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ENHANCED GLUCOSE UPTAKE ACTIVITY OF *ORTHOSIPHON THYMIFLORUS* (ROTH) SLEESEN EXTRACTS IN L6 RAT SKELETAL MUSCLE CELL LINES: AN *IN VITRO* ANTI-DIABETIC STUDY

Saran Mohan M. B.[1]*, Harigovind M.[2], Visakh V. V.[3], Elizabeth M. Abraham[4], Simi S. M.[5]

[1,2,3]Student, Department of Pharmacology, Mar Dioscorus College of Pharmacy, Hermongiri Vidyapeetam, Alathara, Sreekariyam, Thiruvananthapuram.

[4,5]Associate Professsor, Department of Pharmacology, Mar Dioscorus College of Pharmacy, Hermongiri Vidyapeetam, Alathara, Sreekariyam, Thiruvananthapuram.

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***Corresponding Author**

Saran Mohan M. B.

Student, Department of Pharmacology, Mar Dioscorus College of Pharmacy, Hermongiri Vidyapeetam, Alathara, Sreekariyam, Thiruvananthapuram.

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ABSTRACT

Skeletal muscle insulin resistance is a primary defect in Type 2 Diabetes Mellitus, and plants enhancing peripheral glucose uptake are of significant therapeutic interest. This study aimed to investigate the *in vitro* anti-diabetic potential of chloroform and ethanolic extracts of *Orthosiphon thymiflorus* by evaluating glucose uptake in L6 myotubes. Differentiated L6 rat skeletal muscle cells were treated with varying concentrations (25, 50, 100 µg/mL) of chloroform and ethanolic extracts of *O. thymiflorus*. Glucose uptake activity was measured by estimating the residual glucose concentration in the culture medium using the anthrone reagent method and comparing it to untreated control cells and standard insulin. The ethanolic extract exhibited a marked, concentration-dependent increase in glucose uptake. At 100 µg/mL, the ethanolic extract facilitated 68.89% glucose uptake, compared to 66.42% for the chloroform extract and 88.09% for standard insulin at the same concentration, while baseline glucose uptake in untreated cells was 31.22%. These findings demonstrate that the ethanolic extract of *O. thymiflorus* significantly enhances glucose uptake in L6 skeletal muscle cells, indicating potent peripheral insulin-mimetic or insulin-sensitizing

activity. This study provides the first *in vitro* evidence supporting the traditional use of *O. thymiflorus* for managing diabetes mellitus.

KEYWORDS: *Orthosiphon thymiflorus*; Diabetes Mellitus; Glucose Uptake Assay; L6 Cell Line; Skeletal Muscle; *In Vitro* Anti-diabetic; Insulin.

1. INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The global prevalence of diabetes has reached epidemic proportions, with the International Diabetes Federation estimating that over 589 million adults were living with diabetes in 2024, a number projected to rise to nearly 900 million by 2050.[1,2] Type 2 diabetes mellitus (T2DM), which accounts for approximately 90-95% of all diabetes cases, is primarily driven by peripheral insulin resistance in key metabolic tissues, particularly skeletal muscle, adipose tissue, and liver.[3]

Skeletal muscle is the principal site of postprandial glucose disposal, responsible for up to 80% of insulin-stimulated glucose uptake in healthy individuals.[4] This process is mediated by the insulin-regulated translocation of glucose transporter type 4 (GLUT4) from intracellular vesicles to the plasma membrane. In T2DM, defects in the insulin signaling cascade impair GLUT4 translocation, resulting in reduced glucose clearance and chronic hyperglycemia.[5] Therefore, therapeutic strategies aimed at enhancing peripheral glucose uptake in skeletal muscle represent a cornerstone of T2DM management.

While several classes of oral antidiabetic agents exist, including biguanides, sulfonylureas, and thiazolidinediones, their use is often associated with adverse effects such as gastrointestinal intolerance, hypoglycemia, weight gain, and cardiovascular concerns.[6] Consequently, there is a growing interest in identifying safe and effective natural products from medicinal plants that can improve glucose homeostasis through multiple mechanisms, including enhancement of glucose uptake and amelioration of insulin resistance.[7,8]

The genus *Orthosiphon* (Lamiaceae) comprises several species with documented antidiabetic potential. *Orthosiphon stamineus* Benth. (Java tea) has been extensively studied and shown to exhibit hypoglycemic, hypolipidemic, and insulin-sensitizing effects in various experimental models.[9,10] *Orthosiphon thymiflorus* (Roth) Sleesen, a closely related species indigenous to

South India and parts of tropical Asia and Africa, is traditionally employed in Ayurvedic and folk medicine for managing "sugar balance" and metabolic disorders.[11,12] Phytochemical investigations have revealed the presence of bioactive constituents in *O. thymiflorus*, including flavonoids (eupatorin, sinensetin), phenolic acids (rosmarinic acid), and triterpenoids, which have been independently associated with antidiabetic properties.[13,14]

Despite its traditional use and promising phytochemical profile, the antidiabetic activity of *O. thymiflorus* has not been scientifically validated. The L6 rat skeletal muscle cell line is a well-established and widely used *in vitro* model for studying glucose uptake, GLUT4 translocation, and insulin signaling mechanisms.[15] Therefore, the present study was designed to evaluate the *in vitro* antidiabetic potential of chloroform and ethanolic extracts of *O. thymiflorus* whole plant by assessing their ability to enhance glucose uptake in differentiated L6 myotubes.

2. MATERIALS AND METHODS

2.1 Plant Material and Extraction

The whole plant of *Orthosiphon thymiflorus* (Roth) Sleesen was collected from Thiruvananthapuram, Kerala, India, and authenticated by a qualified botanist (Voucher specimen deposited). The plant material was shade-dried, pulverized into a coarse powder, and 60 g was subjected to successive Soxhlet extraction using chloroform (45-55°C, 3-5 days) followed by 95% ethanol (40-50°C, 3-7 days). The extracts were concentrated under reduced pressure using a rotary evaporator and dried to obtain the crude chloroform extract (OTCE) and crude ethanolic extract (OTEE). The percentage yield was calculated, and extracts were stored at 4°C until use.

2.2 Chemicals and Reagents

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), antibiotic-antimycotic solution, bovine serum albumin (BSA), phosphate-buffered saline (PBS), D-glucose, anthrone reagent, and human recombinant insulin were procured from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

2.3 Cell Culture and Differentiation

The L6 rat skeletal muscle myoblast cell line was obtained from the National Centre for Cell Science (NCCS), Pune, India. Cells were maintained in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic solution at 37°C in a humidified atmosphere containing 5%

CO_2. For differentiation into multinucleated myotubes, cells were seeded in 12-well plates and allowed to reach 90-100% confluence. The medium was then replaced with DMEM containing 2% horse serum, and differentiation was allowed to proceed for 6-7 days with medium changes every 48 hours. Differentiation was confirmed by microscopic observation of myotube formation.

2.4 Glucose Uptake Assay

Glucose uptake activity in differentiated L6 myotubes was estimated using the method described by Gupta et al. with slight modifications (16). Differentiated myotubes were serum-starved overnight (18 hours) in serum-free DMEM containing 0.2% BSA. Following starvation, cells were washed once with PBS (pH 7.4) and incubated with 1000 µg/mL glucose in the presence of varying concentrations (25, 50, 100 µg/mL) of the test extracts (OTCE or OTEE) or standard insulin for 1 hour at 37°C.

Untreated control cells received only the glucose-containing medium without any test substance. After the 1-hour incubation, the medium was collected from each well, and the residual glucose concentration was determined using the anthrone-sulfuric acid method.[17]

2.5 Estimation of Residual Glucose (Anthrone Method)

A glucose standard curve was prepared using concentrations ranging from 200 to 1000 µg/mL. To 1 mL of each standard or test sample (diluted appropriately), 4 mL of freshly prepared anthrone reagent (0.2% w/v in concentrated sulfuric acid) was added carefully. The tubes were vortexed and placed in a boiling water bath for 8 minutes. After cooling rapidly to room temperature, the absorbance was measured at 630 nm using a UV-Visible spectrophotometer (Shimadzu UV-1800). The glucose concentration in the test samples was interpolated from the standard curve.

2.6 Calculation of Glucose Uptake

Glucose uptake was calculated as the difference between the initial glucose concentration (1000 µg/mL) and the residual glucose concentration measured in the medium after the 1-hour incubation period. The percentage glucose uptake was calculated using the following formula:

% Glucose Uptake = [(Initial Glucose - Final Glucose) / Initial Glucose] × 100

2.7 Statistical Analysis

All experiments were performed in triplicate, and data are expressed as mean ± standard deviation (SD). Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test using GraphPad Prism software. A p-value < 0.05 was considered statistically significant.

3. RESULTS

3.1 Extraction Yield

The successive Soxhlet extraction yielded **4.15% w/w** for the chloroform extract (OTCE) and **8.0% w/w** for the ethanolic extract (OTEE), indicating a higher proportion of polar extractable constituents in the plant material.

3.2 Glucose Standard Curve

The anthrone method produced a linear standard curve for glucose concentrations ranging from 200 to 1000 µg/mL, with a correlation coefficient (R^2) > 0.99. The regression equation was used for accurate interpolation of residual glucose concentrations in the test samples.

3.3 Effect of Extracts on Glucose Uptake in L6 Myotubes

Both chloroform and ethanolic extracts of *O. thymiflorus* significantly enhanced glucose uptake in differentiated L6 myotubes in a concentration-dependent manner. The ethanolic extract (OTEE) demonstrated consistently superior glucose uptake activity compared to the chloroform extract (OTCE) across all tested concentrations.

At the highest tested concentration of **100µg/mL**, the ethanolic extract facilitated **68.89%** glucose uptake, while the chloroform extract achieved **66.42%** uptake. In comparison, the standard drug **insulin (100µg/mL)** exhibited **88.09%** glucose uptake. The baseline glucose uptake in untreated control cells was **31.22%**.

The detailed results of the glucose uptake assay are presented in **Table 1**.

Table 1: Percentage Glucose Uptake in L6 Myotubes by *O. thymiflorus* Extracts and Insulin.

Treatment Group	Concentration (µg/mL)	Glucose Uptake (%)
Untreated Control	-	31.22 ± 1.5
Standard Insulin	25	56.42 ± 1.2
	50	67.52 ± 1.0
	100	88.09 ± 0.8
Chloroform	25	43.09 ± 1.8

Extract (OTCE)		
	50	54.06 ± 1.4
	100	66.42 ± 1.1
Ethanolic Extract (OTEE)	25	44.26 ± 1.6
	50	55.82 ± 1.3
	100	68.89 ± 0.9

Values are expressed as mean ± SD (n=3).

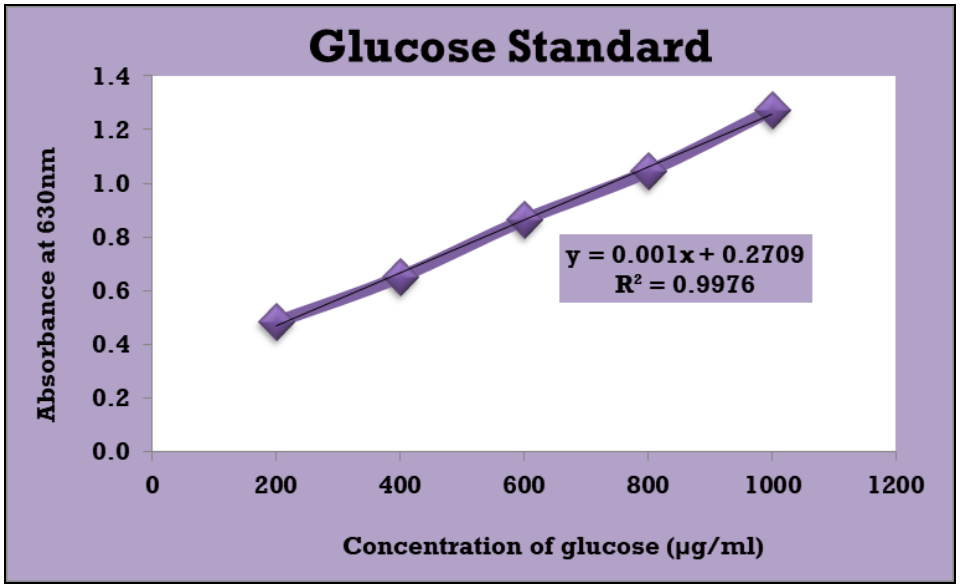

Figure 5.17: Glucose standard graph.

The concentration-dependent increase in glucose uptake for both extracts and insulin is illustrated in **Figure 1**.

1) Sample 1: Chloroform extract (Test Sample).

Table 5.21: Percentage glucose uptake of chloroform extract.

Sample concentration	Absorbance at 630 nm				Glucose concentration (µg/ml)	Percentage Glucose uptake
	Triplicate 1	Triplicate 2	Triplicate 3	Average		
Control (Untreated)	0.950	0.965	0.961	0.959	687.77	31.22
25	0.844	0.840	0.836	0.840	569.10	43.09
50	0.737	0.729	0.725	0.730	459.43	54.06
100	0.600	0.608	0.612	0.607	335.77	66.42

Inference

The glucose uptake was found to be increasing in concentration dependent manner also. The highest glucose uptake activity was shown by the sample at the concentration of 100µg/ml.

Sample 2: Ethanolic extract (Test Sample).

Table 5.22: Percentage glucose uptake of ethanolic extract.

Sample concentration	Absorbance at 630 nm				Glucose concentration (µg/ml)	Percentage Glucose uptake
	Triplicate 1	Triplicate 2	Triplicate 3	Average		
Control (Untreated)	0.950	0.965	0.961	0.959	687.77	31.22
25	0.822	0.829	0.834	0.828	557.43	44.26
50	0.711	0.719	0.708	0.713	441.77	55.82
100	0.572	0.589	0.585	0.582	311.10	68.89

Inference

The glucose uptake was found to be increasing in concentration dependent manner also. The highest glucose uptake activity was shown by the sample at the concentration of 100µg/ml.

4. DISCUSSION

The L6 skeletal muscle cell line is a well-characterized and physiologically relevant *in vitro* model for studying glucose transport and insulin action. Upon differentiation, L6 myoblasts fuse to form multinucleated myotubes that express key proteins of the insulin signaling pathway, including the insulin receptor, insulin receptor substrate-1 (IRS-1), phosphatidylinositol 3-kinase (PI3K), Akt, and the insulin-sensitive glucose transporter GLUT4 (15). Therefore, enhanced glucose uptake in this model is indicative of potential insulin-mimetic or insulin-sensitizing activity of the test substance.

The present study demonstrates, for the first time, that extracts of *Orthosiphon thymiflorus* significantly stimulate glucose uptake in L6 myotubes in a concentration-dependent manner. The ethanolic extract (OTEE) at 100 µg/mL enhanced glucose uptake to 68.89%, which is approximately 2.2-fold higher than the basal uptake observed in untreated control cells (31.22%). While the activity was lower than that of standard insulin at the equivalent concentration (88.09%), the substantial glucose uptake observed with the crude extract is highly promising and underscores the therapeutic potential of this traditionally used medicinal plant.

The superior activity of the ethanolic extract compared to the chloroform extract is consistent with the known phytochemical profile of *O. thymiflorus* and related *Orthosiphon* species. Ethanol, being a polar solvent, efficiently extracts a wide range of bioactive secondary metabolites, including flavonoid glycosides, phenolic acids, and certain saponins, which are

known to possess antidiabetic properties.[18,19] In contrast, chloroform primarily extracts non-polar to moderately polar compounds such as terpenoids, sterols, and some aglycones, which may have a more modest effect on glucose uptake.

Several mechanisms have been proposed to explain the glucose uptake-enhancing effects of plant-derived flavonoids and phenolic compounds. These bioactive molecules can activate AMP-activated protein kinase (AMPK), a key cellular energy sensor that promotes GLUT4 translocation independent of insulin signalling.[20] They may also enhance insulin sensitivity by reducing oxidative stress and inflammation, which are known to impair insulin signaling through serine phosphorylation of IRS-1.[21] Furthermore, certain phytoconstituents can directly stimulate the PI3K/Akt pathway, mimicking insulin's action.[22] Given that *O. thymiflorus* has been shown to be rich in flavonoids such as eupatorin and sinensetin, as well as rosmarinic acid,[13,14] it is plausible that one or more of these mechanisms contribute to the observed enhancement of glucose uptake.

Previous studies on the related species *Orthosiphon stamineus* have demonstrated significant antidiabetic effects, including reduction of fasting blood glucose, improvement of glucose tolerance, and amelioration of insulin resistance in high-fat diet-fed and streptozotocin-induced diabetic rodent models.[9,10] The active constituents identified in *O. stamineus*, such as sinensetin and tetramethylscutellarein, have been shown to activate AMPK and enhance GLUT4 translocation in skeletal muscle cells.[23] The present findings with *O. thymiflorus* align with these observations and suggest that this species may share similar antidiabetic mechanisms.

It is noteworthy that the ethanolic extract of *O. thymiflorus* exhibited a glucose uptake activity that was approximately 78% of the insulin response at the same concentration (100 µg/mL). This level of *in vitro* efficacy in a crude extract is significant and supports further investigation.

5. CONCLUSION

This study provides the first *in vitro* evidence demonstrating that extracts of *Orthosiphon thymiflorus* (Roth) Sleesen, particularly the ethanolic extract, significantly enhance glucose uptake in differentiated L6 rat skeletal muscle myotubes. The ethanolic extract exhibited a marked, concentration-dependent stimulation of glucose transport, achieving 68.89% uptake at 100 µg/mL compared to 31.22% in untreated control cells. This represents a 2.2-fold

increase over basal uptake and approximates 78% of the insulin response at equivalent concentration. The observed activity is likely mediated through insulin-mimetic or insulin-sensitizing mechanisms, potentially involving activation of AMPK and PI3K/Akt signaling pathways, leading to enhanced GLUT4 translocation to the plasma membrane. The superior efficacy of the ethanolic extract correlates with its richer profile of polar bioactive constituents, including flavonoids and phenolic compounds known to modulate glucose homeostasis. These findings offer robust scientific validation for the traditional use of *O. thymiflorus* in managing diabetes mellitus and related metabolic disorders. Further investigations, including *in vivo* studies in diabetic animal models and mechanistic elucidation of the precise molecular pathways and active phytoconstituents responsible for the observed activity, are warranted to fully establish the therapeutic potential of this promising medicinal plant.

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