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Sildenafil Citrate Attenuates Carbon Tetrachloride-Induced Hepatic Fibrogenesis Through Modulation of Oxidative Stress, Inflammation, and Profibrotic Signaling in Rats

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ABSTRACT: Hepatic fibrosis remains a leading cause of global morbidity, and the repurposing of approved pharmacological agents with antifibrotic potential represents a promising therapeutic avenue. This study investigated the fibroprotective efficacy of sildenafil citrate, a phosphodiesterase-5 inhibitor, against carbon tetrachloride (CCl_4)-induced hepatic fibrosis in male Sprague–Dawley rats. Hepatic fibrosis was induced by intraperitoneal injection of CCl_4 (40% v/v in olive oil, 0.5 mg/kg, twice weekly for eight weeks). Sildenafil citrate was administered orally throughout the induction period. Hepatoprotection was assessed through serum biochemistry (alanine aminotransferase [ALT], aspartate aminotransferase [AST], total bilirubin), oxidative stress markers (reduced glutathione [GSH] and malondialdehyde [MDA] in serum and liver tissue), histopathological examination (haematoxylin and eosin and Masson's trichrome staining), and immunohistochemical evaluation of transforming growth factor-beta 1 (TGF-β1), alpha-fetoprotein (AFP), and p53. The administration of CCl_4 resulted in marked hepatocellular injury, evidenced by a 7.79-fold elevation in ALT, a 2.69-fold increase in AST, and a 3.12-fold rise in total bilirubin (all $P < 0.001$). Sildenafil citrate treatment significantly attenuated these disturbances, reducing ALT by 67%, AST by 35%, and total bilirubin by 59% relative to the untreated fibrotic group (all $P < 0.001$). The fibrotic group exhibited profound oxidative stress, characterized by depletion of GSH and accumulation of MDA; sildenafil citrate restored GSH by 4.25-fold (serum) and 2.90-fold (hepatic) and reduced MDA by 41.7% (serum) and 22.4% (hepatic). Histopathological examination revealed that sildenafil citrate achieved an 80.0% reduction in fibrosis area, an 81.0% decrease in the apoptotic index, and a 677.8% increase in regenerating hepatocytes. In addition, immunohistochemically of TGF-β1 expression in stromal cells declined from 15–20% to 5–10%, p53-positive apoptotic cells decreased by approximately 72%, and AFP-positive cells increased approximately by 9.5-fold. These findings demonstrate that sildenafil citrate exerts concurrent antioxidant, anti-apoptotic, antifibrotic, and pro-

regenerative effects in CCl_4-induced hepatic fibrosis, supporting its candidacy as a repurposed antifibrotic agent.

1. INTRODUCTION

Hepatic fibrosis is the pathological consequence of sustained or repeated liver injury, defined by excessive deposition of extracellular matrix (ECM) components, principally type I and type III collagen, within the hepatic parenchyma **(Nasr et al., 2025)**. It represents a common, etiology-independent endpoint of chronic viral, alcoholic, metabolic, autoimmune, and toxic liver diseases, and its progression underlie the transition from reversible parenchymal injury to cirrhosis, portal hypertension, hepatic decompensation, and hepatocellular carcinoma **(Devarbhavi et al., 2023)**. Global Burden of Disease data indicate that cirrhosis incidence rose from 36.92 million cases in 1990 to 58.41 million cases in 2021, and that liver disease now accounts for approximately two million deaths annually worldwide, underscoring the scale of this pathology **(Devarbhavi et al., 2023)**.

At the cellular level, fibrogenesis is driven by activation of hepatic stellate cells (HSCs), which transdifferentiate from a quiescent, vitamin A–storing phenotype into proliferative, contractile, collagen-producing myofibroblast-like cells in response to sustained oxidative stress and inflammatory signaling **(Zhang et al., 2024)**. Transforming growth factor-beta (TGF-β) is the principal fibrogenic cytokine driving this trans-differentiation, acting through the canonical Smad2/3–Smad4 signaling axis to upregulate collagen and α-smooth muscle actin expression, while the inhibitory Smad7 provides physiological restraint on this pathway **(Zhang et al., 2024)**. Progression of fibrosis reflects a chronic imbalance between ECM synthesis by activated HSCs and its degradation by matrix metalloproteinases, whose activity is constrained by tissue inhibitors of metalloproteinases; because this balance can, in principle, be reversed once the fibrogenic stimulus is withdrawn, the fibrotic stage remains the most rational and clinically meaningful target for antifibrotic pharmacotherapy **(Zhang et al., 2024)**. Despite this, no drug has yet been approved specifically for hepatic fibrosis, and current management is confined to controlling the underlying cause of injury together with supportive care **(Gilgenkrantz et al., 2025; Mohammed et al., 2023)**, which sustains active interest in identifying new or repurposed antifibrotic agents.

Experimentally, carbon tetrachloride (CCl4) remains the most widely used hepatotoxin for modelling this process, because its mechanism of injury reproduces the oxidative-inflammatory-fibrogenic sequence seen in chronic human liver disease **(Weber et al., 2003)**. CCl4 is bioactivated by cytochrome P450 2E1 to the trichloromethyl radical ($CCl_3•$), which in the presence of oxygen is further converted to the highly reactive trichloromethyl peroxy radical ($CCl_3OO•$); this radical initiates a self-propagating chain of lipid peroxidation within hepatocyte membranes, producing centrilobular necrosis, glutathione depletion, and release of pro-fibrogenic cytokines that drive HSC activation **(MANIBUSAN et al., 2007; Weber et al., 2003)**. Repeated CCl4 administration is accordingly a well-validated, reproducible model for evaluating candidate hepatoprotective and antifibrotic compounds.

Sildenafil citrate is the citrate salt of sildenafil, a synthetic pyrazolo-pyrimidinone compound with the chemical formula $C_{22}H_{30}N_6O_4S \cdot C_6H_8O_7$ and a molecular weight of 666.7 g/mol. Structurally, sildenafil is a cGMP analogue that competitively occupies the catalytic site of phosphodiesterase type 5 (PDE5), the enzyme responsible for hydrolyzing cyclic guanosine monophosphate (cGMP) to its inactive metabolite, 5'-GMP; sildenafil binds PDE5 with high affinity ($K_i \approx 3.5$ nM) and marked selectivity relative to other phosphodiesterase isoforms **(Terrett et al., 1996)**. The compound was originally developed by Pfizer for angina pectoris and was subsequently repurposed, first for erectile dysfunction under the trade name Viagra, and later, at higher doses, for pulmonary arterial hypertension under the trade name Revatio.

Biologically, sildenafil acts downstream of nitric oxide (NO): NO activates soluble guanylate cyclase to generate cGMP, which activates cGMP-dependent protein kinase (PKG) to relax vascular and visceral smooth muscle; by inhibiting PDE5, sildenafil prolongs and amplifies this cGMP–PKG signal **(Halverscheid et al., 2009)**. This NO–cGMP–PDE5 axis is physiologically active in the hepatic sinusoid, where NO generated by sinusoidal endothelial cells signals to adjacent hepatic stellate cells to maintain sinusoidal relaxation; in chronic liver injury, endothelial NO synthase activity falls, PDE5 activity rises, and HSCs acquire a contractile phenotype, together increasing intrahepatic vascular resistance **(Halverscheid et al., 2009)**.

Beyond this haemodynamic action, sildenafil exerts direct antifibrotic effects at the molecular level. PDE5 expression is itself upregulated in fibrotic liver tissue and in TGF-β1-activated hepatic stellate cells, and PDE5 inhibition activates the cGMP/PKG pathway in a manner that suppresses downstream Akt/NF-κB signalling, thereby restraining stellate-cell activation and collagen deposition. In the CCl4 model specifically, sildenafil has been shown to restore hepatic glutathione and catalase activity, reduce

malondialdehyde-based indices of lipid peroxidation, and down-regulate the mRNA expression of collagen-1α, TGF-β, interleukin-1β, and osteopontin, with corresponding reduction in periportal fibrosis and inflammatory infiltration on histopathology **(Nasr et al., 2025)**. Comparable protection has been reported using intraperitoneal CCl4 injection, where sildenafil pretreatment attenuated the rise in serum liver enzymes (Molehin et al., 2018), and in the bile-duct-ligation cholestatic fibrosis model, where sildenafil's hepatoprotective effect was shown to be dependent on upregulation of silent information regulator 1 (SIRT1) **(Abd El Motteleb et al., 2017)**. An additional, more recently described mechanism involves sildenafil-induced upregulation of endogenous hepatic hydrogen sulfide (H2S) synthesis and activation of the Keap1/Nrf2 antioxidant pathway, providing a further route through which the drug limits oxidative hepatocellular injury **(Alan Albayrak et al., 2023)**.

Two converging facts justify the present investigation. First, hepatic fibrosis remains a leading contributor to chronic liver disease morbidity and mortality worldwide, yet, despite substantial mechanistic progress, no antifibrotic drug has received regulatory approval specifically for this indication, leaving an acknowledged and clinically significant therapeutic gap **(Gilgenkrantz et al., 2025; Mohammed et al., 2023)**. Second, drug repurposing offers a comparatively rapid and economical route toward filling that gap, since molecules already approved for other indications carry an established human safety record and require a shorter translational path than a novel chemical entity **(Mohammed et al., 2023)**. Sildenafil citrate satisfies these criteria precisely: it is inexpensive, orally bioavailable, and has decades of clinical use in erectile dysfunction and pulmonary arterial hypertension, while preclinical evidence, summarized above, indicates that it favorably modulates oxidative stress, inflammation, apoptosis, and hepatic stellate cell activation across more than one experimental model of hepatic fibrosis.

Nevertheless, this preclinical evidence base remains comparatively limited and heterogeneous in dosing regimen, treatment duration, and outcome measures across the small number of published studies. A dedicated investigation of sildenafil citrate against a standardized CCl4-induced hepatic fibrosis protocol in albino rats, incorporating biochemical, oxidative-stress, histopathological, and immunohistochemical endpoints in a single controlled design, is therefore warranted to consolidate the existing evidence and to clarify, more precisely, the extent and mechanism of sildenafil's fibroprotective action, thereby strengthening the scientific basis for its consideration as a repurposed antifibrotic agent.

This study aims to investigate the fibroprotective role of sildenafil citrate against carbon tetrachloride-induced hepatic fibrosis in albino rats, through the following specific objectives:

1. To assess liver function through measurement of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin.

2. To evaluate hepatic oxidative and antioxidant status through measurement of reduced glutathione (GSH) and malondialdehyde (MDA);

3. To examine hepatic architecture and general histopathological changes using hematoxylin and eosin (H&E) staining.

4. To assess the degree of hepatic fibrosis and collagen deposition using Masson's trichrome staining.

5. To characterize the expression of transforming growth factor-beta 1 (TGF-β1), alpha-fetoprotein (α-FP), and the tumor suppressor protein p53 by immunohistochemistry, as markers of fibrogenic activity, hepatic progenitor/regenerative response, and cellular stress response, respectively.

Through this integrated biochemical, molecular, and histopathological approach, the study is intended to provide mechanistic evidence supporting, or refining, the candidacy of sildenafil citrate as a repurposed pharmacological option for the prevention or amelioration of hepatic fibrosis.

2. MATERIALS AND METHODS

2.1. Materials and chemicals

Carbon tetrachloride (CCl_4), as 99% (v/v) colorless liquid, was purchased from Adwic Chemicals Co. (Cairo, Egypt). CCl_4 was dissolved in olive oil in a ratio of 2:3 to yield 40% (v/v). Also, Sildenafil, blue bills, which has the following chemical formula $C_{28}H_{38}N_6O_{11}S$ and molecular weight of 666.70 g/mol and purchased from Pfizer. Sildenafil was suspended in 0.5% carboxymethylcellulose (CMC) for oral administration.

2.2. Animals and ethical approval

The study utilized sixty male Sprague-Dawley rats, each weighing between 200 and 250 grams. These animals were procured from the Animal Research House affiliated with the Faculty of Veterinary Medicine at Mansoura University **MU-ACUC (VM.R.26.10.335**). They were housed in transparent plastic cages under controlled environmental conditions, including a temperature maintained around 30°C and a humidity level of 60 ± 10%. The light/dark cycle was set at 12 hours per phase. Rats were provided with standard laboratory feed and unlimited access to fresh water. Before commencing the experiment, the animals were acclimatized for one week. All experimental procedures adhered to ethical guidelines established and approved by the local experimental ethics committee.

2.3. Hepatic Fibrosis induction

Hepatic fibrosis was induced by intraperitoneal (i/p) injection of CCl4 (40% v/v in olive oil) in a dose of 0.5 mg/Kg body weight of rat twice weekly 8 weeks **(Iredale et al., 1998)**.

2.4. Experimental Design

The rats were randomly allocated into three groups, each comprising 20 animals. Group I served as the control, receiving only 1 mL of 5% carboxymethyl cellulose (CMC) solution orally via gastric gavage for 16 consecutive weeks. Group II was designated as the hepatic fibrosis model, receiving intraperitoneal injections of carbon tetrachloride (CCl_4) at 1 mL/kg of a 40% v/v solution in olive oil, administered twice weekly for 16 weeks. In order to simulate clinical conditions more realistically, treatments aimed at fibrosis mitigation were initiated after 8 weeks of CCl_4 intoxication and continued concurrently with CCl_4 exposure for an additional 8 weeks. Group III was subjected to CCl4 injections alone for 8 weeks, followed by concurrent administration of CCl4 and sildenafil citrate via gastric gavage for another 8 weeks at dose of 10mg/kg/day **(Cadirci et al., 2011)**.

At the end of the experimental period, animals will undergo terminal blood collection under an appropriately approved anesthetic protocol, followed by humane euthanasia in accordance with the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (2020). Twenty-four hours following the final CCl_4 or treatment dose, blood samples were collected from the medial canthus of the eye for subsequent biochemical and oxidative stress analyses. Liver tissue samples were excised and divided into two portions: one placed in normal saline for preparation of liver homogenates for oxidative stress assays, and the other fixed in 10-15% neutral buffered formalin for histopathological study.

2.5. Laboratory analysis

The collected blood was allowed to clot at room temperature (25°C) for 30 minutes, followed by centrifugation at 2000 g for 10 minutes. The supernatant serum was carefully separated, avoiding disturbance of the buffy coat, and stored in a chilled container until analysis on the same day. Excised liver specimens were suspended in physiological saline containing heparin (0.16 mg/mL) to prevent coagulation. The tissues were homogenized at 10% w/v concentration in a Tris-HCl buffer (20 mM, pH 7.4) with 1 nM EDTA. The homogenate was centrifuged at 3000 g for 20 minutes at 4°C, and the clear supernatant was collected while avoiding the floating lipid layer. Samples were maintained on ice for immediate biochemical assays.

2.5.1. Colorimetric assays of serum liver function

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using commercially available kits (Randox, catalog number AL100) following the methods described by **(Reitman & Frankel, 1957)**. Total bilirubin concentration was determined using the protocol by **(Pearlman & Lee, 1974)**.

2.5.2. Oxidative Stress Markers

Reduced glutathione (GSH) levels were quantified spectrophotometrically in both serum and liver homogenates based on the enzymatic colorimetric method outlined by **(BEUTLER et al., 1963)**. Malondialdehyde (MDA) concentration, indicative of lipid peroxidation, was measured in serum and liver tissue according to the colorimetric assay of **(Gaweł et al., 2004).**

2.5.3. Histopathological Evaluation of Hepatic Fibrosis

Periodic core liver biopsies were obtained throughout the experimental timeline, and terminal liver specimens underwent standard histopathological processing. Formalin-fixed tissues were processed using an automated tissue processor (KD-TS6A),

involving a two-step fixation-dehydration protocol. Fixation was performed by immersion in 10% neutral buffered formalin for 48 hours, followed by rinsing in distilled water. Dehydration included sequential immersion in graded ethanol concentrations (70%, 90%, and 100%) with exposure times ranging from 90 to 120 minutes per step. After dehydration, tissues were cleared through xylene baths, embedded in paraffin wax, and sectioned at 4–5 μm thickness. Hematoxylin and eosin (H&E) staining was performed according to standard protocols (Suvarna et al., 2013). In addition, Masson's trichrome stain was applied on serial sections of the same paraffin blocks to corroborate fibrosis extent and detect minimal fibroproliferative activity potentially undetectable by H&E. This staining also facilitated evaluation of the antifibrotic effects of treatments.

2.5.4. Immunohistochemical Analysis

Paraffin sections underwent antigen retrieval via microwaving at 720 W for 25 minutes, followed by overnight incubation at 4°C with primary antibodies targeting transforming growth factor-beta 1 (TGF-β1), alpha-fetoprotein (α-FP), or tumor suppressor protein p53. After PBS washing, sections were incubated with biotinylated secondary antibodies and streptavidin/alkaline phosphatase complexes (Dako Corp.) at 1:200 dilutions. Diaminobenzidine (DAB) was used for chromogenic detection, and counterstaining was performed with hematoxylin. Slides were dehydrated, cleared in xylene, mounted, and examined under high-power light microscopy. Quantification of immunostaining was carried out by measuring the percentage of positively stained areas in seven high-power fields using Image J software (version 1.46a, NIH, USA) **(Ali et al., 2021)**.

2.6. Statistical analysis

The statistical analysis was performed using GraphPad Prism version 8.x (GraphPad Software, LLC). Data are presented as means ± SD. Statistical significance was determined by one-way ANOVA followed by a post-hoc Tukey test.

3. RESULTS

3.1. Sildenafil citrate improved hepatic Architecture and liver function biochemical tests in hepatic fibrosis

The gross examination of liver in fibrosis group showed small liver in size, firm in consistency, yellowish brown in colure, and showed white-yellowish areas on the surface. In addition, multifocal nodules of about 0.1-0.3 cm, distributed all over the hepatic surface beside adhesion of the hepatic capsule with surrounding tissues (Figure 1A) when compared to smooth reddish appearance of liver from the healthy control group (Figure 1A). However, in the Sildenafil citrate group, the liver was apparently normal in size, yellowish brown in color and slightly firm in consistency and the hepatic surface was focally nodular with thick irregular borders **(Figure 1A).**

The quantitative analysis serum ALT activity was markedly elevated following CCl_4 administration. The ALT concentration increased by 7.79-fold change; representing 678.70% percent change relative to the control group ($P < 0.0001$), confirming pronounced hepatic injury following CCl_4 exposure. On the other hand, sildenafil citrate treatment substantially reduced serum ALT activity by 0.33-fold change relative to CCl_4 group ($P < 0.0001$), indicating a marked improvement in the biochemical manifestation of liver injury following sildenafil administration **(Figure 1B)**.

Moreover, serum aspartate aminotransferase (AST) activity was markedly altered following CCl_4 administration. The AST activity revealed significant increase by 2.69-fold change to the control group ($P < 0.0001$), demonstrating substantial hepatic injury following CCl_4 exposure. Treatment with sildenafil citrate reduced the serum AST activity by 0.65-fold change ($P < 0.0002$). relative to the fibrotic group **(Figure 1C)**.

Serum total bilirubin was markedly elevated in the CCl_4-induced liver fibrosis group compared with healthy controls by 3.12-fold increase, corresponding 211.91% elevation relative to the control value ($P < 0.0001$). Sildenafil citrate treatment markedly reduced serum total bilirubin by 0.41-fold relative to the untreated fibrotic group ($P < 0.0001$).

Collectively, these findings indicate that CCl_4 administration produced pronounced hepatocellular injury, as reflected by the marked elevation of both serum aminases and serum total bilirubin, whereas sildenafil citrate exerted a significant hepatoprotective effect by substantially attenuating the CCl_4-induced increase in serum ALT, AST activity, and total bilirubin, although complete biochemical normalization was not achieved.

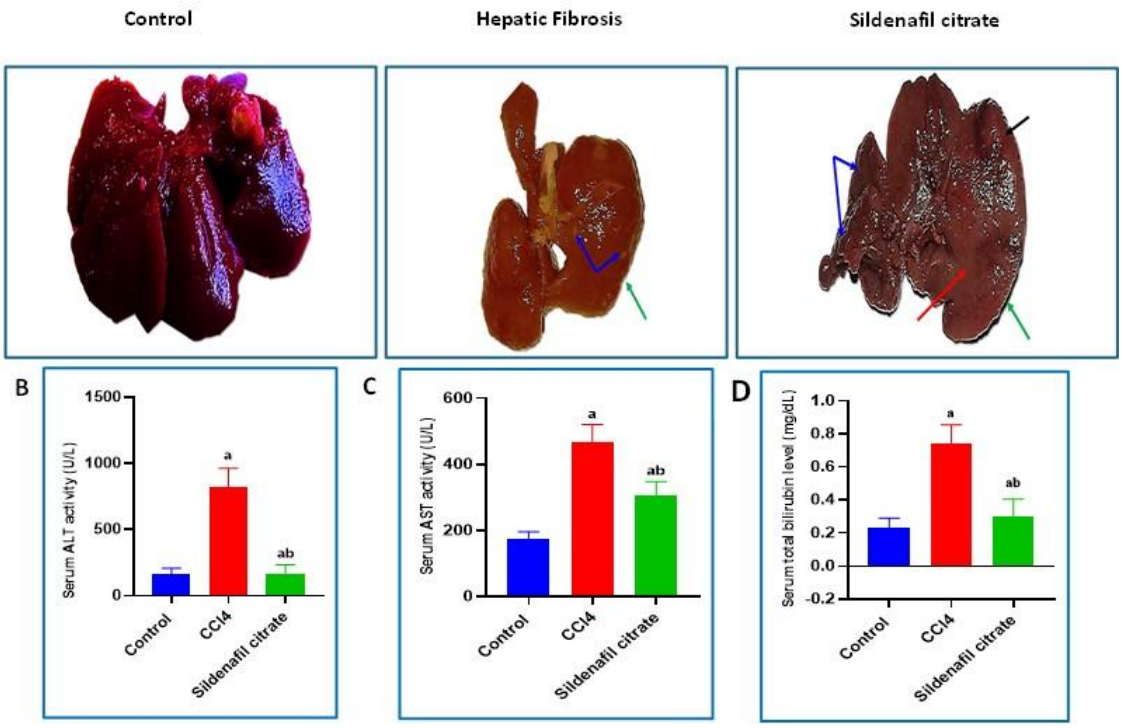


Figure 1: Effect of Sildenafil citrate on hepatic architecture and liver function biochemical tests. **[A]** Liver gross examination images of livers from control, hepatic fibrosis, and liver fibrosis treated with sildenafil citrate groups. **[B]** Serum activity of alanine aminotransferase (ALT). **[C]** Serum activity of aspartate aminotransferase (AST). **[D]** Serum level of total bilirubin, at the end of the study in different groups; a: significant vs control, b: significant vs CCL4. Data are expressed as mean± SD. Significance was set at p≤0.05. Control group: **ALT:** Alanine aminotransferase**, AST:** Aspartate aminotransferase. **a**: significant *vs.* normal control group, **b**: significant *vs.* HCC group.

3.2. Antioxidant Activity Improvement by Sildenafil citrate

Notably, CCl_4 administration produced a pronounced oxidative imbalance, characterized by a substantial depletion of reduced glutathione (GSH) and an elevation of malondialdehyde (MDA) in both serum and hepatic tissue.

Serum GSH decreased by 0.10-fold change relative to control. Similarly, hepatic GSH decreased by 0.21-fold change compared with healthy controls. In parallel with GSH depletion, CCl_4 markedly increased lipid peroxidation. Serum MDA markedly increased by 4.05-fold increase**;** corresponding to 305.16% elevation**.** In addition, hepatic MDA reported marked increase in 1.31-fold change and 30.94% percent change relative to the control group.

On the other hand, Sildenafil citrate treatment markedly improved the CCl_4-induced oxidative imbalance. Serum GSH increased by 4.25-fold increase relative to the CCl_4 group, while hepatic GSH demonstrated 2.90-fold change increase relative to the CCl_4 group. Although GSH concentrations remained below those of healthy controls, these changes indicate substantial recovery of the antioxidant capacity following sildenafil treatment.

Concomitantly, sildenafil citrate reduced MDA concentrations in both compartments. Serum MDA decreased by 41.67% reduction compared with the CCl_4 group, while hepatic MDA decreased by 22.40% reduction relative to untreated fibrosis. Notably, hepatic MDA in the sildenafil-treated group was very close to the control value, indicating marked restoration of hepatic lipid peroxidation status.

Collectively, these findings demonstrate that CCl_4-induced hepatic fibrosis was accompanied by a substantial disruption of redox homeostasis, characterized by depletion of GSH and accumulation of MDA. Sildenafil citrate markedly attenuated these alterations by enhancing GSH availability and reducing lipid peroxidation, supporting a significant improvement in the oxidative stress status associated with CCl_4-induced hepatic injury.

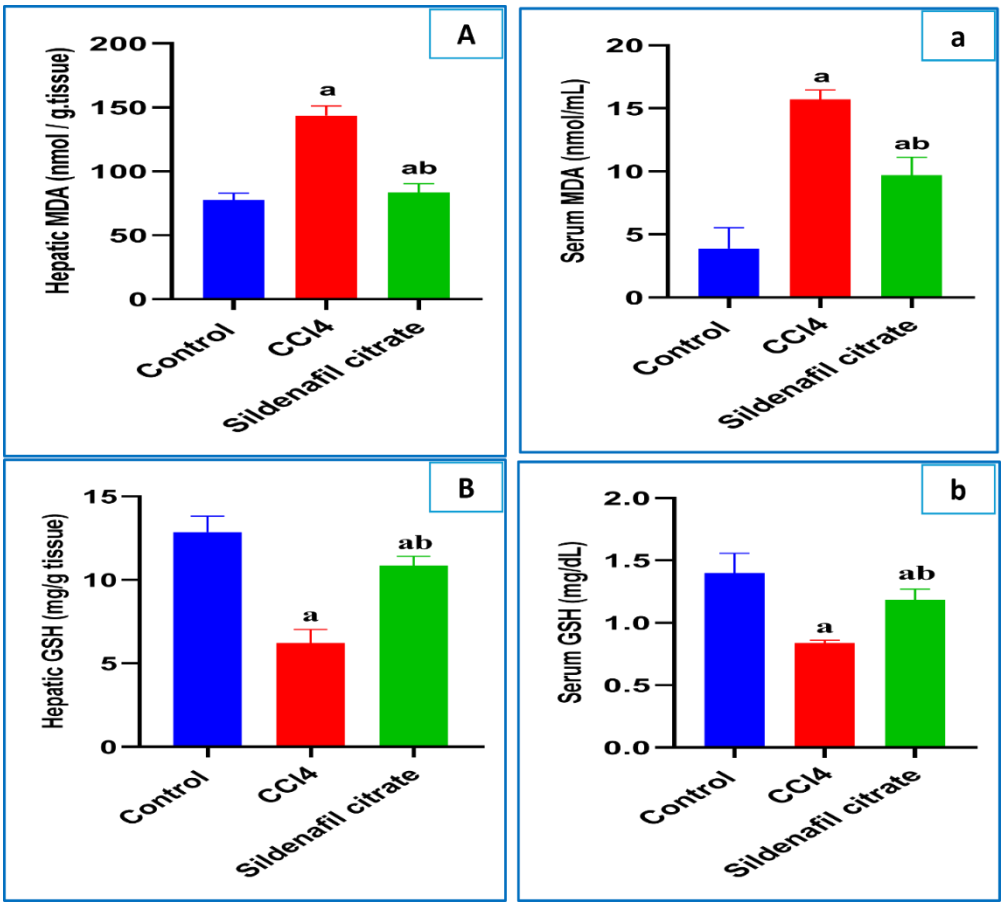


Figure 2: Effect of Sildenafil citrate on antioxidant activity. **[A,a]** Hepatic and serum MDA level in control, hepatic fibrosis, and liver fibrosis treated with sildenafil citrate groups. **[B,b]** Hepatic and serum GSH level in control, hepatic fibrosis, and liver fibrosis treated with sildenafil citrate groups. Data are expressed as mean± SD. Significance was set at p≤0.05. **GSH:** Glutathione, **MDA:** Malondialdehyde. **a**: significant *vs.* normal control group, **b**: significant *vs.* HCC group.

3.3 . Sildenafil citrate improved histopathological findings, and Fibrosis degree in hepatic fibrosis

Microscopic pictures of H&E-stained liver sections in control group showed preserved arrangement of hepatic cords, central veins, portal traid's structure, vascular tributaries, biliary system, Von kupffer's cells and supporting stroma, and sinusoids in the control group **(Figure 3A)**. However, liver sections from the fibrosis group (CCl_4) revealed marked disruption of arrangement of hepatic cords, central veins, and portal areas that characterized by mono-lobular cirrhotic changes represented by fibrous tissue proliferations encircling individual hepatic lobules; most of the hepatic parenchyma was affected with marked portal fibrosis. In addition, marked thick anastomosing fibrous tissue deposition containing congested blood vessels, leukocytic cells, and hemosiderin laden macrophages, forming multiple separate complete hepatic nodules with ballooning degeneration in hepatocytes. The Kupffer cells were mildly or moderately hypertrophied and some of the hepatic cells were apoptotic and others were regenerated **(Figure 3B)**. While liver section from the treated group with sildenafil citrate demonstrated mild to moderate number of hepatocytes, especially periportal showed macrosteatosis. The remaining hepatic parenchyma appeared normal apart from mild hydropic degeneration of some cells. The hepatic lobules were separated by normal fine fibrous strands, and the portal triads were free from the fibrotic changes which were seen in the CCl_4-treated group. Additionally, kupffer cells were moderately hypertrophied and the hepatic sinusoids showed variable number of lymphocytes, monocytes and eosinophils **(Figure 3C)**.

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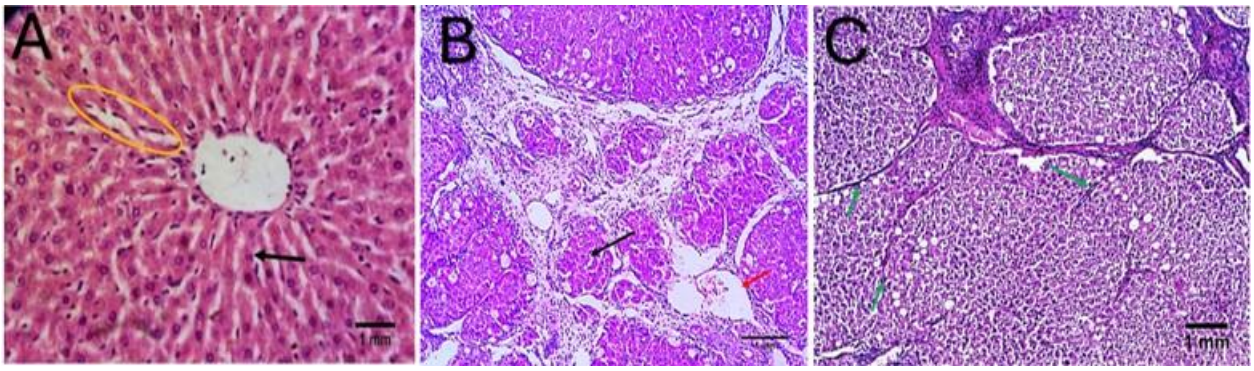


Figure (3): Representative photomicrograph of liver tissue sections stained with of H & E. (A) Photomicrograph of rat's liver group showing normal hepatic architecture that demonstrate preserved hepatic cords, portal triad's structures, vascular tributaries, biliary system, Von kupffer's cells and supporting stroma. (B) Photomicrograph of rat's liver group showing monolobular cirrhotic changes with marked portal fibrosis (red arrow) and fibrous tissue proliferation encircling the individual hepatic lobule (black arrow). (C) Photomicrograph of rat's liver group showing residues of previous fibrosis (green arrows).

Furthermore, co-administration of sildenafil citrate with CCl_4 produced a significant, multi-parameter amelioration of hepatic injury relative to the untreated fibrosis group. The 1.74-fold (42.5%) reduction in macro- and micro-steatosis suggests that sildenafil attenuated the lipid-laden hepatocyte phenotype. The more pronounced 5.27-fold (81.0%) fall in the apoptotic index points to a robust anti-apoptotic effect. In addition, the 5.01-fold (80.0%) reduction in fibrosis area which is the pivotal finding and directly demonstrated that sildenafil suppressed collagen deposition. This magnitude of reduction varied from a severely fibrotic state to a near-baseline level, indicated as substantial antifibrotic effect rather than a marginal one. Most strikingly, regenerating hepatocytes increased 7.78-fold (677.8%) in Group treated with sildenafil, as tissue regeneration is normally suppressed in the setting of ongoing necroinflammation and fibrogenesis, this near eight-fold rise indicates that sildenafil not only limited injury but also created a permissive environment for compensatory hepatocyte proliferation. This pattern aligned with reduced apoptotic and fibrotic burden freeing the tissue to re-enter a regenerative program. Collectively, sildenafil citrate simultaneously reduced steatotic and apoptotic injury, curtailed fibrogenesis, and restored regenerative capacity, supporting its candidacy as a fibro-protective repurposed agent in this model **(Figure 4).**

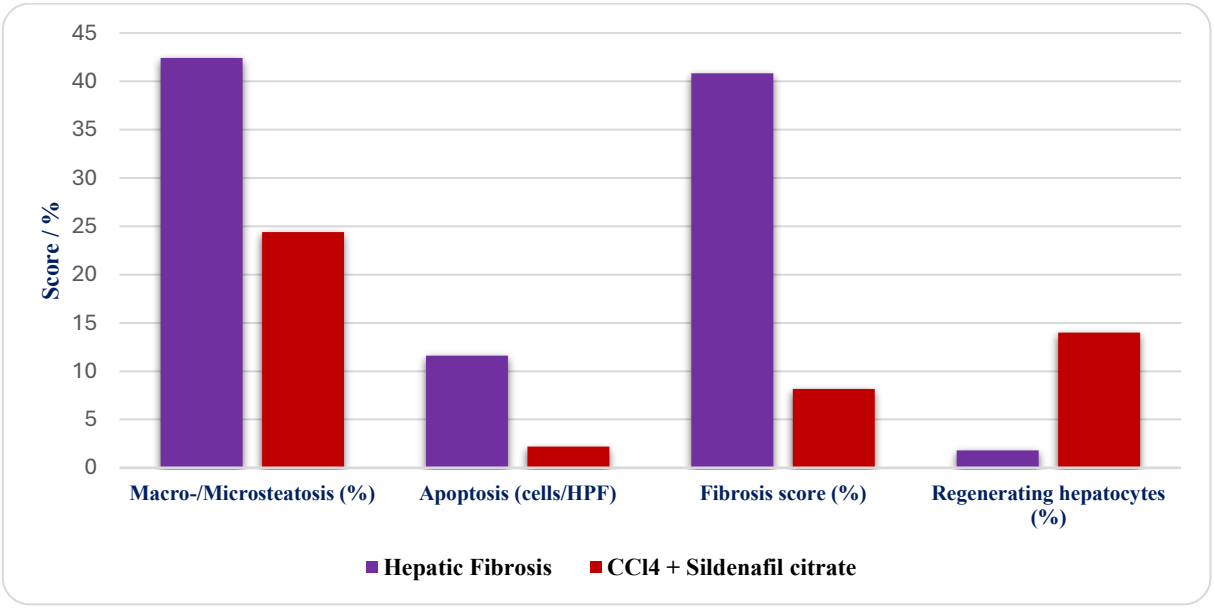


Figure 4: Effect of sildenafil citrate on CCl4-induced hepatic fibrosis: different histopathological changes.

Remarkably, the estimated semi-quantitative most prominent pathological lesions scoring in **figure (5)** revealed biliary hyperplasia, vascular changes, and inflammatory reactions were highly recorded in fibrotic induced group as compared with sildenafil treatment group. However, necrosis was interestingly highly detected in fibrotic group, but it was unrecorded in the treated group.

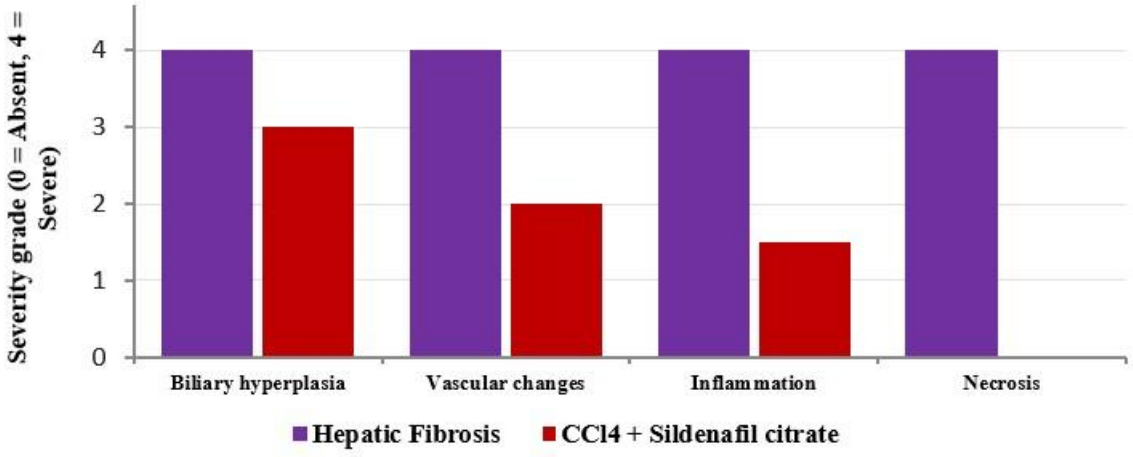


Figure 5: Semiquantitative estimation of the most prominent pathological lesions of hepatic fibrosis.

3.4. Masson's trichrome examination

The control group showed minimal collagen expression just around the central veins. Examination of liver-stained sections of CCl4-induced hepatic fibrosis showed marked fibro-cirrhotic changes with their fibrous bands separating the individual hepatic lobules and appeared blue stained. The portal triads showed heavy fibrosis which appeared as bluish fibrillar martials **(Figure 6 - A)**. Some fibrous strands were seen extending to the intralobular tissue at a variable distance. On the other hand, treatment of rats with sildenafil revealed remnants of fibrosis between hepatic lobules. The fibrotic remnants were mostly seen in portal triads and extended to a variable degree between hepatic lobules **(Figure 6 - B)**.

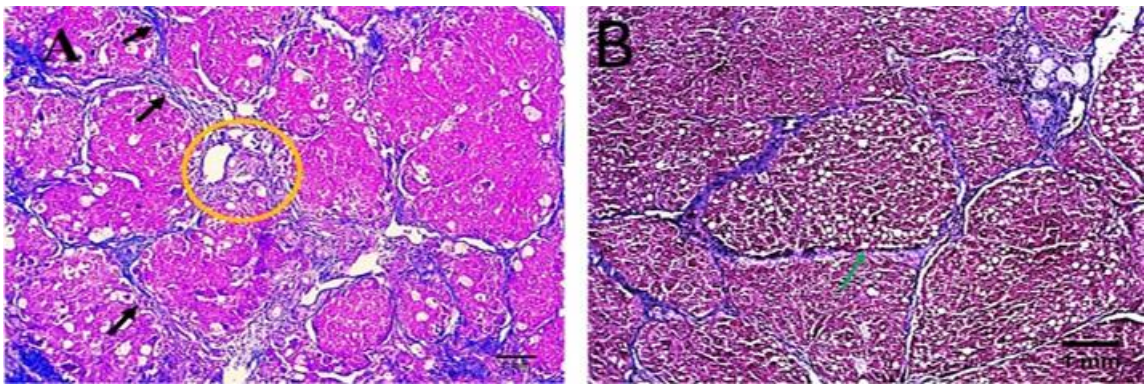


Figure (6): Representative photomicrograph of liver tissue sections stained with of Masson's trichome. (A) Photomicrograph of rat's liver demonstrated marked fibro-cirrhotic changes with their fibrous bands separating the individual hepatic lobules and appeared blue stained. The portal triads showed heavy fibrosis which appeared as a bluish fibrillar martials. Some of fibrous strands were seen extending to the intralobular tissue at a variable distance. (B) Photomicrograph of rat's liver revealed remnants of fibrosis between hepatic lobules and the fibrotic remnants were mostly seen in portal triads and extended to a variable degree between hepatic lobules.

3.5. Sildenafil citrate effect on Alpha-Fetoprotein (AFP) tumor marker Immunoexpression

Hepatocytes of the control group showed complete absence of AFP immunoreactivity, with uniformly negative cytoplasmic staining throughout the hepatic parenchyma **(Figure 7)**. While untreated hepatic fibrosis group revealed AFP immunopositivity

was largely confined to a small subset of stromal cells and regenerating hepatocytes, comprising approximately 1–3% of the examined cell population, and the remaining parenchyma remained negative **(Figure 7)**. On the other hand, combined administration of sildenafil citrate with CCl_4 markedly expanded the AFP-positive cell fraction, with 18–20% of hepatic parenchymal and stromal cells exhibiting positive brownish cytoplasmic immunostaining. Taking the midpoint of each reported range, this corresponds to an approximately 9.5-fold increase in AFP-positive cells in sildenafil citrate group relative to the untreated fibrosis group. Given the established role of AFP as a marker of hepatic progenitor/oval cell activation during liver regeneration, this marked expansion of AFP immunoreactivity is consistent with the substantially higher number of regenerating hepatocytes previously observed histologically in sildenafil group, and suggested that sildenafil promoted a proliferative, regenerative hepatic response alongside its antifibrotic action **(Figure 7)**.

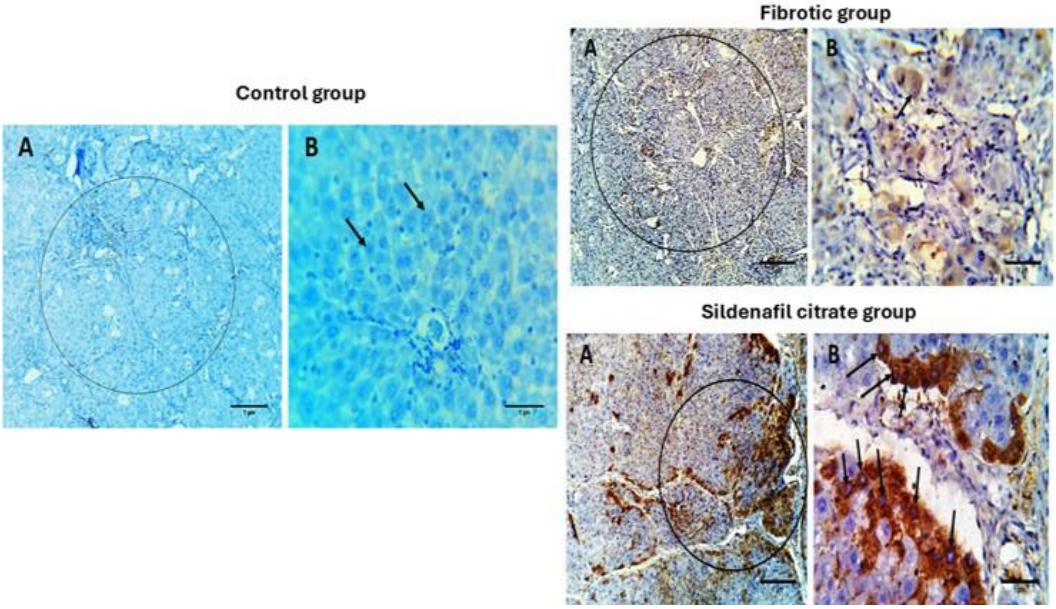


Figure 7: Photomicrograph of rat's liver demonstrated by immunohistochemical stain for αFP. Control group showed: (A) All hepatic cells showing negative stainability for αFP and (B) showed high magnification to demonstrate the negative stainability of the hepatocytes. While in fibrotic group, photomicrograph of rat's liver demonstrated (A) expression of αFP in 1-3% of the stromal cells and regenerated hepatocytes. Immunohistochemical stain for αFP (black circles). (B) High magnification to demonstrate the brownish stainability of the positive cells (black arrows). On the other hand, photomicrograph of rat's liver treated group with sildenafil citrate revealed (A) Expression of αFP in 18-20 % of the hepatic parenchyma and stromal cells. Immunohistochemical stain for αFP (black circles). (B) High magnification to demonstrate the brownish stainability of the positive cells (black arrows).

3.5 Antifibrotic activity of sildenafil citrate (Transforming Growth Factor-β1 (TGF-β1)

The negative control group demonstrated negative TGF-β1 immunostaining in both hepatocytes and the vascular structures of the portal triads **(Figure 8A, B)**. In addition, the hepatic fibrosis group showed a heterogeneous pattern of TGF-β1 expression: peripherolobular hepatocytes displayed positive cytoplasmic staining in 3–5% of cells, while positively stained proliferating stromal cells were more extensive and variable, ranging between 15–20%; the remaining parenchymal cells, together with round cells and mature fibroblasts, were negative. Proliferating vascular endothelial cells were also positively immunolabelled for TGF-β1 in this group **(Figure 8A, B)**. While, the sildenafil-treated group, 10–15% of parenchymal cells and 5–10% of stromal cells demonstrated positive cytoplasmic TGF-β1 immunoreactivity **(Figure 8A, B)**.

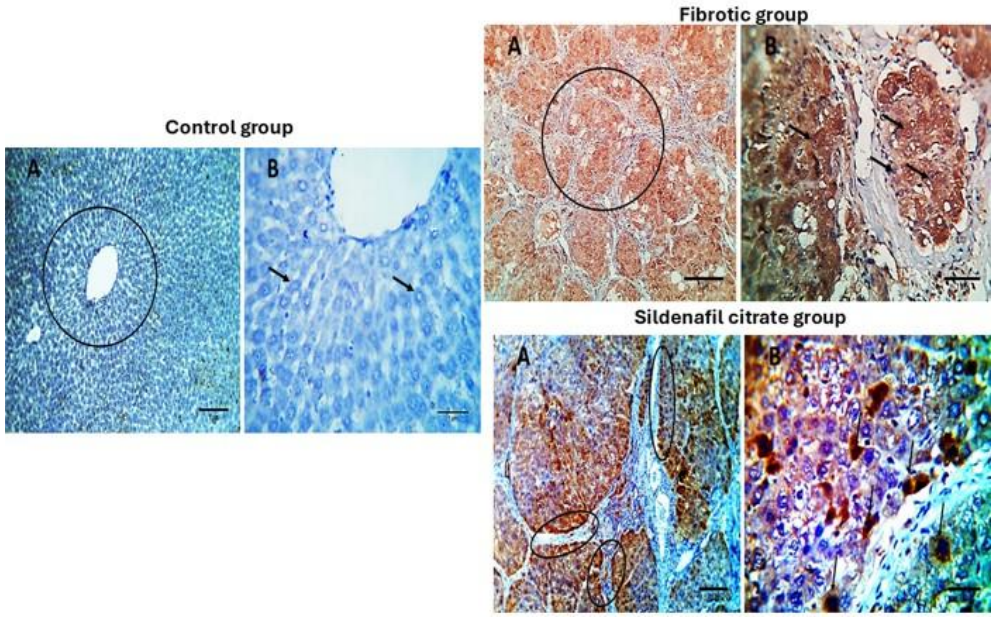


Figure 8: Photomicrograph of rat's liver demonstrated by immunohistochemical stain for TGF-β1. Control group: Photomicrograph of rat's liver of group showed (A) the hepatocytes and the vascular structure of portal triads showing negative stainability for TGF-β1. (B) High magnification to demonstrate the negative stainability of the hepatocytes. While fibrotic group: photomicrograph of rat's liver showed (A) Expression of TGF-β1 in 15-20 % of the hepatic parenchyma and stromal cells. Immunohistochemical stain for TGF-β1 (black circles) and (B) high magnification to demonstrate the brownish stainability of the positive cells (black arrows). Treated group with sildenafil citrate: Photomicrograph of rat's liver revealed (A) expression of TGF-β1 in 10-15% of the parenchymal cells and 15-20% of the stromal cells (black circles). (B) High magnification to demonstrate the brownish stainability of the positive cells (black arrows).

3.6. Anti-apoptotic effect of sildenafil citrate on apoptotic p53 Immunoexpression

In the negative control group, only 1–2% of hepatocytes across the hepatic lobules showed weak, focal p53 cytoplasmic immunopositivity **(Figure 9 A, B)**. This proportion rose substantially in the hepatic fibrosis group, in which 8–10% of hepatocytes and stromal cells displayed small apoptotic cells characterized by densely brownish-stained cytoplasm and condensed pyknotic nuclei, consistent with an apoptotic phenotype **(Figure 9A, B)**. In sildenafil citrate, p53-positive cells were reduced to 2–3% of parenchymal and stromal cells **(Figure 9A, B)**. Based on range midpoints, this represents an approximately 3.6-fold reduction in p53-positive apoptotic cells in sildenafil group relative to the untreated fibrosis group, corresponding to a decrease of nearly 72%. This finding is consistent with the marked reduction in the histological apoptotic index previously reported in sildenafil citrate and supports an anti-apoptotic action of sildenafil citrate against CCl4-induced hepatocellular injury.

Collectively, the three immunohistochemical markers converge on a coherent biological narrative rather than three independent observations. The reduction in p53-positive apoptotic cells indicates that sildenafil citrate limited CCl4-induced hepatocellular injury at the level of cell survival; the marked expansion of AFP-positive cells demonstrates that this injury reduction was accompanied by, and likely enabled, a substantially enhanced progenitor-cell-mediated regenerative response; and the stable-to-modestly-increased parenchymal TGF-β1 immunoreactivity, despite a large reduction in matrix deposition, suggests that sildenafil's antifibrotic action operates chiefly through restraint of TGF-β1 downstream fibrogenic signaling in hepatic stellate cells rather than through suppression of TGF-β1 expression itself, leaving the cytokine's regenerative and tissue-remodeling functions comparatively intact. Together, these findings support a model in which sildenafil citrate shifts the hepatic response to CCl4 injury away from a chronic, apoptosis-driven fibrogenic trajectory and toward a regeneration-dominant, wound-resolving phenotype.

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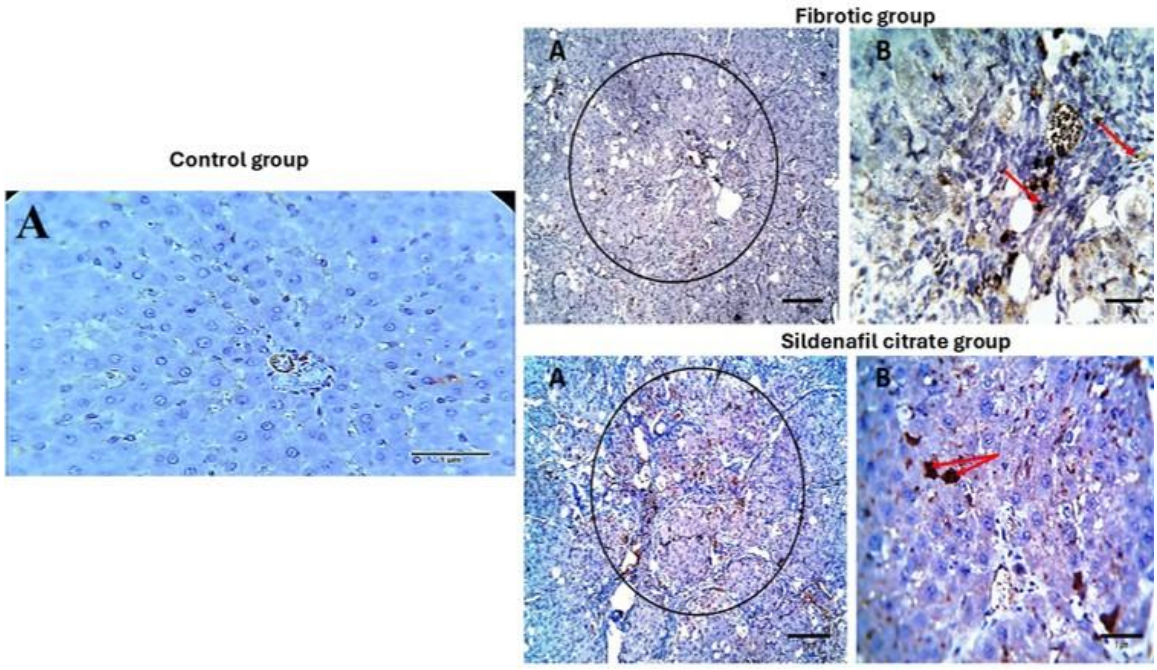


Figure 9: Photomicrograph of rat's liver demonstrated by immunohistochemical stain for P53. Control group: Photomicrograph of rat's liver showed expressions of P53 in 1-2% of the hepatocytes in some hepatic lobules (black circle). While fibrotic group: photomicrograph of rat's liver showed (A) Expression of P53in 8-10% of the hepatocytes and stromal cells. (black circle) (B) High magnification to demonstrate the brownish stainability of the positive cells (red arrows). Treated group with sildenafil citrate: Photomicrograph of rat's liver revealed (A) expression of P53in 2-3% of the hepatocytes and stromal cells. Immunohistochemical stain for P53 (black circle). (B) High magnification to demonstrate the brownish stainability of the positive cells (red arrows).

DISCUSSION

The present study demonstrates that sildenafil citrate, administered concomitantly with CCl_4 over an eight-week induction period, exerts a multi-modal hepatoprotective effect encompassing significant attenuation of biochemical injury, restoration of redox homeostasis, suppression of fibrosis deposition, reduction of apoptotic cell death, and promotion of hepatocellular regeneration. These convergent actions collectively position sildenafil citrate as a compelling candidate for repurposing in the antifibrotic therapeutic target, consistent with the broader contemporary interest in drug repurposing as an efficient strategy for identifying new treatments for chronic liver diseases **(Mohammed et al., 2023)**. The following discussion contextualizes these findings within the existing preclinical literature on phosphodiesterase-5 inhibition in hepatic fibrosis, highlighting both points of convergence and notable discrepancies.

The magnitude of hepatocellular protection observed in this study is noteworthy. CCl_4 administration elevated serum ALT by 7.79-fold and AST by 2.69-fold, reflecting severe hepatocellular membrane disruption and mitochondrial injury consistent with the well-established hepatotoxic mechanism of CCl_4, in which its cytochrome P450 2E1-mediated bioactivation generates the trichloromethyl radical ($\bullet CCl_3$) and trichloromethylperoxyl radical ($\bullet OOCCl_3$), initiating lipid peroxidation cascades that compromise hepatocellular integrity **(MANIBUSAN et al., 2007; Weber et al., 2003)**. Sildenafil citrate treatment substantially attenuated these enzymatic elevations, reducing ALT by 67% and AST by 35% relative to the untreated fibrotic group, and similarly reduced total bilirubin by 59%. These biochemical improvements align closely with several prior investigations. **(Molehin et al., 2018)** employed the same CCl_4 model of hepatotoxicity in rats, reported that sildenafil at 10 and 20 mg/kg significantly reduced serum ALT, AST, ALP, and γ-GT levels, with the higher dose conferring more pronounced protection. Similarly, **Nasr et al., 2025** demonstrated that sildenafil (10 mg/kg) restored ALT, AST, and GGT toward normal values in CCl_4-induced hepatic fibrosis, attributing this effect partly to downregulation of osteopontin gene expression **(Nasr et al., 2025)**. In addition, **Nasr et al., 2025** reported that combined quercetin with sildenafil (50 mg/kg) significantly improved liver function parameters in a chronic ten-week CCl_4 model, with the combination therapy outperforming sildenafil alone **(Nasr et**

al., 2025). Further **Sabra et al.** in 2025, extended these observations by showing that novel nano-formulations of sildenafil, when co-administered with neem extract, produced superior hepatoprotection compared to conventional sildenafil in an oral CCl_4 model, with significant reductions in ALT, AST, and ALP **(Sabra et al., 2025)**. The consistency of these biochemical improvements across diverse studies, including the present one, strengthens the evidence that sildenafil confers genuine hepatoprotection rather than producing a chance or model-specific effect. However, it is important to acknowledge **(Cruz et al., 2013)** who reported a fundamentally different outcome in their murine model of chronic liver disease progression, sildenafil citrate treatment did not affect the progression of the hepatic fibrogenesis and noticeably increased the levels of AST, suggesting a possible toxic effect.

The antioxidant effects of sildenafil citrate observed in this study further substantiate its hepatoprotective profile. CCl_4 administration produced a pronounced depletion of reduced glutathione (GSH) in both serum (0.10-fold of control) and hepatic tissue (0.21-fold of control), concomitant with a marked elevation of malondialdehyde (MDA), a stable end-product of lipid peroxidation, by 4.05-fold (serum) and 1.31-fold (hepatic). Sildenafil citrate treatment substantially restored GSH levels — by 4.25-fold in serum and 2.90-fold in hepatic tissue relative to the fibrotic group — while reducing MDA by 41.7% (serum) and 22.4% (hepatic). These findings are in strong agreement with the existing literature. Furthermore, **Molehin et al. 2018** similarly reported that sildenafil dose-dependently attenuated CCl_4-induced oxidative stress, restoring hepatic GSH and reducing MDA levels, and attributed these effects to the preservation of endogenous antioxidant defenses **(Molehin et al., 2018)**. In alignment with that, N**asr et al. 2025** confirmed that sildenafil restored hepatic GSH and catalase activity while reducing MDA and nitric oxide metabolites in CCl_4-induced fibrosis **(Nasr et al., 2025)**. As well as **Nasr et al. (2025)** demonstrated that the combination of quercetin with sildenafil or pentoxifylline produced a synergistic enhancement of antioxidant capacity, with significant upregulation of Nrf2/ARE-mediated redox defence pathways and downregulation of TLR4/NF-κB-driven pro-oxidant signalling **(Nasr et al., 2025)**. Additionally, **Sabra et al. (2025)** reported that nano-sildenafil formulations significantly reduced hepatic MDA while elevating antioxidant markers **(Sabra et al., 2025)**. The mechanistic basis for these antioxidant effects likely involves the NO–cGMP–PKG signalling axis: by inhibiting PDE5, sildenafil amplifies cGMP-mediated PKG activation, which has been shown to enhance the expression and activity of endogenous antioxidant enzymes while suppressing NADPH oxidase–derived reactive oxygen species **(Alan Albayrak et al., 2023)**. A partial rather than complete normalization or restoration of GSH was observed in the present study, suggesting that while sildenafil substantially recovers antioxidant capacity, residual oxidative stress persists under ongoing CCl_4 challenge — a finding also reported by **(Molehin et al., 2018)**, who noted that even the highest sildenafil dose (20 mg/kg) did not fully restore GSH to control levels.

The antifibrotic effects of sildenafil citrate constitute the central finding of this investigation. Histopathological examination revealed an 80.0% reduction in fibrosis area and a marked regression of collagen deposition on Masson's trichrome staining, transitioning from a severely fibrotic state with bridging septa and cirrhotic nodule formation to a near-baseline architecture with only residual periportal fibrous strands. These morphological improvements are concordant with **(Said et al., 2013)**, who reported that sildenafil (10 mg/kg) significantly reduced hepatic hydroxyproline and collagen content — direct quantitative indices of fibrosis — in thioacetamide-induced cirrhosis in rats, with a 50% reduction in hepatic collagen content after six weeks of treatment. **(Nasr et al., 2025)** also demonstrated that sildenafil reduced the histopathological fibrosis score and hydroxyproline content in CCl_4-induced fibrosis, concomitant with downregulation of osteopontin, a secreted phosphoprotein implicated in HSC activation and extracellular matrix remodeling. At the molecular level, **(Feng et al., 2025)** provided compelling mechanistic evidence that PDE5 inhibition activates the cGMP/PKG pathway, which in turn suppresses the Akt/NF-κB signalling cascade — a critical axis driving hepatic stellate cell activation, proliferation, and collagen synthesis. Although **(Feng et al., 2025)** employed CPD1, a novel PDE5 inhibitor, rather than sildenafil itself, the shared pharmacological target and conserved downstream pathway lend strong mechanistic support to the antifibrotic effects observed herein.

However, the immunohistochemical profile of TGF-β1 in the present study revealed a more nuanced pattern than previously reported. While stromal TGF-β1 expression decreased from 15–20% to 5–10% of cells — consistent with an antifibrotic effect on activated mesenchymal and fibroblast-like cells within the fibrotic septa — parenchymal TGF-β1 expression paradoxically increased from 3–5% to 10–15% of hepatocytes following sildenafil treatment. This divergent behavior between cellular compartments has not, to our knowledge, been previously described in the context of sildenafil and hepatic fibrosis. **(Said et al., 2013)** reported a straightforward reduction in serum TGF-β1 levels with sildenafil treatment in TAA-induced cirrhosis, without compartmental immunohistochemical discrimination. The increase in parenchymal TGF-β1 observed in the present study may reflect a regenerative rather than fibrogenic role of this cytokine. TGF-β1 signaling in hepatocytes has been shown

to participate in liver regeneration under certain contexts, promoting hepatocyte proliferation and survival pathways distinct from its classical fibrogenic actions on stellate cells **(Gilgenkrantz et al., 2025)**. This interpretation is supported by the concurrent 677.8% increase in regenerating hepatocytes and the 9.5-fold expansion of AFP-positive cells observed in the sildenafil-treated group, suggesting that elevated parenchymal TGF-β1 may form part of a coordinated regenerative response rather than indicating treatment failure. This dual role of TGF-β1 — fibrogenic in stromal compartments yet potentially pro-regenerative in parenchymal cells — represents a novel contribution of this study and warrants further investigation at the molecular signaling level.

The anti-apoptotic and pro-regenerative effects of sildenafil citrate, as assessed by p53 and AFP immunohistochemistry, constitute another distinctive aspect of this investigation. The 72% reduction in p53-positive apoptotic cells is consistent with the 81.0% reduction in the histological apoptotic index and aligns with the established role of p53 as a sensor of oxidative DNA damage that triggers apoptotic cell death in response to sustained genotoxic and oxidative stress **(Humpton et al., 2022)**. By reducing oxidative stress and preserving mitochondrial integrity, sildenafil likely attenuates the p53-dependent apoptotic cascade, thereby permitting hepatocyte survival under conditions of ongoing CCl_4-mediated injury. The concurrent 9.5-fold increase in AFP-positive cells is of particular interest. AFP is a well-established marker of hepatic progenitor and oval cell activation during liver regeneration following parenchymal injury **(Iwai et al., 2000)**. Its marked upregulation in the sildenafil-treated group, combined with the near eight-fold rise in regenerating hepatocytes, strongly suggests that sildenafil not only limits ongoing injury but also actively creates a permissive microenvironment for compensatory hepatocyte proliferation — a dual action that, to our knowledge, has not been simultaneously demonstrated by any prior study of sildenafil in hepatic fibrosis. This pro-regenerative dimension adds a hitherto underappreciated facet to the pharmacological profile of sildenafil in the liver.

The discrepancies between the present findings and those of **(Cruz et al., 2013)**, who reported that sildenafil did not affect the progression of the hepatic fibrogenesis and noticeably increased the levels of AST, merit careful consideration. Several methodological factors may account for this discordance. First, **(Cruz et al., 2013)** employed a different hepatotoxic model — chronic CCl_4 administration at a lower frequency (once weekly for six weeks) — which may have produced a fibrotic phenotype with a different balance of necroinflammation, angiogenesis, and matrix deposition. Second, the sildenafil dosing regimen and route of administration differed from the present study, potentially yielding divergent hepatic drug concentrations. Third, and perhaps most importantly, the small sample size in the **(Cruz et al., 2013)** study may have limited statistical power to detect modest treatment effects. Similarly, **(Said et al., 2013)** stated that serum ALT was not significantly reduced by sildenafil despite improvements in AST, ALP, and albumin, suggesting that the aminotransferase response may be model-dependent and influenced by the timing of treatment initiation relative to injury onset. The dose response relationship also warrants consideration **(Nasr et al., 2025)** employed a substantially higher sildenafil dose (50 mg/kg) in combination with quercetin, while **(Molehin et al., 2018)** demonstrated dose-dependent hepatoprotection across 5, 10, and 20 mg/kg, indicating that the antifibrotic efficacy of sildenafil is likely dose-sensitive. Furthermore, **(Sabra et al., 2025)** showed that nano-formulated sildenafil conferred superior hepatoprotection compared to the conventional formulation, suggesting that bioavailability and hepatic drug delivery are critical determinants of therapeutic outcome. These methodological heterogeneities — spanning animal strain, fibrosis model, CCl_4 dose and duration, sildenafil dose and formulation, and treatment timing — collectively underscore the importance of standardizing experimental protocols in this field and help explain why the anti-fibrotic signal, while consistently present across the majority of studies, varies in magnitude.

Several limitations of the present study should be acknowledged. First, only a single dose of sildenafil citrate was evaluated; a dose–response study would more precisely define the therapeutic window and identify the minimum effective dose. Second, while the immunohistochemical markers (TGF-β1, AFP, p53) provide valuable semi-quantitative data, they were not complemented by molecular analyses such as quantitative RT-PCR or western blotting of key fibrogenic signalling pathways (e.g., Akt/NF-κB, Smad2/3, Nrf2/ARE), which would have provided mechanistic depth. Third, the study did not assess portal haemodynamic parameters, and therefore the relative contribution of haemodynamic improvement versus direct antifibrotic action cannot be dissected. Finally, the three-group experimental design, while enabling multiple comparisons, may have benefited from a larger sample size per group to enhance statistical robustness, particularly for the semi-quantitative histopathological scoring.

In conclusion, sildenafil citrate demonstrated significant fibro-protective efficacy in the CCl_4-induced hepatic fibrosis model, exerting concurrent antioxidant, anti-apoptotic, antifibrotic, and pro-regenerative actions. These findings are broadly consistent with the majority of the preclinical literature on PDE5 inhibition in liver fibrosis and extend the evidence base by demonstrating,

for the first time, a sildenafil-associated expansion of AFP-positive hepatic progenitor cells and a compartment-specific dissociation of TGF-β1 expression. The discrepancies with a minority of published reports are attributable to methodological heterogeneity across studies. Collectively, these data support further investigation of sildenafil citrate as a repurposed antifibrotic agent, with future studies addressing dose optimization, combination therapy, and elucidation of the molecular signaling pathways underlying its pleiotropic hepatic effects.

Ethics approval

The study was run under the rules that govern the care and use of laboratory animals and was cleared by the Research Ethics Committee at the Faculty of Veterinary Medicine, Mansoura University, Egypt (Approval code No.: **(VM.R.26.10.335**).

Consent for publication

Available upon request.

Availability of data and material

The data are available upon request by the corresponding authors and can be supplied on reasonable requests.

Competing interests

The authors declare that they have no financial or personal conflicts that might be viewed as shaping the work reported in this manuscript.

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