-3T3-L1 cells.

Values are expressed as the mean ± SD (n=3). Difference between the groups were evaluated by one-way ANOVA followed by Duncan's Post hoc test P<0.05.

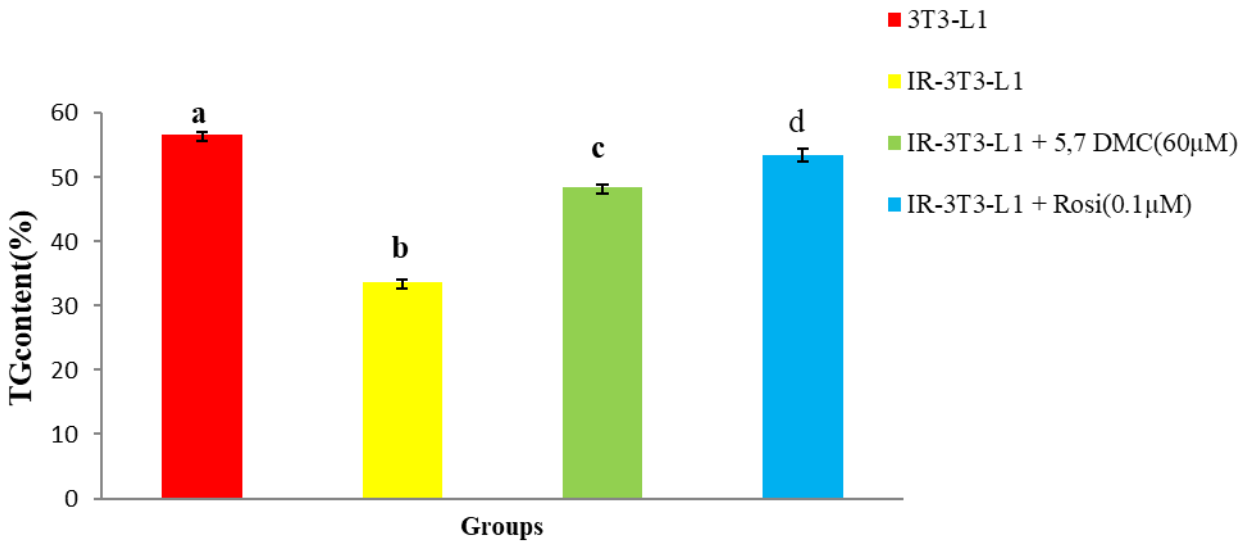
Effect of 5, 7-DMC on intracellular triglyceride content in 3T3-L1 adipocytes

Insulin resistance is a pathological condition characterized by impaired glucose and lipid metabolism, often accompanied by dysregulated adipocyte differentiation and lipid storage. In adipose tissue, proper triglyceride (TG) synthesis and intracellular lipid accumulation are essential for buffering circulating lipids and maintaining metabolic homeostasis. Dysfunctional adipocytes exhibit decreased lipid storage capacity, leading to lipid spillover into non-adipose tissues, which exacerbates insulin resistance and promotes the onset of metabolic disorders such as type 2 diabetes and non-alcoholic fatty liver disease (Choi *et al.*, 2020). At the cellular level, insulin promotes adipogenesis and lipid accumulation via activation of the PI3K/Akt signaling pathway and upregulation of key lipogenic genes. In insulin-resistant adipocytes, this pathway

is disrupted leading to decreased expression and activity of G3PDH with reduced triglyceride accumulation.

In the current study, IR-3T3-L1 adipocytes exhibited significantly decreased intracellular TG levels with reduced lipid droplet accumulation, evidenced by Oil Red O staining compared to normal adipocytes, reflecting impaired adipogenic capacity. Treatment with 5,7-DMC (60 µM) for 24 h resulted in a marked increase in TG content and lipid accumulation suggesting restoration of lipid synthesis under insulin-resistant conditions (Fig. 5 and 6). The effect of 5,7-DMC was comparable to that of rosiglitazone. These results demonstrate that 5,7-DMC promotes adipogenic differentiation and restored intracellular lipid storage in insulin-resistant adipocytes. Recent studies underscore the importance of preserving adipose lipid storage capacity to prevent ectopic lipid deposition and systemic insulin resistance (Trouwborst et al., 2018). Efficient storage of triglycerides in adipocytes prevents lipid overflow to the liver and skeletal muscle, thereby improving metabolic balance (Park et al., 2022). The observed increase in triglyceride and lipid content following 5,7-DMC treatment suggests that it may modulate key adipogenic transcription factors or signaling pathways such as PPARγ or PI3K/Akt to restore adipocyte function. Taken together, these findings highlight the therapeutic potential of 5,7-DMC in ameliorating insulin resistance by enhancing adipogenic capacity and restoring lipid metabolism in dysfunctional adipocytes. Further mechanistic studies are warranted to elucidate the molecular targets through which 5,7-DMC exerts its insulin-sensitizing effects.

Fig. 5. Effect of 5, 7- DMC on TG content in 3T3-L1 cells. Values are expressed as the mean ± SD (n=3). Difference between the groups were evaluated by one-way ANOVA followed by Duncan's Post hoc test P<0.05.



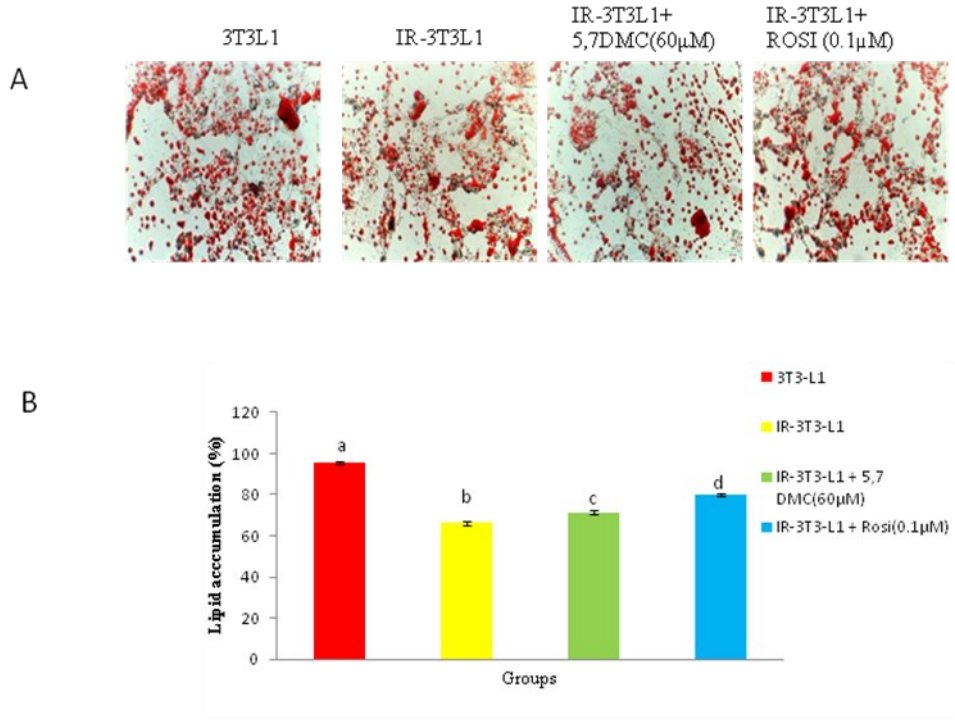


Fig. 6. Effect of 5,7 DMC on lipid accumulation in 3T3-L1 cells A: Staining images of experimental cell lines. **B.** Lipids were extracted and quantified at 520nm. Values are expressed as the mean ± SD (n=3). Difference between the groups were evaluated by one-way ANOVA followed by Duncan's Post hoc test P<0.05.

Binding interaction of 5,7 Dimethoxy coumarin and rosiglitazone against PPARγ

Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear transcription factor and master regulator of adipogenesis. It regulates genes involved in lipid biosynthesis, glucose metabolism, and triglyceride storage. Among the PPAR isoforms (PPARα, PPARß/δ, and PPARγ), PPARγ is predominantly expressed in adipose tissue and is crucial for maintaining adipocyte differentiation and insulin sensitivity (Corrales et al., 2018). Activation of PPARγ enhances glucose uptake and promotes the expression of adipogenic markers, making it a prime therapeutic target in metabolic diseases (Ahmadian et al., 2013). In the present study, molecular docking analysis was performed to investigate the interaction between 5,7-DMC and the ligand-binding domain of PPARγ. The docking score for 5,7-DMC was -6.6 kcal/mol, indicating a favorable binding affinity, while rosiglitazone, a well-established PPARγ agonist, showed a higher docking score of -7.5 kcal/mol (Table1).

Table 1: Binding energy and interacting amino acid residues of 5,7-DMC and rosiglitazone with PPARγ

Ligands	Targets	Binding energy (kcal/mol)	Interacting residues	Intersecting residues	No. of Hydrogen bond interactions
5,7-DMC	PPARγ	-6.6	MET463, LEU465, LEU453, GLN454, PHE282, LYS451, GLN 286 and SER464	MET463, LEU453, GLN454, PHE282	2
Rosiglitazone		-7.5	MET463, LYS457, LEU453, ASP475, LYS474, ILE456, GLN 454, LEU465, PHE 282, VAL 450, TYR 473 and GLN 470.		1

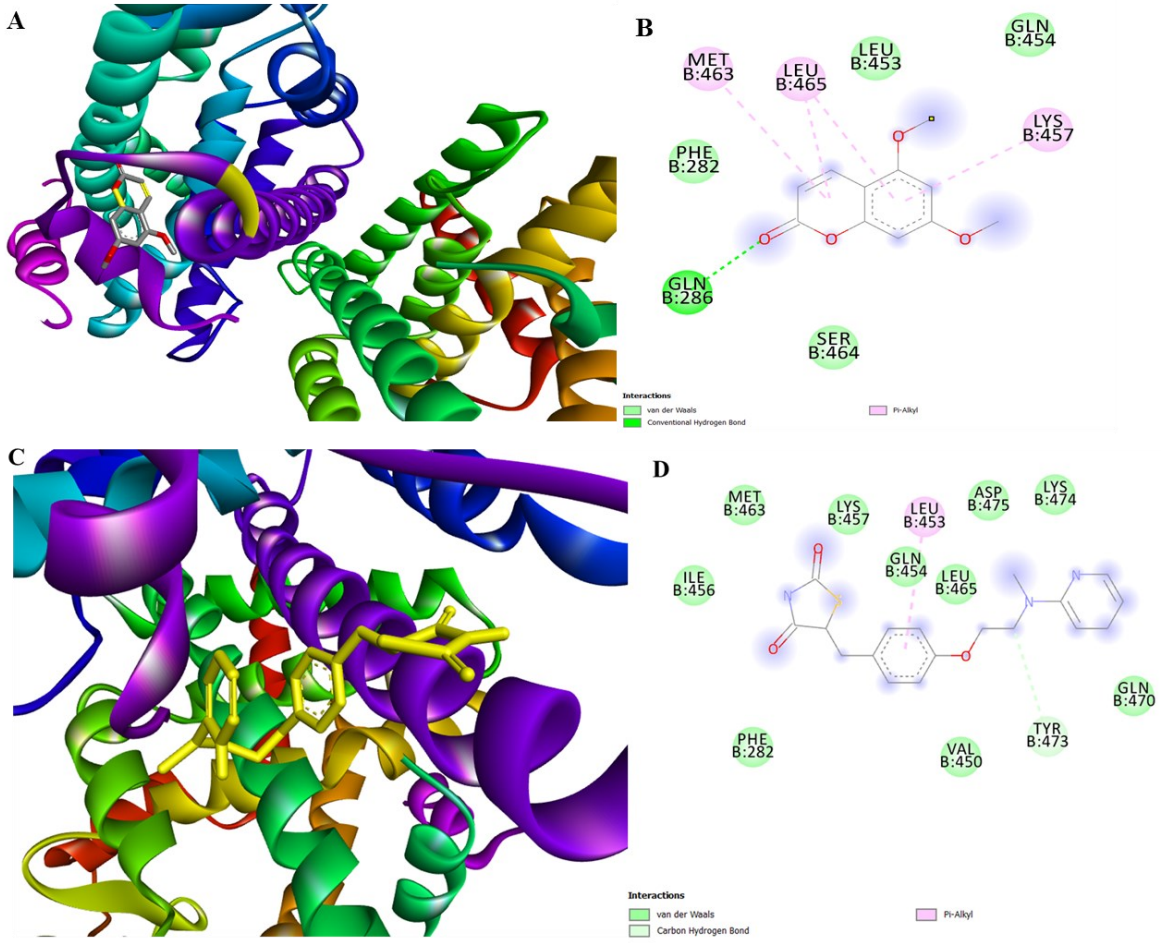


Fig. 7. 3D and 2D interactions of 5,7-DMC (A and B) and rosiglitazone (C and D) with PPARγ.

Despite the slightly lower affinity, 5,7-DMC demonstrated strong interaction with the active site of PPARγ, forming hydrogen bonds with critical residues including MET463, LEU465, LEU453, GLN454, PHE282, LYS451, GLN286, and SER464 (Fig.7B). These residues are known to be crucial for the activation of the receptor and downstream transcriptional regulation of adipogenic genes. The comparable binding interactions of 5,7-DMC and rosiglitazone suggest that 5,7-DMC may mimic the activity of synthetic PPARγ agonists, promoting adipogenesis and improving insulin sensitivity. In line with these findings, experimental data demonstrated that treatment with 5,7-DMC significantly increased glucose uptake and lipid accumulation in IR-3T3-L1 adipocytes. This enhancement in adipocyte functionality was accompanied by elevated triglyceride synthesis, consistent with the role of PPARγ in upregulating genes involved in lipogenesis. The observed effects of 5,7-DMC

are parallel to punicic acid which has been reported to improve glucose uptake and adipogenic differentiation through PPARγ activation (Anushree et al., 2020). Such activation contributes to restoration of adipocyte metabolic function under insulin-resistant conditions. Together, these results indicate that 5,7-DMC interacts efficiently with PPARγ and enhances adipogenesis and lipid storage in insulin-resistant adipocytes. By targeting the PPARγ signaling pathway, 5,7-DMC holds promise as a therapeutic agent for managing metabolic disorders associated with insulin resistance such as obesity and type 2 diabetes.

CONCLUSION

The findings of this study indicated that 5,7-dimethoxy coumarin significantly restored insulin sensitivity and improved adipocyte function under hyperglycemia induced insulin-resistant conditions. The beneficial effects are mediated through its interaction with PPARγ, a key transcription factor involved in adipogenesis and lipid metabolism. As a naturally occurring phytochemical, 5,7-DMC holds promise as a cost-effective and potentially safer alternative to synthetic drugs for managing insulin resistance. Further *in vivo* and clinical studies are warranted.

COMPETING INTEREST

Authors have declared that no competing interest exists.

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