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Sinigrin as a Therapeutic Agent for Diabetes: A Combined In Vitro and In Silico Investigation.

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KEYWORDS

Diabetes mellitus; Sinigrin; glucose uptake potential; Gluconeogenic enzymes; antidiabetic property

ABSTRACT

Type 2 diabetes continues to have a detrimental influence on the health of millions. Insulin resistance, or the inability to respond to insulin and eliminate blood glucose, is a major pathogenic driver of the disease. Skeletal muscle is the principal tissue responsible for maintaining glucose homeostasis through glucose uptake via insulin-dependent and insulin-independent processes. Natural products are a rich source of chemicals for drug discovery, as has long been known. Many groups of secondary metabolites, including glucosinolates are found to possess medicinal properties. Sinigrin which belongs to Brassicaceae family of glucosides is examined for its antidiabetic properties. The effect of sinigrin on the viability of L6 myotubes was evaluated by MTT assay at different sinigrin concentrations. There was more than 95% viability at concentrations of sinigrin up to 100 µM indicating its non-toxic nature of sinigrin. Sinigrin enhanced glucose uptake as a percentage compared to control cells, 106%, 111%, 121%, 132%, and 156% concentrations, 20,40,60, 80 and 100 µM respectively. In addition, glucose uptake was reduced in the presence of LY294002 (PI-3 kinase inhibitor) indicating a major mechanism involving the induction of GLUT4 membrane translocation via the activation of both PI3K/Akt pathway. Sinigrin enhances the uptake of glucose in rat L6 myotubes independent of insulin through the PI 3 kinase signaling pathway. According to computational analysis, sinigrin's interactions and binding energy with PEPCK and G6 pase indicate that the compound has the ability to inhibit both gluconeogenic enzymes. The study fully understands the mechanism of action of sinigrin, and these findings show that sinigrin could be developed into an effective antidiabetic medicine.

1. Introduction

Hyperglycemia has been associated to the development of difficult-to-treat macrovascular illnesses such as cardiovascular disease and amputation, as well as microvascular disorders such as neuropathy, blindness, and diabetic retinopathy (Saeedi et al., 2019, Sun et al., 2022). In contrast to 108 million in 1980, an estimated 422 million people globally had diabetes in 2014. Since 1980, the age-standardized prevalence of diabetes in adults has nearly doubled, rising from 4.7% to 8.5% worldwide (Han et al., 2018). The growing prevalence of diabetes has posed a tremendous challenge to researchers around the globe, who are working tirelessly to create breakthrough medicines that can prevent the progression of the disease .

Skeletal muscle is critical in glucose homeostasis, accounting for the majority (75%) of postprandial insulin-mediated glucose absorption in

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glucose metabolism (Kondash et al., 2020). A number of bioactive compounds such as Isoquercetin, taxifolin, and urolithin A have been shown to enhance glucose uptake (GU) in isolated and incubated rat soleus muscles (Khondo et al., 2024; Rey et al., 2020). Cell lines have been shown to be useful for screening and analysing the mechanisms of several bioactive compounds. L6 myoblasts from rat skeletal muscle tissue have been shown to spontaneously fuse and develop into multinucleated L6 myotubes in culture (Issac et al., 2021). The myotube was used to study the effect of bioactive compounds on muscle function in vitro without having to prepare animal muscle tissue.

Natural products are a rich source of molecules for the development of new drugs. In the series of various bioactive compounds from plant origin, the occurrence of sinigrin, Glucosinolates in cruciferous plants of Brassicaceae family such as Brussels sprouts, broccoli, cabbage, and mustard seeds possess a such as antifungal, antibacterial, bioherbicidal, antioxidant, antimutagenic, anticancer, and anti-inflammatory properties (Maria et al., 2002; Ippoushi et al., 2010 Bhattacharya et al, 2010; Lee et al., 2014; Mazumder et al., 2016). Having known the pharmacological properties of sinigrin, the present study was aimed in evaluating the glucose uptake potential of sinigrin in rat L6 myotubes. In addition, In silico studies were performed to evaluate the possible antidiabetic mechanism of sinigrin

MATERIALS AND METHODS

INVITRO STUDIES

Culture of rat L6 myoblast

The rat L6 myoblast cell line was obtained from NCCS, Pune, India, and was cultured in DMEM. The medium was supplemented with 4.5 g/L glucose, 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin-B (250 ng/ml). Cultures were maintained at 37ºC with 5% CO2 in an incubator. Once the cells had reached 60–70% confluence, differentiation was induced by replacing the growth medium with DMEM supplemented with 2% fetal bovine serum instead of 10% fetal bovine serum (Sinha et al., 2004). Myotube formation was achieved after 6-7 days of media changes every 48 hours. All of the experiments were carried out in differentiated L6 myotubes.

Cell viability assay

The effect on the viability of the L6 myotubes was determined by colorimetric MTT assay (Mosmann, 1983). After overnight fasting with serum-free DMEM, the cells were treated with various concentrations (20, 40, 60, 80, and 100 µM) of sinigrin for 24 h at 37ºC with 5% CO2 in an incubator. After treatment, the culture medium was removed from the wells, and 100 µl of MTT at a concentration of 5 mg/ml in D-PBS was added to each well. After 4 h incubation at 37ºC, MTT in D-PBS was removed and then the formazan crystals were solubilized in 100 µl of 2-propanol. The dye's absorbance was measured at a wavelength of 570 nm. The results were expressed as a percentage of the viability of control cells.

Determination of glucose uptake by cultured rat L6 myotubes

L6 myoblasts were seeded in 24-well tissue culture plates and for differentiation, cells were grown in DMEM with 2% fetal bovine serum for 6-7 days with subsequent change in media for every 48 h. After differentiation, the cells were fasted overnight with serum-free DMEM containing low-glucose and then treated with insulin (100 nM) for 1 h as well as sinigrin (20, 40, 60, 80, and 100 µM) in fresh serum-free DMEM, for 3h. After treatment, glucose concentration in the medium was determined using the glucose oxidase method. The glucose of the wells with cells was subtracted from the glucose of the blank wells to calculate the glucose uptake (Hong et al., 2012). Studies on the effect of LY294002 (PI-3kinase inhibitor), Compound C (AMPK inhibitor) on sinigrin-induced glucose uptake in rat L6 myotubes to elucidate the exact pathway through which sinigrin mediate the glucose uptake. L6 myotubes were treated with sinigrin as previously described in the glucose uptake assay method, either in the presence or absence of 10 µM of LY294002 (PI-3kinase inhibitor), 5 µM of Compound C (AMPK inhibitor). After treatment, glucose concentration in the medium was determined using the glucose oxidase method.

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IN SILICO STUDIES

Protein preparation and optimization

The crystal structure of PEPCK and Glucose 6 Phosphatase from Homo sapiens were retrieved from the RCSB protein data bank (http://www.rcsb.org/pdb). The Hydrogen atoms, solvation parameters, and fragmental volumes to the protein were added and electronic charges were assigned to the protein atoms using the Kollman united atoms force field by using Auto Dock Tool (ADT) –2.0. 2.2.

Ligand preparation and optimization:

Using Chemsketch Software the structure of the Sinigrin was drawn and 3-D structures were generated and optimized. The selected ligands were saved in MDL-MOL format which was subsequently converted using an open Babel molecular converter program (Boyle et al., 2011) and saved in PDB format.

Property Prediction and ADMET Prediction

Physiochemical of the sinigrin were assessed using the PkCSM Server. Properties such as molecular size, rotatable bond, logP, hydrogen bond donor, and acceptor characteristics were predicted.

Membrane permeability, bioavailability, distribution, metabolism, and adsorption (Lipinski's rule of 5 was also predicted using PkCSM (pkCSM (unimelb.edu.au)). It is a web server used for the prediction of pharmacokinetic properties of compounds. (Pires et al., 2015) It is used to predict various parameters that help us to determine the pharmacokinetics of a drug. The parameters include absorption, distribution, metabolism, and excretion. Previously mentioned parameters can be predicted by certain characteristics.

Absorption of a drug can be predicted by predicting its characteristics such as CaCO2 and skin permeability, intestinal absorption, P-glycoprotein substrate or inhibitor. Characteristics such as volume distribution (VD) and permeability of CNS and BBB were utilized to predict the distribution of the drug. For the determination of the Metabolism, Cytochrome P450 model was used. Excretion of the drug was determined by Total clearance and Renal substrate.

Molecular Docking and Visualization

Docking analysis between PEPCK and Glucose 6 Phosphatase against Sinigrin was done using AutoDock Tool to see the affinity (Morris et al., 1998). Many intermediate steps such as making pdbqt files of protein and ligand, grid preparation, and grid map using a grid box were done. A genetic algorithm was used as a search parameter. Lamarckian GA was the output selected. The protein-ligand interaction between glucose 6 phosphatase, PEPCK, and Sinigrin was visualized using Biovia Discovery Studio Visualizer software

RESULTS AND DISCUSSION

The skeletal muscle is the body's largest organ by mass. It also regulates glucose homeostasis, accounting for 80% of postprandial glucose uptake from the blood. Skeletal muscle is required for metabolism, both in terms of glucose uptake and in exercise and metabolic illness (Merz, 2020). Insulin resistance is caused by the desensitization of muscle to the insulin released by the pancreas to elicit glucose uptake, leading to elevated blood glucose levels. Skeletal muscle insulin resistance can develop decades before β-cell loss and clinical T2D (DeFronzo and Tripathy,2009). In muscle cells, glucose transporter type 4 (GLUT4) proteins translocate to the plasma membrane in response to insulin, thus allowing massive entry of glucose into the cell. The ability of muscle cells to respond to insulin by increasing the rate of glucose uptake is one of the standard readouts to quantify muscle cell sensitivity to insulin. Human primary myotubes are a suitable in vitro model, as the cells maintain many features of the donor phenotype, including insulin sensitivity (Chanon et al., 2017).

For differentiation of myoblast into myotubes, the myoblasts were seeded into culture plates and maintained in DMEM containing 2% fetal bovine serum for 6-7 days with subsequent media change for every 48 hours. The effect of sinigrin on the viability of L6 myotubes was evaluated by MTT assay at different sinigrin concentrations (There was more than 95% viability at concentrations of sinigrin up to 100 µM when compared with control survival (Figure 1). This indicates the non-toxic nature of sinigrin

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Myotubes were serum starved overnight and then incubated with the indicated concentrations of morin for 3 hrs. Insulin (100nM was used as a positive control). After treatment, glucose concentration in the medium was determined using the glucose oxidase method. The glucose in the wells with subtracted from the glucose of the blank wells to calculate glucose uptake. Values are expressed as mean± S.E.M of triplicate experiments. P*<0.05, compared with normal control cultures.

The effect of sinigrin on muscle glucose uptake is shown in Figure 2, Sinigrin enhanced glucose uptake as a percentage compared to control cells, 106%, 111%, 121%, 132%, and 156% at concentrations, 20,40,60, 80, and 100 µM respectively. However, sinigrin at concentrations, there was a only slight difference observed in the dosages between 80 and 100 µM when compared to the effect of insulin (100 nM). This data indicates that sinigrin may facilitate metabolic effects in skeletal muscle cells.

Several pathways are involved in the translocation of GLUT4 in skeletal muscles. The most well-characterized is the insulin pathway, in which insulin binds to the insulin receptor in membrane, followed by the activation of downstream signaling molecules, including IRS, PI3K, and Akt. The activation of Akt leads to the translocation of GLUT4 and glucose uptake into cells (Chang et al., 2004). To find out the exact pathway through which sinigrin exerts its action we have further evaluated the uptake of glucose in the presence of PI3k as well as AMPK inhibitors

Activation of the PI3K/AKT pathway starts with the binding of IRS proteins via SH2 domains to PI3 kinase regulatory subunits. This results in the activation of PI3K that phosphorylates phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3). This, in turn, leads to the activation of PIP3-dependent kinases: PDK-1 and PDK-2 and eventually to the activation of AKT/PKB kinase and atypical PKCs (Araya et al., 2003). Subsequently, AKT catalyzes the phosphorylation of AS160 substrate protein that stimulates the translocation of GLUT glucose transporters from the cytoplasmic vesicles onto the cell membrane surface and thereby increases the insulin-dependent transport of glucose into the cell.

Figure 1: Effect of sinigrin on cell viability in L6 myotubes

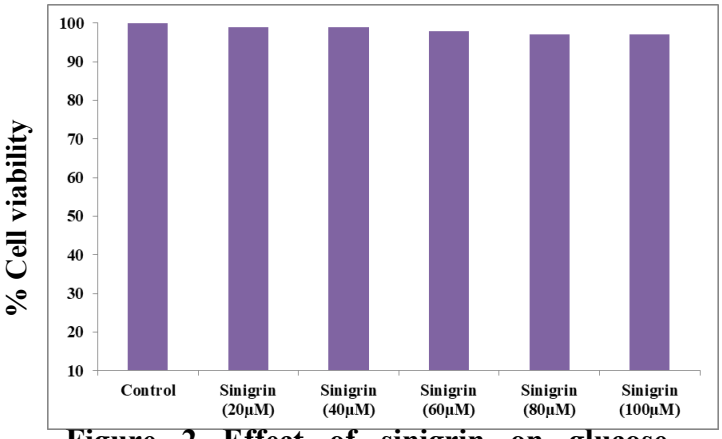
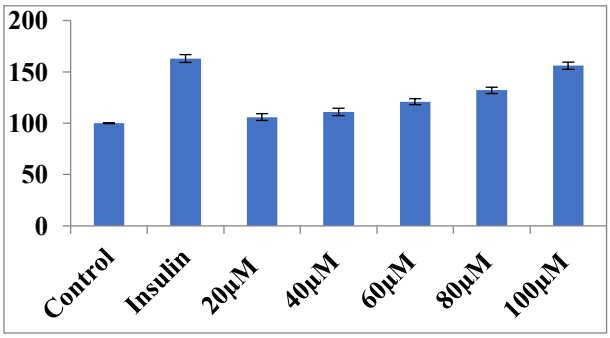


Figure 2 Effect of sinigrin on glucose uptake in rat L6 myotubes



In order to determine whether sinigrin stimulates muscle glucose uptake by increasing the translocation of GLUT4 to the membrane either via insulin-mediated PI-3kinase or exercise mimetics, AMPK, myotubes were treated with sinigrin the presence or absence of LY294002 (PI-3 kinase inhibitor), Compound C (AMPK inhibitor). Figure 3 shows that glucose uptake was not disturbed in the presence of Compound C (AMPK inhibitor) which confirms the fact that sinigrin does not facilitate glucose uptake through the AMPK pathway. Glucose uptake was reduced in the presence of LY294002 (PI-3 kinase inhibitor) indicating a major mechanism involving the induction of GLUT4 membrane translocation via the activation of both PI3K/Akt pathway. Sinigrin enhances the uptake of glucose in rat L6 myotubes independent of insulin through PI 3 kinase signaling pathway.

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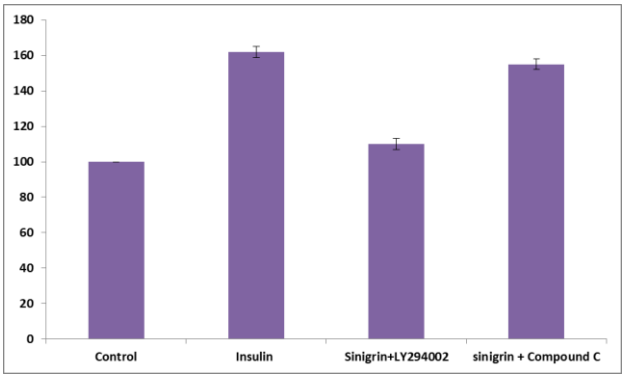
Figure 3: Effect of LY294002 (PI-3kinase inhibitor), Compound C (AMPK inhibitor) on sinigrin Induced glucose uptake in rat L6 myotubes



The results of the present study revealed the fact that sinigrin facilitates glucose uptake through the activation of the phosphatidyl inositol 3 kinase (PI3-K) pathway. In conclusion, sinigrin has potent effects to increase glucose uptake by up-regulating the battery of targets like GLUT 4, PI3 kinase in myotubes. These findings suggest that sinigrin has complementary potency to develop as an antidiabetic agent for the treatment of diabetes mellitus.

***In silico* studies**

Protein structure retrieval

The crystal structures of **PEPCK (PDB ID: 1KHB)** and **Glucose 6 Phosphatase (PDB ID: 9J7V)** were retrieved from the Protein Data Bank (PDB) to analyze their structural and functional characteristics.

Targets	PDB ID	Resolution	Chain
PEPCK	1KHB	1.85 Å	A
Glucose 6 Phosphatase	9J7V	3.30 Å	A

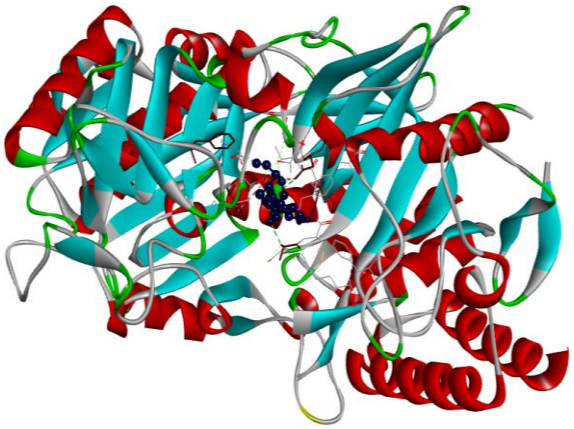


Figure 4a. Solid ribbon model of PEPCK with Sinigrin (scaled ball and stick model) Hydrogen PEPCK with Sinigrin Interactions.

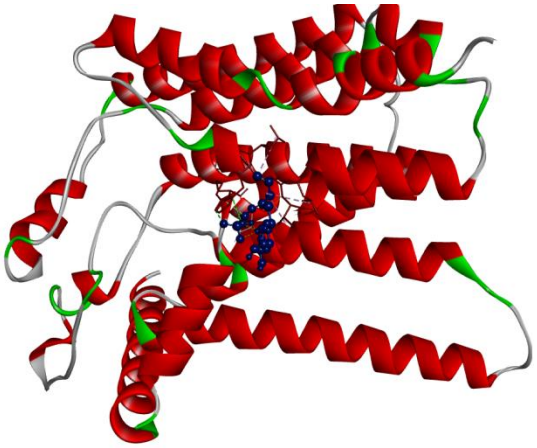


Figure 4b. Solid ribbon model of Glucose 6 Phosphatase with Sinigrin (scaled ball and stick model)

Sinigrin – PkCSM results

The ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) investigation of Sinigrin with PkCSM revealed important pharmacokinetic features. Sinigrin has a molecular weight of 359.378 g/mol, a LogP value of -1.7715, and a large polar surface area (130.542 Å²), indicating its hydrophilic nature. The chemical has minimal permeability across biological membranes, as demonstrated by its poor Caco-2 permeability (-0.669 log Papp) and nil human intestine absorption (% absorbed = 0). Furthermore, it is neither a substrate nor an inhibitor of P-glycoprotein, revealing a minimal interaction with efflux transporters.

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Sinigrin has a low volume of distribution (VDss = -0.366 log L/kg), indicating limited tissue penetration. Furthermore, its low BBB permeability (-1.513 log BB) and CNS permeability (-3.853 log PS) suggest minimal central nervous system (CNS) exposure. Sinigrin's metabolism profile indicates that it is neither a substrate nor an inhibitor of key cytochrome P450 enzymes (CYP2D6, CYP3A4, CYP1A2, CYP2C9, CYP2C19), signifying a low likelihood of metabolic interaction.

In terms of excretion, Sinigrin's total clearance (0.503 log ml/min/kg) indicates modest elimination, and it is not a substrate for renal OCT2 transporters, implying that renal clearance is not the predominant route of elimination. Sinigrin's toxicity profile demonstrates that it is non-toxic in the Ames test, non-hepatotoxic, and does not act as a hERG I/II inhibitor, which reduces worries about cardiotoxicity. The oral rat acute toxicity (LD50) is 1.875 mol/kg, while the chronic toxicity (LOAEL) is 3.291 log mg/kg_bw/day, showing a generally safe profile. Furthermore, Sinigrin does not cause skin sensitization, demonstrating its biocompatibility.

Overall, Sinigrin's pharmacokinetic profile demonstrates poor absorption and CNS penetration, low metabolic interaction, and a favorable safety profile, making it a promising option for future therapeutic development.

Table 1. Molecule properties:

Descriptor	Value
Molecular Weight	359.378
Log P	-1.7715
#Rotatable Bonds	6
#Acceptors	10
#Donors	5
Surface Area	130.542

Table 2 ADMET prediction of Sinigrin:

Property	Model Name	Predicted Value	Unit
Absorption	Water solubility	-2.045	Numeric (log mol/L)
Absorption	Caco2 permeability	-0.669	Numeric (log Papp in 10^{-6} cm/s)
Absorption	Intestinal absorption (human)	0	Numeric (% Absorbed)
Absorption	Skin Permeability	-2.735	Numeric (log Kp)
Absorption	P-glycoprotein substrate	No	Categorical (Yes/No)
Absorption	P-glycoprotein I inhibitor	No	Categorical (Yes/No)
Absorption	P-glycoprotein II inhibitor	No	Categorical (Yes/No)
Distribution	VDss (human)	-0.366	Numeric (log L/kg)
Distribution	Fraction unbound (human)	0.754	Numeric (Fu)
Distribution	BBB permeability	-1.513	Numeric (log BB)
Distribution	CNS permeability	-3.853	Numeric (log PS)
Metabolism	CYP2D6 substrate	No	Categorical (Yes/No)
Metabolism	CYP3A4	No	Categorical

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Property	Model Name	Predicted Value	Unit
	substrate		(Yes/No)
Metabolism	CYP1A2 inhibitor	No	Categorical (Yes/No)
Metabolism	CYP2C19 inhibitor	No	Categorical (Yes/No)
Metabolism	CYP2C9 inhibitor	No	Categorical (Yes/No)
Metabolism	CYP2D6 inhibitor	No	Categorical (Yes/No)
Metabolism	CYP3A4 inhibitor	No	Categorical (Yes/No)
Excretion	Total Clearance	0.503	Numeric (log ml/min/kg)
Excretion	Renal OCT2 substrate	No	Categorical (Yes/No)
Toxicity	AMES toxicity	No	Categorical (Yes/No)
Toxicity	Max. tolerated dose (human)	1.327	Numeric (log mg/kg/day)
Toxicity	hERG I inhibitor	No	Categorical (Yes/No)
Toxicity	hERG II inhibitor	No	Categorical (Yes/No)
Toxicity	Oral Rat Acute Toxicity (LD50)	1.875	Numeric (mol/kg)
Toxicity	Oral Rat Chronic Toxicity (LOAEL)	3.291	Numeric (log mg/kg_bw/day)
Toxicity	Hepatotoxicity	No	Categorical (Yes/No)
Toxicity	Skin Sensitisation	No	Categorical (Yes/No)
Toxicity	T.Pyriformis toxicity	0.285	Numeric (log ug/L)
Toxicity	Minnow toxicity	4.982	Numeric (log mM)

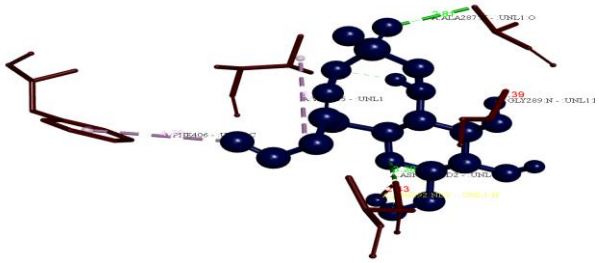

Figure 5. 3D interactions: PEPCK (protein) –blue color stick model; and Sinigrin ligand) red color scaled ball and stick model; green dotted lines – Hydrogen bond interactions

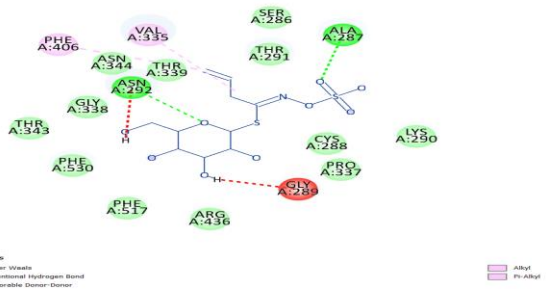

Figure 6: 2D Diagram: Ligand shown in red color line model and Protein interactions are colored depending on their type: pi-alkyl interactions are colored in light purple. Pi-sigma bonds are shown in dark purple color.

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Table 3 Binding interactions of key amino acid residues of PEPCK with sinigrin

Donor – Donor-acceptor interaction	Distance (Å)	Category of Bond	Type
A: ALA287: N -: UNL1:O	2.80617	Hydrogen Bond	Conventional Hydrogen Bond
A: ASN292: ND2 -: UNL1:O	3.27613	Hydrogen Bond	Conventional Hydrogen Bond
A: VAL335 -: UNL1	5.31177	Hydrophobic	Alkyl
A: PHE406 -: UNL1:C	4.69935	Hydrophobic	Pi-Alkyl

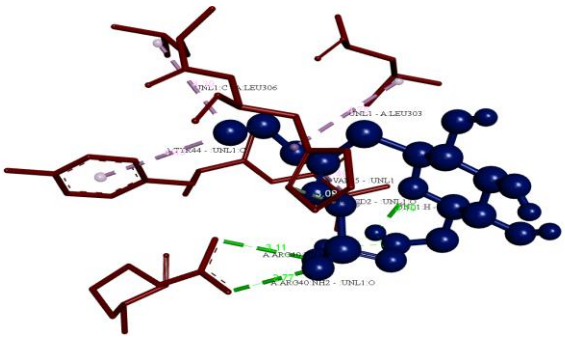

Figure 7. 3D interactions: Glucose 6 Phosphatase (protein) –blue color stick model; and Sinigrin ligand) red color scaled ball and stick model; green dotted lines – Hydrogen bond interactions.

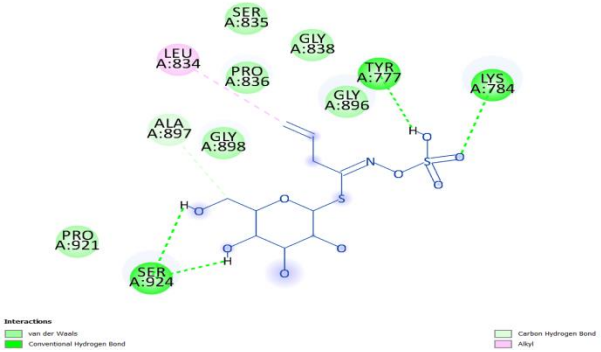

Figure 8: 2D Diagram: Ligand shown in red color line model and Protein interactions are colored depending on their type: pi-alkyl interactions are colored in light purple. Pi-sigma bonds are shown in dark purple color.

Table 4 Binding interactions of key amino acid residues of Glucose 6 Phosphatase with sinigrin.

Donor – Donor-acceptor interaction	Distance (Å)	Category of Bond	Type
A: ARG40: NH1-: UNL1:O	3.10634	Hydrogen Bond	Conventional Hydrogen Bond
A: ARG40: NH2 -: UNL1:O	2.76734	Hydrogen Bond	Conventional Hydrogen Bond
A: UNL1:H -: UNL1:O	2.10357	Hydrogen Bond	Conventional Hydrogen Bond
A: HIS307: CD2 -: UNL1:O	3.01889	Hydrogen Bond	Carbon Hydrogen Bond
A: VAL45 -: UNL1	4.49192	Hydrophobic	Alkyl
A: UNL1 - A: LEU303	4.74931	Hydrophobic	Alkyl
A: UNL1:C - A: LEU306	4.38829	Hydrophobic	Alkyl
A: TYR44 -: UNL1:C	4.43164	Hydrophobic	Pi-Alkyl

Table 5 Sinigrin binding energy (kcal/mol) with different targets

Conformation	Binding Energy	Ligand efficiency	Inhibitory constant, K_i (μm)	Intermolecular energy	vdW + H bond + desolv Energy	Electrostatic energy	Torsional energy	Total internal Unbound

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PEPCK	-4.13	-0.19	936.54	-7.71	-6.85	-0.86	3.58	-5.54
Glucose 6 Phosphatase	-2.92	-0.13	7.22	-6.5	-5.81	-0.7	3.58	-5.79

The coordinated regulation of cellular glucose absorption and endogenous glucose synthesis is critical for maintaining stable blood glucose levels. The liver plays a crucial role in this process by managing the processes of glycogen breakdown (glycogenolysis) and de novo glucose generation (gluconeogenesis) (Hatting et al., 2017). Phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase) are enzymes that limit gluconeogenesis rate (Sato et al., 2011). In addition, G6Pase is a critical enzyme in the last step of the glycogenolytic pathway (Podolin et al., 1999).Abnormally enhanced hepatic gluconeogenesis contributes significantly to fasting hyperglycemia in patients with type 2 diabetes mellitus (T2DM) caused by insulin resistance (Barroso et al., 2024). A significant metabolic effect of glucagon is enhancing the rate of gluconeogenesis, which increases hepatic glucose output. Insulin opposes this hepatic glucagon action and enhances the storage of glucose as glycogen in the liver. Insulin can potently and promptly inhibit the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK), which has long been thought to be the rate-determining enzyme directing GNG flow to G6P, (Pilkis and Granner, 1992, O'Brien et al., 2001, Stalmans et al., 1990).

Sinigrin experiments on L6 myotubes show that it is nontoxic and effectively increases glucose uptake in dose dependant manner. According to computational analysis, sinigrin's interactions and binding energy with gluconeogenic enzymes such as PEPCK and G6 pase indicate that sinigrin has the ability to inhibit both these enzymes which is an important therapeutic strategy to treat diabetes. From the results both *in vivo* and *in silico* studies evidence the fact that sinigrin could be developed into an effective antidiabetic medicine

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