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Promotion of glucose uptake by mulberry leaf extract and its active component 1-deoxynojirimycin in C2C12 cells

Ngoc Han Le Thi^{1†}, Jun Hyung Park^{2†}, Min Ji Han³, Seon Hwa Kim³, Tae Hoon Kim³, Dong-Wook Kim⁴, Seung Hyun Kim^{2*} and Ki Sung Kang^{1*} 

Abstract

Mulberry leaf extract (MLE) is a proprietary extract derived from the leaves of *Morus alba*, contains high concentrations of 1-deoxynojirimycin (DNJ), which inhibits intestinal glucose uptake and improves glucose tolerance. The present study investigated the impact of MLE and DNJ on the inhibition of alpha glucosidase and enhanced glucose uptake in a dose-dependent manner in C2C12 cells. This effect was accompanied by changes in the expression of key glucose transporter proteins, such as glucose transporter type 4 (GLUT4), the phosphorylation states of insulin receptor substrate (p-IRS,) and adenosine monophosphate-activated protein kinase (p-AMPK), which regulate glucose metabolism. These findings highlight the distinct metabolic effects of MLE and DNJ on glucose uptake in skeletal muscle cells, indicating their potential as natural compounds for modulating glucose metabolism. Further investigation of the molecular pathways involved and in vivo studies are required to validate and expand to fully understand their potential therapeutic applications.

Keywords 1-Deoxynojirimycin, MLE extract, Glucose uptake, Skeletal muscle cells

Introduction

Glucose metabolism is an important part of general metabolic health, and skeletal muscles play a key role in this process. Efficient glucose uptake by skeletal muscle cells is required for adequate blood glucose levels and metabolic homeostasis [5]. Dysregulation of glucose uptake and metabolism in skeletal muscles is linked to a variety of metabolic diseases, including type 2 diabetes and obesity. Therefore, understanding the mechanisms governing glucose uptake in skeletal muscle cells is of major importance for the development of possible therapeutic therapies.

A proprietary extract derived from mulberry leaf extract (MLE), contains 1-deoxynojirimycin (DNJ), a strong alpha-glucosidase inhibitor that regulates glucose metabolism [15]. Previous studies have shown that DNJ inhibits intestinal glucose uptake and improves glucose

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tolerance, implying that it may promote metabolic health [17, 29, 34]. However, the effects of MLE and DNJ on glucose uptake in skeletal muscle cells remain poorly understood.

C2C12 cells, a murine skeletal muscle-derived cell line, serve as a widely recognized in vitro system for exploring skeletal muscle biology and associated metabolic processes [18]. These cells are frequently employed to examine diverse aspects of muscle physiology, such as mechanisms regulating glucose metabolism [1, 6]. Furthermore, MLE and its active component, 1-deoxynojirimycin (DNJ), have been investigated for their possible impacts on modulating glucose metabolism [17, 29]. Therefore, the current research focused on evaluating how MLE and DNJ influence alpha glucosidase activity and cellular glucose uptake in C2C12 myotubes. Through alpha glucosidase inhibition assays and glucose uptake measurements, we aimed to clarify the concentration-dependent impacts of these compounds on enzymatic inhibition and glucose absorption [15, 33]. Prior research has highlighted the critical role of glucose transporter proteins, particularly GLUT4 in facilitating glucose uptake within muscle cells [32]. To unravel the mechanism of observed effects, the investigation analyzed the expression patterns of GLUT4 and other relevant transporters. Additionally, it evaluated the participation of pivotal signaling molecules, such as phosphorylated insulin receptor substrate (p-IRS) and phosphorylated AMP-activated protein kinase (p-AMPK), in modulating these metabolic responses [13, 19, 44].

To enhance our understanding of the molecular interactions and pathways affected by these compounds, we used a network-based pharmacological approach. This integrative analysis provides a comprehensive view of the complex interactions between MLE, DNJ, and key signaling pathways involved in both glucose metabolism and muscle cell damage. With the aid of network pharmacology, the complex impacts of MLE and DNJ on multiple targets involved in glucose metabolism and muscle cell damage were better interpreted [3, 16, 42].

Materials and methods

Network Pharmacology analysis

Acquisition of the predicted and disease-related targets of DNJ

The SMILES code for DNJ was obtained from the PubChem database [<https://pubchem.ncbi.nlm.nih.gov/>] (accessed on July 24, 2024) and entered into the SwissTargetPrediction database [<http://www.swisstargetprediction.ch/>] (accessed on July 24, 2024) to determine the predicted targets of DNJ. To identify disease-related targets, we used “glucose uptake” and “skeletal muscle” as a

keyword and searched the GeneCards database [<https://www.genecards.org/>] (accessed on July 24, 2024)].

Acquisition of potential targets

Potential targets were determined by identifying common targets among the predicted targets of DNJ, glucose uptake and skeletal muscle-related targets, which were visualized by generating a Venn diagram using Venny 2.1 [<https://bioinfogp.cnb.csic.es/tools/venny/index.html>] (accessed on July 24, 2024) [31]. The DisGeNET database [<https://www.disgenet.org/search>] (accessed on July 24, 2024) [24] was used to obtain information regarding the specific protein classes of the potential targets.

Construction and analysis of the protein–protein interaction (PPI) network

PPIs of potential targets were analyzed using the STRING database [<https://string-db.org/>] (accessed on July 24, 2024) [27]. The analysis parameters were set at a high confidence of 0.700 and a medium false discovery rate (FDR) stringency of 5%. The resultant data were imported into the Cytoscape software (Ver. 3.10.2) [26] to construct and analyze potential PPI network targets. Three parameters, including “degree” “betweenness centrality,” and “closeness centrality,” were used to estimate the topological features of the network nodes.

Gene ontology (GO) enrichment analysis and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis

GO enrichment analysis and KEGG pathway analysis for potential targets were performed using the DAVID Bioinformatics Resources database, 2021 version [<https://david.ncifcrf.gov/home.jsp>] (accessed July 24, 2024) [8,12]. The FDR error control method (FDR < 0.05) was used to correct the p-value, and a threshold of < 0.05 was set to identify significant pathways. The results were visualized using ImageGP [<https://www.bic.ac.cn/ImageGP>] (accessed July 24, 2024)].

Construction and analysis of the compound–target–pathway (C-T-P) network

An integrated C-T-P network was constructed and analyzed using Cytoscape software (Ver. 3.10.2, Seattle, WA, USA). The general workflow of the network pharmacology analysis is provided in Fig. 1.

Assay of alpha glucosidase activity

Briefly, alpha glucosidase enzyme solution was prepared by dissolving the enzyme in phosphate buffer (pH 6.86) to achieve a final concentration of 0.5 U/mL. The enzymatic assay involved pre-incubating 50 µL of the enzyme solution with 25 µL of the test compounds at different concentrations, diluted in the same buffer (pH 6.86) at

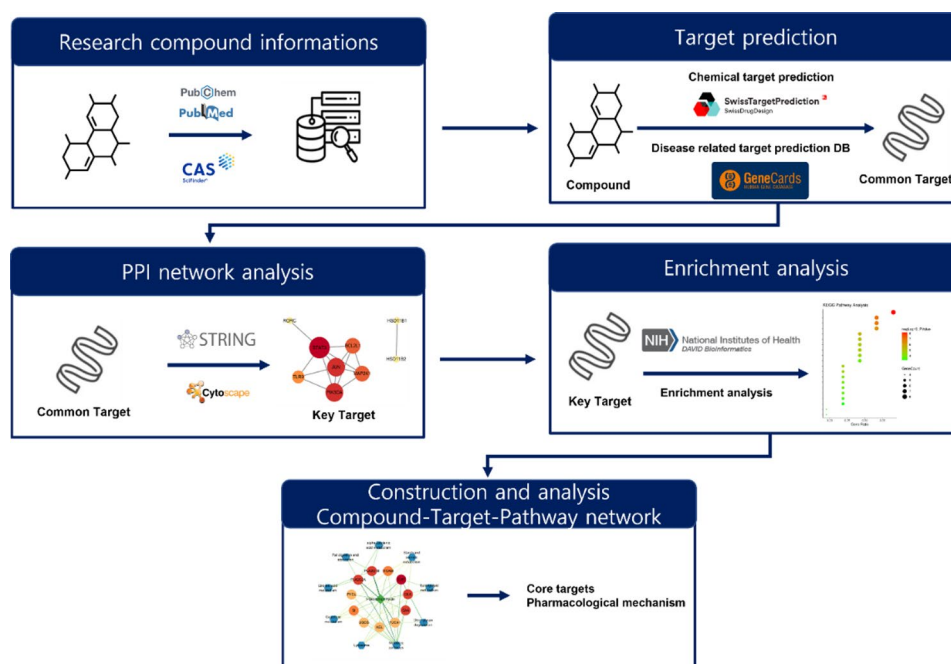


Fig. 1 Schematic of the network pharmacology pipeline

37 °C for 10 min. The reaction was initiated by adding 25 μ L of 4-nitrophenyl α -D-glucopyranoside (p-NPG), yielding a final substrate concentration of 0.625 mM. Following a 10-minute incubation period, the reaction was terminated by introducing 50 μ L of 0.5 M sodium carbonate (Na_2CO_3). The enzymatic activity was quantified by determining the concentration of 4-nitrophenol liberated from p-NPG hydrolysis, which was assessed the absorbance at 405 nm using a microplate reader (PowerWave XS; Bio-Tek Instruments, Winooski, VT, USA).

Reagents

The EZ-Cytox Cell Viability Assay Kit was purchased from ITS BIO (Seoul, Korea). Fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA). The primary anti-bodies for insulin receptor substrate 1 (IRS-1) and GLUT4 and secondary antibodies for phosphorylated-IRS-1 (p-IRS-1), AMPK, and p-AMPK were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture

The mouse C2C12 skeletal muscle cell line was employed in this study. For cell culture, Dulbecco's modified Eagle medium (DMEM) was supplemented with 10% FBS, antibiotics (penicillin/streptomycin), L-glutamine, and sodium pyruvate (Gibco, Waltham, MA USA) and the cells were incubated in an incubator under conditions of 5% CO_2 and 37 °C.

Sample preparation

The standardized MLE was identified as Reducose® (5% DNJ content), was purchased from Phynova (Little Rock, AR, United States). MLE was dissolved in DMSO such that the final concentration of DMSO in the culture media was kept below 0.1%.

Cell viability assay

To evaluate the cytotoxic effects of these compounds C2C12 cells were exposed to various concentrations of MLE (6.25, 12.5, 25, 50, 100, or 200 $\mu\text{g/mL}$) or DNJ (6.25, 12.5, 25, 50, or 100 μM). Cell viability was assessed using the EZ-CYTOX assay. Briefly, cells were cultured in 96-well plates (Corning, New York, USA) at a density of 1×10^4 cells per well in 100 μL of medium and incubated for 24 h. Following this incubation period, cells were treated with the respective compounds or with a vehicle control consisting of medium containing 0.5% (v/v) dimethyl sulfoxide (DMSO) as a control. After treatment, 10 μL of EZ-CYTOX (Daeil Lab Service, Seoul, Korea) was added to each well, and the plates were incubated for an additional 30 min. The absorbance was measured at 450 nm using a microplate reader (PowerWave XS; Bio-Tek Instruments, Winooski, VT, USA). A blank sample was prepared by mixing 100 μL of culture medium with 10 μL of EZ-CYTOX [36].

Glucose uptake assay

C2C12 myotubes were maintained in high-glucose DMEM medium supplemented with 10% FBS (Gibco, Waltham, MA USA), streptomycin, and penicillin (both

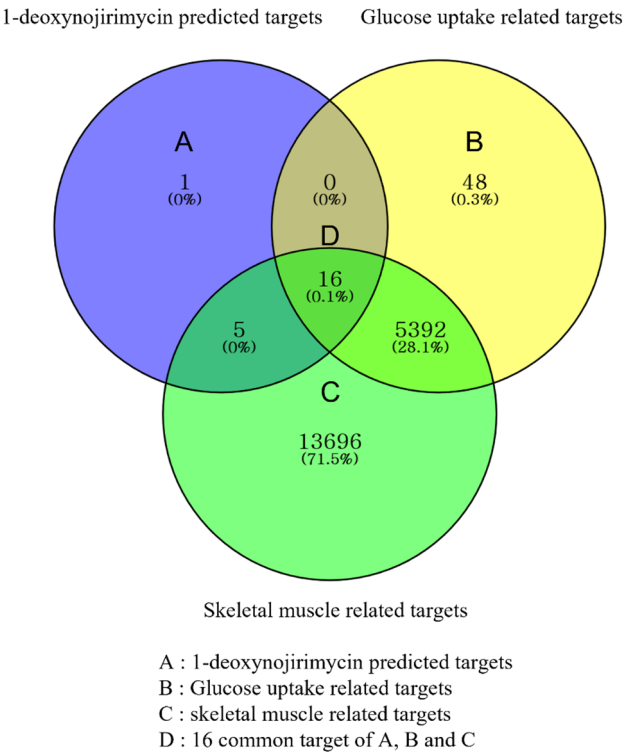


Fig. 2 Venn diagram of DNJ-predicted/glucose uptake and skeletal muscle-related targets. 16 common targets were selected as potential targets

100 µg/mL; Gibco, Waltham, MA USA) at 37 °C in 5% CO₂. To induce myotube differentiation, C2C12 cells were first cultured in DMEM until the cells reached 90% confluence and then transferred to DMEM supplemented with 2% horse serum. Following 5 days of differentiation, fully differentiated cells were incubated with gliclazide (6.25, 12.5, 25, or 50 µM) as the positive control. For the experimental groups, cells were treated with MLE (100–200 µg/mL) or DNJ (50 or 100 µM) in serum-free high-glucose DMEM. Following treatment, cells were exposed to the glucose analog 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose (2-NBDG, 10 µM) for glucose uptake assessment [39]. After 12 h, the culture medium was replaced with phosphate-buffered saline (PBS) and the fluorescence intensities were measured at Ex/Em = 475 nm/550 nm using a Microplate Reader (TECAN Group Ltd., Shanghai, China).

Western blot analysis

C2C12 myoblasts (2 × 10⁵ cells/mL) cultured in 60-mm dishes were exposed to various concentrations of MLE (100–200 µg/mL) or DNJ (50 or 100 µM) for 24 h. Following treatment completion, cell lysates were prepared using radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with 1× protease inhibitor cocktail and 1 mM phenyl methyl sulfonyl fluoride (PMSF) [41, 43]. Proteins (30

Table 1 List of potential DNJ-predicted/glucose uptake and skeletal muscle-related targets

Gene	Description	Rel-evance score
GAA	Alpha Glucosidase	28.99261
UGCG	UDP-Glucose Ceramide Glucosyltransferase	23.0946
MGAM	Maltase-Glucoamylase	17.13668
PYGL	Glycogen Phosphorylase L	14.49694
SI	Sucrase-Isomaltase	14.16237
AGL	Amylo-Alpha-1, 6-Glucosidase, 4-Alpha-Glucanotransferase	12.79472
GANAB	Glucosidase II Alpha Subunit	8.647933
GLA	Galactosidase Alpha	7.699261
GLB1	Galactosidase Beta 1	5.811518
PLA2G2A	Phospholipase A2 Group IIA	5.181933
PLA2G1B	Phospholipase A2 Group IB	3.379941
FUCA1	Alpha-L-Fucosidase 1	2.995494
SLC18A3	Solute Carrier Family 18 Member A3	2.807955
PABPC1	Poly(A) Binding Protein Cytoplasmic 1	2.206785
MAN2B1	Mannosidase Alpha Class 2B Member 1	1.957123
LSS	Lanosterol Synthase	1.244614

µg/lane) were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), subsequently transferred onto polyvinylidene fluoride membranes, and allowed to bind to epitope-specific primary and secondary antibodies. Antibody binding was detected using enhanced chemiluminescence advance western blotting detection reagents (GE Healthcare, Buckinghamshire, UK) following the manufacturer’s protocol, and visualized using an LAS 4000 imaging system (Fujifilm, Tokyo, Japan).

Results and discussion

Network pharmacology analysis

Prediction of targets and screening of potential targets of DNJ

Using the SwissADME target prediction database, we identified 22 predicted targets for DNJ; targets with probabilities of < 0 were excluded. A total of 5497 glucose uptake and 19,109 skeletal muscle-related targets were obtained from the GeneCards database. Among these, 16 targets common to both the predicted targets of DNJ and glucose uptake and skeletal muscle-related targets were selected as potential targets (Fig. 2; Table 1). Considering that skeletal muscle is the primary tissue responsible for glucose uptake and that diabetes has been reported to induce skeletal muscle atrophy and functional decline [24, 31], it is noteworthy that the majority of glucose-uptake-related targets in Fig. 2B were classified as skeletal muscle-associated. Although most previous studies on DNJ have primarily focused on its antidiabetic effects [15, 16], our target prediction results further support this notion by showing that the majority of DNJ-associated

targets are positioned within the overlapping subset between panels B and C. This overlap implies that DNJ may exert its glucose-lowering activity via pathways that are closely related to skeletal muscle function.

PPI network analysis of potential targets

PPI networks were established to obtain a more in-depth understanding of the relationships among target genes. The STRING database was used to construct a PPI network for the 16 potential targets. The network was analyzed and visualized using the Cytoscape software. Network analysis was performed based on three parameters used to determine the importance of different nodes within a complex network: degree, betweenness centrality, and closeness centrality. Degree measures centrality measurement index that calculates the number of connections of a specific node, with a higher degree value indicating the relatively high importance of a node within the network [10]. Betweenness centrality quantifies the extent to which a node lies on the shortest paths between other nodes, and identifies nodes that serve as critical bridges [21]. Closeness centrality reflects the

shortest path length between two nodes, and identifies nodes that are closely connected to others [7]. Network visualization indicates that nodes with higher degrees were represented by larger sizes and colors that transitioned from yellow to red. Edges are displayed with varying widths based on their combined scores. The resultant PPI network, comprising 11 nodes and 14 edges (Fig. 3A), revealed that maltase-glucoamylase (MGAM), which catalyzes the final glucose-releasing step of starch digestion, is the highest-degree node (Table 2).

GO enrichment and KEGG pathway analysis of potential targets

GO enrichment and KEGG pathway analyses were performed using DAVID Bioinformatics Resources 2021 to predict the biological processes and pathways associated with the potential targets, and the results are presented in bubble charts (Fig. 3). As shown in Supplementary Fig. 3B, eight biological process terms were identified using GO enrichment analysis, with potential targets closely related to glycogen catabolic processes. Based on the results of KEGG pathway analysis, nine KEGG

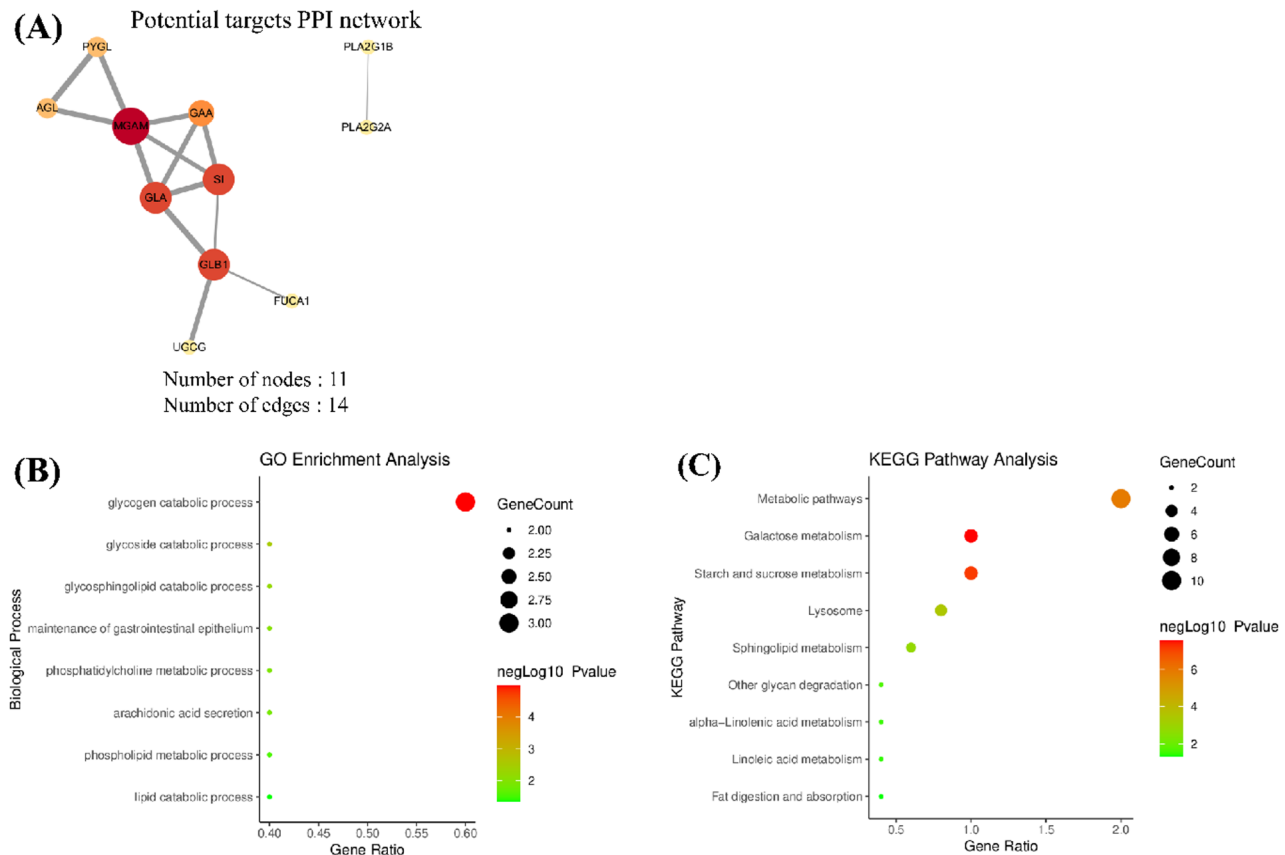


Fig. 3 Protein-protein interaction network of potential targets shows that MGAM, GLE1, GLA, and SI have a degree ≥ 4 , highlighting them as important targets. (A), Bubble chart of GO enrichment, the GO enrichment of the 11 potential targets indicates that glycogen catabolic process was the most significantly enriched term. (B), and KEGG pathway analysis of 11 potential targets indicates that galactose metabolism is the most significantly enriched pathway, whereas metabolic pathways involve the largest number of targets. (C)

Table 2 List of potential targets based on protein-protein interaction network topological analysis

Gene	Degree	Relevance score	Betweenness Centrality	Closeness Centrality
MGAM	5	17.1366	0.4285	0.6153
GLB1	4	5.8115	0.4642	0.5714
GLA	4	7.6992	0.2142	0.6666
SI	4	14.1623	0.2142	0.6666
GAA	3	28.9926	0	0.5333
AGL	2	12.7947	0	0.4210
PYGL	2	14.4969	0	0.4210
FUCA1	1	2.9954	0	0.3809
UGCG	1	23.0946	0	0.3809
PLA2G1B	1	3.3799	0	1
PLA2G2A	1	5.1819	0	1

Table 3 List of KEGG pathway functional annotation chart of potential targets

KEGG pathway	Genes	P-value	Gene Count
Galactose metabolism	MGAM, GLB1, GAA, SI, GLA	2.9E-8	5
Starch and sucrose metabolism	MGAM, AGL, GAA, SI, PYGL	7.4E-8	5
Metabolic pathways	UGCG, MGAM, GLB1, PLA2G1B, AGL, GAA, SI, PLA2G2A, PYGL, GLA	1.4E-6	10
Lysosome	GLB1, GAA, FUCA1, GLA	3.6E-4	4
Sphingolipid metabolism	UGCG, GLB1, GLA	1.6E-3	3
Other glycan degradation	GLB1, FUCA1	2.0E-2	2
alpha-Linolenic acid metabolism	PLA2G1B, PLA2G2A	2.9E-2	2
Linoleic acid metabolism	PLA2G1B, PLA2G2A	3.3E-2	2
Fat digestion and absorption	PLA2G1B, PLA2G2A	4.8E-2	2

pathway terms were identified (Table 3). The potential targets were primarily galactose metabolism, starch and sucrose metabolism, and metabolic pathways (Fig. 3C).

Construction and analysis of the C-T-P network

An integrated C-T-P network incorporating compounds, potential targets, and pathways was constructed using Cytoscape software. This network comprised 21 nodes and 46 edges (Fig. 4). Network visualization provided insights into the complex relationships among the individual components and their pharmacological mechanisms. Green diamond, reddish circular, and blue hexagonal nodes represent DNJ, potential targets, and pathways, respectively. The color of the potential target nodes is indicated in degrees. Thus, a redder node indicates a higher degree value and greater importance in the network. The edge colors represent the edge betweenness values, transitioning from yellow to green with higher

values. Topological analysis showed that the metabolic pathway, galactose metabolism, and starch and sucrose metabolism had high degree values (10, 5, and 5, respectively) among the pathway nodes (Table 4). These pathways exhibited high gene ratios and p-values in KEGG pathway analysis. GLB1 showed the highest degree value of 6 among the target nodes. GLB1 was involved in five pathways at high frequencies, further supporting its importance (Table 5). Similarly, GAA demonstrated substantial network connectivity, appearing in four pathways with the second highest degree value (degree = 5), indicating its pivotal role in glucose uptake. These C-T-P network results indicate that DNJ modulates 11 genes, including GAA, among the glucose uptake-related genes, thereby exhibiting glucose uptake activity by being involved in metabolic pathways, galactose metabolism, and starch and sucrose metabolism.

In Fig. 4, the C-T subnetwork shows that DNJ is highly connected to GLB1 (degree = 6) and to GAA, GLA, PLA2G1B, and PLA2G2A (degree = 5 each), whereas in the T-P subnetwork, metabolic pathways exhibit the highest degree centrality (degree = 10). In addition, the high degree network between GLB1 and GLA suggests the lysosomal degradation of glycosphingolipids, thereby reducing ceramide/glucosylceramide accumulation that would otherwise impair IRS-1/AKT signaling and disrupt GLUT4 translocation in skeletal muscle [9, 22, 25]. Their high centrality thus suggests an indirect mechanistic link to the IRS-1-GLUT4 that regulates muscle glucose uptake.

Inhibitory effects of MLE, DNJ on alpha glucosidase activity

Among 16 potential glucose uptake-related targets, alpha glucosidase is one of them (Table 1). As a results, it suggests that alpha glucosidase is a potential target in metabolic pathway. Next, the inhibitory effects of MLE and DNJ on GAA were evaluated. As shown in Fig. 5A, MLE and DNJ showed activity, with IC₅₀ values of 15.07 µg/mL and 192 µM, respectively. These results indicate that these compounds can be potential alpha glucosidase inhibitors, suggesting the need for further molecular studies.

Effects of the MLE and DNJ on cell viability and glucose uptake in C2C12s

Next, we explored the effect of MLE and DNJ on metabolic pathways. First, the effects of MLE and DNJ on mouse skeletal muscle cells (C2C12 cells) were assessed in terms of cell viability using the EZ-Cytox assay. Cells were treated with varying concentrations of MLE and DNJ for 24 h. MLE (6.25, 12.5, 25, 50, 100, or 200 µg/mL) and DNJ (6.25, 12.5, 25, 50, or 100 µM) treatment did not exhibit significant cytotoxic effects on C2C12 cells. Cell viability remained relatively consistent across all tested

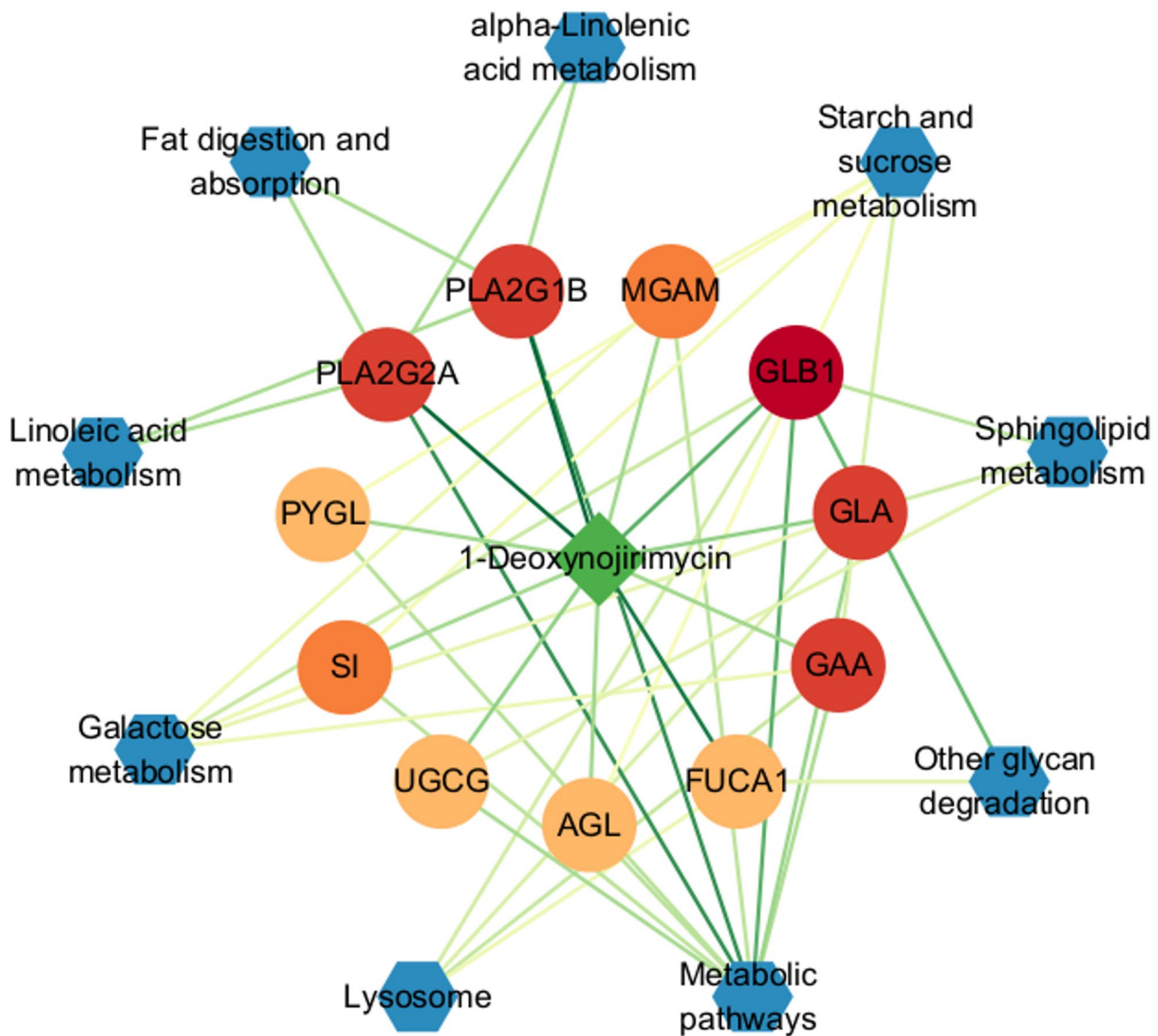


Fig. 4 A C-T-P network for the potential targets of DNJ

Table 4 List of pathways based on C-T-P network topological analysis

Pathway	Degree	Betweenness Centrality	Closeness Centrality
Metabolic pathways	10	0.6452	0.2644
Galactose metabolism	5	0.4255	0.0293
Starch and sucrose metabolism	5	0.3922	0.0209
Lysosome	4	0.4082	0.0221
Sphingolipid metabolism	3	0.3774	0.0087
Other glycan degradation	2	0.3636	0.0036
alpha-Linolenic acid metabolism	2	0.3509	0.0011
Linoleic acid metabolism	2	0.3509	0.0011
Fat digestion and absorption	2	0.3509	0.0011

Table 5 List of potential targets based on C-T-P network topological analysis

Gene	Degree	Betweenness Centrality	Closeness Centrality
GLB1	6	0.5263	0.1334
GAA	5	0.5	0.0576
GLA	5	0.5	0.0632
PLA2G1B	5	0.5	0.1351
PLA2G2A	5	0.5	0.1351
MGAM	4	0.4762	0.0276
SI	4	0.4762	0.0276
AGL	3	0.4545	0.0133
FUCA1	3	0.4545	0.0343
PYGL	3	0.4545	0.0133
UGCG	3	0.4545	0.0226

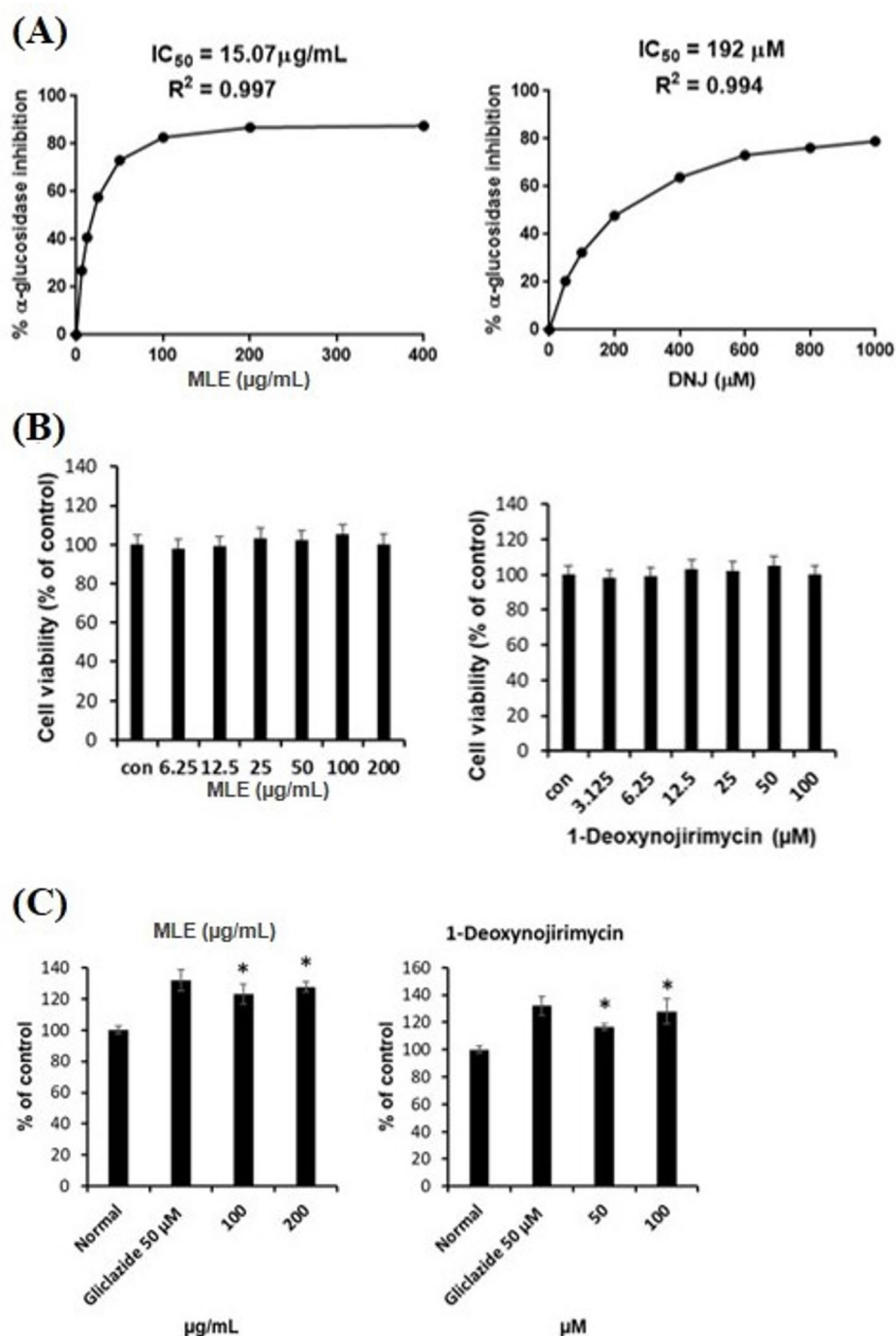


Fig. 5 Glucosidase-inhibitory activities of MLE and DNJ shown using the alpha glucosidase-inhibitory activity assay **(A)**; Effects of MLE and DNJ on C2C12 cells viability **(B)** and the glucose uptake in C2C12 cells **(C)**. The data are presented as the mean $\pm$ standard deviation (SD) of three independent experiments ($n=3$). *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.01$ vs. control group

concentrations of MLE and DNJ compared to that of the control group (Fig. 5B). These results indicate that MLE and DNJ are well-tolerated by C2C12 cells within the tested concentration ranges and do not induce cytotoxicity under the experimental conditions used in this study.

Then, the effects of MLE, DNJ on glucose uptake in C2C12 cells were measured. Cells were treated with varying concentrations of MLE and DNJ for 24 h. Treatment with MLE (100–200 $\mu\text{g/mL}$) and DNJ (50 and 100 μM) significantly increased glucose uptake compared to that of the control group (Fig. 5C). These results indicate that

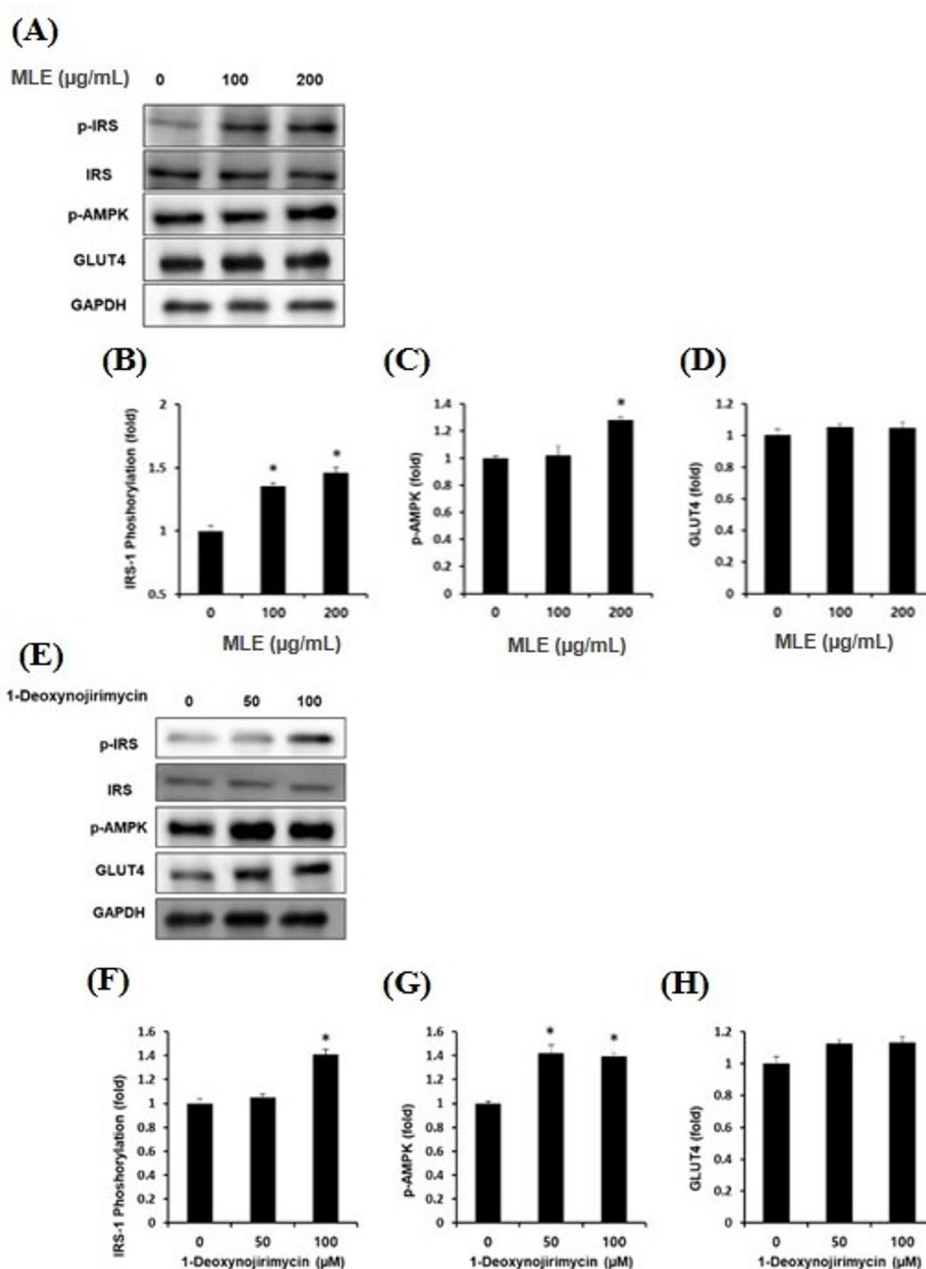


Fig. 6 Effect of MLE, DNJ on the protein expression levels of p-IRS-1, IRS-1, GLUT4, AMPK, and p-AMPK. **(A)** IRS-1, p-IRS-1, GLUT4, AMPK, p-AMPK, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in C2C12 cells treated with 100 and 200 $\mu\text{g/mL}$ MLE for 24 h or untreated. **(B–D)** Each bar graph presents the densitometric quantification of western blotting bands. **(E)** IRS-1, p-IRS-1, GLUT4, AMPK, p-AMPK, and GAPDH in C2C12 cells treated with 50 and 100 μM DNJ for 24 h or untreated. **(F–H)** Each bar graph presents the densitometric quantification of western blotting bands. The data are presented as the mean $\pm$ standard deviation (SD) of three independent experiments ($n = 3$). *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.01$ vs. control group

MLE and DNJ promoted glucose uptake in C2C12 cells within the tested concentration ranges.

Effect of MLE and DNJ on the protein expression of IRS-1, p-IRS-1, GLUT4, AMPK, and p-AMPK

It is known that the presence of MLE and DNJ upregulated the AMPK pathway at the mRNA and protein levels, leading to GLUT4 translocation in mice [20]. Moreover,

MLE and DNJ treatment increased IRS protein expression and improved insulin resistance by activating the insulin signaling pathway and glucose metabolism in the skeletal muscles [14]. Therefore, Western blot analysis was used to investigate the effects of MLE and DNJ on protein expression in C2C12 cells, focusing specifically on the p-AMPK, IRS-1, and GLUT4 signaling pathways (Fig. 6). Treatment of C2C12 cells with MLE and DNJ,

resulted in noticeable alterations in protein expression levels. Quantitative analysis revealed significant changes in the phosphorylation and expression of IRS-1, and p-AMPK. Specifically, treatment with MLE at 100 µg/mL led to a 1.35-fold increase in IRS-1 and phosphorylation level. At 200 µg/mL, MLE treatment resulted in a 1.46- and 1.28-fold increases in IRS-1 and p-AMPK phosphorylation levels, respectively. Similarly, DNJ treatment at 50 µM led to a 1.05- and 1.42-fold increases in IRS-1 and p-AMPK phosphorylation levels, respectively. At 100 µM, DNJ resulted in a 1.41- and 1.39-fold increases in IRS-1 and p-AMPK phosphorylation levels, respectively. However, GLUT4 expression was not significantly increased (1.12 to 1.13) by DNJ treatments. These findings are, to some extent, in line with what could be anticipated from the preceding network pharmacology analysis.

In the present study, although there is no upregulation of GLUT4, MLE and DNJ exhibited the significant enhancement of glucose uptake. This suggests that these compounds may effectively stimulate glucose transport through the enhanced number of the GLUT4 transporters, with no effect on GLUT4 protein levels in skeletal muscle cells [28]. However, it remains to be determined whether the observed enhancement in glucose uptake is driven by direct modulation of skeletal muscle-specific pathways or represents secondary effects of systemic glucose regulation. In addition, we examined the effect of MLE and DNJ on enhancing glucose uptake through p-IRS and p-AMPK expression. The increase in p-IRS and p-AMPK levels in response to MLE and DNJ treatment suggests that these compounds activate insulin signaling and energy-sensing pathways, respectively [2, 35]. The activation of AMPK, a critical energy sensor, has been shown to enhance glucose uptake and fatty acid oxidation in muscle cells, which supports our findings [4, 11].

In summary, we used a network pharmacology approach to obtain a comprehensive view of the molecular interactions and pathways influenced by MLE and DNJ. Network pharmacology analysis enables the interpretation of how natural product-derived compounds exert multitarget actions and modulate protein–protein interaction, providing a systems-level understanding of their pharmacological effects [37, 38, 40]. This approach allowed us to interpret the functional relationships between DNJ-responsive proteins, experimentally validated in our study, and pathways associated with glucose metabolism and skeletal muscle injury [23, 30]. Despite these promising results, the exact molecular mechanisms by which DNJ exerts its glucose-lowering effects remain unclear, including whether DNJ directly targets skeletal muscle or modulates systemic glucose metabolism indirectly. Therefore, further studies are required to clarify these mechanistic uncertainties through in vivo

validation and detailed pathway-focused investigations. Collectively, our findings provide a mechanistic rationale for advancing DNJ as a potential modulator of skeletal muscle-associated glucose regulation.

Abbreviations

AMPK	AMP-activated protein kinase
C-T-P	Compound–Target–Pathway
DMSO	Dimethyl sulfoxide
DNJ	1-deoxynojirimycin
GAA	Alpha glucosidase
GLB1	Galactosidase beta 1
GLUT4	Glucose transporter type 4
IRS-1	Insulin receptor substrate 1
MGAM	Maltase-glucoamylase
MLE	Mulberry leaf extract
p-AMPK	Phosphorylated AMP-activated protein kinase
p-IRS-1	Phosphorylated insulin receptor substrate 1
p-NPG	4-nitrophenyl α-d-glucopyranoside
PPI	Protein–protein interaction

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Not applicable.

Author contributions

S.H.K., and K.S.K. conceived and designed the experiments; N.H.L.T., J.H.P., M.J.H. D.-W.K., S.H.K. and S.H.K. performed the experiments; T.H.K., S.H.K. and K.S.K. analyzed the data; and N.H.L.T., and J.H.P. wrote the manuscript. All authors have read and approved the final manuscript.

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Data availability

Not applicable.

Declarations

Competing Interests

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