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Quercetin mitigates hepatic injury by inhibiting TGF- β /p-Smad3 signaling pathway activation and enhancing AMPK activity in type 2 diabetic rats

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ABSTRACT

Objective: To investigate the effects of quercetin on inflammatory signaling pathways and hepatic oxidative injury using a streptozotocin-induced liver injury model.

Methods: Four groups of 32 rats were used in this study: three groups were given streptozotocin to induce diabetes, and one group was given a normal control. For treatment groups, each group received either metformin (200 mg/kg body weight) or quercetin (50 mg/kg body weight) for a month. The expression of *SREBP1c* was detected by quantitative RT-PCR and fibrosis-related proteins (TGF- β and p-Smad3) was evaluated by Western blot. Furthermore, MCP and IL-1 β were determined by ELISA.

Results: Quercetin significantly reduced insulin and glucose levels. Besides, it reduced the serum levels of ALT, AST, and ALP, improved lipid profile, lowered the MDA level, increased SOD activity, decreased the rise in MCP-1 and IL-1 β , inhibited the TGF- β /Smad3 signaling pathway, decreased α -SMA and *SREBP1c* expression, and increased AMPK ($P < 0.05$).

Conclusions: Quercetin could significantly mitigate hepatic damage by modulating the expression of pro-inflammatory cytokines and fibrosis markers.

KEYWORDS: Quercetin; Metformin; Streptozotocin; Liver injury; TGF- β /Smad signaling; AMPK; SREBP1c

1. Introduction

Due to its high rates of morbidity and mortality, diabetes mellitus (DM) is a serious concern, particularly in developed nations[1].

Diabetes is linked to increased liver-related death and a higher risk of hepatocellular cancer, in addition to the well-established chronic macrovascular and microvascular consequences[2]. Nonalcoholic fatty liver disease (NAFLD) is more common in people with type 2 DM, according to growing data[3,4]. Lipid accumulation eventually causes liver fibrosis, which is a hallmark of NAFLD. In the context of NAFLD, type 2 DM progresses due to a combination of factors, including increased oxidative stress, inflammation, changes in liver lipid metabolism, insulin resistance, and elevated insulin levels[5]. These factors have the potential to lead to cirrhosis and fibrosis in the long run[6]. Hepatic stellate cells are changed into contractile, proliferative, and fibrogenic cells after liver damage, signifying a

Summary

Question: Dose quercetin modulate hepatic injury in diabetic rats?

Findings: Treatment with quercetin significantly reduced fibrosis-related markers (TGF- β 1 and p-Smad3) and modulated metabolic regulators (AMPK and SREBP1c).

Meaning: These findings indicate that quercetin may protect against liver damage in type 2 diabetes, and could be a therapeutic strategy for diabetic liver complications.

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critical phase in the progression of liver fibrosis[7,8].

Weight loss and lifestyle modifications are given priority as initial interventions in current NAFLD management strategies. Therapies including insulin-sensitizing medications[9], antioxidants[10], and metformin[11] had potential to improve NAFLD outcomes and reduce the risk of hepatic fibrosis. Some treatments use proteins linked to islet neogenesis to promote β -cell regeneration and enhance pancreatic islet function[12]. However, some treatments are often associated with adverse outcomes, despite their potential benefits. Therefore, there is a growing interest in researching traditional herbal therapies for the prevention and treatment of diabetes[13], since they are considered to be safer, less toxic, and more accessible. A range of medicinal plants have become more and more popular for managing diabetes[14,15]. Several fruits and vegetables contain the polyphenolic flavonoid quercetin, which has been demonstrated to have significant health advantages[16], including energy production, mitochondrial biogenesis, electron transport chain efficiency, and mitigating mitochondrial malfunction[17]. Besides, its anti-inflammatory, antioxidant, and anti-angiogenic activities have been shown in earlier studies[18].

In liver fibrosis, the TGF- β /Smad signaling pathway is crucial[8]. This study sought to ascertain whether quercetin could ameliorate streptozotocin (STZ)-induced liver damage in a diabetic rat model and the effect of quercetin on the TGF- β /Smad and AMPK signaling pathways.

2. Materials and methods

2.1. Chemicals and reagents

Quercetin and STZ were from Sigma Aldrich Inc., St. Louis, Missouri, United States.

2.2. Induction of diabetes in rats

After an overnight fast, the rats were given a single injection of 35 mg/kg STZ in citrate buffer (pH 4.4, 0.1 mol/L) to induce type 2 DM after a 12-week high-fat diet protocol. An ACCU-CHEK glucometer was used to measure blood glucose levels 72 hours after injection in order to confirm the presence of diabetes. Rats were deemed diabetic and added to the study when their blood glucose levels exceeded 200 mg/dL. Seven days following the STZ injection, blood glucose levels were reassessed to confirm the persistence of diabetes[19].

2.3. Ethical statement

Every procedure followed the ARRIVE guidelines [PLoS Bio

8(6), e1000412, 2010] and the study was approved by Institutional Animal Care and Use Committee Research Ethics Board, Faculty of Veterinary Medicine, Benha University (BUFVTM 04/01/22).

2.4. Animals and experimental design

Female Wistar albino rats were supplied by Nile Company for Pharmaceuticals weighing 180–200 g and were about 8 weeks old. They were kept in cages, with humidity levels below 55%, temperatures between 25 °C and 28 °C, and a 12-hour light/dark cycle. The rats were fed pellets and given unlimited access to water. For the study, 32 rats were divided into four groups: Group I (Control): Normal healthy rats; Group II (STZ): Rats were fed a high-fat diet for 12 weeks and injected with a single dose of STZ (35 mg/kg) to induce diabetes; Group III (STZ + quercetin): STZ-induced diabetic rats received an oral administration of quercetin (50 mg/kg) for one month, following the method of Ola *et al.*[20]; Group IV (STZ + MET): STZ-induced diabetic rats were administered metformin (200 mg/kg b.w.) orally for one month, as a standard treatment according to Al Za'abi *et al.*[21].

Urethane anesthesia was used to euthanize all of the rats, and heart punctures were performed to obtain blood samples. Sera were separated from the blood in order to measure the activity of the aminotransferase enzyme and lipid levels. The liver tissues were taken out, weighed, and sectioned. While some sections were fixed in 10% formalin for histopathological assessment, others were homogenized in phosphate-buffered saline for biochemical analysis of inflammatory and antioxidant markers.

2.5. Biochemical analysis

Serum levels of low-density lipoprotein (LDL), triglycerides (TG), and cholesterol were determined (Biodiagnostic Company in Cairo, Egypt). Superoxide dismutase (SOD) activity and malondialdehyde (MDA) levels were assessed in liver homogenates (Bio-diagnostic in Egypt). Serum activities of alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate transaminase (AST) were measured (Biodiagnostic Company in Cairo). Hepatic levels of monocyte chemotactic protein-1 (MCP-1), total AMP-activated protein kinase (AMPK, catalog No. MBS269938), and rat phosphorylated smad 3 (p-Smad 3, catalog No. MBS1602983) were measured by ELISA assay (My Biosource Inc. in San Diego, California, USA). The serum levels of interleukin-1 β (IL-1 β , catalog No. MBS825017, My Biosource Inc. in San Diego, California, the United States) were determined. Serum glucose levels (BIOMED in Cairo, Egypt) and serum insulin levels were determined by ELISA assay (Merck Millipore in Billerica, MA, USA).

2.6. Detection of gene expression by real-time quantitative polymerase chain reaction (PCR)

TRIzol reagent (Life Technologies, USA) was used for total RNA extraction from 30 mg of liver tissue. The integrity of the RNA was confirmed through 1% agarose gel electrophoresis with ethidium bromide staining. First-strand cDNA was synthesized using reverse transcriptase (Invitrogen) with 1 µg of total RNA as the template. RT-PCR was conducted using the Sequence Detection Program (PE Biosystems, CA) in Applied Biosystems (USA). The PCR conditions included an initial step at 95 °C for 5 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 20 s. Curve analysis was performed at the end of the reaction. Results were normalized using β -actin gene as the housekeeping gene, and the relative expression was determined using the comparative Ct method by Livak and Schmittgen[22]. The primer sequences are listed in Supplementary Table 1.

2.7. Determination of the transforming growth factor beta (TGF- β) and α -smooth muscle actin (α -SMA) by Western blot

2.7.1. Protein extraction

The tissue samples were incubated on ice or in a refrigerator at 4 °C for 5 min after being mixed with a suitable volume of 0-5 mL RIPA lysis buffer. To ensure complete lysis of the cells, the resultant cell suspension was moved to a microfuge tube and vigorously mixed for 30 s. To improve extraction efficiency, this mixture was then gently shaken every ten minutes while incubated on ice for an additional half hour. The cell debris was separated from the lysate by centrifugation at 14 000 rpm for 15 min at 4 °C after the incubation. Then the supernatants were kept in aliquots at -80 °C.

2.7.2. Sample preparation

Extracted protein samples and a molecular weight marker were thawed before the experiment started. A total of 20 µg sample was mixed with an equivalent volume of sample buffer. Five minutes after denaturation at 100 °C, the mixture was centrifuged to eliminate condensation. The membrane was re-probed with a 1:1 000 dilution of β -actin antibody to confirm equal loading of protein samples. The membrane was gently cleaned with deionized water for immunological detection, and then it was blocked for 1 to 2 h at room temperature using 3% BSA in TBST buffer. After that, the membrane was incubated for 2 h at room temperature with the particular primary mouse monoclonal antibody on a slowly rotating platform. After that, 1×TBS-T buffer was used to wash the membrane three times, once for 15 min and twice for 10 min. Horseradish peroxidase-conjugated goat anti-rabbit IgG was then

diluted to 1:5 000 in a blocking solution and incubated for 1 h at room temperature. This was followed by three more washes with 1 ×TBS-T. The membranes were thoroughly cleaned three times for 5 min each in TBS containing 0%-1% Tween-20 after being incubated with the antibodies. After that, they were put on a fresh, flat piece of plastic wrap and covered entirely with TMB solution. The TMB solution was carefully incubated on the membranes for 15 min at room temperature.

2.7.3. Protein concentration determination

The Folin-Lowry method was used to determine the total protein content with BSA as the standard reference. After carefully pipetting 50 µL of the protein solution into a microfuge tube, 500 µL of the working solution which was made in a 100 A:1B:1C ratio was added. To ensure a proper reaction, this mixture was incubated for 10 min at room temperature. The mixture was then shaken right away and incubated in the dark for 30 min after the addition of 50 µL of freshly made Folin Ciocalteu's reagent (diluted from 2 mol/L to 1 mol/L with an equal volume of dH₂O). The protein concentration (mg/dL) can be precisely calculated using a standard curve produced by the ECO Spectro (clinical chemistry analyzer) by measuring the optical density of the sample at 660 nm against a blank.

2.8. Histopathological examination of liver tissues

Liver specimens were first fixed in 10% formal saline, then trimmed, cleaned, and dehydrated in increasing alcohol concentrations for histopathological analysis. Following xylene treatment, the dehydrated samples were embedded in paraffin blocks and cut into slices in 4-6 µm thickness. To be examined under an electric light microscope, these sections were deparaffinized with xylene and stained with Masson's Trichrome stain and hematoxylin and eosin (H & E)[23]. A light microscope with a digital camera (Olympus XC30, Tokyo, Japan) was used. Based on the method by Plaa and Charbonneau[24], a grading scale was used to evaluate the frequency and severity of liver lesions as follows: grade 0 for no obvious injury, grade 1 for hepatocyte swelling, grade 2 for hepatocyte ballooning, grade 3 for lipid droplet accumulation in hepatocytes, and grade 4 for hepatocyte necrosis. A numerical grading system was used to determine the degree of liver fibrosis: 0 denoted no fibrosis, 1 denoted mild fibrosis limited to the central liver lobule, 2 denoted moderate fibrosis with spread central fibrotic areas, and 3 denoted severe fibrosis[25].

2.9. Statistical analysis

SPSS version 20.0 was used to examine the data. One-way ANOVA test and *post hoc* test were used for multiple comparisons. A *P*-value

< 0.05 was used to determine whether significant differences were significant. All data are shown as the mean with standard error.

3. Results

3.1. Effect on glucose and insulin levels of diabetic rats

Compared with normal control rats, the STZ group showed elevated insulin and fasting blood glucose levels (Figure 1). Metformin or quercetin significantly reduced insulin and glucose levels ($P < 0.05$).

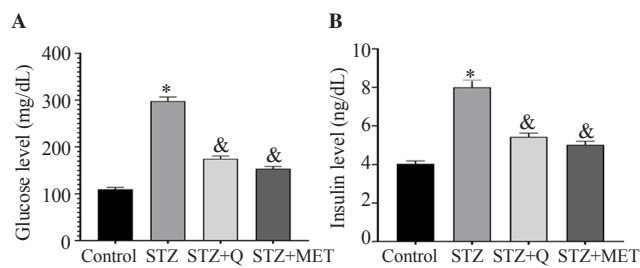


Figure 1. Effect of quercetin (Q) and metformin (MET) on the levels of glucose (A) and insulin (B) in diabetic rats. Data are presented as mean $\pm$ SEM ($n=6$). * $P < 0.05$ versus the control group, & $P < 0.05$ versus the streptozotocin (STZ) group.

3.2. Effect on liver function of diabetic rats

As illustrated in Figure 2, serum ALT, AST, and ALP activities of the STZ group were significantly higher than those of the normal control group ($P < 0.05$). In contrast to the STZ group, quercetin or metformin significantly reduced the serum levels of these liver function markers ($P < 0.05$).

3.3. Effect on lipid profile of diabetic rats

As shown in Figure 3, rats with STZ injections had significantly higher levels of serum cholesterol, TG, and LDL than the normal group. Quercetin or metformin could significantly improve lipid profile compared to the STZ group ($P < 0.05$).

3.4. Effect on oxidative stress in diabetic rats

According to Figure 4, rats with STZ injections had a significantly higher liver MDA level and lower SOD level than control rats. Quercetin or metformin treatment could significantly lower the MDA level and increase SOD activity compared to the STZ group ($P < 0.05$).

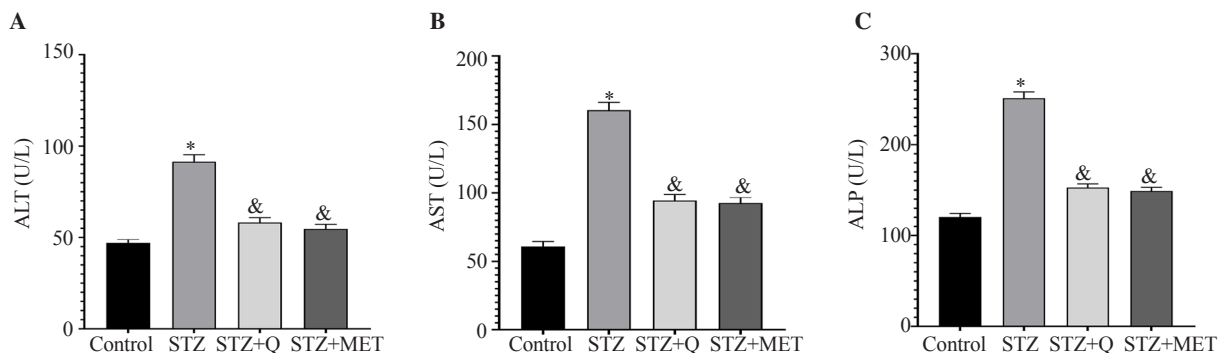


Figure 2. Effect of quercetin on serum levels of alanine transaminase (ALT) (A), aspartate transaminase (AST) (B), and alkaline phosphatase (ALP) (C) of diabetic rats. Data are presented as mean $\pm$ SEM ($n=6$). * $P < 0.05$ versus the control group, & $P < 0.05$ versus the STZ group.

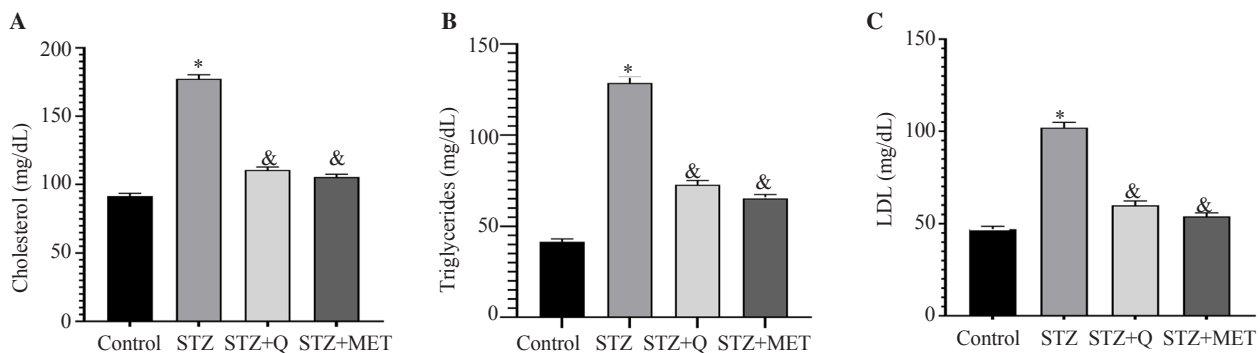


Figure 3. Effect of quercetin on the levels of cholesterol (A), triglycerides (B), and low-density lipoprotein (LDL) (C) in diabetic rats. Data are presented as mean $\pm$ SEM ($n=6$). * $P < 0.05$ versus the control group, & $P < 0.05$ versus the STZ group.

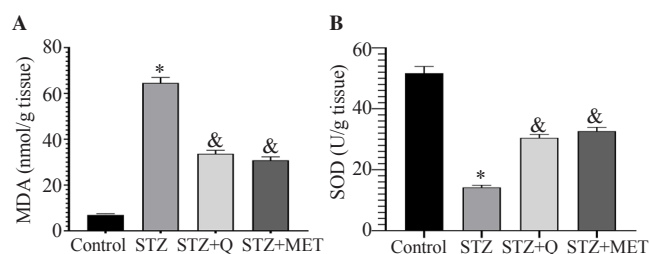


Figure 4. Effect of quercetin on the MDA (A) and SOD (B) level in liver tissue of diabetic rats. Data are presented as mean±SEM ($n=6$). * $P<0.05$ versus the control group, & $P<0.05$ versus the STZ group.

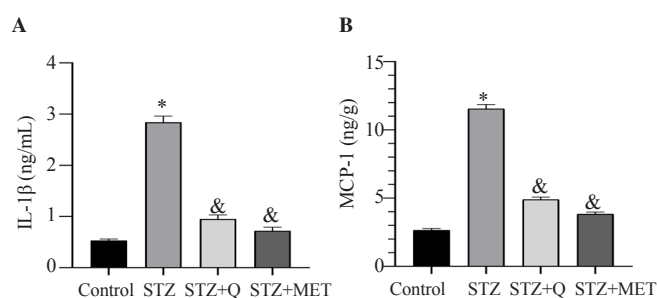


Figure 5. Effect of quercetin on IL-1β level (A) in serum and monocyte chemotactic protein (MCP-1) (B) in liver tissue of diabetic rats. Data are presented as mean±SEM ($n=6$). * $P<0.05$ versus the control group, & $P<0.05$ versus the STZ group.

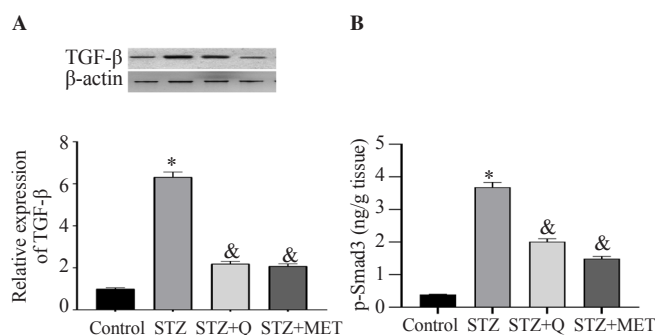


Figure 6. Inhibition of quercetin on TGF-β (A) induced p-Smad3 (B) signaling pathway activation in liver tissue of diabetic rats. Data are presented as mean±SEM ($n=6$). * $P<0.05$ versus the control group, & $P<0.05$ versus the STZ group.

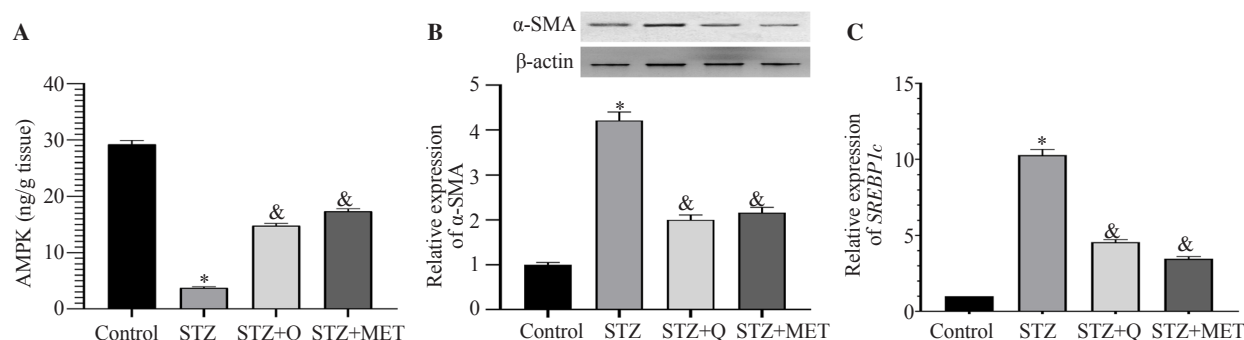


Figure 7. Effect of quercetin on AMP-activated protein kinase (AMPK) (A), α-SMA (B), and gene expression of SREBP1c (C) in liver tissue of diabetic rats. Data are presented as mean±SEM ($n=6$). * $P<0.05$ versus the control group, & $P<0.05$ versus the STZ group.

3.5. Effect on inflammation in diabetic rats

As seen in Figure 5, the levels of IL-1β and MCP-1 in diabetic rats were significantly higher than those of the normal control group. In contrast, the administration of metformin or quercetin decreased the rise in MCP-1 and IL-1β in diabetic rats.

3.6. Effect on TGF-β-induced Smad3 signaling pathway activation in diabetic rats

The TGF-β and p-Smad3 levels in the STZ group were significantly higher than those of the control group (Figure 6). Quercetin or metformin significantly reduced the TGF-β and p-Smad3 levels and inhibited the TGF-β/Smad3 signaling pathway, which was highly active in the liver of diabetics.

3.7. Effect on liver fibrosis regulators

Levels of α-SMA protein and sterol regulatory element-binding protein 1c (SREBP1c) gene expression were higher while AMPK protein was lower in liver tissue of diabetic rats than in the control group (Figure 7). When compared to the STZ group, quercetin or metformin significantly decreased α-SMA and SREBP1c gene expression and increased AMPK ($P<0.05$).

3.8. Histopathological finding

The liver tissue section of the control group showed normal hepatic lobules made up of radiating plates of polygonal cells with prominent round nuclei and eosinophilic cytoplasm vertical to central vein sinusoids were lined by a discontinuous layer of fenestrated endothelial cells with the fine arrangement of Kupffer cells (score 0) (Supplementary Figure 1A). The Masson trichrome-stained histopathological appearance revealed normal hepatocyte morphology and intact portal triad architecture (grade 0) (Supplementary Figure 1B). Animals injected with STZ revealed a massive fatty change of hepatocytes which was characterized by

signet ring cells and appeared as clusters scattering all over hepatic lobules. Apoptosis of hepatocytes was also seen. Disorganization of hepatic cords and hyperplasia of Kuffper cells was also noticed with a score of 4 (Supplementary Figure 1C). Masson trichrome-stained histopathological result showed dilatation of the portal vein and hyperplasia of bile ductules with fibrosis deposition (grade 1) (Supplementary Figure 1D). Animals receiving STZ and quercetin showed swelling of hepatocytes accompanied by granularity of its cytoplasm in hepatic parenchyma. The narrowing of hepatic sinusoids and hyperplasia of Kupffer cells were seen with a score of 1 (Supplementary Figure 1E). Masson trichrome-stained histopathological result showed normal histological structure and delicate fibrous tissues in portal triads (grade 0) (Supplementary Figure 1F). Animals receiving STZ and metformin showed mild swelling of hepatocytes and hyperplasia of Kuffper cells with a score of 1 (Supplementary Figure 1G) and an increase of fibrous tissue proliferation with hyperplasia of the bile duct (grade 1) (Supplementary Figure 1H).

4. Discussion

In a type 2 diabetes rat model, quercetin successfully reduces oxidative stress and inflammation, as well as abnormalities in glucose and lipid metabolism, offering a promising treatment of diabetic liver injury[8]. Through a four-week trial, the results also show a consistent ability of quercetin to lower blood glucose levels, pointing to a possible therapeutic approach for managing diabetes[26]. This study emphasizes how quercetin can reduce liver fibrosis by blocking TGF- β /Smad signaling pathways.

In patients with type 2 diabetes, NAFLD is more prevalent, up to 50% of cases have been reported[26]. The liver plays a critical role in essential metabolic processes like lipogenesis, gluconeogenesis, and cholesterol metabolism. Hepatic steatosis arises when people with type 2 diabetes have issues with the consumption, synthesis, export, and oxidation of free fatty acids[27]. Insulin resistance causes peripheral adipocytes to release free fatty acids, which eventually accumulate in the liver. Our study discovered that quercetin successfully decreased the expression of SREBP1c, which is a crucial mediator between insulin and glucose and linked to glycolysis and lipogenesis[8,28,29].

This study emphasizes the effect of quercetin on abnormalities in lipid metabolism in diabetes rats, which are characterized by high levels of LDL, TG, and cholesterol as well as excessive fat deposition in the liver. These imbalances could lead to oxidative stress, which could result in further structural and functional damage, according to the "two-hit" theory[30]. Quercetin could significantly improve lipid profile.

AMPK is essential as an energy sensor and regulates cellular energy metabolism[31]. This study shows that quercetin significantly

raises AMPK in diabetic rats' liver tissues, suggesting that activating it could enhance liver metabolism and protect against hepatic damage[32]. Additionally, our findings indicate that diabetic rats have raised MDA levels and decreased SOD. Quercetin significantly decreased MDA levels and boosted the activity of oxidative stress-fighting enzymes like SOD, which is similar to the study of Watanabe *et al.* and Oryan *et al.*[33,34].

Hepatocytes with an excess of lipids also experience elevated levels of oxidative stress, which leads to inflammation and the release of pro-inflammatory cytokines like IL-6 and MCP-1[35]. Macrophages infiltrate and more pro-inflammatory cytokines are synthesized as a result of this cascade[36]. According to the current study, there is increased inflammation in the liver of diabetic rats, as evidenced by the significant increases in MCP-1 and IL-1 β , which are decreased by quercetin. Since fibrosis is largely caused by inflammatory cytokines, our research highlights how chronic inflammation in diabetes conditions often occurs before fibrosis. Untreated fibrosis may progress to liver cirrhosis, which may result in life-threatening consequences such as organ failure. One notable antifibrotic effect of quercetin was to reduce the levels of collagen types I and II, which comprise most of the extracellular matrix[37].

Additionally, this study indicated that diabetes was linked to elevated expression of α -SMA, a marker of hepatic stellate cell (HSC) activation. Quercetin could significantly decrease α -SMA. Quercetin may affect HSC activation *via* decreasing α -SMA expression[37].

Furthermore, by secreting TGF- β , which promotes the accumulation of extracellular matrix and cell adhesion molecules, activated HSCs also contribute significantly to liver fibrosis[38]. According to earlier research, TGF- β 1 is crucial for the onset of liver fibrosis[39]. By blocking the TGF- β /Smad signaling pathway, quercetin may reduce liver fibrosis in rats with type 2 diabetes. Besides, in our study, quercetin treatment regulates lipid metabolism in the liver of diabetic rats by downregulating *SREBP1c* gene expression and upregulating AMPK.

To relieve hepatic impairment in diabetes patients, quercetin may be considered as a possible adjuvant[40]. Because of its instability and poor solubility, the use of quercetin in this study is restricted and more study is required to improve its instability and poor solubility.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

Authors' contributions

HFBG, MKM, FSMM, ESAA, and OARAZ contributed to the design and implementation of the research, the analysis of the results, and the writing of the manuscript. All authors discussed the results and commented on the manuscript.

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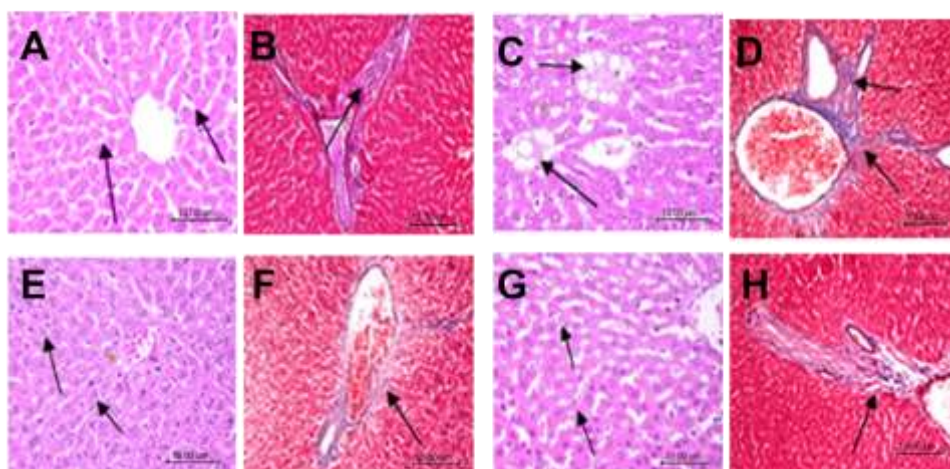
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Supplementary Figure 1. Photomicrograph of the hepatic tissue section. (A) normal hepatic lobules made up of radiating plates of polygonal cells in the normal group (H&E) (arrow); (B) intact portal triads architecture in the normal group (Masson trichrome-stain) (arrow); (C) signet ring cells appearing as cluster scatter in the STZ group (H&E) (arrow); (D) dilatation of portal vein and hyperplasia of bile ductules with fibrosis deposition in the STZ group (Masson trichrome-stain) (arrow); (E) swelling of hepatocytes and narrowing of hepatic sinusoids in the quercetin group (H&E) (arrow); (F) normal histological structure and delicate fibrous tissues in portal triads in the quercetin group (Masson trichrome-stain) (arrow); (G) mild swelling of hepatocytes and hyperplasia of Kuffner cells in the metformin group (H&E) (arrow); (H) mild increase of fibrous tissue proliferation with hyperplasia of bile duct in the metformin group (Masson trichrome-stain) (arrow) ($\times 400$).

Supplementary Table 1. Primer sequences.

Gene	Sequence	Product length (bp)
<i>SREBP1c</i>	Forward: 5'- GACGACGGAGCCATGGATT -3' Reverse: 5'- GGGAAGTCACTGTCTTGGTTGTT -3'	71
<i>β-actin</i>	Forward: 5'- AAGATCCTGACCGAGCGTGG -3' Reverse: 5'- CAGCACTGTGTTGGCATAGAGG -3'	327