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RESEARCH ARTICLE

# Prophylactic Impacts of Lotusine Against Hyperglycaemia-Induced Oxidative Stress in Hepatic Cells Isolated from Diabetic Rats Via Irs-1/Pi3 K/Akt Pathway

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**ABSTRACT**

Lotusine (LT), a compound extracted from *Nelumbo nucifera*, is being studied as a potentially safer, natural therapeutic antioxidant alternative to chemical drugs for diabetes management. The present study aims to investigate LT's protective effects against D-glucose-induced oxidative stress in HepG2 cells, focusing on antioxidant enzyme activities and insulin signaling pathways. Administrating D-glucose (25 mM) to HepG2 cells increased MDA by 56%, indicative of heightened lipid peroxidation, which was significantly ($P<0.05$) reduced by LT, maintaining 90 % of the cell viability. D-glucose also considerably ($P<0.05$) decreased the activity of antioxidant enzymes, where the activity of SOD and CAT decreased by 2.2-fold after 48 hours, and GPx activity dropped by 38% after 48 h of treatment. LT effectively reversed these reductions, boosting antioxidant enzyme activity. Furthermore, LT (200 µg/ml) reduced the activity of α-amylase and α-glucosidase by 80.36% and 82.6%, respectively, with IC50 of 30.60 µg/ml for α-amylase and 36.15 µg/ml for α-glucosidase, compared to acarbose and EGCG. Alongside the downregulating expression of IRS-1 and AKT-2, GLUT4 is upregulated. In silico docking studies highlighted LT's affinity for proteins involved in diabetes pathophysiology. The *in vitro* results indicated that LT might mitigate oxidative stress, enhance antioxidant defenses in hyperglycemia, and be a potential therapeutic agent against hepatic oxidative damage. The *in vivo* study involved four groups of ten rats each. Group 1 was the normal control, Group 2 was the streptozotocin-treated diabetic control, Group 3 received glibenclamide, and Group 4 received a lotus-supplemented diet. All groups were fed a normal control diet for four weeks. Diabetic rats exhibited lower BWG, FCR, and GR; dietary LT enhanced the growth parameters in G3 and G4. Also, the diabetic rats showed reduced serum, liver, and pancreatic vitamin C and E levels, which LT treatment effectively restored. LT significantly ($P<0.05$) increased liver cell protein content to 11.6 mg/g, with a relative increase of 21 % compared to glibenclamide-treated rats; however, diabetic rats showed a significant ($P<0.05$) decrease in protein content.

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## INTRODUCTION

Diabetes mellitus (DM) is a chronic health condition resulting from disturbed insulin emission, function, or both (Salazar-García and Corona, 2021; Sasso *et al.*, 2024). In DM, hyperglycemia is accompanied by dysfunction and failure of different organs and tissues, development of retinopathy, nephropathy, and neuropathy and cardiovascular disorders (Chen *et al.*, 2021; Salazar-García and Corona, 2021). Type 1 (DM1) diabetes results from the autoimmune destruction of pancreatic β-cells, leading to absolute insulin deficiency, while type 2 (DM2) diabetes is characterized by varying degrees of insulin resistance combined with progressive β-cell dysfunction (Aodah *et al.*, 2024; Lucianus *et al.*, 2024).

Nonalcoholic fatty liver disease (NAFLD) plays a significant role in the development of several metabolic disorders, including hypertension, diabetes, abnormal blood lipid levels (dyslipidemia), insulin resistance, and obesity (Alharbi *et al.*, 2024; El-Sayed *et al.*, 2024;

Mueed *et al.*, 2024). This highlights the importance of managing NAFLD in promoting overall health (Reda *et al.*, 2024a). The liver is crucial for drug metabolism, making it particularly susceptible to drug-induced damage. This can manifest as hepatitis, liver fibrosis, liver failure, and ultimately, death. Liver cancer is the second leading cause of cancer-related deaths globally. Therefore, effectively managing NAFLD and minimizing drug-induced liver injury is critical for maintaining liver health and preventing serious health complications (El-Sheekh *et al.*, 2023; Alkafaas *et al.*, 2024; Elsalahaty *et al.*, 2024).

A significant number of therapeutic interventions for diabetic animals can induce hypoglycemia. Therefore, the propagation of studies on herbal plants used in traditional medicine is of great interest, as they may provide potential sources for novel therapeutic agents that could manage and prevent various diseases (AlSuhaymi, 2024; Hossein *et al.*, 2024; El-Saadony *et al.*, 2024a,b; Reda *et al.*, 2024b). Herbs and spices rich in phytochemicals and polyphenolic fractions have antioxidant properties (Askari *et al.*, 2024), and insulin-like effects in glucose utilization (Aodah *et al.*, 2024; Lucianus *et al.*, 2024).

Herbal remedies and their extracts have gained significant global attention for their potential in treating and preventing various diseases (El-Saadony et al., 2024a). The use of herbs for medicinal purposes has a long history, particularly in Asian countries, spanning thousands of years. A recent study revealed that over 65% of European countries utilize herbal remedies (Almuhayawi *et al.*, 2023; Hegazy *et al.*, 2023; Hegazy *et al.*, 2024; Soliman *et al.*, 2024). Over 1,100 medications have been identified as potentially causing liver toxicity. Research suggests that herbal medicines offer significant protective effects for the liver. These beneficial effects are attributed to the presence of various compounds within herbs, such as alkaloids, flavonoids, saccharides, and phenylpropanoids (Saad *et al.*, 2021a,c; El-Hack *et al.*, 2022b; El-Saadony *et al.*, 2023a).

The global prevalence of diabetes is alarmingly high, with the International Diabetes Federation (IDF) Diabetes Atlas reporting that in 2021, approximately 537 million adults (20-79 years) were living with the condition, a number projected to surge to 643 million by 2030 and 783 million by 2045, driven by factors like urbanization, aging populations, and rising obesity (IDF Diabetes Atlas, 10th edition); this widespread issue exhibits regional disparities, with a significant proportion of those affected residing in low- and middle-income countries, and critically, a large percentage of individuals remain undiagnosed, exacerbating the risk of severe health complications (IDF Diabetes Atlas, 10th edition) (IDF, 2025); the IDF Diabetes Atlas serves as a primary resource for these statistics, and it is essential to note that an updated version of the atlas will be released in April of 2025 (Ong *et al.*, 2023). Over the past two decades, diabetes mellitus has become increasingly prevalent in Saudi Arabia, presenting a significant public health challenge. Recent data highlight the substantial burden of this chronic disease regionally (Al Dawish and Robert, 2021). Risk factors for hyperglycemia include age, family history, obesity, physical inactivity, usage of certain medications (e.g., olanzapine), antidepressants, immunosuppressants, and selective estrogen receptor modulators (Wu *et al.*, 2014). Hyperglycemia, characterized by elevated blood glucose, arises from a complex web of risk factors, including genetic predisposition, obesity, and sedentary lifestyles that contribute to insulin resistance; critically, hyperglycemia itself induces oxidative stress, an imbalance of free radicals that damages cells and impairs insulin function, while simultaneously, liver damage, such as from nonalcoholic fatty liver disease (NAFLD), disrupts crucial glucose regulation, and conversely, chronic hyperglycemia further exacerbates liver dysfunction, creating a detrimental cycle; these factors are interconnected, as obesity can fuel both oxidative stress and liver damage, thus compounding hyperglycemia, necessitating a multifaceted management approach (Sapra *et al*., 2021; Shao *et al*., 2005).

Diabetes leads to a disproportionate increase in free radical formation, driven by the activation of the polyol, AGE, PKC, and hexosamine pathways (Sayal, 2024). Increased levels of reactive oxygen species (ROS) can induce damage in protein, lipid, and DNA and promote inflammation by stimulating inflammatory factor secretion from activated inflammatory cells (Arif *et al.*, 2024). Because of the relatively low expression of antioxidant enzymes like catalase (CAT) and superoxide dismutase (SOD) in diabetes, β-cells of the pancreas and other tissues are vulnerable to attack by reactive oxygen species (ROS) when the system is under oxidative stress.

Reactive oxygen species (ROS) at elevated ranges overwhelm the body's natural antioxidant defenses, leading to oxidative stress, which damages cellular components like lipids, proteins, and DNA; such damage facilitates diabetic consequences like retinopathy, neuropathy, and nephropathy (Forrester *et al.*, 2018). Therefore, managing hyperglycemia and mitigating oxidative stress are crucial strategies for preventing diabetes-related complications, highlighting the need for comprehensive diabetes care beyond glycemic control (Bhatti *et al.*, 2022). Catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD), and glutathione peroxidase are examples of enzymatic antioxidants that play a critical role in protecting against ROS by converting free radicals into non-toxic products, thus maintaining cellular redox balance (Zandi and Schnug, 2022).

Flavonoids from medicinal and herbal plants i.e., quercetin, kaempferol, hesperidin, and naringenin, exhibited anti-inflammatory, antioxidant, and antiseptic effects with limited toxicity (El-Kassas *et al.*, 2022; Selim *et al.*, 2022; Al-Quwaie *et al.*, 2023; Mueed *et al.*, 2023). Their high redox potential allows them to effectively scavenge free radicals (Mucha *et al*., 2021; Martemucci *et al.*, 2022).

The antidiabetic mechanisms of herbal plants include enhancing insulin secretion by stimulating pancreatic beta cells, as seen with *Gymnema sylvestre*, and improving insulin sensitivity through herbs like *Berberis aristata* (berberine) and *Momordica charantia* (bitter melon) (Alam *et al.*, 2022; Cortez-Navarrete *et al.*, 2023). They can also inhibit carbohydrate absorption by blocking enzymes such as alpha-glucosidase and alpha-amylase, as demonstrated by *Salacia oblonga* and *Eugenia jambolana* (jamun),

and enhance glucose uptake in peripheral tissues, as observed with *Trigonella foenum-graecum* (fenugreek) (Ghanadian and Taslimi, 2024).

The lotus plant (*Nelumbo nucifera*) has a long history of use, dating back over 2,000 years, as a medicinal herb, a functional food, and a vegetable. These benefits include potential anti-amnesic, antitumor, antioxidant, anti-inflammatory, and hepatoprotective effects (Alagawany *et al.*, 2021; Reda *et al.*, 2021).

Lotus leaves are traditionally used in China to aid in weight management. Such as most herbals, the protective effects of the lotus plant are believed to stem from its bioactive compounds, such as alkaloids and flavonoids (El-Hack *et al.*, 2021; El-Hack *et al.*, 2022a; Salem *et al.*, 2022; El-Saadony *et al.*, 2023b). Lotus seeds are nutritionally rich, containing approximately 25% protein, 65% carbohydrates, and essential minerals like calcium, potassium, sodium, copper, phosphorus, magnesium, manganese, selenium, zinc, and iron.

Lotusine, a prominent flavonoid in various vegetables, fruits, and food products, is recognized for its significant antioxidant capacity. It has been demonstrated to stop cell death and shield DNA from oxidative damage (Ryu *et al.*, 2022; Xu *et al.*, 2022). Previous literature has indicated that extracts from lotus, including procyanidin, leaf, and rhizome extracts, exhibit antidiabetic activity (Zeng *et al.*, 2017; Xiang *et al.*, 2022; De Silva *et al.*, 2023). Khader *et al.* (2020) found that different parts of louts (*Nelumbo nucifera*) had positive effects on diabetic rats; however, there are no studies on the impact of louts on oxidatively stressed hepatic cells from diabetic rats. Therefore, in this study, Lotusine's potent antioxidant properties and the role of oxidative stress in hyperglycemia-induced cellular injury and developing new therapeutic agents require more effort as pharmacotherapeutic options for liver diseases are limited. Therefore, the present study aims to investigate the impacts of Lotusine on hyperglycemia-induced oxidative stress in hepatic cells and diabetic rats

## MATERIALS AND METHODS

**Chemicals:** Penicillin, fetal bovine serum (FBS), streptomycin, and Roswell Park Memorial Institute Medium (RPMI) were obtained from Gibco-BRL (Paisley, UK). Sigma Chemical Co. was the supplier of lotusine and D-glucose (St. Louis, MO, USA). The supplier of the BSA total protein assay kit was Bio-Rad, located in Hercules, California, USA. The source of the trypsin was BDH in England. Fluka (England) supplied potassium chloride and potassium dihydrogen orthophosphate. Sigma Chemical Co. (Poole, Dorset, UK) provided the tertbutyl hydroperoxide (t-BuOOH), glutathione reductase (GR), dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), and bovine serum albumin (BSA).

**Experimental design:** Proper amounts of Lotusine Hydrochloride reference substances were weighed and dissolved in methanol to prepare mixed standard solutions with 1.0 mg/mL concentrations, respectively. The appropriate amount of stock solution was taken and diluted with 0.1% formic acid water + methanol solution (80 + 20) into a series of mixed working solutions with the concentration of 25-50 μg/mL (Lotusine).

Two hundred (n=200) male Wistar (Sprague Dawley) rats weighing 160 ± 20g were employed for this study. The rats were housed under standard laboratory conditions. The laboratory animal facility was maintained under a 12 h light/dark cycle at 23 °C and relative humidity of 20–50%. All the experiment procedures were carried out according to internationally accepted guidelines for using and caring for experimental laboratory animals (ethical code: SCBR-429/2025, Prince Sattam bin Abdulaziz University, Saudi Arabia). All rats were initially fed a standard chow diet during the two weeks of acclimatization and food and water *ad libitum* (Moraal *et al.*, 2012).

Diabetes induction was carried out by a single intraperitoneal injection of streptozotocin (STZ, Sigma-Aldrich, USA) prepared at 50 mg/kg bw. Streptozotocin was prepared in 0.1 M citrate buffer at pH 4.5 and administered to rats fasted overnight, according to Richa *et al.* (2024), with slight procedure modifications to minimize decomposition of the drug, it needs to be prepared in ice-cold citrate buffer and maintained on ice. 5% glucose solution was provided for the rats after six hours of STZ administration for the next 12 hours *post-STZ* injection. The hyperglycemic state was confirmed after 3 days of STZ induction; the rats were marked with hyperglycemia when fasting blood glucose (FBG) ≥ 250 mg/dL and postprandial glucose (PPG) ≥ 350 mg/dL.

200 rats were divided into four groups of ten in, each with five replicates. Group 1, control rats were given normal saline supplements, besides a basal diet, for four weeks. Group 2 consisted of diabetic rats that received the STZ 50 mg/kg bw for 4 weeks and served as diabetic control (diabetic untreated). Group 3 consisted of diabetic rats who received glibenclamide at a dose of 50 mg/kg bw of LT, fed the NC diet for four weeks, and were labeled the glibenclamide-treated diabetic group. Group 4 consisted of diabetic rats that received LT 50 mg/kg.

**Blood sample collection and serum preparation:** At the end of the experiment, the rats were sacrificed by cervical dislocation. The blood samples were collected by cardiac puncture using a sterilized needle and syringe into specimen bottles, allowed to clot, and centrifuged at 3,000 × g for 10 minutes. The serum was decanted, stored at -80°C, and then used for oxidative stress biomarkers assay.

**Tissue homogenate preparation:** The sacrificed rats were dissected to remove the liver and pancreas. A portion of the liver and pancreas was washed in ice-cold sucrose (250 mM) and then homogenized in PB (50 mM, pH 7.4) to obtain a final 10% w/v homogenates solution. The centrifugation of homogenates was at 10,000 × g for 30 min at 4°C, and the supernatants were employed for the assay of oxidative stress biomarkers as well as total protein concentration.

**Cell Culture and Treatment:** The RPMI 1640 medium was supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin to cultivate the HepG2 cell line from diabetic rats. The culture was maintained at 37°C in a humid environment

with 5% $CO_2$. Cells were grown to 70% confluence for the experiments (Alshememry *et al.*, 2022). The cells were separated into five different categories. (1) media containing 5.5 mM D-glucose, (2) media containing 25 mM D-glucose, (3) media containing 25 µM lotusine, (4) media containing 25 mM D-glucose + 25 µM lotusine, and (5) media containing 25 mM D-glucose + 50 µM lotusine. The cells were cultured at 37°C, and each treatment was carried out in triplicates with 5% $CO_2$. After treatments for 48 and 72 hours, cell lysates were gathered and kept cold (-20°C) until needed.

**Cell Viability Assay:** Using the MTT test, cell viability was evaluated following (Alshememry et al., 2022). In 96-well plates, HepG2 cells (12×10^3 cells/well) were incubated for 48 and 72 hours at 37°C with 25 µM and 50 µM D-glucose and/or 25 µM lotusine. Following treatment, for 3.5 hours, cells were incubated in a serum-free media containing 0.5 mg/ml of MTT dissolved in it. After dissolving formazan crystals in 100 µl DMSO, an ELISA reader (Bio-Rad, USA) was set up to measure the absorbance at wavelength 570/650 nm. The ratio of treated cells' absorbance to that of untreated cells (control) was used to determine the vitality of the cells.

**Lipid Peroxidation Inhibitory Activity:** Lipid peroxidation was measured by determining malondialdehyde (MDA) levels (Ohkawa *et al.*, 1979). HepG2 cell lysates (100 µl) treated with D-glucose and/or lotusine were mixed with 0.25 mol/L HCl, 15% trichloroacetic acid, and 400 µl TBA reagent containing 0.375% TBA. After 30 minutes of heating at 95°C and a quick cooling period, the mixture was centrifuged for 15 minutes at 4°C at 8000 ×g. The supernatant's pink absorbance was measured at 532 nm.

**Assays for Antioxidant Enzymes:** Using the Nitro Blue Tetrazolium (NBT) technique, SOD activity was recorded. Glutathione peroxidase (GPx) and catalase activities were assessed as previously described (Carrillo *et al.*, 1991). A total protein test assay (Bio-Rad BSA) was performed to test protein levels following the manufacturer's instructions (Ernst and Zor, 2010).

The GSH concentration was measured in the serum and supernatants of liver and pancreatic tissue homogenates by Hu *et al.*, (2024).The thiol reagent, 5-5'-dithiobis [2-nitrobenzoic acid] (DTNB, Ellman's Reagent) reacts with free sulphydryl groups like glutathione to form 5-thionitrobenzoic acid (TNB) and GS-TNB complex. The relatively stable yellow complex formed can be measured at 412 nm and the concentration of reduced glutathione in the sample is proportional to the colour intensity. The concentration of reduced glutathione is measured in milligram/100 gram (mg/100g) wet tissue or in serum as mg/dl.

**Vitamins**

**Determination of Vitamin C (ascorbic acid) content:** Vitamin C estimation in serum and supernatants of liver and pancreatic tissue homogenates was carried out using the procedure of Ghosh *et al.* (2024). The assay procedure is based on the colorimetric measurement, which involves precipitation with tricarboxylic acid and its estimation in 2, 4 dinitrophenyl hydrazine: thiourea: copper sulfate for color development. The absorbance of the solution was read at a wavelength of 520 nm, and pure ascorbic acid was used to prepare the standard curve. The value of the ascorbic acid was measured in mg/100g wet tissue and serum as mg/dl.

**Vitamin E (α-Tocopherol) content:** Serum Vitamin E content and that of liver and pancreatic tissue supernatant homogenates were determined following Mecheri *et al.* (2024). This colorimetric method quantifies the analyte through saponification, extraction with ethanol ascorbate and KOH, and subsequent estimation. The absorbance was read at 536 nm. The value of Vitamin E was measured in mg/100 gram (mg/100g) wet tissue or in serum as mg/ dl.

**Protein Assay:** Total protein concentration was determined in the liver and pancreatic tissue Supernatants using Lowry et al.'s colorimetric procedure. The principle of the method is based on the peptide nitrogens (s) reactivity with copper (II) ions in an alkaline medium and Folin-Ciocalteau phosphomolybdic phosphotungstic acid and subsequent reduction to give hetero-polymolybdenum blue by oxidation of aromatic acids. Lowry assay maintained a pH of 10-10.5 because of its sensitivity to pH change. The absorbance of the solution was read at 550nm wavelength with bovine serum albumin (BSA) as standard.

**Diabetic enzymes**

**Alpha-Amylase Inhibition (AAI) Assay:** AAI assay was performed as per Sagbo *et al.* (2018). The reaction mixture containing 5 µl of porcine pancreatic enzyme solution and 15 µl of LT diluted in phosphate buffer (1.56-200 µg/ml) at room temperature and incubated for 10 minutes. A starch solution (20 µl) was added, and the mixture was then incubated for 30 minutes at 37°C. After adding 10 µl of 1 M HCl and 75 µl of iodine reagent to stop the reaction, at 540 nm, absorbance was measured. As positive and negative controls, there was acarbose and PBS. The following formula was used to get the %inhibition:

$$Inhibition(\%) = [Abs\,(control) - Abs\,(sample)]/Abs\,(control) * 100$$

**Alpha-Glucosidase Inhibition Assay:** An altered version test for alpha-glucosidase inhibition was used (Sancheti *et al.*, 2010). A 96-well plate was used to incubate the reaction mixture, which contained 5 µl of LT (1.56–200 µg/ml) and 20 µl of alpha-glucosidase solution (50 µg/ml). The mixture was incubated at 37°C for 20 min following adding 10 µl of p-nitrophenyl-α-D-glucopyranoside (PNP-GLUC, 10 mM) and 60 µl of 67 mM $KH_2PO_4$ buffer (pH 6.8). At 405 nm, absorbance was measured when the reaction was halted with 25 µl of 100 mM sodium carbonate ($Na_2CO_3$). As a positive control, epigallocatechin gallate was utilized. The previously mentioned method was used to calculate the % inhibition.

**Growth performance of diabetic rats:** Rats were weighed individually at the beginning and end of the study, and feed intake per cage was recorded daily. Body

weight gain, feed conversion ratio and survival rate were calculated for each animal. Upon completion of the treatment periods, the mice were euthanized by cervical dislocation, and the heart, brain, liver, kidneys, lungs, and spleen were dissected. After removing any excess fat, the relative weight of each organ was determined as a percentage of total body weight (Zhou *et al.*, 2024).

**Real-Time PCR:** Total RNA was extracted using TRIzol reagent (Thermofisher Scientific, USA), The isolated RNA was subsequently used for quantitative real-time PCR (qRT-PCR) analysis. All qRT-PCR reactions were performed using $RT^2$ SYBR® Green mix on a real-time PCR system. The PCR amplification protocol commenced with an initial denaturation step at 95°C for 4 minutes, followed by 35 cycles consisting of denaturation at 95°C for 90 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 90 seconds. The program concluded with a final extension step at 72°C for 10 min. Using NCBI Primer Design, IRS-1, Akt-2, GLUT-4 primers, and GAPDH was used as internal control (Table 1). Real-time PCR was conducted using $RT^2$ SYBR® Green PCR master mix (Applied Biosystems, USA), and relative gene expression changes were calculated using the $2^{-\Delta\Delta CT}$ method.

**Molecular Docking Analysis:** The molecular structures of target receptor binding sites for human enzymes were obtained from the RCSB Protein Data Bank (PDB ID 2zj4). The Schrödinger software (Maestro, UAS) package performed docking calculations. Protein-ligand complexes were visualized and analyzed using PyMOL and Discovery Studio.

**Statistical Analysis:** GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA) was used to compare groups. The data is shown as mean ± SD. A one-way ANOVA and Tukey's multiple comparison test were used to establish statistical significance; P-value of less than 0.05 was deemed significant.

## RESULTS

**Impacts of D-glucose and LT on the HepG2 cells' viability:** As described in Figure 1, treatment with 25 mM and 50 mM D-glucose and lotusine (with or without D-glucose) did not significantly affect ($p>0.05$) the viability of HepG2 cells over 48 and 72 hours, maintaining 85-90% cell viability. Each data point is expressed as a percentage compared to the control (100% cell viability).

**Effect of D-glucose and LT on SOD, MDA, CAT, and GPx:** The impact of D-glucose on SOD activity varied with varying concentrations and time intervals with 25 mM D-glucose significantly ($P<0.05$) reduced SOD activity after both 48 and 72 hours, as shown in Figure 2. However, LT treatment combined with 25 mM D-glucose significantly ameliorated this reduction in SOD activity ($P<0.05$).

HepG2 cells, on exposure to 25 mM D-glucose for 48 hours, showed a 2.2-fold decrease in CAT activity compared to control cells. Notably, after 72 hours, no significant change ($p>0.05$) in CAT levels was observed across all groups. LT treatment mitigated the reduction in CAT activity induced by 25 mM D-glucose at 48 and 72 hours intervals. LT treatment for 48 hours significantly ($P<0.05$) increased CAT activity compared to the control ($P<0.05$).

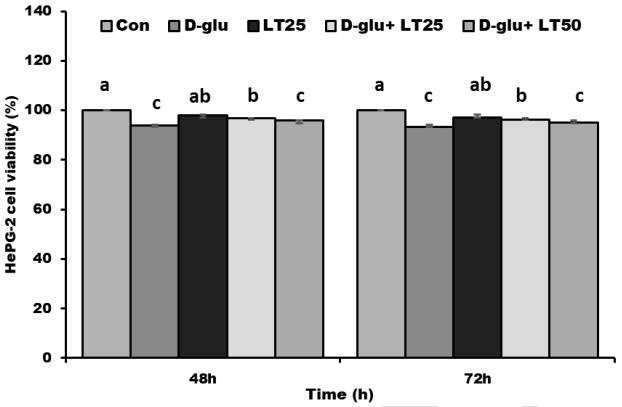


**Fig. 1:** Effects of lotusine (LT) and/or D-glucose on HepG2 cell viability using the MTT test after 48 and 72 hours of treatment. The information is shown as mean ±SD (n=3).

**Table 1:** Primers for defense genes

| Genes | Forwarded (5-3) | Reverse (5-3) |
|---|---|---|
| *IRS-1* | TATCTGCATGGGTGGCAAGG | GGGTAGGCAGGCATCATCTC |
| *Akt-2* | GCTAGGTGACAGCGTACCAC | GGCCTCTCGGTCTTCATCAG |
| *GLUT-4* | TTCCAGCCATGAGCTACGTC | GCAGGAGGACCGCAAATAGA |
| *GAPDH* | CTCTCTGCTCCTCCTGTTCG | ACGACCAAATCCGTTGACTC |

Exposure to 25 mM D-glucose for 48 hours reduced the activity of GPx by 38% as depicted in Figure 2. After 72 hours, GPx activity was reduced by 40% compared to control cells, with no significant changes observed over the extended treatment period. LT treatment combined with 25 mM D-glucose increased GPx activity relative to the control group, with a considerable increase noted at 50 µM after both 48 and 72 hours ($P<0.05$).

HepG2 cells treated with 25 mM D-glucose for 48 hours had a substantial 56% increase in MDA levels, a lipid peroxidation marker, in contrast to the control group ($P<0.05$), as shown in Figure 2. LT dramatically reduced MDA levels in cells exposed to 25 mM D-glucose ($P<0.05$). In HepG2 cells fed with 50 mM D-glucose for 72 hours, MDA levels increased by 41% but decreased GSH by 50 %. However, the LT-supplemented diet-treated rats expressed considerable protection by increasing the GSH levels.

**Vitamin and protein content:** As shown in Table 2, There was a reduction in serum, liver and pancreatic concentrations of vitamin C in G2 compared to G1. However, following treatment with LT supplementation for four weeks, the observed decrease in serum, liver, and pancreatic tissues vitamin C was reversed to near normal values. Also, the diabetic rats (G2) showed significant ($P< 0.05$) reduction in serum, HepG2 cells vitamin E concentrations compared to those in normal control. However, following treatment with LT for four weeks, the observed decrease in serum, liver, and pancreatic tissues vitamin E was reversed to near normal values (Table 2). The protein content in liver cells reached a high level in the LT-treated group (11.6 mg/g) compared to 9.6 mg/g in glibenclamide-treated rats, lower protein content in diabetic rats (1.3 mg/g) with a relative decrease of 300 % compared to control (Table 2).

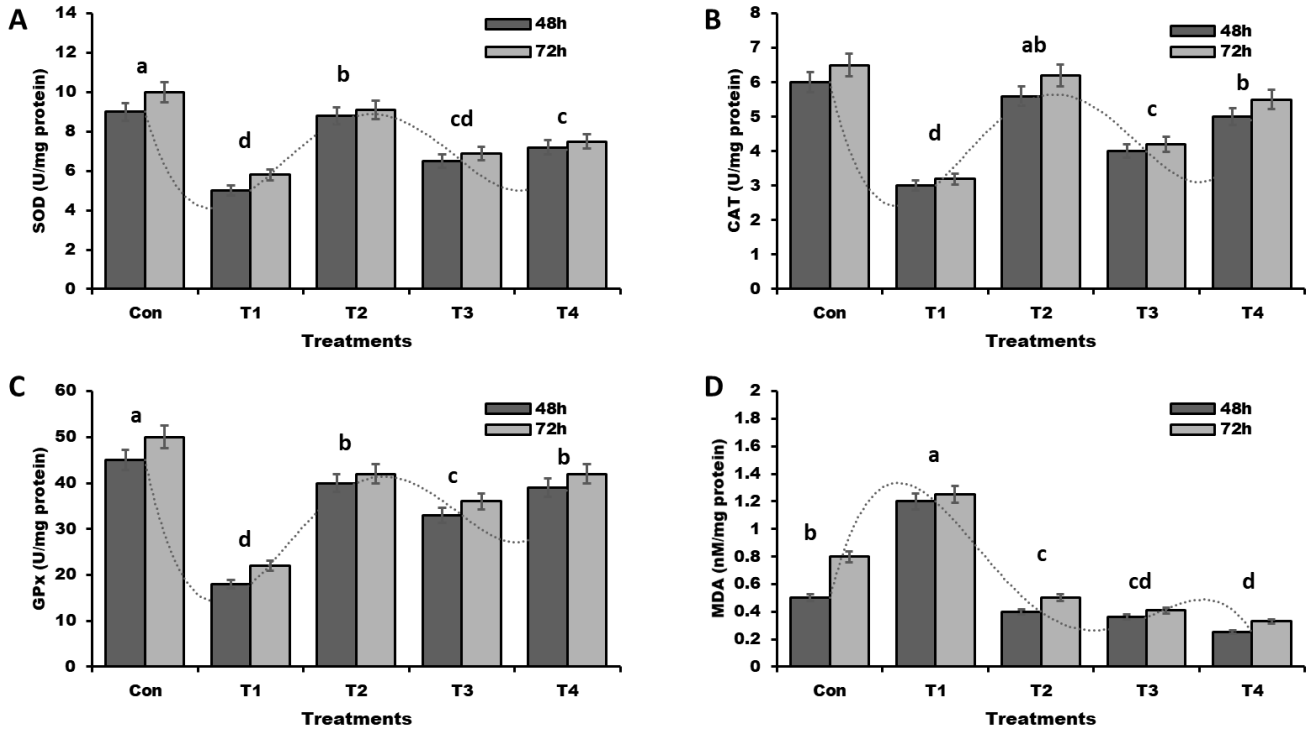


**Fig. 2:** The impact of lotusine on A) SOD, B) MDA, C) CAT and D) GPx. The data is presented as mean ± SD (n=3).

**Alpha-Amylase Inhibition Assay:** LT inhibits alpha-amylase activity dose-dependently (Figure 3). LT (200 µg/ml) exhibited an inhibition rate of 80.36%. The positive control, acarbose, demonstrated a significantly higher (P<0.05) inhibition rate of 90.22%, respectively under similar conditions. The IC50 values for LT and acarbose were 30.60 µg/ml and 11.57 µg/ml, respectively; the IC50 of LT was higher than that of acarbose, indicating that acarbose is more potent at inhibiting alpha-amylase.

**Glucosidase Inhibition Assay:** Figure 4 shows LT inhibits α-glucosidase activity dose-dependently; LT (200 µg/ml) inhibited 82.6% glucosidase activity. The positive control, EGCG, demonstrated higher inhibition rates of 94.7%. The IC50 of LT was higher than that of EGCG (36.15 µg/ml vs. 8.93 µg/ml), indicating that EGCG is more potent at inhibiting alpha-glucosidase (Figure 4).

**Growth performance:** Table 3 shows that STZ-diabetic rats showed the lowest body weight gain (BWG) of 45.3 g, with relative decreases of 22.4 % compared to the control group (P<0.05). The LT-treated rats (G3) recorded the highest BWG; meanwhile, the combined treatment LT+STZ showed the potential of LT in lowering STZ-causing diabetes, enhancing the BWG by 13 % and 45 % compared to control and G2, respectively (P<0.05). There was a slight significant decrease in FCR level values in G1, G3, and G4 groups, compared to the highest value recorded in G2; the latter two groups showed the highest FCR values (P<0.05). Survival rates were the same in all groups, while relative organ weight increased was the highest in G2, which mitigated and decreased in G1, G3 and G4 (P<0.05).

**Table 2:** The effect of LT treatment on the vitamin and protein content of liver cells of diabetic mice (data are mean±SE)

| Treatments | Vitamin C (mg/100g) | Vitamin E (mg/100g) | Total protein (mg/g) |
|---|---|---|---|
| G1 | 230±10.3a | 3600±12.9a | 5.6±0.2c |
| G2 | 100±9.8d | 900±3.8c | 1.3±0.1d |
| G3 | 195±7.7b | 3100±15.8b | 9.5±0.6b |
| G4 | 160±3.6c | 2990±12.6bc | 11.6±0.3a |

Lowercase letters in the same column indicate significant differences at p<0.05. G1, control, G2, STZ-diabitiec rats, G3, LT-recevied rats, and G4 was STZ+LT.

**Table 3:** Effect of dietary LT on growth performance parameters of diabetic rats (data are mean±SE)

| Parameters | G1 | G2 | G3 | G4 |
|---|---|---|---|---|
| LBW (g) | 160±0.0 | 161.2±0.2 | 161.6±0.0 | 160.2±0.0 |
| FBW (g) | 218.1±1.3c | 206±1.5d | 239.4±2.1a | 225.5±1.2b |
| BWG (g) | 58.1±0.5c | 45.3±0.3d | 77.8±0.5a | 65.3±0.8b |
| FCR | 1.55±0.1b | 1.65±0.6a | 1.40±0.3c | 1.45±0.2c |
| SR (%) | 100±0.0 | 100±0.0 | 100±0.0 | 100±0.0 |
| ROW (%) | 2.39±0.1c | 3.6±0.3ab | 2.56±0.3c | 2.49±0.1c |

Values with different letters within a row differ significantly (P<0.05). Live body weight (LBW); Final body weight (FBW); weight gain (BWG), Feed intake (FI), Feed conversion ratio (FCR); survival rate (GR), and relative organ weight (ROW). G1, control, G2, STZ-diabitiec rats, G3, LT-recevied rats, and G4 was STZ+LT.

**mRNA expression in HepG2 cells:** Figure 5 presents the fold change in expression of IRS-1 (A), AKT-2 (B), and Glut-4 (C) proteins across four treatment groups: Control (Con), Metformin (Met), LT25, and LT50, visualized through bar graphs with fitted cubic trendlines ($R^2$=1), demonstrating the effects of these treatments on key proteins involved in insulin signaling and glucose uptake; Metformin consistently and significantly increased the expression of all three proteins, serving as a positive control, while LT50 showed a moderate increase, suggesting a potential therapeutic effect at this higher dose; conversely, LT25 appeared to decrease the expression of all three proteins, indicating a potential adverse effect; the statistical significance of differences

between treatments is implied by the letters (a, b, c, d), and the response curves illustrate a dose-dependent effect of LT, with higher doses correlating with increased protein expression, thus highlighting the influence of these treatments on insulin sensitivity and glucose metabolism.

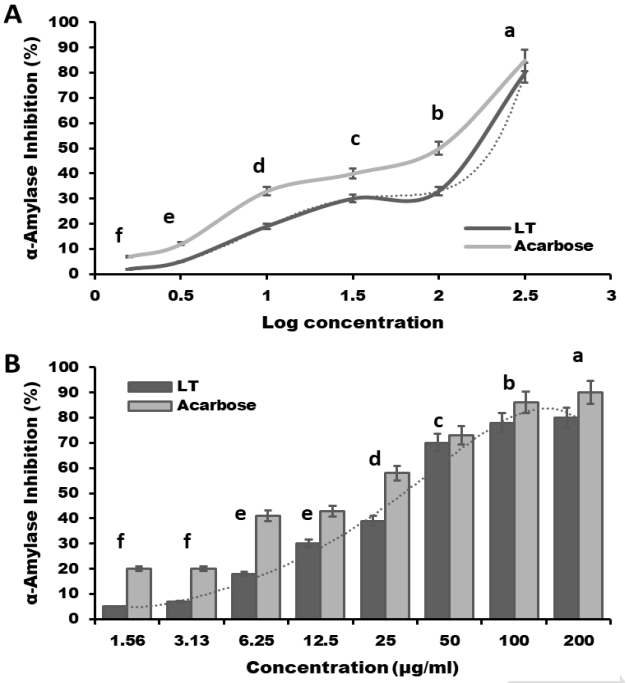


**Fig. 3:** The impact of lotusine on alpha-amylase activity. The data is shown as mean ± SD (n = 3).

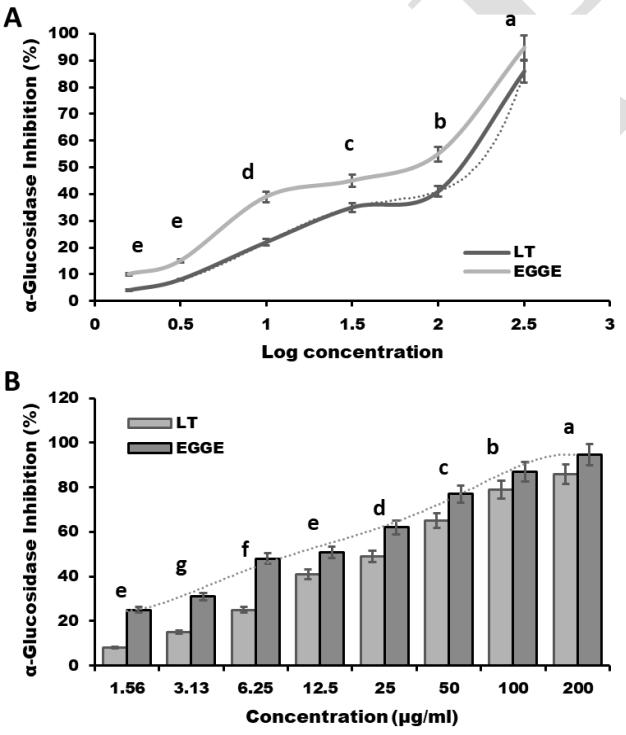


**Fig. 4:** The impact of lotusine on alpha-glucosidease activity. The data is shown as mean ± SD (n = 3).

**In silico Molecular docking analysis**: Table 2 shows the binding affinities of Lotusine to several key proteins involved in diabetes pathophysiology "A: Glucose fructose 6 phosphate amidotransferase (PDB: 2zj4), B: Glycogen Synthase Kinase (PDB: 3f7z), C: aldose reductase (PDB: 3g5e), D: Glucokinase (PDB: 4ixc), E: Hydroxysteroid dehydrogenase (PDB: 4k1l), F: Proliferator-activated receptor gamma (PDB: 3dzy), G: Pyruvate dehydrogenase kinase (PDB: 4mp2)".

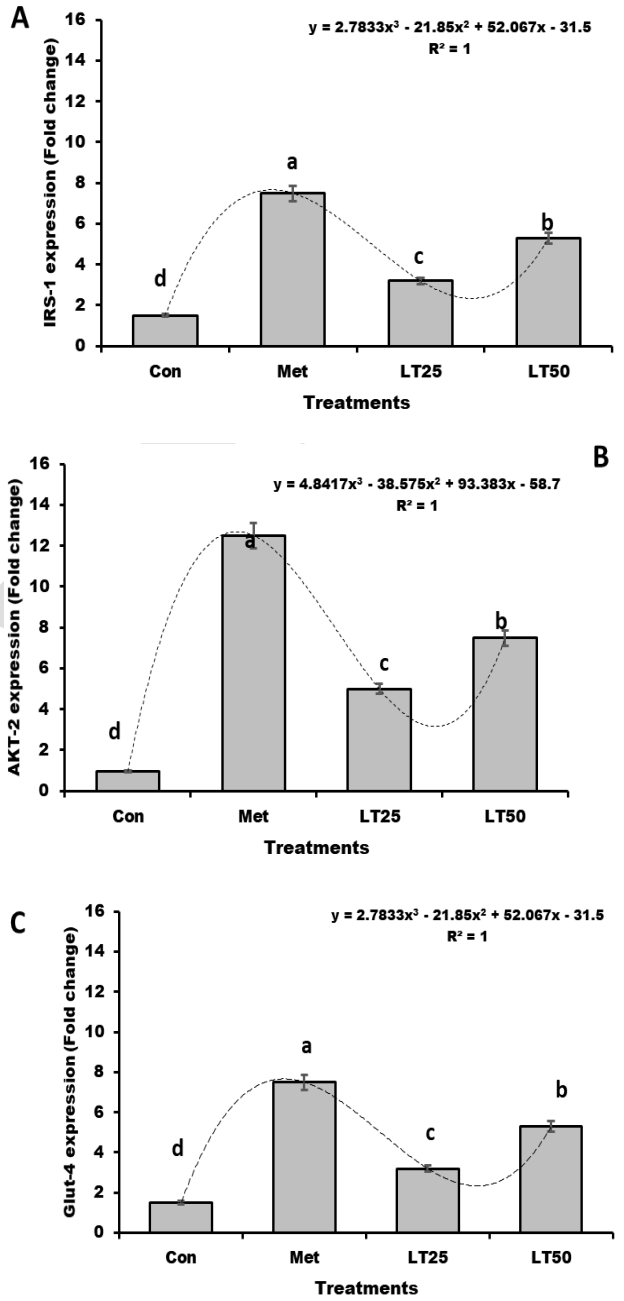


**Fig. 5:** Effect of Lotusine on the mRNA expression of IRS-1 (A), AKT-2 (B), and GLUT-4 (C) in HepG2 cells.

Figure 6 illustrates docking studies of LT with pivotal proteins implicated in diabetes pathophysiology, encompassing glucose-fructose 6 phosphate amidotransferase, glycogen synthase kinase, aldose reductase, glucokinase, hydroxysteroid dehydrogenase, proliferator-activated receptor gamma, and pyruvate dehydrogenase kinase.

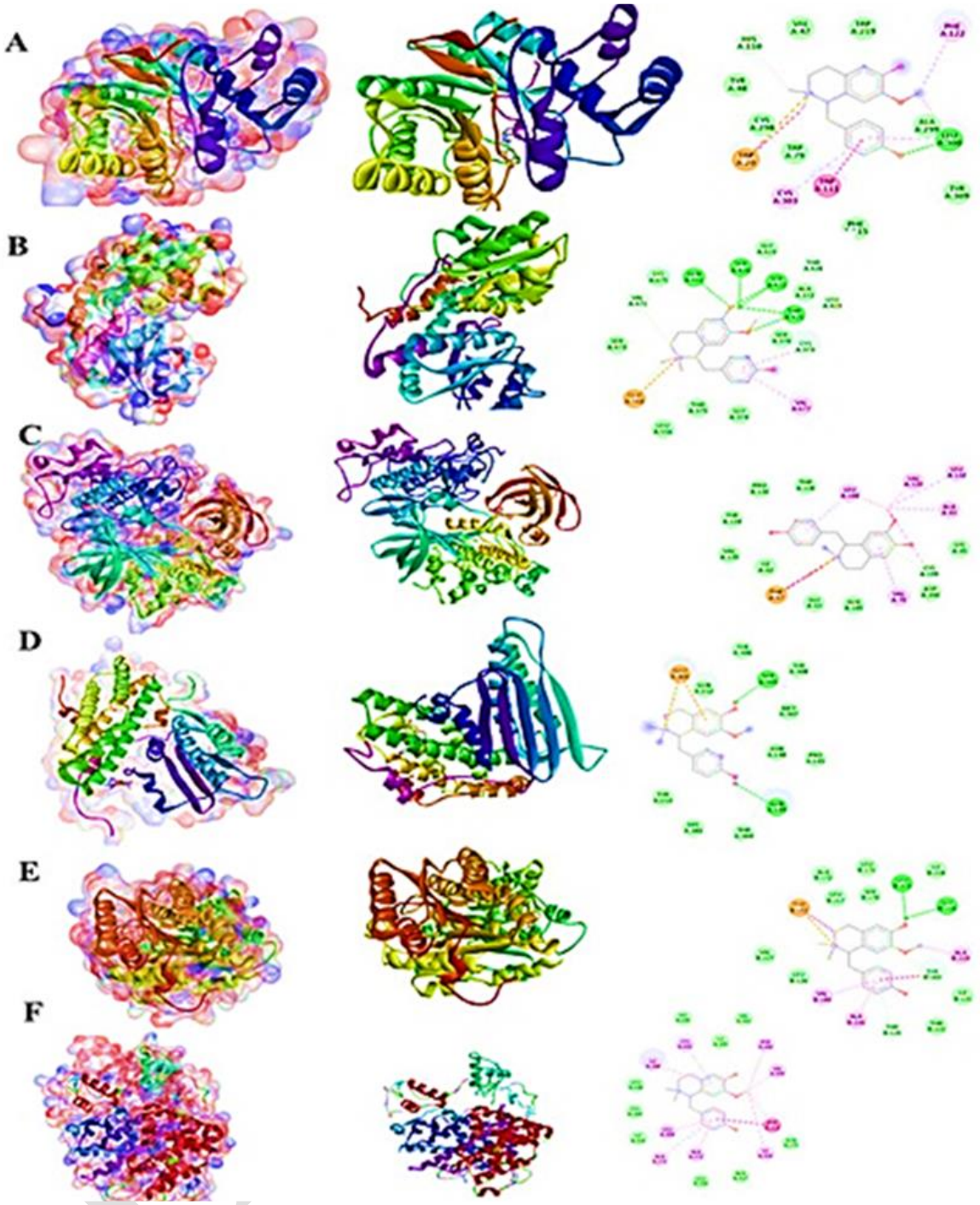


**Fig. 6:** Binding site of Lotusine with "A) Glucosefructose 6 phosphate amidotransferase (PDB:2zj4), B) Glycogen Synthase Kinase (PDB:3f7z), C) aldose reductase (PDB:3g5e), D) Glucokinase (PDB:4ixc), E) Hydroxysteroid dehydrogenase (PDB:4k1l), F) PPAR gamma (PDB:3dzy) and G) Pyruvate dehydrogenase kinase (PDB:4mp2) and ligand interacting residues with different proteins".

## DISCUSSION

Various factors, including a family history of high blood glucose and early onset of diabetes, can influence hyperglycemia (Luo *et al.*, 2024). Lifestyle factors such as a sedentary lifestyle, aging, obesity due to lack of physical activity, an imbalanced diet with a high intake of unhealthy foods, alcohol consumption, and smoking significantly contribute to the rising prevalence of diabetes (Abdulkareem *et al.*, 2024).

Previous studies have demonstrated a decline in antioxidant mechanisms in the livers of diabetic rats (Ziyanok-Demirtas, 2024). In type 2 diabetes (T2DM), insulin resistance, impaired cell function, and poor glucose tolerance lead to the production of reactive oxygen species (ROS), which drive oxidative stress. Naturally occurring antioxidant enzymes, such as catalase (CAT), glutathione (GSH), and nitric oxide (NOx), counteract ROS by degrading them into less harmful substances (Wongmanee *et al.*, 2024). The liver's levels of these antioxidant enzymes are closely linked to the diabetic state. NOx plays a crucial role in carbohydrate metabolism, influencing its availability and contributing to the development of T2DM. It also enhances insulin synthesis, improves blood flow, and lowers blood pressure. Additionally, NOx reduces arterial stiffness and improves blood flow in the carotid artery (Chen *et al.*, 2025).

Oxidative stress affects mitochondrial function by directly impairing oxidative. Phosphorylation. Reactive nitrogen or reactive oxygen species can provoke mitochondrial membrane permeability transition and mitochondrial DNA deletions, activating caspases that lead to cell death. Studies reported that the liver diseases linked with oxidative stress are fatty liver, fibrosis, cirrhosis, chronic hepatitis, and carcinoma, therefore finding natural compounds to mitigate oxidative stress is crucial (El-Saadony *et al.*, 2021; Saad *et al.*, 2021b; Saad *et al.*, 2021d).

The current work sought to evaluate the significance of LT's impact against D-glucose-induced oxidative stress in rat HepG2 cells by emphasizing antioxidant enzyme activity and insulin signalling pathway gene expression. Numerous studies have proved that HepG2 cells are appropriate for analyzing hyperglycemia in vitro (Kheirollahzadeh *et al.*, 2022).

The findings indicate that both Lotusine and D-glucose exhibit no toxic effects on HepG2 cells, as treatment with 25 mM and 50 mM D-glucose, as well as lotusine (with or without D-glucose), did not significantly affect the viability of HepG2 cells over 48 and 72 hours, hence D-glucose at various concentrations over various time points had same impacts on cellular antioxidant enzymes and viability of HepG2 cells, which could be attributed to the possibility that the amount and duration of being exposed to oxidative situations might affect antioxidant enzyme defense capability. Following Majeed *et al.* (2022), who concluded that D-glucose at various concentrations as well as duration points had distinct impacts on cellular antioxidant enzyme sequences, oxidative stress caused by greater concentrations and more time of D-glucose being exposed (50 mM for 72 hr) may outperform an antioxidant enzyme's responses capability in HepG2 cells in comparison to cells exposed to 30 mM D-glucose for 48 hours. This observation aligns with earlier investigations, such as those by, Ryu et al. (2022), reporting the non-toxic nature of LT on HaCaT cells. Moreover, Alangari *et al.*, (2023) highlighted LT's beneficial effects on skin aging post-UV exposure and its potential applications in the food and cosmetic industries.

All enzymes demonstrated a significant increase in their levels in HepG2 cells, a significant 41% increase in MDA levels treated with 50 mM D-glucose for 72 hours, which was dose-dependently and significantly reduced by LT treatment ($P<0.05$). While LT treatment mitigated the reduction in CAT activity induced by 25 mM D-glucose at both 48 and 72-hours intervals ($P<0.05$). When LT treated combined with 25 mM D-glucose increased GPx activity relative to the control group, a significant increase was noted at 50 µM after 48 and 72 hours ($P<0.05$). These results collectively suggest that lotusine possesses potent antioxidant properties, demonstrating its ability to scavenge superoxide radicals, neutralize hydrogen peroxide, detoxify peroxides, and protect against lipid peroxidation. These findings have broad implications given the widespread involvement of oxidative damage in various health issues and diseases. While the antioxidant potential of lotus has been established in previous studies focusing on numerous extracts from different plant parts (Li *et al.*, 2021; Hammi *et al.*, 2022; Yuan *et al.*, 2022), it previously noted that lotusine attenuated oxidative stress induced by the anticancer drug doxorubicin in H9c2 cells (Harishkumar and Selvaraj, 2020). The present study investigated serum, hepatic and pancreatic lipid peroxidation status by measuring malondialdehyde (MDA) levels.

The observed elevation of MDA in diabetics untreated in the blood and tissues studied revealed the degree of cellular lipid peroxidation and is regarded as the biomarker of cellular injury (Senthilkumar *et al.*, 2021). This finding is in line with the reports of Yusuf *et al.* (2020), who presented the increased levels of lipid peroxides in diabetic experimental rats models of liver and pancreatic tissues, respectively. Salazar-García and Corona (2021) suggested that increased tissue MDA is a sign of oxidative stress in degenerative diseases such as diabetes mellitus. Ogunmoyole *et al.* (2021) described MDA as the major end product of peroxidation of lipids and an indicator of damage to the tissue by chain reactions.

According to Singh *et al.*, (2022) oxidative stress secondary products are lipid peroxides unleashed due to the harmful effect of reactive oxygen species (ROS) generated during the peroxidation of lipids in diabetes. In the present studies, food supplementation with LT in diabetic-induced rats notably declined serum, hepatic, and pancreatic lipid peroxide levels. This observation is consistent with Ademosun *et al.* (2023) in which reduction in MDA in tissues of the brain of diabetic rats was reported after treatment with a specific plant fiber, and also Setyawati and Hidayati (2021), who reported a decrease in MDA concentration after treating rat induced by cigarette smoke with orange peel (*Citrus sinensis*) extract. This effect could be traced to LT's free radical scavenging and antioxidant potential. Decreased activities of antioxidant enzymes like Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), and non-enzymatic antioxidants such as reduced glutathione (GSH) concentration have been reported in diabetic experimental animals (Arif *et al.*, 2022). Superoxide dismutase (SOD) and catalase (CAT) are directly involved in the elimination of oxygen species. Superoxide dismutase catalyzes superoxide anion (O2•) conversion to molecular oxygen (O2) and hydrogen peroxide ($H_2O_2$) while CAT completes the detoxification process initiated by SOD with the decomposition of $H_2O_2$ and gives protection to tissues against hydroxyl radicals damage (Yuan *et al.*, 2021). The observed reduced SOD and CAT activities in the diabetic untreated group, agree with the reports of the studies of Asghari *et al.*, (2022) and might probably be due to the elevation of enzymatic antioxidant consumption to compensate for the increased generation of ROS. LT-based food supplement was observed to elevate the activities of SOD and CAT and resulted in reducing oxidative damage. This could be due to a decline in oxidants because of high contents of heterogeneous phytoconstituents such as flavonoids, phenol, and fatty acid antioxidant esters in LT (Martins *et al.*, 2023).

The potential of any compound to hinder the superoxide radical's formation, which is a toxic species, can be considered a good marker to quantitate antioxidant activities. Reduced glutathione is regarded as a major non-protein thiol in living cells, which is key in coordinating the body's antioxidant defense process. It is involved in the cell's defense against xenobiotics and other naturally

occurring harmful substances like free radicals (Averill-Bates, 2023). The present studies observed a decrease in GSH level of diabetes untreated, which is an indication of its raised consumption to neutralize the generated free radicals. Several studies have demonstrated the decline in GSH level in the blood and tissues of experimental diabetic animals (Sarmah and Roy, 2022). Diabetic conditions often lead to decreased glutathione (GSH) levels in both plasma and tissues. This reduction is primarily attributed to the increased consumption of GSH to counteract the elevated oxidative stress associated with diabetes (Ortiz-Avila *et al.*, 2024). This depletion can be attributed basically to the STZ injection into the experimental rats, acting as a diabetes inducer and xenobiotic. The reduction in glutathione concentration could lead to a drastic decrease in the antioxidant status of the rats because reduced glutathione helps recycle antioxidants in the cell, inhibits damage by free radicals, and plays a vital role in detoxifying toxic compounds. The results obtained in the present studies are consistent with what had earlier been reported by Leh and Lee, (2022) on the reduction of antioxidant capacity in plasma of uncontrolled diabetes.

According to Said *et al.* (2021) a high dose of Vitamin A plus Vitamin E supplementation combined with zinc as an adjunct could improve glycemic control, Beta-cell function, and insulin secretion in adult patients with type 2 diabetes mellitus. Mason *et al.* (2021) also reported that Type 1 and Type 2 Diabetes are associated with low Vitamin C status and a high prevalence of hypovitaminosis C in those with Type 2 diabetes mellitus relative to Type 1 diabetes mellitus. Vitamin C, a water-soluble antioxidant that doesn't require enzymes, acts within the aqueous environment to neutralize oxidants and protect against oxidative damage (Vollbracht and Kraft, 2022).

In contrast, vitamin E, another antioxidant, directly interacts with lipid peroxyl radicals, effectively halting the chain reaction of lipid peroxidation. Under normal conditions, propagation of lipid peroxides is suppressed by Vitamin E. Vitamin E with vitamin C inhibit the formation of hydroperoxide, some lipid peroxidation reactions involve metal complexing agents binding transition metals and these inhibit Fenton- and Haber-Weiss-type reactions generating hydroxyl radicals (OH.) from hydrogen peroxide ($H_2O_2$) as well as superoxide anion ($O2^-$) (Packer, 2023; Panda *et al.*, 2023).

The current studies suggest that decreased vitamin C levels in untreated diabetic rats may be linked to its increased consumption in neutralizing reactive oxygen species. A decline in reduced glutathione (GSH) concentration can also contribute to lower vitamin C levels, as GSH plays a crucial role in regenerating vitamin C (Averill-Bates, 2023). Furthermore, the observed reduction in vitamin E concentration of untreated diabetic rats could be linked to the declined vitamin C concentration (Ratnaningtyas *et al.*, 2022). Our results agree with the earlier reports by Shrivastav *et al.* (2023), who showed a significant reduction in endogenous antioxidants vitamins C and E levels in diabetes patients. However, diabetic rats treated with LT supplementation showed significant elevation in liver, serum, and pancreatic Vitamins C and E concentrations. This implies that LT-based food supplements could ameliorate the changed antioxidant status of rats-induced diabetes. This could be traced to the high contents of antioxidant compounds and active phytochemicals such as flavonoids, phenols, tannins, and saponins in LT. Flavonoids possess many pharmacological activities, such as antihyperlipidemic, antidiabetic, and hypoglycemic potentials. The presence of tannins and saponins in medicinal plants can inhibit lipid absorption, consequently ROS that cause the oxidative damage (Żurek *et al.*, 2024).

Currently, numerous antidiabetic medications are implemented for treating or controlling diabetes, and their mechanisms of action are extensively researched. Our finding regarding AAI assay demonstrated that LT concentrations significantly inhibited alpha-amylase activity. The LT (200 µg/ml) exhibited an inhibition rate of 80.36%, in comparison the positive control, acarbose, which demonstrated a significantly higher inhibition rate of 94.7% under similar conditions, indicating that LT effectively inhibits alpha-amylase activity. These results suggest that LT has the potential to control blood sugar levels by slowing down carbohydrate digestion.

No scientific research on lotusin and its effectiveness on amylase was revealed. However, there is research on the impact of other extracts on amylase, as the results noted by Sagbo e*t al.* (2018), who reported that B. elliptica's extracts demonstrate a mild inhibitory effect on alpha-amylase in the study is unlikely to be physiologically appropriate. This implies that B. elliptica's antidiabetic mechanism does not involve inhibiting these enzymes.

Regarding Glucosidase Inhibition Assay findings suggest that LT can slow down the digestion and absorption of carbohydrates. Previous studies have shown that lotus seed extracts act as reversible and uncompetitive inhibitors of α-amylase and α-glucosidase enzymes (Ćorković *et al.*, 2022). Our research corroborates these findings, demonstrating that lotusine (LT), derived from lotus seeds, effectively inhibits these enzymes and retards carbohydrate metabolism.

On the other hand, LT treatment significantly altered mRNA expression levels, with IRS-1 showing a noteworthy increase, comparable to the effect of Metformin, suggesting enhanced insulin sensitivity. AKT-2 and GLUT-4 mRNA levels also exhibited significant changes following LT treatment, indicating potential influences on insulin signaling and glucose transport regulation similar to Metformin. These findings underscore LT's impact on insulin sensitivity markers, though statistical significance was not observed at lower concentrations (LT25). Flavonoids from different plants have shown antidiabetic activities in vivo (Chen *et al.*, 2019; Zheng *et al.*, 2022; Miao *et al.*, 2023). Our finding has similar expression IRS-1, AKT-2 and GLUT-4 mRNA expression.

Docking studies were conducted to investigate the interactions between LT (likely a specific ligand or compound) and key proteins implicated in the pathogenesis of diabetes. These proteins include Glucose-fructose-6-phosphate amidotransferase, Glycogen synthase kinase, aldose reductase, glucokinase, Hydroxysteroid dehydrogenase, Peroxisome proliferator-activated receptor gamma (PPAR-gamma), Pyruvate dehydrogenase kinase (Kaur *et al.*, 2018).

The results indicate that LT has the potential to effectively reduce intracellular glucose levels, which are associated with late-onset diabetic complications like retinopathy, neuropathy, and nephropathy (Rao *et al.*, 2023). Given the adverse side effects and suboptimal pharmacokinetics of synthetic aldose reductase inhibitors, LT represents an interesting feature for more investigation as a safer and more efficient antidiabetic agent following additional experimental validation.

The impact of LT treatment on mRNA levels of AKT-2, IRS-1, and GLUT-4 in HepG2 cells, providing insight into LT's potential role in regulating insulin signaling and glucose homeostasis. LT treatment significantly altered mRNA expression levels, with IRS-1 showing a noteworthy increase, comparable to the effect of Metformin, suggesting enhanced insulin sensitivity. AKT-2 and GLUT-4 mRNA levels also exhibited significant changes following LT treatment, indicating potential influences on insulin signaling and glucose transport regulation similar to Metformin.

The biologically active compounds discovered in LT may be invented into prospective therapeutic compounds against enzymes/proteins that contribute to the emergence and progression of chronic inflammation and for monitoring hyperglycemia.

Identifying therapeutic natural compounds has grown in importance in the drug discovery process. In the current in-vitro anti-inflammatory assays, the LT extracts demonstrated potent membrane stabilization activity. This study additionally provided further proof to establish the anti-inflammatory properties of the four plants, which could be due to the possibility of the substances discovered inhibiting several inflammatory mediators.

Future research should focus on assessing Lotusine efficacy, safety profile, and toxicity in phyto-constituent extracts. These results highlight the need for rigorous clinical trials and preclinical models to investigate LT's role as a nutraceutical intervention bridging dietary components and pharmacological therapies. While these phytochemical findings are promising, further in vivo studies are essential for fully exploring LT's anti-hyperglycemia therapeutic effects through comprehensive research projects.

**Conclusions:** The study concluded that Lotusine exhibits tolerability and hypoglycemic effects. The dose-dependent changes in gene expression observed with LT suggest its potential as an effective antidiabetic agent. Underscores LT's potential as a practical alternative for managing (T2DM), emphasizing the importance of future investigations into its preventive capabilities and effects across different human and animals cell types.

**Competing interests:** The authors declare that they have no competing interests.

**Author contributions:** Nahed S. Alharthi: Conceptualization, Supervision, Project administration, Investigation, Data curation, Writing – review & editing, Investigation, Formal analysis, Methodology, interpretation.

**Ethical approval:** The experimental procedure was done in accordance with the guidelines of the Standing Committee of Bioethics Research (SCBR) at Prince Sattam bin Abdulaziz University, Al-Kharj, Saudi Arabia (Approval no. SCBR-429/2025). The author indicate that all animal experiments comply with the ARRIVE guidelines 2.0 (Animal Research: Reporting of in Vivo Experiments) and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

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