Journal of King Saud University – Science 35 (2023) 102450

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Journal of King Saud University – Science

journal homepage: www.sciencedirect.com

Original article

A synthetic antioxidant molecule, GP13 derived from cysteine desulfurase of spirulina, *Arthrospira platensis* exhibited anti-diabetic activity on L6 rat skeletal muscle cells through GLUT-4 pathway

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ARTICLE INFO

Article history:
Received 9 August 2022
Revised 9 November 2022
Accepted 13 November 2022
Available online 21 November 2022

Keywords:
Diabetics
Oxidative stress
Antioxidant system
L6 myotubes cells
Insulin signaling
Arthrrospira platensis

ABSTRACT

Objectives: Diabetes creates oxidative stress, which damages several organs and causes various problems including hyperglycemia, hyperlipidemia, hypertension, and maybe iron dyshomeostasis. Consequently, antioxidant therapy may be a promising strategy to avoid diabetes and diabetic complications. In the current study, we investigated the activity of the antioxidant GP13 peptide in an *in-vitro* diabetic model.
Methods: All anti-diabetic and antioxidant *in-vitro* tests were performed on differentiated L6 myotubes cells. MTT assay was used to analyze the cytotoxic effect of the GP13 at different concentrations (10 μM to 80 μM) in the L6 cells. The DCFDA fluorescence was performed to confirm the radical scavenging effect of GP13 in the myotubule cells. The cells were treated with different concentrations of GP13 peptide before the enzyme assay was conducted. The differentiated L6 myotubes were kept for serum deprivation for 8 h before being treated with GP13 peptide. The RNA extraction from the L6 myotubes was performed using the Trizol reagent.
Results: Cell viability analysis exhibited the non-toxic nature of GP13 in a dose-dependent manner (10 μM to 80 μM). Antioxidant enzyme, superoxide dismutase activity was 23.25 U/mL in the untreated group, whereas it was only 11.75 U/mL in the group that was exposed to GP13 at 80 μM. The catalase activity at 40 μM was slightly altered in the cells, while the hydrogen peroxide inhibition activity was higher (91.2%) compared to the control group. Additionally, GP13 showed anti-diabetic effects through a dose-dependent increase in glycogen storage (6.1 mM). It was discovered that 40 μM was the ideal concentration for the highest level of activity. Additionally, the genes involved in diabetes-related to antioxidants and the insulin signalling system were investigated.
Conclusion: It is concluded that the GP13 peptide from *A. platensis* is a promising agent for anti-diabetic and antioxidant activities. To treat diabetes and its consequences, we thus propose that GP13 be regarded

Abbreviations: T2D, type 2 diabetes; GLUT4, glucose transporter type 4; P13K, phosphoinositide 3-kinase; ROS, reactive oxygen species; CDS, cysteine desulfurase; DMEM, Dulbecco's Modified Eagle Medium; DCFDA, Dichlorodihydrofluorescin diacetate; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxide; GST, glutathione S-transferase; GCS, glutamyl cysteine synthetase; GRAS, Generally Recognized as Safe.

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.jksus.2022.102450

as a natural lead. The animal model design of this study has limitations, and further research is needed to draw conclusions about its therapeutic relevance to people.

1. Introduction

Diabetes mellitus, a global public health issue, is now becoming an epidemic on a global scale. Diabetes now affects 150 million people worldwide, which is expected to rise to 300 million by 2025 (Bhattacharya et al., 2019; Ousset et al., 2020). Diabetes is a metabolic disease caused by either extreme insulin insufficiency in the system (type 1 diabetes) or systemic opposition to insulin activity (type 2 diabetes (T2D) (Grodsky, 1972; Taylor, 2012). Therefore, reducing insulin intolerance is a massive technique for treating diabetes nowadays. Skeletal muscle is a prominent storage site for postprandial glucose that contributes to insulin opposition in people with diabetes (James et al., 1988; Guru et al., 2022a; Sudhakaran et al., 2022). As an insulin target, muscle cells are critical locations for energy expenditure, consumption, and storage (Guo et al., 2019). Many of these processes, in conjunction with hyperglycemia-induced mitochondrial dysfunction and ER-stress, enhance the production of free radicals, which generate cellular apoptosis and significantly contribute to hyperglycemia consequences (Fig. 1) (Issac et al., 2021b). The peptide derived from various natural sources was reported to show great efficiency in diabetes. These peptide properties are influenced by the number of amino acids present in the sequence (Raju et al., 2020). Oxidative stress causes β-cell damage and inhibition of insulin signals to induce insulin resistance in diabetic patients (Unuofin and Lebelo, 2020). Therefore, the peptide with antioxidant property helps in reducing oxidative stress and lipid peroxidation. The cysteine and glycine amino acids are known to stimulate insulin

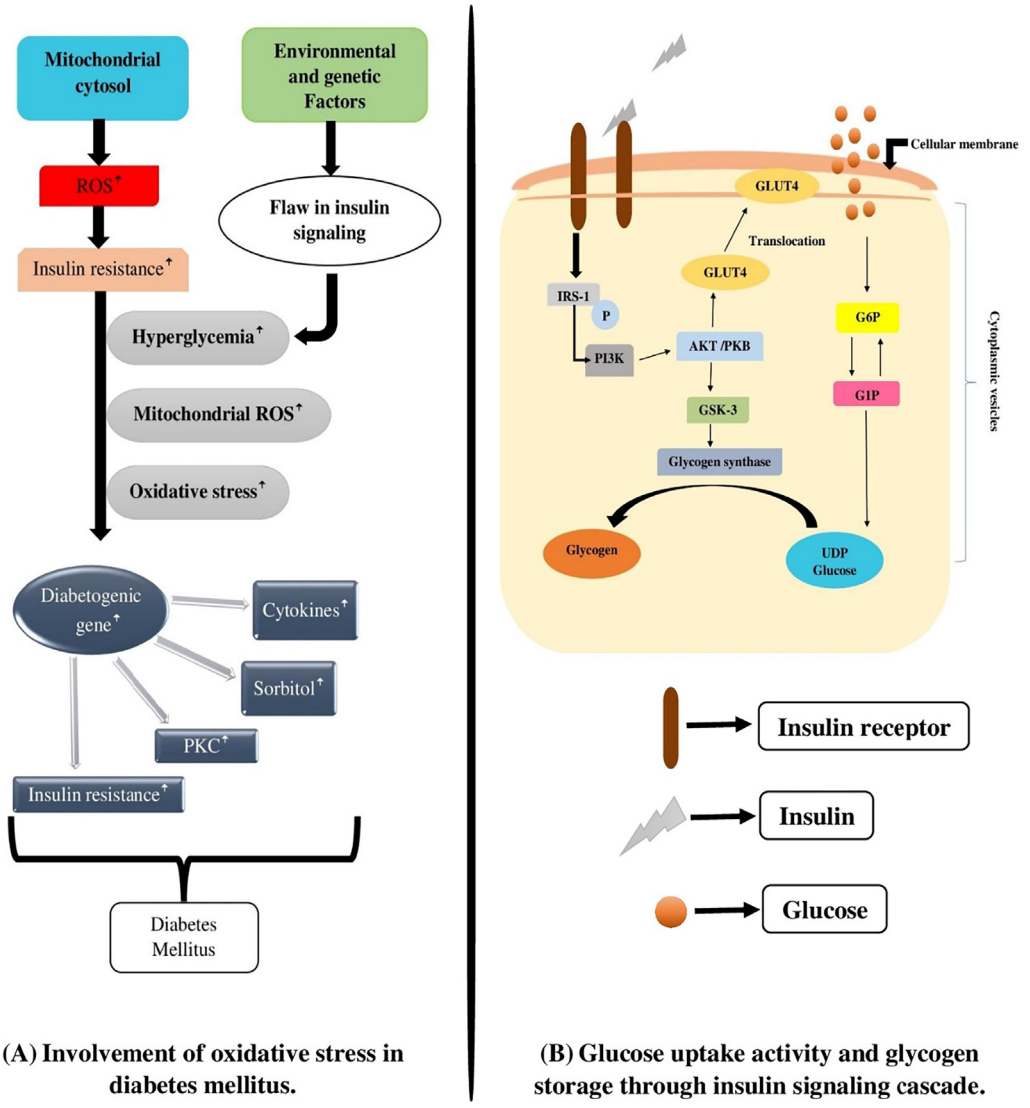

Fig. 1. **(A)** Involvement of oxidative stress in diabetes mellitus; ROS - Reactive oxygen species, PKC - Protein kinase C. **(B)** Glucose uptake activity and glycogen storage through insulin signalling cascade; IR - Insulin receptor, IRS - Insulin receptor substrate, AKT/PKB - Protein kinase B, GSK- Glycogen synthase kinase 3.

secretion from pancreatic β-cells by serving normal glucose metabolism.

Spirulina is gaining popularity due to its amino acid composition, which have antioxidant properties. They are widely used in a number of applications, including biomedical research, dietary components, natural colours for health and beauty products, and possible therapeutic compounds in induced-oxidative stress disorders. It can be used as a natural dietary antioxidant supplement that might be added to cereals, organic food bars, or drinks to help prevent several chronic illnesses caused by free radicals (Li et al., 2020). In perspective with the above facts, in the current work GP13 peptide derived from the cysteine desulfurase (CDS) of *Channa striatus* was investigated for glucose uptake and radical scavenging activity in insulin resistance conditioned L6 cell line.

2. Materials and methods

2.1. In-vitro L6 myoblasts differentiation

Rat skeletal muscle cell line (L6 myoblast) was purchased from the NCCS, Pune with the passage number of 15. The cells were cultured using a T25 flask in the CO_2 incubator. To induce the T2D and oxidative stress condition, the cells were supplemented with DMEM containing 25 mM/L glucose for 5 days. The L6 myoblast cells are converted to L6 myotubes, which are represented by multinucleation in cells. All anti-diabetic and antioxidant *in-vitro* tests were performed on differentiated L6 myotubes cells.

2.2. Cytotoxicity assay

MTT assay was used to analyze the cytotoxic effect of the GP13 at different concentrations (10 μM to 80 μM) in the L6 cell line as reported in our earlier study (Velayutham et al., 2021b) and we used the following formula was used to calculate the proportion of viable cells at each concentration: % Cell viability = OD of treated cells/OD of control cells × 100.

2.3. Dichlorodihydrofluorescin diacetate (DCFDA) staining assay

The DCFDA fluorescence was performed to confirm the radical scavenging effect of GP13 in L6 myotubes cell line. In the 6-well plate, 3.6 x10^5 cells/well were seeded and L6 myoblast cells differentiated to myotubes; they were treated with various concentrations of GP13 (10 μM to 80 μM) and incubated for 24 h as demonstrated by Haridevamuthu et al. (2022b) and Lite et al. (2022).

2.4. Antioxidant enzyme assay

The L6 myotubes cells were treated with different concentrations of GP13 peptide before the enzyme assay was conducted following the procedure of Guru et al. (2021a). The Bradford method was used to measure the sample's protein content after the supernatant had been collected.

2.5. Estimation of glycogen

The differentiated L6 myotubes were kept for serum deprivation for 8 h before being treated with GP13 peptide. A positive control of 100 nM insulin was used, while a stimulant of insulin (10 nM) was given to the cells. The 30% of potassium hydroxide was used in the aspirated cells. After centrifugation of this lysate, the pellet was obtained and treated with anthrone reagent. The absorbance was recorded at 620 nm, and glycogen of various concentrations was utilized as a standard.

2.6. Gene expression studies

The RNA extraction from the L6 myotubes was performed using the Trizol reagent as described by Guru et al. (2022b). We used the primers listed in Table 1 for the expression study. GAPDH was used as a housekeeping gene and the relative gene expression was calculated as explained by Haridevamuthu et al. (2022a).

2.7. Statistics

The study's results are based on an average of three replicates along with a standard deviation. Duncan's multiple tests were performed using Graph Pad Prism 5.0. The statistical variance was considered significant, in case the p-value is < 0.05 or < 0.01.

Table 1
Primers used in the gene expression study.

Gene	Primer	Reference
GAPDH	Forward: 5′-CCACCCATGGCAAATTCCATGGCA-3′ Reverse: 5′-TCTAGACGGCAGGTCAGGTCCACC-3′	(Sahni et al., 1999)
GST	Forward: 5′-TCTGTCTGGACCTGTGTACCTG-3′ Reverse: 5′-GTTGGGAAAGTCTGAGAGGATGC-3′	(Choi et al., 2008)
GPx	Forward: 5′-CCTCAAGTACGTCCGACCTG-3′ Reverse: 5′-CAATGTCGTTGCGGCACACC-3′	(Mouatassim et al., 1999)
GCS	Forward: 5′-CCTTCTGGCACAGCACGTTG-3′ Reverse: 5′-TAAGACGGCATCTCGCTCCT-3′	(Mouatassim et al., 1999)
IRTK	Forward: 5′-ATCTGGATCCCCCTGATAACTGTC-3′ Reverse: 5′-ATGTGGGTGTAGGGGATGTGTTCA-3′	GenBank ID: NM_017071
IRS-1	Forward: 5′-AAGTGGCGGCACAAGTCGAG-3′ Reverse: 5′-CGGGTGTAGAGAGCCACCAG-3′	GenBank ID: XM_002749924
PI3K	Forward: 5′-TGACGCTTTCAAACGCTATC-3′ Reverse: 5′-CAGAGAGTACTCTTGCATTC-3′	(Laville et al., 1996)
GS	Forward: 5′-CGTGGTGAGAGGAAGGAACTGAGC-3′ Reverse: 5′-CCGTCAGACCGTGGAGACA-3′	(Magnoni et al., 2012)
GLUT4	Forward: 5′-CGGGACGTGGAGCTGGCCGAGGAG-3′ Reverse: 5′-CCCCCTCAGCAGCGAGTGA-3′	(Buhl et al., 2001)

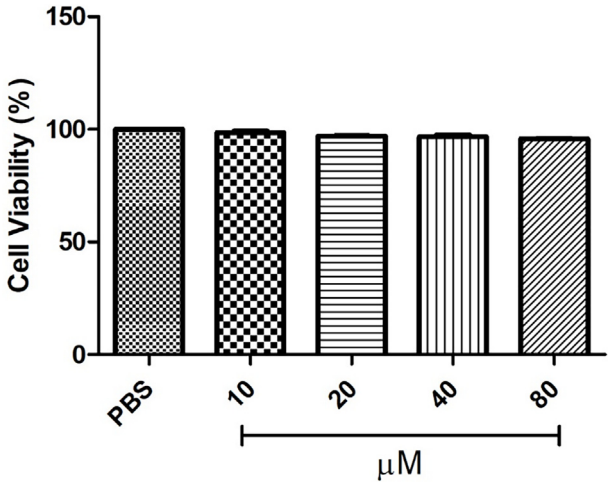

Fig. 2. Cytotoxic analysis on L6 cell line treated with various concentration of GP13 (10 to 80 μM). PBS treated cell was used as the control. Data were expressed as mean ± SD of three independent experiments.

3. Results

3.1. Cell viability of L6 myotube cells

After being exposed for 24 h to different concentrations of the GP13 peptide, ranging from 10 μM to 80 μM, the viability of the L6 myotube cell was evaluated. The untreated cells were employed as the control. More than 90% of the L6 cells treated with GP13 (10–80 μM) exhibited cellular viability (Fig. 2). The results demonstrated that the GP13 peptide had negligible cytotoxic effects at the treatment concentrations.

3.2. Estimation of ROS in L6 cell lines

The effectiveness of GP13 against ROS generation in high glucose (25 mM) induced oxidative stressed L6 myotubes was examined using DCFDA staining. When compared to the untreated L6 myotubes (control), GP13 treated cells had lower ROS levels (Fig. 3). In the control group, higher mean fluorescence intensity was estimated. However, as the peptide concentration increases, a substantial ($p < 0.05$ and $p < 0.01$) decline in ROS levels was seen

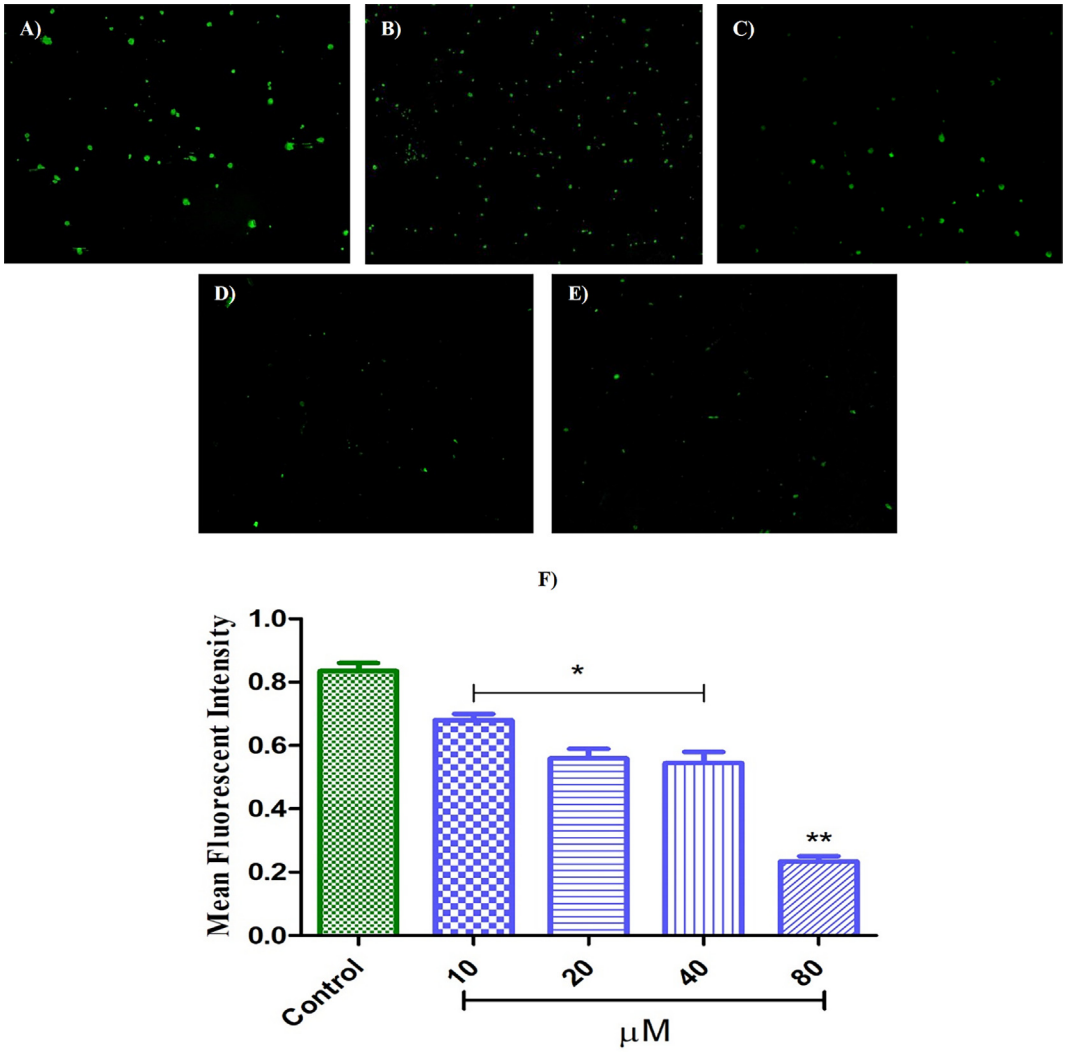

Fig. 3. DCFDA labeling in L6 cells (3.6 x10^5/well) to measure intracellular ROS. **(A)** Control: Induced oxidative stress in L6 cells using 25 mM glucose. **(B)** 25 mM glucose + 10 μM GP13, **(C)** 25 mM glucose + 20 μM GP13, **(D)** 25 mM glucose + 40 μM GP13, **(E)** 25 mM glucose + 80 μM GP13 and **(F)** The fluorescence intensity percentage for the experimental groups. Data presented in mean ± standard deviation (SD) of triplicates. The asterix (*) indicates $p < 0.05$ and double asterix (**) indicates $p < 0.01$ compared to control.

in the GP13 peptide treatment groups. In the 10 μM GP13 exposure group, the ROS mean fluorescence intensity was determined to be 0.59%, but the value dropped to 0.25% in the higher concentration treated group (80 μM). This demonstrates the antioxidant action of the GP13. As a result, high concentrations of ROS within cells were reduced in a concentrated-dependent manner.

3.3. Enzymatic analysis of GP13

SOD is an important antioxidant enzyme that serves as the first line of defence against oxidative damage in all aerobic life. The SOD activity in the untreated group was 23.25 U/mL, while it was significantly reduced in the GP13 exposure group at 80 μM (11.75 U/mL). However, no such difference was identified between GP13 concentrations of 10, 20 and 40 μM, indicating that the GP13 treated group with the higher concentration had better activity (Fig. 4A). At 80 μM of GP13 treated L6 cell line, we noticed a significant increase in hydrogen peroxide inhibition compared to untreated cells (Fig. 4B). The cells revealed minor alterations in the activity of catalase at 40 μM, whereas, the higher concentration (80 μM) showed hydrogen peroxide inhibition activity (91.2 %) compared to the control group.

3.4. Glycogen content in L6 cell line

In comparison to the control, it was discovered that the L6 myotubes treated with GP13 peptide (10 to 80 μM) showed ($p < 0.01$) higher glycogen content (Fig. 5). Glycogen production in L6 myotubes was reported to increase at 40 μM.

3.5. Gene expression analysis

qRT-PCR was used to examine the glutathione expression level. In the L6 myotubes under oxidative stress, the expression levels of glutathione peroxide (GPx), glutathione S-transferase (GST) and glutamyl cysteine synthetase (GCS) were measured (Fig. 6). All antioxidant enzyme genes GST (2.8 fold), GPx (2.0 fold) and GCS (2.2) had the higher expression levels as compared to control. Additionally, qRT-PCR was used to assess the genes implicated in the insulin signalling cascade. The outcomes demonstrated that IRTK (2.5 fold), IRS1 (2.6 fold), PI3K (2.5 fold), GLUT4 (1.6 fold) and GS (1.2 fold) genes were significantly ($p < 0.05$) upregulated in GP13 peptide treatment.

4. Discussion

In this study, we investigated GP13 peptide synthesized from *A. platensis* have proved to exhibit antioxidant properties against oxidative stress (Wu et al., 2016; Sarkar et al., 2021). In this current study, we examined the GP13 anti-diabetic action by suppressing oxidative mediated stress and increasing GLUT4 in L6 cells. This work uses a L6 rat skeletal muscle cell line because glucose uptake predominantly occurs in the skeletal muscle (Issac et al., 2021b).

The test sample was utilized to conduct cytotoxic research against the cell line using the MTT assay. The MTT test helps to assess cytotoxicity based on the formazan crystal formation in the live cells. Fig. 2 demonstrates that no measured concentration of GP13 (10 μM, 20 μM, 40 μM & 80 μM) caused cell toxicity, indi-

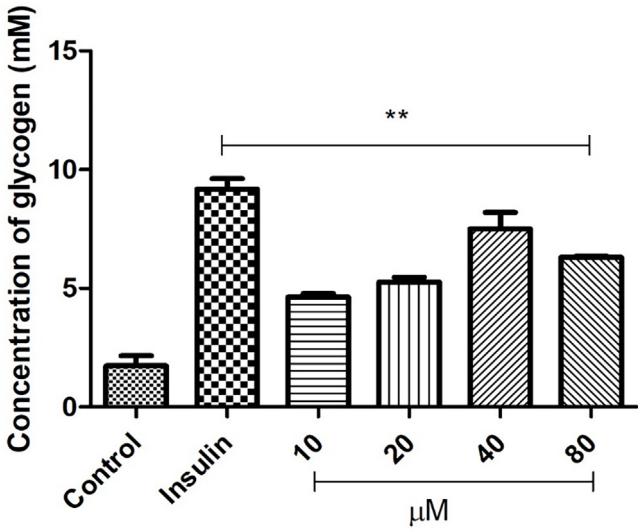

Fig. 5. Dose-response analysis of GP13 (10 to 80 μM) in glycogen synthesis in L6 cell lines. The concentration of glycogen was measured in L6 cell lines tretaed with different doses of GP13. The concentration of glycogen was expressed as mM. Values are statistically significant at $^{**}p < 0.01$ as compared to the control.

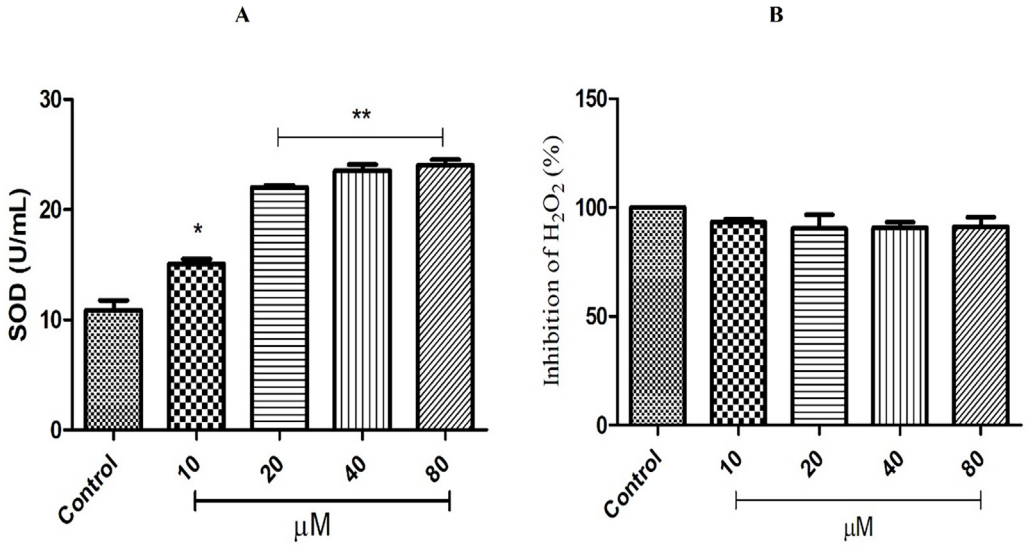

Fig. 4. Effect of GP13 peptide on **(A)** SOD and **(B)** CAT activity in L6 cell line. The peptide treatment in L6 cell line showed a dose-dependent increase in enzyme activity. Data were expressed as mean ± SD of three independent experiments. * denotes $p < 0.05$ and ** denotes $p < 0.01$ as compared to the control.

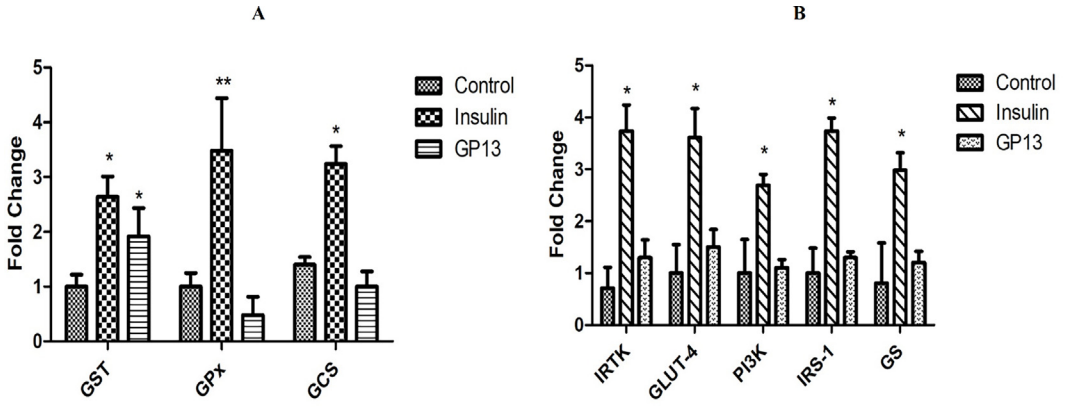

Fig. 6. Effect of GP13 (40 μM) on the mRNA expression of genes implicated in the insulin signalling cascade and antioxidant genes. **(A)** Effect of GP13 on the mRNA expression of GST, GPx, and GCS. **(B)** Effect of GP13 on mRNA expression of IRTK, GLUT-4, PI3K, IRS-1 and GS. $^{*}p < 0.05$ as compared to the control.

cating that it might be utilized as a therapeutic medication and free from any cytotoxic effects. Using DCFDA as a fluorescent probe, the amount of intracellular ROS is measured. Non-ionic, lipophilic, and non-fluorescent DCFDA can diffuse into the cytoplasm and pass through cell membranes. The DCFDA chemical reacts with intracellular ROS generation to increase the fluorescent intensity (Issac et al., 2021a). The fluorescence intensity increased in L6 myotubes when exposed to 25 mM glucose. Previous studies had reported that when the L6 cells were cultured along with high glucose, an increase in oxidative stress and apoptosis was observed, which is consistent with our findings (Udumula et al., 2017). However, a reduction in ROS level was seen in L6 myotubes treated with various doses of GP13 when compared to the control, as shown in Fig. 3. As a result, GP13 reduces the oxidative stress experienced by L6 myotubes and inhibits a rise in intracellular ROS levels. A significant factor in the process of apoptosis is the interaction of ROS with the double bonds of polyunsaturated fatty acids, which results in the generation of lipid hydroperoxides. Such hydroperoxides can decompose to produce a variety of aldehydes (Guru et al., 2021b).

Oxidative stress leads to an imbalance of cellular oxidants and antioxidants. Superoxide and hydrogen peroxide can be removed by enzymes like SOD and CAT (Siddhu et al., 2022). But elevated ROS generation or a feeble antioxidant defence mechanism results in physiological failure, increasing cell damage, and the formation of illness (Manjunathan et al., 2021). In the current work, the L6 myotubes showed lesser CAT and SOD activity when cultivated with 25 mM glucose. However, GP13 pretreatment markedly and dose-dependently raised the degree of antioxidant enzyme activity as shown in Fig. 4. In a similar manner, Wang et al. reported in prior studies that high doses of glucose generated ROS in HepG2 cells, and this ROS condition was effectively inhibited by the peptide from walnut through the SOD and CAT enzyme activation (Velayutham et al., 2021a). Skeletal muscles are crucial for sustaining glucose homeostasis as they are responsible for the majority of insulin-mediated glucose absorption in the post-prandial state (Minakawa et al., 2012). GLUT4 translocation from storage vesicles to the plasma membrane is primarily responsible for the enhanced glucose flow that occurs in response to insulin within skeletal muscle. By promoting GLUT4 and activating the enzyme glycogen synthase for glycogen formation, insulin enhances glucose utilization in the muscle. Glycogen synthase (GS) is inhibited in the skeletal muscle of diabetics with glucose intolerance and insulin resistance (Nikoulina et al., 2001). Based on this, the role of GP13 in L6 myotubes for glycogen production was examined. Glycogen content increased after treatment with various doses of GP13, as illustrated in Fig. 5.

GPx, GST, and GCS gene expression have been studied to determine the precise molecular mechanism of GP13 in their appropriate protection against oxidative damage. In L6 myotubes, the expression of mRNA for GST, GPx, and GCS was downregulated, whereas the group that had been treated with GP13 had increased expression, particularly in GST. Fig. 6A illustrates how activation of the antioxidant genes GST, GPx, and GCS reveals the protective effects of the GP13 peptide in response to oxidative stress. IRTK, GLUT4, PI3K, IRS1, and GS mRNA expression were examined in order to better understand the molecular processes of GP13 by looking at its important targets in insulin signalling. The findings showed that GP13 dramatically raised the expression of IRTK, GLUT4, PI3K, IRS1, and GS in treated cells, illustrated in Fig. 6B. These findings demonstrate that GP13 stimulates insulin to bind to its tyrosine kinase receptor, which causes tyrosine phosphorylation of the insulin receptor substrate and the subsequent activation of PI3K. PKB are the downstream targets of PI3K, and when they are active, they cause GLUT4 to go to the plasma membrane and stimulate glycogen synthase.

This finding concluded that the GP13 peptide from *A. platensis* is a promising agent for anti-diabetic and antioxidant activities. However, further research is needed to draw conclusions about its therapeutic relevance to people.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors would like to extend their sincere appreciation to the Researchers Supporting Project Number (RSP-2021/111), King Saud University, Riyadh, Saudi Arabia.

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