



Hepatoprotective Effect of Quercetin against CCl₄Induced Liver Fibrosis: miRNA-124-3p Regulation in Rat Model

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Abstract: Quercetin (QUE) is a natural flavonoid compound found in many herbal drugs. It has broad biopharmacological effects that are beneficial for treating liver diseases such as liver steatosis, liver fibrosis, and liver cancer. It can mitigate liver damage through its powerful antioxidative, anti-inflammatory, and antifibrotic properties. This study aimed to clarify the molecular regulatory effect of QUE on the expression levels of miRNA-124-3p in liver fibrosis induced by Carbon tetrachloride (CCl₄). Twenty-four adult, male albino rats were randomly allocated into 4 groups (n=6); group I (control group), group II (QUE group; 5mg/kg/day, p.o), group III (CCl₄ group; 2mL/kg, twice a week, SC), and group IV (QUE+CCl₄; received the same previous doses). After eight successive weeks, the rats were sacrificed and hepatic enzymes and oxidative stress biomarkers (MDA, SOD, and CAT) were evaluated using colorimetric methods, while IL-6, TNF- α , and TGF- β 1 levels were measured using ELISA. In addition, the miRNA-124-3p expression levels were determined using qRT-PCR. Histopathological examination was performed. The results revealed that group IV (QUE+CCl₄) treated rats exhibited a significant decrease in serum liver enzyme activities, histopathological alterations, hepatic MDA, and inflammatory markers (IL-6 and TNF- α), as well as a significant increase in hepatic SOD and CAT compared to group III (CCl₄). Moreover, the hepatic levels of TGF-β1 were significantly decreased. In addition, there was an upregulation of miRNA-124-3p expression level. Furthermore, an inverse significant correlation was observed between miRNA-124-3p and TGF-β1. These results revealed that QUE exhibits hepatoprotective effects against CCl₄-induced liver fibrosis by regulating miRNA-124-3p/TGF-β1 molecular axis.

Keywords: Hepatoprotective; Liver Fibrosis; Quercetin; miRNA-124-3p; TGF-β1

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1. INTRODUCTION

Liver fibrosis is an abnormal wound-healing process secondary to a progression of chronic liver diseases represented by parasitic and viral infections, autoimmune hepatitis, alcohol abuse, non-alcoholic steatohepatitis, metabolic ailment, and cholestatic liver injury ¹. Hepatic stellate cells (HSCs) are the central fibrogenic cell type implicated in liver fibrosis ². During liver injury, HSCs are differentiated into proliferative myofibroblasts-like cells, resulting in a massive gathering of extracellular matrix (ECM) proteins leading to distortion of the hepatic physiological architecture ³.

Interestingly, transforming growth factor- β (TGF- β) is a crucial fibrotic moderator tangled in liver fibrosis, significantly influencing its pathophysiology ⁴. It acts as a potent activator of HSCs ⁵, triggers expression of genes that link to fibrosis, and subsequently increases the deposition of

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hepatic ECM components ^{6,7}. It also simultaneously inhibits matrix turnover by suppressing matrix metalloproteinases ⁸. Thus, attenuating TGF- β activity is an attractive target for combating liver fibrogenesis.

Importantly, specific treatments for hepatic fibrosis are lacking. Nevertheless, natural compounds are suggested to slow down or reduce the advancement of the condition. In this context, quercetin (QUE), a prominent flavonoid found abundantly in vegetables and fruits, exhibits a variety of potential pharmacological and biological actions ^{9, 10}.

Recently several researchers have confirmed the effective role of microRNAs (miRNAs) in controlling liver fibrosis 11, 12. MicroRNAs are non-coding conserved, small RNAs of approximately 22 nucleotides in length, that function as regulators of gene expression after transcription. In this regard, miRNAs are integral to a wide range of biological processes, and their aberrant profiles are tied to the genesis and advancement of diverse illnesses, including liver fibrosis 13. Among these miRNAs, miRNA-124 has been shown to play varied functions in both normal and pathological processes, highlighting its importance in cellular regulation and disease mechanisms 14-16. As well as it has also been reported that miRNA-124 controls renal and pulmonary fibrosis ^{17, 18}.

In liver fibrosis, inflammation exhibits a principal role in the pathophysiology of the disease ¹⁹. Numerous investigations have emphasized the potential effect of miRNA-124 in mitigating inflammation by modulating the expression pattern of key inflammatory target genes, thus regulating inflammatory responses ²⁰⁻²². Additionally, the recent study by Zhang in 2021 indicated that TGF- β 1 was a target of miRNA-124 which can inhibit the upregulation of hypertrophic scar fibroblasts ²³.

This work was designed to clarify the molecular regulatory effect of QUE on the expression pattern of miRNA-124-3p and to assess its association with the fibrotic marker (TGF- β 1) in liver fibrosis induced by CCl₄.

2. METHODS

2.1. Chemicals and drugs

Carbon tetrachloride (CCl₄), QUE, and dimethyl sulfoxide (DMSO) were sourced from Sigma-Aldrich (Co. St. Louis, MO, USA). CCl₄ was dissolved in corn oil (50% v/v), while QUE was diluted in 10% DMSO (10mg/2ml). CCl₄ dose was (2mL/kg) ²⁴, while the QUE dose was (5mg/kg) ²⁵. All the other compounds were of the highest available commercial quality.

2.2. Ethical approval

The experimental protocol and animal care procedures followed the guidelines established by Al-Azhar University's Research Ethics Committee - Faculty of Pharmacy (Girls) - Egypt (serial approval number: PhD 328/2022).

2.3. Animals

Twenty-four male albino rats, each weighing $(200 \pm 20 \text{ g})$, were obtained from the Nile Company for Pharmaceuticals & Chemical Industries, Cairo, Egypt. Rats had access to both pellets and tap water without restriction. Rats were adapted to the lab environment for two weeks. They were housed in plastic cages (6 rats/cage) in a standard environment of (12-12 h) light-dark cycle, (25 °C temperature), and (60%-70% humidity).

2.4. Experimental design

Twenty-four rats were arbitrarily allocated into 4 groups (n= 6) as follows: I) Control group: subcutaneously (SC) injected with corn oil (2mL/kg, twice a week) and received oral 10% DMSO (0.1/day), 8 weeks, II) QUE group: received oral QUE (5mg/kg/day, 8 weeks), III) CCl₄ group: SC injected with CCl₄ (2mL/kg, twice a week for 8 weeks), IV) CCl₄ + QUE group: SC injected with CCl₄ (2mL/kg, twice a week) and received oral QUE (5mg/kg/day) for 8 weeks.

Afterward, animals received anesthesia using light ether, and blood samples were obtained from rat's retro-orbital sinuses. These samples were then centrifuged to isolate sera. These sera samples were then kept at -80 °C for subsequent biochemical analysis. The rat's livers were removed and subsequently divided into three portions. The first portion was used for biochemical investigations (oxidative stress biomarkers and TGF- β 1), the second portion was used for microRNA analysis, and the third portion was assigned for histopathological evaluation.

2.5. Assessment of hepatotoxicity indices

Colorimetric determination of the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum was assayed with commercial kits (Biodiagnostics Company, Cairo, Egypt) following the manufacturers' instructions ²⁶.

2.6. Assessment of oxidative stress levels

Spectrophotometric determination of hepatic malondialdehyde (MDA), Catalase (CAT), and superoxide dismutase (SOD) levels were evaluated following the protocol articulated by Ohkawa et al.

²⁷, Sinha ²⁸, and Marklund and Marklund ²⁹, respectively.

2.7. Assessment of pro-inflammatory cytokines

Tumor necrosis factor- α (TNF- α) and interleukin 6 (IL-6) levels were assessed following the manufacturers' instructions using a rat enzymelinked immunosorbent (ELISA) kits (Bioassay Technology Laboratory, Shanghai, China, [Cat. # E0135Ra] & [Cat. # E0764Ra], respectively).

2.8. Assessment of fibrotic marker, TGF-β1:

TGF-β1 levels were measured in liver homogenates following the manufacturers' instructions using a rat sandwich ELISA kit (Cat. # MBS824788, MyBioSource, San Diego, California, USA.).

2.9. Assessment of miRNA-124 gene expressions:

Three steps were carried out to quantify miRNA-124-3p gene expressions:

2.9.1. Isolation of total RNA:

Total RNA from hepatic tissue lysate was extracted with miRNeasy Mini Kit (QIAGEN[®], USA, Cat. # 217004). Then, nuclease-free water (Invitrogen, Germany) was utilized for the isolated RNA for further purification. All RNA aliquots' concentrations (ng/mL) and purities were measured using a NanoDrop®1000 spectrophotometer (ND[®]-1000, Thermo Scientific, Wilmington, DE, USA). The RNA samples were conserved at - 80 °C ³⁰.

2.9.2. Reverse transcription and quantification of miRNA-124-3p expressions using qRT-PCR:

The Complementary DNA (cDNA) synthesis and PCR amplification of miRNA-124 was performed in a single reaction tube using the miRCURY LNATM miRNA PCR Starter kit (QIAGEN®, USA, Catalog no.: 339320). The cDNA was produced efficiently from the extracted RNA using miRCURY RT Enzyme Mix containing a unique thermostable reverse transcriptase. The relative expression profiles of miRNA-124 were determined by a miRCURY SYBR® Green master mix (2X), SYBR Green dye, Taq DNA polymerases, and primers in the presence of ROX dye. The miRNA-124 expression was standardized to a small nuclear RNA RNU6 (U6). The amplification curve was determined from 65°C to 95 °C. The miRNA-124 quantification relative to values of U6 was measured using the $2^{-\Delta Ct}$ values, where $\Delta Ct = (CtmiR-124 - CtU6)$. The fold changes of miRNA-124 were computed by using the $2^{-\Delta\Delta Ct}$ method. The primers utilized were defined in (Table 1).

Table 1. The qRT-PCR primer's sequences

Product	Primer's sequence (5'- 3')
miRNA-124-3p	Forward: 5'-GCGGCCGTGTTCACAGCGGACC-3'
(target)	Reverse: 5'-GTGCAGGGTCCGAGGT-3'
RNU6 (U6)	Forward: 5'-CTCGCTTCGGCAGCACA-3'
(reference)	Reverse: 5-AACGCTTCACGAATTTGCGT-3'

2.10. Histopathological examination

Liver specimens were thoroughly rinsed and subsequently fixed in 10% neutral-buffered formalin. Following fixation, the samples were trimmed, dehydrated in graded alcohol solutions, cleared in xylene, and embedded in paraffin wax. Five μ m sections were obtained using a rotary microtome. These sections were then subjected to staining with hematoxylin and eosin (H&E) before being examined under a light microscope ³¹.

2.11. Statistical Analysis:

The GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA) for Windows® was used for all statistical analysis. The quantitative alteration among the studied groups will be evaluated by a one-way analysis of variance (ANOVA) test followed by post hoc Tukey's test for parametric variables. The correlation between parameters has been assessed via Pearson's correlation coefficient. The criteria for statistical significance were at p < 0.05.

3. RESULTS

3.1. Effect of QUE on hepatic aminotransferases The hepatotoxicity of CCl₄ was evidenced by a 3-fold and 4.5-fold increase in serum AST and ALT levels, respectively, compared to the control group. Interestingly, QUE (5mg/kg) co-treatment significantly decreased their levels by 38.1% and 49%, respectively, relative to the CCl₄ group (Fig. 1A and B).

3.2. Effect of QUE on oxidative stress markers

The effects of CCl₄ and/or QUE (5mg/kg) administration on various oxidative stress markers are illustrated in (Fig. 2A, B, and C). The hepatic MDA levels in the CCl₄ group exhibited a substantial elevation by 4.4-fold relative to the control group. Meanwhile, co-treatment with QUE (5mg/kg) significantly improved this surge by 36.4% in comparison to the CCl₄ group (Fig. 2A). Furthermore, the CCl₄ group exhibited a significant decrease in hepatic SOD and CAT activities by 46.2% and 81.3%, respectively, in comparison to the cCl₄ group, QUE (5mg/kg) co-treatment led to a marked increase in their activities by 1.2-fold and 3.3-fold, respectively (Fig. 2B and C).

Atta et al, Azhar Int J Pharm Med Sci 2025; Vol 5 (2):179-190.



Figure1. Effect of co-treatment with QUE (5mg/kg) on hepatotoxicity markers in response to CCl₄-induced hepatic damage in rats. (A) AST and (B) ALT. All values are shown as mean \pm SD. (a) and (b) Significance when compared to control and CCl₄ groups, respectively at P < 0.05, as determined by the ANOVA test. AST: aspartate transaminase, ALT: alanine transaminase, CCl₄: Carbon tetrachloride, QUE: Quercetin, ns: non-significant.



Figure 2. Effect of co-treatment with QUE (5mg/kg) on indicators of oxidative stress in response to CCl₄induced hepatic damage in rats. (A) MDA, (B) SOD, and (D) CAT. All values are shown as mean \pm SD. (a) and (b) Significance when compared to control and CCl₄ groups, respectively at P < 0.05, as determined by the ANOVA test. MDA: malondialdehyde, SOD: superoxide dismutase, CAT: catalase, CCl₄: Carbon tetrachloride, QUE: Quercetin, ns: non-significant.

3.3. Effect of QUE on pro-inflammatory indicators

A notable characteristic of liver damage attributed to CCl_4 is Inflammation. The CCl_4 group had markedly elevated levels of IL-6 and TNF- α by 2.1-fold and 2.5-fold, respectively, when compared to the control group. Conversely, QUE (5mg/kg) co-treatment significantly decreased the levels of these cytokines by 29.3% and 33.5%, respectively, when compared to the CCl₄ group (Fig. 3A and B).

3.4. Effect of QUE on fibrotic marker, TGF-B1

The CCl₄ group has a significant 3.1-fold elevation in TGF- β 1 levels when compared to the control group. Alternatively, co-treatment with QUE (5mg/kg) significantly decreased its levels by 44.6% compared to the CCl₄ group (Fig. 4).

3.5. Effect of QUE on miRNA-124-3p expression

The expression of miRNA-124-3p decreased significantly by 80.5% in the CCl₄ group when compared to the control group. Conversely, QUE (5mg/kg) co-treatment significantly boosted miRNA-124-3p expression by 2.5-fold in comparison to the CCl₄ group (Fig. 5).

3.6. Correlation between miRNA-124-3p and TGF-β1 in the studied groups

The correlation between miRNA-124-3p level and TGF- β 1 was evaluated using the Pearson correlation coefficient. The results revealed a strong negative correlation between TGF- β 1 and miRNA-124-3p, r= -0.9043 at p< 0.0001 (Fig. 6). Therefore, miRNA-124-3p/TGF- β 1 molecular axis may be effective as a therapeutic target for managing liver fibrosis.

3.7. Effect of QUE on liver histopathological assessment

Liver sections of the control and quercetin-onlytreated groups showed normal histopathological features, with no signs of fibrosis (Fig. 7A and B). On the contrary, the CCl₄ group showed significant pathological alterations, including the presence of thick fibrous bands with moderate inflammatory infiltrate, multiple complete nodules with scattered apoptotic hepatocytes, and marked macro-vesicular steatosis (Fig. 7C). Notably, the CCl₄+QUE group showed significant improvement in general histological features showed as an average central vein with intact hepatocytes in peri-venular area, and a mild degree of steatosis (Fig. 7D).

4. DISCUSSION

Liver fibrosis represents a major global health issue caused by various chronic liver diseases. Hepatocellular carcinoma and end-stage cirrhosis, the two primary contributors to liver-related mortality, are caused by liver fibrosis ¹¹. CCl₄ is recognized as a hepatotoxic agent that induces liver fibrosis in experimental models, and its effects closely resemble the clinical features of human liver cirrhosis, making it a preferred model among the different experimental approaches to studying liver fibrosis ³². Upon CCl₄ administration, significant hepatic injury and dysfunction are observed, as evidenced by an elevation of serum liver transaminases due to the leaking of these enzymes from damaged hepatocytes into the bloodstream, a decrease in protein synthesis, and an alteration in lipid profile 33, 34.

In the current study, serum ALT and AST were substantially augmented in the CCl_4 group compared to the controls. These findings were significantly resolved by the co-administration of QUE. The inhibitory effect of QUE on aminotransferases aligns with prior investigations by **Wang et al. and Ekpo et al.**, who reported that QUE preserves hepatic enzymes homeostasis by functioning as a membrane-stabilizing agent that attenuates liver cell injury ^{25, 35}.

Oxidative stress has emerged as a vital determinant in the pathogenesis of liver fibrosis. It initiates the fibrotic process through several mechanisms: it induces hepatocellular injury, stimulates the release of profibrogenic mediators, promotes recruitment of inflammatory cells, and directly activates HSCs, which collectively contribute to the advancement of fibrotic liver disease ^{36, 37}. The hepatotoxic effects of CCl₄ are primarily attributed to its metabolism by hepatic cytochrome P450 enzymes, which convert CCl₄ into highly reactive trichloromethyl radicals. These radicals initiate a cascade of oxidative processes, including lipid peroxidation, leading to cellular membrane damage and subsequent release of inflammatory mediators ^{38, 39}. MDA serves as a key lipid peroxidation product and a prominent biomarker for cellular oxidative damage, and an increase in MDA concentration reflects an accelerated rate of lipid peroxidation ³⁵. In the current study, MDA level was markedly elevated in the CCl₄ group. This observation aligns with recent research by Abdelghffar et al., who reported a surge in MDA content in CCl₄-intoxicated mice ⁴⁰.

Atta et al, Azhar Int J Pharm Med Sci 2025; Vol 5 (2):179-190.



Figure 3. Effect of co-treatment with QUE (5mg/kg) on inflammatory markers in response to CCl₄-induced hepatic damage in rats. (A) IL-6 and (B) TNF- α . All values are shown as mean \pm SD. (a) and (b) Significance when compared to control and CCl₄ groups, respectively at P < 0.05, as determined by the ANOVA test. IL-6: Interleukin-6, TNF- α : Tumor necrosis factor-alpha, CCl₄: Carbon tetrachloride, QUE: Quercetin, ns: non-significant.



Figure 4. Effect of co-treatment with QUE (5mg/kg) on TGF- β 1 in response to CCl₄-induced hepatic damage in rats. All values are shown as mean \pm SD. (a) and (b) Significance when compared to control and CCl₄ groups, respectively at P < 0.05, as determined by the ANOVA test. TGF- β 1: Transforming growth factor- β eta 1, CCl₄: Carbon tetrachloride, QUE: Quercetin, ns: non-significant.



Figure 5. Effect of co-treatment with QUE (5mg/kg) on miRNA-124-3p expression in response to CCl₄-induced hepatic damage in rats. The expression of miRNA-123-3p is based on qRT-PCR analysis. All values are shown as mean \pm SD. (a) and (b) Significance when compared to control and CCl₄ groups, respectively at P < 0.05, as determined by the ANOVA test. CCl₄: Carbon tetrachloride, QUE: Quercetin, ns: non-significant.



Figure 6. Association of miRNA-124-3p with TGF-β1



Figure 7. Representative photomicrographs of liver sections stained with hematoxylin and eosin (H&E X400). (A) Liver tissues obtained from rats in the control group showing an average portal vein (PV) with an average portal tract (black arrow) and normal intact hepatocytes in the peri-portal area (blue arrow). (B) Liver tissues obtained from rats in the QUE-only-treated group showing an average central vein (CV) with normal intact hepatocytes in the peri-venular area (blue arrow). (C) Liver tissues obtained from rats in the CCl₄ group showing thick fibrous bands with moderate inflammatory infiltrate (black arrow), multiple complete nodules (N) with scattered apoptotic hepatocytes (yellow arrow), and marked macro-vesicular steatosis (red arrow). (D) Liver tissues obtained from rats in the QUE+CCl₄ group showing an average central vein (CV) with normal intact hepatocytes in the peri-venular area (blue arrow) and marked macro-vesicular steatosis (red arrow). (D) Liver tissues obtained from rats in the QUE+CCl₄ group showing an average central vein (CV) with normal intact hepatocytes in the peri-venular area (blue arrow) and marked macro-vesicular steatosis (red arrow). (D) Liver tissues obtained from rats in the QUE+CCl₄ group showing an average central vein (CV) with normal intact hepatocytes in the peri-venular area (blue arrow) and mild macro-vesicular steatosis (red arrow).

Additionally, CCl₄ administration is strongly associated with a marked deficiency in antioxidant capacity. This correlation is observed by significant reductions in key antioxidant molecules and enzymes, including reduced glutathione, CAT, and SOD ^{41, 42}. Our results revealed a substantial reduction in the hepatic profile of SOD and CAT following CCl₄ administration. QUE co-treatment notably ameliorated these alterations, consistent with findings from a previous study by **El-Nekeety et al.**, who demonstrate QUE's potential to restore redox homeostasis due to its ability to reduce lipid peroxidation (MDA) and boost antioxidants production (glutathione peroxidase and SOD) ⁴³.

The interplay between oxidative stress and cytokinemediated inflammation is a crucial step in the progress of liver fibrosis 44. Elevated oxidative stress leads to the secretion of TNF- α , IL-6, and IL-1 β ⁴⁵. In particular, IL-6 has been linked to the severity of liver fibrosis, it can increase HSC survival, proliferation, and activity 45 . Additionally, TNF- α exacerbates hepatocyte injury, promotes the stellate cell's activation and proliferation, enhances the deposition of ECM components, amplifies inflammatory and fibrotic responses, and hence, contributes to liver fibrosis ⁴⁶⁻⁴⁹. In this research, the CCl₄ group presented a notable inflammatory response confirmed by the upsurge of both IL-6 and TNF- α profiles. However, QUE displayed a distinctive anti-inflammatory function via lowering their levels, similar to previous studies ^{50, 51}.

TGF- β 1 is a major contributor to liver fibrosis, which induces the transdifferentiate of stellate cells into fibrogenic myofibroblasts. It plays a role in all phases of disease progression, from the initiation of liver damage via inflammation and fibrosis to excessive hepatocyte apoptosis, cirrhosis, and cancer ^{2, 38, 52}. In this work, it was found that the level of TGF- β 1 was substantially elevated in the rats that were injected with CCl₄ only and its level was decreased with QUE co-treatment, protecting against hepatic fibrosis. These results align with the prior research findings indicating that QUE can attenuate liver fibrosis in LX-2 cells (human immortalized HSC line) by reducing TGF- β 1 expression ⁵³.

MicroRNAs represent another significant factor in hepatic fibrosis pathogenesis. The expression patterns of miRNAs offer a further precise reflection of pathophysiological conditions compared to mRNA expression patterns. Consequently, miRNAs have the potential to function as valuable diagnostic and prognostic biomarkers in hepatic fibrosis, while concurrently acting as targets for antifibrotic agents ^{54, 55}. Several recent research have displayed that QUE has beneficial effects on miRNA expression in fibrotic models ^{11, 56, 57}. Therefore, we proceeded with our investigation by assessing the expression pattern of miRNA-124-3p in CCl₄-induced liver fibrosis and QUE co-administration. MiRNA-124 was found to be downregulated in different fibrotic models ^{58, 59}, as well as in hepatocellular carcinoma (HCC)¹⁴. Moreover, miRNA-124 is known as an antiinflammatory miRNA and recently, it was reported that miRNA-124 overexpression can diminish HSCs cytokines secretion including TNF-a, IL-6, and IL- 1β , consequently decreasing the inflammatory responses 60.

This finding demonstrated the anti-inflammatory role of miRNA-124-3p in liver fibrosis. Furthermore, a recent research study by Mahmoudi et al. verified the impact of miRNA-124 overexpression on the TGF-\u00b31 expression in the pathogenesis of non-alcoholic fatty liver disease. Real-time PCR results revealed that overexpression of miRNA-124 after transfection significantly reduced TGF- β 1 expression ⁶¹. Another recent supportive investigation demonstrated that miRNA-124-3p-enriched exosomes (ExomiRNA-124-3p) treatment reduces collagen accumulation, attenuates inflammation, and inhibits TGF-B1 activity 45.

In line with these studies, our findings showed that miRNA-124-3p expression decreased in CCl₄intoxicated rats, but co-treatment with QUE leads to upregulation of its expression. Moreover, it should be noted that an inverse substantial association was observed between the expression of miRNA-124-3p and TGF- β 1 level, showing that miRNA-124-3p can potentially prevent the progress of hepatic fibrosis via modulating the TGF- β 1. As a result, therapeutic strategies focused on restoring the inappropriate expression of miRNAs are considered novel treatments for liver fibrosis ^{62, 63}. Thus, the restoration of miRNA-124-3p expression can be a promising strategy for ameliorating liver fibrosis.

5. CONCLUSION

QUE alleviates liver fibrosis by inhibiting TGF- β 1 expression through overexpression of miRNA-124-3p. Therefore, the regulation of the miRNA-124-3p/TGF- β 1 axis is a promising therapeutic approach for liver fibrosis.

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