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INVESTIGATING THE POTENTIAL EFFECT OF L-CITRULLINE ON PI3K SIGNALING PATHWAY: *IN SILICO* AND *IN VITRO* EVALUATION

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Insulin resistance is a key feature of type 2 diabetes mellitus (T2DM), resulting from dysfunction in the insulin signaling pathway, which involves critical proteins such as IRS-PI3K-IRS-1-PKC-AKT2 and GLUT4. Metformin, a first-line T2DM treatment, exerts its effects via various mechanisms, but alternative therapies are needed. L-Citrulline, an amino acid with antiglycation and antioxidant properties, has shown potential as a therapeutic agent.
The aim. *This study aims to evaluate the efficacy of L-Citrulline, alongside the well-established antidiabetic drug metformin, in a T2DM model using both in vitro and in silico approaches.*
Materials and methods. *Differentiated L6 skeletal muscle cells, induced with high glucose and insulin concentrations to model insulin resistance, were treated with either L-Citrulline or metformin. The expression of PI3K, a key protein in insulin signaling, was assessed using an ELISA Kit. In silico molecular docking studies were also conducted to examine the binding interactions of L-Citrulline and metformin with PI3K.*
Results. *L-Citrulline treatment significantly increased PI3K concentration levels in insulin-resistant skeletal muscle cells, indicating a potential restoration of insulin signaling. The enhancement in PI3K concentration was comparable to that observed with metformin, validating the effectiveness of L-Citrulline in modulating the PI3K pathway. Molecular docking studies revealed that L-Citrulline formed stable and favorable interactions with PI3K, suggesting strong binding affinity and potential enhancement of its catalytic activity.*
Conclusion. *L-Citrulline demonstrates potential in modulating the PI3K signaling pathway based on both in vitro and in silico findings, indicating a possible role in improving insulin responsiveness in type 2 diabetes mellitus (T2DM). Nevertheless, these results are preliminary, and further in vivo and clinical investigations are needed to confirm its therapeutic relevance*
Keywords: *Insulin resistance, Type 2 diabetes mellitus, L-Citrulline, Metformin, PI3K signaling pathway, ADME, In vitro, In silico, Molecular docking, Skeletal muscle cells*

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1. Introduction

Diabetes Mellitus (DM) is a prevalent metabolic disorder characterized by elevated levels of glucose in the bloodstream, a condition known as hyperglycemia. It results from abnormalities in insulin secretion, impaired insulin sensitivity, or a combination of both [1]. Type 2 diabetes mellitus (T2DM) is a complex metabolic condition characterized by insufficient secretion of insulin from pancreatic beta cells, increased glucose uptake by the liver, and insulin resistance in peripheral tissues such as the liver, skeletal muscle, and adipose tissue [2]. Over the last 30 years, there has been a significant increase in the occurrence of type 2 diabetes across countries with varying income levels. According to the International Diabetes Federation, global diabetes prevalence was estimated at 9.3% in 2019, affecting nearly 463 million individuals. Projections indicate a rise to 10.2% (equivalent to 578 million people) by 2030, with further escalation to 10.9% (reaching 700 million individuals) by 2045 [3]. Insulin, by activating the tyrosine kinase receptor pathway, is crucial in controlling plasma glucose levels and aiding cellular functions like glucose absorption, glycogenesis, and protein synthesis in skeletal muscle tissues [4, 5]. Impaired glucose transport in the skeletal muscle of people with diabetes causes insulin resistance, leading to serious consequences such as high blood sugar levels and elevated cholesterol levels [5]. The translocation of glucose transporter type 4 (GLUT4) to the cell membrane, which is necessary for glucose uptake in skeletal muscle, is controlled by two separate signaling networks: the PI3K/AKT and AMPK pathways. Insulin facilitates this process by stimulating the movement of GLUT4 from inside the cell to the outer membrane, mainly through the activation of the PI3K/AKT pathway [6].

Derivatives of urea, exemplified by the biguanide class of drugs such as metformin, have emerged as primary pharmacotherapeutic interventions for the management of type 2 diabetes mellitus. Metformin exerts its therapeutic effect by enhancing glucose utilization within muscle tissue through a mechanism situated distally from the insulin receptor and influenced by insulin signaling. Notably, metformin demonstrates the capacity to ameliorate glucose metabolism in individuals with type 2 diabetes by augmenting insulin sensitivity in peripheral tissues, particularly in skeletal muscle [7].

L-Citrulline, a nonessential amino acid involved in the urea cycle, has the capacity to elevate nitric oxide (NO) levels in the body upon consumption. Given NO's crucial role in regulating glucose metabolism – and the observed reduction in NO bioavailability among individuals with type 2 diabetes mellitus (T2DM) – enhancing NO production represents a promising strategy for improving glycemic control [7]. Recent clinical and preclinical studies have highlighted L-Citrulline's potential as a nutraceutical supplement with metabolic and cardiovascular benefits. Supplementation with L-Citrulline has been shown to increase NO bioavailability, improve endothelial function, and support glucose utilization in insulin-resistant conditions [8]. Moreover, oral L-Citrulline administration has demonstrated improvements in arterial stiffness and metabolic parameters among patients with obesity and T2DM [9].

In addition to these clinical findings, previous reports have indicated that it possesses antiglycation properties *in vitro*, which means it can suppress the production of advanced glycation end products (AGEs). Furthermore, research has indicated that L-Citrulline has antioxidant properties due to its capacity to reduce the generation of reactive oxygen species (ROS) triggered by oxidative stress. Despite existing evidence of its antidiabetic potential, the effects of L-Citrulline have not been fully elucidated. Accordingly, this study aims to investigate the *in vitro* and *in silico* activity of L-Citrulline on the PI3K signaling pathway [10].

Metformin, a widely used antidiabetic agent, was employed as a reference control. The investigation focused on insulin resistance mediated through the PI3K signaling pathway and included ADME profiling and toxicity risk assessment [5]. Both *in silico* molecular docking and *in vitro* experiments were conducted to comprehensively evaluate the potential of L-Citrulline. In the computational phase of this study, molecular docking was performed using AutoDock Vina 1.2.0 to model the interaction between L-Citrulline and the PI3K isoforms, specifically PI3K-α and PI3K-γ, at the atomic level. Molecular descriptors and pharmacokinetic properties, including absorption, distribution, metabolism, and excretion (ADME), were assessed using the SwissADME and SwissTargetPrediction platforms, based on Lipinski's, Ghose's, and Veber's rules. In addition, *in silico* toxicity predictions were carried out using the OSIRIS Property Explorer. These simulations provided insight into the binding behavior of L-Citrulline with key proteins in the PI3K signaling pathway, enhancing our understanding of its potential role in modulating insulin resistance and contributing to therapeutic strategies for type 2 diabetes mellitus. In the *in vitro* phase, an insulin-resistant cellular model was developed by exposing L6 skeletal muscle cells to elevated levels of insulin and glucose. The L6 cell line was chosen for its strong expression of the PI3K gene, which plays a pivotal role in insulin signaling.

Accordingly, this study aims to investigate the *in vitro* and *in silico* activities of L-Citrulline on the phosphoinositide 3-kinase (PI3K) signaling pathway to provide deeper insight into its potential therapeutic relevance in insulin resistance and type 2 diabetes mellitus (T2DM). The scientific novelty of this work lies in the integrative application of *in vitro* and *in silico* approaches to elucidate the mechanistic role of L-Citrulline in modulating PI3K activity – an aspect that remains underexplored in existing literature. This investigation provides new mechanistic evidence supporting L-Citrulline's potential role in enhancing insulin responsiveness and glucose metabolism.

2. Planning (methodology) of research

1. *In vitro***.** Induce insulin resistance in differentiated L6 skeletal muscle cells using high glucose and insulin concentrations, then treat with L-Citrulline or metformin. Assess PI3K concentration using an ELISA assay to evaluate the effect on the insulin signaling pathway.

2. *In silico* plan. Perform molecular docking studies to analyze the binding affinity and interaction of L-Citrulline and metformin with PI3K using computational tools, supporting the *in vitro* findings with structural insights.

3. Materials and methods

3. 1. Cell culture

Cells of L6 rat skeletal muscle cells (ATCC-CRL-1458) procured from Bio-Focus Scientific, Malaysia, were maintained in DMEM media containing 10% (v/v) FBS and 1% penicillin (100 U/mL). The cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C until they reached 70–80% confluency. Then they were detached using 0.25% (w/v) trypsin- EDTA and 0.05% glucose in PBS. The doubling time of the cells was 22–24 h. Therefore, re- suspended cells were transferred to a new T25 flask in a split ratio of 1:4 (subculture in 2–3 day).

3. 2. Cell differentiation and induction of insulin resistance of L6 cells

Myotube differentiation was induced in L6 rat skeletal cells through differentiation media during incubation. The cultures were incubated in a T-25 flask under high humidity and 5% CO_2 at 37°C. For the induction of insulin resistance, differentiated myotubes were cultured on 96-well microplates at a density of 2×10^4 cells/well. Before experimentation, the myotubes were subjected to a 24-hour pretreatment with DMEM media containing 25 mM glucose and 100 mM insulin. The cells were subsequently transferred to DMEM with low glucose concentration (5 mM) and without insulin for 5 hours. The media enriched with glucose was disposed of after 5 hours and subsequently substituted with media containing 100 mM insulin for an additional period of 30 minutes. L-Citrulline samples at concentrations of 10, 50, and 100 μM were prepared in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% heat-inactivated fetal bovine serum (FBS)**.** The selected concentrations were based on previous reports indicating that L-Citrulline is non-toxic to mammalian cells at micromolar levels. No cytotoxic effects were observed at these concentra-

tions during preliminary assessments, consistent with literature findings. Cell treatments were performed using L-Citrulline, and the cultures were incubated for 24 hours. Following treatment, protein was isolated from the cells for subsequent ELISA analysis**.**

3. 3. Rat Phosphotylinositol 3 kinase ELISA Kit

The L6 myoblast cell line was utilized for the present assay. The ELISA technique was employed to conduct the analysis, using the Rat Phosphotylinositol 3 kinase ELISA Kit (manufactured by Bioassay Technology Laboratory, China). The Rat PI3K antibody has been applied as a pre-coating on the plate. When PI3K from the sample is introduced, it binds to antibodies coated on the wells. Subsequently, the Rat PI3K Antibody that has been biotinylated is added and interacts with PI3K in the specimen. Later, the Streptavidin-HRP is introduced and forms a complex with the Biotinylated PI3K antibody. Following incubation, unbound Streptavidin-HRP is eliminated through a washing procedure. The addition of substrate solution results in the development of color directly proportional to the quantity of Rat PI3K. The reaction was stopped by introducing an acidic stop solution, and subsequently, the absorbance was quantified at a wavelength of 450 nm.

3. 4. *In silico* docking of L-Citrulline and metformin with PI3K protein preparation

The X-ray crystal structures of P13K alpha (PDB ID: 6PYS) and P13K gamma (PDB ID: 5JHB) were obtained from the Protein Data Bank and processed using a standard procedure. The structures were imported into Biovia Discovery Studio 2021 Client. Heteroatoms, such as water, are eliminated. The binding site properties were identified by analyzing the position of the co- crystalized ligand. The ligands (P5J and 6K5) that were co-crystallized with each structure were eliminated. Charges were subsequently introduced, and the structures were preserved as prepared protein. The constructed structures were subsequently imported into AutoDock Tools version 1.5.7 and saved in pdbqt format.

3. 5. Ligand preparation

The molecular structures of L-Citrulline and the standard Metformin were imported into AutoDock Tools version 1.5.7 to be prepared as ligands. The Gasteiger charges were incorporated into the corresponding structures and subsequently saved in the pdbqt format.

3. 6. Redocking of the co-crystallized ligand

The co-crystallized ligand was synthesized using the identical procedure as the other ligands and subsequently inserted into the binding site of the corresponding proteins using the same technique to get the docked positions. In Biovia Discovery Studio 2021 Client, the docked positions were superimposed upon the original poses to calculate the Root Mean Square Deviation (RMSD). A Root Mean Square Deviation (RMSD) value below 2.0 Å was deemed as the validation criterion for the docking process.

3. 7. Molecular docking

The process of molecular docking was conducted using Autodock Vina 1.2.0. The ligands synthesized in the preceding stage were computationally positioned within the binding sites of the produced P13K alpha and P13K gamma structures. The coordinates of the binding site for PI3K alpha were *X*: –18.018853, *Y*: 11.788912, *Z*: 28.460029. For PI3K gamma, the coordinates were *X*: 21.349173, *Y*: –4.074288, *Z*: 20.869615. A grid with dimensions of 40 × 40 × 40 points was used to cover the binding site. The level of exhaustiveness was established as 8. The stance exhibiting the lowest binding energy was determined to be the most advantageous pose. The binding interactions were observed using the Biovia Discovery Studio 2021 Client.

3. 8. Drug-likeness, ADME, and toxicity prediction

The molecular descriptor, ADME parameters, parameters of Lipinski, Ghose, and Veber rules, and molecular target were calculated using the SwissADME server and the SwissTargetPrediction server. Moreover, OSIRIS Property Explorer program was employed for *in silico* toxicity prediction which includes the potential mutagenicity, tumorigenicity, irritant effects, and reproductive toxicity of the hit compounds.

3. 9. Statistical analysis

The outcomes were presented as the average value ± the standard deviation of the mean, based on a minimum of three independent experiments. The process of assessing the data was executed through the utilization of GraphPad Prism 9.0.0, specifically designed. The statistical method of one-way analysis of variance (ANOVA) was utilized to analyze data sets that consisted of more than two conditions. In instances where the results indicated a significant difference ($p < 0.05$), a post hoc analysis was conducted using Dunnett test.

4. Results

Effect of L-Citrulline on the concentration of PI3K.

L-Citrulline was evaluated for its potential effects on insulin resistance using an *in vitro* model. Insulin resistance was induced in L6 skeletal muscle cells through exposure to high concentrations of insulin and glucose. Metformin was used as a standard control to validate the model.

As shown in Fig. 1, the concentration of PI3K were significantly reduced in the diabetic control group compared to the normal (untreated) control group. Treatment with L-Citrulline resulted in an increase in PI3K concentration, indicating a potential restoration of insulin signaling. A similar trend was observed in the metformin-treated group, further supporting the reliability of the experimental model.

The cell culture model demonstrated a statistically significant increase ($p < 0.0001$) in PI3K concentration in the metformin-treated group compared to the diabetic control, confirming the validity of metformin as the pos-

itive control. Among all groups, metformin resulted in the most pronounced PI3K increase. L-Citrulline treatment also resulted in a significant increase in PI3K concentration across all tested concentrations when compared to the diabetic control. At 100 µM, L-Citrulline induced a 55.48% increase in PI3K concentration, while the metformin control showed a 58.92% increase. Although metformin yielded a slightly higher response, the difference in effect between the two was not substantial. Interestingly, even at a lower concentration of 10 µM, L-Citrulline significantly enhanced PI3K concentration, suggesting its potential to restore insulin signaling via the PI3K/Akt pathway. These findings indicate that L-Citrulline may improve insulin sensitivity in insulin-resistant skeletal muscle cells.

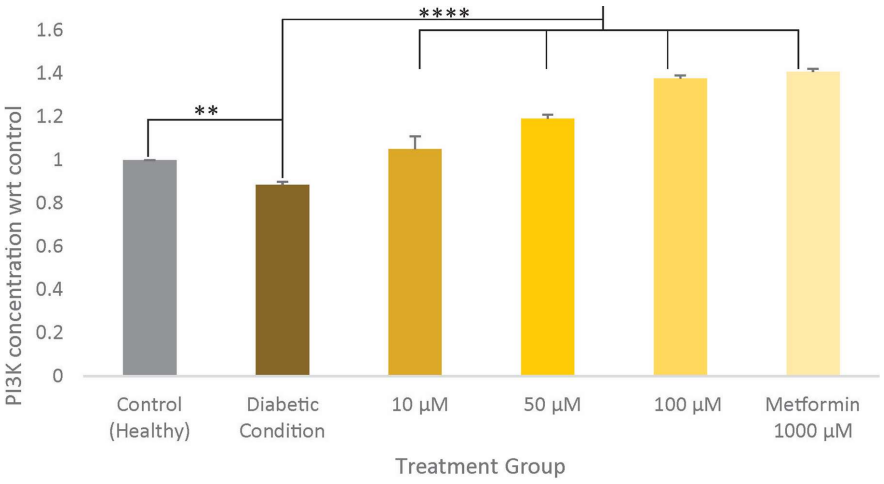


Fig. 1. PI3K concentration levels in healthy group, metformin, and L-Citrulline-treated groups relative to the diabetic condition. Data are presented as mean ± SEM from three independent experiments ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test. Significant differences are indicated relative to the diabetic condition: **** – $p < 0.0001$, *** – $p < 0.001$, ** – $p < 0.01$, $p < 0.05$

In Silico results.

To validate the docking protocol, the co-crystallized ligand was redocked into the binding site, and the resulting pose was compared to the native ligand using root mean square deviation (RMSD). An RMSD value below 2.0 Å is considered indicative of successful docking accuracy. The obtained RMSD values (Table 1) fell within this threshold, confirming the reliability of the docking process. This is further supported by the superimposed ligand poses shown in Fig. 2. Additionally, favorable binding energies were obtained for L-Citrulline with PI3K-α and PI3K-γ using AutoDock Vina 1.2.0, as presented in Table 1.

Based on the examination of binding energies, metformin, serving as the reference compound, showed binding affinities of –5.0 kcal/mol for PI3K gamma and –4.9 kcal/mol for PI3K alpha. In contrast, L-Citrulline exhibited slightly more negative binding energies of –5.4 kcal/mol for PI3K gamma and –5.5 kcal/mol for PI3K alpha. Comparing the binding energies of L-Citrulline and metformin provides valuable insight into their potential interactions with PI3K isoforms. The more negative values observed for L-Citrulline suggest a stronger predicted interaction with both PI3K targets. This may reflect a higher binding stability between L-Citrulline and the receptor sites. These findings support its potential role in modulating insulin signaling via the PI3K pathway.

Table 1

RMSD value of co-crystallized (from PI3K Alpha and Gamma) vs. docked poses

Protein-ligand	RMSD value (Å)
PI3K alpha–P5J	0.392
P13K gamma–6K5	2.022

Table 2

The binding energy of the drugs with PI3K Gamma and PI3K Alpha

Item	Binding energy (kcal/mol)	
	PI3K Gamma	PI3K Alpha
Metformin	–5.0	–4.9
L-Citrulline	–5.4	–5.5

The high binding affinity of L-Citrulline indicates its potential effectiveness as a therapeutic drug that targets these receptors. These receptors are involved in a range of cellular processes and diseases, including as cancer, inflammation, and metabolic disorders.

Subsequent investigations were carried out to scrutinize the binding interactions of L-Citrulline and metformin with PI3K gamma and alpha, depicted in Fig. 3–5. These complexes were visualized using Biovia Discovery Studio Visualizer 2021. Analysis of the binding interactions reveals that metformin engages in conventional hydrogen bonding with residues CYS275, HIS304, and GLU302 of PI3K gamma, and solely with TYR836 and MET811 of PI3K alpha. Additionally, there is a carbon hydrogen bond observed for both receptors: GLU301 for alpha and GLN630 for gamma. These interactions suggest stable anchoring of metformin within the binding pocket of both isoforms, primarily through polar and hydrogen-bonding forces that stabilize the ligand-receptor complex. Evaluation of the binding interactions of L-Citrulline demonstrates conventional hydrogen bonds with LEU1006, GLN1014, SER1008, GLY1007, and ASN677 of PI3K alpha, and with TRP292, HIS304, CYS275, and GLY276 of PI3K gamma. A carbon–hydrogen bond interaction was observed with GLN1014 of PI3K-α, while alkyl interactions were present with ALA822 and LEU823 of PI3K-gamma. These multiple hydrogen-bonding and hydrophobic contacts indicate favorable binding stability and a well-defined orientation within the catalytic domain of PI3K.

Overall, the docking results provide a detailed depiction of the molecular interactions between these

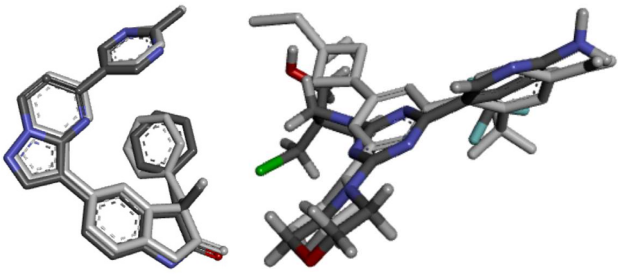
compounds and PI3K isoforms. The presence of multiple stabilizing interactions highlights potential ligand compatibility with the enzyme's active site, suggesting that both compounds could modulate PI3K activity through non-covalent binding mechanisms. Such interaction mapping enhances understanding of the chemical basis underlying PI3K recognition and may support rational design of future modulators targeting this pathway.

ADME analysis.

The tested compound, L-Citrulline, was subjected to preliminary *in silico* analyses of pharmacokinetic parameters and ADMET profile and prediction of biological targets using the web platforms SwissADME, SwissTargetPrediction and OSIRIS Property Explorer.

Due to the structural differences (Fig. 5), all molecular descriptors from the molecular weight up to the TPSA surface and lipophilicity are different.

Despite their inherent distinctions, both Metformin and L-Citrulline adhere to Lipinski's five rules and Veber's rules, indicating favorable oral bioavailability. However, they do not completely satisfy the criteria outlined by Ghose and Muegge for bioavailability. Furthermore, the software SwissTargetPrediction predicts a high likelihood of interaction with diverse molecular targets, including proteases, kinases, oxidoreductase, and family A G protein-coupled receptors for these compounds.



a *b*

Fig. 2. Structure of: *a* – PI3K alpha co-crystallized (from PI3K alpha) vs. docked pose; *b* – PI3K gamma co-crystallized (from PI3K gamma) vs. docked pose

Table 3

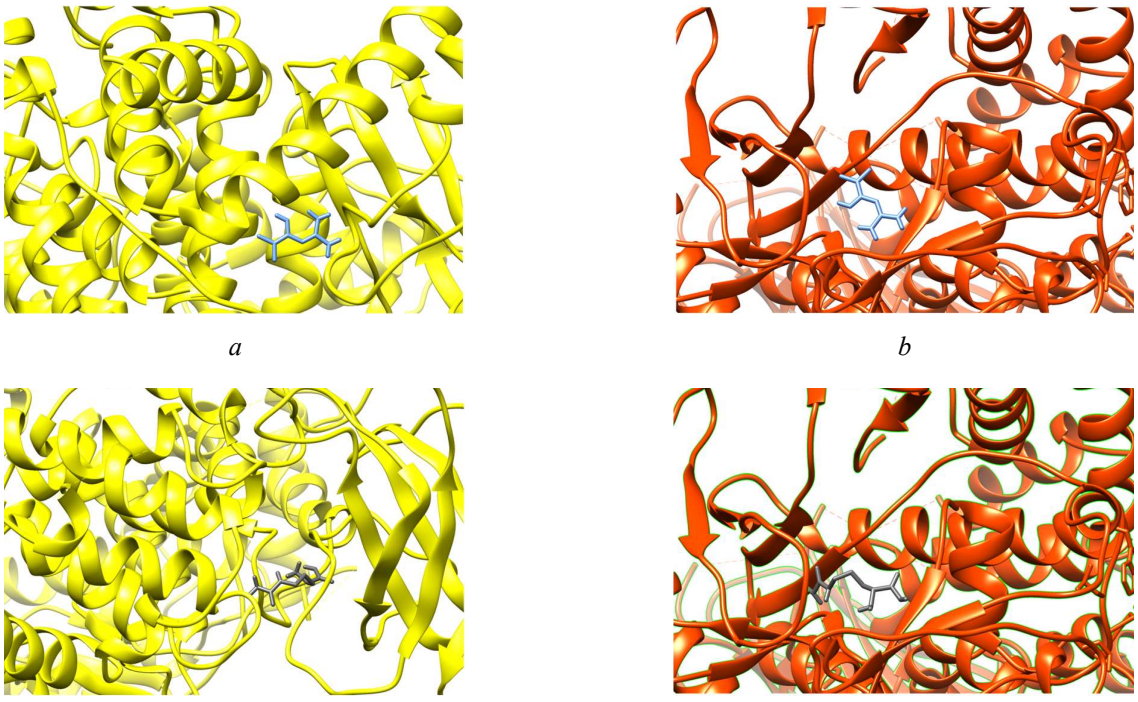
The molecular descriptor and parameters of Lipinski's, Ghose's, and Veber's rules for (1), Metformin (2) L-Citrulline

No.	Molecular mass (*M*), g/mol	H-Bond acceptors	H-bond donor	Rotatable bonds	TPSA, Å^2	Log P consensus	Lipinski's rule	Ghose's rule	Veber's rule	Muegge's rule
1	129.16	2	3	2	91.49	–0.89	+	–	+	–
2	175.19	4	4	6	118.44	–1.82	+	–	+	–

Table 4

Toxicity predictions of Metformin and L-Citrulline

Compounds	Toxicity			
	Mutagenicity	Tumorgenicity	Irritant Effect	Reproductive
Metformin	none	none	none	none
L-Citrulline	none	none	none	none



a *b*

c *d*

Fig. 3. The dock poses of: *a* – metformin with PI3K alpha; *b* – metformin with PI3K gamma; *c* – L-Citrulline with PI3K alpha; *d* – L-Citrulline with PI3K gamma shown as ribbon representation

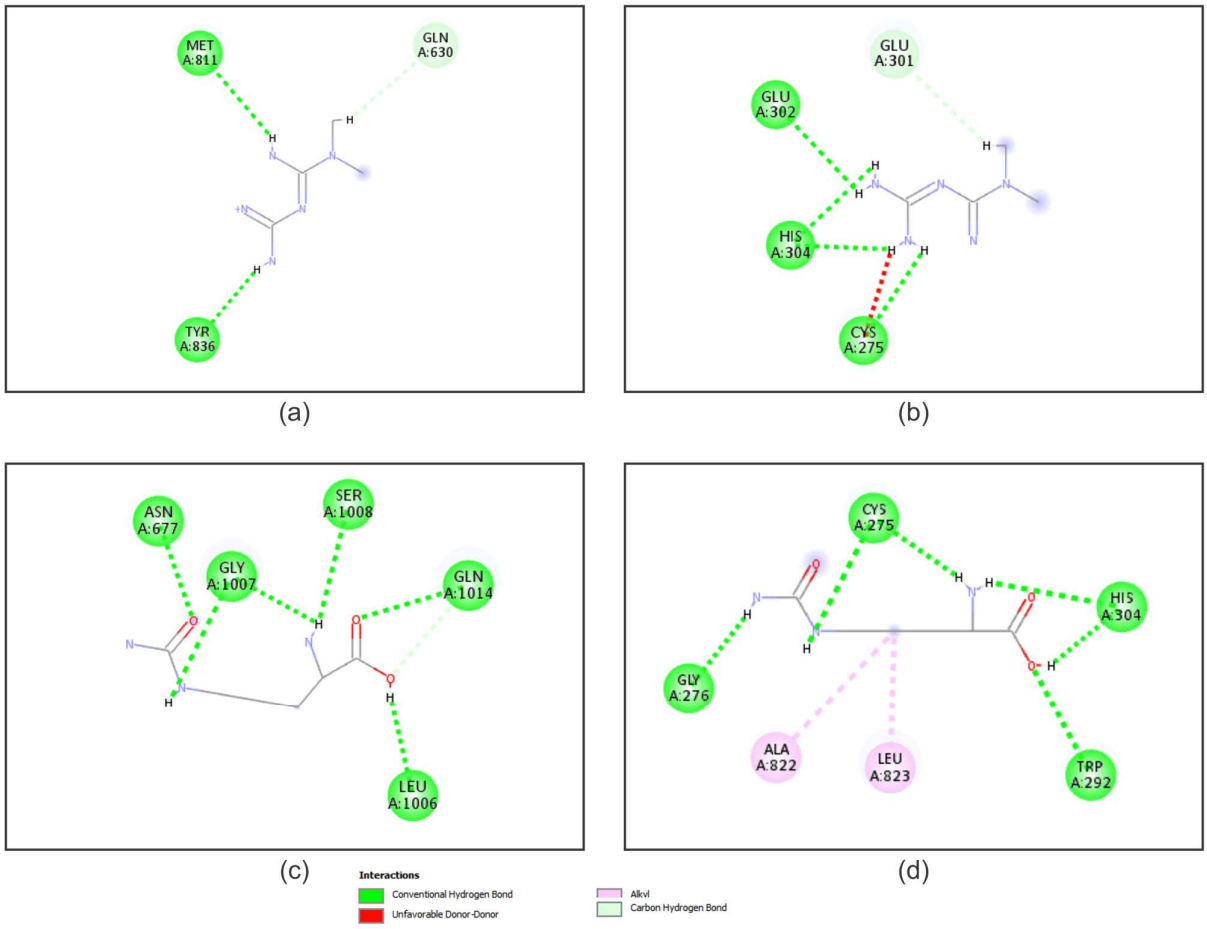


Fig. 4. The structural binding interaction of: *a* – metformin with PI3K alpha; *b* – metformin with PI3K gamma; *c* – L-Citrulline with PI3K alpha; *d* – L-Citrulline with PI3K gamma

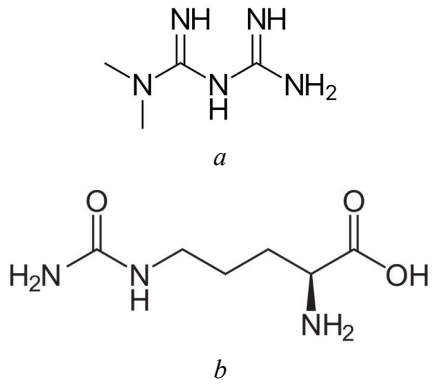


Fig. 5. Chemical structures of: *a* – metformin; *b* – L-Citrulline

According to toxicity prediction in Table 4, both Metformin and L-Citrulline emerge as promising candidates for Type 2 diabetes treatment due to their lack of mutagenic, tumorigenic, and reproductive toxicities. This insight underscores their potential safety profiles, which are crucial considerations in the development of effective pharmaceutical interventions for managing this prevalent metabolic disorder.

5. Discussion

The global burden of type 2 diabetes mellitus (T2DM) continues to rise at an alarming pace, driven by sedentary lifestyles, dietary changes, and increasing insulin resistance among at-risk populations [1, 5]. A fundamental defect in T2DM is the dysregulation of the insulin signaling cascade, particularly involving the phosphoinositide 3-kinase (PI3K)/Akt pathway, which is essential for glucose uptake, glycogen synthesis, lipid metabolism, and protein synthesis [5]. Impairment of this pathway in skeletal muscle cells leads to reduced glucose transport via GLUT4, contributing significantly to hyperglycemia and metabolic inflexibility [11–14].

In this study, we investigated the potential of L-Citrulline to modulate insulin signaling and restore PI3K concentration in insulin-resistant skeletal muscle cells. Metformin was employed as a positive control due to its well-documented insulin-sensitizing effects and its clinical utility as a first-line antidiabetic drug [5]. The L6 skeletal muscle cell line was used as an *in vitro* model, chosen for its relevance in glucose uptake studies and the concentration of insulin-responsive genes such as PI3K. Insulin resistance was induced by prolonged exposure to high concentrations of glucose and insulin, mimicking the metabolic environment of T2DM.

In vitro findings indicated that L-Citrulline induced a dose-dependent elevation in PI3K levels. At a concentration of 10 µM, L-Citrulline significantly increased PI3K concentration by 55.48%, which is remarkably close to the 58.92% increase observed with 100 µM

metformin. These findings suggest that L-Citrulline possesses the potential to restore impaired insulin signaling in diabetic muscle cells. All L-Citrulline-treated groups exhibited statistically significant increases relative to the diabetic control, demonstrating efficacy across the tested concentration range. Moreover, the ability of L-Citrulline to enhance PI3K levels at lower concentrations than metformin may indicate higher potency or efficiency, underscoring the need for further mechanistic studies to elucidate its precise mode of action.

Beyond its role in modulating insulin signaling, L-Citrulline enhances nitric oxide (NO) production through its conversion to L-arginine, thereby promoting endothelial function and vasodilation [15, 16]. These vasodilatory effects have been linked to improved glucose uptake and insulin sensitivity in skeletal muscle via enhanced tissue perfusion and oxygen delivery [16]. Moreover, L-Citrulline's antioxidant and antiglycation activities, as previously reported [8], further expand its therapeutic potential. Oxidative stress and the accumulation of advanced glycation end products (AGEs) are recognized as major contributors to the pathogenesis of T2DM and its vascular and neural complications [17, 18]. By attenuating oxidative damage and inhibiting AGE formation, L-Citrulline may improve insulin responsiveness while conferring protection against long-term diabetic complications [19, 20].

Within the insulin signaling pathway, PI3K is a critical mediator that transmits signals from activated insulin receptors to downstream effectors such as AKT, ultimately facilitating GLUT4 translocation and glucose uptake in skeletal muscle. Glycation of signaling proteins and oxidative modifications from excessive reactive oxygen species can impair PI3K activation and disrupt this signaling cascade [6]. The antiglycation action of L-Citrulline may preserve the structural integrity of PI3K and its upstream activators (e.g., IRS-1), while its antioxidant effects can mitigate oxidative stress that diminishes PI3K phosphorylation. Collectively, these protective mechanisms help sustain PI3K function, thereby supporting effective insulin signaling and maintaining glucose homeostasis.

To complement the cellular findings, *in silico* molecular docking was employed to assess the potential interaction of L-Citrulline with PI3K isoforms (alpha and gamma). The docking results indicated favorable binding energies of –5.5 kcal/mol (PI3K alpha) and –5.4 kcal/mol (PI3K gamma) for L-Citrulline. These values were slightly more negative than those of metformin (–4.9 and –5.0 kcal/mol), suggesting that L-Citrulline may exhibit comparable or even enhanced binding affinity for these target proteins. These interactions provide a molecular rationale for the observed increase in PI3K levels *in vitro*, supporting the hypothesis that L-Citrulline may directly modulate key signaling proteins involved in glucose metabolism.

Furthermore, validation of the docking process through redocking of the co-crystallized ligands yielded root mean square deviation (RMSD) values below 2.0 Å, confirming the reliability and accuracy of the docking methodology used. Such precision strengthens the credibility of the ligand–protein interaction predictions made for both L-Citrulline and metformin. To assess pharmacokinetic suitability, *in silico* ADME (absorption, distribution, metabolism, and excretion) and toxicity profiling was performed. L-Citrulline complied with key drug-likeness rules (Lipinski's, Ghose's, and Veber's), and showed no predicted toxicity or mutagenic potential based on OSIRIS and SwissADME platforms. This reinforces its potential as a safe therapeutic agent with good oral bioavailability and minimal risk of adverse effects – characteristics desirable in both primary and adjunctive diabetes therapies.

Taken together, the findings of this integrated *in vitro–in silico* study suggest that L-Citrulline has the potential to modulate insulin resistance and influence PI3K pathway function in skeletal muscle cells. The observed effects, which are comparable to those of metformin, indicate that L-Citrulline may warrant further investigation in preclinical and clinical models of diabetes. As a naturally occurring, diet-derived compound, L-Citrulline could offer opportunities for development in nutraceutical formulations or functional foods targeting metabolic disorders.

Future studies are recommended to examine the downstream effects of PI3K activation by L-Citrulline, such as Akt phosphorylation and GLUT4 translocation, and to confirm its effects *in vivo*. Furthermore, investigation into synergistic effects with existing antidiabetic agents, including metformin, could pave the way for combination therapies that maximize glucose control while minimizing side effects. This study provides compelling evidence that L-Citrulline, a simple yet biologically active amino acid, holds significant potential in reversing insulin resistance through its regulatory action on the PI3K pathway, antioxidant function, and favorable interaction with key metabolic enzymes. Its dual role as a metabolic modulator and signaling enhancer marks it as a promising agent in the evolving landscape of antidiabetic research.

Practical relevance. The results indicate that L-Citrulline could be developed as a novel therapeutic or nutraceutical agent for improving insulin sensitivity in individuals with T2DM. Its ability to restore PI3K concentration and demonstrate strong binding affinity to PI3K highlights its potential for incorporation into functional foods, dietary supplements, or pharmacological formulations aimed at supporting glycemic control and preventing the progression of insulin resistance.

Research limitations. The present study is limited by its reliance on *in vitro* skeletal muscle cell models and *in silico* molecular docking analyses, which, while valuable for mechanistic exploration, cannot fully replicate the complexity of systemic glucose homeostasis *in vivo*. The scope was further restricted to the PI3K node within the insulin signaling cascade, without assessing downstream or parallel pathways, and without evaluating pharmacokinetic parameters or long-term safety profiles under physiological conditions. This focus on PI3K was intentional, as recent studies have identified it as a key upstream regulator of glucose metabolism and insulin responsiveness. Nevertheless, downstream markers such

as Akt phosphorylation and GLUT4 translocation were not examined; thus, future studies should incorporate these endpoints to comprehensively elucidate the molecular mechanisms underlying *L-citrulline*'s potential antidiabetic effects.

Prospects for further research. Subsequent investigations should employ in vivo models to validate the modulatory effects of L-Citrulline on PI3K signaling within the broader metabolic network, incorporating assessments of glucose uptake, oxidative stress modulation, and glycation end-product inhibition. Clinical translation would benefit from dose–response studies, bioavailability optimization, and exploration of synergistic effects with other bioactive compounds or dietary interventions targeting insulin resistance.

6. Conclusion

The findings of this study, derived from both *in vitro* cellular assays and *in silico* molecular docking simulations, provide compelling evidence for the potential of L-Citrulline as an adjunct therapeutic agent in managing Type 2 Diabetes Mellitus (T2DM) and insulin resistance. Despite the established efficacy of metformin, L-Citrulline exhibited comparable effects in restoring insulin signaling, even at lower concentrations, particularly through the increased of PI3K concentration in skeletal muscle cells. This suggests that L-Citrulline may enhance tyrosine phosphorylation of insulin receptor substrates, leading to activation of the PI3K pathway, a central route for glucose uptake via GLUT4 translocation**.**

In the molecular docking simulations, L-Citrulline demonstrated favorable binding affinities toward both PI3K alpha and PI3K gamma isoforms, comparable to or slightly better than those of metformin. Although L-Citrulline is structurally distinct – a non-proteinogenic amino acid – it effectively docked within the active sites of PI3K, supporting its possible role in modulating key insulin signaling proteins**.** These interactions may contribute to restoring insulin sensitivity under hyperglycemic conditions. Furthermore, *in silico* pharmacokinetic profiling using SwissADME and SwissTargetPrediction revealed that L-Citrulline complies with Lipinski's Rule of Five and Veber's criteria, indicating favorable oral bioavailability and drug-likeness. No violations of major drug-likeness filters were noted, and toxicity predictions did not indicate major concerns, suggesting a safe pharmacological profile.

Collectively, these results suggest that L-Citrulline may exert a dual mechanism of action: first, by directly enhancing insulin signaling via the PI3K axis in skeletal muscle, and second, by mitigating oxidative stress and glycation-related damage – both of which are implicated in insulin resistance and beta-cell dysfunction. These properties make L-Citrulline a promising candidate for further investigation as a supportive or complementary therapy in the management of T2DM.

Conflict of interest

The authors hereby state that they do not possess any conflict of interest with regards to this research, be it financial, personal, authorship-related, or otherwise, that may have an impact on the research and its outcomes as presented in this article.

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Data availability

Data will be made available on reasonable request.

Use of artificial intelligence

Artificial intelligence tools were utilized solely for grammar and language refinement. No AI-generated content was used for data analysis, interpretation, or drawing conclusions.

Authors' contributions

Jessa Marielle U. Paulines: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Statistical analysis, Writing – original draft, Writing – review & editing, Project administration; **Patrick Nwabueze Okechukwu:** Conceptualization, Methodology, Formal analysis, Statistical analysis, Validation, Writing – review & editing, Supervision; **Charlie A. Lavilla, Jr.:** Conceptualization, Methodology, Formal analysis, Funding acquisition, Supervision, Writing – review & editing.

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