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Protective Effects of Crocetin against Thioacetamide-induced Liver Fibrosis in Rats

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ABSTRACT

Background: Liver fibrosis represents irreversible liver destruction. Crocetin, a carotenoid dicarboxylic acid found in saffron, possesses anti-inflammatory, antioxidant and anti-apoptotic properties. This study investigated crocetin's hepatoprotective effects against thioacetamide-induced liver fibrosis in rats, focusing on its potential therapeutic mechanisms.

Methods: Forty male Wistar albino rats were divided into four groups: control (normal saline), crocetin (40 mg/kg, i.p.), thioacetamide (200 mg/kg, i.p., twice weekly for 6 weeks) and thioacetamide+crocetin (pretreatment with crocetin before thioacetamide). Various biochemical and histological parameters were assessed.

Result: Thioacetamide administration increased oxidative stress markers (nitric oxide, malondialdehyde), decreased antioxidants, elevated inflammatory markers (IL-1β, TNF-α, NF-κB) and impaired liver enzymes. Histologically, it caused cirrhotic nodules and extensive necrosis. Crocetin treatment restored antioxidant levels, reduced lipid peroxidation, suppressed inflammatory cytokines, regulated the Bax/Bcl-2 ratio and normalized liver enzyme levels. The protective effects of crocetin were particularly evident in liver tissue morphology, where it significantly prevented the formation of fibrotic bridges, maintained hepatocyte integrity and preserved sinusoidal structure. Additionally, biochemical analysis revealed that crocetin treatment effectively restored glutathione peroxidase and catalase activities to near-normal levels. The study concluded that crocetin effectively mitigates thioacetamide-induced hepatic fibrosis through its antioxidative, anti-inflammatory and anti-apoptotic properties, suggesting potential therapeutic applications in liver disease.

Key words: Antioxidants, Crocetin, Liver fibrosis, Rat, Thioacetamide.

INTRODUCTION

Liver fibrosis represents a significant health challenge in the Kingdom of Saudi Arabia, primarily due to limited therapeutic options (Alqahtani *et al.,* 2020). The condition is characterized by excessive accumulation of extracellular matrix proteins, particularly collagen fibers, which can progress to cirrhosis if left untreated (Elpek, 2014). To study potential treatments, researchers commonly employ thioacetamide, an organosulfur compound, to induce liver fibrosis in experimental animal models (Wallace *et al.,* 2015).

The hepatotoxicity of thioacetamide occurs through its metabolic activation by cytochrome P450 enzymes, which convert it to thioacetamide-S-oxide (Xie *et al.,* 2012). This reactive metabolite subsequently transforms into thioacetamide-sulfdioxide, leading to cellular necrosis and fibrosis through interactions with cellular proteins, lipids and nucleic acids (Sarma *et al.,* 2012). The metabolic activation process generates reactive oxygen species (ROS), triggering oxidative stress that damages cellular components and impairs mitochondrial function, resulting in decreased ATP synthesis and further ROS production (ElBaset *et al.,* 2022).

Thioacetamide-induced liver toxicity initiates inflammatory cascades through the activation of Kupffer cells, which release pro-inflammatory mediators including TNF-α, IL-1 and IL-6 (ElBaset *et al.,* 2022). The inflammatory response involves several key regulators: Nuclear factor erythroid 2-related factor 2 (Nrf2) controls antioxidant protein production, while interleukin-1 (IL-1) modulates inflammatory processes and subsequent fibrosis (Lurie *et al.,* 2015). Nuclear factor-kappa B (NF-κB) plays a crucial role in orchestrating inflammatory cytokine responses (Abdel Rahman *et al.,* 2022).

The combined effects of inflammatory mediators and oxidative stress activate hepatic stellate cells (HSCs), which transform into myofibroblasts - the primary collagen-producing cells in liver fibrosis (Biagini and Ballardini, 1989). The NF-κB pathway's activation by pro-inflammatory cytokines represents a well-documented mechanism in inflammatory regulation (Singh *et al.,* 2021), making these mediators valuable diagnostic markers for assessing tissue injury (Cevik *et al.,* 2017; Cao *et al.,* 2021; Sharma *et al.,* 2024).

The pathogenesis of liver fibrosis involves increased oxidative stress and inflammation, which promote apoptosis by disrupting the balance between pro-apoptotic

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(Bax, caspase-3) and anti-apoptotic (Bcl-2) proteins (Wu *et al.,* 2019). Recent evidence suggests that enhanced hepatocyte apoptosis contributes significantly to fibrosis development (Chakraborty *et al.,* 2012).

In the search for effective therapeutic agents, natural compounds have gained attention. Crocetin, an aglycone derivative of crocin found in saffron, emerges as a promising candidate (Siracusa *et al.,* 2011). This bioactive molecule, also generated through crocin hydrolysis in biological systems, demonstrates significant therapeutic potential through its antioxidant, anti-inflammatory and anti-apoptotic properties (Mousa *et al.,* 2019; Guo *et al.,* 2022). Crocetin's therapeutic mechanisms include free radical scavenging, enhancement of antioxidant defense systems like glutathione peroxidase (Guo *et al.,* 2022) and modulation of inflammatory responses through downregulation of pro-inflammatory cytokines and NF-κB pathway regulation (Sun *et al.,* 2014).

The therapeutic applications of crocetin extend beyond hepatoprotection, showing promise in treating various conditions including myocardial injury (Wang *et al.,* 2014), hepatic failure (Gao *et al.,* 2019), retinal ischemic damage (Ishizuka *et al.,* 2013) and neurodegenerative disorders such as Parkinson's disease (Dong *et al.,* 2020). Given its broad therapeutic potential and multiple beneficial properties, this study aimed to investigate crocetin's hepatoprotective effects against thioacetamide-induced liver fibrosis through comprehensive analysis of biochemical, physiological and histological parameters.

MATERIALS AND METHODS

Animals

This study utilized 40 male Wistar albino rats (10 weeks, 180-220 g), obtained from King Abdulaziz University, Saudi Arabia. To acclimate, the animals were kept in the lab under normal conditions for one week. The laboratory experiments were conducted at the School of Medicine, Taif University (2023-2024).

Drugs and experimental kits

Crocetin (CA-27876-94-4), thioacetamide (CA-62-55-5) and all kits used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Experiment model

Animals were divided into 4 groups (10 each):

- **Control group:** In the control group, rats were administered normal salinefor 6-weeks.
- **Crocetin group:** Rats were administered crocetin (40 mg/ kg, i.p.) every day for 6-weeks (Ma *et al.,* 2024).
- **Thioacetamide group:** According to Eissa *et al.* (2018), rats were treated with thioacetamide (200 mg/kg, ip) twice a week for 6 weeks. Thioacetamide was used in this study to induce hepatic fibrosis.
- **Thioacetamide+crocetin group:** Rats were pretreated with crocetin 1 hour prior to treatment with thioacetamide for 6 weeks.

Analysis of liver function

Albumin was assessed according to Schmidt and Eisenburg (1975). Serum alanine transaminase (ALT; Ca-MAK052), alkaline phosphatase (ALP; Ca-MAK122) and aspartate transaminase (AST; Ca-MAK055) activities were assessed according to Reitman and Frankel (1957). Liver function enzymes (ALT, AST and ALP) were measured in serum/plasma samples.

Analysis of the state of oxidants and antioxidants

The levels of oxidants, including malondialdehyde (MDA; Ca-MAK085) measured as described by Ohkawa *et al.* (1979) and nitric oxide (NO; Ca-MAK454-1KT) according to Green *et al.* (1982), as well as antioxidants, including catalase (CAT; Ca-CAT100-1KT) as described by Aebi (1984), GPx (Ca-MAK437-1KT) according to Paglia and Valentine (1967), GR (GRSA) according to Carlberg and Mannervik (1985), SOD (19160) according to Marklund and Marklund (1974) and glutathione (GSH; Ca-MAK517-1KT) according to Ellman (1959) and Nrf2 (Ca-SAB6010051) according to Bradford (1976). All kits used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). For this purpose, the UV-visible spectrophotometer Shimadzu UV 1800 was used. All oxidative stress markers, antioxidant parameters and inflammatory markers were measured in tissue homogenates.

Evaluation of apoptotic markers

The levels of Bcl2 (Ca-RAB0472), Bax (Ca-RAB0471) and caspase-3 (Ca-CASP3C) in liver tissue were assessed by ELISA following manufacturer's guidelines**.**

Assessment of inflammatory cytokines

In this study, an ELISA was utilized to determine the inflammation cytokine levels. As directed by the maker, ELISA kits were used to measure interleukin-1β (IL-1β Ca-RAB0277), NF-κB (Ca- RAB0671) and tumor necrosis factor-α (TNF-α; Ca- RAB0479).

Histopathology study

In this study, two different stains were used, hematoxylin and eosin, to show histopathological alteration. Masson's trichrome stain was used to confirm liver fibrosis. Liver samples were cut at 4 mm. samples of the liver immersed in a 10% neutral formaldehyde solution for 24 hours to fix them, then dehydrated using high-quality alcohol before embedding them in paraffin. The samples were cut using the microtome to slice the materials into 4 mm thickness. The samples were stained with both stains. An Olympus-BX53 microscope was used to examine the slides and photographs were captured using an Olympus-DP70 camera.

Ethical approval

This study, along with all experimental procedures, was conducted according to the principles outlined by the ethics committee of the University of Taif (Approval No. HAO-02-T-105).

Statistical analysis

The results are presented as the mean ±standard deviation (SD). Statistical analysis was performed using one-way ANOVA, followed by Tukey's post hoc test to assess statistical significance, utilizing SPSS software (version 18). P-values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Analysis of the liver enzymes

Biochemical tests showed that thioacetamide markedly raised ($p<0.05$) levels of AST, ALT and ALP while decreasing levels of albumin in the blood compared with the control group. The crocetin group exhibited no statistically significant changes in these markers as compared with the control group. However, coadministration of crocetin with thioacetamide significantly reduced ($p<0.05$) the liver enzyme disturbance and albumin decrease compared to the thioacetamide-only group (Fig 1). These findings indicate that crocetin offers protection against liver enzyme disturbances induced by thioacetamide.

Antioxidants and lipid peroxidation markers

Analysis of liver tissue antioxidant enzymes showed a significant reduction ($p<0.05$) in SOD, CAT, GPx and GR activity in the thioacetamide-treated group compared to the control group (Fig 2). Co-administration of crocetin with thioacetamide increased SOD, CAT, GPx and GR activity compared with thioacetamide alone. In addition, thioacetamide treatment resulted in increased MDA and NO levels ($p<0.05$), accompanied by a marked decline in levels of GSH and Nrf-2 compared to the control group. Co-administration of crocetin with thioacetamide increased GSH and Nrf-2, accompanied by a notable decrease in levels of NO and MDA compared to the control group (Fig 3).

Anti-inflammatory markers

Compared with the control group, animals treated with thioacetamide showed significantly elevated levels ($p<0.05$) of proinflammatory cytokines (TNF-α, IL-1β and NF-κB). The crocetin-treated rats showed significantly reduced TNF-α, IL-1β and NF-κB levels compared with the thioacetamide group (Fig 4).

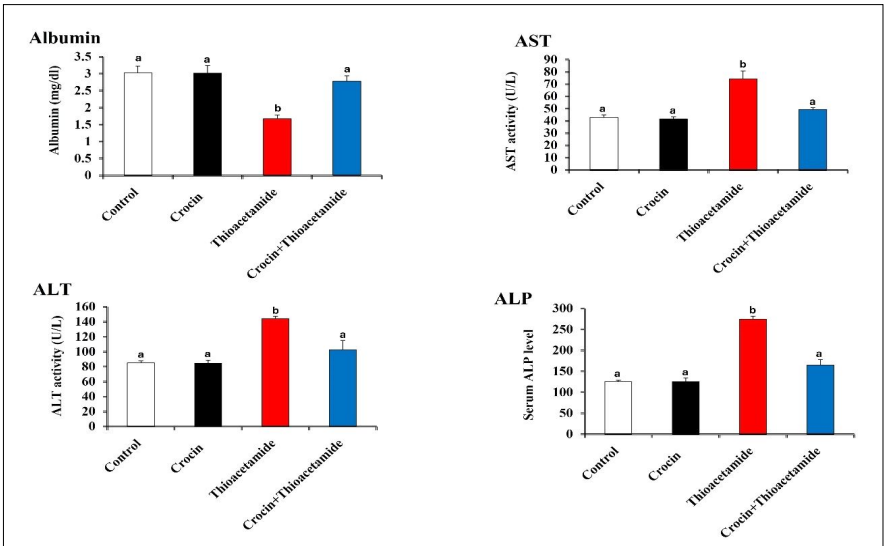
Apoptotic markers

Apoptotic indicators showed significant increases ($p<0.05$) in Bax and caspase-3 levels with marked reduction in Bcl-2 in the thioacetamide group compared to controls. However, crocetin prevented liver cell apoptosis and restored both apoptotic and anti-apoptotic protein levels to baseline (Fig 5).

Histopathology investigation

The control and crocetin-treated groups showed normal liver histology with H andE staining (Fig 6A and C) and normal collagen distribution with Masson's trichrome staining (Fig 7A and C). In contrast, thioacetamide-treated liver sections showed fibrosis characterized by excessive extracellular matrix deposition, cirrhotic nodules, hepatocyte disorganization, necrosis, inflammatory infiltration, vascular distortion and apoptotic changes (Fig 6B). Masson's trichrome staining revealed bridging fibrosis with blue-stained collagen fibers, indicating progression to cirrhosis (Fig 7B). Conversely, Crocetin treatment showed marked improvement in liver histology, with only mild fibroplasia, inflammation (H and E, Fig 6D) and collagen deposition (Masson's trichrome, Fig 7D).

The present study evaluated the therapeutic effectiveness of crocetin in protecting against hepatic



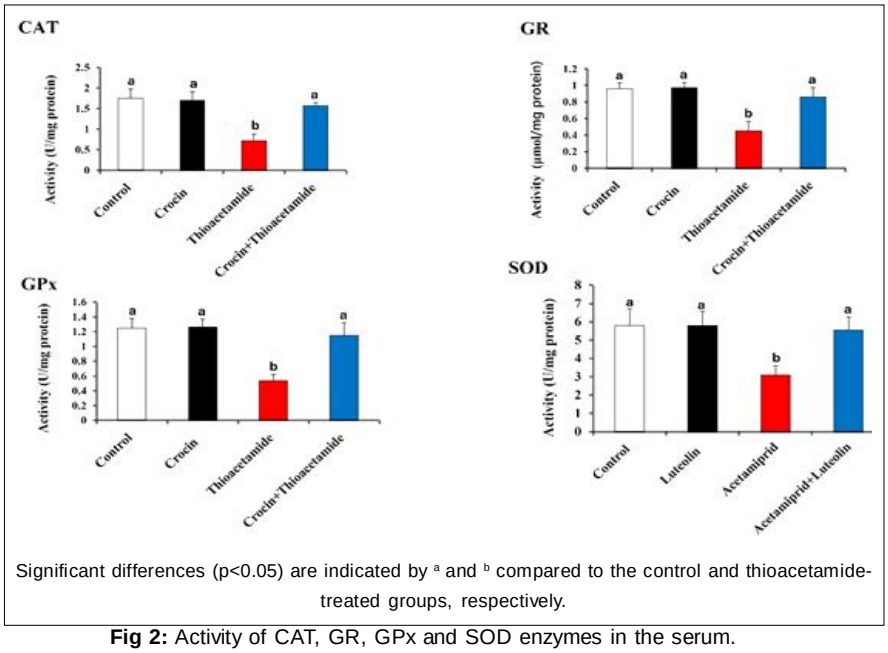
Significant differences ($p<0.05$) are indicated by [a] and [b] compared to the control and thioacetamide-treated groups, respectively.

Fig 1: Albumin content and the levels of AST, ALT and ALP in the serum.

fibrosis induced by thioacetamide. This was achieved by examining several pathways, including oxidative stress, inflammation and apoptosis, as well as conducting histopathological analyses (Dwivedi *et al.,* 2020).

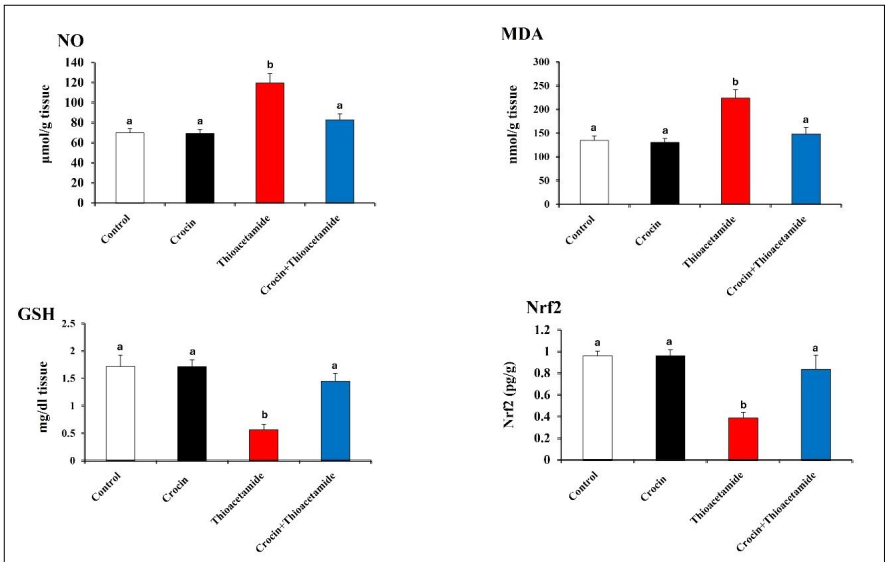
Our study showed that thioacetamide exposure increased ALP, AST and ALT levels and reduced albumin content in rat livers. These results are consistent with previous findings that thioacetamide damages liver cells and alters enzyme levels (Schyman *et al.,* 2018).

Crocetin has been shown to protect liver enzymes from xenobiotic-induced toxicity (Xu *et al.,* 2022). Our study confirmed that crocetin reduces AST, ALT and ALP levels while significantly lowering serological albumin, demonstrating its effectiveness in alleviating thioacetamide-induced liver damage. These findings align with previous studies highlighting crocetin's hepatoprotective effects in various liver diseases (Xu *et al.,* 2022). Thioacetamide treatment in mice elevated AST, ALT and ALP levels while



Significant differences ($p<0.05$) are indicated by [a] and [b] compared to the control and thioacetamide-treated groups, respectively.

Fig 2: Activity of CAT, GR, GPx and SOD enzymes in the serum.



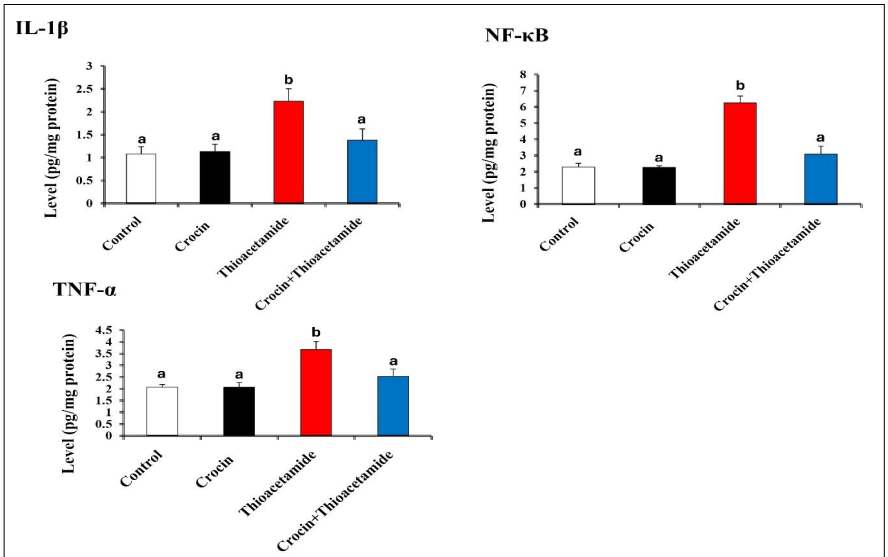
Significant differences ($p<0.05$) are indicated by [a] and [b] compared to the control and thioacetamide-treated groups, respectively.

Fig 3: Levels of NO, MDA, GSH and Nrf-2 in liver tissue.

decreasing albumin content, further emphasizing crocetin's protective role.

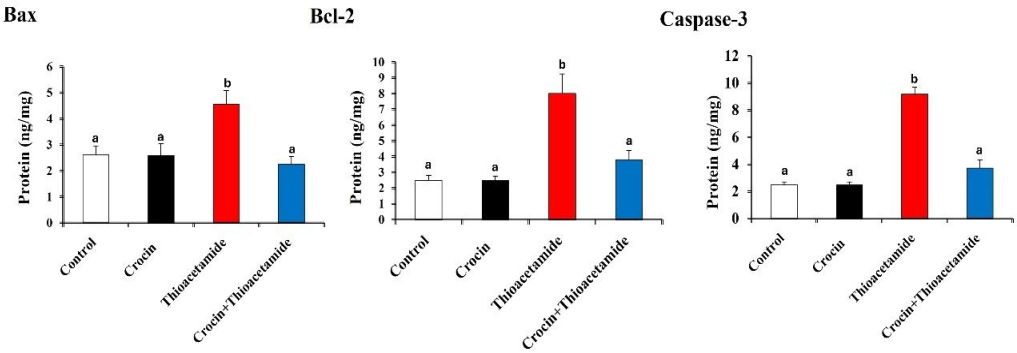
Oxidative stress has been known as the key pathway mechanism causing cellular damage and subsequently leading to liver fibrosis (Sánchez-Valle *et al.,* 2012). Additionally, oxidative stress has been linked to a substantial rise in the generation of ROS, accompanied by a reduction in the body's antioxidant capacity (Gomes *et al.,* 2012). In the current investigation, exposure to thioacetamide resulted in the production of MDA and NO and a significant decrease in the levels of GSH. It also suppressed the activity of SOD, CAT, GPx and GR enzymes. These results were similar to those of (Ibrahim *et al.,* 2023), who found that giving rats thioacetamide (200 mg/kg) intraperitoneally raised levels of MDA and lowered SOD, CAT and GSH.

Crocetin is recognised as a powerful scavenger of ROS, safeguarding cells against the buildup of ROS triggered by oxidative stress (Guo *et al.,* 2022). Furthermore, Sun *et al.* (2014) reported that crocetin, administered orally, mitigates cisplatin-induced hepatotoxicity in mice by reducing oxidative stress *via* suppression MDA levels and NO activity. Also, The findings of this study demonstrate a notable enhancement in the antioxidant state of the hepatic tissue in rats administered with crocetin. These results align with other studies that emphasise the antioxidant properties of



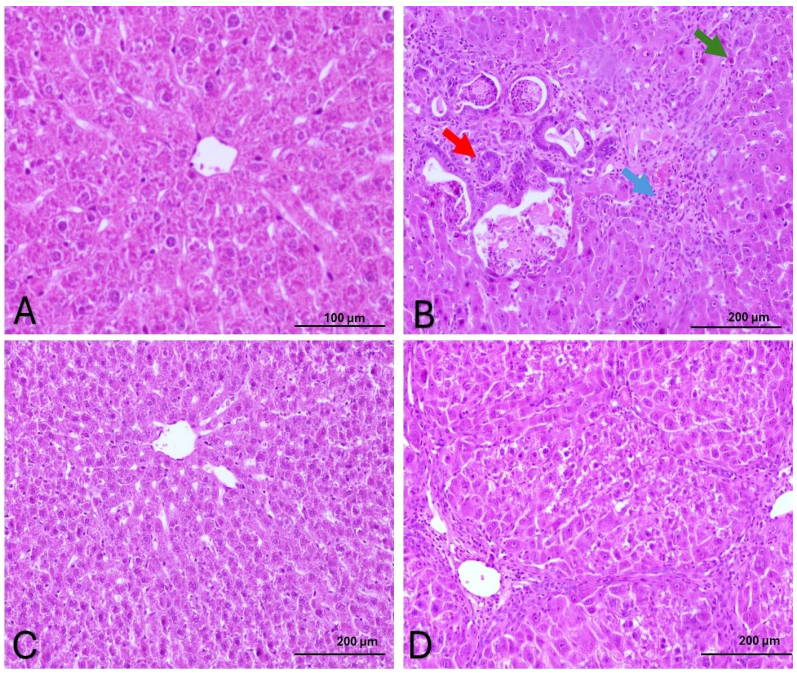
Significant differences ($p<0.05$) are indicated by [a] and [b] compared to the control and thioacetamide-treated groups, respectively.

Fig 4: Level of IL-1β, NF-κB and TNF-α in the liver tissue.



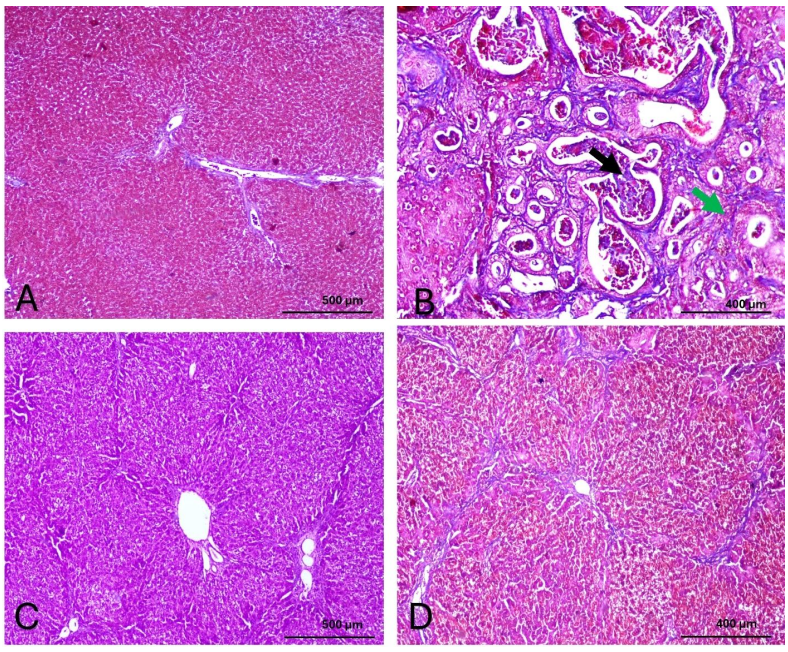
Significant differences ($p<0.05$) are indicated by a and b compared to the control and thioacetamide-treated groups, respectively.

Fig 5: Levels of apoptosis markers Bax, Bcl-2 and caspase-3.



(A, C) show normal hepatic structure. (B) shows cirrhotic nodules (red arrow), necrosis (blue arrow) and apoptosis (green arrow) induced by thioacetamide. (D) shows improved liver architecture after crocetin treatment. H and E, A × 400, B, C and D × 200.

Fig 6: Histopathological liver sections stained with H and E.



(A, C) show normal hepatic structure with minimal collagen deposition. (B) exhibits extensive fibrosis with cirrhotic nodules (black arrow) and dense collagen accumulation (green arrow) induced by thioacetamide. (D) shows reduced fibrosis following crocetin treatment. Masson's trichrome stain, A, B, C and D × 100.

Fig 7: Liver sections stained with Masson's trichrome to assess fibrosis.

crocetin in multiple liver fibrosis models (Xu *et al.,* 2022). The potential of crocetin to decrease MDA and NO levels provides further evidence of its potent antioxidant characteristics.

Under normal conditions, Nrf2 is linked with Keap1 in the cytoplasm of hepatocytes. Thioacetamide exposure causes Nrf2 to dissociate from Keap1, translocate to the nucleus and regulate antioxidant gene transcripts in rats (Dwivedi *et al.,* 2020; Shin *et al.,* 2021). Results of the current work found that thioacetamide inhibited Nrf2 and its target enzymes (SOD, CAT, GPx, GR) in rat livers. Prior research has demonstrated crocetin's ability to mitigate non-alcoholic fatty liver disease progression by attenuating oxidative stress. This action subsequently alle*via*tes inflammation and enhances expression of Nrf2 and H0-1 (Xu *et al.,* 2021). Furthermore, our results showed that coadministration of crocetin increased Nrf2 activity and its associated enzymes, supporting previous findings that crocetin protects hepatocytes from oxidative injury by activating Nrf2 and boosting antioxidant enzyme production (Xu *et al.,* 2021).

Chronic liver disease is marked by ongoing inflammation and liver dysfunction, which can eventually progress to liver cirrhosis. Several studies have reported that thioacetamide exposure induces the release of pro-inflammatory cytokines, including TNF-α and IL-1β (Su *et al.,* 2019; ElBaset *et al.,* 2022). These cytokines regulate inflammatory responses and modulate the growth of liver fibrosis by stimulating inflammatory cells, which in turn promote the formation of fibroblasts that deposit extracellular matrix components (Su *et al.,* 2019). Our findings demonstrate exposure to thioacetamide led to elevated levels of proinflammatory cytokines. These findings align with those stated by (ElBaset *et al.,* 2022), who demonstrated similar increases in TNF-α, IL-1β and NF-κB in the livers of rats treated with thioacetamide.

Several studies indicated the hepatoprotective effects of crocetin against inflammation by regulating inflammatory cytokines (Wang *et al.,* 2014; Gao *et al.,* 2019). Sun *et al.* (2014) reported that crocetin, administered orally, mitigates cisplatin-induced hepatotoxicity in mice by reducing inflammation *via* decreasing levels of TNF-α, IL-1β and NF-κB. Interestingly, our results showed a decrease in the levels of pro-inflammatory cytokines in the group that received crocetin. This finding supports the efficacy of crocetin in reducing inflammation associated with thioacetamide-induced liver fibrosis.

Excessive apoptosis in hepatic tissue is a hallmark of liver fibrosis (Wieckowska *et al.,* 2006). Thioacetamide's harmful effects primarily trigger apoptosis by damaging mitochondria (Staòková *et al.,* 2010). Bcl-2 regulates apoptotic function *via* control of the mitochondrial apoptosis process (Mousa *et al.,* 2019). Our findings suggest that thioacetamide exposure in rats disturbed the balance between pro-apoptotic and anti-apoptotic protein expression. These findings suggest that thioacetamide-induced apoptosis in rats' hepatocytes. This result is steady with Mousa *et al.* (2019), who showed a similar disrupted balance between pro-apoptotic (Bax and caspase-3) and anti-apoptotic (Bcl-2) protein expression following the treatment with thioacetamide (200 mg/kg) in rats. This imbalance is associated with observable histological alterations in hepatic tissues (Enciso *et al.,* 2022). Our results suggest that crocetin restored pro/anti-apoptotic protein balance, demonstrating its cytoprotective effect against thioacetamide-induced liver fibrosis.

Histopathology results of the hepatic tissues of rats in the thioacetamide group revealed variable-sized cirrhotic hepatic nodules and loss of normal hepatic architecture with intervening fibrosis. The presence of these regenerative nodules is characteristic of cirrhotic progression (Biagini and Ballardini, 1989). Our findings are consistent with those reported by Alkreathy and Esmat (2022), who demonstrated that administering thioacetamide (200 mg/kg) to rats induced liver fibrosis. Their study revealed substantial fibrous tissue deposition encircling hepatic lobules, accompanied by mononuclear inflammatory cell infiltration and vacuolar degeneration of hepatocytes. Moreover, they observed extensive fibroplasia surrounding the hepatic lobules, which was highlighted by blue staining with Masson's trichrome technique. The enhancement of the histopathological alteration and the restored of the hepatic architecture of the hepatic architecture to be near normal in the crocetin-treated group confirm its potential as an anti-fibrotic agent.

CONCLUSION

In conclusion, this study provides compelling evidence for crocetin's therapeutic potential in treating liver fibrosis. Through multiple complementary mechanisms, including antioxidant, anti-inflammatory and anti-apoptotic effects, crocetin demonstrated significant hepatoprotective properties against thioacetamide-induced liver damage. The restoration of antioxidant enzyme activities, suppression of inflammatory markers and preservation of liver tissue architecture collectively suggest that crocetin could serve as a promising therapeutic agent for liver fibrosis. These findings not only advance our understanding of crocetin's protective mechanisms but also lay the groundwork for future clinical investigations exploring its potential use in treating chronic liver diseases. Further research is warranted to investigate optimal dosing regimens and potential combinatorial therapies to maximize crocetin's therapeutic benefits in clinical settings.

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Author contributions

The principal author completed this study's research items.

Conflict of interest

All authors declared that there is no conflict of interest.

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